EFFECTS OF PLANT-MICROBIOME INTERACTIONS ON PHYTO- AND BIO-REMEDIATION CAPACITY

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EFFECTS OF PLANT-MICROBIOME INTERACTIONS ON PHYTO- AND BIO-REMEDIATION CAPACITY

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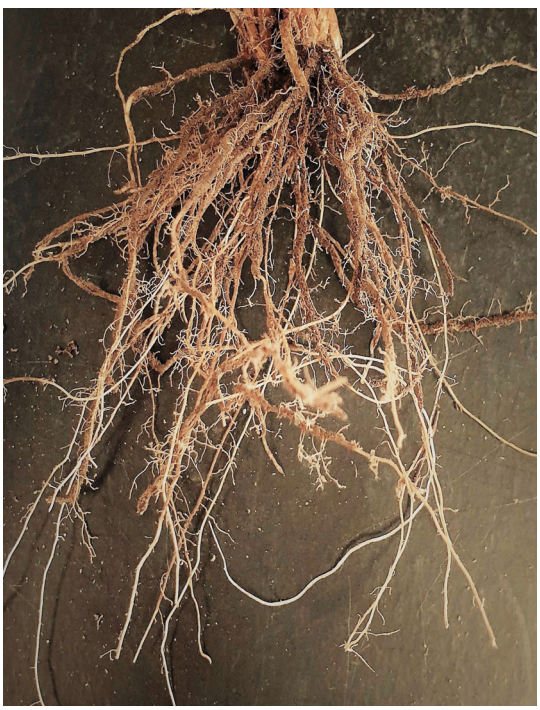

Root apparatus and its interactions with the surrounding environment.
Image by Francesco Guarino.

Bio- and Phyto-remediation have been seen in the past by scientists as two independent "green technologies", employing separately either microorganisms (bacteria and/or fungi), or plants to reclaim polluted soil, water and air. However, in the last decade, the idea has emerged that microorganisms and plants can and have to work synergistically to obtain better results in terms of reclamation performances; hence these two technologies have to be considered the different sides of the same coin. Therefore a single term can be used to refer to both of these technologies: bio-remediation.

The Research Topic articles, collected in this eBook, report the isolation and characterization of bacteria, fungi and endophytes with Plant Growth Promoting features. Moreover, some of these microorganisms have been added to plants to ameliorate their health status when grown in polluted soils and waters; or to realize and improve the water reclamation performance of Constructed Wetlands, a very interesting application of the bio-remediation process.

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frontiers in Plant Science

EDITORIAL
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Editorial: Effects of Plant-Microbiome Interactions on Phyto- and Bio-Remediation Capacity

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Keywords: bio-remediation, interaction, PGPR, endophytes, mycorrhiza, NGS, constructed wetlands

Editorial on the Research Topic

Effects of Plant-Microbiome Interactions on Phyto- and Bio-Remediation Capacity

INTRODUCTION

Bio-remediation is a process that looks at plants and microorganisms (bacteria and fungi) as natural systems that are able to degrade, transform, and even accumulate large quantities of contaminants that are found, naturally or have been introduced into the environment. Emerging evidence indicates that this is possible thanks to strong plant-microorganism interactions that take place preferentially at soil level (Masciandaro et al., 2013).

In the past, plants and microorganisms have been employed separately for bio-remediation. However, in recent years, several studies have demonstrated that they can act synergistically to improve the remediation process of different matrices, such as water, soil, and air (Khan et al., 2018). The rhizosphere is the thin layer of soil (1–2 mm) adherent to the rhizoplane where the majority of soil microbiota resides (Walker et al., 2003). Plants release large amounts of photosynthesis byproducts (up to 30%) into the soil that nourish rhizosphere microorganisms (Canarini et al., 2019). Microorganisms in turn are able to improve cation exchange capacity (CEC) altering soil water pH, and release siderophores and phosphatases, which improve plant nutrition, and even secrete enzymes that are able to reduce plant stress, such as ACC deaminase (Pilon-Smits, 2005). Some fungi have established a broad, strong, and fundamental relationship with plants for more than 400 million years, the so-called endo- and ecto-mycorrhizal symbioses (Remy et al., 1994). Endomycorrhiza have been used to improve phosphorus removal using trees (e.g., willow and poplar) as vegetation filters (Fillion et al., 2011). While other fungi and bacterial species live inside plant tissues and are defined for this characteristic as "endophytes" (Zhang et al., 2019).

Plants and microorganisms have been recently used to ameliorate different phytoremediation processes, such as phyto-extraction (Sessitsch et al., 2013; Vigliotta et al., 2016), phyto-degradation (Feng et al., 2017), and phyto-stimulation (Cicatelli et al., 2017). All these different strategies are fundamental for reclamation of polluted water by means of the constructed wetlands (CWs), a very promising application of bio-remediation developed in the last decade (Wu et al., 2015). In fact, in CWs plants and microorganisms fruitfully interact in the bio-remediation of urban, livestock and industrial wastes. The aim of this research topic was to highlight this specific field of Plant Sciences which, even though very promising, is still in its infancy. The research articles published here address many aspects of bio-remediation, but they all provide evidence that isolated bacteria and fungi, growing in different soils, contaminated or not, can help plants to survive in harsh environments that are often polluted by organic compounds or by inorganic elements.

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In some of the articles published in this eBook, Next Generation Sequencing (NGS) techniques (Derocles et al., 2018) have been applied to study the rhizosphere microbiome. These molecular tools, introduced within the last decade, have not yet fully reached their potential; however, already they allow scientists to discover new and unknown taxa of microorganisms.

The articles published in this eBook focus on plant-bacteria and/or plant-fungi interactions, and the effects that soil pollutants have on the plant microbiota.

Root bacterial communities of several plant species were investigated with the goal of understanding how certain pollutants, present in the soil or water, affect them in terms of biodiversity and composition. Five of the articles of the eBook illustrate how many bacterial strains, belonging to different species or taxa (e.g., *Bacillus*, *Serratia,* etc.), and from which plant species, growing on different soil conditions, have been isolated from the rhizospere. Several of these bacteria strains showed resistance to inorganic pollutants of natural (Caneschi et al. - Brazilian Ironstone) and/or anthropogenic origin (Mesa-Marín et al. - heavy metals, Zappellini et al.- red gypsum landfill), or to organic compounds (Thijs et al. - TNT, Cao et al. - atrazine). The goal of the research reported in these articles was that to identify the ones showing plant growth promoting characters (PGP-Kloepper and Schroth, 1978) in the presence of soil pollutants (Segura et al., 2009). All of the identified bacterial strains were biochemically characterized for their PGP capabilities, such as to solubilise inorganic phosphate, or to produce siderophores, organic acids, IAA, and/or other compounds. The isolates were also molecularly profiled using hyper-variable regions of the 16S rDNA in order to relate them phylogenetically to known bacteria for which these sequences have been already deposited in public databases. Some bacterial strains, either individually or in consortium, exhibited high bio-remediating potential. In particular, they were shown to: (i) reduce plant stress in harsh environments; (ii) increase the uptake, translocation, accumulation, and/or modification of the contaminants to the aerial parts.

Some of the aforementioned articles demonstrated that the addition of microorganisms to the polluted soil had a significant effect on plant biomass production, which is ultimately the most indicative parameter to evaluate the efficacy of the bio-remediation treatment.

Three of the articles published here deal with the effect of mycorrhization on the ability of forest trees (Nadeau et al. - White spruce, Gil-Martínez et al. - Holm Oak) to counteract metal toxicity. Plants were exposed to pollutants of different origin including: trace elements (TE), heavy metals, as in the case of waste rocks (WRs), or fine tailings (FTs) commonly present in abandoned mine sites. Mycorrhization, in particular ecto-mycorrhization, occurs in woody pioneer species such as those mentioned above. Holm oak, however, cannot be considered a forest pioneer but rather a late successional species. Ecto-mycorrhiza improve phosphorus and nitrogen uptake by trees and enhance resistance to diverse stress factors, such as drought, salinity, heavy metals, and pathogens. As in the case of PGPRs, fungal communities are also strongly modified by soil contaminants; in fact, environmental constraints lead to the selection of specific and environmental well-adapted taxa.

Two of the 11 articles deal with endophytic bacteria or fungi. Their presence in herbaceous plants grown in soil containing organic pollutants (Lumactud and Fulthorpe - petroleum hydrocarbons), or metalloids (Lindblom et al. - *Alternaria tenuissima* and selenium) was studied in detail. In the case of endophytic bacteria, it was confirmed once again, as for rhizosphere bacteria, their PGP character and their ubiquity, since they were present in different plant species growing on the same contaminated site. Even though fungi were the first endophyte to be discovered at the beginning of the nineteenth century (De Bary, 1866), little is still known about plant-fungus interactions. Although the article published here on this topic refers to an unusual case (a fungal endophyte of a selenium hyperaccumulator), it confirmed that fungal colonization of plant tissues, organs and reproductive structures (seeds) favors the host survival due to the production of compounds that protect them from high contents of metals or metalloids present in the soil. The endophytes can also protect plants from herbivores, and increase nutrient absorption and biomass.

An interesting application of bio-remediation is that of the Constructed Wetlands. Similar to other bio-remediation strategies, which include bio-degradation, bio-stimulation and bio-augmentation, both rhizosphere and/or endophytic microorganisms can be utilized in establishing CWs. In fact, they have been widely studied, and, in many CWs, the synergic activities of plants and microorganisms (Syranidou et al. - CWs, emerging pollutants and bio-augmentation) have been clearly demonstrated. Therefore, CWs can be considered a realistic alternative to the more traditional techniques employed to reclaim waters polluted by various compounds introduced into the environment by urban, agricultural, and industrial activities.

AUTHOR CONTRIBUTIONS

SC is responsible for organizing the materials and writing the editorial. AC, NF, and PR are responsible for its reading and revising.

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Brazilian Ironstone Plant Communities as Reservoirs of Culturable Bacteria With Diverse Biotechnological Potential

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Extensive mineral extractivism in the Brazilian Iron Quadrangle (IQ) region has destroyed large areas of land, decimating plant species, and their associated microbiota. Very little is known about the microbiota of the region; hence, cultivable bacteria associated with plants of its soils were investigated for their biotechnological potential. Samples were collected from nine plant species and six soils, and 65 cultivable bacterial isolates were obtained. These represent predominantly gram-positive bacilli (70%) capable of producing amylases (55%), proteases (63%), cellulases (47%), indole acetic acid (IAA) (46%), siderophores (26%), and to solubilize phosphate (9%). In addition, 65% of these were resistant to ampicillin, 100% were sensitive to tetracycline, and 97% were tolerant to high arsenic concentrations. Three isolates were studied further: the isolate FOB3 (*Rosenbergiella* sp.) produced high concentrations of IAA *in vitro* in the absence of tryptophan – shown by the significant improvement in plant germination and growth rate where the isolate was present. For isolates C25 (*Acinetobacter* sp.) and FG3 (*Serratia* sp.), plasmids were purified and inserted into *Escherichia coli* cells where they modified the physiological profile of the transformed strains. The *E. coli*::pFG3B strain showed the highest capacity for biofilm production, as well as an increase in the replication rate, arsenic tolerance and catalase activity. Moreover, this strain increased DNA integrity in the presence of arsenic, compared to the wild-type strain. These results help to explain the importance of bacteria in maintaining plant survival in ferruginous, rocky soils, acting as plant growth promoters, and to highlight the biotechnological potential of these bacteria.

IMPORTANCE

The Iron Quadrangle region is responsible for ∼60% of all Brazilian iron production and, at the same time, is responsible for housing a wide diversity of landscapes, and consequently, a series of endemic plant species and dozens of rare species – all of which have been poorly studied. Studies exploring the microbiota associated with

these plant species are limited and in the face of the continuous pressure of extractive action, some species along with their microbiota are being decimated. To understand the potential of this microbiota, we discovered that cultivable bacterial isolates obtained from plants in the ferruginous rocky soil of the Iron Quadrangle region have diverse biotechnological potential, revealing a genetic ancestry still unknown.

Keywords: canga, plasmids, PGPB, protection against ROS, arsenic tolerance

INTRODUCTION

The ironstone plant communities are hotspots for biodiversity and endemism in metal-rich regions. Owing to their restricted distribution and association with the main deposits of iron ore in the world, these hotspots are among some of the most threatened vegetations (Gibson et al., 2010). In southeastern Brazil, these plant communities occur mainly in the Iron Quadrangle (IQ): a mineral province formed by old, geologically complex lands, distributed over an area of $\sim$ 7,200 km^2. This region presents heterogeneity of reliefs, phytophysiognomies, and landscapes, intensely threatened by the high concentration of mineral complexes, which, in turn, generate technological and environmental challenges for the management of immense environmental liabilities, including the recovery of degraded areas (Dorr, 1969; Jacobi et al., 2011; Sonter et al., 2014; Carmo et al., 2017). These attributes have classified the IQ as an area of extremely high importance for the conservation and sustainable use of Brazilian biodiversity (MMA, 2007).

The ironstone outcrop plant communities are associated with iron duricrusts (known as "cangas"), formed from the intense weathering of banded iron formations (BIFs) over millions of years. These plant communities are exposed to conditions that determine severe restrictions for the establishment of plants, such as high ultraviolet (UV) exposure; extreme scarcity of soil, which makes fixing and root growth more difficult; high concentrations of metals, specifically Fe, Mn, and Al; acidity and very low nutrient availability (Jacobi et al., 2007; Schaefer et al., 2015; Carmo and Jacobi, 2016). Moreover, developed soils of metalliferous substrates can promote the selection of plant species resistant to high levels of metals, by means of physiological and morphological adaptations (Vincent and Meguro, 2008).

Plant adaptive mechanisms to abiotic stresses (e.g., salinity, heat, water scarcity, and metal concentration) are complex, involving the reception and translation of signals, followed by genetic and physiological responses. Among the most common adaptive mechanisms are the production of osmolytes, alterations in water transport and the elimination of reactive species (RS) (Rodriguez et al., 2008). However, this adaptation goes beyond genetic and physiological characteristics, depending also on the presence of endophytic or rhizosphere microorganisms, which play a fundamental role in this adaptive process (Souza et al., 2015).

The soil is characterized as an environment replete with microorganisms, including bacteria, fungi, actinomycetes, protozoa, viruses, and algae. Among these, bacteria are the most abundant, and can maintain both beneficial and harmful ecological relationships with plants. Bacteria that benefit plants are called plant growth promoting bacteria (PGPB). These benefits include direct involvement with mechanisms, such as the acquisition of mineral resources (iron, nitrogen, phosphate) or even by the production of molecules that mimic phytorhormones (Glick, 2012; Souza et al., 2015). In addition, PGPB can also indirectly benefit the plant community by reducing the harmful effects of phytopathogens or even by removing xenobiotics from numerous compounds harmful to plant growth (Glick, 2012; Kong and Glick, 2017). Knowledge of the microorganisms of these ancient ecosystems is still incipient.

From an ecological perspective, plants of the IQ are under imminent risk of decimation by anthropic actions, mainly mineral extraction (Jacobi et al., 2011). In this context, loss of flora would most likely induce the loss of specialized microbiota. This makes the IQ an excellent area of study for the prospection of neglected microorganisms.

In order to reduce this gap in biological knowledge, bacteria associated with nine ironstone plant species of the IQ were investigated. After cultivation, isolated bacteria were submitted to a series of biochemical and morphological characterization tests, that allowed the identification of the biotechnological potential in this unexplored genetic heritage.

MATERIALS AND METHODS

Study Area and Sampling of Soil and Plants

In the Iron Quadrangle (IQ), southeastern Brazil (**Figure 1**), the BIFs of the Itabira geological group (2.6–2.1 Ga, paleoproterozoic) and associated cangas stand out (Rosière and Chemale, 2000). In this region, a subtropical climate predominates, which, according to Köppen climate classification, is characterized by dry winters and rainy summers. The average temperature of the coldest month is 18°C and the hottest month is 22°C. The samples were collected from cangas (iron duricrusts) located in Sinclinal Moeda, Nova Lima and Jardim Canada, Minas Gerais (latitude 20°15′83″S, longitude 43°97′41″W). Six samples of soil and nine species of plants were collected: *Lychnophora pinaster* Mart., *Stachytarpheta glabra* Cham., *Baccharis reticularia* DC., *Gomphrena arborescens* L.f., *Symphyopappus compressus* (Gardner) B. L. Rob., *Lagenocarpus rigidus* Nees, *Pleroma heteromallum* D. Don (D. Don), *Peixotoa tomentosa* A. Juss., and *Vellozia compacta* Mart. ex Schult. & Schult.f. (**Figure 1** and **Table 1**).

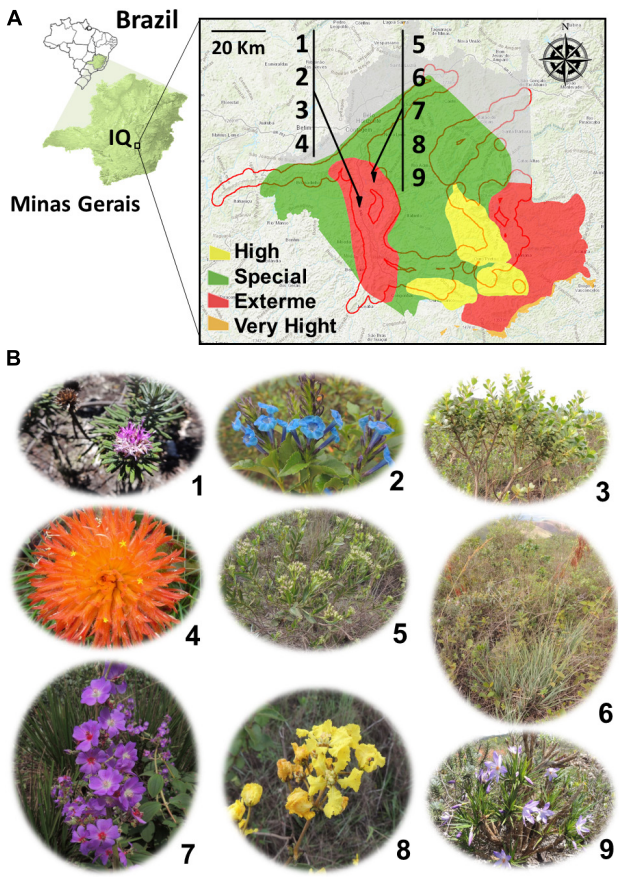

FIGURE 1 | (A) Localization of collection areas for the morphological and biochemical characterization of bacterial isolates obtained from plant species and soils from the Iron Quadrangle (MG) region, southeastern Brazil. The numbers 1–9 indicate the plant species sampled and identified in **Table 1** and shown in **(B)**. The red contour determines the edges of the Iron Quadrangle while the colors yellow, red, orange, and green the degree of importance of the region for preservation of the flora of the Minas Gerais state.

Isolation and Preservation of Microorganisms

The plant samples (stems, roots, and leaves) were initially washed with distilled water. Then, they were immersed in 2.5% sodium hypochlorite solution for 2 min, then washed in 70% ethanol for 2 min. Finally, the samples were washed with sterile distilled water and placed on plates containing Luria Bertani (LB) agar, pH 7.0, containing 0.03 mg/l of antifungal methyl thiophanate. The samples were incubated at 28°C for 3–4 days for bacterial growth. Isolated colonies were collected with sterile toothpicks and grown on nutrient agar (NA). After isolation, the bacterial cultures grown in liquid LB were supplemented with 30% glycerol and stored at −80°C.

Construction of a Matrix Plate for Biochemical Assays

For the large-scale assays, a 96 well array was constructed in which all isolates were grown in casein nutrient agar (CN) for 48 h at 28°C, then transferred using a multi-inoculator to Petri dishes or 96-well plates (according to the related biochemical assay).

Indole Acetic Acid (IAA) Production

To analyze IAA production, the colorimetric method adapted from BRIC and collaborators was used (Bric et al., 1991). The isolates were grown in a U-shaped bottom 96-well plate (Costar^{TM}) containing CN enriched with 5 mM L-tryptophan for 2 days at 28°C. The plates were centrifuged at 0.7 × *g* and 100 µl of the supernatant was transferred to a new 96 well plate. Afterward, 100 µl of Salkowski solution (2 mL 0.5 M $FeCl_3.6H_2O$/l of $HClO_4$) was added to each well and allowed to stand for 2 h. Subsequently, the color change from yellow to brown was verified, indicating hormone production. A calibration curve with different concentrations of IAA was constructed and the absorbance of each sample was quantified by a spectrophotometer with absorbance at 530 nm.

Amylase and Cellulase Production

To determine amylase and cellulase production, yeast nitrogen base (YNB) agar medium containing 6.7 g/l YNB, 2.0 g/l soluble starch, 0.5 g/l cellobiose, 1 g/l carboxymethyl cellulose, and 20 g/l of agar was used. The reactants were solubilized in warm water, the pH adjusted to 7.0, autoclaved for 15 min at 120°C and then poured into 150 mm × 20 mm Petri dishes. Using a multi-inoculator, the isolates from the matrix plate were transferred and grown in this medium for 24 h at 28°C. To reveal the production of amylases, the plate was covered with a lugol solution for 5 min. This solution was then discarded and the production of a transparent halo in the culture medium was evaluated (Strauss et al., 2001). To evaluate cellulase production, a solution of 0.03% Congo Red was added, for 10 min, to the culture medium, followed by washing with a 1 M NaCl solution. Cellulase producing bacteria formed a clear halo on a red background (Strauss et al., 2001).

Siderophore Production

The detection of siderophore production was based on the Schwyn and Neilands method (Schwyn and Neilands, 1987). From the matrix plates, 3 µl of a solution of isolates (0.6–0.8 OD_{600nm}) was transferred to 96-well plates containing 200 µl of CN medium, previously treated with 3% (w/w) 8-hydroxyquinoline. After 2 days at 28°C, the change in color from blue to yellow around the colony indicated the production of siderophores.

Phosphate Solubilization

For phosphate solubilization assays, the NBRIP (National Botanical Research Institute's Phosphate) culture medium was used (Nautiyal, 1999). The isolates were originally grown on a matrix plate containing CN medium, and then ∼3 µl of a solution of isolates (0.6–0.8 OD_{600nm}) was transferred to 150 mm × 20 mm Petri dishes containing NBRIP culture medium. After 3 days at 28°C, the evaluation of the formation of a clear halo in the medium indicated the ability of phosphate solubilization by the isolate.

TABLE 1 | Plants and soils sampled, and bacterial isolates obtained.

	Plant samples			
ID	**Scientific name**	**Canga location**	**Source**	**Isolate name**
1	*Lychnophora pinaster* Mart.	Mãe d'agua (Sinclinal Moeda)	Flower	FA1
	Lychnophora pinaster Mart.	Mãe d'agua (Sinclinal Moeda)	Root	RA1
	Lychnophora pinaster Mart.	Mãe d'agua (Sinclinal Moeda)	Root	RA2
	Lychnophora pinaster Mart.	Mãe d'agua (Sinclinal Moeda)	Root	RA3
	Lychnophora pinaster Mart.	Mãe d'agua (Sinclinal Moeda)	Root	RA4
	Lychnophora pinaster Mart.	Mãe d'agua (Sinclinal Moeda)	Root	RA5
	Lychnophora pinaster Mart.	Mãe d'agua (Sinclinal Moeda)	Rhizosphere	RizA1
	Lychnophora pinaster Mart.	Mãe d'agua (Sinclinal Moeda)	Rhizosphere	RizA2
2	*Stachytarpheta glabra* Cham.	Mãe d'agua (Sinclinal Moeda)	Flower	FG1
	Stachytarpheta glabra Cham.	Mãe d'agua (Sinclinal Moeda)	Flower	FG2
	Stachytarpheta glabra Cham.	Mãe d'agua (Sinclinal Moeda)	Flower	FG3
3	*Baccharis reticularia* DC.	Mãe d'agua (Sinclinal Moeda)	Flower	FB1
	Baccharis reticularia DC.	Mãe d'agua (Sinclinal Moeda)	Flower	FB2
	Baccharis reticularia DC.	Mãe d'agua (Sinclinal Moeda)	Flower	FB3
	Baccharis reticularia DC.	Mãe d'agua (Sinclinal Moeda)	Flower	FB4
	Baccharis reticularia DC.	Mãe d'agua (Sinclinal Moeda)	Leaf	FoB1
	Baccharis reticularia DC.	Mãe d'agua (Sinclinal Moeda)	Leaf	FoB2
	Baccharis reticularia DC.	Mãe d'agua (Sinclinal Moeda)	Leaf	FoB3
4	*Gomphrena arborescens* L.f.	Mãe d'agua (Sinclinal Moeda)	Leaf	FPT1
	Gomphrena arborescens L.f.	Mãe d'agua (Sinclinal Moeda)	Flower	IPT1
	Gomphrena arborescens L.f.	Mãe d'agua (Sinclinal Moeda)	Flower	IPT2
	Gomphrena arborescens L.f.	Mãe d'agua (Sinclinal Moeda)	Flower	IPT3
	Gomphrena arborescens L.f.	Mãe d'agua (Sinclinal Moeda)	Root	RPT1
	Gomphrena arborescens L.f.	Mãe d'agua (Sinclinal Moeda)	Root	RPT2
	Gomphrena arborescens L.f.	Mãe d'agua (Sinclinal Moeda)	Root	RPT3
	Gomphrena arborescens L.f.	Mãe d'agua (Sinclinal Moeda)	Rhizosphere	RizPT1
	Gomphrena arborescens L.f.	Mãe d'agua (Sinclinal Moeda)	Rhizosphere	RizPT2
	Gomphrena arborescens L.f.	Mãe d'agua (Sinclinal Moeda)	Rhizosphere	RizPT3
	Gomphrena arborescens L.f.	Mãe d'agua (Sinclinal Moeda)	Tubercle	TPT1
	Gomphrena arborescens L.f.	Mãe d'agua (Sinclinal Moeda)	Tubercle	TPT2
	Gomphrena arborescens L.f.	Mãe d'agua (Sinclinal Moeda)	Tubercle	TPT3
	Gomphrena arborescens L.f.	Mãe d'agua (Sinclinal Moeda)	Tubercle	TPT4
	Gomphrena arborescens L.f.	Mãe d'agua (Sinclinal Moeda)	Tubercle	TPT5
	Gomphrena arborescens L.f.	Mãe d'agua (Sinclinal Moeda)	Tubercle	TPT6
5	*Symphyopappus compressus* (Gardner) B. L. Rob.	Jardim Canadá (Nova Lima)	Root	RS1
	Symphyopappus compressus (Gardner) B. L. Rob.	Jardim Canadá (Nova Lima)	Root	RS2
6	*Lagenocarpus rigidus* Nees	Jardim Canadá (Nova Lima)	Leaf	LR1
7	*Pleroma heteromallum* D. Don (D. Don)	Jardim Canadá (Nova Lima)	Leaf	TH1
8	*Peixotoa tomentosa* A. Juss.	Jardim Canadá (Nova Lima)	Flower	P1
	Peixotoa tomentosa A. Juss.	Jardim Canadá (Nova Lima)	Flower	P2
9	*Vellozia compacta* Mart. ex Schult. & Schult.f.	Jardim Canadá (Nova Lima)	Leaf	FCE
	Vellozia compacta Mart. ex Schult. & Schult.f.	Jardim Canadá (Nova Lima)	Root	RCE1
	Vellozia compacta Mart. ex Schult. & Schult.f.	Jardim Canadá (Nova Lima)	Root	RCE2
	Vellozia compacta Mart. ex Schult. & Schult.f.	Jardim Canadá (Nova Lima)	Root	RCE3
	Vellozia compacta Mart. ex Schult. & Schult.f.	Jardim Canadá (Nova Lima)	Root	RCE4
Soil samples				
1	Canga 1	Jardim Canadá (Nova Lima)	Soil	C11
	Canga 1	Jardim Canadá (Nova Lima)	Soil	C12
	Canga 1	Jardim Canadá (Nova Lima)	Soil	C13
2	Canga 2	Jardim Canadá (Nova Lima)	Soil	C21

(Continued)

TABLE 1 | Continued

	Plant samples			
ID	**Scientific name**	**Canga location**	**Source**	**Isolate name**
	Canga 2	Jardim Canadá (Nova Lima)	Soil	C22
	Canga 2	Jardim Canadá (Nova Lima)	Soil	C23
	Canga 2	Jardim Canadá (Nova Lima)	Soil	C24
	Canga 2	Jardim Canadá (Nova Lima)	Soil	C25
3	Canga 3	Jardim Canadá (Nova Lima)	Soil	C31
	Canga 3	Jardim Canadá (Nova Lima)	Soil	C32
	Canga 3	Jardim Canadá (Nova Lima)	Soil	C33
4	Canga 4	Jardim Canadá (Nova Lima)	Soil	C41
	Canga 4	Jardim Canadá (Nova Lima)	Soil	C42
	Canga 4	Jardim Canadá (Nova Lima)	Soil	C43
	Canga 4	Jardim Canadá (Nova Lima)	Soil	C44
	Canga 4	Jardim Canadá (Nova Lima)	Soil	C45
5	Canga 5	Jardim Canadá (Nova Lima)	Soil	C51
6	Canga 6	Jardim Canadá (Nova Lima)	Soil	C61
	Canga 6	Jardim Canadá (Nova Lima)	Soil	C62
	Canga 6	Jardim Canadá (Nova Lima)	Soil	C63
	Total of isolates			65

Evaluation of Antibiotic Tolerance

From the matrix plates, 3 μl of a solution of isolates (0.6–0.8 $OD_{600\ nm}$) was transferred to 96-well plates containing 200 μl of CN liquid medium and either 100 μg/ml ampicillin or 30 μg/ml tetracycline. After 3 days at 28°C, the ability of the bacterial strains to grow in the respective antibiotics was evaluated.

Arsenic Tolerance

From the matrix plates, 3 μl of a solution of isolates (0.6–0.8 $OD_{600\ nm}$) was transferred to 150 mm × 20 mm Petri dishes containing LB agar with sodium arsenite ($NaAsO_2$) at 1, 5, and 10 mM concentrations. After 3 days at 28°C, the ability of the bacterial strains to grow in the respective arsenic concentrations was evaluated.

Morphological Characterization

The isolates were grown in LB medium at 28 ± 2°C and 150 rpm in a shaker for 24 h and then Gram stained. The isolates were observed under an optical microscope with 100× magnification and were then characterized based on their morphology and coloration.

Growth Curves

The bacterial isolates were grown in CN medium for 12 h at 28 ± 2°C and at a rotation of 150 rpm. The bacterial density was standardized for all isolates at $OD_{600\ nm}$ = 1.0 ($\sim 10^8$ cells/ml). The cell suspension was then diluted to 1:500 in CN medium and the optical density monitored over time at 600 nm.

Extraction of Plasmid DNA

An isolated colony was inoculated into CN medium and incubated at 28 ± 2°C for 12 h. Plasmid DNA extraction was performed using the QIAprep Spin Miniprep Kit™ extraction kit. For visualization of the extracted plasmid DNA, the samples were submitted to 0.7% agarose gel electrophoresis.

Bacterial Transformation by Electroporation

Escherichia coli strain Stbl2 [F-endA1 glnV44 thi1 recA1 gyrA96 relA1 Δ (lac-proAB) mcrAΔ (mcrBC-hsdRMSmrr) λ-] was inoculated into CN medium at 28 ± 2°C with a rotation of 150 rpm. One hundred milliliter of CN medium was inoculated with 1 ml of the culture, grown for 12 h and the optical density monitored at 600 nm, to ∼ 0.5. Cells were centrifuged at 1.957 × *g* for 5 min at 4°C. Thereafter, the cell pellet was resuspended in 50 ml of cold sterile distilled water and centrifuged twice, as before. The cells were resuspended in 10 m of cold sterile 10% glycerol, incubated on ice for 5 min and centrifuged as above, followed by the addition of 300 μl of 10% sterile glycerol. Forty microliter of the cell suspension was used for each transformation reaction. Plasmid DNA was added to the cell suspension and incubated on ice for 5 min. The mixture was transferred to an electroporation cuvette and subjected to a 1.8 kV pulse. Immediately, 1 ml of SOC medium (2 g/l tryptone, 0.5 g/l yeast extract, 0.05 g/l NaCl, 0.02 g/l KCl, 0.8 g/l glucose, and 40 mM $MgCl_2$) was added to the cell suspension and incubated at 28 ± 2°C overnight. One hundred microliter of cell suspension from each reaction was plated onto LB solid medium containing the appropriate antibiotics.

Plasmid Stability Assay

The plasmid stability assay was performed using the method of Yao et al. (2015). Plasmid-containing *E. coli* Stbl2 cells were inoculated into LB medium supplemented with 100 μg/ml

ampicillin and incubated at 28 ± 2°C and at a rotation of 150 rpm for 24 h. Bacterial suspension aliquots were withdrawn and normalized to optical density equal to 1.0 ($\sim 10^8$ cells/ml), followed by serial dilutions up to 10^{-5}. One hundred microliter of the last dilution was plated in LB solid medium, some of which were supplemented with 100 μg/ml ampicillin, and after 24 h of growth the colonies were counted. Procedures were repeated for 6 consecutive days.

Colony Forming Units Count

Cells were grown in liquid LB medium for 12 h at 28 ± 2°C and shaking at 150 rpm. Bacterial cell density was standardized for all isolates in OD equal to 1 (10^8 cells/ml). The cell suspension was diluted to 1:500 in LB medium and incubated at 28°C and 150 rpm with 1, 2, and 5 mM of sodium arsenite and the optical density was monitored until reaching a value equal or close to 1. The bacterial suspension was diluted to $\sim 10^{-6}$ cells/ml, followed by plating of 100 μl in solid LB. The plates were incubated at 28 ± 2°C for 24 h and the number of forming units was evaluated.

Plant Growth Test

Twenty Santa Clara 5800 tomato seeds were sown in soil prepared with compound perlite and vermiculite (6:4) and stored at 4°C for 3 days in the absence of light, to accelerate germination. After this period, 10 ml of water containing the isolate, in optical density equal to 1.0 ($\sim 10^8$ cells/ml), was inoculated into the soil. The tomato seeds were planted in soil and transferred to the greenhouse of The Federal University of Ouro Preto for 21 days under controlled conditions (26°C and 60% humidity). Then, the germination rate was evaluated and the aerial parts of the plants were measured.

Autoaggregation Assay

This assay was based on the protocol of Alamuri et al. (2010). Cultures of bacterial isolates, grown overnight in CN medium at 28 ± 2°C, were adjusted to the same optical density (OD = 1.0), and 10 ml samples from each culture were transferred to 20 ml sterile tubes. Initially, all cultures were shaken vigorously for 15 s. The tubes remained static throughout the experiment and 100 μl samples from each tube were taken from ∼ 1 cm from the top of the culture and the optical density was evaluated at $OD_{600\ nm}$ every hour.

Biofilm Production Assay

This assay was based on the O'Toole protocol (O'Toole, 2011). Bacterial isolates were grown in LB liquid medium overnight at 28 ± 2°C. Bacterial density was standardized for all isolates in optical density equal to 1.0 ($\sim 10^8$ cells/ml). The samples were then diluted to 1:10 in LB medium and 100 μl was transferred to a 96-well plate and incubated for 12 h at 28°C. After 12 h growth, the plate was washed with distilled water to remove the cells and allowed to dry for 2 h. Then, 125 μl of 0.1% crystal violet solution (CV) was transferred into each well and allowed to stand for 45 min. After incubation the plate was washed again with distilled water and allowed to dry. Then, 125 μl of 95% ethanol was added to each well and left for 45 min for the complete dissolution of the CV. The absorbance was recorded on a plate reader (Perkin Elmer VICTOR X3, Waltham, MA, United States) with a wavelength of 550 nm. For each isolate, seven replicates were performed.

Quantification of Total Arsenic by X-ray Fluorescence Spectroscopy

To evaluate whether the isolates were removing arsenic from the culture medium, the cells were grown in 50 ml XVM2 medium [NaCl 1.16 g/l, $(NH_4)_2SO_4$ 1.32 g/l, 1 mM $CaCl_2$, 5 mM $MgSO_4$, 0.021 g/l KH_2PO_4, K_2HPO_4 0.055 g/l, $FeSO_4$ 0.0027 g/l, fructose 1.8 g/l, sucrose 3.432 g/l, casamino acid 0.003 g/l, pH 7.0], supplemented with 1 mM sodium arsenite for 7 days. Aliquots of 1 ml were collected and centrifuged for 15 min at 5 × *g* and 500 μl of the supernatants were collected for arsenic dosing. For total arsenic dosage, Total Reflection X-ray Fluorescence methodology was used (S2 PicoFox, Bruker, United States). Ten microliters of the sample were placed into quartz plates and the reading was performed with 600 s reading time, current 700 mA and voltage 50 kV.

Scanning Electron Microscopy and Energy Dispersive X-ray (EDX) Analysis

Cells were grown in 50 ml XVM2 medium for 72 h, some containing 1 mM of sodium arsenite. Cells were collected and washed with phosphate buffer pH 7.2 three times, with centrifugation at 1.957 × *g* for 5 min. The cell pellet was resuspended in phosphate buffer supplemented with 1% paraformaldehyde and 2% glutaraldehyde then placed on slides and allowed to dry for 2 h. The slides were dehydrated in an ethanol series of 30, 50, 70, 80, 90, 95, and 100%. The cells were coated with gold for electrical conduction and visualized under the scanning electron microscope (SEM) (Q150RES, Quorum).

Arsenite Oxidase Activity Assay

The assays were performed using (with adaptations) the protocol of Dey et al. (2016). Cells were grown in 50 ml XVM2 liquid medium containing different concentrations of sodium arsenite at 28 ± 2°C and shaking at 150 rpm. Over 7 days, 500 μl of bacterial cultures were collected and centrifuged at 5,000 × *g* for 15 min. One hundred microliter of the supernatant was mixed with 100 μl of 0.1 M $AgNO_3$ solution. Yellow precipitate formation indicated the formation of Ag_3AsO_3 [silver arsenite (As^{3+})], while brown precipitate formation indicated the formation of Ag_3AsO_4 [silver arsenate (As^{5+})].

Dosage of Intracellular Reactive Species

The assays were performed using (with adaptations) the protocol of Kim et al. (2017). Cells were grown in 50 ml LB liquid medium to the optical density equal to 1.0 ($\sim 10^8$ cells/ml). One milliliter of the bacterial culture was collected and centrifuged at 1.957 × *g* for 5 min. The supernatant was discarded, and the cells were washed three times with PBS (pH 7.2) and centrifuged as previously described. The cell pellet was resuspended in 1 ml of PBS at a final concentration of 20 μM of the 2′, 7′-Dichlorodihydrofluorescein (H_2DCFDA) diacetate probe. The samples were incubated for 1 h at 37°C in the absence of

light. The cells were then washed with PBS three times and resuspended in the same buffer, with a final concentration of 5 mM sodium arsenite, for 1 h at 37°C. Subsequently, 150 µl of the bacterial suspension was placed into 96-well plates and fluorescence emission was evaluated in a plate reader (Perkin Elmer VICTOR X3, Waltham, MA, United States) with excitation at 485 nm and emission 535 nm.

Catalase Activity

The assays were based on the method of Aebi (1984). Cells were cultured in 50 ml CN medium at 28 ± 2°C until reaching optical density of 1.0 (∼10^8 cells/ml). Thereafter, the bacterial culture was exposed to 5 mM sodium arsenite for 1 h. Cells were collected and centrifuged at 1.957 × *g* for 15 min, washed three times with PBS buffer (pH 7.2) and the cell pellet was resuspended in 3 ml of PBS. Glass beads were added to the cell suspension and the tube was vortexed seven times for 30 s, with 30 s intervals on ice. One milliliter of the prepared extract was collected and centrifuged at 5 × *g* for 15 min. Twenty microliters of the supernatant was added to the PBS solution with a final concentration of 1 mM H_2O_2. The absorbance reading was performed in a spectrophotometer (Biospectro SP-220) at 240 nm for 3 min. The protein dosage was performed by the Bradford method (Bradford, 1976).

DNA Integrity

Bacterial cultures were grown in 50 ml LB liquid culture medium at 28 ± 2°C and rotated at 150 rpm until reaching the optical density of 1.0 (∼10^8 cells/ml). Thereafter, the cultures were exposed to a final concentration of 0.5, 1, 2, and 5 mM of sodium arsenite for 12 h. After the exposure time, an optical density was normalized to 1.0. A volume of 1.5 ml was collected and centrifuged at 13.22 × *g* for 5 min. The supernatant was discarded and the cells were resuspended in 300 µl of a solution containing 50 mM glucose solution, 25 mM Tris, 10 mM EDTA, pH 8.0. Five microliter of proteinase K (20 mg/ml) and 30 µl of 10% SDS were added. The mixtures were incubated at 37°C for 2 h. Then, an equal volume of chloroform was added, mixed, and centrifuged at 13.22 × *g* for 5 min. The supernatant was collected, an equal volume of chloroform was added and the centrifugation was repeated. The supernatant was then collected and 0.1 volume of 3 M NaCl and 1 volume of ice-cold isopropanol were added. The mixture was allowed to stand for 3 h in the freezer at −20°C, and then centrifuged for 15 min at 13.22 × *g*. The supernatant was discarded again and the precipitate was washed with 70% ethanol. The precipitate was allowed to dry at room temperature and then resuspended in 100 µl of TE buffer (pH 8.0). Integrity of the genomic DNA was evaluated on 1% agarose gel compared to a condition where the isolates were not exposed to sodium arsenite.

Motility Assay

The assays were based on the protocol of Farasin et al. (2017). Bacterial cultures were grown in CN medium at 28 ± 2°C with rotation of 150 rpm until reaching an optical density equal to 1.0 (∼10^8 cells/ml). Then, 10 µl of the bacterial suspension was dropped in LB semisolid medium (0.3% agar) containing 0.2 mM sodium arsenite or sodium arsenate. After 24 h of incubation at 28 ± 2°C, the plates were visually inspected and the diameters of respective colonies were measured.

PCR Assay

Each PCR mixture contained 50 ng of template DNA, 2.5 µl buffer reaction (10X), 1.5 µl dNTP (10 mM), 2.5 µl $MgCl_2$ (25 mM), 0.25 µl *Taq* polymerase (5 units/µl), 8 µl of the mixture Forward (GCTTGGGCATAGGTTGGAGT) and Reverse (GGCTCGACGTTTTTACGCAG) primers (10 pmol/µl) and sufficient water to reach 25 µl. PCR cycles were preceded by denaturation at 94°C for 3 min, followed by 35 consecutive cycles with 45 s at 94°C for denaturation, 45 s at 60°C for annealing and 45 s at 72°C for extension, followed by a final step of extension at 72°C for 3 min using Biocycler thermocycler (Biosystems, United States).

Molecular Identification of Strains

For molecular identification of FG3, C25, and FOB3 strains, the genomic DNA was extracted by the Wizard® Genomic DNA Purification Kit (Promega). To identify the strains, an amplicon was generated by PCR technique for the V4–V5 region of the 16S ribosomal gene. A total volume of 30 µl of reaction was used: 50 ng template DNA, 2.5 µl reaction buffer (10X), 1.5 µl dNTP (10 mM), 2.5 µl MgCl 2 (25 mM), 0.25 µl Taq polymerase enzyme (5 units/µl), 4 µl of the primer mixture Forward (GTGCCAGCMGCCGCGGTAA) and Reverse (CCGTCAATTYYTTTRAGTTT) (10 pmol/µl). PCR cycles were preceded by denaturation at 94°C for 3 min, followed by 35 consecutive cycles with 45 s at 94°C for denaturation, 45 s at 57°C for annealing and 45 s at 72°C for extension, followed by a final extension step at 72°C for 3 min, using 2720 ThermalCycler™ (Applied Biosystems, United States). The amplicons generated by PCR were verified in 1% agarose gels and purified using 20% PEG-8000 in 2.5 M NaCl (Arbeli and Fuentes, 2007). The product obtained was quantified by spectrophotometry using NanoDrop ND 1000™ (NanoDrop Technologies). Sequencing was carried out with the DYEnamicTM™ kit (Amersham Biosciences, United States) in combination with the MegaBACE 1000™ automated sequencing system (Amersham Biosciences, United States). The sequencing reactions were performed with 100–150 ng purified DNA and the reagents in the DYEnamic™ kit (Amersham Biosciences, United States), following the manufacturer's recommendations. The program consisted of 36 cycles with an initial denaturation at 95°C for 25 min, followed by 15 s annealing at 50°C and 3 min of extension at 60°C. After cycling, the reaction product was transferred to a 96-well sequencing plate to be precipitated. For precipitation, the established protocol was the same as previously described by Felestrino et al. (2017b).

Nucleotide Sequence Accession Numbers

The sequences of FG3, C25, and FOB3 were deposited in GenBank, and the identifiers of these sequences are, respectively, MH424466, MH42447, and MH42448.

Statistical Analyses

Statistical analyses were performed using the statistical package GraphPad Prism, version 5.00 (San Diego, CA, United States). The results were submitted to the normality test of Smirnov Kolmogorov and represented as the mean ± SEM. The Student's *t*-test was used to compare pairs of parametric groups while one-way analysis of variance (ANOVA) was used to compare three or more groups with Tukey *post hoc* tests for parametric data. The Kruskal–Wallis test was used to compare Dunn's *post hoc* test data, considering $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

Characterization of Isolates

Two canga regions were selected for collecting plant and soil samples: Canga Mãe d'Água and Canga Jardim Canadá, located in Sinclinal Moeda, and Nova Lima, respectively. The choice was based on the fact that they are inserted in a region of extreme conservation for the flora biodiversity of the state of Minas Gerais (**Figure 1A**). From samples of nine plants, some of which were from ferruginous rupestrian grasslands, restricted from cangas and from six soil samples (**Figure 1B**), 65 isolates were obtained (**Table 1**).

Once isolated and stored in a 96-well matrix plate, a series of qualitative biochemical assays and morphological characterization of these isolates were performed (**Figure 2A**). For these assays, 38 (58.46%) isolates were able to secrete amylases, 31 (47.69%) were able to secrete cellulases, 41 (63.07%) were able to secrete proteases, 30 (46.15%) were able to produce IAA, 17 (26.15%) were able to produce siderophores and only 6 (9.23%) of these isolates were able to solubilize inorganic phosphates (**Figure 2A**). None of the isolates were resistant to the antibiotic, tetracycline, although 44 (64.61%) isolates showed tolerance to ampicillin. Finally, 45 (69.61%) isolates were able to grow in culture medium containing 1 mM sodium arsenite (**Figure 2A**). Regarding morphology, 46 (71%) isolates were classified as bacilli, 10 (15%) as cocci and 9 (13.20%) as streptobacilli, and among these, 53 (81.13%) isolates were identified as Gram-positive and 12 (18.86%) as Gram-negative bacteria (**Figure 2A**). In order to verify the *in vitro* growth profile of these isolates, 11 were randomly selected. From the same cell concentration (~10^8 cells/ml), 10 of these isolates (C25, C33, FOB1, FOB2, FOB3, FG1, FG2, FG3, FA1, RPT1) showed a growth profile higher than *E. coli* when underwent to the same growth conditions. Only the IPT1 isolate showed a growth profile equal to or less than *E. coli* (**Figure 2B**).

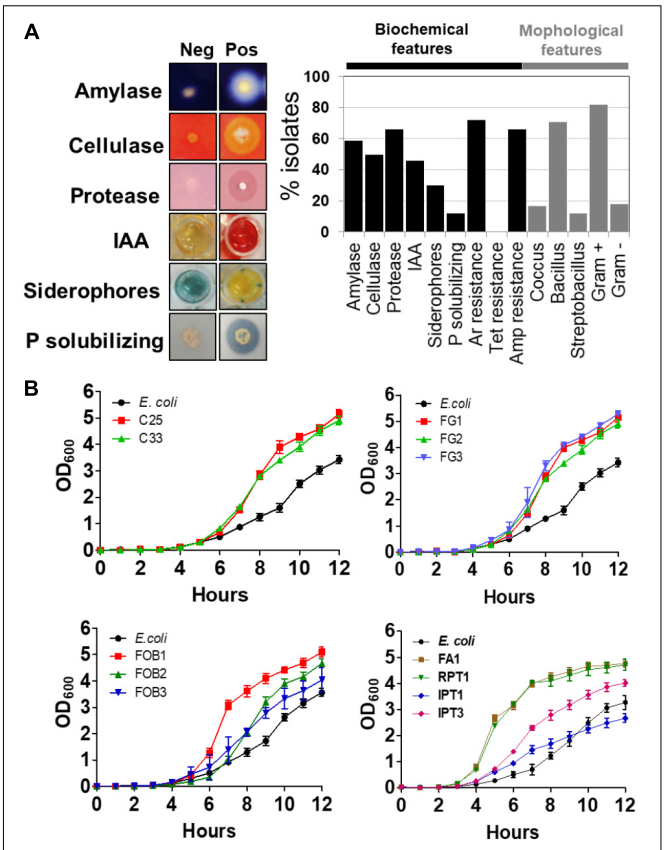

FIGURE 2 | Biochemical and morphological characterization of the isolates using different assays. The results are expressed as the percentage of positive isolates for each assay **(A)**. Comparison of the growth curves of different plant and soil isolates to the control *Escherichia coli* **(B)**.

Evaluation of Potential as Biofertilizers

Among the 30 isolates that presented biofertilizer potential, for producing different concentrations of IAA, 3 were chosen because they presented the capacity to produce siderophores as well. Analysis of the 16S rRNA gene sequence corresponding to the V4–V5 region, showed that FG3, C25, and FOB3 correspond, respectively, to *Serratia* non-pigmented (ID MH424466), *Acinetobacter* (ID MH424467), and *Rosenbergiella* (ID MH424468). These bacterial strains were used for plant growth promotion trials. Initially, it was verified whether the production of IAA and the growth rate of these isolates were dependent on tryptophan and also if that the three isolates showed no significant variation in the growth rate, in the presence or absence of tryptophan (**Figure 3A**). At the same time, it was found that the production of IAA reached a peak of ~ 30 μg/ml after 96 h with isolates C25 and FOB3, whereas the FG3 isolate produced ~20 μg/ml of IAA in this same period, in the presence of tryptophan. However, in the absence of tryptophan, it was verified that only the FOB3 isolate produced significant amounts of IAA (10 μg/ml) within 20 h of culture, stabilizing its production up to 96 h of culture. On the other hand, the isolates C25 and FG3 produced only 2 μg/ml IAA throughout the whole culture period investigated (**Figure 2A**).

Based on these results, the FOB3 isolate was selected as the most promising in the plant growth promotion assay. For plant growth trials, 20 Santa Clara tomato seeds were sown in the presence and absence of the FOB3 isolate, according to methodology. The germination and elongation rates of aerial parts were verified over the course of 20 days. It was observed during all the gauging periods that the development of tomato plants in the presence of the FOB3 isolate was earlier and remained more efficient throughout the investigated period

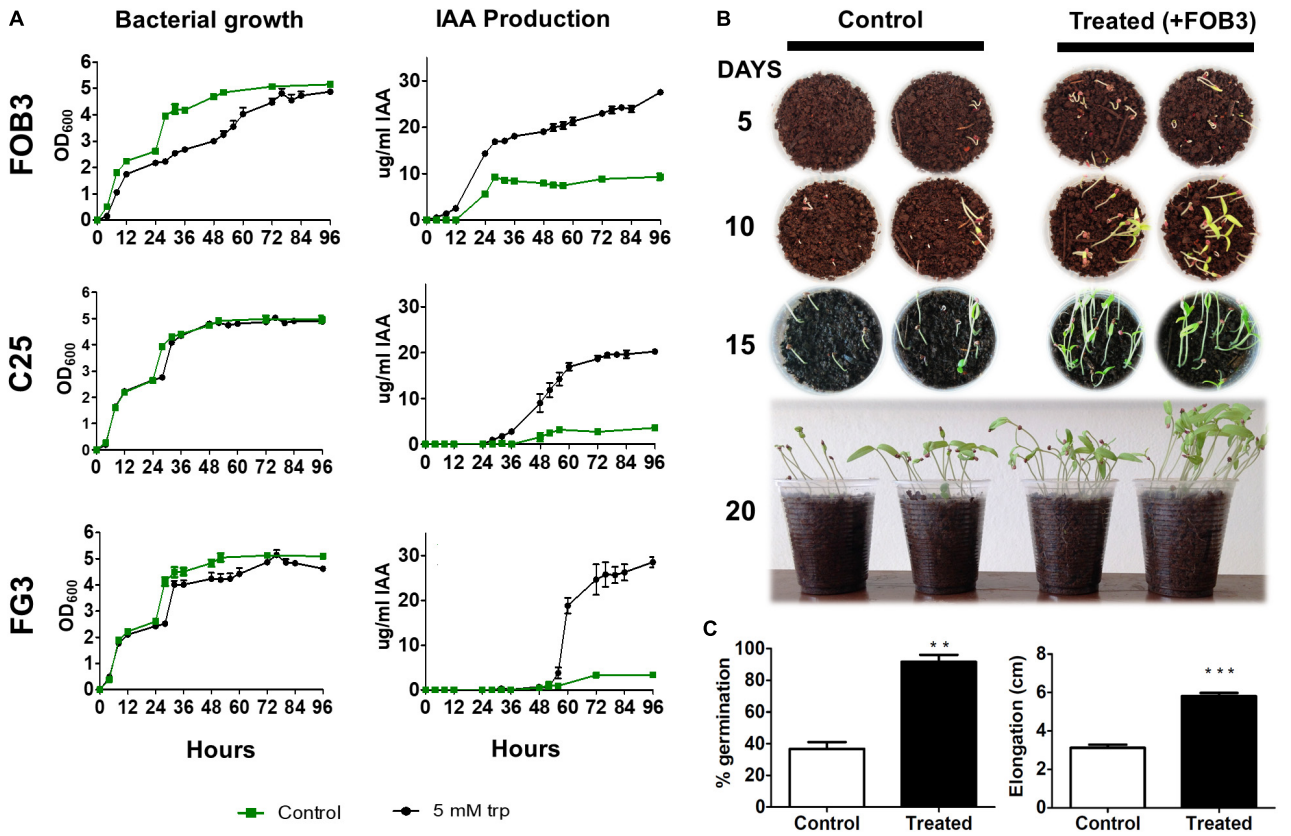

FIGURE 3 | Evaluation of the potential of three bacterial isolates as PGPB. **(A)** Relationship between growth curve and IAA dosage in the presence and absence of tryptophan (Trp). The FOB3 isolate showed the highest rate of IAA production in the absence of Trp. **(B)** Tomato seeds were grown in the absence and presence of the FOB3 bioinoculatory potential and evaluated for 20 days. **(C)** The germination rate and growth of the aerial part were evaluated at the end of 20 days of incubation. The *p*-values were determined by Student's *t*-test and the groups were considered significantly different when $^{**}p < 0.01$ or $^{***}p < 0.001$.

(**Figure 3B**). Plants inoculated with the FOB3 isolate showed a 100% germination rate and around 5.80 cm aerial part elongation compared to 36.7% germination and around 3.10 cm of aerial part elongation in plants not inoculated with this isolate (**Figure 3C**).

Analysis of Plasmid Potential

Since plasmids are often related to both antibiotic and heavy metal tolerance, the possible presence of plasmids was verified for FOB3, C25, and FG3 isolates. Based on the established protocol, plasmids were identified in isolates C25 and FG3, but not in FOB3 (**Figure 4A**). In an attempt to understand the biological potentials associated with these plasmids, they were inserted into *E. coli* by electroporation. The transformed *E. coli* strains were incubated in a rich medium, containing ampicillin and X-gal, and after 24 h of growth it was found that the transformant carrying plasmid of the C25 isolate (*E. coli*::pC25) was resistant to ampicillin but unable to degrade X-gal (**Figure 4B**). The same procedure was performed for the FG3 plasmid, where the presence of white and blue colonies was observed, indicating the possibility of two plasmids. After isolation of these transformed colonies, two differentiated transformants were identified, respectively, named *E. coli*::pFG3A (β-gal+ and Amp+) and *E. coli*::pFG3B (β-gal− and Amp+) (**Figure 4B**). A further extraction of the plasmids from the transformed *E. coli* strains, confirmed that FG3 has two plasmids with small size variation (**Figure 4C**), confirming the qualitative results in the presence of X-gal.

To evaluate the stability of the plasmids in the transformed cells, *E. coli*::pFG3A and *E. coli*::pFG3B were cultured in an LB broth medium supplemented with ampicillin (100 μg/ml), and every 24 h a sample of these cultures was plated on solid LB medium containing antibiotic. It was observed that even after 6 days of culturing (∼450 replications) about 98 and 99% of the transformed cells (*E. coli*::pFG3A and *E. coli*::pFG3B, respectively), maintained the plasmids (**Figure 4D**). In an attempt to verify if the presence of the plasmids could interfere with the growth rate of *E. coli*, growth curves with the transformed strains were performed. It has been found that the presence of plasmid pFG3B in *E. coli* cells is capable of altering the growth rate, reaching the log phase of growth more rapidly compared to wild *E. coli*. In addition, *E. coli*::pFG3B showed a higher growth rate in relation to the wild-type FG3 isolate, a result not observed for the *E. coli*::pFG3A strain (**Figure 4E**).

In order to characterize the potential of these plasmids, the *E. coli* transformed cells were evaluated for biofilm production and autoaggregation, in comparison to the wild strains C25, FG3, and *E. coli*. The wild C25 isolate and *E. coli*::pC25

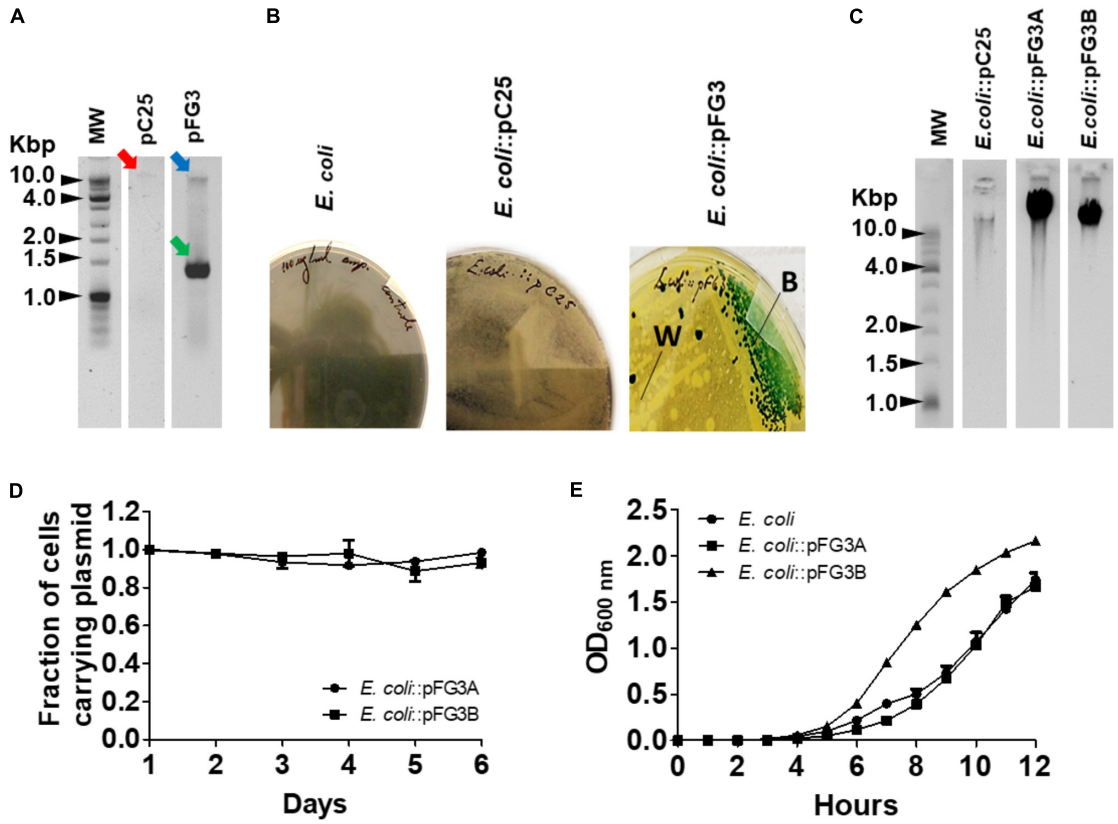

FIGURE 4 | Characterization of the plasmids obtained from the isolates C25 and FG3. **(A)** Analysis of the electrophoretic profile of plasmids extracted from wild strains C25 (red arrow) and FG3 in two conditions, relaxed (blue arrow) and coiled (green arrow). **(B)** Phenotypic results of *E. coli* transformed with the C25 and FG3 plasmids and incubated in LB broth supplemented with X-gal and ampicillin. Two phenotypic profiles were observed for *E. coli*::pFG3, white (W) and blue (B) colonies suggesting the presence of two distinct plasmids in the wild strain. **(C)** Electrophoretic profile of the plasmids from the transformed *E. coli* strains. For C25 a plasmid of the same size as that of the wild strain (pC25) was observed. For FG3 two different plasmids, named FG3A (pFG3A) and FG3B (pFG3B), respectively, were observed. **(D)** Verification of the plasmid stability of transformed strains resistant to ampicillin and grown on LB agar and evaluated over 6 days of growth. **(E)** Growth curves of the transformed strains compared to that of wild-type *E. coli*, demonstrating that the plasmid pFG3B confers increased bacterial replication rate (as observed in **Figure 2B**).

transformant strain did not produce significant amounts of biofilm (**Figure 5A**). In contrast, it was observed that the FG3 isolate produced significantly higher amounts than the wild strains of C25 and *E. coli*. Interestingly, this effect was associated with the presence of the plasmid pFG3B, since the transformant *E. coli*::pFG3B showed levels similar to the wild-type FG3 isolate, an effect not observed for the *E. coli*::pFG3A strain (**Figure 5A**). As for the autoaggregation profile, the strains *E. coli*::pFG3A and *E. coli*::pFG3B were not able to form precipitates. However, *E. coli*::pC25 presented 73% of its aggregated cells and formed a precipitate after 12 h of rest (**Figure 5B**). This variation in biofilm production and autoaggregation potential was confirmed by microscopy, allowing the observation of a biofilm layer around the colony *E. coli*::pFG3B not observed in the other strains, and a greater roughness at the ends of the colony of *E. coli*::pC25, not observed in the other strains (**Figure 5C**).

Analysis of Arsenic Tolerance Profile

Due to high soil metal rates where the isolates were collected, all 65 isolates were challenged for arsenic tolerance. FG3 was one of the most tolerant bacterial strain to the presence of this metalloid (5 mM). In an attempt to understand whether this adaptation was plasmid-dependent, *E. coli*::pFG3A- and *E. coli*::pFG3B-transformed strains were incubated in culture media containing different concentrations of sodium arsenite. It was found that wild *E. coli,* as well as *E. coli*::pFG3A, did not show tolerance to high concentrations of arsenite. In contrast to these results, the *E. coli*::pFG3B-transformed strain presented a tolerance profile similar to the wild strain FG3 (**Figure 6A**).

To better evaluate this tolerance to the metalloid, growth curves at different concentrations of arsenite were performed. The FG3 isolate was shown to be able to grow at concentrations of 1, 2, and 5 mM. It is also noteworthy that at 5 mM, the strain had a longer stationary phase than to the other lower doses tested and then a very quick growth rate compared to the other cultures (**Figure 6B**). The *E. coli*::pFG3B strain was shown to be even more resistant when comparing its growth profile at the same arsenite concentrations to that of the FG3 isolate (**Figure 6C**). Note that the *E. coli*::pFG3B strain showed an almost identical growth profile when exposed to 1 mM of arsenite, while wild

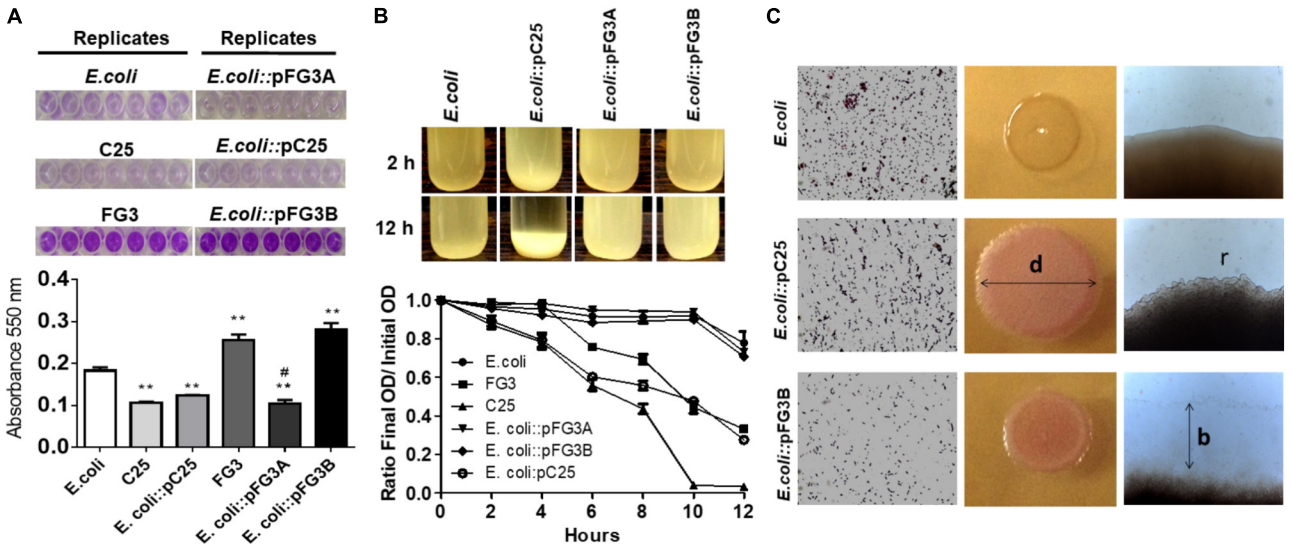

FIGURE 5 | Verification of biofilm production and cell aggregation in transformed strains. **(A)** Analysis of the biofilm production in the wild-type and transformed strains incubated for 12 h and stained with crystal violet (seven replicates). The biofilm production was measured by spectrophotometry at 550 nm. The *p*-values were determined by one way ANOVA followed by Tukey's *post hoc* test and the groups were considered significantly different when $^{**}p < 0.01$ compared to wild *E. coli* and $^{\#}p < 0.05$ compared to wild-type FG3. The high level biofilm production of the wild-type FG3 strain is mediated by the plasmid pFG3B. **(B)** Aggregation profile of the transformed strains after 2 and 12 h of rest in LB broth, starting from a culture with an OD_{600} of 1.0. To quantify this rate of aggregation, an approximate number of cells in the supernatant was estimated by spectrophotometry (OD_{600}), measured every 2 h and expressed as mean ± SEM. *E. coli*::pC25 had an aggregation rate of ∼50% higher relative to wild-type *E. coli* or *E. coli* transformed with plasmids pFG3A and pFG3B, after 10 h. C25 and FG3 wild-types had an aggregation rate of 100 and 50%, respectively. **(C)** Optical microscopy showing aggregation profile altering diameter (d) colony roughness (r) in *E. coli*::pC25 strain and increase in biofilm production (b) around colonies of *E. coli*::pFG3B strain.

E. coli was not able to grow at any concentration of arsenite tested (**Figure 6C**).

To verify the viability of the cells, they were exposed to different concentrations of sodium arsenite, and when they reached the approximate concentration of 10^8 cells/ml, the suspensions were diluted, plated and the colony forming units count was evaluated. It was found that FG3 has the same proportion of viable cells compared to the control at concentrations of 1 and 2 mM of arsenitebut, at the concentration of 5 mM this ratio reduces to approximately half of viable cells (**Figure 6D**). However, it was observed that at all concentrations of arsenite tested, the proportion of viable cells remained constant for *E. coli*::pFG3B (**Figure 6E**). We do not reject the hypothesis that complexes can be formed among the metals, phosphates, and other components present in the growth medium.

Aiming to better understand the relationship between tolerance and the ability to remove arsenic in the medium, wild-type FG3 and *E. coli*::pFG3B strains were cultured in XVM2 medium supplemented with 1 mM arsenite. The supernatants were collected and submitted to total arsenic dosing by the X-ray Fluorescence Spectroscopy method (**Figure 6F**). A culture medium containing 1 mM of arsenite was used as a positive control, and a culture medium without arsenite was used as a negative control. At the end of 7 days of growth, the percentage of removal of the metalloid in solution was quantified, where a low removal efficiency was observed for FG3 (5.78%) and *E. coli*::pFG3B (2.82%).

Since the strains are resistant but do not bioaccumulate arsenic, they may possibly act as biodegraders of the metalloid. To verify this hypothesis, cultures of FG3 cells were grown in XVM2 media containing different concentrations of arsenite and arsenate. Formation of a yellow color precipitate in the presence of silver nitrate indicates formation of silver arsenite (Ag_3AsO_3), while the formation of a brown precipitate indicates the presence of silver arsenate (Ag_3AsO_4). **Figure 6G** shows that with 12 h of experiment there was no formation of silver arsenite at a concentration of 1 mM of arsenite, demonstrating that this species had biotransformed it. At 48 h this result was repeated for a 2 mM concentration of arsenite, and from 96 h again this was repeated at a concentration of 5 mM. With 168 h of cultivation there was a complete disappearance of the precipitate of silver arsenite. Contrasting this arsenite biotransformation, no modification was observed when FG3 was grown in the presence of arsenate under the same conditions.

To investigate whether the presence of this metalloid induces a change in cell morphology, strains FG3 and *E. coli*::pFG3B were grown in culture media containing 1 mM arsenite, and their morphology was evaluated by SEM. As can be seen in **Figure 6H**, no apparent change in the morphology of FG3 or *E. coli*::pFG3B was observed. Similarly, analysis by energy-dispersive X-ray (EDX) was not able to detect the presence of arsenic in the cell composition of either strain. These results corroborate that both FG3 and *E. coli*::pFG3B do not bioaccumulate or adsorb arsenic in their membranes.

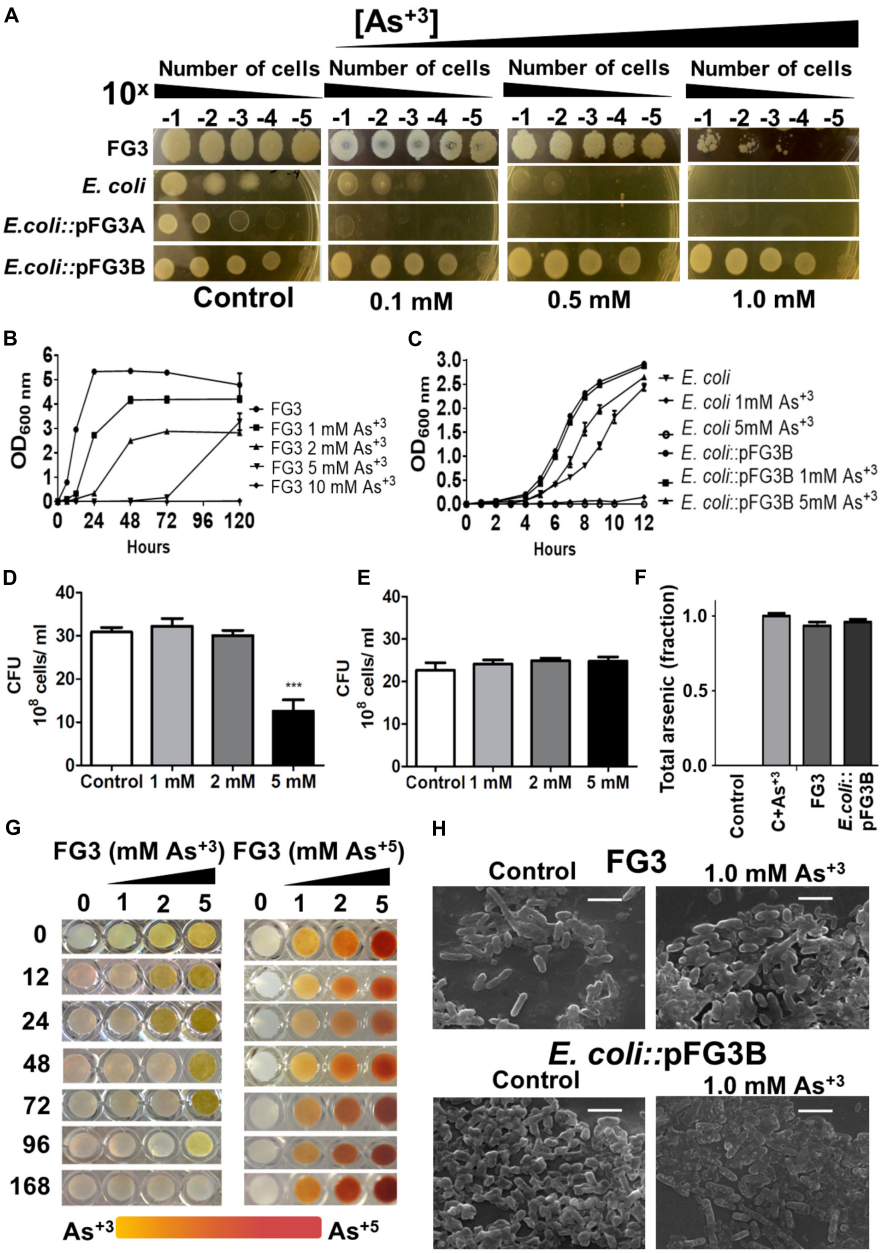

FIGURE 6 | Evaluation of the tolerance, removal, and biotransformation of arsenic. **(A)** Relation between the number of cells and the tolerance to arsenic in culture medium, after 12 h of incubation at 28°C. **(B)** Growth curves of the wild-type FG3 isolate at different concentrations of sodium arsenite. **(C)** Growth curves of wild-type and transformed *E. coli* at different concentrations of sodium arsenite. It is possible to observe that the tolerance to arsenic is mediated by the presence of the plasmid pFG3B. **(D)** CFU of FG3 wild-type submitted in different arsenite concentrations, reiterating the results observed in **(B)**. **(E)** CFU of *E. coli*::pFG3b strain submitted in different arsenite concentrations, reiterating the results observed in **(C)**. **(F)** Analysis of Arsenic removal by wild-type and transformed strains. Data were calculated as ratio of initial to final arsenic concentration **(G)** Verification of the activity of the enzyme arsenite oxidase. FG3 strain was grown in XVM2 medium supplemented with different concentrations of arsenite and sodium arsenate. Over time, samples were collected and the supernatant was mixed with 0.1 M solution of Silver Nitrate in the ratio. **(H)** Strains were grown in XVM2 medium supplemented with 1 mM sodium arsenite for 7 days, dosing of total arsenic in the supernatant was performed by X-ray fluorescence spectroscopy and the cell morphology was examined by scanning electron microscopy. Results are expressed as the mean ± SEM (d) considering $^{***}p < 0.001$. 1:1.

