Joint PhD

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BACTERIAL INOCULATION IN *Helianthus tuberosus* FOR IMPROVING PHYTOREMEDIATION OF METAL-POLLUTED SOILS

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The chemical barrage has been hurled against the fabric of life -a fabric on the one hand delicate and destructible, on the other miraculously tough and resilient, and capable of striking back in unexpected ways.

— Rachel Carson
In *Silent Spring*, (1962)

Resumen

Inoculación de bacterias en *Helianthus tuberosus* para mejorar la fitorremediación de suelos contaminados con metales

La minería y las industrias metalúrgicas, así como el uso de fungicidas y fertilizantes inorgánicos han incrementado los niveles de metales y metaloides en los suelos. Los metales constituyen un problema global debido a su elevada capacidad para acumularse en la cadena trófica, su toxicidad y su persistencia en el medio ambiente, que supone un riesgo para las aguas superficiales y subterráneas. Las tecnologías convencionales de remediación de suelos son elevadamente caras, invasivas y pueden generar problemas ambientales añadidos, como la degradación del propio suelo. Por otro lado, las fitotecnologías son técnicas sostenibles de remediación de suelos que utilizan plantas, microorganismos y enmiendas para reducir la concentración y biodisponibilidad de los contaminantes, y además contribuyen a dar un uso sostenible a zonas en desuso.

Una de las mayores limitaciones de las fitotecnologías, en suelos contaminados con metales, es el largo tiempo necesario para descontaminar el suelo y alcanzar los niveles permitidos. El uso de cultivos energéticos, capaces de acumular metales, puede compensar el tiempo requerido en esta tecnología con la producción de biomasa con valor económico.

Brachypodium distachyon (L.) Beauv. es la primera planta de la familia Poaceae que ha sido secuenciada. Esta planta ha sido propuesta recientemente como modelo para el desarrollo de nuevas energías sostenibles. Aumentar el conocimiento sobre la tolerancia y la acumulación de metal de esta nueva planta modelo puede ayudar a entender los mecanismos de toxicidad a nivel molecular, así como de genes y proteínas implicadas en la respuesta de las plantas a altas concentraciones de metales. El primer objetivo de este trabajo fue estudiar la habilidad de las semillas de *B. distachyon* de germinar y crecer en condiciones *in vitro* con Cd, As (V), Zn o Cr (VI) en comparación con dos cultivos energéticos conocidos por su tolerancia a metales, *Brassica napus* L. y *Helianthus annuus* L. Altas concentraciones de Cd, Zn y As (V) no afectaron a la germinación de las semillas de las especies estudiadas. El máximo nivel de toxicidad se encontró en plantas tratadas con Cr (VI). La biomasa solamente se redujo a altas dosis de los metales estudiados. Teniendo en cuenta los resultados obtenidos, *B. distachyon* mostró una alta capacidad de germinar y crecer en presencia de altas dosis de Cd, As (V) y especialmente de Zn, lo que sugiere que esta planta puede ser un modelo apropiado para

estudiar cultivos energéticos tolerantes a metales, y los mecanismos implicados en la respuesta al estrés por Zn a nivel molecular.

Por otro lado, *Helianthus tuberosus* L. es un cultivo de alta biomasa, recientemente propuesto como candidato para ser utilizado en fitotecnologías de suelos contaminados con metales. Esta especie presenta características agronómicas que pueden ser útiles en fitotecnologías, como su alta capacidad de colonización y adaptación a diferentes tipos de suelo, incluyendo suelos pobres en nutrientes y salinos, así como su resistencia a plagas y enfermedades. Este cultivo se propaga por tubérculos, de forma diferente a los otros cultivos estudiados, lo que complicó su crecimiento en condiciones *in vitro* con agar. Después de sucesivas pruebas en condiciones controladas, la hidroponía con substrato fue elegida como el método más apropiado para evaluar su habilidad para crecer y acumular metales. Este sistema de crecimiento permitió dar soporte al tubérculo y a la vez permitió el desarrollo del sistema radicular en condiciones controladas.

El crecimiento, la acumulación de metal, así como las interacciones entre metales y nutrientes en dos variedades de *H. tuberosus* (VR y D19), conocidas por su elevada producción de biomasa, se evaluaron en este trabajo. Para ello, se realizaron tres experimentos en condiciones de hidroponía en el invernadero con tres mezclas diferentes de metales y metaloides. La variedad D19 acumuló concentraciones más altas de metal que VR, y además mostró una movilización efectiva de Pb a la parte aérea. Aunque ambas variedades mostraron una gran capacidad para crecer en presencia de múltiples metales y metaloides, D19 mostró mejores características que VR para ser utilizada en fitotecnologías.

Las mayores limitaciones para aplicar la fitoextracción en suelos contaminados con metales son la disponibilidad del metal en el suelo y su toxicidad para la planta. La interacción entre plantas y bacterias capaces de promover el crecimiento de plantas puede ayudar a aumentar la producción de biomasa y mejorar la tolerancia de la planta a los metales disminuyendo la fitotoxicidad. El segundo objetivo se centró en aislar y caracterizar las comunidades bacterianas asociadas a *Brassica napus* en un suelo contaminado con Zn, para seleccionar bacterias capaces de promover el crecimiento y la tolerancia a metales de cultivos energéticos de alta biomasa. Un total de 426 cepas bacterianas morfológicamente diferentes fueron aisladas de suelo, rizosfera, raíz y tallo de *B. napus*. Aunque la mayoría de las cepas bacterianas mostraron características que pueden promover el crecimiento de plantas, una cepa de suelo (*Arthrobacter* sp. 222), una cepa de rizosfera (*Staphylococcus* sp. 25) asociada a

B. napus, y cuatro endófitos de raíz (*Pseudomonas* sp. 228, *Serratia* sp. 246, *Pseudomonas* sp. 256, *Pseudomonas* sp. 262) mostraron una elevada actividad ACC deaminasa, producción de sideróforos y ácidos orgánicos, capacidad para solubilizar fosfato y fijar nitrógeno durante los test fenotípicos; y por ello, fueron seleccionadas para ser inoculadas en planta. La inoculación de semillas de *B. napus* con *Arthrobacter* sp. 222 *Pseudomonas* sp. 228, *Serratia* sp. 246, *Pseudomonas* sp. 256 y *Pseudomonas* sp. 262 aumentó el crecimiento de la raíz en presencia de Zn (1000 µM) o Cd (300 µM) en placas de agar verticales.

Las cinco cepas bacterianas que aumentaron el crecimiento de la raíz en plántulas de *B. napus* fueron inoculadas en *H. tuberosus* en condiciones de hidroponía con Cd (0.1mM) y Zn (1mM) con el objetivo de evaluar el efecto de las bacterias sobre el crecimiento, la acumulación de metal y el estrés oxidativo en este cultivo. La inoculación de *Pseudomonas* sp. 228, *Serratia* sp. 246 y *Pseudomonas* sp. 262 aumentó el crecimiento de la variedad D19 de *H. tuberosus* en presencia de Cd o Zn, y la inoculación de *Pseudomonas* sp. 228, *Serratia* sp. 246 y *Pseudomonas* sp. 256 disminuyó el contenido de compuestos reactivos del ácido tiobarbitúrico **(**TBA) en la raíz de plantas crecidas con Zn. Tanto la mejora del crecimiento, como la disminución del estrés inducido por metales observados en plantas inoculadas con bacteria, fueron más pronunciadas en la variedad D19 que en VR.

En un estudio posterior, las cinco cepas bacterianas fueron inoculadas en la variedad D19 de *H. tuberosus* en un suelo contaminado con Cd-Zn para evaluar la eficiencia de estas bacterias en el crecimiento y la acumulación de metal de la planta cuando existe competencia con otros microorganismos del suelo, y los metales y nutrientes se encuentran menos disponibles. En este último estudio, las cepas bacterianas se inocularon de manera individual y en consorcio. La inoculación individual no afectó al crecimiento de *H. tuberosus* en condiciones de suelo, si bien la acción conjunta de las bacterias añadidas como consorcio aumentó la biomasa, la acumulación de Pb y Zn y la actividad de la enzima málica e isocitrato deshidrogenasa en raíz, y glutatión reductasa en hojas. Estos efectos indicaron que se estableció una relación entre las bacterias inoculadas y *H. tuberosus*. Esta relación fue apoyada por la observación en microscopia laser confocal de la bacteria marcada con el plásmido de fluorescencia verde adherida a los pelos radiculares, y por el aumento de los pelos radiculares en plantas de *H. tuberosus* inoculadas con el consorcio.

Los endófitos de raíz de *B. napus* afectaron a la estructura de la raíz de *H. tuberosus*, indicando que las bacterias inoculadas pueden promover el crecimiento en una especie

diferente de la hospedadora inicial. *H. tuberosus* acumuló más de 1000 $mg.kg^{-1}$ de Zn en la parte aérea, en un suelo contaminado con Cd y Zn. Teniendo en cuenta los resultados obtenidos, la variedad D19 de *H. tuberosus* en combinación con el consorcio inoculado puede considerarse una estrategia apropiada para utilizarse en el manejo sostenible de suelos contaminados con metales.

Summary

Bacterial inoculation in *Helianthus tuberosus* for improving phytoremediation of metal-polluted soils

Mining processes and metallurgical industries as well as the use of fungicides and inorganic fertilizers have increased the levels of metal(loid)s in soils. Metals represent a worldwide problem because of their elevated bioaccumulation capacity via food chains, toxicity and persistence in the environment. In addition, their presence in the soil generates a risk of contamination of surface and ground waters. Conventional soil remediation technologies are highly expensive, invasive and may lead to additional environmental problems such as soil degradation. On the other hand, phytotechnologies are sustainable soil remediation techniques that use green plants, microorganisms and amendments to reduce the concentration and bioavailability of contaminants, and also, contribute to a sustainable use of marginal areas.

One of the most critical limitations of using phytotechnologies on metal-contaminated soils is the long time required to clean-up the soil and reach the permitted levels. The use of high biomass crops able to extract metals from soil could compensate the long time required in this technology with the production of valuable biomass.

Brachypodium distachyon (L.) Beauv. is the first pooid grass to be sequenced and has been recently proposed as a model grass for developing new sustainable energy. Increasing the knowledge about metal tolerance and uptake in this new model grass could help to understand the mechanisms of toxicity at molecular level, and genes and proteins involved in the response of plants to high concentrations of metals. The first objective of this work was to study the ability of *B. distachyon* seeds to germinate and grow *in vitro* with Cd, As(V), Zn, or Cr(VI) in comparison with the two well-known metal tolerant energy crops, *Brassica napus* L. and *Helianthus annuus* L. High concentrations of Cd, Zn and As (V) did not affect the seed germination of the studied species. The maximum toxicity level was found in plants treated with Cr (VI). Biomass reduction was only observed at high doses. Taking into account the results, *B. distachyon* showed high capacity to germinate and grow in presence of high doses of Cd, As (V) and specially, of Zn. This suggests that this grass could be a suitable model plant to study energy crops tolerant to this metal, and the mechanisms implicated in the response to Zn stress at molecular level.

On the other hand, *Helianthus tuberosus* L. is a high biomass crop recently proposed as a candidate for use in phytotechnologies on metal polluted soils. This plant species presents agronomic characteristics that could be useful in phytotechnologies such as a high ability of colonization and adaptability to grow in a wide range of soils, including saline and poor soils, and its high resistence to pests and diseases. This crop is propagated by tubers, differently from the other studied crops, which made it difficult to grow under *in vitro* conditions with agar. After successive tests in controlled conditions, the hydroponic conditions with substrate were selected as the most appropriate method to evaluate its ability to grow and accumulate metals. This growth system allowed to hold the tuber and at the same time to develop the root system in controlled conditions.

Plant growth, metal(loid) uptake and the metal(loid)-nutrient interactions of two cultivars of *H. tuberosus* (VR and D19), recognized for their high biomass production were evaluated in this work. For this purpose, three hydroponic experiments with different mixtures of metal(loid)s were performed under greenhouse conditions. D19 accumulated higher concentrations of metals than VR, and showed an effective mobilization of Pb to the above-ground biomass. Although both cultivars showed high capacity to grow in presence of multiple metal(loid)s, D19 showed better characteristics than VR to become a potential candidate for use in phytoextraction.

The main limiting factors to implement phytoextraction in metal-contaminated soils are metal availability and phytotoxicity. The interaction between plant growth-promoting bacteria (PGPB) and plants can enhance biomass production and metal tolerance of the host plant by decreasing phytotoxicity. The second aim was to isolate and characterize the cultivable bacterial community associated with *Brassica napus* growing on a Zn contaminated soil, in order to select cultivable PGPB that might enhance biomass production and metal tolerance of energy crops. A total of 426 morphologically different bacterial strains were isolated from soil, rhizosphere, roots and stems of *B. napus*. Although most of the bacterial strains showed plant-growth promoting characteristics, one strain from bulk soil (*Arthrobacter* sp. 222), one rhizosphere strain (*Staphylococcus* sp. 25) associated with *B. napus*, and four root endophytes (*Pseudomonas* sp. 228, *Serratia* sp. 246, *Pseudomonas* sp. 256, *Pseudomonas* sp. 262) showed the highest production of ACC deaminase activity, siderophores, acetoin, organic acids and capacity to solubilize phosphate and fix nitrogen during the phenotypic test; due to it they were selected to perform the inoculation experiments in the plant. The re-inoculation of *B. napus* seeds with *Arthrobacter* sp. 222, *Pseudomonas* sp. 228, *Serratia* sp. 246,

Pseudomonas sp. 256, *Pseudomonas* sp. 262 improved root growth in the presence of 1000 µM Zn or 300 µM Cd in vertical agar plates.

The five PGP bacterial strains that improved the root growth of *B. napus* seedlings were inoculated in *H. tuberosus* under hydroponic conditions with 0.1mM Cd and 1mM Zn, in order to evaluate their effects on growth, metal uptake and oxidative stress of this crop. The inoculation of *Pseudomonas* sp. 228, *Serratia* sp. 246 and *Pseudomonas* sp. 262 enhanced growth of D19 cultivar-clone in presence of Cd or Zn; and the addition of *Pseudomonas* sp. 228, *Serratia* sp. 246 and *Pseudomonas* sp. 256 decreased the contents of thiobarbituric acid (TBA) reactive compounds in roots of plants exposed to Zn. The improvement of the growth and the decrease of the metal-induced stress that were observed after bacterial inoculation, were more pronounced in D19 cultivar-clone than in VR.

In a further study, the five bacterial strains were inoculated in the cultivar D19 of *H. tuberosus* in a Cd-Zn contaminated to assess their efficiency to improve plant biomass and metal uptake when they have to compete with other microorganisms and metal and nutrients are less available. In this case, the bacterial strains were added individually and as a consortium. The individual inoculation of the strains did not affect *H. tuberosus* growth in soil conditions, but the joint action of the consortium increased the biomass, the Pb and Zn uptake in the roots of *H. tuberosus,* and the activities of malic enzyme and isocitrate dehydrogenase in roots, and of glutathione reductase in leaves. These effects indicate that the bacteria established an interaction with *H. tuberosus*. This interaction was also supported by the egfp-labeled bacteria attached to the root hair surface observed under Confocal Laser Scanning Microscope, and by the stimulation of the root hair development of *H. tuberosus* inoculated with the consortium.

Root endophytic bacteria of *B. napus* affected the root structure of *H. tuberosus,* indicating that the inoculated bacteria can improve plant growth in a species different from their host of origin. *H. tuberosus* accumulated more than 1000 $mg \cdot kg^{-1}$ of Zn in aerial parts from a Cd-Zn contaminated soil. Taking into account the results obtained in this work, *H. tuberosus* D19 in combination with the inoculated consortium could be a suitable strategy to be used in sustainable management of metal contaminated soils.

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Chapter 1

Introduction

1.1. Concern and current status of the metal contaminated soils

Soil is an essential, finite and non-renewable natural resource. With a global population estimated to exceed 9 billion by 2050, compounded by competition for land and water resources and the impact of climate change, our current and future food security hinges on our ability to increase yields and food quality using the soils that are under production today (FAO, 2015). In the International Year of Soils, the FAO suggests the sustainable soil management to retrieve unproductive and degraded soils and transform them into healthy and productive. Healthy soils are the basis for plant growth and biodiversity conservation above and below ground. Moreover, soils help to mitigate the climate change by playing a key role in the carbon cycle, and they store and filter water and improve resilience to climate variability, floods and droughts (FAO, 2015).

Soil health can be affected by external causes as erosion, loss of organic matter (Boardman et al., 1990), loss of structure and biodiversity (Doran and Parkin, 1994) and chemical contamination (Van der Brink, 1995). Soil contamination is nowadays a worldwide environmental problem, which represents also risks for ground and surface waters and food production (Vangronsveld et al., 2009; Mench et al., 2010; Rajaganapathy et al., 2011; FAO, 2015). To assess the potential impact of soil contaminants, it is necessary to take into account their concentration and also their environmental behavior in the soil (European Commission, 2002).

In the European Union the importance of soil protection is recognized in “The Thematic Strategy for the Protection of Soils” (COM, 2006) and within different directives, such as the Directive 2004/34/EC on environmental liability with regard to the prevention and remediation of the environmental damage. This directive established a framework based on the “polluter pays” principle to prevent and remediate the environmental damage. Other EU directives support also the prevention and clean up of contamination, such as the Directive 2008/98/EC relating to waste management and prevention of pollution; Directive 2000/60/EC that establishes a framework in the field

of water policy, including land contamination that causes water pollution; Directive 2002/118/EC on groundwater; Directive 91/676/EEC on nitrates from agricultural sources and Directive 2008/1/EC, concerning integrated pollution prevention and control. According to this last one, industrial and agricultural activities with a high pollution potential should need a permit, which could only be issued if certain environmental conditions were met. Thus, the companies themselves would bear responsibility for preventing and reducing any pollution they might cause. In this context, prevention is an essential approach, taking into account that industrial activities are responsible for over 60% of soil contamination in Europe (Panagos et al., 2013; EEA, 2014). Although the current legislation aims to reduce contamination in the future, there are over 340,000 contaminated sites in the European Union that require urgent remediation actions (EEA, 2014).

Heavy metals and hydrocarbons are the most common pollutants in soils due to anthropogenic activities. In the European Union, heavy metals and mineral oils represent 35% and 24% of the contamination cases, respectively (Figure 1.1) (EEA, 2014). Metals represent a serious problem because of their elevated bioaccumulation capacity via the food chain, and their toxicity and persistence in the environment (Kabata-Pendias, 2011; Garbisu and Alkorta, 2003). Most of them have been described as initiators or promoters of carcinogenic activity in animals (Nriagu, 1988; Järup, L. 2003; Nawrot et al., 2008).

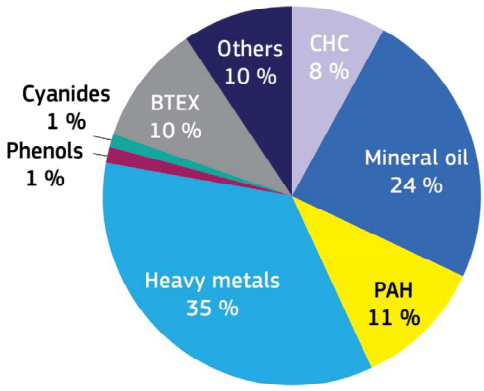

Figure 1.1. Overview of contaminants that affect soils in Europe (EEA, 2014). CHC: Chlorinated Hydrocarbons, PAH: Polycyclic Aromatic Hydrocarbons, BTEX: Aromatic hydrocarbons.

Soil contamination can be diffuse or localized. Diffuse contamination is generally associated with atmospheric deposition, some agricultural practices and inappropriate waste and wastewater recycling and treatment (European Commission, 2006); while localized metal contaminated soils are commonly associated with mining, abandoned industrial sites, accidental release of pollutants or inappropriate waste disposal (Vamerali et al., 2010; Panagos et al., 2013). Mining processes such as coal and metal ore mines, smelting, electroplating and metallurgical industries as well as the use of fungicides, inorganic and phosphate fertilizers have increased the levels of metal(loid)s As, Cd, Cu, Cr, Ni, Pb and Zn in soils (Alloway, 1995; Nagajyoti et al., 2010; Kabir et al., 2012). Cd, Pb and Zn are among the most important metals that cause contamination in soils (Adriano et al., 2004; Vassilev et al., 2004; Gallego et al., 2012). Figure 1.2 shows the concentration of these metals in the topsoil of Europe. Their presence in the environment has been increased at global level during the last decades (Mulligan et al., 2001; Saraswat and Rai, 2011). Due to this, Cd, Pb and Zn have been the subject of numerous works related to soil decontamination until our days, where they still represent an alarming environmental problem (Markus and McBratney, 2001; Broadley et al., 2006; Kabir et al., 2012; Bolan et al., 2013).

Conventional remediation technologies are highly expensive, environmentally invasive and may lead to additional environmental problems such as soil degradation (Pilon-Smits, 2004; Vangronsveld et al., 2009; Conesa et al., 2010). Witters et al. (2012a) estimated that the greenhouse gas emissions, generated by conventional cleanup techniques such as soil vapor extraction or pump and treat, were about 4,700 and 323,456 (total yearly CO_2 emissions), respectively. In spite of these facts, traditional remediation techniques are still the most commonly used for the treatment of contaminated soil in Europe, particularly, the technique of soil excavation and disposal is applied in about 30 % of the sites (EEA, 2014). *In situ* biological techniques represents about 20% in the case of the Netherlands, 15% in Lithuania, 12% in Belgium (Flanders) and France, and 10% in Italy. For the rest of the European countries, including Spain, these technologies are applied on less than 5% of the sites (EEA, 2014). The selection of a remediation alternative depends on the dimension and location of the contamination, soil characteristics, chemical state of the contaminants, the desired final land use as well as environmental and social issues (Mulligan et al., 2001; Vassilev et al., 2004).

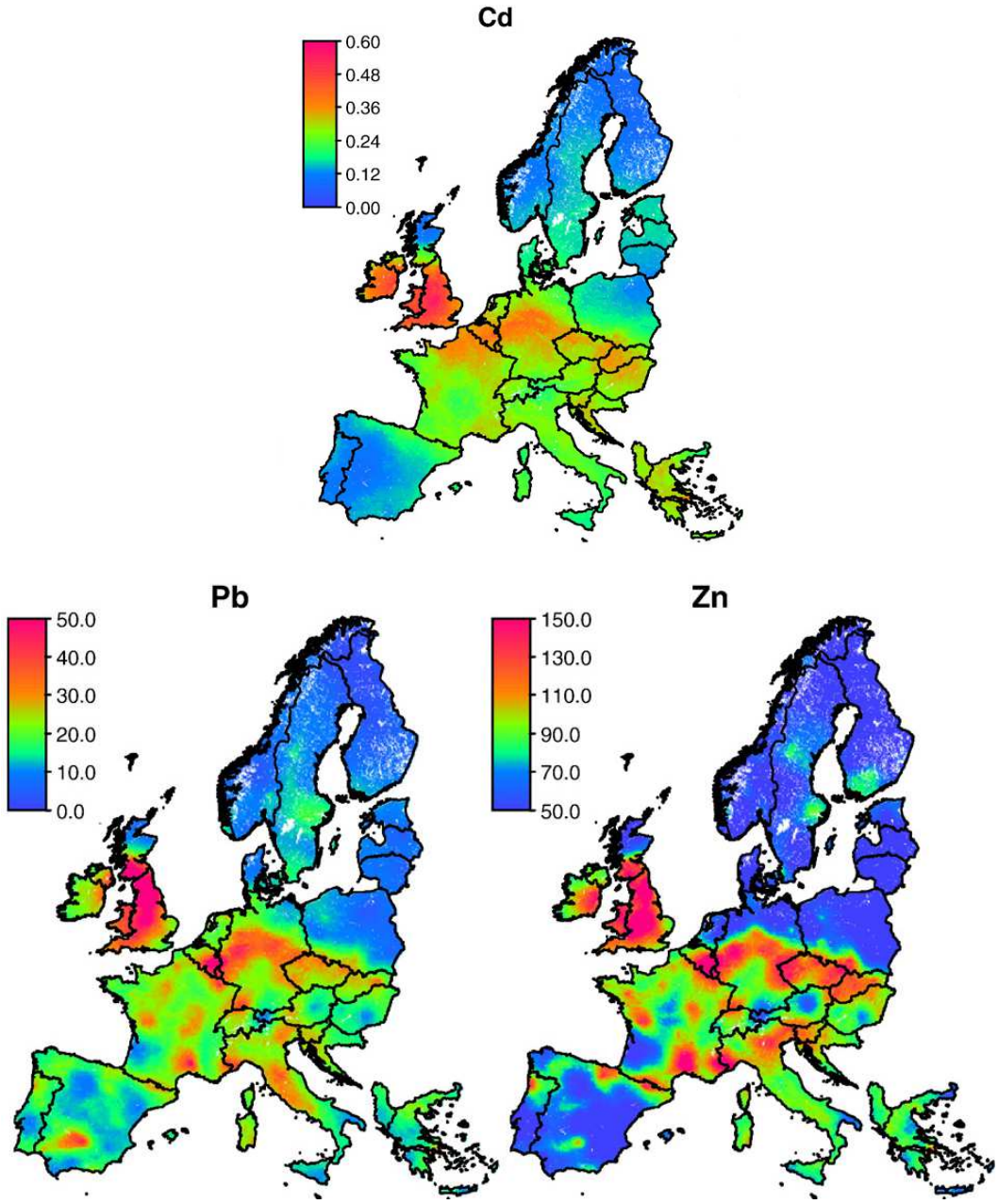

Figure 1.2. Total concentrations of Cd, Pb and Zn in the European topsoil (mg kg^{-1}) (Lado et al., 2008).

In general, remediation alternatives can be grouped in two types of techniques: "site decontamination or clean-up techniques" that remove the contaminants from the soil or "site stabilization techniques" that reduce the availability of the elements (Vangronsveld et al., 2009).

Until now, soil thresholds are based on the total soil metal concentrations in most of existing legislation (Adriano et al., 2004; Mench et al., 2009). Table 1.1 shows the generic reference values adopted in Madrid (Spain) (RD 9/2005, de 14 de enero, Anexo

VII) and Table 1.2 shows the clean-up values adopted in Flanders (Belgium) (VLAREBO, 2009), with regards to metal(oid) concentrations in soils depending on the indented use of the area. The maximum allowed concentration for Cd in agricultural soils is similar in both regions (2 and 3 $mg \cdot kg^{-1}$ in Flanders and Madrid, respectively), however other metals, such as Pb and Zn, show different treshhold values (200 $mg \cdot kg^{-1}$ of Pb, 600 $mg \cdot kg^{-1}$ of Zn in Flanders, and 77 $mg \cdot kg^{-1}$ of Pb, 1170 $mg \cdot kg^{-1}$ of Zn in Madrid). The implementation and unification of soil contamination legislation according to standard criteria is complicated due to the natural differences of the metal contents in soils, and thereby, it must be evaluated considering each type of soil (Reimann and Garrett, 2005). Soil properties such as organic matter content, pH, texture, and CEC differ along geographical areas and they also strongly affect metal availability in the soil (Korte et al., 1976; Kayser et al., 2001). Due to these interactions, the total metal concentration in soils is a useful parameter to indicate the extension of soil contamination, but it does not reflect the risks because the total metal content is not often correlated with its effects to the organisms (Rieuwerts et al., 1998; Vig et al., 2003; Adriano et al., 2004; Meer et al., 2005). In the last decades, metal bioavailability has been suggested as a term which could include these interactions in the definition. In general, metal bioavailability can be defined as the fraction of the total metal content of the soil that can interact with a biological target (Geebelen et al., 2003). Taking this into account, a risk based land management strategy have been proposed based on evaluating the mobility of toxic elements in order to protect public health and environment, even after applying remediation techniques (Witters et al., 2012b; Cundy et al., 2013; Peña-Fernández et al., 2014).

Conventional technologies, although they would be capable of remediating the land within a limited amount of time, tend to destroy soil structure and functionality, which would mean that after remediation the use of that soil would be limited. On the contrary, sustainable soil remediation involves different techniques that present high social acceptance and allow to remediate contaminated sites and water, improvement of food safety, and carbon sequestration as a tool to reduce global warming by maintaining soil properties and structure (Sinha et al., 2007; Robinson et al., 2007; Vangronsveld et al., 2009).

Table 1.1. Generic reference values in Madrid (Spain) as a function of the soil indented use (RD 9/2005, de 14 de enero, Anexo VII, BOE núm. 15, de 18 de enero de 2005).

Element ($mg \cdot kg^{-1}$ DW)	Industrial	Urban	Other uses*
Sb	80	8	0.8
As	40	24	24
Ba	100000	15200	4200
Be	13	2	2
Cd	300	30	3
Co	1500	150	15
Cu	8000	800	80
Cr_{total}	2300	230	90
Sn	100000	46730	46730
Mn	33900	3.90	690
Hg	15	7	5
Mo	1500	150	15
Ni	15600	1 560	405
Ag	500	50	5
Se	3900	390	85
Pb	2700	270	75
Ta	30	3	2
Va	3700	370	37
Zn	100000	11700	1170

*Other uses: agricultural, forestry and livestock.

Table 1.2. Clean-up values ($mg \cdot kg^{-1}$ DW) adopted in Flanders (Belgium) as a function of the soil indented use (VLAREBO, 2009).

Element ($mg \cdot kg^{-1}$ DW)	Natural Park	Agriculture	Residential	Recreational	Industrial
As	45	45	110	200	300
Cd	2	2	6	15	30
Cr^{3+}	130	130	300	500	800
Cu	200	200	400	500	800
Hg	10	10	15	20	30
Ni	100	100	470	550	700
Pb	200	200	400	1500	2500
Zn	600	600	1000	1000	3000

The standard soil sample contains 10% clay, 2% organic matter.

1.2. Sustainable soil remediation: Phytotechnologies

Jaffré et al. (1976), Brooks et al. (1977) and Rascio (1977) were some of the first authors that performed pioneer studies about plants able to accumulate metals from soils. Since then, the potential uses of plants to remediate contaminated soils have been investigated. Phytoextraction is the use of plants to reduce the concentration of toxic metals in soils (Baker, 1981; Chaney, 1997). Nowadays, phytoextraction is grouped

within the term of phytotechnologies (Conesa et al., 2012), which include different strategies that use green plants to remediate *in situ* contaminated soils and represent an alternative to the conventional cleanup techniques, especially in case of large-scale contaminated areas (Pilon-Smits, 2004; Prasad and Freitas, 2003; Vangronsveld et al., 2009). Phytotechnologies are environmentally friendly tools that can improve the food chain safety by reducing the concentration and bioavailability of contaminants, and also, contribute to a more sustainable use of marginal lands maintaining healthy ecosystems (Barceló and Poschenrieder, 2003; Robinson et al., 2003; Schwitzguebel et al., 2009). The increasing interest for this low-cost alternative is reflected in the scientific literature (2040 new results available in the Web of Knowledge in the last five years) and in the numerous experiments that have been performed in field conditions (Marmiroli et al., 2006; Kidd et al., 2009; Bhargava et al., 2012; Conesa et al., 2012; Cundy et al., 2013).

Phytoremediation of metal contaminated soils can be approached with two basic strategies: phytoextraction and phytostabilization (Krämer, 2005). Phytoextraction implies that the contaminant is removed from a specific area, using plants able to accumulate metals in the harvestable parts (Kumar et al., 1995; Salt et al., 1998). Phytostabilization reduces the metal bioavailability and its transfer to the environment through the combined use of plants and soil amendments (Vangronsveld and Cunningham, 1998). Phytoextraction is a suitable alternative in areas with diffuse contamination (Kidd et al., 2009), where metals are superficially presented at a relatively low-medium concentration (Cunningham and Berti, 1993; McGrath et al., 2002). Phytostabilization has to be strictly associated with an adequate monitoring of the contaminated area in order to assure the long-term stabilization (Vassilev et al., 2004; Ruttens et al., 2006). For that reason, several authors consider that phytostabilization should not be the final solution for a contaminated site (McGrath and Zhao, 2003; Vangronsveld et al., 2009). But its use as a step before performing a more definitive remediation would reduce the risk to transfer the contamination to other compartments (Vangronsveld et al., 1995; Mench et al., 2000; Raskin and Ensley, 2000), especially in large areas with high and multi-elemental contamination (Kidd et al., 2009). The established vegetation cover can also reduce the wind blowing of metal-contaminated soil as dust particulates, which also represents a remarkable risk for human health and the surroundings areas (Johnson et al., 1992; Dickinson et al., 2009)

and also contributes to soil aggregation and binding of contaminants (Stomp et al., 1993; Pulford and Watson, 2003).

1.2.1. Application of phytoextraction

To develop an effective phytoextraction, the plant is required to tolerate the concentration(s) of metals accumulated and also, to present fast growth and high accumulation in the above ground biomass (Ebbs and Kochian, 1997; Maxted et al., 2007). Hyperaccumulator plants are able to grow in soils with elevated concentrations of metals and concentrate high levels of metal in their above-ground biomass (Reeves and Brooks, 1983; Pollard et al., 2014). However, most of hyperaccumulators show a low-yield and biomass production (Cunningham and Lee, 1995; Dickinson et al., 2009; Brunetti et al., 2011). The need to obtain an economic resource using this alternative leads to develop different strategies such as phytomining (Brooks et al., 1998) and agro-mining (van der Ent et al., 2015), by means of which the metal could be recycled (McGrath and Zhao, 2003). Phytomining of Ni has shown more potential than other metals (Chaney et al., 2007) due to the high bioavailability of this metal in soils, the high percentages of ultramafic areas in the world (Reeves and Baker, 2000) and the relatively high market price of Ni. *Alyssum murale, A. corsicum* and *Berkheya coddii* have been proposed as effective Ni-hyperaccumulator plants to be used in phytomining of Ni-rich soils that are inappropriate for food and feed production (Dickinson et al., 2009; Bani et al., 2015; van der Ent et al., 2015). Also other species have been mentioned due to their hyperaccumulator ability such as *Arabidopsis halleri*, *Thlaspi caerulescens, Sedum alfredii* for Zn and Cd, and *Pteris vittata* for As, but their use in phytomining is still poorly studied (Lombi et al., 2000; Bert et al., 2002; Pollard et al., 2014).

Biofortification of mineral micronutrients in food crops for the benefit of human nutrition and phytoremediation of metal/metalloid contaminated soils represent another strategy that allow to obtain a valuable resource from phytoextraction (Zhao and McGrath, 2009). Micronutrients such as Fe, Zn and Se are deficient in many diets (White and Broadley, 2005). Recent studies have shown that wild cereal crops, in particular cereals such as *Triticum monococcum* var. *monococcum*, *T. turgidum* var. dicoccum, *T. aestivum* var. *spelta*, *Aegilops tauschii* are able to retain high

concentrations of Fe, Zn and Se in the grain (Lyons et al., 2005; Li et al., 2008). The use of these crops can improve human health and the agricultural productivity (Graham et al., 2007). However, medical tests, toxicity assessment, and appropriate dosages are needed before the biofortified products can be made available for the population (Zhao and McGrath, 2009; Conesa et al., 2012).

The use of high biomass crops tolerant to metals in phytotechnologies is another alternative, and it is known as phytomanagement (Bañuelos et al., 1995; Madejón et al., 2003; Robinson et al., 2007; Domínguez et al., 2008). This allows to obtain renewable energy resources, and at the same time, to remediate metal-contaminated sites that cannot longer be used for food or feed production (Licht et al., 2005; Mlezeck et al., 2010; Witters et al., 2012b). Energy crops are sometimes grown on soils that still can be used for food production (Field et al., 2008); and this could be avoided by assigning the use of contaminated marginal lands as landfills or brown-fields for bio-energy production (Robinson et al., 2003; French et al., 2006). The cultivation of energy crops also could help to mitigate risks as leaching, run-off or erosion (Fässler et al., 2010; Pulford and Watson, 2003) and open new CO_2 abating perspectives (Marmiroli et al., 2006; Dickinson et al., 2009; Witters et al., 2012a; Cundy et al., 2013). Furthermore, these crops can easily be cultivated using established agronomic techniques, which decreases the cultivation cost (Garbisu and Alkorta, 2001). Several energy crops have been proposed as effective phytoextractors to be used on metal contaminated soils. Some of the most competent crops due to their high accumulation capacity are commented as follows. Zn concentrations higher than 1000 mg kg^{-1} have been reported in high biomass crops such as *Brassica* spp. (Marchiol et al., 2004), *Phaseolus vulgaris* (Luo et al., 2005) and *Zea mays* L. (Fellet et al., 2007) grown in metal contaminated soils. Cu concentrations above 500 mg kg^{-1} have been found in *Zea mays*, *Phaseolus vulgaris* (Luo et al., 2008) and *Sorghum bicolor* (Marchiol et al., 2007). Concentrations of Pb greater than 1000 mg kg^{-1} have been described for *Festuca* spp. (Álvarez et al., 2003) and *Medicago sativa* (Pajuelo et al., 2007). Cd concentrations around 50 mg kg^{-1} have been reached in *Zea mays* and several species from Fabaceae and Brassicaceae (Luo et al., 2005). The metal concentrations reached by these crops demonstrate their potential to be used in phytoextraction (Vamerali et al., 2010).

One of the most critical limitations of using phytotechnologies for remediation of metal contaminated soils is the long time required to clean-up the soil and reach the

appropriate thresholds (Mulligan et al., 2001). The production of valuable biomass crops able to extract metals from useless soils could compensate for the long time required by this technology (Van Ginneken et al., 2007; Fässler et al., 2010; Thewys et al., 2010). The promotion of the energy use from renewable sources is one of the main interests of the European Union.

The Directive 2009/28/EC establishes a common framework for the use of energy from renewable sources in order to limit greenhouse gas emissions and to promote cleaner transport. The directive takes into account energy from biofuels produced using raw materials from outside or within the Community but, they should not be produced using raw materials from land with high biodiversity value or with high carbon stock. Their use should contribute to reduce at least 35 % of greenhouse gas emissions. From 1 January 2017, their share in emissions savings should be increased to 50 %. In this context, the exploitation of renewable energy from polluted areas with low ecological value is especially desirable.

1.2.2. Improvement of phytoextraction

Metal availability, uptake and phytotoxicity are the main limiting factors during the application of phytoextraction in metal-contaminated soils (Meers et al., 2008; Weyens et al., 2009b). Phytoextraction of metals can be assisted by two basic strategies: (1) through the addition of chelating-agents that present high affinity for metals and can increase the metal bioavailability (Vangronsveld and Cunningham, 1998; Nowack et al., 2006) and (2) by using plant-associated bacteria that are able to induce metal mobility by different mechanisms such as organic acids or siderophores production (Germida et al., 1998; Glick et al., 2010).

It is well known that the addition of ethylenediaminetetraacetic acid (EDTA) enhances phytoextration (Meers et al., 2005), but due to its high persistence in the environment, toxicity and leaching to the groundwater, other biodegradable chelating-agents have been evaluated such as fresh manure (Walker et al., 2003), [S,S]-ethylenediaminedisuccinic acid (EDDS) (Nowack et al., 2006), iminodisuccinic acid (IDSA), methylglycine diacetic acid (MGDA), and nitrilotriacetic acid (NTA) (Tandy et al., 2004; Fässler et al., 2010). The effectiveness of chelating-agents depends on the

plant species, metals and their respective concentrations in the soil soluble phase (Mench et al., 2009; Mench et al., 2010). Crops like *Salix viminalis* (Klang-Westin et al., 2003), *Brassica juncea* (Do Nascimento and Xing, 2006), *Zea mays* (Kos et al., 2003; Shilev et al., 2007) and *Helianthus annuus* (Solhi et al., 2005) have been proposed as suitable crops to be used in combination with a wide range of chelating-agents due to their high tolerance to metals such as Cd, Pb and Zn. In spite of the high tolerance of these crops, their biomass production decreases after chelate additions due to an increased phytotoxicity, which reduce the volume of valuable biomass (Meers et al., 2008; Neugschwandtner et al., 2008).

As mentioned above, another strategy to improve phytoextraction is the use of plant-associated bacteria. Differently to the use of chelating-agents that are mainly focused on increasing metal uptake; beneficial bacteria also offer the capacity to enhance plant growth and tolerance to toxic metals by decreasing phytotoxicity (Germida et al., 1998; Genrich et al., 2000; Rajkumar et al., 2012). Plant-associated bacteria that have beneficial effects on plant growth were grouped under the name of plant growth-promoting bacteria (PGPB) by Kloepper and Schroth (1978). PGP bacteria form part of the soil microorganisms, but they are mainly present in the rhizosphere, since they are strongly attracted by the nutrient-rich components secreted by the roots (Glick, 2003; Compant et al., 2010). Some microorganisms from the rhizosphere are pathogens for plants (James and Olivares, 1997) but some others, such as PGP bacteria, are able to enhance plant growth (Germida et al., 1998).

Certain PGP bacteria have the capacity to colonize the internal tissues of plants without causing infection or negative effects on their host plant (Misaghi and Donndelinger, 1990; Kloepper and Beauchamp, 1992). These bacteria, known under the name of endophytes, establish endosymbiotic relations with the plant, where the plant receives an ecological benefit from the presence of the symbiont (Quispel, 1992; Lodewyckx et al., 2002). The endophytic relation with the host plant can be in some cases extremely close, and can be transferred to consecutive generations via seeds (Mastretta et al., 2009; Truyens et al., 2015). The inoculation of PGP bacteria in energy crops could stimulate plant growth, increase yield, reduce pathogen infection, as well as reduce biotic or abiotic plant stress, to finally improve the phytoextraction efficiency (Welbaum et al., 2004; van Loon et al., 2005).

1.2.2.1. Bacterial mechanisms to improve plant growth and tolerance

Long-term polluted soils are sources of metal-tolerant microorganisms that show beneficial interactions with the plants that are growing in them (Burd et al., 1998; Gadd, 2010). There are numerous works in which plant-growth promoting (PGP) bacteria have been isolated from plants grown in metal contaminated soils (Ma et al., 2009; Glick et al., 2010; Zhang et al., 2012; Becerra-Castro et al., 2012; Croes et al., 2013). PGP bacteria can improve plant growth through two strategies (Figure 1.3): (1) indirectly by preventing the growth and/or activity of plant pathogens through competition for the niche, nutrients or secreting antibiotics substances or fungal cell wall lysing enzymes (Lugtenberg and Kamilova, 2009; Glick, 2010) or (2) directly by increasing nutrient uptake and growth through different mechanisms such as nitrogen fixation, synthesis of phytohormones (such as auxins, cytokinins, gibberellins, indole-3-acetic acid), solubilization of minerals, and production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick et al., 2003; Rajkumar et al., 2009).

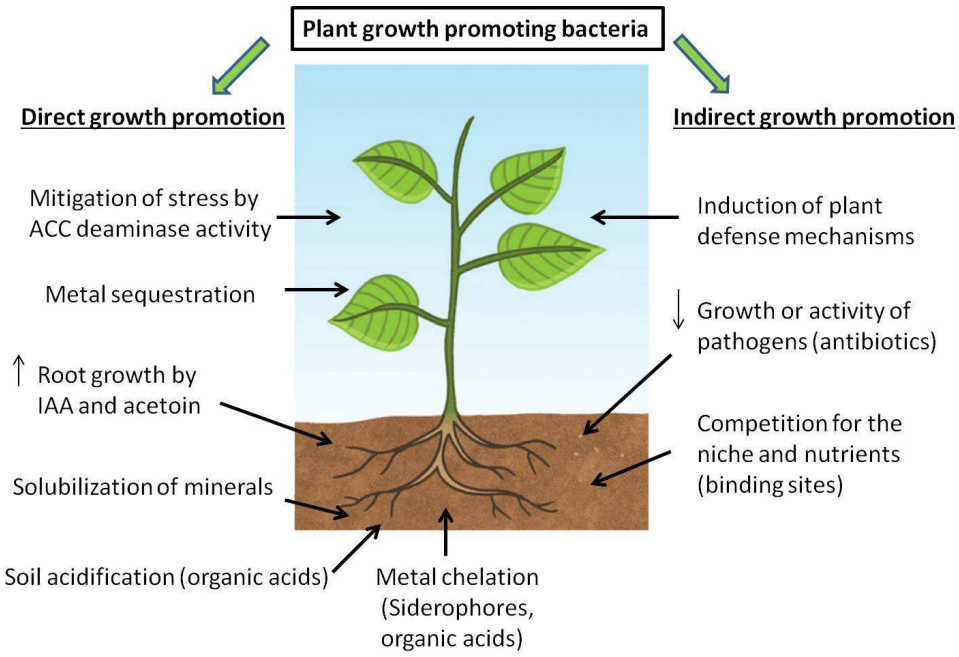

Figure 1.3. Enhance of growth and metal uptake by plant growth-promoting bacteria (Modified from Weyens et al., 2009 and Sessitsch et al., 2013).

Competition of bacteria with pathogens for nutrients and niches in the rhizosphere has been suggested as a possible indirect mechanism to improve plant growth, but the

experimental proof still is lacking (Backman and Sikora, 2008; Lugtenberg and Kamilova, 2009). There are some studies of indirect mechanisms that have demonstrated the importance of siderophores production of *Pseudomonas* spp. in the inhibition of fungal and bacterial pathogens through the competition for Fe (Schippers et al., 1987) and the wide range of antiobiotic substances produced by *Bacillus* spp. that can inhibit the growth of the surrounding bacteria (Ryu et al., 2003). Some plant-associated bacteria can also produce hydrolytic enzymes that cause cell wall lysis, and can be used to control fungal pathogens (Compant et al., 2005). For example, *Pseudomonas fluorescens* produce viscosinamide and tensin, which show a remarkable antifungal activity (Thrane et al., 2000); also, *Streptomyces* sp. produce oligomycin, an inhibitor of the fungal plant pathogen *Phytophthora capsici* that affects several crops (Kim et al., 1999). By using these mechanisms, bacteria compete in the rhizosphere with a wide range of microorganisms for space and nutrients at the root surface, and at the same time protect the plant against pathogens that can affect plant growth and development (Haas and Défago, 2005; Raaijmakers et al., 2009).

Nitrogen, phosphorus and iron are limiting factors in plant growth (Clarkson, 1985; Hopkins, 1995; Hayat et al., 2010). The presence of the enzyme nitrogenase in PGP bacteria is one of the most important bacterial direct mechanisms to improve plant growth. This enzyme can contribute to supply nitrogen required to the plant by fixing atmospheric nitrogen (Roper and Ladha, 1995). Some other PGP bacteria are also able to produce organic acids or phosphatases which solubilize unavailable forms of phosphorus and other nutrients present in the soil by decreasing the pH in the rhizosphere (Kim et al., 1998; Igual et al., 2001). As mentioned above, iron is one of the nutrients that it is often not available for the plant at the required concentrations (Bar-Ness et al., 1991; Dell'Amico et al., 2005). The plant-associated bacteria can solubilize and sequester iron from the soil by producing siderophores, and make this element available for the plant (Glick and Bashan, 1997; Ryan et al., 2008; Barry and Challi, 2009).

The presence of toxic metal concentrations and other types of stress like high salt concentrations or phytopathogens induce elevated ethylene levels in plants, which reduce root growth and development (Glick, 2010; Schellingen et al., 2014). Some PGP bacteria produce the enzyme ACC deaminase which can decrease the ethylene levels in the stressed plant by metabolizing 1-aminocyclopropane-1-carboxylate. This compound

is the precursor of the gaseous hormone ethylene in plants (Yang and Hoffman, 1984; Jacobson et al., 1994). In this way, bacteria able to produce ACC deaminase can reduce the phytotoxicity, due to toxic metals, by enhancing the plant growth in these conditions (Glick, 1998; Belimov et al., 2001; Dell'Amico et al., 2008).

In addition, certain PGP bacteria show the capacity to produce indole-3-acetic acid (IAA). IAA is a phytohormone that has been connected with the enhancement of the growth of lateral and adventitious roots. This increase in root surface can improve the nutrient uptake of the plant, and also the root exudation that stimulates bacterial proliferation on the roots (Dobbelaere et al., 1999). Acetoin (3-hydroxy-2-butanone) production is also present in some plant-associated bacteria. Recently, this volatile compound has been described as stimulator of root development (Glick et al., 2010; Duan et. al., 2013). Similar to IAA, acetoin can increase the root surface, improve the plant nutrient uptake and thereby, the plant growth under metal-stress.

Interesting results have shown that inoculation with specific PGP bacteria isolated from contaminated soils with metals such as Cd and Zn (Sheng et al., 2006; Burd et al., 2000; Dell' Amico et al., 2008; Guo et al., 2011; Islam et al., 2014) can improve plant growth in the presence of both metals. Since PGP bacteria can enhance plant growth by different mechanisms that are acting at the same time, it is difficult to know which mechanism is behind the positive effects on the growth or metal uptake. Several studies have reported the improvement of plant growth after PGP bacterial inoculation. The inoculation of *Pseudomonas* sp. RJ10 and *Bacillus* sp. RJ16, both IAA producers, improved *Brassica napus* growth in presence of Cd (Sheng et al., 2006). Burd et al. (2000) inoculated *Kluyvera ascorbata* SUD165 and a mutant able to overproduce siderophores (*Kluyvera ascorbata* SUD165/26) in canola plants exposed to Zn. They observed that the addition of both strains increased the zinc-inhibited level of chlorophyll, with the siderophore overproducer having the greater effect. The authors suggested that this strain could protect the plant against the inhibitory effect of the metal by providing sufficient iron to the plant. Dell'Amico et al. (2008) reported that the inhibitory effects of Cd were significantly reduced in *Brassica napus* plants due to the inoculation with three bacterial strains able to produce IAA, siderophores and ACC deaminase activity. The biomass production of *Sorghum bicolor* was also increased in the presence of Cd after inoculation of a *Bacillus* sp. SLS18 able to produce siderophores, ACC deaminasa activity and IAA (Luo et al., 2012). The authors

concluded that all of these bacterial mechanisms were involved in the increase of plant growth, by reducing metal phytotoxicity. Mastretta et al. (2009) also observed the positive effect of the seed endophytic bacterial inoculation in the improvement of biomass production of *Nicotiana tabacum* exposed to Cd.

1.2.2.2. Effect of plant-associated bacteria on the metal bioavailabity

Plant-associated bacteria can increase metal availability by excreting siderophores, trace element-chelating organic acids or biosurfactants (Ma et al., 2009; Rajkumar et al., 2012; Sessitsch et al., 2013). Siderophores produced by bacteria can improve plant growth by increasing the Fe concentration in the plant. Siderophores have a higher affinity for Fe(III) than for other elements (Diels et al., 1999). But also, they can increase the plant metal uptake, by forming complexes of low stability with metals as Zn, Cu, Cd (Glick and Bashan, 1997). In this way, the siderophores produced by bacteria can enhance the metal availability to the plants, improving phytoextraction efficiency (Bar-Ness et al., 1991; van der Lelie et al., 2000). Dimkpa et al. (2009) reported that the Cd and Fe uptake increased in *Helianthus annuus* inoculated with *Streptomyces tendae* F4 due to siderophores production. Sheng et al. (2008a) also found an increase in the Pb uptake by *Brassica napus* after inoculation with *Pseudomonas fluorescens* G10 and *Microbacterium* sp. G16 able to produce IAA, ACC deaminase and siderophores. They suggested that the enhancement of Pb uptake could be due to an increase in the Pb solubility, and at the same time, to an enhancement of plant growth by the production of IAA, siderophores and ACC deaminase activity that protect the plant against the inhibitory effects of high concentrations of Pb.

