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A native Zn-solubilizing bacteria from mine soil promotes plant growth and facilitates phytoremediation

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Abstract

Purpose An important bottleneck in phytoremediation technologies, which focus on extraction of potentially toxic trace elements, is the low solubility and bioavailability of metals, a handicap that worsens over time. To overcome this barrier, we explored the possibility of using Zn-solubilizing bacteria with plant growth-promoting characteristics.

Material and methods Zn-tolerant soil bacteria were isolated from an ancient Zn-Pb mine using cultivation media with a high ZnSO₄ concentration (2.5 mM). Among the bacteria able to grow on this medium, those with plant growth-promoting (PGP) characteristics (P and Zn solubilisation and indole-3-acetic acid production) were selected. The best candidates (seven strains) were identified after 16S rDNA sequencing and *Pseudomonas fluorescens* NCBI Accession No. MT218317 was selected for a first microbial-assisted phytoremediation experiment based on its ability to solubilize ZnO. For this purpose, *Sinapis alba* plants growing on potted mine spoil soil were inoculated with the PGP isolate.

Results and discussion Fifteen days after inoculation, both shoot and root growth were higher in inoculated than in un-inoculated white mustard plants. Soil metal contents (total and exchangeable fractions) remained practically invariable. In contrast, the PGP bacteria raised the plant concentrations of some of the micronutrients, together with Cd and Pb. In addition, the bioaccumulation factors for Cd and Zn were higher in the bacteria-inoculated plants.

Conclusions The artificial inoculation of *P. fluorescens* MT218317 promoted the biomass of *S. alba* plants potentiating the phytoextraction of Zn and phytostabilisation of Cd and Pb on a metal-contaminated soil. The novelty of the study is the use of metal-solubilizing microorganism in combination with metal-tolerant plants for improving the restoration of soils containing potentially toxic elements.

Keywords

Plant growth-promoting bacteria, mine soil, *Sinapis alba*, bioavailability, Zn solubilisation

1 INTRODUCTION

During the last century, anthropogenic activities such as industry, mining, and agriculture have increased the toxic elements present in terrestrial, atmospheric, and aquatic ecosystems (Guarino and Sciarrillo 2017). Use of polluted zones is severely limited unless these toxins are removed or inactivated. The use of plants for soil restoration has become one of the best alternatives as it is a low-cost and environmentally friendly practice (Vangronsveld et al. 2009; Jeevanantham et al. 2019).

The success of any phytoremediation technique is based on the bioavailability of metals. Unfortunately, the efficiency of trace element phytoremediation decreases over time due to a decrease in bioavailability (Mench et al. 2009). Soil properties such as pH, total organic carbon, and cation exchange capacity influence the solubility of metals (Basta et al., 2005). Moreover, the extraction efficiency of metal hyperaccumulating plant species is rather limited because of their low biomass, slow growth, and small root system (Kidd et al. 2009). Therefore, metal tolerant species with high biomass are promising candidates for phytoremediation (Rajkumar and Freitas 2008; Bhargava et al. 2012). One such species is *Sinapis alba* (from the Brassicaceae family), an annual plant that can survive under high metal concentrations and shows fast growth in comparison with hyperaccumulating Brassicaceae like *Noccaea caerulescens* (Tolrà et al., 1996). The metal tolerance of *S. alba* for Cd, Cu, Pb, Se, and Zn was confirmed by Fargasova (2004) and its phytoremediator potential was previously mentioned by Evangelou (2007) and Zalewska and Nogalska (2014).

In the last few decades, the use of microbial assisted phytoremediation has been explored to facilitate the trace element bioavailability of soils, ultimately improving phytoremediation efficiency (Pilon-Smits and Freema 2006; de-Bashan et al. 2012; Pinto et al. 2018; He et al. 2020). Several studies have demonstrated that plant-associated microorganisms can contribute to the uptake of potentially hazardous elements by plants (Whiting et al. 2001; Chen et al. 2005; Sheng and Xia 2006; Wang et al. 2007; Dell'Amico et al. 2008; Gopalakrishnan et al. 2015). The plant-microbe interaction in hazardous environments is a win-win strategy for both actors. Root exudates provide a nutrient-rich environment that stimulates microbial activity (de-Bashan et al. 2012), while rhizospheric bacteria can increase metal bioavailability through changes in soil pH, production of chelating compounds, and modification of redox potential (Kidd et al. 2009). Additionally, bacteria can directly enhance the phytoremediation ability of plants by promoting

their growth via hormone synthesis, enzyme secretion, siderophore production, and solubilisation of essential macro- and micronutrients (Glick 2010; Sun et al. 2020). Indeed, bacterial strains that increase metal phytoremediation are characterised as plant growth-promoting bacteria (PGPB) (Ma et al. 2011; Miransari 2011; Marques et al. 2013).

The inoculation of plants with PGPB to improve their phytoremediation efficiency has produced contrasting results: some assays of bacterial inoculation reported a reduction in metal uptake by plants (Chen et al. 2005; Wang et al. 2007; Becerra-Castro et al. 2012; Ma et al. 2015), while others found higher uptake (Plociniczak et al. 2013; Moreira et al. 2014; Ali et al. 2017). Regardless of the strategy, all studies confirmed an increased biomass of inoculated plants. Zinc-solubilizing bacteria have generally been used to improve the productivity and biofortification of crops or alleviate Zn deficiency in soils (Singh et al. 2017; Dinesh et al. 2018; Rehman et al. 2018; Hussain et al. 2020). Enhancing the solubility of metals using microorganisms has been scarcely explored although some studies have pointed out the possibilities of this approach (Saravanan et al. 2007; Li et al. 2010; Mishra et al. 2019).

An adequate selection of bacterial strains is critical for the success of microbial-assisted phytoremediation. Microbes that are both naturally adapted to high metal concentrations and able to mobilize metals in bioavailable but less toxic forms are especially attractive for phytoremediation purposes. The ability to mobilize metals in a plant bioavailable form should be combined with metal tolerance and plant growth-promoting characteristics. In this study, we applied these selection criteria with a focus on zinc (Zn), and isolated seven different bacterial strains from a mining site. After genotypic and phenotypic characterisation, one *Pseudomonas fluorescens* strain was selected as the best candidate for a first microbial-assisted phytoremediation experiment using *Sinapis alba*. The novelty of our work is the use of a Zn-solubilizing bacterium to enhance phytoremediation from metal-rich mining soils contaminated by multiple metals.

