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Rhizosphere response to nickel in a facultative hyperaccumulator

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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 associated with *A. utriculata* from serpentine and non-serpentine sites were collected 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.

Highlights

- Only 10% of hyperaccumulators have their rhizosphere examined

- *A. utriculata* is a facultative Ni-hyperaccumulator that thrives in serpentine soils
- Rhizobiota of *A. utriculata* seems to be limited by serpentine soil conditions
- *Pseudomonas, Streptomyces, T. harzianum* group and *P. ochrochloron* hypertolerate Ni
- Rhizobiota with PGP traits and high Ni tolerance can improve plant phytoextraction

Keywords: microfungal strain, metal uptake, microbiota, PGPR, rhizobacteria, root area.

1. Introduction

The rhizosphere, i.e., the soil-root interface 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).

Among natural metalliferous soils, ultramafic serpentinitic soils usually have geochemical traits, which include an elevated concentration of magnesium (Mg) and iron (Fe), a low calcium (Ca):Mg ratio and elevated concentrations of trace elements such as nickel (Ni) (bioavailable Ni 7 to >100 mg kg^{-1}; total Ni 500-8000 mg kg^{-1}), chromium (Cr), and cobalt (Co) (Reeves and Baker, 2000; Freitas et al., 2004; Ghaderian et al., 2007; Turgay et al., 2012; Van der Ent et al., 2013; Roccotiello et al., 2015a; Kidd et al., 2018). They are also known for their paucity of macronutrients such as nitrogen (N), potassium (K), or phosphorus (P) (Nkrumah et al., 2018).

Specialized plants known as hyperaccumulators are able to live and reproduce on those soils without experiencing toxicity symptoms (Rascio and Navari-Izzo, 2011). Those plants are also cultivated to accumulate more than 1% leaf metals from soils and to translocate them to the harvestable biomass (Chaney et al., 2007). In addition, f*acultative hyperaccumulators able to live on both metalliferous and non-metalliferous sites are of a key importance because of their environmental plasticity respect to metal stress (Pollard et al., 2014) with consequent better*

adaptation to metal-disturbed habitat. 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). . Recently, studies on the role of microbiota in the rhizosphere of metalliferous plants have encouraged the development of remediation technologies employing native soil microorganisms for metal phytoextraction (Zhuang et al., 2007; Lebeau et al., 2008; Ma et al., 2009a; Glick, 2010; Sessitsch et al., 2013; Cabello-Conejo et al., 2014). Besides, 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 (Mengoni et al., 2001; Abou-Shanab et al., 2007; Iram et al., 2012; Turgay et al., 2012; Álvarez-López et al., 2016; Kidd et al., 2017; Thijs et al., 2017;) i.e., able to thrive in metal-rich soil (Gadd, 1992; Lima de Silva et al., 2012).

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 (J.Presl & C.Presl) F.K.Mey. (=Thlaspi c. J.Presl & C.Presl), Pseudomonas acting in synergy with roots of Odontarrhena bertolonii (Desv.) L. Cecchi & Selvi (=Alyssum b. Desv.), Microbacterium and Sphingomonas belonging to the rhizosphere of Odontarrhena muralis complex (=Alyssum m.) and members of the genus Burkholderia collected in the rhizosphere of Pycnandra acuminata (Pierre ex Baill.) Swenson & Munzinger (=Sebertia a. Pierre ex Baill.) and Psychotria douarrei (Beauvis.) Däniker, together with other nickel-resistant strains, like Hafnia alvei, Pseudomonas mendocina, Acinetobacter, Comamonas acidovorans, and Agrobacterium tumefaciens (Schlegel et al., 1991; Stoppel and Schlegel, 1995, Mengoni et al., 2001; Idris et al., 2004; Barzanti et al., 2007; Aboudrar et al., 2013). In addition, 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 (Benizri et al., 2001; Aboudrar et al., 2013; de Souza et al., 2015).

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 (Zotti et al., 2014; Roccotiello et al., 2015b; Cecchi et al., 2017b). Most of them were also metal-accumulator (Zotti et al., 2014; Roccotiello et al., 2015b; Cecchi et al., 2017b). 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). 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).

For instance, a comparison among the serpentine rhizospheric bacterial biodiversity associated with some subspecies of the Ni-hyperaccumulator A. 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).

