BIOTECHNOLOGICAL ADVANCES IN BIOREMEDIATION OF HEAVY METAL POLLUTED SOILS

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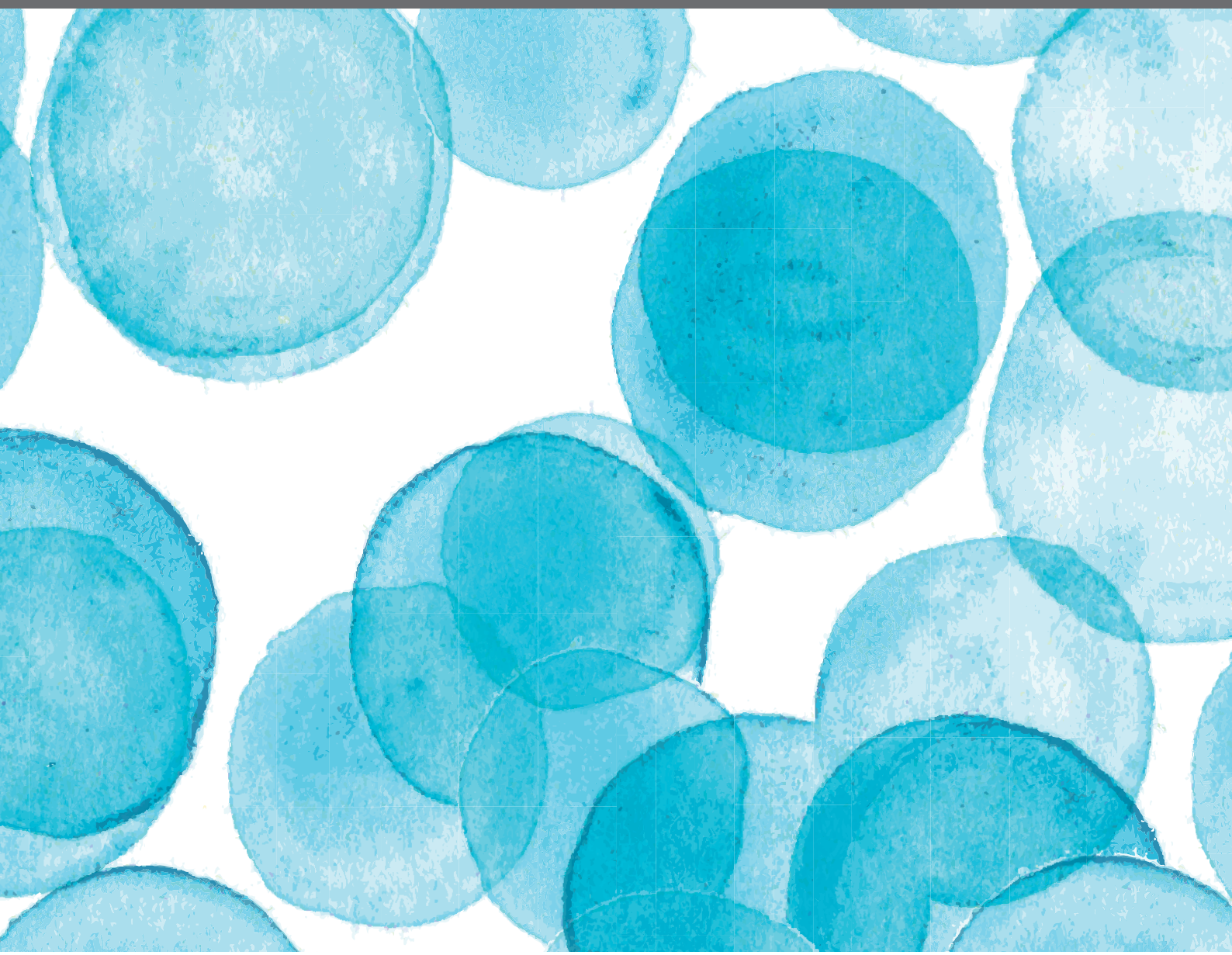

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BIOTECHNOLOGICAL ADVANCES IN BIOREMEDIATION OF HEAVY METAL POLLUTED SOILS

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Effects of Plant Growth-Promoting Bacteria (PGPB) Inoculation on the Growth, Antioxidant Activity, Cu Uptake, and Bacterial Community Structure of Rape (*Brassica napus* L.) Grown in Cu-Contaminated Agricultural Soil

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Previous analyses of plant growth-promoting bacteria (PGPB) combined with the remediation of heavy metal pollution in soil have largely been performed under potting or greenhouse conditions, and *in situ* remediation experiments under field conditions have rarely been reported. In this study, the effects of the metal-resistant PGPB *Microbacterium oxydans* JYC17, *Pseudomonas thivervalensis* Y1-3-9, and *Burkholderia cepacia* J62 on soil Cu pollution under rape remediation were studied in the farmland surrounding the Nanjing Jiuhuashan copper mining region in China. Following inoculation treatment for 50 days, the biomasses of the rape inoculated with strains JYC17, Y1-3-9, and J62 increased, and the total amounts of Cu uptake increased by 113.38, 66.26, and 67.91%, respectively, the translocation factor (TF) of rape inoculated with J62 was 0.85, a significant increase of 70.68%, thus improving the Cu remediation efficiency of the rape. Y1-3-9 and J62 affected the bioavailability of Cu in the soil, and the water-soluble Cu contents were increased by 10.13 and 41.77%, respectively, compared with the control. The antioxidant activities in the rape leaves showed that the tested bacteria increased the contents of antioxidant non-enzymatic substances, including ascorbic acid (ASA) and glutathione (GSH), which were increased by 40.24–91.22% and 9.89–17.67%, respectively, thereby reducing the oxidative stress caused by heavy metals and the contents of thiobarbituric acid-reactive substances (TBARS) and peroxidase (POD). PCR-denaturing gradient gel electrophoresis (PCR-DGGE) was used to analyze the effects of the tested bacteria on the cultivation-dependent and cultivation-independent bacterial communities in the root endosphere and rhizosphere soil of the

rape. The sequencing results of the DGGE bands indicated that the tested bacteria colonized the endosphere and rhizosphere, and they became an important component of the cultivation-dependent bacteria. The canonical correspondence analysis (CCA) of the DGGE profile and similarity cluster analysis showed that the tested bacteria affected the cultivation-dependent and cultivation-independent bacterial communities in the root endosphere and rhizosphere. In this experiment, the effects and mechanisms of the combined plant-microbe remediation under field conditions were preliminarily studied, and the results are expected to provide a theoretical basis for future combined remediation experiments.

Keywords: plant growth-promoting bacteria, plant-PGPB combined remediation, antioxidant activity, bacterial community, field experiment

HIGHLIGHTS

- *In situ* combined plant-PGPB remediation experiments were conducted through field trials.
- Metal-resistant PGPB J62 increased the biomass, Cu uptake and translocated the Cu to harvestable tissues of rape.
- PGPB increased the ASA and GSH contents in the rape leaves and reduced the TBARS and POD contents.
- PGPB colonized the rape endosphere and rhizosphere and altered its bacterial community composition.

INTRODUCTION

Due to mining and heavy metals from sewage irrigation, heavy metal-contaminated cultivated land accounts for approximately one-fifth of the total cultivated land in China. This large-scale heavy metal pollution of farmland has serious consequences for food security in China and necessitates remediation and treatment (Li et al., 2014; Zhao et al., 2015; Duan et al., 2016). Phytoremediation has become an important area of research within soil heavy metal pollution remediation because it does not produce secondary pollution, is a simple operation, and incurs low investment costs (Cunningham et al., 1995; Liu et al., 2018).

During the remediation of heavy metal-contaminated soil, plants grow slowly and have reduced biomass due to heavy metal stress, thus limiting their remediation efficiency (Ullah et al., 2015). There are many types of microorganisms in soil, which are present in large quantities, have high biological activity, and play important roles in the growth of remediation plants in heavy metal-contaminated soils and in the geochemical cycle of heavy metals (Teng et al., 2015; Chen et al., 2017; Jing and Kjellerup, 2018). Bacteria in soil can improve plant nutrition through phosphorus solubilization and nitrogen fixation and through the secretion of plant hormones [indole-3-acetic acid (IAA), etc.], siderophores, and specific enzymes [1-aminocyclopropane-1-carboxylate (ACC) deaminase, etc.], thus promoting the growth of remediation plants and the enrichment of heavy metals under stress as well as improving the heavy metal phytoremediation efficiency in soil. Due to their complementation ability, plant-microbe-combined remediation is currently a research hotspot (Glick, 2003; Ma et al., 2016; Ashraf et al., 2017). To date, researchers have inoculated hyperaccumulator plants such as *Solanum nigrum* (Luo et al., 2011), *Thlaspi caerulescens* (Whiting et al., 2001), and *Sedum plumbizincicola* (Ma et al., 2015), energy plants such as *Zea mays* (Sheng et al., 2012) and *Napier grass* (Wiangkham and Prapagdee, 2018), and high-biomass plants such as poplar (Wang et al., 2011) with plant growth-promoting bacteria (PGPB) to investigate their effects on plant growth and their heavy metal remediation efficiency in soils. However, most investigations of combined plant-PGPB remediation for soil heavy metal pollution have been performed under simple or controllable conditions such as potted plants or greenhouses. Field trials with more complicated environmental conditions have rarely been reported, while field trials are a necessary stage for combined plant-PGPB remediation technology to progress from the laboratory to practical applications, and the related work must be continued (Zhuang et al., 2007; Dary et al., 2010; Kong and Glick, 2017; Ju et al., 2019). Prapagdee and Khonsue (2015) showed that the inoculation of cadmium-resistant *Ralstonia* sp. TISTR 2219 and *Arthrobacter* sp. TISTR 2220 bacteria under field conditions increased cadmium accumulation in the roots, above-ground tissues, and whole plants of *Ocimum gratissimum*. The "*in situ*" remediation experiment conducted by Dary et al. (2010) showed that the inoculation of *Lupinus luteus* with metal-resistant PGPB increased the biomass and reduced the metal accumulation.

Rape is a cruciferous *Brassica* plant, for which many species or genotypes have strong heavy metal accumulation characteristics (Rizwan et al., 2018). Research examining the combined remediation of heavy metal pollution in soil using rape as a remediation plant inoculated with PGPB has been performed under potted or greenhouse conditions, but *in situ* field trials for related research have rarely been reported (Farwell et al., 2006; Sheng and Xia, 2006; Chen et al., 2013; Dąbrowska et al., 2017; Pan et al., 2017). Therefore, the primary objectives of this study were (1) to evaluate the effect of PGPB inoculation on the growth of rape and its enrichment of heavy metals in the field around a region characterized by heavy metal mine tailings, (2) to determine the effect of PGPB on the antioxidant activity of rape during the enrichment of soil heavy metals, and (3) to determine the effect of PGPB inoculation on the composition of the bacterial community in the rape rhizosphere and endosphere. Through the above research, we expected to perform a preliminary exploration

of the PGPB mechanism that improves plant enrichment of heavy metals under field conditions from the perspectives of plant physiological indicators and microbial communities.

MATERIALS AND METHODS

Bacterial Strain and Field Experiment Site

Three metal-resistant bacteria, *Burkholderia cepacia* J62, *Pseudomonas thivervalensis* Y1-3-9, and *Microbacterium oxydans* JYC17, which were stored in our laboratory, can produce plant growth-promoting substances such as indole-3-acetic acid (ranging from 3.3 to 10.8 mg L^{-1}), siderophores, 1-aminocyclopropane-1-carboxylic deaminase (ranging from 8.0 to 307.0 μM α-KB mg^{-1} h^{-1}), and solubilized inorganic phosphate (ranging from 127.0 to 234.0 mg L^{-1}) (Jiang et al., 2008; He et al., 2010; Zhang et al., 2011). *B. cepacia* J62 was isolated from a Pb-contaminated paddy field in Zhejiang, China; *P. thivervalensis* Y1-3-9 was isolated from the leaves of *Mosla chinensis*; and *M. oxydans* JYC17 was isolated from the rhizosphere soils of *Kummerowia striata* grown on Cu mine wasteland. These bacterial isolates can promote plant growth and heavy metal uptake by plants from heavy metal-contaminated soils during pot experiments (Jiang et al., 2008; He et al., 2010; Zhang et al., 2011).

The field experiment site is farmland near Nanjing Jiuhuashan Cu mining, on Funiu mountain in Tangshan town (32°04'N; 119°05'E), Jiangning District of Nanjing, which was polluted by Cu mining. The soil characteristics include a pH of 6.68, organic matter content of 3.04 g kg^{-1}, cation exchange capacity (CEC) of 14.5 cmol kg^{-1}, total nitrogen (N) of 1.41 g kg^{-1}, phosphorus (P) of 13.4 mg kg^{-1}, and potassium (K) of 85.7 mg kg^{-1}, along with heavy metal (Cu, Zn, Pb, and Mn) contents of 1068.25, 133.00, 34.50, and 534.75 mg kg^{-1}. With reference to Soil Environmental Quality Standard Two (GB 15618-1995), the Cu pollution at the field experiment site exceeded grade III for agricultural land (400 mg kg^{-1}).

Introduction of Strains J62, JYC17, and Y1-3-9 and Plant Growth

The field experiments were performed during the season between September and November in 2012. The fields were divided into plots for rape (variety Qinyou-7) planting. The field experiments were divided into four treatments, namely, J62 inoculation, Y1-3-9 inoculation, JYC17 inoculation, and an uninoculated control. Each plot was 52 m^2 (13 m × 4 m) in area, with plants growing in a row spacing of 0.5 m × 0.5 m (**Supplementary Figure S1A**). The field plot experiments were conducted using a randomized arrangement with three replications. For the inoculation, strains J62, JYC17, and Y1-3-9 were grown in LB medium for 18 h at 28°C with continuous shaking at 200 rpm, and the cells were collected by centrifugation at 6,000 rpm for 10 min, washed, and recentrifuged in sterile distilled water to obtain a bacteria inoculum of approximately 10^8 cfu mL^{-1}. The bacterial suspensions (10 mL $plant^{-1}$) were sprayed on the rape rhizosphere. For the uninoculated control, an equal volume of sterile water was added.

Fifty days after the inoculation treatment, five plants were harvested at random from the central parts of each plot (**Supplementary Figure S1B**). Rhizosphere soil was obtained by first gently shaking off the loosely bound soil, while the rhizosphere soil adhering to the root system was isolated by more vigorous shaking or by hand. The above-ground tissues and roots were separated and washed extensively, first in several changes of 0.01 M EDTA and then in distilled water to remove any non-specifically bound heavy metals, and then they were dried at 80°C for 2 days before the root and above-ground tissue dry weights were determined (**Supplementary Figure S1C**). The root and above-ground tissues were digested in a mixture of concentrated HNO_3 and $HClO_4$ (4:1, v/v), and the Cu contents of the samples were determined with an inductively coupled-plasma optical emission spectrometer (ICP-OES) (Optima 2100 DV, Perkin Elmer). The water-soluble and NH_4OAc-extractable Cu concentrations in the rhizosphere soils of the plants were determined by ICP-OES, and the pH of the soil (1:1 w/v water) was determined with a pH meter.

Determination of Antioxidant Enzymes and Thiobarbituric Acid-Reactive Substances (TBARS)

For the enzyme extraction, fresh leaves (0.5 g) were homogenized in 10 mL of ice-cold potassium phosphate buffer (pH 7.0) in an ice bath by grinding using a mortar and pestle. The homogenate was centrifuged at 12,000 g for 20 min at 4°C. The supernatant was stored at 4°C and used to determine the various antioxidant enzymes. The superoxide dismutase (SOD) activity was measured through the photoreduction of nitro blue tetrazolium chloride (NBT) (Dhindsa and Matowe, 1981). The peroxidase (POD) activity was measured according to the method by Rao et al. (1996). The glutathione (GSH) was measured according to Griffith (1980), and the ascorbic acid (ASA) was measured according to Arakawa et al. (1981). The protein content was determined according to the Bradford (1976) method with bovine serum albumin as the standard. The TBARS was measured as described by Jiang and Zhang (2001).

DNA Extracted From Biomass Was Accumulated on an Agar Plate (Cultivation-Dependent) and Extracted Directly From the Endosphere and Rhizosphere Samples (Cultivation-Independent)

Culturable endophytic and rhizosphere bacteria were isolated by traditional plate culture methods. The plant roots were surface-sterilized by sequential immersion in 75% (v/v) ethanol for 2 min and 1% mercuric chloride for 1 min, they were ground with a mortar and pestle in the presence of 5 ml of sterile distilled water, and then they were spread on plates containing 1/5-strength LB medium for 72 h at 28°C to isolate the endophytic bacteria. Samples of rhizosphere soil

weighing 1 g were taken from each treatment, homogenized in 10 mL of 0.85% saline, and serially diluted (10-fold) in the same container, and the aliquots (100 μL) were spread on 1/5 LB medium and incubated for 72 h at 28°C. DNA was extracted from the bacterial biomass on the plates by cetyltrimethylammonium bromide (CTAB) method (Ellis et al., 2003), and the results were considered "cultivation-dependent" samples.

Endophytic bacteria from the plant roots was extracted as described by Sun et al. (2010). The plant roots were surface-sterilized as described above for the isolation of culturable endophytic bacteria, they were ground in a sterilized mortar with 5 mL sodium phosphate buffer, and then they were transferred to tubes that were shaken for 1 h to dislodge the bacterial cells from inside the plant tissue. The bacterial cells were collected by centrifugation at 12000 g for 10 min and resuspended in 550 μL of TE buffer. The total endophytic bacterial DNA was extracted as described by Araújo et al. (2002). The rhizosphere bacterial DNA was extracted based on a modification of a method by Zhou et al. (1996). The resulting extracts were considered "endosphere" and "rhizosphere" samples.

PCR-Denaturing Gradient Gel Electrophoresis (DGGE) Analyses

The DGGE primers GC-341F and 534R were used to amplify the V3 hypervariable region of 16S rRNA genes directly from the DNA samples (Muyzer et al., 1993). The PCRs were performed in a PTC-200 DNA Engine Cycler (Bio-Rad, United States) using the amplification program and reaction conditions described by Chen et al. (2013). For the DGGE analysis, the PCR products generated from each sample were separated on an 8% acrylamide gel with a linear denaturant gradient ranging from 45 to 75% using a DCode^{TM} Universal Mutation Detection System from BIO-RAD, United States. The DGGE was performed using 20 μL of the PCR product in 1× TAE buffer at 60°C, 200 V for 10 min, then 85 V for 12 h. The gels were stained with SYBR Green I (Generay Biotech Co., Ltd., Shanghai, China) in 1× TAE for 30 min, and the gels were scanned with a gel photo GS-800 system (Bio-Rad, United States).

The bands of interest were excised and eluted with 50 μL of ddH_2O for 24 h at 4°C. The resulting solution (1 μL) was used as the target DNA for a subsequent PCR amplification with primers 341F and 534R, and the PCR products were cloned into the pGEM-T Easy vector as described by the manufacturer. The clones were sequenced, and these short fragments were subjected to BLAST-assisted searches of the NCBI database; the closest match of known phylogenetic affiliation was used to assign the bands to taxonomic groups.

Statistical and Cluster Analyses

The microbial community banding profiles on the DGGE gels were analyzed using a Quantity One software package (Bio-Rad Laboratories, Inc., Hercules, United States). The plant growth parameter data were analyzed using a Student–Newman–Keuls test at a significance level of $P < 0.05$. All the statistics were performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, United States).

RESULTS

Effects of Each Strain on the Biomass and Cu Enrichment in Rape

The remediation rape plants grew well in the Cu-contaminated farmland, and the inoculation treatment promoted plant growth. The promotion of above-ground biomass (dry weight) was more obvious than the promotion of root growth. Compared with the plants without inoculation treatment, the above-ground biomass after the inoculation treatment increased by 27.3–59.5%, and the root biomass increased by 8.6–67.2%. The increases in the above-ground biomass of rape inoculated with strains JYC17 and J62 were significantly different from those for the control without inoculation treatment ($P < 0.05$) (**Figure 1**). Simultaneously, the inoculation treatment affected the Cu concentration in the rape to varying degrees. The JYC17 and Y1-3-9 increased the Cu concentration in the roots of the rape, with values of 42.63 and 40.14 mg kg^{-1}, respectively, demonstrating increases of 41.48 and 34.13%, compared with 30.13 mg kg^{-1} in the control group, for which the former difference was statistically significant ($P < 0.05$). Y1-3-9 and J62 increased the Cu concentrations in the above-ground part of the rape by 10.27 and 25.56%, respectively (**Figure 2**). The translocation factor (TF) indicates the capacity of the plants to translocate heavy metals from the roots to the above-ground tissues, which is expressed as the ratio of the heavy metal concentration in the above-ground tissues/the heavy metal concentration in the roots. The experimental results showed that the TFs of plants inoculated with JYC17, Y1-3-9, and J62 were 0.33, 0.41, and 0.85, respectively. Strain J62 significantly increased the TF by 70.68% compared with the control ($P < 0.05$). Although the tested bacteria showed different effects on the Cu concentration in different parts of the rape, the inoculation treatment increased the above-ground biomass of the plant, such that the total amount of Cu enrichment in the rape showed different degrees of improvement compared with the control. The total amounts of Cu enrichment in the roots of each rape plant inoculated with JYC17 and Y1-3-9 were 1193.90 and 727.02 μg $plant^{-1}$, respectively, demonstrating 210.2 and 88.9% significantly higher enrichment compared with the control ($P < 0.05$). The total amounts of Cu enrichment in the above-ground part of each rape plant inoculated with JYC17, Y1-3-9, and J62 were 1296.87, 1232.14, and 1696.69 μg $plant^{-1}$, respectively, demonstrating increases of 63.4, 55.3, and 113.8% compared with the control value of 793.47 μg $plant^{-1}$. The total Cu enrichment in whole plants inoculated with JYC17, Y1-3-9, and J62 increased by 113.4, 66.3, and 67.9%, respectively (**Figure 3**).

Effect of Strain Inoculation on the Soil Bioavailability of Heavy Metals

The bioavailability of heavy metals in the plant rhizosphere is an important factor limiting the efficiency of phytoremediation. The

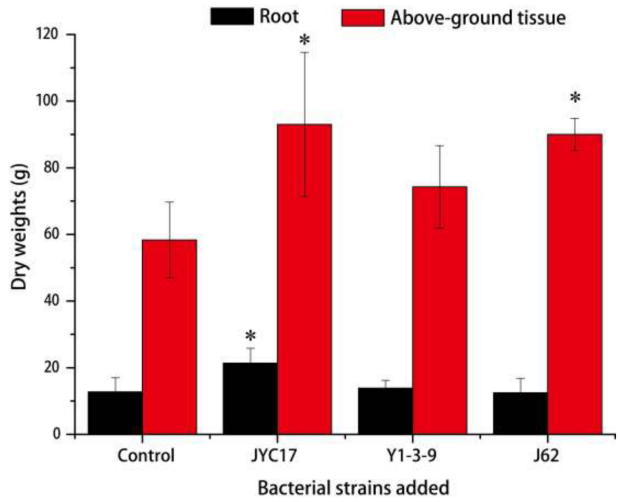

FIGURE 1 | The dry weights of plants that were uninoculated and inoculated with treatments. The error bars are ± the standard deviation. An asterisk (*) denotes a significantly different value between the uninoculated and inoculated treatments ($P < 0.05$).

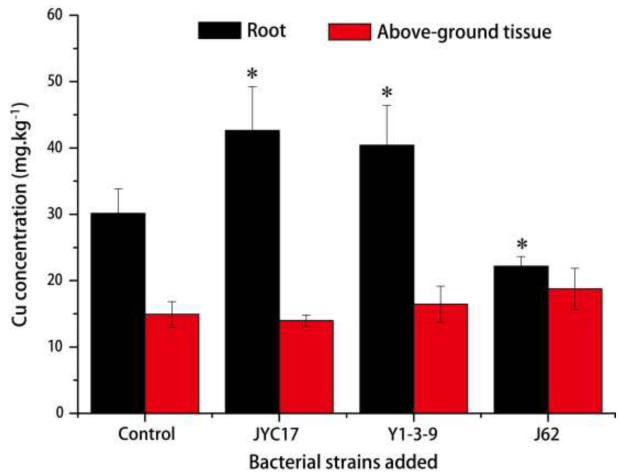

FIGURE 2 | The Cu concentrations in uninoculated and inoculated plant treatments. The error bars are ± the standard deviation. An asterisk (*) denotes a significantly different value between the uninoculated and inoculated treatments ($P < 0.05$).

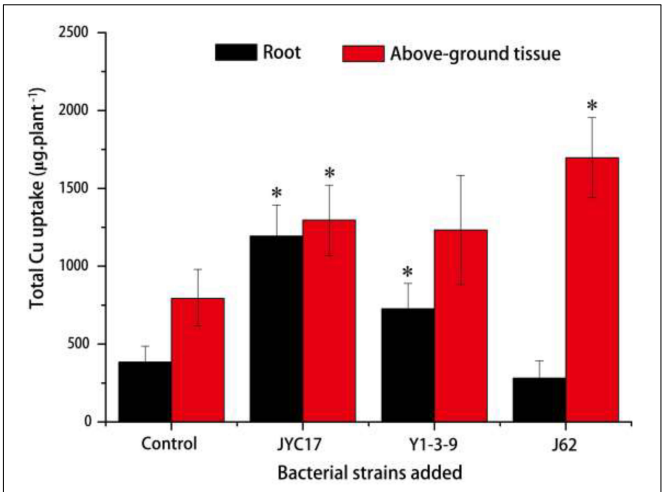

FIGURE 3 | The total Cu uptake by plants in the uninoculated and inoculated treatments. The error bars are ± the standard deviation. An asterisk (*) denotes a significantly different value between the uninoculated and inoculated treatments ($P < 0.05$).

effects of different treatments on the forms of Cu in the rape rhizosphere soil was determined, and the results are shown in **Table 1**. Accordingly, the inoculation using the tested bacteria changed the forms of Cu in the soil. Strains Y1-3-9 and J62 increased the levels of water-soluble Cu in the rape rhizosphere soil to 1.12 and 0.87 mg kg^{-1}, respectively, demonstrating increases of 41.77 and 10.13% compared with the control. The contents of NH_4OAc-extracted and DTPA-extracted Cu after the inoculation treatment were lower than those in the control group, to different degrees. The inoculation showed little effect on the pH of the rhizosphere soil.

The Effect of Strain Inoculation on the Antioxidant Activities in Rape Leaves

Under an environment of heavy metal stress, non-enzymatic substances composed of ASA and GSH can facilitate a plant's elimination of peroxidation. According to the ASA content measurements shown in **Figure 4**, the inoculation using the tested bacteria increased the ASA contents in the rape leaves. The ASA content increased from 456.68 mg kg^{-1} fresh weight (FW) in the control to 873.26 mg kg^{-1} FW in the plants inoculated with JYC17 (91.22% increase), 640.47 mg kg^{-1} FW in the plants inoculated with Y1-3-9 (40.24% increase), and 913.37 mg kg^{-1} FW in the plants inoculated with J62 (80.49% increase). Similar to the ASA results, inoculation using the tested bacteria increased the GSH contents in the rape leaves. The GSH content increased from 373.47 mg kg^{-1} FW in the control to 415.15 mg kg^{-1} FW in the plants inoculated with JYC17 (11.16% increase), 439.46 mg kg^{-1} FW in the plants inoculated with Y1-3-9 (17.67% increase), and 410.41 mg kg^{-1} FW in the plants inoculated with J62 (9.89% increase).

The TBARS content in plants is a physiological indicator of plant stress. The increase in the TBARS content in a plant indicates that the plant is under a certain degree of stress. The TBARS contents in rape leaves inoculated with JYC17, Y1-3-9, and J62 were decreased to different extents compared with the control (10.16, 10.58, and 21.88%, respectively). Similar to the effect of TBARS in plants, the SOD and POD activities in plants exposed to adverse conditions such as heavy metal stress can be enhanced, and the reactive oxygen species (ROS) caused by the stress in the plants can be scavenged. Therefore, they can also be used as a physiological indicator of plant stress. According to the SOD and POD activity assay shown in **Figure 4**, JYC17, Y1-3-9, and J62 inoculation reduced the POD activity in the rape leaves by 9.39, 4.55, and 17.80%, respectively. Concomitantly, inoculating with strain J62 reduced

TABLE 1 | The influence of the test strains on the number of cultivation-dependent bacteria in the rape rhizosphere and endosphere, the water-soluble Cu, NH_4OAc-extractable Cu, DTPA-extracted Cu, and the pH in the rhizosphere soil.

Treatments	Number of endophytic bacteria (cfu g^{-1} fresh weight)	Number of rhizosphere bacteria (cfu g^{-1} soil)	Water-soluble Cu (mg kg^{-1})	NH_4OAc-extracted Cu (mg kg^{-1})	DTPA-extracted Cu (mg kg^{-1})	pH
Control	2.86×10^4	1.64×10^6	0.79 ± 0.08^b	13.7 ± 1.77^a	297.2 ± 12.7^a	7.82
JYC17	4.56×10^4	3.08×10^6	0.54 ± 0.07^c	10.8 ± 3.51^{abc}	243.3 ± 17.9^b	7.84
Y1-3-9	4.45×10^4	1.70×10^6	1.12 ± 0.19^a	8.98 ± 0.95^c	249.6 ± 37.6^b	7.91
J62	1.64×10^4	2.62×10^6	0.87 ± 0.23^b	11.2 ± 1.20^b	228.1 ± 9.17^b	8.13

Means within the same column followed by the same letter are not significantly different at $P < 0.05$, based on the Student–Newman–Keuls test.

the SOD activity in the rape leaves by 5.99%; the remaining two groups showed little change compared with the control. Combined with the changing TBARS pattern in the plants, the results suggested that inoculating with the tested bacteria reduced the physiological indicator of plant stress in the Cu-polluted farmland soil for rape.

Composition of the Cultivation-Dependent and Cultivation-Independent Bacterial Community on the Rape Root Endosphere and Rhizosphere Soil

The band mobility of each sample in the DGGE profile was analyzed using Quantity One analysis software. A total of 23 bands from the cultivation-dependent profile and 43 bands from the culture-independent profile were cut and recovered (**Figure 5**). These bands were sequenced, and a homology alignment was performed with the known sequences in GenBank using BLAST software. The application of accession number HQ603005-HQ603051 was submitted to the GenBank database. Based on the sequencing results for the bands, the composition of the cultivation-dependent bacterial community in the rape root endosphere and its rhizosphere soil in the heavy metal-contaminated farmland near the Cu mine was as follows: Gammaproteobacteria, Firmicutes, Betaproteobacteria, and Actinobacteria, with 10, 7, 3, and 1 bands and proportions of the 21 sequenced bands at 47.6, 33.3, 14.3, and 4.7%, respectively (**Figure 5A** and **Supplementary Table S1**). *Bacillus* spp. in Gammaproteobacteria and *Pseudomonas* spp. in Firmicutes were the dominant populations among the cultivation-dependent bacteria, accounting for 47.6 and 28.6% of the total numbers of sequenced bands, respectively. The bands in the cultivation-independent DGGE profile of the directly extracted DNA samples were sequenced, and they primarily consisted of seven bacterial populations, including Alphaproteobacteria (1 band, 3.8%), Betaproteobacteria (2 bands, 7.7%), Gammaproteobacteria (5 bands, 19.2%), Firmicutes (2 bands, 7.7%), Actinobacteria (2 bands, 7.7%), Bacteroidetes (3 bands, 11.5%), and uncultured bacteria (11 bands, 42.3%) (**Figure 5B** and **Supplementary Table S1**). *Rhodanobacter* spp. and *Pseudomonas* spp. in Gammaproteobacteria and *Burkholderia* sp., *Janthinobacterium* sp. and *Achromobacter* sp. in Bacteroidetes were the dominant populations in the endosphere and rhizosphere samples.

Effects of the Tested Bacteria on the Bacterial Communities in the Endosphere and Rhizosphere

The effects of the tested bacteria on the number of cultivation-dependent bacteria in the rape rhizosphere and endosphere were determined. The results showed that the total number of bacteria in the rhizosphere of the tested plants was 10^6 cfu g^{-1} soil and that the total number of bacteria in the roots was approximately 10^4 cfu g^{-1} fresh weight. Inoculating with Y1-3-9 and JYC17 increased the number of bacteria in the rhizosphere and the roots. Simultaneously, the sequencing results for the DGGE bands showed that the sequence information for bands A15, A16, and A23 corresponded to the test bacteria Y1-3-9, J62, and JYC17, respectively, with a similarity of 100%. Based on the DGGE sequencing results and the counts of cultivation-dependent bacteria, the tested bacteria colonized the rape endosphere and rhizosphere under field conditions (**Table 1**, **Supplementary Table S1**, and **Figure 5A**).

To investigate the effects of the tested bacteria on the bacterial community structure of the rape endosphere and its rhizosphere, the DGGE profile was subjected to a canonical correspondence analysis (CCA), and the results are shown in **Figure 6**. **Figure 6A** shows the CCA of the cultivation-dependent DGGE profile. The cultivation-dependent bacterial community in the endosphere (E-CK) and the cultivation-dependent bacterial community in the rhizosphere (R-CK) in the control group without strain inoculation was separated through axis 1 (36.9%), indicating that the structure of the cultivation-dependent bacterial communities in the root endosphere was significantly different from that of the cultivation-dependent bacterial communities in the rhizosphere soil. Following inoculation with the tested bacteria, the endosphere cultivation-dependent bacteria community and rhizosphere cultivation-dependent bacteria community were clustered together above axis 2 (18.5%) of the CCA profile, in which the endosphere cultivation-dependent bacteria (E-J62) and rhizosphere cultivation-dependent bacteria (R-J62) for strain J62 and the endosphere cultivation-dependent bacteria (E-Y1-3-9) and rhizosphere cultivation-dependent bacteria (R-Y1-3-9) for strain Y1-3-9 were clustered together and separated from those of the control group (E-CK and R-CK). This finding

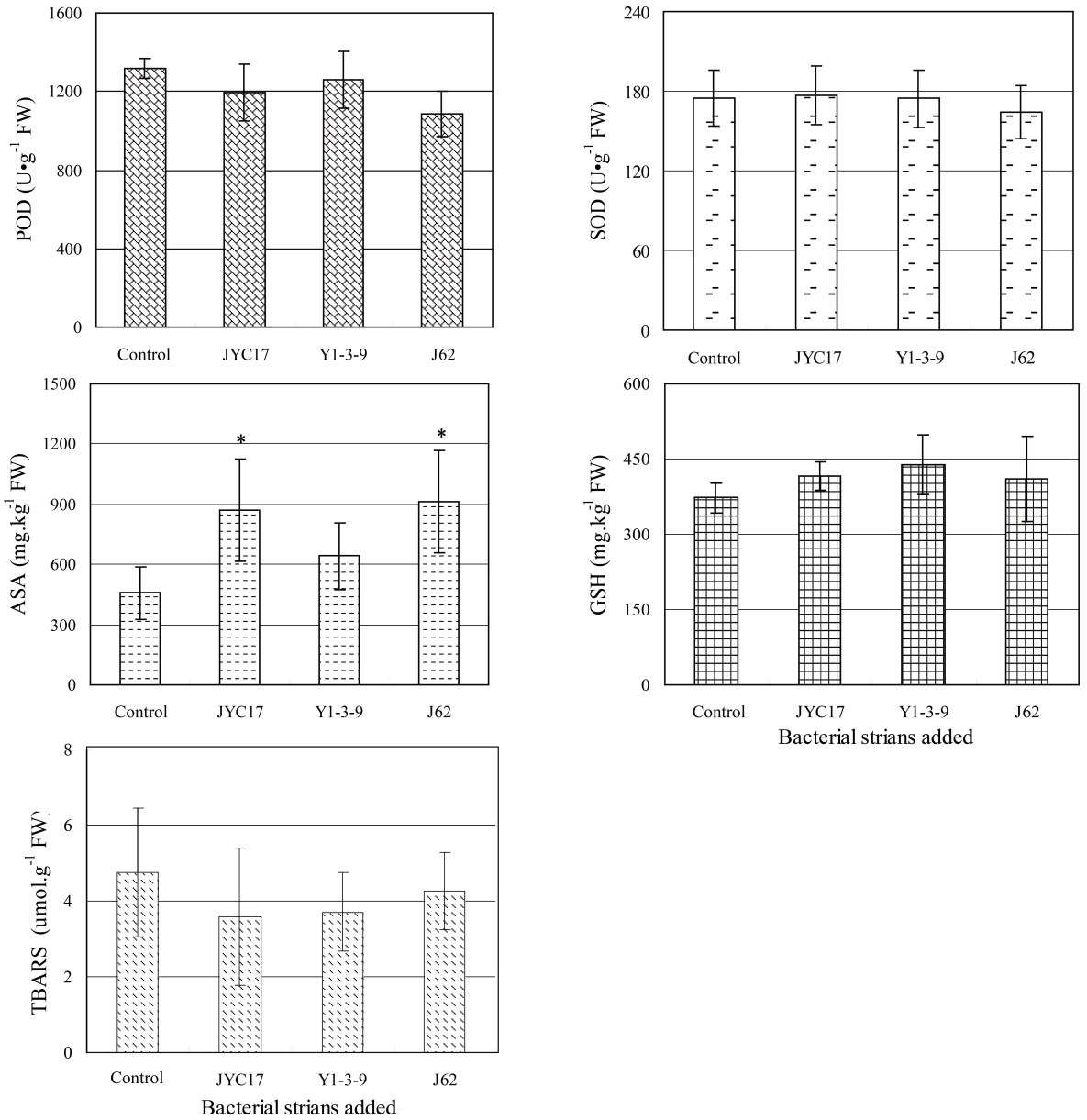

FIGURE 4 | Effect of PGPB inoculation on the leaf POD, SOD, ASA, GSH, and TBARS contents of rape under metal stress. An asterisk (*) denotes a significantly different value between uninoculated and inoculated treatments ($P < 0.05$).

indicates that the test bacteria J62 and Y1-3-9 affected the structure of the cultivation-dependent bacterial communities in the rape endosphere and its rhizosphere and had similar community structures.

Figure 6B shows the CCA of the cultivation-independent DGGE profile. Axis 1 divided the endosphere bacteria and rhizosphere bacteria into two parts at 31.0%, indicating that different sample sources (the rape endosphere and rhizosphere) are the most important factors determining the structure of the bacterial communities. Based on the CCA, the community structure in the control group at day 0 for the endosphere bacteria (E-CK0) and rhizosphere bacteria (R-CK0) was separated from the community structure at day 50 for the endosphere bacteria (E-CK) and rhizosphere bacteria (R-CK), indicating that the bacterial community changed over time. The E-Y1-3-9, E-JYC17, and E-J62 for the rape endosphere following inoculation were separated from each other in the CCA profile, while E-Y1-3-9 was closer to the E-CK of the control group, indicating that inoculation with the tested bacteria affected the bacterial communities of the rape root endosphere and that the degrees of influence from the different strains were different. Similarly, the R-Y1-3-9, R-JYC17, and R-J62 for the rhizosphere after inoculation treatment were clustered together in the CCA profile and separated from the R-CK

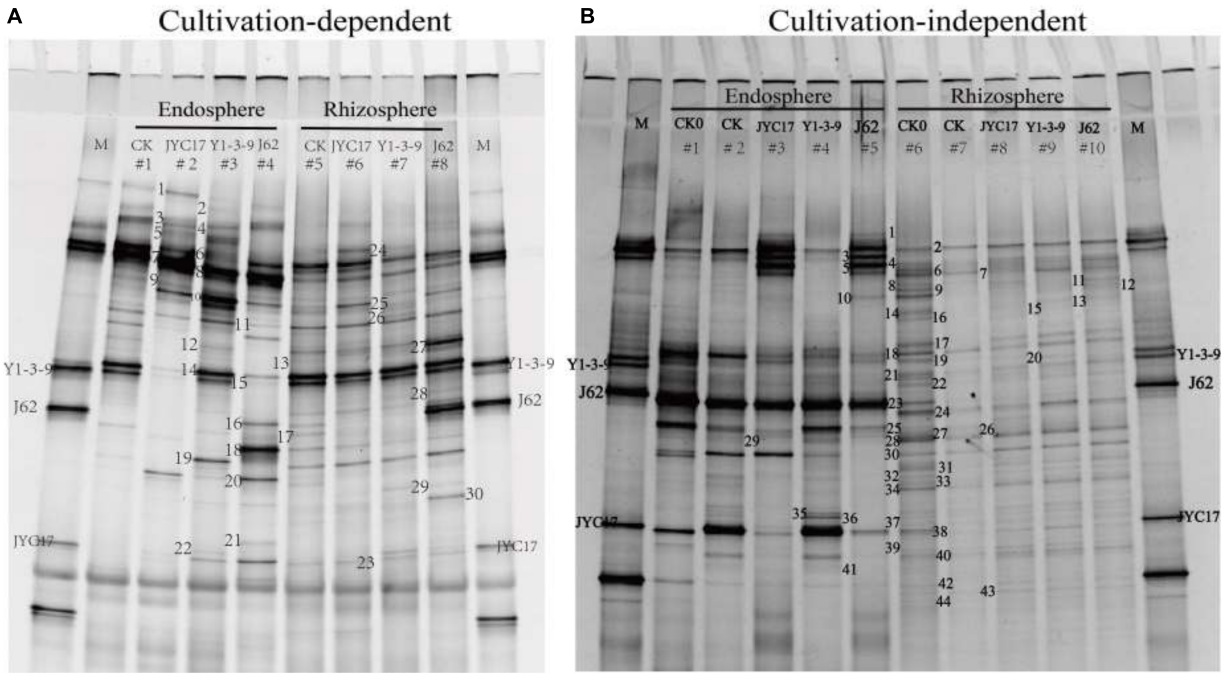

FIGURE 5 | DGGE gels showing diverse 16S rRNA gene fragments amplified from panel **(A)** DNA extracted from biomass accumulated on an agar plate (cultivation-dependent) and panel **(B)** DNA extracted directly from endosphere and rhizosphere samples (cultivation-independent). **(A)** Lanes 1 to 4, endosphere samples from the 50-day uninoculated control (E-CK), 50-day inoculation with JYC17 (E-JYC17), 50-day inoculation with Y1-3-9 (E-Y1-3-9), and 50-day inoculation with J62 (E-J62); lanes 5 to 8, rhizosphere samples from the 50-day uninoculated control (R-CK), the 50-day inoculation with JYC17 (R-JYC17), the 50-day inoculation with Y1-3-9 (R-Y1-3-9), and the 50-day inoculation with J62 (R-J62). **(B)** Lanes 1 to 5, endosphere samples from the 0-day uninoculated control (E-CK0), the 50-day uninoculated control (E-CK), the 50-day inoculation with JYC17 (E-JYC17), the 50-day inoculation with Y1-3-9 (E-Y1-3-9), and the 50-day inoculation with J62 (E-J62); lanes 6 to 10, rhizoplane samples from the 0-day uninoculated control (R-CK1), the 50-day uninoculated control (R-CK2), the 50-day inoculation with JYC17 (R-JYC17), the 50-day inoculation with Y1-3-9 (R-Y1-3-9), and the 50-day inoculation with J62 (R-J62). M, marker lane consisting of 16S rRNA gene fragments from J62, JYC17 and Y1-3-9. The bands in the DGGE profiles were excised and sequenced, and their given numbers correspond to the list in **Supplementary Table S1**.

of the control group, indicating that the inoculation with the tested bacteria affected the bacterial communities of the rape rhizosphere soil.

A bacterial community similarity clustering analysis was performed on the DGGE profile according to the unweighted pair group method with the arithmetic mean (UPGMA) algorithm, which was similar to the CCA results. The cultivation-dependent bacteria in the endosphere samples were clustered at a similarity level of 0.39 and were separated from the rhizosphere samples, indicating that the different sample source was the most important factor affecting the composition of the cultivation-dependent bacterial communities (**Supplementary Figure S2**). Concurrently, the inoculated and non-inoculated samples were separated from each other in the cluster analysis profile, indicating that inoculating with the tested bacteria affected the composition of the cultivation-dependent bacterial communities in the rape endosphere and rhizosphere. Similarly, the cultivation-independent bacteria in the rhizosphere samples were clustered at a similarity level of 0.43 and were separated from the rhizosphere samples. Simultaneously, the inoculated and non-inoculated samples were separated from one another in the cluster analysis profile.

