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REGULAR ARTICLE

Bacterial communities in the rhizosphere of different populations of the Ni-hyperaccumulator *Alyssum serpyllifolium* and the metal-excluder *Dactylis glomerata* growing in ultramafic soils

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Abstract
Aims Compare bacterial communities in non-vegetated soils and in the rhizosphere of Ni-hyperaccumulating or Ni-excluding plants from four serpentine sites of the Iberian Peninsula.
Methods Bacterial communities in non-vegetated soil and in rhizosphere of *Alyssum serpyllifolium*, and *Dactylis glomerata*, were analysed using denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments of the total bacterial community and of *Alphaproteobacteria*, *Actinobacteria* and *Streptomycetaceae*.
Results Rhizosphere bacterial communities of, either the hyperaccumulators or the excluder plants, were significantly different from communities in non-vegetated soils, especially for *Alphaproteobacteria* and *Actinobacteria*. The main differences between the rhizobacterial communities of the hyperaccumulator and of the excluder corresponded to *Alphaproteobacteria* profiles. Communities in non-vegetated soil were significantly influenced by intrinsic soil properties while in rhizosphere soil these factors were replaced by parameters related to nutrient availability. Members of the genera *Blastococcus*, *Geodermatophilus, Modestobacter, Rhodococcus, Pseudonocardia, Devosia* and *Hyphomicrobium* were detected in the rhizosphere of the Ni-hyperaccumulating plants.
Conclusions Bacterial communities significantly differed between hyperaccumulator and excluder rhizosphere and non-vegetated soil. *Actinobacteria* of the *Geodermatophilaceae* family and *Alphaproteobacteria* of the *Hyphomicrobiaceae* family predominated in the rhizosphere profiles of the Ni-hyperaccumulators. *Alphaproteobacteria* could participate in the tolerance of *Poaceae* to trace elements in ultramafic soils.

Keywords DGGE fingerprinting · Bacterial community · Ultramafic soils · Phytoextraction · Nickel · Endemic plants

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Introduction

The soils derived from ultramafic rocks are commonly referred to as ultramafic or serpentine soils (Proctor 1999). These soils are characterised by elevated concentrations of Mg, Fe and trace elements (TE) such as Co, Cr and Ni, a low Ca/Mg ratio, low concentration of essential plant nutrients such as N, P and K and a strong tendency to drought (Brooks 1987; Whittaker 1954). These

characteristics generate a challenging environment for the establishment of plants, which is one of the reasons why serpentine areas often support a high proportion of endemic species (Brooks 1987; Galey et al. 2017; van der Ent et al. 2013).

The ecology of plants inhabiting ultramafic areas has been well documented, with many plant species showing mechanisms of stress-tolerance. In 1981, Baker defined three strategies whereby plants could persist in environments with high levels of toxic metals and classified them as accumulators, excluders and indicators. Some of the plants following an accumulating strategy are able to concentrate extremely high amounts of metals in their shoots and are called hyperaccumulators (Brooks et al. 1977). Among hyperaccumulators, over 450 taxa are able to accumulate Ni (Pollard et al. 2014; van der Ent et al. 2013). The Iberian Peninsula hosts two serpentine-endemic and Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* Desf.: *Alyssum serpyllifolium* subsp. *lusitanicum* from Galicia (NW Spain) and Trás-os-Montes (NE Portugal), and *Alyssum serpyllifolium* subsp. *malacitanum* from Andalucía (S Spain) (López González 1975; Brooks et al. 1981; Menezes de Sequeira and Pinto da Silva 1992). Hyperaccumulating plants are considered good candidates for application in phytoextraction technologies such as the phytomining of soils or substrates naturally enriched or contaminated with metals. On the contrary, excluders are tolerant to high metal concentrations in soil but metals are excluded from uptake into aerial tissues.

The study of microbial communities in ultramafic soils has received less attention. Studies based on traditional culture-dependent methods generally indicate that these soils are characterised by low microbial density, biomass and activity, which has been attributed mainly to the high concentrations of TE (Acea and Carballas 1986; Lipman 1926; Pal et al. 2005). More recently, some authors using molecular analyses detected high microbial diversity in ultramafic soils (Bordez et al. 2016; Mengoni et al. 2004; Oline 2006; Thijs et al. 2017; Lopez et al. 2017). Recently Lopez et al. (2017) using a high-throughput sequencing technique found, in the rhizosphere of the hyperaccumulator *Alyssum murale* growing in ultramafic soils in Greece, a highly diverse bacterial community dominated by *Chloroflexi*. These authors also observed an important influence of Ni availability (which was positively correlated with the relative abundance of *Alphaproteobacteria* and *Actinobacteria*) on the composition of the bacterial communities. Ultramafic soils are particularly interesting for studying natural microbial adaptation to metal toxicity and the evolution of microbial communities in soils naturally enriched in TE, which constitutes a situation very different from that found in anthropogenically contaminated soils (Héry et al. 2003). Most studies assessing the microbiology of ultramafic soils are devoted to the analysis of bacterial communities in the rhizosphere of Ni-hyperaccumulating plants (Visioli et al. 2015).

The rhizosphere represents a complex microenvironment where plant roots and soil interact with microorganisms. Rhizosphere microorganisms play a vital role in the biogeochemical cycling of nutrients and in the speciation and bioavailability of metals for plants (Kuffner et al. 2008; Mendes et al. 2013; Muehe et al. 2015; Philippot et al. 2013). Likewise, plants exert a great influence on microorganisms through the excretion of root exudates (Philippot et al. 2013). These exudates are an important source of nutrients that will be used by soil bacteria for energy and biomass production (Haichar et al. 2008; Ma et al. 2016). Moreover, these exudates take part in the early colonisation of the roots by indigenous soil microorganisms, inducing chemotactic response of rhizosphere bacteria (Dennis et al. 2010; Haichar et al. 2008; Segura et al. 2009). Root exudates are considered the main factor affecting indigenous soil microorganisms and driving the establishment of specific microbial communities in the rhizosphere of different plant species (Berg and Smalla 2009).

Studies of the rhizosphere bacterial communities associated with Ni-hyperaccumulating plants have been mainly focused on the culturable bacterial community and aimed at the isolation and characterisation of bacterial strains with potential use as bioinoculants to improve metal phytoextraction (Abou-Shanab et al. 2003; Álvarez-López et al. 2016; Becerra-Castro et al. 2009; Benizri and Kidd 2018; Idris et al. 2004; Mengoni et al. 2001). Microbial communities associated with non-accumulating plants growing in ultramafic environments have received much less attention (Mengoni et al. 2010). A few authors have conducted comparative studies looking at the culturable bacterial communities associated with either Ni-hyperaccumulators or non-accumulating plants (Álvarez-López et al. 2016; Becerra-Castro et al. 2009; Delorme et al. 2001), while culture-independent techniques have not been used in this type of study. Becerra-Castro et al. (2009) and

Álvarez-López et al. (2016) found higher densities of Ni-tolerant bacteria in the rhizosphere of some populations of the Ni-hyperaccumulator *Alyssum serpyllifolium* than in the rhizosphere of *Dactylis glomerata*, a metal excluding plant, or non-vegetated soil from the same sites. The increase in densities of metal-tolerant bacteria was related to a higher concentration of bioavailable metal in the rhizosphere of the hyperaccumulators (Becerra-Castro et al. 2009). These authors also observed some differences in the taxa isolated from either the rhizosphere of the hyperaccumulating or excluding plants (Álvarez-López et al. 2016). The rhizosphere of the metal hyperaccumulating *Noccaea caerulescens* (syn. *Thlaspi caerulescens*) growing in Zn and Cd contaminated soil was also found to host a higher ratio of metal-resistant bacteria than the non-hyperaccumulating *Trifolium pratense* growing in the same soil (Delorme et al. 2001).