Alyssoides utriculata is an evergreen shrub, Ni-facultative hyperaccumulator, able to concentrate up to 2000 mg kg^{-1} Ni in shoots when growing on serpentine sites (Roccotiello et al., 2010, 2015a; 2015b; 2016). Despite the medium-high ability to concentrate Ni in shoots, this 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. The inoculation of some Ni-tolerant bacterial and fungal strains in the rhizosphere of hyperaccumulators, specifically facultative-hyperaccumulators,

129 can increase the efficiency of phytoextraction by promoting the development of root biomass (Ma et
130 al., 2009a) and enhancing Ni accumulation in plant organs (Ma et al., 2011a).
131 This study is aimed at characterizing the culturable microbiota associated with the rhizosphere of
132 the facultative Ni-hyperaccumulator *A. utriculata* from serpentine and non-serpentine sites and to
133 obtain a collection of bacterial and fungal strains for application in Ni phytoextraction. Ni tolerance
134 of rhizosphere bacteria and fungi and PGPR traits of isolated bacterial strains have been also
135 evaluated.
136 The paper shows the first results of an interdisciplinary approach focused on the assessment of the
137 whole rhizospheric components in a facultative hyperaccumulator.
138

139 **2. Materials and methods**

140 *2.1 Sampling sites, plant and soil sample collection*

141 The facultative Ni-hyperaccumulator *Alyssoides utriculata* and related soil were sampled from
142 serpentine (S) and non-serpentine sites (NS). The S is located at Beigua Geopark (44°27′41.4″N
143 8°40′03″E) in the eastern Ligurian Alps. The soils derived from ultramafic bedrocks like
144 serpentines and eclogitic metagabbros (Capponi and Crispini, 2008; Marsili et al., 2009). The NS
145 site was the locality of Glori in the NW of Liguria (43°57′19″N 7°50′08″ E), geologically
146 characterized by flysch and clay marl (Giammarino et al., 2010).
147 Five shoots of adult plants of *A. utriculata* from non-fruiting branches and roots replicates (1 plant
148 = 1 replicate) were collected in July 2016 (fruiting stage) from the S and NS sites (n = 5 each site).
149 Ni-hyperaccumulation in plants was assessed with colorimetric dimethylglyoxime test (DMG 1% in
150 ethanol 95%, Sigma-Aldrich) (Charlot, 1964), then plants were quantitatively evaluated with ICP-
151 MS.
152

153 *2.2 Rhizospheric soil sampling*

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, samples were processed as described in '*Isolation and identification of culturable bacteria'*.

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 for about eight months.

2.3 Plant and soil sample analysis

In the laboratory, the 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), the leaves and roots were separately powdered using a ball mill (Retsch MM2000, Haan, Germany), before the chemical analyses.

Soil samples were air-dried, particle size distribution analysis was carried out by wet-sieving for the fraction >50 μm and, the composition of the fine fraction (<50 μm) was determined by pipette procedure after dispersion of the sample with sodium hexametaphosphate, $(NaPO_3)_6$.

The pH was measured with the potentiometric method in a 1:2.5 soil:water suspension. The cation exchange capacity (CEC) were determined with $BaCl_2$-triethanolamine at pH 8.2.

177 The soil samples were oven-dried at 60°C for 48 h before being sieved through a 2.0 mm mesh. The
178 soil and plant fractions were analyzed for Ni, Ca, Mg, Co and Cr concentration by means of a
179 Inductively Coupled Plasma Mass Spectrometry (ICP-MS).
180 The accuracy of the results was checked processing BCR-100 'beech leaves' reference material
181 (JRC-IRMM, 2004). Plant and soil metal concentrations were expressed on a dry weight basis
182 (DW).
183
184 *2.4 Isolation and identification of culturable bacteria*
185 Bacteria were extracted from 2.5 g of fresh soil by using 25 ml of sterile saline solution. Aliquots (1
186 ml) were serially diluted with NaCl 0.9% w/v (Khan et al., 2015; Turgay et al., 2012) and spread on
187 Tryptic Soy Agar (TSA, Sigma-Aldrich) (Idris et al., 2004; Vieira and Nahas, 2005; Aboudrar et al.,
188 2013; Rue et al., 2015) and amended with 50 mg l^{-1} of cycloheximide (Sigma-Aldrich) to inhibit the
189 microfungal growth (Visioli et al., 2014).
190 Plates (n= 90, each site and soil type) were incubated in the dark at 27±1°C for 72 h (Barzanti et al.,
191 2007; Sheng et al., 2008; Luo et al., 2011; Aboudrar et al., 2013). Bacterial colonies with distinct
192 morphologies (color, shape, size, opacity, etc.) were selected from the plates and repeatedly re-
193 streaked onto fresh agar medium prepared as previously described to obtain pure bacterial colonies
194 (Turgay et al., 2012; Khan et al., 2015). Relative concentration of identified morphotypes (Hanirah
195 et al., 2015) was also determined and expressed as Colony Forming Units per g of dry soil (CFU g^{-1})
196 (Rue et al., 2015). The most representative morphotypes were selected (n= 30, three replicates
197 each isolate) to perform the DNA sequencing.
198 To identify isolates, DNA extraction from pure bacterial culture was performed by the boiling
199 method (99°C, 10 min). PCR amplification of a 409 bp region was then performed using the
200 universal primers Com1 (5′-CAGCAGCCGCGGTAATAC-3′) and Com2 (5′-
201 CCGTCAATTCCTTTGAGTTT -3′), amplifying positions 519–926 of the *Escherichia coli*
202 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 of DISTAV (University of Genoa, Italy).

