BIOTECHNOLOGICAL POTENTIAL OF PLANT-MICROBE INTERACTIONS IN ENVIRONMENTAL DECONTAMINATION

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PUBLISHED IN: Frontiers in Plant Science and Frontiers in Microbiology

frontiers Research Topics

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ISSN 1664-8714
ISBN 978-2-88963-292-3
DOI 10.3389/978-2-88963-292-3

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BIOTECHNOLOGICAL POTENTIAL OF PLANT-MICROBE INTERACTIONS IN ENVIRONMENTAL DECONTAMINATION

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Citation: Ma, Y., Rensing, C., eds. (2020). Biotechnological Potential of Plant-Microbe Interactions in Environmental Decontamination. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88963-292-3

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frontiers in Plant Science

EDITORIAL
published: 26 November 2019
doi: 10.3389/fpls.2019.01519

Editorial: Biotechnological Potential of Plant-Microbe Interactions in Environmental Decontamination

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Keywords: plant growth promoting microorganisms, plant-microbe-metal interactions, biotechnology, climate change induced-stresses, environmental decontamination

Editorial on the Research Topic

Biotechnological Potential of Plant-Microbe Interactions in Environmental Decontaminaion

Soil contamination with heavy metals and organic contaminants has become a major global environmental problem. Phytoremediation, use of plants to immobilize, extract metals or degrade organic pollutants, provides a cost-effective eco-benign alternative to traditional methods. In most cases, plants act indirectly by stimulating beneficial rhizosphere and endophytic microbes, which could facilitate/accelerate phytoremediation process by improving plant growth, altering soil metal bioavailability or facilitating the degradation of organic pollutants (known as bioaugmentation). Plant growth-promoting microorganisms (PGPM) [e.g., plant growth-promoting bacteria (PGPB), rhizobia, and arbuscular mycorrhizal fungi (AMF)] exhibiting plant growth-promoting (PGP) traits [e.g., synthesis of indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, siderophores, surfactants, nitrogen (N) fixation, solubilization of phosphate (P) and potassium (K)] can enhance plant biomass production. Furthermore, AMF can contribute considerably to the short-term underground carbon (C) sequestration by retaining photosynthate C transferred by their host plant and/or stabilizing soil aggregate in the phytoremediation systems. In the case of organic pollutants, the application of pollutant-degrading bacteria and fungi can improve phytoremediation due to their ability to partially degrade organic pollutants or metabolize pollutant degradation products to CO_2 and water. Regarding heavy metal decontamination, the release of organic acids and acidification of rhizosphere soils by metal-mobilizing microbes may facilitate phytoextraction, whereas the release of root exudates (such as sugars, amino acids, and enzymes) and precipitation of metal-immobilizing bacteria are beneficial to phytostabilization. However, the knowledge on the understanding of soil microbial communities and their functions, plant performance and metabolism, as well as environmental conditions that promote predictable activities of both plants and microbes in polluted soils is far from complete. Therefore, this research topic was launched to advance our knowledge of integrated response of plant-microbe-soil interactions and underlying mechanisms, and review recent progress on how the estimable environmental microbial biotechnologies can be used to boost plant growth and metabolism, appropriate microbial community assembly, and eventually improve phytoremediation efficiency, therefore contributing to translate basic knowledge into sustainable applications.

Plant-microbe interactions play a critical role in plant adaption to metalliferous environments, stimulation of plant growth, and thus can be explored to accelerate microbe-aided phytoremediation. Ma et al. (2016a) extensively reviewed the recent advances to understand the biochemical (e.g., chemotaxis, colonization, beneficial functioning) and molecular mechanisms (e.g., signal and

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: *08 August 2019*
Accepted: *31 October 2019*
Published: *26 November 2019*

Citation:
Ma Y (2019) Editorial: Biotechnological Potential of Plant-Microbe Interactions in Environmental Decontamination. Front. Plant Sci. 10:1519. doi: 10.3389/fpls.2019.01519

volatiles, quorum sensing, chemical signal) of plant-microbe interactions and their potential in the phytoremediation process, which may contribute significantly to the practical application of phytoremediation techniques.

Although the impacts of PGPB on plant growth have been well investigated, limited information is available whether the application of PGPB promotes soil nutrient (e.g., N, P, and K) availability and the growth of fruit crops. A pot experiment containing four treatments was conducted by Shen et al. to evaluate the impacts of the complex PGPB inoculation on soil microflora, *Actinidia chinensis* growth, soil N fixation, and solubility of P and K. The results indicated an improvement of soil nutrient (N, P, and K) bioavailability, plant biomass, and N, P, and K uptake through the complex inoculant, suggesting this complex bacterial inoculant might be utilized as a biofertilizer for increasing soil fertility and thereafter plant growth.

Among PGPB, plant growth-promoting rhizobacteria (PGPR) are the essential component of phytoremediation technologies (Ma et al., 2011). To understand the roles of PGPR in microbe-aided phytoremediation, Fang et al. screened Cu/Zn-resistant PGPR isolates and assessed their bioremediation potential (plant growth enhancement and metal solubilization/tolerance/biosorption). Of those 10 Cu/Zn-resistant ACC-utilizing rhizobacterial isolates with superior PGP traits (e.g., P solubilization, production of IAA and siderophores), *Acinetobacter* sp. FQ-44 was chosen as the most profitable strain due to its abilities to 1) promote *Brassica napus* seedling growth under gnotobiotic conditions; 2) tolerate high concentrations of Cu and Zn; 3) mobilize the greatest amounts of water-soluble Cu, Zn, Pb, and Fe; and 4) adsorb the greatest amounts of Cu and Zn. The findings imply that *Acinetobacter* sp. FQ-44 might be exploited for microbe-aided phytoextraction. This study provided a viable method for screening metal-resistant PGPR that can be used to facilitate/accelerate phytoremediation of multi-metal contaminated soils.

In addition to plant growth promotion (e.g., production of ammonia, ACC deaminase, IAA and hydrogen cyanide) and metal uptake potential, Płociniczak et al. also studied the ability of PGPR *Brevibacterium casei* MH8a to colonize plant tissues using an antibiotic (e.g., rifampicin) as a biomarker. Furthermore, phospholipid fatty acid (PLFA) analysis was used to evaluate the ecological impacts of bioaugmentation on indigenous bacterial communities. The results demonstrated that the introduction of MH8a into soil resulted in its colonization of roots and leaves, enhanced *Salix alba* biomass and metal (Cd, Zn, and Cu) accumulation in roots, and temporary change in the structure of the autochthonous bacterial communities. The findings imply its long-term survival in soil, endophytic and PGP features, bioremediation potential, as well as its temporary impact on indigenous microbes.

Plants are often confronted simultaneously with both biotic and abiotic stresses, resulting in a reduction in plant growth and yield (Ma et al., 2016a). It is crucial to understand the impact of PGPR on multiple-stress amelioration and plant performance. Laksmanan et al. reported that plants subjected to arsenic (As) regime increased their susceptibility to infections of the blast pathogen *Magnaporthe oryzae*; however, inoculation of PGPR *Pantoea* sp. EA106 reduced blast infections and As uptake by rice. This is attributed to the up-regulation of defense-related genes mediated by *Pantoea* sp. EA106. The findings show the first evidence of how rice copes with mixed stress (As and blast infection) regimes.

Apart from the use of PGPR possessing biocontrol properties, developing resistant varieties *via* inserting resistance genes with marker-based breeding is an alternative to reduce and eliminate blast infections. Jain et al. used the resistant near-isogenic line (NIL) of Pusa Basmati-1(PB1) to investigate blast resistance gene *Pi9*-mediated resistance response. Moreover, they performed transcriptome profiling to unravel *Pi9*-mediated resistance mechanisms.

Besides PGPR, endophytic bacteria that colonize healthy plant tissues without causing symptoms have been receiving attention lately for their capacity to promote plant growth and thus phytoremediation. IAA produced by endophytic bacteria is known to play a crucial role in the interactions between plant hosts and their endomicrobes. Chen et al. evaluated the impacts of the bacterial endophyte *Pseudomonas fluorescens* Sasm05 and exogenous IAA on plant physiochemical traits, cadmium (Cd) uptake and the expression of metal transporter genes in *Sedum alfredii* Hance grown in a hydroponic media contaminated with Cd. The results demonstrated that both exogenous IAA and Sasm05 inoculation improved plant growth and photosynthesis; however, Sasm05 had a greater influence on uptake and translocation of Cd, suggesting that under Cd stress Sasm05 may produce IAA to stimulate plant growth and regulate the expression of Cd uptake and transport genes to improve plant Cd uptake.

The application of plant-endophyte symbiotic systems can also be a prospective approach to boost phytoremediation efficiency. Ma et al. (2016b) isolated, characterized and identified a multi-metal resistant and plant growth-promoting endophytic bacterium (PGPE) *Achromobacter piechaudii* E6S and evaluated its role in plant growth, metal uptake and translocation in *Sedum plumbizincicola*. Strain E6S was found to resist high concentrations of various metals (Cd, Zn, and Pb) and exhibit PGP traits (e.g., IAA production and P solubilization), increase soil metal (Cd, Zn, and Pb) bioavailability, and also bind considerable amounts of metal ions (Zn > Cd > Pb) on its cells. In the pot experiments, E6S inoculation enhanced plant biomass and accumulation of Cd, Zn, and Pb, but reduced translocation factor of Cd and Zn. The results indicate that *A. piechaudii* E6S can promote metal rhizoaccumulation and thus phytostabilization efficiency.

Similarly, Visioli et al. assessed the impacts of five specific nickel (Ni) resistant PGPE strains (individual and co-inoculation) on phytoextraction potential of Ni-hyperaccumulator *Noccaea caerulescens*. The results demonstrated that individual bacterial inoculation was not effective in increasing the growth and Ni translocation in *N. caerulescens*, except for *Arthrobacter* sp. Ncr-1 and *Microbacterium* sp. Ncr-8. Co-inoculation of Ncr-1 and Ncr-8 with *N. caerulescens* resulted in dense colonization of roots and leaf epidermal tissues and was more effective in the plant growth promotion, Ni removal from soil and translocation within the plant, together with that of Fe, Co, and Cu. The

findings suggest that tolerance/adaptation of *N. caerulescens* to highly Ni-polluted serpentine soils can be improved by an integrated PGPE community.

Mesa et al. investigated that a high proportion of metal (As, Cu, and Zn) resistant endophytic bacterial strains were the most abundant in soil and tissues of metal bioaccumularor *Spartina maritima* growing in polluted estuaries. These strains possessed multi-enzymatic properties (e.g., amylase, cellulase, chitinase, protease, and lipase) and PGP properties (e.g., N fixation, P solubilization and production of IAA, siderophores, and ACC deaminase). After inoculating *S. maritima* with a consortium of PGPE strains, they found that endophytic inoculation increased plant photosynthesis and intrinsic water use efficiency, but reduced metal accumulation. The findings imply that inoculation of indigenous metal-resistant PGPE can be considered as a practical approach to facilitate/accelerate the adaption/tolerance and growth of *S. maritima* in polluted estuaries, but unsuitable for rhizoaccumulation purposes.

Air pollution results in adverse effects on human health and ecosystems. It is well known that plant leaves can absorb air pollutants, and leaf endophytes can transform contaminants into less or nontoxic forms; however, their integrated capacities for air remediation have been scarcely explored so far. Wei et al. reviewed bioremediation of air pollutants, with a focus on the advances in omics technologies and molecular basis underlying the role of plant leaves and leaf-associated microbes in the reduction of air pollutants, therefore providing theoretical bases for developing phylloremediation to mitigate pollutants in the air.

Besides indigenous microbes, engineering the plant-associated microbiome (PAM) is expected to promote plant survival, growth, and performance in contaminated soils. Yergeau et al. modified the PAM *via* gamma-irradiation followed by soil inoculation, which caused short-term shifts in microbial communities, but lasting impacts on growth traits of *Salix* sp. They hypothesized on the potential of manipulating the PAM to modify target plant characteristics. However, it did not occur in this study. They also highlighted several key factors when engineering the PAM.

Zhang et al. investigated flux profiles of Cd^{2+}, Ca^{2+}, and H^+ in axenic cultures of two *Paxillus involutus* strains, ectomycorrhizal (EM) *Populus* × *canescens*, and non-mycorrhizal (NM) roots using a non-invasive micro-test technique. The results showed that EM *P.* × *canescens* roots maintained high production of H_2O_2 and activity of H^+-pumping, which activated the plasma membrane Ca^{2+} channels and hence stimulated a high Cd^{2+} influx under Cd stress. They proposed a signaling pathway that triggered Ca^{2+} channel-mediated Cd^{2+} influx in the roots of NM *P.* × *canescens* and elucidated the indubitable Cd^{2+} stimulation in EM associations under Cd stress.

The potential ecological impacts of long-term growth of genetically modified plants (GMPs) have received increasing attention. Qi et al. examined fungal diversity associated with 15-year conventional cotton, 10-year *Bacillus thuringiensis* cotton, and 15-year transgenic cotton, and monitored the variation in fungal communities over an annual growth cycle. The results indicate that among different transgenic cultivars or lines variations of microbial diversity could exist, and the unintended variations between conventional and transgenic cotton may be within a generally acceptable range.

It is well known that bacterial ACC deaminase encoded by the gene *AcdS* is regulated differently under different environmental conditions and can help plants alleviate biotic and abiotic stresses by hydrolyzing ACC (a plant ethylene precursor) into ammonia and α-ketobutyrate, therefore reducing ethylene level and providing carbon and N for bacterial growth (Ma et al., 2016a, 2016b). Singh et al. explored current knowledge of bacterial ACC deaminase induced plant physiological changes, mechanism of enzyme action, genetics and distribution and its ecological role, as well as avenues for future research to explore transgenic plants expressing a foreign *AcdS* gene to adapt to environmental stresses.

Most terrestrial plant roots form a symbiosis with AMF, which contributes significantly to nutrient cycling and ecosystem sustainability. To understand the role of AMF in nutrient acquisition and soil stress alleviation, Aliferis et al. attempted to elucidate the changes in the metabolic response of *Salix purpurea* during AMF symbiosis through recording the fluctuation of leaf metabolome. The results revealed that AMF inoculation caused up-regulation of various biosynthetic pathways (e.g., flavonoid, isoflavonoid, phenylpropanoid, chlorophyll, and porphyrin), which had important roles in plant physiology and resistance to various environmental stresses. The fluctuation in leaf metabolism may provide AMF-inoculated *S. purpurea* with a significant advantage when grown in highly polluted soils. The discovered biomarkers of *S. purpurea* response to AMF inoculation and corresponding pathways might be utilized in the biomarker-based selection of *S. purpurea* cultivars with high phytoremediation potential.

Besides PGPB and AMF, certain filamentous fungi (e.g., *Trichoderma* sp.) have great potential to improve plant establishment and thus phytoremediation capacity (Bareen et al., 2012). Teng et al. explored the impacts of Cd-tolerant *Trichoderma reesei* FS10-C on soil fertility and phytoremediation of Cd-polluted soil by *S. plumbizincicola*. The results showed that two inoculation agents containing FS10-C were better on all accounts compared to those without FS10-C. Moreover, solid fermentation powder was proposed as an efficient inoculation agent for FS10-C to improve soil fertility and Cd phytoremediation, as it had the greatest potential to promote plant growth, Cd accumulation, nutrient availability, as well as microbial biomass and activities.

Scientists from basic science to applied science are contributing significantly to a better understanding of physicochemical, molecular, and cellular mechanisms involved in plant-microbe-metal interactions under various abiotic and biotic stresses, which will certainly help develop novel solutions for PGPM-aided phytoremediation and restoration strategies.

AUTHOR CONTRIBUTIONS

YM drafted the editorial text, revised and approved the final version of the editorial text.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 18 May 2015
doi: 10.3389/fpls.2015.00344

Metabolic responses of willow (*Salix purpurea* L.) leaves to mycorrhization as revealed by mass spectrometry and ^{1}H NMR spectroscopy metabolite profiling

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The root system of most terrestrial plants form symbiotic interfaces with arbuscular mycorrhizal fungi (AMF), which are important for nutrient cycling and ecosystem sustainability. The elucidation of the undergoing changes in plants' metabolism during symbiosis is essential for understanding nutrient acquisition and for alleviation of soil stresses caused by environmental cues. Within this context, we have undertaken the task of recording the fluctuation of willow (*Salix purpurea* L.) leaf metabolome in response to AMF inoculation. The development of an advanced metabolomics/bioinformatics protocol employing mass spectrometry (MS) and ^{1}H NMR analyzers combined with the in-house-built metabolite library for willow (http://willowmetabolib.research.mcgill.ca/index.html) are key components of the research. Analyses revealed that AMF inoculation of willow causes up-regulation of various biosynthetic pathways, among others, those of flavonoid, isoflavonoid, phenylpropanoid, and the chlorophyll and porphyrin pathways, which have well-established roles in plant physiology and are related to resistance against environmental stresses. The recorded fluctuation in the willow leaf metabolism is very likely to provide AMF-inoculated willows with a significant advantage compared to non-inoculated ones when they are exposed to stresses such as, high levels of soil pollutants. The discovered biomarkers of willow response to AMF inoculation and corresponding pathways could be exploited in biomarker-assisted selection of willow cultivars with superior phytoremediation capacity or genetic engineering programs.

Keywords: arbuscular mycorrhizal fungi, metabolomics, plant-fungal interactions, plant selection, plant stress responses

OPEN ACCESS

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Specialty section:
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science

Received: *24 January 2015*
Accepted: *30 April 2015*
Published: *18 May 2015*

Citation:
Aliferis KA, Chamoun R and Jabaji S (2015) Metabolic responses of willow (Salix purpurea L.) leaves to mycorrhization as revealed by mass spectrometry and ^{1}H NMR spectroscopy metabolite profiling. Front. Plant Sci. 6:344. doi: 10.3389/fpls.2015.00344

Introduction

Willow (*Salix* spp.) is a highly diverse genera containing fast growing species used for biomass production (Labrecque and Teodorescu, 2003; Djomo et al., 2015), bioenergy and biofuels (Karp et al., 2011), phytoremediation (Guidi et al., 2012), and erosion control (Bariteau et al., 2013). This diversity is mainly due to willows' unique characteristics such as, superior growth rate and extensive fibrous root system, and adaptability to extreme environmental and soil conditions

(Jensen et al., 2009; Vangronsveld et al., 2009). The latter is improved by its symbiotic relationship with obligate biotrophs, the arbuscular mycorrhizal fungi (AMF), present in its rhizosphere (Bamforth and Singleton, 2005; Bonfante and Genre, 2008, 2015; Leigh et al., 2009). AMF are important components of ecosystems forming symbiotic relationships with the roots of the vast majority of plants (Smith and Read, 2008), which contribute to their improved nutrition and stress tolerance, and enhance soil structure (van der Heijden et al., 2006; Vogelsang et al., 2006).

The impact of AMF on willow has been investigated at different levels, however, there is no information on their effect on willows' global metabolism regulation. Increased root length and shoot growth in *Salix repens* has been reported following colonization by the AMF *Glomus mosseae* (van der Heijden, 2001). Also, colonization of *Salix miyabeana* and *Salix viminalis* by *Glomus intraradices* has resulted in increased phosphorus content in stems providing advantages for phytoremediation of heavy metals due to increased biomass (Fillion et al., 2011). The latter has been investigated on the ability of *G. intraradices*-*S. viminalis* interaction to rehabilitate a disturbed and slightly contaminated brownfield (Bissonnette et al., 2010).

AMF colonization is restricted to the root system, however, its effects are detectable, even macroscopically, in the above-ground plant parts (Smith and Read, 2008). New evidence is emerging on the capability of AMF on regulating plant genes involved in metabolic processes such as, defense and hormonal metabolism in shoots and leaves (Fiorilli et al., 2009; Lopez-Raez et al., 2010; Zouari et al., 2014). In addition to the impact on plant growth and resistance, mycorrhization improves the nutritional quality of fruits and leaves of agricultural crops via increased levels of plant secondary metabolites (Toussaint et al., 2007; Baslam et al., 2011), which are important for enhanced plant tolerance to stresses (Jeffries et al., 2003).

Metabolomics for the study of willow is still in its infancy with a handful of studies focusing on the phytochemical properties of willow bark and leaves (Du et al., 2007; Förster et al., 2010; Agnolet et al., 2012), the inhibitory compounds in lignocellulosic willow wood chips hydrolysates (Zha et al., 2014), and the chemical composition of the cuticular wax in relation to biomass productivity (Teece et al., 2008). These studies report on the accumulation of primary and secondary metabolites in the aerial parts of AMF willows. To our knowledge, there are no studies relevant to the effect of the AMF symbiosis on the leaf metabolome of willows, which highlights the need for further research on the mechanism by which it affects willows' metabolism and metabolite allocation to above-ground plant parts.

Within this context, as part of a multidisciplinary research project (GenoRem, http://genorem.ca) aiming at the optimization of willow-AMF symbiosis for phytoremediation purposes, we have undertaken the task of dissecting the effect of AMF on young willows' (*Salix purpurea* L. cv Fish Creek) leaf metabolism. Results will reveal the significance of AMF on willow's metabolism, and its indirect correlation with their phytoremediation capacity, when the willow-AMF system is a component of integrated phytoremediation strategies. For example, up-regulation of certain biosynthetic pathways as a result of AMF colonization could impact the adaptability of the plant and its performance under unfavorable conditions. To achieve this task, an advanced metabolomics/bioinformatics protocol was established employing proton nuclear magnetic resonance spectroscopy (^{1}H NMR), gas chromatography-mass spectrometry (GC/MS), and liquid chromatography-MS (LC/MS) using an LTQ Orbitrap analyzer for the monitoring of the global metabolism regulation of willow in response to AMF colonization. An essential element of the study was the development of a metabolite species-specific library for willow, which accelerated the steps of metabolite identification and biological interpretation of results.

Materials and Methods

Chemicals and Reagents

Chemicals and reagents used for GC/MS sample derivatization [i.e., methoxylamine hydrochloride 99.8%, *N*-methyl-*N*-(trimethyl-silyl)trifluoroacetamide (MSTFA) 98%, and pyridine] and for ^{1}H NMR analysis [i.e., deuterium oxide 99.9% (D_2O) containing 0.05% trimethylsilyl-2,2,3,3-d4-propionic acid sodium salt (TSP)] were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Ethyl acetate, methanol, formic acid, ammonium acetate (Optima grade®), and water (HPLC grade) were purchased from Fisher Scientific Company (Ottawa, ON, Canada). The ProteoMass ESI Calibration Kit MSCAL5 and MSCAL6 (Sigma-Aldrich), and the DRO/GRO Range Calibration Standard (Restek Corporation, Bellefonte, PA, USA, catalog #31832), which is a mixture of 12 alkanes, were used for the calibration and monitoring instruments' performance. The peptide leucine-encephalin (Sigma-Aldrich) was used as internal standard in LC/MS analysis.

Biological Material and Inoculation of Willows with *Rhizophagus irregularis*

Experiments were conducted in the greenhouse of the Institut de Recherche en Biologie Végétale (IRBV) (Montreal, Canada) following a completely randomized design and under controlled conditions; temperature of 20°C at day/18°C at night, relative humidity of 50%, and light intensity of 300 $\mu E/m^2$ s for 16 h per day. The AMF *Rhizophagus irregularis* isolate DAOM-240415 was obtained from the Canadian National Mycological Herbarium (Ontario, Canada) and maintained *in vitro* on modified minimal media (MM) (Bécard and Fortin, 1988) solidified with 0.4% (w/v) gellan gum (Sigma) with carrot roots transformed with Ri T-DNA of *Agrobacterium rhizogenes*. For simplicity from here and onwards the term AMF will be used instead of *R. irregularis*. The inoculum suspension was prepared from mycelia, spores and roots harvested from MM, dissolved in extraction buffer (0.82 mM sodium citrate and 0.18 mM citric acid) and mixed in a blender for 30 s (Hijri and Sanders, 2004). Willow cuttings (*S. purpurea* cv. Fish Creek) of 20 cm length were planted in pots containing an autoclaved mixed substrate composed of peat-moss, sand, and soil (1:1:1, v/v/v) with available P inferior to 10 mg m^{-3} for 6 weeks. Subsequently, seedlings were individually inoculated with 5 mL of inoculum suspension (approximately 500 propagules) whereas

5 mL of autoclaved water were added to non-mycorrhizal control plants that were placed at a distance from the inoculated ones to reduce the possibility of cross-contamination. No fertilizers or amendments were used. Plants were watered to soil capacity with deionized water every 2 days and were harvested 2 weeks following AMF inoculation when stems had reached approximately 1 m in length. There were five plants per treatment, each in a separate pot. Mycorrhizal colonization for willow roots was confirmed by microscopic observation of stained root sections (Vierheilig et al., 1998). The rate of the endomycorrhizal colonization was around 6% of root length, a level of colonization reported for willows (Bissonnette et al., 2010).

Sampling and Metabolite Extraction

The top five fully expanded leaves were harvested per plant. For MS analyses, two plugs were taken from each leaf, and the plugs were pooled (approximately 72 mg of fresh weight). Sample extraction and processing was performed as previously described (Aliferis et al., 2014) using 1 mL of a mixture of methanol:ethyl acetate (50:50, v/v). Following filtering, they were divided into two portions of 0.5 mL in glass autosampler vials for GC/MS and LC/MS analyses. The latter was further divided into two portions (0.25 mL each) for analysis in positive (ESI^+) and negative (ESI^-) electrospray modes. For GC/MS, samples were spiked by adding 20 μL of a ribitol solution (0.2 mg mL^{-1}) in methanol-water (50:50, v/v). Finally, extracts were dried using a Labconco CentriVap refrigerated vacuum concentrator (Labconco, Kansas City, MO, USA) equipped with a cold trap.

For ^1H NMR analyses, pulverized leaf material (100 mg) was lyophilized for 24 h and dissolved in D_2O (1 mL) for the extraction of polar compounds in glass autosampler vials (2 mL). Extracts were sonicated for 25 min and kept under continuous agitation (150 rpm) for 1 h at 24°C. For the removal of debris, samples were centrifuged (12,000 × *g*) for 1 h and the supernatants were subjected to a second centrifugation (12,000 × *g*) for 30 min. Supernatants were then collected and kept at −80°C until the acquisition of ^1H NMR spectra.

Chemical Analyses and Data Pre-processing

Gas Chromatography-mass Spectrometry (GC/MS)

Derivatization of samples for GC/MS analyses was performed as previously described (Aliferis and Jabaji, 2012; Aliferis et al., 2014). Briefly, methoxymation was performed using methoxylamine hydrochloride (80 μL, 20 mg mL^{-1} in pyridine) to the dried extracts, incubated for 120 min at 30°C, followed by silylation using MSTFA (80 μL, 37°C for 90 min). Derivatized samples were transferred to microinserts (150 μL, Fisher Scientific Company), which were placed in glass autosampler vials (2 mL). An Agilent 7890A GC platform (Agilent Technologies Inc. Santa Clara, CA, USA) equipped with a 7693A series autosampler and coupled with a 5975C series mass selective detector (MSD) was employed. Chromatogram acquisition and data pre-processing were carried out with the Agilent MSD Chemstation (v. E.02.00.493). The electron ionization was set at 70 eV and full scan mass spectra were acquired at the mass range of 50–800 Da at 1 scan s^{-1} rate with a 10 min solvent delay. The temperatures were; ion source at 150°C, transfer line at 230°C, and injector at 230°C. Samples (1 μL) were injected using a split ratio of 10:1 into a HP-5MS ultra inert (UI) capillary column (30 m × 250 μm I.D., 0.25 μm film thickness; Agilent Technologies Inc.). Helium was used as the carrier gas (flow rate 1 mL min^{-1}). The temperature of the oven was 70°C stable for 5 min, followed by a 5°C min^{-1} increase to 310°C and finally stable for 1 min.

Liquid Chromatography-mass Spectrometry (LC/MS)

The dried samples were re-dissolved in 160 μL of a mixture of methanol:formic acid (0.1% v/v) (50–50, v/v) or 160 μL of methanol:ammonium acetate (2.5 mM) for analysis in ESI^+ and ESI^-, respectively. Extracts were then transferred to microinserts and placed in glass autosampler vials. An LTQ-Orbitrap MS Classic (Thermo Scientific, San Jose, CA, USA), equipped with a reverse phase Luna®C18(2) column (cat. no. 00F-4251-B0, 150 × 2.0 mm, 3 μm, 100 Å pore size) (Phenomenex, Torrance, CA, USA) and a Security Guard Cartridge (cat. no. KJO-4282, Phenomenex), was used. All experimental events were controlled by the software Xcalibur v.2 (Thermo Scientific). Specifications of the analyzer have been described previously (Aliferis et al., 2014). Samples (10 μL) were injected manually at a flow rate of 1 μL min^{-1} using a syringe (Hamilton, Reno, NV, USA). The gradients used for ESI^+ and ESI^- are displayed in the Supplementary Tables 1, 2, and settings of the LTQ Orbitrap MS in the Supplementary Tables 3, 4, respectively.

Analyses were performed at a mass resolution of 60,000 at *m/z* 400 and spectra were acquired over the range of 80–1200 Da. For selected samples, MS/MS analyses were performed with the normalized collision energy maintained at 35 eV, the activation q set to 0.25 and the activation time to 30 ms. Target ions already selected for MS/MS were dynamically excluded for 15 s. Acquired chromatograms (∗.raw) were processed using the software SIEVE v.2.0 (Thermo Scientific) after setting optimization for ESI^+ and ESI^- (Supplementary Tables 5, 6).

Nuclear Magnetic Resonance (^1H NMR) Spectroscopy

^1H NMR spectra were recorded using a Varian Inova 500 MHz ^1H NMR spectrometer (Varian, Palo Alto, CA, USA) equipped with a ^1H (^{13}C,^{15}N) triple resonance cold probe as previously described (Aliferis and Jabaji, 2010). A total of 128 transients of 64 K data points were acquired per sample with a 90° pulse angle, 2 s acquisition time, and 2 s recycle delay with presaturation of H_2O during the recycle delay. Spectra were Fourier transformed, and the phase and baseline was automatically corrected. Offsets of chemical shifts were corrected based on the reference signal of TSP (0.00 ppm). Processing was performed using the Spectrus Processor and C+H NMR Predictor and Database v.12.01 of ACD Labs (Advanced Chemistry Development, Inc., ACD/Labs, Toronto, Canada).

Quality Control of Metabolomics Analyses

Standard operating procedures (SOP) and quality control (QC) measures were followed throughout the experimental steps to ensure the quality and validity of analyses. For each treatment, a QC sample was obtained by pooling aliquots of

the five biological replications. Additionally, blank samples were analyzed for the detection of possible sources of contamination during the different experimental steps, such as impurities of glassware, reagents, column bleeding, or source contamination. For this purpose, blank samples were processed alongside the experimental samples and were subjected to identical handling. Detected features not related to the biological material being analyzed were excluded from analyses.

To maintain instruments' performance, calibration of the analyzers was performed following the recommended manufacturers' procedures and using calibration solutions. For GC/MS analysis, tuning of the MS detector was performed automatically using the AutoTune function and the DRO/GRO Range Calibration Standard was injected every 10 samples to monitor the performance of the instrument. Additionally, samples were spiked with ribitol in order to monitor possible shifts in retention time and the reproducibility of analyses. For LC/MS analysis, the ProteoMass ESI Calibration Kit (Sigma-Aldrich) was used to cover the range between 138 and 1822 Da for ESI^+ analyses (catalog # MSCAL5), and between 265 and 1880 Da for ESI^- analyses (catalog # MSCAL6). Samples were spiked with the peptide leucine-encephalin in order to monitor shifts in retention time, the performance of the analyzer, and mass errors.

In addition to QC samples, technical replications of randomly selected samples were performed in order to access the reproducibility of analytical conditions. Samples were analyzed in completely randomized order to avoid possible variability caused by inconsistent performance of the analyzers.

Construction of a Species-specific Metabolite Library for Willow

For high-throughput untargeted metabolomics, the use of species-specific metabolite libraries is necessary for the robust deconvolution of the vast amount of the obtained information and the decrease of false discovery rate. Although for many plant species there are comprehensive metabolite libraries (e.g., collection of PlantCyc), for willow such library does not exist. Therefore, we have undertaken the task of developing a species-specific library for willow (Willow MetaboLib v.1.0., http://willowmetabolib.research.mcgill.ca/index.html). For its construction, results from analyses and information from the metabolite libraries of PoplarCyc (http://pmn.plantcyc.org/POPLAR/class-instances?object=Compounds), Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/), PubChem (http://pubchem.ncbi.nlm.nih.gov/), KNapSack (http://kanaya.naist.jp/KNApSAcK/), the European Bioinformatics Institute (EMBL-EBI) (http://www.ebi.ac.uk/), and the literature were retrieved and integrated.

Metabolite Identification

For GC/MS and NMR analysis, the identification of the vast majority of metabolic features was performed at levels 1 (absolute identification) and 3 (tentative identification), whereas for LC/MS analysis, identification was performed at levels 2 (tentative identification) and 3 (tentative identification of compound class) for the majority of metabolic features (Dunn et al., 2013), as described below (**Supplementary Data set 1**).

For GC/MS analysis, mass spectra searches were performed against the library of the National Institute of Standards and Technology, NIST 08 (Gaithersburg, MD, USA). Following the guidelines of the metabolomics standards initiative (MSI) (Sumner et al., 2007), selected metabolites were absolutely identified based on fragmentation patterns and retention times (RT) of authentic chemical standards analyzed on the same GC/MS system with the same analytical method. Tentative identification was performed for metabolites with a very good fit (>90%).

For ^{1}H NMR, identification of metabolites was performed by comparing the recorded chemical shifts and *J*-coupling values to those of analytical standards. Additionally, identification was performed by assigning signals to corresponding metabolites using the ACD/C+H NMR Predictor and Database v.12.01 (ACD/Labs). Using the software, ^{1}H NMR spectra of metabolites can be simulated and their similarities regarding chemical shifts and *J*-coupling values can be used for the identification of unknowns.

Due to its superior analytical capabilities, LTQ Orbitrap MS represents an excellent analyzer for metabolomics. However, the vast amount of obtained information is challenging even when achieving very low mass errors (e.g., <1 ppm). Here, a biologically-driven approach was employed having as main pillar the use of the Willow MetaboLib v.1.0. Putative identification of metabolites was based on targeted searches against the library taken into consideration mass accuracy, and when available, isotope and MS/MS fragmentation patterns.

Finally, to ensure the validity of analyses and metabolite identification, results of metabolite identification and their fluctuation between the two treatments from the different analyzers were cross-validated.

Biomarker Discovery

Following peak deconvolution and integration, GC/MS data were exported to Microsoft® Excel, aligned, and normalized against ribitol for the construction of the data matrix. The data matrix was then exported to SIMCA-P^+ v.12.0.1 (Umetrics, MKS Instruments Inc. Andover, MA, USA) for the detection of trends and corresponding metabolite-biomarkers performing multivariate analyses as previously described (Aliferis and Jabaji, 2012; Aliferis et al., 2014). Principal component analysis (PCA) was initially performed for the overview of the data set and the detection of possible outliers, which could have a leverage on the analysis. The discovery of biomarkers was based on scaled and centered partial least square-discriminant analysis (PLS-DA) regression coefficients ($P < 0.05$), since by PCA the largest sources of variation may not be represented by the computed principal components (PCs). Standard errors were calculated using Jack-knifing (95% confidence interval) (Efron and Gong, 1983). The performance of the obtained model was assessed by the cumulative fraction of the total variation of the *X*'s that could be predicted by the extracted components [$Q^2_{(cum)}$] and the fraction of the sum of squares of all *X*'s (R^2X) and *Y*'s (R^2Y) explained by the current component. Additionally, GC/MS data

were subjected to One-Way ANOVA performing the Student's *t*-test ($P < 0.05$) using the software JMP 8.0 (SAS Institute Inc., NC, USA).

^{1}H NMR spectra were automatically aligned and bucket integrated using a 0.01 ppm bucket width and the Intelligent Bucketing function of the software Spectrus Processor with a width looseness of 50%. For the discovery of biomarkers performing ^{1}H NMR analyses, the processed data were exported to Microsoft® Excel and the obtained data matrix was finally exported to SIMCA-P+ v.12.0.1 for multivariate analysis. PCA was initially performed for the overview of the data set and the detection of possible outliers. The discovery of biomarkers was performed similarly to that of GC/MS data and as previously described (Aliferis and Jabaji, 2010).

For LTQ-Orbitrap MS acquired data, the frames (rectangular regions m/z vs. RT) obtained using the software SIEVE v.2.0 were filtered for isotopes, retaining only the ions corresponding to ^{12}C (Aliferis et al., 2014). In a second step, based on their coefficient of variation distribution (CV) in the reconstructed ion chromatograms (RIC), frames were filtered, retaining those with CV < 0.8. Ion intensities were normalized against the total ion current (TIC). The obtained matrix was exported to Microsoft® Excel and finally to SIMCA-P+ v.12.0.1 for multivariate analysis for the detection of trends and an overview of the analysis. In contrast to GC/MS and ^{1}H NMR data, for the high-throughput analysis and biological interpretation of data, the discovery of biomarkers was performed using the integrated into SIEVE v.2.0 Student's *t*-test ($P < 0.05$).

Metabolic Networking

The global willow metabolome and biomarkers implicated in its interaction with AMF were visualized using the software Cytoscape v.2.8.2 (Smoot et al., 2011) following previously described approach (Aliferis and Jabaji, 2012; Aliferis et al., 2014). For the *ab-initio* construction of networks, the biosynthetic pathways of KEGG were used.

Results and Discussion

The Willow Metabolite Library "Willow Metabolib v.1.0"

The application of metabolomics for the study of plant symbiosis with AMF could provide insights that can be exploited in various fields of science (Fiorilli et al., 2009). Here, for the robust deconvolution of willow's metabolome, an in-house built library for willow, the Willow MetaboLib v1.0 (http://willowmetabolib.research.mcgill.ca/index.html), was constructed. It contains more than 2000 entries with information on molecular formulae, standardized chemical classification, KEGG and PubChem identifiers, and biosynthetic pathways following the KEGG coding system (**Figure 1**). Metabolites are categorized based on their chemical group, and data sets can be downloaded in MS Excel format (.xls) by selecting from the home page the tab "Database," and then the desired chemical group. The website additionally contains information on the related research, photos, and useful links. The library was used for the high-throughput identification of metabolites in the analyzed samples performing LC/MS analyses, and for cross-validation of metabolite identities performing GC/MS and ^{1}H NMR analyses. Such approach is necessary toward the standardization of large-scale metabolomics data deconvolution, reporting, and biological interpretation.

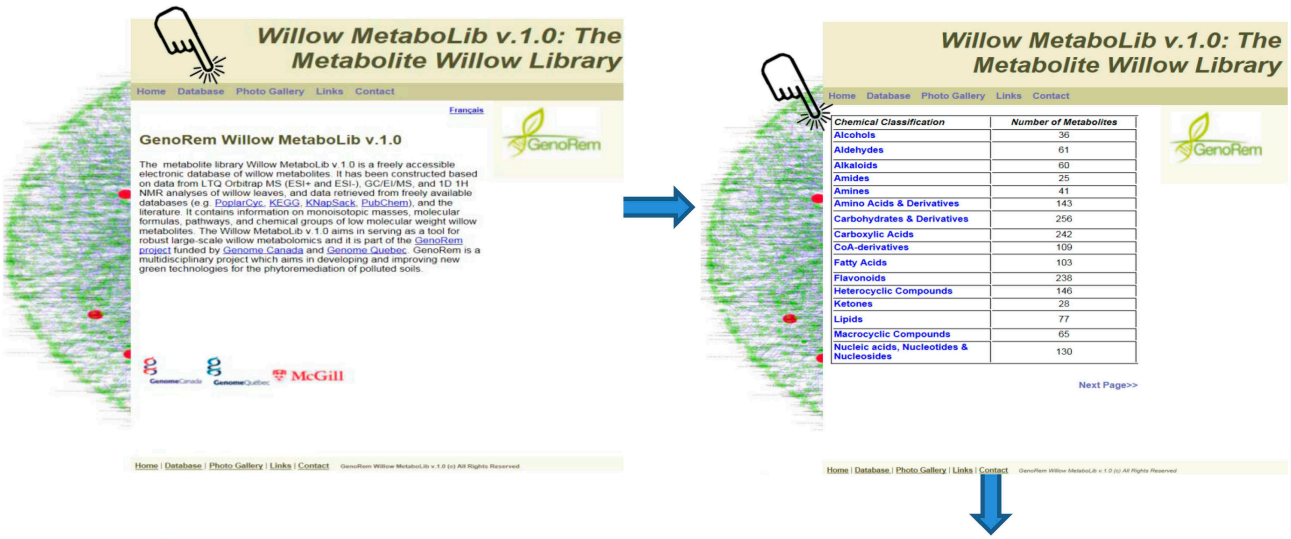
Overview of the Analysis

For untargeted large-scale plant metabolomics, validated, high-throughput metabolite identification and biomarker discovery are challenging tasks. Here, the complementary capabilities of three of the most powerful and commonly employed analyzers in metabolomics (Dunn and Ellis, 2005; Kim et al., 2010) were exploited in order to accelerate and strengthen metabolite identification and expand the metabolome coverage, in combination with the use of the Willow MetaboLib v1.0 (**Figures 1**, **2**). The robustness of the developed bioanalytical protocols is confirmed by the quality of the obtained GC/MS (**Supplementary Figure 1**) and LTQ Orbitrap MS (**Supplementary Figure 2**) chromatograms and ^{1}H NMR spectra (**Supplementary Figure 3**).

GC/MS and ^{1}H NMR were employed in order to monitor mainly the fluctuation in the levels of primary metabolites caused by AMF inoculation. A very good correlation is observed between the results obtained by the two platforms with similar fluctuation patterns in the levels of the commonly identified metabolites (**Supplementary Figure 3** and **Supplementary Data set 1**). For example, NMR analysis revealed higher levels of sugars (**Supplementary Figure 3**, region 4.6–5.6 ppm) and lower levels of glutamine and pyroglutamate in AMF-inoculated willows compared to non-inoculated ones (**Supplementary Figure 3**, region 2.0–2.4 ppm), which is in agreement with results of GC/MS analysis (**Supplementary Data set 1**). In addition, NMR analysis revealed higher levels of aromatic metabolites in AMF-inoculated willows (**Supplementary Figure 3**, region 6.3–8.4 ppm). This is in agreement with results of LTQ Orbitrap analysis, which show high levels of flavonoids and phenolics. Additionally, performing LTQ Orbitrap analysis, fluctuation in secondary metabolites not detected/identified by GC/MS or NMR analysis such as, flavonoids, macrocyclic compounds, and phenolics, was recorded (**Supplementary Data set 1**). The above, justifies the notion of employing more than one analyzer for a comprehensive coverage of plant metabolism, the study of its regulation, and for strengthening our metabolite identification capacity through cross-validation (Aliferis and Jabaji, 2010; Aliferis et al., 2014).

By integration of data from MS analyzers and confirmation by ^{1}H NMR data, 177 significantly up- or down-regulated features were assigned to metabolites or to unique molecular formulae designated as biomarkers in response to AMF inoculation (**Supplementary Data set 1**) ($P < 0.05$). These biomarkers belong to various chemical groups and biosynthetic pathways of willow (**Table 1**). In contrast, approximately 250 identified metabolites or unique molecular formulae did not statistically differ between treatments ($P < 0.05$) (**Figures 3**, **4**).

Partial least squares-discriminant analyses (PLS-DA) performed for GC/MS, ^{1}H NMR, and LTQ Orbitrap MS in



	A	B	C	D	E	F	G
1	Metabolite	KEGG ID	PubChem ID	Molecular Formula	Biosynthetic Pathway	Chemical Classificat	Willow MetaboLib
2	(3E)-3,7-dimethyl-3-octene-1,2,6,7-tetrol	n/a	n/a	C10H20O4	n/a	Alcohol	WMLB-697
3	(R,R)-2,3-butanediol	C03044	225936	C4H10O2	map00650 Butanoate metabolism cutin biosynthesis phosphate acquisition cutin biosynthesis	Alcohol	WMLB-80
4	(R,S)-2,3-butanediol	n/a	220010	C4H10O2	map00650 Butanoate metabolism cutin biosynthesis phosphate acquisition cutin biosynthesis	Alcohol	WMLB-81
5	(S,S)-2,3-butanediol	C03046	439888	C4H10O2	map00650 Butanoate metabolism cutin biosynthesis phosphate acquisition cutin biosynthesis	Alcohol	WMLB-82
6	1,2-dioctanoyl-1,2,3-butanetriol	n/a	44237229	C20H38O5	n/a	Alcohol	WMLB-1415
7	1-diphosinositol-pentakisphosphate	C11174	46173525	C6H19O27P7	map04070 Phosphatidylinositol signaling system	Alcohol	WMLB-2079
8	1-hydroxycumene	n/a	12053	C9H12O	vernolic acid biosynthesis	Alcohol	WMLB-275
9	1-nonanol	C14696	8914	C9H20O	n/a	Alcohol / Fatty Alcohol	WMLB-322
10	2-decanol	C01633	8174	C10H22O	n/a	Alcohol	WMLB-424
11	2-furanmethanol	C20441	7361	C5H6O2	map00365 Furfural degradation	Alcohol	WMLB-91
12	2-methylbutanol	n/a	8723	C5H12O	n/a	Alcohol / Fatty Alcohol	WMLB-67
13	2-phenylethanol	C05853	6054	C8H10O	map00360 Phenylalanine metabolism	Alcohol	WMLB-191
14	3,4-dihydroxyphenylglycol	C05576	91528	C8H10O4	map00350 Tyrosine metabolism	Alcohol	WMLB-500
15	3,5-bisdiphosphoinositol-1D-myo-inositol-(2,3,4,6)tetrakisphosphate	n/a	46173277	C6H20O30P8	inositol pyrophosphates biosynthesis cutin biosynthesis phosphate acquisition	Alcohol	WMLB-2162
16	3-hexenol	C08492	5281167	C6H12O	13 LOX and-13 HPL-pathway cutin biosynthesis	Alcohol / Fatty Alcohol	WMLB-100

FIGURE 1 | The Willow Metabolite Library "Willow MetaboLib v.1.0" (http://willowmetabolib.research.mcgill.ca/index.html). In-house built metabolite library for willow containing more than 2000 entries with information on molecular formulae, standardized chemical classification, KEGG and PubChem identifiers, and biosynthetic pathways following the KEGG coding system. Sets of metabolites can be downloaded in MS Excel® format by first selecting the tab "Database" and then the desired chemical group. The website contains also other useful information on the project and links.

ESI^+ and ESI^- data sets (**Supplementary Figure 4**) revealed in each case two distinct tight clusters, one representing the metabolomes of leaves of inoculated willows and the other that of non-inoculated ones.

Based on the identified biomarkers and applying metabolic networking, the global overview of the willow's leaf metabolic network in response to AMF was obtained (**Figure 3**). Such network represents an excellent tool for studying metabolism regulation based on large-scale data thanks to the wide range of applications that exist for Cytoscape. Following a reverse genetics approach, the network can also provide useful information on the links between metabolites and proteins, which can be further exploited in systems biology approaches (Ghosh et al., 2011; Aliferis and Jabaji, 2012). Results revealed the general disturbance of willow's metabolism with an increase in the majority of metabolites in mycorrhized trees compared to non-mycorrhized ones. Also, the up-regulation of α-linolenic acid metabolism and porphyrin and chlorophyll metabolism is evident in the network (**Figure 3**).

Effect of AMF on the Willow Leaves' Chemical Composition

The high-throughput biological interpretation of large-scale metabolomics data from global profiling experiments is challenging. Nonetheless, the summary of the effect of a treatment on the chemical composition of the biological system being studied could provide a first qualitative overview of the underlying biochemical changes and insights on metabolism regulation in a timely fashion (Aliferis et al., 2014). Here, metabolites belonging to various chemical groups involved in the primary and secondary plant metabolism and symbiosis such as, amino acids, carbohydrates, flavonoids, macrocyclic compounds, phenylpropanoids, and terpenoids, showed a substantial fluctuation 2 weeks following willows' inoculation with AMF (**Table 1**, **Figures 3**, **4**, **5**, and **Supplementary Data set 1**). Such observation is facilitated by the standardized chemical classification of metabolites in the Willow MetaboLib v.1.0. Metabolites belonging to these groups have important and well-established roles in plant physiology, and exhibit bioactivity.

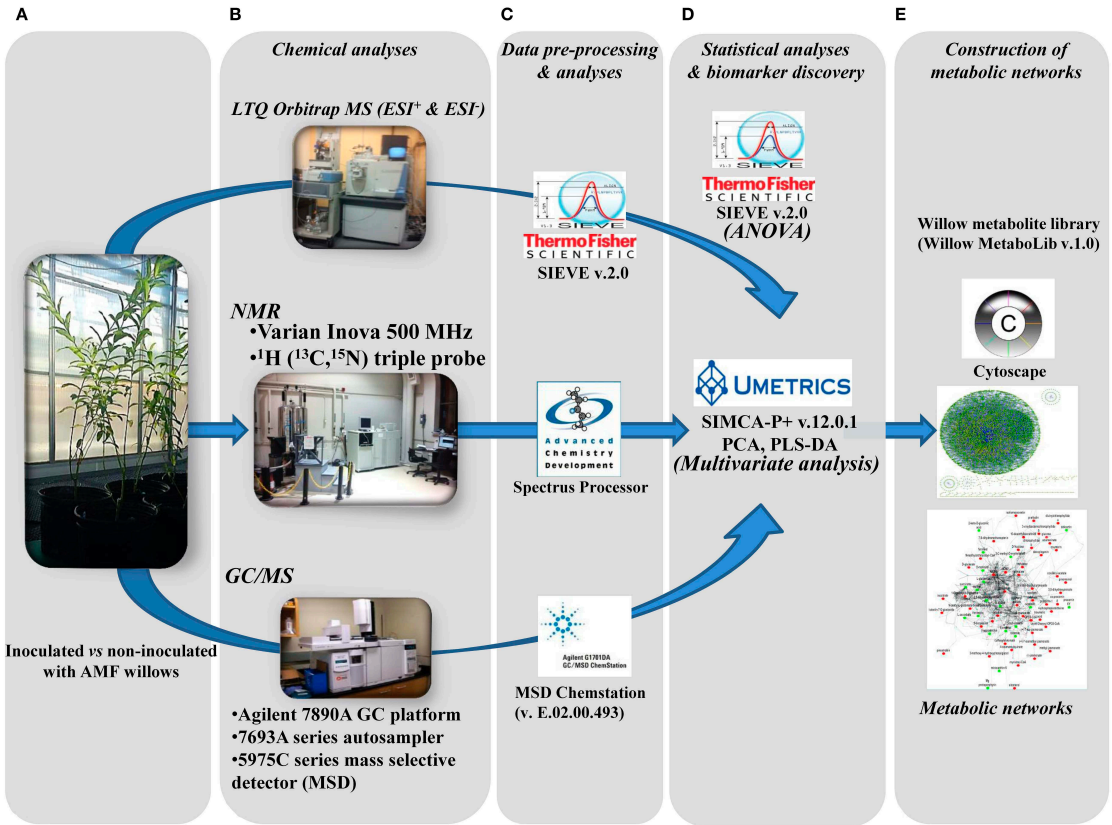


FIGURE 2 | Developed pipeline for the discovery of willow's metabolic biomarkers following inoculation with the AMF *Rhizophagus irregularis*. After sampling **(A)**, four subsequent steps were applied: **(B)** chemical analyses by integrating three analytical platforms, **(C)** data pre-processing and analyses, **(D)** statistical analyses and biomarker discovery, and **(E)** construction of metabolic networks, for the global visualization of changes in the global metabolic network of willow in response to mycorrhization.

Their potential role during AMF-willow interaction is discussed in more detail in the following sections.

The observed general disturbance of the host's metabolism based on the chemical composition of the leaves is not surprising since symbiosis is a complex and dynamic interaction, whose outcome has to maintain the mutualistic relationship of both partners (García-Garrido and Ocampo, 2002; Cavagnaro, 2008). It also confirms that willow's response or the effect of the AMF on the plant is not localized in the roots, but systemic, which is in accordance with observations in various AMF-plant symbiotic systems (Erb et al., 2009; Schweiger et al., 2014).

Effect of AMF on the Willow's Leaf Primary Metabolism

The direct effect of AMF on the primary metabolism of willow leaves is evident mainly by the fluctuation of their content in carbohydrates and amino acids (**Table 1**, **Figure 3**, and **Supplementary Data set 1**).

The mobilization of willow leaf carbohydrates in response to AMF is in agreement with previous observations (Doidy et al., 2012) and indicative of an underlying operating sugar exchange mechanism between willow and AMF. AMF inoculation resulted in higher levels in the vast majority of monosaccharides and lower levels of sugar alcohols in willow leaves compared to those of non-inoculated ones.

Carbohydrates are products of plants' photosynthetic activity, which generally constitute the bulk of organic material to be translocated through the phloem tissue to the different plant parts (Ainsworth and Bush, 2011; Doidy et al., 2012) and play an important role in plant physiology by regulating gene expression (Koch, 1996). Their content could serve as an indicator of the plant's physiological condition, for example, studies have shown that carbohydrates accumulate in plants under drought conditions (Seki et al., 2007). Also, they are the main nutrient source that the AMF depend on during symbiosis. The observed high levels of carbohydrates in AMF mycorrhized willows can be well attributed to the up-regulation of the porphyrin and chlorophyll biosynthetic pathway (**Table 1**, **Figures 3**, **4**, and **Supplementary Data set 1**). The produced monosaccharides are the main carbon source for AMF after their transportation to the roots through monosaccharide transporters (Doidy et al., 2012), as revealed in previous reports (Bonfante and Genre, 2008; Leigh et al., 2009). In addition, the disaccharide α-α-trehalose, whose relative concentration in leaves was significantly increased in response to AMF inoculation, has unique physicochemical properties and is implicated in plant

TABLE 1 | Classification of metabolite-biomarkers of willow's leaves in response to the AMF *Rhizophagus irregularis* according to their participation in metabolic pathways/functions and chemical groups.

Pathways/Metabolite groups	Increased	Decreased	Total
PATHWAYS			
map00250,map00330, map00360 Amino acid Metabolism	2	9	11
map00944 Flavone and flavonol biosynthesis	3	0	3
map00941 Flavonoid biosynthesis	9	1	10
map00943 Isoflavonoid biosynthesis	4	0	4
map00940 Phenylpropanoid biosynthesis	6	1	7
map00020 Citrate cycle (TCA cycle)	1	3	4
map00592 a-Linolenic acid metabolism	5	0	5
map02010 ABC transporters	5	2	7
map00860 Porphyrin and chlorophyll metabolism	8	2	10
map00902 Monoterpenoid biosynthesis	3	0	3
METABOLITE GROUPS			
Amino acids and derivatives	2	7	9
Carbohydrates and derivatives	11	8	19
Carboxylic acids	7	17	24
CoA-derivatives	10	0	10
Flavonoids	31	5	36
Heterocyclic compounds	5	2	7
Macrocyclic compounds	10	3	13
Nucleic acids nucleotides and nucleosides	6	1	7
Fatty acids	4	1	5
Phenolic glycosides	5	1	6
Phenylpropanoids	9	1	10
Terpenoids	7	1	8
Various	15	8	23

Biomarkers are classified as increased and decreased in colonized seedlings compared to controls. The code system of KEGG for pathways is used whereas for the chemical classification of metabolites information was retrieved from the database PubChem. A color gradient was used ranging from dark green (0) to red (36).

responses to various stimuli (Paul et al., 2008). However, since its concentration in the cells is not clear, trehalose may not work as a protective agent in the biological system being studied.

Sugar alcohols play an important role in plant physiology, including protection against osmolytic and oxidative stress, as well as in plant-pathogen interactions (Williamson et al., 2002). Increased content in sugar alcohols has been reported in plants under drought stress (Seki et al., 2007). The pattern by which the content of a mycorrhized plant in sugar alcohols is altered seems to be species-specific (Schweiger et al., 2014). Based on these observations it can be suggested that the observed decrease of sugar alcohols in AMF-mycorrhized willows is an indication that the plants are under a lower stress level compared to the controls.

The amino acid pool of willow leaves decreased in AMF-inoculated willows (**Table 1**, **Figure 3**, and **Supplementary Data set 1**). Similar findings have been reported during the mycorrhizal symbiosis of *G. mosseae* with *Lotus japonicus* (Fester et al., 2011). However, the effect of AMF on the amino acid content of the host do not exhibit a clear pattern (Hodge and Storer, 2014; Souza et al., 2014), which is indicative of the complexity of the undergoing interactions during symbiosis. Additionally, metabolites involved in the amino acid metabolism were detected in lower amounts in mycorrhized compared to non-mycorrhized plants. This could be attributed to the carbon sink effect induced by AMF and the carbon allocation to the roots where the mycorrhizal interaction takes place.

Effect of AMF on the Willow's Leaf Secondary Metabolism

Symbiosis with AMF caused a general disturbance of willow leaves' metabolism (**Table 1**, **Figures 3**, **4**, **5**, and **Supplementary Data set 1**). Results revealed up-regulation of key biosynthetic pathways involved in willow responses to biotic and abiotic stresses and indirectly to adaptation. Here, the effect of AMF inoculation on major biosynthetic pathways of willow is discussed.

Up-regulation of the Phenylpropanoid Biosynthetic Pathway

The up-regulation of the phenylpropanoid pathway and related metabolites of willow as a main response to AMF (**Table 1**, **Figures 3**, **4**, and **Supplementary Data set 1**) is in accordance to previous reports (Morandi, 1996; Pozo et al., 2002). This is a key biosynthetic pathway in plants' physiology, involved, among others, in the biosynthesis of secondary metabolites that play crucial role in responses to stresses (Dixon et al., 2002; Petersen et al., 2010).

Among the identified metabolites, coumaryl acetate, 4-coumaroylquinate, and caffeoyl-shikimate have shown an up-regulation of 1.8, 2.3 and 1.6 fold, respectively, in inoculated plants compared to the controls (**Figure 5**). Caffeoyl-shikimate is also an intermediate in the lignin biosynthetic pathway (Grassmann, 2005). Lignin is a biopolymer that serves as a matrix around the polysaccharide components of plant cell walls, providing to the latter additional rigidity and structural integrity (Bhuiyan et al., 2009).

Similarly, coumarins and their hydroxy forms increased in response to AMF. Coumarins are well-studied plant secondary metabolites with antimicrobial, antioxidant, and hormonal regulatory properties thus, playing multiple roles in plants' physiology (Bourgaud et al., 2006; Stanchev et al., 2010).

Up-regulation of the α -Linolenate Biosynthetic Pathway

The α-linolenate pathway was significantly up-regulated in the presence of AMF (**Table 1**, **Figures 3**, **4**, and **Supplementary Data set 1**). Increased levels of OPC6-CoA, trans-2-enoyl-OPC6-CoA, and jasmonates (JAs; jasmonate, JA and iso-JA) were observed in inoculated AMF plants,

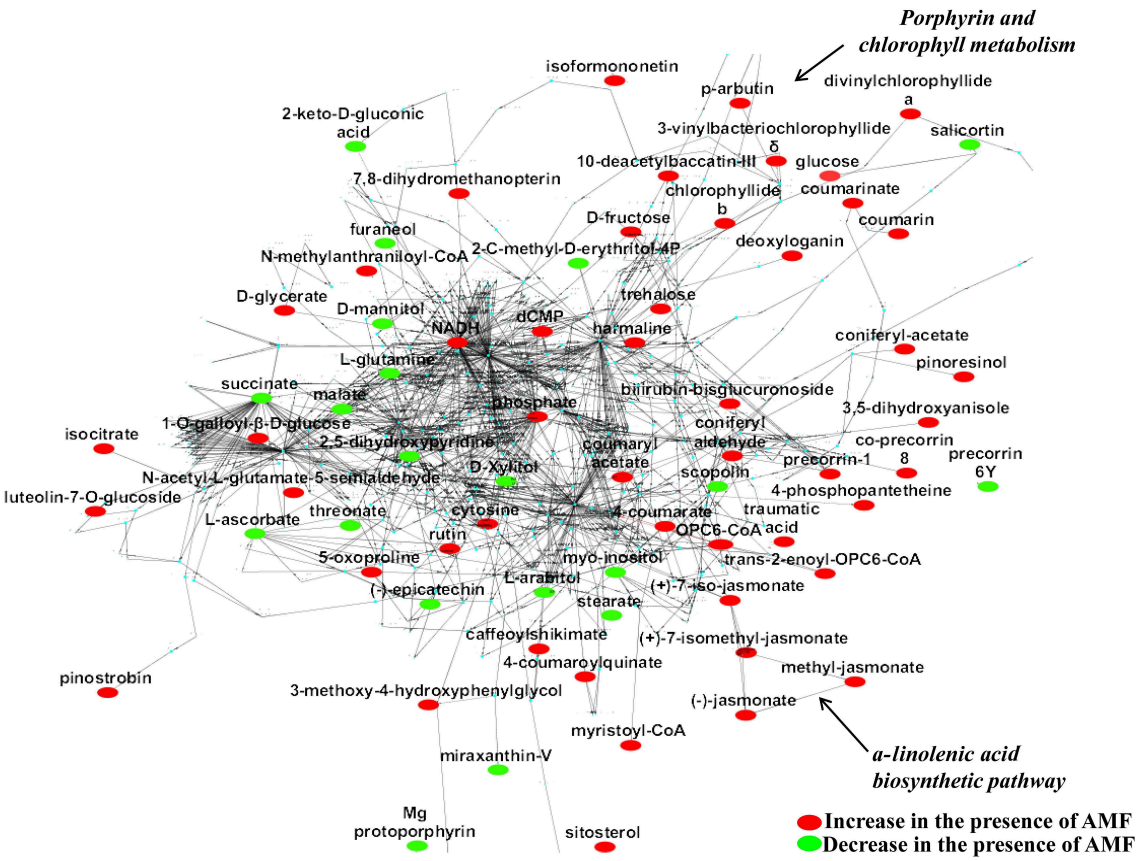


FIGURE 3 | The global willow leaf metabolic network and its perturbation 2 weeks following inoculation with the AMF *Rhizophagus irregularis* visualized using the software Cytoscape. Biomarkers of the symbiosis and possible interconnecting networks between them are displayed. Sections of the *a*-linolenate biosynthetic pathway and porphyrin and chlorophyll metabolism are indicated by arrows. Metabolite fluctuations are displayed using a color code based on *P*-values ($P < 0.05$) performing the Student's *t*-test.

with the highest increase observed for methyl-jasmonates (methyl-jasmonate/methyl iso-jasmonate, Me-JAs) (2.1 fold) compared to controls (**Figure 5**).

During mycorrhization, the regulation of hormonal pathways is altered with jasmonic acid (JA) playing a central role during symbiosis (Jung et al., 2012; León Morcillo et al., 2012). To date, it is not clear to what extent shoot-derived JAs contribute to the regulation of mycorrhizal symbiosis in the roots, however, in analogy we expect the JA hormonal pathway to be differentially regulated in the leaves. Evidence on the involvement of the α-linolenate biosynthetic pathway in defense priming during mycorrhization has been recently established (Van Wees et al., 2008; Pozo et al., 2009; Jung et al., 2012). This induction is attributed to its intracellular signal transduction role leading to mediation of secondary metabolite biosynthesis (e.g., phenolic compounds, terpenes, alkaloids, and isoflavonoids) (Schliemann et al., 2008). Strong positive correlation was observed between endogenous concentration of JAs and trichome density, phenylalanine ammonia-lyase (PAL) activity, and phenols concentrations in mycorrhized tomato plants (Kapoor, 2008). This is in accordance with the observed correlation between the increased levels of JAs and metabolites of the phenylpropanoid pathway of willow leaves following inoculation with AMF. Furthermore, it has been reported that biotic and/or abiotic stresses remodel the plant's membrane fluidity by releasing α-linolenate, which in turn plays a protective role in the photosynthetic apparatus (Upchurch, 2008).

Up-regulation of the Flavonoid, Isoflavonoid, and Flavone Biosynthetic Pathways

Inoculation of willows with AMF significantly increased the concentration of the vast majority of features that correspond to flavonoids including those involved in the flavonoid and isoflavonoid biosynthetic pathways of willow leaves (**Table 1**, **Figures 3**, **4**, and **Supplementary Data set 1**). The up-regulation of these pathways was among the most interesting findings of the present research. Among the identified components of the two pathways, pinostrobin and isoformononetin significantly increased, 1.9-fold and 1.5-fold, respectively, whereas epicatechin was significantly down-regulated (4.9 fold) in mycorrhized plants (**Figure 5**).

Flavonoids are major plant secondary metabolites that play a key role in their physiology by protecting them against biotic and abiotic stresses (Pourcel et al., 2007; Dixon and Pasinetti,

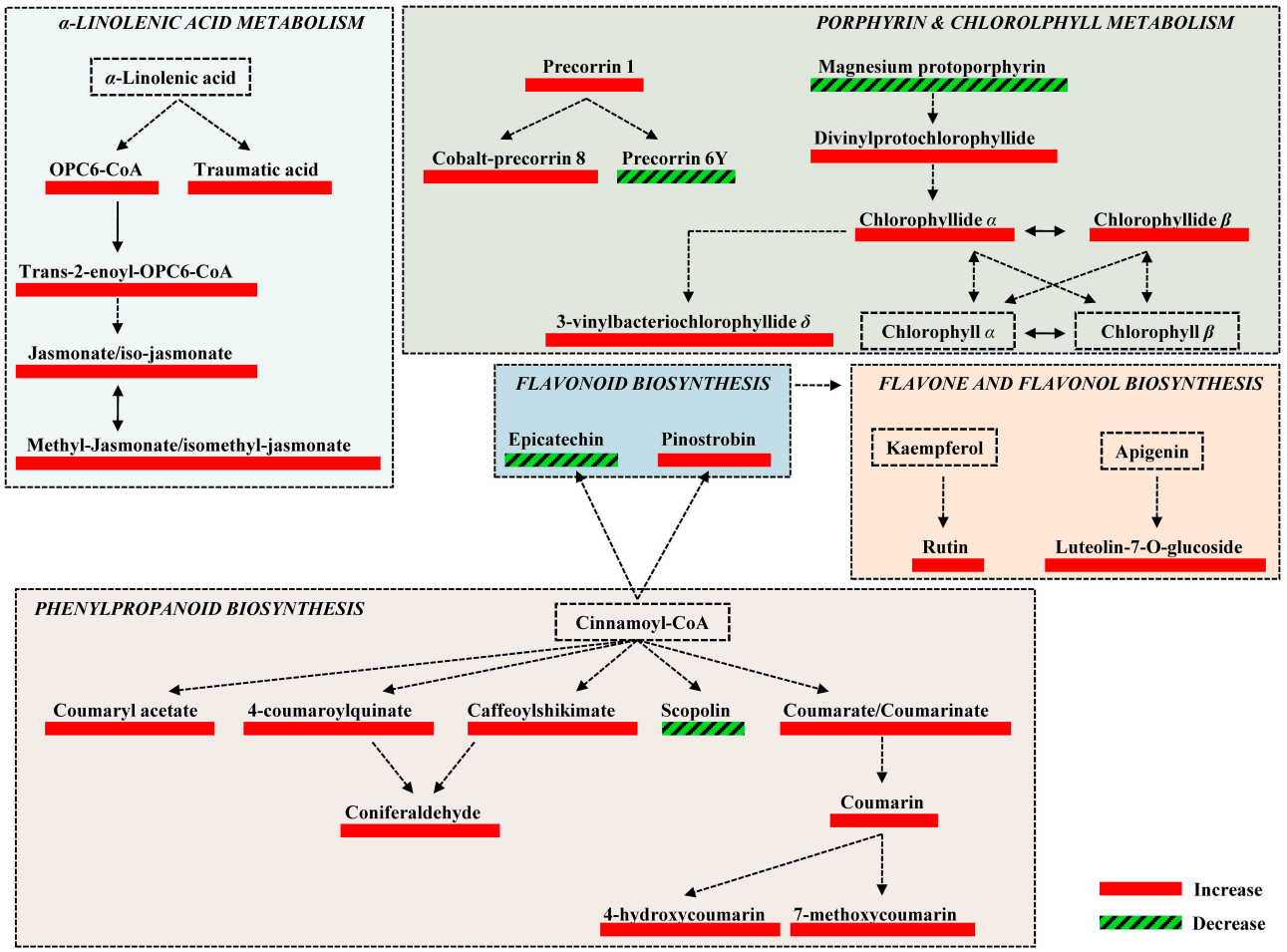


FIGURE 4 | Fluctuations of major biosynthetic pathways of willow leaves 2 weeks post-inoculation with the AMF *Rhizophagus irregularis* infection based on changes in the relative concentration of identified metabolites. Metabolite fluctuations are displayed using a color code based on *P*-values ($P < 0.05$) performing the Student's *t*-test. Five biological replications and a quality control sample were analyzed per treatment. Dashed lines symbolize multi-step or not fully elucidated reactions and solid lines one-step reactions.

2010). Many flavonoids exhibit free radical scavenging and antimicrobial activities (Treutter, 2006; Buer et al., 2010; Cesco et al., 2010; Dixon and Pasinetti, 2010) and in conjugated form, they are also released in the soil as eco-sensing signals for the establishment of suitable symbiotic relationship between plants and rhizobia, AMF or ectomycorrhizal fungi (Shaw et al., 2006; Abdel-Lateif et al., 2012). However, once the AMF-plant association is established, the role of flavonoids in the regulation of mycorrhization is still unclear (Steinkellner et al., 2007). In agreement with our results, flavonoid metabolism in roots and leaves of clover was strongly affected by AMF association, suggesting a strong link between AMF and the regulation of flavonoid metabolism of mycorrhized clover (Ponce et al., 2004).

Focusing on the identified biomarkers, pinostrobin is a potent inducer of antioxidant enzymes (Fahey and Stephenson, 2002) and isoformonetin, a naturally occurring methoxydaidzein, is the product of reaction of daidzein with S-adenosyl-L-methionine (SAM), which is catalyzed by isoflavone-7-O-methyltransferase [EC:2.1.1.150], and its role in plant physiology is not known. Interestingly, isoformonetin has been reported to attract fungal zoospores (Dakora and Phillips, 1996). On the other hand, epicatechin, whose level decreased following AMF inoculation, is the main monomeric unit for proanthocyanidins, a group of polyphenolic compounds with diverse biological and biochemical activities, including protection against predation and pathogen invasion, as well as with an allelopathic function (He et al., 2008).

Finally, in response to inoculation with AMF, the flavonoids rutin and luteolin-7-O-glucoside, which are implicated in the flavone and flavonol biosynthetic pathway, showed a substantial increase of 2.1 and 5 fold, respectively. Both metabolites have been reported as potent antioxidants in *in-vitro* experiments (Süzgeç et al., 2005; Iacopini et al., 2008; Yang et al., 2008). Additionally, luteolin-7-O-glucoside exhibits antimicrobial activity (Chiruvella et al., 2007) and is reported as a plant response to several environmental stresses (Oh et al., 2009; Ahuja et al., 2010).

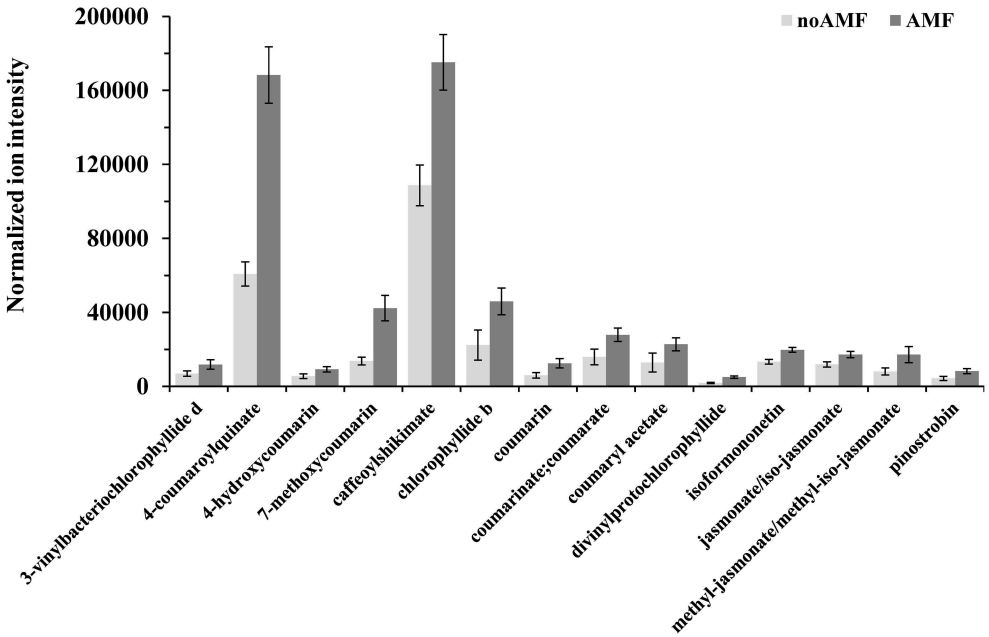


FIGURE 5 | Normalized ion intensities for representative secondary metabolites that serve as biomarkers of willow leaves in response to *Rhizophagus irregularis* 2 weeks post-inoculation. Data were recorded performing liquid chromatography-mass spectrometry analysis using an LTQ-Orbitrap acquiring in the positive (ESI^+) or negative (ESI^-) electrospray modes. Bars represent standard errors performing the Student's *t*-test ($P < 0.05$).

Up-regulation of the Porphyrin and Chlorophyll Biosynthetic Pathway

The increase in the vast majority of the identified metabolites involved in the porphyrin and chlorophyll biosynthetic pathway of willow leaves following inoculation with AMF, is another major finding of the present research (**Table 1**, **Figures 3**, **4**, **5**, and **Supplementary Data set 1**). Such up-regulation of the pathway is likely the cause for the observed higher levels of carbohydrates in the leaves of mycorrhized willows as presented above.

Two of the identified metabolites, chlorophyllide α and chlorophyllide β, are the main precursors of chlorophyll α and β biosynthesis, respectively. The up-regulation of the chlorophyll biosynthetic pathway in AMF-inoculated willows could significantly affect their development, directly and indirectly, through its biosynthetic products and role in defense against pests and pathogens via ROS production. AMF inoculation has been reported to alleviate the content of chlorophyll in plants grown under salinity stress (Sheng et al., 2008; Hajiboland et al., 2010; Latef and He, 2011), which was attributed to the increase in mineral uptake that is mediated via the colonization of roots with AMF.

Not only chlorophylls play a key role in plant growth and are reliable indicators of plant nutrient status, but regulation of their product levels is extremely important being strong photosensitizers generating ROS when they are present in excess (Asada, 2006). In the plant cells, ROS can strengthen the cell walls via cross-linking of glycoproteins (Huckelhoven, 2007) or lipid peroxidation (Montillet et al., 2005). However, it is also evident that ROS are important signaling molecules mediating defense gene activation and controlling various processes including pathogen defense, programmed cell death, and stomatal behavior (Apel and Hirt, 2004; Sharma et al., 2012).

Effect of AMF on Secondary Metabolites with Various Roles in Plant Physiology

In addition to metabolites involved in the abovementioned pathways of willow leaves, metabolites with important roles in plant physiology that belong to various chemical groups and are implicated in several biosynthetic pathways, such as, phenolic glucosides and terpenoids were significantly affected by the AMF inoculation (**Table 1**, and **Supplementary Data set 1**).

Phenolic glycosides of the *Salix* spp. is their most studied group of metabolites due to their bioactivity and role in plant physiology (Förster et al., 2010; Boeckler et al., 2011), with their levels exhibiting a high seasonal variability (Förster et al., 2010). Here, with the exception of salicortin, all identified phenolic glycosides increased in response to AMF inoculation (**Figure 3** and **Supplementary Data set 1**). These metabolites play an important role in plants' defense against pests and also act synergistically with pathogens on herbivores (Boeckler et al., 2011). This is an indication that mycorrhization of willow could improve its resistance against pests. In addition, willows' phenolic glucosides serve as chemoattractants for herbivorous insects (Kolehmainen et al., 1995).

Additionally, increased levels of terpenoids were recorded in leaves of AMF-inoculated willows compared to those of non-inoculated ones. Terpenoids include metabolites that play a key role in the metabolism of arbuscular mycorrhized roots by regulating major biosynthetic pathways such as, the

methylerythritol phosphate pathway and carotenoid biosynthesis (Strack and Fester, 2006). Increased levels of terpenoids are correlated with the production of signaling molecules or protection of root cells against oxidative damage. Several terpenoids function as antioxidants, phytoalexins, and play role in plant defense against pathogens (Grassmann, 2005; Cheng et al., 2007). The induction of these metabolites and their increase following AMF inoculation is expected to have a positive effect on willow's adaptation to stresses.

Concluding Remarks

Here, by the development of a cutting-edge metabolomics/bioinformatics protocol, we investigated in-depth the effects of AMF on willow's leaf metabolism, which could possibly be beneficial for willow when used as a component of integrated phytoremediation strategies. Results unraveled the complexity of AMF-willow interaction, clearly demonstrating beneficial direct and indirect effects on host priming against external stresses as well as by enhancing its growth and productivity. Such interaction is anticipated to provide inoculated plants with a significant advantage over non-inoculated ones by the time that they will be exposed to unfavorable conditions such as, contaminated soil, pests, and pathogens when used as components of a phytoremediation strategy.

Author Contributions

KA and SJ conceived, designed, and executed the experiments. KA, RC, and SJ analyzed the data. KA, RC, and SJ contributed to the writing of the manuscript.

Acknowledgments

The work is part of the research project "GenoRem" (http://genorem.ca) funded by Genome Canada (Grant number 233017) and Genome Quebec. ^{1}H NMR experiments were recorded at the Québec/Eastern Canada High Field NMR Facility, supported by the Canada Foundation for Innovation, the Groupe de Recherche Axé sur la Structure des Protéines (GRASP), McGill University Faculty of Science and Department of Chemistry and PROTEO, The Québec Network for Research on Protein Function, Structure, and Engineering.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00344/abstract

Supplementary Data set 1 | Identified biomarkers of leaves of willow plants 2 weeks following inoculation with *Rhizophagus irregularis* based on GC/MS and LTQ Orbitrap MS analyses.

Supplementary Figure 1 | Total ion chromatograms (TICs) of willow leaves performing GC/MS analysis. Representative metabolites are annotated.

Supplementary Figure 2 | Total ion chromatograms (TICs) of willow leaves performing LC/MS analysis using an LTQ Orbitrap Classic analyzer in the positive (ESI^+) and negative (ESI^-) electrospray modes.

Supplementary Figure 3 | ^{1}H NMR spectra of willow control (A) and mycorrhized (C) willow leaves. Corresponding partial least squares (PLS) coefficient diagram with values of scaled and centered PLS regression coefficients (CoeffCS) is displayed **(B)**. Representative metabolites are annotated.

Supplementary Figure 4 | Partial least squares-discriminant analyses (PLS-DA) PC1/PC2 score plots of GC/MS, ^{1}H NMR and LTQ Orbitrap MS (ESI^+ and ESI^-) metabolite profiles of control (■) and willow leaves 2 weeks following inoculation with the AMF *Rhizophagus irregularis* (●). The ellipse represents the Hotelling T^2 with 95% confidence interval. Five (5) biological replications were used per treatment and one quality control sample (QC) [$Q^2_{(cum)}$; cumulative fraction of the total variation of the X's that can be predicted by the extracted components, R^2X and R^2Y; the fraction of the sum of squares of all *X*'s and *Y*'s explained by the current component, respectively. *PCs*, principal components].

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 10 June 2015
doi: 10.3389/fpls.2015.00438

Trichoderma reesei FS10-C enhances phytoremediation of Cd-contaminated soil by *Sedum plumbizincicola* and associated soil microbial activities

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OPEN ACCESS

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Specialty section:
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science

Received: *05 December 2014*
Accepted: *28 May 2015*
Published: *10 June 2015*

Citation:
Teng Y, Luo Y, Ma W, Zhu L, Ren W, Luo Y, Christie P and Li Z (2015) Trichoderma reesei FS10-C enhances phytoremediation of Cd-contaminated soil by Sedum plumbizincicola and associated soil microbial activities. Front. Plant Sci. 6:438. doi: 10.3389/fpls.2015.00438

This study aimed to explore the effects of *Trichoderma reesei* FS10-C on the phytoremediation of Cd-contaminated soil by the hyperaccumulator *Sedum plumbizincicola* and on soil fertility. The Cd tolerance of *T. reesei* FS10-C was characterized and then a pot experiment was conducted to investigate the growth and Cd uptake of *S. plumbizincicola* with the addition of inoculation agents in the presence and absence of *T. reesei* FS10-C. The results indicated that FS10-C possessed high Cd resistance (up to 300 mg L^{-1}). All inoculation agents investigated enhanced plant shoot biomass by 6–53% of fresh weight and 16–61% of dry weight and Cd uptake by the shoots by 10–53% compared with the control. All inoculation agents also played critical roles in increasing soil microbial biomass and microbial activities (such as biomass C, dehydrogenase activity and fluorescein diacetate hydrolysis activity). Two inoculation agents accompanied by FS10-C were also superior to the inoculation agents, indicating that *T. reesei* FS10-C was effective in enhancing both Cd phytoremediation by *S. plumbizincicola* and soil fertility. Furthermore, solid fermentation powder of FS10-C showed the greatest capacity to enhance plant growth, Cd uptake, nutrient release, microbial biomass and activities, as indicated by its superior ability to promote colonization by *Trichoderma*. The solid fermentation powder of FS10-C might serve as a suitable inoculation agent for *T. reesei* FS10-C to enhance both the phytoremediation efficiency of Cd-contaminated soil and soil fertility.

Keywords: *Trichoderma reesei* FS10-C, cadmium, phytoremediation, *Sedum plumbizincicola*, soil microbial activities

Introduction

Soil contamination by heavy metals (HMs) such as cadmium released from agricultural and industrial activities is an environmental problem worldwide. Potentially toxic HMs are resistant to biodegradation and their persistence thus threatens the environment and public health (Lima et al., 2011; Sahu et al., 2012). A number of methods have been developed for the remediation of metal-contaminated soils and phytoremediation is considered to be a promising technique

because it is cost-effective and environmentally friendly. However, its use in field conditions has been somewhat restricted (Li et al., 2011; Shin et al., 2012; Wu et al., 2012).

In recent years plant-associated bacteria and fungi have been examined for their capacity to enhance the efficiency of phytoremediation (Rajkumar et al., 2012). Numerous filamentous fungi such as *Trichoderma* sp. have aroused increasing interest due to their potential for enhancing the establishment of vegetation and the remediation of metal-contaminated soils (Bareen et al., 2012). Moreover, *Trichoderma* species are characterized by rapid growth, asexual reproduction, effective colonization capacity and low-specificity plant symbiosis (Williams et al., 2003; Harman et al., 2004; Zafar et al., 2007). Certain *Trichoderma* species have been reported to enhance plant growth and metal availability to plants in contaminated soils. Babu et al. (2014b) reported that *Trichoderma virens* PDR-28 increased the dry biomass of maize and its Cd accumulation compared with the control. Similarly, *T. pseudokoningii* increased the biomass and Cd accumulation of pearl millet (Bareen et al., 2012). However, little is known about the effects of *T. reesei* on the phytoremediation of HM contaminated soils.

The objectives of the present study were to explore the effects of *Trichoderma reesei* FS10-C (isolated and preserved previously in our laboratory) on Cd phytoremediation by *Sedum plumbizincicola*, a Cd hyperaccumulator (Wu et al., 2012; Hu et al., 2013), to evaluate soil fertility after phytoremediation, mainly based on microbial biomass and activities, and to determine the colonization ability of *Trichoderma*.

Materials and Methods

Cd Tolerance and Morphological Analysis of *Trichoderma reesei* FS10-C

The Cd tolerance of FS10-C was examined by incubation at 28°C on Potato Dextrose agar (PDA; Tapia et al., 2011) and in corresponding liquid media containing 0, 5, 10, 15, 50, 100, 150, 200, 250, and 300 mg L^{-1} Cd^{2+} ($CdCl_2 \cdot 2.5H_2O$). Colony diameters were measured after growth for 3 days. Mycelia in the conical flasks were collected after 5 days and then dried at 70°C for 24 h before being weighed. Furthermore, the EC_{50} of FS10-C under Cd stress was calculated by the linear interpolation method (Hughes et al., 2001).

The morphological changes in FS10-C under Cd stress were studied by incubating the activated strain in Potato Dextrose (PD) media at 28°C and at 150 rev min^{-1} for 5 days. Equal amounts of mycelia were picked off and added to Czapek's medium (Kexiang et al., 2002) spiked with 0, 10, and 100 mg L^{-1} Cd^{2+} ($CdCl_2 \cdot 2.5H_2O$) respectively for further incubation for 15 h at 28°C and at 150 rev min^{-1}. A moderate amount of mycelium was then extracted, immobilized with 4% glutaraldehyde solution prepared using 0.2 mol L^{-1} phosphate buffered saline (PBS, pH 7.2) for 4 h, and washed with 0.1 mol L^{-1} PBS (pH 7.2) three times. After ethanol dehydration, the ethanol was replaced twice with isoamylacetate, for 15 min each time. Finally, electrical conductivity (15 mA, 90 s) was measured after critical point drying. Treated samples were then observed with a scanning electron microscope (SEM, FEI Quanta 200, Hillsboro, OR, USA) and evaluated qualitatively and quantitatively with an Energy Dispersive X-ray Detector (EDX, INCA E-250, High Wycombe, UK).

Preparation of Inoculation Agents

To apply *Trichoderma* sp. widely in practice the first task is to obtain a large number of *Trichoderma* products. Thus far, the *Trichoderma* agents produced in commercial applications have been intended primarily for spore preparation. In this context, we prepared several inoculation agents using the following preparation methods. The fermentation conditions and proportion parameters were explored in orthogonal experiments.

Trichoderma reesei FS10-C was first activated in PDA media and then prepared as a spore suspension (1×10^6 colony-forming units mL^{-1}). The spore suspension was inoculated onto a sterilized solid matrix (1:20 v/w) and incubated at 28°C for 10 days. The solid matrix consisted of orange peel powder and wheat bran (1:1 w/w) with the moisture content adjusted to 50% w/v using deionized water. This fermented product was designated 'solid fermentation powder of *T. reesei* FS10-C' and its efficacy was examined with and without sterilization (the first and second inoculation agents).

Conidium wettable powder of *T. reesei* FS10-C was the third inoculation agent, consisting of a mixture of 10% *T. reesei* FS10-C conidium powder, 5% sodium dodecyl benzene sulfonic acid, 0.4% vitamin C, 10% kaolinite and 74.6% sodium lignosulphonate. This mixture, without the addition of 10% *T. reesei* FS10-C conidium powder, was used as the fourth inoculation agent. These latter two inoculation agents were diluted 500 times before use.

Sample Collection and Experimental Design

Soil samples were collected from the arable layer (top 15 cm) of a Cd-contaminated agricultural soil located in Xiangtan, Hunan Province, China. The physicochemical properties and Cd content of the soil samples are shown in **Table 1**. The soil was air dried, sieved (2 mm), and mixed thoroughly with 0.15 g kg^{-1} N as $(NH_4)_2SO_4$, 0.20 g kg^{-1} P as NaH_2PO_4

TABLE 1 | Selected physicochemical properties of the soil used in the pot experiment.

Soil property	Value
pH (H_2O)	4.7
Organic matter (g kg^{-1})	32.0
Total N (g kg^{-1})	2.2
Total P (g kg^{-1})	0.5
Total K (g kg^{-1})	11.2
Hydrolyzable nitrogen (mg kg^{-1})	91.0
Available P (mg kg^{-1})	8.6
Available K (mg kg^{-1})	55.0
Cd (mg kg^{-1})	0.53

and 0.30 g kg^{-1} K as KCl. Seedlings of *S. plumbizincicola* were obtained from a heavy-metal polluted area in Zhejiang Province, east China and were ∼5 cm long with a pair of leaves and 4–5 nodes. Healthy plants of uniform size were chosen for the pot experiment after the seedlings had produced roots under incubation in half-strength Hoagland nutrient solution for 2 weeks.

In the pot experiment five treatments were set up in a fully randomized layout, namely: (1) uninoculated *S. plumbizincicola* as control (CK); (2) *S. plumbizincicola* inoculated with solid fermentation powder of *T. reesei* FS10-C (SP); (3) *S. plumbizincicola* inoculated with sterilized solid fermentation powder (SCK); (4) *S. plumbizincicola* inoculated with conidium wettable powder of *T. reesei* FS10-C (WP); (5) *S. plumbizincicola* inoculated with conidium wettable powder without the addition of *T. reesei* FS10-C conidia (WCK). The inoculation method for the SP and SCP treatments was hole fertilization (4% inoculum was added), whereas spray irrigation (100 mL per pot was added at 0, 30, 60, 90, and 120 days respectively) was used for the WP and WCP treatments. Each treatment was set up in quadruplicate, giving a total of 20 pots. Each pot received 1.5 kg soil and five plants.

The plants grew in a growth chamber under controlled light (14-h photoperiod at 1.5×10^4 lux), temperature (25/20°C, light/dark), and humidity (60–70%). Throughout the experiment the plants were watered with deionized water to maintain 70% of water-holding capacity. Shoot and soil samples were collected after incubation for 120 days. Shoot fresh and oven dry weights (DWs) were determined. A portion of the soil samples was air-dried, ground and sieved (0.15 mm) before analysis for pH and available phosphorus (P). Soil pH was measured using a pH meter (520M-01, Thermo Orion, Beverly, MA, USA) and soil available P was determined based on the Olsen method (Olsen et al., 1954). The remaining potion of the soil samples was sieved (<2 mm) for subsequent experiments.

Analysis of Cd in Soil and Plant Samples

Ground plant samples weighing 0.5 g were placed into digestion vials and mixed with 10 mL of $HClO_4$:HNO_3 (2:3 v/v). Dry soil samples (0.25 g) were weighed and mixed with 14 mL of HCl:HNO_3 (4:1 v/v). All samples were digested according to the EPA Method 3050B (EPA, 1996) using the Hot Block Digestion System (SISP, DS-360). After digestion all samples were cooled completely and then diluted to 50 mL. Cd concentrations were measured using atomic absorption spectrophotometry (Varian SpectrAA 220Z). In addition, blank and certified reference materials (GSV-2 for plant analysis, GSS-4 for soil analysis, Chinese geological reference materials) were used for quality control.

Soil Enzyme Activities

Soil dehydrogenase (DHA) activity was assessed by a modification of the method of Singh and Singh (2005). Sieved soil (5 g) was weighed and mixed with 5 mL of 0.5% 2, 3, 5-triphenyltetrazolium chloride (TTC) solution and incubated for 12 h at 30°C in the dark. After incubation triphenylformazan (TPF), formed by the reduction of TTC, was extracted with three batches of 100 mL methanol, shaken at 300 rpm for 1 h and centrifuged at 2,000 rpm for 5 min. The supernatant was filtered and the concentration of TPF was determined spectrophotometrically at 485 nm. Blanks with TTC omitted were included. All results are expressed as μg g^{-1} dw.

Fluorescein diacetate (FDA) hydrolysis activity was determined according to Aira and Domínguez (2014). Briefly, 5 g of sieved soil was incubated for 20 min at 30°C and 200 rpm with 15 mL of 60 mM potassium dihydrogen phosphate buffer (pH 7.6) and 0.2 mL of FDA stock solution (1000 μg mL^{-1}). The reaction was stopped by adding 15 mL of chloroform/methanol (1:1 v/v) and the mixture was gently mixed and centrifuged at 2,000 rpm for 3 min. The supernatant was filtered and read at 490 nm. The results are expressed as μg g^{-1} dw.

Soil Microbial Biomass C

The fumigation-extraction method (Vance et al., 1987) was used to determine soil microbial biomass C according to Beck et al. (1997). Ten grams of sieved soil for chloroform-fumigated and non-fumigated treatments were extracted with 50 mL of 0.5 mol L^{-1} K_2SO_4 and then filtered at 300 rev min^{-1} for 30 min. Organic C in the supernatant was measured using an automated TOC Analyzer. Microbial biomass C was calculated as follows: biomass C = Ec/k_{EC}, where Ec = (organic C extracted from fumigated soil) - (organic C extracted from non-fumigated soil), and k_{EC}, which was used to convert the measured flush of C to biomass C, was 0.45 (Beck et al., 1997; Yao et al., 2003).

Biolog® EcoPlate Analysis of the Soil Microbial Community

Soil bacterial functional diversity was assessed as described by Yao et al. (2003). Briefly, 10 g of sieved soil was added to 100 mL of sterile water in a 250-mL flask and shaken at 180 rpm for 10 min. Ten-fold serial dilutions were made and 150 μL of the final 10^{-3} dilution was added to each well of a Biolog® EcoPlate. The plates were incubated at 28°C for 7 days and color development in each well was recorded as the absorbance at 590 nm using a microplate reader (BioTek μQuant, Winooski, VT, USA) at regular 12-h intervals. Microbial metabolic activity in each microplate, expressed as the average well-color development (AWCD), was determined as follows: AWCD = Σ ODi/31, where ODi is the optical density value from each well (Fang et al., 2009). The McIntosh index (U) was calculated as $U = \sqrt{(\sum ni^2)}$, where ni refers to the absorbance value (McIntosh, 1967). The Shannon–Weaver index (H) was calculated as H = –Σ Pi (ln Pi), where Pi is the ratio of the activity of each substrate (ODi) to the sum of activities of all substrates (Σ ODi; Zhong et al., 2009).

Quantitative Real-Time PCR Assay

The abundance of *Trichoderma* sp. was quantified by real-time PCR (Bio-Rad, Hercules, CA, USA) performed on a Real-Time System and 18S rDNA amplifications were performed in a total volume of 20 μL containing 2 μL of soil microbial DNA, 0.2 μL of each of the primers DG (5′-CTGGCATCGATGAAGAACG-3′) and DT (5′-ATGCGAGTGTGCAAACTACTG-3′) and 10 μL

of SYBR Green I. The qPCR was performed with an initial denaturation and enzyme activation step for 5 s at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 53°C, and 30 s at 72°C and a final extension performed at 72°C for 5 min. Fluorescence measurements were made at the end of each annealing cycle and an additional measuring point at 80°C for 1 s to detect the formation of primer dimers during amplification. A melt curve analysis was performed by raising the temperature from 65 to 95°C in 0.2°C steps for 1 s each. The results are expressed as copies g^{-1} dw.

Data Analysis

To evaluate the phytoremediation effects of *S. plumbizincicola* we defined phytoextraction efficiency (%) as:

$$\frac{\text{The Cd content accumulated in the plant shoots after phytoremediation}}{\text{The initial soil Cd content}}$$

and the removal efficiency (%) as:

$$\frac{\text{The difference of Cd content in the soil between the beginning and end of phytoremediation}}{\text{The initial soil Cd content}}.$$

The pot experiment was performed in quadruplicate and the other experiments in triplicate. All results are presented as the mean ± SD. Data processing and correlation analysis were performed using MS Excel 2003. The results related to Cd phytoremediation and soil properties were analyzed by one-way analysis of variance using SigmaPlot 12.5 and all pairwise multiple comparison analyses (Holm-Sidak method) were performed at the $p < 0.05$ level.

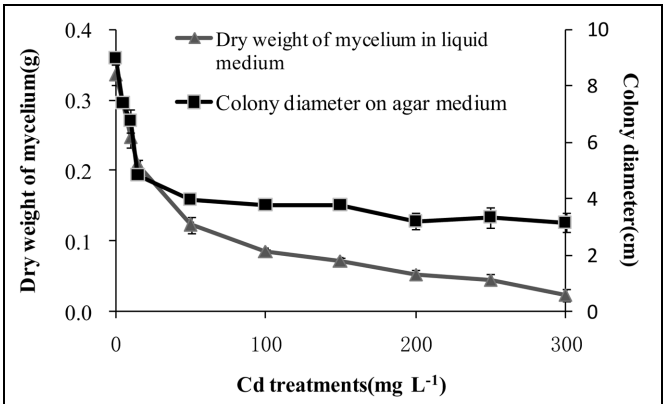

FIGURE 1 | Effect of Cd on the growth of *Trichoderma reesei* FS10-C. Values are mean ± SD of triplicate determinations.

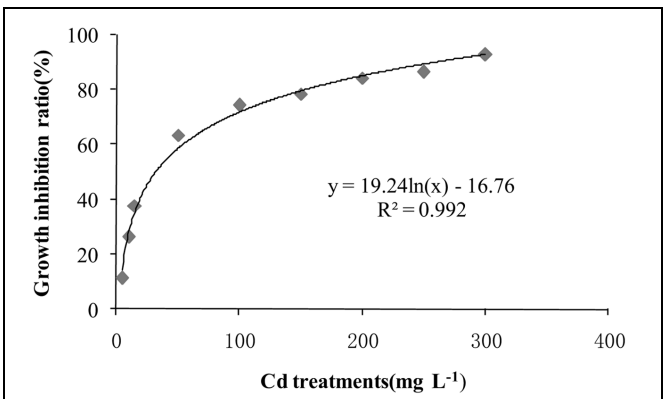

FIGURE 2 | The growth inhibition ratio of *T. reesei* FS10-C in liquid medium.

Results and Discussion

Cadmium tolerance of *T. reesei* FS10-C

After incubation at 28°C for 7 days the growth of *T. reesei* FS10-C on solid and in liquid media with different Cd treatments exhibited similar trends (**Figure 1**). As the Cd concentration increased, *T. reesei* FS10-C experienced more pronounced growth inhibition. However, FS10-C was still able to grow at a concentration of 300 mg L^{-1} Cd^{2+}. The growth inhibition ratio of FS10-C is shown in **Figure 2** and indicates that FS10-C tolerated high Cd stress.

To better understand the Cd tolerance of FS10-C its morphological changes under different Cd treatments were investigated using SEM (**Figure 3**). The results suggested that there was no significant influence on FS10-C growth in the 10 mg L^{-1} Cd treatment compared with the control, although a small irregular fold emerged on the surface of FS10-C mycelia in the 100 mg L^{-1} Cd treatment. Furthermore, EDX analysis showed that the peaks of the four nutrient elements P, S, K, and Fe increased (**Figure 4**).

Cao et al. (2008) found that *T. atroviride* F6 resisted up to 100 mg Cd^{2+} L^{-1} in liquid media. Sahu et al. (2012) indicated that the DW of *T. viride* mycelia was 0.07 g in 200 ppm Cd. In addition, Babu et al. (2014a) showed that *Trichoderma* sp. PDR1-7 survived in 100 mg L^{-1} Cd with 0.9 g dw. Our results showed that *T. reesei* FS10-C survived in 300 mg L^{-1} Cd in both solid and liquid media, indicating that *T. reesei* FS10-C has a high level of Cd tolerance.

Increasing Growth and Cd Uptake by *Sedum plumbizincicola* When Inoculated with *T. reesei* FS10-C

Shoot biomass after 120 days is shown in **Table 2**. The shoot biomass values under the four treatments using inoculation agents were all higher than the control in the following declining sequence: SP > SCK > WP> WCK > CK. Compared with the control, the fresh shoot weights in the SP and SCK treatments were enhanced by 53 and 30%, respectively ($p < 0.05$), and their corresponding DWs were enhanced by 61 and 35% ($p < 0.05$). Shoot DW under the WP treatment (12.81 g pot^{-1}) was also significantly higher ($p < 0.05$) than the control (10.20 g pot^{-1}) but there was no significant increase in fresh shoot weight under the WP and WCK treatments or shoot DW in the WCK treatment.

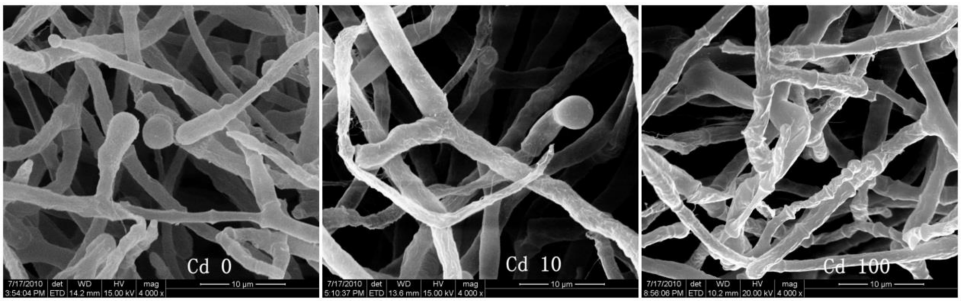

FIGURE 3 | Effect of Cd on the morphology of *T. reesei* FS10-C.

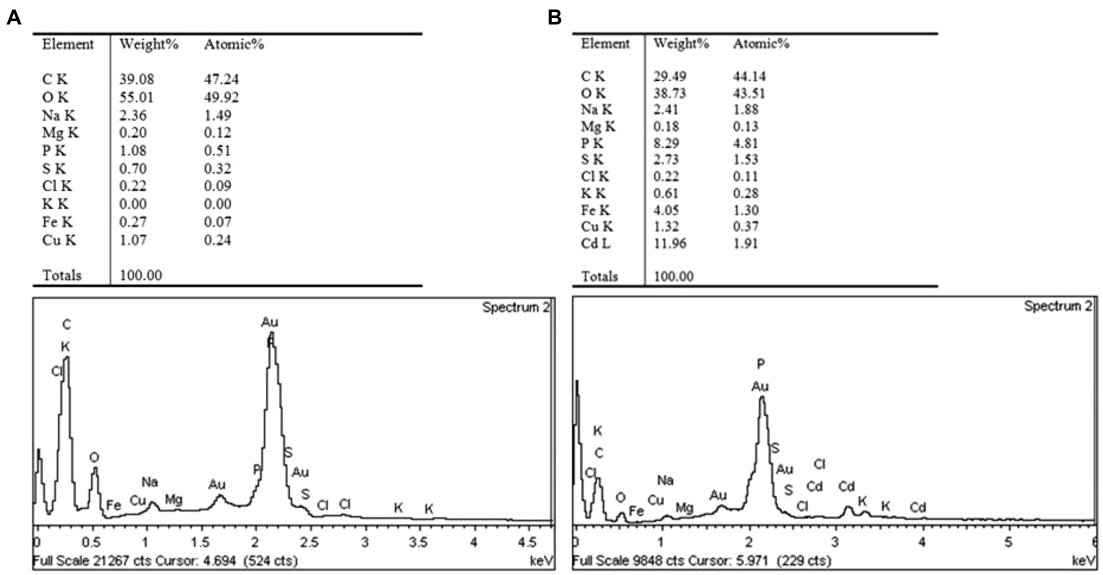

FIGURE 4 | The elemental contents of mycelia under (A) 0 mg Cd L^{-1} and (B) 100 mg Cd L^{-1} treatments.

TABLE 2 | Effect of inoculation agents on shoot biomass and Cd uptake of *Sedum plumbizincicola* and phytoremediation.

Treatment	Fresh weight (g pot^{-1})	Dry weight (g pot^{-1})	Cd uptake (mg pot^{-1})	Cd conc. in soil (mg kg^{-1})	Phytoextraction efficiency (%)	Removal efficiency (%)
CK	123.79 ± 4.39^{c}	10.20 ± 0.15^{c}	0.30 ± 0.04^{b}	0.27 ± 0.02^{b}	37.24 ± 4.51^{b}	49.45 ± 4.43^{b}
SCK	160.76 ± 16.39^{ab}	13.73 ± 0.97^{b}	0.37 ± 0.05^{ab}	0.24 ± 0.01^{ab}	47.12 ± 6.83^{ab}	54.27 ± 1.94^{ab}
SP	189.25 ± 23.35^{a}	16.44 ± 1.86^{a}	0.46 ± 0.05^{a}	0.21 ± 0.01^{a}	58.30 ± 6.37^{a}	60.50 ± 1.24^{a}
WCK	131.50 ± 4.73^{bc}	11.79 ± 0.59^{bc}	0.33 ± 0.06^{b}	0.24 ± 0.03^{ab}	41.02 ± 7.30^{b}	54.40 ± 5.73^{ab}
WP	141.98 ± 12.10^{bc}	12.56 ± 0.73^{b}	0.44 ± 0.03^{a}	0.22 ± 0.01^{a}	54.84 ± 3.48^{a}	58.56 ± 2.39^{a}

Mean values (±SD) followed by different letters with in a column differ significantly at $p < 0.05$.

Shoot Cd uptake is also shown in **Table 2**. Cadmium accumulation under the SP and WP treatments was 0.46 mg pot^{-1} and 0.44 mg pot^{-1} respectively and these values were significantly higher ($p < 0.05$) than the control treatment (0.30 mg pot^{-1}). Cadmium uptake under the SCK and WCK treatments did not increase significantly over the control. A significant decline ($p < 0.05$) was observed in all treatments (including the control) compared with the initial soil Cd content, perhaps attributable to the Cd accumulation potential of *S. plumbizincicola*. The phytoextraction and removal efficiencies of *S. plumbizincicola* alone (the control treatment) were 37.2 and 49.5%, respectively (**Table 2**). After the addition of inoculation agents the phytoextraction efficiency of *S. plumbizincicola* increased by 41.0–58.3% and the removal efficiency increased by 54.3–60.5% (**Table 2**). In addition, SP and WP treatments with *T. reesei* FS10-C showed greater phytoextraction capabilities and removal efficiencies than SCK and WCK treatments without FS10-C.

Our results showed that the declining sequence of shoot biomass among all treatments was: SP > SCK > WP > WCK > CK. It can be concluded that the use of solid fermentation powder as an inoculation agent significantly enhanced ($p < 0.05$) the growth of *S. plumbizincicola* with a distinct advantage over conidium wettable powder. In addition, SP > SCK and WP > WCK showed that the inoculation agents with *T. reesei* FS10-C were more effective than inoculation agents without FS10-C, indicating that FS10-C played an important role in promoting the growth of *S. plumbizincicola*. Higher plant biomass under the SCK treatment as compared to the control indicates possible effects of nutrients and *Trichoderma* secondary metabolites in the inoculation agent for SCK promoting plant growth (Hoyos-Carvajal et al., 2009; Vinale et al., 2009). The sequence of shoot Cd uptake was: SP > WP > SCK > WCK > CK. Cadmium uptake in the SP and WP treatments was significantly higher than the SCK and WCK treatments ($p < 0.05$), indicating that the inoculation agents with *T. reesei* FS10-C were more effective in enhancing Cd uptake by *S. plumbizincicola* than those inoculation agents without FS10-C. In addition, the sequence SP > WP and SCK > WCK showed that higher plant biomass accumulated more Cd.

In general, our study demonstrated that *T. reesei* FS10-C was able to enhance the plant biomass and Cd accumulation of *S. plumbizincicola*, especially with an inoculation agent such as solid fermentation powder of FS10-C. Certain other *Trichoderma* sp. have also been reported to enhance plant growth and Cd uptake under Cd stress. Adams et al. (2007) showed that inoculation with *T. harzianum* Rifai 1295-22 increased the DW of crack willow and Cd accumulation in the shoots by 39 and 24%, respectively. Babu et al. (2014b) found that inoculation with *T. virens* PDR-28 increased the DW of maize shoots and Cd accumulation by 56 and 59%. In our study inoculation with solid fermentation powder of FS10-C increased the DW of *S. plumbizincicola* shoots by 61% and shoot Cd accumulation by 53%, representing an enhancement of Cd phytoremediation. Plant growth promotion by inoculation with FS10-C was mainly attributable to the production of IAA, ACC deaminase and siderophores as well as phosphate solubilization (Gravel et al., 2007; Qi and Zhao, 2013). Qualitative and quantitative analyses of plant growth-promoting traits of FS10-C (Triveni et al., 2013; Kotasthane et al., 2015) have been carried out. The results showed that FS10-C had the ability to produce ACC deaminase and siderophores and to solubilize phosphate (Supplementary Figures S1 and S2; average siderophore production was 68%) and this agrees with the findings of Babu et al. (2014b,c). Increased Cd uptake induced by FS10-C might be attributed to the successful colonization of FS10-C both promoting plant growth to accumulate more Cd and enhancing Cd phytoextraction by altering the solubility, availability and transport of Cd (Song et al., 2015).

Changes in Soil pH and Available P

After 120 days there was a clear decline ($p < 0.05$) in soil available P concentration in all treatments compared with the initial value (8.6 mg kg^{-1}), but there was no significant difference in soil pH (**Table 3**). Compared with the control the pH under the SP treatment increased significantly ($p < 0.05$). Soil available P under the SP and SCK treatments increased substantially ($p < 0.05$) by 107 and 71%, respectively, compared with the control.

TABLE 3 | Soil pH and available P concentration after phytoremediation for 120 days.

Treatment	pH	Available P (mg kg^{-1})
CK	4.63 ± 0.03^{bc}	3.60 ± 0.11^{c}
SCK	4.84 ± 0.14^{a}	6.15 ± 0.46^{b}
SP	4.80 ± 0.06^{ab}	7.43 ± 0.40^{a}
WCK	4.68 ± 0.09^{ac}	2.64 ± 0.19^{d}
WP	4.55 ± 0.06^{c}	3.25 ± 0.09^{c}

Mean values (±SD) followed by different letters with in a column differ significantly at $p < 0.05$.

Soil available P under both SP and SCK treatments was significantly higher than the control ($p < 0.05$), indicating that *T. reesei* FS10-C played important role in enhancing nutrient release into the soil. Furthermore, more soil available P under the SP treatment than SCK showed that the role of FS10-C was more prominent. This was likely due to the phosphate solubilizing capacity of FS10-C. However, the soil available P values under both WP and WCK treatments were lower than the control. This might be attributable to the spore content of FS10-C being quite small in the inoculation agent of the WP treatment and the inoculation agents in both WP and WCP treatments being added by foliar spray which would have made little contribution to the solubilization of P in the soil. The overall decline in soil available P under all treatments compared with the initial value might be attributable to plant nutrient uptake as available P was positively related to plant fresh and DWs with R^2 values of 0.86 and 0.72, respectively. In addition, our results showed that soil pH values also increased under both SP and SCK treatments in addition to soil available P, which differed with the earlier conclusion that soil available P was negatively related to soil pH (Deng et al., 2012; Babu et al., 2014c). This was likely due to the soil pH value being neutralized after the addition of the inoculation agent.

Enhancement of Soil Microbial Activities by Inoculation Agents

Microbial biomass C, DHA and FDA hydrolysis activities under the four treatments with the addition of inoculation agents were enhanced to different degrees after phytoremediation (**Table 4**). The highest increase in soil microbial biomass C (145%, $p < 0.05$) was observed under the SP treatment, followed by SCK and WP treatments with increases of 62 and 62%, respectively ($p < 0.05$). Similarly, the highest DHA activity was found under the SP treatment, followed by SCK, WP and WCK, and all exhibited significantly higher levels than the control with increases of 86, 56, 49, and 20% ($p < 0.05$), respectively. In terms of FDA hydrolysis activity, the four treatments with the addition of inoculation agents were significantly higher than the control, with increases of 69, 69, 49, and 34% ($p < 0.05$). Furthermore, the treatments involving inoculation with conidium wettable powder with or without *T. reesei* FS10-C clearly demonstrated greater ability to increase FDA hydrolysis activity than those inoculated

TABLE 4 | Microbial biomass C and soil enzyme activities after phytoremediation for 120 days.

Treatment	Microbial biomass C $\mu g\ g^{-1}$	DHA activity $\mu g\ g^{-1}$ dw	FDA hydrolysis activity $\mu g\ g^{-1}$ dw × 20 min
CK	217.21 ± 48.96^{c}	55.82 ± 3.05^{d}	17.60 ± 2.78^{c}
SCK	340.15 ± 44.69^{b}	86.67 ± 4.05^{b}	23.18 ± 0.39^{b}
SP	522.51 ± 73.52^{a}	103.80 ± 5.23^{a}	25.83 ± 3.14^{b}
WCK	246.08 ± 12.20^{c}	66.98 ± 2.07^{c}	29.27 ± 2.04^{a}
WP	341.71 ± 26.26^{b}	82.71 ± 1.97^{b}	29.28 ± 1.36^{a}

Mean values (±SD) followed by different letters with in a column differ significantly at $p < 0.05$.

with solid fermentation powder with or without *T. reesei* FS10-C.

The maintenance of soil fertility depends on the microbial biomass and its activities which are of primary importance in nutrient cycling and ecosystem sustainability, and microbial biomass and activities are sensitive to changes in soil HM content (Giller et al., 1998). One of the soil microbiological parameters, microbial biomass C, is considered to be a sensitive indicator of HM toxicity and soil quality (Yao et al., 2003). Our data suggested that all inoculation agents with or without *T. reesei* FS10-C, particularly the solid fermentation powder of *T. reesei* FS10-C, increased microbial biomass C. In addition, soil DHA activity based on the metabolic state of the soil biota (Araujo et al., 2015) is also used to assess soil health (Gil-Sotres et al., 2005). Our results showed that all inoculation agents tested enhanced DHA activity, indicating that the general activities of soil microbes were enhanced. FDA hydrolysis activity is also a good indicator of soil health in the presence of metal contaminants (Frølund et al., 1995; Gil-Sotres et al., 2005). All inoculation agents increased FDA hydrolysis activity, indicating that the metabolism of soil microbes was promoted, thus resulting in the enhanced microbial activities.

Changes in Microbial Community Functional Diversity after Phytoremediation

Changes in AWCD reflecting the oxidative catabolism of the microbial community over time are shown in **Figure 5**. It is clear that AWCD values for all treatments initially increased rapidly until 84 h and then tended to slow down. The average AWCD values for SP, SCK, WP, and WCK treatments were 2.00, 1.97, 1.93, and 1.85 respectively. SP, SCK and WP treatments were significantly higher than the control (1.82, $p < 0.05$) but WCK treatment did not significantly increase compared with the control. The Shannon–Weaver index and McIntosh index are used to indicate microbial community richness (Fang et al., 2009) and evenness (McIntosh, 1967), respectively. The data in **Table 5** indicate that there was no significant difference between the values of Shannon–Weaver index under all treatments. The values of the McIntosh index under all treatments reached a level of 10.68–11.61 (**Table 5**) and the values under SP, SCK, and WP treatments were significantly higher than that in the control ($p < 0.05$).

Microbial community structure is also considered to be a biological indicator of HM stress (Doelman et al., 1994). The Biolog® EcoPlate method combined with other analyses such as the diversity indices has been recommended to evaluate whole soil microbial community functional diversity (Ros et al., 2008). Our data showed that

TABLE 5 | Shannon–Weaver index (H) and McIntosh index (U) based on community level physiological profiles detected using a Biolog EcoPlate.

Treatment	H (84 h)	U
CK	3.371 ± 0.021^{a}	10.681 ± 0.054^{b}
SCK	3.381 ± 0.014^{a}	11.435 ± 0.243^{a}
SP	3.374 ± 0.009^{a}	11.607 ± 0.519^{a}
WCK	3.364 ± 0.012^{a}	10.822 ± 0.259^{b}
WP	3.362 ± 0.024^{a}	11.310 ± 0.262^{a}

Mean values (± SD) followed by different letters within a column differ significantly at $p < 0.05$.

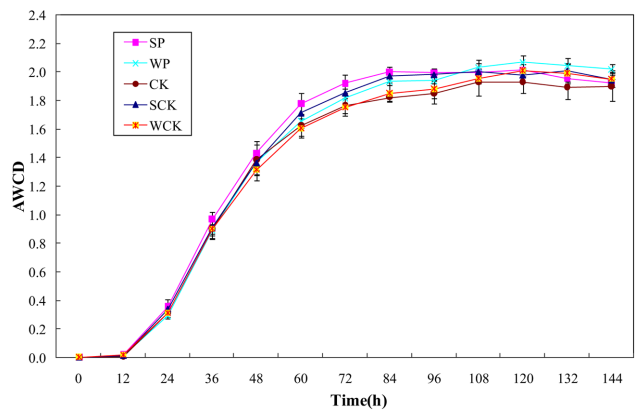

FIGURE 5 | Average well color development (AWCD) values of microbial communities under the different treatments. Values are means ± SD of triplicate measurements.

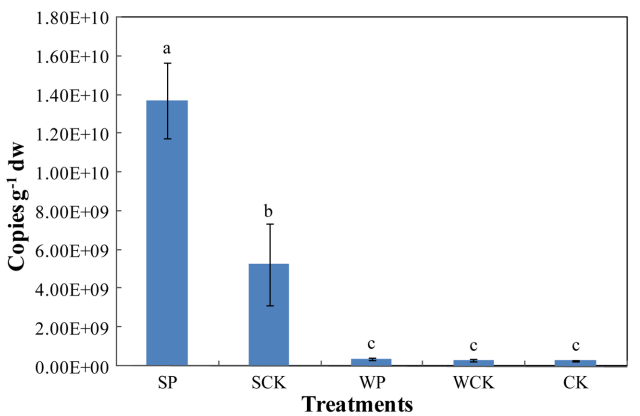

FIGURE 6 | The gene copy numbers of soil *Trichoderma* sp. under the different treatments. Values are means ± SD of triplicate measurements.

the evenness of the soil microbial community was enhanced by all inoculation agents but no significant variation in richness. It was likely due to the low levels of Cd contamination in the soil and the similar experimental conditions in each of the treatments. A similar study also indicated that differences in flora could not be distinguished sensitively by the diversity indices when the experimental conditions did not differ significantly between different treatments (Drenovsky et al., 2008). In addition, the AWCD values were also enhanced by all inoculation agents and found to be closely correlated with microbial biomass C ($R^2 = 0.81$), indicating that microbial biomass and metabolic activity were both enhanced by the addition of the inoculation agents. High concentrations of HMs affect the functional diversity of soil microbial community, resulting in a decline in soil quality (Chander and Brookes, 1993). Our results demonstrated that the soil microbial community might be protected and even enhanced by phytoremediation using *S. plumbizincicola* with all tested inoculation agents, particularly the solid fermentation powder of *T. reesei* FS10-C.

Trichoderma Colonization Ability of Solid Fermentation Powder of *T. reesei* FS10-C

The abundance of *Trichoderma* sp. was estimated via the real-time quantification of its rDNA gene copy numbers (**Figure 6**). The results show that the abundance of *Trichoderma* sp. under SP treatment reached its highest level of 1.37×10^{10} copies g^{-1} dw after phytoremediation, followed by 5.26×10^9 copies g^{-1} dw under the SCK treatment. Both were significantly higher ($p < 0.05$) than the other three treatments (WP: 3.94×10^8 copies g^{-1} dw, WCK: 2.92×10^8 copies g^{-1} dw, CK: 2.98×10^8 copies g^{-1} dw), which were not significantly different from each other.

It is clear that the SCK treatment resulted in a high *Trichoderma* gene abundance, suggesting that the organic substance present in the inoculation agent supplied nutrients and promoted the growth of indigenous *Trichoderma* in the soil. In addition, the *Trichoderma* gene abundance under the SP treatment was significantly higher ($p < 0.05$) than SCK, indicating that FS10-C helped *Trichoderma* sp. to be the most advantaged flora in the soil and enhanced the colonization ability of *Trichoderma*. The *Trichoderma* gene copies were low in the WP and WCK treatments. Possible explanations for this are restriction of the formation of advantageous flora by the decreased FS10-C spore content and the addition of the two inoculation agents (foliar spray) being adverse to the rhizosphere gene expression of FS10-C.

Babu et al. (2014b) indicated that plant growth and HM uptake enhancement were attributable to the successful colonization of *T. virens* PDR-28 in soil. Our results showed that *Trichoderma* gene abundance was positively related to plant DW, Cd uptake, available P, microbial biomass C and DHA activity (R^2 values were 0.84, 0.41, 0.88, 0.85, and 0.72, respectively). Thus, we supposed that the successful colonization of *T. reesei* FS10-C was contributed to the enhancement of plant growth, Cd uptake, nutrient release, microbial biomass and activities. This also indicated that *T. reesei* FS10-C was a good candidate for the enhancement of Cd phytoremediation.

Conclusion

To our knowledge this is the first report demonstrating the potential of *T. reesei* FS10-C to promote plant growth and the Cd removal capacity of *S. plumbizincicola* grown in Cd-contaminated soil. Our results showed that all the inoculation agents tested were able to increase plant biomass and Cd uptake with the simultaneous inoculation of FS10-C compared with inoculation without FS10-C thereby increasing the efficiency of phytoremediation by *S. plumbizincicola*. In addition, nutrition release and microbial activities were promoted, particularly by the solid fermentation powder of FS10-C, indicating an enhancement of soil fertility after phytoremediation. We also found that *Trichoderma* colonization ability played an important role in enhancing plant growth, Cd removal and soil fertility. Solid fermentation powder of *T. reesei* FS10-C showed the greatest *Trichoderma* colonization ability and thus the greatest potential for use as an inoculation agent to enhance Cd phytoremediation. However, field experiments need to be conducted to further verify the practical effects of the application of solid fermentation powder of FS10-C under field conditions.

Acknowledgments

This work was jointly supported by the Science and Technology Service Network Initiative (Project KFJ-EW-ZY-005) and the High Technology Research Development Program of the People's Republic of China (Projects 2012AA06A204 and 2007AA061001). We thank Professor Longhua Wu and his research group at Nanjing Institute of Soil Science for providing the hyperaccumulator seedlings.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00438/abstract

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Conflict of Interest Statement: The Guest Associate Editor Ying Ma declares that, despite having collaborated with the authors Ying Teng, Yongming Luo and Zhengao Li, the review process was handled objectively. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 14 August 2015
doi: 10.3389/fpls.2015.00638

Combined endophytic inoculants enhance nickel phytoextraction from serpentine soil in the hyperaccumulator *Noccaea caerulescens*

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OPEN ACCESS

Edited by:
Ying Ma, University of Coimbra, Portugal

Reviewed by:
Vijai Kumar Gupta, National University of Ireland Galway, Ireland
Alexander A. Kamnev, Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Russia

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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *18 June 2015*
Accepted: *31 July 2015*
Published: *14 August 2015*

Citation:
Visioli G, Vamerali T, Mattarozzi M, Dramis L and Sanangelantoni AM (2015) Combined endophytic inoculants enhance nickel phytoextraction from serpentine soil in the hyperaccumulator Noccaea caerulescens. Front. Plant Sci. 6:638. doi: 10.3389/fpls.2015.00638

This study assesses the effects of specific bacterial endophytes on the phytoextraction capacity of the Ni-hyperaccumulator *Noccaea caerulescens,* spontaneously growing in a serpentine soil environment. Five metal-tolerant endophytes had already been selected for their high Ni tolerance (6 mM) and plant growth promoting ability. Here we demonstrate that individual bacterial inoculation is ineffective in enhancing Ni translocation and growth of *N. caerulescens* in serpentine soil, except for specific strains Ncr-1 and Ncr-8, belonging to the *Arthrobacter* and *Microbacterium* genera, which showed the highest indole acetic acid production and 1-aminocyclopropane-1-carboxylic acid-deaminase activity. Ncr-1 and Ncr-8 co-inoculation was even more efficient in promoting plant growth, soil Ni removal, and translocation of Ni, together with that of Fe, Co, and Cu. Bacteria of both strains densely colonized the root surfaces and intercellular spaces of leaf epidermal tissue. These two bacterial strains also turned out to stimulate root length, shoot biomass, and Ni uptake in *Arabidopsis thaliana* grown in MS agar medium supplemented with Ni. It is concluded that adaptation of *N. caerulescens* in highly Ni-contaminated serpentine soil can be enhanced by an integrated community of bacterial endophytes rather than by single strains; of the former, *Arthrobacter* and *Microbacterium* may be useful candidates for future phytoremediation trials in multiple metal-contaminated sites, with possible extension to non-hyperaccumulator plants.

Keywords: plant growth-promoting endophytic bacteria (PGPE), serpentine soil, nickel, tissue colonization, phytoextraction, *Noccaea caerulescens*, *Arabidopsis thaliana*

Introduction

Phytoextraction has been proposed as a low-cost effective technology for remediation of contaminated soils and for phytomining (Chaney et al., 2007). Phytoextraction implies the cultivation of plants which can accumulate trace metals from contaminated soils and transport them to the above-ground biomass, and then be harvested. Metal hyperaccumulator plants

TABLE 1 | Enzymatic and hormonal activity (mean ± SD, $n = 3$) of bacterial strains isolated from roots of Ni-hyperaccumulator *N. caerulescens* in a previous work (Visioli et al., 2014).

Bacterial strain	Accession number	Closest described relative	Ni resistance			Siderophore production	IAA production (mg L^{-1})	ACC deaminase activity (μM αKB mg^{-1} h^{-1})
			1 mM	3 mM	6 mM			
Ncr-1	KJ92857	*Arthrobacter sp.*	+++	+++	+++	+	25.6 ± 1.3	20 ± 2.5
Ncr-3	KJ92859	*Kocuria rhizophila*	+++	+++	+++	+	14.5 ± 2.8	ND
Ncr-5	KJ92861	*Bacillus sp.*	+++	+++	+++	+	10.6 ± 3.6	11.01 ± 2.0
Ncr-8	KJ92864	*Microbacterium oxydans*	+++	+++	+++	+	20.8 ± 2.4	30 ± 2.9
Ncr-9	KJ92865	*Bacillus amyloliquefaciens*	+++	+++	+++	+	11.3 ± 0.8	ND

ND, not detected; ACC, 1-aminocyclopropane-1-carboxylic acid; αKB, α-ketobutyrate; IAA, indole-3-acetic acid.

are ideal candidates for this technology, thanks to their extraordinary capacity for absorbing and accumulating metals in their shoots without showing symptoms of phytotoxicity (Baker et al., 2000). Unfortunately, most hyperaccumulators do not produce substantial quantities of biomass, thus hampering the management of phytoextraction. Understanding of the mechanisms underlying hyperaccumulation may allow this technology to be transferred to metal-tolerant and high biomass-yielding plants (Ebbs and Kochian, 1998).

In recent years, attention has focused on microorganisms thriving in the rhizosphere or inhabiting the roots of hyperaccumulators which show plant growth-promoting activity (Sessitsch et al., 2013; Visioli et al., 2015). Plant growth-promoting rhizobacteria (PGPR) and endophytes [Plant growth-promoting endophytic bacteria (PGPE)] can also enhance plant tolerance, growth, and survival in stress conditions of metal-rich soils by reducing the nutrient deficiency and phytotoxicity of trace metals. This is achieved by the production of siderophores, carboxylic acids, and solubilisation of phosphates, which increase the mobility of macro- and micronutrients in the rhizosphere (Rajkumar et al., 2012; Bashan et al., 2013a,b). PGPR and PGPE can also promote growth, both indirectly by protecting plants against pathogens and directly by producing phytohormones such as indole acetic acid (IAA), abscisic acid, and gibberellic acid, and by secreting enzymes such as 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which inhibits ethylene production and consequently slows down plant aging. By lowering ethylene levels in plant tissues, ACC-deaminase reduces plant stress and improves growth under metal contamination, this being the main beneficial effect in phytoremediation (Kamnev, 2003; Spaepen et al., 2007; Belimov et al., 2009). The positive effects of PGPR and PGPE on plant growth and metal bioavailability can markedly improve metal accumulation by roots and shoots, thus efficiently influencing phytoremediation of metal-polluted sites (Li et al., 2007; Ma et al., 2009a,b, 2011a; Rajkumar et al., 2009, 2012; Chen et al., 2010; Sessitsch et al., 2013).

TABLE 2 | Physicochemical characteristics of serpentine soil used for growing inoculated and control *N. caerulescens* plants.

Parameters	
pH	7.2–7.5
Organic matter (%)	2.95 ± 0.21
Water content (%)	19.01 ± 0.2
Ca/Mg	0.25 ± 0.0
Total metal concentration (mg kg^{-1})	
Fe	47120 ± 341 (62.5 ± 3.4)
Ni	1244 ± 24 (34.8 ± 3.4)
Zn	71 ± 0.2 (1.4 ± 0.01)
Co	97 ± 1.3 (7.3 ± 0.03)
Cu	11 ± 0.1 (2.3 ± 0.01)

Diethylene triamine pentaacetic acid (DTPA)-extractable fractions are reported in parenthesis.

In many cases, the effects of plant–microbe interactions on growth and metal uptake has been shown to be species- and soil-specific (Sheng et al., 2008; Becerra-Castro et al., 2012; Cabello-Conejo et al., 2014), but interesting results have also been obtained by inoculating Ni-resistant PGPR isolated from the rhizosphere of *Alyssum serpyllifolium* and *Astragalus incanus* on the growth and Ni accumulation of *Ricinus communis*, *Heliantus annus,* and *Brassica juncea,* grown in single and multiple metal-contaminated soils (Ma et al., 2011a,b, 2015a; Jing et al., 2014). Cd- and Pb-mobilizing rhizospheric bacteria also enhanced the uptake of metals in tomato plants (Jiang et al., 2008) and Zn-mobilizing bacteria, isolated from serpentine soils, promoted Zn, Cu, and Ni accumulation in *R. communis* (Rajkumar and Freitas, 2008). Recent studies have further confirmed the potential role of PGPE in metal accumulation. For instance, endophytes from the *Bacillus* genus isolated from the roots of the Zn/Cd hyperaccumulator *Sedum plumbizincicola,* can effectively enhance plant biomass and Cd or Zn uptake (Ma et al., 2015b). A Cd-mobilizing endophytic strain isolated from maize roots can also improve Cd uptake by hyperaccumulator plants of the genus *Amaranthus* (Yuan et al., 2014); *Rahnella* sp. JN6, isolated from *Polygonum pubescens,* can also promote growth and Cd, Pb, and Zn uptake in the biomass species *B. napus* (He et al., 2013).

The increasing number of bacterial strains with beneficial effects on plant growth and metal accumulation traits, isolated from contaminated soil environments, both in the rhizosphere and from the roots of hyperaccumulator plant species, may contribute to the creation of a "bacteria phytoremediation database," to be used to check the effectiveness of bacterial inocula on various high-yielding biomass plant species, for possible future applications in the phytomanagement of polluted sites.

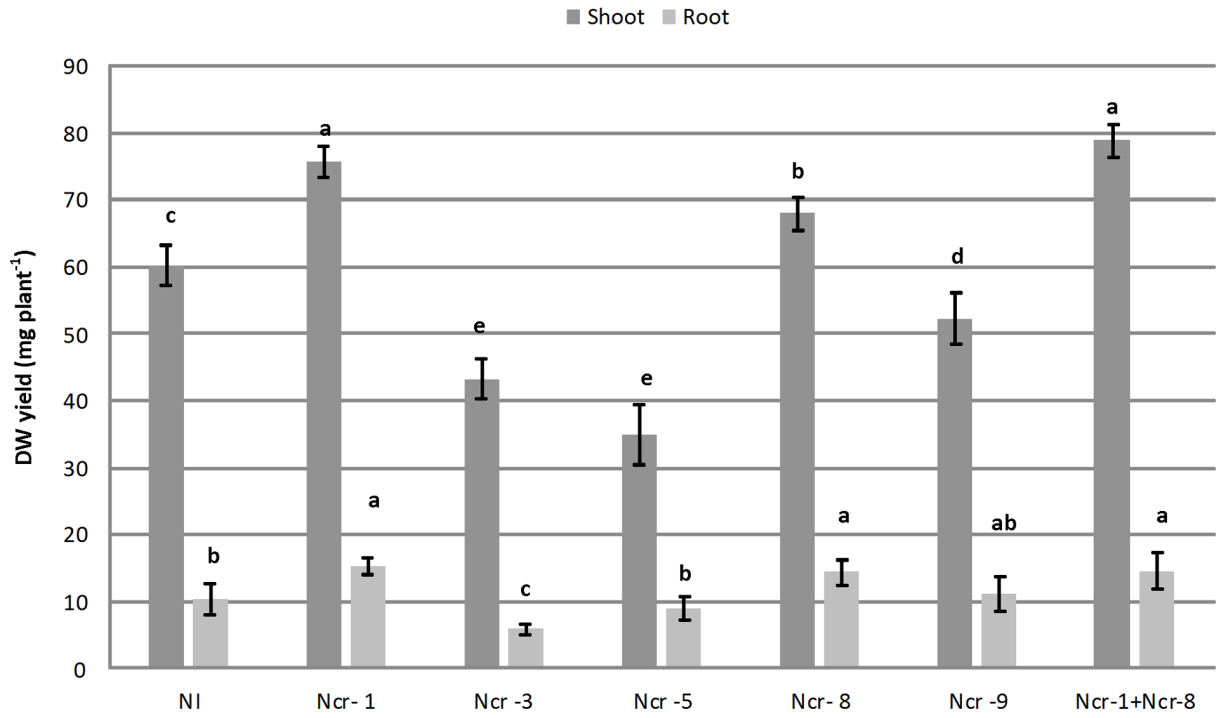

FIGURE 1 | Effects of five endophytic inoculants and co-inoculation of Ncr-1 + Ncr-8 PGPE strains compared with non-inoculated plants (NI) on shoot and root biomass (mg plant^{-1}) of *N. caerulescens* growing on serpentine soil in pot experiment. Data of each plant batch (three replicates) derived from three plants. Values with different letters are significantly different among treatments ($p < 0.05$).

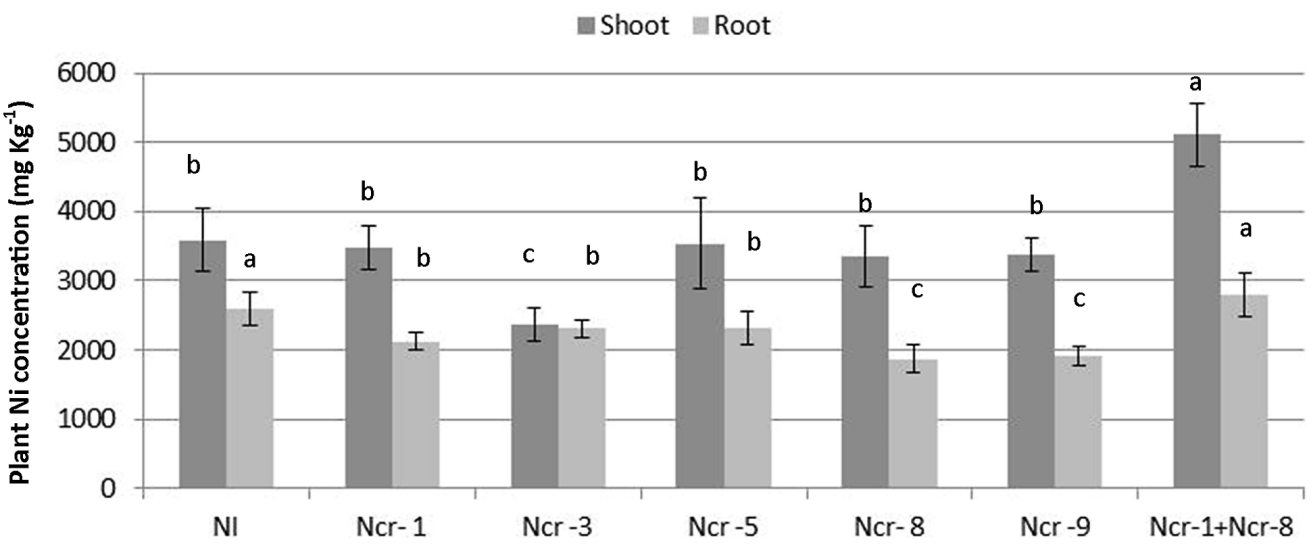

FIGURE 2 | Effects of five endophytic inoculants and co-inoculation of Ncr-1 + Ncr-8 PGPE strains compared with non-inoculated plants (NI) on shoot and root Ni concentration (mg kg^{-1} DW) of *N. caerulescens* on serpentine soil in pot experiment. Data of each plant batch (three replicates) derived from three plants. Values with different letters are significantly different among treatments ($p < 0.05$).