Induction of Redox Processes

To verify if the exposure to these metals induces redox processes in these strains, dosage of RS, consumption of peroxide and DNA integrity were investigated. As can be observed in **Figure 7A**, the production of reactive oxygen species (ROS) occurred in all tested strains when exposed to arsenic, however this increase was more prominent (∼2.5 times) in *E. coli* than in the strains FG3 and *E. coli*::pFG3B (**Figure 7A**). This data was corroborated

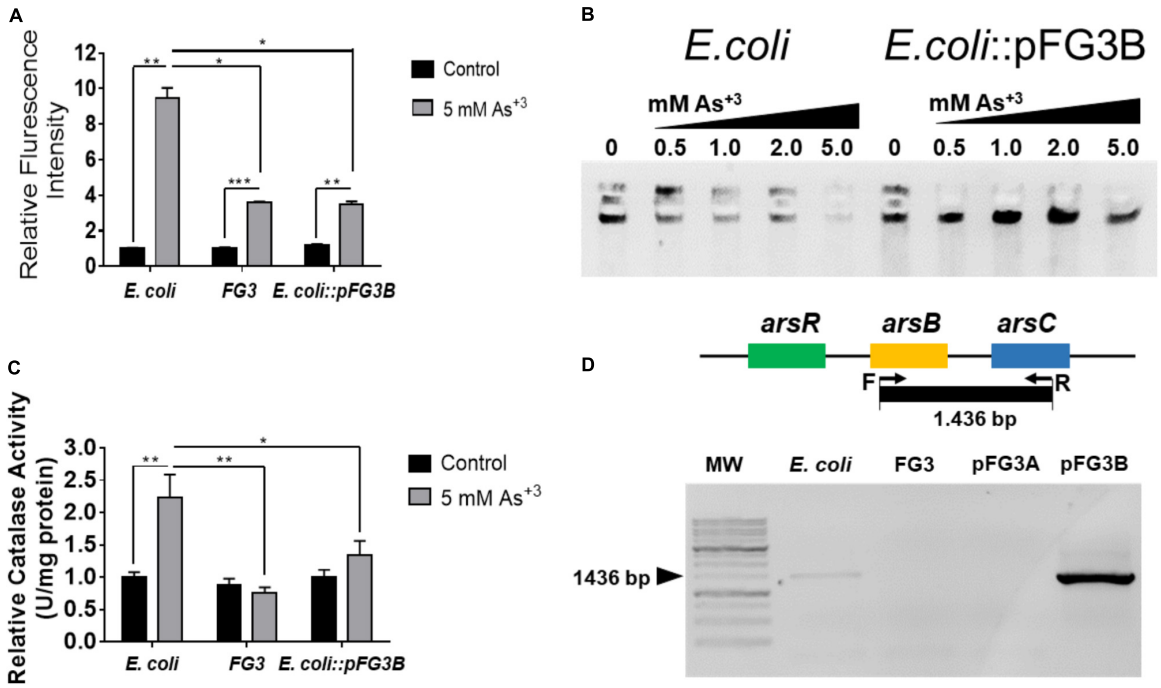

FIGURE 7 | Verification of the induction of redox processes by sodium arsenite. **(A)** Production of reactive oxygen species (ROS) **(B)** catalase activity. It is possible to observe that the profile of catalase activity is proportional to ROS production in the strains investigated. The results were expressed as the mean ± SEM considering $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by one way ANOVA followed by the Tukey's *post hoc* test. **(C)** Evaluation of the integrity of genomic DNA from wild-type *E. coli* and *E. coli*::pFG3B in agarose gel after the treatment with increasing concentrations of arsenic. Note that the DNAs of the transformed strain maintained greater integrity than those of the wild-type strain at all concentrations investigated. **(D)** Amplification of genomic region of the arsB-arsC amplicon by PCR demonstrating that these genes are present in plasmid pFG3B.

by the evaluation of catalase activity under the same conditions established for the H_2DCFDA experiments, indicating that in *E. coli* the induction of redox processes is higher than in the FG3 strain and in transformed *E. coli* (**Figure 7B**).

To verify if intracellular ROS were causing damage to DNA, its integrity was investigated in *E. coli* and in the *E. coli*::pFG3B-transformed strain when underwent to different concentrations of sodium arsenite. As shown in **Figure 7C**, *E. coli* exposure at different concentrations progressively increased the degradation of genomic DNA. On the other hand, it was observed that in the *E. coli*::pFG3B strain, the DNA remains intact even at the highest concentrations of arsenite.

In order to verify whether the arsenic tolerance conferred by plasmid pFG3B is related to the presence of *ars* genes, wild *E. coli* and FG3 strains and the plasmids of the transformed strains *E. coli*:: pFG3A and *E. coli*:: pFG3B were investigated. As it can be observed in **Figure 7D**, an amplification product corresponding to the theoretical amplicon of 1,436 bp was detected in *E. coli* genomic DNA and in plasmid pFG3B. However, no amplification product was observed for the FG3 genomic DNA and the plasmid pFG3A, indicating that the expression of the *ars* genes is involved in the tolerance of the strains to arsenic.

Motility in the Presence of Arsenic

Another strategy for surviving stress is cell motility. *E. coli* and strains resistant to arsenic were evaluated for their swimming-type motility in semisolid media containing 0.2 mM of sodium arsenite or sodium arsenate. Motility was evaluated by the growth area of the colony observed in the culture medium. It was found that both *E. coli* and *E. coli*::pFG3B exposed to arsenite or arsenate did not exhibit swimming-like motility (**Figure 8A**). However, it was observed that, under normal conditions, FG3 presented a larger area of growth compared to *E. coli*, and when exposed to arsenite and arsenate, increased its motility compared to the respective control (**Figure 8B**).

DISCUSSION

The cangas are outcrops formed millions of years ago as a result of the weathering of iron-bearing rocks that are structured in armor that can reach 10s of meters of thickness and extend over 1000s of hectares (Carmo and Jacobi, 2013). In the IQ region, these areas are colonized by plants that present adaptations to limiting conditions such as shallow soils, water deficit, low fertility, high daily thermal amplitudes, high incidence of fire, sun exposure and constant winds (Jacobi et al., 2007). All plants growing on ferruginous outcrops are metallophytes, or specifically pseudo-metalophiles, as many of them are capable of bioaccumulating metals as an anti-herbivory feature (Ribeiro et al., 2017). For these reasons, and given their ecological importance in extreme environments, plants of this environment

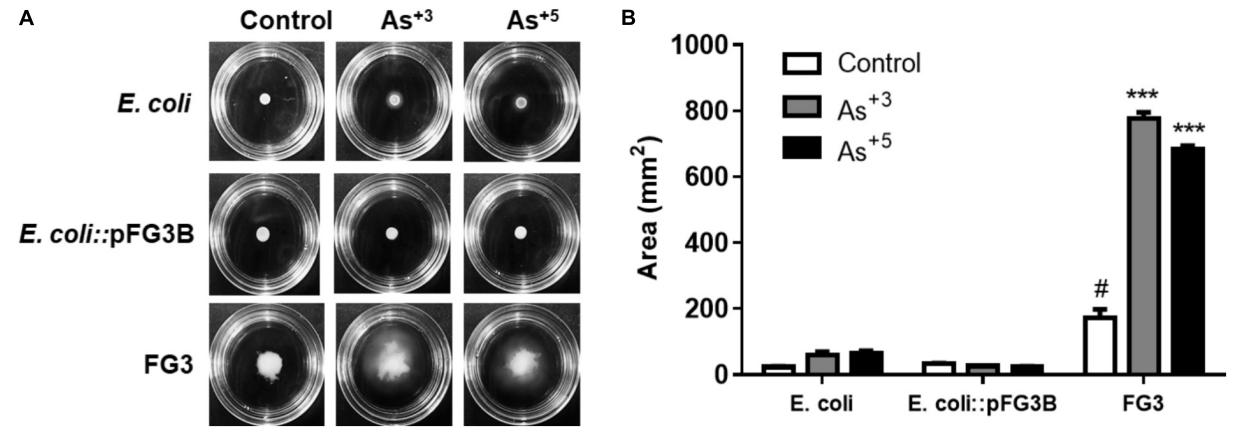

FIGURE 8 | Analysis of arsenic-induced cell motility. **(A)** Swimming motility induced by arsenic in the wild-type FG3 strain, demonstrating that this protection mechanism is not associated with the presence of the plasmid pFG3B. **(B)** Quantification of the motility profile in the strains investigated. $^{***}p < 0.001$ was considered significant in the groups compared to *E. coli* and $^{\#}p < 0.05$ when compared to wild-type FG3 by one way ANOVA followed by Tukey's *post hoc* test.

have been well-studied (Silveira et al., 2016). However, studies that portray the importance of the microbiota associated with these plants are still incipient, and its biotechnological potential is practically unknown.

Recent works developed by our team have demonstrated that the microbial composition associated with these plants is an important factor for such adaptations (Felestrino et al., 2017a,b). Continuing these investigations, this work aimed to understand the biotechnological potential of the cultivable microbiota associated with plants of the ferruginous rupestrian grasslands. For this, 65 bacteria were isolated from plants and plant soils in the state of Minas Gerais.

Initially, the ability of the isolates of this study to act as PGPB was evaluated, and a series of qualitative exploratory tests were proposed. Regarding the production of hydrolytic enzymes, ∼58% of the isolates were amylase producers, 63% cellulases and 47.69% proteases. The secretion of these enzymes in the environment can play a fundamental role in plant growth by promoting the cycling of organic matter in the soil (Choubane et al., 2016). In the case of canga soils, which are reported to have low fertility, these isolates may be of crucial importance in the cycling of essential nutrients for the maintenance of the plants. In addition, they can indirectly act as biocontrollers for other organisms, thus protecting against damage to cellular structures (Glick, 2012). In view of these findings, future research on the potential of these enzymes opens a new perspective of investment related to the microbiota associated with ferruginous rocky fields.

Similarly, the siderophores can act as biocontrollers, produced by about 25% of the isolates. They are low molecular weight molecules that have high affinities for iron and other metals (Cabaj and Kosakowska, 2009; Mahanty et al., 2017) and these molecules can limit the availability of metals to other organisms and plants, also acting as indirect promoters of plant growth (Ahmed and Holmstrom, 2014; Mahanty et al., 2017).

Also investigated in this work were the abilities to solubilize phosphate and to produce IAA by culturable isolates, characteristics desirable for PGPB. A small proportion of the isolates evaluated (9.23%) were able to solubilize inorganic phosphate. However, canga regions have very low concentrations of phosphorus, usually found in the form of phosphates (Vincent and Meguro, 2008; Schaefer et al., 2015). Thus, although the number of isolates with this capacity is limited, those that have it might have fundamental importance for the maintenance and establishment of plants in these regions. Regarding the ability of bacteria to produce IAA, 46.15% of the isolates showed such potential, reaching concentrations ranging from 2 to 10 μg/ml in the absence of tryptophan. The outcrops on the cangas are very hard, which can be an obstacle for deepening the roots of plants (Skirycz et al., 2014), the production of this phytormonium by almost half of the isolates should contribute to the establishment of these plants in such an environment, helping to promote the branching of their roots into the soil in search of nutrients. The set of isolates capable of solubilizing phosphate and producing IAA may be biotechnologically investigated for potential use as agricultural bioinoculants in soils lacking in nutrients, being, therefore, able to establish planting or increase productivity (Adnan et al., 2017).

In an attempt to empirically understand the importance of these bacteria in promoting plant growth, three isolates (C25, FOB3, and FG3), which were shown to be most promising as PGPB, were evaluated for their ability to promote growth in tomato plants. Firstly, the possibility of producing IAA in the absence of the amino acid L-tryptophan, a key precursor for phytonutrient synthesis, was evaluated, as the use of tryptophan-dependent PGPB for IAA synthesis becomes economically unviable for application as bioinoculants in large scale farming or restoration of degraded areas. After 98 h of culture in the presence and absence of tryptophan, it was possible to verify that the FOB3 isolate produced the highest amount of IAA in the absence of the precursor, ∼10 mg/l after 24 h of growth. When inoculated into soil containing tomato seeds, this isolate was able to raise the germination rate from 40 to 100% and increase the aerial part growth by 50%, from 3 to 6 cm after 20 days of growth (**Figure 3**). A study carried out by Khalid et al. (2004), showed

that in the absence and presence of L-tryptophan, PGPBs that were able to produce between 12 and 24.8 mg/l IAA strongly stimulated the growth of wheat plants. Considering that FOB3 in the absence of the precursor was able to produce almost double the concentration of this same phytormonium, and that the result was promising in tomato plants, it is possible that it could also promote growth of other plants of agronomic interest, with the possibility of it being used as a tryptophan-free bioinoculant and would, therefore, have lower implementation costs.

Other outstanding characteristics of the isolates investigated relate to their ability to grow in antibiotic-containing culture media (ampicillin and arsenic at different concentrations). It is well-described in the literature that several plasmids are related to antibiotic and heavy metal tolerance, including arsenic tolerance. Among the three isolates investigated for the production of IAA, only in FOB3 was not observed the presence of an associated plasmid. In C25, one plasmid was obtained (pC25) while two plasmids (pFG3A and pFG3B) from FG3 isolate were obtained, all conferring tolerance to ampicillin in transformed *E. coli* strains.

Plasmid stability data showed that these plasmids have high stability within transformed cells, maintained through generations, which raises the potential of these plasmids as biological vectors. Another peculiar characteristic observed was the ability of the plasmid pFG3B to stimulate the growth of the transformed strain, raising its replication rate significantly, suggesting the existence of genes that directly or indirectly act in the control of the cell cycle. Further identification of such genes may allow new transformation and cloning vectors to be constructed in order to accelerate the production of recombinant enzymes of human interest, for example.

In addition, recent research describes high concentrations of arsenic in the IQ (Costa et al., 2015), mainly located in the center-north region. Chemically, the pentavalent form (arsenate) is more abundant in oxidizing environments, and the trivalent form in reducing environments (Roy and Saha, 2002; Watanabe and Hirano, 2013). It has been reported by Ghosh that one of the possible mechanisms of resistance to arsenate, a more abundant form in the surfaces, coincides with the non-solubilization of phosphates, since these are internalized in the cells through the same carriers, thereby preventing damage to biomolecules (Ghosh et al., 2015). Morphologically, the isolates from this study were classified as gram-positive bacilli. A similar study evaluated the resistance of two *Bacillus* gram-positive bacterial isolates to arsenic (Dey et al., 2016). According to the authors, the thicker wall of gram-positive bacteria may hinder the entry of toxic compounds, such as arsenic, which is consistent with the results found in this work, suggesting that the isolates from the canga regions are highly adapted to the conditions in which they live.

To survive, the bacteria must adapt quickly to changes in environmental conditions. Changes in growth rate must be accompanied by changes in the cell cycle to ensure that cell division is coordinated with mass doubling, chromosomal replication, and chromosomal segregation (Wang and Levin, 2009). It was possible to observe that a large number of the isolates tested had a high replication rate when compared to *E. coli* under normal conditions. We speculate that the bacteria of the canga regions, due to being in constant contact with an environment of high concentrations of toxic metals and few nutrients, need to multiply rapidly as part of a survival mechanism to guarantee their presence and perpetuation in the environment, even under unfavorable growth conditions.

For plant-associated bacteria, biofilm formation is an adaptive strategy to successfully achieve colonization of the host, for example, on leaf or rhizosphere surfaces, or even as a key strategy for pathogenesis, as well as protecting against environmental conditions (Castiblanco and Sundin, 2016). Our results show that, of the strains evaluated (**Figure 4A**), only the FG3 strain as the transforming *E. coli*::pFG3B strain produced significant amounts of biofilm, suggesting that the presence of the plasmid pFG3B directly influences the metabolism of cellular biofilm production. The data so far point to the plasmid pFG3B as a highly specialized molecule in the medium, as part of a mechanism essential to the adaptation of the strain to the canga regions. In addition, it was observed that the strain carrying the plasmid pC25 has a greater ability to autoaggregate compared to the others. Unfavorable growth conditions, or even low metabolic activity, are able to induce cellular aggregation, reflecting a strategy of survival in hostile environments (Bogino et al., 2013). Under favorable growth conditions, pC25 induces a greater capacity for cellular autoaggregation. According to Bogino, the ability of cells to autoaggregate has implications for agriculture in the production of bacteria-based inoculants. Bacterial aggregates can be produced on a large scale and separated in a simpler and easier way, in relation to the dispersive bacteria in the medium (Bogino et al., 2013).

Our data shows that both the FG3 strain and *E. coli*::pFG3B have high tolerance to sodium arsenite, growing even at high concentrations (**Figures 6A–E**). Arsenic removal analysis revealed that resistant strains do not bioaccumulate arsenic within cells or even in the cell membrane, with no apparent structural modifications. In addition, the ability of the FG3 strain to decrease the toxicity of the medium by oxidizing arsenite to arsenate, which was not observed in our experiments, was evaluated. This result may be related to the production of siderophores by the bacterium. According to Ghosh, bacteria producing a high number of siderophores are more resistant to arsenite, while bacteria producing a low number of siderophores are more resistant to arsenate (Ghosh et al., 2015). Our data suggests that siderophores produced by FG3 chelate with arsenite (As^{3+}) in culture media as a way to attenuate the stress caused by this metalloid. The mechanism of resistance most widespread in bacteria is related to the functional presence of the operon *ars*, which can be associated with both chromosomal and plasmid DNA (Kaur and Rosen, 1992; Bachate et al., 2009). The presence of *ars* operon genes was found in plasmid pFG3B, not found in the genome of the FG3 strain, suggesting once again that this plasmid in particular has an important role in the adaptation of this strain to environmental adversity. The presence of arsenic tolerance genes and possibly other mechanisms not evaluated here, such as the production of siderophores and DNA damage response mechanisms, may be acting together to decrease intracellular toxicity and protect cells globally against damage to biomolecules, thus allowing greater resistance and adaptation

to the adverse environment. These results also show that the plasmid pFG3B has prominent characteristics that might be used for biotechnological purposes.

Once bacteria detect toxic compounds around them, they can protect themselves by forming biofilms or by moving to less toxic areas (Farasin et al., 2017). It has been reported in literature that the proteome of the strain *Herminiimonas arsenicoxydans,* combined with transcriptome analysis results, indicated that *H. arsenicoxydans* not only expressed genes for detoxification and stress response against arsenic, but also genes involved in the synthesis of exopolysaccharides, phosphate, and motility (Weiss et al., 2009). It was observed that the FG3 strain is highly sensitive, presenting greater motility when exposed to arsenite and arsenate. This is clearly a mechanism of defense for this bacterium, against the toxicity of arsenic, suggesting that this mechanism is not related to the plasmid pFG3B.

CONCLUSION

Our data demonstrate that the bacterial isolates from the canga regions of the IQ have ecological and biotechnological implications, which may contribute as PGPB in nutrient-poor soils, increasing the growth of plants, or even cycling organic compounds present in the environment through lytic enzymes. Our results also revealed that the plasmid pFG3B has desired characteristics for the biotechnology industry, such as metal tolerance and antibiotic resistance, and accelerating the growth of the host cells. A compilation of these characteristics (high stability, stimulation of the growth rate) for the creation of an expression vector could optimize and accelerate the production of enzymes, demonstrating its high biotechnological potential. The isolates from canga regions have an arsenal of genes with promising biotechnological potential, suggesting the need for further studies aimed at identifying new species, as well as characterizing of new interesting genes.

ETHICS STATEMENT

The authorization for the collection of plant and soil samples was granted by the Instituto Chico Mendes de Conservação da Biodiversidade, issued by the number 54015-3, valid until 03/01/2019.

AUTHOR CONTRIBUTIONS

WC, ÉF, LM, FdC, and LK: designed the work, selected the plant samples investigated and collected the plant samples. WC, ÉF, IV, MV, IC, RA, CL, NF, and AS: performed all biochemical assays. WC, ÉF, LM, FdC, LK, and CG: interpreted findings. WC, LM, FdC, LK, AS, and CG: wrote the paper. WC, IV, IC, RA, CL, NF, AS, MV, FdC, LK, CG, and LM: contributed additional interpretations and general manuscript comments. WC, LM, RA, AS, FdC, LK, and CG: revised the paper.

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The Sycamore Maple Bacterial Culture Collection From a TNT Polluted Site Shows Novel Plant-Growth Promoting and Explosives Degrading Bacteria

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Military activities have worldwide introduced toxic explosives into the environment with considerable effects on soil and plant-associated microbiota. Fortunately, these microorganisms, and their collective metabolic activities, can be harnessed for site restoration via *in situ* phytoremediation. We characterized the bacterial communities inhabiting the bulk soil and rhizosphere of sycamore maple (*Acer pseudoplatanus*) in two chronically 2,4,6-trinitrotoluene (TNT) polluted soils. Three hundred strains were isolated, purified and characterized, a majority of which showed multiple plant growth promoting (PGP) traits. Several isolates showed high nitroreductase enzyme activity and concurrent TNT-transformation. A 12-member bacterial consortium, comprising selected TNT-detoxifying and rhizobacterial strains, significantly enhanced TNT removal from soil compared to non-inoculated plants, increased root and shoot weight, and the plants were less stressed than the un-inoculated plants as estimated by the responses of antioxidative enzymes. The sycamore maple tree (SYCAM) culture collection is a significant resource of plant-associated strains with multiple PGP and catalytic properties, available for further genetic and phenotypic discovery and use in field applications.

Keywords: plant-associated bacteria, *Acer pseudoplatanus*, plant-growth-promoting-bacteria, TNT degradation, culture collections

INTRODUCTION

Terrestrial sites polluted with explosive compounds such as 2,4,6-trinitrotoluene (TNT) are widespread and present persistent environmental problems (Nishino and Spain, 2004; Kulkarni and Chaudhari, 2007). Pollution can occur during manufacturing, transport, storage of obsolete ammunition, burning and detonation operations, especially at military installations (Rieger and Knackmuss, 1995; Singh and Mishra, 2014). The impacts of TNT pollution are exacerbated by the large amounts of water that are required for its production, which eventually end up in aqueous

waste streams that may be discharged on soil or in shallow basins severely polluting soil and (ground)water (Kalafut et al., 1998; Brannon and Pennington, 2002). The TNT concentrations in polluted water and soils can reach as high as 20,000 mg TNT kg^{-1} (Rieger and Knackmuss, 1995). Because TNT is toxic, mutagenic and a potential carcinogen, there is a strong incentive for remediating these locations. The remoteness of military sites, large area, and diffuse spread of explosives pollution, directs attention toward using green and sustainable technologies such as phytoremediation in a way to reduce prohibitive high costs associated with classical dig and dump (Kilian et al., 2001; Letzel et al., 2003; Khan et al., 2013).

Plant roots are continuously exposed to diverse microbial communities and there exist strong interactions and dynamics between the host plant and its microbiome. Exudates released by plant roots can provide nutrients and energy for bacteria, while several community members in turn beneficially influence vegetation dynamics (Chaudhry et al., 2004; Mackova et al., 2009; Knief et al., 2011; Segura and Ramos, 2013). In general, there is a close relationship between the plant host and its rhizosphere microbiota, however, pollution may alter these interactions. Plants may 'call' on pollutant degrading bacteria while other pollutant-sensitive but plant-specific taxa may significantly decrease in abundance (Siciliano et al., 2001; Bell et al., 2014; Yergeau et al., 2014). Native plants that are spontaneously colonizing TNT polluted soils are ideal models to study in an attempt to better understand how bacterial communities respond to environmental pollution.

Owing to the presence of TNT polluted locations worldwide, several efforts have focused on the development of efficient remediation strategies (Clark and Boopathy, 2007; Mulla et al., 2013). Bioremediation has been considered advantageous because bacterial enzymatic reactions are diverse and have the potential to degrade a diverse set of organic compounds (Parke et al., 2000; Ramos et al., 2005; Rylott et al., 2011b). In general, TNT-transformation in aerobic soils is mediated by bacterial nitroreductases found amongst the genera *Achromobacter*, *Enterobacter*, *Klebsiella*, *Pseudomonas,* and others leading to the formation of amino-metabolites which are less bioavailable and less toxic than the parent compound (Boopathy et al., 1994; French et al., 1998; Labidi et al., 2001; Kim et al., 2002; Caballero et al., 2005; Gonzalez-Perez et al., 2007; Neuwoehner et al., 2007; van Dillewijn et al., 2008b; Rahal and Moussa, 2011; Thijs et al., 2014a; Iman et al., 2017). However, natural TNT degradation is extremely slow due to the chemical structure of TNT which renders it particularly resistant to oxidative attack, ring cleavage and thus mineralization (Qasim et al., 2007). In addition, TNT can have a low bio-availability as a consequence of irreversible binding with humic acids, clay and organic matter, and also harsh environmental conditions, e.g., nutrient limitations, co-pollutants and high toxicity, are not promotive of TNT degradation. As such, novel plant-growth promoting strains with TNT-degrading properties ought to be mined to improve current phytoremediation practices.

While one microorganism may play a dominant role in a particular degradation process, the contribution of associated microbial strains in a consortium may be crucial in augmenting phytoremediation effectiveness (Snellinx et al., 2003). Microbial consortia can be the solution to deal with the multi-complexity of TNT polluted soils, as the partnership might synergistically improve plant biomass, root formation, nutrient availability and toxicity reduction. Though these parameters need to be experimentally tested and validated.

In this study, we investigated the bacterial diversity associated with *Acer pseudoplatanus* L. trees growing around a TNT spill basin in a forest soil. Two TNT polluted soils that differed in the level of pollution and soil type were analyzed and compared to a non-polluted soil as reference. We used amplified ribosomal DNA restriction analysis (ARISA)-fingerprinting to assess the community structure of the resident bacterial communities and performed isolation of the characteristic microbiota. We performed 16S rRNA gene characterisation, molecular, enzymatic and phenotypic characterisation of their plant growth promoting and TNT-transformation abilities. In addition, *Agrostis capillaris* L. was used as plant-model to study PGP effects of selected isolates in a consortium under TNT stress.

MATERIALS AND METHODS

Site Description and Sampling

Samples were collected at a military facility in Zwijndrecht, Belgium (51°11′40.0″N; 4°19′29.6″E) on July 8th 2013. Nine young *A. pseudoplatanus* trees were sampled, three from the lower TNT wastewater basin area (LT-site), three from the deeper forest (HT-site), and three from a non-polluted location (C-site). Rhizosphere soil that was held tightly by the roots after shaking to remove loosely adhered soil, was collected. Bulk soil samples (10 kg soil per sample) were taken adjacent to each tree; namely the layer between −10 cm to −30 cm was collected after manually removing the mulch and top-soil-layer. Soil type was sandy-loam (USDA classification), average pH of 6.2 ± 0.8, variable labile organic C-content (HT: 3074 ± 152 mg kg^{-1}; C: 1625 ± 15 mg kg^{-1}; LT: 1079 ± 28 mg kg^{-1}), and variable inorganic nitrate content (HT: 17 ± 1.2 μg g^{-1}; LT: 5.6 ± 0.8 μg g^{-1}; C: 3.8 ± 0.2 μg g^{-1}). TNT-concentrations in the bulk soils were 47 911 ± 1001 mg TNT kg^{-1} at HT and 3021 ± 435 mg TNT kg^{-1} at LT, and 0 for the non-polluted site (limit of detection, 0.2 μg kg^{-1}). TNT-concentrations in the *A. pseudoplatanus* rhizospheres were 397 mg kg^{-1}, 97 mg kg^{-1} and 0 mg kg^{-1} for HT, LT and non-polluted location respectively. Upon arrival to the lab, all samples were processed for culturing on the same day and aliquots for DNA-extraction were frozen at −80°C after homogenization by sieving (2 mm). Remaining soil was refrigerated (4°C) in the dark before physicochemical soil analysis.

DNA-Extraction

DNA was extracted using the PowerSoil DNA extraction kit (MO-BIO, Laboratories, Carlsbad, CA, United States) according to manufacturer's instructions. The grinding step was optimized to a 10 min shredding at 65 hertz using a Retsch MM2000 grinding mill (MA, United States) to recover the

most quantity of high-molecular weight DNA. DNA integrity and purity were checked using agarose gel-electrophoresis and spectrophotometry using a NanoDrop (NanoDrop Technologies Inc., Wilmington, DE, United States).

Automated Ribosomal Intergenic Spacer Analysis (ARISA)

ARISA was performed using the primers ITSF (5′-GTC GTAACAAGGTAGCCGTA-3′) and ITSReub (5′-GCCAAG GCATCCACC-3′), which amplify the 16S-23S rRNA intergenic transcribed spacers, using 1–5 ng/μl of DNA as input, the high-fidelity PCR kit of Roche (FHIFI ROCHE, Vilvoorde, Belgium) and PCR program as described (Cardinale et al., 2004). PCR-products were separated using a DNA-1000 chip and 2100 Bioanalyser (Agilent Technologies, Diegem, Belgium). The acquired ARISA data profiles were processed using StatFingerprints in R[1] with data normalization and background subtraction.

Cultivable Bacteria Isolation

One gram of soil sample was shaken for 1 h in 10 mM phosphate-buffer (per liter: 2.36 g Na_2HPO_4; 1.80 g NaH_2PO_4, 85.0 g NaCl and 200 μl tween 80; pH 6.8), and 100 μl of a 5-fold dilution series was spread on media plates. The selected media comprised: modified 284 minimal medium (Schlegel et al., 1991) supplemented with 200 μl of the organic carboxylic acid mixture EXU ROOT® per liter (Innovak Global, Chihuahua, Mexico), Pseudomonas agar base CM0559 with Pseudomonas CN selective supplement SR0102 (Oxoid Limited, Hampshire, United Kingdom), BBL™ Columbia CNA agar (BD Benelux, Erembodegem, Belgium) with 5% sheep blood for Gram-positive bacteria, and 1/10 869 rich medium for general heterotrophes (Mergeay et al., 1985). At regular time intervals during incubation, colonies were picked, purified and stored at −45°C, in a 15% w/v glycerol with 0.85% w/v NaCl solution. For biolog ECOplate metabolic profiling, the 10^{-2} dilution was used and soils were inoculated in triplicate on each 96 well plate (31 different carbon and amino sources[2]). Absorbance was measured at selected time intervals at 590 nm using the FLUOstar Omega Microplate reader (BMG Labtech, Isogen Life Sciences, Temse, Belgium).

Genotypic Identification of Cultivable Bacteria

DNA was extracted from all isolates using a DNeasy Blood and Tissue kit (Qiagen, Venlo, Netherlands) and typed by 16S Sanger sequencing using the universal prokaryotic 1392R primer (5′ ACGGGCGGTGTGTRC 3′) and the bacteria-specific 26F primer (5′ AGAGTTTGATCCTGGCTCAG 3′) with PCR-conditions as previously described (Barac et al., 2004). The sequences were quality trimmed using Geneious v4.8.5 and classified using the Ribosomal Database Project tool (Wang et al., 2007).

[1]http://cran.at.r-project.org

[2]biolog.com

Functional Assays of Cultivable Bacteria

For the TNT-transformation studies, we used a minimal salts medium previously described by Snellinx et al. (2003) consisting of 50 mM phosphate/NaCl buffer (pH 6.8), trace elements, 53 mg l^{-1} NH_4Cl and 0.3% (w/v) glucose as carbon source (Snellinx et al., 2003). TNT was added after autoclaving to a concentration of 113.56 mg l^{-1} (500 μM) using a stock solution in DMSO (0.5% w/v). For solid media, 15 g Noble agar (BD Diagnostic Systems, Erembodegem, Belgium) was added per liter.

Nitroreductase Enzyme Activity

Bacteria were precultured in standard rich medium (869) at 30°C for 24 h on a shaker (Greiner Bio-One, Wemmel, Belgium), washed and resuspended in sterile 10 mM $MgSO_4$ pH 7.0 to an $OD_{600\ nm}$ of 1. Then, 10 μl was transferred to microplate wells (Greiner Bio-One, Wemmel, Belgium) containing 150 μl minimal salts medium supplemented with 113 mg l^{-1} μM TNT, 53 mg l^{-1} NH_4Cl and 0.3% w/v glucose. The microplates were incubated for 24 h at 30°C under aerobic conditions on a shaker. After 24 h, the reduction of TNT to nitro-reduction products was judged from the orange-yellow colouration of the growth medium by visual scoring. In addition, TNT was assayed by a colorimetric method based on the formation of Meisenheimer complexes after reaction of TNT with sodium sulfite at high pH (Jenkins and Walsh, 1992). Briefly, to 20 μl sample, 180 μl acetone and 20 μl TNT-solution (2 g sodium sulfite and 10 pellets KOH in 10 ml dH_2O) was added and the plate was immediately shaken vigorously. After centrifugation, the absorbance of the supernatants was measured at 540 nm and TNT concentration calculated based on a standard curve prepared from 0 to 500 μM TNT. A red color indicates TNT, colorless TNT reduction products. A score of 1 was given for complete TNT-reduction, 0.5 for incomplete reduction (pink color), and 0 when no TNT reduction was observed. Additionally, nitrite released from TNT was measured using the Griess reagent system (Griess, 1879).

Metabolic Versatility

Bacteria were grown on overnight, resuspended in sterile 10 mM phosphate buffer (10 mM; pH 6.8) to an OD_{600} of 1. Then, 0.5 ml of each strain belonging to the same phyla or Proteobacteria class were mixed together to generate 6 consortia (Actinobacteria, Bacteroidetes, Firmicutes, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, n = 10 strains per consortium). Cultures were diluted 10^{-2} in phosphate buffer and incubated at 4°C overnight. Next, 150 μl of each diluted consortium was inoculated in each well of a Biolog ECO plate and incubated at 30°C for 5 days (n = 3 replicates). Absorbance was measured as described above.

In vitro Plant Growth-Promoting Activity

Bacteria were grown overnight, washed and resuspended in 2 ml sterile $MgSO_4$ solution to obtain a suspension with OD_{600} of 0.5. Twenty microliter of this suspension was used for the inoculation of 96-well microplate assays (Greiner Bio-One, Wemmel, Belgium) for detection of: auxin production using the Salkowski reagent method (Patten and Glick, 2002); siderophore release was determined by using the Chrome azurol S (CAS)

assay (Schwyn and Neilands, 1987); 1-aminocyclopropane-1-carboxylate (ACC)-deaminase activity was estimated by monitoring the amount of α-ketobutyrate generated by the enzymatic hydrolysis of ACC, a precursor of the plant hormone ethylene (Belimov et al., 2005); organic acid production was determined using the organic dye Alizarin red S (Cunningham and Kuiack, 1992), and acetoin production, measured using the Voges–Proskauer assay (Romick and Fleming, 1998). Bacterial chitin solubilisation was evaluated by dropping 10 μl of the bacterial suspension on plates with colloidal chitin, prepared from crab shell chitin (Sigma, Gent, Belgium) as described elsewhere (Hsu and Lockwood, 1975; Uroz et al., 2013). After incubation at 25°C for 7 days, the clearing of the initially turbid medium indicated chitin hydrolysing bacterial isolates. For all the PGP-assays, the bacterial isolates were distributed into classes scored as '0' and '1' depending on their negative or positive responses.

Pot Experiment

Common bent (*A. capillaris*) seeds were surface sterilized and sown at a density of 150 mg seeds per 200 g sand on sterile autoclaved fine-grade sand spiked with 0, 25, or 50 mg TNT kg^{-1}. Sterilized sand and seeds were used to eliminate the effects of indigenous TNT-degrading bacteria, and sand was used to reduce the probability of covalent binding of TNT to soil organic matter and clay particles. TNT was dissolved in acetone and mixed with the sterilized sand. Plants were watered periodically with 1/10 Hoagland solution (Hoagland and Arnon, 1950) and placed in a greenhouse with the following conditions: photoperiod of 14:10 h light:dark, a temperature cycle of 22°C day:18°C night and a relative humidity of 57%. After establishment of the primary roots (1 week after germination), the plants were inoculated by sand drench with a bacterial consortium in autoclaved 10 mM $MgSO_4$ at a concentration of 10^6 cells g^{-1} sand, while non-inoculated plants were watered with the same amount of sterile $MgSO_4$. All pots were replicated in eight pots per condition, no plant controls, no TNT controls, and no inoculation controls were taken along the experiment. From 4 weeks old plants, biomass, root and shoot length were determined. Acetonitrile-extractable TNT-concentrations in the sand substrate were analyzed by HPLC as described (Thijs et al., 2014a). For the analysis of TNT in plant fractions, 1 g dried root or shoot material was crushed into a fine powder and extracted three times with 10 ml methanol before HPLC analyses. Colony forming units (CFUs) were determined according to standard procedures (Feng et al., 2002). For determining the activities of the antioxidative enzymes, 100 mg of root and 100 mg leaf tissue were collected and snap-frozen in liquid nitrogen. The activities of superoxide dismutase, glutathione reductase, glutathione-S-transferase, malate dehydrogenase, catalase and guaiacol peroxidase were determined spectrophotometrically as described previously (Thijs et al., 2014a).

Accession Numbers

All partial 16S rRNA gene sequences of cultivable isolates were submitted to NCBI Genbank and are available under accession numbers MH337876 - MH338035.

RESULTS

Soil Microbial Communities Diverge Depending on TNT Concentration

To obtain initial insights in the total bacterial community structure and diversity, we used automated fingerprint analyses. This revealed that the soil microbiomes of the TNT-polluted samples were significantly different from non-polluted samples (**Figure 1A**) confirmed by the Bray-Curtis cluster analysis (**Figure 1B**). The community shift and reduced diversity can also be observed in the ARISA-electropherograms (**Figure 1C**). In particular, a lower number of peaks corresponding to spacers in the range of 500–1000 bp was observed in the TNT-polluted bulk soils compared to the non-polluted bulk soils. In addition, the broad peak ranging from 900 to 1200 bp in the non-polluted bulk soil was gapped for the low and high TNT-polluted bulk soils, suggesting a diversity decrease. In contrast, spacers of 1200 bp long were increased in polluted samples compared to the non-polluted samples. Associated with these shifts, we recorded a significantly reduced Shannon diversity in polluted bulk soils (LT, 5.9 ± 0.05; HT, 5.8 ± 0.2 vs. C 6.4 ± 0.2) along with significantly reduced evenness in the low TNT-polluted bulk soil (LT, 0.36 ± 0.02 vs. C, 0.54 ± 0.03) (ANOVA, Tukey-HSD, $p < 0.05$). Shannon diversity and evenness were not different between the polluted and non-polluted rhizosphere samples as detected by ARISA. A comparison between bulk and rhizosphere samples, showed that rhizosphere samples were more diverse than the respective bulk soils, (ANOVA, Tukey-HSD, $p < 0.05$).

SYCAM Culture Collection: Isolation and Genotypic Identification

Three hundred morphologically distinct colonies were picked from the plates and sub-cultured, from here on referred to as the SYCAM culture collection. The cultivable subsets were designated 'Lib', for library of CFUs followed by their sampling location identifier. Of these 300, a total of 160 different strains were detected by restriction fingerprinting, and all these were genotyped using 16S rRNA gene Sanger sequencing (see **Supplementary Table S1** for an overview of the sequenced isolates per library). All of the sequences fell into four phyla of which 56% belonged to Proteobacteria, 31% to Actinobacteria, 11% to Firmicutes and 2% to Bacteroidetes (**Figure 2**).

The polluted bulk soils were dominated by the Proteobacteria, while the non-polluted bulk soil community was dominated by Actinobacteria followed by Gammaproteobacteria, Firmicutes and Alpha- and Beta-proteobacteria. In the rhizosphere, Actinobacteria were enriched in all three soil types with the highest proportion in the low TNT polluted rhizosphere (**Figure 2**). In addition, Proteobacteria, Firmicutes and Bacteroidetes were detected in the rhizosphere. Interestingly, there was a significant correlation (rs 0.92, $p < 0.001$) between the TNT concentrations in soil and the number of cultivated Gammaproteobacteria, specifically the genus *Pseudomonas*.

Isolated CFUs from the phylum Actinobacteria were exclusively Actinomycetales, of which the genus *Streptomyces* dominated in LibC and LibLT, while *Arthrobacter* was the

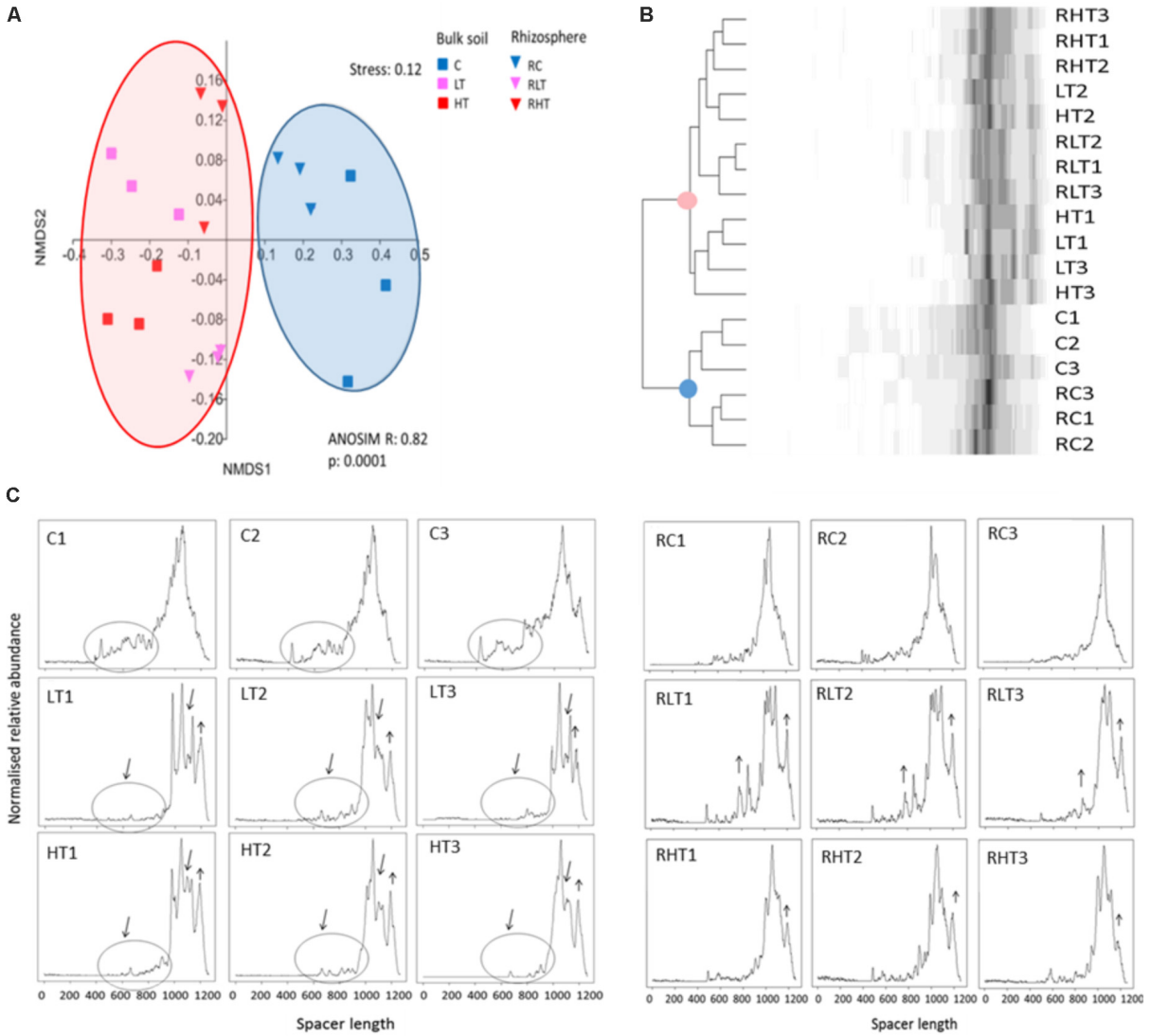

FIGURE 1 | Total microbial communities in polluted soil samples are different from control samples. **(A)** Multivariate analyses of the Bray-Curtis matrix from ARISA profiles. **(B)** Dendrogram of the Bray-Curtis matrix and complete clustering method. **(C)** ARISA electropherograms of bulk soil (left) and rhizosphere (right) with in the X-axis the spacer length (in bp) and in the Y-axis the normalized relative abundance. Sample abbreviations: C, control, LT, low TNT, HT, high TNT location, R, rhizosphere, $n = 9$ samples per condition.

dominant genus in the other libraries except for LibRHT, which was dominated by *Rhodococcus* (**Figure 2**). Betaproteobacteria isolates belonged entirely to the Burkholderiales and were represented only by the genus *Variovorax* in LibC and LibRC, whereas in the polluted soils *Burkholderia*, *Bordetella* (Alcaligenaceae), *Herbaspirillum* and *Achromobacter* were also detected. The Alphaproteobacteria isolates fell into the families Phyllobacteriaceae, Rhizobiaceae, Rhodobacteraceae, and Sphingomonadaceae in LibC, while only 2 families dominated the other libraries. CFUs of the phylum of Firmicutes belonged entirely to the Bacillales.

Counting of the bacterial CFUs on minimal 284 medium revealed that the non-polluted bulk soil contained 3.5×10^6 bacterial CFUs g^{-1} DW soil, whereas the low TNT polluted bulk soil contained a significantly reduced number of cells (2.05×10^5 CFUs g^{-1} DW soil) (ANOVA, Tukey-HSD, $p < 0.05$) (**Supplementary Figure S1**). The highest number of CFUs was obtained for the rhizosphere samples and there was no difference between non-polluted and TNT-communities (av. 5.2×10^6 CFUs g^{-1} DW soil). Enumeration on selective *Pseudomonas* agar showed a significant increase in the number of Pseudomonads in the TNT-polluted bulk soils (LT, 8.6×10^6; HT, 1.04×10^7 CFUs g^{-1} DW soil) compared to the non-polluted bulk soil (4.8×10^6 CFUs g^{-1} DW soil), supporting the results from the pyrosequencing dataset. In contrast, culturing on Gram-positive CNA agar revealed a significant reduction in the number of Gram-positive bacteria in both TNT-polluted bulk soil communities

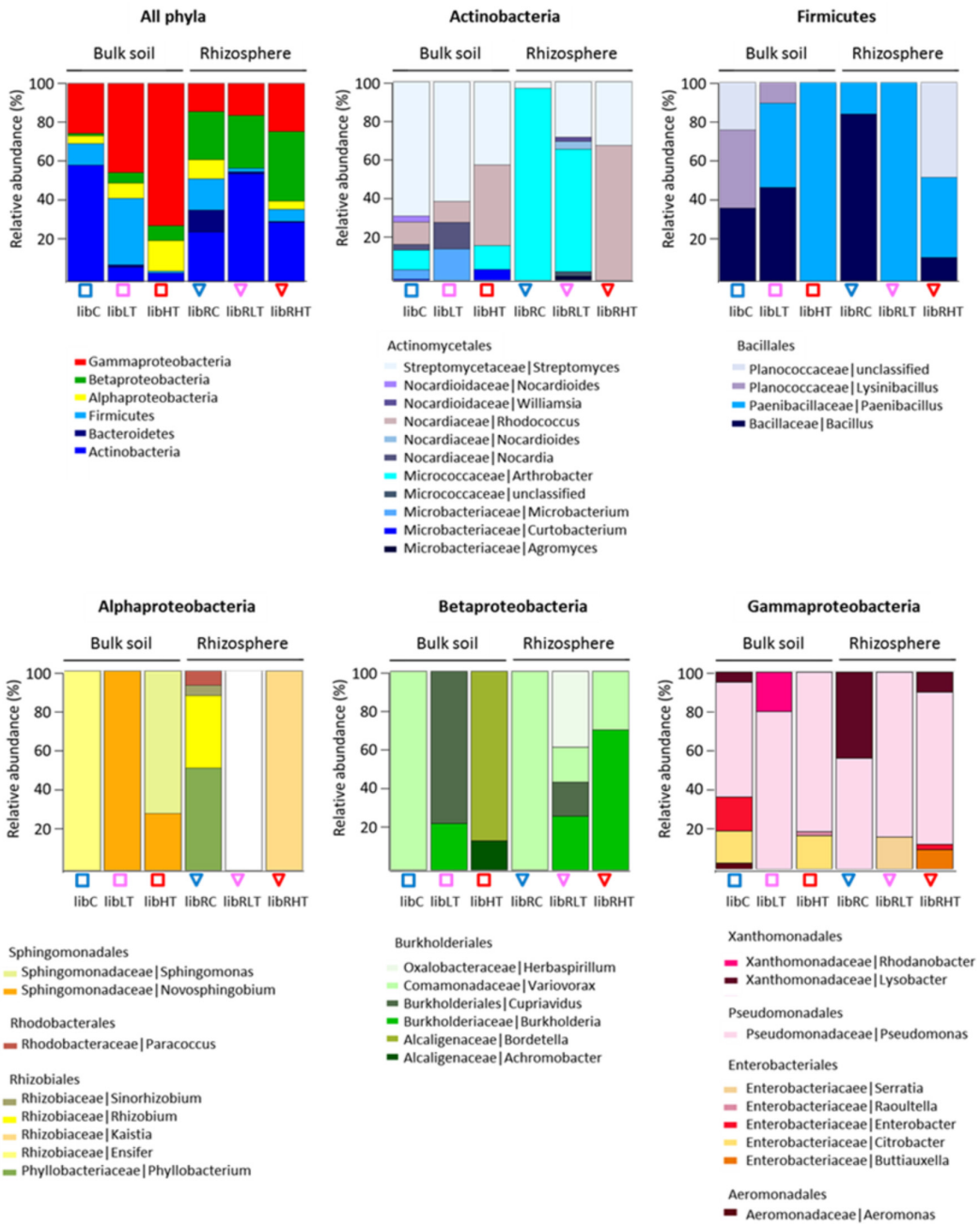

FIGURE 2 | Overview of the genotypic identification and distribution of the isolated CFUs. Phyla and Proteobacteria class level are shown and the distribution of families present among the Actinobacteria, Firmicutes, Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria.

(LT, 2.05×10^5; HT, 1.5×10^5 versus C, 2.27×10^6 CFUs g^{-1} DW soil).

Metabolic Versatility and Plant Growth Promotion Potential

To assess the metabolic versatility of the bacterial strains, the isolates were grouped as 10 isolates per taxon and six different consortia were composed (Actinobacteria, Bacteroidetes, Firmicutes, Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria, see **Figure 3**). Then the bacterial assemblages were separately inoculated into Biolog ECO plates to obtain the carbon-metabolic fingerprint.

Based on the Shannon H' entropy ($p < 0.05$) the Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes were the most metabolically versatile of the cultured isolates; they could use several carbohydrates, carboxylic acids, amino acids, phenols and polymers including many of the common

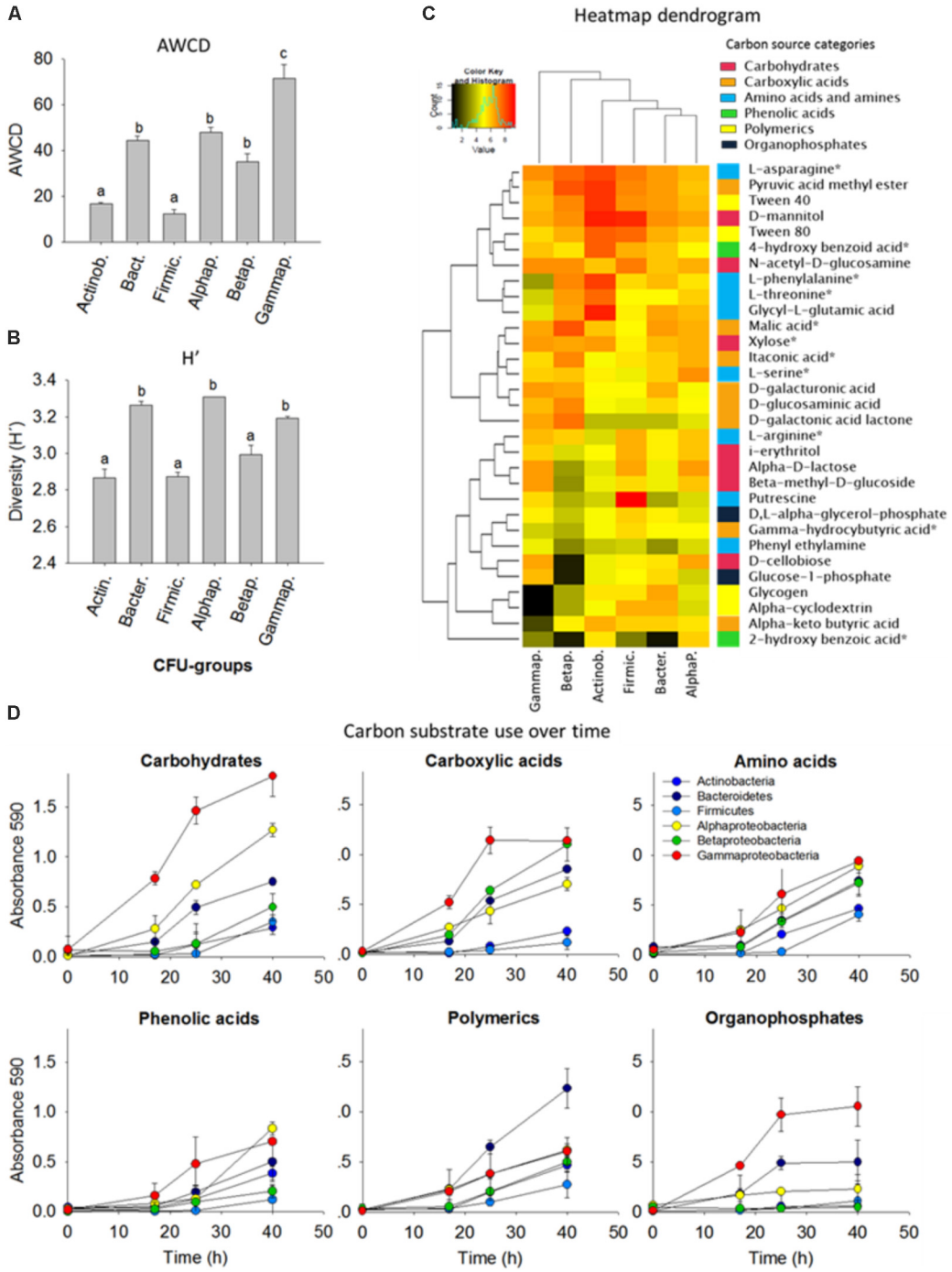

FIGURE 3 | Comparison of carbon substrate utilization activities of isolated colony forming units (CFUs). **(A)** Average well color density (AWCD) (mean ± standard error bars) for carbon substrate utilization. **(B)** Diversity of carbon substrate use as judged by the Shannon index (H'). Different letters (a,b,c) indicate that the means are significantly different (ANOVA, Tukey HSD, $p < 0.05$, $n = 3$). **(C)** Heatmap dendrogram of the carbon substrate utilization based on the area under the absorbance versus time curve AAT/AWCD. Columns represent the bacterial phyla, in rows are the 31 carbon sources. The colored side-bar shows the categories to which the carbon sources belong. Carbon sources with an asterisk are common root exudates. **(D)** Carbon substrate use over time. 10 isolates were grouped per phyla/class and included for the Actinobacteria (*Arthrobacter*, $n = 3$; *Curtobacterium*; *Microbacterium*; *Nocardia*; Nocardioides; *Rhodococcus*, $n = 2$; *Streptomyces*), Bacteroidetes (*Chryseobacterium*, $n = 3$; *Dyadobacter*; *Flavobacterium*, $n = 2$; *Mucilaginibacter*), Firmicutes (*Bacillus*, $n = 4$; *Lysinibacillus*, $n = 2$; *Paenibacillus*, $n = 3$; Planococcaceae), Alphaproteobacteria (*Ensifer*; *Kaistia*; *Novosphingobium*; *Paracoccus*; *Phyllobacterium*; *Rhizobium*; *Sinorhizobium*; *Sphingobium*, $n = 3$), Betaproteobacteria (*Achromobacter*; *Bordetella*; *Burkholderia*, $n = 3$ *Cupriavidus*; *Herbaspirillum*; *Variovorax*, $n = 3$), and Gammaproteobacteria (*Aeromonas*; *Enterobacter*, $n = 2$; *Lysobacter*, $n = 2$; *Pseudomonas*, $n = 4$; *Raoultella*).

plant root-exudates (**Figure 3B**). Gammaproteobacteria showed overall the most rapid growth increase in response to carbon substrates and this was also indicated by the high AWCD value ($p < 0.05$). Firmicutes and Betaproteobacteria showed the lowest AWCD values illustrated by a slow growth after addition of carbohydrates and carboxylic acids (**Figure 3**). Alpha-, Beta- and Gamma-proteobacteria and Bacteroidetes were rapid consumers of small aromatic compounds, which can be released upon degradation of lignin, an important polymer in forest ecosystems. *N*-acetyl-D-glucosamine, the monomeric component of chitin, a major component of fungal cell walls in soils, was utilized by all groups (**Figure 3C**).

All isolates were further evaluated for a diverse set of plant growth promotion features including siderophores production, organic acids production, acetoin synthesis, indole-3-aceteic acid (IAA) and ACC-deaminases, chitin solubilisation potential, and nitroreductase activity. Positive PGP characteristics were distributed over many and a wide variety of the isolates (**Figure 4**), though major differences in relative abundance of certain traits was observed depending on their origin. For example, there was a significant enrichment of nitroreductase producing strains in libHT versus libC (**Figure 4A**). Auxin production can be said to be co-enriched with nitroreductase activity based on the Pearson coefficient of 0.94 (**Figure 4B**). LibLT was enriched for strains producing organic acids and acetoin, which also seem to be highly correlated (Pearson coefficient 0.95). Chitin solubilisation was more abundant in the non-polluted and low polluted bulk soils compared to the high polluted soil.

As with the bulk soil, an enrichment of nitroreductases in the highly polluted rhizospheres was found compared to the non-polluted rhizospheres and low polluted rhizospheres (**Figure 4B**). Auxin and siderophore production were also enriched in libRHT, while acetoin and organic acid production were more enriched in libLRT. Comparison of the rhizosphere fractions with the surrounding bulk soil indicated a higher abundance of chitin solubilizing isolates in the bulk soil and auxin producers in the rhizosphere of the non-polluted soil. Siderophore producing isolates were more enriched in the rhizosphere compared to the bulk soil for HT.

Inspection of the taxonomic distribution to which the isolates belong revealed that TNT-nitroreductase was found mainly in the Gammaproteobacteria group, in particular *Pseudomonas*, next to other plant growth-promoting rhizobacteria (PGPR) including *Enterobacter, Burkholderia, Sphingomonas,* and *Rhodococcus* (**Figure 4B**). The ability to produce auxin was found among the genera *Pseudomonas, Rhodococcus, Arthrobacter, Sphingobium, Bacillus,* and *Enterobacter*. The capacity for organic acid production and acetoin synthesis was associated most often with Firmicutes including *Bacillus* and *Paenibacillus* and further among Actinobacteria, in particular *Streptomyces*, and the Proteobacteria, specifically *Pseudomonas* and *Burkholderia*. Siderophore production and the presence of ACC deaminases were detected for a diverse group of isolates. In contrast, the potential to solubilize chitin was restricted to specific genera including *Streptomyces*, *Bacillus,* and *Aeromonas*.

Characterization of Trinitroaromatic Compound Degraders

To efficiently screen the collection of cultivable isolates for their ability to transform TNT, as a model for trinitroaromatic compounds, a 96-well microplate assay was developed. The assay involves growing pure bacterial cultures separately in individual wells containing minimal medium with TNT (113 mg l^{-1}), NH_4Cl as a source of nitrogen (53 mg l^{-1}), and 0.3% glucose as a source of carbon and energy. Nitroreductase activity was detected based on changes in the color of the medium. The conversion of the TNT-medium from colorless to yellow was indicative for reduction of the nitro-group of TNT resulting in the formation of aminodinitrotoluene (ADNTs) and diaminonitrotoluene (DANTs) (van Dillewijn et al., 2008a,b) (**Supplementary Figure S2**). In addition, production of a brown-orange color can indicate hydrogenation of the aromatic ring to hydride and dihydride-Meisenheimer complexes (Wittich et al., 2008) (**Supplementary Figure S2** and **Supplementary Table S2**).

Of all the isolates tested, the Gammaproteobacteria, in particular the genera *Citrobacter, Enterobacter*, *Pseudomonas, Raoultella* and *Serratia*, and some members of the Alphaproteobacteria mainly *Sphingobium* spp., were found to grow rapidly in the presence of TNT (**Supplementary Tables S1, S2**). This was observed from the orange-colored pigments, presumably caused by Meisenheimer complexes formation, after 4–6 h of incubation and the accumulation of yellow amino reduction products (**Supplementary Figure S2**) after 24 h of growth. Isolates of *Burkholderia* and *Variovorax* (Betaproteobacteria) as well as *Novosphingobium* and *Kaistia* (Alphaproteobacteria) demonstrated slow growth in the presence of TNT and were able to partially reduce TNT, with 50% of the TNT remaining at the termination of the assay (**Supplementary Table S1**). Members of the genus *Rhodococcus* turned the medium to an orange color and accumulated nitrite in the supernatants, suggesting the production of Meisenheimer and hydroxylamino-derivates. The Rhodococci failed, however, to grow with TNT or nitrite (1 mM $NaNO_2$) as sole N-source, and 80% of the TNT remained in the medium after 24 h (data not shown). No growth in the presence of TNT or no effective TNT-transformation were observed for the gram-positives *Arthrobacter*, *Microbacterium* and *Streptomyces* isolates (Actinobacteria), and for Bacilli and Paenibacilli (Firmicutes).

Functional Performance of a Consortium of Selected PGP-Isolates for TNT Transformation in a Pot Experiment

Among the CFUs isolated from the TNT polluted bulk soils and rhizosphere, several representatives were shown to possess multiple PGP and TNT-reductase activities interesting for rhizoremediation (**Supplementary Table S1** and **Figure 4**). To assess the growth-promotion potential of a selection of these bacteria *in planta*, we inoculated the roots of non-exposed and TNT-exposed *Agrostis capillaris* seedlings with a 12-member consortium of isolates from bulk soil and *A. pseudoplatanus* rhizosphere of the TNT polluted location. This consortium consisted of: *Rhocococcus* sp. zw191, *Kaistia*

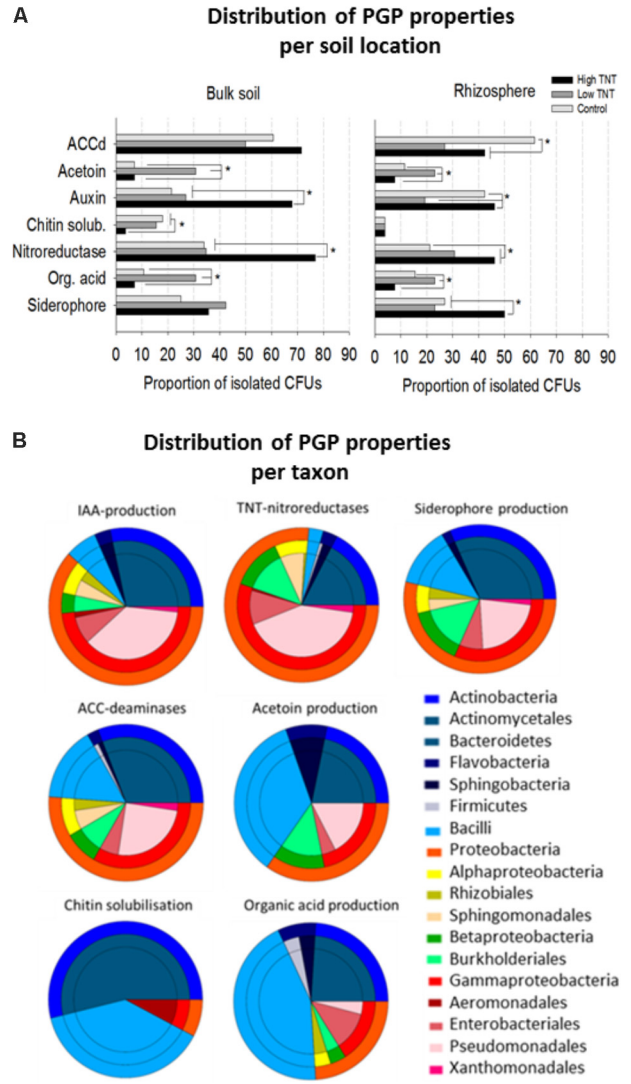

FIGURE 4 | Proportion of bacterial isolates with plant growth-promoting features, nitroreductase activity and their distribution according to origin **(A)**. Comparison of the proportions of bacterial isolates exhibiting *in vitro* PGP-activities and nitroreductase activity. For each assay, all the cultivable isolates per library were tested. Significant differences in activities are marked with an asterisk (Fisher's exact test for pairwise comparisons and FDR correction, p <0.05). **(B)** Pie diagrams showing the cumulative number of taxa with PGP and nitroreductase activity for each category as indicated above the pies. The outer rings represent the phyla and these are further broken down (inner rings) to the order level. For each test (n = 10 bacterial isolates of the same genus were included).

sp. zw161, *Novosphingobium* sp. zw55, *Burkholderia* sp. zw160, *Cupriavidus* sp. zw211, *Herbaspirillum* sp. zw98, *Variovorax* sp. zw90, *Raoultella ornithinolytica* TNT(/zw146), *Pseudomonas* sp. zw94, zw89 and zw38. *Agrostis* was used in this experiment because it shows vigorous growth, has a dense rooting system and is a common grass species on the military sites in Belgium. After 5 weeks, the growth of non-inoculated TNT-exposed plants was significantly inhibited (biomass and root and shoot length) compared to non-exposed control plants, whereas plants inoculated with the bacterial consortium showed a significant improvement of their growth (ANOVA, Holm–Sidak, $p = 0.0001$, $n = 8$) (**Figure 5**). In particular, non-inoculated plants exposed to the highest TNT-concentration (50 mg TNT kg^{-1}) had poorly developed roots (**Figure 5**), while inoculated plants showed a 200–400% increase of both, root weight and length. Differences in root length can be explained by the inoculant used (F:145.1, $p = 0.001$), an interaction term (F:69.6, $p = 0.001$) and pollution level (F:57.1, $p = 0.001$). Differences in root mass were largely explained by a pollution effect (F:49.0, $p = 0.001$) and an inoculum effect (F:26.1, $p = 0.001$). As with the roots, shoot weight of inoculated and TNT-exposed plants doubled compared to non-inoculated TNT-exposed plants. Differences in shoot weight were explained by a bacteria effect (F:255.6, $p = 0.001$) and by pollution (F:93.1, $p = 0.001$).

In addition, we determined the activities of six enzymes involved in cellular defense against oxidative stress in the roots and shoots of TNT-exposed and unexposed plants (**Figure 5A**). A two-way ANOVA for the effect of the treatments on enzyme activity patterns revealed a dominant bacterial effect (F:11.2, $p = 0.0001$), a pollution effect (F:4.4, $p = 0.0002$), and interaction effect (F:3.7, $p = 0.002$), suggesting a differential effect of the bacteria in polluted and non-polluted substrate. Bacterial inoculation explained the lowered glutathione reductase (GR)-activity in the shoots (F:5.8, $p = 0.02$) compared to non-inoculated TNT-exposed plants, whereas a dominant pollution effect explained the high GR-reductase activity in the roots (F:12.2, $p = 0.0004$). Differences in glutathione transferase (GST) activity in shoots and roots were also explained by pollution level (F:7.8, $p = 0.001$ and F:3.8, $p = 0.03$). In particular, GST activity was significantly increased in the highest TNT-exposed plants. Differences in malate dehydrogenase activity in the shoots were influenced by pollution level (F:12.8, $p = 0.0001$), while in the roots, both the effect of pollution (F:11.8, $p = 0.0001$) and the interaction term (F:9.5, $p = 0.0003$) were significant. A dominant bacterial effect (F:38.2, $p = 0.0001$) lowered the superoxide dismutase (SOD) activity in the shoots of TNT-exposed plants relative to the non-inoculated ones, while in the roots both the interaction term (F:7.9, $p = 0.002$) and the bacteria effect (F:4.4, $p = 0.03$) were significant. The activity of catalase in shoots and roots was explained by a significant interaction effect (F:10.8, $p = 0.0006$ and F:10.7, $p = 0.0006$) and pollution effect (F:6.5, $p = 0.006$ and F:6.4, $p = 0.005$). Guaiacol peroxidase (GPOD) activity in shoots was impacted by an interaction term (F: 13.9, $p = 0.0001$) and bacterial effect (F:19.1, $p = 0.0002$) but showed no pollution effect. In contrast, GPOD activity in the roots was enhanced by pollution (F: 6.07, $p = 0.0006$).

At the end of the experiment, extractable TNT-concentrations in the sand were determined (**Figure 5B**). Less than 1% TNT remained in the pots when *Agrostis* was inoculated with consortium ST1, whereas 35–48% of the initial applied TNT was recovered from the non-inoculated pots with *Agrostis*. Control pots without plants and without inoculum still contained more than 80% of original TNT-concentration. The recovery efficiency of TNT from the substrate was 90.1 ± 6.5%, the recovery of initial spiked TNT was 22.01 ± 0.3 mg TNT (for the

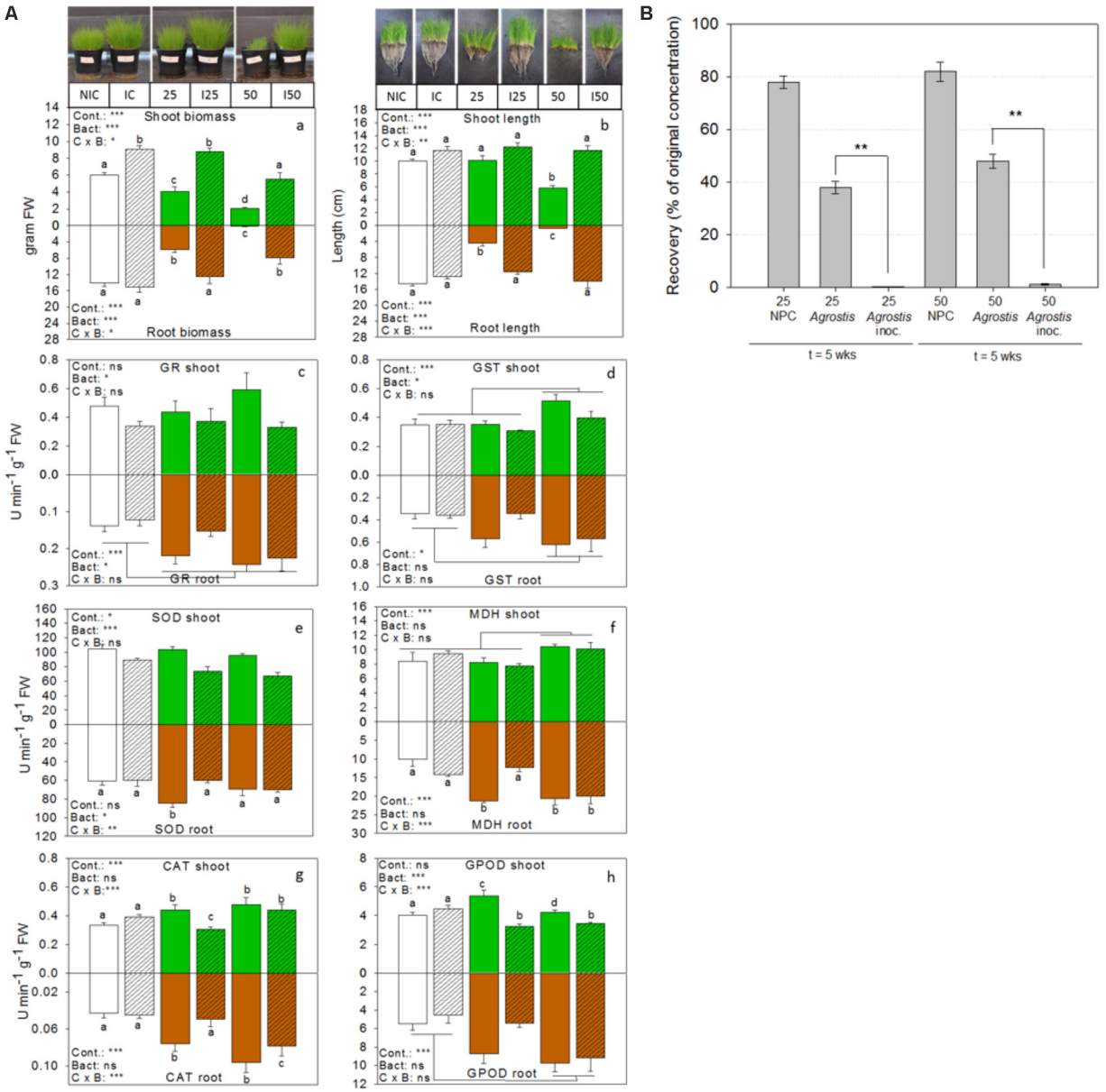

FIGURE 5 | Bioaugmentation with consortium ST1 in the rhizosphere of common bent grass increases biomass, removes TNT and increases plant health. **(A)** Bar graphs showing plant biomass, shoot and root length, and activities of enzymes related to antioxidative defense for inoculated (I) TNT-exposed plants, inoculated non-exposed plants (IC) and non-inoculated non-exposed control plants (NIC). Shoot responses are shown in green (white for the control) and belowground root responses are shown in brown (white for the control). Bacteria inoculation conditions are shown in hatched bars. Enzyme activities are expressed in units per min and gram fresh weight. TNT exposure concentrations were 25 and 50 mg TNT per kg dry weight sand. Values represent average and standard-error of 8 biological replicates per treatment, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, not significant. Cont., control; Bact., bacteria; C × B, contamination x bacteria interaction effect (two-way ANOVA, Holm–Sidak, $n = 8$). **(B)** Percentage of TNT remaining in the substrate after 5 weeks of growth. GR, glutathione reductase; GST, glutathione transferase; SOD, superoxide dismutase; MDH, malate dehydrogenase; CAT, catalase, GPOD, guaiacol peroxidase.

25 mg/kg condition) and 43.70 ± 0.7 mg TNT kg^{-1} (for the 50 mg/kg condition). The TNT-transformation products were only detected in inoculated conditions and only in trace amounts (in all <5%). Roots and shoots were also analyzed for the presence of TNT and its transformation products. TNT was retained in the roots of inoculated plants and only very low concentrations were detected (0.034 mg TNT per gram dry weight root in substrate amended with 50 mg TNT kg^{-1}) and none in the shoots. This was similar for the non-inoculated plants. At the end of the experiment, the numbers of viable bacterial cells per gram rhizosphere were determined in the inoculated pots. These were respectively 2.3×10^5 for the control, 4.32×10^5 for the 25 mg TNT per kg sand conditions and 7.02×10^5 CFUs for the 50 mg TNT per kg soil. Although this was a significant decrease

compared to the initial number (10^6 CFU g^{-1} sand), it was 100 times higher than the non-inoculated substrates (average $<10^3$ CFU g^{-1} sand), suggesting rhizosphere colonization.