DISCUSSION

During the combined plant-microbe remediation of heavy metal pollution in soil, PGPB can regulate the physiological processes of plants and reduce the stress from heavy metals on plants; simultaneously, the strains can activate insoluble heavy metals and phosphorus nutrients in the soil by producing iron carriers and organic acids. These bacteria can change the bacterial community structure of the rhizosphere soil and its endosphere and improve the biomass of the remediation plant, thus improving the efficiency of phytoremediation (Teng et al., 2015; Ma et al., 2016; Ashraf et al., 2017). However, microbes have a strong dependence on the environment, and changes in environmental conditions can modulate the effects of microbes. To date, studies related to the plant-PGPB remediation of heavy metal pollution in soil have primarily been performed under conditions that are relatively easy to control, such as in pots or in a greenhouse. Few studies have examined the effects of these strains under complex field conditions, limiting the practical application of combined plant-PGPB remediation (Zhuang et al., 2007; Dary et al., 2010; Kong and Glick, 2017). The remediation field trials conducted by Prapagdee and Khonsue (2015) and Dary et al. (2010) showed that the inoculation of metal-resistant

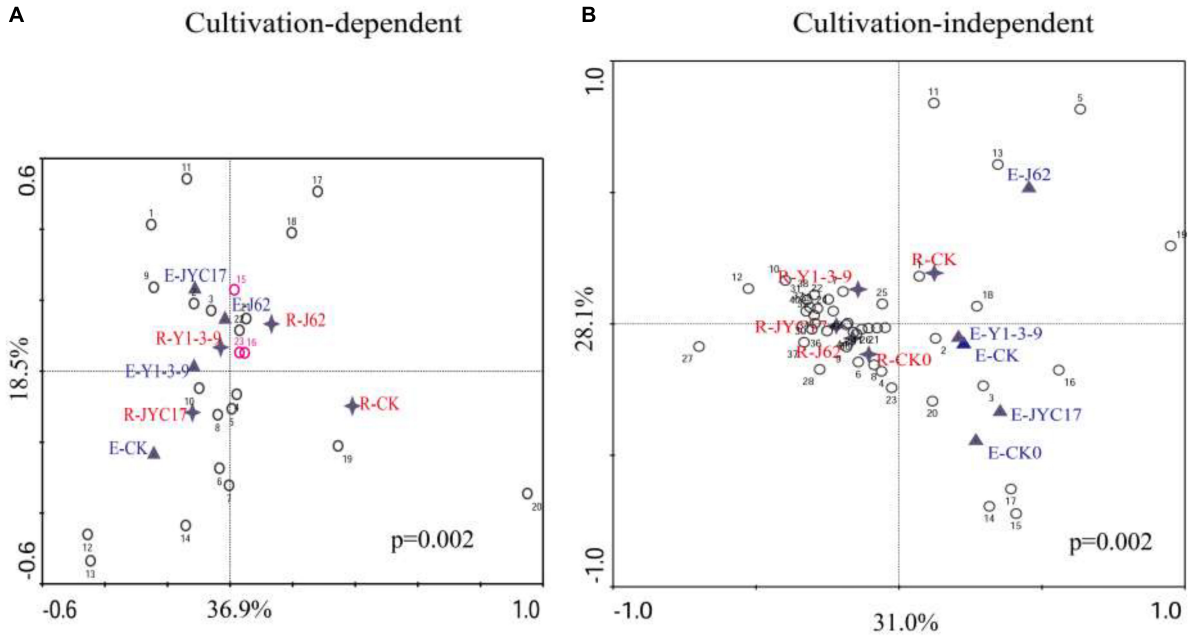

FIGURE 6 | Canonical correspondence analysis (CCA) of DGGE bacterial community profiles from panel **(A)** DNA extracted from biomass accumulated on an agar plate (cultivation-dependent, **Figure 5A**) and panel **(B)** DNA extracted directly from endosphere and rhizosphere samples (cultivation-independent, **Figure 5B**). The values on the axes indicate the total variation percentages explained by each axis. **(A)** Endosphere samples from the 50-day uninoculated control (E-CK), 50-day inoculation with JYC17 (E-JYC17), 50-day inoculation with Y1-3-9 (E-Y1-3-9), and 50-day inoculation with J62 (E-J62); rhizosphere samples from the 50-day uninoculated control (R-CK), the 50-day inoculation with JYC17 (R-JYC17), the 50-day inoculation with Y1-3-9 (R-Y1-3-9), and the 50-day inoculation with J62 (R-J62). The digital numbers represent 23 bands from the cultivation-dependent profile (**Figure 5A**). **(B)** Endosphere samples from the 0-day uninoculated control (E-CK0), the 50-day uninoculated control (E-CK), the 50-day inoculation with JYC17 (E-JYC17), the 50-day inoculation with Y1-3-9 (E-Y1-3-9), and the 50-day inoculation with J62 (E-J62); rhizoplane samples from the 0-day uninoculated control (R-CK1), the 50-day uninoculated control (R-CK2), the 50-day inoculation with JYC17 (R-JYC17), the 50-day inoculation with Y1-3-9 (R-Y1-3-9), and the 50-day inoculation with J62 (R-J62). The digital numbers represent 43 bands from the culture-independent profile (**Figure 5B**).

PGPB improved the biomass of the remediation plant *L. luteus* and achieved the purpose of increasing or decreasing the metal accumulation in *O. gratissimum* and *L. luteus*. The experimental results showed that the combined plant-PGPB remediation of heavy metal pollution in soil has a certain practical value under field conditions. In our previous pot trials, metal-resistant PGPB J62 and Y1-3-9 reduced the Cd stress in rape and increased the total amount of Cd uptake by the plant. The cumulative amount was increased from 6.70 to 40.60% compared with the corresponding control (Chen et al., 2013). The results of the combined remediation field trials showed that inoculating with the tested bacteria promoted rape growth, increased the dry weight of the above-ground tissues and roots, affected the Cu concentration in the rape and increased the total amount of Cu uptake. The total Cu uptake in whole plants inoculated with JYC17, Y1-3-9, and J62 was increased by 113.38, 66.26, and 67.91%, respectively. PGPB can directly or indirectly dissolve insoluble heavy metals through various metabolic activities (Sessitsch et al., 2013). Strains Y1-3-9 and J62 can activate water-soluble Cd in the soil in pot experiments (Chen et al., 2013). Similarly, strains Y1-3-9 and J62 can increase the contents of water-soluble Cu in rape rhizosphere soil under field conditions. The above results indicate that strains JYC17, Y1-3-9, and J62 are effective at promoting the phytoremediation of heavy metal pollution in soil under field conditions and that the rape remediation system and strains Y1-3-9 and J62 have good adaptability, which can be applied to different conditions (potted plants and fields) for different heavy metal types (Cu and Cd pollution), showing a certain application value. However, the current experiments were conducted only for a single rape crop in a single soil contaminated with heavy metals. Experimental research to account for different crops, soil types, and growing environments is needed to investigate the combined remediation of heavy metal pollution in the soil using strains JYC17, Y1-3-9, and J62 under field conditions.

Heavy metals in the soil often cause direct or indirect damage, inhibit the growth of remediation plants, and affect the efficiency of phytoremediation. Studies have shown that PGPB can alleviate oxidative stress and reduced chlorophyll synthesis, impact other physiological and biochemical indicators caused by heavy metals, and improve the adaptability of remediation plants to heavy metal pollution (Tak, 2015; Pan et al., 2016; Chiboub et al., 2018; Rizvi and Khan, 2018). Saleem et al. (2018) found that Pb-tolerant PGPB increased the biomass and Pb uptake of sunflower remediation plants in pot experiments. Simultaneously, the indicators of physiological and antioxidant activities showed that PGPB increased the contents of chlorophyll "a," chlorophyll "b," carotenoids, ascorbate peroxidase, catalase, superoxide

dismutase, glutathione reductase, and proline in sunflowers that were exposed to Pb contamination. These indicators play an important role in improving the photosynthetic activities and alleviating the oxidative stress caused by heavy metals. Similarly, this experiment determined the effects of inoculating the rape with PGPB on the antioxidant indicators under field conditions. The results showed that PGPB inoculation increased the contents of the non-enzymatic antioxidants ASA and GSH by 40.24–91.22% and 9.89–17.67%, respectively. Studies have shown that ASA and GSH are the most important antioxidants in plants, and they play important roles in scavenging the toxic ROS caused by heavy metal stress. The increased contents of these compounds help plants to tolerate various stresses. This result is similar to the findings of Pan et al. (2016) and Islam et al. (2014), who showed that inoculation by the tested bacteria increased the GSH and ASA contents and alleviated the oxidative stress caused by heavy metals. The TBARS, SOD, and POD contents in plants reflect the changes in ROS and hydrogen peroxide free radicals in plant tissues, so they can be used as indicative physiological indicators of plant stress. The TBARS and POD contents in the rape leaves treated with JYC17, Y1-3-9, and J62 were decreased to different degrees compared with the control, indicating that the oxidative stress caused by heavy metals was alleviated in the rape after the inoculation treatment.

Microorganisms are widely distributed in the plant rhizosphere and endosphere, and they play important roles in the phytoremediation of heavy metal-contaminated soil (Ashraf et al., 2017). PCR-DGGE is a modern molecular biology technology for studying microbial communities. It is widely used in microbial ecology investigations in different ecological environments such as plant samples (de Melo Pereira et al., 2012; Chen et al., 2013) and soil (Che et al., 2015; Orlewska et al., 2018). Rhizosphere and endosphere colonizations of PGPB in plants are the primary conditions for its effect. Because of its technical sensitivity, PCR-DGGE technology has been used to study strain colonization in soils or plants (Andreote et al., 2009; Chen et al., 2013; Rilling et al., 2019). Using the PCR products of strains JYC17, Y1-3-9, and J62 as the DGGE markers, and based on the positions of the corresponding bands on the cultivation-dependent and cultivation-independent DGGE profiles and the sequencing information for bands A15, A16, and A23 (**Supplementary Table S1** and **Figure 5**), it was judged that the tested bacteria colonized the rape roots and rhizosphere under field conditions, consistent with the colonization results obtained for strains J62 and Y1-3-9 in the rape rhizosphere and endosphere during previous potting trials (Chen et al., 2013). Studies concerning the interaction of PGPB with microorganisms in soil or the plant endosphere during combined plant-microbe remediation, as well as the impact on the composition of the microbial community in soil, have also been conducted (Jeong et al., 2013; Marques et al., 2013; Liu et al., 2015). Liu et al. (2015) showed that the inoculation of heavy metal-tolerant PGPB during the remediation of Cd-contaminated soil by *S. plumbizincicola* reduced the diversity of the rhizosphere microbial community. Cultivation-dependent bacteria play an important role in the microbial community and its activity. In traditional studies that have examined the composition of cultivation-dependent bacterial communities, cultivation-dependent bacteria are generally first enriched and cultured in a medium, followed by separation, purification, and identification, which is time-consuming and laborious. Based on the results reported by Ellis et al. (2003), the total DNA from bacteria growing on a plate were extracted, and a PCR-DGGE analysis was performed to analyze the effects of the tested bacteria quickly and accurately on the composition of the microbial community of the cultivation-dependent bacteria in the rape rhizosphere soil and root endosphere (**Supplementary Table S1** and **Figure 5**). Using CCA and similarity clustering analyses of the cultivation-dependent DGGE profile, it was concluded that the tested bacteria inoculated with JYC17, Y1-3-9, and J62 under field conditions affected the composition of the cultivation-dependent bacterial communities in the rape root endosphere and rhizosphere soil and that the effects of the different strains were different. These results were consistent with previous findings demonstrating that the cultivation-dependent bacterial community composition of the rape rhizosphere and endosphere was affected by strains J62 and Y1-3-9 under potting conditions (Chen et al., 2013). Similar to the change in the cultivation-dependent bacterial community composition, the cultivation-independent DGGE profile results showed a significant difference between the rape root and rhizosphere. Inoculation with the test bacteria affected the composition of the bacterial communities in the endosphere and rhizosphere to different extents. These results are similar to previous findings showing that inoculation by plant growth-promoting rhizobacteria can alter the bacterial community composition of the plant rhizosphere or endosphere (Andreote et al., 2010; Chen et al., 2013; Liu et al., 2015). These changes in the community composition, especially the colonization of the test bacteria JYC17, Y1-3-9, and J62 with good growth-promoting abilities in the rape roots and rhizosphere, significantly enhanced the proportion of growth-promoting bacteria among the total cultivation-dependent bacteria, which may play an important role in the heavy metal stress tolerance of rape.

CONCLUSION

The results of the *in situ* remediation experiments on rape-promoting bacteria in the Cu-contaminated farmland around heavy metal mine tailings showed that metal-resistant PGPB increased the rape biomass and the total amount of Cu uptake, thus enhancing the Cu enrichment efficiency of rape. Simultaneously, the mechanism of this effect was preliminarily analyzed from the perspectives of the plant physiology and the microbial community structure. The results showed that the test bacteria JYC17, Y1-3-9, and J62 had good growth-promoting abilities, colonized the rape rhizosphere and endosphere, and altered the bacterial community composition of the rape rhizosphere and endosphere. Concurrently, they increased the ASA and GSH contents in the rape and reduced the oxidative stress caused by heavy metals. The above results indicate that the plant-promoting bacteria JYC17, Y1-3-9, and J62 are effective at promoting the phytoremediation of heavy metal pollution in

soil under field conditions, and they could have value for use in specific applications.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

Z-JC designed the experiments and contributed to writing the manuscript. X-MR and S-JG performed the experiments and analyzed the data. HH and YC performed the field sampling. WT and EC measured the heavy metals concentrations in the rape. B-LL and Y-YL reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01455/full#supplementary-material

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Seasonal Microbial Community Characteristic and Its Driving Factors in a Copper Tailings Dam in the Chinese Loess Plateau

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A combined soil bacterial and fungal community survey was conducted for a copper tailings dam in the Chinese Loess Plateau. We investigated the seasonal differences in the composition and function of soil microbial community to examine the key environmental factors influencing soil microorganisms during restorative ecological processes. Significant seasonal differences were found in the community structure of both bacterial and fungal communities. Bacterial community abundance and fungal community (Shannon index) measurements were highest in summer. Soil nitrite nitrogen (NO_2^--N) was the dominant factor influencing both bacterial and fungal communities. The bacterial community composition was significantly affected by NO_2^--N and ammonium nitrogen (NH_4^+-N) in spring, and fungal community structure was significantly affected by soil water content in autumn. Moreover, the fungal community exhibited significant functional feature differences among seasons, whereas bacterial community functional groups remained similar. This study aimed to clarify the adaptation response of microbes applying different approaches used in ecological restoration approaches specific to mining areas, and to identify the natural biofertility capacity of the microbial communities that colonize soil ecosystems.

Keywords: soil bacterial, soil fungal, structure and function, seasonal dynamics, copper tailings dam

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INTRODUCTION

Microorganisms are important in the exchange of mass and energy among the atmosphere, lithosphere, hydrosphere, and biosphere, and they play important roles in global ecological restoration, environmental variation and monitoring, pollution treatments, and biological conservation (Zhu et al., 2017). Microorganisms are ubiquitous throughout all environments, and they are especially important in the restoration of degraded ecosystems in mining areas (Jia et al., 2019). Soil microbes are also important in maintaining the structure of soil, the decomposition of organic matter, the cycle of geochemistry, and the supply of nutrients (Roesch et al., 2007; Falkowski et al., 2008; Douterelo et al., 2010). As an important

component of soil ecosystems, bacteria are essential for the carbon cycle and plant productivity (Johnson et al., 2003). The soil bacterial community composition and diversity are correlated to soil organic carbon transformation processes (Xiao et al., 2015). Fungi are important participants in the decomposition of organic matter and mineral components (Hollister et al., 2010). The magnitude of soil microbial availability is closely associated with the ecological environment they inhabit (Yan et al., 2018). Therefore, any change in the microbial communities of soil can lead to changes in various biochemical processes, thus affecting the stability of degraded ecosystems in mining areas, while also playing a vital role in the efficiency of ecological restoration. Over the course of a year, soil microbes face considerable variation in seasonal environmental factors, including temperature and humidity levels (López-Mondéjar et al., 2015) and the availability of nutrients (Du et al., 2018; Wang et al., 2018). Hence, soil microorganisms are often contingent on dynamic seasonal change characteristics. The seasonal variation in the biomass and structure of soil microorganisms also significantly differs among different ecosystems because the dominance of different environmental factors and the complexity of the comprehensive effects associated with various environmental factors vary among environments (Voříšková et al., 2014; López-Mondéjar et al., 2015).

In the damaged ecosystem of nonferrous mines, a large amount of heavy metals are disposed directly into soil along with waste rock, tailings, and other mineral dust in mining districts and their surrounding areas, which subsequently become the main source of environmental pollution. For instance, the Northern Copper Mine, the largest underground copper mine in China, has an annual output of greater than 7 million tons of ore (Jia et al., 2019). The extensive accumulation of tailings has led to severe pollution and the degeneration of the local ecological environment (Wang et al., 2010). Accordingly, a resolution to this problem is essential, and the best way to resolve this is to effectuate the reasonable and efficient ecological restoration of the copper tailings dam. The ecological functional recovery of soil is the key to such restoration as well as the sustainable development of terrestrial ecosystems (Wang et al., 2013; Jia et al., 2017). Thus, ecological restoration must also focus on soil fertility and the characteristics of dynamic change associated with microbial communities over the course of a whole year.

Although many studies have shown that anthropogenic activities can cause changes in soil microbial structure and diversity, there have been very few studies to date that have reported on the seasonal microbial community characteristics and its driving factors in copper tailings dams. Studies on the seasonal dynamics of the microbial community can improve our knowledge of microbial community ecology in contaminated environments and help to design and implement potential bioremediation strategies by which to address the progressively increasing impacts of xenobiotics in ecosystems (Hoostal and Bouzat, 2016). Therefore, the relationships between environmental conditions and seasonal variations of microbial communities within degraded tailings dam ecosystems warrant further investigation. This study will help to clarify the key environmental factors that affect soil microbial communities during ecological restoration processes in copper tailings dams. Moreover, our experiment explored effective biological indicators in each season during ecological restoration processes in a mining area, while revealing the regular energy transformation and material circulation patterns in soil.

To achieve this objective, we addressed the following questions: (1) How do soil bacterial and fungal communities vary with season changes? (2) What are the special functional bacterial and fungal in a copper tailings dam? (3) What are the dominant environmental factors that affect soil microbial structure and diversity over the course of a year? The aim of this study was to provide an ecological basis for the mechanisms of soil ecosystem restoration and degradation in different seasons, and to strengthen our understanding of soil property and microbial community biodiversity restoration in an environment subjected to pollution.

MATERIALS AND METHODS

Site Description

Construction on the 18 river tailings (latitude 35°15′ ~ 35°17′ N, longitude 118°38′ ~ 111°39′ E) commenced in 1969, which is a constituent of the Northern Copper Mine, situated in the southern region of the province of Shanxi in China (**Figure 1**; Jia et al., 2019). Currently, this copper tailings dam comprises of 16 sub-dams. The study area is marked by four distinct seasons that are contingent on a continental "monsoon" climate. The duration of annual rainfall and temperature information of the study area was shown in **Supplementary Table S1**.

Soil Sampling

The number No. 536 sub-dam of the 18 river tailings dam was selected in July 2017 for investigation. This sub-dam, in its 20th year of restoration, was used for sampling during all four seasons, namely, spring (March), summer (June), autumn (September), and winter (December; Jia et al., 2019). We randomly collected three soil samples from 1 × 1 m plots during each of the four seasons, and each sample plot was spaced greater than 50 m apart. Fresh soil samples were divided into two subsamples after being shifted using a 2 mm sieve. We stored the first subsample (4°C) prior to determining physiological and chemical properties, while we stored the second subsample (−20°C) prior to DNA extraction.

Chemical Properties of Soil

Total carbon (TC), total nitrogen (TN), and total sulfur (TS) content of soil samples were measured using an elemental analyzer (vario EL/MACRO cube, Elementar, Hanau, Germany). Soil water (1:2.5 mass/volume) suspensions were shaken for 30 min prior to measuring soil pH. Gravimetric analysis was used to measure soil moisture. Ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), and nitrite nitrogen (NO_2^--N) in

FIGURE 1 | The panorama of study area **(A)** and the profile **(B)** of sub-dams in copper tailings dam.

soil were measured using the Automatic Discrete Analyzer (CleverChem 380, DeChem-Tech, GmbH, Hamburg, Germany; Jia et al., 2018).

Techniques Used for DNA Extraction, PCR Amplification, and MiSeq Sequencing

We used the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) to extract soil microbial DNA using the manufacturer's protocol. Extracted DNA was quantified using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Primers 515F (5'-GTG CCAGCMGCCGCGG-3') and 907R (5'-CCGTCAATTCMTTT RAGTTT-3') were used to amplify the V4-V5 hyper variable region of the 16S ribosomal RNA (rRNA) bacterial gene. Primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') were used to determine the fungal internal transcribed spacer (ITS) gene copy number of all samples. Finally, sequencing was carried out at Shanghai Majorbio Bio-pharm Technology (Shanghai, China) using the MiSeq platform (Illumina, Inc., USA). The bacterial and fungal sequences have been deposited in the SRA of the NCBI database under the SRA accession: PRJNA600330 and PRJNA605500.

Processing of Sequencing Data

Raw FASTQ files were demultiplexed and quality-filtered using QIIME (version 1.17) under the following criteria: 300-bp reads were truncated at any site receiving an average quality score of <20 over a 50-bp sliding window, and truncated reads shorter than 50-bp were discarded; exact barcode matching, two-nucleotide mismatch in primer matching, and reads containing ambiguous characters were removed; and only sequences that overlapped for more than 10-bp were merged according to their overlap sequence. Reads that could not be merged were discarded. Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (version 7.1, http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene and ITS gene sequences respectively were analyzed using the Ribosomal Database Project (RDP) Classifier[1] against the Silva (SSU128) 16S rRNA and unite 7.0 database with a confidence threshold of 70%.

Statistical Analysis

SPSS Statistics version 20 was used to calculate the data derived from the analyses discussed above. Heatmapping of the top 10 genera in each sample was conducted using the R packages. We used non-metric multidimensional scaling (NMDS) and analysis of similarities (ANOSIM) analysis to investigate differences in bacterial and fungal community structure. Furthermore, we used redundancy analysis (RDA) or canonical correspondence analysis (CCA) to analyze relationships between microbial and environmental factors using Canoco 5.0 (Microcomputer Power, USA). Additionally, one-way ANOVA was used to analyze differences in environmental parameters as well as the alpha diversity (α-diversity) indices and the relative species abundance of the predominant bacterial and fungal species among the four seasons. Structural equation models (SEM) were analyzed using advanced mortar system (AMOS) version 13.0.

RESULTS

Overall Taxonomic Distribution and Microbial Diversity

We extracted DNA from soil samples in the copper tailings dam during all four seasons. Moreover, we used the MiSeq platform to sequence 16S/ITS rRNA genes. For all samples, we obtained a total of 504,999 and 608,880 quality-filtered and chimera-checked 16S/ITS rRNA gene sequences with respective average lengths of 396 and 266 bp. Per sample, we recovered from 32,628 to

[1]http://rdp.cme.msu.edu/

56,152 16S rRNA sequences and from 30,507 to 71,422 fungal ITS rRNA sequences. We also obtained a total of 2,982 bacterial OTUs and 810 fungal OTUs from soil samples based on 97% sequence similarity, which suggests that the sequencing data reflected most microbial diversity in the field. Across all samples, taxonomically classified bacterial OTUs were representative of 32 phyla, 79 classes, 171 orders, 317 families, and 5,629 genera, while fungal OTUs were representative of 6 phyla, 25 classes, 65 orders, 138 families, and 250 genera. The Venn diagram (**Supplementary Figure S1**) reveals that 1884 OTUs were common to bacterial communities, while 150 were common to fungal communities. We compared bacterial and fungal community richness estimators [i.e., Chao1 and abundance-based coverage estimator (ACE)] and diversity index (i.e., Shannon and Simpson) values among the different seasons (**Table 1**). Richness estimators suggested that bacterial community abundance in summer was significantly higher than corresponding values in winter. Shannon indexes showed that fungal communities in summer were higher than in autumn. In addition, Simpson indexes showed that fungal community diversity in summer was significantly higher than those of the other seasons, whereas bacterial community diversity was not significantly different among the four seasons (**Table 1**).

Comparison Between Bacterial and Fungal Communities Among Seasons

The dominant species comprising the bacterial and fungal communities were generally consistent among seasons; however, differences were found in the relative abundance. *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, and *Chloroflexi* were the bacterial phyla with the highest relative abundances (**Figure 2A**). *Ascomycota* and *Basidiomycota* were the dominant fungal phyla (**Figure 2B**). At the order level, the dominant bacterial and fungal community members were *Rhizobiales* and *Pleosporales*, respectively (**Figures 2C,D**). Bray-Curtis dissimilarity based on NMDS and ANOSIM was determined to reveal the dissimilarity of microbial communities among each season (**Figure 3**). ANOSIM revealed significant differences in both bacterial (stress = 0.072; $R = 0.435$; $p = 0.004$) and fungal (stress = 0.088; $R = 0.398$; $p = 0.006$) community structure among the different seasons (**Figure 3**). Moreover, linear regression analysis revealed that fungal and bacterial community composition was significantly positively correlated ($p < 0.001$; **Figure 4**). The top 10 dominant microbial families are shown in **Figure 5**. For the bacterial community, we observed significant differences in norank_c_*Acidobacteria* and *Hyphomicrobiaceae* at a family level (**Figure 5A**). For the fungal community, we observed significant differences in Teratosphaeriaceae and unclassified_c_*Leotiomycetes* at the family level during all four seasons (**Figure 5B**).

We used the PICRUSt and FUNGuild tools to better understand the important roles that bacteria and fungi play in copper tailings dams, respectively (**Figure 6**). Bacteria exhibited similar functional features during different seasons (**Figure 6A**). These functional features mainly included those related to energy production and conversion processes, amino acid transportation and metabolic processes, carbohydrate transportation and metabolic processes, and transcription processes as well as biogenetic and signal transduction processes associated with cell wall, membrane, and envelope mechanisms (**Figure 6A**). However, we observed significant differences in fungal functions among the four seasons. Plant pathogens accounted for 49.8% of the soil fungal OTUs detected in autumn (**Figure 6B**). Animal pathogen, endophyte-lichen parasite, plant pathogen, soil saprotroph, wood saprotroph (26.4%), and dung saprotroph (14.0%) were significantly higher in spring compared with the other three seasons, and parasitic fungi (9.4%) had the highest relative abundance in autumn (**Figure 6B**). Orchid mycorrhizae were only observed in summer. Ectomycorrhizae mainly occurred in winter and spring (**Figure 6B**).

Microbial Community Structure and Environmental Variable Correlations

Soil characteristics varied seasonally (**Table 2**). Soil nutrients (TN and TC) were significantly higher in summer compared with the other seasons ($p < 0.05$), and pH was highest in winter (**Table 2**). Moreover, NO_2^--N was significantly higher in autumn than in spring and summer ($p < 0.05$), but no significant differences were observed in winter. In autumn, the soil water content (SWC) was significantly lower than the other seasons ($p < 0.05$; **Table 2**). Our experiment evaluated the effects of these ecological factors on microbial community structure during different seasons (**Figure 7**). It was found that 36.95% of bacterial

TABLE 1 | Comparison between phylotype coverage and diversity estimators of soil microbial communities of the four seasons.

	Sample	Reads[a]	OTUs[b]	Coverage	Richness estimator		Diversity index	
					ACE	Chao1	Shannon	Simpson
Bacterial	Spring	31109A	1946A	0.987A	2240.01AB	2270.93AB	6.1608A	0.0141A
	Summer	23760B	2049A	0.978B	2443.79A	2447.91A	6.4742A	0.0035A
	Autumn	26604AB	1877A	0.982AB	2261.42AB	2249.20AB	6.2714A	0.0048A
	Winter	26438AB	1856A	0.984A	2145.00B	2152.60B	6.2772A	0.0052A
Fungal	Spring	68327a	268ab	0.9995a	289.50b	288.52b	2.984ab	0.097b
	Summer	36257b	327a	0.9980c	390.55a	383.70a	3.339a	0.087b
	Autumn	34340b	217b	0.9984b	268.37b	264.53b	2.421b	0.197a
	Winter	62343a	244ab	0.9994a	272.45b	273.60b	3.056ab	0.091b

[a]*Provides reads after trimming and chimera removal. The coverage percentage, richness estimators (ACE and Chao1), and diversity indices (Shannon and Simpson) were calculated using the Good's method and the Mothur program, respectively.*

[b]*Operational taxonomic units (OTUs) were defined at a 97% level of similarity. Significant differences between seasons are denoted with letters. (bacteria: A > B > C; fungal: a > b > c).*

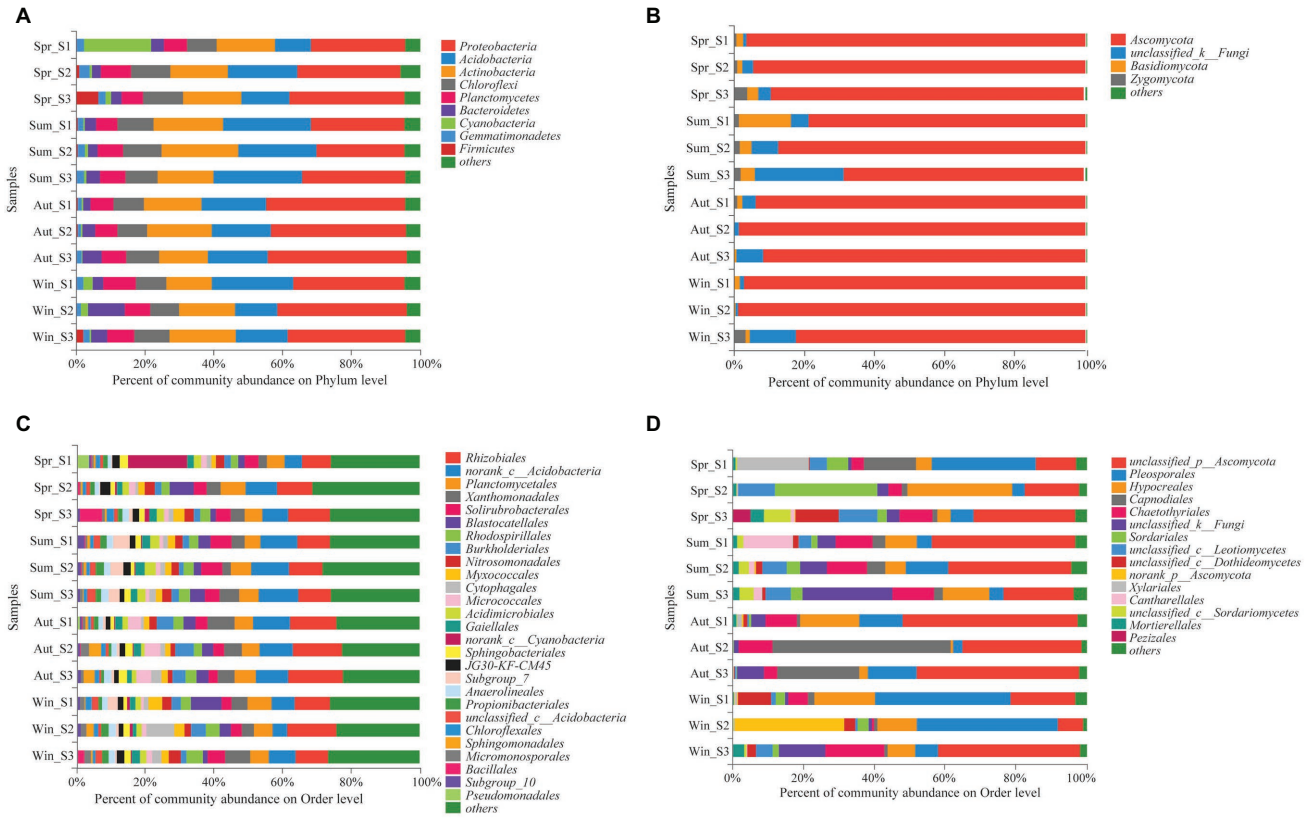


FIGURE 2 | Relative abundance of the dominant phyla **(A,B)** and orders **(C,D)** of bacterial **(A,C)** and fungal **(B,D)** communities in soil (with average relative abundance >2%) among different seasons.

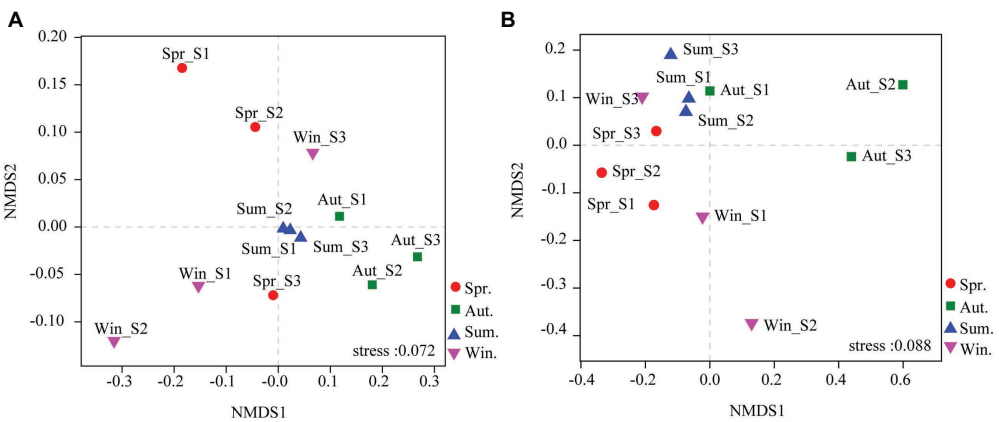


FIGURE 3 | Non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis similarities of bacterial **(A)** and fungal **(B)** community compositions at four seasons. The results indicate a significant influence of season on bacterial and fungal community structures [assessed by a multivariate analysis of similarities (ANOSIM)].

variation could be explained by soil properties (**Figure 7A**). Axis 1 of the RDA plot explained roughly 30.64% of variation, while Axis 2 explained a further 6.31%. The RDA results showed that NH_4^+-N, NO_3^--N, NO_2^--N, and TN were the major controls on the bacterial community structure (**Figure 7A**). Soil properties could explain 27.9% of the variability in fungal community structure (**Figure 7B**), where Axis 1 of the CCA plot explained 15.21% of the variability and Axis 2 explained a further 12.69%. Four soil characteristics were chosen for CCA after redundant variables were removed. As shown in **Figure 7**, SWC, soil temperature (ST), NO_2^--N and pH significantly affected fungal community structure (**Figure 7B**).

The correlation heatmap (**Figure 8**) showed that the relationship between microbial composition and environmental

factors differed between bacterial and fungal communities. For the bacterial community, strains *Nitrospira* and *SBR2076* exhibited significant negative correlations to pH, whereas Phycisphaerae and *Cyanobacteria* were positively correlated to pH. Phycisphaerae exhibited highly significant negative correlations to SWC, ST, salinity and EC (**Figure 8A**). *Acidobacteria* were both significantly and positively correlated to soil TN, TC, and C/N (**Figure 8A**). For the fungal community, both *Cystobasidiomycetes* and *Microbotryomycetes* abundance was positively correlated to pH. Both *Ustilaginomycetes* and *Microbotryomycetes* were negatively correlated to soil sulfur and ST (**Figure 8B**). We constructed a SEM to further quantify the contribution of driving factors to microbial communities (**Figure 9**). Soil NO_2^--N was the dominant factor influencing both the bacterial and fungal communities. Moreover, interactions between NO_3^--N and NH_4^+-N were direct and significant; however, no bacterial and community interactions were observed in soil (**Figure 9**).

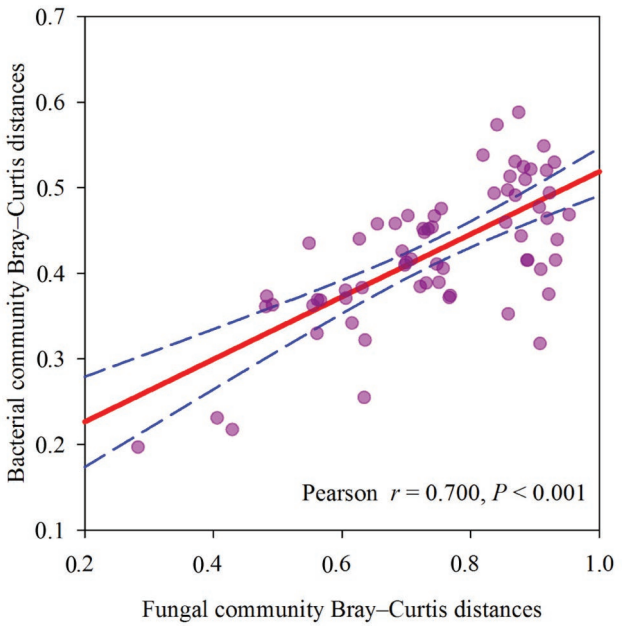


FIGURE 4 | Relationship between soil fungal community Bray-Curtis distances and soil bacterial community Bray-Curtis distances, measured at plot scale across different seasons.

DISCUSSION

Soil Microbial Diversity in a Copper Tailings Dam

Ecological soil functions are based on soil microbial communities. Soil microbes affect soil nutrient cycling and regulation, and they can be used as indicators of soil functions through their participation in soil organic matter's associated decomposition and mineralization processes (Romaniuk et al., 2011). In this study, bacterial richness, fungal richness, and the Shannon index reached their maximum in summer, indicating that this specific season exhibited the highest overall soil microbial activity. This could be because plant photosynthesis was strong in summer. In other words, photosynthetic products enter the soil through root systems, and these products are used by microorganisms as a nutrient source, which promote microbial growth and reproduction (López-Mondéjar et al., 2015). Another possible explanation is that seasonal changes in ST also affect microbial growth. The copper tailings area investigated for this study is warm in summer and cold in winter. In general, microbial activity and species richness increase with an increase in temperature (Sierra et al., 2015), whereas microbial activity decreases with a decrease in temperature (Lipson et al., 2002). This is probably because of a reduction in the lipid fluidity of microbial membranes, resulting in the freezing of intracellular fluids as well as the rupture and death of cells (Yergeau and Kowalchuk, 2008). Therefore, high temperatures in summer provide a relatively stable environment for soil microbial growth.

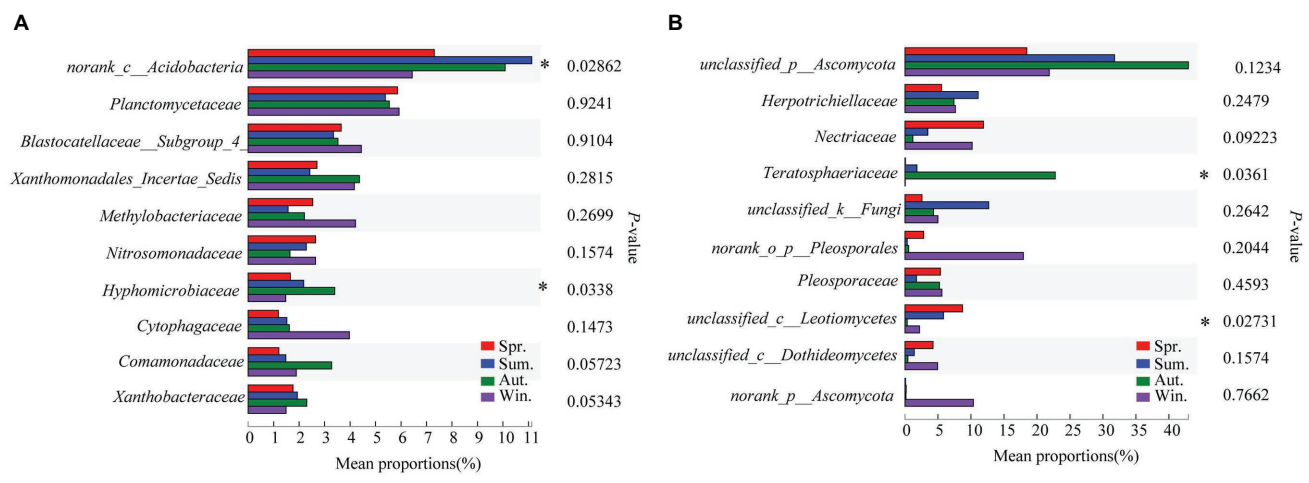


FIGURE 5 | Relative abundances of top 10 bacterial **(A)** and fungal **(B)** families that showed significant differences among seasons. Kruskal-Wallis H test was used to evaluate the significance of differences between the indicated groups. *$p < 0.05$.

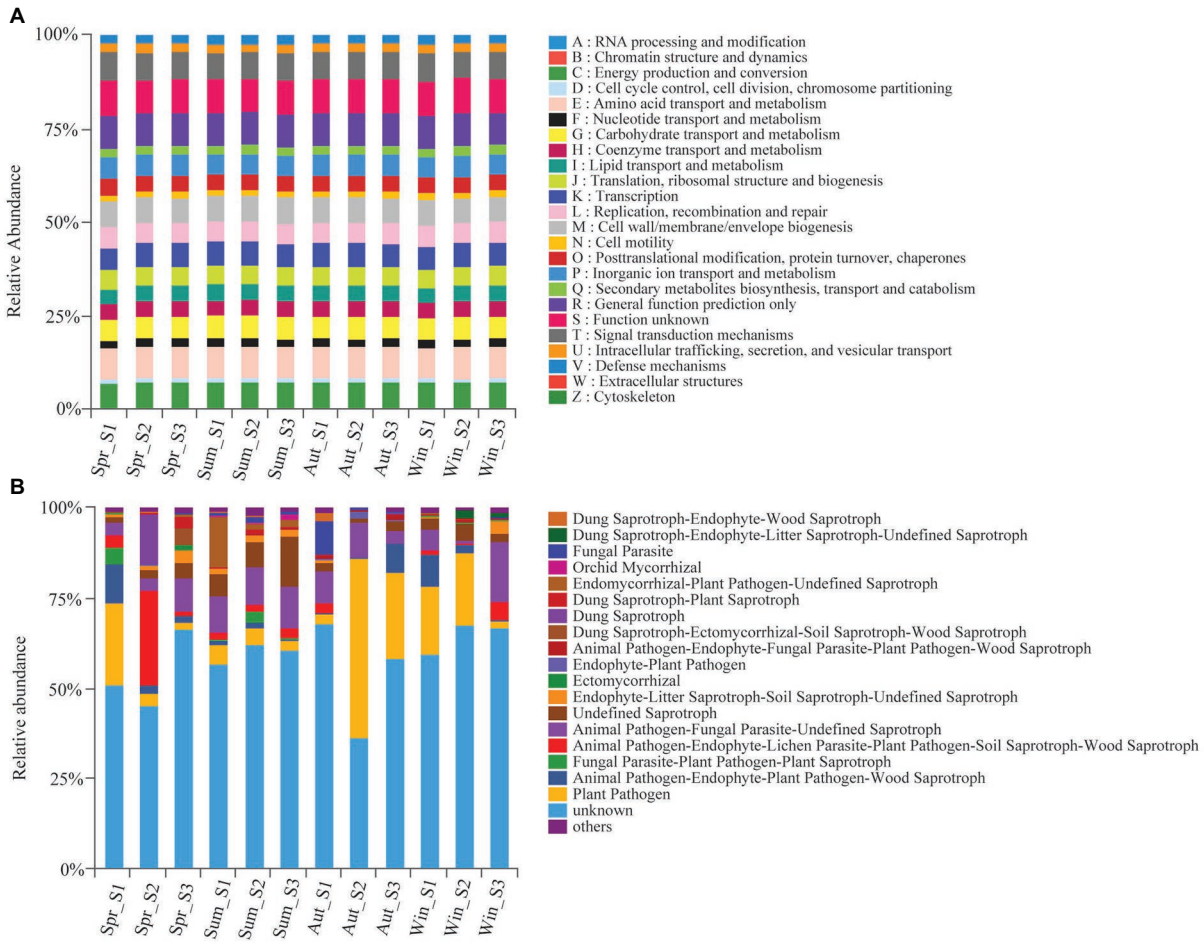


FIGURE 6 | Variations in composition of bacterial **(A)** and fungal **(B)** functional groups inferred by the PICRUSt[10] and FunGuild, respectively.

TABLE 2 | Soil chemical properties of copper tailings dam.

Physical and chemical factors	Spring	Summer	Autumn	Winter
SWC	10.52 ± 0.337b	0.84 ± 0.036c	86.16 ± 0.213a	9.10 ± 1.675b
pH	8.20 ± 0.028b	8.20 ± 0.017b	8.11 ± 0.029b	8.89 ± 0.038a
NH_4^+-N/mg·kg^{-1}	0.40 ± 0.109	0.23 ± 0.086	0.43 ± 0.075	0.19 ± 0.105
NO_3^--N/mg·kg^{-1}	0.15 ± 0.064	0.07 ± 0.008	0.13 ± 0.007	0.11 ± 0.018
NO_2^--N/mg·kg^{-1}	0.01 ± 0.000b	0.01 ± 0.000b	0.01 ± 0.002a	0.01 ± 0.001ab
TN/g·kg^{-1}	0.04 ± 0.003b	0.08 ± 0.005a	0.05 ± 0.005b	0.05 ± 0.006b
TC/g·kg^{-1}	0.92 ± 0.033b	6.10 ± 0.969a	1.03 ± 0.041b	1.04 ± 0.116b
C/N	24.48 ± 2.024b	81.02 ± 16.746a	22.06 ± 1.474b	21.37 ± 2.060b
TS/g·kg^{-1}	0.06 ± 0.006	0.46 ± 0.295	0.14 ± 0.017	0.05 ± 0.002
ST/°C	16.83 ± 1.354b	24.27 ± 0.318a	26.87 ± 0.484a	8.10 ± 1.002c
Salinity/mg·L^{-1}	14.33 ± 3.712b	0.00 ± 0.000c	31.67 ± 1.202a	4.00 ± 1.000c
EC/μs·cm^{-1}	26.33 ± 6.766b	0.00 ± 0.000c	57.67 ± 1.667a	7.33 ± 1.856c

Values represent mean with standard error in parenthesis. Significant differences between sites (Duncan test, $p < 0.05$) are denoted with letters (a > b > c). SWC, soil water content; NH_4^+-N, ammonium nitrogen; NO_3^--N, nitrate nitrogen; NO_2^--N, nitrite nitrogen; TN, total nitrogen; TC, total carbon; TS, total sulfur; C/N, the ratio of carbon and nitrogen; ST, soil temperature; EC, electrical conductivity.