Within this framework, we studied the interaction between the Ni-hyperaccumulator *Noccaea caerulescens* and five PGPE, previously isolated from its roots in a serpentine soil. Bacteria were supplied as the seed inoculum of each strain, both separately and in combination, in order to identify any synergistic mechanism. Characterisation of IAA production and ACC-

TABLE 3 | Nickel translocation factor (TF), calculated as shoot-to-root Ni concentration ratio and bioconcentration factor (BCF), determined as ratio of shoot Ni and total soil Ni concentrations (mean ± SD, *n* = 3) in *N. caerulescens* inoculated with five bacterial strains.

	NI	Ncr-1	Ncr-3	Ncr-5	Ncr-8	Ncr-9	Ncr-1 + Ncr-8
TF	1.4	1.6	1.0	1.5	1.8	1.8	1.8
BCF	2.9 ± 0.1	2.7 ± 0.5	1.9 ± 0.2	2.8 ± 0.3	2.7 ± 0.2	2.7 ± 0.4	4.0 ± 0.5*

**Significant differences ($p < 0.05$) between non-inoculated (NI) and treated plants.*

deaminase activity in these bacteria also showed which of these physiological traits are efficient selection criteria for improving assisted metal phytoextraction. In this study, we documented plant-bacteria interactions by *in vivo* and *in vitro* microscopic observations, and attempted to transfer the technology *in vitro* to the non-hyperaccumulator *Arabidopsis thaliana* with the most efficient bacterial isolates.

Materials and Methods

Bacterial Strains

The endophytic bacteria tested here, Ncr-1, Ncr-3, Ncr-5, Ncr-8, and Ncr-9, had been isolated in a previous study from inners of the roots of *N. caerulescens* plants collected in serpentine soil in the Northern Italian Apennines (Visioli et al., 2014). They were selected from ten different strains on the basis of their greater ability to tolerate high concentrations of Ni in hydroponics (6 mM) and to produce plant growth-promoting substances (**Table 1**).

Bacterial Seed Inoculation and Plant Germination *In Vitro*

Ncr-1, Ncr-3, Ncr-5, Ncr-8, and Ncr-9 strains were grown overnight in 100 mL Luria Bertani (LB) medium at 30°C on a rotary shaker. Cells were collected by centrifugation and suspended in LB medium to obtain a final inoculum density of 10^8 CFU mL^{-1}. *N. caerulescens* seeds were surface sterilized in 50% (v/v) commercial bleach (5% sodium hypochlorite and 0.05% sodium hydroxide) in water for 15 min and then rinsed for 5 min in sterile water three times. Seeds were kept for 2 h in a bacterial suspension of 5×10^8 cells mL^{-1} of each strain or in a bacterial suspension of both Ncr-1 and Ncr-8 strains (5×10^8 cells mL^{-1} of each) and then thoroughly washed in sterilized water. The seeds were then plated on 1× MS (Murashige and Skoog, 1962) agar medium and incubated in a vertical position in an environmentally controlled room (22°C; 16 h/8 h light/dark; 120 μmol m^{-2} s^{-1} PAR, 75% RH) to determine germination and root elongation. The same sterilization and co-inoculation procedures were used for *A. thaliana* seeds with Ncr-1 and Ncr-8 strains.

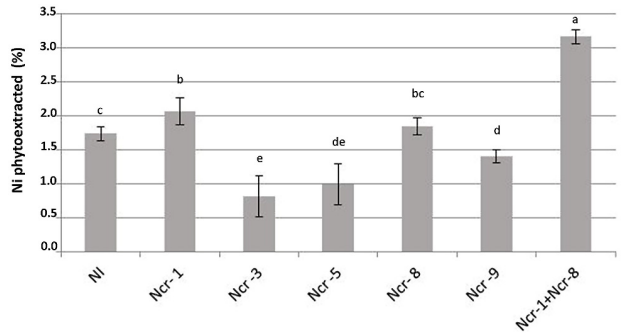

FIGURE 3 | Effects of five endophytic inoculants and co-inoculation of Ncr-1 + Ncr-8 PGPE strains compared with non-inoculated plants (NI) on Ni phytoextraction (% of shoot Ni removed from total soil Ni) by *N. caerulescens* growing on serpentine soil in pot experiment. Data of each plant batch (three replicates) derived from three plants. Values with different letters are significantly different among treatments ($p < 0.05$).

TABLE 4 | Diethylene triamine pentaacetic acid- extractable fractions of metals (mean ± SD, *n* = 3) in rhizosphere soil of inoculated and control *N. caerulescens* plants at harvest (end of experiment).

	Ni mg kg^{-1}	Zn mg kg^{-1}	Co mg kg^{-1}	Cu mg kg^{-1}
NI	34.8 ± 3.4	1.4 ± 0.01	7.3 ± 0.03	2.3 ± 0.01
Ncr-1	35.4 ± 2.5	1.4 ± 0.03	7.4 ± 0.02	2.3 ± 0.02
Ncr-3	34.8 ± 1.3	1.5 ± 0.05	7.3 ± 0.04	2.4 ± 0.03
Ncr-5	34.3 ± 1.2	1.7 ± 0.06	7.3 ± 0.05	2.3 ± 0.07
Ncr-8	36.5 ± 2.9*	1.4 ± 0.10	7.1 ± 0.10	2.4 ± 0.02
Ncr-9	35.8 ± 2.5	1.5 ± 0.05	7.1 ± 0.02	2.2 ± 0.01
Ncr1 + Ncr8	36.2 ± 1.3*	1.5 ± 0.04	7.0 ± 0.01	2.3 ± 0.01

**Significant difference ($p < 0.05$) between inoculated plants and non-inoculated (NI) controls.*

Soil Experiments

The soil used in these experiments was collected at a previously characterized site on Mount Prinzera, a serpentine outcrop in the Northern Italian Apennines (GPS 44.64282°N-10.07951°E; for basic soil properties, see Visioli et al., 2013, and **Table 2**).

Soil pH was measured with a glass electrode from a deionised water suspension (20 g soil/50 mL water) after 1 h agitation and overnight settlement. Organic matter was determined on soils sampled at depths of 10 and 20 cm as LOI (weight loss on ignition; Storer, 1984). Aliquots of 2 g of each sample were placed in closed ceramic crucibles for 3 h at 450°C; organic matter was calculated as the fraction of weight decrease. Soil water content was measured on 20-g samples, placed in closed heat-resistant plastic containers, previously weighed and placed in an oven at 70°C for 24 h. The percentage of water in the sediment was calculated as weight loss from the initial weight. The soil was then sieved (2 mm) and sterilized at 80°C for 24 h.

TABLE 5 | Mean macro- and micro-nutrient concentrations (expressed in g kg^{-1}) in shoots and roots of *N. caerulescens* grown in serpentine soil with five bacterial strains inoculants.

	Ca	K	P	S	Mg	Fe	Zn	Mn	Co	Cu
	g kg^{-1}									
Shoots										
Nl	14.3 ± 2.1	58.7 ± 4.5	3.8 ± 0.33	6.0 ± 1.13	11.7 ± 2.0	0.86 ± 0.11	0.58 ± 0.10	0.38 ± 0.02	0.15 ± 0.01	0.013 ± 0.02
Ncr-1	18.7 ± 7.7	55.1 ± 3.4	3.4 ± 0.45	5.5 ± 0.90	13 ± 1.5	1.37 ± 0.53*	0.34 ± 0.08*	0.35 ± 0.05	0.18 ± 0.02	0.014 ± 0.03
Ncr-3	14.8 ± 3.2	38.8 ± 4.8*	2.7 ± 0.25	4.7 ± 0.34	17 ± 3.6	1.04 ± 0.24*	0.16 ± 0.05*	0.30 ± 0.03	0.13 ± 0.01	0.017 ± 0.02*
Ncr-5	15.8 ± 2.3	48.3 ± 2.3	3.8 ± 0.12	6.4 ± 1.43	16.3 ± 2.8	1.26 ± 0.37*	0.23 ± 0.04*	0.34 ± 0.02	0.17 ± 0.04	0.009 ± 0.01*
Ncr-8	14.2 ± 2.5	52.0 ± 2.2	3.2 ± 0.50	5.2 ± 0.87	12 ± 1.8	1.50 ± 0.22*	0.28 ± 0.03*	0.35 ± 0.03	0.16 ± 0.02	0.016 ± 0.02
Ncr-9	15.3 ± 5.1	51.1 ± 2.8	3.1 ± 0.23	5.1 ± 1.65	12.6 ± 3.0	0.71 ± 0.70	0.28 ± 0.02*	0.32 ± 0.04	0.16 ± 0.02	0.017 ± 0.04*
Ncr1 + Ncr8	16.4 ± 3.2	47.6 ± 3.4	2.5 ± 0.15	6.3 ± 0.34	12.2 ± 2.5	0.80 ± 0.27	0.25 ± 0.04*	1.18 ± 0.06*	0.67 ± 0.02*	0.014 ± 0.03
Roots										
Nl	7.3 ± 0.9	80.3 ± 3.5	4.5 ± 0.26	7.9 ± 0.23	14.8 ± 2.0	2.8 ± 0.1	0.33 ± 0.05	0.45 ± 0.3	0.038 ± 0.01	0.059 ± 0.01
Ncr-1	8.2 ± 1.2	68.9 ± 6.3	6.0 ± 0.37	9.4 ± 0.45	15.0 ± 2.4	3.0 ± 0. 3	0.41 ± 0.04	0.56 ± 0.2	0.060 ± 0.02	0.034 ± 0.02*
Ncr-3	8.8 ± 0.2	127.0 ± 8.0*	5.4 ± 0.25	12.6 ± 1.32*	19.0 ± 1.7*	3.5 ± 0.2	0.35 ± 0.031	0.78 ± 0.2*	0.066 ± 0.02	0.060 ± 0.05
Ncr-5	9.4 ± 1.3	76.8 ± 2.5	4.8 ± 0.10	8.6 ± 0.28	22.3 ± 3.5	5.4 ± 0.3	0.54 ± 0.02	0.84 ± 0.01*	0.088 ± 0.01*	0.021 ± 0.001*
Ncr-8	7.6 ± 0.4	74.7 ± 4.0	4.5 ± 0.31	8.2 ± 0.45	15.3 ± 2.6	3.8 ± 0.4	0.38 ± 0.01	0.44 ± 0.01	0.064 ± 0.01	0.028 ± 0.02*
Ncr-9	6.7 ± 0.5	91.6 ± 3.2	4.6 ± 0.65	8.5 ± 0.32	15.4 ± 1.1	3.7 ± 0.2	0.27 ± 0.01	0.56 ± 0.05	0.051 ± 0.02	0.031 ± 0.02*
Ncr1 + Ncr8	8.2 ± 1.6	89.4 ± 2.8	4.0 ± 0.19	10.5 ± 1.50	13.7 ± 2.1	0.5 ± 0.1	0.32 ± 0.05	0.95 ± 0.03*	0.077 ± 0.07*	0.020 ± 0.001*

*Significant difference ($p < 0.05$) between inoculated plants and non-inoculated (Nl) controls.

Fourteen-day-old *N. caerulescens* seedlings (three per pot), inoculated and non-inoculated with the various bacterial strains, were transferred into PVC pots (upper diameter 110 mm, lower diameter 90 mm, height 100 mm) containing 750 g of soil, with three replicates. The plants were grown in the same conditions as above and the soil was wetted with sterile water and maintained at 25% w/w of water content (corresponding to 50% of its holding capacity). The experiment was set up in September 2014 and lasted 60 days, after which the plants were carefully lifted from the pots and the soil was removed from their roots. Plant roots were immersed in a 10 mM EDTA solution for 30 min and rinsed thoroughly with deionised water to avoid any metal contamination. Shoots and roots were then dried for 3 days at 70°C and root and shoot dry weights (DW) were recorded.

Hundred-mg samples of dried shoots or roots were microwave-acid digested (Milestone ETHOS 900, Bergamo, Italy) by the addition of 7 mL ultrapure grade 6 HNO_3 (65% v/v) and 1 mL suprapur H_2O_2 (30% v/v), according to the EPA 3052 method (USEPA, 1995). The microwave setting reached 200°C in 10 min (step 1), followed by 10 min at 200°C (step 2), and a final air cooling phase down to <30°C. In steps 1 and 2, maximum pressure and power were 45 bar and 1.2 kW, respectively. Samples were then diluted to 25 mL with distilled water, filtered (0.45 μm PTFE) and analyzed by ICP-OES (SPECTRO Ciros Vision EOP, Spectro Analytical Instruments, Kleve, Germany) to reveal metal concentrations. Certified reference materials (ERM-CD281 and BRC-402, JRC-IRMM, Belgium) were used to ensure measurement accuracy. Data are expressed in mg kg^{-1} DW plant material. The Ni translocation factor (TF) was measured as the shoot-to-root metal concentration ratio.

Aliquots of 300 mg of soil collected in the rhizosphere of both inoculated and non-inoculated plants were also dried overnight at 70°C and wet-ashed; total Ni concentration was determined by ICP-OES, with the same procedure as above.

In addition, in order to evaluate Ni, Zn, Cu, and Co bioavailability, the same samples underwent diethylene triamine pentaacetic acid (DTPA) extraction. Bioavailable metals were extracted on 50 g of homogenized air-dried soil through a 100-mL solution of DTPA (1.97 g L^{-1}), calcium chloride bihydrate (1.46 g L^{-1}), and triethanolamine (14.92 g L^{-1}), pH 7.3, shaken for 2 h (60 cycles min^{-1}). Samples were analyzed by ICP-OES after centrifugation (5 min, 2599 × *g*).

The ability of Ncr-1, Ncr-3, Ncr-5, Ncr-8, and Ncr-9 to bioconcentrate Ni in the above-ground biomass of *N. caerulescens* from serpentine soil (BCF: Bioconcentration factor) was calculated as the ratio between shoot Ni concentration and soil pseudo-total Ni concentration. The effect of microbial inoculation on overall Ni phytoextraction efficiency was assessed by taking into account plant growth, and was calculated as the product of DW shoot yield and its Ni concentration.

After log-transformation of the response variables, ANOVA and Tukey's *post hoc* test were used to ascertain differences in root length, root and shoot biomass and metal concentrations.

TABLE 6 | Shoot-to-root concentration ratio (TF) of main macro- and micro-nutrients (mean ± SD, *n* = 3) in *N. caerulescens* grown in serpentine soil with five bacterial strain inoculants.

	Ca	K	P	S	Mg	Fe	Zn	Mn	Co	Cu
					TF					
NI	2.0	0.7	0.8	0.8	0.8	0.3	1.8	0.8	3.9	0.2
Ncr-1	2.3	0.8	0.6	0.6	0.9	0.4	0.8*	0.6	3.0	0.4
Ncr-3	1.7	0.3	0.5	0.4	0.9	0.3	0.5*	0.4	2.0	0.2
Ncr-5	1.7	0.6	0.8	0.7	0.7	0.2	0.4*	0.4	1.9	0.4
Ncr-8	1.9	0.7	0.6	0.6	0.8	0.4	0.7*	0.8	2.5	0.6
Ncr-9	2.3	0.6	0.7	0.6	0.8	0.2	1.1	0.6	3.1	0.6
Ncr1 + Ncr8	2.0	0.5	0.6	0.6	0.9	1.6*	0.8*	1.2*	8.7*	0.7*

**Significant difference ($p < 0.05$) between inoculated plants and non-inoculated (NI) controls.*

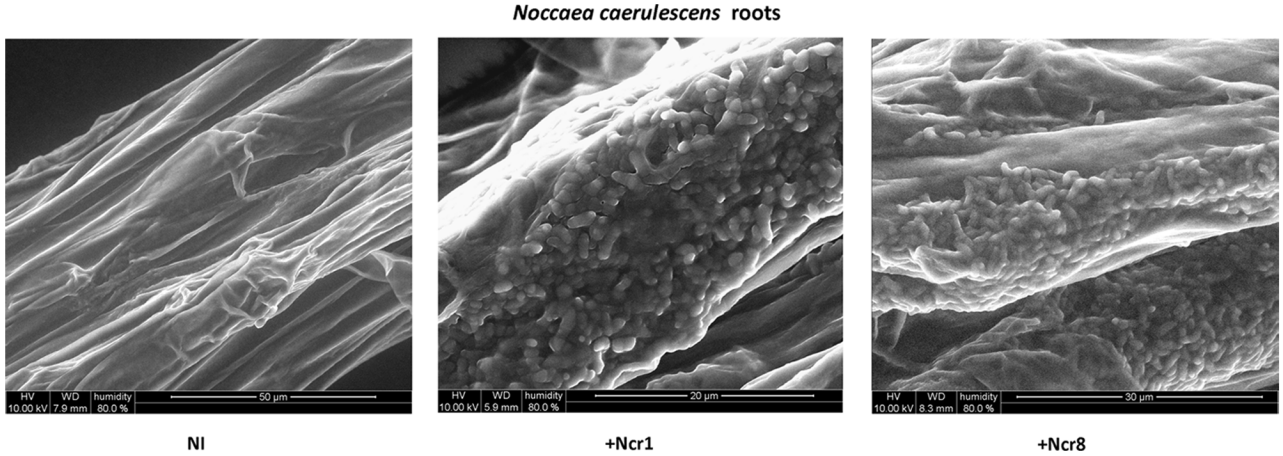

FIGURE 4 | *In vivo* wet-ESEM micrographs of fresh roots from 14-day-old *N. caerulescens* seedlings inoculated with Ncr-1 and Ncr-8 PGPE strains and non-inoculated (NI).

Electron Microscopy Analysis

Fresh roots from 14-day-old *N. caerulescens* seedlings inoculated with Ncr-1 and Ncr-8 were directly collected from the plates and rinsed briefly in sterile water; 5-mm root sections were then excised with a sterile lancet. Unfixed and hydrated samples were directly analyzed under an Environmental Scanning Electron Microscope (ESEM) Quanta^{TM} 250 FEG (FEI, Hillsboro, OR, USA), operating in wet mode conditions. In more detail, samples were placed on double-sided adhesive carbon tape fixed to a pre-cooled metal sample holder, in thermal contact with a Peltier cooling stage maintained at 3°C. Accelerating voltage was 10 kV and the secondary electron signal was collected by a gaseous secondary electron detector (GSED) to generate micrographs for morphological studies. Relative humidity was initially set at 100%, and then slowly decreased to 80% by adjusting chamber pressure.

Optical Microscopy Analysis

Five-mm root and leaf sections were excised with a sterile lancet from *in vitro* 14-day-old *N. caerulescens* seedlings, inoculated and non-inoculated with Ncr-1 and Ncr-8 strains, collected from Petri dishes, and immediately fixed overnight at 4°C in formalin-acetic acid-alcohol (FAA). After this step, samples were dehydrated in a tertiary butyl-alcohol series and gradually embedded in paraffin. They were then cut into sections 5 µm thick with a rotary microtome (Reichert-Jung 2040) and stained with safranin-fast green (Berlyn and Miksche, 1976). Lastly, they were analyzed under a Nikon eclipse E600 microscope mounted with a DS-FIZ camera.

Results and Discussion

Plant Biomass Yield

Solving the problem of interactions between *N. caerulescens* and PGPE in serpentine soil, which is characterized by low levels of essential nutrients and elevated Ni, together with other toxic metals, can be illuminating in understanding plant–microorganism interactions in an extremely adverse environment and the potential use of metal-resistant endophytic bacteria in phytomanagement of metal-polluted sites.

In a previous work, five culturable bacterial endophytes were selected among 10 isolates from *N. caerulescens* roots according to their high resistance to Ni and ability to produce PGP

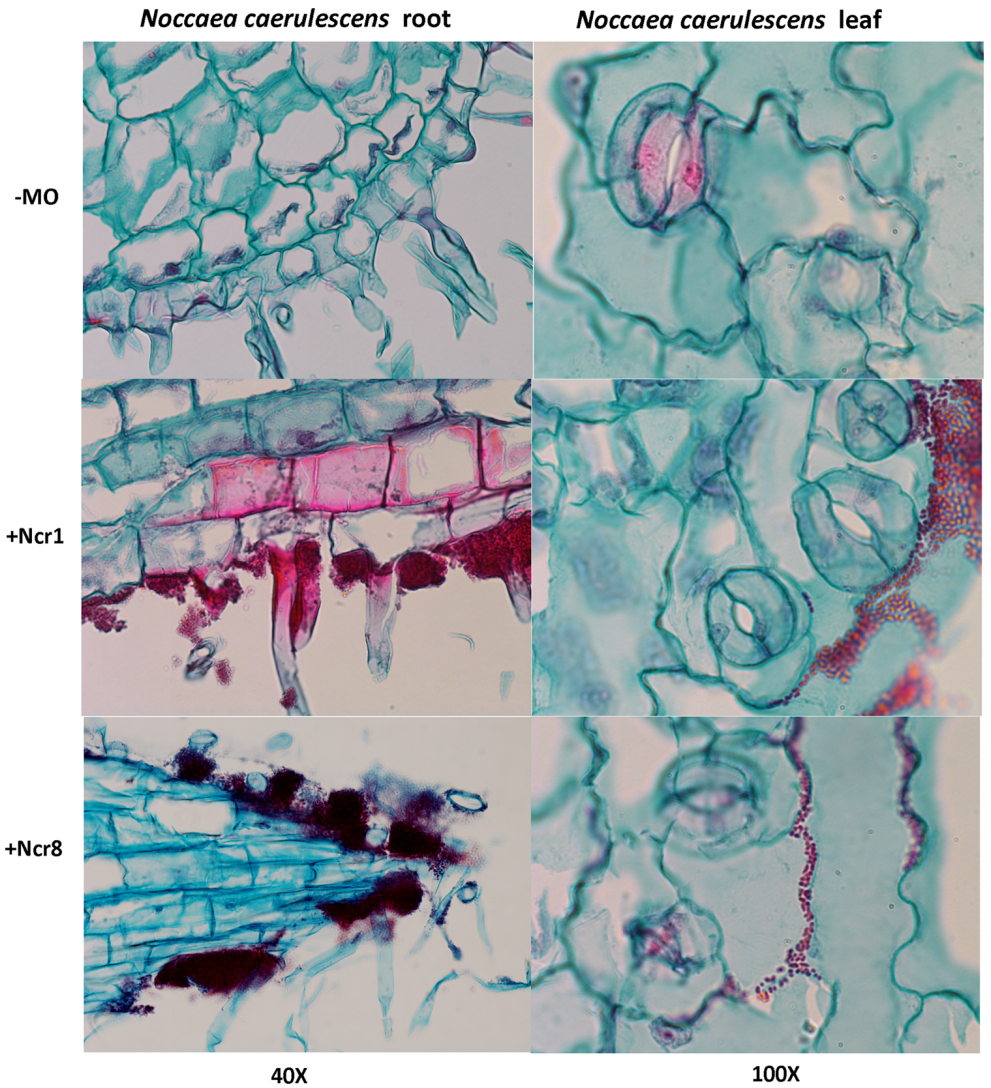

FIGURE 5 | Histological analysis of longitudinal root (left) and leaf (right) sections of 14-day-old *N. caerulescens* seedlings inoculated with Ncr-1 and Ncr-8 PGPE strains and non-inoculated (NI). Bacterial cells were highlighted with safranin and plant cells with fast green staining.

metabolites such as IAA, ACC-deaminase, and siderophores (**Table 1**).

These five inoculants promoted plant growth and Ni translocation in a hydroponic system, enriched with 10 μM Ni and supplied adequately with nutrients for growth (Visioli et al., 2014). Since in the real environment soil properties can greatly affect shoot growth and bacterial root colonization, essential nutrients often being limiting factors, in the present study the performance of these bacteria were further investigated in conditions more similar to those of open fields. The serpentine soil used was the original soil from which the Ni-hyperaccumulator *N. caerulescens* was first collected. The soil has a relatively low content of organic matter (<3%) and neutral pH (**Table 2**). In addition, it has a low Ca/Mg ratio, responsible for Ca uptake inhibition, and high levels of Mg (>300 mg kg^{-1}; Visioli et al., 2013) and several toxic metals, particularly Ni (>1000 mg kg^{-1}). Low levels of available macronutrients N, K, P were further edaphic constraints which severely affect plant growth and reproduction.

Noccaea caerulescens seeds were either inoculated with the five bacterial strains separately, or co-inoculated with strains Ncr-1 and Ncr-8 together, in order to determine the potential additive effects of PGPE on plant growth and Ni accumulation in serpentine soil. Both Ncr-1 and Ncr-8 showed the highest IAA production and AAC-deaminase activity (**Table 1**). Among treatments with single inoculants, only plants treated with Ncr-1 and Ncr-8 showed significant higher shoot biomass compared with untreated controls, i.e., +25 and +12%, respectively (**Figure 1**). Co-inoculum with Ncr-1 and Ncr-8 had a synergistic effect, with a further shoot DW increase of 4 and 16% with respect to Ncr-1 and Ncr-8 alone, respectively. Plant inoculation

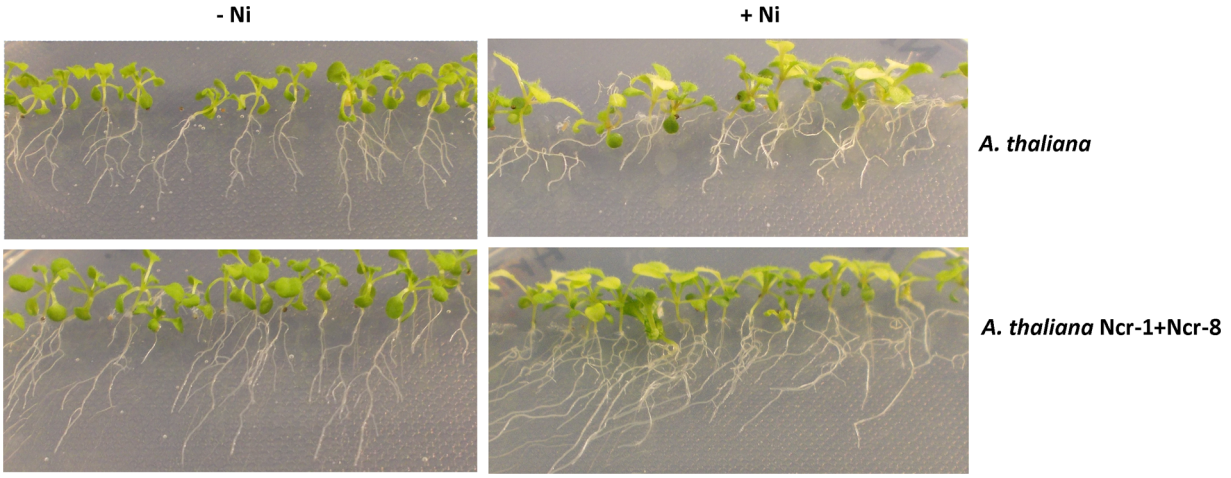

FIGURE 6 | Seven-day-old *Arabidopsis thaliana* seedlings, non-inoculated (NI) or co-inoculated with Ncr-1 and Ncr-8 PGPE strains growing in MS 1× medium, with or without 40 μM $NiSO_4$.

with Ncr-3, Ncr-5, and Ncr-9 produced a significant decrease in shoot and seldom (Ncr-3) in root biomasses, compared with non-inoculated controls, suggesting their poor interaction with *N. caerulescens* (**Figure 1**). The beneficial effects of bacterial inoculants on the growth of metal-exposed plants have often been attributed to the production and transfer to plants of high IAA levels (Spaepen et al., 2007; Dell'Amico et al., 2008). The positive influence of the Ncr-1 + Ncr-8 combination on the biomass yield may be due to their substantial release of auxin, associated with reduced ethylene production through increased ACC-deaminase activity. IAA increases plant growth by promoting cell division or stimulating cell elongation (Spaepen et al., 2007), whereas ACC-deaminase effectively reduces ethylene production by plants, retarding leaf senescence (Glick et al., 1998) and increasing plant yield (Belimov et al., 2009).

The data presented here are consistent with recent results from the literature, evidencing the positive effect on growth of the Ni-hyperaccumulator *Alyssum pintodasilvae* of PGPR and PGPE strains belonging to *Arthrobacter* and *Microbacterium* genera in a serpentine soil (Cabello-Conejo et al., 2014). The beneficial role of PGPE belonging to the *Bacillus* genus on the growth of the Cd/Zn hyperaccumulator *S. plumbizincicola* grown in a multiple-metal contaminated soil has also recently been demonstrated (Ma et al., 2015b).

Among bacterial inoculants, plant growth impairment was particularly evident with strain Ncr-3, since both shoot and root biomass were reduced by 28 and 39%, respectively, compared with non-inoculated plants. Ncr-3 showed great similarity to *Kocuria rhizophila*, and its negative effect on *N. caerulescens* is in contrast with results in recent literature, which reports a positive influence on growth and chromium accumulation in *Cicer arietinum* L, although with a different *K. flava* species isolated from the rhizosphere of chickpea (Singh et al., 2014). Our *Kocuria* strain, coming from the rhizosphere of *N. caerulescens,* was found to have poor IAA production, and lacked ACC-deaminase activity, probably indicating the importance of this hormone and enzymatic activities in establishing positive interactions with plants.

Ni Uptake and Translocation, and Shoot and Root Ionome

The Ni rate in roots of Ncr-1 + Ncr-8 co-inoculated plants and untreated controls was very similar (2.6 ± 0.4 g kg^{-1} vs. 2.7 ± 0.4 g kg^{-1}), although significantly lower values ($p < 0.05$) were observed in all other treatments with single strains. Analysis of shoot tissues confirmed that Ni was significantly higher in the co-inoculated NCr-1 + NCr-8 plants compared with untreated controls (5.1 ± 0.3 g kg^{-1} vs. 3.5 ± 0.2 g kg^{-1}), and the absence of significant differences among control plants and single-strain inoculated plants, with the exception of Ncr-3, which showed reduced rates (2.3 ± 0.1 g kg^{-1}; **Figure 2**). Anyway, shoot Ni rates in *N. caerulescens* were extremely high with all bacteria strains, according with the capability of this Ni-hyperaccumulator species. The shoot-to-root Ni ratio was >1 in all treatments except for Ncr-3 (TF = 1.0; **Table 3**) and was improved with strains Ncr-1 and Ncr-8 and their co-inoculation, confirming the general ability of *N. caerulescens* to accumulate Ni above-ground and involvement of these bacteria in Ni uptake and translocation (**Table 3**).

Nevertheless, Ni soil bioavailability was similar before and after plant growth and did not apparently change in the rhizosphere as a consequence of bacteria inoculation, with an average value of 35.4 mg kg^{-1} as DTPA extractable fraction at end of the experiment (**Table 4**). Compared with non-inoculated controls, inoculants led to some small changes only in Ni phytoavailability, with an increase with Ncr-8 and Ncr1 + Ncr8 co-inoculation. The same effect was observed for Zn, Co, and Cu, with no variations following inoculation.

In the literature, contrasting results are described as regards the effects of rhizosphere bacteria on soil metal mobility.

For instance, *Microbacterium arabinogalactolyticum* was found to increase the soil extractability of Ni (Abou-Shanab et al., 2003, 2006), although no variations were observed with *Arthrobacter nitroguajacolicus* and *Microbacterium* sp. inoculants in serpentine soil with the Ni-hyperaccumulator *A. pintodasilvae* (Cabello-Conejo et al., 2014). The dynamic nature of metal solution-solid phase interactions would explain the absence of a direct correlation between Ni uptake and its DTPA extractable fraction.

Co-inoculation of Ncr-1 and Ncr-8 strains appreciably improved the BCF, i.e., 1.38 times higher than that of untreated controls ($P < 0.05$; **Table 3**). As a consequence, the percentage of Ni removal was significantly higher than in non-inoculated plants (3.2% vs 1.7%); in single-strain inoculated plants, only Ncr-1 showed significantly higher Ni phytoextraction (2.1%; **Figure 3**). Ncr-8 led to the same phytoextraction capacity as controls, whereas Ncr-3, Ncr-5, and Ncr-9 had significantly lower capacities. These results indicate that, with the combined help of two selected bacteria, *N. caerulescens* can take up Ni from the soil more efficiently than in aseptic conditions, confirming previous findings on the stimulation effects of endophytes on metal uptake in higher plants (He et al., 2013; Yuan et al., 2014; Ma et al., 2015b).

As regards other elements, both shoot and root tissues had similar concentrations of the main macronutrients Ca, K, Mg, P, and S between inoculated and untreated plants. The only exception was Ncr-3, which showed a significant decrease in shoot K, together with root K, S, and Mg increases (**Table 5**). Some trace metals also seldom changed between treatments; bacterial inoculation generally led to a substantial Zn decrease in shoots, regardless of bacterial strain, and a generalized Cu decrease in roots, except for Ncr-3 (**Table 5**). The response of Co was peculiar, in that distribution in both shoot and roots only increased with Ncr-1 + Ncr-8 co-inoculation.

As a consequence of such variations in plant metal rates, a general decrease in the shoot-to-root concentration ratio for Zn (i.e., TF) was observed in inoculated plants, whereas only co-inoculation with Ncr-1 and Ncr-8 significantly increased the TF of Fe, Co, and Cu compared with untreated controls and single inoculants (**Table 6**). According to some recent studies, siderophore production and P solubilisation by rhizosphere microrganisms play important roles in increasing the mobility of several trace metals in polluted substrates, thus facilitating their accumulation in plant tissues (Sessitsch et al., 2013). In our case, both Ncr-1 and Ncr-8 had good siderophore production, but real improvements in Ni, Mn, and Co accumulation in the above-ground biomass of *N. caerulescens* were observed when the ability of the two efficient strains were combined. As suggested by Rajkumar et al. (2012), siderophores can chelate the unavailable ferric form of iron in near-neutral pH conditions, allowing more efficient uptake of this metal by *N. caerulescens* roots, and we recorded a slight increase in shoot and root Fe accumulation with some bacterial strains. Instead, the general reduction in shoot Zn of all inoculated treatments was probably due to the mobilization of various metals present at higher concentrations in serpentine soils. These soils are commonly poor in Zn (~70 mg kg^{-1} DW in our case) and competition with other abundant metals such as Ni (>1000 mg kg^{-1} soil DW), Co (>90 mg kg^{-1} soil DW) and Cu may explain the lower uptake and translocation of Zn. This hypothesis was apparently not supported by the stable DTPA-extractable fraction of the most abundant trace metals, but quantification of metal mobility at harvest is a final result which may not have described specific variations during the developmental stages of plants.

Physical Interaction between Plants and Bacteria

An important aspect to consider in root–microbe interactions is the possibility of tracking bacterial growth and plant tissue colonization (Visioli et al., 2015). Some authors have recently followed bacterial colonization after a certain time from inoculation by means of classic microbiological methods, with selection of metal-resistant bacteria. The bacteria are then identified by colony morphology traits, metal-contamination tolerance, and IAA production and ACC-deaminase activity (Ma et al., 2015b). Until now, very few studies have shown physical plant–microbe interactions in the tissues of hyperaccumulator plants. We monitored the colonization and survival of inocula in real environmental conditions by environmental scanning electron *in vivo* microscopy (ESEM). ESEM is a powerful tool which allows observation of biological specimens *in situ* without sample preparation (Stabentheiner et al., 2010). The physical association between the roots of *N. caerulescens* and Ncr-1 and Ncr-8 was analyzed on 14-day-old seedlings from inoculated seeds before the soil experiments were set up. Single Ncr-1 and Ncr-8 strains showed deep colonization in root cavities and deep bacterial root biofilm formation (**Figure 4**). Images obtained after histological staining of 14-day-old *N. carulescens* seedlings inoculated with single Ncr-1 and Ncr-8 bacteria, compared with non-inoculated plants, are shown in **Figure 5**. Both strains adhered closely to the root epidermis and root tips. They were also very abundant on leaf tissues, penetrating the intercellular

TABLE 7 | Root length, fresh plant weight, and Ni content (±SE, $n = 3$) in 7-day-old *Arabidopsis thaliana* seedlings grown *in vitro* under 0 or 40 μM $NiSO_4$, with or without inoculation.

A. thaliana	Root lenght (mm)	Bacterial effect	FW (mg $plant^{-1}$)	Bacterial effect	Ni (mg kg^{-1})	Bacterial effect
NI	3.24 ± 0.2b		45 ± 0.5b			
Ncr-1 + Ncr-8	4.12 ± 0.4a	+27%	52 ± 1.2a	+16%		
NI + Ni	2.94 ± 0.3c		42 ± 2.2b		73 ± 4.3	
Ncr-1 + Ncr-8 + Ni	4.44 ± 0.2a	+51%	55 ± 1.5a	+31%	60 ± 5.3	−17.8%

Data of each plant batch (three replicates) derived from three plants. Values with different letters: significantly different among treatments ($p < 0.05$). NI, non-inoculated plants; FW, fresh plant weight.

spaces between epidermal cells and crowding particularly round the stomata complexes. This finding is noteworthy, because epidermal cells are the primary sites of Ni accumulation in this hyperaccumulator species, although clear exclusion of Ni from guard cells was also recently reported by Mattarozzi et al. (2015).

Ncr-1 and Ncr-8 Co-Inoculum Enhances *Arabidopsis* Root Growth and Tolerance to Ni

The effectiveness of the combination of Ncr-1 and Ncr-8 was also tested in the non-hyperaccumulator *A. thaliana.* **Figure 6** shows non-inoculated vs. Ncr-1 + Ncr-8 co-inoculated 7-day-old seedlings. The treated plants revealed enhanced root and shoot growth both with (40 mM $NiSO_4$) and without Ni contamination (**Table 7**). In addition, when the growing medium was contaminated by Ni, the inoculated plants clearly showed fewer symptoms of phytoxicity than controls, with a ∼50% increase in root length and an ∼30% increase in plant biomass compared with non-inoculated controls. Although bacteria led to reduced Ni concentration in plant tissues, the balance of metal removal was still better than that of controls ($p < 0.05$; **Table 7**).

Although the role of PGPR in promoting growth and Ni uptake in hyperaccumulators has often been reported (Visioli et al., 2015), the protective effect against Ni toxicity exerted by metal-resistant PGPR or PGPE in non-accumulator biomass species has rarely been documented. For instance, Someya et al. (2007) demonstrated that the *Pseudomonas putida* ARB86 strain isolated from a Ni-contaminated soil could increase *Arabidopsis* plant growth and reduce Ni influx. The PGPR *Kluyvera ascorbata* strain, isolated from a Ni-Cu mining area, protected canola and tomato from Ni toxicity, mainly by stimulating root growth, but did not hamper Ni accumulation by the plant (Burd et al., 1998). The positive role played by these bacteria appear to be similar to those of our Ncr-1 and Ncr-8 strains in the presence of Ni, exerting a growth-promoting effect in roots and probably reducing plant stress thanks to reduced ethylene production (see **Table 1**). In our case, the Ni concentration in *Arabidopsis* tissues was reduced as a consequence of bacterial inoculation, probably because of metal dilution in a more elevated biomass or reduction in uptake. The absolute difference between inoculated and control plants of *A. thaliana* was minimal when compared with that of *N. caerulescens,* and *A. thaliana* maintained the characteristics of a non-hyperaccumulator, with a much lower order of magnitude for Ni accumulation (mg kg^{-1} vs. g kg^{-1}).

Extensive research is necessary to examine the possible influence of PGPR and PGPE inoculation on changes of speciation of toxic metals in the rhizosphere and to ascertain whether such changes can alter the accumulation and distribution in plant organs of heavy metals in hyperaccumulator and non-hyperaccumulator plants.

Conclusion

Highly Ni-polluted serpentine soils are populated by a wide range of bacterial species and strains which play an active role in plant adaptations to extreme soil conditions. Culturable root endophytic bacteria represent only the evaluable part of the community of rhizosphere microorganisms, and the involvement of viable but not cultivable (VBNC) bacteria cannot be excluded.

In this paper, we demonstrate that individual PGPE culturable bacteria are ineffective in plant growth and Ni accumulation enhancement, although they were selected for their high Ni resistance. Among selected strains, those belonging to the *Arthrobacter* and *Microbacterium* genera alone led to better plant performance, but revealed a synergistic effect when associated as seed inocula in *N. caerulescens*. Very probably, co-inoculation of various PGPE bacteria partially mimicks the natural conditions of serpentine soils, in which multiple microorganism interactions occur, helping plants to cope with the toxic effects of heavy metals. Co-inoculation can also improve the phytoextraction of various metals at the same time, as we found for Ni, Co, and Cu, which indicates the possibility of exporting the technology to multiple metal contaminated sites. For these purposes, bacterial strain selection is recommended to be based on metal resistance and IAA over productivity, but with particular attention to ACC-deaminase activity, which reduces plant stress, aging and senescence.

Attempts to extend the technology to non-hyperaccumulator plants led to positive results in terms of root and shoot growth in *A. thaliana*, although with low tissue Ni concentration, not comparable with the results from *N. caerulescens*. However, our isolates can contribute to the creation of a "phytoremediating bacteria database," to be tested on high biomass-yielding plant species under multiple metal-contaminated sites for phytoextraction purposes.

Funding

This research was supported by funds to Prof. Anna Maria Sanangelantoni and Dr. Giovanna Visioli from FIL, of the University of Parma Local Funding for Research.

Acknowledgments

The authors would like to thank the Emilia Romagna region (Italy) SITEIA.PARMA Technopole (POR FESR 2007-2013) (NM), Prof. Maria Careri for allowing access to ESEM facilities, and Prof. Roberto Perris for allowing access to optical microscopy facilities. Gabriel Walton is also thanked for revision of the English text.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Microbiology

REVIEW
published: 09 September 2015
doi: 10.3389/fmicb.2015.00937

Biochemistry and genetics of ACC deaminase: a weapon to "stress ethylene" produced in plants

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OPEN ACCESS

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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Microbiology

Received: *27 April 2015*
Accepted: *24 August 2015*
Published: *09 September 2015*

Citation:
Singh RP, Shelke GM, Kumar A and Jha PN (2015) Biochemistry and genetics of ACC deaminase: a weapon to "stress ethylene" produced in plants. Front. Microbiol. 6:937. doi: 10.3389/fmicb.2015.00937

1-aminocyclopropane-1-carboxylate deaminase (ACCD), a pyridoxal phosphate-dependent enzyme, is widespread in diverse bacterial and fungal species. Owing to ACCD activity, certain plant associated bacteria help plant to grow under biotic and abiotic stresses by decreasing the level of "stress ethylene" which is inhibitory to plant growth. ACCD breaks down ACC, an immediate precursor of ethylene, to ammonia and α-ketobutyrate, which can be further metabolized by bacteria for their growth. ACC deaminase is an inducible enzyme whose synthesis is induced in the presence of its substrate ACC. This enzyme encoded by gene *AcdS* is under tight regulation and regulated differentially under different environmental conditions. Regulatory elements of gene *AcdS* are comprised of the regulatory gene encoding LRP protein and other regulatory elements which are activated differentially under aerobic and anaerobic conditions. The role of some additional regulatory genes such as *AcdB* or LysR may also be required for expression of *AcdS*. Phylogenetic analysis of *AcdS* has revealed that distribution of this gene among different bacteria might have resulted from vertical gene transfer with occasional horizontal gene transfer (HGT). Application of bacterial *AcdS* gene has been extended by developing transgenic plants with ACCD gene which showed increased tolerance to biotic and abiotic stresses in plants. Moreover, distribution of ACCD gene or its homolog's in a wide range of species belonging to all three domains indicate an alternative role of ACCD in the physiology of an organism. Therefore, this review is an attempt to explore current knowledge of bacterial ACC deaminase mediated physiological effects in plants, mode of enzyme action, genetics, distribution among different species, ecological role of ACCD and, future research avenues to develop transgenic plants expressing foreign *AcdS* gene to cope with biotic and abiotic stressors. Systemic identification of regulatory circuits would be highly valuable to express the gene under diverse environmental conditions.

Keywords: ACC, *AcdS*, ethylene, abiotic stress, PGPR

Introduction

Plant growth and productivity are limited by several physiological and environmental factors that include availability of macro and micronutrients, physical and chemical properties of soil, plant genotype and growth conditions. Apart from these factors, plant growth and yield are detrimentally affected by diverse biotic and abiotic factors. The latter include stressors such as salt, low and high temperature, drought, water logging, mechanical wounding, the presence of heavy metals and other organic and inorganic toxic compounds (Gamalero and Glick, 2012). The loss incurred due to these factors is estimated to be >50% for most major crop plants (Boyer, 1982; Bray et al., 2000). Thus, abiotic stresses are the major factors that adversely affect the agricultural productivity worldwide. Therefore, world food production needs to be doubled to cope with the ever-growing demand of the population (Tilman et al., 2002). Problems of biotic and nutritional factors can be overcome using pesticides and biofertilizers, respectively but getting rid of calamities arose due to abiotic factors by non-biological means is highly challenging.

Plants respond to above-mentioned stressors by modulating the level of various hormones which in turn induce expression of stress-related proteins required for protection from the deleterious effects of stressors. One of the most common plant hormone that mediates response to the stressors is ethylene. However, when ethylene is produced more than its threshold level, it turns out as "stress ethylene" which is unfavorable in terms of root/shoot proliferation and other growth parameters and, thus hinders plant growth and development. Effect of stress ethylene in plants can be reduced by certain plant-associated bacteria that possess an enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACCD) (Glick, 2007). ACCD breaks down ACC, an immediate precursor of ethylene, to α-ketobutyrate and ammonia resulting into decrease in level of ethylene in plants which in turn resumes root/shoot growth (Honma and Shimomura, 1978; Glick, 2014). This property of attributing tolerance to abiotic stressors by ACCD activity and some additional mechanisms of plant growth promoting bacteria (PGPB) to ameliorate stresses in host plants are referred as "induced systemic tolerance" (Yang et al., 2009). Thus, PGPB equipped with ACCD activity are of utmost importance in reducing the deleterious effect of environmental stressors (**Table 1**). It has been established that generation of stress ethylene is central to the effect of various stressors in plants. Therefore, the present review addresses importance of ethylene in plant physiology, details of biochemistry and genetics of ACC deaminase which reduces the level of stress ethylene in plants and relieve from the deleterious effect of environmental stressors.

Ethylene Biosynthesis and Role in Plant Physiology

The ethylene production in plants depends on the environmental condition as well as the severity of various stresses. The recognition of ethylene as a plant growth regulator originated from observation of premature shedding of leaves, geotropism of etiolated pea seedling when exposed to illuminating gas, and ripening of oranges when exposed to gas from kerosene combustion (Pierik et al., 2006; Glick, 2007). Further studies with the analytical techniques like gas chromatography (GC) elaborated its role in plant growth and development. Ethylene at optimal concentration ($10\,g\,L^{-1}$) is essential in functions related to normal growth and development in plants such as formation of adventitious root and root hairs, acceleration of seed germination, breaking seed dormancy etc. (Arshad and Frankenberger, 1990; Jackson, 1991). However, at a higher concentration ($25\,g\,L^{-1}$), it induces defoliation, inhibition of root elongation, inhibition of nodulation in legumes, leaf senescence, leaf abscission, chlorophyll destruction, and epinasty. Therefore, it is imperative to regulate the ethylene production in roots for normal growth and development of the plants.

The ethylene biosynthesis begins with enzyme ACC synthase that converts S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) and 5′-methylthioadenosine (MTA) latter of which is recycled to L-methionine. The next step is the conversion of ACC to ethylene by ACC oxidase. Several isoforms of ACC oxidase has been reported that show differential activity under different physiological conditions (Abeles et al., 1992). Synthesis of ethylene is affected by a number of different factors including temperature, light, nutrition, gravity, and the presence of various types of biological stressors to which the plant may be subjected (Glick, 2007). Regarding a plant's response to stress, an increased level of ethylene is formed in response to the presence of metals, organic and inorganic chemicals, extreme temperature, ultraviolet light, insect attack, phytopathogens (bacteria and fungi), and mechanical wounding. A model proposed for the synthesis of stress ethylene suggests that two peaks of ethylene are observed after stress exposure to plants. The first small peak of ethylene (Robison et al., 2001; Van Loon et al., 2006; Desbrosses et al., 2009) is believed to be responsible for transcription of genes that encode the plant defensive/protective proteins. The second much larger ethylene peak, termed as "stress ethylene" emerged in response to stresses is detrimental to plant growth and initiates processes like senescence, chlorosis and leaf abscission (Glick, 2007). Reduction in the level of stress ethylene by any chemical or biological treatment can significantly lower the magnitude of stress ethylene and decrease stress-induced damage to the plants (Van Loon et al., 2006). As mentioned above, one of the most common and effective mechanisms to reduce the level of stress ethylene is ACCD mediated degradation of ACC. Following sections deal with functioning and molecular aspects of ACC deaminase.

ACC Deaminase: Biochemical Properties and Mode of Action

ACC deaminase was first discovered in soil microorganism and shown to convert ACC to ammonia and α-ketobutyrate, both of which further metabolized by a microorganism (Honma and Shimomura, 1978). It is a pyridoxal phosphate-dependent

TABLE 1 | PGPR containing ACC deaminase mediated protection in response to various types of stresses.

Bacteria	Host plant	Stress protection	References
Achromobacter piechaudii ARV8	*Lycopersicon esculentum*	Salt	Mayak et al., 2004
Alcaligenes sp., Bacillus pumilus	*Brassica napus*	Heavy metals	Belimov et al., 2001
Burkholderia sp. J62	*Lycopersicon esculentum*	Lead	Jiang et al., 2008
B. phytofirmans PsJN	*Vitis vinifera*	Low temperature	Ait Bakra et al., 2006
Enterobacter aerogenes NBRIK24	*Brassica juncea*	Fly-ash soil	Kumar et al., 2008
E. sakazakii 8MR5	*Zea mays*	Striga-infested soils	Babalola et al., 2003
E. cloacae CAL2	*Brassica napus*	Arsenate	Nie et al., 2002
Kluyvera ascorbata SUD165	*Brassica napus*	Nickel	Burd et al., 1998
K. ascorbata SUD165/26	*Lycopersicon esculentum*	Lead	Burd et al., 2000
Pseudomonas putida UW4	*Lycopersicon esculentum*	Flooding	Grichko and Glick, 2001
P. fluorescens YsS6	*Lycopersicon esculentum*	Salt	Ali et al., 2014
Pseudomonas putida	*Vigna radiata*	Salt	Mayak et al., 1999
P. putida UW4	*Lycopersicon esculentum*	Salt	Yan et al., 2014
Pseudomonas fluorescens	*Arachis hypogea*	Salt	Saravanakumar and Samiyappan, 2007
P. putida	*Lycopersicon esculentum*	Low temperature	Cheng et al., 2007
P. fluorenscens CHA0	*Solanum tuberosum*	Plant pathogen	Wang et al., 2000
P. fluorenscens CHA96	*Cucumis sativus*	Plant pathogen	Wang et al., 2000
P. putida UW4	*Pinus sabiniana*	Plant pathogen	Nascimento et al., 2013
P. putida UW4, *P. putida* HS-2	*Brassica napus*	Nickel	Farwell et al., 2007
P. fluorescens ACC-5	*Pisum sativum*	Drough	Zahir et al., 2008
Pseudomonas sp.	*Pisum sativum*	Drough	Arshad et al., 2008
Sinorhizobium sp. Pb002	*Brassica juncea*	Lead	Di Gregorio et al., 2006
Variovorax paradoxus	*Brassica juncea*	Cadmium	Belimov et al., 2005

enzyme and approximately 3 mol of enzyme-bound pyridoxal phosphate per mol of an enzyme or 1 mol per trimeric subunit (Honma, 1985) are required for enzyme activity. ACC deaminase was first purified from *Pseudomonas sp. strain* ACP and partially purified from *Pseudomonas chloroaphis* 6G5 (Klee et al., 1991) and *Pseudomonas putida* GR12-2 (Jacobson et al., 1994). Enzyme purified from all three sources appears to have similar molecular mass and form. The native size of 110-112 KDa has been reported from *Pseudomonas sp. strain* ACP and 105 KDa for the enzyme from *P. putida* GR12-2. The enzyme is trimeric in form and has an approximate subunit mass of 36,500 daltons.

The absorption spectra of purified ACC deaminase from *Pseudomonas sp*. show different absorption maxima at 416 and 326 nm at pH 6 and pH 9, respectively (Honma, 1985). It is possible that the 326 nm band seen at pH 9 is the active form of ACC deaminase to which substrates and inhibitors bind (Honma, 1985; Jacobson et al., 1994). The observed K_m value of enzyme extracts of different microorganisms at pH 8.5 fall in the range of 1.5–17.4 mM, indicating the low affinity of the enzyme for ACC. The overall efficiency (k_{cat}/k_m) of ACC deaminase is approximately 690 $M^{-1}S^{-1}$ following the second order kinetics. The K_m value of ACC deaminase for ACC has been estimated for enzyme extracts of microorganism at pH 8.5 (Klee et al., 1991). Enzyme activity of ACCD was evaluated over a wide range of pH in many bacterial species, and the highest activity was observed at pH 8.0–8.5 (Zhao et al., 2003). The optimal temperature for ACC deaminase activity of *P. putida* GR12-2 is 30°C (Glick et al., 1998).

ACC deaminase is an inducible enzyme whose synthesis is induced in the presence of its substrate ACC. The minimum level of the substrate for induction was measured as 100 nM in *Pseudomonas* sp. strain ACP and *P. putida* GR12-2. The induction of ACCD is a complex and slow process. It exhibits activity within the first few hours of induction with the substrate, but the activity decreases gradually after initial induction (Walsh et al., 1981; Jacobson et al., 1994). The basal level of enzyme activity is observed in minimal medium supplemented with ammonium sulfate as a nitrogen source. Honma (1983) demonstrated the induced activity after switching the bacteria from nutrient rich medium to minimal medium supplemented with ACC as sole nitrogen source. It illustrates that the induction of enzyme activity is directly correlated with substrate ACC. Apart from ACC, other amino acids such as L-alanine, DL-alanine, D-serine also induce enzyme activity and induce expression of ACCD to some extent. Moreover, the induced level of enzyme activity by both ACC and aminoisobutyric acid was observed to be same in *Pseudomonas* sp. strain ACP (Honma, 1983). Glick et al. (1998) proposed a model for functioning of bacterial ACC deaminase which states that a significant portion of ACC is exuded from plant roots or seeds, taken up by the soil microbes and hydrolyzed to ammonia and α-ketobutyrate. The uptake and hydrolysis of ACC decrease the amount of ACC outside the plant roots. Furthermore, the equilibrium between the internal and external ACC level is maintained through exudation of more ACC into the rhizosphere. Thus, decrease in the level of ACC affects biosynthesis of the stress hormone

ethylene in host plants and stimulate plant growth (Honma et al., 1993; Glick et al., 1998).

Opening of cyclopropane ring of ACC is the main feature of the reaction catalyzed by ACC deaminase. Although the reaction mechanism is not fully understood, nucleophilic addition, and elimination appears to be the most likely routes by which cyclopropane bond is cleaved (Walsh et al., 1981; Ortíz-Castro et al., 2009). ACC deaminase is competitively inhibited by L-isomers of the amino acids such as L-alanine, L-serine, L-homoserine, and L-α aminobutyric acid where the strongest inhibition is seen with L-alanine and L-serine. ACC related compounds such as 2-alkyl -ACC and vinyl-ACC can also function as substrates for ACC deaminase, purified from *Pseudomonas* sp. Strain ACP but the enzyme shows an unusual specificity for D-amino acids and is inactive with any of the L-amino acids or their derivatives (Walsh et al., 1981; Honma, 1985). NMR studies showed that a proton is eliminated from the α-carbon of D-alanine but not from its L-isomer. These findings explain the deamination of D-amino acids and of several β- substituted D-alanines by ACC deaminase and are consistent with the stero-specific cleavage of the cyclopropane ring during ACC deamination (Honma et al., 1993). In the presence of D-alanine, ACC deaminase is inactivated more effectively by the iodoacetamide derivative 1, 5 N-iodoacetamidoethyl-1-aminonapthalene-5-sulfonic acid (1,5-I- AEDANS) than by iodoacetamide. During inactivation, two residues are modified, a thiol group in cysteine residue 162 and the aldimine bond of pyridoxal phosphate with lysine residue 51 (Honma et al., 1993).

Insight into ACC Deaminase Catalyzed Reaction

Based on three-dimensional structures, pyridoxal phosphate enzymes have been classified into four major types: (a) Tryptophan synthase, (b) aspartate aminotransferase, (c) D-amino acid aminotransferase, and (d) alanine racemase. ACC deaminase is a member of pyridoxal 5-phosphate (PLP) dependent enzymes fitting into the tryptophan synthase family. PLP enzymes catalyze a wide range of metabolic reactions such as transamination, deamination, decarboxylation, and eliminations of β and γ substituent groups. The reaction catalyzed by ACC deaminase differs from other PLP-dependent enzymes as the ring cleavage cannot proceed through α-carbanionic intermediate due to lack of abstractable α-hydrogen atom from the substrate ACC. Two types of reactions can be catalyzed by ACC deaminase for a breakdown of ACC. First, abstraction of hydrogen atom and opening of cyclopropane ring by Lys^{51} mediated series of hydrolytic reactions and second, opening of cyclopropane ring by nucleophilic attack on β-carbon atom of ACC followed by β-proton abstraction at the pro-R carbon by a basic residue Lys^{51} (Zhao et al., 2003).

The mechanistic action of ACCD on its substrate is depicted in **Figure 1**. The substrate ACC reacts with internal aldimine [A] resulted from the reaction of PLP (Pyridoxal 5-phosphate) cofactor with Lys residue of ACC deaminase enzyme. This leads to conversion of internal aldimine to external aldimine [C] *via* aminyl intermediate [B] known as trans-aldimination process. These initial mechanistic routes are shared between both proposed mechanisms, i.e., (a) Direct β-hydrogen abstraction and (b) Nucleophilic addition followed by β-hydrogen abstraction.

Ose et al. (2003) proposed that Lys^{51} residue of ACCD causes an initial direct β-hydrogen abstraction of the methylene proton leading to the formation of a quinonoid [1] (**Figure 1**, Route I). The quinonoid [1] undergoes further electronic rearrangement and protonation to form another quinonoid [2]. This is followed by nucleophilic attack by a basic residue on the protein backbone, which ultimately produces 2-aminobut-2-enoate and a quinonoid [3]. These products reversibly undergo hydrolysis to form 2-oxobutanoate and ammonium, regenerating the internal aldimine. In route II, steps are identical to those proposed for the route I up to the production of external aldimine [C] (**Figure 1**, Route II). Ring opening is initiated by nucleophilic attack of a basic residue of protein on the pro-S β-carbon of ACC and a nearby second basic residue located on the protein. It follows the removal of a proton from the pro-R β carbon of ACC which results in the formation of a quinonoid. The remaining mechanistic steps are identical to those of the first mechanism (**Figure 1**, Route II).

Prevalence of ACC Deaminase

The presence of ACC deaminase has been reported in all three domains, i.e., eukarya, bacteria, and archaea. However, ACC deaminase activity is known to be present majorly in different species of bacteria and in some fungi (**Table 2**). ACCD activity has been found in a wide range of gram positive and gram negative bacteria (Nascimento et al., 2014). It has also been reported in strains of *Pyrococcus horikoshii* (Fujino et al., 2004), a hyperthermophilic archaeon. Among eukaryotes, production of ACCD is well evident in some fungi, which include a few species of yeast such as *Hansenula saturnus* (Minami et al., 1998), *Issatchenkia occidentalis* (Palmer et al., 2007), other fungal species namely *Penicillium citrinum* and *Trichoderma asperellum,* and a stramnopile, *Phytophthora sojae* (Jia et al., 1999; Viterbo et al., 2010; Singh and Kashyap, 2012). Recently, ACCD activity has also been observed in certain plants such as *Arabidopsis thaliana,* poplar, and tomato plant (McDonnell et al., 2009; Plett et al., 2009). Presence of ACCD has been confirmed at the molecular level by amplification and sequence analysis of *AcdS*, a structural gene encoding ACCD. The *AcdS* gene is commonly found in Actinobacteria, Deinococcus-Thermus, three classes of Proteobacteria (α, β, and γ), various fungi belonging to Ascomycota and Basidiomycota, and in some Stramenopiles. Although, the presence of ACCD activity has been demonstrated in bacteria belonging to phyla Chlorobi, Bacteroidetes, and Firmicutes but the genes corresponding to ACCD have not been reported yet (Nascimento et al., 2014). On the contrary, putative *AcdS* genes have been reported in *Meiothermus* and *Phytophthora* based on the sequence similarity but there is no record of ACC deaminase activity in these thermophilic strains.

We analyzed the prevalence of *AcdS* gene in IMG (Integrated microbial genomes) database (http://img.jgi.doe.gov/) from Joint

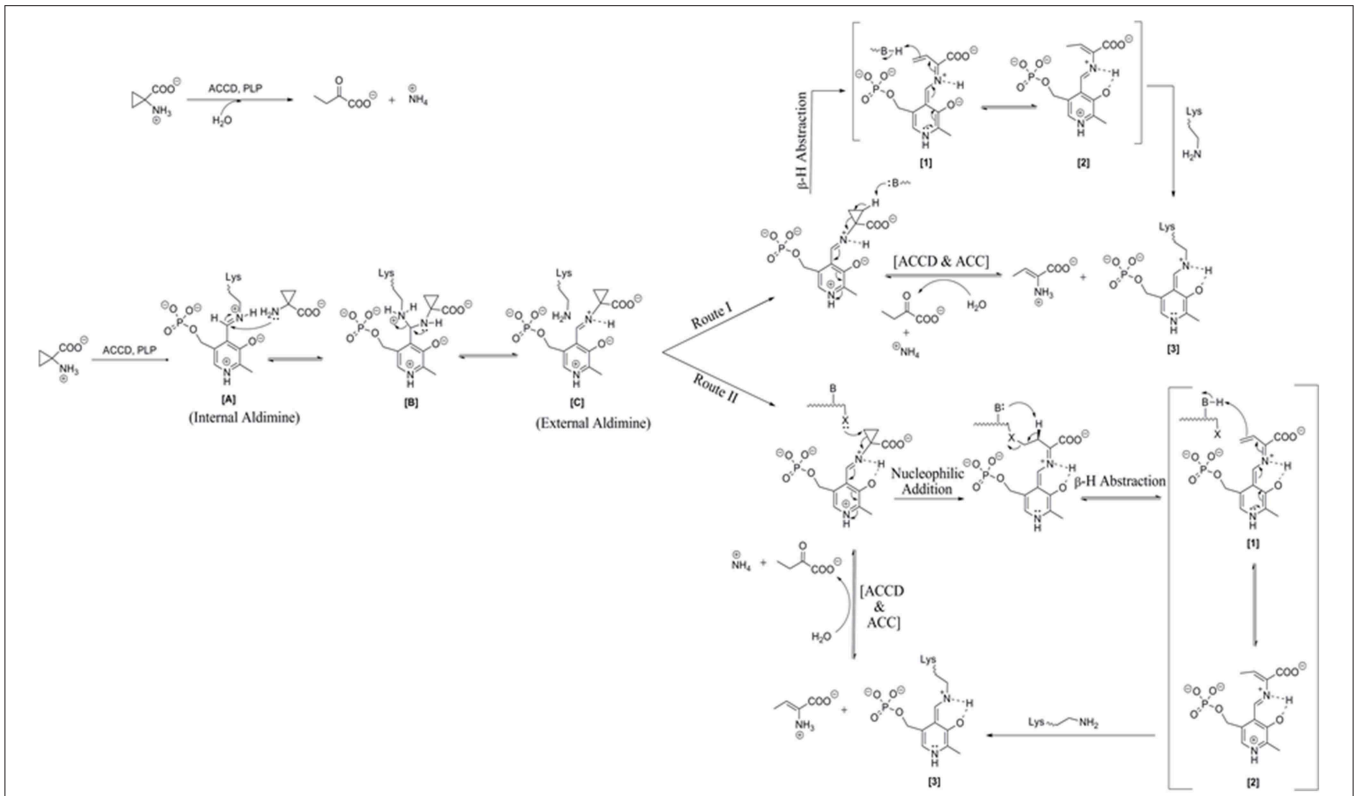

FIGURE 1 | Reaction mechanism catalyzed by microbial ACC deaminase. Route I: direct β-H (Hydrogen) abstraction, Route II: Addition of nucleophiles followed by β-H (Hydrogen) abstraction. Modified figure adapted from the source ref. Hontzeas et al. (2006).

genome Institute (JGI) using locus tag search corresponding to *AcdS* of *P. putida* UW4. The *AcdS* sequences having more than 1000 bp were chosen for further analysis. Altogether, 485 strains belonging to different genera including *Acidovorax, Bordetella, Brenneria, Burkholderia, Collimonas, Cupriavidus, Curvibacter, Dickeya, Herbaspirillum, Halomonas, Lonsdalea, Methylibium, Pantoea, Phytophthora, Polaromonas, Pseudomonas, Ralstonia, Serratia, Tatumella, Variovorax,* and *Xenophilus,* showed presence of *AcdS* gene. Important species belonging to these genera are listed in Supplementary Table 1. These data were extracted from the genomic sequences of organisms from different ecological niches including a human host, bulk soil, plants, and water. For the presence of gene encoding ACCD in other domains (eukarya and archaea), gene search was conducted using the product name as a criterion for the search. It revealed that very few members of archea showed the presence of ACCD gene. It includes strains of *Archaeoglobus fulgidus, Pyrococcus abyssi, Pyrococcus furiosus,* and *Thermococcus nautili.* Analysis of metagenomic database revealed the presence of genes encoding ACCD in various kingdoms, i.e., animalia, chromalveolate, fungi, and plantae of domain Eukarya (**Table 3**). It suggested that among domain Eukarya, ACCD gene is prevalent in members of phylum Ascomycota and Basidiomycota. The IMG database extends our knowledge of the existence of ACCD gene in other higher plants from kingdom plantae which include soybean, potato, maize, and castor oil plants. The presence of ACCD encoding genes in several members of kingdom Animalia seems to be intriguing as no obvious role of ACCD in animals is known (**Table 3**). The presence of ACCD gene in some pathogenic bacteria associated with human and other animals indicates that ACCD may be required for some other unknown function or these bacteria may earlier be plant-associated which later on evolved to colonize animals and other kingdoms as well. Moreover, both bacterial and fungal ACC deaminase shares a common origin and belongs to pyridoxal phosphate-dependent enzyme related to tryptophan-synthase family.

Genetics and Expression of ACC Deaminase

ACC Deaminase Gene

Gene *AcdS* encoding ACC deaminase have been detected in several bacterial and fungal genera as discussed above. More recently, ACC deaminase has been found in wide range of gram-negative bacteria (Belimov et al., 2001; Wand et al., 2001; Hontzeas et al., 2004; Tak et al., 2013), Gram-positive bacteria (Belimov et al., 2001; Timmusk et al., 2011), rhizobia (Ma et al., 2003b; Uchiumi et al., 2004), endophytes (Sessitsch et al., 2002; Rashid et al., 2012), and fungi (Jia et al., 1999). Putative ACC deaminase gene have also been reported several species including *R. leguminosarum bv. Trifoli* (Itoh et al., 1996) and

TABLE 2 | List of microorganism with ACC deaminase activity.

Strain	ACCD activity (nmol α-KB $mg^{-1}h^{-1}$)	References
Achromobacter xylosoxidans A551	400	Belimov et al., 2005
Acidovorax facilis	0.0007	Belimov et al., 2005
A. facilis 4p-6	3080	Belimov et al., 2001, 2005
A. xylosoxidans AF302097	151	Belimov et al., 2001
A. xylosoxidans Bm1	90	Belimov et al., 2005
Alcaligenes sp. AF288728	1172	Belimov et al., 2001
A. xylosoxidans AF302096	555	Belimov et al., 2001
A. xylosoxidans AF288734	305	Dell'Amico et al., 2008
Bacillus pumilus AF288735	760	Belimov et al., 2001
Burkholderia caryophylli	598	Shaharoona et al., 2007b
Enterobacter cloaceae	295	Nadeem et al., 2010
E. aerogenes	341	Nadeem et al., 2007
Escherichia coli DH5α/p4U2	285	Shah et al., 1998
Flavobacterium ferrugineum	405	Nadeem et al., 2007
Methylobacterium sp.	94	Madhaiyan et al., 2006
Mycobacterium sp.	1.14	Dell'Amico et al., 2008
Pseudomonas aeruginosa	153	Zahir et al., 2009
P. bathycetes	501	Nadeem et al., 2007
P. brassicacearum AY007428	972	Belimov et al., 2001
P. chlororaphis	456	Nadeem et al., 2007
P. fluorescens	421	Nadeem et al., 2007
P. fluorescens ATCC 17400/pkK-ACC	157	Shah et al., 1998
P. fluorescens TDK1	349	Zahir et al., 2009
P. fluorescens biotype G	490	Shaharoona et al., 2006a
P. fluorescens biotype F	342	Zahir et al., 2008
P. oryzihabitans AF288732	890	Belimov et al., 2001
P. syringae	440	Nadeem et al., 2007
P. tolaasii	1.16	Dell'Amico et al., 2008
Rhizobium hedysari ATCC 43676	20	Ma et al., 2003b
*R. leguminosarum*128C53K	5	Belimov et al., 2001, 2005
R. leguminosarum 99A1	430	Ma et al., 2003a
Rhodococcus sp. AF 288731	833	Belimov et al., 2001
Rhodococcus sp. strain Fp2	7320	Belimov et al., 2001, 2005
Rhodococcus sp. strain 4N-4	12,970	Belimov et al., 2001, 2005
Serratia ficaria	326	Nadeem et al., 2010
S. proteamaculans	276	Zahir et al., 2009
S. quinivirans SUD165	12	Belimov et al., 2001, 2005
Variovorax paradoxus 3P-3	3700	Belimov et al., 2001, 2005
V. paradoxus 5C-2	4322	Belimov et al., 2001, 2005
V. paradoxus 2C-1	3588	Belimov et al., 2001, 2005
V. paradoxus sp.	1805	Belimov et al., 2005

Mesorhizobium loti MAFF303099 (Kaneko et al., 2002). However, the expression level of ACC deaminase varies from one organism to another. Using a universal pair of primers, a segment of *AcdS* gene has been amplified and analyzed in several environmental isolates (Hontzeas et al., 2005). Several pair of primers has been designed by various researchers to detect the presence of *AcdS* gene in bacteria (Duan et al., 2009; Jha et al., 2012).

TABLE 3 | Distribution of ACC deaminase in domain Eukarya.

Kingdom	Phylum	Species
Animalia	Porifera	*Amphimedon queenslandica*
	Chordata	*Branchiostoma floridae, Ciona intestinalis, Oikopleura dioica*
	Annelida	*Capitella teleta*
	Cnidaria	*Nematostella vectensis*
	Placozoa	*Trichoplax adhaerens Grell-BS-1999*
Chromealveolata	Haptophyta	*Emiliania huxleyi*
	Stramnopile	*Saprolegnia parasitica, Phytophthora ramorum*
	Apicomplexa	*Theileria annulata*
Fungi	Ascomycota	*Arthroderma benhamiae*, Aspergillus clavatus*, A. flavus*, A. fumigatus, Beauveria bassiana, Cercospora sojina, Coccidioides posadasii*, Fusarium oxysporum, Neosartorya fischeri*, Penicillium marneffei*, Talaromyces stipitatus*, Trichophyton verrucosum**
	Basidiomycota	*Cryptococcus neoformans, Gymnopus luxurians, Schizophyllum commune*
Plantae	Lycopodiophyta	*Selaginella moellendorfii*
	Magnoliophyta	*Ricinus communis*, Zea mays, Glycine max, Solanum tuberosum*
	Chlorophyta	*Volvox carteri*

**Putative proteins have been found.*

Complete genetic makeup and function of ACCD gene has been well characterized in only a few bacterial species (Duan et al., 2013). We observed from the data recovered from IMG that nucleotide sequences of *AcdS* gene is very close to other genes namely dcyD and yedO which encode for another PLP-dependent enzyme D-cysteine sulfhydralase. This observation is supported by previous reports where some genes previously identified to encode ACC deaminase were found to encode D-cysteine desulfhydrase activity (Riemenschneider et al., 2005). To differentiate sequences of D-cysteine desulfhydrase from ACC deaminase, Nascimento et al. (2014) analyzed *AcdS* sequences for key protein residues namely Lys51, Ser78, Tyr295, Glu296, and Leu322, known to be important for ACC deaminase activity using *Pseudomonas* sp. UW4 as a reference. Any change in residues at given locations were considered likely to represent D-cysteine desulfhydrase.

Except few, *AcdS* gene in the majority of bacterial species is chromosomal DNA-borne. In symbiotic bacteria *M. loti* (symbiont of *lotus* spp.), ACC deaminase gene is associated with the nitrogen fixation genes and might be regulated by NifA which is known to activate *nif* gene expression in association with the product of *rpoN* gene (Ma et al., 2003a). Moreover, only a small fraction of putative *AcdS* gene has been shown to encode active enzyme (Glick et al., 2013).

Regulation of ACC Deaminase

AcdS is highly regulated and expresses differentially depending on presence or absence of oxygen, concentration of substrate, and

accumulation of products. Except few, mechanism of regulation of *AcdS* gene in different bacterial genera is not well understood. A model for the regulation of ACC deaminase gene in *P. putida* UW4 (earlier known as *Enterobacter clocae* UW4) has been proposed by Li et al. (2000). Regulatory elements for the expression of ACC deaminase gene consist of regulatory gene *AcdR* located 5′ upstream of ACC deaminase structural gene (*AcdS*), promoter regions for binding of regulatory proteins like Lrp box for binding of Lrp protein, AcdB box for binding regulatory protein *AcdB*, FNR box for binding of fumarate and nitrate reductase protein and, CRP box for binding of cAMP receptor protein. In the presence of ACC, LRP forms an active octamer that binds to a complex of ACC and another protein *AcdB* (Cheng et al., 2008). *AcdB* encodes for the glycerophosphoryl diester phosphodiesterase and form complex with ACC. This triparental complex activates transcription of *AcdS* by binding to its promoter region (Li and Glick, 2001). The role of *AcdB* in *AcdS* expression has not been observed in other bacteria characterized for *AcdS* gene expression. ACC deaminase gene is negatively regulated by leucine which is synthesized from α-ketobutyrate, a breakdown product of ACCD catalyzed reaction. As the concentration of leucine increases, it favors formation of inactive LRP dimer form which leads to switching off the transcription of *AcdS* gene (**Figure 2**).

Regulatory machinery for *AcdS* expression varies in different species. Results of IMG database analysis showed that presence of *AcdR* encoding LRP or its homologous sequences is present in the majority of bacteria. In *Bradyrhizobium japonicum* USDA 110 and *Rhizobium leguminosarum bv. Viciae* 128 C53K also, LRP like protein and σ70 promoter are involved in regulation of *AcdS* gene (Kaneko et al., 2002; Ma et al., 2003a). The phylogenetic analysis of *AcdR* gene suggested that *AcdS* and *AcdR* were evolved in a similar manner. In *Burkholderia* sp. CCGE 1002 and *Burkholderia phymatum* STM 815, there is no *AcdR* gene but it has two copies of *AcdS* gene instead, one on the megaplasmid and other on the second chromosome. These shreds of evidence suggest the genomic rearrangement events or gene insertion event in smaller replicons. Regulatory regions of ACC deaminase gene from some bacteria such as *Variovorax parrdoxus* 5C2 and *Achromobacter xylosoxidans* A551 does not contain all these regulatory elements described for *P. putida* UW4.

In *M. loti*, the upstream elements of *AcdS* and *nifH* contain *nifA1* and *nifA2* (regulatory N_2 fixing unit) and σ^{54} RNA polymerase sigma recognition site. It was assumed that expression of ACC deaminase in *M. loti* required the symbiotic nitrogen fixing regulatory gene *nifA2* (Nukui et al., 2006). The *nifA2* encoded protein NifA2 interact with σ^{54} RNA polymerase favoring *AcdS* transcription. The *nifA1* also affect the transcription of *AcdS* gene up to some extent. however, its role in the expression of *AcdS* is not properly understood (Nukui et al., 2006) (**Figure 3**). The expression of *AcdS* gene in root nodules reduces the harmful effect of ethylene induced senescence and elevates the concentration of fixed nitrogen in nodules. ACC deaminase activity is generally assayed in free-living conditions but in *M. loti*, the activity was detected only in symbiotic nodules (Uchiumi et al., 2004). Uchiumi et al. (2004) have reported that mlr5932, an up-regulated gene in Bacteroides, encodes ACC deaminase, which is involved in enhancing nodulation in *Lotus japonicus* plants. It is to be noted that unlike free-living bacteria, ACC deaminase produced by nodule forming *Rhizobia* does not lower the ethylene level throughout the plant and may not be used to protect plants from various stress. Also, the level of

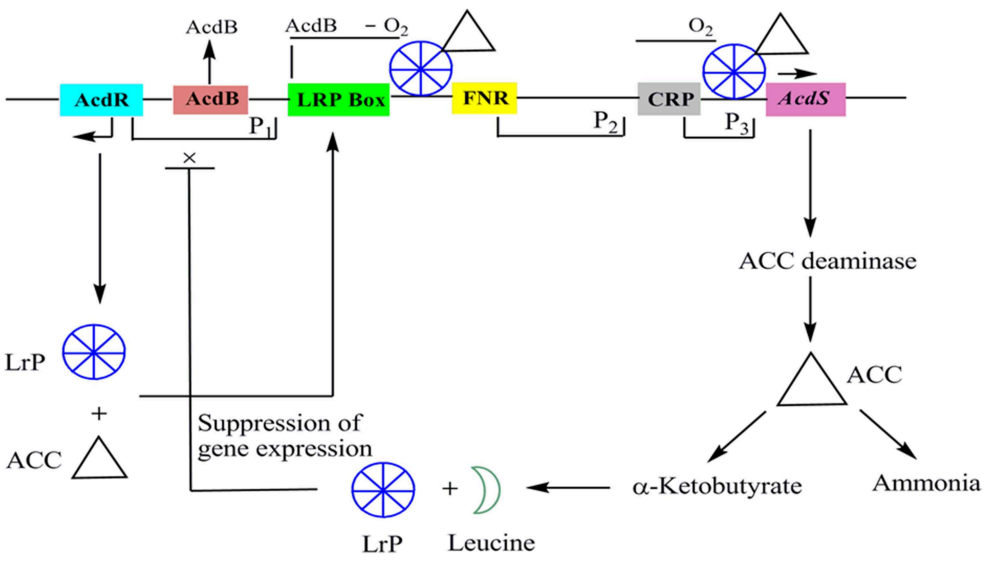

FIGURE 2 | The regulatory circuits of *AcdS* gene expression in *Pseudomonas putida* UW4 and other related bacteria. AcdR, regulatory gene for ACC deaminase; AcdB: encoding for glycerophosphoryl diester phosphodiesterase; LRP, Leucine responsive protein; FNR: fumarate nitrate reductase protein; CRP, c-AMP receptor protein; *AcdS*, gene for encoding ACC deaminase.

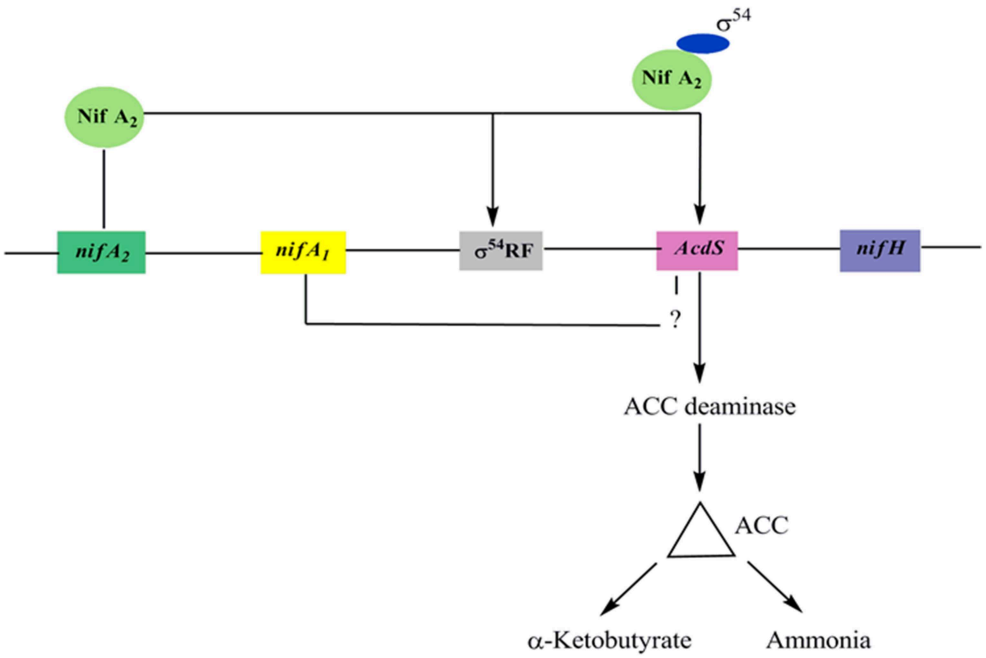

FIGURE 3 | A model for *AcdS* gene regulation in nitrogen fixing *Mesorhizobium* sp. Expression of *AcdS* is positively regulated by $NifA_2$ protein which binds to σ^{54} and switch on transcription of *AcdS* gene. $NifA_1$ is also required in regulation of *AcdS* but its role is not well-understood.

ACCD produced in the nodule is only 2–10% of ACCD produced by free-living bacteria.

In many *Actinobacteria* and *Meiothermus,* GntR protein coding gene is found next to *AcdS* gene. These evidence indicate a possibility that some downstream elements are also involved in regulation of ACC deaminase expression. In some members of these genera, the absence of promoter region strongly suggests that the interaction of *AcdS* gene and some downstream element next to *AcdS* gene is involved in the regulation of *AcdS* gene transcription. In certain species of *Actinobacteria* and *Proteobacteria* like *Brenneria* sp. EniD312, *Burkholderia xenovorans* LB4000 and *Pantoea sp.* At-9B, LysR family of transcription regulatory elements, are found in close vicinity of *AcdS* gene. However, the exact mechanism of regulation of ACC deaminase in these organisms is still poorly known. Additional genetic and biochemical studies are necessary to understand the mechanism of ACC deaminase regulation and functioning in different bacterial genera.

Putative ACC deaminase gene in *M. loti* MAFF303099 does not contain any regulatory elements nor display any enzyme activity when it is induced by ACC (Ma et al., 2003b). Expression of ACC deaminase in *R. leguminosarum bv. viciae* 128C53K is induced by ACC concentrations as low as 1 μM (Ma et al., 2003b). Experimental evidence suggested that introducing ACC deaminase gene as well as its regulatory gene from *R. leguminosarum bv. Viciae* 128C53K to a strain of *Sinorhizobium meliloti* showed greater efficiency in nodulating *Medicago sativa* (alfalfa) (Ma et al., 2004) and latter strain was more competitive in nodulation than the wild type (Ma et al., 2004).

Evolution of ACC Deaminase Gene

Phylogenetic analysis based on *AcdS* gene and protein sequence from different microbial species has been conducted to study the evolution of ACC deaminase gene. Available sequence data revealed that the most of the ancient bacteria belonging to Actinobacteria and Deinococcus-Thermus possess *AcdS* gene in their primary and unique chromosome. The *AcdS* gene in many α-proteobacteria is also found in the chromosomal DNA. The presence of *AcdS* gene has also been observed in plasmid observed on a plasmid DNA in a few bacteria which might have resulted from an event of extensive gene transfer from primary chromosome to plasmid between the members of α-proteobacteria. Location of *AcdS* gene in the second chromosome of *Burkholderia* and in plasmids of *Pseudomonas* reflects the intragenomic transfer of *AcdS* genes from primary chromosome to plasmids. On the other hand, the presence of *AcdS* gene sequence in some fungi like *Fomitopsis pinicola* FP-58527 belonging to Basidiomycota suggested having a bacterial origin. The monophyletic origin of ACC deaminase is evident from the conservation of *AcdS* protein sequences from fungi, Actinobacteria, and α-proteobacteria signify. The presence of *AcdS* gene in some fungal classes and in members of *Stramenopiles* suggest that horizontal gene transfer (HGT) resulted in the transfer of *AcdS* not only between bacteria but also between different domains. To gain additional knowledge about the evolution of ACC deaminase gene, multiple *AcdS* gene sequences and protein sequences were aligned. Based on phylogenetic studies, it was assumed that ACC deaminase belongs to a broader group of pyridoxal phosphate-dependent enzymes that share a common ancestor. It might be possible

that this enzyme was originated as a consequence of specific mutation in its ancestral enzyme gene (Nascimento et al., 2014). Recently, based on alignment of multiple *AcdS* sequence from diverse species, Nascimento et al. (2014) inferred that the continuous vertical transmission of *AcdS* genes might be responsible for the presence of *AcdS* gene in bacteria that are not associated with ACC producing organism. Our analysis of *AcdS* using IMG database and subsequent construction of phylogenetic tree also confer that evolution of *AcdS* gene resulted from vertical gene transfer with occasional HGT. A consensus bootstrap tree was constructed using 465 *AcdS* sequences obtained from IMG database which showed that accept few, *AcdS* of similar species clustered together supporting the above inference (**Figure 4**). However, many earlier studies suggested a role of HGT in the evolution of *AcdS* gene. Intragenomic *AcdS* transfer might play a role in HGT transfer as well as the divergence of *AcdS* gene. Furthermore, these intragenomic transfers may also lead to loss of the gene in many organisms. Phylogenetic analysis of several *AcdS* gene from ACC deaminase bacteria such as *Variovorax paradoxus 5C-2, V. paradoxus 3P-3, and Achromobacter spp.* indicated that the gene evolved through HGT (Hontzeas et al., 2005). Moreover, the presence of *AcdS* gene in the symbiotic island of many *mesorhizobium spp*. like *M. loti* R7A, *M. australicum* WSM2073T, *M. opportunistum* WSM2075T confirms the horizontal transfer of *AcdS* gene (Nascimento et al., 2012b). Even though most of the phylogenetic studies of *AcdS* gene has been done on proteobacteria, presence of ACC deaminase enzyme have also been reported in Actinobacteria (Hontzeas et al., 2005), Firmicutes (Ghosh et al., 2003), and Bacteroidetes (Maimaiti et al., 2007).

Prigent-Combaret et al. (2008) reported that like *AcdS*, *AcdR* also have evolved through HGT. Owing to horizontal (lateral) gene transfer, the juxta-position of *AcdS* and *AcdR* gene has occurred in *Agrobacterium tumefaciens* d3, *B. japonicum* USDA 110, *R. leguminosarum bv. Viciae* 128C53K, *A. xylosoxidans* A551 (Trott et al., 2001; Kaneko et al., 2002; Hontzeas et al., 2005). However, it is not clear that *AcdR* gene was inherited along with the *AcdS* gene or independently. The current knowledge of the phylogeny and evolution of *AcdS* and *AcdR* gene is still incomplete. The presence of *AcdR* in juxta-position to *AcdS* gene in most proteobacteria acquired through a coupled evolution and transmission of these genes. Therefore, more research is required for elucidating the role of protein encoding genes located in the vicinity to *AcdS,* which can focus on a different mechanism of ACC deaminase regulation.

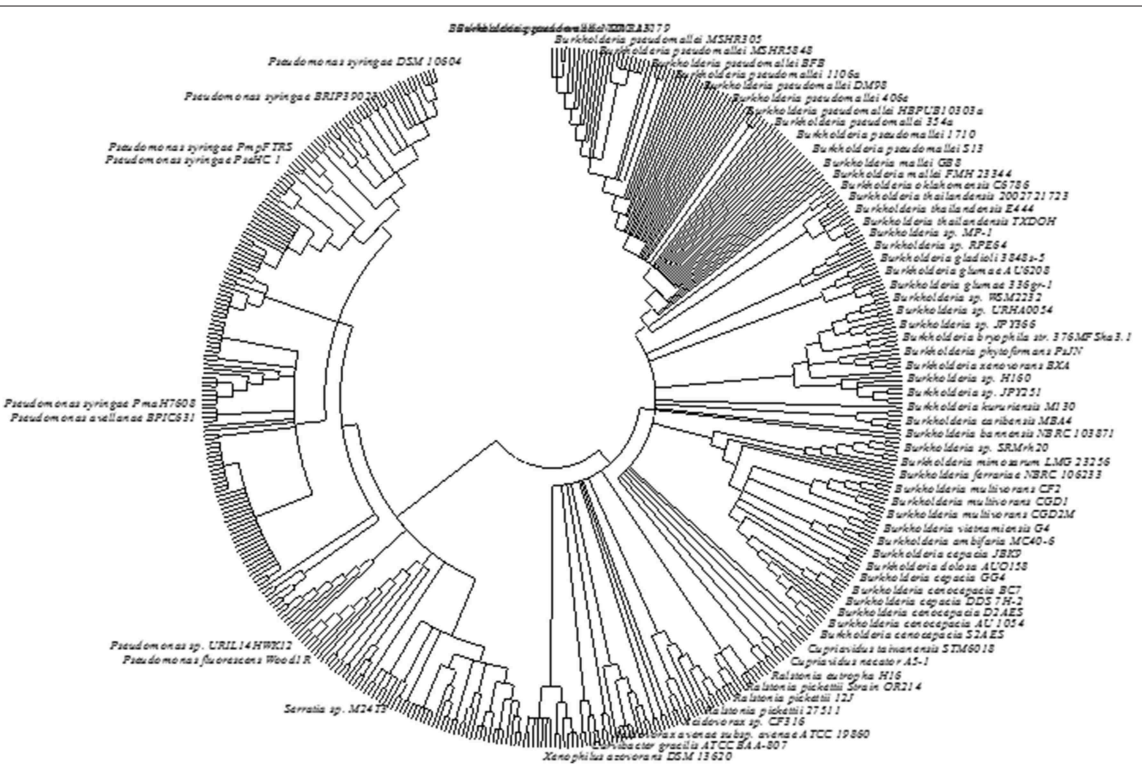

FIGURE 4 | The phylogentic tree constructed from *AcdS* gene sequence of different bacterial strains. The sequence data obtained from IMG database of JGI were used. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. The analysis involved 465 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 958 positions in the final dataset. Label of bacterial strains/species showing values lower than 1% was hidden. Evolutionary analyses were conducted in MEGA6.0.

Ecological Significance of ACC Deaminase Bacteria

The ACC deaminase activity is one of the most common traits among plant growth promoting rhizobacteria (Honma and Shimomura, 1978; Glick, 2005). ACCD bacteria exert its beneficial effect by protecting from the deleterious effect of environmental stressors (Glick, 2014), delay senescence (Ali et al., 2012), exhibit biocontrol activity against variety of phytopathogens in certain plants (Hao et al., 2011), and favor nodulation in legume plants (Nascimento et al., 2012a). Role and importance of bacterial ACCD in plant growth have been described in previous sections. Variation of ACC deaminase activity among microbial species at extreme environmental conditions might be useful in phytoremediation at unusual environmental sites or conditions (Glick, 2005). ACC deaminase bacteria assist associated plants in phytoremediation by biotransformation of toxic elements, rhizodegradation mediated by root exudates, and/or detoxification of heavy metals that allow host plants to survive under adverse conditions. Rhizospheric bacterial community with ACC deaminase can enhance the rate of rhizo-remediation by increasing the root system of the plant as well as increased access to soil by roots. It results in enhanced uptake of inorganic contaminants through modification of root architecture and root uptake system of the plant. Belimov et al. (2005) reported a positive correlation between the increment of bacterial ACC deaminase activity following the accumulation of cadmium in plant tissue and enhanced root growth. Similarly, Rodriguez et al. (2008) observed the enhanced growth of tobacco plants and substantial accumulation of metals from nickel contaminated soil following inoculation of *P. putida* HS-2.

The presence of ACC deaminase in human pathogenic *Burkholderia cenocepacia* J 2315 as well as in plant pathogenic fungi like *Aspergillus spp.* and *Myceliophthora thermophile* suggests that ACC deaminase might play a role in the ecological fitness of these micro-organisms. Role of ACC deaminase in endophytic fungi *P. citrinum* was investigated by Jia et al. (2000) who found accumulation of ACC during mycelial growth and subsequent degradation of ACC by ACC deaminase when the mycelial growth reached the maximum. Importance of ACCD in human pathogenic bacteria is not known, but its role in the pathogenesis of plant pathogens has been studied to some extent. For plant pathogenic microorganisms, it is assumed that production of ACC deaminase may help microbe to overcome ACC mediated plant responses. The presence of ACC deaminase bacteria in the close vicinity of fungal strains might have a role in increased fungal primordial proliferation by reducing ACC levels. Thus, an association of ACC deaminase bacteria plays a significant role in fungal colonization in the extreme soil. Additional advantage of these bacteria is the ability to degrade ACC providing extra nutrients to plant (Nascimento et al., 2014).

Transgenic Plants with ACC Deaminase Activity

The growth enhancement of plant by ACC deaminase bacteria has motivated scientists to transfer this gene into plants as future approach to minimize the deleterious effect of ethylene in plants subjected to adverse environmental conditions (Grichko and Glick, 2001; Robison et al., 2001; Nie et al., 2002). A transgenic Petunia hybrid with ACC deaminase gene maintains a significantly reduced amount of ACC in pollen cells (Lei et al., 1996). Similarly, the transgenic canola plants (*Brassica napus*) with ACC deaminase perform better growth under salinity stress compared to non-transgenic plant (Sergeeva et al., 2006). In a premier study, Reed et al. (1995) transformed two tomato cultivars with *AcdS* gene of *Pseudomonas chlororaphis* which resulted in lengthened duration of fruit ripening as well as significant reduction of stress ethylene production compared to parental line. Klee and Kishore (1992) also observed a significant reduction of ethylene production (77%) and delayed in senescence in tobacco and tomato plants transformed with bacterial *AcdS*. The tomato plant (*Lycopersicon esculentum*) expressing *AcdS* gene of *Pseudomonas* sp. 6GS exhibited reduced ethylene synthesis up to 90% (Klee et al., 1991). A large number of transgenic plants with foreign *AcdS* gene have been genetically engineered to reduce the deleterious ethylene levels in plants (Grichko and Glick, 2000; Robison et al., 2001; Sergeeva et al., 2006; Farwell et al., 2007; Zhang et al., 2008). However, there is a limited report of the performance of transgenic plant containing *AcdS* gene under field condition (Farwell et al., 2006, 2007). Furthermore, future research should focus on (i) field performance of transgenic plant, (ii) their survival and yield under diverse condition, and (iii) genetic re-arrangement for target gene identification for gene insertion and deletion.

Conclusion and Future Prospects

Increasing global warming and environmental pollution in the present scenario have highly affected the agricultural production which is confronted with induced stress generated by both biotic and abiotic factors. Bacteria with ACCD activity are able to mediate the enhanced resistance to biotic stressors and increased tolerance to abiotic stresses in their associated plants. Thus, these bacteria have the potential to promote plant growth under adverse environmental conditions. Therefore, it necessitates exploration of efficient PGPB with strong ACCD activity which can colonize plants effectively and increase plant productivity under actual farming conditions. ACCD is an inducible enzyme which expresses on the availability of ACC and regulated differentially by various physiological factors. This enzyme is tightly regulated. However, precise mechanisms of its regulation are understood only in few bacteria. Lack of information about the regulatory mechanism for expression of ACCD gene in the majority of organisms is a major constraint. The understanding regulatory circuit of ACCD gene will be helpful in the optimal exploitation of ACCD bacteria for enhancing plant productivity. Based on current knowledge, a very few plants like *Arabidopsis*, tomato, poplar have been reported to contain *AcdS* gene. Therefore, future research needs to explore *AcdS* gene in other plant species. The role of ACCD in the plant has been evident in fruit development in tomato and other plants. However, their direct role in stress is not well characterized. Therefore, distribution of ACCD gene in different plants and its possible role in stress amelioration needs to be investigated in detail. Development of transgenic plant overexpressing foreign *AcdS*

gene could be used to overcome stress ethylene generated through stress conditions. Moreover, the exact role of ACCD in bacteria also needs to be investigated in greater detail. The presence of ACCD gene in bacteria other than plant-associated bacteria intrigues its role and suggests the possibility for an alternative function.

Acknowledgments

We are grateful to Department of Biotechnology (File No. BT/PR14527/AGR/21/326/2010) Govt. of India, New Delhi for their support by providing the fund for carrying out the research work. We acknowledge Dr. Pankaj Sharma, Assistant Professor, Department of Biological Sciences, BITS Pilani, for reviewing and editing scientific content and English language of the text.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00937

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Microbiology

GENERAL COMMENTARY
published: 06 November 2015
doi: 10.3389/fmicb.2015.01255

Corrigendum: Biochemistry and genetics of ACC deaminase: a weapon to "stress ethylene" produced in plants

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Keywords: ACC, Acds, ethylene, abiotic stress, PGPR

A corrigendum on

Biochemistry and genetics of ACC deaminase: a weapon to "stress ethylene" produced in plants *by Singh, R. P., Shelke, G. M., Kumar, A., and Jha, P. N. (2015). Front. Microbiol. 6:937. doi: 10.3389/fmicb.2015.00937*

We mistakenly did not cite the reference Gontia-Mishra et al. (2014) in the text. Please find the following corrected paragraph with the given citation in the appropriate place. The full citation is also written below for inclusion in the reference list.

As reviewed in Gontia-Mishra et al. (2014), among eukaryotes, production of ACCD is well evident in some fungi, which include a few species of yeast such as *Hansenula saturnus* (Minami et al., 1998), *Issatchenkia occidentalis* (Palmer et al., 2007), other fungal species namely *Penicillium citrinum* and *Trichoderma asperellum*, and a *stramnopile, Phytophthora sojae* (Jia et al., 1999; Viterbo et al., 2010; Singh and Kashyap, 2012). Recently, ACCD activity has also been observed in certain plants such as *Arabidopsis thaliana*, poplar, and tomato plant (McDonnell et al., 2009; Plett et al., 2009).

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Microbiology

Received: *07 October 2015*
Accepted: *28 October 2015*
Published: *06 November 2015*

Citation:
Singh RP, Shelke GM, Kumar A and Jha PN (2015) Corrigendum: Biochemistry and genetics of ACC deaminase: a weapon to "stress ethylene" produced in plants. Front. Microbiol. 6:1255. doi: 10.3389/fmicb.2015.01255

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Viterbo, A., Landau, U., Kim, S., Chernin, L., and Chet, I. (2010). Characterization of ACC deaminase from the biocontrol and plant growth-promoting agent *Trichoderma asperellum* T203. *FEMS Microbiol. Lett*. 305, 42–48. doi: 10.1111/j.1574-6968.2010.01910.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 21 December 2015
doi: 10.3389/fmicb.2015.01436

Transplanting Soil Microbiomes Leads to Lasting Effects on Willow Growth, but not on the Rhizosphere Microbiome

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Plants interact closely with microbes, which are partly responsible for plant growth, health, and adaptation to stressful environments. Engineering the plant-associated microbiome could improve plant survival and performance in stressful environments such as contaminated soils. Here, willow cuttings were planted into highly petroleum-contaminated soils that had been gamma-irradiated and subjected to one of four treatments: inoculation with rhizosphere soil from a willow that grew well (LA) or sub-optimally (SM) in highly contaminated soils or with bulk soil in which the planted willow had died (DE) or no inoculation (CO). Samples were taken from the starting inoculum, at the beginning of the experiment (T0) and after 100 days of growth (TF). Short hypervariable regions of archaeal/bacterial 16S rRNA genes and the fungal ITS region were amplified from soil DNA extracts and sequenced on the Illumina MiSeq. Willow growth was monitored throughout the experiment, and plant biomass was measured at TF. CO willows were significantly smaller throughout the experiment, while DE willows were the largest at TF. Microbiomes of different treatments were divergent at T0, but for most samples, had converged on highly similar communities by TF. Willow biomass was more strongly linked to overall microbial community structure at T0 than to microbial community structure at TF, and the relative abundance of many genera at T0 was significantly correlated to final willow root and shoot biomass. Although microbial communities had mostly converged at TF, lasting differences in willow growth were observed, probably linked to differences in T0 microbial communities.

Keywords: willow, microbiome engineering, phytoremediation, microbiome transplantation, contaminated soils

OPEN ACCESS

Edited by:
Ying Ma,
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Reviewed by:
Raffaella Balestrini,
Consiglio Nazionale Delle Ricerche, Italy
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Microbiology

Received: *11 June 2015*
Accepted: *01 December 2015*
Published: *21 December 2015*

Citation:
Yergeau E, Bell TH, Champagne J, Maynard C, Tardif S, Tremblay J and Greer CW (2015) Transplanting Soil Microbiomes Leads to Lasting Effects on Willow Growth, but not on the Rhizosphere Microbiome. Front. Microbiol. 6:1436. doi: 10.3389/fmicb.2015.01436

INTRODUCTION

Microorganisms colonize all plant components, and plants interact constantly with this complex microbiome. Between 5 and 20% of a plant's photosynthetic yield is transferred to its microbiome, and this occurs mainly through the roots (Marschner, 1995). As a result of this transfer, the rhizosphere supports much higher bacterial abundance and activity, not only when compared to other plant compartments, but also relative to bulk soil (Smalla et al., 2001; Kowalchuk et al., 2002). However, bacterial diversity in the rhizosphere is generally lower than is observed in bulk

soil (Marilley and Aragno, 1999) while microbial community composition is distinct (Smalla et al., 2001; Kowalchuk et al., 2002; Griffiths et al., 2006; Kielak et al., 2008; Bulgarelli et al., 2012; Peiffer et al., 2013), suggesting a strongly selective environment in the rhizosphere. This selection pressure often varies between plant species (Haichar et al., 2008; Berg and Smalla, 2009) and even genotypes (Lundberg et al., 2012; Sugiyama et al., 2012). This selection pressure results from the exudation of specialized antimicrobials (e.g., flavonoids, salicylic acid, phytoalexins), or compounds that provide carbon (e.g., organic acids, aromatic compounds) and/or nitrogen (e.g., amino acids) to microbes (Badri et al., 2009). An emerging view in the microbial ecology of microbe–host systems is that the host and its microbial inhabitants are an inseparable entity, and actually function as a meta-organism or a holobiont (Bosch and McFall-Ngai, 2011; Vandenkoornhuyse et al., 2015). Interactions between plants and microbes have evolved over millions of years, and these relationships allow the plant–microbe meta-organism to minimize overall stress by, among other mechanisms, deterring pathogens (St-Arnaud and Vujanovic, 2007; Sikes et al., 2009; Mendes et al., 2011), increasing N and P uptake (Richardson et al., 2009), protecting against abiotic stress (Marasco et al., 2012; Selvakumar et al., 2012), and detoxifying the environment (Siciliano et al., 2001). Because of these intricate links, engineering of the plant host without considering the microbiome likely limits the phenotypic optimum that can be achieved (Bell et al., 2014b; El Amrani et al., 2015; Quiza et al., 2015).

Depending on its composition and activity, the plant microbiome can be either beneficial or deleterious to plant health, and shifting this delicate balance has huge implications for plant productivity. Several authors have suggested that optimizing the plant microbiome is a possible solution to the shortage of food on the planet (Morrissey et al., 2004; Glick, 2014). Manipulating the plant microbiome has the potential to reduce the incidence of plant disease (Andrews, 1992; Bloemberg and Lugtenberg, 2001), increase agricultural production (Bakker et al., 2012), reduce the need for chemical inputs (Adesemoye et al., 2009), reduce greenhouse gas emissions (Singh et al., 2010), and increase plant-mediated removal of pollutants (Bell et al., 2014b). One approach to soil microbiome engineering is the use of blanket treatments (e.g., fertilization) to stimulate the whole microbial community, but this may lead to the stimulation of microbes that do not optimally perform targeted functions (Bell et al., 2011). Another is to introduce microorganisms to soil that are capable of performing the desired functions (i.e., bioaugmentation), like polychlorinated biphenyl- (Secher et al., 2013), polycyclic aromatic hydrocarbon- (Baneshi et al., 2014), and diesel- (Chuluun et al., 2014) degradation. However, the abundance and functional diversity of indigenous soil microbes allows them to occupy most available ecological niches, and so attempts to introduce new microorganisms have been met with limited success (Thompson et al., 2005; Gerhardt et al., 2009). Instead, disrupting microbial communities by removing specific taxonomic groups or reducing the overall microbial load may open niches for microbial colonization. Specific inhibitors like antibiotics and fungicides have been used to disrupt soil microbial communities and promote specific functions of interest (Bell et al., 2013; Qiu et al., 2014). For instance, Bell et al. (2013) used two antibiotics to inhibit specific microbial groups in diesel-contaminated soils, and found that using the two antibiotics in combination in nutrient-amended soils resulted in higher diesel degradation rates than controls or soils treated with only one antibiotic. In another study, Qiu et al. (2014) used fungicides in the rhizosphere of cucumber, which resulted in a higher incidence of disease when a pathogen was inoculated, but reduced disease incidence and increased plant growth when the pathogen was inoculated along with an antagonist bacteria. Although, the feasibility and ethics of using such approaches for modifying soil microbiomes in the field is debatable, these studies suggest potential mechanisms by which complex microbiomes can be modified. Other studies demonstrated that inoculation with microbial consortia was a more effective approach than single strain inoculation, as microorganisms appear to work synergistically to efficiently degrade petroleum hydrocarbon contaminants (Alarcón et al., 2008; Afzal et al., 2012). Further factors complicating efforts to engineer plant microbiomes include differences in the physiology and ecology of soil inhabitants, resulting in differential responses of bacterial and fungal activity, growth, and diversity to key rhizosphere parameters like pH (Rousk et al., 2009, 2010) and plant identity (Haichar et al., 2008; Berg and Smalla, 2009).

Willows (*Salix* spp.) have been used as model plants for phytoremediation, as they rapidly produce high amounts of biomass, including an extensive root system capable of stimulating soil microbial communities. One of the keys to effective phytoremediation with willows is the optimization of growth, biomass production, and survival in highly contaminated environments. The goal of the present study was to observe whether a complex microbiome could be transferred from one plant to another, and whether this also transferred certain characteristics of the original plant (growth, biomass production, and survival in a stressful environment). In other words, how much of the plant phenotype is related to the root-associated microbiome? Clonal willow clippings were planted for two generations in soil originating from a hydrocarbon-contaminated field site. First generation willows were planted into the unmodified soil, and soils associated with willows that showed dramatically different growth characteristics were harvested and used to inoculate gamma-irradiated soil from the same site. A second generation of willows was planted into these inoculated soils. We hypothesized that inoculation with the rhizosphere soil of large first-generation willows would result in larger second-generation willows with lower mortality than when inoculating with soil associated with smaller or dying first-generation plants.

MATERIALS AND METHODS

Soil Inoculum

Soil was retrieved from an experiment in which clonal willows (*Salix purpurea* "Fish Creek") were planted into a homogenized highly petroleum-contaminated soil (C10–C50 concentration: 17,500 mg/kg). Most of the introduced willows died; out of 100

initial plants, only 11 were alive after 173 days. The rhizosphere of a large surviving willow (height of 128 cm, shoot fresh weight of 62.00 g, used to inoculate the LA treatment), the rhizosphere of a small surviving willow (height of 80 cm, shoot fresh weight of 43.46 g, used to inoculate the SM treatment) and the bulk soil from a pot in which the willow had died (used to inoculate the DE treatment), were harvested on 21 October 2013 by collecting the soil that remained attached to the root system after vigorously shaking the willows (for the rhizosphere) or by taking a surface soil sample in the middle of the pot (for the bulk soil). These soils represent the three different soil inocula used in subsequent experiments. Soils were transported at 4°C and frozen at −20°C until used for downstream steps.

Experimental Design

Fresh soil was collected at the site of a former petrochemical plant in Varennes, Quebec, Canada, within 2 m of the excavation site of the contaminated soil described above. Soil was mixed thoroughly, transferred in 20 L pails and sent to Nordion (Laval, Quebec, Canada) for gamma irradiation at a dose of 50 kGy, to disrupt the microbiome and minimize the soil microbial load. Following a previous identical irradiation of the same soil, no cultivable microorganisms could be retrieved from the soil (T.H. Bell, unpublished observations), even though bacterial, archaeal, and fungal DNA could be amplified. For each treatment type (DE, LA, and SM treatments), ∼10 kg of irradiated soil was mixed with 1 kg of the different soil inocula and distributed into ten 1 L pots. A control treatment was left uninoculated (CO treatment), resulting in four treatments with 10 replicate pots each. A willow clipping (*S. purpurea* "Fish Creek") was planted to a depth of 10 cm in the middle of each pot. Willow clippings were also planted in 10 pots filled with potting soil to evaluate willow growth under ideal conditions. Pots were placed in a greenhouse (February 18, 2014), and were incubated at a temperature of 20°C during the day and 18°C overnight. High-pressure sodium lamps (430 W) were illuminated for 18 h a day, starting at 6:00 a.m. The position of the pots on the greenhouse bench was determined using a random number generator.

Sampling

Each inoculum type (three samples) was sampled before mixing with irradiated soil and soils from the pots were sampled before planting with willow clippings (February 17, 2014, 4 treatments × 10 replicates = 40 samples at T0). Willow growth was measured on March 21, 2014 (32 days after planting), on April 17, 2014 (59 days after planting), and on May 23, 2014 (95 days after planting). Rhizosphere soils were sampled at the end of the experiment (May 28, 2014, after 100 days, 4 treatments × 10 replicates = 40 samples at TF) by collecting the soil that remained attached to the root system after vigorously shaking the willows. Willow roots and shoots (excluding the original willow cuttings) were also harvested at the end of the experiment, dried at 105°C overnight, and weighed.

DNA Extraction, Amplification, and Sequencing

For the 83 samples, (3 inocula, 4 treatments × 10 replicates at T0 and 4 treatments × 10 replicates at TF), DNA was extracted from an average of 0.352 g of soil using the MoBio Power Soil DNA extraction kit resulting in an average of 1.80 μg of DNA per g of soil at T0 and 5.14 μg of DNA per g of soil at TF (**Figure 1**). Libraries for sequencing were prepared according to Illumina's "16S Metagenomic Sequencing Library Preparation" guide (Part # 15044223 Rev. B), with the exception of using Qiagen HotStar MasterMix for the first PCR ("amplicon PCR") and halving reagents volumes for the second PCR ("index PCR"). The template specific primers were (without the overhang adapter sequence): (1) Archaea 16S rRNA gene: Arch516F (5′-TGYCAGCCGCCGCGGTAAHACCVGC-3′) and A806R (5′-GGACTACVSGGGTATCTAAT-3′), (2) bacterial 16S rRNA gene: F343 (5′-TACGGRAGGCAGCAG-3′) and R803 (5′-CTACCAGGGTATCTAATCC-3′), and (3) fungal internal transcribed spacer (ITS): ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and 5.8A2R (5′-CTGCGTTCTTCATCGAT-3′). The first PCR ("amplicon PCR") was carried out for 30 (bacterial 16S) or 35 (archaeal 16S and fungal ITS) cycles with annealing temperatures of 55°C (archaeal 16S and bacterial 16S) or of 45°C (fungal ITS). Diluted pooled samples were then loaded on an Illumina MiSeq and sequenced using a 300-cycles MiSeq Reagent Kit v2 (Archaeal 16S) or a 600-cycles MiSeq Reagent Kit v3 (bacterial 16S and fungal ITS).

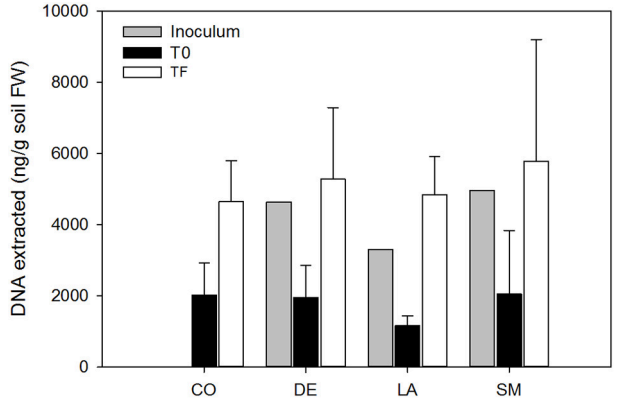

FIGURE 1 | DNA yields from bulk and willow rhizosphere soil samples taken at T0 and TF (after 100 days) for CO, DE, SM, and LA treatments. Error bars represent standard deviation.

Sequence Data Treatment

Sequences were analyzed through our internal rRNA short amplicon analysis pipeline (Tremblay et al., 2015). Common sequence contaminants (i.e., Illumina adapters and PhiX spike-in reads) were first removed from raw sequences using a kmer matching tool (DUK; http://duk.sourceforge.net/). Filtered reads were assembled with the FLASH software (Magoč and Salzberg, 2011). Using in-house Perl scripts, assembled amplicons were then trimmed to remove forward and reverse primer sequences that might be included in some reads. Paired-end assembled amplicons were then filtered for quality: sequences having more than 1 N, an average quality score lower than 30, or more than 10 nucleotides having a quality score lower than 10 were rejected.

OTU generation was done using a three step clustering pipeline. Briefly, quality controlled sequences were dereplicated

at 100% identity. These 100% identity clustered reads were then denoized at 99% identity using USEARCH (Edgar, 2010). Clusters of less than three reads were discarded and remaining clusters were scanned for chimeras using UCHIME *de novo* followed by UCHIME reference using the Broad's Institute 16S rRNA Gold reference database. Remaining clusters were clustered at 97% identity (USEARCH) to produce OTUs; data were then rarefied to 1000 reads.

Taxonomy assignment of resulting bacterial and archaeal OTUs was performed using the RDP classifier with a modified Greengenes training set built from a concatenation of the Greengenes database (version 13_5 maintained by Second Genome), Silva eukaryotes 18S r118 and a selection of chloroplast and mitochondrial rRNA sequences. ITS organisms were classified using the ITS Unite database (version: sh_qiime_release_13.05.2014). Hierarchical tree files were generated with in-house Perl scripts and used to generate training sets using the RDP classifier (v2.5) training set generator's functionality (Wang et al., 2007). With taxonomic lineages in hand, OTU tables were generated and rarefied to 1000 reads. These OTU tables were used for downstream analysis.

Diversity metrics were obtained by aligning OTU sequences on a Greengenes core reference alignment (DeSantis et al., 2006) using the PyNAST aligner (Caporaso et al., 2010). Alignments were filtered to keep only the V4, V7–V8, or V6–V8 part of the alignment. A phylogenetic tree was built from alignment with FastTree (Price et al., 2010). Alpha (observed species) and beta (weighted or unweighted UniFrac and Bray–Curtis distances) diversity metrics and taxonomic classifications were computed using the QIIME software suite (Caporaso et al., 2010; Kuczynski et al., 2010).

Statistical Analyses

All statistical analyses were carried out in R v3.0.2 (R Core Team, 2013). Analysis of variance (ANOVA) and repeated-measures ANOVA was performed using the "aov" function, Spearman rank-order correlation analyses were performed using the "cor.test" function, Permanova was performed using the "adonis" function of the vegan library, and principal coordinate analyses were performed using the "cmdscale" function of the vegan library based on the Bray–Curtis distance calculated from the OTU matrix using the "vegdist" function of the vegan library. Since biomass could only be measured at TF, correlations and permanova analyses were carried out against the relative abundance of genera at TF, but also at T0 to evaluate whether the relative abundance of a genus, the overall community structure, or microbial diversity at T0 could be related to willow growth at TF.

Data Deposition

Raw sequence data produced in this study was deposited in NCBI under the BioProject accession PRJNA301462.

RESULTS

Willow Growth and Soil DNA Yields

All 40 willows planted in the contaminated soil survived over the 100 days of the experiment. However, CO willows showed delayed growth, and were smaller throughout the experiment than those that had been inoculated (**Figure 2A**). DE willows had significantly longer stems ($P < 0.05$) than the CO willows throughout the experiment, while LA willows were only significantly taller ($P < 0.05$) at days 59 and 95, and SM willows treatment were only significantly taller ($P < 0.05$) at day 59 (**Figure 2A**). Furthermore, at day 59, the stems of DE willows were significantly longer ($P < 0.05$) than those from SM willows (**Figure 2A**). At the end of the experiment (TF), there was a significant effect ($P < 0.05$) of inoculation on shoot and root biomass, with significant differences ($P < 0.05$) between the non-inoculated controls (CO) and the inoculated treatments (DE, SM, and LA; **Figure 2B**). Within the inoculated treatments, the DE willows produced significantly more shoot biomass ($P < 0.05$), while root biomass production was comparable across the three inoculation treatments (**Figure 2B**). Willows planted in parallel in non-contaminated potting soil were on average 96.6 cm high, with an average root biomass of 2.81 g and an average shoot biomass of 14.92 g at TF. DNA yields from soil were on average 1.80 μg per g soil for T0 soils, 4.30 μg per g soil for the three

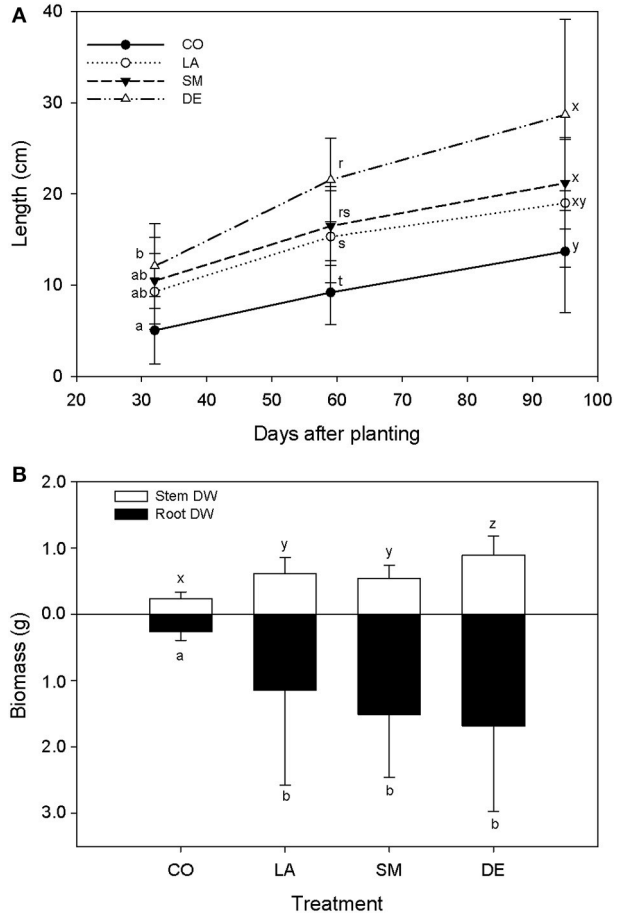

FIGURE 2 | Willow growth throughout the experiment (A) and willow biomass at TF (B) for CO, DE, SM, and LA treatments. Error bars represent standard deviation.

inocula, and 5.14 μg per g soil for TF soils (**Figure 1**). There was a significant difference in DNA yields between T0 and TF samples (repeated-measure ANOVA: $F = 89.84$, $P < 0.001$), but no significant differences were observed between treatments or for the interaction term ($P > 0.05$).

Archaeal Community

At the order level, the archaeal community was very different across inoculum types, with a dominance of the E2 group in the DE inoculum, the *Methanosarcinales* in the LA inoculum, and the *Nitrososphaerales* in the SM inoculum (**Figure 3A**). At T0 (after mixing the inocula with the irradiated soil), the three inoculated treatments were more similar, being co-dominated by *Nitrososphaerales, Methanobacteriales*, E2 group, *Methanosarcinales,* and *Methanomicrobiales* (**Figure 3A**). At TF, the CO soils did not differ markedly from their T0 counterparts, while the DE and LA soils showed an increased dominance of the *Methanosarcinales* and the SM soils showed increased dominance by the *Methanocellales* (**Figure 3A**). The ordination resulting from principal coordinate analysis of Bray–Curtis distances based on OTU relative abundance showed high variability within each of the treatments, especially within the LA and SM rhizospheric soils, while communities from the CO treatment generally clustered together (**Figure 3B**). However, inoculation appeared to have some influence, as the TF inoculated samples generally clustered on the left side of the ordination, and for the SM and LA treatments, the TF samples mostly clustered toward their initial inoculum (**Figure 3B**). Time and treatment had similar effects (similar *F*-ratios) in permanova tests (**Figure 3B**), and when separating T0 and TF samples, the effect of treatment was stronger at TF (**Table 1**). Permanova tests also revealed significant relationships between shoot biomass and archaeal community structure. A slightly stronger link was observed between shoot biomass and the TF community (higher *F*-ratio) (**Table 1**). Diversity was lower in the DE and LA inocula when compared to the SM inoculum (**Figure 3C**). Repeated-measure ANOVA tests demonstrated that archaeal diversity was significantly influenced by treatment type, an effect that was mainly driven by significant differences between the CO, SM, and LA treatments (**Figure 3C**). There was also a significant effect of time on archaeal diversity, with lower diversity in TF samples for all treatments (**Figure 3C**). The initial archaeal diversity (at T0) was significantly correlated with shoot biomass ($r_s = 0.321$, $P = 0.049$), but not root biomass, and no correlations were significant for diversity at TF. Some of the archaeal genera identified at T0 or TF had significant positive or negative correlations with willow biomass (**Table 2**). The relative abundance of *Methanosarcina* at T0 was significantly and positively correlated to root and shoot biomass, while its relative abundance at TF was significantly and positively correlated to root biomass (**Table 2**). Other genera also showed significant correlations with shoot and root biomass and are listed in **Table 2**.

Bacterial Community

The bacterial inocula showed marked differences, with the inocula originating from rhizospheric soil (LA and SM treatments) dominated by *Alpha-*, *Beta-*, and *Gammaproteobacteria*, while the inoculum originating from bulk soil (DE treatment) was dominated by *Bacteroidetes*, with the *Firmicutes*, *Alpha-*, *Beta-*, and *Gammaproteobacteria* present at moderate abundance (**Figure 4A**). The bacterial communities remained variable at T0, with a large dominance of *Firmicutes* in the CO treatment and a dominance of *Proteobacteria* (mainly *Gammaproteobacteria*) in the inoculated treatments (DE, SM, and LA; **Figure 4A**). After 100 days of growth (TF), the bacterial community composition of the willow rhizosphere was remarkably similar between all treatments, with a co-dominance of *Beta-* and *Gammaproteobacteria* (**Figure 4A**). This convergence of the bacterial communities at TF was also visible in the PCoA ordination of Bray–Curtis distances, in which the communities are dispersed at T0 and much more similar at TF (**Figure 4B**). Bacterial communities in the willow rhizosphere at TF were not especially similar to the bacterial communities of the initial inocula (**Figure 4B**). Permanova showed that time was the major factor leading to differences in bacterial composition (highest *F*-ratio), but there were also highly significant effects of the treatments and of the interaction term. When separating the T0 and TF samples, the effect of treatment was significant for both datasets, although the *F*-ratio was much larger for the T0 dataset (**Table 1**). This was also visible in the ordinations. There was also a significant relationship between the bacterial community structure at T0 and TF and root and shoot biomass in permanova tests, with a stronger effect for T0 (higher *F*-ratios; **Table 1**). Bacterial diversity was significantly affected by time, treatment, and the interaction term (**Figure 4C**). Diversity was largest in the CO and DE treatments at T0 and was at its lowest in the CO rhizosphere at TF (**Figure 4C**). Bacterial diversity at T0 was not significantly correlated to root and shoot biomass, but significant correlations were observed at TF between bacterial diversity, shoot biomass ($r_s = 0.650$, $P < 0.001$), and root biomass ($r_s = 0.669$, $P < 0.001$). A variety of bacterial genera showed significant correlations with root and shoot biomass, and the top 10 strongest positive and negative correlations are presented in **Table 3**. Some of the correlations were very strong, with *P*-values well below 1×10^{-5}. Among the most significant positive correlations, many of the identified taxa have previously been reported to be associated with plants.

Fungal Community

The DE inoculum differed markedly from the rhizospheric inocula (LA and SM), harboring relatively more *Sordariomycetes*, *Dothideomycetes*, *Chytridiomycetes,* and *Zygomycota*, and relatively less *Agaricomycetes* and *Pezizomycetes* (**Figure 5A**). Differences between treatments were also visible at T0 and TF, with the CO and DE treatments differing substantially from the LA and SM treatments (**Figure 5A**). Large differences in the dominant class were visible between sampling points and treatments, with the *Agaricomycetes*, *Dothideomycetes*, *Pezizomycetes*, *Sordariomycetes*, *Tremellomycetes,* and *Zygomycota* dominating or co-dominating the various treatments (**Figure 5A**). In the ordination based on principal coordinates analysis of Bray–Curtis distances of OTU tables, a similar story emerged (**Figure 5B**). At T0, the four treatments were clearly distinct in the ordination space, with a few outliers

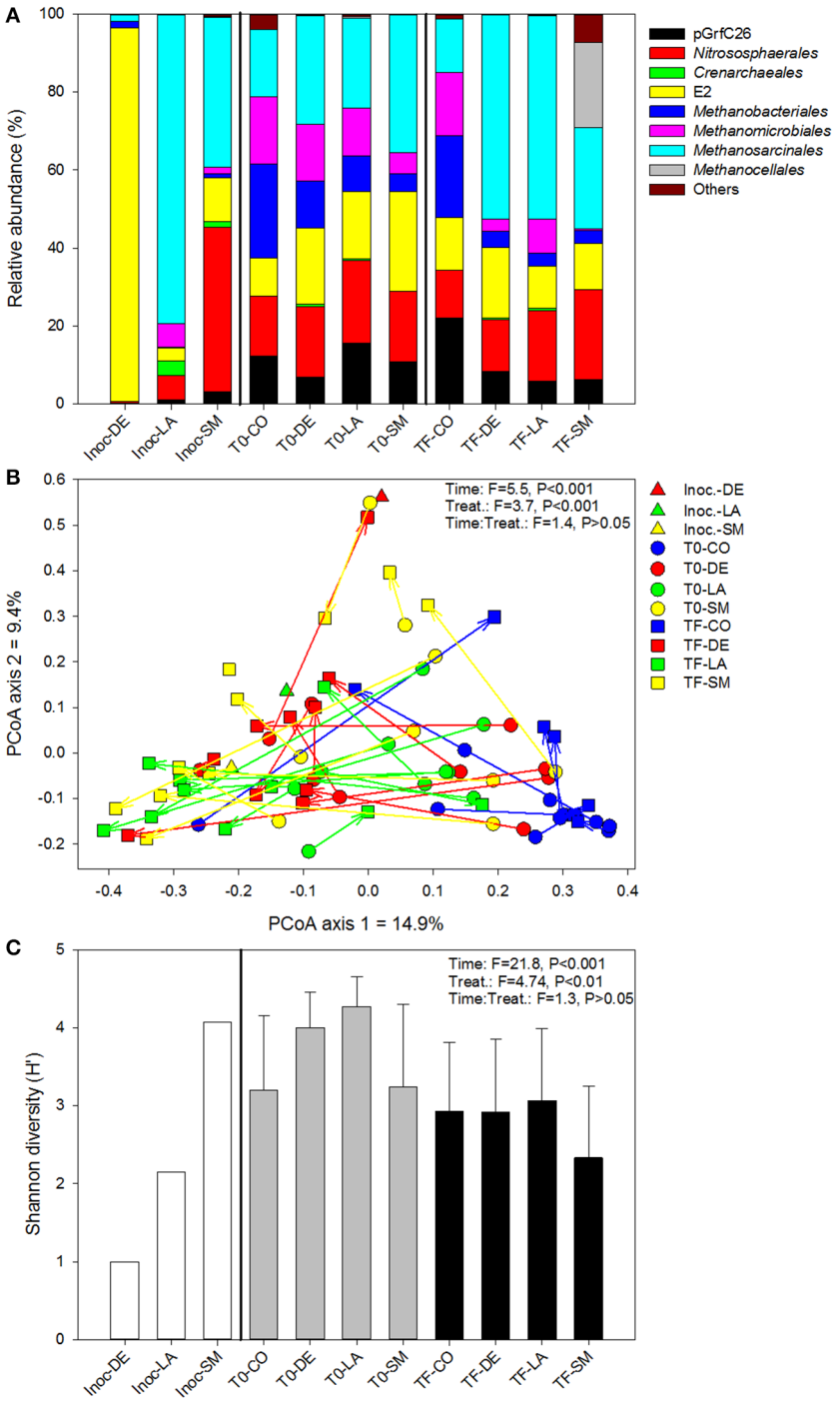

FIGURE 3 | Archaeal community composition (A), structure (B), and diversity (C) for bulk and willow rhizosphere soil samples taken at T0 and TF (after 100 days) for CO, DE, SM, and LA treatments and the original inocula. Vectors are linking samples taken from the same pot at different time points. Error bars represent standard deviation.

(**Figure 5B**). The rhizospheric (SM and LA) inocula and the DE inoculum were also clearly separated in the ordination space (**Figure 5B**). Some of the fungal communities at TF converged toward their respective inocula, with 5/10 samples for the SM treatment, 5/10 samples for the LA treatment, and 10/10 samples for the DE treatment (**Figure 5B**). The LA and SM samples that did not converge toward their initial inoculum and all the DE samples grouped together with the CO samples

TABLE 1 | Permanova analysis.

Time	Factor	*F*-ratio	*P*-values
ARCHAEA			
T0	Treatment	2.18	0.001
T0	Root biomass	1.73	0.065
T0	Shoot biomass	2.49	0.009
TF	Treatment	3.62	0.001
TF	Root biomass	1.45	0.185
TF	Shoot biomass	3.05	0.017
BACTERIA			
T0	Treatment	17.61	0.001
T0	Root biomass	4.71	0.006
T0	Shoot biomass	8.50	0.002
TF	Treatment	4.35	0.001
TF	Root biomass	3.03	0.004
TF	Shoot biomass	4.07	0.001
FUNGI			
T0	Treatment	7.40	0.001
T0	Root biomass	2.33	0.011
T0	Shoot biomass	4.45	0.001
TF	Treatment	2.87	0.001
TF	Root biomass	1.73	0.012
TF	Shoot biomass	2.72	0.001

TABLE 2 | Significant Spearman correlations between the relative abundance of archaeal genera and root or shoot biomass.

Genus	Time	r_S	*P*-values
ROOT BIOMASS—POSITIVE			
Methanosarcina	T0	0.400	0.0127
SAGMA group	TF	0.369	0.0228
Methanosarcina	TF	0.359	0.0268
ROOT BIOMASS—NEGATIVE			
Unid. *Methanomicrobiales*	TF	−0.519	0.0008
Methanobacterium	TF	−0.399	0.0132
pGrfC26	T0	−0.323	0.0476
SHOOT BIOMASS—POSITIVE			
SAGMA group	TF	0.406	0.0114
Methanosarcina	T0	0.381	0.0183
MCGCL group	T0	0.375	0.0204
Methanomassiliicoccus	TF	0.350	0.0312
SHOOT BIOMASS—NEGATIVE			
Unid. *Methanomicrobiales*	TF	−0.515	0.0009
Methanobacterium	T0	−0.390	0.0154
Unid. *Methanomicrobiales*	T0	−0.342	0.0354

Unid., unidentified; OTU in the Greengenes database that was not identified at the genus level. The next lowest taxonomical level for which the OTU was identified is given.

at TF (**Figure 5B**). The CO treatment did not change much through the course of the experiment and samples from T0 and TF were located together in the ordination space (**Figure 5B**). The large effect of the inoculation treatments resulted in a smaller difference between the *F*-ratio for the effect of time and treatment in permanova tests as compared to bacteria and archaea. Separate permanova tests for the effect of treatment on T0 and TF communities revealed highly significant effects, with stronger effects (higher *F*-ratio) for the T0 communities (**Table 1**). Similarly, the relationship between root and shoot biomass and fungal community structure was stronger (higher *F*-ratio) for T0 communities than TF communities (**Table 1**). In terms of diversity, there was a significant effect of time, with significantly higher diversity in T0 samples than TF samples for all treatments (**Figure 5C**). The interaction term was also significant in ANOVA tests, which was due to the fact that the differences in diversity between treatments observed at T0 were no longer visible at TF (**Figure 5C**). Fungal diversity at T0 was not significantly correlated with root or shoot biomass ($P > 0.05$), but there was a significant negative correlation between fungal diversity at TF and shoot biomass ($r_s = -0.322$, $P = 0.045$). The relative abundances of individual genera were also tested for correlation with willow biomass, and the 10 strongest positive and negative correlations are reported in **Table 4**. Most of the strongest positive correlations with willow biomass were fungal genera at T0, while the strongest negative correlations were with fungal genera at TF or T0 (**Table 4**). *Sphaerosporella* showed a particular pattern at TF; it was nearly absent in most samples (0–2.7%), but extremely abundant (58.2–93.7%) in the rhizosphere of the four willows that showed the highest shoot biomass (all from the DE treatment). This resulted in a significant positive Spearman correlation with shoot biomass (**Table 4**).