The aim of this study was to analyse the bacterial community present in the rhizosphere of two Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* Desf. (*Alyssum serpyllifolium* subsp. *lusitanicum* and *Alyssum serpyllifolium* subsp. *malacitanum*) and the metal-excluder plant, *Dactylis glomerata* L., growing in four ultramafic areas in the Iberian Peninsula. The bacterial communities were studied using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments obtained with primers designed for *Bacteria* as well as with group-specific primers. Additionally, multivariate statistical analyses were used to study the relationship between soil properties and the composition of the bacterial communities.

Materials and methods

Study sites and sample collection

Samples were collected in four serpentine areas of the Iberian Peninsula: Barazón (L), in Galicia, NW of Spain (42°51′09″N; 08°01′15″W), Morais (M) and Samil (S), in Trás-os-Montes, NE of Portugal (41°31′21″N; 06°49′20″W and 41°46′48″N; 6°44′47″W, respectively) and Sierra Bermeja (SB) in Andalucía, South of Spain (36°28′48″N; 05°11′52″W). In these serpentine areas, two Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* Desf., endemic to the Iberian Peninsula are found: *A. serpyllifolium* subsp. *lusitanicum* Dudley and P. Silva (commonly referred as *A. pintodasilvae*) in L, M and S sites, and *A. serpyllifolium* subsp. *malacitanum* Rivas Goday (*A. malacitanum*) in SB site (Brooks et al. 1981; Gómez-Zotano et al. 2014; Menezes de Sequeira 1969). The whole plant and root system (including root ball) of 5–7 individuals of the four mentioned *Alyssum* populations were collected. In L, M and S sites 5–7 individuals of the Ni-excluder, *Dactylis glomerata* subsp. *hispanica* (Roth) Nyman were also collected. The rhizosphere soil was separated by gently crushing the root ball and shaking the root system. Tightly held soil (<3 mm from the root surface) was considered as rhizosphere soil. In addition, surface soil samples (0–10 cm) were collected at each site from bare patches at approximately 1 m distance from the sampled *Alyssum* individuals (non-vegetated soil). Aliquots (0.5 g) of the rhizosphere and bulk soil samples were stored at −60 °C until DNA extraction. The analysis of bacterial communities was carried out in the rhizosphere of 4 individuals of each population as well as in 4 replicates of non-vegetated soil from the different serpentine sites. The remaining samples were air-dried and used for physico-chemical analyses, which were also carried out in four replicates as described by Álvarez-López et al. (2016). The main physico-chemical characteristics of the soils at each site are listed in the Table SI 1.

The nomenclature of samples in this study included a first letter indicating the mentioned sampling sites (L, Barazón; M, Morais; S, Samil; SB, Sierra Bermeja), followed by AR, GR or NV, denoting the rhizosphere of *Alyssum*, the rhizosphere of *Dactylis* or non-vegetated soil, respectively.

DNA extraction and PCR amplification of 16S rRNA gene fragments for DGGE

Total soil DNA was extracted from 0.5 g of soil using the PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., California) following the manufacturer's instructions. DNA quality was checked in a 0.8% agarose gel.

The amplification of 16S rRNA gene fragments of *Alphaproteobacteria*, *Actinobacteria* and *Streptomycetaceae* was carried out using a (semi)nested PCR approach. A non-nested approach was used to amplify 16S rRNA gene fragments of the total bacterial community. PCR primers and conditions used are shown in Table SI 2 and PCR reactions were carried out as described by Touceda-González et al. (2017).

DGGE analysis of amplified 16S rRNA gene fragments

A phorU_2 apparatus was used to perform the DGGE analysis (Ingeny, Goes, The Netherlands) of amplified 16S rRNA gene fragments using a double (acrylamide and denaturant) gradient. The gradient was composed of 46.5 to 65.0% of denaturant (100% denaturant corresponds to 7 M urea and 40% (*v*/v) formamide) and 6.2 to 9.0% acrylamide (Gomes et al. 2005). The amount of PCR product used in DGGE was 5 μl, for group–specific fragments, or 7 μl, for total bacterial community fragments. The gels were run in 1× TAE buffer at a constant voltage of 140 V for 17 h at 58 °C, and the gel was stained following a conventional silver staining procedure. After electrophoresis the gels were air-dried and scanned (Epson Perfection V750 Pro, Japan). A marker composed of GC-clamped 16S rRNA gene fragments (positions 984 to 1378) of 7 previously isolated bacterial strains with different electrophoretic mobility was loaded in two lanes on each gel.

Analysis of DGGE fingerprints

The DGGE profiles were analysed using the GelCompar II program, version 6.0 (Applied Maths, Ghent, Belgium). The marker loaded on every gel was used as a reference to convert and normalise the gel images. The pairwise similarities of lanes were calculated for each gel using the Pearson correlation coefficient and the resulting similarity matrices were used for cluster analysis by the unweighted pair-group method using average linkages (UPGMA). The statistical significance of the differences between the microbial community present in the rhizosphere of *Alyssum* or *Dactylis* and in the non-vegetated soil was analysed with a permutation test (Kropf et al. 2004) using the Pearson correlation coefficients similarity matrices. Canonical Correspondence Analysis (CCA), carried out with CANOCO 5 software (Microcomputer Power, USA), was used to relate DGGE fingerprinting information to environmental data (physico-chemical soil properties). The matrices containing DGGE information consisted of relative band intensities calculated by dividing individual band intensity by the sum of all the band intensities in the analysis. Explanatory variables were transformed to a normal distribution by scaling the data obtaining a mean of 0 and a standard deviation of 1. The explanatory variables showing a significant influence on the composition of the bacterial communities ($P < 0.05$) were chosen using manual forward selection (i.e. model building was initiated including the variable that is most significant in the initial analysis, and continued to add variables until the model was not improved by the incorporation of a new variable). The statistical significance of the canonical axes was tested with a Monte Carlo permutation test ($n = 999$). The DGGE bands which were best explained by the model (FitE values >75%) were selected.

