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Julien Ancousture, Xavier Goux, Damien Blaudez, Cristina Gonnelli, Antony van Der Ent, et al.. Endophytic bacteria of Brassicaceae seeds depend on the plant species. *Total Environment Microbiology*, 2025, <10.1016/j.temicr.2025.100006>. <hal-04993767>

HAL Id: hal-04993767

<https://hal.science/hal-04993767v1>

Submitted on 20 Mar 2025

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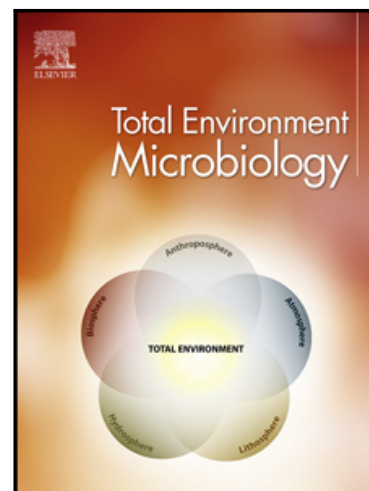
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Journal Pre-proof

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PII: S3050-6417(25)00006-0
DOI: <https://doi.org/10.1016/j.temicr.2025.100006>
Reference: TEMICR 100006



To appear in: *Total Environment Microbiology*

Received date: 30 December 2024
Revised date: 7 March 2025
Accepted date: 13 March 2025

Please cite this article as: Julien Ancousture , Xavier Goux , Damien Blaudez , Cristina Gonnelli , Antony van der Ent , Alexis Durand , Emile Benizri , Endophytic bacteria of Brassicaceae seeds depend on the plant species, *Total Environment Microbiology* (2025), doi: <https://doi.org/10.1016/j.temicr.2025.100006>

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Total Environment Microbiology

Endophytic bacteria of *Brassicaceae* seeds depend on the plant species

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ABSTRACT

Seeds harbor diverse endophytic microbes that can be transmitted to offspring through parental plants. However, studies on the seed microbiomes of metal hyperaccumulators—plants capable of accumulating high levels of specific metals in their tissues—are scarce. This study aimed to characterize the diversity of endophytic bacterial communities in seeds from the Brassicaceae family, which contains the highest number of hyperaccumulating species.

The seed collection analyzed included 65 accessions from 53 hyperaccumulating and 12 non-hyperaccumulating Brassicaceae species, spanning five genera and 24 species. These seeds were collected from various countries (Albania, Austria, France, Greece, Italy, Slovakia, Spain, and Romania) between 2010 and 2021. Using 16S rRNA metabarcoding, we characterized seed

endophytic bacterial communities and investigated potential correlations between bacterial diversity and nickel concentrations in seeds through ICP-MS and μ XRF analyses.

Our findings indicate that plant host taxonomy is the primary determinant of endophytic bacterial community diversity in Brassicaceae seeds. Non-hyperaccumulating plants harbored significantly more diverse bacterial communities than hyperaccumulators. Certain bacterial families, such as Pseudomonadaceae and Nocardiaceae, were more abundant in non-hyperaccumulating Brassicaceae seeds, whereas *Stenotrophomonas* was more prevalent in nickel-rich seeds of hyperaccumulators. These differences suggest that metal accumulation in hyperaccumulator seeds imposes selective pressures, leading to shifts in bacterial community composition.

This work confirms that plant host taxonomy is the primary driver of the seed endophytic bacterial community. However, in hyperaccumulating plants, the high metal concentrations that may occurred in seeds also influence bacterial diversity in this plant organ.

Keywords: endophytes diversity, seeds, hyperaccumulators, Brassicaceae

INTRODUCTION

Seeds are the starting point of a plant's life cycle (Kumar et al., 2024). Microbial communities associated with plants and particularly with their seeds, are often referred to as the 'second genome of the plant' (Vannier et al., 2019), contributing to plant health and resilience (Nelson, 2018; Durand et al., 2021). As pointed out by several authors, seed-associated microbes are important for the fitness of a plant (Langill et al., 2023; Kumar et al., 2024). Therefore, understanding the seed microbial assembly pattern has become crucial due to its multifunctional role in promoting growth and maintaining plant health (Truyens et al., 2013, 2014; Hardoim et al., 2015). While previous studies have primarily focused on mature

plants, microbes can be exceptionally important during the early stages of plant growth (Nelson et al., 2018), and particularly in seeds. To date, most studies have focused on seeds of crop species, such as soybean, peanut, rice, maize, and wheat (Kaga et al., 2009; Johnston-Monje and Raizada, 2011; Rosenblueth et al., 2012; Solanki et al., 2021). These works show that seeds from various plants have diverse endophytic bacterial communities belonging to different genera such as *Acinetobacter*, *Bacillus*, *Micrococcus*, *Rhizobium*, *Paenibacillus*, *Pantoea*, *Pseudomonas* and *Staphylococcus* (Johnston-Monje and Raizada, 2011; Truyens et al., 2015; Nelson, 2018; Wang and Zhang, 2023). Generally, these seed-associated bacterial isolates were found to possess key characteristics such as the production of IAA (Indole-3-acetic acid), siderophores, acetoin, ACC deaminase, and the ability to solubilize tricalcium phosphate (Hardoim et al., 2015). Several studies underlined that inoculation of plants by specific endophytic bacteria play a promising role in offering tolerance to stress-exposed plants. For example, Truyens et al. (2013, 2014) isolated seed endophytic bacteria from *Arabidopsis thaliana* exposed to cadmium (Cd) and showed that inoculation with these bacteria improved root growth of plants during further Cd exposure.

Hyperaccumulators are unique plants capable of attaining metal concentrations in their tissues that can be thousands of times greater in 'normal' plants (van der Ent et al., 2013). These species, found in the Mediterranean region, as well as tropical areas like Brazil, Cuba, New Caledonia, and Southeast Asia (Reeves, 2003), are often used as models to study metal uptake, transport, and sequestration, as well as plant adaptation to extreme environments (Krämer, 2010). The concept of phytomining is based on the use of hyperaccumulators to absorb and transfer metals to their aerial parts, which are then collected as a bio-ore with high concentrations of target metal (Chaney, 2008; Saad et al., 2018). To date, 721 hyperaccumulators species are inventoried in the Global Hyperaccumulator Database (www.hyperaccumulators.org). These hyperaccumulating species are from 52 families. Among

them, the most strongly represented family is *Brassicaceae* (83 species) following by the *Phyllanthaceae* (59 species) (Reeves et al., 2018). Recent studies have, on the one hand, focused on the characterization of the structure and diversity of bacteria associated with the rhizosphere of these particular plants (Lopez et al., 2022) and, on the second hand, on the diversity of endophyte bacteria associated to the hyperaccumulator's tissues (Durand et al., 2021, 2022a, 2022b). Several researches put in evidence that some of the bacteria associated with hyperaccumulating plants (rhizosphere bacteria or endophytes) are able to stimulate the growth of their hosts and protect them against stresses, such as pathogen infections, and drought, and can be consequently considered as plant-growth promoting bacteria (Benizri et al., 2021; Durand et al., 2016; Álvarez-López et al., 2016; Cabello-Conejo et al., 2014).

