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Petra Kidd, Analía Álvarez, Vanessa Álvarez-López, Andrea Cerdeira-Pérez, Beatriz Rodríguez-Garrido, et al.. Beneficial traits of root endophytes and rhizobacteria associated with plants growing in phytomanaged soils with mixed trace metal-polycyclic aromatic hydrocarbon contamination. Chemosphere, 2021, 277, pp.130272. 10.1016/j.chemosphere.2021.130272 . hal-03180392

HAL Id: hal-03180392

<https://hal.science/hal-03180392>

Submitted on 27 Jul 2021

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**Beneficial traits of root endophytes and rhizobacteria associated with plants growing in
phytomanaged soils with mixed trace metal-polycyclic aromatic hydrocarbon contamination**

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ABSTRACT

The diversity of cultivable bacteria associated with plants from phytomanaged soils with mixed trace metal (TM) and polycyclic aromatic hydrocarbon (PAH) contamination in Pierrelaye (France) was evaluated. The emphasis was on the cultivable bacterial community since the overall objective is to obtain bioinoculants to improve the remediation of this type of contaminated site. Root endophytic and rhizosphere soil bacterial counts were determined, and isolates were pooled by amplified rDNA restriction analysis and identified by 16S rDNA sequencing. Isolates were further characterised for the production of plant growth-promoting (PGP) substances, and resistance to TM. The selected strains were evaluated for their ability to degrade PAHs. The potential of cell-free microbial supernatant to increase the mobilisation of PAHs from the polluted soil of Pierrelaye was also evaluated. Proteobacteria and Actinobacteria dominated the collection of isolates, and differences in taxonomic diversity were observed between plant species (*Populus* or *Zea mays*) and depending on the remediation treatment (*Populus* inoculation with mycorrhizae or *Populus* intercropping with *Alnus*). The majority of isolates exhibited at least one of the tested PGP traits, as well as resistance to more than one TM. Several rhizosphere, endophyte and even one bulk soil isolate showed high rates of fluoranthene and pyrene dissipation. The endophyte *Rhizobium* strain MR28 isolated from maize and degrading pyrene produced bioemulsifying molecules capable of improving the availability of PAHs from the soil of Pierrelaye. A selection of the most interesting strains was made for other re-inoculation experiments in order to assess their potential in rhizoremediation processes.

Keywords: organic pollutants; phytomanagement; plant-growth promoting bacteria; *Populus*;
polycyclic aromatic hydrocarbon; trace metals

1. Introduction

Phytomanaging contaminated soils can successfully restore biological fertility and diversity. Phytomanagement of metal-contaminated sites has increasingly been combined with short rotation coppicing (SRC) for the production of bioenergy (Chalot et al., 2020; Mench et al., 2009; Vangronsveld et al., 2009; Ziegler et al., 2019) or fiber-based material (Jeannin et al., 2020). SRC consists of densely planted high yielding poplar and willow species that can be harvested every 2-5 years to produce woody biomass. Willow and poplar species are recognised for their tolerance to metals and, in some species or cultivars, for their potential to extract trace metals (TM) such as Cd and Zn from contaminated soils (Chalot et al., 2020; Kidd et al., 2015). The use of polyclonal stands of *Salix* and *Populus* clones rather than monocultures has also been recommended in the phytomanagement of contaminated soils using SRC. They can offer several benefits in terms of plant productivity and nutrition, soil quality and biodiversity, and pest control (Kidd et al., 2015; Mench et al., 2010). Alder is an important pioneer species of degraded and metal-contaminated soils, and intercropping this woody plant with *Populus* or *Salix* has been proposed as a means of enhancing phytoremediation efficiency (Kidd et al., 2015).

Plants have been shown to promote the abundance of beneficial microorganisms in their environment, and in particular in the endosphere and rhizosphere (Thijs et al., 2017). These mutualistic plant-microbial symbioses can dramatically improve soil phytoremediation processes by promoting plant health and growth and by modifying the mobility and availability of contaminants (Mendes et al., 2013). Plant growth-promoting bacteria (PGPB) can improve plant nutrition by increasing the availability of essential plant nutrients, such as nitrogen (N_2 -fixing organisms), phosphorus (by solubilisation or mineralization through the production of organic acids and/or phosphatases) or iron (by releasing siderophores). They can also directly influence

plant growth and physiology through the production of phyto-hormones [e.g. indoleacetic acid (IAA)].

The application of plant-associated bacterial inoculants is gaining increasing interest in sites of mixed contamination, caused by the presence of organic and inorganic compounds. Polycyclic aromatic hydrocarbons (PAHs) are some of the most widespread organic pollutants; their fate in soils is of great environmental concern due to their potential toxicity, mutagenicity and carcinogenicity (Baoune et al., 2019). Mixed contaminated soils represent a source of potentially beneficial bacteria associated with plants that could be used as part of microbial-assisted remediation strategies targeting this type of site (Weyens et al., 2009). Selection of PAH degraders with metal tolerance and surfactant production abilities, or combining PAH degraders with biosurfactant producers, is of particular interest since surfactants enhance the contaminant solubility (Hickey et al., 2007). Endophytic bacteria can be a great option as bioinoculants because they have the advantage of proliferating in plant tissue, thus facing less competition for nutrients and being protected from the high stress environments of polluted soils (Pawlik et al., 2020). However, few studies have identified PAH-degrading or biosurfactant-producing endophytes.

The objectives of this study were to: (a) assess the cultivable bacterial diversity associated with plants under different phytomanagement options based on poplar SRC established in a contaminated mixed site in Pierrelaye (France), (b) isolate root endophytes and rhizobacteria associated with plants, (c) characterize the bacterial strains for traits promoting plant growth, (d) analyse bacterial strains for PAH degradation capacity, biosurfactant production potential and TM tolerance, and (e) assess the influence of certain biosurfactant producers on the availability of PAHs. The general hypothesis is that certain cultivable bacteria isolated from contaminated sites can produce bioinoculants which can greatly improve the remediation of PAH-contaminated soils.

