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# The parameters determining hyperaccumulator rhizobacteria diversity depend on the study scale

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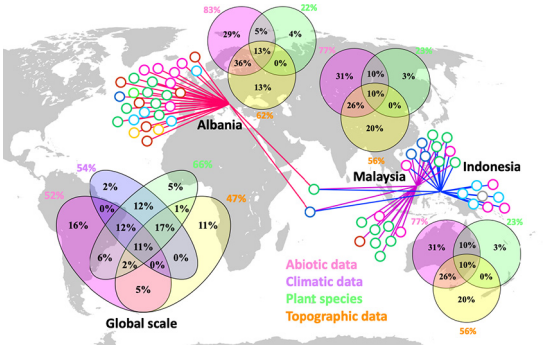
## HIGHLIGHTS

- Climate drives the rhizosphere bacterial community diversity
- At country scale, soil parameters strongly impact the rhizobacterial diversity
- Rhizobacterial diversity is greater in Mediterranean than in tropical regions

## GRAPHICAL ABSTRACT

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## ABSTRACT

Soils harbor some of the most diverse microbiomes on Earth and are essential for both nutrient cycling and carbon storage. Numerous parameters, intrinsic to plant physiology, life history and the soil itself, can influence the structure of rhizomicrobial communities. While our knowledge of rhizosphere microbial diversity is increasing, opinion is divided as to whether the factors that most impact this diversity are abiotic, climatic or plant selection. Here we focused on the rhizosphere bacterial diversity of nickel hyperaccumulator plants (28 species from Mediterranean or tropical climates). We showed, by leveraging 16S Illumina sequencing of 153 ultramafic rhizosphere soils, that bacterial genetic diversity was highest in Mediterranean habitats where plant diversity was the lowest. Concerning those parameters driving this diversity, we demonstrated that climate drives bacterial diversity, in particular with the annual temperature variation. Focusing on each region, we underlined the substantial role of soil physicochemical parameters. Our results highlight the importance of considering spatial scale when explaining bacterial community diversity.

## 1. Introduction

The rhizosphere, inhabited by a complex microbial community, is a narrow soil zone, influenced by plant roots. This zone is home to an overwhelming number of microorganisms and invertebrates and is considered to be one of the most dynamic interfaces on Earth, as it is a focal point of soil-plant-microbe interactions (Hiltner, 1904; Nguyen, 2003; Philippot et al., 2013). The diversity and activity of soil microorganisms are influenced by various abiotic parameters associated with both plant and soil, including soil type, soil texture, soil temperature, altitude, pH, water availability, organic matter content, compaction, drainage and aeration of the soil (Lynch and Whipps, 1991; Rovira, 1956). Concerning biotic factors, plant root deposits (i.e. rhizodeposits) are known to be a key determinant of the structure of the rhizosphere microbial community (Dennis et al., 2010; Walker et al., 2003). These include a wide variety of substances that originate from sloughed-off root cells and tissues, mucilage, volatile substances and soluble lysates and exudates that are released from damaged and intact cells, respectively (Dakora and Phillips, 2002; Uren, 2000). Moreover, rhizodeposits perform key roles in plant defense against pathogens

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(Abbott and Murphy, 2007) and form a basis for chemotaxis to mediate attraction and repulsion of particular microbial species (Kumar et al., 2007). It has been also shown that specific metabolites released into the rhizosphere can trigger multiple responses in different soil microorganisms. For example, plant flavonoids can attract not only symbionts, such as *Bradyrhizobium japonicum,* but also pathogens, such as *Phytophthora sojae* (Philippot et al., 2013). However, plant rhizodeposition can itself be influenced by light intensity, seasonal changes (Dunfield and Germida, 2003), soil properties (Meharg and Killham, 1990; Nguyen, 2003), as well as by plant species or cultivars (Mazzola et al., 2004; Smalla et al., 2001) and even genotype and growth stage (Van Overbeek and Van Elsas, 2008).

Ultramafic outcrops are found worldwide, covering approximately 3% of the terrestrial surface and major outcrops can be found in temperate (e.g. Alps, Balkans, Turkey, California) and tropical regions (e.g. New Caledonia, Cuba, Brazil, Malaysia, Indonesia). Ultramafic soils typically present geochemical peculiarities, which include an elevated concentration of magnesium (Mg) and iron (Fe), a low calcium:magnesium ratio (Ca:Mg) and elevated concentrations of trace elements such as nickel (Ni) which can reach 3600 mg $kg^{-1}$ or more in soils developed over ultramafic rocks (Echevarria, 2018). These ultramafic soils are also known for their deficiency in macronutrients such as nitrogen (N), potassium (K) or phosphorus (P) and are characterized by low organic matter content (Brooks, 1987; Nkrumah et al., 2016). This unusual geochemical composition creates an inhospitable environment for plant growth and ultramafic soils support plant species that have been named hyperaccumulators (Jaffré et al., 1976; Pollard et al., 2014). Hyperaccumulators are able to accumulate in their aerial parts 50 to 500 times greater concentrations of metals than other non-hyperaccumulators (Baker and Brooks, 1989; Chaney et al., 2007).

While our knowledge of the microbial diversity in the rhizosphere is growing, understanding the principles underlying its structuring are today's and tomorrow's challenge. Moreover, opinion is divided as to whether it is edaphic factors or selection by plants that are the greatest determinant of rhizosphere microbiome composition. Many studies have focused on agricultural soils and the factors that affect microbial communities. In agricultural ecosystems, management practices influence soil's physical, chemical, and biological properties, which in turn directly impacts soil microbial composition and behavior (Jangid et al., 2008). For example, tillage practices have been shown to influence microbial community structure, taxonomic composition, microbial abundance and activity, by changing the physicochemical properties of soil (García-Orenes et al., 2013). Nevertheless, microbiologists have paid little attention to the bacterial communities specific to ultramafic areas and only few works have focused on the characterization of the rhizobacterial communities' diversity in hyperaccumulating plants able to grow on nickel-rich soils (Bordez et al., 2016; Lopez et al., 2017, 2019a, 2019b, 2020; Mengoni et al., 2010; Pardo et al., 2018; Visioli et al., 2015). Moreover, concerning the parameters governing this diversity, nothing is known as to which factors are the greatest determinants of hyperaccumulator rhizosphere microbiome composition.