2.5 Screening for PGPR 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 (Penrose and Glick, 2003; Luo et al., 2011). Turbidity indicates positive growth.

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. 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 color highlights IAA production. The optical density was measured using spectrophotometer (Jenway 6300 spectrophotometer) at 530 nm of absorbance and auxin concentration was determined using standard curve of IAA (Ma et al., 2009b; Goswami et al., 2015).The siderophores production 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 solubilization activity 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 $NiSO_4*6H_2O$ (Sigma-Aldrich), at the concentration of 1, 5, 10, 15, and 20 mM (n= 15 each strain).

2.6 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: 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 colonies forming unit (CFU) 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 ITS 1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990; Gardes and Bruns, 1993). The PCR cycling parameters were the following: one cycle of 5 min at 95 °C; thirty-five cycle of 40 s at 94 °C, 45 s at 55 °C, 1 min at 72 °C; one cycle of 10 min at 72 °C; ∞ at 4 °C. 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). These cultures were maintained by agar slants with periodic transfers and then cryoconserved (-20°C).

2.7 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 (n= 15 each strain).

2.8 Data analysis

279 The Bioaccumulation Factor (BF= C_{shoot}/C_{soil}) and the Translocation Factor (TF= C_{shoot}/C_{root}) were
280 calculated.
281 The statistical analyses were performed with Statistica 8.0 (Statsoft Inc.) software.
282 The averages were presented with their standard deviations (SD). The t-test was used to evaluate the
283 differences between serpentine and non-serpentine samples. Significance was considered at the $P <$
284 0.05 level.
285 The differences in the number of bacterial and fungal colonies was evaluated on different sites (S
286 and NS) and substrates (R and B) by means of factorial ANOVA. Data significance was tested with
287 a post hoc Tukey's Honest Significant Difference test (HSD) at $P < 0.05$.
288 The Principal Components Analysis (PCA) was performed as a multivariate display method to
289 visualize the data structure. Significance was considered at the $P < 0.05$ level.
290

291 **3. Results**

292 *3.1 Soil and plant sample analysis*

293 Total nickel concentration in soils (Table 1) is significantly high on S site respect to NS site ($Ni_S >$
294 1000 mg kg^{-1}; Ni_{NS} ~20 mg kg^{-1}, $P < 0.001$), both in R and in B soil. On S site *A. utriculata*
295 hyperaccumulates Ni as expected (Ni_{roots} ~200 mg kg^{-1} Ni_{shoots} ~1200 mg kg^{-1}) (Table 2). T-test
296 reveals clear differences between S and NS sites ($P < 0.05$, Table 1) as regards soil (Ni, Ca, Co),
297 plant roots (Ni, Ca, Mg, Cr) and shoots (Ni, Mg, Co).

Site	Soil type	Element concentration (mg kg^{-1})					CEC meq/100g	pH	Fine Earth fraction %			Parent material
		Ca	Mg	Co	Cr	Ni			Sand	Silt	Clay	
S	R	3249.30±645.54^{a}	9032.93±4878.95	183.06±44.92^{a}	1693.60±308.58	1491.48±624.09^{a}	24.00	7.3	56.10	34.30	9.60	Serpentine
S	B	4267.50±922.94^{a}	11957.34±1876.38	49.74±19.63^{a}	1770.30±137.88	1046.65±404.77^{a}						
NS	R	9288.04±66.76^{b}	7050.32±42.44	13.13±1.62^{b}	27.08±1.63	19.52±0.94^{b}	19.30	8.3	38.4	43.8	17.8	Marl
NS	B	9336.06±54.08^{b}	7095.98±88.31	10.88±4.15^{b}	19.42±6.67	19.46±1.14^{b}						

Table 1 Bulk element concentration (mg kg^{-1}) and main chemical and physical features in soil samples. S: Serpentine site, NS: non-serpentine site, R: Rhizosphere soil, B: Bare soil. Data are mean±SD. n= 5 each site, each soil type. Significant differences were marked with different superscript letters in the same column ($P < 0.05$).