DISCUSSION

This study presents the isolation and detailed characterisation of 300 bacterial strains, called the SYCAM collection, from *A. pseudoplatanus* trees growing on a military site with a long history of explosives pollution. Because of this fact, the site represents a unique resource in terms of finding potentially novel strains with PGP and catalytic activities to be able to deal with multi-compound pollution in a hostile environment with low nutrients. Moreover, plants at such sites are expected to have more close association with their rhizospheric microorganisms, to be able to germinate and grow in polluted soil, in comparison to non-polluted habitats. Therefore, as a first confirmation of our hypothesis, we applied a rapid and inexpensive, though high-throughput, automatic ARISA fingerprinting technique, to get a glimpse of the total microbial communities and their differences or similarities across compartments and locations.

A clear shift was noticed in the bacterial community composition, with the main effect explained along the *x*-axis with TNT pollution and the secondary effect was the separation between bulk soil and rhizosphere, an effect which was much more pronounced for the polluted soils compared to the control site (**Figure 1**). Previous studies also found that TNT induces shifts in bulk soil bacterial communities based on PUFA, DGGE or microarray analysis (Fuller and Manning, 1998; Eyers et al., 2006; George et al., 2008; Travis et al., 2008). In this study, ARISA chip technology has the advantage to be more comparable between sample sets, and to give a higher reproducible result, because of the automatization, and at a fairly high resolution (bands of < 10 bp are distinguished) compared to conventional agarose or polyacrylamide gels. Though the advantage of DGGE compared to ARISA is the separation of isolates based on GC content, and therefore a unique band per strain is expected, whereas the 16S-23S rRNA intergenic spacer region is highly variable amongst strains, but can also be of similar length for very phylogenetically distant strains, so bands are not easily assigned to groups of bacteria (Schmieder and Robert Edwards, personal communication), which doesn't preclude that specific primers targeting specific groups of interest can be used with ARISA, reducing the sample complexity. Though ARISA served still a very useful technique to discriminate diversity patterns and shifts even in a very rich forest soil (**Figure 1B**) and rhizosphere soil.

Dramatic decreases in community richness, Shannon and Simpson diversities were observed in the HT soil, indicating high toxicity experienced by the residing soil microbiome and potentially affecting their functioning. It was also obvious that TNT differentially affects diversity in the rhizosphere. TNT can get complexed with organic compounds exuded by the roots, and form amino-metabolites to humified organic matter, which can decrease its bio-availability and hence toxicity (Hundal et al., 1997; Kreslavski et al., 1999; Thorn et al., 2002). Though, active transformation by different microorganisms in the rhizosphere compared to the bulk soil, can also in part explain the lower TNT concentrations in the rhizosphere and feedback effect on the microorganisms.

As ARISA confirmed our initial expectations that this site harbored different microbial communities compared to a non-polluted forest site, the isolation and characterization of bacterial isolates was straightforward with the aim to obtain a genetic and cultivable pool of plant-associated strains, which may serve high purpose for bioremediation, or other biotechnologies. Our results indicated that using five different media, and especially the medium 284 with synthetic plant root exudates, was appropriate for the isolation of Gamma- and Beta-proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes (**Figure 2**). A very diverse subset of genera was isolated such as *Curtobacterium*, *Kaistia*, *Ensifer*, *Sphingomonas*, *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Actinobacterium*, *Agromyces*, *Aeromonas*, *Pseudomonas*, *Streptomyces*, many of which have previously been described as taxa holding plant growth promoting strains (Compant et al., 2010; Barret et al., 2011; Bruto et al., 2014). Although, the selection of the media did not govern the isolation of new phyla, the diversity of cultured phyla of the SYCAM collection can be expanded in future using additional complementary media, targeting other bacterial groups of interest, as done for the large scale *Arabidopsis thaliana* A*t*-SPHERE culture collection study (Bai et al., 2015).

Bacteria that have a high tolerance to TNT will be favored in TNT-polluted soils. In this study, Gram-negative bacteria were shown to be strongly enriched in the TNT-polluted soils while Gram-positive bacteria were depleted in both TNT-polluted bulk soils and the rhizosphere based on CFU-counts (**Supplementary Figure S1**). Previously, negative effects of TNT on Gram-positive bacteria have been reported and the underlying mechanisms yet remain to be determined (Fuller and Manning, 1997; Eyers et al., 2006). A hypothesis is a covalent cross-linking of cell membrane lipopeptides by TNT inhibiting cell replication (Ho et al., 2004; McBroom and Kuehn, 2007; Cho et al., 2009). Alternatively, gram-negative bacteria may deal with environmental pollution by enhancing the production of exopolymers, as observations for *Pseudomonas putida* HK-6 exposed to TNT show (Lee et al., 2008) or for example for *P. putida* KT2440, by increasing the expression of genes involved in detoxification (antioxidative response pathways, glutathione biosynthesis and nitroreductases) in addition to active efflux pumps to maintain low intracellular TNT-concentrations (Fernandez et al., 2009). Based on our findings of CFU count, it is suggested that the difference in sensitivity toward TNT will most likely affect the strategies used by the bacteria to transform and detoxify TNT, and explain the overall lower or higher fitness.

Several bacterial strains that originated from the polluted soil and rhizosphere (*Rhizobium*, *Burkholderia, Enterobacter, Pseudomonas*, *Sphingomonas, Raoultella*), were able to co-metabolically transform TNT to amino-reduction products (**Figures 3**, **4**). This is not completely unexpected as nitroreductases are fairly diverse distributed in bacteria (Roldan et al., 2008). Many of the bacterial isolated we recovered seem to be able to catalyze the aromatic ring reduction of TNT yielding monohydride- and dihydride-Meisenheimer

products. Especially, the high proportion of *Pseudomonas* spp. with nitroreductase activity in the cultivable collection is highly interesting and demonstrates the usefulness of this strain in TNT-bioremediation. Nitrite was not at all times detected in the supernatants, probably indicating that it is consumed by some strains, as we have shown previously (Thijs et al., 2014b). The formation of Meisenheimer complexes, and diarylamines with the concomitant release of nitrite from TNT is an important metabolic route for TNT detoxification (van Dillewijn et al., 2008b; Wittich et al., 2008; Wittich et al., 2009). This pathway has garnered a significant interest for TNT biotransformation and detoxification (Stenuit and Agathos, 2010). This is also shown by the fact that some of these bacterial nitroreductases have been successfully engineered in plants which significantly improved plant survival, and increased phytoremediation effectiveness (Travis et al., 2007; Rylott et al., 2011a). Further experimental evidence of TNT detoxification and characterisation of the transformation products in many of our isolates are in the pipeline. As an example, the fully genome-sequenced and experimentally characterized isolate, *Raoultella ornithinolytica* strain TNT (Thijs et al., 2014b), was shown to hold the *N*-ethylmaleimide reductase gene, catalyzing TNT-denitration combined with nitrite consumption, only previously described for the laboratory model strains, *Escherichia coli* which lacks the genes to metabolize nitrite as N-source, *Enterobacter cloacae* (Bryant and DeLuca, 1991; Bryant et al., 1991), and *Pseudomonas putida* KT2440 (van Dillewijn et al., 2008a,b). This highlights the genetic potential of this culture collection. On a side, overall Gram-positive bacteria such as *Rhodococcus*, *Bacillus* and *Arthrobacter* did not show growth on TNT or efficient TNT reduction, which corroborates earlier studies (Fuller and Manning, 1997; Thijs et al., 2014a), and thus these taxa are not the candidates to look for in follow-up studies.

The high proportion of cultivable isolates with abilities to produce plant hormones (e.g., auxin, acetoin), release Fe (siderophores) and exhibit ACC-deaminase activity, which plays a role in decreasing ethylene-induced stress (Glick, 2005), indicates the bacteria being studied have adapted to live in close association with the plant host, and in a stressfull environment (**Figure 4**). Also, thirteen chitin-degrading bacteria of the Actinobacteria (*Streptomyces, Bacillus*) were isolated from the soil and rhizosphere samples. Chitinases produced by *Streptomyces* sp. have been shown to suppress the growth of phytopathogenic fungi (Hoster et al., 2005) which can be an interesting property for further research on antifungal activity. ACC-deaminase plays an important role in reducing plant ethylene-induced stress (Glick, 2014); this may explain part of the enhanced plant health effects seen in the inoculated treatments (**Figure 5**). The cultivable community of RLT showed an increased proportion of organic acid producing strains, dominated by Bacilli. Organic acid production by PGPR can improve phosphate solubilisation (Richardson et al., 2009; Bianco and Defez, 2010), which is a limiting plant nutrient, and may contribute to the complexation of toxic metal ions (Cu, Pb, Zn, Cd) (White et al., 1997).

Finally, to get insights into the mechanisms of PGP and TNT-detoxification by rhizospheric bacteria, community members of the low and high TNT-polluted rhizosphere subgroups were investigated in a rhizosphere-colonization experiment to verify if bacteria identified as members of the *Acer* rhizosphere and bulk soil communities were able to promote plant growth, and if bacteria were able to influence plant physiology at the level of their antioxidative defense. It is well known that TNT induces oxidative stress in plant cells leading to the over-production of reactive oxygen species (ROS) (Johnston et al., 2015). Activation of enzymes responsible for reduction, oxidation and conjugation of TNT such as nitroreductases, peroxidases, phenoloxidases and glutathione transferases has been reported in several plant species (Adamia et al., 2006; Brentner et al., 2008; Beynon et al., 2009; Cummins et al., 2011; Gunning et al., 2014). In 2015, researchers found that a mutation in the gene MDHAR6 encoding a monodehydroascorbate reductase offers enhanced TNT tolerance which is very promising, but these mutant lines have not been tested in combination with catalytic and plant-growth promoting consortia (Johnston et al., 2015). In addition, limited evidence exists about how bacteria can influence the activities of antioxidative enzymes in plants native to military locations (Rylott et al., 2011b). Our results showed that after 5 weeks of growth, 35–48% of the TNT remained in pots with the grasses that did not receive a bacterial inoculum, whereas less than 1% TNT was left in pots with grass that was inoculated with the bacterial consortium, suggesting active TNT-transformation by the bacteria in the rhizosphere. In addition, bacterial inoculation increased the above- and below-ground biomass of the plants exposed to 25 and 50 mg TNT kg^{-1} sand, reduced SOD and GPOD-activities in the leaves and affected, to some extent, GR- and GST-activities. Inoculated grasses showed indications of oxidative stress as observed from the elevated activities of antioxidative enzyme. For instance, concentration-dependent responses of catalase and guaiacol peroxidase activity were observed along with an increase in the activity of GST in root tissues. This dose-response relationship between GST and TNT was consistent with earlier observations for poplar trees exposed to TNT (Brentner et al., 2008). The correlation between GST and TNT can be explained by the phase three conjugation reactions according to the green liver model, whereby complexation with glutathione and storage in the central vacuole is used by the plant as detoxification mechanism (Sandermann, 1994). Malate dehydrogenase activity was also increased in response to TNT concentration and indicates increased flux through the tricarboxylic acid cycle, which generates reduced equivalents of NAD(P)H. While the increase of the antioxidative activity in roots may be explained by the local impact of TNT on cell structure and function, the stimulation of antioxidative enzymes in leaf tissues suggests that the presence of bacteria in the rhizosphere indirectly alters leaf physiology or results in the transmission of stress signals from root to leaves as observed for metal-stress (Opdenakker et al., 2012; DalCorso et al., 2013). In addition, this may also be explained by low levels of TNT transported to the aerial parts and stimulating gene expression in shoot and leaf tissues. Other papers have found as well that TNT is only limited transported to plant shoot tissues (Vila et al., 2007; Brentner et al., 2010). The observed increases in the shoot and root weights after inoculation may have resulted from pleiotropic bacterial effects

such as detoxification of TNT in the rhizosphere, changes in plant nutrition, release of plant growth hormones or production of enzymes reducing stress-ethylene levels in the plant (ACC-deaminase) restoring a better balance between pro-oxidants and antioxidants in the plant. Together, these findings indicate that bacteria originating from the *A. pseudoplatanus* rhizosphere and bulk soil, play important roles in growth and survival of plants on TNT polluted soil. Subsequent extrapolation to the field is recommended to study the interactions in the most complex soil environment.

TNT rhizoremediation in natural soils is difficult and slow as a result of high TNT toxicity, abiotic limitations (e.g., bioavailability, heterogeneity of pollution, soil structure, pH and nutrients) and microbial competition. The observation of taxa-specific abundance shifts in the rhizosphere of TNT-soils and the high nitroreductase activity found amongst the cultured members, supports the hypothesis that natural TNT-rhizoremediation by *Acer* trees in the field is indirectly accomplished by the rhizosphere microbes. Hence, phytoremediation has to be considered as a promising strategy. To stimulate phytoremediation, inoculation of plant growth promoting and degradative strains may overcome some of the inherent constraints plants face when colonizing and growing in polluted soils. The isolation and functional characterisation efforts of cultivable members corresponding to important soil groups as *Pseudomonas* spp. with TNT-transforming activity are significant steps forward to reaching our ambition in enhancing the knowledge of plant-associated bacteria. A next useful step would be the evaluation of our strains not only for TNT transformation but also other toxic compounds, like PCBs, and on the other hand prospecting our SYCAM collection for potential antibiotic producing strains, CRISPR-cas loci, multi-strain interaction studies (Serrano-González et al., 2018) and metatranscriptomic analyses. CRISPR-cas engineering of either bacterial genes or the host plant enzymes, e.g., swapping more active gene variants for others, can be a future path in phytoremediation (Basharat et al., 2018). In all, combined metatranscriptomics and analytics will be particularly informative in unraveling the keys to plant growth-promotion and TNT-detoxification in the rhizosphere.

CONCLUSION

In summary, the SYCAM bacterial culture collection holds 300 bacterial isolates with multiple PGP features and catalytic genes, originating from a unique location with high level nitroaromatics explosives pollution. Culture-independent analyses revealed a strong effect of TNT on microbial community composition and diversity in the forest soil, driving distinct communities. In these communities surviving in the harsh conditions, representative strains covering the main phylogenetic groups in soil and rhizosphere were recovered, purified and maintained in culture. Production of auxin, siderophores and the volatile hormone acetoin are a few of the traits multiple strains scored positive for, besides nitroreductase activity to detoxify TNT, but potentially also structural nitroaromatic homologues found in pesticides. Some of the strains in this culture collection have no genome sequenced representatives yet in the database, such as *Agromyces terreus*, and thus this again indicates the many potential novelties this collection holds, also in terms of catalytic functions. Hence, with this new strain repository, available for further phenotypic and *in planta* characterisation in addition to full genome sequencing, we aim to contribute to a better understanding of plant growth promotion, and plant microbiome functioning, to construct consortia which can enhance phytoremediation of the most recalcitrant compounds.

AUTHOR CONTRIBUTIONS

SoT, WS, NW, and JV participated in planning of research, interpretation of obtained results, and manuscript writing. SoT performed all experiments. SaT, BB, and PvD helped with interpretation of obtained results. PS and RC contributed with chemical analyses. JvH, as microbiologist expert, provided valuable feedback on figure layout and structuring of the Results section, and proofread the manuscript for English language.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01134/full#supplementary-material

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PGPR Reduce Root Respiration and Oxidative Stress Enhancing *Spartina maritima* Root Growth and Heavy Metal Rhizoaccumulation

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The present study aims to unravel ecophysiological mechanisms underlying plant-microbe interactions under natural abiotic stress conditions, specifically heavy metal pollution. Effect of plant growth promoting rhizobacteria (PGPR) bioaugmentation on *Spartina maritima in vivo* root respiration and oxidative stress was investigated. This autochthonous plant is a heavy metal hyperaccumulator cordgrass growing in one of the most polluted estuaries in the world. The association with native PGPR is being studied with a view to their biotechnological potential in environmental decontamination. As a novelty, the oxygen-isotope fractionation technique was used to study the *in vivo* activities of cytochrome oxidase (COX) and alternative oxidase (AOX) pathways. Inoculated plants showed decreased antioxidant enzymatic activities and *in vivo* root respiration rates. The reduction in respiratory carbon consumption and the stress alleviation may explain the increments observed in *S. maritima* root biomass and metal rhizoaccumulation after inoculation. For the first time, plant carbon balance and PGPR are interrelated to explain the effect of rhizobacteria under abiotic stress.

Keywords: plant growth promoting rhizobacteria (PGPR), heavy metals, *Spartina maritima*, root respiration, oxygen-isotope fractionation, oxidative stress, carbon balance, bioremediation

INTRODUCTION

Heavy metal pollution in soil is one of the most serious ecological problems all over the world, as it causes negative impacts on the ecosystem and plant, animal and human health (Vassilev et al., 2004; Nagajyoti et al., 2010; Ali et al., 2013). Heavy metals cannot be degraded to harmless products, and consequently persist in the environment indefinitely (Garbisu and Alkorta, 2001). Many remediation strategies have been considered to counter the detrimental effects of metal excess in soil (Sharma and Pandey, 2014). Among them, using metal accumulating plants has been receiving increasing attention due to their good potential for success (Burd et al., 2000).

Spartina maritima (Curtis) Fernald is an indigenous cordgrass that naturally grows in the joint estuary of Tinto and Odiel rivers (SW Spain), one of the most polluted areas by heavy metals in the world (Mesa et al., 2016). This C4 halophyte is an important salt marsh pioneer and ecosystem engineer (Mateos-Naranjo et al., 2010) that possesses a high heavy metal accumulating capacity in tissues, especially in its roots, making it useful for phytoremediation purposes (Redondo-Gómez, 2013). Despite the fact that metal hyperaccumulator plants like *S. maritima* can resist pollution to certain degree, it is well known that exposure to heavy metals triggers a wide range of physiological and biochemical alterations in plants (Singh et al., 2015), ultimately leading to reduced growth and metal phytoaccumulation (Duarte et al., 2013). In this context, the association with plant growth promoting rhizobacteria (PGPR) plays an important role toward enhancement of plant development under heavy metal stress, thus ameliorating phytoremediation capacity of hyperaccumulator plants (Glick, 2010; Rajkumar et al., 2012).

Improvement of *S. maritima* root growth and metal rhizoaccumulation after PGPR inoculation in polluted sediments has been recently demonstrated (Mesa et al., 2015a). This positive effect was mainly mediated by improvement of photosynthetic apparatus performance, linked with a beneficial impact on PSII functionality and chlorophyll concentration. However, despite amelioration in leaf fitness, the bacterial consortium was able to stimulate plant growth and metal uptake chiefly in roots (Mesa et al., 2015a). Taking into consideration that plant growth depends on the accumulation of photosynthetic carbon not consumed during respiration (Ribas-Carbó et al., 2000; González-Meler et al., 2001; Lambers et al., 2008; Del-Saz et al., 2016; Flórez-Sarasa et al., 2016), it is reasonable to study both photosynthesis and respiration processes in *S. maritima* in order to elucidate the manner by which plant growth is increased under PGPR inoculation. With this in mind, it is known that high concentrations of heavy metals greatly alter respiration in plants (Lösch, 2004) by affecting different components of the mitochondrial electron transport chain (mETC) (Keunen et al., 2011). Among them, alternative oxidase (AOX) and cytochrome oxidase (COX) have been, by far, the most studied enzymes of the mETC (Vanlerberghe, 2013). In particular, AOX is thought to play an important role under the stress induced by metal toxicity (Keunen et al., 2011). However, there is no information about the response of the *in vivo* activities of AOX and COX pathways under metal excess and the possible effects of PGPR inoculation on plant respiration under heavy metal stress. Thus, we hypothesize that previously observed beneficial root effect after bacterial bioaugmentation may be linked to a positive carbon balance at root level. It is plausible that microbial inoculation may alter COX and AOX pathways. Indeed, the scales may be tilted in favor of a greater relative effect on AOX component, due to its significance in plant response against stress that comes with heavy metal exposure. Moreover, it should not be forgotten that other defensive responses, including the activities of antioxidant enzymes that reduce reactive oxygen species (ROS) levels, such as catalase (CAT), guaiacol peroxidase (GPX), and superoxide dismutase (SOD) activate under metal stress, and are also susceptible of bacterial regulation (Dimkpa et al., 2009a,b; Das and Roychoudhury, 2014).

The present study links two fields that are rarely combined: the bioremediation/PGPR literature and the respiratory physiology/carbon balance literature. It aims at describing the ecophysiological response, at the organism level, of the autochthonous cordgrass *S. maritima* after PGPR bioaugmentation in natural heavy metal polluted sediments, with a special emphasis on *in vivo* root respiration and oxidative stress. It should provide a new insight into our understanding of plant biology in the context of PGPR-associated phytoremediation, with a view to the biotechnological potential of hyperaccumulator plants in environmental decontamination.

MATERIALS AND METHODS

Plant and Soil Source and Growing Conditions

In June 2016, 10 cm diameter clumps of *S. maritima* were randomly collected from a natural population located in a well-drained gently sloping intertidal low-marsh (mean sea level + 1.30 m relative to Spanish Hydrographic Zero, SHZ) from the Tinto river salt marsh (37°15′N, 6°58′W; SW Spain). Clumps were planted in individual plastic pots (15 cm high × 18 cm diameter), filled with 1 kg of soil from the marsh and placed in a glasshouse with temperatures of 21–25°C, 40–60% relative humidity, natural day light and irrigated with tap water. Pots were kept under these conditions for 1 week and then were randomly assigned to two bioaugmentation treatments (details in the next section). Tap water metal concentrations were: arsenic (As) < 1 μg l^{-1}, cadmium (Cd) < 1 μg l^{-1}, copper (Cu) < 0.01 mg l^{-1}, nickel (Ni) < 5 μg l^{-1}, lead (Pb) < 5 μg l^{-1} and zinc (Zn) < 0.01 mg l^{-1}. Tinto sediment psychochemical properties are given in **Table 1**, which also shows metal threshold values imposed for remedial action.

Bacterial Strains, Inoculant Solution, and Bioaugmentation Treatment

Bacteria used in this work were isolated from the rhizosphere of *S. maritima* grown in the Tinto river estuary, SW Spain (Mesa et al., 2015a). They were identified by PCR amplification and sequencing of the 16S rDNA as *Bacillus methylotrophicus* SMT38 (Accession No. KF962966), *Bacillus aryabhattai* SMT48 (Accession No. KF962976), *Bacillus aryabhattai* SMT50 (Accession No. KF962978), and *Bacillus licheniformis* SMT51 (Accession No. KF962979) (Mesa et al., 2015a). The resistance of these bacteria to different heavy metals and NaCl was determined on plates containing TSA 0.2 M NaCl medium (according to sediment conductivity), both supplemented with increasing concentrations of heavy metals or NaCl from stock solutions (Mesa et al., 2015a). These bacteria showed a high resistance to several heavy metals and metalloids (up to 10 mM Cu, 4 mM Zn, 18 mM As or

TABLE 1 | Concentration of arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn) and physicochemical properties of sediments from Tinto marshes.

	Metal concentration (mg Kg^{-1})					
	As	**Cd**	**Cu**	**Ni**	**Pb**	**Zn**
Tinto sediments	524 ± 31	4.6 ± 0.4	2968 ± 211	34.3 ± 1.5	610 ± 38	2576 ± 192
Nature park soil[2]	>100	>15	>500	>500	>1000	>1000
Agricultural soil[2]	>50	>7	>300	>200	>350	>600

Physico-chemical properties				
Texture[1]	**pH**	**Redox potential (mV)**	**Conductivity (mS cm^{-1})**	**Organic matter (%)**
71/19/10	6.3 ± 0.2	195 ± 12	12.6 ± 0.5	11.5 ± 0.6

Values are means ± SE (n = 6). [1]Texture (silt/clay/sand percentage). [2]Reference threshold values stablished by the local Government to measure pollution severity and to guide corrective actions. Beyond these intervention values, remedial action must be taken in the soil (Junta de Andalucía Consejería de Medio Ambiente, 1999).

20 mM Pb), as well as to NaCl (up to 2 M NaCl). Moreover, these bacteria exhibited multiple plant growth promoting properties, such as nitrogen fixation, phosphate solubilisation, biofilm-forming capacity and production of siderophores and indole-3-acetic acid, demonstrated by several screening tests for plant growth promoting traits (Mesa et al., 2015a). Finally, the four bacterial isolates were cultivated together and none of them showed antagonistic activity against each other (data not shown). To prepare the inoculant solution, bacteria were grown separately in 250 ml Erlenmeyer flasks containing 50 ml of TSB 0.2 M NaCl medium and incubated under continuous gentle shaking at 28°C during 18 h. Then, cultures were centrifuged in 50 ml Falcon tubes at 8000 rpm during 10 min and the supernatant was discarded. Pellets were washed twice with tap water and finally resuspended in tap water to get a suspension with an OD_{600} of 1.0 (ca. 10^8 cells per ml). Then, equal amounts of the four bacterial suspensions were mixed to get the final inoculant solution. Pots were randomly assigned to two treatments (n = 12, 6 pots in each one): control non-inoculated plants and inoculated plants. During the assay, pots were slightly watered with tap water every 2 days. For plant inoculation, every pot was watered with 10 ml of the inoculant solution (ca. 10^9 cells per pot) at the beginning of the experiment (Mesa et al., 2015b).

Plant Biomass and Ions Concentration in Plant Tissues

At the end of the experiment, 30 days after treatment initiation, plants were harvested and separated into roots and shoots and dried at 60°C for 48 h before weighing (n = 6).

For tissues ions concentration, leaf and root samples were randomly collected and successively washed with distilled water in order to remove ions from the free spaces and from its surface prior to analysis (n = 6). After that, leaf and root samples were ground as previously described in Mateos-Naranjo et al. (2008) and digested with 6 ml HNO_3, 0.5 ml HF and 1 ml H_2O_2 at 130°C for 5 h in triplicate. Then, As, Cd, Cu, Ni, Pb, and Zn were measured by inductively coupled plasma (ICP-OES) spectroscopy (ARL-Fison 3410, United States). For quality control, the accuracy and precision of analytical procedure was checked every 5 samples by routine determination of total element concentrations using reference materials from Fisons certified. The average of uncertainty in the determination of elements was in all cases < 2%.

In vivo Root Respiration and Oxygen-Isotope Fractionation Measurements

Respiration and oxygen isotope fractionation measurements were performed in randomly selected root samples (approximately 300 mg fresh weight, FW) of five replicate plants 1 day before complete plants were harvested for growth analysis.

Roots samples were immediately carefully rinsed using a soft water jet and left to air dry during 15 min before to be placed in a 3 ml stainless-steel closed cuvette maintained at a constant temperature of 25°C (Gastón et al., 2003). The respiration cuvette was equipped with two inlets: one connected to the mass spectrometer (Delta XPlus, Thermo LCC, Bremen, Germany), and the other connected to a 2 ml air-tight syringe. Throughout the experiment the syringe was used to both mix the air in the cuvette and to maintain the cuvette at constant pressure. Air samples of 300 µl were sequentially withdrawn from the cuvette and fed into the mass spectrometer. Changes in the $^{18}O/^{16}O$ ratios and oxygen concentration were obtained to calculate the oxygen-isotope fractionation and respiration rates (Ribas-Carbó et al., 2005). The electron partitioning to the alternative pathway (τ_a) was calculated as follows:

$$\tau_a = (\Delta_n - \Delta_c) / (\Delta_a - \Delta_c) \quad (1)$$

Where Δc, Δa are the oxygen-isotope fractionation of the cytochrome (+ SHAM) and alternative (+ KCN) pathway, respectively, and Δ_n, is the oxygen-isotope fractionation of the respiration in the absence of inhibitors. For Δ_a measurements, roots were submerged in a solution of 10 mM KCN for 30 min. In addition, a piece of medical wipe wetted with 10 mM KCN was placed in the cuvette. A value of Δ_a of 28.03 ± 0.03‰ (n = 3) was obtained. For the calculation of Δ_c, roots were submerged in freshly solutions of 25 mM SHAM

for 30 min. A value of Δ_c for 18.0 ± 0.06‰ (n = 3) was obtained.

The individual activities of the cytochrome oxidase pathway, COP (v_{cyt}) and AOX pathway, AOP (v_{alt}) were obtained by multiplying the total oxygen uptake rate (V_t) and the partitioning to each pathway as follows:

$$v_{cyt} = V_t \times (1 - \tau_a)$$

$$v_{alt} = V_t \times \tau_a$$

Antioxidant Enzymes Assays

Enzyme extraction was done following the methodology used by Duarte et al. (2015). At the end of experiment, 500 mg of fresh roots and leaf samples were grounded in 8 ml of 50 mM sodium phosphate buffer (pH 7.6) with 0.1 mM Na-EDTA and were centrifuged at 10,000 × g for 20 min at 4°C to obtain the soluble proteins. Five samples per inoculation treatment were used and three measurements per sample were registered. Catalase (CAT; EC1.11.1.6) activity was measured according to Teranishi et al. (1974), by monitoring the consumption of H_2O_2 and consequent decrease in absorbance at 240 nm (ε = 39.4 mM^{-1} cm^{-1}). The reaction mixture contained 50 mM of sodium phosphate buffer (pH 7.6), 0.1 mM of Na-EDTA and 100 mM of H_2O_2. The reaction was started with the addition of 100 µl of enzyme extract. Guaiacol peroxidase (GPX; EC1.11.1.7) was measured by the method of Bergmeyer et al. (1974), with a reaction mixture consisting of 50 mM of sodium phosphate buffer (pH 7.0), 2 mM of H_2O_2 and 20 mM of guaiacol. The reaction was initiated with the addition of 100 µl of enzyme extract. The enzymatic activity was measured by monitoring the increase in absorbance at 470 nm (ε = 26.6 mM^{-1} cm^{-1}). Superoxide dismutase (SOD; EC1.15.1.1) activity was assayed according to Marklund and Marklund (1974) by monitoring the reduction of pyrogallol and the increase of absorbance at 325 nm. The reaction mixture contained 50 mM of sodium phosphate buffer (pH 7.6), 0.1 mM of Na-EDTA, 3 mM of pyrogallol and Mili-Q water. The reaction was started with the addition of 10 µl of enzyme extract. One enzyme activity was defined as the amount of enzyme capable of inhibiting 50% of the autoxidation of pyrogallol. Control assays were done in the absence of substrate in order to evaluate the auto-oxidation of the substrates. To calculate the enzyme activity per µg of protein, total protein content in leaf and root extracts was determined according to Bradford (1976).

Statistical Analysis

Statistical analyses were carried out using 'Statistica' v. 6.0 (Statsoft Inc.). The differences between means the two inoculation treatments and between leaf and root ions and antioxidative activity at the end of the experiment were made by using one-way analysis of variance (F-test). Finally, Pearson coefficients were calculated between log10 transformed-fold changes of the respiratory and antioxidative variables, in order to assess correlations between them. Data were first tested for normality with the Kolmogorov–Smirnov test and for homogeneity of variance with the Brown–Forsythe test.

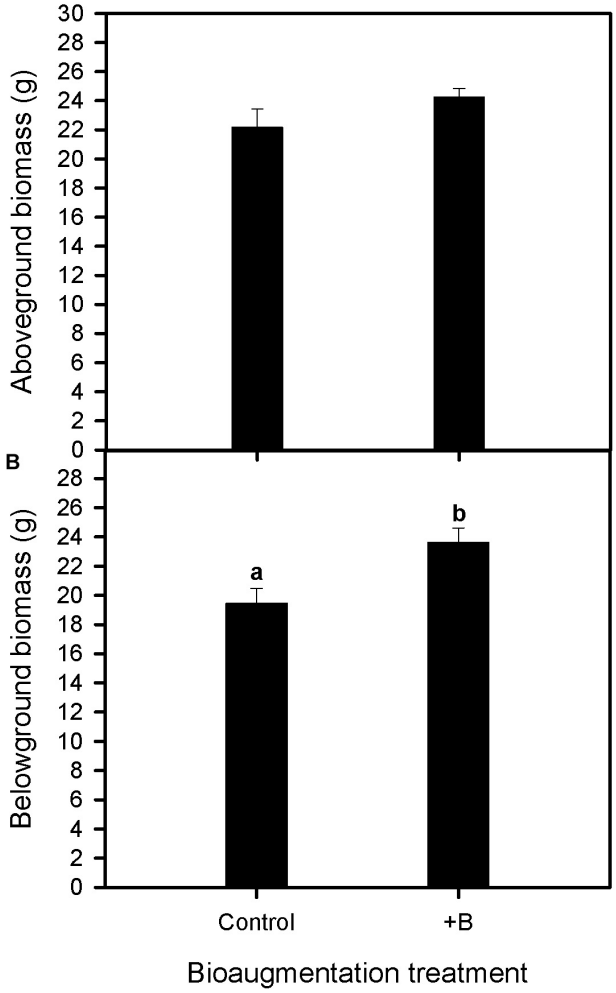

FIGURE 1 | Effect of bioaugmentation with the rhizobacterial consortium (+ B) on aboveground biomass **(A)** and belowground biomass **(B)** in *Spartina maritima* plants grown in natural sediment from Tinto marsh for 30 days. Values are means ± SE (n = 6). Different letters indicate means that are significantly different from each other (F-test, $P < 0.05$).

RESULTS

S. maritima Growth Analysis and Ion Tissues Concentrations

At the end of the experiment (30 days), soil bioaugmentation with the native bacterial consortium increased the belowground biomass of *S. maritima* by about 20% (one-way Anova, $P < 0.05$), whereas no significant differences were recorded for aboveground biomass respect to plants grown without bacterial bioaugmentation (**Figure 1**).

On the other hand, tissue ion concentrations were greater in the roots than in leaves (One-way Anova, $P < 0.05$) and rhizoinoculation treatment favored the capacity of *S. maritima* to accumulate As, Cu, Cd, and Pb in its roots, being the increment

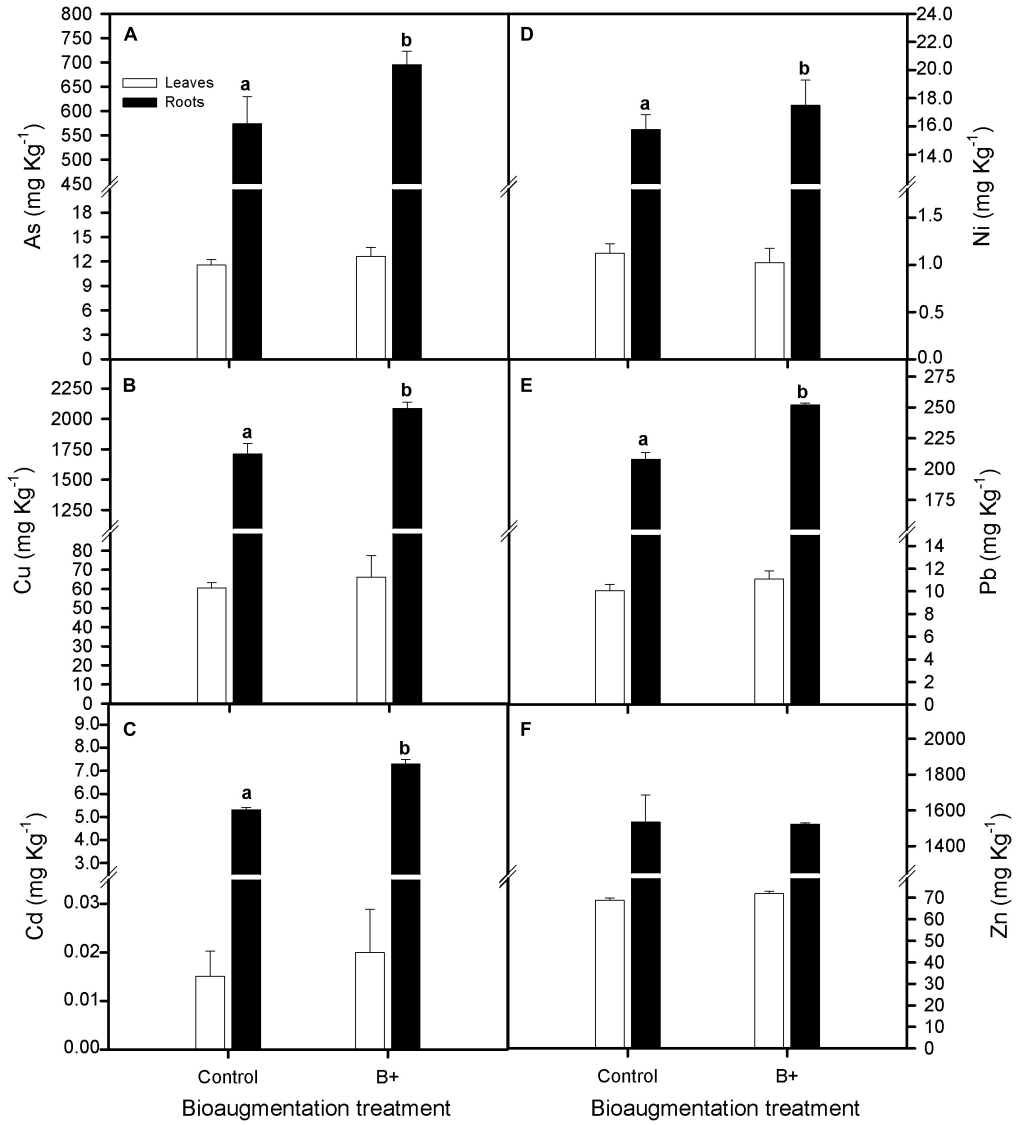

FIGURE 2 | Effect of bioaugmentation with the rhizobacterial consortium (+ B) on total **(A)** arsenic, As, **(B)** copper, Cu, **(C)** cadmium, Cd, **(D)** nickel, Ni, **(E)** lead, Pb, and **(F)** zinc, Zn accumulation for leaves and roots of *Spartina maritima* grown in natural heavy metal polluted sediment from Tinto marsh for 30 days. Values are means ± SE (n = 6). Different letters indicate means that are significantly different from each other (F-test, $P < 0.05$).

in those ions concentrations of 21, 22, 37, and 21%, respectively (one-way Anova, $P < 0.05$; **Figures 2A–C,E**), while root and leaves Ni and Zn ions concentrations did not vary between both inoculation treatments (**Figures 2A–F**).

S. maritima in vivo Root Respiration and Oxygen-Isotope Fractionation Measurements

Our results showed that total respiration rate (V_t) in *S. maritima* roots decreased considerably when the rhizobacterial consortium was used (**Figure 3A**). This reduction reached a 44.1% (one-way Anova, $P < 0.05$, **Figure 3A**). The electron partitioning to the AOX pathway provided a downscaling approach of these differences between both inoculation treatments in respiratory activity. Thus, the lower V_t was accompanied by a notable diminishing of the electron partitioning to the alternative pathway (τ_a) in inoculated plants, being this reduction of 59.2% compared with their non-inoculated counterparts (one-way Anova, $P < 0.01$, **Figure 3B**). Furthermore, it should be highlighted that respiratory activity variation was in greater extent due to higher changes in v_{alt} compared to v_{cyt}. Thus, compared with non-inoculated plants, v_{alt} and v_{cyt} decreased 69.7 and 30.8% respectively (one-way Anova, $P < 0.05$; **Figures 3C,D**).

Antioxidant Enzymes Activity

By the end of the experiment, bacterial bioaugmentation treatment increased considerably soluble protein content both

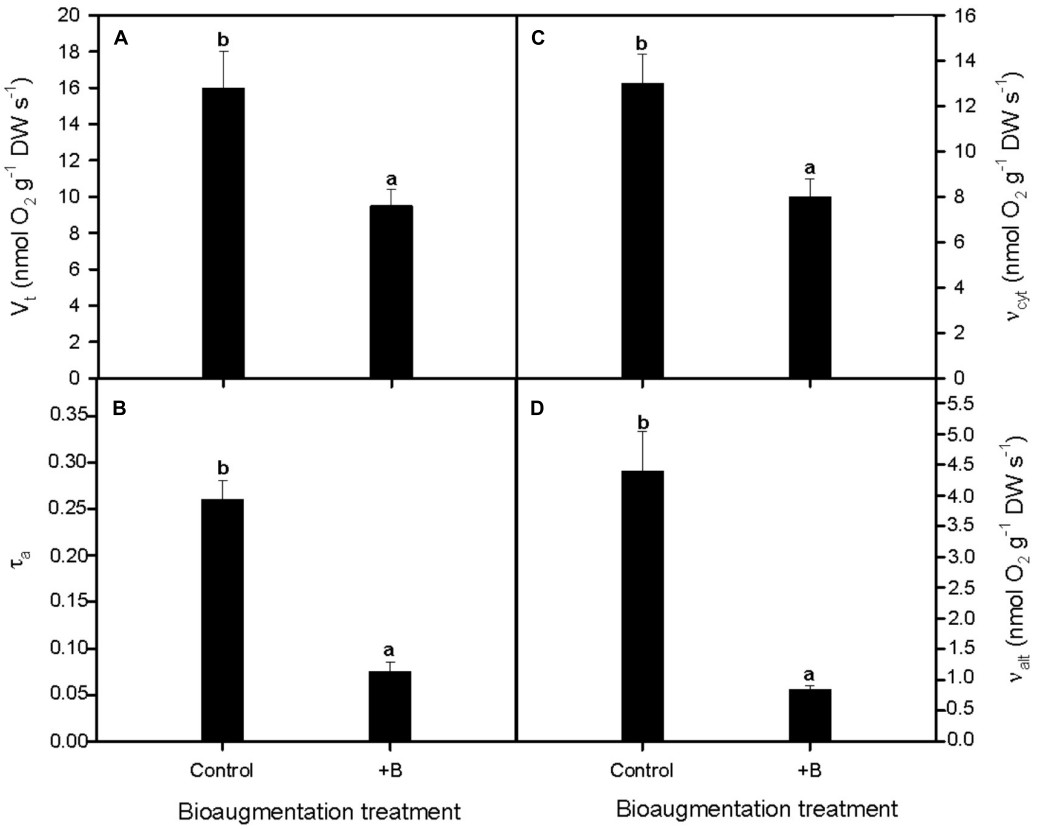

FIGURE 3 | Effect of bioaugmentation with the rhizobacterial consortium (+ B) on *in vivo* root respiratory activities as determined with the oxygen-isotope fractionation technique. **(A)** Total respiration rate (V_t), **(B)** electron partitioning to the alternative pathway (τ_a), and the *in vivo* activities of **(C)** cytochrome oxidase (v_{cyt}) and **(D)** alternative oxidase (v_{alt}) of *S. maritima* grown in natural heavy metal polluted sediment from Tinto marsh for 30 days. Values are means ± SE (n = 5). Different letters indicate means that are significantly different from each other (F-test, $P < 0.05$).

in leaves and roots in S. *maritima* (one-way $\text{Anova}_{\text{leaves and roots}}$, $P < 0.05$; **Figure 4A**). Also, concerning the antioxidative enzymatic activity, we found that CAT, SOD, and GPX activities were higher for roots than for shoots in both inoculation treatments (One-way Anova, $P < 0.05$) and decreased significantly in roots with bioaugmentation treatment (**Figures 4B–D**). Thus, compared with non-inoculated plants, these reductions were of 37, 48, and 43% for CAT, SOD and GPX activities, respectively (one-way Anova, $P < 0.05$; **Figure 4B–D**). Contrarely, bioaugmentation treatment did not have any significative effect on enzyme activities in leaves (**Figure 4B–D**).

DISCUSSION

This work analyses for the first time *in vivo* root activities of COX and AOX pathways during plant respiration in a bioremediation context. As another novelty, the effects of PGPR on the antioxidative enzyme response of *S. maritima* under heavy metal stress are reported. Uncommonly, these studies were carried out under natural heavy metal stress conditions, using natural Tinto estuary sediments, and a non-model plant, given the potential of native *S. maritima* for metal phytoremediation and its utmost important role in estuarine dynamics (Mateos-Naranjo et al., 2010; Redondo-Gómez, 2013).

Bioaugmentation was the inoculation strategy followed. This is, the addition of competent microorganisms, including the reinoculation of soil with indigenous microorganisms directly isolated from the collection site. The rationale behind this approach, supported by several studies (Vogel, 1996; Thompson et al., 2005), is that a strain derived from a population that is temporally and spatially prevalent in a specific type of habitat, is more likely to persist as an inoculum when reintroduced, than one that is transient or even alien to such a habitat, much more so in polluted scenarios like Tinto saltmarsh. Presumably, these strains are already present in the rhizosphere in the uninoculated treatment, and they have demonstrated to be advantageous for plant growth (Mesa et al., 2015a). But their effect is sub-optimal in these marshes sediments, which shows low bacterial diversity (Mesa et al., 2016). In these cases, increasing the bacterial biocatalyst activity offset the advantages of niche fitness (Vogel, 1996; Kuiper et al., 2004).

Plant growth partly depends on the accumulation of carbon not consumed during respiration. Abiotic stress may decrease growth and induce plants to raise respiration rates in order to fuel

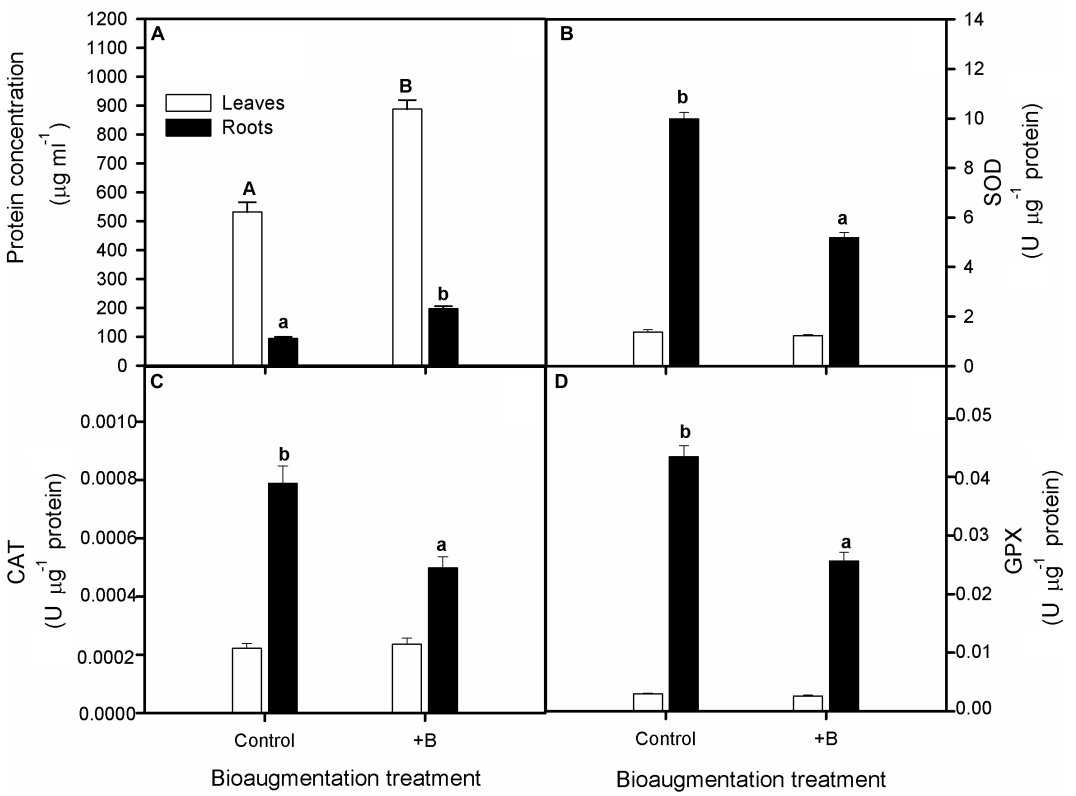

FIGURE 4 | Effect of bioaugmentation with the rhizobacterial consortium (+ B) on soluble protein content **(A)**, catalase, CAT **(B)**, superoxide dismutase **(C)** and guaiacol peroxidase, GPX **(D)** enzymatic activities in leaf and roots randomly selected of *S. maritima* grown in natural heavy metal polluted sediment from Tinto marsh for 30 days. Values are means ± SE ($n = 5$). Different letters indicate means that are significantly different from each other (F-test, $P < 0.05$). Capital letters refer to differences in root while lowercase in leaf.

with ATP the maintenance processes. Thus, the present research is based on the hypothesis that beneficial root effect after bacterial bioaugmentation under heavy metal stress may be linked to a positive carbon balance at root level, related to absolute and/or relative alterations of COX and AOX pathways, as well as the decrease of ROS. It is known that increased respiration is a typical plant response to abiotic stress that may lead to increase ATP production (Jacoby et al., 2011, 2013). Also, the activity of AOX has been largely hypothesized to maintain ROS homeostasis under stress (Vanlerberghe, 2013). However, increased rates of total respiration are associated with higher consumption of carbon, leaving less carbon for growth, which does not favor to phytoremediation purposes. Recently, arbuscular mycorrhizal fungus inoculation has shown to decrease root respiration via COX pathway and increase plant growth (Romero-Munar et al., 2017). Continuing our focus on symbiotic relations, bacteria may also influence root respiration rate by the release of some compounds to the rhizosphere (Adesemoye and Egamberdieva, 2013). In this sense, some authors measured root respiration for bacterial inoculation, generally showing an increased root respiration rate (Hadas and Okon, 1987; Phillips et al., 1999; Zhou et al., 2015; Qin et al., 2016). However, the effect of rhizobacterial bioaugmentation in roots has not been tested considering the electron partitioning between the COX and AOX pathways, much less under heavy metal stress. According to results here presented, inoculated *S. maritima* showed a slower *in vivo* root respiration rate in heavy metal polluted sediments via both the COX and AOX pathways, with particular emphasis on the AOX pathway, which was not observed for mycorrhizal symbiosis (Romero-Munar et al., 2017). The decrease in total respiration suggests that rhizobacterial inoculation induces in roots much less carbon consumption for maintenance purposes. Contrary to what we observed in inoculated plants, the absence of inoculation is associated with faster respiration that ultimately may lead to increase both carbon consumption and respiratory ATP synthesis (Jacoby et al., 2011).

On the other hand, our results revealed that the slower respiration rate was accompanied with lower activities of antioxidant enzymes in bioaugmented *S. maritima* plants, suggesting improved plant tolerance under heavy metal stress after rhizobacterial inoculation (Ahemad, 2012; Mesa et al., 2015b). Some authors state that *Spartina* species possess a well organized and appropriately modulated antioxidative defense system that results in a normal plant development (Martínez Domínguez et al., 2009, 2010). Concretely, *S. maritima* has shown to have antioxidant feedback responses in the presence of heavy metals (Duarte et al., 2013; Van Oosten and Maggio, 2015), but its enzyme response in polluted scenarios after PGPR inoculation

was unknown prior to this study. In this work, rhizobacterial bioaugmentation lessened CAT, SOD, and GPX activities in *S. maritima* roots compared to non-inoculated plants. This is interesting considering that root metal accumulation was higher after inoculation. Then, an increment in the generation of ROS, and consequently antioxidant enzyme activity, would be expected. Although several authors state that rhizobacteria mediates up-regulation of antioxidative enzymes (reviewed in Rajkumar et al., 2012), our data support that bacteria may contribute to the amelioration of abiotic stress not by modulating enzymatic activity, but reducing heavy metal toxicity. For example, siderophores released by the rhizobacterial consortium used (Mesa et al., 2015a) are chelators that may bind metals alleviating their toxicity (Dimkpa et al., 2009a,b). In the same way, selected rhizobacteria produce indoleacetic acid (IAA) (Mesa et al., 2015a), which has a bioprotective effect. Besides, respiration via AOX is thought to play an important role facing the stress induced by metal toxicity and prevents the generation of ROS (Lösch, 2004; Keunen et al., 2011), but our results showed that this pathway was greatly reduced in inoculated *S. maritima*. Collectively, these findings suggest that rhizobacterial inoculation induced a decrease in the formation of cell damaging free radicals, thus reducing the need of plant enzymatic defenses (Dimkpa et al., 2009a,b) and may also explain the lessened root activity of AOX in inoculated roots. In non-inoculated plants, greater activities of antioxidant enzymes and AOX may indicate a higher *S. maritima* sensitivity to heavy metal stress, and such phenomena could contribute to maintain ROS homeostasis.

After PGPR inoculation, the physiological adjustments mentioned above were accompanied by an increase in plant soluble protein content, which generally reflects a good plant physiological status (Gepstein, 1988). In shoots, this fact could be related with the amelioration of photosynthetic parameters observed in our previous research (Mesa et al., 2015b), given the likely importance of some proteins quantity, such as Rubisco, in determining plant photosynthetic capacity (Sybesma, 1983; Gepstein, 1988). Although photosynthesis and respiration are rarely studied together, it is advisable because their pathways are intertwined to constitute the entire bioenergetic plant machinery (Lambers and Ribas-Carbó, 2005). In our previous work, inoculation with the rhizobacterial consortium under the same experimental conditions had a beneficial effect on the photosynthetic apparatus of *S. maritima*, reflected in terms of functionality of PSII, values of F_v/F_m and Φ_{PSII} or chlorophyll pigments (Mesa et al., 2015b). Altogether, increased ATP and carbon availability may permit enhanced biomass formation in inoculated *S. maritima*, which would explain the increment in root growth in both this study and our previous one (Mesa et al., 2015b).

Results presented here are first findings at the organism level that open interesting hypotheses in plant biology. In summary, we suggest that inoculation of *S. maritima* plants with the native PGPR consortium decreased the activity of antioxidant enzymes and plant respiration, notably falling AOX pathway. Together with ameliorated photosynthesis results obtained in a previous work under the same experimental conditions, it may be elucidated that such processes allow *S. maritima* to accumulate more carbon for root biomass formation and increase their heavy metal rhizoaccumulation capacity in polluted soils. More experiments are needed with a view to more specific mechanistic approaches, as well as it would be very interesting to know to what extent the structure of the soil microbiota differs between the inoculated versus non-inoculated treatments. Their elucidation may be highly relevant in heavy metal hyperaccumulator plants like *S. maritima*, given their biotechnological potential in environmental decontamination.

AUTHOR CONTRIBUTIONS

EM-N, JM-M, and ND-S designed the research. JM-M and ND-S performed the experiments and interpreted the data. JM-M and ND-S wrote the manuscript. EM-N, MR-C, SR-G, EP, and IR-L revised the manuscript.

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Insight Into the Variation of Bacterial Structure in Atrazine-Contaminated Soil Regulating by Potential Phytoremediator: *Pennisetum americanum* (L.) K. Schum

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Although plants of the genus *Pennisetum* can accelerate the removal of atrazine from its rhizosphere, the roles played by this plant in adjusting the soil environment and soil microorganism properties that might contribute to pollutant removal are incompletely understood. We selected *Pennisetum americanum* (L.) K. Schum (*P. americanum*) as the test plant and investigated the interaction between *P. americanum* and atrazine-contaminated soil, focusing on the adjustment of the soil biochemical properties as well as bacterial functional and community diversity in the rhizosphere using Biolog EcoPlates and high-throughput sequencing of the 16S rRNA gene. The results demonstrate that the rhizosphere soil of *P. americanum* exhibited higher catalase activity, urease activity and water soluble organic carbon (WSOC) content, as well as a suitable pH for microorganisms after a 28-day incubation. The bacterial functional diversity indices (Shannon and McIntosh) for rhizosphere soil were 3.17 ± 0.04 and 6.43 ± 0.86 respectively, while these indices for non-rhizosphere soil were 2.95 ± 0.06 and 3.98 ± 0.27. Thus, bacteria in the *P. americanum* rhizosphere exhibited better carbon substrate utilization than non-rhizosphere bacteria. Though atrazine decreased the richness of the soil bacterial community, rhizosphere soil had higher bacterial community traits. For example, the Shannon diversity indices for rhizosphere and non-rhizosphere soil were 5.821 and 5.670 respectively. Meanwhile, some bacteria, such as those of the genera *Paenibacillus*, *Rhizobium*, *Sphingobium*, and *Mycoplana*, which facilitate soil nutrient cycling or organic pollutants degradation, were only found in rhizosphere soil after a 28-day remediation. Moreover, redundancy analysis suggests that the soil biochemical properties that were adjusted by the test plant exhibited correlations with the bacterial community composition and functional diversity. These results suggest that the soil environment and bacterial properties could be adjusted by *P. americanum* during phytoremediation of atrazine-contaminated soil.

Keywords: rhizosphere, atrazine, Pennisetum, phytoremediation, microbial succession

INTRODUCTION

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is one of the most widely used herbicides in agriculture. It is primarily applied to control broadleaf weeds in the crops such as maize, sorghum and sugar cane. Though atrazine has been proved highly persistent in the environment with the reported half-life ranges between 10 and 5824 days (Salazar-Ledesma et al., 2018), it also could be metabolized in environment according to microbiological degradation and some kinds of physicochemical process (Sun et al., 2010; Roustan et al., 2014). As a result, atrazine and its metabolites are the most commonly detected pesticide contaminants in groundwater and surface water due to their mobility in soil (Yang et al., 2014). In addition, atrazine has been classified as a priority pollutant since many researches proved it could affect the endocrine system of various kinds of organisms (Lasserre et al., 2009). In addition, there were also some other reports reveal that atrazine also could cause obvious toxic affection on the microorganisms in soil (Muñoz-Leoz et al., 2011; Imfeld and Vuilleumier, 2012). As a result, the soil nutrients cycling, as well as soil physical and chemical properties were also affected simultaneously. Consequently, the toxicity of atrazine has raised serious concerns and innovative strategies for remediating atrazine-contaminated soils are critically needed (Lima et al., 2009; Pandey et al., 2009).

In recent years, phytoremediation has aroused increasing concern in the field of contaminated soil remediation (Hamdi et al., 2012; Wang et al., 2012; Albright et al., 2013; Jagtap et al., 2014). Unlike the conventional physical or chemical technologies for soil remediation, which have the disadvantages of high economic costs, formulation of secondary contaminants and damage to soil organisms, phytoremediation has been considered a cost-effective, environmentally friendly strategy to solve soil contamination (Agnello et al., 2016; Gil-Díaz et al., 2016). Several literatures have been reported that phytoremediation can decrease the residual level of organic contaminates in soil by the interaction between plant roots and the specific microorganisms harbored in the rhizosphere (Cai et al., 2010). The plant-stimulated bioremediation of organic pollutants by rhizospheric microorganisms described above is also termed rhizoremediation (Hussain et al., 2018). Rhizoremediation is the major mechanism for phytoremediation of organic-polluted soil because it stimulates the population growth and activity of degrading microorganisms around roots through the rhizosphere effect (Truu et al., 2015). It is well known that the plant roots can create a nutrient-rich microenvironment for pollutants-degrading microbes, as well as that the microorganisms in rhizosphere can enhance plant growth by providing plant nutrients and protection against the stress caused by contaminants (Hussain et al., 2018; Lacalle et al., 2018). Therefore, the rhizosphere has long been considered as the most biologically active microsites in soil and the organic compounds are degraded here by the stimulated microbial biomass and the activity that is part of the rhizosphere effect (Liu et al., 2014).

Root exudates, which consist of low-molecular-weight carbohydrates, amino acids and organic polymers, can be used as an energy and carbon source by soil microbes during their metabolic processes (Martin et al., 2014). Therefore, root exudation is considered as a potential driving force for stimulated rhizoremediation and is the most important factor affecting microbial drift in the rhizosphere (Hussain et al., 2018). The distinct microorganism community shifts in contaminated soils mentioned above suggest the alteration of microbial catabolic activity and selection of specific microbial strains. Therefore, it is widely considered that root exudates play a distinctive role in shaping the rhizosphere microbiome in polluted soil (Thijs et al., 2016).

The selection of a suitable plant species is an indispensable component of phytoremediation success (Lacalle et al., 2018). It is widely supposed that a plant with the potential for organic pollution remediation may selectively enrich the specific pollutant degraders harbored in rhizosphere by releasing a variety of root exudates (Fang et al., 2001). In addition, the different plant select for certain microorganism or sharpen characteristics, and the types of microorganism that thrive on the rhizoplane mainly depend on the plant types (Chen et al., 2016). This is mainly because the constituents or the concentrations of the root exudates might different between the various species of plants (El Amrani et al., 2015). Therefore, further investigation of the distinct microorganism community or activity stimulating traits by the selected plant used for rhizoremediation is essential to illustrate the phytoremediation mechanism of contaminated soil.

The genus *Pennisetum* has been useful for remediation of soils contaminated with atrazine (Singh et al., 2004). However, little information about the interaction between the *Pennisetum* genus plant roots and the soil microorganisms, especially how this plant affects the soil biochemical properties or bacterial functional diversity and community structure, is available. The objectives of this study were (1) to assess the impact of the *Pennisetum americanum* (L.) K. Schum (*P. americanum*) rhizosphere on atrazine-contaminated soil biochemical properties, and (2) to investigate the variations in the composition, diversity, and functions of bacterial community across the *P. americanum* root-associated compartments, and (3) to examine which environmental factors or soil biochemical properties are important in shaping the structure and carbon substrates utilization diversity of soil bacterial community. All the results could help us better understanding the importance of the interaction between *P. americanum* roots and the soil microorganism during atrazine-polluted soil phytoremediation.

MATERIALS AND METHODS

Soil Samples

The soil used in this research was collected from a farmland in Harbin, Heilongjiang Province which is located in the black soil region of northeast China. The sampled soil was air dried, passed through a 2-mm sieve and detected to be no atrazine. Total organic carbon, ammonium nitrogen, rapidly available phosphorus, rapidly available potassium and pH which are the basic properties of the soil were 20.04 g kg^{-1}, 89.33 mg kg^{-1}, 70.15 mg kg^{-1}, 501.00 mg kg^{-1} and 6.28, respectively.

Experimental Design and Compartmented System

To illustrate the regulatory role of the *P. americanum* rhizosphere on bacterial functional diversity and the bacterial community structure of atrazine-contaminated soil, three treatments were set up: (1) soil without any addition of atrazine or *P. americanum*, which served as the control treatment (CK); (2) soil without planting of *P. americanum* but with addition of 20 mg kg^{-1} atrazine, which served as pollution treatment (PT); (3) soil with planting of *P. americanum* and addition of 20 mg kg^{-1} atrazine as phytoremediation treatment (RT). To separate the non-rhizosphere soil (RN) from the rhizosphere soil (R) in the phytoremediation treatment (RT), a rhizobox was used (**Figure 1**). A 23 μm nylon mesh was used to divide the rhizobox (100 mm length × 100 mm width × 85 mm height) into three sections: the rhizosphere zone, which was in the center of the rhizobox (34 mm in width), and the two non-rhizosphere zones, which were located on the left and right sides of the rhizobox (each 33 mm in width). Four hundred twenty grams of air-dried soil was put in the rhizobox. In the phytoremediation treatment, *P. americanum* was only sown in the central zone. In addition, plastic pot of the same size containing identical amounts of soil were used in the CK and PT treatments, but without dividing these boxes into three parts and without sowing the seeds.

To prepare the soil used for the treatments of PT and RT, atrazine was dissolved in acetone, followed by being completely mixed with a small part of the soil, and then the spiked soil was put in the fume hood to make acetone vaporize thoroughly. Finally, the soil was mixed into a large amount of the soil homogeneously. The final atrazine concentration in the soil was 20 mg kg^{-1} (dry weight).

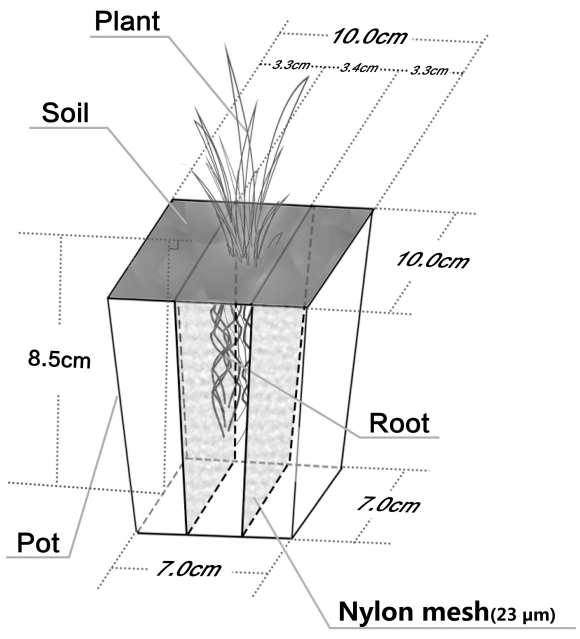

FIGURE 1 | Rhizobox used in phytoremediation treatment (RT). The rhizobox was divided into three parts by a 23 μm nylon mesh. The test plant was only sowed in the middle part and this part represents rhizosphere zone. Additionally, the other two parts in the two side of the rhizobox without sowing test plant were designed as non-rhizosphere.

Seeds of *P. americanum* were soaked in distilled water for 5 h and then were surface-sterilized in 30% H_2O_2 solution for 10 min. Afterward, the sterilized seeds were rinsed several times with deionized water and were placed in a culture dish with moist filter paper for germination overnight at 28°C. The germinated seeds were sown in the rhizosphere zone of the rhizobox mentioned above. Pot experiments were performed in a greenhouse. The temperature of the greenhouse was kept at 27 ± 1°C during the day and 20 ± 1°C during the night. All the samples were watered with distilled water every 2 days to keep the plants at approximately 50% of the water holding capacity. Three replicates were conducted for each treatment mentioned above.

The day of sowing the tested plant seeds described in Section "Experimental Design and Compartmented System" was set as day 0. The soil of CK and PT was sampled on day 0 and the collected samples were named CK0 and PT0, respectively. Additionally, the soil of CK, PT, RN and R were sampled when a 28-day cultural period (as described above) was finished and the collected samples were marked as CK28, PT28, RN28 and R28, respectively. Each sample mentioned above was divided into two sets. One was stored at −20°C until soil microbial community structure and function assessment, and the other was stored at 4°C to measure atrazine concentration and other soil characteristics, such as pH, Eh, catalase activity and urease activity, water-soluble organic carbon and microbial biomass carbon.

Soil Physicochemical Properties and Microbial Biomass Carbon Determination

Some typical physicochemical properties and the microbial biomass carbon of the soil samples mentioned above were detected by the methods described below. (1) Soil pH was determined in water (1:2.5, soil/water) with a pH meter (Rex PHS-3C, China). (2) Soil Eh was measured with an ORP electrode (Rex 501, China). (3) The water- soluble organic carbon (WSOC) was measured using a TOC analyzer (Shimadzu TOC-VCPN, Japan) according to the method reported by Nan et al. (2016). Soil samples (6 g) were shaken with distilled water (ratio of 1:5, w/v) for 1 h at 25°C and 180 r min^{-1} and centrifuged (4500 rpm for 5 min), and the supernatants were filtered through 0.45 μm filter membrane. The extract was analyzed. (4) Soil catalase activity was determined by measuring the hydrogen peroxide (H_2O_2)-catalyzing ability when soil was incubated in H_2O_2 solution. Two grams fresh soil was added to 40 mL hydrogen peroxide solution (0.03%, w/v) and cultured at 37°C, 150 r min^{-1} for 30 min. The enzymatic reaction was stopped by adding 5 mL of 3.0 M H_2SO_4. Then, 25 mL filtrate was titrated by 0.1 M $KMnO_4$ and the soil catalase activity was calculated basing on the change in H_2O_2 concentration as reported by Cao et al. (2015). (5) Soil urease activity was determined by a sodium phenolate and sodium hypochlorite spectrophotometry. Five grams of soil

(wet weight) was placed in tested tube and 1 mL toluene, 10 mL urea (10%, w/v) and 20 mL citrate buffer (pH = 6.7). The mixture was cultured at 37°C for 24 h, then the solution was filtered and measured by spectrophotometer (Shimadzu UV-1800, Japan) at the wavelength of 578 nm (Cao et al., 2015). (6) The soil microbial biomass carbon (MBC) was measured by the chloroform-fumigation-extraction method (Vance et al., 1987). The extracted organic C was determined using the TOC analyze and a K_{EC} of 0.45 was used to convert the difference between the organic C extracted with 0.5 M K_2SO_4 from the chloroform fumigated and unfumigated soil samples.

Soil Microbial Physiological Metabolic Characteristics Analysis

Biolog EcoPlates (MicroPlate., BIOLOG Inc., Hayward, CA, United States) were employed to study the microbial physiological metabolic characteristics. Four grams of soil (wet weight) was added to 36 mL of sterilized 0.85% NaCl/water solution. Tenfold serial dilutions were made and the 10^{-3} dilution was added into the Biolog EcoPlates. Then, the plates were cultured at 25 ± 1°C in the incubator in the dark avoided light for 7 days. Color development in the plates was recorded with an automated microplate reader (Biotek Epoch, United States) every 24 h at 590 nm. Plate readings at 96 h of incubation were used to calculate the average well color development (AWCD), Shannon index (H′), Simpson index(D) and McIntosh index(U), since 96 corresponded to the time of maximal microbial growth that allowed the best resolution among the treatments.

DNA Extraction, PCR and High-Throughput Sequencing

DNA was extracted from the soil samples (0.4 g wet weight) with E.Z.N.A Soil DNA (OMEGA, United States) according to the manufacturer's instructions. The V4 hypervariable region of bacterial 16S rRNA gene fragments were amplified in triplicate from each of the resulting DNA extracts using the primers 515F (5′-GTG CCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The amplification was carried out in 20 μL mixture 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA. The amplification conditions involved an initial denaturing step at 95°C for 2 min followed by 25 cycles (95°C for 30 s, 56°C for 30 s, 72°C for 30 s) and a final extension at 72°C for 5 min.

Amplicons were purified using QIAquick PCR Purification Kit (Qiagen, China) and quantified using QuantiFluor-ST fluorometer (Promega, United States) according to the manufacturer's instructions. Then the qualified libraries mentioned above were sequenced pair-end on the Illumina HiSeq System (Illumina, United States) by the sequencing strategy PE250.

Processing of Sequencing Date

The raw data were quality-filtered using QIIME (version 1.17) with the following criteria: (1) Sequence reads not having an average quality of 20 over a 25 bp sliding window based on the phred algorithm were truncated. Meanwhile, we trimmed and removed the reads with lengths less than 75% of their original length. (2) We removed reads contaminated by adapters (default parameter of 15 base overlapped by reads and adapter, as well as a maximal of 3 bases mismatch allowed). (3) We removed of reads with ambiguous base; (4) removal of low complexity reads that contain more than 10 of the same base consecutively. The filtered paired-end reads were combined to tags based on overlaps by FLASH (v1.2.11). The tags with 97% pairwise identity were binned into operational taxonomic units (OTU) by USEARCH (v7.0.1090). The abundance of each OTU was calculated according to the USEARCH_global method. The most abundant sequence of each OTU was selected as the representative OTU sequence. Taxonomic designation of OTUs was assigned by comparing the representative OTU sequence against the Greengenes database using RDP Classifier (v.2.2). The bacterial community structures diversity of the samples were further analyzed according to the OTU taxonomic richness and number.

Statistical Analysis

The results of the soil typical physicochemical property for each experiment treatment were given as means and standard deviations of three replicates. Statistical significance between treatments was performed using SPSS 19.0 with two-way ANOVA and least significant difference (LSD) at $p < 0.05$.

Principal component analysis (PCA) was performed in Canoco for Windows 4.5 to compare the differences of the microbial physiological metabolic characteristics of the studied treatments based on Biolog EcoPlates data. Redundancy analysis (RDA) was carried out in Canoco for Windows 4.5 to determine which soil environmental variables best explained the changes in the frequency distributions of microbial metabolic functions under various treatments.

The bacterial community structure diversity indices were calculated using Mothur (v1.31.2). Principal coordinate analyses based on pairwise unweighted and weighted UniFrac distances were calculated in the "ade4" package of R software (v3.1.1). The information of common and unique OTUs among various treatments was plotted by "VennDiagram" package of R (v3.1.1). The $\log_{10}$-transformed relative abundance of genus-level OTUs was used to construct a heat map using the "gplots" package for R software (v3.1.1). A hierarchical cluster analysis was performed using BrayeCurtis distances. The relationship between the bacterial community structure and environmental factors was visualized according to redundancy analysis (RDA), which was performed with Canoco for Windows 4.5.

RESULTS

Soil Property and Microbial Biomass Carbon (MBC)

The typical physical and biochemical characteristics, such as pH, Eh, water-soluble organic carbon (WSOC), catalase and

urease activity, and MBC of the soil samples collected during the experimental period are summarized in **Table 1**. CK0 and PT0 only differed in catalase activity (0.56 mg $KMnO_4$ g^{-1} h^{-1} and 0.71 mg $KMnO_4$ g^{-1} h^{-1}, respectively). However, CK, PT, R and RN exhibited various soil physicochemical properties and MBC contents on day 28. Nearly all the indices mentioned above (except Eh and MBC) of R28 were significantly higher than those of CK28 and PT28 ($P < 0.05$). In addition, R28 presented higher pH, catalase activity, urease activity and WSOC than RN28, whereas Eh and MBC were significantly lower in R28 than RN28. Furthermore, CK28 presented higher catalase activity and lower Eh than CK0. On the other hand, PT28 and PT0 only differed in Eh.

Soil Microbial Function Assessment

The results of sampled soil microbial function assessment are shown in **Table 2**. The soil microbial functional diversity indices, such as AWCD, H′, D and U, were not significantly different between CK0 and PT0. In contrast, the soil on day 28 in the four treatment groups exhibited various microbial function diversities. Nearly all the indices mentioned above (except D) of R28 were significantly higher than those of CK28 and PT28 ($P < 0.05$). In addition, R28 presented higher AWCD, H′, and U than did RN28, whereas D was not significantly different between R28 and RN28. Furthermore, CK28 presented lower AWCD, H′, D and U than did CK0. On the other hand, PT28 presented lower AWCD and U than PT0 did.

PCA was performed to reduce the dimensionality of the Biolog EcoPlate data set, as well as to compare the differences in the microbial physiological metabolic characteristics of the researched treatments. The substrate utilization patterns of the researched treatments are shown in **Figure 2A**. The PCA of the substrate utilization patterns extracted two principle components, which explained 74.9% of the total variance together. In addition, the first principle component (PC1) exhibited great power of separation, as it explained 56.4% of the total variance. The PC1 axis showed that the carbon substrate utilization pattern of CK28 was significantly different from that of CK0. Similarly, PT28 exhibited a different carbon substrate utilization pattern from that of PT0. Moreover, PT28 and CK28 were located together, and RN28 was separated from PT28, by PC2. In addition, R28 was completely separated from RN28 and PT28.

Figure 2B shows how the soil microbial community functional diversity varied with the potential explanatory variables. Four RDA axes were extracted, and the eigenvalues for these axes were 0.465, 0.163, 0.054, and 0.022, respectively. In addition, the variance in soil microbial functional data could be better explained by first RDA axis, while the soil microbial functional data exhibited a positive correlation with environmental data, with the correlation coefficient of 0.909. The results of the RDA also suggest that functional microbial groups among various treatments were significantly affected by the studied environmental variables, such as Eh, MBC, catalase activity and urease activity. These environmental variables, respectively, explained 12.3, 8, 5.9, and 4.5% of the total variance in the soil

TABLE 1 | Soil physicochemical properties and microbial biomass carbon.