Excision and sequencing of DGGE fragments

Some of the DGGE bands, which were sample specific or more intense in one sample compared to the others, were excised and sequenced. Bands were cut from the gels by using a sterile scalpel, and immediately placed in a centrifuge tube, broken into small pieces with a sterile pipet tip and incubated overnight at 4 °C in 50 μl of sterile Mili-q water for the elution of DNA. The tubes containing the DGGE bands were centrifuged at 10,000 g for 10 min and the supernatant was carefully recovered. The eluted DNA was then re-amplified using primers F984GC and R1378 as previously described. PCR reamplified fragments were ligated into the pCR™4-TOPO® vector (ThermoFischer) and used for the transformation of One Shot® Top10-competent *Escherichia coli* cells (ThermoFischer). Blue-white screening of transformants was done on LB agar plates containing 50 mg mL^{-1} of ampicillin and top spread with 40 μL of 40 mg mL^{-1} of X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside). Clones were screened for inserts of the correct size by PCR with appropriate primers included in the cloning kit. Three clones from each excised band were sequenced. The obtained sequences were compared to those in the EMBL database by using FASTA at the European Bioinformatics Institute and with those of the GenBank by using nucleotide BLAST to identify closely related gene sequences.

Results

Bacterial community analysis

All PCR amplified fragments of 16S rRNA gene of *Bacteria* (total bacterial community, TC) *Alphaproteobacteria*, *Actinobacteria* and *Streptomycetaceae* generated products which were adequate for analysis by DGGE.

Complex band patterns were obtained for all sites and bacterial groups analysed. In general, there was a good reproducibility between biological replicates both in the rhizosphere and in the non-vegetated soil samples. The UPGMA dendrograms (based on the Pearson similarity coefficients) showed that rhizosphere DGGE profiles of the hyperaccumulator (AR) could be distinguished from those of the excluder rhizosphere (GR) or of the non-vegetated soil (NV) (Fig. 1).

The dendrogram obtained from the similarity matrix of DGGE profiles for the total bacterial community showed a cluster including all samples from the Portuguese sites (Fig. 1a). Within this cluster, the rhizosphere samples of *Dactylis* and *Alyssum* (except for two replicates) were separated into two different groups and the non-vegetated soils, SNV and MNV, also formed two additional separate groups (Fig. 1a). In contrast, samples from Barazón and Sierra Bermeja did not form separate groups. Instead, LAR replicates clustered together and showed low similarity with other samples. SBAR samples also grouped together and surprisingly appeared associated to LNV (Fig. 1a).

In the dendrogram of *Alphaproteobacteria*, the rhizosphere of all *Dactylis* populations clustered within the same clade (Fig. 1b). On the contrary, the rhizosphere of *Alyssum* grouped in two distant clusters, the first including samples from Sierra Bermeja and most of the replicates of Samil and the second containing samples from Barazón and all other replicates of the Portuguese populations. *Alphaproteobacteria* profiles of the non-vegetated soil from Barazón and Sierra Bermeja formed two distant groups with a high similarity between replicates but the non-vegetated soil from Samil and Morais showed more heterogeneous profiles (Fig. 1b).

In the dendrogram of *Actinobacteria*, the samples from the rhizosphere of *Alyssum* populations formed four distant clades (Fig. 1c). In general, the *Alyssum* profiles were also distantly related to the patterns found in the corresponding non-vegetated soils. The profiles of the rhizosphere of *Dactylis* populations also constituted separate clusters. In Barazón and Morais the *Actinobacteria* associated with *Dactylis* were closer to those of the non-vegetated soil than to the *Alyssum* community (Fig. 1c).

In the dendrogram of *Streptomycetaceae*, the samples from different populations tended to group separately. The profiles from Sierra Bermeja formed a cluster (Fig. 1d) and within this the profiles of *Alyssum* rhizosphere (SBAR) clustered separately from those of non-vegetated soil (SBNV). Most of the samples from Portugal, except for the non-vegetated soil from Samil and two rhizosphere replicates from Morais formed a big clade. Within this clade, the samples from Samil were grouped and the rhizosphere of *Alyssum* clustered separately from that of *Dactylis*. The samples from Morais, also tended to group together but the profiles were more heterogeneous. Most samples from Barazón also formed a single cluster and these were more related to the Portuguese populations than to the Sierra Bermeja population. Within this clade the non-vegetated soil profiles (LNV) clustered separately from the rhizosphere profiles.

On a site-by-site level, most of the dendrograms of *Bacteria* DGGE profiles revealed a remarkable separation in community structure between *Alyssum* and *Dactylis* and that of the non-vegetated soil. Based on the permutation tests, differences between the DGGE fingerprints of the rhizosphere, either *Alyssum* or *Dactylis*, and of the non-vegetated soil, were statistically significant, except in the case of some of the *Streptomycetaceae* profiles ($P < 0.05$; Table 1). Generally, the most pronounced dissimilarities corresponded to the profiles of total bacterial community, although the calculated *d-value* for the comparison between *Alyssum* rhizosphere and the non-vegetated soil was maximal for the Alphaproteobacterial profiles in Barazón and for the Actinobacterial profiles in Samil and Sierra Bermeja ($P < 0.05$; Table 1). The fingerprints of total bacterial community, *Alphaproteobacteria* and *Actinobacteria* of the *Alyssum* rhizosphere were also statistically different from those of the *Dactylis* rhizosphere. The dissimilarities reached maxima in the case of Alphaproteobacterial profiles.

The analysis of DGGE profiles from *Alyssum* rhizosphere growing in Barazón, Morais, Samil and Sierra Bermeja revealed that all four rhizosphere soils host significantly different bacterial communities ($P < 0.05$; Table 1), and this was generally the case for all the studied bacterial groups (Fig. SI 1). The lower calculated dissimilarities corresponded to the differences between the two Portuguese populations. The dissimilarities tended to be higher in the case of the *Streptomycetaceae* profiles. In the *Alphaproteobacteria* profiles there was a noteworthy high similarity between SBAR replicates.