Studies focusing on seeds belonging to hyperaccumulators are scarce, despite the fact that research on seed endophytic bacteria from plants surviving on metal-contaminated sites could provide important insights for effective clean-up of contaminated sites in an environmentally sound manner. As pointed by Kumar et al. (2024), beneficial seed endophytic microbes could play an important role in offering tolerance to metal-exposed plants (Wang and Zhang, 2023) and can also provide remediation of metal contaminated soils (Kracmarova-Farren et al., 2023). Moreover, these endophytic bacteria exhibited ACC deaminase activity and production of siderophores and IAA (Truyens et al., 2014) and were able to increase the growth of plants which develop on metal-contaminated site. Recently, Ancousture et al. (2023), studying seed-endophytic bacterial diversity of a wide variety of metal hyperaccumulating plants (able to accumulate mostly nickel (Ni), but also zinc (Zn), lead (Pb) and Cd for some of them) and non-hyperaccumulating plants (93 seed samples from Mediterranean regions, Oceania, South-East Asia) revealed the existence of a core microbiome found in seeds of hyperaccumulating plants. Furthermore, it has been suggested that *Noccaea*

caerulescens, a pseudo-metallophyte species, maintains a conserved bacterial community in its seeds across generations, regardless of changes in the soil environment (Durand et al., 2022a).

This work was based on the same seed collection previously reported on by Ancousture et al. (2023), but in this paper the aim was to focus specifically on seeds belonging to the *Brassicaceae* family as this family contains the greatest number of hyperaccumulating plant species. We hypothesized in this study that plant host taxonomy was the primary factor that drive bacterial community structure in the seed of *Brassicaceae* plants, but that Ni hyperaccumulation in seeds of *Brassicaceae* hyperaccumulating plants was also a driver of the seed endophytic bacterial community structure. The objectives were to characterize the structure and diversity of bacterial communities from a collection of seeds belonging to different genera and to different species of the same genus of hyperaccumulating plants and to non-hyperaccumulating species, all belonging to the *Brassicaceae* family. Moreover, since most of the seeds of this collection came from Ni hyperaccumulating plant species, we used ICP-MS and μ XRF analyses, to determine Ni concentrations in hyperaccumulator seeds with the aim to revealed potential correlations between the seed endophyte bacterial diversity and Ni concentrations in seeds.

EXPERIMENTAL PROCEDURES

Seed collection

This work was based on a collection of seeds that includes 65 seed accessions belonging to 53 hyperaccumulating plants and 12 non-hyperaccumulating plant species, including 5 different genera and 24 species from several countries (Albania, Austria, France, Greece, Italia, Slovakia, Spain and Romania) collected between 2010 to 2021 (Table 1). Among the 83 known *Brassicaceae* hyperaccumulating species, we collected seeds from 16 of them.

Seed sterilization and grinding

The seeds were rinsed and surface sterilized, prior to DNA-extraction, to remove surface contamination. To this aim, 50 mg of each seed samples were placed in a 15-mL Falcon tubes filled with a 1% NaClO solution supplemented with Triton 100 \times 0.1% as a surfactant. After 30 s of incubation, seeds were washed for 30 s with EtOH (96%), and then rinsed five times in sterile deionized water.

Based on the protocol of Sánchez-López et al. (2018), seed sterilization was confirmed by running a PCR targeting the bacterial 16S rRNA gene on the last rinsing water. A recently published procedure by Ancousture et al. (2023) was applied using the following primer sets acquired from Eurofins Genomics, Paris, France: 27f (5'- AGA GTT TGA TCA TGG CTC A- 3') and 1492r (5'- TAC GGT TAC CTT GTT ACG ACT T- 3'). For the PCR we used the DreamTaq™ Green PCR Master Mix (2 \times) kit acquired from Thermo Fisher Scientific, Carlsbad, California.

Sterilized seed samples were ground for 30 s at 30 Hz into a uniform powder using a Mixer Mill (model MM400; Retsch Inc., Newtown, Pennsylvania, USA). To do so, sterilized seeds were placed in 2 mL tubes with 0.5 mm zirconium beads previously rinsed with EtOH (96%) and dried in sterile conditions. The homogenous powders obtained were used for the following steps.

DNA extraction and metabarcoding of endophytic bacterial communities

DNA was extracted from 50 mg of powder. A modified protocol from Healey et al. (2014) was followed to extract total seed DNA using hexadecyltrimethylammonium bromide (CTAB)/alcohol/chloroform. To release DNA, the protocol involved CTAB buffer, thermal shock and enzymatic digestions with proteinase K and α -amylase. The co-extracted RNA was

eliminated with RNase and the coextracted proteins with phase separation using chloroform. The DNA precipitation was obtained with isopropanol and finally purified using a QIAquick® PCR Purification Kit (Qiagen, Germany) and its quality and quantity were checked using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific).

The PCR targeting the V5-V6 hypervariable regions of the 16S rRNA gene were performed with primers 799f (5'- AACMGGATTA GAT ACC CKG - 3') and 1115r (5'- AGG GTT GCG CTC GTT G - 3') excluding chloroplast DNA (Kembel et al., 2014). The PCR reaction was achieved according to the following thermal profile: 3 min at 94°C, followed by 35 cycles of 45 s at 94°C, 30 s at 58°C, 90 s at 72 °C, and a final elongation for 10 min at 72°C. Then, a second PCR was performed by ADNid (France) using Illumina MiSeq adapters and the obtained amplicons were pooled equimolarly.

Synchrotron-based μ XRF experiments

The synchrotron μ XRF experiment was conducted at PETRA III, part of DESY (Deutsches Elektronen-Synchrotron), a source of 6 GeV synchrotron radiation, where the hard X-ray microprobe undulator beamline P06 was used (Boesenberg et al., 2016). More information on instrumental parameters and setup has given in van der Ent et al. (2023). The incident X-ray energy was 17 keV and was focused to $2.1 \times 3.6 \mu\text{m}$ (h \times v) with K-B mirrors, which resulted in a flux of approximately 1.25^{11} ph/s at the focus. For XRF detection, a Vortex ME4 detector in 45° geometry and a prototype 16-element Silicon Drift Ardesia Detector in 315° geometry (Utica et al., 2021) with Xspress 3-pulse processors were used. The XRF event stream was processed with a nonlinear least-squares fitting as implemented in PyMCA (Solé et al., 2007). This resulted in files of elemental distribution in pixel values displayed in the LUT scale 'Fire'. This procedure has been carried out for all hyperaccumulating seeds of the collection.

Elemental analysis

Dried seeds (subsamples of 0.5 g) were acid-digested in 2 ml of concentrated HNO₃ (65%) and 1 mL hydrogen peroxide (30%), and samples were incubated in a block digestion system (DigiPREP, SCP Sciences, Courtaboeuf, France) at 95°C. After 2 h, the solutions were filtered (0.45 µm DigiFILTER, SCP science, Canada) and completed to 5 ml with deionized water, then filtrated to <1 µm. Elemental concentrations were determined by ICP-MS (X Series II Model, Thermo Fischer Scientific, Courtaboeuf, France) for Ni. Since the limited quantity of seeds, this analysis was carried out on 31 seed samples and only one replicate has been done. Quality controls included an internal laboratory standard consisting of aerial parts of *Noccaea caerulescens* with a known Ni concentration (Gonneau et al., 2014).

Bioinformatic and statistical analysis of bacterial diversity

The obtained reads after Illumina amplicons sequencing were assigned to each of the 358 samples sequenced using a unique barcode. Raw sequence pairs were joined at the overlap region using Mothur v.1.40.5 (Schloss et al., 2009). The resulting contigs were filtered by removing homopolymers, ambiguous sequences, inappropriate length sequences, and artifacts (very rare sequences, <10 sequences in the dataset). Good quality sequences were retained if they corresponded to the 16S rRNA gene, were of sufficient length, were non-chimerical, and had at least 8 reads per sequence. Operational taxonomic units (OTUs) were generated using Needleman distance and average neighbor clustering at a distance of 0.03. Taxonomic assignments were made using the SILVA ribosomal RNA databases v1.3.8 (Quast et al., 2013).