	Day 0		Day 28			
	CK0	PT0	CK28	PT28	RN28	R28
pH	6.28 ± 0.06a	6.35 ± 0.10a	6.47 ± 0.01C	6.72 ± 0.03B	6.82 ± 0.03B	7.59 ± 0.11A
Eh(mV)	407 ± 12a	402 ± 7a	371 ± 2B	379 ± 7B	400 ± 2A	373 ± 4B
CAT(mg $KMnO_4$ g^{-1} h^{-1})	0.56 ± 0.01b	0.74 ± 0.03a	0.76 ± 0.07C	0.80 ± 0.09BC	0.89 ± 0.03B	1.16 ± 0.02A
URE(mg NH_4-N g^{-1})	24.06 ± 1.43a	24.73 ± 1.88a	23.63 ± 0.42C	24.31 ± 1.51C	30.29 ± 0.67B	38.81 ± 1.09A
WSOC(mg kg^{-1})	357.75 ± 48.75a	364.17 ± 78.46a	408.69 ± 27.35B	344.65 ± 30.56C	451.06 ± 18.83B	523.60 ± 24.16A
MBC(mg kg^{-1})	269.21 ± 68.87a	232.69 ± 83.06a	254.14 ± 1.94C	331.60 ± 81.37BC	452.06 ± 37.19A	364.31 ± 40.56AB

CAT, catalase; URE, urease; WSOC, water-soluble organic carbon; MBC, microbial biomass carbon; CK, control treatment; PT, pollution treatment (initial atrazine concentration was 20 mg kg^{-1}); RN, non-rhizosphere zone of the phytoremediation treatment; R, rhizosphere zone of phytoremediation treatment. The sampling points were day 0 and day 28, and the day of sowing the plant seeds was set as day 0. The results are given as the means ± SD (n = 3). Different lower-case letters indicate significantly different values between samples of CK and PT at day 0 ($p < 0.05$). Different capital letters indicate significantly different values between samples of CK, PT, RN, and R at day 28 ($p < 0.05$).

TABLE 2 | Soil bacterial community functional diversity indices of all samples.

Treatments		AWCD	H′	D	U
0 day	CK0	0.94 ± 0.06a	3.02 ± 0.08a	0.94 ± 0.00a	6.91 ± 0.39a
	PT0	0.74 ± 0.16a	2.98 ± 0.05a	0.94 ± 0.00a	5.51 ± 0.95a
28 days	CK28	0.13 ± 0.03C	1.83 ± 0.22C	0.74 ± 0.06B	2.08 ± 0.37C
	PT28	0.14 ± 0.06C	2.59 ± 0.42B	0.88 ± 0.07A	1.40 ± 0.35C
	R28	0.96 ± 0.15A	3.17 ± 0.04A	0.95 ± 0.00A	6.43 ± 0.86A
	RN28	0.51 ± 0.03B	2.95 ± 0.06AB	0.94 ± 0.00A	3.98 ± 0.27B

AWCD, average well color development; H′, Shannon index; D, Simpson index; U, McIntosh index; CK, control treatment; PT, pollution treatment (initial atrazine concentration was 20 mg kg^{-1}); RN, non-rhizosphere zone of the phytoremediation treatment; R, rhizosphere zone of phytoremediation treatment. The sampling points were day 0 and day 28, and the day of sowing the plant seeds was set as day 0. The results are given as the means ± SD (n = 3). Different lower-case letters indicate significantly different values between samples of CK and PT at day 0 ($p < 0.05$). Different capital letters indicate significantly different values between samples of CK, PT, RN, and R at day 28 ($p < 0.05$).

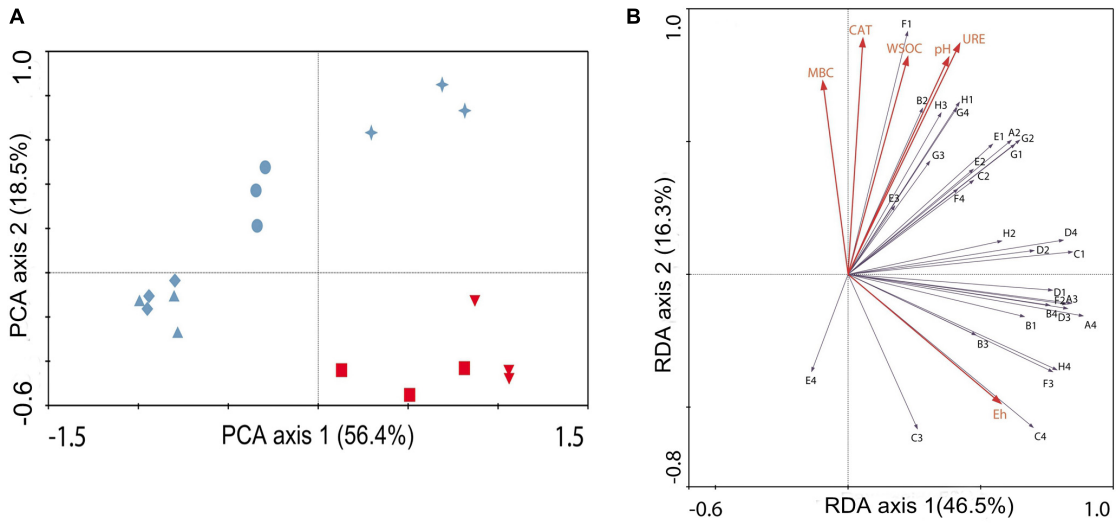

FIGURE 2 | Analysis of functional diversity of bacterial communities in each researched treatment. **(A)** principle component analysis performed with the absorbance values of 31 carbon sources in Biolog ECO plates, measured at 96 h of incubation after adding serial dilutions of soil collected from the treatments. Red downturned triangle (▼) represents CK0, red square (■) represents PT0, blue upturned triangle (▲) represents CK28, blue diamond (♦) represents PT28, blue circle (●) represents RN28 and blue star (✦) represents R28. **(B)** RDA was performed with absorbance values mentioned above and the significant environmental parameters (red arrows, explanatory variables), such as pH, catalase activity (CAT), urease activity (URE), Eh, water soluble organic carbon (WSOC) and microbial biomass carbon (MBC). The arrows show the positions of functional microbial groups (metabolizing specific substrates): A2, β-Methyl-D-glucoside; A3, D-Galactonic acid γ-Lactone; A4, L-Arginine; B1, Pyruvic acid methyl ester; B2, D-Xylose; B3, D-Galacturonic acid; B4, L-Asparagine; C1, Tween 40; C2, I-Erythritol; C3, 2-Hydroxy benzoic acid; C4, L-Phenylalanine; D1, Tween 80; D2, D-Mannitol; D3, 4-Hydroxy benzoic acid; D4, L-Serine; E1, α-Cyclodextrin; E2, *N*-Acetyl-D-glucosamine; E3, γ-Hydroxybutyric acid; E4, L-Threonine; F1, Glycogen; F2, D-Glucosaminic acid; F3, Itaconic acid; F4, Glycyl-L-glutamic acid; G1, D-Cellobiose; G2, Glucose-1-phosphate; G3, α-Ketobutyric acid; G4, Phenylethylamine; H1, α-D-Lactose; H2, D,L-α-Glycerol phosphate; H3, D-Malic acid; and H4, Putrescine.

microbial functional data. In addition, the Eh of soil positively correlated with the use of L- phenylalanine (C4). Urease activity was strongly negatively correlated with the use of L-threonine (E4) and strongly positively correlated with the use of D-xylose (B2). Moreover, the use of the carbon substrates, such as D-malic acid (H3), α-D-lactose (H1) and phenylethylamine (G4), exhibited higher responses to urease activity. Furthermore, other carbon substrates, including L-threonine (E4) and 2-hydroxy benzoic acid (C3), exhibited a lower response in the proximity of the MBC and catalase activity.

Soil Bacterial Community Diversity

A total of 167,992 high-quality 16S rRNA gene tags generated from all samples were clustered into 2686 OTUs. The relative abundances of the OTUs mentioned above at the phylum level are illustrated in **Figure 3A**. In total, 33 identified phyla were observed. *Proteobacteria*, *Actinobacteria,* and *Acidobacteria* were the three dominant phyla in all soil samples. The relative abundance of *Proteobacteria* phyla was significantly higher in PT0 (42.50%) than CK0 (37.70%). Furthermore, the relative abundances of *Actinobacteria* (19.90%), *Verrucomicrobia* (7.54%), *Bacteroidetes* (6.18%), and *Cyanobacteria* (0.33%) in R28 were significantly higher than those of other treatments. In contrast, the relative abundances of *Proteobacteria* and *Acidobacteria* were lower than other treatments. Moreover, *Fibrobacteres* phylum was only found in R28. In addition, the genera *Kaistobacter*, *Candidatus_Nitrososphaera* and *Arthrobacter* were the dominant genera of the present

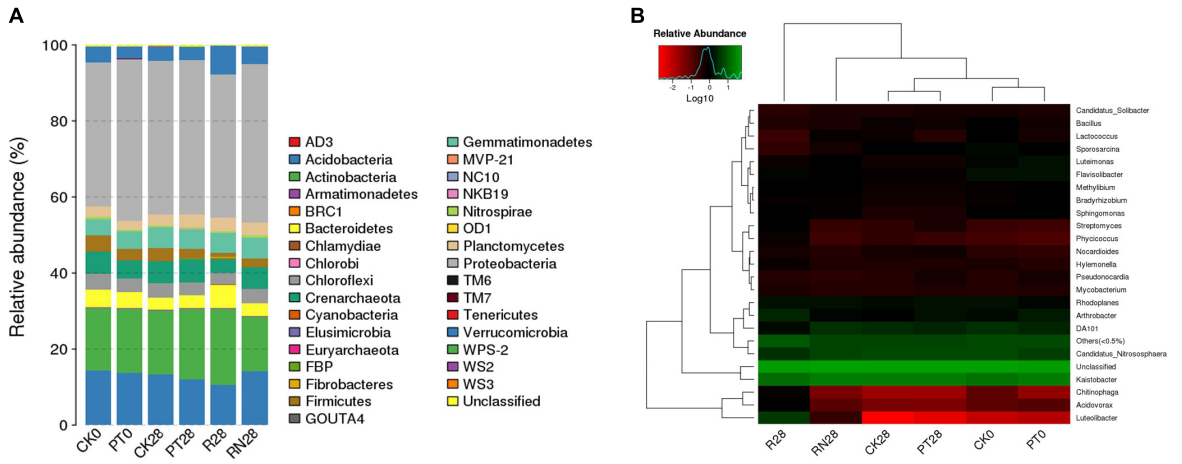

FIGURE 3 | Comparison of the bacterial community structures of the researched treatments at **(A)** phylum level and **(B)** genus level. **(A)** showed the relative abundance of different bacterial phyla by bar plot and **(B)** exhibit the species of clustering heat map based on the relative abundance of different bacterial genus within the researched treatments, including CK, control treatment; PT, pollution treatment; RN, non-rhizosphere zone of the phytoremediation treatment; R, rhizosphere zone of phytoremediation treatment. The sampling point was day 0 (CK0 and PT0) and day 28 (CK28, PT28, R28 and RN28) respectively.

samples. Particularly, the relative abundances of *Luteolibacter*, *Streptomyces Phycicoccus,* and *Chitinophaga* in R28 were significantly higher than those in other samples, while there were lower relative abundances of *Sporosarcina*, *Lactococcus,* and *Kaistobacter* in R28 than other samples (**Figure 3B**).

The alpha bacterial community diversity indices, such as observed species, including Chao, ACE, Shannon-Weaver and Simpson, are shown in **Table 3**. PT0 exhibited lower richness and diversity compared to the CK0 according to the four calculated indices in **Table 3**. Furthermore, significantly greater diversity was observed in R28 compared to CK28, PT28 and RN28 based on their Shannon indices, while the difference in bacterial richness and diversity among CK28, PT28 and RN28 was not obvious. The time period changed the bacterial richness of CK treatments slightly, as the Chao of CK0 and CK28 were 1800.860 and 1790.369, respectively, and the ACE indices were 1860.425 and 1851.134.

PCA was applied to identify the differences in bacterial community structure within all the researched treatments. The two principle components (PC1 and PC2) in **Figure 4A** explained 64.36% of the total variance. In addition, the six treatments of this study clustered into four groups. R28 was widely separated from the other five treatments. However, CK28 and PT28, as well as CK0 and PT0, were grouped together and clustered into two respective groups. RN28 itself was clustered into a new group, but it was located near CK28 and PT28.

RDA analysis was performed to show the effect of main soil physicochemical and biological characteristics on the bacterial communities (phylum level). **Figure 4B** shows that the first two axes explained 86.5% of the total variance, indicating that pH, urease activity, catalase activity, and WSOC were the most influential factors driving the changes in the composition and diversity of the bacterial communities. Specifically, the soil pH was strongly negatively correlated with *Nitrospirae* and *Crenarchaeota*, while it was strongly positively correlated with *Cyanobacteria*, *Fibrobacteres* and *Verrucomicrobia*. Urease activity was strongly negatively correlated with *Acidobacteria*. Catalase activity was strongly negatively correlated with *Chloroflexi* and *Firmicutes* but strongly positively correlated with *Chlorobi* and *Planctomycetes*. *Acidobacteria* and *Firmicutes* exhibited higher responses to WSOC.

DISCUSSION

The plants of the *Pennisetum* genus exhibits tolerance to herbicide atrazine and potential to decrease the atrazine residual level in the rhizosphere (Zhang et al., 2014; Jiang et al., 2016). A higher microbial biomass might be the main reason for the enhanced degradation of atrazine in the rhizosphere (Singh et al., 2004). Therefore, the rhizosphere is widely considered as a hot spot of pollutants rhizoremediation for its higher microbial activity (Velasco et al., 2013). Furthermore, it has been proved that the high biomass and diversity microbita in rhizosphere is mainly due to the interaction of plant and microorganism during the rhizoremediation period (Velasco et al., 2010; Velasco et al., 2013). However, there is little detailed information about the differences in the soil physicochemical properties, microbial metabolize and bacterial community diversitycharacteristics between the rhizosphere and non-rhizosphere soil of *Pennisetum* genus plants in remediation of contaminated soil. This paper is mainly intends to further illustrate the relationships among *P. americanum*, rhizosphere soil physicochemical properties and bacterial community traits during the phytoremediation of atrazine-contaminated soil.

Catalase in soil is responsible for removal of the hydrogen peroxide (H_2O_2) and alleviating the oxidative damage to microorganisms and plants. **Table 1** shows that significantly greater catalase activity was found in PT0 than CK0. This phenomenon might be mainly because of the oxidative stress

TABLE 3 | Bacterial community richness and diversity indices.

Treatments		Chao	ACE	Shannon	Simpson
0 day	CK0	1800.860	1860.425	5.804	0.015
	PT0	1666.950	1722.172	5.601	0.020
28 days	CK28	1790.369	1851.134	5.685	0.018
	PT28	1749.336	1807.963	5.732	0.018
	R28	1736.781	1796.465	5.821	0.013
	RN28	1754.709	1818.818	5.670	0.019

CK, control treatment; PT, pollution treatment (initial atrazine concentration was 20 mg kg^{-1}); RN, non-rhizosphere zone of the phytoremediation treatment; R, rhizosphere zone of phytoremediation treatment. The sampling points were day 0 and day 28, and the day of sowing the plant seeds was set as day 0.

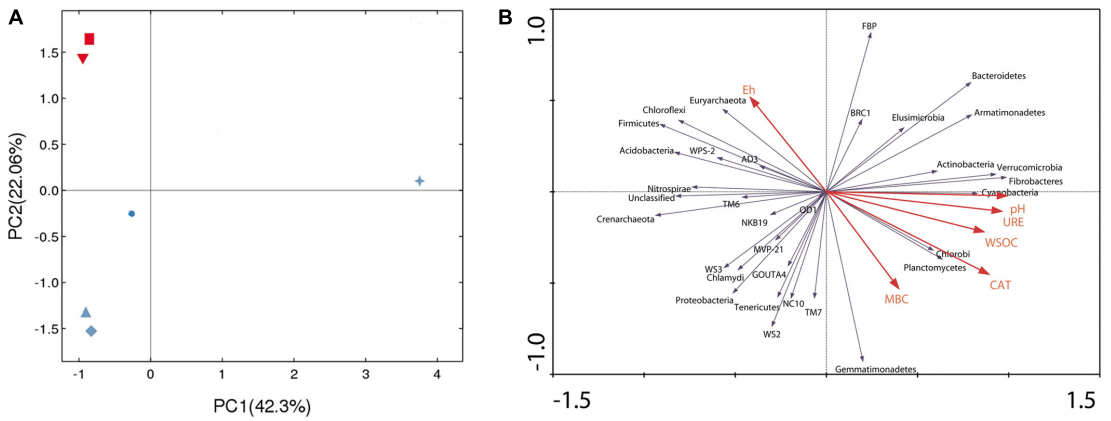

FIGURE 4 | (A) Principle component analysis of soil bacterial community composition based on operational taxonomic units abundance of the different treatments, CK, control treatment; PT, pollution treatment; RN, non-rhizosphere zone of the phytoremediation treatment; R, rhizosphere zone of phytoremediation treatment. The sampling point was day 0 and day 28 respectively. Red downturned triangle (▼) represents CK0, red square (■) represents PT0, blue upturned triangle (▲) represents CK28, blue diamond (♦) represents PT28, blue circle (●) represents RN28 and blue star (✦) represents R28. **(B)** Ordination plots of the results from the redundancy analysis to identify the relationships among the bacterial populations (blue arrows), soil physico-chemical characteristics and microbial biomass carbon (red arrows). CAT, catalase activity; URE, urease activity; WSOC, water soluble organic carbon; MBC, microbial biomass carbon (MBC).

response of soil microorganisms to the addition of atrazine. This inference could be further supported by the well-known viewpoint that atrazine causes oxidative stress on various types of organisms (Zhang et al., 2012; Jiang et al., 2016). Catalase activity could also be used to evaluate the metabolic activity of soil microbial communities (Samuel et al., 2011), and urease activity exhibits a strong correlation with the organic nitrogen transfer ability by microbes. Therefore, the higher catalase and urease activities in R28 suggest that the *P. americanum* rhizosphere could accelerate the metabolic activity of soil microorganisms by releasing various types of nutrient substances (root exudates) to boost the functional microbial survival, or by changing the soil micro-environment to make it favorable to the microorganisms mentioned above. These possibilities are in line with the result that the concentration of water-soluble organic carbon (WSOC), which might be released by the roots of *P. americanum*, in R28 was higher than those of other treatments. They also could be supported by the result that the pH in the rhizosphere of *P. americanum* was much closer to the suitable pH range (6–8) for microorganisms.

The effect of the *P. americanum* rhizosphere on the bacterial catabolic ability was assessed by determining the community-level physiological profiles (CLPP) of soil bacteria using Biolog EcoPlates. Meanwhile, redundancy analysis (RDA) was employed to further investigate the interaction of environmental factors and the carbon-containing substrate utilization characteristics of the soil bacteria (**Figure 3B**). We found that the studied soil physical, chemical and biological properties, such as Eh, MBC, catalase activity and urease activity, exhibited strong positive or negative correlations with the utilization of some types of substrates in the Biolog EcoPlates. Combined with the results described above that the soil physical, chemical and biological properties were affected by the *P. americanum* rhizosphere, it is reasonable to infer that the bacterial catabolic ability of the *P. americanum* rhizosphere might be different from those of other treatments. CLPP-based principal component analysis (PCA) suggested that the substrate utilization pattern of R28 was completely different from other treatments, based on the two principal components in **Figure 3A**. Moreover, the higher bacterial functional diversity indices (AWCD, H′, and U) in R28 revealed that the soil in *P. americanum* rhizosphere exhibited much greater carbon substrate utilization ability, since the indices mentioned above are commonly proposed to measure the functional diversity or catabolic ability of bacterial community (Villeger et al., 2008). These results might partly illustrate why higher organic pollutants removal efficiency could

be found in rhizosphere soil (Li et al., 2011; Blaud et al., 2015).

It has been thought that the soil microbial activity especially the carbon utilization ability could be affected by the species-specific root exudates released from various types of plant species, since the root exudates are the most important sources of readily available carbon for rhizosphere microorganisms (Martineau et al., 2014; Yuan et al., 2016). Because the microorganisms differ in their ability to metabolize and compete for different carbon sources, it is reasonable to consider that the structure of microorganism communities might change during the variation of microorganism functional diversity, as well as the soil physical and chemical properties (Berg and Smalla, 2009). In this paper, high throughput sequencing technology based on the Illumina HiSeq platform was selected to access the bacterial community information of the researched treatments. The redundancy analysis (RDA) based on the bacterial community information and the soil physical and chemical properties further showed that the detected soil properties, such as pH, urease activity, catalase activity, and WSOC, affected the bacterial composition of soil samples collected from various treatments. Since the typical soil physical and chemical properties of the *P. americanum* rhizosphere were different from that of non-rhizosphere treatments (**Table 1**), it also can be concluded that the test plant *P. americanum* could shape the rhizosphere environment, as well as the bacterial community. Additionally, the pincipal component analysis (PCA) of the bacterial community information extracted from the treatments in **Figure 4A** further show that the *P. americanum* rhizosphere exhibited a different bacterial community characteristics. Therefore, a strong evolutionary relationship between *P. americanum* and bacteria might exist in the rhizosphere. These observations are in agreement with results by that Lacalle et al. (2018) that *Brassica napus* plants not only increased the activity of microbial communities in contaminated soils, but also its functional diversity by creating suitable conditions for microbial growth in the rhizosphere.

This study also found that though R28 exhibit a higher bacterial diversity (Shannon indice was 5.821), the greatest bacterial species richness (Chao and ACE) was not found in R28 (**Table 3**). This phenomenon might have been due to the succession of the bacterial communities in the *P. americanum* rhizosphere which triggers an environmental filtering shift of bacteria community composition (Zappelini et al., 2015; Chen et al., 2016). This inference is in line with the data presented in this study that some genera of bacteria, such as such as *Arthrobacter*, *Chitinophaga*, *Streptomyces*, *Sporosarcina* and *Phycicoccus*, were very sensitive to the *P. americanum* rhizosphere environment, as the relative abundances of these genera in R28 were significantly different from those of other treatments. In addition, we found that there were 178 unique OTUs in R28 by comparing the bacterial community composition with those of CK28, PT28 and RN28 (**Figure 5**). Taxonomic analysis indicated that some of the unique OTUs mentioned above belonged to the phyla *Chloroflexi* and *Cyanobacteria*, the order *Acidimicrobiales*, as well as the genera *Paenibacillus* and *Rhizobium*, which can facilitate soil nutrient cycling (Bustamante et al., 2006; Rodrigues et al., 2015; Zimmermann et al., 2015; Redding et al., 2016). In addition, some other OTUs which represent the bacteria with the potential to degrade organic pollutants were found in the *P. americanum* rhizosphere. These OTUs consisted of the genus *Sphingobium* which could participate in the degradation of herbicide (Sun et al., 2008), the genus *Mycoplana* which can decompose 2,4-dichlorophenol (Manikandan et al., 2008), and the family *Sphingomonadaceae*, which degrades aromatic compounds (Lafortune et al., 2009). It is worth to note that the class *Fibrobacteria* and the order *Rhizobiales*, two types of bacteria frequently appearing around the rhizosphere that can decompose fiber and fix nitrogen, respectively, only were detected in R28. These results further suggest that the *P. americanum* also could effectively boost the potential of nutrient metabolism and pollutants degradation in rhizosphere by enhancing the kind or abundance of the bacteria with the corresponding ecology function. It could be greatly supported by the results described above that the abundance of the *Arthrobacter* genus bacteria was obviously enhanced in rhizosphere of *P. americanum* (**Figure 2B**), since much more atrazine-degrading strains has been identified as *Arthrobacter* genus (Zhang et al., 2011). This inference could be further supported by our previous published results that *P. americanum* could obviously accelerate the atrazine removal in soil than that of the treatment without any plant (Zhang et al., 2014). Indeed, further research might continually focus on the variety of microbial functional genes, which responsible for atrazine degradation and soil nutrient cycling, to further illustrate the phytoremediation mechanism of atrazine-contaminated soil by *P. americanum*.

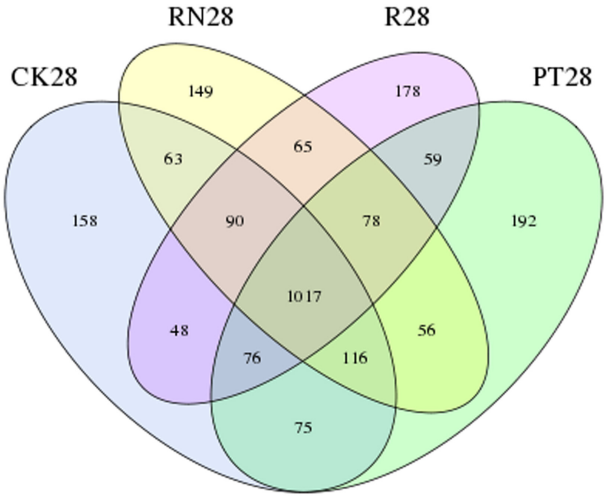

FIGURE 5 | Venn diagram of operational taxonomic units (OTUs) distribution among the samples collected at day 28 of the researched treatments, including CK, control treatment; PT, pollution treatment; RN, non-rhizosphere zone of the phytoremediation treatment; R, rhizosphere zone of phytoremediation treatment. The number in the Venn diagram depicting the shared and unique OTUs present in the four treatments mentioned above.

CONCLUSION

Pennisetum americanum planted in atrazine-contaminated soil shaped the bacterial communities and enhanced the bacterial functional diversity of the rhizosphere by re-shaping the soil physicochemical properties, such as catalase activity, urease activity, WSOC and pH, to be more suitable to soil microorganisms. Additionally, some unique types of bacteria that could facilitate soil nutrient cycling or organic pollutant degradation were only found in the rhizosphere of *P. americanum*. This study provides insight into how that the interaction between the *P. americanum*, soil physicochemical environment as well as the soil bacterial properties (community and functional diversity) plays an important role during the phytoremediation process of atrazine-contaminated soil.

AUTHOR CONTRIBUTIONS

BC, as the first author of this manuscript, was mainly responsible for writing the whole manuscript and analyzing the results about the microorganism community diversity. YZ and ZJ designed the whole experiment together and calculated the data. ML mainly prepared the rhizobox that was used in this research, as well as did much work for soil microbial physiological metabolic characteristics analysis using Biology Eco plates. FY worked on the soil sample collection. ZW detected the soil physicochemical properties of the soil samples. DJ was responsible for the extraction of the soil microorganism DNA and the detection of the soil microbial biomass carbon. In addition, ZJ was also responsible for submitting the manuscript to the journal.

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Streptomyces Dominate the Soil Under *Betula* Trees That Have Naturally Colonized a Red Gypsum Landfill

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The successful restoration of well-engineered tailings storage facilities is needed to avoid mine tailings problems. This study characterized the bacterial communities from vegetated and non-vegetated soils from a red gypsum landfill resulting from the industrial extraction of titanium. A set of 275 bacteria was isolated from vegetated soil and non-vegetated soil areas and taxonomically characterized using BOX-PCR. The study also evaluated the ability of a subset of 88 isolated bacteria on their ability to produce plant growth promoting (PGP) traits [indoleacetic acid (IAA) production, phosphate solubilization, and siderophore production] and their tolerance to potentially toxic elements (PTEs). Twenty strains were chosen for further analysis to produce inoculum for birch-challenging experiments. Principal component analysis (PCA) showed that the set of pedological parameters (pH, granulometry, carbon, organic matter, and Mg content) alone explained approximately 40% of the differences between the two soils. The highest density of total culturable bacteria was found in the vegetated soil, and it was much higher than that in the non-vegetated soil. The *Actinobacteria* phyla dominated the culturable soil community (70% in vegetated soil and 95% in non-vegetated soil), while the phyla *Firmicutes* (including the genus *Bacillus*) and *Bacteroides* (including the genera *Pedobacter* and *Olivibacter*) were found only in the vegetated soil fraction. Additional genera (*Rhizobium, Variovorax,* and *Ensifer)* were found solely in the vegetated soil. The vegetated soil bacteria harbored the most beneficial PGP bacteria with 12% of the isolates showing three or more PGP traits. The strains with higher metal tolerances in our study were *Phyllobacterium* sp. WR140 (RO1.15), *Phyllobacterium* sp. WR140 (R01.34), and *Streptomyces* sp. (R04.15), all isolated from the vegetated soil. Among the isolates tested in challenging experiments, *Phyllobacterium* (R01.34) and *Streptomyces* sp. (R05.33) have the greatest potential to act as PGP rhizobacteria and therefore to be used in the biological restoration of tailings dumps.

Keywords: *Actinobacteria*, red gypsum landfill, birch, plant growth promoting traits, biological restoration

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INTRODUCTION

Mining operations produce significant volumes of waste substrates that originated from the physical or chemical treatment of waste rocks from which ores have been extracted (Kumaresan et al., 2017). Such extractive (anthropogenic) activities produce potentially toxic elements (PTEs) that may contaminate the environment and induce human health problems. Artificial substrates generated by these extractive activities have not been subjected to weathering (Cross et al., 2017) and represent substantial volumes of wastes that are vulnerable to water and wind erosion, and thus potentially transported to long distances (Honeker et al., 2017). They are frequently characterized by a poor physico-hydrological structure, resulting in an unstable geochemical nature, and the presence of potentially toxic chemicals (Wang et al., 2017). Factually, management of tailings plans have often been concentrated on their confinement and containment and little attention has been paid to the long-term alteration of the tailings materials, including changes of their biological, chemical or physical, properties (Santini and Banning, 2016). In addition, they contain abundant by-products, which could be potentially used as amendment in land farming, although they obviously encompass substantial abiotic constraints for the survival of plant and microorganisms.

Revegetation is often encouraged on these tailings since it can efficiently control the erosion of tailings particles by wind and water and may advance the landscape of these waste areas (Mendez and Maier, 2008). The successful restoration of tailings storage amenities may be indeed the best technique to limit mine tailings tragedies (Cross et al., 2017). Salisbury et al. (2017) demonstrated the effectiveness of a vegetative cover to retain some PTE in the upper soil horizons for some decades, and thus playing a considerable function in reclaiming contaminated land. Plant may indeed tolerate PTE contamination through various mechanisms (exclusion, hyperaccumulation traits, and microbe functions) that allow growth and reproduction in such severe environments. Nonetheless, the *in situ* remediation of tailings is likely to necessitate the addition of amendments that may accelerate substrate weathering (Santini and Banning, 2016). The addition of a topsoil to the rooting soil area represents an efficient method to alleviate the abiotic constraints existing in original tailings and is likely to hasten the reappearance of microbial functions (Huang et al., 2012). Choosing plant species that are endemic to the tailings areas is recognized as a suitable choice for a successful revegetation (Wang et al., 2008; Jana et al., 2012; Wanat et al., 2014). Among plant species that readily colonize tailings, *Betula* species have a recognized ability to quickly colonize bare areas and are characterized by their poor affinity for any specific soil category and their capacity to grow in nutrient poor substrates (Atkinson, 1992; Jana et al., 2012).

There is abundant literature on the characterization of microbial communities from forest or agricultural soils contaminated by PTE or polycyclic aromatic hydrocarbon (PAH) (Tardy et al., 2015; Yergeau et al., 2015; Foulon et al., 2016a,b). Microorganisms occurring in mine tailings have also drew significant interest in the past decade, especially in acid mine drainage dumps (Méndez-García et al., 2015; Bruneel et al., 2017; Gupta et al., 2017; Mesa et al., 2017). However, in other environments with different soil characteristics (bauxite and red gypsum), there are considerably fewer studies. Microorganisms are both relevant indicators of ecological functions and facilitators of the soil metabolic activities that are required for further aboveground-plant reestablishment. However, the mechanisms involved on how microorganisms facilitate restoration of degraded lands such as post-mining lands remain poorly understood. Wubs et al. (2016) reported that the addition of microbial inoculum could foster ecosystem restoration, while emphasizing that the origin of the inoculum was a major factor to promote the establishment of plant communities. Therefore, efforts to characterize endogenous microbial communities from these soils are urgently needed to achieve optimal plant recovery.

Among soil bacteria, *Actinobacteria* constitute a group of microorganisms found in high amounts in soils and play key roles in the recycling of natural compounds or xenobiotic such as pesticides and PTE, due to their metabolic capacity (Kieser et al., 2000). Currently, *Actinobacteria* are considered among the most prosperous colonizers in most extreme environments, in contrast to being conventionally considered as endogenous soil and freshwater microorganisms (Álvarez et al., 2017). *Actinobacteria* can directly promote plant growth by supplying the plant with bacterial-synthesized compounds or by facilitating soil nutrient uptake by the plant (Barka et al., 2016). Actinobacteria may also prevent infection by deleterious microorganisms, which is achieved through biocontrol or antagonism toward soil plant pathogens. Despite these recognized traits, the plant growth promoting (PGP) rhizospheric potential of *Streptomyces* has been poorly studied, although there are widely recognized as efficient root colonizers and able to cope with unfavorable growth conditions by forming spores. *Actinomycetes* strains were isolated from birch rhizospheric soils, birch being one of the few native tree able to succeed on a coal mine dump (Ostash et al., 2013).

The present work focused on plant root microbe interactions occurring in a titanium tailings dump that has been naturally recolonized by birch trees, to increase our understanding on how these interactions may be favorable to plant redeployment on such stressful environment. The primary objectives of the present study were: (i) to isolate indigenous bacteria from birch (*Betula* spp.) based on physiological and morphological traits as well as using 16S rRNA gene sequencing, (ii) to test PGP functional traits from isolated bacteria, such as indoleacetic acid (IAA) production, siderophore production, phosphate solubilization and metal tolerance, and (iii) to study the PGP potential of bacterial isolates under controlled conditions.

MATERIALS AND METHODS

Study Site Location

The study site belongs to an 80 ha titanium industry effluent treatment unit located at Thann in northeastern France in the southern part of the Alsace plain (47°47′47.7″N 7°08′18.5″E). The study was carried out in a tailing dump consisting of an embankment, where byproducts produced during the

neutralization of titanium dioxide extraction effluents have been stored since the 1930s. The dump surface studied here has not been used since the early 2000s, which has allowed natural revegetation with flora that is not very abundant and is distributed in a heterogeneous way. We may thus observe heavily vegetated areas and, in contrast, areas completely bare of vegetation. The flora at this dumpsite is almost exclusively dominated by the woody species *Betula* sp.

Sampling

The samples were collected on October 27, 2015. They consisted of samples from two areas, a vegetated and a non-vegetated areas (**Figure 1**). Five birches distributed over the vegetated area were harvested, and the soil fraction adhering to the root system was collected (vegetated soil or VS). For the non-vegetated area, five samples (non-vegetated soil or NVS) were also sampled using an auger at a depth approximately close to that of the root system for the vegetated area. The whole samples were packed on site in plastic bags and transported to the laboratory at a temperature approaching 4°C.

Pedological Characterization

The soils were dried at 40°C and then ground by hand at 2 mm. The soil analyses were carried out by a service provider in accordance with the following French standards for grinding (NF ISO 11464), residual humidity (NF ISO 11465), granulometry (5 fractions – NFX 31-107), pH water + KCl (NF ISO 10390), total organic carbon and organic matter (NF ISO 14235), total nitrogen (NF ISO 13878), CEC Metson (NFX 31-130), bore soluble boiling water (NFX 31-122), oligoelements, K_2O, MgO, CaO, Na_2O (French Norm X 31-108), and total available phosphorus (Joret Hebert method French Norm X 31-161).

In addition, pseudo-total concentrations in the soils were measured using inductively coupled plasma atomic emission spectrometry (ICP-AES, Thermo Fischer Scientific, Inc., Pittsburgh, PA, United States) analysis after the acid digestion of 500 mg of a sample in a microwave digestion system (Mars Xpress, CEM, Saclay, France), using a mix of 2 mL of 67% nitric acid, 6 mL of 34% hydrochloric acid, and 2 mL of 48% hydrofluoric acid. To assess the analytical quality, a standard reference material (loamy clay soil) was used. To determine the extractable fractions of PTE, 5 g of 2 mm sieved soil was dried at 60°C for 48 h (or air-dried) and incubated with 50 mL of 10 mM $CaCl_2$ under agitation (40 rpm) for 2 h at room temperature. The mixture was first filtered with ash-free filters, passed hrough a 0.45 μm mesh, and acidified at 2% (v/v) with HNO_3 prior to ICP-AES analysis.

Microbial Characterization

The vegetated and non-vegetated soil fractions were homogenized in 45 ml of 10 mM $MgSO_4$ and stirred at 100 rpm for 15 min at room temperature. One milliliter was used to perform serial dilutions in 10-fold series, and 100 μL was plated onto a 284-agar medium (Becerra-Castro et al., 2011b) in duplicate dilutions and kept for 7 days at 25°C. The 284 medium contains (per liter): 6.06 g Tris–HCl, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g Na_2SO_4, 0.2 g $MgCl_2.6H_2O$, 0.03 g $CaCl_2.2H_2O$, 0.04 g Na2HPO4.2H2O, and 10 mL Fe(III)NH4 citrate solution (containing 48 mg/100 mL) plus micronutrients (1.5 mg $FeSO_4.7H_2O$, 0.3 mg H_3BO_4, 0.19 mg $CoCl_2.H_2O$, 0.1 mg $MnCl_2.4H_2O$, 0.08 mg $ZnSO_4.7H_2O$, 0.02 mg $CuSO_4.5H_2O$, and 0.036 mg $Na_2MoO_4.2H_2O$) adjusted to a pH of 7. The medium was supplemented with a mixture of different carbon sources: lactate (0.7 g/L), glucose (0.5 g/L), gluconate (0.7 g/L), fructose (0.5 g/L), and succinate (0.8 g/L). Culturable bacterial densities were calculated and expressed as CFU per gram dry soil. Single morphotypes were isolated by plating them twice onto 284 medium-agar plates. The isolates were further stored in cryotubes in a brain heart infusion broth (Roth, D) with 15% glycerol glucosate at −80°C.

Genotypic Characterization

DNA Extraction and BOX-PCR

For DNA preparation, the isolates were grown in the 284 liquid medium at 25°C for 7 days at 250 rpm (Gallenkamp Orbital Incubator). After centrifugation, DNA was extracted from the pellets using an EZNA bacterial DNA isolation kit (Omega Bio-Tek, Inc., Norcross, GA, United States) according to the manufacturer's instructions. The BOX-PCR fingerprinting method was used to group genotypic profiles at a similarity level of 90% as previously described (Becerra-Castro et al., 2011a). BOX reactions were performed in a reaction volume of 25 μL containing 12.5 μL of Ready Mix PCR Master Mix (Thermo Fisher Scientific, Carlsbad, CA, United States), 2 μM BOX A1R primer (5′- CTACGGCAAGGCGACGCTGACG-3′, Eurofins Genomics, Paris, France), and 5 μL of bacterial DNA. DNA amplification was carried out in a thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) under the following conditions: 1 cycle of 5 min at 95°C, 40 cycles of 25 s at 95°C, 35 s at 55°C, and 1.05 min at 72°C with an additional 5 min cycle at 72°C. The amplicons obtained were separated by electrophoresis on a 1.8% agarose gel at 45 V for 3 h. The gel images were analyzed with the software Gel.J (Heras et al., 2015) using the Pearson correlation coefficient and a UPGMA clustering algorithm. VS and NVS bacteria were treated separately.

16S Taxonomic Assignment

PCR was performed on one representative of each BOX group using the following conditions: a volume of 50 μL containing 25 μL AccuStartTM II PCR ToughMix® (2×) (Quantas), 5 μM 27f (*Escherichia coli* positions 8–27, 5′-AGAGTTTGAT CCTGGCTCAG-3′) and 1492r (*E. coli* positions 1,492–1,510, 5′-ACGGTTACC TTGTTACGACTT-3′), which were used to amplify nearly full-length 16S rRNA genes (Mark Ibekwe et al., 2007), and 5 μL of cell lysate. The thermocycling conditions were as follows: 1 cycle of 94°C for 3 min, 40 cycles of 25 s at 94°C, 25 s at 49.4°C, and 1.30 min at 72°C. Alignments were performed using the SILVA website[1].

[1]https://www.arb-silva.de/

Functional Traits

Characterization of PGP Traits

Isolates were screened for their ability to solubilize inorganic phosphate and the production of siderophores, organic acids, and IAA. All of these analyses were conducted for one representative of each BOX group. The ability to solubilize inorganic phosphate was assessed in a modified NBRIP agar medium (1.8%) supplied with 5 g/L of hydroxyapatite and incubated at 28°C for 5 days [10.0 g glucose, 5.0 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, and 0.1 g yeast extract in 1 L deionized water adjusted to a pH of 7.0 modified from Nautiyal, 1999]. A clear halo around the bacterial colony indicated the solubilization of mineral phosphate. 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). All glassware used in this assay was previously cleaned with 30% HNO_3 followed by washing in distilled water (Cox, 1994).

The ability to produce organic acids was tested on an agar medium containing 0.002% bromocresol purple (per liter medium): 10.0 g glucose, 1.0 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, and 0.03 g $CaCl_2.2H_2O$. Colonies forming a yellow halo after 1 day of growth at 28°C indicated a pH change in the medium, and they were considered acid producers. IAA production was evaluated in liquid medium [5.0 g glucose, 1.0 g $(NH_4)_2SO_4$, 2.0 g K_2HPO_4, 0.5 g $CaCO_3$, 0.5 g $MgSO_4.7H_2O$, 0.1 g NaCl, and 0.1 g yeast extract adjusted to a pH of 7 modified from Sheng et al. (2008); supplemented with 0.5 mg/mL tryptophan]. After 5 days incubation at 28°C, the cultures were centrifuged, and the supernatant was incubated with the Salkowski reagent for 25 min. The production of IAA was identified by the presence of red coloring, and isolates were considered IAA producers when the concentration of IAA determined was more than 4 mg/L culture.

Metal Tolerance

Metal tolerance was tested for Cr, Mn, and Zn using 284 agar medium (see above) supplemented with increasing concentrations of Cr [0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 mM; added as $Cr(NO_3)_3.9H_2O$], Zn [1.0, 2.5, 5.0, 10.0, and 25.0 mM added as $Zn(SO_4)_2.7H_2O$] and Mn [5.0, 10.0, and 25.0 mM added as $MnSO_4.H_2O$] and incubated at 28°C for 7 days. The maximal tolerable concentration (MTC) of each metal was recorded for one selected isolate of each BOX-group.

Plant Inoculation Experimental Setup and Post-harvest Analysis

Birch seeds were germinated in a commercial potting mixture. Three-month-old birch seedlings were transplanted into pots containing 200 g of soil collected from the study site. After 1 week of plant adaptation, bacterial inoculation was carried out. Fresh cultures of bacterial strains were grown in an 869 liquid medium (Mergeay et al., 1985) for 24 h, harvested by centrifugation (6,000 rpm, 15 min) and re-suspended in 10 mM $MgSO_4$ to a dry mass weight of 0.5 mg/L. Each pot was inoculated with 10 mL of bacterial suspension. The same amount of sterile 10 mM $MgSO_4$ was added to the non-inoculated pots. Six replicates of each plant species were prepared for each inoculation strain. Plants were watered regularly to maintain soil moisture and incubated in a growth chamber in the following climatic conditions: daylight for 16 h (250–300 $\mu mol\ m^{-2}\ s^{-1}$), day temperature of 22°C, night temperature of 18°C, and day and night humidities of 30%.

After a 3 months growth period, the plants were harvested and the shoot and root dry weight (DW) yields were determined. The plant material was washed in deionized water, oven-dried at 45°C, weighed and ground. The oven-dried plant material was digested in a 2:1 HNO_3:HCl mixture, and the concentrations of P, K, Ca, Mg, Fe, Cd, Pb, and Zn were measured by ICP-AES.

Statistical Analyses

All statistical analyses were performed using R software v. 3.0.2 (R Core Team, 2013). Normality was tested with using Shapiro-Wilk (all data sets), and homoscedasticity was tested with Bartlett's (abiotic dataset) and Levene (biomass dataset, PGP, and metal tolerance traits) tests using R. Data that were normally distributed were analyzed using a parametric test (Student's *t*-test) in R. Data that were not normally distributed were analyzed using a non-parametric Mann–Whitney–Wilcoxon (soil data) or a Kruskal–Wallis (inoculation) test using R. The principal component analysis (PCA) was performed using the R ade4 package. Data expressed as % (PGP and metal tolerance traits) were analyzed using a chi-squared test in R.

RESULTS AND DISCUSSION

Pedological Characterization of the Two Areas

The sampling zone where the vegetation was found (*Betula pendula*) is separated by approximately 60 m from the non-vegetated area (**Figure 1A**). Investigations carried out at the physico-chemical level show that the VS fraction differed significantly from the NVS fraction in several pedological parameters (**Table 1**) and elements (**Table 2**). Physico-chemical analysis revealed that the VS contained significantly less silt and more sand (**Table 1**) and was slightly more acidic than the NVS. It also contained more C and OM. Significant differences between the NVS and VS samples were found for the following parameters: Ti (+25.40% in VS), Mn (+60.04% in NVS), K (+27.66% in NVS), Sb (+28.88% in NVS), As (+32.43% in NVS), and B (+33.31% in NVS). In the $CaCl_2$ extractable fraction, only B, Cr, Fe, K, Mg, Mn, Na, P, S, Si, Sr, Ti, and Zn were detected in significant amounts in this fraction (>0.01% from the total) (**Table 2**). However, for Fe, Mn, and Ti, the extractable fraction accounted for less than 0.01%. Conversely, for Cr, K, Mg, Si, S, and Sr, the extractable fraction accounted for approximately 2–10%. Only the total concentrations of As, B, Mg, Sr, Sb, and Ti differed significantly between the VS and NVS samples, while only the $CaCl_2$ extractable fraction of Mg differed between the two soils.

This set of data indicates that the soil of the tailings dumps is not suitable for revegetation due to its low nutrient content,

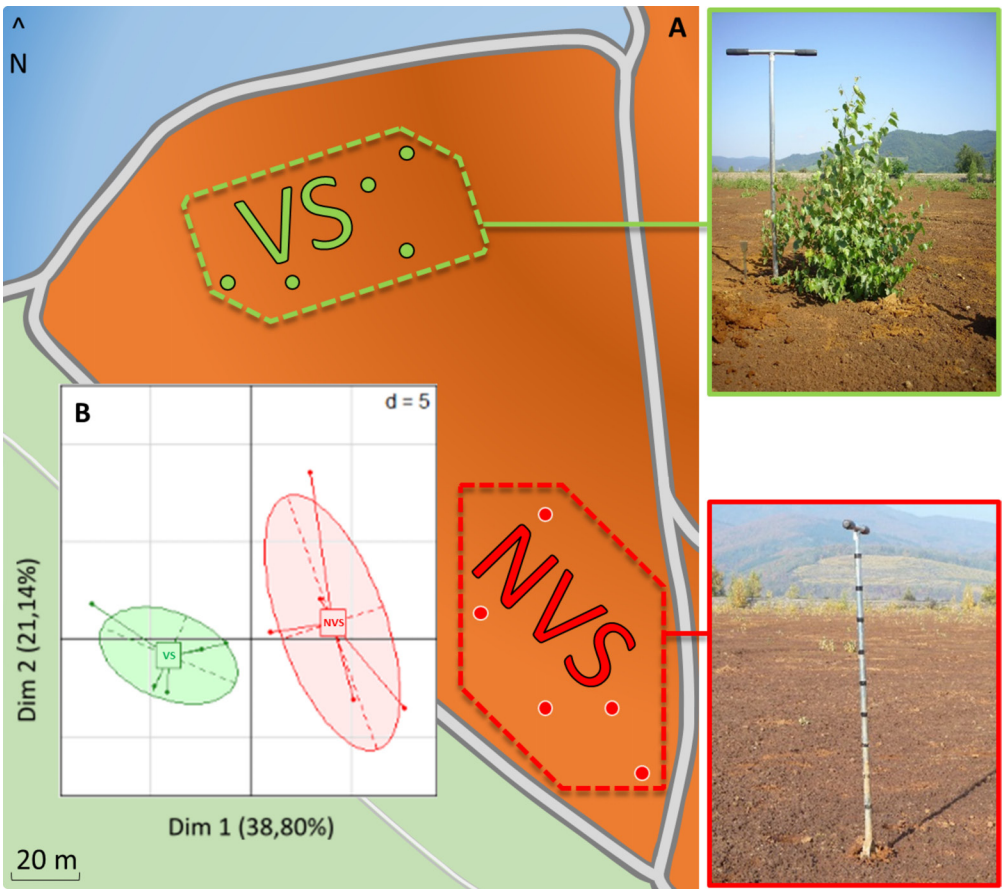

FIGURE 1 | (A) Diagram of the sampling site with soils taken in a vegetated area under birch trees (VS, top-left photo, green dotted line) or soil taken in a non-vegetated area (NVS, bottom-left photo). **(B)** PCA carried out with soil data and soil element concentrations discriminating between the two sampling areas, $n = 5$.

very low N content (below the detection limit) and slightly alkaline pH. These extreme conditions have been shown to suppress tree root growth and to induce leaf chlorosis and decrease biomass production (Wang et al., 2017). The large amount of Fe and Mn, mostly in oxide forms (Carbonell, unpublished data), may also limit the availability of nutrients at this whole area. Previous studies were carried out on the interactions between plant richness and the physicochemical properties of tailings dumps and have identified pH, metal concentration and bioavailability as the major factors that may limit plant establishment on these sites (Santos et al., 2017).

Disparities between the VS and NVS emerged, as illustrated by the PCA (**Figure 1B**). PCA showed that the set of pedological parameters alone explains approximately 40% of the differences between the two soils. The VS also showed a significantly lower pH and higher CEC (**Table 1**). The differences between the VS and NVS could be due to the presence of the *Betula* trees. The lower pH in the vicinity of the birch roots, which could lead to an increase in the CEC in this area, is probably due to the root metabolic activity. Birch trees are known to exude several acids in the millimolar range, especially as monocarboxylic acids (Sandnes et al., 2005). The birch litter may also slightly contribute to the observed enrichment of the vegetated soil in C and OM.

Microbial Characteristics

The VS fraction had the highest density of culturable bacteria density, which was much higher than in the NVS fraction. In the latter samples, the CFUs were five times lower than the CFUs found in the VS (**Figure 2**). A total of 170 (VS) and 105 (NVS) bacteria were isolated in the present study. Based on their BOX-PCR profiles, the isolates were further placed into 53 (VS) and 43 (NVS) distinct groups and were further identified through comparative sequencing of their 16S rDNA. Isolates were recognized to include a total of 16 different bacterial genera, all of which belonging to four bacterial phyla. The Shannon diversity (H′) index was calculated based on the genera, and similar values were found for the VS (H′ = 1.16) and the NVS (H′ = 1.17).

The phyla *Actinobacteria* and *Proteobacteria* were represented in the two soils, while the *Firmicutes* (including the genus *Bacillus*) and *Bacteroides* (including the genera *Pedobacter* and *Olivibacter*) representatives were found only in the VS fraction. The *Actinobacteria* accounted for more than 95%

TABLE 1 | Physico-chemical parameters of vegetated soils (VS) and non-vegetated soils (NVS).

	Non-vegetated soil	Vegetated soil	*P*-value	Test value
Clay (‰)	100.40 ± 6.73	93.60 ± 5.37	0.117	$t = -1.766$
Thin silt (‰)	228.60 ± 38.72	152.20 ± 23.97	0.007**	$t = -3.751$
Large silt (‰)	220.20 ± 17.91	138.80 ± 13.63	5.95E-05***	$t = -8.088$
Thin sand (‰)	169.20 ± 45.65	157.20 ± 29.99	0.638	$t = -0.491$
Coarse sand (‰)	281.80 ± 57.22	458.40 ± 26.90	0.0009***	$t = 6.245$
pH	8.20 ± 0.35	7.84 ± 0.05	0.007**	$W = 25$
pH KCl	8.08 ± 0.36	7.84 ± 0.05	0.009**	$W = 25$
$CaCO_3$ (g/kg)	280.40 ± 115.79	301.60 ± 51.23	0.722	$t = 0.374$
C. org (g/kg)	3.78 ± 0.87	6.88 ± 1.70	0.011*	$t = 3.623$
OM (g/kg)	6.54 ± 1.48	11.90 ± 2.95	0.011*	$t = 3.626$
N tot (g/kg)	<ddl	<ddl		
C/N	NC	NC		
CEC (meq/kg)	26.80 ± 6.06	45.20 ± 11.21	0.017*	$t = 3.228$
K_2Oex (g/kg)	0.00 ± 0.00	0.01 ± 0.01	0.07201	$W = 5$
MgOex (g/kg)	0.76 ± 0.20	0.30 ± 0.16	0.004**	$t = -3.987$
CaOex (g/kg)	76.84 ± 0.44	77.02 ± 0.36	0.500	$t = 0.706$
Na_2Oex (g/kg)	0.02 ± 0.00	0.02 ± 0.00	0.204	$t = -1.407$

*Mean values and standard deviations are provided (n = 5). Normally distributed data were analyzed using the parametric Student's t-test (t-values). Data not normally distributed were analyzed using the non-parametric Mann–Whitney–Wilcoxon (W-values). Significant differences are indicated (*P < 0.05; **P < 0.01; and ***P < 0.001).*

TABLE 2 | Total and $CaCl_2$ extractable element concentrations in vegetated soils (VS) and non-vegetated soils (NVS).

	NVS		VS		Statistics			
	Total (ppm)	$CaCl_2$ extractable (ppm)	Total (ppm)	$CaCl_2$ extractable (ppm)	Total		$CaCl_2$ extractable	
					P-value	Test value	*P*-value	Test value
Al	2, 708 ± 158		3, 010 ± 469		0.309	$W = 7$		
As	6.11 ± 1.24		4.13 ± 0.89		0.022*	$t = -2.90$		
B	5.13 ± 2.68	0.61 ± 0.21	3.42 ± 1.27	0.40 ± 0.12	0.015*	$t = -3.10$	0.101	$t = -1.91$
Ca	194, 267 ± 19462		186, 187 ± 8, 115		0.341	$t = -1.04$		
Cd	0.21 ± 0.21		0.15 ± 0.21		0.824	$W = 14$		
Co	16.24 ± 2.53		21.54 ± 4.33		0.077	$t = 2.11$		
Cr	147.48 ± 43.06	8.26 ± 4.73	186.10 ± 27.67	3.79 ± 0.49	0.156	$t = 1.60$	0.151	$W = 20$
Cu	38.50 ± 2.78		45.78 ± 7.03		0.118	$t = 1.87$		
Fe	41, 559 ± 4, 542	0.99 ± 0.24	52, 198 ± 9, 242	1.13 ± 0.37	0.088	$t = 2.04$	0.526	$t = 0.667$
K	156.23 ± 17.91	6.97 ± 1.53	113.02 ± 31.47	10.39 ± 4.03	0.041*	$t = -2.58$	0.136	$t = 1,774$
Mg	4, 063 ± 1, 336	330.87 ± 67.62	1, 623 ± 372.30	141.35 ± 71.13	0.001**	$t = -4.88$	0.002**	$t = -4.318$
Mn	3, 167 ± 538.06	0.19 ± 0.05	4, 228 ± 1, 023	0.16 ± 0.05	0.112	$t = 1.863$	0.409	$t = -0.872$
Na	76.99 ± 24.88	8.70 ± 1.58	98.54 ± 26.59	7.99 ± 1.75	0.059	$t = 2.397$	0.521	$t = -0.671$
Ni	22.15 ± 16.81		24.61 ± 18.15		0.471	$t = 0.770$		
P	45.57 ± 22.63	0.14 ± 0.01	47.18 ± 24.95	0.17 ± 0.03	0.758	$t = 0.322$	0.104	$t = 1.923$
Pb	19.74 ± 3.06		21.19 ± 2.91		0.841	$W = 11$		
S	94, 917 ± 32445	4, 995 ± 233.56	83, 238 ± 32, 415	4, 808 ± 266.14	0.334	$t = -1.065$	0.272	$t = -1.180$
Sb	4.72 ± 0.62		5.99 ± 0.65		0.016*	$t = 3.157$		
Si	276.27 ± 75.50	9.25 ± 3.07	287.06 ± 84.44	8.14 ± 0.60	0.594	$t = 0.555$	1	$W = 12$
Sr	97.70 ± 11.66	4.57 ± 0.88	107.94 ± 15.36	5.89 ± 0.76	0.151	$W = 5$	0.035*	$t = -2.540$
Ti	5, 234 ± 1, 833	0.25 ± 0.07	6, 564 ± 1, 760	0.27 ± 0.05	0.012*	$t = 3.306$	0.619	$t = 3.306$
Zn	83.70 ± 48.75	0.03 ± 0.02	98.21 ± 52.66	0.02 ± 0.00	0.233	$t = 1.302$	0.786	$W = 9$

*Mean values and standard deviations are provided (n = 5). Normally distributed data were analyzed using the parametric Student's t-test (t-values). Data not normally distributed were analyzed using the non-parametric Mann–Whitney–Wilcoxon (W-values). Significant differences are indicated (*P < 0.05 and **P < 0.01).*

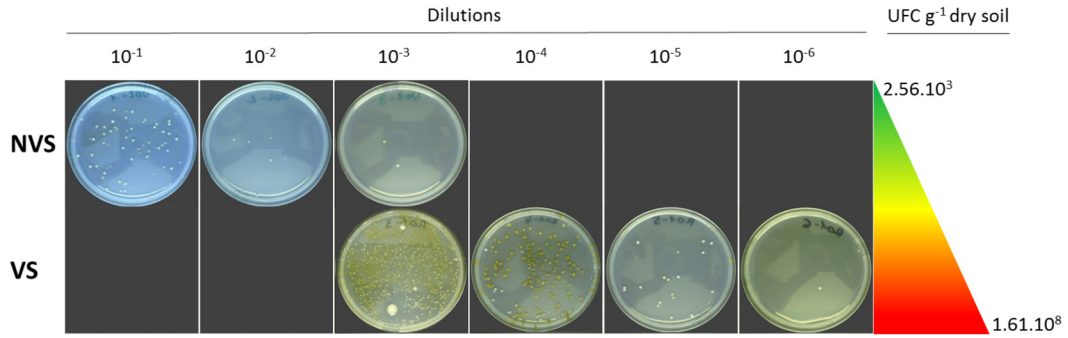

FIGURE 2 | CFU culturable bacterial density expressed as UFC g^{-1} dry soil. The dilution series used to determine the density is represented by the Petri dishes after incubation for the non-vegetated soil (NVS) and the vegetated soil (VS).

in the NVS fraction. Within that phylum, the abundance of each genus differed between the two bacterial populations (**Figure 3**). In both soils, *Streptomyces* dominated and accounted for approximately 70% of the total isolates. Additional *Actinobacteria* were found in the NVS (*Amycolatopsis, Nocardia, Nocardioides,* and *Paenarthrobacter*) but were absent from the VS. The *Rhodococcus* isolates were found only in the VS. Within the *Actinobacteria* phylum, the two soils shared only *Pseudoarthrobacter* and *Arthrobacter* (**Figure 3**). The two soils also shared *Proteobacteria* members, although they were represented to a lower degree in the NVS. However, the two soils shared only *Pseudomonas* and *Phyllobacterium* isolates. *Rhizobium, Variovorax,* and *Ensifer* isolates were detected only in the VS fraction.

The *Actinobacteria* phyla dominated the culturable soil community (70% in the VS and 95% in the NVS). These results agree with other data found in the literature. For example, the rhizospheric soil of PTE-hyperaccumulating plant *Thlaspi caerulescens* hosted bacterial communities that were analyzed and compared with that of contaminated bulk soil (Gremion et al., 2003). The sequences belonging to *Actinobacteria* dominated both the bulk and the rhizosphere soils in that study. Isolates from our soils were restricted to the genera *Streptomyces, Arthrobacter,* and *Rhodococcus*, as previously described (Álvarez-López et al., 2015). *Streptomyces* was also isolated from Mn-contaminated soils (Mo et al., 2017) and from the birch rhizosphere collected on the coal mining dump (Ostash et al., 2013). The rhizosphere from the Ni-hyperaccumulating plant *Alyssum serpyllifolium*

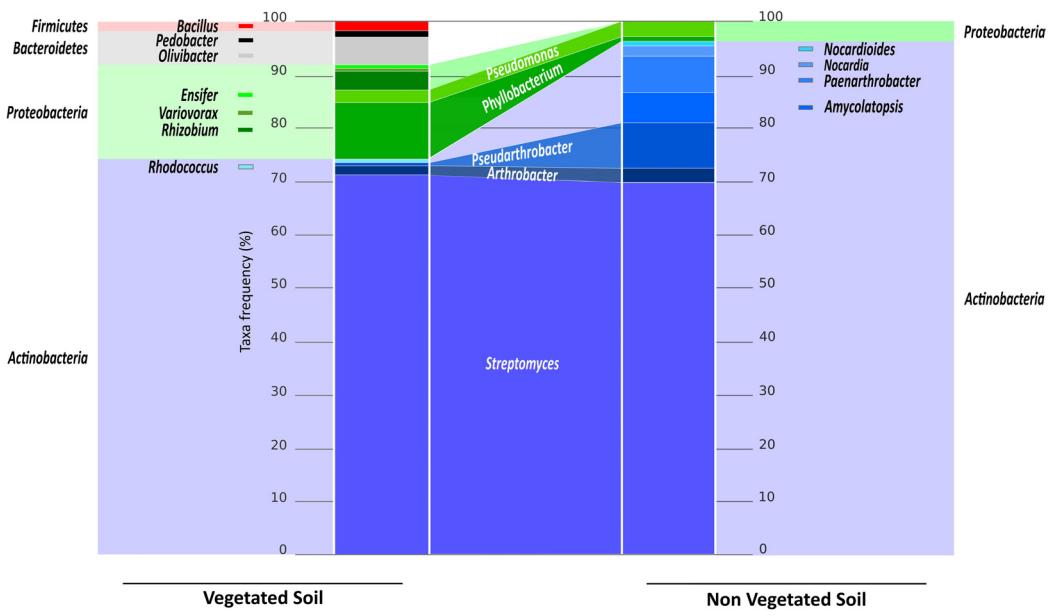

FIGURE 3 | The cultivable bacterial communities from the VS and NVS fractions. The light-colored histograms at the edge represent the abundance of bacterial phyla expressed as a percentage of the total number of bacteria for the VS **(Left)** and the NVS **(Right)**. The dark-colored central histogram represents the abundance of bacterial genera expressed as a percentage of the total number of bacteria for the VS **(Left)** and the NVS **(Right)**. The names in white in the center indicate the shared bacteria between the two habitats.

hosts Ni-resistant bacteria that were predominantly belonging to the *Arthrobacter* and *Streptomyces* genera (Becerra-Castro et al., 2011b). Álvarez et al. (2017) isolated, using culture-dependent methods, and characterized more than 35 *Actinobacteria* genera that were shown to be PTE-tolerant. *Actinobacteria* members tolerant to PTE have been shown to be dominant in PTE-contaminated sites by Margesin et al. (2011), in addition to *Proteobacteria* members, and by Oliveira and Pampulha (2006). Most of these studies agreed on the fact that contaminated soils usually exhibited quantitatively lower culturable bacteria, although *Actinobacteria* were usually less affected by the PTE present in the soil than other culturable heterotrophic bacteria or nitrogen fixers. In addition to *Streptomyces*, *Arthrobacter* was the second most key bacterial genus concerning its PTE-tolerance and thereof it has a real potential for use in bioremediation. Alkaline environments are commonly encountered in soils contaminated with PTE, for instance with Cr. Due to its capacity to tolerate alkaline conditions, Elangovan et al. (2010) suggested the use of both intact *Arthrobacter* cells and cell-free extracts for the bioremediation of alkaline soils contaminated with chromate.

The *Streptomyces* genus, among the order *Actinomycetales* is notably the richest source of natural compounds, including antimetabolites, antibiotics, and antitumor compounds (Bérdy, 2005; Olano et al., 2009; Aigle et al., 2014). For instance, the *Streptomyces* genus produces around 80% of secondary metabolites known to be microbial bioactive compounds (Bérdy, 2005). *Actinobacteria* are able to grow under various life styles such as saprophytes in aquatic environments and soils, or plant commensals such as nitrogen-fixing symbionts. These key features render the *Actinobacteria* well-suited for research-based bioremediation technology.

Functional Traits of the Bacterial Isolates

The PGP properties of the bacterial collection comprising 53 (R) and 43 (S) BOX-PCR groups were tested *in vitro* for PGP traits such as IAA production, nutrient uptake, and the metabolism of bacterial compounds regulating plant growth (**Figure 4**). The bacteria isolated from the vegetated soil harbored the PGP bacteria with the higher beneficial potential, with 12% of the isolates exhibiting three or more PGP traits (**Supplementary Table S1**). The ability to produce IAA was detected in both the isolates from the VS and the NVS fractions, although they were statistically more abundant in the VS. Conversely, the siderophore-producing capacity was higher in the isolates from the NVS. Organic acid-producers and rare phosphate solubilizers were present in both populations and were not significantly different. The production of siderophores by *Streptomyces* isolates has already been shown by Ostash et al. (2013). The genetically and enzymatically based siderophore biosynthesis and transport are well-described in *Streptomyces* (Cruz-Morales et al.,

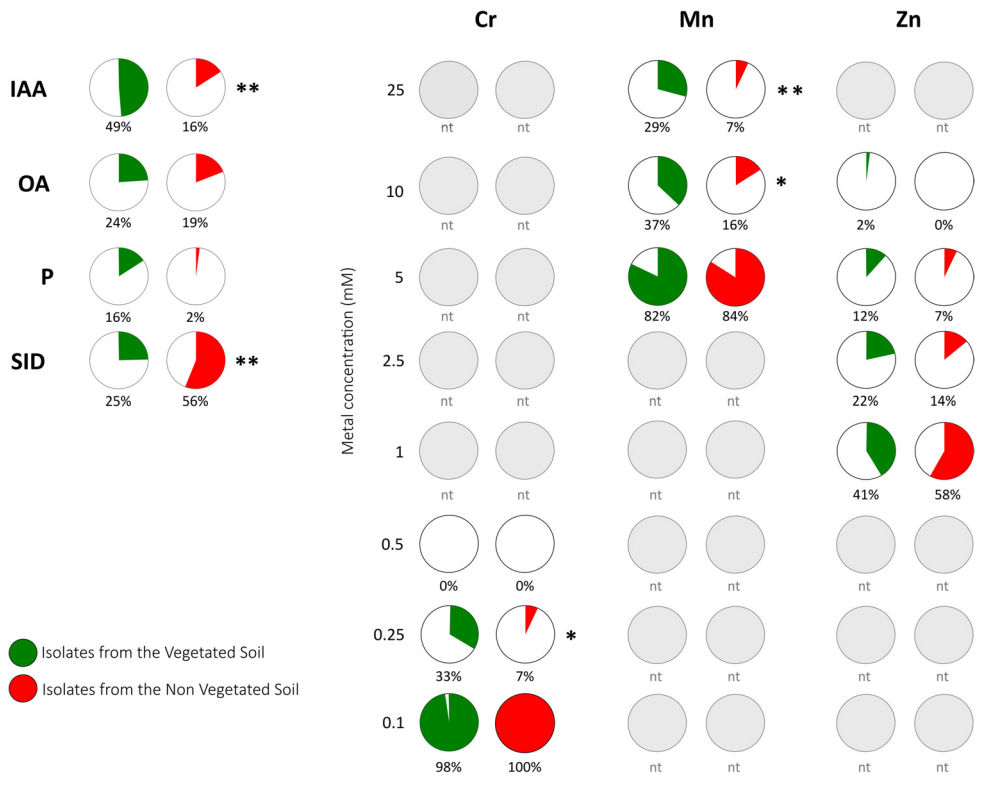

FIGURE 4 | Functional traits of bacterial isolates from the Thann site. Plots representing the percentage of isolates that harbor the positive PGP trait or show metal tolerance. PGP traits were indoleacetic acid (IAA) production, organic acid production (OA), phosphorus solubilization (P), and siderophore production (SID). Metal tolerance was tested for Cr, Mn, and Zn at concentrations ranging from 0.1 to 25 mM. Gray plots indicate non-tested concentrations (nt) for a given metal. Significant differences are indicated (*$P < 0.05$; **$P < 0.01$).

2017). More generally, the *Actinomycetes* group got substantial prominence as PGP microorganism because of its recognized and strong antimicrobial potential and saprophytic behavior dominating numerous soils (Franco-Correa et al., 2010).

In this study, the tolerance to the three metals Zn, Cr, and Mn was investigated (**Table 2**) using the 284 growth medium, and thus our data may only be considered as relative. As indicated in **Figure 4**, the relative order of bacterial toxicity of the three metals was determined as follows: Mn > Zn > Cr. Tolerance to Cr and Zn was considered to be reached at concentrations higher than 0.5 and 1 mM, respectively (Navarro-Noya et al., 2012). In our samples, isolates tolerated Cr concentrations below that threshold that were much lower than that measured for *Cupriavidus metallidurans* (2.5 mM) (Zhao et al., 2012). Conversely, our isolates were more resistant to Zn with an MTC up to 10 mM for some VS isolates. Two percent of our isolates showed an MTC of 10 mM, which is on the same order of magnitude as that determined for the metal-resistant *Cupriavidus metallidurans* (Zhao et al., 2012). Zn-resistant rhizospheric as well as endophytic bacterial isolates of Zn-accumulating *Salix* trees were characterized by Kuffner et al. (2008).

Comparing isolates both from the VS and the NVS samples, the Mn and Cr tolerances were higher for the VS bacteria at the highest concentrations. The strains with the higher metal tolerance in our study were the *Phyllobacterium* sp. WR140 (RO1.15), *Phyllobacterium* sp. WR140 (R01.34) and *Streptomyces* sp. (R04.15), all of which were isolated from the VS fraction. The least tolerant species were isolated from the NVS fraction. The primary tolerant soil bacterium was *Streptomyces flavovirens* (U04.24). To the best of our knowledge, the Mn tolerance in either *Phyllobacterium* or *Streptomyces* has been rarely studied. The *Proteobacteria*, although they were a less abundant species in our isolation experiment, appeared to be the most metal tolerant bacteria (4 over 5 more tolerant). There is abundant literature on Mn oxidation by bacteria (Adams and Ghiorse, 1985; Wang et al., 2009). For instance, the Mn-oxidizing bacterium *Brachybacterium* strain isolated from the deep-sea was able to grow in liquid media supplemented with up to 55 mM MnCl (Wang et al., 2009). Bacterial cells have mechanisms to sense excess metals (Chandrangsu et al., 2017). Generally, the most efficient physiological mechanism that bacteria are exploiting to tolerate excess PTE is efflux. Some of our isolated bacteria (i.e., *Phyllobacterium* sp. WR140) exhibited a Mn MIC higher than that of *Cupriavidus metallidurans* (6 mM) (Zhao et al., 2012). In a previous study, the proportions of metal-tolerant bacterial isolates were primarily represented by Gram-negatives, and the *Proteobacteria* (*Pseudomonas* and *Variovorax* species) dominated (Piotrowska-Seget et al., 2005).

Abbes and Edwerds (1990) evaluated the toxicity of various PTE including Cd, Co, Cu, Cr, Hg, Ni, Zn, and Mn on 34 *Streptomyces* species representative of various taxonomic clusters. Another study described the isolation of several *Streptomyces* strains with resistances to different PTE from contaminated areas, and some exhibited multiple tolerances against different

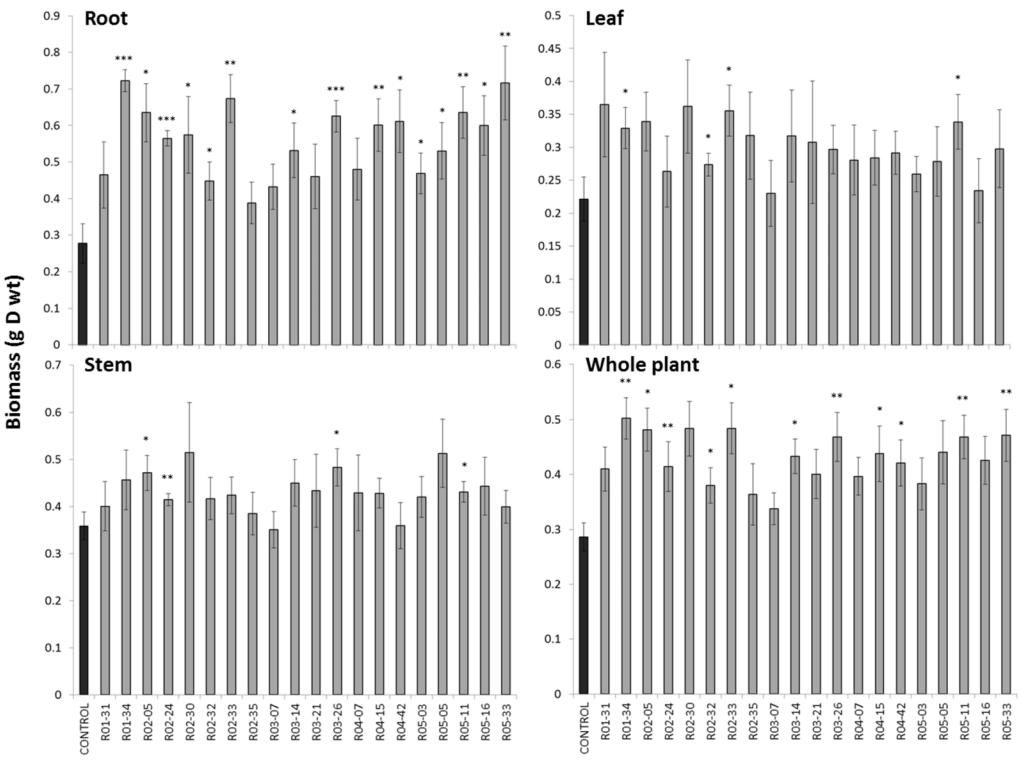

FIGURE 5 | Impact of bacterial inoculation on birch growth. Birch trees were grown for 3 months on the Thann soil inoculated with 20 bacterial isolates collected from the VS fraction. Significant differences are indicated (*$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$).

PTE (Álvarez et al., 2013). Due to the extreme abundance of the genus *Streptomyces* in our study, the further characterization of tolerant *Streptomyces* from this red gypsum dump might lead to a better assessment/new discovery of the physiological mechanisms involved in the metal tolerance in this genus and therefore to ecological applications.

However, we examined the tolerance to the Mn ionic form, while the Mn in the Thann soil was primarily in the form of Mn oxides (Zapata, unpublished results). A recent review also pointed out the use of bacteria in Mn biomining processes, describing distinct, taxonomically distant bacteria that have been described to reduce Mn either by enzymatic or non-enzymatic based mechanisms or Das et al. (2011). Mn is usually reduced to fulfill a nutritional need for soluble forms of Mn and Mn-reducing bacteria belong to either aerobes or facultative anaerobes.