2. Materials and methods

2.1 Soil and plant material

Soil and plant samples were collected from the experimental field site of Pierrelaye (northwest of Paris, France). Long-term (>100 years) irrigation of these agricultural soils with wastewater from Paris resulted in a mixed contamination of both TM (mostly Zn, Cd, Pb and Cu) and organic pollutants (including PAHs). A description of the general soil properties on this site is given by Ciadamidaro et al. (2017), while data of TM concentration in soil were published by Lamy et al. (2006) and Pottier et al. (2015). Two phytomanagement field trials were established on the Pierrelaye site, one in 2007 (project PHYTOPOP) based on the SRC using 14 different poplar cultivars (Foulon et al., 2016a, 2016b; Pottier et al., 2015), the second in 2011 (project BIOFILTREE) to assess the effect of mycorrhizal inoculation by intercropping *Populus* with *Alnus glutinosa* (Ciadamidaro et al., 2017).

The present work is based on rhizosphere soil, plant samples and bulk soil collected from plots with the clone *Populus trichocarpa* × *Populus maximowiczii* Skado [non-inoculated (NI) or inoculated (INOC) with mycorrhizal fungi], from plots with the non-inoculated poplar Skado intercropped with *Alnus* (referred to as intercropped *Populus* plots in the whole manuscript), from plots adjacent to the BIOFILTREE trial planted with maize and from non-planted soil (bulk soil) from the BIOFILTREE field trial. In addition, the rhizosphere soils from *Populus* and bulk soil from the PRPHYTOPOP field trial located also in Pierrelaye were studied. Sampling of soils and plant roots was carried out according to Foulon et al. (2016a, 2016b). Five replicate of each sample were studied.

2.2 Isolation of rhizobacterial and root endophytic bacterial strains

Five grams of bulk soil or fresh rhizosphere soil of five *Populus* and maize plants were suspended in 45 mL sterile sodium hexametaphosphate solution (1%) and shaken for 30 min in an end-over-end shaker. Soil suspensions were diluted in 10-fold series and plated in duplicate onto 284 agar medium (Schlegel et al., 1991) supplemented with 100 µg/mL of cycloheximide. The 284 medium contains (g/L): 6.06 Tris-HCl, 4.68 NaCl, 1.49 KCl, 1.07 NH₄Cl, 0.43 Na₂SO₄, 0.2 MgCl₂·6H₂O, 0.03 CaCl₂·2H₂O, 0.04 Na₂HPO₄·2H₂O, 10 mL Fe(III)NH₄ citrate solution (containing 48 mg/100mL) plus oligoelements (1.5 mg FeSO₄·7H₂O, 0.3 mg H₃BO₄, 0.19 mg CoCl₂·H₂O, 0.1 mg MnCl₂·4H₂O, 0.08 mg ZnSO₄·7H₂O, 0.02 mg CuSO₄·5H₂O, 0.036 mg Na₂MoO₄·2H₂O) adjusted to pH 7. The medium was supplemented with a mixture of different carbon sources (g/L): 0.7 lactate, 0.5 glucose, 0.7 gluconate, 0.5 fructose, and 0.8 succinate. After seven days of incubation at 28 °C, colony forming units (CFUs) were counted and calculated per gram dry weight (DW) soil. Distinct colony morphotypes associated with each sample group were sub-cultured at least three times to ensure purity and cryopreserved.

To isolate the root endophytic bacteria of NI *Populus*, root samples (0.2-0.3 g) were surface-sterilised for 10 min in 2% NaClO solution supplemented with one droplet Tween 80 per 100 ml solution, and were subsequently rinsed three times for 1 min in sterile deionised water. For maize, root samples (0.2-0.3 g) were surface-sterilised by sequential immersion in 5% NaClO solution supplemented with one droplet Tween 80 per 100 ml solution for 10 min and in 75% (v/v) ethanol for 1 min. The surface-sterilisation process was checked by plating aliquots of the third rinsing solution on 869 medium [(g/L): 1.0 tryptone, 0.5 yeast extract, 0.5 NaCl, 0.1 glucose, and 0.035 CaCl₂·2H₂O]. No growth on these control plates after seven days indicated successful sterilisation. The concentrations of NaClO and the sterilisation times were empirically determined in previous studies. After surface sterilisation, the root tissue was macerated in 10 mL of 10 mM MgSO₄ using a Polytron PT1200E homogeniser (Kinematica AG). Ten-fold serial dilutions were plated in duplicate on 1/10 strength 869 agar medium, CFUs were determined as above and calculated per

gram fresh root weight. Distinct colony morphotypes were sub-cultured at least three times to ensure purity and cryopreserved.

2.3 Phenotypic characterisation of bacterial isolates

The tolerance to TM was tested growing the strains at 28 °C for seven days in 284 agar medium (see above) supplemented with Cd, 0.1 mM; Cu, 1.0 mM; Pb, 1.0 mM or Zn 2.5 mM. The ability to solubilize inorganic phosphate was assessed in NBRIP agar medium adjusted to pH 7.0, modified from Nautiyal (1999) supplied with 5 g/L of hydroxyapatite and incubated at 28 °C for 5 days. Yeast extract was added since some strains were unable to grow in yeast-free NBRIP medium. Siderophore production was detected in a modified 284 liquid medium (without Fe) using the Chrome Azurol S (CAS) method described by Schwyn and Neilands (1987). The ability of isolated strains to produce IAA was evaluated in liquid medium, modified from Sheng et al. (2008) supplemented with 0.5 mg/mL of tryptophan. After five days of incubation at 28 °C, cultures were centrifuged and the presence of IAA was evaluated spectrophotometrically (535 nm) after incubating the supernatant with Salkowski reagent for 25 min. Strains were screened for potential biosurfactant production using the qualitative method of Chen et al. (2007).

2.4 Genotypic characterisation of bacterial isolates

For DNA extraction, purified strains were grown in 1/10 strength 869 liquid medium; and genomic DNA was extracted from bacterial cell pellets according to Mergeay et al. (1985). Polymerase chain reaction (PCR) amplification of 16S rRNA genes was carried out in mixtures containing 100 ng/μl DNA, 1× High Fidelity PCR buffer (Invitrogen, Carlsbad, CA, USA), 0.2 mM of each of the four deoxynucleoside triphosphates, 2 mM MgCl₂, 0.2 μM each of the forward

and reverse primers, and 1 U of High Fidelity Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) per 50 µl. The universal 1392R (5'-ACGGGCGGTGTGTRC-3') and the bacteria-specific 26F (5'-AGAGTTTGATCCTGGCTCAG-3') primers were used for prokaryotic 16S rRNA gene amplification. Cycling conditions consisted of: one denaturation cycle at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 45 °C for 45 s, and 72 °C for 1.5 min, and completed with an extension cycle of 10 min at 72 °C. PCR products were purified using the QIAquick PCR purification columns (Qiagen, Valencia, CA, USA) and quantified spectrophotometrically, using the Nanodrop spectrophotometer (ND-1000, Isogen Life Science). The PCR products were subjected to 16S rDNA restriction analysis (ARDRA), used for screening the collection of isolates.

