DESARROLLO DE MICROECONOMÍAS REGIONALES EN LA PRODUCCIÓN DE ACEITES ESENCIALES COSECHADOS EN SUELOS MINEROS

Producto 9: Informe de mecanismos de interacción planta/microorganismo

A. Saran
V. Imperato
L. Fernandez
F. Vannucchi ,
N. M. Steffanie d'Haen
L. J. Merini
P. Gkorezis
J. Vangronsveld
S. Thijs

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El presente documento ha sido preparado por A. Saran, V. Imperato, L. Fernandez , F. Vannucchi , N. M. Steffanie d'Haen, P. Gkorezis L. J. Merini, J. Vangronsveld y S. Thijs

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Resumen producto 9

Las investigaciones sobre las diferentes interacciones planta-microorganismos comprometidas para el segundo año de proyecto como Producto 9, han sido publicadas como artículos científicos en revistas internacionales especializadas. A partir de estos estudios, profundizamos los conocimientos sobre la interacción entre *H. petiolaris* y sus microorganismos asociados (interacción planta-microorganismos). Esto no solo constituye un insumo esencial para comprención de dichos mecanismos, sino la base científica necesaria para la toma racional de deciciones en futuros ensayos de microcosmos, escalados a campo y la consolidación del banco de cepas sobre el que avanzar en formulación de bioinsumos.

A continuación, se presentan ambos artículos, como entregables del Producto 9 comprometido para esta actividad. Considerando que los artículos han sido publicados en inglés y a fin de facilitar la difusión de nuestros resultados en el marco de FONTAGRO, incorporamos sus respectivos resúmenes en español.

Phytostabilization of polluted military soil supported by bioaugmentation with PGP-Trace element tolerant bacteria isolated from Helianthus petiolaris.

El plomo (Pb) y el cadmio (Cd) son los principales contaminantes ambientales siendo la acumulación de estos elementos en suelos y plantas de gran preocupación en la producción agrícola debido a sus efectos tóxicos sobre el crecimiento de los cultivos. Además, estos elementos pueden entrar en la cadena alimentaria y afectar gravemente tanto la salud humana como animal. La bioaumentación con bacterias promotoras del crecimiento de las plantas (PGPB) puede contribuir a un enfoque de remediación eficaz y respetuoso con el medio ambiente, mejorando el desempeño de la planta, la supervivencia y promoción de la fitoestabilización o extracción de elementos en condiciones tan duras como lãs que presentan los suelos mineros.
En este trabajo aislamos y caracterizamos bacterias asociadas a raíces tolerantes a Pb y Cd de *Helianthus petiolaris*, capaces de crecer en un suelo contaminado con Pb / Cd para desarrolar inoculantes que puedan promover el crecimiento de las plantas y también mejorar la eficacia de fitoestabilización o fitoextracción. De la especie de plantas aromáticas *H. petiolaris* se aislaron ciento cinco cepas bacterianas, pertenecientes a ocho especies diferentes, endofíticas y rizosféricas tolerantes a oligoelementos. La mayoría de las cepas mostraron múltiples capacidades PGP, capacidad para inmovilizar oligoelementos en su pared celular y promoción de germinación de semillas. Las especies *Bacillus paramycoides* ST9, *Bacillus wiedmannii* ST29, *Bacillus proteolyticus* ST89, *Brevibacterium frigoritolerans* ST30, *Cellulosimicrobium cellulans* ST54 y *Methylobacterium sp.* ST85 fueron seleccionados para realizar ensayos de bioaumentación en microcosmos de invernadero. Después de 2 meses, las plántulas de girasol (*H. annuus*) cultivado en suelo contaminado e inoculado con *B. proteolyticus* ST89 produjo 40% más de biomasa, en comparación con las plantas de control no inoculadas y acumuló un 20% menos de Pb y un 40% menos Cd en las partes de la planta aérea. Por el contrario, *B. paramycoides* ST9 aumentó la bioacumulación factor (BAF) de Pb tres veces y de Cd seis veces, sin inhibir el crecimiento de la planta. Nuestros resultados indican que, dependiendo de la cepa, la bioaumentación con bacterias beneficiosas específicas puede mejorar el crecimiento de las plantas y reducir la movilidad de los oligoelementos o mejorar la absorción de los oligoelementos.

Palabras Clave: Bioaumentación, bacterias promotoras del crecimiento vegetal (PGPB), oligoelementos, Pb y Cd, suelo contaminado, fitoestabilización

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Phytostabilization of Polluted Military Soil Supported by Bioaugmentation with PGP-Trace Element Tolerant Bacteria Isolated from *Helianthus petiolaris*

Anabel Saran [1,2], Valeria Imperato [2], Lucia Fernandez [1], Panos Gkorezis [2], Jan d'Haen [3,4], Luciano Jose Merini [1], Jaco Vangronsveld [2,5] and Sofie Thijs [2,*]

1 EEA Anguil INTA-CONICET, 6300 Anguil, La Pampa, Argentina; saran.anabel@inta.gob.ar (A.S.); fernandez.lucia@inta.gob.ar (L.F.); merini.luciano@inta.gob.ar (L.J.M.)
2 Environmental Biology, Centre for Environmental Sciences, Hasselt University, Agoralaan Building D, 3590 Diepenbeek, Belgium; valeria.imperato@uhasselt.be (V.I.); panos.gkorezis@uhasselt.be (P.G.); jaco.vangronsveld@uhasselt.be (J.V.)
3 Institute for Material Research (IMO), Hasselt University, Wetenschapspark 1, 3590 Diepenbeek, Belgium; jan.d'haen@uhasselt.be
4 IMEC, Division IMOMEC, Wetenschapspark 1, 3590 Diepenbeek, Belgium
5 Department of Plant Physiology, Faculty of Biology and Biotechnology, Maria Curie Sklodowska University, Akademicka 19, 20-400 Lublin, Poland
* Correspondence: sofie.thijs@uhasselt.be; Tel.: +54-9-2302-648363

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Abstract: Lead (Pb) and cadmium (Cd) are major environmental pollutants, and the accumulation of these elements in soils and plants is of great concern in agricultural production due to their toxic effects on crop growth. Also, these elements can enter into the food chain and severely affect human and animal health. Bioaugmentation with plant growth-promoting bacteria (PGPB) can contribute to an environmentally friendly and effective remediation approach by improving plant survival and promoting element phytostabilization or extraction under such harsh conditions. We isolated and characterised Pb and Cd-tolerant root-associated bacteria from *Helianthus petiolaris* growing on a Pb/Cd polluted soil in order to compose inoculants that can promote plant growth and also ameliorate the phytostabilization or phytoextraction efficiency. One hundred and five trace element-tolerant rhizospheric and endophytic bacterial strains belonging to eight different genera were isolated from the aromatic plant species *Helianthus petiolaris*. Most of the strains showed multiple PGP-capabilities, ability to immobilise trace elements on their cell wall, and promotion of seed germination. *Bacillus paramycoides* ST9, *Bacillus wiedmannii* ST29, *Bacillus proteolyticus* ST89, *Brevibacterium frigoritolerans* ST30, *Cellulosimicrobium cellulans* ST54 and *Methylobacterium* sp. ST85 were selected to perform bioaugmentation assays in greenhouse microcosms. After 2 months, seedlings of sunflower (*H. annuus*) grown on polluted soil and inoculated with *B. proteolyticus* ST89 produced 40% more biomass compared to the non-inoculated control plants and accumulated 20 % less Pb and 40% less Cd in the aboveground plant parts. In contrast, *B. paramycoides* ST9 increased the bioaccumulation factor (BAF) of Pb three times and of Cd six times without inhibiting plant growth. Our results indicate that, depending on the strain, bioaugmentation with specific beneficial bacteria can improve plant growth and either reduce trace element mobility or enhance plant trace element uptake.

Keywords: Bioaugmentation; plant growth promoting bacteria (PGPB); trace elements; Pb and Cd; polluted soil; phytostabilization

1. Introduction

Trace element pollution of agricultural soils and waters has been dramatically increased during the last few decades [1]. Also, human exposure has risen dramatically as a result of an exponential increase in agricultural production of areas with contaminated soil. In recent years, there has been an increasing ecological and global public health concern associated with environmental contamination by these elements [2]. Trace elements such as Pb and Cd are non-essential for living organisms, carcinogenic at low concentrations and are usually originating from mining, smelting, electroplating, petrochemical production [3], and military activities [4].

Due to restrictions on the release of these elements into the environment [5] and the limitations of conventional soil remediation technologies such as high cost, labour intensity, irreversible changes in soil properties, and disturbance of the native soil microflora [6], phytostabilization and phytoextraction received increasing attention as alternative and more environmentally friendly approaches for the remediation of trace element polluted sites [7,8].

Phytostabilization aims to establish a plant cover on polluted sites and to reduce further spread of pollutants and is a common practice around the world to revegetate mine tailings [8,9]. Phytoextraction comprises the process of plant root uptake, root-to-shoot translocation, shoot accumulation and detoxification to concentrate pollutants in harvestable biomass [10,11]. These processes strongly depend on plant growth-promoting bacteria (PGPB), which inhabit the rhizosphere and the internal tissues of plants. Some of the features that these bacteria possess include the production of phytohormones, siderophores, and 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD), as well as nitrogen fixation, solubilisation of insoluble P minerals, and production of antibiotics. Through these mechanisms PGPB can enhance plant tolerance to trace element stress and biomass production [12]. Furthermore, when inoculated into soil (bioaugmentation), such bacteria can influence the bioavailability of trace elements by redox transformations, leading to element mobilization, dissolution, leaching, or immobilization through organic molecule-trace element-binding and precipitation [13].

Sunflower (*Helianthus annuus* L.) is globally one of most important oilseed crops. The popularity of sunflower is driven by its versatility as oil, seed and as an animal feed. Also due to its high drought tolerance and adaptation to a great variety of soils, the sunflower is suitable for cultivation in many regions of the world and is been spreading to many countries including Asia and Africa [14]. Several studies made on this crop have revealed that several elements, including Pb, Cd, copper (Cu), zinc (Zn) and cobalt (Co), accumulate at high concentrations in roots and shoots [15,16] reducing plant growth, biomass, grain yield, and crop quality [17]. However, few attempts have yet been made to use PGPB to alleviate the oxidative stress that excess of these trace elements causes in plants and to reduce trace element mobility controlling the entrance of these elements into the food chain. In their last report the Food and Agriculture Organization of the United Nations (FAO) reported the necessity in the upcoming years to ensure food security and a healthy future for all people and the entire planet [18].

Therefore, the aim of this study was to isolate and characterise Cd- and Pb-tolerant bacteria, to develop inoculants able to enhance plant growth and increase the phytostabilization efficiency of *H. annuus* when cultivated in polluted areas.

2. Materials and Methods

2.1. Isolation of Endophytic and Rhizospheric Trace Element-Tolerant Bacteria

Endophytic bacteria were isolated from surface-sterilized roots of *H. petiolaris* plants growing in soils spiked with up to 1000 mg kg^{-1} of Pb and 100 mg kg^{-1} of Cd from a previous study Saran et al. (2019) [19]. For surface sterilization, roots were immersed in 70% ethanol for 10 sec, then in 2.5% sodium hypochlorite for 30 min, and finally rinsed five times in sterile distilled water. To assess sterility, 100 mL of the water used to rinse the plant tissues were plated onto solid glucose-yeast extract (glucose: 10 g L^{-1}; yeast: 5 g L^{-1}) agar medium; the GY plates were incubated at 30 °C for two days. One g of root tissue was macerated using sterile mortar and pestle in 200 µL of sterile phosphate

buffer (10 mM PBS, pH 7.4). Tissue extracts (100 µL) and their different dilutions were plated onto GY agar medium supplemented with either 10 mg L^{-1} Cd or 100 mg L^{-1} Pb (using $CdCl_2$, $Pb(NO_3)_2$ respectively). After incubation at 30 °C for two days, colonies of varying morphology were picked and repeatedly streaked on GY-trace element containing agar medium until strains were identified as pure. Twenty-two Cd-tolerant isolates and 34 Pb-tolerant isolates were selected and stored on slant agar media for further study.

Isolation of rhizosphere bacteria was performed by washing 1 g of roots with NaCl 0.85% and NaCl 0.85%/Tween 80 0.01% solutions, which were inoculated into sterile GY media supplemented with either 10 mg L^{-1} Cd or 100 mg L^{-1} Pb. After several rounds of enrichment culture in GY-trace element medium and subsequent isolation and purification in GY-trace element agar plates, 28 Cd tolerant isolates and 21 Pb-tolerant isolates were selected and stored on slant agar media for further study.