2 MATERIALS AND METHODS

2.1 Study area

Soil samples were collected around the “Nadal” mine (*Mina d’en Nadal*), also called Can Vergeli Mine (*Mina de Can Vergeli*) (41.895122°, 3.0199100°), 293 m altitude, in the northeast of Catalonia, Spain. The mine is located in the Gavarres Range (Empordà region), which is part of the Catalan Coastal Range. The Gavarres mountains consist of Palaeozoic sedimentary rocks metamorphosed to varying degrees in the late stage (Folch 1992), with intrusions of granite and volcanic acid rock from the Ordovician (Roque and Palli 1994). The geological cross section is completed by lamprophyre rock intrusions across the whole series. These latter rocks are associated with the granite, intruding into existing rocks during the Jurassic.

The whole mine is situated in the oldest Cambrian-Ordovician materials and consists of two units. The first is sandstone with fine and coarse granulometry with alternating white massive quartzites. The second unit constitutes sandstone and lutite, with no fossil record. The whole series presents several metamorphic features. The mined deposits of Zn and Pb are from the lamprophyre rocks that intruded into vertical hydrothermal veins of quartz also containing calcite, barytine, galena, sphalerite, and chalcopyrite (Institut Cartogràfic de Catalunya, Geological map of Palamós 334-2-2).

2.2 Soil samples

Soil samples were collected at different distances (0, 0.5, and 1 m) from the entrance to the mine, in triplicate. Bulk soil samples of 20 cm soil depth, discarding the first 5 cm of surface soil, were directly collected with a sterile soil sampler in plastic bags. Soils were analysed for nutrient content (adapted from Soltanpour and Schwab 1977), pH (Peech 1965; Conyers and Davey 1988), carbonate content (Loeppert et al. 1984), organic matter (Black et al. 1965), water-holding capacity, and soil texture (Reynolds and Topp. 2008). The concentration of metals (Cd, Co, Cu, Fe, Pb, and Zn) and As were measured by inductively coupled plasma optical emission spectrometry (ICP-OES) (model Polyscan 61E; Thermo Jarrell-Ash, Waltham, MA, USA) or by inductively coupled plasma mass spectrometry (ICP-MS) (model Elan 6000; Perkin Elmer-Sciex, Thornhill, Canada) (Bech et al. 2012) after *aqua regia* extraction. Table 1 shows the soil parameters.

For bacterial extraction from the bulk soil, the method of Barillot et al. (2013) was used. To homogenize the amount of each soil, 100 g of bulk soil were added to 1 L of a sterile 0.9% NaCl solution. The soil

suspension was incubated to homogenize the bacterial content in an orbital shaker (300 x g, 90 min, 25 °C) and then centrifuged (200 g, 25 °C, 10 min) in 100 mL sterile tubes to discard sediment soil particles. The supernatant was subsequently roughly filtered through a 1 mm sieve to remove residuals in the suspension. For assessment of the bacterial density of each soil type, 1 mL of the supernatant was diluted in a sterile NaCl 0.9% solution until 10^{-5} . One hundred microlitres of each dilution were spread over the media by surface-scratching in triplicate on Luria Broth (LB)-agar Petri-dishes (90 mm), supplemented with cycloheximide (100 mg/L). The number of colony-forming units (CFU) was determined after six days of incubating the dishes at 25 °C. Plates showing fungal contamination were discarded.

2.3 Isolation of zinc tolerant bacteria

Zinc tolerance was our first selection criterion. For this purpose, the number of CFUs and the growth of bacteria were quantified on normal LB plates and the same medium supplemented with 2.5 mM of soluble Zn (ZnSO_4). To confirm the tolerance to high concentration of Zn, isolates were grown on Tris-minimal medium supplemented with 1, 2.5 and 5 mM of ZnSO_4 . Strains able to grow and develop on the high Zn media (LB and Tris-minimal) were selected for further study; strains were also selected based on morphological differences in form, height, and margin type.

2.4 Bacterial characterisation

2.4.1 Zinc solubilisation

The ability to solubilise Zn from insoluble sources was our second selection criterion. The capacity of the selected Zn-tolerant strains to solubilise ZnO was investigated using the method described by Fasim et al. (2002). Tris-minimal medium was amended with zinc oxide (ZnO) at a concentration equivalent to 0.1% Zn. Freshly grown bacterial liquid cultures were spot inoculated on a plate using sterile toothpicks. Spotted plates were incubated at 28 °C in the dark and the diameters of the halo around the colonies and colonies were measured after seven days. Each isolate was inoculated in triplicate in three sets of experiments. Zinc solubilisation efficiency (SE) was determined according to Nguyen et al. (1992) as $\text{SE} = (\text{diameter of solubilisation halo} / \text{diameter of the colony}) \times 100$.

2.4.2 Phosphate solubilisation

The capacity to solubilise phosphate was assessed using Pikovskaya's (PKV) agar medium (Pikovskaya 1948) containing 0.1% glucose, 0.05% yeast extract, 16 mM $\text{Ca}_3(\text{PO}_4)_2$, 3.8 mM $(\text{NH}_4)_2\text{SO}_4$, 3.4 mM NaCl, 2.7 mM KCl, 0.83 mM MgSO_4 , 0.6 mM MnSO_4 , 0.6 mM FeSO_4 , and 1.5% agar. The pH was adjusted to 7.0 ± 0.2 before sterilization. Finally, the rate of phosphate solubilisation for each strain was calculated by dividing the maximum diameter of the solubilisation halo by the maximum diameter of the colony. This rate was defined as the phosphate solubilisation efficiency.

2.4.3 Siderophore production

The capacity to chelate iron compounds was assessed using the methods of Schwyn and Neilands (1987). In brief, CAS-blue agar was prepared using Chrome Azurol S (CAS) dissolved in distilled water, and mixed with iron (III) solution (1 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 10 mM HCl). Hexadecyltrimethylammonium bromide (HDTMA) was slowly added to the previous solution, while being stirred, to a final concentration of 73 mg/L. The resultant dark blue liquid was autoclaved adding agar (1.5%) and 0.1 M piperazine-N,N'-bis(2-ethanesulfonic acid) (pH 6.8 ± 0.2 adjusted before sterilization). Ten microlitres of freshly grown bacterial liquid culture was spotted on the plates. The siderophore levels produced by the isolates were measured as the diameter of the orange halo produced by the colony after one week, optimizing the pH of the plate and the temperature for each colony. Each isolate was inoculated in duplicate in three sets of experiments. The capacity was calculated by dividing the diameter of the halo by the diameter of the colony.

