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Faculty of Mathematics, Physics and Natural Sciences

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Integrated approach on the rhizosphere response to Nickel in a facultative hyperaccumulator species

Tutor

Prof. Mauro G. Mariotti

Co-tutor

Dr. Enrica Roccotiello

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Dr. Stefano Rosatto

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1. Introduction

The contamination of metals like Nickel (Ni) in the soil represents a serious threat worldwide. To counteract this phenomenon, hyperaccumulator plant species, able to remove metal from soil and store it at high concentration in shoots, are employed for metal phytoremediation purposes. Native microbial communities occurring in the rhizosphere of hyperaccumulators often promote plant growth and metal uptake.

So far, each abiotic and biotic rhizospheric components (soil, root system and microbiota) have been used without considering the reciprocal interactions and the responses to Ni stress as a whole. The present study aims to develop for the first time an innovative and multidisciplinary approach to examine the rhizosphere of Ni-hyperaccumulators as a holistic model, promoting the plant development and the Ni uptake.

A promising approach can be the synergic inoculation of mixed rhizospheric bacterial and fungal strains as natural chelators of metals for plants. This integrated system is feasible owing to the collaboration with the Laboratory of Micology and the Laboratory of Microbiology of Department of Earth, Environmental and Life Sciences (DISTAV), University of Genoa.

Among metalliferous soils, specific attention was given to serpentinitic soil which display extremely hostile conditions (nutrient shortage and concentration of metals - e.g., Ni - highly toxic) for most plants except for hyperaccumulator species.

In the present study, each rhizosphere component was examined before starting the study of microbiota interactions.

The root system of Ni-hyperaccumulators plays a key role in the Ni accumulation. In particular the root surface (area) represents the first zone of potential Ni uptake and it is measured likewise root biomass to assess the response to Ni stress.

Bacterial and fungal communities are involved in plant growth promotion and defence against Ni stress. They are frequently able to alter metal bioavailability in the rhizosphere promoting the plant metal uptake.

The thesis is divided in four parts and it is organized as follows:

Chapter 2 deals the issue of Ni in the soil environment and it briefly mentions the Ni-hyperaccumulators and their role in the soil phytoremediation. Great emphasis is given to rhizosphere as a whole superorganism.

In the end, aim and objectives of the research are stated.

Chapter 3 explains material and methods of this research. Early response to Ni stress in plant development are carried out with micro- and mesocosm germination under Ni stress in the Ni-hyperaccumulator species *Alyssoides utriculata* (L.) Medik, *Noccaea caerulescens* (J. Presl & C. Presl) F. K. Mey. [= *Thlaspi caerulescens.* J. Presl & C. Presl] and *Odontarrhena bertolonii* (Desv.) L. Cecchi & Selvi [= *Alyssum bertolonii* Desv.] and on the related non-accumulator species *Alyssum montanum* L. and *Thlaspi arvense* L., used for comparison. Afterwards, the response to increasing Ni concentrations in terms of root surface area, root and shoot biomass, water content and photosynthetic efficiency was evaluated.

Subsequently, *A. utriculata* was selected as a good candidate to study rhizospheric components because of its Ni-facultative hyperaccumulation traits and its ability to thrive in harsh metalliferous soils. Related rhizosphere and bare soil samples were collected from serpentine and non-serpentine sites.

Plant and soil samples were processed and analysed with specific attention to isolation and identification of culturable microbiota, then selected for their Ni-tolerance and plant growth promoting traits. Later, most performing Ni tolerant bacterial and fungal strains were tested by means of co-growth methods to estimate their potential mutual synergy in a mixed culture to be used as inoculum in the rhizosphere of *A. utriculata*.

Obtained data reported in Chapter 4 were statistically analysed to assess the significant differences between variables, then data structure was visualized through the Principal Components Analysis.

The findings were discussed in Chapter 5, highlighting the importance of this first step of integrated plant-rhizobiota tool, focused on the whole rhizospheric model to improve Ni uptake from polluted soil, although further investigations are required to ascertain the efficiency of the field application.

2. A new rhizospheric approach

2.1 Metals in the environment

Nowadays, environmental pollution by metals represents one of the most dramatic treat in the world. In addition to the natural sources like weathering of minerals, erosion and volcanic activity, the main sources of metal pollution in soils are anthropogenic, like agricultural activity, municipal and industrial wastes and mining (Ali et al., 2013).

Recently, the most frequent definition of "heavy metals" has been used to indicate metals and metalloids characterized by potential toxicity towards environment and living organisms (Dal Corso et al., 2013). Metals are considered as trace elements because of their presence at very low concentrations (i.e., ppb to less than 10 ppm) in various environmental matrices (Kabata-Pendias, 2010). The main characteristics of this contamination can be summarized as: wide distribution, strong latency, irreversibility and remediation hardness (Su, 2014). Besides, the metallic elements are considered systemic toxicants that are known to induce multiple organ damage, even at low levels of exposure (Tchounwou et al., 2012).

The metal accumulation in soils and waters poses a significant risk to human health and environment. The metals concentrate in the body tissues of living organisms (i.e., bioaccumulation) and their concentrations increase as they pass from lower to higher trophic levels (i.e., biomagnification, Ali et al., 2013).

2.2 Nickel: sources, characteristics, functions and toxicity

Nickel is the 24th most abundant element and it occurs either as a free metal in igneous rocks or in combination with other metals like iron (Anjum et al., 2015).

In soil Ni exists both in organic and inorganic form as well as water soluble and as free in soil solution; the acidity of the soil promote the bioavailability of Ni, because low pH values facilitate the metal mobilization towards the plants' roots (Alford et al., 2010; Shahzad et al., 2018).

Nickel is an essential micronutrient and plays a significant role in the development of the seedling (Shahzad et al., 2018). It is involved in the plant growth and in many biological functions (Rahman et al., 2005), it is necessary for the biosynthesis of some proteins and it plays an important role in nitrogen plant assimilation and metabolism as part of many enzymes involved in the process (Mustafiz et al., 2014; Parida et al., 2003). Moreover Ni can protect plant against several biotic and abiotic stresses (Sreekanth et al., 2013).

Ni enters the environment through natural and anthropogenic sources such as metallurgical, chemical and food industries (Shahzad et al., 2018).

Polluted soil may exhibit Ni concentrations of 200-26000 mg kg^{-1} (20- to 30-fold higher than unpolluted areas, Cempel and Nikel, 2006; Izosimova, 2005; Lock et al., 2007).

Excess Ni can be toxic to plants and cause a number of morphological and physiological/biochemical consequences (Anjum et al., 2015).

General symptoms include: inhibition of germination, reduction of biomass, poor branching, irregular shape of the flowers, diminution of the leaf area, staining, chlorosis, necrosis and leaf staining, iron deficiency, disturbed mitosis and general decrease in yield (Ahmad and Ashraf, 2011; Negi et al., 2014; Seregin and Kozhevnikova, 2006).

Ni in the soil competes with other essential ions causing chemical disorders and the formation of chelate complexes with metal ligands (Shahzad et al., 2018). This increases the Ni concentration in plant tissue that causes metabolic perturbations by the alterations of enzymatic activities, inducing oxidative stress that provokes deleterious effects on DNA (Chen et al., 2009; Demchenko et al., 2010), disrupted photosynthesis and ultimately affecting growth and crop yield (Gajewska et al., 2013; Gajewska and Skłodowska, 2009; Negi et al., 2014; Yusuf et al., 2011).

The exposure to Ni or its alloys is very frequent due to its diffusion and uses in everyday life and it is mainly represented by inhalation and dermal contact, with 20% of human population affected by Ni-allergy (Schmidt and Goebeler, 2011; Spiewak et al., 2007; Zambelli et al., 2016). Moreover, as

reported by the epidemiological studies, Ni may induce carcinogenesis and a wide range of genetic alterations in humans (Arita and Costa, 2009; Zambelli et al., 2016, Lightfoot et al., 2017).

2.3 The Ni-hyperaccumulators

In a few plant *taxa* called hyperaccumulators, the concentration of metal(loid)s in aboveground biomass is up to four orders of magnitude higher than in non-hyperaccumulator species and it is associated with a strongly enhanced metal hypertolerance (Krämer, 2010).

The term "hyperaccumulator" was coined by (Jaffré et al., 1976) to indicate the extraordinary ability of *Pycnandra acuminata* Aubrév. [= *Sebertia acuminata* (Pierre ex Baill.) Engl.] to accumulate Ni in its tissues. For the first time Brooks et al. (1977) quantified the accumulation with Ni concentrations > 1000 mg kg^{-1} (0.1%) on dry weight (DW) without showing phytotoxic symptoms; subsequently Reeves (1992) included in this definition only those species that grow in their natural habitat.

About the meaning of the mechanism of metal hyperaccumulation, the tested hypotheses proposed by Alford et al. (2010) are:

- tolerance and subsequent disposal of toxic elements;
- osmotic resistance to drought;
- elemental allelopathy towards against nearly competitors;
- defense against herbivores and pathogens;
- accidental phenomenon.

Hyperaccumulators occur in over 34 families (Verbruggen et al., 2009a), 25% belong to the Brassicaceae family (Brooks, 1998; Rascio and Navari-Izzo, 2011) and by now, 450 Ni hyperaccumulator species have been reported worldwide (van der Ent et al., 2013).

Many hyperaccumulator species are called "strict metallophytes" because they are endemic to metalliferous soils whereas some "facultative metallophytes" can thrive on non-metalliferous soils,

although they are more frequent on metal-rich habitats (Assunção et al., 2003; Baker et al., 2010; Pollard et al., 2014).

The degree of hyperaccumulation of metals significantly varies within and across plant species (Deng et al., 2007; Roosens et al., 2003) but three common traits distinguish a hyperaccumulator species from a related non-hyperaccumulator one:

- the ability of extract metals from soil;
- the fast and effective root-to-shoot metal translocation;
- the ability to sequester and detoxify metals into vacuoles (Rascio and Navari-Izzo, 2011).

The first phase of hyperaccumulation involves active transport or passive metal absorption in the root. Ni can enter the symplast through the uptake sites of the membrane transporters or, alternatively, it may enter the apoplast and then the symplast through low affinity transport systems in the endodermis (Alford et al., 2010; Deng et al., 2018).

The Ni uptake is modulated by some factors such as metal concentration, acidity of the substrate, presence of other metals and organic matter, CEC (cation exchange capacity), soil structure, type of plant and its metabolism (Nishida et al., 2011).

The Ni mobility and the root-to-shoot translocation is affected by the presence of metal-ligands complexes between Ni and organic acids or transporter proteins in root cytoplasm (Boominathan and Doran, 2002; Callahan et al., 2006; Centofanti et al., 2013; Haydon and Cobbett, 2007; Mari et al., 2006; Merlot et al., 2014; Richau et al., 2009).

Ni is translocated via xylem or phloem (Deng et al., 2018) and it is redistributed into the aerial sink organs (young growing tissues) (Milner and Kochian, 2008; Shahzad et al., 2018; Verbruggen et al., 2009a), then the plant activates tolerance and detoxification mechanisms such as vacuolar sequestration and compartmentalization in various subcellular structures (Agrawal et al., 2013; Fryzova et al., 2018; Rascio and Navari-Izzo, 2011) which prevents the toxic effects on the cell (Krämer et al., 2000).

2.4 The phytoremediation

Phytoremediation is a combination of the terms: *phyto* (from Greek, meaning plant) and *remedium* (from Latin, meaning to correct or remove an evil) (Ali et al., 2013).

The concept of phytoremediation (as phytoextraction) suggested by Chaney (1983), allows to restore contaminated soils by anthropogenic sources through the uptake of metals (Chaney et al., 1997; van der Ent et al., 2013).

The purpose of phytoremediation can be threefold: (1) reduce metal bioavailability of pollutants in the environment (phytostabilization); (2) uptake metals from soil and water and translocate and accumulate them in the aboveground biomass (phytoextraction); (3) recover energy and metals with market value from the bio-ore (phytomining). Among the other forms of phytoremediation mentioned by Ali et al. (2013) and Peuke and Rennenberg (2005): phytofiltration is the removal of pollutants from contaminated waters by plants (Sangeeta and Maiti, 2010); phytovolatilization refers to the uptake of pollutants from soil by plants, their conversion to volatile form and subsequent release into the atmosphere (Ali et al., 2013); phytodesalination regards the use of halophytic plants for removal of salts from salt-affected soils (Manousaki and Kalogerakis, 2010); phytodegradation concern the degradation of organic pollutants by plants through enzymes activity (Vishnoi and Srivastava, 2007); rhizodegradation denotes the breakdown of organic pollutants in the soil by rhizospheric microorganisms (Sangeeta and Maiti, 2010).

Promising species for phytoextraction include *Elsholtzia splendens* Nakai ex F.Maek., *Odontarrhena bertolonii, Noccaea caerulescens* and *Pteris vittata* L. (Favas et al., 2014; van der Ent et al., 2013). Species belonging to genera *Haumaniastrum, Eragrostis, Ascolepis, Gladiolus* and *Alyssum* are suitable for phytostabilization purpose (Favas et al., 2014). Regarding phytomining, we can mentioned the Ni recovery in *Alyssum* spp. (Bani et al., 2015a, 2015b; Chaney et al., 2007; Pardo et al., 2018), Cd, Zn and Pb in *Thlaspi* spp. (Eissa, 2016), Cu, Ag and Au in *Brassica* spp. (Eissa, 2016; González-Valdez et al., 2018).

The metal-contaminated soils require specific remediation to avoid ecosystem disruption and harmful effects on human health (Wuana and Okieimen, 2011). The use of plants and associated soil microbes to reduce the concentrations or toxic effects of contaminants in soils (i.e., phytoremediation) can be a sustainable technique to reach this goal (Greipsson, 2011). In fact the phytoremediation technology is a cost-effective, less invasive, green alternative solution to the chemical and physical methods of soil remediation (Kidd et al., 2009; Pilon-Smits, 2005), because many plants are able to uptake organic and inorganic contaminants from the environment causing their detoxification (Ali et al., 2013).

On the other hand, there are some limitations, because a) phytoremediation is effective at low soil depth, where the roots are developed; b) it is a time-consuming process and the success depends on the climatic conditions; c) high concentrations of contaminants in the aerial parts can pose a serious risk to the food chains (Lee, 2013; Shahzad et al., 2018).

Despite the described disadvantages, phytoremediation is recognized as a promising technique for metal remediation of soils. It involves the use of metal hyperaccumulator plant species for the metal uptake from the soil and their deposition in harvestable plants biomass (Chaney et al., 2007; Kamran et al., 2014; Ying Ma et al., 2011).

2.5 The Ni-hyperaccumulator species used

Alyssoides utriculata (Ni in leaves 36–2236 mg kg^{-1} DW) is an evergreen shrub with good biomass. It is a Ni facultative hyperaccumulator (Roccotiello et al., 2015a, b) occurring on both metalliferous and ‘normal’ soils. The species ranges primarily in the northeastern Mediterranean region (Pignatti, 1982). The greater abundance of *A. utriculata* in low-competition serpentine soils compared to adjacent non-serpentine sites, suggests preadaptation tolerance traits (Roccotiello et al., 2015a). It is able to accumulate greater quantities of Ni in the aboveground biomass even in soils with low metal level, compared to the typical serpentine non-hyperaccumulator species (Roccotiello et al., 2016). Despite the medium-high ability to concentrate Ni in shoots, *A. utriculata* species is of a key

importance because it is a native Mediterranean hyperaccumulator that can be exploited for improved phytoremediation purposes in this climate. Nowadays, a deep comparison between serpentine and non-serpentine microbiota of facultative Ni-hyperaccumulators like *A. utriculata* is missing.

Noccaea caerulescens (Ni in the shoots 1000–30000 mg kg^{-1} DW) is a herbaceous biennial plant, found in Europe and in USA (Milner and Kochian, 2008). It has been studied extensively for its ability to hyperaccumulate several metals (Milner and Kochian, 2008). Some populations of genus *Noccaea* (sin. *Thlaspi*) hyperaccumulate Ni in the serpentinitic soil, whereas other populations are capable to uptake Zn and Cd (Assunção et al., 2008), suggesting that hyperaccumulation is monophyletic (Macnair, 2003). *N. caerulescens* also known as 'montane crucifer' (Fones et al., 2019) or 'alpine pennycress' (Halimaa et al., 2014) includes populations that differ in morphological and physiological characteristics, exhibiting a wide range of accumulation and metal tolerance (Visioli et al., 2014b). In Europe, *N. caerulescens* shows three ecological groups that correspond to three edaphic environments (Gonneau, 2014). Two ecological groups are typical of metalliferous soils: the Calamine group develops in soils rich in Zn, Cd and Pb, while the Serpentine group is characterized by populations that thrive in Ni-rich soils derived from serpentinite ultramafic rocks. Finally, the third group includes non-metalliferous populations (Sterckeman et al., 2017).

Odontarrhena bertolonii is a herbaceous perennial plant, endemic of serpentine outcrops of Central Italy (Mengoni et al., 2009). It is able to uptake Ni from 7000 to 12000 mg kg^{-1} in the shoot dry matter (Galardi et al., 2007; Selvi et al., 2017). Some studies have shown that its leaf tissues host a large variety of bacterial strains (Mengoni et al., 2009, 2004) that show a high phenotypic variability depending on the metal tolerance (Barzanti et al., 2007).

2.6 The rhizosphere

The rhizosphere, i.e., the soil-root interface (Hiltner, 1904) for the assimilation of soil elements and the uptake of metals, is characterized by feedback loops of interactions among root processes, physical and chemical soil characteristics and microbial dynamics (Wenzel et al. 2004; Comerford, 2005; Alford et al., 2010; Kidd et al., 2017). The rhizosphere is much more complex than the small volume of soil around the root, indeed it constitutes a system of functionally integrated zones in which abiotic and biotic factors cohabit in a holistic whole (York et al., 2016).

The plant and the associated rhizobiota could be considered as a superorganism that is described as holobiont (Margulis, 1993; Margulis and Fester, 1991; Vandenkoornhuyse et al., 2015). Although we can define the plant holobiont a unit of adaptation and selection processes (Zilber-Rosenberg and Rosenberg, 2008), the implications related to the holobiont concept and its evolution need to be examined (Vandenkoornhuyse et al., 2015).

The interactions between the different components appear to be regulated by a molecular communication system that are currently still understudied albeit they are essential for the sustainable soil management (Lemanceau et al., 2017).

An overview of the individual components is given in the paragraphs 2.6.1, 2.6.2 and 2.6.3.

2.6.1 The soil

The rhizospheric soil consists of a small volume surrounding the root zone (Lazarovitch et al., 2018; York et al., 2016). It is a mixture of mineral and organic material that, in association with roots and microbiota, represents a complex system of mutually integrated zones (York et al., 2016). Its structure and aggregation processes are influenced by the roots (Alford et al., 2010; Schlüter et al., 2018; York et al., 2016).

The rhizosphere contributes to the stability and resistance against soil erosion (Wenzel et al., 2004). Soil chemistry (chemical concentration, pH, redox conditions, etc.) is strongly affected by both the elements uptake and the exudation of organic acids by the root system and soil microorganisms

(Alford et al., 2010). Specifically, the pH decrease can modify the solubility and therefore the bioavailability of metals (Alford et al., 2010; Wenzel et al., 2004). In fact, root exudates can lower the rhizosphere soil pH generally by one or two units over that in the bulk soil (Ali et al., 2013). Moreover, rhizobiota (mainly bacteria and fungi) may significantly increase the bioavailability of metals in soil (Sheoran et al., 2010; Vamerali et al., 2010), which is a critical factor affecting the efficiency of phytoextraction (Ali et al., 2013).

Among natural metalliferous soils, serpentine soils have nutrient deficiency and toxic concentration of metals as Ni (bioavailable Ni: 7 to >100 mg kg^{-1}; total Ni: 500-8000 mg kg^{-1} (Freitas et al., 2004; Ghaderian et al., 2007; Reeves and Baker, 2000; Roccotiello et al., 2015a; Turgay et al., 2012; van der Ent et al., 2013). Worldwide researchers use the term “serpentine” to define abiotic factors such as rocks, soils, but also biotic components such as vegetation and other biota associated with ultramafic outcrops (Rajakaruna et al., 2009).

Serpentine soils provide particularly harsh and hostile conditions for most plant species (Brady et al., 2005), except for some endemic and threatened species (van der Ent et al., 2015) and the presence of some hyperaccumulator species (Mengoni et al., 2010; Pollard et al., 2014).

This "serpentine factor" as mentioned by (Brooks, 1987) is caused by peculiar edaphic conditions as the lack of nutrients (N, P, S, K, Na, Ca) and the high concentration of phytotoxic elements (Ni, Fe, Cu, Co, Cr, etc., Chiarucci and Baker, 2007), wide temperature ranges, occurrence of thin soil layer and consequently low organic content, combined with high surface runoff (Mengoni et al., 2010). Due to their mineralogical and chemical properties, serpentinitic soils have often been overexploited to extract metals from their ultramafic rocks (Pasquet et al., 2018) causing a serious danger to the surrounding ecosystem. In particular, mining activity is responsible for soil degradation and groundwater pollution (Raous et al., 2010), due to metal leaching and active acid mine drainage AMD (Roccotiello et al., 2015b).

2.6.2 The root

The root system provides the structural element of this dynamic microenvironment (Wenzel et al., 2004) determining the plant access to soil-borne elements (Alford et al., 2010).

The root surface plays a significant role in element uptake through membrane transporters and in some hyperaccumulator, the root grows towards trace elements in soil (Alford et al., 2010). This metallophilic behaviour allows plants to mainly develop roots towards metal-rich patches (Hodge, 2004). The induction of root proliferation (i.e. root foraging) in response to Ni, Cd and Zn in soils were reported in few hyperaccumulators, like *Noccaea caerulescens, Thlaspi goesingense* Halácsy, *Sedum alfredii* Hance and *Streptanthus polygaloides* Gray. (Dechamps et al., 2008; Himmelbauer et al., 2005; Liu et al., 2010; Mincey, 2018). Nonetheless, other hyperaccumulators do not show the same positive chemotropism towards metal-spiked soil (Moradi et al., 2009) and for some species there are no information.

Depth and root morphology are also important traits in relation to uptake, although little is known about the relationship between root morphology and metal accumulation (Alford et al., 2010).

Many hyperaccumulators have been described as shallow-rooted (< 0.5 m) and with high proportion of fine roots for the accumulation of elements (Himmelbauer et al., 2005; Keller et al., 2003), but deep-rooted herbaceous species (2 m) exist (Kutschera et al., 1992) and the roots of many arboreal hyper-accumulative species have not yet been examined (Alford et al., 2010).

The release of exudates and acid production (H^+) by roots can alter the metal solubility and consequently the bioavailability in the rhizosphere (Alford et al., 2010; Gonzaga et al., 2009).

At the root surface, specific membrane transporters provide metal uptake sites for soil metals such as Ni (Gendre et al., 2007) which bind metal chelators; some of which can facilitate root-to-shoot translocation or be involved in the metal tolerance (Alford et al., 2010; Rascio and Navari-Izzo, 2011).

Although root depth, morphology and the preferential metal allocation in root may partially explain the high concentration of trace elements in some species of hyperaccumulator (Alford et al., 2010), the relation between the root system and the metal accumulation need to be further examined.

2.6.3 Rhizosphere microbiota: bacteria and fungi

Soil metal contamination exerts selective pressure and changes on microbial communities and functional diversity (Kelly and Tate, 1998; Liao and Xie, 2007). Soil microorganisms (bacteria and fungi) can be metal-tolerant (Álvarez-López et al., 2016; Iram et al., 2012; Kidd et al., 2017; Mengoni et al., 2001; Thijs et al., 2017; Turgay et al., 2012). In addition, they are involved in: a) promoting the growth and development of the root surface area and root hairs (Gadd 2007; Zotti et al. 2014; Cecchi et al. 2017b), b) affecting metal uptake via altered solubility and bioavailability of chemicals (Benizri et al., 2001; Gadd, 2004; Wenzel et al., 2004; Alford et al., 2010; Rajkumar et al., 2013; Johnson and du Plessis, 2015; Kidd et al., 2017), and c) protecting the plant against metal toxicity (Ma et al. 2010). Thanks to those main features, bacteria and fungi from metal-rich soils of natural origin can be exploited for the colonization and bioremediation of metal-polluted soils (Zucconi et al., 2003), suggesting their potential use to help site revegetation (Fomina et al., 2005; Gadd, 2007).

In the rhizosphere of Ni-hyperaccumulators, the optimal plant growth, metal tolerance, and increased Ni uptake are strongly influenced by the native microbial community (Jing et al., 2007; Aboudrar et al., 2013; Rue et al., 2015), improving efficient phytoremediation of metal-contaminated sites (Ma et al., 2009a).

Plant Growth Promoting Rhizobacteria (PGPR) can be found in association with the root system of hyperaccumulators increasing plant growth, biomass development and protecting plant from stresses (Aboudrar et al., 2013; Benizri et al., 2001; de Souza et al., 2015).

Recently, studies on the role of microbiota in the rhizosphere have encouraged the development of remediation technologies employing native soil microorganisms for metal phytoextraction. These

research allow to assess the effects of Ni mobilizing rhizobacteria on the plant growth and Ni uptake in *Alyssum* spp. and *Brassica* spp. (Cabello-Conejo et al., 2014; Glick, 2010; Lebeau et al., 2008; Ma et al. 2009a, 2009b; 2010, 2011; Rajkumar et al., 2013a; Rajkumar and Freitas, 2008a; Sessitsch et al., 2013; Zhuang et al., 2007). However, little is known about the microbial communities associated with the majority of hyperaccumulators, their functions and interactions (Alford et al. 2010; Thijs et al., 2017).

Serpentine bacteria were studied for the ability to mobilize metals and promote plant growth as in the case of *Microbacterium, Arthrobacter, Agreia, Bacillus, Micrococcus, Stenotrophomonas, Kocuria,* and *Variovorax* interacting with the obligate Ni-hyperaccumulating species *Noccaea caerulescens*, *Pseudomonas* acting in synergy with roots of *Odontarrhena bertolonii*, *Microbacterium* and *Sphingomonas* belonging to the rhizosphere of *Odontarrhena muralis* (Waldst. & Kit.) Endl. complex sin. *Alyssum murale* Waldst. & Kit. and members of the genus *Burkholderia* collected in the rhizosphere of *Pycnandra acuminata* (Pierre ex Baill.) Swenson & Munzinger sin. *Sebertia acuminata* Pierre ex Baill. and *Psychotria douarrei* (Beauvis.) Däniker, together with other nickel-resistant strains, like *Acinetobacter* sp., *Hafnia alvei, Pseudomonas mendocina, Comamonas acidovorans*, and *Agrobacterium tumefaciens* (Aboudrar et al., 2013; Barzanti et al., 2007; Idris et al., 2004; Mengoni et al., 2001; Pal et al., 2007).

The Ni-hyperaccumulator species usually host a greater number of Ni-tolerant rhizospheric bacterial strains if compared with those of non-hyperaccumulators growing on the same site or on bare soil (Aboudrar et al., 2007; Abou-Shanab et al., 2003; Idris et al., 2004; Mengoni et al., 2001; Schlegel et al., 1991). For instance, a comparison among the serpentine rhizospheric bacterial biodiversity associated with some subspecies of the Ni-hyperaccumulator *Alyssum serpyllifolium* Desf., the Ni-excluder *Dactylis glomerata* L., and the non-hyperaccumulator *Santolina semidentata* Hoffmanns. & Link, revealed that *A. serpyllifolium* subspecies hosted a greater density of bacteria than the non-hyperaccumulator species (Álvarez-López et al., 2016). This selective increase of Ni-tolerant bacteria in the rhizosphere was correlated with enhanced Ni bioavailability in soil (Becerra-

Castro et al., 2009).

Also rhizosphere fungi have the potential to assist the growth of hyperaccumulator plants in metal-rich soil and to increase their metal uptake (Husna et al., 2017; Thijs et al., 2017). Lately researchers have discovered that the network of fungal hyphae of the soil is involved in the translocation of a large variety of the plant photosynthetic metabolites (Klein et al., 2016).

Many studies were carried out on rhizospheric mycorrhizal communities of serpentine Ni-hyperaccumulator plants (Amir et al., 2013; Husna et al., 2017), but few of them investigated the role of serpentine non-mycorrhizal fungi associated with plant rhizosphere (Pal et al., 2006; Urban et al., 2008). Besides, it is known that fungi affect the composition of the bacterial community in their zone of influence called mycosphere by Warmink and van Elsas, 2008.

Previous studies isolated fungi like *Aspergillus, Botrytis, Clonostachys, Eurotium, Penicillium, Rhodotorula,* and *Trichoderma* from the rhizosphere of the facultative Ni-hyperaccumulator *Alyssoides utriculata* (Roccotiello et al. 2010; 2015a; 2016) growing on serpentine soils. Most of them were also metal-accumulator (Zotti et al., 2014; Roccotiello et al., 2015b; Cecchi et al., 2017b).

Hence, bacteria and fungi contribute in an essential way to biogeochemical cycles of the soil mediating the nutrition, the growth development and the health of the associated plant (Heydari and Pessarakli, 2010; Nazir et al., 2017).

In fact, fungi play the first step in the breakdown of organic matter, producing small molecules which are further decomposed by bacteria occurred in the same soil habitats (Nazir et al., 2017). In turn, bacteria are involved in nitrogen fixation, as well as phosphates solubilization, siderophores formation, phytohormones production (i.e. indole-3-acetic acid, IAA) and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity that are all plant growth promoting traits of rhizobia (Gopalakrishnan et al., 2015). In short, bacteria and fungi together form the basis of soil trophic networks (Rudnick et al., 2015).

Although many soil bacteria and fungi are metal-tolerant and play a key role in mobilization or immobilization of metals (Gadd, 1993), only a few attempts have been made to study the rhizosphere bacteria and non-mycorrhizal fungi of metal hyperaccumulator plants and their role in the tolerance to and uptake of metals by the plants.

Ecologically, the interactions between bacteria and fungi range from symbiotic and mutualistic (Partida-Martinez et al., 2007) to harmful and antagonistic (Scherlach et al., 2013).

In this scenario, the great challenge is to identify the microbiological components involved in these interactions and to understand their pattern (Miransari, 2011). Nowadays, a deep comparison between serpentine and non-serpentine microbiota of facultative Ni-hyperaccumulators like *A. utriculata* is missing. The inoculation of some Ni-tolerant bacterial and fungal strains in the rhizosphere of hyperaccumulators, specifically facultative-hyperaccumulators, can increase the efficiency of phytoextraction by promoting the development of root biomass (Ma et al. 2009a, b) and enhancing Ni accumulation in plant organs (Ma et al. 2011a).

2.7 Aim and objectives

The main aim is to provide basic data for the development of an integrated approach (plants-fungi-bacteria) cost-effective and environmental-friendly to improve the bioremediation of contaminated soils. Specific attention has been given to the interactions between Ni-hyperaccumulator species, native of polluted soils, and the related rhizosphere microbiota. The focus was set on all rhizosphere components to clearly understand how the hyperaccumulators' root and the associated rhizosphere microbiota behaves in presence of Ni.

To this aim, the following objectives were pursued:

- clarify the possible effect of nickel on the first step of plant development, i.e., seed germination of selected Ni-hyperaccumulator species in comparison with non-hyperaccumulators;

- evaluate possible alterations in the development of the root system (morpho-functional response to Ni) in terms of biomass and surface area;
- assess the potential Ni-selectivity of the rhizosphere microbiota through a screening of bacterial and fungal strains from the rhizosphere of a facultative Ni-hyperaccumulator;
- obtain a mixed culture of Ni-tolerant, plant growth promoting bacterial and fungal strains associated with the rhizosphere of a facultative Ni-hyperaccumulator.

This research represents the first step of a multidisciplinary holistic approach focused on the evaluation of all rhizosphere components in a facultative Ni-hyperaccumulator species in the future perspective of using native hyperaccumulator species and the related rhizobiota as natural metal chelators in contaminated soils.

3. Materials and methods

3.1 Plant material

Germination tests were performed with three nickel hyperaccumulator plants (Fig. 1A, B, C) from natural Ni-rich soils: *Alyssoides utriculata, Noccaea caerulescens* and *Odontarrhena bertoloni* and two non-hyperaccumulator plants (Fig. 1D, E) from 'normal' soil: *Alyssum montanum* and *Thlaspi arvense*.

Hyperaccumulator plants were grown from seeds collected according to international guidelines (ENSCONET, 2009), from serpentine soils in Liguria (NW Italy) in July 2016. Samples were harvested from the eastern Ligurian Alps (Voltri Massif, N 44° 28' 49, E 8°40' 44). The presence of Ni in the mother plants was assessed by means of a colorimetric field dimethylglyoxime test (Charlot, 1964; Küpper et al., 2001). All plants yielded a dimethylglyoxime-positive reaction.

A. montanum L. (Fig. 1D) and *T. arvense* L. (Fig. 1E) were the related non-hyperaccumulator species commonly used for comparison in experiments (Agrawal et al., 2013; Broadhurst and Chaney, 2016; Kozhevnikova et al., 2014; Mari et al., 2006; Milner and Kochian, 2008). Seeds were provided by herbarium specimens: *A. montanum* from the Jardin Botanique de Bordeaux FR0BORD120310 - Causse – Méjean, and *T. arvense* were provided by the Botanischer Garten Ulm IPEN XX-0-ULM-1998-F-152.

Figure 1. Test species: Ni-hyperaccumulator A) *A. utriculata* (photograph, S Marsili), B) *N. caerulescens* (photograph, A. Mazzoni) and C) *O. bertolonii* (photograph, S. Marsili); non-hyperaccumulators D) *A. montanum* (photograph, S. Marsili) and E) *T. arvense* (photograph, G. Nicolella).

3.2 Germination test

According to International Seed Testing Association (ISTA), germination is defined as "the emergence and development of the seedling to a stage where the aspect of its essential structures indicates whether or not it is able to develop further into a satisfactory plant under favourable conditions in the soil".

Germination test is often the most reliable way of evaluating the maximum seeds germination potential or viability. Optimal germination conditions should allow the germination of viable seeds.

Knowing the germination rate of plant species is the key to understand how that seed will perform in the field.

Basing on available literature, microcosm and mesocosm experiments were set up to assess plant germination and growth under controlled or semi-natural environmental conditions.

Microcosm test were set up to understand the germination time and rate of the plant species on different substrates without Ni. Tests were performed in a closed system (Petri dishes and veg-boxes).

The mesocosm experiments were carried out to simulate natural environmental conditions, monitoring the germination rate and rhizosphere response of plantlets to Ni stress.

3.2.1 Microcosm

Three type of substrate were placed in Petri dishes (Ø 90 mm): agar 1% (Sigma-Aldrich), mix of peat and river sand (2:1, pH 6.5) and vermiculite mineral substrate (pH 9.6). Each plate was filled with 50 ml of substrate previously sterilized at 120°C for 20 minutes. The substrates were irrigated with sterile deionized water (pH 6.0).

The plant seeds were surface sterilized with sodium hypochlorite (NaClO) 10% for 10 minutes (Baiyeri and Mbah, 2006). Ten seeds per plate of the considered plant species were sown equidistant from each other and three replicates per substrate were carried out (N= 30 seeds per substrate, each species).

The plates were incubated for 4 weeks, at different temperature and photoperiod: 20°C, 8 h light / 16 h darkness for *A. utriculata, O. bertolonii, A. montanum, T. arvense* and 21°C, 12 h light / 12 h darkness for *N. caerulescens* (ENSCOBASE, Ensconet Virtual Seed Bank). Germination data were collected every day in order to evaluate the germination rate, G%= (Number of germinated seeds/Total number of seeds) * 100.

After the primary root emission, images of seedlings were acquired by stereomicroscope and they were processed with LAS EZ software (Fig. 2) to monitor the radicle morphology of test species on different substrates.

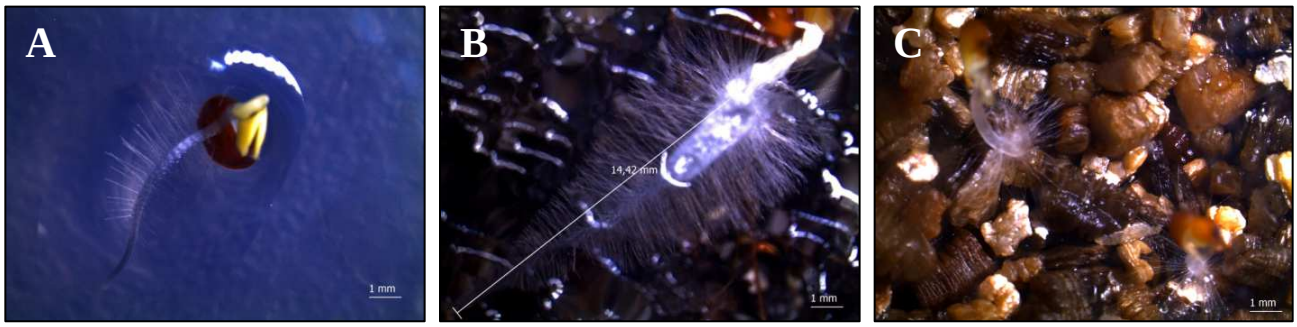

Figure 2. Stereo microscope images of microcosm test. A) Radicle of *A. bertolonii* grown on agar; B) radicle of *A. utriculata* grown on neutral peat-sand mix; C) radicle of *N. caerulescens* on vermiculite.

3.2.2 Mesocosm

After the sterilization at 120°C for 20 minutes, the peat-sand mix was placed inside veg-box (150 ml each veg box), and treated with a solution of 1/2-strength Hoagland's basal salt mixture n.2 (Sigma-Aldrich) containing nickel sulphate hexahydrate ($NiSO_4*6H_2O$, Sigma-Aldrich) at different concentrations of available Ni (0, 2, 12, 25, 50, 100 mg l^{-1}, pH 6.0).

The seeds were surface sterilized with NaClO 10% for 10 minutes (Baiyeri and Mbah, 2006); five seeds per veg-box were sown equidistant each other and three replicates per substrate were carried out (N= 15 seeds per treatment, each species). The veg-box were left in greenhouse in semi-natural conditions with natural photoperiod, T min=19°C, T max=22°C, for 120 days, monitoring weekly to evaluate the germination time (Fig. 3A, B).

At the end of the germination test, plant samples were collected then thoroughly rinsed first with tap water and then with deionized water to remove soil particles and dust.

For each treatment, we evaluated the biomass, in terms of fresh and dry weight.

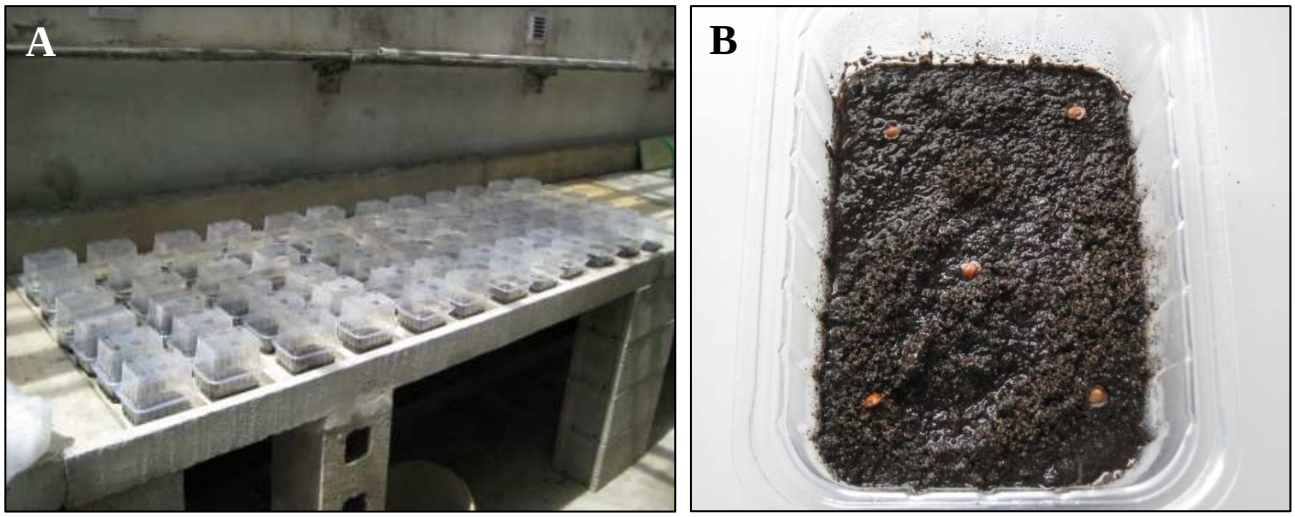

Figure 3. Mesocosm test in greenhouse. A) Veg box filled with neutral peat-sand mix; B) sowing of *A. uticulata* seeds on neutral peat-sand mix. N= 15 seeds per treatment, each species.