2.2. Genotypic Characterization of Cd and Pb Tolerant Strains

Bacteria were grown for 24 h at 30 °C in GY-trace element containing agar medium. Subsequently, total DNA was extracted using a commercial kit (E.Z.N.A; bacterial DNA kit, VWR, Leuven, Belgium). Amplification of the 16S rRNA gene was performed in a final volume of 25 µL containing 1 × Roche high fidelity PCR buffer, 1.8 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of forward and reverse primers (27F, 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492R, 5′-TACGGCTACCTTGTTACGAC-3′), 1.2 U Roche Taq High Fidelity DNA polymerase (Sigma, Leuven, Belgium) and 1 µL of DNA (1 ng μL^{-1}). PCR reaction conditions were an initial denaturation for 5 min at 95 °C, 32 cycles each consisting of denaturation for 1 min at 94 °C, primer annealing for 30 s at 52 °C and extension at 72 °C for 3 min and a final elongation of 10 min at 72 °C (Biorad T100, Brussels, Belgium). Quality of PCR products was assessed by electrophoresis (100 V, 30 min) on a 2% agarose gel stained with GelRed (Biotium, USA). Purified PCR products were sent to Macrogen (Amsterdam, The Netherlands) for Sanger sequencing. Partial 16S rRNA gene sequences obtained were quality trimmed in Geneious v4.8, and blasted against reference nucleotide sequences present in GenBank, NCBI (Table S1).

2.3. In-Vitro PGP Activity of the Strains

Siderophore production was determined in CAS (Chrome Azurol S) agar Petri plates [20]. Plates were incubated for seven days at 29 °C. Bacteria that produced siderophores showed an orange halo around their colonies. Phosphate-solubilizing efficiency of the bacteria was calculated based on the halozones produced around the colonies growing in National Botanical Research Institute's phosphate solid medium prepared according to Nguyen et al. (1992) in Petri plates [21]. Bacterial organic acid production was assessed according to the colorimetric method of Cunningham and Kuiack (1992) [22] after inoculating strains in 800 µL of sucrose tryptone medium in 48 well polyvinylchloride (PVC) plates. Bacterial Indole-3-acetic (IAA) production was evaluated in 1 mL of 1/10 Nutrient Broth with 0.5 g L^{-1} tryptophan. After incubation in 48 well plates, a colorimetric reaction was induced with Salkowski-reagent to assess positive strains [23]. To identify strains that utilize the butylene glycol pathway and produce acetoin, bacteria were inoculated in Methyl Red-Voges Proskauer (MRVP) medium. After 48 h of incubation in 48 well plates, a colorimetric reaction was induced according to Romick and Fleming (1998) [24]. 1-aminocyclopropane-1-carboxylate (ACC)-deaminase activity was evaluated via a modified protocol based on Belimov et al. (2005) [25]. Washed bacterial cells were resuspended in 1 mL minimal salts medium with 10 mM ACC as sole nitrogen source in 48 well plates. After 3 days at 30 °C, bacterial cells were resuspended in 0.1 mL of Tris-HCl buffer (pH 8.5, 0.1 M) and broken by the addition of 1.5 µL of toluene. Subsequently, 1.5 µL of 0.5 M ACC and 100 µL of 0.1 M Tris-HCl buffer (pH 8.5, 0.1 M) were added to prompt ACC deaminase activity, which was stopped by adding 0.5 mL of 0.56 N HCl to check visually the presence of ACC deaminase. Biofilm formation was examined following the method described by O'Toole et al. (2000) [26]. Selected strains were grown on biofilm growth medium (Luria-Bertani, plus 1 mM $MgSO_4$ and 0.1% glucose) in 24 well plates incubated at 29 °C without shaking for 48 h. Biofilms were detected by staining with crystal violet

0.1% w/v. For all the assays not inoculated media were used as a negative control and three replicates were performed per strain tested.

2.4. In-Vitro Trace Element Uptake by Bacteria in Liquid Cultures-Scanning Electron Microscopy (SEM-EDX) Analysis

Batch studies were conducted using 50 mL Falcon tubes that contained 25 mL GY rich medium supplemented with 10 mg L^{-1} of Cd and 100 mg L^{-1} of Pb ($CdCl_2$, $Pb(NO_3)_2$). All tubes were inoculated with 1 mL of a bacterial cell suspension (optical density at 600 nm = 1), which was previously grown in GY medium at 28 °C for 24 h. Samples were incubated at 28 °C and 100 rpm on an orbital shaker. In order to estimate the amounts of trace elements retained by bacterial cells, the trace element concentrations were determined in the supernatant after 72 h of incubation by inductively coupled plasma-atomic emission spectrometry (ICP-OES, Agilent Technologies, 700 series, Belgium). Cultures were first centrifuged 15 min at 4800 rpm. One aliquot of supernatant was filtered (0.22 µm) and used for element determination. Media without the trace elements and media with the elements but not inoculated with bacteria were used as negative controls [27]. For the visualization of bacterial structure, bacterial pellets were washed 3 times with 0.01 M phosphate-buffered saline buffer (PBS, pH 7.0) to remove unbound metals, sugars and proteins. Pellets were resuspended in 2% glutaraldehyde for 1h at room temperature. Afterwards, bacterial samples were centrifuged for 3 min at 3000 rpm and pellets were washed 3 times with milli-Q water. One µL of sample was placed on a sample holder, in carbon conductive tape. Then, samples were coated 30 s with a 15 nm gold layer and analysed using a Scanning Electron Microscope (FEI Quanta 200F FEG-SEM with Thermo Fisher Pathfinder Alpine EDS system with UltraDry Premium (60mm2 active area) EDS detector). Images were taken using an accelerating voltage of 12.5 kV.

2.5. In-Vitro Inoculation on Vertical Agar Plates (VAPs), Germination and Growth Promotion

Seeds of *H. annuus* were sterilized before inoculation by shaking them in 70% ethanol for 5 min, followed by 5% hypochlorite for 5 min, and rinsing five times in sterile, deionised water. Inoculation cultures were prepared by growth in GY-trace element medium at 30 °C for 48 h. Cultures were centrifuged, and pellets were washed twice in sterile 10 mM $MgSO_4$ buffer before being thoroughly resuspended in 10 mL of the saline buffer until an optical density of 1 at 600 nm ($\approx 10^9$ CFU mL^{-1}) [10]. Fifteen seeds were incubated for 60 min at 29 °C on an orbital shaker fully immersed in 10 mL bacterial culture before sowing them in Petri dishes containing a layer of sterile paper and 10 mL of sterile deionised water. Germination was evaluated after 48 h. Three germinated seeds were transferred in Vertical Agar Plates containing Murashige and Skoog basal salts medium and held vertically for two weeks in a growth chamber (16:8 light/dark, photoperiod; 25 °C/19 °C; 400 µM cm^{-2} s^{-1} PAR; 60% relative humidity). Three replicates were performed per strain. Biomass parameters as root and shoot length and fresh and dry weight (DW) were determined after this incubation time.

2.6. Bioaugmentation in Microcosm Assays, Growth Promotion and Element Bio-Accumulation

Germination of commercially available *H. annuus* seeds (EEA-INTA, Anguil, Argentina) was performed in germination trays containing commercial growth substrate (Asef, Osmocote) and controlled conditions of temperature, humidity and watering (25 °C day/19 °C night; 60% relative humidity; 500 mL per day spray watering; PAR = 400 µmol cm^{-2} s^{-1}) in the greenhouse. After 25 days each substrate block was transplanted to pots containing two litres of bulk military polluted soil from North-East Belgium. The physicochemical properties of the sediments and total trace element concentrations are given in Table 1.

Table 1. Physicochemical properties and total cadmium (Cd), copper (Cu), lead (Pb), and zinc (Zn) concentrations of the military soil used in the microcosms assay.

Physicochemical properties			
Texture (%)	**Conductivity (µS cm^{-1})**	**CEC (Meq 100 g^{-1} DW)**	**pH**
sandy-loam	340 ± 1.2	9.2 ± 0.2	6.2 ± 0.8
Trace element concentration (mg kg^{-1})			
Cd	Cu	Pb	Zn
0.42 ± 1.35	1.02 ± 1.16	5.48 ± 0.89	12.43 ± 20.07
Values are mean ± S.E. (n = 24).			

The pot experiment tested the effects of six bacterial strains on the growth and metal accumulation of *H. annuus* seedlings with 6 replications and grown in polluted soil (36 pots). After one week of acclimatization, plants were inoculated with 10 mL of bacterial culture. Inoculation cultures were prepared by growing bacteria in GY-trace element broth at 30 °C for 48 h. Cultures were centrifuged, and cell pellets were washed twice in sterile 10 mM magnesium sulphate buffer, before being thoroughly suspended in 10 mL of the saline buffer until an optical density of 1 at 600 nm [10].

Thirty five days after inoculation, plants were harvested from the pots and roots were thoroughly washed with sterile water to remove any soil particles. Mouth masks were used to avoid human hazard and the wash water was placed in special containers. Samples were oven-dried (60 °C for 1 week), weighed, digested with 70% HNO_3 in a heat block and dissolved in 5 mL of 2% HCl. Trace element concentrations in shoots and roots of *H. annuus* were then determined using inductively coupled plasma-atomic emission spectrometry (ICP-OES, Agilent Technologies, 700 series, Hasselt, Belgium). Blanks (only HNO_3) and standard references (NIST Spinach 1570a) were included. Bio-accumulation factors (BAF) were calculated by dividing the total contents of elements in the plant tissue by the total contents of element in the soil [28].

2.7. Bacterial Survival and Colonization of Plant Tissues in Microcosms-ARISA

DNA extraction from root endophytes was performed by using Invisorb Spin Plant Mini Kit (Stratec Biomedical AG, Berlin, Germany) and soil DNA extractions were done by using the DNeasy PowerSoil Isolation kit (Qiagen, Germantown, MD, USA). Internal transcribed spacer (ITS) regions between 16S rRNA and 23S rRNA were amplified by PCR using ITSF (5-GTCGTAACAAGGTAGCCGTA-3) and ITSReub (5-GCCAAGGCATCCACC-3) primers as previously described by Cardinale et al., (2004) [29]. After amplification, samples were loaded onto Agilent DNA 1000 Chips and analysed using the Agilent 2100 Bioanalyzer® (Agilent Technologies, Santa Clara, CA, USA). Expert Software (Agilent Technologies) was used to digitalize the ARISA fingerprints, resulting in electropherograms in ASCII formats that were processed using the StatFingerprints package [30] in R ×64 3.4.3. Profiles obtained were compared with the profile of the pure inoculated strain.

2.8. Statistical Analysis

Data were analysed by using analysis of variance (ANOVA). When ANOVA showed treatment effect, the Least Significant Difference (LSD) test was applied to make comparisons between the means at $p < 0.05$. ARISA results were analyses using Fingerprint Library in the 2.13.0 version of the R project (The R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Isolation of Endophytic and Rhizospheric Trace Element-Tolerant Bacteria

A total of 105 morphologically different strains were isolated, genotypically identified and subsequently characterized. Fifty-three strains were tolerant to 100 mg L^{-1} Pb, 22 from the rhizosphere

and 31 root endophytes. Forty-nine strains were tolerant to 10 mg kg^{-1} Cd, of which 28 from the rhizosphere and 21 root endophytes.

3.2. Genotypic Characterization of Cd and Pb-Tolerant Strains

Among the 105 morphotypes isolated, 29 different bacteria were identified. Fifteen were Pb tolerant and 14 Cd tolerant. Figure 1 shows the percentages of presence of each strain in the compartment from which it was isolated. Nine genera were identified, which included *Bacillus* (68%), *Brevibacterium* (3%), *Cellulosimicrobium* (3%), *Gordonia* (2%), *Pseudobacter* (5%), *Rhizobium* (3%), *Cupriavidus* (3%), *Klebsiella* (2%), and *Methylobacterium* (3%).

Some strains belonging to the Bacillaceae family, including *B. cereus* ST10/ST60, *B. paramycoides* ST9/ST98, *B. tropicus* ST22/ST77, *B. proteoliticus* ST4/ST89 show tolerance to both elements Cd and Pb, whereas *B. proteoliticus* ST4, *B. safensis* ST11, *B. subtilis* ST7, *B. tropicus* ST22 were isolated from both, rhizosphere and root endosphere.

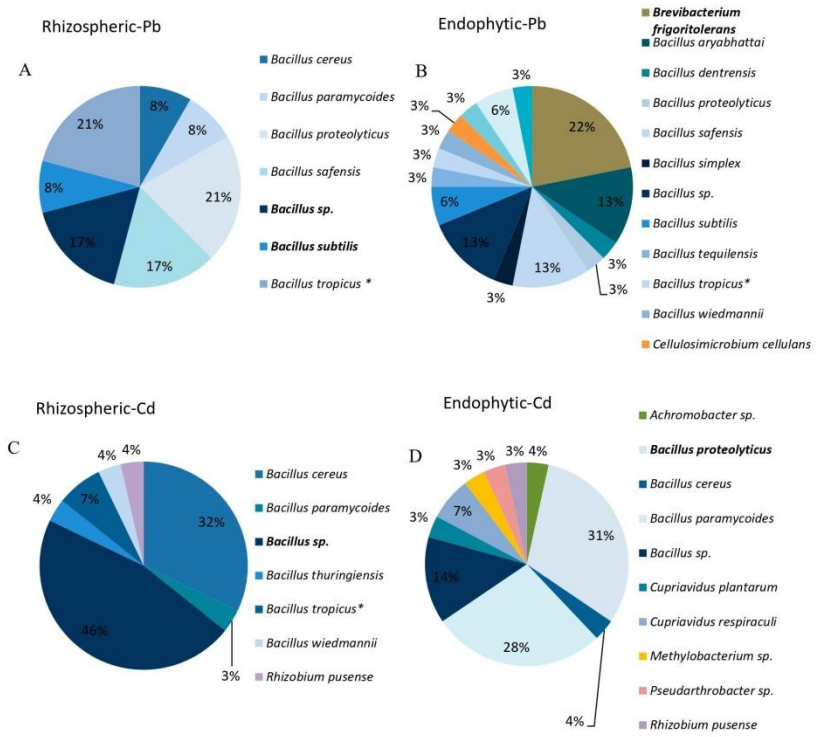

Figure 1. (**A**) Root rhizospheric and (**B**) endophytic Pb tolerant bacterial; and (**C**) root rhizospheric and (**D**) endophytic Cd tolerant bacterial strains; isolated onto heterotrophic glucose-yeast medium, from roots of *H. petiolaris* plants, grown in polluted soils.