2.4.4 IAA production

Auxin (indole-3-acetic acid, IAA) production was quantified according to the method originally proposed by Bric et al. (1991). The bacterial isolates were grown in tubes with a Luria-Bertani (LB) culture medium supplemented with 0.1% Bacto™ Tryptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% L-tryptophan (pH 7.5 ± 0.2). The vials containing the bacterial isolates were incubated at 28 °C for 24 h on a shaker (150 x g). Next, 1.5 mL of the liquid culture was transferred to microtubes, centrifuged at $9,500 \times g$ for 2 min, and 100 μL of the supernatant was transferred to microplate wells together with 100 μL of Salkowski reagent containing 0.5 M FeCl_3 distilled water and concentrated H_2SO_4 at a ratio of 1:50:30 (V/V/V). Absorbance was measured at 530 nm, using a microplate reader Cary 50 (Varian, Walnut Creek, CA, USA), 30 seconds after addition of the Salkowski reagent. A commercial IAA (Sigma-Aldrich, Saint Louis, MO, USA) was used to obtain the standard curve equation. The development of a reddish pink

colour in the samples indicates the production of auxin. The test was completed in triplicate for each isolate.

2.4.5 Other parameters

Other analysed parameters of the selected isolates were Cd tolerance using 200 μ M CdCl₂ plates, capacity to produce fluorescence under UV light, optimum temperature between 15 and 39 °C, and ability to grow from pH 4 to 9.

2.4.6 Genetic characterisation (16S rDNA sequencing)

The 2.5 mM ZnSO₄ tolerant isolates with interesting PGP traits were genetically characterised. The bacterial DNA was extracted from an axenic culture grown on LB agar medium using the FastDNATM SPIN kit (MP Biomedicals, Irvine, CA, USA). The universal primers 27FW (5'- AGAGTTTGATCCTGG CTCAG-3') and 1492R (5'-GTTACCTTGTTACGACTT-3') were used to amplify the 16S rRNA encoding gene. A 50 μ L PCR reaction mixture was prepared containing 100–200 ng of extracted DNA, 1 $\times$ TopTaq buffer, 200 μ M of each dNTP, 200 pM of each primer, and 1.25 U of TopTaq polymerase (Qiagen, Hilden, Germany). After initial denaturation at 94°C for 5 min, each cycle consisted of 1 min denaturation at 94 °C, 1 min annealing at 52 °C, and 1.5 min elongation at 72 °C. After 35 cycles, a final extension step was performed at 72 °C for 10 min. PCR products were separated by electrophoresis on 1% agarose gels. Bands corresponding to approximately 1,450 bp were excised and purified using the NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel, Duren, Germany). After that, the purified PCR products were sequenced. The primers for the sequencing process were 16S-27f (5'- AGRGTTTGATCMTGGCTCAG-3'), 16S-609f (5'- ACTACYVGGGTATCTAAKCC-3'), 16S-907R (5'-AAACTCAAAGGAATTGACGG-3'), and 16S-1492R (5'- GTTACCTTGTTACGACTT-3') using an ABI 3730 48-capillary sequencer (Applied Biosystems, Foster City, CA, USA). PCR reactions for the sequencing were performed using the BigDye® Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Results of the sequencing were aligned and edited using the BioEdit program (Hall 1999) and included in a BLAST search (Basic Local Alignment Search Tool; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences were compared using the database from NCBI (National Center for Biotechnology Information) using the Megablast tool (Altschul et al. 1990). The information for the alignment was included as identity, maximum score, and total score.

2.5 Bacterial inoculation of *Sinapis alba* plants growing on mine soil

2.5.1 Plant culture for bacterial inoculation

Sinapis alba seeds from an annual variety were purchased from Semillas Batlle S.A. (Molins de Rei, Barcelona) and germinated in commercial soil (Compo Sana Universal, Munster, Germany) and perlite (1:1). One week later, seedlings were transferred to alveoli (5×5 tray, 150 cm³ volume/alveolus) that were filled with the Pb-Zn contaminated soil (mine soil, commercial soil, and perlite at 1:1:1). Plants were watered with a modified Hoagland half strength solution (pH 5.5) consisting of 1 mM of Ca (NO₃)₂, 1.25 mM KNO₃, 0.5 mM NH₄H₂PO₄, 0.25 mM MgSO₄, 50 µM KCl, 25 µM H₃BO₃, 2 µM MnSO₄, 0.5 µM NH₄Mo, 0.5 µM CuSO₄, and 20 µM Fe-EDDHA. ZnSO₄ was removed from the solution since the soil already contained a high concentration of Zn. One hundred plants (two seedlings/alveolus) were cultured and divided into two groups: half of them were inoculated with the selected isolate and the rest served as controls. Three-week-old plants were first inoculated with the bacteria and then re-inoculated one week later. Plants were harvested one week after the second bacterial inoculation. The plants were grown in a growth chamber (Conviron® SH10, Controlled Environments Inc., Winnipeg, MB, Canada) under the following conditions: 12/12 h photoperiod, 600 µE m⁻² s⁻¹ photon fluency rate, 23/20 °C day/night temperature, and 60% relative humidity.