The production of organic acids by bacteria is another mechanism that can improve the plant metal uptake, because of decreasing the pH in the rhizosphere, the metal availability for the plants increases (Ström, 1997; Jones, 1998). Moreover, organic acids have received a special interest because they can bind metal ions in the soil solution and thus increase their uptake by plants; thereby, they can play an important role in the mobilization of toxic and essential ions (Saravanan et al., 2007; Li et al., 2010). The stability of the metal-organic acid complexes depends on several soil properties such as organic matter, pH, metal characteristics and nature of the organic acids (Ryan et al., 2001). Among the wide variety of organic acids produced by plant-associated bacteria,

gluconic acid, oxalic acid and citric acid have been the most extensively studied due to their well-known capacity to increase the metal bioavailability (Fasim et al., 2002; Hoberg et al., 2005). Li et al. (2010) isolated *Burkholderia cepacia* from the Cd hyperaccumulator *Sedum alfredii*, and observed that this strain mobilized Cd and Zn in the soil, leading to an increased concentration of these metals in the water-soluble fraction. The authors attributed this effect to the organic acids produced by the bacteria that decreased the soil pH. Wani et al. (2007) studied the mobilization of Pb and Zn by adding three *Bacillus* sp. strains. They observed that bacteria increased the Pb and Zn solubilization in nutrient broth medium, possibly due to the production of organic acids.

Biosurfactants are other important metabolites that can improve metal mobilization, but they have been less studied than organic acids and siderophores (Rajkumar et al., 2012). Biosurfactants are amphiphilic molecules produced by microbes that form complexes with metals at the soil interface, desorb metals from the soil matrix and thus increase metal solubility and bioavailability in the soil solution (Venkatesh et al., 2012: Sheng et al., 2008b). Juwarkar et al. (2007) studied the metal mobilization of *Pseudomonas aeruginosa* BS2 in a column experiment, and reported the production of a biosurfactant (dirhamnolipid) that increase the solubilization of Cd and Pb in a metal contaminated soil. Sheng et al. (2008b) reported that the application of the biosurfactant-producing *Bacillus* sp. J119 significantly enhanced the biomass and the Cd uptake of tomato plants in a soil spiked with Cd.

In contrast, some microorganisms associated with plants can decrease the mobility of metals in the rhizosphere through the production of extracellular polymeric substances, mucopolysaccarides and proteins, or by binding metals to the cell wall to form hydroxides or some other insoluble metal salts (Gadd et al., 1986; González-Chávez et al., 2004; Joshi and Juwarkar, 2009). In this way, the bacteria can also reduce the phytotoxic effects of the metals by improving plant growth (Madhaiyan et al., 2007; Ma et al., 2011; Rajkumar et al., 2009). Several authors have observed this effect in different plants and growth conditions. Marques et al. (2013) observed that the Cd and Zn uptake decreased in roots of *Helianthus annuus* after inoculation with *Chrysiobacterium humi,* previously isolated from a Cd-Zn contaminated soil. This effect was attributed to the fact that some bacteria can share the metal load with the plant, thereby decreasing the metal uptake. Tripathi et al. (2005) observed that the inoculation of *Pseudomonas putida* KNP9 enhanced the biomass of *Phaseolus vulgaris*

grown on a soil spiked with Cd and Pb by decreasing the Cd and Pb uptake in the plant. Vivas et al. (2006) reported that the inoculation of *Brevibacillus* sp. alleviated the toxicity of Zn in *Trifolium repens* by reducing the metal uptake by the plant in a Zn contaminated soil. Aafi et al. (2012) reported that the inoculation with *Serratia* sp. MSMC541 decreased the metal translocation of *Lupinus luteus* in a soil spiked with As, Cd, Pb and Zn. The authors concluded that this strain could protect the plant against metal toxicity, and remarked its potential in phytostabilization of metal contaminated soils.

1.2.2.3. Application and viability of plant growth-promoting bacteria in phytotechnologies

As was mentioned above, several results have shown the potential use of plant growth-promoting bacteria to improve biomass and metal uptake under controlled conditions, both laboratory and greenhouse. In soil conditions, the effectiveness of bacterial inoculation is frequently reduced, mainly due to the competition with other soil microorganisms, but also to the buffer capacity of soils (Compant et al., 2010; Weyens et al. 2010; Lebeau et al., 2011). Only a few studies have evaluated the effectiveness of the bacterial inoculation in field conditions. Yang et al. (2012) obtained very promising results after bacterial inoculation in the field. They reported that addition of As-reducing bacterial strains promoted the growth of *Pteris vittata* and increased the As uptake of this plant in an As-polluted soil by mobilizing the insoluble As forms. Brunetti et al. (2011) observed that the inoculation of *Bacillus licheniformis* BLMB1 strain enhanced Cr and Pb accumulation in shoots of *B. napus* in field conditions. Juwarkar and Jambhulkar (2008) reported that the amendment of Effluent Treatment Plant sludge (ETP sludge) with bio-fertilizer application (including *Rhizobium* and *Azotobacter* species) to mine spoil enhanced the the root development and biomass of *Delbergia sisoo*, *Cassia saemea* and *Tectona grandis*. They observed that this treatment also helped in the improvement of microbiological characteristics for establishment of a better rhizosphere for the plant growth. The addition of organic amendments improves the physico-chemical properties of the soil, and also provides an organic substrate for the proliferation of the inoculated microorganisms (Kumar et al., 2007; Mrozik and Piotrowska-Seget, 2010). This strategy could improve the survival of bacteria which can

enhance plant growth in contaminated soils and thereby, the concentration of metals extracted by the plant.

The colonization, survival and activity are the most limiting factors when bacteria are inoculated in soil conditions (Lugtenberg and Kamilova, 2009; Weyens et al., 2009a,b). The effect of each bacterial inoculation can vary as a consequence of the plant, root exudates and soil properties (Grandlic et al., 2008; de Campos et al., 2013) and also are strongly affected by the competition with indigenous microorganisms (van Veen et al., 1997; Gentry et al., 2004). Besides the competition with other microorganisms, the effectiveness of the inoculation depends also on abiotic stresses, such as fluctuations of temperature, lost of soil moisture, pH, and nutrient availability (van Veen et al., 1997; Mehmannavaz et al., 2001). However, the abiotic characteristics show wide variations depending on the bacteria that are selected to be inoculated (Boopathy, 2000; Hazen and Stahl, 2006). Mrozik and Piotrowska-Seget (2010) emphasized that organic matter is one of the most important soil parameters influencing the effectiveness of inoculation. Thereby, the use of agricultural management that improves the organic matter of the soil is also required to achieve the desired activity of the inoculated microorganisms. In spite of this, further knowledge about survival, steps involved in plant colonization, and compatibility of inoculated microbes and organic amendments are still necessary to use plant growth promoting bacteria in phytotechnologies (Juwarkar and Jambhulkar, 2008; Compant et al., 2010; Lebeau et al., 2011; Rajkumar et al., 2012).

Until now, there is a lack of legislation at European level about the application of bacteria to improve phytoremediation in real conditions. Most of the legislation is focused on the utilization and release to the environment of Genetically Modified Organisms (Directive 2001/18/EC and Directive 2009/41/EC) or in the pathogenicity for humans that present a big number of microorganisms isolated from contaminated soils (Singer et al., 2005; Lebeau et al., 2011). In addition, it is important to note the Directive 98/44/EC on the legal protection of biotechnological inventions, including any material containing genetic information and capable of reproducing itself or being reproduced in a biological system. This directive only addresses the management of patents of biotechnological inventions, but does not authorize the holder to implement or use that invention. The implementation will be regulated by legislation with regards to public health, safety, environmental protection, animal welfare, the preservation of genetic diversity and compliance with certain ethical standards. It is important to note

that there are some projects funded by the European Commission as UMBRELLA (CORDIS, 2013) or GREENLAND that include different plant-based strategies to remediate trace element contaminated soils, including biotechnological approaches as microbial inoculants (Cundy et al., 2013). These projects also provide guidelines for practical application of green technologies that can help to implement the appropriate future legislation.

Although promising advances are being conducted, further research is still necessary to understand the mechanisms of metal-bacteria-plant interactions that take place in the rhizosphere (Dell'Amico et al., 2008; Wenzel, 2009; Glick et al., 2010; Zhang et al., 2011; Chen et al., 2013), and also to evaluate the environmental effects of the dissemination of microorganisms in field conditions, in order to ensure sustainable implementation of this technology (Gentry et al., 2004; Lebeau et al., 2011; Sessitsch et al., 2013). Since phytotoxicity is still a limiting factor in the use of energy crops in phytotechnologies, the addition of organic amendments that can improve the proliferation of the inoculated microorganisms could be a promising strategy to increase the biomass production and at the same time remove gradually the metal concentration in the soil (Kumar et al., 2007; Mrozik and Piotrowska-Seget, 2010). Another possible alternative to increase the inoculation efficiency of plant-associated bacteria is to genetically equip the bacteria with pathways for the synthesis of natural metal chelators, such as citric acid, to increase metal availability for plant uptake or with metal sequestration systems to reduce phytotoxicity and increase metal translocation to aerial plant parts (Sessitsch et al., 2008; Weyens et al., 2009; Ma et al., 2011; Weyens et al., 2013).

1.3. Utilization of *Helianthus tuberosus* in phytotechnologies

1.3.1. General characteristics of the crop

Helianthus tuberosus L. (Asteraceae) is an herbaceous plant (Figure 1.4) that grows as an annual, and is vegetatively propagated by tubers (Serieys et al., 2010). The tubers (Figure 1.5) are formed by thickened branches of underground stems or stolons (Denoroy, 1996). This crop can also be cultivated as a multi-annual from its volunteer re-growth (Hay and Offer, 1992). *H. tuberosus* has 2n=6x=102 chromosomes

corresponding to an alohexaploïd combination of a basic number (n=17). Probably, this species comes from an interspecific cross between *Helianthus* tetraploïd (n=68 chromosomes) and *Helianthus* diploïd (n=34 chromosomes) (Duke et al., 1978).

Figure 1.4. Field collection of *H. tuberosus* cultivar-clones (IMIDRA, Madrid).

Figure 1.5. Tubers of *H. tuberosus*. A: cultivar-clone D19; B: cultivar-clone VR.

It is commonly called Jerusalem artichoke (in english), topinambour (in french) or pataca (in Spanish) (Sánchez-Monge, 1981). Figure 1.6 shows the phenological growth stages of *H. tuberosus.* In the climatic conditions of temperate regions, the "sprouting stage" takes place in March-April, the "stolonization" around May-June and the "flowering stage" (Figure 1.7) in July (in the case of early cultivar-clones, as D19) or in September (in the case of late cultivar-clones, as Violet de Rennes). The "senescence" is frequently associated with the first frosts of the autumn. After maturity the dormancy of the tubers is initiated, which is broken by cold temperature (< 5°C).

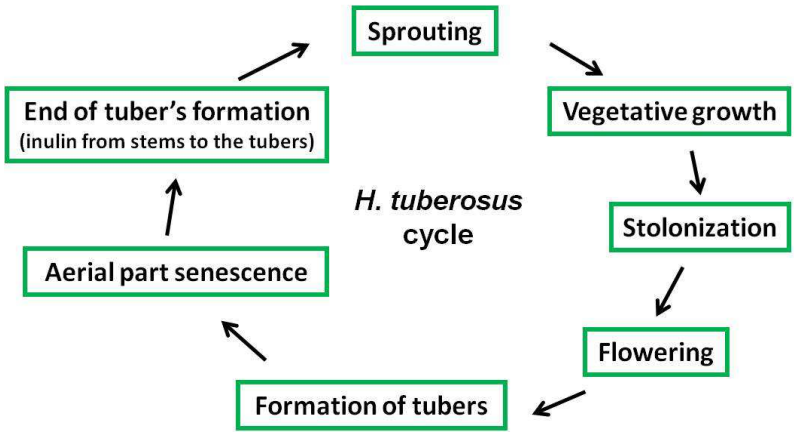

Figure 1.6. Phenological growth stages of *H. tuberosus.*

Figure 1.7. Flowering stage of *H. tuberosus*

H. tuberosus is highly competitive with a high capacity of colonization in the soil in order to capture the resources, which results in repressing the growth of other species (Kays and Nottingham, 2008). This species was considered invasive in numerous European countries, such as Belgium (Belgian Forum on Invasive Species, 2000) and Spain (RD 1628/2011); but in the particular case of Spain, it was declared non-invasive from 2013 (RD 630/2013) due to its increasing interest in bioethanol production at industrial level (Matías et al., 2011; Baldini et al., 2011; Kim and Kim, 2014). Its competitive capacity is related to its high ability to extract nutrients from the soil, especially K and P (Soja et al., 1990) and the efficiency in its use (Zubr and Pedersen,

1993; Sanz-Gallego, 2012). The production costs are low because *H. tuberosus* requires low irrigation and fertilization inputs, as well as shows minimal pest and disease problems (Bajpai and Bajpai, 1991; Denoroy, 1996). Moreover, it is able to grow in saline, poor and alkaline soils (Cosgrove et al., 1991; Long et al., 2010) and resists severe climatic conditions, such as frost or drought (Kim and Kim, 2014). Soil conditions such as pH or texture have slight or no impact on the crop growth (Cors and Falisse, 1980; Denoroy, 1996). These characteristics can be of great interest to produce bio-energy in marginal areas with soils poor in nutrients that cannot be used for food production, thereby avoiding the utilization of healthy soils to produce energy (Robinson et al., 2003; French et al., 2006; Field et al., 2008; Witters et al., 2009). Due to its high ability to propagate, this species has also been used as anti-erosion protection to fix terraces or unstable sand (Denoroy, 1996).

Yields of this crop show a large variability depending on climatic conditions. Total dry weight range from 6-9 (t ha^{-1}) in poor conditions, to 20-30 t ha^{-1} in excellent conditions of radiation and water (Fernández et al., 1991; Zubr and Pedersen, 1993).

Although *H. tuberosus* is native from North America, it was introduced in Europe in 1607, and cultivated especially in France and Germany as consequence of potatoes scarcity during and after the Second World War (Kays and Nottingham, 2008). Nowadays, it is present in almost all Europe, Asia and Australia (Figure 1.8).

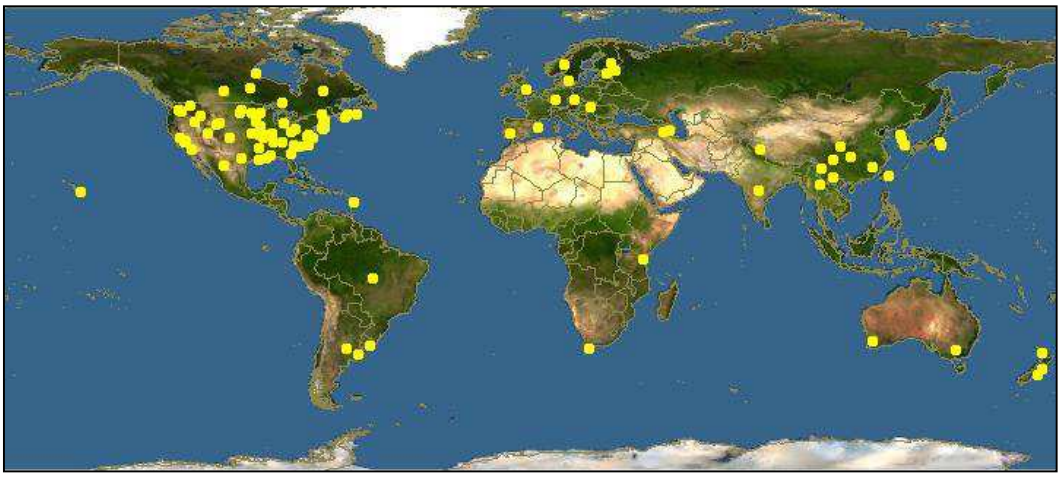

Figure 1.8. Distribution map of *Helianthus tuberosus* L. (Global Biodiversity Information Facility database, GBIF 2013).

1.3.2. Industrial uses

H. tuberosus stores carbon sources as inulin (polymer of fructose), that is used in bio-ethanol production (Rani, 1997; Matías et al., 2011). The initial carbon storage is in the stems (Incoll and Neales, 1970) and after flowering and senescence stages, the carbon is mobilized to the tubers (Figure 1.6), where it is finally stored (Taha et al., 2007; Tassoni et al., 2010). Several studies have determined the metal content in the tubers (Milošević et al., 2012; Sawicka et al., 2013) because the ethanol was traditionally produced from them (Underkofler et al., 1937). Nowadays, some studies have shown the possibility to use also the stems for this aim (Baldini et al., 2003; Curt et al., 2006). Harvesting at the high peak of carbohydrates accumulated in the stems allows to obtain high amounts of fermentable inulin, strongly reducing the costs of the tuber's harvest (Slimestad et al., 2010). Moreover, after harvest, the stems can be stored during several months, while the tubers are quickly affected by microbial activity (Sanz-Gallego, 2012).

This crop has been a model plant in many studies of biochemistry and plant physiology related with the polyamine metabolism and the physiological characteristics during winter dormancy and dormancy breaking of the tubers (Bagni et al., 1983; Serafini-Fracassini et al., 1984; Tassoni et al., 2010). Because of its beneficial nutritional attributes and its use in a wide range of food applications, several studies have been performed in order to enhance the production of inulin under *in vitro* conditions (Rani et al., 1997; Gamburg et al., 1999; Taha et al., 2007). On the other hand, numerous works have studied the carbon distribution in *H. tuberosus*, the agronomic requirements, and its use in bio-ethanol production (Schorr-Galindo and Guiraud, 1997; Somda et al., 1999; Baldini et al., 2003; Curt et al., 2006; Slimestad et al., 2010).

1.3.3. Tolerance to grow in metal contaminated soils

High biomass crops as *Helianthus annuus*, *Brassica* spp., *Nicotiana tabacum*, *Sorghum bicolor* and *Zea mays* have been reported as potential candidates to remediate contaminated soils due to their ability to produce high biomass in the presence of toxic metals (Marchiol et al., 2004; Nouari et al., 2006; Evangelou et al., 2007; Hernández-Allica et al., 2008). *Helianthus tuberosus* L. (Asteraceae) is another high biomass crop recently proposed as candidate to remediate metal contaminated soils, and to obtain a

valuable biomass at the same time. Chen et al. (2011) investigated the Cd tolerance and accumulation of *H. tuberosus* under hydroponic conditions with five doses of Cd (25, 50, 100 or 200 mg L^{-1}). The growth and chlorophyll (chl a, chl b, and total chl) contents were reduced from the lowest dose after 21 days of growth. The phytotoxicity observed occurred as consequence of the high levels of Cd accumulated in the aerial parts (concentrations above 100 mg kg^{-1}). Long et al. (2013) also reported that *H. tuberosus* accumulated 120 mg kg^{-1} of Cd in shoots after 90 days of growing in a soil spiked with 5 mg kg^{-1} of Cd. At this dose, the biomass and chlorophyll content were not reduced in comparison to controls, indicating the tolerance of this crop to this Cd concentration. In a posterior study, Long et al. (2014) characterized the fraction of Cd in the tissues of *H. tuberosus* in a quartz-sand experiment with Hoagland solution and three doses of Cd (2.5, 5.0 and 10 mg L^{-1}). They observed that the increase in Cd concentration in the solution was correlated with an increase in the Cd content of fractions extracted with the following sequential extractants: 80% v/v ethanol, 1M NaCl, deionized water, 2% v/v acetic acid, and 0.6M HCl. The 1M NaCl fraction (correlated with Cd-protein complexes) was high in roots and stems, whereas the 2% v/v acetic acid (correlated with Cd-phosphate complexes) fraction was high in leaves. Based on these results, they proposed that the mobilization from plasma to vacuole after combination with protein may be one of the main mechanisms of Cd-accumulation in *H. tuberosus*.

The tolerance of *H. tuberosus* to other metals such as Cu, Pb and Zn was reported by several authors. Cui et al. (2007) and Chen et al. (2009) collected *H. tuberosus* plants growing in a contaminated soil with 3044 mg kg^{-1} of Pb, 12531 mg kg^{-1} of Cu and 9161 mg kg^{-1} of Zn. In these conditions, *H. tuberosus* accumulated 430 mg kg^{-1} of Pb in roots and 127 mg kg^{-1} of Pb in shoots; 200 mg kg^{-1} of Zn in roots and 206 mg kg^{-1} of Zn in shoots; 191 mg kg^{-1} of Cu in roots and 21 mg kg^{-1} of Cu in shoots. This plant was not able to translocate Pb or Cu in the studied conditions. Zn was the only metal that showed an effective translocation. Although, *H. tuberosus* did not show high extraction capacity, these authors remarked its high ability to grow in highly Pb, Cu and Zn contaminated soils and its potential as biomass producer in these areas. Pogrzeba et al. (2011) reported that *H. tuberosus* accumulated the highest concentration of Pb in aerial part (90 mg kg^{-1} of Pb) from a low Pb-contaminated soil (547 mg kg^{-1} of Pb) in comparison with other energy crops such as *Miscanthus* sp., *Spartina pectinata* or *Elymus elongatus*. This suggests that *H. tuberosus* presents a higher capacity to extract

this metal from soil than the other studied species, but according to the previous authors this crop did not show an effective translocation of Pb.

In addition, Sas-Nowosielska et al. (2008) described *H. tuberosus* as Hg excluder plant in an Hg-contaminated soil, and found higher numbers of heterotrophic, ammonifying soil bacteria, sulphur-amino acid decomposing bacteria and *Pseudomonas* spp. in its rhizosphere in comparison with the surrounding soil. They suggested that these bacteria could play an important role to bind Hg as Hg-sulphide in the root zone avoiding the *H. tuberosus* metal uptake.

Although some works have shown the capacity of *H. tuberosus* to grow in contaminated soils, its use in phytotechnologies to remediate metal contaminated soils is still in its infancy. The low requirements of this crop, its high adaptability to growth in a wide range of soils, and the previous studies with metals as Cd, Pb, Cu, Zn and Hg, suggest its potential use in phytotechnologies, especially in combination with other strategies such as plant growth promoting bacteria that can improve its metal tolerance as well as its effectiveness to accumulate metals in the aerial part. Using farmland to produce biofuel crops reduces the area available for food crops. This adds pressure to free up more land, *e.g.* through deforestation. Deforestation in itself increases greenhouse gas emissions, which may cancel out part or in some cases even all of the beneficial effects of using biofuels. Recently, the Environmental Committee of the European Parliament endorsed a law where the production of traditional biofuels in healthy soils must be limited and new alternative ways should be used to produce “cleaner biofuels” (Environmental Committee of the European Parliament, 2015). The use of energy crops as *H. tuberosus* in metal contaminated soils that cannot be longer used for food or feed production could be an alternative way to produce bioenergy, avoiding the use of healthy soils and deforestation.

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Chapter 2

Objectives

Phytotechnologies still need further research to improve and optimize their effectiveness and application in field conditions. The use of high biomass crops able to extract metals from soil could compensate the long time required for metal phytoextraction with the production of valuable biomass. The cultivation of these crops can also help to mitigate risks as leaching, run-off or wind-erosion and opens new CO_2 abating perspectives. Energy crops are sometimes grown on soils that still can be used for food production; this could be avoided by assigning the use of contaminated marginal lands to produce bio-energy, using energy crops tolerant to metals, and keep healthy soils for food or feed production. The application of this strategy requires the study of crops tolerant to grow and accumulate metals from contaminated soils, and able to produce high biomass in these conditions. Moreover, the cultivation of these crops should present low production costs and requirements.

Phytoavailability, uptake by the roots, translocation to the aerial parts and phytotoxicity of metals are the main limiting factors for application of phytoextraction on metal-contaminated soils. According to the literature, the growth of metal tolerant energy crops that are effective in removing metals from soil is inhibited in the presence of toxic metals, thereby leading to decreased biomass production and thus also the remediation efficiency. Several studies have shown that the use of plant growth-promoting (PGP) bacteria, isolated from metal contaminated soils, can help to improve growth, tolerance and metal uptake of the plant. PGP bacteria can improve plant growth by different mechanisms such as inhibiting growth and activity of plant pathogens, secreting antibiotics substances or fungal cell wall lysing enzymes, supplying nutrients to the plant, producing phytohormones and improving the plant's intracellular tolerance to metals. On the other hand, PGP bacteria can increase the plant availability and thus metal uptake by forming complexes of lower stability with metals as Cd, Cu, Zn, decreasing the soil pH or producing bacterial surfactants that can mobilize metals. Thereby, the combined use of energy crops and PGP bacteria could be a promising strategy to improve the efficiency of phytoextraction, by increasing biomass production and metal uptake in metal contaminated soils.

Taking this into account, **the final aim of this thesis was to evaluate the viability of different energy crops to be used in metal phytoextraction as well as the effects of plant prowth promoting bacterial strains that can enhance the efficiency of this strategy**.

To reach this final aim, the following secondary objectives were established:

1) To compare the growth and metal accumulation of different energy crops (*Brachypodium distachyon* (L.) Beauv, *Brassica napus* L. and *Helianthus annuus* L.) *in vitro* (**Chapter 3**) and in hydroponic cultures (*Helianthus tuberosus* L., **Chapter 4**) with different metal(loid)s commonly found in contaminated soils.

2) To isolate and characterize cultivable bacterial communities associated with *Brassica napus* L. grown on a Zn contaminated soil and evaluate their effects on root growth (**Chapter 5**).

3) To assess the effects of bacterial inoculation on growth, metal uptake and oxidative stress responses of two cultivars-clones (VR and D19) of *H. tuberosus*, under hydroponics conditions with Cd and Zn (**Chapter 6**).

4) To evaluate the effects of the bacterial inoculation on the growth, metal uptake and the metal-induced stress response of D19 cultivar-clone of *H. tuberosus* in a Cd-Zn contaminated soil (**Chapter 7**).

Chapter 3

Screening of energy crops tolerant to metals: Tolerance and uptake of metals at seedlings level

Montalbán, B., García-Gonzalo, P., Pradas del Real, A. E., Alonso, J., Lobo, M. C., Pérez-Sanz, A. 2014. *Brachypodium distachyon* tolerance to metals under *in vitro* conditions: a comparison with two metal tolerant energy crops. Fresenius Environmental Bulletin 23(9): 2086-2092.

3.1. Abstract

Brachypodium distachyon is the first pooid grass to be sequenced and has been recently proposed as model grass for the development of energy crops. The present work reports data concerning the ability of *B. distachyon* seeds to germinate and grow *in vitro* with Cd (0, 0.05, 0.1, 0.3 mM) or As (0, 0.07, 0.1, 0.4 mM) or Zn (0, 0.3, 1, 1.5 mM) or Cr (0, 0.7, 1.5, 2 mM), in comparison with two metal tolerant energy crops (*Brassica napus* and *Helianthus annuus*). *In vitro* tests provided, within a short time, useful information about metal tolerance in plants. High concentrations of Cd, Zn and As (V) did not affect the seed germination of the studied species. The maximum toxicity level was found in plants treated with Cr (VI). Biomass reduction was only observed at high doses. Taking into account the results, *B. distachyon* showed high capacity to germinate and grow in presence of high doses of Cd, As (V) and specially, of Zn.

Keywords: high biomass crops, metal toxicity, metal uptake, phytotechnologies.

3.2. Introduction

The use of energy crops in phytoextraction would allow to obtain renewable energy sources, through sustainable management of marginal areas with the gradual removal or stabilization of the contaminants in polluted soils (Bhargava et al., 2012). *Brassica napus* L. and *Helianthus annuus* L. are high biomass crops, well-known to display a significant tolerance to heavy metals in soils (Hernández-Allica et al., 2008; Adesodun et al., 2010). There are not many studies about their toxicity to Cr (VI) and As (V), as well as their germination and tolerance to different metals in the early stages of growing. *Brachypodium distachyon* (L.) Beauv. is the first pooid grass to be sequenced and has been recently proposed as a model grass for improving food crops and developing new sustainable energy (Bevan et al., 2010; The International Brachypodium Initiative, 2010). Preliminary studies under *in vitro* conditions showed the high capacity of *B. distachyon* to grow in presence of metals (Montalbán et al., 2012). Knowledge concerning metal tolerance and uptake in this new model grass is required as a first step to perform further studies about the mechanisms of toxicity at molecular level, and genes and proteins involved in plants responses to high concentrations of metals in the medium.

Tests on agar medium allow to realize preliminary studies about the plant tolerance to heavy metals during the first stages of growing, taking into account root and shoot elongation, not only germination rate as a parameter (Peralta et al., 2001). *In vitro* test also allows to select potential plants for phytotechnologies and to distinguish between the plant responses and those derived from native microorganisms that are always present in soils (Reynoso-Cuevas et al., 2008). This work compares the ability of *B. distachyon*, with respect to the ability of two metal tolerant energy crops, to germinate and grow *in vitro* with different doses of Cd, Cr (VI), As (V) or Zn.

3.3. Materials and methods

3.3.1. Multielemental analysis of seeds

Certificated seeds of *Helianthus annuus* L. (Asteraceae) and *Brassica napus* L. cv. Nodari (Brassicaceae) were provided by Syngenta Seeds, and Caussade Semences, respectively. *Brachypodium distachyon* L. (Poaceae) seeds were collected from natural

populations in Pozoblanco (Córdoba, Spain). A multielemental analysis of seeds was carried out to know the basal content of elements in the seeds before starting the *in vitro* assays and evaluate if the element concentrations are appropriated for a normal germination of seeds. The seeds were washed in tap water, rinsed in Millipore water and dried at room temperature. According to Aguilar et al. (2010), 10 mg of seeds of each species were individually ground and transferred (0.5g) into a microwave vessel and mixed with 6 mL of HNO_3 (65% Suprapur®) and 1 mL of HClO (70%, Suprapur®). The mixture was digested in a Microwave reaction system, Multiwave 3000, Anton Paar GmbH, Graz (Austria) at 230 °C during 20 min, according to the applications provided by the manufacturer. After cooling, the digests were filtered (Whatman filter paper nº541) and brought up to a volume of 25 mL with Millipore water. Total Zn was determined using a Sequential Inductively Coupled Plasma Atomic Emission Spectroscopy, Liberty AX, Varian, Victoria (Australia) and Cr, As and Cd were determined using an Atomic Absorption Spectrometer AA240Z equipped with Graphite Tube Atomizer GTA 120, Varian, Victoria (Australia).

3.3.2. Seeds and growth conditions

All seeds were surface sterilized by immersing in 7% calcium hypochlorite plus two drops of Tween 20 for 10 minutes, then washed three times with Millipore water and 70% ethanol for 10 minutes. Finally, seeds were rinsed in sterilized Millipore water. Subsequently, fifteen *H. annuus*, twenty-five *B. distachyon* and thirty-five *B. napus* sterilized seeds were set in each of several mason jars containing 250 ml of agar nutrient medium. According to Peralta et al. (2001), the nutrient medium was a modified Hoagland's solution (pH 5.5): Ca $(NO_3)_2 \cdot 4H_2O$ (0.5 mM), KNO_3 (0.75 mM), $NH_4H_2PO_4$ (0.25 mM), $MgSO_4 \cdot 7H_2O$ (0.06 mM), $MnSO_4 \cdot H_2O$ (0.5 µM), H_3BO_3 (6.25 µM), $(NH_4)_6Mo_7O_4$ (0.125 µM), $CuSO_4 \cdot 5H_2O$ (0.025 µM), $ZnSO_4 \cdot 7H_2O$ (0.5 µM), $NaFe^{III}$ EDTA (5 µM), MES buffer (0.5 µM) and agar for microbiology. The medium was autoclaved and four mason jars per treatment were spiked separately with Cd (0, 0.05, 0.1, 0.3 mM as $CdSO_4 \cdot 8H_2O$) or As (0, 0.07, 0.1, 0.4 mM as $Na_2HAsO_4 \cdot 7H_2O$) or Zn (0, 0.3, 1, 1.5 mM as $ZnSO_4 \cdot 7H_2O$) or Cr (0, 0.7, 1.5, 2 mM as $K_2Cr_2O_7$). Metals were filtered through 0.20 µm pore cellulose acetate syringe filters and added to the medium. The process was performed in a vertical laminar flow cabinet under sterile

conditions. The mason jars were put in a growing chamber (SD-1200 VL, Snijders, Tilburg, Netherlands) at 25ºC, photoperiod 12h and 164.527µmol.m^{-2}.s^{-1} photon irradiance, during 15 days (Figure 3.1).

Figure 3.1. *B. distachyon* and *B. napus* grown *in vitro* with 0, 0.3, 1, 1.5 mM Zn.

3.3.3. Analytical procedures in plant samples

After two weeks, germination rate and length of all seedlings were recorded. Whole seedlings were collected and roots were rinsed briefly in 10 mM sodium ethylenediaminetetraacetic acid (Na_2EDTA). Then, plants were washed twice in deionized water, separated into roots and shoots, weighed and dried for 72 h at 60°C. Subsequently, the dried tissues were individually ground and digested (30 mg) by adding 1mL of HNO_3 (65% Suprapur®) and 1mL of $HClO_4$ (70% Suprapur®). The samples were left overnight and heated at 130ºC for 2 h 30 min in a heating block (Dri-Block, DB3D, Techne). After cooling, the volume of the extracts was adjusted to 25 mL with Millipore water. Metal concentrations were analyzed with a Fast Sequential Atomic Absorption Spectrometer AA240FS, Varian, Victoria (Australia). Calibration curves were made from standard solutions of pure metal ions (Scharlau). Arsenic was determined using an Atomic Atomic Absorption Spectrometer AA240Z equipped with Graphite Tube Atomizer GTA 120, Varian, Victoria (Australia). The quality assurance of the digestion and analytical methods was provided by including blanks and certified reference materials (NCS DC73348 Brush Branches and Leaves, China National Analysis Center for Iron and Steel, and CTA-VTL-2 Virginia Tobacco Leaves, Polish Academy of Sciences and Institute of Nuclear Chemistry and Technology) with every set of samples.

3.3.4. Statistical analysis

Statistical analysis of data was made by using the IBM SPSS Statistics 19.0 software. One-way analysis of variance (ANOVA) and Tukey's test were applied. Differences at $P < 0.05$ level were considered significant.

3.4. Results and discussion

3.4.1. Seed analysis

As the first phase of seedling development depends on the amount of mineral nutrients present in seeds (Vreugdenhil et al., 2004), the concentrations of nutrients and metals in seeds of *B. napus*, *H. annuus* and *B. distachyon* (Table 3.1) were analyzed before setting up *in vitro* tests. The analyzed seeds showed mineral contents, Cd and Cr concentrations similar to the values found in certified seeds commonly used in the biodiesel production (Chaves et al., 2010) and in seeds collected from non-polluted soils (Aguilar et al., 2010; Fatima et al., 2013).

Table 3.1. Macro, micronutrient and metal concentrations in seeds of *B. napus*, *H. annuus* and *B. distachyon*.

	B. napus	*H. annuus*	*B. distachyon*
As ($\mu g\ g^{-1}$)	n.d.	n.d.	n.d.
Cd ($\mu g\ g^{-1}$)	0.038 ± 0.006	0.14 ± 0.01	0.10 ± 0.01
Cr ($\mu g\ g^{-1}$)	0.4 ± 0.1	7.4 ± 0.4	19 ± 4
Cu ($mg\ Kg^{-1}$)	74 ± 4	41 ± 5	9.03 ± 0.06
Fe ($mg\ Kg^{-1}$)	92 ± 7	91 ± 9	185 ± 29
Mn ($mg\ Kg^{-1}$)	36 ± 2	20 ± 2	17 ± 1
Zn ($mg\ Kg^{-1}$)	43 ± 2	80 ± 7	33 ± 2
Na ($mg\ Kg^{-1}$)	42 ± 2	25 ± 5	80 ± 5
Ca (%)	0.26 ± 0.02	0.12 ± 0.02	0.16 ± 0.05
K (%)	0.61 ± 0.03	0.71 ± 0.03	0.53 ± 0.03
Mg (%)	0.23 ± 0.01	0.28 ± 0.03	0.138 ± 0.005

Mean values ± SE; n=4. n.d., not detected. As $_{LD} < 0.005\ \mu g\ g^{-1}$

3.4.2. Germination rate

The metal toxicity during the seed germination is a good indicator of the possible tolerance of the adult plant in some species (Buendia-González et al., 2010). Other studies have suggested that germination could be unaffected but subsequent growth could be significantly diminished (Reynoso-Cuevas et al., 2008). The three species showed high germination rates in presence of the studied metals, except for Cr (VI) (Table 3.2). This metal reduced germination rate at high doses, and it was lethal to *H. annuus* seeds. These results are in agreement with Fozia et al. (2008), who observed an important reduction in the germination rate of *H. annuus* from 1.2 mM of Cr (VI), when seeds were germinated in Petri dishes.

Table 3.2. Germination of seeds *in vitro* conditions with Cd, As (V), Zn or Cr (VI).

Metal	Dose (mM)	Germination rate (%)		
		B. distachyon	*B. napus*	*H. annuus*
Zn	0	97 ± 5ns	95 ± 2ab	83 ± 8ns
	0.3	84 ± 7	92 ± 2ab	95 ± 3
	1	90 ± 3	99 ± 2b	90 ± 8
	1.5	89 ± 4	87 ± 1a	93 ± 3
Cd	0	97 ± 5ns	95 ± 2ns	72 ± 3ns
	0.05	95 ± 2	91 ± 1	78 ± 9
	0.1	95 ± 1	91 ± 1	60 ± 11
	0.3	96 ± 2	98 ± 1	67 ± 7
As (V)	0	97 ± 5b	95 ± 2ns	85 ± 6b
	0.07	93 ± 3ab	91 ± 0	88 ± 6b
	0.1	86 ± 3a	81 ± 2	78 ± 7ab
	0.4	83 ± 4a	90 ± 3	53 ± 8a
Cr (VI)	0	97 ± 5c	95 ± 2c	95
	0.7	96 ± 3c	83 ± 1b	0
	1.5	64 ± 7b	74 ± 2ab	0
	2	32 ± 8a	65 ± 1a	0

Different letters mean significant differences after one-way ANOVA and Tukey's test ($P < 0.05$; mean values ± SE; n=4) n.s, means not significant. (Modified from Montalbán et al., 2012).

The toxic effects of Cr (VI) on plant development included alterations in the germination processes as well as in the growth of roots and aerial parts (Hayat et al., 2012). Peralta et al. (2001) also found that 0.7 mM of Cr (VI) reduced the seed

germination of *Medicago sativa* seeds by 50%, in similar conditions to this work. In our results, *B. napus* and *B. distachyon* did not reduce their germination rate in presence of 0.7 mM of Cr (VI). This indicates a high tolerance to this metal during germination, but finally, the length of roots and shoots were reduced drastically after two weeks of growth. This high tolerance during the germination phase could be explained because the seed is a stage in the plant life cycle that is well protected against stresses. However, after imbibition and subsequent vegetative developmental processes, they become stress-sensitive (Hayat et al., 2012). Our results are in agreement with Li et al. (2005), in which the studied seeds still germinated in the presence of high metal concentrations, but the subsequent seedling growth was severely inhibited at much lower concentrations of these heavy metals.

3.4.3. Metal toxicity to seedlings growth

Biomass, root and shoot length were the parameters used to evaluate metal toxicity. In general, these parameters decreased in a dose dependent manner in all the species, but the effect was different depending on metal and plant species. In the case of *H. annuus* growth with Cr (VI), there were no values of elongation, biomass and uptake, because this metal inhibited the germination in the doses tested.

B. distachyon showed high tolerance to 1 mM of Zn in the medium (Figure 3.2, 3.3). The shoot elongation remained constant at the different doses of Zn, and root length and biomass obtained were not significantly different from control values (Figure 3.4a). Indeed, *B. distachyon* accumulated more Zn in aerial part than *B. napus* and *H. annuus* (Table 3.3), without toxicity symptoms. On the other hand, there was a maximum of growth when Zn concentration was 0.3 mM. In this case, the root length significantly increased compared to the control. Peralta et al. (2001) also found a positive effect of Zn in roots of *Medicago sativa* seedlings under *in vitro* conditions. This fact could be explained because Zn is a micronutrient and low increments of this metal increase plant growth.

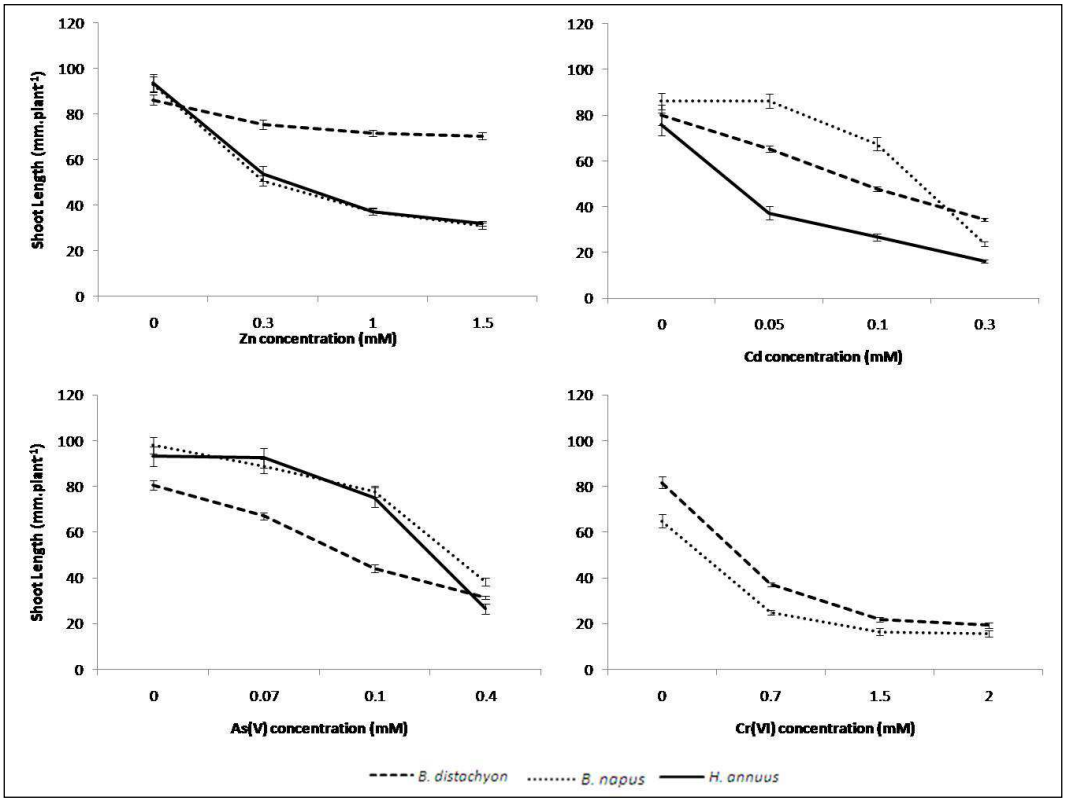

Figure 3.2. Shoot of seedlings after two weeks growing *in vitro* with different metals and doses. Vertical bars represent standard errors.

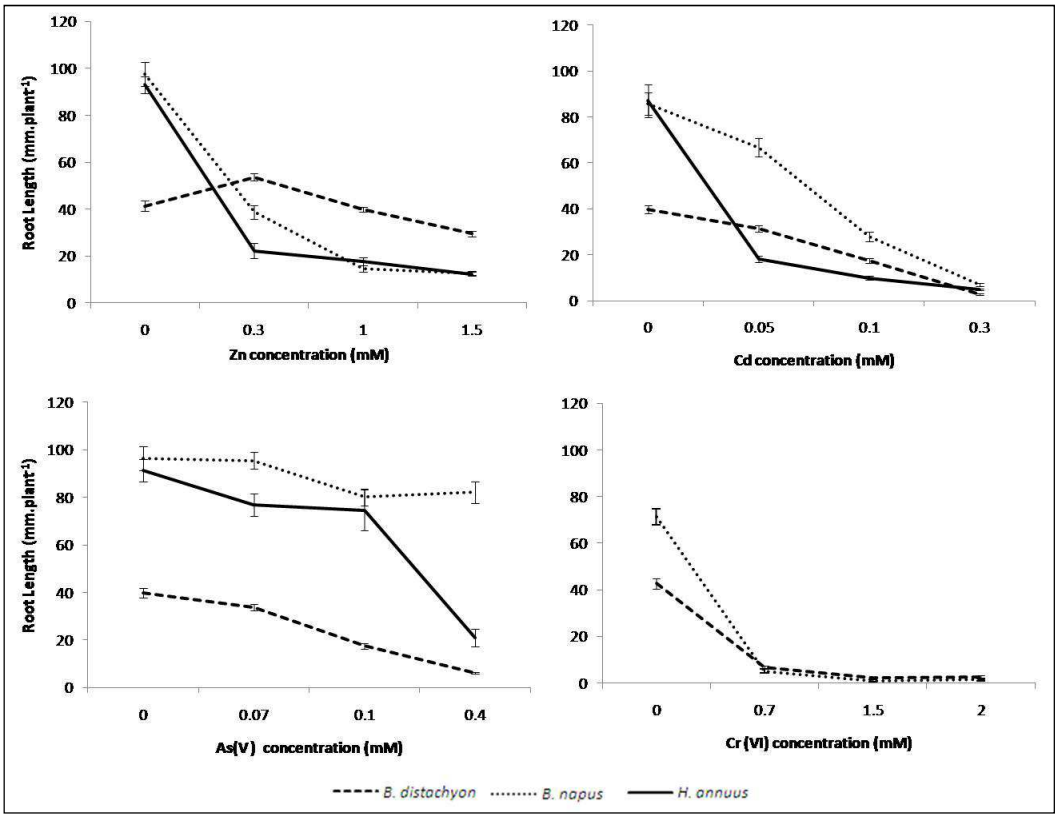

Figure 3.3. Root of seedlings after two weeks growing *in vitro* with different metals and doses. Vertical bars represent standard errors.

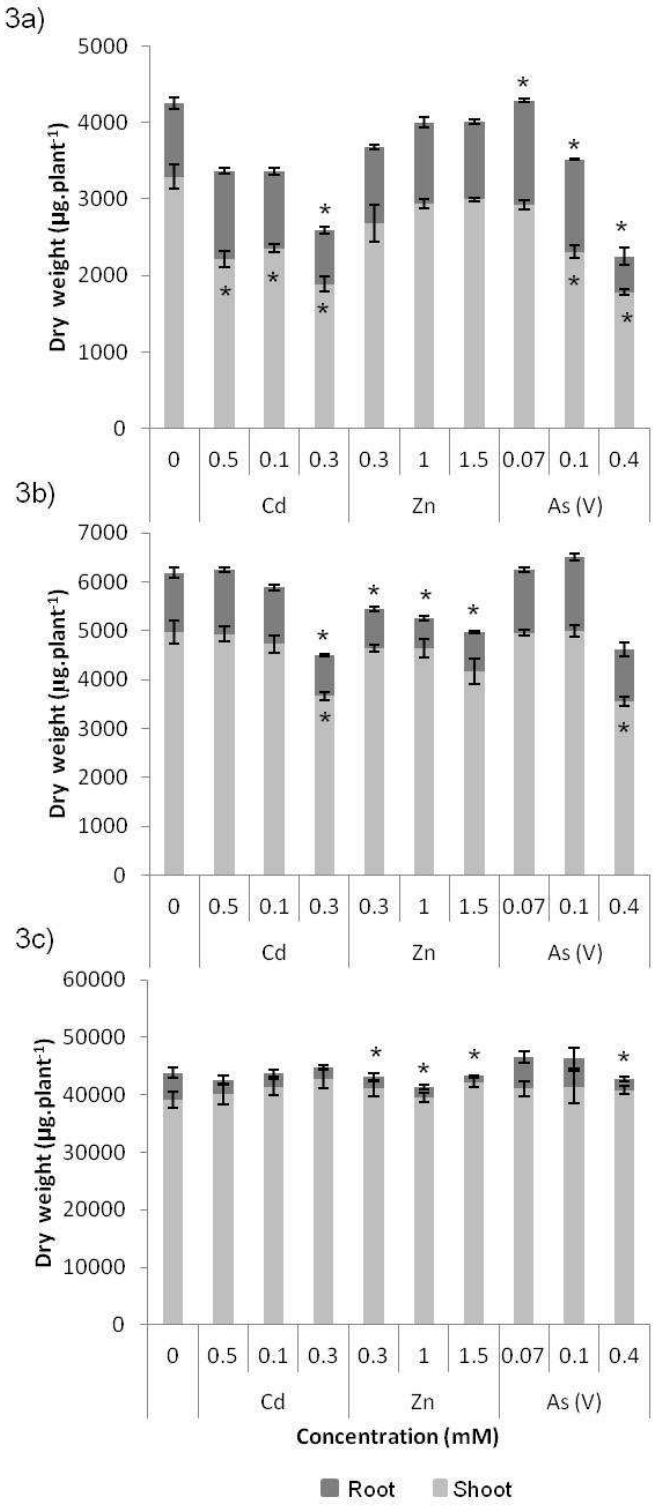

Figure 3.4. Aerial and root biomass obtained after two weeks in presence of metals. 4a) *B. distachyon;* 4b) *B. napus*; 4c) *H. annuus*. Aterisks (*) represent significant differences between the control and doses ($p < 0.05$; mean values ± SE; n=4). Vertical bars represent standard errors.

The shoot and root elongation of *B. napus* and *H. annuus* drastically decreased at the lowest dose of Zn (Figure 3.2, 3.3). The reduction of *B. napus* and *H. annuus* growth in presence of Zn was described by Bernhard et al. (2005) and Hernandez-Allica et al. (2008), under hydroponic conditions. Recent reports showed that an excess of Zn could cause a loss of total chlorophyll, disorganization of the chloroplast and reduce the numbers of thylakoids and grana, hence, the growth could be severely affected (Wang et al., 2009). The maximum toxicity level was found in plants exposed to Cr (VI). The presence of Cr (VI) affected drastically the root and shoot length of *B. distachyon* and *B. napus*, reducing the elongation from the lowest dose (Figure 3.5a, b). However, *B. napus* showed good results in the presence of Cd (Figure 3.6). Its biomass was only reduced in the highest dose (Figure 3.4b), being not different to the control in the other doses, and it was the plant that accumulated the highest concentrations of this metal in aerial parts, with respect to *H. annuus* and *B. distachyon* (Table 3.3).

Table 3.3. Concentrations of Cd, Zn and As in the studied species after 15 days of growth *in vitro* conditions with different metals.

		Shoot metal uptake (mg.kg^{-1} DW)			Root metal uptake (mg.kg^{-1} DW)		
Dose (mM)		*B. distachyon*	*B. napus*	*H. annuus*	*B. distachyon*	*B. napus*	*H. annuus*
Zn	0	42 ± 7a	105 ± 11a	202 ± 28a	153 ± 91a	77 ± 8a	78 ± 19a
	0.3	1065 ± 132b	1504 ± 82c	855 ± 471b	5377 ± 697b	7888 ± 349b	1667 ± 224b
	1	1605 ± 53b	1418 ± 200c	813 ± 260b	8176 ± 419c	12621 ± 1132c	2305 ± 215bc
	1.5	1520 ± 196b	1143 ± 163b	1471 ± 355b	8582 ± 294c	11301 ± 490c	3011 ± 377c
Cd	0	0a	2.2 ± 0.2a	0a	0a	4.7 ± 0.2a	0a
	0.05	28 ± 5b	699 ± 31b	13 ± 3b	824 ± 75b	3418 ± 60b	311 ± 40b
	0.1	27 ± 3b	654 ± 75b	119 ±24c	827 ± 248b	4945 ± 173c	436 ± 35b
	0.3	86 ± 5c	838 ± 235b	604 ± 85d	1360 ± 314b	7236 ± 115d	1573 ± 275c
As (V)	0	0a	0a	0a	0a	0a	0a
	0.07	17 ± 6b	174 ± 27b	107 ± 8b	757 ± 29b	4999 ± 1563b	215 ± 83b
	0.1	48 ± 13b	186 ± 29b	119 ± 4b	809 ± 49b	8773 ± 2236b	405 ± 119bc
	0.4	466 ± 26c	1228 ± 202c	340 ± 61c	12068 ± 4131c	8686 ± 1146b	663 ± 98c

Different letters mean significant differences after ANOVA one-way and Tukey's test ($P < 0.05$; mean values ± SE; n=4).