DISCUSSION

The microbiome of contaminated soils was successfully modified by gamma-irradiation followed by the introduction of various soil inocula. Bacterial and fungal communities from the four treatments were clearly distinct at the beginning of the experiment (T0), with respect to both microbial community composition and diversity. However, after 100 days of willow growth (TF), the original differences were not visible for most treatments, with the exception of the fungal communities for some samples. This convergence of the willow rhizosphere microbiome at TF suggests that the willow rapidly exerts strong selective pressures in the rhizosphere, selecting for a similar microbiome from variable starting microbiomes. This strong selective environment has been reported for other plant species, and resulted in sharp contrasts between the microbial community composition of the rhizosphere and adjacent bulk soil (Smalla et al., 2001; Kowalchuk et al., 2002; Griffiths et al., 2006; Kielak et al., 2008; Bulgarelli et al., 2012; Peiffer et al., 2013). This selective pressure often varies between plant species (Haichar et al., 2008; Berg and Smalla, 2009) and even genotypes (Lundberg et al., 2012; Sugiyama et al., 2012). Here, we observed a relatively low variability in microbiome composition between individual willows possibly because we used a clonal population of willows. This could partly explain the striking convergence in willow rhizosphere communities at TF. For willows planted in contaminated soils, this selection pressure was previously shown to result in an increased expression of

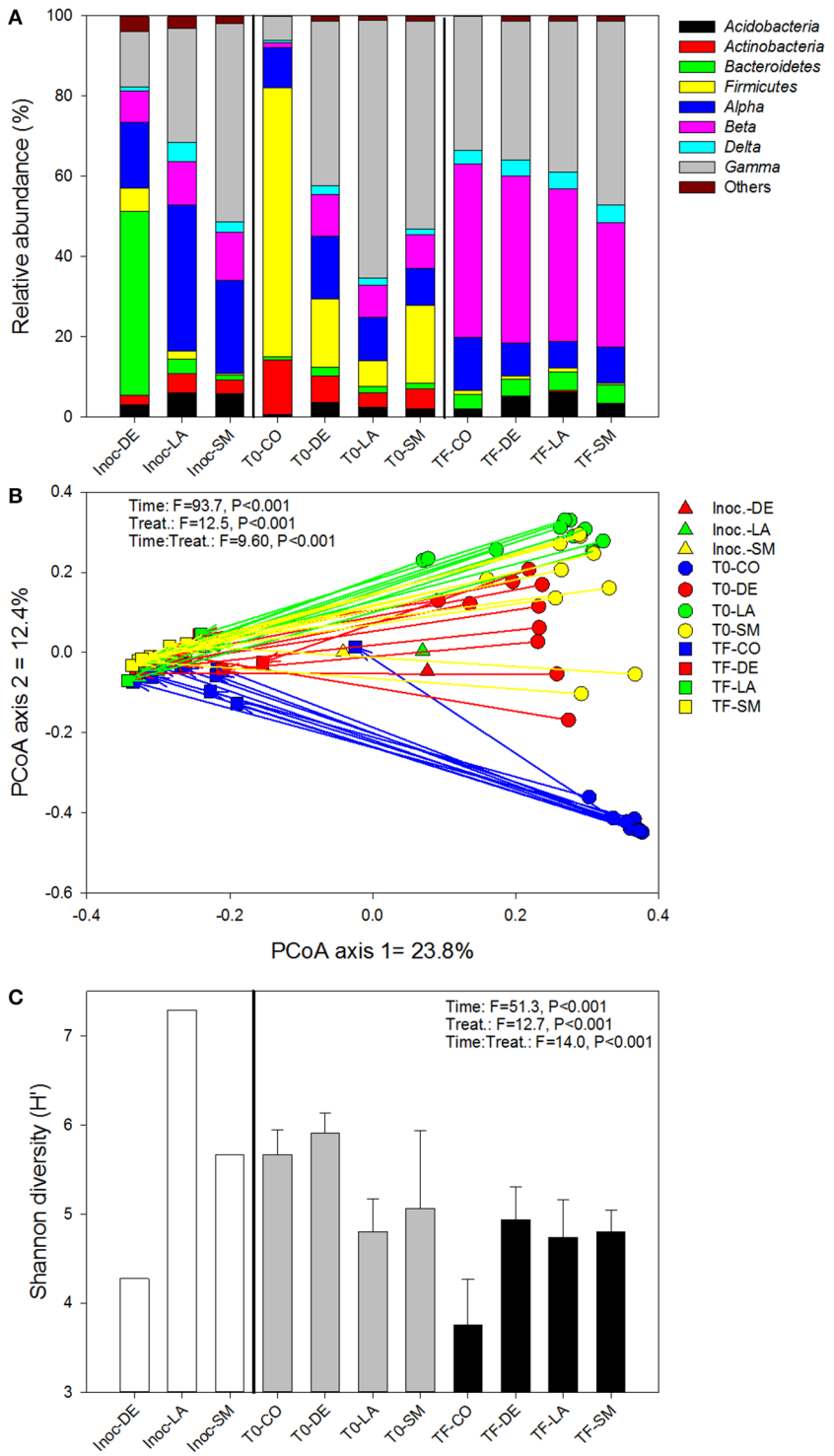

FIGURE 4 | Bacterial community composition (A), structure (B), and diversity (C) for bulk and willow rhizosphere soil samples taken at T0 and TF (after 100 days) for CO, DE, SM, and LA treatments and the original inocula. Vectors are linking samples taken from the same pot at different time points. Error bars represent standard deviation.

microbial genes related to the degradation of hydrocarbons, as well as large shifts in the active microbial community relative to willows planted in non-contaminated soil or contaminated bulk soil (Yergeau et al., 2014; Pagé et al., 2015). Because of this overwhelming rhizosphere effect, the inoculation of a pre-selected microbiome was only effective in the short term,

TABLE 3 | Top 10 most significant Spearman correlations between bacterial genera relative abundance and root or shoot biomass.

Genus	Time	r_S	*P*-values
ROOT BIOMASS—POSITIVE			
Unid. *Alphaproteobacteria*	T0	0.738	5.44×10^{-8}
Unid. *Cytophagaceae*	TF	0.693	7.20×10^{-7}
Unid. *Oxalobacteraceae*	T0	0.658	4.03×10^{-6}
Unid. *Alcaligenaceae*	T0	0.634	1.14×10^{-5}
Aquicella	T0	0.593	5.49×10^{-5}
Unid. *Sinobacteraceae*	TF	0.580	8.83×10^{-5}
Unid. *Ellin329*	TF	0.572	0.000114
Unid. *Betaproteobacteria*	TF	0.568	0.000131
Unid. *DS.18*	T0	0.554	0.000208
Unid. *Alphaproteobacteria*	TF	0.553	0.000217
ROOT BIOMASS—NEGATIVE			
Unid. *OPB41*	T0	−0.585	7.45×10^{-5}
Unid. *Bacillales*	T0	−0.578	9.23×10^{-5}
Unid. *Solirubrobacterales*	T0	−0.568	0.000130
Cohnella	T0	−0.549	0.000243
Pseudoxanthomonas	TF	−0.548	0.000252
Brevibacillus	T0	−0.540	0.000326
Pedobacter	TF	−0.507	0.000835
Ammoniphilus	T0	−0.502	0.000966
Paenibacillus	T0	−0.502	0.000976
Sporosarcina	T0	−0.500	0.00102
SHOOT BIOMASS—POSITIVE			
HB2.32.21	TF	0.683	1.20×10^{-6}
Unid. *Xanthomonadaceae*	T0	0.678	1.56×10^{-6}
Unid. *Betaproteobacteria*	TF	0.667	2.57×10^{-6}
Unid. *Coxiellaceae*	T0	0.635	1.06×10^{-5}
Unid. *Alphaproteobacteria*	T0	0.621	1.94×10^{-5}
Unid. *Ellin6067*	TF	0.605	3.59×10^{-5}
Unid. *Comamonadaceae*	T0	0.590	6.17×10^{-5}
Aquicella	T0	0.589	6.48×10^{-5}
Unid. *Solibacteraceae*	T0	0.585	7.47×10^{-5}
Leptothrix	TF	0.584	7.68×10^{-5}
SHOOT BIOMASS—NEGATIVE			
Unid. *Bacillales*	T0	−0.627	1.51×10^{-5}
Unid. *Peptostreptococcaceae*	T0	−0.597	4.75×10^{-5}
Unid. *Planococcaceae*	T0	−0.567	0.000137
Unid. *Gracilibacteraceae*	T0	−0.561	0.000166
Pilimelia	T0	−0.525	0.000512
Solibacillus	T0	−0.524	0.000523
Unid. *Acidimicrobiales*	T0	−0.523	0.000542
Turicibacter	T0	−0.522	0.000545
Unid. *Thermoactinomycetaceae*	T0	−0.519	0.000596
Unid. *Ruminococcaceae*	T0	−0.516	0.000649

Unid., unidentified; OTU in the Greengenes database that was not identified at the genus level. The next lowest taxonomical level for which the OTU was identified is given.

even though we had disrupted the indigenous soil's microbiome using irradiation. Although, the experimental treatments did not produce lasting microbiome modifications, significant changes were observed in willow biomass production at TF. Many of our results suggest that microbial community composition at TF was a poor indicator of willow growth and biomass production compared with community composition at T0. Thus, the strategy of using irradiation to reduce the microbial load and open niches for microbial colonization successfully modified the starting microbiome of contaminated soil, which led to lasting differences in willow growth.

Our hypothesis was that willows growing in pots inoculated with rhizospheric soil harvested from willows that had grown successfully in contaminated soils would grow more successfully than willows receiving other inoculants. In contrast to our hypothesis, willows growing in soil inoculated with bulk soil (DE treatment) performed better than those growing in pots inoculated with rhizospheric soil (LA and SM). There were no apparent differences in survival rates, as all willows survived throughout the length of the experiment. One possible explanation for the better performance of the DE treatment is that the DE inoculum was in fact bulk soil (since the willow had died) as compared to rhizospheric soil for the LA and SM inocula. As mentioned above, the rhizosphere is a strongly selective environment, which promotes a lower diversity of specialized microorganisms (Marilley and Aragno, 1999), and in fact at T0, the DE pots were more diverse in terms of the bacteria and fungi present. The willows growing in these pots were exposed to a wider diversity of organisms, which may have helped them to initially adapt to the stressful conditions created by the contaminants.

Although, the soil used to pot the second-generation willows was harvested at the exact same location as the soil used for the study that produced the first-generation willows, it should be stressed that the experimental conditions in this study were different: smaller pots, greenhouse vs. outdoor incubation, willows directly planted in soil vs. pre-growth followed by transplantation of clippings, winter vs. spring, irradiated soil vs. fresh soil. This probably explains the observed differences in survival rates, with all the second-generation willows surviving compared to an 89% mortality rate for the first-generation willows. Willows survival in contaminated soil was previously shown to differ markedly depending on field environmental conditions (Guidi et al., 2011). The different experimental conditions also likely modified the rhizosphere–willow association, as well as the composition of the ideal microbiome that would allow optimal growth. Alternatively, rhizosphere communities are known to change over time (Chaparro et al., 2013b) because of shifts in plant exudates (Chaparro et al., 2013a), and the rhizosphere communities that were harvested and used as inocula (6 month old plants) were probably not optimal for willow clipping establishment in soil. Access to a more diverse microbiome might have given an advantage to the DE willows by allowing them to select the best microbiome for the growth conditions and their developmental stage. This suggests a very high specificity of rhizosphere–willow associations under stressful conditions, but a high variability in the composition of the optimal microbiome, depending on growth conditions and plant developmental stage. This further complicates efficient engineering of a beneficial microbiome.

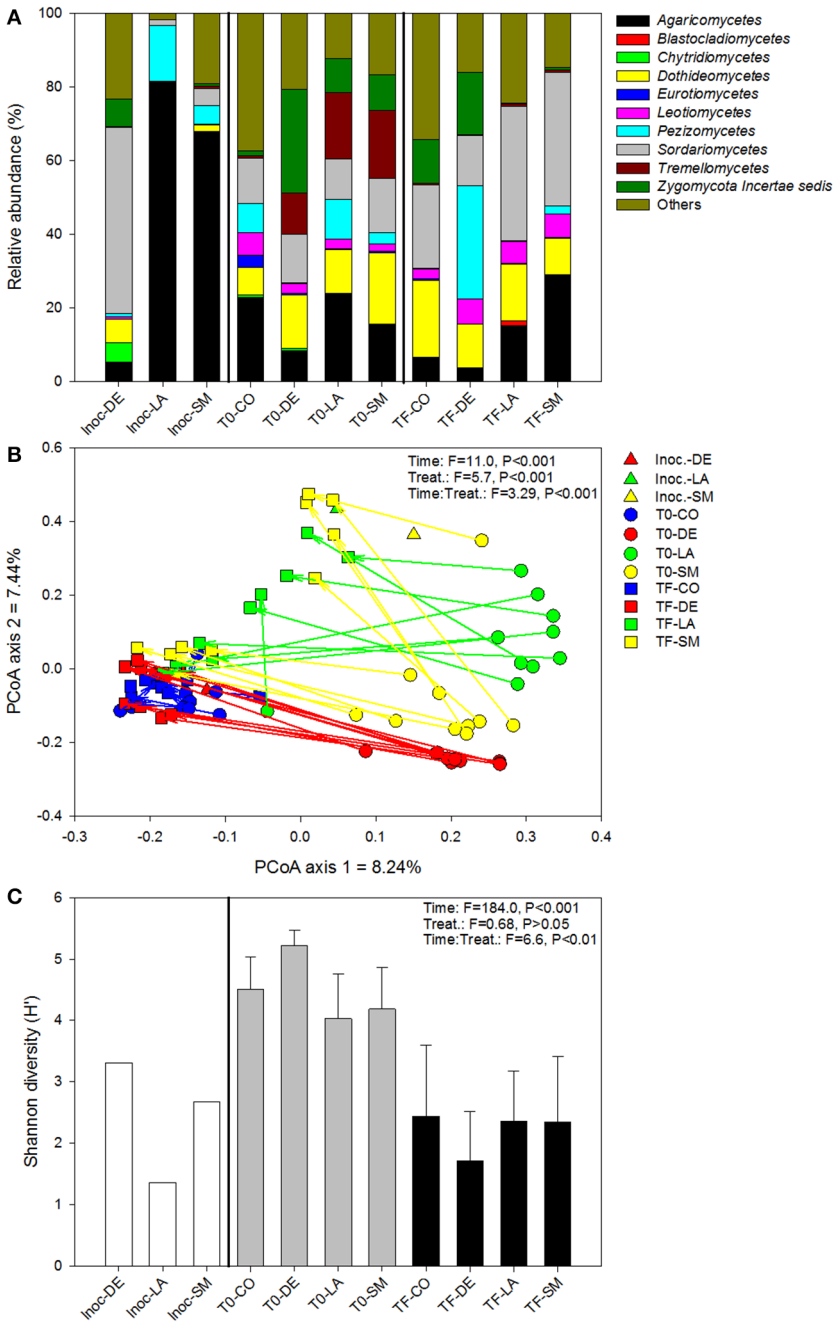

FIGURE 5 | Fungal community composition (A), structure (B), and diversity (C) for bulk and willow rhizosphere soil samples taken at T0 and TF (after 100 days) for CO, DE, SM, and LA treatments and the original inocula. Vectors are linking samples taken from the same pot at different time points. Error bars represent standard deviation.

The willows from the CO treatment showed reduced growth and distinct starting microbial communities compared to the willows from other treatments. One possible explanation could be that certain key microbes required for efficient willow establishment and growth in highly contaminated environments were killed by the irradiation treatment, and could not be recruited in the CO treatment because of the lack of inoculation. Alternatively, some deleterious organisms may have survived irradiation, and rapidly colonized newly available niches. Correlation analyses highlighted some of the potentially beneficial and deleterious organisms that were highly correlated to willow biomass. Consequently, instead of trying to modify whole microbial communities, an alternative approach would be to ensure that beneficial

TABLE 4 | Top 10 most significant Spearman correlations between fungal genera relative abundance and root or shoot biomass.

Genus	Time	r_S	*P*-values
ROOT BIOMASS—POSITIVE			
Mortierella	T0	0.646	6.75×10^{-6}
Epicoccum	T0	0.628	1.44×10^{-5}
Pseudogymnoascus	T0	0.601	4.15×10^{-5}
Valsa	T0	0.579	9.20×10^{-5}
Sporidiobolus	T0	0.481	0.0017
Rosellinia	T0	0.475	0.0019
Cosmospora	TF	0.465	0.0029
Cystofilobasidium	T0	0.462	0.0027
Cosmospora	T0	0.441	0.0044
Cryptococcus	T0	0.418	0.0073
ROOT BIOMASS—NEGATIVE			
Pseudaegerita	T0	−0.613	2.60×10^{-5}
Cladosporium	TF	−0.529	0.0005
Byssochlamys	T0	−0.483	0.0016
Ganoderma	T0	−0.456	0.0031
Talaromyces	T0	−0.414	0.0079
Rhizophagus	T0	−0.364	0.0209
Eupenicillium	TF	−0.351	0.0284
Blastocladiella	TF	−0.349	0.0293
Lignincola	T0	−0.338	0.0330
Pseudogymnoascus	TF	−0.327	0.0419
SHOOT BIOMASS—POSITIVE			
Pseudogymnoascus	T0	0.636	1.04×10^{-5}
Curvularia	T0	0.529	0.0004
Udeniomyces	T0	0.505	0.0009
Mortierella	T0	0.499	0.0010
Trichocladium	T0	0.484	0.0015
Sphaerosporella	TF	0.476	0.0022
Clonostachys	T0	0.472	0.0021
Epicoccum	T0	0.470	0.0022
Mrakia	T0	0.438	0.0047
Leucosporidium	T0	0.431	0.0055
SHOOT BIOMASS—NEGATIVE			
Cladosporium	TF	−0.403	0.011
Geotrichum	T0	−0.382	0.015
Hebeloma	T0	−0.378	0.016
Talaromyces	TF	−0.351	0.028
Rhizophagus	T0	−0.348	0.028
Pseudogymnoascus	TF	−0.346	0.031
Ganoderma	T0	−0.346	0.029
Occultifur	T0	−0.346	0.029
Eupenicillium	TF	−0.335	0.037
Setoseptoria	T0	−0.317	0.046

species are present in high abundance, while restricting the abundance of deleterious taxa. Soil microorganisms can have large effects on plant growth and function (Hoeksema et al., 2010; Glassman and Casper, 2012; Lau and Lennon, 2012), although the relative impact of beneficial and deleterious microorganisms will differ depending on soil type, environmental conditions, and plant species. For instance, mycorrhizal fungi are generally beneficial, but can become parasitic under certain environmental conditions, especially in human-managed ecosystems (Johnson et al., 1997). Accordingly, we found a negative correlation between the mycorrhizal fungi genus *Rhizophagus* and shoot biomass. Alternatively, the correlations between willow biomass and microbial relative abundance could be indirect, through the effect of the microorganisms on other soil organisms or soil physico-chemical characteristics.

The lack of inoculation in the CO treatment also resulted in significantly lower bacterial diversity in the willow rhizosphere at TF. In fact, bacterial diversity at TF was strongly and positively correlated to willow biomass. High community evenness and diversity have been shown to result in healthy soils, high levels of nutrient cycling, increased plant productivity, and reduced stress and disease incidence (Elliot and Lynch, 1994; van Bruggen and Semenov, 2000; Wittebolle et al., 2009; Crowder et al., 2010). A lack of plant community evenness has been associated with reduced plant productivity, possibly due to niches being left vacant and the loss of certain ecosystem services (Wilsey and Potvin, 2000). One way to optimize the willow microbiome might be to provide a soil bacterial community with high diversity and evenness, to allow the willow to select its preferred rhizosphere organisms for optimal growth. This may help to avoid pressures that could lead to selection of suboptimal communities, such as microbial priority effects. However, polluted environments rarely contain diverse or even microbiomes, and one key to effective phytoremediation may be to restore soil bacterial evenness by, for example, soil fertilization, mixing, or aeration before the introduction of plants. Indicative of the importance of restoring soil quality, the willows planted in the contaminated soils only grew to a fraction of the size of those that were grown in parallel in nutrient-rich, well-aerated potting media.

In contrast to bacteria and archaea, the diversity of fungi showed a weak but significant negative correlation with willow shoot biomass and some fungal communities had converged toward the composition of their respective inocula by the end of the experiment. The difference between fungal and bacterial and archaeal communities could be due to the more intimate nature of the relationship between fungi and plants, as many obligate symbionts and pathogens of plants are found in the fungal domain. Previous studies of willows growing in contaminated soil highlighted the stronger link between fungal communities and willow cultivar identity (Bell et al., 2014a) and between fungi and willow growth and zinc uptake (Bell et al., 2015) as compared to bacterial communities. Fungal diversity was also enhanced significantly more by willow introduction than was bacterial diversity, suggesting that phytoremediation may have a disproportionate direct effect on fungi (Bell et al., 2014a). Fungi and bacteria can also be antagonists in the soil environment (De Boer et al., 2005; Rousk et al., 2008; Bonfante and Anca, 2009; Schrey et al., 2012), and competition between these groups has been shown to reduce key soil functions (Siciliano et al., 2009) and microbial growth (Mille-Lindblom et al., 2006;

Meidute et al., 2008). Taken together, these results indicate that the physiological and ecological differences between fungi and bacteria may require domain-specific microbiome engineering strategies.

The relative abundance of certain genera at T0 appeared to play a key role in willow growth. For many of the genera (especially fungi) with the strongest positive correlations with willow growth, it was often their relative abundance at T0 that was most strongly correlated to final willow characteristics. Furthermore, the microbial communities of the different treatments were often dissimilar at T0, closely mirroring eventual differences in willow growth, while the TF communities were more similar to each other, and less strongly related to differences in willow growth. This data strongly implies that the microbiome composition at T0 plays a role in determining eventual willow growth in stressful environments. This is in line with our recent results that show that willow growth and Zn accumulation after 16 months of growth in the field were more strongly related to the abundance of the ectomycorrhizal fungus *Sphaerosporella brunnea* at 4 months than to its abundance at 16 months (Bell et al., 2015).

CONCLUSIONS

Modifying the soil microbiome through gamma-irradiation followed by soil inoculation resulted in short-term shifts in microbial communities, but lasting effects on plant growth characteristics. Our study demonstrated the potential for modifying target plant characteristics through manipulation of the plant-associated microbiome, even though this did not occur as we had hypothesized. This study also highlights several key factors that should be considered when engineering the plant rhizosphere microbiome, including the presence and abundance of keystone species, diversity and evenness of the initial inoculum, ecological differences between fungi and bacteria, environmental conditions, and the plant growth stage that the inoculum originates from.

ACKNOWLEDGMENTS

This project was supported by the Genome Canada and Genome Québec funded GenoRem Project (project number 2510). We are grateful to Pétromont for providing us access to the Varennes field site.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 22 December 2015
doi: 10.3389/fmicb.2015.01450

Endophytic Cultivable Bacteria of the Metal Bioaccumulator *Spartina maritima* Improve Plant Growth but Not Metal Uptake in Polluted Marshes Soils

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Microbiology

Received: *30 July 2015*
Accepted: *04 December 2015*
Published: *22 December 2015*

Citation:
Mesa J, Mateos-Naranjo E, Caviedes MA, Redondo-Gómez S, Pajuelo E and Rodríguez-Llorente ID (2015) Endophytic Cultivable Bacteria of the Metal Bioaccumulator Spartina maritima Improve Plant Growth but Not Metal Uptake in Polluted Marshes Soils. Front. Microbiol. 6:1450. doi: 10.3389/fmicb.2015.01450

Endophytic bacterial population was isolated from *Spartina maritima* tissues, a heavy metal bioaccumulator cordgrass growing in the estuaries of Tinto, Odiel, and Piedras River (south west Spain), one of the most polluted areas in the world. Strains were identified and ability to tolerate salt and heavy metals along with plant growth promoting and enzymatic properties were analyzed. A high proportion of these bacteria were resistant toward one or several heavy metals and metalloids including As, Cu, and Zn, the most abundant in plant tissues and soil. These strains also exhibited multiple enzymatic properties as amylase, cellulase, chitinase, protease and lipase, as well as plant growth promoting properties, including nitrogen fixation, phosphates solubilization, and production of indole-3-acetic acid (IAA), siderophores and 1-aminocyclopropane-1-carboxylate (ACC) deaminase. The best performing strains (*Micrococcus yunnanensis* SMJ12, *Vibrio sagamiensis* SMJ18, and *Salinicola peritrichatus* SMJ30) were selected and tested as a consortium by inoculating *S. maritima* wild plantlets in greenhouse conditions along with wild polluted soil. After 30 days, bacterial inoculation improved plant photosynthetic traits and favored intrinsic water use efficiency. However, far from stimulating plant metal uptake, endophytic inoculation lessened metal accumulation in above and belowground tissues. These results suggest that inoculation of *S. maritima* with indigenous metal-resistant endophytes could mean a useful approach in order to accelerate both adaption and growth of this indigenous cordgrass in polluted estuaries in restorative operations, but may not be suitable for rhizoaccumulation purposes.

Keywords: endophytes, heavy metal, phytoremediation, plant growth promoting bacteria (PGPB), salt marsh, *Spartina maritima*

INTRODUCTION

Environmental pollution by heavy metals is a major concern for authorities (USEPA)[1] due to several reasons: (i) its impact on environment and health, (ii) their high occurrence as a contaminant, (iii) their low solubility and bioavailability, and (iv) their carcinogenic and mutagenic nature (Davis et al., 2011). What is more, they cannot be degraded to harmless products and hence persist in the environment indefinitely (Khan and Doty, 2011). The environmental increase of heavy metal contamination is mainly due to industrial and agricultural activities (mining and smelting of metalliferous ores), waste water irrigation and chemical fertilizers and pesticides abuse (Bradl, 2002). The estuary of the Tinto and Odiel rivers, placed in the province of Huelva (Spain), is known as one of the most contaminated regions throughout the world due to the presence of high amounts of heavy metals in its sediments (especially As, Cu Pb, and Zn) since thousands of years (Nelson and Lamothe, 1993; Davis et al., 2000; Ruiz, 2001; Sáinz et al., 2002, 2004). This region has great environmental interest as well as historical significance (Wilson, 1981). The mining activity, mainly for Cu explotation, together with the later industrialization of the Huelva area, beginning in 1967, have contributed to the current level of pollution of this fluvial-estuarine system (Davis et al., 2000). By contrast, 30 km away from this estuary, is located the Piedras estuary, with absence of relevant anthropic contributions and no significant metallic pollution which therefore maintains its environmental quality (Borrego et al., 2013).

Spartina maritima (Curtis) Fernald belongs to the cordgrass family. It is an indigenous plant naturally growing in the estuary of three close rivers: Tinto, Odiel, and Piedras. It is distributed along the North-African and European coasts, playing an important role in the ecology of the saltmarshes, preserving the structure of intertidal coastal zones as well as defending the coast from erosion. What is more, a good tolerance of *S. maritima* to anthropogenic pollutants, tidal submergence, salinity and drainage has been demonstrated (Mateos-Naranjo et al., 2007, 2010). The utility of this species for biomonitoring coastal systems where it is abundant has also been described (Padinha et al., 2000). On top of that, heavy metals accumulation at different rates in *S. maritima* tissues and rhizosediment allowed concluding that this species could be used for phytostabilization of estuarine sediments (Cambrollé et al., 2008; Redondo-Gómez, 2012). Unfortunately, the invasive *Spartina densiflora* is displacing the native *S. maritima* and colonizing non-restored marshes of the southwest coast of Spain (Castillo et al., 2008). In that situation, it is important not only to learn how to efficiently rehabilitate degraded salt marshes but also how to adequately manage populations of *S. densiflora,* preserving the endangered native plant population (An et al., 2007; Mateos-Naranjo et al., 2012).

In soil metal phytoremediation, plants should be capable to handle with large amounts of heavy metals and reach a high biomass at the same time. Nevertheless, their growth may be limited at high pollution rates, resulting in small size and slow growth rate, lessening their phytoextraction capacity (reviewed in Ali et al., 2013). So that, the beneficial relationships between plants and their associated microbes could be exploited to expedite the production of plant biomass and thereby determine plant metal stabilization (Glick, 2010; Ma et al., 2011; Rajkumar et al., 2012). There is an increasing literature reporting the effect of bacterial inoculation on plant growth and metal uptake. In all these studies, plant-associated bacteria increased plant growth but the effect on metal uptake depended on the specific plant-microorganism partnerships and also on the soil characteristics (Sessitsch et al., 2013; Phieler et al., 2014). Metal bioavailability is often plant species and element specific, and is clearly influenced by metabolites excreted by applied bacteria (Tak et al., 2013; Langella et al., 2014). It has been recently suggested that interactions between halophyte and microorganisms could be useful in phytoremediation strategies in coastal ecosystems (Reboreda and Caçador, 2008; Andrades-Moreno et al., 2014; Mesa et al., 2015b). Although interactions between plants and microbes in the rhizosphere have long been studied by microbial ecologists (de-Bashan et al., 2012), plant growth promoting bacterial (PGPB) endophytes may offer several advantages and are gaining scientific interest (reviewed in Rajkumar et al., 2009). Concerning the genus *Spartina*, several works about their rhizospheric bacterial populations have been published (Lovell et al., 2000; Gamble et al., 2010; Davis et al., 2011; Andrades-Moreno et al., 2014; Mesa et al., 2015a,b), but endophytic bacteria have never been characterized so far.

The aims of this work were (i) to isolate the cultivable endophytes from *S. maritima* growing in salt marshes with different levels of metal contamination, (ii) to characterize them and select the endophytic PGPBs which might be useful for increasing plant biomass production, and (iii) to inoculate wild *S. maritima* seedlings in greenhouse conditions to elucidate the influence of these PGPBs in plant metal uptake in contaminated soils.

MATERIALS AND METHODS

Plant and Soil Sampling and Chemical Analysis

Plant samples of *S. maritima* were harvested in May 2013 from the Tinto (37°13′ N, 6°53′ W), Odiel (37°10′35.2″N 6°55′59.2″W) and Piedras (37°16′09.1″N 7°09′36.4″W) rivers estuaries. Soil samples were collected in the same locations. They were transported to the laboratory and stored at 4°C. Endophytes were isolated within 24 h. For chemical analysis, plant tissues were carefully washed with distilled water and dried at 80°C for 48 h, grounded and homogenized (Redondo-Gómez et al., 2007). Then, samples were acid-digested and the cool residue extracted as described in Cambrollé et al. (2008). Inductively coupled plasma (ICP-AES) spectroscopy (ARLFisons3410, USA) was used to measure elements concentration. Concentrations were expressed as mg/Kg (**Table 1**).

[1]USEPA. US Environmental Protection Agency. http://www.epa.gov

TABLE 1 | Metal(loid) concentrations in mg/Kg for soil samples and *S. maritima* tissues in Tinto, Odiel and Piedras rivers estuaries.

	Location	As	Cd	Co	Cu	Fe	Mn	Ni	Pb	Zn
Root	Tinto	160.54 ± 1.44^{a}	2.15 ± 0.02^{a}	11.57 ± 0.10^{a}	545.47 ± 4.88^{a}	21797.15 ± 194.96^{a}	53.40 ± 0.48^{a}	5.74 ± 0.05^{a}	60.00 ± 0.54^{a}	428.73 ± 3.83^{a}
	Odiel	98.94 ± 0.88^{b}	2.06 ± 0.02^{b}	12.38 ± 0.11^{b}	251.41 ± 2.25^{b}	18850.33 ± 168.60^{b}	124.40 ± 1.11^{b}	6.74 ± 0.06^{b}	72.78 ± 0.65^{b}	779.93 ± 6.98^{b}
	Piedras	29.71 ± 0.27^{c}	0.54 ± 0.00^{c}	2.88 ± 0.03^{c}	62.37 ± 0.56^{c}	7162.19 ± 64.06^{c}	42.01 ± 0.38^{c}	7.04 ± 0.06^{c}	20.80 ± 0.19^{c}	180.91 ± 1.62^{c}
Stem	Tinto	4.95 ± 0.04^{a}	0.24 ± 0.00^{a}	$<0.5 \pm 0.00^{a}$	74.27 ± 0.66^{a}	672.77 ± 6.02^{a}	34.20 ± 0.31^{a}	1.29 ± 0.01^{a}	6.87 ± 0.06^{a}	59.44 ± 0.53^{a}
	Odiel	2.91 ± 0.03^{b}	0.38 ± 0.00^{b}	$<0.5 \pm 0.00^{a}$	39.47 ± 0.35^{b}	1047.61 ± 9.37^{b}	73.85 ± 0.66^{b}	1.68 ± 0.01^{b}	9.53 ± 0.09^{b}	96.24 ± 0.86^{b}
	Piedras	0.47 ± 0.00^{c}	$<0.1 \pm 0.00^{c}$	$<0.5 \pm 0.00^{a}$	3.74 ± 0.03^{c}	602.89 ± 5.39^{c}	46.88 ± 0.42^{c}	1.69 ± 0.02^{b}	1.14 ± 0.01^{c}	26.02 ± 0.23^{c}
Leaf	Tinto	19.68 ± 0.18^{a}	0.19 ± 0.00^{a}	$<0.5 \pm 0.00^{a}$	98.01 ± 0.88^{a}	2692.89 ± 24.09^{a}	53.58 ± 0.48^{a}	2.40 ± 0.02^{a}	25.68 ± 0.23^{a}	105.26 ± 0.94^{a}
	Odiel	9.09 ± 0.08^{b}	0.17 ± 0.00^{b}	0.54 ± 0.00^{b}	51.35 ± 0.46^{b}	3303.44 ± 29.55^{b}	141.32 ± 1.26^{b}	3.49 ± 0.03^{b}	23.80 ± 0.21^{b}	123.98 ± 1.11^{b}
	Piedras	1.04 ± 0.01^{c}	$<0.1 \pm 0.00^{c}$	$<0.5 \pm 0.00^{a}$	7.46 ± 0.07^{c}	1431.08 ± 12.80^{c}	62.53 ± 0.56^{c}	3.08 ± 0.03^{c}	3.46 ± 0.03^{c}	28.03 ± 0.25^{c}
Soil	Tinto	546.0 ± 4.89^{a}	5.23 ± 0.05^{a}	20.54 ± 0.18^{a}	2460.01 ± 22.01^{a}	106231.78 ± 950.17^{a}	326.53 ± 2.92^{a}	43.49 ± 0.39^{a}	701.35 ± 6.27^{a}	2523.69 ± 22.57^{a}
	Odiel	127.0 ± 1.14^{b}	3.49 ± 0.03^{b}	5.24 ± 0.05^{b}	889.11 ± 7.93^{b}	69117.33 ± 537.71^{b}	472.20 ± 4.22^{b}	29.73 ± 0.27^{b}	275.81 ± 2.47^{b}	1655.23 ± 14.80^{b}
	Piedras	3.1 ± 0.03^{c}	0.18 ± 0.00^{c}	$<0.5 \pm 0.00^{c}$	20.99 ± 0.19^{c}	12707.28 ± 113.66^{c}	75.02 ± 0.67^{c}	10.65 ± 0.10^{c}	21.12 ± 0.19^{c}	71.63 ± 0.264^{c}

Values are means ± s.e. (n = 5). Different letters indicate statistical differences for each metal(loid) and tissue between locations ($P < 0.05$).

Isolation and Morphologic Characterization of Cultivable Endophytic Bacteria from *S. maritima* Tissues

Endophytic bacteria were isolated from surface-sterilized leaves, stems and roots of *S. maritima*. The protocol of surface-sterilized was as follows: first surface washing under running tap water to remove soil, insects and other big particles, followed by immersion in 70% (v/v) ethanol for 2 min under slightly shaking, then soaking in 5% (v/v) sodium hypochlorite for 10 min under gentle agitation, and finally 5 rinses in sterile distilled water. Several controls confirmed that the sterilization procedure was effective. On one hand, fragments from each tissue were transferred to commercial tryptic soy agar (TSA) medium (iNtRON Biotechnology, Korea) and modified TSA medium NaCl 0.6 M (approximately seawater salt concentration). On the other hand, duplicates of 100 μL aliquots of the last washing water were also plated in the same way. No bacteria were grown within 5 days incubation at 28°C.

Leafs and roots were macerated separately using a sterile mortar and pestle in a small volume of sterile physiological saline solution, with sterile quartz sand being added to improve the wall disruption. The stems were placed inside sterile pipette tips and then into sterile Falcon tubes. They were centrifuged at 1500 rpm for 5 min, and the obtained liquid was discarded. Then, they were spun at 5000 rpm during 20 min, and the apoplastic fluid was collected. 100 μL of the three resulting tissue extracts were plated onto TSA and TSA 0.6 M NaCl plates, in order to recover halophilic bacteria. Following the incubation during 72–96 h at 28°C, colonies of varying morphology were picked and then subsequently re-streaked on TSA and modified TSA NaCl 0.6 M in order to obtain pure cultures. The morphological characterization was carried out by recording colony characters based on shape, margin, color, surface and consistency followed by Gram staining. TSA NaCl 0.6 M was prepared as described in Mesa et al. (2015a).

Genetic Diversity by BOX-PCR

Genomic DNA extraction and BOX-PCR were performed exactly as described in Mesa et al. (2015a). For BOX-PCR, the BOX A1R primer (5′- CTA CGG CAA GGC GAC GCT GAC G -3′) and 40 ng of DNA as template were used. After PCR, products were electrophoresed using an agarose gel (1.5%) and revealed by UV radiation. The gel was photographed and the images were processed with Phoretix 1D® Software (TotalLab, UK), resulting in dendograms. The similarities in BOX-PCR fingerprints were established by determining the Pearson's product moment correlation coefficient (Jobson, 1991).

Identification of Cultivable Bacteria

The bacteria with most interesting properties were selected for PCR amplification of conserved 16S rRNA and subsequent sequencing and analysis. For PCR amplification, universal primers 16S F8-27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S R1541-1522 (5′-AAGGGAGGTGATCCAGCCGCA-3′) were employed. The reaction mixture contained for 25 μl: 10x Ecogen buffer 2.5 μl, $MgCl_2$ 50 mM 1 μl, dNTPs 10 mM (2.5 mM each)

1 μl, EcoTaq polymerase (5U/μl) 0.2 μl, extracted DNA 1 μl, each primer 10 μM 1 μl and H_2O_{MQ}17.3 μl. The temperature profile was programmed as follows: predenaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 45 s, annealing at 58°C for 45 s, extension at 72°C for 90 s (35 cycles) and final extension at 72°C for 5 min. The amplified products were checked by running on 1% agarose gel and visualized under UV after staining with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Korea). The PCR product was purified with SpeedTools PCR Clean-up kit (Biotools, Spain) and sequencing was done by StabVida Company (Portugal). The EzTaxon server was used to determine 16S rRNA sequence homologies (Chun et al., 2007). Finally, accession numbers from KT036396 to KT036409 were assigned to the sequences deposited in GenBank (**Table 2**).

Bacterial Resistance Against NaCl and Metal(loid)s

The resistance of isolated bacteria toward heavy metals and sodium arsenite was determined on plates containing TSA and modified TSA 0.2M NaCl mediums amended with the following heavy metal stock solutions were employed: $CuSO_4$ 1 M, $ZnSO_4$ 1 M, $NiCl_2$ 0.2 M, $CoCl_2$ 1 M, $CdCl_2$1 M, $Pb(NO_3)_2$0.5 M, and $NaAsO_2$ 0.5 M, as detailed in Mesa et al. (2015a). To establish NaCl tolerance, SW30 stock solution was added to TSA medium. The resistance was expressed as the maximum tolerable concentration (MTC), namely the highest metal or metalloid concentration not impeding bacterial growth.

Screening for Bacterial Enzyme Activity

Strains were tested for amylase, cellulase, lipase, protease, and chitinase activities. They were screened in plates. To detect amylase activity, the isolates were inoculated on starch agar (Scharlab, Spain), and were revealed after incubation by flooding the plates with iodine–potassium iodide solution (lugol). In the case of cellulase, strains were plated onto solid minimal medium M9 supplemented with 0.2% yeast extract and 1% carboxymethyl cellulose (CMC) and was revealed after incubation by flooding the plates with Congo Red solution 1mg/ml for 15 min followed by destaining with sodium chloride 1 M for 15 min. Protease activity was detected by growing the strains in casein agar (Prescott, 2002). All the plates were incubated for 5 days at 28°C and observed for clear zones around the cultures. In the case of lipase activity, strains were grown in Tween agar (Prescott, 2002), plates were incubated for 7 days at 28°C, and looked for the appearance of a precipitate around the strains. Chitinase activity was tested using a minimal medium (per liter, 2.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.7 g $MgSO_4{\cdot}7H_2O$, 0.5 g KCl, 0.13 g yeast extract, 15 g agar, H_2O e.q. to 1 L, pH 7.2) supplemented with colloidal chitin (1.5%). The plates were incubated at 28°C for 7 days until zones of chitin clearing could be seen around the colonies. NaCl concentration in all media was adjusted to 0.2 M by adding filter sterilized SW30 solution after autoclaving to avoid Ca precipitation.

TABLE 2 | Closest species to the fourteen isolates based on the 16S rRNA sequence.

Strain	16S rRNA sequenced fragment (bp)	Accession No.	Related species	Percent identity
SMJ1	1413	KT036396	*Staphylococcus warneri*	99.64
SMJ4	1424	KT036397	*Micrococcus yunnanensis*	99.58
SMJ12	1430	KT036398	*Micrococcus yunnanensis*	99.58
SMJ15	1483	KT036399	*Bacillus selenatarsenatis*	98.09
SMJ17	1458	KT036400	*Bacillus aryabhattai*	99.59
SMJ18	1405	KT036401	*Vibrio sagamiensis*	98.46
SMJ19	1418	KT036402	*Marinomonas alcarazii*	96.18
SMJ20	1352	KT036403	*Marinomonas ostreistagni*	97.40
SMJ24	1407	KT036404	*Vibrio sagamiensis*	98.76
SMJ25	1454	KT036405	*Pseudoalteromonas shioyasakiensis*	99.72
SMJ28	1453	KT036406	*Marinomonas alcarazii*	96.18
SMJ30	1440	KT036407	*Salinicola peritrichatus*	97.29
SMJ32	1347	KT036408	*Salinicola peritrichatus*	97.84
SMJ33	1462	KT036409	*Staphylococcus pasteuri*	99.51

Screening for Bacterial Plant Growth Promoting Traits

Bacterial plant growth promoting traits were recorded as described in Mesa et al. (2015a). Bacterial growth in NFb medium was used to test nitrogen fixation (Dobereiner, 1995). Phosphate solubilization was confirmed on NBRIP medium plates (Nautiyal, 1999) when bacterial growth caused the appearance of surrounding transparent halos. In the same way, orange halos revealed production of siderophores on chrome azurol S (CAS) plates (Schwyn and Neilands, 1987). Plates were always incubated 72 h at 28°C. The synthesis of IAA (indole-3-acetic acid) was colorimetrically estimated as detailed in Mesa et al. (2015a). Presence of ACC deaminase enzyme was detected following the method described in Penrose and Glick (2003). Liquid Dworkin and Foster (DF) mineral medium (Dworkin and Foster, 1958) with 3.0 mM ACC was used to inoculate endophytic strains after enrichment with the same medium with $(NH_4)_2SO_4$ as nitrogen source instead of ACC. The growth on the tubes was checked daily during 3 days at 28°C. As mentioned before, NaCl concentration in all media was adjusted to 0.2 M by adding filter sterilized SW30 solution after autoclaving. Based on the results from this experiment, ACC deaminase activity was determined by monitoring the amount of α-ketobutyric acid generated from the cleavage of ACC (Penrose and Glick, 2003). The reaction was determined by comparing the absorbance at 540 nm of the sample to a standard curve of α-ketobutyrate. Then, total protein concentration of toluenized cells (Bradford, 1976) was estimated using bovine serum albumin (BSA) to produce the protein calibration curve. After determining the amount of protein and α-ketobutyrate, the enzyme activity was calculated based on the μmoles of released α-ketobutyrate per mg of protein per hour.

Pot Inoculation in Greenhouse Conditions

In April 2014, wild plants along with wild soil were collected from the Tinto River estuary, the most contaminated. They were carried in pots, filled with 1 Kg of soil from the marsh, to

the greenhouse facilities at the University of Seville. Pots were randomly assigned to two treatments (non-inoculated control plants; inoculated plants) and placed in the same greenhouse for 30 days ($n = 20$, two treatments with 10 pots each one). During the experimental period the inoculations were performed once a week (four times in total). For that, bacteria were grown separately in 250 ml Erlenmeyer flasks containing 50 ml of TSB medium (iNtRON Biotechnology, Korea) with continuous gentle shaking at 28°C to reach 10^8 cells per ml (18–24 h). Then, cultures were centrifuged at 8000 rpm during 10 min, the supernatant was discarded and pellets were resuspended in 2 L tap water. Hundred milli liter of suspension per pot were used for plant inoculation. Pots were placed in trays (different trays for each treatment) and were lightly watered with tap water every 2 days during the experiment to avoid dryness, since *S. maritima* is a low marsh plant used to tidal flooding.

S. maritima re-colonization Potential of the Endophytic Consortium

In parallel to inoculation treatments, three plants were inoculated with mutant endophytic PGPB in order to confirm they penetrate and colonize plant tissues. *Salinicola* SMJ30 was labeled with the fluorescent protein mCherry by bacterial conjugation. Strains and plasmids used are listed in **Table 3**. This was accomplished by mixing the donor *E. coli* DH5α containing pMP7604, the helper *E. coli* strain containing pRK600 and the recipient strain *Salinicola* SMJ30 in liquid LB medium overnight under slightly shaking at 28°C. Next, 100 μL aliquots were plated onto LB plates containing selective antibiotic for *Salinicola* SMJ30 (rifampicin) and for pMP7604 plasmid (tetracycline). For wild endophytes *Micrococcus* SMJ12 and *Vibrio* SMJ18 no transformants were detected with conjugation, electroporation, heat-shock or freeze-thaw techniques, even with lysozyme pre-treatments. Thus, spontaneous rifampicin and streptomycin resistant mutants of SMJ12 and SMJ18 wild strains were used. They were developed by transfer of high concentrated bacterial overnight liquid cultures in TSA plates amended with 100 μg ml^{-1} of rifampicin and 250 μg ml^{-1} of streptomycin. The mutant strains showing comparable growth with wild type strains were selected. After the inoculation experiment, fluorescent bacteria in plant tissues were visualized in a confocal microscope (Zeiss LSM 7 DUO). Images were obtained using laser excitation 568–585 nm long pass emission and were processed with ZEN Lite 2012 software. Furthermore, 20 days after the last inoculation, plant tissues and soil samples were collected. 100 mg of plant tissues were surface-sterilized and macerated as explained in 2.2. Regarding soil, 100 mg were resuspended in 500 μl sterile physiological saline solution. 100 μl of the obtained extracts were plated into rifampicin and tetracycline plates (for detection of strain SMJ30), rifampicin and streptomycin plates (for strains SMJ12 and SMJ18) and incubated 72 h at 28°C. Colonies obtained were identified by macroscopic and microscopic observation (colony shape, surface and color, Gram staining and determination of motility in a wet mount) and API® identification products (bioMérieux, France) (API® 20 NE for SMJ30 and SMJ18, API® Staph for SMJ12). Then, colonies were counted to estimate colony-forming units (CFU) per gr of tissue or soil.

Plant Growth and Physiological Analysis

At the beginning and at the end of the experiment, three and ten plants per treatment were harvested and dried at 80°C to estimate roots and shoots dry weights and calculate the relative growth rate, RGR (Mateos-Naranjo et al., 2008). Also 30 days after treatment initiation leaf gas exchange and chlorophyll fluorescence parameters were measured in fully expanded leaves ($n = 10$) using an infrared gas analyzer (LI-6400-XT, Li-COR Inc., NE., USA) and a modulate fluorimeter (FMS-2; Hansatech Instruments Ltd., UK), respectively. Thus, net photosynthetic rate (A_N), stomatal conductance (gs), instantaneous water use efficiency (iWUE) and intercellular CO_2 concentration (C_i) were obtained with the following settings: flux light density1500 μmol photons m^{-2} s^{-1}, ambient CO_2 concentration (Ca) 400 μmol mol^{-1} air, leaf temperature of 25°C and 50 ± 5% relative humidity. Minimal fluorescence (F_0), maximum quantum efficiency of PSII photochemistry (F_v/F_m) and quantum efficiency of PSII (Φ_{PSII}) were obtained in light and dark-adapted leaves at midday (1600 μmol photons m^{-2} s^{-1}) according to the protocol described by Mateos-Naranjo et al. (2008).

Chemical Analyses of Plant Tissues

At the end of the experiment, 30 days after treatment initiation, dried leaves and root samples of the 10 replicates plants were ground, and total As, Cd, Cu, Ni, Pb, and Zn concentrations were measured as previously described by Mateos-Naranjo et al. (2008) by inductively coupled plasma (ICP) spectroscopy.

Metal balance in plant and soil was calculated as the ratio between metal accumulated in plant tissues (metal concentration

TABLE 3 | Strains and plasmids used for bacterial conjugation.

Strain or plasmid	Relevant characteristics	Reference or source
Escherichia coli		
DH5α	Host strain used for transformation and propagation of plasmids containing pMP7604, Tc^r	Boyer and Roulland-Dussoix, 1969
DH5α	Helper strain used for propagation of plasmids containing pRK600, Cm^r	Kessler et al., 1992
Salinicola peritrichatus		
SMJ30	Endophyte isolated from *Spartina maritima* stems, Rif^r	This study
PLASMIDS		
pMP7604	pMP6031 derivate harboring mCherry gene under the control of the tac promoter	Lagendijk et al,.2010
pRK600	Cm^r Nm^S, pRK2013 Nmr::Tn9	Finan et al., 1986

in shoots × shoot biomass + metal concentration in roots × root biomass) with regard to metal in soil. This ratio was estimated both at the beginning and at the end of the experiment.

Statistical Analysis

Statistical analyses were carried out using "Statistica" v. 6.0. Comparisons between means of metal(loid) concentration for *S. maritima* tissues in Tinto, Odiel and Piedras rivers estuaries were made by using one-way anova (*F*-test) and between means in different inoculation treatments at the end of the experiment through the Student test (*t*-test).

RESULTS

Concentration of Metal(loid)s in *S. maritima* Tissues in the Tinto, Odiel, and Piedras Rivers Estuaries

Concentration of metals and metalloids in different tissues of *S. maritima* in the three estuaries were determined (**Table 1**). They were similar to data previously published by other authors (reviewed in Redondo-Gómez, 2012). Depending on their final use, there are threshold values for metals in soils (but not for plant tissues) established by Andalusian and Spanish Governments (Consejería de Medio Ambiente, Junta de Andalucía, 1999). Generally, the Tinto salt marsh showed the higher metal concentration, except for Mn, Ni and Zn, greater in Odiel estuary. Regarding plant tissues, metal levels were higher in roots in all the estuaries.

Isolation of Cultivable Endophytic Bacteria from *S. maritima* Tissues in the Tinto, Odiel, and Piedras Rivers Salt Marshes

Bacteria grown on TSA plates showing different colony morphologies were chosen. This resulted in 42 strains. Unfortunately, several strains could not be systematically re-isolated in standard media and could not be characterized properly (Hardoim et al., 2008). Several strains showed identical basic morphology properties; therefore, the number of different strains was reduced to 34. Subsequently, a BOX-PCR was performed in order to avoid redundancy. As shown in **Figure 1**, few bacteria showed identical band profile, and were then discarded. Despite the fact that some strains showed similar profiles, further analyses during the development of this work (see results below) demonstrated distinctive differences. Thus, they were considered different strains. As a result, the scope of the study was finally reduced to 25 strains. For bacterial identification of the strains with most interesting properties, 16S rRNA genes were partially sequenced (**Table 2**).

Distribution of Cultivable Endophytic Bacteria

Distribution of endophytic strains is represented in **Figure 2**. *S. maritima* from Piedras estuary, the less contaminated saltmarsh, showed the scantiest cultivable endophytic diversity (13 strains), while the ones located in the most contaminated area, the Tinto estuary, presented the largest heterogeneity (18 strains), similarly to Odiel estuary (17 strains). Regarding plant tissues (**Figure 2A**), stems harbored from 39 to 46% of the cultivable strains, followed by roots with the 30 and 38%. In contrast, leaves had only 16–29%. Finally, concerning the distribution of cultivable endophytes between different estuaries, 8 out of 25 strains (32%) were common in the three estuaries with different level of pollution (**Figure 2B**). When comparing the estuaries, Tinto and Odiel shared 40% of cultivable endophytes, being the most similar. The origin of each isolate (estuary and plant tissue) is illustrated in Supplementary Table 1.

Endophytes Abiotic Stress Resistance Toward NaCl and Heavy Metals

Resistance to NaCl as well as to several heavy metals (As in sodium meta-arsenite form, Cd, Co, Cu, Ni, Pb, and Zn) was established and represented as maximum tolerable concentration (MTC, the maximum concentration that allows bacterial visible growth) for each strain (Supplementary Table 2). Data are summarized in **Figure 3A**. Resistance to various metals simultaneously was observed for the majority of the isolates, including noteworthy resistance values in several cases. Pb was the most tolerated metal; all the strains resisted over 2 mM, getting to 25 mM for strains SMJ1, SMJ2, SMJ3, SMJ30, SMJ32, and SMJ33. This was followed by Cu, 80% of the strains resisted over 2 mM Cu_2SO_4.Strains SMJ4, SMJ12, and SMJ33 were resistant up to 8–9 mM Cu. Around 50% of the strains were resistant toward Co and Ni as well. Concerning As, some strains presented a high resistance, SMJ10 and SMJ17 arrived to 13 mM, SMJ3 reached 25 mM and the most striking result was SMJ12 reporting resistance to 100 mM $NaAsO_2$. By contrast, the resistance toward Cd of the isolates was not high (scarcely 2 mM). Finally, NaCl tolerance was also studied (supplementary material), which ranged from 0.5 to 3 M, hence most of isolated bacteria could be considered halotolerant.

Enzymatic and PGP Properties of the Endophytic Isolates

The percentage of endophytic strains showing the different enzymatic and PGP properties studied is presented in **Figure 3B**. Regarding enzymatic activities, 21 out of 25 strains exhibited at least one enzyme activity (Supplementary Material). Amilase production was the most common between the isolates (52%), followed by protease (48%), lipase (44%), cellulase (40%), and finally chitinase activity (12%). Notably, strains SMJ4, SMJ14, SMJ17, SMJ20, SMJ21, and SMJ24 had at least 3 of the 5 enzymatic properties assayed, whereas strains SMJ18 and SMJ25 showed all of them. On top of that, SMJ18 produced the biggest halos in plates assay for all the properties. Moving to PGP properties, represented in the same figure as data above, 76% of the strains had at least one PGP property. Overall, 52% were able to solubilize phosphate and produce siderophores, 44% produced IAA, 20% fixed atmospheric nitrogen and only 8% hydrolysed ACC. Concerning this last one, it is important to measure the enzymatic activity, since some strains were able to grow in the minimal medium

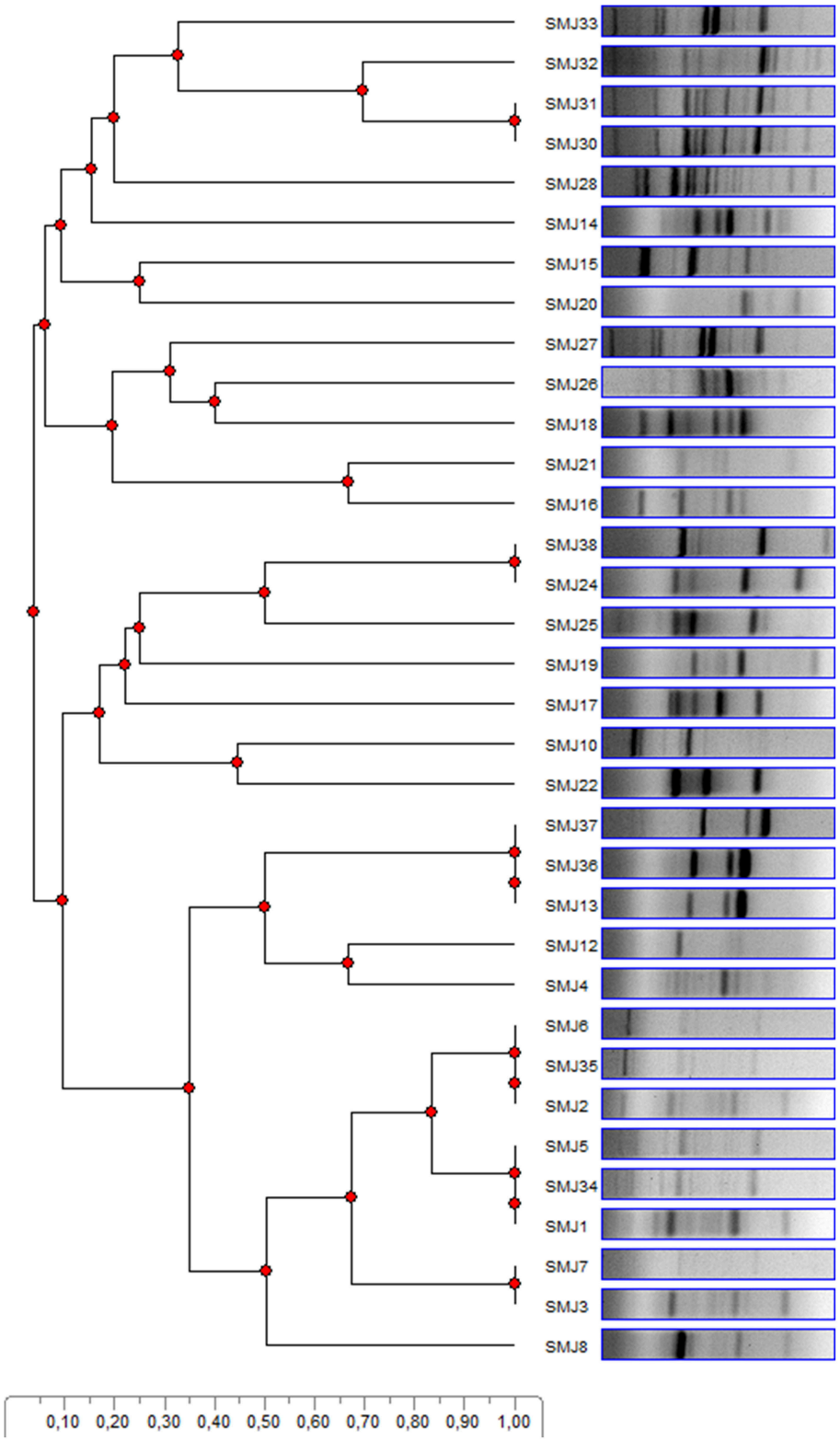

FIGURE 1 | BOX-PCR patterns of the 34 endophytic strains from *S. maritima* tissues in the Tinto, Odiel and Piedras rivers estuaries are compared using a dendogram. Similarity is represented by the scale.

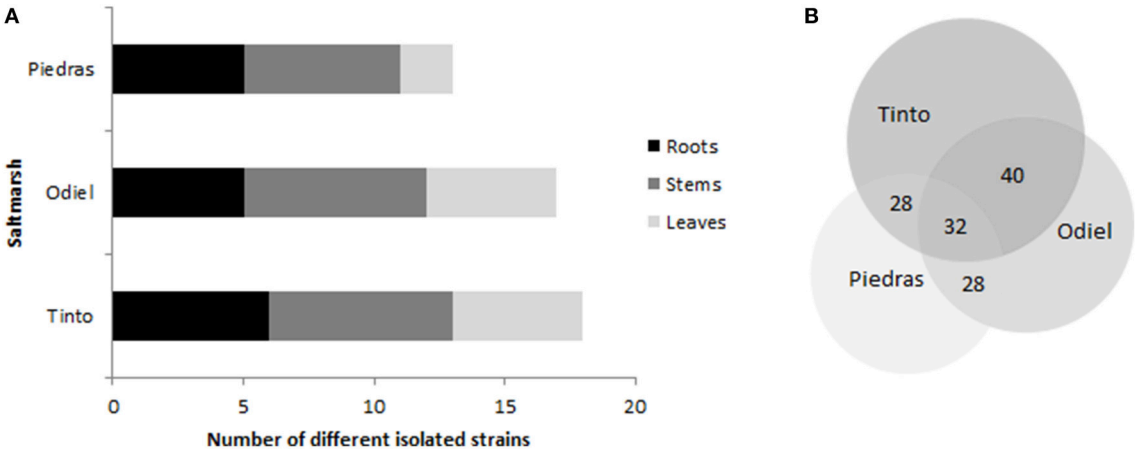

FIGURE 2 | Chart illustrating (A) cultivable endophytes location within distinct plant tissues in each estuary and (B) cultivable endophytes distribution according to the salt marshes expressed as a percentage of total strains isolated (25).

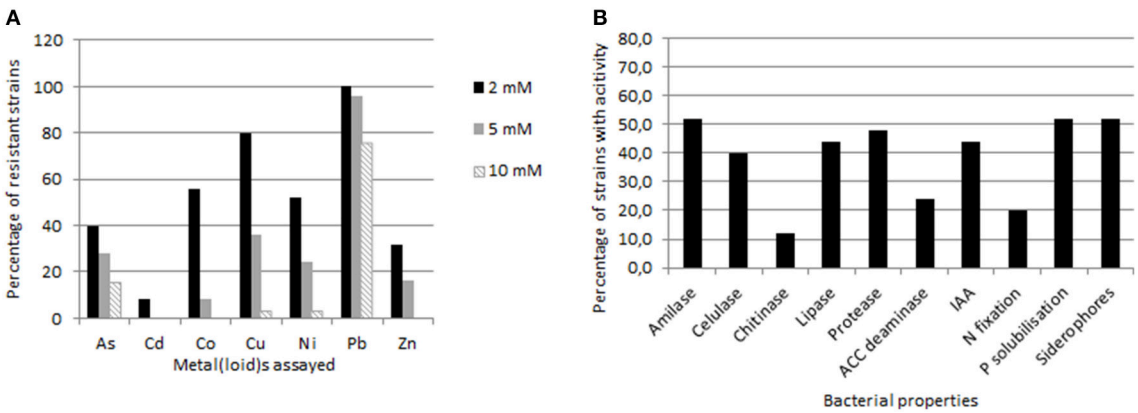

FIGURE 3 | Graphics showing percentages of studied strains (A) with resistance to different heavy metals over concentrations of 2 mM, 5 mM or 10 mM, and (B) with enzymatic and plant growth promoting properties.

with ACC and later gave false positives results. Seven strains were able to grow in the presence of ACC, but only 2 revealed enzymatic activity in the colorimetric assay. Only SMJ28 showed all the properties assayed, while 19% of the strains (SMJ12, SMJ17, SMJ30, and SMJ32) presented 4 out of five properties. SMJ12, SMJ18, and SMJ20 were found to be prominent IAA producers (4.83, 4.52, and 5.18 mg/ml respectively) whereas SMJ30 managed the best siderophores formation capacity and SMJ32 the most notably phosphate solubilization.

Selection of the Best-Performing Endophytic Strains for Pot Inoculation Under Greenhouse Conditions

Micrococcus yunnanensis SMJ12, *Vibrio sagamiensis* SMJ18, and *Salinicola peritrichatus* SMJ30 were selected as the best-performing strains, based on their enzymatic and PGP properties, heavy metals resistance and salt tolerance. While SMJ12 managed the best auxins production and had a marked resistance to As and Cu, SMJ30 had a powerful siderophores formation capacity as well as P solubilization, demonstrating high tolerance to Zn, Pb, and NaCl. Finally, strain SMJ18 was selected by its auxins production and its prominent enzymatic properties, together with a notable resistance to Ni and Co. The three bacterial isolates were cultivated together and no antagonistic activity between them was observed (data not shown). Despite that SMJ28 presented all the PGP properties studied, including the beneficial ACC deaminase activity, its general metal resistance was very poor, probably because it was isolated from the non-contaminated estuary. Then, it was not considered for this bacterial consortium.

Effect of Inoculation on Plant Growth and Physiological Parameters

Plant inoculation with endophytes increased the relative growth rate (RGR) of *S. maritima* 25% after 30 days of treatment

(T-test, $P < 0.05$; **Figure 4A**). This positive effect was restricted to belowground biomass increment (T-test, $P < 0.05$), whereas aboveground biomass did not vary respect to plants grown without bacterial inoculation (**Figure 4B**). Respect to gas exchange measurements, net photosynthetic rate (A_N) values were greater in plants grown in soil inoculated with the endophytes (E+), with an increment of 42% after 30 days of treatment (T-test, $P < 0.05$; **Figure 5A**). Also stomatal conductance (gs) showed a similar trend to that of net photosynthetic rate (A_N) (T-test, $P < 0.05$; **Figure 5B**). Whereas intercellular CO_2 concentration (C_i) values did not differ between treatments after 30 days of experiment, with values c. 135 μmol CO_2 mol^{-1} air in both situations (**Figure 5C**). Furthermore, water use efficiency (iWUE) showed an increment of 15% in plants grown in inoculated soil (E+) after 30 days of treatment (T-test, $P < 0.05$; **Figure 5D**). Finally our fluorescence analysis showed that maximum quantum efficiency of PSII photochemistry (F_v/F_m) and quantum efficiency of PSII (Φ_{PSII}) values at midday were greater in plants grown in soils inoculated with the endophytes (E+) after 30 days of treatment (T-test, $P < 0.05$; **Figures 6A,B**), whereas at dawn both parameters did not show significant differences between treatments with values c. 0.80 in all cases (data not shown).

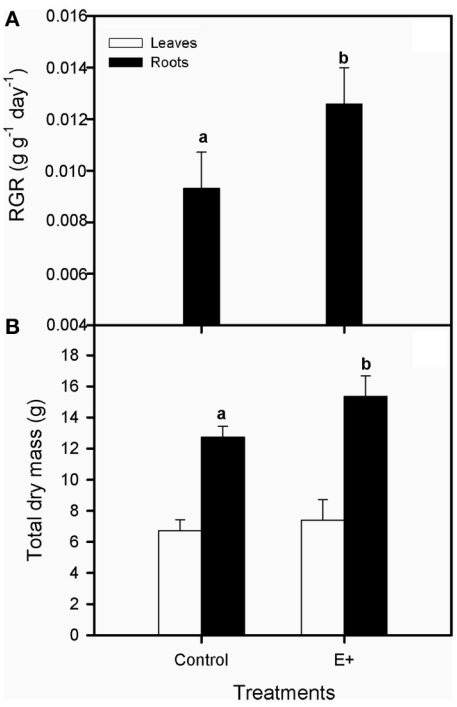

FIGURE 4 | Effect of inoculation (control, without inoculation; E+, inoculations repeated once a week during experimental period) with a bacterial consortium integrated by *Micrococcus yunnanensis* SMJ12, *Vibrio sagamiensis* SMJ18 and *Salinicola peritrichatus* SMJ30 on relative growth rate, RGR (A) and aboveground biomass and belowground biomass (B) in *Spartina maritima* plants grown in natural soil from Tinto marsh for 30 days. Values are means ± s.e. ($n = 10$). Statistical differences between means are indicated by different letters ($P < 0.05$).

Ions Concentrations in Plant Tissues after Inoculation

At the end of the experiment, ion concentrations were greater in roots than in leaves of *S. maritima* in both treatments (t-test, $P < 0.01$; **Figures 7A–F**). Regarding the effect of soil inoculation on tissues ions concentrations, our results revealed that overall, concentration in roots and leaves decreased in plants grown in inoculated soil after 30 days of treatment (T-test, $P < 0.05$; **Figures 7A–F**). Thus, compared to the control, these decreases in roots and leaves ions concentrations (E+), were 22 and 14% for Cu, 15 and 24% for Ni, 28 and 19% for Pb and 19 and 17% for Zn, respectively. Decreases of 20% for As concentration in leaves and 12% for Cd in roots were also recorded. Nevertheless, considering the increments in biomass and the decrease in metal accumulation, no significant differences were observed in total metal balance in plants and soil between inoculated and non-inoculated plants (Supplementary Table 4).

Assessment of Endophytic Colonization in *S. maritima* Tissues

Using confocal laser scanning microscopy (CLSM), mCherry-tagged *Salinicola peritrichatus* SMJ30 cells were visualized from 0.5 mm slices of *S. maritima* stem (**Figure 8**). Roots and leaves fluoresced intensely, hence hindering the visualization of the bacteria. On the other hand, fluorescent colonies grew in TSA plates with rifampicin and tetracycline, while two different types of colonies grew in TSA plates amended with rifampicin and streptomycin. They were identified by morphological traits (macroscopic and microscopic observation) and biochemical traits (API® identification products (bioMérieux, France), obtaining the same results as the strains included in the original consortium. Hence, colonization of *S. maritima* tissues by the endophytic consortium was confirmed. In addition, CFU/gr of tissue and soil were estimated. For soil samples, no strains of SMJ30 or SMJ12 were detected in plates, while 3.5×10^2 CFU of SMJ18 per gram were recorded. Roots were the most populated tissue by the endophytic consortium, as 3.4×10^2 CFU of SMJ30, 2.1×10^3 CFU of SMJ12 and 6.2×10^3 CFU of SMJ18 per gram of tissue were estimated. Regarding the stem, 2.5×10^3 and 2.2×10^2 CFU/gr for SMJ30 and SMJ12, respectively, were registered whereas no colonies of SMJ18 appeared. Finally, leaves were the less re-colonized tissue, 30 and 90 CFU/gr for strains SMJ30 and SMJ12, respectively, were counted and, as the case of the stem, no colonies of SMJ18 were detected.

DISCUSSION

The estuarine sediments are important ecosystems that are largely influenced by plant activity (Almeida et al., 2006). In southwest coast of Spain, *S. maritima* is an endangered indigenous plant frequently used to restore degraded and contaminated salt marshes (Castillo and Figueroa, 2009). This species is included in European and National (Spanish) red lists which propose endangered species to be conserved (Cabezudo et al., 2005), since it is being displaced by the invasive *S. densiflora* (Castillo et al., 2008). Under these circumstances, intervention

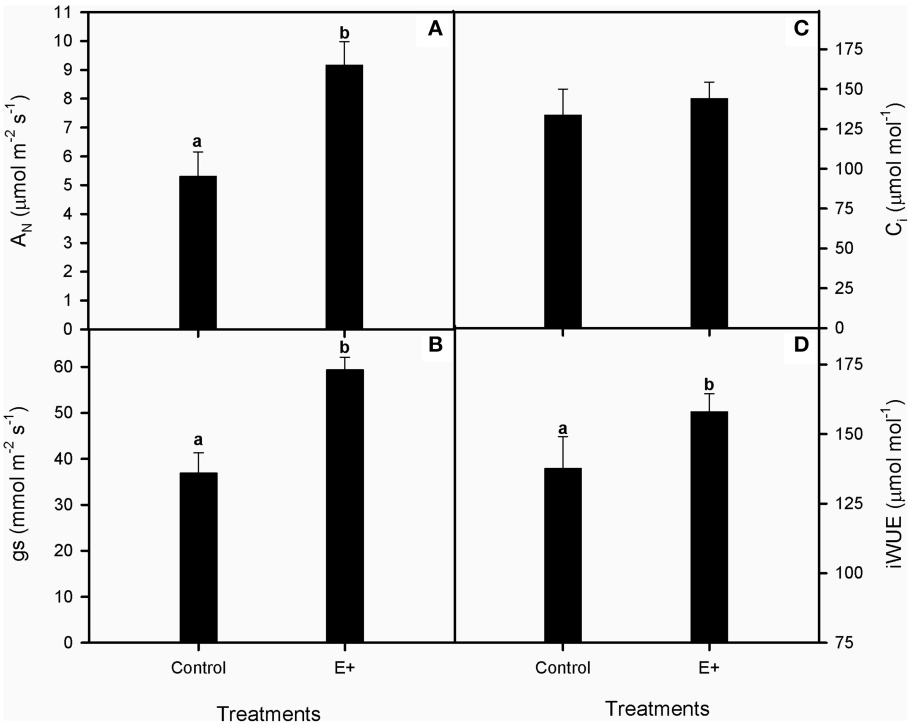

FIGURE 5 | Effect of inoculation with the endophytic bacterial consortium on net photosynthetic rate, A_N (A), stomatal conductance, gs (B), intercellular CO_2 concentration, C_i (C) and intrinsic water-use efficiency, iWUE (D) in leaves of *Spartina maritima* plants grown in natural soil from Tinto marsh for 30 days. Values are means ± s.e. ($n = 10$). Statistical differences between means are indicated by different letters ($P < 0.05$).

is needed. We propose the use of *S. maritima* and the cultivable bacteria associated to this plant as an ecological tool to regenerate contaminated marshes of southwest coast of Spain.

There is a extend literature on the use of PGPB as inoculants for improvement of plant growth in metal contaminated soils. One important conclusion that can be extracted from the analysis of previous results is that certain rhizospheric and endophytic bacteria have the ability to promote plant growth under stress conditions, but the effect of microbial inoculation on plant metal uptake cannot be predicted, since it depends on the specific plant-microbe partnerships and the characteristics of the soil and the contaminant (Sessitsch et al., 2013; Phieler et al., 2014; and references therein). For example, inoculation of plants with *Pseudomonas aeruginosa*, *P. fluorescens* and *Ralstonia metallidurans* strains enhanced Cr and Pb uptake by plants (Braud et al., 2009). Nevertheless, inoculation of *Phaseolus vulgaris* with a *Pseudomonas putida* strain reduced Cd and Pb accumulation in the plant (Tripathi et al., 2005).

The use of PGPR to promote the growth of *Spartina* plants in contaminated or degraded salt marshes have been recently proposed (Andrades-Moreno et al., 2014; Mateos-Naranjo et al., 2015; Mesa et al., 2015b). In one of these works, *S. maritima* wild plants were inoculated with indigenous PGP rhizobacteria (Mesa et al., 2015b). Rhizospheric bacteria increased the capacity of the plant to hyperaccumulate heavy metals by a variety of direct mechanisms, including enhanced heavy-metal mobilization and alleviation of heavy-metal toxicity to the plant, and indirect mechanisms comprising plant growth promotion and improved stress tolerance. Authors concluded that the rhizospheric consortium significantly enhanced the efficiency of metal rhizoaccumulation from natural soils by increasing both *S. maritima* belowground biomass and metal accumulation. Finally, the strategy was proposed as useful to enhance plant adaptation and metal rhizoaccumulation during marsh restoration programs.

Although scientists have mainly focused their research on plant-rhizobacteria interactions, endophytes may offer several competitive advantages over them, from their close and continued contact with plants. Bacterial endophytes are defined as those bacteria that colonize the inner parts of their host plants without causing disease symptoms (Hallmann et al., 1997; Schulz and Boyle, 2006). They have less competition from the surrounding microbes and the plant provides their nutrients. Furthermore, toxic pollutants taken up by the plant may be degraded in planta by endophytes reducing the toxic effects of contaminants in environmental soil on flora and fauna (Khan and Doty, 2011). On top of that, other beneficial effects attributed to endophytes include osmotic adjustment, stomatal regulation, modification of root morphology and enhanced uptake of minerals (Compant et al., 2005; Rajkumar et al., 2009). Finally, some metabolites are not only produced by a single organism, but might be produced by a plant associated with microorganisms (Brader et al., 2014). Hence, attention has focused in the last years on the role of endophytic bacteria in

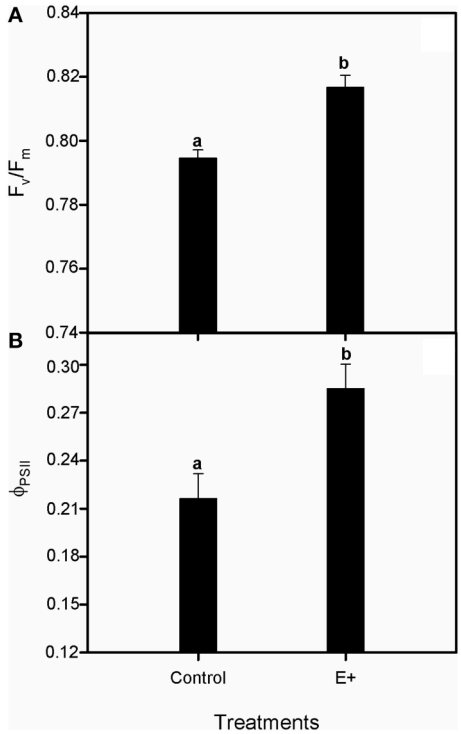

FIGURE 6 | Effect of inoculation with the endophytic bacterial consortium on maximum quantum efficiency of PSII photochemistry, F_V/F_m (A) and quantum efficiency, Φ_{PSII} (B) in leaves of *Spartina maritima* plants grown in natural soil from Tinto marsh for 30 days. Values are means ± s.e. ($n = 10$). Statistical differences between means are indicated by different letters ($P < 0.05$).

phytoremediation of contaminated soils (reviewed by Newman and Reynolds, 2005; Doty, 2008) and their use has been reported by several authors (reviewed in Rajkumar et al., 2009). The interactions between endophytes and hyperaccumulator plants have attracted the attention of several researchers, allowing the study of bacterial communities living on a naturally contaminated environment and their possible biotechnological applications for bioremediation (reviewed in Lodewyckx et al., 2002; Sessitsch et al., 2013; Visioli et al., 2014, 2015; Ma et al., 2015).

In this work, endophytic bacteria from the halophyte cordgrass *S. maritima* growing in polluted salt marshes in southwest Spain were studied. To our knowledge, this is the first work describing the endophytic populations of a halophyte growing in metal contaminated estuaries. On the whole, 25 strains were isolated. It was not surprising the extended bacterial resistance to copper, because is the most common heavy metal whether in soil (up to 3000 mg/kg) (Mesa et al., 2015a) or inside the plant (up to 545.47 mg/kg). Other prominent MTC values were observed for Co (30 mM), Ni (35 mM), Pb (28 mM) or As (100 mM) in the arsenite form, 4–100 times more toxic than arsenate. The amounts of arsenic found in plant tissue are generally proportional to its level in soil. It affects seed germination, and reduces root length and mass (Nie et al., 2002). Arsenic is one of the metal contaminants in soil that requires remediation (Consejería de Medio Ambiente, Junta de Andalucía, 1999), so further studies to describe the arsenite resistance mechanism in this isolate are being developed.

Endophytic enzymatic activities may aid in penetration and colonization of the host plant, as well as intervention in degradation of plant residues and plant nutrient acquisition (Wang and Dai, 2010). Among the isolates, 84% exhibited at least one hydrolytic enzyme activity out of five, thus demonstrating that endophytic bacteria can be an important source of a variety of enzymes. Concerning PGP properties, 76% of the strains had at least one of them. Endophytic PGPB may benefit plant growth increasing the accessibility or supply of major nutrients (Bashan, 1998). The production and modulation of auxins and ethylene play an essential role in plant development and stress tolerance (Brader et al., 2014). In addition, a well-studied form of biofertilization is nitrogen fixation, which is the conversion of atmospheric nitrogen to ammonia (Bloemberg and Lugtenberg, 2001). Moreover, some endophytic PGPB can increase phosphorus availability to the plant through phosphorus solubilization (Kpomblekou-A and Tabatabai, 2003). Finally, regarding biocontrol several mechanisms may be involved, including the production of siderophores or antibiotics (Gaiero et al., 2013).

Considering all the properties studied, the best performing endophytic strains were selected: *Micrococcus yunnanensis* SMJ12, *Vibrio sagamiensis* SMJ18, and *Salinicola peritrichatus* SMJ30. A mixture of naturally cohabitating endophytes may be a better alternative than applying an individual endophyte species, because different species may fulfill different ecological niches (Gaiero et al., 2013). It should also be emphasized that plant re-colonization would be apparently more feasible for endogenous bacteria than for exogenous ones. In this context, colonization of *S. maritima* tissues by the bacterial consortium was demonstrated. Strain SMJ30 was labeled with a fluorescent protein gene marker. This kind of genes has been widely used to visualize and track the colonization patterns of bacterial strains within inoculated host plants (Lagendijk et al., 2010). The presence of inoculated SMJ12 and SMJ18 strains was revealed using double antibiotic resistance selection, since it was not possible to tag these strains with marker genes. On the other hand, results suggested that different endophytic strains had preference for different plant tissues. For example, in this study strain SMJ18 was isolated from roots of *S. maritima* (Supplemetary Table 1) and after re-inoculation no presence of this strain in stems of leaves was observed. Strain SMJ12, isolated from *S. maritima* leaves (Supplemetary Table 1), was detected in all the tissues, probably due to the time spendt by the bacteria in its travel from the soil to the leaves. What is more, each strain is present in greater number in the tissue from which it was first isolated (SMJ18 in roots, SMJ30 in stems and SMJ12 in leaves). Even so, a greater number of CFU/gr was expected for leaves. Another remarkable point is the presence of the consortium in soil. Strain SMJ18, abundant in roots, was also growing in surrounding soil. However, SMJ12 and SMJ30, isolated from leaves and stem respectively (Supplementary Table 1), were not found in soil after 3 weeks of the last inoculation. These bacteria

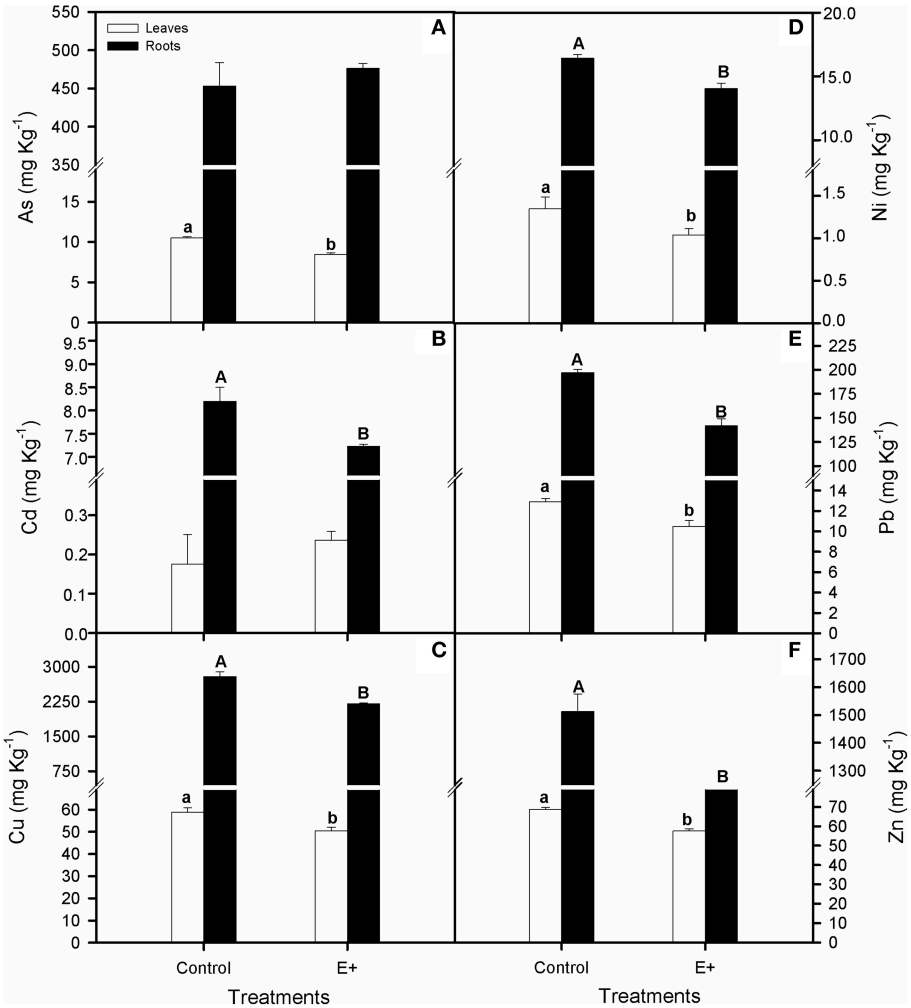

FIGURE 7 | Effect of inoculation with the endophytic bacterial consortium on total arsenic (A), cadmium (B), copper (C), nickel (D), lead (E), and zinc (F) for leaves and roots of *Spartina maritima* plants grown in natural soil from Tinto marsh for 30 days. Values are means ± s.e. ($n = 10$). Different capital letters indicate statistical differences between inoculation treatments in roots and different lower case letters in leaves ($P < 0.05$).

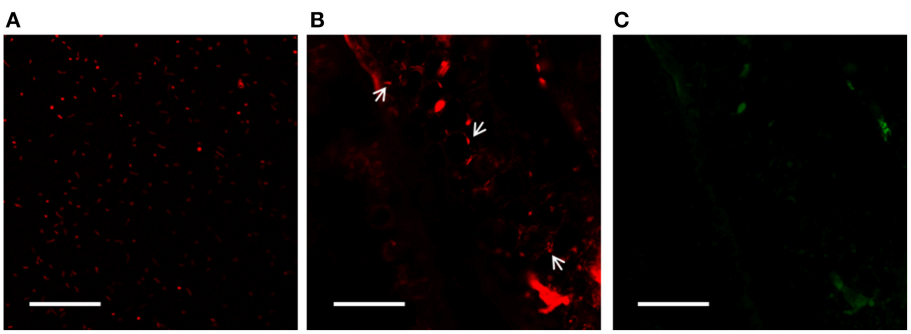

FIGURE 8 | CLSM analysis. Images of mCherry-tagged *Salinicola peritrichatus* SMJ30 strains **(A)** and colonized *Spartina maritima* stems after 25 days of growth and inoculation with the endophytic consortium **(B, C)**. White arrows in **(B)** show bacteria colonizing the stem. Images were taken with different laser conditions to discriminate tissue fluorescence: **(B)** excitation 568–585 nm—long pass emission for red fluorescence; **(C)** excitation 488 nm—emission 522/35 nm for green fluorescence. Images were processed with ZEN Lite 2012 software. Scales bar represent 50 μm.

probably find better growing conditions inside the aerial part of the plant and leave the rhizospheric soil. This data may be considered preliminary, as a better study *in situ* in the salt marsh during a longer period of time is required to establish a well-founded assertion. Usually, greenhouse conditions limit several experimental procedures, as time or tidal flooding.

The inoculation with the selected endophytic bacterial consortium had a positive effect on the growth of *S. maritima*. Despite the fact that the RGR increased, this effect was restricted to belowground biomass. Root elongation has in fact been described as one of the major roles of PGPB (Glick, 2003). There is strong evidence that endophytic PGPB influences overall plant performance, but their detailed effects on photosynthesis, the basis of plant bio-chemical system, under metal stress is very scarce. Hence, in this study the effect of plant inoculation with the endophytic bacterial consortium on the photosynthetic apparatus of *S. maritima* was also analyzed. Obtained results suggested that the increase in growth can be attributed to the improvement in the photosynthetic carbon assimilation. Increased A_N values for the inoculation treatment were associated with an increment in gs. The joint increase in A_N and gs resulted in the augmentation of iWUE of inoculated plants. iWUE reflects the trade-off between CO_2 acquisition for growth and water losses, and is therefore an important indicator of how plants manage water under stress conditions (Tardieu, 2012). Increased root-to-shoot ratio, through increased root growth in inoculated *S. maritima*, would contribute to increase the capacity for water absorption from the soil (Boyer, 1985) and, consequently, iWUE and metal tolerance. Also the functionality of PSII reflected a beneficial effect of inoculation with the endophytic bacterial consortium on the photosynthetic apparatus of *S. maritima*, as indicated by higher values of F_v/F_m and Φ_{PSII} at midday, as well as increased chlorophyll pigments concentrations.

Moving on to metal accumulation, our results showed that the plant growth promotion caused by bacterial inoculation was also accompanied by an overall decrease in the concentration of metals in *S. maritima* tissues, denoting a lower rhizoaccumulation capacity than the one previously described (Cambrollé et al., 2008; Redondo-Gómez, 2012). These results suggest that the endophytic consortium appears to have a protective role against the presence of heavy metals in soil, lessening their uptake by *S. maritima* roots. Since plants were inoculated every week, this result could be due to a rizospheric effect of the bacteria before entering the plant. Metals could be complexed, precipitated and/or adsorbed onto bacterial surface in the rizosphere, thus reducing plant metal availability (reviewed in Sessitsch et al., 2013). Future research is needed to clarify mechanisms behind this effect. The decrement of metal concentration in tissues, as well as the presence of PGP properties in the bacterial consortium used in this experiment could explain the positive effect on *S. maritima* growth. Nevertheless, when total metal balance in plants and soil was estimated, no significant differences were observed between inoculated and non-inoculated plants, since the decrease in metal accumulation was accompanied by the increment in plant biomass. Furthermore, total As and Cd accumulated in inoculated plants was even higher than in non-inoculated plants after 1 month of plant growth (Supplementary Table 4).

In conclusion, we have designed an endophytic consortium that could be useful to promote *S. maritima* adaptation and growth in contaminated salt marshes. Inoculation with this consortium was not useful to increase plant metal accumulation, but could be a complementary strategy during marsh restoration programs, in situations where the increase of plant metal uptake is not desirable. For example, *S. maritima* plants could be first inoculated with the rhizospheric bacterial consortium previously described (Mesa et al., 2015b) and then, once they have loaded their roots with metals, inoculated with endophytic bacteria, facilitating plant growth without increasing metal content in roots in excess. In addition, inoculation with endophytes could also be an adequate strategy to promote *S. maritima* growth in non-contaminated salt-marshes, such as Piedras river estuary. Anyway, it is first necessary to test the effect of bacterial inoculation during longer periods, since conclusions until date have been extracted 30 days after inoculation. For this, a system to keep healthy *S. maritima* plants for more than 1 month in greenhouse conditions is being developed. In addition, an *in situ* experiment for marsh restoration in the south western coast of Spain, using autochthonous plants and the associated PGPB described in this work and those previously reported (Mesa et al., 2015b), is being designed.

AUTHOR CONTRIBUTIONS

JM and EM performed most of the experimental work, data collection and analysis. JM, IR, and EM wrote the manuscript. All authors participated in the design of the study and took part in the evaluation of the results. All authors read and approved the final version of the manuscript to be published.

ACKNOWLEDGMENTS

This project was funded by *Junta de Andalucía* under MARISMA project P11-RNM-7274MO, INIA project RTA2012-00006-C03-03 and VPPI-US project from University of Sevilla. JM acknowledges financial support from the FPU grant (ref. AP2012-1809) awarded by *Ministerio de Educación, Cultura y Deporte*, Spain. Authors are grateful to University of Seville Greenhouse General Services and Microscopy Service (CITIUS) for their collaboration.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01450

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ORIGINAL RESEARCH
published: 04 February 2016
doi: 10.3389/fpls.2016.00075

Bioaugmentation with Endophytic Bacterium E6S Homologous to *Achromobacter piechaudii* Enhances Metal Rhizoaccumulation in Host *Sedum plumbizincicola*

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OPEN ACCESS

Edited by:
Vincenzo Lionetti, Sapienza – Universitá di Roma, Italy

Reviewed by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *25 November 2015*
Accepted: *16 January 2016*
Published: *04 February 2016*

Citation:
Ma Y, Zhang C, Oliveira RS, Freitas H and Luo Y (2016) Bioaugmentation with Endophytic Bacterium E6S Homologous to Achromobacter piechaudii Enhances Metal Rhizoaccumulation in Host Sedum plumbizincicola. Front. Plant Sci. 7:75. doi: 10.3389/fpls.2016.00075

Application of hyperaccumulator-endophyte symbiotic systems is a potential approach to improve phytoremediation efficiency, since some beneficial endophytic bacteria are able to detoxify heavy metals, alter metal solubility in soil, and facilitate plant growth. The objective of this study was to isolate multi-metal resistant and plant beneficial endophytic bacteria and to evaluate their role in enhancing plant growth and metal accumulation/translocation. The metal resistant endophytic bacterial strain E6S was isolated from stems of the Zn/Cd hyperaccumulator plant *Sedum plumbizincicola* growing in metalliferous mine soils using Dworkin and Foster salts minimal agar medium with 1-aminocyclopropane-1-carboxylate (ACC) as the sole nitrogen source, and identified as homologous to *Achromobacter piechaudii* based on morphological and biochemical characteristics, partial 16S rDNA sequence and phylogenetic analysis. Strain E6S showed high level of resistance to various metals (Cd, Zn, and Pb). Besides utilizing ACC, strain E6S exhibited plant beneficial traits, such as solubilization of phosphate and production of indole-3-acetic acid. Inoculation with E6S significantly increased the bioavailability of Cd, Zn, and Pb in soil. In addition, bacterial cells bound considerable amounts of metal ions in the following order: Zn > Cd >Pb. Inoculation of E6S significantly stimulated plant biomass, uptake and bioaccumulation of Cd, Zn, and Pb. However, E6S greatly reduced the root to shoot translocation of Cd and Zn, indicating that bacterial inoculation assisted the host plant to uptake and store heavy metals in its root system. Inoculation with the endophytic bacterium E6S homologous to *A. piechaudii* can improve phytostabilization of metalliferous soils due to its effective ability to enhance *in situ* metal rhizoaccumulation in plants.

Keywords: endophytic bacterium, rhizoaccumulation, multi-metal contamination, phytostabilization, *Sedum plumbizincicola*

INTRODUCTION

Mining activities produce waste tailings containing high levels of metal pollutants that have significant environmental impacts and can affect human health through the food chain (Moreno et al., 2010). During mineral ore processing mine tailings are generated and mostly left without proper management, therefore leading to metal contamination of surrounding soils, with detrimental impacts on the soil microbial community and consequent reduction in ecosystem functioning (Kossoff et al., 2014).

Phytoremediation, application of plants to remove (phytoextraction), stabilize (phytostabilization), or volatilize (phytovolatilization) heavy metals *in situ* in a more attractive and cost-effective manner than the conventional physicochemical technologies, has received increasing attention over the last decades (Raskin and Ensley, 2000). Particularly, phytostabilization of metal contaminated mine tailings, which uses plants that minimize metal accumulation into aboveground tissues, seems to be most promising for remediating polluted sites when phytoextraction is not a feasible option (Mendez and Maier, 2008). Because mine tailings have low nutrient contents, to overcome the limitations of plant establishment, soil amendments with fresh or composted organic matter (biochemical amendments) can enhance plant colonization and reduce metal toxicity and solubility, thereafter improving phytostabilization efficiency (Lee et al., 2011). However, most of those biochemical amendments, such as cyclonic ashes, steel shots. and superphosphate, are toxic to plants and their associated microbes (Ribeiro Filho et al., 2011). Some beneficial bacteria have been successfully employed for environmental applications (so-called bioaugmentation) due to their ability to: (1) promote plant growth by producing beneficial metabolites [e.g., siderophores, indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase] and solubilizing phosphate (P); and/or (2) alter soil metal mobility without disturbing soil ecological structure and function through various mechanisms such as metal biosorption/bioaccumulation, redox reaction, chelation, or complexation (Glick, 2010; Ma et al., 2011a). Amongst all the beneficial features, the production of ACC deaminase is considered as one of the major plant growth promoting traits of bacteria (Ma et al., 2011a). Mesa et al. (2015) reported that the inoculation of autochthonous rhizobacteria stimulated the biomass of native *Spartina maritima*, and enhanced metal (As, Cu, Pb, and Zn) accumulation in the root of plants (rhizoaccumulation) grown in multi-metal contaminated soils. Since bacterial endophytes have more intimate association with host plants than rhizobacteria, they could be reliable bioinoculants for improving metal phytostabilization.

Although the effects of inoculating endophytic bacterial strains on growth promotion of various host and/or non-host plants have been reported (Ma et al., 2011b; Shin et al., 2012; Visioli et al., 2014), little is known on application of endophytes for enhanced phytoremediation of natural non-sterile polluted soils. *Sedum plumbizincicola* is known to hyper-accumulate or extract Cd and Zn from soils (Jiang et al., 2010). Despite the potential of *S. plumbizincicola* to remove various metals from polluted soil, its slow growth is a limitation that needs to be overcome.

The objectives of this study were to: (1) isolate and characterize metal resistant endophytic bacteria that can utilize ACC as the sole nitrogen (N) source; (2) assess plant beneficial activities, metal biosorption and mobilization capacities of a selected endophytic bacterium; (3) examine the effect of the endophytic bacterium on plant growth and metal accumulation/translocation in host *S. plumbizincicola* in non-sterile multi-metal contaminated soils.

MATERIALS AND METHODS

Isolation and Identification of ACC-Utilizing Endophytic Bacterium

Bacterial strains were isolated from tissues of Zn/Cd hyperaccumulator *S. plumbizincicola* growing on metalliferous mine soils in Chunan city of Zhejiang, Southeast of China. Plant samples were washed thoroughly with tap water followed by three rinses with deionized water and then separated into roots, stems, and leaves. Plant organs were sterilized by immersion for 1 min in 70% (v/v) ethanol, and then 3% sodium hypochlorite for 3 min and washed three times with sterile water to remove residual chemicals. To confirm the success of the surface sterilization process, plant tissues were plated on Luria–Bertani (LB) agar plate to detect epiphytic bacteria. No contamination was found. The plant tissues (0.5 g) were ground with a mortar and pestle in 5 mL of sterile deionized water. The appropriate dilutions were plated onto sucrose-minimal salts low-phosphate agar medium supplemented with 100 mg L^{-1} of Cd ($CdCl_2$), Zn ($ZnSO_4$), and Pb [$Pb(NO_3)_2$]. After incubating at 26°C for 5 days, colonies were randomly picked based on distinct colony morphology, purified and re-streaked on the same media. To isolate beneficial endophytes, the growth of all metal resistant isolates was evaluated on Dworkin and Foster salts minimal medium (Dworkin and Foster, 1958) containing ACC as the sole N source.

One bacterial isolate which showed fast growth and capability of utilizing ACC as the sole N source was identified and used for further study. Total DNA was extracted from cells of the selected bacterial isolate using the QuickExtract^{TM} bacterial DNA extraction kit. The 16S ribosomal gene was amplified using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) under the reaction conditions described by Branco et al. (2005). The amplification product (5 μL) was separated by agarose gel (1%, w/v) electrophoresis in TAE buffer (0.04 M Tris acetate, 1 mM EDTA) containing 1 μg mL^{-1} ethidium bromide. Partial nucleotide sequence of the amplified 16S rDNA was determined with automated DNA sequencer and then compared with similar sequences in the GenBank using BLAST.

Biochemical Characterization of Endophytic Bacterium

The bacterial isolate was grown for 5 days at 26°C on LB media supplemented with heavy metal (Cd, Zn, and Pb) at varying concentrations (50–1500 mg L^{-1}). The highest concentration of metal allowing bacterial growth was defined as its resistance level. The antibiotic sensitivity of the isolate was determined by the disk diffusion method (Rajkumar et al., 2008).

The ACC deaminase activity of the isolate was examined by monitoring the concentration of α-ketobutyrate (α-KB) generated through the enzymatic hydrolysis of ACC as described by Honma and Shimomura (1978). The protein content of cell suspensions was determined by the Bradford method (Bradford, 1976). Synthesis of IAA by the strain was assayed as described by Bric et al. (1991) using LB medium with 0.5 mg mL^{-1} of *L*-tryptophan. Bacterial siderophore production was detected by chrome azurol S (CAS) agar plate assay (Schwyn and Neilands, 1987). The P solubilization by the isolate was analyzed in modified Pikovskayas medium (Sundara-Rao and Sinha, 1963) and the solubilized P in the culture supernatant was determined as described by Park et al. (2011).

Metal Mobilization and Biosorption Analyses

In the metal mobilization assay, the physicochemical properties of contaminated agricultural soil collected from Fuyang city of Zhejiang Province, China were: pH (1:1 w/v water) 8.1; organic matter 36.3 g kg^{-1}; cation exchange capacity 11.4 cmol kg^{-1}; Cd 5.9 mg kg^{-1}; Zn 736 mg kg^{-1}; Pb 153 mg kg^{-1}. The soil was air dried, crushed (<2 mm) and then autoclaved at 121°C for 2 h. The bacterial strain was incubated at 27°C with shaking at 200 rpm for 18 h. After adjusting cell density to an OD_{600} of 1.5, 5 mL of culture were centrifuged, then gently washed twice with 0.05 M phosphate buffer (pH 7.2) and three times with sterile deionized water, recentrifuged and finally resuspended in 5 mL sterile deionized water. One milliliter of washed bacterial culture at an OD_{600} of 1 (treatment) or sterile deionized water (control), was added to 1 g of sterilized soil in 50 mL sealed centrifuge tubes. All tubes were weighed and kept at 27°C with 200 rpm in the dark. The total weight was kept constant by adding sterile deionized water to compensate for evaporation. After 7 days, 10 mL of sterile deionized water were added to extract water-soluble metal from soil. The suspensions were centrifuged at 7000 rpm for 10 min and filtered. Metal (Cd, Zn, and Pb) contents of bioavailable fraction in each filtrate were determined using a flame atomic absorption spectrophotometer (AAS; Ma et al., 2011b).

The biosorption of Cd, Zn, and Pb by bacterial cells was evaluated as described by Rajkumar et al. (2009). The bacterium was grown to exponential ($OD_{600} = 1$) at 27°C in LB medium. Cells were harvested by centrifugation at 6000 rpm for 20 min and washed twice with sterile deionized water. The wet biomass was re-suspended in 150 mg L^{-1} of Cd, Zn, or Pb. Cells were harvested after incubation at room temperature for 10 h by centrifugation and the residual metal ion in the supernatant was measured by AAS. The amount of metal biosorbed onto bacterial cells was calculated by subtracting the metal concentration in the supernatant from the original concentration.

Microcosm Experiments

The selected ACC-utilizing strain was initially assessed for its ability to promote plant growth in phytagar media supplied with or without 10 mg Cd L^{-1} using *Brassica napus* as a model plant (Ma et al., 2011b). For pot experiment, the multi-metal contaminated soil described above was used, while agricultural soil from Nanjing, China was used for comparison. The soil was air dried, sieved (2 mm), and stored at 20°C before use. *S. plumbizincicola* seedlings of equal size were obtained from an old Pb/Zn mine in the Zhejiang province of China. Shoot samples with ca. 5 cm were well cleaned with tap water and grown hydroponically in half-strength Hoagland's nutrient solution for a week. Before inoculation, bacterial colony marked with antibiotic resistance was obtained after incubating parental strain onto LB agar containing ampicillin (100 mg L^{-1}) and tetracycline (75 mg L^{-1}), simultaneously. Surface sterilized roots of precultured seedlings were soaked for 2 h in the bacterial culture (OD_{600} of 1.5) or sterile water (controls) and transplanted into 1 L pot filled with 750 g of soil. The seedlings (six plants pot^{-1}) were allowed to grow under greenhouse condition (25 ± 5°C, 16:8 day/night regime). Each treatment was performed in five replicates. After 75 days, plants were carefully removed from the pots and the root surface was cleaned thoroughly with deionized water. Plant root and shoot length, fresh and dry weight were measured. The concentrations of Cd, Zn, and Pb in root and shoot were quantified as described by Ma et al. (2009). The translocation factor (TF) was calculated as the ratio of metal content in the shoots to that in the roots (Malik et al., 2010) and the bioaccumulation factor (BCF) was calculated as the ratio of metal contents in the entire plant to that in the soil (Abdul and Thomas, 2009). The colonization of introduced strain in the rhizosphere and interior tissues of *S. plumbizincicola* was determined using the intrinsic antibiotic marker combined with the dilution-plate method (Ma et al., 2011b).

Statistical Analysis

Student's *t*-test ($p < 0.05$) or Analysis of Variance (ANOVA) followed by the *post hoc* Fisher Least Significant Difference test ($p < 0.05$) were used to compare treatment means. All the statistical analyses were performed using SPSS 17.0.

RESULTS AND DISCUSSION

Isolation and Identification of Metal Resistant and ACC-Utilizing Endophytic Bacterium

Although the interaction between endophytic bacteria and their host plants is not completely understood, some endophytic bacteria isolated from heavy metal hyperaccumulators appear to exert beneficial effects on their hosts (Shin et al., 2012),

such as amelioration of metal stress, stimulation of plant establishment and growth, and biocontrol of phytopathogens (Ma et al., 2011a). Consequently, such beneficial endophytic bacteria could be isolated and selected for their application in assisting phytoremediation of metal contaminated soils (Rajkumar et al., 2009). In this study, we isolated a metal resistant endophytic bacterial strain from stems of Zn/Cd hyperaccumulator *S. plumbizincicola* and assessed its effect as a bioinoculant on multi-metal phytoremediation by its host plant. The initial screening was based on the morphological differences of bacterial colonies and resulted in the isolation of 42 metal (100 mg L^{-1} of Cd, Zn, and Pb) resistant endophytic strains from interior tissues of *S. plumbizincicola*. Out of 42 strains, isolate E6S was specifically chosen based on its fast growth and capability of utilizing ACC as the sole N source.

Based on morphological and biochemical characteristics (**Table 1**), comparative analysis of 16S rDNA sequence and phylogenic analysis (**Figure 1**), strain E6S was identified as being homologous to *Achromobacter piechaudii* (100% similarity). The sequence obtained (858 bp) was submitted to the NCBI databases under the accession number KC151254. Strain E6S was gram-negative, motile, rod shaped and positive for oxidase and catalase. It was able to produce H_2S, utilize citrate and hydrolyze starch (**Table 1**).

TABLE 1 | Morphological, physiological, and biochemical characteristics of endophytic bacterium E6S homologous to *Achromobacter piechaudii*.

Characteristic	E6S
Gram staining	−
Cell shape	Rod
Oxygen requirements	Aerobic
Motile	+
Growth at 15–40°C	+
Growth at 6% NaCl	+
Oxidase	+
Catalase	+
Indole production	−
Voges–Proskauer test	−
H_2S production	+
Nitrate reduction	+
Nitrite reduction	−
Utilization of Arabinose	−
Mannitol	−
Maltose	−
Glucose	−
Citrate	+
Lactate	−
Hydrolysis of Casein	−
Starch	+
Gelatin	−
Esculin	−

+, *positive*; –, *negative*.

Biochemical Properties of Endophytic Bacterium

Under various abiotic stresses (e.g., heavy metals, drought, and salinity), microorganisms face a constant battle for limited resources and try to adapt to unfavorable environmental conditions by acting as stress ameliorators (Hibbing et al., 2010). Hence, strain E6S was tested for the ability to grow on both metal and antibiotic-supplemented agar media. Strain E6S was found to exhibit resistance to heavy metal (Cd, Zn, and Pb) and various antibiotics (**Table 2**). The order of the toxicity of metals to the isolate was found to be Cd > Zn > Pb. This strain exhibited high tolerance to multiple metals, which could be attributed to the fact that it was isolated from tissues of a Zn/Cd hyperaccumulator plant, likely containing high levels of these bioavailable metal ions (Idris et al., 2004). Among six antibiotics tested, strain E6S showed resistance to ampicillin and tetracycline.

Endophytic bacteria can stimulate plant growth directly by solubilizing unavailable nutrients (e.g., P, N, and potassium), sequestering iron by siderophores and phytohormones (e.g., IAA) or indirectly by inducing a systemic resistance in plants against various types of pathogens (Ma et al., 2011b). These features make them perfect choices for improving phytoremediation. Strain E6S was able to produce ACC deaminase, IAA and solubilize P, whereas no biosynthesis of siderophore was found (**Table 2**). In general, bacterial IAA at low level has been implicated in promoting primary root elongation through cell division, however, a high IAA level can inhibit primary root growth but stimulate lateral root formation (Gravel et al., 2007). A low level of IAA production by strain E6S (22 mg L^{-1}) suggests close relationship between plant growth promotion activity (**Table 3**) and IAA production. Strain E6S exhibited high tricalcium phosphate-solubilizing ability (135 mg L^{-1}; **Table 2**), which may compensate for P deficiency-induced plant growth retardation in metal contaminated soil. ACC-utilizing bacteria have been found to prevent the inhibition of root growth by hydrolyzing the ethylene precursor ACC into ammonia and α-KB and inhibiting ACC synthase activity (Arshad et al., 2007). Although strain E6S showed relatively low ACC deaminase activity (11 μm α-KB mg^{-1} h^{-1}), it seemed to be efficient in promoting plant growth under metal stress (**Table 3**). The results suggest that the endophytic bacteria, which can synthesize beneficial metabolites such as IAA, ACC deaminase, and solubilize P should be considered as promising biofertilizers.

Metal Mobilization and Biosorption Potential of Endophytic Bacterium

Endophytic bacterium E6S homologous to *A. piechaudii* displayed the potential for biological mobilization of Cd, Zn, and Pb in multi-metal contaminated soil (**Figure 2**). The inoculation of E6S significantly increased ($p < 0.05$) water extractable Cd, Zn, and Pb in metal contaminated soils by 3.9-, 5.8- and 6.0-fold, respectively, compared to the

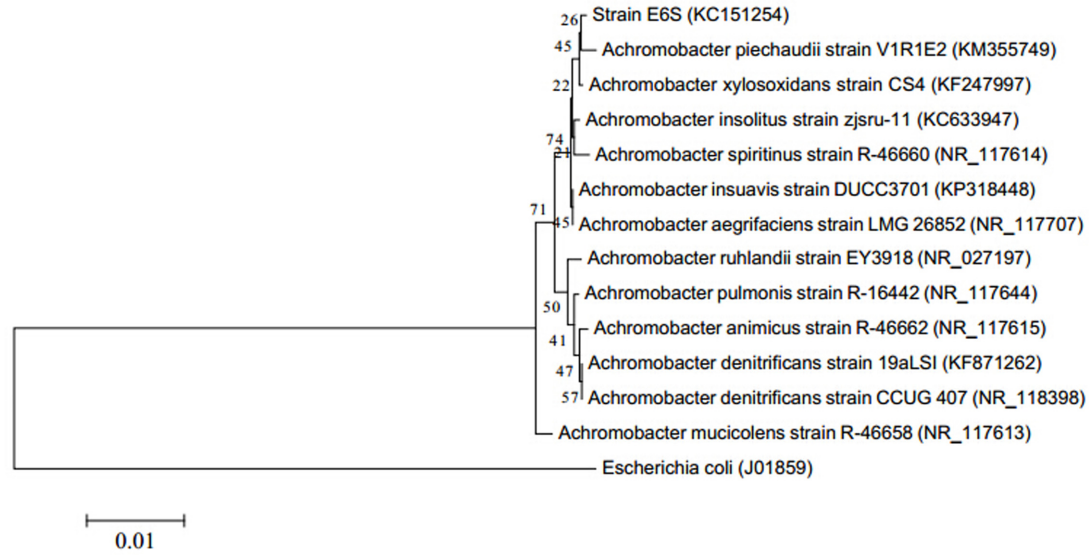

FIGURE 1 | Phylogenetic tree showing the relationship of partial 16S rDNA gene sequences from endophytic bacterium E6S homologous to *Achromobacter piechaudii* with other related sequences from *Achromobacter*. *Escherichia coli* was used as the out-group. The value on each branch is the percentage of bootstrap replications supporting the branch.

TABLE 2 | Heavy metal tolerance, antibiotic resistance, and plant growth promoting traits of endophytic bacterium E6S homologous to *Achromobacter piechaudii*.

Parameter	Unit	E6S
Metal tolerance	mg L^{-1}	
Cd		300
Zn		750
Pb		1200
Antibiotic resistance	mm	
Ampicillin (10 μg)		3 (R)
Tetracycline (30 μg)		9 (R)
Streptomycin (20 μg)		25 (S)
Chloramphenicol (30 μg)		15 (I)
Kanamycin (30 μg)		23 (S)
Plant growth promoting traits		
ACC deaminase	μm α-KB mg^{-1} h^{-1}	11 ± 0.9
P solubilization	mg L^{-1}	135 ± 16
IAA production	mg L^{-1}	22 ± 6
Siderophore (CAS)	cm	nd

R, resistant (<10 mm); I, intermediate (10–15 mm); S, susceptible (>15 mm); ACC deaminase, 1-aminocyclopropane-1-carboxylate deaminase; α-KB, α-ketobutyrate; P, phosphate; IAA, indole-3-acetic acid; CAS, chrome azurol S; nd, not detected.

controls. The observation indicates that strain E6S facilitated the release of metals from non-soluble phase in the soil matrix, thus improving their bioavailability to plants. This may be attributed to organic acid production and phosphate solubilization mediated reduction in soil pH (Ma et al., 2009, 2015).

Biosorption capacity of bacteria plays an important role in reducing metal phytotoxicity by limiting the entry of metal ions into plant cells and may contribute for enhanced plant growth in metal contaminated soils (Ma et al., 2011a). At 150 mg L^{-1} initial metal concentrations, strain E6S was able to remove significant amounts of Cd, Zn, or Pb within 10 h incubation (**Figure 3**). After 8 h incubation, maximum biosorption by E6S was reached, thereafter remaining constant. This was probably due to the achievement of specific equilibrium for metal concentrations. The highest content of metal biosorption was observed with Zn (10.9 mg g^{-1} of cell dry weight), while the lowest was seen with Pb (3.2 mg g^{-1} of cell dry weight). This was probably due to ionic radius of each metal ion (Karakagh et al., 2012), since Zn (0.88 Å) with smaller ionic radius may be more rapidly complexed by bacterial cell wall/membrane compared with Cd (0.97 Å) and Pb (1.2 Å).

Effects of Endophytic Bacterial Inoculation on Plant Biomass

In phytagar assay, inoculation of E6S induced significant increases in fresh and dry weight, ratio of root/shoot dry weight of *B. napus* in both unpolluted and polluted (10 mg L^{-1} Cd) phytagar media (**Table 3**). For instance, E6S induced an increase in fresh and dry weight, and ratio of root/shoot dry weight in Cd polluted media by 65, 47, and 94%, respectively, compared to non-inoculated control. Bacterial inoculation greatly enhanced the biomass production of *B. napus* under non-stressed and metal-stressed conditions. Plants inoculated with strain E6S presented a significantly higher root/shoot ratio than the respective controls, indicating that with the help of endophytic bacterium E6S, plants can acquire nutrients

TABLE 3 | Effects of endophytic bacterium E6S homologous to *Achromobacter piechaudii* on the growth of *Brassica napus* in phytagar assay and *Sedum plumbizincicola* in pot experiment.

Type	Treatment		Relative elongation ratio (RER) of root[a]	RER of shoot[b]	Fresh weight (mg)	Dry weight (mg)	Ratio of root/shoot dry weight[c]
Phytagar assay	Control	Blank	–	–	53.5 ± 3.1 b	2.6 ± 0.0 b	0.74 ± 0.08 b
		E6S	1.62 ± 0.18 b	1.15 ± 0.10 b	67.6 ± 5.3 a	3.2 ± 0.3 a	1.04 ± 0.08 a
	10 mg L^{-1} Cd	Blank	–	–	28.8 ± 4.4 c	1.9 ± 0.3 c	0.32 ± 0.04 c
		E6S	2.78 ± 1.00 a	1.54 ± 0.20 a	47.6 ± 4.2 b	2.8 ± 0.2 b	0.62 ± 0.03 b
Pot experiment	Pristine soil	Blank	–	–	1735 ± 169 z	221 ± 16 z	0.09 ± 0.05 w
		E6S	1.26 ± 0.13 x	1.27 ± 0.10 w	2416 ± 157 y	275 ± 19 y	0.12 ± 0.01 w
	Metal polluted soil	Blank	–	–	6086 ± 403 x	739 ± 24 x	0.02 ± 0.01 x
		E6S	1.89 ± 0.16 w	1.36 ± 0.10 w	8211 ± 481 w	946 ± 51 w	0.03 ± 0.01 x

Average ± standard deviation from five samples. Data of columns indexed by the same letter within each treatment (with or without Cd; pristine or polluted soil) are not significantly different between bacterial treatments according to Fisher's least significant difference (LSD) test ($p < 0.05$).
[a]*RER of root = Mean root length of tested plant/Mean root length of control × 100.*
[b]*RER of shoot = Mean shoot length of tested plant/Mean shoot length of control × 100.*
[c]*Ratio of root/shoot dry weight = Dry weight of root/Dry weight of shoot.*

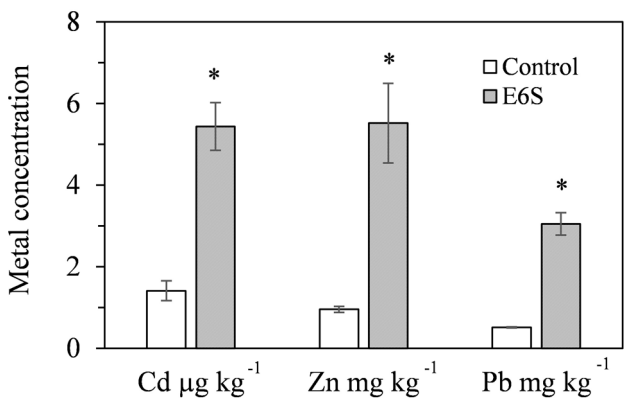

FIGURE 2 | Effect of inoculation with E6S on the mobilization of Cd, Zn, and Pb in soil. Bars represent standard deviations of triplicates. An asterisk (*) denotes a value significantly greater than the corresponding control value according to Student's *t*-test ($p < 0.05$).

more efficiently from soils for plant biomass production, especially for roots. Moreover, the relative elongation rate (RER) of root and shoot in unpolluted media (1.62 and 1.15) were lower than that in polluted treatment (2.78 and 1.54). A possible explanation might be that the endophytic bacteria could exert more efficient functions that help plants to cope with adverse environmental stress (Rajkumar et al., 2009).

In pot experiment, inoculation of E6S significantly improved fresh and dry weight of *S. plumbizincicola* in both pristine and multi-metal polluted soils under non-sterile conditions (**Table 3**). For example, inoculation of E6S considerably increased plant fresh and dry weight by 35 and 28%, respectively, in metal polluted soils. The beneficial effects of E6S on growth of host *S. plumbizincicola* were also observed on growth of non-host *B. napus* in phytagar assay. These effects may be attributed to beneficial metabolites produced by strain E6S, such as IAA and ACC deaminase, which alleviated metal phytotoxicity and thereafter stimulated plant development. Additionally, inoculation of E6S did not significantly influence ratio of root/shoot dry weight in neither pristine nor polluted soils. The presence of metals declined ratio of root/shoot dry weight, compared with non-polluted control. These results concur with the earlier observations of Wan et al. (2012) that elevated metal concentrations mainly impaired root growth, in spite of *S. plumbizincicola* being qualified as a Zn/Cd hyperaccumulator. It is therefore concluded that bioinoculant E6S could serve as biofertilizer for phytoremediation purposes. Interestingly, the fresh and dry weights of *S. plumbizincicola* grown in metal contaminated soil were higher than in pristine soil (**Table 3**). A possible explanation could be that the current levels of heavy metals in soil are not toxic to the plant and can stimulate its growth. This may be attributed to both a beneficial effect of improved metal nutrition and/or the activation of stress scavenging mechanisms, which can help the plant to cope with environmental stresses (Dalcorso et al., 2013).

Endophytic Bacteria-Enhanced Phytostabilization

The changes in metal solubility in polluted soils caused by biological amendments can contribute to facilitate metal accumulation in plants (Ma et al., 2009). Therefore, the effects of metal mobilizing bacterium E6S on metal uptake and translocation by *S. plumbizincicola* were evaluated. In general, inoculation of strain E6S significantly improved plant uptake of Cd, Zn, and Pb (**Table 4**). For instance, strain E6S increased Cd, Zn, and Pb concentration in *S. plumbizincicola* by 32, 37, and 89%, respectively, which is in accordance with significant improvements of BCF of metal (Cd, Zn, and Pb) induced by E6S. This corroborates

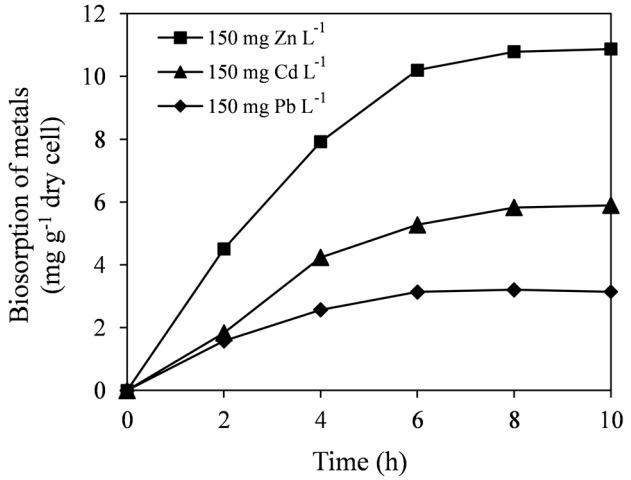

FIGURE 3 | Biosorption of Cd, Zn, and Pb on E6S cells. Bars represent standard deviations of triplicates.

the data shown in **Figure 2** for bacterial metal mobilization, indicating that inoculation of E6S facilitated metal (Cd, Zn, and Pb) bioavailability in soils and thereby their uptake by plants. However, the bacterial inoculation significantly decreased TF of Cd and Zn ($p < 0.05$; **Table 4**) and metal accumulation in shoots (data not shown). The TF was 2.7 and 1.5 (>1) for Cd and Zn in non-inoculated plants, but it decreased to 0.5 and 0.7 (<1) after inoculation with strain E6S. *S. plumbizincicola* has been reported to be a hyperaccumulator for Cd and Zn phytoextraction due to their TF > 1, however, our data showed that the inoculation of strain E6S inhibited the plant-self translocation of metal from roots to shoots and helped plants store metals in their roots, which is desirable for phytostabilization purposes. The present observations indicate that strain E6S effectively increased the bioavailability of metal (Cd, Zn, and Pb) in the rhizosphere soils and also promoted the growth of host *S. plumbizincicola* plants, consequently increasing the total plant metal uptake, while diminishing the translocation of metals from roots to shoots. Results suggest that endophytic bacterium E6S can not only protect the plants against the inhibitory effects of multiple metals, but also effectively improve rhizoaccumulation of toxic metals. Therefore, it can be used to assist phytostabilization of heavy metals in the plant root system. Previously, Srivastava et al. (2013) also reported that *Staphylococcus arlettae* NBRIEAG-6 enhanced As accumulation in roots of *B. juncea* and helped in As phytostabilization. Recently, Sura-de Jong et al. (2015) reported that selenium hyperaccumulators harbor a diverse endophytic bacterial community. It is becoming apparent that some endophytic bacteria play an important role in metal hyperaccumulation and translocation processes, contributing to phytoextraction, while others seem to be effective in improving rhizoaccumulation, contributing to phytostabilization. Future studies are, therefore, needed to access the structure and diversity of bacterial endophytes of *S. plumbizincicola* and their influence in phytoremediation processes.

Bacterial Colonization

After 75 days of inoculation onto plants, strain E6S exhibited high-density colonization of the rhizosphere [3.9×10^5 colony-forming units (CFU) g^{-1}], and fresh roots, stems and leaves (5.4, 3.7, and 0.8×10^3 CFU g^{-1}, respectively) of *S. plumbizincicola*, indicating the great potential of this strain to establish, survive, and develop inside plant tissues and in the root zone. Since beneficial bacteria have great potential to contribute to sustainable plant growth promotion and metal rhizoaccumulation, the colonization and survival properties of introduced beneficial strains are crucial features to evaluate the capacity to assist their host plants in coping with metal stress and therefore phytostabilization efficiency in contaminated sites (Ma et al., 2011a).

CONCLUSION

Endophytic bacterium E6S homologous to *A. piechaudii* isolated from metal hyperaccumulator *S. plumbizincicola* stems was able to facilitate plant growth through the production of IAA and ACC deaminase and solubilization of P. The characterization studies showed that the isolate resisted high concentrations of Cd, Zn, and Pb, via extracellular biosorption in metal containing liquid media and increased water extractable metal

TABLE 4 | Effects of endophytic bacterium E6S homologous to *Achromobacter piechaudii* on metal uptake, translocation factor and bioaccumulation factor by *Sedum plumbizincicola*.

Treatment	Cd			Zn			Pb		
	Plant uptake (mg kg^{-1} dw)	TF	BCF	Plant uptake (mg kg^{-1} dw)	TF	BCF	Plant uptake (mg kg^{-1} dw)	TF	BCF
Control	127 ± 8	2.7 ± 0.3*	21.6 ± 1.4	2262 ± 61	1.5 ± 0.1*	3.1 ± 0.1	114 ± 10	0.2 ± 0.0	0.7 ± 0.1
E6S	168 ± 25*	0.5 ± 0.1	28.4 ± 4.2*	3095 ± 380*	0.7 ± 0.1	4.9 ± 0.2*	215 ± 20*	0.2 ± 0.0	1.4 ± 0.1*

*Average ± standard deviation from five samples. *A value significantly greater than the corresponding control according to Student's t-test ($p < 0.05$). TF, translocation factor; BCF, bioaccumulation factor; dw, dry weight.*

concentration in metal contaminated soils. The inoculation of E6S significantly enhanced plant growth and rhizoaccumulation of Cd, Zn, and Pb by host *S. plumbizincicola*, however, the TFs of Cd and Zn remarkably declined in the presence of bacterial inoculation. Effective metal biosorption and mobilizing capacities as well as the potential to adapt to/survive multi-metal stress conditions along with various plant beneficial traits are clear indications of the advantages of employing this microorganism as a bioinoculant for ameliorating metal phytotoxicity and thus enhancing phytostabilization efficiency. Further work will address the mechanism of the selected bacterial strain contributing to reduce metal translocation from roots to shoots and its effect on the plant biomass yield and metal phytostabilization in field experiments.

AUTHOR CONTRIBUTIONS

YM wrote the manuscript and carried out experiments; CZ and RO analyzed experimental results; HF was the project sponsor; YL designed experiments.

ACKNOWLEDGMENTS

YM and RO wish to acknowledge the support of Fundação para a Ciência e a Tecnologia (FCT) through the research grants SFRH/BPD/76028/2011 and SFRH/BPD/85008/2012 and Fundo Social Europeu. This work is financed by National Funds through the FCT within the Project UID/BIA/04004/2013.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 16 February 2016
doi: 10.3389/fpls.2016.00101

Rhizospheric Bacterial Strain *Brevibacterium casei* MH8a Colonizes Plant Tissues and Enhances Cd, Zn, Cu Phytoextraction by White Mustard

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OPEN ACCESS

Edited by:
Ying Ma,
University of Coimbra, Portugal

Reviewed by:
Rui S. Oliveira,
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Cesar Hugo Hernández-Rodríguez,
Escuela Nacional de Ciencias Biológicas – Instituto Politécnico Nacional, Mexico

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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *31 August 2015*
Accepted: *19 January 2016*
Published: *16 February 2016*

Citation:
Płociniczak T, Sinkkonen A, Romantschuk M, Sułowicz S and Piotrowska-Seget Z (2016) Rhizospheric Bacterial Strain Brevibacterium casei MH8a Colonizes Plant Tissues and Enhances Cd, Zn, Cu Phytoextraction by White Mustard. Front. Plant Sci. 7:101. doi: 10.3389/fpls.2016.00101

Environmental pollution by heavy metals has become a serious problem in the world. Phytoextraction, which is one of the plant-based technologies, has attracted the most attention for the bioremediation of soils polluted with these contaminants. The aim of this study was to determine whether the multiple-tolerant bacterium, *Brevibacterium casei* MH8a isolated from the heavy metal-contaminated rhizosphere soil of *Sinapis alba* L., is able to promote plant growth and enhance Cd, Zn, and Cu uptake by white mustard under laboratory conditions. Additionally, the ability of the rifampicin-resistant spontaneous mutant of MH8a to colonize plant tissues and its mechanisms of plant growth promotion were also examined. In order to assess the ecological consequences of bioaugmentation on autochthonous bacteria, the phospholipid fatty acid (PLFA) analysis was used. The MH8a strain exhibited the ability to produce ammonia, 1-amino-cyclopropane-1-carboxylic acid deaminase, indole 3-acetic acid and HCN but was not able to solubilize inorganic phosphate and produce siderophores. Introduction of MH8a into soil significantly increased *S. alba* biomass and the accumulation of Cd (208%), Zn (86%), and Cu (39%) in plant shoots in comparison with those grown in non-inoculated soil. Introduced into the soil, MH8a was able to enter the plant and was found in the roots and leaves of inoculated plants thus indicating its endophytic features. PLFA analysis revealed that the MH8a that was introduced into soil had a temporary influence on the structure of the autochthonous bacterial communities. The plant growth-promoting features of the MH8a strain and its ability to enhance the metal uptake by white mustard and its long-term survival in soil as well as its temporary impact on autochthonous microorganisms make the strain a suitable candidate for the promotion of plant growth and the efficiency of phytoextraction.

Keywords: phytoextraction, PGPE, heavy metals, *Brevibacterium*, *Sinapis alba* L.

INTRODUCTION

The continued industrialization of countries has led to extensive environmental problems that induce the contamination of soil and water (Rajkumar and Freitas, 2008). Among the pollutants, heavy metals pose a critical concern to human health and the environment (Rajkumar et al., 2009a). Due to the widespread contamination, searching for innovative ways to remove metals from the environment has become a priority in the remediation field (Rajkumar et al., 2009b; Ali et al., 2013). One of the most promising strategies is phytoextraction, which is defined as the use of plants to take up pollutants from contaminated soil (Salt et al., 1998; Glick, 2010; Płociniczak et al., 2013b). The success of metal extraction depends on many factors, but the most important are a plant's ability to uptake and translocate metals to its stems and leaves, metal bioavailability and soil type (Glick, 2010; De-Bashan et al., 2012; Płociniczak et al., 2013a). The results of many studies have shown that the efficiency of heavy metal phytoextraction may be supported by metal-resistant bacteria that belong to the plant growth-promoting bacteria group (PGPB; Glick, 2005; Jing et al., 2007; Li and Ramakrishna, 2011; Li et al., 2012; Sun et al., 2014). PGPB include both rhizospheric (PGPR) and endophytic (PGPE) bacteria (Ma et al., 2011a). Because of the extensive root exudation of many easily degradable compounds, the rhizosphere is a nutrient-rich environment for beneficial bacteria that may colonize the internal tissues of plants and exist as endophytes. Although bacterial plant growth-promoting endophytes (PGPEs) exist in plants to varying degrees, and despite the fact that they are transient, they are often capable of triggering physiological changes that promote the growth and development of plants, even those that are growing in metal-contaminated soil (Rajkumar et al., 2009a).

Generally, the beneficial effects of endophytes are greater than those of many rhizobacteria and these might be enhanced when a plant is growing under either biotic or abiotic stress conditions. It has been shown that PGPE may confer plants with a higher tolerance to heavy metal stress and may stimulate the growth of the host plant through several mechanisms (Ma et al., 2011b). That is one reason why PGPE apart from their application as biocontrol agents and biofertilizers are used to enhance *in situ* phytoextraction (Chen et al., 2010; Becerra-Castro et al., 2011). Heavy-metal-resistant endophytes can enhance plant growth, decrease metal phytotoxicity and affect metal translocation and accumulation in plants and thus play a significant role in the adaptation of plants to a polluted environment (Sessitsch et al., 2013). The inoculation of plants with PGPR/PGPE has been shown to enhance the growth of plants and their development in heavy metals and/or soils contaminated with organic pollutants (Becerra-Castro et al., 2011; Thavasi et al., 2014). Generally, PGPB interact directly or indirectly with a host plant through several mechanisms. These mechanisms involve the production of indole acetic acid (IAA), phytohormones, 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD) and biosurfactants (Arshad et al., 2007; Gravel et al., 2007; Juwarkar et al., 2008; Shoebitz et al., 2009; Li et al., 2012; Sun et al., 2014; Thavasi et al., 2014). Moreover, metal-resistant microorganisms have been shown to increase the availability of heavy metals in soil through soil acidification by producing siderophores and/or by mobilizing metal phosphates (Abou-Shanab et al., 2008; Jing et al., 2012; Płociniczak et al., 2013b).

The aim of this study was to estimate the potential of a metal-resistant strain of *Brevibacterium casei* MH8a to enhance Zn, Cd, and Cu uptake by white mustard under laboratory conditions. Moreover, its potential to promote the growth of plants was also determined. Additionally, the ability of MH8a to colonize the internal tissues of *Sinapis alba* and the ecological consequences of its introduction into soil on autochthonous bacterial communities were also determined.

MATERIALS AND METHODS

Isolation and Identification of Metal-Resistant Bacteria

The *B. casei* MH8a strain was chosen from 12 strains isolated from metal-contaminated rhizosphere soil of *S. alba* L. collected around a non-ferrous steelworks in Dąbrowa Górnicza, Upper Silesia, Poland, which contained the following concentrations of heavy metals: Zn 926, Cd 32, Cu 60, and Ni 150 mg kg^{-1} dry weight (dw). The metal-resistant bacteria were isolated on a 10% Tryptic Soy Agar (TSA) medium that was supplemented with 5 mM of zinc as $ZnCl_2$. The plates were incubated at 28°C for 7 days. Isolated strains were identified using the MIDI microbial identification system (MIDI, Newark, DE, USA) according to the producer's procedure. Identification of MH8a strain was also confirmed based on 16S rRNA gene sequence analysis. For 16S rRNA gene amplification, the universal bacterial primers 8F (5′ AGTTTGATCATCGCTC AG 3′) and 1492R (5′ GGTTACCTTGTTACGACTT 3′) targeting fragment size 1484 bp were used (Pacwa-Płociniczak et al., 2014). The obtained sequence was compared to known 16S rRNA gene sequences using the BLAST server at the National Center for Biotechnology Information (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

DNA sequences were aligned using ClustalW. Phylogenetic analyses were performed by the neighbor–joining (NJ) method to test the support for the phylogeny with a bootstrap analysis based on 1,000 replicates using MEGA software ver. 7.0.

Determination of Metal Minimal Inhibitory Concentration (MIC)

To assess the level of the resistance to heavy metals of the tested strain, the minimal inhibitory concentration (MIC) of Zn, Cu, and Cd was estimated. The MIC values were determined in triplicate in an MES buffered minimal medium (MBMM) as described by Rathnayake et al. (2013). The MBMM medium was supplemented with an increasing content of the metals (1–10 mM of Zn and Cu and 0.5–3 mM of Cd). The MIC was defined as the lowest metal concentration at which bacterial growth was not observed.

Evaluation of Plant Growth-Promoting Activities

Secretion of siderophores by the tested strain was detected using the method described by Schwyn and Neilands (1987) using blue agar plates that contained Chrome azurol S dye (CAS). Orange zones around the colonies on blue agar were considered to be a positive reaction that indicated the production of siderophores.

1-Aminocyclopropane-1-carboxylic acid deaminase activity was assayed according to a modified Honma and Shimomura (1978) method as described by Saleh and Glick (2001). ACCD activity was expressed in nmol of α-ketobutyrate mg^{-1} h^{-1}. The protein concentration of microbial cell suspensions was determined using the Bradford (1976) method.

Indole acetic acid production was assessed using Salkowski's reagent according to the modified method of Bric et al. (1991). The IAA concentration in cultures was calculated using the calibration curve of pure IAA (1–100 μg mL^{-1} of medium) as the standard.

The phosphate solubilizing ability of the tested bacteria was determined on a National Botanical Research Institute's phosphate (NBRIP) agar medium as described by Płociniczak et al. (2013a).

The production of hydrogen cyanide by MH8a was tested using the Lorck (1948) method as shown in Pawlik and Piotrowska-Seget (2015).

The bacterial isolate was tested for the production of ammonia in peptone water according to Cappuccino and Sherman (1992).

Selection of a Rifampicin-Resistant Mutant of MH8a

A spontaneous mutant of the MH8a was selected by plating on a Luria Bertani (LB) medium that was amended with 5 μg mL^{-1} of rifampicin. Next, the growing colony was reinoculated on LB plates with a successively higher content of rifampicin (up to 150 μg mL^{-1} of medium). The stability of rifampicin resistance was confirmed by subculturing $MH8a^{rf}$ five times on an LB medium without antibiotic selection. The cultures were stored in 20% glycerol at −80°C (Płociniczak et al., 2013b). $MH8a^{rf}$ had the same biochemical features as the parental MH8a and was used in pots experiment.