2.5.2 Bacterial culture for plant inoculation

The selected isolate (*Pseudomonas fluorescens*, uab6, NCBI Accession No. MT218317) came from the ‘Nadal/Can Vergeli’ mine soil and, in the initial screening, showed tolerance to a 2.5 mM ZnSO₄ supplemented LB agar medium (Difco Laboratories, Detroit, MI). Additionally, this strain was selected as it showed the highest efficiency to solubilise Zn from an insoluble source (ZnO). A vial of isolate uab6, preserved on 15% glycerol at -80 °C, was extracted and, after reaching room temperature, the bacteria were cultured on LB solid plates for five days. Actively growing colonies were scratched with sterile toothpicks and were inoculated on sterile pots (100 mL) filled halfway with LB broth medium (Difco Laboratories, Detroit, MI). The whole process was carried out under sterile conditions. Pots were placed under agitation (100 ×g) at room temperature and protected from light. After two days, the optical density (620 nm) was checked and adjusted to 0.5. The number of bacteria corresponding to OD 0.5 was counted

with a haemocytometer and was equivalent to 0.702×10^7 CFU/mL. Bacteria from 20 mL of LB (approx. 1.4×10^8 CFU/mL) were recovered by centrifugation at $12.000 \times g$ for 2 min and resuspended in 20 mL of PBS 0.25X. PBS buffer contents (in g/L) were 20 NaCl, 0.5 KCl, 3.6 Na₂HPO₄ 2H₂O, 0.6 KH₂PO₄. Three-week-old plants were inoculated with the bacteria by pouring the PBS bacterial culture directly onto the roots. In the control group, 20 mL of PBS 0.25X buffer was used. One week later, plants were re-inoculated following the same protocol. Each alveolus contained approximately 30 g of mixed soil and the number of CFU/g of soil was estimated as 9.3×10^6 after the two bacterial inoculations. Alveoli from both treatments (bacterial inoculation and control) were placed in separate lower trays to avoid bacterial colonisation of control plants with the selected isolate.

2.5.3 Plant and soil characteristics

One week after the second bacterial inoculation, leaf chlorophyll concentration was quantified with a CCM-300 chlorophyll content meter (Opti-Sciences, Hudson, NH, USA) on three fully expanded leaves per plant. Thereafter, plants were separated into aerial parts and roots. Each part was rinsed twice with distilled water and correctly labelled in paper envelopes, then dehydrated at 60°C for two days; dried plants were then weighed. Approximately 100 mg of dry material from the control and inoculated plants was separated for mineral analysis. Dry tissues (shoots and roots) from 10 replicates were homogenized to a fine powder and then digested (HNO₃:H₂O₂ 69:30%, 5:2 v/v) in a hot-block digestion system (SC154-54-Well Hot Block™, Environmental Express, Charleston, SC, USA).

Soil remaining in the alveoli of control and bacteria inoculated plants, as well as soil that had not been in contact with plants, was analysed to assess the mineral content. Soil samples were sieved through a 2 mm sieve and then analysed using the methods of Soltanpour and Schwab (1977), modified as follows. Ten grams of soil were mixed with 20 mL of 1 M NH₄HCO₃:5 mM DTPA and shaken for 1 h. Extracts were allowed to stand for 1 h and then gravity filtered through filter paper. Additionally, one gram of the three different soils was extracted with *aqua regia*. Both extracts (DTPA and *aqua regia*) were analysed for mineral content (B, Ca, Cd, Cu, Fe, K, Mg, Mn, Na, P, Pb, S, and Zn). Chemical concentration were determined by ICP-OES (Polyscan model 61E, Thermo Jarrell-Ash) or by ICP-MS (model Elan 6000; Perkin Elmer-Sciex) (Bech et al. 2012) for soil and plant samples. The certified material BCR 62 *Olea europaea* was included as a reference plant material for quality control. The certified reference soil CRM 142 R Light Sandy Soil was included for quality control.

Ratios of bioaccumulation factor and translocation factor were also studied for Cd, Pb, and Zn. The bioaccumulation factors were calculated as the ratio between the content of each metal in the plant and the metal concentration in the soil. The translocation factor was calculated as the content of each metal in the shoots relative to the metal content of the roots.

2.6 Statistics

The experiment was conducted in alveolar trays applying two treatments (bacterial inoculation and control). Each alveolus contained two plants but the mean per alveolus was calculated to reduce pseudo-replication. Finally, a set of 25 samples per treatment was statistically analysed for chlorophyll content and dry weight measurements and 10 replicates were analysed for soil and plant mineral content. Data were analysed using the software Statistica 7.0 (StatSoft, Inc., Tulsa, OK, USA). Normal distribution, homoscedasticity, and the independency of errors were checked prior to the analyses and data were log-transformed as needed. To check the statistical differences among groups, a one-way ANOVA was used to examine the effect of bacterial inoculation. Differences at $p < 0.05$ were considered significant.

3 RESULTS AND DISCUSSION

3.1 Soil characteristics

Analysis of soil texture indicated a higher content of sand particles, followed by silt, and finally clay, denoting a sandy loam soil. As expected, the mine soil analysis revealed high concentrations of Zn and Pb (Table 1) due to the long-term exploitation of both metals at the site for commercial interest. Based on these results, the mine soil has a suitable profile to be used as a metal-contaminated soil, and to be restored. The Gavarres Range, where the mine is located, was designated as a natural protected area (*Pla d'Espais d'Interès Natural, PEIN*) by the Catalanian administration in 1992. Environmental decontamination activities are promoted within the *PEIN* framework, and our results could facilitate the effective restoration of soil contaminated with potentially hazardous elements using a metal tolerant plant in consortia with metal-tolerant bacteria. Thus, the technique of phytoremediation combined with rhizobacteria is a plausible tool for restoring soils in this natural protected site.

3.2 Bacteria characterisation

Seven isolates were selected for their capacity to tolerate 2.5 mM of soluble Zn (ZnSO₄). Metal tolerance was confirmed on LB and Tris-minimal media. Isolates were then screened for different PGP traits (Zn and P solubilisation, siderophore and IAA production, and tolerance to Cd) and the results are presented in Table 2. The solubilisation of essential nutrients results revealed that uab1 and uab4 showed the greatest capacity for phosphate solubilisation, while uab6 showed the most efficient solubilisation of ZnO. Insoluble sources of P and Zn are generally converted into soluble forms by the synthesis of bacterial organic acid. The major organic acids produced by PGPB are acetic, citric, formic, gluconic, 2-keto-gluconic, lactic, malic, and oxalic acids (Sadeghzadeh 2013; Tahir et al. 2013; Gouda et al. 2018; Rasul et al. 2019). The production of D-gluconic acid was confirmed only in isolate uab7 (results not shown). The mechanisms responsible for the solubilisation of P and Zn remain unknown for the rest of the isolates and were beyond the scope of this work, but could be a topic of interest for future studies.