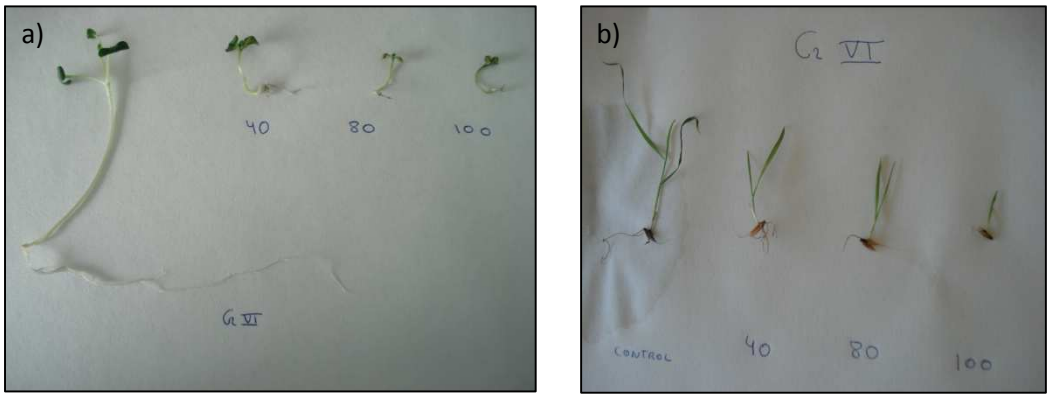

Figure 3.5. *B. napus* (a) and *B. distachyon* (b) grown with different doses of Cr (VI).

Figure 3.6. *B. napus* grown with different doses of Cd.

Many reports suggest that *B. napus* can be a useful candidate for phytoextraction of Cd due to its high above ground biomass in presence of this metal, faster growth and high uptake values (Nouairi et al., 2009; Selvam et al., 2009). *B. distachyon* and *H. annuus* showed also tolerance to Cd concentrations studied, as shown by the biomass values which were not drastically reduced with increasing the doses in medium (Figure 3.4a, c). *H. annuus* plants showed a significant reduction of shoot and root elongation in presence of low doses of Cd, according to Azevedo et al. (2005) and Groppa et al. (2008), but in our study the aerial biomass obtained after two weeks was not affected for any Cd concentrations.

In presence of As (V), *B. distachyon* and *B. napus* showed a progressive reduction in their shoot length with increasing the concentration in the medium (Figure 3.2). In case of *H. annuus*, we did not find significant differences in shoot length between 0 and 0.07 mM of As (V), thereby showing to have more tolerance to As (V) in medium than the

other species. None of the metals affected the aerial biomass of *H. annuus* (Figure 3.4c), suggesting its tolerance to these metals during early development. The high toxicity of As (V) did not affect the root elongation of *H. annuus* and *B. napus* at 0.1 and 0.4 mM, respectively (Figure 3.3). Reductions in germination rate and roots length in response to arsenic exposure has been reported in other metal tolerant plants (Groppa et al., 2008; Gomez et al., 2013). Very low concentrations of As (V) could be beneficial for plant growth, but with increasing concentration, As (V) becomes toxic for plants, causing chlorosis, necrosis, inhibition of growth and finally death (Gulz et al., 2005). Taking this into account, the results of this work report the ability of *B. napus* and *H. annuus* to grow in the presence of high concentrations of As (V), producing in general high values of biomass and uptake in 2-weeks seedlings. These results are in agreement with Liu et al. (2012), who observed a high tolerance of *B. napus* to grow in soil spiked with As (V). More studies about the tolerance mechanisms of these crops in presence of As (V) are necessary in order to evaluate its possible future application as candidates to remediate arsenic polluted lands.

B. napus and *H. annuus* are crops tolerant to Cd, Zn and As; this behavior has been shown in this work under *in vitro* conditions. Considering biomass production, *B. distachyon* showed more sensitive to Cd and As than *B. napus* and *H. annuus*. However, *B. distachyon* showed high tolerance to 1 mM of Zn in the medium. Further studies need to be performed in order to determine the tolerance and toxicity of this new model plant in the presence of these and other metals. A more profound knowledge of this grass might improve the study and application of phytotechnologies in contaminated soils and enable us to produce renewable energy on polluted sites.

3.5. Conclusions

The obtained results show that *in vitro* tests can provide valuable information about the impact of toxic metals on germination, viability and seed metabolism. Because of the high sensitivity of this test, its use as a step prior to soil experiments could help to predict the physiological response of the plant in presence of metals. *B. distachyon* shows mechanisms to avoid metal toxicity during the first stages of growth. This grass species could be a suitable model plant to study energy crops tolerant to metals, and the

mechanisms implicated in the response to metal stress, especially in the presence of high levels of Zn.

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Chapter 4

Metal(loid)s uptake and effects on the growth of *Helianthus tuberosus* cultivar-clones under multi-polluted hydroponic cultures

Montalbán B., Lobo, M. C., Alonso, J., Pérez-Sanz A. 2015. Metal(loid)s uptake and effects on the growth of *Helianthus tuberosus* cultivar-clones under multi-polluted hydroponic cultures. CLEAN-Soil, air, water. DOI: 10.1002/clen.201400630

4.1. Abstract

Helianthus tuberosus is a high biomass crop recently proposed as a candidate for use in phytotechnologies on metal polluted soils. The present work reports data concerning plant growth, metal(loid) uptake and the metal(loid)-nutrient interactions of two cultivar-clones of *H. tuberosus* (VR and D19). Three hydroponic experiments were performed separately: T1: 30 $mg \cdot L^{-1}$ of As(V), Cd, Cr(VI) and Ni; T2: 30 $mg \cdot L^{-1}$ of Cu, Zn, Pb and Cd; T3: 30 $mg \cdot L^{-1}$ of As(V), Cd and Ni. Theoretical estimation of the metal(loid) speciation in the nutrient solution was evaluated by MINTEQA2. The aerial biomass of both cultivar-clones was not significantly reduced with T2 and T3 in comparison to the controls. T1 was the most toxic treatment for *H. tuberosus*, due to the presence of Cr(VI) in the mixture. D19 accumulated higher concentrations of metal in tissues than VR, and showed an effective mobilization of Pb to the stems. Although both cultivar-clones showed high capacity to grow in presence of multiple toxic metal(loid)s, D19 showed better characteristics than VR to become a potential candidate for use in phytotechnologies.

Keywords: Biomass production, Jerusalem artichoke, metal translocation, nutrient status, phytotechnologies.

4.2. Introduction

Industrialization has increased the concentrations of pollutants as metal(loid)s in the biosphere. This presents a global problem for which solutions must be found. Mining processes such as coal mines, smelting, electroplating and metallurgical industries have increased the levels of metal(loid)s As, Cd, Cu, Cr, Ni, Pb and Zn in soils (Kabir et al., 2012). The use of fungicides, inorganic and phosphate fertilizers has also contributed to increase the concentrations of As, Cd, Cr, Ni, Pb and Zn in the surrounding lands of agricultural areas (Nagajyoti et al., 2010). The environmental hazard of metal(loid)s is accentuated by their persistence in the environment and their accumulation in the food chain (Garbisu et al., 2001).

Phytotechnologies are environmentally friendly tools that could improve soil quality by decreasing the concentration and bioavailability of metal(loid)s through the use of plants able to accumulate metal(loid)s in their tissues (Vangronsveld et al., 2001). The utilization of high-biomass crops can help to stabilize or remove toxic metal(loid)s in soils as well as to produce a renewable energy resource on marginal lands that can no longer be used for food and feed production. The long time required to remediate metal polluted soils with plants can be counterbalanced with the abatement of CO_2 and the production of renewable energy with an economic value (Witters et al., 2012). *Helianthus tuberosus* L. (Asteraceae) commonly known as Jerusalem artichoke or topinambur, is a high biomass crop used in bio-ethanol production and vegetatively propagated by tubers (Serieys et al., 2010). Although this crop is native from North America, it is now cultivated in Europe, Asia and Australia. *H. tuberosus* is a salt tolerant plant that can grow in saline, poor and alkaline soils (Long et al., 2010) and is able to resist severe climatic conditions, such as frost or drought (Kim and Kim 2014). Some studies have shown the tolerance of this crop to grow in the presence of metals as Cd, Pb and Zn (Cui et al., 2010; Chen et al., 2011; Long et al., 2013), but its response to multiple metal(loid)s has been poorly investigated. Its cultivation shows low production costs, as well as minimal pest and disease problems (Kays and Nottingham, 2008). All these characteristics make *H. tuberosus* a potential candidate to remediate metal polluted soils, as well as produce a renewable energy resource. The objective of this study was to evaluate the natural ability of two cultivar-clones of *H. tuberosus* (VR and D19) to accumulate metal(loid)s commonly found in polluted sites. Since there are no other studies so far about the effectiveness of this crop to accumulate metal(loid)s as As,

Cu, Cr, Ni, this work provides essential data for evaluating its use in phytoextraction. The experiments were performed in hydroponic culture to maintain all the elements available to the plant, and evaluate the metal interactions that happen during plant uptake. Hydroponic experiments also provide comparable and reproducible data obtained under standardized conditions (Hernández-Allica et al., 2008; January et al., 2008), and avoid the metal retention process that occurs in soils. In order to determine the total concentrations of metals and ligands in the solutions, metal speciation was calculated by using MINTEQA2. Computer programs such as MINTEQA2 have been developed to calculate the equilibrium composition of dilute aqueous solutions and can be used to calculate the distribution of chemical species in natural and synthetic systems. Models such as these predict the chemical form of trace metals in complex media, provided that the chemical composition of the medium is well-defined and valid stability constants for the relevant metal-ligand species are available. Computer modeling of metal speciation in the nutrient solution media, conducted in parallel with plant experiments, may be helpful in interpreting the biological effects of heavy metals (Farrell et al., 1993; Rathnayake et al., 2013).

4.3. Materials and methods

4.3.1. Greenhouse experiment

Tubers of two cultivar-clones of *H. tuberosus* were selected due to their high biomass production (Violet de Rennes shortened as VR, and Blanc précoce commonly called D19). The tubers were collected in May of 2011, in the field collection of IMIDRA (Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario), for use in the experiment (Figure 4.1).

Figure 4.1. Field collection of cultivar-clones of *H. tuberosus* in IMIDRA.

Tuber slices with buds were germinated on humid turf in greenhouse conditions. The temperature during the experiment was 30 ± 4ºC (day) and 20 ± 2 ºC (night). After two weeks, uniform plants (5-7 cm of length) were carefully rinsed in distilled water and transferred to hydroponic culture with coarse perlite and a half strength modified Hoagland's solution (1 mM Ca $(NO_3)_2 \cdot 4H_2O$, 1.5 mM KNO_3, 0.5 mM $NH_4H_2PO_4$, 0.25 mM $MgSO_4 \cdot 7H_2O$, 1 µM $MnSO_4 \cdot H_2O$, 12.5 µM H_3BO_3, 25 µM NaCl, 0.25 µM $(NH_4)_6Mo_7O_4$, 0.05 µM $CuSO_4 \cdot 5H_2O$, 1 µM $ZnSO_4 \cdot 7H_2O$, 10 µM $NaFe^{III}$-EDTA, demineralised water buffered with 1 mM of 2-(*N*-morpholino)ethanesulfonic acid, pH 5.5 ± 0.5).

Three hydroponic experiments were performed separately, each one with a half strength modified Hoagland's solution containing different treatments of metal(loid)s (Figure 4.2). Each metal(loid) was added at 30 $mg \cdot L^{-1}$ concentration. Since there are no studies of *H. tuberosus* in the presence of multiple metal(loid)s under hydroponic conditions, the doses used in these experiments were based on *H. annuus* (January et al., 2008). In the first experiment (Treatment 1, T1), the plants were exposed to As(V) as $Na_2HAsO_4 \cdot 7H_2O$, Cd(II) as $CdSO_4 \cdot 8H_2O$, Cr(VI) as $K_2Cr_2O_7$ and Ni(II) as $NiSO_4 \cdot 6H_2O$. In the second experiment (Treatment 2, T2), the plants were exposed to Cu(II) as $CuSO_4 \cdot 5H_2O$, Zn(II) as $ZnSO_4 \cdot 7H_2O$, Pb(II) as $Pb(NO_3)_2$ and Cd(II) as $CdSO_4 \cdot 8H_2O$. Finally, in the third experiment (Treatment 3, T3), the plants were grown with As(V) as $Na_2HAsO_4 \cdot 7H_2O$, Cd(II) as $CdSO_4 \cdot 8H_2O$ and Ni(II) as $NiSO_4 \cdot 6H_2O$. Control plants were grown only with Hoagland's solution. T1 and T3 were established to evaluate the effectiveness of both cultivar-clones to grow in a multi-polluted mixture from industrial sources, and T2 was set up to mimic pollution from mining. The nutrient solution was replenished daily and completely changed every three days. Aliquots (20 mL) of the nutrient solution were collected before and after each change to check the pH, electrical conductivity and concentrations of metal(loid)s and nutrients. Three plants of each cultivar-clone were set up per tray and four independent trays of each treatment were prepared.

Figure 4.2. Hydroponic experiment with coarse perlite in greenhouse conditions.

4.3.2. Metal speciation in the multi-polluted nutrient solutions

The speciation of metal(loid)s in the multi-polluted nutrient solution was obtained using a software equilibrium speciation model (MINTEQA2 version 3.0 visual basic.NET 2005 compiled by Jon Petter Gustafsson). The variables used to compute metal speciation were pH (buffered with MES) and the total concentration of each component in nutrient solution. The input species were identified as the free metal cations, H^+, and the free ligands. The output data were expressed in terms of activity and percentage of total concentration.

4.3.3. Analytical procedures in plant samples

The plants were harvested after two weeks. Roots were rinsed in 10 mM sodium ethylenediaminetetraacetic acid (Na_2EDTA) and then washed in distilled water. The plants were separated into leaves, stems and roots, then weighed and dried in a forced air oven for 48 h at 60°C. The dry weights were also determined. Subsequently, the dried tissues were individually ground and digested (30 mg) by adding 1mL of HNO_3 (65% Suprapur®) and 1mL of $HClO_4$ (70% Suprapur®). The samples were left overnight and heated at 130ºC for 2 h 30 min in a heating block (Dri-Block, DB3D, Techne). After cooling, the volume of the extracts was adjusted to 25 mL with Millipore water.

Total concentrations of metals and macro/micronutrients were measured in previously acidified samples by flame Atomic Absorption Spectrometer AAS Varian Fast Sequential Model AA240FS. Arsenic was determined using Atomic Absorption Spectrometer Zeeman Atomic AA240Z equipped with Graphite Tube Atomizer GTA120. Calibration curves were made from standard solutions of pure metal ions

(Scharlau). The quality assurance of the digestion and analytical methods was provided by including blanks and certified reference materials (NCS DC73348 Brush Branches and Leaves, China National Analysis Center for Iron and Steel, and CTA-VTL-2 Virginia Tobacco Leaves, Polish Academy of Sciences and Institute of Nuclear Chemistry and Technology) with every set of samples. The recovery percentages were As (~97%), Cd (~95%), Cr (~99%), Cu (~98%), Ni (~110%), Pb (~89%) and Zn (~101%).

4.3.4. Translocation and bioaccumulation factors

The translocation factor (TF) was calculated to determine relative translocation of metal(loid)s from the roots to the aerial parts of the plant (Long et al., 2013):

$TF1 = [metal(loid)]_{stem} / [metal(loid)]_{root}$

$TF2 = [metal(loid)]_{leaf} / [metal(loid)]_{root}$

The bioaccumulation factor (BAF) was also calculated to evaluate the concentration of metal(loid) in the aerial parts with respect to the concentration added to the nutrient solution of each treatment (Sánchez-Pardo and Zornoza, 2014):

$BAF1 = [metal(loid)]_{stem} / [metal(loid)]_{solution}$

$BAF2 = [metal(loid)]_{leaf} / [metal(loid)]_{solution}$

4.3.5. Statistical analysis

Statistical analysis of data was made by using the IBM SPSS Statistics 19.0 software. One-way analysis of variance (ANOVA) and Tukey's test were applied. Differences at $p < 0.05$ levels were considered significant.

4.4. Results and discussion

4.4.1. Estimation of nutrient and metal(loid) concentrations in the multi-polluted solutions

Nutrients and metal(loid)s concentrations were predicted using the visual MINTEQA2 software to quantify the free metal content of each medium (Table 4.1).

Table 4.1. Chemical speciation of metal(loid)s and nutrients in the multi-polluted solutions.

		CONTROL		Treatment 1		Treatment 2		Treatment 3	
		% of total concentration	Activity	% of total concentration	Activity	% of total concentration	Activity	% of total concentration	Activity
Component	Species		µM		µM		µM		µM
Fe^{+3}	$FeEDTA^{-}$	97.1	17.4	63.4	11.2	53.1	9.37	63.4	11.2
	$Fe(OH)^{2+}$	1.25	0.23	20.4	3.60	28.0	4.76	20.5	3.62
	$FeHPO_4^{+}$	0.95	0.17	15.0	2.66	18.9	3.34	15.1	2.67
Cu^{+2}	Cu^{+2}	46.5	0.30	78.3	0.47	78.8	226	78.3	0.46
	$CuEDTA^{-2}$	43.6	0.28	2.92	0.02	1.86	36.1	2.92	0.018
	$CuHPO_4$ (aq)	6.63	0.07	10.1	0.10	9.77	46.2	10.2	0.103
	$CuSO_4$ (aq)	1.78	0.02	6.92	0.007	7.64	5.34	6.72	0.067
Zn^{+2}	Zn^{+2}	93.2	1.20	89.6	1.08	88.9	250	89.5	1.09
Mn^{+2}	Mn^{+2}	93.9	1.20	90.9	1.10	90.3	1.10	90.9	1.10
Cd^{+2}	Cd^{+2}			79.8	130	85.9	141	79.7	131
AsO_4^{-3}	$H_2AsO_4^{-}$			95.4	337			95.5	337
Ni^{+2}	Ni^{+2}			89.7	277			89.7	278
	$NiEDTA^{-2}$			1.40	7.10			1.40	7.10
CrO_4^{-2}	$HCrO_4^{-}$			80.3	425				
Pb^{+2}	Pb^{+2}					76.1	64.8		
	$PbSO_4$ (aq)					15.8	22.2		
	$PbEDTA\text{-}2^{+}$					0.30	0.42		
			mM		mM		mM		mM
K^{+1}	K^{+1}	99.3	2.67	99.0	2.71	99.0	2.62	99.0	2.62
Ca^{+2}	Ca^{+2}	93.4	1.20	88.2	1.07	88.7	1.08	89.3	1.09
Mg^{2+}	Mg^{2+}	96.3	0.31	92.6	0.28	92.2	0.28	92.6	0.28

The metal(loid)s were primarily free in the multi-polluted solutions. The percentages of iron and copper species in solution changed as a consequence of the interactions with other elements. The percentage of iron as Fe-EDTA- decreased in multi-polluted solutions with regards to the control. Ni, Pb and Cd formed organic complexes with EDTA which modified the percentage of Fe, and specially chelated copper in the nutrient solutions. In any case, the concentration of chelated iron in the nutrient solution was sufficient to ensure the plants' nutrition. Other elements as K, Ca and Mg were also estimated because of their role as macronutrient. The free concentration in those media remained relatively constant with the addition of metal to the nutrient solution. Only the percentage of free Ca was slightly lower in multi-polluted solutions than in the control.

The pH and electrical conductivity were determined before and after each change of the nutrient solution to check possible variations in the availability of elements. The pH was maintained at 5.5 ± 0.5 and the electrical conductivity at 1129 ± 160 µS/cm that show that the availability of metal(loid)s and nutrients was constant along the experiment.

4.4.2. Interaction effects of multiple metal(loid)s on plant growth

Biomass production was the parameter used to evaluate the ability of *H. tuberosus* to grow under hydroponic conditions with three different treatments of metal(loid)s. In comparison to the controls, the aerial biomass was not significantly reduced when both cultivar-clones were grown under T2 (Cd, Cu, Pb, Zn) or T3 (As(V), Cd, Ni) (Figure 4.3b,c); in the cultivar VR, the biomass of the stems increased significantly when plants were treated with T3. A significant reduction was observed in the biomass of total plants when both cultivar clones were treated with T1 (As(V), Cd, Cr(VI), Ni) that differs from T3 only by the concentration of Cr(VI) (Figure 4.3a).

In view of the high toxicity found in T1 due to Cr(VI), a new treatment (T3) was set up without this metal to evaluate the tolerance of the other metal(loid)s. Several studies have demonstrated the adverse effect of Cr(III, VI) on plant growth (Singh et al., 2013). The low metal tolerance of sunflower to Cr(VI) was previously reported in other studies (Shahandeh et al., 2000; Montalbán et al., 2014). *H. tuberosus* which is closely related to the sunflower showed the same sensitivity to grow in the presence of Cr(VI), even mixed with other metal(loid)s.

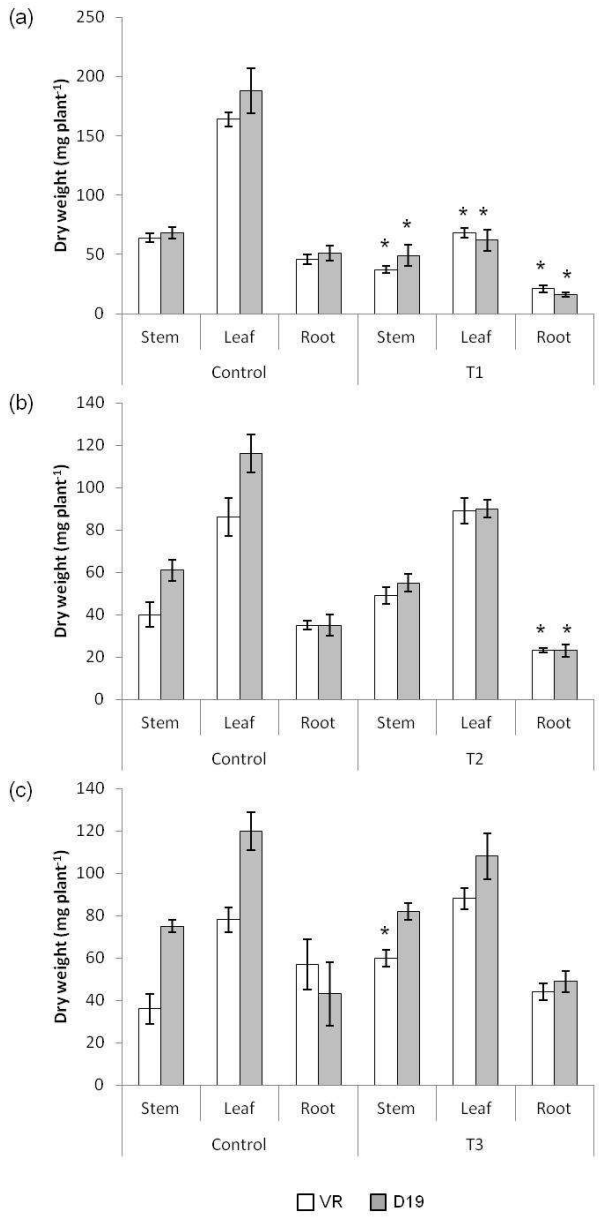

Figure 4.3. Biomass of VR and D19 cultivar-clones under Treatment 1 (a), Treatment 2 (b) or Treatment 3 (c). Asterisks (*) represent significant differences between control and treatments after one-way ANOVA (Tukey's test, $p < 0.05$; mean values ± SE; n=4).

It is well known that Cd is highly phytotoxic even at low concentrations. Chen et al. (2011) observed a significant biomass reduction in all parts of *H. tuberosus* at 5 mg·L^{-1} Cd. The biomass of *H. annuus* was also reduced at 5 mg·L^{-1} of Cd after 15 days of growth (Azevedo et al., 2005). In this work, the biomass was not reduced at 30 mg·L^{-1} of Cd. Apparently, the toxicity of Cd decreased due to the mixture of metal(loid)s, and metal interactions can modify the toxicity of a single metal. Papazoglou et al. (2011)

described the antagonistic effects between Cd and Ni in cardoon plants. Both metals were less toxic for the plant when they were applied in combination rather than individually. The toxicity of Cd, Cu, Pb was also lower in *Cucumis sativus* when the metals were added in ternary combination than separately (An et al., 2004). On the other hand, Sun et al. (2009) showed an antagonistic effect in *Bidens pilosa* grown under co-contamination of As and Cd. The antagonistic effects could take place in T2 (Cd, Cu, Pb, Zn) and T3 (As(V), Cd, Ni), being for that reason less toxic for both cultivar-clones of *H. tuberosus*.

4.4.3. Accumulation of multiple metal(loid)s

Both cultivars were able to accumulate multiple metal(loid)s from the assayed solutions and significant differences were found between cultivars. In general, D19 showed higher concentrations of metal(loid)s in its tissues than VR (Table 4.2). D19 accumulated significantly more Cd, Cr(VI) and Ni in stems under T1, and Cd and As(V) in leaves under T3 than VR. The concentrations of Cd, Cr(VI), Ni and Zn were also higher in roots of D19 than in VR. The relative metal translocation in plants was determined by the TF values. TF values higher than 1 indicate an effective translocation from roots to aerial tissues. In the case of Pb, both cultivar-clones showed TF1 and TF2 values close to 1. D19 showed the highest TF values for this metal (Table 4.2). Pogrzeba et al. (2011) observed the natural ability of *H. tuberosus* to mobilize Pb from roots to the aerial part in soil polluted with Cd, Pb and Zn. Similar results were found in *H. annuus* which also showed a TF value close to 1 at 50 $mg \cdot L^{-1}$ Pb, under hydroponic conditions (Niu et al., 2007). These results suggest that both species have a similar ability to translocate this metal under hydroponic conditions.

The other evaluated elements achieved values lower than 1 of TF1 and TF2, which suggests that the metal(loid)s were retained mainly in the roots. It is important to mention that both cultivar-clones showed TF1 values higher for As than for the other elements (0.57 and 0.68, in VR and D19, respectively), which illustrates the potential capacity of the plant to mobilize this metalloid to the aerial part. The mobility of As and Pb in plants is low and both elements are mainly stored in root cells (Vamerali et al., 2010). Previous studies with *H. annuus* also showed a high capacity to translocate As when the plants were grown under multi-polluted hydroponic cultures with metals as

Cd, Cr and Ni (January et al., 2008). Metal interactions in the root surface could affect the metal uptake by the plant, and once into the plant, translocation and toxicity could be modified (Luo and Rimmer, 1995). January et al. (2008) observed that Cd translocation to aerial parts of *H. annuus* decreased in presence of As, possibly due to competition for phytochelatins. According to them, the presence of Cd in the mixture could increase also the As translocation by *H. tuberosus* under T1 and T3, which explains the high TF1 values found in both cultivar-clones.

The cultivar-clone D19 showed the highest BAF1 values for Cd, Cr(VI) and Ni under T1 (Table 4.2), which indicates a better ability to bioaccumulate these metals in stems with regards to VR. However, VR showed higher BAF1 value for As(V) in T1 than D19. In the case of T2 and T3, the BAF1 and BAF2 values were similar in both cultivars.

The metal selectivity of the plant, based on total metal uptake (Table 4.2), was the same for both cultivar-clones in the case of T1 (Ni > Cd > Cr > As) and T2 (Zn > Cd > Cu > Pb). The selectivity of elements by the plant can be explained through the metal(loid) concentration predicted in the solutions (Table 4.1). Under T1, Ni^{2+} and Cd^{2+} were found as free cations and high concentrations (89.7% and 79.8%, respectively), while, anions such as Cr and As showed the highest activities (425µ for $HCrO_4^-$ and 337µ for $H_2AsO_4^-$). Under T2, the metal selectivity could also be related to the concentrations of cations predicted in the solution, being that Zn, Cd, Cu and Pb were present as Zn^{2+} (89%), Cd^{2+} (80%), Cu^{2+} (79%) and Pb^{2+} (76 %).

In spite of this, complex interactions between metals could affect the accumulation and translocation of metals, through synergistic and antagonistic effects (Beckett et al., 1978). A synergistic effect between Zn and Cu was found by Luo and Rimmer (1995) in spring barley grown on spiked soils with different combinations of metals. On the other hand, Smilde et al. (1992) found antagonism effects between Zn and Cd, since the presence of Zn decreased the Cd uptake by some crops. According to them, the presence of Cu in T2 could help to increase the Zn uptake by *H. tuberosus* and at the same time, the presence of Zn could decrease the Cd absorption.

Table 4.2. Total metal concentrations (mg Kg^{-1} DM) in VR and D19 cultivar-clones of *H. tuberosus*.

Treatments		VR							D19						
		Stem	Leaf	Root	TF1	TF2	BAF1	BAF2	Stem	Leaf	Root	TF1	TF2	BAF1	BAF2
T 1	Cr	94 ± 46a	23 ± 3a	2156 ± 161a	0.04	0.01	3.1	0.8	434 ± 135b	26 ± 5a	2780 ± 104b	0.16	0.01	14.5	0.9
	Ni	257 ± 75a	90 ± 24a	7071 ± 774a	0.04	0.01	8.6	3.0	512 ± 175b	67 ± 8a	8157 ± 733a	0.06	0.01	17.1	2.2
	Cd	68 ± 25a	19 ± 6a	7082 ± 802a	0.01	0.003	2.3	0.6	221 ± 96b	16 ± 3a	7858 ± 929a	0.03	0.002	7.4	0.5
	As	647 ± 87a	150 ± 42a	1138 ± 201a	0.57	0.13	21.6	5.0	349 ± 125b	195 ± 47a	1389 ± 151a	0.25	0.14	11.6	6.5
T 2	Cu	24 ± 6a	18 ± 2a	863 ± 67ab	0.03	0.02	0.8	0.6	18 ± 2a	17 ± 1a	897 ± 40b	0.02	0.02	0.6	0.6
	Cd	63 ± 10ab	33 ± 6a	3320 ± 430ab	0.02	0.01	2.1	1.1	108 ± 34b	27 ± 4a	4075 ± 353b	0.03	0.01	3.6	0.9
	Pb	118 ± 13a	122 ± 6a	142 ± 6a	0.83	0.86	3.9	4.1	127 ± 9a	132 ± 10a	132 ± 11a	0.96	1.00	4.2	4.4
	Zn	166 ± 17a	152 ± 30a	4474 ± 172a	0.04	0.03	5.5	5.1	190 ± 42a	117 ± 19a	5428 ± 318b	0.04	0.02	6.3	3.9
T 3	Ni	1053 ± 78a	86 ± 30a	9645 ± 1058a	0.11	0.01	35.1	2.9	1081 ± 162a	118 ± 7a	11412 ± 553b	0.09	0.01	36.0	3.9
	Cd	512 ± 66a	9 ± 4a	10709 ± 1256a	0.05	0.001	17.1	0.3	497 ± 70a	37 ± 4b	11191 ± 328b	0.04	0.003	16.6	1.2
	As	365 ± 73a	54 ± 11a	760 ± 30b	0.48	0.07	12.2	1.8	403 ± 19a	120 ± 16b	595 ± 97ab	0.68	0.20	13.4	4.0

Different letters represent significant differences per row and cultivar-clone, after one-way ANOVA (Tukey's test, $p < 0.05$; mean values ± SE; n=4). TF: translocation factor. BAF: bioconcentration factor.

Although the metal selectivity was different between cultivar-clones in the case of T3 (Cd > Ni> As for VR, and Ni > Cd > As for D19), the trend of accumulation was similar to T1. Ni and Cd were the most accumulated cations, in spite of the interactions found between metals. These results are in accordance with Chen et al. (2011) that reported the high ability of *H. tuberosus* to accumulate Cd under hydroponic conditions.

4.4.4. Nutrient status

The presence of metal(loid)s affected the nutrient absorption by *H. tuberosus*. In T2, Cu and Zn were not taken into account in the discussion of the nutrient status of the plants, since these metals were added as contaminants. In general, the Cu uptake was higher in plants treated with metal(loid)s than in control plants (Table 4.3), while the Mn uptake showed the opposite trend. Gardea-Torresdey et al. (2004) also observed a reduction in the Mn uptake when *Convolvulus arvensis* was exposed to Cd, Cr(VI) and Cu under *in vitro* conditions. Regarding to Fe and Zn uptake, *H. tuberosus* did not show significant differences between controls and treatments.

In the case of macronutrients, the uptake of Ca was higher in plants treated with metal(loid)s than in control plants, while the concentration of K and Mg was higher in control plants than in plants grown with metal(loid)s (Table 4.4). Gonçalves et al. (2009) also observed a decrease in K and Mg uptake by potato plantlets grown in the presence of 200 µM Cd, under *in vitro* conditions. The polyvalent cations may interfere with the K and Mg uptake (Nagajyoti et al., 2010). For that reason, it is expected that the absorption of these nutrients would be modified in plants treated with metals. No significant differences were found between VR and D19 regarding to macronutrient uptake in control conditions, suggesting than both cultivar-clones have similar nutrient requirements. The differences found between the controls and the treatments were due to the mechanisms used by the plant to maintain the balance of nutrients in presence of the evaluated metal(loid)s. A few studies have been performed about the effect of metals on the nutrient status and mobilization from the root to the aerial parts of *H. tuberosus* and its related *H. annuus* (Ahmad et al., 2011). Different responses of nutrient uptake in other crops have been found among other experiments, depending on the species, mixture of metals, exposure time of the plant to metal or conditions of the experiment (January et al., 2011; Ahmad et al., 2011; Rivelli et al., 2014).

Table 4.3. Total micronutrient concentrations (mg kg^{-1} DM) in VR and D19 cultivar-clones of *H. tuberosus*.

Cultivar-clone		Treatment 1				Treatment 2				Treatment 3			
		Cu	Mn	Fe	Zn	Cu	Mn	Fe	Zn	Cu	Mn	Fe	Zn
VR T	Stem	29 ± 2bc	22 ± 3a	125 ± 25a	113 ± 3a	24 ± 6b	23 ± 1a	125 ± 10a	166 ± 17b	9 ± 2b	25 ± 4a	40 ± 5a	78 ± 12a
	Leaf	33 ± 2b	18 ± 1a	173 ± 20a	105 ± 9a	18 ± 2b	21 ± 1a	139 ± 23a	152 ± 30b	6 ± 1a	23 ± 3a	171 ± 17a	68 ± 15a
	Root	34 ± 3b	34 ± 3a	447 ± 133a	193 ± 12a	863 ± 67b	45 ± 6a	279 ± 58a	4474 ± 172b	33 ± 5b	55 ± 4a	860 ± 103ab	222 ± 12a
VR 0	Stem	21 ± 5ab	38 ± 8b	100 ± 21a	85 ± 6a	4 ± 1a	46 ± 6c	154 ± 18a	28 ± 9a	3.7 ± 0.4a	45 ± 4b	56 ± 4a	29 ± 7a
	Leaf	19 ± 4a	35 ± 5b	180 ± 22a	124 ± 5a	5 ± 1a	32 ± 6b	108 ± 7a	30 ± 6a	2.1 ± 0.2a	30 ± 4a	173 ± 53a	49 ± 8a
	Root	20 ± 6a	55 ± 6b	255 ± 30a	208 ± 24a	7 ± 2a	55 ± 9a	218 ± 17a	52 ± 10a	1.9 ± 0.3a	64 ± 7ab	652 ± 93a	89 ± 19a
D19 T	Stem	33 ± 1c	22 ± 1a	140 ± 17a	110 ± 9a	18 ± 2b	19 ± 2a	158 ± 13a	190 ± 42b	8 ± 2b	31 ± 2ab	71 ± 7a	59 ± 13a
	Leaf	34 ± 2b	24 ± 2ab	182 ± 23a	133 ± 18a	17 ± 1b	16 ± 1a	102 ± 14a	117 ± 19b	6 ± 1a	24 ± 2a	235 ± 25a	77 ± 14a
	Root	40 ± 2b	43 ± 4a	365 ± 35a	210 ± 22a	897 ± 40b	77 ± 13a	604 ± 170a	5428 ± 318c	29 ± 1b	70 ± 3bc	1051 ± 53b	207 ± 15a
D19 0	Stem	17 ± 3a	57 ± 11b	198 ± 74a	234 ± 29a	7 ± 2a	33 ± 2b	149 ± 17a	21 ± 11a	2 ± 1a	115 ± 13c	60 ± 3a	87 ± 32a
	Leaf	16 ± 4a	29 ± 5ab	204 ± 24a	273 ± 7a	5 ± 1a	24 ± 1ab	95 ± 8a	25 ± 8a	5 ± 2a	39 ± 1b	194 ± 14a	71 ± 21a
	Root	19 ± 4a	76 ± 11b	374 ± 49a	247 ± 54a	9 ± 2a	62 ± 3a	309 ± 131a	52 ± 6a	4 ± 2a	102 ± 7c	937 ± 56b	167 ± 60a

Different letters represent significant differences per column, between treated plants (VR T and D19 T) and controls (VR 0 and D19 0), after one-way ANOVA (Tukey's test, $p < 0.05$; mean values ± SE; n=4).

Table 4.4. Total macronutrient concentrations (g $100g^{-1}$ DM) in VR and D19 cultivar-clones of *H. tuberosus.*

Cultivar-clone		Treatment 1			Treatment 2			Treatment 3		
		Ca	K	Mg	Ca	K	Mg	Ca	K	Mg
VR T	Stem	0.14 ± 0.02b	7.7 ± 0.2a	0.11 ± 0.01a	0.09 ± 0.01b	7.7 ± 0.5a	0.14 ± 0.01a	0.13 ± 0.01a	6.4 ± 0.3a	0.16 ± 0.01a
	Leaf	0.18 ± 0.05a	2.2 ± 0.3a	0.27 ± 0.01a	0.18 ± 0.03b	5.6 ± 0.2ab	0.41 ± 0.03a	0.28 ± 0.04c	6.8 ± 0.3a	0.32 ± 0.02a
	Root	0.25 ± 0.04b	2.5 ± 0.2a	0.24 ± 0.02a	0.13 ± 0.01c	4.6 ± 0.2a	0.30 ± 0.02a	0.19 ± 0.04a	1.6 ± 0.2ab	0.16 ± 0.03a
VR 0	Stem	0.06 ± 0.01a	9.0 ± 0.6a	0.17 ± 0.01bc	0.06 ± 0.01a	7.4 ± 0.4a	0.23 ± 0.02b	0.06 ± 0.01a	4.5 ± 1.3a	0.21 ± 0.04ab
	Leaf	0.16 ± 0.02a	8.1 ± 0.3b	0.29 ± 0.01a	0.09 ± 0.01a	6.3 ± 0.3b	0.37 ± 0.04a	0.07 ± 0.01a	6.7 ± 1.8a	0.38 ± 0.02b
	Root	0.04 ± 0.01a	7.0 ± 0.8b	0.26 ± 0.01a	0.06 ± 0.01a	6.1 ± 0.3b	0.32 ± 0.02a	0.19 ± 0.01a	5.8 ± 0.9b	0.26 ± 0.01b
D19 T	Stem	0.08 ± 0.01ab	5.8 ± 0.7a	0.15 ± 0.01ab	0.09 ± 0.01b	6.6 ± 0.6a	0.16 ± 0.01a	0.09 ± 0.04a	5.1 ± 0.4a	0.17 ± 0.01a
	Leaf	0.21 ± 0.03a	4.8 ± 0.2a	0.25 ± 0.02a	0.10 ± 0.01ab	5.1 ± 0.2a	0.31 ± 0.04a	0.22 ± 0.04bc	6.3 ± 0.1a	0.36 ± 0.01b
	Root	0.24 ± 0.02b	1.9 ± 0.3a	0.26 ± 0.02a	0.09 ± 0.01b	4.5 ± 0.2a	0.35 ± 0.02a	0.16 ± 0.02a	1.2 ± 0.1a	0.18 ± 0.01a
D19 0	Stem	0.08 ± 0.01a	5 ± 2a	0.19 ± 0.01c	0.05 ± 0.01a	7.5 ± 0.4a	0.21 ± 0.01ab	0.16 ± 0.04a	4.4 ± 1.2a	0.28 ± 0.03b
	Leaf	0.13 ± 0.03a	7.2 ± 0.2b	0.29 ± 0.02a	0.07 ± 0.01a	5.8 ± 0.2ab	0.31 ± 0.03a	0.09 ± 0.02ab	5.6 ± 1.7a	0.37 ± 0.01b
	Root	0.14 ± 0.03ab	6.5 ± 0.4b	0.26 ± 0.01a	0.07 ± 0.01ab	5.7 ± 0.2b	0.36 ± 0.04a	0.20 ± 0.03a	4.3 ± 1.3b	0.26 ± 0.01b

Different letters represent significant differences per column, between treated plants (VR T and D19 T) and controls (VR 0 and D19 0), after one way ANOVA (Tukey's test, $p < 0.05$; mean values ± SE; n=4).

In general, D19 showed higher concentrations of metal(loid)s in the aerial parts and the roots than VR, but both cultivar-clones showed the same response of nutrient uptake in presence of metals, independent of the treatment. This different response could be due to the presence of a mechanism of VR to avoid the excess of metal(loid)s in the plant, and a better strategy of D19 to tolerate the high levels of these elements in its tissues. The high biomass production of this species in presence of metal(loid)s, in combination with the ability to accumulate them in its tissues, make this plant a promising candidate for use in phytoextraction, especially in the case of Pb, as well as produce a renewable energy resource in polluted sites.

4.5. Conclusions

The results of this work showed that D19 and VR cultivar-clones of *H. tuberosus* did not show a reduced aerial biomass in presence of Cd, Cu, Pb, Zn or As(V), Cd, Ni, added as a mixture of metal(loid)s. Ni, Zn and Cd were the most accumulated metals by both cultivar-clones, but they were mainly retained in the roots. D19 showed an effective mobilization of Pb to the harvestable aerial parts and better capacity to remove metal(loid)s from the studied solutions than VR. Further research must be performed on polluted soils, in order to evaluate the responses of these cultivar-clones to multiple metal(loid)s and their efficiency as an energy crop on polluted sites.

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Chapter 5

Characterization of bacterial communities associated with *Brassica napus* L. growing on a Zn contaminated soil and their effects on root growth

Montalbán, B., Croes, S., Weyens, N., Lobo, M.C., Pérez-Sanz, A., Vangronsveld, J. Characterization of bacterial communities associated with *Brassica napus* L. growing on a Zn contaminated soil and their effects on root growth. Submitted to Environmental Microbiology Reports.

5.1. Abstract

The interaction between plant growth-promoting bacteria (PGPB) and plants can enhance biomass production and metal tolerance of the host plants. The aim of this work was to isolate and characterize the cultivable bacterial community associated with *Brassica napus* growing on a Zn contaminated soil, in order to select cultivable PGPB that might enhance biomass production and metal tolerance of energy crops. The last objective was to evaluate the effect of some of these bacterial strains on root growth of *B. napus* exposed to different concentrations of Zn or Cd. A total of 426 morphologically different bacterial strains were isolated from soil, rhizosphere, roots and stems of *B. napus*. The diversity of the isolated bacterial populations was similar in rhizosphere and roots, but lower in soil and stem compartments. *Burkoholderia*, *Alcaligenes*, *Agrococcus*, *Polaromonas*, *Stenotrophomonas*, *Serratia*, *Microbacterium* and *Caulobacter* were found as root endophytes exclusively. The inoculation of seeds with *Pseudomonas* sp. strains 228 and 256, and *Serratia* sp. strain 246 facilitated the root development of *B. napus* in the presence of 1000 µM Zn, *Arthrobacter* sp. strain 222, *Serratia* sp. strain 246, and *Pseudomonas* sp. 228 and 262 increased the root length in the case of exposure to 300 µM Cd.

Keywords: Plant-associated bacteria, endophytes, inoculation, plant growth-promoting bacteria, bacterial communities.

5.2. Introduction

During the last decades, the number of areas polluted with toxic metals increased due to anthropogenic activities such as mining, metallurgical industries, electroplating, manufacturing of plastics, paint pigments, alloy preparation and batteries, energy and fuel production and application of fertilizers and pesticides (Broadley et al., 2007; Kirkham, 2006). Metals accumulate in the food chain through uptake at primary producer level and subsequently through transfer and bioaccumulation at higher trophic levels (Nagajyot et al., 2010). Phytoextraction is a low cost technology that uses green plants to extract metals from the soil and accumulate them in the harvestable parts of the plants. However, the low biomass production of most hyperaccumulator species, along with their low economic value (Vamerali et al., 2010), have led to a search for higher-biomass hyperaccumulator crops. The use of these crops would couple renewable energy production to the remediation of metal-contaminated soils that can no longer be used for food and feed production (Vangronsveld et al., 2009; Conesa et al., 2012; Witters et al., 2012). Furthermore, these crops can easily be cultivated using established agronomic techniques (Garbisu and Alkorta, 2001).

Metal availability, uptake and phytotoxicity are the main limiting factors of phytoextraction in metal-contaminated soils (Weyens et al., 2009b). In general, metal-tolerant energy crops that are effective in removing metals from soil, reduce their growth in the presence of high concentrations of metals, thereby decreasing both the amount of marketable biomass and the remediation efficiency. The interaction between plant growth-promoting bacteria (PGPB) and plants can enhance biomass production and metal tolerance of the plants (Germida et al., 1998; Genrich et al., 2000; Zhang et al., 2012), decreasing symptoms of phytotoxicity. Some metal tolerant PGPB bacteria from soil, rhizosphere or endophytes have the capacity to promote plant growth by mechanisms such as nitrogen fixation, production of siderophores and phytohormones (such as IAA, indole-3-acetic acid), solubilization of minerals like phosphorous, transformation of nutrients elements, stimulation of root growth, and production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick et al., 2003; Rajkumar et al., 2009; Weyens et al., 2009b). The latter enzyme has received increasing attention because of its role in lowering ethylene levels in a stressed plant. The presence of toxic metals, and other types of stress like high salt concentrations or phytopathogens induce elevated ethylene levels (mainly) causing reduced root growth and development (Glick,

2010; Schellingen et al., 2014). Further, some bacteria can solubilize unavailable forms of toxic metals in soils by excreting organic acids and siderophores (Ma et al., 2009). On the other hand, some plant-associated bacteria can reduce the metal uptake by binding metals to anionic functional groups or to extracellular polymeric substances (Rouch et al., 1995; Ma et al., 2011; Rajkumar et al., 2012). Extending our knowledge about these bacterial characteristics and the action mechanisms of PGPB is important for the development of effective phytotechnologies on metal-contaminated sites (Zhang et al., 2011; Sessitsch et al., 2013).

Brassica napus L. (rapeseed) is a well-known high-biomass crop commonly used for bioenergy production. Many studies have mentioned the tolerance of this crop to toxic metals, such as Cd and Zn, as well as its capacity to accumulate them in their tissues (Marchiol et al., 2004; Grispen et al., 2006; Hernández-Allica et al., 2008). Taking into account the characteristics of this crop, *B. napus* can be a suitable candidate for phytoextraction purposes and to obtain valorizable biomass from land contaminated with Cd and Zn (Marchiol et al., 2004; Croes et al., 2013).

The aim of this work is to isolate and characterize the cultivable bacterial communities associated with *B. napus* growing on a Zn contaminated site, in order to select cultivable PGP bacterial strains that might enhance biomass production and tolerance of energy crops in metal-contaminated sites. Long-term polluted soils are sources of metal-tolerant microorganisms and interesting interactions with the plants that are growing in these soils, such as PGP characteristics (Becerra-Castro et al., 2012). The last objective of this work is to evaluate the effect of PGP bacterial strains on root growth of *B. napus* in the presence of different concentrations of Zn or Cd, using vertical agar plates (VAPs). These assays provide comparable and reproducible data obtained under standardized conditions. In this study, VAPs will be used to perform preliminary inoculation experiments in order to select potential PGP bacteria by studying the root structure (Zhang et al., 1998; Remans et al., 2006), and avoiding competition with other microorganisms (Reynoso-Cuevas et al., 2008).

5.3. Material and methods

5.3.1. Sampling

The sampling area was a Zn contaminated site in the municipality of Lummen (northeast of Belgium). It was previously used by a Zn recycling factory (Figure 5.1). In September 2011, *B. napus* seeds were sown on this site. Soils and plants were sampled in April 2012.

Figure 5.1. Soil sampled in Lummen (Belgium)

5.3.2. Metal concentrations in soils and plants

Metal concentrations were determined in soil and plant samples. The extractable fractions of metals in soil were estimated using 0.1 M $Ca(NO_3)_2$ (Mench et al., 1994). Total concentrations of metals in soil were determined by *aqua regia* digestion (Van Ranst et al., 1999). Plants were washed with distilled water, separated into leaves, stems and roots, and then dried for 48 h at 65°C. Subsequently, the dried tissues were ground and digested according to Weyens et al. (2010). Metal concentrations in the extracts were determined using inductively coupled plasma optical emission spectrometry (ICP-OES). Three replicates of soil and plant samples were analyzed. The quality of the digestion and analytical methods was tested including blanks and certified reference material (NIST Standard Reference Material 1570a, Trace elements in Spinach, U.S. Department of Commerce, National Institute of Standards and Technology) with every set of samples.

5.3.3. Isolation of cultivable bacterial strains

Cultivable bacteria were isolated from bulk soil, rhizosphere soil, roots and stems of *B. napus* following the sampling design of Croes et al. (2015). The isolation procedure was carried out according to Weyens et al. (2009a), but in this work, the chloride solution (1%) and time (1 min) during root surface sterilization were modified. Colony-forming units (cfu) were counted and calculated per gram soil or fresh plant weight. All morphologically different strains were purified using five replicates and then stored at -70°C in a solution with 15% (w:v) glycerol and 0.85% (w:v) NaCl.

5.3.4. Phenotypic characterization

Purified bacterial strains were grown in 869 medium (Mergeay et al., 1985), then washed twice with sterile 10mM $MgSO_4$ (Croes et al., 2013) and tested for their Zn and Cd tolerance and potential plant growth-promoting (PGP) characteristics (phosphate solubilization, nitrogen fixation, ACC-deaminase activity and production of siderophores, organic acids, IAA and acetoin). Strains not able to grow in the test medium were considered as not detectable (nd). Media without bacteria were used as control. The PGP characteristics were screened as described previously by Croes et al. (2013).

All bacterial strains were grown on selective 284 medium with a C-mix (Weyens et al., 2009a) and 1mM of Zn (added as $ZnSO_4$) or 0.8 mM of Cd (added as $CdSO_4$), in order to test the bacterial tolerance to these metals. Tolerance was assessed visually.