Inoculum Preparation

For soil inoculation, the metal-resistant $MH8a^{rf}$ strain was cultured on a Luria-Bertani (LB) medium on an orbital shaker at 120 rpm (28°C) for 24 h. The number of bacteria in the inoculum was established based on the turbidimetry and plating methods. The appropriate volume of bacterial culture was centrifuged (6000 rpm, 21°C, 20 min); the harvested bacteria were washed twice with sterile distilled water and resuspended in 50 mL sterile water.

Experimental Set-Up

The phytoextraction experiment was conducted in laboratory conditions using sandy loam contaminated with heavy metals collected in the vicinity of the non-ferrous steelworks "Mikrohuta" in Dąbrowa Górnicza. Selected physicochemical properties of the tested soil used in the experiments were as follows: pH (H_2O) 5.4 ± 0.1; organic matter 9.6 ± 0.2 g kg^{-1}; total N 284 ± 14 mg kg^{-1}; total P 170 ± 6 mg kg^{-1}; Fe 8792 ± 42 mg kg^{-1}; Cd 32 ± 2.1 mg kg^{-1}; Cu 60 ± 4.6 mg kg^{-1}; Zn 926 ± 32 mg kg^{-1}; Ni 150 ± 11 mg kg^{-1}.

The experiment had a completely randomized block design with three replications that had two treatments (I) plants growing in soil inoculated with the tested $MH8a^{rf}$ strain and (II) control – plants growing in soil inoculated with distilled water that contained thermal inactivated $MH8a^{rf}$ cells instead of the suspension of living bacteria.

Prior to the experiment, the soil was kept for 2 days at room temperature and sieved. Then the soil moisture was adjusted and maintained at 20% which corresponded to 50% of the maximum water holding capacity of the soil. Seeds of *S. alba* L. cv. Nakielska were placed in pots (total volume of 500 mL) that contained 400 g of the soil and kept in a growth room under controlled light (14-h photoperiod at 15,000 lux; temperature 23/18°C light/dark). After 2 weeks, 50 mL of the bacterial solution was poured into the soil up to the number of 10^8 cells of the tested strains g^{-1} dw soil. Plants (five plants per pot) were grown in the conditions described above for 28 days.

Effect of the Bacterial Inoculant on Plant Biomass and Metal Accumulation

After 28 days of incubation, the plants were removed from the pots and shoots (stems and leaves) and roots were weighed separately. Before weighing, the roots were washed carefully using distilled water. The fresh and dw of shoots and roots as well as heavy metal accumulation in the above and underground parts were measured. The metal concentration in the shoots and roots of *S. alba* was determined in triplicate using atomic absorption spectrometry as described in Płociniczak et al. (2013b). The usefulness of *S. alba* in phytoextraction was confirmed by estimating the translocation factor (TF) for the tested metals (Yoon et al., 2006).

Effect of the Bacterial Inoculant on the Structure of Bacterial Community

Changes in the biodiversity and community structure of the autochthonous bacterial populations after soil inoculation with the $MH8a^{rf}$ strain were determined in triplicate at 24 h, 7 and 28 days using the phospholipid fatty acid (PLFA).

Phospholipid fatty acids were isolated from 2 g of fresh soil as described by Pennanen et al. (1999). The fatty acid methyl esters were separated using a gas chromatograph (Hewlett-Packard 6890, USA) with an HP-Ultra 2 capillary column (25 m, 0.22 mm ID) and hydrogen as the carrier gas. PLFA compounds were detected using a flame ionization detector (FID) and identified using MIDI Microbial Identification System software (Sherlock TSBA 6 method and TSBA 6 library; MIDI, Inc., Newark, DE, USA).

Survival of MH8a^{rf} in Soil and its Ability to Colonize Plant Tissues

The number of living MH8a^{rf} cells in soil was determined at 24 h, 7 and 28 days after soil inoculation. In order to estimate the colony-forming units (cfu) of bacteria in soil and plant tissues, the dilution-plate method on Luria-Bertani (LB) agar with the addition of 150 μg mL^{-1} of rifampicin was used. As a control, a suspension of soil inoculated with thermal inactivated MH8a^{rf} cells was plated on LB agar with the addition of an antibiotic.

Plant colonization by MH8a^{rf} was determined 24 h, 7 and 28 days after soil inoculation with the tested strain. Roots, stems and leaves were surface sterilized with 70% ethanol (2 min), 5% sodium hypochlorite (2 min), and 10% hydrogen peroxide (2 min). The samples were rinsed three times in sterile distilled water to remove the disinfectant. The sterilization process was verified by a plating final wash onto a TSA medium. Plates were incubated at 28°C for 7 days. If no microbial growth was found, the surface-sterilization process was recognized as being successful. The roots, stems, and leaves were macerated separately in 5 mL of 0.9% NaCl using a mortar and pestle and a 100 μL suspension was plated onto a TSA medium that contained rifampicin at a concentration of 150 μg mL^{-1} of medium and incubated at 28°C for 7 days (Kukla et al., 2014). At the same time, rifampicin-resistant bacteria were isolated from the control plants grown in soil inoculated with thermal inactivated MH8a^{rf} strain.

Statistical Analysis

Statistical analysis was performed using STATISTICA 10.0 PL software (StatSoft, Tulsa, OK, USA). Analysis of variance (ANOVA) followed by a *post hoc* Least Significant Difference test were conducted in order to identify any significant effects of the introduced strains on the plant biomass as well as on the accumulation of heavy metals in the shoots and roots of *S. alba*. Differences to from the control plants with $p < 0.01$ in the plant inoculation experiments were considered to be significant. For the pot experiments, data were represented as the mean ± standard deviation (SD) of three replicates. For the PLFA experiments principal component analysis (PCA) was used. One-way multivariate analysis of variance (MANOVA) of the PCA-axes values and *post hoc* LSD tests ($p < 0.05$) were applied for the statistical testing of the separation of the profiles along each PC. For the PLFA experiment, data were represented as the mean ± standard deviation (SD) of three replicates.

RESULTS

Identification of Isolate and MIC Values of the Tested Strain

Based on the MIDI-FAME method and 16S rRNA gene sequence analysis, the isolated strain was identified as *B. casei* and designed as MH8a strain. The phylogenetic analysis showed that the 16S rDNA sequence of *B. casei* MH8a (KT951720.1) had a 98 and 97% identity with strains *B. casei* DSM 20657 (NR_041996.1) and *B. casei* NCDO 2048 (NR_119071.1), respectively (**Figure 1**).

The MIC values for Zn, Cd, and Cu reached values of 7, 1.5, and 6 mM, respectively.

Biochemical Characteristic of MH8a (PGP Traits)

Among the tested mechanisms that were considered to be potentially responsible for the support of plant growth and heavy metal phytoextraction, MH8a was able to produce ammonia, hydrocyanic acid and indole 3-acetic acid at a concentration of 3.44 ± 0.09 μg IAA mL^{-1} of medium. The MH8a showed the activity of ACC deaminase at a level of 53.6 ± 1.47 nmol α-ketobutyrate mg^{-1} h^{-1}. *B. casei* MH8a was not able to solubilize $Ca_3(PO_4)_2$ in an NBRIP medium to produce siderophores on a CAS medium.

Effect of Bacteria Inoculation on Plant Biomass and Metal Accumulation

The inoculation of soil with the tested bacterial strain resulted in a significant increase of the biomass of *S. alba* as compared to the control plants (**Figure 2**). MH8a enhanced the fresh and dry weight of shoots by 144 and 51%, respectively, and significantly increased (about 33%) the fresh and dry weight of roots as compared to the control plants.

The concentrations of Zn, Cd, and Cu were significantly higher in plants growing in soil inoculated with MH8a in comparison with the control plants (**Table 1**). MH8a led to a significant increase in Zn (87%), Cd (207%), and Cu (39%) accumulation in shoot tissues. The tested strain also caused a significant increase in the accumulation of metals in the roots of *S. alba*, where the Zn, Cd, and Cu accumulation was 85, 87, and 122% higher as compared to the control plants, respectively.

As is shown by the values of TF, the inoculation of soil with MH8a resulted in a markedly higher Cd translocation from root to shoot. The value of TF for Cd in plants growing in soil treated with MH8a reached a value of 0.91, whereas in the control plants, it was significantly lower (0.55).

Effect of the Bacterial Inoculant on the Structure of Bacterial Community

The inoculation of MH8a into the soil caused temporary changes in the PLFA profiles that were obtained from the treated soil as compared to the control soil (**Figure 3**). All PLFAs were divided into structural classes and among them hydroxylated, cycloprane, and unsaturated fatty acids were considered to be characteristic for Gram-negative bacteria. In turn, the branched fatty acids indicated the presence of Gram-positive bacteria. The inoculation of MH8a into soil caused an increase of branched fatty acids content (4.4%) in PLFA profiles after 24 h. Additionally, a decrease in the percentage of unsaturated fatty acids (3.3%) was also observed. At the same time, the amount of 18:2ω6,9c decreased from 2.5 to 1.9%. Seven days after soil treatment, the percentage of branched fatty acids decreased about 2.6% in the PLFA profiles as compared to samples at 24 h. At the same time, an increase in the percentage of methylated fatty acids was observed. Analysis of the PLFA profiles from 28 days samples (end of the experiment) showed a decrease in the percentage of

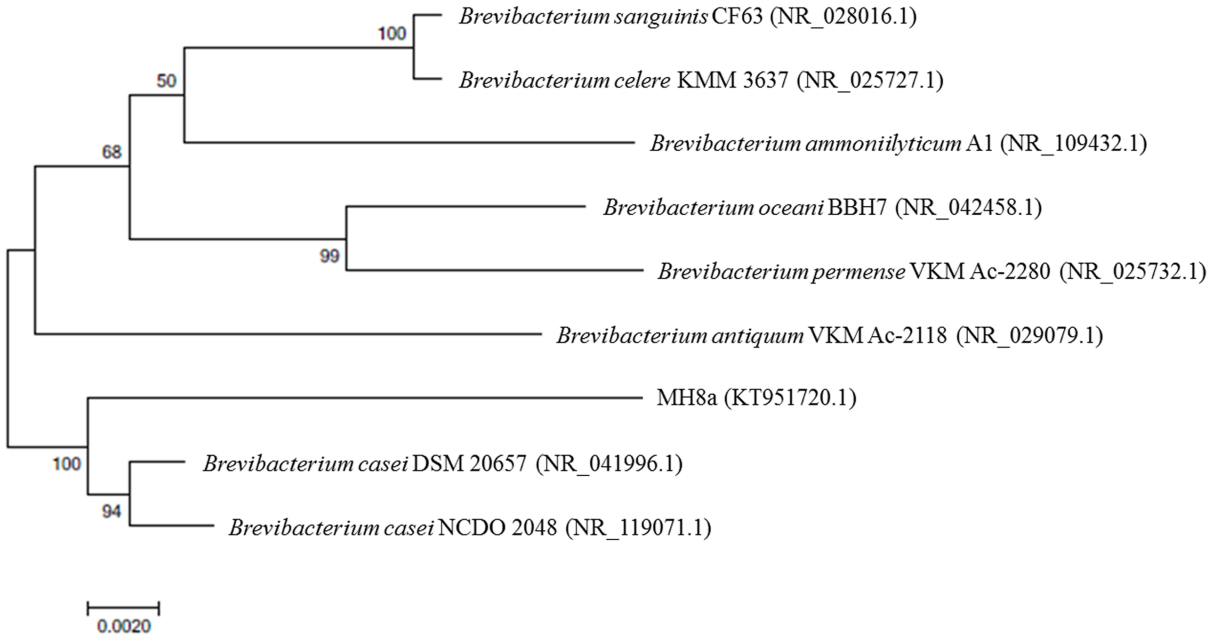

FIGURE 1 | Neighbor–joining phylogenetic tree of bacteria based on a comparison of 16S rRNA gene sequence. Bootstrap values are indicated at the branches from 1,000 replications. GenBank accession numbers are given in brackets.

branched fatty acids and an increase of 18:2ω6,9c as compared with the PLFA profiles from the 24 h and 7 days samples.

Phospholipid fatty acid profiles differed significantly ($p < 0.001$) along PC1, which explained 46.26% of the total variability between the tested samples (**Figure 4**). All PLFA profiles extracted from the soil inoculated with the MH8a strain (24 h, 7 and 28 days) differed significantly as compared to the profiles obtained from the control soil. The most pronounced changes in the bacterial community structure were observed 24 h after soil inoculation with MH8a. Profiles at 24 h also differed significantly from those obtained for soil taken 28 days after soil inoculation with the tested strain. Among the tested samples, PLFA profiles from soil taken at the end of experiment (28 days) had the nearest location to the control samples, but the differences were still statistically significant.

Survival of MH8a in Soil and its Ability to Colonize Plant Tissues

A rifampicin-resistant mutant of the MH8a strain was used for the assessment of its survival in soil and its ability to colonize *S. alba* tissues after soil inoculation. A gradual decrease in cell number in soil was observed during the experimental period (**Figure 5**). At the first sampling time, the number of MH8a cells decreased three-fold (to 3.6×10^7 cfu g^{-1} dw of soil) as compared to the number of bacteria introduced into the soil (10^8 cfu g^{-1} dw of soil). On the last sampling day (28 days), the number of MH8a cells in soil was 4×10^3 cfu g^{-1} of dry soil. The plate inoculation with the plant's suspension showed that no rifampicin-resistant bacteria were isolated from plant tissue 24 h after soil treatment with MH8a. Seven and 28 days after the introduction of MH8a into the soil, the number of bacteria that were isolated from the leaves of *S. alba* reached a value of 4.9×10^3 and 3.6×10^3 cfu g^{-1} of the dw of leaves, respectively. At the same sampling points, the number of MH8a cells in the roots of *S. alba* reached a value of 8.6×10^3 and 9.2×10^3 cfu g^{-1} of the dw of roots, respectively.

DISCUSSION

The plant–bacteria partnership can be applied to increase the phytoremediation efficiency of soil and water contaminated with organic and/or inorganic pollutants (Weyens et al., 2009; Khan et al., 2015). The results of many studies revealed that phytoextraction may be significantly enhanced by various bacteria including PGPB (Jiang et al., 2008; Rajkumar and Freitas, 2008; Dimkpa et al., 2009; Ma et al., 2009; Płociniczak et al., 2013a). Recently, attention has concentrated on the bioaugmentation of soil with bacteria that are characterized by metal resistance and plant growth-promoting traits (Ma et al., 2015).

In the present study, we describe the metal-tolerant bacterial strain *B. casei* MH8a, which was isolated from the rhizosphere of *S. alba* that was growing in heavy metal contaminated soil, as a potential agent in the enhancement of phytoextraction. Moreover, this strain was able to establish a close interaction with *S. alba* by colonizing its tissues.

Endophytic bacteria may improve plant tolerance to heavy metals and plant growth through several biological mechanisms. The beneficial effects of endophytic bacteria are generally attributed to the utilization of ACC, the production of IAA and

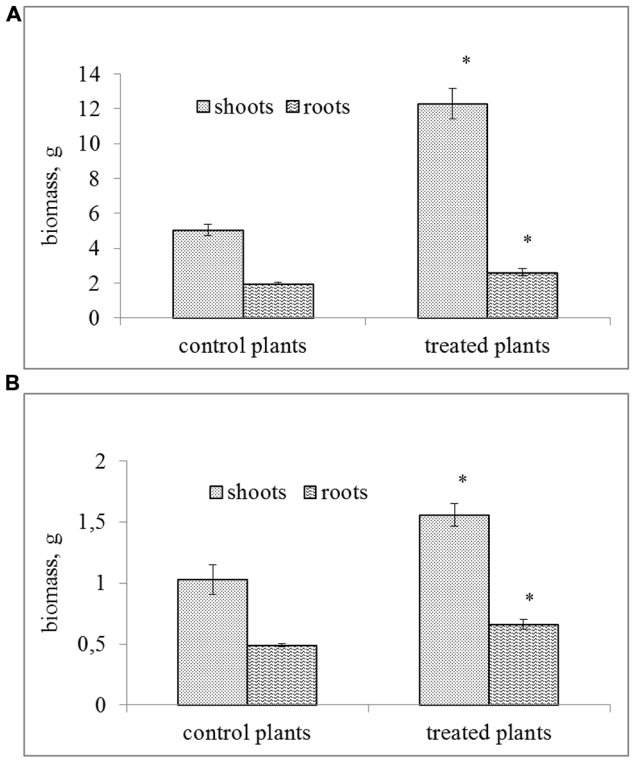

FIGURE 2 | The fresh (A) and dry (B) biomass of the shoots and roots of *Sinapis alba* L. growing in soil inoculated with the MH8a strain (mean ± SD, *n* = 3). *Indicates singnificant differences at the 0.01 probability level.

TABLE 1 | Heavy metal accumulation in the shoots and roots of *Sinapis alba* L. growing in the control soil and soil inoculated with *Brevibacterium casei* MH8a.

Soil treatment		Metal accumulation (mg kg^{-1})		
		Zn	Cd	Cu
Control plants	Shoots	736 ± 21	6.2 ± 0.7	5.1 ± 0.6
	Roots	721.3 ± 36	11.1 ± 0.5	23.1 ± 0.6
Treated plants	Shoots	1375* ± 31	19.2* ± 0.4	7.1* ± 0.3
	Roots	1331* ± 22	20.9* ± 0.5	51.3* ± 1.2

*$p < 0.01$.

siderophores and the solubilization of phosphates (Kolbas et al., 2015). Bacterial ACC deaminase can limit the level of the stress hormone ethylene in plants that are growing in harsh conditions thus increasing a plant's growth. Additionally, the secretion of phytohormones such as IAA can lead to the formation of ACC, which in turn may be converted into ammonium and α-ketobutyrate by endophytic bacteria. The increase in the biomass and metal accumulation in plants that was observed in our studies may be connected with the biochemical features of the MH8a strain. Biochemical analysis of MH8a revealed the activity of several mechanisms that may potentially be responsible for the promotion of plant growth and indirectly for the enhancement of phytoextraction. The ACCD activity at the level of 53.6 ± 1.47 nmol α-ketobutyrate mg^{-1} h^{-1}, IAA production (3.44 ± 0.09 μg IAA mL^{-1}) as well as the release of ammonia and hydrocyanic acid allows the MH8a strain to be classified as a PGPB. It is very difficult to connect the positive effect of MH8a on plant growth and heavy metal phytoextraction with a specific bacterial mechanism. For example, it has been observed that a low level of ACCD activity, approximately ≥20 nmol α-ketobutyrate mg^{-1} protein h^{-1}, is sufficient to enable a bacterium to grow on ACC and to act as a PGPB. Interestingly, organisms with a higher ACCD activity (300–400 nmol α-ketobutyrate mg^{-1} protein h^{-1}) do not necessarily promote root elongation more than the strains that are characterized by a lower enzyme activity (Penrose and Glick, 2003). The secretion of siderophores and phosphate solubilization are also considered to be features of PGPB, but MH8a did not exhibit these activities. Such bacterial mechanisms are essential in niches that are poor in bioavailable forms of iron and phosphorus. During the phytoextraction experiment, symptoms of an Fe and P deficiency were not observed in *S. alba* indicating that bacteria and plants did not suffer from a lack of these nutrients.

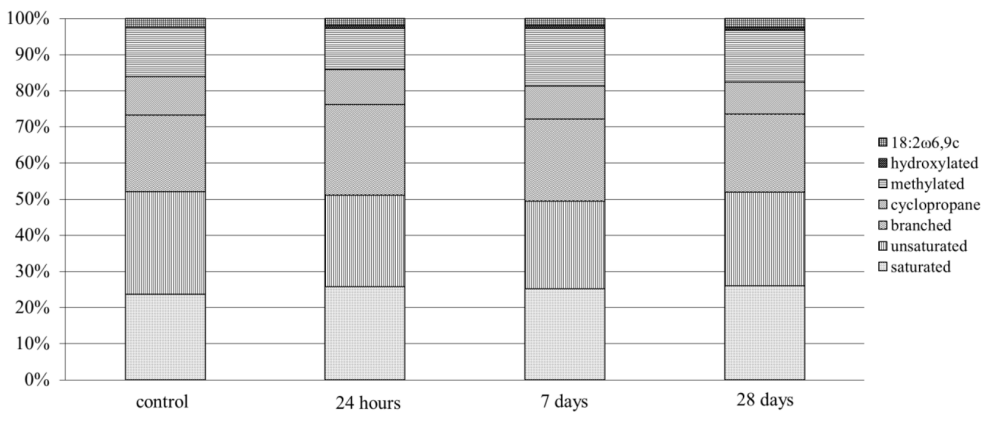

FIGURE 3 | Phospholipid fatty acid (PLFA) profiles derived from the non-inoculated control and soil inoculated with the MH8a strain.

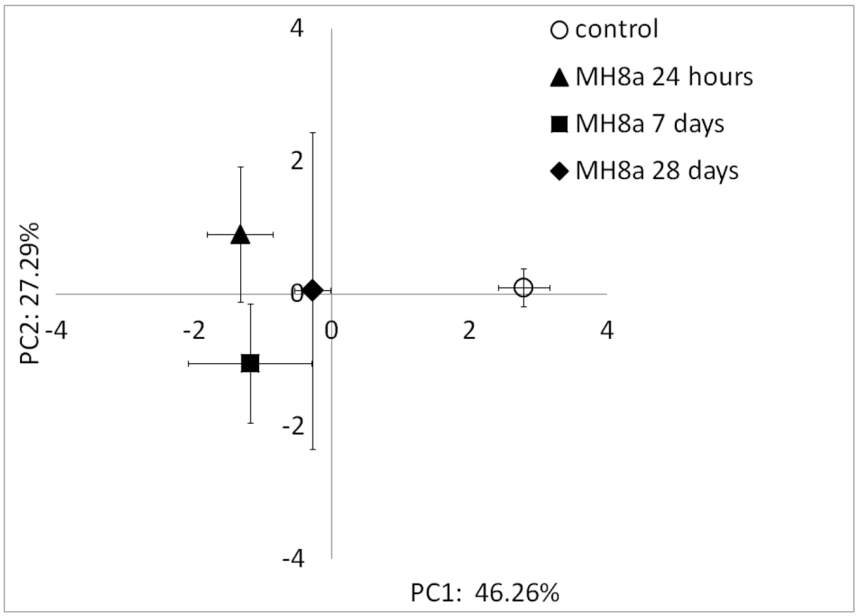

FIGURE 4 | Principal component analysis (PCA) plot of the PLFA profiles extracted from the non-inoculated control and soil inoculated with the MH8a strain. Mean of the PCA-axes values of PLFA profiles ± SD ($n = 3$, $p < 0.05$).

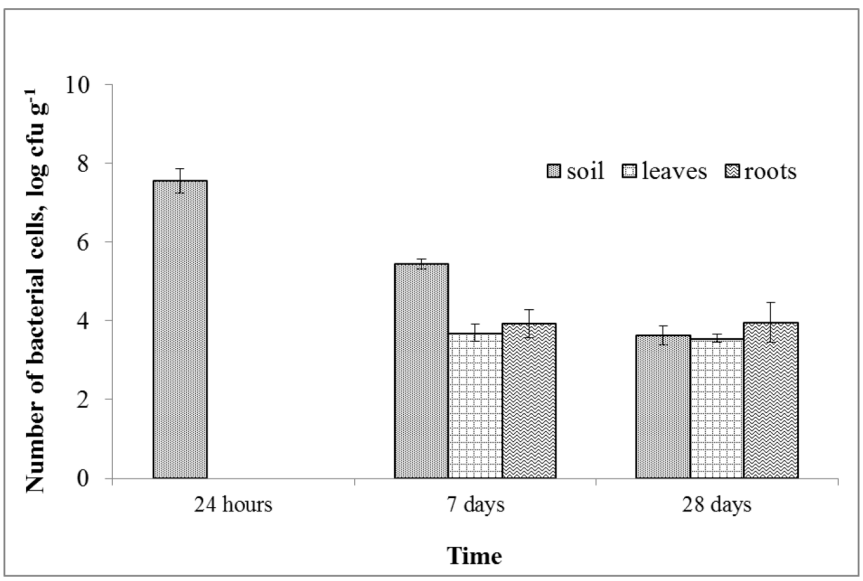

FIGURE 5 | The number of *Brevibacterium casei* MH8a cells isolated from soil and plant tissues 24 h, 7 and 28 days after soil inoculation with MH8a (mean ± SD, $n = 3$).

The effect of MH8a on plant biomass was expressed in the significant increase of *S. alba* weight in the plants that were cultivated in soil treated with the tested strain. This result is in agreement with previous reports describing a greater biomass of *S. alba* growing in soil treated with *Enterobacter intermedius* MH8b and several *Pseudomonas* strains that had also been isolated from heavy metal contaminated soil (Płociniczak et al., 2013a,b). The positive impact of endophytic bacteria on plant biomass has been confirmed by several authors. For instance, Chen et al. (2014) found that the biomass of *Sedum alfredii* treated with the *Sphingomonas* SaMR12 strain was significantly higher as compared with a non-inoculated control. Similarly, Kolbas et al. (2015) confirmed the positive effect of endophytic bacteria (mainly from *Bacillus* and *Rhodococcus* genera) isolated from seeds of *Agrostis capilaris* on the biomass of inoculated plants grown in soil contaminated with Cu

(13–114 mg kg^{-1}). They found that at these Cu concentrations, endophytic inoculants increased shoot biomass between 1.6 and twofold compared to the control plants.

Plants that might be useful in the phytoextraction process, besides possessing a high biomass, should uptake and accumulate heavy metals with a high efficiency. The efficiency of the phytoextraction of metal-contaminated soil can be enhanced by metal-tolerant PGPE (Mastretta et al., 2009; Ma et al., 2011b). In our study we observed that the introduction of the multi-metal-resistant strain MH8a into soil resulted in a significantly higher accumulation of Zn, Cd, and Cu in the shoots and roots of *S. alba*. In comparison with the control plants, the amount of Zn, Cd, and Cu in the shoots of inoculated plants was 87, 207, and 39% higher, respectively. The positive role of metal-resistant PGPE in the enhancement of phytoextraction by *Sedum plumbizincicola* was reported earlier by Ma et al. (2015). Among the tested strains (*Bacillus pumilus* E2S2, *Bacillus* sp. E1S2, *Bacillus* sp. E4S1, *Achromobacter* sp. E4L5 and *Stenotrophomonas* sp. E1L), the most effective strain, *B. pumilus* E252, significantly increased the fresh and dry biomass of plants (37 and 32%, respectively), as well as the plant Cd uptake (43%). In other studies Ma et al. (2011b) showed that the PGPE *Pseudomonas* sp. A3R3 significantly increased the Ni accumulation in *Alyssum serpyllifolium* during microbial-assisted phytoremediation. Similarly, Mastretta et al. (2009) confirmed the positive effect of Cd-resistant *Sanguibacter* sp. on Cd phytoextraction by *Nicotiana tabacum*. Plants growing in soil treated with this strain accumulated a significantly higher amount of Cd, as compared to control plants. It is worth emphasizing that MH8a caused the higher translocation of Cd from the roots to the shoots of plants was confirmed by the higher value of the TF for cadmium (0.91) as compared to the control plants (0.56). This result in combination with the high TF for Zn (1.03) makes the system of white mustard and *B. casei* MH8a especially useful for the phytoextraction of soil contaminated with zinc and cadmium.

It is widely accepted that the inoculants that are used in bioaugmentation should not upset the microbial equilibrium in soil; however, very little is known about the impact of PGPB/PGPE on the indigenous microbial communities in soil. This is particularly important because most of the bacteria that are used in the bioaugmentation process are introduced directly into soil.

In our study the bacterial communities of both the control and inoculated soils were dominated by Gram-negative bacteria. As was indicated by PLFA analysis, the introduction of MH8a caused temporary changes in the structure of the bacterial populations in inoculated soils. The temporary changes in the percentage of branched fatty acids in the PLFA profiles probably resulted from the introduction of the Gram-positive MH8a strain. However, the changes decreased over the period of experiment. This was observed because MH8a was isolated from the same heavy-metal polluted soil that was used in the phytoextraction experiment and was a member of the autochthonous population of microorganisms.

The strain used in our study had the ability to enter a plant's tissues and become an endophyte. One of the problems connected with the use of endophytes as an inoculum is their poor survival in soil and weak recolonization of plants, which are caused by harsh soil conditions and competition between bacterial species. It seems that the bioaugmentation of soil with metal-tolerant PGPB, which have originated from the rhizosphere or bulk soil and are able to colonize plant tissue is an alternative that may reduce the risk of the poor survival of an inoculant.

For the effective enhancement of phytoextraction by bacteria, the bacteria that are introduced should interact closely with plants and exhibit the ability to colonize the rhizosphere and/or interior of plant tissues. The plant apoplast offers different growth conditions, and therefore, different strains originating from rhizosphere can efficiently colonize the plant interior and become endophytes (Khan et al., 2015).

The strain that was used in this study was able to colonize both the rhizosphere soil and plant tissues and this interaction supported the phytoextraction of heavy metals. Seven days after the inoculation of the soil with the MH8a strain, rifampicin-resistant bacteria were detected in the rhizosphere as well as in the roots and leaves of *S. alba*. Similar experiments on bacterial survival in the rhizosphere soil and tissues of apple seedlings was evaluated by Ju et al. (2014), who studied the ability of *Bacillus subtilis* Y-1 to colonize and protect apple seedlings against *Fusarium oxysporum*. Using the plating method, the authors showed that the rifampicin-resistant strain of Y-1 could colonize the rhizosphere and plant tissues within 30 days. The colonization of potato plants by rifampicin-resistant strains of *Paenibacillus* sp. E119 and *Methylobacterium mesophilicum* SR1.6/6 was tested by Andreote et al. (2010). The authors emphasized the role of the shifts in the compositions of plant-associated communities after the successful colonization of plant tissues by the bacterial inoculant. These shifts may lead to differences in the plant's metabolism thereby influencing plant growth and crop yields.

CONCLUSION

A metal-tolerant *B. casei* MH8a strain, due to the activity of several mechanisms that are considered to be important for plant growth-promotion, has the potential to enhance the phytoextraction of heavy metals from contaminated soil. Additionally, MH8a showed a high survival rate after introduction into the soil and was able to colonize the internal tissues of *S. alba*. These features indicate that MH8a in combination with *S. alba* can be regarded as an effective tool for the phytoextraction of Cd and Zn.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENT

The research was supported by grant No. 2013/11/B/NZ9/00152 financed by the National Science Centre, Poland.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer RSO and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 22 June 2016
doi: 10.3389/fmicb.2016.00841

A Complex Inoculant of N_2-Fixing, P- and K-Solubilizing Bacteria from a Purple Soil Improves the Growth of Kiwifruit (*Actinidia chinensis*) Plantlets

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Limited information is available if plant growth promoting bacteria (PGPB) can promote the growth of fruit crops through improvements in soil fertility. This study aimed to evaluate the capacity of PGPB, identified by phenotypic and 16S rRNA sequencing from a vegetable purple soil in Chongqing, China, to increase soil nitrogen (N), phosphorus (P), and potassium (K) availability and growth of kiwifruit (*Actinidia chinensis*). In doing so, three out of 17 bacterial isolates with a high capacity of N_2-fixation (*Bacillus amyloliquefaciens,* XD-N-3), P-solubilization (*B. pumilus*, XD-P-1) or K-solubilization (*B. circulans*, XD-K-2) were mixed as a complex bacterial inoculant. A pot experiment then examined its effects of this complex inoculant on soil microflora, soil N_2-fixation, P- and K-solubility and kiwifruit growth under four treatments. These treatments were (1) no-fertilizer and no-bacterial inoculant (Control), (2) no-bacterial inoculant and a full-rate of chemical NPK fertilizer (CF), (3) the complex inoculant (CI), and (4) a half-rate CF and full CI (1/2CF+CI). Results indicated that significantly greater growth of N_2-fixing, P- and K-solubilizing bacteria among treatments ranked from greatest to least as under 1/2CF+CI $\approx$ CI $>$ CF $\approx$ Control. Though generally without significant treatment differences in soil total N, P, or K, significantly greater soil available N, P, or K among treatments was, respectively, patterned as under 1/2CF+CI $\approx$ CI $>$ CF $\approx$ Control, under 1/2CF+CI $>$ CF $>$ CI $>$ Control or under 1/2CF+CI $>$ CF $\approx$ CI $>$ Control, indicating an improvement of soil fertility by this complex inoculant. In regards to plant growth, significantly greater total plant biomass and total N, P, and K accumulation among treatments were ranked as 1/2CF+CI $\approx$ CI $>$ CF $>$ Control. Additionally, significantly greater leaf polyphenol oxidase activity ranked as under CF $>$ 1/2CF+CI $\approx$ Control $\approx$ CI, while leaf malondialdehyde contents as under Control $>$ CI $\approx$ CF $>$ 1/2CF+CI. In short, the applied complex inoculant is able to improve available soil N, P, and K and kiwifruit growth. These results demonstrate the potential of using a complex bacterial inoculant for promoting soil fertility and plant growth.

Keywords: identification, *Bacillus* spp., N_2-fixation bacterium, K- and P-solubilizing bacteria, malondialdehyde, polyphenol oxidase

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Microbiology

Received: *08 October 2015*
Accepted: *19 May 2016*
Published: *22 June 2016*

Citation:
Shen H, He X, Liu Y, Chen Y, Tang J and Guo T (2016) A Complex Inoculant of N_2-Fixing, P- and K-Solubilizing Bacteria from a Purple Soil Improves the Growth of Kiwifruit (Actinidia chinensis) Plantlets. Front. Microbiol. 7:841. doi: 10.3389/fmicb.2016.00841

INTRODUCTION

High inputs of chemical nitrogen (N), phosphorus (P), and/or potassium (K) fertilizers have been globally used to enhance crop growth and productivity. Meanwhile, the excessive application of chemical N, P, and/or K has resulted in significantly destructive change in soil properties and environmental quality. However, studies pertaining to the relationship between the application of fertilizers and their negative effects on the environment are limited. Therefore, it is timely to explore and identify alternative strategies that can ensure competitive crop yields and environment health while maintaining agro-ecosystem sustainability (Zhang and Ju, 2002; Jiao et al., 2012). Recently, the application of microbial inoculants or plant growth-promoting (PGP) bacteria to soils has been shown to be a promising practice for sustainable production in intensive agricultural production systems around the world (Bhattacharyya and Jha, 2012; Chauhan et al., 2015; Majeed et al., 2015).

Plant growth-promoting bacteria are free-living soil bacteria that can fix or solubilize essential elements in the soil, induce plant host resistance to pathogens, and aggressively colonize the root rhizosphere. Consequently, this results in promotion of better nutrient uptake and thereby enhances plant growth and yield (Chen et al., 2006; Lugtenberg and Kamilova, 2009; Tailor and Joshi, 2014; Zhang and Kong, 2014). The effect of PGP bacteria is mostly explained by their capacity to release metabolites (Majeed et al., 2015). These metabolites are able to: (a) stimulate plant growth by inducing the production and release of plant growth regulators or phytohormones such as indole acetic acid (IAA), cytokinins, and gibberellins; (b) enhance asymbiotic N_2-fixation; (c) solubilize inorganic phosphate and the mineralization of organic phosphate and/or other nutrients; and (d) resist, tolerate or compete with detrimental microorganisms (Lugtenberg and Kamilova, 2009). At present, the application of PGPRs in China has benefited the growth and production of rice, wheat, soybeans, corn, and potato (Chen and Zhang, 2000; Wu et al., 2011; Deng et al., 2012; Gao et al., 2012). The effectiveness of biofertilizers, such as the inoculation of soils with exogenous PGP bacteria, however, has been a hot topic of controversy because various inoculants have shown poor colonization ability, unstable genetic trait, and poor competitive advantage against indigenous soil microorganisms (Du et al., 2008). In particular, information is very limited in the effects of soil microbial inoculants in fruit plantations.

Kiwifruit (*Actinidia chinensis*) has long been recognized as 'the king of fruits' because of its remarkably high vitamin C and balanced mineral composition, dietary fiber, and other metabolites that are beneficial to human health. Kiwifruit is commercially grown in the Sichuan Basin of southwestern China (Li et al., 2015). This basin has an area of 2.6×10^5 km^2 and is located in eastern Sichuan (97°21′E-108°31′E, 26°03′N-34°19′N) and most of the Chongqing Municipal City (105°11′E-110°11′E, 28°10′N-32°13′N). In this region, a purple soil (Eutric Regosol, FAO Soil Taxonomic Classification) is the major agricultural soil. Some PGP bacteria, such as silicate bacteria (Huang et al., 1998; He et al., 2003) and azotobacters (Li et al., 2003; Han et al., 2011) have been isolated from this purple soil. There have been few reports, however, regarding the colonization ability of these PGP bacteria and their compatibility with soil microflora or their ability to stimulate plant growth in the purple soil.

The objectives of this study were to determine (a) if bacterial isolates from the purple soil had a competitive advantage and (b) if the use of the bacterial isolates as a complex soil inoculant could effectively solubilize soil nutrients and thus promote plant growth. In doing so, PGP bacterial isolates were first isolated from the purple soil and identified. The identified isolates were then added to purple soil for evaluating their effects on the release of soil N, P, and K, increase of plant tissue N, P, and K and plant biomass production. The expected findings of this study could increase our understanding of the use of PGP bacteria for improving soil fertility and plant growth.

MATERIALS AND METHODS

Soil Sampling and Site Description

A total of three independent purple soil samples were collected after the harvest of soybean every week in September 2013. Fifteen randomly collected soil cores (10 mm diameter, 0–20 cm depth) were combined into one independent soil sample from each of five plots (200 m^2), where lettuce – eggplant/red pepper – soybean – Chinese cabbage had been rotated for the past 10 years. The collected soils were kept on ice before being transported to the laboratory and stored at 4°C overnight to the next day for the isolation of PGP bacteria. The plots were located at the National Monitoring Station for Soil Fertility and Fertilizer Efficiency, close to the campus of Southwest University, Chongqing (30°26′N, 106°26′E, 266 m above the sea level), China. The area has an annual mean temperature of 18.3°C and a mean precipitation of 1,115 mm. The initial soil properties in 1991 were as follows: pH, 7.5 (water: soil = 2:1), 8.44 g organic matter kg^{-1}, 0.92 g N kg^{-1}, 0.54 g P kg^{-1}, and 16.7 g K kg^{-1}, 43.2 mg available N kg^{-1}, 11.0 mg available P kg^{-1}, and 65.3 mg available K kg^{-1}.

PGP Bacteria Isolation and Determinations of N_2-fixing, P- and K-solubilizing Ability

In each bacterial isolation, a total of 10.0 g of purple soil from each plot was mixed with 90 ml sterilized tap-water and shaken at 120 rpm for 0.2 h at 28 ± 2°C (Zhang et al., 2012). This procedure was repeated five times and the obtained suspensions from each plot were mixed and then diluted with sterilized tap-water. In each 10^{-4}, 10^{-5}, 10^{-6}, or 10^{-7} dilution, 100 μl suspensions were incubated in each plate containing N_2-fixing Ashby, P-solubilizing PKO or K-releasing agar medium for 3–5 days at 28°C (Zhang et al., 2012). Bacterial colonies were then picked up, according to growth speed and transparent zones around, for further purification. All purified isolates were cryopreserved at −80°C in LB containing 40% glycerol. Four replicated bacterial isolations from each plot were performed to

evaluate the bacterial population (CFU g^{-1} soil, Colony Forming Units).

Observation of the presence of N_2-fixing, P- and K-solubilizing bacteria was accorded to Perez et al. (2007), Akhter et al. (2012), and Yi et al. (2012), respectively. Briefly, three replicates of 2 ml purified N_2-fixing, P- or K-solubilizing bacterial isolates (10^8 CFU/ml) were, respectively, added to 100 ml of corresponding medium and incubated at 28 ± 2°C and 120 rpm for 7 days. Then, 10 ml supernatant from each of these incubated cultures were then collected after centrifugation at 5,000 rpm for 10 min. Determination of N, P, and K content in the collected supernatant was accorded to the micro-kjeldahl method (Hendershot, 1985), Mo-Sb colorimetric method (Perez et al., 2007), and flame spectrophotometer method (Yi et al., 2012), respectively. The above-mentioned procedure was repeated three times or batches. A total of 17 bacterial isolates were then initially screened according to their growth curve, pH and temperature tolerance, and N, P, and K content (data not shown). These 17 bacterial isolates included six for N_2-fixing (named as XD-N-1 to XD-N-6), six for P-solubilizing (XD-P-1 to XD-P-6), and five for K-solubilizing (XD-K-1 to XD-K-5) ones.

Phenotypic Identification and 16s rRNA Sequencing of the Obtained Isolates

The determination of phenotypically physiological and biochemical characteristics of these 17 bacterial isolates was accorded to (Samina et al., 2010). These characteristics included cell shape and size, colony pigmentation, Gram staining, spore formation, motility and UV-fluorescence (data not shown), and gelatin liquefaction, glucose produced acid and gas, indole production, methyl red test, nitrate reduction, presence of catalase and oxidase, phenylalaninase, starch hydrolysis, use of citrate, Voges–Proskauer test and xylose produced acid (see data in **Table 1** for three selected isolates).

TABLE 1 | Physiological and biochemical characteristics of the three isolates obtained from a purple soil.

Characteristics	XD-N-3	XD-P-1	XD-K-2
Catalase	+	+	+
Gelatin liquefaction	+	+	+
Glucose produced acid	+	+	+
Glucose produced gas	−	−	−
Indole production	−	−	−
Methyl red test	+	−	+
Nitrate reduction	+	−	+
Oxidase	−	−	+
Phenylalaninase	−	−	−
Starch hydrolysis	+	−	+
Use of citrate	−	+	+
Voges-Proskauer test	+	+	−
Xylose produced acid	+	+	+

*"+" and "−" indicate positive or negative reaction, respectively. Isolate code: XD-K-2, K-solubilizing strain; XD-N-3, N_2-fixing strain 3; XD-P-1, P-solubilizing strain. See **Table 2** for abbreviation details.*

After the phenotypic determination, the genomic DNA of these 17 isolates was extracted using a TAKARA Mini BEST Bacteria Genomic DNA Extraction Kit Ver.3.0 (TAKARA BIO INC.). DNA quality was assessed by OD 260/280 and 260/230 ratio (Beckman Coulter DU 800) and amplified using the universal bacterial 16S rRNA forward primer 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and reverse primer 1492R (5′-TACGGTTACCTTGTTACGACTF-3′). With a *Premix Taq*^{TM} Kit (*TAKARA Taq*^{TM} Version 2.0 plus dye, Takara Bio, Otsu, Japan), the PCR was performed using an ABI PCR Instrument (GenenAmp® 9700). The PCR mixture contained 25.0 μl *Premix Taq*^{TM} (1.25U DNA polymerase, 4 mM Mg^{2+}, 0.4 mM dNTP mixture), 1.0 μl 20 μM forward primer, 1.0 μl 20 μM reverse primer, 1.0 μl template DNA (about 100 ng), and 22.0 μl nuclease-free water to a final volume of 50 μl. After the initial denaturation for 5 min at 95°C, the PCR running was 30 cycles of denaturation for 60 s at 95°C, annealing for 60 s at 55°C, and elongation for 2 min at 72°C, except for 10 min in the final elongation. The PCR products were recovered using an AxyPrepDNA Gel Extraction Kit (Axygen Bio, USA) following the manufacturer's instructions. The amplified partial 16S rRNA gene was a single band (1,300–1,500 bp) by electrophoresis through a 2.0% (w/v) agarose gel and 0.5 mg L^{-1} ethidium bromide. The PCR products were commercially quantified by the Invitrogen Trading Co., Ltd. (ShangHai, China) using the QuantiFluor^{TM}-ST blue fluorescence quantitative system (Promega, China).

The obtained gene sequences were compared with other sequences and the accession numbers (see below in the Result section) were deposited in the GenBank database using the NCBI BLAST[1]. Multiple sequence alignments were performed by CLUSTAL W (MEGA Version 6.06). The phylogenetic tree topology based on re-samplings of 1000 times of the neighbor joining data set was evaluated by the boot strap analysis (MEGA Version 6.06).

Plant Growth Experiment

The selected three isolates of XD-N-3, XD-P-1, and XD-K-2 from the initial 17 screened isolates were used as an inoculant in the plant growth experiment. In doing so, the suspension of XD-N-3, XD-P-1, and XD-K-2, after being separately cultured in LB medium, were mixed together with a ratio of 1:1:1 of CFU into 1.0 kg autoclaved peat (<2 mm) as a complex inoculant (Xi et al., 2009). The total number of effective bacteria was about 3×10^8 cfu.g^{-1} in this complex inoculant. The peat (pH 6.7) contained 437 g organic matter kg^{-1}, 10.2 g N kg^{-1}, 2.23 g P kg^{-1}, and 6.42 g K kg^{-1}.

Four treatments were administered to air-dried and sieved (2 cm) purple soil (**Table 2**): (1) no-fertilizer and no-bacteria inoculant (Control), (2) no-bacteria inoculant and a full-rate of chemical NPK fertilization (CF, N:P:K = 2.6:1:1) as urea (321 mg N kg^{-1}), Ca $(H_2PO_4)_2 \cdot H_2O$ (123.5 mg P kg^{-1}), and KCl (123.5 mg K kg^{-1}), (3) a mixture of the three bacteria as a complex inoculant (CI), and (4) a half-rate of chemical NPK and a full dose of the complex bacterial inoculant (1/2CF+CI).

[1]http://www.ncbi.n1m.nih.gov/blast/Blast.cgi

TABLE 2 | The nutrient composition (mg/kg) of soil treatments utilized in the present study to determine the effect of a complex bacterial inoculants on soil fertility and plant growth.

Treatment	Urea	Calcium superphosphate	Potash	PGP bacterial inoculate
Control	0	0	0	0
CF	321	123.5	119	0
CI	0	0	0	30
1/2CF+CI	160.5	61.75	59.5	30

Control, no-fertilizer and no-inoculant control; CF, no-inoculant and full dose of chemical NPK fertilization; CI, full bacterial complex inoculant; 1/2CF+CI, half-dose of chemical NPK fertilization and full bacterial complex inoculant.

The soil was placed in pots (10 cm × 10 cm, each contained 1.5 kg·soil) and each treatment was represented by four replicates using a fully randomized design. Two uniform, virus-free, tissue-cultured kiwifruit (*A. chinensis* cv. 'Hongyang') plantlets were transplanted into each pot, grown in a greenhouse for 50 days at the Chongqing University of Arts and Science, and then for an additional 40 days in a greenhouse at the College of Resources and Environment, Southwest University, Chongqing, China. Soil moisture was regularly adjusted to 55% water holding capacity with deionized water during the whole period of plant growth. The study was conducted during the spring of 2014.

Analyses of Soil and Plant Samples from the Plant Growth Experiment

Soil samples from each pot were randomly collected around the fine roots of the two kiwifruit plantlets and kept on ice during transport to the laboratory where they were stored overnight at 4°C prior to conducting CFU of bacterial counting analyses. Soil samples for the determination of chemical properties were sieved (1 mm) after being air-dried at room temperature for 7 days and then stored at 4°C prior to analysis. Determinations of soil organic matter (SOM; digestion method), total N, P, and K (micro-Kjeldahl digestion), available N (alkaline diffusion method), available P (0.5 M $NaHCO_3$-extractable P, pH 8.5), available K (1.0 M NH_4OAc)-extractable K) and soil pH (soil:water = 1:1, v/v) were accorded to Page et al. (1982).

At harvest, plant height, ground diameter, and total leaf area were measured. Leaves were collected as described by Lamb and Rubery (1976). Oven-dried (48 h at 70°C) root and shoot samples were weighed and milled with a high-speed multi-function micro-pulverizer (Whirl Type Model Y-60, Hebei, China) prior to analysis. Plant samples were digested by a mixture of 96% H_2SO_4 and 30% H_2O_2. With a Shimadzu Model UV-120-02 spectrophotometer, the digested solution was analyzed for plant N by the Kjeldahl method, P by the molybdate blue colorimetric analysis, and K by the flame photometry (Nanjing Agricultural University, 1994). Leaf polyphenol oxidase (PPO) activity and malondialdehyde (MDA) content were assayed according to Moerschbacher et al. (1988) and Hodges et al. (1999), respectively.

Data Analyses

The pot experiment was only performed once, i.e., without a biological replicate, but with four replicates. Data were hence expressed as means ± SD ($n = 4$) and subjected to ANOVA, and significant differences between treatment means were then compared by the Tukey High Significant Difference (Tukey HSD) Test at $P \leq 5\%$ using SPSS (Version 19.0, New York, USA).

RESULTS AND DISCUSSION

Identification and Characterization of Bacterial Isolates

Three isolates, respectively, identified by their N_2-fixing, and P- and K-solubilizing capacity as XD-N-3, XD-P-1, and XD-K-2 through phenotypic identification and 16s rRNA sequencing, were combined to form a complex inoculant that was used in this study. The N_2-fixing, and P- and K-solubilizing capacity of these three selected bacterial isolates was 91.5 ± 2.69 mg N L^{-1} for the N_2-fixing XD-N-3, 129 ± 11.89 mg P L^{-1} for the P-solubilizing XD-P-1, and 23.2 ± 0.79 mg K L^{-1} for the K-solubilizing XD-K-2. The biochemical characteristics of these three isolates are presented in **Table 1**. All three isolates were fast growing, Gram-positive, and appeared as small, oval rods with a mid-spore, and a thickening capsule (see **Figures 1A–C**). In general, colonies of XD-N-3 were white, semitransparent, regular shaped with a raised elevation and a smooth surface. The average colony diameter was 2–3 mm in 2 days and 6–8 mm in 5 days on Ashby medium (**Figure 1D**). Colonies of XD-P-1 formed an obvious P-solubilizing circle and their average diameter was 2–3 mm in 2 days and 5–6 mm in 5 days on Pikovskaya's medium. The colonies had regular margins, and were moist, transparent, and hard to pick out (**Figure 1E**). Colonies of XD-K-2 formed an obvious K-solubilizing circle and had an average diameter of −2 mm in 2 days and 5–7 mm in 5 days on K-amended selective medium. The colonies were moist, milky white, semitransparent, regular shaped with raised elevation and a smooth surface (**Figure 1F**).

The sequences analysis of 1.5 kb fragment of 16S rRNA genes of the three selected bacterial isolates (XD-N-3, XD-P-1, and XD-K-2) were aligned with other sequences in the GenBank database. The phylogenetic tree of the three bacterial isolates constructed by using their 16s rRNA (**Figure 2**) showed that they were members of genus *Bacillus*. With a similarity of 99, 99, and 98% to respective *Bacillus amyloliquefaciens*, *B. pumilus,* and *B. circulans,* the 16s rRNA gene nucleotide sequence of these three isolates were designated as *B. amyloliquefaciens* XD-N-3, *B. pumilus* XD-P-1, and *B. circulans* XD-K-2, respectively. These isolates were submitted to GeneBank under the accession number KU922934, KU922935, and KU922936, respectively.

Bacillus is one of the most commonly studied genera in plant and soil sciences (Hallmann and Berg, 2006). The isolates belonging to *Bacillus* identified in the present study was similar

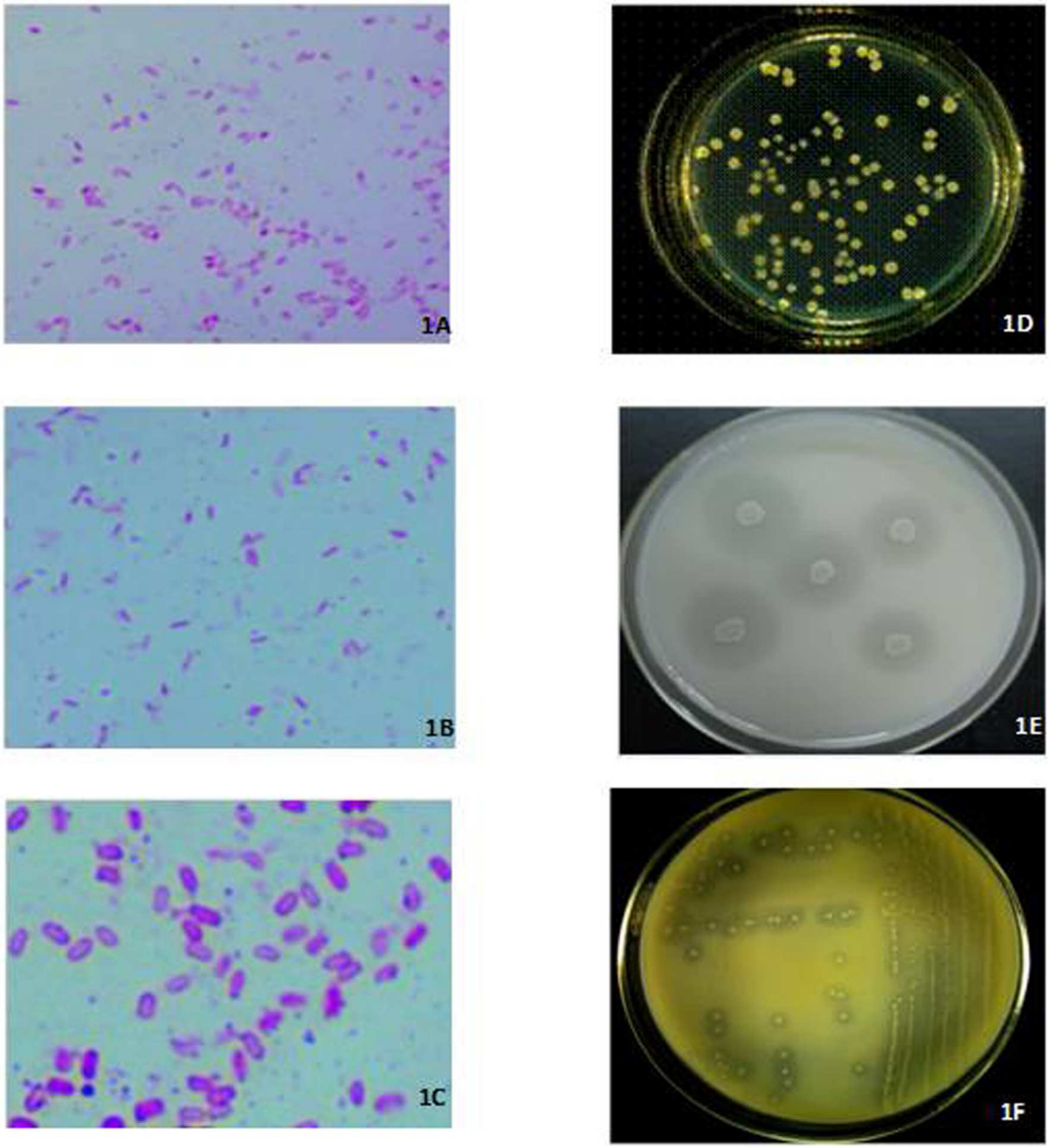

FIGURE 1 | Left: Photomicrographs (1600×) of cells and spores of XD-N-3 (A), XD-P-1 (B), and XD-K-2 (C) grown on LB medium at 28°C for 3 days. Right: *in vitro* colonial morphology of XD-N-3 **(D)** grown on the Ashby medium at 28 ± 2°C after 3 days, and XD-P-1 **(E)**, XD-K-2 **(F)** respectively, grown on the Pikovskaya's and K-amended selective medium at 28 ± 2°C after 5 days. The formation of halo zone around the colonies of **(E)** and **(F)** showed the solubilization of the inorganic phosphate and potassium.

to these identified in root adhering soil of various host plants including peas, clovers (Laguerre et al., 1994). The colonization of the rhizosphere by *Bacillus* sp. during the active plant growth has been reported in maize in France (Lambert et al., 1987) and in Canada (Lalande et al., 1989), in a number of crops incldiung buckwheat, finger millet, frenchbean, and maize (Pal, 1998) and tomato (Mehta et al., 2015) in India, and in wheat in Pakistan (Majeed et al., 2015).

Effects of the Complex Inoculant on the Population of Soil Bacteria

According to above-mentioned methods, three isolates, i.e., XD-N-3, XD-P-1, and XD-K-2, were selected as potential PGP bacteria and combined to produce a complex inoculant. After 90 days of kiwifruit plantlets grown in greenhouse, the level of bacterial growth in the four soil treatments was ranked from highest to lowest as follows: 1/2CF+CI > CI > CF ≈ Control

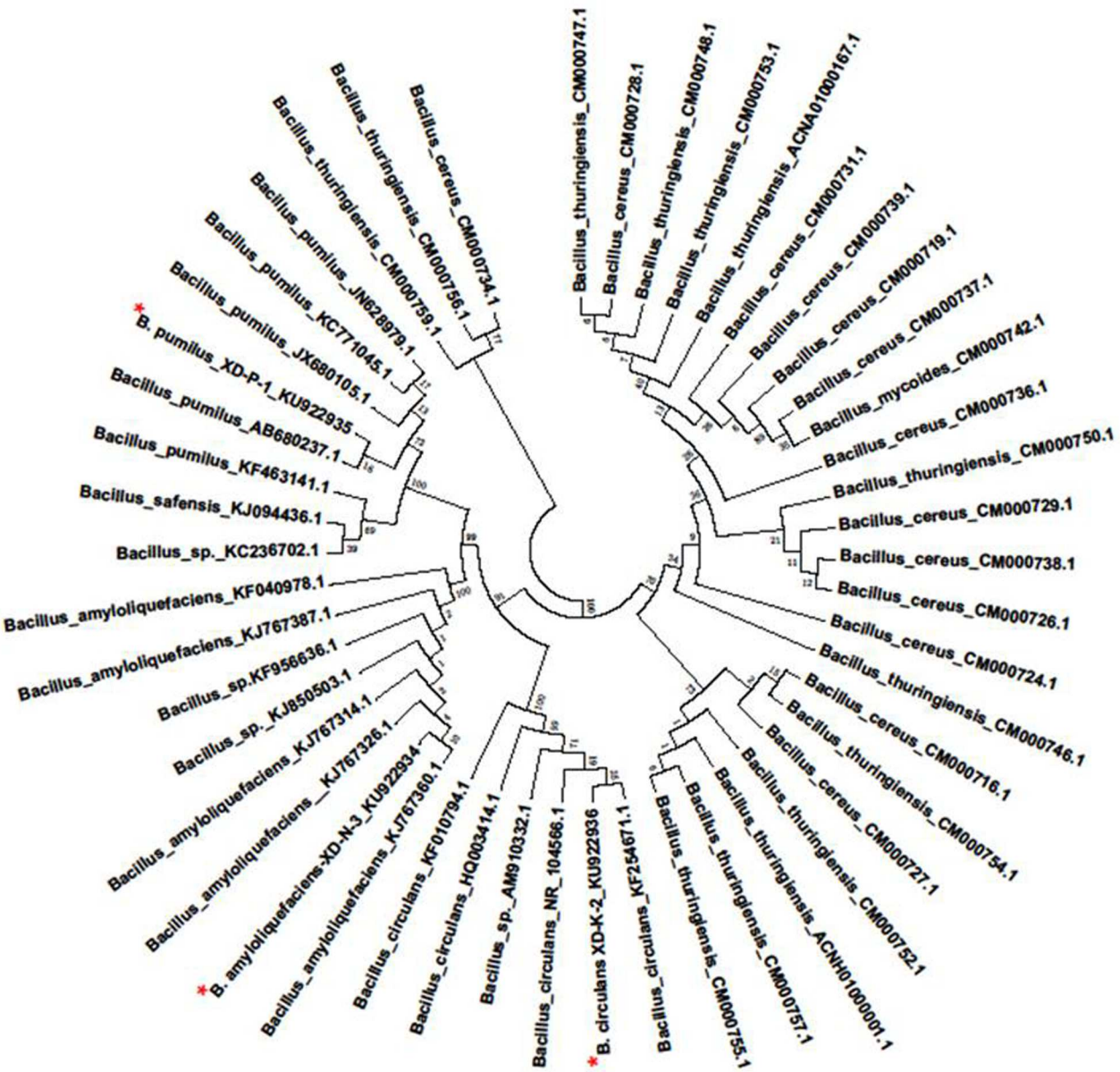

FIGURE 2 | Phylogenetic tree was prepared by Neighbor-joining method of the XD-N-3, XD-P-1, and XD-K-2 which was followed by an asterisk, respectively. Numbers above or below the nodes indicate bootstrap values generated after 1000 replications. Bacteria species and sequences from GeneBank were shown with their accession numbers in the figure. The evolutionary distance was computed using the Maximum Likelihood method and was in the units of the number of base substitutions per site.

for total CFUs, and 1/2CF+CI ≈ CI > CF ≈ Control for N_2-fixing, P-solubilizing -and K-solubilizing bacteria (**Table 3**). These results indicate that there were no significant differences between the Control and CF treatment in regards to total CFUs or the level N_2-fixing, P-, and K-solubilizing bacteria (**Table 3**). Compared to the Control, however, the CFUs of total culturable bacteria, N_2-fixing, P- and K-solubilizing bacteria were strongly enhanced in the CI treatment by 915, 5046, 969, and 729%, respectively. Although the CFUs of culturable bacteria were significantly higher in the 1/2CF+CI treatment than in the CI treatment, there were no significant differences in the CFUs of either N_2-fixing, P- or K-solubilizing bacteria. Additionally, the level of the N_2-fixing, and P/K-solubilizing bacteria were ten-fold greater in the 1/2CF+CI treatment than in the Control and CF treatments. Since the populations of N_2-fixing bacteria, as well as P- and K-solubilizing bacteria were significantly higher in the 1/2CF+CI treatment, these results evidenced that the three isolates, namely *B. amyloliquefaciens* XD-N-3, *B. pumilus* XD-P-1 and *B. circulans* XD-K-2, had ecological adaptation of the tested purple soil.

TABLE 3 | Effect of the complex inoculant on the populations of culturable bacteria in a purple soil.

Treatment	Total Bacteria ($\times 10^6$ cfu• g^{-1} soil)	N_2-fixing bacteria	P-solubilizing bacteria	K-solubilizing bacteria
		($\times 10^3$ cfu• g^{-1} soil)		
Control	3.98 ± 2.28^c	0.55 ± 0.31^b	3.48 ± 2.28^b	7.95 ± 4.56^b
CF	11.63 ± 2.90^c	1.93 ± 0.84^b	6.76 ± 3.02^b	13.53 ± 6.03^b
CI	40.42 ± 4.95^b	28.33 ± 11.62^a	37.24 ± 8.92^a	65.93 ± 6.01^a
1/2CF+CI	64.52 ± 16.05^a	30.52 ± 10.77^a	34.81 ± 10.00^a	55.90 ± 1.57^a

*Data (means ± SD, n = 4) followed by different letters in the same column (a,b) between treatments indicate significant differences at $P \leq 0.05$. See **Table 2** for abbreviation details.*

Effects of the Complex Inoculant on the Availability of Soil Nutrients

The level of soil available N (**Figure 3D**), P (**Figure 3E**), and K (**Figure 3F**) differed significantly between the Control and the CI treatments, indicating that an improvement in soil fertility was achieved through the inoculation of this complex inoculant. The improved level of availability of soil N, P, and K was consistent with results reported in soil grown alfalfa (Han et al., 2011) and vegetable crops of tomato and spinach rotation system (Song et al., 2015). In contrast to our complex inoculant (*B. amyloliquefaciens* XD-N-3, *B. pumilus* XD-P-1, and *B. circulans* XD-K-2), a mixed inoculant of azotobacters, P- and K-solubilizing bacteria was applied by Han et al. (2011), and a mixed inoculant of two strains of *B. subtilis* and two strains of *B. mucilaginosus* was employed by Song et al. (2015).

Significantly differences in SOM were observed between treatments ranked from highest to lowest as follows: 1/2CF+CI (8.38 g·kg^{-1}) > CI ≈ CF ≈ Control (**Figure 3G**). Significant differences in total soil nutrient levels were observed between treatments ranked from highest to lowest as follows: 1/2CF+CI (0.94 g N kg^{-1}) ≈ CI ≈ CF > Control for total N (**Figure 3A**), 1/2CF+CI (0.47 g P kg^{-1}) ≈ CI ≈ CF ≈ Control for total P (**Figure 3B**), and 1/2CF+CI (18.14 g K kg^{-1}) ≤ CF > CI ≈ Control for total K (**Figure 3C**). A pattern of increasing soil available nutrients was observed between the treatments ranked from highest to lowest as follows: 1/2CF+CI (42.93 mg N kg^{-1}) ≈ CI > CF ≈ Control for available N, 1/2CF+CI (11.18 mg P kg^{-1}) > CF > CI > Control for available P, and 1/2CF+CI (110.42 mg K kg^{-1}) > CF ≈ CI > Control for available K. Collectively, these results indicated that the complex inoculant had the capacity to increase soil available N, P, and K (CI vs. Control). In addition, the combination of a

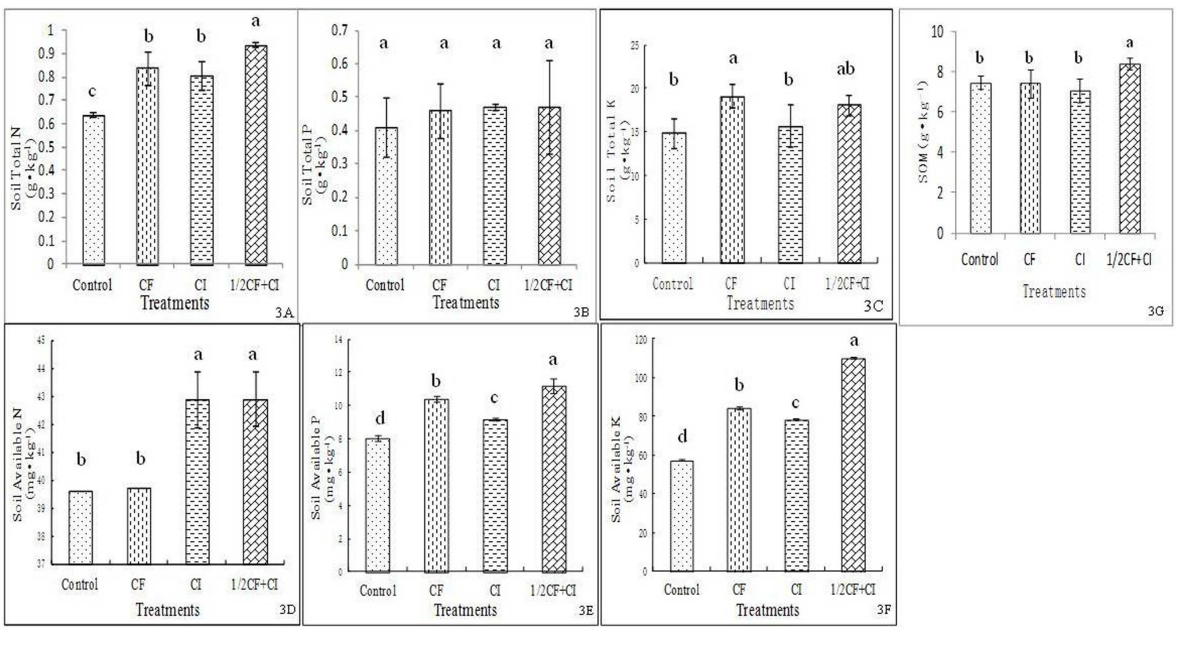

FIGURE 3 | Effect of the complex inoculant on soil total N (A), total P (B) and total K (C), and soil available N (D), available P (E), available K (F) and soil organic matter (G) after 90 days of kiwifruit plantlets grown under greenhouse conditions. Data (means ± SD, $n = 4$) followed by different letters in the same column (a, b) between treatments indicate significant differences at $P \leq 0.05$. Control, no-fertilizer and no-inoculant control; CF, no-inoculant and full dose of chemical NPK fertilization; CI, full bacterial complex inoculant; 1/2CF+CI, half-dose of chemical NPK fertilization and full bacterial complex inoculant.

half-rate of chemical fertilizers with the addition of the complex inoculants containing the three PGP bacterial isolates may have the potential to replace the amount of chemical fertilizer needed to obtain optimum levels of essential nutrients. Importantly, no significant increases in total soil N, P, and K concentrations were observed between the four treatments. Caution should be taken, however, in regards to this claim since the effect of just a 1/2 CF treatment was not examined in the present study. Rong et al. (2014) reported that a biofertilizer consisting of five P-solubilizing isolates and one rhizobium isolate could replace 80% of the chemical fertilizer and produce a similar yield in pea. These results suggested that the inoculated PGP bacteria had contributed to the solubilization of P and/or K either from the soil itself or from the applied chemical P and K fertilizer. The results in N_2-fixation and P-solubilization in the present study are in good accordance with the increase of plant tissue N in maize and soil P availability obtained by three bacterial isolates that showed N_2-fixation and P-solubilizing ability (Zahid et al., 2015). Among these three isolates, two showed 99% similarity with *B. subtilis* and another 99% similarity with *B. megaterium*. The maize was gown under greenhouse conditions along with or without *Bacillus* inoculation, and 1/2NP and full NP fertilization (60 and 45 mg kg^{-1} soil for full N and P rate).

Effects of the Complex Inoculant on Plant Growth

A pattern of an increasing positive impact was observed among treatments ranked from greatest to least as follows: 1/2CF+CI (21.3 cm) ≈ CI > CF > Control for plant height (**Figure 4A**), 1/2CF+CI (0.3 mm) ≈ CI > CF ≈ Control for stem diameter at the ground surface (**Figure 4B**), 1/2CF+CI (104.6 cm^2) > CI > Control > CF for leaf area (**Figure 4C**), 1/2CF+CI (2.43 g·pot^{-1} and 3.40 g·pot^{-1}) ≈ CI > CF > Control for both shoot DW and total plant total biomass, respectively (**Figure 4D**), and 1/2CF+CI (0.97 g·pot^{-1}) ≈ CI > CF ≈ Control for root biomass (**Figure 4E**). In comparison to the Control, plant height, and shoot and root dry weight were significantly increased by 59.8, 83.3, and 33.3% under CF, respectively; while leaf area was significantly decreased by 59.2%.

One of the multiple pathways that PGPRs promote plant growth is to manipulate root growth (Biswas et al., 2000; Lucy et al., 2004). The application of PGP bacteria (CI) resulted in a fairly strong growth-promoting effect on kiwifruit plantlets. For example, a 200 and 125% higher root biomass production was observed under CI, in compared to the Control and CF treatments, respectively. Also compared to the Control, shoot dry weight and plant height both increased significantly by 300 and 118% under CI and by 50 and 50% under CF, respectively.

Interestingly, both shoot and root dry biomass was significantly increased by 118 or 150% under 1/2CF+CI and CF, respectively, compared to the Control (**Figure 4E**). A similar trend was observed between the CI and CF treatments. These results are in accordance with results of plant biomass production obtained in maize (Zahid et al., 2015), Chinese kale (Piromyou et al., 2013) and almost all major vegetables and fruits (Berg, 2009). For instance, compared to the un-inoculated

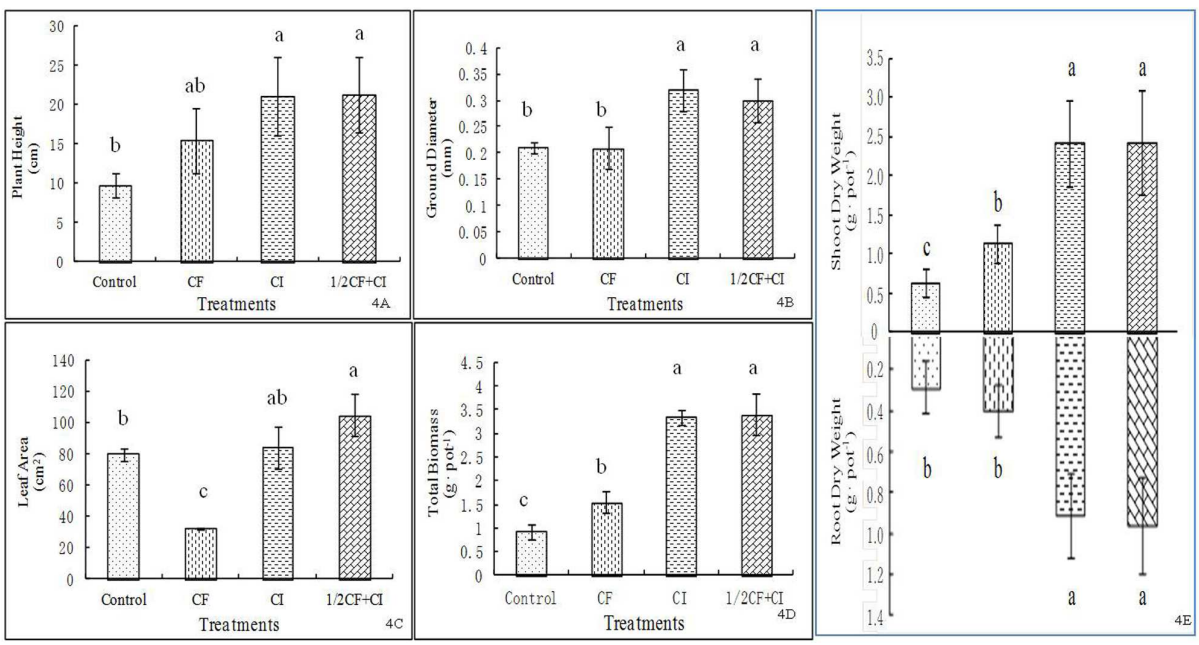

FIGURE 4 | Effect of the complex inoculant on plant height (A), plant ground diameter (B), leaf area (C), total biomass (D) and shoot and root dry weigh (E) of kiwifruit plantlets after 90 days grown under greenhouse conditions. Data (means ± SD, $n = 4$) followed by different letters in the same column (a, b) between treatments indicate significant differences at $P \leq 0.05$. Control, no-fertilizer and no-inoculant control; CF, no-inoculant and full dose of chemical NPK fertilization; CI, full bacterial complex inoculant; 1/2CF+CI, half-dose of chemical NPK fertilization and full bacterial complex inoculant.

plant, biomass production of maize was increased by seven *Bacillus* isolates that showed ability to promote N_2-fixation, P-solubilizing or indole-3-aceticacid production, no matter whether the maize was gown under 1/2NP or full NP fertilization (60 and 45 mg kg^{-1} for full N and P rate) in a greenhouse (Zahid et al., 2015). Similarly, under the same fertilization rate of 1,000 kg compost containing 10 mg N, 29 g P, and 10 mg K kg^{-1}, and 130 kg N, 80 kg P, and 60 kg K ha^{-1} to both pot and field experiments, no effects of the sole compost on plant biomass, but the dual application of compost with *Bacillus* sp. SUT1 did enhance the biomass production of Chinese kale, particularly in the field experiment, indicating a direct growth promotion by *Bacillus* sp. SUT1 (Piromyou et al., 2013). In addition, a number of *Bacillus* isolates, e.g., *B. lichenformis*, *B. megaterium*, *B. subtilis* FZB24, *B. subtilis* GB03, *B. pumilus* GB34, and *B. subtilis* QST716, had been used to not only to promote plant biomass production (almost all major vegetables and fruits), but also to control a variety of bacterial and fungal pathogens (*Botrytis*, *Fusarium*, *Phytophthora*, *Phomopsis*, *Pythium*, and *Rhizoctonia*, etc.; see a review by Berg, 2009). Collectively these studies suggested that the inoculation of soils with PGP bacteria could enhance fertilizer use efficiency (Adesemoye and Kloepper, 2009) by decreasing the level of chemical fertilizers that were required to obtain greater growth and yield (Adesemoye et al., 2009; Rong et al., 2014; Zahid et al., 2015).

The highest levels of N (**Figure 5A**), P (**Figure 5B**), and K (**Figure 5C**) in both shoots and roots were always observed under 1/2CF+CI; with the exceptions for shoot and root N under CF, shoot P under CF and CI, and shoot K under CI. Compared to the CF treatment, N, P, and K concentrations under 1/2CF+CI were increased by 6.8, 12.0, and 40.9% in shoots, or by 16.1, 32.2, and 29.1%, in roots, respectively. Meanwhile, plant total N (**Figure 5D**), P (**Figure 5E**), and K (**Figure 5F**) accumulation in the treatments generally ranked from highest to lowest as follows: 1/2CF+CI (40.79 mg N, 2.95 mg P and 46.01 mg K) ≈ CI > CF > Control, though total plant N accumulation was similar between the CF and CI treatments. These results in kiwifruit are generally consistent with these in pepper and cucumber (Han and Lee, 2006), red pepper (Islam et al., 2013), and sugar beet (Shi et al., 2011). In contrast

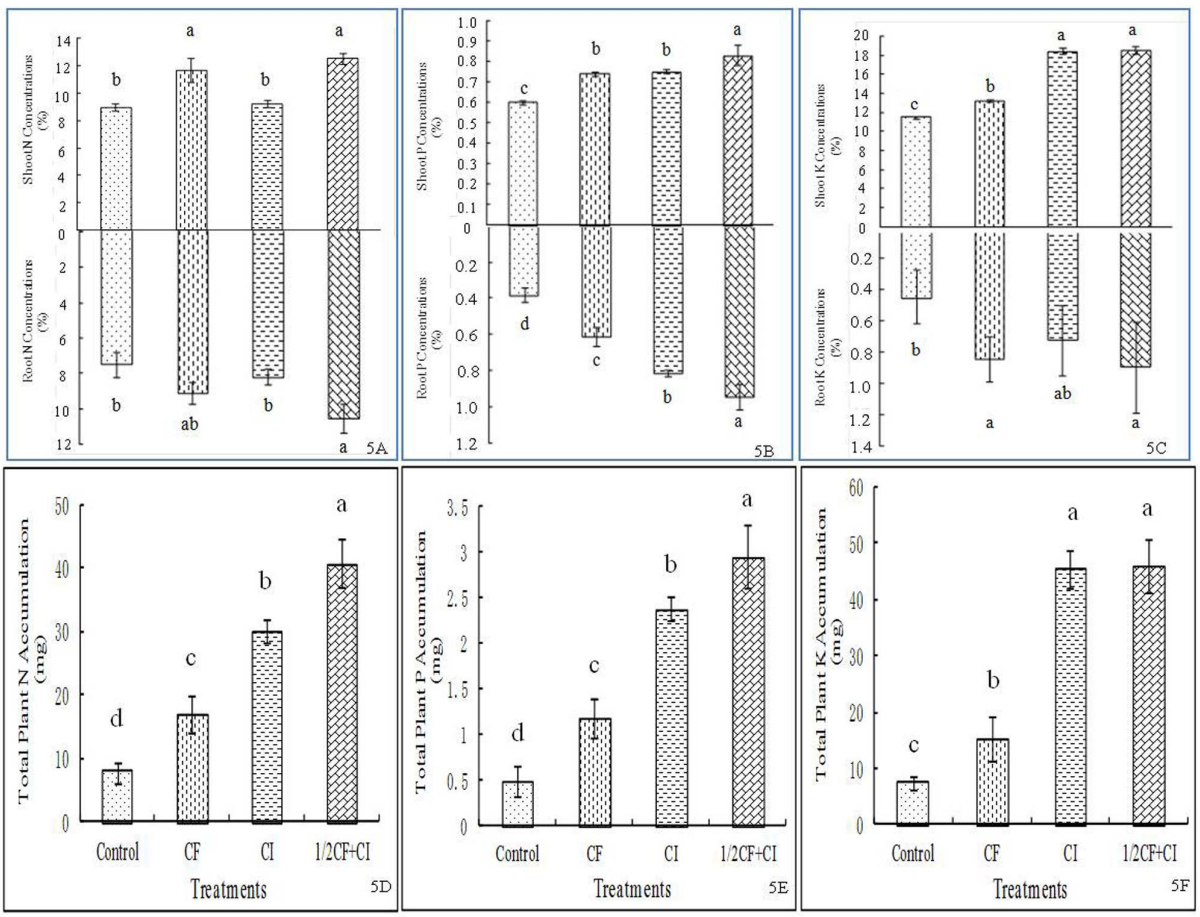

FIGURE 5 | Effect of the complex inoculant on concentrations of plant N (A), P (B), K (C) and accumulations of total N (D), P (E), and K (F) of kiwifruit plantlets after 90 days grown under greenhouse conditions. Data (means ± SD, $n = 4$) followed by different letters in the same column (a, b) between treatments indicate significant differences at $P \leq 0.05$. Control, no-fertilizer and no-inoculant control; CF, no-inoculant and full dose of chemical NPK fertilization; CI, full bacterial complex inoculant; 1/2CF+CI, half-dose of chemical NPK fertilization and full bacterial complex inoculant.

to our triple inoculation with *B. amyloliquefaciens* XD-N-3, *B. pumilus* XD-P-1, and *B. circulans* XD-K-2, the inoculation with both P- and K-solubilizing *B. megaterium* var. *phosphaticum* and *B. mucilaginosus* resulted in significantly greater soil P and K availability (Han and Lee, 2006). Meanwhile, inoculation with the N_2-fixing bacteria *Pseudomonas* sp. RFNB3 (Islam et al., 2013) and *Acinetobacter johnsonii* strain 3–1 (Shi et al., 2011) had resulted in significantly greater uptake of N, P, and K, shoot and root biomass production. Collectively, these studies from this study and other studies indicate that PGP bacteria, used as biofertilizers, have the potential to reduce the application rate of chemical fertilizers. Caution should be taken to this claim, however, since the sole application of the 1/2 CF treatment was not examined in the present study.

Effects of the Complex Inoculant on Leaf PPO Activity and MDA Content

Leaf PPO activity between treatments ranked from significantly highest to lowest as follows: CF (4.24 U/g·min FW) > 1/2CF+CI (2.58 U/g·min FW) > Control (1.81 U/g·min FW) ≈ CI (1.67 U/g·min FW; **Figure 6A**). PPO is a plant respiratory chain terminal oxidase, which catalyzes the oxidation of phenolic compounds to highly toxic quinones that play an important role in plant disease resistance (Richter et al., 2012). Thus the relatively greater leaf PPO activity under 1/2CF+CI might indicate the potential to apply this complex inoculant to enhance plant disease resistance. However, leaf PPO activity is also induced by plant pathogens such as the early blight disease pathogen in tomato (Narendra et al., 2015), the Panama Wilt Resistance pathogen in banana (Kavino et al., 2014), and the

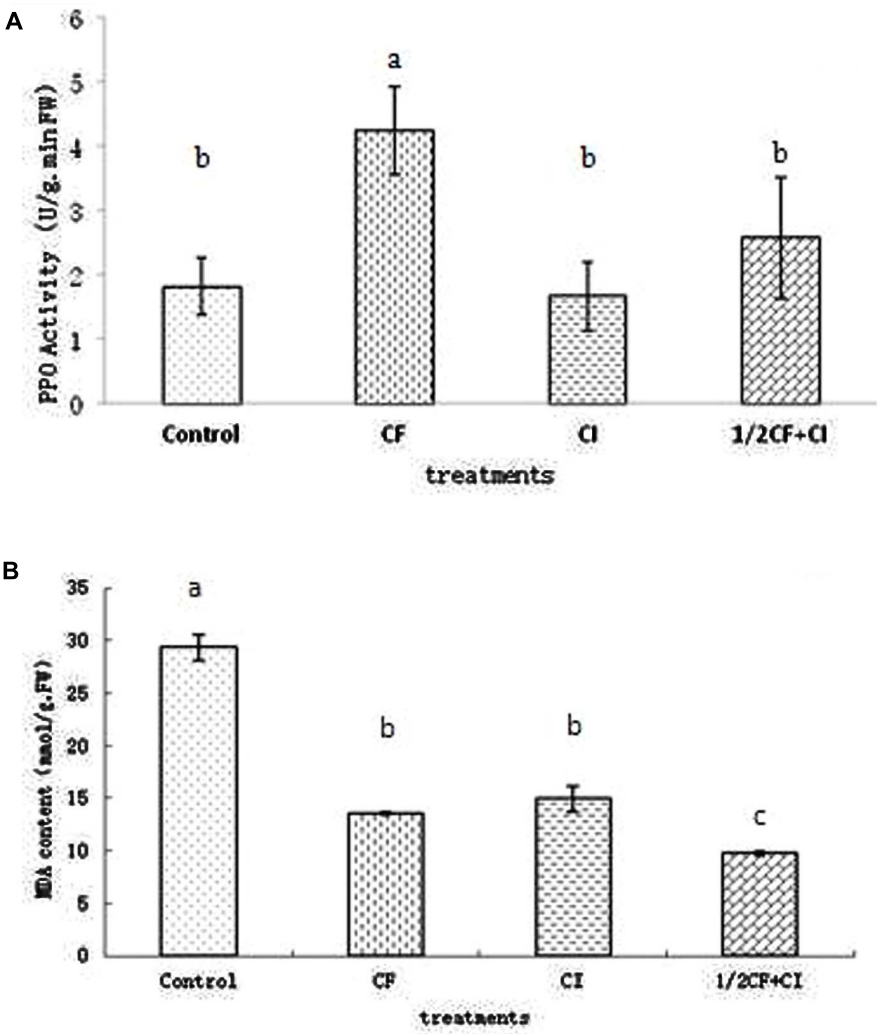

FIGURE 6 | Activity of leaf polyphenol oxidase (PPO; A) and content of Malondialdehyde (MDA; B) in 90-days-old kiwifruit seedlings. Data (means ± SD, $n = 4$) followed by different letters (a, b) between treatments indicate significant differences at $P \leq 0.05$.

Fusarium-wilt pathogen in eggplant and tomato (Altinok et al., 2013; Mei et al., 2014).

Leaf MDA between treatments ranked from significantly highest to lowest as follows: Control (29.33 mmol/g·FW) > CI (14.94 mmol/g·FW) ≈ under CF (13.61 mmol/g·FW) > under 1/2CF+CI (9.79 mmol/g·FW; **Figure 6B**). In general, MDA content is negatively correlated with plant stress resistance and increases in response to stress conditions such as soil salinization (Shukla et al., 2012; Xun et al., 2015), and drought (Wang et al., 2012). Thus, the comparatively lower leaf MDA content in kiwifruit plantlets under 1/2CF+CI and CI might suggest that this complex bacterial inoculant could provide a protective effect to plants under stress conditions.

CONCLUSION

Three selected PGP bacterial isolates, *B. amyloliquefaciens* XD-N-3, *B. pumilus* XD-P-1, and *B. circulans* XD-K-2, which were isolated from a purple soil, exhibited a strong capacity to fix N_2, and solubilize P and K, respectively. Results from a pot experiment with kiwifruit plantlets demonstrated the positive effects of the complex inoculant (a mixed combination of these three *Bacillus* isolates). Specifically, increased available soil N, P, and K in purple soil were observed, in addition to a promotion of leaf N, P, and K levels. Consequently, these effects resulted in an increased plant biomass, leaf PPO activity and MDA. Results of the present study indicated that this complex inoculant might have potential to be used as a bio-fertilizer for improving soil fertility and plant growth. Future studies should focus on (a) the development of a formulation for the complex inoculant that optimizes the ratio of inoculum and the carrier (peat) matrix; (b) a complete biosafety analysis of the use of PGP bacteria agents in field applications.

AUTHOR CONTRIBUTIONS

HS wrote the manuscript. XH School of Plant Biology, University of Western Australia, Crawley 6009, Australia, re-written this manuscript. YL director of Collaborative Innovation Center of Special Plant Industry, this project sponsor. YC and JT carried out experiments. TG Lab manager of Chongqing Key Laboratory of Soil Multi-scale Interfacial Process, designed experiments.

ACKNOWLEDGMENTS

This research was supported by the Achievement Transfer Program of Higher Education Fund of Chongqing Education Commission (KJZH: 14215). The authors greatly appreciate Dr. Michael Wisniewski in the USDA-ARS-Appalachian Fruit Research Station for his critical proofreading and language editing.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

REVIEW
published: 23 June 2016
doi: 10.3389/fpls.2016.00918

Biochemical and Molecular Mechanisms of Plant-Microbe-Metal Interactions: Relevance for Phytoremediation

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Plants and microbes coexist or compete for survival and their cohesive interactions play a vital role in adapting to metalliferous environments, and can thus be explored to improve microbe-assisted phytoremediation. Plant root exudates are useful nutrient and energy sources for soil microorganisms, with whom they establish intricate communication systems. Some beneficial bacteria and fungi, acting as plant growth promoting microorganisms (PGPMs), may alleviate metal phytotoxicity and stimulate plant growth indirectly via the induction of defense mechanisms against phytopathogens, and/or directly through the solubilization of mineral nutrients (nitrogen, phosphate, potassium, iron, etc.), production of plant growth promoting substances (e.g., phytohormones), and secretion of specific enzymes (e.g., 1-aminocyclopropane-1-carboxylate deaminase). PGPM can also change metal bioavailability in soil through various mechanisms such as acidification, precipitation, chelation, complexation, and redox reactions. This review presents the recent advances and applications made hitherto in understanding the biochemical and molecular mechanisms of plant–microbe interactions and their role in the major processes involved in phytoremediation, such as heavy metal detoxification, mobilization, immobilization, transformation, transport, and distribution.

Keywords: plant growth promoting microorganisms, root exudates, heavy metals, molecular bases, phytoremediation

OPEN ACCESS

Edited by:
Abdul Latif Khan, University of Nizwa, Oman

Reviewed by:
Carmen González Bosch, Universitat de València, Spain
Abdur Rahim Khan, Kyungpook National University, South Korea

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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *19 January 2016*
Accepted: *09 June 2016*
Published: *23 June 2016*

Citation:
Ma Y, Oliveira RS, Freitas H and Zhang C (2016) Biochemical and Molecular Mechanisms of Plant-Microbe-Metal Interactions: Relevance for Phytoremediation. Front. Plant Sci. 7:918. doi: 10.3389/fpls.2016.00918

INTRODUCTION

Soil contaminated with heavy metals has become a serious worldwide problem due to geologic and anthropogenic activities. Heavy metals are non-degradable and thus persist indefinitely in the environment. As an alternative to physical and chemical methods, the use of hyperaccumulating plants and beneficial microbes has been a promising approach to clean up metal contaminated soils through extraction (phytoextraction), stabilization (phytostabilization), and/or transformation (phytovolatilization) process (Lebeau et al., 2008; Glick, 2010).

Root exudates and microorganisms are important components of rhizosphere ecology and play important roles in changing the bioavailability of metals and nutrients. Root exudates provide

microbes an abundant source of energy and nutrients for microbes, and in return, microbes stimulate exudation from plant roots. In the co-evolutionary process, plants and their associated microbes coexist or compete for survival in the changing environment, and their relationships, either beneficial or detrimental are of significant importance for both partners. Root exudates are known to enhance mobility of metals and nutrients by (i) acidification due to proton (H^+) release or by forming organic/amino acid-metal/mineral complexes; (ii) intracellular binding compounds (e.g., phytochelatins, organic acids, and amino acids); (iii) electron transfer by enzymes in the rhizosphere (e.g., redox reactions); and (iv) indirectly stimulating rhizosphere microbial activity (e.g., survival, growth, propagation, and functioning), therefore enhancing phytoremediation efficiency (Ström et al., 2002; Pérez-Esteban et al., 2013; Sessitsch et al., 2013; **Figure 1**).

Microbes can enhance phytoremediation in different manners: expedite plant biomass, increase (phytoextraction) or decrease (phytostabilization) metal availability in soil, as well as facilitate metal translocation from soil to root (bioaccumulation) or from root to shoot tissues (translocation; Ma et al., 2011a, 2013; Rajkumar et al., 2012; **Figure 1**). In metal rich natural (serpentine soil) and anthropogenic contaminated habitats (e.g., mine waste and fly ash), microbes are able to tolerate considerable high concentrations of metals, and to evolve resistance strategies (Kumar and Nagendran, 2009; Ma et al., 2015c). There are several advantages in using plant growth promoting microorganisms (PGPMs) rather than chemical amendments in phytoremediation, because the microbial metabolites produced in the rhizosphere *in situ* are biodegradable and less toxic (Rajkumar et al., 2012). Metal resistant PGPM have been widely investigated for their potential to improve plant growth, alleviate metal toxicity, and immobilize/mobilize/transform metals in soil, which may help to develop new microbe-assisted phytoremediation and restoration strategies. Arbuscular mycorrhizal fungi (AMF), particularly those isolated from metalliferous sites, are capable of boosting plant growth (Orłowska et al., 2013) and nutrient uptake (Guo et al., 2013), reduce metal induced toxicity (Meier et al., 2011), change metal availability through alteration of soil pH (Rajkumar et al., 2012) and affect metal translocation (Hua et al., 2009). During greenhouse studies with sunflower grown in soils contaminated with three different Cd concentrations, successful AMF colonization by *Rhizophagus irregularis* resulted in an enhanced phytoextraction of Cd, whereas *Funneliformis mosseae* enhanced phytostabilization of Cd and Zn (Hassan et al., 2013). These results indicate that the two root associated AMF may adopt several mechanisms to trigger either metal mobilization or immobilization, therefore contributing directly to phytoextraction or phytostabilization processes (Ma et al., 2011a). In addition, plant growth promoting bacteria (PGPB) possessing single or multiple traits such as alleviation of metal toxicity (metal resistant bacteria), alteration of metal availability (metal immobilizing or mobilizing bacteria), production of siderophores [siderophores producing bacteria (SPB)], phytohormones [indole-3-acetic acid (IAA) producing bacteria] and biochelator (organic acid- or biosurfactants-producing bacteria), fixation of nitrogen [nitrogen fixing bacteria (NFB)],

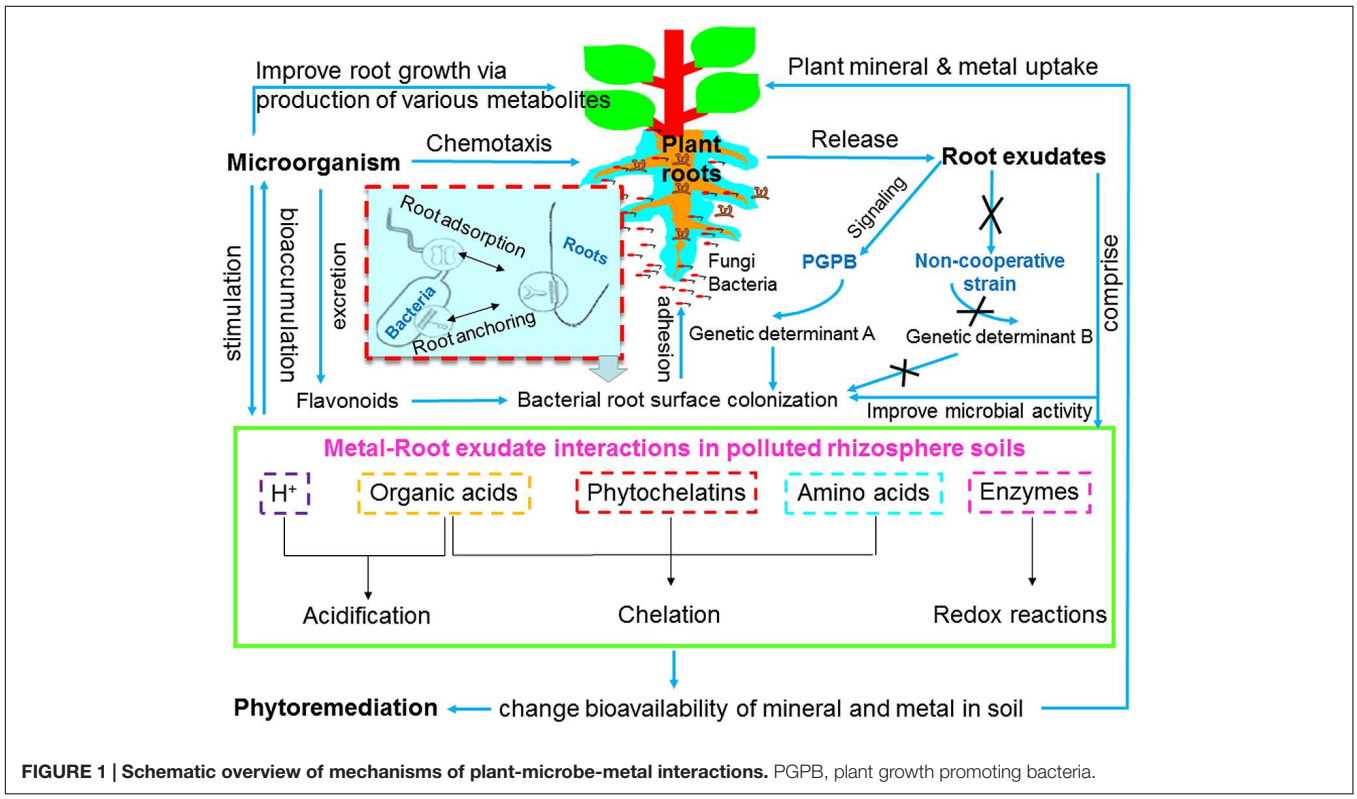

FIGURE 1 | Schematic overview of mechanisms of plant-microbe-metal interactions. PGPB, plant growth promoting bacteria.

and solubilization of mineral nutrients (phosphate or potassium solubilizing bacteria) have been widely proposed as effective bioinoculants for microbe-assisted phytoremediation (Ma et al., 2011a, 2015a,b,c; Rajkumar et al., 2012; Ahemad and Kibret, 2014; **Figure 2**). Ma et al. (2011a) have extensively reviewed the diversity and ecology of metal resistant PGPB and their potential as phytoremediation enhancing agents in metal contaminated soils. Due to the dual role of these microbial inoculants, the inoculation of PGPB can lead to increased plant biomass and enhanced metal mobilization or immobilization in soil.

The interactions between root exudates and microorganisms in the rhizosphere have been recognized as a critical component of plant growth in phytoremediation (Badri et al., 2009; Segura et al., 2009). However, the mechanisms underlying plant-microbe-metal interactions remain elusive. This article attempts to review the recent advances and applications toward understanding the biochemical and molecular mechanisms of plant–microbe interactions and their role in metal phytoremediation processes. In the following sections, we will elaborate on the mechanisms underlying plant-microbe-metal interactions in the rhizosphere, namely: (1) plant–microbe interactions (molecular signaling and perception, quorum sensing (QS), and establishment of associative symbiosis; **Figure 2**); and (2) heavy metals versus plant–microbe interactions (role of plant-microbe-metal interactions in metal detoxification, mobilization, immobilization, transformation, transport, and distribution; **Figure 3**).

PLANT–MICROBE INTERACTIONS

Root exudates are defined as organic chemicals released by living and intact root system in certain stages of plant growth. The components of root exudates and their rhizosphere functions are summarized in **Table 1**. Root exudates play an important role in phytoremediation, as they induce the capability of host plants to actively adapt to and survive under metal stress conditions by either allelopathic functions (affecting the growth of rhizosphere microbes and other plants), or detoxification process (including adsorption, chelation, transformation, and inactivation of metals). In addition, root exudates, particularly organic acids are able to bind metal ions, therefore influencing metal mobility, solubility and bioavailability in soil (Chiang et al., 2011; Luo et al., 2014; **Figures 1** and **3**). Kim et al. (2010) reported that citric acid and oxalic acid from *Echinochloa crusgalli* significantly enhanced both translocation and bioaccumulation of metals (Cd, Cu, and Pb), suggesting that organic acids can be considered natural chelating agents to enhance phytoextraction. However, some components of root exudates do not influence metal availability or have a negative impact on metal mobilization (Zhao et al., 2001). The low molecular weight organic acids (LMWOAs), such as oxalate, released by both ectomycorrhizal and non-mycorrhizal seedlings of Scots pine contributed to metal immobilization through formation of stable metal complexes in soil (Johansson et al., 2008).

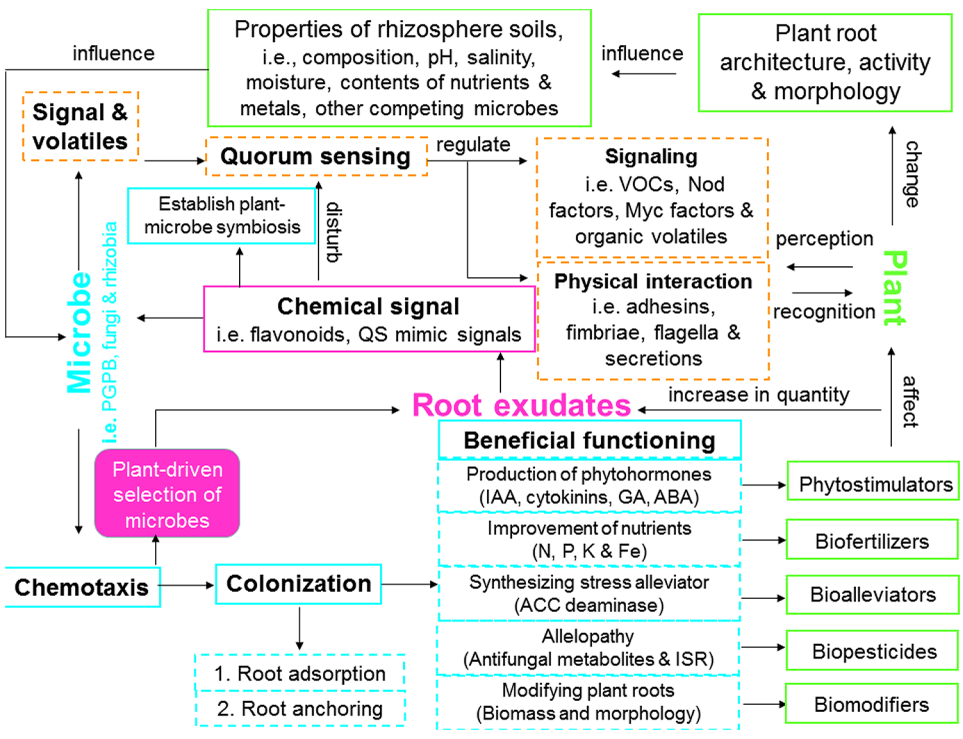

FIGURE 2 | Model showing plant–microbe interactions including signaling and communication, establishment and functioning of associative symbiosis that can be involved in enhancing phytoremediation efficiency. QS, quorum sensing; ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylate; GA, gibberellic acid; IAA, indole-3-acetic acid; ISR, induced systemic resistance; VOCs, volatile organic compounds.

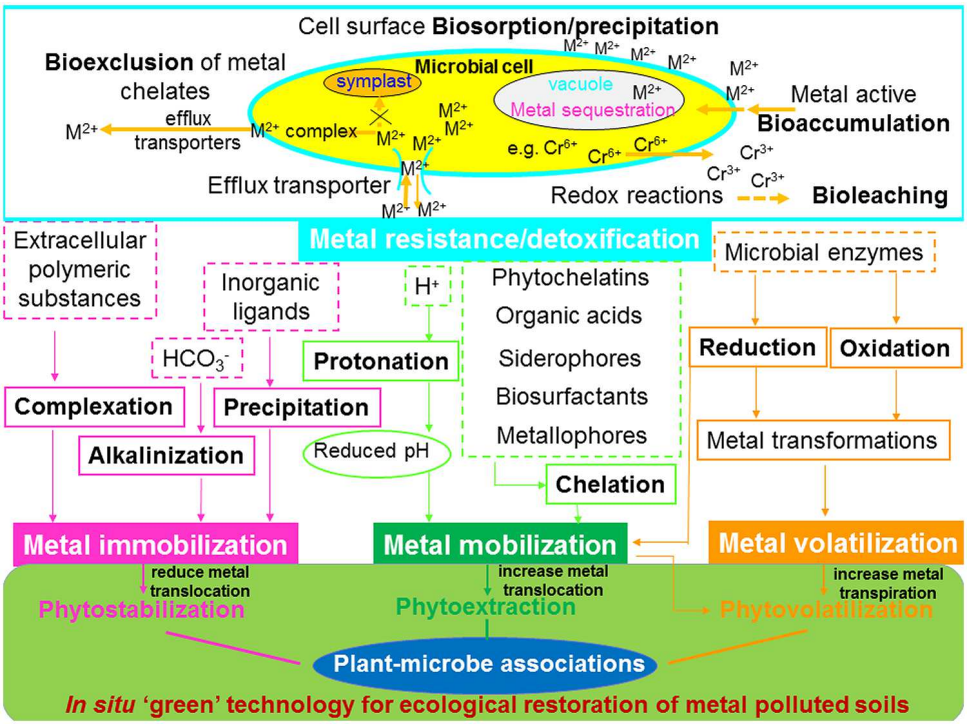

FIGURE 3 | Effects of functioning of plant-microbial associations on the biogeochemical cycling of heavy metals and their implication for phytoremediation. M^{2+}, divalent metal ion.

In general, plants possess the ability to select their own root microflora from the surrounding soil and each particular plant species has a characteristic group of associated microbes (Hartmann et al., 2009). This process is most likely to be linked directly to the quantity and composition of root exudates as well as properties of rhizosphere soil. Based on coevolutionary pressures, interactions between plants and their associated microbes are highly dynamic in nature (Chaparro et al., 2013; **Figure 2**). In the rhizosphere, plants can effectively communicate with their neighboring soil microorganisms by exuding chemicals or signals (signaling molecules and their perception, QS), while their associated microbes may establish an efficient associative symbiosis with plants by triggering host functional signals (e.g., microbial chemotaxis and colonization; Doornbos et al., 2012; Drogue et al., 2012; Bulgarelli et al., 2013; **Figure 2**).

Signaling Molecules and Their Perception

Extensive communication between plants and microbes via various signaling molecules plays a significant role in maintaining the growth of both partners. These include plant-released chemical signals that are recognized by the microbes and microbial signals and volatiles that trigger changes in plant physiology (**Figure 2**).

Plant-Released Signals

Root exuded flavonoids are known as the key signaling components in a number of plant–microbe interactions (e.g., mycorrhiza formation, establishment of legume-rhizobia symbiosis; Steinkellner et al., 2007; **Figure 2**). Flavonoids play a significant role in AMF spore germination, hyphal growth, differentiation, and root colonization in AMF-plant interactions (Badri et al., 2009; Mandal et al., 2010). At the initial stage of AMF-plant association, flavonoids exhibit an AMF fungal genus- and even species specific effect on the pre-symbiotic hyphal growth (Scervino et al., 2005b). By linking alterations of flavonoid pattern in mycorrhizal roots to the developmental stage of AMF symbiosis, Larose et al. (2002) observed intermediate levels of flavonoids in roots during root penetration and the early establishment of AMF, whereas, high levels of flavonoids (such as phytoalexin and medicarpin) at a later stage of root colonization. Once plants are well-colonized by AMF, the flavonoid pattern is dramatically changed and this change plays a regulatory role in plant-AMF interaction (Badri et al., 2009). Moreover, the stimulatory effects of flavonoids on AMF growth might be compromised, because each flavonoid can also exert a negative or neutral effect on different fungi due to its specificity involved in mycorrhizal symbiosis formation (Scervino et al., 2005a). So far, during pre-symbiotic growth, the role of flavonoids and other phenolic acids in AMF association is still unclear.

Apart from their function in the AMF-host symbiosis, flavonoids are able to promote the growth of host-specific rhizobia by serving as chemoattractants and inducers of nodulation (*nod*) genes involved in the synthesis of lipochitin–oligosaccharide signaling molecules, the Nod factors (Perret et al., 2000; Mandal et al., 2010). The flavonoids released by plant

TABLE 1 | Components of root exudates and their roles in the rhizosphere.

Classification	Species	Rhizosphere function	Reference
Organic acids	Acetic, aconitic, adipic, butyric, citric, cyclic, formic, fumaric, gluconic, glutaric, glycolic, glyoxylic, hydroxybutyric, indole-3-acetic, isocitric, lactic, maleic, malic, malonic, oxalic, piscidic, propionic, pyruvic, succinic, tartaric, valeric	Nutrient and energy sources, chemoattractant signals to microbes, chelators/adsorbents of insoluble mineral nutrients, acidifiers of soil, *nod* gene inducers, antibacterial agents	Mucha et al., 2005; Magdziak et al., 2011; Ramachandran et al., 2011; Vranova et al., 2013
Amino acids	α-alanine, β-alanine, γ-aminobutyric acid, arginine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, glycine, histidine, homoserine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan	Nutrient and energy sources, chelators of insoluble mineral nutrients, chemoattractant signals to microbes	Yao et al., 2005; Ma et al., 2009a,b, 2011a; Ahemad and Kibret, 2014; Glick, 2014
Saccharides	Arabinose, fructose, fucose, galactose, glucose, lactose, mannose, raffinose, rhamnose, ribose, sucrose, xylose	Nutrient and energy sources, anchoring of bacteria to plant surfaces	Guibaud et al., 2005; Juwarkar et al., 2007; Sheng et al., 2008; Slaveykova et al., 2010; Venkatesh and Vedaraman, 2012
Phenols	Caffeic acid, ferulic acid, flavonoids/bioflavonoids, *N*-hexanoyl-D,L-homoserine-lactone, 7-hydroxy-6-methoxy coumarin, isoflavonoids, neoflavonoids, pyrocatechol, quercetin, strigolactones, styrene	Nutrient and energy sources, chemoattractant signals to microbes, chelators of insoluble mineral nutrients, microbial growth promoters, *nod* gene inducers or inhibitors in rhizobia, inductors of resistance against phytopathogens	Dakora and Phillips, 2002; Zhao et al., 2005; Steinkellner and Mammerler, 2007; Steinkellner et al., 2007; von Rad et al., 2008; Hofmann, 2013
Enzymes	Amylase, DNase, phosphatase, polygalacturonase, protease, RNase, sucrase, urease, xylanase	Release of phosphorus from organic molecules, transformations of organic matter in soil	Loh et al., 2002; Gonzales-Chavez et al., 2004; Ahemad and Kibret, 2014; Wu et al., 2014
Vitamins	*p*-aminobenzoic acid, ascorbic acid, biotin, β-carotene, folic acid, niacin, pantothenate, pyridoxine, riboflavin, thiamin, thioctic acid, tocopherol, vitamin B12	Stimulation of plant and microbial growth, nutrient source, resistance to soil pathogens, facilitation of organic pollutant degradation, induction of plant–microbe symbioses	Kafkewitz et al., 1996; Bertin et al., 2003; Vranova et al., 2013
Others	Bilineurine, bradyoxetin, glomalin, inositol, nicotinic acid, rhamnolipids, somatropin, surfactants	Stimulation of plant and microbial growth, regulators of symbiotic expression of nodulation genes (*nod*, *nol*, *noe*)	Loh et al., 2002; Vijayan et al., 2013

roots are recognized by rhizobial *nodD* proteins, transcriptional regulators that bind directly to a signaling molecule and are able to synthesize and export *nod* genes. Upon exposure to Nod factors, the root hair cells infection and nodule formation in the host are stimulated. Therefore, specific flavonoids induce not only *nod* gene expression, but also rhizobial chemotaxis and bacterial growth (Bais et al., 2006). This specificity enables rhizobia to recognize their correct host plants and then attach to the root hairs. In addition, some other flavonoid related compounds, such as isoflavonoids (e.g., daidzein and genistein) and plant flavone (e.g., luteolin) can also effectively induce rhizobial *nod* gene expression (Juan et al., 2007). To our knowledge, the data on the implication of flavonoid as signaling compounds in other plant–microbe interactions are relatively limited.

Microbial Signals

Free-living microbes (e.g., PGPB, fungi, and rhizobia) are able to alter the chemical composition of root exudates and thus plant physiology via releasing of various signaling molecules, such as volatile organic compounds (VOCs), Nod factors, Myc factors, microbe-associated molecular patterns (MAMPs) and exopolysaccharides (Goh et al., 2013; **Figure 2**). Bacterial VOCs (such as acetoin and 2,3-butanediol) can establish the communication with plants, trigger plant defense and growth promotion mechanisms by enabling host plants to colonize nutrient (e.g., sulfur and iron) poor soils (Bailly and Weisskopf, 2012), which are common in phytoremediation scenarios. Hofmann (2013) recently demonstrated that VOCs released by *Bacillus* B55 significantly contributed to *Nicotiana attenuata* sulfur nutrition. Ortíz-Castro et al. (2009) have extensively reviewed the occurrence, function and biosynthesis of bacterial volatiles and their role in positive plant–microbe interactions. The available information indicates that VOC emission has a crucial impact in most PGPMs of PGPB by acting as bioprotectants [via induced systemic resistance (ISR); Ryu et al., 2004], biopesticides (via antibiotic functions; Trivedi and Pandey, 2008) and phytostimulators (via triggering hormonal signaling networks; Zhang et al., 2008; **Figure 2**). These functions can contribute to improve plant growth, which is fundamental for successful phytoremediation. Ryu et al. (2004) reported that

VOCs secreted by *Bacillus subtilis* and *B. amyloliquefaciens* can activate ISR in *Arabidopsis* seedlings challenged with the soft-rot pathogen *Erwinia carotovora* subsp. *carotovora*. Moreover, some bioactive VOCs produced by PGPB such as ammonia, butyrolactones, hydrogen cyanide, phenazine-1-carboxylic acid, alcohols, etc. are able to affect mycelium growth and sporulation of different fungal species (Kai et al., 2009). In this regard, VOCs can be used for communication between bacteria and their eukaryotic neighbors. Furthermore, signaling molecules synthesized by AMF (Myc factor) and rhizobia (Nod factors) are able to modulate root system architecture (such as stimulation of lateral root branching, formation of new organs and nodule), therefore facilitating symbiotic infections or nodule organogenesis in the course of evolution (Olah et al., 2005; Maillet et al., 2011). The Nod factor signaling pathway can be also affected by the Myc factor, leading to AMF formation (Maillet et al., 2011). In addition, plants have evolved a large set of defense mechanisms after pathogen perception via the plant innate immune systems pattern recognition receptors. Phytopathogen recognition can be achieved through MAMPs, which are known as biotic elicitors of non-specific immunity in plants (Newman et al., 2013). Varnier et al. (2009) found that novel MAMPs (rhamnolipids) released from *Pseudomonas aeruginosa* protected grapevine against pathogenic fungi. Recently, MAMPs from three PGPB (*Stenotrophomonas maltophilia*, *Chryseobacterium balustinum,* and *Pseudomonas fluorescens*) were able to trigger germination and metabolism of *Papaver somniferum* (Bonilla et al., 2014).

Quorum Sensing

Quorum sensing is a bacterial cell–cell communication process, whereby a coordinated population response (such as monitoring of population density, collective alteration of bacterial gene expression) is controlled by diffusible signaling molecules produced by individual bacterial cells (Daniels et al., 2004; **Figure 2**). The QS induced processes such as sporulation, competence, antibiotic and biofilm production, have been widely documented in plant–microbe interactions (Williams and Camara, 2009). QS signals, such as *N*-acyl-L-homoserine lactones (AHLs) are the essential components of this communication system. AHL quorum signals can enhance or inhibit diverse phenotypes depending on the bacteria being beneficial or pathogenic (Ortíz-Castro et al., 2009). AHLs are commonly found in many Gram-negative pathogenic bacteria (e.g., *P. aeruginosa*, *Rhizobium radiobacter,* and *Erwinia carotovora*) and/or PGPB (e.g., *Burkholderia graminis* and *Gluconacetobacter diazotrophicus*; Cha et al., 1998), which can be used to control a broad range of bacterial traits (such as symbiosis, virulence, competence, conjugation, motility, sporulation, biofilm, and antibiotic production; Fuqua et al., 2001). Conversely, bacterial AHLs can be recognized by plants, thereafter modulating tissue-specific gene expression, plant growth homeostasis, and defense response (Daniels et al., 2004). Recently, Pérez-Montaño et al. (2011) reported that similar pattern of AHLs (e.g., *N*-octanoyl homoserine lactone and its 3-oxo and/or 3-hydroxy derivatives) released by rhizobia *Sinorhizobium fredii*, *Rhizobium etli,* and *R. sullae* were involved in interactions with their host legumes. Similarly, von Rad et al. (2008) demonstrated that the contact of *Arabidopsis thaliana* roots with the bacterial QS molecule *N*-hexanoyl-homoserine lactone (C6-HSL) caused distinct transcriptional changes in legume tissues. Moreover, the AHL mimic compounds (e.g., furanones signals) secreted by higher plants (such as soybean, rice, and barrel clover) and other eukaryotic hosts can disrupt or manipulate QS-regulated behaviors among bacterial population (Pérez-Montaño et al., 2013). The AHL mimics can antagonize AHL-type behaviors by binding to the AHL receptor (e.g., LuxR) due to their structural similarities to bacterial AHLs, therefore affecting bacterial AHL-signaling (Bauer and Mathesius, 2004). Plants may adopt AHL mimics to communicate with specific bacteria to protect them from pathogens. In addition, root exudates (e.g., flavonoid and genistein) play an important role in bacterial QS communication, since they can chemotactically attract rhizobia toward, adhere to and colonize legume roots, as well as regulate expression of rhizobial nodulation genes [such as *nod* and rhizosphere-expressed (*rhi*) genes] in plant tissues (Loh et al., 2002). Strikingly, QS can be prevented (so called quorum quenching) by bacterial VOCs that can significantly affect the AHL output by the producer strain, neighboring pathogenic and/or beneficial rhizobacteria (Dong et al., 2001; Chernin et al., 2011).

The information discussed above suggests that plants and bacteria have acquired mechanisms to sense and respond to each other's signaling molecules. Further research is needed to select the key plant and microbial signaling molecules and study their functions of mediating this interkingdom communication in the rhizosphere, which could develop novel strategies to enhance phytoremediation.

Establishment of Associative Symbiosis

Microbial Chemotaxis

Root exudates are composed by diverse compounds (**Table 1**), which act as chemoattractant signals and/or sources of carbon and nitrogen for microbes (Bais et al., 2006; Compant et al., 2010), therefore creating a unique environment in the rhizosphere. However, the rhizomicrobiome composition differs according to root exudate composition, as it changes along the root system due to plant genotypes and development stages (Chaparro et al., 2013). Consequently, the microbial chemotaxis toward particular root-exuded compounds is an important trait for plant-driven selection of microbes and their colonization (Doornbos et al., 2012; **Figure 2**). It has been established that plants can elicit crosstalk to microorganisms by secreting root exudates as signaling molecules, enabling colonization by some beneficial bacteria while inhibiting the other pathogenic bacteria (Rudrappa et al., 2008; Compant et al., 2011). Rudrappa et al. (2008) demonstrated that malic acid secreted from roots of *Arabidopsis thaliana* recruits the rhizobacterium *B. subtilis* to the root and this interaction plays a significant role in protecting the plant against the foliar pathogen *Pseudomonas syringae*. The demonstration that roots selectively exude organic compounds to effectively signal bacteria and fungi underlines the role of plant metabolites in recruitment of beneficial microbes and in plant–microbe interactions. In addition to chemotaxis, electrotaxis toward

electric gradients generated by plant roots is considered as a possible mechanism for initiating rhizobacterial colonization (Lugtenberg and Kamilova, 2009).

Microbial Colonization

Root colonization is the competitive process and critical step in establishment of plant–microbe association, which is potentially affected by characteristics of both host plants and their associated microbes (Reinhold-Hurek and Hurek, 2011). In general, the process of microbial colonization in plant rhizosphere or tissue interior includes: migration toward root (root adsorption), surface attachment (root anchoring), distribution along root, as well as survival and population growth (**Figures 1** and **2**). For microbial endophytes, one more step is required that is entry into plant tissue interior and formation of micro-colonies either inter- or intracellularly (Ma et al., 2011b, 2015a; Wang et al., 2016).

Bacteria are able to rapidly and adequately alter their cell surface, thereby effectively attaching and colonizing plant roots. In the colonization process, a significant role is played by cell-surface biopolymers including proteins, glycoproteins, glycopeptidolipids, and other macromolecular metabolites (Kamnev, 2008). Among some of cell-surface protein characterized, such as bacterial major outer membrane protein (MOMP) and lectin, their involvement along with adhesion (flagella-driven chemotaxis toward root exudates), root adsorption and cell aggregation of bacteria in host recognition and root colonization process has been demonstrated (Lugtenberg and Kamilova, 2009; Compant et al., 2010). Furthermore, type IV pili and twitching motility are also involved in plant root colonization process. Böhm et al. (2007) studied the role of the type IV pili in rice colonization process of *Azoarcus* spp. using a deletion mutant of pilA and insertional mutant of pilT that was abolished in twitching motility. The results demonstrated that the retractile force mediated by PilT is unessential for bacterial colonization of plant surface, but the twitching motility is necessary for plant invasion and tissue interior establishment. Moreover, the role of two component system of *Pseudomonas fluorescens* ColR/S in competitive tomato root tip colonization has been reported. ColR/S system regulates methyltransferase/WapQ operon and maintains the outer membrane integrity for efficient colonization (De Weert et al., 2009). The colonization process is important to understand the plant-bacteria interactions and the potential of bacteria to establish themselves in the plant environment as biofertilizers, biocontrol agents, and facilitators of phytoremediation processes.

Microbial colonization can be traced by tagging them with certain molecular marker such as green fluorescent protein or β-glucosidase followed by microscopy (Reinhold-Hurek and Hurek, 2011). Although, understanding of the molecular mechanism involved in microbial colonization process is not well-understood, resemblance of colonization methods between pathogenic bacteria and PGPM have been suggested by Hardoim et al. (2008).

Functioning of Associative Symbiosis

Plant–microbial communication is a critical process to characterize the belowground rhizosphere zone, which can be beneficial to host plants and microbes. The metal resistant beneficial microbes (bacteria and AMF) are often used as bioinoculants to affect metabolic functions and membrane permeability of root cells and thus to enhance the establishment, growth and development of remediating plants in metal contaminated soils through: (1) facilitating mineral phytoavailability (N, P, K, Ca, and Fe) by acting as biofertilizers; (2) modulating phytohormones balance by acting as phytostimulators; (3) reducing ethylene synthesis by acting as stress bioalleviators; (4) preventing deleterious effects of phytopathogens via production of antifungals and ISR, by acting as biopesticides; and (5) modifying root biomass and morphology by acting as biomodifiers (Lugtenberg and Kamilova, 2009; Glick, 2010; Ma et al., 2011a; Miransari, 2011; Rajkumar et al., 2012; Ahemad and Kibret, 2014; **Figure 2**).

Currently, a numbers of studies have manifested that some beneficial microbes can help plants acquire sufficient mineral nutrients (such as N, Ca, Fe, Mg, and P) in metal contaminated soils, therefore develop longer and prosperous root system and get better established during the early growth stage, which is highly desirable in heavy metal phytoremediation (Ahemad and Kibret, 2014; **Figure 2**). Examples are NFB (such as rhizobia, rhizobacteria, and endophytic bacteria) and nutrient-absorbing AMF, which can improve the fertility of polluted soils for plant growth by catalyzing the reduction of atmospheric N_2 to biologically available ammonium (Navarro-Noya et al., 2012). Wani et al. (2007) reported that the inoculation of *Vigna radiata* L. with the NFB *Bradyrhizobium* sp. RM8 conferred tolerance to plants grown under metal stress by enhancing N concentration in roots and shoots. Similarly, the AMF *Glomus* spp. benefited plant growth and nutrient (N, P, and K) uptake by leguminous trees grown on Pb/Zn mine tailings. Further, P is one of the major macronutrients required for plant growth, however, most P compounds are not readily soluble in soil and are hence unavailable to plants (Harris and Lottermoser, 2006). The insoluble P compounds in soil can be solubilized by enzymes, organic acids and/or chelating agents excreted by both plants and microbes. One example are P solubilizing microbes (PSMs), which are widely used as inoculants to improve P uptake and plant yield by dissolving various sparingly insoluble P sources with a decrease in the rhizosphere pH (Jeong et al., 2013). Some microorganisms have the ability to couple biologically liberated P with the formation of metal phosphate biominerals, through either the accumulation of high concentrations of P cleaved from glycerol 2-phosphate at microbial cell surface, or the microbial P cycling process (Lloyd and Lovley, 2001). Strikingly, the distribution and activity of phosphate solubilizing bacteria (PSB) and their subsequent effects on P solubilization are determined by exogenous P concentration in soil (Sharma et al., 2013). In the presence of soluble P in soil, the solubilization of insoluble P by some PSB can either be repressed or not affected. However, there are few studies on the number of total PSB and their ability to solubilize P in soil. In addition, microbes can also precipitate highly insoluble metal sulfides, leading to the removal of toxic metals from solutions. This may provide a more attractive option for microorganisms to increase their resistance to metals *per se*. Sharma et al. (2000) reported that

Klebsiella planticola precipitated cadmium through the formation of sulfide from thiosulfate. Moreover, siderophores and protons can also be specifically produced by soil microorganisms in response to iron (Fe) deficiency in soil. Recently, the role of siderophore producing microbes (SPMs) such as bacteria and fungi in Fe acquisition by different plant species as well as the mechanisms behind their promotion of Fe acquisition have been widely studied (Kajula et al., 2010; Ma et al., 2011c; Gaonkar and Bhosle, 2013). Ma et al. (2011c) demonstrated that the bacterial catechol and hydroxamate siderophores produced by *Psychrobacter* sp. SRS8 enhanced the growth of *Ricinus communis* and *Helianthus annuus* in Ni contaminated soil by a simultaneous enhancement of Fe solubilization and uptake. Bacterial siderophores also play a crucial role in the generation/regulation of hormones in plants under metal stress. Chelation through binding toxic metals to siderophores triggers the enhancement of plant Fe uptake capacity and the decrease of free metal ion concentration, thus leading to the attenuation of hormone synthesis inhibition (Dimkpa et al., 2008). Bacteria, such as *Arthrobacter* sp. and *Leifsonia* sp. (Actinobacteria), *Polaromonas* sp. and *Janthinobacterium* sp. (Betaproteobacteria), were previously reported to accelerate the dissolution and mobilization of mineral nutrients (such as Fe, Mn, and K) for soil fertility (Abdulla, 2009; Uroz et al., 2009).

Plant associated microbes can also produce phytohormones such as IAA, cytokinins, gibberellic acid, abscisic acid and others, even under stress conditions, thereby modulating the hormonal balance in plants and their response to stress (Ma et al., 2011a; Ullah et al., 2015; **Figure 2**). The IAA synthesized by microbes, together with endogenous plant IAA, cannot only stimulate root exudation and proliferation of lateral and adventitious roots, but also induce the synthesis of ACC synthase (Glick, 2010). Recently, Chen B. et al. (2014) found that the IAA producing endophytic bacterium *Sphingomonas* SaMR12 increased IAA concentration in plant tissues and the growth of *Sedum alfredii*. Similarly, Ma et al. (2013) assessed the potential of *Phyllobacterium myrsinacearum* RC6b to produce IAA in culture media containing various concentrations of L-tryptophan. The results indicated that although L-tryptophan is a precursor for bacterial growth and IAA production, high concentrations of L-tryptophan (3, 4, and 5 mg mL^{-1}) exert negative effects on bacterial IAA production. The IAA synthesized by RC6b induced significantly greater root growth of *Sedum plumbizincicola* than that of non-inoculated control plants. Furthermore, AMF colonization also has positive effects on plant cell growth and division as a result of fungal hormones production. Yao et al. (2005) demonstrated that inoculation of the AMF *Glomus intraradices* and *Gigaspora margarita* onto seedlings of *Litchi chinensis* significantly increased the IAA and isopentenyl adenosines concentrations in shoots and roots. The changes in endogenous phytohormones level were responsible for morphological alteration induced by AMF inoculation.

Besides the above described plant beneficial mechanisms, under stress conditions soil microorganisms can also enhance plant growth through the synthesis of ethylene production inhibitors [e.g., 1-aminocyclopropane-1-carboxylate (ACC) and rhizobitoxine; Vijayan et al., 2013; Glick, 2014], antimicrobial enzymes (e.g., chitinases, phytoalexins, β-1,3-glucanase, callose, phenolics, and lysozyme; González-Teuber et al., 2010; Saima et al., 2013) and polysaccharides [e.g., exopolysaccharides and extracellular polymeric substances (EPSs); Ashraf et al., 2004; Naseem and Bano, 2014], thus enabling plants to mitigate the negative impact of both biotic (fungi or harmful insects) and abiotic stresses (such as flooding, drought, salinity, and heavy metals; **Figure 2**). One of the key traits related with plant growth promotion is the production of ACC deaminase by PGPB that hydrolyses the plant ethylene precursor ACC into ammonia and α-ketobutyrate (Glick et al., 2007; Glick, 2014). The inoculation of seven Pb-resistant and ACC deaminase-producing endophytic bacterial isolates onto *Brassica napus* grown in metal contaminated sands was found to increase the dry weights of shoots (ranging from 39 to 71%) and roots (from 35 to 123%), compared to the non-inoculated control (Zhang et al., 2011). ACC deaminase-producing microorganisms are able to dilute the negative impact of metal-induced ethylene production in plants, avoiding plant growth inhibition or even death (Glick et al., 2007). Rhizobitoxine is another inhibitor of ethylene synthesis, which cannot only minimize the negative effects of stress-induced ethylene production on nodulation, but also induce foliar chlorosis in soybeans (Vijayan et al., 2013). Prasanna et al. (2013) found that a novel strain *Brevibacillus laterosporus* produced two extracellular chitinase (89.6-kDa four domain and 69.4-kDa two domain) that contribute to its antifungal and insecticidal activities. In addition, some exopolysaccharides-producing plant growth promoting rhizobacteria (PGPR) such as *Proteus penneri*, *P. aeruginosa*, and *Alcaligenes faecalis* were proved to alleviate water stress and improve plant biomass under drought stress (Naseem and Bano, 2014).

Notwithstanding the above, inoculation of efficient fungi and bacteria in compatible host-microorganism-site combination can significantly contribute to modify root morphology and improve plant biomass, which could be a main support for a successful biotechnological application in phytoremediation. AMF play an important role in improving plant establishment, owing to their role in enhancing nutrient uptake and the longer term development of plant root system (Jankong and Visoottiviseth, 2008; Jiang et al., 2016). For example, some species of the genera *Achromobacter*, *Azospirillum*, *Burkholderia*, *Methylobacterium,* and *Psychrobacter* can increase some root morphological traits (Madhaiyan et al., 2007; Molina-Favero et al., 2008; Ma et al., 2009a,b).

In general, plant associated microorganisms are able to promote plant establishment, growth and development by resorting to any one or more of the above mentioned mechanisms. Therefore, those PGPM can be applied not only in agricultural soils for food production, but also in stressful environments for phytoremediation purposes. The effectiveness of PGPM for promoting plant growth depends on the intimate interaction with their host plant and soil characteristics besides their inherent capabilities (Gamalero et al., 2010; Nadeem et al., 2014).

Plant growth promoting microorganisms display various plant-beneficial features, suggesting that the accumulation of the corresponding genes could have been selected in these microbes.

The accumulation of the genes contributing to plant beneficial functions might be an intrinsic feature of PGPM. Future studies should focus on discovering preferential associations occurring between certain genes contributing to phytobeneficial traits, which could provide new insights into plant-PGPM interactions.

ROLE OF PLANT-MICROBE-METAL INTERACTIONS IN PHYTOREMEDIATION

The discovery of plant-microbe-metal interactions sustains the importance of plant–microbe interactions in the biogeochemical cycling of metals and in their application in phytoremediation. It is essential to consider the appropriate combination of plants and microbes involved in applied processes for enhanced phytoremediation efficiency, in order to obtain the best performance from the existing microbe-based technologies. The plant-microbe-metal interactions spanning from both macropartner (higher plants) and micropartner (microorganism) to heavy metals is nevertheless a crucial step in understanding plant metal uptake during geo-bio-interactions (**Figure 3**).

Advances in understanding plant–microbe interactions on metal tolerance and detoxification, together with their functioning on the biogeochemical cycling of heavy metals including metal mobilization/immobilization, translocation and transformation, have led to the development of improved metal bioremediation processes (Bruins et al., 2000; Ma et al., 2011a). The influence of bacterial and fungal activity on metal mobilization or/and immobilization and its use for bioremediation has been reviewed by several researchers (Khan, 2005; Ma et al., 2011a; Rajkumar et al., 2012; Sessitsch et al., 2013; Ahemad and Kibret, 2014). The activities of PGPM, such as metal bioaccumulation, bioleaching and bioexclusion are involved in causing adaptation/resistance/tolerance of microbial communities to heavy metal rich environments. In general, processes such as acidification, chelation and protonation lead to mobilization of metals, whereas precipitation, alkalinization, and complexation cause metal immobilization. However, chemical transformation can cause metal mobilization and/or immobilization. A schematic illustration of the effects of plant–microbial association on the biogeochemical cycling of heavy metals and its implications for phytoremediation is presented in **Figure 3**.

Metal Detoxification

Heavy metal tolerance/resistance in plants and microbes is a vital prerequisite for plant metal accumulation and microbe-assisted phytoremediation. When plants are subjected to high level of metal contaminants, the stress triggers the plants' inter-linked physiological and molecular mechanisms in adapting to stressful environment. Mechanisms involved in plant metal tolerance include plant cell wall binding, active transport of ions into cell vacuoles, intracellular complexation with peptide ligands such [as phytochelatins (PCs) and metallothioneins (MTs)], as well as sequestration of metal-siderophore complexes in root apoplasm or soils (Miransari, 2011; **Figure 3**). Among the tolerance mechanisms employed by metal-accumulating plants, the exudation of various compounds, especially LMWOAs is able to stimulate microbial growth, solubilize insoluble or sparingly soluble mineral nutrients (e.g., P, Fe, and Zn), and detoxify some metals (e.g., As, Cd, and Pb; Tu et al., 2004; Li et al., 2013). Root exudation of LMWOAs has been considered one of the most important strategies developed by plants to tolerate high metal concentrations, due to their ability to exclude metals and metalloids (e.g., As, Cr, Cd, and Pb) through chelation in the rhizosphere or apoplast, thereby preventing the metal ions from entering the cell symplast (Magdziak et al., 2011). Some studies found that the diverse LMWOAs, such as citric, oxalic, malic, and succinic acid exuded by agricultural plants under metal stress play a significant role in alleviating metal phytotoxicity (Evangelou et al., 2006; Yuan et al., 2007; Meier et al., 2012).

Moreover, plant associated AMF and bacteria are capable of inducing metal translocation and distribution, thus metal allocation to the inner root parenchyma cells (Kaldorf et al., 1999). The mechanisms involved in microbial metal resistance are summarized schematically in **Figure 3**: (1) cell surface biosorption/precipitation of metals; (2) active efflux pumping of metals out of the cell via transporter system; (3) sequestration of metals in intracellular compartments (mainly cell vacuole); (4) exclusion of metal chelates into the extracellular space; and (5) enzymatic redox reaction through conversion of metal ion into a non-toxic or less toxic state.