Soil Microbial Community Composition and Function

The dominant bacterial phyla and class in the copper tailings dam were *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, and *Chloroflexi*, which are consistent with the findings of Lauber et al. (2009). Our study found that seasonal variation significantly affected the dominant soil bacteria in the copper tailings dam. The relative abundance of *Bacteroidetes* was lower than

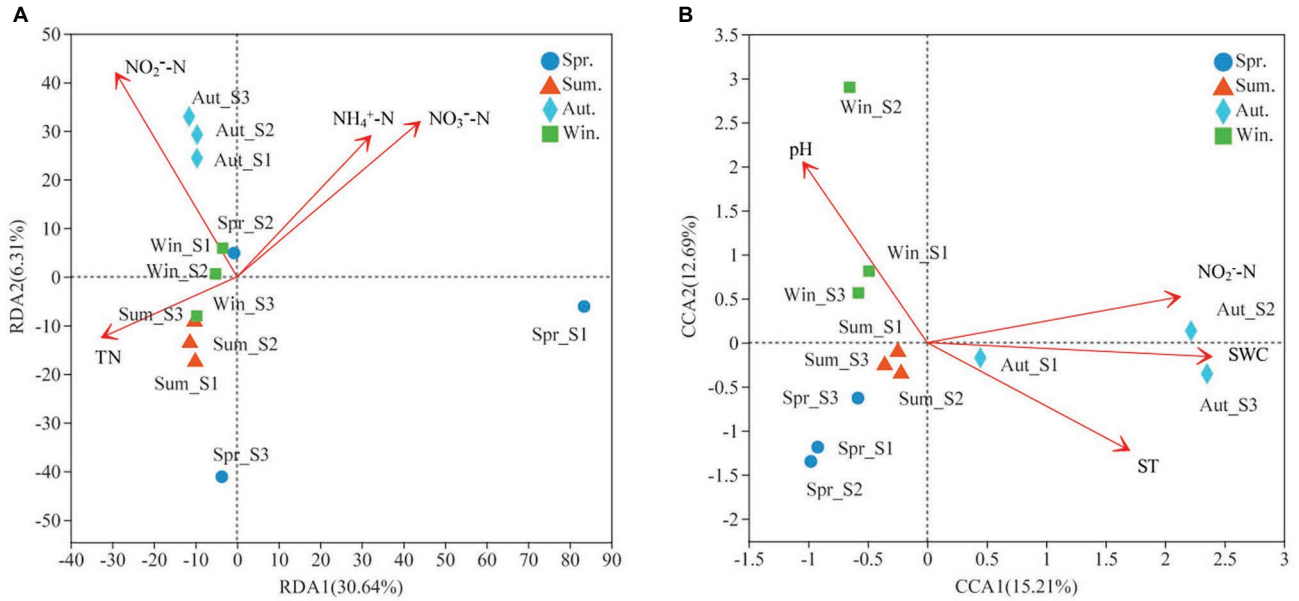


FIGURE 7 | RDA or CCA of the soil bacterial **(A)** and fungal **(B)** community structure and environmental variables at four seasons. Only the environmental variables which were significantly correlated with RDA1, or CCA1 were shown in figures.

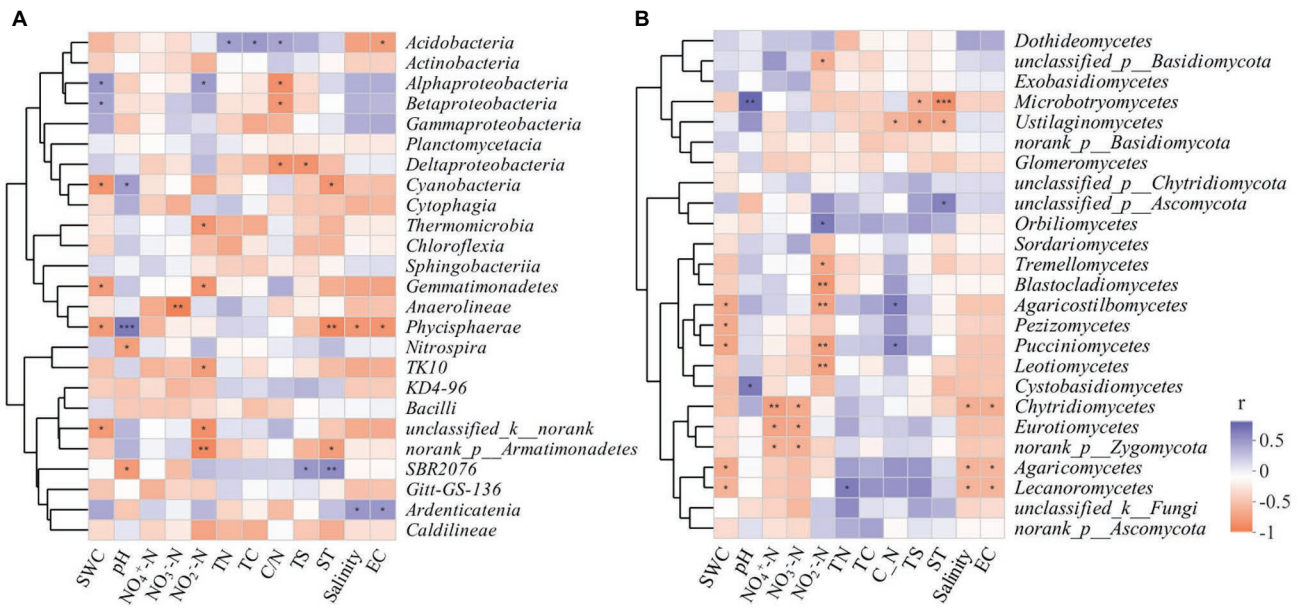
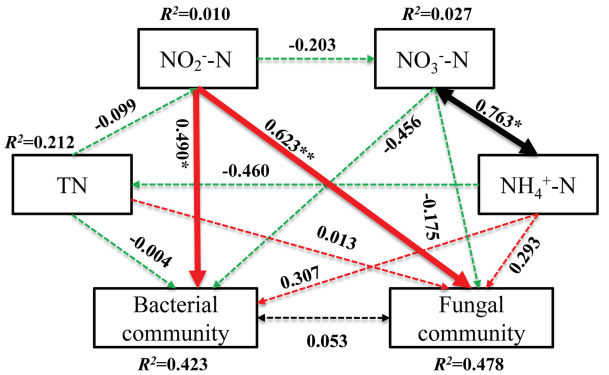


FIGURE 8 | Spearman correlation heatmap of the top 25 soil bacterial **(A)**, fungal **(B)** classes and soil properties. X and Y axis are environmental factors and classes. R in different colors to show, the right side of the legend is the color range of different r values. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Acidobacteria, and the relative abundance of *Bacteroidetes* was highest in winter (**Figure 2**). Margesin and Schinner (1994) reported that *Bacteroidetes* are cold-tolerant bacteria that have unique physiological mechanisms to resist low temperatures, which enables members of this phylum to subsist in cold environments. *Acidobacteria* are an oligotrophic bacterial phylum, while *Bacteroidetes* have entropic characteristics (Mccaig et al., 1999; Fazi et al., 2005; Dion, 2008). The results from this study could also have been caused by soil nutrient depletion in the copper tailings dam, and soil nitrogen becoming a limiting factor in the study area (**Table 2**).

Ascomycota and *Basidiomycota* were the dominant fungal phyla during all four seasons (**Figure 2B**). They were the main decomposers in soil, while also playing a vital role in



χ^2=1.360; *P*=0.507; GFI=0.962; AIC=39.360 ;RMSEA=0.000

FIGURE 9 | Structural equation model (SEM) illustrating the effects of soil properties on microbial communities. Continuous and dashed arrows represent the significant and non-significant relationships, respectively. Adjacent numbers that are labeled in the same direction as the arrow represents path coefficients, and the width of the arrow is in proportion to the degree of path coefficients. Green and red arrows indicate positive and negative relationships, respectively. Black arrows indicate integrations. R^2 values indicate the proportion of variance explained by each variable. Significance levels are denoted with $^*p < 0.05$ and $^{**}p < 0.01$. Standardized total effects (direct plus indirect effects) calculated by the SEM are displayed below the SEM. The low chi-square (χ^2), non-significant probability level ($p > 0.05$), high goodness-of-fit index (GFI > 0.90), low Akaike information criteria (AIC), and low root-mean-square errors of approximation (RMSEA < 0.05) listed below the SEMs indicate that our data matches the hypothetical models.

nutrient cycling. The fungal community exhibited clear seasonal change characteristics, which is consistent with the findings of Andreetta et al. (2012), which showed that microbial communities exhibited seasonal variability resulting from seasonal variation in environmental conditions. The relative abundance of *Basidiomycota* in the copper tailings dam was highest in summer, which is consistent with the findings of Chen et al. (2016). This is because the energy that flows from roots to soil promotes plant growth and subsequently variations in mycorrhizal fungi that surround the root zone area in summer. Most *Basidiomycota* can form mycorrhizae with roots, while only a limited number of *Ascomycota* members can form mycorrhizae. Moreover, most *Ascomycota* are saprophytes. In this study, the relative abundance of *Ascomycota* was high during all four seasons. The response of *Ascomycota* to environmental stress was relatively stable, because it was the dominant phylum in multi-contaminated and non-contaminated ecosystems, as shown by Ventorino et al. (2016).

The fungal community exhibited significant differences in functional features among the four seasons, but no clear differences in bacterial functional groups were observed in this study. The different seasonal responses between the community functions of soil bacteria and soil fungi could result from the lower sensitivity that fungi have to all environmental changes. This is because fungal generation is generally slower than that of bacteria, and thus responds more slowly to soil disturbances (Fiorentino et al., 2018). József et al. (2015) reported that the abundance of saprophytic fungi increases with an increase in temperature, while the abundance of ectomycorrhizal fungi generally decreases with an increase in temperature. Our study also showed that the abundance of saprophytic fungi was high in summer, and the abundance of symbiotic fungi (i.e., ectomycorrhizal fungi) was high in winter (**Figure 6**). This could be because rising temperatures result in an increase in microbial abundance along with organic matter decomposition in summer (Sistla et al., 2013). The role that saprophytic fungi play in organic matter decomposition is particularly important. Competition exists between functional fungal groups. Pathogenic and saprophytic soil fungi were closely bound to roots throughout both the non-growing and growing seasons. Ectomycorrhizal fungi (such as *Thelephoraceae* and *Tuberaceae*) gradually replaced other fungal groups and ultimately became the dominant fungal communities during the growing season (Jumpponen et al., 2010). However, the relative abundance of symbiotic fungi increased in winter in this study. This is likely to be because soil samples, excluding root tips or plant samples, were collected specifically for fungal community analysis. Moreover, spores and mycelia in soil were less affected by plant development.

Soil Microbial Community and Soil Factor Relationships

In this study, RDA analysis showed that the available soil nitrogen content significantly affected bacterial community structure (i.e., NH_4^+-N, NO_3^--N, and NO_2^--N). Soil available nitrogen was a leading factor in the seasonal variation of soil bacterial communities. This suggested that the adaptability of bacterial communities to soil nutrient availability varied, resulting in seasonal bacterial community variation. Hu et al. (2002) reported that the relative abundance of soil microbes was significantly positively correlated to soil nutrients. Increases in soil nutrients will promote the growth of microorganisms and thus, to a certain extent, microbial abundance represents the quality of soil biological fertility. Soil pH affects soil microbial biological activities by influencing physiochemical soil properties and the composition of the soil matrix. In our study, we found that Phycisphaerae and *Cyanobacteria* were positively correlated to pH. *Cyanobacteria* are known to colonize plant roots (Gantar et al., 2010; Lundberg et al., 2012), which can encourage plant growth (Prasanna et al., 2009). Meanwhile, *Cyanobacteria*, owing to its N-fixation ability, are a key source of inorganic N for plants (Franche et al., 2009). *Alphaproteobacteria* and *Betaproteobacteria* were positively correlated to soil C/N, and were significant negative correlations to SWC (**Figure 8A**). It has reported that *Proteobacteria* was the most stress-tolerant phylum under conditions of heavy soil contamination (Eva-Maria et al., 2011). Moreover, *Proteobacteria* have previously exhibited considerable diversity in morphology, physiology, and metabolic processes (Zavarzin et al., 1991), suggesting that this bacterial can adapt to different environments by physiological and metabolic regulation processes. We also found *Acidobacteria* were positively correlated to soil TC, TN, and C/N (**Figure 8A**). *Acidobacteria* can degrade complex lignin and cellulose to provide soil nutrients (Lynd et al., 2002; Pankratov et al., 2011).

Furthermore, studies have shown that variation in both long-term and short-term SWC can alter the soil fungal

community structure (Pasternak et al., 2013; Evans et al., 2014; Hartmann et al., 2017; Engelhardt et al., 2018). Our study found that ST, SWC, NO_2^--N, and soil pH were all important factors influencing soil fungal community variation (**Figure 7**). Moreover, *Agaricomycetes* and *Lecanoromycetes* were negatively correlated to SWC, salinity, and EC (**Figure 8B**). *Agaricomycetes* act as important decomposers, producing both hydrogen peroxide and enzymes, resulting in the degradation of complex plant compounds, such as cellulose and lignin (Kameshwar and Qin, 2016). Soil fungi possess strong decomposition abilities. The effects of soil fungi on labile organic carbon mainly depend on a variety of enzymes that decompose organic matter, particularly recalcitrant organic matter. We further found that *Basidiomycota* and *Ascomycota* were the dominant fungal phyla during all four seasons. These two fungal phyla possess critical genes that can encode cellulose decomposition enzymes and promote carbon conversion processes (Hannula et al., 2012; Bastida et al., 2013). *Basidiomycota* mainly depend on plant litter or soil organic matter as their primary carbon source, and members of this phylum participate in soil carbon transformation processes (Jiang et al., 2006).

In future studies, *Cyanobacteria*, *Proteobacteria*, *Acidobacteria*, *Basidiomycota*, and *Ascomycota* could be inoculated into plant or soil in the process of bioremediation, and studied their effects on plant growth and physiology, so as to better restore contaminated sites *via* a combined microbial and plant interaction. This combined investigation of seasonal microbial communities in a copper tailings dam offers an opportunity to further elucidate on microbial adaptations under ecological restoration in mining areas, allowing us to better understand the ability of the microbial community to colonize soil ecosystems by means of natural biofertility. More studies are still required to further understand the resistance and tolerance of such species as well as the molecular mechanisms involved in the adaptation of spontaneous microbial biodegraders in the contaminated soil of copper tailings dams.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

TJ conceived and designed the experiments. TG, YY, and RW performed the experiments. BC contributed new reagents. TJ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01574/full#supplementary-material.

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Impact of Plant Growth Promoting Bacteria on *Salicornia ramosissima* Ecophysiology and Heavy Metal Phytoremediation Capacity in Estuarine Soils

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Salicornia ramosissima is a C_3 halophyte that grows naturally in South Western Spain salt marshes, under soil salinity and heavy metal pollution (mostly Cu, Zn, As, and Pb) caused by both natural and anthropogenic pressure. However, very few works have reported the phytoremediation potential of *S. ramosissima*. In this work, we studied a microbe-assisted phytoremediation strategy under greenhouse conditions. We inoculated plant growth promoting (PGP) and heavy metal resistant bacteria in pots with *S. ramosissima* and natural non-polluted and polluted sediments collected from Spanish estuaries. Then, we analyzed plant ecophysiological and metal phytoaccumulation response. Our data suggested that inoculation in polluted sediments improved *S. ramosissima* plant growth in terms of relative growth rate (RGR) (32%) and number of new branches (61%). *S. ramosissima* photosynthetic fitness was affected by heavy metal presence in soil, but bacteria inoculation improved the photochemical apparatus integrity and functionality, as reflected by increments in net photosynthetic rate (21%), functionality of PSII (F_m and F_v/F_m) and electron transport rate, according to OJIP derived parameters. Beneficial effect of bacteria in polluted sediments was also observed by augmentation of intrinsic water use efficiency (28%) and slightly water content (2%) in inoculated *S. ramosissima*. Finally, our results demonstrated that *S. ramosissima* was able to accumulate great concentrations of heavy metals, mostly at root level, up to 200 mg Kg^{-1} arsenic, 0.50 mg Kg^{-1} cadmium, 400 mg Kg^{-1} copper, 25 mg Kg^{-1} nickel, 300 mg Kg^{-1} lead, and 300 mg Kg^{-1} zinc. Bioaugmentation incremented *S. ramosissima* heavy metal phytoremediation potential due to plant biomass increment, which enabled a greater accumulation capacity. Thus, our results suggest the potential use of heavy metal resistant PGPB to ameliorate the capacity of *S. ramosissima* as candidate for phytoremediation of salty polluted ecosystems.

Keywords: bioaugmentation, halophyte, Odiel, Piedras, photosynthesis, growth, water, electron transport

INTRODUCTION

Environmental pollution has become a major public concern over the last century. Specially, heavy metal content in water and soil has increased due to rapid worldwide industrial development (Usman et al., 2018). This problem is further compounded by soils simultaneously affected by salinity because of climate change and irrigated agriculture (Liang et al., 2017). To overcome this, phytoremediation has been largely studied and suggested as an environmentally friendly and low-cost clean-up method. However, a great proportion of the known phytoremediators are glycophytes, and they cannot survive the combination of salt and heavy metal pollution long enough to be effective (Wang et al., 2014). Conversely, halophytes are able to survive in saline environments (Flowers and Colmer, 2008) and are naturally adapted to tolerate metals, compared to glycophytes (Van Oosten and Maggio, 2015; Liang et al., 2017). Thus, halophytes may represent excellent candidates for phytoremediation of heavy metals in soils with high salinity (Anjum et al., 2014), as have been demonstrated by several species from the genera *Atriplex, Tamarix, Sporobolus, Juncus, Suaeda*, etc. (Manousaki and Kalogerakis, 2011; Redondo-Gómez, 2013). Among processes that halophytes use for phytoremediation are: (i) phytoextraction (plants take metals from the soil and transport and concentrate them in harvestable above-ground tissues), (ii) phytovolatilization (plants take water soluble metals and release them as they transpire the water), (iii) phytostabilization (plants completely immobilize the metals through accumulation by roots or precipitation within the rhizosphere), (iv) rhizofiltration (plants absorb, concentrate and precipitate pollutants of water), (v) phytoaccumulation (pollutants are accumulated in plants' biomass), and (vi) phytodegradation (pollutants are degraded into insoluble or non-toxic compounds) (Anjum et al., 2014). Phytoremediation strategy is based on major plants, but also on associated microbes and their processes. In this sense, plant growth promoting bacteria (PGPB) bioaugmentation has been proposed as a strategy to improve innate heavy metal phytoremediation capacity in halophytes, since it has been proven to ameliorate plant growth, stress tolerance and phytoremediation potential (Backer et al., 2018). Indeed, positive results have been recently obtained for *Spartina, Arthrocnemum,* and *Suaeda* species, which incremented their root metal phytoaccumulation capacity when they were PGPB-treated (Mateos-Naranjo et al., 2015; Mesa et al., 2015b; Navarro-Torre et al., 2017a; Gómez-Garrido et al., 2018).

An ideal case of study in this matter can be found in Odiel salt marsh (SW Spain). In this area, halophytes grow in soils containing salt and heavy metals. Recent studies in Odiel soils registered around 200 mM NaCl and approximately 150 mg Kg^{-1} As, 3 mg Kg^{-1} Cd, 5 mg Kg^{-1} Co, 900 mg Kg^{-1} Cu, 30 mg Kg^{-1} Ni, 300 mg Kg^{-1} Pb, and 1700 mg Kg^{-1} Zn (Mesa et al., 2016). Pollution levels in the estuary are caused by both natural and anthropogenic pressure (Mesa et al., 2016). *Salicornia ramosissima* J. Woods is a C_3 halophyte that grows naturally in Odiel estuary area. It is a widespread plant on the European coastline, including salt marshes of the Iberian Peninsula, where it usually occupies the higher reaches of the salt marsh and is a pioneer species in the colonization of the intertidal zones of such habitats (Davy et al., 2001). These features, together with the potential for metal bioremediation of the genus *Salicornia* observed by some authors (Ozawa et al., 2010; Sharma et al., 2010; Kaviani et al., 2017a,b; Lou et al., 2020), make *S. ramosissima* an excellent candidate for land restoration. For example, root exudates from *Salicornia europaea* could form Pb-complexes which help Pb-stabilization and, therefore, remediation (Pan et al., 2012). Also, Shrestha et al. (2006) reported in *Salicornia bigelovii* 2.2-fold more biogenic volatile Se compared to the control. However, the metal accumulation ability of the species *S. ramosissima* has been scarcely studied. Only a few works reported *S. ramosissima* phytoaccumulation abilities. Pedro et al. (2013) concluded that this species may be useful for phytoaccumulation and phytostabilization, since plants have a considerable bioaccumulation potential and were able to bioaccumulate Cd mainly in the roots, acting like a sink for this metal and preventing it from becoming available to other organisms. Pérez-Romero et al. (2016) confirmed the traits of Cd accumulation and tolerance of *S. ramosissima*, and stated that this tolerance could be due to many of essential steps of its photosynthetic pathway were tolerant to Cd excess. On the other hand, PGPB treatments have been never tested on *S. ramosissima* with phytoremediation purposes. In a previous work, we isolated PGPB from the rhizosphere of *S. ramosissima* growing in SW Spain salt marshes and designed a bacterial consortium for inoculation (Mesa-Marín et al., 2019c). We hypothesize that *S. ramosissima* may accumulate heavy metals in its tissues when it grows in a polluted soil, and that bioaugmentation treatment with the bacterial inoculum mentioned above may improve growth of *S. ramosissima* and its metal accumulation capacity. Thus, this is the first work that studies the phytoremediation capacity of PGPB-treated *S. ramosissima*.

Then, this work aimed at (1) assessing the tolerance to heavy metals of the bacteria selected for inoculation, in order to ensure they can be used in polluted sediments, (2) describing heavy metal accumulation capacity of *S. ramosissima* growing in natural salt marsh sediments, and (3) analyzing *S. ramosissima* response to PGPB inoculation in terms of growth, photosynthetic fitness, and metal accumulation.

MATERIALS AND METHODS

Plant and Soil Source

Seeds of *S. ramosissima* were collected in December 2017 from Odiel (37°15′N, 6°58′W; SW Spain) and Piedras (37°16′09.1″N 7°09′36.4″W; SW Spain) marshes. Later, they were kept in darkness at 4°C up to 3 months. Prior to the experiment, seeds were surface-disinfected and germinated as indicated in Mateos-Naranjo et al. (2015). Seedlings were planted in perlite (in 11 cm diameter × 9 cm high pots), in a glasshouse with 40–60% relative humidity, natural daylight and 21–25°C. 20% Hoagland's solution (Hoagland and Arnon, 1938) was used to irrigate pots as necessary. For our experiment, two natural marshy soils were used. Soil from the Piedras estuary (Huelva, SW Spain), with no anthropogenic influence and thereby non-polluted, was

used to grow *S. ramosissima* control plants, and soil from the Odiel estuary (Huelva, SW Spain) was used to grow plants under polluted conditions. Metal concentration of both soils is available in **Table 1**. Arsenic, copper and lead concentrations were especially high in Odiel salt marshes (ca. 800, 1000, and 1100 mg Kg^{-1}, respectively), constituting 60×, 30×, and 35× times, respectively, compared to Piedras sediments.

Bacterial Tolerance Against Heavy Metals

Bacteria used as an inoculum in this experiment were isolated from *S. ramosissima* rhizosphere, growing in the Tinto salt marsh (SW Spain, 37° 13′ 51.40″ N 6° 54′ 28.40″ W) (Mesa-Marín et al., 2019c). They were identified as *Vibrio neocaledonicus* SRT1, *Thalassospira australica* SRT8 and *Pseudarthrobacter oxydans* SRT15, and they were selected among other rhizosphere isolates based on their outstanding Plant Growth Promoting (PGP) properties (Mesa-Marín et al., 2019c). This bacterial consortium showed nitrogen fixation abilities, phosphate solubilization and biofilm-forming capacity, as well as production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, indole-3-acetic acid (IAA), and siderophores (**Table 2**; Mesa-Marín et al., 2019c). For this work, heavy metal tolerance of these bacteria was analyzed. Single strains were streaked on TSA 0.2 M NaCl medium (in line with salt marshes soil conductivity) and supplemented with increasing concentrations of heavy metals and metalloids from stock solutions: $NaAsO_2$ 0.5 M, $CdCl_2$ 1 M, $CuSO_4$ 1 M, $CoCl_2$ 1 M, $NiCl_2$ 0.2 M, $ZnSO_4$ 1 M, and $Pb(NO_3)_2$ 0.5 M (in order to avoid Pb precipitation when mixing with TSA, the same concentration of EDTA needs to be added to the plates). Tolerance was assessed for each strain and heavy metal separately. After incubation at 28°C during 72 h, maximum tolerable concentration (MTC) was expressed for each strain and element, namely the maximum concentration of a metal or metalloid that permitted bacterial growth in plates.

Experimental Treatments

Pots containing Piedras and Odiel sediment were planted with *S. ramosissima* and assigned randomly to two bacterial bioaugmentation treatments: non-inoculated (control) and inoculated with the selected PGPB (n = 40, 2 soils × 2 bacterial treatments = 4 treatments, 10 pots per treatment). Pots were put in trays with tap water down to a depth of 1 cm. During the experimental period, 30 days, water level in the trays were checked every 2 days. Inoculated pots were watered with 50 ml of a bacterial suspension at the beginning of the experiment, while control pots were watered with 50 ml of tap water. Inoculant suspensions were prepared as described in Mesa-Marín et al. (2019c). Finally, bacteria pellets were resuspended in tap water until it was obtained a suspension with OD_{600} = 1 (approx. 10^8 cells per ml) for each strain. Equal amounts of each strain were mixed to obtain the final OD_{600} = 1 inoculant suspension. The final volume of inoculant suspension was 1 L, to water 20 pots with 50 ml each.

Salicornia ramosissima Growth and Water Status Analysis

Six *S. ramosissima* plants were harvested before the initiation of the experiment and the rest were collected after 30 days of bioaugmentation treatment. The relative growth rate (RGR) of *S. ramosissima* was calculated (Mesa et al., 2015b). Also, all *S. ramosissima* height and number of ramifications were recorded at the beginning and the end of the experimental period. Furthermore, water content (WC) of primary branches (n = 9 per each soil and bacterial treatment combination) were calculated as follow:

$$WC = [(FW - DW)/FW] \times 100$$

where FW = fresh weight of the branches and DW = dry weight after oven-drying at 80°C for 48 h.

Salicornia ramosissima Photosynthetic Performance Analysis

One day before plants were harvested, gas exchange parameters were measured on primary branches (n = 10, per each soil and bacterial treatment combination) using an infrared gas analyzer (LI-6400, LI-COR Inc., Neb., United States) in an open system. It was equipped with a light leaf chamber (Li-6400-02B, Li-Cor Inc.). Thus, stomatal conductance (g_s), intercellular

TABLE 1 | Metal(loid) concentrations in mg Kg^{-1} in sediments from Odiel to Piedras salt marshes used in this study.

Location	As	Cd	Cu	Ni	Pb	Zn
Piedras	14.22 ± 0.41	0.20 ± 0.12	37.35 ± 1.43	24.28 ± 0.47	31.95 ± 0.16	108.57 ± 3.75
Odiel	853.54 ± 34.45	2.19 ± 0.7	1076.39 ± 58.55	28.77 ± 1.48	1139.85 ± 58.76	809.92 ± 40.06

Values are means ± S.E.

TABLE 2 | Plant growth promoting traits for bacterial consortia used in this study (Mesa-Marín et al., 2019c).

Strain	Nitrogen fixation[a]	Phosphate solubilization[b]	Siderophores production[b]	IAA production (mg/mL)	Biofilm production[a]	ACC deaminase activity (μ mol α -cetog h^{-1} mg $prot^{-1}$)
SRT1	+	10	20	5.65	+	–
SRT8	–	–	–	–	+–	1.24
SRT15	+	9	–	20.99	+	–

[a] *(+) presence or (–) absence of growth;* [b] *halo diameter in mm.*

CO_2 concentration (C_i), net photosynthetic rate (A_N), and instantaneous water use efficiency ($_i$WUE; ratio between A_N and g_s) were obtained with the following leaf chamber settings: photon flux density (PPFD) of 1000 μmol photons m^{-2} s^{-1} (with 15% blue light to maximize stomatal aperture), CO_2 concentration (C_a) surrounding leaf of 400 μmol mol^{-1} air, air temperature of 24 ± 1°C, relative humidity of 45 ± 5%, and vapor pressure deficit of 2.0–3.0 kPa.

Furthermore, chlorophyll fluorescence measurements were performed in the same branches of gas exchange analysis using a FluorPen FP100 (Photo System Instruments, Czechia). Thus, the chlorophyll *a* fast kinetics, or JIP-test (or Kautsky curves), which depicts the rate of reduction kinetics of various components of PSII, were measured in 30 min dark-adapted branches (n = 6, per each soil and bacterial treatment combination), using the pre-programmed OJIP test implemented in the pre-programmed protocols of the FluorPen. Maximum quantum efficiency of PSII photochemistry (F_v/F_m), absorbed energy flux (ABS/CS), trapped energy flux (TR/CS), electron transport energy flux (ET/CS), and dissipated energy flux (DI/CS) per reaction center derived for OJIP were calculated (Strasser et al., 2004).

Chemical Analyses of *Salicornia ramosissima* Tissues

Salicornia ramosissima roots and leaves were carefully washed with distilled water at the end of the experiment to eliminate ions from their surface before the analysis. At the end of the experiment, dried roots and leaves were ground (Mateos-Naranjo et al., 2008). 0.5 g sub-samples were taken from the roots and the leaves of the ten replicate plants. Phosphorus (P), sodium (Na), magnesium (Mg), manganese (Mn), calcium (Ca), potassium (K), arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn) were measured by inductively coupled plasma (ICP) spectroscopy (ARL-Fison 3410, United States) in tissues and soil.

Statistical Analysis

Statistics were analyzed with "Statistica" v. 10.0 (StatSoft Inc.). Generalized linear models (GLM) aided to analyze the interactive effects of soil pollution and bacterial treatments (as categorical factors) on the growth, chlorophyll fluorescence and gas exchange of *S. ramosissima* plants (as dependent variables), followed by a LSD (*post hoc*) test for multiple comparisons analysis. Nutrient and heavy metal content in *S. ramosissima* leaves and roots after different bioaugmentation treatments were compared by the Student test (*T*-test), and values were analyzed for each tissue by using one-way ANOVA (*F*-test). Data normality was tested with the Kolmogorov–Smirnov test and data homogeneity of variance with the Brown–Forsythe test. Tukey (*post hoc*) tests were used for identification of important contrasts. In all cases, a significance level of $p < 0.05$ was used.

RESULTS

Rhizobacteria Tolerance to Heavy Metals

Bacteria selected in this work for bioaugmentation treatments were *Vibrio neocaledonicus* SRT1, *Thalassospira australica* SRT8, and *Pseudarthrobacter oxydans* SRT15. They were isolated from *S. ramosissima* rhizosphere growing in heavy metal polluted Tinto salt marsh (SW Spain). Isolation was conducted by pour-plating a mix of rhizospheric soil and physiological saline solution (NaCl 0.9% w/v) on tryptic soy agar (TSA) NaCl 0.2 M and further streaking of single colonies in the same medium, with incubations at 28°C for 24–48 h (Mesa-Marín et al., 2019c). SRT1 exhibited nitrogen fixation and phosphate solubilization, as well as the capacity to produce siderophores and biofilms. SRT8 produced ACC deaminase and SRT15 showed the best auxins production (**Table 2**). In this work, heavy metal tolerance of these strains was tested (**Table 3**). Strain SRT1 showed the broadest tolerance, with particular emphasis on As, Cd, and Co. Overall, the three strains showed a notable tolerance to Zn (ranging from 1 to 3 mM), Pb (from 3 to 5 mM), Ni (up to 9 mM), Cu (up to 3 mM), and Co (from 3 to 13 mM).

Soil Bioaugmentation Effect on *Salicornia ramosissima* Growth and Water Content

After 30 days of treatment bacterial inoculation improved *S. ramosissima* RGR compared to non-inoculated plants (**Figure 1A**). Soil bioaugmentation incremented RGR 45% in non-polluted Piedras sediments and 32% in polluted Odiel sediments (GLM $_{inoc}$, $p < 0.05$). Also, at the end of the treatment, inoculated *S. ramosissima* showed significant increments of stem branches compared to control (**Figure 1B**), 32% in non-polluted sediments and 61% in polluted sediments (GLM $_{soilxinoc}$, $p < 0.05$). However, for *S. ramosissima* stem height there was no statistically significant difference in any case (data not shown). Plant water content (**Figure 1C**) did not vary with inoculation in non-polluted sediments. Conversely, it was higher in inoculated plants grown in polluted sediments (GLM $_{soil,inoc}$ $p < 0.05$).

Soil Bioaugmentation Effect on *Salicornia ramosissima* Photosynthetic Apparatus Performance

As shown in **Figure 2**, *S. ramosissima* growing in polluted sediments had higher $_i$WUE, and lower A_N, g_s, and C_i,

TABLE 3 | Maximum tolerable concentration (MTC) in mmoles L^{-1} (mM) for each rhizobacteria strain and metal(loid) tested.

Strain	As ($NaAsO_2$)	Cd ($CdCl_2$)	Co ($CoCl_2$)	Cu ($CuSO_4$)	Ni ($NiCl_2$)	Pb ($Pb(NO_3)_2$)	Zn ($ZnSO_4$)
SRT1	5	4	13	3	9	5	3
SRT8	<1	0.3	3	3	1	5	1
SRT15	<1	<0.1	5	1	9	3	1

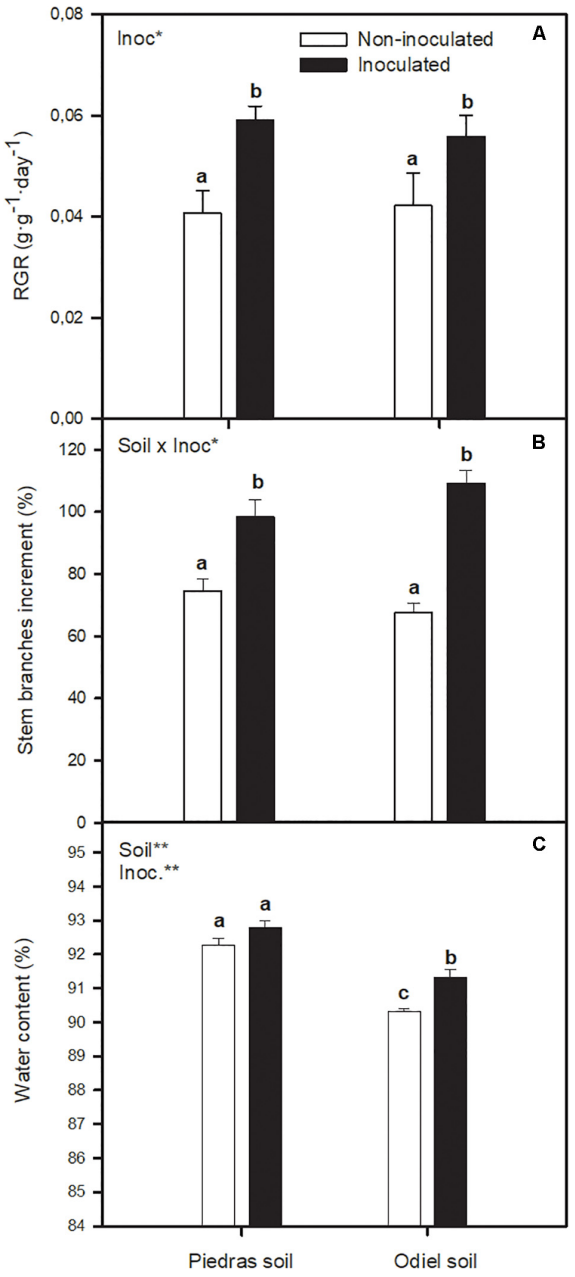

FIGURE 1 | Effect of soil bioaugmentation treatments (non-inoculated and inoculated) with a bacterial consortium integrated by *Vibrio neocaledonicus* SRT1, *Thalassospira australica* SRT8 and *Pseudarthrobacter oxydans* SRT15 on **(A)** relative growth rate, RGR, **(B)** number of stem branches and **(C)** water content of *Salicornia ramosissima* plants grown in natural soil from non-polluted Piedras and polluted Odiel marshes for 30 days. Values are means ± S.E. ($n = 10$). "Inoc" or "Soil" in the upper left corner of the panel indicates main or interaction significant effect (*$p < 0.1$, **$p < 0.01$). Different letters indicate means that are significantly different from each other ($p < 0.05$).

compared to plants growing in non-polluted sediments (T-test, $p < 0.05$). Soil bioaugmentation with rhizobia did not altered *S. ramosissima* A_N, g_s, C_i, and $_i$WUE in non-polluted soil (**Figures 2A–D**). Conversely, soil bioaugmentation in polluted sediments significantly incremented A_N and $_i$WUE, while lowered C_i (GLM $_{soil,inoc,soil \times inoc}$ $p < 0.05$; **Figures 2A–D**). **Figure 3** illustrates changes in chlorophyll fluorescence parameters after 30 days of treatments. In this case, F_m and F_v/F_m showed a clear pattern of statistically significant variation in both sediments and inoculation treatments. Both parameters decreased for inoculated plants in non-polluted soil, while they increased after inoculation in polluted soils (GLM $_{soil,soil \times inoc}$, $p > 0.05$; **Figures 3A,B**). Finally, focusing on OJIP derived parameters (**Figure 4**), we found that bioaugmentation treatments and soil pollution degree did not affect ABS/CS and ET/CS values (GLM, $p > 0.05$; **Figures 4A,B**), while TR/C values decreased in polluted soil in similar degree at both inoculation treatments (GLM $_{soil}$, $p < 0.05$; **Figure 4C**). Contrarily, there was an increment in DI/RC values under metal pollution conditions but this augmentation was more accused in non-inoculated plants (GLM $_{soil \times inoc}$, $p > 0.05$; **Figure 4D**).

Soil Bioaugmentation Effect on *Salicornia ramosissima* Tissues Nutrient Profile

At the end of the experiment, rhizobacterial consortium addition altered nutrient profile in a greater extent in leaves than in roots of *S. ramosissima* (two-way Anova, $p < 0.01$; **Table 4**). Soil bioaugmentation in non-polluted sediments produced greater concentrations of Ca, Mg, Mn, and Na in *S. ramosissima* leaves than their non-inoculated counterparts. Conversely, soil bioaugmentation in polluted soil only increased Na concentrations in leaves (two-way Anova, $p < 0.01$; **Table 4**). It is noteworthy that rhizobacterial treatment increased in both cases Na uptake in leaves 16%.

Salicornia ramosissima Heavy Metal Accumulation Capacity and Effect of Soil Bioaugmentation

Salicornia ramosissima growing in polluted sediments accumulated great concentrations of heavy metals in its tissues, compared to plants growing in non-polluted sediments (**Tables 4**, **5**). This natural bioaccumulation capacity was particularly noticeable in *S. ramosissima* roots, reaching concentrations of 31, 14 or 17 times more As, Cu, and Pb, respectively, than plants growing in non-polluted sediments (T-test, $p < 0.01$; **Table 6**). Ni concentration was similar in both cases, as Ni concentration in both soils was the same (**Table 1**).

Bioaugmentation effect on *S. ramosissima* metal accumulation capacity was studied in polluted sediments (**Figure 5**). Concentration of ions was higher in roots than in *S. ramosissima* leaves (T-test, $p < 0.01$; **Figures 5A–L**). Our data revealed that there was no statistical difference in metal concentration per Kg of *S. ramosissima* roots or leaves between inoculation conditions after 30 days of experiment (one-way ANOVA, $p > 0.05$; **Figures 5A–F**). However, when *S. ramosissima* biomass gaining was taken into account for each treatment at the end of the experiment, results varied. This is, *S. ramosissima* plants inoculated with the rhizobacterial consortium, which

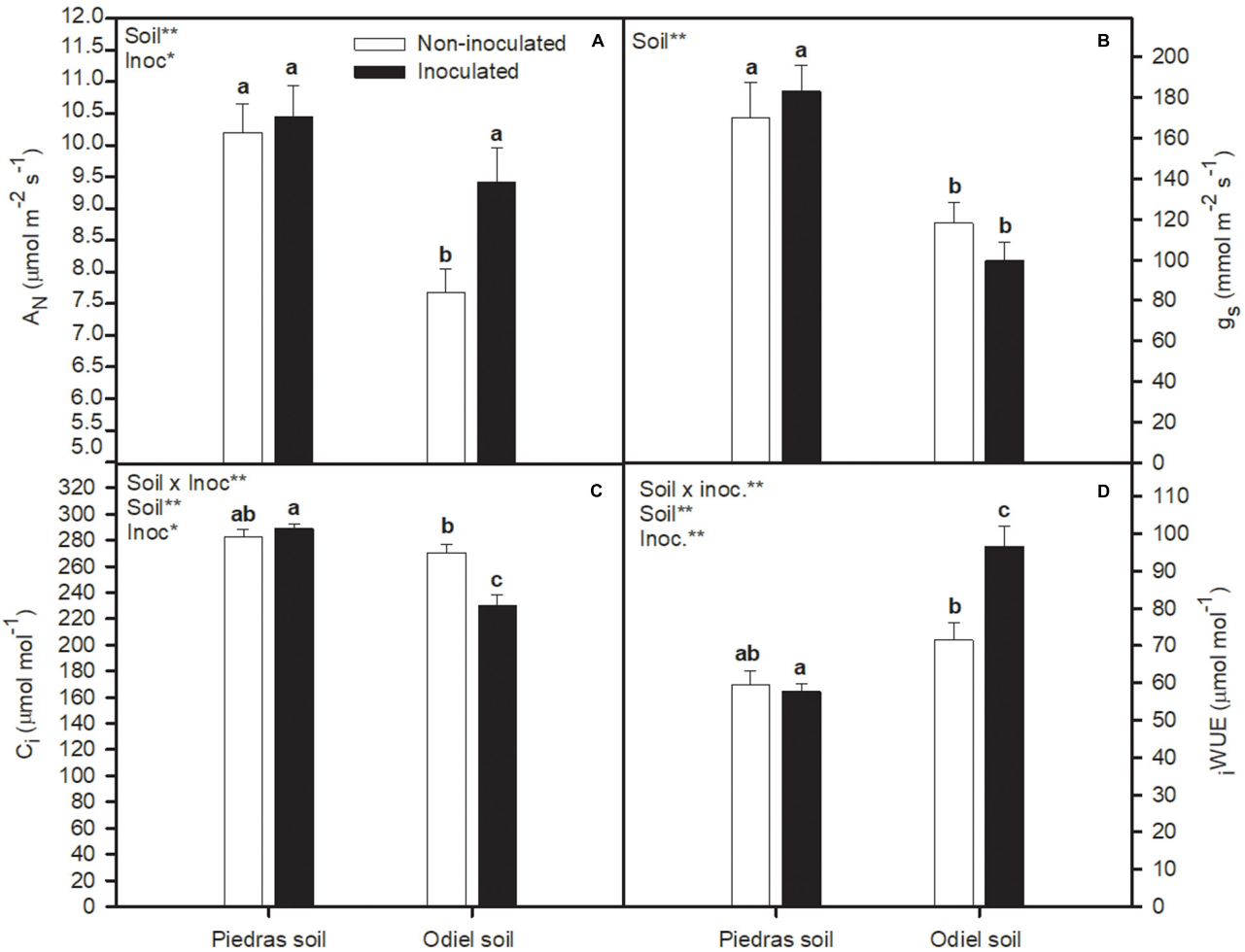

FIGURE 2 | Effect of soil bioaugmentation on *S. ramosissima* **(A)** net photosynthetic rate, A_N, **(B)** stomatal conductance, g_s, **(C)** intercellular CO_2 concentration, C_i, and **(D)** instantaneous water-use efficiency, ¡WUE, after 30 days of treatment. Values are means ± S.E. (n = 10). "Inoc" or "Soil" in the upper left corner of the panel indicates main or interaction significant effect (*$p < 0.1$, **$p < 0.01$). Different letters indicate means that are significantly different from each other ($p < 0.05$).

showed greater growth rates than the non-inoculated ones, showed higher total metal content than the control for all metals assayed, except for Cd (one-way ANOVA, $p < 0.05$; **Figures 5G–L**).

DISCUSSION

In this work, we used two sediments from SW Spain salt marshes where *S. ramosissima* grows naturally, with historically well-known different level of heavy metal pollution. Metal concentration in Piedras salt marsh, without anthropogenic influence, remains very similar to that reported in the last decade (Mesa et al., 2016). On the contrary, data obtained in Odiel salt marshes sediments revealed that the concentration of lead and arsenic has increased more than two-fold compared with data compiled in the last decade, while cadmium and zinc concentrations have decreased (Mesa et al., 2016). Spanish Government established threshold values for metal clean-up intervention in soil (de Andalucía, 1999). Concentration of As (ca. 800 mg Kg^{-1}), Cu (ca. 1000 mg Kg^{-1}), and Pb (ca. 1100 mg Kg^{-1}) in Odiel sediments surpassed threshold values that Spanish Government stablished as intervention limits, this is, 100, 500, and 1000 mg Kg^{-1}, respectively (de Andalucía, 1999). This fact highlights the importance of looking into efficient eco-friendly restoration strategies in these areas, which have a high ecological value. In this work, we studied a proposal of phytoremediation with PGPB-assisted *S. ramosissima*. This is in line with our previous studies on PGPB treatments with several *Spartina* genus in the same area (Mateos-Naranjo et al., 2015; Mesa et al., 2015b).