5.3.5. Genotypic characterization

Total genomic DNA was extracted from all purified morphologically different bacterial strains by the DNeasy® Blood and tissue kit (Qiagen, Valencia, CA, USA). Polymerase chain reaction (PCR) amplification of the 16S rRNA genes of the extracted DNA was carried out using the universal primers, 16S-prokaryotic-R (5'-ACGGGCGGTGTGTRC-3') and 16S-prokaryotic-F (5'-AGAGTTTGATCCTGGCTCAG-3') according to Weyens et al. (2009a). PCR products were directly used for ARDRA and sequencing. For amplified 16S rDNA restriction

analysis (ARDRA), 10µl of the PCR products were digested with the restriction endonuclease HpyCH4 IV (New England Biolabs, Beverly, MA, USA) and separated by electrophoresis as described by Weyens et al. (2009a). Bacterial strains with the same ARDRA patterns were grouped within each compartment (stem, root, rhizosphere, soil). The PCR products of one representative strain per compartment were purified according QIAlquick 96 PCR Purification Kit (Qiagen, Valencia, CA, USA). Purified 16S rRNA genes were sent for sequencing by Macrogen (Korea) with an Automatic Sequencer 3730XL. Sequenced strains were identified by means of Sequence Match at the Ribosomal Database Project II. All strains had a sequence match score higher than 0.900.

5.3.6. Effects of inoculation on root growth

Certified seeds of *B. napus* L. cv. Nodari (Syngenta Seeds) were surface sterilized by immersing in 0.1% sodium hypochlorite for 1 minute, then washed three times with Millipore water. Some sterilized seeds were grown during 3 days at 30°C on 869 rich solid medium (Mergeay et al., 1985) in order to verify the sterilization process. Seeds were considered sterile when no bacterial growth was observed. Bacterial strains were grown on 869 liquid medium for 12h at 30°C, centrifuged at 3000 rpm during 10 min, washed two times and resuspended in 10mM $MgSO_4$. Sterilized seeds were immersed in a bacterial suspension (10^8 cfu mL^{-1}) for 1h at room temperature. Subsequently, the seeds were placed in Petri dishes with 1/10 diluted 869 solid medium for 1 day at 25°C in darkness (Figure 5.2). Finally, the inoculated seeds were putted in square vertical plates (Zhang et al., 1998) with MS medium spiked separately with 300 and 1000 µM of Zn ($ZnSO_4.7H_2O$) and 50 and 300 µM of Cd ($CdSO_4.8H_2O$). The metal doses used in this experiment were chosen according to the tolerance shown by *B. napus* in the presence of different doses of Cd and Zn under *in vitro* conditions (Montalbán et al., 2014). Non-inoculated seeds were immersed in 10mM $MgSO_4$ and were used as controls in plates with MS medium non-spiked (Control) and spiked (Non-inoculated).

Figure 5.2. Placement of sterilized *B. napus* seeds in square vertical agar plates under the laminar flow chamber.

All plates were set up vertically in a growth chamber at 23ºC/12 ºC and 12 h of photoperiod (Figure 5.3). After 5 days, the root systems in vertical plates were scanned (Figure 5.4), and root length was determined after analysis of scanned images using the Optimas Image Analysis Software 6.0 (MediaCybernetics) according to Remans et al. (2006).

Figure 5.3. Vertical agar plates set in a growth chamber.

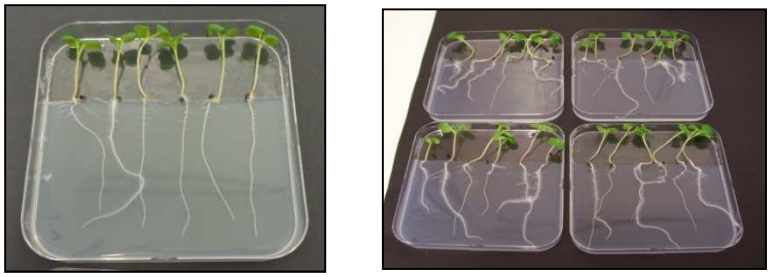

Figure 5.4. *B. napus* seedlings after 5-days of growth placed on the scanner.

5.3.7. Statistical analysis

Statistical analysis of the VAPs results was done using the IBM SPSS Statistics 19.0 software. One-way analysis of variance (ANOVA) and Tukey's test were applied in this case. Differences at $p < 0.05$ level were considered significant. Genotypic information was subjected to correspondence analysis (CA), a principal component analysis related ordination technique based on chi-square distances, illustrating correlations between compartments to evaluate the isolation procedure (Croes et al., 2015). CA was done using the statistical software package R (http://cran.at.r-project.org).

5.4. Results and discussion

5.4.1. Soil

The total and Ca $(NO_3)_2$-extractable Zn and Cd concentrations in the soil are shown in Table 5.1. When background values and clean up values for metals in agricultural soils were compared with the Flemish legislation on soil remediation (VLAREBO, 2009), only total Zn concentrations were higher than normal. Also the Ca $(NO_3)_2$-extractable Zn concentration was high in comparison to non-polluted soils.

Table 5.1. Total and Ca $(NO_3)_2$-extractable metal concentrations (mg kg^{-1} soil), pH and organic matter (OM) content of the soil on the experimental field. Results are mean ± SE of composite soil samples (depth: 0-25 cm).

	Zn	Cd	Cu	Pb	OM (%)	pH-KCl
	($mg.kg^{-1}$)					
Total concentration	343 ± 16	0.36 ± 0.04	35 ± 2	185 ± 10	1.9	5.6 ± 0.1
$Ca(NO_3)_2$-extractable	81 ± 20	0.15 ± 0.02	0.18 ± 0.01	0.38 ± 0.02		
Background values[a]	25-70	0.1-0.5	3-15	5-40	2.3	
Remediation values[b]	333	2	120	200	2	

[a] 'Normal range' values in sandy soils in Flanders according to De Temmerman et al. (2003).
[b] Clean up values for remediation of a 'standard' agricultural soil (2% organic matter and 2 % clay) according to the Flemish legislation on soil remediation (Vlarebo, 2009).

5.4.2. Bacteria isolated from *B. napus* growing on a Zn contaminated site

A total of 426 morphologically different cultivable bacterial strains were isolated from bulk soil, rhizosphere soil, roots and stems of the *B. napus* plants (Annex I). The number of different genera was similar in rhizosphere and root, but lower in soil and stem compartments (Table 5.2). The lower diversity of bacteria found in soil with respect to rhizosphere and roots can be explained as a rhizospheric selection by the plant on its surrounding bacterial community (Gomez-Balderas et al., 2014). The number of cultivable strains found in rhizosphere soil was two orders of magnitude higher than in bulk soil and roots. The decline of the bacterial numbers from the rhizosphere to the roots and soil was also observed by other authors considering *B. napus* associated bacteria (Germida et al., 1998; Croes et al., 2013). This high density of cultivable bacteria in the rhizosphere is due to root exudates that provide a high amount of organic carbon directly to microbial populations, in comparison with the slow decomposition of recalcitrant organic matter in the bulk soil (Soderberg and Bååth, 1998). Microorganisms indeed are attracted by carbohydrates, amino acids and organic acids that are present in the rhizosphere as root exudates and mucilage-derived components (Compant et al., 2010).

On the other hand, the number of endophytic bacteria recovered from the roots was four orders of magnitude higher than in stem samples (Table 5.2). The numbers of endophytes found in roots and stems are in accordance with earlier reports (Lodewyckx et al., 2002). This high density of bacteria in the lower parts of the plant with respect to upper parts was previously reported by Fisher et al. (1992). This suggests that the colonization of the plant interior took place via the root system, through natural and artificial wound sites, root hairs and epidermal junctions (Weyens et al., 2009a; Becerra-Castro et al., 2011), and mainly during the first stages of root development when the tissues are still undifferentiated (Hallmann et al., 2001). Moreover, some studies have shown an active penetration of endophytes through enzymatic degradation of plant cells (Lodewyckx et al., 2002; Truyens et al., 2015).

Table 5.2. Total numbers of colony-forming units (cfu) per gram fresh weight of soil, rhizosphere and *B. napus* tissues isolated from a Zn contaminated site.

Compartment	cfu g^{-1} fresh weight
Soil	$61.5 \times 10^4 \pm 52.8 \times 10^4$ (8)
Rhizosphere	$30.3 \times 10^6 \pm 15.3 \times 10^6$ (20)
Root	$12.5 \times 10^4 \pm 10.1 \times 10^4$ (17)
Stem	$60.7 \times 10 \pm 57.0 \times 10$ (3)

Mean values ± SE, n=3 independent replicates. The numbers of different bacterial genera are marked in parentheses.

5.4.3. Genotypic characterization

In total, 33 different bacterial genera were identified. The pie diagrams in Figure 5.5 show the diversity and relative abundance of bacterial genera present in each compartment. Each color and number relates to a different bacterial genus and subdivided colors represent bacterial genera with different accession number. Eight different genera of bacteria were identified in bulk soil, with *Arthrobacter* (45.9%) and *Bacillus* (41.7%) as dominant genera in this compartment. The high abundance of *Arthrobacter* sp. was not surprising, taking into account that it is considered to be one of the most predominant members of cultivable soil microorganisms (Hanbo et al., 2004). Moreover, this genus has been found in high abundance in Zn-polluted soils by other authors (Dell' Amico et al., 2005). *Bacillus* was also reported as a dominant genus in Cu-Pb-Zn contaminated soil by Ellis et al. (2003). Twenty different genera of bacteria were identified in the rhizosphere, where *Variovorax* (47.2%), *Arthrobacter* (16.9%) *Bacillus* (10.5%), and *Staphylococcus* (8.8%) were most dominant. The presence of these genera in the rhizosphere of *B. napus* was observed previously by other authors (Germida et al., 1998; Croes et al., 2013). Endophytic bacterial strains identified in roots belonged mainly to the genera *Pseudomonas* (33.8%), *Burkholderia* (30.6%), *Variovorax* (21.1%) and *Pedobacter* (7.4%). In stems, *Pedobacter* (66.7%) and *Micrococcus* (27.9%) dominated the cultivable bacterial population.

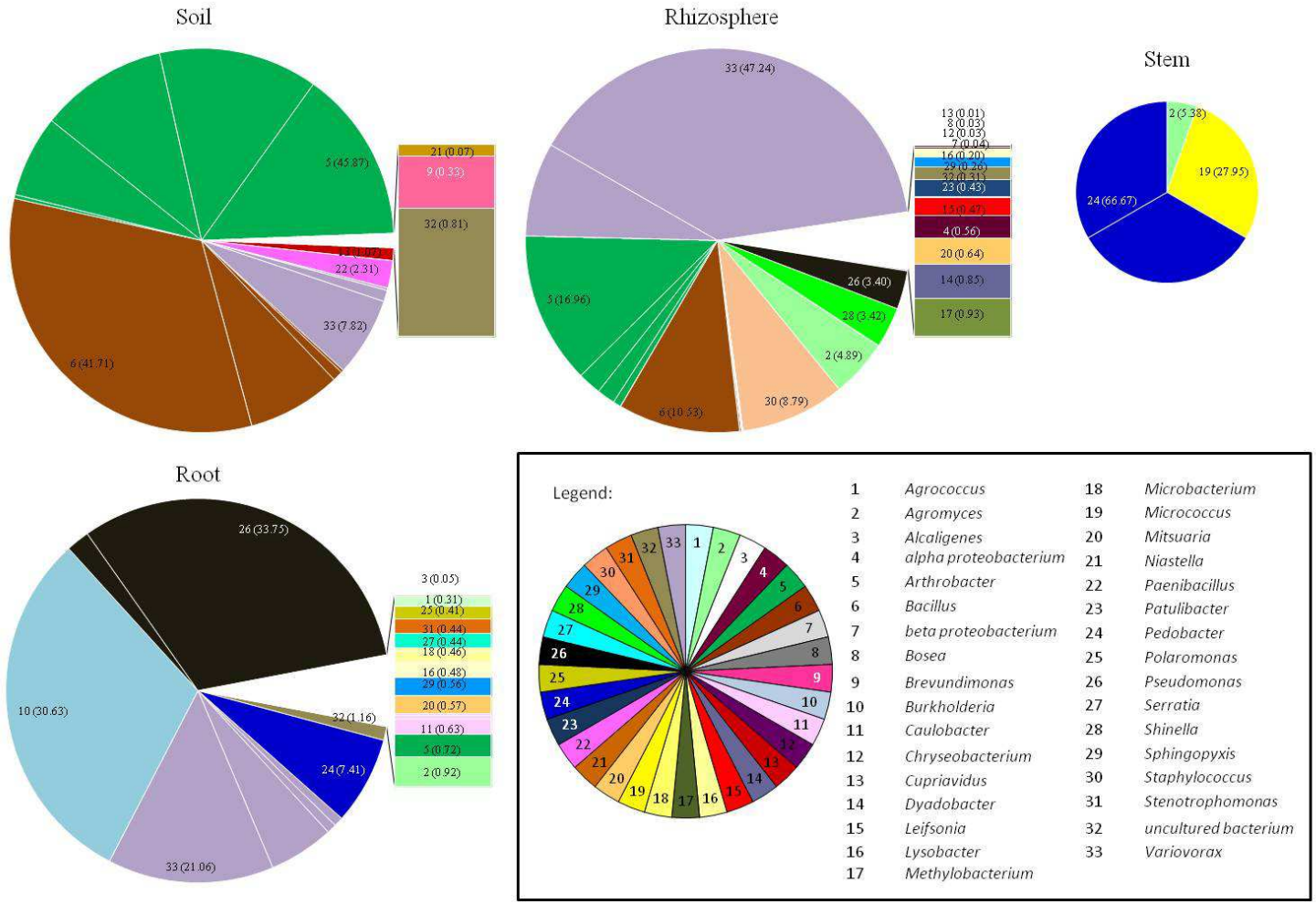

Figure 5.5. Diversity and abundance of cultivable bacteria isolated from bulk soil, rhizosphere, root and stem of *B. napus* plants grown in a Cd-Zn contaminated site. Abundance percentages are shown in parentheses. Bacterial strains with abundances lower than 1% are shown next to the pie diagram.

Although *Pseudomonas, Bacillus, Enterobacter and Agrobacterium* are the most commonly isolated bacterial genera (Becerra-Castro et al., 2011), in this work, *Enterobacter* and *Agrobacterium* were not isolated from any of the compartments under investigation. Field studies of metal contaminated soils have shown that high levels of metals can modify the structure of microbial communities and decrease microbial diversity (Kelly et al., 2003; Dell' Amico et al., 2005).

Paenibacillus, Niastella and *Brevundimonas* were only found in the bulk soil. Gomez-Balderas et al. (2014) and Croes et al. (2013) also isolated the genus *Brevundimonas* from a Zn-Cd contaminated soil, but both studies did not report it in non-contaminated soils, indicating the eventual adaptation of this genus to Zn and Cd contaminated sites. On the contrary, *Arthrobacter* and *Variovorax* were present in all compartments studied, except in the stem.

Staphylococcus, *Shinella*, *Bosea*, *Chryseobacterium*, *Proteobacterium* a/b, *Patulibacter*, *Leifsonia*, *Dyadobacter* and *Methylobacter* were found only in the rhizosphere. However, *Agromyces, Pseudomonas*, *Lysobacter*, *Sphingopyxis* and *Mitsuaria* were present in the rhizosphere and also as root endophytes. *Agromyces* was also found to be a stem endophyte. *Burkholderia*, *Alcaligenes*, *Agrococcus*, *Polaromonas*, *Stenotrophomonas*, *Serratia*, *Microbacterium* and *Caulobacter* were root endophytes exclusively. *Serratia* species were also found as root endophytes in the Cd-hyperacumulator *Solanum nigrum* by Chen et al. (2012). In this study, *Pedobacter* was found as root and stem endophyte, however, *Micrococcus* was exclusively present in the stem. *Micrococcus* sp. were also found by Velazquez et al. (2008) in stems of sugarcane and by Germida et al. (1998) in roots of *Brassica napus*, confirming the ability of this genus to colonize plant tissues.

The genera *Cupriavidus, Niastella, Agromyces, Shinella, Bosea, Proteobacterium, Lysobacter, Shingopyxis, Patulibacter, Mitsuaria, Dyadobacter, Methylobacter, Alacaligenes, Agrococcus* and *Polaromonas* were isolated in this work (Figure 5.5), but were not reported by other authors in bacterial communities associated with *B. napus* (Croes et al., 2013; Germida et al., 1998). This can be due to (a) specific soil factors that could affect the rhizosphere populations (Bulgarelli et al., 2012), and also (b) to the different concentrations of the (available) metals in the soil. Moreover, (c) the surface sterilization method employed, (d) the growth medium used for isolation (Lodewyckx et

al., 2002) or (e) the different stages of growth of the host plant at the moment of sampling could also affect the bacterial populations (de Campos et al., 2013).

Croes et al. (2013) reported that the most dominant root cultivable endophytes in *B. napus* were the genera *Pseudomonas*, *Pedobacter* and *Variovorax*. These genera were found in this work in similar percentages. Taking into account that the seeds sown in our field originated from the same seed stock as used by Croes et al. (2013), this indicates that the host plant might be able to maintain some seed endophytes.

According to the correspondence analysis (Figure 5.6), the mean cultivable rhizosphere bacterial community was more correlated (CC = 0.43) with the mean soil bacterial community than with the endophytic communities found in the roots and stems (CC = 0.35 and -0.08, respectively). This suggests that the root exudates have less influence on the community composition in the rhizosphere than the soil characteristics. Root and stem bacterial communities showed a low correlation coefficient (CC = 0.06), indicating the presence of different bacterial genera. Several authors reported significant differences in bacterial communities between below-ground and above-ground plant parts, demonstrating that the organs of the plants have different bacterial communities associated with them (Lindow and Brandl, 2003; Izumi et al., 2008; Weyens et al., 2009a; Croes et al., 2013).

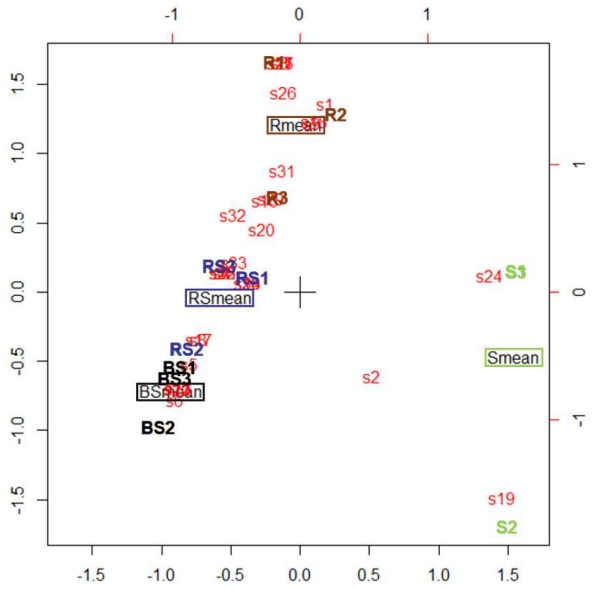

Figure 5.6. Correspondence analysis of bacterial communities isolated from soil, rhizosphere and *B. napus* samples. s1-s33 represent a bacterial genus (see legend in Figure 5.5). BS (Bulk soil, in black), RS (Rhizosphere soil, in blue), R (Root, in brown), S (Stem, in green). Three repetitions of each compartment were used to make the mixed sample (mean).

5.4.4. Phenotypic characterization

A high percentage of stem endophytes showed tolerance to 1mM Zn, but they were not able to grow on 284 medium supplemented with C-mix and 0.8 mM Cd (Table 5.3). The Zn concentrations in leaf, stem and root of *B. napus* collected in the field were 1013 ± 207, 1301 ± 196 and 941 ± 138 mg.kg dry weight^{-1}, respectively. The Cd concentrations were 1.4 ± 0.5, 1.6 ± 0.6 and 1.2 ± 0.3 mg.kg dry weight^{-1} in leaf, stem and root, respectively. The fact that the concentration of Zn in the stems was elevated might explain why the endophytes were highly tolerant to Zn. In contrast to the bacterial strains isolated from the stems, 21.7 % of the root endophytes were tolerant to Cd. The percentages of rhizosphere and soil strains that were tolerant to 0.8mM Cd and 1mM Zn were similar for both compartments. Croes et al. (2013) compared the metal tolerance of bacterial strains isolated from a Zn/Cd-contaminated field and a control field. They observed that the highest numbers of bacterial strains tolerant to 1.6 mM Cd and 2.5 mM Zn originated from the contaminated field. They suggested that the concentrations of metals present in the soil stimulated the presence of bacteria tolerant to both metals.

Table 5.3. Phenotypic characterization of all purified bulk soil, rhizosphere, root and stem isolated bacteria.

	Soil	Rhizosphere	Root	Stem
SID	1.86	3.89	23.76	0.00
OA	36.68	5.53	20.79	53.79
ACC	68.39	18.94	69.25	0.26
IAA	35.38	6.49	47.21	26.76
Acetoin	5.57	10.32	7.07	20.00
P sol	4.09	1.63	17.39	0.00
N_2 fix	1.05	1.05	1.20	53.33
Zn (1 mM)	23.28	25.40	6.18	40.00
Cd (0.8 mM)	22.27	25.07	21.68	0.00

Data are relative abundances expressed in percentages of the total number of cultivable bacteria isolated per gram fresh weight bulk soil, rhizosphere, roots and stem. Bacterial strains were tested for metal resistance (Cd or Zn) and potential plant growth-promoting characteristics: Phosphorus solubilization (P sol), nitrogen fixation (N_2 fix), production of siderophores (SID), organic acids (OA), ACC deaminase (ACC), indole-3-acetic acid (IAA) and acetoin (Acetoin).

Stem endophytes could not solubilize phosphorus or produce siderophores (Table 5.3). Many of them were able to fix nitrogen and produce organic acids. The highest percentages of bacterial strains able to solubilize phosphorus and produce siderophores, IAA and ACC deaminase were found in the roots. On the contrary, the highest percentage of bacterial strains capable of producing acetoin was found in the stems. Our

results suggest that the production of organic acids, IAA and ACC deaminase are important bacterial characteristics in soil and roots, however, siderophore production occurs predominantly in root bacteria.

A comparison of phenotypic characteristics of bacterial communities that were isolated in different studies is complicated due to the large variation that exist between different plant species, growth conditions and concentrations of metals in the plant (Chen et al., 2012). However, it is known that toxic metals in soils can stimulate the production of bacterial siderophores that can decrease metal toxicity by providing iron to the plant (Dell' Amico et al., 2005). Moreover, the production of IAA and ACC deaminase can stimulate root growth in presence of metals and also the root exudation that promotes the bacterial proliferation in the rhizosphere (Glick et al., 2010). Our results support these hypotheses, due to the fact that most of the bacterial strains isolated from the Zn contaminated site show potential PGP characteristics (Table 5.3).

5.4.5. Inoculation of *B. napus* seeds with PGPB

Six Zn-tolerant and/or Cd-tolerant bacterial strains (isolated from bulk soil, rhizosphere and roots of *B. napus* growing on the Zn contaminated site) were selected according to their PGP characteristics (Table 5.4) to be inoculated in *B. napus* seeds. Root length was the parameter used to evaluate the effects of the bacteria on the growth and tolerance of the seedlings to both metals. The architecture of a root system is determined by the intrinsic developmental program but also, by external biotic and abiotic stimuli (Zhang et al., 1998), such as the presence of toxic metals in the growth medium. Root growth has been often used to evaluate the plant tolerance to metals (Peralta et al., 2001, Azevedo et al., 2005), and it is one of the best markers to evaluate the effect of PGP bacteria on plant growth (Pattern and Glick, 2002).

Table 5.4. Metal tolerance and PGP characteristics of selected bacteria for inoculation in *B. napus* seeds.

Comp.	Strain	Identification	Accesion	Zn 1mM	Cd 0.8mM	Fe0µM	Fe0.25µM	OA	ACC	IAA	Ace	Psol	N fix
Rh	25	*Staphylococcus* sp.	AB009944	+++	+++	-	-	+++	-	-	+	-	-
Soil	222	*Arthrobacter* sp.	EU086826	+++	+++	-	-	++	+++	-	-	-	+
Root	228	*Pseudomonas* sp.	GU595312	++	++	+	+	+	++	+	-	++	-
Root	246	*Serratia* sp.	HM596429	+++	+++	+	+	++	+++	++	-	-	-
Root	256	*Pseudomonas* sp.	GU595312	+++	+	+	+	-	+	++	+	+++	++
Root	262	*Pseudomonas* sp.	GU595312	+	+	++	-	+	+++	++	+	-	-

Compartment of origin of the strain (Comp.), Rhizosphere (Rh), growth in the presence of Zn (1mM) and Cd (0.8mM), siderophores (Fe0µM and Fe0.25µM), Organic acids (OA), ACC (ACC deaminase activity), IAA (indole-3-acetic acid), Ace (Acetoin), phosphate solubilization (Psol), nitrogen fixation (N fix). + low, +++ high production.

B. napus did not exhibit significant differences in root length between seedlings grown with low concentrations of Zn or Cd (300 µM and 50 µM, respectively) and controls (Figure 5.7). However, root length decreased at higher doses of both metals (1000 µM Zn and 300 µM Cd). Cd is highly phytotoxic, even at low concentrations (Groppa et al., 2008). On the contrary, zinc (Zn) is an essential micronutrient, required by plants to grow, but it becomes toxic at higher levels (Marques et al., 2013). The positive effect of bacterial inoculation of the seeds on root growth was observed only at high doses of both metals. *Pseudomonas* sp. strain 228 and 256, and *Serratia* sp. strain 246, significantly increased root length of *B. napus* when exposed to 1000µM Zn (Figure 5.7a). *Arthrobacter* sp. strain 222, *Serratia* sp. strain 246, *Pseudomonas* sp. 228 and 262 significantly increased root length of *B. napus* seedlings in the presence of 300 µM Cd in comparison to non-inoculated ones (Figure 5.7b).

In general, the inoculated bacterial strains were able to produce siderophores, indole-3-acetic acid (IAA) and exhibited ACC deaminase activity (Table 5.4). *Pseudomonas* sp. strain 228 and *Serratia* sp. strain 246 also were capable of producing organic acids, and *Pseudomonas* sp. strain 256 could produce acetoin and fix nitrogen. Moreover, both *Pseudomonas* strains could solubilize phosphorous. *Arthrobacter* sp. strain 222 and *Pseudomonas* sp. strain 262 showed high ACC deaminase activities and moderate production of organic acids. *Pseudomonas* sp. strain 262 was also capable of producing acetoin. *Arthrobacter* sp. strain 222 was the only strain that did not possess the capacity to produce siderophores or IAA, but was able to fix nitrogen.

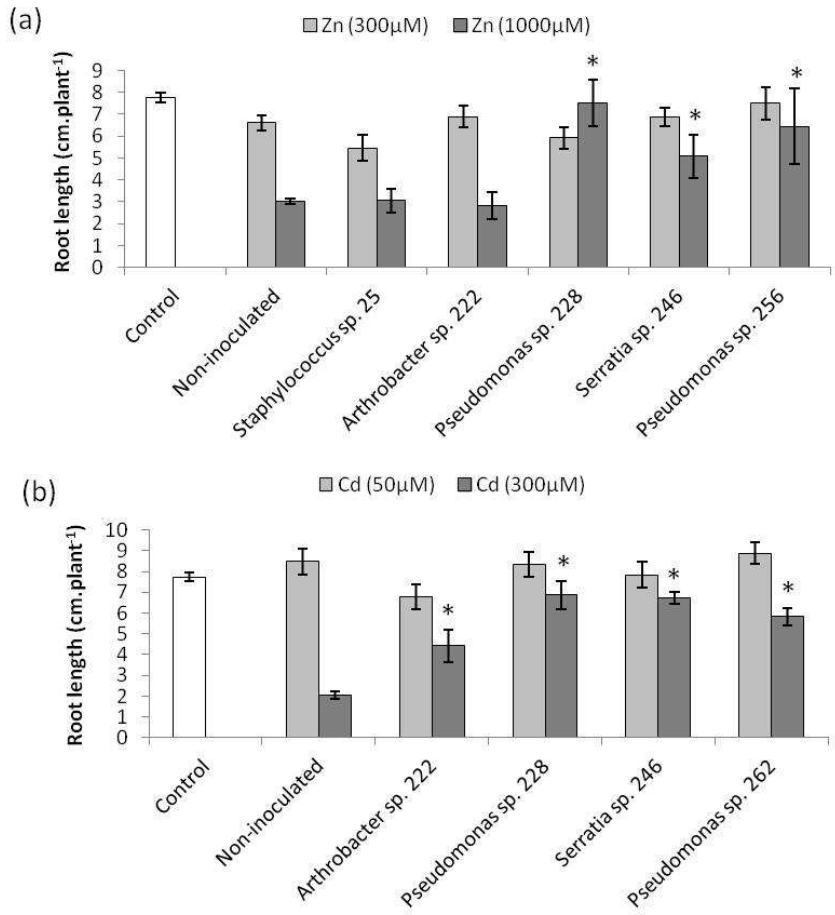

Figure 5.7. Root length of 5-days *B. napus* seedlings after inoculation of PGP bacterial strains in presence of (a) Zn and (b) Cd. Asterisks (*) represent significant differences between non-inoculated and inoculated plants after one-way ANOVA and Tukey's test ($p < 0.05$; mean values ± SE; n=4).

A positive effect due to inoculated bacterial strains on root growth of Cd-exposed *B. napus* was also observed by Sheng et al. (2006) after inoculation with *Pseudomonas* sp. and *Bacillus* sp., both being IAA producers. Many studies have reported positive effects of inoculated bacteria on the plant growth (Glick, 2010). IAA (indole-3-acetic acid) and acetoin (3-hydroxy-2-butanone) production have been shown to stimulate root formation (Duan et al., 2013), and ACC (1-aminocyclopropane-1-carboxylic acid) deaminase activity to protect against the growth inhibiting effects of various stresses such as toxic metals (Glick, 2003). The PGP characteristics of the inoculated bacterial strains played an important role in the root growth of *B. napus* seedlings in the presence of toxic concentrations of Zn and Cd. Further studies are necessary in order to investigate the growth-promoting properties of these bacterial strains in real soils where

there is competition between indigenous microorganisms and where nutrients are present in more recalcitrant forms.

5.5. Conclusions

The isolation of bacteria associated with *B. napus* growing on a Zn contaminated site led to the identification of metal-resistant bacterial strains with potential PGP characteristics. Seed inoculation of selected soil and endophytic bacteria facilitated root growth of *B. napus* seedlings in presence of toxic concentrations of Cd and Zn. Future work should be performed in real soil conditions to evaluate the effects of these bacterial strains on plant growth and metal uptake, in order to select bacterial strains that can be used to improve remediation and biomass production on contaminated soils.

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Chapter 6

Inoculation of plant growth-promoting bacteria in Cd and Zn exposed *Helianthus tuberosus* L. under hydroponic conditions

6.1. Abstract

Plant growth-promoting bacterial strains isolated from *Brassica napus* were inoculated in two cultivar-clones (VR and D19) of *Helianthus tuberosus*, in order to evaluate their effects on growth, metal uptake and oxidative stress, under hydroponics conditions with 0.1 mM Cd and 1 mM Zn. *Pseudomonas* sp. 228, *Serratia* sp. 246 and *Pseudomonas* sp. 262 enhanced the growth of D19 cultivar-clone in presence of Cd and Zn. Only *Pseudomonas* sp. 228 increased Cd uptake. On the other hand, the addition of *Serratia* sp. 246, *Pseudomonas* sp. 256 and 228 decreased the content of TBA reactive compounds in roots of D19 cultivar-clone grown in presence of Zn. The roots of VR also showed lower levels of TBA reactive compounds in plants inoculated with *Pseudomonas* sp. 228. The improvement of growth and the decrease of metal-induced stress were more pronounced in the D19 cultivar-clone than in VR. After observation with confocal microscopy, we found that the *B. napus* endophyte egfp-*Pseudomonas* sp. 262 did not colonize the root interior of *H. tuberosus*, under the studied conditions. However, the bacterial strains were attached to the roots and root hair surfaces of *H. tuberosus*, suggesting that interaction between them was established.

Keywords: high biomass crop, Jerusalem artichoke, metal toxicity, green fluorescent protein, phytotechnologies.

6.2. Introduction

Helianthus tuberosus L. (Asteraceae) is a high biomass crop used in bio-ethanol production and vegetatively propagated by tubers (Serieys et al., 2010). Recent studies have shown the tolerance of this crop to metals as Cd, Pb and Zn (Cui et al., 2007; Chen et al., 2011; Long et al., 2013; Montalbán et al., 2015). Moreover, its cultivation involves low production costs and minimal disease problems (Denoroy, 1996; Kays and Nottingham, 2008). All these characteristics make *H. tuberosus* a promising candidate to remediate metal polluted soils, as well as to produce a renewable energy resource.

Metal availability, uptake and phytotoxicity are the main limiting factors during the application of phytotechnologies in metal-contaminated soils (Mulligan et al., 2001; Weyens et al., 2009a). Interactions between metal tolerant crops and beneficial bacteria may increase the efficiency of the phytoextraction, enhancing biomass production, metal uptake and tolerance of the plants to heavy metals (Germida et al., 1998; Genrich et al., 2000; Rajkumar et al., 2012). Plant growth-promoting bacteria (PGPB) can improve plant growth through two strategies: indirectly by preventing growth and activity of plant pathogens by producing antibiotics or through competition for space and nutrients (Lugtenberg and Kamilova, 2009), or directly by increasing nutrient uptake and growth through different mechanisms such as nitrogen fixation (Roper and Ladha, 1995), synthesis of phytohormones (as IAA, indole-3-acetic acid) (Dobbelaere et al., 1999), solubilization of minerals, and production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick, 1998; Glick et al., 2003). Some microorganisms also present metal-resistance/sequestration systems, by means of which, the bacteria are able to produce natural chelators that can contribute to metal detoxification (Diels et al., 1999).

Moreover, some bacteria can increase metal and nutrients availability by excreting organic acids, that decrease pH values in rhizosphere, or enhancing the Fe(III) mobility and other cations through siderophores production (Glick and Bashan, 1997; Fasim et al., 2002). On the contrary, plant-associated bacteria can adsorb metals by binding them to anionic functional groups or to extracellular polymeric substances of the cell wall (Rouch et al., 1995; Madhaiyan et al., 2007; Vivas et al., 2006). This leads to a reduced metal uptake and translocation inside the plant, improving the growth through the decreased phytotoxicity (Ma et al., 2011; Rajkumar et al., 2012).

The PGP bacterial strains used in this study were isolated from soil and *Brassica napus* growing on a Zn contaminated site. Some of these bacterial strains increased the root length of *Brassica napus* seedlings in presence of Cd and Zn under *in vitro* conditions (see Chapter 5). In the literature, many studies have evaluated host plants re-inoculated with their associated isolated bacteria. However, some studies have shown that bacteria isolated from metal tolerant plants promoted the growth of plants from different taxonomic groups (Ma et al., 2011; Sheng et al., 2012; He et al., 2013; Sessitsch et al., 2013), and showed high levels of colonization in plant tissues, other than the original host. Hydroponic experiments provide comparable and reproducible data obtained under standardized conditions, avoiding the metal retention process that occurs in soils (Hernández-Allica et al., 2008; January et al., 2008). Several works have been performed under these conditions in order to evaluate the effects of bacteria on growth, metal uptake and TBA reactive compounds production using different plants and metals (Rajkumar et al., 2009; Wan et al., 2012; Pandey et al., 2013). This approach allows to evaluate the effects of the bacteria on plant growth when there are no other parameters involved that can mask the bacterial effects, such as the competition with other microorganisms (Compant et al., 2010).

The bacterial inoculation strategy is one of the most critical steps in phytotechnology applications (Weyens et al., 2009b). The colonization of the bacteria has to be appropriate to promote beneficial effects in plant growth and metal uptake (Lugtenberg et al., 2001). Improving the knowledge about PGP bacteria colonization processes and plant-bacteria interactions is necessary to develop an effective inoculation (Compant et al., 2009). The use of fluorescent proteins in non-invasive microscopy is a well-established and valuable tool in biology and biotechnology (Lagendijk et al., 2010). Labeling with enhanced green fluorescent protein (egfp) has allowed to study the colonization pattern of bacteria in numerous studies (Bloemberg and Lugtenberg, 2001; Germaine et al., 2004; Weyens et al., 2012). Gfp has been described as a good marker for studying bacterial behavior at the cell level in the rhizosphere and endophytes (Bloemberg et al., 2000; Newman et al., 2003).

Since *Helianthus tuberosus* could be an appropriate candidate for both phytoremediation and as an energy crop producer; the aim of this work was to evaluate the effects of PGP bacterial strains isolated from *B. napus* on growth, metal uptake and oxidative stress of two cultivars (VR and D19) of *H. tuberosus*, under Cd and Zn

exposed hydroponic conditions. A root endophyte of *B. napus* (*Pseudomonas* sp. 262) with PGPB characteristics was selected to be labeled with the enhanced green fluorescent protein (egfp):tetracycline® plasmid in order to study the bacterial colonization in the roots of *H. tuberosus* under hydroponic conditions. The colonization pattern was investigated by Confocal Laser Scanning Microscopy.

6.3. Material and methods

6.3.1. Plant material

Tubers of two cultivar-clones of *H. tuberosus* (Violet de Rennes shortened as VR, and Blanc précoce commonly called D19), were collected in spring in the field collection of IMIDRA (Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario; Madrid, Spain) to perform the hydroponic experiments. The tubers were maintained during two weeks at 4ºC for vernalization. After this period, the tubers were vigorously washed in tap water to remove the adhered soil, before to set up the experiments.

6.3.2. PGP bacterial strains

Cultivable bacteria were isolated from soil, rhizosphere, root and stem of *Brassica napus* growing on a Zn contaminated site in Belgium as it was described in Chapter 5. Three Zn-tolerant bacterial strains (*Serratia* sp. strain 246, *Pseudomonas* sp. strain 228, *Pseudomonas* sp. strain 256) and four Cd-tolerant bacterial strains (*Arthrobacter* sp. strain 222, *Pseudomonas* sp. strain 228, *Pseudomonas* sp. strain 262 and *Serratia* sp. strain 246) were selected to inoculate in *H. tuberosus*, according to their PGP characteristics (Table 6.1). Bacterial strains were grown in 869 liquid medium (Mergeay et al., 1985) at 30°C under shaking. The bacterial suspension (10^8 cfu mL^{-1}) was added into the pots after the appearance of the first roots (5 days after the sowing).

6.3.3. Inoculation of PGP bacterial strains in *H. tuberosus*

Tuber slices with buds were germinated in 1L plastic pots filled with moist quartz sand and placed in a growth chamber at 25°C/12°C, 14/12h of photoperiod. Two slices with buds were set up per pot and four independent replicates of each treatment were

provided. The following treatments were established: (i) Control: plants grown in sand without metal and bacteria; (ii) Non-inoculated: plants grown with metal (Cd or Zn); (iii) plants inoculated with bacterial strains (*Arthrobacter* sp. 222, *Pseudomonas* sp. 228, 262 and *Serratia* sp. 246) and grown with Cd; and finally (iv) plants inoculated with bacterial strains (*Pseudomonas* sp. 228, 256 and *Serratia* sp. 246) and grown with Zn.

After one week of growth, the seedlings were fertilized with ½ diluted Hoagland's solution (1 mM Ca $(NO_3)_2 \cdot 4H_2O$, 1.5 mM KNO_3, 0.5 mM $NH_4H_2PO_4$, 0.25 mM $MgSO_4 \cdot 7H_2O$, 1 µM $MnSO_4 \cdot H_2O$, 12.5 µM H_3BO_3, 0.25 µM $(NH_4)_6Mo_7O_4$, 0.05 µM $CuSO_4 \cdot 5H_2O$, 1 µM $ZnSO_4 \cdot 7H_2O$, 10 µM $NaFe^{III}$-EDTA, demineralised water buffered with 1 mM of 2-(*N*-morpholino)ethanesulfonic acid, pH 5.5±0.5) spiked with 0.1mM of Cd (added as $CdSO_4.8H_2O$) or 1mM of Zn (added as $ZnSO_4.7H_2O$). Control plants were grown only with ½ diluted Hoagland's solution. The metal tolerance of *Helianthus tuberosus* has been poorly studied in the literature, due to this, the metal doses used in this experiment were chosen according to the tolerance shown by *Helianthus annuus* in the presence of different doses of Cd and Zn under *in vitro* conditions (see Chapter 3). After the appearance of the first roots (5 days after the sowing), the bacterial suspension (10^8 cfu mL^{-1}) in buffer (10 mM $MgSO_4$) was added into the pots. Buffer (10 mM $MgSO_4$) without bacteria was added to the controls. After three weeks of growth in presence of Cd or Zn, plants were harvested (Figure 6.1). One plant per pot was used to determine dry weight and metal concentration. The other one was used to determine the concentration of thiobarbituric acid (TBA) reactive compounds.

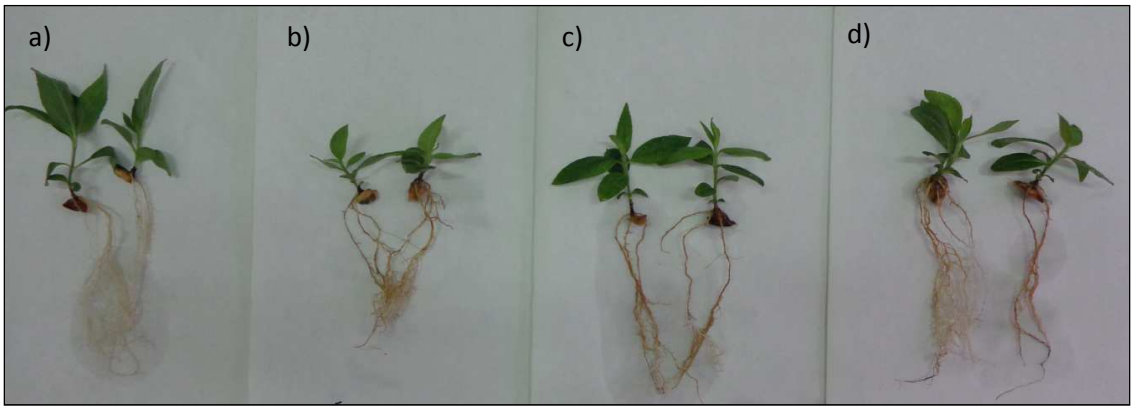


Figure 6.1. D19 cultivar-clone of *H. tuberosus* after 3 weeks of growing in presence of 0.1mM Cd. (a: control; b: non-inoculated; c: inoculated with *Serratia* sp. 246; d: inoculated with *Pseudomonas* sp. 262).

6.3.4. Plant analysis

After harvest, the roots were rinsed briefly in 10 mM sodium ethylenediaminetetraacetic acid (Na_2EDTA) to remove the adhered metal containing particles, and then washed in distilled water. Plants were separated into leaves, stems and roots, weighed and dried in a forced air oven for 48 h at 60°C to determine the dry weight. Subsequently, the dried tissues were individually ground and digested (30 mg) by adding 1mL of HNO_3 (65% Suprapur®) and 1mL of $HClO_4$ (70% Suprapur®). The samples were left overnight and heated at 130ºC for 2 h 30 min in a heating block (Dri-Block, DB3D, Techne). After cooling, the extracts were brought up to 25 mL with Millipore water (Figure 6.2) to measure total concentrations of metals and macro/micronutrients by flame Atomic Absorption Spectrometer AAS Varian Fast Sequential Model AA240FS. The quality assurance of the digestion and analytical methods was tested including blanks and certified reference materials (NCS DC73348 Brush Branches and Leaves, China National Analysis Center for Iron and Steel, and CTA-VTL-2 Virginia Tobacco Leaves, Polish Academy of Sciences and Institute of Nuclear Chemistry and Technology) with every set of samples. The recovery percentages for metals were: Cd (~95%) and Zn (~101%).

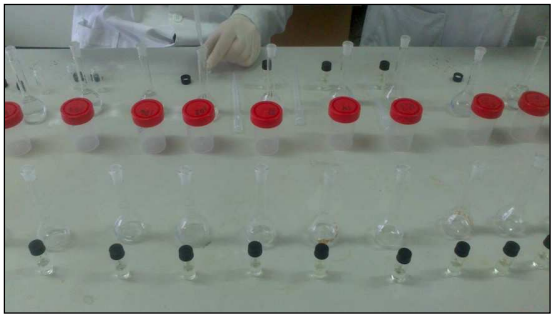


Figure 6.2. Metal extracts brought up with Millipore water in flasks of 25 mL.

6.3.5. Estimation of lipid peroxidation: thiobarbituric acid (TBA) reactive compounds

The membrane lipid peroxidation of plant tissues was evaluated in terms of the content of thiobarbituric acid reactive (TBA) reactive compound according to the method of Reilly and Aust (2001), modified by Catalá et al. (2010). Samples of leaves and roots were frozen in liquid N_2 during harvest and stored at -80 °C. Fresh samples of leaves

and roots (0.2 g) were homogenized on ice with 1 mL of deionised water and centrifuged at 16,000 g for 10 min at 4°C. The supernatants were removed, and the pellets were re-suspended in 500 µL of 0.01% butylated hydroxy-toluene (BHT) in 80% ethanol. Then, 900 µL of TBA (2.57×10^{-2}M), TCA (9.18×10^{-1}M) and HCl (3.20 M) were added to each sample. Samples were vortexed, incubated in a water bath at 70°C for 30 min, cooled on ice and then centrifuged at 16,000 g for 10 min at 4°C. The absorbance of supernatants was measured at 532 nm, and at 600 nm to eliminate the interference of soluble sugars in the samples. The calibration curve was carried out with every set of samples, using 1,1,3,3-Tetraethoxypropane (TEP) as precursor of malondialdehyde (MDA). Absorbances were determined by UV–vis light spectrophotometer (Thermo Spectronic Helios Alpha).

6.3.6. Evaluation of the colonization process: *in situ* bacterial localization

6.3.6.1. Bacterial strains and growth conditions

The receptor, *Pseudomonas* sp. 262 was grown in 284 minimal medium (Schlegel et al., 1961) with 0.4 mM of Cd at 30°C. The donor, *Escherichia coli* strain dH5a carrying the egfp pMP4655 plasmid was grown in 869 medium (Mergeay et al., 1985) supplemented with 20µg ml^{-1} tetracycline at 30°C. The helper, *E. coli* strain dH5a carrying the pRK2013 plasmid was grown in 869 medium at 30°C. Donor and helper were constructed in the Institute of Biology Leiden, Leiden University (Netherlands) (Bloemberg et al., 2000).

6.3.6.2. Introduction of the egfp: tetracycline into *Pseudomonas* sp. strain 262.

Triparental mating was carried out in order to label *Pseudomonas* sp. strain 262 with enhanced green fluorescent protein (egfp): tetracycline® plasmid. The bacterial strains were grown in 869 medium at 30°C under shaking. Growth curves were obtained by diluting an overnight culture in order to check the time needed to reach the optical density (OD) appropriated for conjugation. The OD was measured at 660 nm every 30 min using Visible Diode Array Spectrophotometer, Novaspec Plus, Amersham Biosciences. Once the OD was reached (Donor and helper OD 0.3-0.4, and receptor OD 0.7), the bacterial strains were centrifuged at 3000 rpm during 10 min, and then added to the mating filter in a Petri dish with 869 medium. After the conjugation, 284 minimal

medium supplemented with 0.4mM of Cd and tetraclycline (20µg ml^{-1}) was used to isolate the receptor labeled strains. Fluorescence of the strains was checked in a Nikon 80i fluorescence microscope (High-pressure Mercury Lamp. Excitation filters: 465-495 nm, dichroic mirror 505 nm, emission filter 515-555 nm. Objectives used: 40x/0.95 Air Plan Apo WD 0.14 mm and 100x/1.25 Oil Plan Apo WD 0.17 mm).

6.3.6.3. Inoculation of egfp *Pseudomonas* sp. strain 262 in roots of *H. tuberosus*

Tuber slices with buds of *Helianthus tuberosus* cultivar D19 were grown in hydroponic culture with coarse perlite and a half strength modified Hoagland's solution (1 mM Ca $(NO_3)_2 \cdot 4H_2O$, 1.5 mM KNO_3, 0.5 mM $NH_4H_2PO_4$, 0.25 mM $MgSO_4 \cdot 7H_2O$, 1 µM $MnSO_4 \cdot H_2O$, 12.5 µM H_3BO_3, 0.25 µM $(NH_4)_6Mo_7O_4$, 0.05 µM $CuSO_4 \cdot 5H_2O$, 1 µM $ZnSO_4 \cdot 7H_2O$, 10 µM $NaFe^{III}$-EDTA, demineralised water buffered with 1 mM of 2-(*N*-morpholino)ethanesulfonic acid, pH 5.5 ± 0.5) under greenhouse conditions (25°-30°C temperature and 70-90% relative humidity). The bacterial suspension (10^8 cfu mL^{-1}) was added into the pots (0.2L) after appearance of the first roots (5 days after sowing). Four repetitions were set up.

6.3.6.4. Confocal laser scanning microscopy

After 48 h of incubation, plant roots were washed to remove weakly bound bacterial cells and, subsequently, intact root preparations were observed with a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) mounted on an Axiovert 200M. The objective used was 40x1.1 water immersion (Zeiss LD C-Apochomat 40×/1.1WKorr UV–VIS-IR).

Excitation was performed at the 488 nm line of an Arion laser source. Backward GFP signal was filtered using a 500–550 nm band pass filter. Images were edited using the software Zen 2009 Light Edition (Carl Zeiss MicroImaging GmbH, Jena, Germany).

6.3.7. Statistical analysis

Statistical analysis of data was made by using the IBM SPSS Statistics 19.0 software. One-way analysis of variance (ANOVA) and Duncan's test were applied. Differences at $p < 0.05$ levels were considered significant.

6.4. Results and discussion

6.4.1. Biomass and metal uptake

The presence of Cd and Zn significantly decreased the biomass of both *H. tuberosus* cultivar-clones in comparison to the control plants (Figure 6.3). In particular, the aerial biomass decreased by 57% and the roots by 67% in plants exposed to Cd; and the reductions reached 70% and 50% in aerial and root biomass, respectively, when plants were grown in presence of Zn. However, some of the inoculated bacterial strains enhanced the plant growth under metal exposure. In presence of Zn, inoculation of *Pseudomonas* sp. 228 significantly increased the aerial and root biomass of D19 cultivar-clone. *Serratia* sp. 246 also increased the aerial biomass of VR under Zn exposure. Regarding Cd, the addition of *Pseudomonas* sp. 262 and *Serratia* sp. 246 significantly increased the aerial biomass of D19. Previous studies have shown the positive effect of PGP bacterial inoculation on plant growth under metal exposure (Zaidi et al., 2006; Sheng and Xia, 2006; Sheng et al., 2008).