Samples were rarefied to 1000 representative sequences using the 'sub.sample' function in Mothur. Out of the 358 samples, 12 were removed due to a low number (< 1000) of detected sequences. The 'Good's coverage' was calculated using the 'summary.single' function in Mothur for the remaining 346 samples. To evaluate the dissimilarity between the endophyte community

of each sample, the Bray Curtis distance ($k = 3$) was calculated based on the standardized (Wisconsin double) and square root transformation of OTU abundance and represented using a 2-dimensional Non-Metric Multi-Dimensional Scaling (NMDS) plot. The percentage of Coverage was calculated using the formula $C = [1 - (n / N)] * 100$ (%), where 'n' represents the number of OTUs and 'N' represents the total number of sequences to confirm that the sequencing depth was sufficient to provide a satisfactory representation of the bacterial communities in our samples (Good, 1953). The alpha diversity indices estimated included: observed OTU richness, Chao estimation of OTU richness (Chao, 1984), Shannon-Wiener diversity index, inverse Simpson diversity index, and a Shannon and Simpson index-based measure of evenness. This was performed using 'summary.single' function of Mothur on the subsample dataset.

Statistical analyses of sequencing data were conducted using R v.4.3.0 in the RStudio environment (v.2023.03.0+386). Normality was assessed with Shapiro's test and homoscedasticity with Bartlett's test. Multivariate analyses were performed using the 'metaMDS' function and the 'vegan' package (v.2.6.2) with NMDS. The 'vegan' package's 'anosim' function was used to conduct an Analysis Of SIMilarities (ANOSIM) and obtain p -values (significance levels) and R-values (strength of factors on samples).

A network analysis was done using Cytoscape software (version 3.10.1; Shannon et al., 2003) to visualize the shared OTUs between plant species (> 1% of relative abundance).

RESULTS

Plant host taxonomy and SEB communities

To visually assess the bacterial endophyte community structure composition at the phylum and sub-phylum levels across different plant species, Circos (version 0.69-2, Krzywinski et al., 2009) was utilized to generate Fig. 1. As the different plant species did not

have the same number of samples and to make the visualization and comparison easier between the studied plant species, the data used were normalized and consequently, each plant species representation had the same size in the graphic. The bacterial subphylum with higher abundance on average in the seeds samples was Gamma Proteobacteria (representing $70\% \pm 26$ of the total bacterial community) followed by Actinobacteria ($8.9\% \pm 9.4$), Alpha Proteobacteria ($7.5\% \pm 15.4$), Unclassified Bacteria ($7.4\% \pm 6.1$), Bacteroidota ($6.0\% \pm 12.2$), Unclassified Proteobacteria ($3.9\% \pm 3.9$) and Firmicutes ($3.2\% \pm 6.2$). The bacterial communities were highly different between the studied plant species, which is why the standard deviation was rather high for most of the presented results. Although the Gamma Proteobacteria was the dominant phylum in the seed endophyte bacterial (SEB) communities of most plants, we observed changes for some seed samples. For *O. moravensis*, the dominant phylum was Alpha Proteobacteria (representing 78% of the total bacterial community), while for both *O. alpestris* and *O. argentea*, the main phylum was Gamma Proteobacteria representing respectively, 80.2% and 92% of the total bacterial communities. All three species studied belonging to *Bornmuellera* genera harbored SEB communities with a high relative abundance of Bacteriodota. For several others seed collected from plant species, mostly *Brassica* species and *Noccaea caerulescens*, one of the dominant phyla was Actinobacteria. Otherwise, *O. bertolonii* seeds contained equivalent proportions of three phyla: Gamma Proteobacteria (39.3%), Firmicutes (27.2%) and Actinobacteria (28.9%). The three species of *Brassica* (*B. juncea*, *B. napus* and *B. oleracea*), which are non-hyperaccumulating plants, were characterized by two dominant phyla, Actinobacteriota (representing 21.1%, 20.2% and 24.2%, respectively) and Gamma Proteobacteria (representing 51.4%, 45.4% and 40.1%, respectively), while in two other non-hyperaccumulators (*R. sativus* and *O. tavolarea*), Gamma Proteobacteria was the only major phylum with 89.8% and 86.6%, respectively.

To assess the dissimilarity of the bacterial community composition from the different plant seed samples, a NMDS was carried out based on Bray-Curtis distance (Fig. 2). This NMDS resulted in a stress value of 0.203, which could be considered as an “acceptable” representation of the dataset in a reduced dimension. Indeed, stress < 0.05 provides a good representation in reduced dimensions, < 0.1 is great, < 0.2 is good and over 0.3 is insufficient (Clarke and Ainsworth, 1993). A clear clustering was observed between samples, depending on the plant genera from which seeds were collected. Indeed, the *Noccaea* seeds all grouped together on the left of this graphical representation, and this underlined a similarity in their bacterial community diversity. Additionally, the seeds from *Odontarrhena* clustered together on the right of this graphical representation, indicating similar bacterial community diversity, while *Bornmuellera* and *Brassica* samples were grouped on the bottom part. An ANOSIM was performed to test the effect of the plant genera on the variation in the bacterial communities (Table 2). This analysis revealed that the genera of the considered plants significantly affected the bacterial communities, as shown by the R-value of 0.45 (p -value < 0.001). A second ANOSIM was performed to estimate if the plant species also affected the bacterial communities. As shown by the R-value of 0.57 (p -value < 0.001), we underlined that this model had a high power of explanation. In addition, the plant strategy (hyperaccumulators vs. non-hyperaccumulators) had significant effect on the structure of SEB communities, but with very low R-value underlying a weak correlation (p -value = 0.02, R-value = 0.06). Finally, the plant type (wild vs. domestical plant) resulted in a low R-value with non-significant effect (p -value = 0.29, R-value = 0.02).

Shared and specific OTUs among plant species

A co-occurrence network analysis was performed on the 36 major OTUs (> 1% of relative abundance) allowing a better visualization of the OTUs that were common or specific

to the seeds of the different plant species (Fig. 3). OTUs were mainly affiliated to Gamma Proteobacteria (20 OTUs, 55.6%), Actinobacteria (4 OTUs, 11.1%), Bacteroidia (4 OTUs, 11.1%), Unclassified Bacteria (3 OTUs, 8.3%), Alpha Proteobacteria (2 OTUs, 5.6%), Unclassified Proteobacteria (2 OTUs, 5.6%), Bacilli (1 OTU, 2.8%). The OTUs belonging to Gamma Proteobacteria were distributed throughout the network and were generally present in seeds of all species, whether hyperaccumulating or not. Of the 20 OTUs belonging to Gamma Proteobacteria, only 7 OTUs were exclusively in seeds of hyperaccumulators (OTU0015, OTU0018, OTU0020, OTU0024, OTU0026, OTU0027 and OTU0040), while 2 were found in non-hyperaccumulators (OUT0186 and OTU0058). The other ones were shared whatever the plant strategy. Most of the OTUs belonging to Actinobacteria (3/4, 75%) were only found in non-hyperaccumulating *Brassica* species (OTU0072, OTU0077, OTU0154), while the two OTUs belonging to Unclassified Proteobacteria (OTU0119 and OTU0120) were shared by both hyperaccumulators and non-hyperaccumulating species. Among the nineteen OTUs that were shared by all species (52.8%), 11 (57.9%) belonged to Gamma Proteobacteria, while 2 (10.5%) belonged respectively to Unclassified Proteobacteria or Unclassified Bacteria, and 1 (5.3%) belonged to Alpha Proteobacteria, Bacilli, Actinobacteria, and Bacteroidia. Seeds belonging to the four *Brassica* species and to *R. sativus* were the only ones containing the OTU0058, OTU0072, OTU0077, OTU0084, OTU0185 and OTU0186, which appear specific to these agricultural crops and absent in seeds from wild hyperaccumulating plants. Among agricultural crops, seeds of *B. napus* had the highest number of different OTUs (i.e., 21), while seeds of hyperaccumulator species such as *O. chaldicica* and *O. euboica* contained respectively only 20 and 5 OTUs. Our analysis showed that Gamma Proteobacteria were the most abundant sub-phylum of microbes observed in this study, with Unclassified Enterobacterales, Unclassified Oxalobacteraceae and *Stenotrophomonas* as the most common genera.