Consequently, the aim of this study is to first improve our knowledge of the structure and diversity of rhizobacterial communities of hyperaccumulator plants and secondly to understand, at different scales of study, which parameters drive the diversity of hyperaccumulator's rhizobacteria.

## 2. Methods

### 2.1. Field collection and soil physicochemical parameters

Three different countries with different climates were prospected during fieldtrips (Table 1; www.accuweather.com). In March 2015 (Lopez et al., 2019b), during the fieldwork on the ultramafic soils on the Weda Bay Island (Indonesia), 10 different plant species of hyperaccumulators were identified and sampled. Forty-five rhizosphere soil samples were collected from 16 sites. These hyperaccumulators were: *Aristolchia* sp., *Barringtonia* sp., *Cerbera floribunda*, *Ficus trachypison*, *Glochidion moluccanum*, *Macaranga* sp., *Planchonella roxburghioides*, *Prunus* sp., *Rinorea* aff. *bengalensis* et *Trichospermum morotaiense* (Supplementary Table 1). In October 2015 (Lopez et al., 2019a), rhizosphere soil samples were collected from 11 sites in the major ultramafic massif of Shebenik, spreading over the Librazhd and Pogradec districts (Albania). Four different hyperaccumulator plant species were gathered: *Odontarrhena chalcidica*, *O. smolikana*, *O. rigida* and *Noccaea ochroleuca* (Supplementary Table 1). In July 2017 (Lopez et al., 2020), rhizosphere soil samples were collected in Malaysia from 10 sites. Sixty samples were collected corresponding to 15 plant species belonging to 9 botanical families. These hyperaccumulators were: *Actephila alanbakeri*, *Flacourtia kinabaluensis*, *Glochidion* sp., *G.* cf. *rubrum*, *Macaranga* cf. *lowii*, *Mischocarpus sundaikus*, *Phyllanthus balgooyi*, *P. securinegoides*, *Psychotria sarmentosa*, *Rinorea* sp., *R. bengalensis*, *R. javanica*, *Timonius* sp., *Walsura pinnata*, *Xylosma luzonensis* (Supplementary Table 1). In order to collect rhizosphere soil samples from the different plant species, the plant roots were extracted from the soil with their adhering soil. For woody species, a part of the root system was also extracted. Then, these roots were shaken to remove the non-adhering soil to the roots. A small subsample of the recovered soils (about 1–2 g) was placed as fast as possible at −20 °C for future genomic DNA extractions. The rest of the different soil samples was kept at 4 °C pending physicochemical analyses.

**Table 1**
Climate data from the three prospected countries. Mean and range are provided for the temperature. Rainfall and snowfall are the cumulative data of the year.

| Measures | Albania | Indonesia | Malaysia |
|---|---|---|---|
| Climate (Köppen classification) | Csa: Mediterranean | Af: Equatorial | Am: Monsoon |
| Temperature (°C) | 17.1 [−2–30] | 27.4 [23–33] | 27.6 [24–32] |
| Annual rainfall | 567 | 6975 | 1132 |
| Annual snowfall | 15 | 0 | 0 |
| Weather station | Prrenjas (2015) | Kudat (2015) | Limni (2017) |

Fresh rhizosphere soil samples collected from each hyperaccumulator plant were sieved (2 mm) and the soil moisture content was determined by heating sub-samples to 105 °C until constant weight was attained. The following analyses were carried out on dried soils, after heated them at 40 °C for 48 h. The potentially phytoavailable elements (i.e. chemically extractable) were extracted with a DTPA-TEA solution (0.005 M diethylene triamine pentaacetic acid, 0.01 M calcium chloride dihydrate, 0.1 M triethanolamine, pH 7.3) (Lindsay and Norvell, 1978), and the concentrations in the solutions extracted were measured with ICP-AES (Inductively Coupled Plasma-Atomic Emission Spectrometer; ICP-AES, Liberty II, Varian). Rhizosphere soil samples (500 mg subsample) were acid-digested using freshly prepared Aqua Regia (6 mL 37% hydrochloric acid and 2 mL 70% nitric acid per sample) for a 2-h period and then diluted with distilled water to 50 mL before ICP-AES analysis. Exchangeable elements (associated with the soil cation exchange capacity, i.e. CEC) were extracted in 0.0166 M Co (NH3)6Cl3 at a soil solution ratio of 1:20 (2.5 g:50 mL) and after 1 h's shaking, according to international ISO standard 23,470 (ISO 23470:2007). Soil pH was measured using a pH meter in a soil-water suspension (soil to water ratio = 1:5). Total C and N and organic C were quantified by combustion at 900 °C with a CHNS analyser (vario MICRO cube, Elementar Analysensysteme GmbH).

### 2.2. Microbial diversity by high-throughput 16S rRNA amplicon sequencing

Genomic DNA extractions from 0.5 g of fresh rhizosphere soil samples were obtained using the FastDNA™ SPIN kit for Soil (MP Biomedicals, France), in accordance with the manufacturer's protocol. Concentrations of DNA solutions were measured with a spectrophotometer (SmartSpec Plus spectrophotometer, BIO-RAD) and adjusted to 1.66 ng $\mu L^{-1}$. Barcoded amplicon sequencing was performed using the modified primers S-D-Bact-0909-a-S-18 (5′-ACTCAAAKGAATWGACGG-3′) and S- *-Univ-*-1392-a-A-15 (5′-ACGGGCGGTGTGTRC-3′) targeting a 484 bp fragment of the 16S rRNA gene V6-V8 region (Klindworth et al., 2013). The primer modification consisted of the incorporation of the Nextera XT® transposase sequence (Illumina Inc., San Diego, USA) in the 5′ end of the forward and

reverse primers, then adding four random nucleotides to the forward primer to increase the nucleotide diversity (Bartram et al., 2011). Amplicons were generated using the Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs Inc., Ipswich, USA). The PCR reaction was achieved according to the following thermal profile: 30 s at 98 °C, followed by 22 cycles of 5 s at 98 °C, 30s at 58 °C, 30s at 72 °C, and finally 2 min at 72 °C. Amplicons were purified with AMpure magnetic beads (Agencourt, Beckman Coulter Inc., Fullerton, USA) and further quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The concentration of the amplicons was re-adjusted to 1.66 ng $\mu L^{-1}$ and 1 μL of each library was used as a template in a second PCR, where the Nextera XT® barcodes and the Illumina adapters necessary for hybridization to the flow cell were added using the Nextera XT Index kit. The conditions for the second PCR were 30 s at 98 °C, followed by 8 cycles of 10 s at 98 °C, 30 s at 55 °C, 30 s at 72 °C, and a final elongation at 72 °C during 2 min. The resulting amplicons were purified with AMpure magnetic beads (Agencourt) and pooled in equimolar concentrations. The final concentration of the library pool was determined with a KAPA SYBR® FAST Universal qPCR Kit (Kapa Biosystems, Wil- mington, USA). The libraries obtained were mixed with Illumina-generated PhiX control libraries (5%) and sequenced with the MiSeq Reagent Kit V3–600 cycles (Illumina Inc., San Diego, USA).