3.3. In-Vitro PGP Activity of the Strains

The in vitro PGP traits of 28 bacterial isolates are shown in Table 2. Twelve strains were positive for all the qualitative PGP traits tested. *Bacillus aryabhattai* ST25, *Brevibacterium frigoritolerans* ST30 and *Klebsiella varicola* ST106 exhibited the highest capacities to solubilise tricalcium phosphate. Only

two strains, *Bacillus cereus* ST10 and *Bacillus wiedmannii* ST29 were able to produce siderophores. IAA production was detected in six isolates, ranging from 25.94 to 81.47 μg mL^{-1} and *Bacillus cereus* ST10 was one of the highest biofilm producers.

Table 2. Plant growth-promoting properties of trace element-tolerant bacteria.

Strain	ACCD	Acetoin	[a] OA	[b] P-Solub.	[b] SID	[c] IAA	[d] Biofilm	pH	[e] Element Conc.
Lead tolerant									
Bacillus sp. ST1	+	+	+	0.06 ± 0.01 *	0.16 ± 0.01 **	0.00 ± 0.00	0.01 ± 0.00	4.27 ± 0.18 *	5.91 ± 0.05 **
Bacillus proteoliticus ST4	+	+	+	0.06 ± 0.01 *	0.26 ± 0.02 **	43.61 ± 1.03 *	0.03 ± 0.00 *	4.23 ± 0.28 *	5.67 ± 0.05 *
Bacillus subtilis ST7	-	+	-	0.23 ± 0.01 **	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.02 *	4.22 ± 0.08 *	6.31 ± 0.09 **
Bacillus paramycoides ST9	+	+	+	0.19 ± 0.01 **	0.16 ± 0.01	0.00 ± 0.00	0.02 ± 0.00	4.20 ± 0.15 *	5.85 ± 0.07 **
Bacillus cereus ST10	+	+	+	0.09 ± 0.01 *	0.18 ± 0.01 **	0.00 ± 0.00	0.02 ± 0.00	4.16 ± 0.07 *	6.19 ± 0.05 **
Bacillus safensis ST11	-	+	+	0.08 ± 0.01 *	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	4.15 ± 0.07 *	6.02 ± 0.04 **
Bacillus tropicus ST22	+	+	+	0.06 ± 0.01 *	0.20 ± 0.01 **	0.00 ± 0.00	0.01 ± 0.00	4.21 ± 0.18 *	6.01 ± 0.07 **
Bacillus aryabhattai ST25	+	-	+	0.34 ± 0.02 **	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	4.21 ± 0.32 *	6.03 ± 0.05 **
Brevibacterium frigoritolerans ST30	-	+	+	0.37 ± 0.02 **	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	4.26 ± 0.18 *	5.20 ± 0.12 *
Bacillus wiedmannii ST29	+	+	+	0.05 ± 0.01 *	0.53 ± 0.04 **	0.00 ± 0.00	0.01 ± 0.00	4.25 ± 0.04 *	5.09 ± 0.08 *
Bacillus tequilensis ST34	-	+	-	0.04 ± 0.00 *	0.14 ± 0.01 **	0.00 ± 0.00	0.03 ± 0.00 *	4.254 ± 0.08 *	5.30 ± 0.10 *
Bacillus dentrensis ST38	-	+	-	0.05 ± 0.01 *	0.11 ± 0.01 *	0.00 ± 0.00	0.02 ± 0.00	4.17 ± 0.07 *	6.58 ± 0.22 **
Bacillus simplex ST43	+	-	+	0.01 ± 0.00	0.00 ± 0.00	54.30 ± 2.08 *	0.02 ± 0.00	4.24 ± 0.08*	5.79 ± 0.53*
Gordonia terrae ST51	+	-	+	0.08 ± 0.01 *	0.22 ± 0.02 **	0.00 ± 0.00	0.04 ± 0.00 *	4.18 ± 0.07 *	8.22 ± 0.432 **
Cellulosimicrobium cellulans-ST54	+	+	+	0.10 ± 0.01 *	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.02 *	4.21 ± 0.27 *	6.22 ± 0.12 **
Negative control (GYPb)	-	-	-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	6.08 ± 0.02	0.49 ± 0.08
Cadmium tolerant									
Bacillus cereus ST60	+	+	+	0.01 ± 0.00	0.10 ± 0.01 *	0.00 ± 0.00	0.29 ± 0.06 **	5.08 ± 0.35 *	7.67 ± 0.33 *
Bacillus tropicus ST77	+	+	+	0.00 ± 0.00	0.38 ± 0.03 **	50.08 ± 2.00 **	0.03 ± 0.00 *	4.97 ± 0.027 *	7.89 ± 0.09
Rhizobium pusense ST80	-	-	+	0.15 ± 0.01 **	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.00 *	6.83 ± 0.44	3.48 ± 0.05 **
Methylobacterium ST85	+	+	+	0.20 ± 0.01 **	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	6.98 ± 0.42	5.00 ± 0.12 **
Pseudobacter sp. ST86	+	+	+	0.26 ± 0.02 **	0.00 ± 0.00	30.25 ± 1.98 *	0.03 ± 0.00 *	6.05 ± 0.26	4.08 ± 0.13 **
Bacillus sp. ST87	-	+	+	0.08 ± 0.01 *	0.10 ± 0.01 *	0.00 ± 0.00	0.04 ± 0.00 *	4.70 ± 0.15 *	7.98 ± 0.21
Bacillus proteolyticus ST89	+	+	+	0.03 ± 0.00	0.38 ± 0.03 **	0.00 ± 0.00	0.13 ± 0.02 *	4.99 ± 0.28 *	7.74 ± 0.21 *
Bacillus cereus ST90	+	+	+	0.12 ± 0.01 *	0.60 ± 0.04 **	0.00 ± 0.00	0.04 ± 0.00 *	4.89 ± 0.019 *	6.29 ± 0.14 *
Achomobacter sp. ST95	-	+	+	0.10 ± 0.01 *	0.24 ± 0.02 **	0.00 ± 0.00	0.10 ± 0.02 *	6.98 ± 0.38	4.87 ± 0.28 **
Bacillus paramycoides ST98	+	+	+	0.02 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.00 *	4.97 ± 0.15 *	7.87 ± 0.14 *
Cuprividus plantarum ST102	+	+	+	0.09 ± 0.01 *	0.31 ± 0.02 **	25.94 ± 1.02 *	0.02 ± 0.00	4.86 ± 0.13 *	7.87 ± 0.9 *
Klebsiella variicola ST106	+	+	+	0.36 ± 0.02 **	0.00 ± 0.00	81.47 ± 4.55 **	0.07 ± 0.02 *	3.57 ± 0.17 **	7.95 ± 0.12
Negative control (GYCd)	-	-	-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.6 ± 0.03	8.27 ± 0.12

Values are mean ± S.E. (n = 3); [a] Organic acid; [b] solubilized area (cm^2); [c] μg IAA mL^{-1} of medium; [d] Measured by optical density (OD600); [e] μg mL^{-1} metal in supernatant; + positive; - negative. Values in the same column followed by a * are significantly different from the control at $p \leq 0.05$ by Anova and Tukey test. Values with different number of * are significantly differents.

3.4. In-Vitro Trace Element Uptake in Culture Media and Scanning Electron Microscopy (SEM-EDX) Analysis

All Pb tolerant strains decreased the pH of the medium after 48 h of incubation. Due to this, Pb solubility and its concentration in the supernatant increased (Table 2). *Gordonia terrae* ST51 was the strain that solubilized Pb the most, eight times more than the negative control and between 2 and 3 times more than the other strains ($p < 0.05$). Some Cd tolerant strains (e.g., *Klebsiella varicola* ST106 and *Cupriavidus plantarum* ST102) acidified the medium and increased the Cd concentration in the supernatant. However, some other strains (e.g., *Rhizobium pusense* ST80, *Pseudobacter* sp. ST89 and *Achomobacter* sp. ST95) increased the Cd concentration in the supernatant with about 50% compared with the negative control although the pH did not change.

Interaction between trace elements and other components of the medium is playing an important role in the sequestration of the trace elements. If we compare the nominal concentrations in the growth medium, 10 mg L^{-1} Cd and 100 mg L^{-1} Pb, with the trace element concentrations in the supernatant of negative controls (Table 2), only 0.5% of the Pb added to the medium is available in the supernatant at the conditions established.

Bacillus paramycoides ST4, *Brevibacterium frigoritolerans* ST30, *Bacillus wiedmannii* ST29, *Cellulosimicrobium cellulans* ST54, *Methylobacterium* sp. ST85 and *Bacillus proteolyticus* ST9 were selected to visualize its structure and elements interaction by SEM-EDX (Figure 2). These strains were the most plant growth promoting, did not have adverse effects on seed germination and developed well in vitro. It is clear that Cd and Pb were present in/on the bacterial cell wall. The highest signal intensity of Pb was found around *B. paramycoides* ST4 strains, while around *Brevibacterium frigoritolerans* ST30 the signal intensity of Pb was half of it and Pb was not detected in *Cellulosimicrobium cellulans*. The biofilm capability of *Cellulosimicrobium cellulans* made it difficult to visualize individual cells.