Bioaccumulation

Plant associated microorganisms have been documented to contribute to plant metal resistance through bioaccumulation mechanisms involving interaction and concentration of toxic metals in the biomass of either non-living (biosorption) or living (bioaccumulation) cells (Ma et al., 2011a; Rajkumar et al., 2012; **Figure 3**). Bioaccumulation is a process of intracellular accumulation of metals. It comprises two stages: metabolism-independent passive biosorption (e.g., physical and chemical adsorption, metal ion exchange, chelation, coordination, surface complexation, and microprecipitation), and metabolism-dependent active bioaccumulation (e.g., transport of metal ions into microbial cells including complex permeation, carrier mediated ion pumps and endocytosis; Chojnacka, 2010). Although, the majority of original research has recently focused on biosorption concerning the binding metals by waste materials, the renewable biosorbents (living or dead cells of bacteria, fungi, and plant etc.) have proven to be efficient and economical for the removal of toxic metals from both polluted aqueous solutions and soils (Alluri et al., 2007; Ma et al., 2013). Recently, Ma et al. (2015b) observed that the metal resistant *Bacillus* sp. SC2b was capable of adsorbing significant amounts of metals (e.g., Cd, Pb, and Zn) and bacterial inoculation ameliorated metal toxicity through biosorption, thus exhibiting a protective effect on host plant growth. Dissimilarly, Zafar et al. (2007) studied biosorption of Cr and Cd by metal resistant filamentous fungi *Aspergillus* and *Rhizopus* and found no direct relationship between metal tolerance and biosorption properties of these fungi. The bioaccumulation process is more complex than biosorption and it requires metabolic activity of living cells involving intracellular sequestration (MTs and PCs binding), extracellular precipitation,

metal accumulation and complex formation (Gadd, 2004). This process is highly determined by the operational conditions, especially by the presence of metals in the growth medium, as high metal concentrations can inhibit bacterial growth and their bioaccumulation capacity (Chojnacka, 2010). Metal bioaccumulation by various microbes has been widely described in the literature and it was demonstrated that bioaccumulation mechanism can be accounted for both reduced plant metal toxicity and uptake (Ma et al., 2011a; Deng and Wang, 2012; Mishra and Malik, 2013). Velásquez and Dussan (2009) carried out studies on metals (Co, Hg, Fe, and As) biosorption and bioaccumulation in living biomass as well as biosorption in dead cells of different *Bacillus sphaericus* strains. The biosorption and bioaccumulation processes performed by living cells of the two most tolerant strains were similar. Biosorption in surface molecules (e.g., S-layer proteins) contributes to entrap metal ions either in living or dead cells, whereas bioaccumulation through helper proteins is involved in the incorporation of essential nutrients (e.g., P and S; Suarez and Reyes, 2002) and/or metal reduction through enzymatic processes (Elangovan et al., 2005).

Bioleaching

Mesophilic bacteria [such as sulfur-oxidizing bacteria (SOB; e.g., *Acidithiobacillus thiooxidans*, *A. caldus*, and *A. albertis*)] and iron-oxidizing bacteria (FeOB; e.g., *A. ferrooxidans* and *Leptospirilum ferrooxidans*; Wong et al., 2004), thermophilic bacteria (e.g., *Archeans* sp., *Sulfobacillus thermosulfidooxidans, S. ambivalens*, *S. brierleyi,* and *Thiobacter subterraneus*; Kletzin, 2006) as well as heterotrophic bacteria (e.g., *Acetobacter*, *Acidophilum*, *Arthrobacter,* and *Pseudomonas*) and fungi (e.g., *Penicillium*, *Aspergillus* and *Fusarium* and *Trichoderma*; Mulligan and Galvez-Cloutier, 2003) are able to bioleach heavy metals from sludge, sediments and soils, therefore alleviating metal phytotoxicity directly or indirectly through various metabolic activities such as oxidation, reduction, complexation, adsorption, or dissolution (Pathak et al., 2009; **Figure 3**). Kumar and Nagendran (2009) demonstrated that the acidophilic SOB *Acidithiobacillus thiooxidans* created acidic conditions favorable for bioleaching/removal of metals (e.g., Cd, Cr, Cu, Fe, Pb, and Zn). The capability of bioleaching depends on bacterial species. In general, acidophilic bacteria are more capable for metal bioleaching than neutrophilic bacteria (Navarro et al., 2013).

Bioexclusion

Microbial active transport or efflux of toxic metals from their cytoplasm represents the largest category of metal resistance systems (**Figure 3**). Non-essential metals such as Cd and As, generally enter the cell through either non-ATPase or ATPase-linked nutrient transport systems that are highly specific for the imported cation or anion (Nies and Silver, 1995), whereas active transport of essential metal ions (e.g., Cu^{2+}) from bacterial cells can be achieved through an ATPase efflux mechanism (Bruins et al., 2000). Nies (2003) has intensively reviewed the mechanisms of efflux-mediated heavy metal resistance in prokaryotes by elucidating the action and physiological functions and distribution of metal-exporting proteins such as P-type ATPases, cation diffusion facilitator and chromate proteins, NreB- and CnrT-like resistance factors. Possession of the highly specialized mechanisms makes a bacterium metal resistant.

Metal Mobilization

It is well-known that strong binding of metals to soil particles or precipitation accounts for the insolubilization of a significant fraction of metals in soil and consequently contributes to their unavailability for plant uptake. The mobility and solubilization of metals in soil have been recognized as critical factors in affecting the practical efficiency of phytoextraction (Ma et al., 2009a, 2013). In this regard, metal mobilizing microbes can be used to modify rhizosphere habitat, hence, influencing metal element speciation and mobility in soil through biogeochemical cycling processes of heavy metals, mainly including acidification, protonation and chelation (Glick, 2010; Ma et al., 2011a; Rajkumar et al., 2012; Sessitsch et al., 2013; **Figure 3**).

Acidification

Soil pH is a key factor affecting the content and solubility/mobility of metals in soil. The mobility of most metals decreases with increasing pH (Richards et al., 2000). Soil pH is generally influenced by activities of both plants and microorganisms, and vice versa. The hydrogen ions excreted by plant roots can displace heavy metal cations that are adsorbed on soil particles, leading to acidification of the rhizosphere. Root exudates can lower the pH of rhizosphere by one or two units over that in bulk soil (Sheoran et al., 2011), therefore enhancing soil metal mobility and plant metal bioavailability in soil solution (Alford et al., 2010; Kim et al., 2010). Chen B. et al. (2014) pointed out that the inoculation of endophytic bacterium *Sphingomonas SaMR12* regulated quantity of root exudates (organic acids) from *S. alfredii*, thus substantially improving Cd bioavailability and plant absorption facility. A recent study reported that *P. myrsinacearum* RC6b significantly increased metal uptake by *S. plumbizincicola.* This was attributed to its ability to produce organic acid and solubilize insoluble tricalcium phosphate (Ma et al., 2013).

Protonation

Soil microbes can also acidify their environment by exporting protons to replace heavy metal cations at sorption sites (Rajkumar et al., 2012; **Figure 3**). In order to understand, predict and optimize such processes, there have been extensive attempts to model the interactions between protons, metal ions and bacterial surfaces, as well as to characterize them using spectroscopy. Giotta et al. (2011) studied the interaction of Ni^{2+} with surface protonable groups of *Rhodobacter sphaeroides* by using attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy. The results revealed that carboxylate moieties on the bacterial surface play a significant role in extracellular biosorption of Ni^{2+}, establishing relatively weak bonds with metal ion.

Chelation

Upon chelation, organic chelator compounds released from both plants and microorganisms are able to scavenge metal ions from sorption sites and heavy metal-bearing minerals, thus protecting

them from resorption (Gadd, 2004). To date, the natural organic chelators are known as metal-binding compounds, organic acid anions, siderophores, biosurfactants, and metallophores (Sessitsch et al., 2013).

The metal chelation through the induction of metal-binding peptides (MTs and PCs) can eliminate the phytotoxic effect of free metal ions, allowing for metal uptake, sequestration, compartmentation, xylem loading, and transport within the plant (Cai and Ma, 2002). Phytochelatins, the heavy metal-binding peptides, are synthesized from the tripeptide glutathione and/or through an enzymatic reaction catalyzed by PCs synthase (Solanki and Dhankhar, 2011). The production of PCs is immediately induced by heavy metal exposure, which is positively correlated with metal accumulation in plant tissues (Pal and Rai, 2010; **Figure 3**). In contrast to PCs, MTs, small cysteine-rich and metal-binding proteins, play essential roles in activities of various organisms (e.g., animals, plants, eukaryotic and prokaryotic microorganisms) such as metal detoxification and homeostasis through scavenging reactive oxygen species (Leitenmaier and Küpper, 2013). Bolchi et al. (2011) pointed out that the mycorrhizal fungus *Tuber melanosporum* polypeptides such as MTs (TmelMT) and PC synthase (TmelPCS) were capable of conferring an enhanced tolerance to essential (Cu and Zn) and non-essential (Cd, As, and Hg) thiophilic metal ions in yeast. Nevertheless, it is known that MTs also occur in AMF and that genes encoding several enzymes for PCs synthesis can be activated in mycorrhizal roots, therefore enhancing photosynthetic activity in mycorrhizal plants exposed to metal stress. However, these metal chelation mechanisms cannot make a major contribution to metal tolerance strategies operating in AM symbiosis (Rivera-Becerril et al., 2005).

Fe is an important micronutrient and its concentration in soil is often below the level necessary to support robust plant and microbial life due to its low solubility, especially under metal stress. Therefore, plants surmount challenges to acquire sufficient Fe through three mechanisms, namely, Strategy I (Fe solubilization by all dicots and monocots via rhizosphere acidification); Strategy II [secretion of phytosiderophores (PSs) and uptake Fe^{3+}-PS]; and Strategy III (uptake Fe^{3+}-microbial siderophores by plants). Many studies have shown that PSs are able to solubilize and transport metals by chelation, and thus being secreted into the rhizosphere through a potassium-mugenic acid symporter (Sakaguchi et al., 1999). It has been proved that the siderophores produced by microorganisms have a higher affinity for metals than PSs. Hence, microbes may develop strategies to solubilize metals for a more efficient uptake by plants (**Figure 3**). Recently, Yuan et al. (2014) demonstrated that inoculation with the endophytic bacterium *Rahnella* sp. JN27 promoted Cd solubilization in metal-amended soils through the release of siderophores, therefore facilitating Cd uptake by Cd-hyperaccumulators *Amaranthus hypochondriacus* and *A. mangostanus*.

The organic acids released from both host plants and microbes have been proposed to be involved in various processes occurring in the rhizosphere, including nutrient acquisition, mineral weathering, heavy metal detoxification, and mobilization/solubilization in soil (Rajkumar et al., 2012; **Figure 3**). Organic acids excreted by plant roots, such as malate, citrate, acetate, and oxalate, are widely recognized to be responsible for dissolving the solid phase metals in soil through complexation reaction and thus making them available for plant uptake (Berkelaar and Hale, 2003; **Table 1**; **Figure 1**). Mucha et al. (2005) found that the malonate and oxalate released by *Juncus maritimus* acted as complexing agents of trace metals, and were responsible for the enhanced metal mobility and availability in soil. However, the excretion of organic acids by microorganisms has a somehow more profound effect on the stimulation of metal release than the direct effect of root secretions (Amir and Pineau, 2003). Exudates of LMWOAs from microbial populations, including both aliphatic and phenolic acids, have great potential to improve metal solubilization processes (Rajkumar et al., 2012). A recent study by Chen L. et al. (2014) demonstrated that the organic acids producing endophytic bacterium *Pseudomonas* sp. Lk9 improved soil mineral nutrition (Fe and P) and metal availability by accelerating host-mediated LMWOAs secretion, thereby significantly enhancing shoot biomass production of *Solanum nigrum* and accumulation of metals (Cd, Cu, and Zn) in aerial plant parts. Nevertheless, AMF have not been shown to produce organic acids. However, the specific protein glomalin produced by AMF seems to be efficient in sequestering heavy metals outside the mycelium (Gonzales-Chavez et al., 2004).

Biosurfactants (BSs) are amphiphilic compounds either produced on microbial cell surfaces or excreted extracellularly, and contain hydrophobic and hydrophilic moieties that reduce surface and interfacial tensions (**Figure 3**). The structures of BSs are usually composed of one or more compounds, such as mycolic acid, glycolipids, polysaccharide-lipid complex, lipoprotein or lipopeptide, phospholipid, or the microbial cell surface itself (Pacheco et al., 2011). Due to their amphiphilic structure, BSs are able to create complexes with metals at the soil interface and desorb metals from soil matrix to the soil solution, hence, increasing metal solubility and bioavailability in metal polluted soils (Sheng et al., 2008). Therefore, the use of metal resistant bacterial strains capable of producing BSs has been considered as a promising strategy to improve the removal of heavy metals from soils (Rajkumar et al., 2012; Venkatesh and Vedaraman, 2012). Recently, surfactin from *B. subtilis*, sophorolipids from *Torulopsis bombicola*, and di-rhamnolipids and rhamnolipids from *P. aeruginosa* have been employed to remove metals from contaminated soils (Mulligan et al., 2001; Juwarkar et al., 2007; Venkatesh and Vedaraman, 2012). Although, some studies have reported the potential of microbial BSs to facilitate metal mobilization, the mechanisms (such as ion exchange, precipitation-dissolution, and counterion binding) involved in the interaction among biosurfactants producing microbes, plants, and metals have been scarcely demonstrated.

Metallophores are low molecular weight organic ligands released from microorganisms, which can regulate the intracellular metal concentrations to avoid toxicity or maintain appropriate concentrations for their growth (**Figure 3**). Deicke et al. (2013) reported that metallophores (e.g., protochelin and azotochelin) excreted by the NFB *Azotobacter vinelandii* bound metal cations and oxoanions in its extracellular medium, therefore increasing the metal (Fe and Mo) bioavailability to

bacterial cells, which can subsequently recruit the complexes. The efficient quantification of those metal complexes is crucial for essential cofactors of nitrogenase (e.g., Fe and Mo) homeostasis, N_2 fixation dynamics and N_2 cycle. This strategy allows microbes to control metal speciation in their environment, thereby increasing heavy metal bioavailability to the N_2 fixers.

Metal Immobilization

Some microbes can also reduce plant metal uptake or translocation to aerial plant parts by decreasing metal bioavailability in soil via precipitation, alkalinization, and complexation processes (**Figure 3**).

Precipitation

Certain plant associated microorganisms have the ability to promote the enzymatically catalyzed precipitation of radionuclides (e.g., U, Tc) and toxic metals (e.g., Cr, Se) by microbial reduction processes, which show considerable promise for phytoremediation of metal contaminated soils (Payne and DiChristina, 2006). Oves et al. (2013) reported that the inoculation of Cr reducing bacterium *P. aeruginosa* OSG41 onto chickpea grown in Cr^{6+} contaminated soils significantly decreased Cr uptake by 36, 38, and 40% in roots, shoots and grains, respectively, with a concomitant increase in plant growth performance compared with non-inoculated control. The results indicate that bacteria possessed the ability to protect host plant against the inhibitory effect of high concentration of Cr^{6+} by reducing mobile and toxic Cr^{6+} to non-toxic and immobile Cr^{3+} in the soil. Besides, insoluble mineral forms of radionuclides and metals can also be immobilized either directly through an enzymatic action (Pagnanelli et al., 2010), or indirectly by bacterial Fe oxidation or interactions between microbial inorganic acid (e.g., hydrogen sulfide, bicarbonate, and phosphate; Park et al., 2011; Zhou et al., 2013). The microbial inorganic acid can rapidly react with certain dissolved metals (such as Cu, Fe, Zn, and Pb) to form insoluble precipitates. Noticeably, anaerobic reduction of sulfur by sulfate-reducing bacteria (SRB) has been proved to be a promising treatment of a variety of S-containing and metal rich mining drainage and industrial effluents (Zhou et al., 2013). Similarly, Park et al. (2011) reported that application of PSB reduced Pb availability in contaminated soils though the release of P from insoluble P compounds. In this sense, the ability of bacteria to solubilize minerals, promote plant growth, and immobilize metals in soil makes them a promising choice for phytostabilization of metal contaminated soils.

Alkalinization

Some AMF and bacteria (e.g., SRB and cyanobacteria) exhibit the ability to absorb metals through substratum alkalinization activity, therefore affecting the metal stability in soil (Büdel et al., 2004). The alkalinizing effect induced by AMF through the release of OH^-, can result in an active nitrate uptake by microbes and a reduction in metal phytoavailability in the rhizosphere by secreting glomalin (Giasson et al., 2008). AMF can act as metal sinks to reduce the mobile and available metal cations in soil, thereby creating a more suitable environment for plants growing in metal contaminated soils (Göhre and Paszkowski, 2006). Hu et al. (2013) observed that inoculations of AMF *Glomus caledonium* and *G. mosseae* onto *S. alfredii* and *Lolium perenne* significantly decreased soil DTPA-extractable Cd by 21–38% via alkalinization process, thus facilitating both extraction and stabilization of Cd in the *in situ* treatment of Cd-contaminated acidic soil.

Complexation

The excretion of EPSs by plant associated microbes is of particular importance to form a protective barrier against harmful effects through metal biosorption (Slaveykova et al., 2010; Hou et al., 2013; **Figure 3**). The mechanisms involved in metal biosorption onto to EPS include metal ion exchange, complexation with negatively charged functional groups, adsorption and precipitation (Zhang et al., 2006). Guibaud et al. (2005) found that the EPS extracted from bacterial cultures were less able to complex metals than those from sludge. However, the bacterial EPS exhibited great capacity to bind metals and protect bacteria from metal stress. In addition, AMF can produce an insoluble metal-sorbing glycoprotein (glomalin) that reduces metal mobility or sequesters metals and it could be considered for metal biostabilization in soil (Vodnik et al., 2008). Wu et al. (2014) investigated the role of glomalin-related soil protein (GRSP) that is used to estimate AMF-derived glomalin in soils in sequestering Pb and Cd in an *in situ* field experiment. It was found that after 140 days GRSP bound Pb accounted for 0.21–1.78% of the total Pb, and for Cd, 0.38–0.98% of the total Cd content in the soil. Although, the metal-binding levels are insignificant compared to soil organic matter basis such as humic and fulvic acids, GRSP greatly influenced metal uptake in contaminated soils. Therefore, it is clear that AMF can reduce metal mobility in soil by excreting glomalin, however, studies concerning the structures and mechanisms of glomalin are still required to provide further knowledge.

Metal Transformation

The activity and importance of microbes in participating in biogeochemical redox processes, which lead to diverse chemical transformation of metal contaminants, have been documented in previous studies (Chatterjee et al., 2009; Olegario et al., 2010; Majumder et al., 2013). Heavy metals, such as As, Cr, Hg, Fe, Mn and Se, are most commonly subjected to microbial oxidation and reduction reactions, thereby altering their speciation and mobility in soil and simultaneous reducing metal phytotoxicity (Kashefi and Lovley, 2000; O'Loughlin et al., 2003; Amstaetter et al., 2010; Olegario et al., 2010; **Figure 3**). In general, metals such as Cu and Hg are more soluble in their lower oxidation state, whereas the oxidation states of metals such as Cr, As, and Se are more soluble and toxic (Ross, 1994). Majumder et al. (2013) reported that the metal resistant As-oxidizing bacteria *Bacillus* sp. and *Geobacillus* sp. isolated from arsenic-contaminated soils efficiently oxidized mobile toxic As^{3+} to immobile less toxic As^{5+}. In addition, Cr reduction is other example for the precipitation of metallic ions in aqueous solutions or soils. Chatterjee et al. (2009) pointed out that the inoculation of Cr-resistant bacterium *Cellulosimicrobium cellulans* decreased Cr^{6+}

uptake in the shoot and root of green chili by 37 and 56%, compared with non-inoculated controls. This is due to the ability of bacteria to reduce the mobile and toxic Cr^{6+} to immobile and non-toxic Cr^{3+} in the soil. Therefore, metal reducing or oxidizing microbes are able to reduce the phytotoxic effects of metals by transforming a metal contaminant into a non-bioavailable state in the rhizosphere, reflecting the suitability of these microbes for phytotransformation.

Metal Transport and Distribution

The translocation of metals from plant roots to the above-ground parts varies considerably depending on plant species and metals. Different metals are differently mobile within a plant, e.g., Cd and Zn are more mobile than Cu and Pb. During the transportation through the plant, metals are bound largely on the cell walls of roots, leading to high metal concentration in plant roots. The metal chelation with ligands (e.g., organic acids, amino acids, and thiols) facilitates the metal movement from roots to shoots (Zacchini et al., 2009; **Figure 3**). Due to the high cation exchange capability of the xylem cell, the metal movement is severely retarded when the metals are not chelated by ligands. Organic acids are involved in the Cd translocation in *Brassica juncea* (Salt et al., 1995), whereas, histidine is involved in the long distance Ni translocation in hyperaccumulator *Alyssum lesbiacum* (Solanki and Dhankhar, 2011). Since most heavy metals can only be transferred by forming organic compounds-metal complexes (Maser et al., 2001), a variety of organic ligands secreted by microbes can change the exiting forms and distribution of metals through combination with metals in plants, consequently facilitating the metal translocation from roots to their shoots and therefore improving phytoextraction efficiency (Sheng et al., 2008). Saravanan et al. (2007) reported that the Zn mobilizing *Gluconacetobacter diazotrophicus* helped in the efficient solubilization of the insoluble Zn compounds by producing 5-ketogluconic acid. Similarly, Ma et al. (2013) reported that application of metal resistant PGPB *P. myrsinacearum* RC6b effectively mobilized metals (Cd, Zn and Pb) in soil and significantly increased metal accumulations (Cd and Zn) in shoots of *S. plumbizincicola*. Besides, a few studies found that the growth and metal accumulation in the above ground part of plants have been improved by using combinations of plant associated microorganisms. Zimmer et al. (2009) found that inoculation of *Salix viminalis x caprea* with ectomycorrhiza associated bacteria *Micrococcus luteus* and *Sphingomonas* sp. in combination with the fungus *Hebeloma crustuliniforme* increased the total Cd and Zn accumulation in shoot up to 53 and 62%, respectively, and consequently led to an increase in phytoextraction of Cd and Zn in these fungal-bacterial inoculant combinations. Similarly, De Maria et al. (2011) observed that after inoculation with the rhizobacteria *Streptomyces* sp. and *Agromyces* sp. plus the fungus *Cadophora finlandica* onto *Salix caprea*, shoot concentrations of Cd and Zn were mostly increased, indicating higher translocation of metals from roots to shoots.

As discussed above, the functions of specific PGPM contributing to metal availability in soils have been studied at the laboratory scale. However, more attention should be given to the application of multi-functional PGPM or multi-strain inoculation exhibiting stress resistance and plant beneficial traits when used as bioinoculants with remediating plants at the field scale.

CONCLUSION AND RECOMMENDATIONS

In this review, the most important properties of plants and microbes as well as mechanisms underlying plant-microbe-metal interactions in phytoremediation were discussed through the following aspects: (1) providing deep insights on biochemical and molecular mechanisms of plant–microbe interaction, which could contribute to evolution dynamics of microbial consortia; (2) demonstrating the effectiveness of microbes devoted to hold potential stress-reducing properties and conferring their host plants metal stress resistance by acting as bioprotectants; (3) improving knowledge of how beneficial plant–microbe association may contribute to develop microbial inoculants and to promote plant yield respecting ecosystem biodiversity and safety by acting as biofertilizer; and (4) elucidating the mechanisms of cooperative plant–microbe interactions during metal detoxification, mobilization, immobilization, accumulation, translocation and transformation, which could contribute to the implementation of phytoremediation technologies.

The future research should be focused on: (1) the mechanism of plant-microbe-metal interactions under stressful environmental conditions; (2) the effectiveness of co-inoculation of PGPB and AMF response to multiple biotic and/or abiotic stress; (3) the identification of functional genes of beneficial microbes for growth enhancement and metal metabolism; (4) the optimization of techniques for application in large scale polluted fields; and (5) the exploration of commercial production of bioinoculants for use in metal decontamination.

AUTHOR CONTRIBUTIONS

YM developed the ideas and wrote the manuscript; RO and CZ revised the manuscript; HF was the project sponsor.

ACKNOWLEDGMENTS

YM and RO wish to acknowledge the support of Fundação para a Ciência e a Tecnologia (FCT) through the research grants SFRH/BPD/76028/2011 and SFRH/BPD/85008/2012 and Fundo Social Europeu. This work was financed by national funds through FCT within the scope of Project UID/BIA/04004/2013.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 12 July 2016
doi: 10.3389/fpls.2016.01023

Assessing Fungal Population in Soil Planted with *Cry1Ac* and *CPTI* Transgenic Cotton and Its Conventional Parental Line Using 18S and ITS rDNA Sequences over Four Seasons

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OPEN ACCESS

Edited by:
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Reviewed by:
Lei Zhang,
North Carolina State University, USA
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *04 May 2016*
Accepted: *28 June 2016*
Published: *12 July 2016*

Citation:
Qi X, Liu B, Song Q, Zou B, Bu Y, Wu H, Ding L and Zhou G (2016) Assessing Fungal Population in Soil Planted with Cry1Ac and CPTI Transgenic Cotton and Its Conventional Parental Line Using 18S and ITS rDNA Sequences over Four Seasons. Front. Plant Sci. 7:1023. doi: 10.3389/fpls.2016.01023

Long-term growth of genetically modified plants (GMPs) has raised concerns regarding their ecological effects. Here, FLX-pyrosequencing of region I (18S) and region II (ITS1, 5.8S, and ITS2) rDNA was used to characterize fungal communities in soil samples after 10-year monoculture of one representative transgenic cotton line (TC-10) and 15-year plantation of various transgenic cotton cultivars (TC-15mix) over four seasons. Soil fungal communities in the rhizosphere of non-transgenic control (CC) were also compared. No notable differences were observed in soil fertility variables among CC, TC-10, and TC-15mix. Within seasons, the different estimations were statistically indistinguishable. There were 411 and 2 067 fungal operational taxonomic units in the two regions, respectively. More than 75% of fungal taxa were stable in both CC and TC except for individual taxa with significantly different abundance between TC and CC. Statistical analysis revealed no significant differences between CC and TC-10, while discrimination of separating TC-15mix from CC and TC-10 with 37.86% explained variance in PCoA and a significant difference of Shannon indexes between TC-10 and TC-15mix were observed in region II. As TC-15mix planted with a mixture of transgenic cottons (Zhongmian-29, 30, and 33B) for over 5 years, different genetic modifications may introduce variations in fungal diversity. Further clarification is necessary by detecting the fungal dynamic changes in sites planted in monoculture of various transgenic cottons. Overall, we conclude that monoculture of one representative transgenic cotton cultivar may have no effect on fungal diversity compared with conventional cotton. Furthermore, the choice of amplified region and methodology has potential to affect the outcome of the comparison between GM-crop and its parental line.

Keywords: farmland ecosystem, fungal diversity, genetically modified cotton, indicator taxa, pyrosequencing, seasonality

INTRODUCTION

Genetically modified plants (GMPs) have an improved quality and higher yield than unmodified plants do. The GMPs that have been developed and marketed currently include transgenic rice, transgenic cotton and transgenic corn. The global commercial cultivation of transgenic crops has increased from 1.7 million hectares in 1996 to 170 million hectares in 2012 in 28 countries (Clive, 2013), including US, Brazil, and Australia. In 2006, cotton and maize expressing *Bacillus thuringiensis* proteins were grown on 32.1 million hectares worldwide (Romeis et al., 2006). Because cotton is an economically important crop worldwide, a pesticidal property was introduced into cotton by expressing an insect-resistant protein of *B. thuringiensis* into the cotton genome. It was reported that *B. thuringiensis* caused an osmotic imbalance or opening of ion channels which activated cell death. It is specifically lethal to Lepidopteran and coleopteran insects (Melo et al., 2016). The insect resistance was greatly improved in GM cotton, which resulted in high yields of cotton and reduced use of insecticides. The GM cotton expressing Cry1Ab/c has been cultivated commercially for more than a decade in China, and it currently represents 71.5% of the total cotton grown because of its low production costs (Ronald, 2014; Carrière et al., 2015).

Because GMPs were first commercialized in 1994, they were welcomed by farmers and consumers, but there remained strong concern regarding the potential impact of GMPs on the environment. In 2001, the US Environmental Protection Agency (EPA; Washington, DC, USA) reassessed *B. thuringiensis* crops that had been accepted for agricultural use for six years (from 1995 to 2001). Investigations have been conducted to evaluate whether GMPs affect the natural environment, including invasiveness, non-target species, the potential of transgenes to "escape" into the environment, and the development of resistance to transgene-derived proteins (Jin et al., 2014). Although the *B. thuringiensis* protein is effective in controlling certain pests, it is important to examine its effects on non-target organisms in the soil. It has been reported that *B. thuringiensis* plants have little impact on the soil biota, such as earthworms, collembolans, and the general soil microflora (Li et al., 2011, 2012; Fließbach et al., 2012; Li and Liu, 2013), and these plants confer an environmental advantage over that require insecticides. However, it is not clear whether *B. thuringiensis* in root exudates influence soil microbial communities directly or indirectly (Li et al., 2015) and how a mixture of GMPs shape microbial communities. Preliminary research has indicated that the contents of root exudates differ significantly between GM cotton and conventional cotton. Fungi are directly exposed to GMP roots, and thus, strong feedback due to the interactions between fungi and GMPs would occur, influencing production and vegetation dynamics. In 1929, fungal pathogens caused a 10% loss of cereal yield according to German authorities (Fisher et al., 2012). In Indian, fungal diseases are regarded as the most important factor contributing to yield losses. The techniques of genetic transformation to develop transgenic resistant to fungal diseases have been even developed (Denning and Bromley, 2015). Therefore, investigation of fungal diversity should be welcomed for illuminating the interaction between GMPs and fungi. The populations of cultivable fungi have increased in some parts of GMPs (Hawes et al., 2012; Li et al., 2015), and therefore, the increase in verticillium wilt and fusarium wilt in *B. thuringiensis* cotton may be related to fungi. To validate this hypothesis, a traditional technique is the use of AMF (arbuscular mycorrhizal fungi), which are considered to be an excellent indicator of the possible ecological impact of GMPs (Meyer et al., 2013; Birkett and Pickett, 2014). However, more diverse information may be lost when AMF are selected as the only target. In addition, controlled laboratory conditions may not represent the actual environment, and thus, research based on AMF alone is insufficient. To better understand the linkage between fungal communities and GMPs, it is necessary to investigate the composition of fungal communities in soil planted with conventional and *B. thuringiensis* cotton.

Several methods may be used to detect fungal diversity. Although DGGE, RFLP, ARDRA, and clone sequencing methods (Anderson et al., 2014; Buscardo et al., 2015; Hu et al., 2015) permit the detection of fungal diversity, the communities identified using these methods appeared to be less taxonomically rich, and changes in the relative abundance of species were easy to overlook based on plate counts of cultivable organisms (Epstein, 2013). Using the clone sequencing method, it was difficult to determine how many clones were required to represent the diversity of a single sample. The best technique is next-generation DNA sequencing (NGS), which allows the sequencing of millions of DNA fragments in parallel. As pyrosequencing-based NGS provides the power to sequence a read length of more than 500 bp (Hol et al., 2015; Hossain et al., 2015; Kazeeroni and Al-Sadi, 2016), we employed this sequencing tool for the comprehensive investigation of the microbial community composition in soil planted with conventional cotton for 15 years (CC), monoculture of one representative *B. thuringiensis* cotton line for 10 years (TC-10), and a mixture of transgenic cotton cultivars for 15 years (TC-15mix).

MATERIALS AND METHODS

Sample Collection and Physicochemical Analyses

This study was conducted in a cotton farm in Baibi town, Anyang, Henan Province, China, which belonged to the Cotton Research Institute (CRI) of the Chinese Academy of Agricultural Sciences (CAAS). The experimental field had a temperate continental monsoon climate with a mean annual rainfall of 556.8 mm and mean annual sunshine hours and temperature of 2228.8 h and 14.1°C, respectively. The soil samples were collected from three experimental fields planted with conventional cotton for 15 years (CC), monoculture of one representative transgenic *B. thuringiensis* cotton line for 10 years (TC-10), and a mixture of transgenic cotton cultivars for 15 years (TC-15mix, **Table 1**) in the seeding stage (S, 26 April), bud stage (B, 13 July), blooming stage (Bl, 22 August) and boll opening stage (Bo, 17 October) in 2011. Each field was separated by a distance of 100 m in this farm to achieve equivalent environments. Fifteen meters were

TABLE 1 | Planting information for three cotton fields.

Treatment	Cotton variety	Foreign gene expressed	Planting time
CC	Zhongmian-35	–	1997 up to now
TC-10	Zhongmian-41	Cry 1Ac and CPTI	2002 up to now
TC-15mix	Mixture of Zhongmian-29, 30, and 33B	Cry 1A	1997–2001
	Zhongmian-41	Cry 1Ac and CPTI	2002 up to now

permitted at both ends of every treatment to eliminate marginal field effects on soil sampling. Each type of treatment field was established in quintuplicate (S and B stages) or triplicate (Bl and Bo stages), and each replicate plot was 0.4 hectares. After the weeds and leaves were removed from the surface, soil sub-samples were collected between 0 and 20 cm deep using a soil auger with a diameter of 4 cm. Three replicate soil sub-samples per plot were collected according to the checkerboard method (Committee of handbook of soil fauna research methods, 1998) using a sterile tube and immediately placed on ice for transport to the laboratory. Then the three replicate sub-samples were pooled together and mixed adequately for subsequent DNA extraction and elemental analysis (Supplementary Table S1). The samples were maintained at −80°C for elemental analysis within 7 days according to the National Standard of the People's Republic of China. DNA sequencing of each sample used for elemental analysis was performed.

DNA Extraction and Pyrosequencing

A total of 48 samples (16 each from one type of treatment field) were analyzed in this study. Genomic DNA extracted from 0.25 g soil sample was prepared for pyrosequencing using the MO BIO Power Soil DNA Extraction kit according to the manufacturer's protocol (MO BIO Laboratories, Carlsbad, CA, USA). An additional step was included before the final elution step in which the DNA was incubated at 65 °C for 5 min. Finally, the DNA was eluted and collected in 50 μL C6 buffer (provided in the kit). The region I primers (18S) contained the Roche Life Science A or B Titanium sequencing adapter (italicized) followed immediately by a unique 10-base barcode sequence (BBBBBBBBBB) and finally the 3′ end of the primer: R1-F 5′-*CAT CTC ATC CCT GCG TGT CTC CGA CTC AG* BBB BBB BBB BGA TAC CGT CGT AGT CT-3′ (FF700) and R1-R 5′-*CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG* AGC CAT TCA ATC GGT AGT-3′ (FR1) (Vainio and Hantula, 2000). The region II (ITS1, 5.8S and ITS2) primers contained the Roche Life Science A or B Titanium sequencing adapter (italicized) followed immediately by a unique 10-base barcode sequence (BBBBBBBBBB) and finally the 3′ end of the primer: R2-F 5′-*CAT CTC ATC CCT GCG TGT CTC CGA CTC AG*B BBB BBB BBB GAG GCA ATA ACA GGT CTG TGA TGC-3′ (NS7) and R2-R 5′-*CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG* TCC GCA GGT TCA CCT ACG GA-3′ (NS8) (White et al., 1990).

The pyrosequencing PCR mixtures contained 1.25 U of *Taq* polymerase (Takara Biotechnology, Dalian, Liaoning, China), 2.5 μL of 10× PCR buffer supplied by the manufacturer, 0.5 μL of dNTPs (10 mM), 1 μL of 10 μM reverse primer, 1 μL of 10 μM forward primer, 2 μL of DNA template and water up to 25 μL. The amplification was conducted under the following conditions: an initial denaturation at 95°C for 5 min, 35 cycles at 95°C for 20 s, 58°C for 30 s and 72°C for 45 s, and a final extension at 72°C for 7 min. Negative control reactions without template were consistently performed.

The amplicons were visualized using 1.5% (w/v) agarose gels stained with ethidium bromide and purified with AMPure XP Beads (Beckman Coulter, Danvers, MA, USA) according to the manufacturer's protocol, followed by concentration and size distribution analysis using DNA 1000 chips on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany). Sequencing was conducted on a GS-FLX Titanium pyrosequencer (Roche Life Sciences, Branford, CT, USA) at the Beijing Genomics Institute (Shenzhen, Guangdong, China).

Microbial Community Analyses via rDNA Gene Sequencing

The sequences were parsed by barcodes using the Mothur software packages (1) to sort sequences with exact matches to the specific 10-bp barcodes into different samples (one unambiguous mismatch to the sample and two mismatches to the PCR primer were permitted), (2) to trim off adapters, barcodes and primers using default parameters, and (3) to remove sequences containing ambiguous 'N' homopolymers exceeding 10 bp or with a length shorter than 200 bp (Griffen et al., 2012; Zhang et al., 2012). Denoised sequences were generated by the 'Shhh.flows' command in the Mothur platform to remove sequences that were likely due to pyrosequencing errors. All of the sequences were aligned using a NAST-based sequence aligner to a custom reference based on the SILVA alignment. It was ensured that all of the sequences overlapped in the same alignment space by trimming the ends of each sequence such that all of the sequences started and ended at the same alignment coordinates. All of the sequences were then pre-clustered to permit one base difference in one hundred bases to form a more abundant sequence. Chimeric sequences were then identified using UCHIME (http://drive5.com/uchime). All of the sequences were aligned in the Silva alignment using NAST and then classified using the RDP (Ribosomal Database Project) classifier with an RDP confidence threshold of 80% or greater in the Silva database. The clean dataset was clustered into a molecular operational taxonomic unit (OTU) with a 97% identity threshold using the average neighbor clustering algorithm. The control samples used to validate the abundance data were provided. Both regions were treated equivalently.

OTU richness was determined as the number of OTUs present in a sample. Shannon and Chao 1 were calculated using EstimateS based on 100 randomizations with at least 75% of the sequence selected at one time (Caporaso et al., 2010; Colwell et al., 2012). Shannon diversity was exponentially transformed Exp (H). The relationships between OTU richness, Shannon and soil elements (TOC, TN, TP, and TK) were explored by calculating Pearson's correlation coefficients among each pair of variables.

OTU richness and Shannon among CC, TC-10, and TC-15mix were compared using *t*-tests.

The data were normalized as a percent of the total for each taxon per sample, and the taxa were averaged for region I and region II. The cluster analysis was conducted to group the fungal communities from different soil samples based on the generated OTUs using RDP Complete Linkage Clustering from the merged pool of sequences from all of the samples. The names of the fungal sequences from each sample were specifically encoded to identify their sources in the merged sequence pool. The cluster analysis was then conducted using the unweighted-pair group method with arithmetic mean (UPGMA) based on the distance metric of UniFrac calculated by QIIME (Stoll et al., 2014). It was constructed by aligned, representative sequences for OTUs at 3% cut-off level using Weighted UniFrac with the default settings. Then the similarity cut-off levels clustered the soil samples into several main groups.

PCoA (principal coordinate analysis) was conducted based on the OTUs described above. PCoA is a phylogenetically independent method, and weighted and unweighted UniFrac distances were generated from normalized data in a beta-diversity pattern using a QIIME analysis pipeline. The plots were generated based on the weighted and unweighted UniFrac distance metric (Blaser et al., 2013).

To identify any significant differences among the taxonomic groups from the three soil types, the conventional cotton control samples were compared with the transgenic samples using a Mann-Whitney-Wilcoxon signed-rank test (function wilcox_test in the coin package of R). The obtained *P*-values were adjusted based on a Benjamini-Hochberg false discovery rate correction (function p.adjust in the stats package of R) (Griffen et al., 2012).

RESULTS

Soil Fertility Variables

TOC (total organic carbon), TN (total nitrogen), TP (total phosphorus), and TK (total potassium) were analyzed. As shown in Supplementary Table S2, no notable differences were observed in the concentrations of TOC, TN, TP, or TK across the fields (48 soil specimens), with calculated averages of 15.27 ± 2.22, 1.221 ± 0.301, 0.780 ± 0.083, and 14.77 ± 1.31 g/kg, respectively. Therefore, the transgenic cottons had no effects on soil fertility, and farming management practices appeared to be almost equivalent among the three fields.

Composition and Diversity of Fungal Communities in the Soil

Based on the two sets of primers that were used to analyze fungal diversity in the references (White et al., 1990; Vainio and Hantula, 2000), two different regions (designated "I" and "II") were sequenced for each sample using the barcoded pyrosequencing platform to yield 1 147 486 sequences. Among these sequences, 323 270 (region I) and 199 301 (region II) qualified sequences were generated from 47 (region I) and 48 (region II) samples, respectively. The numbers of qualified sequences per sample were, on average, 6878 ± 2590 (region I) and 4152 ± 1539 (region II), ranging from 2449 to 13497 and from 1176 to 7892, respectively. Of all the qualified sequences, four fungal phyla were identified and the remaining sequencing data were grouped as unclassified fungi and non-fungi (others). The percentages of fungal sequences in the full pyrosequencing dataset were 70% and 61%, respectively, for region I and II. All but 30 and 39% of the sequences could be classified at the domain level (Algae, Alveolata, Amoebozoa, Cryptophyta, Euglenozoa, Metazoa, Viridiplantae) based on the Silva database in region I and region II, respectively, and these sequences also belonged to eukaryotes (Supplementary Tables S2—S5). The percentages of shared taxa in the two regions were 100, 94, 86, 77, and 62% in region I and 100, 77, 60, 40, and 32% in region II, respectively, at the level of the phylum, class, order, family, and genus.

Figure 1 shows the relative abundance of the fungal phyla, unclassified fungi and non-fungi (others) in the overall communities of the soil samples from different groups (CC, TC-10, and TC-15mix) in region I and region II, respectively. In region I (**Figure 1A**), excluding the non-fungi (others), Ascomycota represented the dominant lineage in each group, accounting for 58, 62, and 69% of all sequences in the CC, TC-10, and TC-15mix groups, respectively. Fungi_incertae_sedis accounted for 1.0 and 1.4% of the CC and TC-10 groups, respectively, but decreased to 0.16% in the TC-15mix group. The relative abundance of Basidiomycota and Glomeromycota were 0.12% and 0.13% in TC-15mix, respectively, but reduced to 0.018% and 0.013% in CC, and to 0.014% and 0.022% in TC-10. These data suggested that the relative abundance of Fungi_incertae_sedis, Basidiomycota and Glomeromycota varied largely in the CC, TC-10, and TC-15mix groups and that the relative abundance of the unclassified fungi was nearly equivalent among the samples from the three groups.

In region II (**Figure 1B**), excluding the non-fungi (others), Ascomycota still represented the most dominant lineage in each group, and the relative abundance of Fungi_incertae_sedis also decreased considerably in TC-15mix. In contrast to region I, the relative abundance of Basidiomycota and Glomeromycota from all groups was close to 1%, which was approximately 10 times higher than those in region I. This result indicates that the two regions encompass a different range of species and that each region should be assessed individually in the data analysis.

Taxa Richness

Approximately 95% (region I) and 69% (region II) of the fungal sequences could be classified at the fungal phylum level (Ascomycota, Basal_fungal_lineages, Basidiomycota and Glomeromycota) using the RDP classifier (80% threshold). A total of 411 (region I) and 2067 (region II) fungal OTUs were observed across all of the sampling locations and seasons. Among the locations, 110 (region I) and 320 (region II) fungal OTUs were present in soil samples from CC and TC, respectively. Although a majority of the OTUs were scattered throughout the samples, their frequencies were much lower. The data indicated that microbial populations tended to have a long tail of less abundant taxa even after rigorous sequence denoising and the exclusion of singletons. Among 35 or more samples, there were 15 (region I) and 22 (region II) OTUs (**Table 2**).

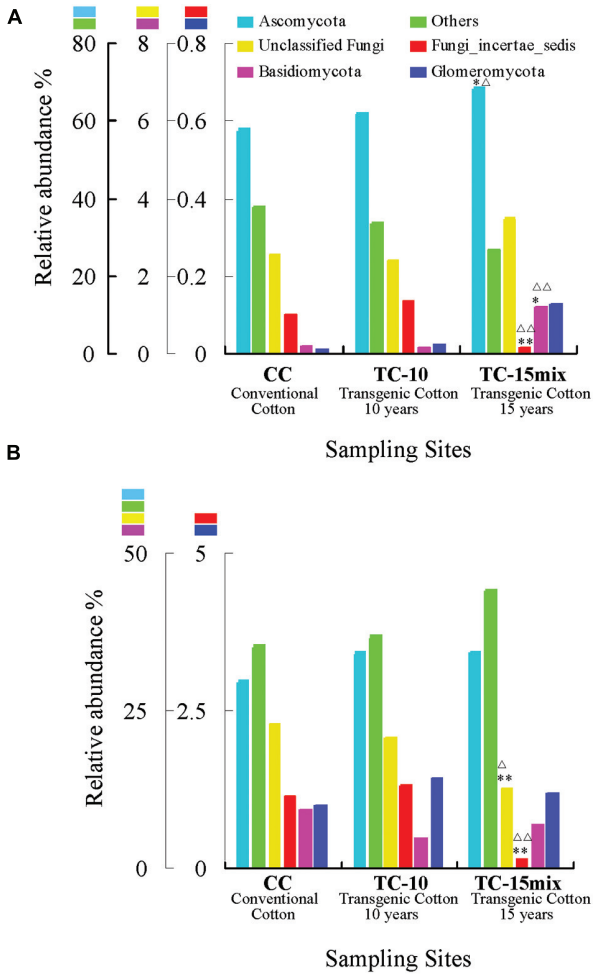

FIGURE 1 | Relative abundance (%) of fungal phyla in the overall communities of soil samples from different groups (CC, TC-10, and TC-15mix) in region I (A) and region II (B). (A) Others include Alveolata, Metazoa, Unclassified_eukaryota, Viridiplantae, and Stramenopiles. **(B)** Others include Algae, Alveolata, Amoebozoa, Cryptophyta, Euglenozoa, Metazoa, Viridiplantae, and Unclassified_eukaryota. *Significant difference with a *P*-value of <0.05 in comparison with CC; **Significant difference with a *P*-value of <0.01 in comparison with CC. $^{\Delta}$Significant difference with a *P*-value of <0.05 in comparison with TC-10; $^{\Delta\Delta}$Significant difference with a *P*-value of <0.01 in comparison with TC-10.

The species diversity and combination of homogeneity and richness were reflected by the Chao 1, OTU richness and Shannon. Within seasons, the different estimations were statistically indistinguishable (**Supplementary Figure S1A**), and there were no significant differences among the separate fields (**Supplementary Figure S1B**, $P > 0.05$). The *t*-test also showed both OTU richness and Shannon had no significant difference between CC and TC-10, except for Shannon index between TC-10 and TC-15mix in region II (Supplementary Table S6). Within regions, region II accumulated OTUs at a higher rate across both sampling sites and seasons. Pearson's correlation coefficients showed that no elements were significantly correlated with diversity indexes (Supplementary Table S7).

Taxonomic Coverage and Indicator Taxa

In region I, 64% of the highly abundant fungal taxa shown in **Table 2** were identified at different sampling sites (CC-soil, TC-10-soil, and TC-15mix-soil), and these taxa were present in more than half of the samples. However, in region II, there were 54, 47, and 40 taxa in the CC-soil, TC-10-soil, and TC-15mix-soil, respectively, and each taxon was present in more than half of the samples from the same sites. In total, 19 orders of fungi encompassing distinct evolutionary lineages and a diversity of morphologies were discovered (**Table 3**). Based on the two regions, the percentages of taxa shared in CC and TC were 100% and 100%, 100% and 86%, 89% and 81%, 91% and 80%, and 84% and 76% in region I and region II at the level of the phylum, class, order, family and genus, respectively. Abundant families of soil fungi in region I and region II were shown in **Figures 2A,B** respectively. Both regions contained only a few fungi that differed in abundance among the soil samples from CC, TC-10 and TC-15mix at the level of the family. For example, in region II, Sarcosomataceae, Myxotrichaceae and mitosporic_Tremellale were highly abundant in the soil samples from TC-15mix, while Pleosporaceae and Choanephoraceae represented the majority in CC.

The abundance of rare genera specific to CC, TC-10, or TC-15mix was less than 0.065%. Some individual genera with significant differences in abundance among CC, TC-10, and TC-15mix over four seasons (lines with different colors) were shown in **Figure 3**. Both regions contained the genera *Geomyces* and *Poitrasia*, and importantly, each season provided a similar pattern of abundance among CC, TC-10, and TC-15mix. The present study also showed that the abundance of *Alternaria* (region II) and *Chaetomium* (region I) decreased by approximately 30% on average in TC-10 in comparison to CC in all four sampling periods and increased to the same level as CC in TC-15mix. In contrast, for *Poitrasia* (in both regions) and *Petalosphaeria* (region I) at the blooming stage, the abundance increased significantly (approximately 60-fold) in TC-10 compared with CC and decreased to the same level as CC in TC-15mix. Although the abundance of some genera showed significant difference between CC and TC, the proportion of them was less than 10%.

Similarity Analysis of Samples from the Three Sampling Sites

The similarity of the 47 samples in region I and 48 samples in region II were evaluated by cluster analysis and principle coordinate analysis (PCoA), respectively. Based on the fungal communities, the soil samples could be clustered into several groups in cluster analysis (**Supplementary Figure S2**). An ambiguous cluster version among samples from CC, TC-10, and TC-15mix was shown in region I. In region II almost all samples from CC separated from those in TC-15mix except for No.33 (branch IV) and 31 (branch II), while CC and TC-10 clustered together. PCoA was also performed to elucidate similarities among different soil samples based on OTUs and exhibited similar observations with cluster analysis. As shown in **Figure 4** (UniFrac at a 3% cutoff), no obvious clusters among the

TABLE 2 | The most abundant fungal taxa in samples of different sampling sites identified at the OTU level.

OTU rank	Region I					Region II				
	Assigned taxonomy	Frequency				Assigned taxonomy	Frequency			
		CC	TC-10	TC-15mix	Total		CC	TC-10	TC-15mix	Total
1	*Mycosphaerella*	100	100	100	47	*Strobiloscypha*	100	100	100	48
2	*Geomyces*	100	100	100	47	Fungi;other	100	100	100	48
3	*Pezizales*	100	100	100	47	Fungi;other	100	100	100	48
4	*Chaetomium*	100	100	100	47	*Geomyces*	100	100	100	48
5	*Halosphaeriaceae*	100	100	100	47	Fungi;other	100	100	100	48
6	*Verticillium*	100	100	100	47	*Dothideomycetes_incertae_sedis*	100	100	93.8	47
7	*Talaromyces*	93.8	93.8	100	45	Fungi;other	100	93.8	93.8	46
8	*Pleospora*	87.5	100	93.3	44	*Agaricales*	100	87.5	93.8	45
9	*Dikarya;other*	87.5	87.5	100	43	*Kirschsteiniothelia*	93.8	93.8	87.5	44
10	*Pestalosphaeria*	81.3	100	93.3	43	Fungi;other	87.5	93.8	87.5	43
11	*Dikarya;other*	87.5	87.5	86.7	41	*Dothideomycetes*	87.5	93.8	87.5	43
12	*Dothideomycetes*	87.5	81.3	86.7	40	*Agaricomycotina*	87.5	81.3	93.8	42
13	*Pezizomycotina*	87.5	81.3	73.3	38	*Alternaria*	93.8	75.0	87.5	41
14	*Coniosporium*	100	100	20.0	35	*Sordariomycetes*	93.8	87.5	75.0	41
15	*Poitrasia*	81.3	56.3	86.7	35	*Pezizomycotina*	87.5	81.3	87.5	41
16	*Ascomycota*	100	100	6.7	33	*Pleospora*	81.3	75.0	93.8	40
17	*Eurotiomycetidae*	37.5	62.5	100	31	*Pezizales*	100	100	43.8	39
18	*Lecanoromycetes*	62.5	87.5	33.3	29	Fungi;other	93.8	87.5	62.5	39
19	Fungi;other	75.0	50.0	53.3	28	*Pezizomycotina*	100	93.8	50.0	39
20	*Pezizomycotina*	62.5	62.5	53.3	28	*Filobasidiales*	93.8	62.5	81.3	38
21	Fungi;other	56.3	75.0	40.0	27	*Coniosporium*	75.0	81.3	81.3	38
22	*Dikarya;other*	43.8	43.8	86.7	27	Fungi;other	100	81.3	56.3	38
23	*Talaromyces*	62.5	43.8	60.0	26	*Glomeromycetes*	62.5	81.3	87.5	37
24	Fungi;other	62.5	43.8	33.3	22	*Pleosporales*	87.5	75.0	68.8	37
25	*Sarcoscyphaceae*	37.5	37.5	60.0	21	*Pezizales*	81.3	93.8	50.0	36
26	*Ascomycota*	18.8	18.8	60.0	15	*Trichocomaceae*	81.3	87.5	56.3	36
27						*Poitrasia*	93.8	100	25.0	35
28						Dikarya;other	87.5	87.5	43.8	35
29						Fungi;other	62.5	87.5	68.8	35
30						*Agaricomycetes*	68.8	75.0	68.8	34
31						*Basidiomycota*	87.5	81.3	37.5	33
32						*Sordariomycetes*	50.0	62.5	87.5	32
33						Fungi;other	75.0	68.8	50.0	31
34						*Paracoccidioides*	62.5	87.5	37.5	30
35						*Halosphaeriaceae*	68.8	62.5	56.3	30
36						*Agaricomycotina*	62.5	81.3	43.8	30
37						*Paracoccidioides*	37.5	50.0	87.5	28
38						*Glomeromycetes*	56.3	56.3	56.3	27
39						Fungi;other	62.5	43.8	62.5	27
40						*Nectria*	56.3	37.5	68.8	26
41						*Agaricomycetes*	37.5	56.3	68.8	26
42						*Basidiomycota*	68.8	68.8	18.8	25
43						*Sporobolomyces*	87.5	62.5	0.0	24
44						Dikarya;other	43.8	56.3	50.0	24
45						Fungi;other	62.5	50.0	31.3	23
46						Hypocreales	68.8	37.5	37.5	23
47						*Basidiomycota*	50.0	56.3	37.5	23
48						*Dothideomycetidae*	56.3	25.0	56.3	22
49						*Tremellomycetes*	87.5	43.8	6.3	22

(Continued)

TABLE 2 | Continued

OTU rank	Region I					Region II				
	Assigned taxonomy	Frequency				Assigned taxonomy	Frequency			
		CC	TC-10	TC-15mix	Total		CC	TC-10	TC-15mix	Total
50						*Strobiloscypha*	56.3	56.3	18.8	21
51						Fungi;other	87.5	25.0	18.8	21
52						*Strobiloscypha*	75.0	37.5	12.5	20
53						*Dothideomycetes*	18.8	31.3	75.0	20
54						*Pezizomycotina*	37.5	31.3	56.3	20
55						*Basidiomycota*	37.5	37.5	50.0	20
56						*Glomeromycetes*	50.0	43.8	25.0	19
57						*Leptosphaeria_maculans_complex*	50.0	31.3	37.5	19
58						*Basidiomycota*	62.5	43.8	6.3	18
59						Dikarya;other	62.5	31.3	6.3	16
60						Fungi;other	31.3	50.0	18.8	16
61						*Agaricomycetes*	43.8	50.0	6.3	16
62						Dikarya;other	50.0	25.0	25.0	16

soil samples were observed in region I. PC1 axes separated TC-15mix from CC and TC-10 with 37.86% explained variance in region II.

DISCUSSION

Although recent research has improved our understanding of microbial communities associated with GMPs (Weinert et al., 2010; Li et al., 2014), studies investigating these pair-wise differences must be examined within a wider context of natural variation for a long planting time (Szénási et al., 2014). This study aimed to elucidate whether transgenic cotton influenced soil fungal communities in natural ecosystem more than 10 years after planting. Moreover, we examined the effects of plant growth stage using two amplified regions to assess the influence of seasonal variation and microbial lineages. Given the current state of our knowledge, no data are available regarding the responses of fungal communities in two amplified regions for the annual cycle of *B. thuringiensis* cotton. We started our investigation with elemental analyses in CC, TC-10, and TC-15mix to determine whether genetic modifications had effects on soil fertility, which in turn may affect the fungal diversity. It was found that genetic modifications did not influence soil fertility, and the elements were not related with taxa richness (Supplementary Tables S1 and S7). Soil fertility analyzed in this study had no interaction with fungal community.

Although many primers are valuable for investigating fungal diversity, unfortunately none of them can capture all diversity in the fungal kingdom with a high specificity (Stukenbrock and McDonald, 2008; Chen et al., 2014; Ene and Bennett, 2014; Schadt and Rosling, 2015). Here, for improved diversity, two primer pairs rather than one were employed to amplify 18S and ITS regions. For instance, *Mycosphaerella*, *Chaetomium*, and *Verticillium* were dominant in region I, while Strobiloscypha and Dothideomycetes_incertae_sedis were included in region II (**Figure 2**; **Tables 2** and **3**). The inconsistency of the communities between the two regions resulted in some differences in their abundance at the higher level (e.g., unclassified fungi, other and Basidiomycota, as detailed in **Figure 1**). This result was due to much more stratified and complex communities and proportions of unclassified reads in the higher taxonomy. Thus, the different primer combinations may have resulted in variable proportions, especially at the higher taxonomic level. Both primer pairs detected not only independent fungal diversity but also some overlapping taxa. Additionally, the similar spatiotemporal variations in abundance of overlapping taxa validated the accuracy of abundance. For instance, a clear tendency for the rate of change in the relative abundance of Fungi_incertae_sedis in the three fields in region I was in perfect agreement with that in region II, because almost all of the reads for Fungi_incertae_sedis in the two regions were assigned to one taxon, *Poitrasia*. Strong consistencies could also be obtained for Pleosporaceae and Myxotrichaceae at the family level (**Figure 2**). Consequently, the results from two regions yielded a consistent and mutually complementary diversity. Indeed, both regions were necessary to characterize the fungal communities in CC, TC-10, and TC-15mix.

It was found that more than 75% of the highly abundant taxa were stable in soil planted with transgenic and conventional cottons. Ascomycota was the largest phylum of fungi, which is consistent with a previous publication (Hibbett and Taylor, 2013). No significant differences were observed in abundance of phyla between CC and TC-10 except for TC-15mix (**Figure 1**). Our results were consistent with the previous publications, suggesting that monoculture of one transgenic cotton line may have no effect on fungal phylum composition in comparison with conventional cotton (Griffiths et al., 2000; Koch et al., 2015; Zhou et al., 2016). However, a remarkable difference was observed between TC-15mix and groups of CC and TC-10 at phylum level. Researches showed cultivar type influenced microbial community structure (İnceoğlu et al., 2010; Dias et al., 2012). Even in the context of

TABLE 3 | Classes of fungi and their representation.

	Region I						Region II					
	Class	**Number of OTUs**	**Description**	**CC**	**TC-10**	**TC-15mix**	**Class**	**Number of OTUs**	**Description**	**CC**	**TC-10**	**TC-15mix**
1	Sordariomycetes	66	Plant pathogens and saprobes	16	16	15	Pezizomycetes	222	Mushrooms and molds	16	16	16
2	Pezizomycetes	40	Mushrooms and molds	16	16	15	Dothideomycetes	162	Molds	16	16	16
3	Dothideomycetes	26	Molds	16	16	15	Agaricomycetes	150	Mushrooms and polypores	16	16	16
4	Ascomycota	21	Mushrooms and polypores	14	9	13	Glomeromycetes	146	Plant root biotrophs	16	16	16
5	Eurotiomycetes	13	Molds	16	16	15	Leotiomycetes	95	Plant pathogens and saprobes	16	16	16
6	Glomeromycetes	12	Plant root biotrophs	5	6	8	Tremellomycetes	63	Yeasts	16	13	14
7	mitosporic_Ascomycota	8	Molds	1	1	1	Sordariomycetes	59	Plant pathogens and saprobes	16	16	16
8	Mucorales	7	Molds	16	16	6	Eurotiomycetes	37	Molds	16	14	16
9	Agaricomycetes	5	Mushrooms and polypores	1	3	4	Basal_fungal_lineages	20	Molds	16	16	8
10	Saccharomycetes	4	Yeasts	7	2	1	Microbotryomycetes	12	Yeasts	14	13	6
11	Lecanoromycetes	3	Lichenized fungi	11	14	5	mitosporic_Ascomycota	10	Molds	15	15	15
12	Leotiomycetes	2	Plant pathogens and saprobes	16	16	15	Taphrinomycetes	7	Plant pathogens	2	3	5
13	Tremellomycetes	2	Yeasts	1	1	1	Cystobasidiomycetes	7	Yeasts	1	0	0
14	Cystobasidiomycetes	1	Yeasts	0	0	1	Lecanoromycetes	6	Lichenized fungi	6	6	5
15	Exobasidiomycetes	1	Plant pathogens	0	0	1	Saccharomycetes	6	Yeasts	7	4	2
16	Fungi;other	85		16	16	15	Exobasidiomycetes	3	Plant pathogens	0	2	3
17	Dikarya;other	74		16	16	15	Dacrymycetes	2	Jelly fungi	2	2	0
18	Pezizomycotina;other	28		16	16	15	Agaricostilbomycetes	1	Molds	0	0	1
19	Basidiomycota;other	13		16	16	15	Pucciniomycetes	1	Molds	1	0	0
20							Fungi;other	403		16	16	16
21							Dikarya;other	284		16	16	16
22							Ascomycota;other	59		16	16	16
23							Pezizomycotina;other	168		16	16	16
24							Basidiomycota;other	79		16	16	16
25							Agaricomycotina;other	64		16	16	16
26							Pucciniomycotina;other	1		16	16	16

The abundances of OTUs which were identified to class were summed. The representative ecologies for those OTUs were given.

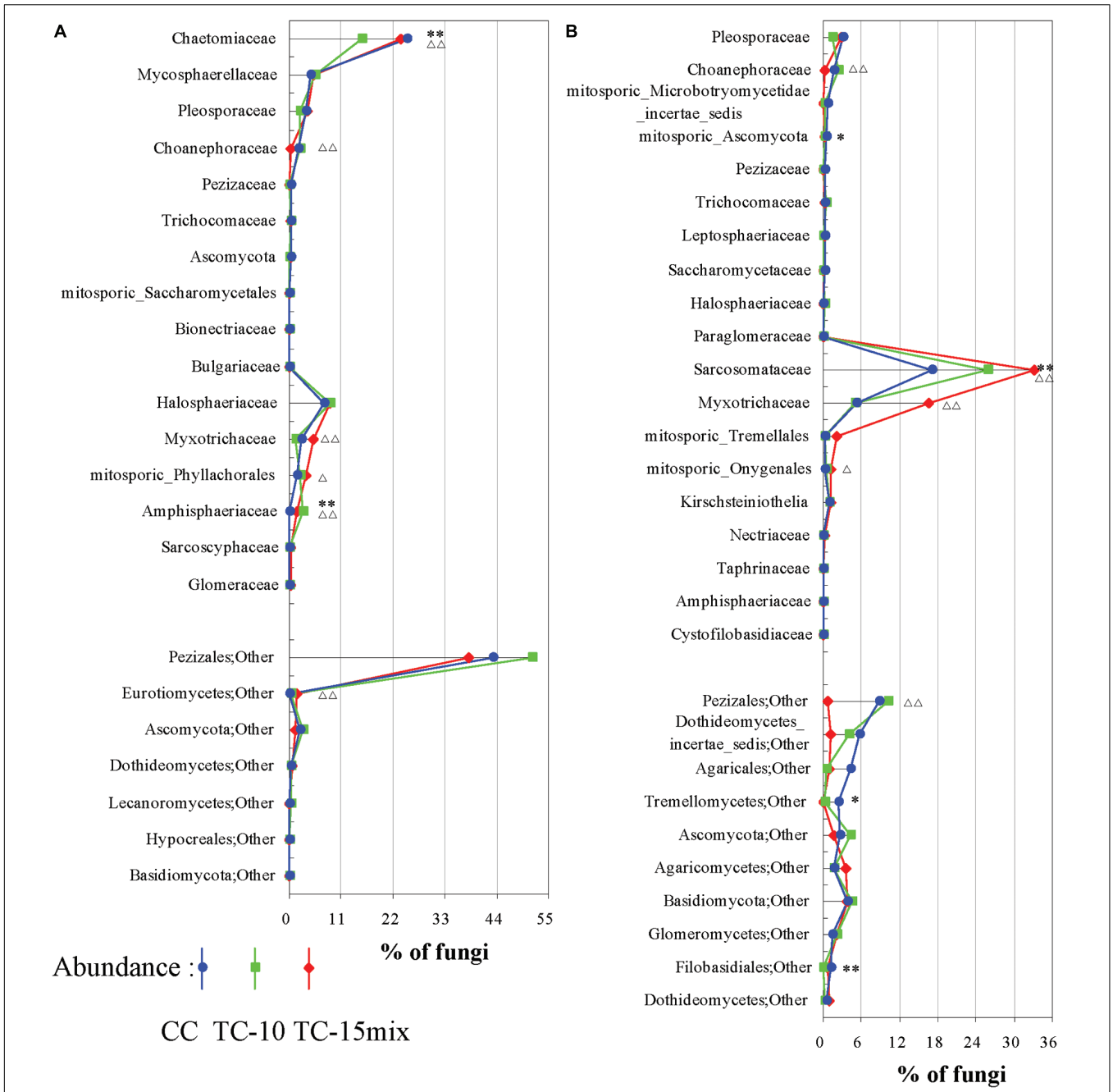

FIGURE 2 | Differences among CC, TC-10, and TC-15mix at the family level. (A) Region I; **(B)** Region II. The graphs show families with differences ≥0.03% and abundances ≥0.03% in the CC and TC samples. Some of the families could not be divided into lower levels and were represented by others according to their respective orders. *Significant difference with a *P*-value of <0.05 between CC and TC-10; **Significant difference with a *P*-value of <0.01 between CC and TC-10; $^{\Delta}$Significant difference with a *P*-value of <0.05 between CC-10 and TC-15mix; $^{\Delta\Delta}$Significant difference with a *P*-value of <0.01 between CC-10 and TC-15mix.

transgenic crops, different genetic modifications may generate non-desirable phenotypic alterations (Ricroch et al., 2011; Li et al., 2015). Both TC-10 and TC-15mix planted with transgenic cottons, while TC-15mix planted with a mixture of genetically modified cottons for over 5 years and appeared to have a different fungal composition from TC-10, suggesting that the variations introduced by different genetic modifications may be greater than those between transgenic and conventional cottons. Similar observations had been made in both regions. Based on the current data, it is still premature to predict the likelihood of dissimilar fungal diversity in TC-15mix from CC and TC-10. Further clarification is necessary by monitoring the fungal dynamic changes in sites planted in monoculture of various transgenic cottons.

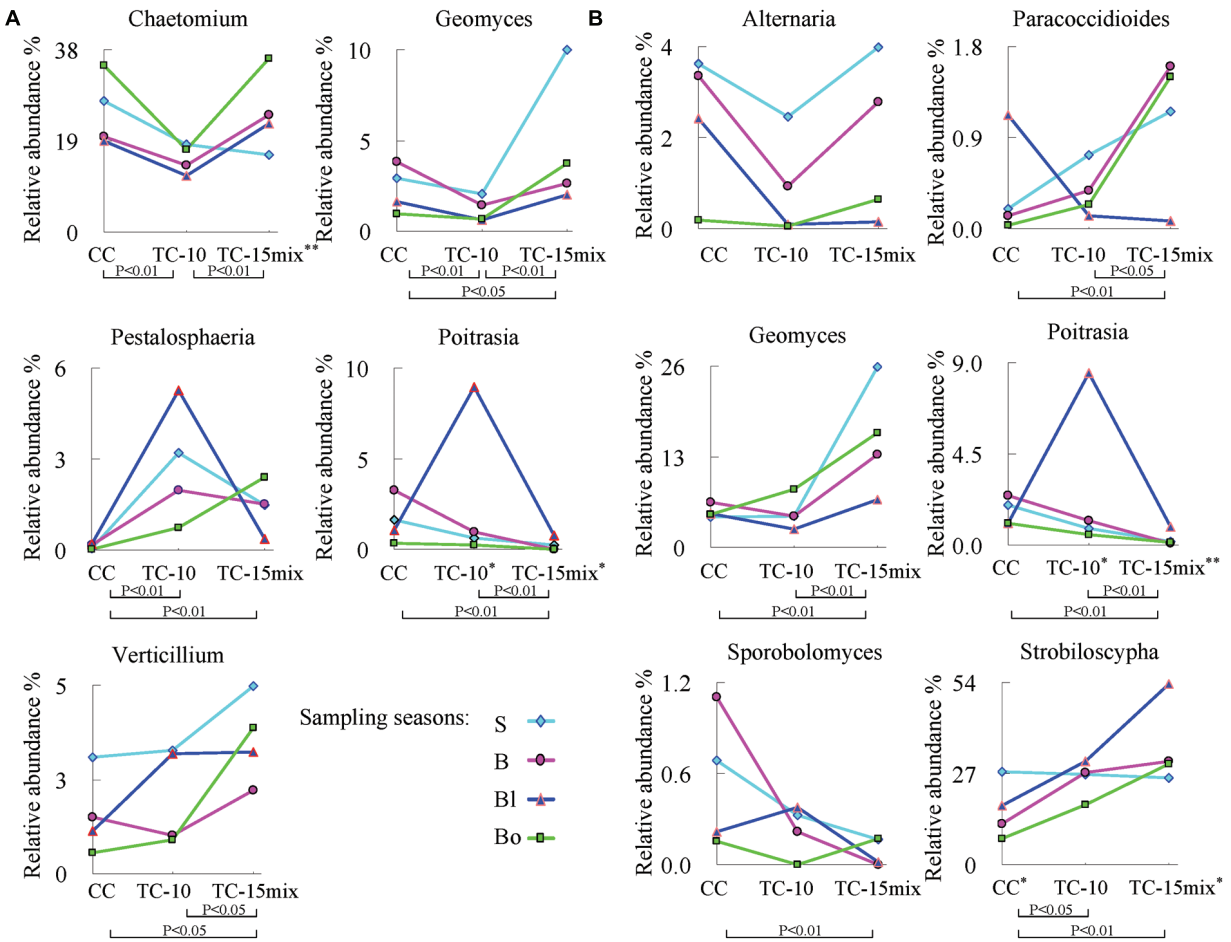

FIGURE 3 | Significant differences among CC, TC-10, and TC-15mix at the genus level during different seasons ($P < 0.05$). (A) Region I; **(B)** Region II. The graphs show the levels for genera with frequencies ≥30 in all samples (48 for region I and 47 for region II) and abundances ≥0.01%. S: seeding stage (26 April); B: bud stage (13 July); Bl: blooming stage (22 August); Bo: boll opening stage (17 October) in 2011. *$P < 0.05$ among the four seasons in the corresponding site. The double asterisks represent significant difference with a P-value of <0.01.

Examinations of the diversity index and community structure data across the samples can help to further illuminate whether transgenic cotton influenced fungal diversity. No discrimination was observed in diversity indexes or fungal communities among the four seasons within one treatment field. The differences in abundance among the four seasons within one field were much smaller than those among the three treatment fields (Supplementary Tables S2—S4; **Supplementary Figure S2**). No significant difference of diversity indexes and no distinct discrimination were observed between CC and TC-10 in both regions (**Figure 4**; Supplementary Table S6; **Supplementary Figures S1** and **S2**). The results revealed that the structures of communities between CC and TC-10 were quite similar. However, Shannon index in TC-10 was statistically different from that in TC-15mix in region II (Supplementary Table S6). Pearson's correlation analysis showed differences in diversity indexes were not affected by soil fertility variables. Furthermore, the discriminations between TC-10 and TC-15mix were detected in PCoA and consistent across four seasons (**Figure 4B**). The data showed a similar pattern to that seen at phylum level, and suggested variations among genetic modifications within GMPs may have an effect on microbial diversity, like variations among conventional cultivars (Dias et al., 2012). Thus, fungal diversities were dissimilar between CC and TC-15mix in cluster analysis and PCoA.

Within the amplified regions, as the two pairs of primers targeted different fungal lineages (**Figure 2**; **Tables 2** and **3**), the discrimination of diversity appeared to be more obvious in region II. As no research of microbial diversity associated with planting various transgenic cottons for such a long time has been carried out, further investigation is necessary to clarify this.

Monoculture of one transgenic cotton line had no effect on fungal diversity in comparison with conventional cotton, while fungal population dynamics among CC, TC-10, and TC-15mix were observed. Despite our current understanding of plant-soil community interactions, the mechanism by which *B. thuringiensis* plants drive fungal community dynamics is not well understood. For example, the long-term variation

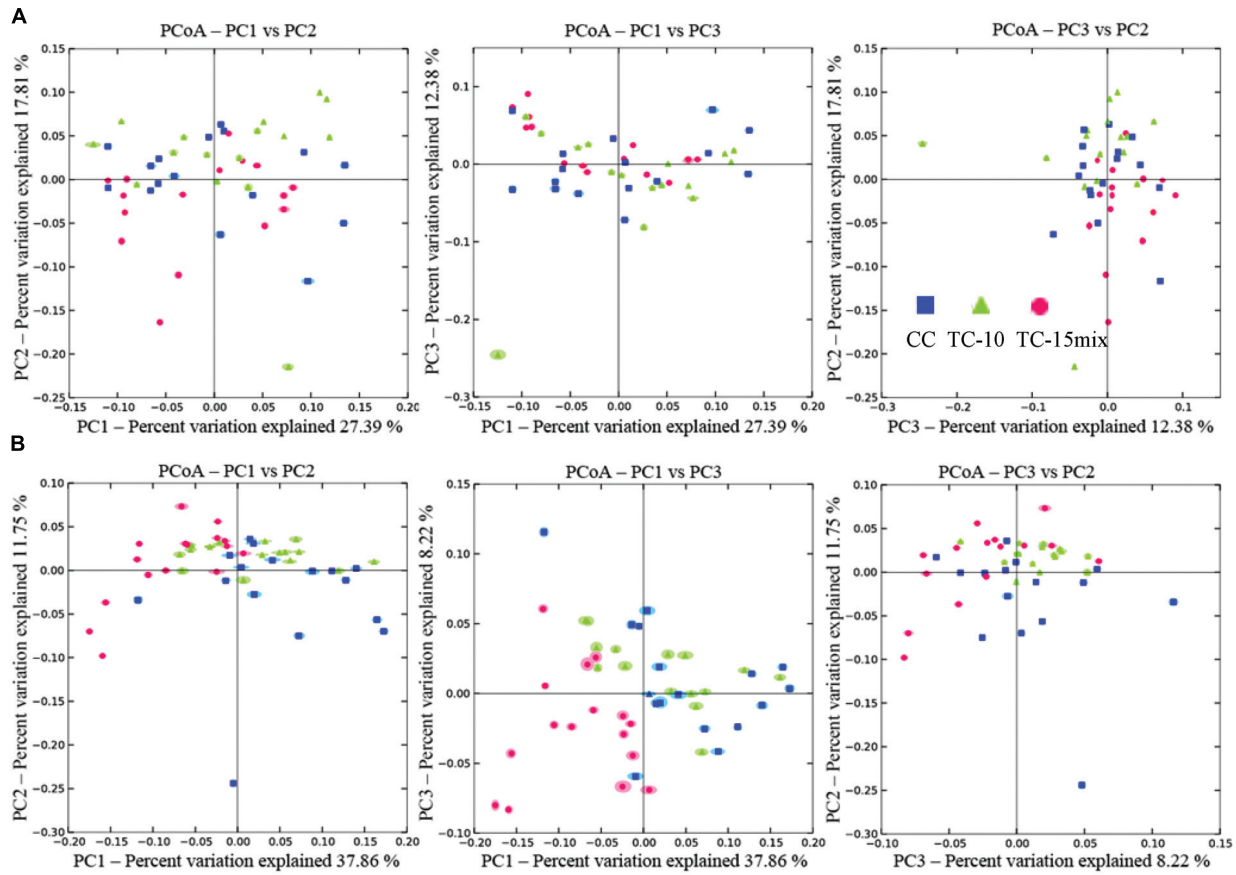

FIGURE 4 | Principal coordinate analysis of soil samples by weighted UniFrac. (A) Region I; **(B)** Region II.

in the plant-specific selection of microbial population in the rhizosphere is unclear. The collected data revealing frequencies of 81% for the dominant taxa showed no obvious tendency among CC, TC-10, and TC-15mix (**Table 2**), while the frequencies of taxa belonging to No. 17 (region I) and 41 (OTU rank order) were highest in TC-15mix and lowest in CC, and the taxa belonging to No.14 and 24 displayed an opposite trend. Similar variations could also be observed in No. 19, 23, 35, and 37 (region II). This indicated such a few taxa may be vulnerable to the plants and soil systems.

The key and indicator taxa are vulnerable to the crop species and may have a crucial role both in clarifying the potential influence of GMPs (Kowalchuk et al., 2003) and in maintaining the soil dynamics. There have been studies on indicators associated with GMPs (Bruinsma et al., 2003; Sarkar et al., 2009; Arango et al., 2014; Cotta et al., 2014), however, the majority of the researches lacked the power to thoroughly estimate rhizosphere microbial diversity and monitor dynamic nature of indicators. In this study, GS-FLX platform gave a better understanding of the dynamic nature of mycorrhizal fungal taxa which were sensitive to disturbances. Myxotrichaceae demonstrated significant differences in abundance between CC and TC ($P < 0.05$), and their cellulolytic ability facilitated the penetration of root cortical cell walls (Dalpé, 1989). In addition, some other families were identified that deserve further investigation (**Figure 2**). Several individual genera with significantly different variations in abundance among CC, TC-10, and TC-15mix were analyzed in **Figure 3**. A similar trendline of variation was observed among CC, TC-10, and TC-15mix within the four seasons. It was suggested that there was a higher abundance of *Verticillium* in TC ($P = 0.018$ for CC vs. TC-15mix, $P = 0.045$ for TC-10 vs. TC-15mix; **Figure 3A**), which is likely to threaten *B. thuringiensis* cotton with wilt diseases (Fradin and Thomma, 2006). This result potentially correlated with the unexpected traits of GMPs reported by several groups, including lower yields and an enhanced susceptibility to pathogens (Pasonen et al., 2004; Zeller et al., 2010). Little information is available for *Geomyces*, except that *Geomyces destructans* is relevant to white-nose syndrome (WNS) in bats (Lorch et al., 2011). *Pestalosphaeria*, a potent plant pathogen, represents the sexual stage of *Pestalotiopsis* (Kang et al., 1999), which causes leaf spots, needle blight and tip blight (Pirone, 1978; Keith et al., 2006). It presents an initial increasing and then decreasing finger period with the exception of Bo. The abundance of *Paracoccidioides* ($P = 0.006$ for CC vs. TC-15mix, $P = 0.039$ for TC-10 vs. TC-15mix) increased from CC to TC-15mix during the sampling periods for S, B, and Bo. The protein TasHyd1 expressed by *Trichoderma* was found to be

harmful to plants, participating in plant root attachment and colonization (Viterbo and Chet, 2006). A Class I hydrophobin from one species of *Paracoccidioides* was found to express the protein Pbhyd1, which is similar to TasHyd1 (Albuquerque et al., 2004). Thus, the increase in *Paracoccidioides* in TC may elevate the disease risk for *B. thuringiensis* cotton. However, *Trichoderma* and *Verticillium* produce bisorbicillinoids that react synergistically with one another and also increase the disease risk of plants (Abe et al., 2000; Harned and Volp, 2011). Interestingly, the abundance of *Paracoccidioides* displayed a trend that was similar to *Verticillium* in different soil types. This finding suggests that *Paracoccidioides* possess a considerable capacity for plant root attachment and cooperative activity with *Verticillium*. Furthermore, *Paracoccidioides brasiliensis*, a human pathogenic fungus, is an etiological agent of paracoccidioidomycosis (Franco, 1987; Felipe et al., 2005). Recent publications provide very little information regarding the functions of *Poitrasia* ($P < 0.002$ for CC vs. TC-10 and for TC-10 vs. TC-15mix in both regions) and *Strobiloscypha*. The abundance of *Sporobolomyces,* a type of phyllosphere fungi, was lowest in TC-15mix during all three sampling stages ($P = 0.001$ for CC vs. TC-15mix). This fungi is beneficial to plants and antagonistic to *Cochliobolus sativus*, *Septaria nodorum* and *Penicillium expansum*, which cause blue mold and storage decay in fruits (Fokkema and Van der Meulen, 1976; Bashi and Fokkema, 1977). The decrease in *Sporobolomyces* in TC-15mix could result in an increase in some pathogenic fungi such as *Paracoccidioides* and *Verticillium*. Regarding the fungi *Alternaria* and *Chaetomium*, a line through the point appeared in TC-10. *Alternaria* species are responsible for at least ten types of plant diseases (Acland et al., 1998; van Wees et al., 2003), and *Chaetomium* reverses the effects of some fungal pathogens such as *Venturia inaequalis* and *Fusarium oxysporum* (Di Pietro et al., 1992; Lee and Hanlin, 1999). Because *Chaetomium* was present in lower amounts in TC-10, a higher abundance of pathogenic *Pestalosphaeria* was detected. A comprehensive view of the patterns of behavior in the indicator taxa in the four sampling seasons revealed almost equivalent tendencies in the variation patterns for each corresponding season. The relative abundance of the communities recovered using each region revealed almost the same dynamic change in CC, TC-10, and TC-15mix at the higher taxonomic level (*Geomyces* and *Poitrasia* in **Figure 3**). These findings revealed the actual variation tendency in the corresponding taxa, with the exception of some individual trends that might be associated with the growth characteristics of a particular taxon. Although changes in the relative abundance of fungi in soil are complex, potential indicators with differences in abundance between conventional and transgenic cottons are valuable for assessing plant-induced perturbations.

CONCLUSION

This study for the first time provided fungal diversity associated with *B. thuringiensis* cottons planted for more than 10 years based on 18S and ITS regions, and monitored the variation in fungal communities over an annual cycle of cotton growth. The diversity indexes and grouping patterns revealed no obvious differences between CC and TC-10, suggesting monoculture of one transgenic cotton cultivar had no effect on fungal diversity in comparison with conventional cotton. However, TC-15mix planting with various transgenic cottons implied dissimilar fungal diversities to those in CC and TC-10, especially in region II. This suggested that variations of microbial diversity may exist among different transgenic cultivars or lines, and the unintended variations between transgenic and conventional cottons may fall into the generally acceptable range. Also, fungal lineages obtained by amplified regions influenced the biodiversity evaluation. Thus further research should devote particular attention to variations among genetic modifications within GMPs and amplified regions. Meanwhile informative indicators might be important for monitoring their local environments.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: GZ and BL. Performed the experiments: XQ, QS, and BZ. Analyzed the data: XQ, QS, YB, and HW. Wrote the paper: LD and XQ. Revised and approved the final version of the paper: GZ and LD.

ACKNOWLEDGMENTS

This research is supported by the National Key Science and Technology Special Project (No.2014ZX08012-005); the National Natural Science Foundation of China (No. 31300706); Jiangsu Provincial Natural Science Foundation (No. BK20151445); General Financial Grant from the China Postdoctoral Science Foundation (No. 2012M512179, No. 2013T60962); the Fundamental Research Funds for the Central Universities (No. 2015ZD008); the Open Project Program of MOE Key Laboratory of Drug Quality Control and Pharmacovigilance (No. DQCP2015MS02) and sponsored by Qing Lan Project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01023

FIGURE S1 | Rarefaction curves for the estimated OTU richness across sampling sites (A) and sampling seasons (B) in the two regions. CC: samples collected from soil amended with conventional cotton. TC-10: samples collected from soil amended with transgenic cotton for 10 years. TC-15mix: samples collected from soil amended with various transgenic cottons for 15 years. S, seeding stage; B, bud stage; Bl, blooming stage; Bo, boll opening stage.

FIGURE S2 | Cluster analysis based on the UniFrac distance of soil samples at a 3% cut-off level. (A) Phylogenetic tree; **(B)** Phylogenetic tree displayed using a circular display. The dot lines showed the similarity cutoff levels to cluster the soil samples into several main groups in **(A)**. In order to exhibit the cluster pattern more clearly, **(A)** was performed as the circle format of **(B)**. The details about sample number were shown in Supplementary Table S1. TC-15mix clustered more closely in the branches of Groups II and III, and Group IV contained TC-15mix samples excluding only one from CC in region I. In Region II CC and TC-10 clustered together in Groups I and III, and TC-15mix were separate from CC.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 30 September 2016
doi: 10.3389/fpls.2016.01487

Screening and Evaluation of the Bioremediation Potential of Cu/Zn-Resistant, Autochthonous *Acinetobacter* sp. FQ-44 from *Sonchus oleraceus* L.

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The quest for new, promising and indigenous plant growth-promoting rhizobacteria and a deeper understanding of their relationship with plants are important considerations in the improvement of phytoremediation. This study focuses on the screening of plant beneficial Cu/Zn-resistant strains and assessment of their bioremediation potential (metal solubilization/tolerance/biosorption and effects on growth of *Brassica napus* seedlings) to identify suitable rhizobacteria and examine their roles in microbes-assisted phytoremediation. Sixty Cu/Zn-resistant rhizobacteria were initially isolated from *Sonchus oleraceus* grown at a multi-metal-polluted site in Shanghai, China. From these strains, 19 isolates that were all resistant to 300 mg·L^{-1} Cu as well as 300 mg·L^{-1} Zn, and could simultaneously grow on Dworkin–Foster salt minimal medium containing 1-aminocyclopropane-1-carboxylic acid were preliminarily selected. Of those 19 isolates, 10 isolates with superior plant growth-promoting properties (indole-3-acetic acid production, siderophore production, and insoluble phosphate solubilization) were secondly chosen and further evaluated to identify those with the highest bioremediation potential and capacity for bioaugmentation. Strain S44, identified as *Acinetobacter* sp. FQ-44 based on 16S rDNA sequencing, was specifically chosen as the most favorable strain owing to its strong capabilities to (1) promote the growth of rape seedlings (significantly increased root length, shoot length, and fresh weight by 92.60%, 31.00%, and 41.96%, respectively) under gnotobiotic conditions; (2) tolerate up to 1000 mg·L^{-1} Cu and 800 mg·L^{-1} Zn; (3) mobilize the highest concentrations of water-soluble Cu, Zn, Pb, and Fe (16.99, 0.98, 0.08, and 3.03 mg·L^{-1}, respectively); and (4) adsorb the greatest quantities of Cu and Zn (7.53 and 6.61 mg·g^{-1} dry cell, respectively). Our findings suggest that *Acinetobacter* sp. FQ-44 could be exploited for bacteria-assisted phytoextraction. Moreover, the present study provides a comprehensive method for the screening of rhizobacteria for phytoremediation of multi-metal-polluted soils, especially those sewage sludge-amended soils contaminated with Cu/Zn.

Keywords: ***Sonchus oleraceus*, plant-growth-promoting rhizobacteria, Cu/Zn-resistant, bioremediation, *Acinetobacter***

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Edited by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *20 April 2016*
Accepted: *20 September 2016*
Published: *30 September 2016*

Citation:
Fang Q, Fan Z, Xie Y, Wang X, Li K and Liu Y (2016) Screening and Evaluation of the Bioremediation Potential of Cu/Zn-Resistant, Autochthonous Acinetobacter sp. FQ-44 from Sonchus oleraceus L. Front. Plant Sci. 7:1487. doi: 10.3389/fpls.2016.01487

INTRODUCTION

Heavy metal pollution of soils has become a global environmental concern. Even essential biological trace elements, such as Zn and Cu, can be toxic or lethal to organisms at high concentrations (Ouzounidou, 1995). Unlike organic compounds, heavy metals in soils cannot be mineralized or broken down to less toxic forms (Chen et al., 2014). A large proportion of heavy metals are generally bound to organic and inorganic soil components or exist as insoluble precipitates, and are thus unavailable for root uptake by field-grown plants (Raskin et al., 1994). Therefore, developing appropriate strategies for the remediation of heavy-metal-polluted soils demands urgent attention from the perspectives of environmental conservation and human health (Aboushanab et al., 2006).

Phytoremediation, an emerging, challenging, and solar-driven *in situ* technology with lower cost and enhanced environmental friendliness in comparison to conventional physicochemical technologies, has received increasing attention from ecological researchers (Kumar et al., 1995). However, this plant-based technique is generally time-consuming, because most hyperaccumulators identified thus far are generally small-biomass and slow-growing (Rajkumar and Freitas, 2008a). Moreover, its efficiency is often limited by the metal bioavailability in soil, plant roots development, and plant tolerance to a particular metal (Pilon-Smits, 2005). Thus, developing alternative strategies that can improve the efficiency of phytoremediation are necessary.

Several researchers have suggested biotechnological approaches and proposed to incorporate plant-associated microorganisms (rhizospheric, endophytic bacteria, and mycorrhizal fungi) into phytoextraction systems (Rajkumar and Freitas, 2008a; Ma et al., 2009a; Sessitsch et al., 2013). In such systems, the plants and rhizosphere are two key factors that make phytoremediation a viable *in situ* technology. On one hand, the plants to be used for remediation of metal-polluted soils must be qualified with tolerance to at least one metal, high competitiveness, fast growth, and large biomass (Glick, 2010). On the other hand, the rhizosphere, as an important soil-plant interface, provides a complex dynamic microenvironment where root-associated microorganisms form unique communities that have a high potential to detoxify hazardous waste compounds (De Souza et al., 1999; Alford et al., 2010). Moreover, the particular microbial community with high activity and large contact area probably acts as a source of microbial chelates (Kärenlampi et al., 2000). Thus, the microorganism-assisted phytoremediation potential, as well as the mechanisms by which rhizobacteria enhance phytoremediation efficiency, has been attracting increasing research interest lately.

Among the plant-associated microbes, plant growth-promoting rhizobacteria (PGPR) are considered a major component of phytoremediation technology (Glick, 2003). They have capacity of plant growth-promoting (PGP) and improving phytoremediation by various mechanisms, including: fixation of atmospheric nitrogen, utilization of 1-aminocyclopropane-1-carboxylic acid (ACC), production of siderophores and antipathogenic substances, production of plant growth regulators, transformation of nutrient elements (Glick et al., 1999), bacteria-induced metal chelation (Adediran et al., 2015), and synthesis of cysteine-rich peptides (Adediran et al., 2016). Thus, inoculation with metal-resistant PGPR, particularly indigenous PGPR (Kozdrój et al., 2004), can improve the efficiency of heavy metal phytoremediation (Ma et al., 2011; Rajkumar et al., 2012). Therefore, researchers need to isolate and screen competitive and effective PGPR (Paau, 1989) that are well adapted to the conditions of a particular site (Sheng and Xia, 2006). Although PGPR play important roles in phytoremediation strategies, studies on Cu/Zn-resistant PGPR in this area remain very limited (Lucy et al., 2004), particularly field studies. Thus, more laboratory and field studies are needed to advance existing research.

Sonchus oleraceus is a cosmopolitan weed species native to Europe and central Asia (Hutchinson et al., 1984) that grows readily and adapts to diverse environments in many countries (Holm et al., 1977). In China, *S. oleraceus* is also widely distributed as an annual and roadside pioneer plant. It is one of few species found at disrupted locations, such as oil well sites in oilfields and barren lands (Xiong et al., 1997). Furthermore, *S. oleraceus* is regarded as the most suitable candidate for the removal of Zn and Cd from soils (Khan et al., 1998).

Despite numerous reports about rhizobacteria-enhanced phytoremediation of heavy metals (Sheng and Xia, 2006; Dell'Amico et al., 2008; Płociniczak et al., 2016), little information is available about effects Cu/Zn-resistant bacteria from the rhizosphere of *S. oleraceus* on plant growth and heavy metal bioavailability/biosorption in multi-metal-polluted soils. Thus, the quest for novel, beneficial and indigenous rhizobacteria among different plant species grown in multi-metal-polluted environments is very meaningful. In addition, to assess the potential rhizospheric mechanisms underlying the effects on plant growth and uptake and translocation of heavy metals, we explored the biochemical characteristics [production of indole-3-acetic acid (IAA), ACC deaminase (ACCD), and siderophores; and solubilization of inorganic phosphate] of selected bacteria. Furthermore, diverse genera of PGPR could affect plant growth in different ways, because the PGP effect could be plant- and/or PGPR-specific. Thus, our main objectives were to: (1) isolate and preliminarily screen Cu/Zn-resistant and ACCD-containing bacteria from the rhizosphere of *S. oleraceus* grown in multi-metal-polluted soils; (2) select indigenous PGPR with superior PGP traits that could effectively increase plant biomass under unfavorable conditions; and (3) evaluate the bioremediation potentials of different PGPR (Cu/Zn/Pb/Cd/Fe-solubilization, Cu/Zn-tolerance/biosorption and effects on the growth of rape) to identify more-suitable rhizobacteria and examine the effects of selected bacteria on plant growth and metal uptake/translocation in *Brassica napus* via sand culture experiments.

MATERIALS AND METHODS

Sampling, Treatment, and Characterization of Soils and Plants

Soils were randomly sampled from a depth of 0–20 cm in the Jiading Wastewater Disposal Plant (31°22′32″ N, 121°09′57″ E), located at Shanghai, China. The soils used in this study were mixtures of sewage sludge and waste residue, and contaminated with multiple heavy metals. Before the experiments commenced, soil samples pretreatmented were air-dried for 1 month and sieved (4 mm) to remove as many plant materials, soil macrofauna, and stones as possible. The soil subsamples were then passed through a 2-mm stainless steel sieve, and subjected to physicochemical chatracterization according to standard methods (Lu, 1999), some of which are listed in **Table 1**.

Native *in situ S. oleraceus* plants were also randomly selected from the same wastewater disposal plant at which the multi-metal-polluted soils were collected. Soon after returning to the laboratory, the rhizospheric soils of *S. oleraceus* (2 cm radius around the roots) were collected by gently shaking the roots (Wenzel et al., 2003) to remove loosely attached soils and stored in a refrigerator at 4°C until further use.

Isolation and Preliminary Screening of Cu/Zn-Resistant and ACC-Utilizing Rhizobacteria

Rhizobacteria were isolated from *S. oleraceus* according to the protocol of Jiang et al. (2008). Sixty pure isolates were initially isolated and stored in 30% (v/v) glycerol at –80°C until further analysis (Wei et al., 2009). Viable bacterial populations, including total and resistant bacteria were counted by the plate count method. The CFU/g of fresh soil is presented in **Table 1**. After isolation, all isolates were further streaked on two Luria–Bertani medium (LB) agar plates containing either Zn or Cu (100 to 500 mg·L^{-1}, respectively) and monitored for growth. All plates were incubated in triplicate at 30°C for 48 h.

In order to obtain Cu/Zn-resistant PGPR, 46 isolates that were all simultaneously resistant to 300 mg·L^{-1} Cu and 300 mg·L^{-1} Zn were further tested for their ability to grow on Dworkin–Foster (DF) salt minimal medium containing ACC (denoted ADF) as a sole nitrogen source (Dworkin and Foster, 1958). The DF medium containing $(NH_4)_2SO_4$ (Rajkumar and Freitas, 2008b) (denoted NDF) and without a nitrogen source were used as controls. We also analyzed the ACCD activity of cell-free extracts analyzed by quantifying the amount of α-ketobutyrate according to a modified method of Honma and Shimomura (1978). After preliminary screening, 19 isolates that were resistant to both 300 mg·L^{-1} Cu and 300 mg·L^{-1} Zn, and simultaneously growing on ADF were selected for further evaluation of PGP parameters (secondary screening).

TABLE 1 | Physicochemical and microbiological properties of the tested soils.

Parameter	Data (means ± SE, $n = 3$)
Soil texture	Sandy loam soil
pH (H_2O, 1:2 w/v)	7.71 ± 0.05
Cation exchange capacity (cmol·kg^{-1})	15.26 ± 0.03
Organic matter (%)	2.63 ± 0.02
Electric conductivity at 25°C (mS·cm^{-1})	3.04 ± 0.04
Total N (mg·kg^{-1})	1620.35 ± 113.00
Total Fe (mg·kg^{-1})	297.10 ± 0.13
Total Zn (mg·kg^{-1})	1263.78 ± 0.43
Total Pb (mg·kg^{-1})	153.26 ± 0.05
Total Ni (mg·kg^{-1})	65.21 ± 0.02
Total Cr (mg·kg^{-1})	206.61 ± 0.11
Total Cd (mg·kg^{-1})	3.03 ± 0.01
Total Hg (mg·kg^{-1})	0.31 ± 0.03
Total Cu (mg·kg^{-1})	650.10 ± 0.21
Total As (mg·kg^{-1})	11.54 ± 0.03
Total cultivable bacterial count[a]	$2.55 \pm 3.28 \times 10^{11}$
Zn-resistant bacterial count	$9.63 \pm 2.08 \times 10^{8}$
Cu-resistant bacterial count	$7.79 \pm 7.56 \times 10^{9}$

[a]*Expressed as colony-forming units (CFU) per gram of fresh soil.*

Evaluation of PGP Properties

Synthesis of IAA by the 19 isolates was quantified as described by Bric et al. (1991), using LB broth supplemented with 0.5 mg·mL^{-1} L-tryptophan. The IAA concentrations were calculated using a calibration curve of pure IAA as the standard (Sigma, USA). Bacterial siderophore production was detected and quantified by the chrome azurol S (CAS) analytical method (Schwyn and Neilands, 1987). According to this assay, the siderophore levels were defined as the A/A_r ratio and a smaller A/A_r ratio indicated higher siderophore output (Sheng et al., 2008). The phosphate-solubilizing ability of the isolates was analyzed in Pikovskaya's medium (Pikovskaya, 1948) supplemented with 0.5% tricalcium phosphate. The soluble phosphate in the supernatant was quantified by the Mo-blue method (Watanabe and Olsen, 1965). After secondary screening, 10 functional strains with superior PGP traits were selected for further evaluation of bioremediation potential (the third screening).

Evaluation of Bioremediation Potential by Functional Strains

Activation of Soil Metals by Functional Strains

Batch experiments on the effects of the 10 functional isolates on metal mobility in soil were conducted in triplicate 50-mL scaled polypropylene centrifuge tubes according to Chen et al. (2005). Briefly, pure cultures of functional strains were centrifuged at 8000 rpm for 10 min after 20 h of growth, washed twice in phosphate buffer (pH 7.0), and re-suspended in sterile distilled water. One milliliter of each washed bacterial suspension ($OD_{600} = 1.0 \pm 0.05$) or sterile water (control) was added to the 1 g of autoclaved soils. All tubes were weighed, wrapped in brown paper, and placed on an orbital shaker at 180 rpm and 28°C. After 1 week, the tubes were weighed again to compensate for evaporation. Sterile water (10 mL) was then added to extract water-soluble metals. The soil suspensions were vibrated at 25°C for 2 h and centrifuged at 10,000 rpm for

10 min. The resulting supernatants were filtered through a 0.22-μm membrane filter for determination of pH and water-soluble Cu/Zn/Pb/Cd/Fe. The metal concentrations were determined by inductively coupled plasma-mass spectrometry (ICP-MS, SPECTRO).

Minimum Inhibitory Concentration (MIC) of Functional Strains

To check the extent of resistance, we used the secondly selected isolates to determine the lowest concentration of Cu and Zn that completely inhibited the growth of bacterial strains, termed as the minimum inhibitory concentration (MIC). Isolates were streaked in triplicate on LB agar media supplemented with varying concentrations (600 to 1000 mg·L^{-1}) of Cu and Zn, respectively. For each strain and each metal, the lowest concentration that inhibited visible growth at 28°C within 3 days was determined.

Metal Biosorption Analyses

The biosorption of Cu and Zn by bacterial cells was evaluated as described by Hernández et al. (1998) with some modifications. Bacterial cells obtained from the bacterial cultures (grown in LB broth at 28°C, $OD_{600} = 1.0 \pm 0.05$) were harvested by centrifugation at 8000 rpm for 20 min and washed twice with sterile deionized water. The harvested cells were re-suspended in 150 mg·L^{-1} of Cu or Zn. An uninoculated solution was used as the control. After incubation at room temperature for 10 h, the cells were harvested following centrifugation and the residual metal ions in the supernatant were measured using a flame atomic absorption spectrophotometer (Varian Spectra model AA240FS; USA). The amount of metal absorbed by the bacterial cells was calculated by subtracting the metal concentration in the supernatant from the original concentration.

In vivo Plant Growth Promotion Assay

Growth promotion of the secondly selected isolates was tested according to Patten and Glick (2002) with some modifications. Seeds of *B. napus* var. Zhongyou-1 were surface-sterilized with a mixture of absolute ethanol and 30% hydrogen peroxide (1:1, v/v) for 20 min, and washed twice with sterile distilled water before being transferred to sterile filter paper in a Petri dish. Seed sterility was monitored by incubating the seeds on LB agar at 30°C and aseptically placed on moistened filter paper. Then 6 mL of each bacterial suspension ($OD_{600} = 0.5 \pm 0.02$) or sterile distilled water (uninoculated control) was added to glass Petri dishes with two-double filter paper. After incubation of closed Petri dishes for 7 days at 28°C in the dark, the root length, shoot length, fresh weight, and number of seedlings that had sprouted within 3 days were determined. The assay was performed twice with two dishes (10 seeds per dish) for each treatment. After the third screening, among the 10 functional strains, S44 with the highest bioremediation potentials was selected for genetic identification.

Genetic Identification of S44

Genomic DNA of S44 was extracted as per a previously reported protocol (Araújo et al., 2002), and used as a template in 16S rDNA PCR amplification with universal primers 27F (5′-GAGTTTGATCACTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′) (Byers et al., 1998). PCR amplification was performed in a DNA Engine Thermal Cycler (PTC-200, BioRad, USA) under the reaction conditions described by (Branco et al., 2005). The amplified product was purified with a DNA Purification Kit and sequenced at HuaDa Biotechnology Company (Shanghai, China). The partial 16S rDNA sequences obtained were matched with nucleotide sequences in GenBank using the BLAST tool[1]. Neighbor joining phylogenetic trees were constructed after calculation of a maximum composite likelihood distance matrix using the MEGA 4.0 software (Tamura et al., 2007).

Sand Culture Experiment

Based on the results of the third screening, the *Acinetobacter* sp. FQ-44 was selected for preliminarily exploring roles of the plant-rhizobacteria partnership in heavy metal remediation. Surface-sterilized seeds of *B. napus* were pregerminated on sterile filter paper in a Petri dish. After germination (4 days), uniform seedlings were selected and soaked for 2 h in the bacterial culture (OD_{600} of 1.0 ± 0.05) or sterile water (control). Six seedlings were subsequently transplanted into a plastic pot (top diameter 85 mm, bottom diameter 65 mm, and height 105 mm) containing sterilized vermiculite and saturated with sterile half-strength Hoagland's nutrient solution (Barac et al., 2004). One week after transplantation, seedlings were thinned to three per pot and subjected to various concentrations of Cu (2, 5, and 10 mg/L). Three replicates were conducted for each treatment. The plantlets were allowed to grow under greenhouse conditions (25 ± 5°C, 16:8 day/night regime). After 45 days, plants were carefully removed from the pots and root surfaces were immersed in 0.01 M EDTA for 30 min, and then rinsed thoroughly with deionized water to remove any surface adsorbed metals. Fresh and dry weights were measured and the concentrations of Cu in roots and shoots were determined using a flame atomic adsorption spectrophotometer. The translocation factor (TF) was calculated as the ratio of metal concentration in the shoots to that in the roots (Liu et al., 2009) and the bioaccumulation factor (BCF) was calculated as the ratio of metal contents in the entire plant to that in the soil (Bu-Olayan and Thomas, 2009).

Statistical Analyses

Results for each treatment were expressed as means ± SD. Significant differences between parameters were tested using the *post hoc* Fisher's protected least significant difference (LSD) test after one-way ANOVA. All statistical analyses, including the Pearson's correlation analysis, were conducted using SPSS 18.0 (SPSS Inc., USA). Unless otherwise indicated, significant level was set at $P < 0.05$. Graphical analyses were performed on SigmaPlot 11.0 (Jandel Scientific, USA).

[1]www.ncbi.nlm.nih.gov

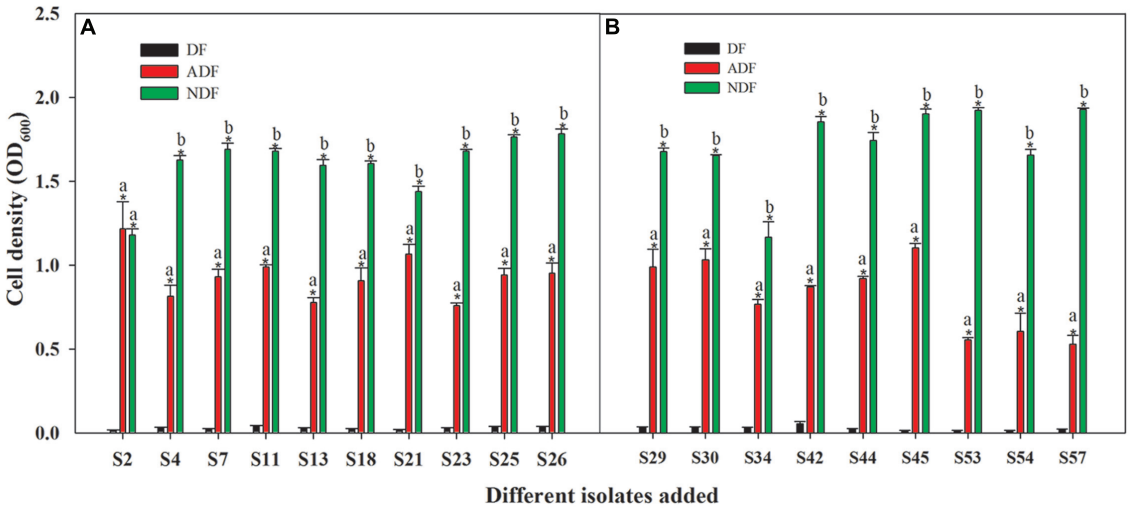

FIGURE 1 | Capacity for growth of 19 Cu/Zn-resistant isolates on ADF (A,B). Different small letters denote significant differences between treatments and an asterisk (*) represents a significantly greater value on ADF and NDF compared to DF ($P < 0.05$).

RESULTS AND DISCUSSION

Isolation and Preliminary Screening of Cu/Zn-Tolerant and ACC-Utilizing Rhizobacteria

Before preliminary screening and identification, 60 cultivable isolates that were simultaneously resistant to 50 mg·L^{-1} of Zn and 50 mg·L^{-1} Cu, were isolated initially from the rhizosphere of *S. oleraceus* and named S1–S60. These bacterial isolates were autochthonous to the metal-polluted site and were thus more suitable for *in situ* phytoremediation of the multi-metal-polluted soils. As reported, rhizobacteria isolated from multi-metal-polluted natural environments can be constitutively or adaptively resistant to increasing metal concentrations, as they have adapted to such environments (Nies, 2003).

Soil microbes with generally higher metal resistance are the preferred choice for phytoremediation studies. Our results indicate that most of the isolates tested were resistant to different concentrations of Zn and Cu (Supplementary Table S1). Among all isolates, 34 were simultaneously resistant to 400 mg·L^{-1} Zn and 400 mg·L^{-1} Cu, among which some were even tolerant of 500 mg·L^{-1} Zn or Cu; whereas 46 isolates were able to simultaneously resist 300 mg·L^{-1} Zn and 300 mg·L^{-1} Cu. To obtain more plant beneficial strains, these 46 isolates were selected for further testing of their ACC utilization ability.

Among those 46 isolates, 19 isolates grew significantly better on ADF and NDF than on DF ($P < 0.05$) (**Figure 1**). Although these isolates grew well on ADF and NDF, their growth without a nitrogen source was limited (**Figure 1**). Thus, these 19 rhizobacteria had the potential to utilize ACC as a sole nitrogen source. Moreover, they had the ability to grow on ADF to produce ACCD (**Figure 1**; Supplementary Table S2), which was supported by earlier observations that ACC-utilizing bacteria could generally produce ACCD. As reported, ACC-utilizing bacteria have been found to facilitate plant growth by producing ACCD that hydrolyzes the ethylene precursor ACC into α-ketobutyrate and ammonia (Glick, 2005) in the presence of salts or heavy metals (Belimov et al., 2005; Zahir et al., 2009). Consequently, these ACC-utilizing isolates could be important for PGPR-mediated phytoremediation.

Screening of Functional Strains with Superior PGP Ability

Various PGP characteristics could contribute to reduced metal stress and increased growth in their host plants (Ma et al., 2011; Rajkumar et al., 2012). In our study, all 19 ACC-utilizing isolates had inherent abilities of IAA production, siderophore production, and insoluble phosphate solubilization (Supplementary Table S2). Out of 19 isolates, 10 with superior PGP traits were selected for statistical analyses (**Table 2**), because each had three indices that were all ranked in the top 10.

As shown in **Table 2**, S44, the best IAA producer (29.57 mg·L^{-1}) in our study, produced significantly more IAA than the other nine strains ($P < 0.05$). As reported similarly, *Enterobacter ludwigii* BNM 0357 released about 30 μg IAA mL^{-1} (Shoebitz et al., 2009). In addition, the IAA production abilities of all 10 isolates might be within a reasonable range for observable PGP effects (Ma et al., 2009b) that might contribute to increased plant biomass. As reported, a low IAA production by PGP bacteria promotes primary root elongation, whereas a high level inhibit primary root growth (Xie et al., 1996). Our rape inoculation experiments also indicated the 10 moderate IAA producers were able to increase root length, which was generally promoted by IAA-producing rhizobacteria (Patten and Glick, 1996). Moreover, Pearson's correlation analysis also revealed that IAA was significantly positively correlated with the fresh weight of seedlings ($r = 0.70$, $P = 0.02$).

TABLE 2 | PGP features of functional strains and pH of solutions in the phosphate solubilization experiment.

Functional strains	IAA synthesis (mg·L^{-1})	Siderophore production (A/A_r)[a]	Phosphate solubilization (mg·L^{-1})[b]	pH
S21	10.55 ± 0.08d	0.13 ± 0.01ab	53.34 ± 1.29b	6.34 ± 0.18a
S23	6.48 ± 0.24bc	0.29 ± 0.01bcd	34.21 ± 1.06a	7.42 ± 0.03d
S25	7.01 ± 0.58c	0.40 ± 0.09d	36.36 ± 1.41a	6.97 ± 0.10bc
S26	3.45 ± 0.29a	0.25 ± 0.05abcd	35.00 ± 0.96a	7.17 ± 0.23cd
S29	4.42 ± 0.15ab	0.10 ± 0.01a	39.42 ± 1.38a	7.39 ± 0.03d
S30	5.10 ± 0.16abc	0.12 ± 0.05a	35.45 ± 1.06a	7.39 ± 0.07d
S42	20.17 ± 0.26e	0.34 ± 0.09cd	39.76 ± 0.82a	7.52 ± 0.01d
S44	29.57 ± 0.95g	0.29 ± 0.04bcd	74.75 ± 1.48c	6.76 ± 0.03b
S45	25.15 ± 0.56f	0.34 ± 0.02cd	55.38 ± 1.41b	7.19 ± 0.08cd
S57	9.81 ± 0.20d	0.23 ± 0.02abc	55.81 ± 1.55b	7.41 ± 0.01d

Data of columns by the same letter are not significantly different between bacterial treatments according to the Fisher's protected LSD test ($P > 0.05$).
[a]*Siderophore production: little, 0.8–1.0; low, 0.6–0.8; moderate, 0.4–0.6; high, 0.2–0.4; very high, 0–0.2.*
[b]*Concentration of phosphorus.*

TABLE 3 | Correlations between solubilization factors and water-soluble heavy metals.

Correlations	Solubilization factors			Water-soluble heavy metals				
	pH	Phosphate solubilization	Siderphores	Cu	Zn	Pb	Cd	Fe
pH	1.00	−0.10	−0.42	0.47	−0.57	−0.06	−0.51	0.13
Phosphate solubilization	−0.10	1.00	0.07	0.44	0.50	0.47	0.26	0.54
Siderphores	−0.42	0.07	1.00	0.05	0.67	0.45	0.24	.08
Cu	0.47	0.44	0.05	1.00	0.39	0.06	−0.22	0.49
Zn	−0.57	0.50	0.67	0.39	1.00	0.31	0.34	0.37
Pb	−0.06	0.47	0.45	0.06	0.31	1.00	−0.24	0.00
Cd	−0.51	0.26	0.24	−0.22	0.34	−0.24	1.00	0.61
Fe	0.13	0.54	0.08	0.49	0.37	0.00	0.61	1.00

Data of columns are pearson's correlation coefficient.

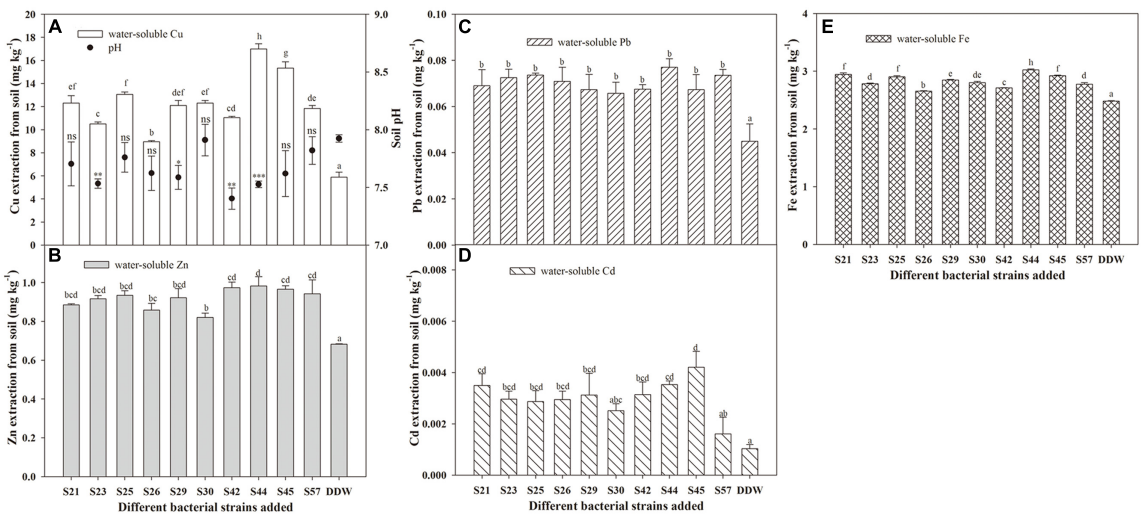

FIGURE 2 | Effects of inoculation with 10 isolates on mobilization of Cu (A), Zn (B), Pb (C), Cd (D), and Fe (E) in autoclaved soil. Values are expressed as means ± SE, $n = 9$. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns, no significant difference. Different letters above the bar indicate significant differences among treatments at the level of $P < 0.05$ according to the Fisher's protected LSD test.

TABLE 4 | MIC of the secondly selected rhizobacteria.

Metals	MIC (mg·L^{-1})									
	S21	**S23**	**S25**	**S26**	**S29**	**S30**	**S42**	**S44**	**S45**	**S57**
Zn	400	600	500	700	500	600	800	800	800	700
Cu	600	800	700	900	800	800	1000	1000	1000	800

Siderophores, another important PGPR-released metabolites, indirectly alleviate heavy metal toxicity by increasing the supply of iron to plants (Burd et al., 2000), thereby facilitating plant growth. In our study, siderophore production was highest in S29 among the 10 isolates, whereas it was lowest in S25 (**Table 2**). Furthermore, siderophores were responsible for the mobilization of insoluble metals such as Fe (**Table 3**) and were positively correlated with three growth parameters (Supplementary Table S3), although this was not significant ($P > 0.05$). Our results concurring with the earlier observations also show that siderophores produced by rhizosphere microorganisms could supply iron to plants via Fe-siderophore complexes under iron-limited conditions (Crowley et al., 1988) and inoculation with a siderophore-producing strain promotes plant growth (Tripathi et al., 2005).

Another crucial PGP mechanism is phosphate solubilization, through which microbes enhance P availability to the host plant and thereby contribute to plant–bacteria interactions and PGP effects in metal-polluted soils (Zaidi et al., 2006). Our findings indicate that phosphate solubilization was positively correlated with all growth parameters ($r = 0.45$, 0.18, and 0.51 for root length, shoot length, and fresh weight, respectively) (Supplementary Table S3). Moreover, Rajkumar et al. (2009) also reported that phosphate solubilization in the rhizosphere greatly contributes to the PGP effects of bacteria. In addition, the highest phosphate-solubilizing ability was also observed in S44 (74.75 mg·L^{-1}), which was significantly higher than other nine isolates ($P < 0.05$, **Table 2**).

The foregoing analyses indicate that these isolates were able to facilitate the growth of *B. napus* probably through these PGP traits. Consequently, the screening of soil bacteria with superior PGP abilities in a multi-metal-polluted environment is one key step in phytoremediation studies.

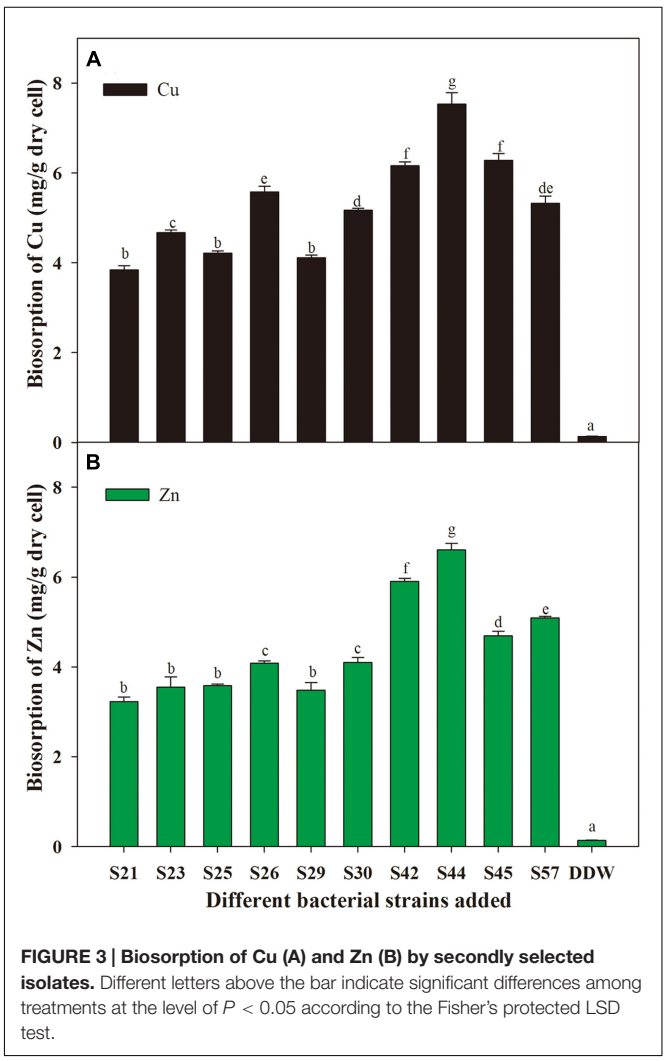

FIGURE 3 | Biosorption of Cu (A) and Zn (B) by secondly selected isolates. Different letters above the bar indicate significant differences among treatments at the level of $P < 0.05$ according to the Fisher's protected LSD test.

Final Choice of S44

Effects of Functional Strains on the Mobility of Soil Metals

Besides PGP traits, successful phytoremediation also depends mainly on metal bioavailability in the soil (Shallari et al., 2001). Therefore, to obtain effective metal-mobilizing strains, we further evaluated the ability of 10 isolates to increase water-soluble Cu, Zn, Cd, Pb, and Fe concentrations in soils. As expected, the presence of bacteria resulted in increased concentrations of water-extractable Cu, Zn, Pb, Cd, and Fe in autoclaved soil compared to axenic soil (**Figure 2**). These results suggest that the 10 Cu/Zn-resistant isolates had metal-solubilizing potential in heavy metal-polluted soil, thereby increasing metal bioavailability. As reported, Soil microorganisms can affect metal mobility and availability via the release of siderophores (Braud et al., 2009) and solubilization of metal phosphates (Aboushanab et al., 2006). Our results also indicate that siderophore and phosphate solubilization were both positively correlated with concentrations of water-soluble Cu, Zn, Pb, Cd, and Fe (**Table 3**).

Although all 10 isolates had the potential to facilitate the release of non-labile-phase Cu, Zn, Cd, Pb, and Fe from sterile soils, their effects actually differed (**Figure 2**). For example, the greatest amounts of water-soluble Cu, Zn, Pb, and Fe released in the soil were all found in S44, which were 16.99, 0.98, 0.08, 3.03 mg·L^{-1}, respectively, but that of water-soluble Cd was observed in S45. Moreover, inoculation with S44 significantly

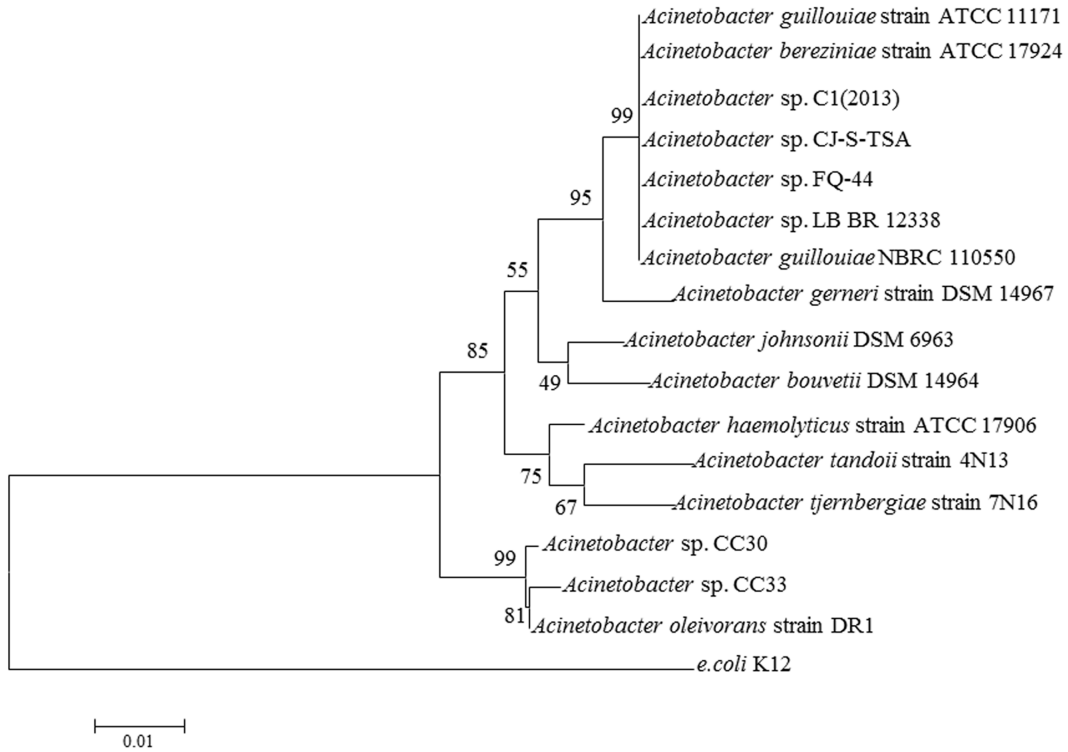

FIGURE 4 | Neighbor joining phylogenetic tree analysis of *Acinetobacter* sp. FQ-44 with closely related strains from GenBank and relevant reports. The scale bar represents 0.01 substitutions per site.

TABLE 5 | Growth parameters of *Brassica napus* L. seedlings in sterile filter paper following infection with 10 isolates.

Functional strains	Root length (cm)	Shoot length (cm)	Fresh weight (mg)[a]	Germination (%)	Vigor index[b]
Control	4.19 ± 0.58a	5.58 ± 0.12ab	73.40 ± 0.52a	80.00	7.85 ± 0.09a
S21	5.27 ± 0.66ab	6.44 ± 0.51abc	86.60 ± 1.39abc	82.50	9.63 ± 0.24b
S23	5.59 ± 0.55abc	7.52 ± 0.41c	94.90 ± 1.82bcd	80.00	10.49 ± 0.15bc
S25	5.37 ± 0.72ab	6.73 ± 0.79abc	82.10 ± 2.09ab	85.00	10.29 ± 0.20bc
S26	6.34 ± 0.71bcde	5.26 ± 0.28a	87.90 ± 0.96bc	90.00	10.44 ± 0.24bc
S29	6.37 ± 0.57bcde	7.56 ± 0.59c	97.10 ± 0.78bcd	87.50	12.19 ± 0.15de
S30	7.07 ± 0.34bcde	7.05 ± 0.83bc	93.13 ± 1.94bcd	87.50	12.35 ± 0.23ef
S42	7.37 ± 0.54cde	7.88 ± 0.55c	100.83 ± 1.79cd	77.50	11.82 ± 0.14cde
S44	8.07 ± 0.31e	7.31 ± 0.24c	104.20 ± 1.06d	90.00	13.84 ± 0.11fg
S45	5.84 ± 0.40abcd	6.68 ± 0.47abc	100.10 ± 1.30cd	85.00	10.63 ± 0.12bcd
S57	7.68 ± 0.55*de*	7.96 ± 0.32c	95.60 ± 1.31bcd	90.00	14.08 ± 0.14h

Values are expressed as means ± SE, n = 10. Different letters in the same column indicate significant differences among treatments at the level of $P < 0.05$ according to the Fisher's protected LSD test.

[a]*Fresh weight of seedling.*

[b]*Vigor index = germination (%) × seedling length (root length + shoot length).*

increased the concentrations of water-soluble Cu, Zn, Pb, Cd, and Fe in soil by 1.88-, 0.44-, 0.71-, 2.50-, and 0.22-fold, respectively, compared to the control. Furthermore, the soil pH following inoculation with S44 dropped significantly compared to the control ($P < 0.05$; **Table 2**).

In addition, mobilization characteristics differed among the metals (**Figure 2**), which could be explained by the physicochemical properties of the various metals, metal-microbe interactions, as well as the unordered competition between metals. However, some isolates, such as S44 and S45, that exhibited high mobilization of one metal, were also remarkably capable of mobilizing other metals.

MIC of Functional Strains

The preliminary resistance results showed that some isolates were able to grow in higher concentrations of all tested metals.

Thus, to determine the extent of resistance, we assessed the Cu and Zn MICs of the secondly selected isolates. Our toxicity tests show that S26, S42, S44, S45, and S57 tolerated relatively high levels of Cu and Zn (**Table 4**). Moreover, among the 10 functional strains, S42, S44, and S45 had the highest Cu (1000 mg·L^{-1}) and Zn (800 mg·L^{-1}) MICs. This high tolerance of Cu and Zn could be attributed to the fact that these bacteria were isolated from the sewage-amended soils containing high levels of Cu and Zn. However, strain S21 was less tolerant of Cu (600 mg·L^{-1}) and Zn (400 mg·L^{-1}). In addition, the present results also indicate that Zn was more toxic to the isolates than Cu, which was different from some previous studies (Hassen et al., 1998; Jiang et al., 2008; Guo et al., 2011).

Metal Biosorption Potential of Secondly Selected Isolates

With respect to microbial remediation, it is very important to determine whether selected bacteria have the capacity for metal uptake. Our results indicate that different isolates exhibited different capacities for biosorption of the metal ions tested (**Figure 3**). Moreover, S44 exhibited the highest potentials to remove Cu (7.53 mg·g^{-1} dry cell) and Zn (6.61 mg·g^{-1} dry cell), and absorbed significantly more Cu and Zn than the other nine isolates ($P < 0.05$). Thus, application of the effective metal-solubilizing/absorbing S44 would be helpful for improving microbe-assisted phytoremediation. As reported, the biosorption capacity of bacteria plays an important role in reducing metal phytotoxicity by limiting the entry of metal ions into plant cells, and might contribute to enhanced plant growth in metal-contaminated soils (Ma et al., 2011). Furthermore, it should be noted that the biosorption ability for Cu was higher than that for Zn (**Figure 3**). One possible explanation could be that Cu (0.72 Å) with smaller ionic radius might be more rapidly complexed by bacterial cell wall/membrane compared to Zn (0.88 Å) (Karakagh et al., 2012). Another explanation probably was that Zn was more toxic to these isolates than Cu.

Effects of Functional Strains on Rape Growth

After the 10 representative isolates infecting sterile *B. napus* L. seeds, seed germination was neither significantly inhibited nor stimulated. For example, seed germination after inoculation with S23 was equal to that of the control (**Table 5**).

A deeper understanding of plant-microbe interactions is complicated, but applicable to microbe-assisted phytoremediation. In our study, seeds inoculated with the various isolates all had longer roots compared to the control (**Table 5**). Moreover, the most significant increase in root length was observed with S44 (92.60%, $P < 0.05$). Although the maximum shoot elongation was observed with S57, inoculation with S44 significantly increased shoot length by 31.00% ($P < 0.05$), compared to the control. Furthermore, the maximum promoting effect on fresh weight was also observed with S44, showing a significant increase by 41.96% ($P < 0.05$; **Table 5**). In addition, the highest seed vigor index was observed with S57 followed by S44 and S30, all exhibiting

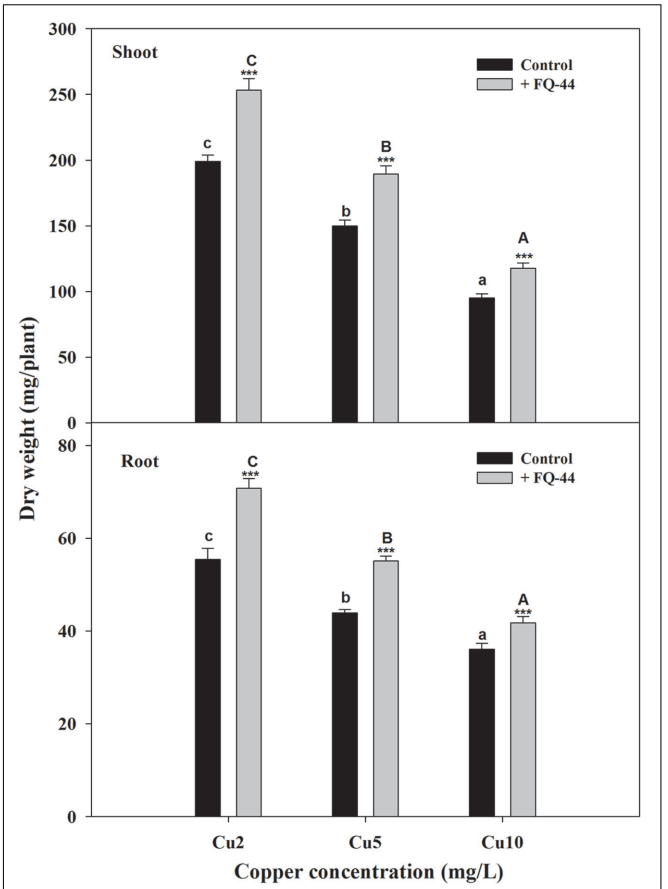

FIGURE 5 | Effects of FQ-44 on dry weight of root and shoot in *Brassica napus* cultivated in different concentrations of Cu. Values are expressed as means ± SE, $n = 9$, ***$P < 0.001$. Different letters above the bar indicate significant differences within the same microbial treatments (control and inoculation) at the level of $P < 0.05$, according to the Fisher's protected LSD test. Cu2, 2 mg/L Cu; Cu5, 5 mg/L Cu; Cu10, 10 mg/L Cu.

significant effects ($P < 0.05$). The foregoing results indicate that S44 has higher potential to facilitate the growth of *B. napus*.

Although the selected isolates showed PGP effects, these responses were not evaluated in the presence of metal stress, which would more effectively demonstrate PGPR-mediated phytoremediation. Of the 10 functional strains, S44 was selected as the most active strain (tolerance of up to 800 mg·L^{-1} Zn and 1000 mg·L^{-1} Cu, adsorption/solubilization of the largest quantities of Cu and Zn, the maximum root length and fresh weight-promoting effects) for molecular identification.

Molecular Identification of Strain S44

S44 was identified as a species of *Acinetobacter* sp. by 16S rDNA gene sequencing and was named *Acinetobacter* sp. FQ-44. The highest sequence similarity (99%) and the phylogenetic tree in **Figure 4**, based on 16S rDNA sequences reveal a relationship between FQ-44 and other relevant bacteria reported. The 16S rDNA sequences (1443 bp) of FQ-44 were deposited in GenBank under accession No. KU206487.

TABLE 6 | Effects of FQ-44 on accumulation, uptake, BCF, and TF of Cu in *B. napus* cultivated in the presence of Cu at various concentrations.

Microbial treatments	Cu treatments (mg/L)	Cu root concentrations (mg/kg DW)	Cu shoot concentrations (mg/kg DW)	TF	Cu root contents (μg)	Cu shoot contents (μg)	BCF
Control	2	74.39 ± 2.61a	51.36 ± 1.34a	0.69	4.15 ± 0.18a	10.14 ± 0.13a	0.48
	5	165.14 ± 3.62b	81.32 ± 2.07b	0.49	7.30 ± 0.13b	11.91 ± 0.19b	0.38
	10	258.67 ± 4.34c	126.47 ± 3.26c	0.49	9.34 ± 0.33c	12.06 ± 0.30b	0.21
FQ-44	2	77.22 ± 2.93nsA	53.44 ± 1.26nsA	0.69ns	5.47 ± 0.16***A	13.53 ± 0.14***A	0.63**
	5	161.58 ± 3.75nsB	85.78 ± 3.69*B	0.53*	8.89 ± 0.18***B	16.47 ± 0.26***B	0.50**
	10	239.11 ± 4.80**C	155.41 ± 3.25***C	0.65***	9.94 ± 0.22nsC	18.26 ± 0.38***C	0.28*

Values are the means ± SE, n = 9. The asterisk (*) denotes a significant difference compared to the control treatment. $^{ns}p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. *Data of columns indexed by the different letters within the same microbial treatments (control and inoculation) are significantly different according to Fisher's protected LSD test ($p < 0.05$).*

Influence of FQ-44 on Growth and Cu Uptake by *B. napus*

The plant–bacteria partnership can be applied to increase the phytoremediation efficiency of soil and water contaminated with organic and/or inorganic pollutants (Khan et al., 2015). Therefore, the effects of metal-mobilizing FQ-44 on growth and metal uptake/translocation by *B. napus* were evaluated. As expected, FQ-44 significantly increased the dry weight of *B. napus* cultivated in different concentrations of Cu (**Figure 5**). In general, inoculation with FQ-44 significantly increased plant uptake of Cu (**Table 6**), which is consistent with significant improvements of BCF of Cu induced by FQ-44. Moreover, FQ-44 also significantly increased the TF of Cu ($P < 0.05$, **Table 6**), besides the Cu concentration of 2 mg/L. Yoon et al. (2006) also demonstrated that plants with a greater BCF and TF have the potential for use in heavy metal phytoextraction. The above results suggest that FQ-44 can be used to facilitate the phytoextraction of Cu. Previously, Rojas-Tapias et al. (2012) also reported that *Acinetobacter* sp. CC30 significantly enhanced Cu uptake by sunflowers. Moreover, Jing et al. (2014) reported that *Enterobacter* sp. JYX7 and *Klebsiella* sp. JYX10 significantly improved Zn uptake by *B. napus*. Recently, Płociniczak et al. (2016) also reported that *Brevibacterium casei* MH8a colonized white mustard plant tissues and enhanced Cu and Zn phytoextraction.