Data collected in our experiment showed that *S. ramosissima* responded to bioaugmentation with a bacterial consortium composed by *Vibrio neocaledonicus* SRT1, *Thalassospira australica* SRT8, and *Pseudarthrobacter oxydans* SRT15 (Mesa-Marín et al., 2019c), as could be seen from diverse physiological parameters. On one hand, PGPB bioaugmentation had in both non-polluted and polluted sediments a positive impact on

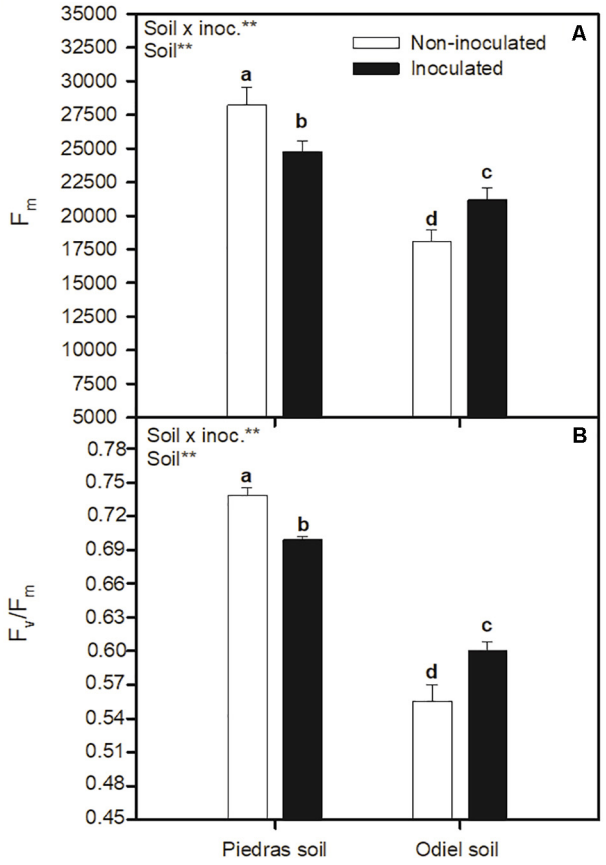

FIGURE 3 | Effect of soil bioaugmentation on *S. ramosissima* **(A)** maximal fluorescence level in the dark-adapted State, F_m, and **(B)** maximum quantum efficiency of PSII photochemistry, F_v/F_m, after 30 days of treatment. Values are means ± S.E. (n = 10). "Inoc" or "Soil" in the upper left corner of the panel indicates main or interaction significant effect (**$p < 0.01$). Different letters indicate means that are significantly different from each other ($p < 0.05$).

S. ramosissima growth, reflected by RGR results and number of *S. ramosissima* stem branches. However, height of plants did not vary with bioaugmentation. Increased plant growth may be due to the well-known beneficial properties on plant development and stress tolerance of PGPB (Glick, 2012). In particular, the use of the three PGP strains mentioned above allowed *S. ramosissima* to benefit from six PGP properties: siderophores, IAA and ACC deaminase production, phosphates solubilization, biofilm formation and nitrogen fixation (Mesa-Marín et al., 2019c). IAA is a phytohormone with a crucial role in cell differentiation and division, and therefore in root development (Duca et al., 2014). Siderophores sequester and solubilize iron from the soil, facilitating to the plant enough acquisition of this element, especially when there are great amounts of other competing metals (Neilands, 1995). Also, bacterial phosphate solubilizing activity provides plants with a bioavailable form of phosphorus, from immobilized inorganic and organic P compounds that can be found in soil (Glick, 2012). Nitrogen fixing bacteria provide assimilable N to the plant (de Souza et al., 2015). Bacteria with the enzyme ACC deaminase degrade the plant ethylene precursor, diminishing ethylene levels in a stressed or developing plant, especially in the presence of salinity excess, and therefore, alleviating plant damage (Glick, 2014; Mesa-Marín et al., 2019b). Finally, there is evidence of the role of biofilms in the presence of heavy metals, by keeping them concentrated, partitioned and immobilized, minimizing the environmental hazards (Bogino et al., 2013). On the whole, *Vibrio neocaledonicus* SRT1, *Thalassospira australica* SRT8, and *Pseudarthrobacter oxydans* SRT15 may improve *S. ramosissima* nutrient acquisition and stress tolerance to heavy metals, which reflects in an increment in RGR and branch development. In our previous works with *Spartina* species growing in Piedras, Odiel, and Tinto sediments (SW Spain), *Spartina densiflora* and *Spartina maritima* also registered increments in their growth rates after PGPB bioaugmentation, in line with the obtained for *S. ramosissima* (Mateos-Naranjo et al., 2015; Mesa et al., 2015b).

On the other hand, the positive effect of bacteria inoculation was also supported by improvement in photosynthetic parameters of *S. ramosissima*. Overall, heavy metal pollution clearly affected *S. ramosissima* photosynthetic metabolism, as observed from lower net photosynthetic rate (A_N) values in control plants growing in polluted sediments compared to non-polluted. In this case, unlike growth, PGPB bioaugmentation had only significant effect in polluted conditions. PGPB inoculation induced a higher plant photosynthetic performance, indicated by greater A_N values. Furthermore, the photosynthetic improvement in inoculated *S. ramosissima* may be related to the greater integrity and functionality of the photochemical apparatus, reflected in terms of functionality of PSII, as indicated by the higher values of F_m and F_v/F_m after 30 days of bioaugmentation. Higher ETR values in inoculated *S. ramosissima* growing in polluted soil demonstrated the maintenance of the functionality of the electron transport chain, reinforcing the positive impact of PGPB inoculation. This fact may ensure enough electrons for carbon fixation through photochemical pathway, which is indeed reflected by A_N values under those suboptimal polluted conditions (Strasser et al., 2004).

Other physiological effects observed in this work were $_i$WUE and water content augmentation in inoculated *S. ramosissima* in polluted sediments. $_i$WUE is a well-established indicator of plant water managing under conditions of stress (Tardieu, 2012). In a similar manner, greater root growth in *S. ramosissima* inoculated with bacteria may aid to enhance water absorption capacity and, therefore, water content, iWUE and metal tolerance. In line with these results, previous works from authors of this study reported that bioaugmentation with indigenous PGPB incremented $_i$WUE and other photosynthetic parameters like A_N and functionality of PSII in halophytes *S. densiflora*, *S. maritima*, and *Arthrocnemum macrostachyum* (Mateos-Naranjo et al., 2015; Mesa et al., 2015a,b; Navarro-Torre et al., 2017a,b; Paredes-Páliz et al., 2017; Mesa-Marín et al., 2019a).

Finally, our results revealed that *S. ramosissima* could be considered a heavy metal phytoaccumulator halophyte, as reflected by high concentrations of As (200 mg Kg^{-1}), Cd (0.50 mg Kg^{-1}), Cu (400 mg Kg^{-1}), Ni (25 mg Kg^{-1}), Pb

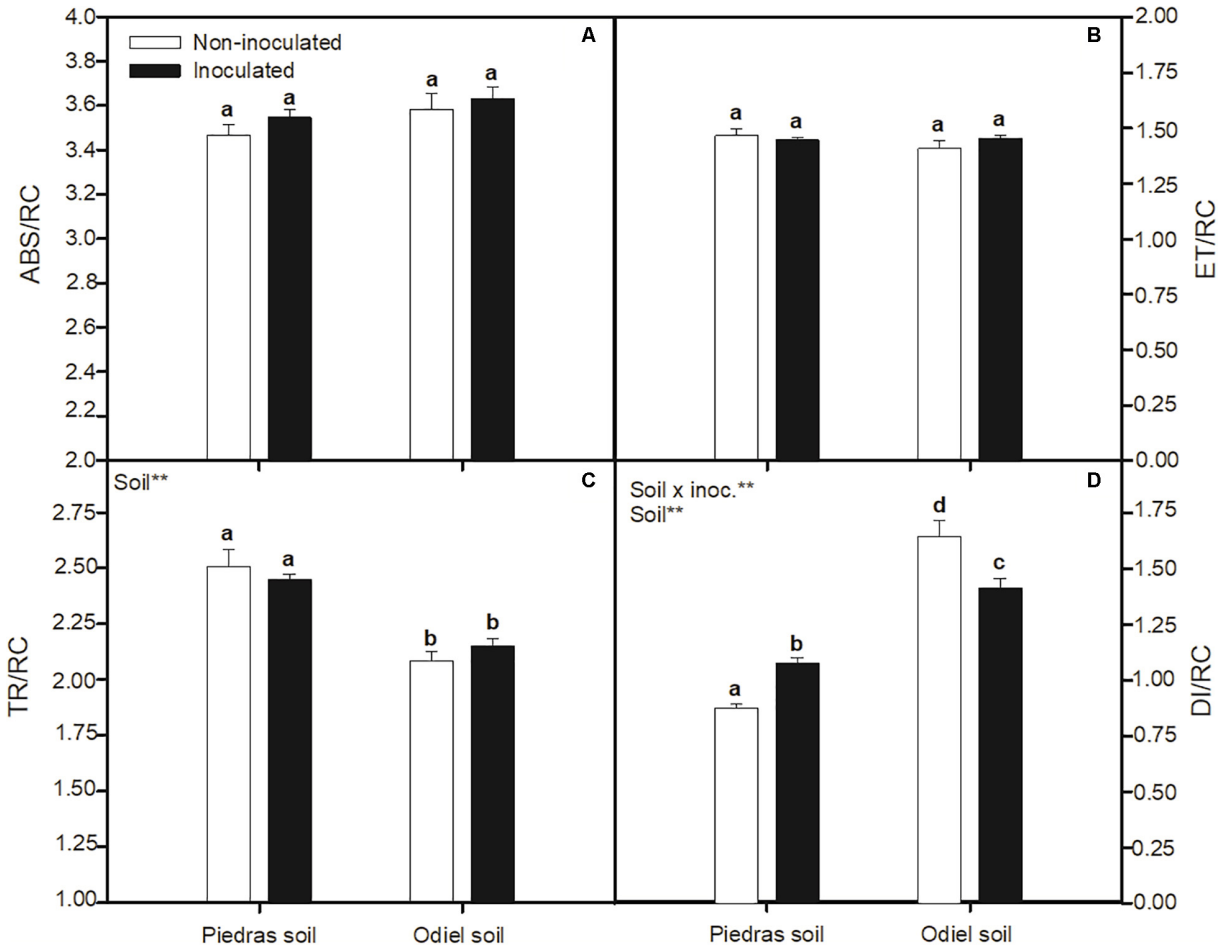

FIGURE 4 | Effect of soil bioaugmentation on *S. ramosissima* **(A)** ABS/RC, **(B)** ET/RC, **(C)** TR/RC, and **(D)** DI/RC after 30 days of treatment. Values are means ± S.E. (n = 10). "Inoc" or "Soil" in the upper left corner of the panel indicates main or interaction significant effect (**$p < 0.01$). Different letters indicate means that are significantly different from each other ($p < 0.05$).

TABLE 4 | Total calcium (Ca), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), and phosphorous (P) concentrations for *Salicornia ramosissima* tillers and roots after 30 days of bioaugmentation treatment in non-polluted Piedras and polluted Odiel sediments.

Soil, tissue and treatment	Ca (mg g^{-1})	K (mg g^{-1})	Mg (mg g^{-1})	Mn (mg Kg^{-1})	Na (mg g^{-1})	P (mg g^{-1})
Piedras						
Leaf						
Control	0.34 ± 0.01^{a}	1.42 ± 0.06^{a}	0.53 ± 0.01^{a}	141.16 ± 4.55^{a}	11.03 ± 0.44^{a}	0.23 ± 0.02^{a}
Inoculated	0.43 ± 0.01^{b}	1.45 ± 0.07^{a}	0.64 ± 0.01^{b}	174.80 ± 3.56^{b}	13.18 ± 0.45^{b}	0.24 ± 0.01^{a}
Root						
Control	0.26 ± 0.02^{a}	1.19 ± 0.08^{a}	0.38 ± 0.02^{a}	267.76 ± 1.27^{a}	1.57 ± 0.09^{a}	0.08 ± 0.00^{a}
Inoculated	0.24 ± 0.01^{a}	1.21 ± 0.05^{a}	0.40 ± 0.02^{a}	258.69 ± 4.91^{a}	1.52 ± 0.06^{a}	0.07 ± 0.00^{a}
Odiel						
Leaf						
Control	0.31 ± 0.04^{a}	1.28 ± 0.16^{a}	0.39 ± 0.05^{a}	67.57 ± 7.81^{a}	9.35 ± 0.37^{a}	0.25 ± 0.03^{a}
Inoculated	0.38 ± 0.03^{a}	1.28 ± 0.10^{a}	0.45 ± 0.04^{a}	71.28 ± 3.89^{a}	11.44 ± 0.46^{b}	0.27 ± 0.01^{a}
Root						
Control	0.40 ± 0.04^{a}	1.41 ± 0.01^{a}	0.32 ± 0.01^{a}	349.97 ± 13.82^{a}	1.26 ± 0.06^{a}	0.21 ± 0.01^{a}
Inoculated	0.34 ± 0.03^{a}	1.34 ± 0.05^{a}	0.31 ± 0.01^{a}	292.09 ± 5.06^{b}	1.16 ± 0.02^{a}	0.19 ± 0.00^{a}

Values are means ± SE. n = 3 and different letters indicate means that are significantly different from each other (p < 0.05).

TABLE 5 | Metal(loid) concentrations in mg Kg^{-1} found in *S. ramosissima* leaves and roots growing in non-polluted Piedras sediments after 30 days of experiment.

Tissue	Treatment	As	Cd	Cu	Ni	Pb	Zn
Leaves	Non-inoculated	0.44 ± 0.17^{a}	$<0.01 \pm 0.00^{a}$	8.08 ± 0.59^{a}	0.43 ± 0.12^{a}	0.73 ± 0.16^{a}	18.56 ± 1.05^{a}
	Inoculated	0.47 ± 0.13^{a}	$<0.01 \pm 0.00^{a}$	9.46 ± 0.19^{a}	1.07 ± 0.29^{b}	0.99 ± 0.28^{a}	26.30 ± 0.80^{b}
Root	Non-inoculated	8.21 ± 0.48^{a}	0.13 ± 0.10^{a}	29.60 ± 1.07^{a}	26.23 ± 2.27^{a}	17.68 ± 1.01^{a}	102.20 ± 3.98^{a}
	Inoculated	10.73 ± 0.53^{a}	0.23 ± 0.11^{a}	34.38 ± 1.01^{b}	33.44 ± 0.93^{b}	21.72 ± 0.79^{b}	99.47 ± 1.53^{a}

Values are means ± S.E. Different letters indicate statistical differences for each metal(loid) and tissue between bioaugmentation treatments.

TABLE 6 | Distribution of arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn) in estuarine soils of our study and *S. ramosissima* in natural conditions (non-inoculated).

Odiel vs. Piedras	As	Cd	Cu	Ni	Pb	Zn
Soil	60	10	29	1	36	7
S. ramosissima roots	31	4	14	1	17	3
S. ramosissima leaves	18	1	2	1	14	2

The table displays the times that every heavy metal is more concentrated in polluted Odiel sediments and S. ramosissima growing in them, compared to non-polluted Piedras sediments and S. ramosissima growing in them. For example, arsenic was 60 times more concentrated in Odiel than in Piedras soil, and S. ramosissima growing in Odiel soil accumulated 31 times more arsenic in roots and 18 in leaves than S. ramosissima growing in Piedras soil.

(300 mg Kg^{-1}), and Zn (300 mg Kg^{-1}) recorded in roots of plants growing in polluted sediments (with approximately As 800 mg Kg^{-1}, Cd 2 mg Kg^{-1}, Cu 1000 mg Kg^{-1}, Ni 30 mg Kg^{-1}, Pb 1000 mg Kg^{-1}, and Zn 800 mg Kg^{-1}). Moreover, it is important to note that accumulation takes place mainly at root level. More specifically, root concentration of the analyzed metals in *S. ramosissima* growing in polluted Odiel sediments was approximately 20, 50, 20, 25, 30, and 15 times, respectively, higher than in leaves. Other authors also observed Cd accumulation capacity in this species (Pedro et al., 2013; Pérez-Romero et al., 2016). In the same line, As, Cd, Li, Ni, and Pb tolerance and accumulation have been noted by several authors for other *Salicornia* species, like *Salicornia brachiata*, *S. europaea,* and *Salicornia iranica* (Ozawa et al., 2010; Sharma et al., 2010; Kaviani et al., 2017a,b; Lou et al., 2020). Toxic metals are thought to enter root cells by means of the same uptake processes that move essential micronutrient metal ions, as for example competitive transport of Cd via voltage-gated cation channels. Also, these species seem to use different mechanisms for restricting the transport of toxic elements within the plant, including sub-cellular compartmentalization of the metal, namely in vacuoles, and the sequestration of the metal by specially produced organic compounds, like phytochelatins, concentrating metal in the plants roots (Pedro et al., 2013). Low metal translocation rates does not mean lower phytoremediation capacity, but different phytoremediation strategies. Thus, *S. ramosissima* may be appropriate for phytostabilization purposes, nor for phytoextraction. Bacteria inoculation with the selected consortium incremented heavy metal phytoaccumulation in *S. ramosissima*. However, we observed that this effect was not due to an amelioration of *S. ramosissima* accumulation ability, but to an increment in plant biomass, which permits a greater accumulation of heavy metals. This effect was also observed by Mesa et al. (2015a) after bioaugmentation with PGP endophytic bacteria in *S. maritima* growing in polluted Tinto salt marsh sediments. Nevertheless, other studies concluded that bioaugmentation with PGP rhizobacteria in halophytes *A. macrostachyum*, *Suaeda vera* and hyperaccumulators *S. maritima* and *S. densiflora* incremented significantly root metal phytoaccumulation ability (Mateos-Naranjo et al., 2015; Mesa et al., 2015b; Navarro-Torre et al., 2017a; Gómez-Garrido et al., 2018). Bacteria used in this work may increase plant tolerance through different mechanisms. For example, higher ETR values recorded in inoculated plants may suggest that several defense mechanisms could be activated, like the dissipation of energy excess as photorespiration or heat (Duarte et al., 2013), helping plants to reduce physiological stress when they are exposed to heavy metals. However, most of the energy absorbed would not take the photochemical pathway (Flexas et al., 2012). This fact affects photosynthetic productivity and, therefore, growth in plants not inoculated, which would support our experimental growth results. Also, PGPB may induce plant enzymatic antioxidant defense, as has been demonstrated by several authors (Kong et al., 2015; Mesa-Marín et al., 2018). Besides, PGPB may confer metal resistance by chemical detoxification, accumulation, transformation and sequestration of heavy metals, altering their phytoavailability in contaminated soils and thus diminishing the metal phytotoxicity (Kong and Glick, 2017). Moreover, numerous studies have suggested that inoculation of plants with PGPB that produce ACC deaminase and IAA, as is currently the case, may play an important role in improving metal phytoremediation by increasing heavy metal tolerance. However, most of these studies did not provide definitive and mechanistic proof of the direct involvement of these compounds (Kong and Glick, 2017). Indeed, there is a wide lack of knowledge regarding to specific mechanism underlying these effects, that opens a broad area of research.

A fact that is important to be noted is that PGPB bioaugmentation with this consortium did not promote in *S. ramosissima* higher accumulation rates of heavy metals in leaves, nor translocation from roots to leaves. This is relevant because *S. ramosissima* may be used as leafy vegetable (Ventura and Sagi, 2013; Ventura et al., 2015; Patel, 2016; Barreira et al., 2017) and it is may be considered a cash-crop halophyte

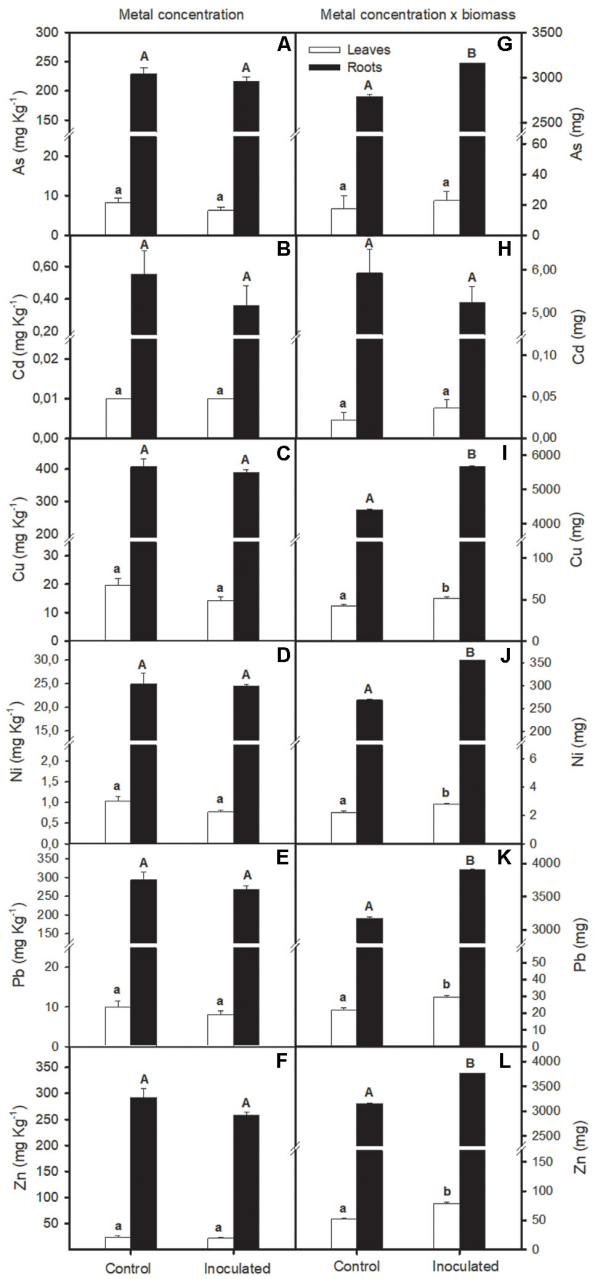

FIGURE 5 | Effect of soil bioaugmentation in polluted Odiel sediments on *S. ramosissima* heavy metal accumulation after 30 days of treatment initiation. In the left column, total **(A)** arsenic, As, **(B)** cadmium, Cd, **(C)** copper, Cu, **(D)** nickel, Ni, **(E)** lead, Pb, and **(F)** zinc, Zn, for leaves and roots of *S. ramosissima*. In the right column, total concentration values have been normalized for *S. ramosissima* biomass after 30 days of treatment **(G–L)**. Values are means ± S.E. (n = 3). Different capital letters indicate statistical differences between inoculation treatments in roots and different lower case letters in leaves ($p < 0.05$).

in scenarios of climate change (Mesa-Marín et al., 2019c). In several studies, bacteria promoted metal translocation from roots to shoots, mainly through chemical transformation of metals that increment their bioavailability (reviewed in Kong and Glick, 2017). In this case, bacteria did not promote metal translocation to leaves. Lastly, a remarkable result was also that bioaugmentation increased Na concentration in leaves, as observed by other authors for the halophyte *A. macrostachyum* (Navarro-Torre et al., 2017a,b). This may be connected plant osmoregulation by salt uptake, as suggested previously by Redondo-Gómez et al. (2010).

As an approach for practical purpose, it would be interesting to study the effect of PGPB bioaugmentation over time. Important considerations are the survival and colonization potential of the inoculated strains, as well as the potential ecological risks of introducing non-native plant and microbial species into field sites. In this sense, it should be highlighted that rhizobacteria used as an inoculum in this experiment were previously isolated from *S. ramosissima* rhizosphere in SW Spain salt marshes. The rationale behind inoculation with native microorganisms isolated from the sampling site and plant is that a bacterial strain from a population that is spatially and temporally prevalent in a concrete habitat, has more chances to persist when reintroduced by inoculation, than others being alien or transient to such habitat, especially in polluted conditions like Odiel salt marsh (Vogel, 1996). Non-native inoculants may have poor survival and colonization ability, and therefore they may be not sufficient to effectively support phytoremediation over time. In a long period, it could be expected that inoculated bacteria successfully colonize the rhizosphere and effectively facilitate plant metal uptake over time (Ma et al., 2015), and even the microbial community diversity in the rhizosphere may decrease (Liu et al., 2015).

In conclusion, our data suggested that inoculation with heavy metal resistant PGPB improved *S. ramosissima* plant growth, photosynthetic fitness, intrinsic water use efficiency and water content in polluted sediments. Also, we observed that *S. ramosissima* was able to accumulate great concentrations of heavy metals, mostly at root level, and that bioaugmentation incremented *S. ramosissima* heavy metal phytoremediation potential due to plant biomass increment achieved after inoculation, which enabled a greater accumulation capacity. Thus, our results claim the potential of using inoculation with heavy metal resistant PGPB to strengthen the capacity of *S. ramosissima* as candidate species in phytoremediation of salty degraded ecosystems.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JM-M: conceptualization, methodology, formal analysis, writing – original draft, and writing – reviewing and editing. JP-R: methodology, formal analysis, and writing – reviewing and editing. SR-G: resources, funding acquisition, and writing – reviewing

and editing. IR-L and EP: resources and writing – reviewing and editing. EM-N: methodology, formal analysis, resources, supervision, funding acquisition, and writing – reviewing and editing. All authors contributed to the article and approved the submitted version.

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A Bacterium Isolated From Soil in a Karst Rocky Desertification Region Has Efficient Phosphate-Solubilizing and Plant Growth-Promoting Ability

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Phosphorus in the soil accessible to plants can easily be combined with calcium ion, the content of which is high in karst rocky desertification (KRD) regions, thereby resulting in a low utilization efficiency of phosphorus. The application of phosphate-solubilizing bacteria (PSB) from the KRD region would facilitate enhanced phosphate availability in the soil. In the present study, the strains belonging to *Acinetobacter*, *Paraburkholderia*, and *Pseudomonas* with efficient phosphate-solubilizing ability were isolated from fruit tree rhizosphere soils in KRD regions. Particularly, *Acinetobacter* sp. Ac-14 had a sustained and stable phosphate-solubilizing ability (439–448 mg/L, 48–120 h). Calcium carbonate decreased the phosphate-solubilizing ability in liquid medium; however, it did not affect the solubilization index in agar-solidified medium. When cocultivated with *Arabidopsis thaliana* seedling, Ac-14 increased the number of lateral roots, fresh weight, and chlorophyll content of the seedlings. Metabolomics analysis revealed that Ac-14 could produce 23 types of organic acids, majorly including gluconic acid and D-(-)-quinic acid. Expression of Ac-14 glucose dehydrogenase gene (*gcd*) conferred *Pseudomonas* sp. Ps-12 with a sustained and stable phosphate-solubilizing ability, suggesting that the production of gluconic acid is an important mechanism that confers phosphate solubilization in bacteria. Moreover, Ac-14 could also produce indole acetic acid and ammonia. Collectively, the isolated Ac-14 from KRD regions possess an efficient phosphate-solubilizing ability and plant growth-promoting effect which could be exploited for enhancing phosphorus availability in KRD regions. This study holds significance for the improvement of soil fertility and agricultural sustainable development in phosphorus-deficient KRD regions.

Keywords: phosphate-solubilizing bacteria, plant growth-promoting, *Acinetobacter*, karst rocky desertification, organic acid

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INTRODUCTION

There are three karst centers worldwide: in the European Mediterranean, the Dinaric karst regions of the Balkan Peninsula, and in Southwest China. In karst regions, the processes of soil erosion, vegetation loss, exposure of rocks, and the appearance of a rocky landscape are termed as karst rocky desertification (KRD, Wang et al., 2004; Jiang et al., 2014). KRD is considered as one of the

most severe ecological problems worldwide (Wang et al., 2004; Tang et al., 2019). The degraded environment and low productivity in KRD regions hinders local economic growth and affects the people living in these regions (Jiang et al., 2014). Karst topography is a special landscape that is developed on carbonate rocks such as limestone, dolomite, or marble, and rocky desertification mainly occurs in carbonate rocks areas. Bare carbonate rocks generally produce Ca^{2+} and HCO_3^- in soil (White, 1997), wherein Ca^{2+} can combine with $H_2PO_4^-$ to form insoluble phosphate, which is inaccessible for plants.

Phosphorus (P) is one of the major essential macronutrients for plants and is an indispensable component of nucleic acids (RNA and DNA), proteins, phospholipids, and cofactors (such as ATP). To provide plants with nutritional requirements, P generally relies on the application of chemical P fertilizers to the soil. Nevertheless, these chemical P fertilizers are easily fixed by Ca^{2+}, Fe^{3+}, and Al^{3+} in the soil, thereby resulting in low utilization efficiency (Wei et al., 2018); on the other hand, extensive usage of chemical P fertilizer may lead to numerous environmental issues, such as soil compaction and water pollution, and may increase economic burden (Sharma et al., 2013; Wang et al., 2019). Considering the issue arising due to low efficiency of chemical P fertilizers, it is crucial to improve P availability in soil, particularly in the KRD regions.

Total P is abundant, but available P is generally low in soil (Wei et al., 2016). We previously reported that the community structure of rhizospheric bacteria is remarkably influenced by water-soluble phosphorus (WSP) in rocky desertification areas (Xie et al., 2019). Microorganisms play a pivotal role in the cycle of soil nutrients, wherein the phosphate-solubilizing bacteria (PSB) can convert insoluble phosphates into soluble forms that are available for plants. Recently, low-input agriculture has gained immense interest from researchers, with focus given to the development and use of commercial biological inoculants to the increase the availability of key nutrients, particularly P, to crop plants (Owen et al., 2015). PSB have been isolated from various environmental areas, including solid waste compost, metal-contaminated soil, activated sludge, and saltern sediments (Park et al., 2011; Zhu et al., 2011; Wei et al., 2016; Yu et al., 2019); however, no report is available regarding PSB isolated from the fruit tree rhizosphere soil in KRD regions. As native PSB have the advantage of colonizing in the local soil (Wang et al., 2018), PSB strains isolated from KRD soil would, therefore, have special significance and could be utilized in KRD ecological restoration and agricultural development.

The mechanisms of PSB on phosphate solubilization are complex. The redox activity of microorganisms, production of CO_2, secretion of siderophores, enzymes, and organic acid, and nitrogen assimilation were considered to be PSB mechanisms that could transform insoluble P to soluble forms (Rodríguez and Fraga, 1999; Sharma et al., 2013; Owen et al., 2015). In general, production of low molecular weight organic acids is the main phosphate-solubilizing mechanism of PSB (Rodríguez and Fraga, 1999; Ludueña et al., 2018). Gluconic acid has immense importance and is mainly produced by the glucose dehydrogenase (GCD) which is encoded by *gcd* gene (Liang et al., 2020). Besides the solubilizing insoluble phosphates, most PSB can also produce plant growth-regulating substances, such as indole acetic acid (IAA) and ammonia, to promote plant growth (Ludueña et al., 2018; Wang et al., 2019).

To develop and restore the agricultural and ecological environment in KRD regions, it is necessary to isolate PSB strains that are suitable for the native soil that also present an efficient phosphate-solubilizing ability and plant growth-promoting effect in KRD soil. The present study aimed to isolate novel PSB strains from the rhizosphere soil of fruit trees in the KRD regions in Southwest China and to analyze characteristics and mechanisms of phosphate solubilization and plant growth-promotion. In this study, we isolated a strain of PSB (*Acinetobacter* sp. Ac-14) from the fruit tree rhizosphere soils in the KRD region which could solubilize phosphate and promote plant growth and found that gluconic acid plays an important role in maintaining sustained and stable phosphate solubilization. Our findings will provide a theoretical foundation for understanding the phosphate solubilization mechanism of PSB and offer a basis for the application and development of ecofriendly, high-yield agriculture in KRD regions.

MATERIALS AND METHODS

Collection of Soil Samples and Isolation of PSB Strains

Soil samples were collected from the rhizosphere soil of fruit tree in KRD and non-KRD (NKRD) regions (**Table 1**), according to the method described previously (Xie et al., 2019). After adding sterile distilled water (soil sample: water = 1:10, w/v), the samples were shaken at 180 r/min for 30 min under 28 ± 2°C. After serial dilution (10^{-1}-10^{-6}) with sterile distilled water, 100 μL of soil solution was placed on modified National Botanical Research Institute's Phosphate (NBRIP) agar-solidified medium (Nautiyal, 1999, in **Supplementary Material and Methods**), and incubated at 28 ± 2°C for 6 days. The strains with clear halos were selected, halo zone diameter (D) and colony diameter (d) were determined, and solubilization index (SI) (the ratio of D/d) was calculated to roughly evaluate their phosphate-solubilizing capacities (Teng et al., 2019). The isolated strains were purified with modified NBPIP and AT salts medium (Nautiyal, 1999, in **Supplementary Material and Methods**) and stored with 20% glycerol in a refrigerator at −70°C.

Identification and Characterization of the Isolated PSB Strains

16S rDNA sequencing was used to identify the isolated strains (Zhu et al., 2011; Biswas et al., 2018; Yu et al., 2019). Genomic DNA was extracted from the isolated strains and the 16S rDNA was amplified via polymerase chain reaction (PCR) using universal primers 27F (5′-agrgtttgatcmtggctcag-3′, where r, a or g; m, a or c) and 1522R (5′-aaggaggtgatccarccrca-3′). Each PCR reaction comprised 1.2 μL deoxynucleotide triphosphate (dNTPs, each 2.5 mM), 1 μL template DNA, 0.18 μL each primer (10 μM), and 0.075 μL Ex DNA polymerase (5 U/μL, TaKaRa Co., Ltd., Dalian, China). The products were detected

TABLE 1 | The soil samples and number of isolated and sequenced PSB strains from the fruit tree rhizosphere soil.

Soil No.	Region	Code	Latitude	Longitude	Altitude/ m	County, province	Tree species	Tree age/year	No. of isolated PSB	No. of sequenced PSB	PSB species by 16S rDNA			
											Ac.	*Pa.*	*Ps.*	Others
1	KRD	EJ1	30°18′49″N	109°29′07″E	400	Enshi, Hubei	*Citrus reticulata*	3	39	2	0	0	2	0
2		GJ1	28°01′44″N	108°28′52″E	593	Yinjiang, Guizhou	*Citrus reticulata*	2	36	3	0	0	2	1
3		GJ2	28°01′46″N	108°28′55″E	588	Yinjiang, Guizhou	*Citrus reticulata*	2	43	11	**6**	0	0	5
4		GJ3	28°01′49″N	108°28′54″E	587	Yinjiang, Guizhou	*Citrus reticulata*	5	104	17	1	0	13	3
5		GY1	28°01′25″N	108°29′07″E	517	Yinjiang, Guizhou	*Citrus maxima* (Burm) Merr.	10	68	2	0	0	2	0
6		HT1	28°27′40″N	109°29′52″E	550	Huayuan, Hunan	*Amygdalus persica* L.	3	19	2	0	0	2	0
7		HZ1	28°26′33″N	109°28′49″E	550	Huayuan, Hunan	*Ziziphus jujuba* Mill.	3	17	4	0	0	4	0
8		NY1	28°12′38″N	112°35′42″E	388	Ningxiang, Hunan	*Citrus aurantium* L.	10	22	2	0	0	**2**	0
9		NY2	28°12′38″N	112°35′42″E	388	Ningxiang, Hunan	*Citrus aurantium* L.	10	17	1	0	0	0	1
10		WG1	30°46′16″N	114°12′24″E	21	Wuhan, Hubei	*Osmanthus fragrans* (Thunb.) Lour.	5	56	5	0	0	5	0
11		XB1	26°32′08″N	110°45′41″E	365	Xinning, Hubei	*Castanea mollissima* BL.	5	30	5	0	**4**	0	1
12		XQ1	26°39′46″N	110°57′52″E	427	Xinning, Huibei	*Citrus sinensis* L. Osbeck	10	59	2	0	0	1	1
13		XQ2	26°31′46″N	110°45′03″E	353	Xinning, Hubei	*Citrus sinensis* L. Osbeck	10	16	5	0	0	1	4
14		YH1	29°00′33″N	108°57′31″E	333	Youyang, Chongqing	*Zanthoxylum schinifolium* Sieb. et Zucc	5	27	0	0	0	0	0
15		YY1	28°56′38″N	109°56′38″E	330	Youyang, Chongqing	*Citrus maxima* (Burm.) Merr.	1	17	6	1	0	0	5
16	NKRD	CX1	43°54′13″N	125°20′31″E	187	Changchun, Jilin	*Armeniaca vulgaris* Lam.	10	59	11	0	0	11	0
17		CX2	43°54′13″N	125°20′37″E	211	Changchun, Jilin	*Armeniaca vulgaris* Lam.	20	107	8	0	0	8	0
18		CX3	43°49′13″N	125°16′41″E	251	Changchun, Jilin	*Armeniaca vulgaris* Lam.	10	69	3	0	0	2	1
Total		/	/	/	/	/	/	/	805	89	8	4	55	22

KRD, karst rocky desertification; NKRD, non-karst rocky desertification; Ac, Acinetobacter; Pa, Paraburkholderia; Ps, Pseudomonas.

via agarose gel electrophoresis and the 1.5 kb band was purified using a Gel/PCR extraction kit (Tiangen Biotech Co., Ltd., Beijing, China). The purified 16S rDNA fragment was ligated to pMD19-T Simple vector (TaKaRa Co., Ltd., Dalian, China), and then transformed into *Escherichia coli* DH5α competent cells. Positive clones were selected for sequencing, and the conservative sequences from numerous sequences were used for BLASTx with the NCBI database to identify the species. A phylogenetic tree was constructed using the neighbor-joining method with MEGA ver. X.

Strains were inoculated in Luria–Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, and NaCl 5 g/L) and incubated overnight under 180 r/min at 28 ± 2°C. Thereafter, the cells were collected via centrifugation at 5,000 r/min for 5 min. After washing with phosphate buffer saline (NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.44 g/L, KH_2PO_4 0.24 g/L, pH = 7.4) 2–3 times, the samples were fixed with 2.5% glutaraldehyde at 4°C for at least 8 h, dehydrated gradually with 30, 50, 70, 80, 90, and 100% ethanol (twice), and then dissolved in absolute ethanol. The solution was dropped on the cover glass, dried at room temperature, and fixed to the scanning electron microscope (SEM) column after spraying gold. The morphology was scanned under SEM (QUANTA 200, FEI Company, Hillsboro, OR, United States). In total, 30 cells from each sample were randomly selected to measure the cell size using ImageJ. Gram staining was performed using the Gram stain kit (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China).

Evaluation of Phosphate-Solubilizing Characteristics of the Isolated PSB Strains

The sequenced PSB strains were incubated in LB medium until A_{600} = 1.8; thereafter, 10% (v/v) inoculum amount was transferred to liquid NBRIP medium and incubated under 180 r/min at 28 ± 2°C for 24 h. The cultures were centrifuged at 8,000 r/min for 10 min and the P content of the supernatant was detected via Mo-blue method (Murphy and Riley, 1962). The NBRIP medium inoculated with the same amount of LB was used as a negative control.

Moreover, the pH value of the supernatant and the A_{600} value of bacteria were determined at 6, 12, 24, 48, 72, 96, and 120 h after inoculation. pH was detected by a pH meter (PH400, Alalis Instruments Technology Co., Ltd., Shanghai, China). A_{600} value was measured by a spectrophotometer (TU-1810S, Beijing Purkinje General Instrument Co., Ltd., Beijing, China). To eliminate the effect of calcium phosphate in the culture medium on A_{600} value, the precipitate obtained by centrifuging the cultures was washed with equal volumes of 0.1 mM hydrochloric acid (Xiang et al., 2011).

Different concentrations of calcium carbonate ($CaCO_3$) (0, 0.5, 1.0, 1.5, and 2.0 g/L) were added to NBRIP liquid medium to detect its effect on the growth of the isolated PSB strains. The SI value was evaluated on NBRIP agar-solidified medium containing $CaCO_3$.

To evaluate the NH_4^+ assimilation of *Acinetobacter* sp. Ac-14, equal amounts of NH_4Cl, $NaNO_3$, and KNO_3 were used to substitute $(NH_4)_2SO_4$ in the NBRIP medium. After 24 h culture, the soluble P content, pH value, and A_{600} value of the supernatant of the cultures were determined.

Evaluation of Plant Growth-Promoting Ability of the Isolated PSB Strains

Arabidopsis thaliana (Columbia) seeds were sterilized with 3% sodium hypochlorite for 1 min, washed with sterile water for 5–6 times, and sown on 1/2 Murashige and Skoog (MS) [Murashige and Skoog basal medium with vitamins (Duchefa Biochemie, NLD) 2.202 g/L, 2-(N-morpholino) ethanesulfonic acid (Genview, United States) 0.5 g/L and sucrose 10 g/L, pH = 5.7] agar-solidified medium. After preserving at 4°C for 3 days, the plates were vertically placed in a light incubator (22°C, 16 h light, 8 h dark) for 7 days. The seedlings were then transferred to NBRIP agar-solidified medium, and inoculated with PSB (10 μL, A_{600} = 0.05) around the roots. The plates were positioned vertically and cultured in a light incubator. After cocultivation for 7 and 14 days, the number of lateral roots, primary root length, fresh weight, and chlorophyll content of the seedlings were measured. The chlorophyll content was determined according to the method described by Xu et al. (2018). The seedlings inoculated with LB were used as the control.

Untargeted Metabolomics of Ac-14

The Ac-14 strain was incubated in LB medium until A_{600} = 1.8, and thereafter, 10% (v/v) inoculum amount was transferred to liquid NBRIP medium and incubated under 180 r/min at 28 ± 2°C for 24 h. The cultures were then centrifuged at 8,000 r/min for 10 min, the supernatants were frozen in liquid nitrogen, and metabolomics were evaluated using liquid chromatography tandem mass spectrometry (LC-MS/MS) method by Novogene Bioinformatic Technology Co., Ltd. (Beijing, China). The detailed protocols are described in **Supplementary Material and Methods**. The supernatants from NBRIP medium inoculated with the same amount of LB were used as negative control.

Cloning and Expression of the Ac-14 *gcd*

The genome of *Acinetobacter* sp. Ac-14 was sequenced by PacBio's Single Molecule Real-Time (SMRT) sequencing technology. The sequence was submitted to NCBI GenBank (accession number CP063769). The *gcd* gene (2,406 bp, in **Supplementary Material and Methods**) was amplified via PCR using primers GCD-F (5′-agaattcatgaatcaaccta cttcaagatcagg-3′, underlined is *Eco*RI site) and GCD-R (5′-aggatccttatttgttatctggtaaggcataagcc-3′, underlined is *Bam*HI site). For expression vector, the *gcd* was cloned into pBBR1MCS-2 plasmid[1]. The recombinant plasmid was then introduced into *Pseudomonas* sp. Ps-12. Ps-12 carrying the recombinant plasmid pBBR1MCS-2-gcd was named Ps-12 (gcd). The phosphate-solubilizing ability, pH value, and A_{600} value of Ps-12 or Ps-12 (gcd) were detected in the NBRIP liquid medium.

[1]http://www.addgene.org/85168/

Determination of IAA and Ammonia of Ac-14

The Ac-14 strain was incubated overnight in LB medium under 180 r/min at 28 ± 2°C. The bacteria were then collected via centrifugation at 5,000 r/min for 2 min. After washing twice with LB liquid medium containing 5 mg/mL tryptophan, the suspension was inoculated into LB (containing 5 mg/mL tryptophan) liquid medium (A_{600} = 0.1). Thereafter, the bacteria were incubated at 28 ± 2°C and 180 r/min for 120 h. Two mL of the culture was collected every 24 h and centrifuged at 12,000 r/min for 1 min. Next, 1 mL of supernatant was mixed with 2 mL of Salkowski's reagent (2% 0.5 M $FeCl_3$ in 35% $HClO_4$ solution) (Biswas et al., 2018). After reacting at room temperature under dark conditions for 30 min, indole acetic acid (IAA) was determined by measuring A_{530}. The LB medium containing 5 mg/mL tryptophan was used as negative control.

The collected bacteria were washed twice with peptone water (peptone 10 g/L, NaCl 5 g/L, pH 7.0 ± 0.2). The biomass was adjusted to A_{600} = 0.1 using peptone water, and incubated under 180 r/min at 28 ± 2°C for 120 h. Culture samples were collected every 24 h and centrifuged at 12,000 r/min for 1 min. The supernatant was then reacted with Nessler's reagent (Marques et al., 2010; Orhan, 2016) to determine ammonia by measuring A_{420}. Peptone water was used as a negative control.

Statistical Analysis

All the experiments concerning data comparisons were performed three times. Statistical analyses were performed using the S-N-K method of one-way ANOVA or independent samples *t*-test (95% confidence) with IBM SPSS Statistics 22.0 (SPSS Inc., Chicago, IL, United States). Values with different lowercases represented a significant difference at $P < 0.05$. * or ** indicated significant difference for the *t*-test ($P < 0.05$ or $P < 0.01$).

RESULTS

Isolation of PSB Strains From the Fruit Tree Rhizosphere Soil in KRD Regions

In total, 805 PSB strains were isolated from 18 fruit tree rhizosphere soil samples. Of these, 570 PSB strains were from KRD regions in Southwest China, and 235 PSB strains were from the NKRD regions (**Table 1**). Moreover, 89 strains (67 and 22 strains from KRD and NKRD, respectively) with different colony morphologies and larger SI values were screened after 16S rDNA sequencing. *Pseudomonas* sp. was present in both KRD and NKRD region soils, whereas *Acinetobacter* sp. and *Paraburkholderia* sp. only appeared in some soil samples of KRD regions (**Table 1**).

The phosphate-solubilizing ability of the 89 strains was evaluated by the Mo-blue method. Of these, 22 PSB strains could dissolve more than 300 mg/L of phosphate. Among these 22 strains, seven belonged to *Acinetobacter*, one belonged to *Paraburkholderia*, and 14 belonged to *Pseudomonas* (**Supplementary Table S1**). The *Acinetobacter* sp. Ac-14 isolated from the KRD region (GJ2, Yinjiang, Guizhou) revealed the highest phosphate-solubilizing ability.

One strain from each genus summarized in **Supplementary Table S1** with the highest phosphate-solubilizing ability was selected for further study (**Table 2**). The colony morphology observation revealed that *Acinetobacter* sp. Ac-14 was white and circular with an SI of 2.37; *Paraburkholderia* sp. Pa-3 was white in the middle and translucent outside with an irregular edge and SI of 2.29; *Pseudomonas* sp. Ps-12 strain was yellow and circular with an SI of 1.66 (**Supplementary Figure S1**). The three strains were rod-shaped and gram-negative bacteria but were different sizes (**Supplementary Figure S1**). Phylogenetic tree by 16S rDNA sequencing confirmed that the three strains belonged to different groups (**Supplementary Figure S2**).