Site	Plant sample	Element concentration (mg kg^{-1})				
		Ni	Ca	Mg	Co	Cr
S	Shoot	1241.37±245.67^{a}	10142.29±1942.62	16342.84±2502.94^{a}	29.97±11.75^{a}	9.70±6.39
S	Root	203.04±49.75^{c}	2886.33±372.81^{a}	2374.12±228.19^{c}	1.46±1.17	3.76±1.53^{a}
NS	Shoot	10.64±0.45^{b}	9220.26±1766.01	21258.69±135.74^{b}	6.64±1.38^{b}	3.90±1.90
NS	Root	5.95±0.25^{d}	1317.18±252.2^{b}	2024.64±12.93^{d}	0.59±0.28	1.61±0.07^{b}

Table 2 Bulk element concentration in shoots and roots of the facultative nickel-hyperaccumulator *Alyssoides utriculata* harvested from Serpentine (S) and non-serpentine (NS) sites. Data are mean±SD. n= 5 each site, each plant sample. Significant differences were marked with different superscript letters in the same column ($P < 0.05$).

In the serpentine site, the Bioaccumulation Factor (BF) for Ni is 0.8, while the Translocation Factor (TF) is greater than 6.

Strain name	Microbiota type	NCBI accession number	Taxonomy rank	Most closely related 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	*Streptomyces* 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 3 The diversity (strain name, accession number, *taxon*) of isolated culturable bacteria and fungi from the rhizosphere (R, n= 5) of *Alyssoides utriculata* growing on serpentine (S, n= 5) and non-serpentine soil (NS, n= 5) and from the adjacent bare soil (B, n= 5).

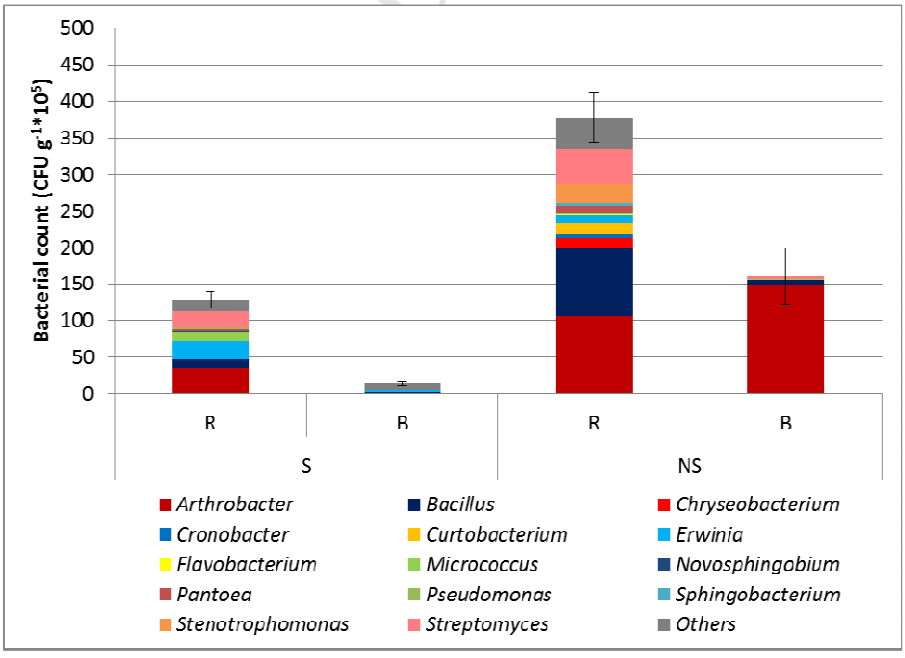
317 *3.2 Isolation and identification of culturable bacteria*

318 In the R soil the bacterial colonies from S and NS site are significantly more abundant than B soils
319 (Figure 1). Bacterial counts from NS site are higher than that from S site.

320 Among all the isolated bacteria, 30 recurring morphotypes are counted and identified with a
321 morphological approach followed by a molecular identification. Among the isolates, the most
322 frequent strain is *Arthrobacter* which accounts for about 30% of average bacterial count of the
323 rhizospheric S site (SR). Besides, there is a prevalence of *Enterobacteriaceae* which accounts for
324 about 20% of the total bacterial colonies isolated. Among others isolates, the most recurrent are
325 *Bacillus*, *Erwinia*, and *Streptomyces*. The bacterial count indicates that *Novosphingobium*, *Pantoea*,
326 and *Stenotrophomonas* are present in the SR and NSR soil, while the metallophilic *Pseudomonas*
327 thrives only in SR soil (Table 3).

328 Post-hoc test reveals a very significant difference between SR and NSR soils ($P < 0.001$), and
329 between NSR and NSB soils ($P < 0.001$), in terms of total culturable bacterial count (CFU g^{-1}).