For ARDRA, aliquots of the PCR products were digested overnight at 37 °C with 1 U of the four-base-specific restriction endonuclease HpyCH4 IV in 1×NEB buffer 1 (New England Biolabs, Beverly, MA, USA). The digestion products obtained were separated by electrophoresis in a 1.5 % agarose gel, and visualized by red safe staining and UV illumination. Bacterial strains with the same ARDRA patterns were grouped using the Pearson correlation coefficient and UPGMA clustering algorithm of the Gel Compar Bionumerics programme (Bionumerics Version 5.1, Applied Maths, Belgium). One representative strain of each group was selected for sequencing. Sequence data were checked using the Chromas v.1.45 software (Technelysium Pty. Ltd., Australia) and assessed for similarity with sequences of the Ribosomal Database Project (Cole et al., 2009).

2.5 PAH degradation capacity

Based on their plant growth promoting characteristics, a selected number of isolates were cultivated in minimal salt medium (MSM) with a mixture of phenanthrene and fluoranthene and

pyrene (Sigma-Aldrich). The PAHs were chosen on the basis of the Pierrelaye soil analyses (Supplementary Table 1). The composition of the MSM was as follows: 100 mL phosphate buffer [$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (140 g/L), KH_2PO_4 (20 g/L)], 10 mL salt solution [$(\text{NH}_4)_2\text{SO}_4$ (100 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (20 g/L)], 1 mL $\text{Ca}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ (4.62 g/L), 28 mL FeNH_4 enriched with 18% Fe (48 mg/100mL), 1 mL oligoelements solution (1L: HCl 37% 5.2 mL, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 1.5 g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.08 g, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.1 g, H_3BO_3 0.3 g, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.19 g, CuSO_4 0.004 g, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 0.024 g, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.036 g), 860 water. A stock solution of the three PAHs dissolved in acetone (120 $\mu\text{g/mL}$) was added to test tubes containing 4 mL of MSM to reach a final concentration of 2 μg of PAHs per ml medium. The test tubes were left uncapped for 30 min to allow evaporation of the acetone prior to the microbial inoculation inside a laminar sterile hood.

To prepare the bacterial inoculants, strains were cultivated in 1/10 strength 869 liquid medium for two days, harvested by centrifugation (4000 x g, 15 min, 4 °C), washed two times and re-suspended in 10 mM MgSO_4 to an optical density of 1.0 at 600 nm (about 10^7 - 10^8 cells per mL). Tubes inoculated with 100 μL of each bacterial strain suspension were incubated at 28 °C and 150 rpm for two, four and seven days. Non-inoculated tubes with MSM were used as a control and four replicates of each treatment were prepared.

PAHs were extracted with 10 mL of hexane added to each test tube in an end-over-end shaker for 30 min. Extracts were dried with anhydrous Na_2SO_4 and then stored at -20 °C for posterior chromatographic analysis.

The concentration of PAHs was determined using a gas chromatograph (Varian 450-GC) and a mass spectrometer with ion trap (Varian 220-MS) equipped with an automatic sampler (CP-8400, Varian Inc., Palo Alto, CA, USA). The split/splitless injector worked at 300 °C and in splitless mode. Sample injection volume was 1 μL . The chromatographic column was a Thermo Trace Gold TG-5SILMS (30 m x 0.25 mm x 0.25 μm) of 5% phenyl methyl polysiloxane. The column oven temperature was varied as follows: 60 °C held for 5 min, followed by an increase of 8 °C

min⁻¹ up to 300 °C which was held for 4 min. The carrier gas was helium at constant flow of 1.5 mL min⁻¹.

The mass spectrometer operated in MS/MS mode, ionization of the molecules was carried out by electronic impact (EI) and the transfer line temperature and the ion trap temperature was fixed at 280 °C and at 220 °C, respectively.

2.6 Influence of a biosurfactant on PAH availability

The potential effect of a microbial biosurfactant on the soil PAHs availability was tested using a cell free culture supernatant as soil extractant. The MR28 strain was cultured in 1/10 strength 869 liquid medium (30 °C, 150 rpm) for two days, and then centrifuged at 9,000 × g for 10 min. The supernatant was separated and filtered at 0.22 µm to remove bacterial cells.

Soil from the Pierrelaye site was air-dried and sieved to <2 mm. Four g of soil were placed in separate glass test tubes sterilized (Alvarez et al., 2015) and extracted with 10 mL of the filtered culture supernatant. The extraction was performed by shaking the tubes at 150 rpm, 30 °C for five days. Control tubes were prepared in parallel with soil samples treated with 869 liquid medium (1/10 strength) filtered at 0.22 µm instead of culture supernatant. Four replicates of each treatment were performed. The soil extracts were separated from soil particles by centrifugation (9,000 × g, 10 min, 4 °C) and the PAHs in the extracts were obtained with 10 ml of hexane by 60 min of agitation in an end-over end shaker. PAHs in hexane were determined as explained above.

3. Results

3.1 Abundance, identification and phylogenetic analysis of cultivable rhizosphere and root endophytic bacteria

274
275 The densities of cultivable bacteria in soil (bulk and rhizosphere) and root endophytes (poplar vs.
276 maize) are presented in Table 1. Rhizobacterial densities did not differ significantly between plant
277 species but were up to one logarithmic unit higher than those found in bulk soils. On the other
278 hand, densities endophytes in the root of poplar or maize were not significantly different, but were
279 between two to three logarithmic units lower than rhizobacterial densities (Table 1).