PSB Strains, Particularly Ac-14, Had Sustained and Stable Phosphate-Solubilizing Ability Even Under $CaCO_3$ Condition

To assess the phosphate-solubilizing characteristics of the isolated strains, their phosphate-solubilizing ability in liquid NBRIP medium, as well as the pH and bacterial A_{600} values, were determined. As illustrated in **Figure 1**, for Ac-14, the soluble P concentration in the medium rapidly elevated within 12 h, and then slowly increased between 12 and 48 h, and was further maintained at a high level (439–448 mg/L, 48–120 h). The pH value of the medium dropped rapidly from 7.3 to 4.5 within 6 h and was then maintained at a low level. The growth of biomass rapidly elevated to 1.2 within 12 h, and then slowly increased until it reached the stationary phase. Thus, the phosphate-solubilizing ability revealed a similar change as that of A_{600}, whereas it revealed an inverse relation with the pH value. Furthermore, the lowest pH value appeared earlier than that of the maximum value of soluble P content and A_{600}.

For Pa-3 (**Figure 1**), the soluble P concentration in the medium rapidly increased and further decreased, with the highest value (365 mg/L) at 24 h; moreover, soluble P could not be detected at 72 h. In contrast, the pH value rapidly decreased within 12 h and then increased. The growth of biomass rapidly elevated within 12 h, then increased slowly, and then decreased slowly. The phosphate-solubilizing ability, pH value, and growth of biomass of Ps-12 revealed a similar change tendency to that of Pa-3 (**Figure 1**).

As rocky desertification mainly occurs in carbonate rock areas, we studied the phosphate-solubilizing ability of the isolated PSB strains under $CaCO_3$ condition (**Figure 2**). $CaCO_3$ markedly decreased the phosphate-solubilizing ability of the strains, and its effect was increased as concentration increased. At a concentration of 2 g/L, soluble P could not be detected in the supernatant; however, Ac-14 still had a higher phosphate-solubilizing ability than Pa-3 and Ps-12 under each $CaCO_3$ concentration condition. Under 0.5 g/L $CaCO_3$ (**Figure 3**), the changes in the phosphate-solubilizing ability, pH value, and A_{600} value of the three PSB strains were similar to those without $CaCO_3$, except for the lower maximum phosphate-solubilizing abilities than those without $CaCO_3$ (**Figure 1**). The effect of

TABLE 2 | The colony characteristics of *Acinetobacter* sp. Ac-14, *Paraburkholderia* sp. Pa-3, and *Pseudomonas* sp. Ps-12 from KRD regions in Southwest China.

Characteristics	Ac-14	Pa-3	Ps-12
Color	White	White in the middle with translucent around the outside	Yellow
Surface	Glossy	Moist	Glossy
Shape	Circular	Irregular edge	Circular
Uplift/shape	Raised	Uneven	Flat
Margin	Smooth	Smooth	Smooth
SI value	2.37 ± 1.09	2.29 ± 0.39	1.66 ± 0.94
Cell size (μm)	(0.58 ± 0.05) × (1.03 ± 0.16)	(0.49 ± 0.04) × (1.99 ± 0.17)	(0.54 ± 0.06) × (1.66 ± 0.19)
Gram character	Negative	Negative	Negative
Genus (by 16S rDNA)	*Acinetobacter*	*Paraburkholderia*	*Pseudomonas*

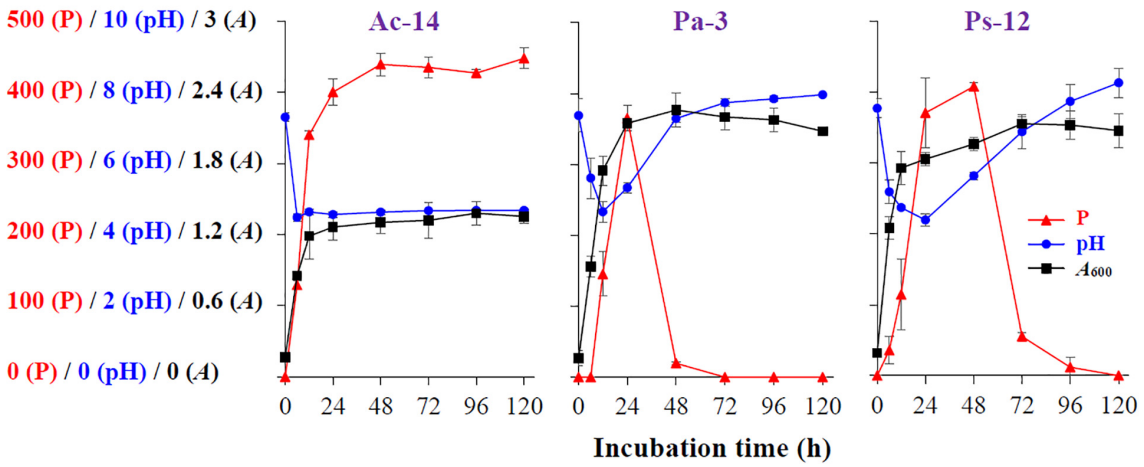

FIGURE 1 | The time-curve of the soluble P content (P, mg/L), pH value (pH), and A_{600} value (*A*) of *Acinetobacter* sp. Ac-14, *Paraburkholderia* sp. Pa-3, and *Pseudomonas* sp. Ps-12.

$CaCO_3$ on phosphate-solubilizing ability was limited in liquid medium, but not in agar-solidified medium (**Supplementary Figure S3** and **Supplementary Table S2**).

Moreover, the effect of several insoluble phosphate sources on the phosphate-solubilizing ability of the aforementioned three PSB strains was studied. When $Ca_3(PO_4)_2$ was substituted with equal amounts of $AlPO_4$ or $FePO_4$ (data not shown) in liquid NBRIP medium, soluble P could not be detected in the medium inoculated with Ac-14, Pa-3, and Ps-12, thus indicating that the three strains can only dissolve $Ca_3(PO_4)_2$.

Inoculation of Ac-14 to Roots of *Arabidopsis thaliana* Seedlings Promoted Vegetative Growth

Since Ac-14, Pa-3, and Ps-12 had a strong ability to dissolve phosphate, their effect on plant growth needs to be explored. Therefore, we inoculated the strains in the roots of *A. thaliana* seedlings and observed the growth status. When Ac-14 was cocultured for 7 days, the number of lateral roots, fresh weight, and chlorophyll content began to increase; however, no change was observed in the primary root length (**Supplementary Figures S4A-D**). On the 14th day, the seedlings inoculated with Ac-14 grew vigorously and had developed lateral roots (**Figure 4A**). The fresh weight was increased 6.5-fold, and the chlorophyll content increased 46.5-fold (**Figures 4B,C**), compared to that without inoculation, thus indicating that Ac-14 could remarkably promote the growth of *A. thaliana* seedlings. Pa-3 and Ps-12 were also effective but had a lesser impact on the growth of *A. thaliana* seedlings compared to that of Ac-14.

We further assessed the effect of Ac-14 on the growth of *A. thaliana* seedlings in a medium containing 0.5 g/L $CaCO_3$. The seedlings inoculated with Ac-14 presented remarkable growth when cocultured for 7 days compared to that without Ac-14 (**Supplementary Figures S4E-H**). On the 14th day, the fresh weight of the seedlings inoculated with Ac-14 increased 2.1-fold, while the chlorophyll content increased 5-fold, compared to that without inoculation (**Figure 5**). These results indicated that inoculation of Ac-14 in the roots of *Arabidopsis* seedlings could promote vegetative growth in the presence and absence of $CaCO_3$.

Ac-14 Produced 23 Types of Organic Acids, While Gluconic Acid and D-(-)-Quinic Acid Increased Mostly

To detect the metabolites of Ac-14 related to phosphate solubilization, we performed untargeted metabolomics

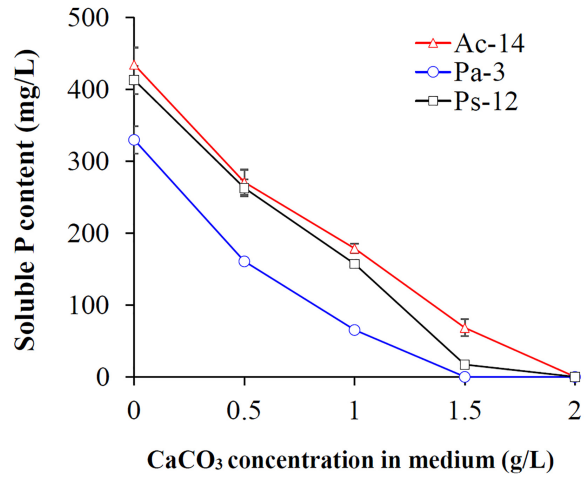

FIGURE 2 | Effect of different concentrations of $CaCO_3$ on the phosphate-solubilizing ability of strains Ac-14, Pa-3, and Ps-12.

(**Supplementary Figure S5**). In total, 752 metabolites were detected; of these, 294 metabolites were detected under LC-MS/MS (ESI-) scan model, whereas 458 metabolites were detected under LC-MS/MS (ESI+) scan model (see sheet "All metabolites" in **Supplementary Excel S1**). According to fold change $\geq$ 1.5 or FC $\leq$ 0.667, VIP $>$ 1, and P-value $<$ 0.05, 124 differential metabolites were identified in Ac-14 compared to that without inoculation; of these, 56 metabolites (37 metabolites were significantly increased and 19 metabolites were significantly decreased) were detected under ESI- model, and 68 metabolites (30 metabolites were significantly increased and 38 metabolites were significantly decreased) were detected under ESI+ model (**Supplementary Figure S5**; sheet "Differential metabolites" in **Supplementary Excel S1**). As organic acids were produced during bacterial phosphate solubilization, the differences in organic acids were further analyzed. Ac-14 could produce 23 types of organic acids (13 kinds of organic acids were increased under ESI- model and 10 kinds of organic acids were increased under ESI+ model) with the concentration increasing from 4.1-fold to 462.2-fold. Among these organic acids, gluconic acid (366-fold) and D-(-)-quinic acid were remarkably increased (462-fold) (**Table 3**). These results indicated that Ac-14 could produce abundant organic acids.

Expression of Ac-14 *gcd* Gene Conferred Ps-12 With Sustained and Stable Phosphate-Solubilizing Ability

To confirm the phosphate-solubilizing ability of *Acinetobacter* sp. Ac-14 related with gluconic acid, we cloned the Ac-14 *gcd* gene and expressed it in *Pseudomonas* sp. Ps-12. As illustrated in **Figure 6**, expression of *gcd* gene in Ps-12 could maintain a sustained and stable soluble P concentration (448 mg/L, 48 h; 438 mg/L, 120 h), whereas Ps-12 without *gcd* revealed a maximum soluble P level at 48 h (393 mg/L), then gradually decreased, and was further undetected at 120 h. As a result,

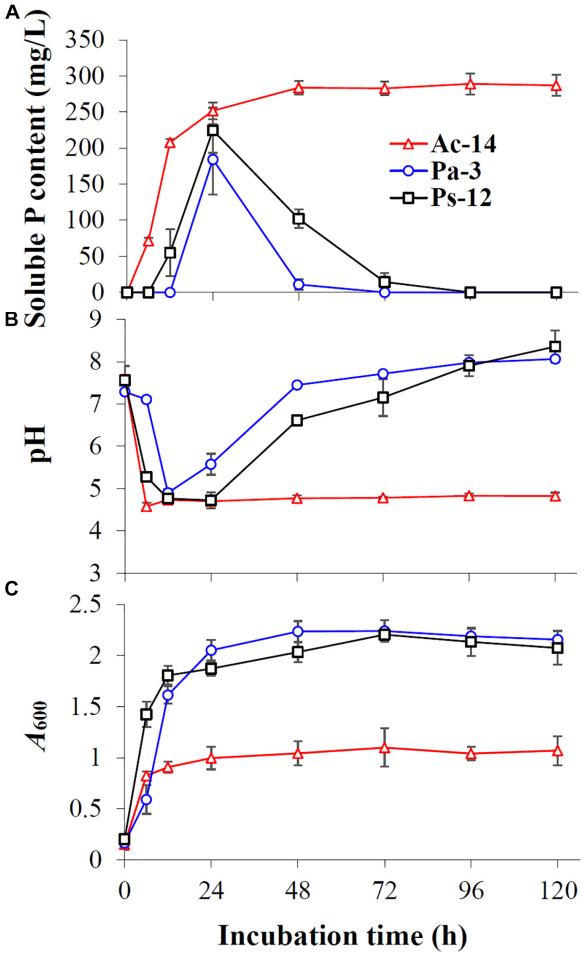

FIGURE 3 | The time-curve of **(A)** the soluble P content, **(B)** pH value, and **(C)** A_{600} value of the strains Ac-14, Pa-3, and Ps-12 in NBRIP medium containing 0.5 g/L $CaCO_3$.

Ps-12 with *gcd* could maintain a lower pH (4.1–4.2) during the experiment period; however, that without *gcd* presented decreased pH within 24 h, which then gradually increased. Ps-12 with *gcd* had a lower biomass than that without *gcd*. These results confirmed that the production of gluconic acid is an important mechanism conferred on bacteria with sustained and stable phosphate solubilization.

Phosphate Solubilization of Ac-14 Did Not Occur via NH_4^+ Assimilation

NH_4^+ assimilation can release protons and results in a decreased pH. Whether the sustained and stable phosphate-solubilizing ability of Ac-14 is related to NH_4^+ assimilation remains unclear. Hence, the phosphate-solubilizing ability, pH value, and A_{600} value under different nitrogen sources [$(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$, or KNO_3] were determined. No significant difference was observed under different nitrogen sources (**Supplementary Figure S6**), indicating that the phosphate-solubilizing ability of Ac-14 did not occur due to NH_4^+ assimilation.

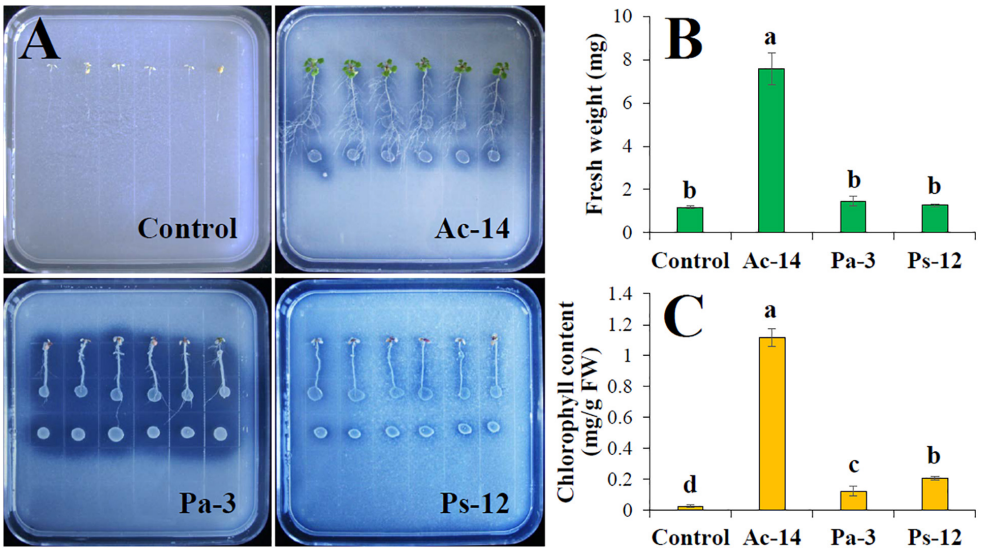

FIGURE 4 | Effects of the strains Ac-14, Pa-3, and Ps-12 on the growth of *Arabidopsis thaliana* seedlings in NBRIP agar-solidified medium (cocultivation for 14 days). **(A)** The growth status, **(B)** fresh weight per plant, and **(C)** chlorophyll content of *A. thaliana*. Different lowercase letters indicated statistically significant differences ($P < 0.05$).

Ac-14 Produced IAA and Ammonia

Whether other mechanisms besides phosphate solubilization are involved in the growth promoting effect of Ac-14 remains unclear. Hence, the plant growth promoting traits (like production of IAA and ammonia) for Ac-14 were detected. IAA was increased with the incubation time, and the concentration reached 25.1 mg/L at 120 h. Furthermore, ammonia was also increased and then maintained at a stable level during the incubation period, and the concentration was 231 mg/L at 120 h (**Figure 7**).

Ac-14 Grew in a Wide Range of pH

As Ac-14 can produce organic acids, the environmental pH range it can withstand remains unclear. Hence, the growth status of Ac-14 under different pH values was explored (**Supplementary Figure S7**). The strain grew best at pH 5–7, and the A_{600} value was approximately 2.3. At pH 8–9, A_{600} value was approximately 2.2. When pH was 4.5 or 10, A_{600} value was about 2.1. When pH was 11, the growth of the strain was severely affected and A_{600} value was only 0.12. When pH was less than 4 or greater than 12, the bacteria could not grow. This result indicated that Ac-14 could grow at various pH ranges.

DISCUSSION

Acinetobacter sp. Ac-14 With Phosphate-Solubilizing Ability Was Successfully Isolated From KRD Soil

P is an important macro-element in plant nutrition. PSB can transform insoluble P into an available form in the soil and has immense applications in ecoagriculture. Due to the limitations of microbial ecological adaptability, the utilization of native microorganisms to develop biological fertilizer has obvious advantages (Wang et al., 2018). Therefore, it is important to isolate PSB that efficiently dissolves phosphate and promotes plant growth from KRD soil samples. In this study, 805 PSB strains were isolated from the rhizosphere soil of fruit trees. Of these, 570 were from 15 soil samples in the KRD regions in Southwest China, while 235 were isolated from three soil samples from NKRD regions. Further analysis revealed that *Acinetobacter* sp. and *Paraburkholderia* sp. were uniquely distributed in fruit tree rhizosphere soil of KRD regions. *Acinetobacter* sp. Ac-14 could efficiently dissolve phosphate and promote plant growth. Therefore, Ac-14 has a potential application in ecological restoration and development in KRD regions.

Previous studies reported that *Pseudomonas*, *Bacillus*, and *Rhizobium* are the most efficient phosphate solubilizers (Rodríguez and Fraga, 1999). In recent years, *Acinetobacter* was reported to have a high phosphate-solubilizing ability. *Acinetobacter calcoaceticus* YC-5a has a strong ability for solubilizing insoluble phosphate by producing organic acid and some plant growth-promoting factors such as IAA and siderophores. Moreover, this bacterium exhibits strong resistances to lead and antibiotics (Ren et al., 2013). *Acinetobacter* sp. YU-SS-SB-29, isolated from monazite sand, exhibits high phosphate solubilization and tolerance to uranium (Sowmya et al., 2014). Furthermore, *Acinetobacter* was also reported to effectively degrade toxic organic compounds (Li et al., 2020). In this study, *Acinetobacter* sp. Ac-14 isolated from fruit tree rhizosphere soil of KRD region had a high phosphate-solubilizing ability, further confirming that *Acinetobacter* can dissolve phosphate. Whether the bacterium is capable of resisting

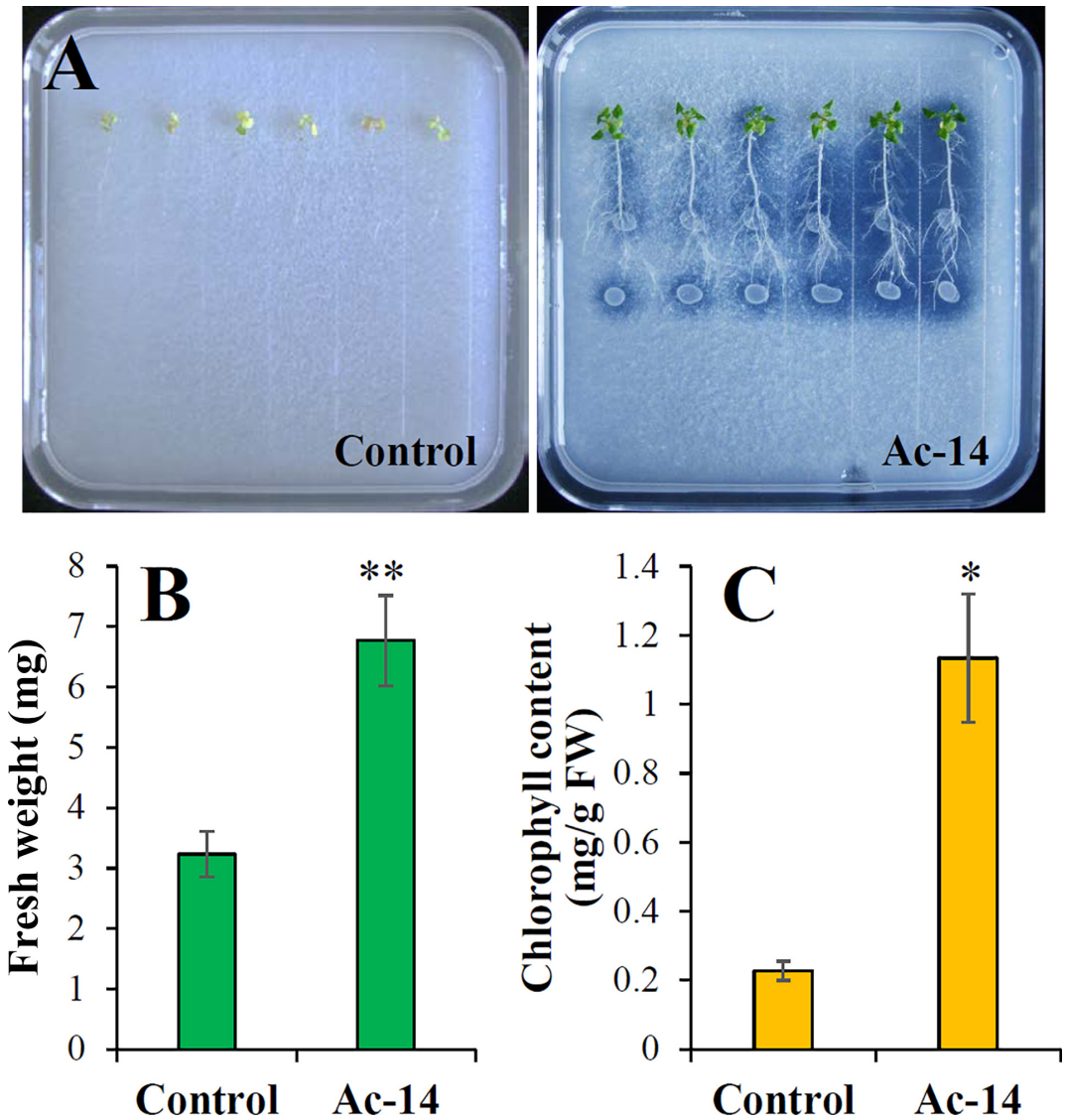

FIGURE 5 | Effect of *Acinetobacter* sp. Ac-14 on the growth of *Arabidopsis thaliana* seedlings in NBRIP agar-solidified medium containing 0.5 g/L $CaCO_3$ (cocultivation for 14 days). **(A)** The growth status, **(B)** fresh weight per plant, and **(C)** chlorophyll content of *A. thaliana*. $^{*}P < 0.05$; $^{**}P < 0.01$.

metal and degrading toxic organic compounds needs to be further studied.

Production of Gluconic Acid Is the Core Mechanism of Ac-14 During Phosphate Solubilization

The mechanisms of PSB involved in phosphate solubilization are complex, and various mechanisms have been proposed; of these, low pH is common. In the present study, Ac-14 revealed efficient, sustained, and stable phosphate-solubilizing ability, with the pH value was maintained at a low level. These findings are in accordance with previous studies (Lin et al., 2006; Wang et al., 2019). For Pa-3 and Ps-12, the phosphate-solubilizing ability was initially increased and then decreased with the incubation time, whereas the pH value was initially decreased and then increased. Therefore, the phosphate solubilization mechanism of these three strains may relate to pH changes; however, the changes occurring in the three strains are not exactly the same, thereby indicating differences in the mechanism of phosphate solubilization. Similar changes of pH and phosphate solubilization to that of Pa-3 and Ps-12 were reported previously (Teng et al., 2019). The maximum A_{600} of Ac-14 was remarkably lower than that of Pa-3 and Ps-12, which was presumably because low pH in the environment inhibited bacterial proliferation. In the NBRIP liquid medium containing $CaCO_3$, the phosphate-solubilizing ability of the three PSB strains was decreased. This may occur because abundant Ca^{2+} combined with soluble P to form insoluble phosphate; however, the changed tendencies of phosphate-solubilizing ability, pH value, and A_{600} under

TABLE 3 | Comparison of organic acids between cultures inoculated with *Acinetobacter* sp. Ac-14 and non-inoculation under LC-MS/MS.

Name	FC	*P*-value	VIP	ESI−/+
D-(-)-quinic acid	462.2	3.17E-05	4.7	+
Gluconic acid	366.0	3.13E-05	3.7	−
Indole-5-carboxylic acid (98%)	142.5	1.98E-04	3.1	−
D-galactonic acid	119.8	1.82E-05	3.7	+
Pyrophosphate	111.1	8.50E-06	3.0	−
(R)-lipoic acid	60.5	1.82E-04	3.2	+
Mesaconic acid	51.6	7.52E-04	2.5	−
2-methylsuccinic acid	40.5	1.75E-03	2.9	+
Indole-3-lactic acid	39.0	2.08E-05	2.3	−
2-hydroxy-2-methylbutanedioic acid	34.9	2.09E-03	2.2	−
6-phosphogluconic acid	32.6	8.44E-04	2.7	+
2-(2-hydroxy-3-methylbutanamido)-4-methylpentanoic acid	27.2	8.80E-05	2.5	+
Kojic acid	16.5	1.71E-03	2.2	+
Kinic acid	15.5	6.28E-04	1.7	−
Suberic acid	12.1	1.77E-03	1.6	−
2-(acetylamino)-4-(methylthio) butanoic acid	9.7	7.48E-04	1.4	−
Uric acid	9.1	8.48E-05	1.7	+
Elaidic acid	7.6	5.72E-03	1.3	−
2-ketoadipic acid	7.0	2.65E-02	1.3	−
4-oxododecanedioic acid	6.3	5.18E-04	1.4	+
D-α-Hydroxyglutaric acid	5.9	1.09E-02	1.1	−
3-[(methoxycarbonyl) amino]-2,2,3-trimethylbutanoic acid	5.3	3.82E-03	1.1	−
4-(2,3-dihydro-1,4-benzodioxin-6-yl) butanoic acid	4.1	5.49E-04	1.1	+

FC, fold change; P-value from univariate analysis (t-test); VIP, variable importance in the projection (VIP) value of the first principal component of PLS-DA model.

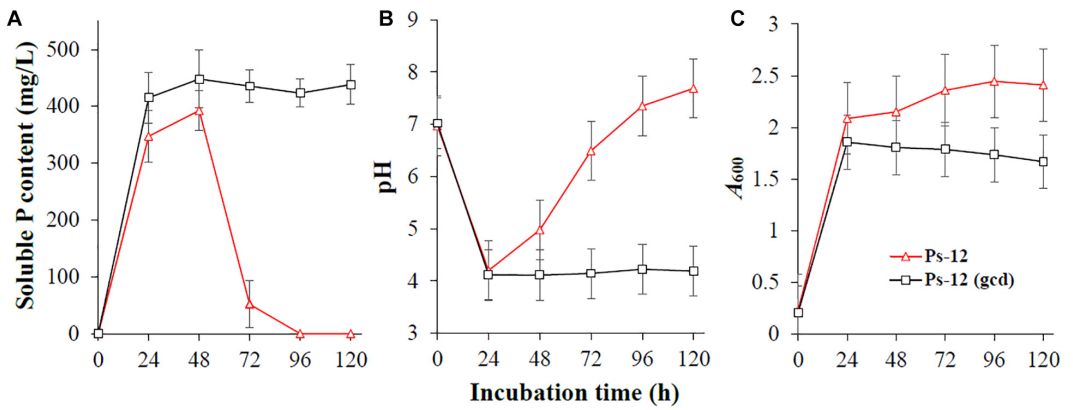

FIGURE 6 | The effects of *Acinetobacter* sp. Ac-14 *gcd* gene on **(A)** the soluble P content, **(B)** pH value, and **(C)** A_{600} value of *Pseudomonas* sp. Ps-12.

$CaCO_3$ condition were almost similar to those without $CaCO_3$. Interestingly, we also found that inhibition of $CaCO_3$ did not affect the phosphate-solubilizing ability in the agar-solidified medium, which might be due to the poor mobility of $CaCO_3$ in this medium. Therefore, application of PSB strains as biological fertilizers in the soil may not be affected by $CaCO_3$ in KRD soils.

Furthermore, it was reported that low pH could occur due to production of organic acids and assimilation of NH_4^+ (Rodríguez and Fraga, 1999; Sharma et al., 2013; Owen et al., 2015; Zhu et al., 2018). In this study, metabolomics analysis revealed that Ac-14 could substantially increase 23 types of organic acids; of these, gluconic acid, one of the most common organic acid metabolites (Lin et al., 2006; Rodríguez et al., 2006; Ludueña et al., 2018), was highly increased. It was reported that gram-negative bacteria produce gluconic acid during the extracellular oxidation of glucose through GCD (Liu et al., 1992; Goldstein, 1995), the lowered pH value and changed reduction potential are considered reasons for the dissolution of tricalcium phosphate (Lin et al., 2006). As a Gram-negative bacterium, Ac-14 may lower the pH value by producing gluconic acid, after excluding the possibility of NH_4^+ assimilation. This was further demonstrated by the genetic engineering approach that suggested that *Pseudomonas*

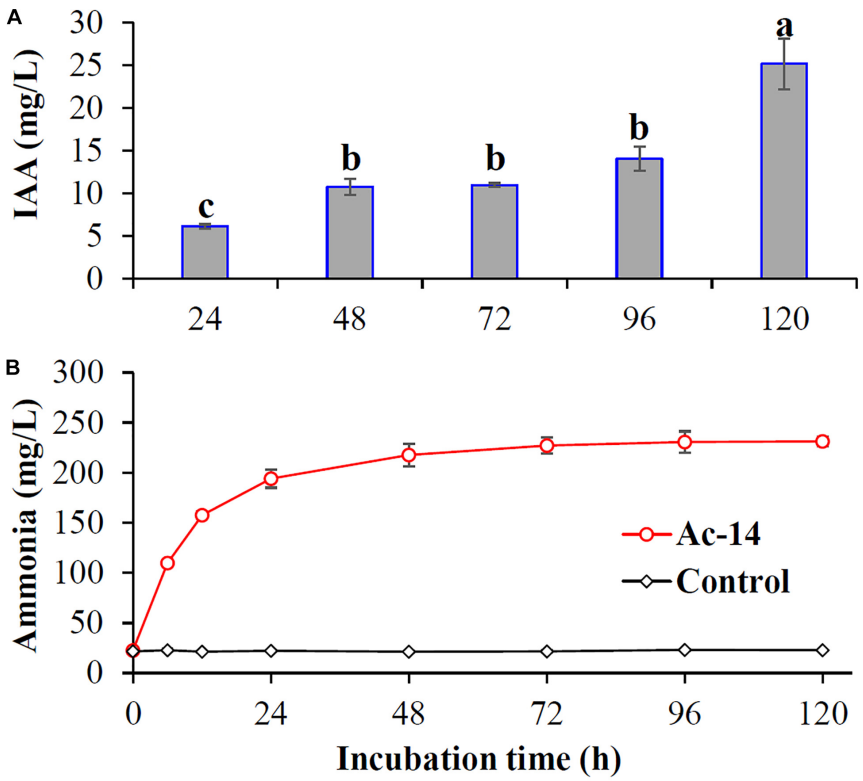

FIGURE 7 | The content of **(A)** IAA and **(B)** ammonia produced by *Acinetobacter* sp. Ac-14. Different lowercase letters indicated statistically significant differences ($P < 0.05$).

sp. Ps-12 expressing Ac-14 *gcd* gene could maintain sustained and stable phosphate-solubilizing ability. Besides the common gluconic acid, D-(-)-quinic acid was also highly increased. D-(-)-quinic acid is an efficient low molecular mass organic acid chelator. It can bind with metal ions (Menelaou et al., 2009) and acts as a metabolite with an antioxidant function (Gargallo-Garriga et al., 2015), which may confer the bacterium with tolerance and/or metal-degrading ability. The exact role of D-(-)-quinic acid in phosphate solubilization, and other mechanisms, needs to be further studied.

Ac-14 Promotes the Growth of *A. thaliana* Seedling by Phosphate Solubilization and Produces IAA and Ammonia

It was reported that PSB could promote the growth of numerous plants, such as *Lolium perenne*, *Zea mays*, *Vigna radiata*, Chinese cabbage, etc. (Owen et al., 2015; Biswas et al., 2018; Wang et al., 2019). In general, PSB enhances the root growth of plants and the yield of crops by increasing P availability (Sharon et al., 2016; Ku et al., 2018). In this study, we explored the effects of Ac-14, Pa-3, and Ps-12 on the growth of *A. thaliana* seedlings. The number of lateral roots, fresh weight, and chlorophyll content were remarkably increased when *A. thaliana* was inoculated with Ac-14, whereas the leaves of *A. thaliana* without inoculation gradually turned white and died. However, not all PSB strains can promote plant growth (Bashan et al., 2013). In this study, Pa-3 or Ps-12 could not significantly promote *A. thaliana* seeding growth. Furthermore, the process of insoluble P-solubilization is affected by several factors including soil type, nutritional richness of the soil, pH, moisture, and others (Rodríguez and Fraga, 1999; Chen et al., 2006; Alori et al., 2017; Wei et al., 2018). On the other hand, PSB would compete with other soil microflora (Wei et al., 2018). Whether Ac-14 could successfully colonize in KRD regions soil and exhibit phosphate-solubilizing ability and growth-promoting activities needs further research.

In this study, we revealed that the Ac-14 strain could produce certain growth-promoting substances such as IAA and ammonia. As a phytohormone, IAA promotes root growth to achieve a large surface area that facilitates nutrient absorption from the soil (Davies, 2010; Biswas et al., 2018; Wang et al., 2019). It was reported that some PSB strains, such as *A. calcoaceticus* YC-5a, *Bacillus* sp., *Staphylococcus* sp., and *Serratia* sp. could also produce IAA (Ren et al., 2013; Biswas et al., 2018; Ludueña et al., 2018). Ammonia is a nitrogen source that can be taken up by plants and could promote plant growth. It was reported that all test strains isolated from plant root nodules of different rhizospheric soils in the vicinity of Aligarh could produce ammonia (Ahmad et al., 2008). Three PSB strains isolated from earthworms (*Metaphire posthuma*) could also produce ammonia (Biswas et al., 2018). Therefore, Ac-14 promoting

A. thaliana seedling growth might also relate to the secretion of IAA and ammonia.

CONCLUSION

In this study, we isolated PSB strains from the fruit tree rhizosphere soil in the KRD regions in Southwest China. A novel PSB strain, *Acinetobacter* sp. Ac-14, presented efficient, sustained, and stable phosphate-solubilizing ability, and could also promote plant growth. The strain dissolved insoluble phosphate by producing 23 types of organic acids; of these, gluconic acid played an important role in the solubilization process. Ac-14 could also produce plant growth-promoting substances such as IAA and ammonia. Therefore, Ac-14 may adapt to the high calcium environment in KRD regions and has the potential to be utilized in improving the fertility of KRD regions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

XC and DC designed the experiments. JX, ZY, GW, WX, and CL performed the experiments. JX, ZY, and DC analyzed the data. JX, XC, and DC wrote the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.625450/full#supplementary-material

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Litter Decomposition of *Imperata cylindrica* in a Copper Tailing Areas With Different Restoration History: Fungal Community Dynamics and Driving Factors

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Microorganisms drive litter decomposition while maintaining the chemical cycle of ecosystems. We used the dominant vegetation (*Imperata cylindrica*) in the mining area selected for this study for this experiment to explore fungal community characteristics, key fungal groups, and their associative driving factors during *I. cylindrica* litter decomposition. Maximum litter C/N values occurred 100 days after the commencement of the decomposition experiment during all different recovery years in this copper tailings area. Heavy metals in litter [copper (Cu), zinc (Zn), plumbum (Pb), and cadmium (Cd)] accumulated gradually with decomposition. The dominant fungal phyla observed in the community were Ascomycota and Basidiomycota, while the classes Sordariomycetes and Eurotiomycetes significantly increased as litter decomposition progressed. Degrees of connectivity and interaction between fungal communities were highest during the early litter decomposition stage. Sordariomycetes, Dothideomycetes, and Leotiomycetes all played critical roles in maintaining fungal community relationships. The effect of physicochemical properties and enzyme activities in *I. cylindrica* litter was significant on the dominant fungi, while driving factors that affected fungal communities differed over different recovery stages. Total nitrogen (TN), heavy metals, pH, and enzyme activities in the little were significantly correlated with fungal community composition. Litter properties throughout the litter decomposition process mainly affected the dynamics of the fungal community structure. The main environmental factors that affected fungal community structure were copper content and pH. *Dichotomopilus*, *Trichoderma*, *Knufia*, *Phialophora*, *Oxyporus*, and *Monocillium*, which all played important roles in litter decomposition, positively correlated with heavy metals, sucrase, and catalase. Finally, results from this study will help us better clarify litter decomposition mechanisms in degraded ecosystems as well as provide a scientific basis for improving species cycling and nutrient transformation efficiency in mining ecosystems.

Keywords: fungal community, litter properties, decomposition dynamics, *Imperata cylindrica*, copper mining area

INTRODUCTION

Although mineral resources are the basis of national economic planning, mining production activities over long periods will have a severe impact on the ecological environment of mining areas. Furthermore, mining activities destabilize the integrity of mountain structures and vegetation coverage while destroying existing landforms (Huang, 2020). The construction of mining areas also displaces enormous amounts of soil while also disrupting the daily lives of residents (Wang, 2020). Additionally, vast amounts of wastewater and solid waste material are produced during mineral resource processing, which has had a catastrophic effect on local water resources and soil ecosystems (Yan and Feng, 2010). In other words, soil organic matter and the nutrient quality of mining areas are extremely poor.

Phytoremediation is a popular method used to alleviate soil organic matter and soil nutrient content degradation in mining areas. It is used to improve the carbon sequestration capacity of aerial components of plants, the root secretion capacity of plants, and the microbial decomposition capacity of plant litter in mining areas (Meng et al., 2018). Plant litter accumulates year by year with vegetation restoration in mining areas. Litter decomposition is a critical factor in ecosystem nutrient transformation processes. Litter properties and microbial community characteristics are also important factors that determined litter decomposition rates. Additionally, environmental factors have important effects on litter decomposition processes. Accordingly, this study explored the biological and abiotic factors that take place during the litter decomposition process in a Chinese copper tailings mining area to help improve species cycling and nutrient conversion efficiency. This study also offers an ecological restoration approach that can be used in copper tailings areas under severe heavy metal pollution.

Saprophytic fungi are widely known to be the main litter decomposers, which secrete extracellular enzymes that decompose cellulose, lignin, and other macromolecular compounds. Fungi are known to have effective and efficient enzymatic systems, some of which can produce extensive hypha. Fungal hyphae penetrate litter where they subsequently decompose carbohydrates, pectin, lignin, and cellulose. At the same time, enzymes in litter can help alter the structure and chemical composition of litter while decomposing complex organic matter into soluble amino acids or other small molecular compounds (Pfeiffer et al., 2013). Most fungi that belong to the Ascomycota phylum can secrete cellulase and hemicellulase, which both play critical roles in litter decomposition (Snajdr et al., 2011). Additionally, Basidiomycetes species are the main decomposers of lignin, and their relative abundance has been shown to peak during the latter stage of litter decomposition (Wang et al., 2019a,b). Moreover, it is known that fungal community succession takes place during litter decomposition. *Aureobasidium*, *Mucor*, and *Aspergillus* are the dominant fungal genera during the early litter decomposition stage, being able to utilize simple sugars and starch. However, over time these fungi genera are gradually replaced by cellulose-decomposing and lignin-decomposing fungi, such as *Trichoderma*, *Marasmius*, and *Mycena*, all of which can secrete cellulase, peroxidase, and polyphenol oxidase. These fungi also play important roles in cellulose and lignin degradation during litter decomposition (Yan, 2011). *Mortierella* and *Penicillium* are known to dominate during the latter litter decomposition stage (Zhang et al., 2003). However, although many relevant studies have focused on natural ecosystems, little is known about litter decomposition characteristics within degraded environments, such as mining areas.

The Northern Copper Mine, Shanxi Province, is China's largest mine not based on coal, which producing 7 million tons of copper tailings annually (Wang et al., 2018). Through soil analysis, a previous study had determined that the soil of this copper tailings area has been contaminated with various heavy metal elements, the most important being arsenic (As), cadmium (Cd), copper (Cu), plumbum (Pb), chromium (Cr), and zinc (Zn; Jia et al., 2019). Dominant plant species include *I. cylindrica*, *Bothriochloa ischaemum*, and *Artemisia sacrorum* to name a few, all of which yield enormous amounts of litter at the end of the growing season. Based on this, we conducted a 460 day *in situ* litter decomposition experiment. As the study object, *I. cylindrical* litter was selected to explore the dynamics of fungal community structure and composition during different decomposition stages using high-throughput sequencing. We also measured physicochemical properties and extracellular enzyme activities in litter. The objective of this study was to answer the following three questions: (1) How did decomposition rates and nutrient content change during litter decomposition in this copper tailings area? (2) In what way did fungal community characteristics and their interactive relationships dynamically change? (3) What were the driven factors of fungal community succession in litter during the litter decomposition process?

MATERIALS AND METHODS

Site Description and Litter Sampling

Initial construction on the Shibahe tailings dam (lat 35°15′ ~ 35°17′ N, long 118°38′ ~ 111°39′ E) began in 1969. This copper tailings dam is a branch of the expansive Northern Copper Mine in China's Shanxi Province. The Shibahe tailings dam is currently subdivided into 16 smaller sub-dams. The region is under the influence of a continental monsoon climate, where the average annual temperature is 13.5°C and annual precipitation is 631 mm (Jia et al., 2019). Our experiments were conducted at three of the 16 three sub-dams: the S516 sub-dam, the S536 sub-dam, and the S560 sub-dam, whose representative phytoremediation years are 50, 22, and 5 (**Supplementary Table S1**; Jia et al., 2019).

In April 2019, we collected a total of nine *I. cylindrica* litter samples from each of the three sub-dams. Litter samples were then divided into two, where one was used as initial litter (D0) for high-throughput sequencing and the other was naturally air-dried to use in litter decomposition bags. The size of the nylon mesh bags was 20 cm × 20 cm, with an aperture

of 1 mm × 1 mm. A total of 8 g of *I. cylindrica* litter was placed into each nylon mesh bag. In May 2019, three sample plots were established in each sub-dam for which three mesh bags were added to each plot. This provided a total of 27 litter bags (**Supplementary Figure S1**). Litter samples were collected after being allowed to decompose for 100 days (D100), 200 days (D200), and 460 days (D460). This provided a total of 36 litter samples. These littler samples were subsequently divided into two. One was stored (−20°C) for high-throughput sequencing, and the other was stored (4°C) to ascertain physiochemical properties and enzyme activities.

Mass Litter Residual Rate and Chemical Properties

Litter bags were collected, and sediment removed, after which the fresh weight of litter (Wi) was measured. A portion of the samples was dried at 65°C to a constant weight, and water content (P) was measured. The mass residual rate of litter was then calculated (Zhang et al., 2019). The mass residual rate (%) = W_i*(1-P)/W_0. Here, W_0 denotes the dry weight of the initial litter bag.

An elemental analyzer (vario EL/MACRO cube, Elementar Analysensysteme GmbH, Hanau, Germany) was used to measure total carbon (TC) and total nitrogen (TN) content in litter samples. Before measuring litter pH, litter water (1:20 mass/volume) suspensions were shaken for 30 min (Fioretto et al., 2000). Heavy metal (Cu, Zn, Pb, and Cd) concentrations in the litter were measured using atomic absorption spectrometry (Agilent Technologies 200 Series AA, United States). Additionally, 3,5-Dinitrosalicylic acid colorimetry was used to measure litter sucrose and cellulase content; phenol-sodium hypochlorite colorimetry was used to measure urease content; potassium permanganate titration was used to measure catalase content; and the disodium phenyl phosphate colorimetric method was used to measure phosphatase content (Jia et al., 2019).

DNA Extraction, PCR Amplification, and Miseq Sequencing

Before being filtered through sterile membranes (0.2 μm pore size; Millipore, Jinteng, Tianjin, China), we washed litter samples three separate times in a sterile phosphate buffer solution (PBS: NaCl, KCl, Na_2HPO_4, and KH_2PO_4). Filtered samples were then sealed in sterile centrifuge tubes before extracting microbial DNA. Following the manufacturer's instructions, the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, United States) was used to extract microbial DNA in litter samples. Additionally, we used the NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States) for DNA quantification. Primers ITS1F (5′-CTTGGTCATTTAGAG GAAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) were used as the internal transcribed spacer (ITS) gene copy numbers for all samples. Sequencing was done at Shanghai Majorbio Bio-pharm Technology (Shanghai, China), using the MiSeq platform (Illumina, Inc., United States). We submitted raw sequencing data to the National Center for Biotechnology Information Sequence Read Archive[1] under the project accession number PRJNA764552.

Statistical Analysis

QIIME was used to integrate the original FASTQ format data (Caporaso et al., 2010). USEARCH (ver. 7.[2]) was used for the verification and removal of chimeric sequences. The operational taxonomic unit (OTU) partition threshold was established at a 97% sequence similarity of the classification results, after which it was used for fungal community diversity and relative abundance calculations. To procure species classification data that correspond to each OTU, the RDP Classifier[3] was employed for the classification and analysis of each OTU sequence. Using the UNITE8.0 fungi database, the reliability threshold was determined to be 70%.

SPSS ver. 24.0 was used to analyze the physicochemical properties of litter during different decomposition stages, while Duncan's multiple range test was used in one-way ANOVA. A Student's *t* test was used to compare both fungal diversity and richness during the different decomposition stages. Moreover, we applied non-metric multidimensional scaling (nMDS) analysis to ascertain the fungal community structure based on Bray–Curtis dissimilarity, while we used analysis of similarities (ANOSIM) to ascertain intergroup differences. Additionally, we applied variance inflation factor analysis to eradicate high multicollinearity in environmental factors employing the "vegan package" in R3.5.3. We further employed Canoco 5.0 (Microcomputer Power, United States) for redundancy analysis. Finally, Gephi (an interactive platform) was used to analyze and envisage networks. Co-correlation network properties, such as the average degree, the network density, the average clustering coefficient, the network diameter, and the average path length, were calculated (Jiao et al., 2020). The higher that the average degree, the average clustering coefficient, and the network density were, the closer the network connection was assumed to be. Additionally, a lower path length and network diameter were indicative of a closed network connection (Ma et al., 2016).