330



331

332 **Figure 1.** Average bacterial count expressed as Colony Forming Unit (CFU g^{-1}) and diversity (genus, comprising
333 different strains) in the rhizospheric (R) soil of facultative Ni-hyperaccumulator *A. utriculata* and adjacent bare (B) soil
334 on serpentine (S) and non-serpentine (NS) site. Data are mean±SD. N=90 each site, each soil type.

335

336 *3.3 PGPR traits and Ni-tolerance in bacteria*

337 Among the serpentine bacterial isolates, 8 strains belonging to 5 genera show to possess more than
338 one PGPR activity (Table 4): *Pseudomonas*, *Stenotrophomonas*, *Streptomyces*, *Pantoea* and
339 *Erwinia.* Specifically, strain SERP14 and SERP25 (*Pantoea* sp.) have all four traits and they exhibit
340 the highest production of IAA at 530 nm of absorbance.

341 SERP1 (*Pseudomonas* sp.), SERP2 (*Stenotrophomonas* sp.), and SERP19 (*Erwinia* sp.) show a
342 great synthesis of siderophores as well as *Pantoea* previously cited, while SERP3, SERP4 and
343 SERP23 (*Streptomyces* sp.) can solubilize phosphate as SERP19 (*Erwinia* sp.) and genus *Pantoea*
344 and to grow on ACC as the sole source of N.

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

348

Strain name	**Microbiota type**	**ACC deaminase[a]**	**IAA synthesis (mg l^{-1})**	**Siderophores Production[b] (halo Ø, mm)**	**Phosphate solubilization[c] (halo Ø, mm)**
SERP1	*Pseudomonas* sp.	-	6.0±0.1	+ (90)	+ (5)
SERP2	*Stenotrophomonas* sp.	-	4.8±0.1	+ (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.	+	10.2±0.2	+ (90)	+ (4)
SERP19	*Erwinia* sp.	-	-	+ (90)	+ (5)

SERP21	*Novosphingobium* sp.	-	-	-	+ (2)
SERP23	*Streptomyces* sp.	+	-	-	+ (2)
SERP25	*Pantoea* sp.	+	20.5±0.3	+ (90)	+ (15)
SERP26	*Micrococcus* sp.	-	-	-	-

Table 4 Plant growth promoting traits of culturable bacteria isolated from the rhizosphere of *A. utriculata* on Serpentine soil (S), n= 8 each strain. The halo refers to Petri dishes of Ø 90 mm. [a] Growth on ACC: - none; + turbidity; [b] Siderophores production: - no halo; + halo; [c]Phosphate solubilization: - no halo; + halo.

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

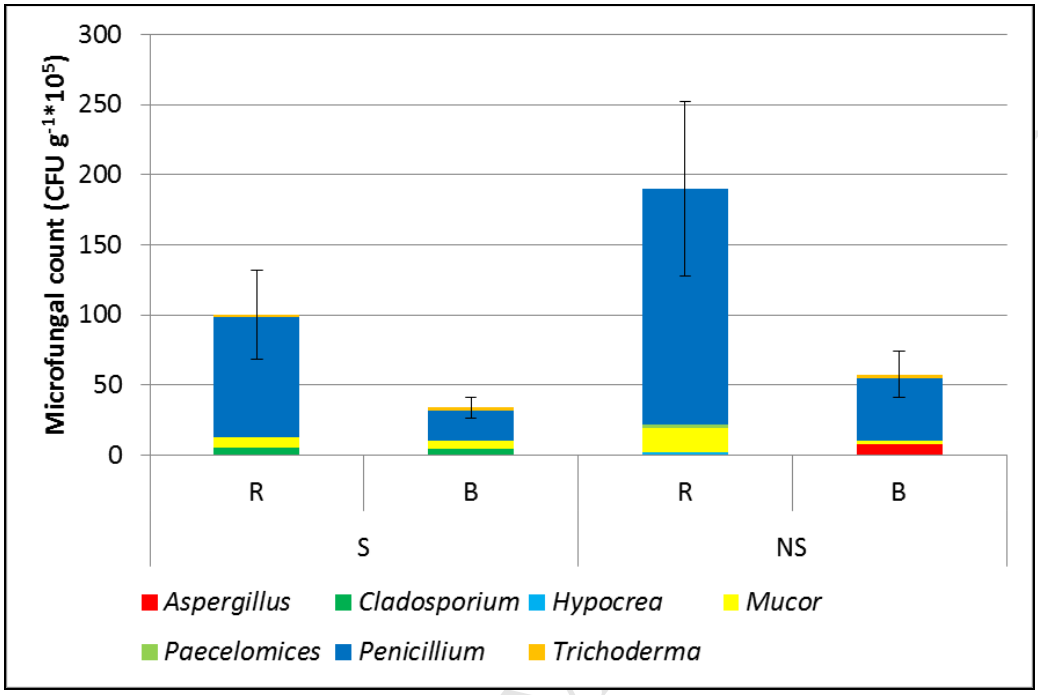
Table 5 Nickel tolerance (mM) of culturable PGPR isolated from the rhizosphere of *A. utriculata* growing on the Serpentine soil (S), n= 15 each strain. Growth: - absence; + presence

3.4 Isolation and identification of culturable fungi

Figure 2 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 3).