### 2.3. Bioinformatic and statistical analyses

The obtained sequence reads were de-multiplexed, quality-trimmed (sequences of low quality (limit = 0.05) were removed, (sequences with no ambiguous nucleotides were allowed and those with a minimum length of 400 nucleotides were kept) and the ones with at least two reads were assigned to OTUs at 97% similarity using a CLC Genomics Workbench 7 and Usearch (v7.0.1090_win64) pipeline (Edgar, 2010). Taxonomic affiliation was carried out with the SILVA database with a confidence threshold of 80% (Silva.nr_v123, https://www.arb-silva.de/). This Targeted Locus Study project has been deposited at DDBJ/ENA/ GenBank under the accessions references KBRT00000000 and KBWT00000000. The versions described in this paper are the first versions, KBRT01000000 and KBWT01000000. In order to be able to compare the data from each rhizosphere sample, the high-throughput sequencing results were normalized to the sample with lowest total counts (3352 reads). Alpha diversity was studied using the QIIME software (Quantitative Insights Into Microbial Ecology, version 1.8.0; Caporaso et al., 2010). Beta diversity was studied with a NMDS (Non-metric MultiDimensional Scaling) using the vegan package (version 2.5–2; Dixon, 2003) in R. After QIIME pipelining, a network analysis was performed using Cytoscape software (version 3.8.2; Shannon et al., 2003). The statistical analyses were carried out on R software (version 3.3.1). TukeyHSD tests were performed on relative abundances to highlight significant differences between the different samples at $p$-value <0.05. Variance partitioning analysis (VPA) was used to determine the contributions of soil properties (pH, cations exchange capacity, organic carbon and five DTPA-extractable Co, Fe, Mn, Ni and Zn), topography (elevation and exposition) and climate (annual temperature and rainfall), and plant species, as well as interactions between them, on the variation of the bacterial community diversity. VPA were performed in R with the vegan package.

## 3. Results

### 3.1. Soil physicochemical properties

Here, following the fieldwork, we collected a total of 153 rhizosphere soil samples of 28 different hyperaccumulator plant species belonging to 16 different botanical families, from ultramafic soil in areas under Mediterranean (Albania) and tropical climates (Indonesia and Malaysia). These samples varying on the one hand, by their elevation and on the other hand, by their soil physicochemical parameters (Supplementary Table 1). In Indonesia, rhizosphere soils showed the highest total and DTPA-extractable Ni concentrations (respectively 7000 and 200 mg $kg^{-1}$), higher than the mean values calculated for all soil samples (total Ni: 3900 mg $kg^{-1}$ and DTPA Ni: 200 mg $kg^{-1}$). The lowest total Ni (3200 mg $kg^{-1}$) and DTPA-extractable Ni (180 mg $kg^{-1}$) concentrations were measured in Albanian ultramafic soils. The soil pH from the different soils ranged from 5.2 to 8.3 with a mean of 6.7. Malaysian soils showed the highest organic carbon, with a mean of 8.3%, while the mean was 5.9% all samples combined, regardless the sampling region.

### 3.2. Bacterial community diversity

#### 3.2.1. Composition of the bacterial diversity

An Illumina sequencing approach was undertaken to characterize the bacterial diversity. A total of 13,882,622 bacterial sequences were obtained from the 153 rhizosphere soil samples collected. 13,924 OTUs were found for all soil samples and were separated into 42 phyla. Major bacteria relative abundance (>1%) at the phyla level is presented in Fig. 1. The major lineages of the total sequences, in all regions considered, were Proteobacteria (36%), Acidobacteria (19%) and Actinobacteria (13%). The highest relative abundance of Proteobacteria was detected in soil samples collected in Malaysia (42%), although samples from Indonesia presented the highest relative abundance of Acidobacteria (18%) and Actinobacteria (15%). Apart for these three phyla, we can underline significant differences between the regions with high relative abundances of Gemmatimonadetes (10%), Chloroflexi (7.5%) and Bacteroidetes (7.2%) for soil samples from Albania in a Mediterranean climate, while in Indonesia and Malaysia, under a tropical climate, Planctomycetes (7.6%), Chloroflexi (4.8%) and Nitrospirae (4.1%) were relatively more abundant. Thus, depending on the climatic region considered, Chloroflexi were well represented, as Gemmatimonadetes in a Mediterranean climate, and Nitrospirae and Planctomycetes in a tropical one.

The α-diversity indices (number of observed OTUs, Chao1 and Shannon indexes) were calculated for the rhizosphere soils of the three countries (Table 2). The samples contained between 459 and 5797 OTUs out of a total of the 13,924 OTUs identified. Chao1 was between 661 and 6716. Thus, the percentage of coverage was at least 68.5%. The Shannon index varied from 7.1 to 10. The samples from Albania had the largest α-diversity indices (4195 for the number of observed OTUs, 5017 for Chao1 and 9.6 for Shannon index), whereas, on average, the rhizosphere soils of Indonesia had the lowest index values (2354, 2914 and 8.9 for observed OTUs, Chao1 and Shannon, respectively). The indices for the Malaysian samples showed intermediate values to the previous ones. So, we found that taxonomic diversity of bacteria peaked at mid-latitudes and declined towards the Equator. Indeed, bacterial diversity was the highest in a Mediterranean biome (latitude 40°N) and the lowest in tropical areas (latitude 0.6°N in Indonesia and 6°N in Malaysia).

To assess the similarity of the bacterial community composition from the different rhizosphere soil samples, a NMDS (Non-metric MultiDimensional Scaling) was carried out (Fig. 2). A clear clustering was observed between samples from Albania, under a Mediterranean climate, and samples from Indonesia and Malaysia under tropical climates. Indeed, the Albanian samples were grouped all together on the right of this graphical representation, this underlined a similarity in their bacterial community diversity. Additionally, the samples from Malaysia and Indonesia were clustered together, indicating similar bacterial community diversity for these samples whether from Indonesia or Malaysia.