For each species and treatment, roots were scanned and the resulting images were processed with ImageJ software (Abràmoff et al., 2004; Rasband, 1997) that performed the estimation of the surface area (Fig. 4), for both hyperaccumulator and non-hyperaccumulator species. Image analysis systems would be developed not only to record the shape of root systems at a specific time point but also to explore different aspect of the soil-root interactions, to provide Root Structure Analysis (RSA), on the mechanisms of root growth and the morpho-physiological responses over time, analyzing the interactions with environment and investigating rhizosphere traits and processes (Downie et al., 2015).

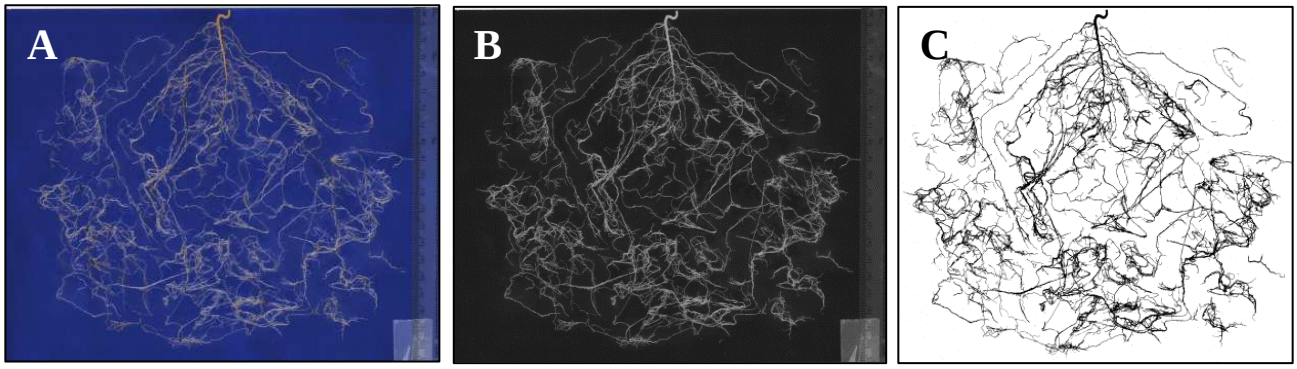

Figure 4. Phases of the image acquisition by ImageJ software. A) Root of *A. utriculata* at Ni 0 mg kg^{-1} scanned and B) converted in RGB (Red, Green, Blue) format, then C) converted to binary system.

3.3 Pot experiment

3.3.1 Evaluation of root area, biomass and plant water content

The greenhouse pot test with different Ni treatments was performed to better simulate natural growth conditions compared to germination tests on Petri dishes or in veg-boxes.

The peat-sand mix (2:1) was chosen as growing substrate; the substrate was sterilized at 120°C for 20', oven dried at 60°C. The final pH of substrate was measured by mixing an aliquot of soil with deionized water (ratio 1:3), and to obtain a pH of 6 - 6.5, slaked lime with Ca and Mg was added to the dry soil. After the assessment of pH, the soil water content at field capacity on volume basis (Bandyopadhyay et al., 2012) was assessed to calculate the Water Holding Capacity (WHC): 100 ml of water was added to 100 ml of dry soil placed in a funnel on a graduated cylinder. After waiting at least 1 hour until the last drop, the WHC (%) was calculated based on the volume of water retained by the soil. Finally, the soil was transferred to 10 cm Ø pot.

To evaluate the root surface response to increasing concentration of available Ni, soil was homogeneously hydrated with a 70% WHC solution of 1/4 strength Hoagland's basal salt mixture n.2 (Sigma-Aldrich) and metallic salt ($NiSO_4*6H_2O$) (Dechamps et al., 2008; Moradi et al., 2009) was added to obtain increasing concentrations of available Ni: 0, 50, 100, 200, 500, 1000 mg l^{-1} respectively. Afterwards, seeds were surface-sterilized with sodium hypochlorite (NaClO) 10% for 10' (Baiyeri and Mbah, 2006) and placed in pot (one seed per box, five replicates each concentration).

Pots were transferred to greenhouse and the plants were grown in semi-natural conditions at controlled temperature (T= 19-22°C) for 120 days, replenishing the plants with deionized water and monitoring plant growth two times a week (Fig. 5).

During the third month of growth, the water-soluble fertilizer Leader N-P-K (20-10-20 + MgO + Me) was solubilised in deionised water at the concentration of 0.5 g l^{-1} and supplied for each pot once a week for one month.

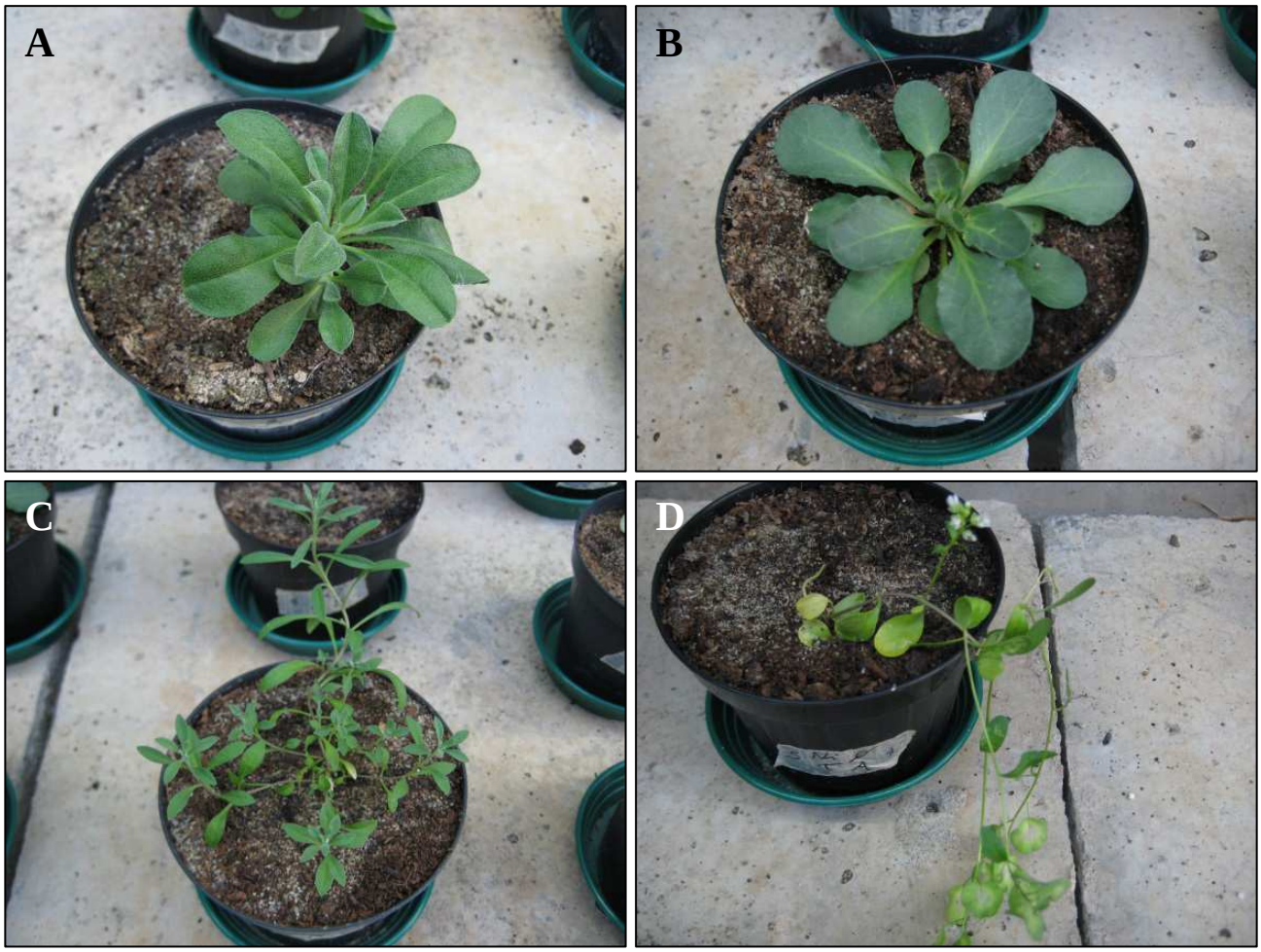

Figure 5. Control plant species grown in pots after 4 months: Ni-hyperaccumulators A) *A. utriculata*, B) *N. caerulescens*; non-hyperaccumulators C) *A. montanum*, D) *T. arvense*. N= 30 each species.

At the end of the test, Ni accumulation in leaves was evaluated via colorimetric dimethylglyoxime test (DMG 1%, Sigma-Aldrich, in ethanol 95%, Charlot, 1964) for each species and treatment. One mature leaf each plant was collected and placed in a solution of 1% DMG in ethanol 95°. Leaves turn red when a positive reaction occurs (high amount of Ni is stored in leaf epidermis).

Each plant was gently removed from the substrate, washed with tap water and then with deionised water, divided into root and shoot and weighed for fresh biomass. Roots were scanned and the resulted images were processed and analysed as in paragraph 3.2.2 to assess the root surface area. Finally, DW were determined after oven-drying (60°C, 48 h). Leaves were powdered using a ball mill (Retsch MM2000, Haan, Germany), preceding XRF analysis.

The chemical characterization of the dried samples was carried out on the granulometric fraction < 2 mm by using a X-ray field portable spectrophotometer (X-MET7500 FP-EDXRF Analyser, Oxford Instruments) that allows non-invasive and non-destructive analyses, providing information about chemical composition of the shoots. Quantitative analyses were obtained from trace level (ppm) to 100% for elements with atomic number ≥ 12 and the data quality level of the analyses was defined according to the Method 6200 of the U.S. Environmental Protection Agency (EPA, 2007). This procedure was performed thanks to the collaboration with Geospectra s.r.l, spin off of the University of Genoa. It represents an efficient, alternative approach to traditional laboratory analysis, allowing the measurement of the concentration of a wide range of chemical elements.

3.3.2 Photosynthethic efficiency

Chlorophyll fluorescence is a simple and non-invasive measurement technique of photosystem activity (PSII) in which the light energy absorbed by chlorophyll is partly re-emitted as light (Murchie and Lawson, 2013).

To estimate the plant physiological condition at the end of the growth period, 10 measurements of photosynthetic efficiency in each plant were performed on leaves with digital fluorimeter Handy-Pea (Hansatech Instruments, Fig. 6A). It provides the high time resolution essential in performing measurements of fast chlorophyll fluorescence induction kinetics. Leaves samples were covered with the leafclip which has a small shutter plate to keep close when the clip is attached so that light is excluded, and dark adaptation takes place (Fig. 6B). After 20 minutes the chlorophyll fluorescence signal received by the sensor head during recording is digitised within the Handy PEA control unit using a fast Analogue/Digital converter.

The fluorescence transient is a tool to characterize and screen photosynthetic samples (Strasser et al., 2000). General parameters recorded by fluorimeter are:

- F0 is the minimum fluorescence value and represents emission by excited chlorophyll *a* molecules in antenna structure of Photosystem II.

- Fm is the maximum fluorescence value obtained after the application of a saturation pulse to the dark-adapted leaf.
- Fv is the variable fluorescence and it denotes the variable component of the recording and relates to the maximum capacity for photochemical quenching.
- Fv/Fm is widely used to indicate the maximum quantum efficiency of Photosystem II. It is a sensitive indication of plant photosynthetic performance.
- Tfm is used to express the time at which the maximum fluorescence value (Fm) was reached.
- Area above the fluorescence curve between F0 and Fm is proportional to the pool size of electron acceptors.
- P.I. (Performance Index) is essentially an indicator of sample vitality.

Figure 6. Fluorescence transient analysis. A) Handy-Pea fluorometer; B) dark adaptation of leaves samples as the first step of the chlorophyll fluorescence measurement; C) Kautsky Induction curve (Brestic & Zivcak, 2013) must be plotted on a logarithmic axis to observe the polyphasic rise to Fm.

The excitation light consisted of a 1 second (s) pulse of ultra-bright continuous red radiation (650 nm peak wavelength), provided by an array of three light-emitting diodes focused on a leaf surface of 5 mm^2 at an intensity of 3500 µmol photons per square meter (m^2) per second (s). The analysis of the transient was based on the fluorescence values measured at 50 µs (F0), 2 ms, 30 ms, and

maximal (Fm) after about 300 ms (Roccotiello et al., 2016; Strasser et al., 2000). The fluorescence induction is well represented by Kautsky Induction curve (Fig. 6C, Brestic and Zivcak, 2013).

The fluorescence emission provides information about the phytochemical efficiency and the heat dissipation; therefore, the presence of any type of stress results in photoinhibition and a low Fv/Fm ratio (Murchie and Lawson, 2013). Therefore a quick screening of the photosynthetic efficiency shows the trend of growth and plant yield (Furbank et al., 2009; Montes et al., 2007).

Data on photosynthetic efficiency and plant physiological performance, obtained from the averages of measurements on the test species, have been processed with PEA-Plus software (Hansatech Instruments).

3.4 Characterization of culturable rhizobiota

3.4.1 Sampling sites and sample collection

The facultative Ni-hyperaccumulator *A. utriculata* and related soil were sampled from serpentine (S) and non-serpentine sites (NS). The S site in the Beigua Geopark (44°27′41.4″N 8°40′03″E) is geologically located in the extreme East of the Ligurian Alps. The soils derived from ultramafic bedrocks like serpentines and eclogitic metagabbros (Capponi and Crispini, 2008; Marsili et al., 2009). The NS site was the locality of Glori in the NW of Liguria (43°57′19″N 7°50′08″ E), geologically characterized by flysch and clay marl (Giammarino et al., 2010). Each site was sampled for plant (*A. utriculata*) and soil (rhizospheric and bare) (Fig. 7).

Five shoots of adult plants of *A. utriculata* from non-fruiting branches and roots replicates (1 plant = 1 replicate) were collected in July 2016 (fruiting stage) from the S and NS sites (N= 5 each site).

Ni-hyperaccumulation in plants was assessed with colorimetric dimethylglyoxime test (DMG 1% in ethanol 95%, Sigma-Aldrich) (Charlot, 1964) on leaves, as described previously, then plants were quantitatively evaluated with ICP-MS.

Rhizosphere soil from S and NS site was collected from five different plants (N= 5 each site). Plants were carefully dug out with an intact root system and the soil tightly adhering to the roots

was collected. The rhizosphere soil was obtained by agitating roots and sampling the soil still attach to the roots according to Khan et al. (2015). Each rhizospheric soil sample was placed into a plastic bag to avoid microbial mixing between the soils and transported into a refrigerated box. In laboratory, aliquots of soil were processed for the isolation of culturable microbiota.

No vegetation was observed in the bare soil from (Khan et al., 2015) S and NS site. Bulk soil samples were collected from five different points which were 20 m away from the vegetation to a depth of approximately 15 cm.

Bare and rhizospheric soil samples not immediately processed were stored at -20°C.

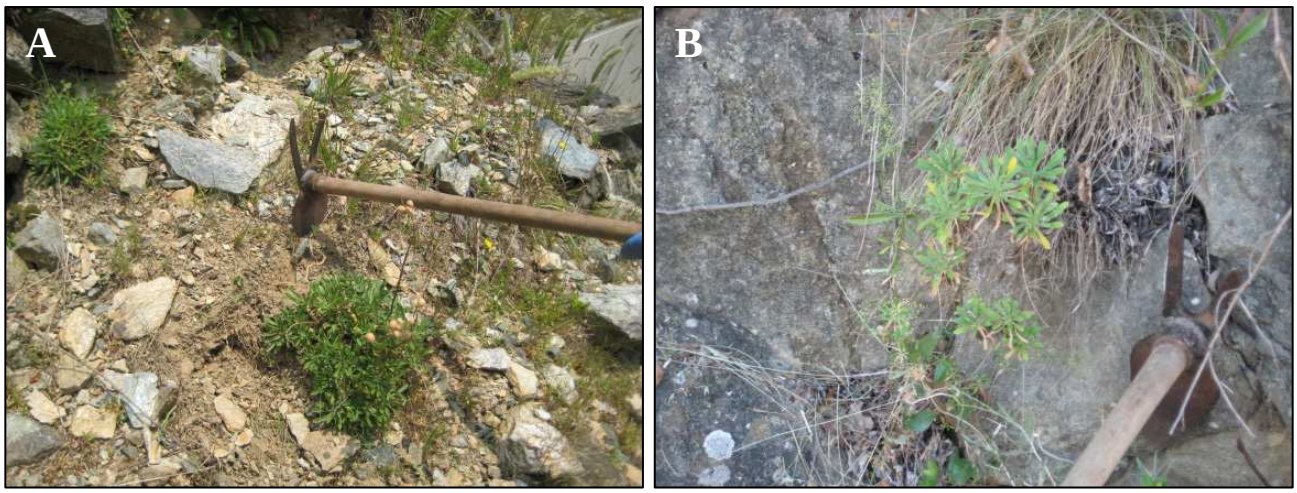

Figure 7. Sampling of *Alyssoides utriculata* and related rhizosphere soil from A) Serpentine and B) Non-Serpentine site.

3.4.2 Plant and soil sample analysis

In the laboratory, plant samples were thoroughly rinsed first with tap water and then with deionized water to remove dust and soil particles. After oven-drying (60°C, 48 h), leaves and roots were separately powdered using a ball mill (Retsch MM2000, Haan, Germany), before the chemical analyses.

Soil samples were oven-dried at 60°C for 48 h before being sieved through a 2.0 mm mesh. Soil and plant fractions were analysed for Ni, Ca, Mg, Cu and Zn concentration by means of a Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

The accuracy of the results was checked processing BCR-100 'beech leaves' reference material (JRC-IRMM, 2004). Plant and soil metal concentrations were expressed on a dry weight basis (DW).

3.4.3 Isolation and identification of culturable bacteria

Bacteria were extracted from 2.5 g of fresh soil by using 25 ml of sterile saline solution. Aliquots (1 ml) were serially diluted with NaCl 0.9% w/v (Khan et al., 2015; Turgay et al., 2012) and spread on Tryptic Soy Agar (TSA, Sigma-Aldrich) added with $NiSO_4*6H_2O$ (Sigma-Aldrich) to obtain the Ni-concentration of 0 (control), 1, and 5 mM, respectively, and amended with 50 mg l^{-1} of cycloheximide (Sigma-Aldrich) to inhibit the microfungal growth (Visioli et al., 2014a). Plates (N= 180 each site) were incubated in the dark at 27±1°C for 72 h (Barzanti et al., 2007). Bacterial colonies with distinct morphologies (colour, shape, size, opacity, etc., Fig. 8) were selected from the plates and repeatedly re-streaked onto fresh agar medium prepared as previously described, to obtain pure bacterial colonies (Barzanti et al., 2007; Khan et al., 2015; Luo et al., 2011; Turgay et al., 2012; Zhu et al., 2014) (Fig. 9).

Relative concentration of identified morphotypes was also determined and expressed as Colony Forming Units per g of dry soil (CFU g^{-1}) (Rue et al., 2015). The most representative morphotypes were selected (N= 30, three replicates each isolate) to perform the DNA sequencing.

To identify isolates, DNA extraction from pure bacterial culture was performed by the boiling method (99°C, 10 min). PCR amplification of a 409 bp region was then performed using the universal primers Com1 (5′-CAGCAGCCGCGGTAATAC-3′) and Com2 (5′-CCGTCAATTCCTTTGAGTTT -3′), amplifying positions 519–926 of the *Escherichia coli* numbering of the 16S rRNA gene (Schwieger and Tebbe, 1998). Each PCR was performed in a

total volume of 15 µl in micro-test tubes (Eppendorf s.r.l., Milan, Italy). Reaction mixtures contained 1× PCR buffer with 1.5 mM of magnesium chloride ($MgCl_2$), deoxynucleoside triphosphate solution (200 mM each dATP, dCTP, dGTP and dTTP), primers Com1 and Com2 (0.5 mM each), and 2.5 U of DNA polymerase (FastStart High Fidelity enzyme blend, Sigma Aldrich srl, Milan, Italy). The temperature profile for the PCR was as follows: an initial step of 10 min at 95°C, followed by denaturation for 1.30 min at 94°C, annealing for 40 s at 55°C and primer extension for 40s at 72°C. After the 35th cycle, the extension step was prolonged for 10 min to complete synthesis of all strands, and then the samples were kept at 4°C until analysis.

Amplified fragments from the PCR reaction were purified using the High Pure PCR product purification kit (Roche Diagnostics, Mannheim, Germany) and sequenced using the automated ABI Prism 3730 DNA sequencer (Applied Biosystems). 16S rRNA gene sequence similarity was determined using the BLAST function of the CLC Genomics workbench (version 9.5.1).

The sequences were submitted in the NCBI GenBank® database under the accession numbers from MG661811 to MG661840.

The isolated strains were cryoconserved at -80°C in 20% glycerol in Luria Bertani (LB, Sigma-Aldrich) broth in the Laboratory of Microbiology (DISTAV, University of Genoa, Italy).

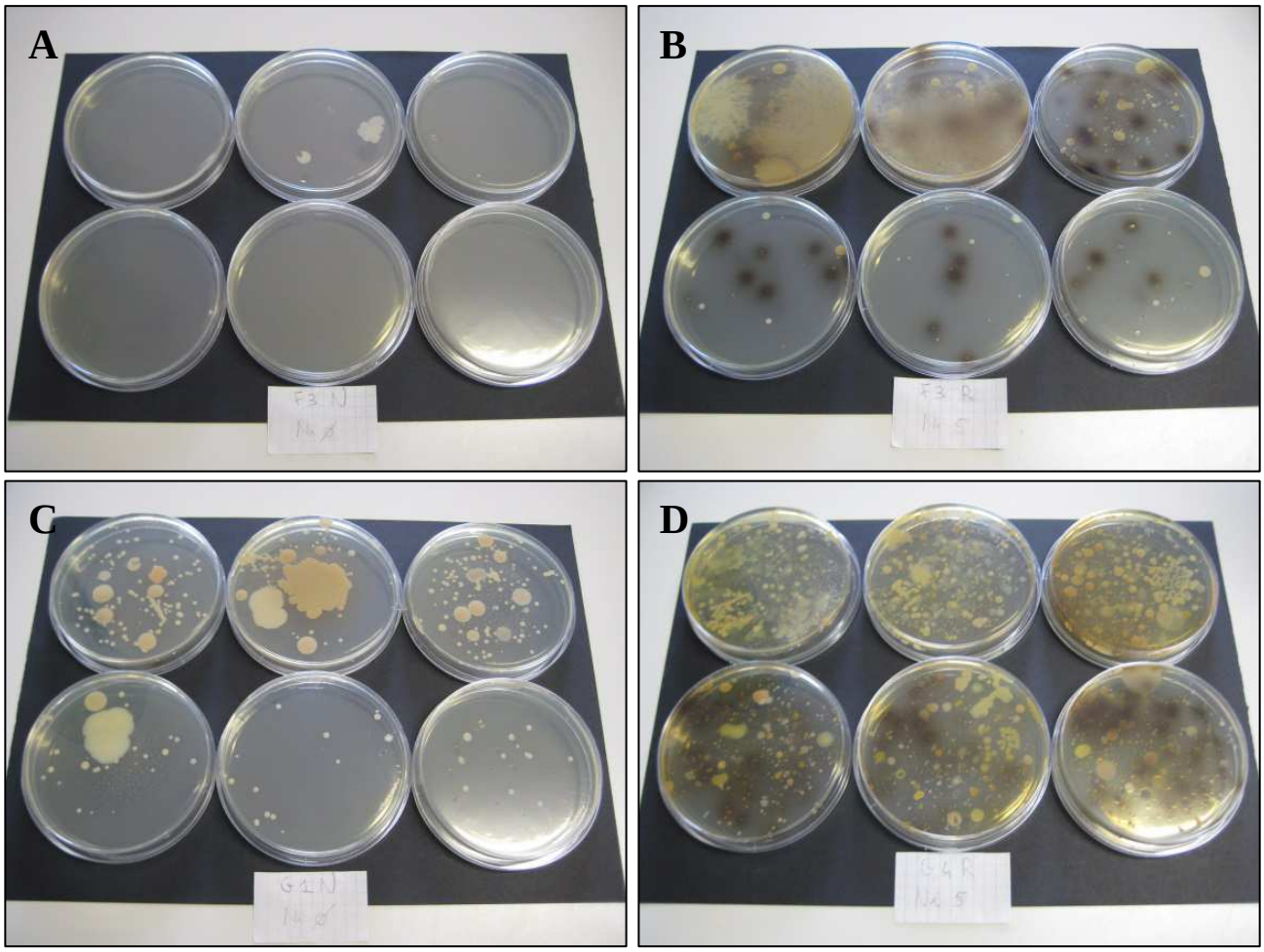

Figure 8. Bacterial colonies diversity on TSA isolated from Serpentine (A, B) and Non-Serpentine (C, D) site. Sample of bare soil (A, C) and rhizosphere soil (B, D). The bacterial colonies are significantly more abundant on R soil than B soil and in the NS site compared to site S. The presence of Ni (B, D) develops a clear black halo around some colonies identified as belonging to genus *Streptomyces*. N=180 each site.

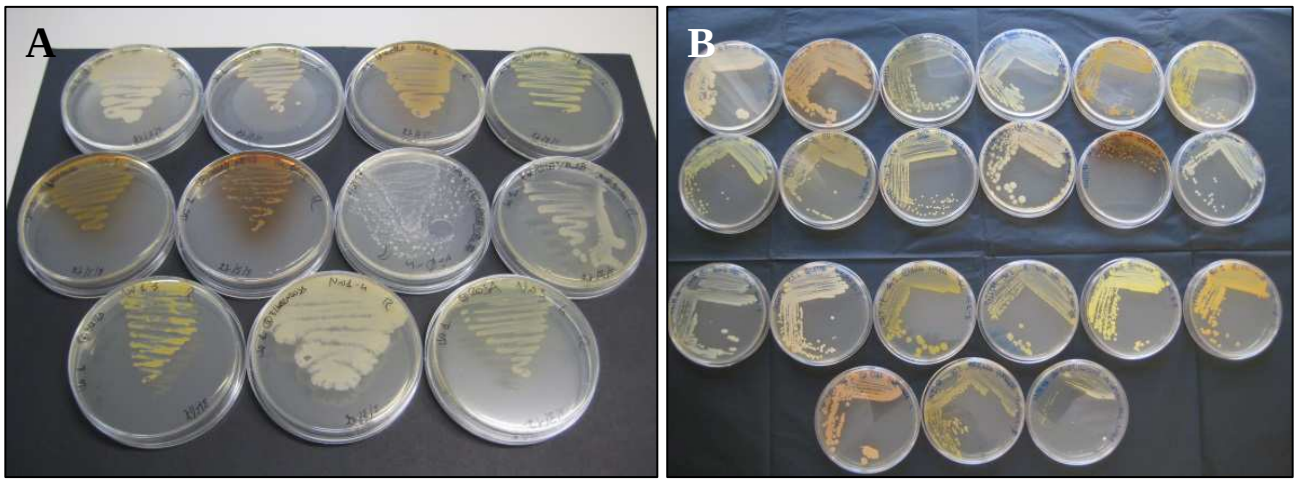

Figure 9. Pure bacterial colonies re-streaked onto agar isolated from A) Serpentine site and B) Non-Serpentine site. N=180 each site.

3.4.4 Screening for PGP traits and Ni-tolerance in bacteria

Serpentine Bacterial strains were screened for their ability to grow on 1-Aminocyclopropane-1-Carboxylic Acid (ACC) as the sole N source, to produce indole-3-acetic acid (IAA) and siderophores and solubilize phosphorous. For ACC deaminase activity, following an incubation of 24 h in Tryptic Soy Broth (TSB, Sigma-Aldrich) at 28°C, bacterial suspension was harvested by centrifugation (4500 g x 10 min) then a 1-ml aliquot was transferred to 50 ml sterile Dworkin and Foster (DF) mineral medium (Dworkin and Foster, 1958) added with 300 µl of ACC (Alfa Aesar) instead of $(NH_4)_2SO_4$ as nitrogen source. A 0.5 M solution of ACC (labile in solution) was previously filter-sterilized through 0.2 µm pore size membrane and added to DF medium. The salts minimal medium without N was used as control. The solution was incubated at 28°C for at least 24 h (Luo et al., 2011; Penrose and Glick, 2003). Turbidity indicates positive growth (Fig. 10A).

Auxin IAA production was estimated using a spectrophotometric method (Bric et al., 1991). Isolates were grown in TSB amended with tryptophan (trp, 1 mg/ml broth) at 32±2°C for 4 days. The surnanant was mixed with Salkowski Reagent (1 ml 0.5 M $FeCl_3$ in 50 ml 35% $HClO_4$) in the ratio of 1:1. After 25-30 min at room temperature, the development of pink colour underlines IAA production (Fig. 10B). The optical density was measured using spectrophotometer (Jenway 6300 spectrophotometer) at 530 nm of absorbance and auxin concentration was estimated using standard curve of IAA (Goswami et al., 2015; Naveed et al., 2014).

The siderophores production (Fig. 10C) was determined after 5 days of incubation at 30°C on Chrome Azurol Sulfonate (CAS) agar (Schwyn and Neilands, 1987), through the development of red-orange halo around the colony (Durand et al., 2016).

Phosphate solubilisation activity (Fig. 10D) was assessed by the formation of a clear halo around the colony on Pikovskaya's agar medium (Khan et al., 2015).

Afterwards, each PGPR isolate was tested for metal tolerance on Tryptic Soy Agar (TSA, Sigma-Aldrich) spiked with $NiSO_4*6H_2O$ at the concentration of 10, 15, and 20 mM respectively, in addition to concentrations previously tested.

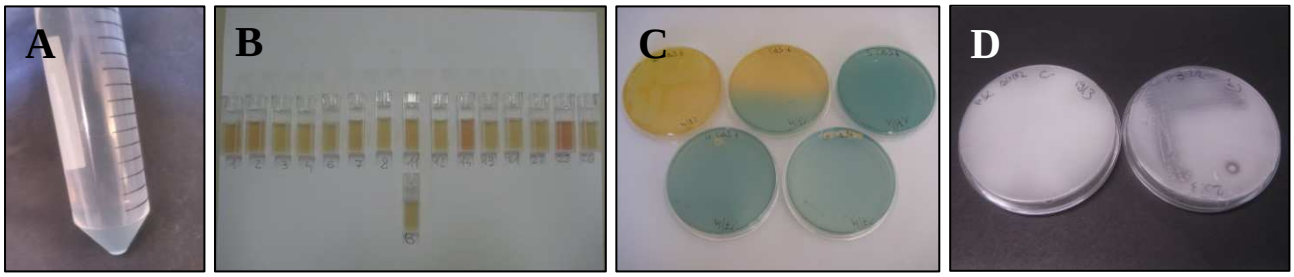

Figure 10. Evaluation of Plant Growth Promoting (PGP) traits. A) Turbidity indicates 1-Aminocyclopropane-1-Carboxylic Acid (ACC) deaminase activity in falcon tube; B) development of pink color in cuvettes denotes synthesis of indole-3-acetic acid (IAA); C) red-orange halo around bacterial colonies in some Petri dishes shows the production of siderophores; D) the clear zone around bacterial growth on plate compared to control demonstrates the phosphate solubilization activity. N= 8 each strain.

3.4.5 Isolation and identification of culturable fungi

Fungi were counted and isolated through a dilution plate technique (Cecchi et al., 2017b; Greco et al., 2017; Zotti et al., 2014) by using two different culture media (Fig. 11A): Malt Extract Agar added with Chloramphenicol (MEA+C) and Rose Bengal agar (RB) (Greco et al., 2017). The dilution was obtained by mixing 1 g of soil with 100 ml of sterile water. Each sample was plated in duplicate, for each dilution (1:50.000 and 1:100.000). The plates were then incubated at 24±1°C, in the dark, for 14 days and checked daily.

The Colony Forming Unit per g of dry soil (CFU g^{-1}) were counted for each fungal strain grown in plates. Then, these strains were isolated and subcultured onto Malt Extract Agar (MEA). The pure cultures were maintained on MEA slants and kept at 4°C.

All the fungal isolates were initially identified by a polybasic approach on the base of their micro-macromorphological, physiological, and molecular characteristics. The morphological identification was confirmed by molecular analysis. PCR amplification of β-tubulin gene was performed using Bt2a and Bt2b primers (Glass and Donaldson, 1995) and ITS region amplification using universal primers ITS1F and ITS4 (White et al., 1990; Gardes and Bruns, 1993). The taxonomic assignment of the sequenced samples was carried out by means of the BLASTN algorithm thus allowing us to compare the sequences obtained in our study with the ones available in the GenBank database. The

sequences were submitted in the NCBI GenBank® database under the accession numbers from MG836709 to MG850983.

The isolated strains were conserved in the culture collection of Mycological Laboratory of DISTAV (University of Genoa, Italy, Fig. 11B). These cultures were maintained by agar slants with periodic transfers and then crioconserved (-20°C).

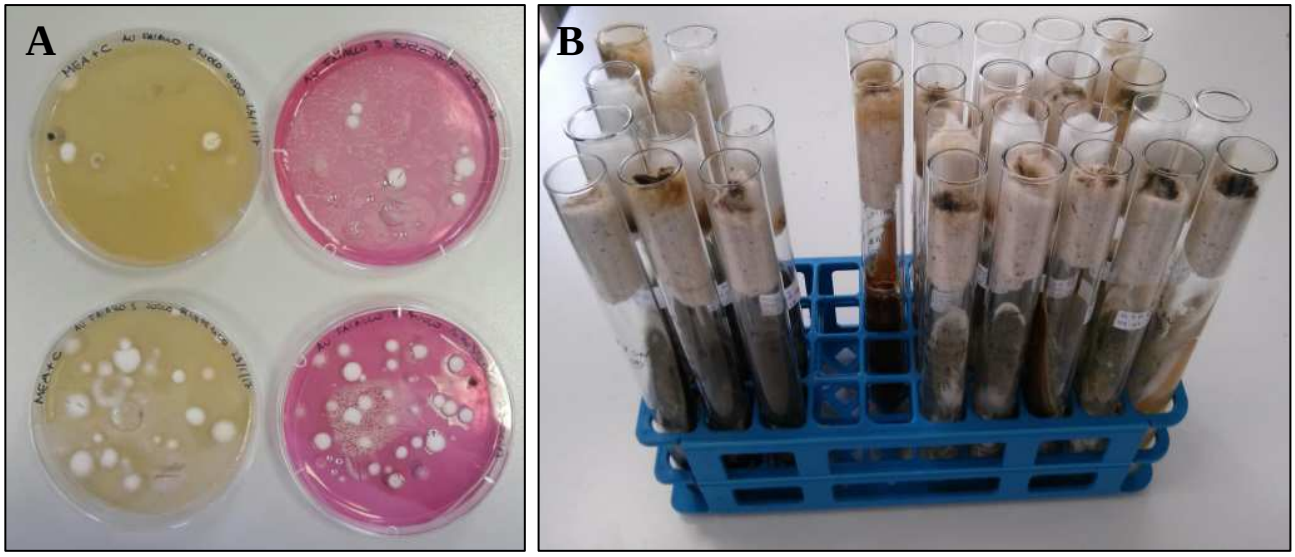

Figure 11. Screening of fungal strains. A) Isolation of serpentinitic fungi on Malt Extract Agar (MEA) and Rose Bengal agar (RB); B) conservation of the fungal strains in tubes. N= 40 each site.

3.4.6 Screening for Ni-tolerance in fungi

Each fungal strain isolated from S site was tested for metal tolerance on Malt Extract Agar (MEA, Sigma-Aldrich) spiked with $NiSO_4*6H_2O$ at the concentration of 1, 5, 10, 15, and 20 mM.

3.5 Co-growth of bacteria and fungi

3.5.1 Preliminary test

The bacterial and fungal strains were isolated on different types of substrate (Fig. 12) to screen the most suitable agar for their growth among Czapek Yeast extract Agar (CYA), Czapek's agar (CZ), Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), Sabouraud (SAB) and Tryptic Soy Agar (TSA).

The growth curves of bacteria were calculated by spectrophotometric analysis of the Absorbance (Optical Density, OD) at a wavelength of 600 nm at regular time intervals (1 h).

Among the eight bacterial strains characterized by PGP traits, *Pseudomonas* sp. (SERP1) and *Streptomyces* sp. (SERP4) were selected because of their tolerance to high concentrations of $NiSO_4$ *$6H_2O$. At the same time, among serpentinitic fungi, *Penicillium ochrochloron* Biourge (Serp03S) and *Trichoderma harzianum* Rifai (Serp05S) were selected for the following tests on the basis of bibliographic data (Cecchi et al., 2017b) on metal uptake and Ni tolerance test.

The selected microbiota was examined to understand if there is an antagonism activity (Agamennone et al., 2018; Trivedi et al., 2008) or if bacteria and fungi grow together without inhibition.

For each test, three methods were used onto two different substrates (CYA and TSA, pH 6), three replicates each method (N= 30):

- agar overlay method;
- agar plug method;
- distance growth method.

Agar plates with bacterium and with fungus alone were used as a control.

Bacteria isolates were grown in cell culture flasks containing TSB until reaching the exponential phase. SERP1 was incubated overnight at 26±1°C, while SERP4 was maintained for 72 h at 26±1°C, then revived 1:20 in the same growth medium and finally incubated for 30 h.

On the other hand, fungi were plated on MEA agar and kept at 24±1°C until conidiogenesis and potential spore formation.

At the starting time of the test, Optical Density (OD_{600}) of the growing culture was recorded and compared with concentration (approximately 10^8 CFU ml^{-1}). Bacterial cells pellet were washed twice in 1x Phosphate-Buffered Saline (PBS) solution pH 7.4 and then concentrated (van Ditmarsch and Xavier, 2011).

In addition to the pure fungal culture on MEA agar, an aliquot of well-grown fungal colony was diluted in distilled sterile water and fungal concentration was estimated by cell counts using a Bürker chamber (Kopecká, 2016).

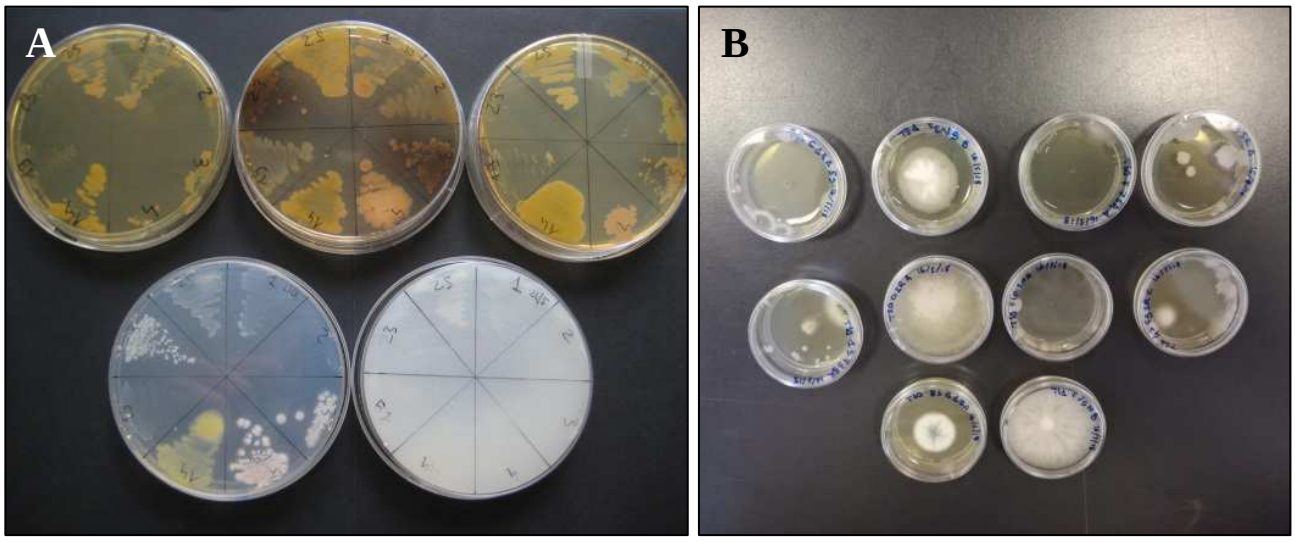

Figure 12. Bacterial and fungal strains tested on different agar plates. A) Bacterial strains grown on Malt Extract Agar (MEA), Czapek Yeast extract Agar (CYA), Sabouraud (SAB), Potato Dextrose Agar (PDA) and Czapek's agar (CZ) (from left to right respectively). N= 40; B) fungal strains grown on Tryptic Soy Agar (TSA) at pH 6.0.N= 10.