Penicillium is the most abundant strain comprising the 84% of total colonies, and about 85% of isolates from SR soils. Although genus *Cladosporium* has a low frequency (2% of the total), it was exclusively isolated from S soils. We observed a slightly greater abundance of microorganisms in R

365 than B soil and in NS than S site. Post-hoc test reveals a significant difference ($P < 0.01$) between
366 NSR and NSB.
367



368

369 **Figure 2.** Microfungal count expressed as Colony Forming Unit (CFU g^{-1}) and diversity (genus, comprising different
370 strains) in the rhizospheric (R) soil of facultative Ni-hyperaccumulator *A. utriculata* and adjacent bare (B) soil on
371 serpentine (S) and non-serpentine (NS) site. Data are mean±SD. N= 20 each site, each soil type.

372

373 *3.5 Ni-tolerance in fungi*

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

378

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 6. Nickel tolerance (mM) of culturable fungi isolated from the rhizosphere of *A. utriculata* growing on the Serpentine soil (S), n= 15 each strain.

3.6 Plant and rhizosphere microbiota response to soil metals

The S and NS samples constitute two well-defined cluster, highlighting a good homogeneity of the starting samples (Fig. 3A). The Loadings plot in Figure 3B show the samples data (element concentration in plant and soil and microbiota count) distribution on the first two principal components. Most of the elements in soil, root and shoot (especially Ni and Co) is associated with positive scores on PC1. Both Loading plots presented explain a total variance of 76.07%. It is noteworthy that Ca is the only soil element associated with negative scores of PC1; indeed, S soil samples are characterized by scarcity of nutrients such as Ca and presence of phytotoxic elements such as Ni and Co. Microfungal and bacterial counts are associated with negative scores on PC1, without a clear response respect to soil element.

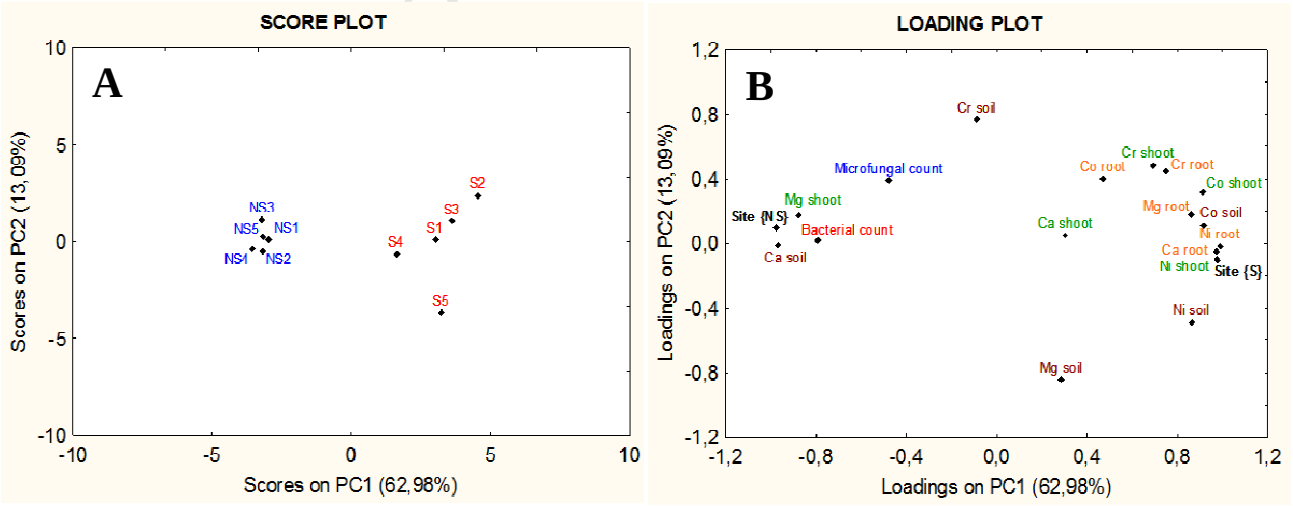


Figure 3. Score plot and Loading plot of a Principal Component Analysis. A) Score plot of 10 R soil samples distributed in S (red) and NS (blue) sites; B) loading plot of the bacterial and the fungal counts and element concentrations in soil and plant. The samples are distinguished by different colors depending on the origin, brown: soil, orange: root, green: shoot, red: bacteria, blue: fungi. Factor 1 (62.98% variation explained) vs Factor 2 (13.09% variation explained). Variables are indicated by symbol ♦.