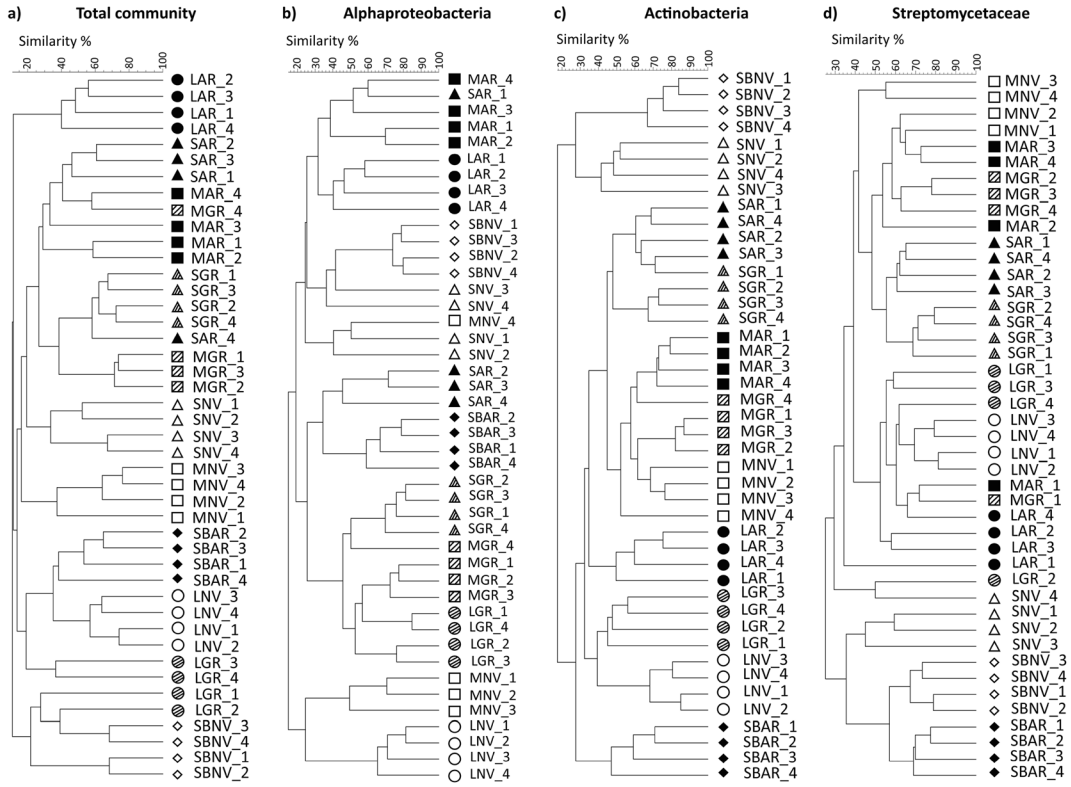

Fig. 1 UPGM dendrogram based on Pearson's correlation similarity matrices of the (**a**) total bacterial, (**b**) *Alphaproteobacteria*, (**c**) *Actinobacteria* and (**d**) *Streptomycetaceae* communities from the rhizosphere of *Alyssum* (AR) and *Dactylis* (GR) (except in SB site) and the non-vegetated soil (NV) in all four serpentine areas studied of the Iberian Peninsula (Barazón (L), Morais (M), Samil (S) and Sierra Bermeja (SB))

Relationship between environmental variables and the bacterial community DGGE patterns

CCA was used to investigate the correlation between environmental variables and the bacterial community data from DGGE patterns.

The data corresponding to the rhizosphere of the two plant species and to the non-vegetated soils were analysed separately and the explanatory variables obtained in the CCAs were compared (Figs. 2 and SI 2). In the CCA of DGGE band patterns of the different bacterial groups the variance explained by axis 1 and 2 was generally around 30%, although in the *Alphaproteobacteria* CCA of the *Alyssum* rhizosphere (Fig. 2a) and in the *Actinobacteria* CCA of *Alyssum* and *Dactylis* rhizosphere (Fig. 2d, e), only one axis was extracted by the analysis and the variance explained was lower (between 13 and 22%). The environmental variables contributed to explaining the variation in the community of all studied bacterial groups (the species-environment correlations for axes 1 and 2 were generally >0.885 and > 0.812, respectively).

In general, the CCA biplots defined by axes 1 and 2 (Figs. 2c, f and Figs. SI 2 c and SI 2 f) showed a separation of the samples of non-vegetated soils from the four serpentine sites studied. A similar grouping was observed for the samples of the *Dactylis* rhizosphere except in the CCA biplot of the Actinobacterial band patterns (Figs. 2b, e and Figs. SI 2 b and SI 2 e). In the case of *Alyssum* rhizosphere, the samples from Barazón were generally separated from all other *Alyssum* samples, although in the CCA of the total bacterial community samples from Sierra Bermeja appeared together with those of Barazón (Figs. 2a, d and Figs. SI 2 a and SI 2 d).

The variables which contributed most to explaining variations in the bacterial community depended on the type of soil and on the bacterial group. In the case of the total community patterns of the non-vegetated soil (Fig. SI 2 c), the variables with the highest intraset correlations were exchangeable Mg and K and CEC. Nevertheless, exchangeable K was absent in the CCA of samples of *Alyssum* rhizosphere (Fig. SI 2 a) and

Table 1 Dissimilarity values (d) obtained using the Kropf permutation test between DGGE patterns of bacterial communities in the rhizosphere soil of *Alyssum* and *Dactylis* and in non-vegetated soils from serpentine areas of Barazón, Morais, Samil and Sierra Bermeja. Asterisks indicate significant differences at $P \leq 0.05$

DOMAIN/GROUP		*Bacteria*	*Alphaproteobacteria*	*Actinobacteria*	*Streptomyces*
Rhizosphere effect	LAR-LNV	51.0*	71.9*	44.5*	5.2
	LGR-LNV	30.9*	16.5*	23.4*	23.8*
	MAR-MNV	52.1*	38.3*	18.2*	6.6
	MGR-MNV	34	27.9*	18.8*	15.7*
	SAR-SNV	42.6*	18.8*	63.8*	23.1*
	SGR-SNV	56.1*	35.8*	58.2*	38.5*
	SBAR-SBNV	41.3*	48.7*	80.4*	17.5*
Hyperaccumulator vs. Excluder	LAR-LGR	32.0*	55.9*	30.8*	15.0
	MAR-MGR	30.5*	48.9*	28.2*	0.5
	SAR-SGR	35.1*	36.5*	27.9*	12.0*
Hyperaccumulator	LAR-MAR	30.5*	12.3*	32.1*	42.8
	LAR-SAR	31.9*	27.4*	25.6*	32.0*
	LAR-SBAR	34.3*	39.1*	30.4*	41.5*
	MAR-SAR	13.0*	22.5*	14.3*	17.6*
	MAR-SBAR	23.6*	37.6*	31.4*	30.7*
	SAR-SBAR	39.1*	22.7*	25.2*	37.8*

CEC absent in the CCA of *Dactylis* rhizosphere (Fig. SI 2 b). Additionally, in the latter case, total Ni concentration also showed a high loading factor on axis 2.

The environmental variables which mostly influenced *Alphaproteobacteria*, *Actinobacteria* and *Streptomycetaceae* community composition of the non-vegetated soil were total organic C, total Ni concentration and available Mg (Figs. 2c, f and SI 2 f). In the CCA of *Streptomycetaceae* CEC also showed high loading factors on both axes 1 and 2 (Fig. SI 2 f). However, the variables explaining the variation in these bacterial groups in the rhizosphere of *Alyssum* and *Dactylis* were different. In the rhizosphere of *Alyssum* only CEC showed a high intraset correlation with axis 1 of the CCA of *Alphaproteobacteria* and *Actinobacteria*, while in the *Streptomycetaceae* CCA, CEC presented a high loading factor on axis 1 and EDTA-extractable Co on both axes 1 and 2 (Figs. 2a, d and SI 2 d). In the rhizosphere of *Dactylis*, EDTA-extractable Ni and exchangeable Mg contributed to explaining the variation in *Alphaproteobacteria*, exchangeable K affected the variation of *Actinobacteria*, while in the *Streptomycetaceae* CCA the variables with highest intraset correlations were EDTA-exchangeable Ni, Co and Cr (Figs. 2b, e and SI 2 e).

Separate CCA analyses were also carried out for each bacterial group combining data from the non-vegetated soil with either *Alyssum* or *Dactylis* rhizosphere (Fig. SI 3). The variance explained by axes 1 and 2 was generally low (15–18%). In these analyses the environmental variables more related with the bacterial composition were different in the case of *Alyssum* or *Dactylis* rhizosphere. In the CCA biplots of *Alphaproteobacteria*, *Actinobacteria* and *Streptomycetaceae* the samples of non-vegetated soil were separated from those of *Dactylis* rhizosphere (Fig. SI 3a to 3f). The variables that contributed most to this separation were CEC and available K. In the case of *Alyssum* rhizosphere, the separation from non-vegetated samples was not consistent and available K was absent among the significant variables (Fig. SI 3a, 3c, 3e and 3 g).