3.5.2 Co-growth methods

Agar overlay

For this method, 100 µl of fungal liquid culture (10^8 CFU ml^{-1}) were stroked on solid medium CYA and TSA pH 6.0 and subsequently 100 µl of bacterial suspension (10^8 CFU ml^{-1}) was inoculated at the centre of the Petri dishes.

Agar plug

For this method, a disc (Ø 5 mm) from well-grown lawn of the fungus was cut and transferred on 100 µl of bacterial suspension (10^8 CFU ml^{-1}) previously stroked on agar.

Distance growth

In this method, the agar plug (5 mm) and the bacterial suspension (10^8 CFU ml^{-1}) were inoculated 3 cm away from each other on the same plate.

In similar manner, bacterial suspension and agar plug from the well-grown lawn of fungus were used as positive control. The plates of three methods were then incubated at 26±1°Cand the growth of the isolates was monitored daily for 10 days.

Scanning Electron Microscopy

To better observe the well-grown mix culture of microbiota after 14 days-incubation on CYA agar, the isolates were fixed in phosphate 10% buffered formalin and dehydrated in ethanol series (Roccotiello et al., 2010). Samples were then mounted on SEM stubs, sputter-coated with gold and viewed with a VEGA3 TESCAN SEM at HV20.0 kV, using BackScattered Electrons (BSE).

3.6 Data analysis

The statistical analyses were performed with Statistica 8.0 (Statsoft Inc.) software.

The averages were presented with their standard deviations (SD). Nonparametric tests were used to avoid data transformation. Normality of parameters were evaluated with the Shapiro-Wilk test. Correlations between variables were analysed using Spearman's correlation coefficient (ρ) using different level of significance (α: 0.05, 0.01, 0.001), since data exhibit a non-normal statistical distribution.

The difference between different sites (S and NS) and substrates (R and B) was estimate by means of Kolmogorov-Smirnov (K-S) two samples test.

The Principal Components Analysis (PCA) was performed as a multivariate display method to visualise the data structure. Significance was considered at the $p < 0.05$ level.

The mobility of Ni from soils towards the roots of *A. utriculata* and the ability to translocate root-to-shoot Ni were assessed, respectively, by means of the Bioaccumulation Factor (BF), and the Translocation Factor (TF) on a dry weight basis (Roccotiello et al., 2016). BF is the ratio of metal concentration in the plant shoots to the initial soil concentration of the metal (C_{shoot}/C_{soil}, Zhang et

al., 2010), while TF indicates the ratio between metal concentration in the shoot and plant root (C_{shoot}/C_{root}) as indicated by Mehes-Smith and Nkongolo (2015).

Moreover, the R/S ratio for both fresh and dry biomass and the water content (100 * DW/FW) in root and shoot were evaluated (Roccotiello et al., 2016).

4. Results

4.1 Germination test

4.1.1 Microcosm

The germination test shows that the mean germination rate (G%) appears high (G> 75%, Fig. 13) in *A. utriculata* (mean germination= 83,8%, Fig. 13A) and *A. montanum* (mean germination= 76,6%, Fig. 13D) after 28 days for analysed substrates. Germination rate of *N. caerulescens* (mean germination= 56,0%, Fig. 13B) reached 100% on vermiculite, while *O. bertolonii* had the lowest germination rate (mean germination= 42,9%, Fig. 13C) compared with the other species.

The correlation between G% and the substrate of each species is shown in Table 1 *O. bertolonii* shows a highly negative correlation between the germination rate and two substrates (agar and vermiculite, $p< 0.001$). *O. bertolonii* was not considered suitable for the following Ni treatment due to its low-performance observed in the microcosm test. On the contrary, in *A. utriculata* the G% reveals highly significant positive correlations both with agar and with peat-sand mix ($p< 0.001$). Vermiculite seems to improve the germination of *N. caerulescens* ($p< 0.01$), while the peat-sand mix promotes the germination of *A. utriculata* ($p< 0.001$), but it hinders the germination of *N. caerulescens* ($p< 0.001$).

Substrate	**Germination rate (G%)**			
	***A.u.*++**	***N.c.*++**	***O.b.*++**	***A.m.*+**
Agar	0.59***	-0.31*	-0.61***	0.32*
Peat-sand mix	0.57***	-0.74***	NS	NS
Vermiculite	NS	0.43**	-0.69***	NS

Table 1. Spearman's rank correlations coefficients between growth substrates and Germination rate (G%) of test species. N= 30 per substrate, each species. *A.u.: A. utriculata, N.c.: N. caerulescens, O. b. Odontarrhena bertolonii, A.m.: A. montanum.*
++ hyperaccumulator species, + non-hyperaccumulator species
*p< 0.05**, p< 0.01 ***, p< 0.001, NS Not Significant

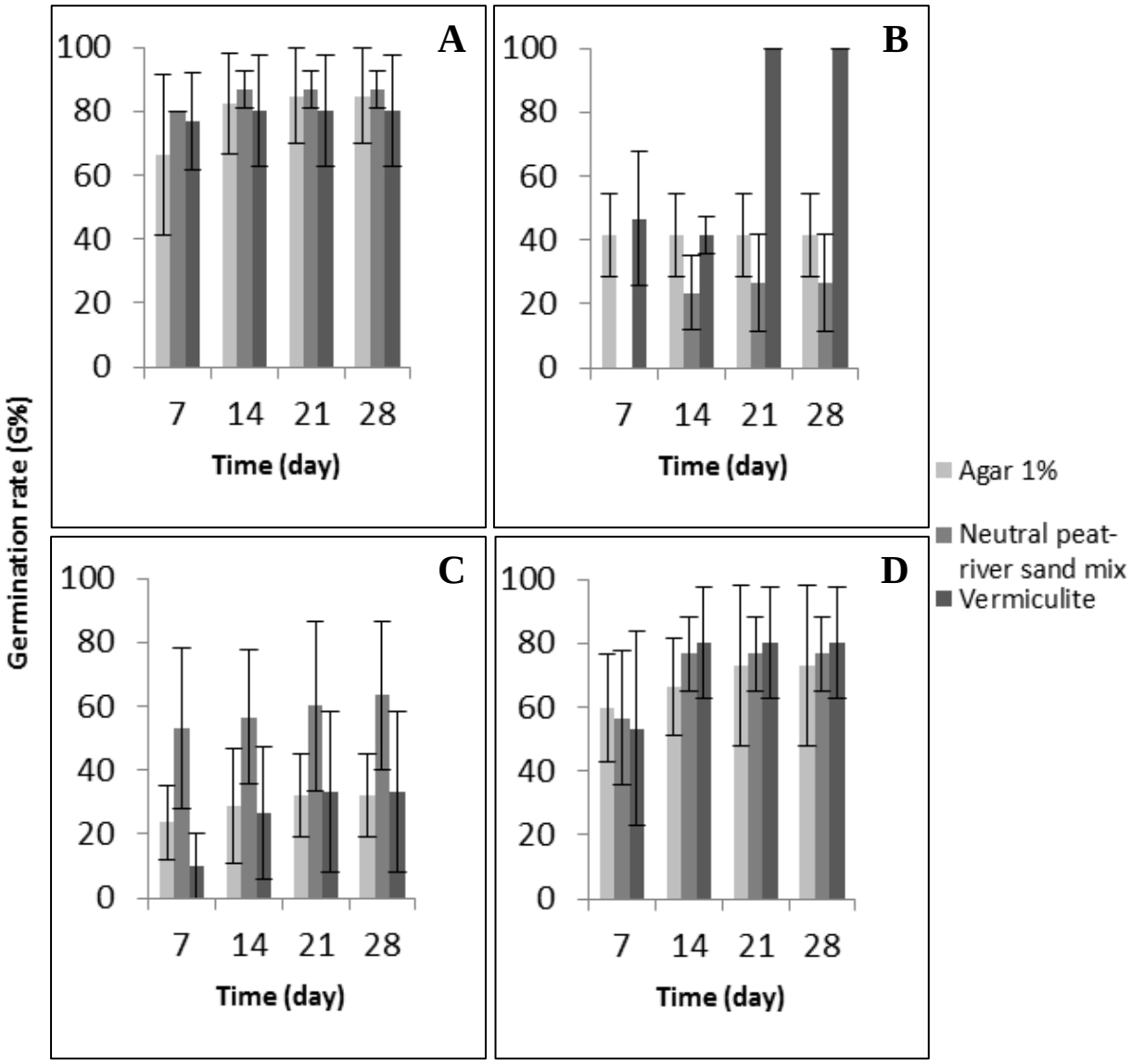

Figure 13. Germination rate (G% of seeds that sprout). of test hyperaccumulator species: A) *A. utriculata*, B) *N. caerulescens*, C) *O. bertolonii* and non-hyperaccumulator species, D) *A. montanum*. Data are mean±SD. N= 30 per substrate, each species.

4.1.2 Mesocosm

Despite of the previous microcosm test, the mean germination rate is low in all treatments (G< 60%).

On neutral peat-sand mix, *A. utriculata* exhibits a low germination rate at the highest Ni concentration: G= 20% and 26,7% for Ni 50 and Ni 100 mg l^{-1} respectively (Fig. 14A) compared to the control (G= 73,3%) as well as *N. caerulescens* (G= 40% and 46,7% for Ni 50 and Ni 100

respectively, Fig. 14B), compared to the control Ni 0 (G= 80%), while in the non-hyperaccumulator species, a variation in terms of germination rate is not observed (Fig. 14C, D).

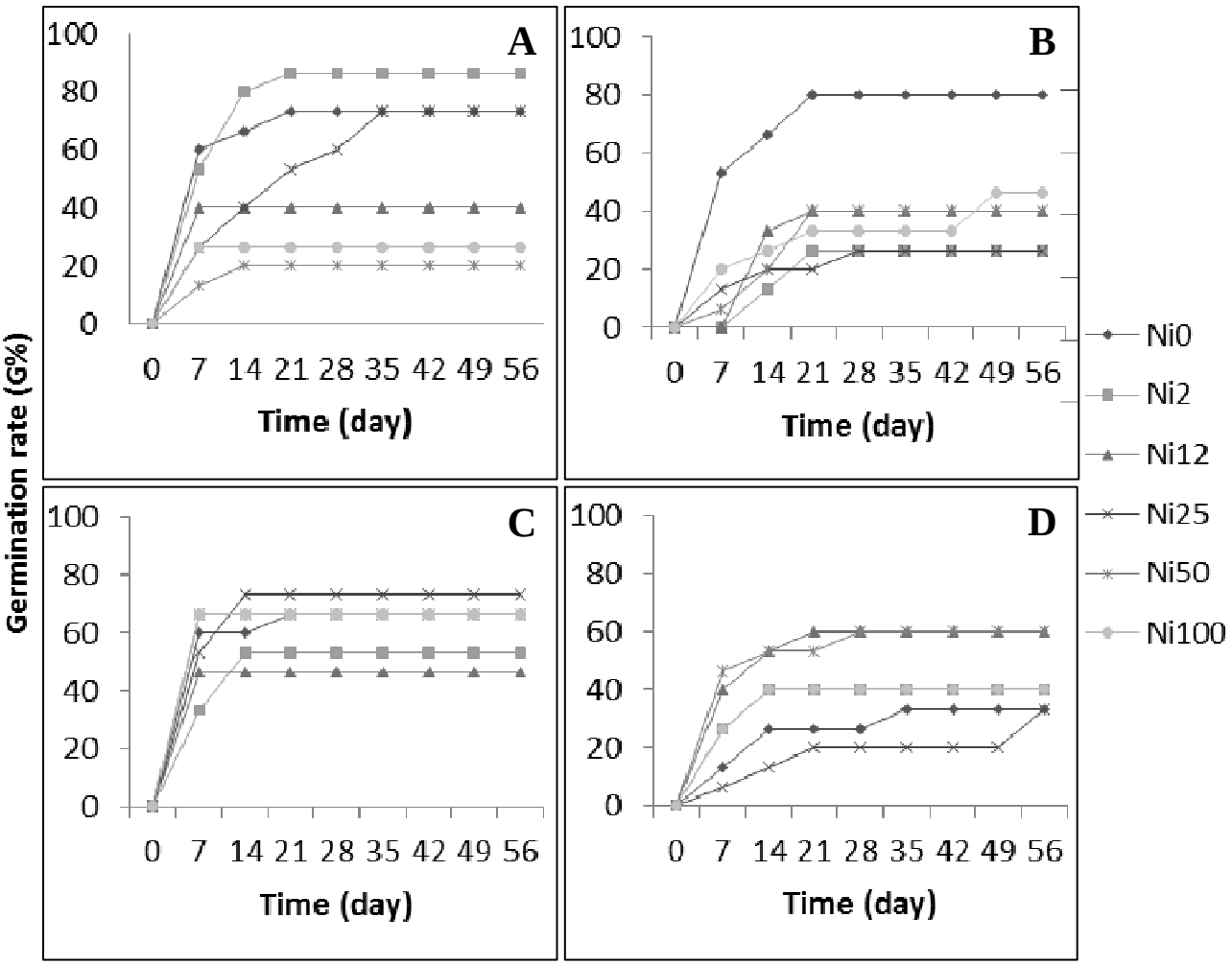

Figure 14. Germination rate (G%) of test hyperaccumulator species: A) *A. utriculata*, B) *N. caerulescens* and non-hyperaccumulator species C) *A. montanum* and D) *T. arvense* on neutral peat-sand mix. Data are mean. N= 15 per treatment, each species.

Hyperaccumulator species do not show significant differences in terms of root surface area between treatment and control (Fig. 15A, B) while the non-hyperaccumulator species show a clear dose-response effect by Ni (Fig. 15C, D) ($p < 0.001$).

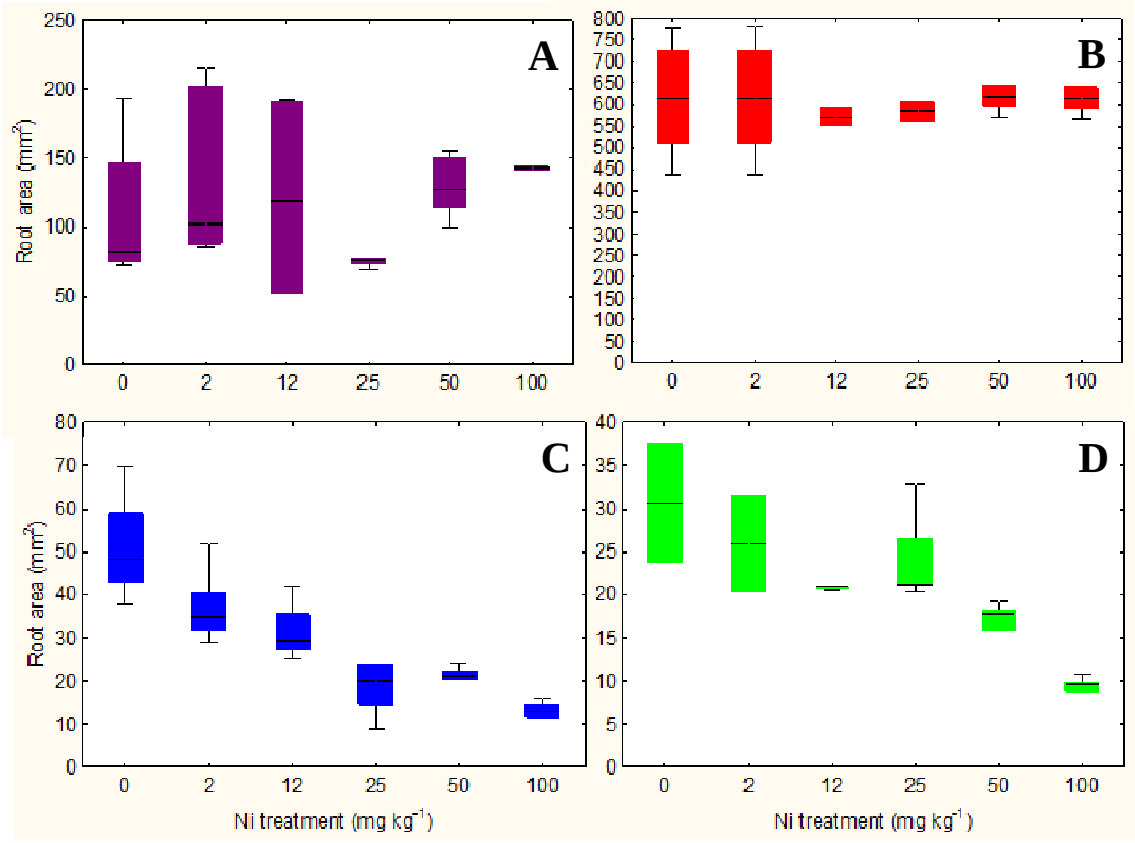

Figure 15. Box-and-whisker plots showing the root surface area (mm^2) of test hyperaccumulator species at increasing Ni concentrations (0-100 mg kg^{-1}): A) *A. utriculata*, B) *N. caerulescens* and non-hyperaccumulator species C) *A. montanum*, D) *T. arvense*. In each box, the central line marks the median of the data; the box edges represent the first and third quartiles; the whiskers show non-outlier range. N= 15 per treatment, each species.

Consistently with previous analysis, the root biomass of hyperaccumulator species do not seem to be affected by Ni, while a sharp decrease in dry biomass was observed at increasing Ni concentrations in *A. montanum* ($p< 0.01$) and *T. arvense* ($p< 0.001$) (Fig. 16).

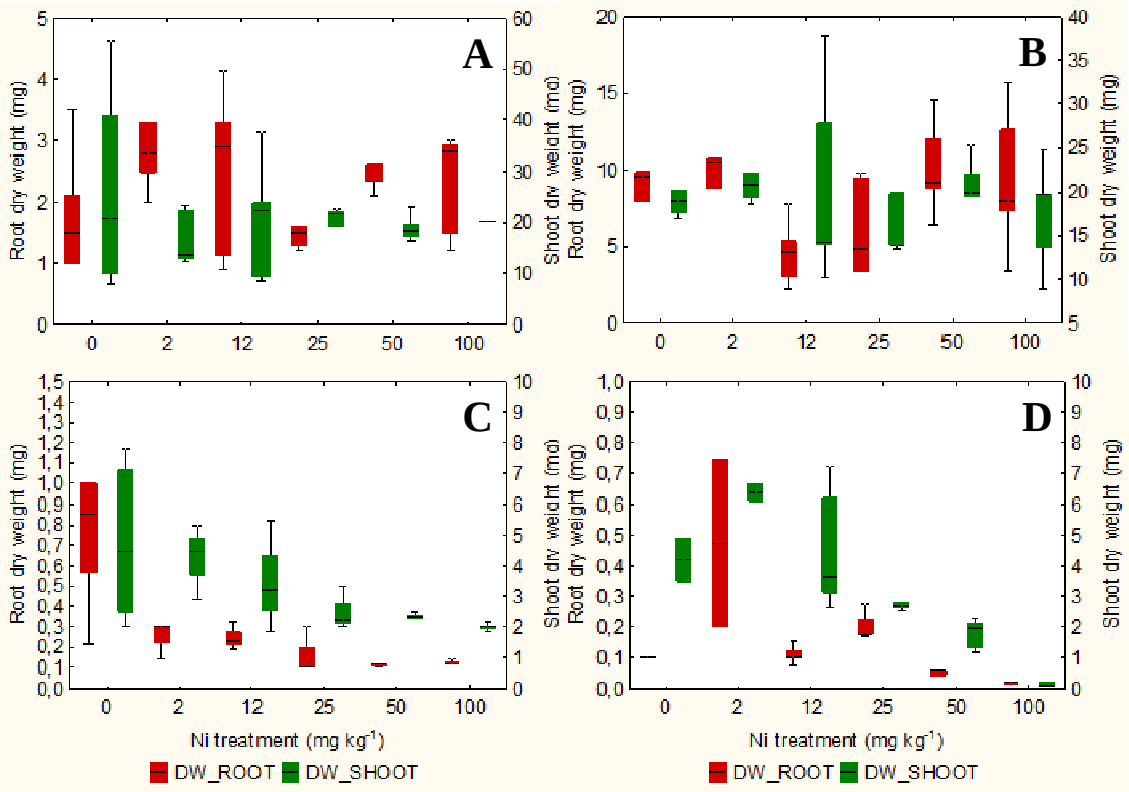

Figure 16. Box-and-whisker plots showing the root and shoot dry biomass (mg) of test hyperaccumulator species at increasing Ni concentrations (0-100 mg kg^{-1}): A) *A. utriculata*, B) *N. caerulescens* and non-hyperaccumulator species C) *A. montanum*, D) *T. arvense.* In each box, the central line marks the median of the data; the box edges represent the first and third quartiles; whiskers show non-outlier range. N= 15 per treatment, each species.

Table 2 reveals that the hyperaccumulator species are not affected by the Ni treatment, while the non-hyperaccumulator species show a significant negative correlation ($p < 0.001$) between Ni and the analyzed biological parameters.

Biological parameters	**Ni treatment**			
	***A.u.*++**	***N.c.*++**	***A.m.*+**	***T.a.*+**
Root surface area	NS	NS	-0.87***	-0.79***
FW_root	NS	NS	-0.58***	-0.59***
DW_root	NS	NS	-0.76***	-0.63***
FW_shoot	NS	NS	-0.86***	-0.87***
DW_shoot	NS	NS	-0.65***	-0.85***
R/S FW	NS	NS	NS	NS
R/S DW	NS	NS	NS	0.55**
% water R	NS	NS	NS	NS
% water S	NS	NS	0.72***	-0.85***

Table 2. Spearman's rank correlations coefficients between Ni concentration and biological parameters of test species. N= 15 per treatment , each species. *A.u.: A. utriculata, N.c.: N. caerulescens, A.m.: A. montanum, T.a.: T. arvense.*
++ hyperaccumulator species, + non-hyperaccumulator species
*p< 0.05, **p< 0.01, ***p< 0.001, NS Not Significant

4.2 Pot experiment

4.2.1 Evaluation of root area, biomass and plant water content

The red colour intensity of the leaves shown by the 1% DMG test enhances with increasing Ni concentration, suggesting a nickel accumulation in the leaf epidermis for both the hyperaccumulator species at 100 mg kg^{-1} of Ni concentration.

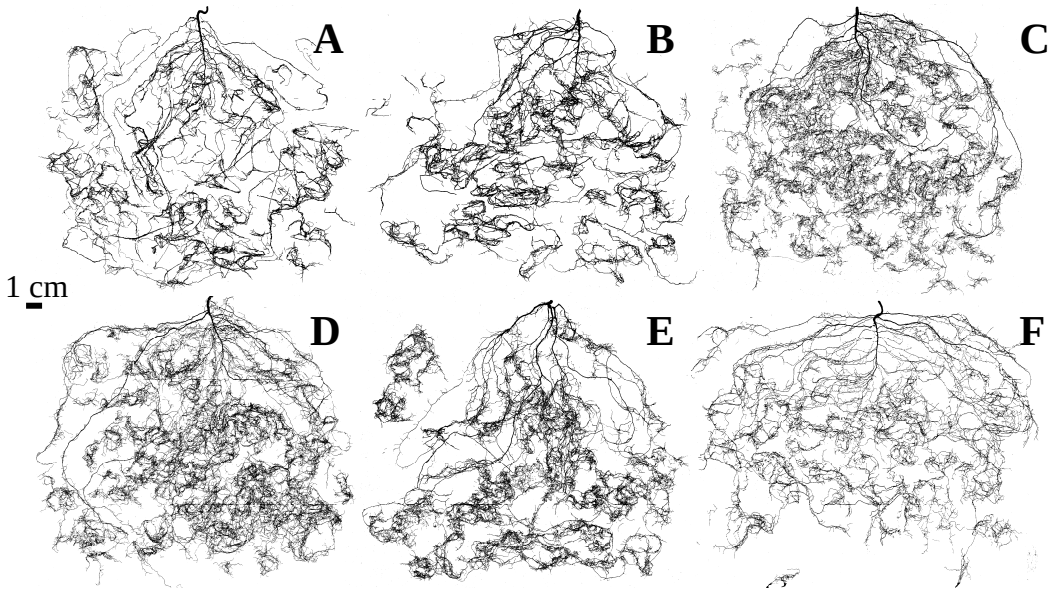

Figure 17. Root system of *A. utriculata* scanned and processed with ImageJ software at different concentrations of Ni (mg kg^{-1}); A) Ni 0, B) Ni 50, C) Ni 100, D) Ni 200, E) Ni 500, F) Ni 1000. N= 20 each treatment.

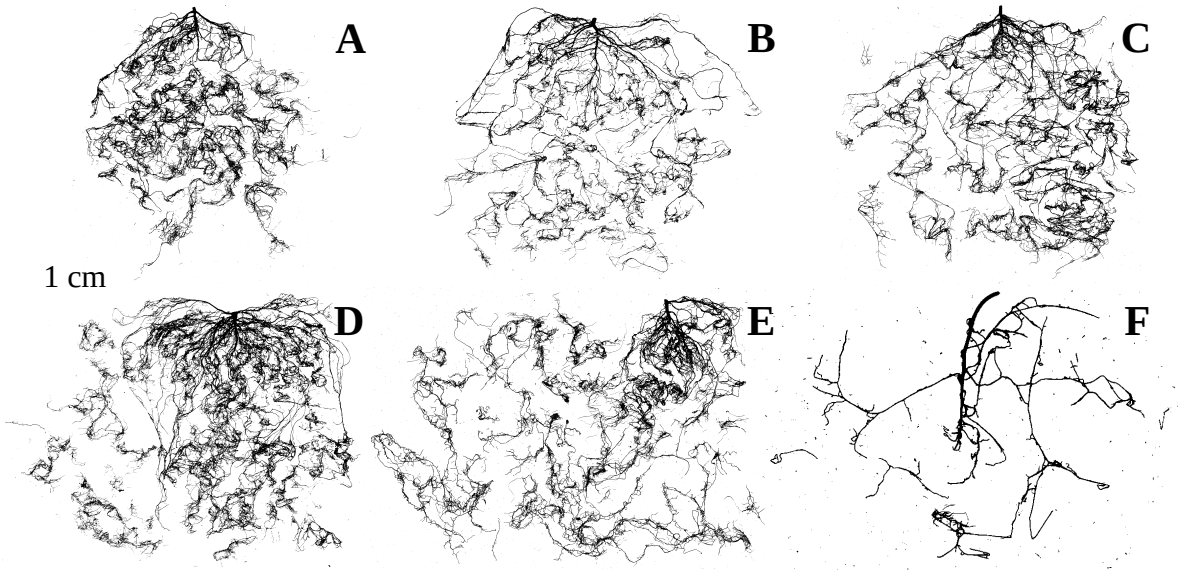

Figure 18. Root system of *N. caerulescens* scanned and processed with ImageJ software at different concentrations of Ni (mg kg^{-1}); A) Ni 0,B) Ni 50, C) Ni 100, D) Ni 200, E) Ni 500, F) Ni 1000. N= 20 each treatment.

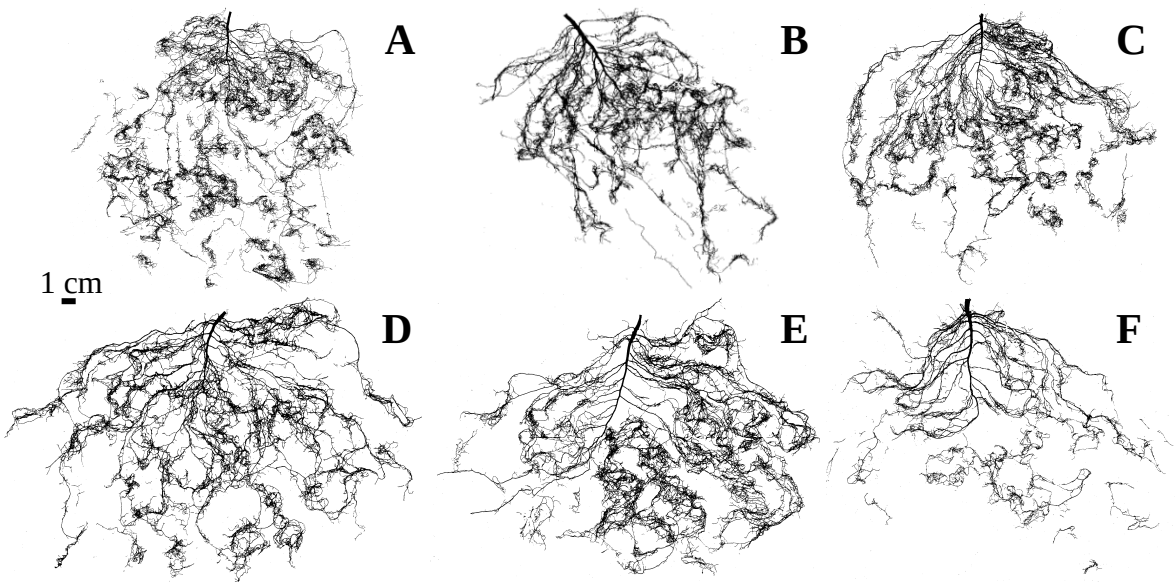

Figure 19. Root system of *A. montanum* scanned and processed with ImageJ software at different concentrations of Ni (mg kg-1); A) Ni 0, B) Ni 50, C) Ni 100, D) Ni 200, E) Ni 500, F) Ni 1000. N= 20 each treatment.

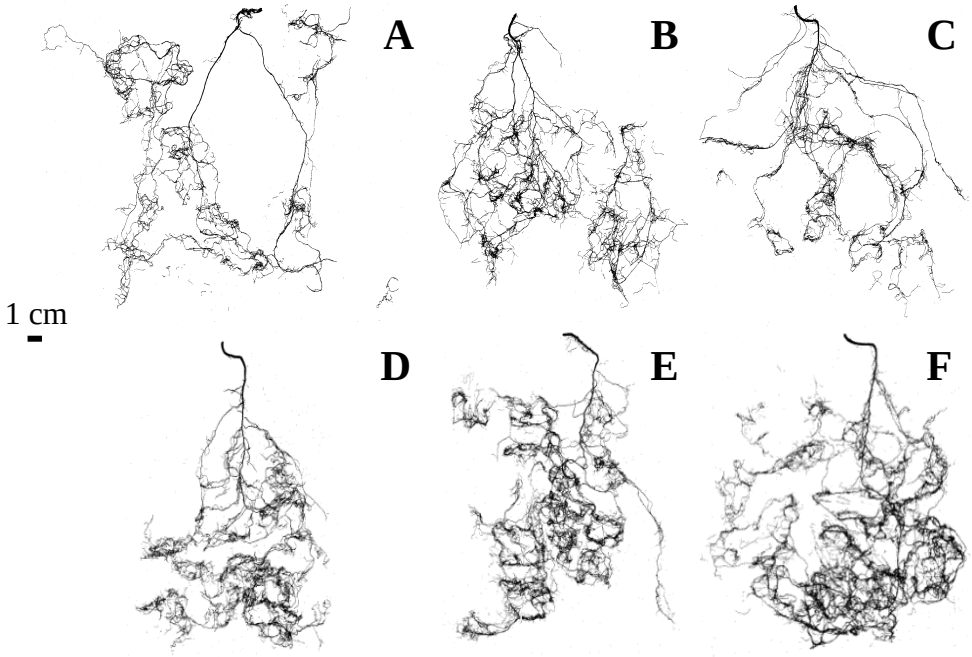

Figure 20. Root system of *T. arvense* scanned and processed with ImageJ software at different concentrations of Ni (mg kg^{-1}); A) Ni 0, B) Ni 50,C) Ni 100,D) Ni 200, E) Ni 500, F) Ni 1000. N= 20 each treatment.

The qualitative observation of the roots of the test species scanned with the ImageJ software does not clarify any relationship between the root surface area and the increasing concentrations of Ni from 0 mg kg^{-1} to 1000 mg kg^{-1} (Fig. 17-20) but the graph in Figure 21 shows the non-

hyperaccumulator *T. arvense* as the only species significantly affected by the presence of Ni, with an increase of the root surface area ($p < 0.01$).

The analysis in non-accumulator *T. arvense* (Fig. 21D, 22D) show an increase in terms of root surface area and root dry weight ($p < 0.01$) at increasing Ni concentrations, which is less evident for the aboveground organs ($p < 0.05$). Interesting to note the significant increase in root ($p < 0.01$) and shoots ($p < 0.01$) biomass of the facultative Ni-hyperaccumulator *A. utriculata* (Fig. 22A), although this is not supported by an increase of the root surface (Fig. 21A).

N. caerulescens and *A. montanum* do not exhibit significant differences in terms of root surface area and biomass, except for *N. caerelescens* at Ni1000 (Fig. 21B, C, Fig. 22B, C).

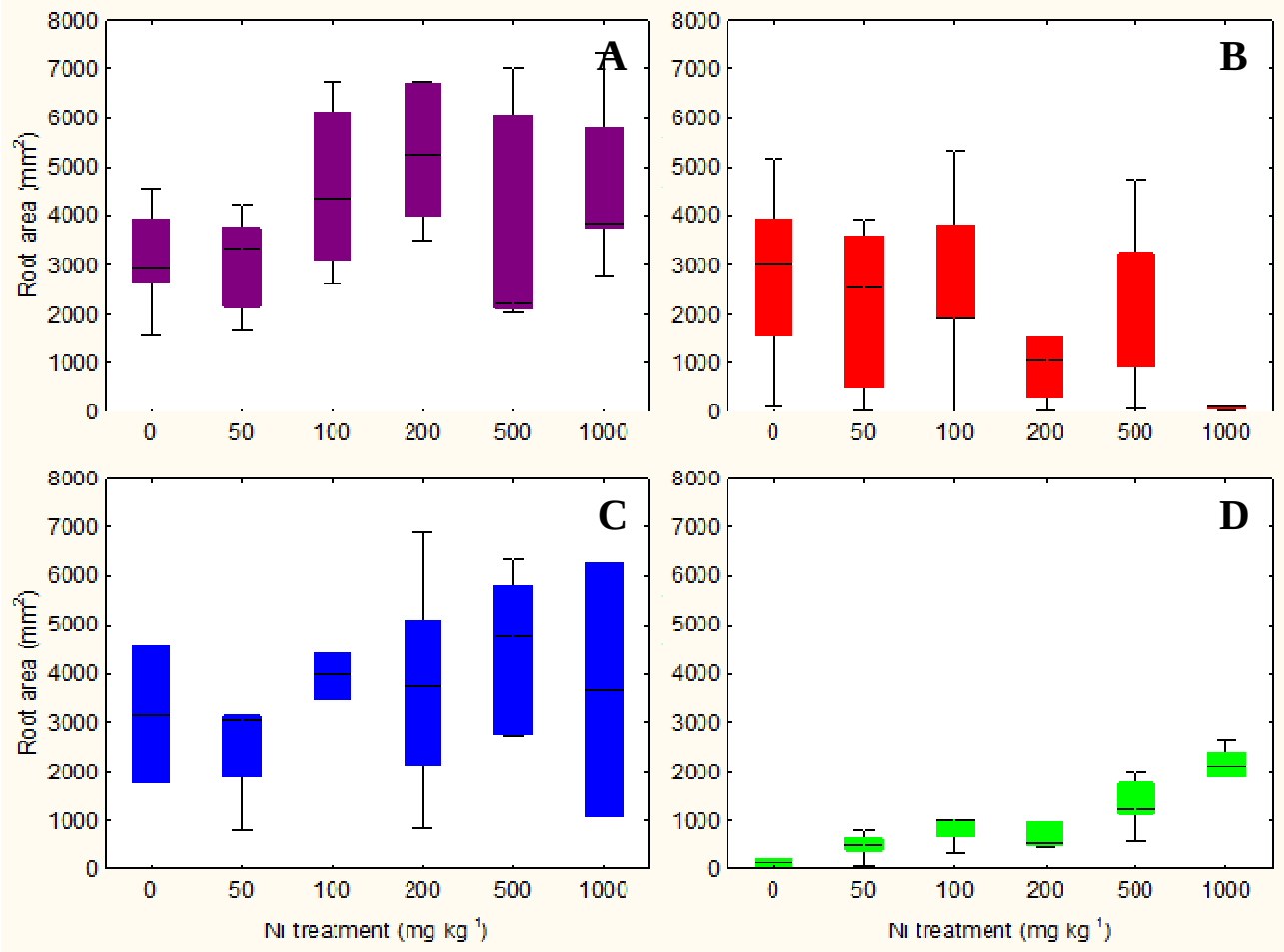

Figure 21. Box-and-whisker plots showing the root surface area (mm^2) of test hyperaccumulator species at increasing Ni concentrations (0-1000 mg kg^{-1}): A) *A. utriculata*, B) *N. caerulescens* and non-hyperaccumulator species C) *A. montanum*, D) *T. arvense*. In each box, the central line marks the median of the data; the box edges represent the first and third quartiles; whiskers show non-outlier range. N= 20 each treatment.

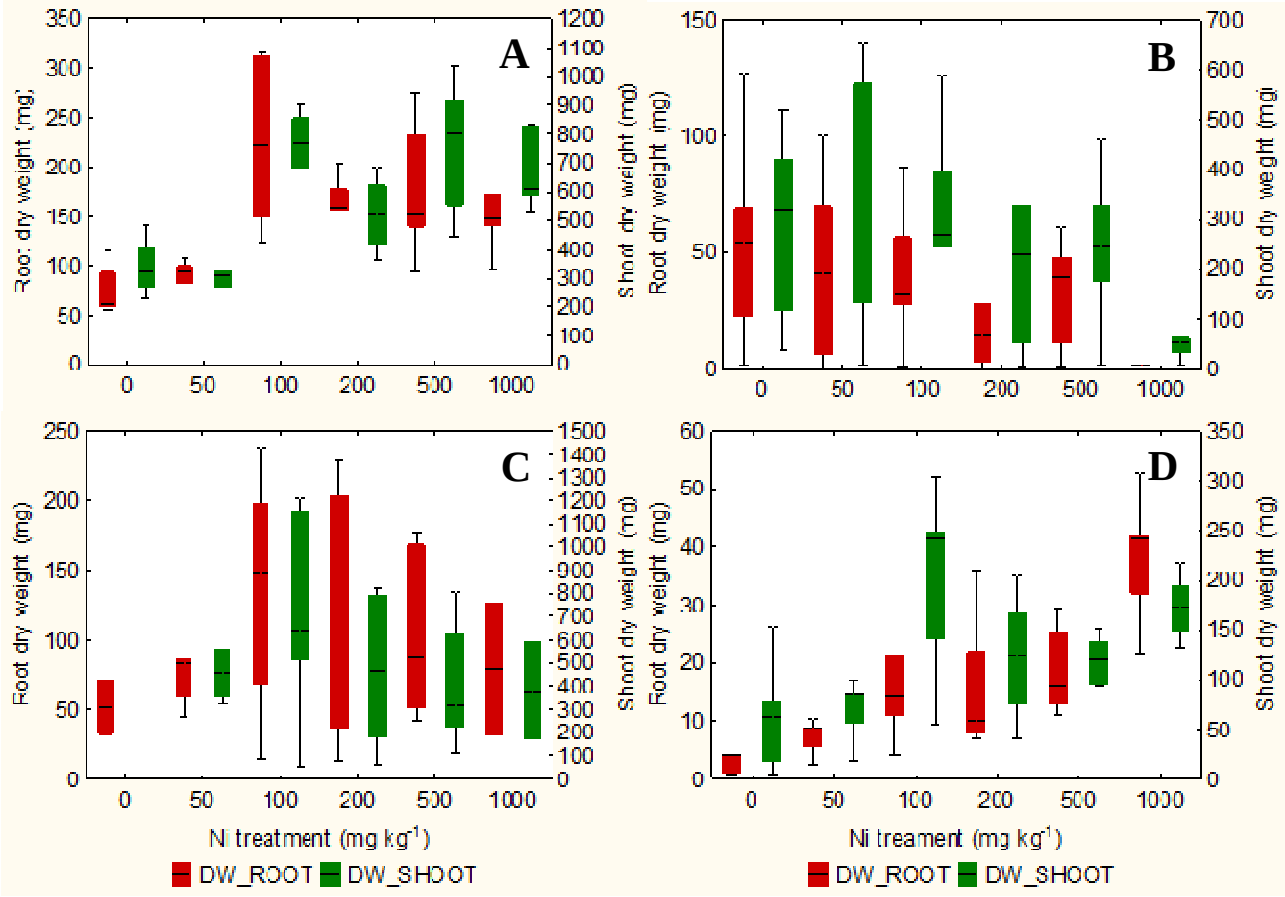

Figure 22. Box-and-whisker plots showing the root and shoot dry biomass (mg) of test hyperaccumulator species at increasing Ni concentrations (0-1000 mg kg^{-1}): A) *A. utriculata*, B) *N. caerulescens* and non-hyperaccumulator species C) *A. montanum*, D) *T. arvense*. In each box, the central line marks the median of the data; the box edges represent the first and third quartiles; whisker show non-outlier range. N= 20 each treatment.