280 A total of 644 different colony morphotypes was isolated, including 327 rhizobacterial strains,
281 161 strains from bulk soil and 156 root endophytic strains (108 from NI *Populus* and 48 from *Zea*
282 *mays*). The diversity of cultivable bacteria was estimated using ARDRA and 16S rDNA
283 sequencing. At a similarity of >90% ARDRA profiles of isolates from PHYTOPOP were allocated
284 into 64 and 28 ARDRA groups for rhizobacteria associated with *Populus* and for bacteria from the
285 bulk soil, respectively. In the case of the ARDRA profiles of the bacteria isolated from the
286 rhizosphere of *Populus* of the different BIOFILTREE plots, were allocated in the following
287 groups: 32 groups for INOC *Populus*, 33 groups for NI *Populus*, 28 groups for intercropped
288 *Populus* and 29 ARDRA groups for bulk soil. Rhizobacterial isolates associated with *Zea mays*
289 adjacent to the BIOFILTREE plots were classified in 19 ARDRA groups which reflects a lower
290 variety of taxa compared to rhizobacterial isolated from *Populus*.

291 The ARDRA profiles of root endophyte isolates from BIOFILTREE plots were divided into 31
292 and 14 groups for *Populus* (NI) and *Zea mays*, respectively. Thus, the cultivable root endophytic
293 community of *Populus* showed a greater variety of taxa than that of *Zea mays*.

294 The 16S rDNA of one representative isolate per ARDRA group was partially sequenced.
295 Gram-positive and Gram-negative bacteria were adequately represented in samples from both
296 PHYTOPOP and BIOFILTREE plots (Table 2). In the PHYTOPOP field trial, isolates belonging
297 to the phylum Actinobacteria dominated the collections obtained from both the *Populus*
298 rhizosphere and the bulk soil. Within this phylum, most of the isolates were classified as

Streptomyces, although the genus *Rhodococcus* was also well represented in the rhizosphere of *Populus*. Firmicutes was the second most dominant phylum in the PHYTOPOP samples. In both the *Populus* rhizosphere and the bulk soil, most of the isolated Firmicutes belonged to the genera *Bacillus* or *Paenibacillus*. Proteobacterial strains were also found: 19% of the rhizobacterial isolates associated with *Populus* and <10% of strains from the bulk soil. The variety of Proteobacteria was higher in the rhizobacterial community of *Populus* in which isolates belonging to the Alpha, Beta- and Gamma-proteobacteria were found. Gamma-proteobacteria was not represented in the collection of the bulk soil.

The collections of isolates obtained from the rhizosphere of *Populus* growing in the BIOFILTREE plots were also dominated by members of the phylum Actinobacteria. Actinobacteria isolated from the rhizosphere of NI *Populus* grown in monocultures mainly belonged to the genera *Streptomyces*, *Rhodococcus* and *Arthrobacter*. On the other hand, in the collections of INOC or intercropped *Populus*, these genera, in particular *Streptomyces*, were also represented but the variety of Actinobacteria was higher: members of the genera *Microlunatus*, *Nocardioideis*, *Micrococcus*, and *Microbacterium* were identified in the rhizosphere of INOC *Populus* whereas *Microbacterium*, *Kribbella*, *Knoella* and *Agromyces* were found in the rhizobacterial community of intercropped *Populus*. By comparing the *Populus* plots and intercropped *Populus* plots, the genera *Kribella* and *Agromyces* were only found in the later. In the collections of NI and INOC *Populus* grown in monoculture, Firmicutes were mainly represented by *Bacillus* strains, while in the samples collected in intercropped *Populus* plots, the three genera *Paenibacillus*, *Bacillus* and *Lysinibacillus* were similarly represented.

Cultivated Proteobacteria from the rhizosphere of *Populus* included members of the Alpha-, Beta- and Gamma- classes and the variety of taxa was higher in NI than in INOC *Populus*. In fact, strains of the *Rhizobiales*, *Ensifer*, *Pseudochrobactrum* and of *Lysobacter* were isolated only from NI samples. The collection obtained from the rhizosphere of intercropped *Populus* plots included

Alpha and Betaproteobacteria; however, most of the isolates were affiliated to genera different than those found in monoculture plots. In the intercropped *Populus* plots, strains of the *Rhizobiales*, *Devosia*, *Mesorhizobium* and *Rhizobium* represented about 1/3 of the cultivated Proteobacteria. Regarding the phylum Bacteroidetes, strains of *Flavobacterium* and *Chitinophaga* were cultivated from NI *Populus* rhizosphere and isolates of *Pedobacter* were obtained from INOC plots. Bacteroidetes were absent in the collection of intercropped *Populus* (Table 2).

Unlike the rhizobacterial communities of *Populus*, in the rhizosphere of maize grown on the BIOFILTREE plots, Proteobacteria predominated, while a lower percentage of Actinobacteria was found (Table 2). The percentage of isolates affiliated to both Firmicutes and Bacteroidetes was 12.5%. The variety of Proteobacteria and Actinobacteria was lower in maize rhizosphere than in *Populus* rhizosphere. Proteobacteria of the Alpha-, Beta- and Gamma classes were isolated and the strains were classified in five different genera: *Rhizobium*, *Variovorax*, *Duganella*, *Pseudomonas* and *Lysobacter*. The isolated Actinobacteria were classified as *Streptomyces*, *Rhodococcus* and *Arthrobacter*. The Bacteroidetes and Firmicutes were only represented by members of the genera *Flavobacterium* and *Bacillus*, respectively.

The taxonomic breakdown of the root endophytic communities of NI *Populus* and *Zea* mays from BIOFILTREE plots was in line with that observed in corresponding rhizosphere soils: 45% of the root endophytes of *Populus* were classified as Proteobacteria, 32.3% as Actinobacteria and 23% as Firmicutes, while the maize root endophytes were clearly dominated by members of the Proteobacteria (75%). In maize root the proportion of isolates classified as Actinobacteria and Firmicutes were 7% and 8%, respectively (Table 2).

3.2 Plant growth-promoting characteristics of rhizosphere and root endophytic bacteria

The majority of the isolates obtained from *Populus* and maize plants had at least one of the PGP traits tested (Supp. Fig. 1A). Soil rhizosphere bacteria were generally able to produce IAA (>75% of all isolates) and the higher IAA-producers were mostly associated with *Populus* rather than *Zea mays*. However, the majority of these bacteria produced low levels of IAA (<5 µg/mL), while the higher IAA-producers were mainly associated with *Populus* rather than *Zea mays*. In addition, greatest number of higher IAA-producers was observed in the intercropped *Populus* plots (8 strains produced 5-20 µg/mL IAA and these were identified as *Bacillus* sp., *Rhizobium* sp. and *Streptomyces* sp.). In contrast, maize rhizobacteria harboured a greater number of bacterial strains producing siderophore (approx. 75% of isolates). Only 30% of the isolates obtained from intercropped *Populus* plots showed this trait. P-solubilizers were predominantly associated with INOC *Populus* (no P-solubilizers were found in NI *Populus*) or with maize. The ability to produce biosurfactants was less common among isolate collections: between 5 to 20% of rhizosphere soil isolates exhibited this trait, and the greatest number of biosurfactant-producing strains were found in rhizosphere soil of INOC *Populus* or maize. In general, between 30 and 60% of isolates were able to grow in the presence of Cd, Cu, Pb or Zn (Supp. Fig. 1B).