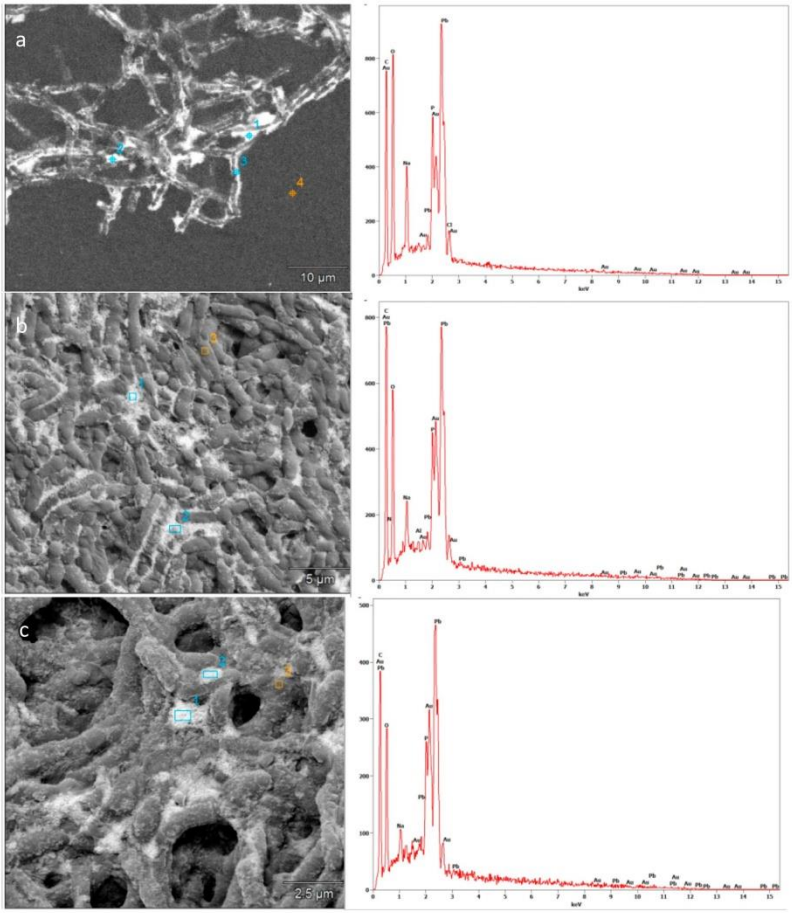

Figure 2. *Cont.*

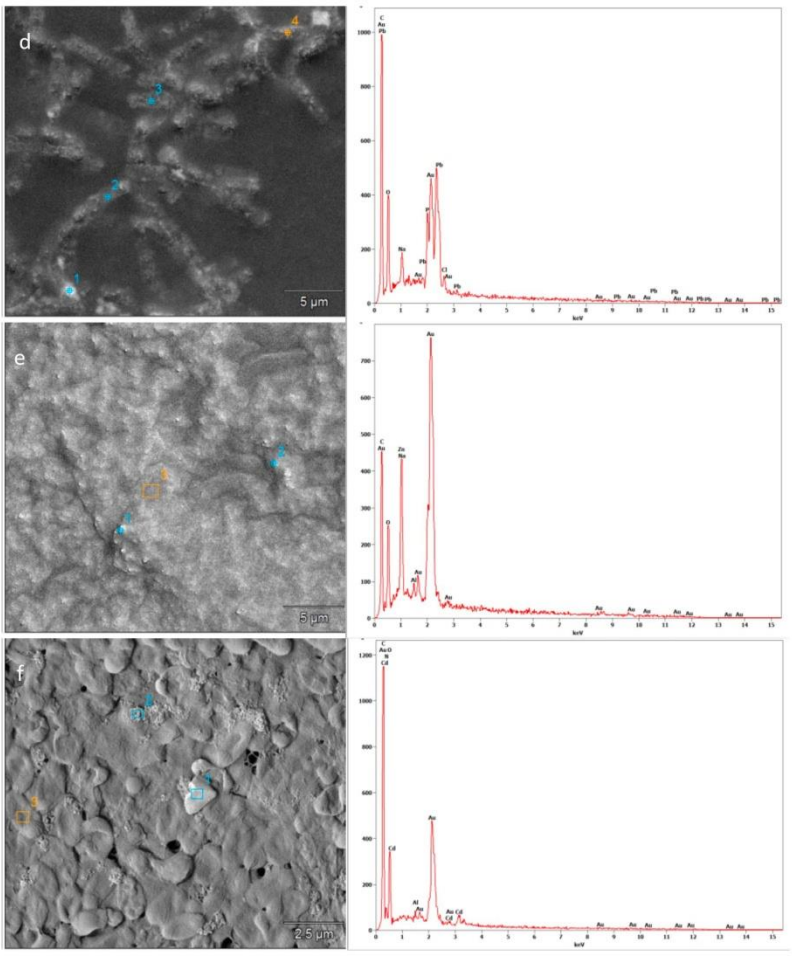

Figure 2. SEM images of bacterial colonies (**a**) *B. paramycoides* ST4 (**b**) *B. proteolyticus* ST9 (**c**) *B. wiedmnni* ST29 (**d**) *Brevibacterium frigoritolerans* ST30 (**e**) *Cellulosimicrobium cellulans* ST54 (**f**) *Methylobacterium* ST85. Numbers in blue colour follow by squares or dots represent the specific locations where EDX-spectra were taken, characterised by more electron dense (bright) metal plaques on the bacterial cells, while the orange area, is a reference location, either background or bacterial cell surface outside of a bright area. Next to each SEM image is the spectrum correspond to the location number one.

3.5. Germination and Growth Promotion on Vertical Agar Plates (VAPs)

After 48 h of incubation, germination of *H. annuus* seeds inoculated with the different strains was 20%–80% higher in comparison to the control seeds (Figure 3). However, some bacterial strains inhibited plant biomass development at the inoculum concentration of 10^9 CFU mL^{-1} that was used. After two weeks, seedlings inoculated with *Klebsiella varicola* ST106, *Achromobacter* sp. ST95, *Bacillus cereus* ST90, *Pseudobacter* sp. ST86, *Gordonia terrae* ST51 and *Bacillus simplex* ST43 showed a 50% lower dry weight in comparison to the negative controls.

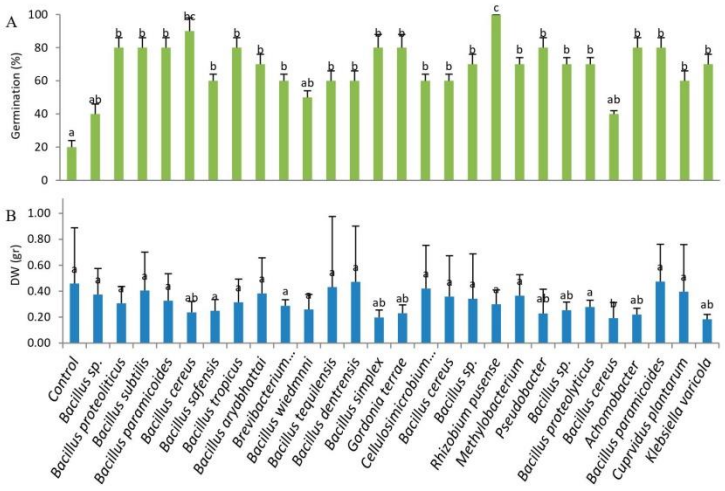

Figure 3. (**A**) Germination percentage of seeds of *H. annuus* inoculated with individual strains and (**B**) biomass development as total dry weight (DW) of plant per plate after two weeks of incubation in VAP systems. Error bars are S.E. (n = 15); Values followed by the same letter(s) are not significantly different at $p \leq 0.05$ by Anova and Tukey test.

3.6. Growth Promotion and Element Bio-Accumulation in Microcosm Systems

H. annuus seedlings were planted into pots filled with trace element polluted military soil and inoculated respectively with *Bacillus paramycoides* ST4, *Brevibacterium frigoritolerans* ST30, *Bacillus wiedmannni* ST29, *Cellulosimicrobium cellulans* ST54, *Methylobacterium* sp. ST85 *and Bacillus proteolyticus* ST9. After 35 days, plants were harvested, and biomass parameters were determined (Table 3). The stem length of plants inoculated with *Bacillus proteolyticus* ST9 was 57% higher compared to the control plants. Furthermore, *Bacillus proteolyticus* ST9 bio-accumulation factors (BAF) of Pb and Cd were respectively 20% and 40% lower. Also, inoculation with *Bacillus wiedmanni* ST29 lowered the bioaccumulation of Cd by 40%. Inoculation with *Cellulosimicrobium cellulans* ST54, on the contrary, increased the accumulation factor of Pb six times and of Cd even 15 times compared to non-inoculated control plants. Also, *Bacillus paramycoides* ST4 increased the trace element accumulation factors: a factor three for Pb and a factor six for Cd.

Table 3. Stem length (cm), dry weight (g), concentrations (mg g^{-1}) and Bio-Accumulation Factors (BAF) of Pb and Cd in roots and leaves of *H. annuus* seedlings inoculated with PGP, Pb-, and Cd-tolerant bacterial strains.

Strain	Stem Length (cm)	Dry Weight Stem (g)	Pb (mg Kg^{-1})		Cd (mg Kg^{-1})		BAF	
			Root	Leaves	Root	Leaves	Pb	Cd
Bacillus paramycoides ST4	4.7	2.00 ± 1.03	21.40 ± 1.61	9.06 ± 1.60	0.31 ± 0.23	0.02 ± 0.04	3.14 c	25.63 d
Bacillus wiedmanni ST29	5.7	1.14 ± 0.35	40.14 ± 5.88	12.97 ± 4.96	<DL [a]	0.24 ± 0.19	1.09 ab	2.46
Brevibacterium frigoritolerans ST30	7.3	2.21 ± 1.40	55.90 ± 18.93	4.95 ± 3.22	0.02 ± 0.31	0.22 ± 0.05	1.66 b	4.83 b
Cellulosimicrobium cellulans ST54	6.3	1.86 ± 0.93	35.77 ± 6.74	3.62 ± 1.25	0.33 ± 0.48	0.17 ± 0.12	6.17 d	61.28 e
Methylobacterium sp. ST85	5.3	1.66 ± 1.11	20.02 ± 1.51	3.51 ± 2.77	0.51 ± 0.17	0.14 ± 0.02	1.84 b	18.69 c
Bacillus proteolyticus ST9	11.0	2.50 ± 0.51	15.60 ± 1.54	3.40 ± 1.03	0.37 ± 0.26	0.21 ± 0.08	0.73 a	2.45 a
Control	7.0	1.85 ± 0.43	28.89 ± 2.04	3.87 ± 1.25	0.43 ± 0.25	0.08 ± 0.01	0.92 a	4.15 b

Values are mean ± S.E. (n = 6); BAF values in the same column followed by the same letter(s) are not significantly different at $p \leq 0.05$ by Anova and Tukey test; [a] <DL: below detection limit (0.05 mg/kg); in grey are highlighted strains that colonized *H. annuus* plants.

3.7. Bacterial Survival and Colonization of Plant Tissues in Microcosms-ARISA

Community-specific profiles obtained from rhizosphere (soil) and root endosphere (root) of the plants that were inoculated in greenhouse were compared with the profiles obtained for each strain individually (blue arrow) to estimate survival and colonization (Figure 4).

Cellulosimicrobium cellulans ST54, *Brevibacterium frigoritolerans* ST30 and *Methylobacterium* ST85 apparently did not colonize the *H. annuus* root endosphere and also did not survive in the rhizosphere. *Cellulosimicrobium cellulans* ST54 was also not detected in control plants growing in polluted soil not inoculated and not sterilized (Figure 4, heatmap G). Taken together, this suggests that this strain should be highly host specific and can only colonize the plant species from which it was isolated. Because the trace element tolerant strains used in this work did not originate from *H. annuus*, but from *H. petiolaris*, the metabolites produced by this species probably did not act as effective attractants for this strain.

A significant increase in the accumulation factor of Pb and Cd was found on those plants inoculated with *Cellulosimicrobium cellulans* ST54 even when this strain was not present on the community profiles. However other strains were found colonizing these plants and could be responsible for the increase in the bioaccumulation.

The other strains could be detected in both, root endosphere and rhizosphere 35 days after inoculation. Control plants (not inoculated) grown in polluted soil were also colonised by the same species: *Baclilus paramycoides*, *Methylobacterium and Bacillus proteolyticus* were found in the root endosphere and *Bacillus wiedmannii* was detected in the rhizosphere.

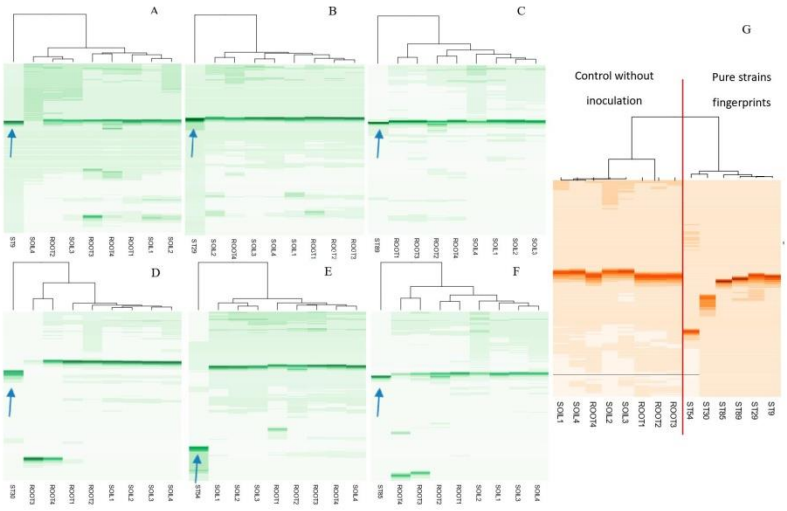

Figure 4. Heatmaps of strain recolonization in rhizosphere (soil) and root endosphere (root) of (**A**) *B. paramycoides* ST9 (**B**) *B. wiedmannii* ST29 (**C**) *B. proteolyticus* ST89 (**D**) *Brevibacterium frigoritolerans* ST30 (**E**) *Cellulosimicrobiumcellulans* ST54 (**F**) *Methylobacterium* ST85. Blue arrows indicate the pure inoculated strain fingerprint. (**G**) Control plants growing in polluted soil without inoculation and pure strains fingerprints. A complete linkage algorithm was used to perform a cluster analysis of Bray-Curtis dissimilarity matrices inferred from the ARISA value.

4. Discussion

One hundred and five endophytic and rhizospheric trace element-tolerant bacteria were isolated from roots of *H. petiolaris* plants, grown in Pb/Cd polluted soils. To the best of our knowledge, there are

no earlier reports about the isolation of endophytic and rhizospheric bacteria from this plant species. Though, there exist many studies reporting the isolation of trace element tolerant bacteria from other species such as *Arabidopsis* [31], *Brassica napus* [32,33], *Thlaspi (syn. Noccaea) caerulescens* [34], *Nicotiana tabacum* [35]. However, some bacteria genera isolated in our study (i.e., *Bacillus*, *Mhetylobacterium*, *Cellulosimicrobium and Rhizobium*) have already been reported in studies made on other plants species harvested from trace elements polluted soils [36–41].

Most earlier reports found that bacterial density/diversity decreases from outside to the root interior [42,43], while in our study the number of cultivable trace element tolerant strains isolated from the root endosphere of *H. petiolaris* plants was almost double that isolated from the rhizosphere.

The PGP traits of the bacterial isolates were determined to identify the most promising strains to improve plant survival and to promote element phytostabilization or extraction. Almost 90% of the isolates acidified the medium in our report, which directly influenced element solubility. These strains were organic acid producers or biofilm producers and most of them also solubilised phosphate. Several authors relate the solubilisation of inorganic insoluble phosphate by microorganisms to the production of organic acids and chelating oxoacids from sugars [44]. Siderophores production was another capability found in those strains that modified pH of the medium and the element bioavailability. It has been demonstrated that siderophores can complex a variety of trace element ions, and biosurfactants which enhance trace element removal from polluted soils [45]. *B. proteolyticus* ST89 significantly increased shoot biomass of *H. annuus* in the greenhouse and in vitro it was one of the strains that produced more IAA. Production of IAA by strains from the rhizosphere and endosphere of different crops, peanut, maize, wheat, and rice was already reported in a number of studies [31,46]. Biofilm producing strains were sought due to their ability to enhance bacterial attachment to the roots and concentrate trace elements into their extracellular polymeric structure [47,48]. However, in our report one of the strains that in vitro was one of the highest biofilm producers (*Cellulosimicrobiumcellulans* ST54) was not able to colonize *H. annuus* rhizosphere in the greenhouse.