No siderophore production was observed for isolates uab3, uab4, or uab6, while similar levels of release were registered for the rest of the isolates. Siderophore production is one of the bacterial processes to increase tolerance to metal toxicity. However, other mechanisms are also associated with metal resistance in non-siderophore producers, for example metal sequestration in cell walls and metallothionein and phytochelatin production (Ojuederie and Babalola 2017). The lack of siderophore production by uab6 was unexpected as it was classified as *Pseudomonas fluorescens* and this species produces pyoverdine, a yellow-green pigment responsible for fluorescence. This pigment is a powerful Fe (III) scavenger and the major siderophore that satisfies the iron needs of these bacteria (Meyer 2000).

All the selected strains produced IAA, although across a wide range. Two extreme strains could be distinguished: uab7 and uab2, with the highest and lowest IAA production, respectively. The rest of the isolates can be considered intermediate IAA producers, although there were up to two-fold differences in their ability to synthesize the hormone. Ahemad and Kibret (2014) observed that 80% of rhizosphere microorganisms from different crops have the capacity to produce and release auxins. Similarly, IAA secretion seems to be a common trait in soil bacteria from metal-contaminated areas (Ahmad et al. 2008; Wani et al. 2008; Xie et al. 2015).

The rest of the analysed parameters were useful to optimize bacterial growth (optimum temperature and pH), and others were used to classify some of the isolated strains (fluorescence). Some of the results of the characterisation process are shown in Supplementary Figure 1, including the ability of isolate uab2 to

solubilise P (a), solubilisation of ZnO (b), fluorescence emitted by uab6 (*P. fluorescens*) under UV light conditions (c), and siderophore production of six isolates (d).

Sequences of the 16S rDNA region for the seven isolates showed identities ranging from 97 to 100% in the NCBI database (Table 3). Uab1 was identified as *Bacillus thuringiensis*, uab2, uab3, and uab7 belong to the genus *Serratia* and uab4, uab5, and uab6 are *Pseudomonas* spp. Genera *Pseudomonas* and *Bacillus* typically appear in the list of most used microorganisms for metal remediation purposes (see Ojuederie and Babalola 2017 and Verma and Kuila 2019). The genus *Serratia* has been less well explored, although some species are also cited for their contributions to metal extraction from contaminated soils (Carlot et al. 2002; Kuffner et al. 2008; Chen et al. 2010; Ji et al. 2012; Li et al. 2016). Finally, isolate uab6 (*P. fluorescens*) was selected for the plant inoculation experiment as it revealed the greatest ability to solubilise Zn from an insoluble source and showed intermediate abilities for the other analysed parameters (Table 2).

3.3 Effect of the PGP bacteria inoculation on *Sinapis alba* plants

Phytoremediation assisted by microbes has been described as a modern biotechnological technique, leading to faster and more efficient phytoextraction and phytostabilisation (Ojuederie and Babalola 2017). For these techniques, the use of metal tolerant plants that produce high biomass is increasingly common. Among these plants, white mustard has specifically been designated as tolerant to different potentially toxic elements (Fargasova 2004; Evangelou et al. 2007; Zalewska and Nogalska 2014). Application of rhizosphere isolates of *P. fluorescens* has been used to complement the remediation role of plants (Li and Ramakrishna 2011; Gupta et al. 2018). Based on previous research, we conducted an assay to assess whether our *P. fluorescens* isolate induced phytoextraction or phytostabilisation by *S. alba*.

3.3.1 Plant growth and chlorophyll content

Inoculation with *Pseudomonas fluorescens* (uab6) increased the aerial biomass of *S. alba* (Fig. 1). The dry weight of aboveground tissues two weeks after inoculation confirmed this observation, and significant increases in shoot and root dry weight were registered in bacteria inoculated plants, compared with those of uninoculated plants (Fig. 2a and b). Bio-inoculation induced a 2-fold biomass increase for shoots and 1.2-fold increase for roots compared with those of control plants. These results confirm the ability of *P. fluorescens* (uab6) to promote the growth of *S. alba* and the direct root application of this soil bacteria to

induce plant growth. A positive increase in plant biomass in the presence of *Pseudomonas* species was described previously: Dell'Amico et al. (2008) found a 54% increase in fresh biomass of *Brassica napus* shoots and Rajkumar and Freitas (2008) revealed promotion of plant growth and metal protection in *Brassica juncea*. A recent study showed a significant increase (122%) in shoots of *Silene vulgaris* in soil treated with living cells of *Pseudomonas* sp. H15 (Plociniczak et al. 2019). The observed promotion of plant growth could be explained by direct mechanisms such as the ability to produce IAA and efficiency to solubilise essential nutrients (Table 2). However, other indirect mechanisms of growth promotion such as biocontrol agents and detoxification of metals (Glick 2012) cannot be dismissed.

The mine soil induced a visible chlorosis in the cultivated plants (Fig. 1). Chlorosis remained after bacterial inoculation and chlorophyll concentrations were similar in both treatments (Fig. 2c). Chlorophyll degradation is commonly associated with metal toxicity but Abroudar et al. (2013) observed fading of chlorosis after the inoculation of *Noccaea caerulea* growing on a Ni-contaminated soil with metal-resistant rhizosphere bacteria. Bacteria induced a 1.5-fold increase in biomass in the inoculated *Noccaea* plants but, in contrast to our experiment, the metal concentration was not increased compared with the control.

3.3.2 Plant mineral nutrition and metal uptake

Plant inoculation with *P. fluorescens* uab6 had a considerable influence on the mineral content of white mustard roots and shoots (Table 4). Macronutrients remained unaltered but some micronutrients, such as B, Cu, and Zn, were enhanced after the bio-inoculation. The increase was influenced by the type of tissue; B was increased in the upper plant parts while Cu and Zn were higher in the roots. Benefits to crop nutrition associated with PGPB have been previously reported, with increases in both macro- and micronutrients (Raklami et al. 2019).

Tissue concentrations of Cd and Pb also increased in the bacteria inoculated plants (Table 4). Lead enhancement was only registered in the roots, while Cd increased in both the roots and shoots. As far as we know, our results indicate, for the first time, the positive effect of PGPB inoculation on root uptake of lead in white mustard. Despite the higher concentration of potentially toxic elements in plant tissues of inoculated plants, growth was notably stimulated and light phytotoxic effects, in the form of chlorosis, were not exacerbated. Previous studies of *S. alba* inoculated with PGPB strains reported microbe-stimulated accumulation of Zn, Cd, and Cu (Plociniczak et al. 2013, 2016). However, the Zn and Cd

concentrations achieved in the *S. alba* plants inoculated with our *P. fluorescens* strain were substantially higher than those previously reported. In our experiment, the increased biomass combined with the high levels of Cd and Zn in the aerial parts of bacteria-inoculated plants indicate the beneficial extractive ability of this plant-bacteria combination.