Inoculation Tests

A set of 20 bacterial strains isolated from the VS was chosen to carry out an inoculation pot experiment. The bacterial strains were selected from our previous experiments (section "Functional Traits of the Bacterial Isolates") and those with the best functional traits (PGP traits, siderophore production and metal resistance) were retained, as detailed in **Supplementary Table S1**. The selected bacterial genera were also representative of the most important phyla in our study and, when possibly, previously tested in the literature. After 3 months of growth, the biomass of the birch plants either inoculated or non-inoculated was analyzed (**Figure 5**). Among the 20 isolates, five increased the total biomass production significantly ($P < 0.01$), and an additional set of six isolates increased the total biomass production significantly ($P < 0.05$) compared with uninoculated control. This was primarily due to an increase in the root biomass, while the shoot biomass was only slightly affected (**Figure 5**). We also measured the elements in birch leaves and found no significant effect of the inoculated bacteria (data not shown).

Among the isolates tested, the *Phyllobacterium* sp. isolate (R01.34) that showed the greatest performance on birch also exhibited multiple PGP traits, including the production of IAA and the solubilization of P. PGP rhizobacteria and bacterial endophytes have huge potential to increase the bioremediation of PTE-contaminated sites (Chen et al., 2010; Luo et al., 2011; Xinxian et al., 2011; Burges et al., 2017). Ma et al. (2013) demonstrated the similar performance of *Phyllobacterium myrsinacearum* on *Sedum plumbizincicola* growth, although this strain increased metal transfer to the shoot, which was not observed in our study. However, another *Phyllobacterium* strain examined in our study did not demonstrate any phytoremediation relevant features, which indicates intragenera variability. A *Pseudomonas* isolate also significantly increased birch biomass production. Similarly, Huang et al. (2016) isolated a Cd-resistant *P. aeruginosa* from a Cd-contaminated oil field, and the inoculations of Cd-polluted soil with that strain significantly elevated the shoot and root biomass. The *Variovorax* isolate (R05-11) also exhibited PGP traits in our study. Similarly, the inoculation of hyperaccumulating plants by *Variovorax paradoxus* isolated from plant rhizosphere significantly increased root biomass (Durand et al., 2018). However, growth promotion was not always linked to functional traits in our study. For instance, *Streptomyces* (R05-33) showed significant growth promotion effects without possessing any significant functional traits. In general, we found that *Streptomyces* strains did not show a great potential to promote plant growth. Other potential traits, such as the release of volatile organic compounds (VOCs), which are very well known in the genus *Streptomyces* (Dias et al., 2017), could be responsible for this beneficial effect. The *Olivibacter soli* (R04-07) isolate exhibited all functional traits while having no growth promoting effects. In greenhouse studies (Alekhya and Gopalakrishnan, 2017), *Cicer arietinum* plant growth was increased by *Streptomyces* due to root length and weight promotion, increase production of nodules, increase production of shoot biomass, pods, and pod weight as compared with the non-inoculated control, demonstrating the colonizing capability of bacteria belonging to this genera.

In addition to the *Arthrobacter* and *Rhodococcus* genera, *Streptomyces* bacteria have received considerable consideration for being used in efficient biotechnological method to clean up contaminated ecosystems. In addition to their physiological diversity, *Streptomyces* isolates may be appropriate for being used as soil inoculants since they are able to rapidly grow as mycelium in semi-selective substrates and their capacity to be genetically transformed (Álvarez et al., 2017). However, most of the studies described in this review paper concerned pesticide-degrading *Actinobacteria*. In the study by Ali et al. (2017), bioremediation was performed on PTE-contaminated mining soils by inoculation using *Streptomyces pactum*. Metal extraction amount data established that the strain Act12 stimulated the PTE uptake and transfer in *Brassica juncea* above ground tissues.

CONCLUSION

This study demonstrates that vegetated soils from red gypsum tailings dumps exhibited a higher bacterial diversity compared with non-vegetated soils, based on a culture-dependent method. The number of bacterial isolates having the capacity to produce IAA was higher for bacteria from the vegetated soil, while siderophore production was higher in the bacteria from the non-vegetated soil. Mn and Cr tolerance was also higher for the bacteria isolated from the VS samples. The potential of some bacterial isolates to promote birch growth was observed, although it was not always linked to PGP traits. However, the results of the inoculation tests and the dominance of some *Streptomyces* (within the VS *Actinobacteria* population) and *Phyllobacterium* (within the VS *Proteobacteria* population) associated with *Betula* growing on this soil suggests that those bacteria are involved in the early establishment of woody species in the dump. They appear to be good candidates to identify new approaches for the management of tailings dumps. The *Phyllobacterium* sp. isolate (R01-34) and *Streptomyces* (R05-33) appeared to be promising alternatives for improved inocula and their application at field levels.

AUTHOR CONTRIBUTIONS

MC, CZ, VA-L, and NC planned and designed the research. VA-L and CZ performed the experiments and conducted the fieldwork. MC, VA-L, CZ, NC, and CG analyzed the data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01772/full#supplementary-material

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Fungal Endophyte *Alternaria tenuissima* Can Affect Growth and Selenium Accumulation in Its Hyperaccumulator Host *Astragalus bisulcatus*

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Endophytes can enhance plant stress tolerance by promoting growth and affecting elemental accumulation, which may be useful in phytoremediation. In earlier studies, up to 35% elemental selenium (Se^0) was found in Se hyperaccumulator *Astragalus bisulcatus*. Since Se^0 can be produced by microbes, the plant Se^0 was hypothesized to be microbe-derived. Here we characterize a fungal endophyte of *A. bisulcatus* named A2. It is common in seeds from natural seleniferous habitat containing 1,000–10,000 mg kg^{-1} Se. We identified A2 as *Alternaria tenuissima* via 18S rRNA sequence analysis and morphological characterization. X-ray microprobe analysis of *A. bisulcatus* seeds that did or did not harbor *Alternaria*, showed that both contained >90% organic seleno-compounds with C-Se-C configuration, likely methylselenocysteine and glutamyl-methylselenocysteine. The seed Se was concentrated in the embryo, not the seed coat. X-ray microprobe analysis of A2 in pure culture showed the fungus produced Se^0 when supplied with selenite, but accumulated mainly organic C-Se-C compounds when supplied with selenate. A2 was completely resistant to selenate up to 300 mg L^{-1}, moderately resistant to selenite (50% inhibition at ~50 mg Se L^{-1}), but relatively sensitive to methylselenocysteine and to Se extracted from *A. bisulcatus* (50% inhibition at 25 mg Se L^{-1}). Four-week old *A. bisulcatus* seedlings derived from surface-sterilized seeds containing endophytic *Alternaria* were up to threefold larger than seeds obtained from seeds not showing evidence of fungal colonization. When supplied with Se, the *Alternaria*-colonized seedlings had lower shoot Se and sulfur levels than seedlings from uncolonized seeds. In conclusion, *A. tenuissima* may contribute to the Se^0 observed earlier in *A. bisulcatus*, and affect host growth and Se accumulation. A2 is sensitive to the Se levels found in its host's tissues, but may avoid Se toxicity by occupying low-Se areas (seed coat, apoplast) and converting plant Se to non-toxic Se^0.

These findings illustrate the potential for hyperaccumulator endophytes to affect plant properties relevant for phytoremediation. Facultative endophytes may also be applicable in bioremediation and biofortification, owing to their capacity to turn toxic inorganic forms of Se into non-toxic or even beneficial, organic forms with anticarcinogenic properties.

Keywords: hyperaccumulation, selenium, endophyte, *Alternaria*, *Astragalus*, x-ray analysis

INTRODUCTION

Selenium is not only toxic at elevated concentrations but also an essential micronutrient for many organisms including humans. The gap between Se deficiency and toxicity is narrow, and both are problems worldwide. Selenium is toxic due to its similarity to sulfur (S). Selenium readily replaces S in proteins, interfering with their function (Stadtman, 1990). In the Western United States, where many soils have elevated Se concentrations, chronic ingestion of high-Se plants by livestock has been reported to result in large livestock losses (Rosenfeld and Beath, 1964; Wilber, 1980).

Selenium serves no known essential function in plants, nor in fungi (Zhang and Gladyshev, 2009). In some microbes and fungi, Se is potentially used as a weak electron acceptor under anaerobic conditions (Heider and Böck, 1993). Selenium can also be beneficial to plants: it has been reported to increase growth and antioxidant activity (Hartikainen, 2005). At higher levels, Se offers plants protection against a wide variety of herbivores (Hanson et al., 2003; Freeman et al., 2006a).

Plants readily take up and assimilate Se into organic compounds, due to the similarities of Se and S (Schiavon and Pilon-Smits, 2017b). Hyperaccumulators can accumulate and tolerate up to 15,000 mg Se kg^{-1}, and are also unique in that they preferentially take up Se over S and allocate Se to the reproductive tissues, i.e., flowers and seeds (Quinn et al., 2011a; Valdez Barillas et al., 2012; El Mehdawi et al., 2018). Selenium accumulation in plants can be used for phytoremediation as well as biofortification (Schiavon and Pilon-Smits, 2017a).

Several hypotheses have been proposed for why plants hyperaccumulate toxic elements like Se: inadvertent uptake, drought tolerance, elemental tolerance, allelopathy, and elemental defense against herbivores and pathogens (Boyd and Martens, 1992). For Se hyperaccumulators, the evidence for the elemental defense hypothesis is well supported. Selenium has been shown to protect plants from a variety of generalist, Se-sensitive herbivores, for a review see El Mehdawi and Pilon-Smits (2012). There is also evidence that hyperaccumulators may deposit Se in the surrounding soil as a form of elemental allelopathy against Se-sensitive neighboring plants (El Mehdawi et al., 2011a).

While Se-sensitive ecological partners suffer in their interactions with Se hyperaccumulators, Se-resistant partners may exploit the high-Se niche offered by hyperaccumulator plants. Se-resistant herbivores have been found to feed on hyperaccumulator seeds and leaves. In some of these herbivores resistance is based on tolerance and in others it is based on exclusion (Freeman et al., 2006a, 2010; Valdez Barillas et al., 2012). Furthermore, Se-tolerant neighboring plants of hyperaccumulators in the field were shown to benefit from their proximity to hyperaccumulators: they exhibited enhanced Se levels, which made them less susceptible to herbivory (El Mehdawi et al., 2011b). Selenium tolerance in these ecological partners was often associated with the accumulation of organic Se (e.g., methylselenocysteine, MeSeCys) in their tissues. Selenium hyperaccumulators also contain mostly MeSeCys, which may explain their extreme Se tolerance. MeSeCys cannot be incorporated into protein, and thus Se toxicity is avoided (Terry et al., 2000).

Relatively little is known about how Se affects the plant–microbe interactions of hyperaccumulators. Depending on whether the associated microbe lives in the rhizoplane (surface of roots), phyllosphere (surface of leaves), or as endophyte (inside plant tissues), it may experience different Se levels, and with that, Se toxicity (Valdez Barillas et al., 2011, 2012). The microbe's relationship with the plant may involve pathogenicity, mutualism, and commensalism. Some microbes may perform beneficial functions for the hyperaccumulator: stimulating growth, aiding in nutrient and water acquisition, or fighting off pathogens. In hyperaccumulators, microbes may also affect the acquisition, speciation, and accumulation of the hyperaccumulated element (de Souza et al., 1999; Di Gregorio et al., 2006; Alford et al., 2010).

There is evidence that Se can protect plants from Se-sensitive microbial pathogens. In a study with non-hyperaccumulator *Brassica juncea*, Se was shown to protect plants from two Se-sensitive fungal pathogens, *Alternaria brassicicola* and a *Fusarium oxysporum* (Hanson et al., 2003). There is also evidence for the presence of Se-resistant microbes that live in association with hyperaccumulators (Wangeline et al., 2011). A litter decomposition experiment on seleniferous soil revealed that there were more culturable microbes (colony forming units per gram) on high-Se leaf litter from hyperaccumulators than on low-Se litter from related species collected from the same site (Quinn et al., 2011b). This finding may suggest that specialist Se-resistant decomposing microbes are present at seleniferous sites. Furthermore, a Se-resistant Rhizobacterium apparently lives in association with the hyperaccumulator *Astragalus bisulcatus* (Fabaceae), since this species produces high-Se nodules (Valdez Barillas et al., 2012). This bacterium may affect plant Se speciation, since the nodules accumulated a high fraction of elemental Se (Se^0) (Valdez Barillas et al., 2012; Alford et al., 2014). Other endophytic bacteria were found to colonize this and other hyperaccumulators, which were

also found to produce elemental Se (Staicu et al., 2015; Sura-de Jong et al., 2015).

Interestingly, roots of Se hyperaccumulators collected from the field contained high fractions of Se^0 (up to 35%) while greenhouse-grown counterparts contained exclusively organic selenocompounds with a C-Se-C configuration (Se attached to two organic groups, Lindblom et al., 2013a). Based on these findings it was hypothesized that microbes are responsible for the production of Se^0 observed in hyperaccumulators in their natural habitat. To test this hypothesis, hyperaccumulator plants were grown from surface-sterilized seeds and inoculated with several root-associated fungi shown earlier to be able to produce Se^0. However, no significant effect on plant Se speciation was observed (Lindblom et al., 2013b, 2014).

In this study we test another hyperaccumulator-associated fungus that appears to be a seed-transmitted endophyte that asymptomatically colonizes stems and leaves of *A. bisulcatus* in their natural habitat. It was found to emerge regularly from surface-sterilized seeds of *A. bisulcatus*, and small spored *Alternaria* species could readily be cultured from surface-sterilized stem and leaf tissue (Valdez Barillas et al., 2012). This endophytic fungus clearly has a close association with the hyperaccumulator and thus maximal opportunity to impact plant Se speciation. In this work we identify this fungal endophyte using a combination of molecular and morphological characters, characterize the Se-related properties of the pure isolate (Se tolerance, Se metabolic properties), and test its impact on plant Se speciation, Se accumulation, and growth.

MATERIALS AND METHODS

Biological Material

Astragalus bisulcatus seeds were collected in Pineridge Natural Area, Fort Collins, CO, United States during 2008–2011. The seeds were stored in coin envelopes inside a silica gel desiccator at 4°C until use. For endophyte isolation, the seeds were first surface-scarified using a scalpel blade and surface-sterilized with 50% bleach for 5 min, then rinsed three times with sterile water. Seeds were transferred to petri dishes with half strength water agar and were allowed to germinate at room temperature. Fungal mycelia growing from the seed were then transferred to half-strength malt extract agar (0.5 MEA, Difco, Detroit, MI, United States) via hyphal tipping. Hyphal tipping was repeated at least two times to ensure the fungal was a pure culture. The isolate was designated as A2.

Sample Preparation for X-Ray Microprobe Analyses

Astragalus bisulcatus seeds were surface-sterilized as described above and germinated on 0.5 strength Murashige and Skoog (1962) basal salts agar medium containing 30 mg L^{-1} Na_2SeO_4. Two seeds were selected for X-ray microprobe analyses: one that showed the presence of A2 fungal mycelium and one that did not. These seeds were frozen at −80°C until analysis.

Agar plugs (0.5 cm × 0.5 cm) of A2 fungal mycelia were transferred to liquid malt extract medium containing 30 mg L^{-1} Na_2SeO_4 or 30 mg L^{-1} Na_2SeO_3. Sections of approximately 3 mm^3 of A2 fungus mycelia were washed briefly in 1 mM sulfate to removed adsorbed Se. Each section was immediately placed inside a separate 0.5 ml centrifuge plastic tube, frozen in liquid nitrogen, and stored at −80°C.

Determination of Fungal Se Tolerance

For the analysis of A2 fungal tolerance to different selenocompounds, the fungus was cultivated under continuous fluorescent light at 22°C in sealed Petri dishes containing 0.5 strength MEA supplemented with Na_2SeO_4 or Na_2SeO_3 at 0, 10, 30, or 300 mg L^{-1}. Fungal tolerance was also tested on different concentrations of MeSeCys (0, 10, 30, 60, 150 mg L^{-1}) in 0.5 MEA as well as on extract made from the flowers of *A. bisulcatus* added at these same Se concentrations to 0.5 MEA.

Fungal Identification

The A2 fungus was grown on V-8 juice agar with continuous light in unsealed plates. Potato carrot agar was used for slide culture conditions and comparison colony conditions with a 8 h light - 16 h dark cycle in unsealed plates. Morphological characterization was carried out as described below in the results section.

For molecular identification of the A2 fungus, DNA extraction, Polymerase chain reaction (PCR) and sequencing were done using the ITS 1 and 4 primers (White et al., 1990), following the protocol by Vincelli and Tisserat (2008).

Analysis of Plant Growth and Se Accumulation as Influenced by Endophytic *Alternaria*

Seeds of *A. bisulcatus* were first scarified for 10 min with concentrated sulfuric acid, and then further surface-sterilized by rinsing for 20 min in 20% bleach, followed by five 10-min rinses in sterile water. Seeds were then germinated on sterile filter paper under continuous light at 23°C in a plant growth cabinet. Upon germination, seedlings were separated into those that naturally contained the endophyte and two that did not, and transferred to culture tubes containing autoclaved potting soil. Half of the seedlings in each group, (A2-associated and seeds without A2), were watered with 80 μM selenate in liquid 0.5 MS medium while the other half were given medium without Se. The culture tubes were sealed with breathable tape and opened only to add fresh medium. There were ten replicates per treatment (40 total). The experiment was terminated after 4 weeks. At that point half of the replicates in the control group had died (i.e., the group without the fungus and without Se added).

Elemental Analysis

At harvest the plant roots were washed and then dried for 48 h at 45°C. Samples were digested in nitric acid as described by Zarcinas et al. (1987). Inductively coupled plasma atomic emission spectrometry (ICP-AES) was used to determine Se and S concentrations in the acid digest (Fassel, 1978).

X-Ray Microprobe Analyses

Elemental distribution and chemical speciation in the tissues were determined using μ X-ray fluorescence mapping (XRF) and μ X-ray absorption near-edge structure (XANES) spectroscopy, respectively, at the Advanced Light Source beamline 10.3.2 of the Lawrence Berkeley National Lab (Marcus et al., 2004). Frozen samples were transferred onto a Peltier stage kept at −25°C to reduce potential beam radiation damage. μXRF elemental maps were recorded at 13 keV, using a 15 μm (H) × 6 μm (V) beam, 15 μm × 15 μm pixel size, 50 ms dwell time per pixel. The chemical forms of Se in particular areas of interest were further investigated using Se K-edge XANES, at the tissue locations indicated in **Figures 1**, **4**. XANES provides information about the oxidation state and, when compared to well-characterized Se standard compounds, information about its chemical speciation (Pickering et al., 1999). XRF maps and XANES spectra were recorded with a seven element Ge solid state detector (Canberra, ON, Canada). Spectra were deadtime corrected, pre-edge background subtracted, and post-edge normalized using standard procedures (Kelly et al., 2008). Red amorphous elemental selenium (white line position set at 12660 eV) was used to calibrate the spectra. Least square linear combination (LSQ) fitting of Se XANES spectra was performed in the 12630–12850 eV range, using a library of standard seleno-compounds. As Se standards a library of 52 compounds was used (Fakra et al., 2018). All data processing and analyses were performed with a suite of custom LabVIEW (National Instruments, Austin, TX, United States) programs available at the beamline. Se valence-state scatter plots of the sample and standard compounds data were also obtained using

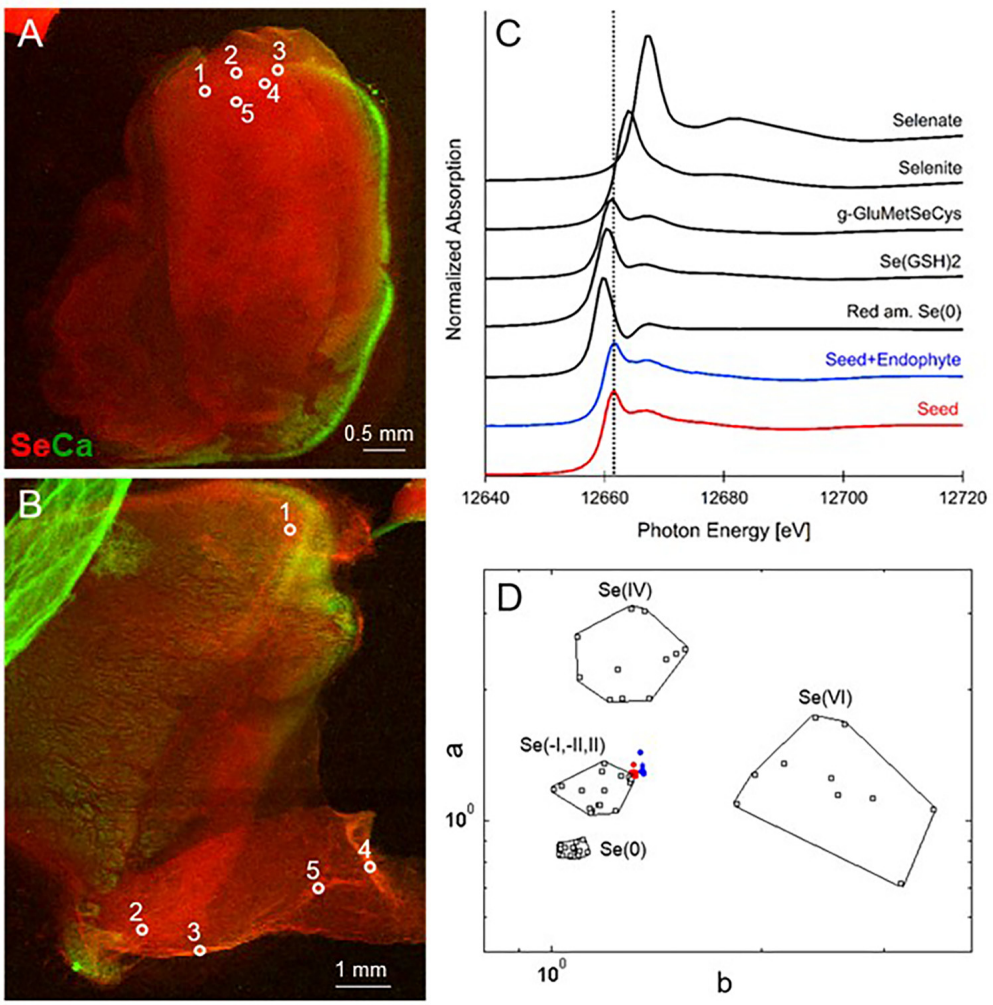

FIGURE 1 | Selenium distribution and speciation in germinating *A. bisulcatus* seeds. **(A,B)** are bicolor-coded XRF maps showing the distribution of Se (in red) and Ca (in green). **(A)** Seed not containing fungal endophyte A2, **(B)** seed containing A2. Locations where XANES spectra were collected are indicated by white circles. The seedlings are oriented with their radicle at the bottom, pointing left **(A)** or right **(B)** and the cotyledons toward the top. Selenium is clearly localized in the seed embryo; the seed coat contains Ca. **(C)** Average XANES spectrum obtained from five locations for each sample, compared to Se-bearing standards. **(D)** Se valence-state scatter plot obtained from XANES spectra of the seed (red dots), seed+endophyte (blue dots) compared to Se standards (black empty squares). The hexagonal datapoints correspond to the average spectrum for each sample.

MATLAB, following methods described in details elsewhere (Fakra et al., 2018).

Statistical Analysis

The software JMP-IN (3.2.6, SAS Institute, Cary, NC) was used for statistical data analysis. Analysis of variance followed by a *post hoc* Tukey Kramer test was used when comparing averages of Se content and averages of plant biomass among selenium treated and untreated *A. bisulcatus* replicates. A student *t*-test was used for pairwise comparisons between two means (using an alpha error = 0.5). It was verified that the assumptions underlying these tests (normal distribution, equal variance) were met.

RESULTS

X-Ray Microprobe Analysis of Seeds

When seeds of *A. bisulcatus* were surface-sterilized and germinated on sterile filter paper, about half of them contained an endophytic fungus, which was designated A2. Germination trials typically have shown around 50% infestation by *Alternaria* in *A. bisulcatus* germinated seeds based on visual estimation. To characterize the distribution and chemical speciation of Se in *A. bisulcatus* seeds, XRF and XANES analysis were performed on non-colonized vs. *Alternaria*-colonized seeds. Also, a valence plot was made, for a quick comparison of the fungal Se data with Se standards of known valence. Regardless of the presence or absence of fungus, Se was found in the embryo but not detected in the seed coat (**Figure 1**). There was no clear difference in Se speciation between *Alternaria*-colonized and uncolonized seeds (**Table 1**). Both contained predominantly (86–90%) organic Se with C-Se-C configuration, that fitted best with the Se standard γ-glutamyl-methylSeCys but may also include other C-Se-C compounds like SeMet or methyl-SeCys. In both seeds, there were small fractions of other selenocompounds that correspond with Se(IV) and Se(VI) oxidation states (forms of selenite and selenate, respectively) that fitted best with various metal selenate standards (Zn, Fe, and Cu selenate, particularly). The micro-XRF spectra (MCA) that were collected on each Se XANES spot indeed detected Ca, Fe, Zn, and Cu; at the energy we were exciting the sample with (13 keV), we were not very sensitive to elements below Ca (such as K, Cl, and S).

TABLE 1 | *Astragalus bisulcatus* seed Se speciation results obtained from least squares linear combination fitting (LCF) of the XANES spectra collected at the spots shown (as white circles) in **Figure 1**, using 52 standard seleno-compounds.

Seed	NSS (xE-4)	C-Se-C (%)	Fe-Se(IV) (%)	Zn selenate (%)
No endophyte				
Spot 1	3.9	92	4	2
Spot 2	3.8	90	5.5	1.5
Spot 3	6.1	89.2	3	5
Spot 4	3.7	95.3	1.4	1.3
Spot 5	4.1	93.4	1.9	2.5
Mean		92.0	3.2	2.5
SEM		1.1	0.7	0.7
With endophyte				
Spot 1	7.7	76	12.6	9
Spot 2	1.8	90	3.3	5.2
Spot 3	6.4	87.7	3.6	5.5
Spot 4	6.25	87	4	6
Spot 5	5.6	93	2	3
Mean		86.7	5.1	5.7
SEM		2.9	1.9	1.0

The best LCF was obtained by minimizing the normalized sumsquares residuals [NSS = 100 × $\Sigma(\mu_{exp} - \mu_{fit})^2/\Sigma(\mu_{exp})^2$], where μ is the normalized absorbance. Error on percentages is estimated to be ±10%. Replicates represent individual spectra obtained from different locations in the seeds. SEM: standard error of the mean. The Fe-Se(IV) corresponds to the standard mandarinoite.

The identity of A2 was initially investigated by DNA sequencing of the internal transcribed spacer region of ribosomal genes (ITS 1 and 4) of the small ribosomal subunit. As shown in **Figure 2A**, the sequence from A2 showed 100% sequence similarity with the known plant pathogen *Alternaria tenuissima* and 99.6% similarity with *Alternaria astragali*, a rhizosphere fungus associated with *A. bisulcatus* (Wangeline and Reeves, 2007). Small-spored *Alternaria*, particularly *A. alternata* and *A. tenuissima*, are difficult to distinguish using solely molecular techniques (Andrew et al., 2009), so morphological characteristics were included for identification (Simmons, 2007). The references were updated accordingly (Simmons, 2007). To date *A. tenuissima* is grouped among other small-spored *Alternaria* that show no association between host, geographic origin and phylogenetic lineage, and is considered by some as an unresolved group associated with *Alternaria alternata* (Andrew et al., 2009).

Fungal Isolate A2 Taxonomic Description

The A2 fungal strain was identified as *A. tenuissima* (Nees and T. Nees: Fr.) Wiltshire, anamorph (no known teleomorph), with the following specific morphology.

Colony at 7–8 days, PCA and V-8: colony 5–6 cm diameter. On PCA is a surface network of interwoven radial hyphae, gray to buff with little sporulation and no discernible rings. Also present is a dense mat of reduced aerial hyphae. The reverse is dark green to black and uniform. The colony on V-8 is similar to PCA. Mycelium cottony, with an advancing colony edge of approximately 10 mm. Colony has dark mycelial rings alternating with dense aerial rings when infrequently present. Mycelia gray, buff, and medium to dark brown. Mycelia layers upward with increasing age. The reverse is dark green to black.

Conidiophores (40×) produced on V-8 range on average between 130–155 μm in length (**Figures 2C,D**). Juvenile conidia are ovoid and have no definable beak, while most mature into a body narrowly ellipsoid with a long beak. The conidiophores are mostly simple but may have low amounts of branching (1–2), sometimes proliferating at 1–2 conidiogenous sites. Conidia chains of 2–6 (8) are produced (most common 4). Older areas of mycelial growth can have a distinctive rope-like formation, commonly up to or less commonly surpassing 11 μm wide.

Conidium bodies (100×) are ovoid or long ellipsoid; commonly with a long beak or less commonly with no distinctive

A

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A2                      ----------------CTGGACCTCTCGGGGTTACAGCCTTGCTGAAT 32
Alternaria_astragali    ----------------CTGGACCTCTCGGGGTT-CAGCCTTGCTGAAT 31
Alternaria_tenuissima   CATAAATATGAAGGCGGGCTGGACCTCTCGGGGTTACAGCCTTGCTGAAT 50
                                        ********************* **************

A2                      TATTCACCCTTGTCTTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCC 82
Alternaria_astragali    T-TTCACCCTTGTCTTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCC 80
Alternaria_tenuissima   TATTCACCCTTGTCTTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCC 100
                        * ************************************************

A2                      ACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAAC 132
Alternaria_astragali    ACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAAC 130
Alternaria_tenuissima   ACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAAC 150
                        ************************************************

A2                      AAATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA 182
Alternaria_astragali    AAATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA 180
Alternaria_tenuissima   AAATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA 200
                        ************************************************

A2                      TGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTG 232
Alternaria_astragali    TGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTG 230
Alternaria_tenuissima   TGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTG 250
                        ************************************************

A2                      AATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGC 282
Alternaria_astragali    AATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGC 280
Alternaria_tenuissima   AATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGC 300
                        ************************************************

A2                      ATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGC 332
Alternaria_astragali    ATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGC 330
Alternaria_tenuissima   ATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGC 350
                        ************************************************

A2                      GTCTTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCG 382
Alternaria_astragali    GTCTTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCG 380
Alternaria_tenuissima   GTCTTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCG 400
                        ************************************************

A2                      GCCTACTGGTTTCGGAGCGCAGCACAAGTCGCACTCTCTATCAGCAAAGG 432
Alternaria_astragali    GCCTACTGGTTTCGGAGCGCAGCACAAGTCGCACTCTCTATCAGCAAAGG 430
Alternaria_tenuissima   GCCTACTGGTTTCGGAGCGCAGCACAAGTCGCACTCTCTATCAGCAAAGG 450
                        ************************************************

A2                      TCTAGCATCCATTAAGCCTTTTTTTCAACTTTTGACCTCGGATCAGGTAG 482
Alternaria_astragali    TCTAGCATCCATTAAGCCTTTTTTTCAACTTTTGACCTCGGATCAGGTAG 480
Alternaria_tenuissima   TCTAGCATCCATTAAGCCTTTTTTTCAACTTTTGACCTCGGATCAGGTAG 500
                        ************************************************

A2                      GGATACCGCTGAACTTAAGCATATCA---------- 509
Alternaria_astragali    GGATACCGCTGAACTTAAGCATATCA---------- 507
Alternaria_tenuissima   GGATACCGCTGAACTTAAGCATATCAATAAGCGGAGGAA 540
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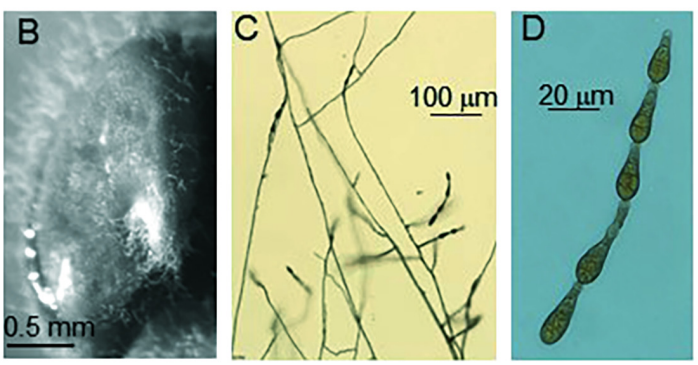

FIGURE 2 | Information used to identify A2 fungus. **(A)** ITS sequence alignment with *Alternaria astragali* (99%) and *Alternaria tenuissima* (100%). **(B)** Surface-sterilized *A. bisulcatus* seed with A2 endophyte mycelia emerging from the seed coat. **(C)** Conidiophores and conidia of A2, and **(D)** magnified conidia.

beak. Conidium length, with beak, ranging from 14 to 39 μm with 2–5 transverse septa, width ranging from 6 to 11 μm with 0–3 longitudinal septa, and beak length from 1.5 to 17 μm. Noteworthy is that mature conidia have beaks >10 μm, and continue to divide, becoming septate in both the beak (2–3 cells) and body gaining additional longitudinal septa. Also noteworthy that roughly 50% of conidia have zero longitudinal septa. Few conidium walls are rough or thickened.

Selenium Tolerance of the *A. tenuissima* A2 Strain

The Se tolerance of the A2 fungus to different forms and levels of Se when grown on MEA is shown in **Figure 3**. The fungus was most tolerant to selenate: its growth was still 90% of the control when supplied with 300 mg Se L^{-1} (**Figure 3A**). The fungus was also fairly tolerant to selenite: it showed 50% inhibition around 100 mg Se L^{-1} (**Figure 3B**). To test A2 growth as a function of plant-derived Se, flower material was extracted in water and added to the growth medium at different dilutions. The Se concentration in the extract was determined using ICP-AES. The fungus was significantly inhibited by the plant extract, showing 50% inhibition around 25 mg Se L^{-1} (**Figure 3C**). To test whether this was likely due to the Se, particularly C-Se-C, or (also) to other growth inhibiting compounds in the flowers, fungal growth was also determined as a function of MeSeCys concentration. When pure MeSeCys was added to the medium, the growth of A2 was 50% inhibited around 20 mg Se L^{-1} (**Figure 3D**), i.e., A2 growth was similarly inhibited by pure MeSeCys and by the Se extracted from *A. bisulcatus*.

X-Ray Microprobe Analysis of A2 Mycelium Grown on Medium With Selenate or Selenite

X-ray microprobe analysis was carried out on mycelia of A2 that was grown on fungal growth media spiked with selenate or selenite. XRF maps and XANES spectra are shown in **Figure 4**, as well as a valence plot for a quick comparison of the fungal Se data

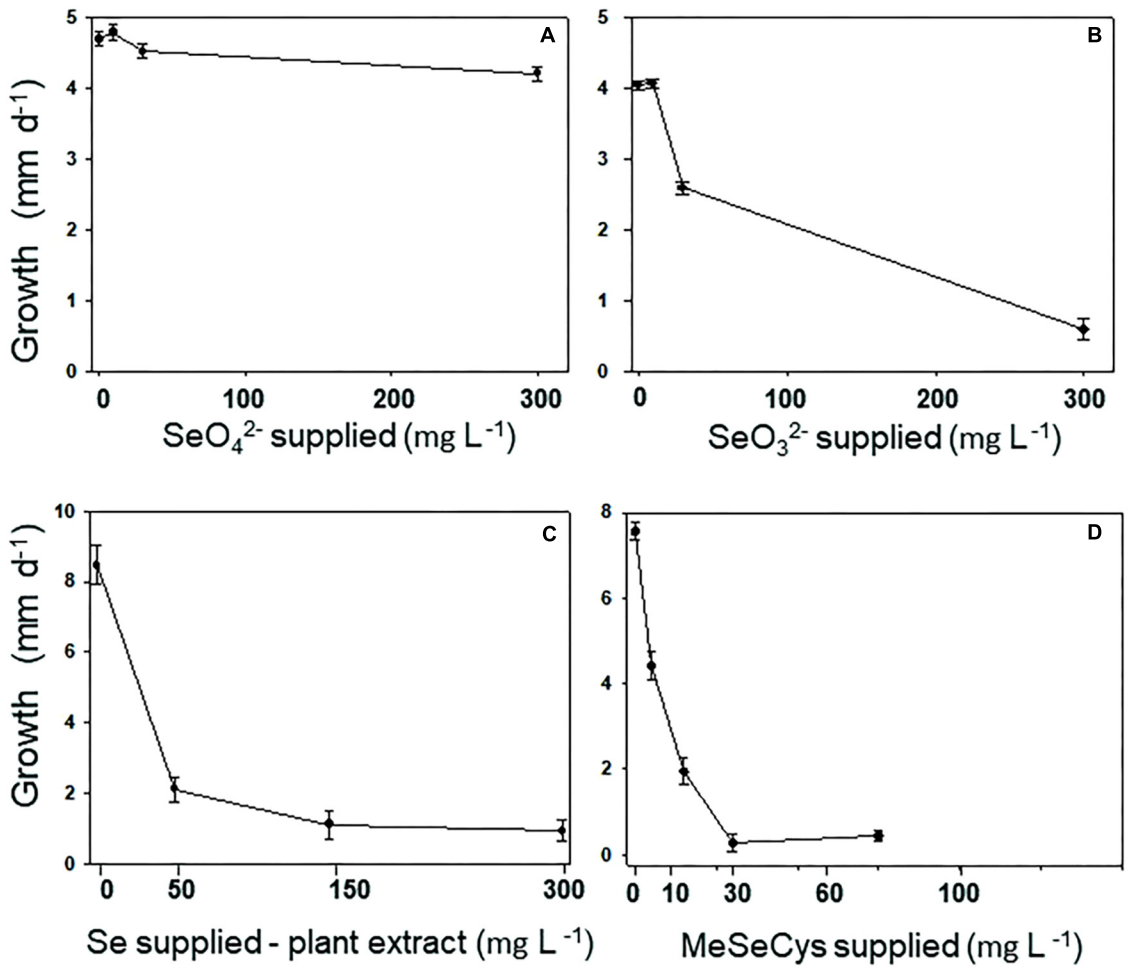

FIGURE 3 | Fungus A2 is resistant to selenate, moderately resistant to selenite, but relatively sensitive to organic MeSeCys and hyperaccumulator-derived Se (= MeSeCys). A2 resistance was measured as growth of the colony per day on varying concentrations of **(A)** selenate, **(B)** selenite, **(C)** extract from the flowers of *Astragalus bisulcatus*, and **(D)** methyl-selenocysteine.

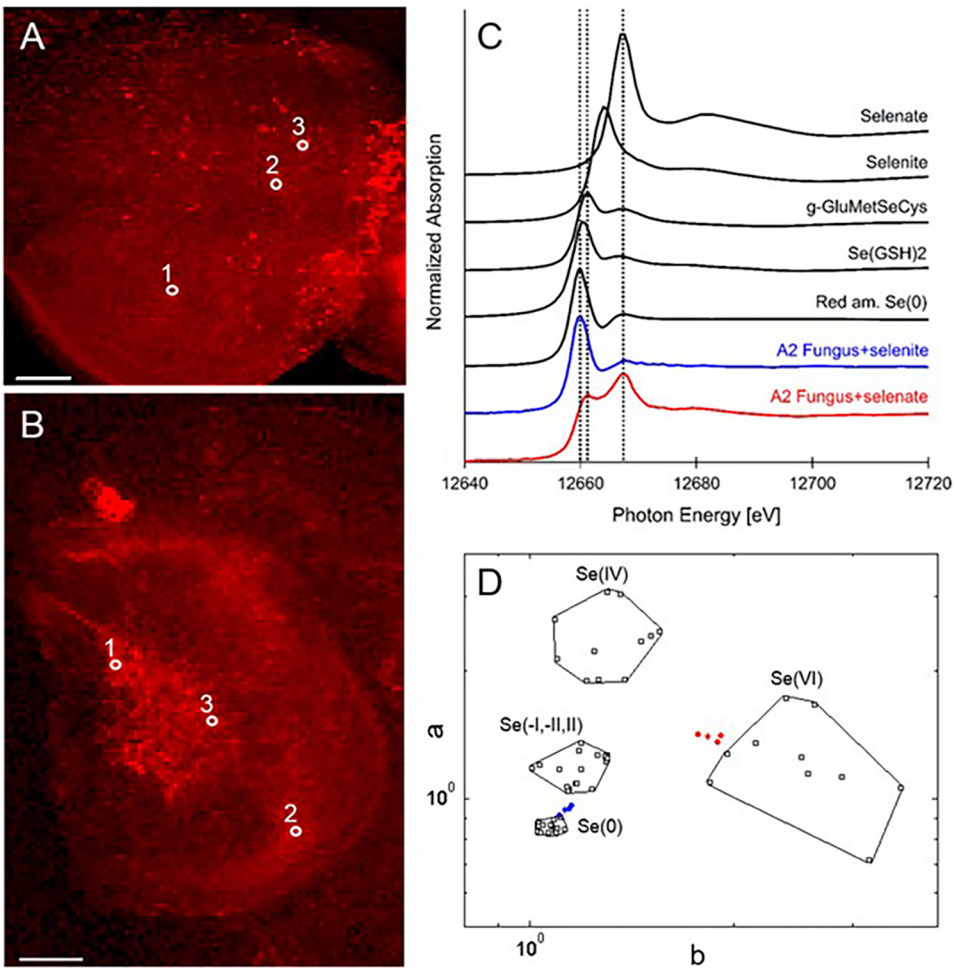

FIGURE 4 | Selenium distribution and speciation in A2 fungal material (mycelium) collected after growth on MEA medium spiked with selenate or selenite. **(A)** XRF Se map of A2 supplied with selenate. **(B)** XRF Se map of A2 supplied with selenite. Scale bars are 500 microns. **(C)** Average XANES spectrum obtained from three locations for each sample (white circles in **A,B**), compared to Se standards. **(D)** Se valence-state scatter plot obtained from XANES spectra of A2 supplied with selenate (in red), A2 supplied with selenite (in blue), compared to Se standards (in black). The hexagonal datapoints correspond to the average spectrum for each sample.

TABLE 2 | Selenium speciation results obtained from least squares LCF of experimental XANES spectra collected from the locations shown in **Figure 4** with standard seleno-compounds.

A	**NSS (xE-4)**	**C-Se-C (%)**	**MeSeCys (%)**	**Se(GSH)2 (%)**	**Mg/Cu selenate (%)**	**SeCl4 (%)**
A2 selenate						
Spot 1	2.2	68	0	7	29	0
Spot 2	5.7	60	0	11	31	0
Spot 3	7.0	74	74	0	22	7
Average		67	25	6	27	2
B*	**NSS (xE-4)**	**Red Se(0) (%)**	**Black Se(0) (%)**	**Gray Se(0) (%)**	**Se(0) total (%)**	**Se(GSH)2 (%)**
A2 selenite						
Spot 1	2.3	81	0	11	92	8
Spot 2	3.0	30	45	0	75	25
Average		55	23	6	83	17

*The best LCF was obtained by minimizing the normalized sumsquares residuals [NSS = 100 × $\Sigma(\mu_{exp} - \mu_{fit})^2/\Sigma(\mu_{exp})^2$], where μ is the normalized absorbance. Error on percentages is estimated to be $\pm$10%. XANES spectra were collected on Alternaria sp. originally isolated from Astragalus bisulcatus seeds, grown on malt extract agar with 30 mg L^{-1} sodium selenate (SeO_4^{2-}) or sodium selenite (SeO_3^{2-}). Replicates represent individual spectra obtained from mycelial mass. *Spot 3 not reported; too noisy to fit.*

with Se standards of known valence. The detailed Se speciation results are listed in **Table 2**. When the fungus was supplied with selenite (SeO_3^{2-}), 83% of Se in the mycelia was present as Se^0; the remainder was best matching the seleno-diglutathione (Se-GSH_2) standard. When supplied with selenate (SeO_4^{2-}) the fungus accumulated a large fraction (67%) of Se as C-Se-C, as well as a substantial fraction of selenate (27%), and a minor fraction of Se-GSH_2 (6%).

Effect of *A. tenuissima* (A2) on *A. bisulcatus* Growth and Se and S Accumulation

Seeds with hyphae emerging during germination were separated from those that did not show hyphae, and the two groups were cultivated on sterile peat moss for 4 weeks with or without selenate, the main form of bioavailable Se found in soil. The A2-containing seedlings had reached a twofold to threefold greater dry weight compared to uncolonized seedlings (**Figures 5A,B**). The addition of Se did not significantly affect *A. bisulcatus* growth for either group (**Figures 5A,B**). The presence of the A2 fungus was also associated with reduced shoot Se and S levels (**Figures 5C,D**).

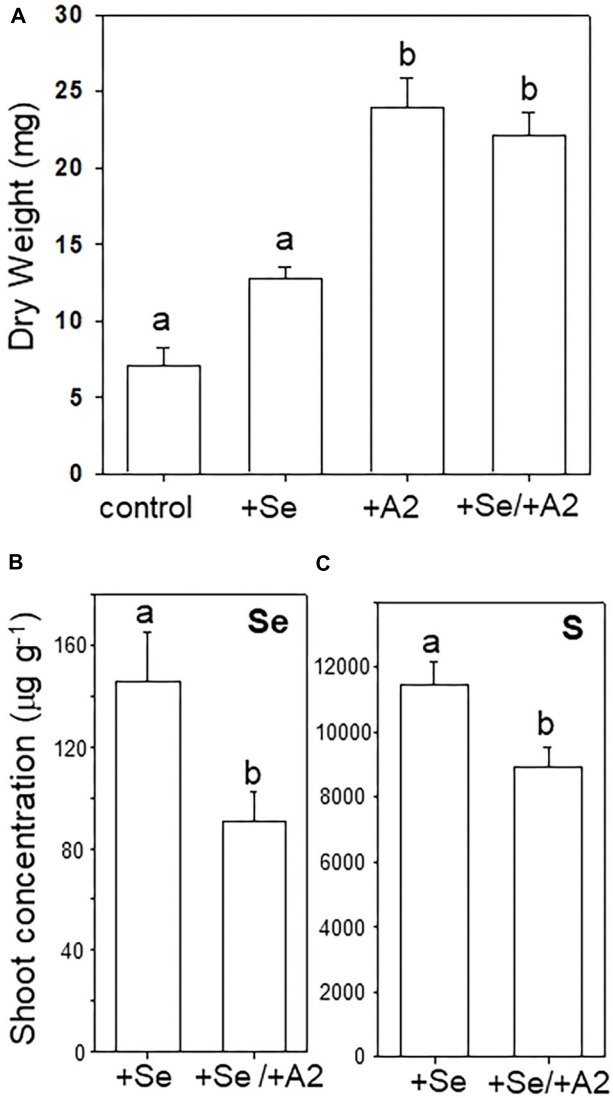

FIGURE 5 | Effect of the presence of *Alternaria* endophyte on growth and Se and S accumulation in its host, Se hyperaccumulator *A. bisulcatus*. **(A)** Plant biomass. **(B)** Shoot Se concentration, **(C)** shoot sulfur concentration. Different letters above bars indicate significant differences ($p < 0.05$) between treatment means ($n = 10$).

DISCUSSION

Hyperaccumulators of toxic elements are an interesting potential resource for discovery of microbes with properties useful in phytoremediation or bioremediation. Like all plants, Se hyperaccumulators harbor a variety of endophytic and rhizosphere bacteria and fungi (Wangeline et al., 2011; Sura-de Jong et al., 2015). While Se hyperaccumulator *A. bisulcatus* accumulates Se mainly in organic C-Se-C forms (methyl-SeCys, especially), up to 30% elemental Se^0 was reported in the roots and stem of mature field-collected plants, as well as in seedlings germinated from field-collected seeds (Valdez Barillas et al., 2012; Lindblom et al., 2013a). The A2 fungal strain, identified here as *A. tenuissima*, may contribute to this fraction of Se^0 in *A. bisulcatus*. It could be cultured from about 50% of field-collected, surface-sterilized *A. bisulcatus* seeds. In addition, small-spored *Alternaria* species, of which *A. tenuissima* is a member, could be cultured from surface-sterilized roots and stems of *A. bisulcatus*. In pure culture supplied with selenite, the A2 fungus was shown here to be capable of producing Se^0. While *A. tenuissima* is known as a potential plant pathogen with a wide host range (Mishra and Parakash, 1975), there is no evidence from this study that it acts as a pathogen on *A. bisulcatus*. *Alternaria*-containing seedlings grew better than seedlings not containing this endophyte, so it actually may be growth-promoting. However, it is possible that the relationship between *A. bisulcatus* and A2 depends on the conditions, particularly the Se level, the overall nutrient supply and the health status of the plant.

The Se speciation in germinating *Alternaria*-colonized or uncolonized *A. bisulcatus* seeds was similar: 86–90% C-Se-C. This was likely MeSeCys and glutamyl-MeSeCys, as has been previously reported for seeds (Nigam and McConnell, 1969); MeSeCys has also been found to be the main form of Se in leaves (Freeman et al., 2006b) and flowers of this species (Valdez Barillas et al., 2012). In contrast, an earlier study by Valdez Barillas et al. (2012) found a seed in a late-stage *Alternaria* infestation to contain 22% Se^0, both in the seed and the mycelium growing from the seed. These results suggest that this *Alternaria* can convert the C-Se-C in the seed to Se^0, perhaps as a tolerance mechanism. Grown in pure culture, A2 produced Se^0 when supplied with selenite. Conversion of more toxic forms of Se to insoluble, inert Se^0 is known to be a tolerance mechanism for many microbes (Gharieb et al., 1994 and citations therein; Gadd, 1993; Lovely, 1993). A2 appears to have a different Se

resistance mechanism for selenate, since it contained a variety of organic selenocompounds (C-Se-C) when supplied with this form of Se. The tolerance of A2 to selenate was much higher than for selenite. Surprisingly, although A2 grew very well on seeds that contained upward of 1,000 mg Se kg^{-1} (Galeas et al., 2007; Quinn et al., 2011a), it was already 50% inhibited by 25 mg kg^{-1} Se extracted from *A. bisulcatus* flowers, as well as by 20 mg kg^{-1} MeSeCys. A possible explanation for the ability of A2 to successfully grow on these high-Se plants could be that A2 occupies areas of the plant where there is relatively less Se, such as the interface between the seed coat and the seed embryo, and in the apoplast. As shown here, the seed coat contains very little Se, and in earlier studies energy dispersive X-ray analysis of hyperaccumulator leaves revealed that Se is generally stored in the vacuole in Se hyperaccumulators, and not in the apoplast (Freeman et al., 2006c, 2010). Thus, A2 may not encounter toxic Se levels in the living plant, like it does when grown on pure selenocompounds or homogenized plant extract.

The ITS sequence alignment identification of the fungus revealed an interesting similarity to another fungal-symbiont of *A. bisulcatus*, *A. astragali* (A3), which was originally isolated from the rhizoplane of surface-sterilized roots (Wangeline and Reeves, 2007). An additional *Alternaria* species, *A. seleniiphila* (A1) was isolated from the rhizoplane of hyperaccumulator *Stanleya pinnata* (Wangeline and Reeves, 2007). Both A1 and A3 were characterized for Se tolerance and speciation by Lindblom et al. (2013a). The Se-related characteristics of A2 are somewhat similar to A1 and A3. All are capable of reducing selenite to Se^0 and all are fairly tolerant to selenate. All three also stimulated the growth of their hyperaccumulator host.

Perhaps related to its effect on Se speciation toward more insoluble Se^0 in roots, the A2-containing *A. bisulcatus* seedlings showed significantly lower Se and S levels in their shoots. In previous studies where hyperaccumulators were inoculated with the related *Alternaria* species A1 and A3, there was a reduction in root-to-shoot translocation (Lindblom et al., 2013b, 2014). The same may be the case for A2; the root biomass was too small to determine root elemental concentrations. The possible mechanism for reduced translocation could be the production of Se^0 in the rhizosphere or inside the root apoplast, trapping Se in a non-soluble and therefore non-translocatable form. In this context it is interesting to note that pure A2 cultures produced mainly C-Se-C compounds from selenate (the form provided in the seedling study), and elemental Se from selenite. Thus, if the A2 endophyte produced elemental Se in the root, the plant may have reduced the selenate to selenite first.

This study helps us understand the ecology of *Alternaria* fungi in relation to various hosts. This fungus is best known for its capacity to colonize different hosts including many domesticated crop species, where it may or may not act as a pathogen. Zou et al. (2018), however, report an *Alternaria* sp. fungal endophyte that acted as a plant growth promoting fungus. The increases in shoot and root biomass observed in that study were attributed to a plant metabolic upregulation induced by the fungal endophyte. In this non-domesticated Se hyperaccumulator *A. tenuissima* behaves asymptomatically similarly to other vertically transmitted fungal endophytes, perhaps due to the host's elemental defense (the high levels of this toxic element may negatively affect pathogens) and limited access to nutrients by the host's apoplast. However, it is apparently capable of colonizing the hyperaccumulator's tissues and perpetuating its genetic line by keeping its host alive and colonizing the seeds. This is another example of *Alternaria's* phenotypic plasticity, showing its ability to colonize a diverse range of hosts via different mechanisms and under different types of ecological interactions.

AUTHOR CONTRIBUTIONS

SL performed the experiments and wrote the manuscript. AW and BD helped with the fungal identification and advised concerning fungal cultivation. SL, SF, and JVB helped with X-ray microprobe analysis. EP-S oversaw the project and helped with manuscript preparation.

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Endophytic Bacterial Community Structure and Function of Herbaceous Plants From Petroleum Hydrocarbon Contaminated and Non-contaminated Sites

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Bacterial endophytes (BEs) are non-pathogenic residents of healthy plant tissues that can confer benefits to plants. Many Bacterial endophytes have been shown to contribute to plant growth and health, alleviation of plant stress and to in-planta contaminant-degradation. This study examined the endophytic bacterial communities of plants growing abundantly in a heavily hydrocarbon contaminated site, and compared them to those found in the same species at a non-contaminated. We used culture- dependent and independent methods to characterize the community structure, hydrocarbon degrading capabilities, and plant growth promoting traits of cultivable endophytes isolated from *Achillea millefolium*, *Solidago Canadensis,* and *Daucus carota* plants from these two sites. Culture- dependent and independent analyses revealed class Gammaproteobacteria predominated in all the plants regardless of the presence of petroleum hydrocarbon, with *Pantoea* spp. as largely dominant. It was interesting to note a >50% taxonomic overlap (genus level) of 16s rRNA high throughput amplicon sequences with cultivable endophytes. PERMANOVA analysis of TRFLP fragments revealed significant structural differences between endophytic bacterial communities from hydrocarbon-contaminated and non-contaminated soils—however, there was no marked difference in their functional capabilities. *Pantoea* spp. demonstrated plant beneficial characteristics, such as P solubilization, indole-3-acetic acid production and presence of 1-aminocyclopropane-1-carboxylate deaminase. Our findings reveal that functional capabilities of bacterial isolates being examined were not influenced by the presence of contamination; and that the stem endosphere supports ubiquitous BEs that were consistent throughout plant hosts and sites.

Keywords: bacterial endophytes, hydrocarbon degradation, plant growth promoting bacteria, oil field, stem endosphere, plant microbiome

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INTRODUCTION

Plants form associations with a multitude of structurally and functionally diverse beneficial microorganisms that can provide them with selective advantages. Among these beneficial associates are bacterial endophytes (BEs) - non-pathogenic bacteria that reside within the living tissues of plants without conferring them harm. Many BEs have been reported to support growth, improve

plant health and alleviate stress (Azevedo et al., 2000; Hardoim et al., 2008; Glick and Stearns, 2011; Mitter et al., 2013).

A growing body of literature demonstrates evidence of some BEs' ability to mineralize petroleum hydrocarbon components (Phillips et al., 2008, 2009; Andria et al., 2009; Yousaf et al., 2010, 2011; Afzal et al., 2012; Kukla et al., 2014). Notably, Yousaf et al. (2010, 2011) found endophytic strains of *Enterobacter ludwigii* and *Pantoea* could successfully colonize plants Italian ryegrass and birdsfoot trefoil, and therein mineralize hydrocarbon, and express genes for hydrocarbon degradation. Improved contaminant degradation was correlated with increased numbers of pollutant-degrading bacteria (Yousaf et al., 2011).

A study on the bacterial epiphytes and their potential to bioremediate hydrocarbon pollutants in the atmosphere revealed significantly higher hydrocarbon degrading epiphytes isolated from a polluted environment compared to those isolated from a pristine environment (Ali et al., 2012). Oliveira et al. (2014) reported that the level of hydrocarbon pollution in salt marsh sediments was the determining factor in endophytic community composition, however, it is still unclear if this holds true for stem endophytic bacteria from plants in grassland or terrestrial systems.

In contaminated environments, BEs can also help plants tolerate contaminant-induced stress by releasing 1-aminocyclopropane-1-carboxylate (ACC) deaminase that decreases ethylene production (Glick, 2004). Some BE strains produce indole-3- acetic acid and solubilize inorganic phosphates, thereby promoting plant growth (Sheng et al., 2008; Dashti et al., 2009; Becerra-Castro et al., 2011). Recently, the proposal that BEs possessing both contaminant-degrading and plant growth promoting capabilities would be more likely to succeed in cleaning up organic contaminants recognizes increasing need for this research (Khan et al., 2013; Afzal et al., 2014; Kukla et al., 2014).

Oil Springs, Ontario, Canada (N42°46. 267, W82°05.539), sits above a naturally occurring near surface oil deposit. Oil has been seeping to the surface and forming gum patches since human recorded history in the area. Hand-dug wells and pumps were established as the 1850's and the pumps still produce oil to this day. During pump services, oil frequently spills onto the nearby soils leading to total petroleum hydrocarbon concentrations in the spill areas from 45,000–300,000 ppm. Despite the known toxicity of petroleum hydrocarbon, several common plant species grow abundantly in these soils.

Plants naturally produce alkanes, aromatic hydrocarbons and other compounds that share structural similarities with many organic pollutants; and some of these compounds are intermediates of degradation pathways that are produced during catabolism of organic contaminants. Bacteria that are in intimate association with plants are known to consume plant exudates, and are deemed capable in degrading organic contaminants. Hence, the role of plant bacterial partners in remediating soils contaminated by organic compounds has been well documented (Weyens et al., 2009; Vangronsveld et al., 2011; Khan et al., 2013; Afzal et al., 2014; Gkorezis et al., 2016; Ijaz et al., 2016).

For all these reasons, we hypothesized that endophytic bacterial communities of these Oil Springs plants might be contributing significantly to their adaptation to petroleum hydrocarbons (PHCs) toxicity. We predicted that (1) these plants harbor high numbers of endophytic bacteria that are able to mineralize PHC and (2) these plants harbor high numbers of endophytic bacteria with plant growth promoting capabilities. To this end we compared the endophytic bacterial populations found in herbaceous species thriving at Oil Springs to those found in the same species growing at a control, uncontaminated site.

MATERIALS AND METHODS

Site Description and Sampling

The hydrocarbon contaminated site is a natural oil seep field located at Oilsprings, Ontario (N42°46. 267, W82°05.539). Soils have silty clay loam soil texture, with pH of 6.8–7.3, and extractable total P and total N were 21 mg/L and 0.44% dry soil, respectively. The non-hydrocarbon contaminated site is located around 80 km away at Komoka, Ontario (N42°56.850, W81°23.697), which is an uncontaminated meadow with undetectable levels of PHCs. The soil was silty loam soil, a pH of 7.7 and extractable total P and total N of 5.3 mg/L and 0.20% dry soil, respectively.

Three pumping wells with recurring spillage were randomly chosen as sampling locations across a 650-acre oil field; whereas in non-contaminated site, three random sampling locations were chosen across a 500 m transect line. Three plant species belonging to—*Achillea millefolium*, *Solidago canadensis,* and *Daucus carota* (at least five individual plants of each plant species to minimize spatial individual plant variation), of the same size and maturity, were sampled. These plants were chosen as these were seen growing at both the hydrocarbon and non-hydrocarbon contaminated sites. All plants were immediately placed in Ziploc bags and into a 4°C cooler box for transport to the lab.

TABLE 1 | Mean values ($N = 3$) of colony forming units and species richness using TRFLP fragments of endophytic bacterial communities per gram fresh weight of plant tissues recovered from *Daucus carota*, *Achillea millefolium*, and *Solidago canadensis* from petroleum hydrocarbon (HC) contaminated and non-contaminated sites, standard deviation in parenthesis.

	Plant species	CFU/g	Phylotype richness (TRFLP fragments)
HC contaminated site	*Daucus carota*	2620 (1015)[a]	10 (1.4)[a]
	Achillea millefolium	1667 (1193)[a]	12.7 (2.3)[a]
	Solidago canadensis	1960 (1061)[a]	12 (1)[a]
NON-HC contaminated site	*Daucus carota*	1233 (929)[a]	11.7 (2.9)[a]
	Achillea millefolium	2200 (1386)[a]	12 (2)[a]
	Solidago canadensis	2433 (404)[a]	12.7 (2.1)[a]

Means with different letters are significantly different at $P < 0.05$.

Culture-Based Analysis: Endophyte Isolation and Identification

In each replicate, 15–20 g of stem tissues were surface- sterilized using a series of washes (70%ethanol; 1.2%bleach with 0.1% Tween 20; followed by six washes of sterile distilled water). To test the efficacy of sterilization, an aliquot of the last wash was plated onto agar plates and sterilized stem samples were imprinted onto both Reasoner's 2A (R2A) and Tryptic Soy Agar (TSA) media. The sterilized stems were then macerated in a sterilized Waring blender vessel at 2,0000 rpm using sterile 60 ml 50 mM Tris-HCl and heterotrophic bacteria were isolated by plating 100 µL on R2A and TSA plates. The media plates were incubated at 28°C for a period of 1–4 weeks. Individual bacterial colonies were isolated and grown into pure culture. Lysates were made from pure colonies by boiling 2 loopfuls of 1 µL sterile disposable loops in 100 µL sterile distilled water for 7 min. One microliter of lysate was used as template in a PCR reaction using 16SrRNA primers 27F (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1492R (5′-TACCTTGTTACGACTT-3′; Frank et al., 2008). The PCR reaction was as follows: 20µL reactions with a final concentration of 0.5 mM of the forward primer and reverse primer, 1.5 mM MgCl2, 200 mM of each dNTP, 2.5 units of HotStarTaq Plus DNA polymerase (Qiagen, Canada). The PCR amplifications were carried out in a PTC-200 thermal cycler (MJ Research Inc.) with the following conditions: initial denaturing at 95°C for 5 min followed by 35 cycles of: denaturing at 95°C for 1 min, annealing at 56°Cfor 1 min and extension at 72°C for 1 min; final extension at 72°C for 10 min. The

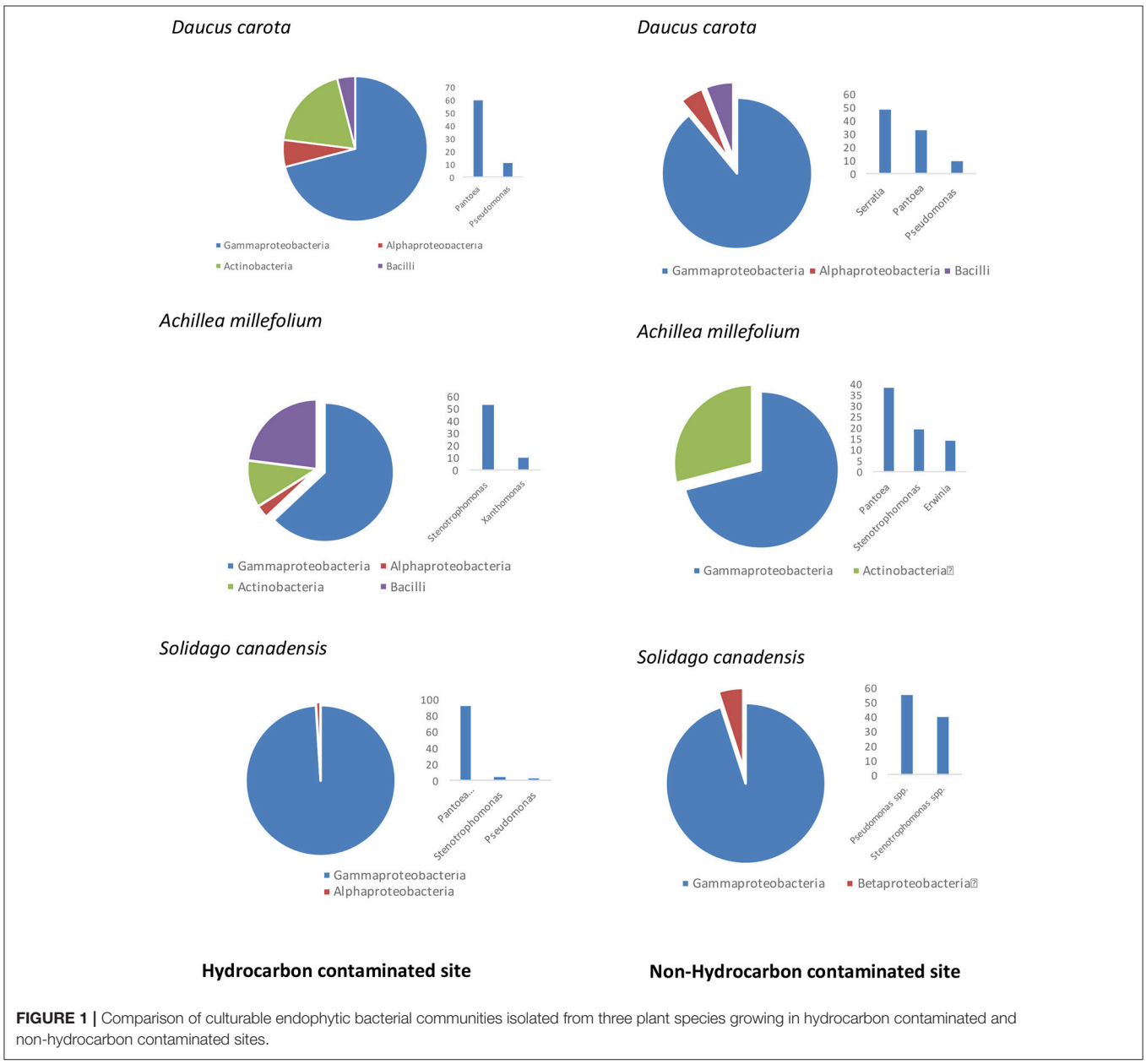

FIGURE 1 | Comparison of culturable endophytic bacterial communities isolated from three plant species growing in hydrocarbon contaminated and non-hydrocarbon contaminated sites.

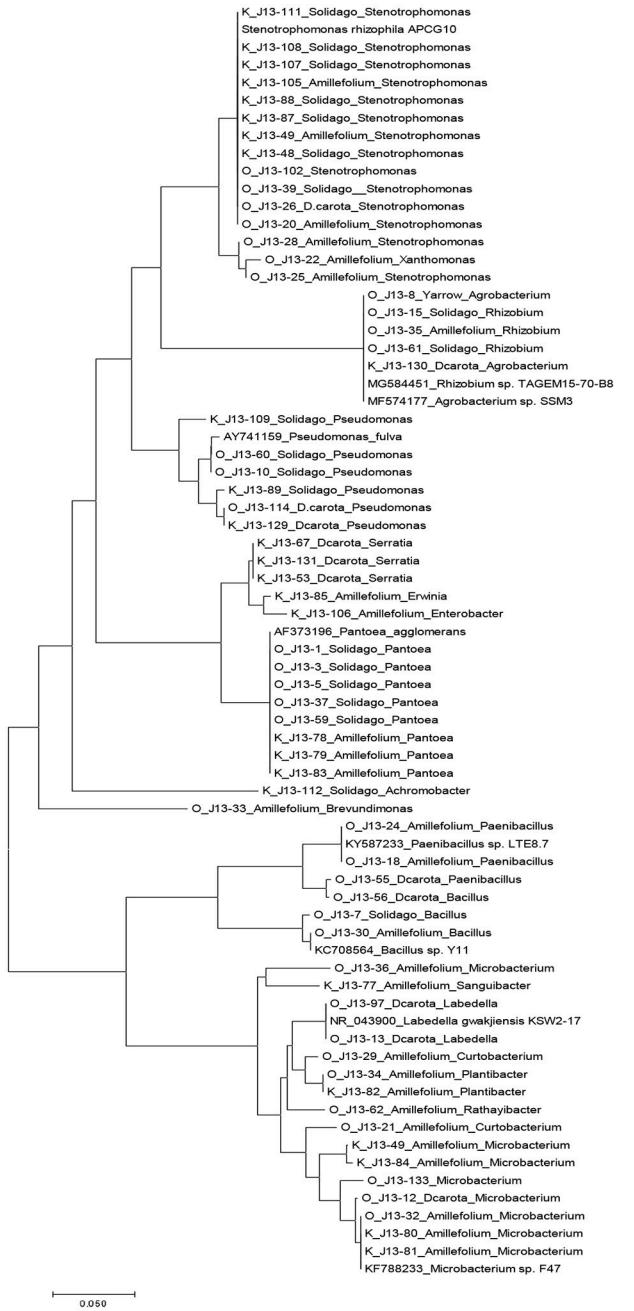

FIGURE 2 | Phylogenetic tree based on partial 16s sequences inferred using the Neighbor-Joining method. The evolutionary distances were computed using the maximum composite likelihood method in MEGA7 (Kumar et al., 2016). Strains that do not have J13 labels are sequences from Genbank database.

resultant amplicons were purified and subsequently submitted for Sanger sequencing at The Centre for Applied Genomics (TCAG) sequencing facility (Toronto, Canada) using 27F (5′-AGAGTTTGATYMTGGCTCAG-3′) primer. The identity of the isolates was determined using the most similar 16S rDNA sequences with the Ribosomal Database Project; sequences were deposited in GenBank with accession numbers: MH470404 - MH470471.

Screening for Hydrocarbon Degrading Potential of Bacterial Endophytes

Mineralization of hydrocarbon by individual endophytes was quantified using rapid growth based colorimetric assays and also via PHC loss measurements in liquid cultures via Gas Chromatography-Flame Ionization Detector (GC-FID). These methods are detailed elsewhere (Lumactud et al., 2016).

Isolates were screened for the presence of known catabolic genes for enzymes: alkane hydroxylase (AlkB), using primers alkBwf 5′-AAYAC NGCNCAYGARCTNGGVCAYAA-3′ and alkBwr 5′-GCRTGRT GRTCHGARTGNCGYTG-3′ that targets groups belonging to *Acinetobacter*, *Pseudomonas* and *Rhodococcus* (Wang et al., 2010). PCR conditions were initial denaturing at 94°C for 4 min followed by 32 cycles of: denaturing at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min; final extension at 72°C for 10 min. Catechol 2,3-dioxygenase (C23O) genes were assayed using primers C23O-F-AGGTGCTCGGTTTCTACCTGGCCGA and C23O-R-ACGGTCATGAATCGTTCGTTGA G (Luz et al., 2004) using PCR conditions- initial denaturing at 94°C for 4 min followed by 30 cycles of: denaturing at 94°C for 1 min, annealing at 60°C for 1 min and extension at 7°C for 1 min; final extension at 72°C for 3 min. Primers that were used for cytochrome P450-type alkane hydroxylase gene assay were F- GTSGGC GGCAACGACACSAC and R- GCASCGGTGGATGCCGAA GCCRAA, following the conditions described in (Arslan et al., 2014). Catechol 1, 2 dioxygenase genes assays were done using the primers—cat1,2-F-ACVCCVCGHACCATYGAAGG and cat1,2-R- CGSGTNGCAWANGCAAAGT following the PCR conditions as described elsewhere (El Azhari et al., 2010).

Evaluation of Plant Growth Promoting Abilities and 1-Aminocyclopropane-1-Carboxylate Deaminase Gene (ACCD)

All isolates were assessed for production of indole-3-acetic acid (IAA), solubilization of inorganic phosphate and the presence of the 1-aminocyclopropane-1-carboxylate deaminase gene. IAA production by bacterial isolates both in the presence and absence of L-tryptophan (L-TRP) was measured following the method described by Gordon and Weber (1951). The phosphate solubilization ability of the isolates was determined on a Pikovskaya agar medium. The presence of a clear zone around the bacterial colonies indicates the solubilization of phosphate. The halo size was calculated by subtracting the colony diameter from the total diameter. This assay was done in duplicate. 1-aminocyclopropane-1-carboxylate deaminase gene (*acdS*) was assayed following Blaha et al. (2006).

Culture-Independent Community Analysis

Total community DNA was extracted from plant macerates using FastDNA SPIN Kits (MP Biomedicals) following manufacturer's instructions with modifications (addition of 100 μL of protein

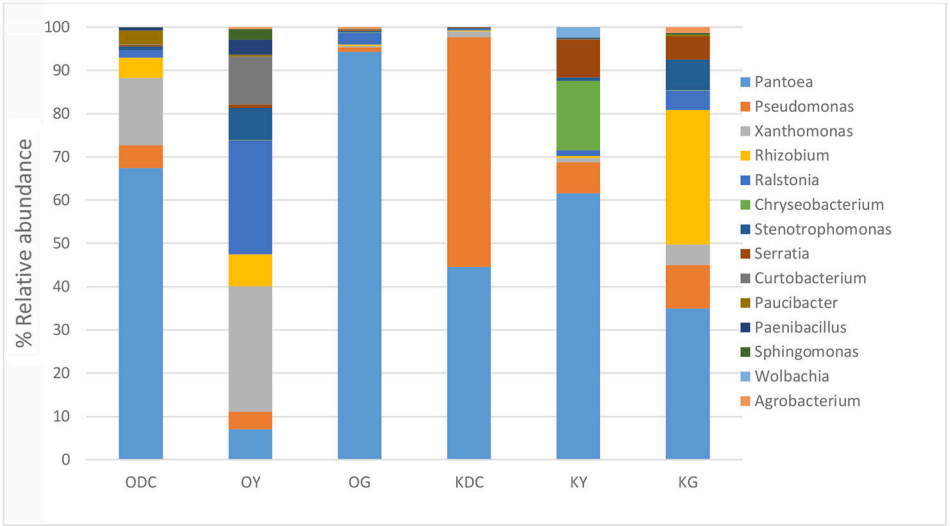

FIGURE 3 | Analysis of culture-independent stem endophytic communities at genus level isolated from plants growing in hydrocarbon contaminated (sample name starts with O for Oil Spring) and non-hydrocarbon contaminated soils (sample name starts with K for Komoka)—ODC, Oil Spring Daucus carota; OY, Oil Spring yarrow (*A. millefolium*); OG, Oil Spring goldenrod (*S. canadensis*); KDC, Komoka Daucus carota; KY, Komoka yarrow; KG, Komoka goldenrod.

TABLE 2 | Alpha-diversity.