Sequence analysis of dominant DGGE bands

Some prominent and characteristic bands observed in the DGGE profiles of the rhizospheres or the non-vegetated soil were excised and processed for their nucleotide sequence analysis (Fig. 3).

In the DGGE gel of total bacterial community few of the excised bands could be sequenced (Fig. 3a). Most of

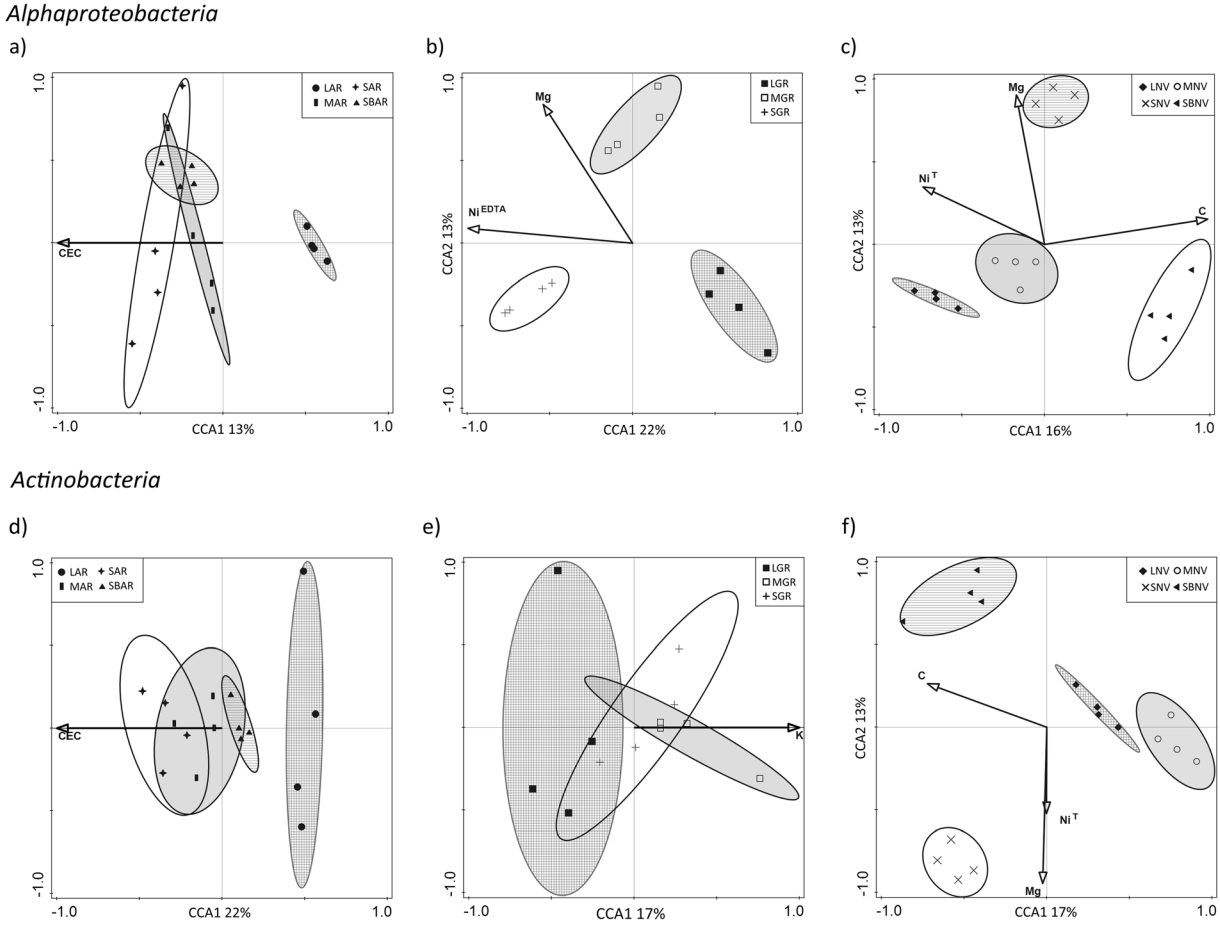

Fig. 2 Canonical correspondence analysis biplot with 95% confidence ellipses of *Alphaproteobacteria* and *Actinobacteria* DGGE patterns and physico-chemical parameters (represented by arrows) of a) the two *Alyssum* subspecies rhizosphere, b) the *Dactylis* rhizosphere and c) the non-vegetated ultramafic soils. CEC, cation exchange capacity ($cmol_c\ kg^{-1}$); Mg, exchangeable Mg ($cmol_c\ kg^{-1}$); K, exchangeable K ($cmol_c\ kg^{-1}$); C, Organic C percentage; Total concentration of Ni ($mg\ kg^{-1}$); Ni_{EDTA}, EDTA-extractable Ni concentration ($mg\ kg^{-1}$)

the sequences obtained were classified within phylum *Actinobacteria*, although none of them could be affiliated to specific organisms (Table SI 3).

In the case of *Alphaproteobacteria* and *Actinobacteria*, in general, the sequence of clones obtained from one band showed a high similarity and were identified as belonging to the same or phylogenetically close organisms (Tables 2 and 3).

Several bands characteristic of the *Alyssum* rhizosphere were cut from the *Alphaproteobacteria* DGGE gel (Fig. 3b). One additional prominent band present in SBNV was also analysed. The sequence of most of the prominent bands analysed showed high similarity with the 16S rRNA gene sequence of *Devosia* spp. and *Hyphomicrobium* spp. belonging to *Hyphomicrobiaceae* family (Table 2).

In the *Actinobacteria* DGGE gel, prominent bands characteristic of the rhizosphere of the different *Alyssum* populations, (Fig. 3c) as well as some bands from the profiles of non-vegetated soil of Samil and Sierra Bermeja and of the rhizosphere of *Dactylis* from Barazón and Morais, were analysed. The sequences of bands obtained from the *Alyssum* rhizosphere were highly similar to those of *Modestobacter*, *Blastococcus* or *Geodermatophilus* genera of the family *Geodermatophilaceae* (Table 3). Bands with sequences closer to that of *Rhodococcus erythropolis*, *Mycobacterium* or *Pseudonocardia* were also prominent in the *Alyssum* rhizosphere profiles. Intense bands identified as from *Mycobacterium* and *Pseudonocardia* were detected in the profiles of *Dactylis* rhizosphere and of non-vegetated soil, although their electrophoretic