#### 3.2.2. Major OTUs present in the three different country

A network analysis was performed on the 57 major OTUs (1 to 2.5% of relative abundance) allowing a better visualization of the OTUs that are common or specific to the different ultramafic soils collected from the 3 regions studied (Fig. 3 and Supplementary Table 2). OTUs were mainly affiliated to Proteobacteria (19 OTUs, 33%), Acidobacteria (14 OTUs, 25%) and Actinobacteria (8 OTUs, 14%). The OTUs belonging to the Proteobacteria, Acidobacteria and Nitrospirae phyla (5 OTUs, 8.8%) were distributed throughout the network and were present in all three countries, while Actinobacteria OTUs were not found in Malaysian rhizosphere soils. A

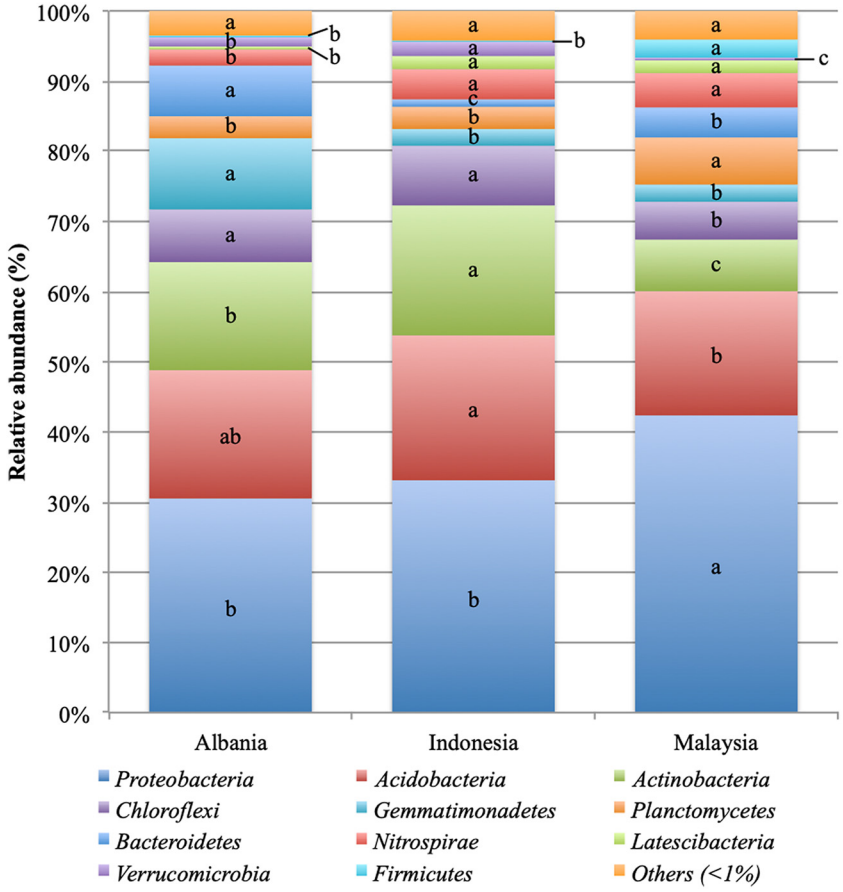


**Fig. 1.** Relative abundance of bacterial phyla identified in the rhizosphere soils of hyperaccumulator plants sampled in Albania, Indonesia and Malaysia (%). "Others (<1%)" refers to Armatimonadetes, BJ-169, BRC1, Candidatus_Berkelbacteria, Chlamydiae, Chlorobi, Cyanobacteria, Deinococcus-Thermus, Elusimicrobia, FBP, FCPU426, Fibrobacteres, GAL15, Gracilibacteria, Hydrogenedentes, Ignavibacteriae, Microgenomates, Nitrospinae, Omnitrophica, Parcubacteria, Peregrinibacteria, RBG-1, Saccharibacteria, SBR1093, Spirochaetae, Tectomicrobia, Tenericutes, TM6, WS2, WS6, WWE3 and unclassified bacteria.

total of 28 OTUs were found only in rhizosphere soil samples from Albania, which represent 49% of the major OTUs, including two Chloroflexi OTUs (OTU_6 and OTU_38) and the only Bacteroidetes OTU (OTU_1) affiliated to the *Flavobacterium* genus. Moreover, Albania possess six of the seven OTUs affiliated to the Gemmatimonadetes phylum. Malaysia and Indonesia shared a close diversity with 10 OTUs present only in these two countries against nine OTUs only from Malaysia and eight from Indonesia, including the only Verrucomicrobia OTU (OTU_188) belonging to the Chtoniobacterales order. The only two OTUs common to the three ultramafic areas were a Nitrospirae (OTU_4) and a Proteobacteria affiliated to the *Bradyrhizobium* genus (OTU_70). The OTU_4 is also the most represented OTU with a relative abundance of 2.5% on average.

### 3.3. Factors driving bacterial diversity

#### 3.3.1. All countries combined

Variance partitioning analysis (VPA) was used to determine at higher scale (Mediterranean and tropical sites combined), the contributions of several factors including soil properties (pH, DTPA-extractable elements, cations exchange capacity, organic carbon – eight variables), topography (elevation and exposition), plant species found in each country prospected, and climate (annual temperature and rainfall), as well as interactions between these factors, with the aim of determining which parameters are the primary determinants of hyperaccumulator rhizosphere microbiome composition (Fig. 4). At the level of the three countries studied, all considered, variables explained 50% of the observed variation. Considered alone, abiotic data explained 8.1% of the variation observed, while topographic data explained 5.4%, plant species 2.4% and climatic data 1.1%. However, taking into consideration the full explanatory percentage given by each variable, plant species explains the highest percentage, with 32% followed by climate with 27%. Therefore, interaction between plant species and climate data accounted for 60% and appeared as the main driver of the rhizobacteria diversity at the study scale.

**Table 2**
Alpha-diversity indices for each country studied. Values followed by the same letter are not significantly different at $p \leq 0.05$.

| Moyenne | Observed OTUs[†] | Chao1 | Shannon |
|---|---|---|---|
| Albania | 4195 (2190–5797) a | 5017 (2871–6716) a | 9.59 (8.51–10.05) a |
| Indonesia | 2354 (459–3693) c | 2914 (661–4527) c | 8.89 (7.13–9.61) c |
| Malaysia | 3127 (1427–3920) b | 3844 (1584–4775) b | 9.24 (7.28–10.08) b |

[†] OTU: Operational Taxonomic Unit.