Although FQ-44 showed PGP effects on rape and enhanced phytoextraction of Cu, its colonization and survival properties are crucial features to evaluate its capacity for promoting sustainable plant growth and cope with metal stress in contaminated sites (Ma et al., 2011). Therefore, future studies using pot experiments containing *in situ* soils are needed to examine the specific effects of selected FQ-44 on the growth of host plants, and to determine whether it has the advantage of rhizosphere colonization.

CONCLUSION

In the present study, the selection of Cu/Zn-resistant FQ-44 isolated from *S. oleraceus* was evaluated through three inter-causal screenings. Our results indicate that FQ-44 has potential to facilitate *B. napus* growth and enhance phytoextraction of Cu by sand culture experiment, which could be attributed to beneficial PGP traits; increased concentrations of water-soluble Cu, Cd, Zn, Pb, and Fe; and tolerance and adsorption of Cu and Zn that effectively improved microbe-assisted phytoremediation. Consequently, these advantages confer bioinoculant properties to FQ-44 that would be helpful for enhancing phytoremediation efficiency of multi-metal-polluted soils, particularly Cu/Zn-contaminated soils. Moreover, the proposed approach to screening in the present study could be useful for the isolation of effective strains and improvement of phytoremediation.

Although FQ-44 possessed PGP traits to facilitate *B. napus* growth and critical bioremediation potentials, in many cases PGP bacteria failed to induce the desired effects, when applied in a natural environment. Further research will address: (1) the interactions between FQ-44 and host plants; (2) the colonization potential of FQ-44 and mechanisms contributing to increased plant biomass and metal uptake/translocation by pot experiment containing *in situ* soils; and (3) the roles of FQ-44 in field phytoremediation experiments.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: XW, QF, ZF, and YX. Conducted the work: QF. Analyzed the data: XW, QF, ZF, and YX. Contributed reagents/materials/analysis tools: XW, ZF, YX, and KL, YL. Wrote the manuscript: QF.

FUNDING

This research was supported by a grant from the National Key Research and Development Program of China (2016YFC0502700), the Major project of Chinese National Social Science (No: 14ZDB140) and the National Science Foundation of China for Yong Scholars (No: KRH1829152).

ACKNOWLEDGMENTS

We specially thank the reviewers and the editor for providing their constructive comments that greatly improve

the manuscript. We also thank GTP and CC for their assistance in the experiment and XYL for help with data analysis. We would like to thank Shanghai Academy of Agricultural Sciences for kindly donating the seeds of *B. napus* for this experiment.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01487

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 13 October 2016
doi: 10.3389/fpls.2016.01514

Killing Two Birds with One Stone: Natural Rice Rhizospheric Microbes Reduce Arsenic Uptake and Blast Infections in Rice

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Our recent work has shown that a rice thizospheric natural isolate, a *Pantoea sp* (hereafter EA106) attenuates Arsenic (As) uptake in rice. In parallel, yet another natural rice rhizospheric isolate, a *Pseudomonas chlororaphis* (hereafter EA105), was shown to inhibit rice blast pathogen *Magnaporthe oryzae*. Considering the above, we envisaged to evaluate the importance of mixed stress regime in rice plants subjected to both As toxicity and blast infections. Plants subjected to As regime showed increased susceptibility to blast infections compared to As-untreated plants. Rice blast pathogen *M. oryzae* showed significant resistance against As toxicity compared to other non-host fungal pathogens. Interestingly, plants treated with EA106 showed reduced susceptibility against blast infections in plants pre-treated with As. This data also corresponded with lower As uptake in plants primed with EA106. In addition, we also evaluated the expression of defense related genes in host plants subjected to As treatment. The data showed that plants primed with EA106 upregulated defense-related genes with or without As treatment. The data shows the first evidence of how rice plants cope with mixed stress regimes. Our work highlights the importance of natural association of plant microbiome which determines the efficacy of benign microbes to promote the development of beneficial traits in plants.

Keywords: aboveground, arsenic, belowground, microbiome, *M. oryzae*, rhizosphere

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *19 June 2016*
Accepted: *26 September 2016*
Published: *13 October 2016*

Citation:
Lakshmanan V, Cottone J and Bais HP (2016) Killing Two Birds with One Stone: Natural Rice Rhizospheric Microbes Reduce Arsenic Uptake and Blast Infections in Rice. Front. Plant Sci. 7:1514. doi: 10.3389/fpls.2016.01514

INTRODUCTION

Rice is the food for over half the world's population and contributes as much as 80% of the daily caloric intake in many South Asian countries (Dawe et al., 2010). Tragically, paddy rice grown in South East Asia and particularly in the arsenic (As) hotspots accumulates inorganic As leading to elevated arsenic in rice grain, contributing to large-scale mass As-poisoning (Huq, 2008). Severe As intoxication results in skin lesions and neurological injury. Chronic low-level exposure increases incidence of multiple cancers and causes disfigurement and recurring diarrhea. This is exacerbated by the fact that elevated As concentrations in soil is phytotoxic and can contribute to decreased grain fill, lowered yield and reduced food availability (Abedin et al., 2002; Panaullah et al., 2009). Novel strategies are needed to simultaneously decrease the As content and increase nutritional value in rice grains.

The effects of beneficial rhizospheric microbes on soil structure and chemistry are well known (Bais et al., 2006), but little is known about the physiology and biochemistry underlying the interactions between bacteria and elemental cycling in soil that influence plant yield and productivity. Plant-associated bacteria within the rhizosphere have the capability to modulate the uptake of elements, including Fe and As. Rhizospheric microbes can alter the rhizosphere geochemistry, potentially rendering As less soluble by tying it up with Fe oxides (Zhu et al., 2013). We have identified a suite of nonpathogenic, rice-associated bacteria, *Pantoea sp.* (EA106) from roots of rice grown in North American rice paddy fields that have shown they promote healthy rice growth and enhance the oxidizing potential of the rhizosphere (Lakshmanan et al., 2015). This creates a microenvironment where As is conceptually less available for plant assimilation in the immediate vicinity of the root by being tightly bound to Fe oxides that form on or near the plant roots, so-called Fe-plaque. It is shown that application of EA106 increases Fe mobilization to roots leading to formation of Fe-plaques and reduced As uptake to shoots (Lakshmanan et al., 2015).

Rice is the principal food crop for more than half of the world's population, but yields are reduced significantly due to disease pressure. Each year rice blast disease caused by *Magnaporthe oryzae* destroys enough rice to feed an estimated 60 million people (Zeigler et al., 1994). In Asia, rice is grown widely under rain-fed, lowland conditions and approximately 45% of the total rice area is not irrigated (Manickavelu et al., 2006; Dawe et al., 2010). Therefore, crops grown in this condition are subjected to both abiotic and biotic stresses, and data show that rice is more susceptible to *M. oryzae* during drought stress (Mosquera et al., 2009). Significant progress has been made to generate plant lines with improved resistance, but the pathogen rapidly overcomes plant-encoded resistance. Chemical pesticides also offer marginal protection from the disease, and there are limited biocontrol strategies against any foliar pathogens of rice. Previously, we have discovered a microbe from rice rhizosphere, *Pseudomonas chlororaphis* (EA105) which attenuates *M. oryzae* in *vitro* and *in vivo* (Spence et al., 2014a,b, 2015). The microbes naturally associate with healthy rice roots and are readily cultured and introduced to axenically-grown rice or rice previously infected with other microbes.

Plants are often confronted with both biotic and abiotic stress leading to loss of productivity and yield (Ben Rejeb et al., 2014). It is often argued that combination of both abiotic and biotic stress responses in plants may be beneficial to plant performance against biotic stress responses (AbuQamar et al., 2009; Ben Rejeb et al., 2014). Interestingly, plants exposed to bacterial pathogens lead to stress responses while dealing with one specific stress is often referred to as "cross tolerance" (Suzuki et al., 2012). Conventionally, plants usually compartmentalize while confronting more than one stress response at time. It is argued that plants are able to defend themselves facing one stress and simultaneously become more resistant to other stress regimes (Bowler and Fluhr, 2000). For example, it is shown that mechanical wounding in plants often results in increased resistance against abiotic stress such as salinity response (Capiati et al., 2006). Pre-exposure of plants against *Pseudomonas syringae* leads to insect tolerance in tomato against *Helicoverpa zea* (Stout et al., 1999). Along similar lines, exposure of plants against ozone increases resistance against *P. syringae* strains (Sharma et al., 1996; Borsani et al., 2001). The results were similar wherein plants were challenged with biotic stress first and then exposed to abiotic stress regime. It is shown that, plants exposed to aerial pathogens close stomata leading to drought tolerance (Goel et al., 2008). Interestingly, exposure of plants to benign microbiome also changed the aboveground physiology leading to resistance against the biotic stress (Reviewed by Lakshmanan et al., 2014; Pieterse et al., 2014). Conversely, plants interacting with simultaneous biotic and abiotic stress may lead to trade-offs increasing plant growth and fitness. The signaling pathways involved in plants exposed to multiple stresses are still being researched and how signaling pathways relate to traits for plant fitness and protection needs to be elucidated.

We have identified a suite of nonpathogenic, rice-associated bacteria (EA106 and EA105) from roots of rice grown in rice paddy fields and have shown that they promote healthy rice growth and enhance the oxidizing potential of the rhizosphere and induce plant protection against rice blast fungus (Spence et al., 2014a,b, 2015; Lakshmanan et al., 2015). The incidence of evaluating two different stress regimes in a model plant system is new. We have examined the fitness of rice plants exposed to As and *M. oryzae* infections simultaneously. The goal of our study was to see if plants exposed to As show variation in its response against *M. oryzae* infections. The impact of benign microbes under multiple stress regimes for defense-related genes and As uptake was also evaluated.

MATERIALS AND METHODS

Plant Growth Conditions

Rice cultivar Nipponbare seeds were provided by Genetic Stocks—Oryza (GSOR) Collection Dale Bumpers National Rice Research Center, Stuttgart, Arkansas, USA. Hyper-susceptible genotype Seraceltik (a gift from the Donofrio lab; University of Delaware, DE) was used for *M. oryzae* infections. Seeds were de-husked, surface sterilized with 50% commercial laundry bleach for 5 min and rinsed three times with sterile water. The surface sterilized seeds were transferred on a germination paper disk (single paper disk per plate and moist with 5 ml of sterile water) in a Petri dish and incubated on culture rack at room temperature ($25 \pm 2^{\circ}C$) with a photoperiod of 12 h dark and 12 h light ($130 \pm 20\,\mu mol\ m^{-2}s^{-1}$) for 7 days. Uniform-size seedlings were selected and used for hydroponic cultivation and *in vitro* experiments

Preparation of Bacterial EA106 and EA105

Bacterial strains EA105, a *Pseudomonas chlororaphis* (Spence et al., 2014a), and EA106, a *Pantoea sp* (Lakshmanan et al., 2015) were isolated from rice cultivar M-104 grown in the paddy soil by Dr. Venkatesan Sundaresan's lab, University of California (Davis). Freezer-stored glycerol stocks of EA106 and EA105 were streaked onto low-salt Luria-Bertani (LB) plates ($10\,g\ L^{-1}$

Tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl) and incubated at 30°C for overnight. An LB liquid culture was made with a single colony from plates and after 12 h incubation at 30°C with 180 rpm, when the OD_{600} reached 0.8–1.0, bacterial cells were centrifuged and washed in 10 mM $MgCl_2$ to remove the medium and centrifuged and re-suspended in water to obtain desired inoculation density.

Hydroponic Cultivation of Rice

Rice cultivar Seraceltik plants grown hydroponically as described by Lakshmanan et al. (2015). Briefly, 7-day-old, *in vitro*-germinated seedlings were transplanted into the hydroponic system that contained 8 L of rice nutrient solution. The hydroponic pots were incubated for 21 days in a growth chamber at 22 ± 2°C temperature, 14/10 h of light/dark photoperiod, 130 ± 20 μmol $m^{-2}s^{-1}$ light intensity and 80% relative humidity and the solution was renewed every 7 days. Then, plants were treated with 5 μM As(III), or EA105 (OD_{600} = 0.02) or EA106 (OD_{600} = 0.02) or combinations of all and grown for an additional 7 days, during which the pH was checked and adjusted daily (pH 6.0–6.5). After 7 days of treatments, samples were collected for *M. oryzae* infection and analyses for total As content.

M. oryzae Infection Assay

Wildtype fungi *M. oryzae* 70-15, a fully sequenced, filter paper stocks stored at −20°C were inoculated onto oatmeal agar plates (OM: oatmeal 50 gL^{-1} and agar 15 gL^{-1}) for germination and to establish starter cultures. Fungus grown on OM plates were transferred to complete medium (CM) containing sucrose (10 gL^{-1}), cas-amino acids (6 gL^{-1}), yeast extract (6 gL^{-1}), and 1 mL of *Aspergillus nidulans* trace elements and kept in dark at 25°C for 7 days. The cultures were subsequently transferred to OM agar for 10 days for sporulation. Conidia from *M. oryzae* grown on OM agar plates were harvested, filtered through miracloth, and counted using a hemacytometer. Concentration of the conidial suspension was adjusted to 1×10^4 spores mL^{-1}. For *M. oryzae* infections, 7 days post treatment of Arsenic and rhizobacteria, hydroponically grown plant, the second youngest fully developed leaf was cut and affixed to a large 150 mm diameter petri dish, on top of moistened paper towels and treated with 3–20 μL droplets of spores were placed along the length on each leaf. Spore droplets were wiped after 24 h incubation in the dark at 25°C. After 120 h post treatment, length and width of lesions were measured also photographed.

Exposure of As against *M. oryzae* and Other Non-host Fungi

The non-host pathogenic fungi, *Fusarium equisetti* and *Geotrichum candidum* were acquired from Nancy Gregory at the University of Delaware and were grown on potato dextrose agar (PDA) for 7 days in dark at 25°C. Arsenic at different concentrations [(0–2000 μM As(III) or As(V)] were used to test against both *M. oryzae* and non-host fungi. Five mm plugs of fungal mycelia were placed in the center of the plates and plates were sealed with parafilm and put in the dark in a 25°C incubator. Photographs were taken after 5 days and the diameter of the mycelium growing out from the plug was measured. Percentage (%) inhibition was calculated by the formula:% inhibition = [(C–T) × 100)/C], where C = fungal diameter (cm) in the control plate, and T = fungal diameter (cm) in the As treated plates.

Quantification of Total As Content

Plants grown in an 8-L hydroponic system for 21 days and treated with As(III), EA105 and EA106 as mentioned above were used for quantification of arsenic. After 7 days of treatments, the root, and shoot samples were collected and dried at 65°C for 3 days and weighed. The total concentrations As in the leaves and seeds were measured by ICP-OES at UD soil testing lab, University of Delaware.

Spore Germination and Appresoria Formation in *M. oryzae* Treated with As

M. oryzae spores were grown on oatmeal agar and harvested and adjusted to concentration of 1×10^5 spores mL^{-1}. Then treated with mock, 5 μM, 10 μM, and 100 μM of As(III) and 50 μl were placed on the hydrophobic (Pho) surface of sterile GelBond. The GelBond films were placed in petri dishes with wet filter disks in the center to promote humidity. Plates were sealed and incubated in the dark for 12 h. Germination percentages were calculated after 6 h incubation, and appressoria formation was determined after 12 h. Appresoria formation was imaged using a Zeiss Axioscope2 light microscope.

RNA Extraction and Semi-Quantitative-RT-PCR Analyses

Five uniform-size 7-days-old, *in vitro*-germinated seedlings of rice cultivar Nipponbare were transplanted to a magenta GA7 plant tissue culture box containing 25 ml of rice nutrient solution and grown for 14 days. Then, plants were transferred to rice nutrient solution with 5 μM As(III), or EA105 (OD_{600} = 0.02) or EA106 (OD_{600} = 0.02) or combinations of all. Post 24 h of treatment total RNA was isolated from pool of five whole seedlings using the Bio Basic EZ-10 Spin Column Plant RNA Mini-Prep Kit. The possible genomic DNA contaminant in RNA extract was removed using turbo DNA-free™ kit (Ambion). The quantity of total RNA was determined using NanoDrop. First-strand complementary DNAs were synthesized from 500 ng of total RNA using High Capacity cDNA Reverse Transcription Kit. PCR was carried out using standard Taq Polymerase (New England Biolabs) using the gene specific primers (Supplementary Table 1). PCR amplifications were performed using PCR mixture (15 μL) that contained 1 μL of RT reaction product as template, 1 × PCR buffer, 200 μM dNTPs (Fermentas GmbH), 1 U of Taq DNA polymerase (New England Biolab), and 0.1 μM of each primer depending on the gene (Supplementary Table 1). PCR was performed at initial denaturation at 94°C for 4 min, 24, or 26 or 28 cycles (30 s at 94°C; 30 s at 60°C; 30 s at 72°C), and final elongation (8 min at 72°C) using a Bio-rad thermal cycler (Supplementary Table 1). We performed a negative control containing RNA instead of cDNA to rule out any possible genomic DNA contamination. The PCR products were electrophoresed on 1.4% agarose gel, stained with ethidium bromide and documented in a gel documentation system; the

bands were quantified using ImageJ. Each band was normalized against the intensity obtained with the same cDNA using the actin primers.

Data analysis

Data were presented as mean with standard error. The statistical software JMP11 was used to analyses the data. The data were analyzed by one-way analysis of variance (ANOVA), and *post hoc* mean separations were performed by Tukey's HSD test and results were considered to be statistically different when $p < 0.05$.

RESULTS

As Treatment Increases Susceptibility of Rice against *M. oryzae*

To evaluate the impact of As treatment on rice blast infections, 21 days old Seraceltik seedlings were treated with 5 μM As(III). Post 7 days treatment of 5 μM As(III), leaves of rice seedlings were subjected to *M. oryzae* infections. The *M. oryzae* were prepared following the protocol published in Spence et al. (2014a). Plant pre-treated with As(III) showed increased susceptibility against blast infections in terms of increased lesion size compared to untreated and mock plants (**Figures 1A,B**). Plant treated with As(III) showed significant As-uptake in aerial shoots/leaves compared to the mock treatment (**Figures 1C,D**). Roots showed more accumulation of total As compared to shoots, the levels of As recorded *in planta* were in accordance with the published reports (Das et al., 2016). We also tested the total As content in the rice grains post As amendment. The Nipponbare genotype was primed with EA106 and treated with As(III) for the total As grain content. Nipponbare seedlings primed with EA106 and treated with As(III) showed significant reduction in grain As content compared to untreated mock plants (Supplementary Figure 1).

Exposure of As against *M. oryzae* and Other Plant Pathogenic Fungi

Both species of As [As(III) and A(V)] are potentially toxic to both prokaryotes and eukaryotes (Styblo et al., 2000). Previously, we have observed toxic effects of As on rice plants

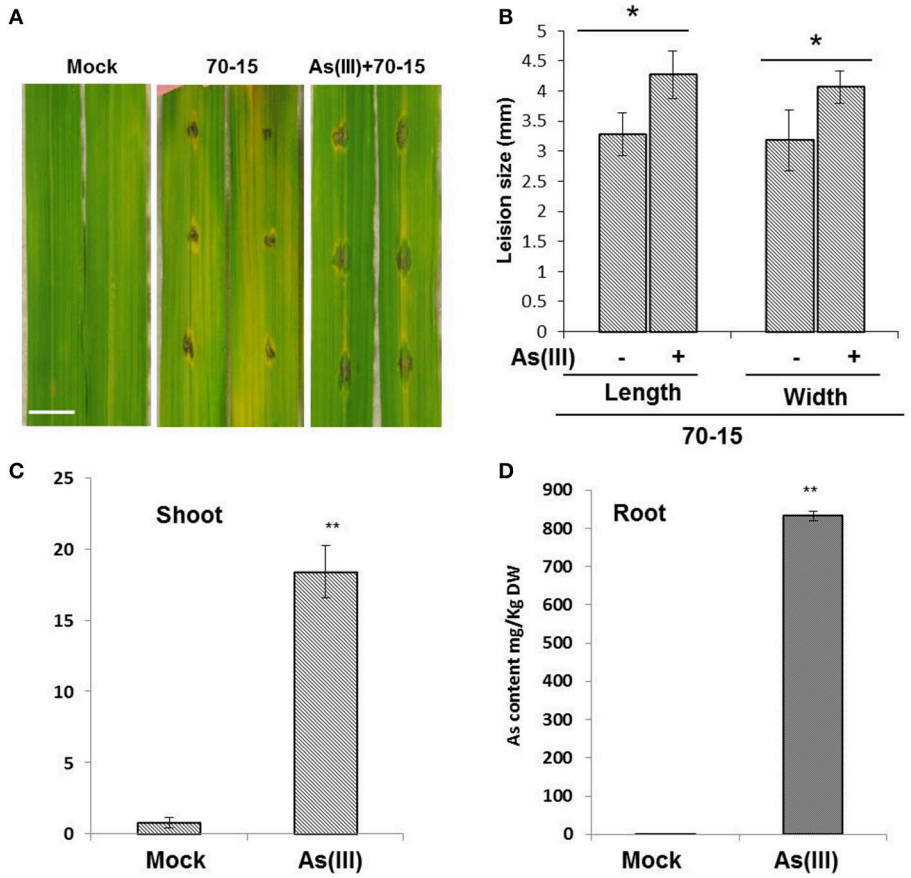

FIGURE 1 | As(III) treatment increases susceptibility of rice against *M. oryzae*. Rice cultivar Seraceltik was germinated on water and transferred to 8-L system containing rice nutrient solution. After 21 days the plants were treated with and without As(III) (5 μM) and incubated for 7 days. Uniform leaves were infected with *M. oryzae* 70-15 tested for their ability to form lesions. **(A)** Representative photographs were taken 5 days post-treatment (Scale bar = 10 mm). **(B)** Lesion size measured by length and width. **(C,D)** ICP-MS quantification of total As content in root and shoots of rice plants. Experiments with three replicates were repeated twice. Error bars indicate standard error. A means comparison was done within lesion length and within lesion width. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$; two-tailed Student's *t*-test.

grown hydroponically (Lakshmanan et al., 2015). To test if As inflicts toxics effects on rice blast pathogen, *M. oryzae* was exposed to increasing concentrations of both As(III) and As(V) (0–2 mM). As was supplemented in the media and a fungal plug was inoculated, radial growth post 5 days was estimated and photographed. *M. oryzae* showed significant tolerance against As(III) and As(V) at 100 μM levels (**Figures 2A,B**). Interestingly, *M. oryzae* showed increased tolerance against As(V) compared to As(III) at 2 mM levels (**Figures 2A,B**). To check the specificity of fungal tolerance against As, other non-host fungal strains were also evaluated. *Fusarium equisetti* and *Geotrichum candidum* were checked against As(III) and As(V) toxicity. In stark contrast, *F. euqisetti* showed increased susceptibility against As(III) and As(V) compared to *M. oryzae*, concentration of As over 50 μM were toxic to growth of *F. quisetti* (Supplementary Figure 2). Interestingly, *G. candidum* showed increased tolerance against both As(III) and As(V) (Supplementary Figure 2A,B).

As Treatment and Appressoria Formation

Since an increased tolerance of *M. oryzae* against As was observed, spore germination and appressoria formation in *M. oryzae* post As(III) treatment was evaluated. *M. oryzae* was exposed to increasing concentration of As (0–100 μM) and spore germination and appressoria formation was evaluated per the published protocol (Spence et al., 2014a). No significant reduction in spore germination and appressoria formation was observed post As (III) treatment in *M. oryzae* (**Figure 3**).

Rhizobacterial Inoculation and Blast Infection in As Treated Rice Plants

Previously, it was shown that two different natural rice isolates from rice Rhizosphere impact both As uptake and blast infections in rice (Spence et al., 2014a; Lakshmanan et al., 2015). Isolates such as EA105 and EA106 were shown to reduce blast infections and As uptake respectively in rice plants. In here, it was envisaged to treat rice plants with the co-inoculation of natural isolates (EA105, EA106, and together) to evaluate plants response against mixed stress regime of As and blast infections. Three-week old rice plants were root-primed with EA105, EA106 and a mixed inoculum of EA105 + EA106 per the published protocol (Spence et al., 2014a; Lakshmanan et al., 2015). Plants were simultaneously subjected to As(III) (5 μM) treatment. Post 7 days

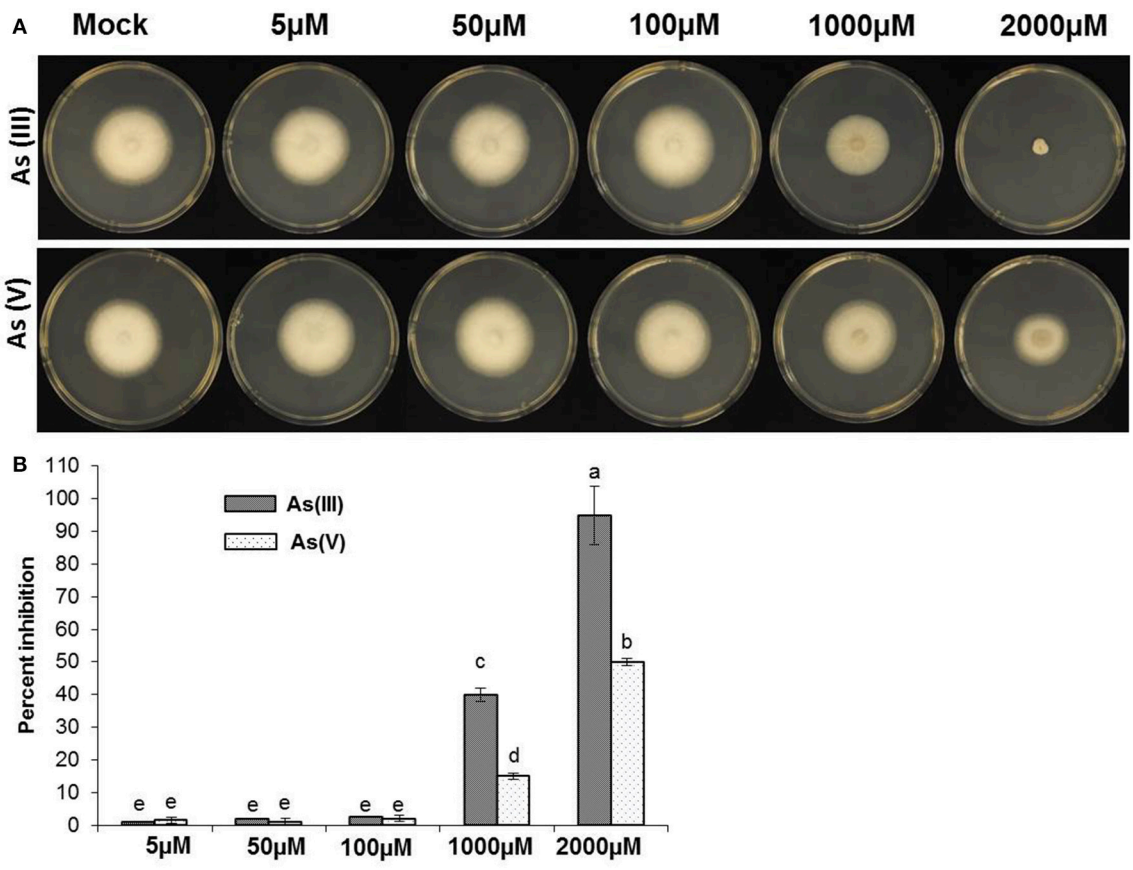

FIGURE 2 | Exposure of As against *M. oryzae*. 5 mm size of fungal plug from 7 days grown *M. oryzae* 70-15 was transferred to CM plates with different concentration (0, 5, 50, 100, 1000, and 2000 μM) of As(III) and As(V) and incubated for 5 days in dark. **(A)** Representative image of the fungal inhibitory effect seen when 70–15 was growth on CM plates with As(III) or As(V). **(B)** Degree of inhibition of *M. oryzae* 70–15 by adding As(III) or As(V) on CM agar plats. Each treatment had five replicates and repeated twice. Error bars indicate standard error. Different letters indicate statistically significant differences between treatments (Tukey's HSD).

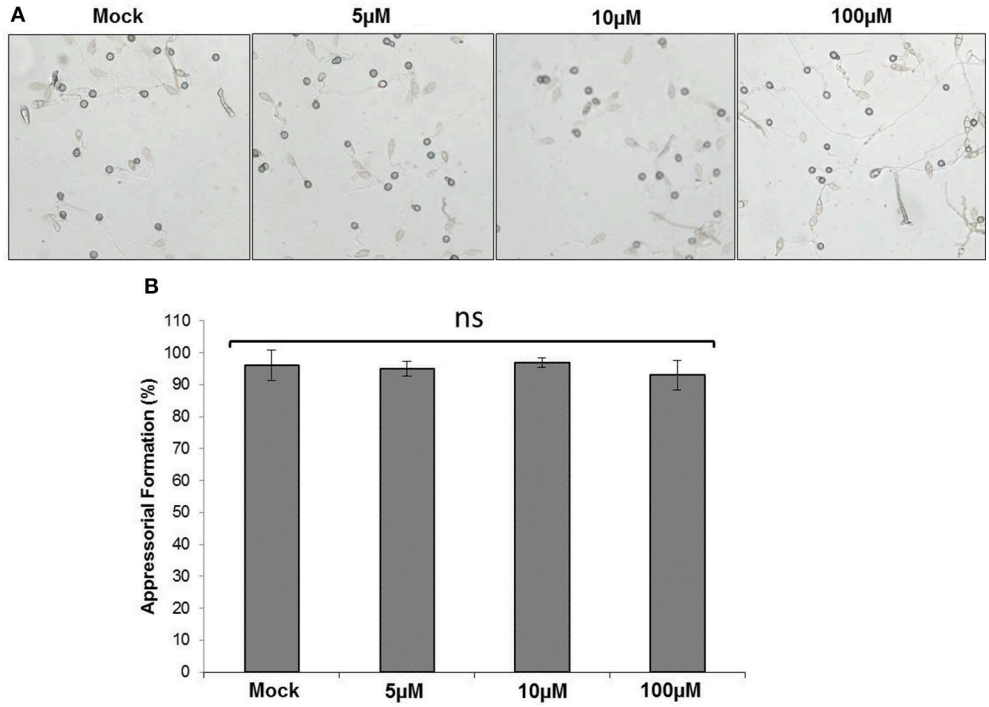

FIGURE 3 | Effect of As treatment on appressoria formation. Spores of *M. oryzae* 70-15 were treated with different concentration (0, 5, 10, and 100 μM) As(III) and incubated for 12 h. **(A)** Representative image of appressorial formation. **(B)** Degree of appressorial formation. Each treatment had five replicates and repeated twice with similar results. Error bars indicate standard error. No statistical differences between treatments (Tukey's HSD).

of inoculation and As administration plants were subjected to blast infections. In accordance with the earlier observations both EA105 and EA106 reduced disease incidence and blast infection in rice compared to mock untreated plants (**Figure 4**). Plants primed with As in soil and co-inoculated with EA105/106 or EA105 + 106 showed increased susceptibility to blast infections compared o As-unprimed plants (**Figure 4**). Interestingly, EA105 and As treated plants showed more susceptibility to blast compared to mock and lone EA105 treated plants (**Figure 4**). Co-inoculation of EA105 and 106 in an As rich environment lead to decrease in blast incidence compared to EA105 or mock treated plants (**Figure 4**).

Rhizobacterial Inoculation Modulates as Uptake

The results showed that plants pre-treated with As revealed increased disease incidence and progression against rice blast fungus *M. oryzae*. Interestingly, plants pretreated with As and bacterial inoculum EA106 showed reduced disease incidence. To elucidate how bacterial inoculation impacted As uptake, we used both EA105 and 106 inoculation in rice plants. Three-weeks old rice plants (Nipponbare) were supplemented with As(III) (5 μM) and were co-inoculated with EA105, EA106, and EA105+EA106 ($OD_{600} = 0.02$). Post inoculations plants were analyzed for total As content in roots and shoots. Expectedly, EA106 inoculation led to less uptake of As in the aerial parts of the plants compared to mock plants (**Figures 5A,B**). Contrastingly, EA105 treatment didn't impact the As uptake and was similar to untreated plants (**Figures 5A,B**). Interestingly, plants co-inoculated with EA105 and EA106 showed significant reduction in shoot As content compared to mock and EA105 treated plants (**Figures 5A,B**). The total root As content also showed reduction with EA106 treatment but was not significant under the EA105 and EA106 co-inoculation regime (**Figures 5A,B**).

Transcriptional Response of Defense-Related Genes in As and As + Microbial Inoculum Treated Rice Plants

To evaluate the effect of As treatment on rice plant defense, transcriptional response of classical defense-related genes were evaluated. Rice plants (21 days) grown under sterile conditions were subjected to As(III)(5 μM) and rhizobacterial treatments. Total RNA was isolated from rice plants subjected to As and As + rhizobacterial inoculum treatment and expression of defense related genes such as Pathogenesis-Related protein (*PR1*), Jasmonate Resistant (*JAR1*) and Ethylene Insensitive$_3$-Like gene (*EIL1*) were evaluated. Expectedly, lone treatments with EA105 and EA106 induced defense response in rice plants (Supplementary Figure 3). In addition, As(III) treatment failed to induce the expression of *PR1, EIL1* and *JAR1* (Supplementary Figure 3). The co-inoculation of As with microbial inoculums (EA106 and EA106 + EA105) negated any significant changes in expression of defense-related genes (Supplementary Figure 3). Interestingly, plants treated with As and EA105 showed

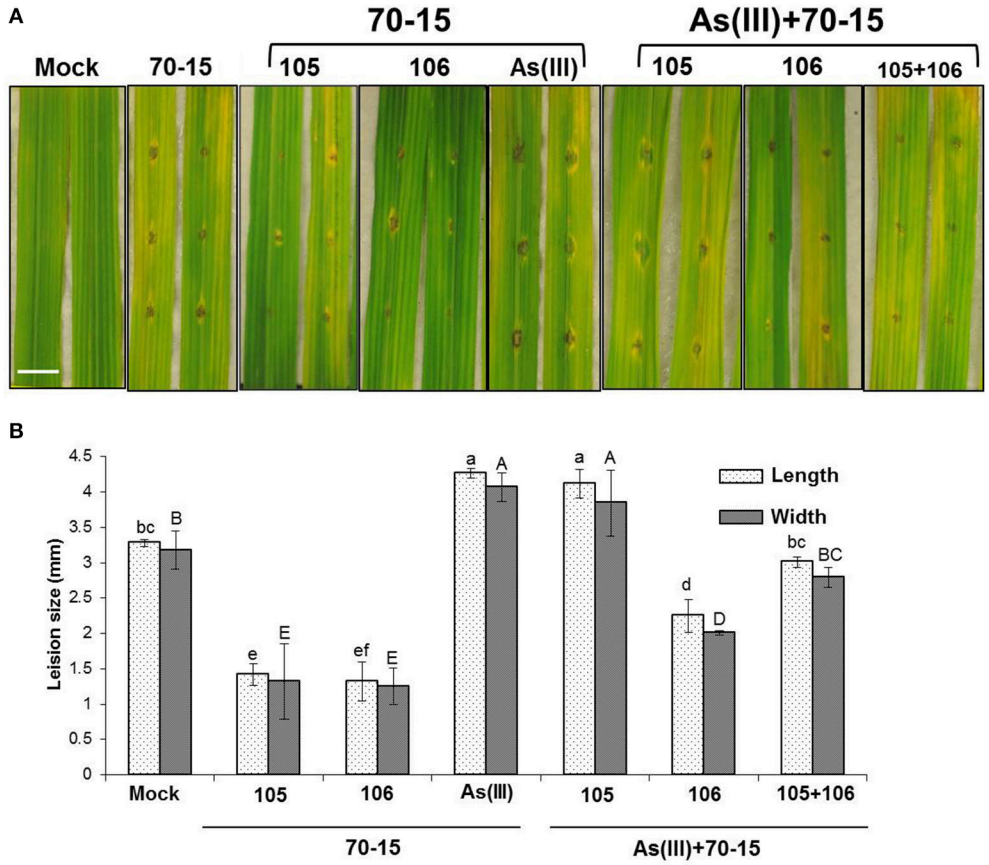

FIGURE 4 | Microbial inoculation and As treatment modulates blast infection in rice plants. Rice cultivar Seraceltik was germinated on water and transferred to 8-L system containing rice nutrient solution. After 21 days the plants were treated with natural isolates EA105 or EA106 with or without (5 μM) As(III) and incubated for 7 days. Two leaves per plants were used for *M. oryzae* 70-15 cut leaf assay. **(A)** Representative images for *M. oryzae* 70-15 infection after 5 days post-treatment (Scale bar = 10 mm). **(B)** Degree of *M. oryzae* 70-15 infection measured by lesion size measured by length and width. Each treatment had five plates and repeated twice with similar results. Error bars indicate standard error. Different letters indicate statistically significant differences between treatments (Tukey's HSD).

downregulation in PR1 expression compared to lone EA105 treatments (Supplementary Figure 3).

DISCUSSION

Influence of natural sediment of heavy metals including arsenic (As), cadmium (Cd), aluminum (Al) have remained as a significant environmental predicament with a negative probable impact on human health and plant productivity. Arsenic is a highly toxic heavy metal and, when present in the environment in excessive amounts, can cause serious damage to all organisms-including plants. The rhizosphere of the plant is crucial microenvironment and may have the greatest influence on the bacterial community in the soil and leading to As bioavailability and uptake into plants. We have identified a suite of nonpathogenic, rice-associated bacteria, *Pantoea sp.* (EA106) from roots of rice grown in North American rice paddy fields and have shown that they promote healthy rice growth and enhance the oxidizing potential of the rhizosphere (Lakshmanan et al., 2015). In addition, in our efforts to characterize cultivable microbiome from rice, we isolated benign microbe, *Pseudomonas sp.* (EA105) that can attenuate rice blast fungus *M. oryzae* infections (Spence et al., 2014a, 2015). The application of using benign microbes may protect plants against both biotic and abiotic stress on rice. Our previous studies showed that application of rice roots with EA106 induces iron-plaque formation abating As uptake (Lakshmanan et al., 2015). Plants are constantly challenged with both biotic and abiotic stress in nature, and the implications of combined stress regimes on plants are very poorly understood. Earlier studies involving combined stress regimes in plants showed that priming plants with one stress regime may induce resistance against a different stress response (Ben Rejeb et al., 2014). We speculated that rice plants exposed to As may show modulation in its response to rice blast infections. As expected, As pre-treated plants when challenged with *M. oryzae* infections revealed increased susceptibility against blast (**Figure 1**). Arsenic exposure to rice seedlings and lettuce plants modulate the structure of cellular membranes, effecting their permeability, leading to oxidative

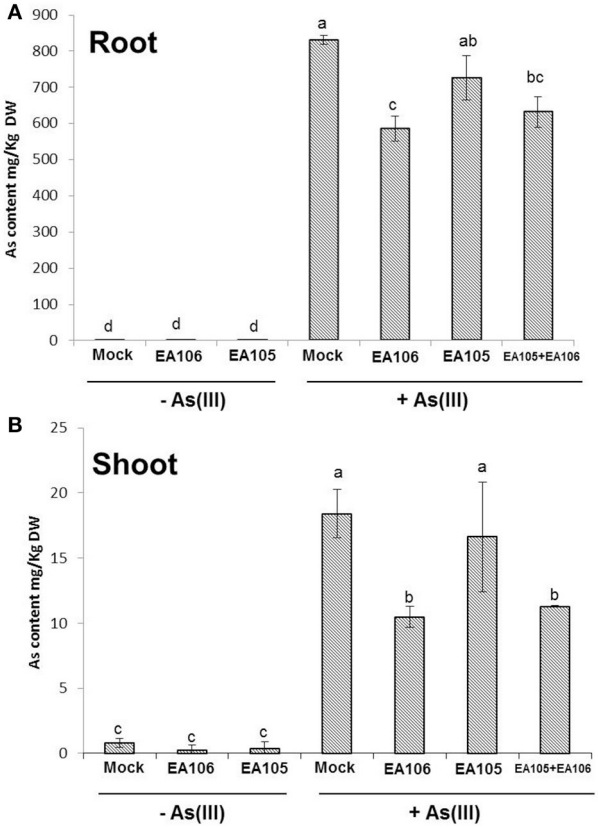

FIGURE 5 | Rhizobacterial inoculation modulates As uptake. Rice cultivar Seraceltik were germinated on water and transferred to 8-L system containing rice nutrient solution. After 21 days the plants were treated with natural isolates EA105 or EA106 with or without (5 μM) As(III) and incubated for 7 days. ICP-MS quantification of As content in roots **(A)** and shoots **(B)** of rice plants. Each treatment had three plants and repeated twice with similar results. Error bars indicate standard error. Different letters indicate statistically significant differences between treatments (Tukey's HSD).

bursts, and modulates antioxidant systems and plant become weaker and more susceptible to infection (Shri et al., 2009; Tuli et al., 2010; Gusman et al., 2013). It is shown that tomato plants exposed to As modifies peroxidase responses leading to increased infections by Cucumber mosaic virus (CMV) (Miteva et al., 2005). There is evidence that plants do respond differently to a simultaneous stress compared to a standalone stress regimes. In this research, we showed that Arsenic accumulation in rice plants elevated the growth of fungus blast pathogen and the questions pertaining to how an uptake of As modifies aboveground plant response is discussed.

The invasiveness of rice blast fungi *M. oryzae* on hosts is well characterized. Spore germination, formation of appressoria as well as vegetative growth is critical for infection (Dean et al., 2012). The factors such as spore germination and appressoria formation in *M. oryzae* are also critically regulated by variety of environmental stressors including nutrients and interactions with other microbes (Mathioni et al., 2011). While almost nothing is known about *M. oryzae* interactions with heavy metals, it is tempting to speculate that the persistence of rice blast fungus may get affected by toxic elements such as As. Interestingly, rice blast fungus *M. oryzae* (up to 100 μM) and other non-host fungi *F. equisetti* (up to 50 μM) and *G. candidum* (up to 100 μM) showed tolerance when treated with As(III) and As(V) (**Figure 2**, Supplementary Figure 2). Our data showed that As (at near biological concentration) inflicted very limited toxicity on mycelial growth, spore germination and appresoria formation in *M. oryzae*. The effect of both biotic and abiotic components other than classical fungicides is rarely tested on the physiology and growth of rice blast fungus *M. oryzae* (Hörger et al., 2013). Previously, we reported that plant growth regulator abscisic acid (ABA) triggers *M. oryzae* spore germination and appressoria formation (Spence et al., 2015). Interestingly, *M. oryzae* biosynthesize ABA to increase its virulence in terms of appresoria formation. Lack of ABA biosynthetic genes led to decrease in appressoria formation and loss of virulence on rice plants (Spence et al., 2014a, 2015). Likewise, As and plant growth hormones like auxins, cytokinins negated any influence on spore germination and appressoria formation (**Figure 3**).

Several metal hyper-accumulating plant species such as *Noccaea caerulescens* is resistant to aboveground pests such as slugs, locusts and caterpillars(Pollard et al., 2002; Behmer et al., 2005; Jiang et al., 2005) Other Nickel (Ni) and As hyper-accumulating plants such as *Brassica juncea* and *Pteris vitatta* reveals resistance against aphids and grasshoppers (Boyd and Jhee, 2005; Rathinasabapathi et al., 2007). To add to the complexity of these unusual interactions, researchers were not able to reproduce the anti-herbivory effect in field grown plants of *N. caerulescens* (Noret et al., 2007). It is been argued that the herbivory may not be a driving force for the plants to evolve to hyper-accumulate toxic elements (Hörger et al., 2013). Interestingly, there is also evidence that herbivores may use toxic metals for their own defense (Freeman et al., 2009). In contrast to the impact of heavy metal hyper-accumulation on herbivory, there have been very few studies reporting the effect of heavy metals on pathogen defense or vice versa. Studies in Ni-hyperaccumulators such as *N. caerulescens* and *Alyssum* species have been shown to be resistant to oomycetes infections (Boyd et al., 1994; Ghaderian et al., 2000). It is argued that the metal defense against pathogens is primarily dependent on mode of infection. In hyperaccumulator plants the toxic metal is usually bound to a ligand and stored in special organelles such as vacuoles (Hörger et al., 2013). Biotrophic and hemi-biotrophic fungi and bacteria may experience apoplastic metals and necrotrophic fungi which targets and disrupts cell organelles may get exposed organelle-bound metals (Hörger et al., 2013). Non-hyperaccumulators such as rice are exposed to both As rich environment and the rice blast. As is usually stored in the aerial tissues bound to a ligand such as phytochelatins (PC). Very little is known about As cellular and sub-cellular localization in non-hyper accumulator plants. It is assumed that non-hyperaccumulators localize As-PC complexes in vacuoles (Vögeli-Lange and Wagner, 1990). Rice blast fungus *M. oryzae* acts as both a biotroph and hemi-biotroph and requires different cellular machinery to

invade plants under each life stages (Koeck et al., 2011). Our results showed that *M. oryzae* to be resistant against biological concentration of As. We speculate that *M. oryzae* under both biotrophic or hemi-biotrophic life stage may get As exposure *in planta*. It also known that host vacuole maintenance under the biotrophic invasion by *M. oryzae* plays a key role in blast infections (Mochizuki et al., 2015). The disruption of cellular organelles during mycelial penetration by *M. oryzae* may release As from the vacuole, which may also be toxic to plants (**Figure 6**).

In recent years, microbes or microbial based products are commercially used for plant growth promotion, plant protection, and bioremediation (Reviewed by Bhattacharyya and Jha, 2012; Bashan et al., 2014; Gaur et al., 2014; Nadeem et al., 2014; Lakshmanan et al., 2015; Kumari et al., 2016; Vejan et al., 2016) More recently, it was shown that natural isolates will have more implication on plant health and fitness by compatible interaction and robust colonization with the same host compared to unrelated isolates (Chen et al., 2013; Spence et al., 2014a; Lakshmanan et al., 2015). In a similar way, we have isolated a bacterium, EA105 from rice rhizospheric soil which showed direct *in vitro* inhibition of *M. oryzae* vegetative growth as well as an ability to interfere with the formation of appressoria, a structure that is critical during *M. oryzae*'s invasion of rice (Spence et al., 2014a). In addition, our work also showed isolation of yet another rice rhizospheric isolate, and EA106 which induces Fe-plaque formation and abates As uptake in roots (Lakshmanan et al., 2015). We hypothesized that microbial inoculums that attenuates As uptake and rice blast infection in rice may modulate disease progression in rice plants exposed to mixed stress regime. As explained in the earlier sections that As treatments alleviates *M. oryzae* infections. However, when As treated plants were co-inoculated with natural rice isolates it lead to decrease in disease severity (**Figure 4**). Interestingly, EA105

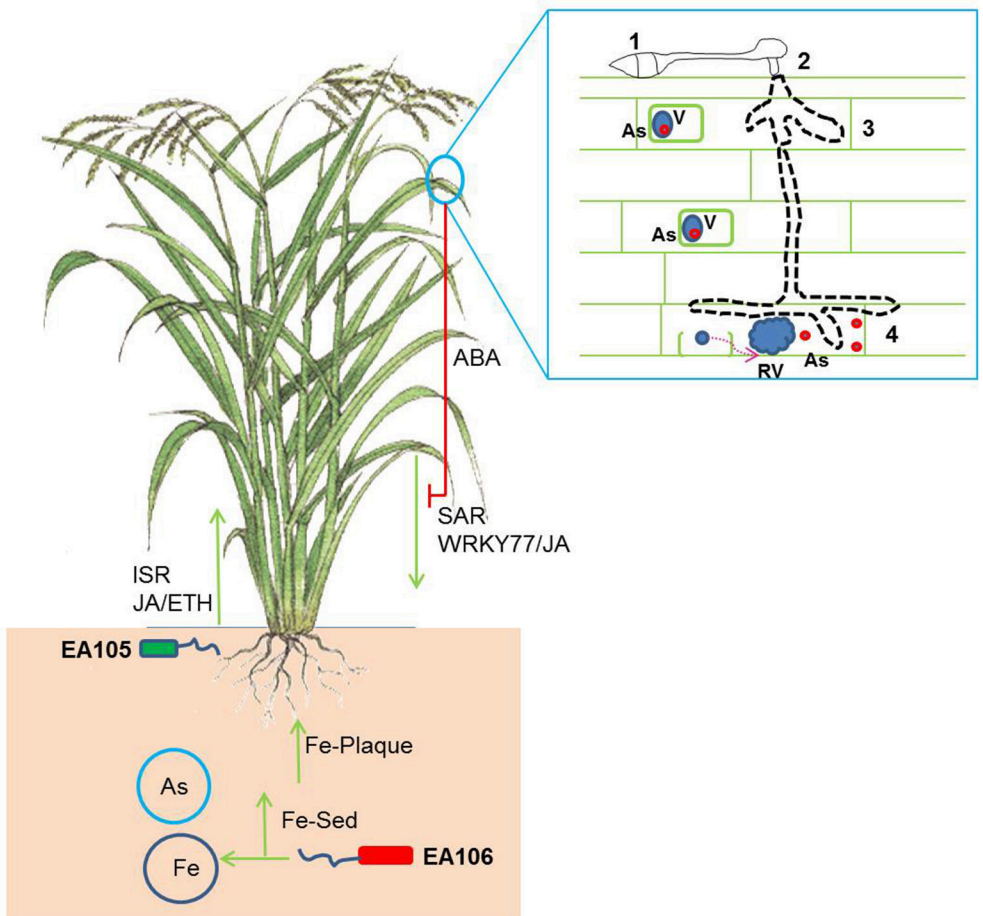

FIGURE 6 | A schematic showing the complex multi-trophic interaction in rice subjected to rhizospheric microbes, As-rich environment and blast infection. The natural rice isolates EA105 and EA106 trigger induced systemic defense response (ISR) against rice blast infection. EA106 mobilizes rhizospheric iron (Fe) *via* siderophore (Fe-Sed) activity and triggers Fe-plaque formation on roots impeding As mobilization in plants. *M. oryzae* infection also induces systemic acquired defense (SAR) response in plants leading to resistance against blast infection. In addition, blast fungus suppresses SAR by producing abscisic acid (ABA). The implication of As (red circles) cellular localization may also be modulated under *M. oryzae* infection. As localized in specialized organelles (blue circles- v = vacuoles) may get released in planta post disruption of cellular organelles (RV = ruptured vacuoles) by rice blast. The boxed panel shows different stages of infection of rice blast: 1. Germ tube and appresorria formation; 2–3. Peg formation and mycelial growth; 4. Cellular rupture by mycelial growth and As release.

shows direct antagonistic activity against *M. oryzae* compared to EA106 (Spence et al., 2014a). In contrast, both EA105 and EA106 induce ISR against *M. oryzae* (Spence et al., 2014a). It has been clearly shown that rice rhizospheric isolates EA105 and EA106 activates resistance by induction of defense signaling molecules such as salicylic acid (SA), jasmonic acid (JA), or ethylene (ETH) (Chisholm et al., 2006; Fu and Dong, 2013; Pieterse et al., 2014; Spence et al., 2014a). Recently showed that natural isolates of rice rhizopheric soil (Spence et al., 2014a,b, 2015) and bulk soil isolates (Shimoi et al., 2010) induces JA/ET/SA related genes and attenuates *M. oryzae* infections. The specificity of rhizobacterial treatment of EA105 and EA106 to elicit PR related genes expression, thereby enhancing plant fitness, suggests that plants acts and responds differently when exposed to multiple stressors. Our data clearly showed that a co-inoculation of two different rice rhziospheric isolates could induce defense against multiple stressors (As and rice blast) in rice. The induction of classical PR defense genes post EA105/EA106 treatment may contribute toward resistance against blast infections. We have shown previously that one of the natural rice isolate EA106 abates As uptake in rice as it induces Fe-plaque formation in roots which may impede As uptake (Lakshmanan et al., 2015). Surprisingly, EA106 also induced PR genes as EA105 which would have contribute to defense against blast. Interestingly, EA105 which protects rice against blast infections didn't reduce As uptake. Surprisingly, co-treatment of As with EA105 reduced the intensity of PR1 expression compared to EA105 lone treatments. At this juncture, we don't know if As toxicity could be linked to pathogen defense in rice. These two responses may very well be compartmentalized in plants and may only have downstream impact on each other. The ability of rice blast fungus to switch from biotroph to a hemi-biotroph mode may lead to As release from the cellular organelles to cytoplasm leading to accelerate cell death and reduction in plant growth (**Figure 6**). Induction of PR genes and defense response post microbial inoculum treatment may play a role in delaying pathogen ingression and release of As in the apoplast.

In this study, we showed for the first time that As accumulation in rice plant tissue leads to increased susceptibility to blast fungus *M. oryzae*. The pre-treatment of rice isolates EA105 and EA106 significantly increased plant fitness by arresting the growth of blast fungus *M. oryzae*. Moreover, As uptake from root to shoot and As accumulation in shoot and grain decreased significantly as a result of the rhizobacterial inoculation. This study further strengthens the case for the use of natural isolates as a biologicals to mitigate biotic and abiotic stress response in plants. The identified natural isolates of rice microbiome can be employed for further characterization of key genes pertaining to defense responses when grown in As contaminated soil. Further research mainly focused on transcriptional and biochemical changes in rice plants growing As-contaminated soil exposed to *M. oryzae*, and co-inoculation of EA105 and EA106 provides insights into use of mixed beneficial microbes. The use of plant growth-promoting rhizobacteria such as EA105 and EA106 offer promise to be a better alternative as biocontrol agent for potential application in sustainable rice production of As-contaminated and *M. oryzae* predominant rice cultivated areas.

AUTHOR CONTRIBUTIONS

HPB conceived the research. VL and JC conducted the experiments and drafted the manuscript. HPB provided suggestions and improvements on the manuscript. All authors read and approved the manuscript.

ACKNOWLEDGMENTS

HPB acknowledges the support from DE-EPSCoR seed grant.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01514

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 06 January 2017
doi: 10.3389/fpls.2016.01975

Paxillus involutus-Facilitated Cd^{2+} Influx through Plasma Membrane Ca^{2+}-Permeable Channels Is Stimulated by H_2O_2 and H^+-ATPase in Ectomycorrhizal *Populus* × *canescens* under Cadmium Stress

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *14 October 2016*
Accepted: *13 December 2016*
Published: *06 January 2017*

Citation:
Zhang Y, Sa G, Zhang Y, Zhu Z, Deng S, Sun J, Li N, Li J, Yao J, Zhao N, Zhao R, Ma X, Polle A and Chen S (2017) Paxillus involutus-Facilitated Cd^{2+} Influx through Plasma Membrane Ca^{2+}-Permeable Channels Is Stimulated by H_2O_2 and H^+-ATPase in Ectomycorrhizal Populus × canescens under Cadmium Stress. Front. Plant Sci. 7:1975. doi: 10.3389/fpls.2016.01975

Using a Non-invasive Micro-test Technique, flux profiles of Cd^{2+}, Ca^{2+}, and H^+ were investigated in axenically grown cultures of two strains of *Paxillus involutus* (MAJ and NAU), ectomycorrhizae formed by these fungi with the woody Cd^{2+}-hyperaccumulator, *Populus* × *canescens,* and non-mycorrhizal (NM) roots. The influx of Cd^{2+} increased in fungal mycelia, NM and ectomycorrhizal (EM) roots upon a 40-min shock, after short-term (ST, 24 h), or long-term (LT, 7 days) exposure to a hydroponic environment of 50 μM $CdCl_2$. Cd^{2+} treatments (shock, ST, and LT) decreased Ca^{2+} influx in NM and EM roots but led to an enhanced influx of Ca^{2+} in axenically grown EM cultures of the two *P. involutus* isolates. The susceptibility of Cd^{2+} flux to typical Ca^{2+} channel blockers ($LaCl_3$, $GdCl_3$, verapamil, and TEA) in fungal mycelia and poplar roots indicated that the Cd^{2+} entry occurred mainly through Ca^{2+}-permeable channels in the plasma membrane (PM). Cd^{2+} treatment resulted in H_2O_2 production. H_2O_2 exposure accelerated the entry of Cd^{2+} and Ca^{2+} in NM and EM roots. Cd^{2+} further stimulated H^+ pumping activity benefiting NM and EM roots to maintain an acidic environment, which favored the entry of Cd^{2+} across the PM. A scavenger of reactive oxygen species, DMTU, and an inhibitor of PM H^+-ATPase, orthovanadate, decreased Ca^{2+} and Cd^{2+} influx in NM and EM roots, suggesting that the entry of Cd^{2+} through Ca^{2+}-permeable channels is stimulated by H_2O_2 and H^+ pumps. Compared to NM roots, EM roots exhibited higher Cd^{2+}-fluxes under shock, ST, and LT Cd^{2+} treatments. We conclude that ectomycorrhizal *P.* × *canescens* roots retained a pronounced H_2O_2 production and a high H^+-pumping activity, which activated PM Ca^{2+} channels and thus facilitated a high influx of Cd^{2+} under Cd^{2+} stress.

Keywords: ectomycorrhizal fungi, *Paxillus involutus*, MAJ, NAU, Cd^{2+}-hyperaccumulator, poplar, NMT

INTRODUCTION

The presence of highly toxic cadmium (Cd^{2+}) in the environment is a serious threat to human health as heavy metals can be enriched in plants and eventually enter the human body through the food chain (Nawrot et al., 2006; Kaplan et al., 2011). The genus *Populus* spp. is of particular interest for phytoremediation of Cd^{2+} pollution (Sell et al., 2005; Krpata et al., 2008, 2009; Kieffer et al., 2009; He et al., 2011, 2013, 2015; Ma Y. et al., 2014), due to its widespread distribution, rapid growth, and genotypic differences in response to ion-specific stress (Chen and Polle, 2010; Polle et al., 2013; Chen et al., 2014; Polle and Chen, 2015). *Populus tremula* (Kieffer et al., 2009) and *Populus* × *canescens* (He et al., 2011) have been recently identified as woody Cd^{2+}-hyperaccumulators. Cd^{2+} enrichment in these poplars (Kieffer et al., 2009; He et al., 2011; Ma Y. et al., 2014) exceed the threshold of 100 μg Cd^{2+} g^{-1} DW that has commonly been defined for hyperaccumulation (Milner and Kochian, 2008; Krämer, 2010). He et al. (2013) demonstrated that *P.* × *canescens* could detoxify Cd^{2+} by its sequestration in the bark.

In nature, poplar roots form symbioses with mycorrhizal fungi (Danielsen et al., 2012, 2013). For example, colonization of *P.* × *canescens* roots with the ectomycorrhizal fungus *Paxillus involutus* improves growth, primes for increased stress tolerance, increases nutrition, and regulates the ion balance under salt stress (Schützendübel and Polle, 2002; Gafur et al., 2004; Langenfeld-Heyser et al., 2007; Luo et al., 2009, 2011; Li J. et al., 2012; Ma X. et al., 2014). A notable finding was that *Paxillus involutus* ectomycorrhizas enhance both Cd^{2+} uptake and tolerance in *P.* × *canescens* (Ma Y. et al., 2014). Thus, ectomycorrhizal poplar plants offer a great potential for phytoremediation of Cd^{2+}-polluted soils (Sell et al., 2005; Krpata et al., 2008, 2009; Luo et al., 2014; Ma Y. et al., 2014).

Cd^{2+} is generally believed to enter plant cells through high affinity transporters responsible for the uptake of divalent cations (Cu^{2+}, Co^{2+}, Fe^{2+}, Ca^{2+}, Mn^{2+}, and Zn^{2+}; Liu et al., 1997; Clemens et al., 1998; Cohen et al., 1998; Hirschi et al., 2000; Thomine et al., 2000; Zhao et al., 2002; Cosio et al., 2004; Clemens, 2006; Roth et al., 2006). Cd^{2+} can even induce nutrient deficiencies by competing with the uptake of essential elements (Zhao et al., 2006; Papoyan et al., 2007; DalCorso et al., 2008; Gallego et al., 2012; Baliardini et al., 2015). On the other hand, elevated Ca^{2+} levels suppress Cd^{2+} uptake in different ecotypes of *Sedum alfredii* also supporting competition of Cd^{2+} uptake with nutrient cations (Lu et al., 2010). Transcript levels of the transporters involved in Cd^{2+} uptake and transport have been investigated in herbaceous and woody species (Kim et al., 2006; Plaza et al., 2007; Krämer, 2010; Migeon et al., 2010; Mendoza-Cózatl et al., 2011; Lin and Aarts, 2012). In poplar plants, a variety of heavy metal transporters, such as ZRT-IRT-like proteins (ZIP2, ZIP6.2), natural resistance associated macrophage proteins (NRAMP1.1, NRAMP1.3), ATP-binding cassette transporter C1 (ABCC1), heavy metal ATPase 4 (HMA4), ATP-binding cassette transporter in mitochondria (ATM3), have been suggested to play pivotal roles in Cd^{2+} transport and detoxification (Ma Y. et al., 2014; He et al., 2015). In addition to these heavy metal transporters, ion channels in the plasma membrane (PM) that are permeable to Cd^{2+} contribute the Cd^{2+} uptake (Li et al., 2012a; Sun et al., 2013a,b; He et al., 2015). High external Cd^{2+} concentrations establish a large electrochemical gradient facilitating the rapid movement of Cd^{2+} ions through Cd^{2+}-permeable channels. Perfus-Barbeoch et al. (2002) suggested that Cd^{2+} enters root cells via plasma membrane (PM) Ca^{2+} channels.

Ca^{2+} channels in the PM have been characterized by electrophysiological measurements involving incorporation of plasma-membrane vesicles into planar lipid bilayers (PLB, White, 2000) and patch clamping (Perfus-Barbeoch et al., 2002). According to their electrophysiological properties, the channels can be divided into depolarisation-, hyperpolarisation-, elicitor-activated, and voltage-insensitive channels (Thuleau et al., 1998; White, 2000). These channels display different sensitivities to typical inhibitors of Ca^{2+} channels, such as La^{3+}, Gd^{3+}, TEA, and verapamil. Specifically, verapamil and TEA inhibit depolarisation-activated Ca^{2+} channels, such as the wheat root channel rca (Piñeros and Tester, 1997; White, 1998), and rye root voltage-dependent cation channel 2, VDCC2 (White, 1998). La^{3+} shares a high similarity to another trivalent cation, Gd^{3+}. Both cations are able to inhibit three distinct classes of Ca^{2+} channels, including depolarisation-activated Ca^{2+} channels, rca (Piñeros and Tester, 1997; White, 1998), hyperpolarisation-activated Ca^{2+} channels (HACCs) in onion bulb epidermis (Pickard and Ding, 1993), voltage-insensitive channels such as Arabidopsis root epidermal non-selective cation channels (NSCCs; Demidchik et al., 2002), and large-conductance elicitor-activated channel (LEAC) in parsley cell suspension (Zimmermann et al., 1997). Ca^{2+} channels in the PM are permeable to divalent (including Ca^{2+}, Mg^{2+}, Ba^{2+}, Sr^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}; Cosgrove and Hedrich, 1991; Ping et al., 1992; Pickard and Ding, 1993; Thuleau et al., 1994a,b; Gelli and Blumwald, 1997; Zimmermann et al., 1997; White, 1998; Grabov and Blatt, 1998, 1999) and monovalent cations (Na^{+}, K^{+}, Cs^{+}, Li^{+}, Rb^{+}; Cosgrove and Hedrich, 1991; Pickard and Ding, 1993; Zimmermann et al., 1997; Piñeros and Tester, 1997; White, 1998). In accordance with the suggestion that Cd^{2+} ions can be transported into cells through Ca^{2+} channels (Perfus-Barbeoch et al., 2002; Gallego et al., 2012; Li et al., 2012b) the permeability for Cd^{2+} through wheat VDCC2 was detected when the plasma membrane derived from root cells was incorporated into PLB (White, 1998). Using the whole-cell patch-clamp technique, Perfus-Barbeoch et al. (2002) confirmed that Cd^{2+} permeates through the PM Ca^{2+} channels in Arabidopsis guard cells. The Cd^{2+} influx was effectively blocked by Ca^{2+} channel blockers, e.g., $LaCl_3$ and verapamil in *Suaeda salsa* (Li et al., 2012a), *Populus euphratica* (Sun et al., 2013b), and *P. tremula* × *P. alba* (He et al., 2015), further indicating that Cd^{2+} ions penetrate into plant cells through Ca^{2+}-permeable channels.

It is possible that hydrogen peroxide (H_2O_2) stimulates the entry of Cd^{2+} through PM Ca^{2+} channels as the activity of these channels has been shown to be stimulated by H_2O_2. Pei et al. (2000) found that H_2O_2 activates the PM Ca^{2+} channels, leading to a subsequent rise of cytosolic Ca^{2+} in *Arabidopsis* guard cells. Demidchik et al. (2007) observed a transient increase

of Ca^{2+} influx in the root epidermis when exogenous H_2O_2 was applied to *Arabidopsis thaliana*. In NaCl-stressed *P. euphratica* cells, Ca^{2+} influx through Ca^{2+} channels was activated by H_2O_2 (Sun et al., 2010). Recently, H_2O_2 was shown to accelerate Cd^{2+} influx in *P. euphratica* cells, while the H_2O_2-stimulated Cd^{2+} influx was blocked by $LaCl_3$ (Sun et al., 2013b; Han et al., 2016). Moreover, the application of a H_2O_2 scavenger, catalase, lowered the Cd^{2+} influx across the PM in Cd^{2+}-stressed *P. euphratica* cells (Sun et al., 2013b). In Cd^{2+}-treated *P. euphratica* cells, hydrogen sulfide was found to reduce Cd^{2+} influx through down-regulation of H_2O_2-stimulated Cd^{2+} transport across the PM Ca^{2+} channels (Sun et al., 2013b). H_2O_2 is not only produced in Cd^{2+}-stressed poplar cells (Sun et al., 2013b; Han et al., 2016) and roots (Ma Y. et al., 2014; He et al., 2015), but is also massively enriched in *Populus* × *canescens*–*Paxillus involutus* ectomycorrhizal associations (Gafur et al., 2004; Langenfeld-Heyser et al., 2007). Thus, it can be speculated that the fungal-elicited H_2O_2 accelerates the entry of Cd^{2+} through PM Ca^{2+} channels. However, this hypothesis needs to be clarified by further electrophysiological investigations.

In addition to H_2O_2, the PM H^+-ATPase plays a crucial role in accelerating Cd^{2+} transport in poplar roots (Ma Y. et al., 2014; He et al., 2015). He et al. (2015) demonstrated that the net Cd^{2+} influx was pH-dependent in poplar roots and effectively blocked by inhibitors of H^+-pumps. Ma Y. et al. (2014) showed that the active PM H^+-ATPase-driven Cd^{2+} uptake is a major factor for increased Cd^{2+} accumulation in ectomycorrhizal (EM) poplar plants. They suggested that the EM-induced transcripts of *HA2.1* and *AHA10.1* genes, encoding PM H^+-ATPases in *P.* × *canescens*, may result in H^+-pump-stimulated Cd^{2+} enrichment (Ma Y. et al., 2014). In agreement with this suggestion transgenic poplars that were more Cd^{2+} tolerant by overexpression of γ-glutamylcysteine synthetase, showed upregulated transcript levels of *VHA1.1*, *HA2.1* and *AHA10.1* and a high Cd^{2+} uptake rate (He et al., 2015). The PM H^+-ATPases maintain a H^+ gradient across the membrane to promote active transport of essential elements across the PM (Beritognolo et al., 2007; Ma et al., 2010; Sun et al., 2010; Luo et al., 2013). Increased H^+-pumping activities have been well characterized in arbuscular mycorrhizal associations (Ramos et al., 2005; Rosewarne et al., 2007) and in ectomycorrhizal associations formed by *Paxillus involutus* (strains MAJ and NAU) with *Populus* × *canescens* (Li J. et al., 2012). We have previously shown that the upregulated H^+-pumping activities in *Paxillus involutus*-*Populus* × *canescens* symbiosis resulted in enhanced Ca^{2+} uptake and enrichment (Li J. et al., 2012). Demidchik et al. (2002) proposed that voltage modulation of the co-existing NSCC/HACC by PM H^+-ATPase would be a potent regulator for Ca^{2+} entry to the root cell cytoplasm. The high H^+-pumping activity leads to hyperpolarization of the PM and, thus, may increase Cd^{2+} influx through hyperpolarisation-activated Ca^{2+} channels. However, it is unknown whether the PM H^+-ATPases could stimulate the entry of Cd^{2+} through Ca^{2+}-permeable channels in ectomycorrhizal plants.

The two *P. involutus* strains, MAJ and NAU, form different colonization structures with *P.* × *canescens* roots (Gafur et al., 2004). Strain MAJ forms a typical hyphal mantle and Hartig net with roots of *P.* × *canescens*, while NAU is unable to intrude between the host cells and forms only a hyphal mantle ensheathing the root tips (Gafur et al., 2004). The colonization of *P.* × *canescens* roots with the competent strain MAJ results in enriched Cd^{2+} levels under Cd^{2+} stress (Ma Y. et al., 2014). Whether the incompatible fungal isolate NAU also affects the Cd^{2+} entry into *P.* × *canescens* host plants needs to be clarified.

In this study, we used a non-invasive micro-test technique (NMT) to measure fluxes of Cd^{2+}, Ca^{2+} and H^+ in Cd^{2+}-stressed roots of non-mycorrhizal (NM) and ectomycorrhizal *P.* × *canescens* plants colonized with *Paxillus involutus* strains, MAJ and NAU. The aim was to elucidate whether the Cd^{2+} influx through Ca^{2+}-permeable channels is stimulated by H_2O_2 and H^+-ATPase in ectomycorrhizal roots since the ectomycorrhizas exhibit enhanced H_2O_2 production and upregulated H^+-pumping activity. NMT microelectrodes measure the ion fluxes on the surface of the tissues, which are either the plant root cells for the NM plants or the fungal hyphae forming the mantle structure ensheathing the roots. To discriminate between potentially different Cd^{2+} effects on fungus and plant roots, fluxes of Cd^{2+}, Ca^{2+} and H^+ were examined for pure fungal mycelia of the two *P. involutus* isolates, MAJ and NAU, in addition to flux recordings on NM and EM roots. Furthermore, flux profiles of Cd^{2+} and Ca^{2+} were recorded in *P. involutus*-inoculated roots after 7 days of co-culture. The aim was to determine whether flux profiles of mature EM associations resemble the pattern of those from host roots at early stages of fungal colonization when the host is known to activate transient defense responses in contrast to the mature ectomycorrhizal symbioses (Duplessis et al., 2005).

MATERIALS AND METHODS

Fungus and Plant Cultures for EM Colonization

The *Paxillus involutus* isolates MAJ and NAU, obtained from the Büsgen Institute: Institute of Forest Botany and Tree Physiology (Göttingen University, Germany), were grown on 2% modified Melin Norkrans (MMN) agar medium ($g \cdot L^{-1}$): KH_2PO_4 0.5, $(NH_4)_2SO_4$ 0.25, $MgSO_4 \cdot 7H_2O$ 0.15, $CaCl_2 \cdot 2H_2O$ 0.05, NaCl 0.025, $FeCl_3 \cdot 6H_2O$ 0.01, thiamine HCl 0.0001, glucose 10, malt extract 3, pH 5.2 (Gafur et al., 2004; Li J. et al., 2012). Prior to the colonization, the fungi were pre-grown on the agar culture medium for 1 week in petri dishes (diameter 90 mm) and kept in darkness at 23°C.

Plantlets of *Populus* × *canescens* (a hybrid of *Populus tremula* × *Populus alba*) were propagated by micropropagation as described by Leple et al. (1992). Regenerated *P.* × *canescens* plants were grown for 3–4 weeks on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Uniform plants with sufficient roots were used for ectomycorrhization. The colonization of *P.* × *canescens* with *Paxillus involutus* strains MAJ and NAU was followed the procedures described by Gafur et al. (2004). In brief, rooted plantlets from sterile culture were placed on the MMN agar medium in the presence or absence of EM mycelium. After fungal inoculation, the petri dishes were

sealed with Parafilm and covered with aluminum foil to keep the roots in darkness. During the period of incubation, the temperature in the climate chamber was maintained at 23°C with a light period of 16 h (6:00 AM–22:00 PM). Photosynthetic active radiation (PAR) of 200 μmol m^{-2} s^{-1} was supplied by cool white fluorescent lamps. After 1 month of inoculation, EM and NM root tips for anatomical investigations were embedded, stained, and photographed as described previously (Gafur et al., 2004). EM and NM plants with similar height and growth performance were used for $CdCl_2$ treatment.

Liquid Culture of Fungi

Liquid culture of *P. involutus* was grown as previously described (Ott et al., 2002; Langenfeld-Heyser et al., 2007; Li J. et al., 2012). In brief, mycelium from the agar plate was homogenized, transferred into 100 mL of liquid medium (pH 4.8) in flasks, and incubated on a rotary shaker in darkness (150 rpm, 23°C). *P. involutus* in submerged culture grew in the form of compact spherical masses of mycelium (pellets). For Cd^{2+} shock treatment, sterile filtered $CdCl_2$ solutions were added to achieve final concentrations of 50 μM. After ST (24 h) or LT (7 days) treatment, axenic cultures of MAJ and NAU were used for steady flux measurements of Cd^{2+}, H^+, and Ca^{2+}.

Cadmium Treatment

Ectomycorrhizal and non-mycorrhizal plants were carefully removed from MMN agar medium. Rooted plantlets were cultivated in individual pots containing hydroponic MS nutrient solution (MS medium without agar and sucrose) (Murashige and Skoog, 1962). Plants were covered with plastic bags to reduce the rapid water loss in a growth room. NM and EM plantlets were subjected to 50 μM $CdCl_2$ for a short-term (ST) exposure, 24 h or a long-term (LT) exposure for 7 days. The required amount of $CdCl_2$ was added to the MS nutrient solution. Control plants were treated in the same manner without the addition of $CdCl_2$. The plants were maintained at 23°C with a light period of 16 h (6:00 AM–22:00 PM) and PAR was 200 μmol m^{-2} s^{-1}. Plants were continuously aerated by passing air to hydroponic MS nutrient solution, which was regularly renewed. Steady fluxes of Cd^{2+}, Ca^{2+} and H^+ in NM and EM roots were examined after 24 h and 7 days of $CdCl_2$ treatment. In addition, ST-induced alterations of Cd^{2+} and Ca^{2+} fluxes were also examined in non-inoculated and *P. involutus*-inoculated roots after 7 days of co-culture.

Measurements of Net Cd^{2+}, Ca^{2+}, and H^+ Fluxes

Preparations of Ion-Selective Microelectrodes

Non-invasive Micro-test Technique (NMT-YG-100, Younger USA LLC, Amherst, MA01002, USA) with ASET 2.0 (Sciencewares, Falmouth, MA 02540, USA) and iFluxes 1.0 Software (Younger USA, LLC, Amherst, MA 01002, USA) was used to monitor fluxes of Cd^{2+}, Ca^{2+} and H^+ in EM and NM roots (Sun et al., 2009a,b; Sun et al., 2013a,b; Ma X. et al., 2014). Ion-selective electrodes were prepared as described in Sun et al. (2009a, 2013a) and Ma X. et al. (2014). Briefly, pre-pulled and silanized glass micropipettes (diameter 4–5 μm, XY-DJ-01; Xuyue (Beijing) Science and Technology Co. Ltd., Beijing, China) were back-filled with backfilling solution [Cd^{2+} microelectrodes: 10 mM $Cd(NO_3)_2$ and 0.1 mM KCl; Ca^{2+} microelectrodes: 100 mM $CaCl_2$; H^+ microelectrodes: 40 mM KH_2PO_4 and 15 mM NaCl, pH 7.0] to a length of 1.0 cm from the tip. Then the micropipettes were front-filled with 15 μm columns of selective liquid ion exchange cocktails (LIXs) (Cd: Fluka 20909, Sigma–Aldrich, St Louis, MO, USA; Ca: Fluka 21048; H: Fluka 95293 Fluka Chemie GmbH, Buchs, Switzerland). An Ag/AgCl wire electrode holder (XYEH01-1; Xuyue Sci. and Tech. Co., Ltd.) was inserted in the back of the electrode to create an electrical contact with the electrolyte solution. DRIREF-2 (World Precision Instruments, Inc., Sarasota, FL, USA) was used as the reference electrode (CMC-4). Prior to the measurements, ion-selective microelectrodes for the target ions were calibrated by the following standard solution:

(1) Cd^{2+}: 0.01, 0.05, 0.1 mM (Cd^{2+} concentration was 0.05 mM in the measuring solution);
(2) Ca^{2+}: 0.1, 0.5, 1.0 mM (Ca^{2+} was 0.2 mM in the measuring buffer);
(3) H^+: pH 4.2, 5.2, 6.2 (pH of the measuring solution was adjusted to 5.2 with KOH and HCl for root samples).

Electrodes were used when the Nernstian slopes in ranges of 29 ± 3 mV/decade (Cd^{2+}, Ca^{2+}) and 58 ± 5 mV/decade (H^+). The flux rate was calculated on the basis of Fick's law of diffusion:

$$J = -D\,(dc/dx),$$

where J is the ion flux in the x direction, D is the ion diffusion coefficient in a particular medium, dc represents the ion concentration difference, dx is the microelectrode movement between two positions, and dc/dx represents the ion concentration gradient. As part of the NMT system, ASET software [Science Wares (East Falmouth, MA, USA) and Applicable Electronics], was used for data and image acquisition, preliminary processing, control of three-dimensional electrode positioner and stepper-motor-controlled fine focus of the microscope stage.

Experimental Protocols for Steady-State Flux Measurements

Cd^{2+}, Ca^{2+}, and H^+ fluxes were non-invasively measured by moving the ion-selective microelectrode between two positions close to the materials in a preset excursion (30 μm for excised roots and fungal mycelia) at a programmable frequency in the range of 0.3–0.5 Hz. *P. involutus* mycelia, EM and NM roots from the ST and LT $CdCl_2$ treatments were rinsed with re-distilled water for 2–3 times, and then incubated in the basic measuring solution to equilibrate for 25 min. The concentration gradients of Cd^{2+}, Ca^{2+}, and H^+ were measured as previously described (Li J. et al., 2012; Lu et al., 2013; Sun et al., 2013a,b).

(1) Cd^{2+} measuring solutions: 0.1 mM KCl, 0.1 mM $MgCl_2$, 0.05 mM $CaCl_2$ and 0.05 mM $CdCl_2$, pH was adjusted to 5.2 with KOH and HCl;

(2) Ca^{2+} measuring solutions: 0.1 mM NaCl, 0.1 mM $MgCl_2$, 0.1 mM KCl, and 0.2 mM $CaCl_2$, pH was adjusted to 5.2 with KOH and HCl;
(3) H^+ measuring solutions: 0.1 mM NaCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 0.5 mM KCl, pH 5.2 was adjusted with KOH and HCl.

The steady fluxes of roots were then recorded 100 μm from the apex and conducted along the root axis until 2300 μm at intervals of 200–300 μm. The fluxes of each measuring point in apical regions were continuously recorded for 6–8 min. For *P. involutus* mycelia, Cd^{2+}, Ca^{2+}, and H^+ fluxes were measured around the surface of pelleted hyphae over a recording period of 30 min.

Transient Flux Recording

Paxillus involutus fungal mycelia and roots sampled from EM and NM plants were immobilized in the measuring solutions of Cd^{2+} (0.1 mM KCl, 0.1 mM $MgCl_2$, 0.05 mM $CaCl_2$, pH 5.2); Ca^{2+} (0.1 mM NaCl, 0.1 mM $MgCl_2$, 0.1 mM KCl, and 0.2 mM $CaCl_2$, pH 5.2) and H^+ (0.1 mM NaCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 0.5 mM KCl, pH 5.2) for 25 min equilibration. Then the steady-state fluxes in fungal mycelia and the root apical region (100 μm from the root apex) were continuously recorded for 5 min prior to the $CdCl_2$ shock. $CdCl_2$ stock (100 μM) was slowly added to the measuring solution using a pipette until the final Cd^{2+} concentration reached 50 μM. Afterward, transient kinetics of Cd^{2+}, Ca^{2+}, and H^+ were restarted and continued for 40 min. The data measured during the first 1–2 min was discarded, due to the effects of the diffusing stock solution. The high flux of Cd^{2+}, Ca^{2+}, and H^+ during the following 2 min was defined as peaking values.

Effects of H_2O_2 on $CdCl_2$-altered transient kinetics of Cd^{2+} and Ca^{2+} were also examined in NM and EM roots. Following the $CdCl_2$ shock (50 μM) as described above, H_2O_2 (1.0 mM) was introduced to the measuring solution and transient kinetics of Cd^{2+} and Ca^{2+} were recorded for 20 min.

Fungal mycelia were exposed to 50 μM $CdCl_2$ to induce a shock. Cd^{2+}, Ca^{2+}, and H^+ fluxes were monitored over a continuous recording period of 40 min. For transient flux kinetics, the data measured during the first 1–2 min were discarded due to the diffusion effects of stock addition.

Effects of Ca^{2+} on Sensitivity of Cd^{2+} Electrodes

To determine whether Ca^{2+} ions compete with Cd^{2+} to penetrate across PM Ca^{2+}-permeable channels, the effects of additional Ca^{2+} ions on Cd^{2+} electrodes was examined. Cd^{2+} calibrating solutions were added with 0, 0.01, 0.025, 0.05, 0.1, 0.2, 0.5, 1.0, or 2.0 mM Ca^{2+}. Then Cd^{2+} microelectrodes were calibrated in Ca^{2+}-supplemented solutions as described above. Moreover, the Nernst slope and intercept of the Cd^{2+} electrodes were calibrated in the measuring solution containing 0.1 mM KCl, 0.1 mM $MgCl_2$, and 0.05 mM $CaCl_2$.

Flux Oscillations

Oscillations in membrane-transport activity are ubiquitous in plant response to salinity, temperature, osmotic, hypoxia, and pH stresses (Shabala et al., 2006). In our study, rhythmic (ultradian) flux oscillations in NM and EM *P.* × *canescens* roots were not noticeable as that observed in herbaceous species (Shabala et al., 1997, 2003, 2006; Shabala and Knowles, 2002). This finding is presumably due to a lower growth rate of woody roots compared with crop species (Li J. et al., 2012). The flux oscillations of the measured ions, e.g., H^+, Ca^{2+}, and Cd^{2+}, were more like fluctuations as previously reported in poplar roots (e.g., Na^+, K^+, H^+, and Ca^{2+}; Li J. et al., 2012). In this study, H^+, Ca^{2+}, and Cd^{2+} fluxes were recorded for 6–8 min at each point, which is long enough to cover oscillatory periods of measured ions.

Inhibitor and Stimulator Treatment

In this study, the effects of Ca^{2+}, pH, H_2O_2, and PM transporter and channel inhibitors on Cd^{2+}-altered ion flux profiles were examined in fungal mycelia and roots (NM and EM). Briefly,

Series 1: Ca^{2+} channel inhibitors. NM and EM roots were pre-treated with or without $LaCl_3$ (5 mM; Sun et al., 2010; Li et al., 2012b), $GdCl_3$ (500 μM, Demidchik et al., 2007, 2009; Sun et al., 2012), TEA (50 μM, White, 1998; Li J. et al., 2012), or verapamil (20 μM, Li et al., 2012a; He et al., 2015) for 24 h in the presence and absence of 50 μM $CdCl_2$. Fungal mycelia of the two *P. involutus* isolates, MAJ and NAU, were subjected to 0 or 5 mM $LaCl_3$ treatment for 24 h supplemented with or without 50 μM $CdCl_2$.

Series 2: Ca^{2+}. After being subjected to Cd^{2+} stress ($CdCl_2$, 50 μM) for 24 h, NM and EM roots were then exposed to 25, 50, or 100 μM $CaCl_2$ for flux recordings in the presence of $CdCl_2$.

Cd^{2+} and Ca^{2+} fluxes in Series 1 and 2 were measured along root axes, 100–2,300 μm from the apex, at intervals of 200–300 μm. In *P. involutus* mycelia, Cd^{2+} and Ca^{2+} fluxes were continuously measured around the surface of pelleted hyphae over a recording period of 30 min.

Series 3: Hydrogen peroxide. NM and EM roots were sampled and immobilized in Cd^{2+} or Ca^{2+} measuring solutions for transient flux recordings in the apical region (100 μm from the root apex). The steady-state fluxes were continuously recorded for 10–20 min prior to the $CdCl_2$ shock. $CdCl_2$ stock (100 μM) was slowly added to the measuring solution until the final Cd^{2+} concentration reached 50 μM and transient kinetics of Cd^{2+} and Ca^{2+} were continuously for 20–30 min. Afterward, H_2O_2 (1.0 mM) was slowly added to the measuring solution and transient kinetics of Cd^{2+} and Ca^{2+} were restarted and continued for 20 min.

Series 4: ROS scavenger. NM and EM roots were pre-treated with or without 1, 3-Dimethyl-2-thiourea (DMTU, 5 mM, Chung et al., 2008; Sun et al., 2010) for 24 h in the presence and absence of 50 μM $CdCl_2$. Then Cd^{2+} and Ca^{2+} fluxes were measured along root axes, 100–2,300 μm from the apex, at intervals of 200–300 μm.

Series 5: External pH. NM and EM roots were pre-treated with 50 μM $CdCl_2$ for 24 h prior to flux measurements. Cd^{2+} and Ca^{2+} fluxes along root axes (100–2,300 μm from the apex) were recorded in Cd^{2+} or Ca^{2+} measuring solutions at pH 5.2, 6.2, or 7.2, respectively.

Series 6: PM H^+-ATPase inhibitor. NM and EM roots were pre-treated with or without sodium orthovanadate (500 μM, Sun

et al., 2010; Lu et al., 2013) for 24 h in the presence and absence of 50 μM $CdCl_2$. Then H^+, Cd^{2+}, and Ca^{2+} fluxes were measured along root axes, 100–2,300 μm from the apex, at intervals of 200–300 μm. *P. involutus* isolates, MAJ and NAU, were exposed to 0 or 500 μM sodium orthovanadate for 24 h prior to a 30-min of continuous recording of H^+ flux.

Measurements of Net H_2O_2 Fluxes

An H_2O_2-sensititive microelectrode [tip diameter 2–3 μm, XY-DJ-502, Xuyue (Beijing) Science and Technology Co. Ltd., Beijing, China] was used to monitor H_2O_2 fluxes in EM and NM roots. H_2O_2 microelectrodes were prepared according to the method described by Twig et al. (2001). Before the measurement, H_2O_2 microelectrode was polarized at +0.60 V against an Ag/AgCl reference electrode. Thereafter, the microelectrodes were calibrated by the standard solution: 0.01, 0.1 and 1 mM H_2O_2. Roots sampled from control and $CdCl_2$ (50 μM,30 min)-treated EM and NM plants were immobilized in the measuring solution (0.1 mM NaCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 0.5 mM KCl, pH was adjusted to 5.2 with KOH and HCl) and equilibrated for 25 min. The fluxes were recorded 100 μm from the apex and conducted along the root axis until 2300 μm, at intervals of 200–300 μm, and then calculated.

Data Analysis

Ionic fluxes were calculated using the program JCal V3.2.1, a free MS Excel spreadsheet, which was developed by the Yue Xu[1]. The experimental data were subjected to SPSS (SPSS Statistics 17.0, 2008) for statistical tests and analyses. Unless otherwise stated, $P < 0.05$ was considered as significant.

RESULTS

Cd^{2+}-Altered Ion Flux Profiles in *Paxillus involutus,* and Roots of NM and EM Poplar

Cd^{2+} Fluxes

We recorded transient Cd^{2+} kinetics upon Cd^{2+} shock at the root apex (100 μm from the root tip; **Figure 1A**), where a vigorous ion flux (e.g., Na^+, K^+, Ca^{2+}, Cd^{2+}, Cl^-) is usually observed in woody and herbaceous plants (Sun et al., 2009a,b; Li J. et al., 2012; Lu et al., 2013; Han et al., 2014). The addition of $CdCl_2$ (50 μM) caused an immediate Cd^{2+} influx in both EM and NM roots which declined with increasing duration of Cd^{2+} exposure (40 min; **Figure 1A**). The peak and mean flux rate of Cd^{2+} in EM roots with MAJ were significantly (13.5 and 38.8%) higher than in NM roots or NAU-colonized roots (**Figure 1A**). Similar to the Cd^{2+} kinetics in EM roots, an instantaneous increase in the Cd^{2+} influx was detected in pure *P. involutus* mycelia after $CdCl_2$ exposure (50 μM; **Figure 1A**). However, the fungal Cd^{2+} influx remained constant over the recording period (40 min; **Figure 1A**) with significantly higher flux rates in MAJ (75.4 pmol cm^{-2} s^{-1}) than in NAU (25.9 pmol cm^{-2} s^{-1}).

After ST (24 h) or LT (7 days) exposure to 50 μM $CdCl_2$ in hydroponic conditions, steady-state Cd^{2+} flux was recorded along root axis (100–2,300 μm from the apex) at intervals of 200–300 μm (**Figure 2**). In NM roots, ST and LT stress caused a net Cd^{2+} influx with an overall mean of 28.9 pmol cm^{-2} s^{-1} along the whole measured distance; LT treatment resulted in a higher flux rate at the region 100–1,000 μm from the apex than at more distant root positions (**Figure 2**). A similar trend was observed in the Cd^{2+}-stressed EM roots, though mean Cd^{2+} fluxes in MAJ- and NAU-ectomycorrhizal roots were 43.1 and 32.0% higher than those of the NM roots under ST and LT stress (**Figure 2**). The mycelia of the two *P. involutus* strains, MAJ and NAU, exhibited a stable Cd^{2+} influx under ST and LT stress, although the $CdCl_2$-induced Cd^{2+} influx was typically higher under LT conditions, 68.9 pmol cm^{-2} s^{-1}, compared with ST treatment, 27.1 pmol cm^{-2} s^{-1} (**Figure 2**). Cd^{2+}-induced alterations of Cd^{2+} flux were also examined in non-inoculated and *P. involutus*-inoculated roots after 7 days of co-culture. NAU- and MAJ-colonized roots showed larger flux rates than non-inoculated roots after ST Cd^{2+} stress (Supplementary Figure S1A).

Our data show that *P. involutus* mycelia and EM roots both exhibited an enhanced Cd^{2+} uptake upon Cd^{2+} shock, ST, or LT treatment (**Figures 1A** and **2**). Unexpectedly, the Cd^{2+} influx in EM roots did not show a high correlation to the flux rate of Cd^{2+} in fungal hyphae under various treatments (shock, ST, or LT, Supplementary Figure S2). However, a relatively high correlation between EM and NM roots was observed especially in response to Cd^{2+} shock (Supplementary Figure S2). This result supports that in the ectomycorrhizal symbioses the continuous Cd^{2+} entry detected by NMT microelectrodes depends on the uptake capacity of inner root cells and that in the plant–fungal interaction divergent regulation of fungal Cd^{2+} transport compared with pure mycelium must take place.

Ca^{2+} Fluxes

In the absence of Cd^{2+} stress, poplar roots exhibited a net Ca^{2+} influx, with a greater flux rate in MAJ- and NAU-ectomycorrhizal roots, 26.9 pmol cm^{-2} s^{-1}, than in NM roots, 9.6 pmol cm^{-2} s^{-1} (**Figure 1B**). Similarly, the mycelia of the two strains exhibited a stable and steady influx of Ca^{2+} (162.3 pmol cm^{-2} s^{-1}), which is ca. 6.0-fold higher than that detected in EM roots (**Figure 1B**). $CdCl_2$ shock (50 μM) caused a transient Ca^{2+} efflux in NM and EM roots with maximum values ranging from 10.9 to 14.8 pmol cm^{-2} s^{-1} (**Figure 1B**). Thereafter, the direction shifted toward an influx and the mean flux over the recording period then declined in EM roots, or displayed a net efflux in NM roots (**Figure 1B**). In contrast to NM and EM roots, Cd^{2+} addition markedly increased the Ca^{2+} influx in the hyphae of pure mycelium, typically with higher flux rates in strain NAU than in MAJ in the first 20 min of Cd^{2+} application (**Figure 1B**). Under ST and LT treatment, Cd^{2+} stress caused a marked decline of Ca^{2+} influx along the root axis (**Figure 3**). MAJ- and NAU-ectomycorrhizal roots maintained 40.5 and 20.6% higher Ca^{2+} fluxes than NM roots under ST and LT stress (**Figure 3**). In the hyphae of the two fungal strains, the Ca^{2+} influx was enhanced by ST and LT treatments (**Figure 3**), similar to the shock treatment (**Figure 1B**). We observed that the

[1]http://www.xuyue.net/

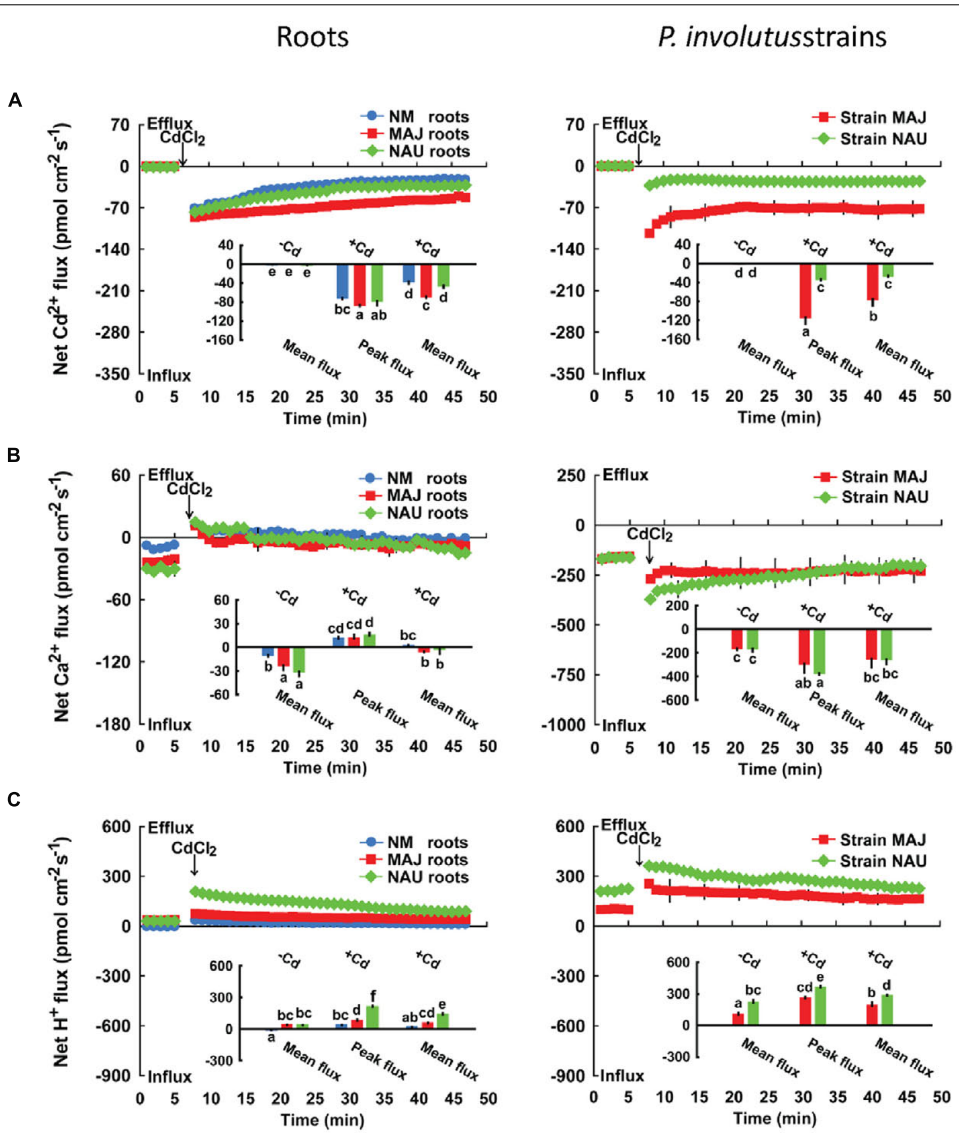

FIGURE 1 | Effects of $CdCl_2$ on transient kinetics of Cd^{2+}, Ca^{2+}, and H^+ in *Populus* × *canescens* roots and *Paxillus involutus* strains MAJ and NAU. Cd^{2+} **(A)**, Ca^{2+} **(B)**, and H^+ **(C)** kinetics were recorded before and after the required amount of 50 μM $CdCl_2$ was introduced into the measuring chamber. Prior to the $CdCl_2$ shock, steady-state fluxes of Cd^{2+}, Ca^{2+}, and H^+ in ectomycorrhizal (MAJ and NAU) and non-mycorrhizal (NM) *P.* × *canescens* roots (measuring site was ca. 100 μm from the root tip) and *P. involutus* isolates were monitored for approximately 5 min. Transient kinetics of Cd^{2+}, Ca^{2+}, and H^+ were recorded after the required amount of 50 μM $CdCl_2$ was introduced into the measuring solution. Inserted sections show the peaking and/or mean values of Cd^{2+}, Ca^{2+}, and H^+ flux before (−Cd) and after (+Cd) the addition of $CdCl_2$. Columns represent the mean of four to five individual plants or axenic EM cultures (pelleted hyphae), and bars represent the standard error of the mean. Different letters, a, b, c, d, e, and f, indicate significant difference at $P < 0.05$ between treatments.

flux rate in the two strains declined with increasing duration of hydroponic culture regardless of control and Cd^{2+} treatments (**Figure 3**). Non-inoculated *P.* × *canescens* roots exhibited a net Ca^{2+} influx under unstressed control conditions and the Ca^{2+} influx was stimulated by 7 days inoculation with MAJ and NAU (Supplementary Figure S1B). ST-treated *P. involutus*-inoculated roots retained higher Ca^{2+} influx than non-inoculated roots although the Ca^{2+} influx in poplar roots was lowered by Cd^{2+} stress (Supplementary Figure S1B).

It has been suggested that the Ca^{2+} enrichment in EM roots was associated with the *P. involutus* fungal hyphae exhibiting a high capacity for Ca^{2+} uptake (**Figures 1B** and **3**; Li J. et al., 2012; Ma X. et al., 2014). However, the Ca^{2+} influx in EM roots was not evidently correlated to the flux rate of Ca^{2+} in fungal hyphae under Cd^{2+} shock, ST, or LT (Supplementary Figure S3). Unexpectedly, the Ca^{2+} flux in EM roots was even negatively correlated to the flux rate of Ca^{2+} in fungal hyphae after a shock treatment (Supplementary Figure S3). The observed correlation of Ca^{2+} fluxes between EM roots and NM roots (Supplementary Figure S3) supports that the Ca^{2+} flow was mainly the consequence of host roots in the Cd^{2+}-stressed ectomycorrhizal symbioses.

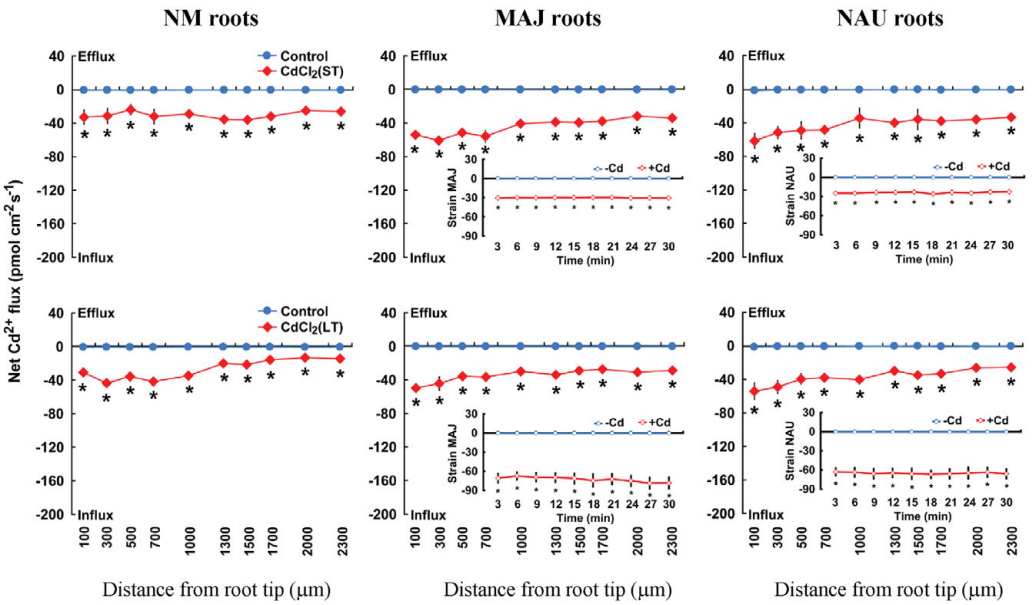

FIGURE 2 | Effects of $CdCl_2$ on steady Cd^{2+} fluxes in *Populus* × *canescens* roots and *Paxillus involutus* strains MAJ and NAU. *P. involutus* isolates, ectomycorrhizal (MAJ and NAU) and non-mycorrhizal (NM) *P.* × *canescens* plants were subjected to short-term (ST, 24 h) and long-term (LT, 7 d) exposure to 50 μM $CdCl_2$, respectively. Control roots and axenic mycelia were well fertilized but treated without $CdCl_2$. Cd^{2+} fluxes in poplar roots were measured along root axis, 100–2,300 μm from the apex, at intervals of 200–300 μm. Cd^{2+} fluxes of *P. involutus* isolates MAJ and NAU were measured along the surface of pelleted hyphae over a recording period of 30 min. Inserted sections show the Cd^{2+} fluxes in *P. involutus* isolates after short-term (ST, 24 h) or long-term (LT, 7 days) $CdCl_2$ treatment. Each point is the mean of 4–5 individual plants or axenic EM cultures (pelleted hyphae), and bars represent the standard error of the mean. Asterisks denote significant difference at $P < 0.05$ between treatments.

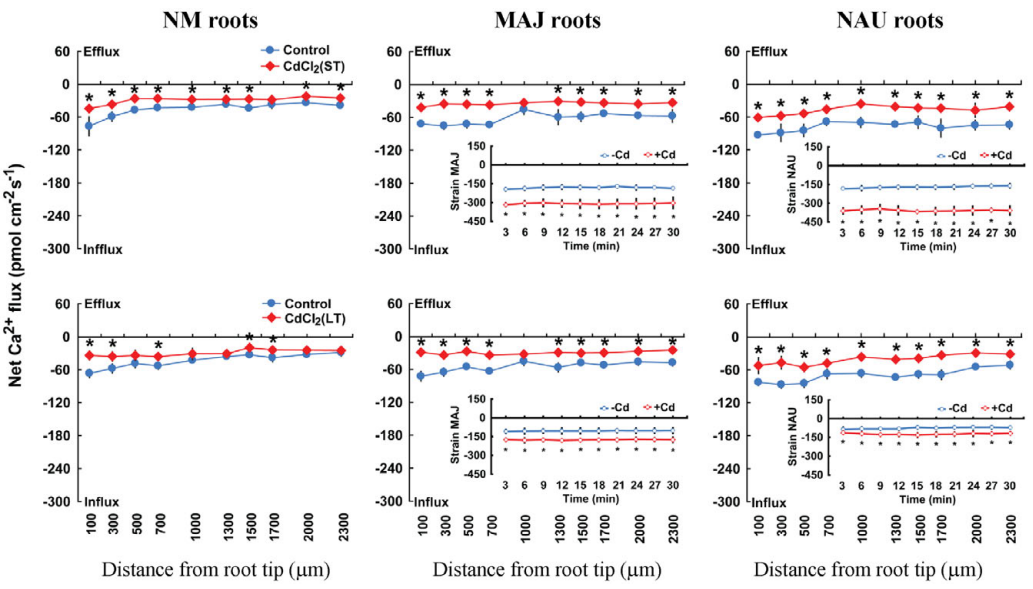

FIGURE 3 | Effects of $CdCl_2$ on steady Ca^{2+} fluxes in *Populus* × *canescens* roots and *Paxillus involutus* strains MAJ and NAU. *P. involutus* isolates, ectomycorrhizal (MAJ and NAU) and non-mycorrhizal (NM) *P.* × *canescens* plants were subjected to short-term (ST, 24 h) and long-term (LT, 7 days) exposure to 50 μM $CdCl_2$, respectively. Control roots and axenic mycelia were well fertilized but treated without $CdCl_2$. Ca^{2+} fluxes in poplar roots were measured along root axis, 100–2,300 μm from the apex, at intervals of 200–300 μm. Ca^{2+} fluxes of *P. involutus* isolates MAJ and NAU were measured along the surface of pelleted hyphae over a recording period of 30 min. Inserted sections show the Ca^{2+} fluxes in *P. involutus* isolates after short-term (ST, 24 h) or long-term (LT, 7 days) $CdCl_2$ treatment. Each point is the mean of 4–5 individual plants or axenic EM cultures (pelleted hyphae), and bars represent the standard error of the mean. Asterisks denote significant difference at $P < 0.05$ between treatments.

Correlations between Cd^{2+} and Ca^{2+} Fluxes

We analyzed the correlation between Cd^{2+} and Ca^{2+} fluxes as NM and EM roots took up these elements with a similar flux rate (**Figures 1A,B**, **2** and **3**). Under ST and LT stress conditions, the total flux rates of Cd^{2+} and Ca^{2+} in the presence of Cd^{2+} ($=\Sigma_{Ca^{2+}+Cd^{2+}}$ with a molar ratio of Cd^{2+} to Ca^{2+} of 1:1) were 37.8–77.4 (NM), 54.1–96.2 (MAJ), and 53.7–122.1 pmol cm^{-2} s^{-1} (NAU), as calculated on the basis of **Figures 2** and **3** (Supplementary Figure S4). The relationships between $\Sigma_{Ca^{2+}+Cd^{2+}}$ and Ca^{2+} flux in the absence of $CdCl_2$ [$\Sigma_{Ca^{2+}(-Cd^{2+})}$] were highly significant and close to 1 for NM and MAJ colonized roots and slightly increased to 1.4 for NAU colonized roots (**Figure 4**). These suggest that the entry of Cd^{2+} and Ca^{2+} is mainly through the same pathway in NM and EM roots, mostly likely through Ca^{2+}-permeable channels in the PM (see below).

H^+ Fluxes

In the absence of $CdCl_2$, EM roots showed a typical H^+ efflux at the apex, which was 7.6-fold higher than that in NM roots (**Figure 1C**). $CdCl_2$ (50 μM) shock stimulated H^+ efflux in both NM and EM plants with a stronger response in EM than in NM roots (**Figure 1C**). Pure MAJ and NAU mycelia exhibited a net H^+ efflux under control conditions similar to that observed for MAJ- and NAU-colonizing roots (**Figure 1C**). However, in pure mycelia the fluxes were 4.8-fold higher than in EM roots (**Figure 1C**). After exposure to $CdCl_2$ (50 μM), hyphae exhibited a transient increase in the H^+ efflux, which then remained constant during the period of recording (40 min; **Figure 1C**). Compared with strain MAJ, strain NAU exhibited higher H^+ efflux irrespective of control or $CdCl_2$ shock treatments (**Figure 1C**).

Steady-state recordings on EM roots showed that the pattern of H^+ flux in ST-stressed roots (50 μM $CdCl_2$, 24 h) differed from those subjected to LT Cd^{2+} exposure (50 μM $CdCl_2$, 7 days). Under ST conditions, $CdCl_2$ (50 μM) stimulated H^+ efflux in EM plants, whereas under LT conditions, EM roots showed a pronounced H^+ influx (**Figure 5**). In NM roots, $CdCl_2$ (50 μM) decreased H^+ influx upon ST exposure or shifted it to a net H^+ efflux under LT stress conditions (**Figure 5**). The pattern of H^+ flux in the fungal mycelia differed from that in EM roots under ST stress (**Figure 5**). ST treatment reduced the efflux of H^+ from the two fungal strains, which is contrast to EM roots where an enhanced H^+ efflux was observed (**Figure 5**). LT stress caused a pronounced shift of H^+ efflux to influx into pure mycelia of the two strains, similar to the finding in LT-stressed EM roots (**Figure 5**).

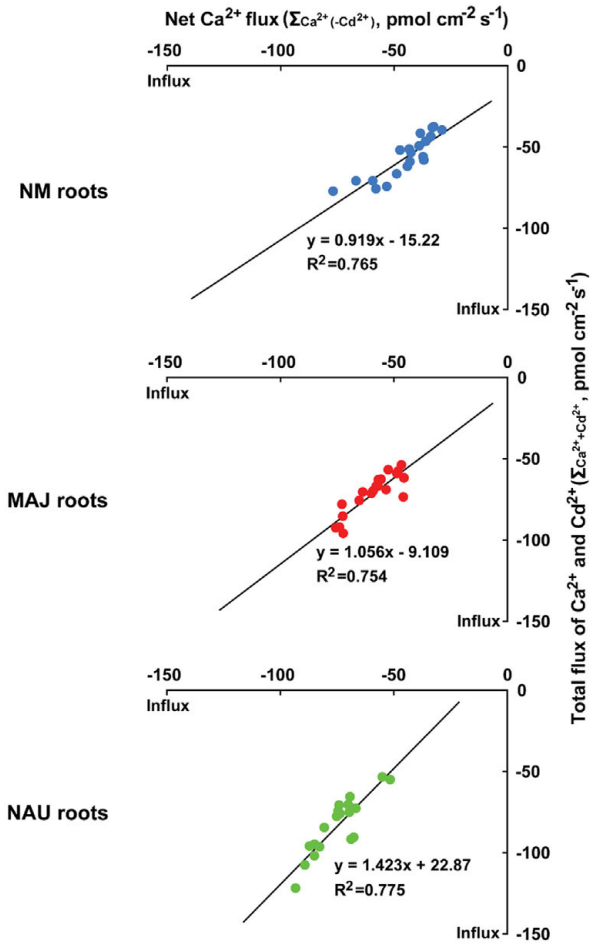

FIGURE 4 | The correlation between total fluxes of Ca^{2+} and Cd^{2+} ($\Sigma_{Ca^{2+}+Cd^{2+}}$) in the presence of $CdCl_2$ (50 μM, +Cd) and Ca^{2+} flux [$\Sigma_{Ca^{2+}(-Cd^{2+})}$] in the absence of $CdCl_2$ (-Cd) in roots of ectomycorrhizal (MAJ and NAU) and non-mycorrhizal (NM) *Populus* × *canescens*. Ectomycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants were subjected to short-term (ST, 24 h) and long-term (LT, 7 days) exposure to 50 μM $CdCl_2$, respectively. Control roots were well fertilized but treated without $CdCl_2$. Ca^{2+} and Cd^{2+} fluxes were measured along root axis, 100–2,300 μm from the apex, at intervals of 200–300 μm. Each point is the mean of 4–5 individual plants.

Cd^{2+}-Altered Flux Profiles of H_2O_2 in EM Roots

H_2O_2-sensitive microprobes were used to detect the H_2O_2 response to Cd^{2+} exposure in NM and EM roots. In the absence of Cd^{2+}, NM roots exhibited a stable H_2O_2 efflux (0.7–1.5 pmol cm^{-2} s^{-1}) along the root axis; the mean flux rate increased 2.4-fold in response to Cd^{2+} treatment (50 μM $CdCl_2$, 30 min, **Figure 6**). Ectomycorrhization of poplar roots with *P. involutus* stains, MAJ and NAU, resulted in a significant increase of H_2O_2 efflux along the roots (**Figure 6**). However, upon $CdCl_2$ exposure EM roots displayed decreased H_2O_2 efflux in contrast to NM roots (**Figure 6**).

Effects of Ca^{2+}, H_2O_2, pH, and PM Transporter and Channel Inhibitors on Cd^{2+}-Altered Ion Flux Profiles in EM Roots

Ca^{2+} and Ca^{2+} Channel Inhibitors

Here, pharmacological experiments were carried out to test whether putative Ca^{2+} channels inhibitors could inhibit Cd^{2+} influx in poplar roots. Four typical Ca^{2+} channels

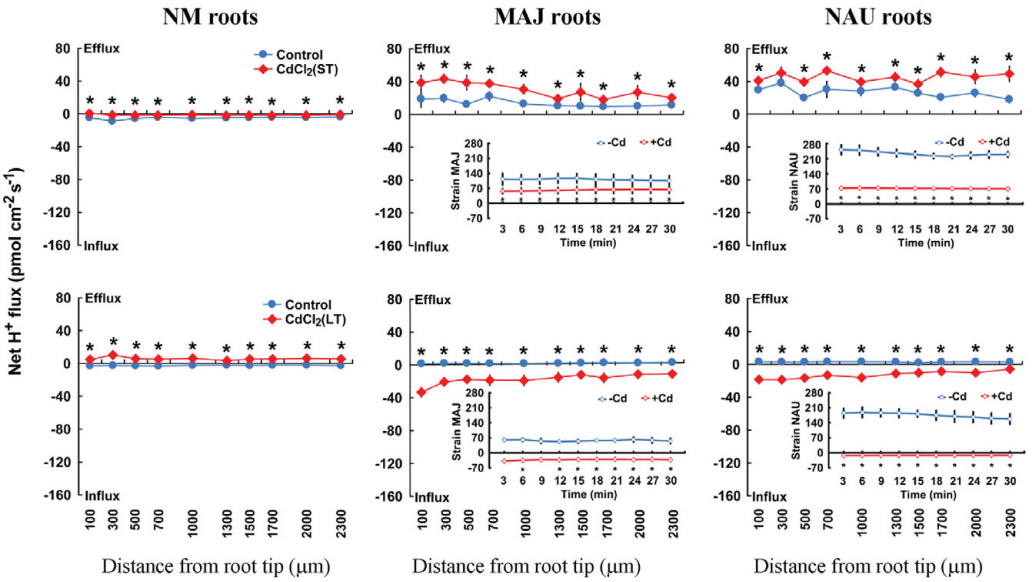

FIGURE 5 | Effects of $CdCl_2$ on steady H^+ fluxes in *Populus* × *canescens* roots and *Paxillus involutus* strains MAJ and NAU. *P. involutus* isolates, ectomycorrhizal (MAJ and NAU) and non-mycorrhizal (NM) *P.* × *canescens* plants were subjected to short-term (ST, 24 h) and long-term (LT, 7 days) exposure to 50 μM $CdCl_2$, respectively. Control roots and axenic mycelia were well fertilized but treated without $CdCl_2$. H^+ fluxes in poplar roots were measured along root axis, 100–2,300 μm from the apex, at intervals of 200–300 μm. H^+ fluxes of *P. involutus* isolates MAJ and NAU were measured along the surface of pelleted hyphae over a recording period of 30 min. Inserted sections show the H^+ fluxes in *P. involutus* isolates after short-term (ST, 24 h) or long-term (LT, 7 days) $CdCl_2$ treatment. Each point is the mean of 4–5 individual plants or axenic EM cultures (pelleted hyphae), and bars represent the standard error of the mean. Asterisks denote significant difference at $P < 0.05$ between treatments.

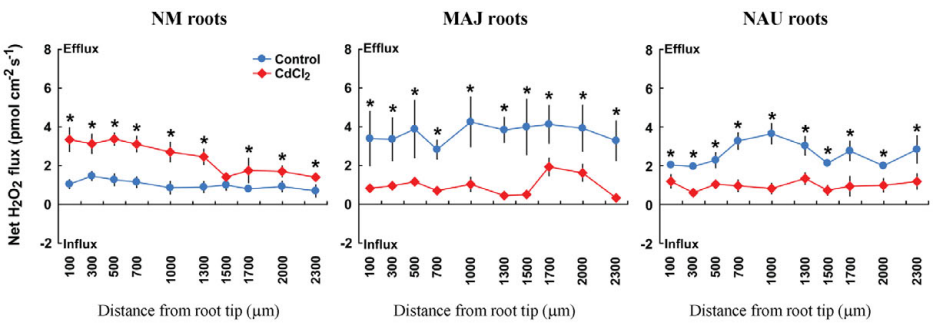

FIGURE 6 | Effects of $CdCl_2$ on steady H_2O_2 flux in roots of ectomycorrhizal (MAJ and NAU) and non-mycorrhizal (NM) *Populus* × *canescens* plants. Ectomycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants were subjected to 50 μM $CdCl_2$ for 30 min. Control roots were well fertilized but treated without $CdCl_2$. H_2O_2 flux was measured along root axis, 100–2,300 μm from the apex, at intervals of 200–300 μm. Each point is the mean of 4–5 individual plants, and bars represent the standard error of the mean. Asterisks denote significant difference at $P < 0.05$ between treatments.

inhibitors, $LaCl_3$, $GdCl_3$, verapamil, and TEA effectively inhibited Ca^{2+} influx in NM and EM roots, regardless of Cd^{2+} treatments (**Figure 7A**, Supplementary Figures S5A, S6A, and S7A). $LaCl_3$ restricted Cd^{2+} influx in $CdCl_2$-treated NM and EM roots (**Figure 7B**). This suggests that Cd^{2+} is taken up through Ca^{2+}-permeable channels because La^{3+} is able to block various types of Ca^{2+}-permeable channels, including depolarisation-, hyperpolarisation-, elicitor-activated, and voltage-insensitive channels (Weiss, 1974; Pickard and Ding, 1993; Gelli and Blumwald, 1997; Piñeros and Tester, 1997; Zimmermann et al., 1997; White, 1998, 2000). Moreover, the other three Ca^{2+}-permeable channel inhibitors, $GdCl_3$, verapamil, and TEA, diminished Cd^{2+} influx to a similar extent as $LaCl_3$-treated plants (**Figure 7B**, Supplementary Figures S5B, S6B, and S7B). Similarly, in the pure *P. involutus* mycelia, $LaCl_3$ also effectively restricted influx of Ca^{2+} and Cd^{2+} or induced net efflux (Supplementary Figure S8).

Additionally, a co-application of Cd^{2+} and Ca^{2+} suppressed the entry of Cd^{2+} in NM and EM roots, and the restriction increased with the increasing fraction of Ca^{2+} in the mixture (Ca^{2+}: Cd^{2+} = 1:2, 1:1, 2:1; **Figure 7C**). The mean Cd^{2+} flux decreased by 95.7% (NM), 72.1% (MAJ), and 45.5% (NAU) at a ratio of Ca^{2+}:Cd^{2+} = 2:1, compared to a those with a higher

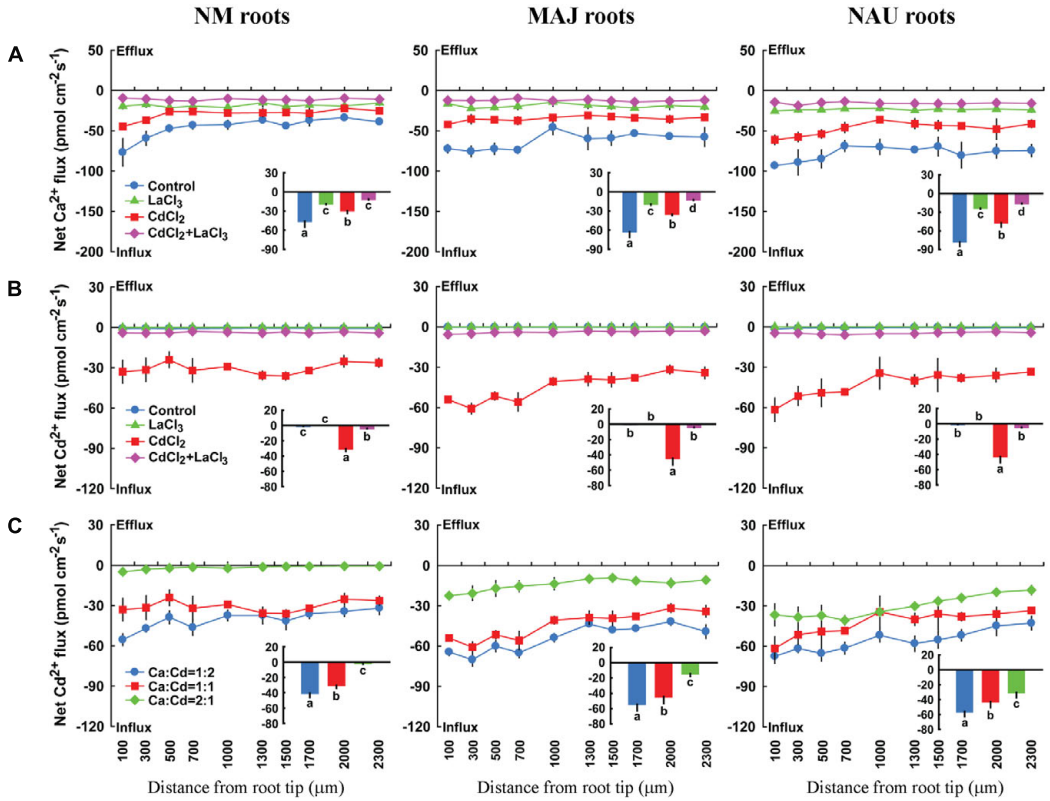

FIGURE 7 | Effects of $LaCl_3$ and external Ca^{2+} on steady Cd^{2+} and/or Ca^{2+} fluxes in roots of ectomycorrhizal (MAJ and NAU) and non-mycorrhizal (NM) *Populus* × *canescens* plants under Cd^{2+} stress. (A,B) Ectomycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants were subjected to 50 μM $CdCl_2$ for 24 h in the presence and absence of 5 mM $LaCl_3$. Control roots were well fertilized but treated without $CdCl_2$ or $LaCl_3$. **(C)** Ectomycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants were subjected to 50 μM $CdCl_2$ for 24 h prior to Cd^{2+} flux recordings in the presence of $CaCl_2$ (25 μM, 50 μM, or 100 μM; the ratio of Ca^{2+}:Cd^{2+} was 1:2; 1:1, and 2:1). Ca^{2+} **(A)** and Cd^{2+} **(B,C)** fluxes were measured along root axes, 100–2,300 μm from the apex, at intervals of 200–300 μm. Each point is the mean of 4–5 individual plants and bars represent the standard error of the mean. Inserted sections show the mean flux rates and different letters, a, b, c, and d, indicate significant difference at $P < 0.05$ between treatments.

Cd^{2+} fraction, Ca^{2+}:Cd^{2+} = 1:2 (**Figure 7C**). These results suggest that the divalent cations, Cd^{2+} and Ca^{2+}, competitively permeated the plasma membrane through Ca^{2+} channels. The lower reduction in Cd^{2+} influx in EM than in NM roots in the presence of Ca^{2+} (**Figure 7C**) reflects the high flow of Cd^{2+} through the activated Ca^{2+} channels.

We observed that the presence of Ca^{2+} in the measuring solution marginally lowered the Cd^{2+} signals (14.7–26.0%) detected by the Cd^{2+} microelectrodes filled with Cd^{2+} liquid ion exchanger (LIX) (Supplementary Table S1). In the absence of Ca^{2+}, the working voltage of microelectrodes and the detected Cd^{2+} signals in Cd^{2+}-treated roots were unstable and fluctuated greatly during the period of recording (data not shown). This behavior is presumably caused by the plant response to nutrient deficiency in the root medium (Li J. et al., 2012). In our study, Cd^{2+} electrodes exhibited higher sensitivity at 0.05 mM Ca^{2+} in the absence and presence of 0.1 mM K^+ and 0.1 mM Mg^{2+} (Supplementary Table S1). The presence of nutrients, K^+, Ca^{2+}, and Mg^{2+}, did not affect the accuracy of our conclusions relating to Cd^{2+} fluxes in NM and EM roots.

H_2O_2 and ROS Scavenger

To investigate whether Cd^{2+} entry through Ca^{2+}-permeable channels is activated by H_2O_2, we examined the effects of hydrogen peroxide and the ROS (reactive oxygen species) scavenger DMTU on Cd^{2+} and Ca^{2+} fluxes. Transient kinetic recordings showed that Cd^{2+} shock caused an immediate increase of Cd^{2+} influx but enhanced Ca^{2+} efflux in NM and EM roots (**Figure 8**). The flux rates of Cd^{2+} and Ca^{2+} decreased with prolonged exposure time (**Figure 8**). Notably, Cd^{2+} influx markedly increased upon H_2O_2 shock (1.0 mM) in both NM and EM roots (**Figure 8A**). However, the Cd^{2+}-elicited Ca^{2+} efflux was reduced by H_2O_2 in EM roots or shifted to a net influx in NM roots (**Figure 8B**). These results suggest that H_2O_2 stimulated the entry of Cd^{2+} and Ca^{2+}, presumably through the plasma membrane Ca^{2+} channels of the roots.

Ca^{2+} influx in NM and EM roots were suppressed by the ROS scavenger, DMTU (5 mM), irrespective of the presence and absence of Cd^{2+} (**Figure 9A**). Similarly, the supplement of DMTU significantly reduced the influx of Cd^{2+} in NM and EM roots (**Figure 9B**). These data indicated that H_2O_2 play a crucial role in accelerating the influx of Ca^{2+} and Cd^{2+}, which

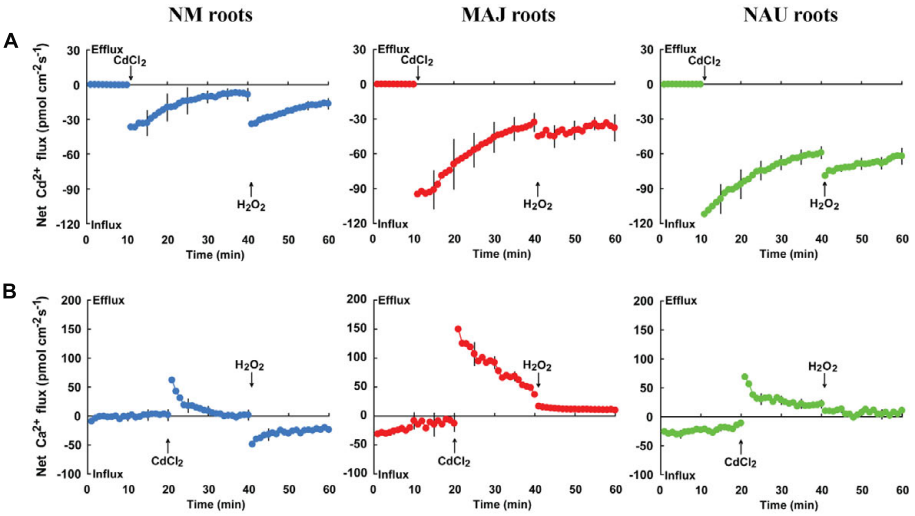

FIGURE 8 | Effects of $CdCl_2$ and H_2O_2 on transient kinetics of Cd^{2+} and Ca^{2+} in roots of ectomycorrhizal (MAJ and NAU) and non-mycorrhizal (NM) *Populus* × *canescens*. Cd^{2+} **(A)** and Ca^{2+} **(B)** kinetics were recorded before and after the required amount of 50 μM $CdCl_2$ or 1.0 mM $H_2O_2$was introduced into the measuring chamber. Prior to the $CdCl_2$ shock, steady-state fluxes of Cd^{2+} and Ca^{2+} were monitored at the apex (measuring site was ca. 100 μm from the root tip) for approximately 10–20 min. Transient kinetics of Cd^{2+} and Ca^{2+} were recorded after the required amount of 50 μM $CdCl_2$ was introduced into the measuring solution. After 20–30 min continuous recording of Cd^{2+} and Ca^{2+} fluxes, Cd^{2+} and Ca^{2+} kinetics were recorded for 20 min after 1.0 mM H_2O_2 was introduced into the measuring solution. Each point represents the mean of 4–5 individual plants and bars represent the standard error of the mean.

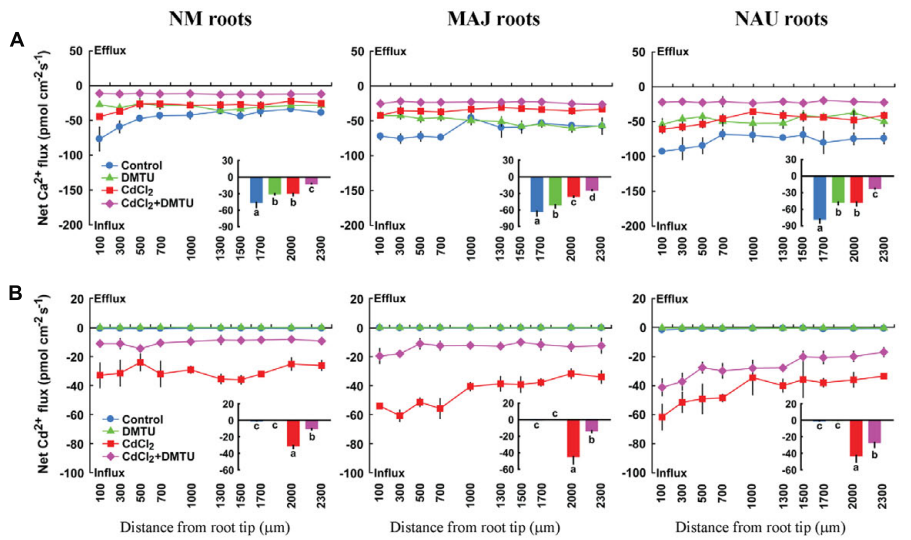

FIGURE 9 | Effects of DMTU on steady Ca^{2+} and Cd^{2+} fluxes in roots of ectomycorrhizal (MAJ and NAU) and non-mycorrhizal (NM) *Populus* × *canescens* under Cd^{2+} stress. Ectomycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants were subjected to 0 or 50 μM $CdCl_2$ for 24 h in the presence or absence of 5 mM DMTU. Ca^{2+} **(A)** and Cd^{2+} **(B)** fluxes were measured along root axes, 100–2,300 μm from the apex, at intervals of 200–300 μm. Each point is the mean of 4–5 individual plants and bars represent the standard error of the mean. Inserted sections show the mean flux rates and different letters, a, b, c, and d, indicate significant difference at $P < 0.05$ between treatments.

is accordance to the results obtained by direct H_2O_2 applications (**Figure 8**).

External pH and H^+-ATPase Inhibitor

Fluxes of Cd^{2+} and Ca^{2+} depend on external pH. An acidic environment accelerated Cd^{2+} and Ca^{2+} influxes in both NM and EM roots with the strongest influx at pH 5.2 and the lowest at pH 6.2 or a neutral pH, 7.2 (**Figure 10**). Moreover, we noticed that the pH effects on fluxes of Cd^{2+} and Ca^{2+} were more pronounced in NM roots than in EM roots (**Figure 10**). Compared to an acidic environment (pH 5.2), the mean flux rate of the divalent cations decreased by 45.8% (Ca^{2+}) and 38.8% (Cd^{2+}) in EM roots under pH 6.2–7.2 (**Figure 10**). In NM roots, the increasing pH lowered Cd^{2+} influxes by 56.5% or even

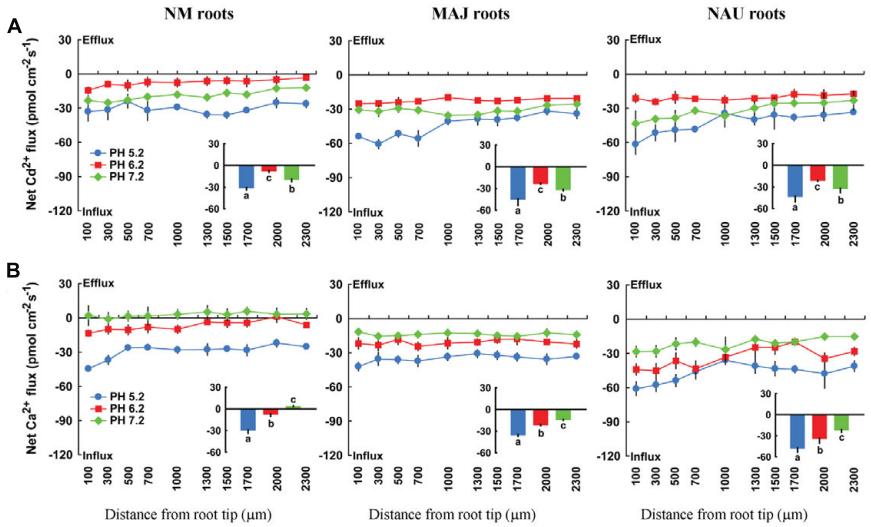

FIGURE 10 | Effects of pH on steady Cd^{2+} and Ca^{2+}fluxes in roots of ectomycorrhizal (MAJ and NAU) and non-mycorrhizal (NM) *Populus* × *canescens* under Cd^{2+} stress. Ectomycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants were subjected to 50 μM $CdCl_2$ for 24 h prior to flux recordings at pH 5.2, 6.2 or 7.2. Cd^{2+} **(A)** and Ca^{2+} **(B)** fluxes were measured along root axes, 100–2,300 μm from the apex, at intervals of 200–300 μm. Each point is the mean of four to five individual plants and bars represent the standard error of the mean. Inserted sections show the mean flux rates and different letters, a, b, and c, indicate significant difference at $P < 0.05$ between treatments.

reversed the rectifications of Ca^{2+} (influx → efflux) at a neutral pH, 7.2 (**Figure 10**). The less reduced influx of Ca^{2+} and Cd^{2+} in EM roots at pH 6.2 or 7.2 was due to the high H^+-pumping activity in the PM (see below).

Sodium orthovanadate (500 μM), the specific inhibitor of PM H^+-ATPase, increased the H^+ influx in NM roots slightly, but caused a drastic shift from H^+ efflux toward influx in both EM roots and *P. involutus* mycelia, irrespective of Cd^{2+} treatment (**Figure 11A**, Supplementary Figure S9). Sodium orthovanadate significantly reduced the Cd^{2+} influx along the roots in Cd^{2+}-treated NM and EM plants (**Figure 11B**). In the absence of Cd^{2+}, the PM H^+-ATPase inhibitor reduced Ca^{2+} influx in NM and MAJ-ectomycorrhizal roots or shifted to efflux in NAU-ectomycorrhizal roots (**Figure 11C**). The inhibition of Ca^{2+} influx by sodium orthovanadate was more pronounced in the presence of Cd^{2+}: the H^+-pump inhibitor reversed the rectifications of Ca^{2+} from influx to efflux in NM and EM roots (**Figure 11C**).