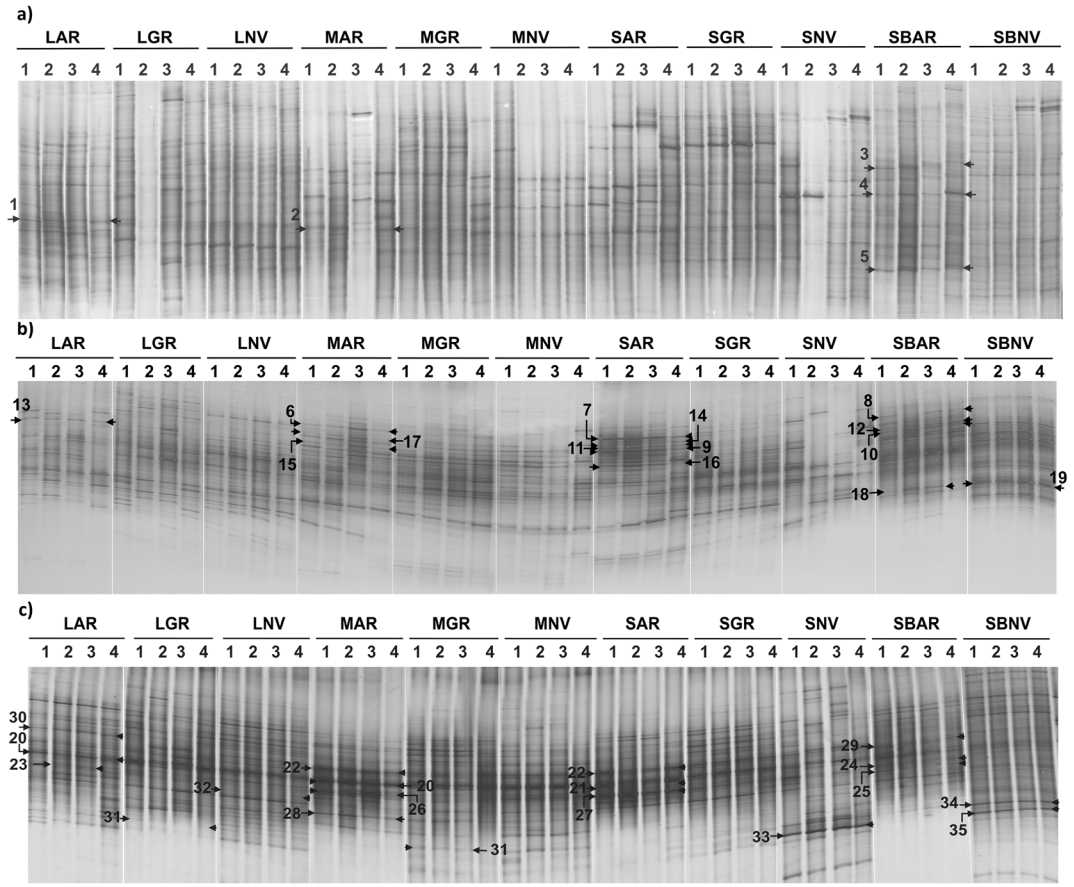

Fig. 3 DGGE fingerprinting from *A. serpyllifolium* (LAR, MAR, SAR, SBAR) and *D. glomerata* (LGR, MGR, SGR) rhizosphere soil and non-vegetated soil (LNV, MNV, SNV, SBNV) from four ultramafic sites in the Iberian Peninsula. (**a**) Total bacterial community (*Bacteria*), (**b**) *Alphaproteobacteria* and (**c**) *Actinobacteria*. Arrows indicate bands that were dominant and/or characteristic of each sample

mobility was different than those detected in the *Alyssum* rhizosphere profiles. Other prominent bands in the profiles of non-vegetated soil were identified as derived from *Tsukamurella* sp. and *Arthrobacter* sp. (Table 3).

Discussion

In this study, DGGE analysis of 16S rRNA gene fragments has been applied to study the bacterial communities in non-vegetated soil and in the rhizosphere of Ni-hyperaccumulating (subspecies of *Alyssum serpyllifolium*) and the excluder (*Dactylis glomerata*) growing in four serpentine areas in the Iberian Peninsula. In the four studied sites, the bacterial communities in the rhizosphere soils significantly differed from the communities in non-vegetated soils, and this was observed at different taxonomic levels (*Bacteria*, *Alphaproteobacteria*, *Actinobacteria* and *Streptomycetaceae*).

Several studies have shown prominent differences between the microbial communities in non-vegetated and rhizosphere soils (Costa et al. 2006; DeAngelis et al. 2008; Gomes et al. 2001; Smalla et al. 2001). The rhizosphere is a stable and favourable environment that favours the settlement of soil microorganisms and acts selectively on the composition and structure of microbial communities (Steer and Harris 2000). Numerous studies have demonstrated increased microbial biomass and activity in the rhizosphere, which has mainly been related to the release of rhizodeposits by plant roots, principally as root exudates (Berg and Smalla 2009; Brimecombe et al. 2001; Hartmann et al. 2009). Root exudates act as signalling molecules and are a major source of available carbon for microbial proliferation (Brimecombe et al. 2001; Hartmann et al. 2009).

Table 2 Sequence analysis of bands excised from *Alphaproteobacteria* community DGGE gels from *A. serpyllifolium* and *D. glomerata* rhizosphere samples and from the non-vegetated soils, collected in four ultramafic areas in the Iberian Peninsula

Band	Clone	Sample	Family	Closest phylogenetic relatives	Similarity
6	B6C1	MAR	*Hyphomicrobiaceae*	*Devosia sp.*	99%
7	B7C1	SAR	*Hyphomicrobiaceae*	*Devosia sp.*	98%
8	B8C1	SBAR	*Hyphomicrobiaceae*	*Devosia sp.*	97%
	B8C2	SBAR	*Hyphomicrobiaceae*	*Rhizomicrobium sp.*	95%
9	B9C1	SAR	*Hyphomicrobiaceae*	*Devosia sp.*	99%
10	B10C1	SBAR	*Hyphomicrobiaceae*	*Devosia sp.*	96%
	B10C2	SBAR	*Hyphomicrobiaceae*	*Hyphomicrobium sp.*	96%
11	B11C1	SAR	*Hyphomicrobiaceae*	*Devosia sp.*	98%
12*	B12C1	SBAR	*Hyphomicrobiaceae*	*Devosia sp.*	98%
13	B13C1	LAR	*Hyphomicrobiaceae*	*Devosia sp.*	99%
14	B14C1	SAR	*Hyphomicrobiaceae*	*Devosia sp.*	99%
15*	B15C1	MAR	*Hyphomicrobiaceae*	*Devosia sp.*	96%
16	B16C1	SAR	*Hyphomicrobiaceae*	*Devosia sp.*	98%
17	B17C1	MAR	*Caulobacteraceae*	*Brevundimonas sp.*	99%
18*	B18C1	SBAR	*Hyphomicrobiaceae*	*Hyphomicrobium sp.*	96%
19*	B19C1	SBNV	*Hyphomicrobiaceae*	*Hyphomicrobium sp.*	94%

*The sequence of one of the clones analysed did not match that of the other clones from the same band: BC12C2, *Caulobacter* sp. BC15C2, *Rhizobium*, B18C2, *Rubellimicrobium* sp. B18C2 *Bradyrhizobium* sp.. Similarities 94%, 97%, 93 and 96%, respectively

Many soil microbes are carbon limited, whereby a quick response to root-induced changes in the rhizosphere soil is expected (DeAngelis et al. 2008). In our study, the organic matter content and dissolved organic C tended