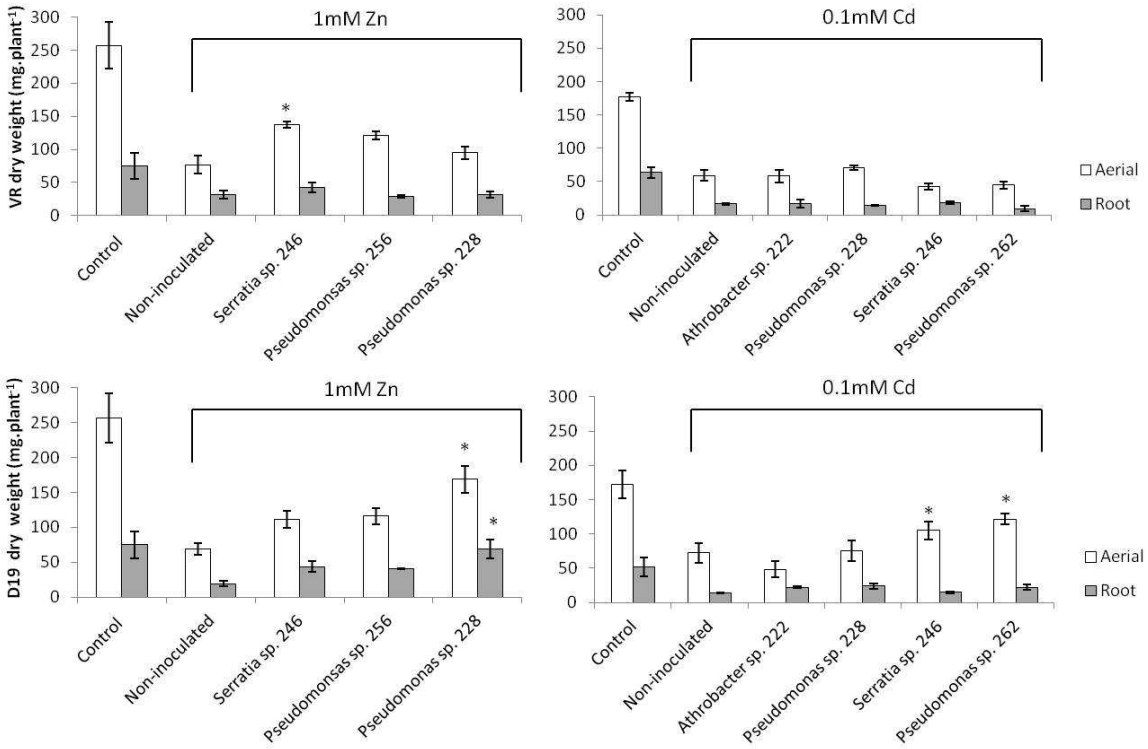


Figure 6.3. Dry weight (mg.plant^{-1}) of VR and D19 *H. tuberosus* cultivar-clones after 3 weeks of growth with 1mM Zn or 0.1mM Cd, under hydroponic conditions. (*) shows significant differences between inoculated and non-inoculated after Duncan's test, $p < 0.05$; mean values ± SE; n=4.

Table 6.1. Metal tolerance and PGP characteristics of selected bacterial strains for inoculation in *H.tuberosus* under hydroponic conditions with Cd and Zn.

Comp.	Strain	Identification	Accesion	Zn 1mM	Cd 0.8mM	Fe0µM	Fe0.25µM	OA	ACC	IAA	Ace	Psol	N fix
Soil	222	*Arthrobacter* sp.	EU086826	+++	+++	-	-	++	+++	-	-	-	+
Root	228	*Pseudomonas* sp.	GU595312	++	++	+	+	+	++	+	-	++	-
Root	246	*Serratia* sp.	HM596429	+++	+++	+	+	++	+++	++	-	-	-
Root	256	*Pseudomonas* sp.	GU595312	+++	+	+	+	-	+	++	+	+++	++
Root	262	*Pseudomonas* sp.	GU595312	+	+	++	-	+	+++	++	+	-	-

Compartment of origin of the strain (Comp.), growth in the presence of Zn (1mM) and Cd (0.8mM), siderophores (Fe0µM and Fe0.25µM), Organic acids (OA), ACC (ACC deaminase activity), IAA (indole-3-acetic acid), Ace (Acetoin), phosphate solubilization (Psol), nitrogen fixation (N fix). + low, +++ high production.

In our work, the inoculated bacterial strains showed plant growth-promoting characteristics as production of indole-3-acetic acid, acetoin and ACC-deaminase activity that could improve the plant growth (Table 6.1). The production of IAA and acetoin can stimulate root formation (Duan et. al., 2013, Glick et al., 2010), and thereby increase the nutrients absorption by the plant. ACC (1-aminocyclopropane-1-carboxylic acid) deaminase activity can reduce the ethylene levels generated as consequence of stress, improving the growth of the plant in presence of toxic metals (Glick, 2003). It is important to mention that the endophytic bacterial strains that increased the growth in *H. tuberosus* also increased the root length of *Brassica napus* seedlings in vertical agar plates with Cd and Zn (See Chapter 5). This suggests that these endophytes are able to be beneficial for plants from different families and genera and in this case a specific relation with the initial host plant is not necessary.

The bacterial inoculation affected the metal uptake of both cultivar-clones of *H. tuberosus* (Table 6.2). The Zn concentration significantly decreased in roots of VR cultivar-clone inoculated with *Pseudomonas* sp. 228. In D19 cultivar-clone no significant differences were found between inoculated and non-inoculated plants. However it was observed that the Zn concentration tended to decrease in roots of inoculated plants of both cultivars, although no significant differences were found in these cases. In case of Cd, the effect was more variable. The inoculation of *Pseudomonas* sp. 228 significantly increased Cd concentration in roots of D19 compared to non-inoculated plants. In contrast, the addition of *Pseudomonas* sp. 262 and *Arthrobacter* sp. 222 decreased the Cd concentration in the roots of VR. *Serratia*

sp. 246 and *Pseudomonas* sp. 262 also decreased the concentration of Cd in the aerial parts of VR and roots of D19, respectively.

Metals are almost entirely available to plants grown on hydroponic culture. Thereby, the effect of the bacteria on the plant uptake could be masked because of the high metal uptake that usually occurs in hydroponic cultures. Wan et al. (2012) did not observe significant differences in the Cd uptake by hydroponically grown *Solanum nigrum* after *Serratia nematodiphila* LRE07 inoculation in presence of high Cd concentrations. These authors concluded that the effect of the strain was more significant at low concentrations (10µM of Cd).

Table 6.2. Total metal concentrations ($mg.kg^{-1}$ DM) in two cultivar-clones of *H. tuberosus* grown in absence (control) and in presence of 1mM Zn or 0.1mM Cd.

Treatments		VR		D19	
		Zn			
		Aerial	Root	Aerial	Root
Zn	Control	58 ± 14a	40 ± 10a	75 ± 23a	41 ± 5a
	Non-inoculated	1533 ± 149b	4533 ± 945c	1097 ± 175b	3862 ± 1063bc
	Serratia sp. 246	1155 ± 23b	4195 ± 355bc	1283 ± 207b	3455 ± 1767b
	Pseudomonas sp. 256	1349 ± 183b	4368 ± 442bc	1554 ± 299b	3484 ± 651b
	Pseudomonas sp. 228	975 ± 154b	2237 ± 368b	1317 ± 177b	3504 ± 1167b
		Cd			
Cd	Control	0.43 ± 0.09a	1.2 ± 0.2a	0.6 ± 0.1a	0.5 ± 0.2a
	Non-inoculated	152 ± 10c	1118 ± 177def	106 ± 44bc	889 ± 196cde
	Arthrobacter sp. 222	83 ± 6bc	492 ± 85bc	58 ± 8b	631 ± 140bc
	Pseudomonas sp. 228	112 ± 23bc	1250 ± 320ef	106 ± 18bc	1365 ± 145f
	Serratia sp. 246	24 ± 4b	908 ± 314cde	129 ± 45c	798 ± 65bcd
	Pseudomonas sp. 262	145 ± 29c	487 ± 57bc	81 ± 5bc	383 ± 107b

Different letters represent significant differences per column and cultivar-clone, after Tukey's test, $p < 0.05$; mean values ± SE; n=4.

On the other hand, the decrease in Cd and Zn uptake by inoculated plants could be due to the capacity of some bacteria to adsorb and immobilize toxic ions from the solution through the production of extracellular polysaccharides and proteins that can bind and precipitate metals (Burd et al., 1998). It this way, the bacteria also can reduce the phytotoxic effects of the metals improving the plant growth (Madhaiyan et al., 2007; Ma et al., 2011; Rajkumar et al., 2009). Several authors have observed this effect in

different plants and growth conditions. Marques et al. (2013) observed that the Cd and Zn uptake decreased in roots of *Helianthus annuus* after inoculation with *Chrysiobacterium humi* isolated from a Cd-Zn contaminated soil. They attributed this effect to the fact that some bacteria can share the metal load with the plant, decreasing thereby the metal phytoextraction. Tripathi et al. (2005) observed that the growth of *Phaseolus vulgaris* enhanced with the inoculation of *Pseudomonas putida* KNP9 in a soil spiked with Cd and Pb. They suggested that the increase of growth was possibly due to the decrease of the metal uptake by the plant. Vivas et al. (2006) reported that the inoculation of *Brevibacillus* sp. alleviated the toxicity of Zn in *Trifolium repens* by reducing the metal uptake by the plant growing on a Zn contaminated soil. Aafi et al. (2012) reported that inoculation with *Serratia* sp. MSMC541 decreased the metal translocation of *Lupinus luteus* in a soil spiked with As, Cd, Pb and Zn. They concluded that this strain can protect the plant against metal toxicity by reducing their uptake and thereby, promoting the plant growth. In our work, the plant biomass of D19 increased in presence of *Pseudomonas* sp. 228 under Zn exposure, and after addition of *Pseudomonas* sp. 262 and *Serratia* sp. 246 in presence of Cd. In the case of VR, the biomass was also increased in plants inoculated with *Serratia* sp. 246 in presence of Zn. The concentration of both metals tended to decrease in roots of plants inoculated with these bacteria, although this decrease was only significant in the case of D19, after inoculation with *Pseudomonas* sp. 262 in presence of Cd. Taking into account this trend, we support the hypothesis that in these conditions the bacteria could improve the plant growth through decreasing metal uptake and thereby phytotoxicity.

6.4.2. Nutrient status

In general, the inoculated bacterial strains had no effect on the nutrient uptake of both cultivar-clones (Table 6.3 to Table 6.6), but there were some exceptions. Macro-nutrients as Na and Ca were significantly lower in roots of VR and D19, respectively, when plants were inoculated with *Serratia* sp. 246, *Pseudomonas* sp. 228 and 256 in presence of 1mM of Zn (Table 6.3). In the case of 0.1mM of Cd, the addition of *Arthrobacter* sp. 222, *Serratia* sp. 246, *Pseudomonas* sp. 228 and 262 decreased the K concentrations in aerial parts of VR (Table 6.4). Also, the Na concentration in the aerial part of D19 was significantly lower after inoculation of *Serratia* sp. 246.

Table 6.3. Macro-nutrient concentration ($g \cdot 100g^{-1}$ DM) in aerial part and roots of two cultivar-clones of *H. tuberosus* grown in absence (control) and presence of Zn (1 mM).

			Zn (1mM)			
		Control	Non-inoculated	*Serratia* sp. 246	*Pseudomonas* sp. 256	*Pseudomonas* sp. 228
			Aerial			
VR	Ca	2.8 ± 0.5 c	1.8 ± 0.2 abc	1.6 ± 0.18 abc	1.5 ± 0.2 ab	1.7 ± 0.3 abc
	K	5.7 ± 0.8 b	4.1 ± 0.7 a	3.0 ± 0.5 a	2.8 ± 0.3 a	2.7 ± 0.4 a
	Mg	0.7 ± 0.1 c	0.52 ± 0.05 abc	0.49 ± 0.09 abc	0.40 ± 0.05 ab	0.45 ± 0.09 ab
	Na	0.09 ± 0.03 b	0.06 ± 0.01 a	0.09 ± 0.02 ab	0.09 ± 0.01 ab	0.09 ± 0.01 ab
D19	Ca	2.5 ± 0.5 bc	1.5 ± 0.3 ab	1.2 ± 0.4 a	1.3 ± 0.4 a	1.2 ± 0.3 a
	K	4.0 ± 0.5 b	2.9 ± 0.5 a	2.7 ± 0.4 a	2.7 ± 0.2 a	2.8 ± 0.19 a
	Mg	0.8 ± 0.1 bc	0.36 ± 0.09 a	0.33 ± 0.06 a	0.37 ± 0.07 a	0.42 ± 0.06 ab
	Na	0.09 ± 0.01 ab	0.08 ± 0.01 ab	0.07 ± 0.01 ab	0.04 ± 0.01 a	0.05 ± 0.01 a
			Root			
VR	Ca	0.04 ± 0.01 a	0.6 ± 0.2 ab	0.22 ± 0.02 ab	0.30 ± 0.05 b	0.24 ± 0.04 ab
	K	1.5 ± 0.5 ns	1.8 ± 0.4	1.50 ± 0.07	1.4 ± 0.1	1.5 ± 0.1
	Mg	0.29 ± 0.04 ns	0.4 ± 0.07	0.15 ± 0.02	0.16 ± 0.03	0.15 ± 0.02
	Na	0.21 ± 0.04 ab	0.36 ± 0.02 b	0.10 ± 0.01 a	0.11 ± 0.01 a	0.12 ± 0.01 a
D19	Ca	0.11 ± 0.02 ab	0.58 ± 0.19 c	0.23 ± 0.03 ab	0.19 ± 0.05 ab	0.08 ± 0.04 a
	K	1.6 ± 0.5 ns	2.3 ± 0.5	1.3 ± 0.2	1.4 ± 0.1	1.9 ± 0.6
	Mg	0.3 ± 0.1 ns	0.13 ± 0.04	0.16 ± 0.02	0.15 ± 0.02	0.14 ± 0.05
	Na	0.16 ± 0.04 ab	0.16 ± 0.05 ab	0.12 ± 0.01 a	0.12 ± 0.02 a	0.09 ± 0.03 a

Different letters represent significant differences per row and cultivar-clone, after Tukey's test, $p < 0.05$; mean values ± SE; n=4. ns: not significant.

Table 6.4. Macro-nutrient concentration ($g \cdot 100g^{-1}$ DM) in aerial part and roots of two cultivar-clones of *H. tuberosus* grown in absence (control) and presence of Cd (0.1mM).

			Cd (0.1mM)				
		Control	Non-inoculated	*Athrobacter* sp. 222	*Pseudomonas* sp. 228	*Serratia* sp. 246	*Pseudomonas* sp. 262
Aerial							
VR	Ca	2.7 ± 0.1 d	1.9 ± 0.5 bc	1.2 ± 0.1 ab	0.9 ± 0.1 ab	0.6 ± 0.1 ab	1.1 ± 0.1 ab
	K	13 ± 1 d	12.9 ± 0.4 d	9 ± 1 bc	5 ± 1 a	3.8 ± 0.2 a	8 ± 1 b
	Mg	0.65 ± 0.04 cd	0.57 ± 0.04 abc	0.56 ± 0.06 abc	0.37 ± 0.07 a	0.44 ± 0.06 ab	0.60 ± 0.07 bcd
	Na	0.014 ± 0.003 a	0.031 ± 0.008 ab	0.05 ± 0.01 ab	0.017 ± 0.003 a	0.029 ± 0.008 a	0.05 ± 0.01 ab
D19	Ca	2.4 ± 0.6 cd	1.4 ± 0.5 abc	1.1 ± 0.3 ab	1.1 ± 0.1 ab	1.6 ± 0.2 abc	0.7 ± 0.1 a
	K	11 ± 1 cd	7 ± 2 b	7 ± 2 bc	8 ± 1 b	8.2 ± 0.3 bc	5 ± 1 a
	Mg	0.79 ± 0.09 d	0.53 ± 0.09 abc	1.1 ± 0.1 abc	1.10 ± 0.02 abc	1.6 ± 0.06 cd	0.53 ± 0.09 abc
	Na	0.027 ± 0.007 ab	0.042 ± 0.009 ab	0.04 ± 0.01 bc	0.04 ± 0.01 ab	0.08 ± 0.02 c	0.032 ± 0.008 ab
Root							
VR	Ca	0.38 ± 0.04 ab	0.4 ± 0.1 abc	0.29 ± 0.05 a	0.58 ± 0.03 bc	0.4 ± 0.1 abc	0.5 ± 0.1 c
	K	7 ± 1 a	5 ± 1 bc	3 ± 1 ab	5 ± 1 abc	4 ± 1 ab	3 ± 1 a
	Mg	0.29 ± 0.05 ab	0.17 ± 0.03 ab	0.17 ± 0.02 ab	0.24 ± 0.01 ab	0.17 ± 0.02 ab	0.23 ± 0.02 ab
	Na	0.06 ± 0.01 ab	0.1 ± 0.01 ab	0.16 ± 0.05 ab	0.08 ± 0.02 ab	0.072 ± 0.003 ab	0.09 ± 0.01 ab
D19	Ca	0.37 ± 0.04 ab	0.6 ± 0.2 bc	0.46 ± 0.03 abc	0.6 ± 0.1 bc	0.6 ± 0.1 bc	0.6 ± 0.1 bc
	K	4 ± 1 ab	4 ± 1 ab	3 ± 1 ab	3 ± 1 ab	3 ± 1 ab	2.5 ± 0.6 a
	Mg	0.15 ± 0.04 b	0.15 ± 0.03 a	0.15 ± 0.01 a	0.21 ± 0.05 ab	0.2 ± 0.02 ab	0.15 ± 0.03 a
	Na	0.15 ± 0.04 b	0.099 ± 0.004 ab	0.07 ± 0.01 a	0.09 ± 0.01 ab	0.09 ± 0.02 ab	0.08 ± 0.01 ab

Different letters represent significant differences per row and cultivar-clone, after Tukey's test, $p < 0.05$; mean values ± SE; n=4. ns: not significant.

Micro-nutrient concentrations were also modified in some cases after bacterial inoculation. The addition of *Serratia* sp. 246, *Pseudomonas* sp. 228 and 256 significantly decreased the Cu and Fe concentration in roots of VR and D19 respectively, when plants were grown with Zn (Table 6.5). The concentrations of Cu in roots of VR were also lower after inoculation with *Arthrobacter* sp. 222, *Serratia* sp. 246 and *Pseudomonas* sp. 262 under Cd exposure (Table 6.6). However, *Serratia* sp. 246 increased the Fe content in the aerial part of VR in presence of Zn. This last strain also increased the plant biomass of VR in presence of Zn. The decrease in the nutrient concentration found in the other cases, could be related to a bacterial mechanism of metal sequestration and/or biosorption. Microorganisms have developed complex mechanisms of metal resistance that can affect the metal and nutrients availability (Nies, 1999, Bruins et al., 2000). PGB bacteria can sequestrate elements through extracellular production of polysaccharides or by fixing elements such as Fe or Cu on the membrane, cell wall or capsule in the form of hydroxides or some other insoluble metal salts (Chen and Cutright, 2003; Kidd et al., 2009; Ma et al., 2011). The bacterial surfaces present polarizable groups that can interact with cations, being responsible of the binding capacity (Vechio et al., 1998). In our work, the excess of Cd and Zn could induce the bacterial mechanisms of metal resistance that reduce the metal availability and also the solubility of other nutrients that could be precipitated on the cell surface.

The siderophore synthesis is stimulated in presence of toxic metals, in order to supply the appropriate amount of ions to the plant and reduce the phytotoxicity symptoms (Glick and Bashan, 1997; Rajkumar et al., 2009). This PGP characteristic plays an important role under soil conditions, where the nutrients are present in for plants unavailable forms, so it can be expected that under hydroponical conditions the effect of the bacteria in the nutrient uptake is less visible, since the Fe is supplied in the appropriate concentration with the nutrient solution. Thereby, the differences observed in the nutrient absorption could also be due to the imbalance of nutrients created by the presence of metals in the solution.

Table 6.5. Micro-nutrient concentration (mg.kg^{-1} DM) in aerial part and roots of two cultivar-clones of *H. tuberosus* grown in absence (control) and presence of Zn (1 mM).

			Zn (1mM)			
		Control	Non-inoculated	*Serratia* sp. 246	*Pseudomonas* sp. 256	*Pseudomonas* sp. 228
			Aerial			
VR	Cu	13.8 ± 1.6 abc	17.5 ± 1.3 c	14.1 ± 1.8 abc	14.1 ± 1.3 abc	17.3 ± 1.9 c
	Fe	194.1 ± 23.0 e	89.2 ± 12.6 bc	148.4 ± 13.7 d	130.5 ± 26.7 cd	82.8 ± 25.1 abc
	Mn	11.3 ± 1.7 abc	14.3 ± 2.8 abc	10.8 ± 0.7 ab	8.5 ± 1.0 a	16.6 ± 6.1 abc
D19	Cu	16.8 ± 2.8 bc	10.4 ± 1.4 a	10.6 ± 0.6 ab	13.4 ± 2.0 abc	10.4 ± 4.1 a
	Fe	145.1 ± 13.8 a	80.2 ± 11.2 abc	44.5 ± 13.6 ab	35.5 ± 6.8 a	67.3 ± 8.3 ab
	Mn	44.5 ± 4.0 d	15.7 ± 3.1 abc	16.7 ± 6.3 abc	21.1 ± 5.8 bc	22.6 ± 5.3 c
			Root			
VR	Cu	17.3 ± 5.3 ab	18.9 ± 2.6 c	9.8 ± 0.3 ab	12.4 ± 2.6 b	11.4 ± 0.6 ab
	Fe	118.0 ± 36.4 ab	102.2 ± 18.9 ab	91.9 ± 7.8 ab	83.3 ± 5.9 ab	83.2 ± 8.1 ab
	Mn	2.2 ± 0.2 a	2.4 ± 0.1 ab	1.79 ± 0.05 ab	1.6 ± 0.1 a	1.8 ± 0.3 ab
D19	Cu	10.4 ± 1.5 ab	10.6 ± 3.0 ab	9.3 ± 3.0 ab	13.5 ± 2.6 b	6.8 ± 1.1 a
	Fe	141.5 ± 27.1 b	113.6 ± 21.7 b	71.5 ± 6.7 a	63.4 ± 6.7 a	62.4 ± 11.3 a
	Mn	6.1 ± 0.7 c	4.5 ± 1.3 bc	2.8 ± 0.2 ab	3.0 ± 0.5 b	3.3 ± 0.1 ab

Different letters represent significant differences per row and cultivar-clone, after Tukey's test, $p < 0.05$; mean values ± SE; n=4.

Table 6.6. Micro-nutrient concentration (mg.kg^{-1} DM) in aerial part and roots of two cultivar-clones of *H. tuberosus* grown in absence (control) and presence of Cd (0.1mM).

			Cd (0.1mM)				
		Control	Non-inoculated	*Athrobacter* sp. 222	*Pseudomonas* sp. 228	*Serratia* sp. 246	*Pseudomonas* sp. 262
Aerial							
VR	Cu	7.4 ± 1.0 abc	8.0 ± 2.0 abc	3.0 ± 1.0 a	3.5 ± 0.3 ab	8.0 ± 0.9 abc	8.8 ± 1.3 bc
	Fe	65.9 ± 8.7 de	33.2 ± 10.3 abcd	11.6 ± 7.1 a	25.0 ± 4.7 abc	20.7 ± 7.0 ab	48.1 ± 9.0 abcd
	Mn	11.8 ± 1.1 c	3.2 ± 0.4 ab	2.4 ± 0.6 a	1.5 ± 0.4 a	1.5 ± 0.6 a	8.0 ± 2.1 b
D19	Cu	10.5 ± 2.3 c	9.4 ± 1.4 c	5.1 ± 0.8 abc	8.2 ± 1.6 abc	6.6 ± 1.2 abc	10.2 ± 3.3 c
	Fe	93.2 ± 16.9 e	48.7 ± 10.1 bcd	41.4 ± 7.7 abcd	32.0 ± 6.7 abcd	52.3 ± 12.8 cd	47.4 ± 9.4 bcd
	Mn	12.5 ± 2.1 c	3.8 ± 0.5 ab	1.3 ± 0.3 a	1.1 ± 0.3 a	2.6 ± 0.2 a	1.5 ± 0.4 a
Root							
VR	Cu	14.8 ± 1.3 bc	19.5 ± 4.3 c	4.4 ± 1.4 a	17.1 ± 2.6 bc	11.3 ± 5.8 ab	9.8 ± 0.8 ab
	Fe	193.8 ± 46.9 c	62.4 ± 13.7 b	63.2 ± 2.1 b	65.6 ± 13.2 b	60.0 ± 25.4 b	68.8 ± 16.4 b
	Mn	3.4 ± 0.9 c	0.9 ± 0.3 a	1.0 ± 0.2 ab	2.6 ± 0.6 abc	1.0 ± 0.1 ab	3.2 ± 0.7 bc
D19	Cu	10.2 ± 1.4 ab	10.3 ± 3.1 ab	5.5 ± 0.8 a	10.1 ± 3.1 ab	10.8 ± 1.6 ab	3.0 ± 1.1 a
	Fe	154.8 ± 49.6 c	34.3 ± 7.6 ab	47.3 ± 5.7 ab	68.7 ± 19.0 ab	52.7 ± 12.2 ab	10.1 ± 0.8 a
	Mn	2.7 ± 0.7 abc	1.5 ± 0.7 abc	1.9 ± 0.4 abc	2.2 ± 1.0 abc	0.5 ± 0.2 a	1.1 ± 0.4 ab

Different letters represent significant differences per row and cultivar-clone, after Tukey's test, $p < 0.05$; mean values ± SE; n=4.

6.4.3. Lipid peroxidation

TBA reactive compounds are produced as a consequence of peroxidation of lipids in the membrane. This process is initiated by free radicals due to oxidative stress. An increase of the content of TBA reactive compounds is an indicator for physiological stress (Li et al., 2013). Many studies have reported that levels of TBA reactive compounds are increased in plants exposed to metals as Cd, Zn, Pb (Wang et al., 2009; Bauddh et al., 2012, Li et al., 2013).

In the present work, the exposure to 1 mM Zn and 0.1 mM Cd significantly enhanced the TBA reactive compounds in roots of both cultivar-clones of *H. tuberosus* after 3 weeks of growth, since significant differences were found between controls plants and plants exposed to metals (Figure 6.4a, b). However, there were no significant differences in the content of TBA reactive compounds in leaves of plants grown in presence of metals in comparison to control plants. Nouairi et al. (2009) also observed this effect in leaves of *Brassica juncea* grown with 50 µM Cd. According to them, this observation could be related with an interesting mechanism of tolerance of the plant to avoid the oxidative stress generated by the presence of metals in the leaves. The reduction of the concentration of TBA reactive compounds has been described by some authors to result from an increase in the anti-oxidative enzyme activities, which reduce H_2O_2 levels and the damage in the membrane (Zhang et al., 2007).

On the other hand, the addition of *Serratia* sp. 246, *Pseudomonas* sp. 256 and 228 decreased the content of TBA reactive compounds in roots of D19 cultivar-clone grown in presence of Zn (Figure 6.4a). The roots of VR also showed lower levels of TBA reactive compounds in plants inoculated with *Pseudomonas* sp. 228. In the case of Cd, no significant differences were found regarding the content of TBA reactive compounds between inoculated and non-inoculated plants (Figure 6.4b). The decrease in the content of TBA reactive compounds after PGP bacteria inoculation has been reported by several authors in different plants. Pandey et al. (2013) reported that the inoculation of *Ochrobactrum* strain CdSP9 decreased the content of TBA reactive compounds in *Oryza sativa* exposed to Cd under hydroponic conditions. Wan et al. (2012) also observed that the addition of *Serratia nematodiphila* LRE07 decreased the concentration of TBA reactive compounds in *Solanum nigrum* in presence of Cd under hydroponic conditions.

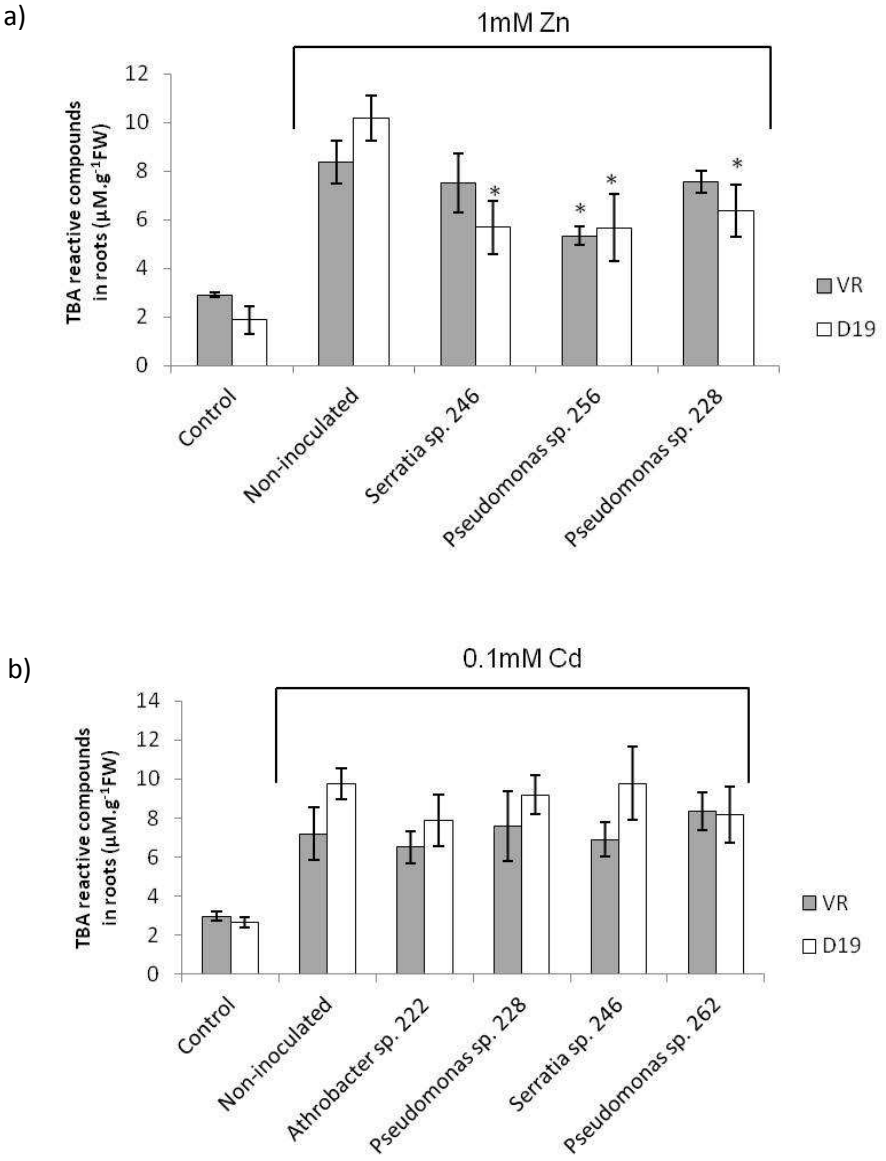


Figure 6.4. TBA reactive compounds ($\mu M \cdot g^{-1}$ FW) in roots of VR and D19 *H. tuberosus* cultivar-clones after 3 weeks of growth with 1mM of Zn (a) and 0.1mM Cd (b), under hydroponic conditions. (*) shows significant differences between inoculated and non-inoculated after Duncan's test, $p < 0.05$; mean values ± SE; n=4.

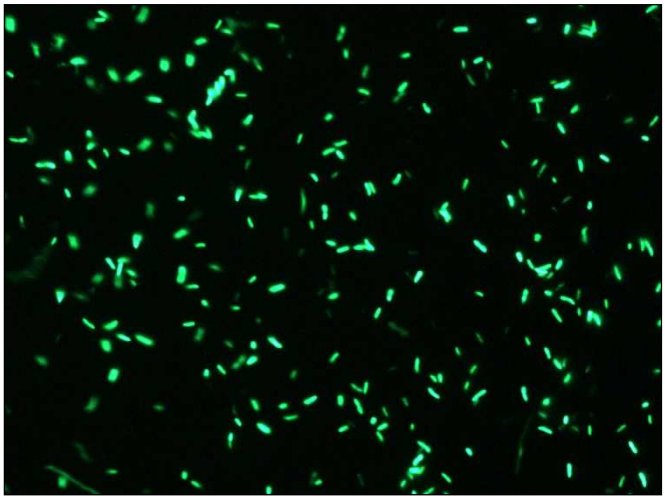
These results suggest that the inoculated bacteria can assist the plant to control the oxidative damage under metal exposure. In spite of this, it is important to mention that in our study the concentration of Zn tended to decrease in inoculated plants, and this fact could also explain the decrease in the content of TBA reactive compounds in plants inoculated with bacteria.

6.4.4. Colonization of egfp: tetracycline® *Pseudomonas* sp. 262 in the roots of *H. tuberosus*

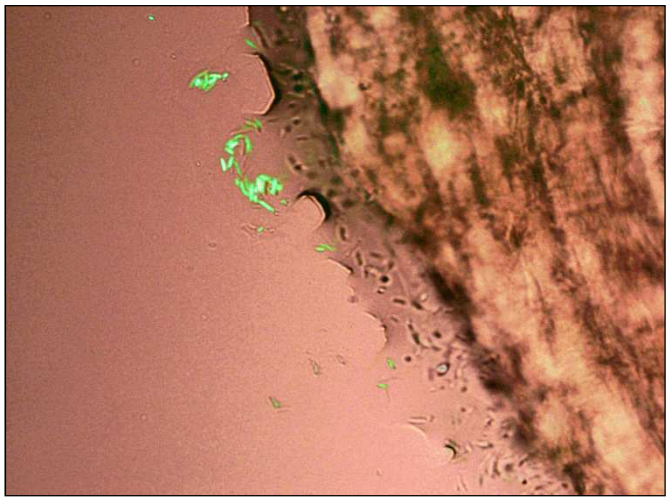
Pseudomonas sp. strain 262 was able to grow with 0.8 mM of Cd and showed the capacity to produce siderophores (in the absence of iron), organic acids, indole acetic acid, acetoin and ACC deaminase (Table 6.1). Moreover, this bacterial strain increased the aerial biomass of D19 cultivar-clone of *H. tuberosus* when exposed to 0.1 mM Cd. Taking this into account, this strain was selected to be labeled with the enhanced green fluorescent protein (egfp): tetracycline® plasmid in order to study the bacterial colonization in the roots of *H. tuberosus*.

Figure 6.5A shows that the conjugation was effective, since *Pseudomonas* sp. 262 showed fluorescence after blue light (488 nm) excitation, and was able to grow in the presence of tetracycline ($20 \mu g.ml^{-1}$). In Figure 6.5B and C the bacterial strains can be visualized around the root of *H. tuberosus* after inoculation under hydroponic conditions. In Figure 6.6A, egfp-*Pseudomonas* sp. 262 can be visualized as single cells attached to the root hair surface. Bacterial cells were not found in the root interior when the root was transversally and longitudinally explored (Figure 6.6B and C). Root exudates under hydroponic conditions vary from soil, modifying the amount of nutrients available to the bacteria (Compant et al., 2010) and also, the colonization pattern. Ma et al. (2011) reported that *Pseudomonas* sp. A3R3 isolated from roots of *Alyssum serpyllifolium* showed a high level of colonization in shoot and root interior of *Brassica juncea*. He et al. (2013) also observed that *Rahnella* sp. JN6 originally isolated from *Polygonum pubescens* could colonize the root, stem and leaf tissues of *Brassica napus*. *Rahnella aquatilis* SPb, an endophytic bacterial strain from *Ipomoea batatas* was inoculated in hybrid poplar and increased the growth of the cuttings with respect no non-inoculated, showing the beneficial effects of the strains in the plant growth of another plant not related with the initial host plant (Khan and Doty, 2009).

A)



B)



C)

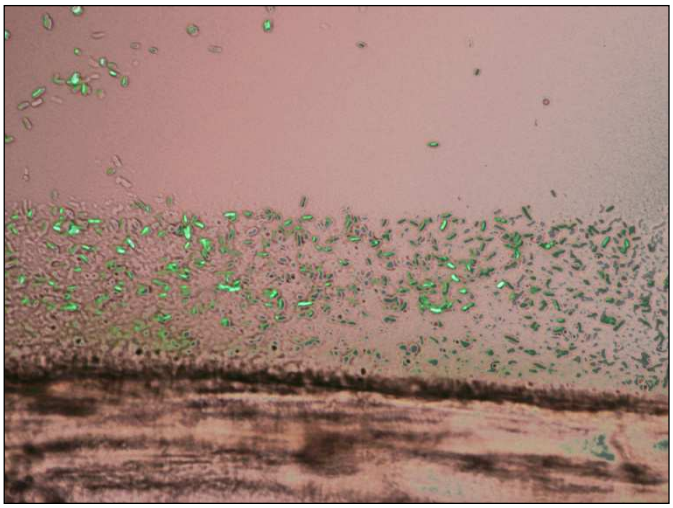
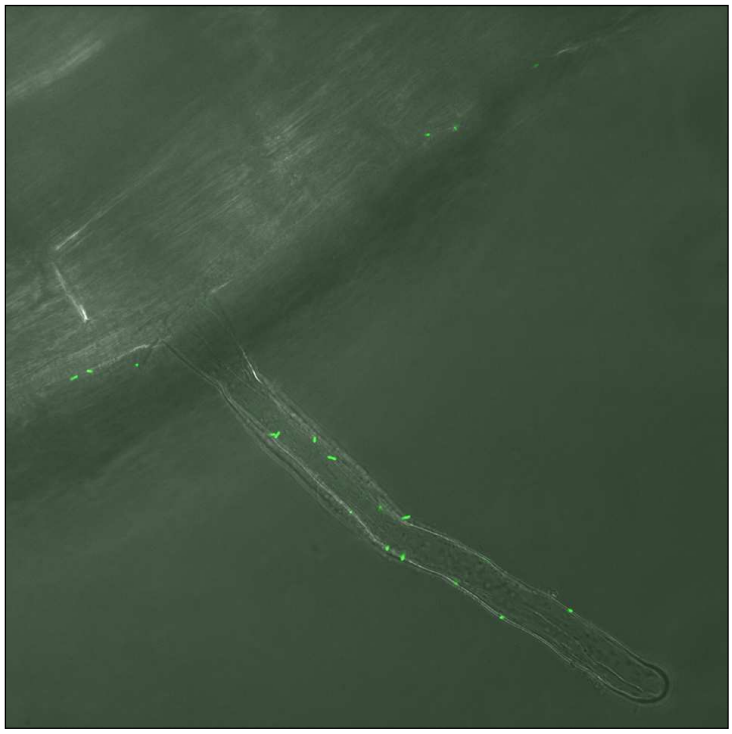
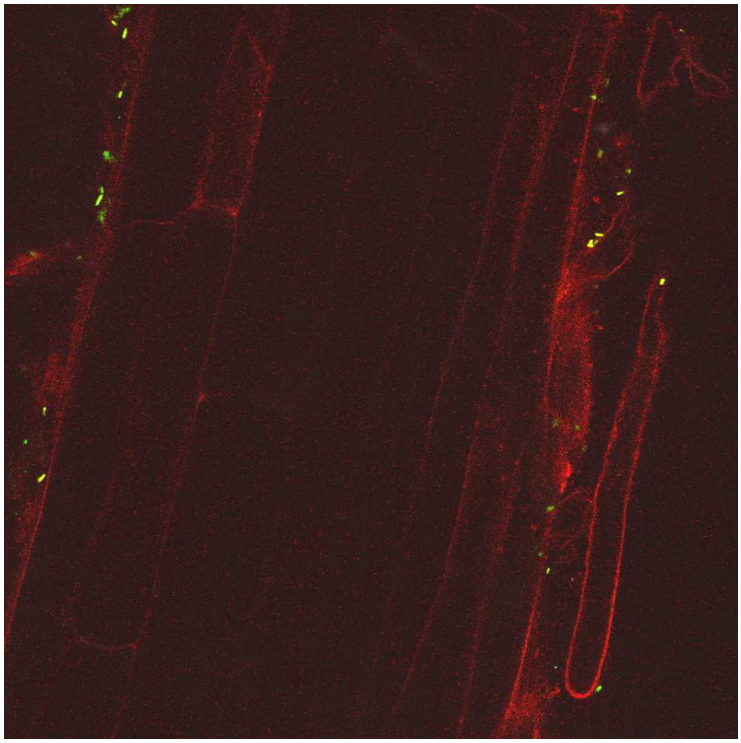


Figure 6.5. A: Solution with egfp-labeled *Pseudomonas* sp. strain 262 with blue light (488 nm) excitation. **B, C**: egfp-*Pseudomonas* sp. strain 262 and root of *H. tuberosus* 1week-plants. The inoculation was carried out in hydroponic conditions with coarse perlite, under greenhouse conditions. The picture was taken two days after the inoculation.

A)



B)



C)

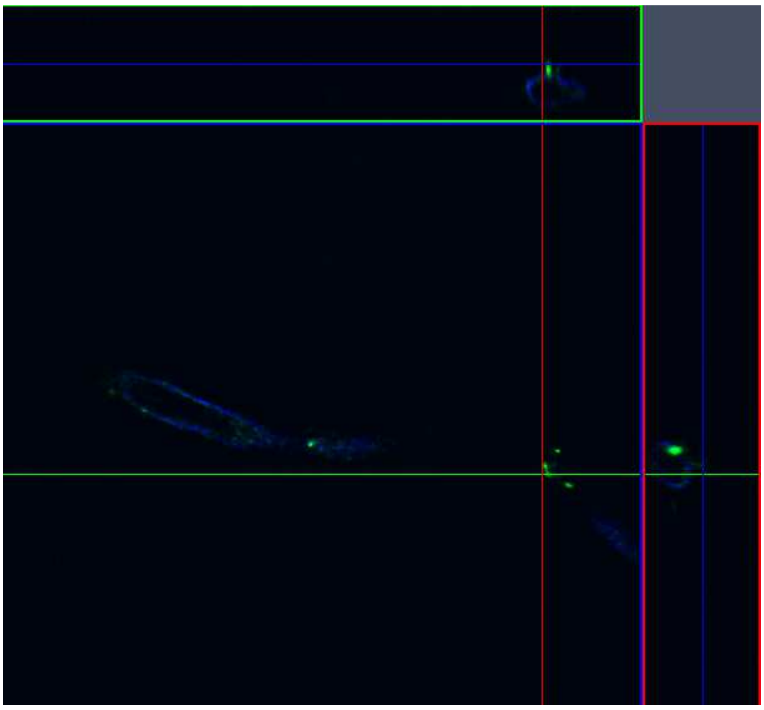


Figure 6.6. Confocal image of live egfp-labeled *Pseudomonas* sp. strain 262 in the root of a freshly prepared intact *Helianthus tuberosus* root. Bacteria with blue light (488 nm) excitation. **A**: Single cells attached to the root hair. **B**: Longitudinal cut of the root (in red), single cells attached to the root surface. **C**: Ortho-image of the root (in blue).

Although the endophytic colonization of bacteria in non-host plants has been reported in numerous works, in our study, the bacterial strain egfp-labeled did not colonize the root interior of *H. tuberosus* in the studied conditions. Weyens et al. (2012) compared the colonization of wild-type *Pseudomonas putida* W619 and the same gfp-labeled strain in *Populus deltoids* x (*trichocarpa* x *deltoids*). They observed that the colonization of the gfp-labeled strain was lower than the wild-type, and concluded that gfp-labeling can result in a different colonization pattern in comparison with the wild-type. In this work, the inoculated bacterial strains were attached to the root hair surface. It is important to mention that this bacterial strain increased growth of D19 cultivar-clone of *H. tuberosus* under 0.1mM Cd exposure, in hydroponic conditions. This positive effect on the growth and the visual result of the bacteria attached to the root hair surface show that there was a clear interaction between bacteria and plant during the experiment.

6.5. Conclusions

The effect of the bacterial strains on *H. tuberosus* growth was different depending on the metal, inoculated bacterial strains and cultivar-clone in our experimental conditions. The improvement of growth and the decrease of the metal-induced stress were more pronounced in D19 cultivar-clone than in VR. Three endophytes of *Brassica napus* enhanced the growth of D19 cultivar-clone in presence of Cd and Zn. Only *Pseudomonas* sp. 228 increased Cd uptake. After observation using confocal microscopy, we could observe that egfp-*Pseudomonas* sp. 262 did not colonize the root interior of *H. tuberosus*, under hydroponic conditions. However, the bacterial strains were attached to the roots and roots hairs surface of *H. tuberosus*, suggesting that interaction between them was established. D19 in combination with *Pseudomonas* sp. 228, *Serratia* sp. 246 and *Pseudomonas* sp. 262 could be a suitable strategy to be used on Cd-Zn contaminated soils due to the improvement of biomass reached by bacterial inoculation. Additional experiments in soils need to be performed in order to evaluate the efficiency of this strategy when there is competence with other microorganisms and the edaphic process interferes with the metal and nutrients availability.

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Chapter 7

Improvement of growth of *Helianthus tuberosus* L. on a metal-contaminated soil by exploiting plant growth-promoting bacteria

7.1. Abstract

Five plant growth-promoting bacterial strains isolated from *Brassica napus* were inoculated in *Helianthus tuberosus* (D19 cultivar-clone) growing on a Cd-Zn contaminated soil in order to assess their efficiency to improve plant biomass and metal uptake. The bacterial strains were added individually and as a consortium. Consortium inoculation increased the biomass, the Pb and Zn uptake in the roots of *H. tuberosus*, and the activities of the malic enzyme and isocitrate dehydrogenase. Also, the glutathione reductase activitiy was higher in leaves of plants inoculated with the consortium. A stimulatory effect on the root hair development was observed after stereomicroscopic observation of plants inoculated with the consortium. Root endophytic bacteria of *Brassica napus* affected the root structure of *H. tuberosus*, indicating that the inoculated bacteria can improve plant growth in a plant species different from the original host plant. *H. tuberosus* showed able to accumulate more than 1000 $mg.kg^{-1}$ of Zn in the aerial parts when grown on a metal contaminated soil. Taking into account the results obtained in this work, *H. tuberosus* in combination with the inoculated consortium could be a feasible strategy to be applied on metal contaminated soils, and mainly in Zn phytoextraction.

Keywords: High biomass crop, Jerusalem artichoke, root hair, consortium, nutrient status.

7.2. Introduction

Environmental pollution by heavy metals has increased due to anthropogenic and industrial activities (Alloway, 1995; Kabir et al., 2012), being nowadays a global problem. According to the European Environment Agency (EEA, 2014), heavy metals represent 35% of the contaminants that affect soils in Europe. Many of these soils have been marginalized because of risk-based legislation (Mench et al., 2010) and they require urgent decontamination actions (Vamerali et al., 2010; Panagos et al., 2013). Some metals are essential nutrients for organisms (Kabata-Pendias, 2011), but in general, they are toxic at high concentrations because they induce oxidative stress and replace metal co-factors in enzymes, affect the biological function of molecules and cause metabolic disorders (Stohs and Bagchi, 1995). Moreover, heavy metals cannot be degraded, they are persistent in the environment and can be accumulated via food chains (Garbisu and Alkorta, 2001; Nawrot et al., 2010).

In the Campine region (northeast of Belgium and south of Netherlands), zinc ore smelters have increased the total concentrations of Cd, Pb and Zn in the agricultural lands due to atmospheric deposition (Witters et al., 2012a). Cd, Zn and Pb represent a serious problem because soil pH is relatively low and the metals bioavailability is high in these areas (Ruttens et al., 2010). Soil thresholds are based on total soil metal concentrations (Adriano et al., 2004), but the ecotoxicological risk in soils is also determined by the metal speciation and bioavailability. Both parameters define the metal concentration that can interact with a biological target (Kayser et al., 2001; Meers et al., 2005). Phytoextraction is a low-cost alternative that uses plants to decrease the concentration and bioavailability of metals and to improve the fertility of the soil (Baker et al., 1994, Chaney et al., 1997). This technology is especially efficient in areas with diffuse and superficial contamination (0-50cm of the soil), such as areas polluted due to aerial deposition (Cunningham and Berti, 1993; Vangronsveld et al., 2009). One of the most critical limitations of this technology is the long time required to clean-up the soil and reach appropriate thresholds (Mulligan et al., 2001). The use of high biomass crops able to extract metals from soil could compensate for the long time required in this technology with the production of valuable biomass (Van Ginneken et al., 2007; Fässler et al., 2010a; Witters et al., 2012b).

Helianthus tuberosus L. (Asteraceae) is a high biomass crop used in bio-ethanol production and vegetatively propagated by tubers (Serieys et al., 2010). Its cultivation shows low production costs and minimal disease problems (Denoroy, 1996; Kays and Nottingham, 2008). Recently, some studies have shown promising results concerning the ability of this crop to grow in presence of metals such as Cd, Pb and Zn (Cui et al., 2007; Chen et al., 2011; Long et al., 2013; Montalbán et al., 2015). In spite of this, its response to metals in contaminated soils is still poorly investigated. All these characteristics make *H. tuberosus* a potential candidate to be used in phytotechnologies, as well as to produce a renewable energy resource.

Plant growth-promoting (PGP) bacteria associated with metal tolerant crops may increase the efficiency of phytoextraction by enhancing biomass production and tolerance of the plants to heavy metals (Germida et al., 1998; Genrich et al., 2000; Rajkumar et al., 2009). These bacteria can improve plant growth (1) indirectly by preventing the growth and/or activity of plant pathogens through competition for space and nutrients (Lugtenberg and Kamilova, 2009), or (2) directly by increasing nutrient uptake and growth through different mechanisms such as nitrogen fixation (Roper and Ladha, 1995), synthesis of phytohormones (as IAA, indole-3-acetic acid) (Dobbelaere et al., 1999), solubilization of minerals, and production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick, 1998; Glick et al., 2003). Some microorganisms are equipped with metal-resistance/sequestration systems, by means of which the bacteria are able to produce natural chelators that can contribute to metal detoxification (Diels et al., 1999). Moreover, some bacteria can increase availability of metals and nutrients by excreting organic acids, that decrease pH in the rhizosphere, or enhancing the Fe(III) mobility and other cations through siderophores production (Glick and Bashan, 1997; Fasim et al., 2002).

The objective of this work was to evaluate the effects of PGP bacterial inoculation on the growth, root structure and metal uptake of D19 cultivar-clone of *H. tuberosus* in a Cd-Zn contaminated soil. The activity of enzymes of the intermediary metabolism and antioxidant enzymes were determined to evaluate the effect of bacterial inoculation on the metal-induced plant stress.

7.3. Material and methods

7.3.1 Pot experiment

7.3.1.1 Soil characterization

A sandy metal polluted soil (86% sand, 9% silt, 5% clay) was collected in the municipally of Lommel (51°12'41'' N; 5°14'32'' E), (Belgium) about 500 m NE of the zinc smelter of Balen.

Fresh soil samples were taken from the surface layer (20-25 cm depth), mixed and homogenized into one composite sample. A soil subsample of 2 kg was dried and sieved through a 2-mm sieve and characterized as follows: electrical conductivity (EC), pH-H_2O and pH-KCl (1 M KCl) were determined in a ratio 1/2.5 soil:water (Walinga et al., 1989). Total N Kjeldahl, was measured according to ISO 11261. Available nutrients (Ca, K, Mg and P) and metals were extracted using 0.1 M $Ca(NO_3)_2$ (Mench et al., 1994). Organic matter content was determined by Walkley-Black method (Allison, 1965). CEC was determined after saturating the soil with NH_4^+, according to Van Ranst et al. (1999). Total soil trace elements were determined using inductively coupled plasma optical emission spectrometry (ICP-OES), after acid digestion in a microwave system (Milestone, 1200 MEGA) according to Van Ranst et al. (1999). Quality of the analyses was verified by including blanks and a reference soil (CRM 143 R SEWAGE SLUDGE AMENDED SOIL, Community Bureau of Reference - BCR N° 230).

7.3.1.2. Pore water solution analysis

Pore water samples of each treatment were taken using Rhizon Soil Moisture Samplers (Rhizon SMS MOM; Eijkelkamp Agrisearch Equipment, Giesbeek, The Netherlands). The rhizons (10cm length, pore diameter 0.12 - 0.18 µm) were inserted into the pot at an angle of 45° (Figure 7.1). Soil pore water solution was extracted by attaching 30 ml plastic syringes to each Rhizon (Meers et al., 2005). Approximately 10 ml of each pore water solution sample was taken to analyze the bioavailable fraction of metals and nutrients, in previously acidified samples with 1 mL HNO_3 (65% Suprapur®). In total, three samplings of pore water were performed. Samples were taken three days after each bacterial inoculation.