SEB community diversity of hyperaccumulating and non-hyperaccumulating plants

Despite the fact that the NMDS analysis revealed that the major driver of the SEB communities was the plant host taxonomy, the analysis of alpha diversity in the communities highlighted significant differences between the group of hyperaccumulating plants and non-hyperaccumulating plant species (Table 3). The seeds of non-hyperaccumulators exhibited bacterial communities with average richness indicators significantly greater than those observed for hyperaccumulators, with a decrease of 26% of observed OTUs and 14% of OTUs estimated with Chao1. In addition, the evenness indexes within the SEB communities showed that non-hyperaccumulators hosted significantly more even communities in their seeds compared to hyperaccumulators. Indeed, evenness based on the Shannon index was 25% lower in seeds from hyperaccumulators and the evenness based on Simpson index was also 23% lower. Finally, the analysis of the Shannon and Simpson diversity indices revealed that the seeds of non-hyperaccumulating plants harbored greater bacterial diversity than those of hyperaccumulators plants. Indeed, the average Shannon index associated with the bacteria in the seeds of hyperaccumulators plants was significantly lower with a decrease of 29% and similarly the average inverse Simpson index was decreasing of 51% in hyperaccumulating seeds compared to non-hyperaccumulating species. This overall decrease in diversity in the seeds of hyperaccumulating plants was characterized by the reduction in the average relative abundance of certain OTUs compared to the seeds of non-hyperaccumulators, while other OTUs are favored (Fig. 4). There were 9 OTUs more abundant with a change of at least 2 points of percentage of relative abundance in seeds of hyperaccumulators when compared to seeds of non-hyperaccumulators (OTU0018, OTU0023, OTU0034, OTU0042, OTU0054, OTU0058, OTU0072, OTU0077 and OTU0084). By contrast, there were 10 OTUs more abundant with a change of at least 2 points of percentage of relative abundance in seeds of non-

hyperaccumulators when compared to seeds of hyperaccumulators (OTU001, OTU0010, OTU11, OTU0012, OTU0019, OTU0024, OTU0026, OTU0027, OTU0029 and OTU0030).

Ni hyperaccumulation in seeds of hyperaccumulators and diversity of SEB communities

Using ICP-MS analyses, our results revealed seeds of hyperaccumulators with very contrasted Ni concentrations from 2.67 to 6360 mg kg⁻¹ (Table 1). Moreover, using ICP-MS and μ XRF analyses and focusing on specific hyperaccumulator seeds, we observed that among the seed collection, some plants of the same species (*B. emarginata* 5 and *B. emarginata* 6; *N. caerulescens* 1 and *N. caerulescens* 2) have contrasted Ni concentrations (respectively, 4403.9 and 57.8 for *B. emarginata* 5 and *B. emarginata* 6 and 1982.2 and 2.67 for *N. caerulescens* 1 and *N. caerulescens* 2) (Table 1). This observation was confirmed by the μ XRF analyses (Fig. 5), which revealed contrasted Ni concentrations. Bacterial communities were compared between, in the one hand, *B. emarginata* 5 and *B. emarginata* 6 and, on the other hand *N. caerulescens* 1 and *N. caerulescens* 2 (Fig. 6A, 6B).

These comparisons revealed 3 OTUs more abundant with a change of at least 0.5 points of percentage of relative abundance in seeds of *B. emarginata* with high Ni concentrations when compared to seeds of the same plant species with low Ni concentrations in seeds (OTU0023, OTU0067 and OTU0111). In contrast, there were 4 OTUs more abundant with a change of at least 0.5 points of percentage of relative abundance in seeds with low Ni concentrations when compared to seeds of *B. emarginata* with high concentrations of Ni (OTU0018, OTU0030, OTU0063 and OTU0063). Additionally, there were 3 OTUs more abundant with a change of at least 0.5 points of percentage of relative abundance in seeds of *N. caerulescens* with high Ni concentrations when compared to seeds of the same species with low Ni concentration in seeds (OTU0024, OTU0026 and OTU0059). In contrast there were 2 OTUs more abundant with a change of at least 0.5 points of percentage of relative abundance in seeds with low Ni

concentrations when compared to seeds of *N. caerulescens* with high concentrations of Ni (OTU0059, OTU0081 and OTU0083). Among all of the OTUs, the OTU0023, a *Stenotrophomonas*, was the most enriched OTU in Ni hyperaccumulating plant seeds, but also the most enriched OTU in both *B. emarginata* and *N. caerulescens* seeds with high Ni concentrations.

DISCUSSION

Our study showed that Gamma Proteobacteria was the major sub-phylum present in the studied seeds whether these belonged to hyperaccumulating or non-hyperaccumulating plant species. These results were in accordance with those underlined by several authors: in general, Proteobacteria, and especially Gamma Proteobacteria, were found to be the dominant endophytic phylum isolated from a wide variety of plant species, while Actinobacteria, Firmicutes and Bacteroidetes were much less represented (Rosenblueth and Martínez-Romero, 2006). Similarly, studying 14 populations of seeds from hyperaccumulating plants belonging to the species *N. caerulescens* (*Brassicaceae*), Durand et al. (2020) reported that the main phyla and sub-phyla were Gamma Proteobacteria (56.6%) > Alpha Proteobacteria (32.2%) > Actinobacteria (7.9%) > Firmicutes (3.8%).

Diverse factors affect the seed endophytic community. Several studies underlined that plant host taxonomy could be a driver of the SEB community (Granér et al., 2003; Xu et al., 2014; Durand et al., 2021; Choi et al., 2022), while some studies could not detect this effect (Fürnkranz et al., 2012). Studying the seed endophytic bacteria in natural populations of *Capsella bursa-pastoris*, a *Brassicaceae* model species of weed ecology, Choi et al. (2022) suggested that the two populations studied, likely two different genotypes (Choi et al., 2019), were the major driver of the structure of the endophytic community of seeds. Durand et al. (2020) showed that the factor that best explained dissimilarities of the endophytic bacterial

communities associated with hyperaccumulating plant seeds collected from nonmetalliferous or calamine soils, was the plant's genetic subunit that exist among the *Noccaea caerulescens* species French populations (Gonneau et al., 2017). These slight variations in the host plant genetics were enough to significantly explained the distribution of OTUs in the SEB communities and was a better driver of the SEB community structure than the host plant environments. The results obtained in the present study seemed to confirm these previous observations. Although, our results revealed that Gamma Proteobacteria were common to almost all seeds sampling from hyperaccumulators or non-hyperaccumulating plants, some phyla and even more some OTUs seemed to be more specific to certain plant species. This underlined that plant host taxonomy affected the SEB community. For instance, *Brassica* species harbored mostly Actinobacteriota and Gamma Proteobacteria, while *Bornmuellera* more Bacteroidota and Gamma Proteobacteria. In the same way, Alpha Proteobacteria seemed to be more specific to *O. moravensis*. We also observed specific OTUs linked to specific plant species. Indeed, the seeds belonging to the three *Bornmuellera* species were the only ones harboring OTU0018, OUT0027, OTU0029 and OTU0030. The OTU0154 was only present in the four *Brassica* species, while OTU0026 was only found in the three species of *Noccaea*. Notably, the OTU0040 was the only one found in *O. muralis*. which is a Ni tolerant plant species, but not a hyperaccumulator (Bettarini et al., 2021).