In the case of root endophytes, 20-25% of root isolates were strains producing siderophore (Supp. Fig. 1A). P-solubilisers were again more associated with roots of *Zea mays* than *Populus*, and up to 40% of maize root endophytes were able to solubilise mineral phosphate. Producers of biosurfactants were also less frequent and were mainly associated with *Populus* and identified as *Bacillus*. Only two strains isolated from maize roots were capable of producing biosurfactants and were identified as *Rhizobium* and *Pseudomonas*.

The most frequently observed PGP trait was the ability to produce IAA (although the majority of the strains produced <5 µg/mL IAA), and a higher number of these IAA-producing strains were associated with *Populus* rather than maize. With the exception of Cu, metal-resistant strains were

more frequent in root endophytes from *Populus* than maize (50-60% of *Populus* isolates could grow in the presence of Cd, Pb and Zn compared to 15-20% of maize isolates) (Supp. Fig. 1B).

3.3 PAHs degradation

Bacterial strains belonging to different collection of isolates were evaluated for their ability to degrade phenanthrene, fluoranthene and pyrene as the only C source. Isolates showing at least two PGP traits (including biosurfactant production) as well as at least one metal tolerance were selected for this purpose (Table 3).

Thus, eleven bacterial isolates including endophyte, rhizospheric and bulk soil strains were assessed for their ability to degrade PAHs. Mean concentrations of phenanthrene, fluoranthene and pyrene in the non-inoculated medium were 1248.6 ± 53.1 $\mu\text{g/L}$, 1531.5 ± 119.6 $\mu\text{g/L}$ and 1726.9 ± 131.4 $\mu\text{g/L}$, respectively (Figure 1). The mean phenanthrene concentration decreased after seven days of testing with most of the strains. The final concentrations were between 815.3 ± 182.6 $\mu\text{g/L}$ and 1236.2 ± 88.6 $\mu\text{g/L}$; however, the differences were not statistically significant compared to the non-inoculated medium ($p>0.05$) (Figure 1A). A notable exception was recorded by the strain *Bacillus* sp. B32.23, which by the fourth day of cultivation exhibited a significant decrease in phenanthrene concentration (758.0 ± 111.7 $\mu\text{g/L}$ vs 1248.6 ± 53.1 $\mu\text{g/L}$, in B32.23 tubes and in the non-inoculated control, respectively) ($p<0.05$). On the fourth day of the experiment, the observed decrease in fluoranthene concentration was even more pronounced especially in the case of the strain *Bacillus* sp. B32.23 (773.6 ± 136.9 $\mu\text{g/L}$ residual fluoranthene, $p<0.05$). At the end of the incubation, the strains *Streptomyces* sp. Sk.2012, *Bacillus* sp. Sk1.23, *Rhodococcus* sp. Sk12.6, *Microbacterium* sp. Sk11.4 and *Bacillus* sp. Sk2.3 also reduced the fluoranthene concentration in the culture media ($p<0.05$). In the case of these isolates pronounced fluoranthene dissipation took place between day four and day seven of incubation (Figure 1B). Again, at the end of the

experiment, the lowest fluoranthene concentration ($734.8 \pm 347.0 \mu\text{g/L}$) was found in tubes inoculated with *Bacillus* sp. B32.23, although similar concentration of residual fluoranthene was detected in tubes inoculated with the strains *Streptomyces* sp. Sk.20.12, *Bacillus* sp. Sk1.23, *Rhodococcus* sp. Sk12.6, *Microbacterium* sp. Sk11.4 and *Bacillus* sp. Sk2.3 strains ($p>0.05$).

A significant decrease in the pyrene concentration was observed in the presence of the strain *Bacillus* sp. B32.23 on the second day of the incubation (Fig. 1C) ($p<0.05$). On the fourth day, the pyrene concentration was also significantly reduced by *Pseudomonas* sp. MR47 and *Sphingomonas* sp. SR62. In addition to *Sphingomonas* sp. SR62 and *Bacillus* sp. B32.23 strains, a pronounced decrease in residual pyrene was recorded towards the end of the experiment in tubes inoculated with strains *Rhizobium* sp. MR28, *Microbacterium* sp. Sk11.4, *Rhodococcus* sp. Sk12.6, *Bacillus* sp. Sk1.23, *Streptomyces* sp. Sk20.12 and *Bacillus* sp. Sk2.3. As in the case of fluoranthene, the dissipation of pyrene by these isolates was only detected at the end of the incubation. For instance, in the case of strain Sk11.4, pyrene concentration dropped sharply from $1723.0 \pm 49.5 \mu\text{g/L}$ at day four, to $968.0 \pm 191.6 \mu\text{g/L}$ at day seven. The lowest residual pyrene concentration was detected in the presence of *Bacillus* sp. B32.23 strain ($843.1 \pm 407.3 \mu\text{g/L}$), although this value was not significantly different than those observed for isolates MR28, Sk11.4, Sk12.6, Sk1.23 and Sk20.12 ($p>0.05$).

3.4 Influence of a biosurfactant on PAH availability

The maize-endophyte strain *Rhizobium* sp. MR28 was selected for its high capacity to produce bioemulsifiers. Thus, the cell-free culture supernatant of *Rhizobium* sp. MR28 was evaluated as a soil extractant to test if bioemulsifiers potentially released to the growth medium could increase the bioavailability of PAHs in Pierrelaye's soil. Nine different PAHs were detected in the soil extracts, and the concentration of four of them, namely naphthalene, fluorine, anthracene and

phenanthrene was significantly higher in extracts obtained from MR28 cell-free growth medium than in those obtained with sterile culture medium (control) ($p<0.05$) (Fig. 2).