Figure 7.1. Rhizons SMS MOM inserted into the pots.

7.3.1.3. Plant material

Tubers of D19 cultivar-clone (Blanc précoce) of *H. tuberosus* were collected in the field collection of IMIDRA (Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario; Madrid, Spain) to perform the soil experiment. The tubers were maintained during two weeks at 4ºC for vernalization before setting up the experiments.

7.3.1.4. PGP bacterial strains

Cultivable bacteria were isolated from rhizosphere, root and stem of *Brassica napus* grown in Zn contaminated soil (See Chapter 5). The bacteria were genotypically characterized, and tested for metal resistance and potential plant growth-promoting characteristics (nitrogen fixation, phosphate solubilization, ACC deaminase activity, production of siderophores, organic acids, acetoin and indole-3-acetic acid). Five bacterial strains (*Arthrobacter* sp. strain 222, *Pseudomonas* sp. strain 228, *Pseudomonas* sp. strain 256, *Pseudomonas* sp. strain 262 and *Serratia* sp. strain 246) were selected to inoculate in *H. tuberosus* D19 cultivar-clone, according to their PGP characteristics (Table 7.1). The bacterial strains were inoculated individually and as consortium (comprising the five bacterial strains). Bacterial strains were grown in 869 liquid medium (Mergeay et al., 1985) at 30°C under shaking.

Table 7.1. Metal tolerance and PGP characteristics of selected bacteria for inoculation in *H.tuberosus* under hydroponic and soil conditions.

Comp.	Strain	Identification	Accesion	Zn 1mM	Cd 0.8mM	Fe0µM	Fe0.25µM	OA	ACC	IAA	Ace	Psol	N fix
Soil	222	*Arthrobacter* sp.	EU086826	+++	+++	-	-	++	+++	-	-	-	+
Root	228	*Pseudomonas* sp.	GU595312	++	++	+	+	+	++	+	-	++	-
Root	246	*Serratia* sp.	HM596429	+++	+++	+	+	++	+++	++	-	-	-
Root	256	*Pseudomonas* sp.	GU595312	+++	+	+	+	-	+	++	+	+++	++
Root	262	*Pseudomonas* sp.	GU595312	+	+	++	-	+	+++	++	+	-	-

Compartment of origin of the strain (Comp.), growth in presence of Zn (1mM) and Cd (0.8mM), siderophores (Fe0µM and Fe0.25µM), Organic acids (OA), ACC (ACC deaminase activity), IAA (indole-3-acetic acid), Ace (Acetoin), phosphate solubilization (Psol), nitrogen fixation (N fix). + low, +++ high production.

7.3.1.5. Growth conditions

The pot experiment was carried out in a greenhouse under controlled conditions. Temperature and humidity were regulated by an automatic ventilating and heating system (25°-30°C temperature, 70-90% relative humidity). Tuber slices with buds were grown in 2L plastic pots filled with Cd-Zn contaminated soil during 4 weeks. The treatments established are shown in Table 7.2. A total of 120 pots were used in the experiment. Two slices with buds were set up per pot. Plants were watered every three days. The bacterial suspension (10^8 cfu mL^{-1}) in tap water was added into the pots after the appearance of the first roots (5 days after sowing). Moreover, bacteria were inoculated once per week, during three weeks.

After four weeks from the first inoculation, plants were harvested, washed thoroughly in tap water and rinsed twice in distilled water. One of the sampled plants was dried to analyze biomass, and total metal concentration (Figure 7.2). The other one was frozen in liquid N_2 to measure the enzyme activities. At the end of the experiment, soil was sampled to analyze the physicochemical properties, including availability of nutrients and metals.

Table 7.2. Experimental design of the pot experiment.

Treatments	nº pots
Soil	10
Soil + *Arthrobacter* sp. 222	5
Soil + *Pseudomonas* sp. 228	5
Soil + *Serratia* sp. 246	5
Soil + *Pseudomonas* sp. 256	5
Soil + *Pseudomonas* sp. 262	5
Soil + Consortium	5
Soil + *H. tuberosus*	20
Soil + *H. tuberosus* + *Arthrobacter* sp. 222	10
Soil + *H. tuberosus* + *Pseudomonas* sp. 228	10
Soil + *H. tuberosus* + *Serratia* sp. 246	10
Soil + *H. tuberosus* + *Pseudomonas* sp. 256	10
Soil + *H. tuberosus* + *Pseudomonas* sp. 262	10
Soil + *H. tuberosus* + Consortium	10

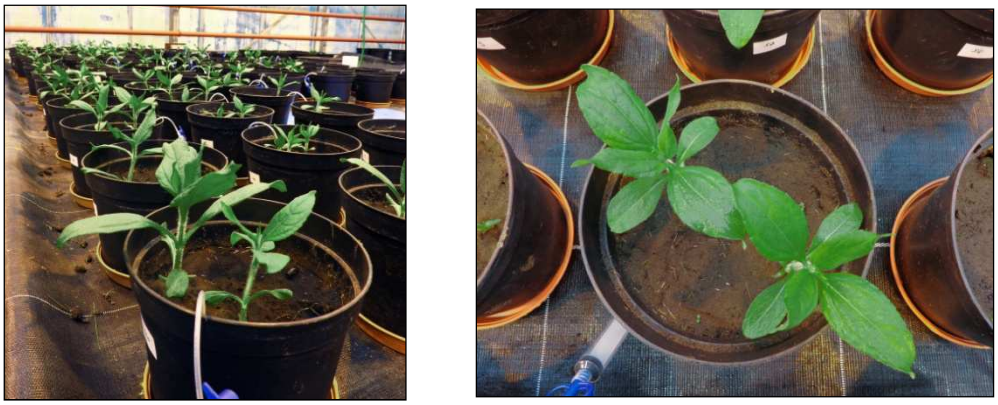

Figure 7.2. *H. tuberosus* plants growing under greenhouse conditions.

7.3.1.6. Enzymatic activities

Roots and the last completely developed leaves of each plant were sampled for enzyme measurements. The plant material was frozen in liquid N_2 during harvest and stored at -80 °C until analysis. Frozen samples (200 mg) of leaves and roots were homogenized under ice-cold conditions using a mortar and pestle. The extraction was performed with 5 mg of insoluble polyvinyl pyrolidone (PVP) in 2 ml 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM DDT and 1 mM EDTA. Subsequently, the extracts were centrifuged during 10 min at 16100 g, 4°C. The enzyme activities were measured spectrophotometrically (Shimadzu UV-1800, UV Spectrophotometer, Shimadzu

Scientific Instruments Inc., Columbia, MD, USA) in the supernatant at 25°C. The glutathione reductase (GR, EC 1.6.4.2) capacity was determined based on the reduction of GSSG in presence of NADPH at 340 nm. NAD(P)H-depending enzymes as malic enzyme (ME, EC 1.1.1.40), NADP-dependent isocitrate dehydrogenase (ICDH, EC 1.1.1.42) and glutamate dehydrogenase (GDH, EC 1.4.1.2) were monitored also at 340 nm (Bergmeyer et al., 1974). Peroxidase (EC 1.11.1.9) activity was analyzed using guaiacol or syringaldazine as a substrate. Guaiacol peroxidase (GPOD) and syringaldazine peroxidase (SPOD) activity were measured at 436 and 530 nm, respectively (Bergmeyer et al., 1974; Imberty et al., 1984).

7.3.1.7. Plant analysis

The plants were separated into leaves, stems and roots, then weighed and dried in a forced air oven for 48 h at 60°C. The dry weights were determined. The dried tissues were grounded and digested according to Weyens et al. (2010). Metal and nutrient concentrations were measured in the digested extracts using inductively coupled plasma optical emission spectrometry (ICP-OES). Quality of the analyses was verified by including blanks and a reference material (Spinach certified reference material; NIST SRM 1570a). The recovery percentages for metals were: Cd (~95%), Pb (~89%) and Zn (~101%).

7.3.1.8. Translocation factor

The translocation factor (TF) was calculated to determine relative translocation of the metals from the root to the aerial part of the plant (Long et al., 2013).

$$TF = [metal]_{aerial\ part} / [metal]_{root}$$

7.3.2. Root morphological analysis

A second experiment was performed in order to compare the root structure of non-inoculated plants with plants inoculated with the consortium. The experiment was carried out in a greenhouse under controlled conditions as mentioned above (25°-30°C temperature, 70-95% relative humidity). Tuber slices with buds of *H. tuberosus* cultivar-clone D19, were grown in 1L plastic pots with Cd-Zn contaminated soil of

Lommel. Two slices with buds were planted per pot and five replicates per treatment were set up. The plants were inoculated with *Arthrobacter* sp. strain 222, *Pseudomonas* sp. strain 228, *Serratia* sp. strain 246, *Pseudomonas* sp. strain 256 and *Pseudomonas* sp. strain 262 as a consortium. Bacterial strains were grown in 869 liquid medium (Mergeay et al., 1985) at 30°C under shaking. The bacterial suspension (10^8 cfu mL^{-1}) in tap water was added into the pots after the appearance of the first roots (5 days after sowing). Once a week, inoculation was carried out. Plants were watered every three days. After two weeks of growth, plants were harvested carefully. Roots were immersed in water to remove the soil, and then the root tips were dipped in crystal violet solution (0.075% in 70% ethanol) during 1 min (Dobbelaere et al., 1999). Subsequently, the roots were rinsed in water during 2 min, and observed with a Zoom stereomicroscope (Nikon SMZ800).

7.3.3. Statistical analysis

The data were analyzed by a General Lineal Model and Duncan's test using the statistical package IBM SPSS version19.0. The values given in the tables and figures indicate mean values±standard error (SE). Differences at $p < 0.05$ levels were considered significant. Dunnett's test was used to evaluate significant differences in the case of dry weight and enzyme activities due to the experimental design established (double amount of controls than treatments).

7.4. Result and discussion

7.4.1. Soil characterization

The properties of the soil used in this experiment are shown in Table 7.3. Metal bioavailability depends on the physical and chemical properties of the element, but it is also highly affected by the soil properties (Kayser et al., 2001). High metal bioavailability represents a serious environmental problem because of metal leaching to the groundwater (Meers et al., 2007; Ruttens et al., 2010). The concentration of Cd and Zn extracted with $Ca(NO_3)_2$ indicated that both metals are relatively soluble, potentially bioavailable, and mobile in the soil. The sandy soil texture (with only 5 % of clays), and low pH (5.5) also favour the mobility of both metals in soils (Korte et al., 1976). However, Pb could be immobilized by the organic matter. Although the acid pH

increases the Pb solubility, its mobilization is mainly regulated by the organic matter of the soil (Kabata-Pendias, 2011). Logan et al. (1997) reported the elevated binding capacity that present Pb with the components of the soil organic matter even at pH values around 5.

The total concentrations of Cd (10 $mg \cdot kg^{-1}$), Pb (210 $mg \cdot kg^{-1}$) and Zn (631 $mg \cdot kg^{-1}$) are above the "normal" values (0.5 $mg \cdot kg^{-1}$ of Cd, 40 $mg \cdot kg^{-1}$ of Pb, and 70 $mg \cdot kg^{-1}$ of Zn) for sandy soils in Flanders (De Temmerman et al., 2003). The values are also exceeding the Soil Clean-up Standards (2 $mg \cdot kg^{-1}$ of Cd, 200 $mg \cdot kg^{-1}$ of Pb, and 333 $mg \cdot kg^{-1}$ of Zn) established by the Flemish legislation on soil remediation (VLAREBO, 2009). The Pb concentration is on the limit of the legislation. However, Cd and Zn concentrations are far above of the permitted levels.

Table 7.3. Characteristics of the contaminated soil used in the pot experiment.

Soil properties		
pH-KCl		5.5
pH-H_2O		6.3
EC ($dS\ m^{-1}$)		0.07
CEC ($cmolc\ kg^{-1}$)		7.6
Total organic matter (%)		1.6
Total N Kjeldahl (%)		0.11
$Ca(NO_3)_2$ extractable ($mg\ kg^{-1}$):		
Macronutrients:	K	64
	Mg	31
	Na	304
	P	12
Micronutrients:	Cu	0.41
	Fe	1.9
	Mn	0.81
Metals:	Cd	1.4
	Pb	0.78
	Zn	72
Total concentration ($mg\ kg^{-1}$):		
	Cd	10
	Pb	210
	Zn	631

CEC- cation exchange capacity; EC-electrical conductivity.

7.4.2. Effect on biomass and metal uptake

Figure 7.3 shows the dry weight of *H. tuberosus* grown in the Cd-Zn contaminated soil. The independent inoculation of each bacterial strain did not affect the dry weight of *H. tuberosus*. However, the dry weight of leaves and roots significantly increased when plants were inoculated with the consortium (Figure 7.3, 7.4).

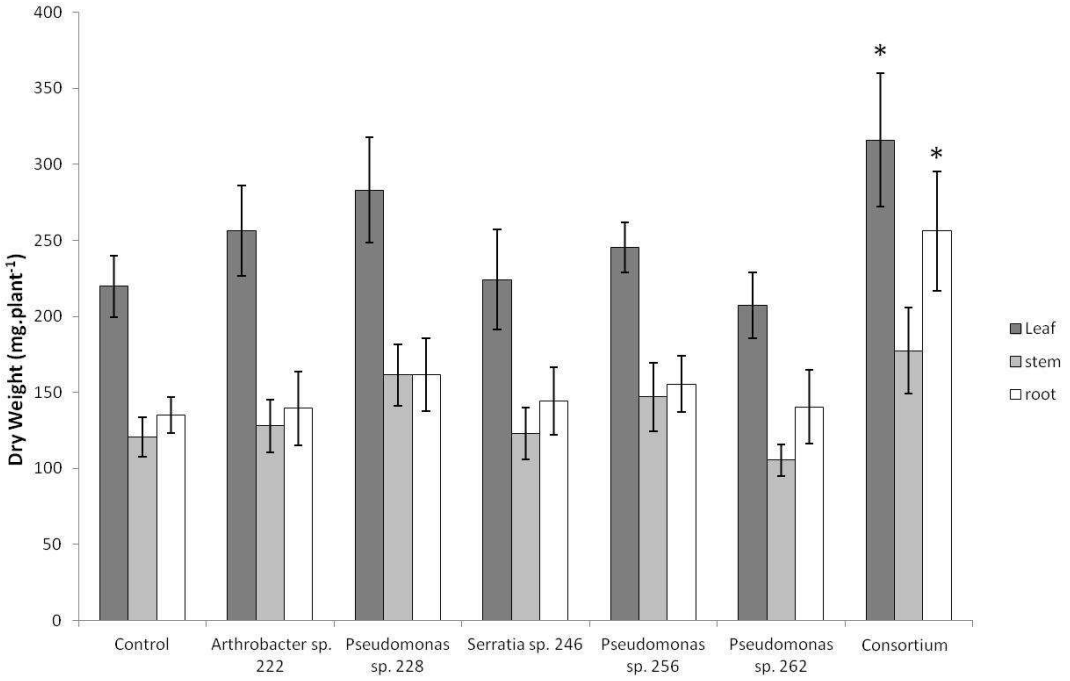

Figure 7.3. Dry weight (mg.plant^{-1}) of *H. tuberosus* grown in a Cd-Zn contaminated soil. (*) shows significant differences after Dunnett's test, $p < 0.05$; mean values ± SE; $n_{(control)} = 20$, $n_{(bacteria\ inoculated)} = 10$.

Figure 7.4. Control plants on the left, and plants inoculated with consortium on the right.

The competition between the inoculated bacterial strains and the endemic soil microorganisms could reduce the effect of the inoculated bacteria on the plant growth (Weyens et al., 2009; Compant et al., 2010). This competition could be minimized by using the bacterial consortium. The results are in accordance with other authors that observed the potential of some bacterial strains acting together as a consortium versus the individual application of the same bacterial strains. Malekzadeh et al. (2012) observed that the inoculation of a consortium formed by *Bacillus mycoides* and *Micrococcus roseus* strains increased the Cd uptake in maize more than the individual inoculation of these strains in a pot experiment with a Cd-contaminated soil. Biomass production of *Brassica juncea* grown on Pb–Zn mine tailings was enhanced upon inoculation with a PGPR consortium consisting of *Azotobacter chroococcum* HKN5, *Bacillus megaterium* HKP-1, and *Bacillus mucilaginosus* HKK-1 (Wu et al., 2006). Langella et al. (2014) reported a significant enhancement of the biomass and uptake of *Festuca rubra* and *Agrostis capillaris* after inoculation with a bacterial consortium of 10 different bacterial strains in a pot experiment with a multi-polluted soil. All these data suggest that in soil conditions, where the competition with other microorganisms is high, the use of consortia and the interactions that occur between bacteria seem to have a significant advantage.

In general, the addition of the bacterial strains significantly decreased metal accumulation in the leaves of *H. tuberosus* (Table 7.4). The concentration of Pb and Zn decreased in the leaves of *H. tuberosus* inoculated with *Arthrobacter* sp. 222, *Pseudomonas* sp. 256, *Pseudomonas* sp. 262 and the consortium. Leaves of plants inoculated with *Serratia* sp. 246 also showed lower concentrations of Zn than leaves of control plants. Cd accumulation was less affected by inoculation of the bacterial strains. The concentration of this element in leaves was only decreased with the inoculation of *Arthrobacter* sp. 222. In the stems, no significant differences were found regarding Cd and Pb uptake of inoculated and control plants. Only in the case of Zn, the concentration in stems decreased after addition of *Pseudomonas* sp. 256 and the consortium. Some authors have reported that certain plant-associated bacteria can adsorb metals, and in this way reduce the plant metal uptake and also the translocation inside the plant. Madhaiyan et al. (2007) observed that inoculation with *Methylobacterium oryzae* and *Burkholderia* sp. reduced Ni and Cd accumulation of tomato plants grown in a Ni and Cd polluted soil. Vivas et al. (2006) also found that inoculation of *Trifolium repens* with

Brevibacillus sp. B-I decreased Zn uptake when plants were grown in a Zn spiked soil. Marques et al. (2013) reported similar effects in *Helianthus annuus* plants, in which Cd and Zn uptake decreased after inoculation with *Chrysiobacterium humi* in soil conditions. The reduction of the metal uptake was attributed to an increased metal biosorption by the bacteria through binding metals to anionic functional groups or its chelation by extracellular polymeric substances, siderophores and organic acids (Rouch et al., 1995; Ma et al., 2011a; Rajkumar et al., 2012). Moreover, bacteria can modify the metal bioavailability by producing ammonia or organic bases that induce the metal precipitation in the rhizosphere or via metal reduction/oxidation (Chen and Cutright, 2003).

In our work, the joint action of the consortium enhanced the Zn and Pb uptake in roots of *H. tuberosus*, suggesting a different behavior of the strains when they were added individually or in a consortium. Only *Pseudomonas* sp. 262 was able to increase the Pb uptake in roots of *H. tuberosus* when it was individually inoculated. According to this, *Pseudomonas* sp. 262 could play an important role in the consortium with regards to Pb uptake. This strain showed a higher capacity to produce siderophores than the rest of the bacterial strains tested (Table 7.1), which could affect to the metal uptake by the plant. Braud et al. (2009) observed an increase in Pb and Cr uptake of maize plants from a contaminated soil after inoculation with *Pseudomonas aeruginosa*, which showed a high ability to produce siderophores. Jiang et al. (2008) also reported an increase in the Cd and Pb uptake by maize and tomato plants after inoculation with *Burkholderia* sp. J62 able to produce siderophores. Siderophores possess higher affinity for Fe(III) than for other elements (Diels et al., 1999). However, they can form complexes of lower stability with metals such as Zn, Cu, Cd and Pb, enhancing their accumulation by the plant (Glick and Bashan, 1997).

The metal translocation factor and accumulation patterns vary considerably for each element, plant and growth condition (Alloway, 1995; Hernández-Allica et al., 2008; Dickinson et al., 2009). The translocation factor was calculated to evaluate the metal mobility in *H. tuberosus* (Table 7.4). Only Zn showed an effective translocation (TF values ≥ 1).

Table 7.4. Total metal concentration (mg.kg^{-1} DM) in D19 cultivar of *H. tuberosus* after 4 weeks of growth in a Cd-Zn polluted soil.

	Cd				Pb				Zn			
	Leaf	Stem	Root	TF	Leaf	Stem	Root	TF	Leaf	Stem	Root	TF
Control	7.3 ± 0.8 b	5.9 ± 0.6 ab	33.1 ± 4.6 ns	0.4	16.4 ± 1.1 b	9.4 ± 1.1 ns	82.4 ± 5.3 a	0.3	1048.3 ± 63.4 c	379.9 ± 31.4 b	830.8 ± 93.9 a	1.7
Arthrobacter sp. 222	4.8 ± 0.5 a	6.5 ± 0.6 ab	34.2 ± 4.8	0.3	12.3 ± 1.3 a	7.7 ± 0.4	97.3 ± 13.6 ab	0.2	740.5 ± 94.2 ab	338.8 ± 39.9 ab	1047 ± 114.2 ab	1.0
Pseudomonas sp. 228	6.7 ± 0.7 ab	6.6 ± 0.8 ab	44.2 ± 5.3	0.3	16.2 ± 1.6 b	8.6 ± 1.1	109.8 ± 7.9 ab	0.2	927.9 ±76.8 bc	310.1 ± 45.1 ab	1145.1 ± 122.6 ab	1.1
Serratia sp. 246	6.5 ± 0.7 ab	7.3 ± 0.9 ab	32.1 ± 3.8	0.4	13.4 ± 0.9 ab	7.9 ± 1.2	86.4 ± 8.7 a	0.2	766.0 ± 71.4 ab	287.5 ± 43.3 ab	856.5 ± 104.1 a	1.2
Pseudomonas sp. 256	6.9 ± 0.5 ab	8.4 ± 1.2 b	33.4 ± 3.7	0.5	11.5 ± 1.1 a	10.4 ± 1.5	87.1 ± 4.6 a	0.3	711.9 ± 88.7 ab	272.0 ± 21.9 a	912.1 ± 98.7 ab	1.1
Pseudomonas sp. 262	6.6 ± 0.9 ab	7.1 ± 0.4 ab	32.1 ± 3.8	0.4	11.7 ± 0.5 a	9.1 ± 0.8	135.6 ± 24.1 b	0.2	607.7 ± 21.0 a	288.6 ± 25.1 ab	880.7 ± 106.0 ab	1.0
Consortium	7.3 ± 0.6 b	4.8 ± 1.1 a	45.5 ± 3.2	0.3	12.2 ± 0.4 a	8.2 ± 1.7	136.2 ± 21.9 b	0.1	724.2 ± 62.4 ab	227.7 ± 30.1 a	1247.6 ± 120.9 b	0.8

Different letters represent significant differences per column after Duncan's test, $p < 0.05$; mean values ± SE; n=5. TF: translocation factor. ns: not significant.

Zn is an essential micronutrient with moderate mobility inside the plant; while Pb has a low mobility because it is tightly bound in root cells (Malone et al., 1974; Meyers et al., 2008). Chen et al. (2009) collected *H. tuberosus* plants grown in a smelter-contaminated soil (silty clay loamy, pH 6.2) with 3044 $mg.kg^{-1}$ of Pb. In these conditions, *H. tuberosus* accumulated 430 $mg.kg^{-1}$ of Pb in roots, and 127 $mg.kg^{-1}$ of Pb in shoots. Although, this plant was not able to translocate Pb in field conditions, these authors reported its high ability to grow in highly Pb contaminated soils.

The concentration of Zn in the aerial part of *H. tuberosus* (Table 7.4) exceeded the phytotoxicity values (>100-400 $mg \cdot kg^{-1}$) proposed by Kabata-Pendias (2011) in aerial tissues of a wide range of plants. In the case of Cd, the concentrations were within the values described as excessive or toxic (5-30 $mg \cdot kg^{-1}$). This suggests that *H. tuberosus* shows an appropriate mechanism to tolerate these metals in its tissues. Long et al. (2013) evaluated the chlorophyll content in *H. tuberosus* in presence of 10 $mg.kg^{-1}$ of Cd in a spiked soil, and concluded that the photosynthetic organs of *H. tuberosus* are tolerant to Cd stress. Our results are also in agreement with Cui et al. (2007) that observed the high ability of *H. tuberosus* to extract Zn from soils that are contaminated with Cd, Cu, Pb and Zn, in comparison with other crops. In this work, *H. tuberosus* was able to accumulate ≈ 1000 $mg.kg^{-1}$ Zn in the aerial parts. Similar values were found in herbaceous crops considered competent Zn phytoextractors, such as *Brassica* spp. (1400-2000 $mg \cdot kg^{-1}$, *Phaseolus vulgaris* (1400 $mg \cdot kg^{-1}$) and *Zea mays* (1200 $mg \cdot kg^{-1}$) grown in contaminated soils (Vamerali et al., 2010).

7.4.3. Nutrient status

In general, the bacterial inoculation did not affect to the macro-nutrient status in roots and leaves of *H. tuberosus* plants grown in the experimental conditions described in this study (Table 7.5). The concentrations of Mg, P and K in stems decreased when plants were treated with the consortium and individually with *Pseudomonas* sp. 228 and 256. Also, the presence of *Serratia* sp. 246 decreased the K concentration in stems. In contrast, the concentration of Na increased in stems of plants inoculated with *Pseudomonas* sp. 256, 262 and with the consortium. Na was the only element that increased in the stems after bacterial inoculation, the rest of the nutrients were lower in stems of plants inoculated with bacteria than in controls. The leaves of *H. tuberosus*

accumulate high concentrations of mineral nutrients and proteins; while the stems are rich in carbohydrates and fibers (Seiler et al., 1990; Denoroy et al., 1996). In this work, the low nutrient accumulation in the stems could be also accentuated by the binding capacity of bacteria, and thereby, the significant differences were found in stems but not in leaves or roots.

Micro-nutrient concentrations were affected by the inoculated bacteria (Table 7.6). The Cu concentration was lower in leaves and stems of inoculated plants than in controls. The Mn concentration decreased in roots of inoculated plants, although significant differences were only found in the case of *Pseudomonas* sp. 262. The Fe concentration in the leaf was also reduced in presence of bacteria, except with *Pseudomonas* sp. 228. The roots of plants inoculated with the consortium also showed lower concentrations of Fe than roots of control plants. The inoculated bacterial strains showed the capacity to produce siderophores during the phenotypic characterization in selective medium (Table 7.2). However, the micro-nutrient uptake was not increased due to bacterial inoculation under soil conditions. The inoculation of plants with bacteria able to produce siderophores could also reduce the uptake of trace elements, depending on the plant, bacteria and metal (Sessitsch et al., 2013). Dimpka et al. (2008) observed a reduction of Ni accumulation by cowpea plants inoculated with *Streptomyces acidiscabies* which was able to produce siderophores. Fe and Mn can also be immobilized on the bacterial surface in the form of hydroxides or some other insoluble metal salts due to the siderophores production (Ma et al., 2011a).

In the literature, the mineral nutrition of *H. tuberosus* has been poorly investigated. Most of the studies are focused on the nutrient content in the tubers but not in the rest of the plant. For this reason, the nutrient concentrations of *H. tuberosus* are compared with previous references of *Helianthus annuus*, since it is the closest related species (Serieys et al., 2010). The concentrations of Mg and K were below the concentrations considered as optimal for *H. annuus* growth (Fässler et al., 2010b). On the contrary, Ca and P concentrations were within the adequate ranges.

Table 7.5. Total macro-nutrient concentrations (g· 100g^{-1} DM) in D19 cultivar of *H. tuberosus* grown in a Cd-Zn contaminated soil.

	Ca			K			Mg		
	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root
Control	0.14 ± 0.01 ns	0.60 ± 0.05 ns	0.42 ± 0.02 ns	0.36 ± 0.01 ns	0.35 ± 0.05 b	0.31 ± 0.02 ns	0.023 ± 0.002 ns	0.08 ± 0.1 b	0.14 ± 0.01 ns
Arthrobacter sp. 222	0.13 ± 0.02	0.60 ± 0.08	0.46 ± 0.04	0.34 ± 0.04	0.29 ± 0.04 ab	0.27 ± 0.02	0.022 ± 0.003	0.06 ± 0.01 ab	0.15 ± 0.02
Pseudomonas sp. 228	0.15 ± 0.01	0.48 ± 0.06	0.50 ± 0.03	0.35 ± 0.02	0.21 ± 0.03 a	0.29 ± 0.04	0.022 ± 0.001	0.05 ± 0.01 a	0.18 ± 0.01
Serratia sp. 246	0.15 ± 0.01	0.55 ± 0.09	0.45 ± 0.01	0.38 ± 0.02	0.21 ± 0.04 a	0.28 ± 0.02	0.023 ± 0.003	0.06 ± 0.01 ab	0.14 ± 0.01
Pseudomonas sp. 256	0.13 ± 0.01	0.45 ± 0.07	0.41 ± 0.02	0.38 ± 0.02	0.18 ± 0.04 a	0.33 ± 0.04	0.021 ± 0.002	0.04 ± 0.01 a	0.16 ± 0.02
Pseudomonas sp. 262	0.14 ± 0.01	0.60 ± 0.05	0.43 ± 0.03	0.38 ± 0.01	0.28 ± 0.03 ab	0.28 ± 0.02	0.021 ± 0.002	0.07 ± 0.01 ab	0.13 ± 0.01
Consortium	0.13 ± 0.01	0.45 ± 0.07	0.47 ± 0.03	0.35 ± 0.02	0.19 ± 0.03 a	0.27 ± 0.04	0.019 ± 0.001	0.05 ± 0.01 a	0.17 ± 0.02

Na			P		
Leaf	Stem	Root	Leaf	Stem	Root
0.95 ± 0.07 ab	0.77 ± 0.21a	2.10 ± 0.22 b	0.42 ± 0.02 ns	0.45 ± 0.04 b	0.30 ± 0.04 ns
1.10 ± 0.14 b	0.61 ± 0.11 a	2.17 ± 0.28 b	0.34 ± 0.04	0.35 ± 0.02 ab	0.27 ± 0.03
1.26 ± 0.54 b	0.80 ± 0.26 a	1.84 ± 0.37 ab	0.39 ± 0.03	0.28 ± 0.03 a	0.30 ± 0.01
0.75 ± 0.09 a	0.69 ± 0.12 a	2.10 ± 0.27 b	0.38 ± 0.03	0.30 ± 0.05 ab	0.26 ± 0.03
1.22 ± 0.43 b	2.21 ± 0.38 b	1.25 ± 0.13 a	0.39 ± 0.04	0.25 ± 0.05 a	0.35 ± 0.03
0.62 ± 0.14 a	1.35 ± 0.19 b	1.11 ± 0.28 a	0.40 ± 0.03	0.37 ± 0.03 ab	0.28 ± 0.03
1.23 ± 0.31b	2.19 ± 0.48 b	1.17 ± 0.45 a	0.34 ± 0.02	0.24 ± 0.03 a	0.30 ± 0.04

Different letters represent significant differences per column after Duncan's test, $p < 0.05$; mean values ± SE; n=5. ns: not significant.

Table 7.6. Total micro-nutrient concentrations ($mg \cdot kg^{-1}$ DM) in D19 cultivar of *H. tuberosus* grown in a Cd-Zn contaminated soil.

	Cu			Fe			Mn		
	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root
Control	18.7 ± 1.1 b	6.4 ± 0.4 b	13.8 ± 0.7 a	195.6 ± 25.5 c	31.3 ± 5.0 ns	211.3 ± 39.1 b	5.8 ± 0.6 a	23.3 ± 2.2 b	23.1 ± 4.1 b
Arthrobacter sp. 222	13.7 ± 2.3 a	5.1 ± 0.3 ab	16.3 ± 2.0 abc	134.1 ± 21.3 ab	25.2 ± 2.7	126.6 ± 42.6 ab	24.5 ± 1.3b	10.9 ± 3.0a	14.3 ± 2.3 ab
Pseudomonas sp. 228	15.0 ± 0.7 a	4.7 ± 0.4 a	17.7 ± 1.6 abc	168.9 ± 28.6 bc	27.4 ± 3.7	146.1 ± 60.4 ab	26.4 ± 2.3b	13.6 ± 3.1ab	12.9 ± 3.2 ab
Serratia sp. 246	13.9 ± 1.1 a	4.6 ± 0.6 a	14.6 ± 0.6 ab	96.1 ± 26.6 ab	29.9 ± 4.2	186.9 ± 36.1 ab	12.9 ± 4.3ab	16.4 ± 6.2ab	16.6 ± 0.6 ab
Pseudomonas sp. 256	13.0 ± 0.5 a	4.6 ± 0.8 a	15.9 ± 0.7 abc	97.3 ± 7.6 a	32.9 ± 7.7	250.4 ± 31.9 b	17.2 ± 6.7ab	23.9 ± 7.1b	17.1 ± 5.2 ab
Pseudomonas sp. 262	11.8 ± 1.3 a	5.5 ± 0.4 ab	19.5 ± 1.7 bc	75.2 ± 16.7 a	40.0 ± 8.4	115.1 ± 56.1 ab	6.2 ± 3.2a	20.1 ± 6.0b	11.0 ± 3.1 a
Consortium	12.1 ± 0.3 a	4.1 ± 0.5 a	21.0 ± 2.7 c	79.1 ± 3.9 a	28.2 ± 9.1	50.6 ± 11.6 a	14.6 ± 5.5ab	9.5 ± 2.6a	19.5 ± 3.7 ab

Different letters represent significant differences per column after Duncan's test, $p < 0.05$; mean values ± SE; n=5. ns: not significant.

Micronutrients showed values within the adequate concentration range for *H. annuus* growth (Fässler et al., 2010a). The plant nutrient absorption in soils depends on several factors such as soil properties, nutrient availability, plant requirements and its capacity to absorb them (Baker, 1981). Nutrient concentrations vary between species and also depend on plant age at the harvest time (Fässler et al., 2010b). The nutrient absorption is high in the three first months of growing and begins to decrease after this period in most of the plants (Smith et al., 1997). In *H. tuberosus* the deficiency in K or Mg disturbs the tuber morphogenesis more than the aerial growth (Soja et al., 1990). Thus, the biomass of *H. tuberosus* was not diminished in our experiment since the plants were growing during one month, despite of the low concentrations of K and Mg found. In general, the plants did not show deficiency symptoms along the experiment. *H. tuberosus* can grow in poor soils and its cultivation does not require necessarily fertilization (Kays and Nottingham, 2008). This crop is able to grow in most soils, including sandy soils, and for that reason it has been used as anti-erosion to fix terraces or unstable sand (Denoroy, 1996). The high adaptability of this plant to a wide variety of soils makes of this crop a suitable candidate to be used in phytotechnologies.

7.4.4. Effect of the inoculated bacteria in the activity of antioxidants enzymes

Plants have efficient mechanisms to avoid metal toxicity in metabolically active compartments of the cells (Ma et al., 2005; Pongrac et al., 2009). It is well known that metals induce oxidative stress in plants and increase the production of reactive oxygen species (ROS). The levels of ROS are controlled by the action of antioxidant enzymes (Nehnevajova et al., 2012; Lin and Aarts, 2012). The activities of antioxidant enzymes such as glutathione reductase (GR), guaiacol peroxidase (GPOD), syringaldazine peroxidase (SPOD), and also the activities of enzymes related to the Krebs cycle such as isocitrate dehydrogenasa (ICDH), glutamate dehydrogenase (GDH) and malic enzyme (ME) were measured as representative enzymes involved in the response to Cd-Zn exposure (Vangronsveld and Clijsters, 1994; Noctor and Foyer, 1998; Sytar et al., 2013). The results showed differences in the enzyme activities in root and leaf, and also depending on the bacterial strain that was inoculated (Figure 7.5 and 7.6). The enzymatic activity was measured in roots and leaves of *H. tuberosus* after 4 weeks of

growth in a Cd-Zn contaminated soil. Significant differences between inoculated and non-inoculated plants were found only in the activities of ME, ICDH and GR.

The ME and ICDH activities were significantly higher in roots of plants inoculated with the consortium than the other treatments (Figure 7.5). Since the activity of both enzymes is induced by metal toxicity (Vangronsveld and Clijsters, 1994) to compensate the decrease of ATP and NADPH generated by the metal-sensitive photosynthetic reactions (Ernst, 1980), the high enzymatic activity observed could be related with the higher concentrations of Pb and Zn that were accumulated in roots of plants inoculated with the consortium in comparison to control plants (Table 7.4). This increase in the metal concentration in the plant tissues may be associated with an efficient enzymatic system that allows the assimilation of toxic metals into the plant.

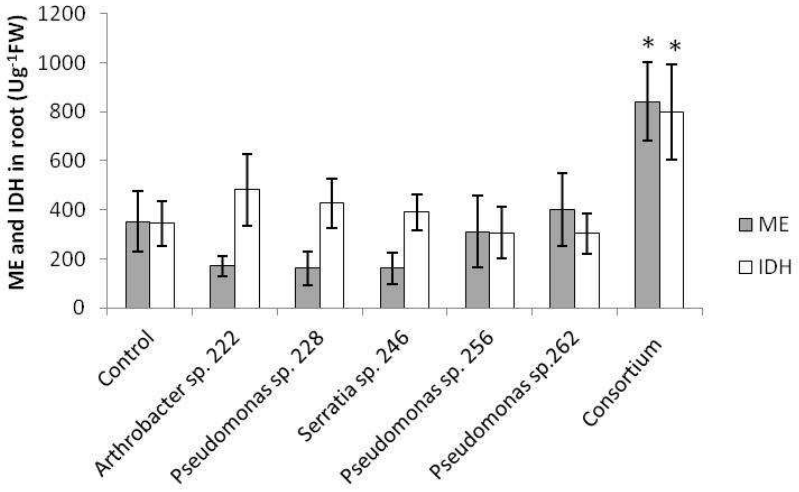

Figure 7.5. Malic enzyme (ME) and isocitrate dehydrogenase (IDH) activity ($U.g^{-1}$ FW) in root of *H. tuberosus* grown in a Cd-Zn contaminated soil. (*) shows significant differences after Dunnett's test, $p < 0.05$; mean values ± SE; $n_{(control)} = 10$, $n_{(bacteria\ inoculated)} = 5$.

GR converts the oxidized form of glutathione (GSSG) to GSH. This enzyme plays a key role in the antioxidant defense processes, by reducing GSSG, thus allowing a high GSH/GSSG ratio to be maintained (Bhaduri and Fulekar, 2012; Sytar et al., 2013). In our work, the GR activities were higher in leaves of plants inoculated with the consortium and *Pseudomonas* sp. 262 than in the other conditions (Figura 6a).

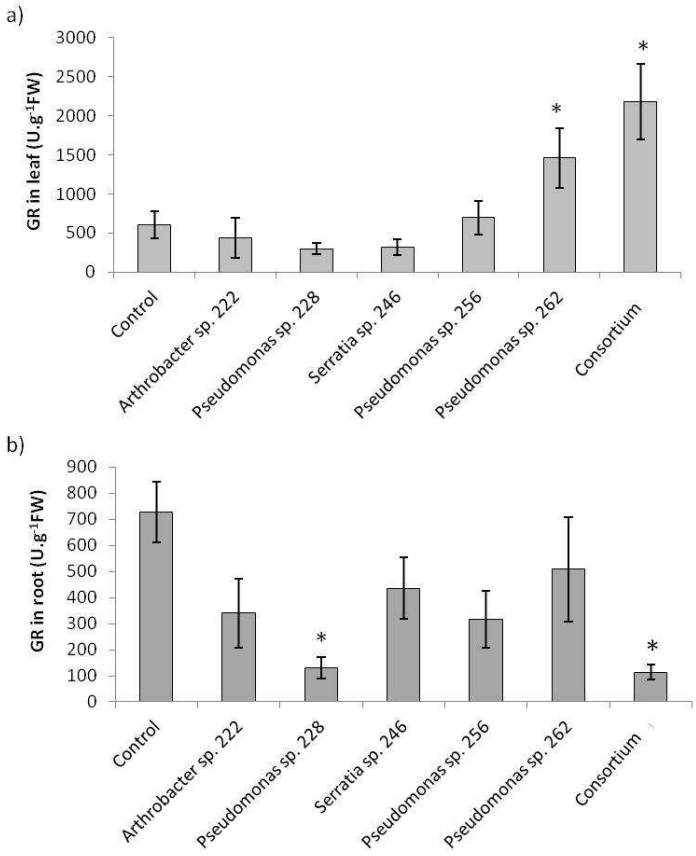

Figure 7.6. Glutathione reductase (GR) activity (U.g^{-1} FW) in leaf (a) and root (b) of *H. tuberosus* grown in a Cd-Zn contaminated soil. (*) shows significant differences after Dunnett's test, $p < 0.05$; mean values ± SE; $n_{(control)} = 10$, $n_{(bacteria\ inoculated)} = 5$.

In both cases the plants accumulated higher levels of Zn and Pb in the roots than controls, but this increase was not observed in leaves. García et al. (2006) and Nehnevajova et al. (2012) also found increased GR activities in leaves of *H. annuus* under Cd and Zn excess. Cd and Zn induce the antioxidative defense mechanisms in plants by different pathways, but when both metals are present in combination, Zn plays a synergistic role in Cd-induced antioxidative defense because of its role as cofactor (Lin and Aarts, 2012). In this way, the presence of Zn in combination with Cd increases the activities of antioxidant enzymes, such as GR (Cherif et al., 2011; Sanaeiostovar et al., 2012). Numerous authors have reported the increase of the activity of enzymes related with the anti-oxidative stress of plants in the presence of metals (Weckx and Clijsters, 1997; Fornazier et al., 2002; Verma and Dubey et al., 2003; Cuypers et al., 2011). However, the increase of the antioxidant responses in order to avoid the negative consequences of metal stress is limited. The exposure to elevated concentrations of

reactive metals could result in decreasing the activities of antioxidative enzymes (Bhaduri and Fulekar, 2012). As shown in Figure 7.6b, the GR activity was significantly lower in roots of *H. tuberosus* inoculated with *Pseudomonas* sp. 228 and the consortium than in control plants. Rosa et al. (2003) also observed that the GR activity decreased in roots of *Vicia faba* when plants were exposed to high concentrations of Cd; by contrast the GR activity increased when the Cd concentrations diminished. They suggested that the decrease of GR in roots could be due to an increase of the metal toxicity for the plant. Metals generate reactive radicals, resulting in cellular damage such as depletion of enzyme activities (Stohs and Bagchi, 1995). Bielawski and Joy (1986) and Gallego et al. (1996) also observed a decreased GR activity in *Pisum sativum* and *H. annuus*, respectively, exposed to Cu, Zn and Cd. According to this, the GR in roots of *H. tuberosus* could be depleted due to Cd and Zn excess.

7.4.5. Phytovailability of metals in soil

The phytoavailability depicts the soluble fraction in the soil that is available for plants (Sposito, 1989; Song et al., 2004; Meers et al., 2007). PGP bacteria have the ability to increase the phytoavailability of nutrients and metals in soils through organic acids and siderophores production or phosphorous solubilization. Numerous works have shown that bacteria could solubilize metals and nutrients in soils, and improve the metal uptake by the plant (Sessitsch et al., 2013). Chen et al. (2013) observed that the water-soluble Cd contents in the rhizosphere soils of the rape plants inoculated with *Burkholderia* sp. J62 and *P. thivervalensis* Y-1-3-9 increased from 59% to 237% compared to the controls, after two months of growth. However, some authors observed the opposite effect. Madhaiyan et al. (2007) reported that the metal availability in soil decreased after bacterial inoculation, suggesting that the bacteria could immobilize metals in the rhizosphere. Kidambi et al. (1995) mentioned that the presence of metals enhanced the microbial exopolymer production that can bind metals and nutrients. This behaviour has an advantage for the bacteria to resist toxic concentrations of metals.

In this context, the pH, electrical conductivity and phytoavailability of metals and nutrients were evaluated in soil for each treatment (Table 7.7). The electrical conductivity was not significantly affected by the bacterial inoculation. The pH of the soil used in this study was relatively low, making it more difficult to observe the effect

of the bacteria on the availability of elements in a short period experiment. However, some significant differences were found between bare soil and soil with *H. tuberosus*. The pH-water of the soil increased in *H. tuberosus* inoculated with *Arthrobacter* sp. 222, in comparison with the bare soil inoculated with the same bacteria. In this case, the combination plant-bacteria increased the pH; which could reduce the metal and nutrient uptake of the plant. On the contrary, the pH-KCl decreased with *Pseudomonas* sp. 256 and 262 inoculated in bare soil in comparison with the control, suggesting the capacity of both strains to acidify the pH when they are not associated to the plant. The soil pH-KCl also decreased in *H. tuberosus* inoculated with the consortium, but in this case the differences were not significant. The trend observed is in agreement with the high accumulation of Zn that was found in the consortium. The decrease in the pH of this treatment could increase the Zn availability, improving the uptake of this metal.

Significant differences were found only in the case of Cd, K and Mn extracted by $Ca(NO_3)_2$ (Table 7.7). The other elements were not affected by bacterial inoculation. The concentration of $Ca(NO_3)_2$ extractable Pb is not shown in Table 7.7 because it was below the detection limits. The Cd availability decreased in pots with *H. tuberosus* in comparison with bare soil, possibly due to the plant uptake, but no significant differences were observed between inoculated bacteria. The K availability was also significantly lower in soils with *H. tuberosus* than in bare soil, due to the uptake by the plant. *H. tuberosus* requires high levels of K and Ca during its growth (Cassells and Deadman, 1993). As it was mentioned above, this crop is highly effective in extracting these nutrients from the soil. This can explain the significant decrease found in the $Ca(NO_3)_2$ extractable K concentration of soils with *H. tuberosus*. The Mn availability increased in *H. tuberosus* inoculated with *Pseudomonas* sp. 262 in comparison to controls, suggesting a possible ability of this strain to solubilize this nutrient.

Pore water solution has been described as useful tool to assess the readily available metal fractions in soil experiments (Chapman et al., 2002; Prokop et al., 2003; Shan et al., 2003). In particular, rhizon samplers have been used in numerous studies to extract the nutrients and trace elements potentially available for the plant from the soil (Knight et al., 1998; Cabrera, 1998; Meers et al., 2006). Rhizon samplers were inserted immediately next to the plant with the objective of modelling the elements available in the rhizosphere of *H. tuberosus*. Three samplings were performed along the experiment and differences between them were statistically evaluated (Annex II).

Table 7.7. Physicochemical properties of the soil in each treatment.

Treatments	EC (dS m^{-1})	pH-water	pH-KCl	Ca $(NO_3)_2$-extractable (mg.kg^{-1})									
				Cd	Cu	Fe	K	Mg	Mn	Na	P	S	Zn
Soil (Control)	**0.071 ± 0.002ab**	**6.34 ± 0.01abcd**	**5.57 ± 0.03ef**	**1.36 ± 0.02c**	**0.41 ± 0.01ns**	**1.9 ± 0.1ns**	**63.8 ± 0.8c**	**30.6 ± 0.5ns**	**0.81 ± 0.04ab**	**304 ± 16ab**	**12.1 ± 0.3ab**	**136 ± 15ab**	**72 ± 1ab**
soil+ *Arthrobacter* sp. 222	0.073 ± 0.007b	6.32 ± 0.03abc	5.59 ± 0.04f	1.35 ± 0.02c	0.40 ± 0.02	1.9 ± 0.1	65.5 ± 1.8c	31.3 ± 0.5	0.80 ± 0.02ab	303 ± 23ab	11.1 ± 0.5ab	115 ± 16ab	73 ± 2ab
soil+ *Pseudomonas* sp. 228	0.064 ± 0.004a	6.34 ± 0.04abcd	5.54 ± 0.05cde	1.27 ± 0.03bc	0.40 ± 0.04	1.8 ± 0.2	60.9 ± 0.5c	29.4 ± 0.6	0.74 ± 0.04a	270 ± 31ab	13.5 ± 1.5ab	114 ± 14ab	69 ± 3ab
soil + *Serratia* sp. 246	0.072 ± 0.006ab	6.39 ± 0.03bcd	5.53 ± 0.03bcdef	1.26 ± 0.03bc	0.39 ± 0.01	1.5 ± 0.1	60.5 ± 2.5c	30.5 ± 0.4	1.05 ± 0.15ab	319 ± 31ab	10.5 ± 0.2a	102 ± 14ab	71 ± 1ab
soil + *Pseudomonas* sp. 256	0.063 ± 0.002a	6.24 ± 0.03a	5.47 ± 0.02bcd	1.27 ± 0.02bc	0.38 ± 0.01	1.5 ± 0.1	59.0 ± 1.1c	30.6 ± 0.2	0.87 ± 0.05ab	252 ± 32a	11.8 ± 0.5ab	134 ± 18ab	75 ± 1ab
soil + *Pseudomonas* sp. 262	0.060 ± 0.004a	6.25 ± 0.02ab	5.45 ± 0.01ab	1.26 ± 0.03bc	0.39 ± 0.03	1.5 ± 0.1	59.9 ± 1.6c	31.3 ± 0.8	0.93 ± 0.08ab	269 ± 19ab	12.9 ± 0.1ab	91 ± 09a	75 ± 3ab
soil + Consortium	0.065 ± 0.008ab	6.40 ± 0.03cd	5.55 ± 0.02def	1.18 ± 0.02ab	0.39 ± 0.02	1.9 ± 0.1	60.3 ± 2.2c	31.7 ± 1.2	0.87 ± 0.09ab	334 ± 45ab	11.4 ± 0.5ab	104 ± 21ab	67 ± 2a
soil + *H. tuberosus* (Control)	**0.072 ± 0.005ab**	**6.39 ± 0.02bcd**	**5.46 ± 0.01abc**	**1.22 ± 0.01ab**	**0.45 ± 0.04**	**2.0 ± 0.6**	**35.6 ± 4.2ab**	**31.9 ± 0.2**	**1.21 ± 0.13ab**	**339 ± 23ab**	**12.8 ± 1.8ab**	**140 ± 23ab**	**72 ± 1ab**
soil + *H. tuberosus* + *Arthrobacter* sp. 222	0.067 ± 0.002a	6.47 ± 0.06d	5.50 ± 0.01bcde	1.26 ± 0.03bc	0.44 ± 0.03	1.6 ± 0.2	31.2 ± 2.0ab	31.6 ± 0.5	0.92 ± 0.07ab	309 ± 17ab	11.6 ± 0.8ab	110 ± 19ab	74 ± 2ab
soil + *H. tuberosus* + *Pseudomonas* sp. 228	0.061 ± 0.006a	6.29 ± 0.05abc	5.50 ± 0.03bcdef	1.23 ± 0.05bc	0.42 ± 0.03	2.1 ± 0.3	33.6 ± 5.6ab	31.3 ± 0.9	1.60 ± 0.14bc	305 ± 37ab	13.4 ± 0.4b	123 ± 14ab	74 ± 1ab
soil + *H. tuberosus* + *Serratia* sp. 246	0.071 ± 0.004ab	6.46 ± 0.02d	5.50 ± 0.01bcde	1.26 ± 0.04bc	0.41 ± 0.02	1.6 ± 0.1	43.2 ± 3.3b	30.9 ± 0.3	1.50 ± 0.11bc	357 ± 22ab	11.4 ± 0.3ab	150 ± 15ab	73 ± 2ab
soil + *H. tuberosus* + *Pseudomonas* sp. 256	0.073 ± 0.005b	6.27 ± 0.03abc	5.45 ± 0.01ab	1.22 ± 0.02bc	0.41 ± 0.01	1.7 ± 0.1	36.6 ± 2.4ab	28.7 ± 1.9	1.08 ± 0.11ab	343 ± 62ab	11.9 ± 0.2ab	166 ± 31b	70 ± 1ab
soil + *H. tuberosus* + *Pseudomonas* sp. 262	0.068 ± 0.002ab	6.30 ± 0.05abc	5.50 ± 0.02bcdef	1.16 ± 0.04ab	0.42 ± 0.03	2.0 ± 0.1	31.6 ± 6.4ab	32.7 ± 0.6	1.90 ± 0.27c	374 ± 45b	12.6 ± 0.5ab	141 ± 18ab	70 ± 1ab
soil + *H. tuberosus* + Consortium	0.073 ± 0.010b	6.28 ± 0.05abc	5.38 ± 0.03a	1.17 ± 0.02ab	0.42 ± 0.01	1.8 ± 0.1	32.3 ± 7.3ab	30.6 ± 0.9	1.00 ± 0.08ab	336 ± 46ab	12.0 ± 0.4ab	141 ± 07ab	70 ± 1ab

Different letters represent significant differences per column after Duncan's test, $p < 0.05$; mean values ± SE; n=4.