	Sample	Observed	Shannon	Chao1
Hydrocarbon-contaminated sites	OG	113	1.9	239
	OY	270	3.2	481
	ODC	237	3.2	442
Non-hydrocarbon-contaminated sites	KG	201	3.0	297
	KY	141	2.6	279
	KDC	123	2.7	172

precipitation solution solution to the lysing solution and two additional SEW-S washes of the bound DNA). Community structure and taxonomic diversity were examined using Terminal Restriction Fragment Length Polymorphism (TRFLP) of PCR-amplified 16S rRNA gene fragments. The genomic DNA was initially amplified with universal bacterial primers 27F (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1492R (5′-TACCTT GTTACGACTT-3′) using conditions as above. The resultant amplicons were digested with restriction enzymes PvuII and MscI (NEB Canada) to minimize amplification of chloroplasts and mitochondria (Shen and Fulthorpe, 2015). One microliter of the resultant digested product was used as template in PCR reaction (same conditions as above) using 16S rRNA fluorescein labeled primers, 27F-FAMand 1492R-HEX (LifeTechnologies, Canada). The generated amplicons were digested with restriction enzyme MspI and sent to the Agriculture and Food laboratory at the University of Guelph for fragment analysis.

FAM labeled terminal fragments were used to determine phylotype densities and richness after fragments <60 bp in size were omitted in the analysis. The Microsoft Excel macro Treeflap (Rees et al., 2005), obtained from http://urbanstreams.net/index.php/the-treeflap-macro/, was used to round the fragment sizes to the nearest one base pair and to align the fragments of the same size from different samples. The height data was converted to % abundance based on total fluoresence and any fragments that represented <1% abundance were omitted.

16s rRNA Gene Sequencing Analysis

DNA amplification of the16s rRNA gene sequencing of triplicate pools of samples (6 samples) was performed at Molecular Research LP (Shallowwater, Texas, USA) on an illumina MiSeq following the manufacturer's guidelines. The 16s rRNA gene V4 hypervariable region was amplified using the PCR primers 515/806 with barcode on the forward primer. PCR reactions were prepared using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. 16s sequence data were trimmed, denoised, and chimera depleted with default parameters using Qiime pipeline v.1.8 (Caporaso et al., 2010). 16S rRNA taxonomy was assigned using RDP classifier trained using the greengenes input files provided by Qiime (DeSantis et al., 2006). Raw sequencing data can be retrieved from the Short Read Archive under the study accession- PRJNA475746.

Data Analyses

Analysis of Variance tests (ANOVAs) were used to compare total culturable heterotrophic bacteria and phylotype richness of each plant species, thereafter, a *post-hoc* test for was done using bonferroni correction. All the above-mentioned data analyses and graphs were made using Microsoft excel. A non-metric multidimensional scaling (NMDS) based on Bray Curtis

dissimilarities was used for the ordination of TRFLP dataset. Permutational ANOVA was then carried out using the function Adonis in the vegan package (Oksanen et al., 2012) for R studio (RStudio Team, 2015).

RESULTS

Endophytic bacterial communities were isolated from the stem endosphere of *Daucus carota* (wild carrot), *Achillea millefolium* (yarrow) and *Solidago canadensis* (goldenrod). Community characterizations were done using culture- dependent and independent means. Phenotypic characterization assays were done on the culturable fraction of endophytic bacterial strains.

Community Characterizations

Culturable Communities

An average of ∼2000 CFU/g of fresh stem tissue were recovered from the plants. There were no significant differences of culturable endophytes abundance and TRFLP phylotype richness (**Table 1**). **Figure 1** illustrates the culturable endophytic bacterial communities isolated from three plant species in hydrocarbon (HC) and non-HC contaminated sites. Gammaproteobacteria were predominant in all the plants and at both sites. Additional phyla were recovered from the plants growing in HC contaminated site. An additional phylum (Actinobacteria) was recovered from wild carrot at the contaminated site. The phyla Alphaproteobacteria and Firmicutes, absent in yarrow from non-HC contaminated site, were retrieved from yarrow in HC contaminated site. Gammaproteobacteria and Alphaproteobacteria were recovered from goldenrods in HC contaminated site while Gammaproteobacteria and Betaproteobacteria were recovered in non-hydrocarbon contaminated site. Similar predominant taxa were recovered in each plant species regardless of contamination. *Pantoea* and *Pseudomonas* spp. dominated the culturable flora of the wild carrot plants from both sites. *Stenotrophomonas*

FIGURE 4 | Principal coordinate analyses of axes 1 and 2 using weighted unifrac distances.

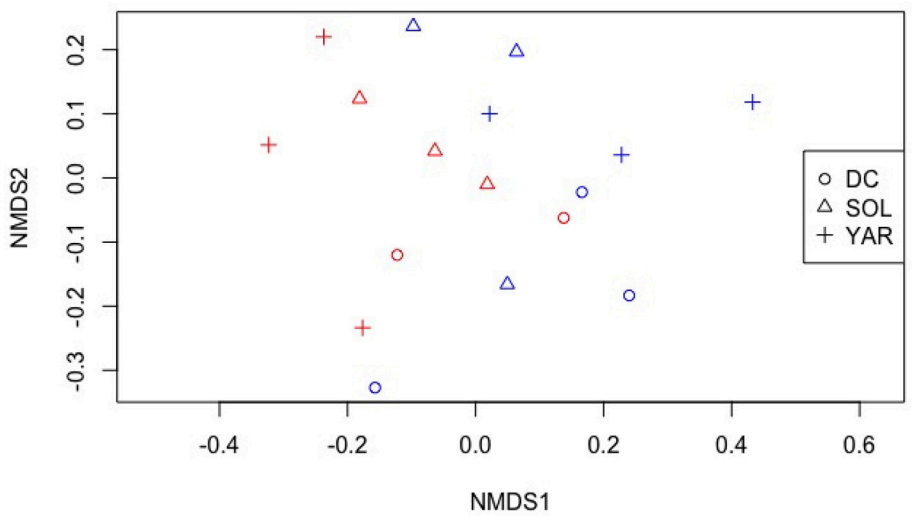

FIGURE 5 | Non-metric multidimensional scaling of TRFLP profiles of bacterial endophytic communities from different plant species. Stress: 0.14. DC- Daucus carota (Wild carrot) SOL- Solidago canadensis (Goldenrod) YAR- Achillea millefolium (Yarrow). Red color- contaminated site Blue color- non-contaminated site.

spp. were recovered in yarrow plants from both sites, whereas, both *Pseudomonas* and *Stenotrophomonas* were recovered from goldenrods at both sites. *Pantoea* spp., having been retrieved from all the plants regardless of contamination seemed to be ubiquitous. Phylogenetic structure of the 16s rRNA revealed grouping of cultivable bacterial endophytes regardless of plants host species and contamination (**Figure 2**).

Culture-Independent Communities

Figure 3 illustrates 16s rRNA amplicon sequencing showed Gammaproteobacteria to dominate all the samples in all the plant species and at both sites. *Pantoea* predominated all the plants at both sites except in yarrow plants at HC contaminated site where they were predominated by *Ralstonia* and *Xanthomonas*. At non-HC site,*Daucus carota* were also predominated by *Pseudomonas*, while *Rhizobium* predominated the goldenrods. Alpha diversity indices are presented on **Table 2** with non-HC samples showing, on average, a generally lower indices. Principal coordinate analyses of weighted unifrac distances as an indication of beta diversity revealed that irrespective of petroleum hydrocarbon contamination of the site, the bacterial communities in the stem endosphere of *Daucus carota* plants were different from the rest of the plant samples (**Figure 4**).

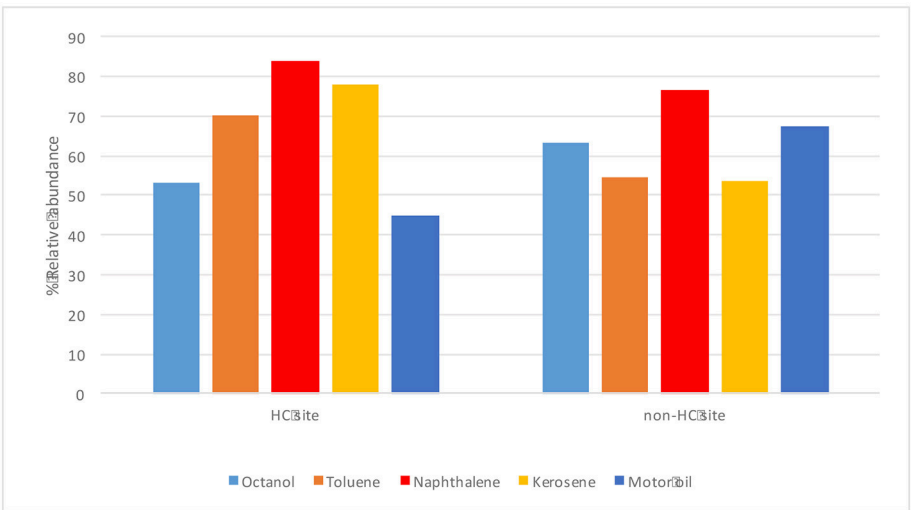

FIGURE 6 | Hydrocarbon degrading potential (through colorimetric mineralization assay) of different petroleum hydrocarbon substrates by endophytic bacterial isolates from hydrocarbon contaminated and non- hydrocarbon contaminated sites.

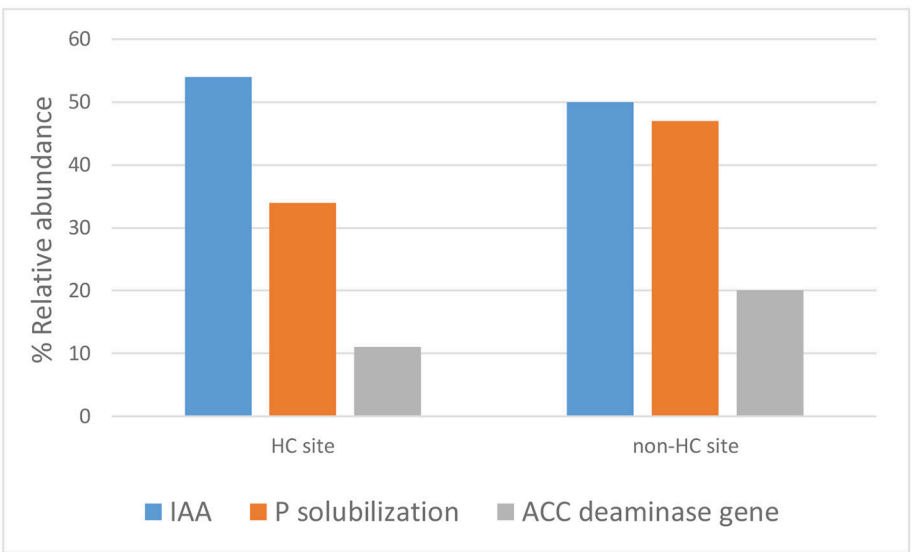

FIGURE 7 | Plant growth promoting and stress resistance potential of bacterial endophytes isolated from hydrocarbon contaminated and non- hydrocarbon contaminated site.

TABLE 3 | Plant growth promoting and hydrocarbon degrading potentials of bacterial endophytes isolated from hydrocarbon and non-hydrocarbon contaminated sites.

Site	Plant	Closest type strain				Hydrocarbon degrading genes		GC FID mineralization activity	
			P solubilization	IAA	ACC deaminase gene	*alkB*	Cat1,2	Toluene	Napthalene
Hydrocarbon contaminated site	*Achillea millefolium*	*Agrobacterium tumefaciens*	−	−	−	−	−	−	4.6
		Bacillus spp.	±	±	−	−	−	±, 9.5	−
		Curtobacterium spp.	−	+	−	−	−	−	−
		Microbacterium oxydans	+	−	−	−	−	−	−
		Paenibacillus illinoisensis	−	+	−	−	−	na	na
		Paenibacillus sp	++	−	−	−	−	na	na
		Plantibacter flavus	++	+	−	−	−	−	−
		Rhizobium sp	−	−	+	−	−	−	−
		Stenotrophomonas chelatiphaga	−	−	−	+	−	−	−
		Stenotrophomonas sp.	−	−	−	+	−	12.7	−
		Xanthomonas campestris	−	−	+	−	−	−	−
		StrainJ13–19	−	−	−	−	−	86.2	−
		StrainJ13–31	−	+	−	−	−	22	−
	Daucus carota	*Microbacterium testaceum*	+	+	−	−	−	−	−
		Paenibacillus amylolyticus	−	±	−	−	−	±, 9.2	−
		Pantoea agglomerans	+	+	±	−	−	−	−
		Labedella gwakjiensis	na	±	−	−	−	−	−
	Solidago canadensis	*Pantoea agglomerans* spp.	+	±	−	−	−	±, 3.8	−
		Pseudomonas fulva	−	+	−	−	−	−	−
		Pseudomonas st J13–60	−	+	+	−	+	9.2	−
		Stenotrophomonas spp.	−	+	±	−	−	−	−
		StrainJ13–38	+++	+	+	−	−	−	−
Non–hydrocarbon contaminated site	*Achillea millefolium*	*Erwinia*	+++	+	+	−	−	−	−
		Microbacterium hydrocarbonoxydans	−	−	−	−	−	4.3	−
		Microbacterium hydrocarbonoxydans J13–84	−	+	−	−	−	−	−
		Pantoea agglomerans J13–78	+	−	+	−	−	−	−
		Pantoea agglomerans J13–79	+	+	+	−	−	69.6	48.2
		Pantoea agglomerans J13–83	++	+	−	−	−	−	−
		Plantibacter cousiniae	−	+	−	−	−	−	−
		Sanguibacter inulinus	−	−	−	−	−	24.4	20.4
		Stenotrophomonas rhizophila	−	−	−	−	−	19.0	16.7
	Daucus carota	*Pantoea agglomerans* J13–52	+	−	−	−	−	−	−
		Pantoea agglomerans J13–66	+	+	−	−	−	19.4	9.8
		Pseudomonas putida str 129	na	na	+	+	+	−	−
		Pseudomonas str J13–114	+	+++	−	−	+	−	−
		Serratia ficaria	+	−	−	−	−	−	−
		Serratia plymuthica	+	+	−	−	−	−	−
		Strain J13–74	+	+++	+	−	−	−	−
		Strain J13–76	+++	−	−	−	−	22.7	17.5
	Solidago canadensis	*Achromobacter* sp.	+	+					
		Pseudomonas putida J13–89	+	+++	+	−	+	−	−
		Pseudomonas str J13–109	+	−	+	−	−	−	−
		Stenotrophomonas maltophilia	−	±	−	−	−	−	−
		Stenotrophomonas rhizophila	−	±	−	−	−	−	−
		Stenotrophomonas str J13–108	−		+	−	−	−	−

na, not assayed; -, not detected or no significant loss under GC-FID mineralization acitivity. ±, indicates that some isolates being tested were negative. +, means positive for the assay; ++, strongly positive; +++, very strongly positive.

TABLE 4 | % similarity of 16S rRNA of representative OTUs (repseq) and culturable isolates.

Samples	Taxa	OTU representative sequence	% relative abundance		16s rRNA isolate strain	% similarity (16S rRNA)
			16s rRNA amplicons (OTUs)	16s rRNA isolate		
ODC	*Pantoea*		68.8	60		
		Repseq197			O_J13-9	99.2
	Pseudomonas		5.3	11		
		Repseq493			O_J13-114	100
	Xanthomonas		16			
	Stenotrophomonas		1			
	Rhizobium		5			
OY	*Pantoea*		7.2			
	Pseudomonas		4.1			
	Xanthomonas		29	10		
		Repseq592			O_J13-22	100
	Stenotrophomonas		7	53		
		Repseq517			O_J13-28	96.2
					O_J13-20	95.1
	Rhizobium		7			
		Repseq515			O_J13-35	98.9
	Curtobacterium		11.3	3		
		Repseq83			O_J13-29	96.6
					O_J13-21	99.6
	Paenibacillus		3.5	8		
		Repseq110			O_J13-18	100
OG	*Pantoea*		96.2	92		
		Repseq197			O_J13-17	99.6
					O_J13-59	99.6
					O_J13-3	99.6
					O_J13-1	99.6
					O_J13-37	99.6
					O_J13-5	99.6
	Pseudomonas		1.1	3		
		Repseq493			O_J13-10	97.7
					O_J13-60_	97.7
	*Rhizobium**	Repseq515	-	1	O_J13-15	98.9
					O_J13-61	98.9
KDC	*Pantoea*		45.0	32		
	Pseudomonas		53.5	9		
		Repseq493			K_J13-129	95.9
	Xanthomonas		1	-		
	*Serratia**	repseq616	-	48	K_J13-53	96.2
					K_J13-131	95.5
KY	*Pantoea*		63.2	38		
		Repseq197			K_J13-79	99.2
					K_J13-83	99.6
	Pseudomonas		7.4	-		
	Xanthomonas		1	-		
	Stenotrophomonas		1	19		
		Repseq517			K_J13-50	95.1
					K_J13-105	95.1

(Continued)

TABLE 4 | Continued

Samples	Taxa	OTU representative sequence	% relative abundance		16s rRNA isolate strain	% similarity (16S rRNA)
			16s rRNA amplicons (OTUs)	16s rRNA isolate		
	*Serratia**	repseq616	9.0	-		
KG	*Pantoea*		35.3	-		
	Pseudomonas		10.2	55		
		Repseq493			K_J13-89	97.7
					K_J13-109	98.3
	Xanthomonas		5	-		
	Stenotrophomonas		7	40		
		Repseq517			K_J13-107	95.1
					K_J13-108	95.1
					K_J13-8	94.7
					K_J13-88	94.7
					K_J13-48	95.1
					K_J13-111	95.1
	Rhizobium		31	-		
	*Serratia**	repseq616	5.4	-		

% relative abundances of OTUs and isolates per taxa in each sample were also shown.
Serratia isolates absent in KG and KY, but present in KDC, still showing >95 % similarity.*
Rhizobium isolates absent in samples with OTU abundance, still showing >95 % similarity when compared with isolates from other samples.*

We examined the endophytic community assemblages using nonmetric multidimensional scaling of TRFLP fragments as shown in **Figure 5**. Though there was no visually distinct grouping of plant species and location, permutational multivariate analysis of variance revealed significant differences of endophytic bacterial phylotypes of differing plant species ($P < 0.05$, F model $= 1.78$, $R^2 = 0.45$). There was also a significant interaction effects of plant species and location ($P < 0.05$, F model $= 2.21$, $R^2 = 0.12$).

Functional Characterizations

A total of 99 isolates, of which 54 isolates (*Solidago*- 16 *Daucus*- 14 *Achillea*- 24 and 45 (*Solidago*- 16 *Daucus*- 15 *Achillea*- 14) from HC contaminated and non-HC contaminated sites, respectively, were assayed for their hydrocarbon degradation capabilities, production of indole acetic acid, P solubilization, presence of hydrocarbon degrading and ACC deaminase genes.

The endophytes isolated from non-HC contaminated site did not show marked difference from those isolated from HC contaminated site in their hydrocarbon degradation ability on various petroleum hydrocarbon substrates (**Figure 6**). Catechol 2,3-dioxygenase and P450 genes were not detected in any of the isolates. **Figure 7** shows plant growth promoting and stress resistance capabilities of the bacterial endophytes. Results show that 54% and 50% of isolates from HC and non-HC contaminated sites, respectively, were able to produce indole acetic acid in the presence of tryptophan. The endophytes were also able to solubilize inorganic phosphate into soluble form. Results revealed higher number of percent relative abundance of P solubilization ability in non-HC site at 47% compared to 34% from HC site. The ACC deaminase production potential of the isolates was evaluated through presence of *acdS* gene, 11% of the isolates tested from HC contaminated site possessed acc deaminase genes, while 20% from non-HC contaminated site. **Table 3** shows the results of the functional characterization of bacterial endophytic strains grouped according to plant species and location. We also showed mineralization activity of bacterial endophytes on toluene and naphthalene using GC-FID method; it is interesting to note that there was no difference in mineralization pattern between HC contaminated and non-HC contaminated sites.

DISCUSSION

To the best of our knowledge, this is the first report that looked at community composition, structure and function of BEs in pioneer plants growing in both chronically contaminated with high levels of petroleum hydrocarbon and non-hydrocarbon contaminated sites. Moreover, this study looked at both the culture- dependent and independent endophytic bacteria in the stem endosphere, a largely understudied ecological niche in plant-bacterial system. Since root endophytes are highly derived from the rhizosphere, we focused on endophytes present in stems tissue as we are most interested in those that are selected by the plant and not the soil ecosystem. The plants are not only exposed to both soluble HC from the soil, but also to volatile HC from atmosphere at the site. The study of the stem microflora was assumed to not only be reflective on both these selection

pressures from HC contamination but also of adaptation to the plant interior.

Our results show significant differences in community composition, through TRFLP fragments, across plant species and plant host-contamination interaction. This finding was in agreement with the previous studies where bacterial community structure differs with presence of contaminants and differing hydrocarbon levels at sites with simulated contamination (Phillips et al., 2009; Afzal et al., 2011; Kukla et al., 2014). PCoA of 16s amplicon sequencing showed separation of *D. carota* stem endophytes from the rest of the samples. It is interesting to note that *D. carota* plant belongs to family Apiaceae, while *A. millefolium,* and *S. canadensis* belong to family Asteraceae. Although inconclusive, it seems that plant host families influence bacterial diversity of the stem endosphere.

For both culture-dependent and independent community composition, we found that Gammaproteobacteria, particularly those genera from family Enterobacteriacea dominated the plant endospheric communities regardless of plant host species and contamination. Genera from family Enterobacteriaceae was also found to predominate the endosphere rather than the rhizosphere of plants in Athabasca oilsands reclamation sites (Mitter et al., 2017). Gammaproteobacteria was also reported to be predominant in the endosphere of ginseng plants (Khan Chowdhury et al., 2017). Except for *Achillea millefolium* in contaminated site, which was dominated by *Xanthomonas* and *Ralstonia*, *Pantoea* were abundant in all the plants. The *Pantoea* sp. in this study were closely related to *P. agglomerans* and *P. vagans*. *P. vagans*, which was formerly reported as *P. agglomerans* and *Erwinia herbicola*, is a common plant epiphyte and has been reported to control fire blight caused by the related enterobacterium *Erwinia amylovora* (Smits et al., 2010). *P. agglomerans* was reported as a potential plant growth promoting endophytic diazotroph for deep water rice (Verma et al., 2001) and many other plants. In this present study, *Pantoea* spp. demonstrated production of IAA and phosphorus solubilization.

It was interesting to discover a >50% overlap (genus level) of culture-independent and cultivable endophytic bacterial community structure. As shown in **Figures 1**, **3**, predominating culture-dependent bacterial endophytes were also predominant using culture-independent techniques, revealing high cultivability of these BEs. **Table 4** shows % similarity of 16S rRNA sequences from representative OTUs of the predominating taxa and isolates from each sample. This result corroborated the previous reports of Chelius and Triplett (2001) where there was a 48% overlap of culture-independent and cultivable bacterial communities in the maize roots.

Phosphate is an essential plant nutrient with low bioavailability in soil which is unavailable to the plants. It is well known that improved nutrient uptake of plants is mediated by plant-associated microorganisms. In this study case, the majority of the P solubilizing bacteria were *Pantoea* species.

Indole-3-acetic acid (IAA)—a plant growth hormone, is synthesized by a large number of plant associated bacteria (Long et al., 2008; Merzaeva and Shirokikh, 2010; Khan et al., 2012). In this study, *Pantoea* spp., *Pseudomonas* spp. and *Stenotrophomonas* showed evidence of IAA production. Other bacteria are well known for their production of ACC deaminase. This enzyme is highly influential in the plant environment because it hinders the production of ethylene. Under stressful conditions, production of ethylene is induced; this induction then inhibits plant growth. BEs are known to hinder ethylene biosynthesis through the expression of the enzyme ACC (1-aminocyclopropane-1-carboxylate) deaminase encoded by the *acds* gene that converts the ethylene precursor ACC to α-ketobutyrate and ammonia (Glick, 2005, 2015; Sun et al., 2009; Glick and Stearns, 2011). In this study, a few isolates were found to be putative ACC deaminase producers. These were from species of *Rhizobium*, *Xanthomonas*, *Pantoea*, *Pseudomonas,* and *Stenotrophomonas* from HC contaminated site; and species of *Erwinia*, *Pseudomonas* and *Pantoea* from non-HC contaminated site.

We expected to find functional differences between the bacterial endophytes isolated from HC and non-HC contaminated sites. However, there was no marked difference on functional capabilities of stem bacterial endophytes isolated from either contaminated or non-contaminated site. Particularly unexpected was virtual absence of known alkane hydroxylase and catechol 2,3-dioxygenase genes in the strain collection. Genomic analyses of *Microbacterium foliorum* 122 and *Plantibacter flavus* 251, bacterial endophytes that are common among the plants in this study, revealed that there were no known genes for classical toluene and naphthalene metabolism despite the fact that both these strains demonstrated utilization of toluene and naphthalene substrates (Lumactud et al., 2017a,b). Further research work is needed to delve deeper into the metabolic characteristics of these endophytes as new hydrocarbon degrading enzymes may possibly be involved.

In summary we found very few differences in adaptive traits in the endophytes of Oil Springs and the control site Komoka. The stem as a habitat may have protected the bacterial endophytes from the stress that is occurring outside the plants, which is likely one of the reasons why there was no marked difference of functional capabilities between contaminated and non-contaminated sites. The selective pressure for the endophytes is more determined by the plant interior than by the exterior contaminants.

AUTHOR CONTRIBUTION

RL conceptualized the project, performed field and laboratory work, analyzed data, wrote the manuscript with supervision and editing by RF.

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Ectomycorrhizal Fungal Communities and Their Functional Traits Mediate Plant–Soil Interactions in Trace Element Contaminated Soils

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There is an increasing consensus that microbial communities have an important role in mediating ecosystem processes. Trait-based ecology predicts that the impact of the microbial communities on ecosystem functions will be mediated by the expression of their traits at community level. The link between the response of microbial community traits to environmental conditions and its effect on plant functioning is a gap in most current microbial ecology studies. In this study, we analyzed functional traits of ectomycorrhizal fungal species in order to understand the importance of their community assembly for the soil–plant relationships in holm oak trees (*Quercus ilex* subsp. *ballota*) growing in a gradient of exposure to anthropogenic trace element (TE) contamination after a metalliferous tailings spill. Particularly, we addressed how the ectomycorrhizal composition and morphological traits at community level mediate plant response to TE contamination and its capacity for phytoremediation. Ectomycorrhizal fungal taxonomy and functional diversity explained a high proportion of variance of tree functional traits, both in roots and leaves. Trees where ectomycorrhizal fungal communities were dominated by the abundant taxa *Hebeloma cavipes* and *Thelephora terrestris* showed a conservative root economics spectrum, while trees colonized by rare taxa presented a resource acquisition strategy. Conservative roots presented ectomycorrhizal functional traits characterized by high rhizomorphs formation and low melanization which may be driven by resource limitation. Soil-to-root transfer of TEs was explained substantially by the ectomycorrhizal fungal species composition, with the highest transfer found in trees whose roots were colonized by *Hebeloma cavipes*. Leaf phosphorus was related to ectomycorrhizal species composition, specifically higher leaf phosphorus was related to the root colonization by *Thelephora terrestris*. These findings support that ectomycorrhizal fungal community composition and their functional traits mediate plant performance in metal-contaminated soils, and have a high influence on plant capacity for phytoremediation of contaminants. The study also corroborates the

overall effects of ectomycorrhizal fungi on ecosystem functioning through their mediation over the plant economics spectrum.

Keywords: ecosystem processes, heavy metal, microbiome, phytoremediation, *Quercus ilex* subsp. *ballota* (holm oak), root economics spectrum, symbiosis, trace element transfer

INTRODUCTION

There is an increasing consensus that microbial communities have an important role in mediating ecosystem processes. In recent years, and thanks to the development of molecular approaches, several studies have focused on the interaction between plants and soil microbial communities to reveal the potential of microbes to drive vegetation diversity and dynamics (Bever, 2003; Wardle et al., 2004; van der Heijden et al., 2015; Erktan et al., 2018; Rutten and Gómez-Aparicio, 2018). As vegetation determines how ecosystems function to a large extent, plant microbiomes indirectly affect the provision of multiple ecosystem services (Friesen et al., 2011; Van der Putten et al., 2013). In addition, some studies have highlighted the existence of feedback processes between plants and soil organisms (Bever et al., 2010; Brinkman et al., 2010), suggesting not only the potential of microbes to modify plant communities but also the role of plant communities and their traits at structuring microbial community compositions (de Vries et al., 2012; Aponte et al., 2013; Bauman et al., 2016; López-García et al., 2017).

Although the effect of plant hosts on their microbiomes has often been studied from a taxonomic point of view (Aponte et al., 2010; de Vries et al., 2012; Kurm et al., 2018), little is known about how soil microbial functional traits are affecting the functioning of plant species. It is debatable whether the features of microbes associated to individual plants (species composition and trait distribution) can be actually defined as plant traits, as they are not heritable features, according to the definition of Garnier et al. (2016). Often, microbial traits in the root microbiome are referred as "biotic root traits" (Bardgett et al., 2014). Recently, some authors have considered the use of traits in the root microbiome as an extension of the plant species phenotype for explaining functional changes in plant communities along environmental gradients and it has been included in a multidimensional root trait framework (Navarro-Fernández et al., 2016; Weemstra et al., 2016).

The influence of the plant microbiome from a trait-based perspective usually requires assessment of individual species in communities (Díaz et al., 2007), and this has been proven to be very challenging when working with microbes (see Crowther et al., 2014). According to the current thinking on ecological assembly, recording traits at individual species level will allow to differentiate between response and effect traits (Zirbel et al., 2017). The links between the response of microbial community-level traits to environmental conditions and the effects of these microbial traits changes on plant functioning is an important knowledge gap to be filled in current microbial ecology studies, although the existence of these links have been predicted previously (see Koide et al., 2014).

Mycorrhizal fungi are recognized for their importance for plant foraging of soil resources (Tibbett and Sanders, 2002; van der Heijden et al., 2015; Köhler et al., 2018), particularly in plant species with relatively thick absorptive roots (Eissenstat et al., 2015; Liu et al., 2015). Coevolution of plant and fungal partners has been recently suggested by Chen et al. (2018), based on their description of a root-fungal functional complementarity in nutrient foraging. However, how mycorrhizal and plant traits are interrelated, for example aligned into the common root economics spectrum framework, and how mycorrhizal traits mediates soil–plant relationships are still open questions that need to be addressed (Weemstra et al., 2016).

This mycorrhiza–root association improves plant health by enhancing resistance to diverse stresses like drought, salinity, heavy metals and pathogens, among others (van der Heijden et al., 2015). Therefore, mycorrhizal mediation on plant performance might be especially important in highly stressful environments, such as trace element (TE) contaminated soils. In these soils, mycorrhizal fungi enhance plant nutrition, stress tolerance and soil structure and, consequently, promote the recovery of the functions in the degraded soil (van der Heijden and Scheublin, 2007; Firmin et al., 2015). Association with mycorrhizal fungi can also play an important role in the transfer of TEs through the soil–root continuum, an issue of special relevance for the management of TE contaminated sites. For instance, the phytostabilization approach is a phytoremediation technology that combines the use of soil amendments and plants to immobilize pollutants into the soil, thus reducing the risks of transfer of these pollutants through the aboveground food web (Mendez and Maier, 2008). A prerequisite to apply this approach to large contaminated areas is that the plants used to remediate the soil can retain TEs at the rhizosphere level, and do not accumulate them into their aboveground biomass (Bolan et al., 2011; Madejón et al., 2018a). In relation to this, ectomycorrhizal (ECM) fungi may provide protection against metal toxicity through avoidance (i.e., extracellular precipitation, biosorption to cell walls, reduced uptake) and sequestration (i.e., intracellular chelation, compartmentation into fungi vacuoles) (Hartley et al., 1997; Jentschke and Godbold, 2000; Bellion et al., 2006). Therefore, phytoremediation of TE polluted soils can be facilitated by ECM fungi as they adapt to TE stress promoting the host growth (Wen et al., 2017).

In this study, we aimed to elucidate the role of ECM community in the plant nutritional status and the transfer of TEs through the soil-root-leaf continuum in a large-scale phytoremediation case study. Holm oaks (*Quercus ilex* subsp. *ballota*) root and leaf functional traits were analyzed in trees growing on remediated soils exhibiting a gradient of anthropogenic TE contamination. Relationships between

nutrient/TE concentrations in plants and the structure on the ECM communities were evaluated. Ectomycorrhizal community composition and morphological traits along the same gradient of soil contamination were previously reported by López-García et al. (2018). Here, relationships between ECM, soil, root, and leaf variables were explored in order to understand the importance of the ECM community assembly in the soil–plant relationships in holm oak trees.

We hypothesized that (i) plant traits (i.e., morphological and chemical) of holm oak would change along TE gradient; (ii) ECM fungal communities, would partly mediate plant response to TEs, and thus a significant fraction of the plant nutrient status and transfer of TEs from soils to leaves will be explained by ECM variables (either species composition or functional traits) (iii) ECM fungal communities lead the intraspecific variation of root functional traits.

MATERIALS AND METHODS

Study Area

The study was conducted at the Guadiamar Green Corridor (SW Spain), an area affected by a large mining accident in 1998 (the Aznalcóllar mine spill; Madejón et al., 2018b). The failure of a large tailings storage facility was one of the largest mining accidents in Europe to the date, which provoked the release of ca. 6 hm^3 of metalliferous tailings (water and sludge) over 55 km^2 of the Guadiamar River basin. As a result, soils were severely polluted with several TEs, mainly As, Cd, Cu, Pb, Tl, and Zn (Cabrera et al., 1999). After the accident, a large scale soil remediation program was conducted, which included the removal of the deposited sludge and the soil surface using heavy machinery, followed by the application of organic matter and calcium-rich amendments to immobilize TEs into the soil. The affected lands, mostly under agricultural production until the mining accident, were purchased by the Regional Administration, and then afforested using native tree and shrub species (Domínguez et al., 2008). Despite these remediation operations, contamination levels were highly variable across short distances in the Corridor and some patches are still highly degraded, due to acid drainage of the remnants of the sludge that lead to soil acidification and to a high solubility of toxic TEs (Domínguez et al., 2016).

The climate of the study area is typically Mediterranean, with mild rainy winters and warm dry summers. Average annual temperature is 19°C (minimum monthly mean of 9°C in January, and maximum of 27°C in July) and annual average rainfall is 484 mm. The study area harbors soils with different geology adding additional variation to the patchily distributed levels of TEs. Typical bedrock types at the North of the Corridor are slate and schist, and derived soils are naturally acidic. In the South (further than 15 km away from the mine) geological substrate tends to be dominated by calcarenite and marls originating neutral to calcareous loam soils. Potential vegetation is dominated by sclerophyllous Mediterranean forests, in particular by ECM holm oak in the alluvial terraces.

Sampling Design

The study was conducted in April 2016, 16 years after the application of soil amendments and the plantation of the former agricultural lands with native trees and shrubs. Holm oak was the target species of the study, given that it was intensively used to afforest the alluvial terraces of the affected area. Four sites were selected along a gradient of soil pollution across the affected area. A site location map and a general description of these soils as well as their classification is provided in López-García et al. (2018). Site 1 and Site 3 were located at the North of the corridor, while Site 2 and site 4 were located at the South of the Corridor. At each site, 10 holm oak trees were randomly selected (N = 40 trees). All these trees were planted at the same time (Autumn 2000) and with similar seed provenance.

For each tree, roots (and their associated ECM fungi) were sampled by carefully tracing from the stems of the tree to the roots belowground in the four cardinal directions. Around 200 g of root material was collected from each direction, i.e., subsamples. Root samples were used to characterize the main root functional traits and the ECM community (see López-García et al., 2018 for ECM characterization). Soil samples (0–20 cm depth) were taken with an auger from the four directions under each tree canopy and were pooled to a total of 500 g to make a composite sample per tree. Likewise, fully expanded leaf samples were taken from the four cardinal directions of the tree canopies to obtain a composite sample of leaves for each tree.

Soil Chemical Analyses

Soil chemical analyses were conducted for the study reported in López-García et al. (2018). Soil samples were air-dried and sieved to <2 mm for chemical analysis. Soil pH, Ca, K, P, NH_4, NO_3, total C, total N and total TEs were measured, following the methodologies described in that paper. For this study, available concentrations of S and TEs were also analyzed. Sulfur and TEs were extracted from samples (<60 μm) with a 0.01 M $CaCl_2$ solution (Houba et al., 2000) and analyzed by inductively coupled plasma spectrophotometry (ICP-OES) using a Varian ICP 720-ES (simultaneous ICP-OES with axially viewed plasma).

Soil Enzyme Activities

The activity of three extracellular enzymes involved respectively in C, N, and P cycling [β-glucosidase (BGL), N-acetylglucosaminidase (NAG), and acid phosphatase (ACP)] were measured as indicators of microbial activity in the collected soils. These enzymes were analyzed colorimetrically by incubation with p-nitrophenyl-linked substrates at 37°C for 1 h, according to Tabatabai and Bremner (1969); Tabatabai (1982), Parham and Deng (2000), respective methods.

ECM Species Composition and Functional Traits

Molecular analysis of ECM in root samples, as well as quantification of ECM functional traits (abundance of rhizomorphs, emanating hyphae, and melanin content) were conducted by López-García et al. (2018). Briefly, a composite sample of 28 root fragments per tree was obtained by selecting

the seven longest root fragments in each of the four root subsamples collected from each tree. A random individual root tip per root fragment was photographed for posterior trait quantification (presence of emanating hyphae and rhizomorph and colorimetric estimation of melanization, see López-García et al., 2018, Appendix 1). Community weighted means (CWMs), i.e., the averaged value for these traits per tree, was calculated as the number of root tips exhibiting emanating hyphae or rhizomorphs divided by the total number of quantified root tips (Lepš et al., 2011). The color value was averaged between the 28 root tips of each tree for having an overall estimation of the ECM melanization of the community. The remaining material was used for the quantification of the percentage of root length colonized by ECM fungi, using the gridline intersect method (Brundrett et al., 1996; Navarro-Fernández et al., 2016). All these data was reported by López-García et al. (2018) and was included in the statistical analyses in order to evaluate the influence of ECM communities on holm oak status.

A small portion of each root tip was cut and immersed separately into 10 μl of Extraction Solution (Extract-N-Amp^{TM} Plant PCR Kit by Sigma-Aldrich) and the protocol of the manufacturer was followed to extract its DNA. PCR amplification was conducted using primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) following the procedure described in López-García et al. (2018), and Sanger sequenced. Sequences were blasted against the UNITE database (Kõljalg et al., 2013) and those found to correspond to ECM fungi were grouped by genera or family (see López-García et al., 2018 for details) and compared against the UNITE database (Kõljalg et al., 2013) for their taxonomic placement and Species Hypothesis determination. The number of root tips belonging to each root was used as abundance data.

Plant Functional Traits

Root and leaf functional traits were measured specifically for this study, following the protocol described in Pérez-Harguindeguy et al. (2013). Morphological root traits included specific root length (SRL), specific root area (SRA), and root dry matter content (RDMC) and were measured with WinRHIZO 2009 (Regent Instruments, Quebec, CA, United States). Specific leaf area (SLA) and leaf dry matter content (LDMC) were measured in a subsample of 10 leaves per tree: leaves were scanned and analyzed with Image-Pro 4.5 (Media Cybernetic, Rockville, MD, United States).

After sampling, we selected 10 separated leaves from each tree and washed them with deionized water to determine the Chlorophyll Content Index (CCI) with a SPAD-502 chlorophyll meter (Minolta Camera, Co. Ltd., Osaka, Japan) taking three measurements per leaf.

Subsamples of roots and leaves collected from each tree were used for chemical analysis. This root material can be considered as the symbiotic combination of plant and fungi tissues. These subsamples were washed with distilled water, dried at 70°C for at least 48 h, and ground. Total C and N were determined by using a Flash 2000 HT elemental analyzer (Thermo Scientific, Bremen, Germany). Trace elements (As, Cd, Cu, Fe, Mn, Ni, Pb, and Zn) and macronutrients (S, P, K, Ca, and Mg) were determined by ICP-OES after digestion of plant tissues by wet oxidation with concentrated HNO_3 in a Digiprep MS block digester (Domínguez et al., 2008).

Data Analysis

In order to explore the relationships among ECM and plant variables we conducted a preliminary selection of key variables to be included in subsequent multivariate and modeling analyses. As the aim of the work was to evaluate whether plant performance (nutrient status and TE accumulation) is mediated by ECM communities in these soils, the subset of variables used as predictor variables included soil background properties and TEs, ECM species composition and ECM traits. The subset of response variables included TEs transfer from soil to root and leaves, root traits and leaf traits (**Supplementary Figure 1**). Soil variables and the characterization of ECM community, published by López-García et al., 2018, were used for the analysis of the present study.

A preliminary analysis of variance (ANOVA) to compare differences in soil, root, and leaf variables among sampling sites was performed. We checked for normality and homoscedasticity of data, and when assumptions were not met data were log or square root transformed. When these assumptions were met a Tukey's Honest *post hoc* test followed. Otherwise, a non-parametric Kruskal–Wallis test and a Dunn's test corrected by Bonferroni *post hoc* were performed.

Due to the dataset complexity, and in order to remove correlations and to reduce collinearity between soil variables, a principal component analysis (PCA) was performed to select a non-collinear subset of soil TEs to be used as predictors of plant traits in subsequent statistical analysis. Original data was log-transformed for normalization. Most correlated TEs with the first two axes of each PCA were selected for subsequent analyses.

In order to reduce ECM fungal species composition into two dimensions, a principal coordinate analysis (PCoA) was performed with the operational taxonomic units (OTUs) matrix (Legendre and Gallagher, 2001). The first two PCoA axes were selected (**Supplementary Table 1**) (Pinheiro and Bates, 2000; Zuur, 2009).

To evaluate the influence of soil and ECM variables on plant nutritional status and its functional traits we applied both correlational analysis and linear mixed models. In order to understand the relationships between the response and predictor variables, we first performed Pearson's correlation tests, adjusted with Benjamini-Hochberg correction (Benjamini and Hochberg, 1995). Those soil and ECM variables showing a significant correlation with plant variables were considered as fixed effect factors in univariate linear mixed effect models, with sampling site as random factor. The significant variables from univariate models were included additively in multivariate models, however variance inflated factors (VIFs) were calculated and variables with VIF > 3 were removed to avoid collinear predictors (Zuur et al., 2010). Models were compared against a null model, assuming no influence of any of these predictors on plant variables. The best and most parsimonious predictive models were selected based on the Akaike information criterion corrected for small sample sizes (AICc; Burnham and Anderson, 2002). Selected models were

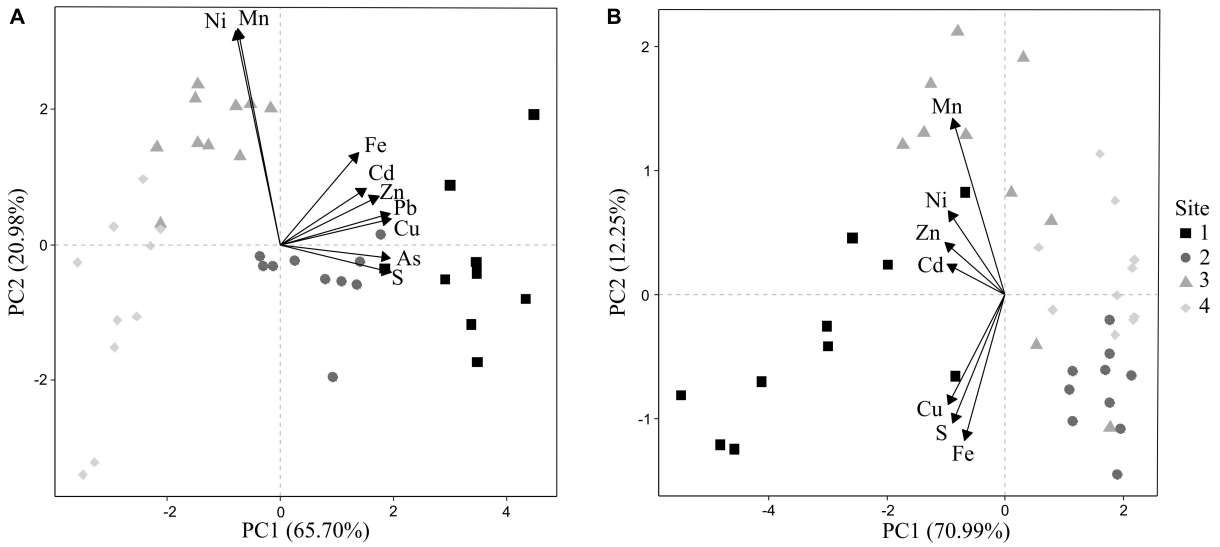

FIGURE 1 | Principal component analysis (PCA) ordination of **(A)** soil total trace elements (TEs) and **(B)** soil available TEs, sampled at 0-20 cm depth under holm oak trees (*N* = 40) and classification by site.

fitted, and marginal and conditional R^2 values were computed. Marginal R^2 (R^2_{LMMm}) is the variance explained by fixed factors, while conditional R^2 (R^2_{LMMc}) is variance explained by both fixed and random factors (Nakagawa and Schielzeth, 2013). Requirements for normality and homoscedasticity of residuals were fulfilled in all the selected models.

All statistical analyses were carried out using the R software v.3.3.2 (R Core Team, 2016), using packages *ggplot2* (Wickham, 2009), *MuMIn* (Barton, 2017), *nlme* (Pinheiro et al., 2016), *psych* (Revelle, 2017), and *vegan* (Oksanen et al., 2016).

RESULTS

Soil Characterization

As reported by López-García et al. (2018), soil pH was significantly different among sites; sites 2 and 4 showed a significantly higher pH than site 3 and, specially, than site 1 (**Supplementary Table 2**). Available TEs levels decreased from Site 1 to Site 4. About soil nutrients, sites 1 and 3 showed significantly higher NH_4, NO_3, total N and organic C than sites 1 and 4. Calcium concentration was significantly higher at site 2 with respect to the other sites. Phosphorus contents were not significantly different among sites (**Supplementary Table 2**). All soil enzyme activities presented the highest activity at site 3 and NAG and ACP activities were found significantly lower at site 2 (**Supplementary Table 2**).

Reduction of Trace Element and Community Composition Variables for Model Analysis

Soil total TEs PC1 and PC2 ordination axis explained most of the total variance (86.68%) in the chemical composition of soils (**Figure 1A**). Axis 1 and 2 represented the variation of two clear groups of TEs which were orthogonal to each other. Axis 1 correlated well with total As, Cd, Cu, Pb, S and Zn, which tended to covariate. Axis 2 showed a high covariance between Mn and Ni. Likewise, the first two axes of available TEs explained most of the total variance (83.24%) (**Figure 1B**), being Zn and Mn the most correlated with axes PC1 and PC2 respectively. The final selected TEs included in the subsequent analyses were: total As, Fe and Mn, and available Mn and Zn concentrations. Available Cd was not chosen because some of the samples were below the detection limits. Lower guideline values (LGVs) for contaminated soils (Ministry of the Environment Finland, 2007) were exceeded for As, Cu, and Pb at site 1 (**Supplementary Table 2**).

Fifty five OTUs were recorded belonging to ECM fungal species in 494 successfully sequenced root tips (published in López-García et al., 2018). In summary, these taxa comprised 14 families and 19 genera. The presence of rare species was common among the study: 19 of 55 OTUs were only identified in one root tip (**Supplementary Figure 2**). Two species, *Hebeloma cavipes* and *Thelephora terrestris* dominated the communities with 83 and 61 root tips, respectively (**Supplementary Figure 2**). The first two axes of PCoA of the ECM fungal communities explained a 25.08% of the variance in community composition. PCoA axis 1 (13.36%) showed a gradient from rare to abundant species (**Figure 2**). A clear pattern was also found in PCoA axis 2 (11.72% of explained variance) showing a transition of ECM fungal communities from *Thelephora terrestris* to *Hebeloma cavipes*.

Relationships Between Soil and ECM Variables and Root Traits

In general, nutritional root status was found to be more affected by biotic factors than by abiotic ones when univariate models

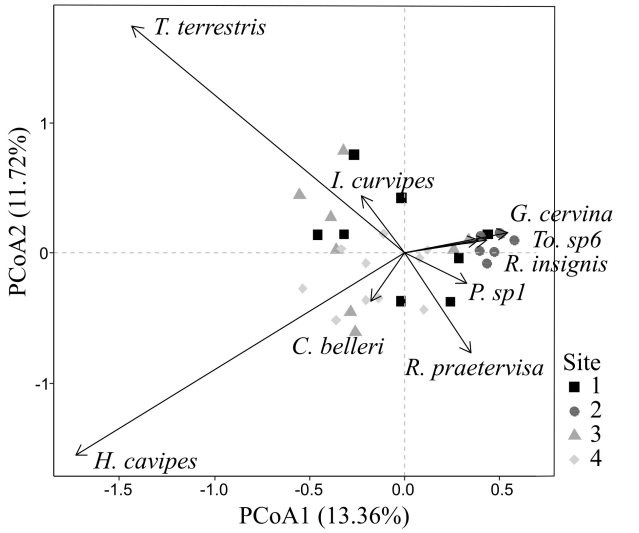

FIGURE 2 | Principal coordinate analysis ordination of ECM fungal species in symbioses with holm oak roots and classification by site. Arrows indicate signicant ECM species ($p < 0.05$).

were run. Root C was the variable that was best explained by the considered predictors (**Table 1**). Soil Ca and available Mn (Estimate = 0.10) were those variables explaining the greatest variation in root C (univariate models), followed closely by melanization (Estimate = −0.23) (**Table 1**). Species composition PCoA1 (Estimate = −1.13), PCoA2 (Estimate = −1.14), and rhizomorph formation (Estimate = 0.08) also presented an effect on root C but to a lesser extent. Soil Ca content presented a negative effect on C root concentration. ECM species composition (PCoA1 and PCoA2 scores) was also related to root C; a higher root C was observed where *Hebeloma cavipes* species was dominant. In terms of biotic CWM traits, a low melanin content and high rhizomorph presence were also affecting root C (univariate models).

Root N was best explained by single total Zn, which exerted a positive effect (**Table 1**). In terms of biotic effects, species composition PCoA1 had a marginally significance influence (Estimate = 0.05). Root C:N ratio corroborated the role of total Zn on root N, as Zn was found negatively correlated to C:N ratio. The best model for root C:N included species composition PCoA1, exerting a negative effect, which confirmed that the presence of the most abundant species, *Hebeloma cavipes* and *Thelephora terrestris*, negatively influenced root N content. Root P was not explained by any abiotic factor but was positively affected by ECM species composition PCoA1 (**Figure 3A** and **Table 1**). As well as root N, root P was found to be lower when *Hebeloma cavipes* and *Thelephora terrestris* species were abundant in roots, therefore the symbioses with other species, here considered as rare due to their lower abundance, probably improved the nutritional status of holm oak roots in terms of P. Root N:P ratio was not significantly explained by any abiotic or biotic factor.

Both morphological root traits, SRA and RDMC were better explained by species composition PCoA1 than by any abiotic

TABLE 1 | Univariate and multivariate linear mixed models showing significant soil and ECM fungi fixed effects for each of the root traits and model explained variance.

Response variable		Individual effects of soil factors				Individual effects of ECM fungal factors				Combining significant effects into the best predictive model					
		Nutrients and EA		Trace elements		PCoA axis 1 and 2		Fungal traits CWM		Linear mixed effect models				Variance	
Root	Variable	*p*	Variable	*p*	Variable	*p*	Variable	*p*	Model	*SE*	*t*	*p*	R^2_{LMMm}	R^2_{LMMc}
C (%)	Ca	<0.001	Av. Mn	<0.001	PCoA1	0.048	Melanization	0.003	C = 45.43 – 0.001 Ca	0.0003	−4.11	<0.001	0.31	0.31
					PCoA2	0.044	Rhizomorph	0.047						
N (%)	–	–	Total Zn	0.006	(PCoA1)	(0.053)	–	–	N = 0.25 + 0.0006 Total Zn	0.0002	2.90	0.006	0.19	0.19
C:N	–	–	Total Zn	0.010	PCoA1	0.012	–	–	C:N = 141.70 – 29.64 PCoA1	11.07	−2.68	0.012	0.22	0.40
P (%)	–	–	–	–	PCoA1	0.009	–	–	P = 0.09 + 0.02 PCoA1	0.007	2.80	0.009	0.21	0.22
SRA (m^2 kg^{-1})	–	–	–	–	PCoA1	0.045	–	–	SRA = 9.47 + 0.62 PCoA1	0.30	2.10	0.045	0.12	0.12
RDMC (mg g^{-1})	–	–	–	–	PCoA1	0.006	–	–	RDMC = 427.04 – 22.04 PCoA1	7.40	−2.98	0.006	0.22	0.22

SRA, specific root area; RDMC, root dry matter content; Av., available; CWM, community weighted mean; EA, enzyme activity; SE, standard error; R^2_{LMMm}*, marginal variance;* R^2_{LMMc}*, conditional variance.*

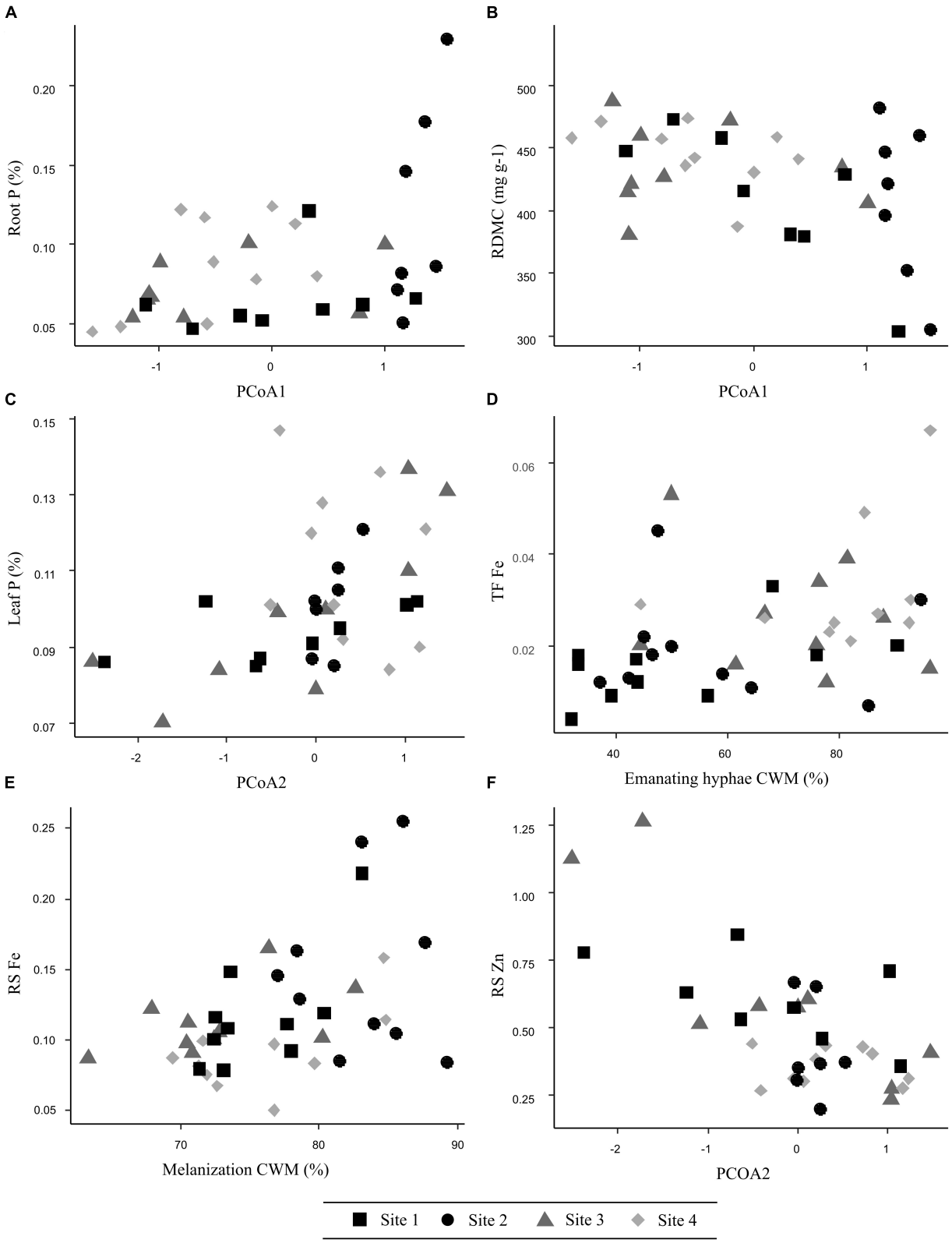

FIGURE 3 | Relationship between selected key ECM fungal species composition and traits and their effects on **(A)** root P, **(B)** root dry matter content, **(C)** leaf P, **(D)** translocation factor Fe, **(E)** soil-to-root Fe transfer, and **(F)** soil-to-root Zn transfer in studied sites.

factors, but their effects were opposite (**Table 1**). The presence of the most abundant species, *Hebeloma cavipes* and *Thelephora terrestris* reduced SRA but increased RDMC (**Figure 3B**), while SRL was not significantly explained by any of the measured soil or ECM fungal factors. In summary, a key effect of species composition was found for those variables related to root nutrition variables. The abundance of *Thelephora terrestris* and, in special, *Hebeloma cavipes* species seemed to be related to high C, C:N ratio and RDMC values, and low N, P and SRA values in holm oak roots.

Marginal and conditional R^2 for all the response variables, except C:N ratio, were similar. Variance explained by conditional R^2 for the C:N ratio response almost doubled the marginal R^2 (**Table 1**).

Relationships Between Soil and ECM Variables and Transfer of Trace Elements to Roots

Transfer of TEs from soil to root seemed to be mainly driven by biotic factors: species composition PCoA2 and melanization CWM (**Table 2**). The soil-to-root transfer of As (RS As) was related to species composition PCoA2 and ECM melanization (**Table 2**). A high abundance of *Hebeloma cavipes* species and high melanin content seemed to be associated to a high As transfer to roots. A negative relationship with soil As was also found (Estimate = −0.0004). The soil-to-root transfer of Fe (RS Fe) was positively explained by soil NO_3 and melanization (**Figure 3E** and **Table 2**). Species composition PCoA1 was also positively related (Estimate = 0.026), meaning that in those soils where rare species were abundant, Fe transfer to roots was higher. Rhizomorphs formation was negatively related (Estimate = −0.01). The soil-to-root transfer of Mn (RS Mn) was best explained by abiotic variables, namely soil Ca and soil Mn, which were negatively associated with this transfer (**Table 2**). Species composition PCoA2 showed an individual negative effect on Mn transfer (Estimate = −0.06), therefore the abundance of *Hebeloma cavipes* species in the soil was found to be positively related to Mn transfer from soils to roots. The soil-to-root transfer of Zn (RS Zn) was negatively affected by species composition PCoA2 (**Figure 3F** and **Table 2**). Therefore, as previously found for As, a higher abundance of *Hebeloma cavipes* species increased the soil-to-root transfer of Zn. In this case, the significant effect of melanization was negative (Estimate = −0.01), opposite to the As and Fe transfers. Soil Ca (Estimate = −0.00009) showed an individual negative effect on soil-to-root transfer of Zn.

Marginal and conditional R^2 showed similar percentage of variances for Fe, Mn, and Zn transfer but transfer of As was more explained by the site random effect (conditional R^2) than the biotic fixed effects (**Table 2**).

Relations of Soil Factors With Translocation of Trace Elements to Leaves

Translocation of TEs from roots to leaves were explained by different abiotic and biotic factors (**Table 2**), depending on the element. Translocation of As was not significantly explained by any individual abiotic or biotic factor. Due to the non-significant fixed effect of the model for the response variable translocation factor of As, a covariate Cu transfer was studied. Translocation factor of Cu was highly explained by soil Cu and species composition PCoA2 (R^2_{LMMm} = 0.55; R^2_{LMMc} = 0.76). Soil Cu contamination showed a significant negative effect on the Cu translocation ($p < 0.001$) while PCoA2 showed a significant positive effect ($p = 0.013$), therefore Cu translocation was favored on *Thelephora terrestris* dominated soils. Iron translocation from roots to leaves was only significantly explained by the biotic emanating hyphae, showing a positive relationship (**Figure 3D** and **Table 2**). Translocation factor of Mn was significantly related to NAG enzyme activity and species composition PCoA1, being this last variable the most explicative, showing a positive effect (Estimate = 8.54) (**Table 2**). Translocation factor of Zn was only significantly explained by soil Zn, however, Zn showed a negative effect on Zn transfer (**Table 2**).

High differences between marginal and conditional R^2 variance were found for all TE translocation factors, except for Fe (**Table 2**).

Relations of Soil Factors With Leaf Traits

Nutritional status of holm oak leaves were, in general, highly affected by soil P and ECM fungal species composition (PCoA1 and PCoA2 factors; **Table 3**). Leaf C was highly explained by a combination of abiotic and biotic factors (soil As and species composition PCoA1 factors, **Table 3**). Both predictor variables showed a strong positive relationship with leaf C. Emanating hyphae was also found to influence leaf C content, but negatively (Estimate = −0.09), when univariate relationships were analyzed. Leaf N was significantly influenced by soil P (**Table 3**) which explained a high proportion of variance of leaf N. No biotic factor was identified as significant for leaf N. Leaf C:N ratio was also highly explained by soil P but a negative effect was observed, in coherence with leaf N effects. A biotic effect was significantly found in relation to species composition PCoA1. The positive effect (Estimate = 2.04) of PCoA1 on this ratio showed consistency with model effects on leaf C. In summary, the results from these models showed a higher leaf C content and, therefore a higher C:N ratio, in those sites with particular abiotic characteristics (high As contamination and low soil P), and associated with certain biotic features: low abundance of *Hebeloma cavipes* and *Thelephora terrestris*, and low emanating hyphae.

Leaf P was best explained by species composition PCoA2 alone, which had a positive effect on this response variable (**Figure 3C** and **Table 3**). Soil P and available Mn had also a significant influence on leaf P, according to abiotic univariate models; soil P had a positive effect (Estimate = 0.0007) while Mn availability showed a negative effect (Estimate = −0.0005) on leaf P. Leaf N:P ratio was best predicted by species composition PCoA2 alone; the negative effect between PCoA2 and this the ratio corroborates the previous leaf P results. No abiotic variables were found to have a significant effect on leaf N:P ratio. To summarize, a higher leaf P and a lower N:P ratio were found

TABLE 2 | Univariate and multivariate linear mixed models showing significant soil and ECM fungi fixed effects for each of the soil-to-root (RS) transfer and translocation factor (TF) and model explained variance.

Response variable	Individual effects of soil factors				Individual effects of ECM fungal factors				Combining significant effects into the best predictive model					
	Nutrients and EA		Trace elements		PCoA axis 1 and 2		Fungal traits CWM		Linear mixed effect models				Variance	
Transfer	Variable	*p*	Variable	*p*	Variable	*p*	Variable	*p*	Model	*SE*	*t*	*p*	R^2_{LMMm}	R^2_{LMMc}
RS As	–	–	Total As	0.036	PCoA2	0.008	Melanization	0.050	RS As = −0.32 − 0.04 PCoA2	PCoA2 0.01	PCoA2 −3.54	PCoA2 0.001	0.27	0.63
									+ 0.006 Melanization	Mel 0.002	Mel 2.90	Mel 0.007		
RS Fe	NO_3	<0.001	–	–	PCoA1	0.002	Melanization	0.004	RS Fe = −0.12 + 0.009 NO_3	NO_3 0.002	NO_3 4.39	NO_3 < 0.001	0.48	0.51
							Rhizomorph	0.021	+ 0.003 Melanization	Mel 0.0009	Mel 2.99	Mel 0.005		
RS Mn	Ca	0.003	Total Mn	0.004	PCoA2	0.039	–	–	RS Mn = 0.61 − 0.00005 Ca −0.0003 Total Mn	Ca 0.00001 Total Mn 0.00009	Ca −4.09 Total Mn −3.83	Ca < 0.001 Total Mn < 0.001	0.44	0.44
RS Zn	Ca	<0.001	–	–	PCoA2	<0.001	Melanization	0.041	RS Zn = 0.50 − 0.16 PCoA2	0.03	−5.29	<0.001	0.46	0.51
TF Fe	–	–	–	–	–	–	Hyphae	0.026	TF Fe = 0.009 + 0.0002 Hyphae	0.0001	2.32	0.026	0.13	0.13
TF Mn	NAG	<0.001	–	–	PCoA1	0.004	–	–	TF Mn = 3.62 + 1.82 PCoA1	0.58	3.15	0.004	0.16	0.75
TF Zn	–	–	Total Zn	0.007	–	–	–	–	TF Zn = 0.98 − 0.002 Total Zn	0.0007	−2.88	0.007	0.22	0.61

NAG, N-acetyl-glucosaminidase; CWM, community weighted mean; EA, enzyme activity; SE, standard error; R^2_{LMMm}*, marginal variance;* R^2_{LMMc}*, conditional variance.*

TABLE 3 | Univariate and multivariate linear mixed models showing significant soil and ECM fungi fixed effects for each of the leaf traits and model explained variance.

Response variable	Individual effects of soil factors				Individual effects of ECM fungal factors				Combining significant effects into the best predictive model					
	Nutrients and EA		Trace elements		PCoA axis 1 and 2		Fungal traits CWM		Linear mixed effect models				Variance	
Leaf	Variable	*p*	Variable	*p*	Variable	*p*	Variable	*p*	Model	*SE*	*t*	*p*	R^2_{LMMm}	R^2_{LMMc}
C (%)	–	–	Total As	0.014	PCoA1	0.010	Hyphae	<0.001	C = 48.56 + 1.93 PCoA1 + 0.019 Total As	PCoA1 0.50 Total As 0.006	PCoA1 3.86 Total As 2.87	PCoA1 < 0.001 Total As 0.008	0.51	0.51
N (%)	P	<0.001	–	–	–	–	–	–	N = 1.20 + 0.001 P	0.002	4.45	<0.001	0.35	0.35
C:N	P	<0.001	–	–	PCoA1	0.048	–	–	C:N = 42.10 – 0.32 P	0.09	–3.47	<0.001	0.25	0.26
P (%)	P	0.035	Av. Mn	0.007	PCoA2	0.007	–	–	P = 0.10 + 0.009 PCoA2	0.003	2.91	0.007	0.21	0.21
N:P	–	–	–	–	PCoA2	0.039	–	–	N:P = 13.30 – 0.90 PCoA2	0.42	–2.16	0.039	0.13	0.13
SLA (m^2 kg^{-1})	NAG	0.002	–	–	–	–	–	–	SLA = 4.38 + 1.10 NAG	0.33	3.35	0.002	0.24	0.24
CCI (SPAD)	P	<0.001	–	–	–	–	–	–	CCI = 44.37 + 0.30 P	0.07	4.38	<0.001	0.30	0.49

SLA, specific leaf area; CCI, Chlorophyll Content Index; NAG, N-acetyl-glucosaminidase; Av., available; CWM, community weighted mean; EA, enzyme activity; SE, standard error; R^2_{LMMm}, marginal variance; R^2_{LMMc}, conditional variance.

in soils with high P, low Mn availability and dominance of *Thelephora terrestris* over *Hebeloma cavipes* species.

Morphological trait SLA was best related with soil NAG enzyme activity, while CCI was significantly related to soil P. For both SLA and CCI no mycorrhizal variables were significant predictors of their variance (**Table 3**). In addition, no significant variables were found to explain LDMC variation.

Marginal and conditional R^2 for all the leaf response variables were akin except for CCI which presented a higher conditional variance (**Table 3**).

DISCUSSION

In this study we aimed to quantify the influence of ECM fungal communities on certain plant morphological and chemical traits, and to assess whether they may influence host status. The scenario chosen for this purpose was a TE contaminated area in which the effect of the abiotic factors, including the TE contamination and the soil background variables, on the community composition and functional traits of ECM fungi had been already tested (López-García et al., 2018). Hence, since the abiotic environment was indeed shaping the ECM communities, any effect of the latter on plant traits must be interpreted as a mediated effect of the ECM fungi on soil–plant relationships. In general, we found that ECM community composition and traits explained more than the abiotic environment for most of the measured plant traits.

Root Functional Traits

Root systems are known to show a high plasticity in their development depending on soil local heterogeneity (Ostonen et al., 2007). In this study, we found several significant relationships between soil variables and root traits in holm oak trees with similar age and origin, which suggests high root plasticity in response to the studied environmental gradient. We further found that root functional traits were highly explained by the ECM community (in terms of both fungal species and traits), which corroborates the important mediation role of ECM on plant status and performance, and the need of incorporating symbiotic traits into the analysis of root traits (Weemstra et al., 2016).

In relation to the root economics spectrum, we could align the presence of abundant species of ECM (*Hebeloma cavipes* and *Thelephora terrestris*) with conservative positions into the root economics spectrum, i.e., exhibiting conservative traits such as a high C:N ratio and a low N and P content, and consequently a high C content, high RDMC and low SRL (de la Riva et al., 2016, 2018). The basidiomycete *Thelephora terrestris* is a common symbiotic ECM fungus (Marx et al., 1984; Menkis and Vasaitis, 2011) with beneficial effects for trees growing under stressful conditions, such as those that prevail in mine areas and reclamation sites (Lee and Koo, 1983), given that it protects the host by decreasing metal (Cu) transfer from soil to roots (Van Tichelen et al., 2001). Although *Hebeloma* spp. have been frequently found in heavy-metal contaminated soils (Colpaert et al., 2011) the abundant *Hebeloma cavipes*

taxa is associated in the study area with soils with a low level of TE contamination (López-García et al., 2018). In terms of ECM traits, a high rhizomorph formation and low melanin content characterized those ECM fungi (i.e., *Thelephora terrestris* and *Hebeloma cavipes*) that were colonizing roots showing the most conservative traits. The presence of rhizomorphs, which functionally increases water and phosphate uptake through a long-distance exploration mechanism (Agerer, 2001), may be a consequence of resource limitations, hence constituting a conservative trait. Although melanin plays a role in protecting the root cells against high concentrations of heavy metals in the soil (Gadd and de Rome, 1988) these ECM fungi may prevent toxicity with other mechanisms (Bellion et al., 2006).

In the opposite edge of the root economics spectrum, we found roots colonized mostly by rare species and showing more acquisitive features, i.e., a high N and P concentration, a low RDMC and high SRA (de la Riva et al., 2016, 2018). These root traits might be indicating less resource limitations, probably due to higher soil nutrient contents and thus less dependency on rhizomorphs for nutrient acquisition. The fact that these roots belong to trees growing in soils with a high level of TE contamination could explain the higher degree of melanization of these fungi, in order to avoid TE toxicity.

In this study, we might have anticipated that the adverse soil chemical conditions posed by the contamination episode could have modulated root acquisition strategies, with roots growing in the most contaminated soils showing a more conservative strategy. However, conservative root traits were related to low soil TE (Zn) concentrations. On one hand, it is possible that the *a priori* concern about TE contamination as the main factor of stress for plant performance is masked by other sources of stress, such as water or nutrient limitations. Recently, López-García et al. (2018) found that soil background properties and TEs concentrations explained the same proportion of variance in ECM species composition, which support this concept. On the other hand, although the root economics spectrum is associated to nutrient absorption and soil fertility, here we found that other factors such as ECM community composition and TE contamination could support the multidimensional root trait framework. Mycorrhizal fungi have a fundamental role in acquiring resources but also protecting plants from the negative impact of some sources of biotic and abiotic stress. Trace element contamination seems to be independent from root economics spectrum, which indicates the existence of a multidimensional framework that includes other processes different from those related to nutrient uptake (Weemstra et al., 2016).

Soil-Root-Leaf Transfer of Trace Elements

Trace element mobility through the soil-root-leaf continuum depends on several factors, and obviously initial concentrations in the soil is one of them (Kabata-Pendias, 2004). Despite that the range of soil TE concentrations in our environmental gradient was relatively large (for example, total As concentrations ranged from 6.83 to 286 mg kg^{-1}), accumulation of TEs in oak leaves was relatively low, and leaf TE concentrations were within the normal ranges (except Mn levels; over 400 mg kg^{-1}) and below the levels that can be toxic to plants (Madejón et al., 2002). This confirms that holm oak is a suitable species for the phytostabilization of contaminated soils, given its ability to prevent TE accumulation into aboveground biomass (**Supplementary Table 2**). Previous work under controlled greenhouse conditions showed that this species has a capacity to retain and tolerate high concentrations of some TEs (Cd) into fine roots (Domínguez et al., 2009). Mechanisms involved in TE retention into the root system include adsorption onto roots, or precipitation within the rhizosphere (Pulford and Watson, 2003; Wong, 2003). The pectin in the cell wall are the main constituents allowing metal binding due to their carboxyl groups, which have a high cation exchange capacity (Franco et al., 2002). In the present study, the soil-to-root transfer of As, Mn, and Zn was highly explained by ECM fungal species composition and traits, which suggests that interactions with fungi play an important role at determining the capacity of this species to retain TEs into its roots. The highest transfer of these TEs from soil to roots (plant and fungi tissue) was observed in trees whose roots were colonized by *Hebeloma cavipes*. In contrast, soil-to-root transfer of Fe presented a different trend, with the highest transfer being recorded in roots colonized by rare species taxa. This confirms that the mechanisms by which mycorrhizal fungi participate in metal uptake by plants can differ for each element and each fungal species (Godbold et al., 1998; Jentschke and Godbold, 2000).

Melanization was corroborated as a trait with a role in the protection of plants against heavy metals (Gadd and de Rome, 1988), as it was highly positively related to TE transfer to roots, although the relationship between melanin content and transfer of TEs from soils to roots differed across elements. Melanization was positively related to As and Mn but negatively related to Zn transfer. These opposite trends could indicate that roots are subjected to multiple constraints (Weemstra et al., 2016) in these multi-metal contaminated soils, and that different elements affect differently to these ECM traits.

Leaf Functional Traits

Resource availability directly impacts functional traits such as SLA and leaf N and P content (Friesen et al., 2011). It was expected that ECM fungal mediation would increase resource acquisition by plants by accessing to organic forms unavailable to plants and by more efficient foraging (Friesen et al., 2011).

Leaf C is captured via photosynthesis, therefore C uptake is not mediated by ECM fungi. But assimilation of C into plant tissue might be affected by a range of factors, such as nutritional status and water stress, in which ECM community may play certain role (Cornelissen et al., 2001), as explored here. A high positive relationship was found between ECM species composition (mainly, in relation to the presence of rare species) and leaf C and C:N ratio; that could be an indirect effect of ECM community composition through its effects on root functional traits. Leaf P and N:P ratio were related to ECM species composition as well, specifically high leaf P was related to the root colonization by *Thelephora terrestris*. This is in agreement with Van Tichelen et al. (2001), who showed that *Thelephora terrestris*

played a central role in the P nutrition of the host plant in a P-limited and Cu-contaminated soil.