#### 3.3.2. At country scale

At the scale of each country (Albania, Indonesia and Malaysia, considered separately), the variation in the bacterial community structure was mainly partitioned by soil physicochemical parameters, whatever the areas studied and the plant species found in each country (Fig. 5). Indeed, bacterial diversity in samples from Albania (Fig. 5a) was mainly explained by rhizosphere physicochemical parameters, with 16% of the explanation specifically related to abiotic variables (pH, DTPA-extractable elements, cations exchange capacity and organic carbon), yet 46% of explanation when considering the influence of abiotic parameters in association with the other variables. Topographic data, when considered alone, explained a small percentage of the variation

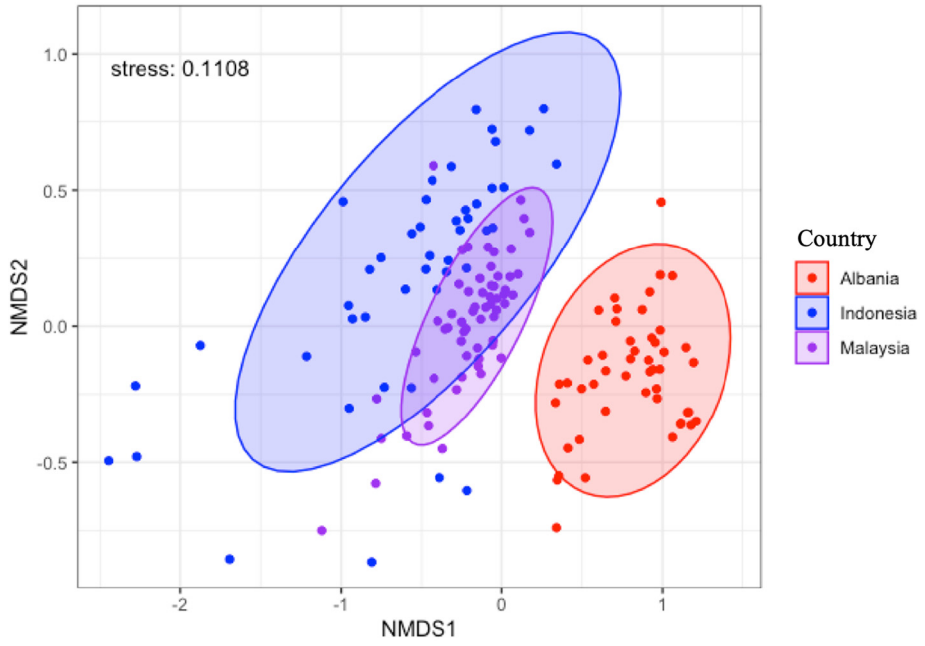


**Fig. 2.** Non-metric multidimensional scaling (NMDS) plot of bacterial communities at OTUs level calculated with the Bray Curtis method. Each point represents a sample. The color of each point corresponds to the country sampled. The confidence area of the ellipses is 0.95 and stress of the representation is shown in the upper left corner.