Concentrations of K, Na, Mg and Zn in the pore water solution varied along the experiment in the treatments with plant. In general, the concentration of K decreased in the last sampling in comparison with the first sampling in rhizospheric soil of *H. tuberosus*. This decrease in K concentration is also in agreement with the high capacity of *H. tuberosus* to extract K from the soil, as mentioned above. In contrast, the concentration of Na increased in the last sampling compared to the first sampling. Taking into account that this increase also occurred in control plants, we can hypothesize that the plant exudates were playing a more important role in the Na availability than the bacteria. The concentrations of Mg and Zn decreased in the last sampling in comparison with the first sampling in plants inoculated with *Pseudomonas* sp. 228, but both elements were not modified in the rest of the cases. Considering the treatments in bare soil, only *Arthrobacter* sp. 222 affected the mobility of the elements in the soil. The concentrations of Ca, Cd, Mg, S and Zn decreased in the last sampling in comparison with the first sampling in soil inoculated with this strain. This bacterial strain is the only one that comes from the soil, so it is expected to have more pronounced effects on bare soil than the rest of the strains that were isolated from the roots.

Every sampling was studied separately, but due to the homogeneity observed, the values were expressed as mean values of the three samplings (Table 7.8). In general, the concentrations of Ca, Cd, K, Mg and Na in the pore water solution were higher in bare soil than in pots with *H. tuberosus* due to the fact that these elements were accumulated by the plant. Although this decrease was only significant in the case of K, similar trends were observed for the other elements. In case of Fe, the effect was different. The inoculation of the consortium in bare soil decreased the Fe concentration in pore water, however, the joint effect of the consortium and the plant increased the Fe concentration in comparison with the other treatments. Although the Fe was present in the pore water solution, it was not taken up by the plants as it is shown in Table 7.6, possibly because it was precipitated at pH-water 6.3.

Table 7.8. Mean values of the three samplings of pore water solutions ($mg.L^{-1}$) per treatment.

Treatments	Pore water solution										
	Ca	**Cd**	**Cu**	**Fe**	**K**	**Mg**	**Mn**	**Na**	**P**	**S**	**Zn**
soil (Control)	**61 ± 9ab**	**0.025 ± 0.003ab**	**0.052 ± 0.007c**	**0.11 ± 0.04ab**	**30 ± 3d**	**6.1 ± 1.2ab**	**0.03 ± 0.02ns**	**76 ± 4de**	**1.2 ± 0.1ns**	**63 ± 6ns**	**1.9 ± 0.3ab**
soil+ *Arthrobacter* sp. 222	67 ± 3ab	0.025 ± 0.001ab	0.044 ± 0.002abc	0.09 ± 0.01ab	29 ± 2d	5.9 ± 0.3ab	0.03 ± 0.01	73 ± 1bcd	1.0 ± 0.1	56 ± 2	1.9 ± 0.1ab
soil+ *Pseudomonas* sp. 228	64 ± 12ab	0.023 ± 0.002ab	0.042 ± 0.003a	0.10 ± 0.03ab	28 ± 3cd	5.5 ± 1.2ab	0.03 ± 0.01	75 ± 6de	1.1 ± 0.1	59 ± 7	1.8 ± 0.2ab
soil + *Serratia* sp. 246	52 ±4a	0.027 ±0.006b	0.048 ± 0.007abc	0.10 ± 0.04ab	27 ± 5cd	6.3 ± 2.1b	0.04 ± 0.02	73 ± 6bcd	1.3 ± 0.2	56 ± 7	1.6 ± 0.1a
soil + *Pseudomonas* sp. 256	67 ± 15ab	0.025 ± 0.003ab	0.045 ± 0.004abc	0.13 ± 0.06ab	30 ± 4d	5.8 ± 1.3ab	0.03 ± 0.02	63 ± 4a	1.2 ± 0.1	54 ± 5	2.0 ± 0.3b
soil + *Pseudomonas* sp. 262	65 ± 5ab	0.023 ± 0.001ab	0.047 ± 0.003abc	0.11 ± 0.03ab	27 ± 1cd	5.8 ± 0.4ab	0.04 ± 0.02	68 ± 5abcd	1.1 ± 0.1	54 ± 4	1.9 ± 0.1ab
soil + Consortium	69 ±9b	0.024 ± 0.003ab	0.042 ± 0.003a	0.08 ± 0.01a	29 ± 3d	5.8 ± 1.0ab	0.02 ± 0.01	83 ± 8e	1.1 ± 0.1	62 ± 9	1.9 ± 0.3ab
soil + *H. tuberosus* (Control)	**53 ± 6a**	**0.022 ± 0.001ab**	**0.052 ± 0.004bc**	**0.14 ± 0.04ab**	**20 ± 3ab**	**4.4 ± 0.6a**	**0.05 ± 0.02**	**66 ± 5abc**	**1.2 ± 0.1**	**56 ± 9**	**1.7 ± 0.1ab**
soil + *H. tuberosus* + *Arthrobacter* sp. 222	55 ± 6ab	0.021 ± 0.002a	0.043 ± 0.002ab	0.09 ± 0.01ab	14 ± 2a	5.0 ± 0.6ab	0.02 ± 0.01	74 ± 5cde	1.3 ± 0.1	58 ± 5	1.6 ± 0.1a
soil + *H. tuberosus* + *Pseudomonas* sp. 228	53 ± 7a	0.020 ±0.002a	0.045 ± 0.003abc	0.13 ± 0.05ab	22 ± 5bc	4.5 ± 0.6ab	0.04 ± 0.02	64 ± 2ab	1.4 ± 0.1	50 ± 8	1.6 ± 0,2a
soil + *H. tuberosus* + *Serratia* sp. 246	51 ± 13a	0.021 ± 0.004ab	0.046 ± 0.001abc	0.11 ± 0.03ab	18 ± 4ab	4.9 ± 0.7ab	0.02 ± 0.01	74 ± 5cde	1.2 ± 0.1	62 ± 8	1.6 ± 0.3a
soil + *H. tuberosus* + *Pseudomonas* sp. 256	55 ± 5ab	0.021 ±0.002a	0.048 ± 0.003abc	0.14 ± 0.03ab	19 ± 2ab	4.8 ± 0.4ab	0.02 ± 0.01	67 ± 4abcd	1.3 ± 0.1	53 ± 6	1.7 ± 0.2ab
soil + *H. tuberosus* + *Pseudomonas* sp. 262	54 ± 7ab	0.022 ± 0.002ab	0.044 ± 0.005abc	0.09 ± 0.01ab	17 ± 2ab	4.9 ± 0.6ab	0.02 ± 0.01	70 ± 2abcd	1.3 ± 0.1	55 ± 7	1.8 ± 0.2ab
soil + *H. tuberosus* + Consortium	57 ± 9ab	0.022 ± 0.003ab	0.049 ± 0.003abc	0.17 ± 0.05b	17 ± 3ab	5.4 ± 0.9ab	0.05 ± 0.02	72 ± 3abcd	1.4 ± 0.1	58 ± 8	1.9 ± 0.3ab

Different letters represent significant differences per column after Duncan's test, $p < 0.05$; mean values ± SE; n=12.

7.4.6. Effect of consortium inoculation on the root morphology of *H. tuberosus*

The inoculation of the consortium promoted the root hair development in *H. tuberosus* in comparison to the control plants (Figure 7.7A, B). Plant growth-promoting activity of some rhizosphere microorganisms has been related to the production of substances that modify root development (Bloemberg and Lugtenberg, 2001). De Souza et al. (1999) and López-Bucio et al. (2007) observed an increase in the root hairs formation after bacterial inoculation in *Brassica juncea* and *Arabidopsis thaliana*, respectively. Dobbelaere et al. (1999) also observed an increase in the root hair formation of wheat seedlings after inoculating *Azospirillum* sp., and attributed this effect to its IAA production. Four of the bacterial strains of our consortium (*Pseudomonas* sp. 228, 256, 262 and *Serratia* sp. 246) showed the capacity to produce IAA (Table 7.1), which could play an important role in the root hair development. Moreover, two of these bacterial strains (*Pseudomonas* sp. 256 and 262) were able to produce acetoin. This volatile compound has also been described as root growth promoter (Ryu et al., 2003; Duan et. al., 2013, Glick et al., 2010). PGP-bacteria can improve plant growth under metal exposure by increasing the root surface area and hair production (Sessitsch et al., 2013), in this way also the nutrient and metal uptake by the plant can be facilitated. Bacteria can enhance root growth directly affecting to the root-system architecture by secretion of plant growth-promoting substances such as auxins and cytokinins or in an indirect way, by the production of antibiotics, which promote plant growth by inhibiting the growth of harmful microorganisms in the rhizhosphere (López-Bucio et al., 2007).

It is important to mention that four of the bacterial strains used to form the consortium (*Pseudomonas* sp. 228, 256, 262 and *Serratia* sp. 246) were isolated as root endophytes from *Brassica napus* (See Chapter 5). The increase of root hairs in *H. tuberosus* after inoculation with these bacterial strains, also demonstrates that bacterial strains originating from other hostplants can affect the root structure of plants from a different taxonomic group, having in this case not necessarily a specific relation with the initial host plant. This result is in agreement with other studies that observed positive effects of bacteria isolated from metal tolerant plants on the growth of plants from different families (Ma et al., 2011b; Sheng et al., 2012; He et al., 2013).

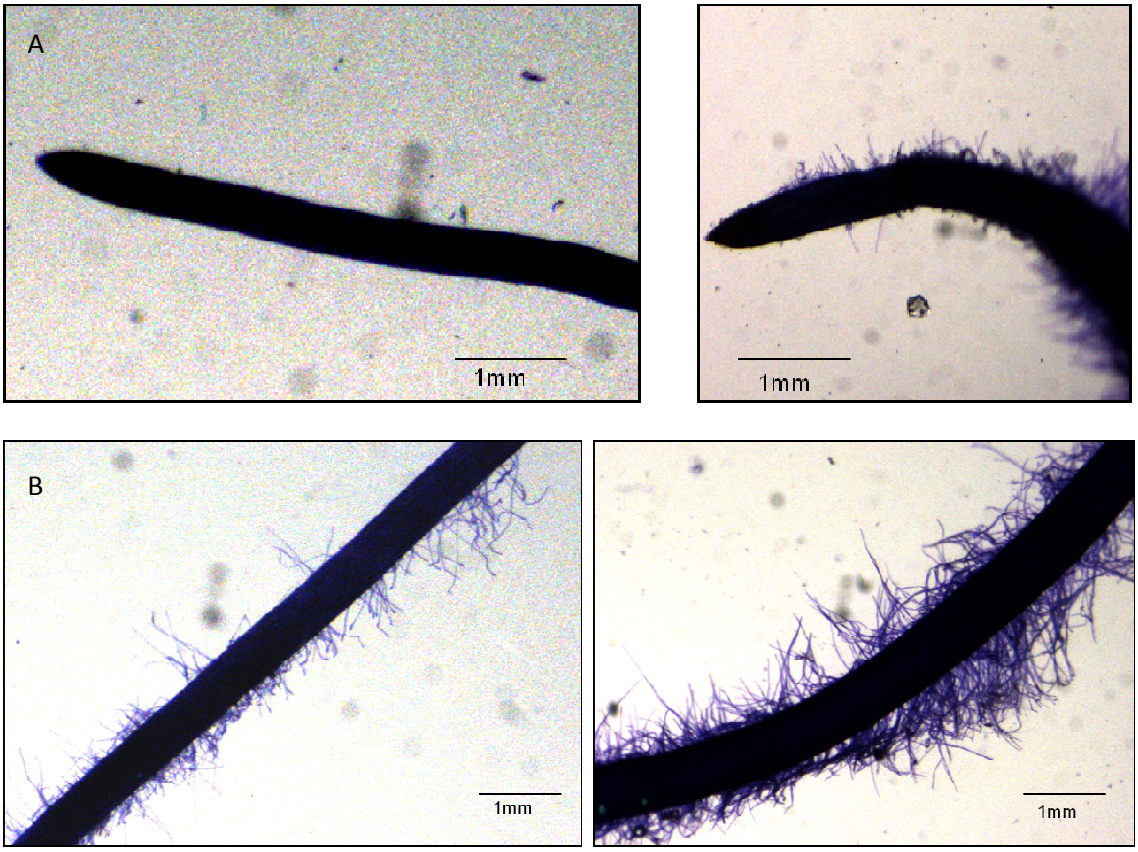

Figure 7.7. Effect of consortium inoculation on the root hair development of 2-week-old *H. tuberosus* plants. Left roots represent controls and right roots are taken from inoculated pots with the consortium. A: root tip; B: immediately after root tip.

7.5. Conclusions

Inoculation of the consortium increased the biomass of *H. tuberosus* D19 cultivar-clone growing in a Cd-Zn contaminated soil, and also the Pb and Zn uptake in the roots after one month of growing. *Pseudomonas* sp. 262 increased the Pb uptake in the roots when it was individually inoculated. *H. tuberosus* inoculated with the consortium showed high activity of malic enzyme, isocitrate dehydrogenase in roots and glutathione reductase in leaves that could be related with the assimilation of metals into the plant. The consortium inoculation stimulated root hair development of *H. tuberosus* D19 cultivar-clone. Bacterial strains isolated from *Brassica napus* roots affected the root structure of *H. tuberosus* indicating that beneficial bacterial effects do not have to be necessarily limited to their initial host plant. *H. tuberosus* D19 cultivar-clone in combination with the inoculated consortium could be a promising tool on contaminated

soils with Cd, Pb and Zn that allows to increase biomass production and thereby, the amount of metal extracted by the plant. *H. tuberosus* could be a suitable crop to be used as Zn phytoextractor.

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Chapter 8

General discussion and conclusions

8.1. General discussion

One of the most critical limitations of metal phytoextraction is the long time required to clean-up metal contaminated soils and reach the appropriate thresholds. The use of high biomass crops able to extract metals from soil could compensate for the long time required applying this technology with the production of valuable biomass and with an improvement of the soil properties (Dickinson et al., 2009; Fässler et al., 2010; Thewys et al., 2010; Witters et al., 2012). To develop this strategy, the plant is required to tolerate the concentrations of metals accumulated in its tissues without significant biomass reduction, especially in the aerial parts that will be harvested to produce bio-energy (Maxted et al., 2007; Vangronsveld et al., 2009).

Brassica napus and *Helianthus annuus* are high biomass crops well-known because of their high capacity to grow on soils contaminated with Cd, Pb and Zn (Hernández-Allica et al., 2008; Adesodun et al., 2010). However, there are not many studies about their toxicity to Cr (VI) and As (V), as well as their germination rate and tolerance to these metals in the early stages of growing. On the other hand, *Brachypodium distachyon* is the first pooid grass to be sequenced and has been recently proposed as a model grass for improving food crops and developing new sustainable energy (Bevan et al., 2010). Our work provides the first data with regards to the metal tolerance of *B. distachyon* during the first stages of growing. Increasing the knowledge about metal tolerance and uptake in this new model grass could help to understand the mechanisms of toxicity at the molecular level, and genes and proteins involved in the response of plants to high concentrations of metals. The ability of *B. distachyon* seeds to germinate and grow *in vitro* with Cd, As(V), Zn, or Cr(VI) was compared with the two well-known metal tolerant energy crops, *Brassica napus* and *Helianthus annuus,* in order to evaluate its capacity to grow in the presence of metals.

The experiments conducted under *in vitro* conditions allowed to realize preliminary studies concerning the metal tolerance of *B. distachyon*, *B. napus* and *H. annuus* during

their first growth stages. The plant tolerance was evaluated based on the germination rate and shoot and root length in agar medium according to Peralta et al. (2001), Buendia-González et al. (2010) and Di Lonardo et al. (2011). These experiments in sterile conditions allow to distinguish the natural plant responses from others derived from the relation with microorganisms (Reynoso-Cuevas et al., 2008). Moreover, agar medium does not modify the root growth as hydroponics because it is a solid medium, and avoids the transfer of the seedlings after germination that may cause damage to the roots, since seeds can germinate and grow in the same medium (Tschan et al., 2009). Our results showed that plant growth decreased in a dose dependent manner in all species, but the effect was different according to metal and plant species. Considering shoot growth, *H. annuus* and *B. napus* exhibited similar responses to grow in the presence of Zn. Both crops decreased their shoot length at 0.3 mM of Zn. Our results are in agreement with Bernhard et al. (2005) and Hernández-Allica et al. (2008) who also reported the reduction of *B. napus* and *H. annuus* growth in the presence of high Zn concentrations under hydroponic conditions. However, *B. distachyon* showed a high capacity to grow with 1 mM of Zn in the medium. The shoot length remained the same at the different doses of Zn, and root length did not differ significantly from the control values, indicating the high tolerance of this grass to Zn.

As it was expected, *B. napus* showed a higher capacity to grow in the presence of 0.05 mM of Cd than the other two species. Many studies have remarked the high biomass production of *B. napus* in the presence of Cd concentrations that are toxic for most of the crops, being proposed for this reason as Cd phytoextractor by several authors (Nouairi et al., 2009; Selvam et al., 2009). Our work reported also the ability of *B. napus* and *H. annuus* seedlings to grow in the presence of 0.07 and 0.1 mM of As (V). *B. distachyon* was more sensitive to As (V) than the other two species: since its biomass was reduced from the lowest dose that was applied. Our results are in agreement with Liu et al. (2012), Chaturvedi et al. (2006) and Zhong et al. (2011) who observed the capacity of *Brassica* spp. to grow in As (V) spiked soils, and also with Marchiol et al. (2007) who reported the ability of *H. annuus* to grow in the presence of As in field conditions. In spite of these works, Vamerali et al. (2010) and Cavalca et al. (2013) remarked that the literature about the tolerance of energy crops to As is still limited, making it necessary to perform more studies about the ability of high biomass crops to

grow in the presence of this metalloid, in order to evaluate their possible future application on arsenic contaminated sites.

The biomass of the three crops strongly decreased in the presence of the lowest dose of Cr (VI). The high sensitivity that these crops showed to this metal dose indicates that the doses used were too high to test the tolerance of these crops. Fozia et al. (2008) also observed that the shoot length of *Helianthus annuus* was reduced by 50% in a spiked soil with 60 mg/kg of Cr (VI). Terzi et al. (2014) evaluated the Cr (VI) tolerance of different cultivars of *Brassica napus* under hydroponic conditions. They reported that the biomass was also reduced by 40% and 70% depending on the cultivar in the presence of 100µM of Cr (VI). The conducted *in vitro* experiments provided useful information about the impact of toxic metals on the first stages of plant growth. The comparison of our results with other works performed in different conditions showed that these tests are appropriated to evaluate the plant tolerance to metals in a short term. According to Peralta et al. (2001), Reynoso-Cuevas et al. (2008) and Buendía-González et al. (2010), the use of *in vitro* tests as a preceding step to soil experiments could help to predict the physiological responses of plants in the presence of metals. In addition, these experiments allowed to evaluate the tolerance of the new model grass, *B. distachyon.* This grass showed a high tolerance to Zn during the first growth stages, suggesting that it could be a suitable model plant to study energy crops tolerant to this metal, and the mechanisms involved in the responses to Zn stress at molecular level.

Recently, the Environmental Committee of the European Parliament endorsed a law where the production of traditional biofuels in healthy soils must be limited and new alternative ways should be used to produce biofuels (Environmental Committee of the European Parliament, 2015). The use of healthy soils to produce bio-energy reduces the area available for food and feed crop production. This increases pressure to free up more land, such as deforestation. Deforestation in itself increases greenhouse gas emissions, which may level off part or in some cases even eliminate the beneficial effects of using biofuels. The use of energy crops able to grow in metal contaminated soils could be an alternative way to produce bio-energy, avoiding the utilization of healthy soils to produce energy (Robinson et al., 2003; French et al., 2006; Field et al., 2008; Witters et al., 2009). Based on the need to find energy crops able to grow in metal contaminated soils with high biomass production and low requirements of cultivation, *H. tuberosus* was selected to evaluate its effectiveness to be used in phytotechnologies.

Although some authors have shown the ability of this plant to grow in the presence of metals (Cui et al., 2007; Chen et al., 2011; Long et al., 2013), its response to metals has been poorly studied.

H. tuberosus is propagated by tubers, differently from the other crops, which made it difficult to grow under *in vitro* conditions with agar. After successive tests in controlled conditions, a hydroponic system with substrate was selected as the most appropriate method to evaluate its ability to grow and accumulate metals, according to Chen et al. (2011). This growth system allowed to hold the tuber and at the same time to develop the root system in controlled conditions.

Under hydroponic conditions, *H. tuberosus* showed a similar response to *H. annuus* to grow with 0.1 mM Cd and 1 mM Zn, since plant growth was reduced below 50% in both crops. Similar behavior between both species was expected because they are closely related crops (Kays and Nottingham, 2008; Serieys et al., 2010). However, its propagation by tubers provides interesting advantages, such as the high ability of colonization and capture resources in the soil, the high adaptability to grow on poor soils and resist severe climatic conditions, such as frost or drought (Denoroy, 1996; Kim and Kim, 2014), which can be useful to reduce the production costs in phytotechnologies.

H. tuberosus grew well in the presence of multiple metal(loid)s under hydroponic conditions, since its above-ground biomass was not reduced in the presence of Cd, Cu, Pb, Zn or As(V), Cd and Ni, added as a mixture. The speciation analysis showed that metals and nutrients were present in species available to the plants. In addition, the pH and electrical conductivity were determined before and after each change of the nutrient solution to check possible variations in the availability of elements. The pH was maintained at 5.5 ± 0.5 and the electrical conductivity at 1.11 ± 0.16 $dS.m^{-1}$ showing that the availability of metal(loid)s and nutrients was constant along the experiment. In the studied conditions, complex interactions among metals could reduce the toxicity of the multi-polluted mixture (An et al., 2004; Sun et al., 2009; Papazoglou et al., 2011) through antagonistic effects, and also increase the metal uptake through synergistic interactions (Smilde et al., 1992; Luo and Rimmer, 1995). These interactions could explain the high concentration of metal(loid)s found in the plant tissues at the end of the experiment. Plant growth was strongly affected by the concentration of Cr (VI) that was

used in the experiment, suggesting that this concentration was too elevated to evaluate the tolerance of this crop to Cr (VI).

The two cultivar-clones (VR and D19) of *H. tuberosus* used in this study have been highlighted by their high yield in field conditions (Serieys et al., 2010; Sanz-Gallego, 2012). With the aim of selecting the best candidate for phytotechnologies, the responses of both cultivar-clones to metals were evaluated. Both cultivar-clones showed differences in metal accumulation where, D19 showed an effective mobilization of Pb to the harvestable parts and the best capacity to remove metal(loid)s from the studied solutions. Pogrzeba et al. (2011) reported the ability of *H. tuberosus* to mobilize Pb to the aerial parts in comparison with other energy crops, and also Cui et al. (2007) and Chen et al. (2009) observed the natural capacity of *H. tuberosus* to grow in a contaminated soil with elevated concentration of Pb. According to these results, this crop could be a promising candidate to obtain biomass and at the same time, remove metals from the soil, especially in Pb contaminated areas.

Both cultivar-clones showed similar nutrient requirements, since no significant differences were found between them in control conditions. D19 showed higher concentrations of metal(loid)s in the aerial parts and the roots than VR, but both cultivar-clones showed the same response of nutrient uptake in the presence of metals, independent of the treatment. This different response to metal could be due to the presence of a mechanism of VR to avoid the excess of metal(loid)s in the plant, and a better strategy of D19 to tolerate the high levels of these elements inside its tissues. Taking this into account, D19 presents characteristics that are more appropriate to be used as metal phytoextractor.

It is well known that the main limiting factors to implement phytoextraction in metal-contaminated soils are metal availability, metal uptake and translocation by the plant and phytotoxicity (Meers et al., 2008; Weyens et al., 2009). The interaction between plant growth-promoting bacteria (PGPB) and plants can enhance biomass production and metal tolerance of the host plant by decreasing phytotoxic symptoms (Genrich et al., 2000; Rajkumar et al., 2012). Several studies have shown that the production of bacterial siderophores, IAA, ACC deaminase and metal-resistance/sequestration systems are stimulated by metal exposure (Glick 1998; Diels et al., 1999; Dell' Amico et al., 2005). In our study, a total of 426 morphologically different cultivable bacterial

strains were isolated from bulk soil, rhizosphere soil, roots and stems of the *B. napus* plants grown in a metal contaminated soil. Most of the bacterial strains showed potential plant-growth promoting characteristics during the phenotypic tests in selective media, suggesting that metal contaminated soil exerts a selective pressure for bacteria able to tolerate metals and to produce beneficial compounds that can improve plant growth, and at the same time improve the proliferation of bacteria associated to the plant.

Although most of the bacterial strains showed plant-growth promoting characteristics, one bacterial strain from bulk soil (*Arthrobacter* sp. 222) associated to *B. napus*, and four root endophytes (*Pseudomonas* sp. 228, *Serratia* sp. 246, *Pseudomonas* sp. 256, *Pseudomonas* sp. 262) showed the highest production of ACC deaminase activity, indole-3-acetic acid, siderophores, acetoin, organic acids and capacity to solubilize phosphate and fix nitrogen. For this reason, these strains were selected to be inoculated as potential plant-growth promoting bacterial strains. The inoculation of the selected bacterial strains in *B. napus* seeds enhanced the root growth in the presence of concentrations of Cd and Zn that were highly toxic for the non-inoculated seedlings. These data confirm the ability of the selected strains to improve the plant growth in presence of toxic metals. At doses of 1000 µM Zn and 300 µM Cd, the five bacterial strains played an important role in the root development during the first stages of the plant growth. However, at doses of 300 µM Zn and 50 µM Cd, *B. napus* did not show toxicity symptoms and the bacteria did not enhance root growth. These data are in agreement with the hypothesis indicated above, which suggests that the plant growth-promoting bacterial mechanisms are stimulated under metal-induced stress. In the experiment with *B. napus*, the metal doses of 300 µM Zn and 50 µM Cd did not generate toxicity symptoms to the plant, and neither induced the bacterial PGP characteristics. Thereby, the growth promoting effects of the bacteria on the *B. napus* growth were only observed when plants were exposed to toxic concentration of metals.

In the literature, many authors have evaluated the inoculation of host plants with their associated bacteria (Germaine et al., 2004; Li et al., 2007; Gosh et al., 2011; Sessitsch et al., 2013), but some studies have also shown that bacteria isolated from metal tolerant plants can also promote the growth of plants from different taxonomic groups (Ma et al., 2011; Sheng et al., 2012; He et al., 2013). In the present study, bacterial strains associated with *B. napus* were also able to increase the biomass of *H. tuberosus* under

hydroponic conditions with increased Cd and Zn concentrations, and also when bacteria were added as consortium in a Cd-Zn contaminated soil. In addition to plant growth promotion, the inoculated strains also decreased the production of TBA reactive compounds and stimulated the activities of glutathione reductase, malic enzyme and isocitrate dehydrogenase. These effects on the plant physiology indicated that the bacteria established an interaction with *H. tuberosus* D19. This close interaction was also supported by the egfp-bacteria attached to the root hair surface and by the stimulation of the root hair development of plants inoculated with bacteria.

Gfp has been described as a practicable marker to study the bacterial behavior at the cell level in the rhizosphere or inside plant tissues (Bloemberg et al., 2000; Newman et al., 2003; Germaine et al., 2004). However, some authors have reported that the colonization of the gfp-labeled strain is lower than the wild-type, and thereby the labelling modified the colonization pattern of the wild-type (Weyens et al., 2012). Under hydroponic conditions, the egfp-*Pseudomonas* sp. 262 was attached to the root hair surface but it was not found inside the root of *H. tuberosus* D19. However, we cannot discard that the wild type could show different behavior and be able to colonize the tissues inside the root in other conditions. In spite of this, the obtained results illustrate that there was a clear interaction between bacteria and *H. tuberosus* D19, and that, in the presence of metals the bacterial strains isolated from *B. napus* can also establish an interaction with plants that are not taxonomically related.

Under hydroponic conditions, the inoculation of the bacterial strains had a more pronounced effect on the D19 cultivar-clone than on the VR. Inoculation of the bacterial strains improved the growth of D19 in the presence of Cd and Zn, while in the case of VR cultivar-clone the increase in growth was only produced under Zn-exposure. In case of Cd, the bacterial strains did not affect the growth of VR plants. In addition, D19 showed a higher biomass production than VR, a better capacity to extract metals from the multi-polluted solutions, and tolerated them inside the tissues. Based on the results obtained in the hydroponic experiments, D19 was chosen as a more suitable candidate to be inoculated with the selected bacterial strains in a Cd-Zn polluted soil.

As it was expected, the effects of the bacterial strains inoculated separately on the plant growth were more visible under hydroponic than in soil conditions. In the hydroponic experiment the metals were completely available to the plant, which generated a higher

phytotoxicity than in soil conditions. Thereby, the differences between inoculated plants and controls were less pronounced in soil than in hydroponic conditions. This fact could mask the positive effect of the bacteria on the growth when they were inoculated separately in the soil. Moreover, in soil there are numerous parameters involved that can decrease the effects of the inoculated bacteria, the competition with other microorganisms being one of the most important (Compant et al., 2010; Lebeau et al., 2011). The colonization, survival and activity are the most important factors when the bacteria are inoculated in soil conditions (Lugtenberg and Kamilova, 2009). In spite of this, the consortium inoculation improved the growth of *H. tuberosus* D19 in a Cd-Zn contaminated soil, showing that in soil conditions, where the competition with other microorganisms is high, the use of consortia and the interactions that occur between strains seem to provide a significant advantage. These results are in accordance with other authors that observed the potential of some bacterial strains acting together as a consortium versus the individual application of the same bacterial strains (Wu et al., 2006; Malekzadeh et al., 2012; Langella et al., 2014).

PGP bacteria can enhance plant growth through different mechanisms that are acting at the same time. For this reason it is difficult to know which mechanisms are behind the increased plant growth and metal uptake. In this study, the inoculation of *Pseudomonas* sp. 228, *Pseudomonas* sp. 262 and *Serratia* sp. 246 enhanced the growth of *H. tuberosus* under hydroponic conditions in the presence of Cd and Zn. The metal concentrations of both elements decreased in roots of plants inoculated with these bacterial strains. This decrease was only significant in plants inoculated with *Pseudomonas* sp. 262, but similar trends were observed in the other two strains. These data could support the hypothesis that in the studied conditions the bacteria could improve the plant growth through decreasing metal uptake and phytotoxicity symptoms by sharing the metal load with the plant. Similarly to metals, the decrease in the amount of nutrients observed in *H. tuberosus* inoculated with these bacteria could also be related with the bacterial mechanism of metal sequestration and/or biosorption. Microorganisms have developed complex mechanisms of metal resistance that can affect metals and nutrients availability (Nies, 1999; Bruins et al., 2000). PGB bacteria can sequestrate metals and nutrients through extracellular production of polysaccharides or by fixing these elements on the membrane, cell wall or capsule in the form of hydroxides or some other insoluble metal salts (Chen and Cutright, 2003; Kidd et al.,

2009; Ma et al., 2011). The bacterial surfaces present polarizable groups that can interact with cations, being responsible for the binding capacity (Vecchio et al., 1998). In the present study, the excess of Cd and Zn added in the nutrient solution could induce the bacterial mechanisms of metal resistance that reduce the metal availability, and also the solubility of other nutrients that could be precipitated in the cell surface. These results are in agreement with the studies of Madhaiyan et al. (2007), Vivas et al. (2006) and Marques et al. (2013) that also observed a decrease in the metal uptake of plants inoculated with different bacterial strains. According to them, bacteria can protect the plant against metal toxicity by reducing their uptake and phytotoxicity, and thereby, promoting the plant growth.

In soil conditions, the joint action of the consortium enhanced the Zn and Pb uptake in roots of *H. tuberosus* D19, indicating a different behavior when the strais were added individually or in combination. *Pseudomonas* sp. 262 was the only strain that increased the Pb uptake in roots of *H. tuberosus* D19 when it was individually inoculated. This suggests that *Pseudomonas* sp. 262 could be playing a more important role in the consortium with regards to Pb uptake than the other bacterial strains. *H. tuberosus* D19 inoculated with the consortium showed the highest concentrations of Zn and Pb in the roots. The decrease in the soil pH, and the increase in the root hair development could help to enhance the metal uptake by the plant. This increase in the metal uptake could induce the activities of glutathione reductase, malic enzyme and isocitrate dehydrogenase in order to protect the plant against metal induced oxidative stress, which can explain the high enzymatic activity observed in the roots of plants inoculated with the consortium.

Based on the results obtained in the phenotypic tests, the bacterial strains that formed the consortium could improve the growth and metal uptake of *H. tuberosus* D19 by producing ACC deaminase, indole-3-acetic acid, acetoin, siderophores, and organic acids. The production of these plant-growth promoting substances was not evaluated under soil conditions. However, the high capacity of these bacteria to produce PGP substances in the selective media, and the positive effects of the inoculation with respect to non-inoculated conditions, indicates that these characteristics can be related to the improvement of growth and metal uptake. In particular, indole-3-acetic acid and acetoin have been associated with the enhancement of the growth of lateral and adventitious roots. Both compounds can increase the root hair development, and thereby the root

surface, improving the element uptake, and also the root exudation that stimulates bacterial proliferation on the roots (Dobbelaere et al., 1999). Siderophores and organic acid production can also play an important role in the increase of the metal uptake by the plant, since the soil pH was acidifying, and the concentration of Fe in pore water solution increased in case *H. tuberosus* D19 was inoculated with the consortium. According to the results obtained in the pot soil experiment, we hypothesize that the bacterial strains acting as consortium increased plant growth and metal uptake in a Cd-Zn contaminated soil by decreasing phytotoxicity to metals, and by increasing the root surface through the enhancement of root hair development.

H. tuberosus D19 showed an effective translocation of Zn, leading to an accumulation of more than 1000 $mg.kg^{-1}$ Zn in the aerial parts from the Cd-Zn contaminated soil used in this study. Similar values of accumulation were found in herbaceous crops considered competent Zn phytoextrators in soil conditions (Vamerali et al., 2010), suggesting that *H. tuberosus* could be used as Zn phytoextractor. The results are also in agreement with Cui et al. (2007) who observed the high ability of *H. tuberosus* to extract Zn from contaminated soils, in comparison with other crops. The capacity of this crop to translocate Zn in a Cd-Zn contaminated soil, and the ability observed under multi-polluted hydroponic conditions to extract Pb from the solutions, suggest the use of this crop on soils contaminated with both metals. Besides this, the combination of this crop with the evaluated bacterial consortium could enhance the biomass production and the phytoextraction of both metals in contaminated areas not suitable for feed and food production.

In addition to its tolerance to grow in the presence of toxic metals, *H. tuberosus* presents agronomic characteristics required in phytotechnologies, such as high ability to extract nutrients from the soil, particulary K, and being efficient in its use (Soja et al., 1990; Sanz-Gallego, 2012). *H. tuberosus* requires high levels of K and Ca during its growth (Cassells and Deadman, 1993). This requirement was observed in the pot soil experiment. During the experiment, the K and Ca availability decreased in the rhizospheric soil of *H. tuberosus* D19, due to the consumption by the plant. In spite of this, *H. tuberosus* D19 showed concentrations of nutrients such as Mg and K considered too low to reach an appropriate growth of the plant in long-term conditions. In *H. tuberosus* the deficiency in nutrients disturbs the tuber morphogenesis more than the aerial growth (Soja et al., 1990). Thus, the biomass of *H. tuberosus* D19 was not

diminished in our experiment since the plants were growing during one month, despite of the low concentrations of some nutrients. In the studied soil, fertilization could be required to reach a reasonable biomass production of this crop in long-term experiments. Moreover, after addition of the bacterial consortium, the levels of nutrients as Mg, K, P, Fe and Cu decreased in the plant. This also suggests that an extra input of nutrients could enhance the plant growth and also the bacterial proliferation.

In spite of this, it is important to mention that *H. tuberosus* can grow in poor soils (Kays and Nottingham, 2008), including sandy soils (Denoroy, 1996). Soil pH or texture have only slight or no impact on the crop growth (Cors and Falisse, 1980), thereby this crop can be easily cultivated in a wide range of soils (Cosgrove et al., 1991; Kim and Kim, 2014). The high adaptability of this plant to a wide variety of soils makes of this crop a suitable candidate to be used in phytotechnologies.

The bacterial inoculation after the appearance of the first roots permits to evaluate the bacterial effects on the development of the plant during the first weeks of growth under metal exposure. The beneficial effects of PGP bacteria, in particular added as consortium, on the growth and metal uptake of *H. tuberosus* D19 in short-term exposure have been demonstrated in this study. Short-term inoculation experiments allow to study the bacterial mechanisms involved in plant growth and metal mobilization, and to select potential bacterial strains able to improve biomass in real conditions (Madhaiyan et al., 2007; Jiang et al., 2008; Braud et al., 2009). Further studies in soil must be performed with *H. tuberosus* in combination with the selected consortium in order to ensure the sustainable implementation of this technology for producing biomass and at the same time remediate contaminated soils. The survival of the consortium must be evaluated in long-term experiments, and in combination with an appropriate fertilization that can assure optimal plant growth and bacterial proliferation. The use of *H. tuberosus* D19 in combination with the selected consortium could be a suitable strategy to be used in phytoextraction of contaminated soils with Zn and Pb.

8.2. General conclusions

1. *B. distachyon* is able to grow in the presence of high doses of Zn during the first growth stages. This grass could be a suitable model plant to study energy crops tolerant to this metal, and the mechanisms implicated in the response to Zn stress at molecular level.

2. VR and D19 cultivar-clones of *H. tuberosus* show different responses to multiple metal(loid)s exposure:

- VR seems to possess mechanisms to avoid the entry of metals inside the tissues.

- D19 presents a better capacity to take up metals and tolerates them inside the plant.

3. The selected PGP bacterial strains can improve plant growth, even in a different species than the initial host plant.

4. The bacterial strains inoculated as consortium increase growth and metal uptake of the D19 cultivar-clone of *H. tuberosus* in a Cd-Zn contaminated soil by decreasing phytotoxicity to metals and by increasing root hair development.

5. *H. tuberosus* D19 responds to Pb and Zn excess by increasing the activities of malic enzyme and isocitrate dehydrogenase in roots, and glutathione reductase in leaves.

6. *H. tuberosus* D19 is a suitable crop to be used in metal contaminated soils and mainly as Zn phytoextractor.

7. The inoculation of the evaluated consortium improves the biomass production of *H. tuberosus* D19 and thereby, the amount of metals extracted by this crop.

8. Bacteria-assisted phytoextraction using *Heliantus tuberosus* could be a feasible strategy to remediate metal contaminated soils and obtain at the same time valuable biomass.

8.3. References

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ANNEX I

Genotypic and phenotypic characteristics of all bacterial strains isolated from soil (So), rhizosphere (Rh), roots (R) and stems (S) of *Brassica napus* plants growing in a Cd-Zn polluted soil. Bacterial strains were tested for metal resistance (0.8 mM Cd or 1mM Zn) and potential plant growth-promoting characteristics: production of ACC deaminase (ACC), acetoin (Acetoin), siderophores (Fe0 and Fe 0.25), indole-3-acetic acid (IAA), nitrogen fixation (N fix), organic acids (OA), and phosphorus solubilization (P sol). Comp: compartment; nd: not detected.

Comp	Strain	Identification	Accesion	Cd 0.8	Zn 1	ACC	Acetoin	Fe0	Fe0.25	IAA	N fix	OA	Psol
So 1	160	Arthrobacter	AJ785759	nd	nd	++	-	-	-	-	nd	-	-
So 1	161	Arthrobacter	AJ785759	+++	+++	-	-	-	-	-	-	-	-
So 1	162	Arthrobacter	AJ785759	++	+++	+	-	-	-	-	nd	-	-
So 1	163	Arthrobacter	AJ785759	-	-	+	-	-	-	-	-	-	-
So 1	164	Arthrobacter	AJ785759	++	++	-	-	-	-	-	-	-	-
So 3	204	Arthrobacter	AJ785759	+++	+++	+	-	-	-	-	-	-	-
So 3	193	Arthrobacter	EU086811	+++	+++	-	-	-	-	-	-	-	-
So 1	171	Arthrobacter	EU086826	-	-	+	-	-	-	-	-	-	-
So 1	177	Arthrobacter	EU086826	-	-	+	-	-	-	-	-	-	-
So 3	212	Arthrobacter	EU086826	-	-	-	nd	-	-	-	-	-	-
So 3	213	Arthrobacter	EU086826	-	-	-	nd	-	-	-	-	-	-
So 3	214	Arthrobacter	EU086826	-	-	-	nd	-	-	-	nd	nd	-
So 3	215	Arthrobacter	EU086826	-	-	+	-	-	-	-	-	-	-
So 3	216	Arthrobacter	EU086826	+	+++	-	-	-	-	-	-	-	+
So 3	222	Arthrobacter	EU086826	+++	+++	-	-	-	-	+++	-	-	+
So 3	196	Arthrobacter	FN908760	+++	+++	+	-	-	-	-	-	-	-
So 3	197	Arthrobacter	FN908760	+++	+++	+	-	-	-	-	-	-	+
So 3	198	Arthrobacter	FN908760	+++	+++	+	-	-	-	-	-	-	-
So 3	217	Arthrobacter	FN908760	+	+++	-	-	-	-	-	-	-	-
So 3	223	Arthrobacter	FN908760	+++	+++	-	-	+	+	-	-	+	-
So 3	224	Arthrobacter	FN908760	nd	nd	nd	nd	nd	nd	nd	-	nd	-
So 3	218	Arthrobacter	HQ398376	-	-	+	-	-	-	-	-	-	-
So 3	219	Arthrobacter	HQ398376	nd	nd	nd	nd	nd	nd	nd	-	nd	-
So 3	220	Arthrobacter	HQ398376	nd	nd	++	nd	nd	nd	nd	-	nd	-
So 1	176	Bacillus	AJ494727	nd	nd	-	-	-	-	-	nd	-	-
So 2	178	Bacillus	DQ207365	+	-	++	+	+	+	-	nd	-	-
So 2	179	Bacillus	DQ207365	+++	-	++	-	+	+	-	-	-	-
So 2	180	Bacillus	DQ207365	+++	+++	++	-	+	+	-	-	-	-
So 2	181	Bacillus	DQ207365	-	-	-	-	-	-	-	+	-	-
So 3	206	Bacillus	DQ207365	nd	nd	-	nd	nd	nd	-	-	-	-
So 3	226	Bacillus	DQ207365	-	-	-	-	nd	nd	nd	-	nd	-
So 2	189	Bacillus	EF173317	nd	nd	+	-	-	-	+	nd	+	-
So 1	170	Bacillus	EF528275	-	-	+	++	nd	nd	-	-	-	-
So 1	172	Bacillus	EF528275	+++	+++	nd	nd	nd	-	nd	-	nd	-

So 2	182	Bacillus	EF528275	-	-	+	++	nd	nd	-	+	-	-
So 2	183	Bacillus	EF528275	++	+++	++	-	-	-	-	+	-	-
So 3	190	Bacillus	EF528275	-	+	nd	nd	nd	nd	nd	nd	nd	-
So 3	192	Brevundimonas	DQ177489	-	-	-	-	-	-	-	-	-	-
So 3	194	Cupriavidus	HQ880684	-	++	-	-	-	-	-	nd	-	-
So 3	225	Cupriavidus	HQ880684	-	+++	+	nd	-	-	++	nd	-	-
So 3	203	Niastella	GQ339899	nd	nd	+	-	-	-	-	-	-	-
So 3	195	Paenibacillus	AY289507	-	-	+	+	+	-	-	-	+	+
So 3	210	Paenibacillus	AY289507	-	-	+	-	-	-	-	-	+	-
So 3	211	Paenibacillus	AY289507	-	-	++	-	-	-	-	-	+	-
So 3	221	Paenibacillus	AY289507	-	-	+	-	+	+	+	+	++	+
So 3	199	uncultured bacterium	HM813165	nd	++	-	nd	-	-	+++	nd	+	-
So 3	200	uncultured bacterium	HM813165	nd	nd	-	nd	nd	nd	nd	-	nd	-
So 2	184	Variovorax	AB098595	++	+++	-	-	+	-	-	nd	-	-
So 1	165	Variovorax	CP001635	++	+++	-	-	-	-	-	nd	-	-
So 1	166	Variovorax	CP001635	nd	nd	+	-	-	-	-	nd	-	-
So 1	167	Variovorax	CP001635	nd	nd	-	-	-	-	-	nd	-	-
So 3	207	Variovorax	EU934231	-	-	+	-	-	-	-	nd	-	-
So 3	208	Variovorax	EU934231	+	-	+	-	-	-	-	-	+	++
Rh 1	21	Agromyces	AY158025	-	-	-	-	-	-	-	nd	-	-
Rh 1	35	Agromyces	AY158025	-	-	-	nd	-	-	-	nd	-	-
Rh 1	37	Agromyces	AY158025	-	-	nd	nd	nd	nd	-	nd	nd	-
Rh 1	38	Agromyces	AY158025	-	-		-	-	-	-	nd	-	-
Rh 1	40	Agromyces	AY158025	-	-	nd	-	nd	nd	-	nd	-	-
Rh 1	41	Agromyces	AY158025	-	-	nd	-	nd	nd	-	nd	-	-
Rh 1	42	Agromyces	AY158025	-	-	nd	-	nd	nd	-	nd	nd	-
Rh 1	43	Agromyces	AY158025	-	-	nd	-	nd	nd	-	nd	nd	-
Rh 1	44	Agromyces	AY158025	-	-	-	nd	-	-	-	nd	-	-
Rh 3	122	Agromyces	AY452074	-	-	+	+	nd	nd	-	+	-	+
Rh 3	130	alpha proteobacterium	AB074623	+++	+++	-	-	-	-	-	-	-	-
Rh 3	131	alpha proteobacterium	AB074623	+	-	nd	nd	nd	nd	-	nd	-	-
Rh 3	132	alpha proteobacterium	AB074623	nd	nd	nd	-	-	-	-	nd	-	-
Rh 3	139	alpha proteobacterium	AB074623	-	-	-	nd	-		-	-	-	-
Rh 3	143	Arthrobacter	AB089841	-	+++	-	-	-	-	-	-	-	-
Rh 3	144	Arthrobacter	AB089841	++	+++	+	-	-	-	-	-	-	-
Rh 3	145	Arthrobacter	AB089841	+++	+++	+	-	-	-	++	-	-	+
Rh 3	146	Arthrobacter	AB089841	+++	+++	+	-	nd	nd	-	-	-	-
Rh 3	147	Arthrobacter	AB089841	+++	+++	+	-	-	-	-	-	-	-
Rh 2	434	Arthrobacter	AB648946	+	+++	++	-	-	-	-	-	-	-
Rh 2	62	Arthrobacter	EU086826	-	+	+	-	-	-	-	+	+	-
Rh 2	66	Arthrobacter	EU086826	-	+++	-	-	-	-	-	+	-	-
Rh 2	67	Arthrobacter	EU086826	++	+++	-	-	-	-	-	nd	-	-
Rh 2	68	Arthrobacter	EU086826	+++	+++	-	-	-	-	-	-	-	-

Rh 2	69	Arthrobacter	EU086826	++	+++	-	-	-	-	-	-	-	-
Rh 2	70	Arthrobacter	EU086826	++	++	-	-	-	-	-	-	-	-
Rh 2	71	Arthrobacter	EU086826	+	+++	-	-	-	-	-	-	-	-
Rh 2	72	Arthrobacter	EU086826	-	+++	-	-	-	-	-	-	-	-
Rh 2	73	Arthrobacter	EU086826	-	+++	-	-	-	-	-	-	++	-
Rh 2	74	Arthrobacter	EU086826	-	-	-	-	-	-	-	+	+	-
Rh 2	75	Arthrobacter	EU086826	+++	+++	-	-	-	-	-	-	-	-
Rh 2	79	Arthrobacter	EU086826	+	+	nd	-	-	nd	-	-	+	-
Rh 2	80	Arthrobacter	EU086826	-	+++	-	-	-	-	-	-	-	-
Rh 2	81	Arthrobacter	EU086826	+	++	-	-	-	-	-	+	+	-
Rh 2	83	Arthrobacter	EU086826	++	++	-	-	-	-	-	-	+	-
Rh 2	84	Arthrobacter	EU086826	-	-	-	-	-	-	-	nd	-	-
Rh 3	123	Arthrobacter	EU086826	-	+++	-	-	-	-	-	-	-	+
Rh 3	137	Arthrobacter	EU086826	nd	nd	+	-	-	-	-	-	+	+
Rh 3	138	Arthrobacter	EU086826	-	+++	-	-	-	-	-	-	+	++
Rh 3	148	Arthrobacter	EU086826	+++	+++	+	-	-	-	-	-	-	+
Rh 1	14	Arthrobacter	FN908760	-	++	-	-	-	-	-	-	++	-
Rh 1	15	Arthrobacter	FN908760	-	-	-	nd	-	-	-	-	-	-
Rh 1	16	Arthrobacter	FN908760	-	-	-	+	-	-	-	-	+++	-
Rh 1	17	Arthrobacter	FN908760	++	+++	-	-	-	-	-	-	-	-
Rh 1	18	Arthrobacter	FN908760	++	+++	-	-	-	-	-	-	-	-
Rh 1	22	Arthrobacter	FN908760	-	+++	-	-	-	-	-	-	-	-
Rh 1	23	Arthrobacter	FN908760	+	+++	-	-	-	-	+	-	-	+
Rh 1	52	Arthrobacter	FN908760	++	+++	-	nd	-	-	-	nd	-	-
Rh 1	53	Arthrobacter	FN908760	++	+++	-	nd	-	-	-	nd	-	-
Rh 2	63	Arthrobacter	FN908760	-	++	-	nd	-	-	-	-	-	-
Rh 2	82	Arthrobacter	FN908760	+++	+++	-	-	-	-	-	+	-	-
Rh 2	92	Arthrobacter	FN908760	++	+++	-	-	-	-	-	-	-	-
Rh 2	93	Arthrobacter	FN908760	++	-	-	-	-	-	-	-	-	-
Rh 2	94	Arthrobacter	FN908760	+++	+++	-	-	-	-	-	-	-	-
Rh 2	95	Arthrobacter	FN908760	+++	+++	-	-	-	-	-	-	-	-
Rh 2	96	Arthrobacter	FN908760	nd	nd	+	-	-	-	-	-	-	-
Rh 2	100	Arthrobacter	FN908760	-	-	+++		-		-	-	++	-
Rh 3	149	Arthrobacter	FN908760	+++	+++	-	-	-	-	++	-	-	+
Rh 2	78	Arthrobacter	HQ398376	-	+++	-	-	-	-	-	-	-	-
Rh 3	133	Bacillus	AY947532	nd	nd	nd	-	-	nd	-	-	+	-
Rh 3	134	Bacillus	AY947532	-	+++	-	-	-	-	-	-	-	-
Rh 1	1	Bacillus	DQ445268	-	+	-	+	-	-	-	-	++	-
Rh 1	2	Bacillus	DQ445268	-	-	++	-	++	++	++	nd	-	-
Rh 1	3	Bacillus	DQ445268	-	-	++	++	++	++	+	-	++	-
Rh 1	4	Bacillus	DQ445268	-	+	-	++	-	-	-	-	++	-
Rh 1	5	Bacillus	DQ445268	-	+	-	+	-	-	-	-	++	-
Rh 3	116	Bacillus	EF173317	-	+++	-	-	-	-	-	-	+	-
Rh 3	117	Bacillus	EF173317	-	-	-	-	+	+	-	-	-	-
Rh 3	118	Bacillus	EF173317	-	+	+	-	+	+	+	++	+	++
Rh 3	119	Bacillus	EF173317	-	-	+	++	-	-	-	-	+	+