Plants are performing a continuous carbon and nutrient investment in order to maintain the key leaf functions (i.e., photosynthesis) (Poorter and Bongers, 2006). Leaf N is responsible of the photosynthetic machinery, especially Rubisco, and leaf P is found in nucleic acids, lipid membranes and bioenergetic molecules (Wright et al., 2004), therefore both are key chemical traits. Leaf N correlated positively with CCI and this result agreed with that an optimal leaf N is essential for photosynthesis (de la Riva et al., 2016). Leaf N, P and CCI have shown a high positive relationship to soil P. A phosphorus limitation in soils has been previously registered in the study area (Domínguez et al., 2010) and it is known that leaf nutrient traits are more closely linked to soil P under limiting conditions (Niinemets and Kull, 2003; Liu et al., 2010; Chen et al., 2011).

The leaf traits SLA and CCI were not related to ECM fungal species composition or traits. These functional traits are related to light capturing functions (Niinemets and Sack, 2006) which here have been found not to be mediated by ECM, but affected by soil variables (i.e., NAG and P).

Feedback Effect of the Symbiosis ECM Fungi–Host Plant

Pollution by TEs may favored the dominance of tolerant ECM fungal species, altering the ECM fungal composition (Hui et al., 2011; Op De Beeck et al., 2015). Abundant *Thelephora* and *Hebeloma* taxa have been previously found in areas contaminated by different TEs such as Cd, Cu, Mn, Pb, or Zn (Hartley et al., 1997; Van Tichelen et al., 2001; Hui et al., 2011; Huang et al., 2014; De Oliveira and Tibbett, 2018). Therefore, there may be a selection of these ECM species which are able to tolerate TE contamination probably through extracellular and intracellular mechanisms (Jentschke and Godbold, 2000; Bellion et al., 2006; Ciadamidaro et al., 2017). These ECM species would protect the host plant by decreasing TE transfer and shaping plant functional traits (Van Tichelen et al., 2001; Bauman et al., 2016). Although this study has not studied how plant communities and their traits are also responsible of structuring ECM communities composition, previous studies (de Vries et al., 2012; Aponte et al., 2013; Bauman et al., 2016; López-García et al., 2017) have found the existence of feedback processes. Due to the ecological complexity of the soil–plant interaction system, further research is needed to understand the ECM fungi and host plant relevant traits, as well as genetic variation, which allow the establishment of the host plant in TE contaminated soils. Finally, a better understanding of the symbiosis would improve the planning and outcomes of phyto- and myco-remediation strategies (Ali et al., 2017; Ciadamidaro et al., 2017).

CONCLUSION

The analysis of root and leaf traits, as well as ECM communities and soil physico-chemical properties in a large-scale phytoremediated area, revealed that plant functions, expressed as variations in plant traits, can be affected in similar extents by the abiotic and the biotic environment that surround and interact with each individual plant. We could identify some ECM fungal community traits that were highly related to the studied plant variables (root traits, nutrient status, and TE accumulation), in a greater extent than the abiotic environment. In some cases, such as the transfer of As, Mn and Zn, the best explanatory variable was directly related to the composition of the ECM community, suggesting species-specific mechanisms of interactions between holm oak and ECM fungi. ECM traits co-varied with the root economics spectrum, as ECM rhizomorphs and melanization traits were related to the acquisitive-conservative root spectrum. Future studies on plant–soil interactions in contaminated soils should therefore consider that critical processes, such as nutrient assimilation and TE accumulation into biomass, can be largely mediated by ECM fungi.

AUTHOR CONTRIBUTIONS

MG-M, ÁL-G, CN-F, MD, and TM designed the study and conducted the sampling. MG-M, ÁL-G, CN-F, MD, and RK conducted the laboratory analyses. MG-M, ÁL-G, and MD conducted the data analyses. MG-M wrote the manuscript with contributions from all the authors.

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Mycorrhizae and Rhizobacteria on Precambrian Rocky Gold Mine Tailings: I. Mine-Adapted Symbionts Promote White Spruce Health and Growth

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White spruce [*Picea glauca* (Moench) Voss] is a commercially valuable boreal tree that has been known for its ability to colonize deglaciated rock tailings. Over the last decade, there has been an increasing interest in using this species for the revegetation and successful restoration of abandoned mine spoils. Herein, we conducted a glasshouse experiment to screen mycorrhizal fungi and rhizobacteria capable of improving the health and growth of white spruce seedlings growing directly on waste rocks (WRs) or fine tailings (FTs) from the Sigma-Lamaque gold mine located in the Canadian Abitibi region. After 32 weeks, measurements of health, growth, and mycorrhizal colonization variables of seedlings were performed. Overall, symbionts isolated from roots of healthy white spruce seedlings growing on the mining site, especially *Cadophora finlandia* Cad. fin. MBN0213 GenBank No. KC840625 and *Pseudomonas putida* MBN0213 GenBank No. AY391278, were more efficient in enhancing seedling health and growth than allochthonous species and constitute promising microbial symbionts. In general, mycorrhizae promoted plant health and belowground development, while rhizobacteria enhanced aboveground plant biomass. The observed beneficial effects were substrate-, strain-, and/or strains combination-specific. Therefore, preliminary experiments in control conditions such as the one described here can be part of an efficient and integrated strategy to select ecologically well-adapted symbiotic microorganisms, critical for the success of a long-term revegetation program.

Keywords: mycorrhizae, mine waste, *Picea glauca*, rhizobacteria, plant growth, plant health

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INTRODUCTION

Mining operations generate an enormous volume of waste materials that are difficult to dispose of. With more than 200 active sites, the Canadian mineral extraction industry produces over 1,000 million tons of solid waste per year (Statistics Canada, 2012; Mining Association of Canada, 2016). Prior to the first legislation in the 1970s, proper mine closure plans were not required, and residues were usually stored on adjacent wasteland where they constitute a very challenging substrate for the regeneration of natural ecosystems.

In Val-d'Or, Québec, the Sigma-Lamaque gold mine has been in operation since 1935. No mine closure plan was in place, coarse WRs and FTs cover 150 ha within the city limits that must now be efficiently revegetated. To do so, the revegetation of this area considered at low risk for contamination (Beauregard et al., 2012; Callender, 2014; Nadeau et al., 2016) with native species can be a successful strategy (Jackson et al., 1995; Larchevêque et al., 2013; Nadeau et al., 2016). White spruce is a dominant species of the boreal forest. Due to its ability to repopulate harsh environments and promote the subsequent establishment of a self-sustaining and more diverse ecosystem (Sutton, 1973), it is commonly used for land reclamation (Renault et al., 2004; Leewis et al., 2013; Onwuchekwa et al., 2014; Schoenmuth et al., 2015; Frerichs et al., 2017). A few healthy white spruce [*Picea glauca* (Moench) Voss] seedlings found naturally regenerating on the mine tailings revealed a mycorrhizal fungal community distinct from the neighboring ecosystems (Nadeau et al., 2016). Beneficial microorganisms discovered from the rhizosphere of seedlings can significantly ease plant growth and development – a major asset in a nutrient-depleted substrate like the Sigma-Lamaque gold mine tailings (Nguyen et al., 2006; Hoeksema et al., 2010). For instance, in tailings of a copper mine, fungal inoculation enhanced Japanese red pine (*Pinus densiflora*) seedlings performance (Zong et al., 2015). Similarly, in western Canada, Onwuchekwa et al. (2014) have shown that the inoculation of white spruce and jack pine (*Pinus banksiana*) with several fungal species (*Hebeloma crustuliniforme*, *Suillus tomentosus*, *Laccaria bicolor*) improved plant survival on oil sand tailings.

In addition to symbiotic fungi, rhizobacteria were also isolated from the rhizosphere of white spruce host naturally regenerating on the Sigma-Lamaque mining site. Like mycorrhizae, bacterial strains can increase plant performance as observed in coniferous tree species (Cardoso et al., 2011). As a matter of fact, the use of biofertilizers in agriculture is gaining popularity worldwide (Humphry et al., 2007; Baset Mia and Shamsuddin, 2010; Damir et al., 2011; Hrynkiewicz and Baum, 2011).

In nature, positive interactions between plant host and its symbionts occur through a number of mechanisms. Whether a beneficial microorganism is a biocontrol agent (Kropp and Langois, 1990; Pieterse et al., 2003), improves root development, water, and nutrient uptake (Blum et al., 2002; Allen, 2007; Vayssières et al., 2015) and/or limits the uptake of toxic compounds (Chaudhry et al., 2005), it co-exists with other organisms within the microbiome. Combinations of microbial strains or species may be neutral or even profitable to the plant host. However, some fungal and bacterial species can also behave like antagonists (Artursson et al., 2006; Uroz et al., 2007; Uroz et al., 2009). Plant–microbe interactions evolve with the development of a more complex ecosystem, with soil weathering and aging of the plant host (Mummey et al., 2002; Allen E.B. et al., 2003; Allen M.F. et al., 2003; Elliott et al., 2007).

White spruce has a substantial potential to be used in the phytorestoration of mine tailings. Because it is highly sensitive to transplanting shock (Nienstaedt and Zasada, 1990), the selection of adequate symbionts to improve the establishment of young seedlings could determine the success of a revegetation program with this species. Moreover, the role of mycorrhizal fungi and rhizobacteria in tree physiology on Precambrian metamorphic rocks of the Canadian Shield has never been studied. In the context of evaluating a new selection strategy for site-specific reforestation, we investigated the potential of selected cultivable fungi and rhizobacteria to improve the performance of white spruce seedling on mine tailings under glasshouse conditions. Thus, two hypotheses were formulated. First, the combined inoculation of seedlings with fungi and rhizobacteria improves the growth and overall health of seedling. Second, the use of native strains isolated directly from the mining site may give better results than the allochthonous ones.

MATERIALS AND METHODS

Seed Germination and Seedling Growth

White spruce seeds were germinated in Styroblock containers. Cavities (9.5 mL capacity) were filled with a peat–vermiculite–perlite substrate (80:15:5). The trial was conducted in a greenhouse at the Université Laval (Quebec City, QC, Canada). The greenhouse was disinfected with a bleach solution prior to the experiment. To favor seedling establishment and nutrition before inoculation, plants were fertilized 2 weeks after germination with a commercial solution (20N-8P-20K). Three weeks after germination, seedlings were transferred into 1.75 L pots filled with WRs or FTs collected from Sigma-Lamaque gold mine (Val-d'Or, QC, Canada). The mine residues are considered to have low risk of contamination (Taner et al., 1986; Beauregard et al., 2012) but soil chemical composition analyses of four randomly selected samples indicate an absence of nitrogen source (NO_3^-, NO_2^-, or NH_4^+), low concentration of elements important for plant growth (P: 0.203 ± 0.123(SE) g/kg; K: 0.096 ± 0.003 g/kg) and relatively important concentration of metals (Fe: 14.5 ± 0.5 g/kg; Ca: 22.3 ± 0.5 g/kg; Mg: 4.2 ± 0.2 g/kg; and Al: 5.7 ± 0.1 g/kg); arsenic (8.75 ± 0.25 mg/kg); and cyanides (4.6 ± 0.6 mg/kg). The pH of tailings was relatively alkaline with values varying between 8.55 and 8.68. Throughout the 32-week-long experiment, seedlings were watered daily at field capacity. Greenhouse conditions for optimal growth of white spruce seedlings were set at an alternating temperature of 25/20°C (day/night). Seedlings received artificial light with light intensity of 400 lux (5.56 $\mu E\ m^{-2}\ s^{-1}$) for 16 h/day.

Bacterial and Fungal Inoculation

Three bacterial strains were selected for this experiment. One commercial strain of *Azotobacter chroococcum* ATCC 9043 was purchased from CEDARLANE Laboratories, Ltd. (Burlington, ON, Canada). Two (*Pseudomonas putida* MBN0213 GenBank No. AY391278 and *Rhizobium radiobacter* MBN0213 GenBank No. FR828334) were isolated from the rhizosphere of healthy white spruce host naturally regenerating on coarse WRs of the mining site following the method described by Mazinani et al. (2013), a combination of the soil paste and the direct sowing of single soil grains on Mannitol-agar medium selective isolation methods for nitrogen-fixing bacteria. Bacteria were not screen

for metal resistance. For accurate identification, DNA extraction, PCR amplification using standard 16S rRNA primers 27f and 1492r (Peace et al., 1994), and DNA sequencing were performed following the method employed by Herter et al. (2011).

For maximum cell production before inoculation, bacteria were cultivated in suspension cultures under aseptic conditions at 30°C for 7 days. *P. putida*, *R. radiobacter*, and *A. chroococcum* were respectively grown in Tryptic soybean broth – Difco medium, a liquid yeast extract mannitol medium and a liquid Waksman medium following the method developed by Agri-Tech (Aurangabad-Maharashtra, India). Bacterial cells were harvested after centrifugation (20 min, 4000 rpm at 4°C) and resuspended in sterile water until the inoculant reached a concentration of 10^8 CFU mL^{-1}. Ten milliliter of the inoculant was applied onto roots of 4-week-old white spruce seedlings two times within 14 days in order to increase rhizospheric colonization success.

Three mycorrhizal fungi displaying compelling *in vitro* growth and tolerance to mine tailings were chosen for this experiment (Nadeau, 2014). *Hebeloma crustuliniforme* UAMH5247, from the Centre for Forest Research genomic and microbial collections[1], was isolated from white spruce roots in a natural forest stand of the boreal forest in Canada. Both *Tricholoma scalpturatum* Tri. scalp. MBN0213 GenBank No. KC840613 and *Cadophora finlandia* Cad. fin. MBN0213 GenBank No. KC840625 were isolated from healthy naturally regenerating white spruce seedlings on Sigma-Lamaque gold mine coarse tailings (Nadeau et al., 2016).

The inoculum was produced by cultivating fungal mycelia in a liquid Melin Norkrans medium at 23°C under aseptic shaking conditions. After 2 months, the mycelia were collected and rinsed with sterile water to discard excess nutrients. Blended mycelia were mixed with sterile water (ratio 1:10) to obtain a final concentration $\geq 5 \times 10^5$ viable propagules mL^{-1}. White spruce seedlings were inoculated with 5 ml of the inoculant when they were 3-week-old and a second time 4 weeks later to increase root inoculation success. The inoculum was released into the root zone using an analog adjustable-dispenser.

Experimental Design and Treatments

The experimental design was a randomized complete block (RCB) with three crossed fixed factors (*Tailing type* × *fungi* × *bacteria*). Tailing type was composed of two levels: WR and FT. Fungal factor had a total of four levels: none (noF), *H. crustuliniforme* (*Hc*), *T. scalpturatum* (*Ts*), and *C. finlandia* (*Cf*). Bacteria also had four levels: none (noB), *P. putida* (*Pp*), *R. radiobacter* (*Rr*), and *A. chroococcum* (*Ac*). There were 32 treatments with three replicates in each of the four blocks for a total of 384 experimental units. Each replicate was randomly assigned to experimental units within blocks. Every experimental unit consisted of a 1.75-L pot filled with tailings containing one white spruce seedling. Experimental units within blocks were separated by a thin piece of plastic to avoid cross bacterial contamination. Each block was surrounded by two guard rows to maintain the most homogeneous environmental conditions possible in all experimental units. **Supplementary Figure S1** gives detailed layout and illustrations of the experimental design.

Measurements of Seedling Survival Rate and Nutrient Content Analyses

Detailed descriptions of seedling survival rate and nutrient content analyses are presented in the companion paper (Nadeau et al., 2018).

Measurements of Seedling Health and Growth

At the end of the glasshouse experiment, seedlings were brought into a growth chamber an hour before measuring chlorophyll fluorescence. Photochemical efficiency (Fv/Fm) was measured in a dark environment using a portable fluorometer PAM-2000 with the data acquisition software DA-2000 (Heinz Walz, Effeltrich, Germany). Briefly, the foliage was placed under the fluorescence booster for recording Fv/Fm data.

Needles were excised from stems, weighted, and individually positioned on a transparent plastic plate prior to scanning (WinSEEDLE PRO LA2400 scanner system and software, Regent Instruments, Inc., Quebec City, QC, Canada) were used to determine specific surface foliar areas (SSFA) of green, yellow, brown, dark-red, and light-red foliar tissues. Percentages of healthy-green foliage and dark-red foliage were calculated by comparing their SSFA with the sum of all SSFAs.

Stems were weighted and measured with a 15-cm ruler. Roots were washed gently with tap water in a 2-mm mesh sieve to remove all soil particles and thereafter weighed. The percentage of fungal colonization was calculated after manual counting under a microscope as the ratio of mycorrhizal root tips number to total root tips number multiplied by 100. Subsequently, roots were transferred onto a transparent plastic plate. WinRHIZO PRO LA2400 scanner system and software (Regent Instruments, Inc., Quebec City, QC, Canada) were used for measuring total root length, volume, and number of root tips.

For dry biomass analyses, white spruce seedling roots, shoots, and needles were dried at 65°C for 7 days. Percentage of water content was calculated by subtracting dry biomass from wet biomass, dividing the result by wet biomass and then multiplying by 100.

Statistical Analyses

Differences Among Treatments

All the statistical analyses were conducted with the SAS software (SAS Institute Inc., 2012). Seedling health, growth, and percentage of fungal root colonization data were subjected to three-way analyses of variance (*Tailing type* × *fungi* × *bacteria*) using PROC GLM. Proper transformations were performed when needed. Log transformations were performed with total root length, number of root tips and dry biomass data. Arcsine transformation was used with the photochemical efficiency variable. Finally, non-parametric analyses (Wilcoxon rank sum test and *post hoc* test) was conducted on the percentage of dark-red foliage and percentage of roots colonized by fungus because it was not possible to meet normality and/or homoscedasticity

[1]http://www.cef-cfr.ca/index.php?n=CEF.Collections

assumptions even after transformations. The non-parametric tests were performed using PROC NPAR1WAY. Significance for all analyses was set at $\alpha = 0.05$ ($P \leq 0.05$). Means and standard errors of each treatment were calculated for all health, growth, and colonization variables.

Correlation Analyses

Correlations between the percentage of colonized roots and other health and growth variables were investigated using PROC CORR. Furthermore, correlation analyses between health variables (photochemical efficiency, percentage of healthy green foliage, and percentage of dark-red foliage) and growth variables (root, stem, and needle dry biomass) were performed in order to determine if there was a relationship between white spruce seedling health and growth. For these analyses, individual data were used. Significance for all Pearson correlation coefficients (r) was set at $\alpha = 0.05$ ($P \leq 0.05$).

RESULTS

Effect of Symbiotic Association on Plant Health

After the 32 weeks of glasshouse trial in Sigma-Lamaque gold mine tailings, seedling exhibited contrasting phenotypes (**Figure 1A**). Seedling growth and health clearly benefitted (**Figures 1B,C**) from symbiotic associations that prove to be successful (**Figures 1D–F**).

Belowground, the inoculation of seedlings with one of the two native mycorrhizal fungi *T. scalpturatum* and *C. finlandia*, increased root water content by 6 and 4%, respectively, when compared to non-inoculated control plants (**Figure 2A**, left panel; *P*-values < 0.0001). Despite a 2% increase, seedlings inoculated with *H. crustuliniforme* did not differ statistically from control. For the bacterial treatments, only *A. chroococcum*-associated seedlings outperformed non-inoculated control plants (**Figure 2A**, right panel; *P*-values = 0.003).

The proportion of dark-red foliage is a health-related variable indicative of element toxicity; a higher percentage value indicates reduced seedling health. In the control seedlings, almost one-third ($31 \pm 2\%$; *P*-values < 0.0001) of seedling foliage was dark red. This suggests the importance of the symbiotic associations with either fungal partner or a bacteria strain for plant health in a severely disturbed environment. Indeed, plants inoculated with *C. finlandia* exhibited significantly less dark-red foliage than controls (**Figure 2B**, left panel; *P*-value < 0.0001). The proportion of healthy green foliage on seedlings inoculated with *C. finlandia* and *T. scalpturatum* was significantly greater than control plants without fungal inoculation (**Figure 2C**, left panel; *P*-value < 0.0001). The benefit of the allochthonous fungus *H. crustuliniforme* was not significant, a trend we also observed for photochemical efficiency (**Figure 2D**, left panel).

For bacterial treatments (**Figures 2C,D**, right panels; *P*-values < 0.0001), only the *A. chroococcum* commercial strain significantly increased the proportion of healthy-green foliage and to a slightly lower extent, association with the locally sourced-bacteria *R. radiobacter* compared to control and *P. putida* treatments (**Figure 2B**, right panel; *P*-value < 0.0001).

Photochemical efficiency (Fv/Fm) measures the capacity of the photosystem apparatus to capture light energy. From 0.56 in control plants, Fv/Fm ratio did not improve in *H. crustuliniforme*-associated plants (0.58) but reached 0.63 and 0.70 in plants associated with *T. scalpturatum* and *C. finlandia* (*P*-value < 0.0001). Association with the *A. chroococcum* commercial strain also improved photochemical efficiency compared to *P. putida*-associated plants (*P*-value = 0.0048). In fact, *P. putida* did not improve any of the four health-related parameters measured.

Effect of Symbiotic Association on Plant Growth

A contrario, bacterial treatments, especially site-specific species, significantly improved aboveground growth (**Figure 3**). After 32 weeks, seedlings inoculated with *P. putida* had significantly greater needle biomass (*P*-value < 0.0001), stem biomass (*P*-value = 0.0048), and stem length (*P*-value = 0.0217) than control plants not associated with a bacterial strain.

Surprisingly, no aerial growth parameter was directly influenced by mycorrhizal fungi treatment. However, careful observation of the belowground growth was much more informative. For the number of root tips per plant, there was an interaction between the three factors *Tailing type* × *fungus* × *bacteria* (**Figure 4**; *P*-value = 0.0128), while

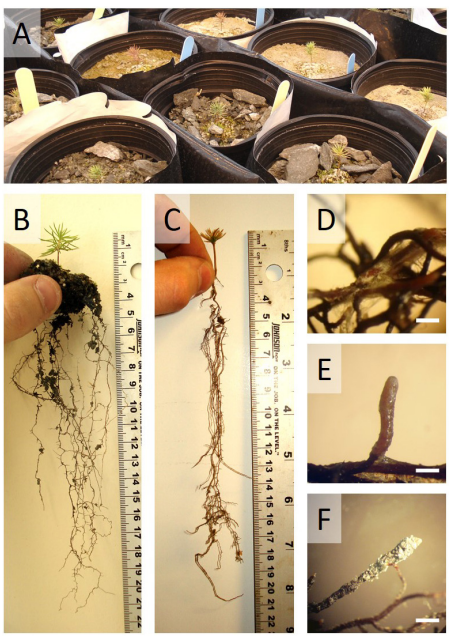

FIGURE 1 | White spruce seedling after 32 weeks of growth in waste rocks or fine tailings from the Sigma-Lamaque gold mine. **(A)** Partial view of the glasshouse experiment; typical examples of **(B)** a healthy white spruce seedling and **(C)** an unhealthy white spruce seedling; root tips colonized by **(D)** *Hebeloma crustuliniforme*, **(E)** *Tricholoma scalpturatum*, and **(F)** *Cadophora finlandia*.

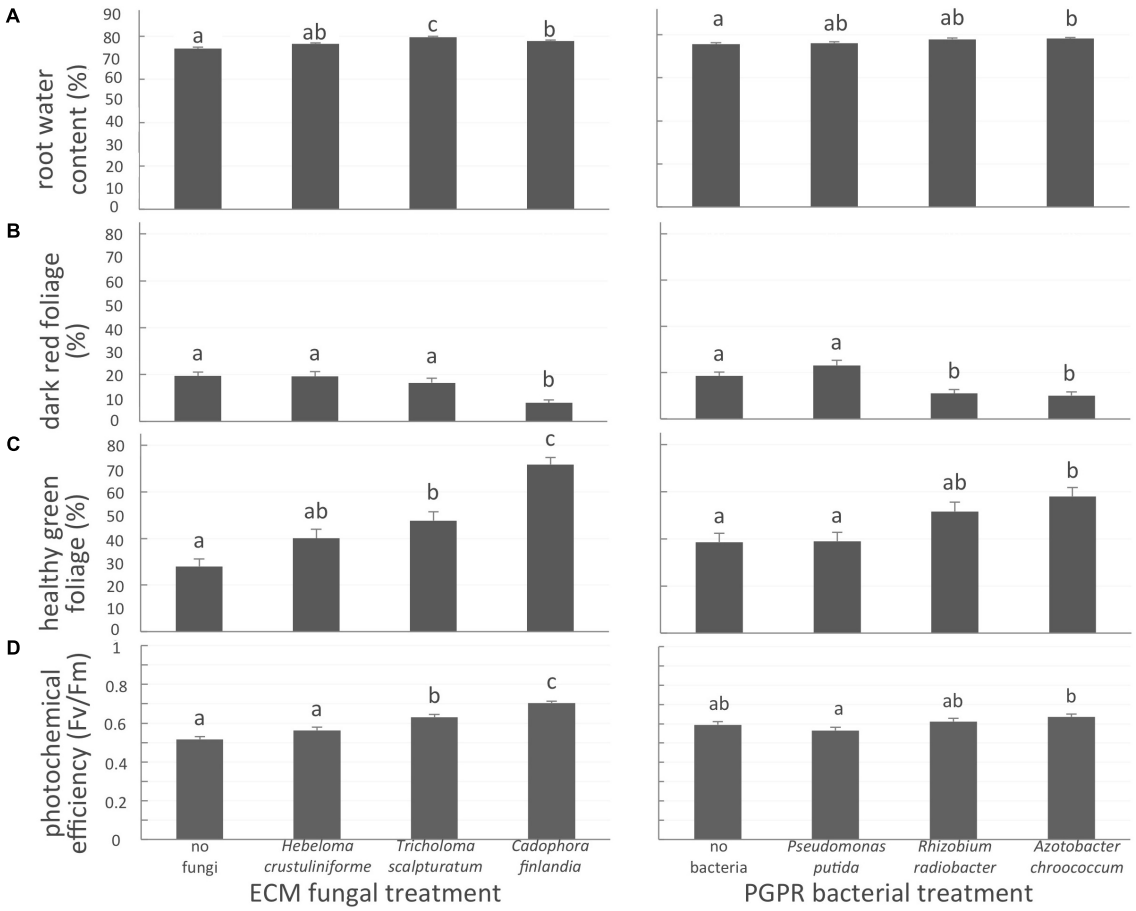

FIGURE 2 | White spruce health related parameters affected by symbiotic associations. **(A)** Root water content (%); **(B)** proportion of dark-red foliage (%); **(C)** proportion of healthy green foliage (%), and **(D)** photochemical efficiency (Fv/Fm ratio) were affected by both mycorrhizal fungal treatment and bacterial treatment after 32 weeks of growth. Values are means ± SE. Different letters indicate significant difference.

root tips number was <400 in plants grown on WR and without microorganism associations: this number increased significantly by 60 to 140% (**Figure 4**, "WR" labeled bars on the left) with the inoculation of at least one symbiont (fungus, bacteria, or both). The association with native fungi were the most beneficial: on WRs, number of root tips went consistently above average (**Figure 4**, see dashed line) when plants were associated with *T. scalpturatum*. On FTs (**Figure 4**, "FT" labeled bars on the right), the number of root tips increased by 20 to 100% (from 461 when plants were grown without a partner) but only when seedlings grew in association with some specific symbiont combinations. On this tailing type, the association with *T. scalpturatum* was also beneficial but the inoculation of rhizobacteria was not sufficient to notice a significant increase; neither did the allochthonous fungus *H. crustuliniforme* alone and several other fungus × bacteria combinations.

Mycorrhizal Root Colonization Rate

Root colonization was greater on WRs (45%) than on FTs (37%) (**Figure 5A**; P-value < 0.0001). The percentage of root tips colonized was not influenced by bacterial treatment (P-value = 0.3194) but varied between the different mycorrhizal fungi (**Figure 5B**; P-value < 0.0001). For instance, seedlings associated with *C. finlandia* displayed the greatest percentage of colonized root tips (63%).

Interestingly, the proportion of root tips colonized correlated with health and growth variables in this experiment (**Table 1**). The proportion of healthy green foliage, photochemical efficiency, root growth parameters (total biomass, volume, stem length, and root tips number), stem and finally needle dry biomass correlated positively with the percentage of root tips colonized. However, those relationships broke for several growth parameters (number of root tips, stem, or needle dry biomass) when plants were associated with the allochthonous *H. crustuliniforme* and tended to be significantly much stronger with *C. finlandia* than with *T. scalpturatum*.

DISCUSSION

In the present study, tree-symbiont associations proved to be beneficial to the establishment of white spruce seedlings on

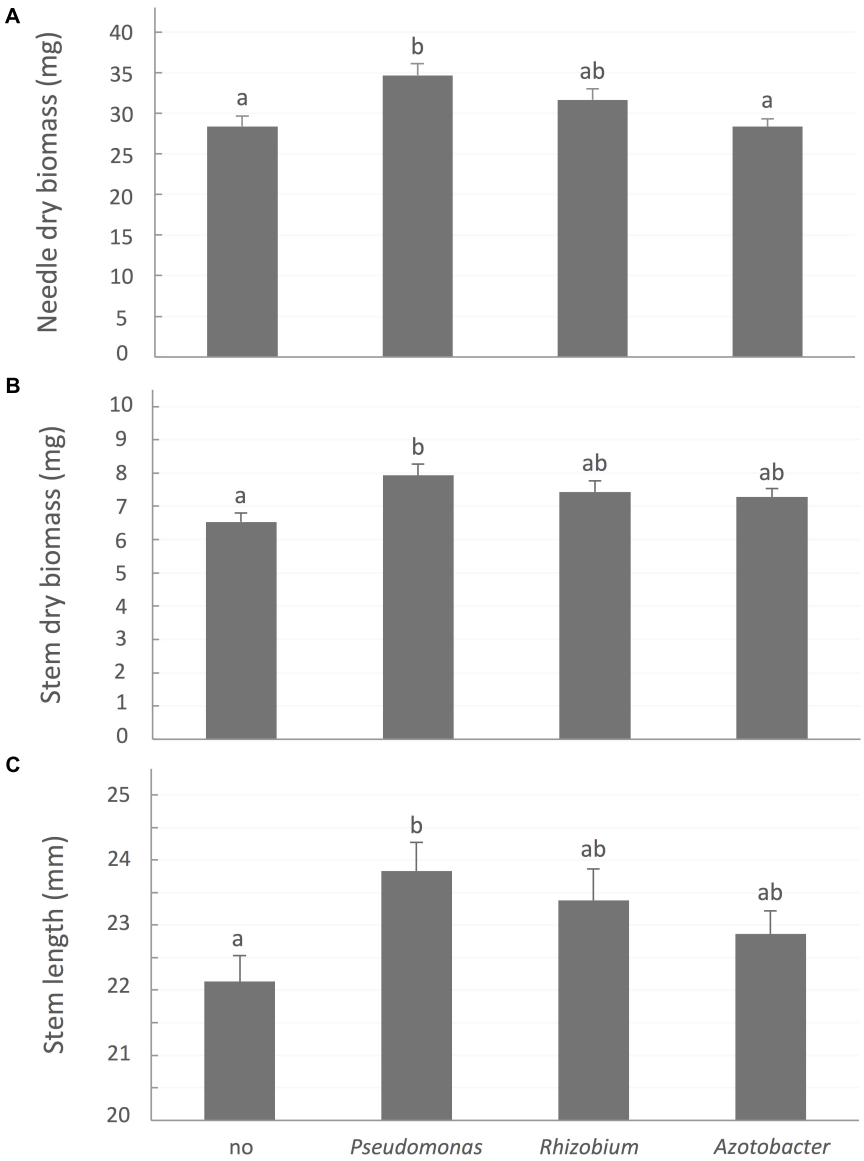

FIGURE 3 | White spruce growth parameters affected by association with bacteria. **(A)** Needle dry biomass (mg), **(B)** Stem dry biomass (mg), and **(C)** stem length (mm) were affected by rhizobacterial treatments after 32 weeks of growth. Values are means ± SE. Different letters indicate significant difference.

waste material of the Sigma-Lamaque gold mine. Mine-adapted mycorrhizae and rhizobacteria were respectively capable of improving plant health and growth under glasshouse conditions.

Microbial Symbiotic Association Improved Overall Plant Health

Azotobacter chroococcum and to a lesser extent *R. radiobacter* were more successful in enhancing white spruce seedlings health but much less than mycorrhizal fungi and *C. finlandia* in particular. Indeed, seedling health was greatly improved by the two fungi *C. finlandia* and *T. scalpturatum* isolated from the mining site. Inoculation with *H. crustuliniforme* neither improved root water uptake nor other plant health parameters, albeit Onwuchekwa et al. (2014) have demonstrated that *H. crustuliniforme* increased white spruce water uptake on oil sand tailings. Moreover, the same strain we used in this study had proven to be beneficial to seedlings grown on peat moss and sand mix under salt stress conditions (Mushin and Zwiazek, 2002). But as a drought intolerant strain isolated from a natural boreal forest stand (Coleman and Bledsoe, 1989), *H. crustuliniforme* may not be well-suited to grow in mine tailings, an environment very prone to water stress. Native *C. finlandia* and *T. scalpturatum* fungi may be better adapted, thereby capable of enhancing seedling health. Both species are commonly found in high abundance and frequency, in heavy metal polluted sites (Krpata et al., 2008; Gorfer et al., 2009). Extreme, arid, or toxic soil conditions lead to the evolution

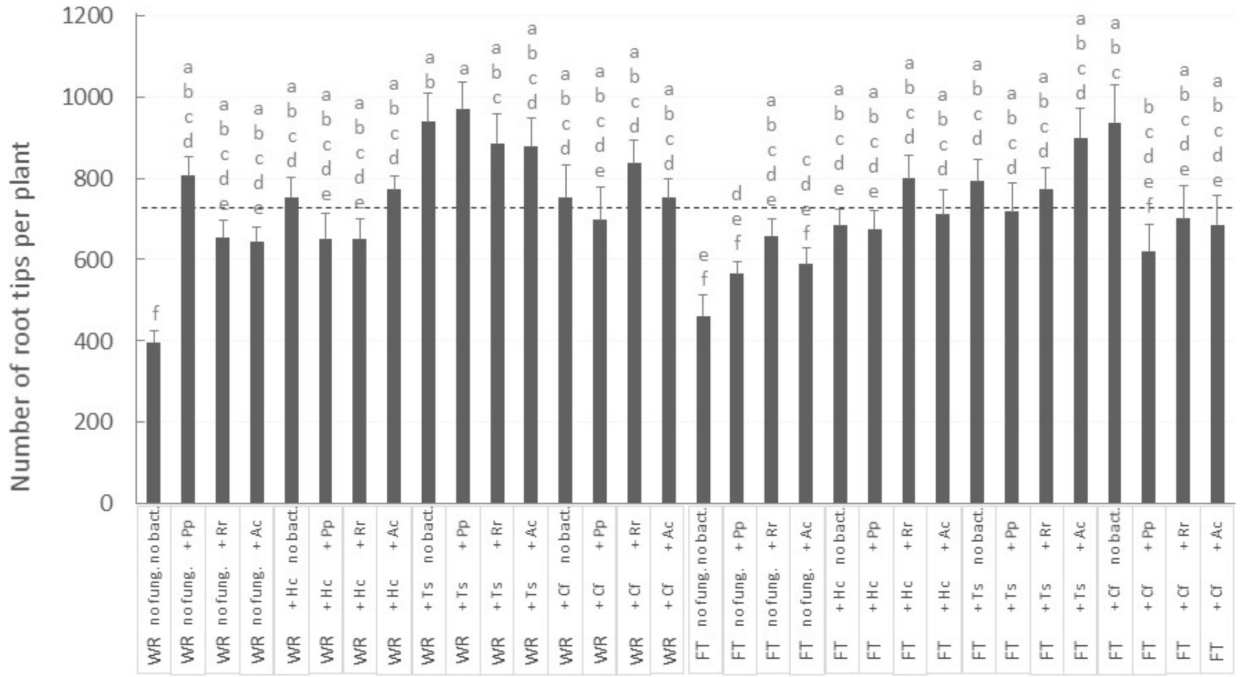

FIGURE 4 | Number of white spruce root tips after 32 weeks of growth in 32 different conditions. There was an interaction between the three factors (*P*-value = 0.0128): tailing type (WR, waste rocks; FT, fine tailings); mycorrhizal fungi (no fung., no fungi; +Hc, *Hebeloma crustuliniforme*; +Ts, *Tricholoma scalpturatum*; +Cf, *Cadophora finlandia*); and bacteria (no bact., no bacteria; +Pp, *Pseudomonas putida*; +Rr, *Rhizobium radiobacter*; +Ac, *Azotobacter chroococum*). The two large light gray bars indicate average root tips number on waste rocks and FTs. Values are means ± SE, same letters are not significantly different at $\alpha = 0.05$, Tukey test.

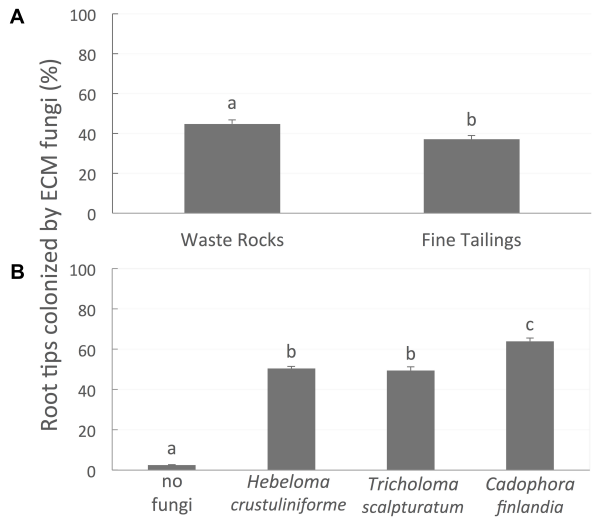

FIGURE 5 | The proportion of root tips colonized by mycorrhizal fungi. **(A)** Figure is different on waste rocks than on fine tailings, **(B)** diverge between the different fungi inoculated. Values are means ± SE. Different letters indicate significant difference.

of tolerant strains, an adaptation indispensable for both tree and fungal survival (Colpaert et al., 2011). Like for many fungi (Douhan et al., 2011), the genetic diversity of *C. finlandia* is still unknown but, under conditions of high heavy metal concentrations, it has the capability to enhance the expression of several genes encoding extracellular and plasma membrane proteins potentially involved in detoxification processes (Gorfer et al., 2009). *T. scalpturatum* is a generalist species with high genetic diversity at the local scale (Carriconde et al., 2008), whereas, *H. crustuliniforme* has low intraspecific genetic diversity (Aanen et al., 2000).

The ability of mycorrhizae to improve seedling health is indeed site-specific and positively associated with its capacity to grow on a given substrate. Root colonization rate was much lower on FTs than on WRs, the substrate on which plants performed the best. In a preliminary *in vitro* experiment (unpublished), we have found that *C. finlandia* produced the highest mycelial biomass on poor liquid medium amended with mine tailings followed by *T. scalpturatum* and then *H. crustuliniforme*. Accordingly, in the present study, plants inoculated with *H. crustuliniforme* were the ones with the least colonized root system compared to *T. scalpturatum* and *C. finlandia* inoculated ones, the later being the most colonized. *C. finlandia* is also the one symbiont that alleviated the most Ca and Fe deleterious effects *in vivo* (Nadeau et al., 2018). Health-related parameters were strongly and positively correlated with root colonization rates of the two fungi isolated from the mining site but only to a smaller extent with mycorrhization rate of *H. crustuliniforme*, the fungus that presents the lowest potential for reforestation program in gold mine WR and FTs.

TABLE 1 | Pearson correlation coefficient (*r*) and their associated *P*-values calculated for the proportion of root tips colonized by different mycorrhizal fungi and measured health and growth variables.

	Proportion of healthy green foliage	Photochemical efficiency
Cadophora finlandia	**$r = 0.75$** (P-value < 0.0001)	**$r = 0.67$** (P-value < 0.0001)
Tricholoma scalpturatum	**$r = 0.71$** (P-value < 0.0001)	**$r = 0.54$** (P-value < 0.0001)
Hebeloma crustuliniforme	**$r = 0.47$** (P-value < 0.0001)	**$r = 0.33$** (P-value $= 0.0014$)

	Root dry biomass	Total root volume	Total root length	Root tips number	Stem dry biomass	Needle dry biomass
Cadophora finlandia	**$r = 0.56$** (P-value < 0.0001)	**$r = 0.59$** (P-value < 0.0001)	**$r = 0.49$** (P-value < 0.0001)	**$r = 0.34$** (P-value $= 0.0013$)	**$r = 0.38$** (P-value $= 0.0004$)	**$r = 0.34$** (P-value $= 0.0014$)
Tricholoma scalpturatum	**$r = 0.43$** (P-value < 0.0001)	**$r = 0.47$** (P-value < 0.0001)	**$r = 0.41$** (P-value < 0.0001)	**$r = 0.26$** (P-value $= 0.015$)	**$r = 0.28$** (P-value $= 0.0078$)	**$r = 0.21$** (P-value $= 0.0451$)
Hebeloma crustuliniforme	**$r = 0.37$** (P-value $= 0.0003$)	**$r = 0.41$** (P-value < 0.0001)	**$r = 0.35$** (P-value $= 0.0008$)	$r = 0.15$ (P-value $= 0.1714$)	$r = 0.2$ (P-value $= 0.0606$)	$r = 0.12$ (P-value $= 0.2653$)

Significant correlation at $\alpha \leq 0.05$. Statistically significant values ($P < 0.05$) are given in bold.

Improvement of Plant Growth Is Associated With Symbiont Type and Source

Despite its huge effect on plant health, mycorrhization did not enhance seedling aerial growth in this experiment. Moreover, mycorrhization rate of native species correlated well with root growth but much less with aerial growth parameters. Early root colonization could have a carbon cost that negates the aboveground seedling growth during the first growing season but enhances it the subsequent years (Rygiewicz and Andersen, 1994). An idea supported by the fact that several treatments with at least one symbiont yielded a higher number of fine root tips on white spruce seedlings than the control without symbiont. Mycorrhizal fungi and rhizobacteria such as species of the genus *Pseudomonas* and *Azotobacter* produce auxins that alter considerably the host root morphology (Rajkumar et al., 2009; Etemadi et al., 2014). On that account, hormone production by the investigated symbionts plays probably a very important role in plant growth behavior. The production of a higher number of root tips by seedlings inoculated with mycorrhizal fungi and/or rhizobacteria may be highly beneficial to white spruce trees by allowing extra uptake of water and nutrients.

The inoculation of white spruce with *P. putida*, a rhizobacteria strain isolated from the rhizosphere of healthy white spruce seedlings naturally regenerating on the mining site, was the only treatment that increased considerably seedling aerial growth. Beall and Tipping (1989) and O'Neill et al. (1992) have also reported that *P. putida* enhanced jack pine and spruce aerial growth.

Though the beneficial effects of *A. chroococcum* or *R. radiobacter* have been extensively demonstrated in both woody (Leyval and Berthelin, 1993; Karthikeyan and Sakthivel, 2011) and non-woody (Aquilanti et al., 2004; Humphry et al., 2007; Baset Mia and Shamsuddin, 2010) plant species, neither of the commercial strain of *A. chroococcum* nor the indigenous *R. radiobacter* influenced plant growth according to our results. However, to the best of our knowledge, the present study is the first to investigate the impact of native rhizobacteria on gold mine tailings, a completely different environment than agricultural fields and forest stands. Indeed *A. chroococcum* may not be adapted to tailing conditions as much as *P. putida* in that perspective. However, unlike *P. putida*, both *A. chroococcum* and *R. radiobacter* had appreciable effects on seedling health suggesting a much complex explanation for their limited plant growth promoting ability. For that reason, bacterial selection must be cautiously done in order to identify strains that have the potential to be effective in the field.

Selection of Symbiotic Partners for Successful Mine Reforestation Programs

In conclusion, our initial on-site sampling strategy proves to be effective: mycorrhizal fungi and rhizobacteria isolated from roots of healthy white spruce seedlings naturally regenerating on the mining site proved to be remarkably more efficient than allochthonous species in enhancing seedling health and growth when planted on mine tailings. Within the 32 weeks of glasshouse trial, native fungi, *C. finlandia*, *T. scalpturatum*, and the native rhizobacteria *R. radiobacter* promoted seedling health through better root colonization rate. The native rhizobacteria *P. putida* was the sole symbiont that distinctly improve seedling aerial growth but not seedling health. Allochthonous fungus, *H. crustuliniforme* had little effects on seedling performance, whereas plants benefited from the inoculation of *A. chroococcum*. Specifically, the allochthonous rhizobacteria *A. chroococcum* improved seedling root water uptake, especially when paired with mycorrhizal fungi. As discussed in a companion paper (Nadeau et al., 2018), a symbiont (or symbiont combination) capacity to modulate plants access to otherwise limited (water, nutrients) or toxic compounds is directly linked to the success of the white spruce seedling establishment.

Since its soil chemical composition is already well-documented, the Sigma-Lamaque gold mine at Val-d'Or

is at one and the same time a land that must be revegetated once its exploitation is completed and a potential testing ground to validate the several steps involved in the development of a new green technology (Nadeau, 2014). The comprehensive analysis of the entire ecosystem – of which the present study is an important component – should unravel the significance of each parameter for the success of an integrated reforestation program including the soil chemical composition and the isolation, selection, validation, and large-scale production of the best plant–symbiont combinations (Nadeau and Khasa, 2016).

AUTHOR CONTRIBUTIONS

MBN and DK conceived and designed the experiments. MBN performed the experiments. MBN, JL, and DK analyzed the data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01267/full#supplementary-material

FIGURE S1 | Illustrations and design of the experiment. Mycorrhizal fungi and rhizobacteria used in this **(A)** glasshouse experiment were either **(B)** native from the Sigma-Lamaque gold mine site [*Tricholoma scalpturatum* (Ts), *Cadophora finlandia* (Cf), *Pseudomonas putida* (Pp), *Rhizobium radiobacter* (Rr)] or isolated from a natural forest stand [*Hebeloma crustuliniforme* (Hc)] or of commercial origin for *Azotobacter chroococcum* (Ac). White spruce saplings were either planted in **(C)** waste rocks (WRs) or in fine tailings (FTs). **(D)** Randomized complete block (RCB) design with three crossed fixed factors: tailing type (FT in regular font, WR in bold); fungi [none (noF) in black; Ts, green font; Cf, blue font; Hc, red font]; and bacteria [none (noB), no background; Rr, background in red; Pp, background in blue; Ac, background in green] for a total of 32 treatments, 4 blocks, 3 replicates per treatment per block, and 384 experimental units.

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Mycorrhizae and Rhizobacteria on Precambrian Rocky Gold Mine Tailings: II. Mine-Adapted Symbionts Alleviate Soil Element Imbalance for a Better Nutritional Status of White Spruce Seedlings

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In the context of a phytorestoration project, the purpose of this study was to assess the respective contribution to the nutritional status of *Picea glauca* seedlings of ectomycorrhizae and rhizobacteria native or not to the Sigma-Lamaque gold mine wastes in northern Quebec, Canada. In a glasshouse experiment, inoculated plants were grown for 32 weeks on coarse waste rocks or fine tailings obtained from the mining site. The survival, health, growth, and nutritional status of plants were better on coarse waste rocks than on fine tailings. Fe and Ca were especially found at high levels in plant tissues but at much lower concentrations on waste rocks. Interestingly, inoculation of microsymbionts had only minimal effects on N, P, K, and Mg plant status that were indeed close or within the concentration range encountered in healthy seedlings. However, both fungal and bacterial treatments improved Fe and Ca concentrations in plant tissues. Fe concentration in the foliage of plants inoculated with the fungi *Tricholoma scalpturatum* Tri. scalp. MBN0213 GenBank #KC840613 and *Cadophora finlandia* Cad. fin. MBN0213 GenBank #KC840625 was reduced by >50%. Both fungi were isolated from the mining site. The rhizobacteria, *Azotobacter chroococcum,* also improved plant Fe level in some cases. Regarding Ca nutritional status, the native bacterial strain *Pseudomonas putida* MBN0213 GenBank #AY391278 was the only symbiont that reduced foliar content by up to 23%. Ca concentration was negatively correlated with the fungal mycorrhization rate of seedling roots. This relation was especially strong ($r = -0.66$, p-value ≤ 0.0001) in the case of *C. finlandia*. Also, a similar relationship existed with root Fe concentration ($r = -0.44$, p-value ≤ 0.0001). In fact, results showed that seedling performance was more correlated with elevated Ca and Fe concentration *in planta* than with nutrient deficiency. Also, native microsymbionts were capable of regulating seedling nutrition in the poor substrate of the Sigma-Lamaque gold mine tailings.

Keywords: mycorrhizae, land reclamation, mine waste, nutrition, *Picea glauca*, rhizobacteria, toxicity

INTRODUCTION

Anthropogenic activities such as mining of the Precambrian gold ores create severely disturbed ecosystems: most nutrients and minerals are trapped in the rock tailings, the only soil left. The situation is very similar to the natural process that occurs after glaciation retreat (Taner et al., 1986; Balogh-Brunstad et al., 2008) – yet, over time, many species recolonize this low fertility environment where organic matter is inexistent (Hobbie et al., 1998). Should it be cautiously designed, phytorestoration is an appropriate on-site management strategy for the reclamation of mine residues. Phytoremediation is an efficient, economically and ecologically sound solution (Nadeau, 2014; Vodouhe and Khasa, 2015).

Jumpponen et al. (2002) have studied the occurrence of ectomycorrhizae (ECM), the first mycorrhizal fungi present in primary succession (Trowbridge and Jumpponen, 2004), on the forefront of a retreating glacier. They found that pioneer plants were only able to thrive on the rock tailings in association with ECM. Like alder or spruce trees, all studied species (*Salix commutata, S. phylicifolia, Abies lasiocarpa, Larix lyallii, Pinus contorta,* and *Tsuga mertensiana*) are commonly known to form mycorrhizal symbiotic relationships (Dixon and Buschena, 1988; Nienstaedt and Zasada, 1990; Dahlberg, 2001; Quoreshi et al., 2007; Roy et al., 2007; Smith and Read, 2008). To thrive in harsh post-glacial conditions or human-made new ecosystems, plants have co-evolved with their microsymbionts capable of scavenging nutrients from rocks or fixing atmospheric nitrogen in exchange for plant photosynthetic carbon sources (Allen et al., 2003a,b; Khan, 2006; Roy et al., 2007; Quoreshi and Khasa, 2008).

One of the most characteristic trees of the boreal forest, white spruce (*Picea glauca*) has been known as a plastic species because of its wide distribution across North America and its ability to recolonize areas at the end of glaciation (Nienstaedt and Zasada, 1990). It can support extremely diverse site conditions in terms of temperature, moisture, light exposure or soil type (Sutton, 1973). White spruce seedlings were sporadically found regenerating on the Sigma-Lamaque gold mine tailings at Val d'Or, Abitibi, Quebec. This rocky soil is essentially made of biotite, an iron-rich mica (Taner et al., 1986). While considered at low risk for contamination (Beauregard et al., 2012), the mine waste material – a pile of coarse rocks and a basin full of fine ground tailings –, constitute a rather hostile environment for vegetation establishment because of its unbalanced mineral content, alkaline pH, poor soil structure, and water holding capacity of the substrate. However, the rhizosphere of the healthy seedlings found on site revealed a community of fungi that is distinct from the adjacent nursery, forest edge, and natural forest ecosystems (Nadeau and Khasa, 2016).

By expanding the surface absorption of their hosts, fungi have the ability to increase water uptake and to extract insoluble forms of nutrients otherwise unavailable to the plant host (Balogh-Brunstad et al., 2008). Mycorrhizal associations have a tremendous potential to be used for land reclamation albeit some species and strains may be better adapted than others to the extreme site conditions of the Sigma-Lamaque mine tailings (Callender et al., 2016). Likewise, nitrogen-fixing bacteria have been found in the soil of all ecosystems including the boreal forest (Marshall, 2000). The inoculation of plant roots with plant growth promoting rhizobacteria (PGPR) of different genera including *Acetobacter, Agrobacterium, Bacillus, Burkholderia*, *Mycobacterium*, *Pseudomonas*, *Rhizobium, Sphingomonas,* and *Staphylococcus* has previously been shown to increase mineral accessibility to plants (reviewed in Rodríguez and Fraga, 1999; Tilak et al., 2005; Uroz et al., 2009; Dynarski and Houlton, 2018). For instance, *Pseudomonas* and *Bacillus* strains associated with maize roots of the Himalayan mountains showed marked P-solubilization activity (Zahid et al., 2015). Similarly, *Burkholderia glathei* inoculated on Scots pine roots (*Pinus sylvestris*) significantly increased plant Mg and K uptake from biotite substrate (Calvaruso et al., 2006). But in all these studies, the benefits conferred by the microsymbiotic association are unequal and site-, strain-, and/or plant specific. Although numerous reports list mycorrhizal fungi and PGPR that have been screened for their potential as biofertilizers in agriculture (see most recent references: Amin and Latif, 2016; Sritongon et al., 2017; Zahoor et al., 2017), the information on spruce trees interacting with a combination of fungal and bacterial symbionts under harsh conditions such as abandoned mine tailings of the Canadian north has never been studied before. In order to develop an efficient microbial consortium, it is necessary to test different microorganisms for the challenging stress and the synergistic interactions between fungi and bacteria in a way to form a basis of a cumulative impact on plant establishment.

Thus, to rapidly reforest the mining site, the main objective of the study was to assess the efficiency of selected allochthonous and indigenous mycorrhizal and bacterial symbionts in promoting adequate plant nutrition and in limiting toxicity of such an unfavorable substrate. A greenhouse experiment was conducted in which the establishment of white spruce seedlings on waste rocks or fine tailings of Sigma-Lamaque gold mine was evaluated. Both root and foliar nutrient concentrations (N, P, K, Ca, Mg, Fe) of white spruce seedlings were recorded after 32 weeks of growth during which seedling survival, health and growth parameters were also monitored (Nadeau et al., 2018). Results were comprehensively analyzed to refine our working hypothesis that favors the use of well-adapted microsymbionts directly isolated from the mining site over allochthonous ones.

MATERIALS AND METHODS

Experimental Design and Treatments

Detailed descriptions of the experimental design, treatments, growth and health measurements are presented in the companion paper (Nadeau et al., 2018).

Briefly, tailings collected directly from Sigma-Lamaque gold mine were used for this glasshouse trial. Three-week-old white spruce seedlings were grown in waste rocks or fine tailings and inoculated with or without a mycorrhizal fungus isolated from healthy naturally regenerating white spruce seedlings on Sigma-Lamaque gold mine coarse tailings (Nadeau and Khasa, 2016; Nadeau et al., 2016): *Tricholoma scalpturatum* Tri. scalp. MBN0213 GenBank #KC840613 or *Cadophora finlandia* Cad. fin.

MBN0213 GenBank #KC840625; or *Hebeloma crustuliniforme* UAMH5247 from the Centre for Forest Research genomic and microbial collections[1] and with or without nitrogen-fixing bacteria [*Pseudomonas putida* MBN0213 GenBank #AY391278, *Rhizobium radiobacter* MBN0213 GenBank #FR828334 isolated from the mining site; or *Azotobacter chroococcum* ATCC 9043 purchased from CEDARLANE Laboratories Ltd. (Ontario, Canada)]. The experiment was run for 32 weeks in a glasshouse at the Université Laval under semi-controlled conditions.

The study was designed as a randomized complete block (RCB) with three crossed fixed factors (*Tailing type* × *ECM fungi* × *PGPR*). There were 32 treatments including the controls with three replicates randomly placed within each of the four blocks for a total of 384 experimental units consisting of a 1.75 L pot containing one white spruce seedling (**Supplementary Figure S1**).

Measurements of Seedling Health and Growth

Detailed description of growth and health measurements are presented in the companion paper (Nadeau et al., 2018). Briefly, we used a portable fluorometer PAM-2000 with the data acquisition software DA-2000 (Heinz Walz, Effeltrich, Germany) to measure photochemical efficiency, we used WinSEEDLE to determine specific surface foliar areas (SSFA) of green, yellow, brown, dark red, and light red foliar tissues. Fresh needles, stem and roots were measured, weighted, and/or observed under a microscope to calculate the level of fungal colonization. Needles, stem and roots were dried for 7 days at 65°C to calculate the percentage of water content [(Fresh weight – Dry Weight)/Fresh weight]*100.

Monitoring of Seedling Survival and Nutrient Content Analyses

Seedling survival was assessed every 4 weeks through visual observations. Seedlings were considered dead when light red needles had no green color left.

For nutrient content analysis, replicates within treatments were pooled together in each block. Seedlings roots and needles were ground separately in a Wiley Mill. Samples were digested in concentrated H_2SO_4 and 50% H_2O_2. Chemical analyses of nitrogen (N), potassium (P), phosphorus (K), magnesium (Mg), calcium (Ca), and iron (Fe) in roots and needles were performed on the digested tissues following techniques outlined in Kalra (1998) and Quoreshi and Khasa (2008). Other micronutrients were not measured because their concentrations in tailings were neither a limiting nor a toxic factor.

Statistical Analyses

Differences Among Treatments

As described in the compagnion paper (Nadeau et al., 2018), all the statistical analyses were conducted with the SAS software (SAS Institute Inc., 2012). Survival data were quantified in percentage and compared using χ^2 test with PROC FREQ. Nutrient data were subjected to three-way analyses of variance (tailing type × fungi × bacteria) using PROC GLM. No transformation was necessary for root N, P, K, Ca, and Mg concentrations, and foliar K, Ca, and Mg concentrations. Log transformations were performed with root Fe concentration, and foliar N, P, and Fe concentrations.

Significance for all analyses was set at $\alpha = 0.05$ ($P \leq 0.05$). Means and standard errors of each treatment were calculated for all variables.

Correlation Analyses

Correlations between the percentage of roots colonized by fungi and nutrient concentration variables were investigated using PROC CORR. Correlation analyses between health variables (photochemical efficiency, percentage of healthy green foliage, and percentage of dark red foliage), growth variables (root, stem, and needle dry biomass), and nutritional variables (N, P, K, Mg, Ca, and Fe concentrations in roots and foliage) were performed in order to determine which soil elements and concentration affected positively or negatively seedling health and growth. For these analyses, block means per treatment were used. Significance for all Pearson correlation coefficients (r) was set at $\alpha = 0.05$ ($P \leq 0.05$).

RESULTS

Plant Establishment on Mine Tailings

Seedling mortality on both soil types began 8 weeks after the experiment started. While seedling mortality stabilized during the 16th week for the waste rock treatment, on fine tailings the percentage of seedling survival stopped decreasing during the 28th week only (**Figure 1**). Difference started to be significant after 20 weeks (p-value = 0.0158).

Most of the growth and health-related variables measured were independently affected by tailing type in the same manner

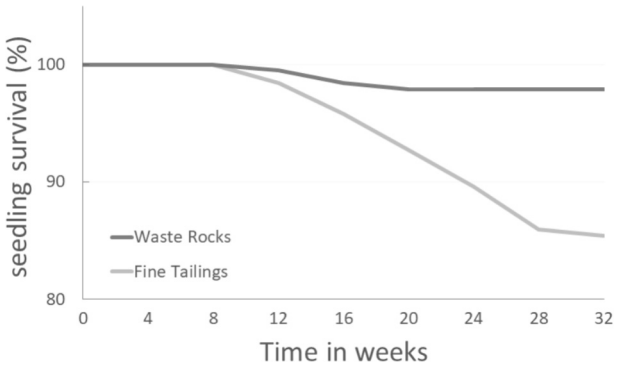

FIGURE 1 | White spruce seedling survival percentage over time on two different gold mine tailing types. White spruce seedlings were grown for 32 weeks on waste rocks (WT) or fine tailings (FT). Seedling survival rates (%) were monitored and compared. After 12, 16, 20, 24, 28, and 32 weeks, the p-values were 0.5622, 0.1261, 0.0158, 0.0007, <0.0001, and <0.0001 (Chi-square test; $\alpha = 0.05$), respectively.

[1] http://www.cef-cfr.ca/index.php?n=CEF.Collections

(**Table 1**). Seedlings grew much faster and healthier on waste rocks than on fine tailings. Aboveground, measured parameters diverged by up to 20% between the two treatments. Needle biomass, stem biomass and stem length were significantly higher for seedlings planted on waste rocks than those on fine tailings (all p-values < 0.0001). Belowground, the root system also benefited more from waste rocks tailing. Root biomass, root length, root area and root volume data of plants grown on waste rocks were 12%, 13%, 15%, and 17% higher, respectively, than those grown on fine tailings (all p-values < 0.001).

In agreement with survival rate and growth data, we found that plant health was also affected by tailing type. The percentage of dark red foliage (a bronzing symptom that can be associated with toxic mineral concentration) was much lower in seedlings grown on coarse waste rocks than on fine tailings (p-value < 0.0001). Plant-water status was also improved for seedlings planted on waste rocks than for seedlings planted on fine tailings that exhibit a more conservative water-balance. On fine tailings, root water content was lower (p-value $= 0.0278$) and plants strictly maintained higher shoot water content (p-value < 0.0001) than on coarse waste rocks.

Influence of Mineral Uptake on Plant Physiology

Among the above-mentioned health and growth parameters, several were correlated with nutrient concentrations that could diverge widely in this experiment from concentration ranges encountered in perfectly healthy seedlings. This is particularly true for Ca and Fe for which concentrations were systematically recorded at levels exceeding the normal level by up to seven and 40 times but also for other elements [**Table 2**, measured concentration ranges diverging from the ones observed in healthy seedlings (Van den Driessche, 1991) are indicated in bold].

TABLE 1 | White spruce seedlings growth and health-related parameters affected by tailing type.

	Waste rocks	Fine tailings
Needle dry biomass (mg)	33.9 ± 0.8^a	27.2 ± 0.9^b
Stem dry biomass (mg)	7.9 ± 0.2^a	6.6 ± 0.2^b
Stem length (mm)	24.2 ± 0.3^a	21.7 ± 0.3^b
Root dry biomass (mg)	51 ± 1.4^a	44.7 ± 1.4^b
Total root length (mm)	214.2 ± 5^a	186 ± 4.9^b
Total root area (mm^2)	29.2 ± 0.8^a	24.7 ± 0.7^b
Total root volume (mm^3)	0.32 ± 0.01^a	0.27 ± 0.01^b
Dark red foliage (%)	13.9 ± 1.3^b	18.1 ± 1.4^a
Root water content (%)	77.6 ± 0.4^a	76.2 ± 0.4^b
Shoot water content (%)	48.4 ± 0.3^b	51 ± 0.3^a

White spruce seedlings were grown for 32 weeks on waste rocks (WT) or fine tailings (FT). Belowground ground, root dry biomass (mg), root volume (mm^3) and total root length (cm) were affected by tailing type treatment. Aboveground, growth-related parameters affected by tailing type treatment were needle dry biomass (mg), stem dry biomass (mg) and stem length (mm). Proportion of dark red foliage (%) and root water content (%) were affected by tailing type treatment. Values are means ± SE. Different letters indicate significant difference for each growth and health-related parameter between waste rocks and fine tailings.

Growth parameters were negatively correlated with increased concentrations of Ca and N encountered in seedling roots and with concentrations of root Fe, and K, and with N in needles. Negative correlations were also observed between root dry biomass and needle Fe concentration; between stem dry biomass and needle Ca and Mg concentrations; between needle dry biomass and the needle concentrations of Ca, Mg and Fe. For health-related parameters, the percentage of dark red foliage was positively correlated with increasing concentrations of nutrients in roots (Ca, N, and Fe) and needles (K, Fe); it was negatively correlated with root K concentration. Conversely, the percentage of healthy green foliage was positively correlated with an increased root K concentration and negatively correlated with the concentration of Ca, N, and Fe in roots and with needle K and Ca concentrations.

The strongest relations of correlation were observed for both growth and health-related parameters with N and Ca concentrations. The negative correlations between N concentration in roots with root, stem and needle dry biomass were of moderate strength ($-0.53 \leq r \leq -0.44$). The negative coefficients of correlation between dry biomass and Ca concentrations in roots and in needles were especially strong ($r = -0.63$, p-value ≤ 0.0001 and $= -0.59$, p-value ≤ 0.0001, respectively). Finally, root Ca concentration did also affect at a slightly more moderate level the percentage of dark red foliage ($r = 0.47$, p-value ≤ 0.0001) and of healthy green foliage ($r = -0.42$, p-value ≤ 0.0001).

A Better Nutrition Status for Plants Grown on Coarse Waste Rocks

Apart from P for which concentration did not vary significantly during the experiment and remained only slightly below or within normal concentration range observed in healthy coniferous seedlings (**Supplementary Figure S2**, optimum concentration range indicated by the two dashed lines), tailing type influenced all tested element concentrations in roots and/or needles (**Figures 2–4**). For all elements, maxima were systematically measured in roots of plants grown on fine tailings but not on coarse waste rocks.

In general, N concentration was only slightly below optimum and benefited significantly from fine tailing treatment in both roots and needles (**Figures 2A,B**, p-values < 0.0001) where it exceeded a normal concentration range. A similar pattern was observed for K. In roots, values were within normal ranges (**Figure 2C**; p-value $= 0.0112$) and reached a maximum in needles of seedlings growing on fine tailings (**Figure 2D**; p-value < 0.0001). Soil Mg concentration was high and seedling root uptake was higher than optimum on both types of soil but particularly on coarse waste rocks (**Figure 3A**; p-value < 0.0001). But because translocation factor (**Supplementary Figure S3**) was also much lower on waste rocks, it resulted in an opposite pattern in needles: Mg concentration was normal and even lower than in fine talings-grown plants (**Figure 3B**; p-value < 0.0001). The concentrations of Ca and Fe were especially high (**Figure 4**). Compared to those growing on fine tailings, seedlings growing on waste rocks exhibited lower concentrations of Ca in roots

TABLE 2 | Pearson correlation coefficient (*r*) and their associated *p*-values calculated for physiological parameters and the measured root and needle nutrition variables.

		Root concentration						Needle concentration					
		N	**P**	**K**	**Ca**	**Mg**	**Fe**	**N**	**P**	**K**	**Ca**	**Mg**	**Fe**
Concentration range (g/kg) observed in healthy seedlings (Van den Driessche, 1991)		12–15	1–2	3–10	2–5	1–2	5.10^{-3}–0.01	12–15	1–2	3–10	2–5	1–2	5.10^{-3}–0.01
Concentration range (g/kg) measured		**9–28**	0.5–2	3–9	**10–20**	**2–5**	**0.3–4**	**7–40**	0.4–3	**3–16**	**10–34**	1–2.8	**0.1–1**
Root dry	r	**−0.53**	**0.19**	0.11	**−0.63**	0.11	**−0.26**	**−0.2**	0.04	**−0.26**	−0.1	−0.12	**−0.19**
biomass	*p-value*	**<0.0001**	**<0.05**	0.2	**<0.0001**	0.2	**<0.01**	**<0.05**	0.6	**<0.01**	0.3	0.2	**<0.05**
Stem dry	r	**−0.44**	0.1	−0.06	**−0.58**	0.06	**−0.22**	**−0.23**	−0.04	**−0.24**	**−0.3**	**−0.18**	−0.14
biomass	*p-value*	**<0.0001**	0.3	0.5	**<0.0001**	0.5	**<0.05**	**<0.05**	0.6	**<0.01**	**<0.001**	**<0.05**	0.1
Needle dry	r	**−0.49**	0.03	−0.16	**−0.54**	0.01	**−0.25**	**−0.38**	−0.16	**−0.25**	**−0.59**	**−0.35**	**−0.18**
biomass	*p-value*	**<0.0001**	0.7	0.1	**<0.0001**	0.9	**<0.01**	**<0.0001**	0.1	**<0.01**	**<0.0001**	**<0.0001**	**<0.05**
Total root length	r	**−0.51**	**0.29**	0.05	**−0.65**	0.05	**−0.48**	**−0.35**	0.17	**−0.32**	−0.04	−0.02	**−0.53**
	p-value	**<0.0001**	**<0.001**	0.6	**<0.0001**	0.6	**<0.0001**	**<0.0001**	0.05	**<0.001**	0.7	0.8	**<0.0001**
Plant height	r	**−0.48**	0.17	−0.13	**−0.62**	0.05	**−0.33**	**−0.43**	0.00	**−0.33**	**−0.39**	**−0.19**	**−0.52**
	p-value	**<0.0001**	0.06	0.2	**<0.0001**	0.6	**<0.001**	**c**	0.9	**<0.001**	**<0.0001**	**<0.05**	**<0.0001**
Dark red	r	**0.28**	−0.15	**−0.19**	**0.47**	−0.06	**0.26**	0.01	−0.13	**0.26**	−0.15	0.12	**0.21**
foliage (%)	*p-value*	**0.0011**	0.0937	**0.0347**	**<0.0001**	0.4841	**0.0036**	0.8893	0.1435	**0.0035**	0.0903	0.1691	**0.0197**
Healthy green	*r*	**−0.2**	0.16	**0.24**	**−0.42**	0.1	**−0.19**	0.11	**0.23**	**−0.23**	**−0.21**	−0.13	−0.12
foliage (%)	*p-value*	**0.02520.28**	0.0627−0.15	**0.0071−0.19**	**<0.00010.47**	0.2487−0.06	**0.03260.26**	0.22320.01	**0.0095**−0.13	**0.00860.26**	**0.0198**−0.15	0.14160.12	0.0197**0.21**
Dark red foliage (%)	*p-value*	**0.0011**	0.0937	**0.0347**	**<0.0001**	0.4841	**0.0036**	0.8893	0.1435	**0.0035**	0.0903	0.1691	**0.0197**

Optimal and measured concentration ranges are given for indicative purposes. Measured concentration ranges above normal as well as significant correlations coefficients at $\alpha \leq 0.05$ are in bold.

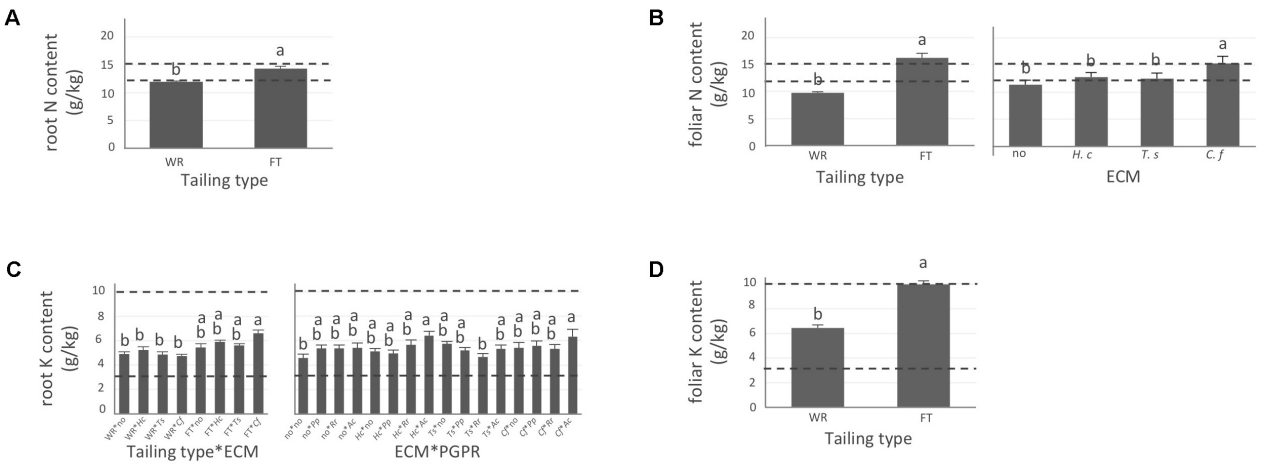

FIGURE 2 | Nitrogen and potassium concentrations of white spruce roots and needles. White spruce seedlings were grown for 32 weeks on waste rocks (WT) or fine tailings (FT) with or without mycorrhizal fungi inoculation (noF = no fungus, *+Hc* = *Hebeloma crustuliniforme*, *+Ts* = *Tricholoma scalpturatum*, and *+Cf* = *Cadophora finlandia*) and with or without inoculation of rhizobacteria (noB = no bacteria, *+Pp* = *Pseudomonas putida*, *+Rr* = *Rhizobium radiobacter*, and *+Ac* = *Azotobacter chroococcum*). **(A)** Root nitrogen (N) content (g/kg) was affected by tailing type treatment (p-value < 0.0001); **(B)** Foliar N content was independently affected by tailing type and fungal association (p-values < 0.0001). **(C)** For root potassium (K) concentration (g/kg), there was an interaction between the two factors "Tailing type x fungi" (p-value = 0.0112) and between the two factors "fungi x Bacteria" (p-value = 0.0358); **(D)** foliar K concentration was affected by tailing type (p-value < 0.0001). Dotted lines indicate optimal concentration ranges (Van den Driessche, 1991). Values are means ± SE. Different letters indicate significant difference.

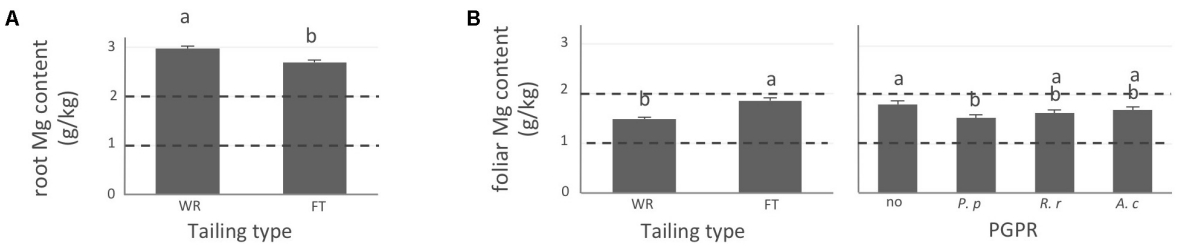

FIGURE 3 | Magnesium concentration of white spruce roots and needles. White spruce seedlings were grown for 32 weeks on waste rocks (WT) or fine tailings (FT) with or without mycorrhizal fungi inoculation (noF = no fungus, *+Hc* = *Hebeloma crustuliniforme*, *+Ts* = *Tricholoma scalpturatum*, and *+Cf* = *Cadophora finlandia*) and with or without inoculation of rhizobacteria (noB = no bacteria, *+Pp* = *Pseudomonas putida*, *+Rr* = *Rhizobium radiobacter*, and *+Ac* = *Azotobacter chroococcum*). **(A)** Root magnesium (Mg) content (g/kg) was affected by tailing type treatment (p-value < 0.0001); **(B)** foliar Mg content was independently affected by tailing type and by bacterial association (p-values; <0.0001 and $= 0.0058$). Dotted lines indicate optimal concentration ranges (Van den Driessche, 1991). Values are means ± SE. Different letters indicate significant difference.

and needles (**Figures 4A,B**; p-values < 0.0001). The aboveground concentration of Ca differed by 23% in seedlings planted on waste rocks compared to seedlings on fine tailings. A similar pattern was observed for Fe in roots (in interaction with bacterial factor, **Figure 4C**; p-values $= 0.0206$) and in needles (**Figure 4D**; p-value < 0.0001) where concentration was 38% lower in plants grown on waste rocks and dropped clearly compared to root concentration (compare y-axis scale ranges, **Figures 4C,D**).