In our study, among the OTUs shared by all the plant species, we found OTU0014 (Unclassified Enterobacterales (95)), OTU0016 (Unclassified Enterobacterales (97)), OTU0023 (*Stenotrophomonas* (97) and OTU0034 (Unclassified Oxalobacteraceae (100). Thus, one might speculate that these organisms form a core microbiota which is conserved across hyperaccumulating and non-hyperaccumulating *Brassicaceae* plant species. Enterobacterales are a group of gram-negative bacteria with strong degradation ability for organic compounds, which dominate in many plants endophytic microflora and are the endophytic core microflora

of many plant seeds; members of this order are related to plant health and/or disease (Cernava et al., 2019; Bziuk et al., 2021). Consequently, it may maintain *Brassicaceae* growth by promoting plant growth or assisting plants to resist pests and diseases (Hao et al., 2022), even if, as pointed by Martens and Boyd (1994), the ability of the hyperaccumulators to accumulate high levels of metal in their tissue already provide “elemental defense” against plant natural enemies. *Stenotrophomonas* was also one of the most common OTUs observed in the studied *Brassicaceae* seed collection. *Stenotrophomonas* is a ubiquitous bacterial genus adaptable for different environment and associated with plant roots (Kumar et al., 2023; Ryan et al., 2009; Schmidt et al., 2012; Garin et al., 2024; Zhao et al., 2024). Additionally, *S. rhizophila* was found in the epidermis and absorbent hairs of the roots, from sterilized seeds of *Solanum lycopersicum* (cv. *Avicenna*) indicating the presence of this bacteria in the seed endosphere (Ryan et al., 2009). This bacterial genus is referred to as a potential PGPR with advantageous effects because of its capacity to produce siderophores, the ability to solubilize phosphate, and the generation of phytohormones (Suckstorff et al., 2003; Ulrich et al., 2021; Kumar et al., 2023). Moreover, *Stenotrophomonas* species have been proven to be efficient microbial biocontrol agents showing both antagonistic activity against plant pathogens (Lottmann et al., 1999; Minkwitz and Berg, 2001) and entomopathogenic activity against insect pests. OTU0034 Unclassified Oxalobacteraceae (100) was also shared by all seeds of the plant species studied. Oxalobacteraceae is a family within the order Burkholderiales in the subclass of Beta Proteobacteria. Among this family, several genera, such as *Massilia*, exhibited *in vitro* attributes related to plant growth promotion, including IAA production (Kuffner et al., 2010), siderophore production (Hrynkiewicz et al., 2010) and *in vitro* antagonism towards plant pathogens (Weinert et al., 2010).

The more restricted bacterial diversity observed in the seeds of hyperaccumulating plants compared to nH plants could be explained by the accumulation of trace elements in these

organs. In conventionally grown plants (corn, sunflower, wheat, and soybean), seeds can contain metals such as Ni, Cd, or Zn (Banjac et al., 2021), but the concentrations are significantly higher in the seeds of hyperaccumulating plants, ranging from 100 to 1000 times more concentrated for Ni, Cd, and Zn (Kachenko et al., 2009). At high concentrations in tissues, these metals are toxic and entail changes in the endosphere bacterial communities (Durand et al., 2023). It is possible that a stronger selective pressure was exerted in the seeds of hyperaccumulators, leading to a less diverse community of metal tolerant bacteria. Indeed, as pointed by Yao et al. (2022), who studied the endophytic bacterial community of *Robinia pseudoacacia*, a plant species for metal phytoremediation, metals have prevailing impacts on the endophytic community structures and the alpha diversity of endophyte communities, inhabiting root, stems and leaves, decreased. In our study, we clearly demonstrated a decrease in the diversity indices of SEB communities for hyperaccumulating plants compared to non-hyperaccumulating plants, with some OTUs particularly abundant in hyperaccumulator seeds, such as OTU0023 *Stenotrophomonas*. However, it is difficult to conclude that this decrease in endophytic bacterial diversity is the result of selection pressure related to metal accumulation in the seeds of hyperaccumulating plants. Indeed, as we have demonstrated, the plant species is the major determinant of the composition of SEB communities and not the plant strategy (hyperaccumulating vs non-hyperaccumulating).

Nevertheless, in our collection of Ni-hyperaccumulating plant seeds, several samples had contrasting Ni concentrations in their seeds despite the same plant species origin. Indeed, one sample belonging to the plant species *Bornmuellera emarginata* and another one belonging to *Noccaea caerulea*, had concentrations reaching values higher than 1000 mg kg⁻¹, which are by definition concentrations characteristic of those found in hyperaccumulator shoots (Baker et al., 1989). By contrast, in two other samples belonging to the two previously mentioned species, very low Ni concentrations were observed. The environment, notably the

soil on which the plants have grown, should explain the different Ni concentration in seeds of the same species. In the case of *B. emarginata* seeds, relatively few changes were observed in the SEB communities, despite difference in Ni concentration. By contrast, for *N. caerulescens*, relative abundance of some OTUs decreased (OTU0083) or increased (OTU0024 and OTU0026) depending on Ni concentrations in the seeds. Both for *B. emarginata* and *N. caerulescens*, observed differences in the SEB communities were attributed to the differences in Ni concentrations in the seeds, since SEB communities were essentially preserved from soil influence (Durand et al., 2022b). Finally, plant genetic was the main driver of SEB diversity, however Ni hyperaccumulation in seeds may also appeared as a driver.

Among the OTUs, *Stenotrophomonas* (OTU0023, OTU0024, and OTU0026) were the most abundant in the SEB communities of hyperaccumulating plants and also in seeds containing high Ni concentrations. A comparative genomic analysis of 213 strains of *Stenotrophomonas* revealed resistance to metals and ubiquitous PGP traits (Zhao et al., 2024) and members of *Stenotrophomonas* genus are well known for surviving in nutrient-deficient and trace element-rich rhizosphere soil (Ryan et al., 2009). Moreover, a strain of *Stenotrophomonas* has been shown to accumulate Ni and to be resistant to Ni concentrations over 1000 mg kg⁻¹ (Aslam et al., 2020). In addition, in the literature, *Stenotrophomonas maltophilia*, *S. plymuthica* and *S. rhizophila* were the most studied species of *Stenotrophomonas*, and those species may have ecosystem functions in the sulfur and nitrogen cycles, and in degradation of complex compounds and pollutants (An and Berg, 2018). Those species possessed metals efflux pumps and multi tolerances (Sun, 2021). Several strains were even able of Ni biosorption (Wierzba, 2015), for example *S. maltophilia* and *S. plymuthica* strains accumulated Ni up to 300.3 and 422.6 mg g⁻¹ dried cells, respectively (Ettoumi et al., 2015). In fact, *S. rhizophila* was found more represented in the rhizosphere of the Ni hyperaccumulator *Noccaea caerulescens* growing in serpentine soil compared to the

surrounding bulk soil (Mattarozzi et al., 2017). Otherwise, using an amendment of manure and a *Stenotrophomonas* strain previously isolated from *Odontarrhena serpyllifolia*, Ghasemi et al. (2018) showed a significant increase in aerial dry biomass and Ni yield in aerial parts of *Odontarrhena serpyllifolia* and *O. bracteate* (Ni hyperaccumulators). In the same way, in the rhizosphere of indigenous plants growing in ultramafic soil, the *Stenotrophomonas* genus was significantly correlated with nitrate reduction activity (Koner et al., 2023). Furthermore, Bashandy et al. (2020) found that *S. maltophilia* exhibited both nitrate reduction and nitrogen fixation activities, which improved the growth of *Brassica napus* L., and as shown by Akhtar et al. (2018), a strain of *Stenotrophomonas* enhanced the growth of the shoots and roots in *Raphanus sativus* L. in soil with high Ni concentration.