In the facultative Ni-hyperaccumulator *A. utriculata*, the Ni concentration is positively correlated with fresh and dry shoot biomass ($p < 0.001$) and with the root dry biomass ($p < 0.01$). In *N. caerulescens* Ni seems to positively affects the root water content ($p < 0.01$) (Table 3).

Non-hyperaccumulator species behave differently: while in *A.montanum* the presence of Ni does not seem to affect the considered biological parameters, *T. arvense* shows a positive correlation between Ni and the root surface area and biomass, the root: shoot biomass ratio, the water content of the aerial organs ($p < 0.001$) and the shoot dry weight ($p < 0.01$) (Table 3).

Biological parameters	Ni treatment (mg kg^{-1})			
	***A.u.*++**	***N.c.*++**	***A.m.*+**	***T.a.*+**
Total Area	NS	NS	NS	0.74***
FW_root	NS	NS	NS	0.66***
DW_root	0.53**	NS	NS	0.78***
FW_shoot	0.61***	NS	NS	NS
DW_shoot	0.61***	NS	NS	0.48**
R/S FW	NS	-0.42*	NS	0.68***
R/S DW	NS	NS	NS	0.64***
% water R	NS	0.54**	NS	NS
% water S	NS	NS	NS	0.60***

Table 3. Spearman's rank correlations coefficients between Ni concentration and biological parameters of test species. N= 20 each treatment. *A.u.: A. utriculata, N.c.: N. caerulescens, A.m.: A. montanum, T.a.: T. arvense.*
++ hyperaccumulator species, + non-hyperaccumulator species
*p< 0.05, ** p< 0.01, ***p< 0.001, NS Not Significant

A clear difference in terms of Ni uptake between the hyperaccumulator species exists, as indicated in Table 4. At the maximum Ni concentration, the facultative hyperaccumulator *A. utriculata* accumulates ~1000 mg Ni kg^{-1} in the aboveground biomass, while *N. caerulescens* is able to accumulate seven times higher Ni concentration (6798 mg Ni kg^{-1}) compared to the other species.

In the non-hyperaccumulator species, the shoot metal uptake at Ni 1000 is significantly lower compared hyperaccumulators and the range varies between 100 and 200 mg kg^{-1}

Species	Ni treatment	Ni concentration (mg kg^{-1})
***A. utriculata* ++**	0	18.33±2
	1000	999.67±8
***N. caerulescens* ++**	0	76.67±3
	1000	6798±24.33
***A. montanum* +**	0	4±1.33
	1000	127.33±4
***T. arvense* +**	0	0
	1000	177±5

Table 4. XRF chemical analysis of Ni concentration measured on shoots of test species *A. utriculata, N. caerulescens, A. montanum* and *T. arvense*. N= 10 each species.
++ hyperaccumulator species, + non-hyperaccumulator species

4.2.2 Photosynthetic efficiency

The curves represented by the Figure 23 are plotted on a logarithmic axis to observe the fluorescence over time up to the maximum fluorescence value. On the other hand, spider (or radar)

plots (Fig. 24) provide a method of comparing the spread of individual parameter values for selected records within a data set and a visualisation of which parameters have greater sensitivity to certain type of stress.

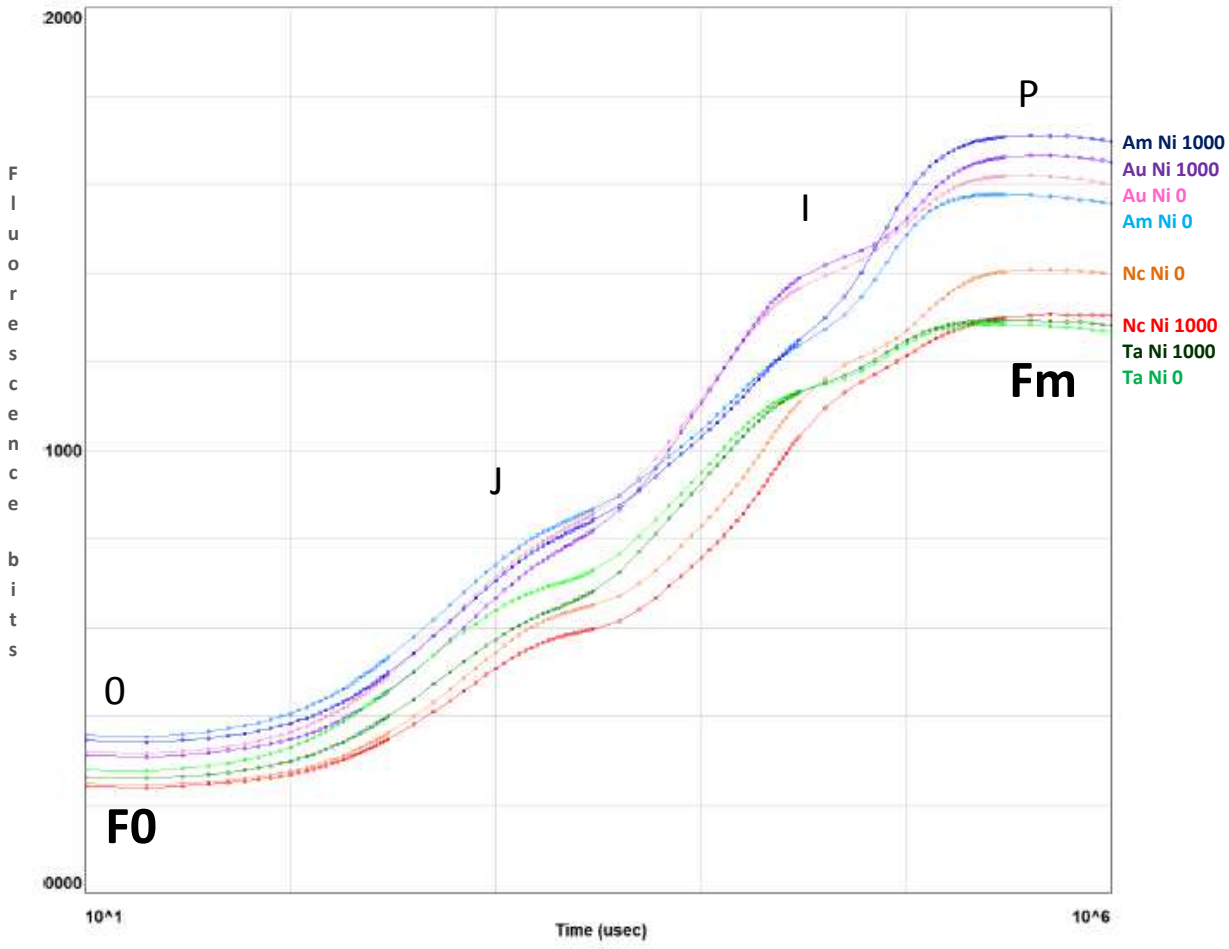

Figure 23. Fluorescence transient analysis of test species distinguished by colour of the curves (purple: *A. uticulata*, red: *N. caerulescens*, blue: *A. montanum*, green: *T. arvense*) at Ni 0 mg kg^{-1} (light color) and Ni 1000 mg kg^{-1} (dark color). The peaks are denoted by letters 0, J, I, P and correspond to fluorescence values measured at 50 ms (F0, step 0), 2 ms (step J), 30 ms (step I), and maximal (Fm, step P). Data are the mean of ten measurements per plant. N= 100 each species.

Figure 23 summarizes the transient fluorescence analysis for controls and treatments at Ni 1000 mg kg^{-1}. The curves represent the average values of samples measured for the indicated species at Ni 0 (light colour) and Ni 1000 mg kg^{-1} (dark colour). The peaks represent the fluorescence values measured at 50 µs (0), 2 ms (J), 30 ms (I) and maximal (P) (Strasser et al., 2004).

Through the evaluation of the Fv/Fm ratio, the Photosystem II efficiency was assessed for the test species. Instead, P.I. is an index of the vitality of the sample: it expresses the potential capacity for energy conservation.

Only *N. caerulescens* shows a greater Fm in the control than at the maximum Ni concentration, while *A. utriculata* and *A. montanum* exhibit the highest values of Fm.

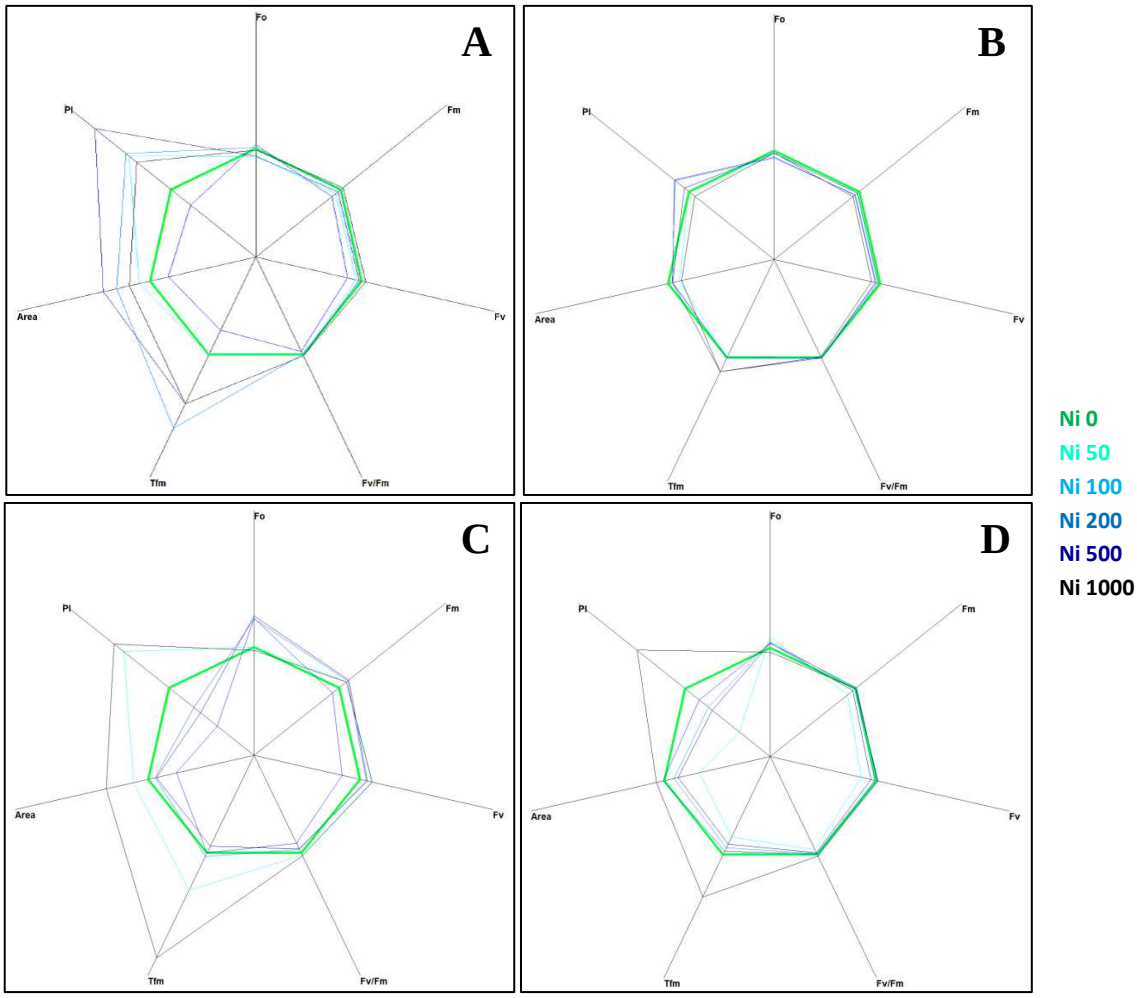

Figure 24. Fluorescence transient analysis of test species represented by spider plots: A) *A. uticulata*, B) *N. caerulescens*, C) *A. montanum*, D) *T. arvense*. Increasing concentrations of nickel correspond to darker shades of blue; green identifies the control. Data are standardized for Ni 0 mg kg^{-1}. Data are the mean of ten measurements per plant. N= 300 each species.

Figure 24 represents a set of spider plots showing the differences between the test species at increasing Ni concentration in terms of recorded parameters.

Each axis of the plot corresponded to one of the seven parameters (F0, Fv, Fm, Fv/Fm, Tfm, Area and P.I.) and the average value of each parameter for the control species was used as the comparative value.

In *A. utriculata* (Fig. 24A), Tfm, Area and P.I. increase in treatment ($p < 0.001$, $p < 0.001$ and $p < 0.05$ respectively). The parameters do not seem to differ considerably in *N. caerulescens*, while in non-hyperaccumulators there is a reduction in the P.I. except for Ni 50 mg kg^{-1} (*A. montanum*, Fig. 24C) and Ni 1000 mg kg^{-1} (both species, Fig. 24C, D). Clear differences are observed between the Ni treatments in non-accumulators. In particular, Tfm and P.I. reach the maximum value at Ni 1000 mg kg^{-1}.

Data summarized in Table 5 show that in *A. utriculata*, Performance Index (P.I., $p < 0.05$) and the time to reaching Fm (Tfm, $p < 0.001$) increase at increasing Ni concentrations. Similarly, the latter enhances ($p < 0.001$) in *N. caerulescens*, although the maximum fluorescence (Fm) decrease ($p < 0.01$), and there are no significant difference in terms of P.I.. In *A. montanum* a positive correlation between dark level fluorescence (F0) and Ni ($p < 0.001$) and between the latter and Fm ($p < 0.05$) is observed. On the other hand, in the same conditions, *T. arvense* exhibits a sensible reduction of F0 ($p < 0.01$), but a highly significant positive correlation ($p < 0.001$) between Ni and the maximum efficiency of PSII (Fv/Fm), the P.I. and the Tfm.

Parameters	**Ni treatment**			
	***A.u.*++**	***N.c.*++**	***A.m.*+**	***T.a*+**
F0	NS	NS	0.25***	-0.16**
Fm	NS	-0.17**	0.15*	NS
Fv/Fm	NS	NS	-0.22**	0.24***
Tfm (ms)	0.38***	0.37***	NS	0.25***
Area	0.30***	NS	NS	0.19***
P.I.	0.13*	NS	-0.21**	0.31***

Table 5. Spearman's rank correlations coefficients between Ni concentration and fluorescence parameters of test species (F0: dark level fluorescence, Fm: maximum fluorescence, Fv/Fm: maximum efficiency of PSII, Tfm: time (ms) to reaching Fm, P.I. Performance Index). N= 300 each species. *A.u.: A. utriculata, N.c.: N. caerulescens, A.m.: A. montanum, T.a.: T. arvense.*
++ hyperaccumulator species, + non-hyperaccumulator species.
*p< 0.05, **p< 0.01, *** p< 0.001, NS Not Significant

4.3 Characterization of culturable rhizobiota

4.3.1 Plant and soil sample analysis

Total nickel concentration in soils (Table 6) is significantly high on S site respect to NS site (Ni_S> 1000 mg kg^{-1}; Ni_{NS} ~20 mg kg^{-1}, $p< 0.01$), both in R and in B soil. On S site *A. utriculata* hyperaccumulates Ni as expected (Ni_{roots}~ 200 mg kg^{-1} Ni_{shoots}~ 1200 mg kg^{-1}, Table 7). Among other soil elements, only Ca has a concentration significantly high in the NS site compared with S site ($p< 0.01$, Table 6).

Site	Soil type	Element concentration (mg kg^{-1})				
		Ni	Ca	Mg	Cu	Zn
S	**R**	1491.48±624.09	3249.30±645.54	9032.93±4878.95	85.37±42.90	76,57±25.69
S	**B**	1046.65±404.77	4267.50±922.94	11957.34±1876.38	88.64±40.57	84.42±19.81
NS	**R**	19.52±0.94	9288.04±66.76	7050.32±42.44	21.48±3.35	53.22±0.92
NS	**B**	19.46±1.14	9336.06±54.08	7095.98±88.31	20.46±1.66	52.90±1.99

Table 6. Bulk element concentration in soil samples (mg kg^{-1}). S: Serpentine site, NS: Non-Serpentine site, R: Rhizosphere soil, B: Bare soil. Data are mean±SD. N= 5 each site, each soil type.

Site	Plant sample	Element concentration (mg kg^{-1})				
		Ni	Ca	Mg	Cu	Zn
S	**Shoot**	1241.37±245.67	10142.29±1942.62	16342.84±2502.94	14.06±2.30	45.88±9.25
S	**Root**	203.04±49.75	2886.33±372.81	2374.12±228.19	16.56±11.89	28.46±8.11
NS	**Shoot**	10.64±0.45	9220.26±1766.01	21258.69±135.74	7.10±0.55	21.24±0.36
NS	**Root**	5.95±0.25	1317.18±252.29	2024.64±12.93	16.32±1.26	14.28±0.36

Table 7. Bulk element concentration in shoots and roots of the facultative nickel-hyperaccumulator *A. utriculata* harvested from Serpentine (S) and Non-Serpentine (NS) sites. Dara are mean±SD. N= 5 each site, each plant sample.

Kolmogorov-Smirnov two-sample test reveals marked differences between S and NS sites examined ($p< 0.05$) as regards Ni, Cu, Zn, Ca and Mg ($p< 0.05$).

In the S site, the bioaccumulation factor ($BF= C_{shoot}/C_{soil}$) of *A. utriculata* is approximately 1 (0.8), while the Translocation Factor ($TF= C_{shoot}/C_{root}$) is greater than 6 while in the NS site BF is ~0.5 and TF is approximately 2 (1.8).

4.3.2 Isolation and identification of culturable bacteria

Bacterial counts from NS site are higher than that from S site in terms of bacterial strain diversity, without a clear Ni selectivity among Ni 0, 1 and 5 mM except for the black halo around some colonies isolated from rhizospheric soil that is clearly visible in the presence of Ni. In the R soil the bacterial colonies from S and NS site are significantly more abundant than B soils (Fig. 25) at all Ni concentrations (0, 1, 5 mM, $p < 0.05$).

Among all the isolated bacteria, 30 morphotypes are counted and identified with a morphological approach followed by a molecular identification. Among the isolates, the most frequent strain is *Arthrobacter* which accounts for about 27% of average bacterial count of the rhizospheric S site (SR). Besides, there is a prevalence of *Enterobacteriaceae* which accounts for 18% of the total bacterial colonies isolated at Ni 0. Among others isolates, the most recurrent are *Bacillus*, *Erwinia*, and *Streptomyces*. The bacterial count indicates that *Novosphingobium*, *Pantoea*, and *Stenotrophomonas* are present in the SR and NSR soil, while the metallophilic *Pseudomonas* thrives only in SR soil (Table 8).

There are no significant correlations between the total culturable bacterial count isolated at Ni 0 mM (CFU g^{-1}) and the bulk element concentration in native soils (mg kg^{-1} DW), except for the significant positive correlation with Zn ($r = 0.90$, $p < 0.05$) in the SR soil.

Kolmogorov-Smirnov two-sample test reveals marked differences between S and NS sites examined ($p < 0.05$) in terms of culturable bacteria ($p < 0.05$). Similarly, bacterial count differ significantly between R and B substrates ($p < 0.05$).

Strain name	Microbiota type	NCBI accession number	Taxonomy rank	Most closely related genus/species (sequence similarity)	Soil compartment
SERP1	Bacterium	MG661811	Genus	*Pseudomonas* sp. (99%)	SR
SERP2	Bacterium	MG661822	Genus	*Stenotrophomonas* sp. (99%)	SR, NSR
SERP3	Bacterium	MG661833	Genus	*Streptomyces* sp. (99%)	SR
SERP4	Bacterium	MG661835	Genus	*Streaptomyces* sp. (99%)	SR, SB, NSR, NSB
SERP5	Bacterium	MG661836	Family	Enterobacteriaceae	SR
SERP6	Bacterium	MG661837	Genus	*Bacillus* sp. (99%)	SR, SB, NSR, NSB
SERP7	Bacterium	MG661838	Genus	*Bacillus* sp. (99%)	SR, SB
SERP8	Bacterium	MG661839	Genus	*Bacillus* sp. (99%)	SR, SB, NSR, NSB
SERP9	Bacterium	MG661840	Genus	*Bacillus* sp. (99%)	SB
SERP10	Bacterium	MG661812	Genus	*Bacillus* sp. (99%)	SB, NSR, NSB
SERP11	Bacterium	MG661813	Genus	*Arthrobacter* sp. (99%)	SR, SB, NSR, NSB
SERP12	Bacterium	MG661814	Genus	*Bacillus* sp. (99%)	SR, SB, NSR, NSB
SERP13	Bacterium	MG661815	Genus	*Chryseobacterium* sp (99%)	NSR
SERP14	Bacterium	MG661816	Genus	*Pantoea* sp. (99%)	SR, NSR
SERP15	Bacterium	MG661817	Genus	*Micrococcus* sp. (99%)	NSB
SERP16	Bacterium	MG661818	Genus	*Bacillus* sp. (99%)	NSB
SERP17	Bacterium	MG661819	Genus	*Streptomyces* sp. (99%)	SR, SB, NSR, NSB
SERP18	Bacterium	MG661820	Family	Enterobacteriaceae	SB, NSR
SERP19	Bacterium	MG661821	Genus	*Erwinia* sp. (99%)	SR, SB, NSR, NSB
SERP20	Bacterium	MG661823	Genus	*Flavobacterium* sp. (98%)	NSR
SERP21	Bacterium	MG661824	Genus	*Novosphingobium* sp. (100%)	SR, SB, NSR
SERP22	Bacterium	MG661825	Genus	*Curtobacterium* sp. (99%)	NSR
SERP23	Bacterium	MG661826	Genus	*Streptomyces* sp. (99%)	SR, SB, NSR, NSB
SERP24	Bacterium	MG661827	Genus	*Leucobacter* sp. (99%)	NSR
SERP25	Bacterium	MG661828	Genus	*Pantoea* sp. (100%)	SR, NSR
SERP26	Bacterium	MG661829	Genus	*Micrococcus* sp. (100%)	SR, SB, NSR, NSB
SERP27	Bacterium	MG661830	Genus	*Cronobacter* sp. (99%)	NSR
SERP28	Bacterium	MG661831	Genus	*Stenotrophomonas* sp. (99%)	NSR
SERP29	Bacterium	MG661832	Genus	*Sphingobacterium* sp. (100%)	NSR
SERP30	Bacterium	MG661834	Genus	*Massilia* sp. (100%)	NSR
Serp01S	Fungus	MG836709	Species	*Cladosporium cladosporioides* (100 %)	SR, SB
Serp03S	Fungus	MG850978	Species	*Penicillium ochrochloron* (99 %)	SR, SB
Serp04S	Fungus	MG850979	Species	*Penicillium canescens* (100 %)	SR, NSR
Serp05S	Fungus	MG836710	Species	*Trichoderma harzianum* (99 %)	SR, SB, NSB
Serp06S	Fungus	MG850980	Species	*Aspergillus niger* (100 %)	SB, NSB
Serp08S	Fungus	MG850981	Species	*Penicillium lanosum* (98%)	NSR
Serp11S	Fungus	MG850982	Species	*Penicillium atramentosum* (99 %)	NSR, NSB
Serp13S	Fungus	MG850983	Species	*Penicillium canescens* (99 %)	SR, NSR

Table 8. The diversity (strain name, accession number, taxon) of isolated culturable bacteria and fungi from the rhizosphere (R, N= 10) of *A. utriculata* growing on Serpentine and Non Serpentine soil and from the adjacent bare soil (B, N= 10).

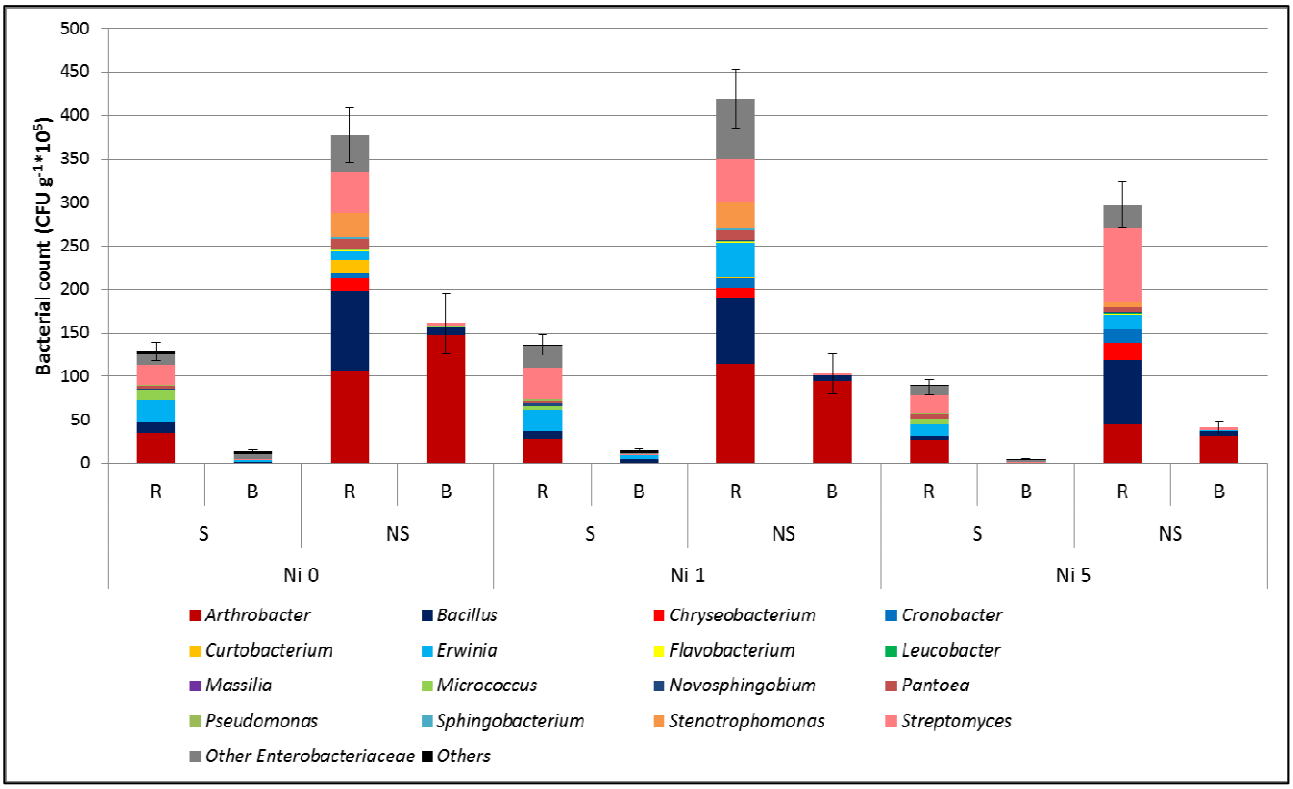

Figure 25. Average bacterial count expressed as Colony Forming Unit (CFU g^{-1}) and diversity (genus, comprising different strains) in the rhizospheric (R) soil of facultative Ni-hyperaccumulator *A. utriculata* and adjacent bare (B) soil on serpentine (S) and non-serpentine (NS) site. Data are mean±SD. N= 180 each site.

4.3.3 PGP traits and Ni-tolerant bacteria

Among the serpentine bacterial isolates, 8 strains belonging to 5 genera show to possess more than one PGPR activity (Table 9): *Pseudomonas*, *Stenotrophomonas*, *Streptomyces*, *Pantoea* and *Erwinia*. Specifically, strain SERP14 and SERP25 (*Pantoea* sp.) have all four traits and they exhibit high production of IAA at 530 nm of absorbance.

SERP1 (*Pseudomonas* sp.), SERP4 (*Streptomyces* sp.) and SERP19 (*Erwinia* sp.) show a great synthesis of siderophores as well as *Pantoea* previously cited, while SERP3, SERP4 and SERP23 (*Streptomyces* sp.) can solubilize phosphate as SERP19 (*Erwinia* sp.) and genus *Pantoea* and to grow on ACC as the sole source of N.

All the bacterial colonies considered highlight a metal-tolerance at low Ni concentrations (up to 5 mM of Ni, Table 10). Half of these isolates tolerate concentrations up to 10 mM of Ni and only SERP01 (*Pseudomonas* sp.) and SERP04 (*Streptomyces* sp.) can be cultivated on Ni 15 mM.

Strain name	Genus	ACC deaminase	IAA synthesis	Siderophores (halo Ø, mm)	Phosphate solubilization (halo Ø, mm)
SERP1	*Pseudomonas* sp.	-	+	+ (90)	+ (5)
SERP2	*Stenotrophomonas* sp.	-	+	+ (90)	-
SERP3	*Streptomyces* sp.	+	-	-	+ (3)
SERP4	*Streptomyces* sp.	+	-	+ (12)	+ (3)
SERP6	*Bacillus* sp.	-	-	-	-
SERP7	*Bacillus* sp.	-	-	+ (15)	-
SERP8	*Bacillus* sp.	-	-	-	-
SERP11	*Arthrobacter* sp.	-	-	+ (45)	-
SERP12	*Bacillus* sp.	-	-	-	-
SERP14	*Pantoea* sp.	+	+	+ (90)	+ (4)
SERP19	*Erwinia* sp.	-	-	+ (90)	+ (5)
SERP21	*Novosphingobium* sp.	-	-	-	+ (2)
SERP23	*Streptomyces* sp.	+	-	-	+ (2)
SERP25	*Pantoea* sp.	+	+	+ (90)	+ (15)
SERP26	*Micrococcus* sp.	-	-	-	-

Table 9. Plant growth promoting traits of culturable bacteria isolated from the rhizosphere of *A. utriculata* on Serpentine soil (S), N= 8 each strain. The absorbance was recorded at 530 nm. Growth: - absence; + presence.

Strain name	Genus	Ni 0	Ni 1	Ni 5	Ni 10	Ni 15	Ni 20
SERP1	*Pseudomonas* sp.	+	+	+	+	+	-
SERP2	*Stenotrophomonas* sp.	+	+	+	+	-	-
SERP3	*Streptomyces* sp.	+	+	+	+	-	-
SERP4	*Streptomyces* sp.	+	+	+	+	+	-
SERP14	*Pantoea* sp.	+	+	+	-	-	-
SERP19	*Erwinia* sp.	+	+	+	-	-	-
SERP23	*Streptomyces* sp.	+	+	+	-	-	-
SERP25	*Pantoea* sp.	+	+	+	-	-	-

Table 10. Nickel tolerance (mM) of culturable PGPR isolated from the rhizosphere of *A. utriculata* growing on the Serpentine soil (S), N= 15 each strain. Growth: - absence; + presence.

4.3.4 Isolation and identification of culturable fungi

Figure 26 summarizes the fungal presence in S and NS sites both in R and B soils. Fungi preferably colonize NSR soil respect to SR. The most recurrent genera are *Aspergillus*, *Penicillium*, *Cladosporium*, and *Trichoderma* (Table 8).

Genus *Penicillium* is the most abundant, comprising the 84% of total colonies. Although genus *Cladosporium* has a low frequency (2% of the total), it was exclusively isolated from S soils. In particular, *Penicillium canescens* Sopp represented the most abundant species (36% of total colonies).

There are no correlations between the presence of microfungi (CFU g^{-1}) and the bulk element concentration in native soils (mg kg^{-1} DW).

As for bacteria, Kolmogorov-Smirnov two-sample test exhibits clear differences between R and B substrates in terms of culturable fungal abundance ($p< 0.05$). Moreover, the fungi are positively correlated with Ni_{root} ($p< 0.05$).

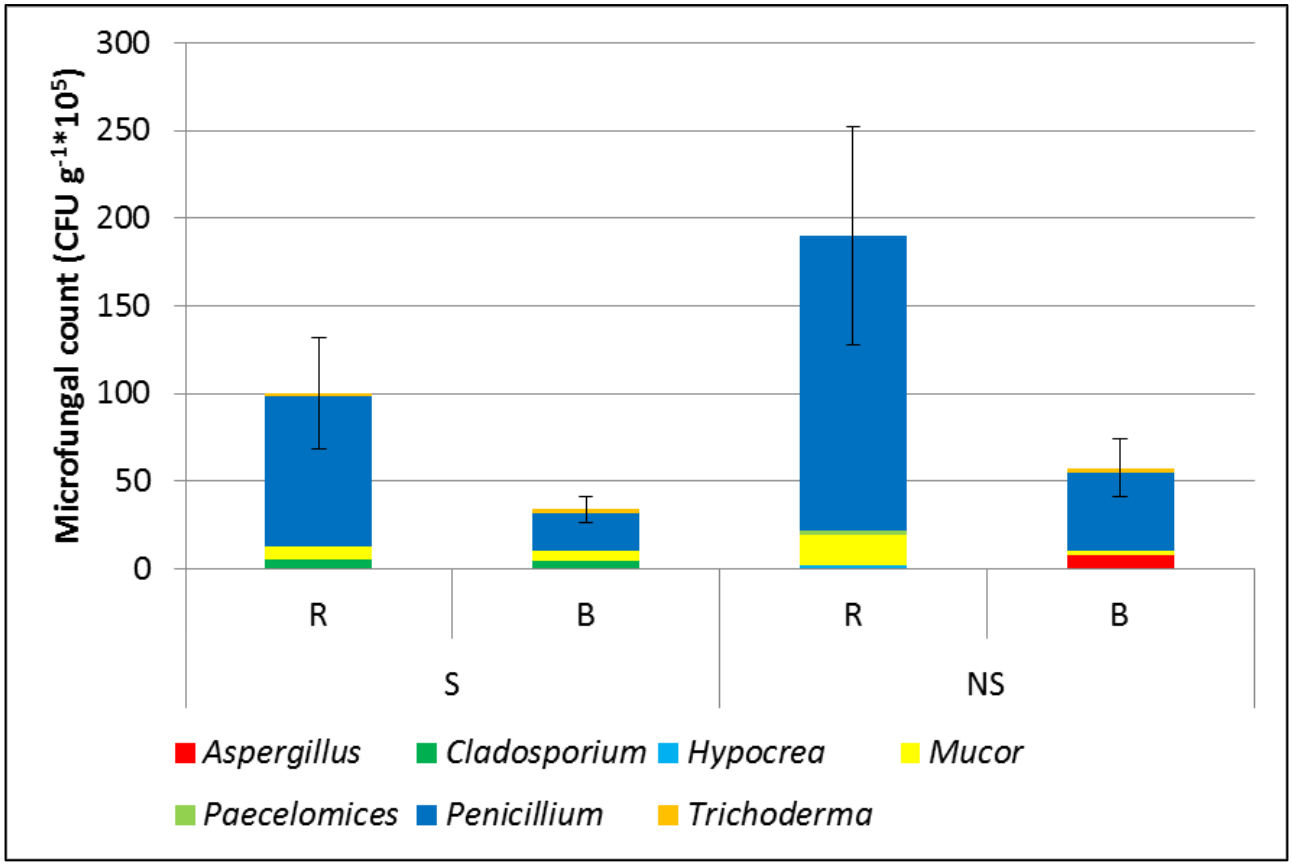

Figure 26. Microfungal count expressed as Colony Forming Unit (CFU g^{-1}) and diversity (genus, comprising different strains) in the rhizospheric (R) soil of facultative Ni-hyperaccumulator *A. utriculata* and adjacent bare (B) soil on serpentine (S) and non-serpentine (NS) site. Data are mean±SD. N= 40 each site.

4.3.5 Ni-tolerant fungi

Among the isolated S fungal strains, *Cladosporium cladosporoides* (Fresen.) G.A. de Vries does not grow in the presence of Ni, *Trichoderma harzianum* and *Penicillium canescens* tolerate up to 5 mM of $NiSO_4*6H_2O$, while *Penicillium ochrochloron* is able to tolerate up to 20 mM (Table 11).

Strain name	Species	Ni 0	Ni 1	Ni 5	Ni 10	Ni 15	Ni 20
Serp01S	*Cladosporium cladosporoides*	+	-	-	-	-	-
Serp03S	*Penicillium ochrochloron*	+	+	+	+	+	+
Serp04S	*Penicillium canescens*	+	+	+	-	-	-
Serp05S	*Trichoderma harzianum*	+	+	+	-	-	-

Table 11. Nickel tolerance (mM) of culturable fungi isolated from the rhizosphere of *A. utriculata* growing on the Serpentine soil (S), N= 15 each strain. Growth: - absence; + presence.

4.3.6 Plant-soil-microbiota relationship

S and NS samples constitute two fairly well-defined cluster, highlighting a good homogeneity of the starting samples (Fig. 27A). The Loadings plot in Figure 27B show the samples data distribution (element concentration in plant and soil and microbiota count) on the first two principal components.

Despite the less uniform pattern, most of the elements in soil, root and shoot (especially Ni) is associated with negative scores of PC1 (Fig. 28A, B). Both Loading plots presented explain a total variance of 75.02%.

It is noteworthy that Ca is the only soil element associated with positive scores on PC1; indeed, S soil samples are characterized by scarcity of nutrients such as Ca and presence of phytotoxic elements such as Ni. Differently, the separation of the bacterial and fungal strains is less evident, but at least four mixed groups of bacteria and fungi are present without specific interactions with analysed elements except the genus *Bacillus* which appears (as *Chryseobacterium* sp.) negatively correlated with Ni_{soil} ($p< 0.05$) and Cu_{soil} ($p< 0.01$) and positively correlated with Ca_{soil} ($p< 0.001$).

Among the microorganisms selected by means PGPR tests and the Ni tolerance test, there is a highly significant positive correlation between *Stenotrophomonas* sp. and *Streptomyces* sp. ($p< 0.01$), between these two bacterial strains and the fungus *P. canescens* ($p< 0.001$ and $p< 0.01$ respectively) and between the two fungal strain *P. ochrochloron* and *T. harzianum* group ($p< 0.05$). As opposed to genus *Bacillus, C. cladosporoides* is negatively correlated with Ca (and Mg) concentration in the soil ($p< 0.01$ for both) and positively with Cu_{soil} ($p< 0.05$).

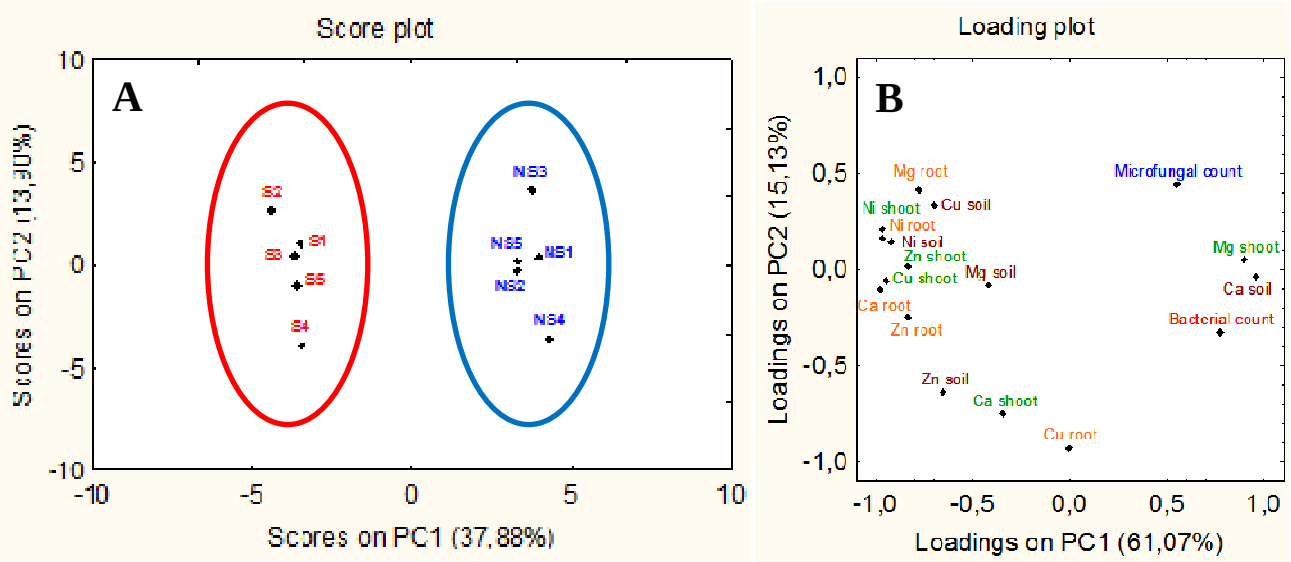

Figure 27. Score plot and Loading plot of a Principal Component Analysis. A) Score plot of 10 R soil samples distributed in the two sites (red: S site; blue NS site); B) loading plot of microbiota and element concentration data in R soil. Samples are indicated by different colour (brown: element concentration in soil, orange: element concentration in roots, green: element concentration in shoots, red: bacterial count, blue: microfungal count). Variables are indicated by symbol ♦.