The beneficial impacts of metal-tolerant bacteria in plants after bio-inoculation have frequently been reported. Some studies have described a reduction in metal uptake by plants due to the ability of the bacteria to sequester them (Chen et al. 2005; Wang et al. 2007; Becerra-Castro et al. 2012; Ma et al. 2015). Such metal removal can be conducted through processes of precipitation, biosorption, and enzymatic transformation that converts metals to less innocuous forms (Khan et al. 2009) and this induces a reduced availability of metals to plant roots. All these mechanisms decrease metal concentrations in the soil, which is an interesting approach for crop cultivation. However, for phytoremediation purposes, higher metal bioavailability is desirable. Numerous studies have demonstrated the improved uptake of metals as a result of bacterial inoculation (Plociniczak et al. 2013, 2016, 2019; Moreira et al. 2014; Ali et al. 2017). The potential mechanisms behind the increased uptake of metals could be the promotion of plant growth via hormone synthesis, enzyme secretion, siderophore production, and solubilisation of essential macro- and micronutrients (Verma et al. 2001; Glick 2010). In our study, inoculation of *S. alba* plants with *P. fluorescens* uab6 mitigated the potential deleterious effects of extremely high Zn and Cd tissue concentrations and stimulated growth, probably by favouring maintenance of adequate levels of essential nutrients and the production of growth-stimulating phytohormones.

From our results and those of previous studies (Fargasova 2004; Evangelou et al. 2007; Vanek et al. 2010; Zalewska and Nogalska 2014), we can assert that *S. alba* performs well in soils with high metal burdens and is able to accumulate considerable amounts of metal in its shoots.

3.3.3 Variation in soil metal concentrations

During our relatively short experiment, the mineralogical composition of soil showed only small changes due to bacterial inoculation (Table 5). Among the 12 analysed elements, K and S were exclusively increased in the extractable soil fraction after the PGPB were applied. In fact, the presence of plants in the soil, but not the bacteria, induced the highest changes in nutrient levels. This result may be due to the capacity of the plant to exude organic compounds, the reduction in soil pH, and the dissolution of solid phases (Dakora and Phillips 2002). Moreover, the presence of plants could have stimulated the activity of

microorganisms naturally present in the soil, shifting towards a more metal-tolerant microbial community (Becerra-Castro et al. 2012). Kuffner et al. (2010) found an increase in the total bacterial population in the rhizosphere of different plants grown in metal-contaminated sites, which induced better solubilisation of different inorganic compounds. In fact, it has been proposed that a supply of PGPB can make a greater contribution to the maintenance of soil fertility in metal-contaminated soils than in conventional agricultural management practices (Khan et al. 2009).

3.3.4 Bioaccumulation and translocation factors for Cd, Pb, and Zn

Notable differences between bioaccumulation and translocation factors between inoculated and uninoculated plants were observed (Fig. 3). Regardless of the presence of bacteria, the plants registered ratios higher than unity, highlighting the metal-tolerant characteristics previously described. However, inoculated plants had higher Zn translocation factors and higher Zn and Cd bioaccumulation factors than uninoculated plants. No influence on Pb was observed (not shown). The mobility of Zn was moderate within plants but we observed a positive effect of bio-inoculation, which induced a higher translocation of this metal. In fact, the bacterial treatment caused a shift in the Zn TF of 2.5, meaning that Zn was effectively translocated to the shoots (Fayiga and Ma 2006). In contrast, Cd is easily distributed within plants (Nwosu et al. 1995), while Pb has limited mobility and mainly accumulates in the roots (Xiong 1998). *Pseudomonas fluorescens* inoculation did not stimulate translocation from the roots to the shoots for any of the metals. Recent studies reported that bacterial treatment induced higher TF (Pb and Zn) in artificially metal-amended soils (El-Meihi et al. 2019; Rehman et al. 2019). This difference in results may be due to the use of two different bacterial species, which could cause a synergistic effect, and/or the use of artificially amended soil, in which metal mobility may be higher than in the studied ancient mine soil.

4 CONCLUSION

Pseudomonas fluorescens (uab6, NCBI Accession No. MT218317) is a metal-tolerant isolate from the soil of an ancient Zn-Pb mine. The PGP traits of this soil bacterium promoted the biomass of *S. alba* plants grown on mine soil. Moreover, the bacteria were able to mobilize Zn from insoluble sources and stimulated metal acquisition (Cd, Cu, Pb, and Zn) by white mustard plants. Therefore, we propose that this isolate is a potential agent for use in the restoration of soils containing potentially toxic elements using metal-tolerant plants. This plant-bacterium partnership will potentiate the phytoextraction of Zn and

phytostabilisation of Cd and Pb. The role of the bacteria in concert with hyperaccumulating plants is a topic of interest for future investigations.

Mining soils are good sources for the isolation of PGP and metal-tolerant bacteria since seven isolates were extracted from the mine soil in this study. Isolates belonging to the genera *Bacillus*, *Pseudomonas*, and *Serratia* offer different abilities to produce IAA and siderophores, as well as to solubilise Zn and P. The isolate selected to be artificially inoculated was *P. fluorescens* (uab6), which showed the greatest ability to solubilise Zn from insoluble sources. Increased metal concentrations in bio-inoculated plants without phytotoxic effects can be explained by a better bioavailability of micronutrients or by production of beneficial compounds induced by the bacteria. Further studies are required to understand the specific bacteria-synthesised metabolites involved in plant growth promotion and phytoremediation efficiency.