CONCLUSION

Focusing on the SEB communities of a wide variety of Brassicaceae plants, our results underlined that plant host taxonomy was a major factor driving the diversity of bacteria living in the studied seeds. Some phyla appeared to be common to almost all seed samples from hyperaccumulators or non-hyperaccumulating plants. However, by contrast some bacterial phyla and more precisely some OTUs appear to be specific to certain plant species. Among the bacterial diversity identified, the genus *Stenotrophomonas*, was revealed as one of the most common OTUs observed in the all the studied Brassicaceae seed collection. In addition, the relative abundance of this genus revealed to be the most enriched in bacterial endophyte seeds of hyperaccumulators compared to non-hyperaccumulators. Our results also demonstrate that the genus *Stenotrophomonas*, was favored in seeds of hyperaccumulators with high Ni concentrations. This genus already known to be capable of seed colonization, has been well studied for its PGP properties, its Ni tolerance and its Ni biosorption potential. This genus

isolated from hyperaccumulating plant seeds may shelter good candidates for bioaugmentation process in phytomanagement program.

Our results confirm that host taxonomy was a major factor driving the diversity, but also provided evidence suggesting a selection pressure exerted on seed endophytes related to high Ni concentrations in the seeds of hyperaccumulating plants. This may have led to seed adapted PGP endophytes selection. The role of other factors that may drive the bacterial diversity in seeds of hyperaccumulators needs to be elucidated, for example, the impact of the distribution of Ni in the seed tissues on the selection pressure exerted on the SEB communities.

AUTHOR CONTRIBUTIONS

Emile Benizri and Alexis Durand contributed to initiate the study. Emile Benizri performed field sampling and sample processing. Julien Ancousture analyzed the data with inputs from Alexis Durand and Xavier Goux. Cristina Gonnelli contributed to establish the seed collection. Damien Blaudez and Antony van der Ent examined the Ni quantification in seeds and conducted the synchrotron experiment. Emile Benizri and Alexis Durand wrote the first draft of the paper and led revisions. All authors commented and revised the final draft.

ACKNOWLEDGMENTS

The authors acknowledge Aida Bani from the University of Tirana, Maria Konstantinou from the International Hellenic University, which provided some seeds for the collection. The authors also thank Cristina Gonnelli, Andrea Coppi, Giovanni Bacci and their colleagues from the Università di Firenze for their renewed collaboration and for providing several seeds. This work is included in the scientific program of the GISFI research consortium (Groupement d'Intérêt Scientifique sur les Friches Industrielles). We acknowledge DESY (Hamburg,

Germany), a member of the Helmholtz Association HGF, for the provision of experimental facilities. Parts of this research were carried out at PETRA III and we would like to thank Dennis Brueckner for assistance in using beamline P06.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have non-competing interests.

DATA AVAILABILITY STATEMENT

The data of this study are available from the corresponding author, Emile Benizri, upon reasonable request.

FUNDING

The Fondation pour la Recherche sur la Biodiversité (FRB) is acknowledged for funding the Master's Degree scholarship of J. Ancousture. The authors acknowledge Earth-Environment Observatory of Lorraine (OTELo) that provided the financial support (project CoreEndoSeed).

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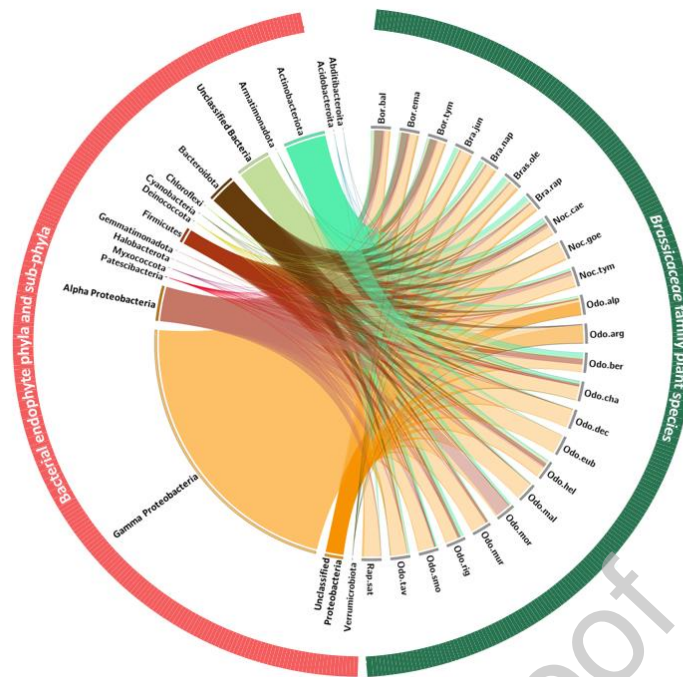


Fig. 1. Bacterial endophyte community structure composition at the phylum and sub-phylum levels for the different studied plant species. To make the visualization and comparison easier between the studied plant species, the number of representative for each plant species were normalized to the same number.

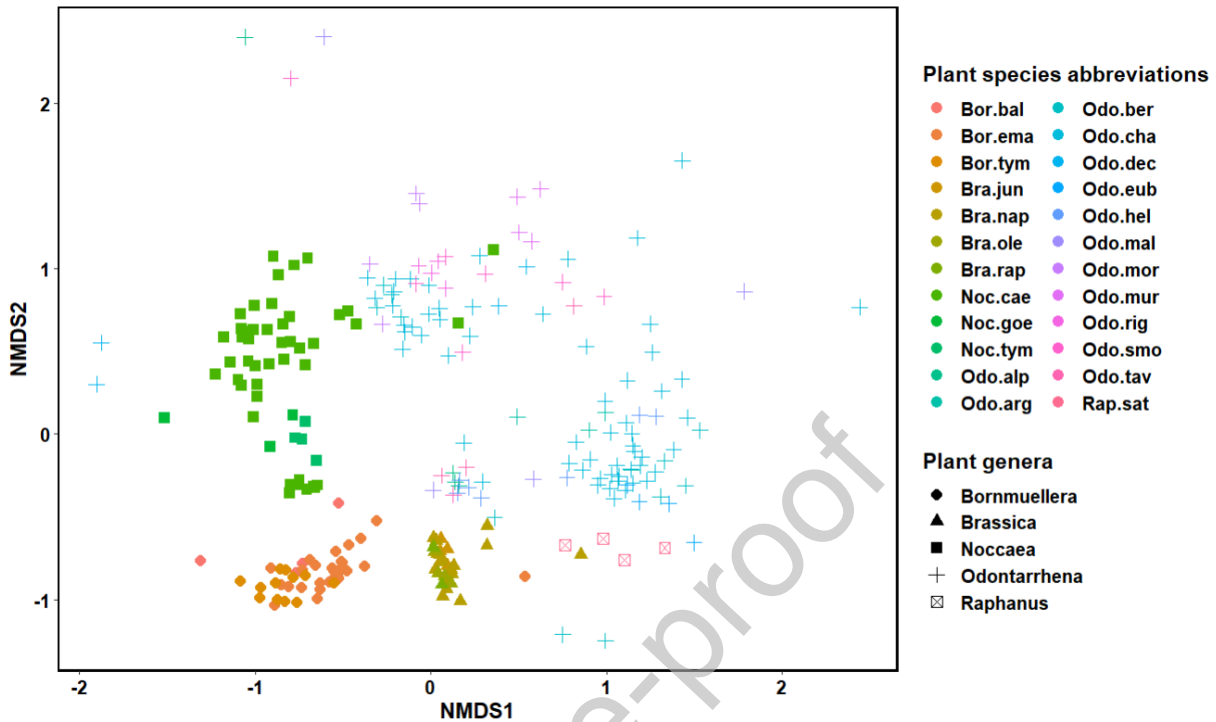


Fig. 2. Non-metric multidimensional scaling (NMDS) plot of bacterial communities. Each point coordinate depends on the diversity the bacterial community described in each sample, for a total of 65 samples (see Table 1). The shape of each point is based on the plant genera from which seeds have been collected and colors correspond to the plant species. Stress of the representation in reduced dimensions is shown in the bottom right corner.