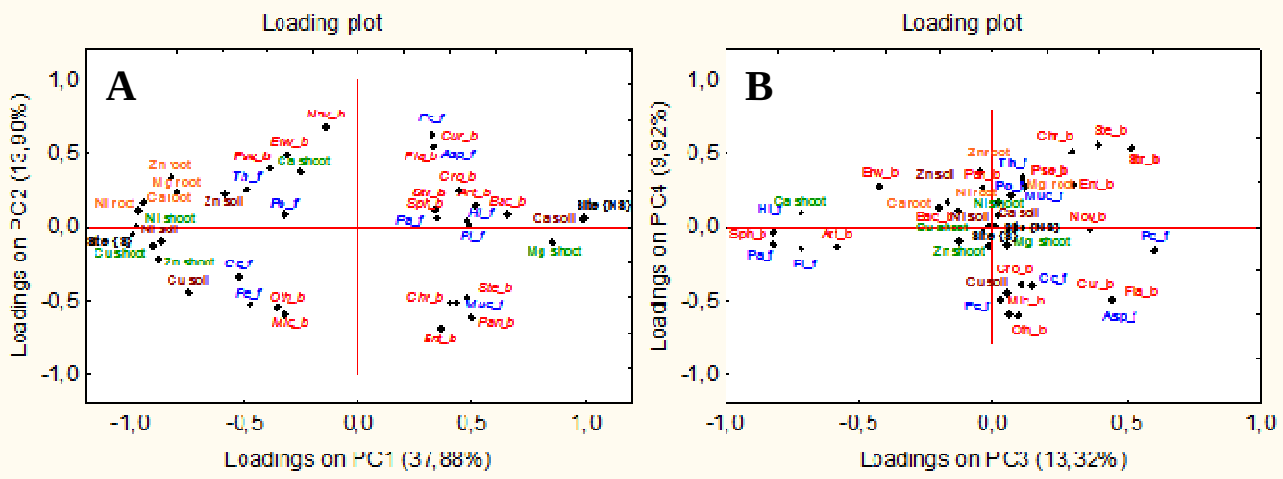

Figure 28. Loadings plot of a Principal Component Analysis including the abundance of bacterial and fungal taxa and element concentration in plant and soil data obtained from R soil. A) Factor 1 (37.88% variation explained) vs Factor 2 (13.90% variation explained); B) Factor 3 (13,32% variation explained) vs Factor 4 (9,92% variation explained). In bold S and NS sites; the samples are distinguished by different colours depending on the origin, brown: soil, orange: root, green: shoot, red: bacteria, blue: fungi. Variables are indicated by symbol ♦.

4.4 Co-growth of bacteria and fungi

4.4.1 Preliminary test

At the end of the preliminary tests, CYA and TSA buffered at pH 6.0 proved to be the most suitable agar substrates for the growth of bacterial and fungal strains.

The calculation of the growth curves associated with plate culture for the eight serpentinitic bacteria allowed to evaluate the time lapse useful to reach the exponential phase of the bacterial growth.

Among the isolated selected for the co-growth experiment, fungal strains exhibited increased growth on the CYA substrate compared to TSA pH 6.0 as noted in the Figure 29C, D, whereas bacteria thrive on both agar culture media.

The species belonging to the genus *Pseudomonas* are Gram negative bacilli, straight or slightly curved and their size range from 1.5 to 5 µm x 0.5-1 µm approximately. They are motile due to the presence of one or more polar flagella and they are obligatory aerobes, mainly oxidase and catalase positive; usually the colonies are colourless, although pigmentations of white and white-dirty colour are frequent (Fig. 30A, Henry et al. 2011).

Streptomyces are a group of non-motile, Gram-positive bacteria characterized by a slow multiplication by fragmentation similar to filamentous mycetes but are considered bacteria because of their prokaryotic cell structure and small size (diameter rarely higher than 1 µm, Bergey et al., 2001). In *Streptomyces* sp. SERP4, as shown in Figure 30B, the macroscopic mycelium morphology includes pellets and clump formation that are correlated with secondary metabolite production (Manteca and Yagüe, 2018).

Regarding microfungi, the colony of *P. ochrochloron* on CYA appears round, grooved in a radial pattern white to light grey in the margin once maturation is reached (Fig. 30C).

The colonies of this *P. ochrochloron* exhibit fast growth rate and are variously branched, with asymmetrically and/or symmetrically branched conidiophores (Houbraken et al., 2011) and this is clearly evident in the Figure 35.

On the other hand *Trichoderma* isolates (Fig. 30D) produce rough and globose to subglobose (Hassan et al., 2014; Shah et al., 2012) conidia (2.8 x 2.6 µm) and their colour was yellow or green (Savitha and Sriram, 2015); conidia production is greater at the centre compared to the margins (Hassan et al., 2014).

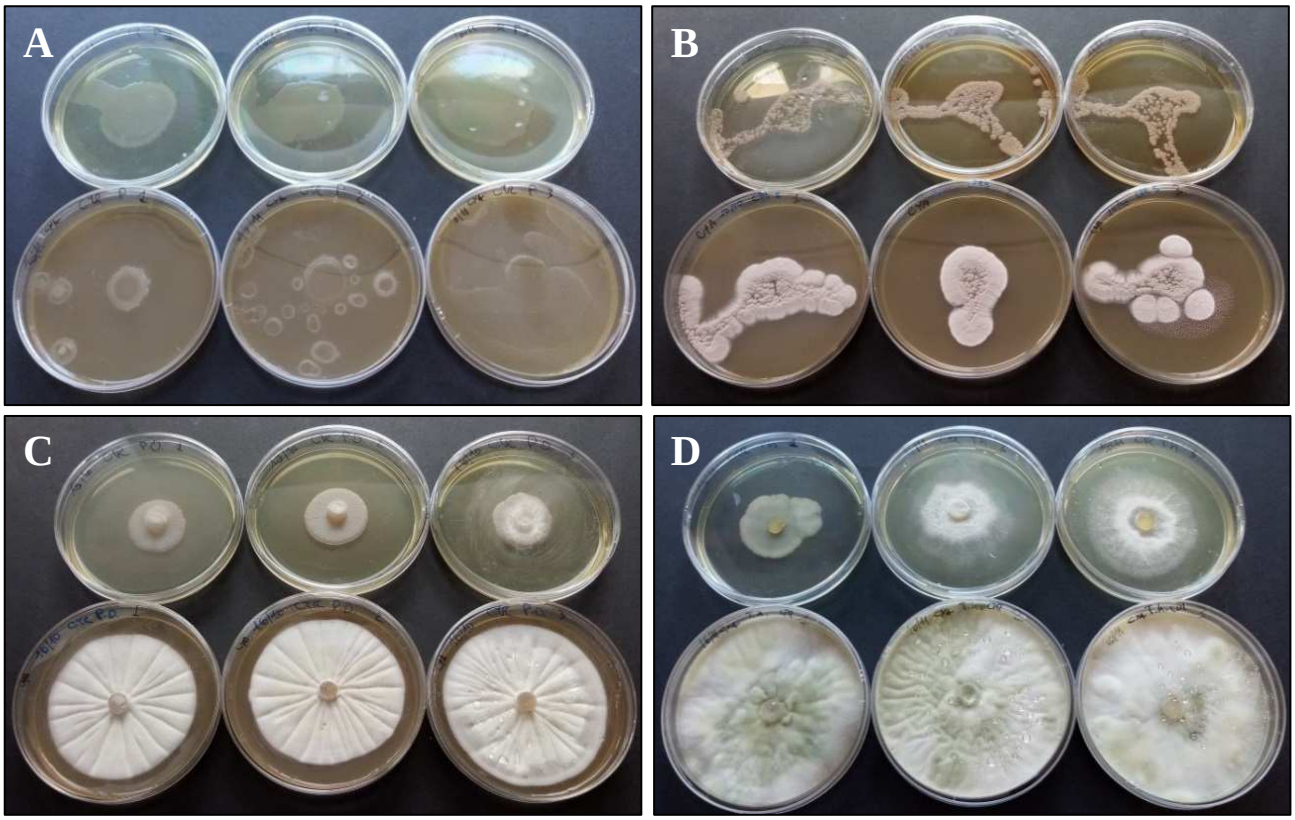

Figure 29. Positive growth petri dishes (3 replicates of TSA pH 6.0 and 3 replicates of CYA) with bacterial strains A) *Pseudomonas* sp. SERP1, B) *Streptomyces* sp. SERP4 and fungal strains C) *P. ochrochloron* Serp03S, D) *T. harzianum* group Serp05S. N=30 each test.

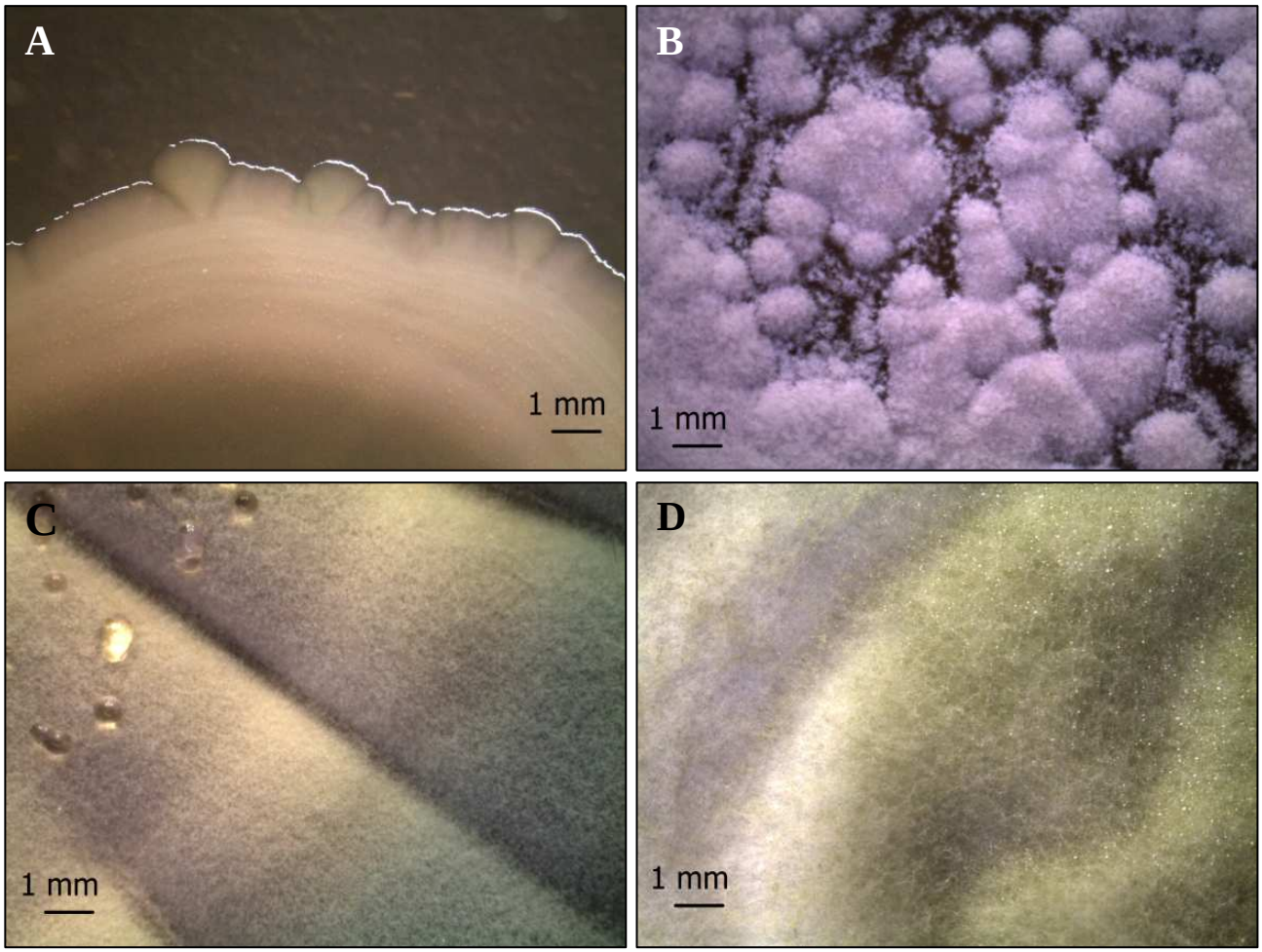

Figure 30. Stereo Microscope images of positive growth of A) *Pseudomonas* sp. SERP1, B) *Streptomyces* sp. SERP4, C) *P. ochrochloron* Serp03, D) *T. harzianum* group Serp05S.

4.4.2 Co-growth methods

a) *Pseudomonas* sp. (SERP1) vs *P. ochrochloron* (Serp03S).

The agar overlay method (Fig. 31A, 32A) on CYA does not seem to clarify any relationship between the two organisms. The bacterium develops where it has been inoculated, while the microfungus grow better around bacterium. After 10 days of incubation the fungus reaches maturation, assuming a grey-black colour.

In the agar plot (Fig. 31B, 32B) and in the distance growth (Fig. 31C, 32C) methods the contact between the two organisms develops a round structure with concentric rings that could mean a sort of growth mix.

b) *Pseudomonas* sp. (SERP1) vs *T. harzianum* (Serp05S):

a suspected antagonistic activity was found between the two organisms, because in the agar overlay method the fungus do not thrive where the bacterium was inoculated (Fig. 31D, 32D). *Vice versa* in other treatments (Fig. 31E, F, 32E, F) the fungus covers almost the entire agar plate, but its growth appears to be disturbed: in the last distance growth method, fungal strain is confined in its half of the plate, suggesting an inhibitory property of the bacterium.

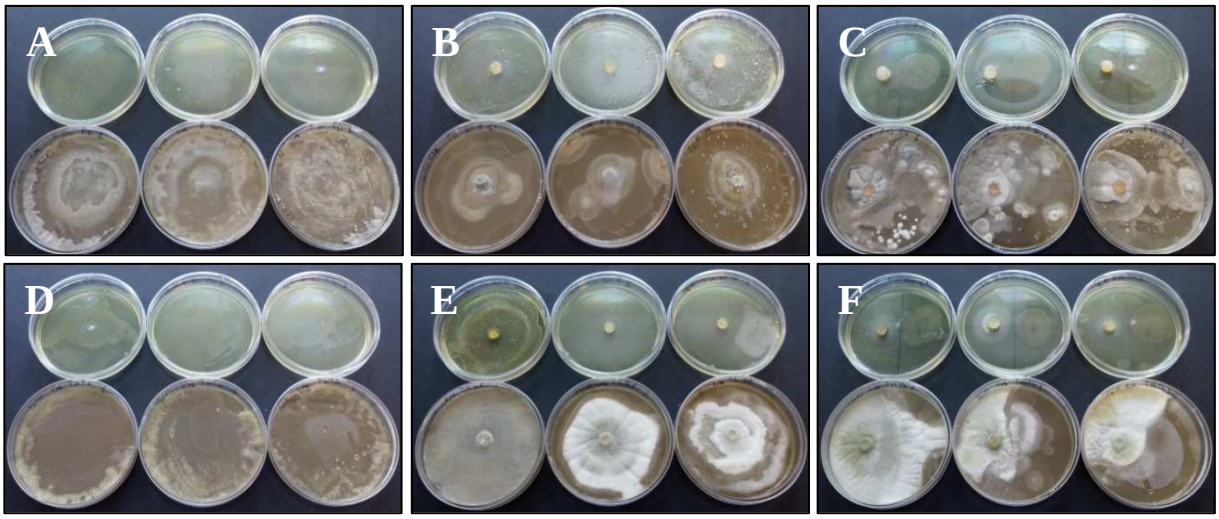

Figure 31. Qualitative evaluation of bacteria and fungi growth test: *Pseudomonas* sp. SERP1 and *P. ochrochloron* Serp03S (A, B, C), *Pseudomonas* sp. SERP1 and *T. harzianum* group Serp05S (D, E, F), by means of agar overlay method (A, D), agar plot method (B, E) and distance growth method (C, F). N= 30 each test.

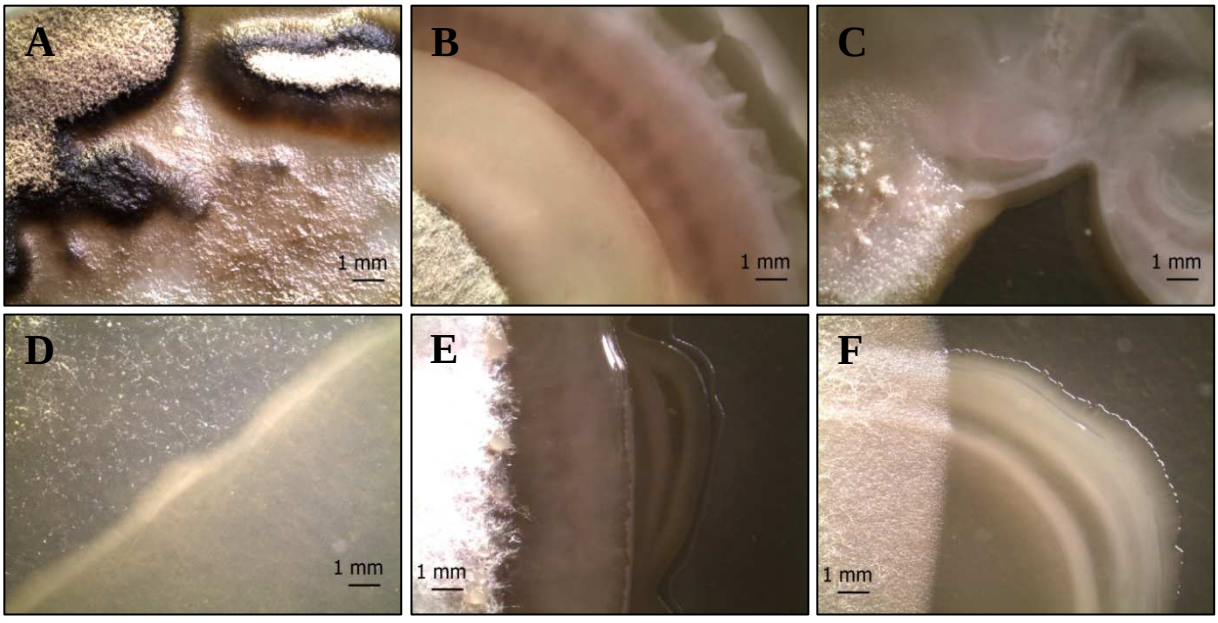

Figure 32. Stereo Microscope images of culturable mix of *Pseudomonas* sp, SERP1 and *P. ochrochloron* Serp03S growth test (A, B, C); culturable mix of *Pseudomonas* sp. SERP1 and *T. harzianum* group Serp05S (D, E, F). Agar overlay method (A, D); agar plot method (B, E); distance growth method (C, F).

c) *Streptomyces* sp. (SERP4) vs *P. ochrochloron* (Serp03S)

there is not an antagonistic effect. In fact, the mix of *Streptomyces* and *P. ochrochloron* covers almost the entire agar plate, assuming a grey-white colour both in the overlay and plot test (Fig. 33A, B, 34A, B). No zones of inhibition are visible in the distance growth method (Fig. 33C, 34C) where bacteria grow above the fungus.

d) *Streptomyces* sp. (SERP4) vs *T. harzianum* (Serp05S)

the bacterial colonies appear smaller than the control and the fungus reaches maturation late. The bacterium grows only in the centre of the plate in the overlay agar test (Fig. 33D, 34D), while the fungus appears to be inhibited in the agar plot test (Fig. 33E, 34E). Bacteria and fungus tolerate each other, as shown by the contact between the two microorganisms (Fig. 33F, 34F), but they remain distinct.

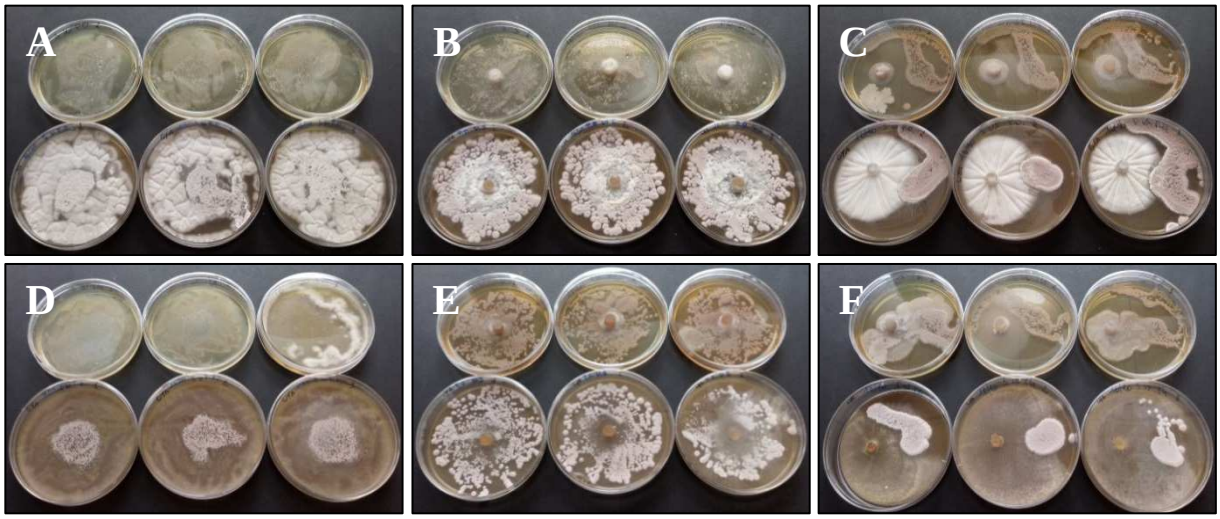

Figure 33. Qualitative evaluation of bacteria and fungi growth test: *Streptomyces* sp. SERP4 and *P. ochrochloron* Serp03S (A, B, C), *Streptomyces* sp. SERP4 and *T. harzianum* group Serp05S (D, E, F), through agar overlay method (A, D), agar plot method (B, E) and distance growth method (C, F). N= 30 each test.

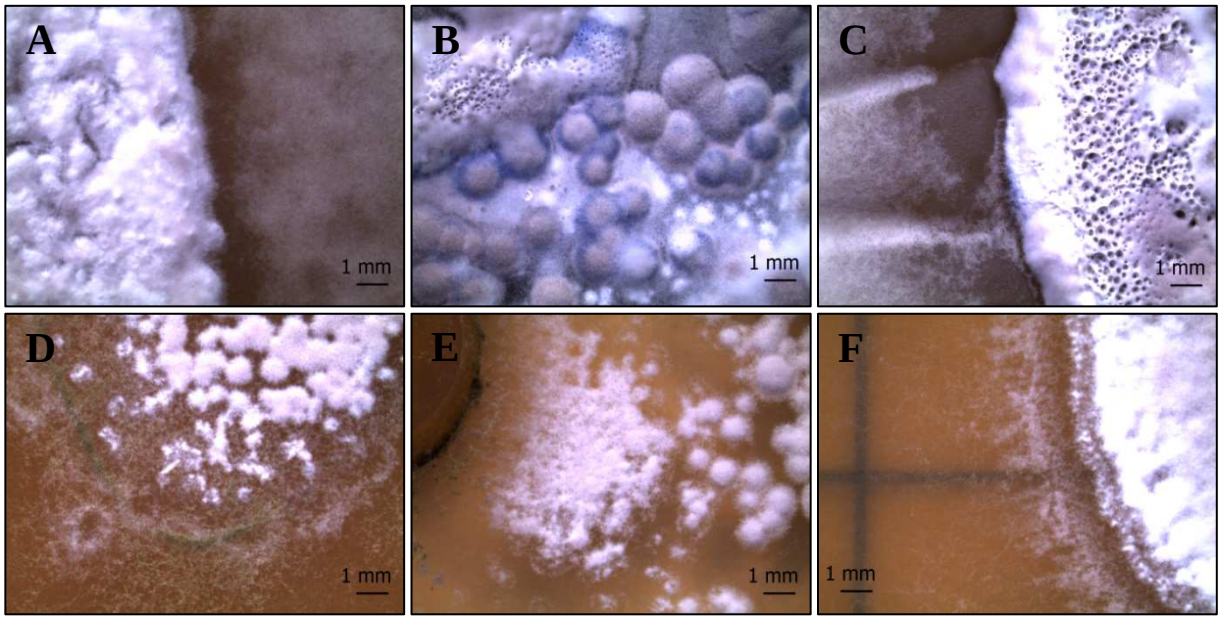

Figure 34. Stereo Microscope images of culturable mix of *Streptomyces* sp, SERP4 and *P. ochrochloron* Serp03S growth test (A, B, C); culturable mix of *Streptomyces* sp. SERP4 and *T. harzianum* group Serp05S (D, E, F). Agar overlay method (A, D); agar plot method (B, E); distance growth method (C, F). N= 30 each test.

As mentioned earlier, the in-depth observation of the *Pseudomonas* SERP1- *P. ochrochloron* Serp03S mix with the Optical Microscope magnified at 40X (Fig. 35) shows the fungal conidiophore branching patterns mostly symmetrical and biverticillated (Visagie et al., 2014). On the other hand, the gram staining allowed to underline the presence of the bacterium. *Pseudomonas* sp. is a Gram-negative bacterium, it has a thin layer of cell wall, so it is counter-stained pink by safranin (Fig. 36). However also the conidiophores of *P. ochrochloron* are clearly visible, stained with a bright red colour.

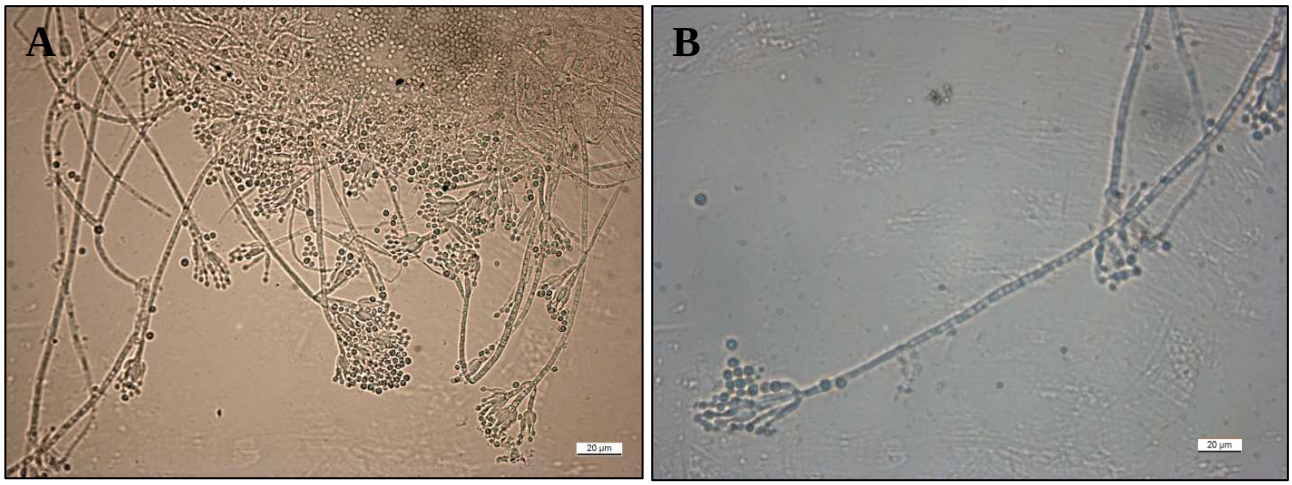

Figure 35. Optical Microscope images (40X) of A) mono- and biverticillate branched conidiophores of *P. ochrochloron* Serp03S in the co-growth method and B) maturation of conidia on conidiophores in detail.

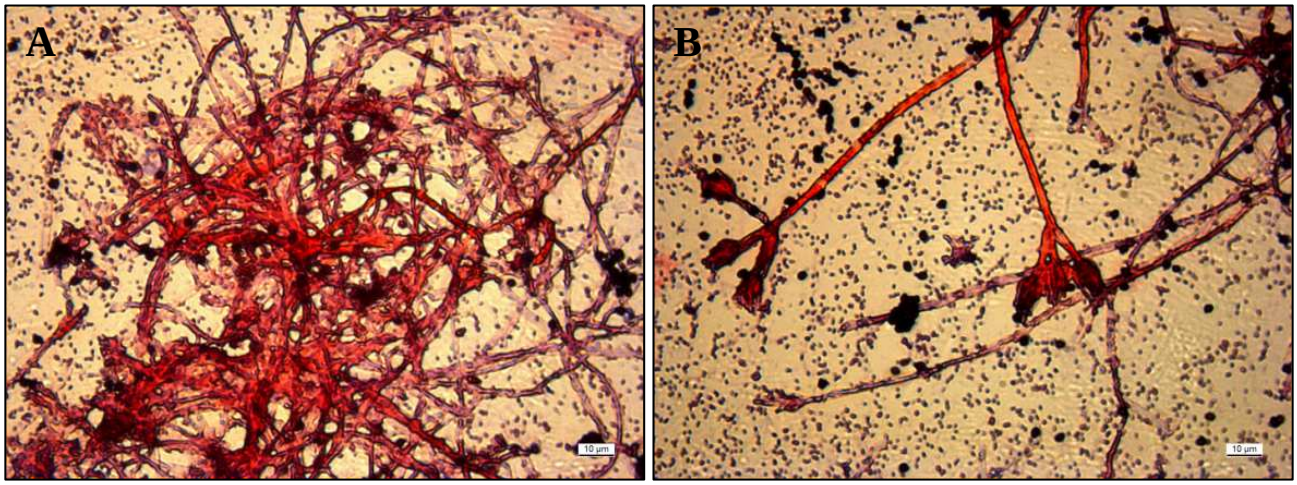

Figure 36. Gram staining images of *Pseudomonas* SERP1 and *P. ochrochloron* Serp03S by Optical Microscope at 63X magnification. In detail A) the mass of fungal hyphae and B) the branched conidiophores of *P. ochrochloron* surrounded by *Pseudomonas* sp. cells that do not retain the crystal violet stain used in the Gram-staining method (pink-red rods).

Scanning Electron Microscopy

The backscattered (BSE) SEM imaging of SERP1 (*Pseudomonas* sp.) and Serp03S (*P. ocrhrochloron*) highlights clusters of fungal hyphae enveloped by groups of flagellate *Pseudomonas* sp. SERP1 (Fig. 37A, B). Symmetric, mostly biverticillated, conidiophores (Fig. 37C, D) are often covered on the tip by bacterial colonies adhering by means of adhesive filaments that provide a compact structure to bacterial biofilm consisting of live and dead cells. Fungal spherical conidia are free or still borne on specialized stalks of the conidiophore that is characterized by typical "vial"-shaped cell called phialide (Fig. 37D, E).

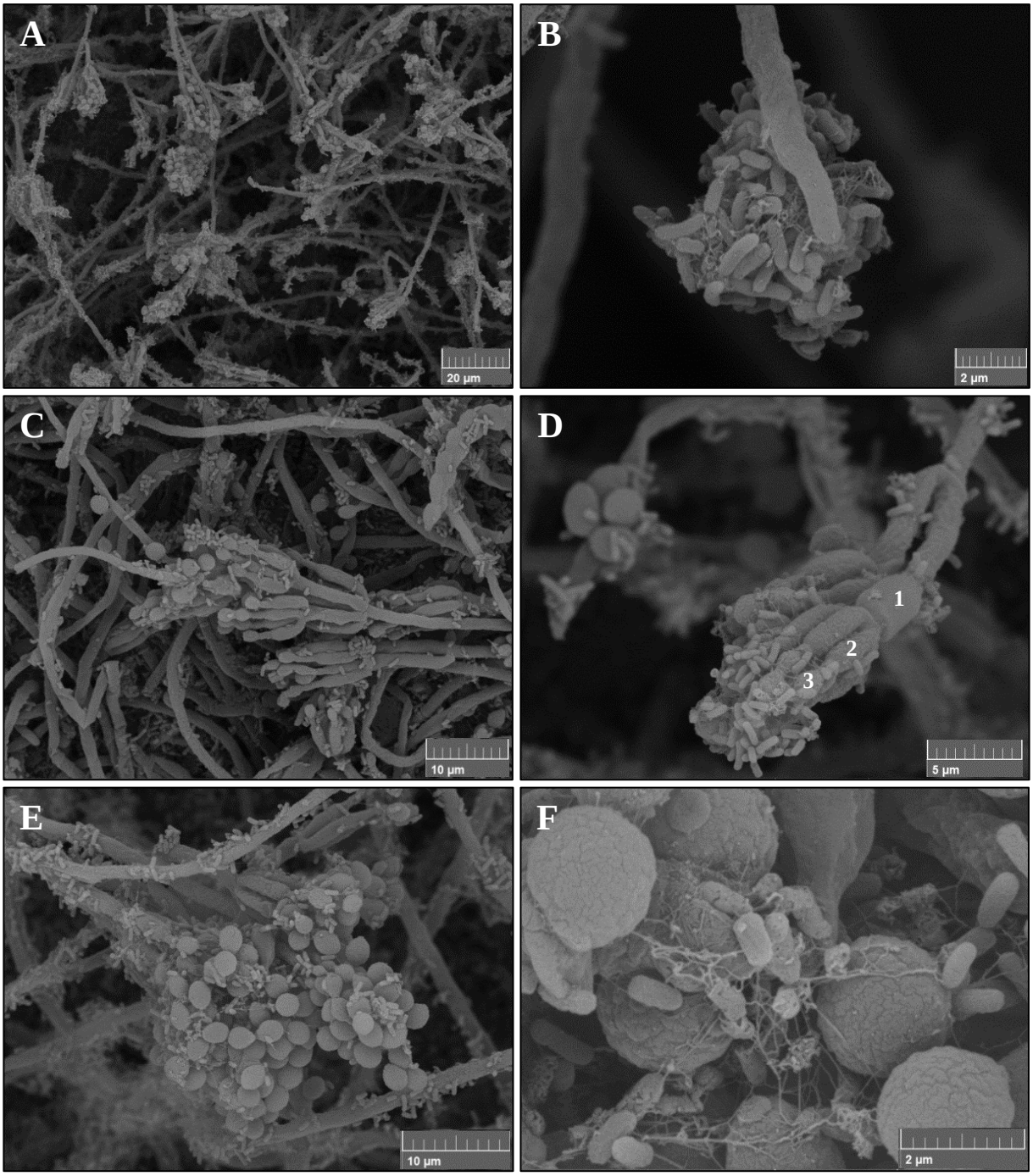

Figure 37. Backscattered electron (BSE) SEM images of the mixed culture of *Pseudomonas* sp. SERP1 and *P. ochrochloron* Serp03S: A) tangle of fungal hyphae covered by bacteria. Magnification 1.90 kx, Working Distance 10.51 mm; B) growing hyphal tip sorrounded by cluster of bacteria jointed by means of adhesins. Magnification 19.8 kx, Working Distance10.84 mm; C) large group of branching biverticillated conidiophores. Magnification 4.63 kx, Working Distance 10.66 mm; D) detail of branch (1) metula (2) interfaces with bacteria adherent to phialides (3). Magnification 10.6 kx, Working Distance 10.51 mm; E) mature conidia on conidiophores. Magnification 20.0 kw, Working Distance 10.57 mm; F) fungal conidia (2 µm diameter) and bacterial colonies (1 µm length), some of which in death phase; the rough surface of the conidia seems to encourage the adhesion of the bacteria cells.

5. Discussion

The development of integrated approach plant-bacteria-fungi, to improve bioremediation of soil contaminated by metals like Ni, needs to examine potential responses and interactions between plant roots and rhizosphere microbiota.

Seed germination highlighted different level of response to this metal at earliest stage of plant development, although *O. bertolonii*, *A. utriculata* and *N. caerulescens* could have preadaptation tolerance traits to Ni that allow hyperaccumulators to grow on low-competition serpentine soils (Milner and Kochian, 2008; Roccotiello et al., 2015a; Selvi et al., 2017).

The microcosm test was used as an early screening to assess the viability and germinability of the seeds to establish the best performing species suitable for Ni treatments. *O. bertolonii* has always low seed germination. This could be linked to restricted growing conditions in relations to its limited distribution area (Corem et al., 2009). *N. caerulescens* has a slower germination rate compared to the other species but this is the same with germination rate of Ni-amended soil suggesting an earlier preadaptation tolerance trait. On the contrary seed germination of the facultative hyperaccumulator *A. utriculata* was affected by increasing Ni concentrations maybe because it requires a longer time to adapt to Ni that commonly inhibit seed germination and seedlings development in concentration-dependent and species-dependent way (Kranner and Colville, 2011). These results are in accordance with literature data, highlighting that Ni at low concentrations can stimulate germination in some cases (Hossein Khoshgoftarmanesh and Bahmanziari, 2012). At the same time low Ni amounts can stimulate root elongation (Carlson et al., 1991) while high concentrations can decrease root length (Soudek et al., 2010).

As described by Pavlova et al. (2018), differences on seed germination were found between Ni-hyperaccumulator species *Alyssum markgrafii* O.E.Schulz and *Alyssum murale*, an obligate and facultative hyperaccumulator respectively. Microcosm test showed that in spite of their accumulation ability, high Ni concentrations can induce a marked inhibition of the seed germination process compared to control, while a slightly Ni concentration may stimulate the germinability.

Therefore further investigations can be useful since timing affects the growth of the seedling, the survival and the fitness of the whole species (Donohue et al., 2010; Fenner and Thompson, 2005; Simons and Johnston, 2000; Verdú and Traveset, 2005).

The effects of metal concentration on plant growth and biomass are often used to assess phytotoxicity in non-hyperaccumulator species (Ryser and Sauder, 2006) or to evaluate differences in trace element concentration from inoculated and uninoculated plants (Al Agely et al., 2005; Trotta et al., 2006).

At the highest concentration of available Ni in mesocosm test, hyperaccumulator species do not show macromorphological alterations in terms of root area and plant biomass, while the non-hyperaccumulator species were Ni-affected suggesting that increasing Ni concentration in soil hindered root development as indicated by Moradi et al. (2009).

In the pot test the non-hyperaccumulator *A. montanum* shows constant root area and plant biomass respect to mesocosm. Surprisingly, root biomass and root area of *T. arvense* significantly increase with Ni treatments (but 2,5 time less than hyperaccumulators) despite other studies showed a disrupted growth in non-accumulator plants (i.e. *Lactuca sativa* L., Pollard et al., 2014) or severe root biomass reduction in non-accumulator *Cicer arietinum* L. (Moradi et al., 2009). A possible explanation is that the pot test lasted longer time than mesocosm test causing a sort of adaptation of the involved species, but further investigations will clarify this point.

At the highest Ni concentration in pot, both hyperaccumulator species gave positive reaction to DMG test that highlight a Ni translocation to aboveground biomass (Nkrumah et al., 2018) as expected. In addition, *A. utriculata* significantly increased aboveground biomass while *N. caerulescens* was comparable to the control, as indicated by Pollard et al. (2014) and similarly to what reported by Moradi et al. (2009) for *Berkheya coddii* Roessler.