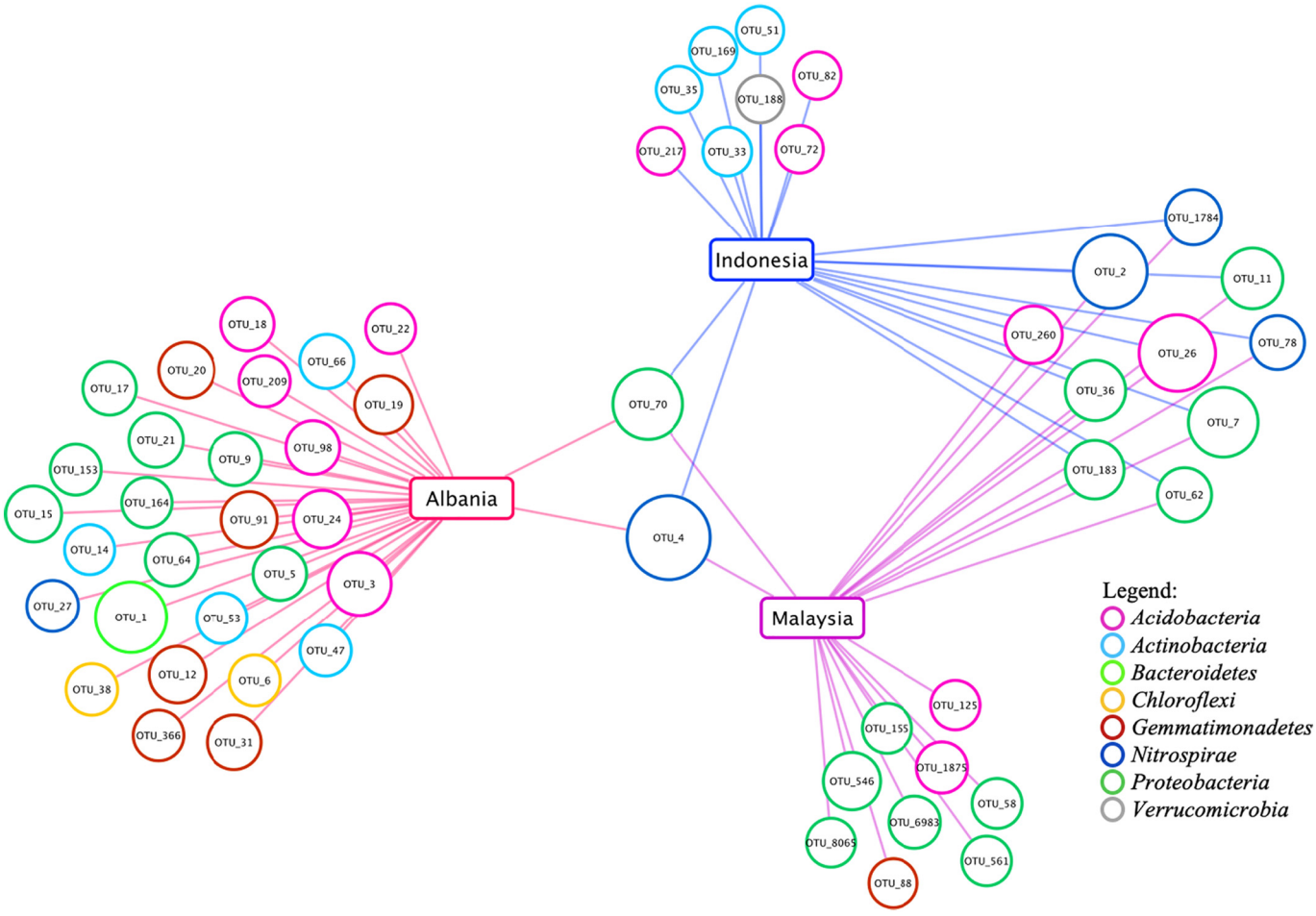


**Fig. 3.** Bacterial network analysis of the major OTUs (> 1%), categorized by country samples. The relative abundance ranging from 1% to 4.7%. Squares represent country and circles represent individual OTUs. The line color indicates the presence of an OTU with a relative abundance >1% in a country.

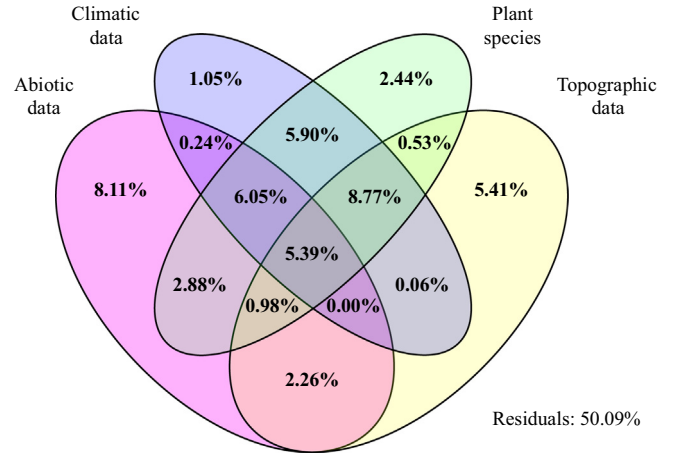


**Fig. 4.** Variance partitioning analysis at the scale of the three studied countries considered all together.

observed and only accounted for 7.4%. On the other hand, the percentages of explanation only relating to the plant species (2.0%) or to plant species in association with other variables (11%) were low, underlying their weak influence on bacterial diversity. Whatever the tropical area considered (Indonesia or Malaysia), the physicochemical properties of the rhizosphere were the best determinants of the hyperaccumulator root microbiome composition (Fig. 5b, c). The bacterial diversity of rhizosphere samples was explained by pH, DTPA-extractable elements, cation exchange capacity and organic carbon, which accounted for 15% and 21%, in Malaysia and Indonesia, respectively, reaching in total 38% and 33% in association with the other variables. Topographic data (elevation and exposition) were the main drivers of bacterial diversity in Malaysia, with 10% of the explanation, whereas plant species had only a weak influence both in Indonesia (0%) and Malaysia (1.5%).

## 4. Discussion

Numerous parameters, intrinsic to the plant's physiology, life history and the soil itself can influence the structure and diversity of rhizomicrobial communities. However, regarding the question of which factor has the greatest effect on microbial community structure and diversity, opinions diverge. Some works have underlined the predominant role of soil physicochemical parameters, while others consider climate or the capacity of plants to thrive as the major driver.

### *4.1. Bacterial diversity in the rhizosphere of hyperaccumulators*

In this study, our sampling, covering 153 different rhizosphere soils from three different ultramafic regions characterized by contrasting climates, confirmed previous results that the three major phyla were Proteobacteria, Acidobacteria and Actinobacteria, whether in environments with high metal concentrations or not (Delmont et al., 2012; Oline, 2006), regardless of the study site. Indeed, at a global level, the best represented phyla were Proteobacteria (35%), followed by Acidobacteria (19%) and Actinobacteria (15%). We found that Proteobacteria was the dominant phylum in the hyperaccumulator rhizosphere for all countries studied, as observed in many other soil types, including multi-contaminated soils (Gołębiewski et al., 2014), naturally metal-rich soils (Lopez et al., 2019a), agricultural soils (Yang et al., 2017) or even forest soils (Uroz et al., 2010). The bacteria belonging to this phylum have been defined as copiotrophic and are known to prefer carbon-rich environments, such as rhizospheres (Yang et al., 2017). The Actinobacteria phylum was also predominant in all the rhizosphere soils sampled and this can be due to its adaptation to toxic concentrations of trace elements (Abou-Shanab et al., 2003).