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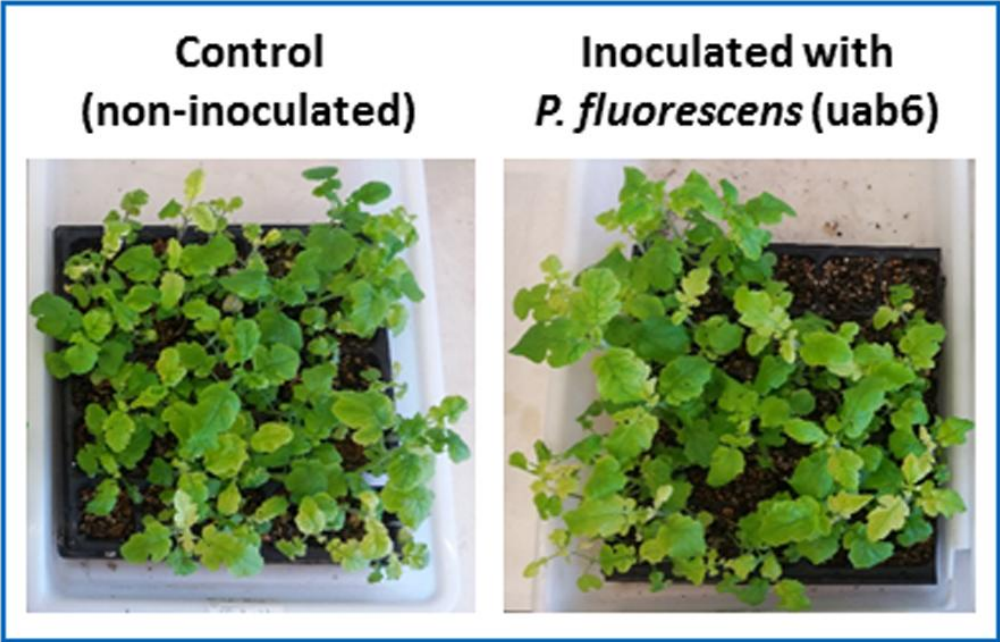
FIGURE CAPTIONS

Fig. 1 Upper view of *Sinapis alba* plants un-inoculated and inoculated with *Pseudomonas fluorescens* (uab6, NCBI Accession No. MT218317) 15 days after the first inoculation. Bacteria were re-inoculated one week after the first inoculation. Plants were growing on a Pb-Zn mining soil mixed with commercial soil and perlite (1:1:1).

Fig. 2 Shoot dry weight (a), root dry weight (b), and chlorophyll content (c) of *Sinapis alba* plants un-inoculated (Ctrl) and inoculated (Inoc) with *Pseudomonas fluorescens* (uab6, NCBI Accession No. MT218317). Chlorophyll content was measured 15 days after the first bacterial inoculation. Dry weight was recorded after two days at 60° C. Error bars correspond to the standard error (n = 25). * and ** indicate statistically significant differences among treatments at $P < 0.05$ and 0.01, respectively.

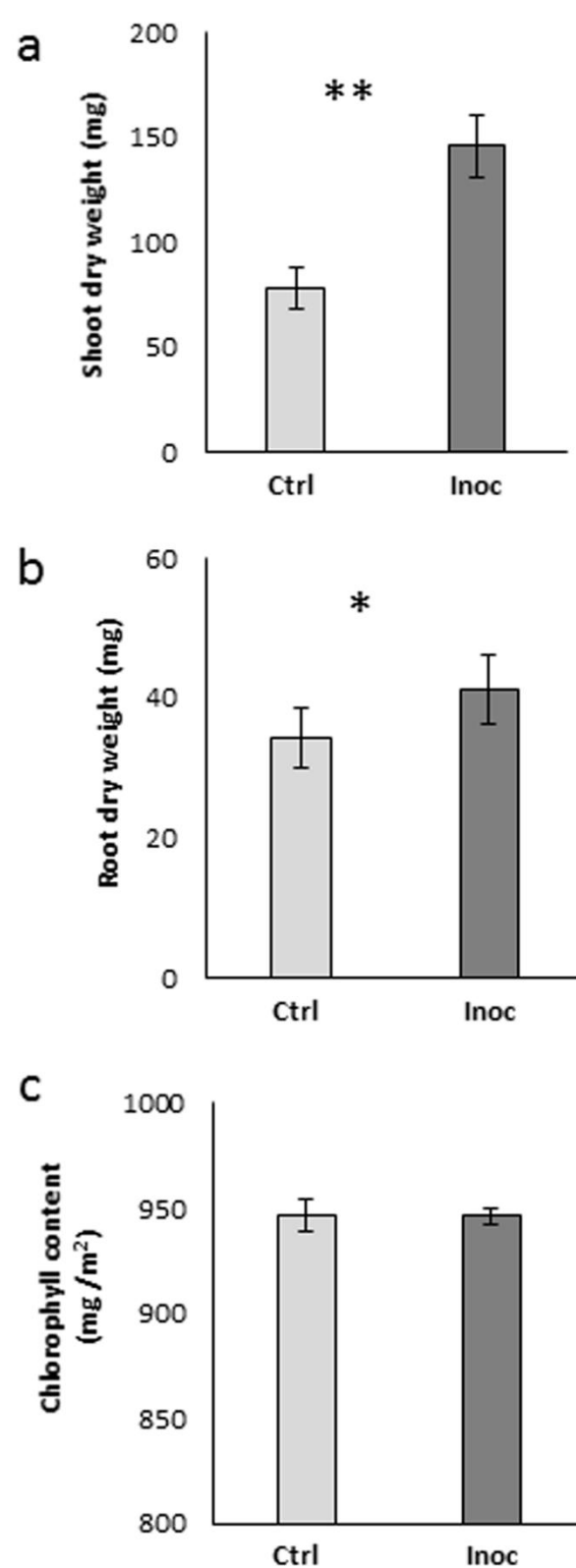
Fig. 3 Translocation factors for Cd (a) and Zn (c) and bioaccumulation factors for Cd (b) and Zn (d). Translocation factor is the metal concentration in shoots divided by the metal concentration in roots. Bioaccumulation factor is the metal concentration in plants divided by the metal concentration in the soil. Both parameters were calculated for *Sinapis alba* plants un-inoculated (Ctrl) and inoculated (Inoc) with *Pseudomonas fluorescens* (uab6, NCBI Accession No. MT218317). Error bars correspond to the deviation error (n=10). * and ** indicate statistically significant differences among treatments at $P < 0.05$ and 0.01, respectively.