Interaction between trace elements and the components of the medium played an important role in the sequestration of the trace elements in our in vitro study. Pb precipitation is common in culture media as Pb readily complexes with phosphates and hydroxides [38]. In vitro trace element uptake by our isolates in culture media indicated that the strains modified the pH and the element solubility, making them more or less bioavailable for sequestration or internalization. Some *Pseudomonas* and *Bacillus* have been reported to perform maximum removal in the pH range of 6–8 [49,50] and *Klebsiella* sp. was reported by Prapagdee et al., 2013 [51] to remove 62% of Cd at an ion concentration of 25 mg L^{-1}.

In vitro inoculations of *H. annuus* seeds on VAPs showed the capability of some of our strains to enhance germination and development of seedlings. Increased plant growth after bacterial inoculation was reported to be mainly due to the synthesis of plant growth compounds such as IAA and ACC-deaminases, which stimulate elongation of shoots and roots [52,53]. Nevertheless, too high IAA concentrations can result in an unbalanced plant growth or in deleterious effects on root development [54]. In our study, *Klebsiella varicola* ST106 produced the highest concentrations of IAA (Table 2) and this strain had some of the most adverse effects on plant biomass development (Figure 3).

This is the first report describing the effects of *Bacillus paramycoides* and *Bacillus proteolyticus* on plant growth promotion and trace element uptake of plants in microcosm systems. *Bacillus wiedmanni* isolated from a landfill with electrical waste in China was earlier described by Chen et al., (2018) [55] as a species able to reduce Pd (II) under both aerobic and anaerobic conditions. *Methylobacterium* sp. was isolated by Koo et al., (2007) [56] from the rhizosphere of plants growing in crude oil and trace element polluted soil and Madhaiyan et al., (2007) [57] reported that inoculation with methylotrophic bacteria decreased trace element uptake by tomato plants and at the same time increased plant biomass. *Cellulosimicrobium* sp. was reported by Karthik et al., 2017 [58] as a Cr-reducing bacterium able to promote plant growth of alfalfa under trace element stress conditions and to enhance alfalfa trace element uptake. Our study is the first report about Pb tolerance of *Cellulosimicrobium cellulans*.

Despite the high PGP potential of some strains as confirmed under laboratory conditions, the results of pot and/or field experiments are often ambiguous [59]. In our study strains that displayed in vitro PGP capabilities, didn't express it when they were inoculated in the greenhouse. The interaction between the inoculated strain(s) and the autochthonic soil microbiota is an important factor that influences the success of bioaugmentation [60].

The complexity of the interaction between microorganisms, soil and plants was highlighted by Ghasemi et al. (2018) [61], who found that strains isolated from the rhizosphere of *Odontarrhena serpyllifolia* supported the phytoextraction of Ni by different *Odontarrhena* species (*O. bracteata*, *O. inflata*, *O. serpyllifolia*) with different efficiencies, which was dependent on the plant species, soil type, and bacterial inoculant.

5. Conclusions

This research provides a collection of Pb and Cd tolerant-PGP bacterial strains. Among those, we selected six promising strains, *Bacillus paramycoides* ST9, *Bacillus wiedmanni* ST29, *Bacillus proteolyticus* ST89, *Brevibacterium frigoritolerans* ST30, *Cellulosimicrobiumcellulans* ST54, *Methylobacterium* sp. ST85 and used them in microcosm experiments. They showed high colonisation capabilities after inoculation, colonising the root endosphere of *H. annuus*. Inoculation with *B. proteolyticus* ST89 significantly increased shoot biomass and lowered trace elements uptake from a polluted military soil. This strain is promising for application in crops that are cultivated in moderately trace element polluted soils to improve plant growth under such harsh field conditions ensuring food security. Inoculation with *B. paramycoides* ST9, on the other hand, led to an increase of Pb and Cd concentrations in *H. annuus* seedlings, so we do not recommend this strain to be inoculated in crops, but it could be used in other plant species for phytoextraction. Further studies are needed to elucidate the interaction mechanisms between these bacterial strains, trace elements, and plants. In the future, inoculation experiments with consortia will be performed to assess the potential of these promising strains in both phytostabilization and phytoextraction, depending on the remediation strategy that will be chosen.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/2/204/s1. Table S1: Strain identification based on the 16S rRNA partial sequence.

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Bioaugmentation with PGP-trace element tolerant bacterial consortia affects Pb uptake by Helianthus annuus grown on trace element polluted military soils

En este estudio, buscamos componer consorcios de bacterias promotoras del crecimiento vegetal (PGP) y tolerantes a oligoelementos, para mejorar el crecimiento de las plantas e inhibir la absorción y translocación de oligoelementos, permitiendo eventualmente el cultivo de cultivos lucrativos en suelos contaminados con oligoelementos, reduciendo los riesgos de entrada de estos elementos en la cadena alimentaria. Girasol (*Helianthus annuus* L.) se cultivó en dos suelos militares contaminados (MS1 y MS2) en microcosmos de invernadero y se inoculó con tres consorcios bacterianos diferentes (C1, C2, C3). El crecimiento y estado fisiológico del las plantas no se vieron afectados durante el experimento con la inoculación. Después de 2 meses, las plantas fueron cosechadas. El consorcio C2 y C3 disminuyó la bioacumulación en los brotes, de Pb en un 80% a un 85%, respectivamente cuando las plantas se cultivaron en el MS1, incluso a concentraciones por debajo del límite de detección en plantas cultivadas en MS2. Las diferencias en la captación y la localización (sub) celular de Pb y Cd en aislados bacterianos seleccionados fueron investigadas *in vitro* mediante TEM-EDX. La absorción de Pb se observó mediante cultivos de *Bacillus wiedmanni* ST29 y *Bacillus paramycoides* ST9. Mientras que la adsorción en la pared celular bacteriana fue observado por *Bacillus paramycoides* ST9 y la retención en la matriz extracelular por *Cellulosimicrobium cellulans* ST54.

Palabras Clave:
Cultivo comercial, inoculación, suelos contaminados

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Bioaugmentation with PGP-trace element tolerant bacterial consortia affects Pb uptake by *Helianthus annuus* grown on trace element polluted military soils

A. Saran[a,b], V. Imperato[b], L. Fernandez[a], F. Vannucchi[c], N. M. Steffanie[d], J. d'Haen[e,f], L. J. Merini[a], J. Vangronsveld[b,g], and S. Thijs[b]

[a]EEA Anguil INTA-CONICET, La Pampa, Argentina; [b]Environmental Biology, Centre for Environmental Sciences, Hasselt University, Diepenbeek, Belgium; [c]Institute of Life Science, Scuola Superiore Sant'Anna, Pisa, Italy; [d]Zoology: Biodiversity and Toxicology, Centre for Environmental Sciences, Hasselt University, Diepenbeek, Belgium; [e]Institute for Material Research (IMO), Hasselt University, Diepenbeek, Belgium; [f]Division IMOMEC, IMEC, Diepenbeek, Belgium; [g]Department of Plant Physiology and Biophysics, Faculty of Biology and Biotechnology, Maria Curie Sklodowska University, Lublin, Poland

ABSTRACT

In this study, we sought to compose consortia of plant growth-promoting (PGP) and trace element tolerant bacteria, to improve plant growth and inhibit uptake and translocation of trace elements, eventually allowing the cultivation of profitmaking crops on trace elements polluted soils, reducing the risks of entrance of these elements into the food chain. Sunflower (*Helianthus annuus L.*) was grown on two polluted military soils (MS1 and MS2) in greenhouse microcosms and inoculated with three different bacterial consortia (C1, C2, C3). Growth and physiological status of the plants were unaffected during the experiment with the inoculation. After 2 months, plants were harvested. Consortium C2 and C3 decreased Pb shoot bioaccumulation by respectively 80–85% when plants were grown in the MS1 and even to concentrations below detection limit in plants grown in MS2. Differences in uptake and (sub)cellular localization of Pb and Cd in selected bacterial isolates were investigated *in vitro* by TEM-EDX. Pb absorption was observed by *Bacillus wiedmanni* ST29 and *Bacillus paramycoides* ST9 cultures. While adsorption at the bacterial cell wall was observed by *Bacillus paramycoides* ST9 and retention in the extracellular matrix by *Cellulosimicrobium cellulans* ST54.

KEYWORDS

Commercial crop; inoculation; polluted soils

Introduction

Land reserved for military use accounts for approximately 6% of the Earth's terrestrial surface. Military activities cause serious environmental harm, including soil pollution with trace elements, fuels, solvents and explosives (Zentelis and Lindenmayer 2015). In particular, trace elements that accumulated in soil and (ground) water cannot be degraded are adversely affecting ecosystems, including soil microbial communities, plants, animals, and human health (Román-Ponce *et al.* 2016). Military areas are the second largest source of Pb contamination ranging from 10 to 60,000 tons of annual depositions in different countries (Ahmad *et al.* 2011). Several studies have reported Pb contamination in military shooting range soils exceeding total concentrations of 10,000 mg kg^{-1} (Spuller *et al.* 2007; Cao *et al.* 2009), and exceeding the U.S. EPA screening criteria of 400 mg Pb kg^{-1} (USEPA 1996b). Since many of these military soils are used for pasture after decommission or during periods of no shooting activity, and some are even used for food crop production (Kettler and Schenk 2006), the risks associated to trace element transfer into the human food chain is an important issue (Robinson *et al.* 2008).

Sunflower (*Helianthus annuus* L.) is globally one of most important oilseed crops (Dalchiavon *et al.* 2018). The popularity of sunflower is driven by its versatility as oil, seed and animal feed. Also due to its high drought tolerance and adaptation to a great variety of soils, sunflower is suitable for cultivation in many regions around the world and has been introduced into many countries including Asia and Africa (Jošić *et al.* 2015). Several studies on sunflower have revealed that trace elements, including Cd, Cu, Pb and Zn are accumulate at high concentrations in roots and shoots (Alaboudi *et al.* 2018; Govarthanan *et al.* 2018; Thijs *et al.* 2018) reducing plant growth, biomass, grain yield and crop quality (Ahmad *et al.* 2011; Ramzani *et al.* 2016).

Uptake of trace elements does not only depend on the bioavailability of the elements in soil and the expression of element transporters and detoxification genes of the plant, but also on the plant-associated microbiota (Vangronsveld *et al.* 2009; Thijs *et al.* 2017). Considering that the indigenous rhizosphere microflora may increase plant element uptake and plant fitnessit is possible, at least to a certain

CONTACT A. Saran saran.anabel@inta.gov.ar, anabel.saran@uhasselt.be EEA Anguil INTA-CONICET, La Pampa, Argentina; Environmental Biology, Centre for Environmental Sciences, Hasselt University, Diepenbeek, Belgium.

Supplemental data for this article can be accessed at publisher's website.

Table 1. Soil texture, pH-H_2O and total cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn) concentrations (mg kg^{-1}) of the military soils (MS1 and MS2) used in the pot experiment.

Physicochemical properties						
	Texture	pH-H_2O	Cd	Cu	Pb	Zn
MS1	Sandy-loam	6.2 ± 0.8	6.31 ± 1.35	380.50 ± 60.20	3800.05 ± 300.45	320.05 ± 20.10
MS2	Clay	3.9 ± 0.6	1.50 ± 0.80	470.60 ± 50.01	250.00 ± 10.20	820.20 ± 1.30
RS	Sandy	6.1 ± 0.6	0.24 ± 0.03	13.20 ± 2.30	15.10 ± 1.70	26.90 ± 2.20
RM N°143	–	–	65 ± 12.44	115.30 ± 25.54	214 ± 4.25	1035 ± 30.76

Values are mean ± S.E. ($n = 24$). RM N°143 R: Reference Material, trace elements in a sewage sludge amended soil, Commission of the European Communities. Recoveries 85–110%.
RS is reference soil from the same region.

extent, to influence element accumulation in the shoots by selecting the appropriate microbial consortia in the rhizosphere (Vangronsveld *et al.* 2009; Muehe *et al.* 2015). When such bacteria are inoculated in the rhizosphere (bioaugmentation), they can influence the plant availability of trace elements by redox transformations, leading to element mobilization, dissolution, leaching, or immobilization through organic molecule-trace element binding and precipitation (Gadd 2004; Kidd *et al.* 2009; Sessitsch *et al.* 2013; Kidd *et al.* 2017). Few attempts have been made to use PGPB consortia to alleviate plant oxidative stress, and to reduce trace element mobility controlling the uptake and translocation of these elements into crops, and food chain (Khanna *et al.* 2019; Ren *et al.* 2019).