4. Discussion

The use of microorganisms for treating soils contaminated with TM or organic compounds is an environmentally safe technology which can be improved by plants via the stimulation of microbial growth (Baoune et al., 2019). In the present study, the cultivable bacterial diversity associated with plants established in a mixed contaminated site in Pierrelaye (France) under different SRC-based phytomanagement options was evaluated.

Rhizobacterial densities did not differ significantly between plant species (poplar vs maize) but were up to one logarithmic unit higher than those found in bulk soils. This is a common observation, which can be related, among many other factors with a greater availability of carbon in the rhizosphere (Kidd et al., 2008). The main driver for the rhizosphere effect is the massive root supply of organic substrates to the soil, which can increase the bioavailability of PAHs but also induce a selection of rhizospheric microbial inhabitants (Arslan et al., 2014; Kidd et al., 2009). Root exudates and mucilage-derived nutrients attract large numbers of organisms to the rhizosphere environment. However, the endophytic competence/colonization is limited to specific bacterial species (Durand et al., 2017). Densities of endophytes in the root of poplar or maize were not significantly different, but were between two to three logarithmic units lower than in the rhizosphere. The lower diversity of the endophytic communities compared to soil communities can be explained by the oligotrophic environment present inside plants, leading to a restriction of bacteria capable of using the available resources (Baoune et al., 2018).

According to the partially sequenced 16S rDNA, both Gram-positive and Gram-negative bacteria were widely represented in the samples from the PHYTOPOP and BIOFILTREE trials suggesting that the variety of different taxa may reflect the adaptation of the community to the contaminated soil of Pierrelaye over several decades (Bourceret et al., 2016). The dataset obtained for the roots and soil samples in this work corroborates other previous studies carried out at the same site (Pierrelaye) on native tree species (poplar and willow), revealing the dominance of Proteobacteria and Actinobacteria (Durand et al., 2017; Foulon et al., 2016).

The collection of isolates obtained from the rhizosphere of *Populus* was dominated by members of the phylum Actinobacteria. Gram-positive bacteria, and in particular the Actinobacteria, have frequently been found to dominate cultivable bacterial communities of TM contaminated soils and natural soils rich in TM, such as serpentine soils (Álvarez-López et al., 2016; Becerra-Castro et al., 2012). Several actinobacterial inoculants, selected for their PGP traits, have been used successfully in bioaugmentation assays to enhance TM extraction (phytoextraction and phytomining) (Becerra-Castro et al., 2013; Cabello-Conejo et al., 2014). Actinobacteria are also known for their ability to remove xenobiotics, such as pesticides, from aquatic and terrestrial environments (Álvarez et al., 2017).

Significant reductions in CaCl₂-extractable TM concentrations under poplar-planted soils at the Pierrelaye site were observed compared to unplanted soils or to baseline values prior to the implementation of the phytomanagement trials (Foulon et al., 2016a; Ciadamidaro et al., 2017). Mycorrhizal inoculation also significantly increased the biomass yield of the *Populus* Skado clone at the Pierrelaye site (Ciadamidaro et al., 2017). Foulon et al. (2016a) reported that higher bacterial diversity and richness indices were observed in poplar soils compared to unplanted soils, which is in agreement with the results of the present study. In the present study, the bacterial communities associating with the INOC *Populus* were found to be significantly different from the NI *Populus* in the BIOFILTREE plots. At the phyla level, the dominant bacterial groups remained

the same (Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria) but, surprisingly, the variety of cultivable bacteria was lower when the trees had been previously inoculated (INOC) by a mycorrhizal consortium. Inoculation led to a decrease in the relative abundance of members of the Bacteroidetes and Proteobacteria, and to an increase in those belonging to Firmicutes.

A clear enrichment in the Proteobacteria phylum in the endospheric compartment has been recorded in previous publications (Afzal et al., 2014). Such as in the case of the rhizosphere, a higher variety of isolates was observed in the root endophytic community of *Populus* compared to that of maize. Blain et al. (2017) characterized the endophytic root bacteria associated with the natural vegetation growing on a hydrocarbon-contaminated site. In agreement with our results, the authors found that the bacterial orders more represented within these communities were Rhizobiales, Pseudomonadales, Burkholderiales, Sphingomonadales and Actinomycetales. Members of these orders seem to be well adapted to PAH contaminated soils and useful for remediation strategies (Blain et al., 2017).

Bioaugmentation with PGP and PAH-degrading bacterial strains has been shown to improve dissipation of PAHs in contaminated soils. Recent studies have identified rhizosphere and endophytic bacteria which have the potential to degrade PAH and are widely distributed in the organs and rhizosphere of plants growing in PAH-polluted sites (Baoune et al., 2018; Pawlik et al., 2020). Nevertheless, studies on endophytes or rhizosphere bacteria with PAH degradation capacity are still rare. In the present work, several bacterial strains were evaluated for their ability to degrade phenanthrene, fluoranthene and pyrene. As was previously mentioned, all the strains selected to evaluate their capacity to degrade PAHs showed at least two PGP traits and were tolerant to at least one TM. Among the fluoaranthene degrading bacteria, the actinobacterium *Streptomyces* sp. Sk2012 was found to be able to produce the highest IAA amount among all the isolates assessed. The IAA is the most important phyto-hormone responsible for root development stimulating plant cell proliferation and plant cell elongation (Glick, 2014). Another fluoranthene

degrading actinobacterium, *Rhodococcus* Sk12.6, was the only soil strain capable of solubilizing phosphate, a rare trait for members of the phylum as they are rarely described for their ability to produce the organic acid responsible for the solubilization of phosphate (Jog et al., 2014). It is important to note that two endophytes (*Sphingomonas* SR62 isolated from *Populus* and *Rhizobium* sp. MR28 isolated from maize) were among the best pyrene degrading strains. According to Afzal et al. (2014), endophytic bacteria with PGP and pollutant degradation traits performed better than bacteria with only one of these characteristics. In addition, the PAH-degrading activity could be considered itself as a PGP characteristic, because contaminants, have a negatively effect on plant development.