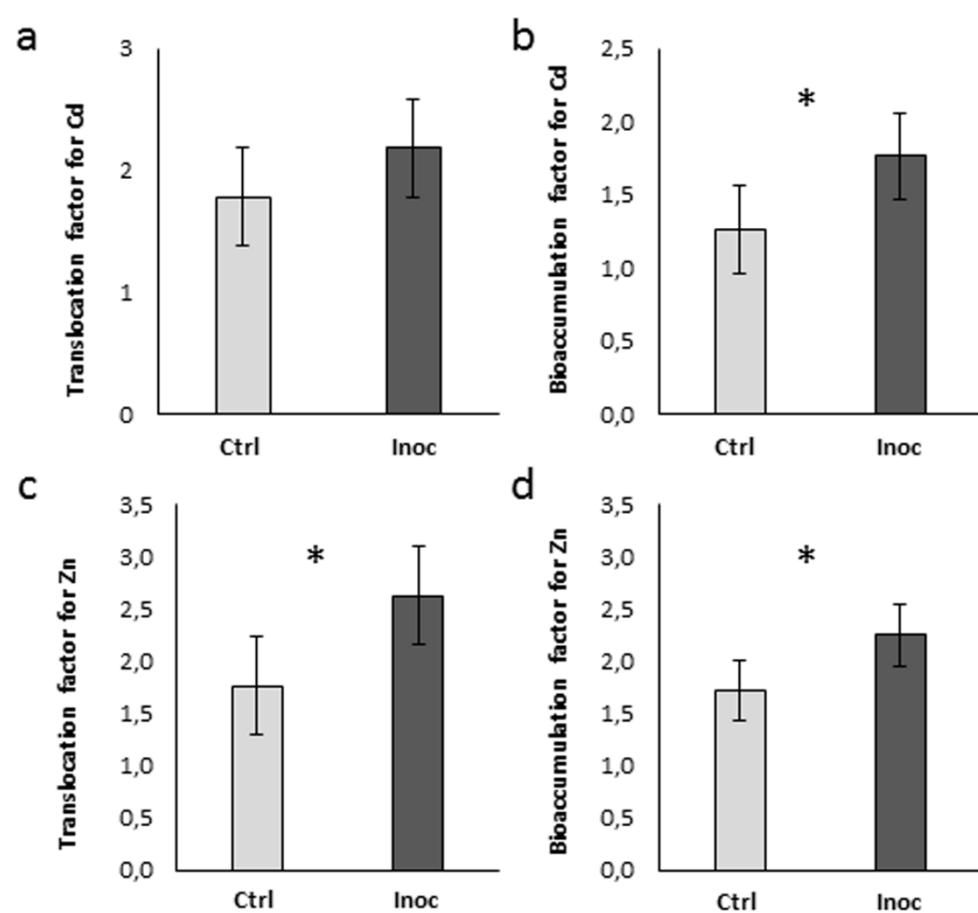
Supp Fig. 1 Ability of isolate uab2 to solubilise P (a), fluorescence emitted by *P. fluorescens* (uab6, NCBI Accession No. MT218317) under UV light conditions (b), solubilisation of ZnO (c), and siderophore production of six isolates (d).



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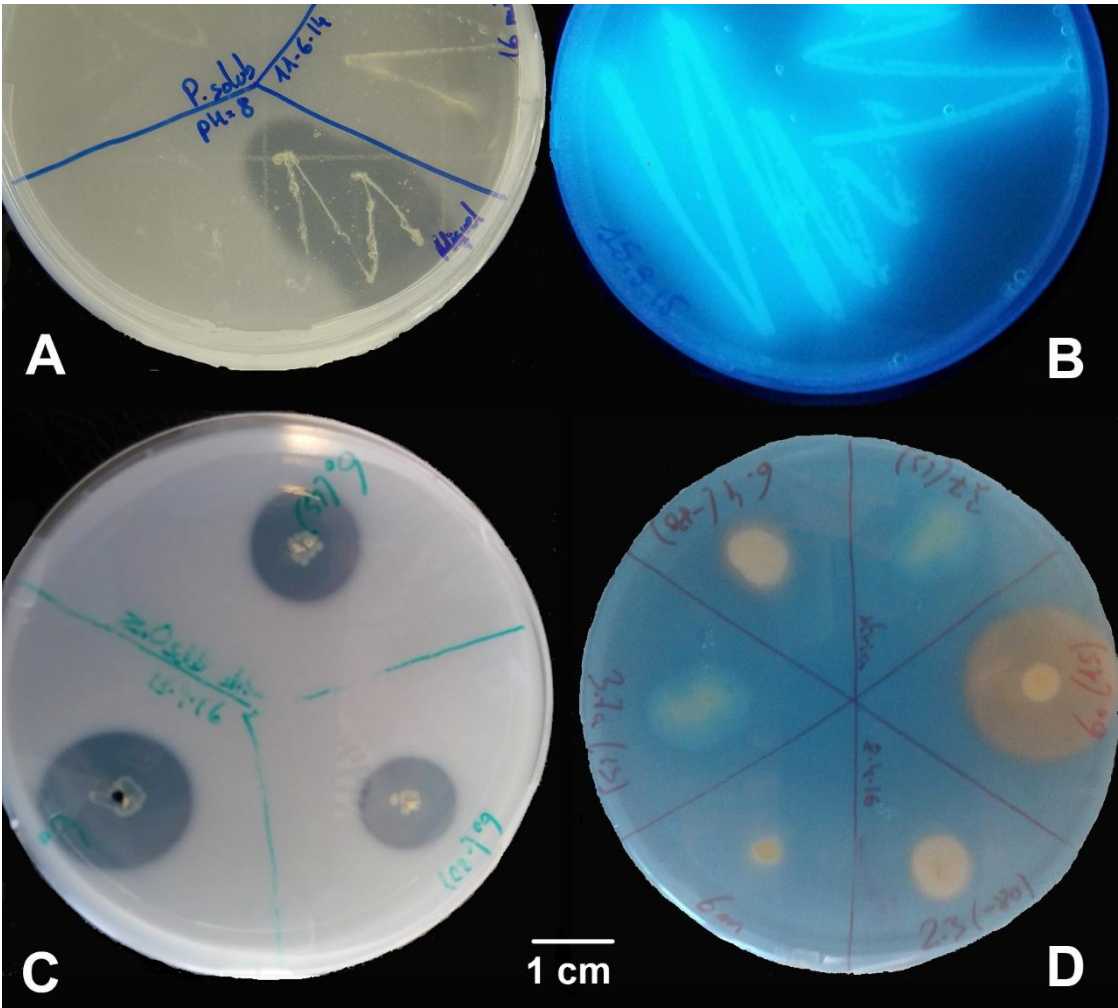




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