The previously reported PGP trace element tolerant strains *Bacillus paramycoides* ST9, *Brevibacterium frigotolerans* ST28, *Bacillus wiedmannii* ST29, *Cellulosimicrobium cellulans* ST54, *Methylobacterium sp.* ST85 and *Bacillus proteolyticus* ST89 isolated from the rhizosphere and endosphere of *Helianthus petiolaris* roots (Saran *et al.* 2020) were proposed as suitable candidates to improve trace element phytostabilization of trace element polluted soils. In the present study, as a continuation of the aforementioned, we hypothesized that bacterial consortia composed of these selected strains can play an important role in trace element uptake, accumulation and transport, as well as in the mitigation of trace element phytotoxicity.

Material and methods

Consortia design

The individual members of the consortia were selected based on their PGP properties and trace element bioacumulation capabilities (Saran *et al.* 2020). We composed three consortia, consortium (C1) comprised of *Brevibacterium frigoritolerans* ST28, *Bacillus wiedmannii* ST29 and *Bacillus paramycoides* ST9, consortium (C2) included *Methylobacterium* sp. ST85, *Bacillus proteolyticus* ST89 and *Cellulosimicrobium cellulans* ST54 and a third consortium contained all of them together (C3). The criteria for grouping them together were based on their PGP or metal bioacumulation properties. *Bacillus paramycoides* ST9 in our previous study (Saran *et al.* 2020) was able to increase *H. annuus* Pb and Cd bioaccumulation factor (BAF), and we joined this strain together with one siderophore producer (*Bacillus wiedmannii* ST29) and one phosphate solubilizer (*Brevibacterium frigotolerans* ST28). In contrast, *Bacillus proteolyticus* ST89 decreased *H. annuus* Pb and Cd BAF, we joined this strain together with one biofilm producer (*Cellulosimicrobium cellulans* ST54) and one ACCD producer (*Methylobacterium* sp. ST85). We also grouped all of them together (consortium 3) to evaluate the synergism or antagonism of these capabilities when the bacteria are growing together in the plant rhizosphere. Each strain was cultivated separately in 50 ml of GY medium at 30 °C during 48 h and continuous shaking to reach 10^8 CFU/ml. Then, cultures were centrifuged for 15 min at 4000 rpm and pellets were washed three times with 50 ml of 10 mM $MgSO_4$ solution, and centrifuged at the same conditions. Washed pellets were re-suspended in 30 ml of 10 mM $MgSO_4$ and mixed in 5 ml Falcon tubes (Płociniczak *et al.* 2016). Finally, consortia C1, C2 and C3 were composed by mixing equal proportions of the cultures of each strain in a final volume of 50 ml sterile tap water.

Soil used in the pot experiment

Polluted soils (top layer, 0–20 cm deep) were collected from two military fields in the North-East of Belgium (MS1 and MS2). Physicochemical characteristics of the collected soils are listed in Table 1. Total trace element concentrations were determined using the USEPA 3051 HNO_3-microwave assisted digestion method (Environmental Protection Agency [EPA] 1994).

Experimental design

Germination of commercial *H. annuus* seeds (EEA-INTA, Argentina) was performed in germination trays containing commercial growth substrate (CS) (Asef, Osmocote; Belgium) and controlled conditions of temperature, humidity and watering (25 °C day/19 °C night; 60% relative humidity; 500 ml per day spray watering; PAR = 400 µmol $cm^{-2}s^{-1}$) in the greenhouse. After 25 days each substrate block was transplanted to pots containing two liters of military soil (MS1 and MS2) and one liter of commercial substrate. The new concentration of the soil was nominal calculated, values are providing in Supplementary Table S1. In the pot experiment, we tested the effects of three bacterial consortia on the growth and element accumulation of *H. annuus* seedlings with 6 replicates per consortium treatment and grown in two military soils; negative controls consisted of plants grown on the polluted military soils but without inoculation (48 pots in total). After one week of

acclimatization, each plant was inoculated with 50 ml of each consortium.

Photosynthetic parameters, biomass and trace element accumulation

Physiological responses such as light adapted quantum yield of PS(II) (Y(II)) and dark adapted maximum potential quantum efficiency of PS(II) (Fv/Fm) were measured during the experiment using a Plant Stress Kit (Ecotechnic, ADC BioScientific Ltd, Belgium). Total chlorophyll was monitored using a CCM-200 plus Chlorophyll Content Meter (OPTI-SCIENCE, INC, USA). Three measures were taken per plant. Sixty days after inoculation, plants were harvested from the pots and washed three times with sterile water to remove any soil particles. Shoots and roots samples were oven-dried (60 °C for 1 week), weighed, digested with 70% HNO_3 in a heat block and dissolved in 5 ml of 2% HCl using the USEPA 3050B Acid Digestion of Sediments, Sludges, and Soils (Environmental Protection Agency [EPA] 1996a). Total trace element concentrations in shoots and roots of *H. annuus* were determined using ICP-AES (ICP-AES, 700 series, Agilent Technologies, Belgium). Blanks (only HNO_3) were included. The bioaccumulation factor (BAF) was calculated as follows Rafati *et al.* (2011), using the ratio of metal concentration in the plant roots (C roots) to the soil (C soil) to calculate the BAF roots and the ratio of total metal concentration in plant shoots (C stem + leaves) to the soil to calculate the BAF shoots.

$$BAF\ roots = \frac{C\ roots}{C\ soil}$$

$$BAF\ shoots = \frac{C\ stem + leaves}{C\ soil}$$

Rhizosphere bacterial diversity after inoculation

DNA extraction from the rhizosphere was performed by using the Invisorb Spin Plant Mini Kit (Stratec Biomedical AG, Berlin, Germany). From each pot, five subsamples (5 g of soil tightly adhered to the roots per subsample) were collected at a depth of 0–10 cm. The subsamples were mixed in the laboratory to obtain a composite sample. Internal transcribed spacer (ITS) regions between 16S rRNA and 23S rRNA were PCR amplified by using ITSF (5-GTCGTAACAAGGTAGCCGTA-3) and ITSReub (5-GCCAAGGCATCCACC-3) primers as previously described by Cardinale *et al.* (2004). After amplification, samples were loaded into Agilent DNA 1000 Chips and analyzed using the Agilent 2100 Bioanalyzer® (Agilent Technologies, Santa Clara, CA, USA). Expert Software (Agilent Technologies) was used to digitalize the ARISA fingerprints, resulting in electropherograms in ASCII formats that were processed using the StatFingerprints package (Michelland *et al.* 2009) in R version 3.4.3. Profiles obtained were compared to evaluate modifications on the rhizosphere microbial communities with the inoculation of specific consortia.

Uptake by and localization of trace elements in bacteria in liquid cultures

Batch studies were conducted using individual cultures of each bacteria, 50 ml Falcon tubes containing 25 ml GY rich medium (glucose: 10 g L^{-1}; yeast: 5 g L^{-1}; pH 7), supplemented with 10 mg L^{-1} of $CdCl_2$ and 100 mg L^{-1} of $Pb(NO_3)_2$. All tubes were inoculated with 1 ml of overnight grown bacterial cell culture (OD_{600}: 1), in triplicate. Samples were incubated at 28 °C and 100 rpm on an orbital shaker (Laboshake, Gerhardt, Germany). In order to determine the proportion of trace elements retained by bacterial cells, trace elements were measured in the supernatants and cell pellet after 72 h of incubation, by inductively coupled plasma atomic emission spectrometry (ICP-AES, 700 series, Agilent Technologies, Belgium). Cultures were first centrifuged 15 min at 4800 rpm. One aliquot of the supernatant was filtered (0.22 µm) and used for element determination. The pellet was washed 3 times with 0.01 M phosphate-buffered saline buffer (PBS, pH 7.0) to remove unbound element, sugars and proteins. Element determination by ICP (ICP-AES, 700 series, Agilent Technologies, Belgium) was done by digestion of the dry pellets with 70% HNO_3 in a heat block and finally dissolved in 5 ml of 2% HCl. Media with the element but not inoculated with bacteria were used as negative control.

For the transmission electron microscopy (TEM-EDX, Philips CM12, Amsterdam, Netherlands) analysis of the bacterial cells, cell pellets were washed in PBS and fixed in 2% glutaraldehyde made with PBS buffer for 1 h at 4 °C. Subsequently, bacterial samples were centrifuged for 3 min at 3000 × g and pellets were washed one time with PBS and two times with milli-Q water. The samples were dehydrated with ethanol/water mixtures, until absolute ethanol. Then 100% acetone was used. For the impregnation a mixture of acetone and resin 1:1 has been used. The polymerization was performed with a mixture of Epon/Araldite in silicone forms and left in the oven for 72 h at 60 °C. The sections of the cell pellets were cut using Leica FCS ultramicrotome (Leica Biosystems, Nussloch, Germany).

Statistical analysis

Statistical analysis was performed using R software (StatSoft, Tulsa, USA). Analysis of variance (ANOVA) followed by a Tukey post-hoc test were conducted in order to identify any significant effects of the introduced consortia on the plant biomass and physiological status. Non-parametric test was selected for further analyses because of the large heterogeneity of the variances. Non-parametric Kruskal-Wallis and Tukey test was used to determine the effects of the introduced consortia on the accumulation of trace elements. Differences between the non-inoculated plants and those inoculated with consortia at $p < 0.05$ were considered significant. For the pot experiments, data were represented as the mean ± standard deviation (SD) of six replicates.

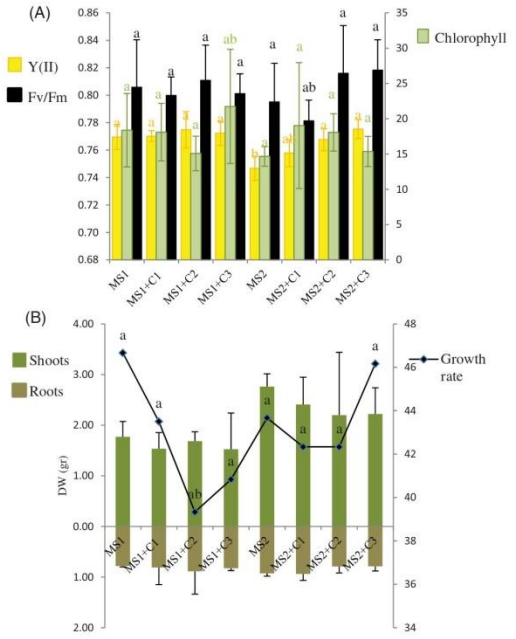

Figure 1. (A) Light adapted quantum yield (Y(II)), dark adapted maximum potential quantum efficiency (Fv/Fm) and total chlorophyll, and (B) dry weights and growth rate of H. annuus after a growth period of 60 days in MS1 and MS2 soils. Error bars are S.E. ($n = 6$). Values in the lines followed by the same letter and color are not significantly different at $p \leq 0.05$ by ANOVA and Tukey test.

Results

In the greenhouse, *H. annuus* seedlings were planted in pots filled with MS1 and MS2 and inoculated with consortia C1, C2 or C3. To assess the effects of the consortia on plant growth and health, photosynthetic activity was used as a proxy during the experiment. Results showed that neither the light adapted quantum yield (Y(II)), nor dark adapted maximum potential quantum efficiency (Fv/Fm) of inoculated *H. annuus* plants showed significant differences between the non-inoculated and inoculated plants on MS1 soil (Figure 1(A)). For MS2 soil, Y(II) was higher for *H. annuus* plants inoculated with C2 and C3 compared to the non-inoculated ones ($p \leq 0.05$). Lastly, when grown in MS1, an increase of the total chlorophyll content was observed in plants inoculated with the C3 compared to the non-inoculated ones, however the difference was not statistically significant (Figure 1(A)).

After 60 days, plants were harvested and biomass parameters were determined (Figure 1(B)). Growth rate was 25% lower when the plants were grown on MS1 and inoculated with the C2. There were no statistically significant differences in the dry weight (DW) of roots and shoots between inoculated and non-inoculated plants.

Pb was the element found in the highest concentration in the plant tissue of *H. annuus* followed by Zn, Cu and Cd (Table 2). However, a lack of normal distribution was observed principally with Cd and Pb accumulation. In some treatments the deviation obtained was greater than the concentration average of the element in the plant tissue. Plants grown in MS1 soil translocated Pb to the aerial parts, while those growing in MS2 soil, mainly retained this element in the roots. The opposite happed with Cd, plants grown in MS2 soil translocated Cd to the aerial parts, while those growing in MS1 soil, mainly retained this element in the roots. Consortium C1 significantly increased Cd and Zn concentration in roots of plants grown in the MS2. While C3 significantly decreased Cd concentration in roots of plants grown in the MS1 soil and Pb concentration in roots of plants grown in the MS2 soil. In shoots, only statistically significant differences were observed in the concentration of Pb. All the consortia tested decreased the Pb concentration in shoots in both military soils.

Based on the final nominal trace element concentrations in potting soil (supplementary Table S1) and the concentration measured after harvest in shoots and roots, the respective bioaccumulation factors (BAF) were calculated (Table 3). Cd was the most accumulated element by this plant species followed by Zn, Cu and Pb. After inoculation with the C2 consortium the shoot BAF of Pb decreased with 80% and even to concentrations below the detection limit when plants were grown in the MS1 and MS2 respectively. Also inoculation with C3 lowered the Pb shoot BAF in plants grown in the MS1 with about 85% and to concentrations below the detection limit in plants grown in the MS2.