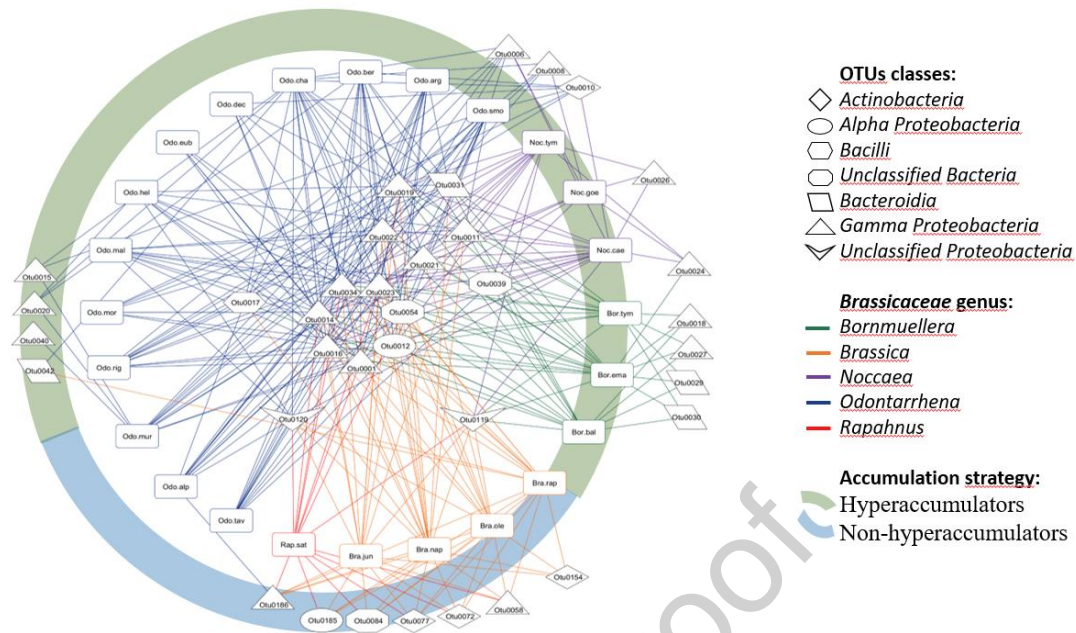


Fig. 3. Analysis of bacterial networks focusing on the major OTUs (over 1%) within the *Brassicaceae*. Geometric shapes denote OTU classes, colored links indicate taxonomic affiliations within the *Brassicaceae*, and colored semi-circles represent accumulation strategies.



Fig. 4. Comparisons of relative abundance variations (percentage points) between the most contrasted OTUs in SEB communities of Hyperaccumulator (H) versus non-Hyperaccumulators (nH). All OTUs with more than 2 points of variations were shown. nH: non-hyperaccumulator seeds, H: hyperaccumulator seeds. For each OTU, significant differences are indicated by * for p-value below 0.001, ** for p-value below 0.01, * for p-value below 0.05 and † for p-value below 0.10.**

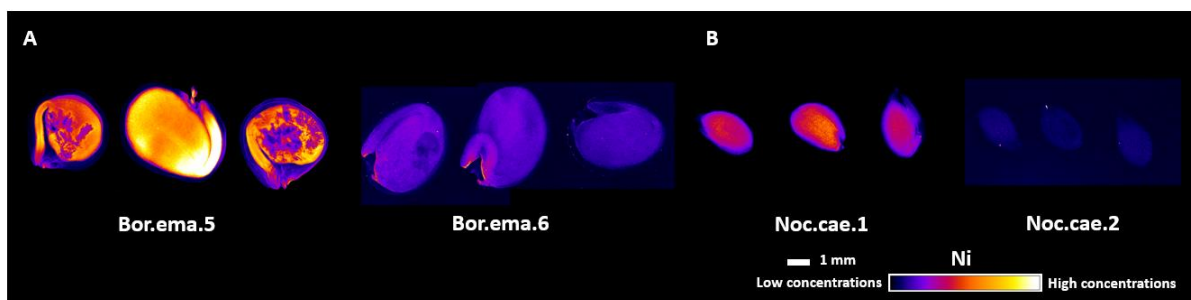


Fig. 5. Synchrotron μ XRF elemental maps showing the distributions of Ni in intact dry seeds of *Bornmuellera emarginata* and *Noccaea caerulescens*. **A:** *Bornmuellera emarginata* Bor.ema.5 (high Ni concentration in seeds) versus Bor.ema.6 (low Ni concentration in seeds), and **B :** *Noccaea caerulescens* Noc.cae.1 (high Ni concentration in seeds) versus Noc.cae.2 (low concentration in seeds).

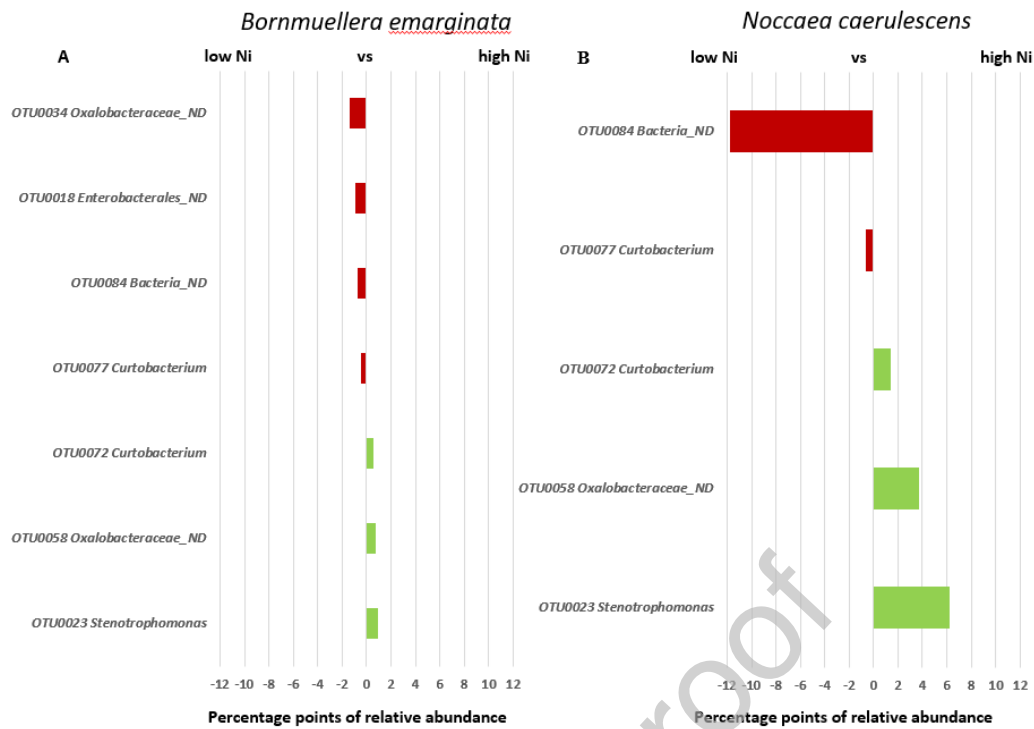


Fig. 6. Comparisons of relative abundance variations (percentage points) between the most contrasted OTUs in SEB communities of A: *Bornmuellera emarginata* Bor.ema.5 (high Ni concentration in seeds) versus Bor.ema.6 (low Ni concentration in seeds), and B : *Noccaea caerulea* Noc.cae.1 (high Ni concentration in seeds) versus Noc.cae.2 (low concentration in seeds). All OTUs with more than 0.5 points of variation were shown.