Table 3 Sequence analysis of bands excised from *Actinobacteria* community DGGE gels from *A. serpyllifolium* and *D. glomerata* rhizosphere samples and from the non-vegetated soils, collected in four ultramafic areas in the Iberian Peninsula

Band	Clone	Sample	Family	Closest phylogenetic relatives	Similarity
20	B20C1	MAR	*Geodermatophilaceae*	*Geodermatophilus* sp.	91%
21	B21C1	SAR	*Geodermatophilaceae*	*Blastococcus* sp.	95%
22	B22C1	SAR, MAR	*Geodermatophilaceae*	*Geodermatophilus* sp.	91%
23*	B23C1	LAR	*Geodermatophilaceae*	*Blastococcus* sp.	95%
24*	B24C1	SBAR	*Geodermatophilaceae*	*Modestobacter* sp.	97%
25	B25C1	SBAR	*Geodermatophilaceae*	*Blastococcus* sp.	97%
26	B26C1	MAR	*Corynebacterineae*	*Rhodococcus erythropolis*	97%
27	B27C1	SAR	*Corynebacterineae*	*Rhodococcus erythropolis*	99%
28	B28C1	MAR	*Mycobacteriaceae*	*Mycobacterium* sp.	98%
29	B29C1	SBAR	*Mycobacteriaceae*	*Mycobacterium* sp.	95%
30	B30C1	LAR	*Pseudonocardiaceae*	*Pseudonocardia* sp.	92%
31	B31C1	LGR, MGR	*Mycobacteriaceae*	*Mycobacterium* sp.	94%
32	B32C1	LNV	*Pseudonocardiaceae*	*Pseudonocardia* sp.	97%
33	B33C1	SNV	*Tsukamurellaceae*	*Tsukamurella* sp.	98%
34	B34C1	SBNV	*Micrococcaceae*	*Arthrobacter* sp.	99%
35	B35C1	SBNV	*Mycobacteriaceae*	*Mycobacterium* sp.	93%

*The sequence of one of the clones analysed did not match that of the other clones from the same band: BC23C2, *Mycobacterium* sp. BC24C2, *Nocardioides* sp. Similarities 95 and 97%, respectively

to be higher in the rhizosphere than in the non-vegetated soil. In addition, in low nutrient soils (such as serpentine soils) a stronger dependence of the root microbiome on plant exudates is expected (Dakora and Phillips 2002; Marschner et al. 2004).

Here, the rhizosphere effect was similar or more pronounced for *Alphaproteobacteria* and/or *Actinobacteria* than for the total bacterial community. Several authors found that the intensity of the rhizosphere effect depends on the bacterial group under investigation (Costa et al. 2006; Garbeva et al. 2004; Gomes et al. 2001). Gomes et al. (2001) found a strong seasonal population shift in the rhizosphere community of maize cultivars in the DGGE patterns of total bacteria, *Alpha-* and *Beta-proteobacteria*, and to a lesser extent for *Actinobacteria*. In this study, we focused on *Actinobacteria* and *Proteobacteria* bacterial groups based on the results obtained in previous culture-dependent studies which found that the culturable rhizosphere bacterial communities of the Ni-hyperaccumulating *Alyssum* in the Iberian Peninsula were dominated by members of these two phyla (Álvarez-López et al. 2016; Becerra-Castro et al. 2009; Becerra-Castro et al. 2011). Members of *Proteobacteria* and *Actinobacteria* are considered as abundant inhabitants of the rhizosphere (Philippot et al. 2013). The *Proteobacteria* phylum includes numerous fast-growing bacteria, known as r strategists, which have the capacity to use a wide range of root exudates as carbon substrate (Fierer et al. 2007; Philippot et al. 2013) as well as numerous plant symbiotic and plant growth promoting bacteria able to fix atmospheric nitrogen (Gomes et al. 2001; McCaig et al. 1999).

The UPGMA dendrograms and the permutation test, showed significant differences between the bacterial communities associated with the hyperaccumulator and excluder plants in the serpentine sites analysed. Several studies have highlighted the influence of different plant species on the composition and structure of rhizosphere bacterial communities (Costa et al. 2006; DeAngelis et al. 2008; Dias et al. 2012; Haichar et al. 2008; Hartmann et al. 2009; Smalla et al. 2001; Steer and Harris 2000). Smalla et al. (2001), detected plant species-specific bacterial communities in the rhizosphere of three important agricultural plant species, strawberry, oilseed rape and potato. Haichar et al. (2008) worked with plant species belonging to the same families as *Alyssum* (*Brassicaceae*) and *Dactylis* (*Poaceae*) and found that the differences between the rhizosphere communities of rape (*Brassicaceae*) and wheat (*Poaceae*) were more pronounced in the case of bacteria using root exudates than when analysing other bacteria present in the rhizosphere.

The hyperaccumulating *Alyssum* and the excluder *Dactylis* have developed opposing mechanisms to cope with high concentrations of TE which are naturally present in the ultramafic soils where they proliferate. Hyperaccumulators accumulate high concentrations of TE in their shoots, while excluders limit the absorption and transfer of metals to the aboveground biomass (Baker 1981). Several authors have suggested that root exudates, especially chelating organic acids, may play an important role in Ni acquisition by hyperaccumulators (Benizri and Kidd 2018; Wenzel et al. 2003). *Poaceae* are known for their capacity to produce compounds such phytosiderophores, which have a high affinity for iron and are also able to chelate other micronutrients. Some authors found differences in the composition and chelating properties of organic compounds in the rhizosphere of hyperaccumulators and excluders (Cattani et al. 2009; Wenzel et al. 2003). In the sites studied here, the concentration of dissolved organic carbon in the rhizosphere of *Dactylis* was higher (in Barazón and Morais) or similar (in Samil) than in the rhizosphere of *Alyssum*. However, we did not study the exudate composition of these two plant species. Changes induced by plants in the soil surrounding the roots are also affected by the root system architecture (Hinsinger and Courchesne 2007; Schwartz et al. 1999). Differences in root size and morphology between *Alyssum* and *Dactylis* may also have an impact on the rhizosphere bacterial community.

The dissimilarities between the rhizosphere communities of the two plants reached their maximum in the case of *Alphaproteobacteria*. Moreover, Alphaproteobacterial profiles of the rhizosphere of different *Dactylis* populations showed a high similarity and clustered together separately from the other soils. Gomes et al. (2010) observed that the soil amendment with non-contaminated and Cd- and Zn-contaminated sludges induced a more pronounced alteration of the *Alphaproteobacteria* community in arable soils than in soils covered by *Poaceae* (permanent grass ley). The differences observed were related to the potential plasmid-mediated transfer of heavy metal resistance within *Alphaproteobacteria* (Sandaa et al. 1999, 2001). Lopez et al. (2017) also found a positive correlation between Ni availability and the relative abundance of some *Alphaproteobacteria* such as

members of the family *Hyphomonadaceae*, in ultramafic soils in Greece. *Alphaproteobacteria* may play a role in the mechanisms of exclusion or contribute to the tolerance of *Poaceae* to the high concentrations of TE in ultramafic soils.