Community-specific profiles obtained from the plant rhizospheres were compared to find out if the bioaugmentation with specific consortia modified the indigenous rhizosphere community profile. A complete linkage algorithm was used to perform a cluster analysis of Bray–Curtis dissimilarity matrices inferred from the fingerprints profiles. Due to the high sensitivity of the automated sequencer, the Bioanalyser software allowed us to detect between 12 and 28 peaks in the electropherograms (bands in the heatmaps) per profile. The structure of the profiles, characterized by the number and length distribution of major bands (peaks of highest relative fluorescence intensity), varied principally between soil type (MS1 and MS2) (Figures 2 and 3).

For *H. annuus* plants growing on the MS1 soil (Figure 2), inoculation shifted the rhizosphere community profile slightly. All rhizosphere samples showed two high bands, so inoculation did not disorder the more abundant taxa but the less abundant taxa (bands below these two upper bands) vanished when plants were bioaugmented with the consortia. There were no differences in the community profiles between the different inoculations.

Based on the heatmaps and clustering performed, the rhizosphere community profiles of *H. annuus* plants growing on the MS2 did not change substantially after bioaugmentation of any of the consortia (Figure 3). All rhizosphere samples taken showed two high bands, so inoculation did not disturb the more abundant rhizosphere taxa. These bands have the same length distribution as the ones that appeared on the MS1 soil (Figure 2). However, the less abundant taxa were specific for each sample (individual

Table 2. Concentrations (mg kg^{-1}) of Cd, Cu, Pb and Zn in roots and shoots of *H. annuus* seedlings inoculated with C1, C2 and C3, growing in two military polluted soils (MS1 and MS2).

	Roots				Shoots			
	Cd	Cu	Pb	Zn	Cd	Cu	Pb	Zn
MS1	0.19 ± 0.14^{a}	0.49 ± 0.16^{a}	8.06 ± 3.51^{a}	1.43 ± 0.34^{a}	nd	0.72 ± 0.14^{a}	11.63 ± 6.89^{b}	3.68 ± 0.81^{a}
MS1 + C1	0.19 ± 0.10^{a}	0.56 ± 0.40^{a}	15.32 ± 13.12^{a}	1.68 ± 0.38^{a}	nd	0.89 ± 0.18^{a}	2.87 ± 2.99^{ab}	4.98 ± 1.33^{a}
MS1 + C2	0.21 ± 0.12^{a}	0.36 ± 0.19^{a}	14.49 ± 17.91^{a}	1.77 ± 0.64^{a}	nd	0.78 ± 0.11^{a}	2.01 ± 1.37^{a}	3.89 ± 0.81^{a}
MS1 + C3	nd	0.27 ± 0.17^{a}	7.01 ± 3.55^{a}	1.59 ± 0.20^{a}	nd	0.75 ± 0.08^{a}	1.03 ± 0.64^{a}	4.17 ± 0.74^{a}
MS2	0.21 ± 0.08^{a}	0.27 ± 0.15^{a}	6.95 ± 2.49^{a}	3.52 ± 0.48^{a}	0.16 ± 0.17^{a}	0.56 ± 0.08^{a}	0.89 ± 0.33	5.05 ± 1.41^{a}
MS2 + C1	0.49 ± 0.08^{b}	0.44 ± 0.23^{b}	6.45 ± 3.30^{a}	4.96 ± 1.60^{b}	0.35 ± 0.43^{a}	0.74 ± 0.25^{a}	nd	7.59 ± 4.09^{a}
MS2 + C2	0.35 ± 0.14^{a}	0.24 ± 0.13^{a}	3.93 ± 2.42^{a}	3.61 ± 0.77^{a}	0.56 ± 0.62^{a}	0.58 ± 0.25^{a}	nd	6.41 ± 2.78^{a}
MS2 + C3	0.18 ± 0.04^{a}	0.16 ± 0.10^{a}	nd	2.42 ± 0.71^{a}	0.30 ± 0.29^{a}	0.58 ± 0.12^{a}	nd	4.94 ± 1.65^{a}

Values are mean ± S.E. ($n = 6$); values in a column followed by the same letter(s) are not significantly different at $p \leq 0.05$ by Kruskal-Wallis and Tukey test; nd: not detected.
Limits of detection (LD) Cd = 0.012 mg Kg^{-1}, Cu = 0.006 mg Kg^{-1}; Pb = 0.054 mg Kg^{-1}; Zn = 0.16 mg Kg^{-1}.

Table 3. Bio-Accumulation Factors (BAF) of Cd, Cu, Pb and Zn in roots and shoots of *H. annuus* seedlings inoculated with C1, C2 and C3, growing in two military polluted soils (MS1 and MS2).

	BAF roots				BAF shoots			
	Cd	Cu	Pb	Zn	Cd	Cu	Pb	Zn
MS1	0.045 ± 0.03^{a}	0.002 ± 0.0006^{a}	0.003 ± 0.001^{a}	0.007 ± 0.002^{a}	–	0.003 ± 0.001^{a}	0.005 ± 0.003^{b}	0.017 ± 0.004^{a}
MS1 + C1	0.045 ± 0.02^{a}	0.002 ± 0.0002^{a}	0.006 ± 0.005^{a}	0.008 ± 0.002^{a}	–	0.003 ± 0.001^{a}	0.001 ± 0.002^{ab}	0.023 ± 0.006^{a}
MS1 + C2	0.05 ± 0.03^{a}	0.001 ± 0.0007^{a}	0.006 ± 0.007^{a}	0.008 ± 0.003^{a}	–	0.003 ± 0.000^{a}	0.001 ± 0.000^{a}	0.018 ± 0.003^{a}
MS1 + C3	–	0.001 ± 0.0006^{a}	0.003 ± 0.001^{a}	0.007 ± 0.001^{a}	–	0.003 ± 0.000^{a}	0.000 ± 0.000^{a}	0.019 ± 0.003^{a}
MS2	0.21 ± 0.08^{a}	0.001 ± 0.0005^{a}	0.04 ± 0.015^{a}	0.006 ± 0.001^{a}	0.16 ± 0.17^{a}	0.002 ± 0.000^{a}	0.005 ± 0.002	0.009 ± 0.002^{a}
MS2 + C1	0.49 ± 0.08^{b}	0.001 ± 0.0007^{a}	0.04 ± 0.019^{a}	0.009 ± 0.002^{b}	0.35 ± 0.43^{a}	0.002 ± 0.001^{a}	–	0.014 ± 0.007^{a}
MS2 + C2	0.35 ± 0.14^{a}	0.001 ± 0.0004^{a}	0.02 ± 0.014^{a}	0.006 ± 0.001^{a}	0.56 ± 0.62^{a}	0.002 ± 0.001^{a}	–	0.012 ± 0.005^{a}
MS2 + C3	0.18 ± 0.04^{a}	0.0005 ± 0.0003^{a}	–	0.005 ± 0.001^{a}	0.30 ± 0.29^{a}	0.002 ± 0.000^{a}	–	0.009 ± 0.003^{a}

Values are mean ± S.E. ($n = 6$); values in a column followed by the same letter(s) are not significantly different at $p \leq 0.05$ by Kruskal-Wallis and Tukey test.

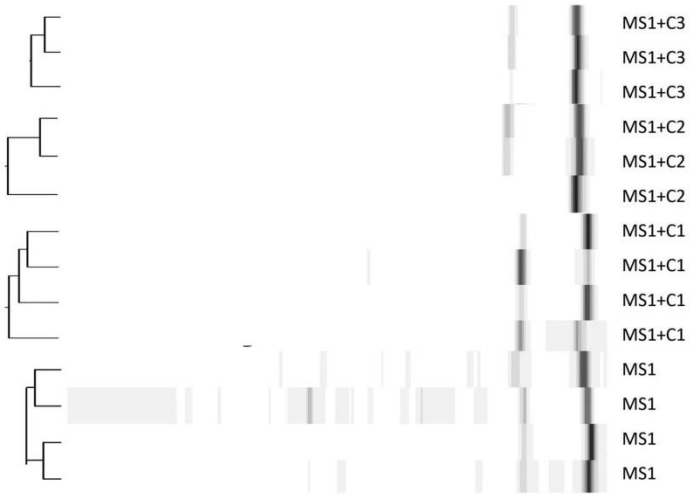

Figure 2. Heatmaps of rhizosphere bacterial communities of *H. annuus* plants growing on MS1, comparison between plants inoculated with specific consortia and non-inoculated plants. A complete linkage algorithm was used to perform a cluster analysis of Bray-Curtis dissimilarity matrices inferred from ARISA values.

plant) even between those plants that were inoculated with the same consortium.

The Shannon diversity index was higher in the rhizosphere of non-inoculated plants that were growing in the MS1 than those growing in the MS2 (Table 4). Bioaugmentation with the consortia decreased the diversity of the rhizosphere of plants growing on the MS1. On the contrary, a higher diversity index was observed when plants were growing on the MS2, mainly when they were inoculated with the C1 and C3 consortia.

Uptake by and localization of trace elements in individual bacteria in liquid cultures was analyzed *in vitro*. After 48 h of incubation, the pH of the medium was clearly lower. As expected, the solubility of Pb and its concentration in the supernatant increased correspondingly to this decrease of the pH (Table 4). *Bacillus proteolyticus* ST89 was the strain

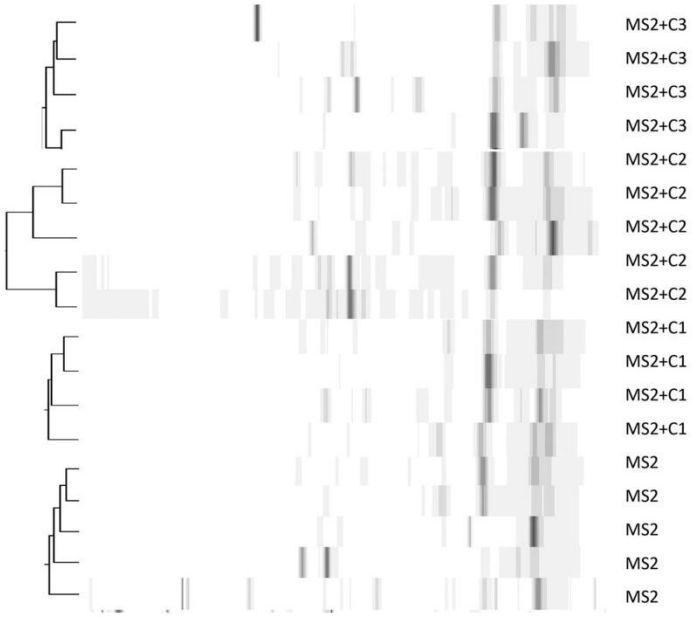

Figure 3. Heatmaps of rhizospheric bacterial communities of *H. annuus* plants growing on MS2, comparison between plants inoculated with specific consortia and control plants not inoculated. A complete linkage algorithm was used to perform a cluster analysis of Bray-Curtis dissimilarity matrices inferred from ARISA values.

Table 4. Diversity index calculated from ARISA fingerprints of *H. annuus* plants inoculated with C1, 2 and 3 and non-inoculated (MS1 and MS2).

Samples	Peaks	Shannon	Samples	Peaks	Shannon
MS1	23 ± 3.2^{a}	0.12 ± 0.02^{a}	MS2	25 ± 3.1^{a}	0.10 ± 0.01^{a}
MS1 + C1	12 ± 1.4^{b}	0.09 ± 0.01^{b}	MS2 + C1	28 ± 4.0^{a}	0.16 ± 0.02^{b}
MS1 + C2	12 ± 1.4^{b}	0.08 ± 0.01^{b}	MS2 + C2	17 ± 2.3^{b}	0.11 ± 0.01^{a}
MS1 + C3	13 ± 1.5^{b}	0.07 ± 0.02^{b}	MS2 + C3	24 ± 2.5^{a}	0.17 ± 0.02^{b}

Values are mean ± S.E. ($n = 6$); Values in a column followed by the same letter(s) are not significantly different at $p \leq 0.05$ by ANOVA and Tukey test.

that retained the most Pb in the bacterial pellet, followed by *Bacillus paramycoides* ST9, four times more than the other strains ($p < 0.05$). *Methylobacterium* ST85, the only Cd tolerant strain, increased the pH of the medium and did not retain Cd in the bacterial cell pellet.

The TEM images of bacteria cultures (Figure 4) allow to observe the (sub)cellular localization of Pb and Cd. *Bacillus wiedmanni* ST29 (Figure 4(d)) and *Bacillus proteolyticus* ST89 (Figure 4(c)) were fixating Pb at their cell wall surface, which was also observed in *Bacillus paramycoides* ST9 (Figure 4(a)) in a lesser extent. Internalization of Pb into the cell was observed by *Bacillus wiedmanni* ST29 (Figure 4(d)) and *Bacillus paramycoides* ST9 (Figure 4(a)) cultures. *Cellulosimicrobium* ST54 (Figure 4(b)), retains a significant concentration of Pb outside the cells. While in *Methylobacterium* ST85, the only strain tolerant to Cd (Figure 4(e)), no Cd was detected neither inside nor attached to the cell wall. The morphology of *Brevibacterium frigoritolerans* ST28 made it impossible to visualize individual cells. In Supplementary Figure S1 the EDX spectra are presented.