The phenotypic plasticity of the facultative hyperaccumulator species *A. utriculata* may depend on epigenetic modifications enabling a rapid adaptation to metal stress (Mirouze and Paszkowski, 2011; Ou et al., 2012; Wang et al., 2016) or adaptation to edaphic conditions (Adamidis et al.,

2014). This may led to a greater development of *A. utriculata* in the aboveground biomass where Ni accumulates. The significant increase of root biomass at increasing Ni treatment was not yet appreciated, even if *A. utriculata* had no negative effects on biomass, development and plant water content under Ni stress (Roccotiello et al., 2016). This aspect can probably be related to the species ability to concentrate metals in its aboveground biomass without display toxicity symptoms (Roccotiello et al., 2016).

Considering that Ni-hyperaccumulators sequester Ni in shoots and by contrast Ni-excluders store Ni in roots, we could hypothesize in agreement with Seregin et al. (2014) that roots systems of both hyperaccumulators *A. utriculata* and *N. caerulescens* do not show differences at increasing Ni because they are more able to tolerate metal, accumulating Ni in aboveground organs, while *T. arvense* specifically act as excluder increasing mostly root area and biomass to sequester metal.

Consistently with the biomass results, the maximum primary photochemical efficiency of PSII, Fv/Fm, and the Performance Index, P.I.(Strasser et al., 2000) do not differ in the hyperaccumulator species at increasing Ni concentrations, while it significantly declines in *A. montanum.* It could be due to the great sensitivity to the fluctuations of environmental abiotic factors (Živčák et al., 2015), such as Ni stress. Both parameters increase in *T. arvense*, although Fm is lower than the other species. A low Tfm may indicate sample stress (Strasser et al., 2000) which causes the Fm to be achieved much earlier than expected, similar to how happen in *A. montanum.*

A comprehensive explanation could concern a Ni adaptation of *A. utriculata* to Ni input during the plant growth due to a possible phenotypic plasticity (Visioli et al., 2012). The same will probably occurs to *T. arvense*, specifically at the root level. However, epigenetic aspects involved in plant adaptation to strongly stressed environments and detoxification mechanisms need to be verified by further investigations (Verbruggen et al., 2009b).

To obtain a mixed culture of Ni-tolerant, plant-growth-promoting microorganisms associated with the rhizosphere of the facultative Ni-hyperaccumulator *A. utriculata*, the first step was the identification of the plant-soil relations and the characterization of culturable microbiota associated

with serpentine and non-serpentine populations.

The comparison between serpentine and non-serpentine soils, plants and related microbiota revealed overall distinction resulting in two well-distinct groups. Bacterial and fungal strains are limited by serpentine soil conditions resulting less abundant in S than NS site with preferential colonization of rhizospheric soil. The difference in terms of element concentration is significant between the two sites (S and NS) because of the high level of Ni and a Ca-deficiency and the low Ca/Mg ratio (0.36) typical of harsh serpentinitic environmental conditions (Ghasemi et al., 2015). However, chemical differences between rhizospheric and bare soils are not significant. As expected, only *A. utriculata* from serpentine soils shows typical hyperaccumulator traits (shoot Ni >1000 mg/kg; BF~1, TF>>1,) (Reeves, 1992; van der Ent et al., 2013) confirming its accumulator traits (Roccotiello et al., 2015a, 2016).

Bacteria and fungi in the rhizosphere of *A. utriculata* are commonly more abundant than in bare soils as shown for other hyperaccumulators (Lopes et al., 2016; Wenzel et al., 2004). This trait known as 'rhizosphere effect' implies that the plant exuding a large number of compounds that can be used as nutrition sources by microbes to proliferate and colonize the root surrounding area (Morgan et al., 2005; Segura et al., 2009; Smalla et al., 2001; Wenzel et al., 2004). Among the identified bacterial morphotypes, genera *Arthrobacter, Bacillus, Erwinia, Micrococcus, Novosphingobium, Pantoea, Pseudomonas,* and *Streptomyces* are distinguished as they have already been isolated on serpentine soil from the rhizosphere of Ni-hyperaccumulator plants (Abou-Shanab et al., 2003; Idris et al., 2004; Ma et al., 2009a; Mengoni et al., 2001; Turgay et al., 2012; Visioli et al., 2015a). We cannot exclude that those bacterial and fungal strains can induce promoting effects on the growth of *A. utriculata* in S site immobilising Ni instead of increasing metal uptake, as in the case of *N. caerulescens* (Aboudrar et al., 2013) but further studies will clarify this point. The presence of some PGPR bacterial strains like *Pseudomonas* sp. and *Streptomyces* sp. able to grow up to Ni 15 mM and microfungi up to Ni 3 mM (Cecchi et al., 2017b; Schlegel et al., 1991) could support this idea.

The PGPR activities of the selected strains potentially support the growth of *A. utriculata*, reducing metal stress and increasing Ni uptake and accumulation in aerial parts as observed for rhizospheric bacteria isolated from Ni-hyperaccumulator *Alyssum serpyllifolium* (Ma et al., 2009b, 2011) and *Brassica juncea* (L.) Czern (Rajkumar et al., 2013b; Rajkumar and Freitas, 2008b; Zaidi et al., 2006). *Pseudomonas* SERP1 can help plants produce more biomass by providing them the IAA that directly stimulates the plant cell division and elongation and root initiation (Prapagdee et al., 2013) in addition to siderophores production and P-solubilisation. Previous studies revealed a high biomass yield in *Brassica* spp. inoculated with a serpentine selected strain of *Pseudomonas* sp. (Freitas et al., 2004), emphasizing the protective role of this bacterium against the inhibitory effects of Ni and the increasing of Ni uptake by plants (Ma et al., 2009c; Rajkumar and Freitas, 2008b).

Genus *Streptomyces* sp. is predominant in soil samples (Mengoni et al., 2001) and Ni-tolerant as *Arthrobacter* (up to 10 mM Ni in the plate test, Kuffner et al. (2010). *Streptomyces* sp. SERP4 shows ACC deaminase activity, siderophores production and phosphate solubilisation. All these aspects suggest that specific strains showing good activity, colonization potential and PGP traits, will be useful in enhancing bioavailability, phytoextraction or phytostabilization performance by plants (Sessitsch et al., 2013).

The isolated microfungal strains of *Penicillium*, *Aspergillus*, *Trichoderma*, and *Mucor* are commonly saprotrophic microfungi with species found in metal-polluted habitats worldwide (Gadd and Fomina, 2011; Kubatova et al., 2002; Massaccesi et al., 2002). Among the isolates, *Penicillium canescens* Sopp was a typical species of rhizosphere able to live until 50 cm depth in soil and *P. ochrochloron* is known for its high tolerance to copper and other metals (Domsch et al., 2007). It is used for biodegradation of triphenylmethane dye cotton blue, which is very recalcitrant to biodegradation (Shedbalkar et al., 2008).

Moreover, *Penicillium expansum* Link represents a cosmopolitan species able to colonize polluted soils and extreme environments and to bioaccumulate high metal concentrations (Di Piazza et al., 2017). Among *Penicillium, Mucor, Cladosporium,* and *Trichoderma* isolated on serpentine soils,

only *Trichoderma* strain, belonging to the *harzianum* group, was also isolated from the rhizosphere of *A. utriculata* in metal-contaminated sites. *T. harzianum* is a saprophytic fungus that grows in soil (Demirel et al., 2005); it is used as a biocontrol against soil and plant pests and pathogens (Degenkolb et al., 2008; Hanson, 2005; Madsen et al., 2007). By means of the extracellular laccase production described by Hölker et al. (2002), Zafra and Cortés-Espinosa (2015), this species is able to degrade PAHs. This fungus tolerates Ni up to 500 mg l^{-1} (Cecchi et al., 2017b; Roccotiello et al., 2015b) and other metals (Cecchi et al., 2017a; Zotti et al., 2014) with great bioaccumulation capacity (up to 11000 mg Ni kg^{-1} as described by Cecchi et al. (2017b).

Moreover, *Aspergillus* strain belonging to the *niger* group represents one of the more studied fungus about biocorrosion, bioalteration, and bioaccumulation of toxic metals, due to the high amount of secondary organic acid production (Gadd, 2007).

Although the culturable techniques are not representative of the total phylogenetic diversity of soil microbiota (i.e. less than 1% of bacteria can be cultured in laboratory, Pham and Kim (2012), the characterization of culturable bacterial and fungal strains is essential for future field studies using bioinoculants as natural chelators in the rhizosphere of hyperaccumulator and facultative hyperaccumulator species. Because of the PGP traits, the high tolerance to Ni and the positive correlation with other strains, *Pseudomonas* sp. SERP1, and *Streptomyces* sp. SERP4, *P. ochrochloron* Serp03S and *T. harzianum* Serp05, were used for the subsequent co-growth test. This technique allow to obtain for the first time a mixed culture of microorganisms associated with the rhizosphere of a facultative Ni-hyperaccumulator evaluating their reciprocal behaviour (i.e. antagonistic, Agamennone et al., 2018; Trivedi et al., 2008 or synergic activities).

Streptomyces sp. SERP4 thrives with both tested fungi, but the *Pseudomonas* sp. SERP1 - *P. ochrochloron* Serp03S culture mix was even more noteworthy. In the agar plug and in the distance growth methods, the bacterial colony assumed an atypical shape around the fungal strain, showing a mixed growth of both microorganisms. The interface between bacterial strain *Pseudomonas* sp SERP1 and fungal strain *P. ochrochloron* Serp03S does not show any mutual inhibition halo.

The Gram staining underlines the high number of bacteria that surrounded clusters of hyphae. The presence of conidiophores at the apex of fungal hyphae and bacterial colonies specifically adherent to their surface was clearly revealed by Scanning Electron Microscopy, allowing for the first time to assess the morphological features of the mature bacterium-fungus mix culture. The development of bacterial biofilms and the abundant presence of asexual non-motile mitospores (conidia) proves that both organisms have reached together the maturation phase without showing clear macro- and micromorphological signs of reciprocal suffering or inhibition.

The synergic effects of *A. utriculata* selected microbiota can play a key role in plant adaptations to severe soil condition (Visioli et al., 2015b) that contribute to the hyperaccumulator phenotype (Visioli et al., 2015a). Using microbiota as inoculum of the rhizosphere could increase the root development and the metal uptake and translocation to shoots improving in a self-sustaining and environmental-friendly way the phytoremediation of metal-contaminated soils by facultative hyperaccumulators.

Root inoculation with selected Ni-tolerant bacteria and fungi may represent a powerful tool in phytotoremediation, although transferring this technology to the field requires further bacteria-fungi mix growth studies and field validation.

6. Conclusion

At the early stage of plant development, the strict accumulator *N. caerulescens* do not change the germination rate and time. On the contrary, the facultative hyperaccumulator *A. utriculata* seems to be affected in the first phase of seed germination and root development. However, at the plantlets and young plant level, *A. utriculata* reveals a great development of root and shoot biomass consistently with the species ecophysiological response in terms of Performance Index.

Bacteria and fungi were significantly more abundant in rhizosphere than in bare soil and were dominated by genera *Arthrobacter, Bacillus, Erwinia, Micrococcus, Novosphingobium*, *Pantoea*, *Pseudomonas,* and *Streptomyces Penicillium*, *Aspergillus*, *Trichoderma*, and *Mucor.*

Interestingly, the genus *Pseudomonas* was only found in rhizospheric serpentine soils (1.6 % of total serpentinitic rhizospheric colonies isolated) and with *Streptomyces* sp. showed highest Ni-tolerance up to 15 mM. Among serpentine bacterial isolates, eight strains belonging to five genera showed at least one PGPR activity. Similarly, *Trichoderma* strain, belonging to the *harzianum* group (1.7 % of the total microfungal count), exhibits great Ni-tolerance and high Ni uptake ability.

The rhizosphere of *A. utriculata* can be an excellent model to enhance Ni uptake and plant growth using a microbiota associated with its root system.

To date, considering the encouraging achieved results, we can consider the use of an integrated approach (plants-fungi-bacteria) cost-effective and environmental-friendly to improve metal uptake and the bioremediation of contaminated soils. This complex multidisciplinary research has shown that a holistic approach to soil pollution allows to extend the perspectives and to overcome the current studies focused on the single rhizospheric components, reinforcing the accomplished results and the field applications. Just assuming the entire rhizosphere as a whole of components and interactions it is possible to better understand its structure and function.

So the proposed model is an example of integration of different disciplines aimed to studying the dynamic microenvironment known as the rhizosphere in the perspective of trying to provide guidance for using Ni-hyperaccumulator species and the associated rhizobiota to remediate Ni contaminated soils.

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"Enrica, I have a message for you… Do not undermine your steady faith for the Research. Believe in the Force…of MYPHYTO!"

Annexes

Annex 1

III Workshop nazionale
BONIFICA, RECUPERO AMBIENTALE E SVILUPPO DEL TERRITORIO: ESPERIENZE A CONFRONTO SUL FITORIMEDIO

ISPRA - 17 - 18 marzo 2016
Via Vitaliano Brancati, 48, 00144 Roma
PHYTOREMEDIATION: UN APPROCCIO RIZOSFERICO

Rosatto, S.[a], Roccotiello, E.[a]*, Cecchi, G.[b], Di Piazza, S.[b], Greco, G.[b], Zotti, M.[b], Mariotti, M.G.[a]

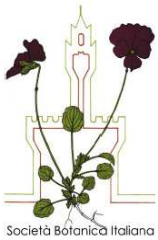
DISTAV – Dipartimento di Scienze della Terra, dell'Ambiente e della Vita, Università degli Studi di Genova, Corso Europa 26, I-16132, Genova
[a] *Laboratorio di Biologia Vegetale.*
[b] *Laboratorio di Micologia.*
**Autore per corrispondenza: enrica.roccotiello@unige.it*

Key-words: *Alyssoides utriculata*, metalli, microbiota, rizosfera

L'interfaccia suolo-radice di specie vegetali iperaccumulatrici rappresenta la prima zona di scambio e potenziale captazione di contaminanti. Tuttavia i processi rizosferici di *taxa* ipertolleranti restano ancora in gran parte inesplorati. Attraverso la caratterizzazione del rizoplano e degli xenobiotici ad esso legati, nonché delle componenti rizosferiche microbiche e fungine, è possibile migliorare l'efficacia dell'assorbimento di contaminanti metallici.
Di seguito si intende illustrare in sintesi parte degli studi svolti per standardizzare e trasferire in campo una metodica a basso costo ed ecosostenibile per siti contaminati da attività antropiche, avvalendosi di specie vegetali native e delle componenti rizosferiche ad esse associate.
Studi condotti in collaborazione fra botanici e micologi e il coinvolgimento di altre competenze hanno portato all'individuazione di organismi ipertolleranti vegetali e fungini presenti in un sito minerario dismesso, caratterizzato da un'intensa contaminazione di Fe e Cu e di altri elementi a concentrazioni potenzialmente tossico-nocive (es., Ti, Mn, Co, Ni, V, Cr, Cu, Zn e Cd) eccedenti i limiti di legge (D. Lgs. 152/2006) [1].
Fra le specie vegetali native dell'area di studio, è stata scelta e valutata la capacità di legame ed assorbimento radicale nell'iperaccumulatrice di Ni *Alyssoides utriculata* (L.) Medik. [2].
Sono stati esaminati campioni di suolo provenienti dalla rizosfera di *A. utriculata*, da cui sono stati isolati alcuni ceppi microfungini.
I risultati attuali evidenziano la grande capacità di flora e micoflora di accumulare selettivamente specifici metalli. In particolare, *A. utriculata* è in grado di accumulare più di 1000 mg Ni Kg^{-1} a livello delle parti aeree. Il Ni presente nella radice è per il 20% legato alla superficie radicale e per il restante 80% captato al suo interno [2].
Tra i ceppi fungini isolati, *Trichoderma harzianum* Rifai, mostra un'elevata tolleranza al Ni, fino a 500 mg l^{-1} e una capacità di accumulo fino a 11000 mg Ni Kg^{-1} [3].
A seguito dei test in microcosmo sarà avviato un monitoraggio in continuo dell'apparato radicale tramite un sistema *rhizobox* 2D, progettato in modo da favorire la manipolazione e l'osservazione dell'ambiente radicale rispetto all'apporto di nutrienti o fattori perturbanti, quali sostanze a concentrazioni tossiche. Saranno valutati differenti parametri radicali (es. lunghezza, diametro, tasso di crescita, numero di radici laterali), oltre che prelevate soluzioni circolanti ed essudati radicali. Verrà inoltre determinata l'interazione del microbiota inoculato con l'apparato radicale e l'influenza di quest'ultimo sulla tolleranza e la capacità di accumulo di metalli in *A. utriculata*.
I risultati ottenuti consentiranno la messa a punto di un sistema integrato che porterebbe il microbiota del suolo ad agire come chelante naturale nei confronti dei metalli, alleviando lo stress che essi provocano alla pianta e favorendo la ricolonizzazione di suoli disturbati.

Bibliografia:
1. Roccotiello et al. 2015. Biodiversity in ecosystem - Linking structure and functions, pp. 563-582.
2. Roccotiello et al. 2015. Chemosphere 119: 1372-1378.
3. Cecchi et al. Unpublished.

Annex 2



111° Congresso della Società Botanica Italiana
III international Plant Science Conference

Roma 21-23 settembre 2016

3.6 A RHIZOSPHERE APPROACH TO MITIGATE SOIL EROSION AND POLLUTION

STEFANO ROSATTO[1], ENRICA ROCCOTIELLO[1]*, GRAZIA CECCHI[2], SIMONE DI PIAZZA[2], GIUSEPPE GRECO[2], MIRCA ZOTTI[2], MAURO GIORGIO MARIOTTI[1]
DISTAV – Department of Earth, Environment and Life Sciences, University of Genoa, Corso Europa 26, I-16132, Genova. [1]Laboratory of Plant Biology, [2]Laboratory of Micology.
[1]*Corresponding author: enrica.roccotiello@unige.it

Environmental pollution by metals represents a severe risk to human health and to the environment in urban and peri-urban areas, where the main sources of metal pollution are mining, municipal and industrial wastes and agricultural activities [1, 2]. In a few plant *taxa* called hyperaccumulator, the concentration of metal(loid)s in aboveground biomass is up to four orders of magnitude higher than in non-hyperaccumulator species and it is associated with a strongly enhanced metal hypertolerance [3]. The rhizosphere, defined as the soil-root interface, is the micro-ecosystem where roots access soil trace elements [4] and represents the first area of potential metal uptake. However, there is a general lack of knowledge about hyperaccumulators, particularly with respect to rhizosphere processes [4].
The aim of this study is to characterize the rhizosphere of selected hyperaccumulator and non-hyperaccumulator species in order to improve metal hyperaccumulation via root uptake.
Metal-tolerant plant and fungi were selected in metalliferous sites with high concentration of potentially toxic elements (i.e. Ni, Cr Co and Zn) exceeding the law limits (D. Lgs. 152/2006). Among the studied species, the nickel-hyperaccumulators *Alyssoides utriculata* (L.) Medik. and *Thlaspi caerulescens* J. & C. Presl. were selected as target species. *A. utriculata* is able to accumulate over 1.000 mg Ni kg^{-1} (DW). Nickel uptaken by roots is adsorbed to rhizoplane (20%) or absorbed (80%) [5].
Besides, soil and rhizosphere samples were examined and some fungal strains were isolated. Among these, a *Trichoderma harzianum* Rifai strain exhibits high Ni tolerance, up to 500 mg l^{-1} and high uptake ability, up to 11.000 mg Ni kg^{-1} [6].
Seed germination tests were carried out in veg-box with germination substrate spiked with Ni (0, 10, 50, 100, 200, 400 mg Ni kg^{-1}, respectively) on target species using non-hyperaccumulator species (i.e., *Alyssum montanum* L. and *Thlaspi arvense* L.) for comparison.
The same substrates were used in mesocosm 3D experiment in pots to evaluate the root elongation and the root anatomy under Ni hyperaccumulation.
Improving root surface increases metal uptake, prevent soil erosion and reduces the spread of metal pollutants, favoring the remediation of peri-urban dismissed industrial sites.

1) H. Ali, E. Khan, M. A. Sajad (2013). "Phytoremediation of heavy metals-Concepts and applications". Chemosphere 91: 869-881.
2) F. De Nicola, D. Baldantoni, L. Sessa, F. Monaci, R. Bargagli, A. Alfani (2015). "Distribution of heavy metals and polycyclic aromatic hydrocarbons in holm oak plant–soil system evaluated along urbanization gradients". Chemosphere 134 (2015) 91–97.
3) U. Krämer (2010), "Metal hyperaccumulation in plants". Annual reviews Plant Biology 61: 517-34.
4) E. R. Alford, E. A. H. Pilon-Smits, M. W. Paschke (2010). "Metallophytes - a view from the rhizosphere". Plant Soil 337: 35-50.
5) E. Roccotiello, H. C. Serrano, M. G. Mariotti, C. Branquinho (2015). "Nickel phytoremediation potential of the Mediterranean *Alyssoides utriculata* (L.) Medik." Chemosphere 119: 1372-1378.
6) G. Cecchi, E. Roccotiello, S. Di Piazza, A. Riggi, M. G. Mariotti, M. Zotti. "Sustainable nickel bioremediation by microfungi", submitted.

Annex 3

SOCIETÀ ITALIANA DI BIOLOGIA SPERIMENTALE

Dipartimento di Scienze Mediche Veterinarie, Università di Bologna
Via Tolara di Sopra 50, 40064 Ozzano dell'Emilia, Bologna

89° Convegno della SOCIETÀ ITALIANA DI BIOLOGIA SPERIMENTALE

"Clima e Vita"

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METAL-TOLERANT PLANT RESPONSE TO SOIL CONTAMINATION

S. Rosatto[1*], E. Roccotiello[1], M. Zotti[2], M.G. Mariotti[1]

[1]Laboratory of Plant Biology; [2]Laboratory of Micology, DISTAV – Department of Earth, Environment and Life Sciences, University of Genoa, Genova, Italy

**E-mail: stefano.rosatto@gmail.com*

The global climate is predicted to change drastically over the next century. From literature it is clear that certain climate change scenarios will have effects on metal phytoremediation and plant–microorganism interactions, which are increasingly being explored [1, 2]. The hyperaccumulator plants actively take up large amounts of metals from the soil at concentrations 100–1000-fold higher than in other species, showing no symptoms of phytotoxicity, resulting in a strong metal-hypertolerance [3]. However, there is a lack of knowledge about hyperaccumulators, particularly as regards rhizosphere processes [4]. The aim of this study is to assess the metal-tolerant plant response to abiotic stress by nickel (Ni) through seed germination tests and through the evaluation of potential morpho-functional root alterations using hyperaccumulator and non-hyperaccumulator species under controlled growing conditions. Growing substrates were spiked with Ni at different concentrations. The Image J analysis of roots was used to evaluate parameters like root elongation, surface area and number of lateral roots. Furthermore, Ni-hyperaccumulator plants and soil samples were collected on metalliferous soils to characterize the rhizospheric microbiota. The presence of Ni seems to determine a general decrease of seed germination and a greater root development in hyperaccumulator species, compared to non-hyperaccumulator species. Moreover, the bacterial isolations show a greater number of bacterial colonies in the rhizosphere soils compared to bare soils. The development of an integrated system plant-rhizobiota, using the rhizobiota as a natural metal-chelator could improve metal uptake, alleviating the nickel stress and promoting the recolonization of metal-polluted areas.

References:

1. Compant S, M. van der Heijden GA, Sessitsch A. Climate change effects on beneficial plant-microorganism interactions. FEMS Microbiol Ecol 2010;73:197-214.
2. Rajkumar M, Vara Prasad MN, Swaminathan S, Freitas H (2012). "Climate change driven plant–metal–microbe interactions. Environ Int 2013;53:74-86.
3. Rascio N, Navari-Izzo F. Heavy metal hyperaccumulating plants. How and why do they do it? And what makes them so interesting? Plant Sci 2011;180:169-81.
4. Ali H, Khan E, Sajad MA. Phytoremediation of heavy metals. Concepts and applications. Chemosphere 2013;91:869-81.

Annex 4

SOCIETÀ ITALIANA DI BIOLOGIA SPERIMENTALE

Dipartimento di Scienze Mediche Veterinarie, Università di Bologna
Via Tolara di Sopra 50, 40064 Ozzano dell'Emilia, Bologna

89° Convegno della SOCIETÀ ITALIANA DI BIOLOGIA SPERIMENTALE

"Clima e Vita"

MULTIPLE BIOASSAYS TO EVALUATE ECOTOXICITY OF POLLUTED SUBSTRATES

E. Roccotiello[1*§], A. Amaroli[2§], E. Giacco[3§], S. Rosatto[1], D. Dozza[1], L. Pane[3], M.G. Mariotti[1]

[1]Laboratory of Plant Biology; [2]Laboratory of Protozoology; [3]Laboratory of Ecotoxicology and Planctology, DISTAV, Department of Earth, Environment and Life Sciences, University of Genoa, Genoa, Italy

§These authors equally contributed to the research

**E-mail: enrica.roccotiello@unige.it*

Potential ecotoxicity of three tunnel excavation materials conditioned with two different foaming conditioners (P and F, respectively) and lubricant were screened using a multi-endpoint bioassay approach. A modified OECD artificial soil was used as control soil (C) to screen only the effect of each conditioner. Acute toxicity bioassays were conducted with a selected set of test species (*Dictyostelium discoideum, Daphnia magna, Hordeum vulgare* and *Lactuca sativa*). Tests were done immediately after adding conditioners (t_0), and after one (t_7) and two weeks (t_{14}) from P and F addition. Although substrates showed no toxic effect, the sensitivity of the test species and the toxicity endpoints varied. The *D. discoideum* were not affected by F exposure, showing morphology and fission rate rhythm (FRR) similar to that of C. Conversely, at t_0 P inhibits the FRR, while t_7 and t_{14} were comparable with C for cell division and morphology. The *D. magna* values of immobilization percentage (I%) were always lower than 10% (*i.e.*, comparable with C), with the exception of one substrate+P in which I=10%. The germination index of *L. sativa* and *H. vulgare* ranged around 80% (*i.e.*, no phytotoxicity) and the same trend was observed for mean root length. No significant time-effect (*i.e.*, increasing conditioner biodegradation) were recorded with the exception of one substrates, with values that turned high at t_{14}. Thus, substrates+conditioners were not toxic but with different sensitivity showed by multiple bioassays that revealed that this approach is suitable to be applied for a quick and exhaustive screening of soil toxicity.

Annex 5

Rhizosphere response to nickel stress in hyperaccumulator and non-hyperaccumulator species

Stefano Rosatto[1], Enrica Roccotiello[1], Grazia Cecchi[2], Mirca Zotti[2] and Mauro Mariotti[1]

DISTAV – Department of Earth, Environment and Life Sciences, University of Genoa, Italy
[1]Laboratory of Plant Biology
[2]Laboratory of Micology

stefano.rosatto@gmail.com

Abstract

Rhizosphere, where roots access soil trace elements, represents the first area of potential metal uptake. The aim of this study is to assess the plant response to nickel in terms of root biomass development, and possible selectivity of rhizosphere microbiota. Test species were Ni-hyperaccumulators: *Alyssoides utriculata*, *Alyssum bertolonii*, *Thlaspi caerulescens* and non-hyperaccumulators: *A. montanum* and *T. arvense*. Soils were spiked with different concentration of Ni (0-1000 mg kg^{-1}) both in microcosm and in mesocosm. Root surface and fresh and dry biomass were evaluated. Various 10-fold dilutions of soil samples were plated on Tryptic Soy Agar added with Ni (0-5 mM) to determine the number of culturable bacteria and on Malt Extract Agar (MEA) to isolate vital fungal strains by a dilution plate technique. Except *A. utriculata*, highest Ni concentration affected root development in terms of root branching, surface area and biomass. In rhizosphere soil, bacteria colonies are more abundant than in bare soil; native soil microfungi were screened for Ni tolerance and a *Trichoderma harzianum* Rifai strain was selected as Ni hyperaccumulator (up to 11000 mg kg^{-1}). The native rhizosphere microbiota will be used to evaluate possible plant tolerance to Ni stress.

Keywords: *bacteria, fungi, metal uptake, microbiota, root.*

Annex 6

Seed germination under nickel stress in hyperaccumulator and non-hyperaccumulator species

Enrica Roccotiello, Stefano Rosatto, Alex Riggi and Mauro Giorgio Mariotti

DISTAV – Department of Earth, Environment and Life Sciences, University of Genoa, Italy
Laboratory of Plant Biology

enrica.roccotiello@unige.it

Abstract
High nickel (Ni) levels exert toxic effects on seed germination, but literature on this topic in hyperaccumulators is very few. The aim of the study was to test the Ni effect on seed germination of hyperaccumulators and related species both in native and amended soils. Seeds of *Alyssoides utriculata* were collected from 3 populations in NW Italy on native soils (A, serpentine; B, serpentine with Fe and Cu contamination; C, non-serpentine) and sown on them and on neutral growing substrates used as control. Experiments were replicated in microcosm (n=400) and in mesocosm (n=3000). We also investigated the seed germination of hyperaccumulators *A. utriculata*, *Alyssum bertolonii*, *Thlaspi caerulescens* and on non-hyperaccumulators *A. montanum* and *T. arvense* (n=90, each species) on soils spiked with different Ni concentrations (0-1000 mg kg^{-1}). In native soils, the germination index (GI) of *A. utriculata* is significantly high in microcosm, while in mesocosm, GI<50% under both Ni and drought stress. No significant effects are shown in relations with native soils. Among main results, in soils amended with Ni seed germination rate (G%) in *A. utriculata* and in *T. caerulescens* do not show significant differences under Ni stress.

Keywords: *abiotic stress, native soil, germination index, germination rate.*

Annex 7

Histology and body growth of newborn earthworms *Dendrobaena veneta* (Rosa, 1886) exposed to nickel

Sara Ferrando[1], **Enrica Roccotiello[2]**, Andrea Amaroli[1,2], Lorenzo Gallus[1], Marino Rottigni[1], Stefano Rosatto[1], Chiara Gambardella[3], Mauro Mariotti[1]

[1]*Department of Environmental, Earth, and Life Science (DISTAV), University of Genoa, Italy*
[2]*Department of Surgical Science (DISC), University of Genoa, Italy*
[3]*ISMAR, CNR, Italy*

enrica.roccotiello@unige.it

Abstract

Soils are complex systems thus the evaluation of the toxicity of a substance on soil organisms requires to standardize the soil-related influences. For that purpose, the use of defined, artificial soils has been widely accepted. In the present study, we investigated the effects of Nickel (Ni) on newborn earthworm *Dendrobaena veneta* in artificial soil (peat:sand 2:1).
Different concentration of Ni were added to the artificial soil (Ni 0, 2.5, 12.5, 25, 50, 100 mg kg^{-1}), and 3.0 g (dry weight) of control and Ni-enriched soils were used for each Petri dish, where earthworms were reared individually. Newborns of *D. veneta*, within 12h after the cocoon hatching, were weighted and randomly assigned to different treatment groups. The weight of the earthworms was then measured every fourth day, for 60 days of treatment. During that time, the soil was periodically soaked with distilled water, and added oat flour. The optimal amount of oat flour supply for one *D. veneta* specimen, from hatching to two-months, was determined in previous experiments. After the 60 days of experimental treatment, specimens were anesthetized on melting ice and fixed in paraformaldehyde 4% for standard histological methods: sections of the whole body was obtained after paraffin embedding and observed after hematoxylin-eosin, Masson's trichrome, Alcian-PAS, and Sirius Red.
The concentration of Ni 25 mg kg^{-1} was the lowest, among the tested ones, to determine a lower growth rate in *D. veneta*, which appeared to be significant after the first 16 days. Tissue damages were observed after 60 days of treatments. These preliminary results allow suggesting the weight increasing rate of newborns *D. veneta* as a sensitive parameter for Ni pollution and further studies will be focused on a better characterization of nickel effects on *D. veneta* body growth.
Further studies will be done on serpentine soils with the same experimental design of the present study.

Keywords: *ecotoxicology, earthworms, metals, soil.*

Annex 8

112° Congresso della Società Botanica Italiana

IV INTERNATIONAL PLANT SCIENCE CONFERENCE (IPSC)

Parma, 20 - 23 September 2017

2.4 = RHIZOSPHERE RESPONSE TO NICKEL IN HYPERACCUMULATOR AND NON-HYPERACCUMULATOR SPECIES

Stefano Rosatto[1] (stefano.rosatto@gmail.com), Enrica Roccotiello[1], Grazia Cecchi[2], Mirca Zotti[2], Mauro Mariotti[1]

DISTAV – Department of Earth, Environment and Life Sciences, University of Genoa, Corso Europa 26, 16132, Genova. [1]Laboratory of Plant Biology; [2]Laboratory of Micology

Rhizosphere, as an important interface of soil and plant, plays a significant role in phytoremediation of contaminated soil by metals, in which, microbial populations are known to enhance phytoremediation processes (1). However, more information on the microbial communities and their functions associated with the majority of hyperaccumulators are required (2).

Bacteria in the rhizosphere are involved in the accumulation of potentially toxic trace elements into plant tissues (1). Additionally, microfungi are essential in colonizing and detoxifying metal-contaminated soil ecosystems and consequently have environmental and economic significance (3).

The aim of this study is to assess the plant response to nickel (Ni) in terms of root biomass and surface development, and to select rhizosphere microbiota with affinity to Ni.

Test species were Ni-hyperaccumulators: *Alyssoides utriculata* (L.) Medik., *Thlaspi caerulescens* J. Presl & C. Presl and non-hyperaccumulators: *Alyssum montanum* L. and *T. arvense* L.. Soils were amended with different concentration of Ni (0-1000 mg kg^{-1}) both in microcosm and in mesocosm (Fig. 1). Root surface development and fresh and dry biomass were evaluated. Various 10-fold dilutions of soil samples were plated on Tryptic Soy Agar added with Ni (0, 1, 5 mM) to determine the number of culturable bacteria (Fig. 2) and on Malt Extract Agar (MEA) and on Rose Bengal agar (RB) to isolate vital fungal strains (Fig. 3) by a dilution plate technique.

Hyperaccumulators do not seem to be affected by nickel stress; on the contrary non-hyperaccumulators exhibit a decrease in terms of root surface area and fresh and dry root and shoot biomass.

In rhizosphere soil of *A. utriculata* bacteria colonies are more abundant than in bare soil but without a clear selectivity by nickel at increasing Ni concentrations. Native soil fungi were screened for Ni tolerance and a strain of *Trichoderma harzianum* group was selected as Ni hyperaccumulator (up to 11000 mg Ni kg^{-1} in dry biomass) suggesting its possible employ in a bioremediation protocol able to provide a sustainable reclamation of broad contaminated areas (4).

The native rhizosphere microbiota can be useful to evaluate possible plant tolerance to Ni stress. Bacteria and microfungi can be inoculated in the hyperaccumulators' rhizosphere to alleviate Ni stress and to increase metal uptake

Fig. 1
Greenhouse mesocosm test.

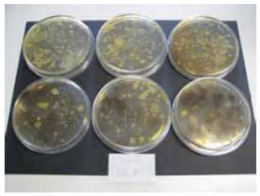

Fig. 2
Bacterial isolation.

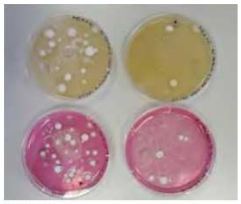

Fig. 3
Microfungal strains isolation.

1) Jing, Y., He, Z., Yang, X., 2007. Role of soil rhizobacteria in phytoremediation of heavy metal contaminated soils. J. Zhejiang Univ. Sci. B 8, 192–207

2) Thijs, S., Langill, T., Vangronsveld, J., 2017. Chapter Two - The Bacterial and Fungal Microbiota of Hyperaccumulator Plants: Small Organisms, Large Influence, in: Vangronsveld, A.C. and J. (Ed.), Advances in Botanical Research, Phytoremediation. Academic Press, pp. 43–86

3) Gadd, G.M., 2007. Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. Mycol. Res. 111, 3–49

4) Cecchi, G., Roccotiello, E., Piazza, S.D., Riggi, A., Mariotti, M.G., Zotti, M., 2017. Assessment of Ni accumulation capability by fungi for a possible approach to remove metals from soils and waters. J. Environ. Sci. Health Part B 52, 166–170

Annex 9

112° Congresso della Società Botanica Italiana

IV INTERNATIONAL PLANT SCIENCE CONFERENCE (IPSC)

Parma, 20 - 23 September 2017

2.1 = EFFECT OF NICKEL STRESS ON SEED GERMINATION IN HYPERACCUMULATOR PLANTS AND RELATED SPECIES

Enrica Roccotiello[1] (enrica.roccotiello@unige.it), Stefano Rosatto[1], Alex Riggi[1], Denise Dozza[1], Mirca Zotti[2], Mauro Mariotti[1]

[1]Laboratory of Plant biology, Department of Earth, Environmental and Life Sciences (DISTAV), University of Genoa, Viale Benedetto XV, 5, 16132 Genova, Italy; [2]Laboratory of Micology, Department of Earth, Environmental and Life Sciences (DISTAV), University of Genoa, Corso Europa 26, 16132 Genova, Italy

High level of available nickel (Ni) in soil exert toxic effects on seed germination of common plants. Most studies are focused on metal accumulation in seedlings and adult plants of crop and hyperaccumulator species under different Ni level, but very few of them on the first stages of seed germination.

As a consequence, the main goal of the present research is to investigate the effect of low-to-high available nickel concentration on seed germination in hyperaccumulator and non-hyperaccumulator species both in native and amended soils.

We investigated the seed germination of the facultative hyperaccumulator *Alyssoides utriculata* (L.) Medik., the obligate hyperaccumulators *Odontarrhena bertolonii* Desv. L. Cecchi&Selvi and *Thlaspi caerulescens* J.Presl & C.Presl and in the non-hyperaccumulator related species *Alyssum montanum* L. and *T. arvense* L. (n=90, each species) on soils spiked with different Ni concentrations (0-1000 mg kg^{-1}). In addition, seeds of *A. utriculata* were collected from 3 populations in NW Italy on native soils (S, serpentine; M, mine site with high Fe and Cu; NS, non-serpentine) and sown on them and on neutral growing substrates used as control. Experiments were replicated in growth chamber in Petri dishes (seeds, n=400), in mesocosm at water holding capacity of WHC=80% and in mesocosm in semi-natural condition with a WHC of 50% (seeds, n=4000). The ecotoxicological and ecophysiological response of the seedlings were investigated as well in terms of root elongation and photosynthetic performance.

We found significant differences in germination rate (G%) between hyperaccumulator and related species up to 100 mg kg^{-1} of available Ni (tolerance). The germination time is longer under semi-natural conditions. Native soils are related to significant lower germination rate. Germination rate is not affected by soil Ni level, and the same for root elongation in hyperaccumulator species. Accumulation is soil-dependent, not population-dependent.

Germination stages are particularly important for successful subsequent development of a crop for phytoremediation.

Annex 10

113° Congresso della Società Botanica Italiana

V INTERNATIONAL PLANT SCIENCE CONFERENCE (IPSC)

Fisciano (SA), 12 - 15 September 2018

3.4 = Rhizosphere microbiota responses to nickel stress

Stefano Rosatto[1], Enrica Roccotiello[1], Grazia Cecchi[2], Mirca Zotti[2] and Mauro Mariotti[1]
DISTAV – Department of Earth, Environment and Life Sciences, University of Genoa, Corso Europa 26, 16132, Genova.
[1]Laboratory of Plant Biology, [2]Laboratory of Micology. [1*]Corresponding author: stefano.rosatto@gmail.com

The serpentine soils, characterized by high level of metals like Ni, Cr, Co, Mn (1,2), and low levels of N, P, K, Ca (3), provide inhospitable habitat for many plant species (4,5), except for hyperaccumulators, able to store metals such as nickel (Ni) in aboveground biomass (6). Despite the high number of research on plants growing on serpentine substratum, the interest on the root system of hyperaccumulators, and in its interactions with the other components of the rhizosphere is quite recent (7). The rhizosphere plays a crucial role in hyperaccumulation, since plant root-associated bacteria and fungi provide beneficial effects on their host, improving the efficiency of phytoremediation processes (7).
This study aims at characterizing the microbiota associated with the rhizosphere of the facultative Ni-hyperaccumulator *Alyssoides utriculata* (L.) Medik. from serpentine and non-serpentine sites, and at obtaining a screening of bacterial and fungal strains which are capable to promote metal uptake, and hence allow plant development. Culturable bacteria and fungal strains were isolated on agar by a dilution plate technique from the rhizosphere of *A. utriculata*, as well as from bare soil samples. Microbiota isolated from serpentine soil were selected on the basis of their Plant Growth-Promoting Rhizobacteria (PGPR) properties, and Ni tolerance.
Isolated strains from the rhizosphere of plants that grow on serpentine soils were evaluated for their ACC deaminase activity, production of phytohormone IAA, synthesis of siderophores, phosphate solubilizing capacity, and Ni tolerance, up to 20 mM of nickel sulphate hexahydrate ($NiSO_4*6H_2O$) on agar.
Eight tested bacterial isolates were positive for more than one plant growth-promoting character. The rhizobacteria *Pantoea* exhibits all PGP activities, showing high production of IAA and siderophores, such as *Pseudomonas*. The solubilization of phosphates is mainly observed in *Pantoea* and *Erwinia*, while *Streptomyces* grows better on ACC as the sole source of N. Only two strains (*Pseudomonas* and *Streptomyces*) are able to tolerate up to 15 mM $NiSO_4*6H_2O$.
Among fungal strains, *Trichoderma harzianum* Rifai group exhibits Ni tolerance (up to 500 mg l^{-1} of $NiSO_4*6H_2O$), and high bioextraction capability (more than 10000 mg kg^{-1}) (8).
Bacteria and fungal communities associated with root system could be useful to alleviate metal stress, and to promote plant growth and Ni uptake, through the development of an integrated plant-microbiota system.