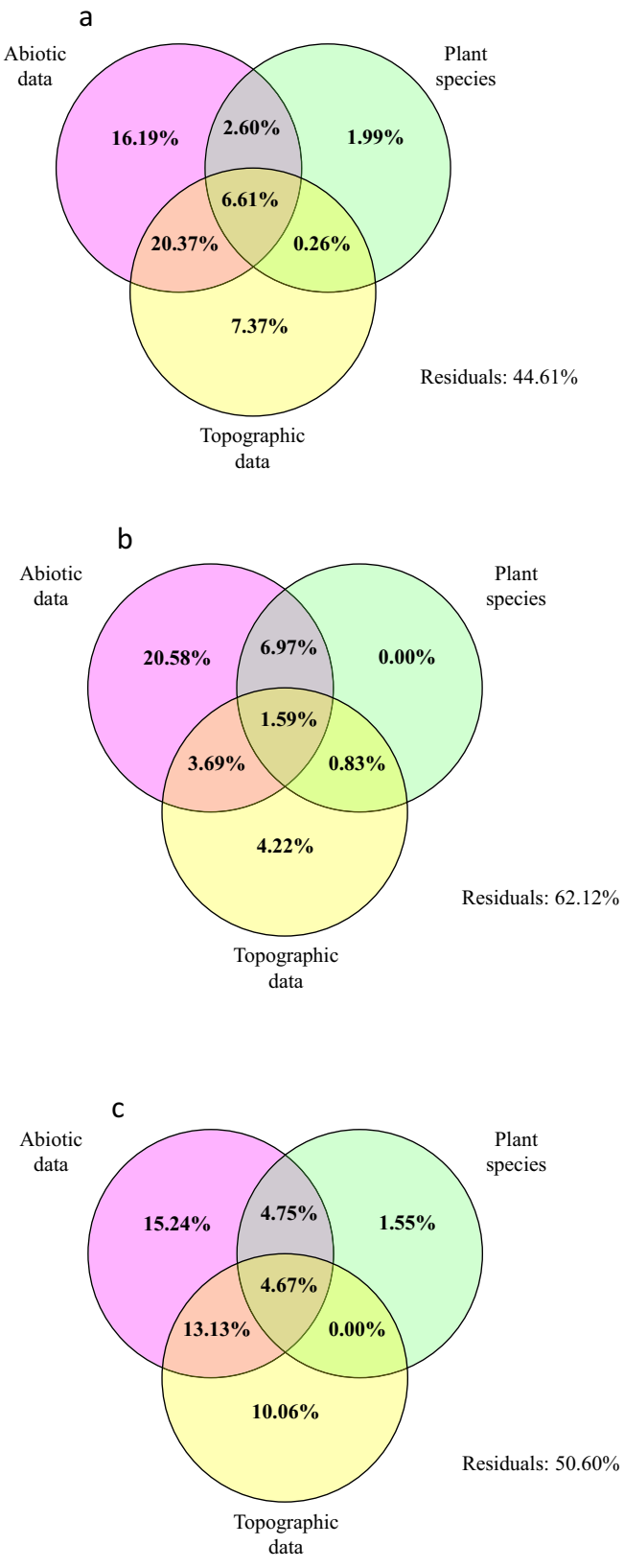


**Fig. 5.** Variance partitioning analysis at the scale of each country considered separately a) Albania, b) Indonesia, c) Malaysia.

Chloroflexi phylum had an average relative abundance of around 7% for all the rhizosphere soils sampled. Our results were in accordance with several studies which showed a proportion of Chloroflexi ranging from

1.7 to 10.3% (Chodak et al., 2013; Gołębiewski et al., 2014; Yasir et al., 2015). This phylum is however, well represented in extreme environments such as hot springs (10 to 15%) or hypersaline lakes (21 to 39%) (Boomer et al., 2002; Nübel et al., 2002; Yamada and Sekiguchi, 2009). Ultramafic soils, deficient in essential nutrients but, conversely, rich in heavy metals, are considered to be stressful environments due to their extreme mineral composition for many microorganisms (Mengoni et al., 2010; Pal et al., 2005). This could therefore explain the strong representativeness of this phylum in ultramafic soils. In addition, this phylum was found to be predominant in samples of Ni mine cuttings (Herrera et al., 2007) and in the rhizosphere of *O. chalcidica* growing on Greek ultramafic soils (Lopez et al., 2017).

Concerning the other abundant phyla in the rhizosphere of the plants sampled, certain peculiarities emerge from our study, depending on the country considered. Indeed, the Latescibacteria phylum had a relative abundance greater than 1% only in Indonesia (2.5%) and Malaysia (1.8%). This phylum is often found in negligible proportions in soils, yet here, on the contrary, represented 4.4% of the relative abundance of telluric bacterial communities in a field cultivated without tillage in the region of La Pampa in Argentina (Farag et al., 2017). The climate of this region of South America is of the humid subtropical type (Cfa), showing similarities to the tropical climates of Indonesia and Malaysia, which are characterized by low temperature differences and an annual rainfall exceeding 1000 mm. In addition, the work of Farag et al. (2017) underlined a relative abundance of about 5% for the Nitrospirae phylum. For the reasons given above, the similarity of climates between their study area and the sampling areas that we surveyed in Indonesia and Malaysia, could explain the strong presence of this phylum. Indeed, the relative abundances that we have established here are similar, respectively 5.2% for Indonesia and 4.9% for Malaysia, and which are significantly statistically higher than those found in Albania (2.4%).

The Gemmatimonadetes are more abundant in Albania, which had the most arid climate, of the three areas studied, with the lowest recorded annual precipitation (567 mm). The relative abundance of Gemmatimonadetes in this country is relatively high, reaching 10% regardless of the site studied. This is in line with the observations by DeBruyn et al. (2011), which showed that the highest proportions of Gemmatimonadetes are found in arid regions. These results suggested an adaptation to low humidity environments by bacteria belonging to this phylum. Indeed, other studies have emphasized that the relative abundances of Gemmatimonadetes are inversely correlated with soil moisture (Chanal et al., 2006; Costello et al., 2009).

### 4.2. Shared OTUs between countries

Of the 13,924 OTUs common to all three countries studied, 57 were found to have a relative abundance greater than 1% in at least one of the countries prospected. These OTUs mainly belonged to either the Proteobacteria phylum (genera *Bradyrhizobium*, *Rhizobacter* and *Azospira*), the Actinobacteria phylum (genera *Streptomyces*, *Gaiella* and *Solirubrobacter*) or to the Acidobacteria one (mainly the Blastocatellaceae family). The OTU, affiliated with the *Bradyrizobium* genus, is one of the two common to all the studied rhizospheres with a relative abundance of nearly 2.5%. Bacteria affiliated at the genus *Bradyrhizobium* have the ability to form nodules with legumes, but are found in the rhizosphere environment of many plant species (Sachs et al., 2009). The strong representativeness of this bacterial genus can be explained by certain peculiarities of the studied ultramafic soils. Indeed, they were rich in trace metal elements and, moreover, had a relatively acidic pH. However, bacteria affiliated with the *Bradyrhizobium* genus thrive in acidic pHs and can be found in environments rich in trace metal elements (Ozawa et al., 1999). The other OTU shared between the 3 countries is a Nitrospirales. Even if the Nitrospirae phylum was not the most represented phylum in the three country (ranging from 2.3% in Albania to 4.8% in Malaysia), the OTU_4 affiliated to the Nitrospirales Order (family 0319-6A21; same as the OTUs numbers 2, 78 and 1784 found only in Indonesia and Malaysia) was the one with the highest relative abundance (mean of 2.5). The work of Lavoie et al. (2017) presented this family as dominant in caves, which may indicate a preponderant role of this OTU in the nitrogen cycle. Its high abundance in the rhizosphere of hyperaccumulator plants could be explained by a low amount of N in ultramafic soils, where these bacteria could therefore play a role in transferring soil N to plants (Mehrani et al., 2020).