4. Discussion

Only 10% of hyperaccumulators have their rhizosphere examined (Alford et al., 2010; Visioli et al., 2015). Up to date facultative hyperaccumulators have been poorly studied (Pollard et al., 2014) and no facultative Ni-hyperaccumulator Mediterranean plant species have been studied in combination with their rhizosphere microbiota for phytoremediation purposes.

The differences between serpentine and non-serpentine sites in terms of element concentration is significant because of the serpentine higher level of Ni, Co, Cr, the Ca-deficiency, and the low Ca/Mg ratio (0.36) typical of harsh serpentinitic environmental conditions (Ghasemi et al., 2015; Kidd et al., 2018). The differences in terms of leaf Ni and Co concentration, in *A. utriculata*, between sites, could reflect soil bioavailability of these elements (Reeves, 2006; Ghasemi and Ghaderian, 2009). As expected, only *A. utriculata* from serpentine soils shows typical hyperaccumulator traits (shoot Ni >1000 mg kg^{-1}; TF>>1, BF~1)(Reeves, 1992; van der Ent et al., 2013).

Even the culturable microbiota of the facultative Ni-hyperaccumulator *A. utriculata* seems to be limited by the serpentine soil conditions resulting less abundant than in non-serpentine site with preferential colonization of rhizospheric soil. The higher microbiota density in serpentine rhizosphere respect to bare soil is a common feature of hyperaccumulators (Mengoni et al., 2001; Wenzel et al., 2004; Álvarez-López et al., 2016; Lopes et al., 2016; Benizri and Kidd, 2018). This result partially reflects the

'rhizosphere effect'. It 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; Rovira, 1965; 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-hyperaccumulating plants (Mengoni et al., 2001; Abou-Shanab et al., 2003; Idris et al., 2004; Ma et al., 2009; Turgay et al., 2012; Visioli et al., 2015).

Bacterial and fungal strains are limited by serpentine soil conditions. This is probably due to the highly spatial and temporal heterogeneous soil structure (Abbott et al., 2015; Jansson and Hofmockel, 2018) which is affected by elements and nutrients distribution (i.e. C, N, P, Xue et al., 2018; Zhang et al., 2018). We cannot exclude that this bacterial and fungal community, native of serpentinitic soil may induce promoting effects on the growth of *A. utriculata*, immobilizing or uptaking Ni, as already demonstrated in *N caerulescens* (Aboudrar et al., 2013). Further studies will clarify this point. The presence of some PGPR bacterial strains like *Pseudomonas* sp. and *Streptomyces* sp. that can be cultivated on Ni 15 mM and microfungi able to grow on Ni up to 3 mM (Cecchi et al., 2017b) could support this idea.

Most of culturable bacterial strains exhibit one or more plant growth-promoting activities that potentially support the growth of *Alyssoides utriculata*, reducing metal stress and increasing Ni uptake and accumulation in aerial parts. One third of serpentinitic isolates shows ACC deaminase that determines the reduction of ethylene stress with inhibition of plant development at rhizosphere level (Glick, 2010; Glick and Stearns, 2011; Sessitsch et al., 2013) and produces IAA that enhances the root surface area and plants nutrients uptake (Shagol et al., 2014). Moreover, the synthesis of IAA can stimulate plant cell proliferation and cell elongation can induce the transcription of the enzyme that catalyses the synthesis of ACC (ACC sintase) (Khan et al., 2015).