1) Cheng C-H, Jien S-H, Iizuka Y, Tsai H, Chang Y-H, Hseu Z-Y. Pedogenic Chromium and Nickel Partitioning in Serpentine Soils along a Toposequence. Soil Sci Soc Am J. 2011 Mar 1;75(2):659–68.
2) Oze C, Fendorf S, Bird DK, Coleman RG. Chromium Geochemistry of Serpentine Soils. Int Geol Rev. 2004 Feb 1;46(2):97–126.
3) Brooks RR. Serpentine and its vegetation: a multidisciplinary approach. Ecol Phytogeogr Physiol Ser USA [Internet]. 1987 [cited 2018 May 29]; Available from: http://agris.fao.org/agris-search/search.do?recordID=US8918938
4) Brady KU, Kruckeberg AR, Jr HDB. Evolutionary Ecology of Plant Adaptation to Serpentine Soils. Annu Rev Ecol Evol Syst. 2005;36(1):243–66.
5) Chiarucci A, Robinson BH, Bonini I, Petit D, Brooks RR, Dominicis VD. Vegetation of tuscan ultramafic soils in relation to edaphic and physical factors. Folia Geobot. 1998 Jun 1;33(2):113–31.
6) Krämer U. Metal Hyperaccumulation in Plants. Annu Rev Plant Biol. 2010;61(1):517–34.
7) Mengoni A, Schat H, Vangronsveld J. Plants as extreme environments? Ni-resistant bacteria and Ni-hyperaccumulators of serpentine flora. Plant Soil. 2010 Jun 1;331(1–2):5–16.
8) Cecchi G, Roccotiello E, Piazza SD, Riggi A, Mariotti MG, Zotti M. Assessment of Ni accumulation capability by fungi for a possible approach to remove metals from soils and waters. J Environ Sci Health Part B. 2017 Mar 4;52(3):166–70.

Annex 11

Ferrara Fiere Congressi, 19-21 Settembre 2018

Prove di phytoremediation nell'ottica di un approccio integrato pianta-funghi-batteri

Roccotiello Enrica[1*], Rosatto Stefano[1], Mariotti Mauro[1], Puccillo Giorgio[2], Zotti Mirca[3]

DISTAV – Dipartimento di Scienze della Terra, dell'Ambiente e della Vita, Università degli Studi di Genova, Corso Europa 26, I-16132 Genova
[1]Laboratorio di Biologia Vegetale e [3]Laboratorio di Micologia - DISTAV
[2]ptech s.r.l., Via Anton Maria Maragliano 10/1, 16121 Genova
[1*]Corresponding author: enrica.roccotiello@unige.it

La rizosfera, come interfaccia suolo-radice, svolge un ruolo significativo nella *phytoremediation* di suoli contaminati da metalli. Si tratta di un micro-ecosistema in cui le radici hanno accesso agli elementi del suolo (1) e rappresenta la prima area di potenziale captazione di metallo in piante iperaccumulatrici.
Nonostante sia noto da letteratura che le comunità microbiche presenti a livello rizosferico siano potenzialmente in grado di incrementare le performance di *phytoremediation* (2), le interazioni fra le componenti rizosferiche di *taxa* iperaccumulatori restano ancora in gran parte inesplorate. I nostri studi e sperimentazioni sono volti a valutare la risposta delle piante ai metalli con particolare riferimento al nichel (Ni) in termini di sviluppo di biomassa e superficie radicale, e selezionare il microbioma rizosferico idoneo per incrementare l'accumulo di metalli.
Le piante iperaccumulatrici di Ni *Alyssoides utriculata* (L.) Medik., *Noccaea caerulescens* (J.Presl & C.Presl) F.K.Mey. e i non iperaccumulatori *Alyssum montanum* L. *e Thlaspi arvense* L. sono stati selezionati quali specie test in micro- e mesocosmo e trattati su suoli contaminati con differenti concentrazioni di Ni (0-1000 mg kg^{-1}). Parallelamente si è proceduto ad isolare la componente batterica e fungina da campioni di suolo rizosferico di *A. utriculata* (3).
Il microbioma rizosferico testato si prevede possa essere utilizzato a livello della rizosfera di piante iperaccumulatrici per ottenere una *bioaugmentation*. Questo potrà consentire la messa a punto di un sistema integrato pianta-funghi-batteri che porterà il microbioma del suolo ad agire come chelante naturale nei confronti dei metalli al fine di alleviarne lo stress ed incrementarne l'accumulo, promuovendo attivamente la ricolonizzazione di suoli disturbati.

1. E. R. Alford, E. A. H. Pilon-Smits, M. W. Paschke (2010). "Metallophytes - a view from the rhizosphere". Plant Soil 337: 35-50.

2. Jing, Y., He, Z., Yang, X., 2007. Role of soil rhizobacteria in phytoremediation of heavy metal contaminated soils. J. Zhejiang Univ. Sci. B 8, 192–207.

3. Rosatto, S., Roccotiello, E., Cecchi G., Zotti M., Mariotti, M. Rhizosphere response to nickel stress in hyperaccumulator and non-hyperaccumulator species. The 9th International Conference on Serpentine Ecology 4-9th June 2017, Tirana and Pogradec, Albania.

Annex 12

Rhizosphere response to nickel in a facultative hyperaccumulator

Stefano Rosatto, Enrica Roccotiello, Giuseppe Greco, Simone Di Piazza, Grazia Cecchi, Mirca Zotti, Luigi Vezzulli, Mauro Mariotti

Abstract

This study faces the characterization of the culturable microbiota of the facultative Ni-hyperaccumulator *Alyssoides utriculata* to obtain a collection of bacterial and fungal strains for potential applications in Ni phytoextraction.

Rhizosphere soil samples and adjacent bare soil were collected from *A. utriculata* from serpentine and non-serpentine sites together with plant roots and shoots. Rhizobacteria and fungi were isolated and characterized genotypically and phenotypically. Plants and soils were analyzed for total element concentration using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Serpentine and non-serpentine sites differ in terms of elements concentration in soil, plant roots and shoots. Ni and Co are significantly higher on serpentine site, while Ca is more abundant in non-serpentine site.

Bacteria and fungi were significantly more abundant in rhizosphere than in bare soil and were dominated by genera *Arthrobacter*, *Bacillus* and *Streptomyces*, *Penicillium* and *Mucor*. The genus *Pseudomonas* was only found in rhizospheric serpentine soils (< 2 % of total serpentine isolates) and with *Streptomyces* sp. showed highest Ni-tolerance up to 15 mM. The same occurred for *Trichoderma* strain, belonging to the *harzianum* group (< 2 % of the total microfungal count) and *Penicillium ochrochloron* (< 10 % of the total microfungal count, tolerance up to Ni 20 mM). Among serpentine bacterial isolates, 8 strains belonging to 5 genera showed at least one PGPR activity (1-Aminocyclopropane-1-Carboxylic Acid (ACC) deaminase activity, production of indole-3-acetic acid (IAA), siderophores and phosphate solubilizing capacity), especially genera *Pantoea*, *Pseudomonas* and *Streptomyces*. Those microorganisms might thus be promising candidates for employment in bioaugmentation trials.

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References

Aboudrar, W., Schwartz, C., Benizri, E., Morel, J.L., Boularbah, A., 2007. Soil Microbial Diversity as Affected by the Rhizosphere of the Hyperaccumulator Thlaspi Caerulescens Under Natural Conditions. International Journal of Phytoremediation 9, 41–52. https://doi.org/10.1080/15226510601139417

Aboudrar, W., Schwartz, C., Morel, J.L., Boularbah, A., 2013. Effect of nickel-resistant rhizosphere bacteria on the uptake of nickel by the hyperaccumulator <Emphasis Type="Italic">Noccaea caerulescens</Emphasis> under controlled conditions. J Soils Sediments 13, 501–507. https://doi.org/10.1007/s11368-012-0614-x

Abou-Shanab, R., Angle, J., Delorme, T., Chaney, R., Van Berkum, P., Moawad, H., Ghanem, K., Ghozlan, H., 2003. Rhizobacterial effects on nickel extraction from soil and uptake by Alyssum murale. New Phytologist 158, 219–224. https://doi.org/10.1046/j.1469-8137.2003.00721.x

Abou-Shanab, R.I., Delorme, T.A., Angle, J.S., Chaney, R.L., Ghanem, K., Moawad, H., Ghozlan, H.A., 2003. Phenotypic characterization of microbes in the rhizosphere of alyssum murale. International Journal of Phytoremediation 5, 367–379. https://doi.org/10.1080/15226510309359043

Abràmoff, M.D., Magalhães, P.J., Ram, S.J., 2004. Image processing with ImageJ. Biophotonics international 11, 36–42.

Adamidis, G.C., Aloupi, M., Kazakou, E., Dimitrakopoulos, P.G., 2014. Intra-specific variation in Ni tolerance, accumulation and translocation patterns in the Ni-hyperaccumulator Alyssum lesbiacum. Chemosphere 95, 496–502.

Agamennone, V., Roelofs, D., Straalen, N.M. van, Janssens, T.K.S., 2018. Antimicrobial activity in culturable gut microbial communities of springtails. Journal of Applied Microbiology 125, 740–752. https://doi.org/10.1111/jam.13899

Agrawal, B., Czymmek, K.J., Sparks, D.L., Bais, H.P., 2013. Transient Influx of nickel in root mitochondria modulates organic acid and reactive oxygen species production in nickel hyperaccumulator Alyssum murale. J. Biol. Chem. 288, 7351–7362. https://doi.org/10.1074/jbc.M112.406645

Ahmad, M.S.A., Ashraf, M., 2011. Essential Roles and Hazardous Effects of Nickel in Plants, in: Whitacre, D.M. (Ed.), Reviews of Environmental Contamination and Toxicology, Reviews of Environmental Contamination and Toxicology. Springer New York, New York, NY, pp. 125–167. https://doi.org/10.1007/978-1-4614-0668-6_6

Al Agely, A., Sylvia, D.M., Ma, L.Q., 2005. Mycorrhizae Increase Arsenic Uptake by the Hyperaccumulator Chinese Brake Fern (Pteris vittata L.). Journal of Environmental Quality 34, 2181–2186. https://doi.org/10.2134/jeq2004.0411

Alford, É.R., Pilon-Smits, E.A.H., Paschke, M.W., 2010. Metallophytes-a view from the rhizosphere. Plant and Soil 337, 33–50. https://doi.org/10.1007/s11104-010-0482-3

Ali, H., Khan, E., Sajad, M.A., 2013. Phytoremediation of heavy metals—Concepts and applications. Chemosphere 91, 869–881. https://doi.org/10.1016/j.chemosphere.2013.01.075

Álvarez-López, V., Prieto-Fernández, Á., Becerra-Castro, C., Monterroso, C., Kidd, P.S., 2016. Rhizobacterial communities associated with the flora of three serpentine outcrops of the Iberian Peninsula. Plant Soil 403, 233–252. https://doi.org/10.1007/s11104-015-2632-0

Amir, H., Lagrange, A., Hassaïne, N., Cavaloc, Y., 2013. Arbuscular mycorrhizal fungi from New Caledonian ultramafic soils improve tolerance to nickel of endemic plant species. Mycorrhiza 23, 585–595. https://doi.org/10.1007/s00572-013-0499-6

Anjum, N.A., Singh, H.P., Khan, M.I.R., Masood, A., Per, T.S., Negi, A., Batish, D.R., Khan, N.A., Duarte, A.C., Pereira, E., Ahmad, I., 2015. Too much is bad—an appraisal of phytotoxicity of elevated plant-

beneficial heavy metal ions. Environ Sci Pollut Res 22, 3361–3382. https://doi.org/10.1007/s11356-014-3849-9

Arita, A., Costa, M., 2009. Epigenetics in metal carcinogenesis: nickel, arsenic, chromium and cadmium. Metallomics 1, 222–228. https://doi.org/10.1039/B903049B

Assunção, A.G.L., Bleeker, P., ten Bookum, W.M., Vooijs, R., Schat, H., 2008. Intraspecific variation of metal preference patterns for hyperaccumulation in Thlaspi caerulescens: evidence from binary metal exposures. Plant Soil 303, 289–299. https://doi.org/10.1007/s11104-007-9508-x

Assunção, A.G.L., Schat, H., Aarts, M.G.M., 2003. Thlaspi caerulescens, an attractive model species to study heavy metal hyperaccumulation in plants. New Phytologist 351–360. https://doi.org/10.1046/j.1469-8137.2003.00820.x@10.1111/(ISSN)1469-8137.HeavymetalsandplantsAug2003

Baiyeri, K.P., Mbah, B.N., 2006. Surface sterilization and duration of seed storage influenced emergence and seedling quality of African breadfruit (Treculia africana Decne). African Journal of Biotechnology 5, 1393–1396.

Baker, A.J., Ernst, W.H., van der Ent, A., Malaisse, F., Ginocchio, R., 2010. Metallophytes: the unique biological resource, its ecology and conservational status in Europe, central Africa and Latin America. Ecology of industrial pollution 7–40.

BAKER, A.J.M., 2000. Metal hyperaccumulator plants : A review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils. Phytoremediation of Contaminated Soil and Water.

Baker, A.J.M., Brooks, R., 1989. Terrestrial higher plants which hyperaccumulate metallic elements. A review of their distribution, ecology and phytochemistry. Biorecovery. 1, 81–126.

Bandyopadhyay, K., Aggarwal, P., Chakraborty, D., Pradhan, S., Narayan Garg, R., Singh, R., 2012. Practical Manual on Measurement of Soil Physical Properties Practical.

Bani, A., Echevarria, G., Sulçe, S., Morel, J.L., 2015a. Improving the Agronomy of Alyssum murale for Extensive Phytomining: A Five-Year Field Study. International Journal of Phytoremediation 17, 117–127. https://doi.org/10.1080/15226514.2013.862204

Bani, A., Echevarria, G., Zhang, X., Benizri, E., Laubie, B., Morel, J.L., Simonnot, M.-O., 2015b. The effect of plant density in nickel-phytomining field experiments with *Alyssum murale* in Albania. Aust. J. Bot. 63, 72–77. https://doi.org/10.1071/BT14285

Barzanti, R., Ozino, F., Bazzicalupo, M., Gabbrielli, R., Galardi, F., Gonnelli, C., Mengoni, A., 2007. Isolation and Characterization of Endophytic Bacteria from the Nickel Hyperaccumulator Plant Alyssum bertolonii. Microb Ecol 53, 306–316. https://doi.org/10.1007/s00248-006-9164-3

Becerra-Castro, C., Monterroso, C., García-Lestón, M., Prieto-Fernández, A., Acea, M.J., Kidd, P.S., 2009. Rhizosphere Microbial Densities and Trace Metal Tolerance of the Nickel Hyperaccumulator Alyssum Serpyllifolium Subsp. Lusitanicum. International Journal of Phytoremediation 11, 525–541. https://doi.org/10.1080/15226510902717549

Benizri, E., Baudoin, E., Guckert, A., 2001. Root Colonization by Inoculated Plant Growth-Promoting Rhizobacteria. Biocontrol Science and Technology 11, 557–574. https://doi.org/10.1080/09583150120076120

Bergey, D.H., Boone, D.R., Castenholz, R.W., Garrity, G.M., 2001. Bergey's manual of systematic bacteriology. Vol. 1, Archaea and the deeply branching and phototrophic bacteria. Springer.

Boominathan, R., Doran, P.M., 2002. Ni-induced oxidative stress in roots of the Ni hyperaccumulator, Alyssum bertolonii. New Phytologist 156, 205–215. https://doi.org/10.1046/j.1469-8137.2002.00506.x

Brady, K.U., Kruckeberg, A.R., Jr, H.D.B., 2005. Evolutionary Ecology of Plant Adaptation to Serpentine Soils. Annual Review of Ecology, Evolution, and Systematics 36, 243–266. https://doi.org/10.1146/annurev.ecolsys.35.021103.105730

Brestic, M., Zivcak, M., 2013. PSII Fluorescence Techniques for Measurement of Drought and High Temperature Stress Signal in Crop Plants: Protocols and Applications, in: Rout, G.R., Das, A.B. (Eds.), Molecular Stress Physiology of Plants. Springer India, India, pp. 87–131. https://doi.org/10.1007/978-81-322-0807-5_4

Bric, J.M., Bostock, R.M., Silverstone, S.E., 1991. Rapid In Situ Assay for Indoleacetic Acid Production by Bacteria Immobilized on a Nitrocellulose Membrane. Appl. Environ. Microbiol. 57, 535–538.

Broadhurst, C.L., Chaney, R.L., 2016. Growth and Metal Accumulation of an Alyssum murale Nickel Hyperaccumulator Ecotype Co-cropped with Alyssum montanum and Perennial Ryegrass in Serpentine Soil. Front Plant Sci 7, 451. https://doi.org/10.3389/fpls.2016.00451

Brooks, R.R., 1998. Plants that Hyperaccumulate Heavy Metals, CAB. Pub. UK.

Brooks, R.R., 1987. Serpentine and its vegetation: a multidisciplinary approach. Ecology, phytogeography and physiology series (USA).

Brooks, R.R., Lee, J., Reeves, R.D., Jaffre, T., 1977. Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. Journal of Geochemical Exploration 7, 49–57. https://doi.org/10.1016/0375-6742(77)90074-7

Cabello-Conejo, M.I., Becerra-Castro, C., Prieto-Fernández, A., Monterroso, C., Saavedra-Ferro, A., Mench, M., Kidd, P.S., 2014. Rhizobacterial inoculants can improve nickel phytoextraction by the hyperaccumulator <Emphasis Type="Italic">Alyssum pintodasilvae</Emphasis>. Plant Soil 379, 35–50. https://doi.org/10.1007/s11104-014-2043-7

Callahan, D.L., Baker, A.J.M., Kolev, S.D., Wedd, A.G., 2006. Metal ion ligands in hyperaccumulating plants. J Biol Inorg Chem 11, 2–12. https://doi.org/10.1007/s00775-005-0056-7

Capponi, G., Crispini, L., 2008. Note illustrative alla Carta Geologica d'Italia alla scala 1:50.000. Foglio 213 - 230 "Genova".

Carlson, C.L., Adriano, D.C., Sajwan, K.S., Abels, S.L., Thoma, D.P., Driver, J.T., 1991. Effects of selected trace metals on germinating seeds of six plant species. Water, Air, and Soil Pollution 59, 231–240.

Cecchi, G., Marescotti, P., Piazza, S.D., Zotti, M., 2017a. Native fungi as metal remediators: Silver myco-accumulation from metal contaminated waste-rock dumps (Libiola Mine, Italy). Journal of Environmental Science and Health, Part B 52, 191–195. https://doi.org/10.1080/03601234.2017.1261549

Cecchi, G., Roccotiello, E., Piazza, S.D., Riggi, A., Mariotti, M.G., Zotti, M., 2017b. Assessment of Ni accumulation capability by fungi for a possible approach to remove metals from soils and waters. Journal of Environmental Science and Health, Part B 52, 166–170. https://doi.org/10.1080/03601234.2017.1261539

Cempel, M., Nikel, G., n.d. Nickel: A Review of Its Sources and Environmental Toxicology 8.

Centofanti, T., Sayers, Z., Cabello-Conejo, M.I., Kidd, P., Nishizawa, N.K., Kakei, Y., Davis, A.P., Sicher, R.C., Chaney, R.L., 2013. Xylem exudate composition and root-to-shoot nickel translocation in Alyssum species. Plant Soil 373, 59–75. https://doi.org/10.1007/s11104-013-1782-1

Chaney, R.L., 1983. Plant uptake of inorganic waste. Land treatment of hazardous wastes.

Chaney, R.L., Angle, J.S., Broadhurst, C.L., Peters, C.A., Tappero, R.V., Sparks, D.L., 2007. Improved Understanding of Hyperaccumulation Yields Commercial Phytoextraction and Phytomining Technologies. Journal of Environmental Quality 36, 1429–1443. https://doi.org/10.2134/jeq2006.0514

Chaney, R.L., Malik, M., Li, Y.M., Brown, S.L., Brewer, E.P., Angle, J.S., Baker, A.J., 1997. Phytoremediation of soil metals. Current Opinion in Biotechnology 8, 279–284. https://doi.org/10.1016/S0958-1669(97)80004-3

Charlot, G., 1964. Colorimetric Determination of Elements, Principles and Methods: By G. Charlot. Elsevier.

Chen, C., Huang, D., Liu, J., 2009. Functions and Toxicity of Nickel in Plants: Recent Advances and Future Prospects. CLEAN – Soil, Air, Water 37, 304–313. https://doi.org/10.1002/clen.200800199

Chiarucci, A., Baker, A.J.M., 2007. Advances in the ecology of serpentine soils. Plant Soil 293, 1–2. https://doi.org/10.1007/s11104-007-9268-7

Comerford, N.B., 2005. Soil Factors Affecting Nutrient Bioavailability, in: Nutrient Acquisition by Plants, Ecological Studies. Springer, Berlin, Heidelberg, pp. 1–14. https://doi.org/10.1007/3-540-27675-0_1

Corem, S., Carpaneto, A., Soliani, P., Cornara, L., Gambale, F., Scholz-Starke, J., 2009. Response to cytosolic nickel of Slow Vacuolar channels in the hyperaccumulator plant Alyssum bertolonii. Eur Biophys J 38, 495. https://doi.org/10.1007/s00249-008-0400-2

DalCorso, G., Manara, A., Furini, A., 2013. An overview of heavy metal challenge in plants: from roots to shoots. Metallomics 5, 1117–1132. https://doi.org/10.1039/C3MT00038A

de Souza, R., Ambrosini, A., Passaglia, L.M.P., 2015. Plant growth-promoting bacteria as inoculants in agricultural soils. Genet Mol Biol 38, 401–419. https://doi.org/10.1590/S1415-475738420150053

Dechamps, C., Noret, N., Mozek, R., Draye, X., Meerts, P., 2008. Root allocation in metal-rich patch by Thlaspi caerulescens from normal and metalliferous soil—new insights into the rhizobox approach. Plant Soil 310, 211. https://doi.org/10.1007/s11104-008-9648-7

Degenkolb, T., von Döhren, H., Fog Nielsen, K., Samuels, G.J., Brückner, H., 2008. Recent Advances and Future Prospects in Peptaibiotics, Hydrophobin, and Mycotoxin Research, and Their Importance for Chemotaxonomy of Trichoderma and Hypocrea. Chemistry & Biodiversity 5, 671–680. https://doi.org/10.1002/cbdv.200890064

Demchenko, N.P., Kalimova, I.B., Demchenko, K.N., 2010. Effect of nickel at high concentration on proliferation of quiescent center cells and initiation of lateral root primordia in wheat seedlings. Russ J Plant Physiol 57, 438–447. https://doi.org/10.1134/S1021443710030179

Demirel, R., Ilhan, S., Asan, A., Kinaci, E., Oner, S., 2005. Microfungi in cultivated fields in Eskişehir provience (Turkey). Journal of Basic Microbiology 45, 279–293. https://doi.org/10.1002/jobm.200410526

Deng, D.M., Shu, W.S., Zhang, J., Zou, H.L., Lin, Z., Ye, Z.H., Wong, M.H., 2007. Zinc and cadmium accumulation and tolerance in populations of Sedum alfredii. Environmental Pollution, Environmental Pollution in China 147, 381–386. https://doi.org/10.1016/j.envpol.2006.05.024

Deng, T.-H.-B., van der Ent, A., Tang, Y.-T., Sterckeman, T., Echevarria, G., Morel, J.-L., Qiu, R.-L., 2018. Nickel hyperaccumulation mechanisms: a review on the current state of knowledge. Plant Soil 423, 1–11. https://doi.org/10.1007/s11104-017-3539-8

Di Piazza, S., Cecchi, G., Cardinale, A.M., Carbone, C., Mariotti, M.G., Giovine, M., Zotti, M., 2017. Penicillium expansum Link strain for a biometallurgical method to recover REEs from WEEE. Waste Management, Special Thematic Issue: Urban Mining and Circular Economy 60, 596–600. https://doi.org/10.1016/j.wasman.2016.07.029

Domsch, K.H., Gams, W., Anderson, T.H., 2007. Compendium of soil fungi, 2nd taxonomically revised edition by W. Gams. IHW, Eching.

Donohue, K., Rubio de Casas, R., Burghardt, L., Kovach, K., Willis, C.G., 2010. Germination, Postgermination Adaptation, and Species Ecological Ranges. Annual Review of Ecology, Evolution, and Systematics 41, 293–319. https://doi.org/10.1146/annurev-ecolsys-102209-144715

Downie, H.F., Adu, M.O., Schmidt, S., Otten, W., Dupuy, L.X., White, P.J., Valentine, T.A., 2015. Challenges and opportunities for quantifying roots and rhizosphere interactions through imaging and image analysis. Plant Cell Environ 38, 1213–1232. https://doi.org/10.1111/pce.12448

Durand, A., Piutti, S., Rue, M., Morel, J.L., Echevarria, G., Benizri, E., 2016. Improving nickel phytoextraction by co-cropping hyperaccumulator plants inoculated by plant growth promoting rhizobacteria. Plant Soil 399, 179–192. https://doi.org/10.1007/s11104-015-2691-2

Dworkin, M., Foster, J.W., 1958. EXPERIMENTS WITH SOME MICROORGANISMS WHICH UTILIZE ETHANE AND HYDROGEN1. J Bacteriol 75, 592–603.

Eissa, M.A., 2016. Phytomining.

EPA, U., 2007. Method 6200: Field Portable X-Ray Fluorescence Spectrometry for the Determination of Elemental Concentrations in Soil and Sediment.

Favas, P.J., Pratas, J., Varun, M., D'Souza, R., Paul, M.S., 2014. Phytoremediation of soils contaminated with metals and metalloids at mining areas: potential of native flora, in: Environmental Risk Assessment of Soil Contamination. IntechOpen.

Fenner, M., Thompson, K., 2005. The ecology of seeds. Cambridge University Press.

Fomina, M.A., Alexander, I.J., Colpaert, J.V., Gadd, G.M., 2005. Solubilization of toxic metal minerals and metal tolerance of mycorrhizal fungi. Soil Biology and Biochemistry 37, 851–866. https://doi.org/10.1016/j.soilbio.2004.10.013

Fones, H.N., Preston, G.M., Smith, J.A.C., 2019. Variation in defence strategies in the metal hyperaccumulator plant Noccaea caerulescens is indicative of synergies and trade-offs between forms of defence. R Soc Open Sci 6. https://doi.org/10.1098/rsos.172418

Freitas, H., Prasad, M.N.V., Pratas, J., 2004. Analysis of serpentinophytes from north–east of Portugal for trace metal accumulation—relevance to the management of mine environment. Chemosphere 54, 1625–1642. https://doi.org/10.1016/j.chemosphere.2003.09.045

Fryzova, R., Pohanka, M., Martinkova, P., Cihlarova, H., Brtnicky, M., Hladky, J., Kynicky, J., 2018. Oxidative Stress and Heavy Metals in Plants. Rev Environ Contam Toxicol 245, 129–156. https://doi.org/10.1007/398_2017_7

Furbank, R.T., Caemmerer, S. von, Sheehy, J., Edwards, G., 2009. C4 rice: a challenge for plant phenomics. Functional Plant Biol. 36, 845–856. https://doi.org/10.1071/FP09185

Gadd, G.M., 2007. Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. Mycological Research 111, 3–49. https://doi.org/10.1016/j.mycres.2006.12.001

Gadd, G.M., 1993. Interactions of fungip with toxic metals. New Phytologist 124, 25–60. https://doi.org/10.1111/j.1469-8137.1993.tb03796.x

Gadd, G.M., Fomina, M., 2011. Uranium and Fungi. Geomicrobiology Journal 28, 471–482. https://doi.org/10.1080/01490451.2010.508019

Gajewska, E., Niewiadomska, E., Tokarz, K., Słaba, M., Skłodowska, M., 2013. Nickel-induced changes in carbon metabolism in wheat shoots. Journal of Plant Physiology 170, 369–377. https://doi.org/10.1016/j.jplph.2012.10.012

Gajewska, E., Skłodowska, M., 2009. Nickel-induced changes in nitrogen metabolism in wheat shoots. Journal of Plant Physiology 166, 1034–1044. https://doi.org/10.1016/j.jplph.2008.12.004

Galardi, F., Mengoni, A., Pucci, S., Barletti, L., Massi, L., Barzanti, R., Gabbrielli, R., Gonnelli, C., 2007. Intra-specific differences in mineral element composition in the Ni-hyperaccumulator Alyssum bertolonii: a survey of populations in nature. Environmental and experimental botany 60, 50–56.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular ecology 2, 113–118.

Gendre, D., Czernic, P., Conéjéro, G., Pianelli, K., Briat, J.-F., Lebrun, M., Mari, S., 2007. TcYSL3, a member of the YSL gene family from the hyper-accumulator Thlaspi caerulescens, encodes a nicotianamine-Ni/Fe transporter. The Plant Journal 49, 1–15. https://doi.org/10.1111/j.1365-313X.2006.02937.x

Ghaderian, S.M., Mohtadi, A., Rahiminejad, R., Reeves, R.D., Baker, A.J.M., 2007. Hyperaccumulation of nickel by two Alyssum species from the serpentine soils of Iran. Plant Soil 293, 91–97. https://doi.org/10.1007/s11104-007-9221-9

Ghasemi, R., Chavoshi, Z.Z., Boyd, R.S., Rajakaruna, N., 2015. Calcium : magnesium ratio affects environmental stress sensitivity in the serpentine-endemic Alyssum inflatum (Brassicaceae). Aust. J. Bot. 63, 39–46. https://doi.org/10.1071/BT14235

Giammarino, S., Orezzi, S., Rosti, D., Fannucci, F., Morelli, D., De Stefanis, A., 2010. Note Illustrative della Carta Geologica d'Italia alla scala 1: 50.000-Foglio 258–271 Sanremo.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. 61, 1323–1330.

Glick, B.R., 2010. Using soil bacteria to facilitate phytoremediation. Biotechnology Advances 28, 367–374. https://doi.org/10.1016/j.biotechadv.2010.02.001

Gonneau, C., 2014. Distribution, écologie et évolution de l'hyperaccumulation des éléments en traces par Noccaea caerulescens (PhD Thesis). Université de Lorraine.

Gonzaga, M.I.S., Ma, L.Q., Santos, J.A.G., Matias, M.I.S., 2009. Rhizosphere characteristics of two arsenic hyperaccumulating Pteris ferns. Science of The Total Environment 407, 4711–4716. https://doi.org/10.1016/j.scitotenv.2009.04.037

González-Valdez, E., Alarcón, A., Ferrera-Cerrato, R., Vega-Carrillo, H.R., Maldonado-Vega, M., Salas-Luévano, M.Á., Argumedo-Delira, R., 2018. Induced accumulation of Au, Ag and Cu in Brassica napus grown in a mine tailings with the inoculation of Aspergillus niger and the application of two chemical compounds. Ecotoxicol. Environ. Saf. 154, 180–186. https://doi.org/10.1016/j.ecoenv.2018.02.055

Gopalakrishnan, S., Sathya, A., Vijayabharathi, R., Varshney, R.K., Gowda, C.L.L., Krishnamurthy, L., 2015. Plant growth promoting rhizobia: challenges and opportunities. 3 Biotech 5, 355–377. https://doi.org/10.1007/s13205-014-0241-x

Goswami, D., Thakker, J.N., Dhandhukia, P.C., 2015. Simultaneous detection and quantification of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) produced by rhizobacteria from l-tryptophan (Trp) using HPTLC. Journal of Microbiological Methods 110, 7–14. https://doi.org/10.1016/j.mimet.2015.01.001

Greco, G., Capello, M., Cecchi, G., Cutroneo, L., Di, P., Zotti, M., 2017. Another possible risk for the Mediterranean Sea? Aspergillus sydowii discovered in the Port of Genoa (Ligurian Sea, Italy). Marine Pollution Bulletin 122, 470–474. https://doi.org/10.1016/j.marpolbul.2017.06.058

Greipsson, S., 2011. Phytoremediation. Nature Education Knowledge 3, 7.

Halimaa, P., Blande, D., Aarts, M.G.M., Tuomainen, M., Tervahauta, A., Kärenlampi, S., 2014. Comparative transcriptome analysis of the metal hyperaccumulator Noccaea caerulescens. Front Plant Sci 5. https://doi.org/10.3389/fpls.2014.00213

Hanson, J.R., 2005. The chemistry of the bio-control agent, Trichoderma harzianum [WWW Document]. https://doi.org/info:doi/10.3184/003685005783238372

Hassan, M., Gaber, A., El-Hallous, E., 2014. Molecular and Morphological Characterization of Trichoderma harzianum from different Egyptian Soils. Wulfenia journal 0.26.

Haydon, M.J., Cobbett, C.S., 2007. Transporters of ligands for essential metal ions in plants. New Phytologist 174, 499–506. https://doi.org/10.1111/j.1469-8137.2007.02051.x

Heydari, A., Pessarakli, M., 2010. A review on biological control of fungal plant pathogens using microbial antagonists. Journal of Biological Sciences 10, 273–290.

HILTNER, L., 1904. Ober neuter erfahrungen und probleme auf dem gebiete der bodenbakteriologie unter besonderer berucksichtigung der grundungung und brache. Arbeiten der Deutshen Landwirschafthchen Gesellschaft 98, 59–78.

Himmelbauer, M.L., Puschenreiter, M., Schnepf, A., Loiskandl, W., Wenzel, W.W., 2005. Root morphology of Thlaspi goesingense Hálácsy grown on a serpentine soil. Journal of Plant Nutrition and Soil Science 168, 138–144. https://doi.org/10.1002/jpln.200420434

Hodge, A., 2004. The plastic plant: root responses to heterogeneous supplies of nutrients. New Phytologist 162, 9–24. https://doi.org/10.1111/j.1469-8137.2004.01015.x

Hölker, U., Dohse, J., Höfer, M., 2002. Extracellular laccases in ascomycetesTrichoderma atroviride andTrichoderma harzianum. Folia Microbiol 47, 423–427. https://doi.org/10.1007/BF02818702

Hossein Khoshgoftarmanesh, A., Bahmanziari, H., 2012. Stimulating and toxicity effects of nickel on growth, yield, and fruit quality of cucumber supplied with different nitrogen sources. Journal of Plant Nutrition and Soil Science 175, 474–481.

Houbraken, J., López-Quintero, C.A., Frisvad, J.C., Boekhout, T., Theelen, B., Franco-Molano, A.E., Samson, R.A., 2011. Penicillium araracuarense sp. nov., Penicillium elleniae sp. nov., Penicillium penarojense sp. nov., Penicillium vanderhammenii sp. nov. and Penicillium wotroi sp. nov., isolated from leaf litter. International Journal of Systematic and Evolutionary Microbiology 61, 1462–1475. https://doi.org/10.1099/ijs.0.025098-0

Husna, Tuheteru, F.D., Arif, A., 2017. Arbuscular Mycorrhizal Fungi and Plant Growth on Serpentine Soils, in: Arbuscular Mycorrhizas and Stress Tolerance of Plants. Springer, Singapore, pp. 293–303. https://doi.org/10.1007/978-981-10-4115-0_12

Idris, R., Trifonova, R., Puschenreiter, M., Wenzel, W.W., Sessitsch, A., 2004. Bacterial Communities Associated with Flowering Plants of the Ni Hyperaccumulator Thlaspi goesingense. Appl. Environ. Microbiol. 70, 2667–2677. https://doi.org/10.1128/AEM.70.5.2667-2677.2004

Iram, S., Parveen, K., Usman, J., Nasir, K., Akhtar, N., Arouj, S., Ahmad, I., 2012. Heavy metal tolerance of filamentous fungal strains isolated from soil irrigated with industrial wastewater. Biologija 58. https://doi.org/10.6001/biologija.v58i3.2527

Izosimova, A., 2005. Modelling the interaction between calcium and nickel in the soil-plant system. Bundesforschungsanstalt für Landwirtschaft (FAL).

Jaffré, T., Brooks, R.R., Lee, J., Reeves, R.D., 1976. Sebertia acuminata: A Hyperaccumulator of Nickel from New Caledonia. Science 193, 579–580. https://doi.org/10.1126/science.193.4253.579

Jing, Y., He, Z., Yang, X., 2007. Role of soil rhizobacteria in phytoremediation of heavy metal contaminated soils. J. Zhejiang Univ. - Sci. B 8, 192–207. https://doi.org/10.1631/jzus.2007.B0192

Kabata-Pendias, A., 2010. Trace Elements in Soils and Plants, Fourth Edition. CRC Press.

Kamran, M.A., Amna, Mufti, R., Mubariz, N., Syed, J.H., Bano, A., Javed, M.T., Munis, M.F.H., Tan, Z., Chaudhary, H.J., 2014. The potential of the flora from different regions of Pakistan in phytoremediation: a review. Environ Sci Pollut Res 21, 801–812. https://doi.org/10.1007/s11356-013-2187-7

Keller, C., Hammer, D., Kayser, A., Richner, W., Brodbeck, M., Sennhauser, M., 2003. Root development and heavy metal phytoextraction efficiency: comparison of different plant species in the field. Plant and Soil 249, 67–81.

Kelly, J.J., Tate, R.L., 1998. Effects of Heavy Metal Contamination and Remediation on Soil Microbial Communities in the Vicinity of a Zinc Smelter. Journal of Environmental Quality 27, 609–617. https://doi.org/10.2134/jeq1998.00472425002700030019x

Khan, M.U., Sessitsch, A., Harris, M., Fatima, K., Imran, A., Arslan, M., Shabir, G., Khan, Q.M., Afzal, M., 2015. Cr-resistant rhizo- and endophytic bacteria associated with Prosopis juliflora and their potential as phytoremediation enhancing agents in metal-degraded soils. Front Plant Sci 5. https://doi.org/10.3389/fpls.2014.00755

Kidd, P., Barceló, J., Bernal, M.P., Navari-Izzo, F., Poschenrieder, C., Shilev, S., Clemente, R., Monterroso, C., 2009. Trace element behaviour at the root–soil interface: Implications in phytoremediation. Environmental and Experimental Botany 67, 243–259. https://doi.org/10.1016/j.envexpbot.2009.06.013

Kidd, P.S., Álvarez-López, V., Becerra-Castro, C., Cabello-Conejo, M., Prieto-Fernández, Á., 2017. Chapter Three - Potential Role of Plant-Associated Bacteria in Plant Metal Uptake and Implications in Phytotechnologies, in: Cuypers, A., Vangronsveld, J. (Eds.), Advances in Botanical Research, Phytoremediation. Academic Press, pp. 87–126. https://doi.org/10.1016/bs.abr.2016.12.004

Klein, T., Siegwolf, R.T.W., Körner, C., 2016. Belowground carbon trade among tall trees in a temperate forest. Science 352, 342–344. https://doi.org/10.1126/science.aad6188

Kopecká, M., 2016. Microtubules and Actin Cytoskeleton of Cryptococcus neoformans as Targets for Anticancer Agents to Potentiate a Novel Approach for New Antifungals. CHE 61, 117–121. https://doi.org/10.1159/000437134

Kozhevnikova, A.D., Seregin, I.V., Erlikh, N.T., Shevyreva, T.A., Andreev, I.M., Verweij, R., Schat, H., 2014. Histidine-mediated xylem loading of zinc is a species-wide character in Noccaea caerulescens. New Phytol. 203, 508–519. https://doi.org/10.1111/nph.12816

Krämer, U., 2010. Metal Hyperaccumulation in Plants. Annual Review of Plant Biology 61, 517–534. https://doi.org/10.1146/annurev-arplant-042809-112156

Krämer, U., Pickering, I.J., Prince, R.C., Raskin, I., Salt, D.E., 2000. Subcellular localization and speciation of nickel in hyperaccumulator and non-accumulator Thlaspi species. Plant Physiol. 122, 1343–1353.