In summary, the nutritional status of plants grown on coarse waste rocks was in general significantly better than those grown in fine tailings. It was closer or within concentration range of healthy white spruce seedlings for N and K, the availability of which was limited in Sigma-Lamaque gold mine tailings; Mg concentration fell within normal concentration range in needles; while Ca and Fe concentrations were indeed above optima but at significantly lower levels compared to plants grown on fine tailings.

Influence of Symbiotic Association on Plant Nutrition

As shown in the companion paper (Nadeau et al., 2018), root mycorrhization rate was also greater on waste rocks than on fine tailings, and much higher when plants were inoculated with the fungus *C. finlandia* originally isolated from the mining site. Albeit the concentrations of only a few elements were influenced by mycorrhizal inoculation, *C. finlandia* is a good ectomycorrhizal fungal candidate: only plants inoculated with *C. Finlandia* differ from controls regarding foliar N concentration (**Figure 2B**), root K concentration (**Figure 2C**) and foliar Fe content (**Figure 4D**).

The percentage of root tips colonized by *H. crustuliniforme* had a negative effect on root N concentration (**Table 3**) and was positively correlated with root P concentration (as it was for plants colonized with *T. scalpturatum*). On fine tailings only, the association with *C. finlandia* resulted in a positive translocation factor from the roots to the needles (**Supplementary Figure S3**). *C. finlandia* was also the one symbiont for which the negative correlation between root mycorrhization rate and root concentration of Ca was the strongest (r = 0.66, p-value $\leq$ 0.0001). It was the only one for which root mycorrhization rate was negatively correlated with root Fe concentration: the more roots were colonized the least Ca and Fe they uptake from a soil where both are found in excess. However, in roots, none of the above-mentioned nutrients was influenced by fungal treatment. Both fungi and rhizobacteria affected the aboveground Fe concentration. There was an interaction between the two treatments (**Figure 4D**; p-value = 0.0472). Compared to seedlings without symbionts, inoculation with either *C. finlandia* alone or with both the fungus *T. scalpturatum* and the rhizobacterial strain *A. chroococcum* reduced by half the accumulation of Fe in needles while no other associations improved foliar plant Fe status.

Indeed, association with *A. chroococcum* alone resulted in the highest foliar Fe concentration. *R. radiobacter* treatment also had a negative impact on foliar Fe concentration (systematically > 0.4 g/kg except in combination with *T. scalpturatum*). Besides foliar Fe measurements, it also had a significant impact on root K, foliar Mg, foliar Ca, and root

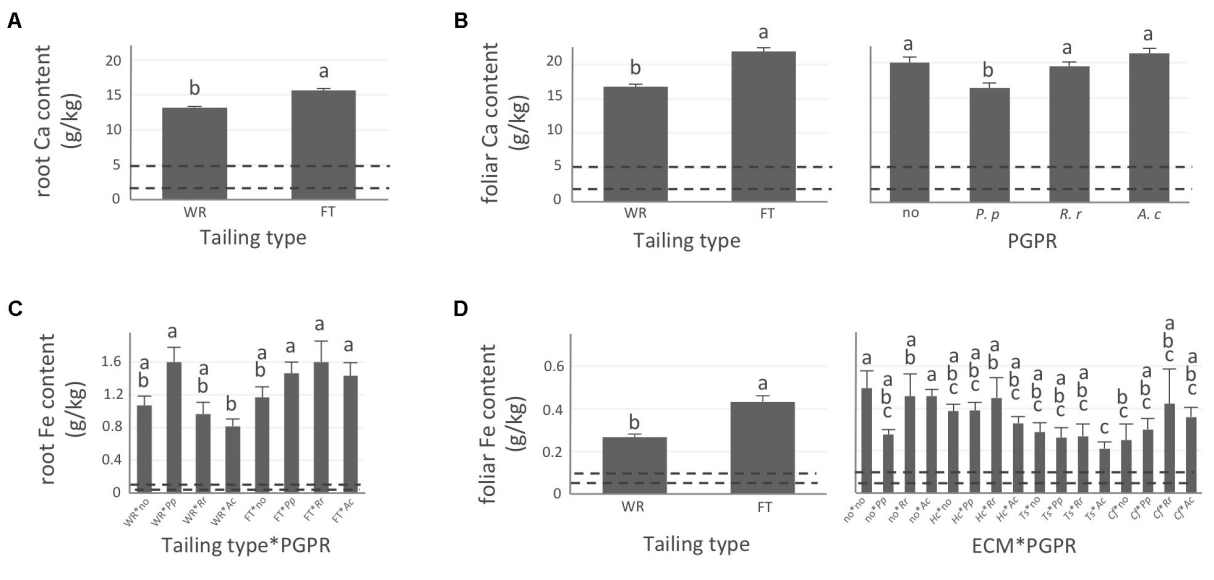

FIGURE 4 | Calcium and iron concentrations of white spruce roots and needles. White spruce seedlings were grown for 32 weeks on waste rocks (WT) or fine tailings (FT) with or without mycorrhizal fungi inoculation (noF = no fungus, *+Hc* = *Hebeloma crustuliniforme*, *+Ts* = *Tricholoma scalpturatum*, and *+Cf* = *Cadophora finlandia*) and with or without inoculation of rhizobacteria (noB = no bacteria, *+Pp* = *Pseudomonas putida*, *+Rr* = *Rhizobium radiobacter*, and *+Ac* = *Azotobacter chroococcum*). **(A)** Root calcium (Ca) content (g/kg) was affected by tailing type treatment (p-value < 0.0001); **(B)** Foliar Ca concentration was independently affected by tailing type and by bacterial association (p-values < 0.0001); **(C)** for root iron (Fe) concentration (g/kg), there was an interaction between the two factors "Tailing type x bacteria" (p-value = 0.0206); **(D)** Foliar Fe concentration was affected by tailing type treatment (p-value < 0.0001), there was an interaction between the two factors "fungi × bacteria" (p-value = 0.0472). Dotted lines indicate optimal concentration ranges (Van den Driessche, 1991). Values are means ± SE. Different letters indicate significant difference.

TABLE 3 | Pearson correlation coefficient (r) and their associated p-values calculated for the proportion of root tips colonized by different fungi and measured root and needle nutrition variables.

		Root content						Needle content					
		N	**P**	**K**	**Ca**	**Mg**	**Fe**	**N**	**P**	**K**	**Ca**	**Mg**	**Fe**
Cadophora	*r*	−0.3	0.28	−0.21	**−0.66**	0.14	**−0.44**	−0.32	0.05	**−0.43**	−0.11	−0.12	−0.09
finlandia	*p-value*	0.0942	0.1156	0.2426	**<0.0001**	0.4354	**0.0109**	0.0764	0.7959	**0.0137**	0.5308	0.5217	0.6092
Tricholoma	*r*	−0.21	**0.42**	0.05	**−0.49**	0.23	−0.23	0.04	−0.02	−0.15	0.25	−0.11	−0.3
scalpturatum	*p-value*	0.2448	**0.0175**	0.7757	**0.0047**	0.2048	0.207	0.8268	0.8905	0.4071	0.1705	0.5419	0.0966
Hebeloma	*r*	**−0.49**	**0.39**	0.26	**−0.49**	0.34	−0.23	−0.2	0.33	−0.17	−0.22	−0.27	−0.29
crustuliniforme	*p-value*	**0.0042**	**0.0283**	0.1567	**0.0047**	0.0594	0.1957	0.2744	0.0661	0.3384	0.2369	0.1357	0.1097

Significant correlations coefficients at $\alpha \leq 0.05$ are in bold.

Fe concentrations. On waste rocks, *A. chroococcum* clearly benefitted white spruces reducing by half root Fe concentration (**Figure 4C**, p-value = 0.0206) but the same strain had no effect on fine tailings. In the interaction with fungus, the bacterial strain also influenced K concentration in roots. Values remained within the normal range in all cases, but plants inoculated with *A. chroococcum* exhibited higher K concentrations (**Figure 2C**; p-value = 0.0358), a minimum average K concentration was observed for plants associated with both *T. scalpturatum* fungus and *R. radiobacter* bacterium. At the foliar level, Mg and Ca concentrations (**Figures 3B**, **4B**, p-values = 0.0058 and < 0.0001, respectively) were reduced by *P. putida* inoculation, while plants treated with *A. chroococcum* exhibited concentrations similar to those of plants treated with *R. radiobacter* or to non-inoculated white spruce seedlings. To conclude, the impact of rhizobacteria factor on overall plant nutritional status is complex: *A. chroococcum* improved Fe nutrition in some cases, *P. putida* improved both foliar Mg and Ca concentrations, and *R. radiobacter* was the only tested strain with no positive effect.

DISCUSSION

The results showed the substantial effect of microsymbiont inoculation on white spruce seedlings growing on Sigma-Lamaque gold mine tailings. In agreement with our main hypothesis, the nutritional status of plants associated with native strains of fungi and rhizobacteria was significantly improved.

Effect of Tailing Type and Particle Size on Plant Performance

In this experiment, seedlings performed much better on waste rocks than on fine tailings with respect to survival rate, health and growth. On coarse waste rocks, plants also tend to a more balanced mineral content.

Tailings do not contain any nitrogen source and only small concentrations of phosphorus and potassium. Thus one may think macro-elements availability is the limiting factor for plant survival on the mining site. However, increasing concentrations of N, P, or K in plant tissue were not associated with improvements in seedling health and growth. On the contrary, high root and foliar N concentrations were negatively correlated with seedling biomass or percentage of healthy green foliage. While macro-element concentrations in plants grown on waste rocks were within or only slightly below normal concentration ranges, N and K were measured at significantly higher levels in plants on fine tailings, mineral weathering being normally higher in finer particle substrates (Modak et al., 2001). In some cases, N concentration actually exceeded by more than twice the maximum observed in healthy coniferous trees (Van den Driessche, 1991).

Likewise, the concentrations of calcium, magnesium and iron – found in abundance in the substrates – were in general lower in roots and/or needles of plants grown on fine tailings than on waste rocks. Ca and Fe concentrations were systematically present at levels largely above the normal concentration ranges. Indeed, in this study, correlation analyses highlight the negative impact of excessive mineral uptake (Ca and Fe but also N) on plant growth- and health-related parameters. Because minerals are less mobile in coarser size residues, white spruce seedlings may perform better on waste rocks than on fine tailings of the Sigma-Lamaque gold mine – where high element concentration rather than nutrient deficiency could limit plant establishment and consequently the development of an effective phytorestoration program.

Allochthonous Microsymbionts Fail to Improve Plant NPK Nutrition

In our study, microsymbionts played an important role in reducing Ca and Fe level but the concentrations of most other elements and especially the primary macro-elements N–P–K, were not improved by the different inoculants. Microsymbiotic benefits are often specific to the site, the strain or even to the host involved. For instance regarding nitrogen nutrition, the ability of *Azotobacter* sp. to fix nitrogen is highly dependant on phosphate availability (Brown et al., 1962; Dynarski and Houlton, 2018); not all strains of *Rhizobium radiobacter* are capable of fixing nitrogen (Humphry et al., 2007); and the fungus *C. finlandia* was found important for N nutrition of Norway spruce (*Picea abies*, Mrnka et al., 2009) but not for scots pine (*Pinus sylvestris*, Alberton et al., 2010). Regarding phosphorus nutrition, foliar P does not usually differ between mycorrhizal and non-mycorrhizal seedlings, although hyphae are known to be more effective than roots at P uptake (Smith and Hinckley, 1995). Adeleke et al. (2012) also found that the inoculation of seedlings with ECM fungi enhanced potassium mobilization but did not increase foliar K concentration.

In our experiment, none of the bacterial treatments allowed the increase of seedling N uptake. Only foliar N concentration was significantly increased by the association with the fungus *C. finlandia*, albeit other treatments, including the one without symbionts, fall within normal concentration range. Because the tailings were not sterilized to mimic field conditions, we suspect that some native diazotrophic bacteria may have already been present (Dynarski and Houlton, 2018). The high pH of the substrate makes it a favorable environment (Brown et al., 1962) for the growth and development of several species capable of improving plant N nutrition (Damir et al., 2011). Interestingly, when an excessive concentration of nitrogen was measured (associated with disturbance of seedling health and growth), it was often in non-inoculated plants or in plants inoculated with allochthonous microsymbionts. In the context of multi-partite interactions like the ones we established or more complex ones that could occur in the rhizosphere of trees planted on the mine tailings, N-fixing free-living bacteria naturally occurring *in situ* may coexist better with inoculated fungi or rhizobacteria originating from the same ecosystem (Uroz et al., 2007). Fungi and bacteria already present in the mine tailings (Nadeau and Khasa, 2016) could outcompete microsymbionts that are not well-adapted, hence limiting plant N nutrition and the successful revegetation with allochtonous microsymbionts.

The tested microsymbiotic associations also failed to clearly improve P and K concentrations in roots where they were

measured under (for P) or within normal concentration range (for P and K). Detailed analysis of the data helped us to refine our strategy and to consider, in addition to mycorrhizal fungi inoculation, the use of phosphorus amendment otherwise the only macro-element present in limited quantities in the substrate and within the plant tissues. Indeed, an increasing concentration of P in plant was shown to benefit seedling growth and health, and also correlated with *T. scalpturatum* and *H. crustuliniforme* root colonization rate but not with *C. finlandia*, a fungus yet well adapted to the mining site.

Selection of Native Microsymbiotic Partners for the Successful Alleviation of Element Deleterious Effects

Among nutrients investigated, plant Ca concentration displayed the strongest negative correlation relationships with general seedling growth and health, followed by iron.

Regarding Mg concentration (measured at a high level in the substrate), on average, it was effectively found above normal concentration range in roots but not in needles. In needles, high levels of Mg impact growth and may lead to premature fall (Marschner, 1995).

Calcium, also found in excess in the mine tailings, is incidentally known to alleviate Mg toxicity (Brooks, 1987; Chiarucci et al., 1998). Moreover, the concentrations of both Mg and Ca in needles were low in presence of the bacterial strain *Pseudomonas putida*, which is well characterized for its ability to provide plants with iron through the massive production of siderophores (Bar-Ness et al., 1992). However, *P. putida* is a versatile species with a broad ability to several traits for adaptations (Matilla et al., 2011; Udaondo et al., 2013). The strain MBN0213 used in this study originated from the mining site. Similar to mycorrhizal fungi that produce calcium oxalate crystals to sequester toxic amounts of Ca (Snetselaar and Whitney, 1990; Arocena et al., 1999), strain MBN0213 may have developed specific mechanisms to reduce Ca toxicity.

Rhizobacteria inoculation did not clearly improve Fe distribution within plant tissues; only specific combinations resulted in low concentrations. In needles, the allochthonous *Azotobacter chroococcum* reduced Fe concentration when inoculated in consortium with *T. scalpturatum* but not when inoculated alone (in which case average Fe concentration equaled those found in non-inoculated seedlings or in seedlings inoculated with the siderophores-producing *P. putida*). In summary, *P. putida* MBN0213 was the most promising rhizobacterium tested. Although it neither reduced excessive Fe concentration nor improved N–P–K nutrition, it was the only bacterium that minimized Mg and Ca concentrations *in planta*, with the level of Ca being the most limiting factor we identified in this study.

Native fungi of the mining site are also of great interest, especially *C. finlandia*. It is the fungus that colonized seedling roots most efficiently and enhanced seedling health (Nadeau et al., 2018). Also, it was the only microsymbiont whose root colonization rate was negatively correlated with Fe root concentration and the most strongly correlated with Ca root uptake. Therefore, *C. finlandia* is capable of alleviating the deleterious effects of excessive Fe and Ca. On moderately polluted soil, the study of De Maria et al. (2011) demonstrated that an efficient microbial consortium formed by the dual inoculation of *C. finlandia* and PGPR enhanced goat willow (*Salix caprea*) phytoextraction efficiency. In the context of the Sigma-Lamaque gold mine tailing revegetation program, we should be able to exploit the synergistic interactions between fungi and bacteria the present study reveals.

AUTHOR CONTRIBUTIONS

MN and DK conceived and designed the experiments. MN performed the experiments. MN, JL, and DK analyzed the data and contributed in drafting the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01268/full#supplementary-material

FIGURE S1 | Illustrations and design of the experiment. Mycorrhizal fungi and rhizobacteria used in this **(A)** glasshouse experiment were either **(B)** native from the Sigma-Lamaque gold mine site (*Tricholoma scalpturatum* (Ts), *Cadophora finlandia* (Cf); *Pseudomonas putida* (Pp), *Rhizobium radiobacter* (Rr)), or isolated from a natural forest stand (*Hebeloma crustuliniforme* (Hc)) or of commercial origin for *Azotobacter chroococcum* (Ac). White spruce saplings were either planted in **(C)** waste rocks or in fine tailings. **(D)** Randomized complete block (RCB) design with three crossed fixed factors: tailing type [fine tailing (FT) in regular font, waste

rocks (WT) in bold]; fungi [none (noF) in black, Ts: green font, Cf: blue font, Hc: red font]; and bacteria [none (noB): no background, Rr: background in red, Pp: background in blue, Ac: background in green] for a total of 32 treatments, 4 blocks, 3 replicates per treatment per block, and 384 experimental units.

FIGURE S2 | Phosphorus (P) concentration of white spruce roots and needles. White spruce seedlings were grown for 32 weeks on waste rocks (WT) or fine tailings (FT) with or without mycorrhizal fungi inoculation (noF = no fungus, *+Hc = Hebeloma crustuliniforme*, *+Ts = Tricholoma scalpturatum*, and *+Cf = Cadophora finlandia*) and with or without inoculation of rhizobacteria (noB = no bacteria, *+Pp = Pseudomonas putida*, *+Rr = Rhizobium radiobacter*, and *+Ac = Azotobacter chroococcum*). Dotted lines indicate optimal concentration ranges (Van den Driessche, 1991). Values are means ± SE.

FIGURE S3 | Translocation factors. White spruce seedlings were grown for 32 weeks on waste rocks (WT) or fine tailings (FT) with or without mycorrhizal fungi inoculation (noF = no fungus, *+Hc = Hebeloma crustuliniforme*, *+Ts = Tricholoma scalpturatum*, and *+Cf = Cadophora finlandia*) and with or without inoculation of rhizobacteria (noB = no bacteria, *+Pp = Pseudomonas putida*, *+Rr = Rhizobium radiobacter*, and *+Ac = Azotobacter chroococcum*).

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frontiers in Plant Science

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Responses of the Endophytic Bacterial Communities of *Juncus acutus* to Pollution With Metals, Emerging Organic Pollutants and to Bioaugmentation With Indigenous Strains

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Plants and their associated bacteria play a crucial role in constructed wetlands. In this study, the impact of different levels of pollution and bioaugmentation with indigenous strains individually or in consortia was investigated on the composition of the endophytic microbial communities of *Juncus acutus*. Five treatments were examined and compared in where the wetland plant was exposed to increasing levels of metal pollution (Zn, Ni, Cd) and emerging pollutants (BPA, SMX, CIP), enriched with different combinations of single or mixed endophytic strains. High levels of mixed pollution had a negative effect on alpha diversity indices of the root communities; moreover, the diversity indices were negatively correlated with the increasing metal concentrations. It was demonstrated that the root communities were separated depending on the level of mixed pollution, while the family *Sphingomonadaceae* exhibited the higher relative abundance within the root endophytic communities from high and low polluted treatments. This study highlights the effects of pollution and inoculation on phytoremediation efficiency based on a better understanding of the plant microbiome community composition.

Keywords: wetland plant, *J. acutus*, endophytic bacterial community, metals, emerging organic contaminants

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INTRODUCTION

Constructed wetlands (CWs) are a promising alternative for treating various chemical compounds and for preventing their dispersion in the environment (Verlicchi and Zambello, 2014). They are engineered, state of the art sustainable systems used to treat effluents rich in pharmaceutical and personal care products by exploiting plant-bacteria interactions in combination with

Abbreviations: ARGs, antibiotic resistance genes; BPA, bisphenol A; CIP, ciprofloxacin; EOCs, emerging organic contaminants; SMX, Sulfamethoxazole.

physicochemical processes (Zhang et al., 2014). Recently, many studies have focused on investigating the efficiency of wetland plants to remove EOCs in hydroponic systems and the potential effects of these compounds on the plant physiological status (Dordio et al., 2011; Liu et al., 2013; Christofilopoulos et al., 2016). They are also efficient in removing metals from various influents such as industrial wastewater (Khan et al., 2009), landfill leachate (A et al., 2017), and acid mine drainage (Wu et al., 2015).

In such systems, the selection of both, the appropriate plant species and their associated rhizospheric and endophytic microbiota, significantly influence the performance of the CW (Guittonny-Philippe et al., 2014; Li et al., 2014). Rhizospheric microorganisms can enhance xenobiotic transformations *ex planta* and improve nutrient uptake, while the endophytic community is important for transforming the organic compounds *in planta*, thus reducing their toxicity and evapotranspiration of water-soluble and volatile compounds (Barac et al., 2004; Taghavi et al., 2005; Afzal et al., 2014). The contribution of plant-associated microorganisms to metal phytoremediation has also been highlighted through promoting plant growth in metal polluted areas, influencing metal uptake and translocation, and increasing the metal bioavailability by secretion of ligands and organic acids (Sessitsch et al., 2013; Ma et al., 2016a).

Only a few studies attempted to explore the bacterial communities associated with wetland plants and even fewer to describe the responses of such communities to mixed and highly polluted environments (Su et al., 2015; Zhao et al., 2015; Zhang D. et al., 2016; Syranidou et al., 2017b). Moreover, information relevant to the impacts of pollutants on the endophytic bacteria of wetland plants is scarce. In such complicated environments, it is unclear whether the type and level of pollution, the plant species, the application of biostimulant bacteria, or a multifactor combination influence the phytoremediation potential and underlying endophytic assemblages. However, in order to improve the performance of CWs, it is important to address these questions.

Previously, we showed that inoculation of the wetland plant *J. acutus* with a selected endophytic bacterial consortium removed emergent contaminants and metals faster and more efficiently compared to non-inoculated plants (Syranidou et al., 2016). Moreover, bioaugmentation with a tailored endophytic consortium enhanced phytoremediation efficacy, and the microbes seemed to alleviate the stress induced by the pollutants, especially at the high concentration treatments. The consortium consisted of strains tolerant to metals (Zn, Ni, Cd) and emerging contaminants (BPA, CIP, SMX) and they have been characterized as potential degraders of these contaminants (Syranidou et al., 2017b). Because insights in this important part was lacking completely, we decided to perform a deeper analyses and study the total endophytic community response. In this study, the effect of mixed pollutants and bioaugmentation with single indigenous endophytic strains or in a consortium was addressed on the endophytic bacterial community of *J. acutus*, under different levels of mixed pollutants.

MATERIALS AND METHODS

Experimental Design

Three endophytic strains (leaf B1- *Sphingomonas* sp. U33, root B2- *Bacillus* sp. R12, root B3- *Ochrobactrum* sp. R24) (Syranidou et al., 2016) were inoculated separately and as a consortium to beakers (n = 10 for every treatment) with *J. acutus* plants. One week later, two different concentrations of metals (Zn, Ni, Cd), bisphenol-A (BPA) and two antibiotics [ciprofloxacin (CIP) and sulfamethoxazole (SMX)] were added. More specifically, 50 μg L^{-1} CIP, 250 μg L^{-1} SMX, 5 mg L^{-1} BPA, 200 mg L^{-1} Zn, 20 mg L^{-1} Ni, and 1 mg L^{-1} Cd were added to the low concentration treatments and 100 μg L^{-1} CIP, 500 μg L^{-1} SMX, 10 mg L^{-1} BPA, 400 mg L^{-1} Zn, 40 mg L^{-1} Ni, and 2 mg L^{-1} Cd were used in the high concentration treatments. Further, in four treatments no pollutants were added in order to investigate the potential effects of inoculation on the endophytic community in the absence of pollutants. A two-factor design study was followed with factor 1 pollutant concentration (three levels, zero, low, high), and factor 2 bioaugmentation treatments (five levels, no inoculation, strain 1, strain 2, strain 3, consortium). In total, there were five different treatments (one non-inoculated control and four bioaugmented treatments) concerning the inoculation effect and three different concentrations of the mixture of pollutants (one without pollutants-NO, one low concentration-LC and one high concentration-HC) concerning the pollution effect. The experiment lasted for 21 days and was irrigated with 50 mL tap water every week. A schematic representation of the experimental design in provided in **Supplementary Figure S1**.

Sample Collection

Fresh root (0.3 g) and leaf (1 g) tissue samples (n = 3 for every treatment) were collected for DNA extraction at the end of the experiment. In order to sterilize the outer surface, the plant plants were immersed in 70% ethanol for 30 s and subsequently in 2% NaClO solution supplemented with one droplet Tween 80 per 100 mL solution for 10 min. Subsequently, they were rinsed three times with sterile distilled water for 1 min and 100 μL of the last rinsing solution were streaked on 869 plates (Mergeay et al., 1985) and incubated for 7 days at 30°C to verify the surface sterility. The maceration of the plant samples was performed with liquid nitrogen and total DNA was extracted using the Invisorb® Spin Plant Mini Kit (STRATEC Molecular GmbH, Berlin, Germany).

16S rRNA Gene Amplicon Libraries Preparation

The forward 799F primer (AACMGGATTAGATACCCKG) and the reverse primer 1193R (ACGTCATCCCCACCTTCC) were used for the amplification of the V5–V7 hypervariable region of the bacterial 16S rRNA gene, producing a ∼400 bp fragment (Schlaeppi et al., 2014). The primer pair was selected based on the 2 bp mismatch at the 3′-end of the 799F primer with the chloroplastidal DNA. After the first PCR, the bacterial DNA was selected over the not always present mitochondrial DNA (approximately 800 bp). The bacterial amplicons were collected and purified from agarose with the QIAquick gel extraction kit

(Qiagen, Venlo, Netherlands). For multiplexed pyrosequencing, a sample-specific 10 bp barcode (MID) was fused to the forward primer, followed by the key and a Lib-L Adaptor A sequence. Every PCR reaction contained 1 × FastStart High Fidelity Reaction buffer (Roche) with 1.8 mM $MgCl_2$ (Roche), 200 μM of each dNTP (Roche), 250 nM forward primer, 250 nM reverse primer, 1.25 U FastStart High Fidelity Taq DNA polymerase (Roche), 1 μL DNA template and RNase free water until a total volume of 25 μL. The PCR conditions were: an initial denaturation step of 2.5 min at 95°C, 35 (1st PCR) or 20 (2nd PCR) cycles of denaturation of 1 min at 94°C, annealing for 40 s at 53°C and extension for 40 s at 72°C, and a final extension step of 7 min at 72°C. The bacterial amplicons produced by the second PCR were purified and the concentration of purified DNA was determined with the Quant-iT Picogreen dsDNA assay kit (Life Technologies Europe, Ghent, Belgium) according to the manufacture's protocol. Equimolar mixtures of different samples were prepared. For checking the amplicons, 1 μL of the library was loaded on a DNA-chip (DNA 1000 kit, Agilent Technologies, Diegem, Belgium) and analyzed on a 2100 Bioanalyzer (Agilent Technologies, Diegem, Belgium). The libraries were clonally amplified using the emPCR Lib-L kit and then sequenced using the Roche 454 GS-FLX Plus Life Sciences Genome Sequencer at Macrogen, Seoul, South Korea.

16s rRNA Gene Sequences Analysis

Bacterial amplicons were quality filtered and trimmed using the DADA2 v1.8 pipeline in R v3.4 (Callahan et al., 2016) with the adapted following read settings: filtering criteria (max N = 0, Max EE = 2, TruncQ = 2, trimming of the first 15 bp, and fixed trunclength of 300 bp). Prediction of Absolute sequence variants (ASVs) was performed with DADA2, homopolymer gap penalty was set to −1 and band size was equal to 32, followed by de novo chimera removal. The high quality reads were classified against the SILVA v132 training dataset (Quast et al., 2013; Yilmaz et al., 2014; Glöckner et al., 2017) using the naive Bayesian classifier method in DADA2. The standard flowgram format (SFF) files were deposited in the NCBI Sequence Read Archive (SRA) under the accession number SRP158657.

Metal Analysis in Plant Parts

Juncus acutus roots and leaves (n = 10 for every treatment) were washed with tap water (3×) followed by washing with distilled water in order to remove any adhered particles. Next, they were dried at 50°C for 4 days. Dried plant samples (0.2 g) were digested with 9 mL HNO_3 (>69%, Sigma-Aldrich) and diluted with ultrapure water and centrifuged. Supernatants were subsequently filtered (0.45 μm, Whatman), diluted at 1:10 (v/v) with ultrapure water and analyzed by ICP-MS (ICP-MS 7500cx coupled with Autosampler Series 3000, both from Agilent Technologies).

Colonization and Distribution of Endophytes Within Host Plants

Strains were tagged with fluorescent proteins as previously described (Sánchez-López et al., 2018) in order to monitor their colonization to the host plant under similar experimental conditions. The labeled strains were inoculated (10^9 cells mL^{-1}) to plants with and without addition of pollutants. Their colonization efficiency was investigated with a confocal laser microscope Ultra VIEW VoX, PerkinElmer (Zaventem, Belgium) using an excitation wavelength of 561 nm (red) for mCherry, and 405 (Dapi) for plant cell walls while the confocal pictures were analyzed using ImageJ software and Amira 3D visualization software version 6.1.0 (FEI Visualization Sciences Group, Hillsboro, OR, United States) as previously described (Sánchez-López et al., 2018).

PCR and qPCR

The occurrence of various ARGs such as the sulfamethoxazole (*sul*I) and ciprofloxacin resistance genes [*qnrA, qnrS* and *aac(6′)-Ib*] was screened via polymerase chain reaction (PCR) detection assays. The primers sul1-F (5′-CTT CGA TGA GAG CCG GCG GC-3′) and sul1-R (5′-GCA AGG CGG AAA CCC GCG CC-3′) (Jacobs and Chenia, 2007), aac-F (5′-TTGCGA TGCTCTATGAGTGGCTA-3′) and aac-R (5′-CTCGAATGCCTGGC GTGTTT-3′) (Park et al., 2006), qnrA-F (5′-GAT AAA GTT TTT CAG CAA GAG G-3′) and qnrA-R (5′-ATC CAG ATC GGC AAA GGT TA-3′) and qnrS-F (5′-GTA TAG AGT TCC GTG CGT GTG A-3′) and qnrS-R (5′-GGT TCG TTC CTA TCC AGC GAT T-3′) (Mao et al., 2015) were used for the detection of *sul*I, *aac(6′)-lb-cr*, *qnrA*, and *qnrS* genes, respectively. PCR assays were performed as previously described (Park et al., 2006; Jacobs and Chenia, 2007; Mao et al., 2015). Since only the *sul*I was detected in the endophytic communities, the abundance of this gene was estimated using a StepOne Plus System (Applied Biosystems Inc., Foster City, CA, United States). qPCR reaction was performed in a 20 μL volume mixture and conducted in 96 well plates containing 10 μL of SYBR Green Dye (Applied Biosystems), 0.2 μM of each primer and 2 μL of template DNA. The detailed protocol was as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at 55°C for 60 s and 72°C for 2 min. All samples and standards were amplified in triplicates. For the standard curve, six-fold serial dilution of the *sul*I gene isolated from the B3 endophytic strain was performed. The amplification efficiency and coefficient (r^2) was 110% and 0.98, respectively. Melting-curve and a 1.5% agarose gel were used for assuring the specificity of the products.

Statistical Analysis

Statistical analysis was performed with R v3.3.2 (R Development Core Team, 2016). Differences in the metal concentrations and alpha diversity indices in different plant parts among the treatments were estimated with an analysis of variance (two-way ANOVA). After detecting significant differences, a multiple Tukey comparison test was performed. Correlation analysis between the diversity indices and the different metal concentrations was performed in R [package: Hmisc and corrplot (Wei and Simko, 2017)], based on Pearson's product moment correlation coefficient. Processed amplicon sequencing data were analyzed using the Bioconductor package phyloseq v1.19.1 (McMurdie and Holmes, 2013). The raw data was used prior to alpha-diversity analyses. For beta-diversity analyses, an inclusion

threshold of 2% prevalence was used, data was normalized by total sum scaling and expressed in relative abundance %, followed by a *log*(1+x) transformation. Community dissimilarities were represented by non-metric multidimensional scaling (NMDS) using the Bray–Curtis distance and by Principal coordinate analysis (PCoA) using weighted and unweighted UniFrac distance.

To evaluate the similarity of community assemblages among the samples, PERMANOVA (R-vegan function Adonis) was performed onto the Bray–Curtis dissimilarity matrix with $n = 999$ permutations. Mantel test analysis was performed in order to detect any significant correlation between the presence of metals under different levels of mixed pollution and endophytic bacteria composition using the Bray–Curtis dissimilarity matrix. LEfSe [Linear Discriminant Analysis (LDA) Effect Size] was used to detect the bacterial taxonomic biomarkers across the different treatments (Segata et al., 2011). In addition, differentially abundant taxa were identified using DESeq2 v1.14.1 analyses in R (Love et al., 2014). The Venn diagrams were generated according to Wang et al. (2016).

RESULTS

The effects of bioaugmentation with endophytic bacteria on the efficiency of phytoremediation of mixed pollutants was investigated with promising results (Syranidou et al., 2016). In this study, the effects of mixed pollutants and bioaugmentation on the root and leaf endophytic communities were investigated in depth to obtain a better understanding of the interactions between microbes and their host plants during wastewater treatment.

Metal Uptake by Plants

The metal concentrations in *J. acutus* roots and leaves tended to increase in the treatments with high concentrations of mixed pollution (**Supplementary Figures S2–S4**) while roots accumulated higher concentrations of metals compared to the leaves in every treatment. Bioaugmentation through inoculation with indigenous endophytic bacteria increased Zn concentrations in roots and leaves and two-way ANOVA revealed significant effects of the level of pollution as well as of inoculation on the metal concentrations in plants. When exposed to 200 mg L^{-1} Zn, all the inoculants significantly enhanced the phytoextraction capacity of the plants compared to the non-inoculated ones. At elevated Zn concentrations, plants inoculated with strain B1, B3 and the consortium accumulated significantly higher amounts of Zn in the roots while B1, B2, and B3 inoculated plants accumulated significantly more Zn in the leaves in comparison to the non-inoculated plants, indicating an increase in the translocation factor. With respect to nickel, the beneficial effects of bioaugmentation on the phytoextraction capacity of *J. acutus* were less pronounced. Nevertheless, significant effects on plant Ni concentration were observed depending on the metal concentrations they were exposed to. At low Ni concentrations, the B1 inoculated plants accumulated significantly more Ni in the roots in comparison to the non-inoculated plants while a significantly higher Ni concentration was detected in the leaves of the plants inoculated with the consortium. When exposed to 40 mg L^{-1} Ni, a significant increase in the Ni concentration in the roots of B1 inoculated plant was observed in comparison to the non-inoculated plants. The B1, B2 and B3 inoculated plants contained significantly higher Ni concentrations in their leaves in comparison to the non-inoculated plants.

Cadmium was not detected in leaves of all plants regardless of the initial exposure concentration and inoculation effort. In roots, there was no significant difference in Cd accumulation capacity of inoculated and non-inoculated plants at low and high Cd exposure.

Responses of Endophytic Bacterial Communities

In order to investigate the interactions between the host plant and its associated endophytic microbial community during phytoremediation of mixed polluted water as well as in case of bioaugmentation with indigenous endophytic strains, the total DNA was extracted from *J. acutus* leaves and roots and was analyzed using high-throughput sequencing. A total of 358035 16S rRNA gene sequences were obtained from 66 samples, with average read length of 353 bp. After the sequence quality filtering, denoising and removing of all chimeric sequences 63 samples remained with 231202 assembled high-quality sequences and 2680 ASVs recorded.

High levels of mixed pollution had a negative effect on alpha diversity indices of the root communities (**Figure 1A**). In particular, pollution significantly lowered the Shannon diversity (F: 8.8, $p = 0.001$) as well as all the indices. Significant differences were detected between the indices of the communities from highly polluted with the communities of plants exposed to low pollution and no pollution, while no significant differences were noticed between the low and non-polluted treatments. The Simpson diversity index significantly differed in communities exposed to low pollution and no pollution. The effect of various inoculants on Shannon diversity was minimal (F: 1.1, $p = 0.37$), as well as on all the indices.

Correlation analysis revealed that the number of observed species, phylogenetic diversity, and Shannon and Simpson diversity of the root communities negatively correlated with increased concentrations of metals in roots (**Figure 1B**). The number of observed species was negatively affected by nickel, zinc and cadmium concentrations in the root compartment (Ni: $p < 10^{-4}$, $r = -0.53$, Zn: $p < 10^{-4}$, $r = -0.56$, Cd: $p < 10^{-4}$, $r = -0.53$), as well as the Simpson diversity index (Ni: $p < 10^{-4}$, $r = -0.73$, Zn: $p < 10^{-4}$, $r = -0.77$, Cd: $p < 10^{-4}$, $r = -0.77$). Similarly, the increased metal concentrations decreased significantly the Shannon diversity (Ni: $p < 10^{-4}$, $r = -0.71$, Zn: $p < 10^{-4}$, $r = -0.76$, Cd: $p < 10^{-4}$, $r = -0.74$).

The *J. acutus* root endophytic communities seemed to alter in function of the level of pollution. As seen in **Figure 2A**, separate groups are formed, while this separation is statistically significant ($p = 0.001$). Each group involved samples of plants exposed to the same concentration of the mixture of metals

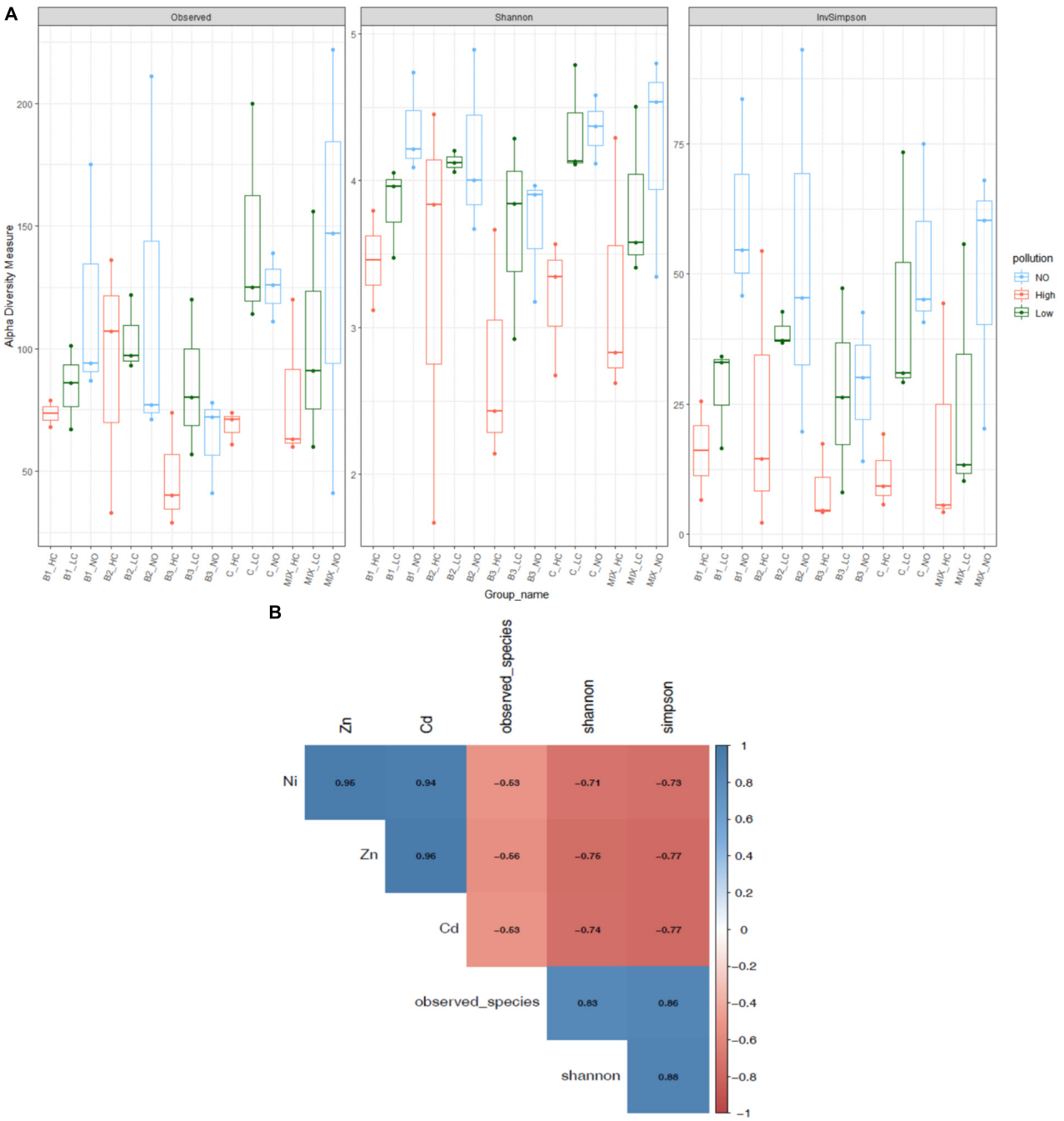

FIGURE 1 | Alpha diversity indices of root endophytic communities among the different bioaugmented treatments **(A)** and the correlation of them with the metal concentration in the roots (the significant correlation coefficients are indicated) **(B)**. The scale colors denote whether the correlation is positive (closer to 1, blue circles) or negative (closer to –1, red circles) between the diversity indices and metal concentrations.

and emerging organic pollutants (High, Low, NO), indicating an induced shift in the root community composition. Root endophytic communities from the non-polluted treatments were clearly different from the others, whereas a slight overlap was noticed between communities of plants exposed to low and high pollution. With respect to the composition of the leaf endophytic community, no clear pattern was detected (**Supplementary Figure S5**). It appears that almost all samples are grouped together regardless of the level of pollution or inoculation effort.

Mantel correlation coefficients indicated that significant relationships existed between root communities of plants exposed to different concentrations of metals (**Figure 2B**). When the root communities from the different inoculation treatments were

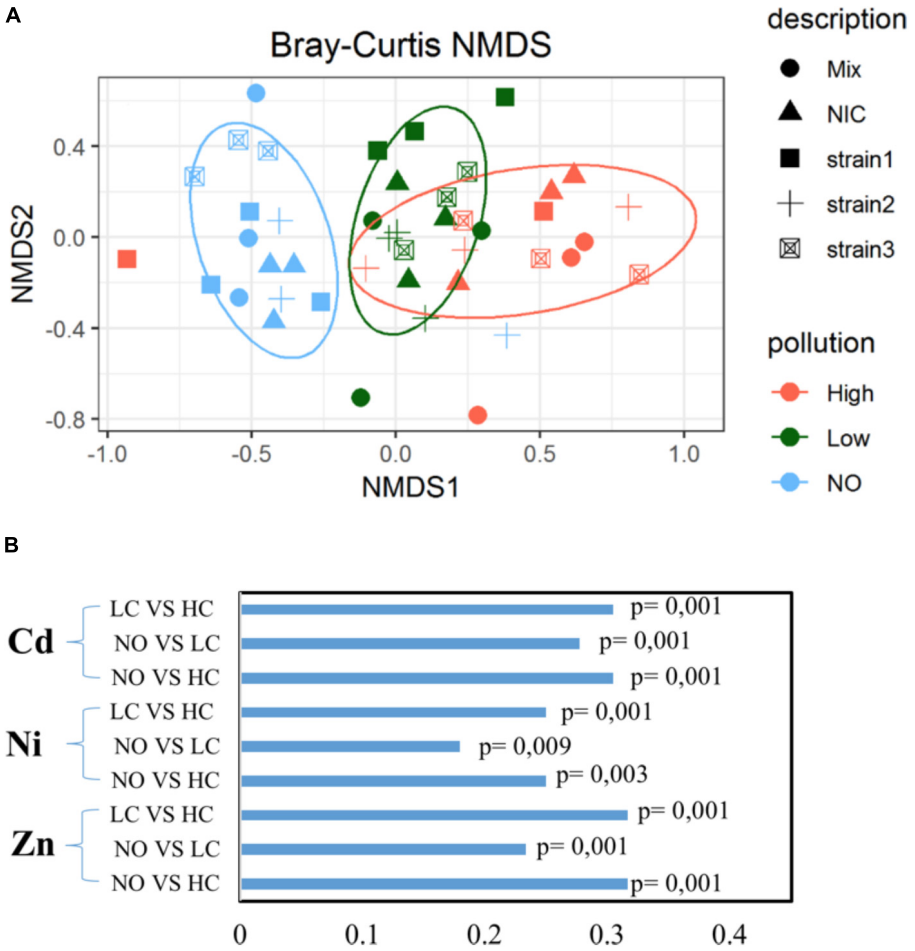

FIGURE 2 | Similarity of the root endophytic communities across a mixed pollution gradient **(A)**. Distance between the samples is based on similarity of the community composition visualized in non-metric multidimensional scaling (NMDS). **(B)** Phylogenetic correlation of root bacterial communities across metal gradient in roots using the Mantel test (999 permutations).

investigated separately, different correlations between endophytic assemblages and the exposure concentration of pollutants were revealed. The root endosphere of B2 inoculated plants was significantly correlated with the increasing metal concentrations in this plant compartment, while no association was observed between the other endophytic communities and each metal, indicating treatment-specific responses.

Endophytic Community Composition

In this study, 16 phyla were detected in the endosphere of *J. acutus*; overall the phylum Proteobacteria dominated the endophytic bacterial community. The root community consisted mainly of Alphaproteobacteria followed by Gamma-proteobacteria, and members of Bacteroidia, Fibrobacteria, and Actinobacteria (**Figure 3**).

The abundance of Proteobacteria was significantly increased in root communities exposed to elevated concentrations of mixed pollution, while the phyla Bacteroidetes and Fibrobacteres significantly decreased. In accordance, the abundance of Alphaproteobacteria was significantly increased in roots of plants grown in high polluted treatments in comparison to unpolluted ones, while the abundance of Fibrobacteria, Bacteroidia, and Deltaproteobacteria was significantly decreased. Within the root endophytes, those affiliated with the order Sphingomonadales were enriched in the *J. acutus* roots exposed to high pollution whereas those affiliated with Flavobacteriales were enriched in roots exposed to low pollution. The family *Sphingomonadaceae* exhibits the higher relative abundance within the root endophytic communities from high and low polluted treatments while the abundance of the families *Pseudomonadaceae* and *Rhizobiaceae* is higher in roots of plants grown in absence of pollutants.

The phylum Proteobacteria dominated almost all leaf communities; in general endophytic leaf community abundance profiles showed more variable patterns compared to the root communities. The most abundant classes were Alphaproteobacteria followed by Gamma-proteobacteria, and members of Bacteroidia, Fibrobacteria and Actinobacteria, while Bacilli and Saccharimonadia exhibited high relative abundance (**Figure 4**). It is important to notice that the class

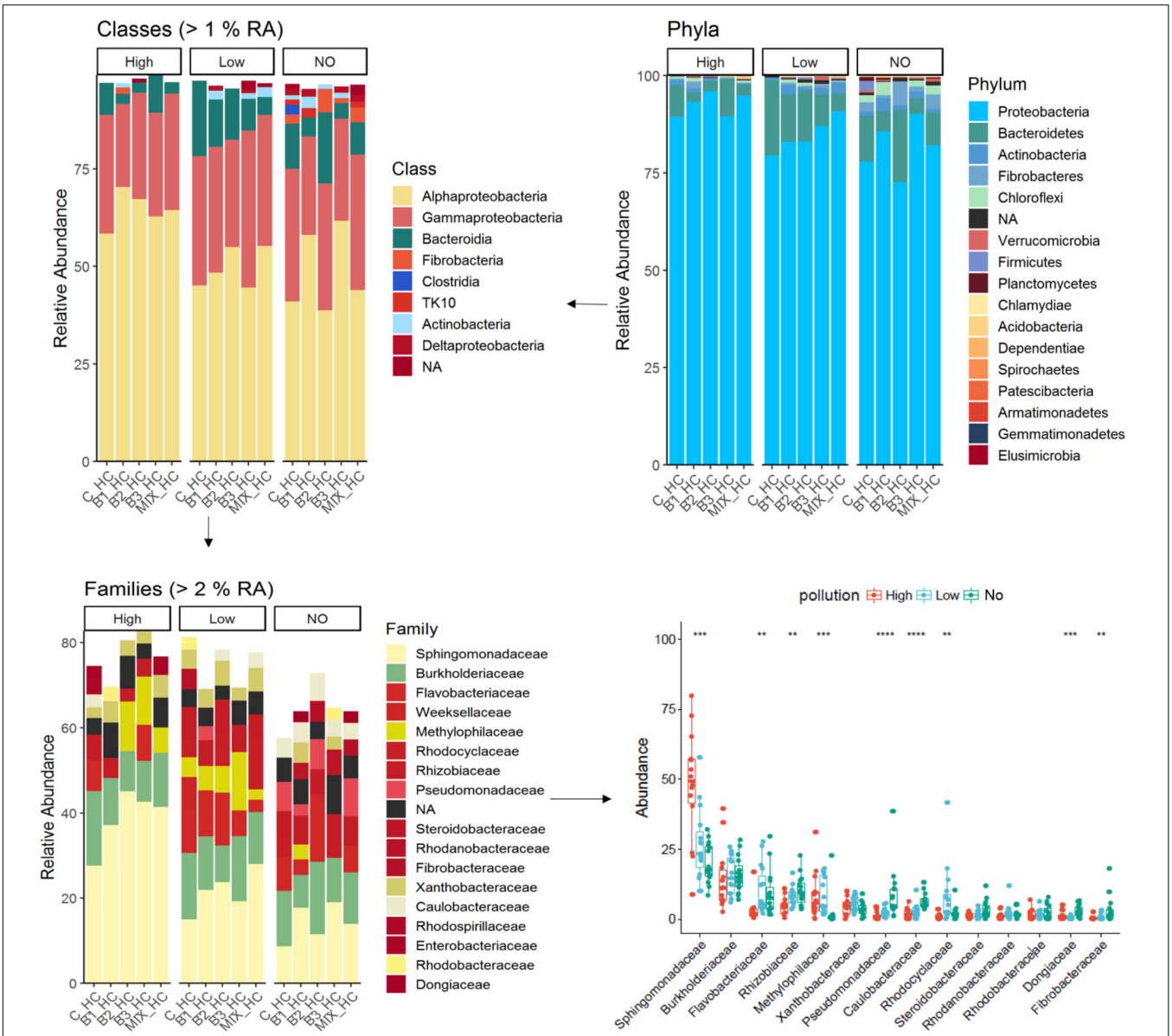

FIGURE 3 | Overview of the relative populations of root endophytes with respect to phylum, class, and family under the different level of pollution.

Bacilli dominated the leaf community of B2 inoculated plants that were not exposed to pollution which is in accordance with the inoculant. Similarly, the relative abundance of the B1 strain was higher in the leaf endophytic communities of unexposed plants in comparison to exposed ones. As seen in **Figure 5**, the endophytic stain B3 could efficiently colonize the roots surface of *J. acutus* in the presence of mixed pollution.

Changes in the community members were observed in response to increasing levels of pollution (**Figure 6**). The root communities from plants exposed to different concentrations of mixed pollution were compared in order to identify the potential indicator taxa. Only 85 root ASVs (LEfSE, $p < 0.05$, $\log_{10}$ LDA score >3.5) were enriched or depleted across the pollution gradient. More specifically, members of Proteobacteria (affiliated to *Burkholderiaceae*, *Micromonosporaceae*, *Xanthobacteraceae,* and *Rhodocyclaceae*) and Bacteroidetes (affiliated to *Flavobacteriaceae*) were enriched at the low level of pollution. ASVs assigned to the families such as *Xanthobacteraceae*, *Dongiaceae*, *Xanthomonadaceae*, *Rhizobiaceae* and *Caulobacteraceae*, *Devosiaceae*, *Fibrobacteraceae*, *Pseudomonadaceae* were discriminative for roots of plants grown in the absence of pollution. In the roots of plants exposed to high pollution, the genera *Herminiimonas*, *Methylophilus*, *Cupriavidus*, *Novosphingobium,* and *Oligotropha* were enriched. According to Venn diagrams, a high number of ASVs is shared among the root communities exposed to different levels of pollution (**Figure 7**). Similarly, the high percentage of ASVs can

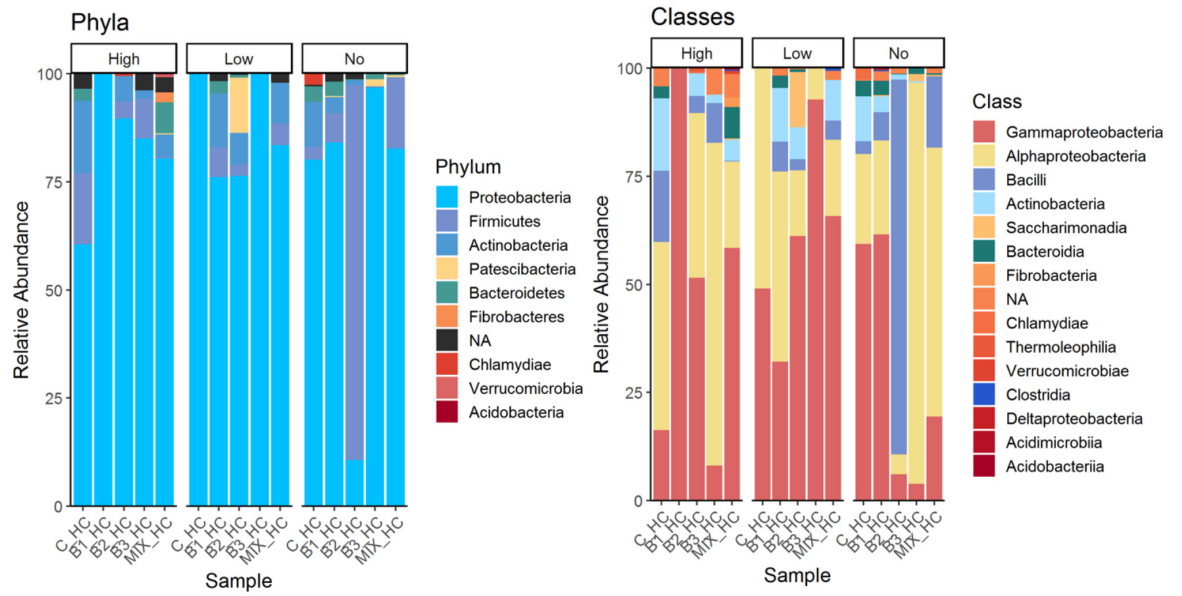

FIGURE 4 | Overview of the relative populations of leaf endophytes with respect to phylum and class under the different level of pollution.

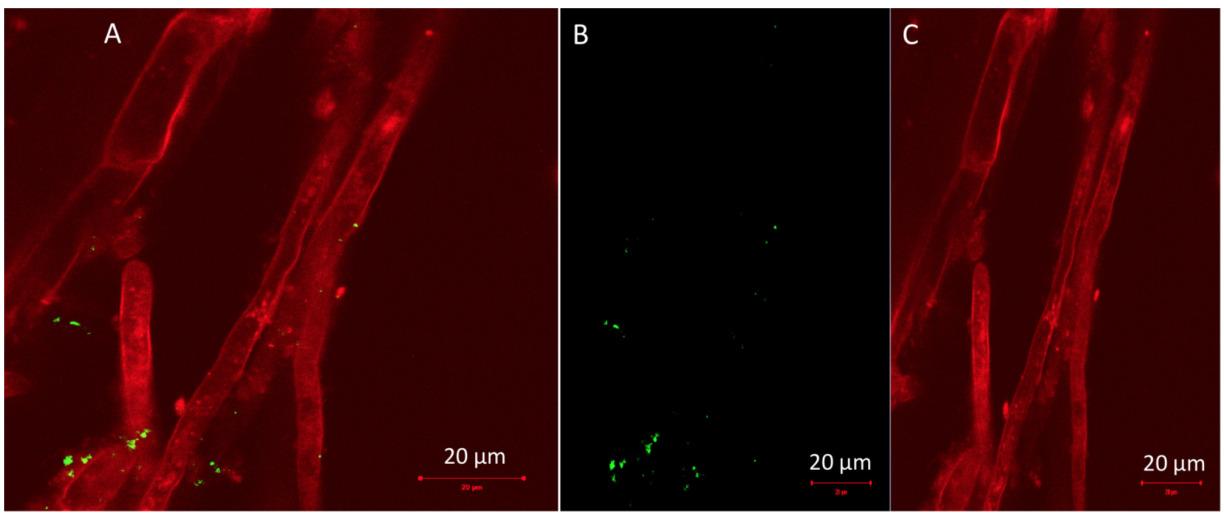

FIGURE 5 | Confocal laser scanning micrographs of **(A)** gfp-tagged B3 strain colonizing roots of *Juncus acutus*, **(B)** solution with live gfp-labeled B3 bacteria, and **(C)** root tissue autofluorescence.

be found in both plant compartments along the pollution gradient.

qPCR

One sulfonamide gene (*sul*I) was detected in the endosphere of roots of plants exposed to mixed pollution, while it was not found in the roots of non-exposed plants (**Figure 8**). Fluoroquinolone resistant genes [*qnrA*, *qnrS*, and *aac(6′)-Ib-cr*] were not found in roots. At low concentrations of pollutants, the abundance of *sul*I gene showed significantly higher values in non-inoculated roots while it was not detected in plants inoculated with the consortium. When elevated concentration of pollutants was added to the aqueous phase, the abundance of SMX resistance genes increased in B1 and B3 inoculated plants as well as plants inoculated with the consortium after 21 days of incubation. In non-inoculated and B2 inoculated plants, the *sul*I abundance remained stable at the two levels of pollution.

DISCUSSION

Applying phytotechnologies to sites with mixed pollution is a complex issue, since possible interactions between organic xenobiotics and metals may occur as well as with the associated

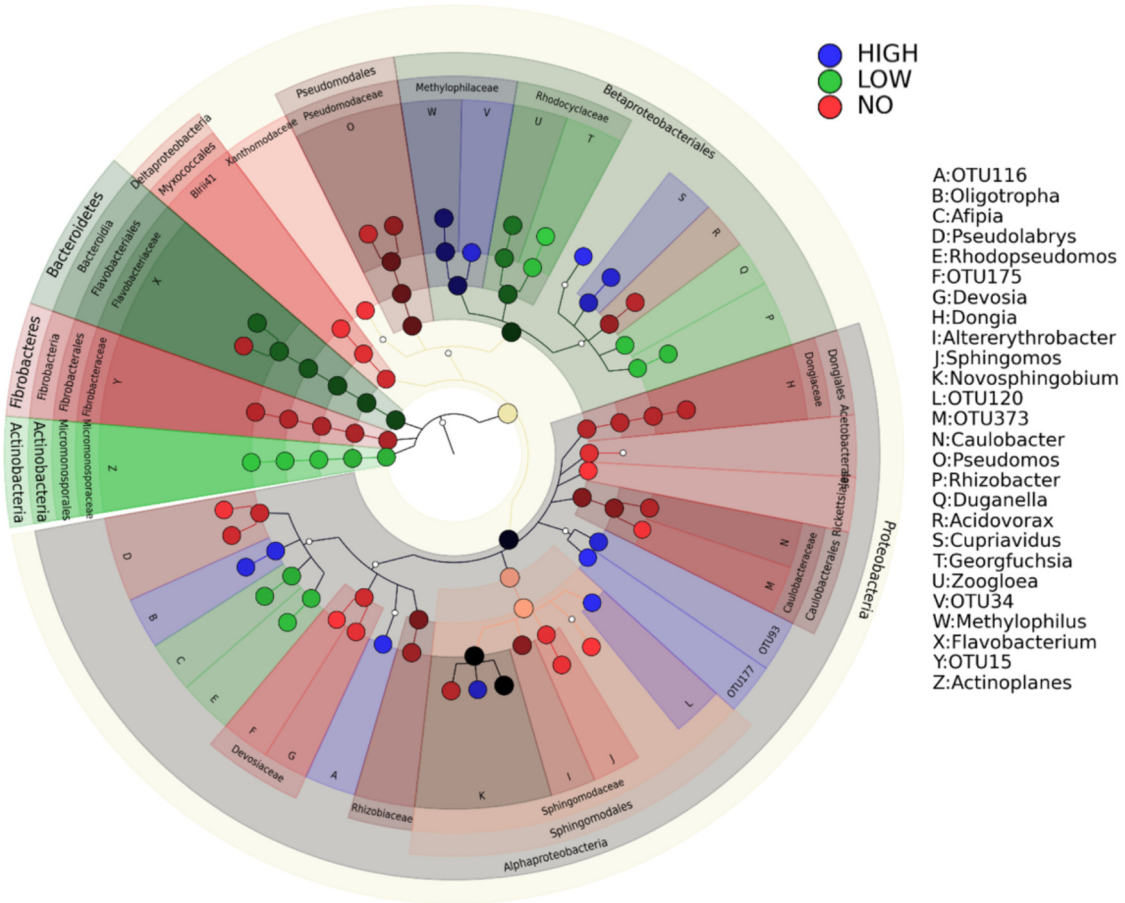

FIGURE 6 | Biomarkers of the endophytic community of roots exposed to high and low concentration of pollutants and of the roots in unpolluted treatments. LEfSe was used to validate the statistical significance and the size effect of the differential abundances of taxa (Kruskal–Wallis and Wilcoxon rank-sum $p < 0.05$ and LDA score >3.5). In the cladogram, the class, order, and family are presented and the genus is represented using letters.

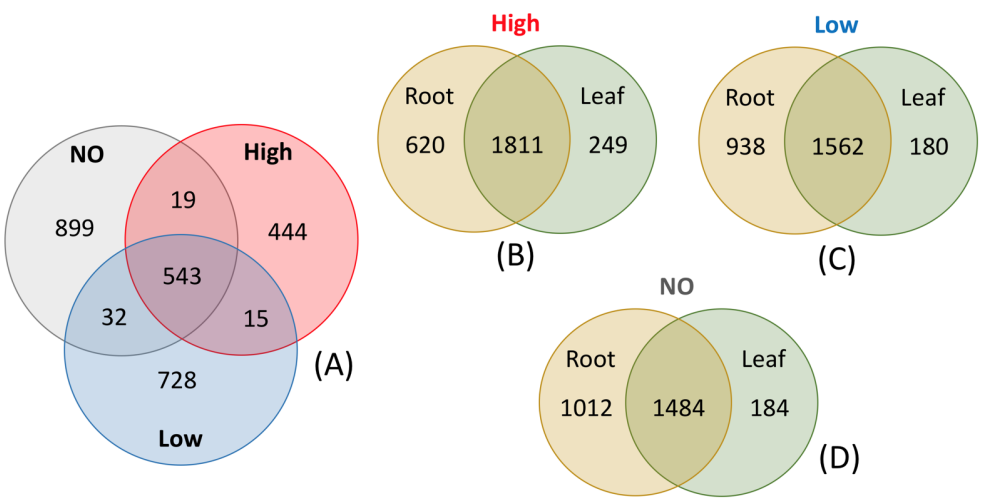

FIGURE 7 | Venn diagrams of root endophytic communities exposed to a mixed pollution gradient **(A)**, and endophytic communities of *J. acutus* exposed to high **(B)**, low **(C)** pollution as well as no pollution **(D)**.

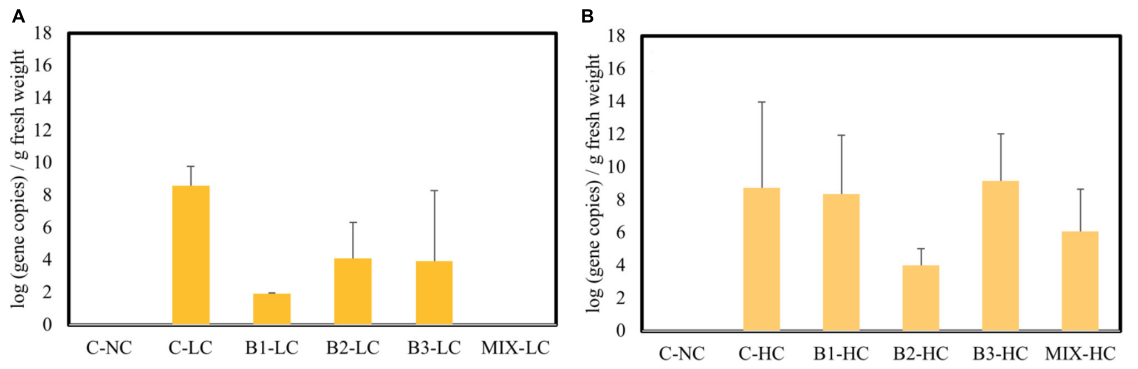

FIGURE 8 | The abundance of *sulI* gene in the root interior of plants exposed to low **(A)** high **(B)** concentration mixture of pollutants (n = 3); data with asterisk are significantly different (p < 0,05) compared to the non-inoculated plants.

microorganisms. Moreover, the presence of different pollutants causes toxicity thus affecting plant growth and performance (Chirakkara et al., 2016). Taking into consideration that the presence of a mixture of pollutants is the case in almost every CW, new approaches to improve the wastewater remediation efficiency of CWs need to be investigated. For example, choosing the appropriate plant is a crucial issue; *J. acutus* has been characterized as a promising wetland plant for both, metals and organics remediation (Syranidou et al., 2017a).

Bioaugmentation with indigenous endophytic bacteria influenced positively metal concentration in plant tissues at both levels of mixed pollution; they significantly enhanced Zn concentrations inside the plant while they also increased Ni concentrations to a lesser extent (**Supplementary Figures S2–S4**). Earlier, it was also shown that bioaugmentation with endophytic bacteria stimulates the host to uptake metals (Ma et al., 2015, 2016b; Visioli et al., 2015) while at the same time no effect of metal accumulation was demonstrated by other studies (Mesa et al., 2015). Metal tolerant bacteria expressing plant growth properties (ACC deaminase, siderophores, IAA) were inoculated to *J. acutus* as they may enhance its ability to accumulate Zn and Ni in the internal tissues by reducing the stress caused by metal toxicity. Endophytic bacteria can alter the phytoextraction capacity of their host plant through several mechanisms; they can influence the uptake, translocation, accumulation, transformation, and detoxification of metals (Ma et al., 2016a). Often, the inoculation of bacterial strains possessing plant growth promoting traits along with tolerance to the target compound has a positive phytoremediation outcome (Rajkumar et al., 2009; Afzal et al., 2014). Our results corroborate the before-mentioned observations. After considering the total amount of metals accumulated in the whole plant biomass, it was demonstrated that (in almost all cases) significantly higher amounts of Ni and Zn were accumulated inside inoculated plants in comparison to non-inoculated ones. It is worth mentioning that the consortium had the most pronounced effect on the concentrations of all three metals in *J. acutus*: the consortium inoculated plants contained approximately three times higher Ni, two times higher Zn and 1.5 times higher Cd concentrations in comparison to the non-inoculated ones. Utilizing a variety of metal tolerant strains ensured that many PGP characteristics will be expressed since the single inoculants are able to produce a limited number of traits.

The bacterial community is an important factor in determining the removal rates in CWs (Meng et al., 2014), and is strongly affected in terms of functionality and diversity by the presence of helophytes (Fernandes et al., 2015; Button et al., 2016). The type and the concentration of pollutants are also significant factors that shape the plant associated communities; negative trends in diversity of endophytic bacteria have been described in response to increased levels of pollutants (Peng et al., 2013; Su et al., 2015). High metal concentrations were reported to have negative or adverse effects on the microbial community (Khan et al., 2010; Mucha et al., 2013). In the more sensitive environment such as CW in comparison to soils, multi-metal pollution caused changes in the bacterial community (Zhang C. et al., 2016). A significant negative correlation between the alpha indices of the root endophytic community and the metal concentration was observed (**Figure 1**). Moreover, the root communities were separated in response to the level of pollution; communities from the non-polluted treatments were clearly different from the others, whereas a slight overlap was observed between communities of low and high-polluted treatments (**Figure 2**). Similarly, significant differences were detected in the cultivable endophytic communities isolated from *Halimione portulacoides* across a gradient of metal(loid) pollution (Fidalgo et al., 2016). A significant dissimilarity has also been revealed between the DDE-exposed and non-exposed root endospheres of *Cucurbita pepo* (Eevers et al., 2016).

For the leaf endosphere, a convergence among the leaf composition has been demonstrated and it may be attributed to the lower metal concentrations in this plant compartment (**Figure 4**). This suggests that this compartment is affected to a lesser extent by the mixed pollution. However, the cultivable leaf endophytic communities of *Spartina alterniflora* were reported to vary among plants harvested from oil polluted sites (Kandalepas et al., 2015). Most likely, the limited dispersal possibilities of seeds along with the oil presence and stochastic phenomena contribute

to this dissimilarity. Host plant, sampling site and time have been identified to be significant factors that influence the leaf community at non-polluted areas (Ding et al., 2013).

Proteobacteria dominated the cultured endophytic community of several wetland plants growing in CWs (Calheiros et al., 2016). In our study, the family *Sphingomonadaceae* exhibits the higher relative abundance within the root endophytic communities from high and low pollution treatments (**Figure 3**). This is not surprising since this family comprises of well-known genera for their ability to degrade a variety of organic pollutants (Acosta-González et al., 2015; Waigi et al., 2017). This family together with the *Methylophilaceae*, *Burkholderiaceae,* and *Xanthobacteraceae* are the marker families in the root microbiome at high pollution treatments (**Figure 6**). Members of the family *Methylophilaceae* has been found to exhibit higher abundances in metal polluted soils (Kou et al., 2018). Increase in the abundance of the families *Burkholderiaceae* and *Xanthobacteraceae* has been demonstrated in the enrofloxacin and ceftiofur acclimated communities from rhizosphere sediments of CWs (Alexandrino et al., 2017). It is important to mention that the abundance of ASVs affiliated with the family *Pseudomonadaceae* decreased significantly in the roots exposed to low and high pollution although this family is frequently isolated from plants at polluted sites (Afzal et al., 2014).

The plant itself in combination with the environmental parameters such as the type and concentration of pollutants can selectively enrich specific genotypes of endophytic bacteria (Siciliano et al., 2001). For example, the concentration of genes encoding for PAH-ring hydroxylating dioxygenases was stimulated in endophytic communities isolated from plants growing in more polluted areas (Oliveira et al., 2014). Moreover, the abundance of these genes was significantly higher in a root endosphere of plants exposed to phenanthrene in comparison to the control community (Hong et al., 2015). In our study, an antibiotic resistance gene to sulfamethoxazole was identified in the root endosphere of plants that were exposed to pollution and in the B3-inoculated strain while the presence of this gene was below detection level in the roots of non-exposed plants (**Figure 8**). Further experiments will reveal to what extent these genes can enhance the *in planta* sulfamethoxazole degradation, since the antibiotic can be dispersed within the plant parts. However, it is difficult to attribute this *in planta* enhancement of functional traits either to plant or to pollutant selection. For sure, the host plant plays an important role, influencing the endosphere composition and thus developing species specific relations in a polluted environment (Phillips et al., 2008; Lumactud et al., 2016). In unpolluted environments, it has been demonstrated that the host genotype affects the endophytic community to a lesser extent than the soil type or the soil microbial community (Bulgarelli et al., 2012; Lundberg et al., 2012). The 16S rRNA gene pyrosequencing of bulk soil, rhizosphere, and root samples of eight *Arabidopsis* ecotypes grown in two soil types revealed that among all the 778 OTUs, only 12 OTUs showed host genotype–dependent quantitative enrichment of the root endophytes (Lundberg et al., 2012).

Although the pollution has been characterized as a significant factor that influences the plant microbiome, inoculation of indigenous endophytic bacterial strains did not alter the endophytic community structure, which is in accordance with other studies (Conn and Franco, 2004). Despite the fact that differences were detected in the metal concentrations in the plants, inoculation of indigenous bacterial strains was not strongly influencing the community. In accordance with this observation, inoculation with *Burkholderia phytofirmans* PsJN to maize cultivars did not change the shoot and rhizosphere communities (Touceda-González et al., 2015). However, a strong effect on the soil microbial community has been observed after inoculation with a soil strain, while inoculation with a consortium had minor effects (Festa et al., 2016).

Until now, only few studies have investigated the effects of host genotype–dependent variation on the root bacterial microbiota profiles and the responses of endophytic communities to pollution with high-resolution techniques. In case of CWs, the studies mainly focus on the soil and rhizosphere compartment (Fernandes et al., 2015; Yi et al., 2016). This study contributes to the understanding of host-pollutant interactions and the endosphere which in turn should aid us in improving the efficiency of CWs. However, more studies are required to shed light on the underlying mechanisms that drive the interactions between plants and their bacterial community in response to increased levels of stress.

CONCLUSION

Engineering the plant endosphere towards enhancing the efficiency of the host is expected to expand CW applications. Bioaugmentation with indigenous endophytic bacterial strains was shown to improve the metal phytoextraction potential of the wetland plant *J. acutus*. The increased concentration of the mixture of pollutants seemed a crucial factor that decreased the diversity and shaped the root endosphere communities while the inoculation effort had minor impact. Moreover, the diversity of the root communities showed a significant correlation with the metal concentrations in this plant part as well as the community composition, while the extent of this correlation varied among the inoculated plants. In contrast, the leaf communities seemed to remain unchanged across the pollution gradient the plants were exposed to. Specific ASVs were enriched in the root compartment in response to high levels of mixed pollution. However, it has not yet been examined whether plants growing in multi-polluted soils/water alter the survival potential of specific resistant and/or beneficial microbes. Thus, it is crucial to explore the diversity, distribution, and activity of endophytic microbial communities associated with various plant species in phytoremediation studies and monitor the changes induced due to pollution. To the best of our knowledge, this is the first study that uses high throughput analysis in order to elucidate the responses of endophytic communities to different levels of mixed pollution, including metals and emerging organic contaminants. More studies are needed to reveal the underlying mechanisms that drive the

synergistic relationships between plants and their endophytic bacteria in order to exploit this symbiosis towards more robust and resilient phytoremediation technologies.

AUTHOR CONTRIBUTIONS

ES performed most of the experimental work and wrote the first draft manuscript. ST helped with the amplicon libraries preparation, the analysis of the metagenomic data, and with the discussion of the statistics. ES, ST, and IP monitored the colonization efficiency of the strains. MA performed the qPCR analysis. ES, ST, NW, DV, JV, and NK contributed in the design of the experiments. ST, NW, DV, JV, and NK helped with the proofreading, and revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01526/full#supplementary-material

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