RESULTS

Litter Properties Throughout the Decomposition Process

The decomposition rate of *I. cylindrica* litter was higher during the early decomposition stage but then decreased after 100 days of decomposition (**Supplementary Figure S2**). During the latter decomposition stage, the range of the remaining litter mass was from 27.05 to 67.14%, and the mass residual rate of litter in the S536 sub-dam was significantly higher compared to the S560 and S516 sub-dams ($p < 0.05$).

The nutrient content of litter decreased as litter decomposition progressed ($p < 0.05$). The C/N ratio reached a peak after 100 days of decomposition and then significantly decreased. During the

[1]https://www.ncbi.nlm.nih.gov/sra
[2]http://drive5.com/usearch/
[3]http://rdp.cme.msu.edu

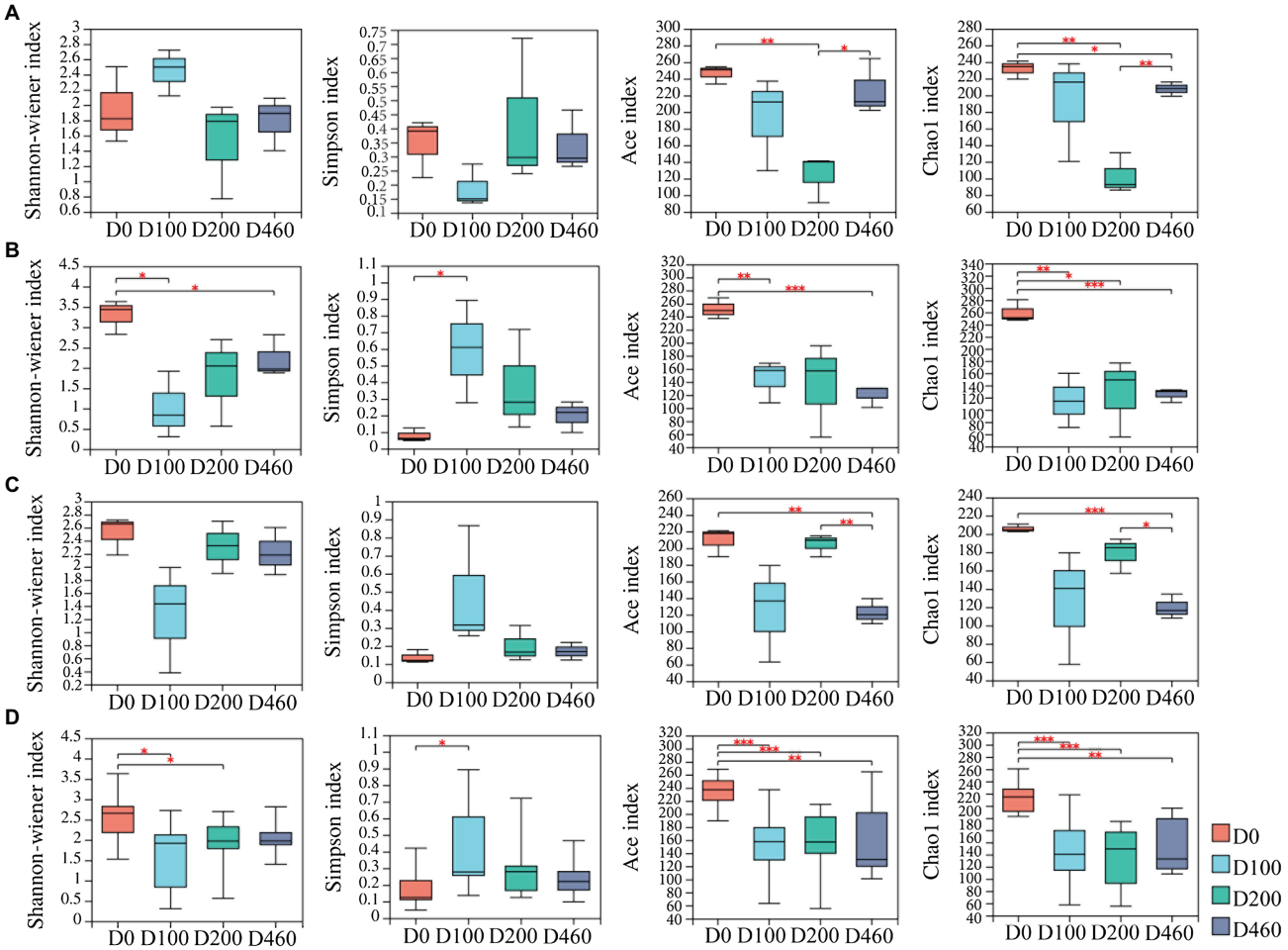

FIGURE 1 | The dynamics of litter fungal community diversity indexes in S516 **(A)**, S536 **(B)**, S560 **(C)**, and the whole copper mining area **(D)**. Significance levels were denoted with $^{**}p<0.01$ and $^{***}p<0.001$.

early decomposition stage, the litter C/N ratio in the S536 sub-dam was significantly higher compared to the other sub-dams. Heavy metal (Cu, Zn, Pb, and Cd) content gradually accumulated as decomposition progressed. and except for Zn, these heavy metals significantly increased throughout the decomposition process ($p<0.05$). Moreover, litter pH increased throughout the decomposition process in the S516 sub-dam ($p<0.05$), reaching a maximum after 200 days of decomposition in the S536 and S560 sub-dams (**Supplementary Table S2**).

No consistent change was observed in *I. cylindrica* litter enzyme activities throughout the entire decomposition process in all sub-dams (**Supplementary Table S3**). However, catalase activity in litter significantly exceeded the initial decomposition stage (D0) in the S536 and S560 sub-dams. Litter cellulase activity in these two sub-dams was significantly higher at D100 than the other decomposition stages, while urease activity reached a maximum at D200 (**Supplementary Table S3**). Sucrase activity in litter significantly increased throughout the decomposition process in the S536 sub-dam, while it first increased before decreasing again throughout the decomposition process in the S516 and S560 sub-dams ($p<0.05$). Compared to the other sub-dams, sucrase activity in the S560 sub-dam was significantly higher ($p<0.05$) after 200 days of decomposition (D200).

Taxonomic Distribution and Fungal Diversity

A total of 993 fungal OTUs were obtained from litter samples based on 97% sequence similarity. Specie richness (i.e., the ACE and Chao1 indices) of the litter fungi community decreased at D200 before increasing again in the S516 sub-dam ($p<0.05$). The Shannon, ACE, and Chao1 indices decreased after 100 days of decomposition (D100) in the S536 sub-dam ($p<0.05$). However, the richness index of the fungi community in litter decreased at the D460 stage in the S560 dam ($p<0.05$; **Figure 1**).

Results from this study showed that common OTU numbers in each sub-dam throughout the different decomposition stages were 100 (S516), 30 (S536), and 32 (S560), accounting for 10.02, 5.23, and 6.78% of the total OTUs, indicating compositional differences in fungi communities during the different

decomposition stages. We found 215 and 146 special OTUs in the litter, accounting for 37.46 and 30.93% of total OUTs at the D0 stage in the S536 and S560 sub-dams, respectively, and 76 special OTUs in the litter were found at the D0 stage in the S516 sub-dam. This was indicative of the significant differences between litter fungi communities and litter decomposition processes (**Figure 2**).

Fungal Community Composition Among the Different Litter Decomposition Stages

Ascomycota and Basidiomycota were the dominant fungi in *I. cylindrica* litter, with a 99.41% relative fungal community abundance. Ascomycota was the dominant fungi during the initial litter decomposition stage (D0), with an 88.68% relative abundance. At a class level, Dothideomycetes was the dominant fungal group at the D0 stage, with a 49.19% relative abundance (**Figures 3, 4**). Dothideomycetes abundance decreased as litter decomposition progressed ($p < 0.05$; **Figures 3, 4**). On the other hand, Sordariomycetes significantly increased as litter decomposition progressed ($p < 0.05$). Similarly, Eurotiomycetes also increased with litter decomposition and was affected by litter decomposition in the S516 sub-dam ($p < 0.05$; **Figures 3, 4**). Overall, the relative abundance of Agaricomycetes peaked at D100 (**Figure 3**).

We observed significant differences in the composition of fungal communities in the litter at a genus level. In the S516 sub-dam, *Cladosporium* was the dominant fungal genus during the initial litter decomposition stage (D0), and it decreased as litter decomposition progressed ($p < 0.05$; **Figure 4E**). However, *Cercophora*, *Scytalidium*, *Dichotomopilus*, and *Knufia* all increased as litter decomposition progressed. The dominant fungi genera were *Talaromyces* and *Trichoderma* during the intermediate and later stages of litter decomposition, respectively (**Figure 3**). Overall, we observed significant differences among *Trichoderma*, *Cladosporium*, *Alternaria*, and *Knufia* throughout the different decomposition stages ($p < 0.05$; **Figure 4H**).

Correlations Among Different Fungal Communities

We constructed a genus level-based fungal community co-correlation network to explore changes in fungal community relationships during the litter decomposition process (**Figure 5**). Results showed that the fungal community network contained 120 nodes and 270 edges during the initial decomposition

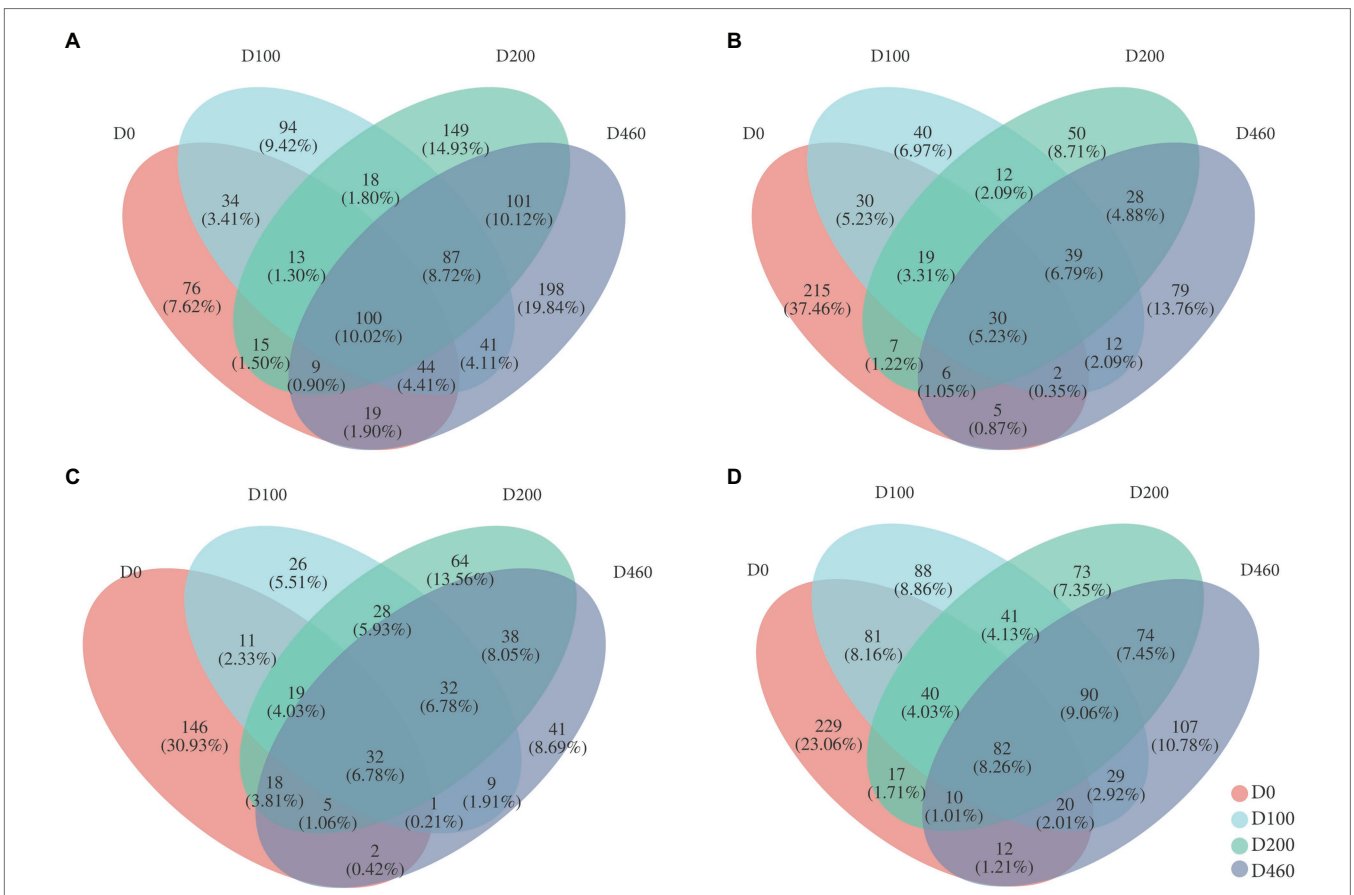

FIGURE 2 | Venn diagrams of litter fungi at OTU level in S516 **(A)**, S536 **(B)**, S560 **(C)**, and the whole copper mining area **(D)**. The percentage written in parentheses means the special species as a percentage of total OTUs.

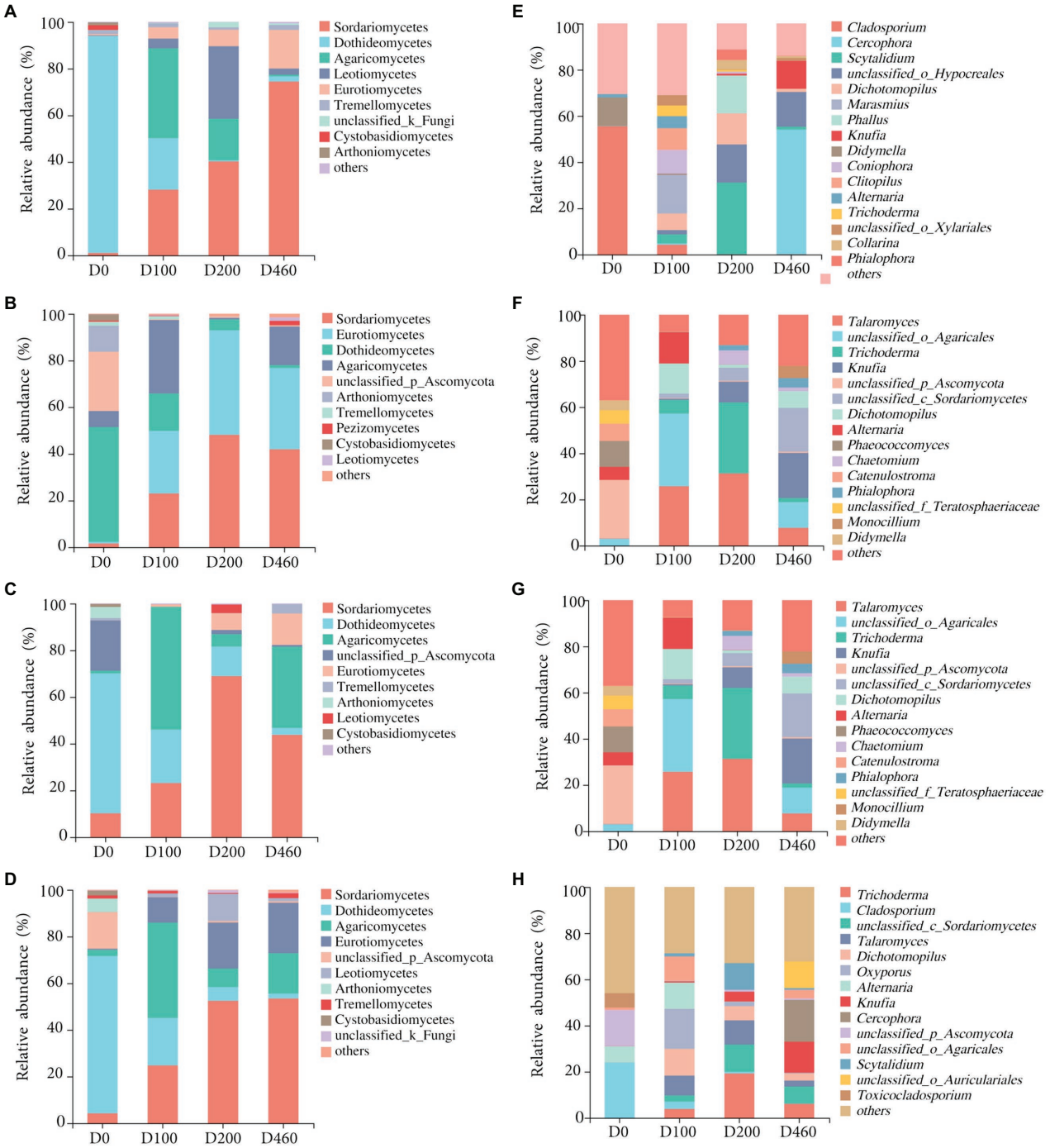

FIGURE 3 | Litter fungal community compositions with relative abundance greater than 1 and 4% at class **(A-D)** and genus level **(E-H)**, respectively, in S516 **(A**,**E)**, S536 **(B**,**F)**, S560 **(C**,**G)**, and the whole copper mining area **(D**,**H)**.

stage (D0). The number of nodes and edges increased after 100 days of decomposition (D100) but decreased after 200 days of decomposition (D200). Throughout the different decomposition stages, the proportions of positive fungal community correlations reached 100%, indicating that fungal communities coexisted during the litter decomposition process. Results showed that the average degree, the network density, and the average clustering coefficient of the fungal network were all highest at the D100 stage, indicating that degrees of connectivity and interaction among fungal communities were highest during the early litter decomposition stage. Additionally, to a certain extent, the degree of modularization reflected the degree of

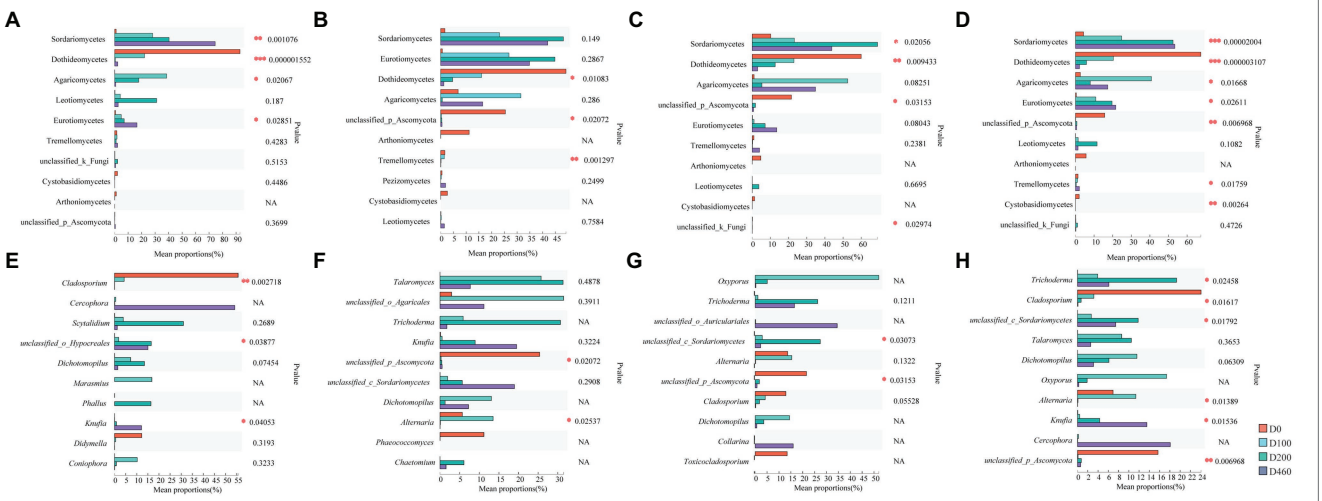

FIGURE 4 | Difference of dominant fungi classes **(A-D)** and genera **(E-H)** at four litter decomposition stages in S516 **(A**,**E)**, S536 **(B**,**F)**, S560 **(C**,**G)**, and the whole copper mining area **(D**,**H)**. Significance levels were denoted with $^{**}p<0.01$ and $^{***}p<0.001$.

community functional diversity. The degree of fungal network modularization during the different decomposition stages was relatively high, which indicated that the functional diversity of the fungal community was high during the litter decomposition process (**Table 1**). The key fungal groups that maintained fungal network connectivity also changed during the litter decomposition process. *Botrytis* had the highest betweenness centrality (BC) value at the D0 stage. *Cladostachy*s was gradually replaced by *Clonostachys* and *Phaeosphaeri*a as litter decomposition progressed. The role that *Devriesia* played was important in maintaining relationships among fungal communities at the D460 stage (**Table 2**).

Fungal Community Structure and Environmental Variable Correlations

At an OUT level, nMDS analysis was conducted on fungal communities at all decomposition stages. Results showed that *I. cylindrica* litter decomposition significantly affected fungal community structure during the different recovery stages (S516: $R^2=0.623$, $p=0.001$; S536: $R^2=487$, $p=0.003$; S560: $R^2=0.614$, $p=0.001$; **Figure 6**). Canonical correlation analysis results showed that physicochemical properties and enzyme activities in litter significantly affected the dominant fungi in litter ($p<0.05$), while driving factors affecting fungal communities in litter also varied during the different recovery stages (**Figure 7**). In the S516 sub-dam, driving factors that caused changes to the fungal community structure were Zn ($R^2=0.776$, $p=0.003$), C/N ($R^2=0.740$, $p=0.006$), TC ($R^2=0.734$, $p=0.004$), and Pb ($R^2=0.662$, $p=0.016$). Moreover, the key factors that affected fungal community structure in the S536 sub-dam were Cu ($R^2=0.731$, $p=0.001$) and Zn ($R^2=0.579$, $p=0.016$). Driving factors that significantly affected the fungal community structure in the S560 sub-dam were Cu ($R^2=0.896$, $p=0.002$), TC ($R^2=0.667$, $p=0.01$), and C/N ($R^2=0500$, $p=0.041$). Overall, TN, heavy metal content (Cu, Zn, Pb, and Cd), pH, and enzyme activities (sucrose and catalase) significantly correlated with fungal community composition ($p<0.05$). Variance partitioning analysis results showed that litter properties and extracellular enzyme activities accounted for 13.15 and 7.10% of fungal community structure, respectively. Litter properties during the litter decomposition process primarily affected changes in fungal community structure, while the Cu content ($R^2=0.877$, $p=0.002$) and pH level ($R^2=0.866$, $p=0.002$) were the main ecological factors that affected fungal community structure (**Supplementary Figure S3**).

In this study, *Trichoderma* and *Clitopilus* positively correlated with sucrase in the S516 sub-dam (**Figure 8A**), which can potentially promote cellulose decomposition. *Boubovia*, *Knufia*, and *Phialophora* positively correlated with sucrase, and *Marchandiomyces* significantly correlated with cellulase in the S536 sub-dam ($p<0.05$; **Figure 8B**), indicating the important roles that these fungi groups may play in maintaining ecosystem carbon cycling. Generally, *Dichotomopilus*, *Trichoderma*, *Knufia*, *Phialophora*, *Oxyporus*, and *Monocillium* significantly and positively correlated with heavy metal content ($p<0.05$), indicating that these genera possess a certain heavy metal tolerance. Moreover, these fungal genera significantly and positively correlated with sucrase and catalase content ($p<0.05$), indicating the critical role that they play in litter decomposition within this copper tailings area (**Figures 8C,D**).

DISCUSSION

Characteristics of Litter Decomposition and Nutrient Release During Different Recovery Stages

Litter decomposition rates closely correlate with their corresponding chemical properties, while TC, TN, total phosphorus (TP), and

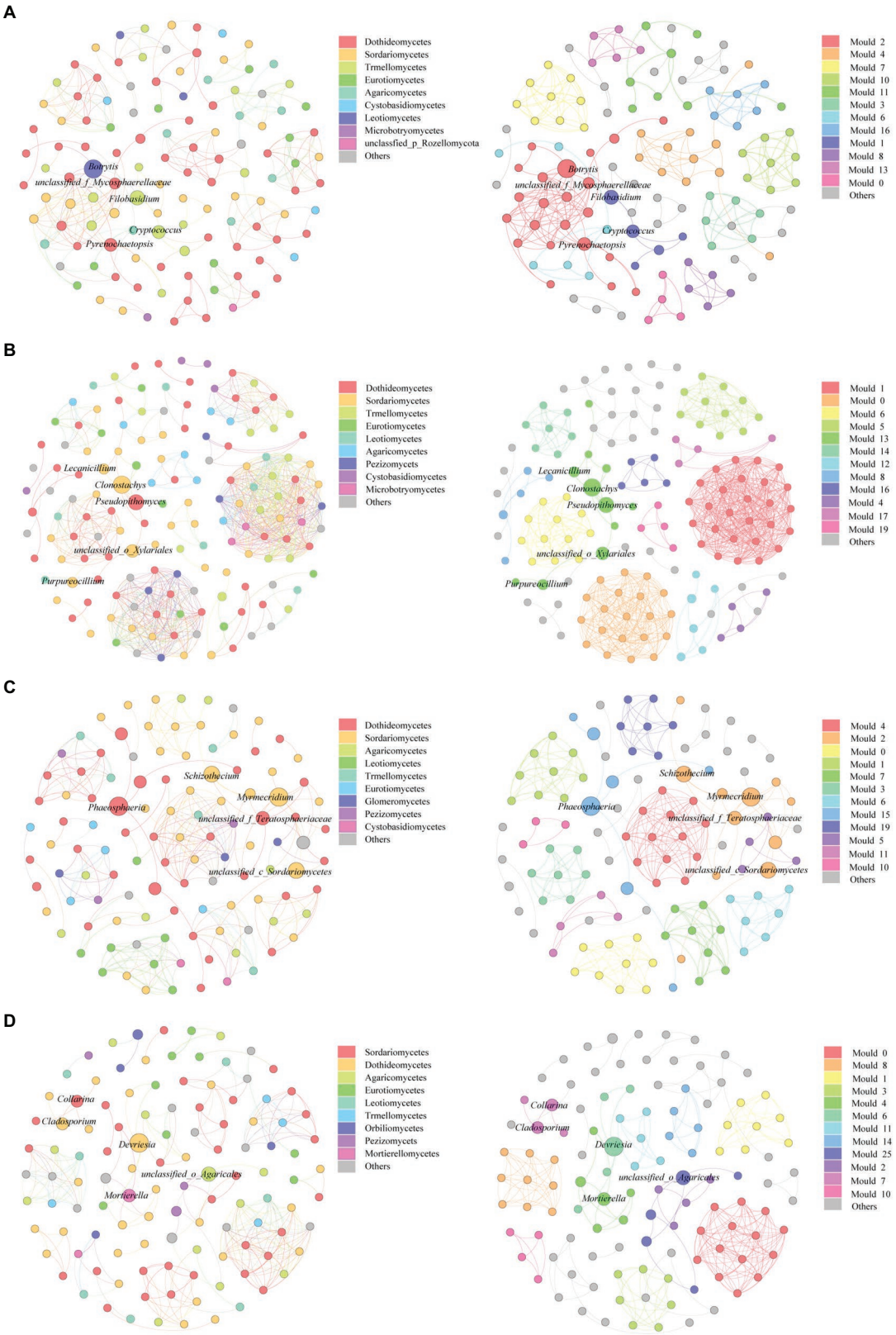

FIGURE 5 | Change of fungal community co-correlation network at D0 **(A)**, D100 **(B)**, D200 **(C)**, and D460 **(D)**. Each node represented a fungal genus, and the line represented a significant correlation between two genera ($p < 0.001$). The nodes in the left figure of each decomposition stage were colored by class level, and the nodes in the right figure were colored by module. The top 10 litter fungal classes and modules were defined, and the other microbial were defined as others.

TABLE 1 | Properties of litter fungal community correlation network as decomposition progressed.

Topological properties	D0	D100	D200	D460
Nodes	120	148	112	114
Edges	270	761	300	265
Average degree	4.500	10.284	5.357	4.649
Network density	0.038	0.07	0.048	0.041
Modularity	0.867	0.708	0.871	0.84
Average clustering coefficient	0.87	0.94	0.912	0.93
Average path length	2.261	1.124	1.21	1.067
Positive correlation	100%	100%	100%	100%

TABLE 2 | Key fungal genera in correlation network during the litter decomposition process.

Decomposition stage	Genus	Class	BC
D0	*Botrytis*	Leotiomycetes	143.0
	Filobasidium	Tremellomycetes	88.0
	Pyrenochaetopsis	Dothideomycetes	71.0
	Cryptococcus_f_ Tremellaceae	Tremellomycetes	69.0
	unclassified_f_ Mycosphaerellaceae	Dothideomycetes	48.0
D100	*Clonostachys*	Sordariomycetes	29.0
	Pseudopithomyces	Dothideomycetes	23.5
	unclassified_o_Xylariales	Sordariomycetes	16.0
	Lecanicillium	Sordariomycetes	9.0
	Purpureocillium	Sordariomycetes	9.0
D200	*Phaeosphaeria*	Dothideomycetes	12.0
	Myrmecridium	Sordariomycetes	11.7
	unclassified_c_ Sordariomycetes	Sordariomycetes	9.0
	Schizothecium	Sordariomycetes	9.0
	unclassified_f_ Teratosphaeriaceae	Dothideomycetes	6.3
D460	*Devriesia*	Dothideomycetes	5.0
	unclassified_o_Agaricales	Agaricomycetes	3.0
	Mortierella	Mortierellomycetes	2.3
	Cladosporium	Dothideomycetes	2.0
	Collarina	Sordariomycetes	2.0

ecological stoichiometry in a litter affect litter decomposition rates (Sun et al., 2019). It has been reported that litter decomposition rates positively correlate with the initial TN and TP content in the litter but negatively correlate with total organic carbon, lignin, and cellulose content (Cao et al., 2016). In this study, the initial TN content in *I. cylindrica* litter in the S516 and S560 sub-dams is significantly higher compared to the S536 sub-dam, providing more N for microbial growth. This could potentially be the reason that litter decomposition rates in the S516 and S560 sub-dams were higher compared to the S536 sub-dam. Results show that litter decomposition strongly affected Cu, Zn, Pb, and Cd accumulation, which is consistent with a previous study (Sheng, 2009). It has been reported that multivalent metal ions can form highly stable complexes with humus during litter decomposition (Yue et al., 2019). Therefore, more metal ions bind to humus as litter decomposition progress. This indicates that litter may act as an effective metal storage pool; namely, litter absorbs metals from contaminated soil, which may be highly significant in the remediation of heavy metal contaminated soil.

Fungal Community Succession During Litter Decomposition

Results show that fungal community diversity and richness significantly decreased as litter decomposition progressed. It has been reported the role that fungal communities play primarily occurs during the early litter decomposition stage (Baldrian, 2017). Thus, fungal richness gradually decreases as litter decomposition progresses. Dominant litter microbes are the primary decomposers in ecosystem material cycling processes, which determines litter decomposition rates (Gavito et al., 2021). Ascomycota is the dominant fungal phylum during the early litter decomposition stage, a phylum that is considered an early fungal decomposer (Zhang et al., 2017). Moreover, in this study Ascomycota is the dominant fungal phylum in all samples and is gradually replaced by Basidiomycota as litter decomposition progressed, which consistent with results from a previous study (Purahong et al., 2016). Dothideomycetes is known to secrete a variety of cellulase and hemicellulose (Wang et al., 2019a,b). Moreover, sugars, soluble starch, and cellulose in litter gradually decomposed as litter decomposition progressed, which lead to an increase in lignin accumulation that results in the colonization of *Aspergillus* and other microbial groups during the latter litter decomposition stage, namely microbial groups that have the capacity to decompose lignin (Bonanomi et al., 2019). *Cladosporium* and *Alternaria* are the dominant fungal genera during the early litter decomposition stage, while *Trichoderma* and *Knufia* significantly increase during the litter decomposition process. Being widely distributed throughout the soil, *Cladosporium* and *Alternaria* can utilize small-sized molecular sugars and starch. *Trichoderma* is a typical cellulolytic fungus with strong cellulose- and hemicellulose-decomposing abilities (Yan, 2011). In this study, *Trichoderma* significantly correlates with heavy metals, sucrase, and catalase, which indicate that this genus possesses a certain level of heavy metal tolerance. Additionally, *Trichoderma* is a highly significant fungal genus in litter decomposition in heavy metal contaminated areas.

Microbial Interactions During Litter Decomposition

The D100 stage yields the maximum node and edge number values and connectivity values in the fungal network, which is indicative of the complexities of interactions between the fungal communities when reaching their highest values. The nutrient content of litter is higher during the early decomposition stage, and an adequate amount of nutrients promote an increase in microbial biomass, providing greater opportunities for species to interact (Zhou et al., 2020). Results show that resource and information transmission rates are higher, and an increase in functional diversity is higher compared to a relatively simple network (Wagg et al., 2019). Additionally, complex microbial networks also improve fungal tolerance to environmental interference while being able to maintain stable microbial communities (Shang et al., 2018). Overall, litter fungi community

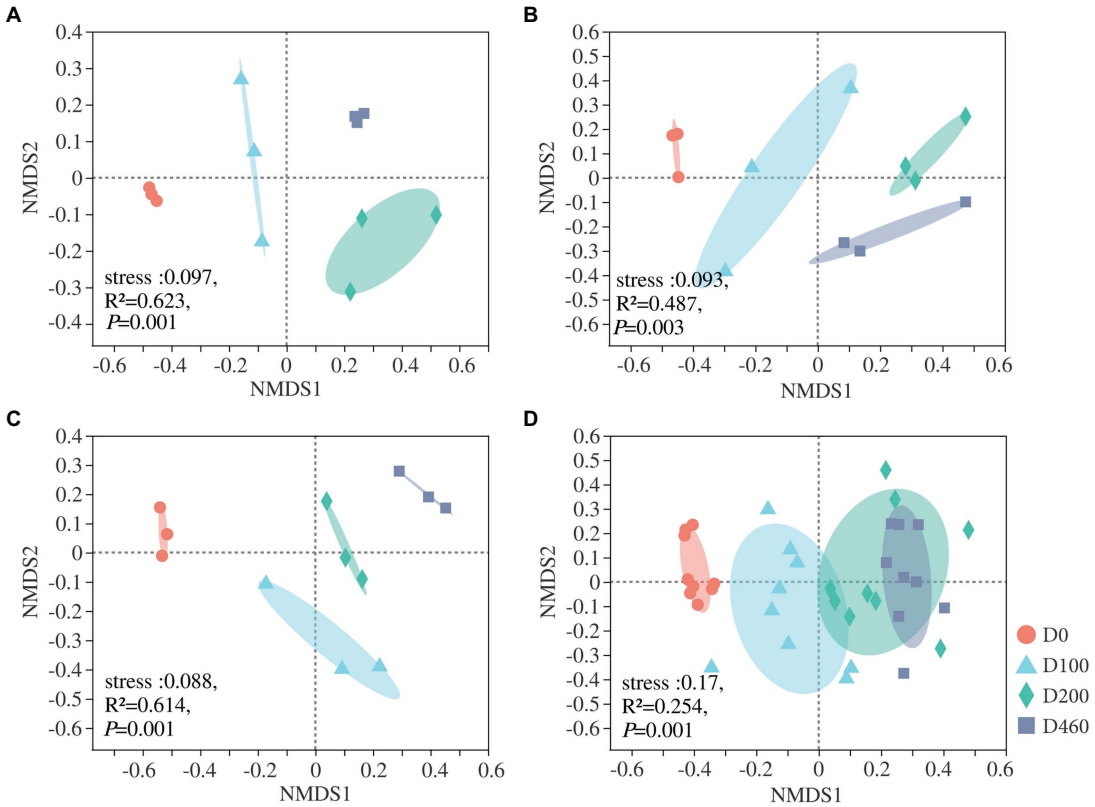

FIGURE 6 | The nMDS of litter fungal community at four decomposition stages in S516 **(A)**, S536 **(B)**, S560 **(C)**, and the whole copper mining area **(D)**.

stability is highest during the early litter decomposition stage, after which interactions among fungal communities decrease, further confirming that fungal communities may be most active during the prophase stage of litter decomposition.

Furthermore, BC values reflect how nodes connect to other nodes. Generally, the larger the BC value is, the more important role that the node plays in maintaining stable network connectivity (Chen et al., 2019). On a class level, results show that Sordariomycetes and Dothideomycetes gradually replace Leotiomycetes as litter decomposition progressed. This is consistent with the changes observed in fungal community composition. This consistency shows that the interactive ability of more abundant fungi is higher within fungal communities (Zhan et al., 2021). However, it must be noted that the key fungi within our network analysis are based on statistical analysis and therefore do not represent real-world relationships among fungal communities, which remain to be verified (Freilich et al., 2018). Therefore, future studies should selectively exclude some key species in verifying the role they play in species interactions and microbial community functions (Zheng et al., 2021).

Litter Fungal Community and Litter Factor Relationships

Environmental factors (e.g., litter quality, pH, temperature, and water content) significantly affect the fungal community structure during litter decomposition processes (Chen et al., 2020a, 2021). Results from this study show that the nutrient content, the heavy metal content, and the pH level in litter significantly correlate with fungal community composition. Additionally, the Cu content and the pH level are driving factors of fungal community structure. It has been previously reported that TC and TN content in litter significantly correlates with fungal community structure (Zeng et al., 2017). Moreover, N content contributes to the synthesis of proteins and nucleotides as well as the synthesis of other macromolecules that are vital to physiological microbial functions (Purahong et al., 2015). Thus, N typically acts as a limiting factor in litter decomposition, while also affecting the microbial community structure associated with N transformation processes (Lucas-Borja et al., 2019). In soil ecosystems, pH has been reported to be a key environmental factor that affects microbial community composition and has a considerable effect on microbial community structure and functional pathways (Lucas-Borja et al., 2019). A previous study has shown that the fungal community structure and the functional pathway were both mainly driven by litter pH and TC during litter decomposition (Wang et al., 2019a,b), indicating that pH also plays an important role in litter microbial community assemblages.

Clearly, at low concentrations, heavy metals are necessary for microbial growth, and the role they play in a variety of biological metabolic processes is important (Chen et al., 2020b). However, at higher concentrations, heavy metals impact microbial

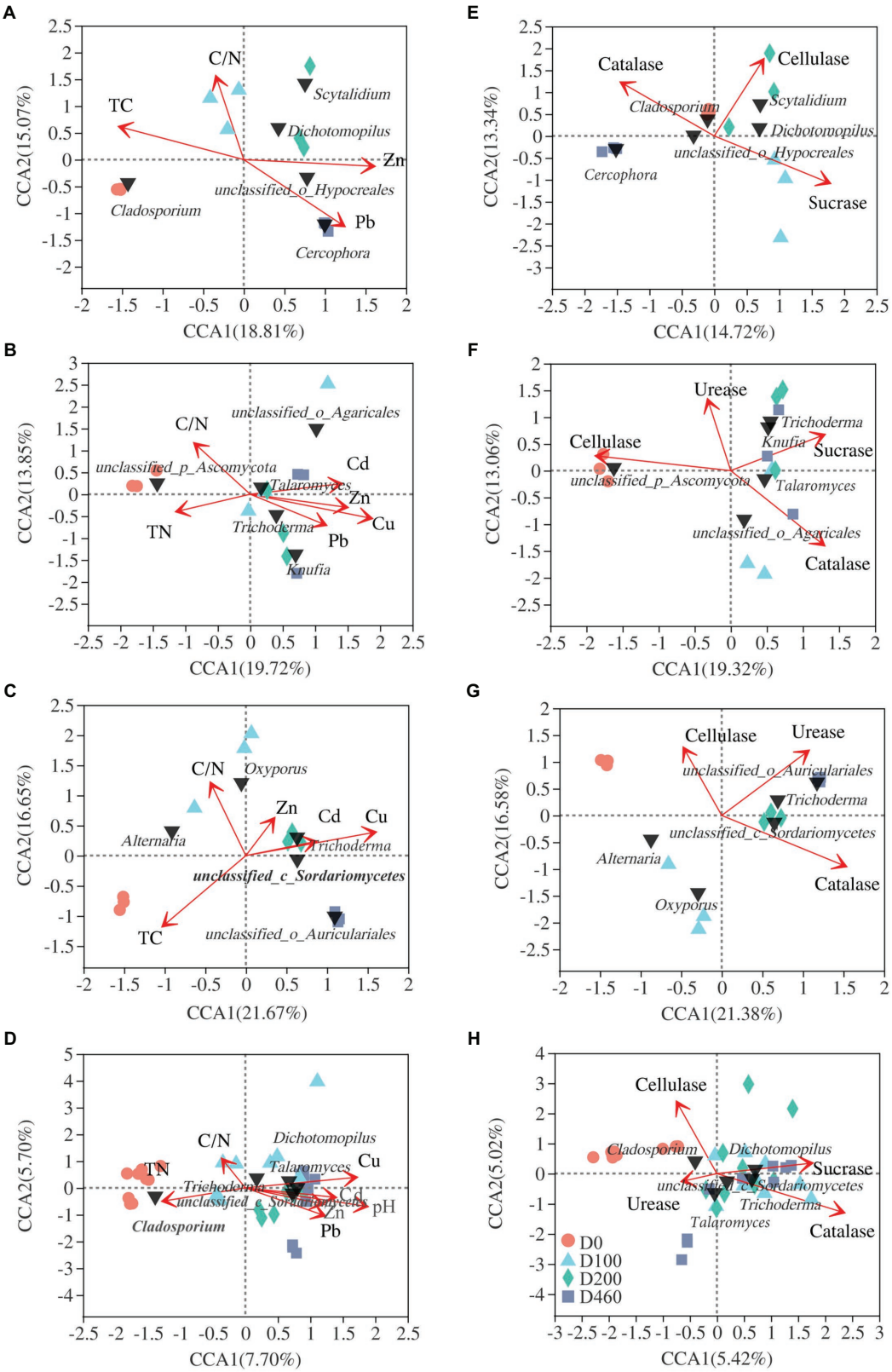

FIGURE 7 | CCA analysis of the top 5 fungal genera (black triangle) and litter properties **(A-D)** and enzyme activities **(E-H)** and fungal community structure in S516 **(A**,**E)**; S536 **(B**,**F)**; S560 **(C**,**G)**; and the whole copper mining area **(D**,**H)**.

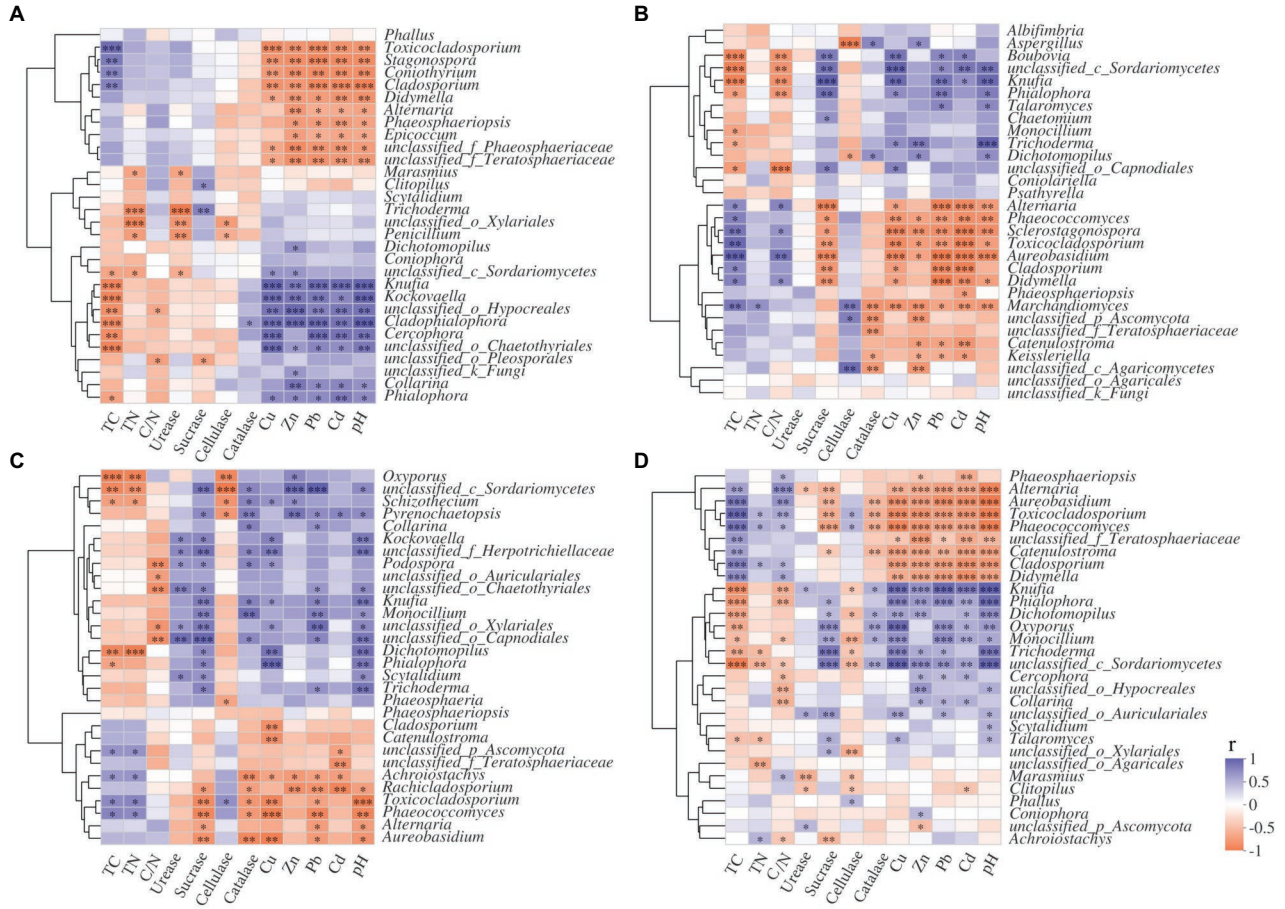

FIGURE 8 | Correlation analysis of litter properties and dominant fungi genera in S516 **(A)**, S536 **(B)**, S560 **(C)**, and the whole copper mining area **(D)**. Different colors denoted the relative coefficients. Significance levels were denoted with $^{**}p < 0.01$ and $^{***}p < 0.001$.

composition, functional genes, and microbial diversity (Chen et al., 2018). Results from this study show that *Dichotomopilus*, *Trichoderma*, *Knufia*, *Phialophora*, *Oxyporus*, and *Monocillium* significantly and positively correlated with heavy metal, sucrase, and catalase content ($p < 0.05$). It can therefore be concluded that soil heavy metal content may affect extracellular enzyme activities by instigating changes in fungal community composition, which will ultimately have an effect on *I. cylindrica* litter decomposition.