DISCUSSION

Colonization of *P.* × *canescens* Roots with *Paxillus involutus* Stimulates Cd^{2+} Uptake under Cd^{2+} Stress

The woody Cd^{2+}-hyperaccumulator *P.* × *canescens* exhibited a vigorous Cd^{2+} uptake after a 50 μM $CdCl_2$ shock (40 min), ST (24 h), and LT (7 days) treatment (**Figures 1A** and **2**). The result is consistent with previous findings where *P.* × *canescens* roots exhibited a high Cd^{2+} uptake after 40 days of $CdSO_4$ exposure (50 μM, Ma Y. et al., 2014). Similarly, a high entry of Cd^{2+} was recorded in hyperaccumulating ecotypes of *Sedum alfredii* (Lu et al., 2010; Sun et al., 2013a) and *Suaeda salsa* under Cd^{2+} stress (Li et al., 2012a). An important result was that EM roots exhibited higher Cd^{2+} influx than NM roots irrespective of Cd^{2+} stress conditions, shock, ST, and LT (**Figures 1A** and **2**). Substantial evidence indicates that Cd^{2+} can be enriched in ectomycorrhizal plants (Sell et al., 2005; Baum et al., 2006; Krpata et al., 2008, 2009; Sousa et al., 2012; Ma Y. et al., 2014). The enhanced Cd^{2+} uptake in EM roots is partly due to the capacity of the fungus to take up Cd^{2+} because $CdCl_2$ shock resulted in a net Cd^{2+} influx in the mycelia of the two *P. involutus* strains and the flux rate increased with the prolonged duration of $CdCl_2$ treatment from 24 h to 7 days (**Figures 1A** and **2**). In liquid cultures, *P. involutus* cultures also showed high capacities for Cd^{2+} accumulation (Ott et al., 2002). *P. involutus* could bind Cd^{2+} onto the cell walls or accumulate the metal in the vacuolar compartment (Blaudez et al., 2000; Ott et al., 2002). Moreover, the ectomycorrhizal fungus appears to detoxify high concentrations of Cd^{2+} by (i) the chelation of metal ions in the cytosol with thiol-containing compounds, e.g., glutathione, phytochelatins, or metallothioneins (Courbot et al., 2004; Jacob et al., 2004), and (ii) activation of antioxidative defense system (Jacob et al., 2001; Ott et al., 2002). Our pharmacological data revealed that Cd^{2+} entered the fungal hyphae mainly through PM Ca^{2+} channels because the influx was suppressed by $LaCl_3$, a Ca^{2+} channel blocker (Supplementary Figure S8B). Therefore, Cd^{2+} enriched by ectomycorrhizal hyphae is thought to be transferred to the host roots, probably through the apoplastic space during the period of Cd^{2+} stress.

There were marked differences between the two strains in Cd^{2+} uptake given the shock treatment (**Figure 1A**). Pure fungal mycelium of MAJ accumulated Cd^{2+} with a higher rate

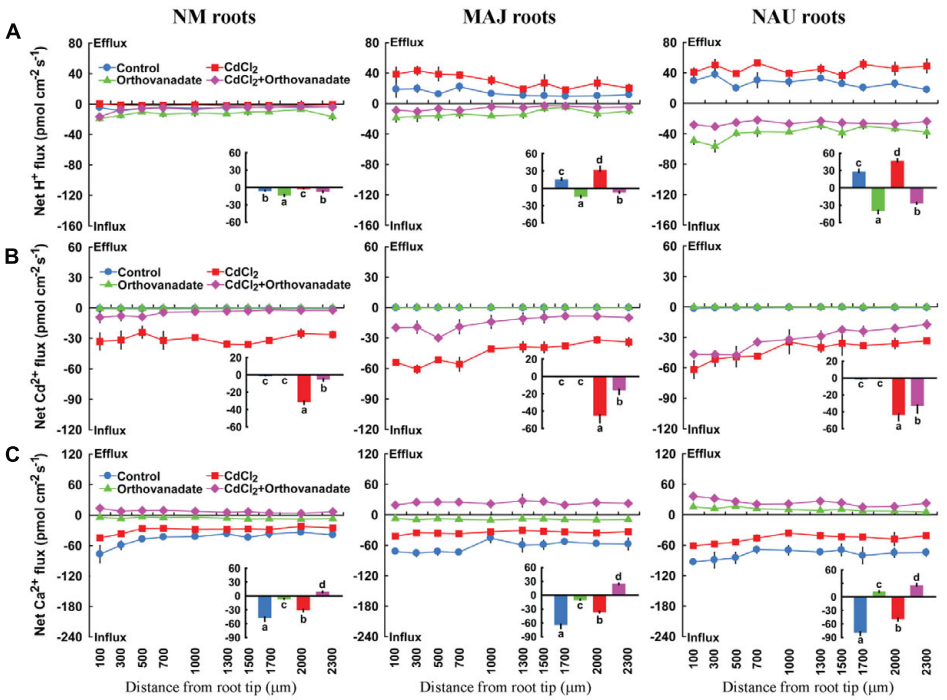

FIGURE 11 | Effects of sodium orthovanadate on steady H^+, Cd^{2+}, and Ca^{2+} fluxes in roots of ectomycorrhizal (MAJ and NAU) and non-mycorrhizal (NM) *Populus* × *canescens* under Cd^{2+} stress. Ectomycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants were subjected to 0 or 50 μM $CdCl_2$ for 24 h in the presence and absence of 500 μM sodium orthovanadate. H^+ **(A)**, Cd^{2+} **(B)**, and Ca^{2+} **(C)** fluxes were measured along root axes, 100–2,300 μm from the apex, at intervals of 200–300 μm. Each point is the mean of 4–5 individual plants and bars represent the standard error of the mean. Inserted sections show the mean flux rates and different letters, a, b, c, and d, indicate significant difference at $P < 0.05$ between treatments.

than NAU (**Figure 1A**). In the *P. involutus*-ectomycorrhizal symbioses, the incompatible fungal isolate NAU is unable to induce a functional ectomycorrhizae while MAJ forms a typical Hartig net with the roots of *P.* × *canescens* (Gafur et al., 2004). Thus, in MAJ-colonized roots the host cells might have been more accessible to Cd^{2+}. In accordance, MAJ roots exhibited a higher influx than NAU roots after the onset of $CdCl_2$ shock (**Figure 1A**). However, Cd^{2+} influx into NAU-colonized roots was similar to that of MAJ-colonized roots during ST or LT Cd^{2+} treatment (**Figure 2**). This was likely due to (i) similar capacities for Cd^{2+} uptake of MAJ and NAU hyphae during a 24-h or 7-days of Cd^{2+} exposure (**Figure 2**), or (ii) similar uptake capacity of the fungus-ensheathed inner root cells (**Figure 2**). The observed correlation between EM and NM roots showed that the continuous Cd^{2+} flow was mainly the consequence of host roots in the Cd^{2+}-stressed ectomycorrhizal symbioses during a prolonged period of Cd^{2+} exposure (24 h to 7 days; Supplementary Figure S2).

Paxillus involutus-Ectomycorrhizas Enhance Cd^{2+} Influx through Ca^{2+}-Permeable Channels in the Plasma Membrane

Our data revealed that the entry of Cd^{2+} is likely mediated through PM Ca^{2+} channels in the fungal hyphae and poplar roots, and *P. involutus*-ectomycorrhizas facilitated the channel-mediated Cd^{2+} influx under Cd^{2+} stress. The experimental evidence for these conclusions is briefly listed below.

(1) The addition of Cd^{2+} resulted in an immediate influx of Cd^{2+} in NM roots, and the flux was more pronounced in EM roots (**Figure 1A**). Rapid entry of Cd^{2+} is generally through PM ion channels that are permeable to Cd^{2+} (The first 1–2 min flux recordings were discarded to diminish the diffusion effect of stock addition in roots and fungal mycelia). Our pharmacological data revealed that the net Cd^{2+} influx in $CdCl_2$-stressed NM and EM roots was strongly suppressed by typical Ca^{2+} channel blockers, such as $LaCl_3$, $GdCl_3$, verapamil, and TEA (**Figure 7B**, Supplementary Figures S5B, S6B, and S7B). Moreover, in *P. involutus* mycelium the $CdCl_2$-elicited influx of Cd^{2+} was also inhibited by $LaCl_3$ (Supplementary Figure S8B). These results suggest that under $CdCl_2$ stress Cd^{2+} enters fungal and root tissues through PM Ca^{2+} channels.

(2) Cd^{2+} treatments (shock, ST, and LT) affected the uptake of Ca^{2+} in poplar roots (**Figures 1B** and **3**), while the influx of Cd^{2+} declined with increasing the concentration of Ca^{2+} when NM and EM roots were subjected to the concomitant application of Cd^{2+} and Ca^{2+} (**Figure 7C**). Similarly, the Cd^{2+} influx was affected by the presence of Ca^{2+} in two contrasting (hyperaccumulating and

non-hyperaccumulating) *Sedum alfredii* ecotypes (Lu et al., 2010). It was suggested that Ca^{2+} and Cd^{2+} ions compete for the binding sites of transporters (Gussarsson et al., 1996; Rodríguez-Serrano et al., 2009). Our transient kinetics showed that Cd^{2+} exposure blocked the Ca^{2+} influx and caused an immediate change in the rectification of Ca^{2+} from influx to efflux (**Figures 1B** and **8B**). This suggests that Cd^{2+} ions competed with Ca^{2+} to penetrate across PM Ca^{2+} channels that are permeable to divalent cations (Perfus-Barbeoch et al., 2002).

(3) In ST- and LT-stressed NM and EM roots, the total flux rates of Cd^{2+} and Ca^{2+} in the presence of Cd^{2+} ($\Sigma_{Ca^{2+}+Cd^{2+}}$) were nearly equal to the flux rate of Ca^{2+} in the absence of Cd^{2+} stress ($\Sigma_{Ca^{2+}(-Cd^{2+})}$; Supplementary Figure S4). Moreover, the correlations between $\Sigma_{Ca^{2+}+Cd^{2+}}$ and $\Sigma_{Ca^{2+}(-Cd^{2+})}$ (**Figure 4**) suggest that Cd^{2+} ions enter NM and EM roots mainly through Ca^{2+}-permeable channels in the PM.

Collectively, under $CdCl_2$ stress Cd^{2+} ions could penetrate the PM Ca^{2+} channels in fungal hyphae and in *P.* × *canescens* roots. At present we cannot exclude the possibility that Cd^{2+} penetrated the PM through transporters for Cd^{2+} (Ma Y. et al., 2014; He et al., 2015) or other nutritional ions (Gussarsson et al., 1996; Cohen et al., 1998; Zhao et al., 2002; Cosio et al., 2004; Clemens, 2006), because (1) the four types of Ca^{2+} channel inhibitors applied here were not able to fully block the Cd^{2+} influx in NM and EM roots (**Figure 7B**, Supplementary Figures S5B, S6B and S7B), and (2) the total flux of Cd^{2+} and Ca^{2+} ($\Sigma_{Ca^{2+}+Cd^{2+}}$, molar ratio of Cd^{2+} to Ca^{2+} is 1:1) under ST and LT Cd^{2+} stress was 10.9–27.7% higher than the flux rate of Ca^{2+} under non-Cd^{2+} conditions (**Figure 4**, Supplementary Figure S4). This implies that a small fraction of Cd^{2+} ions penetrated the PM through other channels and transporters.

Plasma membrane Ca^{2+} channels in *P. involutus* hyphae maybe more permeable to Cd^{2+} compared to the channels in *P.* × *canescens* roots as the fungal mycelium displayed a typical higher Ca^{2+} influx than poplar roots under control and Cd^{2+}-stress conditions (**Figures 1B** and **3**). We cannot discriminate between the channels of the fungus and those of the plant in the ectomycorrhizal symbiosis, but the Cd^{2+} and Ca^{2+} fluxes in EM roots appear to mainly reflect the response of the host plants to Cd^{2+} stress because (1) EM roots exhibited a different pattern from the *P. involutus* mycelia in enhancing Ca^{2+} and Cd^{2+} uptake under hydroponic Cd^{2+} conditions. Cd^{2+}-shocked MAJ and NAU fungal strains usually displayed a stable Cd^{2+} influx with the exception of an initial transient increase (**Figure 1A**). However, EM roots showed a declined Cd^{2+} influx over the duration of Cd^{2+} exposure, similar to the Cd^{2+} kinetics in NM roots (**Figure 1A**). Moreover, the Cd^{2+} influx in the mycelia of the two *P. involutus* strains increased with the prolonged $CdCl_2$ exposure from 24 h to 7 days (from 23.9 to 72.7 pmol cm^{-2} s^{-1}; **Figure 2**). In contrast, the Cd^{2+} fluxes in EM roots were relatively stable under ST (43.7 ± 8.4 pmol cm^{-2} s^{-1}) and LT treatments (35.9 ± 6.0 pmol cm^{-2} s^{-1}; **Figure 2**). ST, LT, and Cd^{2+} shock increased the Ca^{2+} influx in *P. involutus* mycelia, while the Ca^{2+} influx in EM roots was declined by these Cd^{2+} treatments (**Figures 1B** and **3**). (2) NMT data showed that ion fluxes in mature *P.* × *canescens*–*P. involutus* symbiotic associations bear a striking resemblance to the ST inoculated roots (Supplementary Figure S1). Similar findings have been previously reported in a salt stress study where *P.* × *canescens* roots were inoculated with *P. involutus* for 10 and 20 days (Ma X. et al., 2014). At early stages of fungal co-culture the Cd^{2+} and Ca^{2+} influx is mostly the result of host properties. Therefore, the Cd^{2+} and Ca^{2+} stimulation in *P. involutus*-ectomycorrhizal roots reflects the enhanced root uptake ability. (3) The correlation analyses revealed that Cd^{2+} and Ca^{2+} influxes in EM roots show a significant relationship with NM roots but not with fungal mycelia under various Cd^{2+} treatments (shock, ST, and LT; Supplementary Figures S2 and S3). Taken together, these data suggest that the continuous flow of Cd^{2+} and Ca^{2+} in EM roots detected by NMT microelectrodes was largely driven by the host and that the fungal partner enhanced fluxes leading to enriched Cd^{2+} and Ca^{2+} concentrations.

The observed patterns of Cd^{2+} and Ca^{2+} fluxes upon Cd^{2+} exposure could be explained by channel-mediated ion fluxes. NMT data show that the Ca^{2+} flux in EM roots was negatively correlated with the Ca^{2+} influx in fungal hyphae upon Cd^{2+} shock treatment (Supplementary Figure S3). This is presumably the result of Cd^{2+}-Ca^{2+} competition across the Ca^{2+} channels in the root PM. After being exposed to Cd^{2+} shock, Ca^{2+} entry was enhanced in the hyphae (**Figure 1B**). However, the fungal hyphae which were enriched in Ca^{2+} ions, were unable to deliver Ca^{2+} to the root cells because the Cd^{2+} ions competitively inhibited the entry of Ca^{2+} through the PM channels. As a result, the high influx of Ca^{2+} through fungal hyphae led to an apparently greater Ca^{2+} efflux in Cd^{2+}-exposed EM roots (**Figures 1B** and **8B**).

Paxillus involutus colonization enhanced the uptake of Cd^{2+} under shock, ST, and LT stress, compared to NM roots (**Figures 1A** and **2**). The increased entry of Cd^{2+} is likely due to the activation of PM Ca^{2+} channels in the ectomycorrhizas. The stimulated Ca^{2+} influx by *P. involutus* inoculation revealed the activation of PM Ca^{2+} channels since the ectomycorrhiza-enhanced entry of Ca^{2+} was suppressed by Ca^{2+} channel blockers ($LaCl_3$, $GdCl_3$, verapamil, or TEA; **Figure 7A**, Supplementary Figures S5A, S6A, and S7A). The activated PM Ca^{2+} channels allowed the entry of Cd^{2+} in addition to Ca^{2+} under Cd^{2+} stress (**Figures 1A** and **2**).

Hydrogen Peroxide Induced by $CdCl_2$ and Fungal Colonization Stimulates Cd^{2+} Influx through PM Ca^{2+} Channels

After being subjected to $CdCl_2$ exposure, NM roots displayed an increased H_2O_2 efflux along the root axis (**Figure 6**). It is well documented that Cd^{2+} induced accumulation of H_2O_2 in pine roots (Schützendübel et al., 2001), *P* × *canescens* roots (Schützendübel et al., 2002), and in suspension cultures of tobacco (Piqueras et al., 1999) and *P. euphratica* (Sun et al., 2013b; Han et al., 2016). H_2O_2 efflux was evident in EM roots irrespective of the presence or absence of Cd^{2+} treatments (**Figure 6**). Our results suggest that the Cd^{2+} influx through PM

Ca^{2+} channels is stimulated by H_2O_2 in NM and EM roots. The experimental evidence and explanations are briefly listed here.

(1) H_2O_2 (1.0 mM) exhibited an enhancement on Ca^{2+} influx in NM and EM roots (**Figure 8B**). Pei et al. (2000) showed that H_2O_2 (0.05–5.0 mM) activates Ca^{2+} currents through PM Ca^{2+} channels of *Arabidopsis thaliana* guard cells. Moreover, H_2O_2 increased Ca^{2+} influx across the PM in *P. euphratica* cells (Sun et al., 2010), roots of *A. thaliana* (Demidchik et al., 2007) and mangroves (Lu et al., 2013). Furthermore, the H_2O_2-stimulated entry of Ca^{2+} in *P. euphratica* cells was inhibited by $LaCl_3$ (Sun et al., 2010). In this study, Cd^{2+} influx in NM and EM roots was significantly enhanced after exposure to 1.0 mM H_2O_2 (**Figure 8A**). This finding is in agreement with Sun et al. (2013b) and Han et al. (2016), who found that H_2O_2 (3.0 mM) stimulated entry of Cd^{2+} into *P. euphratica* cells. In addition, the Cd^{2+} influx was blocked by $LaCl_3$ in $CdCl_2$-stressed roots (**Figure 7B**). These results suggest that H_2O_2 stimulates the influx of Cd^{2+} and Ca^{2+} through Ca^{2+}-permeable channels in the PM.

(2) The H_2O_2 induction of Cd^{2+} resembles the pattern of Ca^{2+} kinetics in response to H_2O_2 (**Figure 8**). Moreover, Cd^{2+} and Ca^{2+} influx in NM and EM roots were both suppressed by the ROS scavenger, DMTU (**Figure 9**). Similarly, Sun et al. (2013b) showed that the entry of Cd^{2+} into *P. euphratica* cells was reduced when a H_2O_2 scavenger, catalase, was applied.

Taken together, these results suggest that Cd^{2+} and Ca^{2+} ions enter NM and EM roots by the same pathway involving PM Ca^{2+} channels that are activated by Cd^{2+}-elicited H_2O_2.

The high Cd^{2+} influx in EM roots resulted from the pronounced activation of PM Ca^{2+} channels that were stimulated, at least in part, by the fungal-elicited H_2O_2. Compared to NM roots, MAJ- and NAU-ectomycorrhizal roots displayed a significant higher H_2O_2 efflux in the absence of Cd^{2+} stress (**Figure 6**), suggesting that the inoculation with *P. involutus* caused a strong production of H_2O_2 in EM roots. This finding agrees with Gafur et al. (2004) and Langenfeld-Heyser et al. (2007), who detected strong H_2O_2 accumulation in the outer hyphae mantle of compatible (MAJ) and incompatible (NAU) interactions. H_2O_2 production in the hyphae is suggested to regulate host's root growth, defense against other invading microbes, and increasing plant-innate immunity (Salzer et al., 1999; Gafur et al., 2004). In our study, H_2O_2 produced in the ectomycorrhizae accelerated the influx of Ca^{2+} in the absence of Cd^{2+}, whereas it increased entry of Cd^{2+} in the presence of high external Cd^{2+} (**Figure 8**). ROS scavenging by DMTU simultaneously decreased Ca^{2+} and Cd^{2+} influxes along the root axis of EM plants (**Figure 9**). These observations suggest that H_2O_2 produced in compatible (MAJ) and incompetent (NAU) ectomycorrhizal associations activated Ca^{2+} permeable channels, which allowed the entry of Cd^{2+} under Cd^{2+} stress.

We noticed that the H_2O_2 efflux in MAJ and NAU-ectomycorrhizal roots was lowered by Cd^{2+} stress (**Figure 6**). This reduction may have resulted from the activation of antioxidant enzymes and increased amounts of ROS scavengers produced as a defense response. It has been repeatedly shown that the antioxidant enzyme activities are activated under heavy metal stresses (Schützendübel et al., 2001, 2002; Rozpądek et al., 2014; Chen et al., 2015; Tan et al., 2015). The enhanced activities of superoxide dismutase, peroxidase, catalase, and ascorbate peroxidase play an important role in scavenging the Cd^{2+}-elicited H_2O_2 in plants (Garg and Aggarwal, 2012; Anjum et al., 2015; Tan et al., 2015). To combat Cd^{2+}-induced superoxide and H_2O_2, *P.* × *canescens* plants were found to rely mainly on antioxidant enzymes and the formation of the potential radical scavenging molecules, such as proline, sugar alcohols and soluble phenolics (He et al., 2011). However, the lowered H_2O_2 efflux in EM roots (**Figure 6**) did not reduce the Cd^{2+}-elicited entry of Cd^{2+}, because (i) the fungal-elicited H_2O_2 had already activated the Ca^{2+}-channels before the Cd^{2+} addition, and/or (ii) the H_2O_2 level is still high enough to activate the channels under Cd^{2+} stress. The observation that stressed EM roots still contain high concentrations of H_2O_2 in the hyphae (Langenfeld-Heyser et al., 2007) supports these speculations.

PM H^+-ATPase Activated by Cd^{2+} and Fungal Colonization Stimulates Cd^{2+} Influx through PM Ca^{2+} Channels

In addition to H_2O_2, PM H^+-ATPase activated by Cd^{2+} and enhanced by fungal colonization also accelerated Cd^{2+} influx through PM Ca^{2+} channels in NM and EM roots. PM H^+-ATPases pump protons into the external medium to maintain an electrochemical H^+ gradient across the PM (Blumwald et al., 2000; Zhu, 2003). Krämer (2010) suggested that H^+-ATPases play an important role in adaptation of plants to heavy metal stress. The finding that the net H^+ efflux in fungal mycelia and EM roots was markedly reduced by a specific inhibitor of PM H^+-ATPase (sodium orthovanadate) in the presence and absence of Cd^{2+} stress (**Figure 11A**, Supplementary Figure S9) supports that the vigorous H^+ efflux is the consequence of H^+-ATPase activity. Accordingly, the increased H^+ efflux upon Cd^{2+} shock (NM, MAJ and NAU roots; **Figure 1C**), ST (MAJ and NAU roots; **Figure 5**) and LT stress (NM roots; **Figure 5**) indicates the activated H^+-pumping activity. In NM and EM roots, Cd^{2+} exposure led to a marked upregulation of *HA2.1* and *AHA10.1*, two important genes encoding PM H^+-ATPases (Ma Y. et al., 2014). The activation of PM H^+-ATPase by Cd^{2+} is likely associated with the Cd^{2+}-elicited H_2O_2, since (i) H_2O_2 increased H^+ pumping activity in *P. euphratica* callus cells (Sun et al., 2010), in roots of *P. euphratica* (Sun et al., 2010) and secretor and non-secretor mangrove species (Lu et al., 2013; Lang et al., 2014), and (ii) the expression of genes encoding PM H^+-ATPase are stimulated by H_2O_2 in *Cucumis sativus* roots (Janicka-Russak and Kabala, 2012; Janicka-Russak et al., 2012).

The activated PM H^+-ATPase enabled NM and EM roots to maintain an acidic environment, which favors the entry of Cd^{2+} across the PM (**Figure 10A**). Similarly, He et al. (2015) showed that pH 5.5 accelerates Cd^{2+} influx into poplar roots compared to pH 4.0 or pH 7.0. Moreover, the Cd^{2+} influx was markedly suppressed by the application of sodium vanadate, an

inhibitor of PM H^+-ATPase (**Figure 11B**). These results indicate that the PM H^+-pumps play a crucial role in enhancing the entry of Cd^{2+} (Ma Y. et al., 2014). Accordingly, NMT profiles of NM and EM roots showed that the maximum influx of Ca^{2+} was observed at pH 5.2 (**Figure 10B**), and that Ca^{2+} influx was blocked by sodium vanadate (**Figure 11C**). Therefore, we infer that Cd^{2+} activated H^+-pumping in the PM, which led to hyperpolarization of the PM and increased Cd^{2+} influx through hyperpolarization-activated Ca^{2+} channels (HACCs). However, at present we cannot exclude the possibility that Cd^{2+} ions also penetrated through depolarization-activated (DACCs) and voltage-independent Ca^{2+} channels (VICCs), because the inhibitor of PM H^+-ATPase, sodium vanadate, could not fully block the Cd^{2+} influx in NM and EM roots (**Figure 11B**). It has been shown that NSCCs co-exist with HACCs in the root cell plasma membrane to mediate the entry of Ca^{2+}, but the two Ca^{2+} influx routes differ in their voltage sensitivity (Demidchik et al., 2002).

Ectomycorrhizal *Populus* × *canescens* show highly activated H^+-pumping activity in the PM, which favors the Cd^{2+} influx through HACCs. Our NMT data showed that colonization of *P.* × *canescens* with *P. involutus* caused a marked H^+ efflux (**Figures 1C**, **5**, and **11A**), suggesting that the fungal colonization could activate the PM H^+-ATPase in ectomycorrhizas. This is consistent to our previous studies (Li J. et al., 2012; Ma X. et al., 2014). It has been documented that some host PM H^+-ATPase isoforms show high activity in arbuscular mycorrhizal associations (Ramos et al., 2005; Rosewarne et al., 2007). Obviously, H^+-pumping activity was activated by Cd^{2+} shock and ST exposure, as the H^+ efflux in MAJ- and NAU-ectomycorrhizal roots were significantly higher than the NM roots (**Figures 1C** and **5**). Increased abundance of *HA2.1* and *AHA10.1* encoding PM H^+-ATPase in ectomycorrhizas compared to NM roots of *P.* × *canescens* were suggested to lead to higher activities of PM H^+-ATPases (Ma Y. et al., 2014). The highly activated PM H^+-ATPase, on the one hand maintains a more suitable acidic environment to promote the Cd^{2+} and Ca^{2+} influx across the PM (**Figure 10**) and on the other hand, provides an electrochemical H^+ gradient for PM hyperpolarization, thus increasing Cd^{2+} influx via HACCs. Accordingly, the Cd^{2+}-stimulated Cd^{2+} and Ca^{2+} in the *P. involutus* mycelia (**Figures 1–3**) was associated with the activated H^+ pumps since Cd^{2+} treatment markedly upregulated the transcription of PM H^+-ATPase 1 (Jacob et al., 2004).

Importantly, the H_2O_2 produced in the ectomycorrhizal associations may accelerate the Cd^{2+} through the PM H^+-ATPase-mediated HACCs. Whole-cell patch clamp recordings of *Arabidopsis* guard cells showed that the PM hyperpolarization only activates Ca^{2+} currents in the presence of H_2O_2 (50 μM to 5 mM), and the Ca^{2+} current amplitudes increase with increasing H_2O_2 concentrations (Pei et al., 2000). Demidchik et al. (2007) showed that application of H_2O_2 (10 mM) to the external PM face of elongation zone epidermal protoplasts resulted in the appearance of a hyperpolarization-activated Ca^{2+} permeable conductance. In mature epidermal protoplasts, PM HACCs were activated only when H_2O_2 was present at the intracellular membrane face, and channel opening probability increased with intracellular H_2O_2 concentrations at hyperpolarized voltages

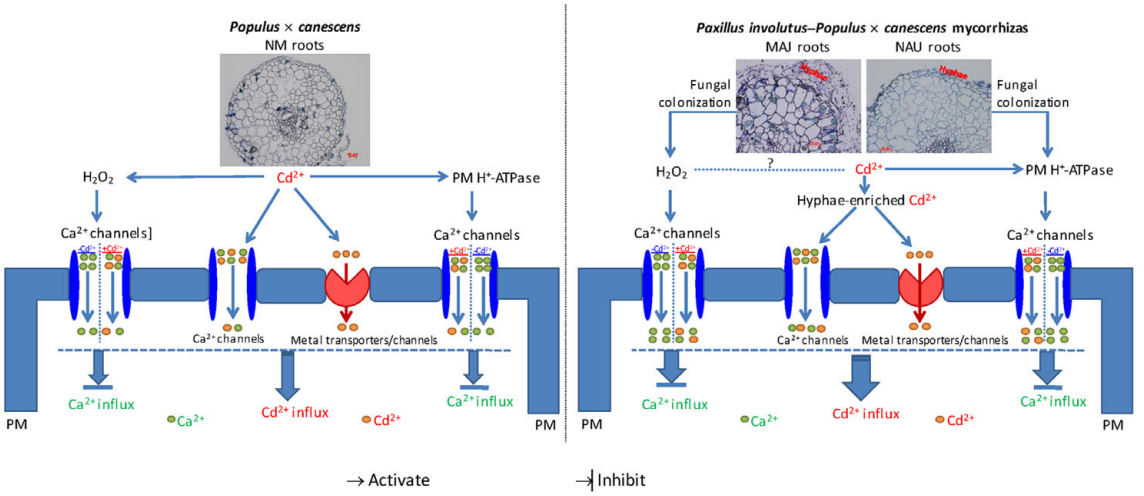

FIGURE 12 | Schematic models showing Cd^{2+} influx through plasma membrane (PM) Ca^{2+} channels that stimulated by H_2O_2 and H^+-ATPase in *Paxillus involutus*-ectomycorrhizal (MAJ and NAU) and non-mycorrhizal (NM) *Populus* × *canescens* roots under Cd^{2+} stress. High external Cd^{2+} facilitates the rapid movement of Cd^{2+} along its electrochemical gradient into fungal and plant cells. Cd^{2+} ions penetrated the ectomycorrhizal fungal hyphae and poplar roots through PM Ca^{2+} channels and other metal transporters or channels. The PM Ca^{2+} channels mediate the entry of Ca^{2+} in the absence of Cd^{2+} (−Cd) while allow the entry of Cd^{2+} in the presence of Cd^{2+} ions (+Cd). The Cd^{2+}-permeable Ca^{2+} channels were activated by H_2O_2 and H^+-pumping activity. Thus the Cd^{2+}-elicited H_2O_2 and active H^+-pumps favored the Cd^{2+} influx through Ca^{2+} channels in NM roots and *P. involutus*-ectomycorrhizas. In ectomycorrhizas, Cd^{2+} enriched in hyphae is thought to be delivered to the host roots. Moreover, the colonization of *P.* × *canescens* roots with the fungal strains MAJ and NAU stimulates H_2O_2 production and increases H^+-pumping activity, and thus accelerates Cd^{2+} entry through Ca^{2+} channels under excessive Cd^{2+}. Cd^{2+} ions competitively enter Ca^{2+} channels, and thus diminish the entry of Ca^{2+}, leading to a marked Cd^{2+} enrichment in ectomycorrhizal roots under Cd^{2+} stress.

(Demidchik et al., 2007). A massive presence of H_2O_2 was demonstrated in the outer hyphae mantle of *P. involutus* symbiosis (Gafur et al., 2004; Langenfeld-Heyser et al., 2007) and obviously could be released from the hyphae into the surrounding medium (**Figure 6**). Therefore, we suppose that in ectomycorrhizal *P.* × *canescens*, H_2O_2 elicited by fungal colonization stimulated Cd^{2+} influx through the HACCs that had been activated by *P. involutus* colonization. In addition, we found that Cd^{2+} influx in NAU-roots was less restricted than in MAJ-roots by DMTU and sodium orthvanadate (**Figures 9B** and **11B**). The difference in the sensitivity to antagonists of H_2O_2 and PM H^+-ATPase indicates the involvement of voltage-independent Ca^{2+} channels (VICCs) in the mediation of Cd^{2+} uptake in NAU-roots, in addition to the dominant Cd^{2+} entry through HACCs.

We noticed that LT stress in hydroponic conditions caused a pronounced shift of H^+ efflux toward an influx in EM roots (**Figure 5**). LT-stressed *P. involutus* mycelia exhibited a trend similar to that in EM roots (**Figure 5**). These results imply that ectomycorrhization activated an H^+/Cd^{2+} antiport to reduce excessive Cd^{2+} uptake and accumulation under prolonged stress conditions (Sun et al., 2013b). Similarly, we have previously shown that NaCl-treated *P. euphratica* roots retain an active PM Na^+/H^+ antiport to avoid the excessive buildup of Na^+ when exposed to LT salinity (Sun et al., 2009a,b). Here, the rate of H^+/Cd^{2+} antiport could not be determined, because our NMT data only show the net flux of the target element across the PM, instead of an unidirectional flux. In addition, EM roots were able to avoid the ROS burst in Cd^{2+} environments (**Figure 6**), probably because these roots were characterized by elevated H_2O_2 production (Gafur et al., 2004). Therefore, EM roots are likely to control the Cd^{2+} influx through the H_2O_2-activated PM Ca^{2+} channels, thus avoiding an excessive accumulation of the heavy metal ions under prolonged period of Cd^{2+} stress.

CONCLUSION

High external Cd^{2+} facilitates the rapid movement of Cd^{2+} along its electrochemical gradient into fungal and plant cells. Based on pharmacological evidence, we conclude that Cd^{2+} ions mainly penetrated the ectomycorrhizal fungal hyphae and poplar roots through PM Ca^{2+} channels. Because the entry of Cd^{2+} could not be fully blocked by various Ca^{2+} channel inhibitors ($LaCl_3$, $GdCl_3$, verapamil, and TEA), our results indicate that Cd^{2+} ions also entered the root and fungal cells through other metal transporters or channels. Our flux measurements show that the Cd^{2+}-permeable Ca^{2+} channels were activated by H_2O_2 and H^+-pumping activity. Altogether based on the current and literature data, we propose a signaling pathway that triggers Ca^{2+}-channel-mediated Cd^{2+} influx in NM *P.* × *canescens* roots and explains the pronounced Cd^{2+} stimulation in ectomycorrhizal associations under Cd^{2+} stress. As shown in **Figure 12**, the Cd^{2+}-elicited H_2O_2 and active H^+-pumps favored the Cd^{2+} influx through Ca^{2+} channels in NM roots and *P. involutus*-ectomycorrhiza, while these channels mediate Ca^{2+} influx in the absence of Cd^{2+} stress. In ectomycorrhizas, Cd^{2+} enriched in hyphae is thought to be delivered to the host roots. Moreover, the colonization of *P.* × *canescens* roots with the fungal strains MAJ and NAU stimulates H_2O_2 production and increases H^+-pumping activity, and thus accelerates Cd^{2+} entry through Ca^{2+} channels, in particular through HACCs, under excessive Cd^{2+}. Cd^{2+} ions competitively enter Ca^{2+} channels, and thus diminish the entry of Ca^{2+}, leading to a marked Cd^{2+} enrichment in ectomycorrhizal roots under Cd^{2+} stress.

AUTHOR CONTRIBUTIONS

YhZ and SC conceived the original screening and research plans; SC supervised the experiments; YhZ, GS, YnZ, ZZ, and NL performed most of the experiments; SD, JS, JL, JY, NZ, RZ, and XM provided technical assistance to YhZ, GS and YnZ; YhZ designed the experiments and analyzed the data; YhZ conceived the project and wrote the article with contributions of all the authors; SC and AP supervised and complemented the writing. All authors have read and approved the manuscript.

FUNDING

The research was supported jointly by the Research Project of the Chinese Ministry of Education (grant no. 113013A), the German Science Foundation (DFG) to AP, the Guest Lecturer Scheme of Georg-August Universität Göttingen (Germany), the Alexander von Humboldt-Stiftung (Germany), travel grants by the Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz (BMELV), the National Natural Science Foundation of China (grant nos. 31570587, 31270654), the key project for Oversea Scholars by the Ministry of Human Resources and Social Security of PR China (grant no. 2012001), the Program for Changjiang Scholars and Innovative Research Teams in University (grant no. IRT13047), the Program of Introducing Talents of Discipline to Universities (111 Project, grant no. B13007), and Basic and Frontier Research Plan of Henan Province (No. 132300410399).

ACKNOWLEDGMENTS

We thank Ms. Christine Kettner (Georg-August Universität Göttingen) and Dr. Ulrike Lipka (Georg-August Universität Göttingen) for their excellent technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01975/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 23 February 2017
doi: 10.3389/fpls.2017.00093

Understanding Host-Pathogen Interactions with Expression Profiling of NILs Carrying Rice-Blast Resistance *Pi9* Gene

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Edited by:
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Reviewed by:
Oswaldo Valdes-Lopez, National Autonomous University of Mexico, Mexico
Manish Kumar Pandey, International Crops Research Institute for the Semi-Arid Tropics, India

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Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: 17 August 2016
Accepted: 16 January 2017
Published: 23 February 2017

Citation:
Jain P, Singh PK, Kapoor R, Khanna A, Solanke AU, Krishnan SG, Singh AK, Sharma V and Sharma TR (2017) Understanding Host-Pathogen Interactions with Expression Profiling of NILs Carrying Rice-Blast Resistance Pi9 Gene. Front. Plant Sci. 8:93. doi: 10.3389/fpls.2017.00093

Magnaporthe oryzae infection causes rice blast, a destructive disease that is responsible for considerable decrease in rice yield. Development of resistant varieties via introgressing resistance genes with marker-assisted breeding can eliminate pesticide use and minimize crop losses. Here, resistant near-isogenic line (NIL) of Pusa Basmati-1(PB1) carrying broad spectrum rice blast resistance gene *Pi9* was used to investigate *Pi9*-mediated resistance response. Infected and uninfected resistant NIL and susceptible control line were subjected to RNA-Seq. With the exception of one gene (*Pi9*), transcriptional signatures between the two lines were alike, reflecting basal similarities in their profiles. Resistant and susceptible lines possessed 1043 (727 up-regulated and 316 down-regulated) and 568 (341 up-regulated and 227 down-regulated) unique and significant differentially expressed loci (SDEL), respectively. Pathway analysis revealed higher transcriptional activation of kinases, WRKY, MYB, and ERF transcription factors, JA-ET hormones, chitinases, glycosyl hydrolases, lipid biosynthesis, pathogenesis and secondary metabolism related genes in resistant NIL than susceptible line. Singular enrichment analysis demonstrated that blast resistant NIL is significantly enriched with genes for primary and secondary metabolism, response to biotic stimulus and transcriptional regulation. The co-expression network showed proteins of genes in response to biotic stimulus interacted in a manner unique to resistant NIL upon *M. oryzae* infection. These data suggest that *Pi9* modulates genome-wide transcriptional regulation in resistant NIL but not in susceptible PB1. We successfully used transcriptome profiling to understand the molecular basis of *Pi9*-mediated resistance mechanisms, identified potential candidate genes involved in early pathogen response and revealed the sophisticated transcriptional reprogramming during rice-*M. oryzae* interactions.

Keywords: rice blast, *Magnaporthe oryzae*, host-pathogen interaction, near isogenic lines, RNA-Seq, WRKY, jasmonic acid, ethylene

INTRODUCTION

Rice is among the most important staple foods, contributing 23% of total calories consumed globally. Over 600 million tons of rice is produced annually from 150 million hectares of rice paddies. Asia is responsible for 92% of that production, with three-quarters of the output from India and China (Maclean et al., 2002). Rice is the model system for monocotyledons because its genome has been fully sequenced (International Rice Genome Sequencing Project, 2005; 430 Mb), the availability of high-density genetic maps, genome-wide microarrays (Jung et al., 2007), and genetic transformation methods. Both biotic and abiotic stresses affect rice growth. Among biotic stresses, infection by *Magnaporthe oryzae* (a hemibiotrophic fungus) causes rice blast disease, resulting in a 20–100% global crop loss, an amount that can feed 60 million people (Sharma et al., 2012).

The development of new molecular tools and availability of model plant-fungal systems have increased our understanding of the mechanisms underlying fungal infections. Rice blast is one of the model diseases under study because annotated genome sequences are available for both organisms (*M. oryzae*: Dean et al., 2005; rice: Ohyanagi et al., 2006). The complete genome sequences of the cultivated rice strains *Oryza sativa* L. ssp. japonica and ssp. indica also allow for genome-wide transcriptome analyses (Jiao et al., 2009; Sato et al., 2011).

The gene-for-gene hypothesis operates in the rice-*M. oryzae* system (Flor, 1971). Infection on the rice plant starts with fungal spore attachment to the leaf, followed by germination and appressoria formation within 24 h to penetrate the leaf cuticle, invading epidermal cells (Talbot, 2003; Skamnioti and Gurr, 2009). Effector-triggered immunity (ETI) occurs when the *M. oryzae Avr* (avirulence) protein recognizes rice *R* (resistance) proteins, leading to a hypersensitive response that stops fungal growth. Pathogen triggered immunity (PTI) is triggered if fungal pathogen-associated molecular patterns (PAMP; e.g., chitins) is recognized by rice pattern-recognition receptors (PRR) and fungal hyphae spread inside plant causing disease symptoms (Liu et al., 2010; Chen and Ronald, 2011). Of the two defense responses, ETI is stronger and faster (Tao et al., 2003). Rice-blast resistant varieties can eliminate pesticide use and minimize crop losses, but high pathogen variability and little mechanistic understanding of *R*-mediated resistance pathways mean that newly developed resistant cultivars are often susceptible after only a few years. Currently, over 100 rice-blast *R* genes (50% indica, 45% japonica, 4% wild species) have been mapped, but only 25 have been characterized and cloned (Sharma et al., 2016).

Studying transcriptome dynamics provides insight into functionally important genomic elements, their expression patterns, and their regulation in different developmental stages, tissues, and environmental stressors (Wang et al., 2011). Several methods are available for transcriptome isolation and quantification, including expressed sequence tag (EST) library sequencing, serial analysis of gene expression (SAGE), and SuperSAGE. However, these methods have numerous disadvantages, including low throughput, cloning bias, low sensitivity, and high cost. Microarray hybridization avoids some of these issues and thus sees common use, but the technique is prone to high background noise and requires known gene sequences, meaning it cannot identify novel transcribed regions. Fortunately, next-generation RNA sequencing technology (mRNA-Seq) is now available as a high-throughput method for simultaneously sequencing, mapping, and quantifying transcriptome reads. It is a robust tool for identifying rare or novel transcripts, alternative splice junctions and transcription start sites (Zhang et al., 2010).

Monogenic or near-isogenic lines (NILs) that differ in a single rice-blast resistance gene are useful as differential varieties in pathogenicity tests and as genetic resources in rice breeding programs. However, because the development and phenotyping process is time-consuming and laborious, such lines exist only for a few genes. Among these is *Pi9*, a broad-spectrum rice-blast resistance gene that encodes a nucleotide-binding site–leucine-rich repeat (NBS-LRR) protein and is part of a multigene family on chromosome 6. This gene is widely used in pyramiding programs for increasing broad-spectrum resistance to *M. oryzae*, and is more effective than 14 other NILs against several virulent *M. oryzae* strains (Imam et al., 2014). For example, *Pi9*+*Pita* is an effective combination for incorporation in Indian rice varieties (Khanna et al., 2015a). Numerous studies have examined the expression profiles of rice defense response genes (Vergne et al., 2008; Sharma et al., 2016). However, transcriptome profiling studies of rice NILs upon *M. oryzae* infection are few in number (Sharma et al., 2016). Earlier transcriptome profiling of NIL IRBL22 carrying *Pi9* gene upon *M. oryzae* infection in the background of a japonica cultivar LTH was performed using microarray which provides information only about the known genes (Wei et al., 2013). Here, we have used novel monogenic lines containing *Pi9* in the background of Pusa Basmati1 (PB1), a variety released in 1989 as the first high-yielding, semi-dwarf, photoperiod-insensitive, and superior quality scented rice line. This NIL (PB1+*Pi9*) shows broad-spectrum resistance against 100 diverse strains of *M. oryzae* from eastern (Variar et al., 2009; Imam et al., 2014) and northern (Khanna et al., 2015b) India. This is the first NIL carrying *Pi9* gene in the background of any scented rice and serves an excellent biological material for understanding the molecular basis of rice-*Magnaporthe* interactions characterized by RNA-seq.

The objectives of this study were to identify early transcriptional changes in the PB1+*Pi9* compared with PB1 after 24 h post-infection (hpi) with *M. oryzae* and to find the unique set of genes that were regulated only in PB1+*Pi9* compared to PB1, in providing resistance against *M. oryzae* in PB1+*Pi9*. Our results will improve understanding of the molecular pathways and interactions during *Pi9*-meditated resistance.

MATERIALS AND METHODS

Plant Material and Growth Condition

The experiment was performed using resistant NILs of PB1 (*O. sativa* L. ssp indica) containing *Pi9* and its susceptible counterpart line PB1. The seeds of BC3F6 generation of PB1+*Pi9* NIL was used. The seeds of both rice lines were surface-sterilized and germinated via soaking in water at 37°C for 5 days. Seedlings were transferred to pots filled with sterile soil under standard

growth conditions of 16 h light (115 μ Mol m^{-2} s^{-1}) and 8 h dark at 25 ± 2°C. Two-week-old healthy plants (four-leaf stage) were used for inoculation with highly virulent isolate Mo-nwi-53 of *M. oryzae*.

Fungal Inoculation

Magnaporthe oryzae was maintained on oatmeal agar and Mathur's media at 25 ± 1°C for 15 days. Conidia of *M. oryzae* were collected from culture plates via rinsing with 0.25% gelatin. Conidia were filtered with two layers of gauze and their concentration adjusted to 10^5 spores/ml using a hemocytometer. The mock plants were sprayed only with 0.25% gelatin. An atomizer was used for fine spraying so that spore suspension was retained on the leaves. Both lines (PB1+*Pi9* and PB1) were phenotyped against several *M. oryzae* strains collected from eastern and northern India. Finally, *Magnaporthe oryzae* strain Mo-nwi-53 (from northwest India) was used for inoculation. Three biological replicates were performed for *M. oryzae* and mock inoculation on both resistant and susceptible lines at four-leaf stage. The inoculated plants were placed in a darkened (covered) humid chamber for 24 h at 25 ± 1°C and 90% relative humidity. Leaf tissues were collected from fully expanded leaves of each rice line 24 hpi and frozen in liquid nitrogen. Some leaves from each pot of PB1+*Pi9* and PB1 were kept for disease development (Mackill and Bonman, 1992). On PB1 leaves, spindle-shaped lesions, characteristic of rice blast, and a disease rating of 5 (on a 0–5 scale) were observed after seven days of incubation. In PB1+*Pi9*, a hypersensitive response was observed within 2 days of infection (**Figure 1A**).

RNA Isolation and Library Preparation for Illumina Hiseq 1000

Total RNA was extracted from leaf tissues using Spectrum™ Plant Total RNA Kit (Sigma) following manufacturer protocol. The quantity and quality of RNA was measured using Nanodrop-1000 (Thermo Fischer Scientific), and quality was further assessed in the Agilent 2100 Bioanalyzer (Agilent Technologies). Samples with RNA Integrity Number (RIN) > 8.5 were used for library preparation. Total extracted RNA samples (5 μg) from each biological replicate were used to isolate poly(A) mRNA and to prepare DNA libraries using TruSeq RNA preparation protocol v.2 (Low Throughput protocol, Illumina, Inc., USA). Library quality and size were assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies) using a high sensitivity DNA Kit. Paired-end sequencing was performed with the TruSeq SBS Kit v3-HS (Illumina, Inc.) on Illumina HiSeq 1000. The CASAVA pipeline 1.7 was used for data processing, de-multiplexing and Bcl conversion. Illumina filters were kept for passing all samples reads. Read quality was checked in FastQC. Low-quality reads and adapters were removed using Trimmomatics (Bolger et al., 2014). The data were deposited as GSE81906.

Bioinformatics Analysis

Raw reads were filtered and checked for sequence contaminants using Trimmomatic and FastQC. To build index of the reference genome (*O. sativa* japonica) bowtie2-build was used. The spliced-read mappers Tophat v2.0.9 (Trapnell et al., 2009) is built on the ultrafast short read mapping program bowtie. Tophat was used to map reads individually for each biological replicate in PB1+*Pi9* and PB1 against *O. sativa* japonica group, cultivar Nipponbare; MSU release 7. During alignment of reads with tophat the following parameters were used read mismatch of 2, read gap length of 2, read edit-distance of 2, splice mismatches 0, minimum intron length of 50, maximum intron length of 500,000 maximum multihits 20, maximum insertion and deletion length of 3. The aligned reads obtained from Tophat were analyzed in Qualimap (García-Alcalde et al., 2012) and visualized on IGV. Qualimap was used to obtain reads mapped to exons (including splice-junction reads mapped to exon ends) for each biological replicate of both lines. Cufflink v2.1.1 was used for assembly into transcripts with reference annotation to guide assembly (Trapnell et al., 2010). The following parameters were used in cufflinks for abundance estimation, average fragment length of 200, fragment length standard deviation of 80 and unlimited alignment allowed per fragment. Cuffdiff was used to quantify transcripts with the merged transcript assembly as well as to detect differentially expressed genes (DEG). Quantification of transcript was done in terms of fragment per kilobase of transcript per Million mapped reads (FPKM), false discovery rate of 0.05 was used for testing and pooled dispersion method was used to estimate dispersion model. Cuffdiff output was analyzed in CummeRbund. The significant differentially expressed loci (SDEL) were identified from all expressed loci after applying multiple corrections (FDR adjusted $p \leq 0.05$). The SDEL unique to PB1+*Pi9* or PB1 with $\log_2$ fold change ≥2 were used for further analysis (**Figure 2**). Heat maps were generated using R package, heatmap2. Pearson correlation coefficients were calculated in R. Venn diagrams were generated in InteractiVenn.

Pathway Analysis

Pathway analysis was performed in MapMan version 3.1.1 from the Max Planck Institute of Molecular Plant Physiology, Germany (Thimm et al., 2004). Non-redundant SDEL unique to PB1+*Pi9* and PB1 were classified and their functional annotation was visualized via searching against the *Oryza sativa* TIGR7 database.

Singular Enrichment Analysis

Singular enrichment analysis (SEA) was performed on SDEL unique to PB1+*Pi9* and PB1 using AgriGo (Du et al., 2010). Significant GO terms were identified in biological and molecular function category in PB1+*Pi9* and PB1.

Co-expression Network Analysis

Co-expression network analysis of proteins corresponding to significant differentially expressed loci (SDEL; FDR adjusted $p \leq 0.05$ & $\log_2$ fold change ≥2) unique to PB1+*Pi9* in response to biotic stimulus. In the co-expression network, circular node represents the protein encoded by the respective SDEL was performed with STRING 10 (Szklarczyk et al., 2015). Proteins were represented as a node with a specific number. Nodes were connected by interconnecting lines that represent the source by which protein interactions were derived. Source of protein interaction were represented with black, pink, green

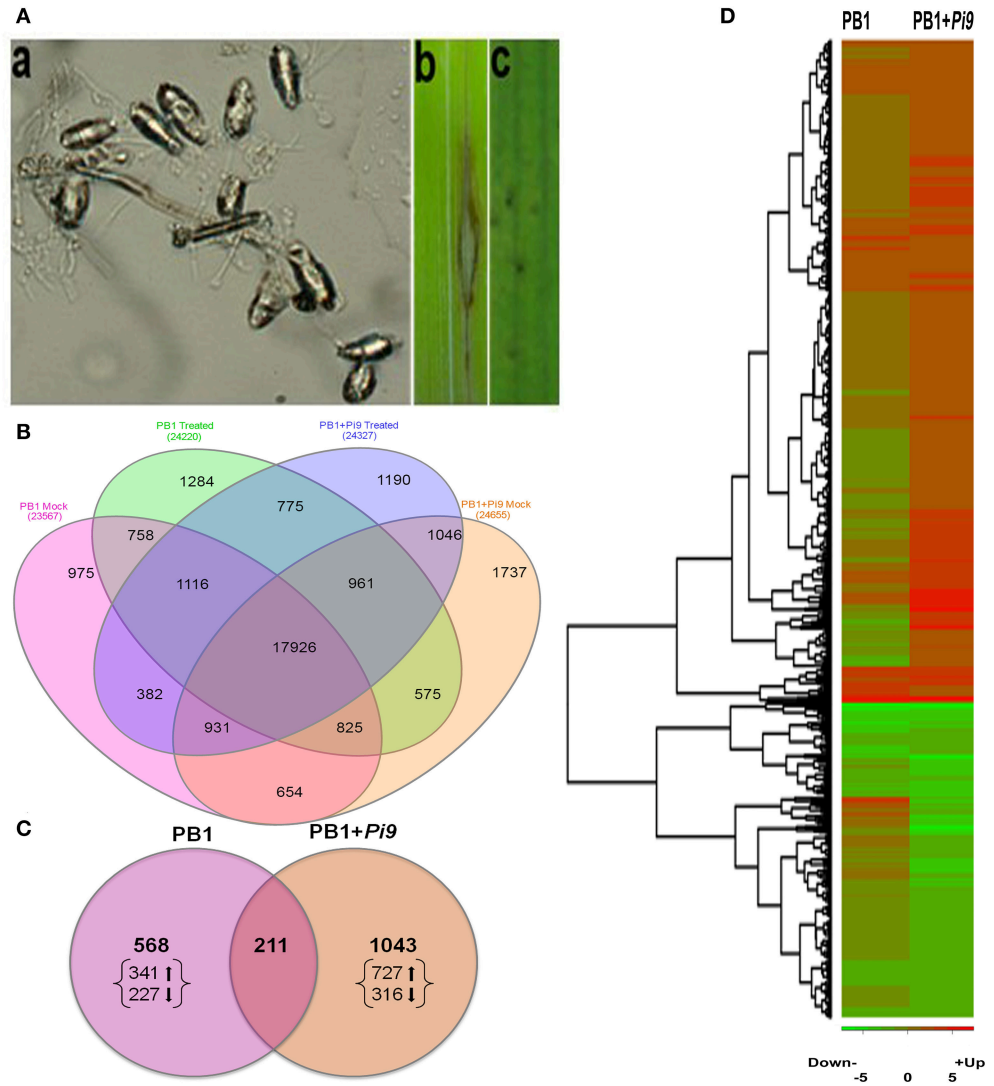

FIGURE 1 | Disease interaction and expression profile of resistant (PB1+*Pi9*) and susceptible (PB1) lines 24 hpi with *Magnaporthae oryzae*. (A) a: *M. oryzae* spore, b: compatible reaction of PB1 line, and c: Incompatible reaction of PB1+*Pi9* NIL. **(B)** Expressed loci common between PB1+*Pi9* and PB1 with *M. oryzae* inoculation and without *M. oryzae* inoculation (mock). **(C)** Significant (FDR adjusted $p \leq 0.05$) differentially expressed loci ($\log_2$ fold change ≥ 2) common across PB1+*Pi9* and PB1. **(D)** Heat map of significant (FDR adjusted $p \leq 0.05$ & $\log_2$ fold change ≥ 2) differentially expressed loci of PB1+*Pi9* and their respective $\log_2$ fold change in PB1. Red represents up-regulated loci and green represents down-regulated loci.

and blue lines that represent co-expression, experimental data, text mining, and homology respectively. Confidence of each interaction between proteins was obtained in terms of total score.

Real Time PCR Validation

Real time PCR (qRT-PCR) was used to validate SDEL obtained from RNA-Seq. Primers were designed using PrimerQuest (IDT) and were listed in Supplementary Table 1. The ProtoScript M-MuLV First Strand cDNA synthesis kit (NEB) was used to synthesize cDNA. The reaction was performed in the LightCycler® 480 II PCR system (Roche) with a volume of 10 μL, containing 5 μL of SYBR Green I Master, 0.2 μL of forward primer, 0.2 μL reverse primer, 2 μL of 1:5 diluted cDNA template and RNAase free water. Actin was used as internal control. The efficiency of RT-PCR was calculated in both control and target samples. Fold change was calculated using $2^{-\Delta\Delta CT}$ method.

RESULTS AND DISCUSSION

Differentially Expressed Genes in Rice-Blast Resistant NIL (PB1+*Pi9*)

To study early defense mechanisms against *M. oryzae* in rice, RNA-Seq was performed on PB1+*Pi9* NIL and susceptible control PB1 24 hpi. The PB1+*Pi9* NIL was developed with

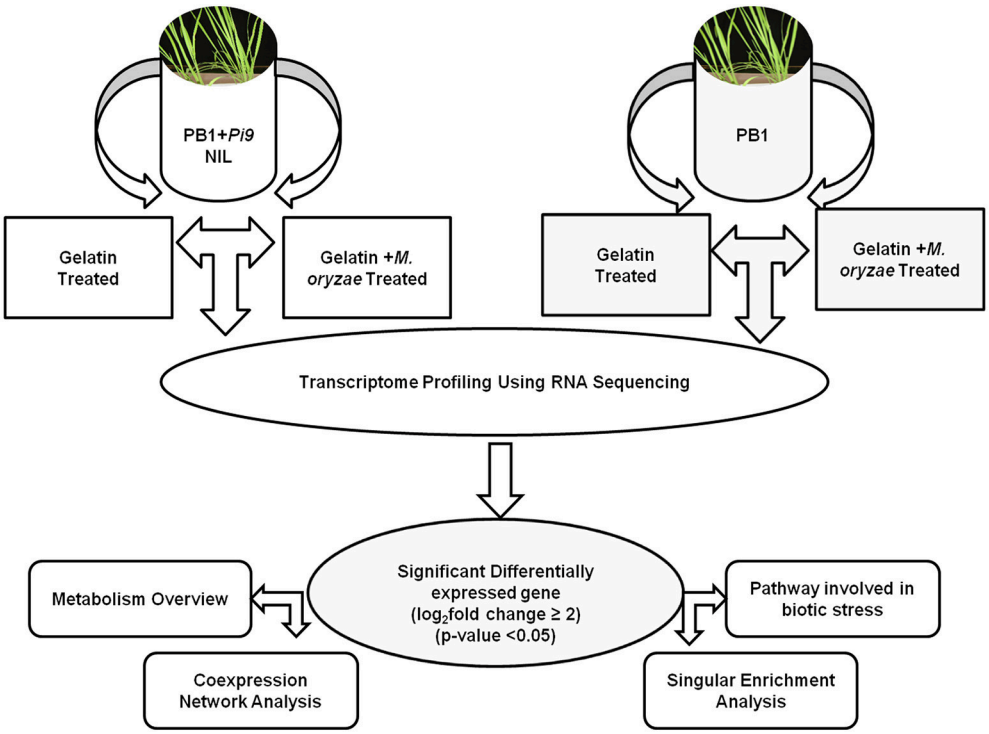

FIGURE 2 | *Magnaporthe* infection and RNA-Seq workflow of PB1+*Pi9* and PB1.

marker-assisted backcross breeding (Khanna et al., 2015b). The functional marker NBS2-Pi9 195-1 (Qu et al., 2006) and gene-linked marker AP5659-5 (Fjellstrom et al., 2006) were used to confirm the presence of *Pi9* in the NIL. Background selection was performed to minimize linkage drag and maximize recipient parent recovery, which was upto 95.6% (Khanna et al., 2015b). *Pi9* is a constitutively expressing gene regardless of pathogen infection; however, post-transcriptional reprogramming allows the *R* gene to activate downstream processes involved in biotic stress only after *M. oryzae* infection (Qu et al., 2006). Here, the PB1+*Pi9*-*M. oryzae* interaction was observed incompatible, possibly mediated by ETI, whereas the PB1-*M. oryzae* interaction was compatible and likely represented PTI (**Figure 1A**).

The paired-end transcriptome sequencing was performed for NILs on Illumina Hiseq 1000. Approximately 30 million pairs of filtered 101 base-pair reads were obtained from each biological replicate (Supplementary Table 2). Around 90% of these pre-processed reads aligned to RGAP7 (ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/). Multiple hits to RGAP7 occurred for around 1–2 million reads (Supplementary Table 2). The biological replicates of treated and untreated samples from both the lines were highly correlated (Pearson's $R > 0.6$), indicating strong reproducibility (Supplementary Tables 3, 4). Between PB1+*Pi9* and PB1, 20,778 (with inoculation) and 20,336 (without inoculation) commonly expressed loci were found (**Figure 1B**), suggesting minimal background noise exists between these two lines.

Among commonly expressed loci, 74% (17,926) of the loci were common between NIL PB1+*Pi9* and PB1, with and without pathogen treatment, reflecting basal similarities between their transcriptional profiles, despite differing in *Pi9* presence. Wei et al. (2013) reported similar results for the same model system, as did Tao et al. (2003) for *Arabidopsis thaliana-Pseudomonas syringae* interactions. Together, these data indicate that the *O. sativa-M. oryzae* interaction is quantitative, like other plant-pathogen relationships. Total 8418 (4914 up-regulated and 3504 down-regulated) and 3336 (1837 up-regulated and 1494 down-regulated) SDEL were obtained in the PB1+*Pi9* and PB1 rice lines 24 hpi, respectively (Supplementary Figure 1). Among them 2027 SDEL were common between PB1+*Pi9* and PB1. The number of SDEL with $\log_2$ fold change ≥ 2 were 1254 (850 up-regulated and 404 down-regulated) and 781 (466 up-regulated and 313 down-regulated) in PB1+*Pi9* and PB1, respectively (**Figure 1D**). Among them, 211 SDEL were found common between resistance and susceptible lines (**Figure 1C**). The SDEL exclusively regulated in PB1+*Pi9* were 1043 (727 up-regulated and 316 down-regulated) and in PB1 were 568 (341 up-regulated and 227 down-regulated) respectively. The proportion of up and down-regulated unique SDEL was higher in PB1+*Pi9* than in PB1. The SDEL with $\log_2$ fold change ≥ 2 unique to PB1+*Pi9* or PB1 were used for further analysis.

Identification of Genes for Primary Defense Response through Respiratory Burst

Plants use ROS as stress-signal transduction molecules, and their accumulation plays a central role in plant stress response (Fujita et al., 2006; Ton et al., 2009), inducing unique ROS-responsive genes (Gadjev et al., 2006). In infected plants, respiratory burst is the earliest and most rapid defense response which involves generation of active ROS (primarily superoxide and H_2O_2) to control pathogen spread (Grant et al., 2000). NADPH oxidase also plays a central role in ROS generation. ROS causes lipid peroxidation and membrane damage (Montillet et al., 2005). Major ROS scavenging enzymes (redoxin, glutathioredoxin, catalase, and peroxidase) have to restrict ROS dependent damage and fine tune ROS signaling (Mittler et al., 2004). Peroxidase catalyze ROS during last step of cell wall fortification to polymerize lignin (Wally and Punja, 2010) and cross–linking of cell-wall components. This makes the cell wall stronger to fight against the invading pathogen. Our results showed differential expression of three categories of respiratory-burst genes, namely, redox state, peroxidases, and glutathione S-transferases (**Figure 3** and Supplementary Table 5). Five redoxin genes were found up-regulated in PB1+*Pi9* while one is down-regulated. Among seven peroxidase precursors found in PB1+*Pi9*, six were up-regulated and one is down-regulated. All glutathione S transferase loci were found up-regulated in PB1+*Pi9*. Previous studies have demonstrated the up-regulation of 10 peroxidase genes in *M. oryzae*-infected rice (Sasaki et al., 2004) and a 16-fold up-regulation of class III peroxidases in the transgenic rice line TP-*Pi54* using microarray (Gupta et al., 2012). For example, *M. grisea* infection triggered the differential expression of 34 GST genes in a susceptible rice line (Ribot et al., 2008). Under pathogen attack, many plants differentially regulate GSTs (Alvarez et al., 1998; Wagner et al., 2002). The relative expression of peroxidase (LOC_Os08g02110), thioredoxin (LOC_Os07g29410), and cytochrome P450 (LOC_Os06g39780) was validated with real time PCR for infected and uninfected samples of PB1+*Pi9* after 24 hpi (**Figure 4**, Supplementary Table 6, and Supplementary Figure 2). The qRT-PCR results also support the transcriptome data. Although PB1 shows compatible reaction, many ROS scavenging genes were differentially expressed after *M. oryzae* infection. Total five redoxin, five peroxidase precursors and three GSTs were up-regulated (Supplementary Figure 3). This may be a result of primary ROS burst after infection as stated by Lamb and Dixon (1997). In PB1+*Pi9* and PB1, major loci of redoxins, peroxidases and glutathione S transferases were showing similar trend of up-regulation. This reflects that in both PB1+*Pi9* (incompatible interaction) and PB1 (compatible interaction) upon *M. oryzae* attack, unique set of respiratory burst responsive loci were activated. However, during incompatible interaction

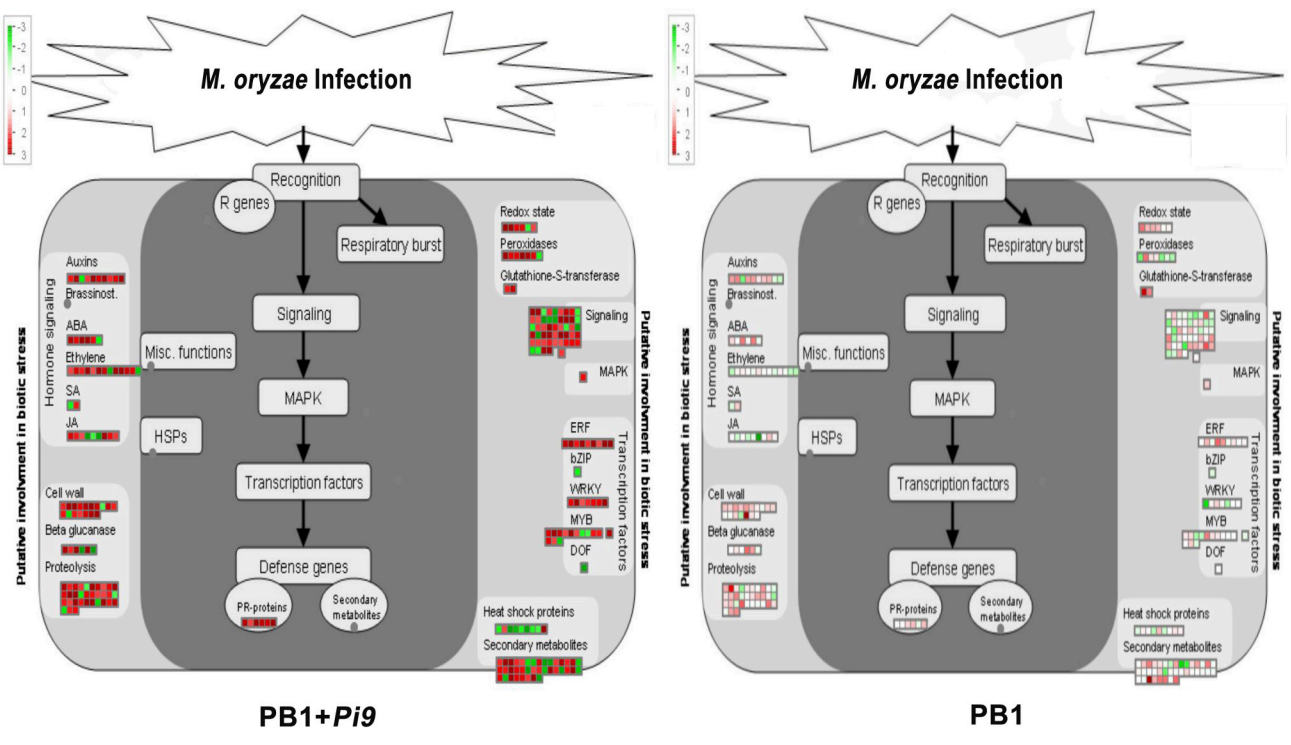

FIGURE 3 | MapMan overview of significant (FDR adjusted $p \leq 0.05$ & $\log_2$ fold change ≥ 2) differentially expressed loci (SDEL) involved in biotic stress pathway, unique to PB1+*Pi9*, upon *M. oryzae* infection. SDEL are binned to MapMan functional categories and values represented as $\log_2$ fold change values. Red represents up-regulated loci and green represents down-regulated loci. JA, jasmonic acid; SA, salicylic acid; bZIP, basic region-leucine zipper; ERF, ethylene response factor; MAPK, mitogen-activated protein kinase; PR-protein, pathogenesis-related protein.

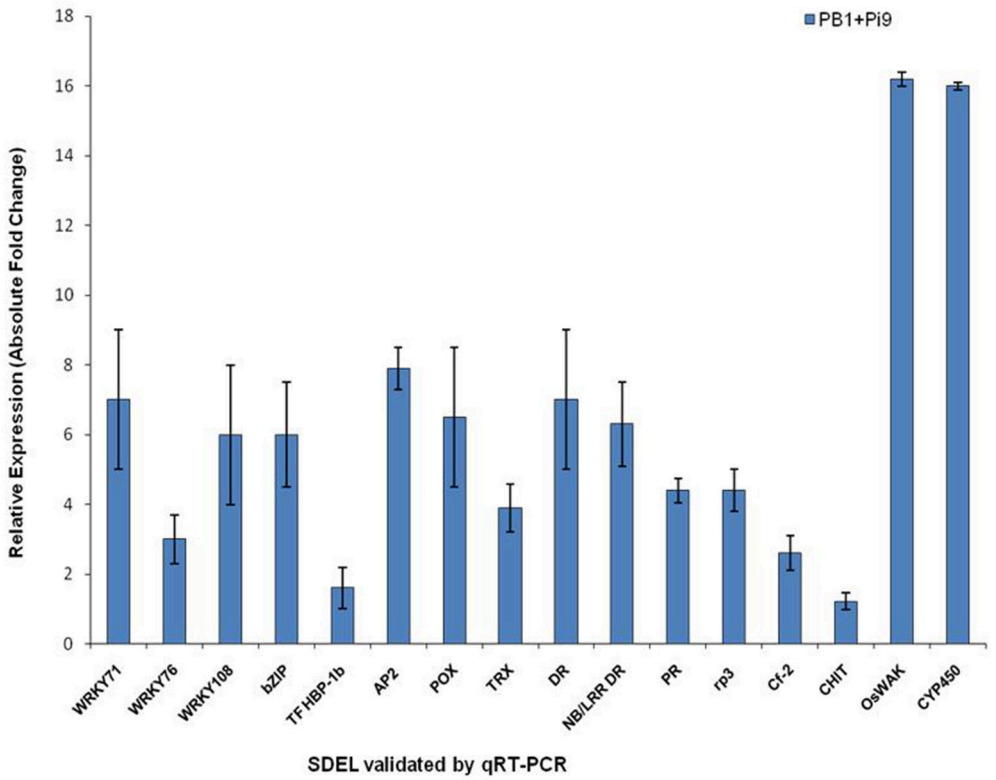

FIGURE 4 | The qRT-PCR validation of significant differentially expressed loci (SDEL) in PB1+*Pi9* upon *M. oryzae* infection. Blue bar represents the absolute fold change of PB1+*Pi9*. Actin was used for transcript normalization. Standard error bar show the standard deviation for three triplicate assay. Abbreviations: WRKY71, WRKY71 transcription factor (LOC_Os02g08440); WRKY76, WRKY76 transcription factor (LOC_Os09g25060); WRKY108, WRKY108 transcription factor (LOC_Os01g60600); bZIP, bZIP transcription factor domain (LOC_Os07g48660); TF HBP-1b, transcription factor HBP-1b (LOC_Os01g06560); AP2, AP2 domain containing protein (LOC_Os02g45420); POX, Peroxidase (LOC_Os08g02110); TRX, thioredoxin (LOC_Os07g29410); DR, disease resistance protein SlVe2 precursor (LOC_Os01g06836); NB/LRR DR, NB-ARC/LRR disease resistance protein (LOC_Os04g43440); PR, pathogenesis-related Bet v I (LOC_Os08g28670); rp3, rp3 protein (LOC_Os12g03080); Cf-2, Cf-2 (LOC_Os01g06876); CHIT, CHIT1-Chitinase (LOC_Os02g39330); OsWAK, OsWAK95- OsWAK receptor-like protein kinase (LOC_Os10g02250); CYP450, cytochrome P450 (LOC_Os06g39780).

ROS accumulation starts with lower amplitude followed by sustained phase of much higher amplitude, while in compatible interaction there is only transient low amplitude single phase ROS accumulation.

Host Hormonal Regulation during *M. oryzae* Infection

The burst of jasmonic acid (JA) and ethylene (ET) is well characterized in plant-pathogen interactions. Beside JA and ET, salicylic acid (SA) also acts as important signal molecules in biotic stress responses (Pieterse et al., 2009). SA primarily involved in resistance to biotrophic pathogens, whereas JA and ET influence necrotroph resistance (Roberts et al., 2011). In PB1+*Pi9*, one loci (LOC_Os06g20920) of SAM-dependent carboxyl methyl transferases (SAMT) is up-regulated whereas other (LOC_Os06g20790) is down-regulated (**Figure 3** and Supplementary Table 7). SAMT converts SA into methyl salicylate (MeSA). During pathogen attack MeSA acts as airborne signal involved in intra and inter plant communication (Shulaev et al., 1997). Earlier it was shown that exogenous SA treatment did not induce *M. oryzae* resistance in young rice plants (four-leaf stage), but it induces in adults eight-leaf stage (Iwai et al., 2007). Here, plants from PB1+*Pi9* and PB1 were infected at the four-leaf stage; therefore, SA did not change much significantly in response to *M. oryzae*.

JA biosynthesis and signal transduction related six genes were up-regulated in PB1+*Pi9* post infection (**Figure 3** and Supplementary Table 7). Among six up-regulated loci, four were involved in JA biosynthesis, out of which, two were lipoxygenase (LOX) that converts the Linolenic acid to hydroperoxy derivatives and two were 12-oxophytodienoate reductase (similar to OPR) that converts 12-oxo phytodienoic acid to 3-oxo-2-cyclopentane-1-octanoic acid during JA biosynthesis. The rest two up-regulated loci were zinc finger protein (LOC_Os07g42370) involved in JA signal transduction and CBS domain containing membrane protein (LOC_Os09g02710) similar to loss of the timing of ET and JA biosynthesis 2 gene (LEJ2). CBS domain containing protein is reported to regulate thioredoxin system (Yoo et al., 2011).

Ten ET-metabolism-related unique loci were up-regulated and two were down-regulated in infected PB1+*Pi9* (**Figure 3** and Supplementary Table 7). Among 10 up-regulated loci four were 1-aminocyclopropane-1-carboxylate oxidase proteins similar to ethylene forming enzyme (AT1GO5010), four were AP2 domain containing protein involved in ethylene signal transduction and two were ethylene responsive proteins. The down-regulated loci were AP2 domain containing protein and ethylene responsive protein in PB1+*Pi9*.

In PB1 only one locus related to JA & ET biosynthesis were found up-regulated (Supplementary Figure 3). The induction of several genes involved in JA and ET signaling and biosynthesis was quantitatively higher in PB1+*Pi9* than in PB1. The above trend leads to enhance JA-ET signaling in PB1+*Pi9*. Previous several reports indicated that JA is a strong activator of resistance against hemibiotrophs *M. oryzae* and *X. oryzae pv. oryzae* (Deng et al., 2012; Yamada et al., 2012; Riemann et al., 2013). During transcriptome profiling of resistant (IRBL18, IRBL22) and susceptible (LTH) lines after *M. oryzae* infection, eight out of 34 enzymes involved in JA biosynthesis were up-regulated (Wei et al., 2013). It was observed that exogenous application of ET inhibitor and generator respectively induced and suppressed rice-blast infection (Singh et al., 2004). Activation of ET emission was found little earlier in incompatible compared to compatible interaction (Iwai et al., 2006). Additionally, ET-overproducing rice transformants increased resistance to both the fungal pathogen *Rhizoctonia solani* and *M. oryzae* (Helliwell et al., 2013). Normally, ETI response involves redundant activities of both SA and JA-ET pathways (Tsuda et al., 2009). However, when SA signaling is not active, substantial resistance to pathogen is contributed by JA-ET pathway (Dodds and Rathjen, 2010). Our results reflect that the increased level of ET and JA production in PB1+*Pi9* helps in providing resistance to *M. oryzae* compared to PB1.The induction in level of JA-ET hormone in PB1+*Pi9* might be controlling the expression of defense genes by regulating the abundance of transcription factors.

Kinase-Mediated Signaling

Plant signal perception and activation of downstream processes is crucial for the innate immunity. Defense signaling involves molecules such as receptor-like kinases (RLKs), MAPK, and calmodulin-related calcium sensor protein. In PB1+*Pi9* 29 receptor kinases (RK) were found to be differentially expressed (**Figure 3** and Supplementary Table 8). Previous research in IRBL18 and IRBL22 (resistant NILs), 103 receptor kinases (subfamily RLK and WAK) were up-regulated, while one was down-regulated (Wei et al., 2013). In Digu rice, 48 SDEL of receptor kinases were found upon *M. oryzae* infection (Li et al., 2016). Further, in a transgenic rice line carrying rice-blast resistance gene *Pi54* (Gupta et al., 2012), as well as resistant NILs IRBL18 and IRBL22 (Wei et al., 2013), signaling-related genes were highly up-regulated post *M. oryzae* infection, compared with susceptible controls.

During infection, WAK triggers innate immune response through detecting fungal cell wall-associated oligogalacturonides (Brutus et al., 2010). From OsWAK subfamily four OsWAK (LOC_Os02g56370, LOC_Os09g38850, LOC_Os09g38910, LOC_Os10g02250) and one OsMAPK (LOC_Os05g49140) were up-regulated in PB1+*Pi9* (**Figure 3** and Supplementary Table 8). OsWAK receptor-like protein kinase (OsWAK95, LOC_Os10g02250) was validated with real time PCR in infected and uninfected samples of PB1+*Pi9* (**Figure 4**, Supplementary Table 6, Supplementary Figure 2). Upon *M. oryzae* infection several calcium dependent kinase were also activated. In PB1+*Pi9* eight calmodulin related calcium sensor protein and three G-proteins were up-regulated. The most highly up-regulated receptor kinase in PB1+*Pi9* after *M. oryzae* infection is LOC_Os06g13320.1 with FPKM value of 0.6 (3.5-fold; **Figure 3** and Supplementary Table 8). A previous microarray analysis of blast-infected rice revealed high up-regulation of OsWAK71 and OsWAK25 (Wei et al., 2013). During transcriptional profiling of resistant vs. susceptible lines, the former exhibited more up-regulated WAKs (Bagnaresi et al., 2012). Mitogen-activated protein kinases are conserved signaling molecules that transduce extracellular stimuli into intra-cellular responses. Active MAPK further activates the downstream transcription factors (TF). Earlier up-regulation of four MAPK transcripts, as well as MAP3K.3 and MAP3K.1 isoforms, were observed only in a resistant rice line (Bagnaresi et al., 2012). In PB1 few receptor kinases were differentially expressed (Supplementary Figure 3). But these kinases were different from kinases differentially expressed in PB1+*Pi9*. So, the signaling mediated by kinases in PB1 is not able to provide resistance against *M. oryzae*. The expression trend of kinases in mediating signaling to provide resistance against *M. oryzae* in PB1+*Pi9* reflects that up-regulation of several subfamilies of kinases, calcium sensor and G protein that positively regulate the activation of transcription factors. Few kinases being down-regulated negatively regulate the defense response in PB1+*Pi9*. This also shows that the positive and negative regulation by kinases being differentially expressed in PB1+*Pi9* together mediated stronger ETI- responses in PB1+*Pi9* compared to PB1, with a basal response similar to those of earlier studies.

Regulation by Transcription Factors

Defense signaling pathways trigger the action of transcription factors such as WRKY, MYB, and ERF. We observed up-regulation of seven WRKY loci in PB1+*Pi9* 24 hpi (**Figure 3** and Supplementary Table 9). Notable up-regulated WRKYs in PB1+*Pi9* include WRKY47 (LOC_Os07g48260) with FPKM value of 17.8 after infection, which was reported to enhances resistance against *M. oryzae* in transgenic rice lines along with other WRKY genes (Wei et al., 2013). WRKY71 (LOC_Os02g08440) exhibited up-regulation with FPKM value of 406.7 (2.5-fold) after infection in PB1+*Pi9*. Its over-expression was shown to enhance resistance against bacterial pathogen *X. oryzae* by triggering defense-related genes, including chitinases, PR-5 and peroxidases (Liu et al., 2007). WRKY42 was up-regulated in PB1+*Pi9* with FPKM value of 5.9 (3.8-fold) after infection also shown to induce ROS production in rice (Han et al., 2014). WRKY76 (LOC_Os09g25060) which suppresses PR-gene induction and affects phytoalexin synthesis (Yokotani et al., 2013); was also up-regulated with

FPKM value of 11.2937 (2.3-fold) in PB1+*Pi9* after infection. WRKY28 (LOC_Os06g44010), which was up-regulated here after *M. oryzae* infection, shown to activate OsPR10 gene by its over expression and provide resistance against *X. oryzae* (Peng et al., 2010). Several microarray studies have found that *M. oryzae* infection induces numerous WRKY genes like WRKY76, WRKY47, WRKY45, WRKY55, WRKY53, WRKY62, and WRKY71 (Akimoto-Tomiyama et al., 2003; Chujo et al., 2007; Zhang et al., 2008; Wei et al., 2013). Clearly, WRKYs were key to pathogen resistance in rice, via the formation of a transcriptional network. Therefore, up-regulation of OsWRKY family members were implicated in the regulation of transcriptional reprogramming associated with early response to *M. oryzae*. Another class of transcription factors involved in plant stress response is MYBs, which also influence the metabolism, differentiation, and development (Ambawat et al., 2013). In PB1+*Pi9*, 10 MYBs were up-regulated and two were down-regulated (**Figure 3** and Supplementary Table 9). Specifically, defense-related roles of MYBs include phenylpropanoid biosynthesis (Dubos et al., 2010), biotic stress induced ROS production (Heine et al., 2004) and immune signaling (Ramalingam et al., 2003; Rasmussen et al., 2012). In PB1+*Pi9*, four AP2-domain containing proteins were up-regulated and five dehydration responsive element binding proteins (DREB) were up-regulated (**Figure 3** and Supplementary Table 9). Transcription factors containing ERF/AP2 domains directly regulate PR gene expression (Gu et al., 2002; Oñate-Sánchez and Singh, 2002; Zarei et al., 2011). The ERF transcription factors integrate signals received from JA and ET (Lorenzo et al., 2003).

In PB1 only one WRKY and six MYB loci were found to be up-regulated while four MYB loci were down-regulated (Supplementary Figure 3). The relative expression of WRKY71 (LOC_Os02g08440), bZIP transcription factor domain containing protein (LOC_Os07g48660), AP2 domain containing protein (LOC_Os02g45420), transcription factor HBP-1b (LOC_Os01g06560), WRKY108 (LOC_Os01g60600), and WRKY76 (LOC_Os09g25060) was validated with real time PCR in infected and uninfected samples of PB1+*Pi9* (**Figure 4**, Supplementary Table 6 and Supplementary Figure 2).

Activation of Primary and Secondary Metabolites

Carbohydrate, lipid, and protein metabolism, as well as photosynthesis, produce primary metabolites in plants. In contrast, secondary metabolites include phenylpropanoids, lignin, phenolics, waxes, terpens, and flavanoids. Pathogen infection affects host lipid metabolism. We observed the up-regulation of all six loci involved in phospholipid-biosynthesis in infected PB1+*Pi9*. Among six loci four (LOC_Os01g50030, LOC_Os01g50032, LOC_Os05g47540, LOC_Os05g47545) were similar to phosphoethanolamine-N-methyltransferase that converts S-adenosyl methionine to S-methionine homocysteine during phospholipid biosynthesis (**Figure 5** and Supplementary Table 10). Rest two up-regulated loci were phosphatidate

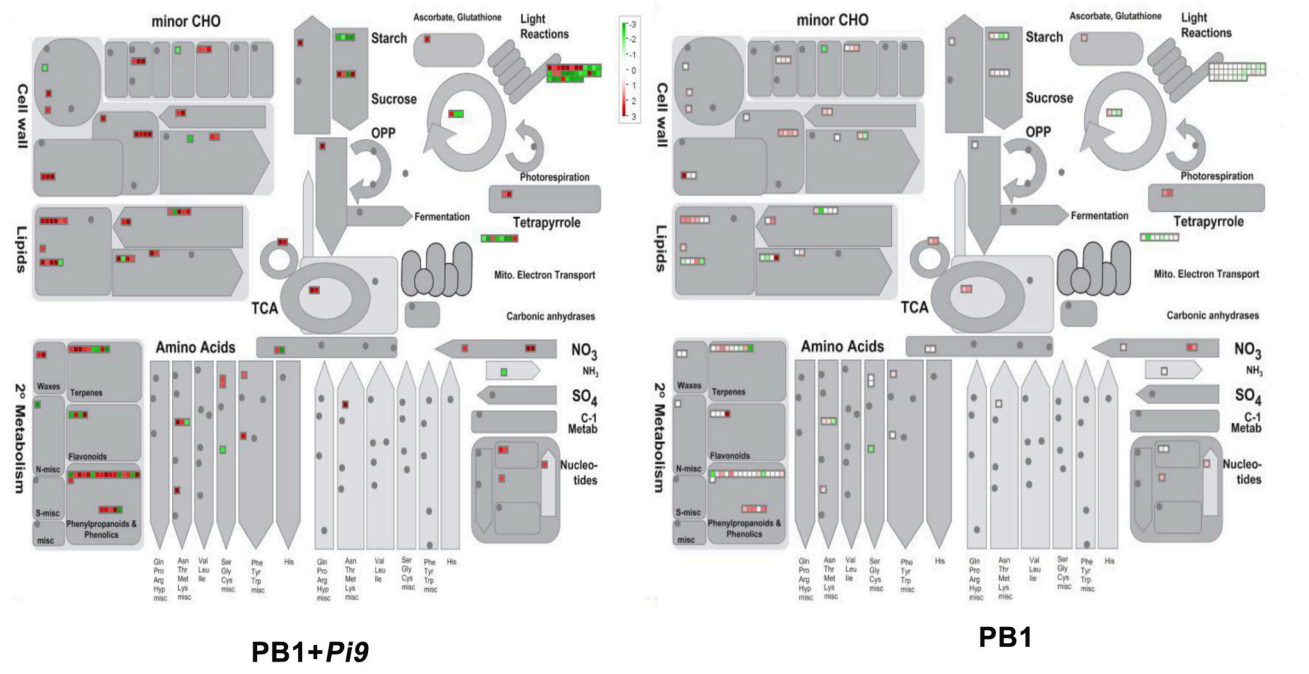

FIGURE 5 | Metabolism overview of significant differentially expressed loci (FDR adjusted $p \leq 0.05$ & $\log_2$ fold change ≥2) unique to PB1+*Pi9*. Metabolism overview covers the primary and secondary metabolism, upon *M. oryzae* infection in PB1+*Pi9*. SDEL are binned to MapMan functional categories and values represented as log2-transformed values. Red represents up-regulated loci and green represents down-regulated loci.

cytidylyltransferase (LOC_Os10g17990) and diacylglycerol kinase (LOC_Os04g54200) involved in phospholipid biosynthesis (Li-Beisson et al., 2013). Phosphatidic acid (PA) formed during phopsholipid synthesis induce generation of ROS in *Arabidopsis* (Sang et al., 2001) and rice (Yamaguchi et al., 2003). PA activates MAPK and induces expression of defense related genes (Yamaguchi et al., 2005) and plays positive role in mobilizing defense response (Yamaguchi et al., 2009). A locus of sphingolipid delta desaturase (LOC_Os02g42660) was found two-fold up-regulated in PB1+*Pi9*. Sphingolipids induce ROS production that leads to programmed cell death (Brodersen et al., 2002; Shi et al., 2007). Genes involved in fatty acid (FA) synthesis and elongation were also highly up-regulated in PB1+*Pi9* (**Figure 5** and Supplementary Table 10). Four loci involved in FA synthesis and elongation were found up-regulated in PB1+*Pi9*. Among four up-regulated loci two were ketoacyl CoA synthase involved in both long chain FA and wax biosynthesis (Todd et al., 1999). Rest two were 3-oxoacyl synthase and acyl desturase. Fatty acid plays role in regulating enzyme activity that were involved in generation of signal molecules in plant defense (Shah, 2005). Two loci involved in fatty acid desaturation namely omega-3 and omega-6 fatty acid desaturase (LOC_Os07g23430, LOC_Os03g18070) were up-regulated in PB1+*Pi9*. These enzymes were responsible for the synthesis of polyunsaturated fatty acids (PUFA), which act as antimicrobial agents and signaling molecules (Iba, 2002; Turner et al., 2002; Weber, 2002; Yaeno et al., 2004). Oxylipins, another antimicrobial compound, were synthesized from PUFA through the action of lipoxygenase. Two loci of lipoxygenase (LOC_Os08g39840, LOC_Os04g37430) were up-regulated in PB1+*Pi9* (**Table 1**). In plants, oxylipins

TABLE 1 | The significant differentially expressed loci (SDEL; FDR adjusted $p \leq 0.05$ & $\log_2$ fold change ≥2) of chitinase, glucanase, lipoxygenase, LTP (lipid transfer protein) and pathogenesis-related proteins, unique to PB1+*Pi9*, upon *M. oryzae* infection.

SDEL	PB1+*Pi9*			PB1		
Chitinase	**FPKM Mock**	**FPKM *M. oryzae* Treated**	**Fold Change**	**FPKM Mock**	**FPKM *M. oryzae* Treated**	**Fold Change**
LOC_Os02g39330	6.9	105.2	3.9	59	106.9	0.9
LOC_Os10g39680	19.2	113.9	2.6	53.9	65.6	0.3
GLYCOSYL HYDROLASE						
LOC_Os04g51460	3.1	61.7	4.3	49.4	62.9	0.3
LOC_Os01g64100	0.1	0.8	3.8	1.1	0.2	−2.4
LOC_Os02g01590	0	0.5	3.6	1	1.1	0.2
LOC_Os01g51570	46.5	511	3.5	222	233.3	0.1
LOC_Os05g41610	4.6	38	3	19	52.5	1.5
LOC_Os06g48160	0.6	4.3	2.9	3.9	6.7	0.8
LOC_Os01g71340	136.6	1018.3	2.9	185.7	180.2	0
LOC_Os01g64110	4.8	30.9	2.7	15.1	8.6	−0.8
LOC_Os02g33110	13.8	57.8	2.1	59.6	61.4	0
LIPOXYGENASE						
LOC_Os08g39840	67.6	335.5	2.3	197.3	95.8	−1
LOC_Os04g37430	2.8	11.3	2	13.4	12.5	−0.1
LTP						
LOC_Os04g52260	13.7	765.3	5.8	685.1	2661.3	2
LOC_Os03g50960	1.5	40.2	4.7	60.8	113.1	0.9
LOC_Os04g33920	0.1	1.9	3.8	1.3	4.7	1.9
LOC_Os05g06780	0.6	4.4	2.9	8.2	12.4	0.6
LOC_Os03g01320	2.4	18.7	3	16.5	32.4	1
LOC_Os04g46830	0.1	0.4	2.7	0	0.2	2.7
LOC_Os07g18990	19	95.6	2.3	76.6	66.3	−0.2
LOC_Os07g19000	3.3	16.2	2.3	18.6	16.3	−0.2
LOC_Os10g40520	4.4	18.8	2.1	6.2	7.4	0.3
PR-PROTEINS						
LOC_Os01g06836	0.2	2.1	3.7	9.9	22.6	1.2
LOC_Os08g28670	0.5	5.5	3.4	0.5	0.8	0.7
LOC_Os04g43440	4.7	30.7	2.7	34.9	21.8	−0.7
LOC_Os04g50700	3.2	16.6	2.4	10	34.5	1.8
LOC_Os01g53090	18.9	98	2.4	25.2	11.6	−1.1

The expression value of each locus was measured in terms of fragment per kilobase of transcript per million mapped reads (FPKM) with (M. oryzae) and without (Mock) treatment in both PB1+Pi9 and PB1 along with their respective $\log_2$ fold changes (FC).

were potent signaling molecule in defense response. Five loci of lipases were found up-regulated in PB1+*Pi9*, among which three loci (LOC_Os08g04800, LOC_Os04g56240, LOC_Os07g34420) were lipases and two were phospholipases (LOC_Os06g40170, LOC_Os05g07880) (**Figure 5** and Supplementary Table 10). Phospholipases catalyze hydrolysis of phospholipid to release free fatty acid for synthesis of JA during plant defense response (Shah, 2005).

Infection also alters protein degradation and modification. The consecutive action of three protein classes (E1s, E2s, E3s; Komander, 2009) mediates the process of ubiquitination. PB1+*Pi9* exhibited differential regulation of genes related to F-Box and RING subcomplex of E3 ligase in ubiquitin-dependent degradation (**Figure 3** and Supplementary Table 11). The present results were in agreement with previous data in which it has been found that a UPS ubiquitin proteasome system protein OsBBI1 with E3 ligase activity possesses broad-spectrum resistance to *M. oryzae* (Li et al., 2011). JA-ET hormones induced in PB1+*Pi9* may be regulating expression of defense genes by TF through regulated protein degradation. Photosynthesis in rice is compromised upon *M. oryzae* infection because the pathogen competes with the host for photosynthates (Bastiaans and Kropff, 1993). Several studies reported down-regulation of photosynthesis-related genes during *M. oryzae* attack (Vergne et al., 2007; Bagnaresi et al., 2012). Many genes from the light reaction of photosynthesis were differentially expressed in PB1+*Pi9* (**Figure 5**). Similarly, an earlier study indicated that the down-regulation of photosynthesis-related genes reflects the usage of energy and resources to defend against invading pathogens (Bolton, 2009).

Secondary metabolites were the products of specialized metabolic pathways that were important in defense but not essential to plant existence. Several enzymes like phenyl ammonia lyase (PAL), coumarate CoA ligase (4CL), cinnamyl alcohol dehydrogenase (CAD) and caffeoyl CoA-o-methyl transferase (CCoAOMT) involved in phenyl propanoid (PP) pathway were found up-regulated in PB1+*Pi9* upon *M. oryzae* attack (**Table 2**). Two loci, each of PAL and CCoAOMT that convert phenylalanine to cinnamic acid and caffeoyl-CoA to feruloyl-CoA respectively were found up-regulated in PB1+*Pi9*. Two loci of AMP binding domain were found up-regulated that is similar to 4-coumarate CoA ligase (4CL) and were involved in several conversions during PP pathway. In PB1+*Pi9* two loci of dehydrogenase similar to cinnamyl alcohol dehydrogenase (CAD7) were found up-regulated. And CAD enzyme converts aldehyde to alcohol during PP pathway. Peroxidases found up-regulated in PB1+*Pi9* were involved in conversion of alcohol to lignin during PP pathway (**Figure 5** and Supplementary Table 12). The phenylpropanoid pathway is critical to plant defense because it is involved in synthesis of phytoalexin (include isoflavonoids, terpenoids, alkaloids etc), lignin, flavanoids, coumarins, phenylpropanoid esters, and cutin synthesis. Phytoalexin has antimicrobial activity and lignin acts as a physical barrier against pathogens (Maher et al., 1994; Dixon et al., 2002). During pathogen attack, flavonoid biosynthesis and accumulation is enhanced (Treutter, 2005). In a previous study of resistant NIL, 19 out of 80 enzymes related to phenylpropanoid biosynthesis and 6 out of 14 genes related to shikimate biosynthesis were up-regulated (Wei et al., 2013). Additionally, secondary metabolites were highly enriched in resistant IRBL18 and IRBL22 compared with a susceptible control line after *M. oryzae* infection (Wei et al., 2013).

TABLE 2 | Number of up-regulated and down-regulated significant differentially expressed loci (FDR adjusted $p \leq 0.05$ & $\log_2$ fold change ≥2) unique to PB1+*Pi9* and PB1 respectively and found in different categories of enzymes involved in phenyl propanoid pathway.

Category of enzymes involved in phenyl propanoid pathway	PB1+*Pi9*		PB1	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated
PAL (phenyl ammonia lyase)	2	0	0	0
4CL (coumarate CoA ligase)	2	0	1	0
HCT	0	1	0	0
CCoAOMT (caffeoyl CoA-o-methyl transferase)	2	1	0	0
CAD (cinnamyl alcohol dehydrogenase)	2	1	1	0
PER (peroxidase)	6	1	5	3
Total	14	4	7	3

Plant susceptibility increases with down-regulation of phenylpropanoid pathway genes (Bhuiyan et al., 2009; Naoumkina et al., 2010) that were regulated mainly by MYB transcription factors (Chen et al., 2006; Zhao and Dixon, 2010). As already described, MYBs were highly up-regulated in PB1+*Pi9*, correlating with phenylpropanoid biosynthesis. In PB1+*Pi9* peroxidase which catalyze phytoalexin and lignin biosynthesis were also found highly up-regulated. The up-regulated loci involved in primary and secondary metabolism were different in PB1+*Pi9* from PB1 with variable magnitudes. In PB1, very few loci involved in primary and secondary metabolite synthesis and degradation were altered upon *M. oryzae* infection (Supplementary Figure 4 and **Table 2**). Only two loci involved in PP pathway namely AMP domain binding protein (similar to 4CL enzyme) and dehydrogenase (similar to CAD9) were found up-regulated in PB1. This shows that during primary and secondary metabolism, metabolites formed in PB1 were not able to provide resistance while in PB1+*Pi9* the higher activation of enzymes involved in primary and secondary metabolism for example phospholipid and fatty acid biosynthesis that induce defense genes expression by activating MAPK and higher accumulation of antimicrobial compounds like PUFA, oxylipin, phytoalexin and phenol also prevent growth of *M. oryzae* in PB1+*Pi9*.

Pathogenesis Response Genes

Class III plant peroxidases (EC 1.11.1.7) were well-known pathogenicity-related (PR) proteins, involved during host plant defense were highly induced upon *M. oryzae* attack in PB1+*Pi9*. Similarly, two chitinases and nine glycosyl hydrolase were up-regulated in PB1+*Pi9* (**Figure 3** and **Table 1**). Out of these,

chitinase (LOC_Os02g39330) with FPKM value of 105 (3.9-fold) and glycosyl hydrolase (LOC_Os04g51460) with FPKM value of 511 (4.4-fold) after *M. oryzae* infection were found to be highly up-regulated in PB1+*Pi9*. The loci of glycosyl hydrolase were moderately similar to β-1,3glucanases. Chitinases (PR-3 class) and β-1,3-glucanases (PR-2 class) were two protein groups that inhibit fungal growth through hydrolytic degradation (Mauch et al., 1988; Woloschuk et al., 1991; Sela-Buurlage et al., 1993). Chitinase family protein precursor (CHIT1-LOC_Os02g39330) was validated with real time PCR in infected and uninfected samples of PB1+*Pi9* (**Figure 4**, Supplementary Table 6, and Supplementary Figure 2). Chitinase and β-1,3-glucanases induction helps in strengthening the cell wall by inhibiting fungal growth in PB1+*Pi9* to provide resistance. Transgenic tobacco seedlings with constitutive up-regulation of chitinases and β-1,3-glucanases were resistant to fungal pathogens (Broglie et al., 1991; Zhu et al., 1994). Furthermore, transgenic rice lines with over-expression of family 19 chitinase contributed to increased fungal resistance (Lin et al., 1995; Nishizawa et al., 1999; Datta et al., 2001).

Pathogenesis-related proteins were induced in response to pathogen infection and thus often used as markers to identify plant defense. The PR Bet v I family encodes genes homologous to PR10 (Radauer et al., 2008). In infected PB1+*Pi9 two* PR Bet v I family proteins (LOC_Os08g28670 and LOC_Os04g50700) were highly up-regulated. In addition, two disease resistance proteins (LOC_Os04g43440 & LOC_Os01g06836) were up-regulated in PB1+*Pi9* (**Figure 3** and **Table 1**). Disease resistance protein SlVe2 precursor (LOC_Os01g06836), pathogenesis-related Bet v I family protein (LOC_Os08g28670), NB-ARC/LRR disease resistance protein (LOC_Os04g43440), rp3 protein (LOC_Os12g03080) and Cf-2 (LOC_Os01g06876) were validated with real time PCR in infected and uninfected samples of PB1+*Pi9* after *M. oryzae* infection (**Figure 4**, Supplementary Table 6, and Supplementary Figure 2). During infection, PR-10 proteins were induced in various plant species and exhibit ribonuclease activity (Mcgee et al., 2001; Kim et al., 2004, 2008). PR genes were shown to be expressed in the resistant Digu line compared with the susceptible LTH line (Li et al., 2016). Lipid transfer proteins (LTP) from the PR-14 family exhibit antifungal activity. PB1+*Pi9* exhibited up-regulation of nine LTP loci 24 hpi (**Table 1**). Earlier it was shown that differential expression of rice LTP in response to *M. grisea* was higher in incompatible compared with compatible interaction (Broekaert et al., 1997; van-Loon and van-Strien, 1999; Kim et al., 2006). In susceptible line (PB1) none of the chitinases, β-1,3-glucanases and LTP showed up-regulation (Supplementary Figure 3). In PB1+*Pi9,* the activation of PR and disease resistance proteins leads to synthesis of antimicrobial compounds (e.g., secondary metabolites) that arrest pathogen growth. These results showed expanded transcriptional activation of different metabolic pathways and PR genes during the early response of rice to *M. oryzae*. Furthermore, it indicates that downstream transcriptional regulation may be controlled by *Pi9*, which provides resistance to PB1 against *M. oryzae*.

Singular Enrichment Analysis

Singular enrichment analysis (SEA) was performed to understand the biological processes and molecular functions of genes which plays important role in *Pi9*-mediated resistance in PB1+*Pi9*. Enriched biological processes and molecular function were found for PB1+*Pi9* and PB1. Eight biological processes (BP) and four molecular functions (MF) were found significantly (corrected $p < 0.01$) enriched among 1043 SDEL uniquely found in PB1+*Pi9* (**Figure 6** and **Table 3**). Among eight biological processes 151, 216, and 69 SDEL belonging to

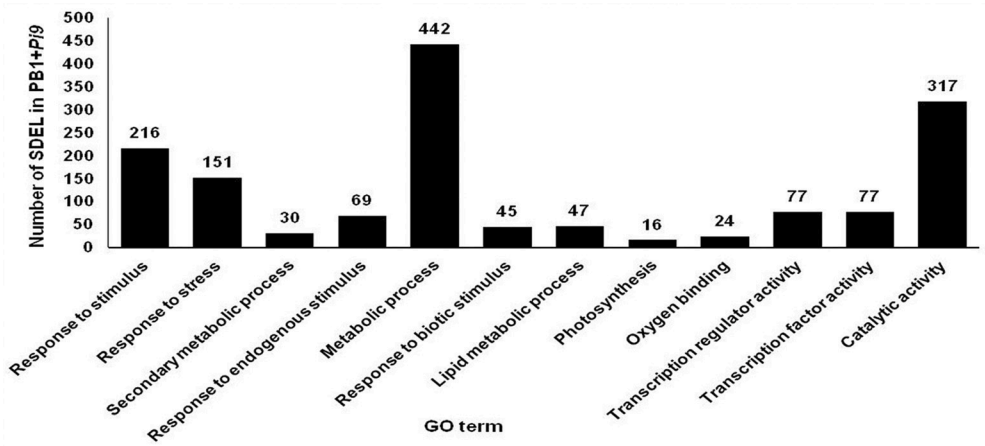

FIGURE 6 | Number of significant differentially expressed loci (SDEL; FDR adjusted $p \leq 0.05$ & $\log_2$ fold change ≥2) unique to PB1+*Pi9* present in different biological processes and molecular functions of all significant GO terms. GO terms for biological processes are response to stimulus (GO:0050896), response to stress (GO:0006950), secondary metabolic process (GO:0019748), response to endogenous stimulus (GO:0009719), response to biotic stimulus (GO:0009607), lipid metabolic process (GO:0006629), photosynthesis (GO:0015979) with *p*-value of 1.00E-08, 1.30E-06, 0.00067, 0.002, 0.0099, 0.025, 0.025, 0.056, respectively. GO terms of molecular function are oxygen binding (GO:0019825), transcription regulator activity (GO:0030528), transcription factor activity (GO:0003700) and catalytic activity (GO:0003824) with *p*-value of 0.00027, 0.0014, 0.0014, 0.016, respectively.

TABLE 3 | The number of significant differentially expressed loci (SDEL; FDR adjusted $p \leq 0.05$ & $\log_2$ fold change ≥2) unique to PB1+*Pi9* present in different gene ontology (GO) terms obtained by Singular Enrichment Analysis (SEA).

GO Accession	Term type	GO Term	Number of SDEL in PB1+*Pi9*	Corrected *p*-value
GO:0050896	P	Response to stimulus	216	1.00E-08
GO:0006950	P	Response to stress	151	1.30E-06
GO:0019748	P	Secondary metabolic process	30	0.00067
GO:0009719	P	Response to endogenous stimulus	69	0.002
GO:0008152	P	Metabolic process	442	0.0099
GO:0009607	P	Response to biotic stimulus	45	0.025
GO:0006629	P	Lipid metabolic process	47	0.025
GO:0015979	P	Photosynthesis	16	0.056
GO:0019825	F	Oxygen binding	24	0.00027
GO:0030528	F	Transcription regulator activity	77	0.0014
GO:0003700	F	Transcription factor activity	77	0.0014
GO:0003824	F	Catalytic activity	317	0.016

P, biological processes; F, molecular function.

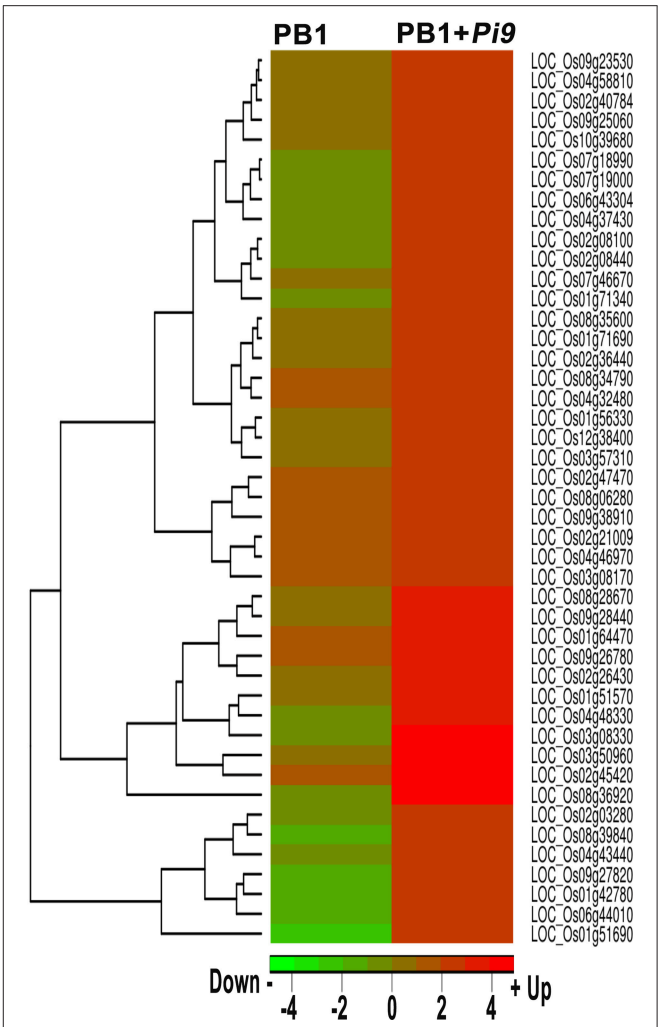

FIGURE 7 | Heat-map of significant differentially expressed loci (FDR adjusted $p \leq 0.05$ & $\log_2$ fold change ≥2) unique to PB1+*Pi9* present in the gene ontology term, response to biotic stimulus (GO:0009607) and their respective $\log_2$ fold change in both PB1+*Pi9* and PB1. Red represents up-regulated loci and green represents down-regulated loci.

the response to stress, response to stimulus and response to endogenous stimulus GO term, respectively. These results reflect that large numbers of loci were being differentially regulated in response to stress in PB1+*Pi9* upon *M. oryzae* infection. During pathway analysis in PB1+*Pi9*, significant enrichment of up-regulated genes is observed in both primary metabolism and secondary metabolism. The similar trend was reflected in SE analysis in PB1+*Pi9* with 489 and 30 SDEL falling in primary and secondary metabolic processes respectively. In PB1+*Pi9,* 16 SDEL were falling under photosynthesis GO term. This shows that photosynthesis related genes were affected upon *M. oryzae* attack because both host and pathogen were fighting for the photosynthates. Among three molecular functions in PB1+*Pi9* 77, 24, 77, and 317 SDEL were found in transcriptional regulation activity, oxygen binding, transcription factor activity and catalytic activity respectively. Overall, PB1+*Pi9* have several unique SDEL involved in transcriptional regulation by different transcription factors suggesting that resistance involves the action of many genes regulating different biological processes. Pathway analysis demonstrated higher transcriptional regulation in PB1+*Pi9* compared with PB1, corresponding results were observed in SE analysis (Supplementary Figure 5 and Supplementary Table 13).

In the present study, these data provide a higher degree of confidence regarding the regulatory role of a single rice-blast resistance gene present in PB1+*Pi9*. The GO term directly associated with defense response to fungal infection was "response to biotic stimulus" (GO:0009607) was found significantly enriched only in PB1+*Pi9*. In PB1+*Pi9*, SDELs involved in biotic stimulus were highly up-regulated and they include transcriptions factors, glycosyl hydrolase, cytochrome P450, zinc finger proteins, lipoxygenase, disease resistance proteins and pathogensis related proteins (**Figure 7** and Supplementary Table 14) These SDEL were important candidates for blast resistance in the rice-*Magnaporthe* pathosystem, and network analysis was thus performed to understand their co-regulation.

Co-expression Network of SDEL Unique to PB1+*Pi9* in Response to Biotic Stimulus

SDEL identified in response to biotic stimulus (GO:0009607) during enrichment analysis were significantly up-regulated only in PB1+*Pi9*. The co-expression network analysis of these important candidate SDEL helps in understanding *Pi9* mediated resistance. In the network, each node represents a protein encoded by a SDEL (**Figure 8** and Supplementary Table 15).

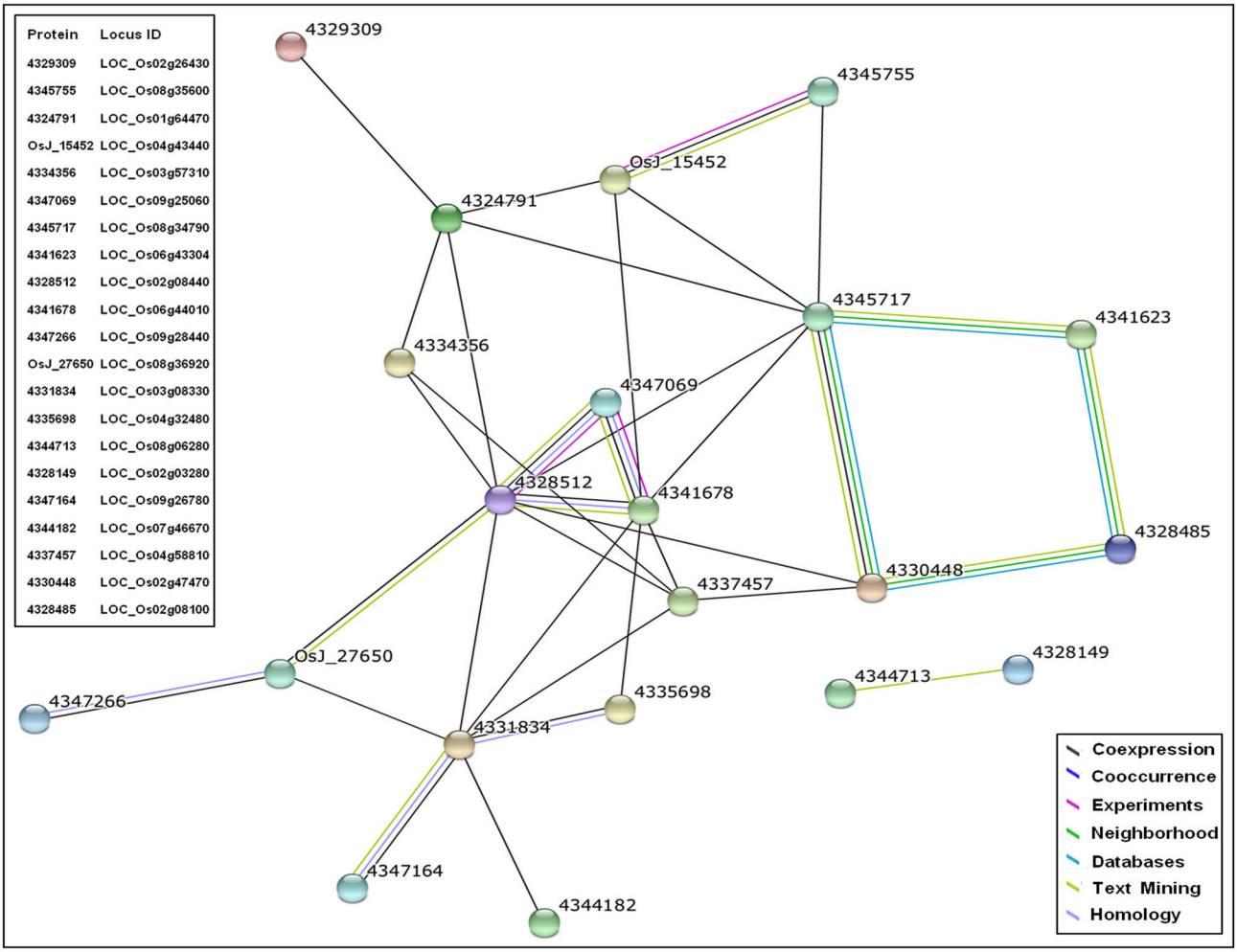

FIGURE 8 | Co-expression network of proteins corresponding to significant differentially expressed loci (SDEL; FDR adjusted $p \leq 0.05$ & $\log_2$ fold change ≥2) unique to PB1+*Pi9* in response to biotic stimulus. In the network, circular node represents the protein encoded by the respective SDEL and the interconnecting lines represent the source by which protein interactions are derived. The protein interactions are derived from coexpression, concurrence, experiments, neighborhood, database, text mining, and homology sources. All the interactions represented have score greater than 0.4 that shows medium confidence for the network.

Several interactions in the network were derived from co-expression and text mining source. The total score of each interaction in the network is above 0.4, so the overall network is of medium confidence. Several interactions in the network have total score above 0.7 so they represent the high confidence interactions (Supplementary Table 15).

The network revealed co-expression of three zinc finger proteins 4335698 (LOC_Os04g32480), 4347164 (LOC_Os09g26780), and 4331834 (LOC_Os03g08330) from the STRING database. These zinc finger proteins were involved in the KEGG plant-pathogen interaction pathway (osa04626) and plant hormone signal transduction pathway (osa04075) of rice (**Table 4**). In the network (supported by experimental data), three WRKY proteins 4328512 (OsWRKY28), 4347069 (OsWRKY71), 4341678 (OsWRKY76) were co-expressed. These WRKYs have zinc finger domains and show homology across related species. The orthologous genes for OsWRKY71 and OsWRKY76 in *A. thaliana* is AtWRKY40, whereas OsWRKY28 has two orthologous genes, AtWRKY60 and AtWRKY18. The STRING database report showed that WRKY40 and WRKY18 possessed confidence co-expression scores of 0.695; both also interact with the W box, an elicitor responsive cis-acting element. Functional and physical interaction between WRKY40, WRKY18, and WRKY60 has been reported in *A. thaliana* (Xu et al., 2006). These three WRKYs were structurally similar and induced by pathogen attack. Triple mutants of all three WRKYs (WRKY40, WRKY18, WRKY60) were resistant to *P. syringae* compared with wild type (Xu et al., 2006). Thus, network analysis showed that in PB1+*Pi9* NIL, OsWRKY71, OsWRKY76, OsWRKY28 were co-expressed with zinc finger proteins (4335698, 4347164, 4331834). Proteins 4345717 (LOC_Os08g34790) and 4328485 (LOC_Os02g08100) involved

TABLE 4 | The number of significant differentially expressed loci (FDR adjusted $p \leq 0.05$ & $\log_2$ fold change ≥2) unique to PB1+*Pi9* NIL, found in different KEGG pathways present in coexpression network of proteins.

Pathway ID	Pathway	Number of loci	*p*-value
4626	Plant-pathogen interaction	3	1.27E-04
940	Phenylpropanoid biosynthesis	3	1.33E-04
130	Ubiquinone and other terpenoid-quinone biosynthesis	2	2.33E-04
1110	Biosynthesis of secondary metabolites	5	3.65E-04
4075	Plant hormone signal transduction	3	4.60E-04
360	Phenylalanine metabolism	2	2.86E-03
1100	Metabolic pathways	5	6.85E-03

in phenylpropanoid biosynthesis (osa00940), biosynthesis of secondary metabolites (osa01110), as well as ubiquinone and other terpenoid-quinone biosynthesis (osa00130) also show co-expression in the network (**Table 4**). Overall the network contains zinc finger proteins, WRKY, kinases, AMP binding domain proteins, cytochrome P450 and disease resistance proteins. These important candidates in the co-expresion network help in predicting a model of *Pi9* mediated resistance in PB1+*Pi9*. The network indicates that upon *M. oryzae* infection in PB1+*Pi9*, signaling molecule like zinc finger proteins (LOC_Os04g32480, LOC_Os09g26780, LOC_Os03g08330) involve in plant hormone signal transduction and plant pathogen interaction pathway triggers the downstream WRKY TFs (LOC_Os02g08440, LOC_Os06g44010, LOC_Os09g25060). These WRKY TFs activate the transcription of disease resistance protein (LOC_Os04g43440) and biosynthesis of secondary metabolites like phenylpropanoid and terpenoids related genes (LOC_Os08g34790, LOC_Os02g08100). This co-expression network of genes involved in biotic stimulus improves our understanding of genome-wide co-expression and suggest protein-level interactions among genes in PB1+*Pi9* NIL to provide *Pi9*-mediated resistance.

Pi9-Mediated Resistance in PB1+*Pi9*

The present study confirms that a single functional blast-resistant gene (*Pi9*) in PB1+*Pi9* activates a cascade of defense response genes, leading to incompatible interaction between host and pathogen. The resistant NIL displays a broad spectrum of transcriptional changes upon *M. oryzae* attack. The pathway analysis of SDEL revealed that genes involved in cell wall fortification, respiratory burst, kinase signaling, hormone signaling, WRKY, MYB, and ERF transcription factors, defense response genes (peroxidases, glucanases, chitinases, laccases, lipoxygenase, phenyl ammonia lyase, PR proteins) were highly activated in PB1+*Pi9* than in PB1. The activation of defense response genes results in the synthesis of antimicrobial secondary metabolites, inhibiting the spread of *M. oryzae* in PB1+*Pi9*. We also proposed a model showing how *Pi9*-mediated regulators controls incompatible interaction against *M. oryzae* infection (**Figure 9**).

The role of WRKYs in the *O. sativa-M. oryzae* interaction had been previously reported in several studies. In the present study, MYB, bZIP and ERF were also observed to mediate downstream resistance response 24 hpi, along with WRKY genes. Increased expression of JA/ET signaling genes in PB1+*Pi9* showed that *M. oryzae* is regulated via a pathway between the typical SA regulation of biotrophs and JA regulation of necrotrophs. The pathway analysis reflects the increased complexity of the PB1+*Pi9* defense response, compared with that of PB1, the former involving intricate mechanisms of cell wall fortification and ROS mediated signaling upon *M. oryzae* attack. The effector molecule released from *M. oryzae* bind to *Pi9* gene and activates the downstream signaling by kinases and hormones that activates the transcription factors during the initial infection phase. These transcription factors activate the transcription of defense response genes and formation of secondary metabolites. The secondary metabolites like phytoalexin and phenols act as antimicrobial compounds and stops *M. oryzae* growth in PB1+*Pi9,* unlike PB1. Singular enrichment analysis of SDEL also supported the pathway analysis data, indicating that the genes involved in signaling, response to biotic stimulus, biological regulation, transcriptional regulation, as well as primary and secondary metabolism were enriched in PB1+*Pi9* compared with PB1.

Earlier reports using rice NIL to study rice-*M. oryzae* interaction mainly used microarray technology (Sharma et al., 2016). The transcriptional profiling using microarray has limited range of expression which identifies only known genes, while RNA sequencing has greater dynamic range of detection along with identification of novel candidate genes (Wang et al., 2009; Agarwal et al., 2010; Xu et al., 2012). The level of capture in transcriptome data is clearly reflected by comparison of upregulated genes found in different studies on rice trancriptome 24 hpi with *M. oryzae*. It has been observed that numbers of differentially expressed genes were higher in both compatible and incompatible interactions using RNA-seq compared to microarray. In PB1+*Pi9* and PB1, the number of SDEL were 1254 and 779 ($\log_2$ fold change ≥2; FDR adjusted $p \leq 0.05$), respectively, while the number of SDEL in IRBL22 and LTH 24 hpi upon *M. oryzae* infection were 649 and 131 (absolute fold change ≥2; $p < 0.05$), respectively (Wei et al., 2013). This is probably due to the sensitivity of technology to capture all transcripts, differences in the background of both the NILs and also the strain of *M. oryzae* used for infection. In our study in-depth transcriptional level changes covered signaling mediated by receptor kinases, wall associated kinases, calmodulin related calcium sensor proteins and G-proteins; hormone signaling mediated by JA/ET; MYB, bZIP, and ERF transcription factors along with WRKY; activation of enzymes involved in lipid metabolism like phospholipid and fatty acid biosynthesis; activation of defense response genes and accumulation of antimicrobial compounds like

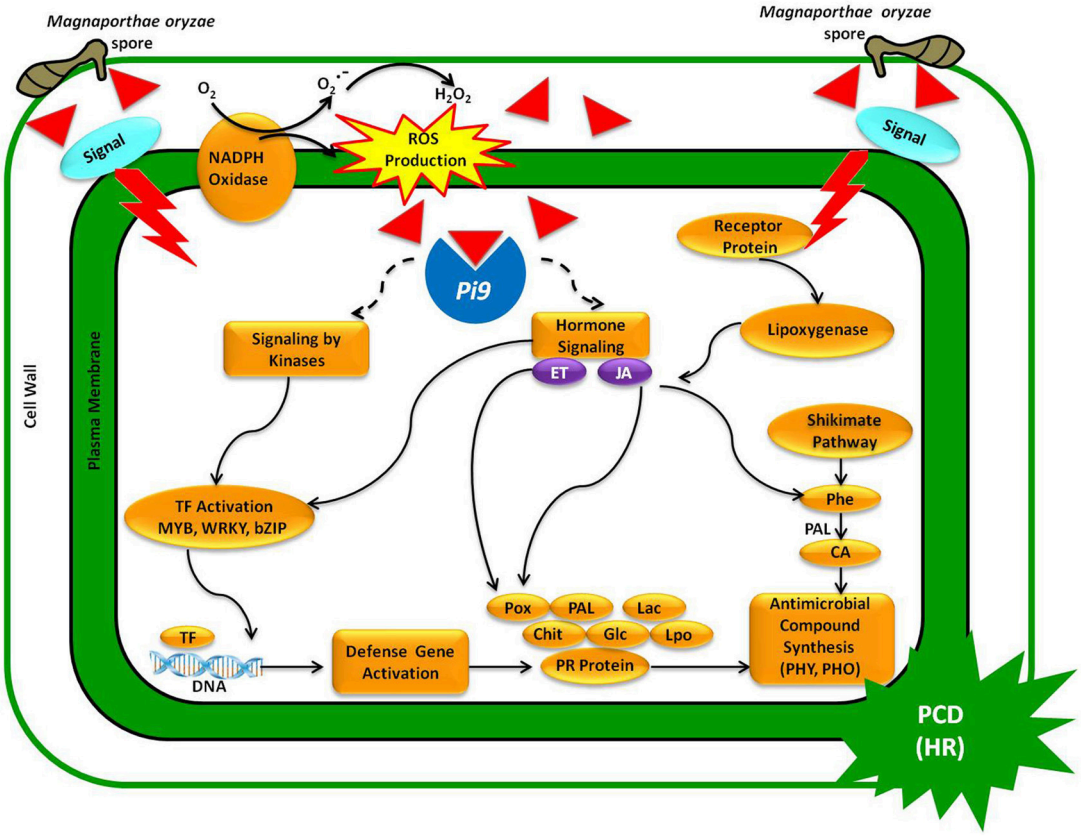

FIGURE 9 | A model representing *Pi9*-mediated resistance upon *M. oryzae* infection in PB1+*Pi9*. Upon germination, *M. oryzae* spores penetrate the leaf cuticle, where upon fungal effectors are recognized by the R (*Pi9*) protein. In turn, *Pi9* triggers ROS production and activating signaling molecules (kinases and hormones). Signaling molecules then activate transcription factors (MYB, WRKY, bZIP) that trigger the expression of defense response genes (*Pox, Pal, Lac, Chit, Glc, Lpo*), leading to the synthesis of antimicrobial compounds (PHY, PHO) that curtails *M. oryzae* growth. Inverted red triangle represents effector proteins secreted by germinating *M. oryzae* spores invading cell wall. Abbreviations: ROS, reactive oxygen species; HR, hypersensitive response; PCD, programmed cell death; TF, transcription factor; ET, ethylene; JA, jasmonic Acid; PR proteins, pathogenesis-related proteins; CA, cinnamic acid; PAL, phenylalanine ammonia lyase; Glc, glucanases; Chit, chitinases; LPO, lipoxygenases; POX, peroxidases; Lac, laccases; PHO, pheolics; PHY, phytoalexin.

PUFA, oxylipin and phenol that prevent the growth of *M. oryzae*.

CONCLUSIONS

The present study deciphered the transcriptional snapshot of blast resistant NIL PB1+*Pi9* at 24 hpi with *M. oryzae*. In this line, singular enrichment analysis showed that SDELs involved in biotic stimulus were highly up-regulated. The co-expression network of proteins involved in biotic stimulus (GO:0009607) played an important role in understanding broad-spectrum resistance against *M. oryzae* in PB1+*Pi9*, as these genes were down-regulated in PB1. Several proteins in the network were involved in both plant-pathogen interaction and plant hormone signal transduction pathways. The support from both transcriptional and co-expression interaction data indicates that these are prominent candidates for blast resistance in the rice-*Magnapothe* pathosystem. These important candidates can be manipulated to modify important pathway and enhance disease resistance. Thus, current study revealed additional transcriptional changes via modulating genome-wide transcriptional regulation using three important methods namely pathway, SEA and co-expression analysis, along with qRT-PCR validation of important candidates in rice NIL (PB1+*Pi9*), 24 hpi upon *M. oryzae* infection and helped in unraveling mechanism of broad-spectrum blast resistance mediated by *Pi9* gene.

AUTHOR CONTRIBUTIONS

TS: Conceived and designed the experiments; AS and GK: Provided biological material; PJ: Performed the experiments and analyzed data; PS: Fungal culture maintenance and figure editing; RK: Real time validation of some genes; TS, PJ and AUS: Wrote the paper; VS: Designing of work; AK: Developed resistant Pi9-NIL.

ACKNOWLEDGMENTS

The financial assistance received from National Agricultural Innovation Project, ICAR (C4/C1071) and Incentivizing Research in Agriculture, ICAR by TS is gratefully acknowledged. TS is also thankful to Department of Science and Technology, Govt. of India for JC Bose National Fellowship. PJ is thankful to the Council of Scientific & Industrial Research (CSIR) for providing Senior Research Fellowship (SRF).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00093/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

REVIEW
published: 28 July 2017
doi: 10.3389/fpls.2017.01318

Phylloremediation of Air Pollutants: Exploiting the Potential of Plant Leaves and Leaf-Associated Microbes

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: *31 March 2017*
Accepted: *12 July 2017*
Published: *28 July 2017*

Citation:
Wei X, Lyu S, Yu Y, Wang Z, Liu H, Pan D and Chen J (2017) Phylloremediation of Air Pollutants: Exploiting the Potential of Plant Leaves and Leaf-Associated Microbes. Front. Plant Sci. 8:1318. doi: 10.3389/fpls.2017.01318

Air pollution is air contaminated by anthropogenic or naturally occurring substances in high concentrations for a prolonged time, resulting in adverse effects on human comfort and health as well as on ecosystems. Major air pollutants include particulate matters (PMs), ground-level ozone (O_3), sulfur dioxide (SO_2), nitrogen dioxides (NO_2), and volatile organic compounds (VOCs). During the last three decades, air has become increasingly polluted in countries like China and India due to rapid economic growth accompanied by increased energy consumption. Various policies, regulations, and technologies have been brought together for remediation of air pollution, but the air still remains polluted. In this review, we direct attention to bioremediation of air pollutants by exploiting the potentials of plant leaves and leaf-associated microbes. The aerial surfaces of plants, particularly leaves, are estimated to sum up to 4×10^8 km^2 on the earth and are also home for up to 10^{26} bacterial cells. Plant leaves are able to adsorb or absorb air pollutants, and habituated microbes on leaf surface and in leaves (endophytes) are reported to be able to biodegrade or transform pollutants into less or nontoxic molecules, but their potentials for air remediation has been largely unexplored. With advances in omics technologies, molecular mechanisms underlying plant leaves and leaf associated microbes in reduction of air pollutants will be deeply examined, which will provide theoretical bases for developing leaf-based remediation technologies or phylloremediation for mitigating pollutants in the air.

Keywords: air pollution, nitrogen dioxides, ozone, particulate matter, phylloremediation, phyllosphere, sulfur dioxide, volatile organic compounds

INTRODUCTION

Air pollution is referred to as the presence of harmful or poisonous substances in the earth's atmosphere, which cause adverse effects on human health and on the ecosystem. Major air pollutants include particulate matters (PMs), nitrogen oxides (NO_2), sulfur dioxide (SO_2), ground-level ozone (O_3), and volatile organic compounds (VOCs) (Archibald et al., 2017).

Various effects of some common air pollutants on human comfort and health are presented in **Table 1**, ranging from respiratory illness, cardiovascular disease to bladder and lung cancer (Kampa and Castanas, 2008).

The world has experienced unprecedented urban growth during the last three decades. Urban population is expected to increase at 2.3% per year in developing countries from 2000 to 2030 (Brockherhoff, 2000; United Nations, 2000, 2004; UNFPA, 2004). Urbanization is often associated with rapid economic growth. For example, China's urbanization grew from 17.92% in 1978 to 52.57% in 2012, and China's gross domestic products (GDPs) increased from 454.6 billion Chinese Yuan in 1980 to 51,894.2 billion Yuan in 2012 (Zhao and Wang, 2015). The increased economic growth has been accompanied with elevated energy consumption. China's energy consumption, primarily fossil fuels like coal, increased from 602.75 million tons in 1980 to 3,617.32 million tons in 2012 (Zhao and Wang, 2015). The increased combustion of fossil fuels with relatively low combustion efficiency along with weak emission control measures have resulted in drastic increases in air pollutants, such as PMs, SO_2, NO_2, O_3, and VOCs. Per unit of GDPs in 2006, China emitted 6–33 times more pollutants than the United States (US). As a result, air quality has become a major focus of environmental policy in China. India experiences similar situations as China. Urbanization coupled with rapid economic development in India increased energy consumption and also air pollution in some megacities (Gurjar et al., 2016). For example, PM_{10} in Delhi was almost 10 times of the maximum PM_{10} limit at 198 $\mu g\ m^{-3}$ in 2011 (Rizwan et al., 2013). Concentrations of major pollutants in the air of some selected cities are present in **Table 2**.

The World Health Organization (WHO) air quality guidelines stated that the mean limits for annual exposure to $PM_{2.5}$ (particle diameters at 2.5 μm or less) and PM_{10} (particle diameter at 10 μm or less) are 10 $\mu g\ m^{-3}$ and 25 $\mu g\ m^{-3}$, respectively; and the limits for 24-h exposure are 25 $\mu g\ m^{-3}$ and 50 $\mu g\ m^{-3}$, respectively. The limit for 8-h exposure to O_3 is 100 $\mu g\ m^{-3}$. Annual mean for NO_2 is 40 $\mu g\ m^{-3}$ or 200 $\mu g\ m^{-3}$ for 1 h, and 24-h exposure to SO_2 is 20 $\mu g\ m^{-3}$ or 500 $\mu g\ m^{-3}$ for 10 min (WHO, 2006). The results presented in **Table 2** suggest that residents in some of the listed cities were exposed to air contamination far beyond the limits set by WHO. PMs have become the most pressing environmental problems in China and India. For example, during the first quarter of 2013, China experienced extremely severe and persistent haze pollution that directly affected about 1.3 million km^2 and about 800 million people (Huang et al., 2014). Of which daily average concentrations of $PM_{2.5}$ measured at 74 major cities exceeded the Chinese pollution standard of 75 $\mu g\ m^{-3}$, which is approximately twice that of the US EPA (United States Environmental Protection Agency) standard of 35 $\mu g\ m^{-3}$, for 69% of days in January, with a record-breaking daily concentration of 772 $\mu g\ m^{-3}$ (Huang et al., 2014).

Recent studies from the International Agency for Research on Cancer showed that there were 223,000 deaths in 2010 due to air pollution-resultant lung cancer worldwide, and air pollution has become the most widespread environmental carcinogen (International Agency for Research on Cancer, 2013). The WHO reported that around 7 million people died of air pollution exposure directly or indirectly in 2012. This data was more than double previous estimates and confirmed that air pollution has become a substantial burden to human health and is the world's largest single environmental health risk (WHO, 2014). Additionally, air pollution also harms animals, plants, and ecological resources including water and soils (Vallero, 2014; Duan et al., 2017).

MEASURES FOR REDUCING AIR POLLUTION

To reduce air pollution, the first step is to eliminate or reduce anthropogenic-caused emissions. The second step is to remediate existing pollutants. Different strategies, policies, and models for air pollution abatement have been proposed or implemented (Macpherson et al., 2017). For example, the Chinese government has imposed restrictions on major pollution sources including vehicles, power plants, transport, and industry sectors (Liu et al., 2016) and promulgated the "Atmospheric Pollution Prevention and Control Action Plan" in September 2013, which was intended to reduce $PM_{2.5}$ by 25% by 2017 relative to 2012 levels (Huang et al., 2014). Science-based technologies have been developed for control of air pollutants, such as diesel particulate filters (Tsai et al., 2011) and activated carbon filtering as adsorbent for xylene and NO_2 (Guo et al., 2001). Catalytic oxidization and chemisorption methods have been used for indoor formaldehyde removal (Pei and Zhang, 2011; Wang et al., 2013). Photocatalysis as one of the most promising technologies has been used for eliminating VOCs (Huang et al., 2016).

Air pollutants can also be mitigated through biological means, commonly referred to as biological remediation or bioremediation. It is the use of organisms to assimilate, degrade or transform hazardous substances into less toxic or non toxic ones (Mueller et al., 1996). Plants have been used for remediation of pollutants from air, soils, and water, which has been termed as phytoremediation (Cunningham et al., 1995; Salt et al., 1995; Huang et al., 1997). Microbes such as bacteria and fungi are also capable of biodegrading or biotransforming pollutants into non toxic and less toxic substances, which is known as microbial biodegradation (Ward et al., 1980; Ma et al., 2016). Microbes as heterotrophs occur nearly everywhere, including plant roots and shoots. Both roots and shoots have been reported to be able to remediate air pollutants (Weyens et al., 2015; Gawronski et al., 2017), but little credit has been given to microbe activity.

Plant shoots or the above-ground organs of plants colonized by a variety of bacteria, yeasts, and fungi are known as phyllosphere (Last, 1955). However, most scientific work on phyllosphere microbiology has been focused on leaves (Lindow and Brandl, 2003). This review is intended to explore the potential of plant leaves and leaf-associated microbes in bioremediation of air pollutants, or simply known as phylloremediation. Phylloremediation was first coined by Sandhu et al. (2007), who demonstrated that surface-sterilized leaves took up phenol, and leaves with habiated

TABLE 1 | Major air pollutants and their effects on human comfort and health.