The CCA analysis indicated that the main environmental factors influencing the bacterial community in the non-vegetated soil differed from those affecting the communities in the rhizosphere of *Alyssum* and *Dactylis*. In the non-vegetated soils, the *Alphaproteobacteria*, *Actinobacteria* and *Streptomycetaceae* communities were affected by available Mg and soil characteristics such as the total organic matter content and total Ni concentration. However, in the rhizosphere soils the influence of total Ni and organic matter content was replaced by other parameters related to nutrient availability (available K or Mg in the rhizosphere of *Dactylis*, and CEC in the rhizosphere of *Alyssum*). In the *Dactylis* rhizosphere, but not in the *Alyssum* rhizosphere, the available concentration (extractable with EDTA) of TE also contributed towards shaping the communities of *Alphaproteobacteria* and *Streptomycetaceae*. The results suggest a potential role of the rhizosphere bacterial community on plant nutrition and, in the case of the excluder, in TE tolerance. Despite similar levels of available K in the rhizosphere of both *Dactylis* and *Alyssum*, this variable contributed towards shaping the bacterial communities associated with the excluder but not with the hyperaccumulator. This result could be related to the contrasting strategies of these plants and their associated microorganisms for scavenging nutrients in ultramafic soils.

Some dominant bands were excised from DGGE gels with the aim of identifying members of the bacterial community characteristic of the different samples analysed. We observed that some bands, particularly those from the total community profiles, were constituted by fragments from organisms phylogenetically very distant which indicates that a large diversity might be hidden behind a single DGGE band. Similar observations were made by several authors (Costa et al. 2006; Gomes et al. 2001; Schmalenberger and Tebbe 2003; Smalla et al. 2001). Furthermore, bands excised from different positions in the gels were found to be associated with organisms of the same genus. Kušar and Avguštin (2012) obtained similar results when optimising the DGGE band identification method.

The DGGEs targeting different bacterial groups allowed for the detection of organisms that were not found in the total community analysis, enhancing the level of resolution of the PCR-DGGE technique. The sequences of bands characteristic of *Alyssum* rhizosphere excised from the actinobacterial DGGE gels were found to be originated from members of the genera *Blastococcus* sp., *Geodermatophilus* sp. and *Modestobacter* sp. (all three belonging to the family *Geodermatophilaceae*), *Mycobacterium* sp., *Rhodococcus* sp. and *Pseudonocardia* sp. The bacteria affiliated to the family *Geodermatophilaceae* have the ability to colonise poor substrates, such as rocks and extreme soils of hot and cold deserts and present modest growth requirements (Normand et al. 2014). Members of *Geodermatophilaceae* possess enzymatic tools to adapt to extreme environments. Essoussi et al. (2010) observed that the esterase activities in some members of this family, showed a high flexibility under high temperature, alkaline pH and high cationic concentration. Gtari et al. (2012) found a higher resistance to TE in *Blastococcus* than in *Geodermatophilus obscurus* or *Modestobacter multiseptatus*. The bands with a sequence similar to that of *Pseudonocardia* sp. were excised from both the non-vegetated ultramafic soil and the rhizosphere of *Alyssum*, but band intensity was higher in the rhizosphere profiles. Bacteria from this genus have been isolated and commonly detected in association with plants (Franco and Labeda 2014). Álvarez-López et al. (2016) also isolated strains of *Lentzea* sp., from the rhizosphere of the *Alyssum*. In the two Portuguese populations, a band characteristic of the *Alyssum* rhizosphere showed a sequence similar to that of *Rhodococcus erythropolis*. Members of the genus *Rhodococcus* are widely distributed in soils, have a saprophytic lifestyle and they are known to have a role in organic matter turnover and in the degradation of xenobiotics (Goodfellow 2014). *Rhodococcus* strains represented up to 10% of the isolates obtained (Álvarez-López et al. 2016; Becerra-Castro et al. 2011) from the rhizosphere of *A. serpyllifolium* subsp. *lusitanicum* and subsp. *malacitanum* growing in the Iberian Peninsula. *Rhodococcus* sp. were also found in the rhizosphere of the hyperaccumulator, *Noccaea goesingensis* (Idris et al. 2004).

Lopez et al. (2017), when analysing the rhizosphere bacterial community of the Ni-hyperaccumulator *Alyssum murale* growing in serpentine soils in Greece, found that the relative abundance of *Actinobacteria*,

particularly of members of the family *Gaiellaceae*, was positively correlated with Ni availability. Gremion et al. (2003) also observed that *Actinobacteria*, in particular members of *Rubrobacteria* genus, are a dominant part of the metabolically active population in the rhizosphere of the Cd and Zn hyperaccumulator *Noccaea caerulescens*.

In this study, members of the *Hyphomicrobiaceae* family (*Alphaproteobacteria*) were detected in the rhizosphere of *Alyssum*, in particular members of the genera *Devosia* and *Hyphomicrobium*. One *Devosia* strain was isolated from the rhizosphere of *A. serpyllifolium* subsp. *lusitanicum* by Becerra-Castro et al. (2011). Genes for both nitrogen fixing and nodulation were found in *Devosia neptuniae* isolated from an aquatic leguminous plant (Rivas et al. 2002, 2003). Other *Devosia* strains were cultivated from root nodules; however, the role of these organisms in N_2 fixation has not been demonstrated. Some *Hyphomicrobiaceae* bacteria such as members of *Hyphomicrobium* are characterised by the presence of prosthecate, monopolar or bipolar semi-rigid filamentous appendages that confer them the ability to survive in nutrient poor habitats as the prosthecate increase the cells surface area to increase uptake of nutrients (Oren and Xu 2014). *Hyphomicrobiaceae* bacteria, including members of the genera *Devosia* and *Hyphomicrobium* were among the most represented *Alphaproteobacteria* in the rhizosphere of Ni-hyperaccumulator *Alyssum murale* (Lopez et al. 2017). Mengoni et al. (2004) also detected some *Alphaproteobacteria* characteristic of the rhizosphere of the Ni-hyperaccumulator *Alyssum bertolonii*, although the method used (T-RFLP of 16S rRNA gene fragments) did not offer detailed taxonomic information.

Overall, in the four ultramafic sites studied the rhizosphere bacterial communities were significantly different from the communities in non-vegetated soils and this was observed at different taxonomic levels. The rhizobacterial communities associated with the hyperaccumulators and excluder plants also differed significantly. The sequences of bands characteristic of *Alyssum* rhizosphere indicated that *Actinobacteria* of the family *Geodermatophilaceae* (genera *Blastococcus, Geodermatophilus*, or *Modestobacter*) and of genera *Rhodococcus* and *Pseudonocardia*, as well as *Alphaproteobacteria* of genera *Devosia* and *Hyphomicrobium*, are likely to be relevant members of the rhizobacterial community associated with this hyperaccumulator plant. Regarding the Ni-excluder *Dactylis*, the clustering of *Alphaproteobacteria* rhizospheric profiles of different populations and the high dissimilarity found with *Alphaproteobacteria* in the rhizosphere of the hyperaccumulating plants suggest that members of this class could play a role in the mechanisms of exclusion or contribute to the tolerance of *Poaceae* to the high concentrations of TE in metal enriched soils.

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