CONCLUSION

In this study, we found that significant differences were observed in litter decomposition characteristics among the different sub-dams, while the litter decomposition mass was significantly lower in the S536 sub-dam compared to the other sub-dams. *Ascomycetes* was the dominant species in the fungal community during the initial litter decomposition stage. Fungal community diversity significantly decreased as litter decomposition progressed, while degrees of interaction and stability of the fungal communities were highest during the early litter decomposition stage. Finally, significant differences were observed in fungal community structure during the different decomposition stages. The Cu content and the pH value were the key driving factors of fungal community assemblages.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

TJ conceived and designed the experiments. TG and XW performed the experiments. BC contributed new reagents. TJ wrote the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.780015/full#supplementary-material

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REVIEW
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Recent Developments in Microbe–Plant-Based Bioremediation for Tackling Heavy Metal-Polluted Soils

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Soil contamination with heavy metals (HMs) is a serious concern for the developing world due to its non-biodegradability and significant potential to damage the ecosystem and associated services. Rapid industrialization and activities such as mining, manufacturing, and construction are generating a huge quantity of toxic waste which causes environmental hazards. There are various traditional physicochemical techniques such as electro-remediation, immobilization, stabilization, and chemical reduction to clean the contaminants from the soil. However, these methods require high energy, trained manpower, and hazardous chemicals make these techniques costly and non-environment friendly. Bioremediation, which includes microorganism-based, plant-based, microorganism-plant associated, and other innovative methods, is employed to restore the contaminated soils. This review covers some new aspects and dimensions of bioremediation of heavy metal-polluted soils. The bioremediation potential of bacteria and fungi individually and in association with plants has been reviewed and critically examined. It is reported that microbes such as *Pseudomonas* spp., *Bacillus* spp., and *Aspergillus* spp., have high metal tolerance, and bioremediation potential up to 98% both individually and when associated with plants such as *Trifolium repens, Helianthus annuus*, and *Vallisneria denseserrulata*. The mechanism of microbe's detoxification of metals depends upon various aspects which include the internal structure, cell surface properties of microorganisms, and the surrounding environmental conditions have been covered. Further, factors affecting the bioremediation efficiency and their possible solution, along with challenges and future prospects, are also discussed.

Keywords: bioremediation, beneficial microorganisms, heavy metals, phytoremediation, soil management

INTRODUCTION

With the onset of the twentieth century, human beings have witnessed advancement in technologies related to food production, health, infrastructure, transport, and communications. Such activities require a vast quantity of new materials and energies destroying natural environmental components and the production of huge quantities of wastes resulting in environmental degradation (Mani and Kumar, 2014). The presence of toxic metals and metalloids

in the waste generated from the industrial, domestic, and agricultural sectors causes significant damages to the ecosystem and associated lives (Pourret et al., 2016; Goyal et al., 2020; Leong and Chang, 2020). The contaminants are highly mobile and soluble, thus possessing the capability to be bioaccumulated in the food chain and causing serious damage with increasing tropic levels (Petavratzi et al., 2005; Zerizghi et al., 2020). When these contaminants enter the human body, they can cause various life-threatening diseases such as cancer, kidney and bone diseases, cardiovascular diseases, hypertension, low birth weight, Alzheimer diseases, and atherosclerosis (Nawrot et al., 2006; Ahern et al., 2011; Bernhoft, 2012; Flora et al., 2012; Muszynska and Hanus-Fajerska, 2015; Lee et al., 2017). Metal accumulated in biological tissues is hard to remove due to its non-biodegradability, and it becomes a major concern to global health (Ayangbenro and Babalola, 2017). Metal contamination leads to the alteration in soil physicochemical and biological properties such as an increase in bulk density and soil pH, as well as a decrease in soil fertility and water holding capacity, microbial diversity and soil enzyme activity (Wuana and Okieimen, 2011; Jin et al., 2019; Saha and Bauddh, 2020). They are also responsible for the alteration in microbial communities, leading to disturbing the proper function of the biogeochemical cycle and imbalance in the ecosystem (De Quadros et al., 2016; Feng et al., 2019). Heavy metals like As, Hg, Ni, Cr, Pb, and Cu can cause multiple indirect and direct effects on plant growth, such as chlorosis, necrosis, root injury, reduced carotenoid concentration, oxidative stress, inhibition of enzyme activities, osmotic imbalance, decreased photosynthetic activities, and imbalance of the nutrients (Lewis et al., 2001; Mascher et al., 2006; Shaibur et al., 2009; Yadav, 2010; Hasan et al., 2017; Sachan and Lal, 2017). Further, due to these environmental effects of metals, there are incessant efforts made to sustainably eliminate this toxic and excess amount of metals for stabilizing the ecosystem.

Various physicochemical techniques (such as extraction, immobilization, stabilization, coagulation, electrodialysis, vitrification, reverse osmosis, ion exchange, chemical reduction, evapotranspiration, and precipitation) have already been practicing to reduce metal contamination (Ali et al., 2013; Gupta and Kumar, 2017). However, these techniques are costly, require high energy, harsh chemicals with low removal efficiency, and can generate secondary environmental pollution (Tang et al., 2007; Acheampong et al., 2010; Ali et al., 2013; Gupta and Diwan, 2016; Suman et al., 2018). Therefore, there is a continuous demand for environmental friendly remediation methods that can be helpful to reduce its harmful effects on the environment.

Bioremediation is an ecologically sound technique that requires the use of green plants, microorganisms including fungi, bacteria, yeast, and algae or their enzymes to help the polluted sites return to their original states (Chakraborty et al., 2012; Mani and Kumar, 2014). The late 19th century ascertained to be the golden period for bioremediation. With further improvement, the 20th century marked the beginning of research in the field of microbial ecology, involving the identification and isolation of microbes that have the potential to degrade pollutants, e.g., *Candidatus accumulibacter* that is capable of accumulating excess amount of phosphorus as polyphosphates in their cells from the sewage treatment plants (Seviour et al., 2003). Later, the delineation of catabolic pathways to break pollutants, the genomic construction of recombinant microbes tailored to eliminate metals, and the application of molecular techniques to understand microbial activities have been explored (Siezen and Galardini, 2008; Ramos et al., 2011).

Soil microorganisms play an essential role in stabilizing soil macroaggregates by producing polysaccharides to maintain soil architectural patterns for plant productivity (Ghose, 2005). Such microorganisms including numerous species of bacteria, fungi, yeast, and algae contribute significantly to the decomposition and stabilization of inorganic and organic pollutants (Fulekar et al., 2012; Rahman et al., 2015; Leong and Chang, 2020). A number of studies have highlighted that various natural and genetically engineered microorganisms (GEM) such as *Bacillus cereus*, *Chlorella pyrendoidosa, B. cereus* XMCr-6, *Pseudomonas veronii* 2E, *P. aeruginosa, Serratia marcescens, Sacharomyces cerevisiae, Penicillium canescens Spirogyra* sp., *Spirullina* sp., and *Cladophora* sp. are responsible to remediate HMs such as Cd, Pb, As, Cr, Mn, Cu, U, Se, and Zn from contaminated land and water (Lee and Chang, 2011; Kumar et al., 2011b; Hrynkiewicz et al., 2012; Kanmani et al., 2012; Mane and Bhosle, 2012; Mani and Kumar, 2014; Farhan and Khadom, 2015; Lívia et al., 2015; Ojuederie and Babalola, 2017; Verma and Kuila, 2019).

There is a need for characterization and regular assessment of various contaminated sites such as mining dumpsites, nuclear waste, surface wastewater, sewage sludge pump sites, agricultural soils, and various industrial and commercial dumping zones. Recently a number of research studies and literature reviews have been focused on the phytoremediation potential of particular plant species and selected metals with different microorganisms or particular microorganism-based remediation strategies (Raza et al., 2020; Yan et al., 2020; Wang et al., 2021; Hao et al., 2021; Sharma et al., 2021).

In this review, we have covered some new aspects and dimensions of bioremediation of heavy metal-polluted soils. Here, we have reviewed the recent literature published mainly between the year 2019–2021. There is a critical examination of the bioremediation potential of different microorganisms, especially bacteria and fungi individually and in association with plants. Further, the different mechanisms adopted by the microorganisms to detoxify HMs have also been discussed. Moreover, the study attempts to explore the knowledge about field applications with several case studies, factors affecting bioremediation, challenges, as well as future prospects have been covered.

METHODOLOGY

The relevant literature was searched and collected from the online database using Scopus, Web of Science, Google, Google Scholar, Springer Nature, Frontiers, Taylor and Francis, Science Direct, etc. The keywords used for the literature search

include bioremediation, phytoremediation, phytoextraction, phytomanagement, remediation using living organisms, remediation through plant/microorganism, plant–microbe association for heavy metal removal, etc. In addition, particular focus journals such as International Journal of Phytoremediation, Bioremediation Journal, Frontiers in Microbiology, Journal of Environmental Management, Frontiers in Plant Science, Science of the Total Environment, Chemosphere, Water, Air, & Soil Pollution, Environmental Science and Pollution Research, Microbial Research, etc. were browsed volume-wise for track the relevant papers until July 2021. The literature includes journal articles, books, book chapters, conference papers, proceedings, and technical reports were referred in this review paper from which 92.91% were published between the years 2010 to 2021. In total, more than 400 documents were examined individually and eliminated the quotative and duplicate papers (Qi et al., 2018). Out of which 254 documents were selected for reference in this work.

BIOREMEDIATION

Bioremediation is an emerging and highly acceptable practice for restoring heavy metal contaminated soils, because of its environment friendly and low cost as compared to other conventional methods such as dredging, capping, and incineration that are often very costly and ineffective when metal concentration level is low and often generates a significant amount of toxic byproducts (Ekperusi and Aigbodion, 2015; Ayangbenro and Babalola, 2017). A study has been shown that it costs about 100–500 USD/ton for cleaning metal-polluted sediments and soils through landfilling and chemical treatment, and 90–870 USD/ton for vitrification, whereas about 15–200 USD/ton for bioremediation and 5–40 USD/ton for phytoremediation (Meier et al., 2012). It estimates that bioremediation can save 50–65% for cleaning one acre of Pb-contaminated soil compared to traditional excavation and landfill (Blaylock et al., 1997; Chibuike and Obiora, 2014). In addition, bioremediation is a non-invasive method that can remove contaminants permanently, leave the environment intact, and can be hybridized with chemical and physical treatments (Mani and Kumar, 2014). The bioremediation processes rely entirely on natural biological potency. The majority of bioremediation methods depends on several parameters such as soil structure, pH of the polluted sites, moisture content, type of the pollutants, nutrient supplement, microbial diversity, the temperature of treatment sites, and oxygen availability (Atagana et al., 2003; Thapa et al., 2012; Mangunwardoyo et al., 2013; Mani and Kumar, 2014). Bioremediation can occur naturally in a polluted site, which is called natural attenuation.

Lombi and Hamon (2005) have divided bioremediation into '*in-situ*' and '*ex-situ*' strategies. *In-situ* or on-site bioremediation is the most preferred option for removing contaminants from polluted soil and water. In the *in-situ* process, the soils remain confined to their initial location throughout the reclamation process, ending up in minimal site disturbance, fewer public health risks associated with excavation and off-site transport of contaminated soil, and reduced the overall cost over other remediation technologies (Hellekson, 1999; Lombi and Hamon, 2005). The *in-situ* bioremediation is broadly classified into two types, intrinsic and engineered bioremediation (Hazen, 2010). Intrinsic bioremediation takes place through the stimulations of indigenous microorganisms by supplying them with nutrients and oxygen to boost their metabolic activity. This is an unstimulated, unmanipulated, and unenhanced biological remedy of contaminates. Whereas for engineered bioremediation, a specific type of microorganisms or genetically engineered bacteria are introduced into the contaminated place to accelerates the degradation process by creating a conducive physicochemical condition (Kumar et al., 2011).

On the other side, *ex-situ* bioremediation methods require the excavation of polluted soil and water from its original location for the treatment. This is further categorized as a solid-phase system and slurry phase system. Solid-phase bioremediation includes contaminated waste such as industrial waste, domestic waste, municipal solid waste, and sewage sludge with organic waste including manure, leaves, and agricultural waste. The treatment process includes composting, soil biopile, hydroponics, and land farming, which create suitable conditions for indigenous anaerobic and aerobic microorganisms to boost the reclamation process (Kumar et al., 2011; Rayu et al., 2012). From which in hydroponics methods plants are grown in the mineral nutrient solution. Nowadays, this method is a common step for screening the suitable plant for phytoremediation by characterization of its response to heavy metal stress. On the other hand, slurry phase bioremediation is a speedy process where contaminated soils are mixed with additives and water in a bioreactor to create an appropriate environment for microorganisms to eliminate the contaminants.

MECHANISMS OF BIOREMEDIATION

Both *in-situ* and *ex-situ* remediation methods work on the principle of biotransformation/biodegradation, removal, mobilization, immobilization, or decontamination of various pollutants from the environment through the action of microorganisms (bacteria, fungi, and yeast) and plants (Abatenh et al., 2017). Microbes use chemical contaminants as an energy source during biotransformation and metabolize the target contaminant into useable energy *via* redox reactions. There are usually less harmful by-products or metabolites released back into the environment compared to the primary pollutants. For instance, microorganisms can degrade petroleum hydrocarbons through aerobic respiration in the presence of oxygen. The hydrocarbon gets oxidized by losing electrons, whereas the oxygen reduces by gaining electrons. Water and carbon dioxide are formed as a by-product of this redox reaction (Nester et al., 2001).

The microorganisms play an important role in HM remediation from the contaminated soil as they have acquired various mechanisms to tolerate the toxic effects of HMs.

Microorganisms can sequester, precipitate, biosorb, and change the oxidation states of various metals (Ndeddy Aka and Babalola, 2016; Yin et al., 2019; Rizvi et al., 2020; Ibrahim et al., 2021). Metal sequestration happens by cell wall components and by intercellular metal bindings peptides and proteins such as metallothionein, phytochelatins with bacterial siderophores (Ojuederie and Babalola, 2017; Balzano et al., 2020). Microorganisms convert the toxic metal into a less toxic or innocuous form with the help of enzymes (such as dioxygenases, peroxidases, and oxidoreductases). The mechanisms applied by microorganisms to remove HMs from the contaminated soil or convert to less toxic form have been presented in **Figure 1**. However, the biosorption mechanism is based on two way: first depends on cell metabolism and second on the location of the cell where the HM is removed.

Three key bioremediation ingredients are (1) the presence of a contaminant, (2) the acceptor of electrons, and (3) the existence of microorganisms that can degrade a specific contaminant. Generally, the biodegradation process is easy for the naturally occurring contaminant or those have chemical similarities with naturally occurring compounds. It is due to the potential of microorganisms to destroy the contaminants. For instance, petroleum hydrocarbons are naturally derived chemical products, therefore microorganisms are habituated for these contaminants and can degrade them easily. Different approaches applied in the microbial remediation process such as bioattenuation, biostimulation, bioaugmentation for removing the toxic pollutants from the contaminated land, have been described below.

Bioattenuation

The contaminants are converted to less harmful or immobilized forms during bioattenuation. Such processes of immobilization and transformation are primarily attributed to microbial biodegradation and biological transformation (Smets and Pritchard, 2003), and, to some degree, to reactions with naturally occurring chemicals and geological media sorption. Contaminant-specific processes of natural attenuation are considered as methods for the remedy of fuel components [e.g., biosparging of benzene, toluene, ethylbenzene, and xylene (BTEX)], but not for other various classes of contaminants (e.g., sulfide and ferrous iron; Atteia and Guillot, 2007).

Biostimulation

This includes modification in environmental parameters, such as restricting nutrients supplement such as slow-release fertilizers, biosurfactants, and biopolymers (Kumar, 2019), which helps to remove the heavy metal, hydrocarbons and oil contaminants (Junior et al., 2019; Sun et al., 2019, 2021). It also enhances the bioavailability of Cu, Cd, Pb, and Zn, heavy metal uptake, translocation, and biodegradation rate of hydrocarbons, pesticides and herbicides by naturally existing microorganisms present on the site (Lim et al., 2016; Kumar, 2019). There are various fertilizers available as nutrients for microbes to stimulate, e.g.,

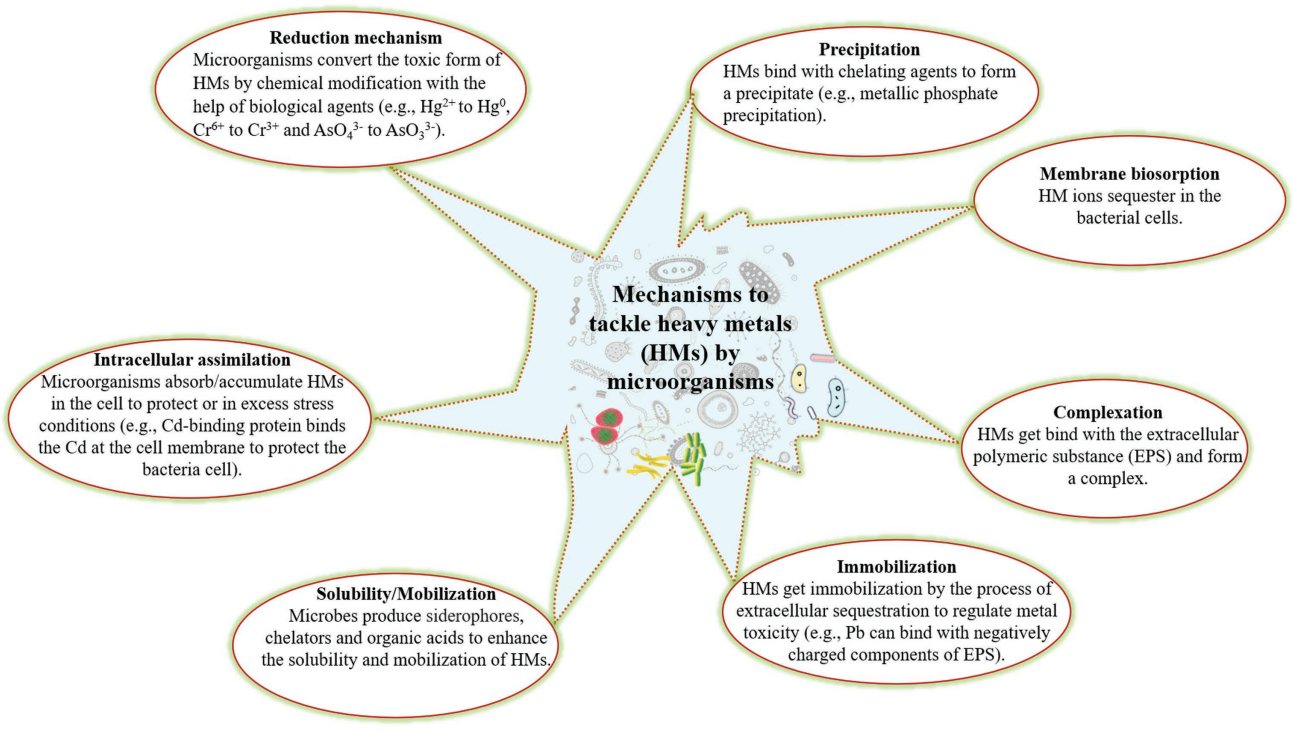

FIGURE 1 | Different microorganisms mechanisms to tackle the HMs from the soil.

water-soluble $NaNO_3$, KNO_3, NH_3NO_3, slow-release customizable, max-bac, IBDU, and oleophilic Inipol EAP22, MM80, F1, S200.

Bioaugmentation

Bioaugmentation basically increases the heavy metal removal efficiency by introducing the pre-grown microorganisms. In this process, natural/exotic/engineered microbes are incorporated artificially in the heavy metal contaminated soil (Hassan et al., 2019, 2020a). Microbes are collected from the remediation site, separately cultured, genetically grown, and returned to the location. This process helps increase the growth and population of microorganisms, which enhance the solubility, mobility, accumulation of HMs, and increase the remediation efficacy (Atigh et al., 2020). However, it also reduces the risk of these pollutants either through chemically altering their chemical structure or by decreasing their bioavailability (Mandal et al., 2016; Hassan et al., 2019; Zanganeh et al., 2021). Recently this method is applied to various HM contaminated soil using different types of bacteria and fungal strains which include *Oscillatoria* sp., *Leptolyngbya* sp., *Portulaca oleracea, Perenniporia subtephropora, Aspergillus niger* MH541017*, Daldinia starbaeckii, Tremates versicolor*, and *Tremates versicolor* (Atigh et al., 2020; Hassan et al., 2020a; Zanganeh et al., 2021).

PLANT-BASED BIOREMEDIATION

Plants are used for bioremediation either alone or in combination with microbes (Ramos et al., 2005) instead of depending on microbes and their efficacy in achieving bioremediation of any contaminated medium. The application of green plants to clean up any contaminated medium or surface is not a novel concept. Plants were proposed for treating the wastewater around 300 years ago (Hartman, 1975). Presently a number of plant species such as *Amaranthus spinosus, A. hypochondriacus Chrysopogon zizanioides, Brassica juncea*, *Ricinus communis, Chromolaena odorata, Ageratum conyzoides, Ipomoea carnea, Prosopis juliflora, Lantana camara, Parthenium hysterophorus, Fagopyrum esculentum, Odontarrhena chalcidica, Tagetes patula, T. erecta, and Odontarrhena chalcidica,* have been identified which helpremediate HM contaminated soil (Bauddh and Singh, 2012; Bauddh and Singh, 2015; Huang et al., 2019; Chen et al., 2020a; Raza et al., 2020; Biswal et al., 2021; Cui et al., 2021; Gonzaga et al., 2021; Nugroho et al., 2021; Singh et al., 2021). In addition, plants like *Nicotiana tabacum*, *Arabidopsis thaliana, Beta vulgaris* and *Sedum alfredii* have been genetically modified with suitable bacterial genes from *Caenorhabditis elegans, Saccharomyces cerevisiae, Streptococcus thermophilus, Pseudomonas fuorescens* and employed for remediating the targeted contaminants (Daghan et al., 2013; Liu et al., 2015a; Wang et al., 2019; Nedjimi, 2021). For instance, mercury (Hg) reductase bacterial genes, e.g., merA and merB have been applied in plants for the detoxification of methyl-Hg (Li et al., 2020a). In addition, various biostimulators, such as manure and organic amendments (e.g., various plant biochar, biosolids, and litter) are used in this plant-based bioremediation. Use of different chelators such as citric acid, ethylene diamine tetraacetic acid (EDTA), [S,S]-ethylenediaminedisuccinic acid (EDDS), ethylenediamine-di-o-hydroxyphenylacetic acid (EDDHA), diethylenetriaminepentaacetic acid (DTPA), ethylene glycol tetraeacitic acid (AGTA), nhydroxyethylenediaminetriacetic acid (HEDTA), fulvic acids, salicyclic acid, and tartaric acid control metal sorption, and precipitation through the formation of metal chelate complexes, which consequently enhance the bioavailability of these metals and also improve phytoextraction efficiency (Caporale and Violante, 2016; Acuña et al., 2020; Saleem et al., 2020). The addition of chelates in soils can move more metals into soil solution *via* the suspension of precipitated compounds and desorption of sorbed species. Plants can also naturally produce various phytosiderophores, organic acids, and carboxylates, which can enhance metal mobility, solubility, and bioavailability in soils, thus increasing the phytoremediation potential of plants (Vithanage et al., 2012; Gupta and Singh, 2017). For instance, *Miscanthus sinensis* can detoxify Al by producing various phytosiderophores such as citric acid, malic acid, and chlorogenic acid and stored the metal in cell walls (Haruma et al., 2019).

Plant-based bioremediation is considered a potential tool for the accumulation, transformation, and immobilization of a low level of contaminants (Rayu et al., 2012). The mechanisms behind plants facilitate the reclamation of the polluted soils and groundwater are presented in **Table 1**. The approach of plant-based bioremediation has several merits such as cost-effectiveness, public acceptance, and the ability to remove inorganic and organic contaminants simultaneously. In a study, mixed mercury-trichloroethylene (Hg-TCE) pollutants are removed by transgenic alfalfa plants pKHCG co-expressing human P450 2E1 (CYP2E1) genes and glutathione S-transferase (GST; Zhang et al., 2013). A major synergistic effect caused by simultaneous expression of CYP2E1 and GST leads to increased accumulation and resistance of heavy metal–organic complex pollutants. Another study by Tammam et al. (2021) found that the plant *Glebionis coronaria* can eliminate Pb from the contaminated soil. It is also recorded that the foliar spray of Indole-3-acetic acid (IAA) and gibberellic acid (GA3) enhanced the growth significantly and increase the phytostabilization capacity of the studied plant. The application of bamboo biochar with the *Salix psammophila* to remediate the multi-metal contaminated soil, enhance the translocation factor (TF) and bioconcentration factors (BCF) of Cd, Cu and Zn (Li et al., 2021a). The higher TF for Zn ($TF > 1$) and BCF for Cd ($BCF > 1$) makes *S. psammophila* a potential candidate for the phytoremediation in BBC amendment soil. Recently several studies found that the application of nanoparticles such as Ag nanoparticles (AgNPs), nano-TiO_2 particles, nanoscale zero-valent iron (nZVI), salicylic acid nanoparticles (SANPs) and magnesium oxide (MgO) nanoparticles along with plants *Zea mays, Glycine max, Isatis cappadocica, Lolium perenne, Boehmeria nivea* and *Raphanus sativus* enhance the growth and phytoextraction of HMs Cd and Pb (Khan and Bano, 2016; Singh and Lee, 2016; Gong et al., 2017; Souri et al., 2017; Huang et al., 2018; Hussain et al., 2019).

TABLE 1 | List of various phytoremediation mechanisms and plant species used in various process.

Technique	Mechanism	Plant used	Plant parts	Surface medium	References
Phytoextraction	Uptake and accumulation of heavy metal into plant tissues with subsequent elimination of the plants	*Brassica juncea Amaranthus hypochondriacus, Thlaspi caerulescens*	Roots, Shoot, Leaves	Soils	Odoh et al., 2019; Cui et al., 2021; Singh et al., 2021
Phytodegradation/ Rhizodegradation	Enzyme catalysed metabolism by rhizosphere-dwelling microorganisms to transform organic contaminant into simpler molecules	*Rhizophora mangle, Salix viminalis, Vetiveria zizanioides, Typha latifolia*	Roots, Leaves	Surface water, Groundwater	Sampaio et al., 2019; Papadopoulos and Zalidis, 2019; Nedjimi, 2021
Phytostabilization	Decreases the mobility and migration of soil contaminants	*Atriplex undulata, Salix alba, Glebionis coronaria*	Roots	Soils,Groundwater, Mine tailing	Mataruga et al., 2020; Li et al., 2021; Tammam et al., 2021
Rhizofiltration	Uptake of metals *via* plant roots	*Eichhornia crassipes, Lemna minor, Pistia stratiotes*	Roots	Surface water, Water pumped	Kodituwakku and Yatawara, 2020; Singh et al., 2021
Phytovolatilization	Removal of pollutants such as selenium, mercury, volatile hydrocarbons *via* evapotranspiration processes	*Arundo donax, Stanleya pinnata, Brassica juncea, B. Napus*	Roots, Leaves	Soils, Groundwater	Guarino et al., 2020; Hasanuzzaman et al., 2020; Yan et al., 2021
Phytostimulation	Phytostimulation (a symbiotic relationship that exists between plants and several soil microorganisms) is developed for the remediation of polychlorinated biphenyl (PCBs)	*Brassica campestris, Zea mays, glycine max*	Roots	Soils	Zahoor et al., 2017; Bilal et al., 2020

Plants are effective in extracting inorganic and organic pollutants from the ground through the roots, they can also be transported and accumulated (phytoextraction/accumulation) in the harvestable parts of the plant (Pranaw et al., 2020). Transpiration to the atmosphere *via* leaf stomata (phytovolatilization) occurs in some instances (Rascio and Navari-Izzo, 2011). Phytodegradation of organic compounds are metabolized by plants in three sequential steps (namely transformation, conjugation, compartmentalization, respectively) with the aid of enzymes, e.g., cytochrome (CY) P450 and GT–glycosyltransferase (GT), which results in the storage of contaminant in the vacuole, incorporation into the cell wall, or excretion from the cell. In addition, plant-associated microorganisms in the rhizosphere (rhizodegradation) can degrade organic contaminants (Truu et al., 2015). By releasing root exudates and other compounds (e.g., organic acids) to the surrounding soil along with providing a surface for microbe colonization, plants can promote the biodegradation of pollutants, thereby contributing to the increased density and metabolic activity of microorganisms (rhizosphere effect) and contaminant bioavailability. Plant supplements nutrients to endophytic bacteria and stimulates catabolic gene expression. In turn, endophytic bacteria degrade organic contaminants, thereby reducing phytotoxicity and producing hormones (Shukla et al., 2020).

Since metal bioavailability in soils is relatively poor under most conditions, plants have very active metal uptake systems that utilize transporter molecules such as Zn-regulated transporter protein, Cu transporter protein, etc. (Krämer et al., 2007). In addition, plants are capable of acidifying the soil and mobilize soil-bound metals by secreting metal-chelating molecules to the surrounding soil, such as siderophores (catechol and hydroxymate), organic acids (e.g., citrate and malate), biosurfactants (rhamnolipids), protons from the root exudates (Yan et al., 2020; Bruno et al., 2021). Heavy metals cannot be biodegraded inside the plant, unlike organic contaminants, but can only be converted from one oxidation state/organic complex to another. It ends up in metal accumulation inside the plant. There are nearly 450 hyperaccumulator plants varies from annual to perennial herbs, shrubs, and trees (e.g., *Brassica juncea, Zea mays, Ricinus communis, nicotiana tabacum, Helianthus annuus, Pteris vittata, Thlaspi caerulescens, Russian thistle, Sesbania drummondii, Salix matsudana, Populus deltoides*), which have been identified to accumulate, metabolize and depollute extraordinary high concentration of metal ions (such as Cd, Pb, Ni, Co, Mn, Zn) in their above-ground tissues (Meagher, 2000; Padmavathiamma and Li, 2007; Shah and Nongkynrih, 2007; Sheoran et al., 2009; Palanivel et al., 2020).

MICROORGANISM-BASED BIOREMEDIATION

The capacity of microorganisms to degrade contaminants depends on their metabolic system through which the pollutants alter to innocuous form *via* the redox process (Jan et al., 2014). They help plants alleviate metal toxicity by sequestration of metals in cell wall components, alteration of the biochemical pathway to block metal uptake, reduction of the intercellular metal concentration *via* a precise efflux system, and conversion of poisonous metals to a less harmful state (Jan et al., 2014;

Ojuederie and Babalola, 2017). Microorganisms (such as bacteria and fungi) play a vital role in the microbial bioremediation process. In addition, microorganisms contain several genes located in transposons and plasmids, which encode heavy metal resistant proteins and transporters. Recently, Kang et al. (2016) found that four bacterial strains, namely *Enterobacter cloacae* KJ-46, *E. cloacae* KJ-47, *Sporosarcina soli* B-22, and *Viridibacillus arenosi* B-21 had synergistic effects on the remediation of Cd, Pb, and Cu from contaminated soil. Moreover, the combination of bacteria strains shows greater resistance and efficacy for metal bioremediation compared to a single strain after 48 h of experiments. Microbes secrete several metabolites that play a significant role in bioremediation of contaminated sites (Sobrinho et al., 2013; Dixit et al., 2015; Coelho et al., 2015; Ahemad, 2019; **Figure 2**).

Bacteria generate siderophores that can diminish metal bioavailability and are subsequently eliminated from contaminated land (Ahemad, 2019). It is recorded that bacterial cell can alter their morphology to increase the production of siderophores for promoting the intercellular accumulation of metals (Manoj et al., 2020). Chibuike and Obiora (2014) found that a sulfate-reducing bacterium *Desulfovibrio desulfuricans* can alter sulfate to hydrogen sulfate, which further reacts with HMs (Cd and Zn) and then form insoluble metal sulfides. The biomolecules of microbial cell walls contain negatively charged functional groups such as phosphate, hydroxyl, and carbonyl, which bind quickly with toxic metal ions and help them in bioremediation (Coelho et al., 2015; Dixit et al., 2015). Besides, bacteria can be grown and survive in any control and intense environmental conditions, making them a perfect bioremediation agent (Srivastava et al., 2015).

Likewise, fungi can be grown in harsh environmental conditions and detoxify metal ions by accumulation, valence transformation, and extra and intracellular precipitations (Ayangbenro and Babalola, 2017). In addition, fungi act as a promising biocatalyst in the bioremediation process, where they absorb toxic chemicals into their spores and mycelium. Recently, Hassan et al. (2020a) showed the bioremediation capability of fungal consortia of *Ascomycota* and *Basidiomycota*, suggesting fungal bioaugmentation helps decontaminate heavy metal from contaminated land. A number of investigations are carried to study the microorganism bioaccumulation and biosorption capacity for effectively remediate metal-contaminated environment (**Table 2**).

Recently researchers have been isolated various heavy metal resistance microorganism from contaminated lands, mining dumping and abandoned sites, industrial waste dumping yards, and the rhizosphere of plants growing in metal-contaminated sites (Banerjee et al., 2019; Aguilar et al., 2020; Akhter et al., 2020; Nurfitriani et al., 2020; Din et al., 2021; Sharma and Shukla, 2021b). The isolated bacterial genera (such as *Arthrobacter, Enterobacter, Corynebacterium, Stenotrophomonas, Bacillus*, and *Pseudomonas*) and fungi (such as *Aspergillus flavus, Aspegillus awamori, Saccharomyces cerevisiae, Phanerochaete chrysosporium, Penicillium oxalicum*, and *Trichoderma viride*) play a significant role in bioremediation process. Bacteria and fungi precisely used to eliminate the specific metals in recent years have been reviewed and presented in **Tables 3, 4**, respectively.

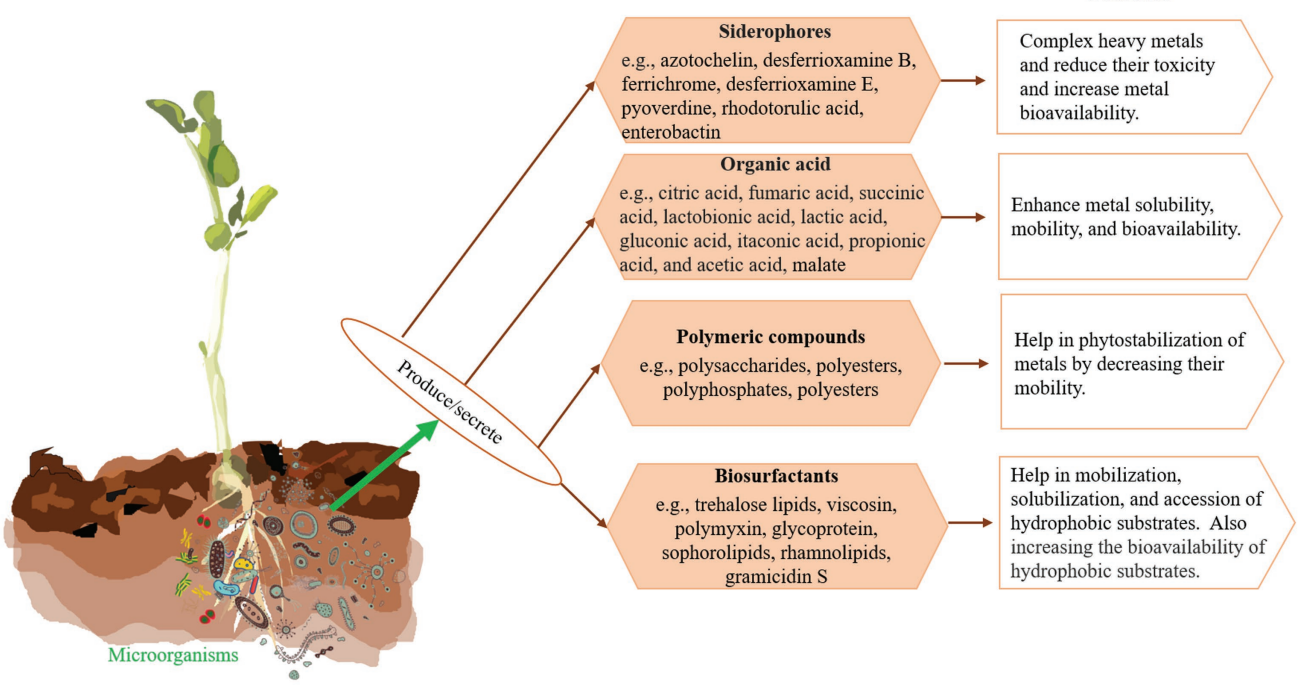

FIGURE 2 | Microorganisms produce/secrete different compounds and their role in bioremediation.

TABLE 2 | Different microorganisms and their bioaccumulation and biosorption capacity.

Microorganism(s)	Contaminant(s)	Remarks	References
Scenedesmus acutus, Chlorella pyrenoidosa	Cd	*C. pyrenoidosa* and *S. acutus* accumulated 3 and 1.5% of Cd and biosorbed 97 and 98.5% of Cd, respectively.	Chandra et al., 2020
Aspergillus spp.	Cd, Cu	The removal efficiency for Cu and Cd was recorded >90%. The biosorption potential of living and dead cells for Cd was 0.1977 and 0.1772 mg g^{-1} and for Cu it was 5.3676 and 18.661 mg g^{-1}, respectively.	Hasgül et al., 2019
Streptomyces K11	Zn	The bioaccumulation capacity was 4.4 mmol g^{-1}. The maximum biosorption capacity recorded was 0.75 mmol g^{-1}.	Sedlakova-Kadukova et al., 2019
Bacillus xiamenensis PbRPSD202	Pb, Cd, Cr, As, Ni, Cu, and Zn	The maximum Pb biosorption capacity for living and dead biomass of *B. xiamenensis* shows 216.75 and 207.4 mg g^{-1}, respectively.	Mohapatra et al., 2019
Aspergillus flavus SFL	Cr	The intercellular accumulation of *A. flavus* SFL was 50% more than the reference strain.	Vajpai et al., 2020
Phanerochaete chrysosporium	Cd^{+2}, Ni^{+2}	The accumulation efficiency of *P. chrysosporium* for Cd^{2+} and Ni^{2+} was 96.23 and 89.48%. The maximum biosorption capacity for Cd^{+2} and Ni^{+2} recorded 71.43 and 46.50 mg g^{-1}, respectively.	Noormohamadi et al., 2019
Pseudomonas azotoformans JAW1	Cd, Pb, and Cu	Metal accumulation occurs on the cell surface (biosorption). The maximum adsorption found of Cd, Pb, and Cu by 98.57, 88.57 and 69.76%, respectively. The removal level achieved the highest in order of Pb (78.23%), Cu (63.32%), and Cd (44.67%).	Choińska-Pulit et al., 2018
Aspergillus tamari, Simplicillium subtropicum, Aspergillus niger, Fusarium solani,	Cu	Although *A. tamari* and *S. subtropicum* growth rate was low, the intake of Cu per unit of biomass is high compare to two other species.	Ong et al., 2017
Ensifer adhaerens OS3	Cd, Cr, Ni, Pb, Cu, and Zn	The maximum accumulation was recorded for Ni (95%) and lowest for Pb (74%) and in order of Ni > Cu > Zn > Cr > Cd > Pb. Biosorption capacity recorded in order of Zn > Cr > Cd > Ni > Cu > Pb.	Oves et al., 2017

PLANT–MICROBE ASSOCIATED REMEDIATION

The microorganism-plant-based remediation has gain popularity currently due to its higher removal efficiency compared to plant-based remediation process. These microorganisms are involved in the various biochemical process such as carbon and nitrogen mineralization, nitrogen fixation, and decomposing organic matter, which contributes to soil formation, nutrient cycling and transfer of energy. Soil microorganisms are also affected by HMs in contaminated areas. However, with continuous exposer, they tend to tolerate and develop unique features with few specific microbial populations. These types of specific microbes can be employed for remediating toxic metals from contaminated lands. Further, soil microorganisms that form a symbiotic association with host plants are the most successful species in the soil reclamation process. The mycorrhizal fungi form intimate symbiotic relationship with host plants, which have been applied in many bioremediation processes (Yang et al., 2015; Gunathilakae et al., 2018; González-Chávez et al., 2019; Rubin and Görres, 2021). The arbuscular mycorrhizae as the most well-known symbiotic fungi are frequently used in phytoremediation due to their ubiquity in soil. They can develop several mechanisms to tolerate high metal concentrations in soils, thus promoting plant growth (Janoušková et al., 2005; Fasani et al., 2018). In addition, plant growth-promoting bacteria (PGPB) can also stimulate plant growth activities and help plants cope with the contaminated ecosystem. They can enhance plant growth through direct and indirect mechanisms that are discussed in the separated section below.

There are two aspects of plant–microbe-based bioremediation process. First of all is the microorganisms help the host plant sustain in the harsh environmental condition by providing nutrients. Second, the plant plays a critical role by maintaining favorable environmental conditions such as improving soil organic matter, available P, K, and N, where soil microorganisms can thrive and enhance the reclamation process. Recently, a number of studies have been highlighted both side benefits of the plant–microbe-based bioremediation process. A study recorded that planting of *Trifolium repens* in heavy metal contaminated sites improves soil enzymatic activities (Lin et al., 2021). Wang et al. (2021) also showed that plantation of *Salix* in Cd contaminated soil increased beneficial microorganisms diversity, such as genera of bacteria include *Arthrobacter, Bacillus, Flavobacterium, Niastella, Novosphingobium, Niabella*, *Anaeromyxobacter, Rmlibacter, Solitalea,*

TABLE 3 | Metal bioremediation potential of bacteria strains.

Targeted heavy metal	Bacteria used	Remarks	References
Cd and Pb	*Enterobacter cloacae, Klebsiella edwardsii* and *Pseudomonas aeruginosa*	*P. aeruginosa* showed the highest bioremediation potential compared to the other two with 58.80 and 33.67% of remediation in 50 mg Cd L^{-1} and 300 mg Pb L^{-1}, respectively.	Oziegbe et al., 2021
Pb and Ni	*Ochrobactrum intermedium* BPS-20 and *Ochrobactrum ciceri* BPS-26	*O. intermedium* BPS-20 and *O. ciceri* BPS-26 accumulated Pb by 85.34 and 71.20% and Ni by 74.87 and 88.48%, respectively.	Sharma and Shukla, 2021a
Pb	*Bacillus cereus BPS-9*	BPS-9 strains recorded the highest Pb accumulation potential of 79.26% and the biosorption capacity was 193.93 mg g^{-1}.	Sharma and Shukla, 2021b
Cr, Pb, and Ni	*Klebsiella pneumoniae* MB361, *Stenotrophomonas* sp. MB339, and *Staphylococcus* sp. MB371	The percentage of accumulation increase gradually with time and increased biomass. The highest removal was recorded by MB339 with Pb (85.30%), and Ni (48.78%), followed by MB361 with Cr (83.51%), while MB371 sorbed Pb by 88.33%.	Aslam et al., 2020
Ni	*Pseudomonas* sp. P21, *Stenotrophomonas* sp. S20, and *Sphingobium* sp. S42	Bacterial strains S20 and P21 show high tolerant levels to Ni up to 400 mg L^{-1}, while S42 removed 33.7% of metal.	Chen et al., 2020
Hg	*Fictibacillus nanhainensis* SKT-B and *Bacillus toyonensis* PJM-F1	*F. nanhainensis* SKT-B accumulated the highest level of Hg followed by *B. toyonensis* PJM-F1 with 82.25 and 81.21%, respectively.	Nurfitriani et al., 2020
Co and Ni	*Anoxybacillus mongoliensis*	The highest accumulation by bacteria recorded for Co and Ni was 274.9 and 268.5 mg g^{-1}, respectively. Further, increasing activities of superoxide dismutase (SOD) and catalase (CAT) were also recorded.	Akkoyun et al., 2020a
Pb, Cd, and Ni	*Rhizopus stolonifer* and *Bacillus megaterium*	When growing the bacteria separately, *R. stolonifer* and *B. megaterium* recorded maximum uptake of Cd and Ni by 479.10 and 501.05 mg L^{-1}, respectively. Overall *B. megaterium* uptake a higher concentration of combined HMs.	Njoku et al., 2020
Cr	*Bacillus cereus* AVP12 and *Bacillus cereus* NC7401	The highest Cr accumulation potential of AVP12 and NC7401 strains isolated from the contaminated sites was 181.0 and 107.5 mg L^{-1}, respectively. While for the same strains AVP12 and NC7401 isolated from non-polluted sites were 92.59 and 62.11 mg L^{-1}, respectively.	Akhter et al., 2020
Hg and Pb	*Exiguobacterium profundum*	The highest bioaccumulation of Pb and Hg for *E. profundum* were 54.35 and 37.56 mg g^{-1}, respectively.	Akkoyun et al., 2020b
Cr, Ni, and Pb	*Lactobacillus plantarum* MF042018	It shows high tolerance against the Ni and Cr up to 500 and 100 ppm, respectively. The biosorption capacity of MF042018 was recorded very high for Cd and Pb at pH 2.0 and temperature 22°C after 1 h.	Ameen et al., 2020
As	*Bacillus cereus* and *Lysinibacillus boronitolerans*	The bacterial strains P2IIB, P1C1Ib and P2Ic resistant to 3,000 mg L^{-1} of As. The bacteria culture removes 85.72% of arsenate and 71.88% of arsenite from the medium.	Aguilar et al., 2020
Cr	*Bacillus cereus*	The bacteria strain can tolerate Cr_{2000} (2,000 mg L^{-1}) Cr(VI) and can completely decrease Cr_{200} under heterotrophic conditions within 16 h. It is recorded that Cr(VI) was effectively reduced to Cr(III).	Banerjee et al., 2019
As	*Ochrobactrum ciceri* SW1 and *Exiguobacterium profundum* PT2	Both bacterial strains increased production of EPS in the presence of As, which help to sequester arsenic.	Saba et al., 2019
Hg, Cd, Pb, Cu, Ni, and Zn	*Escherichia coli* K-12	The bacterial strain can absorb different types of metal ions. It can absorb more than 30 varieties of metal ions *via* its outer membrane.	Jin et al., 2018
Cd	*Cupriavidus necator* GX_5, *Sphingomonas sp.* GX_15, and *Curtobacterium* sp. GX_31	The highest removal capacity of Cd recorded in order of GX_31, GX_15 and GX_5 with 86.06, 53.88 and 25.05%, respectively.	Li et al., 2018

Devosia, Mesorhizobium Nitrospira, Thermomonas, Flavisolibacter, Pedomicrobium, Lysobacter, Rubrivivax Phyllobacterium, and mycorrhizal genera of fungi include *Actinomucor, Conocytes, Amanita, Cryptococcus, Xylaria, Ramicandelaber, Spizellomyces, Sporobolomyces, Rhodotorula Umbilicaria, Claroideoglomus, Tilletiopsis,* and *Cirrenalia* in plant rhizosphere.