A wide range of bacteria are able to degrade low molecular weight PAHs, while relatively few microorganisms are able to degrade high molecular weight recalcitrant PAHs (Ghosal et al., 2016). Interestingly, although fluoranthene and pyrene have been used as a model of high molecular weight PAHs, several isolates studied here were able to dissipate these compounds. On the other hand, one of the most frequently cited and generally accepted constraints for PAHs bioremediation is the low water solubility of these compounds which results in low bioavailability for pollutant-degrading organisms (Makkar and Rockne 2003). It has been hypothesized that the synthesis and release of bioemulsifying compounds is one of the numerous microbial adaptations to access poorly soluble substrates such as hydrocarbons (Uzoigwe et al., 2015). In the present work, the cell-free growth medium of the pyrene-degrading endophyte *Rhizobium* sp. MR28 showed higher capacity for solubilising PAHs from contaminated Pierrelaye soil than sterile culture medium used as control. Hickey et al. (2007) found that biosurfactants considerably increased the desorption of PAHs from soil and improved their biodegradation by *Pseudomonas alcaligenes* PA-10. Kuppusamy et al. (2017) isolated two strains belonging to the genera *Bacillus* and *Pseudomonas* from animal faeces which completely degraded phenanthrene, pyrene and benzo-a-pyrene after 6,

7 and 40–50 days, respectively. These isolates were found to produce a biosurfactant of rhamnolipid nature upon PAH degradation.

In a phytoremediation scenario, endophytic bacteria with specific metabolic capabilities might be able to degrade organic pollutants and reduce both phytotoxicity and evapotranspiration of volatile compounds. For instance, Sheng et al. (2008) found that the endophytic *Enterobacter* sp. 12J1 could efficiently remove pyrene and improve maize and wheat growth in pyrene contaminated soils. Recently, Pawlik et al., (2020) demonstrated that in an aged petroleum hydrocarbon polluted soil, the method of inoculation of endophytic strains determines the efficiency of the phytoremediation process.

The inoculation of plants grown in mixed contaminated sites such as Pierrelaye with the group of bacterial strains studied could be a successful phytotechnology to improve SRC-based phytomanagement options.

5. Conclusion

The cultivable bacterial diversity associated with trees under different SRC-based phytomanagement options in a PAH and TM contaminated site in Pierrelaye (France) was assessed.

The collections of isolates were dominated by Proteobacteria and Actinobacteria. Differences in taxonomic composition were observed between plant species and depending on the phytomanagement option. A greater variety of taxa was found in the endophytic community of *Populus* than inside the root of *Zea mays*.

On the basis of the PGP characteristics analysed, the tolerance to TM and the ability for increasing the dissipation of different PAHs, the following strains could be selected as potential interesting candidates for further re-inoculation experiments: *Bacillus* sp. B32.23, *Streptomyces*

sp. Sk.2012, *Rhodococcus* sp. Sk12.6, *Microbacterium* sp. Sk11.4, *Bacillus* sp. Sk1.23, *Bacillus* sp. Sk2.3, *Sphingomonas* sp. SR62, *Pseudomonas* sp. MR47 and *Rhizobium* sp. MR28.

Acknowledgements

This work was supported by the Agence Nationale de la Recherche (ANR) under the PRECodd (ANR-06-ECOT-O15-01-PHYTOPOP) and the Blanc International (ANR-10-INTB-1703-01-BIOFILTREE) projects.

This article is dedicated to the memory of our dearly missed colleague and friend Petra Kidd (1972-2020).

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Figure 1. Mean concentrations of PAHs detected in the culture medium after growing a selected group of strains from bulk soil, rhizosphere and roots of poplar and maize plants from Pierrelaye. (A) phenanthrene; (B) fluoranthene; (C) pyrene. The mean concentrations of non-inoculated samples (NI) are indicated by a continuous line. Bars with an asterisk indicate that they were statistically different from the NI control ($p < 0.05$, Tukey's post-test). MR and SR: endophyte strains from maize and poplar Skado (respectively) from the BIOFILTREE plots; Sk: rhizosphere strains from poplar Skado from PHYTOPOP plots; B: strain from bulk soil (PHYTOPOP plots).

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Figure 2. PAHs detected before use the cell-free culture supernatant of *Rhizobium* sp. MR28 as soil extractant, isolated from the Pierrelaye site. Asterisk indicates significant differences among treatments ($p < 0.05$ Tukey's post-test)

828 Table 1. Description of samples from Pierrelaye soil which were used for the isolation of
829 bacterial strains.

Plot and sample name	Description*
<i>Phytopop</i>	
Bulk soil	Isolation of soil bacteria
Poplar Skado	Isolation of rhizobacteria
<i>Biofiltree</i>	
Bulk soil	Isolation of soil bacteria
Maize	Isolation of rhizobacteria & root endophytes
Poplar Skado NI**	Isolation of rhizobacteria & root endophytes
Poplar Skado INOC**	Isolation of rhizobacteria
Poplar Skado/ Alder	Isolation of rhizobacteria

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831 *Five replicates of each sample were studied

832 **NI: non-inoculated plots with mycorrhiza; INOC: inoculated plots with mycorrhiza

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836 Table 2. Taxonomic affiliation of culturable strains isolated from bulk soil, rhizosphere and

Plot name	PHYTOPOP		BIOFILTREE						
Treat ment	Bulk soil PHYTOPOP	<i>Populus</i> rhizospher e	Bulk soil BIOFIL TREE	<i>Populus</i> rhizosphere			<i>Zea mays</i> rhizos phere	<i>Zea mays</i> root	<i>Populus</i> root
Phylu m	Actinobacteria								
Total (%)	47%	51%	15%	48%	47%	54%	31%	17%	32%
Gene ra (%)	Strepto myces (25%)	Streptomyc es (29%)	Strepto myces (8%)	Streptomyc es (23%)	Strepto myces (13%)	Strepto myces (25%)	Strepto myces (19%)	Microba cterium (17%)	Strepto myces (13%)
	Rhodoc occus (4%)	Rhodococc us (8%)	Rhodoc occus (8%)	Rhodococc us (10%)	Rhodoc occus (7%)	Rhodoc occus (4%)	Rhodoc occus (6%)		Microba cterium (16%)
	Arthroba cter (2%)	Arthrobact er (2%)		Arthrobact er (10%)	Arthroba cter (7%)	Arthroba cter (4%)	Arthrob acter (6%)		Mycoba cterium (3%)
	Nocardi oides (4%)	Nocardioi d es (6%)		Promicrom onospora (3%)	Nocardi oides (7%)	Agromy ces (7%)			
	Microco ccus 2%	Promicrom onospora (2%)		Lentzea (3%)	Microco ccus (3%)	Microba cterium (4%)			
	Kribbell a (2%)	Longispora (2%)			Microba cterium (7%)	Kribbella (7%)			
	Longisp ora (2%)	Okibacteriu m (2%)			Microlun atus (3%)	Knoellia (4%)			
	Mycoba cterium (6%)	Williamsia (2%)							
	Phylu m	Firmicutes							
Total (%)	39%	27%	31%	13%	30%	14%	13%	8%	23%
Gene ra (%)	Bacillus (33%)	Bacillus (24%)	Bacillus (31%)	Bacillus (13%)	Bacillus (27%)	Bacillus (4%)	Bacillus (12%)	Paeniba cillus (8%)	Bacillus (16%)
	Paeniba cillus (6%)	Paenibacill us (3%)			Paeniba cillus (3%)	Paeniba cillus (7%)			Paeniba cillus (6%)
						Lysiniba cillus (4%)			