Kranner, I., Colville, L., 2011. Metals and seeds: Biochemical and molecular implications and their significance for seed germination. Environmental and Experimental Botany, Metal(loid) tolerance in plants and lichens 72, 93–105. https://doi.org/10.1016/j.envexpbot.2010.05.005

Kubatova, A., Prasil, K., Vanova, M., 2002. Diversity of soil microscopic fungi on abandoned industrial deposits. Cryptogamie. Mycologie 23, 205–219.

Kuffner, M., De Maria, S., Puschenreiter, M., Fallmann, K., Wieshammer, G., Gorfer, M., Strauss, J., Rivelli, A. r., Sessitsch, A., 2010. Culturable bacteria from Zn- and Cd-accumulating Salix caprea with differential effects on plant growth and heavy metal availability. Journal of Applied Microbiology 108, 1471–1484. https://doi.org/10.1111/j.1365-2672.2010.04670.x

Kutschera, L., Lichtenegger, E., Sobotik, M., 1992. Wurzelatlas mitteleuropäischer Grünlandpflanzen. G. Fischer.

Lazarovitch, N., Vanderborght, J., Jin, Y., van Genuchten, M.T., 2018. The Root Zone: Soil Physics and Beyond. Vadose Zone Journal 17. https://doi.org/10.2136/vzj2018.01.0002

Lebeau, T., Braud, A., Jézéquel, K., 2008. Performance of bioaugmentation-assisted phytoextraction applied to metal contaminated soils: A review. Environmental Pollution 153, 497–522. https://doi.org/10.1016/j.envpol.2007.09.015

Lee, J.H., 2013. An overview of phytoremediation as a potentially promising technology for environmental pollution control. Biotechnol Bioproc E 18, 431–439. https://doi.org/10.1007/s12257-013-0193-8

Lemanceau, P., Barret, M., Mazurier, S., Mondy, S., Pivato, B., Fort, T., Vacher, C., 2017. Chapter Five - Plant Communication With Associated Microbiota in the Spermosphere, Rhizosphere and Phyllosphere, in: Becard, G. (Ed.), Advances in Botanical Research, How Plants Communicate with Their Biotic Environment. Academic Press, pp. 101–133. https://doi.org/10.1016/bs.abr.2016.10.007

Liao, M., Xie, X.M., 2007. Effect of heavy metals on substrate utilization pattern, biomass, and activity of microbial communities in a reclaimed mining wasteland of red soil area. Ecotoxicology and Environmental Safety 66, 217–223. https://doi.org/10.1016/j.ecoenv.2005.12.013

Lightfoot, N.E., Berriault, C.J., Seilkop, S.K., Conard, B.R., 2017. Nonrespiratory mortality and cancer incidence in a cohort of Canadian nickel workers. Archives of Environmental & Occupational Health 72, 187–203. https://doi.org/10.1080/19338244.2016.1197879

Liu, F., Tang, Y., Du, R., Yang, H., Wu, Q., Qiu, R., 2010. Root foraging for zinc and cadmium requirement in the Zn/Cd hyperaccumulator plant Sedum alfredii. Plant Soil 327, 365–375. https://doi.org/10.1007/s11104-009-0060-8

Lock, K., Van Eeckhout, H., De Schamphelaere, K.A.C., Criel, P., Janssen, C.R., 2007. Development of a biotic ligand model (BLM) predicting nickel toxicity to barley (Hordeum vulgare). Chemosphere 66, 1346–1352. https://doi.org/10.1016/j.chemosphere.2006.07.008

Lopes, L.D., Pereira e Silva, M. de C., Andreote, F.D., 2016. Bacterial Abilities and Adaptation Toward the Rhizosphere Colonization. Front Microbiol 7. https://doi.org/10.3389/fmicb.2016.01341

Luo, S., Chen, L., Chen, J., Xiao, X., Xu, T., Wan, Y., Rao, C., Liu, C., Liu, Y., Lai, C., Zeng, G., 2011. Analysis and characterization of cultivable heavy metal-resistant bacterial endophytes isolated from Cd-hyperaccumulator Solanum nigrum L. and their potential use for phytoremediation. Chemosphere 85, 1130–1138. https://doi.org/10.1016/j.chemosphere.2011.07.053

Ma, Y., Prasad, M.N.V., Rajkumar, M., Freitas, H., 2011. Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. Biotechnology Advances 29, 248–258. https://doi.org/10.1016/j.biotechadv.2010.12.001

Ma, Y., Rajkumar, M., Freitas, H., 2009a. Isolation and characterization of Ni mobilizing PGPB from serpentine soils and their potential in promoting plant growth and Ni accumulation by Brassica spp. Chemosphere 75, 719–725. https://doi.org/10.1016/j.chemosphere.2009.01.056

Ma, Y., Rajkumar, M., Freitas, H., 2009b. Inoculation of plant growth promoting bacterium Achromobacter xylosoxidans strain Ax10 for the improvement of copper phytoextraction by Brassica juncea. Journal of Environmental Management 90, 831–837. https://doi.org/10.1016/j.jenvman.2008.01.014

Ma, Y., Rajkumar, M., Freitas, H., 2009c. Improvement of plant growth and nickel uptake by nickel resistant-plant-growth promoting bacteria. Journal of Hazardous Materials 166, 1154–1161. https://doi.org/10.1016/j.jhazmat.2008.12.018

Ma, Ying, Rajkumar, M., Luo, Y., Freitas, H., 2011. Inoculation of endophytic bacteria on host and non-host plants—Effects on plant growth and Ni uptake. Journal of Hazardous Materials 195, 230–237. https://doi.org/10.1016/j.jhazmat.2011.08.034

Ma, Y., Rajkumar, M., Vicente, J.A.F., Freitas, H., 2010. Inoculation of Ni-Resistant Plant Growth Promoting Bacterium Psychrobacter sp. Strain SRS8 for the Improvement of Nickel Phytoextraction by Energy Crops. International Journal of Phytoremediation 13, 126–139. https://doi.org/10.1080/15226511003671403

Macnair, M.R., 2003. The hyperaccumulation of metals by plants. Advances in Botanical Research 40, 63–105.

Madsen, A.M., Hansen, V.M., Meyling, N.V., Eilenberg, J., 2007. Human exposure to airborne fungi from genera used as biocontrol agents in plant production. Ann Agric Environ Med. 14, 5–24.

Manousaki, E., Kalogerakis, N., 2010. Halophytes present new opportunities in phytoremediation of heavy metals and saline soils. Industrial & Engineering Chemistry Research 50, 656–660.

Manteca, Á., Yagüe, P., 2018. Streptomyces Differentiation in Liquid Cultures as a Trigger of Secondary Metabolism. Antibiotics (Basel) 7. https://doi.org/10.3390/antibiotics7020041

Margulis, L., 1993. Symbiosis in cell evolution. Microbial communities in the archaean and proterozoic Eons.
Margulis, L., Fester, R., 1991. Symbiosis as a source of evolutionary innovation: speciation and morphogenesis. Mit Press.
Mari, S., Gendre, D., Pianelli, K., Ouerdane, L., Lobinski, R., Briat, J.-F., Lebrun, M., Czernic, P., 2006. Root-to-shoot long-distance circulation of nicotianamine and nicotianamine-nickel chelates in the metal hyperaccumulator Thlaspi caerulescens. J. Exp. Bot. 57, 4111–4122. https://doi.org/10.1093/jxb/erl184
Marsili, S., Roccotiello, E., Carbone, C., Marescotti, P., Cornara, L., Mariotti, M.G., 2009. Plant Colonization on a Contaminated Serpentine Site. Northeastern Naturalist 16, 297–308. https://doi.org/10.1656/045.016.0522
Massaccesi, G., Romero, M.C., Cazau, M.C., Bucsinszky, A.M., 2002. Cadmium removal capacities of filamentous soil fungi isolated from industrially polluted sediments, in La Plata (Argentina). World Journal of Microbiology and Biotechnology 18, 817–820. https://doi.org/10.1023/A:1021282718440
Mehes-Smith, M., Nkongolo, K.K., 2015. Physiological and Cytological Responses of Deschampsia cespitosa and Populus tremuloides to Soil Metal Contamination. Water Air Soil Pollut 226, 125. https://doi.org/10.1007/s11270-015-2382-x
Mengoni, A., Barzanti, R., Gonnelli, C., Gabbrielli, R., Bazzicalupo, M., 2001. Characterization of nickel-resistant bacteria isolated from serpentine soil. Environmental Microbiology 3, 691–698. https://doi.org/10.1046/j.1462-2920.2001.00243.x
Mengoni, A., Grassi, E., Barzanti, R., Biondi, E.G., Gonnelli, C., Kim, C.K., Bazzicalupo, M., 2004. Genetic diversity of bacterial communities of serpentine soil and of rhizosphere of the nickel-hyperaccumulator plant Alyssum bertolonii. Microbial ecology 48, 209–217.
Mengoni, A., Pini, F., Huang, L.-N., Shu, W.-S., Bazzicalupo, M., 2009. Plant-by-Plant Variations of Bacterial Communities Associated with Leaves of the Nickel Hyperaccumulator Alyssum bertolonii Desv. Microb Ecol 58, 660–667. https://doi.org/10.1007/s00248-009-9537-5
Mengoni, A., Schat, H., Vangronsveld, J., 2010. Plants as extreme environments? Ni-resistant bacteria and Ni-hyperaccumulators of serpentine flora. Plant Soil 331, 5–16. https://doi.org/10.1007/s11104-009-0242-4
Merlot, S., Hannibal, L., Martins, S., Martinelli, L., Amir, H., Lebrun, M., Thomine, S., 2014. The metal transporter PgIREG1 from the hyperaccumulator Psychotria gabriellae is a candidate gene for nickel tolerance and accumulation. J Exp Bot 65, 1551–1564. https://doi.org/10.1093/jxb/eru025
Milner, M.J., Kochian, L.V., 2008. Investigating Heavy-metal Hyperaccumulation using Thlaspi caerulescens as a Model System. Ann Bot 102, 3–13. https://doi.org/10.1093/aob/mcn063
Mincey, K., 2018. Ecological and Genetic Investigations of the Nickel Hyperaccumulator Streptanthus polygaloides (Brassicaceae).
Miransari, M., 2011. Interactions between arbuscular mycorrhizal fungi and soil bacteria. Appl Microbiol Biotechnol 89, 917–930. https://doi.org/10.1007/s00253-010-3004-6
Mirouze, M., Paszkowski, J., 2011. Epigenetic contribution to stress adaptation in plants. Current opinion in plant biology 14, 267–274.
Montes, J.M., Melchinger, A.E., Reif, J.C., 2007. Novel throughput phenotyping platforms in plant genetic studies. Trends in Plant Science 12, 433–436. https://doi.org/10.1016/j.tplants.2007.08.006
Moradi, A.B., Conesa, H.M., Robinson, B.H., Lehmann, E., Kaestner, A., Schulin, R., 2009. Root responses to soil Ni heterogeneity in a hyperaccumulator and a non-accumulator species. Environmental Pollution 157, 2189–2196. https://doi.org/10.1016/j.envpol.2009.04.015

Morgan, J. a. W., Bending, G.D., White, P.J., 2005. Biological costs and benefits to plant–microbe interactions in the rhizosphere. J Exp Bot 56, 1729–1739. https://doi.org/10.1093/jxb/eri205

Murchie, E.H., Lawson, T., 2013. Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. J Exp Bot 64, 3983–3998. https://doi.org/10.1093/jxb/ert208

Mustafiz, A., Ghosh, A., Tripathi, A.K., Kaur, C., Ganguly, A.K., Bhavesh, N.S., Tripathi, J.K., Pareek, A., Sopory, S.K., Singla-Pareek, S.L., 2014. A unique Ni2+ -dependent and methylglyoxal-inducible rice glyoxalase I possesses a single active site and functions in abiotic stress response. The Plant Journal 78, 951–963. https://doi.org/10.1111/tpj.12521

Naveed, M., Mitter, B., Yousaf, S., Pastar, M., Afzal, M., Sessitsch, A., 2014. The endophyte <Emphasis Type="Italic">Enterobacter</Emphasis> sp. FD17: a maize growth enhancer selected based on rigorous testing of plant beneficial traits and colonization characteristics. Biol Fertil Soils 50, 249–262. https://doi.org/10.1007/s00374-013-0854-y

Nazir, R., Mazurier, S., Yang, P., Lemanceau, P., van Elsas, J.D., 2017. The Ecological Role of Type Three Secretion Systems in the Interaction of Bacteria with Fungi in Soil and Related Habitats Is Diverse and Context-Dependent. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.00038

Negi, A., Singh, H.P., Batish, D.R., Kohli, R.K., 2014. Ni+2-inhibited radicle growth in germinating wheat seeds involves alterations in sugar metabolism. Acta Physiol Plant 36, 923–929. https://doi.org/10.1007/s11738-013-1471-y

Nishida, S., Tsuzuki, C., Kato, A., Aisu, A., Yoshida, J., Mizuno, T., 2011. AtIRT1, the Primary Iron Uptake Transporter in the Root, Mediates Excess Nickel Accumulation in Arabidopsis thaliana. Plant Cell Physiol 52, 1433–1442. https://doi.org/10.1093/pcp/pcr089

Nkrumah, P.N., Echevarria, G., Erskine, P.D., van der Ent, A., 2018. Nickel hyperaccumulation in Antidesma montis-silam: from herbarium discovery to collection in the native habitat. Ecological Research 1–11.

Ou, X., Zhang, Y., Xu, C., Lin, X., Zang, Q., Zhuang, T., Jiang, L., von Wettstein, D., Liu, B., 2012. Transgenerational inheritance of modified DNA methylation patterns and enhanced tolerance induced by heavy metal stress in rice (Oryza sativa L.). PloS one 7, e41143.

Pal, A., Ghosh, S., Paul, A.K., 2006. Biosorption of cobalt by fungi from serpentine soil of Andaman. Bioresource Technology 97, 1253–1258. https://doi.org/10.1016/j.biortech.2005.01.043

Pal, A., Wauters, G., Paul, A.K., 2007. Nickel tolerance and accumulation by bacteria from rhizosphere of nickel hyperaccumulators in serpentine soil ecosystem of Andaman, India. Plant Soil 293, 37–48. https://doi.org/10.1007/s11104-007-9195-7

Pandey, N., Sharma, C.P., 2002. Effect of heavy metals Co2+, Ni2+ and Cd2+ on growth and metabolism of cabbage. Plant Science 163, 753–758. https://doi.org/10.1016/S0168-9452(02)00210-8

Pardo, T., Rodríguez-Garrido, B., Saad, R.F., Soto-Vázquez, J.L., Loureiro-Viñas, M., Prieto-Fernández, Á., Echevarria, G., Benizri, E., Kidd, P.S., 2018. Assessing the agromining potential of Mediterranean nickel-hyperaccumulating plant species at field-scale in ultramafic soils under humid-temperate climate. Science of the Total Environment 630, 275–286.

Parida, B.K., Chhibba, I.M., Nayyar, V.K., 2003. Influence of nickel-contaminated soils on fenugreek (Trigonella corniculata L.) growth and mineral composition. Scientia Horticulturae 98, 113–119. https://doi.org/10.1016/S0304-4238(02)00208-X

Partida-Martinez, L.P., Monajembashi, S., Greulich, K.-O., Hertweck, C., 2007. Endosymbiont-Dependent Host Reproduction Maintains Bacterial-Fungal Mutualism. Current Biology 17, 773–777. https://doi.org/10.1016/j.cub.2007.03.039

Pasquet, C., Monna, F., van Oort, F., Gunkel-Grillon, P., Laporte-Magoni, C., Losno, R., Chateau, C., 2018. Mobility of Ni, Co, and Mn in ultramafic mining soils of New Caledonia, assessed by kinetic EDTA extractions. Environ Monit Assess 190, 638. https://doi.org/10.1007/s10661-018-7029-0

Pavlova, D., Vila, D., Vila, K., Bani, A., Xhaferri, B., 2018. EFFECT OF NICKEL ON SEED GERMINATION OF ALYSSUM SPECIES WITH POTENTIAL FOR PHYTOMIMING IN ALBANIA. Fresenius Environmental Bulletin 27.

Penrose, D.M., Glick, B.R., n.d. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiologia Plantarum 118, 10–15. https://doi.org/10.1034/j.1399-3054.2003.00086.x

Peuke, A.D., Rennenberg, H., 2005. Phytoremediation: Molecular biology, requirements for application, environmental protection, public attention and feasibility. EMBO reports 6, 497–501. https://doi.org/10.1038/sj.embor.7400445

Pham, V.H.T., Kim, J., 2012. Cultivation of unculturable soil bacteria. Trends Biotechnol. 30, 475–484. https://doi.org/10.1016/j.tibtech.2012.05.007

Pignatti, S., 1982. Flora d'Italia. 3 vols. Bologna: Edagricole.

Pilon-Smits, E., 2005. Phytoremediation. Annual Review of Plant Biology 56, 15–39. https://doi.org/10.1146/annurev.arplant.56.032604.144214

Pollard, A.J., Reeves, R.D., Baker, A.J.M., 2014. Facultative hyperaccumulation of heavy metals and metalloids. Plant Science 217–218, 8–17. https://doi.org/10.1016/j.plantsci.2013.11.011

Prapagdee, B., Chanprasert, M., Mongkolsuk, S., 2013. Bioaugmentation with cadmium-resistant plant growth-promoting rhizobacteria to assist cadmium phytoextraction by Helianthus annuus. Chemosphere 92, 659–666. https://doi.org/10.1016/j.chemosphere.2013.01.082

Rahman, H., Sabreen, S., Alam, S., Kawai, S., 2005. Effects of Nickel on Growth and Composition of Metal Micronutrients in Barley Plants Grown in Nutrient Solution. Journal of Plant Nutrition 28, 393–404. https://doi.org/10.1081/PLN-200049149

Rajakaruna, N., Harris, T.B., Alexander, E.B., 2009. SERPENTINE GEOECOLOGY OF EASTERN NORTH AMERICA: A REVIEW. Rhodora 111, 21–108.

Rajkumar, M., Freitas, H., 2008a. Effects of inoculation of plant-growth promoting bacteria on Ni uptake by Indian mustard. Bioresource Technology 99, 3491–3498. https://doi.org/10.1016/j.biortech.2007.07.046

Rajkumar, M., Freitas, H., 2008b. Influence of metal resistant-plant growth-promoting bacteria on the growth of Ricinus communis in soil contaminated with heavy metals. Chemosphere 71, 834–842. https://doi.org/10.1016/j.chemosphere.2007.11.038

Rajkumar, M., Ma, Y., Freitas, H., 2013a. Improvement of Ni phytostabilization by inoculation of Ni resistant Bacillus megaterium SR28C. Journal of Environmental Management 128, 973–980. https://doi.org/10.1016/j.jenvman.2013.07.001

Rajkumar, M., Prasad, M.N.V., Swaminathan, S., Freitas, H., 2013b. Climate change driven plant–metal–microbe interactions. Environment International 53, 74–86. https://doi.org/10.1016/j.envint.2012.12.009

Raous, S., Becquer, T., Garnier, J., Martins, É. de S., Echevarria, G., Sterckeman, T., 2010. Mobility of metals in nickel mine spoil materials. Applied Geochemistry 25, 1746–1755. https://doi.org/10.1016/j.apgeochem.2010.09.001

Rascio, N., Navari-Izzo, F., 2011. Heavy metal hyperaccumulating plants: How and why do they do it? And what makes them so interesting? Plant Science 180, 169–181. https://doi.org/10.1016/j.plantsci.2010.08.016

Reeves, R.D., 1992. The hyperaccumulation of nickel by serpentine plants. The vegetation of ultramafic (serpentine) soils 253–277.

Reeves, R.D., Baker, A.J., 2000. Metal accumulating plants. Phytoremediation of toxic metals using plants to clean up the environment. Raskin I, Ensley BD (eds.) John Wiley & Sons Inc, New York.

Richau, K.H., Kozhevnikova, A.D., Seregin, I.V., Vooijs, R., Koevoets, P.L.M., Smith, J.A.C., Ivanov, V.B., Schat, H., 2009. Chelation by histidine inhibits the vacuolar sequestration of nickel in roots of the hyperaccumulator Thlaspi caerulescens. New Phytologist 183, 106–116. https://doi.org/10.1111/j.1469-8137.2009.02826.x

Roccotiello, Enrica, Manfredi, A., Drava, G., Minganti, V., Mariotti, M.G., Berta, G., Cornara, L., 2010. Zinc tolerance and accumulation in the ferns Polypodium cambricum L. and Pteris vittata L. Ecotoxicology and environmental safety 73, 1264–1271.

Roccotiello, E., Marescotti, P., Di Piazza, S., Cecchi, G., Mariotti, M.G., Zotti, M., 2015. Biodiversity in Metal-Contaminated Sites–Problem and Perspective–A Case Study. significance 16, 26–28.

Roccotiello, E., Serrano, H.C., Mariotti, M.G., Branquinho, C., 2016. The impact of Ni on the physiology of a Mediterranean Ni-hyperaccumulating plant. Environ Sci Pollut Res 23, 12414–12422. https://doi.org/10.1007/s11356-016-6461-3

Roccotiello, Enrica, Serrano, H.C., Mariotti, M.G., Branquinho, C., 2015. Nickel phytoremediation potential of the Mediterranean Alyssoides utriculata (L.) Medik. Chemosphere 119, 1372–1378. https://doi.org/10.1016/j.chemosphere.2014.02.031

Roccotiello, E., Zotti, M., Mesiti, S., Marescotti, P., Carbone, C., Cornara, L., Mariotti, M.G., 2010. Biodiversity in metal-polluted soils. Fresenius Environmental Bulletin 19, 2420–2425.

Roosens, N., Verbruggen, N., Meerts, P., Ximénez-Embún, P., Smith, J. a. C., 2003. Natural variation in cadmium tolerance and its relationship to metal hyperaccumulation for seven populations of Thlaspi caerulescens from western Europe. Plant, Cell & Environment 26, 1657–1672. https://doi.org/10.1046/j.1365-3040.2003.01084.x

Rudnick, M.B., van Veen, J.A., de Boer, W., 2015. Baiting of rhizosphere bacteria with hyphae of common soil fungi reveals a diverse group of potentially mycophagous secondary consumers. Soil Biology and Biochemistry 88, 73–82. https://doi.org/10.1016/j.soilbio.2015.04.015

Rue, M., Vallance, J., Echevarria, G., Rey, P., Benizri, E., 2015. Phytoextraction of nickel and rhizosphere microbial communities under mono- or multispecies hyperaccumulator plant cover in a serpentine soil. Aust. J. Bot. 63, 92–102. https://doi.org/10.1071/BT14249

Ryser, P., Sauder, W.R., 2006. Effects of heavy-metal-contaminated soil on growth, phenology and biomass turnover of Hieracium piloselloides. Environmental Pollution 140, 52–61. https://doi.org/10.1016/j.envpol.2005.06.026

Sangeeta, M., Maiti, S.K., 2010. Phytoremediation of metal enriched mine waste: a review. American-Eurasian Journal of Agricultural & Environmental Sciences 9, 560–575.

Savitha, M.J., Sriram, S., 2015. Morphological and molecular identification of Trichoderma isolates with biocontrol potential against Phytophthora blight in red pepper. Pest Management In Horticultural Ecosystems 21, 194-202–202.

Scherlach, K., Graupner, K., Hertweck, C., 2013. Molecular Bacteria-Fungi Interactions: Effects on Environment, Food, and Medicine. Annual Review of Microbiology 67, 375–397. https://doi.org/10.1146/annurev-micro-092412-155702

Schlegel, H.G., Cosson, J.-P., Baker, A.J.M., 1991. Nickel-hyperaccumulating Plants Provide a Niche for Nickel-resistant Bacteria. Botanica Acta 104, 18–25. https://doi.org/10.1111/j.1438-8677.1991.tb00189.x

Schlüter, S., Blaser, S.R.G.A., Weber, M., Schmidt, V., Vetterlein, D., 2018. Quantification of Root Growth Patterns From the Soil Perspective via Root Distance Models. Front Plant Sci 9. https://doi.org/10.3389/fpls.2018.01084

Schmidt, M., Goebeler, M., 2011. Nickel allergies: paying the Toll for innate immunity. J Mol Med 89, 961. https://doi.org/10.1007/s00109-011-0780-0

Schwieger, F., Tebbe, C.C., 1998. A New Approach To Utilize PCR–Single-Strand-Conformation Polymorphism for 16S rRNA Gene-Based Microbial Community Analysis. Appl. Environ. Microbiol. 64, 4870–4876.

Schwyn, B., Neilands, J.B., 1987. Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry 160, 47–56. https://doi.org/10.1016/0003-2697(87)90612-9

Segura, A., Rodríguez-Conde, S., Ramos, C., Ramos, J.L., 2009. Bacterial responses and interactions with plants during rhizoremediation. Microbial Biotechnology 2, 452–464. https://doi.org/10.1111/j.1751-7915.2009.00113.x

Selvi, F., Carrari, E., Colzi, I., Coppi, A., Gonnelli, C., 2017. Responses of serpentine plants to pine invasion: Vegetation diversity and nickel accumulation in species with contrasting adaptive strategies. Science of The Total Environment 595, 72–80. https://doi.org/10.1016/j.scitotenv.2017.03.249

Seregin, I.V., Erlikh, N.T., Kozhevnikova, A.D., 2014. Nickel and zinc accumulation capacities and tolerance to these metals in the excluder Thlaspi arvense and the hyperaccumulator Noccaea caerulescens. Russ J Plant Physiol 61, 204–214. https://doi.org/10.1134/S1021443714020137

Seregin, I.V., Kozhevnikova, A.D., 2006. Physiological role of nickel and its toxic effects on higher plants. Russ J Plant Physiol 53, 257–277. https://doi.org/10.1134/S1021443706020178

Sessitsch, A., Kuffner, M., Kidd, P., Vangronsveld, J., Wenzel, W.W., Fallmann, K., Puschenreiter, M., 2013. The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. Soil Biology and Biochemistry 60, 182–194. https://doi.org/10.1016/j.soilbio.2013.01.012

Shah, S., Nasreen, S., Sheikh, P.A., 2012. Cultural and morphological characterization of Trichoderma spp. associated with green mold disease of Pleurotus spp. in Kashmirm. Res. J. Microbiol 7, 139–144.

Shahzad, B., Tanveer, M., Rehman, A., Cheema, S.A., Fahad, S., Rehman, S., Sharma, A., 2018. Nickel; whether toxic or essential for plants and environment - A review. Plant Physiol. Biochem. 132, 641–651. https://doi.org/10.1016/j.plaphy.2018.10.014

Shedbalkar, U., Dhanve, R., Jadhav, J., 2008. Biodegradation of triphenylmethane dye cotton blue by Penicillium ochrochloron MTCC 517. Journal of Hazardous Materials 157, 472–479. https://doi.org/10.1016/j.jhazmat.2008.01.023

Sheoran, I.S., Singal, H.R., Singh, R., 1990. Effect of cadmium and nickel on photosynthesis and the enzymes of the photosynthetic carbon reduction cycle in pigeonpea (Cajanus cajan L.). Photosynth Res 23, 345–351. https://doi.org/10.1007/BF00034865

Sheoran, V., Sheoran, A.S., Poonia, P., 2010. Role of Hyperaccumulators in Phytoextraction of Metals From Contaminated Mining Sites: A Review. Critical Reviews in Environmental Science and Technology 41, 168–214. https://doi.org/10.1080/10643380902718418

Simons, A.M., Johnston, M.O., 2000. Variation in seed traits of Lobelia inflata (Campanulaceae): sources and fitness consequences. American Journal of Botany 87, 124–132.

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H., Berg, G., 2001. Bulk and Rhizosphere Soil Bacterial Communities Studied by Denaturing Gradient Gel Electrophoresis: Plant-Dependent Enrichment and Seasonal Shifts Revealed. Appl. Environ. Microbiol. 67, 4742–4751. https://doi.org/10.1128/AEM.67.10.4742-4751.2001

Soudek, P., Katrušáková, A., Sedláček, L., Petrová, Š., Kočí, V., Maršík, P., Griga, M., Vaněk, T., 2010. Effect of heavy metals on inhibition of root elongation in 23 cultivars of flax (Linum usitatissimum L.). Archives of environmental contamination and toxicology 59, 194–203.

Spiewak, R., Pietowska, J., Curzytek, K., 2007. Nickel: a unique allergen – from molecular structure to European legislation. Expert Review of Clinical Immunology 3, 851–859. https://doi.org/10.1586/1744666X.3.6.851

Sreekanth, T.V.M., Nagajyothi, P.C., Lee, K.D., Prasad, T.N.V.K.V., 2013. Occurrence, physiological responses and toxicity of nickel in plants. Int. J. Environ. Sci. Technol. 10, 1129–1140. https://doi.org/10.1007/s13762-013-0245-9

Sterckeman, T., Cazes, Y., Gonneau, C., Sirguey, C., 2017. Phenotyping 60 populations of Noccaea caerulescens provides a broader knowledge of variation in traits of interest for phytoextraction. Plant Soil 418, 523–540. https://doi.org/10.1007/s11104-017-3311-0

Strasser, R.J., Srivastava, A., Tsimilli-Michael, M., 2000. The fluorescence transient as a tool to characterize and screen photosynthetic samples. Probing photosynthesis: mechanisms, regulation and adaptation 445–483.

Strasser, R.J., Tsimilli-Michael, M., Srivastava, A., 2004. Analysis of the chlorophyll a fluorescence transient, in: Chlorophyll a Fluorescence. Springer, pp. 321–362.

Su, C., 2014. A review on heavy metal contamination in the soil worldwide: Situation, impact and remediation techniques. Environmental Skeptics and Critics 3, 24.

Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy Metal Toxicity and the Environment, in: Luch, A. (Ed.), Molecular, Clinical and Environmental Toxicology, Experientia Supplementum. Springer Basel, pp. 133–164. https://doi.org/10.1007/978-3-7643-8340-4_6

Thijs, S., Langill, T., Vangronsveld, J., 2017. Chapter Two - The Bacterial and Fungal Microbiota of Hyperaccumulator Plants: Small Organisms, Large Influence, in: Cuypers, A., Vangronsveld, J. (Eds.), Advances in Botanical Research, Phytoremediation. Academic Press, pp. 43–86. https://doi.org/10.1016/bs.abr.2016.12.003

Trivedi, P., Pandey, A., Palni, L.M.S., 2008. In vitro evaluation of antagonistic properties of Pseudomonas corrugata. Microbiological Research 163, 329–336. https://doi.org/10.1016/j.micres.2006.06.007

Trotta, A., Falaschi, P., Cornara, L., Minganti, V., Fusconi, A., Drava, G., Berta, G., 2006. Arbuscular mycorrhizae increase the arsenic translocation factor in the As hyperaccumulating fern Pteris vittata L. Chemosphere 65, 74–81. https://doi.org/10.1016/j.chemosphere.2006.02.048

Turgay, O.C., Görmez, A., Bilen, S., 2012. Isolation and characterization of metal resistant-tolerant rhizosphere bacteria from the serpentine soils in Turkey. Environ Monit Assess 184, 515–526. https://doi.org/10.1007/s10661-011-1984-z

Urban, A., Puschenreiter, M., Strauss, J., Gorfer, M., 2008. Diversity and structure of ectomycorrhizal and co-associated fungal communities in a serpentine soil. Mycorrhiza 18, 339–354. https://doi.org/10.1007/s00572-008-0189-y

Vamerali, T., Bandiera, M., Mosca, G., 2010. Field crops for phytoremediation of metal-contaminated land. A review. Environ Chem Lett 8, 1–17. https://doi.org/10.1007/s10311-009-0268-0

van der Ent, A., Baker, A.J.M., Reeves, R.D., Pollard, A.J., Schat, H., 2013. Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. Plant Soil 362, 319–334. https://doi.org/10.1007/s11104-012-1287-3

van der Ent, A. van der, Rajakaruna, N., Boyd, R., Echevarria, G., Repin, R., Williams, D., 2015. Global research on ultramafic (serpentine) ecosystems (8th International Conference on Serpentine Ecology in Sabah, Malaysia): a summary and synthesis. Aust. J. Bot. 63, 1–16. https://doi.org/10.1071/BT15060

van Ditmarsch, D., Xavier, J.B., 2011. High-resolution time series of Pseudomonas aeruginosa gene expression and rhamnolipid secretion through growth curve synchronization. BMC Microbiology 11, 140. https://doi.org/10.1186/1471-2180-11-140

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., Dufresne, A., 2015. The importance of the microbiome of the plant holobiont. New Phytol 206, 1196–1206. https://doi.org/10.1111/nph.13312

Verbruggen, N., Hermans, C., Schat, H., 2009a. Molecular mechanisms of metal hyperaccumulation in plants. New Phytologist 181, 759–776. https://doi.org/10.1111/j.1469-8137.2008.02748.x

Verbruggen, N., Hermans, C., Schat, H., 2009b. Molecular mechanisms of metal hyperaccumulation in plants. New Phytologist 181, 759–776. https://doi.org/10.1111/j.1469-8137.2008.02748.x

Verdú, M., Traveset, A., 2005. Early emergence enhances plant fitness: a phylogenetically controlled meta-analysis. Ecology 86, 1385–1394.

Visagie, C.M., Houbraken, J., Frisvad, J.C., Hong, S.-B., Klaassen, C.H.W., Perrone, G., Seifert, K.A., Varga, J., Yaguchi, T., Samson, R.A., 2014. Identification and nomenclature of the genus Penicillium. Studies in Mycology 78, 343–371. https://doi.org/10.1016/j.simyco.2014.09.001

Vishnoi, S.R., Srivastava, P.N., 2007. Phytoremediation–green for environmental clean, in: Proceedings of Taal2007: The 12th World Lake Conference. p. 1021.

Visioli, G., D'Egidio, S., Sanangelantoni, A.M., 2015a. The bacterial rhizobiome of hyperaccumulators: future perspectives based on omics analysis and advanced microscopy. Front Plant Sci 5. https://doi.org/10.3389/fpls.2014.00752

Visioli, G., D'Egidio, S., Vamerali, T., Mattarozzi, M., Sanangelantoni, A.M., 2014a. Culturable endophytic bacteria enhance Ni translocation in the hyperaccumulator Noccaea caerulescens. Chemosphere 117, 538–544. https://doi.org/10.1016/j.chemosphere.2014.09.014

Visioli, G., Gullì, M., Marmiroli, N., 2014b. Noccaea caerulescens populations adapted to grow in metalliferous and non-metalliferous soils: Ni tolerance, accumulation and expression analysis of genes involved in metal homeostasis. Environmental and experimental botany 105, 10–17.

Visioli, G., Vamerali, T., Mattarozzi, M., Dramis, L., Sanangelantoni, A.M., 2015b. Combined endophytic inoculants enhance nickel phytoextraction from serpentine soil in the hyperaccumulator Noccaea caerulescens. Front Plant Sci 6. https://doi.org/10.3389/fpls.2015.00638

Visioli, G., Vincenzi, S., Marmiroli, M., Marmiroli, N., 2012. Correlation between phenotype and proteome in the Ni hyperaccumulator Noccaea caerulescens subsp. caerulescens. Environmental and Experimental Botany 77, 156–164. https://doi.org/10.1016/j.envexpbot.2011.11.016

Wang, H., He, L., Song, J., Cui, W., Zhang, Y., Jia, C., Francis, D., Rogers, H.J., Sun, L., Tai, P., 2016. Cadmium-induced genomic instability in Arabidopsis: Molecular toxicological biomarkers for early diagnosis of cadmium stress. Chemosphere 150, 258–265.

Warmink, J.A., van Elsas, J.D., 2008. Selection of bacterial populations in the mycosphere of *Laccaria proxima*: is type III secretion involved? The ISME Journal 2, 887–900. https://doi.org/10.1038/ismej.2008.41

Wenzel, W.W., Lombi, E., Adriano, D.C., 2004. Root and Rhizosphere Processes in Metal Hyperaccumulation and Phytoremediation Technology, in: Heavy Metal Stress in Plants. Springer, Berlin, Heidelberg, pp. 313–344. https://doi.org/10.1007/978-3-662-07743-6_13

White, T.J., Bruns, T., Lee, S., Taylor, J.L., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 18, 315–322.

Wuana, R.A., Okieimen, F.E., 2011. Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. Isrn Ecology 2011.

York, L.M., Carminati, A., Mooney, S.J., Ritz, K., Bennett, M.J., 2016. The holistic rhizosphere: integrating zones, processes, and semantics in the soil influenced by roots. J Exp Bot 67, 3629–3643. https://doi.org/10.1093/jxb/erw108

Yusuf, M., Fariduddin, Q., Hayat, S., Ahmad, A., 2011. Nickel: An Overview of Uptake, Essentiality and Toxicity in Plants. Bull Environ Contam Toxicol 86, 1–17. https://doi.org/10.1007/s00128-010-0171-1

Zafra, G., Cortés-Espinosa, D.V., 2015. Biodegradation of polycyclic aromatic hydrocarbons by Trichoderma species: a mini review. Environ Sci Pollut Res 22, 19426–19433. https://doi.org/10.1007/s11356-015-5602-4

Zaidi, S., Usmani, S., Singh, B.R., Musarrat, J., 2006. Significance of Bacillus subtilis strain SJ-101 as a bioinoculant for concurrent plant growth promotion and nickel accumulation in Brassica juncea. Chemosphere 64, 991–997. https://doi.org/10.1016/j.chemosphere.2005.12.057

Zambelli, B., Uversky, V.N., Ciurli, S., 2016. Nickel impact on human health: An intrinsic disorder perspective. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1864, 1714–1731. https://doi.org/10.1016/j.bbapap.2016.09.008

Zhang, Z., Rengel, Z., Meney, K., 2010. Cadmium Accumulation and Translocation in Four Emergent Wetland Species. Water Air Soil Pollut 212, 239–249. https://doi.org/10.1007/s11270-010-0339-7

Zhu, L.-J., Guan, D.-X., Luo, J., Rathinasabapathi, B., Ma, L.Q., 2014. Characterization of arsenic-resistant endophytic bacteria from hyperaccumulators Pteris vittata and Pteris multifida. Chemosphere 113, 9–16. https://doi.org/10.1016/j.chemosphere.2014.03.081

Zhuang, X., Chen, J., Shim, H., Bai, Z., 2007. New advances in plant growth-promoting rhizobacteria for bioremediation. Environment International 33, 406–413. https://doi.org/10.1016/j.envint.2006.12.005

Zilber-Rosenberg, I., Rosenberg, E., 2008. Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. FEMS microbiology reviews 32, 723–735.

Živčák, M., Olšovská, K., Slamka, P., Galambošová, J., Rataj, V., Shao, H.B., Brestič, M., 2015. Application of chlorophyll fluorescence performance indices to assess the wheat photosynthetic functions influenced by nitrogen deficiency. Plant, Soil and Environment 60, 210–215.

Zotti, M., Di Piazza, S., Roccotiello, E., Lucchetti, G., Mariotti, M.G., Marescotti, P., 2014. Microfungi in highly copper-contaminated soils from an abandoned Fe–Cu sulphide mine: Growth responses, tolerance and bioaccumulation. Chemosphere 117, 471–476. https://doi.org/10.1016/j.chemosphere.2014.08.057

Zucconi, L., Ripa, C., Alianiello, F., Benedetti, A., Onofri, S., 2003. Lead resistance, sorption and accumulation in a Paecilomyces lilacinus strain. Biol Fertil Soils 37, 17–22. https://doi.org/10.1007/s00374-002-0555-4

Consulted websites:

https://www.actaplantarum.org./

http://enscobase.maich.gr/

http://data.kew.org/sid/