448 Five bacterial strains produce siderophores and this could suggest the ability to alter metal solubility
449 in the rhizosphere stimulating the plant growth (Lodewyckx et al., 2002; Idris et al., 2004; Tripathi
450 et al., 2005; Barzanti et al., 2007; Ma et al., 2009b) but further investigations can clarify this point.
451 Half of the tested bacteria is positive for the phosphate solubilization activity which increase the
452 availability of P for the plant (Fitz and Wenzel, 2002) and the metal solubility through the bacterial
453 phosphatase-mediated dissolution of metal phosphates (Alford et al., 2010). In addition, our study
454 shows that the PGPR strains isolated on serpentine soils often exhibit a high tolerance to Ni (up to
455 15 mM in the culture medium) according to Schlegel et al. (1991). We found *Pseudomonas* only in
456 the rhizospheric soil of the S site suggesting potential metallophilic behavior. This genus is known
457 for its PGP traits (Li and Ramakrishna, 2011; Ma et al., 2011) and Ni tolerance (growing up to 10
458 mM Ni, Turgay et al. 2012). Previous studies revealed a high biomass yield in *Brassica* spp.
459 inoculated with a serpentine selected strain of *Pseudomonas* (Freitas et al. 2004), highlighting the
460 protective role of this bacterium against the inhibitory effects of Ni (Rajkumar and Freitas, 2008a;
461 Ma et al., 2009). Moreover, an increased uptake of Ni, Cu and Zn was recorded in the root system
462 of *Ricinus communis* L. inoculated with *Pseudomonas* (Rajkumar and Freitas, 2008b). Inoculation
463 of metal-tolerant serpentinitic strains such as *Bacillus* sp. and *Pseudomonas* sp. seems to be
464 effective, directly by increasing the accumulation of metal in plant tissues and indirectly stimulating
465 of plant growth, protecting the plant from the inhibition of growth given by soil Ni, thus reducing
466 its toxicity (Ma et al., 2009b; Rajkumar et al., 2013; Rajkumar and Freitas, 2008a; Zaidi et al.,
467 2006).
468 Although genera *Arthrobacter*, *Bacillus*, and *Streptomyces* were isolated from all soil type, some
469 authors have shown their beneficial effects on different plants growing on disturbed soils types
470 (Dimkpa et al., 2008, 2009; Gray and Smith, 2005; Y. Ma et al., 2011). All these aspects suggest
471 that specific strains showing good activity, colonization potential and PGPR traits will be useful in
472 enhancing bioavalability, phytoextraction and phytostabilization performance by plants (Sessitsch et
473 al., 2013).

The lower amount of microfungal strains in bare respect to rhizosphere soils can be related to the lower availability of nutrients, water, and oxygen commonly provided by the rhizosphere (Söderström, 1975; Kjøller and Struwe, 1982). The isolated microfungal strains of *Penicillium*, *Aspergillus*, *Trichoderma*, and *Mucor* are commonly saprotrophic microfungi with species found in metal-polluted habitats worldwide (Kubatova et al., 2002; Massaccesi et al., 2002; Gadd and Fomina, 2011). 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. This work confirmed the capability of this fungal strain to uptake Ni as described by Roccotiello et al. (2015b) and Cecchi et al. (2017b). *T. harzianum* was known to hyperaccumulate Ni up to 500 mg L^{-1} (Roccotiello et al. 2015b) and other metals (Cecchi et al., 2017a, 2017b; Zotti et al., 2014) with an amazing bioaccumulation capacity (up to 11,000 mg Ni kg-1) (Cecchi et al., 2017b). Moreover, *Penicillium canescens* and P. *ochrochloron* also showed the capacity to tolerate Ni up to 5mM and 20 mM, respectively. The first is a typical rhizospheric species able to live until 50 cm depth in soil, while the second is known for its high tolerance to copper and other metals (Domsch et al., 2007). Besides, *P. expansum* represents a cosmopolitan species able to colonize polluted soils and extreme environments and to bioaccumulate high metal concentrations (Cecchi et al., 2017b; Di Piazza et al., 2017). 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. The microbiota characterization of the facultative hyperaccumulator species plays a key role because this could help isolate the culturable bacteria and fungi that

contribute to the hyperaccumulator phenotype (Visioli et al., 2015), using microbiota as inoculum of the rhizosphere to improve the root development, the metal uptake, and the phytoremediation of metal contaminated soils. The rhizosphere of *A. utriculata* can be an excellent model to enhance Ni uptake and a better growth using a microbiota associated with its root system.

5. Conclusion

The characterization of the culturable microbiota of the facultative Ni-hyperaccumulator *Alyssoides utriculata* provide a collection of bacterial and fungal strains for potential applications in Ni phytoextraction.

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 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, *P. ochrochloron* and *Trichoderma* strain belonging to the *harzianum* group exhibit great Ni-tolerance. This latter also has a 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. This multidisciplinary research on the dynamic microenvironment known as the rhizosphere has the perspective to provide guidance for using Ni-hyperaccumulator species and the associated rhizobiota to remediate Ni contaminated soils.

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- Only 10% of hyperaccumulators have their rhizosphere examined
- *A. utriculata* is a facultative Ni-hyperaccumulator that thrives in serpentine soils
- Rhizobiota of *A. utriculata* seems to be limited by serpentine soil conditions
- *Pseudomonas, Streptomyces, P. ochrochloron* and *T. harzianum* group hypertolerate Ni
- Rhizobiota with PGP traits and high Ni tolerance can improve plant phytoextraction