Pollutants	Adverse effects on human health	References
PMs[a]	Heart and lung cancer, risk of bladder cancer, nervous systems and respiratory illness	Anderson et al., 2012; Kumar et al., 2013; WHO, 2013; Kelly and Fussell, 2015
O_3	Breathing problems, asthma, reduction of lung function, and lung diseases	Amann, 2008
NO_2	Asthmatic bronchitis, reduced lung function growth, respiratory infections, and airway obstruction	Lambert et al., 1993; Lambert, 1996; Eberlein-Konig et al., 1998
SO_2	Eye irritation, infections of the respiratory tract, coughing, mucus secretion, asthma and chronic bronchitis, and cardiac disease	Qin et al., 1993
CO	Neuropsychological impairment, headache, fatigue, dizziness, and nausea, fetal damage, myocardial ischemia	Dahms et al., 1993
PAHs[b]	Lung cancer	Fugas and Sega, 1995
Volatile organic compounds	Asthma, nocturnal breathlessness, sensitization reactions, respiratory tract, mucous membrane irritation, central nervous system symptoms, headache, drowsiness, fatigue, confusion, lethargy, and dizziness	Molhave, 1991; Wallace, 1991; Wieslander et al., 1996
Formaldehyde	Sneezing, coughing, minor eye irritation, irritant of the skin, respiratory tract, and nasopharyngeal cancer	Wieslander et al., 1996; Morgan, 1997; Eberlein-Konig et al., 1998
Radon	Lung cancer, acute myeloid and acute lymphoblastic leukemia	Steindorf et al., 1995
Tobacco smoke	Irritation of eye, nose, and throat, asthma, lung cancer, bronchitis, pneumonia, bronchiolitis, acute childhood lower respiratory tract illnesses, and tuberculosis	Lin et al., 2007
Asbestos	Skin irritation, lung cancer, mesothelioma, and asbestosis	McDonald, 1991

[a] *Particulate matters.*
[b] *Polycyclic aromatic hydrocarbons.*

TABLE 2 | Concentrations of some major air pollutants in the air of selected cities.

City (country)	Year	$PM_{2.5}$ ($\mu g\ m^{-3}$)	PM_{10} ($\mu g\ m^{-3}$)	O_3 ($\mu g\ m^{-3}$)	NO_2 ($\mu g\ m^{-3}$)	SO_2 ($\mu g\ m^{-3}$)	CO ($mg\ m^{-3}$)	References
Ho Chi Minh City (Vietnam)	2007	–	74.00	40.00	18.90	30.30	–	Phung et al., 2016
Patras (Greece)	2011	–	42.10	84.20	47.10	–	0.90	Karagiannidis et al., 2014
Quetta (Pakistan)	2009	160.28	370.52	–	97.05	50.00	3.80	Ilyas et al., 2009
Beijing (China)	2015	78.50	104.82	–	50.49	16.86	1.25	Chen et al., 2016
Shanghai (China)	2015	55.54	75.64	–	45.23	18.40	0.86	Chen et al., 2016
Shenzhen (China)	2015	32.83	55.37	–	32.94	8.08	1.01	Chen et al., 2016
Guangzhou (China)	2015	44.38	65.91	–	45.46	14.67	0.99	Chen et al., 2016
Rome (Italy)	2015	19.50	27.60	42.30	48.70	1.00	0.60	Battista et al., 2016
Lucknow City (India)	2012	91.10	217.35	–	74.10	12.30	0.20	Lawrence and Fatima, 2014

microbes or a inoculated bacterium were able to biodegrade signficantly more phenol than leaves alone. Previous reports also documented that both plant leaves and leaf-associated microbes mitiagted air pollutants, such as azalea leaves and the leaf-associated *Pseudomonas putida* in reducing VOCs (De Kempeneer et al., 2004), leaves of yellow lupine plants along with endophytic *Burkholderia cepacia* for toluene reduction (Barac et al., 2004), and poplar leaves and the leaf-associated *Methylobacterium* sp. decreased xenobiotic compounds (Van Aken et al., 2004). Phyllo originated from Greek word of phullon, meaning leaf. Thus, phylloremediation should be defined as a natural process of bioremediation of air pollutants through leaves and leaf-associated microbes, not the microbes alone.

PLANT LEAVES AND PHYLLOSPHERE

Leaves are the primary photosynthetic organs with distinctive upper surface (adaxial) and lower surface (abaxial) (**Figure 1**). The upper surface has a layer ($<$0.1–10 μm) of waxy cover called cuticle (Kirkwood, 1999). Wax contents and compositions frequently differ among plant species. The primary function of cuticle is to prevent evaporation of water from leaf surfaces, and it is also the first barrier for the penetration of xenobiotics. The leaf surface is filled with trichomes, which are epidermal outgrowths in various forms. Trichomes play roles in mechanical defense because of their physical properties and also in biochemical defense due to the secretion of secondary metabolites (Tian et al., 2017). Epidermis cells are directly underneath the cuticle layer

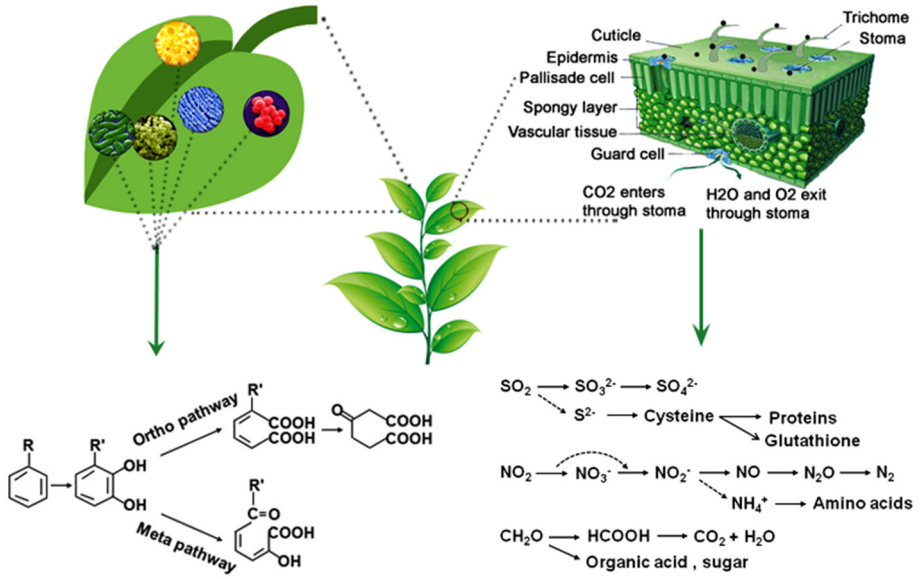

FIGURE 1 | A schematic illustration of phyllosphere. The middle panel represent an aerial part of a plant. Right panel shows a magnified schematic cross section of a leaf where leaf surface and trichomes can retain particulate matter (PMs) and stomata adsorb or absorb PMs as well as how leaves can assimilate SO_2, NO_2, and CH_2O (formaldehyde) to simple organic compounds, amino acids, or proteins. The left panel depict a magnified leaf surface with bacteria, which can biodegrade or transform volatile organic compounds to less toxic or nontoxic ones like benzene and its derivatives that can be degraded through Ortho pathway or Meta pathway.

in which stomata often occur. Xylem and phloem are situated within the veins of leaves as the plant vascular system, which are connected from root tips to leaf edges. There is a layer of compactly arranged cells around the vein called bundle sheath regulating substance circle around the xylem and the phloem. Xylem transports water and nutrients from roots to shoots, and phloem transports assimilated products from source and sink tissues. Under the epidermis, there are mesophyll cells in two layers: column-like palisade cells and loosely packed spongy cells. The air spaces among the spongy cells promote gas exchange, and photosynthesis takes place in chloroplasts packed in the mesophyll cells. The underside of leaves also has a layer of epidermal cells where most stomata are located. There are two guard cells surround the stomata, and stomatal pore opening and closure is regulated by changes in the turgor pressure of the guard cells. Stomata regulate the flow of gases in and out of leaves and also able to adsorb or absorb other chemicals.

Leaves also play pivotal roles in supporting phyllosphere microbes (Bringel and Couee, 2015). The phyllosphere is estimated to have area up to 4×10^8 km^2 on the earth and is the home for up to 10^{26} bacterial cells (Kembel et al., 2014). Phyllosphere bacterial communities are generally dominated by Proteobacteria, such as *Methylobacterium* and *Sphingomonas*. *Beijerinckia, Azotobacter*, *Klebsiella,* and Cyanobacteria like *Nostoc*, *Scytonema*, and *Stigonema* also reside in the phyllosphere (Vacher et al., 2016). Population of γ-Proteobacteria such as *Pseudomonas* could be high as well (Delmotte et al., 2009; Fierer et al., 2011; Bodenhausen et al., 2013; Kembel et al., 2014). Dominant fungi in the phyllosphere include Ascomycota, of which the most common genera are *Aureobasidium*, *Cladosporium*, and *Taphrina* (Coince et al., 2013; Kembel and Mueller 2014). Basidiomycetous yeasts belonging to the genera *Cryptococcus* and *Sporobolomyces* are also abundant in phyllosphere (Cordier et al., 2012; Ottesen et al., 2013). The microbes can be epiphytic by living on the surface of plant organs and/or endophytic occurring within plant tissues without causing apparent disease.

Plant species significantly influence the composition of a phyllosphere community (Whipps et al., 2008). In a study of 56 different tree species, Redford et al. (2010) reported that different species harbor distinct microbial communities in phyllosphere. This principle was also confirmed for trees in temperate and tropical climates and for Mediterranean perennials (Lambais et al., 2006; Kim et al., 2012; Vokou et al., 2012; Kembel et al., 2014; Laforest-Lapointe et al., 2016). Using high-throughput sequencing technology, Kembel and Mueller (2014) studied fungal communities on leaves of 51 tree species in a lowland tropical rainforest in Panama and reported that fungal communities on leaves were dominated by the phyla Ascomycota, which accounted for 79% of all sequences, followed by Basidiomycota (11%) and Chytridiomycota (5%). More than half of the variation in fungal community composition could be explained by plant species differences. Leaf chemistry and morphology as well as plant growth status and mortality were closely related to fungal community structure (Kembel and Mueller, 2014). These results may suggest that different tree species host different fungal communities. Additionally, microbial compositions within plant species may differ due to geographic locations (Finkel et al., 2012; Qvit-Raz et al., 2012; Rastogi et al., 2012). The differences could be caused by

climatic variation (Finkel et al., 2011) or due to the limited dispersal of the colonizing taxa (Finkel et al., 2012; Qvit-Raz et al., 2012). Furthermore, phyllosphere microbial community may differ between urban and non-urban locations (Jumpponen and Jones, 2010) and also differ by seasons (Redford and Fierer, 2009).

ROLES OF LEAVES AND PHYLLOSPHERE MICROBES IN AIR REMEDIATION

The close association between plant species and specific microbial communities in the phyllosphere suggests their adaptation and coevolutionary relationships. Recent studies show that leaf bacterial diversity mediates plant diversity and ecosystem function relationships (Laforest-Lapointe et al., 2017). We hypothesize that a long-lasting exposure of leaves and leaf-associated microbes to air pollutants could result in plants or microbes individually or coordinately developing mechansims for adapting to the polluted substances. Such mechanisms may include leaf adsorption or absorption and pollutant assimilation as well as microbial biodegradation, transformation or metabolic assimilation of the substances. The coordination between leaves and micriobes could be synergistic or antagonistic. **Table 3** presents plant-supported microbes that are able to biodegrade or biotransform air pollutants, primarily organic compounds. However, information regarding phyllospere microbes in remediation of PMs, SO_2, NO_2, and O_3 is scarce, suggesting relatively limited research has been devoted to microbial roles. Thus, the current knowledge on phylloremediation of PM, SO_2, NO_2, and O_3 is mostly come from plants.

Remediation of PMs

As mentioned above, PMs have become the most dangerous pollutants in some countries. Chemical species of PMs, derived from the available data over China included SO_4^{2-}, NO_3^-, NH_4^+, organic carbon, and elemental carbon, which were in a range of 2.2–60.9, 0.1–35.6, 0.1–29.8, 1.5–102.3, 0.2–37.0 $\mu g\ cm^{-3}$ in $PM_{2.5}$, and 1.6–104.6, 0.5–46.6, 0.2–31.0, 1.7–98.7, and 0.3–26.8 $\mu g\ cm^{-3}$ in PM_{10}, respectively (Zhou et al., 2016). $PM_{2.5}$ is the major component of PM_{10}, accounting for 65%. PMs are also composed of microorganisms. In a study of PMs in Jeddah, Saudi Arabia (Alghamdi et al., 2014), the average concentrations of PM_{10} and $PM_{2.5}$ were 159.9 and 60 $\mu g\ cm^{-3}$, respectively and the concentrations of O_3, SO_2, and NO_2 averaged 35.73, 38.1, and 52.5 $\mu g\ cm^{-3}$, respectively. Microbial loads were higher in PM_{10} than $PM_{2.5}$. *Aspergillus fumigatus* and *Aspergillus niger* were the common fungal species associated with PMs. Microbes were also found in PMs in Austria (Haas et al., 2013), including fungi from genera *Aspergillus*, *Cladosporium*, and *Penicillium* and aerobic mesophilic bacteria. Using metagenomic methods, Cao et al. (2014) identified 1,315 distinct bacterial and archaeal species from 14 PM samples collected from Beijing, China. The most abundant phyla were Actinobacteria, Proteobacteria, Chloroflexi, Firmicutes, Bacteroidetes, and Euryarchaeota. Among them, an unclassified bacterium in the nitrogen fixing, filamentous bacteria genus Frankia was the most abundant, and the most abundant classified bacterial species appeared to be *Geodermatophilus obscures*. The abundance of airborne bacteria was reported to be in a range from 10^4 to 10^6 cells m^{-3} depending on environmental conditions (Bowers et al., 2011), and materials of biological origin might account for up to 25% of the atmospheric aerosol (Jaenicke, 2005). Ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB), and complete ammonia oxidizers (Comammox) were identified in $PM_{2.5}$ collected from the Beijing-Tianjin-Heibei megalopolis, China (Gao et al., 2016). Of which *Nitrosopumilus* subcluster 5.2 was the most dominant AOA, *Nitrosospira multiformis* and *Nitrosomonas aestuarii* were the most dominant AOB, and the presence of Comammox was revealed by the occurrence of *Candidatus* Nitrospira inopinata. The mean cell numbers of AOA, AOB, and *Ca.* N. inopinata were 2.82×10^4, 4.65×10^3, and 1.15×10^3 cell m^{-3}, respectively. The average maximum nitrification rate of $PM_{2.5}$ was 0.14 $\mu g\ (NH4^+\text{-}N)\ [m^3\ air\ h]^{-1}$ (Gao et al., 2016). AOA might account for most of the ammonia oxidation, followed by Comammox, while AOB were responsible for a small part of ammonia oxidation. The assay of nitrification activity was performed in laboratory conditions (Gao et al., 2016). However, the nitrification potential of such bacteria in PMs after being deposited on leaf surfaces is unknown. We hypothesize that the nitrification process could be more active once such PM-containing bacteria settled on leaves. Further investigation on nitrification of PM-associated bacteria in the phyllosphere could provide insight into how the phyllosphere could potentially act as manufactories in the nitrification of ammonia.

The current literature regarding phylloremediation of PMs has been primarily focused on plant leaves. Plant canopy is a sink for PMs. This is due to the fact that leaves are in the air and they span more than 4×10^8 km^2 on a global scale, which is about 78.4% of the total surface area of the earth; leaves thus physically act as a natural carrier for PMs. Leaves differ greatly in surface structure and metabolic secreted substances as well as microbial composition. The amount of surface waxes and compositions show different capacity to retain and embrace PMs. Sæbø et al. (2012) studied leaves of 22 trees and 25 shrubs in accumulation of PMs in Norway and Poland and found that PM accumulation differed by 10 and 15 folds depending on plant species in the two locations and also positive correlations occurred among PM accumulation, leaf wax contents, and leaf hair density. Thirteen woody species were examined by Popek et al. (2013) during a 3-year period, and total amount of PMs captured by leaves ranged from 7.5 mg cm^{-2} by *Catalpa bignonioides* to 32 mg cm^{-2} by *Syringa meyeri*. Leaf wax contents were significantly correlated with the amount of PMs on leaves. Among the PMs captured, 60% was washable by water, and 40% could be washed by chloroform only, suggesting that the PMs were embraced in waxes. Using two photon excitation microscopy (TPEM), Terzaghi et al. (2013) investigated leaves of stone pine (*Pinus pinea*), cornel (*Cornus mas*), and maple (*Acer pseudoplatanus*) in capture and encapsulation of PMs. The authors found that particles ranging from 0.2 to 70.4 μm were visualized on leaves, of which $PM_{2.6}$ was the dominant size across plant species. Particle less than 10.6 μm were encapsulated in the cuticle. Plant species

TABLE 3 | Plant-supported microbes that are able to biodegrade or biotransform air pollutants.

Plants	Microbes	Pollutant	References
Acorus calamus var. *angustatus*	*Methylobacterium* sp.	Methanol	Iguchi et al., 2012
Amaranthus cruentus	*Alcaligenes feacalis* and *Alcaligenes* sp.11SO	Naphthalene and Phenanthrene	Ljs, 2016
Annona muricana	*Pseudomonas* sp, *Alcaligenes* sp, and *Micrococcus roseus*	Hydrocarbon	Ilori et al., 2006
Arabidopsis thaliana	*Hyphomicrobium* sp,	Chloromethane	Nadalig et al., 2011
Arabidopsis thaliana	*Achromobacter xylosoxidans* F3B	Phenolic pollutants	Ho et al., 2012
Arachis hypogaea	*Arthrobacter nitroguajacolicus*, *Achromobacter xylosoxidans, Basea thiooxidans, Microbacterium natoriense, Kocuria rosea, Dyadobacter fermentans,* and *Klebsiella pneumoniae*	n-alkanes	Al-Awadhi et al., 2012
Azalea indica	*Pseudomonas putida* TVA8	Toluene	De Kempeneer et al., 2004
Boerhavia diffusa	*Rhodococcus corynebacteriodes* and *Exiguobacterium arabatum*	n-alkanes	Al-Awadhi et al., 2012
Bougainvillea buttiana	*Enterobacter cloacae* LSRC11, *Staphylococcus* sp. A1 and *Pseudomonas aeruginos*	Xylene	Sangthong et al., 2016
Calystegia soldanella	*Hyphomicrobium* sp.	Methanol	Iguchi et al., 2012
Chenopodium album	*Burkholderia fungorum*	n-alkanes	Al-Awadhi et al., 2012
Chenopodium murale	*Gordonia polyisoprenivorans*	n-alkanes	Al-Awadhi et al., 2012
Chenopodium murale	*Flavobacterium* sp., *Halomonas* sp., and *Arthrobacter* sp.	Volatile hydrocarbons	Ali et al., 2015
Chrysopogon zizanioides	*Achromobacter xylosoxidans* F3B	BTEX compounds (benzene, toluene, ethylbenzene, and xylene)	Ho et al., 2013
Clitoria ternatea	*Bacillus cereus*	Formaldehyde	Khaksar et al., 2016b
Conocarpus lancifolius	*Flavobacterium* sp., *Halomonas* sp., and *Arthrobacter* sp.	Volatile hydrocarbons	Ali et al., 2015
Cucumis sativus	*Arthrobacter ureafaciens, Arthrobacter aurescens,* and *Microbacterium natoriense*	n-alkanes	Al-Awadhi et al., 2012
Cynodon spp.	*Rhodococcus* sp. and *Pseudomonas* sp.	Volatile oil hydrocarbons crude oil, n-hexadecane, or phenanthrene	Sorkhoh et al., 2011
Eichhornia crassipes	*Methylophilus* sp.	Methanol	Iguchi et al., 2012
Ervatamia divaricata	*Alcaligenes feacalis* and *Alcaligenes* sp.11SO	Naphthalene and Phenanthrene	Ljs, 2016
Fraxinus excelsior		Trichloroethylene, Toluene	Weyens et al., 2009a
Fraxinus pennsyhanica	*Acinetobacter* sp., *Alcaligenes* sp., and *Rhodococcus* sp.	Phenol	Sandhu et al., 2009
Gazania rigens	*Methylobacterium populi, Gordonia lacunae, Dietzia maris, Microbacterium oleivorans,* and *Pseudomonas stutzeri*	n-alkanes	Al-Awadhi et al., 2012
Gazania rigens	*Flavobacterium* sp., *Halomonas* sp., and *Arthrobacter* sp.	Volatile hydrocarbons	Ali et al., 2015
Gossipium hirsutum 'MCU12'	*Methylobacterium gossipiicola* sp. nov.	Methanol and dichloromethane	Madhaiyan et al., 2012)
Hibiscus rosa-sinensis	*Alcaligenes feacalis* and *Alcaligenes* sp.11SO	Naphthalene and Phenanthrene	Ljs, 2016
Hedera spp.	*Hymenobacter* sp. and *Sphingomonadaceae* sp.	PMs	Smets et al., 2016
Ixora chinensis	*Alcaligenes feacalis* and *Alcaligenes* sp.11SO	Naphthalene and Phenanthrene	Ljs, 2016
Ixora spp.	*Acinetobacter* sp. and *Pseudomonas* sp. *Pseudoxanthomonas* sp., and *Mycobacterium* sp.	Polycyclic aromatic hydrocarbons	Yutthammo et al., 2010
Ixora spp.	*Pseudomonas* sp., *Microbacterium* sp., *Rhizobium* sp., and *Deinococcus* sp.	Phenanthrene	Waight et al., 2007
Keteleeria davidiana	*Methylobacterium* sp. and *Methylophilus* sp.	Methanol	Iguchi et al., 2012
Lolium multiflorum Lam.	*Pseudomonas* sp. Ph6-gfp	Phenanthrene	Sun et al., 2014
Lolium multiflorum var. Taurus	*Pseudomonas* sp. ITSI10, ITRI15, ITRH76, and BTRH79	Hydrocarbon	Yousaf et al., 2010
Lolium perenne	*Pseudomonas* sp.	Petroleum hydrocarbons	Kukla et al., 2014
Lotus corniculatus var. Leo	*Pseudomonas* sp. ITSI10, ITRI15, ITRH76, and BTRH79	Hydrocarbon	Yousaf et al., 2010

(Continued)

TABLE 3 | Continued

Plants	Microbes	Pollutant	References
Magnifera indica	*Pseudomonas* sp., *Alcaligenes* sp., and *Micrococcus roseus*	Hydrocarbon	Ilori et al., 2006
Malus pumila	*Arthrobacter* sp.	4-chlorophenol	Scheublin and Leveau, 2013
Mattiola incana	*Acinetobacter calcoacelicus, Pseudomonas putida, Planomicrobium glaciei,Arthrobacter agilis, Kucuria turfanensis, Pseudomonas geniculate, Chyseobacterium taeanense, Flavobacterium ahuensis,* and *Microbacterium oxydans*	n-alkanes	Al-Awadhi et al., 2012
Mesembryanthemum nodifloru	*Nesterenkonia jeotgali, Nesterkonia lacusekhoensis,* and *Agrococcus terreus*	n-alkanes	Al-Awadhi et al., 2012
Oryza sativa	*Methylobacterium* sp.	Methanol	Knief et al., 2012
Phaseolus vulgaris	*Microbacterium* sp. and *Citrobacter freundii*	Crude oil, phenanthrene and n-octadecane	Ali et al., 2012
Phaseolus vulgaris	*Arthrobacter chlorophenolicus*	4-chlorophenol, hydroquinone	Scheublin et al., 2014
Phaseolus vulgaris	*Pseudomonas* sp. CF600	Phenol	Sandhu et al., 2007
Phragmites australis	*Hyphomicrobium* sp.	Methanol	Iguchi et al., 2012
Picea abies		Trichloroacetic acid	Forczek et al., 2004
Pisum sativum	*Pseudomonas putida* VM1441(pNAH7)	Polyaromatic hydrocarbons (PAHs)	Germaine et al., 2009
Pisum sativum	*Microbacterium* sp. and *Rhodococcus* sp.	Crude oil, phenanthrene and n-octadecane	Ali et al., 2012
Pisum sativum	*Agromyces fucosus, Agrococcus jenensis, Paenibacillus polymixa, Bacillus cereus, Bacillus megaterium, Brevibacillus brevis, Bacillus, Neolsonii, Bacillus subtilis, Arthrobacter ramosus, Microbacterium imperial, Bacillus endophyticus,* and *Cellulosimicrobium cellulans*	n-alkanes	Al-Awadhi et al., 2012
Poaceae spp.	*Hyphomicrobium* sp.	Methanol	Iguchi et al., 2012
Populus deltoides	*Pseudomonas putida* W619-TCE	Trichloroethylene (TEC)	Weyens et al., 2009b
Populus deltoids × *nigra*	*Methylobacterium* sp.	2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine	Van Aken et al., 2004
Populus trichocarpa deltoides	*Burkholderia cepacia* VM1468	Toluene	Taghavi et al., 2005
Pyrus calleryansa	*Methylobacterium* sp. and *Methylophilus* sp.	Methanol	Iguchi et al., 2012
Quercus phillyraeoides	*Methylobacterium* sp. and *Methylophilus* sp.	Methanol	Iguchi et al., 2012
Quercus robu		Trichloroethylene, toluene	Weyens et al., 2009a
Salix discolor clone S-365	*Pseudomonas putida* PD1	Polycyclic aromatic hydrocarbons (PAHs)	Khan et al., 2014
Salix purpurea clone 94006	*Pseudomonas putida* PD1	Polycyclic aromatic hydrocarbons (PAHs)	Khan et al., 2014
Salsola baryosma	*Halomonas marisflava* and *Salinococcus hispanicus*	n-alkanes	Al-Awadhi et al., 2012
Sonchus oleraceus	*Flavobacterium* sp., *Halomonas* sp., and *Arthrobacter* sp.	Volatile hydrocarbons	Ali et al., 2015
Tecoma stans	*Afipia genosp* and *Microbacterium hydrocarbonoxydans* and *Bacillus subtilis*	n-alkanes	Al-Awadhi et al., 2012
Tecoma stans	*Flavobacterium* sp., *Halomonas* sp. and *Arthrobacter* sp.	Volatile hydrocarbons	Ali et al., 2015
Vicia faba	*Flavobacterium* sp., *Halomonas* sp. and *Arthrobacter* sp.	Volatile hydrocarbons	Ali et al., 2015
Vicia faba	*Rhodococcus* sp. and *Pseudomonas* sp.	Volatile oil hydrocarbons crude oil, n-hexadecane, or phenanthrene	Sorkhoh et al., 2011
Vigna unguiculata	*Microbacterium arabinogalactanolyticum* and *Pseudomonas oryzihabtans*	n-alkanes	Al-Awadhi et al., 2012
Viola x *wittrockiana*	*Methylobacterium* sp. and *Methylophilus* sp.	Methanol	Iguchi et al., 2012
W. religiosa	*Acinetobacter* sp., *Pseudomonas* sp., *Pseudoxanthomonas* sp. and *Mycobacterium* sp.	Polycyclic aromatic hydrocarbons	Yutthammo et al., 2010
Zamioculcas zamiifolia	*Bacillus cereus*	Formaldehyde	Khaksar et al., 2016a
Zamioculcas zamiifolia	*Pseudomonas aeruginosa* and *Bacillus cereus*	Ethylbenzene	Toabaita et al., 2016
Zea mays	*Pseudomonas* sp. CF600	Phenol	Sandhu et al., 2007

differed in particle retention and encapsulation, which were attributed to leaf characteristics, cuticle chemical composition and structure.

Leaf physical characteristics such as leaf shape, hairs or trichomes, and stomata significantly affect PM accumulation. Needle leaves were reported to accumulate more $PM_{2.5}$ than broad leaves (Terzaghi et al., 2013; Chen et al., 2017). The effectiveness was attributed to the higher capture efficiency and higher Stoke's numbers of needles compared to those of broad leaves (Beckett et al., 2000). Additionally, small individual leaf area and abundant wax layer also contribute to the effectiveness (Chen et al., 2017). Leaf trichomes have been shown to increase $PM_{2.5}$ accumulation. The trichome density was positively correlated with amount of $PM_{2.5}$ accumulated on leaves, and plant species with abundant hairs, such as *Catalpa speciosa*, *Broussonetia papyrifera*, and *Ulmus pumila* were able to retain more $PM_{2.5}$ than those with fewer hairs (Chen et al., 2017). The adaxial surface of leaves accumulated more PMs than the abaxial leaf surface (Baldacchini et al., 2017), which is probably due to the fact that the abaxial surface in general has few trichomes and less rough surface. Stomata may play some roles in accumulation of PMs. The length of stomata ranges from 10 to 80 μm and densities varies from 5 to 1,000 mm^{-2} depending on plant species and environmental conditions (Hetherington and Woodward, 2003). Stomatal pore areas range from 46 to 125 μm^2 (Peschel et al., 2003; Dow et al., 2014), thus stomata could retain or adsorb either $PM_{2.5}$ or PM_{10}. A study of PM deposition on leaves of five evergreen species in Beijing, China showed that PM diameter up to 2 μm was in the stomatal cavity (Song et al., 2015). Rai (2016) studied the effects of PMs on 12 common roadside plant species and found that stomatal sizes were reduced due to air dust deposition, but plant growth was not affected, suggesting the potential of plants in adsorbing air pollutants.

Growing evidence has suggested that plant leaves are able to capture PMs and act as biofilters. On average, the upper leaf surface of 11 plant species intercepted 1,531 particles per mm^{-2} (Wang et al., 2006). Needles of *Pinus sylvestris* accumulated 18,000 mineral particles per mm^2 (Teper, 2009). Upper leaves of *Hedera helix* captured about 17,000 particles per mm^2 (Ottele et al., 2010). Trees removed 1,261 tons of air pollutants in Beijing, of which 772 tons were PM_{10}(Yang et al., 2005). In New Zealand, urban trees removed 1,320 tons of particular matter annually due to the existence of woodlands in Auckland (Cavanagh and Clemons, 2006). Nowak et al. (2014) showed trees within cities removed fine particles from the atmosphere and consequently improved air quality and human health. Tree effects on $PM_{2.5}$ concentrations and human health are modeled for 10 U.S. cities. The total amount of $PM_{2.5}$ removed by trees varied from 4.7 tons in Syracuse to 64.5 tons in Atlanta in the U.S annually. All the reported removal of PMs is attributed to plant leaves. It is unknown at this time if phyllosphere microbes could break down the PMs on leaves and if mineral elements released from the broken PMs could become plant nutrients. Considering the fact that the microbes can biodegrade a wide range of substances including petroleum, we hypothesize that some microbes should be able to break down PM. Future research in this regard will be conducted, and identified microbes could be used for PM reduction.

Remediation of SO_2

Sulfur dioxide (SO_2) was among the first air pollutants identified to harm human health and ecosystems. The combustion of fossil fuels has substantially increased SO_2 in the air. China has contributed to about one-fourth of global SO_2 emission since 1990 (Zhang et al., 2013). The emission of SO_2 from Guangdong province totaled 1,177 Gg in 2007, of which 97% was emitted by power plants and industries (Lu et al., 2010). SO_2 can be oxidized photochemically or catalytically to sulfur trioxide (SO_3) and sulfate (SO_4^{2-}) in the air (Bufalini, 1971). With the presence of water, SO_3 is converted rapidly to sulfuric acid (H_2SO_4), which is commonly known as acid rain. While in sulfur assimilation, SO_4^{2-} is reduced to organic sulfhydryl groups (R-SH) by sulfate-reducing bacteria, fungi, and plants. Sulfur oxidizing bacteria such as *Beggiatoa* and *Paracoccus* are able to oxidize reduced sulfur compounds like H_2S to inorganic sulfur, and thiosulfate to form sulfuric acid (Pokoma and Zabranska, 2015). Sulfate reducing bacteria like *Archaeoglobus* and *Desulfotomaculum* can convert sulfur compounds to hydrogen sulfide (H_2S). Oxidation of H_2S produces elemental sulfur (S°), which is completed by the photosynthetic green and purple sulfur bacteria and some chemolithothrophs. Further oxidation of elemental sulfur produces sulfate. Sulfate is assimilated through the sulfate activation pathway, which is consisted of three reactions: the synthesis of adenosine 5′-phosphorylation of (APS), the hydrolysis of GTp, and the 3′-phosphorylation of APS to produce 3′-phosphoadenosine 5′-phosphosulfate (PAPS) (Sun et al., 2005). In *Mycobacterium tuberculosis*, the entire sulfate activation pathway is organized into a single complex (Sun et al., 2005). Additionally, sulfate reducing bacteria have been shown to use hydrocarbons in pure cultures, which can be used for bioremediation of benzene, toluene, ethylbenzene, and xylene in contaminated soils (Muyzer and Stams, 2008). Such bacteria may also colonize leaf surfaces and could be used for remediation of air pollutants.

Plant leaves absorb SO_2 via stomata. At apoplastic pH, it is hydrated and oxidized successively to sulfite and sulfate, both of which can inhibit photosynthesis and energy metabolism if they accumulate to a high concentration. Such inhibition can cause SO_2 toxicity. Symptoms include interveinal chlorosis and necrosis in broad-leaved species, and chlorotic spots and brown tips in pine conifers (Rennenberg, 1984). Until the 1970s, SO_2 was considered to be a key contributor of acid rain causing forest dieback (Bloem et al., 2015). Interestingly, when the Clean Air Acts came into action in the 1980s, the reduction in atmosphere SO_2 resulted in sulfur (S) deficiency in crops, particularly *Brassica* species. The S deficiency was responsible for the increased incidence of disease caused by *Pyrenopeziza brassicae* (Bloem et al., 2015). The explanation is that plants could become injured in a SO_2 concentration range from 131 to 1,310 $\mu g\, m^{-3}$; plants, however, can rapidly assimilate SO_2 and H_2S into reduced sulfur pools such as cysteine and sulfates as illustrated in **Figure 1**. A recent transcriptome analysis of *Arabidopsis* responses to SO_2 showed that plant adaptation to SO_2 evokes a

comprehensive reprogramming of metabolic pathways including NO and reactive oxygen species (ROS) signaling molecules, and also plant defense response pathways (Zhao and Yi, 2014). The importance of this study revealed that plant responses to SO_2 stress is at the transcription level with initial activation of cross tolerance and followed by sulfur assimilation pathways. Cysteine metabolism in particular is associated with the network of plant stress responses, thus improving plant growth in soils where sulfur supply is limited (Bloem et al., 2015). It has been shown that an atmospheric level of 79 ng m^{-3} SO_2 could contribute to 10–40% of leaf sulfur assimilation (De Kok et al., 2007; Zhao et al., 2008). Elevated SO_2 concentrations around natural CO_2 springs have been documented to enhance accumulation of sulfur metabolites and proteins in surrounding vegetation (Rennenberg, 1984). Therefore, plants can be selected for growing in SO_2 polluted environments (Chung et al., 2010). In 2000, about 42.62 Mg of SO_2 was removed from the atmosphere by urban trees in Guangzhou, China (Zhang et al., 2013). Additionally, S metabolism can be genetically engineered for improving plant resistance to SO_2. Transgenic tobacco plants overexpressing cysteine synthase or serine acetyltransferase gene were highly tolerant to SO_2 and sulfite (Noji et al., 2001).

Remediation of NO_x

There are several oxides of nitrogen (N) in the atmosphere: nitrogen dioxide (NO_2), nitric oxide (NO), nitrous oxide (N_2O), nitrogen trioxide (N_2O_3), and nitrogen trioxide (N_2O_5). Among them, the USEPA regulates NO_2 only because it is the most prevalent form of NO_x generated anthropogenically (USEPA, 1999). NO_2 also participates in the formation of ozone (O_3) and NO. NO_x emissions in China increased rapidly from 11.0 Mt in 1995 to 26.1 Mt in 2010. Power plants, industry, and transportation were major sources of NO_x emissions, accounting for 28.4, 34.0, and 25.4% of the total NO_x emissions in 2010, respectively (Zhou et al., 2013). The total NO_x emissions in China are projected to increase 36% based on the 2010 value by 2030.

A group of bacteria like *Azotobacter* and *Rhizobium* and fungi such as mycorrhizas are capable of fixing atmospheric N. *Cyanobacteria* are able of using a variety of inorganic and organic sources of combined N, like nitrate, nitrite, ammonium, urea or some amino acids. These microbes are often associated with plant roots. Nitrifying bacteria including species from the genera *Nitrosomonas*, *Nitrosococcus*, *Nitrobacter*, and *Nitrococcus* oxidize ammonia to hydroxylamine, and nitrite oxidoreductase oxidizes nitrite to nitrate. Nitrifying bacteria thrive in soils, lakes, rivers, and streams with high inputs and outputs of sewage, wastewater and freshwater because of high ammonia content. Phyllosphere diazotrophic bacteria, like *Beijerinckia*, *Azotobacter*, and *Klebsiella* and also Cyanobacteria, such as *Nostoc*, *Scytonema*, and *Stigonema* can use atmospheric dinitrogen (N_2) as a source of nitrogen (Whipps et al., 2008). N_2 is fixed by the nitrogenase enzyme encoded by *nif* genes, and the gene *nifH* has been widely used for analysis of their community structure (Fürnkranz et al., 2008; Rico et al., 2014). The abundance of N_2-fixing bacteria was also reported to improve drought tolerance, suggesting their adaptability to plants grown in different environmental conditions (Rico et al., 2014).

Plants absorb gaseous NO_2 more rapidly than NO because NO_2 reacts rapidly with water while NO is almost insoluble. The uptake of NO_2 per unit leaf area was reported to be nearly three times that of NO when the two gases occurred in the same concentration (Law and Mansfield, 1982). As a result, NO_2 has been considered to be more toxic than NO. Visible symptoms resulting from NO_2 exposure are relatively large, irregular brown or black spots. However, phytotoxicity of NO_2 is rare and much less than SO_2 and O_3. This is due to the fact that NO_x are plant nutrients. When NO and NO_2 are absorbed and dissolved in the extracellular solution of leaves, they form nitrate (NO_3) and NO_2 in equal amounts and proton (H^+). NO_3 is then utilized by plants in the same way as it is absorbed from roots and used as a nitrogen source for synthesizing amino acids and proteins (**Figure 1**). Foliar absorption of NO_2 varies widely depending on plant species. Morikawa et al. (1998) studied 217 herbaceous and woody species in uptake of NO_2 and found that plant species differed by 657 folds in NO_2 uptake and assimilation. The most efficient woody plants included *Eucalyptus viminalis*, *Populus nigra*, *Magnolia kob*u, and *Robinia pseudoacacia*, and the most herbaceous plants include *Erechtites hieracifolia*, *Crassocephalum crepidioides*, and *Nicotiana tabacum* (Morikawa et al., 1998).

Nitrogen dioxide could be a plant signal molecule that improves plant growth. Morikawa et al. (2004) reported that about one-third of NO_2-derived N absorbed by leaves was converted into a previously unknown Kjeldahl-unrecoverable organic nitrogen, which comprise a novel heterocyclic Δ2 1,2,3 thiadiazoline derivative and nitroso- and nitro-organic

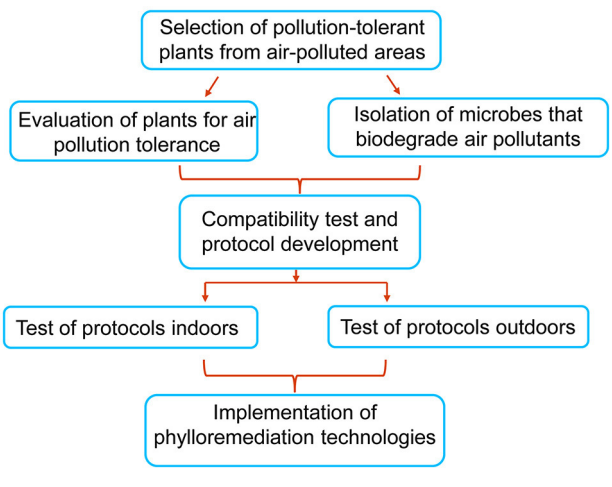

FIGURE 2 | A general outline for developing phylloremediation technologies. Plants species and microbes should be selected from air polluted areas. Selected plants should be evaluated for their ability to adsorb or absorb air pollutants, and concurrently microbes are screened for biodegradation or biotransformation of pollutants. The selected plants and microbes are tested for synergistic effects on the reduction of particular air pollutants. Based on the test results, specific plant-microbe combinations that can remove one or more air pollutants are identified, and protocols are formulated for evaluating their effectiveness in removal pollutants indoors and outdoors. Effective protocols will be developed into phylloremediation technologies for use in reducing air pollutants.

compounds (Miyawaki et al., 2004; Morikawa et al., 2005). These results indicate that NO_2 is not only known as a pollutant or a supplemental source of N, but also acts as an airborne reactive nitrogen species signal (Morikawa et al., 2004, 2005). This is in agreement with the reports that endogenously produced NO_x such as NO act as a vital plant signal (Wendehenne et al., 2001; Neill et al., 2003). To further analyze atmospheric NOx effects on plants, Morikawa et al. (2003) determined if plants could use NO_2 as a fertilizer and concomitantly reduce NO_2 concentrations. The authors found that application of 282 $\mu g\ m^{-3}$ NO_2, equivalent to the heavily polluted urban air, to plants for 10 weeks almost doubled the biomass, total leaf area, the contents of carbon (C), N, S, phosphorus (P), potassium (K), calcium (Ca), and magnesium (Mg) as well as free amino acid contents and crude proteins (Morikawa et al., 2003). The mass spectrometric analysis of the $^{15}N/^{14}N$ ratio showed that N derived from NO_2 comprised less than 3% of total plant N, meaning that the contribution of NO_2-N to total N was relatively low. These results imply that NO_2 could be a multifunctional signal to stimulate plant growth, nutrient uptake, and metabolism (Takahashi et al., 2005).

Remediation of O_3

Anthropogenic O_3 is primarily generated from the reaction of atmospheric O_2 with ground-state O (3P) radicals that result from the photolytic dissociation of ambient NO_2. Thus, the presence of NO and NO_2 in the lower atmosphere is closely linked with ground-level of O_3. In China, O_3 levels increased at a rate of 2.2 $\mu g\ m^{-3}$ per year from 2001 to 2006. Average O_3 concentrations in Beijing varied from 45 to 96.2 $\mu g\ m^{-3}$ depending on locations (Wan et al., 2014). In Shanghai, 1-h average concentration of O_3 was 54.2 $\mu g\ m^{-3}$. O_3 level increased during spring, reached the peak in late spring and early summer, and then decreased in autumn and finally dropped in winter. The highest monthly average O_3 concentration (82.2 $\mu g\ m^{-3}$) in June was 2.7 times greater than the lowest level (30.4 $\mu g\ m^{-3}$) recorded in December (Zhao et al., 2015).

Ozone is considered an effective antimicrobial agent against some bacteria and fungi (Sharma and Hudson, 2008). There have been no reports on microbial-mediated O_3 reduction. However, in a study of O_3 effects on phyllosphere fungal populations, Fenn et al. (1989) found that a chronic exposure of mature Valencia orange trees (*Citrus sinensis*) to O_3 or SO_2 for 4 years decreased populations of phyllosphere fungi. In a same experiment conducted by the authors, a short-term fumigation of O_3 to giant sequoia (*Sequoiadendron giganteum*) and California black oak (*Quercus kelloggii*) did not significantly affect the numbers of phyllospere fungi. Plant absorption of O_3 is mainly through stomata, O_3 is easily dissolved in water and reacts with apoplastic structures and plasma membranes to form reactive oxygen species (ROS), such as O_2^-, H_2O_2, and OH radical. The O_3 or ROS can disturb cell membrane integrity and attack sulfhydryl (SH) groups or ring amino acids of protein, thus causing phytotoxicity. Injury symptoms include white, yellow or brown flecks on the upper surface of leaves. The threshold concentrations that cause a 10% reduction in yield are 80 $\mu g\ m^{-3}$ for sensitive crops and 150 $\mu g\ m^{-3}$ for the most resistant crops. Adaptation of plants to O_3 stress has resulted in plants developing mechanisms against O_3 toxicity. First, O_3 can be removed from the air by chemical reactions with reactive compounds emitted by vegetation, particularly monoterpenes (Di Carlo et al., 2004). Second, semi-volatile organic compounds, such as different diterpenoids exuded by trichomes on leaves are an efficient O_3 sink (Jud et al., 2016). Tobacco leaves can secret diterpenoid cis-abienol, which acts as a powerful chemical protection shield against stomatal O_3 uptake by depleting O_3 at the leaf surface. As a result, O_3 flux through the open stomata is strongly reduced (Jud et al., 2016). As to O_3 absorbed by leaves, an oxidative burst occurs as the initial reaction to O_3, followed by activation of several signaling cascade and plant antioxidant systems including ascorbate-glutathione cycle and antioxidant enzymes to alleviate the oxidative burden resulting from O_3 exposure (Vainonen and Kangasjarvi, 2015).

Remediation of VOCs

VOCs are organic chemicals that have a low boiling point and a high vapor pressure at room temperature causing large numbers of molecules to evaporate into the surrounding air. VOCs are numerous and ubiquitous including naturally occurring and anthropogenic chemical compounds. VOCs participate in atmospheric photochemical reactions contributing to O_3 formation and also play a role in formation of secondary organic aerosols, which are found in PMs. The strong odor emitted by many plants consists of green leaf volatiles, a subset of VOCs called biogenic VOCs, which emit exclusively from plant leaves, the stomata in particular. Major species of biogenic VOCs include isoprene, terpenes, and alkanes.

Anthropogenic VOCs include large groups of organic chemicals, such as formaldehyde, polycyclic aromatic hydrocarbons (PAHs), and BTX (benzenes, toluene, and xylenes). The most significant sources of formaldehyde are engineered wood products made of adhesives that contain urea-formaldehyde (UF) resins. BTX come from painting and coating materials used for interior decoration and refurbishment. Motor-vehicle exhausts, tobacco smoke, and heating also contribute to the presence of VOCs. A great concern over VOCs has been indoor air quality. Indoor formaldehyde in recently renovated homes ranged from 0.14 to 0.61 mg m^{-3}, and benzene, toluene, and xylenes were 124.0, 258.9, and 189.7 $\mu g\ m^{-3}$, respectively (Hao et al., 2014). The formaldehyde concentration is 65–100% higher than indoor air quality standards of China. Formaldehyde and BTX as main indoor VOCs contribute to the so-called "sick building syndrome" (Brown et al., 1994; Wieslander et al., 1996; Wargocki et al., 2000; Berg et al., 2014). This review regarding VOCs is thus emphasized on indoor air quality.

As early as in the 1970s, NASA (U.S. National Aeronautics and Space Administration) conducted research on the use of foliage plants for remediation of air quality in space shuttles. Foliage plants are those with attractive foliage and/or flowers that are able to survive and grow indoors (Chen et al., 2005). Results showed that foliage plants removed nearly 87% of air pollutants from sealed chambers within 24 h (Wolverton et al., 1984, 1989; Cruz et al., 2014a). For example, each plant of peace lily (*Spathiphyllum* spp. 'Mauna Loa') removed 16 mg of formaldehyde, 27 mg of trichloroethylen, and 41 mg of benzene from sealed chambers

after a 24-h exposure to the respective chemical. Generally, plants absorb gaseous pollutants via leaf stomata. Some of the VOCs are recognized as xenobiotics by plants, and they are detoxified through xenobiotic metabolism, involving oxidoreductase or hydrolases, bioconjugation with sugars, amino acids, organic acids, or peptides, and then removed from the cytoplasm for deposition in vacuoles (Edwards et al., 2011). In addition to plant leaves, rhizosphere microbes also contribute to reduction of VOCs under interior environments (Llewellyn and Dixon, 2011). Using a dynamic chamber technique, Xu et al. (2011) investigated formaldehyde removal by potted foliage plants and found that formaldehyde removal was attributed not only to the formaldehyde dehydrogenase activities of plant leaves but also to the absorption and metabolism by microorganisms in the rhizosphere. Such bacteria have been isolated from soils, water, and different tissues of plants in polluted environments. Many pure cultures of bacteria, including various strains of *P. putida*, have been evaluated for biodegradation of air pollutants. Some fungi strains are also able to use volatile aromatic hydrocarbons as sole source of carbon and catalyze degradation reactions (Prenafeta-Boldú et al., 2001; Kennes and Veiga, 2004; Jin et al., 2006). Here we mainly discuss phylloremediation of formaldehyde, benzene, toluene, and xylene as well as phenols and PAHS.

Formaldehyde

Formaldehyde is a colorless, flammable gas or liquid that has pungent and suffocating odor. It poses a significant danger to human health due to its high reactivity with proteins and DNA, thus formaldehyde is known to be a human carcinogen. Plants can directly absorb formaldehyde and transform it to organic acids, sugars or CO_2 and H_2O (**Figure 1**). Giese et al. (1994) exposed shoots of *Chlorophytum comosum* to 8.5 mg m^{-3} gaseous [^{14}C]-formaldehyde over 24 h and found that about 88% of the recovered radioactivity was associated with plant metabolites as ^{14}C, which had been incorporated into organic acids, amino acids, free sugars, lipids, and cell wall components. Formaldehyde responsive genes were identified from golden pothos (*Epipremnum aureum*) (Tada et al., 2010). Glutathione (GSH)-dependent formaldehyde dehydrogenase (FADH) and formate dehydrogenase (FDH) can detoxify formaldehyde to formate and further to carbon dioxide (Tada and Kidu, 2011). A wide range of foliage plants have been documented to be able to remove formaldehyde. Kim et al. (2010) exposed 86 species of foliage plants individually to 2 μl L^{-1} formaldehyde in sealed chambers and found that formaldehyde removed per cm^2 leaf area in 5 h ranged from 0.1 to 6.64 mg m^{-3}, depending on plant species. The most efficient species in removal of formaldehyde include *Osmunda japonica*, *Selaginella tamariscina*, *Davallia mariesii*, and *Polypodium formosanum*. Surprisingly, these efficient plants belong to pteridophytes, commonly known as ferns and fern allies. Why this group of plants is more efficient than the other foliage plants in formaldehyde removal deserves further investigation.

Formaldehyde can also be assimilated as a carbon source by bacteria (Vorholt, 2002). Such assimilation occurs in *Methylobacterium extorquens* through the reactions of the serine cycle (Smejkalova et al., 2010), in *Bacillus methanolicus* through the RuMP cycle (Kato et al., 2006), and in *Pichia pastoris* through the xylulose monophosphate cycle (Lüers et al., 1998). Some fungi also assimilate formaldehyde. Yu et al. (2015) isolated a fungal strain (*Aspergillus sydowii* HUA), which was able to grow in the presence of formaldehyde up to 2,400 mg l^{-1} and the specific activity of formaldehyde dehydrogenase and formate dehydrogenase were as high as 5.02 and 1.06 U mg^{-1}, respectively, suggesting that this fungal isolate could have great potential for removing formaldehyde. Some of the bacteria and fungi used to colonize roots can also colonize leaves and could be used for phylloremediation of formaldehyde in the air (Khaksar et al., 2016a).

BTX

BTX refers to benzene, toluene, and three xylene isomers [ortho– (or o–), meta– (or m–), and para– (or p–)], which are major components of gasoline. Due to their low water solubility and acute toxicity and genotoxicity, BTX components have been classified as priority pollutants by the USEPA (Eriksson et al., 1998). Plants leaves can absorb BTX mainly through stomata, which are converted to phenol or pyrocatechol, and subsequently to muconic acid and fumaric acid (Ugrekhelidze et al., 1997). Foliage plants, such as *Dracaena deremensis* and *Spathiphyllum* spp. have been documented to remove BTX indoors (Wolverton et al., 1984, 1989; Wood et al., 2006; Mosaddegh et al., 2014). Liu et al. (2007) fumigated 73 plant species with 478.5 μg m^{-3} benzene gas and found that 23 of the 73 species showed inability to reduce fumigated benzene, the rest varied in benzene reduction, ranging from 0.1 to 80%. The most efficient plant species were *Crassula portulacea*, *Hydrangea macrophylla*, and *Cymbidium* 'Golden Elf'. Foliage plants that are effective in removal of toluene include *H. helix*, *Philodendron* spp., *Schefflera elegantisima*, and *Sansevieria* spp. (Kim et al., 2011; Sriprapat et al., 2013; Cruz et al., 2014b). The wax of *Sansevieria trifasciata* and *S. hyacinthoides* is rich in hexadecanoic acid, which could pay an important role in absorption of toluene (Sriprapat et al., 2013). Sriprapat et al. (2014) also evaluated plant absorption of xylene. The tested 15 plant species were able to remove xylene with removal efficiency ranging from 59.1 to 88.2%, of which *Zamioculcas zamiifolia* was the most efficient species.

Bacteria including some strains of *Rhodococcus rhodochrous* (Deeb and Alvarez-Cohen, 1999), *Alcaligenes xylosoxidans* (Yeom and Yoo, 2002), and *P. putida* (Alagappan and Cowan, 2003) and also fungal cultures of *Cladophialophora* sp. (Prenafeta-Boldú et al., 2002) are able to degrade BTX (**Figure 1**). Many *Pseudomonas* species are leaf colonists and some are plant pathogens (Dulla et al., 2005). BTX are actual growth substrates for a number of organisms, such as *P. putida* (Inoue et al., 1991). In a study of bioremediation of airborne toluene, De Kempeneer et al. (2004) found that the time required for 95% reduction of the initial toluene concentration of 339 mg m^{-3} was 75 h by *Azalea indica* plants along. Such reduction by the plants inoculated with *P. putida* TVA8 under the identical conditions was only 27 h. Subsequent additions of toluene further increased the removal efficiency of plants inoculated with the bacterial strain, but the toluene-removal rate was comparably

low in plants without inoculation. Hence, inoculation of the leaf surface with *P. putida* TVA8 was considered to be essential for rapid removal of toluene. These results clearly demonstrated the importance of both plant leaves and leaf-associated microbes in phylloremediation of indoor air pollutants. The genetics and biochemistry of strains F1 and mt-2 of *P. putida* have been intensively studied (Harayama and Rekik, 1990; Horn et al., 1991; Timmis et al., 1994; Aemprapa and Williams, 1998). Such information could be important for exploring these strains for effective removal of air pollutants.

Air Borne Phenols and Polycyclic Aromatic Hydrocarbon (PAHs)

Air borne phenols are a class of chemical compounds containing a hydroxyl group bonded directly to an aromatic hydrocarbon group, whereas PAHs are hydrocarbon comprising only carbon and hydrogen with multiple aromatic rings. Phenol and PAHs are major air pollutants in urban areas, and some PAHs have been considered carcinogenic. It has been reported that *Bacillus cereus* can degrade phenol via meta-cleavage pathway (Banerjee and Ghoshal, 2010). *Pseudomonas* sp. CF600 can mineralize phenol on bean and maize leaves by dmp catabolic pathway (Sandhu et al., 2007). Sandhu et al. (2007) directly measured phenol degradation by natural phyllosphere communities. Leaves were collected from trees growing in an area that was known to have high concentrations of VOCs. Unsterilized and surface-sterilized leaves were then exposed to radiolabeled phenol in closed chambers for 24 h and the amount of phenol degradation was compared. The phenol degradation by the non-sterilized leaves was significantly greater than the degradation by the sterilized leaves, indicating that degradation of VOCs was enhanced by the presence of the phyllosphere communities. This work indicates that plant leaves can accumulate phenols, which may be subsequently available for bacteria in the phyllosphere for degradation.

Plant leaves can absorb atmospheric PAHs. A study on deciduous forest in Southern Ontario, Canada, confirmed that amounts of phenanthrene, anthracene, and pyrene were reduced within and above the forest canopy during bud break in early spring (Choi et al., 2008). Plant species differ in removal of PAHs, the differences could be attributed to specific morphological and chemical constitutions of plants as well as leaf-associated microbes. Phyllosphere bacteria on 10 ornamental plant species were studied based on their diversity and activity toward the removal of PAHs (Yutthammo et al., 2010). The phyllosphere hosted diverse bacterial species including *Acinetobacter*, *Pseudomonas*, *Pseudoxanthomonas*, *Mycobacterium*, and unculturable ones, of which PAH degrading bacteria accounted for about 1–10% of the total heterotrophic phyllosphere populations depending on plant species. The analysis of bacterial community structures using PCR and denaturing gradient gel electrophoresis showed that each plant species had distinct band patterns, suggesting that the bacterial communities are closely associated with leaf morphology and chemical characteristics of ornamental plant species. Furthermore, branches of fresh leaves of selected plant species were evaluated in sealed chambers for removal of a mixture of PAHs (acenaphthene, acenaphthylene, fluorene, and phenanthrene). Bacteria on unsterilized leaves of all tested plants showed an enhanced removal of phenanthrene. Bacteria on leaves of *Wrightia religiosa* in particular were able to reduce all the tested PAHs (Yutthammo et al., 2010). Therefore, phyllosphere bacteria on ornamental plants may play an important role in natural attenuation of airborne PAHs and plant species differ in supporting microbes in PAH removal.

DEVELOPMENT OF PHYLLOREMEDIATION TECHNOLOGIES

This review has documented that plant leaves and leaf-associated microbes individually can reduce air pollution and the combination of the two generally exhibits enhanced remediation of air pollutants. Since air pollution never before has become such an urgent problem in countries like China and India, now is the time to seriously consider all options for reducing the pollutants. Phylloremediation is a natural and environmentally friendly way of bioremediation of air contaminants. Our proposal for developing phylloremediation technologies is outlined in **Figure 2**, which includes (1) selection and evaluation of appropriate plant species and microorganisms that are tolerant to pollution and able to remove one or more air pollutants; (2) testing and analysis of the compatibility of plant leaf surfaces with isolated microbes for synergetic interactions in reduction of pollutants in laboratories, in simulated indoor environments, and in outdoor settings; (3) analysis of experimental data and development of phylloremediation technologies; and (4) implementation of the technologies for remediation of air in both indoor and outdoor environments.

Plant Selection

Plants should be selected from four categories: (1) trees, (2) shrubs or small tress, and (3) ground cover plants for use in outdoor environments as well as (4) foliage plants for indoor environments. Trees are referred to as perennial plants with elongated stems or trunks, supporting branches and leaves. Shrubs (or small trees) are those small to medium-sized woody plants that grow under some degree of shaded conditions. Ground covers are any plants that can grow over an area of ground and they can grow below the shrub layer including turfgrass and other woody and herbaceous selections. Foliage plants are those which can grow and survive indoors for interior decoration.

Plant species not only differ greatly in adsorption, absorption, and assimilation of air pollutants but also vary significantly in pollution tolerance. Air pollution tolerance index has been used for evaluation of plants specie in response of pollutants (Singh et al., 1991). Information generated by the index is useful, but the index may require revision for better reflecting the ability of plants in tolerance of air pollutants. An initial large-scale evaluation of plants from the four categories should be conducted for identifying candidate species that are able to tolerate PMs, O_2, SO_2, NO_x, and VOCs individually or collectively and can also substantially retain or assimilate these

pollutants. Plants should also tolerate abiotic stresses, such as drought, heat, and cold, and biotic stresses like plant pathogens. Leaves of plants should be able to support one or more selected microbes. Trees should have a relatively fast growth rate. Needle-leaved plants should be particularly considered. As mentioned before, needles are rich in waxes for capturing PMs, and they are also used as as passive bio-samplers to determine polybrominated diphenyl ethers (Ratola et al., 2011). Broad-leaved plants should have more hairs or trichomes and more stomata with a large canopy. Leaf water and nutritional contents, leaf cuticular wax composition, hairs or trichomes, and surface physical characteristics should be suitable for microbial colonization. Shrubs and ground cover plants should have similar leaf physical and chemical properties but be able to tolerate slight shade. For foliage plants, they should substantially tolerate shade and can survive and grow under indoor low-light conditions.

Plant species possessing the aforementioned traits should be selected from particular regions where plants survive and thrive under heavily polluted environments. The rationale is that plants that are able to grow in the polluted environments may develop mechanisms for adaptation to the stressful conditions. Thus, some regions of China and India could be ideal locations for initial selection of plant species. Plants have been documented to tolerate multiple stresses, which include induced cross tolerances and the ability of particular variants to resist multiple distinct stresses. Reactive oxygen species are key molecular signals produced in response to multiple stresses, which are aimed at the maintenance of cellular equilibrium (Perez and Brown, 2014). Glutathione-S-transferase (GST) genes play an important role in the maintenance of ROS equilibrium. Salicylic acid, jasmonic acid, and ROS interplay in the transcriptional control of multiple stresses. Additionally, omics technologies should be used for identifying molecular mechanisms in regulation of plant responses to multiple stresses. Such information, particularly transcriptional factors, key regulatory genes or enzymes should be incorporated into the plant selection processes.

Genetic engineering is an option for improving plants to remediate air pollutants (Abhilash et al., 2009). Genes listed in **Table 4** can be used for generating transgenic plants. Cysteine synthase is a key enzyme to utilize H_2S and SO_2 as a sulfur source to synthesize cysteine. Overexpression of cysteine synthase in rice was shown to enhance sulfur assimilation upon exposure to a high level of H_2S (Yamaguchi et al., 2006). Nitrite reductase catalyzes the six-electron reduction of nitrite to ammonium. Transgenic *Arabidopsis* plants bearing chimeric spinach *NiR* gene enhanced nitrite reductase activity and NO_2 assimilation (Takahashi and Morikawa, 2001). Cytochrome P450 2E1 has strong and specific capacity of decomposing organic pollutants in animal bodies. Transgenic tobacco plants overexpressing *CYP2E1* gene showed increased ability to detoxify broad classes of pollutants such as chlorinated solvents and aromatic hydrocarbons (James et al., 2008). Unlike tobacco, poplar (*Populus tremula* × *Populus alba*) plants are a fast-growing tree species with large canopies. Poplar plants overexpressing a mammal *CYP2E1* exhibited increased metabolism and enhanced removal of organic pollutants from hydroponic solution and the air (Doty et al., 2007). Some genes from microbes can also be used for engineering transgenic plants for phylloremediation. The ribulose monophosphate (RuMP) pathway is one of the formaldehyde-fixation pathways found in microorganisms (Orita et al., 2006). The key enzymes of this pathway are 3-hexulose-6-phosphate synthase (HPS), which fixes formaldehyde to D-ribulose 5-phosphate (Ru5P) to produce D-arabino-3-hexulose 6-phosphate (Hu6P) and 6-phospho-3-hexuloisomerase (PHI), and then converts Hu6P to fructose 6-phosphate (F6P) (Orita et al., 2006; Chen et al., 2010). Co-expression of HPS and PHI in tobacco plants resulted in 20% reduction of formaldehyde compared to the control plants (Chen et al., 2010). In another study, a chlorocatechol 1,2-dioxygenase gene (*tfdC*) derived from the bacteria *Plesiomonas* was introduced into *Arabidopsis thaliana* (Liao et al., 2006). Transgenic plants showed enhanced tolerances to catechol, an aromatic ring. Transgenic plants were also able to remove a large amount of catechol from their media and highly efficient in convertion of catechol to cis,cis-muconic acid, suggesting that degradative genes derived from microbes can be used to produce transgenic plants for bioremediation of aromatic pollutants in the environment (Liao et al., 2006).

Selected plants should be evaluated in controlled environmental chambers to measure their capacity for tolerance and also assimilation of air pollutants. Seedlings could be exposed to particular pollutants or a mixture of pollutants in different concentrations and durations. Plant responses to the exposures could quickly evaluated based on stomatal conductance, net photosynthetic rate, the maximum quantum efficiency of photosystem II using the new LI-COR6800. Their morphological appearance, i.e., leaf greenness, leaf size, and plant height and canopy dimension compared to control treatments should be evaluated. The ability of plants to remove pollutants should be tested using GC-MS. For evaluation of plant responses to PM, in addition to the mentioned plant characteristics, leaf morphology, particularly leaf surface characters should be examined under microscopes and stomatal size and density recorded. If needed, isotopic labeling techniques could be used to track the fate of particular compounds. The evaluation results once analyzed and compared, plants that tolerate stresses and are able to adsorb or absorb or assimilate pollutants could be identified from each type of plants for subsequent compatiablity tests with selected microbes.

Microbe Selection

Cultivable bacteria only account for a small fraction of the total diversity in the phyllosphere, which has greatly hampered the use of some valuable microbes. New approaches, such as the use of improved culture and advanced devices (i-Chip), co-culture with other bacteria, recreating the environment in the laboratory, and combining these approaches with microcultivation should be employed to convert more uncultivable bacteria into cultured isolates in the laboratory (Nichols et al., 2010; Stewart, 2012; Müller and Ruppel, 2014). Similar to plant selection, initial microbial selection could be carried out in areas where plants have been contaminated by air pollutants.

TABLE 4 | Genes from different sources have been demonstrated to be able to remediate air pollutants in transgenic plants.

Gene name	Source	Transgenic plants	Pollutant	References
3-hexulose 6-phosphate and 6-phospho-3-hexuloisomerase	*Mycobacterium gastri* MB19	*Arabidopsis thaliana* and *Nicotiana tabacum*	Formaldehyde	Chen et al., 2010
Biphenyl-Chlorobiphenyl Dioxygenase	*Burkholderia xenovorans*	*Nicotiana tabacum*	4-chlorobiphenyl	Mohammadi et al., 2007
Chlorocatechol 1,2-dioxygenase	*Plesiomonas* spp.	*Arabidopsis thaliana*	Catechol	Liao et al., 2006
CYP450 2E1	*Oryctolagus cuniculus*	*Populus tremula* × *populous alba*	Trichloroethylene, benzene, and chloroform	Doty et al., 2007
CYP450 2E1	*Homo sapiens*	*Nicotiana tabacum*	Trichloroethylene, and ethylene dibromide	Doty et al., 2000
CYP450 2E1	*Homo sapiens*	*Nicotiana tabacum*	Trichloroethylene, benzene, toluene, vinyl chloride, chlorotoluene, and chloroform	James et al., 2008
Cysteine synthase	*Nicotiana tabacum*	*Nicotiana tabacum*	SO_2	Noji et al., 2001
Glutathione reductase	*Escherichia coli*	*Populus sieboldii* × *P. grandidentata*	SO_2	Endo et al., 1997
Mn-peroxidase	*Coriolus versicolor*	*Nicotiana tabacum*	Phencyclidine	Iimura et al., 2002
Nitrite reductase	*Spinacia oleracea*	*Arabidopsis thaliana*	NO_2	Takahashi and Morikawa, 2001
O-acetylserine(thiol) lyase	*Triticum aestivum*	*Nicotiana tabacum*	Hydrogen sulfide	Youssefian et al., 1993
O-acetylserine(thiol) lyase	*Triticum aestivum*	*Nicotiana tabacum*	SO_2	Youssefian et al., 2001
Peroxidases	*Lycopersicon esculentum*	*Nicotiana tabacum.*	Phenol	Sosa Alderete et al., 2009

In coordination with plant selection, microbes could be isolated from leaves of plants identified in plant selection. This is because the pollutants may exert selective pressures to phyllosphere microbial diversity. For example, bacterial communities hosted by *Platanus* × *acerifolia* leaves from different locations of Milan (Italy) were analyzed by high throughput sequencing. The results showed that biodiversity of bacterial communities decreased but hydrocarbon-degrading populations increased along the growing season, which suggest that air contaminants might play an important role in the selection of phyllospheric populations in urban areas (Gandolfi et al., 2017).

A particular attention should be given to endophytic microbes. There are about 300,000 plant species on the earth; each plant could host one or more endophytes (Petrini, 1991; Strobel and Daisy, 2003). Endophytes are resided inside plant tissues and generally have no harmful effects on plants. Endophytic bacteria that colonize leaves could be particularly desirable as they could not be washed away by precipitation. Recent advances in endophyte-assisted remediation have been reviewed (Khan and Doty, 2011; Stepniewska and Kuzniar, 2013; Ijaz et al., 2016; Syranidou et al., 2016). Endophytic *B. cereus* ZQN5 isolated from natural *Zamioculcas zamiifolia* leaves enhanced ethylbenzene removal rate on sterile *Z. zamiifolia* (Toabaita et al., 2016). Microbes could also be isolated from the rhizosphere of plants contaminated by air pollutants as more endophytism occurs in roots (Ijaz et al., 2016). Some of leaf endophytes could be initially established in roots and subsequently transported to shoots. Khaksar et al. (2016a) reported that some microbes isolated from roots can also colonize leaf surfaces. An endophytic strain of *B. cereus* ERBP from roots of *Clitoria ternatea* was able to colonize the leaf surface of *Z. zamifolia*. During a 20-d fumigation with formaldehyde, the inoculation of ERBP did not interfere with the natural shoot endophytic community of *Z. zamiifolia*. ERBP inoculated *Z. zamiifolia* exhibited a significantly higher formaldehyde removal efficiency when compared to the non-inoculated plants.

Microbes, once identified and cultured, could be engineered to improve phylloremediation capacity (**Table 5**). A pTOM toluene-degradation plasmid from *B. cepacia* G4 was introduced into *Bacillus cepacia* L.S.2.4, a natural endophyte from yellow lupine (*Lupinus arboreus*; Barac et al., 2004). After the engineered bacteria were inoculated into aseptic lupine seedlings, the recombinant endophytics degraded 50–70% more toluene and provided much more protection against the phytotoxic effects of toluene than that obtained from soil bacteria (Barac et al., 2004). Horizontal genes can transfer among plant-associated endophytic bacteria in plants. Poplar was inoculated with the yellow lupine endophyte *B. cepacia* VM1468, which contains the pTOM-Bu61 plasmid coding for constitutively expressed toluene degradation (Taghavi et al., 2005). Inoculated plant growth was enhanced in the presence of toluene, and the amount of toluene release via evapotranspiration was also reduced. Although no inoculated strains were detected in the endophytic community, there was horizontal gene transfer of pTOM-Bu61 to different members of the endogenous endophytic community (Taghavi et al., 2005). The TCE-degrading strain *P. putida* W619-TCE also can be engineered

TABLE 5 | Genes from microbes have been demonstrated to be able to remediate pollutants in transgenic microbes.

Gene name	Source	Transgenic organism	Pollutant	References
BphA1	*Burkholderia xenovorans* LB400	*Pseudomonas pseudoalcaligenes*	Aromatic hydrocarbons and pentachlorobenzene	Suenaga et al., 2010
C23O	*Pseudomonas aeruginosa* zI1f4	*Bacillus subtilis*	Phenol	Yang et al., 2012
Camphor monooxygenase and a hybrid dioxygenase	*Pseudomonas putida*	*Alcaligenes*	Pentachloroaniline	Iwakiri et al., 2004
Catechol 2, 3-dioxygenase	*Pseudomonas aeruginosa* SZH16	*Pseudomonas fluorescens* P13	Phenol	Yang et al., 2012
Hemoglobin	*Vitreoscilla* sp.	*Pseudomonas putida*	Benzene, toluene and xylene	Kahraman and Geckil, 2005
Hemoglobin	*Vitreoscilla* sp.	*Xanthomonas maltophilia*	Benzoic acid	Liu et al., 1996
Phenol hydroxylase	*Escherichia coli*	*Pseudomonas putida*	Trichloroethylene	Fujita et al., 1995
Phenol Hydroxylase	*Ralstonia* sp.	*Ralstonia* sp.	Trichloroethylene	Ishida and Nakamura, 2000
Pro U operon	*Escherichia coli*	Microbial consortium	Hydrocarbon	Kapley et al., 1999
Tod and xyl	*Pseudomonas putida*	*Deinococcus radiodurans*	Toluene	Brim et al., 2006
Toluene dioxygenase	*Deinococcus radiodurans*	*Deinococcus radiodurans*	Toluene and Trichloroethylene	Lange et al., 1998
Toluene o-monooxygenase	*Burkholderia cepacia*	*Pseudomonas fluorescens*	Trichloroethylene	Yee et al., 1998
Xyl and lux gene cassette	*Pseudomonas putida*	*Pseudomonas putida*	Xylene	Kong et al., 2005

via horizontal gene transfer in poplar plants (Weyens et al., 2009b).

Efforts on microbe selection should also be placed on the identification of microbes that could remediate PM, SO_2, NO_2, and O_3. As mentioned above, a group of microbes can assimilate SO_2 and NO_2, further research should explore those microbes for effective assimilation of the two pollutants. Thus far, it appears that no information is available regarding microbial remediation of PM and O_3, which may not be the case in the nature. Extensive research should be conducted to determine if nature has offered microbes that can break down PMs and can also biodegrade or biotransform O_3.

Selected microbes could be domesticated by growing them in different cultures varying in pH, carbon source, temperature, and O_2 to identify appropriate culture media and conditions for maximizing their growth. Morphological characterization and internal transcribed spacer rDNA analysis should be conducted to determine their phylogenetic relationships with other microbes. Their ability to biodegrade particular or a group of air pollutants should be evaluated in the laboratory. Microbial characteristics including their utilization of organic compounds, decomposition rate of pollutants, adaptability, competition, and growth rate should be recorded and analyzed. Competitive strains that show promise in bioremediation should be identified. A series of bacterial and filamentous fungal genomes have been sequenced recently. More than hundreds of bacterial and fungal transcriptomic and proteomic datasets are available. With the advent of increasingly sophisticated bioinformatics and genetic manipulation tools, mechanisms underlying the biodegradation or transformation of pollutants by the isolated microbes could be elucidated. This information, in turn, will significantly improve our understanding of the microbes and provide us with molecular bases for manipulation of the microbes for enhancing phylloremediation.

Evaluation of the Compatibility between Plant Leaves and Microbes

Plants selected from the four categories should be inoculated with selected microbes to determine the compatibility of each selected microbe with each selected plant species. The test could begin first in laboratory settings using entire leaves in designated chambers or utilizing young seedlings in relative large growth chambers to evaluate if inoculated microbes could grow on leaf surfaces and if the specific inoculation affects plant growth. Compatible combinations would be exposed to pollutants at different concentrations and durations to determine the potential for pollutant reduction. A microbe that is compatible with one plant species may not be compatible with another. For example, *B. cereus* ERBP isolated from roots of *C. ternatea* was compatible with the leaf surface of *Z. zamifolia* but not with the leaf surface of *Euphorbia milii*. ERBP-colonized *Z. zamifolia* grew well and showed high efficiency in removal of formaldehyde, but ERBP-colonized *E. milii* were less effective in removal formaldehyde and the plants exhibited stress symptom (Khaksar et al., 2016a). Laboratory evaluation will generate a large number of plant-microbe combinations that are specifically effective in removal of a particular pollutant or a particular group of pollutants. Bacteria would be propagated using bioreactors and corresponding plants would be propagated through either cuttings or tissue culture. The plants would be transplanted into greenhouses or specific regions with air pollution for testing the effectiveness of the combinations in real-world situations.

Plants and microbe combinations that pass the real-world test will be investigated using the next-generation sequencing (NGS) technologies (metagenomics, metatranscriptomics, metaproteomics, and metabolomics) and the rapid evolution of SIP (Stable isotope probing) for identifying molecular mechanisms underlying microbial and plant interactions in facilitation of phylloremediation. The compatibility evaluation and molecular analysis would ultimately result

in the development of protocols for culturing microbes and producing corresponding plants. Some protocols will be catered to trees, others used for shrubs or small trees. Some would be effective for improving groundcover plants, and some will be used for indoor foliage plants. Effectiveness of each protocol in remediation of particular or general pollutants would be determined using the model described by Nowak et al. (2006). If the test is to be conducted in a large scale, satellite image acquisition and analysis should be used. The analysis of the data will finally validate the protocols, i.e., particular plants can be inoculated with a specific group of microbes for use in remediation of a particular pollutant or a mixture of pollutants.

Implementation of Phylloremediation Technologies

The protocols will be implemented for phylloremediation. We propose three types of plantscape: (1) manufactory plantscape, (2) urban plantscape, and (3) interior plantscape. The plantscape for manufactories and cities should have three levels of greening: the sky with trees, the ground with groundcover plants, and shrubs in between. Additionally, climber plants can be used to build green walls and small trees and shrubs as well as groundcovers can be used to build green roofs. For interior plantscape, each room should have a minimum of one potted foliage plant. Foliage plants can also be used to install green walls in interior environments for enhance remediation of indoor air pollutants.

The implementation of phylloremediation technologies should also take landscape design concepts into consideration, resulting greenbelts, green parks, green walls that fulfill roles not only for air remediation but also for recreation. Depending on the occurrence of pollutants and the scale and degree of the overall pollution, relevant protocols to the particular situations would be implemented. The remediation efficiency could be monitored over time using specific models in connection with satellite imagine data to determine how much of individual pollutants have been removed.

CONCLUSION

Air pollution is real, and it is adversely affecting human comfort and health and jeopardizing the ecosystem. The causes are multidimensional including increased population, urbanization, and industrialization accompanied with increased energy consumption and economic growth along with weak regulation, deforestation, and climate change. A recent article published by Cai et al. (2017) suggested that circulation changes including the weakening of the East Asia winter monsoon induced by global greenhouse gas emission contribute to the increased frequency and persistence of the haze weather conditions in Beijing, China. This claim could be true. The fact is that air pollutants released anthropogenically has caused the global warming. Our attention nevertheless should focus on how to control the emissions and how to remediate the pollutants. Although rhizosphere (roots and root associated microbes) contributes greatly to remediation of air pollutants, in this review, we specifically discuss phylloremediation. The role of plant leaves and leaf-associated microbes in remediation of air pollutants has not been well explored. Using the Urban Forest Effects Model, Yang et al. (2005) studied the influence of the urban forest on air quality in Beijing, China and found that the 2.4 million trees in the central part of Beijing removed 1,261.4 tons of pollutants from the air in 2002, of which 720 tons were PM. Nowak et al. (2014) has shown that computer simulations with local environmental data reveal that trees and forests in the contiguous US removed 17.4 million tons (t) of air pollution in 2010, with human health effects valued at 6.8 billion US dollars. Such forest-aided remediation might have avoided more than 850 incidences of human mortality and 670,000 incidences of acute respiratory problems.

We believe that phylloremediation is an environmentally friendly, cost effective way of remediation of air pollutants. The key component of this technology lies in plants. It is plants that can adsorb or absorb pollutants and plants that support microbes in biodegradation or biotransformation of pollutants. To develop phylloremediation technologies, some basic questions should be addressed: (1) Anatomical, physiological, biochemical and molecular mechanisms underlying plant responses to each pollutant should be investigated. Previous research has documented plant responses to pollutants such as NO_x, SO_2, O_3, and VOCs, but the research was largely intended to identify how plants were injured. We need to exploit why many plants are tolerant to the pollutants, what are the underling mechanisms, and how can we manipulate the mechanisms for increased tolerance and for use in phylloremediation. There is little information regarding plant responses to PM. Do plants simply adsorb PM? What are the fates of stomatal absorbed PM? (2) Phyllosphere microbes are still largely a mystery and many are not culturable. Methods for collection, identification, and cultivation should be developed. Some microbes isolated from the rhizosphere can also be used for leaf colonization. Mechanisms for biodegradation and transformation of pollutants have been mentioned in this review. However, we still do not know if there are microbes that can remediate PM and O_3. An important question that should be immediately addressed is the roles of microbes within the PM. Do the microbes become active once settled on leaves? Do they have the ability to break down the PM? With the advances of omics, these questions will be answered, and new strains with high efficiency in breaking down pollutants are expected to be isolated and utilized. (3) A large scale and intensive test for the compatibility among identified plants and identified microbes should be carried out. Specific plant-microbe groups or combinations that can effectively reduce one or more pollutants should be identified, tested, and confirmed in real-world situations and corresponding protocols for using each combination should developed. (4) New methods for analyzing dynamic changes of air pollutants in the atmosphere should be developed and standardized for monitoring the effectiveness of the phyllosphere technologies. (5) Research and development of phyllosphere technologies is a multidisciplinary project requiring collaboration among researchers with different academic backgrounds at regional, national, and international levels. Nature has offered healthy

alternatives for remediation of air pollution; we should collaborate with nature as a partner to restore nature's identity.

AUTHOR CONTRIBUTIONS

All authors contributed to the acquisition and interpretation of available literature and the conception of the work. JC, SL, and XW wrote the manuscript, and all authors reviewed and revised the manuscript and approved this final version. XW and SL contributed equally to this work.

ACKNOWLEDGMENTS

The authors would like to thank the Fujian Science and Technology Key Projects (2013NZ0002-1B) for "Construction of High-level University program of Fujian Agriculture and Forestry University" and for "Construction of High-level Horticulture Science Discipline" (612014007) for supporting this study. The appreciation also extends to Mr. He Hong at College of Art and Landscape Architecture, Fujian Agriculture and Forestry University for assistance in preparation of **Figure 1**.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 19 December 2017
doi: 10.3389/fmicb.2017.02538

The Effects of the Endophytic Bacterium *Pseudomonas fluorescens* Sasm05 and IAA on the Plant Growth and Cadmium Uptake of *Sedum alfredii* Hance

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Edited by:
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Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Microbiology

Received: *31 March 2017*
Accepted: *06 December 2017*
Published: *19 December 2017*

Citation:
Chen B, Luo S, Wu Y, Ye J, Wang Q, Xu X, Pan F, Khan KY, Feng Y and Yang X (2017) The Effects of the Endophytic Bacterium Pseudomonas fluorescens Sasm05 and IAA on the Plant Growth and Cadmium Uptake of Sedum alfredii Hance. Front. Microbiol. 8:2538. doi: 10.3389/fmicb.2017.02538

Endophytic bacteria have received attention for their ability to promote plant growth and enhance phytoremediation, which may be attributed to their ability to produce indole-3-acetic acid (IAA). As a signal molecular, IAA plays a key role on the interaction of plant and its endomicrobes. However, the different effects that endophytic bacteria and IAA may have on plant growth and heavy metal uptake is not clear. In this study, the endophytic bacterium *Pseudomonas fluorescens* Sasm05 was isolated from the stem of the zinc (Zn)/cadmium (Cd) hyperaccumulator *Sedum alfredii* Hance. The effects of Sasm05 and exogenous IAA on plant growth, leaf chlorophyll concentration, leaf Mg^{2+}-ATPase and Ca^{2+}-ATPase activity, cadmium (Cd) uptake and accumulation as well as the expression of metal transporter genes were compared in a hydroponic experiment with 10 μM Cd. The results showed that after treatment with 1 μM IAA, the shoot biomass and chlorophyll concentration increased significantly, but the Cd uptake and accumulation by the plant was not obviously affected. Sasm05 inoculation dramatically increased plant biomass, Cd concentration, shoot chlorophyll concentration and enzyme activities, largely improved the relative expression of the three metal transporter families ZRT/IRT-like protein (ZIP), natural resistance associated macrophage protein (NRAMP) and heavy metal ATPase (HMA). Sasm05 stimulated the expression of the *SaHMAs* (*SaHMA2*, *SaHMA3,* and *SaHMA4*), which enhanced Cd root to shoot translocation, and upregulated *SaZIP,* especially *SaIRT1,* expression to increase Cd uptake. These results showed that although both exogenous IAA and Sasm05 inoculation can improve plant growth and photosynthesis, Sasm05 inoculation has a greater effect on Cd uptake and translocation, indicating that this endophytic bacterium might not only produce IAA to promote plant growth under Cd stress but also directly regulate the expression of putative key Cd uptake and transport genes to enhance Cd accumulation of plant.

Keywords: hyperaccumulator, gene expression, metal transporter, plant growth-promoting bacteria (PGPB), Cd

INTRODUCTION

Plant growth-promoting bacteria (PGPB) have recently attracted wide attention, because PGPB can effectively increase the plant biomass and the efficiency of heavy metal phytoextraction (Rajkumar et al., 2009; Glick, 2010, 2012, 2014; Bashan et al., 2013; Ma et al., 2016; Santoyo et al., 2016). Many studies have shown that the supplementary effects of PGPB on heavy metal phytoextraction were related to their capacity to promote plant growth, the production of indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, siderophores, antibiotics and phosphorus (P) solubilization (Penrose and Glick, 2003; Khalid et al., 2004; Hynes et al., 2008; Glick, 2010, 2012, 2014; Bashan et al., 2013). For instance, *Pseudomonas azotoformans* ASS1 could protect plants against abiotic stresses and help plants to thrive in semiarid ecosystems, accelerate the phytoremediation process in metal-polluted soils, and significantly enhance the chlorophyll content and improve the accumulation, bio-concentration factor and biological accumulation coefficient of metals (Ma et al., 2017).

As a phytohormone, IAA is known to be involved in root imitation, cell division and cell enlargement (Teale et al., 2006). It can not only found in plants but also reported to be synthesized in microorganisms (Kazan, 2013). The inoculation of IAA-producing endophytic bacteria has been demonstrated as a promising way to enhance plant biomass, root length, root tip number and root surface area (Chen et al., 2014a; Ali et al., 2017). The regulation of IAA secreted by PGPB and the plant is considered to be an important cause of growth promotion. For example, the bacterial endophyte *Sphingomonas* sp. LK11 isolated from the leaf of *Tephrosia apollinea*, which produces IAA (11.23 ± 0.93 μM mL^{-1}) and gibberellins, promoted the growth of tomato (Khan et al., 2014). IAA produced by PGPB might play an extremely important role as a growth regulating substance that drives root hair and cotyledon cell expansion during seedling development (Strader et al., 2010). And even though the concentration of IAA production from various endophytic bacteria are different, IAA synthesis in both plant and microbe were affected by their interaction (Jasim et al., 2014). For instance, an average of 35 μg mL^{-1} IAA was produced in the five endophytic bacteria isolated from *Piper nigrum*, the yield of IAA was drastically increased around 20-fold to 869 μg mL^{-1} which can be due to the induction of the endophytic IAA biosynthetic pathway by the host plant metabolites (Jasim et al., 2014). Additionally, several reports showed that endophytes may also alter plant auxin synthesis (Kazan, 2013). And the cross-border regulation of microbes and their products on the plant IAA signal system is considered as the main mechanism that promotes lateral root development and relieves plant stress (Glick, 2012; Duca et al., 2014). Recent results indicated that IAA-overproducing endophytes had shown many transcriptional changes naturally occurring in nitrogen-fixing root nodule (Defez et al., 2016) and the high expression of *nifH* gene coding for the nitrogenase iron protein, moreover, they could increase nitrogenase activity of rice (Defez et al., 2017). Except for growth promoting, IAA can also affect plant heavy metal uptake and translocation. In Arabidopsis, exogenous auxin could enhance Cd^{2+} fixation in the root cell wall, decrease Cd^{2+} translocation from root to shoot thus to alleviate Cd toxicity (Zhu et al., 2013). Therefore, the IAA producing trait of endophytic bacteria may have multiple consequences in plant–microbe interaction.

Many studies have demonstrated that plants take up Cd primarily by the iron (Fe), zinc (Zn), manganese (Mn), and calcium (Ca) pathways in the roots (Clemens et al., 2013). IRT1 is metal ion transporter with a broad substrate range localized in the plasma membrane in *Arabidopsis thaliana* that can transport Fe, Zn, Mn, Cd, and cobalt (Co) (Korshunova et al., 1999). IRT1 is expressed in the plasma membrane of root epidermal cells (Vert, 2002) and is likely to be involved in Cd uptake. Other metal transporters of the ZRT-IRT-like Protein (ZIP) family (e.g., AtZIP1 and AtZIP2) have been indicated to play a role in Zn and Mn uptake (Milner et al., 2013). In *Arabidopsis thaliana*, P_{1B}-type ATPases are known as Heavy Metal ATPase (HMA) and are the major transporters for root-to-shoot Cd translocation (Wong and Cobbett, 2009). In the HMA family, HvHMA2 is a plasma membrane P1B-ATPase from barley that functions in Zn/Cd root-to-shoot transport (Barabasz et al., 2013), AtHMA3 participates in the vacuolar storage of Cd (Morel et al., 2009), and AtHMA4 encodes the export protein responsible for loading Zn and Cd into the xylem vessels, thus controlling root to shoot translocation in Arabidopsis (Wong and Cobbett, 2009). We recently isolated and functionally characterized a tonoplast-localized SaHMA3 gene from *Sedum alfredii* Hance (*S. alfredii*), which had a significantly higher constitutive expression level. SaHMA3 is a Cd-specific transporter with the ability to transport both Cd and Zn (Zhang et al., 2016). Similar results were also obtained in *S. plumbizincicola* (Liu et al., 2017). In addition to ZIP and the HMA family, the Natural Resistance Associated Macrophage Protein (NRAMP) family play an important role in regulating metal ion transport (Sasaki et al., 2012). The plasma membrane-localized OsNramp5 is a major transporter for Cd uptake in rice (Ishimaru et al., 2012; Sasaki et al., 2012). AtNramp3 and AtNramp4 are located in vascular tissues and are both related to the mobilization of vacuolar Cd (Thomine et al., 2003; Lanquar et al., 2010). In Arabidopsis, *Nramp1, 3, 4,* and *6* were shown to confer Cd sensitivity in yeast (Thomine et al., 2000; Cailliatte et al., 2009).

Recent studies showed that PGPB could also regulate host plant gene expression. Defez et al. (2017) showed that the inoculation of IAA-overproducing endophytes could significantly up-regulate nitrogenase activity. *Bacillus altitudinis* WR10 could up-regulate the expression of many genes encoding ferritins, which alleviated iron deficiency in plants (Sun et al., 2017). Our results also showed that the inoculation of the IAA-producing endophytic bacteria SaMR12 can not only increase the leaf chlorophyll content as well as the iron and magnesium uptake but also regulate the expression of the three above mentioned heavy metal transporter genes in *S. alferdii* (Pan et al., 2017). However, few studies were compared the effects of PGPB and exogenous IAA on metal transporter gene expression.

S. alfredii is a native of China and a Zn/Cd hyperaccumulator. Based on the data from RNA-seq (Gao et al., 2013), we cloned a series of transporter genes (Zhang et al., 2016), but

the mechanism of Cd hyperaccumulation in this plant is still not fully understood. Many endophytic bacteria have been isolated (Zhang et al., 2012) and they might also contribute to Cd hyperaccumulation in plants. In the present study, we investigated (1) the effects of the endophytic bacterium Sasm05 on plant growth and Cd uptake; (2) the effects of IAA and its transport inhibitor naphthylphthalamic acid (NPA) on plant growth and Cd uptake; and (3) the effects of Sasm05, IAA and NPA on the expression of selected transporter genes, to elucidate the functional contribution of IAA to plant–endophytic bacteria interactions.

MATERIALS AND METHODS

Plant Materials and Endophytic Bacterium Sasm05

Plant Materials

The plant *S. alfredii* was collected from an old Pb/Zn mined site in Quzhou city, Zhejiang Province of China (Yang et al., 2004), and healthy and uniform shoots were selected and cultured hydroponically. After growth for 12 days in distilled water, the plants were subjected to 4 days exposure to one-half strength Hoagland nutrient solution, continuously aerated and renewed every 3 days. Plants were grown in an environmentally controlled growth chamber with a temperature range of 23–26°C, relative humidity 60% and light intensity of 180 μM m^{-2} s^{-1} during a 14/10 h day/night duration. Single shoot tips were excised and grown as described above for another generation to remove the heavy metals.

Isolation and Identification of Sasm05

Healthy plants of *S. alfredii* together with soil were collected from six different points of the mined site, put into a sterile bag and sealed. After come back to the lab, the bacteria were isolated immediately and the other samples were stored at 4°C. The whole plant was washed with tap water for 30 min, and the roots, rotten leaves as well as diseased tissues were removed, and thus only the green healthy parts were preserved. Then the whole process was carried out in a super clean bench. First the plant tissue was washed with distilled water at least three times, 3 min each time. Later the washed tissue was immersed in 75% ethanol to maintain 3 min, sterile water washed 3 times, and then soaked in of 3% NaOCl (Cl^- concentration) for 3 min, sterile water washed for five times. The obtained surface sterile tissues were placed on the sterilized filter paper, and the excess water was absorbed. The stems were sliced into thin slices and laid on the solid culture dish containing 20 mL Petri plates of Luria–Bertani's (LB) medium. The sealed film was used to seal the culture dish and placed in 30°C for dark culture. In order to verify the effectiveness of the *in vitro* sterilization process, 200 μL washed water of the last time was evenly coated on the LB solid medium, and no colony growth treatment was used as effective surface sterilization. The colonies on the plant tissue were picked out with inoculation needle, purified in LB solid medium, and cultured at 30°C for 3 days, then the monoclonal strain was obtained (Supplementary Materials). A single colony was placed on a LB solid medium, 3 replicates per plant, cultured for 48 h and stored at 4°C for further analysis.

After purification and pathogenicity identification, single clone of candidate endophytic bacteria were selected and inoculated to 10 mL liquid LB medium, cultured at 37°C overnight. 1 mL bacteria suspension were put into a 1.5 mL centrifuge tube, 12000 rpm, 3 min. Then the precipitate were collected and washed by sterile water for two times. The genomic DNA was extracted with a rapid bacterial genomic DNA isolation kit (Sangon Biotech, China). Universal primers for bacteria were used for polymerase chain reaction (PCR) amplification; the forward primer was (27f: 5′-AGAGTTTGATCCTGGCTCAG-3′) and the reverse primer was (1492r: 5′-GGTTACCTTGTTACGACTT-3′). The amplified DNA was purified with a DNA purification kit (Sangon Biotech) and sequencing was performed at the Huada biotechnology company (Guangzhou, China). The 16S rDNA sequence was compared with sequences in the GenBank database using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool - nucleotide (BLASTn) program[1] (Weisburg et al., 1991). The strain was named Sasm05 and has been preserved in the China General Microbiological Culture Collection Center with preservation number is CGMCC 12173 (Supplementary Materials).

The ability of bacteria to produce siderophore and ACC deaminase as well as heavy metal resistance were investigated according to Sheng et al. (2008). And IAA production and phosphate solubilizing enzyme were determined according to Tiwari et al. (2016).

GFP Labeling and Colonization of Sasm05

The green fluorescent protein (GFP) was used to label Sasm05 according to Zhang et al. (2013). Sasm05 was inoculated into 250 mL Erlenmeyer flasks containing 150 mL sterilized LB liquid medium and cultivated aerobically in an orbital rotary shaker (200 rpm) at 30°C for 24 h. The cells were collected by centrifugation, washed three times with 0.85% sterile saline, and resuspended to an OD = 1.0. Uniform plant seedlings were selected, and the roots were immersed in the labeled Sasm05 suspension for 2 h, then transferred to 10 μM Cd-containing Hoagland medium. The roots were imaged by Laser Scanning Confocal Microscopy (LSCM) 6 d after inoculation.

Experiment Design and Analysis

After 4 weeks of preculture, the roots of uniform young plants were immersed in each treatment for 4 h: (1) Control treatment (sterilized deionized water), (2) Sasm05 (10^7 CFU /mL), (3) Sasm05 and 10 μM IAA transport inhibitor (NPA), (4) 1 μM IAA, (5) 1 μM IAA and 10 μM NPA, and (6) 10 μM NPA, then exposed to 10 μM Cd Hoagland nutrient solution with (treatments 3, 5, and 6) or without NPA (treatments 1, 2, and 4). 4 plants were transferred into a 2.5 L black plastic bucket as one treatment, and each treatment was repeated six times.

[1] http://blast.ncbi.nlm.nih.gov

Plant Harvest and Weights

The hydroponic plant cultures were harvested after 7 days for gene expression analysis or 30 days for Cd uptake analysis, and the shoots and roots were washed with deionized water. The fresh weights (FW) and the dry weights (DW) of the roots and shoots were recorded before and after oven-drying at 65°C for 48 h.

Cd Concentration Determination

The plant roots were soaked in 20 mM Na_2-EDTA for 15 min to remove the Cd ions adhering to the root surfaces. The shoots and roots were dried and powered as much as possible. A final sample of 0.1 g was digested by adding 5 mL HNO_3 and 1 mL $HClO_4$ in a boiled Polytetrachloroethylene cup at 180°C for 8 h. The digested samples were diluted with deionized water, filtered through a filter membrane (13 mm, 0.22 μm), and the Cd concentration was determined using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent 7500a, United States).

Chlorophyll Concentration Determination

Acetone and ethyl alcohol were mixed in a ratio of 2:1, fresh leaf (without veins and stalks) samples (0.20 g each) were placed into this mixture (20 mL) in the dark for 24 h before the extracts were measured for the absorbance at wavelengths of 663 and 645 nm using an ultraviolet spectrophotometer (Lambda 350V-vis, PerkinElmer, Singapore) (Chen et al., 2014b). The Chl concentration (g kg^{-1}) was calculated using the following equations: Total Chl = [8.02 × A663] + [20.21 × A645].

Enzyme Activity Determination

For Ca^{2+}-ATPase, 3 mL of total reaction mixture containing 0.3 mL of crude mitochondrial extract, 30 mmol L^{-1} Tris-HCl buffer (pH 8.0), 3 mmol L^{-1} Mg_2SO_4, 0.1 mmol L^{-1} Na_3VO_4, 50 mmol L^{-1} $NaNO_3$, 3 mmol L^{-1} $Ca(NO_3)_2$ and 0.1 mmol L^{-1} ammonium molybdate was employed. The reaction was initiated by the addition of 100 μL of 30 mmol L^{-1} trichloroacetic acid after 20 min of incubation at 37°C. One unit of Ca^{2+}-ATPase activity was defined as the release of 1 mol of phosphorus in absorbance per minute at 660 nm under the assay conditions (Jin et al., 2013).

For Mg^{2+}-ATPase, a reaction mixture containing 50 mM Tris-HCl, pH 8.8, 33% methanol, 4 mM ATP, 4 mM $MgCl_2$ and thylakoids at 30 μg Chl/mL in total volume of 1.0 mL was employed. After incubation at 37°C for 2 min, the reaction was terminated by the addition of 0.1 ml of 20% TCA, then the release of Pi was determined (Ren et al., 1995).

Relative Gene Expression Analysis

Shoot and root tissues were collected after 7 days of treatment and immediately frozen in liquid nitrogen prior to total RNA extraction. Total RNA was isolated using TRIzol (Invitrogen). The first-strand cDNA was synthesized with a 10 μL reaction system according to the instructions for the TAKARA PrimeScript RT Reagent Kit (Perfect for Real Time) (TaKaRa Biotechnology, Dalian, China). For a real-time RT-PCR analysis, 1 μL 10-fold-diluted cDNA was used for the quantitative analysis of gene expression performed with SYBR PremixExTaq (Takara), and the pairs of gene-specific primers used were the same as in Pan et al. (2017). The expression data were normalized to the expression of actin (forward: 5′-TGTGCTTTCCCTCTATGCC-3′; reverse: 5′-CGCTCAGCAGTGGTTGTG-3′).

A Mastercycler ep realplex2 Real Time PCR machine (Eppendorf, Hamburg, Germany) with the default program (2 min at 50°C and 10 min at 95°C followed by 40 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s.) were employed for quantitative RT-PCR analysis with a reaction mixture volume of 20 μL in an optical 96-well plate. A control was also included in each plate with 2 μL of RNase-free water as a template. Three technical replicates were contained in each plate. Specificity verification of the PCR amplification dissociation and the PCR efficiency curves were determined for each candidate reference gene prior to the quantitative RT-PCR evaluation of these genes in *S. alfredii*. The relative quantification analysis was performed using the comparative ΔΔCt method and the formula was: Fold induction = $2^{-\Delta\Delta C_T}$, as described by Winer et al. (1999). To evaluate the gene expression level, the results were normalized using Ct values obtained from actin cDNA amplifications run on the same plate.

Statistical Analysis

Three replicates were used and analyzed independently for each treatment. The data was analyzed using OriginPro 8, and it was analyzed statistically by a one-way analysis of variance (ANOVA). Significantly different means were indicated by Fisher's least significant difference (LSD) test and Duncan's multiple range test at the $P < 0.05$ level.

RESULTS

Sasm05 Isolation, *gfp*-Tagging and Its Colonization

Sasm05, an endophytic bacterium isolated from surface sterilized stems of *S. alfredii*, was identified as a gram-negative bacterium and a *Pseudomonas fluorescens* by 16S rRNA array. It could utilize tryptophane for growing and produce IAA (15–50 μg mL^{-1}). It also could utilize ACC as the sole nitrogen source and show relatively high levels of ACC deaminase activity (**Table 1**).

Sasm05 was successfully tagged by GFP (**Figure 1A**). Laser scanning confocal microscopy (LSCM) showed that under 10 μM Cd treatment, *gfp*-tagged Sasm05 colonized the root surface (**Figure 1B**) and evenly distributed in the plant stem as shown by the cross-section profile of the stem (**Figure 1C**).

Effect of IAA and Sasm05 on the Plant Growth

Plants were exposed to 10 μM Cd for 30 days and no visible phytotoxicity of Cd was observed. The shoot and root biomass (expressed as the fresh weight) were significantly ($p < 0.05$) enhanced by 20% and 45% by Sasm05 inoculation (**Figure 2**). IAA treatment could increase the shoot biomass by 19% but had no obvious effect on root biomass (**Figure 2**). However, the plant biomass was significantly inhibited by 64% and 70% ($p < 0.05$)

in the 10 μM NPA treatment, and IAA or Sasm05 could alleviate this inhibition to some extent (**Figure 2**).

Effect of IAA and Sasm05 on Cd Concentration of Plant

After inoculation with Sasm05, the Cd concentration in *S. alfredii* was significantly increased by 19% in the shoots and 59% in the roots, and even with an additional NPA treatment, the Cd concentration was still increased by 13% in the shoots and 37% in the roots (**Figure 3**). However, IAA treatment could not increase the Cd concentration compared to the control either whether NPA was added or not. In the NPA treatment, the Cd concentration was significantly decreased by 17% in the shoots and no changes were observed in the roots.

Cd accumulation in *S. alfredii* was significantly increased by 60% in the shoots and 46% in the roots with Sasm05 treatment (**Figure 4**). In the shoots, IAA or a combined NPA treatment showed no effect on the Cd accumulation, while it decreased by 60% when treated with NPA alone. In the roots, Sasm05 combined with NPA could increase Cd accumulation by 21%, while IAA combined with NPA or treated with NPA alone decreased the Cd accumulation by 17 and 25%, respectively.

Effect of IAA and Sasm05 on Leaf Chlorophyll Concentration

Both Sasm05 and IAA had a positive effect on the content of chlorophyll ($P < 0.05$), which was increased by 24–44 and 20% with Sasm05 and IAA treatments, respectively (**Figure 5**). Combined with NPA treatment, Sasm05 showed a higher chlorophyll concentration (18%) than when combined with an IAA treatment. Treatment with NPA alone resulted in a reduction of the chlorophyll concentration by 40%, indicating that NPA can inhibit the synthesis of chlorophyll (**Figure 5**).

Effect of IAA and Sasm05 on Leaf Mg^{2+}-ATPase and Ca^{2+}-ATPase

ATPases are the main transport proteins and energy source. Ca^{2+}-ATPase was often affected by changes in the growth environment. In this experiment, both Sasm05 and IAA treatment could significantly increase the activity of Ca^{2+}-ATPase by 32% and 37%, and IAA treatment was better than Sasm05 inoculation. All the NPA treated plants had lower Ca^{2+}-ATPase levels than the control, which were decreased by 22% when combined with Sasm05, by 27% when combined with IAA, and by 38% when treated with NPA alone (**Figure 6**).

The Mg^{2+}-ATPase activity was significantly enhanced by 47% and 22% in the Sasm05 and Sasm05 + NPA treatments, respectively (**Figure 6**). The biological activity of the Mg^{2+}-ATPase was also enhanced by 46% by IAA, but it was significantly decreased by 33% in the IAA + NPA treatment (**Figure 6**).

TABLE 1 | Characteristics of the endophytic bacterium *Pseudomonas fluorescens* Sasm05.

Strain	Data
Bacteria name	Sasm05
Genus affiliation	*Pseudomonas*
Source	Stem
16S rRNA gene accession number	NC_007492.2
Heavy metal resistance (mg L^{-1})	$Pb^{2.5}$, Zn^{30}, $Cd^{2.0}$
IAA-production	+
ACCase production	+
Phosphate solubilization	−
Siderophore production	+
Cellulase activity	−
Pectinase activity	−
Antibiotics (μg mL^{-1})	$Amp^{r(100)}$

The Relative Expression Levels of Metal Transporter Genes

The quantitative RT-PCR results revealed that the transcripts of these metal transporter genes were highly induced by Sasm05, and the *IRT1* transcript levels in the Sasm05 treatment in the shoots and the roots were 17-fold and 9-fold higher, respectively (**Figure 7K**). After inoculation with Sasm05, the expression of *SaHMAs* (*SaHMA2*, *SaHMA3* and *SaHMA4*), *SaNramps* (*SaNramp1*, *SaNramp3*, *SaNramp6*), and *SaZIPs* (*SaZIP2*, *SaZIP3*, *SaZIP4* and *SaZIP11*) in the shoots and *SaHMAs* (*SaHMA2* and *SaHMA3*), *SaNramps* (*SaNramp3* and *SaNramp6*), as well as *SaZIPs* (*SaZIP2*, *SaZIP4* and *SaZIP11*) in the roots was significantly increased (**Figures 7A–K**). IAA treatment had no obvious effect on most genes, but it also increased the expression of *SaNramp3*, *SaNramp6* and *SaZIP4* in the shoots and *SaNramps* (*SaNramp1*, *SaNramp6*), *SaZIPs* (*SaZIP4*, *SaZIP11* and *SaIRT1*) in the roots (**Figures 7A–K**). On the contrary, the expression levels of *SaHMAs* (*SaHMA2* and *SaHMA4*), *SaNramps* (*SaNramp1*, *SaNramp3* and *SaNramp6*), *SaZIPs* (*SaZIP3*, *SaZIP4,* and *SaZIP11*) in the shoots and *SaHMAs* (*SaHMA2*, *SaHMA3* and *SaHMA4*), *SaNramps* (*SaNramp1* and *SaNramp3*), *SaZIPs* (*SaZIP2*, *SaZIP11*, and *SaIRT1*) in the roots were reduced by the addition of NPA (**Figures 7A–K**). However, the addition of NPA and IAA increased the expression levels of *SaHMAs* (*SaHMA2* and *SaHMA4*), *SaNramps* (*SaNramp1*, *SaNramp3,* and *SaNramp6*), *SaZIP4* in the shoots and *SaHMAs* (*SaHMA3* and *SaHMA4*), *SaNramps* (*SaNramp1*, *SaNramp3* and *SaNramp6*), *SaZIPs* (*SaZIP4*, *SaZIP11*, *SaIRT1*) in the roots (**Figures 7A–K**).

DISCUSSION

The Plant Growth-Promoting Effects of PGPB and IAA

Previous studies showed that endophyte inoculation improved plant biomass and root growth (Zhang et al., 2013; Chen et al., 2014a,b; Cocozza et al., 2014). In the hydroponic experiment, both Sasm05 and IAA could promote plant growth and enhanced shoot biomass. IAA is a regulator known to stimulate both rapid (e.g., increases in cell elongation) and long-term (e.g., cell division and differentiation) responses in

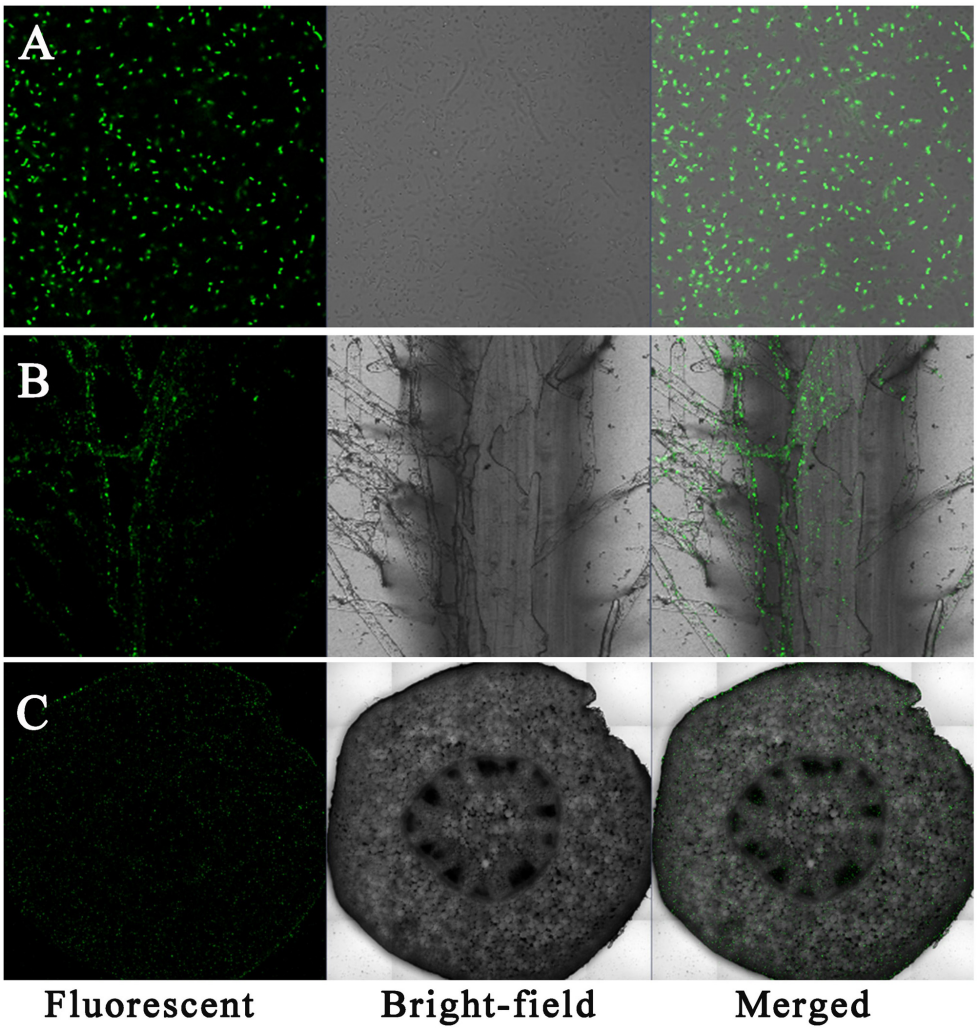

FIGURE 1 | Colonization of *gfp*-tagged Sasm05 in plant. **(A)** *gfp*-tagged Sasm05 strains on root surface, **(B)** colonized in the roots and **(C)** colonized in the stems. LSCM images show bacterial GFP fluorescence in green. Bars = 25 μM.

plants (Shi et al., 2009). ACC deaminase can lower plant ethylene levels and thus stimulate plant growth (Glick et al., 2007). Furthermore, siderophores are able to bind metals, improving plant growth and enhancing phytoremediation processes (Rajkumar et al., 2010). Therefore, it was reasonable that Sasm05 treatment showed greater improvement in plant growth and root biomass compared to the exogenous IAA treatment.

As a transport inhibitor of IAA, NPA significantly decreased the root IAA concentration while it increased the shoot IAA concentration (Gong et al., 2014). Ljung et al. (2001) conducted an experiment in which 40 mM NPA was added to the medium, demonstrating that NPA decreased the amount of IAA and leaf expansion probably by feedback inhibition of IAA biosynthesis. Root length and lateral root development was significantly inhibited after the application of 10 μM NPA compared with the absence of NPA (Casimiro et al., 2001). Further study revealed that the role of NPA is not only related to auxin transport but also closely related to the content of ethylene; the inhibitory effect of NPA on the growth of plant roots may be related to an increase in the ethylene content (Rashotte et al., 2000; Keller et al., 2004; Ruzicka et al., 2007). Here, the root biomass was significantly reduced by NPA, which was consistent with the discovery of Barkoulas et al. (2008), for it might have a significant inhibitory effect on the plant lateral roots (Gong et al., 2014). NPA treatment prevents the lateral roots from penetrating the hypodermis due to the hardening of hypodermis cell walls and the disturbance of gravitropism (Ogawa et al., 2017).

Effect of Sasm05 and IAA on Mg^{2+}-ATPase and Ca^{2+}-ATPase

Indole-3-acetic acid is the most common, naturally occurring plant hormone in the auxin class (Marathe et al., 2017). Apart from IAA produced by plants, some bacteria hosted in

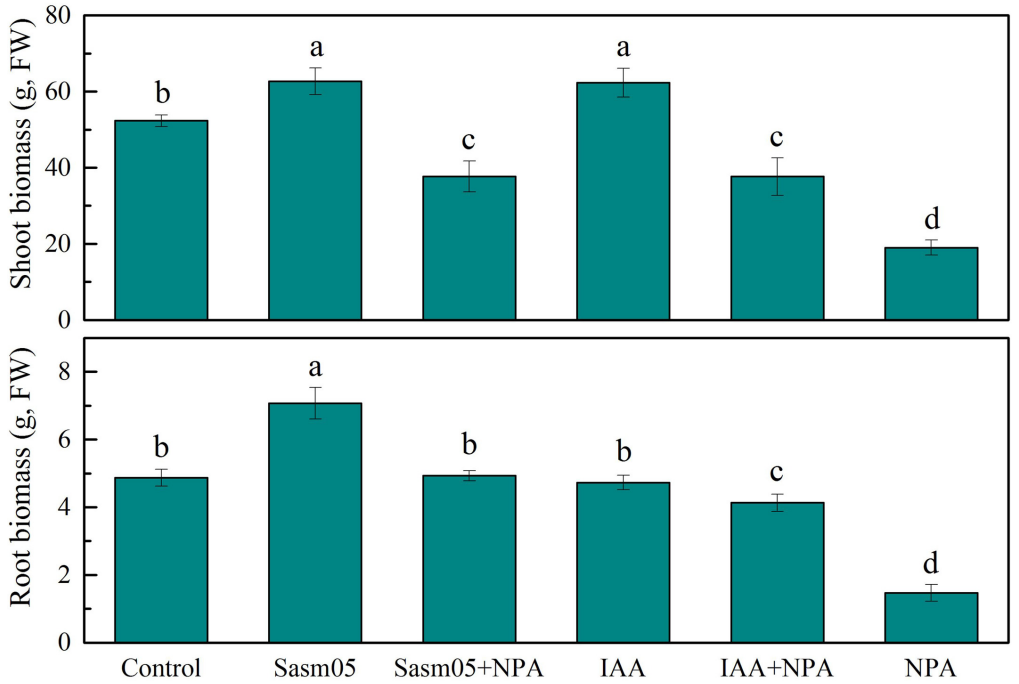

FIGURE 2 | Effects of Sasm05 and IAA on the plant biomass. Error bars represent the standard deviation (SD) of three individual replicates. The different letters on the error bars indicate significant differences among the treatments at $p < 0.05$.

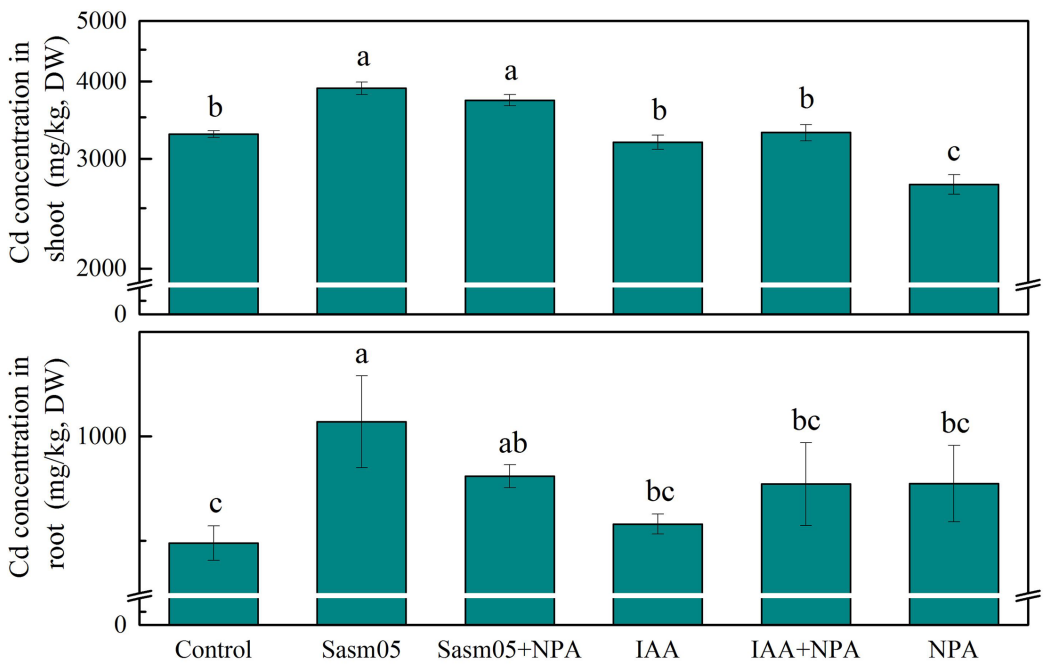

FIGURE 3 | Effects of Sasm05 and IAA on Cd concentration. Error bars represent the standard deviation (SD) from of three individual replicates. The different letters on the error bars indicate significant differences among the treatments at $p < 0.05$.

plant showed the ability to produce IAA and promote plant growth. The IAA produced by *Pseudomonas fluorescens* HP72 is proposed to act as a stimulator of cell proliferation and elongation and enhance the host uptake of minerals and nutrients from the soil (Suzuki et al., 2003). This promotion effect was outstanding when the plant was grown in a stressed environment,

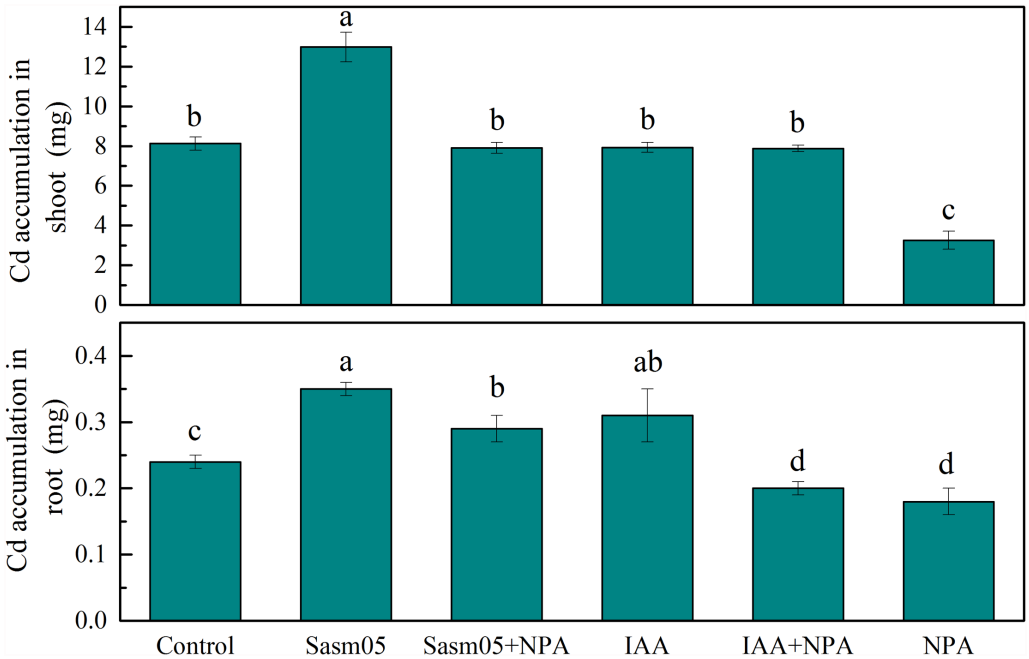

FIGURE 4 | Effects of Sasm05 and IAA on Cd accumulation. Error bars represent the standard deviation (SD) from of three individual replicates. The different letters on the error bars indicate significant differences among the treatments at $p < 0.05$.

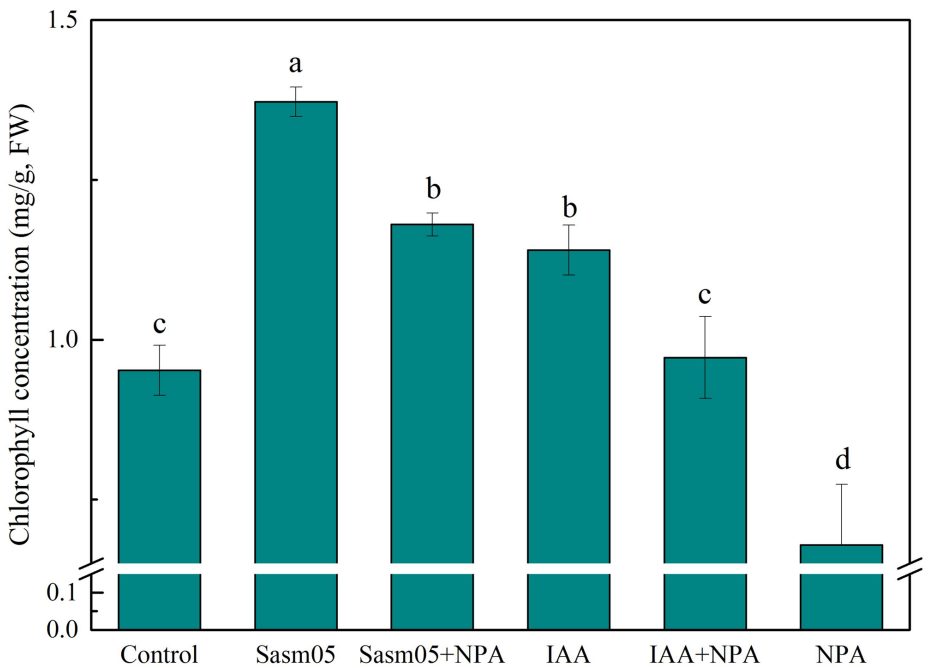

FIGURE 5 | Effects of Sasm05 and IAA on plant chlorophyll concentration. Error bars represent the standard deviation (SD) from of three individual replicates. The different letters on the error bars indicate significant differences among the treatments at $p < 0.05$.

such as stress from water, temperature, salt, pollutants, and so on (Husen et al., 2016; Ma et al., 2016). Thus, growth parameters including chlorophyll synthesis were suppressed under a stressed environment, and the effect of the stress was greater in plants that received no additional IAA treatment than on those treated with exogenous IAA or IAA-producing PGPB. Inoculation with *Pantoea alhagi* sp. nov. could promote chlorophyll production in the leaves compared to non-inoculated

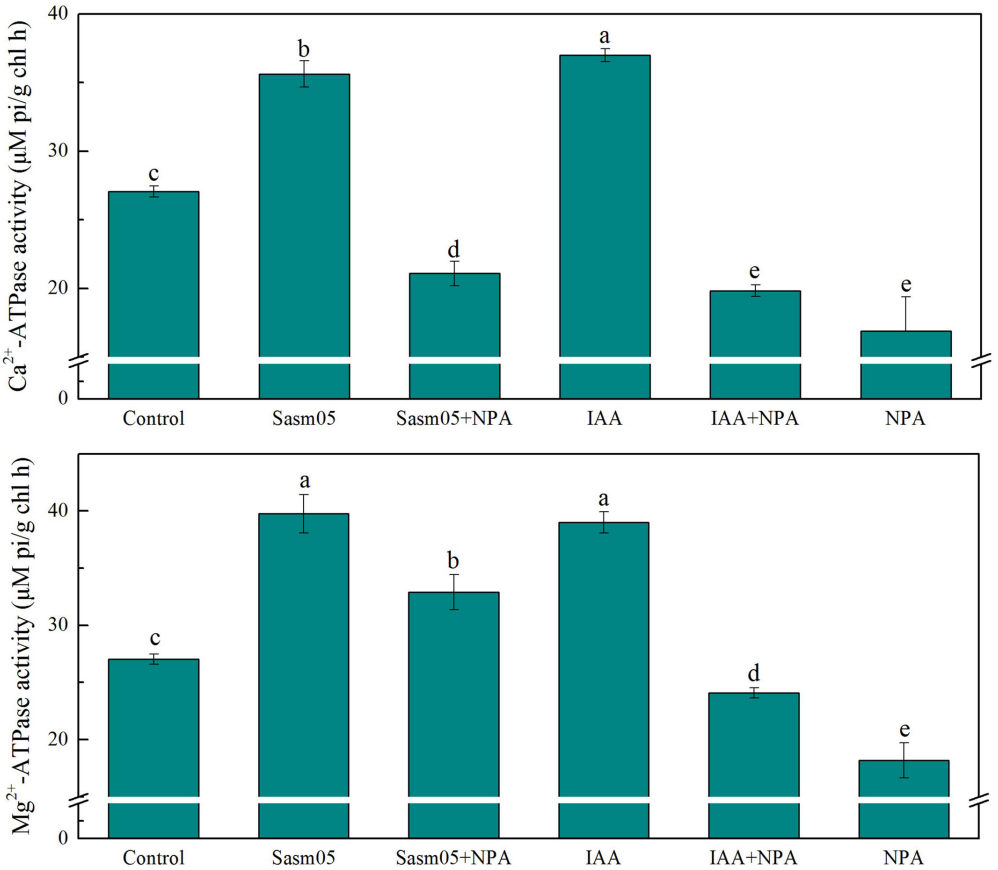

FIGURE 6 | Effects of Sasm05 and IAA on leave Mg^{2+}-ATPase activity and Ca^{2+}-ATPase activity. Error bars represent the standard deviation (SD) from of three individual replicates. The different letters on the error bars indicate significant differences among the treatments at $p < 0.05$.

control plants under similar water stress conditions (Chen et al., 2017). Consistently, the addition of Sasm05 and IAA could also significantly increase the chlorophyll concentration (**Figure 5**).

Mg^{2+} and Ca^{2+} are essential to the integrity of the cellular membrane and the intracellular adhesives, and the function of the Ca^{2+}-ATPase and Mg^{2+}-ATPase are important in the maintenance of the intracellular calcium and magnesium level (de la Torre et al., 2000; Lin et al., 2016). In the present study, the activity of Ca^{2+}-ATPase and Mg^{2+}-ATPase were measured, and it was found that both Sasm05 and IAA can activate Ca^{2+}-ATPase and Mg^{2+}-ATPase (**Figure 6**), indicating that both of them can stimulate photosynthesis. The IAA treatment had a growth-stimulating effect via the simulation of the electrogenic activity of the plasmalemma membrane potential H^{+}-ATPase and the hormonal effects were mediated by a transient elevation in the level of free Ca^{2+} in the cytosol and generation of reactive oxygen species (Kovaleva et al., 2016). However, all NPA treatments sharply decreased the activity of Ca^{2+}-ATPase and Mg^{2+}-ATPase, as well as decreasing the leaf chlorophyll content (**Figure 5**), which illustrated that IAA and its transport had dramatic effects on the improvement of plant photosynthesis.

The Effects of Sasm05 and IAA on Cd Uptake and Transport

Although a lot of literatures indicated that PGPB can increase heavy metal uptake in plant, the opposite results were also not unusual. Mesa et al. (2015) found that endophytic cultivable bacteria of the metal bioaccumulator *Spartina maritima* improve plant growth but not metal uptake in polluted marshes soils. Here we found that Sasm05 increase Cd concentration in both root and shoot while exogenous have no significant effects (**Figure 3**). Therefore, we assumed that it should be related with transporter gene expression regulation.

Many metal transporters and homologous genes involved in metal transport in plants have been identified by genetic and molecular techniques, such as sequence comparison. A suite of metal-sensing regulatory proteins from bacterial sources orchestrated metal homeostasis by allosterically coupling the selective binding of target metals to the activity of the DNA-binding domains for detecting and responding to toxic levels of heavy metals (Furukawa et al., 2015). Although our previous data showed that the IAA-producing PGPB *Sphingomonas* SaMR12 could affect the gene expression of transporters (Pan et al., 2017), the effects of exogenous IAA and the auxin transport inhibitor

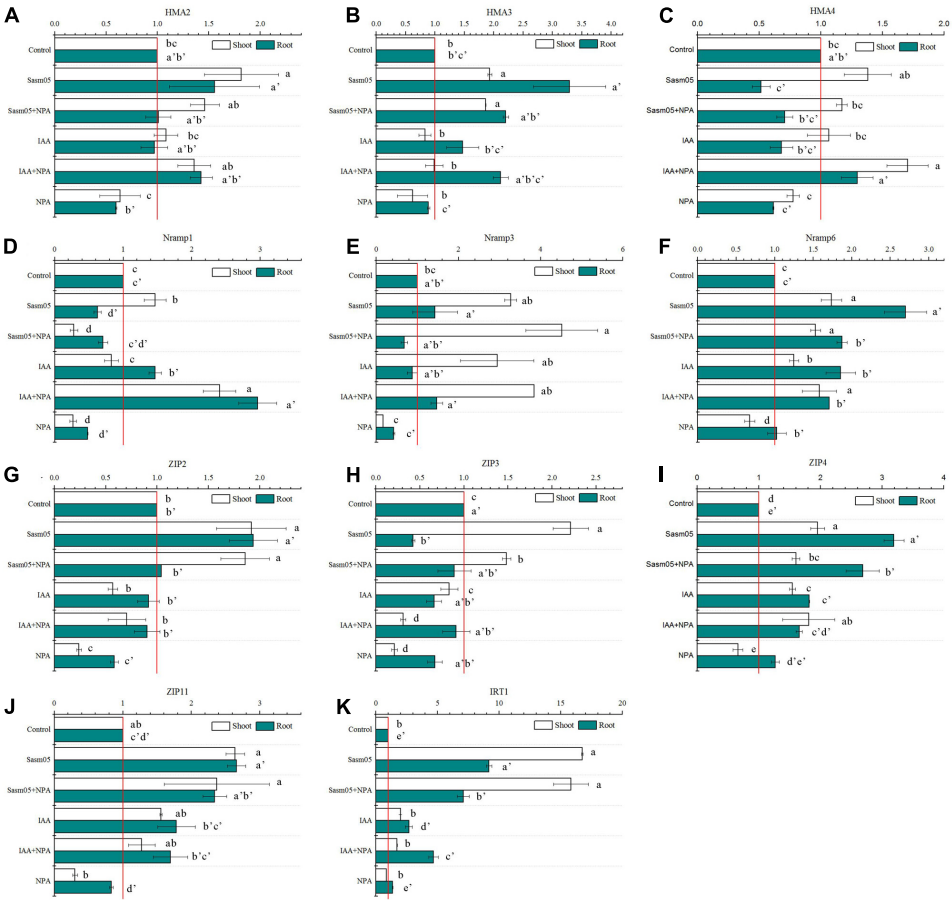

FIGURE 7 | Effects of Sasm05 and IAA on relative expression levels of SaHMAs **(A–C)**, SaNramps **(D–F)** and SaZIPs **(G–K)** genes. Error bars represent the standard deviation (SD) from of three individual replicates. The different letters on the error bars indicate significant differences among the treatments at $p < 0.05$.

were the first time compared. In Arabidopsis, AtHMA2 and AtHMA4 are essential for Zn and Cd translocation from the roots to the shoots (Eren, 2004; Verret et al., 2004). AtHMA3 is another heavy metal ATPase transporter that is located in the tonoplast and is associated with Cd and Zn tolerance (Morel et al., 2009). And in *Cucumber*, *CsHMA3*, *CsHMA4* were predominantly expressed in the roots and up-regulated by excess Zn and Cd (Migocka et al., 2015). SaHMA3 of *S. alfredii* was a Cd transporter, constitutively expressed in both shoots and roots, and encoded tonoplast-localized proteins (Zhang et al., 2016). Although no significantly induced expression of *SaHMA2*, *SaHMA3* and *SaHMA4* was observed after addition of IAA or NPA, Sasm05 inoculation increased their expression levels except for *SaHMA4* in the roots (**Figures 7A–C**), indicating that Sasm05 might enhance Cd root to shoot translocation by regulation these genes.

In Arabidopsis, six *AtNramp1-6* genes encode the NRAMP proteins. AtNramp1 played an important role on plant iron homoeostasis (Curie et al., 2000). AtNramp3 and AtNramp4 are localized to the vacuolar membrane and encode tonoplastic proteins with redundant functions (Lanquar et al., 2005). They are metal transporters with a broad range of substrate specificities including Fe, Cd, and Zn (Lanquar et al., 2005, 2010). AtNramp6 is an intracellular Cd transporter that functions inside the cell either by mobilizing Cd from its storage compartment or by taking up Cd into a cellular compartment where it becomes toxic (Cailliatte et al., 2009). However, in rice, OsNramp5 has been reported to be a major transporter for Cd uptake (Ishimaru et al., 2012; Sasaki et al., 2012), and the expression of *OsNramp5* was increased in the roots and shoots in the presence of Cd (Ishimaru et al., 2012). In this research, we found the variation of the transcription levels of *SaNramp1*, *SaNramp3,* and *SaNramp6* in the different treatments are not uniform (**Figures 7D–F**), indicating the function of these genes may also differ. NPA greatly inhibited the expression of these genes, while Sasm05 and IAA increased their expression. Those results indicated that Sasm05 and IAA might help the plant alleviate Cd toxicity to the cells, but more data about the functions of these genes in *S. alfredii* are needed.

Present studies have indicated that the ZIP family is the main membrane transporter responsible for possible Cd^{2+} uptake and transport (Zhu et al., 2013). As expected, the expression levels of ZIP family genes in the shoots and roots were all up-regulated by the Sasm05 treatment, except for *ZIP3* in the roots

(**Figures 7G–K**), suggesting that Sasm05 causes more Cd^{2+} to enter the cell. Although the relative expression levels of other genes varied limited in fivefold, the expression of *IRT1* was dramatically induced by Sasm05 by 17-fold in the shoots and 9-fold in the roots (**Figure 7K**), suggesting that Sasm05 promotes plant Cd uptake and transport through up-regulating the expression of the ZIP genes, especially *IRT1*.

CONCLUSION

In conclusion, with exposure to Cd, both the endophytic bacterium Sasm05 and exogenous IAA can promote the growth and photosynthesis of *S. alfredii*, and Sasm05 has greater effects on the enhancement of phytoextraction compare with IAA treatment. Moreover, Sasm05 can upregulate the expression of key transporters for Cd uptake, root to shoot translocation as well as detoxification, although IAA can also improve the expression of some transporter genes involved metal uptake and detoxification. These results indicated that the beneficial plant-endophytic bacterial interaction of Sasm05 is but not limited to IAA production. This study analyzed for the first time the effects of PGPB Sasm05 and exogenous IAA on phytoremediation of Cd contaminated soil, through which not only cleared the improvement from IAA treatment, but also illustrated related mechanisms of phytoremediation enhancement from Sasm05. These results will guide our subsequent study on plant growth promoting endophytic bacteria and their application for realizing efficient phytoremediation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. In this research, YF, XY, and BC designed the experiment; BC, SL, YW, FP, QW, and JY performed the experiment; SL, BC, and QW analyzed the data and drafted the manuscript; YF, BC, XX, FP, and KK revised the manuscript.

ACKNOWLEDGMENTS

This research was supported by the National Science Foundation for Post-doctoral Scientists of China (2016M591999), the National Natural Science Foundation of China (No. 41771345), and the National Key Research and Development Projects of China (2017YFD0801104 and 2016YFD0800801).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02538/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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