Table 1

Collection of hyperaccumulating and non-hyperaccumulating plant seeds belonging to *Brassicaceae* family. The taxonomic code is composed by the first 3 letters of the genus followed by the 3 letters of the plant species. ND = not enough seeds to be determined. Last column shows Ni concentration in seeds determined by ICP-MS.

Taxonomic code	Genus and species	Ni concentration in seeds (mg kg⁻¹)	Location
Hyperaccumulators			
Bor.bal.1	<i>Bornmuellera baldacci</i> subsp. <i>Markgrafii</i>	1182.2	Greece
Bor.ema.1	<i>Bornmuellera emarginata</i>	3711.1	Greece
Bor.ema.2	<i>Bornmuellera emarginata</i>	5532.6	Greece
Bor.ema.3	<i>Bornmuellera emarginata</i>	2961.7	Greece
Bor.ema.4	<i>Bornmuellera emarginata</i>	2003.2	Greece
Bor.ema.5	<i>Bornmuellera emarginata</i>	4403.9	Greece
Bor.ema.6	<i>Bornmuellera emarginata</i>	57.8	Greece
Bor.ema.7	<i>Bornmuellera emarginata</i>	4030.5	Greece
Bor.tym.1	<i>Bornmuellera tymphaea</i>	2551.2	Greece
Bor.tym.2	<i>Bornmuellera tymphaea</i>	4913.9	Greece
Bor.tym.3	<i>Bornmuellera tymphaea</i>	2690.3	Greece
Noc.cae.1	<i>Noccaea caerulescens</i>	1982.2	Spain
Noc.cae.2	<i>Noccaea caerulescens</i>	2.7	Slovakia
Noc.cae.3	<i>Noccaea caerulescens</i>	ND	France
Noc.cae.4	<i>Noccaea caerulescens</i>	ND	France
Noc.cae.5	<i>Noccaea caerulescens</i>	ND	France
Noc.cae.6	<i>Noccaea caerulescens</i>	ND	France
Noc.cae.7	<i>Noccaea caerulescens</i>	ND	France
Noc.cae.8	<i>Noccaea caerulescens</i>	ND	France
Noc.cae.9	<i>Noccaea caerulescens</i>	ND	France
Noc.cae.10	<i>Noccaea caerulescens</i>	ND	France
Noc.cae.11	<i>Noccaea caerulescens</i>	ND	France
Noc.cae.12	<i>Noccaea caerulescens</i>	ND	France
Noc.goe.1	<i>Noccaea goesingense</i>	ND	Austria
Noc.tym.1	<i>Noccaea tymphaea</i>	2239.6	Greece
Odo.arg.1	<i>Odontarrhena argentea</i>	3758.1	Italia
Odo.ber.1	<i>Odontarrhena bertolinii</i>	3783.1	Italia
Odo.ber.2	<i>Odontarrhena bertolinii</i>	ND	Italia
Odo.ber.3	<i>Odontarrhena bertolinii</i>	ND	Italia
Odo.cha.1	<i>Odontarrhena chalcidica</i>	4008.4	Greece
Odo.cha.2	<i>Odontarrhena chalcidica</i>	3098.0	Greece
Odo.cha.3	<i>Odontarrhena chalcidica</i>	4978.6	Greece
Odo.cha.4	<i>Odontarrhena chalcidica</i>	6355.3	Greece
Odo.cha.5	<i>Odontarrhena chalcidica</i>	7132.3	Greece
Odo.cha.6	<i>Odontarrhena chalcidica</i>	716.3	Greece
Odo.cha.7	<i>Odontarrhena chalcidica</i>	5623.0	Greece
Odo.cha.8	<i>Odontarrhena chalcidica</i>	5178.4	Greece
Odo.cha.9	<i>Odontarrhena chalcidica</i>	2349.7	Albania
Odo.cha.10	<i>Odontarrhena chalcidica</i>	5409.5	Albania
Odo.cha.11	<i>Odontarrhena chalcidica</i>	ND	Albania
Odo.cha.12	<i>Odontarrhena chalcidica</i>	ND	Albania

Odo.cha.13	<i>Odontarrhena chalcidica</i>	ND	Albania
Odo.cha.14	<i>Odontarrhena chalcidica</i>	ND	Greece
Odo.cha.15	<i>Odontarrhena chalcidica</i>	ND	Greece
Odo.dec.1	<i>Odontarrhena decipiens</i>	ND	Albania
Odo.eub.1	<i>Odontarrhena euboea</i>	4152.0	Greece
Odo.hel.1	<i>Odontarrhena heldreichii</i>	4986.7	Greece
Odo.hel.2	<i>Odontarrhena heldreichii</i>	3068.5	Greece
Odo.mal.1	<i>Odontarrhena malacitana</i>	ND	Spain
Odo.mor.1	<i>Odontarrhena moravensis</i>	ND	Albania
Odo.rig.1	<i>Odontarrhena rigida</i>	ND	Albania
Odo.smo.1	<i>Odontarrhena smolikana</i>	ND	Greece
Odo.smo.2	<i>Odontarrhena smolikana</i>	ND	Albania

Non-hyperaccumulators

Bra.jun.1	<i>Brassica juncea</i>	ND	France
Bra.nap.1	<i>Brassica napus</i>	ND	France
Bra.nap.2	<i>Brassica napus</i>	ND	France
Bra.nap.3	<i>Brassica napus</i>	ND	France
Bra.nap.4	<i>Brassica napus</i>	ND	France
Bra.nap.5	<i>Brassica napus</i>	ND	France
Bra.ole.1	<i>Brassica oleracea</i>	ND	France
Bra.rap.1	<i>Brassica rapa</i>	ND	France
Odo.alp.1	<i>Odontarrhena alpestris</i>	ND	Italia
Odo.mur.1	<i>Odontarrhena muralis</i>	ND	Romania
Odo.tav.1	<i>Odontarrhena tavolarea</i>	ND	Italia
Rap.sat.1	<i>Raphanus sativus</i>	ND	France

Table 2

ANalysis Of SIMilarities (ANOSIM) is similar to an ANOVA hypothesis test. It shows p -value (i.e., significance levels of clustering) and R (i.e., the level of dissimilarities between clusters, where 1 is maximum dissimilarity). This ANOSIM was performed on the four factors: plant type (wild or domestical plant), plant strategy (hyperaccumulating or non-hyperaccumulating plant), plant genus, plant species.

Factors	R	p-value
Plant type	0.022	0.293
Plant strategy	0.063	0.020
Genus	0.453	0.001
Species	0.570	0.001

Table 3

Analysis of alpha diversity in the communities highlighted differences between the group of hyperaccumulating (H) plants and non-hyperaccumulating (nH) plants. Means were given with standard error between bracket. Statistical differences (*p*-value) were determined with an analysis of variance (ANOVA) and a post-hoc Tukey.

		H plants	nH plants	<i>p</i>-value
Richness	Observed OTUs	52.5 (± 1.4)	70.9 (± 4.8)	3.4 10 ⁻⁵
	Chao1 estimator	91.1 (± 4.2)	106.2 (± 6.9)	8.7 10 ⁻³
Evenness	Shannon's even.	0.441 (± 0.007)	0.588 (± 0.012)	7.9 10 ⁻⁶
	Simpson's even.	0.106 (± 0.016)	0.137 (± 0.026)	2.1 10 ⁻⁴
Diversity	Shannon's index.	1.77 (± 0.08)	2.51 (± 0.18)	2.2 10 ⁻⁶
	Inverse of Simpson's index	5.0 (± 0.3)	10.2 (± 0.8)	2.5 10 ⁻¹⁰

CONFLICT OF INTEREST STATEMENT

The authors declare that they have non-competing interests.