837 roots of *Populus* and maize growing in the PHYTOPOP and BIOFILTREE field trials of

838 Pierrelaye (France).

839 *NI: non-inoculated plots with mycorrhiza; INOC: inoculated plots with mycorrhiza

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Plot name	Phytopop		Biofiltree						
Treatment	Bulk soil Phytopop	<i>Populus</i> rhizosphere	Bulk soil Biofiltree	<i>Populus</i> rhizosphere			<i>Zea mays</i> rhizosphere	<i>Zea mays</i> root	<i>Populus</i> root
				NI*	INOC*	Intercropped <i>Alnus</i>			

841 Table 2 (continuation)

Phylum	Proteobacteria								
Total (%)	10%	19%	31%	29%	20%	32%	44%	75%	45%
Class	α-Proteobacteria								
Genera (%)	Methylobacterium (2%)	Methylobacterium (2%)	Ensifer (15%)	Pseudochrobactrum (3%)	Sphingopyxis (3%)	Rhizobium (4%)	Rhizobium (6%)	Rhizobium (17%)	Rhizobium (3%)
	Microvirga (2%)	Microvirga (2%)		Ensifer (3%)		Devosia (4%)		Devosia (3%)	
	Ensifer (2%)	Bosea (2%)		Sphingomonas (3%)		Mesorhizobium (4%)		Bosea (6%)	
		Sphingomonas (2%)						Sphingomonas (3%)	
		Roseomonas (2%)						Tardiphaga (10%)	
		Phylobacterium (2%)							
Class	β-Proteobacteria								
Genera (%)	Variovorax (2%)	Cupriavidus (3%)	Variovorax (8%)	Variovorax (6%)	Variovorax (3%)	Variovorax (11%)	Variovorax (19%)	Achromobacter (8%)	
	Duganella (2%)	Massilia (2%)		Massilia (3%)		Achromobacter (4%)	Duganella (6%)		
Class	γ-Proteobacteria								
Genera (%)		Pseudomonas (3%)	Lyso bacter (8%)	Pseudomonas (3%)	Pseudomonas (3%)	Unclassified Oxal * (4%)	Pseudomonas (6%)	Pseudomonas (17%)	Pseudomonas (16%)
		Pseudoxanthomonas (2%)		Pseudoxanthomonas (6%)	Pseudoxanthomonas (7%)	Shinella (4%)	Lysobacter (6%)		Steroidobacter (3%)
				Lysobacter (3%)					

*NI: non-inoculated plots with mycorrhiza; INOC: inoculated plots with mycorrhiza

Plot name	Phytopop		Biofiltree			
Treatment	Bulk soil Phytopop	<i>Populus</i> rhizosphere	Bulk soil Biofiltree	<i>Populus</i> rhizosphere		
				NI*	INOC*	Intercrop <i>Alnus</i>
Phylum	Bacteroidetes					
Total (%)	4%	3%	23%	10%	3%	
Genera (%)	Chryseobacterium (2%) Terrimonas (2%)	Chryseobacterium (3%)	Chryseobacterium (15%)	Pedobacter (8%) Chitinophaga (3%)	Flavobacterium (6%)	Pedobacter (3%)

Table 2 (continuation)

*NI: non-inoculated plots with mycorrhiza; INOC: inoculated plots with mycorrhiza

Table 3. Bacterial strains isolated from the Pierrelaye field trials and selected for PAHs degradation tests, based on their plant growth promoting traits, surfactant production and metal resistance ability. They were isolated from bulk soil, from the rhizosphere of poplar or maize, or from the endosphere of poplar.

Isolate	Identification (16S rDNA) and ID*	Origin	Sider.	Surf.	SoluP	Cd 0.1 mM	Cu 1mM	Pb 1 mM	Zn 2.5 mM	IAA (µg/mL)
SR15	<i>Bacillus</i> MW036883	Endophyte poplar	+	+	-	-	-	-	+	6.58
SR18	<i>Bacillus</i> MW036882	Endophyte poplar	-	+	-	+	-	+	+	2.52
SR62	<i>Sphingomonas</i> MW036888	Endophyte poplar	-	-	+	+	-	+	+	10.8
MR28	<i>Rhizobium</i> MW036864	Endophyte maize	+	+	+	+	+	+	-	1.38
MR47	<i>Pseudomonas</i> MW036871	Endophyte maize	-	+	+	+	-	-	-	2.89
Sk2.3	<i>Bacillus</i> MW037076	Rhizosphere poplar	-	+	-	-	-	+	-	2.07
Sk12.6	<i>Rodococcus</i> MW037075	Rhizosphere poplar	-	+	+	+	+	+	+	5.35
Sk20.12	<i>Streptomyces</i> MW037077	Rhizosphere Poplar	+	-	-	+	-	-	+	12.13
Sk1.23	<i>Bacillus</i> MW036928	Rhizosphere Poplar	+	+	-	+	-	-	-	5.14
Sk11.4	<i>Microbacterium</i> MW037063	Rhizosphere poplar	+	+	-	+	-	-	-	0.81
B32.24	<i>Bacillus</i> MW037184	Bulk soil	+	+	-	+	-	+	-	8.08

Sider: siderophore-producers; Surf: biosurfactant-producers; PSolub: inorganic P solubilizers;

IAA: indoleacetic acid-producers

(*) ID: GenBank Accession number