Rh 2	97	Bacillus	EF528288	+++	+++	-	+++	nd	nd	-	-	-	-
Rh 2	98	Bacillus	EF528288	-	-	+	+++	nd	nd	-	-	-	-
Rh 2	99	Bacillus	EF528288	-	-	+	++	nd	nd	-	-	-	-
Rh 3	120	Bacillus	EF528288	-	++	+	++	nd	nd	nd	-	-	-
Rh 3	121	Bacillus	EF528288	-	-	-	-	-	-	-	-	-	-
Rh 3	125	Bacillus	EF528288	-	+++	-	-	-	-	+++	nd	-	-
Rh 3	126	Bacillus	EF528288	+	+++	nd	-	-	-	-	-	-	+
Rh 3	127	Bacillus	EF528288	+++	+++	nd	-	-	-	-	-	-	-
Rh 3	128	Bacillus	EF528288	+++	+++	-	-	-	-	-	-	-	-
Rh 3	129	Bacillus	EF528288	+++	+++	-	-	-	-	-	-	-	-
Rh 3	427	Bacillus	EF528288	++	+++	-	-	-	-	-	+	-	-
Rh 3	428	Bacillus	EF528288	-	-	++	-	-	-	-	+	-	-
Rh 1	45	Bacillus	FM173268	-	-	-	-	-	-	-	-	-	-
Rh 2	60	Bacillus	JF713459	nd	nd	-	-	-	-	-	nd	-	-
Rh 3	112	beta proteobacterium	DQ535028	-	-	+	-	-	-	-	nd	-	-
Rh 2	64	Bosea	AB542375	nd	nd	+	-	-	-	-	nd	-	-
Rh 2	76	Bosea	AB542375	-	-	++	-	-	nd	-	nd	-	-
Rh 3	113	Chryseobacterium	JF700385	-	+++	nd	-	-	-	-	-	-	-
Rh 3	114	Chryseobacterium	JF700385	-	-	-	-	-	-	-	nd		-
Rh 1	11	Cupriavidus	HQ880684	+++	+++	-	-	-	-	-	nd	-	-
Rh 1	12	Cupriavidus	HQ880684	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Rh 1	46	Dyadobacter	HQ231938	nd	nd	-	nd	-	-	-	-	-	-
Rh 1	47	Dyadobacter	HQ231938	-	-	+	-	+	+	+	nd	-	-
Rh 3	105	Leifsonia	FR749830	+++	+++	-	-	-	-	-	-	+	+
Rh 3	151	Leifsonia	HQ530518	-	-	+	-	-	-	-	-	-	+++
Rh 3	101	Lysobacter	EU440724	+++	+++	-	nd	nd	nd	-	nd	-	-
Rh 3	102	Lysobacter	EU440724	nd	nd	nd	nd	nd	nd	nd	nd	nd	-
Rh 3	103	Lysobacter	EU440724	nd	nd	nd	nd	-	-	-	-	-	-
Rh 3	124	Lysobacter	EU440724	+++	-	+	-	-	-	-	-	-	-
Rh 3	135	Lysobacter	EU440724	++	+++	-	nd	-	nd	-	-	-	-
Rh 3	136	Lysobacter	EU440724	nd	nd	-	nd	nd	nd	-	-	-	-
Rh 2	86	Methylobacterium	AF293375	-	-	-	-	-	-	-	nd	nd	-
Rh 1	8	Mitsuaria	AB560607	nd	nd	-	-	-	-	-	nd	-	-
Rh 1	19	Mitsuaria	AB560607	nd	nd	-	-	-	-	-	nd	-	-
Rh 1	20	Mitsuaria	AB560607	-	-	-	-	-	-	-	nd	-	-
Rh 1	56	Mitsuaria	AB560607	-	-	-	-	-	-	-	nd	-	-
Rh 3	152	Mitsuaria	AB560607	-	-	nd	-	+	+	-	nd	-	-
Rh 3	153	Mitsuaria	AB560607	nd	nd	-	-	-	-	-	nd	-	-
Rh 3	115	Patulibacter	EU710748	-	-	-	-	-	-	-	++	+	-
Rh 3	140	Patulibacter	EU710748	-	+++	-	-	-	-	-	++	+	-
Rh 3	141	Patulibacter	EU710748	-	+++	-	-	-	-	-	++	+	+
Rh 3	142	Patulibacter	EU710748	-	+++	-	-	-	-	-	++	+	-
Rh 3	154	Patulibacter	EU710748	nd	nd	-	-	-	-	-	++	+	-
Rh 3	155	Pseudomonas	GU595312	-	+++	++	-	+	+	-	nd	-	+
Rh 1	7	Pseudomonas	HQ242754	-	-	++	-	-	-	+	nd	-	-
Rh 2	77	Pseudomonas	HQ242754	-	-	++	-	+	+	++	nd	-	-

Rh 1	57	Pseudomonas	JF772537	-	-	+++	-	+	+	+	nd	-	-
Rh 1	58	Pseudomonas	JF772537	-	-	+	-	+	+	+	nd	-	-
Rh 1	59	Pseudomonas	JF772537	-	-	+	-	+	+	+	nd	-	-
Rh 1	54	Shinella	AB238789	nd	nd	-	-	-	-	+	nd	-	-
Rh 1	55	Shinella	AB238789	+	+++	-	-	-	-	-	-	-	-
Rh 2	61	Shinella	AB238789	nd	nd	-	nd	-	nd	nd	nd	-	-
Rh 1	24	Sphingopyxis	AB161684	-	-	-	nd	-	-	-	nd	-	-
Rh 1	25	Staphylococcus	AB009944	+++	+++	-	+	-	-	-	-	+++	-
Rh 1	26	Staphylococcus	AB009944	+++	+++	-	-	-	-	-	-	-	-
Rh 1	27	Staphylococcus	AB009944	+++	+++	-	-	-	-	-	nd	-	-
Rh 1	28	Staphylococcus	AB009944	+++	+++	-	-	-	-	-	nd	-	-
Rh 1	29	Staphylococcus	AB009944	+++	+++	-	-	-	-	-	nd	-	-
Rh 1	30	Staphylococcus	AB009944	++	+++	++	nd	-	-	-	nd	-	-
Rh 1	31	Staphylococcus	AB009944	++	+++	-	nd	-	-	-	nd	-	-
Rh 1	32	Staphylococcus	AB009944	++	+++	-	-	-	-	-	nd	-	-
Rh 1	33	Staphylococcus	AB009944	+	+++	+	-	-	-	-	nd	-	-
Rh 1	34	Staphylococcus	AB009944	++	+++	-	nd	-	-	-	-	-	-
Rh 1	36	Staphylococcus	AB009944	-	-	-	nd	nd	nd	-	nd	-	-
Rh 1	39	Staphylococcus	AB009944	-	-		-	-	-	-	nd	-	-
Rh 2	85	uncultured bacterium	JF181263	-	-	++	-	nd	-	+	-	++	-
Rh 2	65	Variovorax	AB098595	nd	nd	-	-	-	-	-	nd	-	-
Rh 2	87	Variovorax	AB552859	nd	nd	nd	nd	nd	nd	nd	-	nd	-
Rh 2	88	Variovorax	AB552859	nd	nd	-	-	-	-	-	nd	-	-
Rh 2	89	Variovorax	AB552859	nd	nd	-	-	-	-	-	nd	-	-
Rh 2	90	Variovorax	AB552859	nd	nd	nd	nd	nd	nd	nd	nd	nd	-
Rh 2	91	Variovorax	AB552859	nd	nd	-	-	-	-	-	nd	-	-
Rh 3	157	Variovorax	AB552859	nd	nd	nd	-	-	-	-	nd	-	-
Rh 3	158	Variovorax	AB552859	nd	nd	-	-	nd	nd	-	-	-	-
Rh 3	159	Variovorax	AB552859	nd	nd	-	-	-	-	-	nd	-	-
Rh 1	48	Variovorax	CP001635	nd	nd	-	-	-	-	-	nd	-	-
Rh 1	50	Variovorax	CP001635	nd	nd	-	-	-	-	-	nd	-	-
Rh 1	51	Variovorax	CP001635	nd	nd	-	-	-	-	-	nd	-	-
Rh 3	110	Variovorax	CP001635	-	-	-	++	nd	nd	-	+	-	+
Rh 3	150	Variovorax	CP001635	+++	+++	-	-	-	-	-	nd	-	-
R 2	296	Agrococcus	EU169180	-	-	nd	nd	nd	nd	nd	-	nd	-
R 2	297	Agrococcus	EU169180	-	-	nd	-	nd	nd	nd	-	nd	-
R 2	298	Agrococcus	EU169180	++	-	++	-	-	-	+	nd	-	++
R 3	324	Agromyces	AY452074	-	+++	++	-	-	-	-	+	-	+
R 3	325	Agromyces	AY452074	-	+++	+	-	-	-	-	+	+	-
R 3	339	Agromyces	AY452074	-	-	++	nd	-	-	-	-	-	-
R 3	340	Agromyces	AY452074	++	+++	++	-	-	-	-	-	-	-
R 3	341	Agromyces	AY452074	++	+++	+	-	-	-	-	-	-	-
R 3	342	Agromyces	AY452074	++	+++	++	-	-	-	-	-	-	-
R 3	351	Agromyces	AY452074	-	-	+++	-	-	-	-	+	-	-
R 1	242	Alcaligenes	AJ509012	+	++	++	-	-	-	-	-	-	-

R 2	299	Arthrobacter	HQ398376	-	-	+	-	-	-	-	-	-	-
R 2	300	Arthrobacter	HQ398376	++	+++	+	-	-	-	-	-	-	-
R 2	301	Arthrobacter	HQ398376	++	+++	+	-	-	-	-	+	-	-
R 2	302	Arthrobacter	HQ398376	+	+++	+	-	-	-	-	-	-	-
R 2	303	Arthrobacter	HQ398376	+	+++	+	-	-	-	-	-	-	-
R 1	275	Burkholderia	FJ786047	-	-	+	-	-	-	+	nd	-	-
R 2	313	Burkholderia	FJ786047	nd	nd	++	-	-	-	+	-	-	-
R 2	314	Burkholderia	FJ786047	nd	nd	++	-	-	-	+	nd	-	-
R 2	315	Burkholderia	FJ786047	nd	nd	++	-	-	-	-	-	-	-
R 2	316	Burkholderia	FJ786047	-	-	+	-	-	-	+++	nd	-	-
R 3	357	Burkholderia	FJ786047	nd	nd	+	-	-	-	+	nd	-	-
R 3	358	Burkholderia	FJ786047	nd	nd	++	-	-	-	-	nd	-	-
R 3	359	Burkholderia	FJ786047	nd	nd	++	-	-	-	-	nd	-	-
R 3	360	Burkholderia	FJ786047	nd	nd	++	-	-	-	-	nd	-	-
R 3	361	Burkholderia	FJ786047	-	-	++	-	nd	nd	-	-	-	-
R 3	371	Burkholderia	FJ786047	nd	nd	-	-	-	-	-	-	-	-
R 3	375	Burkholderia	FJ786047	nd	nd	+++	-	-	nd	-	-	-	-
R 3	386	Burkholderia	FJ786047	nd	nd	++	-	-	-	-	-	-	-
R 3	435	Burkholderia	FJ786047	nd	nd	-	nd	nd	nd	-	-	-	-
R 3	388	Burkholderia	FJ786047	nd	nd	++	-	nd	nd	nd	nd	nd	-
R 1	240	Caulobacter	AJ227759	nd	-	nd	nd	nd	nd	nd	-	nd	-
R 1	241	Caulobacter	AJ227759	-	+++	+	nd	-	-	-	nd	-	-
R 1	239	Caulobacter	EF612347	nd	-	+	nd	-	-	-	-	+	-
R 1	267	Caulobacter	JF722654	nd	-	+	-	-	-	+++	-	-	-
R 1	268	Caulobacter	JF722654	-	+++	+	nd	-	-	-	-	-	-
R 1	269	Caulobacter	JF722654	-	+++	+	nd	-	+	-	-	-	-
R 3	326	Lysobacter	EU440724	nd	nd	nd	nd	nd	nd	nd	-	nd	-
R 3	327	Lysobacter	EU440724	-	+++	-	-	-	-	-	nd	-	-
R 3	328	Lysobacter	EU440724	nd	nd	nd	nd	nd	nd	nd	-	nd	-
R 3	329	Lysobacter	EU440724	nd	nd	nd	-	nd	nd	-	-	-	-
R 2	304	Microbacterium	EF540477	-	-	+	nd	nd	nd	-	-	-	-
R 3	331	Microbacterium	JF915347	-	-	++	-	-	-	-	+	+	+
R 3	321	Mitsuaria	AB560607	nd	nd	-	-	+	+	-	nd	-	-
R 3	322	Mitsuaria	AB560607	-	-	-	-	-	-	-	nd	-	-
R 3	323	Mitsuaria	AB560607	nd	nd	nd	nd	nd	nd	nd	-	nd	-
R 3	382	Mitsuaria	AB560607	nd	nd	-	-	-	-	-	nd	-	-
R 2	317	Pedobacter	AY599663	nd	nd	-	++	-	-	-	-	-	-
R 3	354	Pedobacter	AY599663	nd	nd	-	+	-	-	-	-	-	-
R 3	355	Pedobacter	AY599663	-	-	-	+	-	-	-	-	-	-
R 3	356	Pedobacter	AY599663	nd	nd	nd	nd	nd	nd	nd	-	nd	-
R 1	277	Polaromonas	AB245355	nd	-	++	-	+	nd	+	-	nd	-
R 2	286	Pseudomonas	DQ453821	+	-	+	-	-	-	+	-	-	+
R 2	287	Pseudomonas	DQ453821	+	-	+	-	-	-	+	-	-	+
R 2	288	Pseudomonas	DQ453821	+	-	++	-	-	-	+	-	-	+
R 2	289	Pseudomonas	DQ453821	+	-	++	-	+	+	+	nd	-	-
R 2	290	Pseudomonas	DQ453821	+	-	++	-	-	-	+	nd	-	++

R 2	291	Pseudomonas	DQ453821	+	-	++	-	-	-	+	+	-	++
R 2	292	Pseudomonas	DQ453821	+	-	+	-	-	-	+	-	-	++
R 2	430	Pseudomonas	DQ453821	-	-	+++	-	+	+	+	nd	-	-
R 2	431	Pseudomonas	DQ453821	-	-	+++	-	+	+	+	nd	-	-
R 2	432	Pseudomonas	DQ453821	-	-	+++	-	+	+	+	nd	-	-
R 2	293	Pseudomonas	DQ453821	+	-	++	-	-	-	+	-	-	-
R 1	228	Pseudomonas	GU595312	++	++	++	-	+	+	+	-	++	++
R 1	229	Pseudomonas	GU595312	++	-	+	-	+	+	+	-	++	-
R 1	230	Pseudomonas	GU595312	++	-	+	-	+	+	+++	-	+	-
R 1	231	Pseudomonas	GU595312	++	-	++	-	+	+	+	-	++	-
R 1	232	Pseudomonas	GU595312	++	-	+	-	+	+	+	-	+	-
R 1	233	Pseudomonas	GU595312	++	-	+	-	+	+	+	-	+	-
R 1	234	Pseudomonas	GU595312	++	-	+++	-	+	+	+	-	+	+
R 1	235	Pseudomonas	GU595312	++	-	+++	-	+	+	+	-	+	+
R 1	236	Pseudomonas	GU595312	+	-	-	-	+	+	+	-	+	-
R 1	237	Pseudomonas	GU595312	-	-	+++	-	+	+	+	-	-	-
R 1	250	Pseudomonas	GU595312	+++	-	+	-	+	+	+	-	++	-
R 1	251	Pseudomonas	GU595312	+	-	+	-	+	+	+	-	++	-
R 1	252	Pseudomonas	GU595312	++	++	+	-	+	+	-	-	++	-
R 1	254	Pseudomonas	GU595312	++	++	+	-	+	+	+	-	++	++
R 1	255	Pseudomonas	GU595312	+	++	++	-	+	+	+	-	++	++
R 1	256	Pseudomonas	GU595312	+	+++	+	+	+	+	++	++	+	+++
R 1	257	Pseudomonas	GU595312	-	-	+	-	+	+	+++	-	+	+++
R 1	258	Pseudomonas	GU595312	-	++	-	nd	+	+	+++	-	-	-
R 1	259	Pseudomonas	GU595312	nd	-	-	-	-	-	-	nd	-	-
R 1	261	Pseudomonas	GU595312	+++	+++	+	-	+	+	++	nd	-	-
R 1	262	Pseudomonas	GU595312	+++	++	+	-	+	+	+	-	++	-
R 1	263	Pseudomonas	GU595312	+++	-	+	-	+	+	+	-	++	+
R 1	264	Pseudomonas	GU595312	++	+	+	-	+	+	+	-	++	+
R 1	265	Pseudomonas	GU595312	++	-	+	-	+	+	+	-	++	++
R 1	266	Pseudomonas	GU595312	-	-	++	-	+	+	+	-	+	-
R 1	433	Pseudomonas	GU595312	++	-	-	-	+	+	-	nd	++	-
R 1	270	Pseudomonas	GU595312	++	-	-	-	+	-	+	-	++	+
R 1	274	Pseudomonas	GU595312	nd	-	-	-	-	-	+++	-	-	-
R 1	276	Pseudomonas	GU595312	-	-	+	nd	-	-	+	-	-	-
R 1	278	Pseudomonas	GU595312	++	-	+	-	+	+	+	-	++	++
R 1	279	Pseudomonas	GU595312	++	-	+	-	+	+	+	-	++	++
R 1	280	Pseudomonas	GU595312	nd	-	nd	nd	nd	nd	nd	-	++	+
R 1	281	Pseudomonas	GU595312	nd	-	+	-	+	+	+	-	nd	+
R 1	282	Pseudomonas	GU595312	++	-	+	-	+	+	+	-	++	++
R 1	283	Pseudomonas	GU595312	++	-	++	-	nd	nd	-	-	++	-
R 1	284	Pseudomonas	GU595312	nd	-	++	-	-	-	+	-	-	-
R 1	285	Pseudomonas	GU595312	nd	-	++	-	-	-	-	-	-	-
R 2	295	Pseudomonas	GU595312	-	-	+	-	+	+	++	-	-	-
R 2	307	Pseudomonas	GU595312	-	-	++	-	-	-	++	-	-	-
R 2	308	Pseudomonas	GU595312	++	-	++	-	-	-	+	-	-	++

R 2	309	Pseudomonas	GU595312	++	-	+	-	-	-	+	-	-	++
R 2	310	Pseudomonas	GU595312	-	-	+	-	+	+	+	-	-	-
R 2	311	Pseudomonas	GU595312	-	-	+	-	+	+	++	-	-	-
R 2	312	Pseudomonas	GU595312	++	-	++	-	-	-	+	-	-	++
R 3	374	Pseudomonas	GU595312	-	+++	+	-	+	+	-	-	-	-
R 3	376	Pseudomonas	GU595312	-	-	++	-	-	-	+	-	-	-
R 3	385	Pseudomonas	GU595312	-	-	+	-	-	-	+++	-	-	-
R 1	243	Serratia	HM596429	+++	-	++	++	+	+	+++	++	++	+
R 1	244	Serratia	HM596429	+++	+++	++	-	+	+	++	-	-	-
R 1	245	Serratia	HM596429	+++	-	+	+	+	+	+++	++	++	-
R 1	246	Serratia	HM596429	+++	+++	+++	-	+	+	++	-	++	-
R 1	249	Serratia	HM596429	+++	-	++	+	+	+	+++	++	++	-
R 1	238	Sphingopyxis	AB161684	-	+	-	nd	-	-	-	nd	-	-
R 3	318	Sphingopyxis	AB161684	-	+++	-	-	-	-	-	nd	-	-
R 3	319	Sphingopyxis	AB161684	-	+++	++	-	-	-	+++	-	-	-
R 3	320	Sphingopyxis	AB161684	-	+++	-	-	-	-	-	-	-	+
R 3	330	Sphingopyxis	AB161684	-	-	-	nd	-	-	-	nd	-	-
R 3	336	Stenotrophomonas	AJ551165	nd	nd	++	-	-	-	-	nd	-	-
R 3	346	Stenotrophomonas	AJ551165	-	-	+++	-	-	-	-	nd	-	-
R 3	347	Stenotrophomonas	AJ551165	-	-	+++	-	-	-	+++	-	-	-
R 1	273	uncultured bacterium	HM813165	-	-	+	-	+	+	++	nd	-	-
R 1	271	Variovorax	AB552859	nd	-	-	-	-	-	-	nd	-	-
R 1	272	Variovorax	AB552859	-	-	nd	nd	nd	nd	++	-	-	-
R 2	305	Variovorax	AB552859	nd	nd	-	-	-	-	-	nd	-	-
R 3	333	Variovorax	AB552859	nd	nd	nd	nd	nd	nd	nd	-	nd	-
R 3	334	Variovorax	AB552859	nd	nd	nd	nd	nd	nd	nd	-	nd	-
R 3	335	Variovorax	AB552859	nd	nd	nd	nd	nd	nd	nd	-	nd	-
R 3	343	Variovorax	AB552859	nd	nd	nd	-	nd	+	nd	-	nd	-
R 3	344	Variovorax	AB552859	nd	nd	nd	nd	nd	nd	nd	-	nd	-
R 3	345	Variovorax	AB552859	nd	nd	nd	nd	nd	nd	nd	-	nd	-
R 3	349	Variovorax	AB552859	nd	nd	-	-	-	-	-	nd	-	-
R 3	350	Variovorax	AB552859	nd	nd	-	-	-	-	-	nd	-	-
R 3	362	Variovorax	AB552859	nd	nd	nd	-	-	-	-	-	-	-
R 3	363	Variovorax	AB552859	-	-	nd	-	-	-	-	nd	-	-
R 3	364	Variovorax	AB552859	-	-	-	-	-	-	-	-	-	-
R 3	366	Variovorax	AB552859	-	-	-	-	-	-	-	nd	-	-
R 3	367	Variovorax	AB552859	nd	nd	nd	nd	nd	nd	nd	-	nd	-
R 3	377	Variovorax	AB552859	-	-	+	-	nd	-	-	-	-	-
R 3	378	Variovorax	AB552859	-	-	+	-	-	nd	-	-	-	-
R 3	379	Variovorax	AB552859	nd	nd	+	-	nd	nd	-	nd	-	-
R 3	380	Variovorax	AB552859	nd	nd	nd	nd	nd	nd	-	-	-	-
R 1	247	Variovorax	CP001635	-	-	-	-	-	-	-	nd	-	-
R 1	248	Variovorax	CP001635	-	-	+	-	-	+	-	nd	-	-
R 1	253	Variovorax	CP001635	nd	-	-	-	-	-	-	nd	-	-
R 1	260	Variovorax	CP001635	-	-	-	-	-	nd	-	nd	-	-
R 2	306	Variovorax	CP001635	-	+	nd	nd	nd	nd	nd	-	nd	-

R 3	352	Variovorax	DQ3256487	-	-	++	-	nd	nd	-	-	-	-
R 3	353	Variovorax	DQ3256487	nd	nd	++	-	nd	nd	-	-	-	-
R 3	368	Variovorax	DQ3256487	-	-	-	-	-	+	-	-	-	-
R 3	369	Variovorax	DQ3256487	-	-	-	-	-	-	-	nd	-	-
R 3	372	Variovorax	DQ3256487	-	-	-	-	-	-	-	-	-	-
R 3	383	Variovorax	DQ3256487	-	-	-	-	-	-	-	-	-	-
R 3	384	Variovorax	DQ3256487	nd	nd	nd	-	-	-	-	-	-	-
R 3	392	Variovorax	DQ3256487	nd	nd	+	-	nd	nd	-	nd	-	-
R 3	365	Variovorax	GU181268	-	-	++	-	nd	nd	-	-	-	-
S 2	403	Agromyces	AY452074	-	-	-	-	-	-	+	-	-	-
S 2	404	Agromyces	AY452074	-	-	-	-	-	-	+	-	-	-
S 2	405	Agromyces	AY452074	-	-	+	nd	-	-	+	-	-	-
S 2	406	Agromyces	AY452074	-	-	-	-	-	-	++	-	-	-
S 2	407	Agromyces	AY452074	-	-	-	-	-	-	-	-	-	-
S 2	408	Agromyces	AY452074	-	-	-	-	-	-	+	-	-	-
S 2	409	Agromyces	AY452074	-	-	-	-	-	-	-	-	-	-
S 2	410	Agromyces	AY452074	-	-	-	-	-	-	-	-	-	-
S 2	411	Agromyces	AY452074	-	-	-	nd	-	-	-	-	-	-
S 2	412	Agromyces	AY452074	-	-	-	-	-	-	-	-	++	-
S 2	413	Agromyces	AY452074	-	-	-	-	-	-	-	-	-	-
S 2	414	Agromyces	AY452074	-	-	-	nd	-	-	-	-	-	-
S 2	415	Agromyces	AY452074	-	-	-	-	-	-	+	-	-	-
S 2	398	Micrococcus	GU595336	-	-	-	-	-	-	+++	-	-	-
S 2	399	Micrococcus	GU595336	-	-	-	-	-	-	+++	-	-	-
S 2	400	Micrococcus	GU595336	-	-	-	-	-	-	++	-	-	-
S 2	401	Micrococcus	GU595336	-	-	-	-	-	-	+++	-	-	-
S 2	402	Micrococcus	GU595336	-	-	-	-	-	-	++	-	-	-
S 2	416	Micrococcus	GU595336	-	-	-	-	-	-	+++	-	-	-
S 2	417	Micrococcus	GU595336	-	-	-	-	-	-	+	-	-	-
S 2	418	Micrococcus	GU595336	-	-	-	-	-	-	+	-	-	-
S 2	419	Micrococcus	GU595336	-	-	-	-	-	-	+	-	-	-
S 2	420	Micrococcus	GU595336	-	-	-	-	-	-	+	-	-	-
S 2	421	Micrococcus	GU595336	-	-	-	-	-	-	+	-	-	-
S 2	422	Micrococcus	GU595336	-	-	-	-	-	-	+	-	-	-
S 2	423	Micrococcus	GU595336	-	-	-	nd	-	-	-	-	nd	-
S 2	424	Micrococcus	GU595336	-	-	-	nd	-	-	+++	-	-	-
S 2	425	Micrococcus	GU595336	-	-	-	nd	-	-	-	-	-	-
S 3	426	Pedobacter	AY599663	-	+++	-	-	-	-	-	+	+	-
S 1	393	Pedobacter	HM224489	nd	nd	nd	nd	nd	nd	nd	-	nd	-
S 1	394	Pedobacter	HM224489	nd	nd	nd	nd	nd	nd	nd	-	nd	-
S 1	395	Pedobacter	HM224489	nd	nd	-	+	-	-	-	+	+	-
S 1	396	Pedobacter	HM224489	-	-	-	+	-	-	-	+	+	-
S 1	397	Pedobacter	HM224489	nd	++	-	+	-	-	-	+	+	-

ANNEX II

Statistical analyses per treatment to evaluate differences between samplings of pore water solution along the pot soil experiment.

Soil

Ca

Duncan[a,b,c]

Sampling	N	Subset 1
1	3	54,4067
2	3	63,0467
3	3	65,3433
Sig.		,443

Mg

Duncan[a,b,c]

Sampling	N	Subset 1
2	3	5,6133
3	3	5,8967
1	3	6,6933
Sig.		,582

Cd

Duncan[a,b,c]

Sampling	N	Subset 1
2	3	,02333
3	3	,02433
1	3	,02867
Sig.		,330

P

Duncan[a,b,c]

Sampling	N	Subset 1
3	3	1,0600
2	3	1,1600
1	3	1,2600
Sig.		,441

K

Duncan[a,b,c]

Sampling	N	Subset 1
1	3	28,8633
2	3	30,5467
3	3	31,0300
Sig.		,642

S

Duncan[a,b,c]

Sampling	N	Subset 1
1	3	54,3333
2	3	59,9767
3	3	72,4467
Sig.		,080

Na

Duncan[a,b,c]

Sampling	N	Subset 1
1	3	70,6633
2	3	74,4333
3	3	82,9200
Sig.		,068

Zn

Duncan[a,b,c]

Sampling	N	Subset 1
2	3	1,5867
3	3	1,9000
1	3	2,0800
Sig.		,250

Soil+ *Arthrobacter* sp 222

Ca

Duncan[a,b,c]

Sampling	N	Subset 1	Subset 2
3	3	60,5333	
2	3	63,5767	
1	3		77,5467
Sig.		,588	1,000

Na

Duncan[a,b,c]

Sampling	N	Subset 1	Subset 2	Subset 3
1	3	64,6100		
2	3		71,9933	
3	3			82,5800
Sig.		1,000	1,000	1,000

Cd

Duncan[a,b,c]

Sampling	N	Subset 1	Subset 2
2	3	,02300	
3	3	,02300	
1	3		,02933
Sig.		1,000	1,000

P

Duncan[a,b,c]

Sampling	N	Subset 1
2	3	1,0200
3	3	1,0233
1	3	1,0533
Sig.		,711

K

Duncan[a,b,c]

Sampling	N	Subset 1
3	3	28,7467
2	3	29,1000
1	3	29,9200
Sig.		,651

S

Duncan[a,b,c]

Sampling	N	Subset 1	Subset 2
1	3	49,8233	
2	3	55,7667	
3	3		63,6867
Sig.		,097	1,000

Mg

Duncan[a,b,c]

Sampling	N	Subset 1	Subset 2
3	3	5,2633	
2	3	5,4733	
1	3		6,8800
Sig.		,683	1,000

Zn

Duncan[a,b,c]

Sampling	N	Subset 1	Subset 2
3	3	1,7567	
2	3	1,7600	
1	3		2,1567
Sig.		,977	1,000

Soil+ *Pseudomonas* sp 228

Ca

Duncan[a,b,c]

Sampling	N	Subset 1
2	3	57,2700
3	3	61,3633
1	3	73,0333
Sig.		,414

Na

Duncan[a,b,c]

Sampling	N	Subset 1
1	3	70,8500
2	3	72,0133
3	3	83,5633
Sig.		,158

Cd

Duncan[a,b,c]

Sampling	N	Subset 1
2	3	,02167
3	3	,02200
1	3	,02567
Sig.		,275

P

Duncan[a,b,c]

Sampling	N	Subset 1
3	3	1,0767
1	3	1,1533
2	3	1,2033
Sig.		,533

K

Duncan[a,b,c]

Sampling	N	Subset 1
2	3	26,6833
1	3	28,0600
3	3	28,1533
Sig.		,706

S

Duncan[a,b,c]

Sampling	N	Subset 1
2	3	54,3867
1	3	56,7733
3	3	65,5300
Sig.		,301

Mg

Duncan[a,b,c]

Sampling	N	Subset 1
2	3	4,8067
3	3	5,1733
1	3	6,4767
Sig.		,367

Zn

Duncan[a,b,c]

Sampling	N	Subset 1
2	3	1,6133
3	3	1,7033
1	3	1,9700
Sig.		,344

Soil + *Serratia* sp. 246

Ca

Duncan[a,b,c]

Sampling	N	Subset 1
3	3	52,0567
2	3	52,6000
1	3	52,7000
Sig.		,898

Na

Duncan[a,b,c]

Sampling	N	Subset 1
1	3	67,8967
2	3	70,4400
3	3	81,4833
Sig.		,206

Cd

Duncan[a,b,c]

Sampling	N	Subset 1
3	3	,02633
1	3	,02733
2	3	,02733
Sig.		,914

P

Duncan[a,b,c]

Sampling	N	Subset 1
3	3	1,1800
2	3	1,2800
1	3	1,4500
Sig.		,405

K

Duncan[a,b,c]

Sampling	N	Subset 1
1	3	26,5367
2	3	27,4967
3	3	28,1033
Sig.		,839

S

Duncan[a,b,c]

Sampling	N	Subset 1
1	3	52,6633
2	3	54,0167
3	3	62,5600
Sig.		,394

Mg

Duncan[a,b,c]

Sampling	N	Subset 1
2	3	5,9667
3	3	5,9833
1	3	6,9767
Sig.		,754

Zn

Duncan[a,b,c]

Sampling	N	Subset 1	Subset 2
2	3	1,4533	
1	3		1,6433
3	3		1,6567
Sig.		1,000	,841

Soil + *Pseudomonas* sp. 256

Ca

Duncan[a,b,c]

Sampling	N	Subset 1
3	6	56,3117
2	6	64,0483
1	6	76,7433
Sig.		,074

Na

Duncan[a,b,c]

Sampling	N	Subset 1
1	6	60,5517
2	6	66,5450
3	6	69,3483
Sig.		,200

Cd

Duncan[a,b,c]

Sampling	N	Subset 1
3	6	,02233
2	6	,02383
1	6	,02667
Sig.		,086

P

Duncan[a,b,c]

Sampling	N	Subset 1
1	6	1,1483
3	6	1,1567
2	6	1,1917
Sig.		,728

K

Duncan[a,b,c]

Sampling	N	Subset 1
3	6	26,6017
2	6	28,8000
1	6	29,3117
Sig.		,332

S

Duncan[a,b,c]

Sampling	N	Subset 1
2	6	50,6983
1	6	52,1250
3	6	60,0133
Sig.		,129

Mg

Duncan[a,b,c]

Sampling	N	Subset 1	Subset 2
3	6	4,9000	
2	6	5,5350	5,5350
1	6		6,9300
Sig.		,470	,124

Zn

Duncan[a,b,c]

Sampling	N	Subset 1
3	6	1,7500
2	6	1,8867
1	6	2,2117
Sig.		,065

Soil + Consortium

Ca

Duncan[a,b,c]

Sampling	N	Subset 1
2	3	67,0000
3	3	67,9033
1	3	72,6500
Sig.		,700

Na

Duncan[a,b,c]

Sampling	N	Subset 1
1	3	71,7067
2	3	81,9500
3	3	94,3533
Sig.		,097

Cd

Duncan[a,b,c]

Sampling	N	Subset 1
2	3	,02367
3	3	,02367
1	3	,02467
Sig.		,804

P

Duncan[a,b,c]

Sampling	N	Subset 1
3	3	1,0733
1	3	1,1467
2	3	1,1533
Sig.		,624

K

Duncan[a,b,c]

Sampling	N	Subset 1
1	3	28,2867
2	3	29,8567
3	3	30,1567
Sig.		,665

S

Duncan[a,b,c]

Sampling	N	Subset 1
1	3	54,5267
2	3	62,4700
3	3	67,6767
Sig.		,342

Mg

Duncan[a,b,c]

Sampling	N	Subset 1
2	3	5,5067
3	3	5,6167
1	3	6,2033
Sig.		,659

Zn

Duncan[a,b,c]

Sampling	N	Subset 1
2	3	1,8167
3	3	1,8567
1	3	1,9900
Sig.		,671

Soil + *H. tuberosus*

Ca

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	47,4025
2	4	50,0275
1	3	64,6733
Sig.		,096

Cd

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	,02050
2	4	,02150
1	3	,02400
Sig.		,091

K

Duncan[a,b,c]

Sampling	N	Subset 1	Subset 2
3	4	13,5575	
2	4		22,4425
1	3		25,6833
Sig.		1,000	,423

Mg

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	4,0750
2	4	4,3275
1	3	4,8200
Sig.		,438

Na

Duncan[a,b]

Sampling	N	Subset 1	Subset 2
1	3	51,9867	
2	4	63,7550	63,7550
3	4		78,5200
Sig.		,168	,094

P

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	1,1425
2	4	1,2650
1	3	1,3400
Sig.		,200

S

Duncan[a,b]

Sampling	N	Subset 1
1	3	44,0567
2	4	55,9650
3	4	64,2400
Sig.		,190

Zn

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	1,5800
2	4	1,6275
1	3	1,8367
Sig.		,144

Soil + *H. tuberosus* + *Arthrobacter* sp. 222

Ca

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	45,6300
2	4	52,7125
1	3	69,9867
Sig.		,053

Na

Duncan[a,b]

Sampling	N	Subset 1	Subset 2
1	3	61,4800	
2	4	71,3900	
3	4		86,3025
Sig.		,146	1,000

Cd

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	,01825
2	4	,02075
1	3	,02533
Sig.		,055

P

Duncan[a,b]

Sampling	N	Subset 1
1	3	1,2167
3	4	1,3200
2	4	1,3500
Sig.		,155

K

Duncan[a,b,c]

Sampling	N	Subset 1	Subset 2
3	4	9,0525	
2	4	15,2350	15,2350
1	3		20,2867
Sig.		,073	,131

S

Duncan[a,b,c]

Sampling	N	Subset 1
2	4	57,5675
3	4	58,5375
1	3	59,6700
Sig.		,803

Mg

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	4,1325
2	4	4,7225
1	3	6,3500
Sig.		,068

Zn

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	1,3950
2	4	1,6125
1	3	1,6900
Sig.		,184

Soil + *H. tuberosus* + *Pseudomonas* sp. 228

Ca

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	48,1550
2	4	50,4100
1	3	63,9600
Sig.		,120

Na

Duncan[a,b]

Sampling	N	Subset 1	2	3
1	3	52,9500		
2	4		63,7350	
3	4			73,3075
Sig.		1,000	1,000	1,000

Cd

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	,01725
2	4	,02000
1	3	,02333
Sig.		,118

P

Duncan[a,b]

Sampling	N	Subset 1
1	3	1,3500
3	4	1,5475
2	4	1,9950
Sig.		,335

K

Duncan[a,b,c]

Sampling	N	Subset 1	2
3	4	12,8525	
2	4	23,6675	23,6675
1	3		31,7733
Sig.		,148	,264

S

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	45,1050
2	4	48,9425
1	3	58,6100
Sig.		,354

Mg

Duncan[a,b,c]

Sampling	N	Subset 1	2
3	4	3,4275	
2	4	4,4875	4,4875
1	3		5,8700
Sig.		,209	,113

Zn

Duncan[a,b,c]

Sampling	N	Subset 1	2
3	4	1,2950	
2	4	1,6150	1,6150
1	3		1,9633
Sig.		,167	,137

Soil + *H. tuberosus* + *Serratia* sp. 246

Ca

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	47,1350
1	3	50,3700
2	4	56,5450
Sig.		,542

Na

Duncan[a,b]

Sampling	N	Subset 1	Subset 2
1	3	55,1433	
2	4	72,5675	72,5675
3	4		90,0300
Sig.		,074	,074

Cd

Duncan[a,b]

Sampling	N	Subset 1
1	3	,01767
2	4	,02200
3	4	,02350
Sig.		,361

P

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	1,1700
2	4	1,2575
1	3	1,2833
Sig.		,402

K

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	14,8100
2	4	19,2900
1	3	21,3833
Sig.		,308

S

Duncan[a,b]

Sampling	N	Subset 1
1	3	49,2833
2	4	60,0800
3	4	72,7425
Sig.		,351

Mg

Duncan[a,b]

Sampling	N	Subset 1
1	3	4,5500
2	4	4,9175
3	4	5,2025
Sig.		,731

Zn

Duncan[a,b]

Sampling	N	Subset 1
1	3	1,2333
2	4	1,6425
3	4	1,8025
Sig.		,314

Soil + *H. tuberosus* + *Pseudomonas* sp. 262

Ca

Duncan[a,b,c]

Sampling	N	Subset 1
3	3	47,4933
2	4	49,4050
1	3	67,8967
Sig.		,107

Na

Duncan[a,b,c]

Sampling	N	Subset 1	Subset 2	Subset 3
1	3	58,2700		
2	4		69,8350	
3	3			80,2833
Sig.		1,000	1,000	1,000

Cd

Duncan[a,b,c]

Sampling	N	Subset 1
3	3	,02033
2	4	,02050
1	3	,02667
Sig.		,069

P

Duncan[a,b,c]

Sampling	N	Subset 1
1	3	1,2400
2	4	1,2900
3	3	1,3267
Sig.		,329

K

Duncan[a,b,c]

Sampling	N	Subset 1	Subset 2
3	3	10,8700	
2	4	16,3400	
1	3		23,6067
Sig.		,072	1,000

S

Duncan[a,b]

Sampling	N	Subset 1
2	4	53,5300
1	3	53,7967
3	3	59,2167
Sig.		,644

Mg

Duncan[a,b,c]

Sampling	N	Subset 1
3	3	4,3033
2	4	4,4525
1	3	6,2100
Sig.		,095

Zn

Duncan[a,b]

Sampling	N	Subset 1
2	4	1,5725
3	3	1,6433
1	3	2,2467
Sig.		,066

Soil + *H. tuberosus* + Consortium

Ca

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	51,4725
2	4	52,0975
1	3	70,7200
Sig.		,164

Na

Duncan[a,b]

Sampling	N	Subset 1	Subset 2
1	3	61,2533	
2	4	70,2775	
3	4		81,0550
Sig.		,058	1,000

Cd

Duncan[a,b,c]

Sampling	N	Subset 1
2	4	,01975
3	4	,02200
1	3	,02567
Sig.		,277

P

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	1,2700
2	4	1,4075
1	3	1,4133
Sig.		,507

K

Duncan[a,b,c]

Sampling	N	Subset 1	Subset 2
3	4	10,9150	
2	4	17,3400	17,3400
1	3		24,9867
Sig.		,219	,151

S

Duncan[a,b,c]

Sampling	N	Subset 1
2	4	52,4425
1	3	56,9367
3	4	63,3725
Sig.		,506

Mg

Duncan[a,b,c]

Sampling	N	Subset 1
3	4	4,7600
2	4	4,8275
1	3	6,9667
Sig.		,139

Zn

Duncan[a,b,c]

Sampling	N	Subset 1
2	4	1,6875
3	4	1,7550
1	3	2,2133
Sig.		,209

Curriculum vitae

Blanca Montalbán Ginés **email:** blanca.montalban@gmail.com

Current position

Position: PhD candidate

Joint PhD between Universidad Autónoma Madrid and Hasselt University

Supervisors: Mª Carmen Lobo Bedmar, Araceli Pérez-Sanz, Nele Weyens and Jaco Vangronsveld.

Education

Degree in Biology. Faculty of Sciences. Universidad Autónoma Madrid (2008).

> Final Project: Changes in the Curculionidae (Coleoptera: Curculionoidea) diversity over a quarry's ecological restoration in the area of Yepes (Toledo, Spain)
>
> In: Entomology Department, Faculty of Sciences, UAM.

Conservation Biology Official Master. Faculty of Biology. Universidad Complutense Madrid (2010).

> Final Project: Ecological risk produced by metal species dispersion in the Portman Bay mining complex (Murcia, Spain)
>
> In: Environmental Sciences Center, Environmental Pollution Department, CSIC.

Professional Experience

Pre-doctoral grant from INIA (Spanish Science and Innovation Ministry) (2010-2014)

> In: Agroenvironmental Sciences Department, IMIDRA, Madrid, Spain.

Technician Support Research in the project: "Entomopathogenic nematode (NEP) indigenous strains bioecological characterization. Its interest in developing sustainable technologies for insects and nematodes control" (2009).

> In: Environmental Pollution Department, Environmental Sciences Center. CSIC, Madrid, Spain.

Research Stages Abroad

Centre for Environmental Sciences, Hasselt University (Belgium). 9.5 months (2014)

> Prof. Dr. Jaco Vangronsveld and Dr. Nele Weyens.

Soil and Physical Sciences, Lincoln University (New Zealand). 3 months (2013).

> Prof. Dr. Brett Robinson

Scientific publications

Montalbán, B., Croes, S., Weyens, N., Lobo, M.C., Pérez-Sanz, A., and Vangronsveld, J. Characterization of bacterial communities associated with *Brassica napus* L. growing on a Cd-Zn contaminated soil and their effects on root growth. Submitted to *Environmental Microbiology Reports*.

Montalbán, B., Lobo, M. C., Alonso, J., Pérez-Sanz, A. Metal(loid)s uptake and effects on the growth of *Helianthus tuberosus* cultivar-clones under multi-polluted hydroponic cultures. *CLEAN - Soil, Air, Water 2015*. DOI: 10.1002/clen.201400630.

Montalbán, B., García-Gonzalo, P., Pradas del Real, A. E., Alonso, J., Lobo, M. C., Pérez-Sanz, A. *Brachypodium distachyon* tolerance to metals under in vitro conditions: a comparison with two metal-tolerant energy crops. *Fresenius Environmental Bulletin 2014, 23(9): 2086-2092.*

Montalbán, B., Pradas del Real, A. E., García, P., Lobo, M. C., Pérez-Sanz, A. Germination and Early Development of *Brassica napus* and *Brachypodium distachyon* Grown with Zn, Cr (VI), As (V) or Cd: Preliminary Results. *Acta Phytopathologica et Entomologica Hungarica 2012,* 47 (2): 363-371.

Campos-Herrera, R., Piedra-Buena, A., Escuer, M., **Montalbán, B.**, Gutiérrez, C. Effect of seasonality and agricultural practices on occurrence of entomopathogenic nematodes and soil characteristics in La Rioja (Northern Spain). *Pedobiologia 2010,* 53: 253-258.

Conferences

Montalbán B., Lobo, M. C., Croes, S., Weyens, N., Vangronsveld, J., Pérez-Sanz, A. Improvement of growth of *Helianthus tuberosus* L. by soil and root endophytic bacteria on a Cd-Zn contaminated soil**.** Poster presentation. 11th International Phytotechnologies Conference. Creta, Greece. 2014.

Montalbán, B., Lobo, M. C., Pérez-Sanz, A. Effects of endophytic, rhizosphere and soil bacteria on the growth of *Brassica napus* in presence of Cd and Zn. Poster presentation. 9th Iberian and 6th Iberoamerican Congress on Environmental Contamination and Toxicology. Valencia, Spain. 2013

Gil-Díaz, M., Alonso, J., **Montalbán, B**., Rodríguez-Valdés, E., Pinilla, P., Lobo, M.C. Reducing mobility of arsenic in a brownfield soil using stabilized zero□valent iron nanoparticles. Poster presentation. 9th Iberian and 6th Iberoamerican Congress on Environmental Contamination and Toxicology. Valencia, Spain. 2013

Pérez-Sanz, A., Pradas del Real, A. E., **Montalbán, B**., García Gonzalo, P., Tardío, J., Lobo, M. C. Evaluation of different species from *Rumex* genus to phytotechnologies. Poster presentation. 9th International Phytotechnologies Conference. Hasselt, Belgium. 2012

Pérez-Sanz, A., Pradas del Real, A. E., **Montalbán, B**., García Gonzalo, P., Lobo, M. C. Especies silvestres en recuperación de suelos. Oral presentation. XIV Simposio Hispano-Luso de Nutrición Mineral de las Plantas. Nutriplanta. Madrid, Spain. 2012

Montalbán, B., Pérez-Sanz, A., Pradas del Real, A. E., Plaza, A., Lobo, M. C. *Helianthus tuberosus* L. grown with multiple pollution in greenhouse conditions. Poster presentation. 6th Society of Environmental Toxicology and Chemistry (SETAC) World Congress. Berlin, Germany. 2012

Pradas del Real, A. E., **Montalbán, B**., García-Gonzalo, P., Lobo, M. C., Pérez-Sanz, A. Exploring the phytoremediation potential of *Rumex pulcher* L. using agar based medium. Oral presentation.

Sustainable Approaches to Remediation of Contaminated Land in Europe (SARCLE, 2011) /Contaminated Site Management in Europe (CSME, 2011) Gent, Belgium. 2011

Montalbán, B., Pérez-Sanz, A., Pradas del Real, A. E., García, P., Plaza, A., Lobo, M. C. Germination and early development of *Brassica napus* L. and *Brachypodium distachyon* (L.) Beauv. grown with Zn, Cr(VI), As(V) or Cd. Poster presentation. International Conference on Agri-Environmental Chemistry and Toxicology. Budapest, Hungary. 2011

Fajardo, C., Martín, M., **Montalbán, B**., Gil-Díaz, M., Pérez-Sanz, A., Lobo, M.C. Use of iron nanoparticles (FeNP) in environmental remediation processes: impact on bacterial populations. Poster presentation. III Congress of Industrial Microbiology and Microbial Biotechnology. Madrid, Spain. 2010

Seminars

Montalbán, B. 2015. Bacterial inoculation in *Helianthus tuberosus* for improving phytoremediation of metal-polluted soils**.** Doctoral Program in Agricultural Chemistry, Universidad Autónoma de Madrid.

Montalbán, B. 2013. Isolation and characterization of bacterial communities associated to *Brassica napus* L. in a Cd-Zn contaminated soil. Doctoral Program in Agricultural Chemistry, Universidad Autónoma de Madrid.

Montalbán, B. 2011. Germination, tolerance and metal uptake during the first stages of growth of *Brachypodium distachyon* (L.) Beauv. and *Brassica napus* L. EIADES Annual Conference. Finca El Encín. Ponencia. Alcalá de Henares, Madrid.

Language

English (Advanced level in reading, speaking and writing)

Participation in Research Projects

Environmental impact assessment and recovery of the natural environment in contaminated sites. 01/09/2010 - 30/05/2014. Consejeria de Educación, Comunidad de Madrid. EIADES PROGRAM S2009/AMB-1478

Use of wild grasses to recover degraded soil. Evaluation of plant tolerance to heavy metal as function of soil characteristics. 01/09/2010 - 31/12/2012. Spanish Minister of Science and Innovation-INIA (RTA2009-00150-00-0)

Ecological risk of metals from mineral deposits. 01/01/2010 - 01/09/2010. Spanish Minister of Science and Innovation. CSIC (CGL–2009–14686–C01–00 BOS)

Bioecological characterization of indigenous strains of entomopathogenic nematode and its interest in developing sustainable technologies for insects and nematodes control. 16/04/2009 - 31/12/2009. Spanish Minister of Science and Innovation. CSIC (CICYT CGL2005-07611)

Courses

Environmental geology and geochemistry. Remediation of polluted soils. (100 hrs.) Universidad Autonoma Madrid. Spain. June 2010

Soil degradation and remediation. (56 hrs.) CIEMAT, Madrid. Spain. September 2009

Pyrenees flora and vegetation. (50 hrs.) Pyrenean Ecology Institut, CSIC. Huesca. Spain. July 2009

Environmental stress assessment and conservation-oriented modelling methods. (40 hrs.) Pyrenean Ecology Institut, CSIC. Huesca. Spain. July 2009

Experimental Sciences Statistics. (25 hrs.) CSIC, Madrid. Spain. June 2009

Scientific Culture: popularization and communication in science. (25 hrs.) CSIC, Madrid. Spain. June 2009