Discussion

Studies on sunflower (*Helianthus annuus*) have revealed that several elements, including Pb, Cd, Cu, Zn and Co, can accumulate at high concentrations in roots and shoots (Alaboudi *et al.* 2018; Govarthanan *et al.* 2018) inhibiting plant growth and lowering biomass production, grain yield, and crop quality (Ahmad *et al.* 2011; Ramzani *et al.* 2016). Our findings agree with these reports, *H. annuus* was able to accumulate Cd, Zn, Cu and Pb (Table 2). Lee *et al.* (2013) and Forte and Mutiti (2017) reported that *H. annuus* is less efficient for taking up Pb in its tissues compared to other elements. The large fluctuations of trace element content that we observed specifically for Cd and Pb (Table 2) were also observed in other plants species and reported by Duman and Obali (2008) and Polechońska *et al.* (2017).

Few attempts have been made to use PGPB consortia to reduce trace element mobility controlling the uptake and translocation of these elements into the crops (Khanna *et al.* 2019; Ren *et al.* 2019). The consortia proposed and tested in our study showed different efficiencies depending on the soil type on which they were applied. The MS1 soil contained more organic matter and had a higher total Pb concentration than the MS2 soil (Table 1). The differences found between the effects of the consortia generated in the different types of soil may be due to MO and the content of total trace element in each soil. Inoculation of consortium C2 and C3 decreased significantly the Pb shoot BAF when plants were grown in the MS1 and MS2 (Table 3). However, growth and physiological status of the plants inoculated

were not significant different from those non-inoculated (Figure 1). In previous studies (Saran *et al.* 2020), *B. proteolyticus* ST89 inoculated alone managed to reduce Pb bioaccumilation and increase the biomass develop of *H. annuus* plants. Also in other crops, like rice, a decreased As-toxicity was reported after inoculation of a synthetic consortium of plant growth-promoting rhizobacteria (*Pseudomonas putida*) and algae (*Chlorella vulgaris*) (Awasthi *et al.* 2018). Also some Pb-tolerant bacteria were found to play specific roles in the growth of Pb-exposed plants. For example, the endophyte *Bacillus* sp. MN3-4 increased Pb (II) accumulation in *Alnus firma*, and *Pseudomonas fluorescens* G10 and *Mycobacterium* sp. G16 promoted growth and reduced Pb toxicity in *Brassica napus* (Shen *et al.* 2008; Shin *et al.* 2012).

An important factor influencing the success of bioaugmentation is the interaction between the inoculated strain(s) and the autochthonous microflora (Pacwa-Płociniczak *et al.* 2016). In our study, the inoculation did not disturb considerably the natural microbial profiles of the plants (Figures 2 and 3). What could have influenced in the non-promotion on the plant growth (Figure 1(b)) and in little or no differences in root element concentrations (Table 2). The more abundant taxa did not change after bioaugmentation of specific consortia. A modification of the bacterial community structure caused by inoculation could be buffered by ecosystem resilience, which is driven by the level of diversity and interactions of the plant-soil-biota (Trabelsi and Mhamdi 2013). Several studies describe the impact of inoculation on soil microbial communities (Salamone *et al.* 2010; Schumpp and Deakin 2010). Some of them show qualitative and quantitative effects of the inoculation on the rhizosphere bacterial diversity (Schwieger and Tebbe 2000). Others, however, like Lerner *et al.* 2006 reported no obvious effects on the bacterial communities of maize in two different soils and in different growth systems after inoculation with *Azospirillum brasilense*.

In *in vitro* batch studies, *Cellulosimicrobium cellulans* ST54 and *Bacillus paramycoides* ST9 acidified the medium in liquid culture, which directly influenced element solubility (Table 5). These strains were also identified as organic acid producers (Table 5). Mehta and Nautiyal (2001) relate the solubilization of inorganic insoluble phosphate by microorganisms to the production of organic acids and chelating oxoacids from sugars. This capability can also influence the bioavailability of metals in the soil. Siderophore production and biofilms production were other traits found for strains that modify element bioavailability like *Bacillus wiedmannii* ST29 and *Bacillus proteolyticus* ST89.

Using TEM-EDX we could localize the (sub)cellular element localization in the selected strains. *B. wiedmanni* ST29, *B. proteolyticus* ST89 and *B. paramycoides* ST9 were able to retain Pb at their cell wall surface (Figure 4). Cabuk *et al.* (2006) reported that hydroxyl and carboxyl groups from the cell wall, as well as nitrogen-based bio-ligands including amide and sulfonamide, can be involved in the binding of Pb on the cell surface of *Bacillus* sp. ATS. Other possible Pb binding complexes are siderophores (Naik and Dubey 2011). Apart from iron, siderophores may interact with other elements outside the cell. They have been associated with Pb complexation by *Pseudomonas aeruginosa* 4EA and by the endophyte *P. putida* KNP9, isolated from mung bean (*Phaseolus vulgaris*) (Tripathi *et al.* 2005).

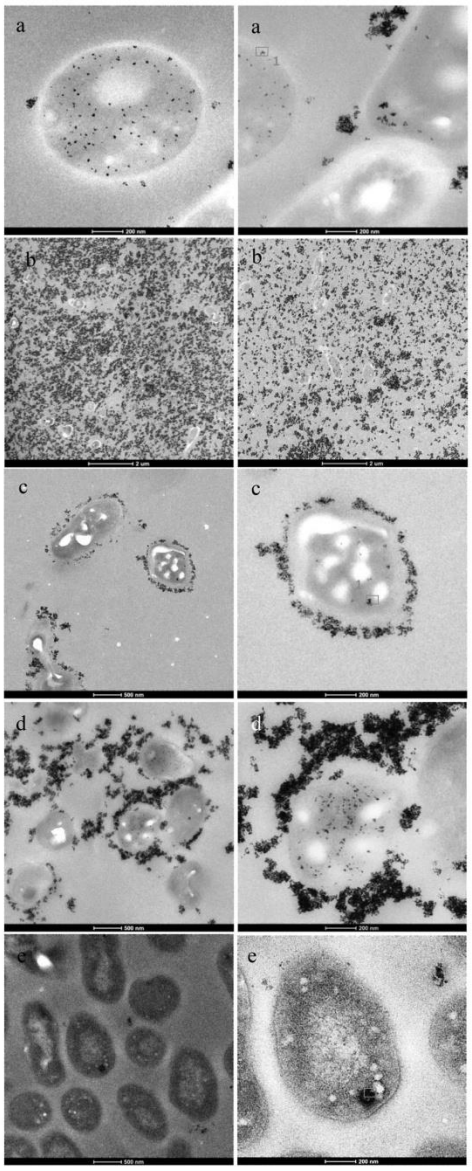

Figure 4. TEM images of bacterial strains exposed to Pb (a) *Bacillus paramicoides* ST9 (b) *Cellulosimicrobium* ST54 (c) *Bacillus proteolyticus* ST89 (d) *Bacillus wiedmanni* ST29 and Cd (e) *Methylobacterium* ST85. Numbers in blue color follow by squares represent the specific locations where EDX-spectra were taken (see also Supplementary figure S1).

Table 5. *In vitro* uptake of Pb and Cd by bacteria in liquid cultures and their plant growth-promoting properties described by Saran *et al.*, 2020.

Strain	pH	Pb		Plant growth-promoting properties (Saran et al. 2020)					
		Supernatants (mg l^{-1})	Pellet (mg kg^{-1})	ACCD	Acetoin	[a]OA	[b]P-Solub.	[c]SID	[d]Biofilm
Cellulosimicrobium cellulans ST54	4.89 ± 0.2^{a}	18.82 ± 2.13^{a}	55.85 ± 4.78^{c}	+	+	+	0.10 ± 0.01^{c}	0.00 ± 0.00^{c}	0.07 ± 0.02^{a}
Bacillus paramycoides ST9	4.34 ± 0.15^{a}	16.57 ± 2.98^{a}	159.17 ± 6.45^{ab}	+	+	+	0.19 ± 0.01^{b}	0.16 ± 0.01^{b}	0.02 ± 0.00^{b}
Bacillus wiedmannii ST29	5.75 ± 0.40^{ab}	11.36 ± 2.02^{b}	67.65 ± 4.97^{c}	+	+	+	0.05 ± 0.01^{cd}	0.53 ± 0.04^{a}	0.01 ± 0.0^{0b}
Brevibacterium frigotolerans ST30	6.11 ± 0.22^{b}	$14{,}58 \pm 2.24^{b}$	39.26 ± 4.15^{d}	−	+	+	0.37 ± 0.02^{a}	0.00 ± 0.00^{c}	0.02 ± 0.00^{b}
Bacillus proteolyticus ST89	6.04 ± 0.17^{b}	13.15 ± 2.09^{b}	184.17 ± 12.42^{a}	+	+	+	0.03 ± 0.00^{d}	0.38 ± 0.03^{ab}	0.13 ± 0.02^{a}
Abiotic control	5.75 ± 0.25^{ab}	0.49 ± 0.08^{c}	0.08 ± 0.03^{f}						
	Cd								
Methylobacterium ST85	8.38 ± 0.32^{b}	6.08 ± 1.67^{a}	0.22 ± 0.09^{a}	+	+	+	0.20 ± 0.01^{b}	0.00 ± 0.00^{c}	0.01 ± 0.00^{b}
Abiotic control	6.6 ± 0.03^{a}	8.27 ± 0.12^{ab}	$0{,}20 \pm 0.18^{a}$						

Values are mean ± S.E. ($n = 3$); values in a column followed by the same letter(s) are not significantly different at $p \leq 0.05$ by ANOVA and Tukey test.
[a]Organic acid; [b]solubilized area (cm^2); [c]µg IAA ml^{-1} of medium; [d]Measured by optical density (OD600); + positive; − negative. Abbreviations: ACCD: 1-aminocyclopropane-1-carboxylate deaminase; OA: organic acids; P-solub: P solubilization; SID: siderophore production.

In Table 5, B. *proteolyticus* ST89 was reported as a siderophore producer. The highest accumulation of Pb was found outside *Cellulosimicrobium* ST54 cells (Figure 4(b)), reported as a biofilm producer in Table 5. Lead binding by extracellular polymers has been reported for *Bacillus firmus*, *Pseudomonas* sp. (Salehizadeh and Shojaosadati 2003), *Cyanobacteria* (Paperi *et al.* 2006) and *Halomonas* sp. (Amoozegar *et al.* 2012). In this case, extracellular polymers produced by the latter strain were highly Pb specific, and adsorbed ten times more Pb than other elements such as Cd, Co, Ni, Zn and Cu (Aguilera *et al.* 2008). Pb can also enter bacterial cells through the transporters used for essential divalent elements such as Mn(II) (Laddaga *et al.* 1985) and Zn(II) (Grass *et al.* 2002). Absorption of Pb was observed for *B. wiedmanni* ST29 and *B. paramicoides* ST9 (Figure 4). These are some capabilities through which consortium C2 and C3 could be retaining Pb and avoiding its translocation to the shoots tissue (Table 3).

Further research is necessary to elucidate the localization of these strains in the soil matrix, as well as the specific mechanisms or genetic pathways involved in the bacteria-plant deliver of trace element and chemicals to understand the direct influence on improved plant nutrition and growth.

Conclusion

Inoculation by consortia C2 and C3 reduced Pb shoot bioaccumulation in *H. annuus* plants growing on two military soils polluted with different concentrations of trace elements and possessing different physiochemical properties. This could be attributed to element internalization and adsorption at the bacterial cell wall found by electron microscopy images of *in vitro* cultures of these strains. However, biomass develop and plant physiology were not promote by the inoculation of any consortia tested. As well as the soil bacterial community structure was not significantly altered. Consequently, bioaugmentation with the appropriate PGP and trace element tolerant consortia can affect trace element uptake by plants. However, further studies are necessary to understand the bacteria-plant deliver of trace element as well as the plant physiology response, to decrease the risk of entrance of these elements into the human food chain.

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Statement of novelty

The presence of trace elements such as Cd, Cu, Pb and Zn in the topsoil of post-military soils negatively affects soil and water resources and decreas biodiversity, imposing substantial threats to human health.

Our results indicate that bioaugmentation with specific PGP and trace-element tolerant bacteria consortia can be a promising approach to revalue these areas or other soils moderately polluted with trace elements, by reducing the risks of entrance of Pbinto the food chain.

Supplemental online material

In this section you can find Supplementary Table S1- Nominal concentration of the elements in the soil after the dilution performed on the pots and Supplementary Figure S1- EDX spectrums of bacterial strains exposed to Pb (a) *Bacillus paramicoides* ST9 (b) *Cellulosimicrobium* ST54 (c) *Bacillus proteolyticus* ST89 (d) *Bacillus wiedmanni* ST29 and Cd (e) *Methylobacterium* ST85.

ORCID

A. Saran http://orcid.org/0000-0002-4008-7759
L. J. Merini http://orcid.org/0000-0002-2533-8601

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Instituciones participantes

Secretaría Técnica Administrativa

Con el apoyo de:

www.fontagro.org
Correo electrónico: fontagro@fontagro.org