### 4.3. Bacterial community explanation when all regions were considered

Focusing on two contrasted climatic areas, Mediterranean and tropical, plant species and climate played a major role in the structure and the diversity of the rhizosphere bacterial communities of the sampled Ni hyperaccumulators. In fact, at OTU level, the NMDS analysis underlined a close rhizosphere bacterial community diversity shared between Indonesia and Malaysia hyperaccumulator plants, which was different from this of the Albanian samples. In addition, at phyla level, the strongest factor driving the bacterial diversity was the temperature (and the annual temperature variation), allowing a distribution of soil samples according to whether they come from a climate Csa (Mediterranean: temperate climate with well-defined seasons and a dry summer, Albania), Af (Equatorial: tropical climate with high precipitations, Indonesia) or Am (Monsoon: tropical climate with a high rainfall period, Malaysia). Our observations corroborated recent studies (Bahram et al., 2018), which showed that bacterial genetic diversity is dependent on climate and thus latitude. Latitude is a relevant integrative parameter, accounting for both the climate and vegetation type. This could explain why for the three countries considered together, plant species explained 32% of the rhizobacterial diversity variation followed by climate with 27% and that the variation was mostly explained by interactions between plant species and climate data, which accounted for 59%.

### 4.4. Country scale bacterial community explanation

This study, carried out on the scale of a climatic region, showed that the soil physicochemical parameters were of paramount importance for the structuring and diversity of bacterial communities. Indeed, whether they were considered alone or in combination with other parameters, soil physicochemical data explained about 82% of the rhizosphere bacterial diversity, in accordance with other studies (Buée et al., 2009; Marschner et al., 2004; Seldin et al., 1998). Indeed, studies have demonstrated that soil has a profound influence on the assembly of bacterial (and also mycorrhizal fungal) communities in the rhizosphere, because the complex physicochemical characteristics of soils affect plant physiology and root exudation patterns, which in turn influence the composition of the rhizosphere microbiota (Philippot et al., 2013).

The second factor explaining the diversity of rhizosphere bacterial communities recorded in this study, corresponded to topographic data of sampling sites, namely exposure and altitude. In Indonesia, topography had the lowest explanatory percentage compared to the total explanation, i.e. 27%, whereas this was 56% in Malaysia and 62% in Albania. It was also in Indonesia that the altitudinal gradient had the lowest influence. This could be explained by the sampled sites in Indonesia, where only one site was at an elevate altitude (981 m asl), whereas the other sites ranging from 0 to 239 m asl. Due to that, altitude affected the relative abundance of many phyla in Albania (sites ranging from 587 to 1524 m asl) and Malaysia (sites ranging from 340 to 1410 m asl), but this was more moderate in Indonesia, where only Proteobacteria and Armatimonadetes were favored at high altitudes, while relative abundances of Actinobacteria and Nitrospirae were lower. On the other hand, regardless of the origin of soil samples, *Bacteroidetes* were the only phylum never influenced by altitude. Siles et al. (2017) and Xu et al. (2015) highlighted the relationship between bacterial diversity and the altitudinal gradient, in relation to the degradation of organic matter. The bacteria belonging to the Bacteroidetes phylum are known to have colonized all types of environments and have great genome plasticity, which allows them to break down diverse carbon sources (Thomas et al., 2011). Thus, the

relative abundance of this phylum was not affected whatever the altitude or the state of the organic matter.

### 4.5. Albania with a Mediterranean climate appears as a great reservoir of bacterial diversity

It is recognized that plant diversity in the tropics is higher than in temperate regions, with a greater plant diversity in equatorial rainforests than in any other type of vegetation (Gentry, 1988). This is the case not only in the Amazon, but also on the island of Borneo, where the largest number of tree species has been estimated (Ashton, 1992; Ter Steege et al., 2000). Nevertheless, we found that the measured rhizosphere bacterial communities α-diversity (Shannon index) was the highest for Albania and statistically higher than those for Malaysia, which was also higher than those estimated in Indonesia. This observation was confirmed by the number of OTUs, with a greater number in the rhizosphere ultramafic soils of Albania than those estimated in Indonesia and Malaysia. Moreover, based on shared OTUs, samples from Albania are phylogenetically distant from Indonesia and Malaysia. Thus, it is clear from these analyses that the European rhizosphere environment (Albania) is a greater reservoir of bacterial diversity than the tropical environments (Indonesia and Malaysia). This confirms the study by (Bahram et al., 2018), which shows that the diversity of bacterial communities (apprehended by the Shannon index) was greater in temperate regions than in tropical regions. The wider annual range of precipitation and temperature in the Mediterranean climate with the associated changes in soil moisture may favor higher soil bacterial diversity. These bacterial populations could be more adapted to contrasting climatic conditions compared to those present under tropical climates, with a higher soil moisture contents during all the year. In this study, we found that taxonomic bacterial diversity peaked in Albania at latitude 40°N and declined towards the Equator (latitude 0.6°N in Indonesia and 6°N in Malaysia), which emphasized that latitude could be a good integrative parameter for highlighting different bacterial diversity patterns on a worldwide scale.

## 5. Conclusion

In our study, after studying 153 ultramafic rhizosphere soils collected from hyperaccumulator plants, we have demonstrated that parameters determining hyperaccumulator rhizobacteria diversity depend on the study scale. Here, the diversity of bacterial communities was highest in Mediterranean habitats where plant diversity was the lowest, in comparison with tropical regions. Moreover, latitude, which is correlated with climate and vegetation types, has been found to be the major driver of soil microbial communities considering all our sampling areas under Mediterranean and tropical climates. In contrast, on the local scale, microbial community structure and diversity has been shown to be largely affected by abiotic variables rather than by plant species. Local physicochemical conditions play a significant role in shaping the bacterial rhizobiomes. Unexpectedly, the effect of soil properties strongly dominates plant properties, such as the composition of root exudates, in determining the characteristics of rhizobiomes.

In order to confirm the role of latitude on the structure of bacterial community diversity from hyperaccumulators, it would be necessary to extend the study to ultramafic regions in other latitudes, such as Philippines (latitude 14°N), Goiás in Brazil (latitude 15°S) or New Caledonia (latitude 20°S). Based on a collection of samples from a larger panel of ultramafic areas, it would be possible to build a predictive model of bacterial diversity according to latitude.

## CRediT authorship contribution statement

Séverine Lopez: formal analysis, investigation, data curation, writing – original draft, visualization.

Jean Louis Morel: writing – review and editing, conceptualization.

Emile Benizri: writing – review and editing, conceptualization, supervision.

## Declaration of competing interest

All authors disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted that might inappropriately influence, or be perceived as influencing, their work.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.155274.

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