Plant Growth-Promoting Bacteria

It is well known that PGPB can enhance phytoremediation efficiency (Ma et al., 2016; Lin et al., 2021; Kumar et al., 2021a). The PGPB may directly prompt root proliferation and improve plant growth and fitness, plant metal resistance, uptake and translocation of nutrients and metals, and protect plants from phytopathogens (Ma et al., 2011; Gupta et al., 2013; Fasani et al., 2018) by producing and secreting various organic acids, polymeric compounds, chelators, and hormones such as indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, polysaccharides, glomalinand, azotobactin, azotochelin, alcaligin E, pyochelin, coelichelin, ferrioxamine B, and pyoverdin, which are responsible for decrease the soil pH and enhance the metal bioavailability, whereas the polymeric compounds help in phytostabilization

of metals by decreasing their mobility (Chen et al., 2017). The chelators work as metal-binding ligands to enhance metal bioavailability, improve root-shoot translocation and metal uptake capacity, and facilitate intracellular heavy metal accumulation in organelles (Yan et al., 2020). Inoculation of ACC deaminase-producing PGPB showed extensive root and shoot density along with increased biomass and phytoremediation efficiencies (Arshad et al., 2007; Yan et al., 2020). It is found that *Bacillus* sp. XZM lowers As toxicity to the plant by producing a higher amount of extracellular polymeric substance (EPS), siderophore, and IAA (Irshad et al., 2020). Some of PGPB, such as *Pseudomonas, Micrococcus, Erwinia, Azospirillium, Flavobacterium, Azotobacter, Chromobacterium*, and *Agrobacterium* have been applied in the phytoremediation process (Bhattacharyya and Jha, 2012; Ma et al., 2019). Ma et al. (2016) isolated two droughts resistant serpentine PGPB *Pseudomonas reactans* Ph3R3 and *Pseudomonas libanensis* that showed high resistance to different HMs (Cd, Cr, Pb, Cu, Ni, and Zn), salinity, extreme temperature, and antibiotics. Both strains significantly enhanced plant growth, pigment content, and leaf relative water, and also translocation and bioconcentration factors for Cu and Zn under the drought condition.

Further, PGPB are found to be an important player in remediating the HM contaminated marine ecosystems. The study by Mesa-Marín et al. (2020) recorded that inoculation of *Thalassospira australica* SRT8, *Vibrio neocaledonicus* SRT1 and *Pseudarthrobacter oxydans* SRT15, with *Salicornia ramosissima* improved the relative plant growth rate and the number of new branches by 32 and 61%, respectively, when planted in the HM contaminated estuarine soil. The inoculation of PGPB also helps to accumulate the highest concentration of HMs like As, Cd, Cu, Ni, Pb and Zn in the root and subsequently enhance the phytoremediation potential of *S. ramosissima*. In one another study inoculation of *Bacillus flexus* KLBMP 4941 with coastal halophytes *Limonium sinense* under the salt stress ecosystem shows positive effects on the hostplant survival and growth and it can be employed for phytoremediation of saline soils (Xiong et al., 2020). Two PGPB namely *Bacillus cereus* strain P2 and *Planomicrobium chinense* strain P1 isolated by Khan et al. (2018) and inoculated with *Helianthus annus* for phytoremediation of HMs in drought conditions found a significantly positive result. The study confirmed that the application of PGPB and salicylic acid significantly increased the rhizosphere accumulation of Cd, Pb, Ni by 84, 66 and 65%, respectively. In addition, inoculation of PGPB significantly enhanced the root length, shoot length, root fresh, and dry weight by 68, 60, 61, and 63%, respectively. Likewise, in various studies different types of PGPB such as *Bacillus subtilis, Bacillus thuringiensis, Ensifer meliloti* RhOL6 and RhOL8*, Bacillus megaterium, Pseudomonas sp.* DSP17 and *Proteus sp.* DSP1 have been applied along with organic and inorganic amendments found enhanced remediation of HMs from different types of soils which include sandy soil, arid and semi-arid soils (Khan and Bano, 2018; Raklami et al., 2019; Khodaverdiloo et al., 2020).

Generally, associations of leguminous plants with PGPB have also been applied in the phytoremediation process of highly metal-contaminated sites (Hao et al., 2014). But recently, this remediation method is used in less or moderately metal-contaminated agriculture soil (Saadani et al., 2019). Recently, Saadani et al. (2019) found that the inoculation of PGPB with *Sulla coronaria* and *Vicia faba* L. var. *minor* showed a higher metal accumulation in legumes grown in low contaminated agriculture soil compared to non-inoculated legumes. After the cultivation of symbiotic legumes, soil fertility is positively affected with higher organic content (phosphorous and nitrogen) and soil decomposition rate. The rhizobium-legume symbiosis relationship between high metal-resistant *Sinorhizobium meliloti* CCNWSX0020 and plant *Medicago lupulina* has been successfully used in the study for efficient bioremediation of HMs (Lu et al., 2017). It is also recorded that the bacterial strain's extracellular polymeric substances help to immobilize Cu^{2+}. The genetically engineered rhizobium-legume symbiont is also used to remediate the As contamination from the soil. A study by Zhang et al. (2017) inserted the arsenite [As (III)] S-adenosylmethionine methyltransferase gene (*Crars*M) derived from alga *Chlamydomonas reinhardtii* in *Rhizobium leguminosarum* bv. *trifolii* strain R3 and check the As methylation capacity by symbiosis with red clover found a positive result in the test. Likewise, Tsyganov et al. (2020), applied two transgenic strains of *Rhizobium leguminosarum* bv. *viciae*, 3,841-PsMT2 and 3,841-PsMT1 to pea plants (*Pisum sativum*) for the study of Cd tolerance and accumulation in plants. The study concludes that the pair of legume-rhizobia may be applied for phytostabilization purposes.

Arbuscular Mycorrhizal Fungi

AMF are mostly found in terrestrial plant roots by forming the symbiotic association. In the root cortex, the fungus colonizes and develops a thick extended mycelium around the roots, which acts as an intermediatory connection between plants and soils and helps absorb nutrients from soils (Kernaghan, 2005; Reinhardt, 2007). AMF are also found in highly disturbed ecosystems or polluted soils (Cornejo et al., 2008; Yan et al., 2020). AMF can confer plant metal resistance (Singh, 2012; Xu et al., 2012; Curaqueo et al., 2014; Gunathilakae et al., 2018). AMF is a tremendous biological interest due to its positive effects on symbiotic relationships and remediation capability. Further, it has been exploring in every way to employ AMF for stabilizing the metals in contaminated land. The mycorrhizal plants enhance metal phytostabilization by metal sequestration in roots and hyphae. The metals confined to soils make them less bioavailable. Thus, the toxic effects of metals on other living microorganisms are alleviated.

Many studies have been conducted to investigate the role of AMF in phytoremediation (**Table 5**). Liu et al. (2015b) conducted a study on Cd uptake capacity of *Solanum nigrum* inoculated with *Glomus versiforme* BGC GD01C (Gv) in different Cd concentrations soil. They found that the inoculation of *G. versiforme* highly improved the total Cd uptake in plants at different Cd concentrations. Many researchers have attempted

TABLE 4 | Metal bioremediation potential of fungi strains.

Target heavy metal	Fungi used	Remarks	References
Cd	*Penicillium chrysogenum* FMS2	The highest tolerance level recorded for *P. chrysogenum* FMS2 was 1,000 mg L^{-1}. The fungal strain can survive in the wide environmental condition such as temperature and pH range between 15–35°C and 4.0–12.0, respectively. The Cd removal capacity of fungi was approximately 49% in 15 days of exposure.	Din et al., 2021
Cd, Cu, Ni, Pb, and Zn	*Ganoderma lucidum*	The concentration of Pb, Zn, Ni, Cu and Cd in contaminated soil were 4,490, 147, 27.7, 19.4 and 2.18 mg kg^{-1} and *G. lucidum* accumulated 138, 29.8, 3.48, 3.69 and 1.01 mg kg^{-1} of respective metal after inoculated in contaminated soil.	Ipeaiyeda et al., 2020
Pb	*Aspergillus niger, Penicillium oxalicum*, and *Trichoderma asperellum*	*Trichoderma, Penicillium* and *Aspergillus* accumulate Pb ions by 75.29, 66.77, and 56.82%, respectively.	Mariconi et al., 2020
Pb, Ni, and Zn	All isolated fungi, *Ascomycota* and *Basidiomycota*	The highest bioremoval capacity for Ni and Pb was 52 and 44% from the bioaugmented soil with all isolated fungi. While for Zn, the maximum removal was 36% in *A. consortium*-treated soil. Overall, Pb and Ni removal efficacy in order of isolated fungi > *Basidiomycota* > *Ascomycota*, whereas for Zn it was *Basidiomycota* > all isolated fungi > *Ascomycota*.	Hassan et al., 2020a
As, Cr, Cu, Mn, and Fe	All isolated fungi, *Ascomycota* and *Basidiomycota*	Fungal consortia show the highest tolerance index of 1.0 for Cr, Cu and Fe in agar medium. Further, the consortium of all isolated fungi shows the removal capacity of As, Mn, Cr, and Cu by 77,71, 60 and 52%, respectively.	Hassan et al., 2020b
As	21 fungal strains including *Humicola* sp.	All the isolated fungal strains can tolerate up to 5,000 mg L^{-1} AsV. The accumulation capacity of fungi biomass ranged between 0.146 to 11.36 g kg^{-1} and volatilization of As between 0.05 to 53.39 mg kg^{-1} biomass. *Humicola* sp. recorded the highest biovolatilization capacity by 53.39 mg kg^{-1}.	Tripathi et al., 2020
Hg	*Penicillium spp.* DC-F11	DC-F11 fungal strain detoxified Hg *via* extracellular sequestration through precipitation and adsorption.	Chang et al., 2020
Hg	*Aspergillus* sp. A31, *Lindgomycetaceae* P87, *Curvularia geniculata* P1, and *Westerdykella* sp. P71	All four species of endophytic fungi remove up to 100% of Hg in a species-dependent manner from the culture medium.	Pietro-Souza et al., 2020
Cd	*Aspergillus fumigatus*	*A. fumigatus* showed the highest tolerance against Cd with a removal percentage of 74.76 and uptake capacity of approximately 5.02 mg gm^{-1}.	Talukdar et al., 2020
Cd and Pb	*Simplicillium chinense* QD10	Cd biosorption occurs with forming Cd-chelate and Pb mainly adsorbed by extracellular polymeric substances (EPA).	Jin et al., 2019
Cu, Cd, Pb, and Zn	*Alternaria chlamydosporigena, Trichoderma harzianum, Acremonium persicinum, Fusarium verticillioides, Seimatosporium pistaciae,* and *Penicillium simplicissimum*	*T. harzianum* was found the maximum tolerant against Cd, Cu and Pb. *A. persicinum* and *P. simplicissimum* record the highest biosorption and accumulation of HMs.	Mohammadian et al., 2017
Cd, Cr, Cu, Ni, and Zn	*Beauveria bassiana*	It removed 84% multi-metal from the mixture sample while individual metal removal capacity was 61–75%. *B. bassiana* removed the metal *via* accumulation and sorption processes.	Gola et al., 2016
Cu, Pb	*Aspergillus flavus* and *A. niger*	The biosorption of Cu and Pb by *A. flavus* and *A. niger* was recorded 81.8 and 83.1%, respectively, during the initial 10 min.	Iram et al., 2015

to explore more possibilities to remediate the contaminants from the stressed environment. Recently, a study conducted by Hao et al. (2021) showed that the phytoremediation potential of *Zea mays* inoculated with *Claroideoglomus etunicatum* grown in Lanthanum (La) contaminated soils enhanced bacterial diversity including *Agrococcus, Lysobacter, Planomicrobium, Microbacterium, Streptomyces, Saccharothrix, Penicillium*, and other unclassified bacteria and fungi like *Penicillium*. This study confirmed that AMF can regulate the rhizosphere fungal and bacterial diversity to foster beneficial microorganisms that help the plant sustain. Further, an investigation is undertaken in Ni contaminated saline soil for remediation using *Helianthus annuus* inoculated with plant beneficial bacteria (*Pseudomonas libanensis* TR1) and AMF (*Claroideoglomus claroideum* BEG210; Ma et al., 2019). The study found that the bacteria and fungi alone or in combination, significantly increase plant growth, physiological parameters, and accumulation of Ni and Na^+, thus contributing significantly to Ni Phytostabilization, Na^+ and Ni detoxification, and Na^+ exclusion. Therefore, bioaugmentation with PGPB with AMF can be used as a useful strategy for reclaiming metal-contaminated saline soil.

TABLE 5 | Role of microorganisms in the removal of heavy metals by plants.

Targeted heavy metal	Microorganisms used	Host plant	Remarks	References
Bacteria				
Cd, Cu, Ni, Pb, and Zn	*Bacillus cereus* TCU11	*Zea mays*	TCU11 significantly enhanced the biomass, chlorophyll, carotenoids, proline, phenolics, protein and antioxidant enzymes. It also increased the translocation of metals except for Ni. Overall, it improves the phytoremediation efficiency.	Bruno et al., 2021
Cu	*Pseudomonas lurida* EOO26	*Helianthus annuus*	Inoculation of EOO26 increased the Cu accumulation in roots and leaves by 8.6 and 1.9-fold, respectively, and total plant uptake by 2.6-fold compared to the uninoculated plants.	Kumar et al., 2021a
Cd, Pb, and Cr	*Adhaeribacter, Kaistobacter, Lysobacter, Pontibacter, Flavisolibacter, Bacillus*	*Trifolium repens*	*Kaistobacter, Lysobacter* and *Pontibacter* significantly helped in metal accumulation, whereas the other three species enhanced plant growth.	Lin et al., 2021
Cd	*Micrococcus* sp., *Arthrobacter* sp.	*Chlorophytum amaniense, C. comosum*	*Micrococcus* sp. increased the production of biomass of both plants. Both the bacterial strains boost phytoextraction of Cd.	Sangsuwan and Prapagdee, 2021
Cu	*Pseudomonas* sp. TR15a, *Bacillus aerophilus* TR15c	*Helianthus annuus*	The consortium of bacteria significantly increased the dry biomass, germination, root and shoot Cu accumulation by 64, 32, 47 and 75%, respectively.	Kumar et al., 2021b
Cu, Cd, Pb, and Zn	*Bacillus* subtilis, *Bacillus licheniformis* - BC *Streptomyces pactum* Act12 -ACT	*Brassica juncea*	Co-inoculation of bacteria increased the enzyme activity, metal bioavailability, plant growth and phytoextraction capacity of *B. juncea.*	Jeyasundar et al., 2021
Cd	*Lelliottia jeotgali* MR2, *Klebsiella michiganensis* TS8	*Miscanthus floridulus*	Strain TS8 enhanced plant growth and declines the total Cd in the rhizosphere, while MR2 significantly increased the translocation of Cd from root to shoot parts.	Liu et al., 2021a
Cu, Cd, Pb, and Zn	*Bacillus cereus* MG257494.1*, Alcaligenes faecalis* MG966440.1 *Alcaligenes faecalis* MG257493.1	*Sorghum vulgare*	The bacteria consortium increased the microbial activity and reduced metal bioaccumulation in the plant and its root. It also controlled the metals bioaccumulation factor (BAF) in plants and the rhizosphere.	Abou-Aly et al., 2021
Cd, Pb, and Cr	*Pseudomonas putida* RE02	*Trifolium repens*	The inoculation RE02 improved the seed germination tailing, soil fertility and the uptake of total heavy metal by 30.03–574.58%.	Liu et al., 2021b
Cd and Mn	*Enterobacter* sp. FM-1	*Polygonum lapathifolium* L.*, Polygonum hydropiper* L.	Inoculation of bacteria increased soil bioavailability of Cd and Mn significantly and lowered the soil pH, resulting in an increase in metal accumulation in both the plants.	Li et al., 2020
Sb	*Pseudomonas fuorescens*	*Trifolium repens*	The application PGPB with nZVI significantly enhanced Sb accumulation capacity of *T. repens.*	Zand et al., 2020
As	*Cupriavidus basilensis* r507	*Pteris vittate*	*P. vittata* accumulated up to 171% of As, when inoculated with the bacterial strain.	Yang et al., 2020
Pb	*Micrococcus luteus*	*Chromolaena odorata*	*M. luteus* inoculated with *C. odorata* can be applied to remediate the moderately Pb-fuel oil contaminated mild saline soil.	Jampasri et al., 2020
As	*Bacillus* sp. *XZM*	*Vallisneria denseserrulata*	The symbiosis between the plant and bacteria significantly enhanced As uptake and removal capacity. In addition, 85% arsenic found as As (III) and >77% stored in vacuole of leaves cells.	Irshad et al., 2020
Al	*Chaetomium cupreum*	*Miscanthus sinensis*	The bacteria produced siderophore called oosporein that supports seedling growth and increased Al tolerance and accumulation.	Haruma et al., 2019
As	*Azospirillum brasilense* Az39, *Bradyrhizobium japonicum* E109	*Glycine max*	The mortality of plants reduced with an increase in plant growth, nodule number and nitrogen content. As translocation to aerial parts also decreased, thus it enhances the phytostabilization potential of *G. max.*	Armendariz et al., 2019
Cd, Pb Cr, Cu, and Zn	*Mesorhizobium loti* HZ76, *Ensifer adhaerens* HZ14, *Rhizobium radiobacter* HZ6	*Robinia pseudoacacia*	Treatment with *M. loti* HZ76 results in significantly increased nodule number. Overall, the addition of bacteria strains enhanced the phytoremediation efficiency.	Fan et al., 2018

(Continued)

TABLE 5 | Continued

Targeted heavy metal	Microorganisms used	Host plant	Remarks	References
Cd, Pb, and Zn	*Streptomyces* sp. Strain B1, B2, B3	*Salix dasyclados* L.	Bioaugmentation with bacteria significantly enhanced plant biomass and decreased oxidative stress. B1 strain record the high potential for phytoextraction due to its highest ability for siderophore secretion.	Złoch et al., 2017
Pb and U	*Enterobacter sp.* HU38, *Pantoea stewartii* ASI11, *Microbacterium arborescens* HU33	*Leptochloa fusca*	The bacterial consortia increased metal accumulation capacity by 58–97% and 53–88% for Pb and U, respectively.	Ahsan et al., 2017
Fungi				
Cd and Zn	*Rhizophagus irregularis* (FR717169)	*Phragmites australis*	Under Zn stress, the fungi helped increase the activities of ascorbate peroxidase (APX) and SOD. Under Cd stress, CAT, peroxidase (POD), SOD and APX increased significantly. The translocation factor of Zn and Cd reduced by 10–57 and 17–40%, respectively.	You et al., 2021
Cd	*Funnelliformis mosseae*	*Solanum nigrum, Oryza sativa*	Intercropping with fungi enhanced growth and Cd accumulation of *S. nigrum.* The treatments help reduce the Cd level in rice parts with a maximum reduction in brown rice by 64.5%.	Yang et al., 2021
Cd	*Blastocladiomycota, Chytridiomycota, Mortiriellomycota, Tilletiopsis, Sporobolomyces, Cryptococcus, Conocytes,Umbilicaria, Amanita, Xylaria, Cirrenalia*	*Salix*	The presence of fungi showed a positive correlation with Cd accumulation. The study recorded that a higher fungal number contributes to high biomass.	Wang et al., 2021
La	*Claroideoglomus etunicatum*	*Zea mays*	The AMF promoted nutrient uptake and growth of *Z. mays* in various La stressed soil. It also increased the root and shoot fresh and dry weight significantly. The shoot concentration of La decline significantly by 51.53% and increased root concentration by 30.45%.	Hao et al., 2021
Cd, As, and Pb	*Glomus mosseae*	*Pisum sativum*	Inoculation with *G. mosseae* enhanced plant growth, the concentration of carbohydrates, photosynthetic pigments, nitrogen and defense antioxidants. This symbiosis can be employing for onsite remedy of Cd- and Pb-polluted soil.	Chaturvedi et al., 2021
Cr	*Rhizophagus irregularis*	*Brachiaria mutica*	AMF enhanced the photosynthetic performance by increasing the chlorophyll, carotenoid, proline, protein content and activities of antioxidant enzymes. It also improves the tolerance index, transportation index and bioconcentration factor of *B. mutica*.	Kullu et al., 2020
Hg	*Aspergillus* sp. A31, *Lindgomycetaceae* P87, *Curvularia geniculata* P1 *and Westerdykella sp.* P71	*Aeschynomene fluminensis, Zea mays*	The tolerance capacity of plants for the Hg^{2+} was improved after the inoculation of fungi. The biomass of the plants increased along with the reduction in soil Hg concentration. Further, the soil Hg level reduced in *A. fluminensis* by 57.14% inoculated with P87.	Pietro-Souza et al., 2020
As	21 fungal strains including *Humicola* sp.	*Bacopa monnieri*	*Humicola* sp. enhanced the plant growth and bacoside content and can use as a realistic and potential mitigation strategy for reducing the As level in the cropping system.	Tripathi et al., 2020
As	*Piriformospora indica*	*Artemisia annua*	The inoculation of fungi helped the plant to accumulate significantly high concentration of As in roots than shoots. In addition, overall biomass, artemisinin, flavonoids, peroxidase and SOD were increased significantly.	Saeed-ur-Rahman et al., 2020

(Continued)

TABLE 5 | Continued

Targeted heavy metal	Microorganisms used	Host plant	Remarks	References
Cd, Pb, and Zn	*Cenococcum geophilum* (Cg, KY075873.1), *Laccaria* sp. (L1, KY075876.1,), *Pisolithus* sp.1 (P1, KY075877.1), *Pisolithus* sp. 2 (P2, MN422052)	*Pinus sylvestris*	Inoculation of fungi increased the survival rates of plants by enhancing the biomass, photosynthetic rate, transpiration rate, stomatal conductance, mineral nutrients and intercellular CO_2 concentration. Further, *P. sylvestris* accumulated a higher concentration of Cd, Pb and Zn than non-ectomycorrhizal seedlings.	Liu et al., 2020
As	*Rhizophagus, Funelliformis*	*Pteris vittata*	*Rhizophagus and Funelliformis* inoculation improved the plant growth and increased the fresh and dry weight of aerial parts by 44 and 37%, respectively. The BAF for inoculated plants was 7.6 while for uninoculated it was recorded 6.0.	Cantamessa et al., 2020
Cd and Pb	*Simplicillium chinense* QD10	*Phragmites communis*	The amendments of *S. chinense* QD10 significantly increased the phytoextraction of metal by 28.6–48.0% of *P. communis*.	Jin et al., 2019
Cd	*Acaulospora Laevis, Glomus monosporum, G. clarum, Gigaspora nigra*	*Trigonella foenumgraecum*	Inoculation of AMF enhanced the plant growth parameters, protein and chlorophyll contents. The TF of plants was also reduced significantly.	Abdelhameed and Metwally, 2019

FACTORS AFFECTING BIOREMEDIATION EFFICIENCY

The most important factor affecting bioremediation efficiency is site characteristics. Secondly, environmental factors such as water content, temperature, pH, nutrient availability, moisture content, and pollutant bioavailability can also hinder the efficiency of bioremediation (Freitas et al., 2013; Azubuike et al., 2016; Khodaverdiloo et al., 2020; Leong and Chang, 2020). Apart from this, the bioremediation process is a complex system that is optimized and controlled by many factors. The interactions among the contaminants, microbes, nutrient availability and environmental factors affect the bioavailability and biodegradation of the contaminants.

Site Characteristics

The first and most important factors which affect the bioremediation process are the site location and its characteristics. The extent and type of contaminants present in the location determine the remediation efficiency (Abatenh et al., 2017). These factors can be overcome and managed by sufficient prior investigation and characterization of sites before implementing the remediation process.

Temperature

Temperature is an important factor that determines the survival and growth of the microorganism and the composition of hydrocarbon (Yang et al., 2009). It plays a critical role in the microbe-assisted remediation process by affecting both the physical and chemical states of contaminants present in the polluted sites and interrupting the microbial metabolisms, growth rate, soil matrix, and gas solubilities (Megharaj et al., 2011). It is recorded that high temperature destroys the cell metabolic activity of bacteria and affects the process of bioaccumulation (Javanbakht et al., 2014). Furthermore, the temperature can speed up or slow down the remediation process as microbial physiological properties are highly influenced by temperature. The interaction between fungal membrane binding sites and heavy metal ions depends on the temperature. Temperature also affects the configuration and stability of fungal membrane by chemical moieties ionization (Oka et al., 2005). Jin et al. (2019) showed that the biosorption efficiency of *S. chinense* QD10 for Cd and Pb was highest at 30°C by 60.4 and 38.3%, respectively. But it significantly declined when the temperature increased to 45°C. The microbial adsorption is also affected by temperature (Timková et al., 2018).

pH

pH has its own impacts on the metabolic activity of microorganisms which can increase or decrease the removal process. Bioremediation can be applied in a wide range of pH. However, a pH of 6.5 to 8.5 is considered the maximum potential for remediating the most terrestrial and aquatic systems (Abatenh et al., 2017). The pH value influences the biosorption process by dissociation of functional groups on the fungal membrane and affects heavy metal mobility and solubility (Wang et al., 2014). It was observed that the Cd biosorption capacity of *Exiguo bacterium* sp. enhanced with increased pH up to 7.0 and remained neutral when the pH was higher than 7.0 (Park and Chon, 2016). The microbial adsorption is also affected pH and ionic strength (Timková et al., 2018).

Nutrient Availability

Likewise, nutrient concentration, availability, and type are also important for microbial growth and activity in the bioremediation process. The fundamental elements (such as carbon, nitrogen, and phosphorous) help the microbes produce the necessary enzymes to break down the pollutants. The lower level of nutrient availability affects the plant and microorganisms, which ultimately affects the bioremediation rate and effectiveness. In this condition balancing the essential

nutrient such as nitrogen (N) and phosphorus (P) can enhance the bioremediation efficacy through optimizing the bacterial C:N:P ratio (Abatenh et al., 2017). In the colder environment, the supply of an appropriate quantity of nutrients enhances the metabolic activity of microorganisms, which leads to an increase in the remediation rate (Phulia et al., 2013; Couto et al., 2014). It has been reported that an excessive amount of nitrogen in the contaminated medium resulted in microbial inhabitation (Varjani and Upasani, 2017). Further, the higher concentration of nitrogen, phosphorus, and potassium hinders the biodegradation efficiency of hydrocarbon contaminants.

Moisture Content

The microorganisms can be adversely affected by the soil moisture content. Moisture affects the rate of pollutant metabolism *via* influencing the amount and type of soluble materials as well as the pH and osmotic pressure of the terrestrial and aquatic sites (Abatenh et al., 2017).

Type/Nature of Microorganism and Plant

The existence of unsuitable microorganisms or the inadequate presence of suitable microorganisms in the contaminated sites affects the bioremediation efficiency. Apart from this, the microbial biophysical process also influences bioaccumulation as the process is metabolically dependent and uses cellular energy for metal uptake. It depends on the microbial biochemical features, genetic and physiological ability, internal structure, cell surface properties such as charge changes, and surrounding environmental conditions (Srinath et al., 2002; Vijayaraghavan and Yun, 2008; Issazadeh et al., 2013). Razmi et al. (2021) found that phytoremediation efficiency was influenced by various biological and chemical factors. For the plant-based remediation, the important factors consider for selecting the suitable plants includes the root system, it may be tap or fibrous roots depending on the depth of the contaminants, above-ground biomass, which should not preferable for livestock consumption, survival, and adaptation of plants and the plant growth (Azubuike et al., 2016). However, the role of plant type in the phytoremediation of Cd, Pb, Ni, and Zn has been considered as the prime factor. Similarly, the maximum biosorption efficiency for most of the fungal strains was found under their optimal growth conditions (Iram et al., 2015).

Water Content

In general, microorganisms require water activity values between 0.9–1.0 for metabolism and growth. Most of the bacteria grow optimally at the upper limits of water activity values (Sharma, 2019). Therefore, the water content in contaminated land is an essential factor that may affect the bioremediation rate. Recently, Khodaverdiloo et al. (2020) highlighted that water deficiency, sodicity, and salinity are also important factors that affect bioremediation efficiency.

Pollutant Bioavailability

The low bioavailability of HMs in the contaminated soil greatly affected the bioremediation efficiency. The bioavailability of contaminants is controlled by various physicochemical processes such as sorption, diffusion, desorption, and dissolution. This problem can be managed using various surfactants and chelating agents, which enhance the bioavailability of HMs for microbial degradation and plant uptake. Various types of organic and inorganic chelating agents are applied recently such as ethylenediamine tetraacetic acid (EDTA), [S,S]-ethylenediaminedisuccinic acid (EDDS), ethylenediamine-di-ohydroxyphenylacetic acid (EDDHA), diethylenetriaminepentaacetic acid (DTPA), nhydroxyethylenediaminetriacetic acid (HEDTA) citric acid, acetic acid, and malic acid. Application of these chelating agents has successfully proven that it effectively forms a complex with HMs and increases the bioavailability (Sarwar et al., 2017).

CHALLENGES AND FUTURE PROSPECTS

The bioremediation methods are diverse and show effectiveness in restoring the polluted sites contaminated with multiple HMs. However, there are some important factors to be considered before implementing bioremediation practices. There is a need for regular investigation and assessment of the level of HMs and other pollutant concentrations in the contaminated sites before proposing bioremediation. The selection of an appropriate type of microbes and plant species is a very hefty task for the sites where the presence of multi-metals and other organic pollutants at the same site. Secondly for the plant-based bioremediation, the presence of volatile metals and metalloids such as Si, Hg, and As in the site may get volatilized into the atmosphere in their toxic form which may affect the living organisms. Third, if edible plants are used for bioremediation purposes, there is a risk that they can be consumed by animals, insects and which may further contaminate the food chain and ultimately reach humans and cause serious health complications. For this, nonedible and nonpalatable phytoremediator plant species can be preferred or in the case of the edible plants, proper protection during cultivation, and harvesting must be taken to avoid future complications. With the presence HMs deeper into the ground where plant roots cannot reach, *in situ* phytoremediation becomes difficult.

Further research, assessment, and investigation are required to enhance our knowledge and understanding of best management practices for efficient bioremediation of HMs. There is a need for futuristic clarification of mechanisms, metabolites, and novel approaches/methods are required. For simple and efficient plant-based bioremediation, utilization of hyperaccumulator plants to efficiently remove of HMs from the contaminated soil need novel strategies for its further progress. This can be achieved in two ways, first by finding and validating the various diversity of new hyperaccumulator plant species, and second by developing the hyperaccumulator plant using genetic engineering. In addition, we can consider the hyperaccumulator plants with deep root plants for, e.g., woody plants or tree such as *Populus* × *canescens, Rinorea bengalensis, Schima superba* and *Pycnandra acuminata* with high translocation rate, high biomass and growth rates and more tolerant plant species.

Biotechnological intervention including genetic engineering, for example, the rate-limiting step in a known metabolic pathway

can be manipulated genetically to enhance the transfer and biodegradation rates, or by introducing a completely new metabolic pathway into the microbe for higher accumulation of HMs or degradation of recalcitrant compounds. In addition, overexpression of foreign genes into a non-tolerant plant with having higher biomass for HM remediation from the soil may be a feasible strategy. The advanced way to study hologenomics of plants microorganism will be helpful for the manipulation of microbial niches which help to enhance the resistance against toxic metal contamination. For multi-metal contaminated and multi-stress environmental conditions, there is a need to development of suitable amendments to enhance the survival of the suitable plant species. Although there are several organic and inorganic amendments and metal chelators are available there is a need for further investigation to find out more suitable and eco-friendly amendments which can be applied for the treatment of multi-metal contaminated and multi-stressed soil. There is a necessity for coordination and contribution of researchers, scientists, policymakers, government, industrial sectors, and individuals that can help to success and reliability of bioremediation.

CONCLUSION

Man-made activities have been introducing a high amount of toxic metals into the environment, affecting the life processes of all living organisms in direct and indirect ways. It has been reported that more than one type of heavy metal is simultaneously present in the contaminated land and the available conventional methods are not significantly efficient to detoxify the pollutants compared to the bioremediation process. It has been proved that bioremediation methods are easily affordable compared to other physicochemical remediation techniques. A number of bacterial and fungal strains have been isolated and identified from different metal-contaminated and mining abandoned soils in recent years. *Pseudomona*s spp., *Bacillus* spp., *Aspergillus* spp., and *Penicillium* spp. are found frequently and show high metal tolerance and bioremediation potential. Currently, bioremediation has been practiced in various contaminated sites globally with varying degrees of success. Recently by applying the various plants and microorganisms to remediate the contaminants from the environment has been noted like Alaska oil spill remediation, China's Aleutian island bioremediation operation and other decontamination cases of HMs from the industrial and agricultural fields. The addition of proper supplements and enhancing environmental conditions are the prime concern for the significant yield of bioremediation. To overcome the above problem, the addition of organic matter and a consortium of microorganisms can enhance microbial metabolic activity and may improve bioremediation potential. In addition, more investigations are still required to screen the more suitable microorganisms, hyperaccumulator plants that will have a high capacity to tolerate multi-metal contaminated and multi-stress environmental conditions sites and accumulate multi-metals at once. Further attention will be required to plant–microbe-based bioremediation strategies to identify the novel plant–microbe pairs that will have high metal removal efficiency along with creating a favorable environment to accommodate other microbial diversity for indirectly improving the soil health. Additionally, further research on the application of nanomaterials and biochar along with microbes to enhance bioremediation efficiency is needed.

AUTHOR CONTRIBUTIONS

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

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Plant-Mycorrhizal Fungi Interactions in Phytoremediation of Geogenic Contaminated Soils

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Soil contamination by geogenic contaminants (GCs) represents an imperative environmental problem. Various soil remediation methods have been successfully employed to ameliorate the health risks associated with GCs. Phytoremediation is considered as an eco-friendly and economical approach to revegetate GC-contaminated soils. However, it is a very slow process, as plants take a considerable amount of time to gain biomass. Also, the process is limited only to the depth and surface area of the root. Inoculation of arbuscular mycorrhizal fungi (AMF) with remediating plants has been found to accelerate the phytoremediation process by enhancing plant biomass and their metal accumulation potential while improving the soil physicochemical and biological characteristics. Progress in the field application is hindered by a lack of understanding of complex interactions between host plant and AMF that contribute to metal detoxification/(im)mobilization/accumulation/translocation. Thus, this review is an attempt to reveal the underlying mechanisms of plant-AMF interactions in phytoremediation.

Keywords: phytoremediation, arbuscular mycorrhizal fungi, metal contaminated soils, metal transporters, genes

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INTRODUCTION

As a result of rampant industrial activities, geogenic contaminants (GCs) have intruded in almost all spheres of the environment, including soil, water, air, and plants (Sandeep et al., 2019). Globally, the soils of >20 million hectares of land in 10 million sites are contaminated, and more than 50% of them are polluted with GCs (He et al., 2015). The root cause of this recurring problem of GC pollution seems to be the increased rate of industrialization, urbanization, mining, milling, fossil fuel burning, agrochemicals that release a wide range of GCs, and metalloids into the environment (Sandeep et al., 2019; Zhang et al., 2019). Leachates of municipal solid waste landfills in poor waste disposal systems contain elevated concentrations of GCs and metalloids, which are also responsible for contaminating soil soil-crop systems (Vongdala et al., 2019). The concentrations of GCs in soils may also be enhanced by applying inorganic and organic fertilizers, organic manure, pesticides, and herbicides (Dharma-Wardana, 2018; Reboredo et al., 2019). Several studies reported toxic metals accumulations in plant food samples harvested from contaminated soils, indicating that contaminated soils become the pathway of GCs to crops (Emurotu and Onianwa, 2017; Chaou et al., 2019; Kibet et al., 2019; Liang et al., 2019; Afonne and Ifediba, 2020). These released GCs

are biomagnified in living beings of the higher trophic levels once they enter the food chain *via* the ingestion of food and vegetable.

Removal of GCs from the contaminated sites may be attained by various traditional techniques, such as detonation, incineration, soil excavation, soil washing, chemical precipitation, etc., which are very costly and adversely affect ecosystem functioning (Dermont et al., 2008). Recently, a widely used phytoremediation technique, the use of plants to extract, sequester, and detoxify pollutants, has been reported to be effective, non-intrusive, inexpensive, aesthetically pleasing, and socially accepted technology to remediate polluted soils (Kachout et al., 2009; Pandey and Bauddh, 2018). Soil amendment by using microorganisms, especially arbuscular mycorrhizal fungi (AMF) are efficient in accelerating the phytoremediation process (Ma et al., 2016). AMF inoculation is regarded as a promising tool in biotechnology for the sustainable remediation of hazardous contaminants (Schneider et al., 2017). Certain aspects in AMF associated phytoremediation, such as the response of plant and AMF species, the role of different soil parameters on their association, etc., needs to be well explored. Providing an in-depth literature review on the mechanisms responsible for plant-mycorrhizal fungi interactions in a lucid manner separates it from previous related work. Therefore, this paper expounds on the feasibility of a cost-effective and green method of AMF-assisted GC phytoremediation. Further, the mechanisms of action involved in plant-mycorrhizal fungi association for GC remediation from the contaminated sites have also been discussed.

Methodology

The literature cited in this review ranged from 1904 to 2021. However, the majority of the articles targeted were from journal articles, book chapters, and books published between 2011 and 2021. The relevant literature surveyed were studied employing Google, Google Scholar, Web of Science, Research Gate, and Scopus using various keywords such as phytoremediation, arbuscular mycorrhizal fungi, metal contaminated soils, metal transporters, genes. Further, especially focused journals were Annual review of plant biology, Frontiers in Microbiology, Current Opinion in Toxicology, Journal of Plant Physiology, Plant Physiology, etc., were browsed for digging deeper into the relevant literature until 2021. Subsequently, we have examined the publication individually and eliminated the quotative and duplicate papers. Out of the total literature documents yielded, we have selected and referred to 168 articles. Out of which, total journal articles were 161 followed by six book chapters and one book. Around 51% of the cited documents were of the years 2011–2021. To the best of our knowledge, this article is an updated review article that focused and covered all dimensions of plant-mycorrhizal fungi interactions in metal phytoremediation.

ESTABLISHMENT OF MUTUALISTIC SYMBIOSIS

Soil can facilitate a conducive environment for interaction among diverse and highly complex microbial communities and is considered as a "safe haven" for them. Hiltner (1904) was the first soil biologist who defined the rhizosphere as a hyperactive "zone of contact" around the plant root system in the soil where microbes live and contribute to plant health. The findings of various studies suggested that rhizosphere processes are affected by exudates of plant roots and rhizosphere microorganisms (Kamilova et al., 2006; Kumar et al., 2007). Root exudates are involved in important functions, such as inducing plant defense response against pathogenic microorganisms (Abbott and Murphy, 2003) and providing a basis for chemotaxis to attract and repel microbial species and populations (Kumar et al., 2007), keeping the soil wet and moist, altering the chemical properties of the soils, mobilizing the nutrients, inhibiting the growth of competitor plants, and stabilizing soil aggregates around the roots. Root exudates mainly consist of carbon-based compounds (Bais et al., 2006), including low molecular weight compounds (e.g., amino acids, organic acids, sugars, phenolic, and several secondary metabolites), and high molecular weight compounds (e.g., mucilaginous substances and proteins; Badri and Vivanco, 2009).

The fungus-plant association fosters plant growth and boosts root development (Janeeshma and Puthur, 2020; Tiwari et al., 2020). Based on the basis of morphological characteristics, mycorrhiza is classified into five groups such as ecto-, ericoid, arbutoid, arbuscular, orchid, and monotropoid (Wang and Qiu, 2006). Among them, AMF is considered as most effective in promoting plant growth and development in the ecosystem by speeding up the processes of nutrient absorption. AMF starts symbiosis before they reach the host plant roots. During this pre-infection stage, plant roots release signal molecules (e.g., branching factors), which are responsible for the fast growth and branching of hyphae, followed by the differentiation of fungal adhesion structures. In reciprocation of branching factors, AMF may release signal molecules (e.g., Myc factors) that can induce both molecular and cellular responses and thus ensure successful AMF root colonization (Maillet et al., 2011). Positive results of this symbiosis are attributed to physiological changes of host plants, including hormonal equilibrium, transcriptional profile, primary, and secondary metabolism (López-Ráez et al., 2010).

PERFORMANCE OF MUTUALISTIC SYMBIOSIS

Amongst several mutualistic symbioses, the arbuscular mycorrhizal symbiosis is considered as one of the significant determinants for plant health and soil fertility in terrestrial ecosystems (Jeffries et al., 2003). The fine hyphae that spread into the soil and absorb minerals more effectively than plant roots alone, and the presence of the fungi constantly decreases soil-borne fungi and nematode root attacks (Smith and Read, 2008). AMF may play important role in plant growth in metal contaminated soils (Hildebrandt et al., 2007) by acting as bioalleviator and/or biofertilizer (**Figure 1**). In addition, the large and dense mycelial network established by AMF improves the stability of soil particles through the excretion of glomalin (an insoluble and hydrophobic protein material) and soil proteins associated with glomalin, thus inhibiting disaggregation

of soil organic carbon and water (Bedini et al., 2009; Hallett et al., 2009). AMF colonization can affect vegetative (Miller et al., 1987) and sexual reproduction of plants by influencing the number of inflorescences, production of seeds and fruits, and offspring vigor (Nuortila et al., 2004). These different attributes of AMF may contribute to protect endangered plants (Bothe et al., 2010). Following are some of the attributes that have been briefly discussed.

Bioalleviator

The reactive oxygen species (ROS) formation accelerates under biotic and abiotic stresses (Hasanuzzaman et al., 2013; Bauddh and Singh, 2015; Jajic et al., 2015; Sachdev et al., 2021). ROS generation in plants has been reported as long-distance signals in response to various stresses (Mittler, 2017). To minimize the toxic effects of ROS, plants possess effective ROS-scavenging systems involving both enzymatic (e.g., ascorbate peroxidase and superoxide dismutase) and non-enzymatic (e.g., glutathione and ascorbic acid) ROS actions (Hasanuzzaman et al., 2013; Sachdev et al., 2021). Few researchers have reported that ROS generation occurs during early symbiotic interactions between AMF and host plant roots (Fester and Hause, 2005; Kiirika et al., 2014). To mitigate its potentially toxic effects, there must be a balance between ROS generation and scavenging. In plants, redox homeostasis, antioxidant signaling, and continuous production or removal of ROS at the cellular level are considered as stress signals (Jajic et al., 2015).

The plant-microbe symbiotic associations play a crucial role in alleviating biotic and abiotic stresses such as heat, salinity, drought, metals, and extreme temperatures (Goh et al., 2013; Schouteden et al., 2015). Studies on AMF mediated stress tolerance and increased growth of host plants have been the pivotal research on plant stress responses (Tahat and Sijam, 2012). Plant-AMF interactions can improve plant growth and health by controlling the generation and scavenging of ROS (e.g., H_2O_2, superoxide radicals, alkoxy radicals, singlet oxygen, perhydroxyl radicals, etc.) under biotic and abiotic stresses (Goh et al., 2013; Nath et al., 2016). For instance, a significantly higher amount of ROS is generated due to GC stress, therefore causing oxidative damage to the cellular structures of plants (Yang et al., 2015b). In response to such oxidative stress, plant-AMF associations can activate numerous antioxidant enzymes (e.g., thioredoxin, superoxide dismutase, glutathione peroxidases, and catalase) for scavenging the generated ROS to protect against cell damage (Hashem et al., 2018).

Biofertilizer

AMF are considered natural biofertilizers, because they help the host plants to develop their root system for absorption of water and essential nutrients in exchange for photosynthetic products and to protect plants against harmful pathogens (Berruti et al., 2016; Gao et al., 2020; Basiru et al., 2021). It is well documented that, the AMF-pant association has countless benefits in terms of healthy soil conditions and increased crop productivity (Berruti et al., 2016). Therefore, AMF are considered as the most important biotic soil components, the impoverished or missing AMF can lead to a less efficient ecosystem functioning (Berruti et al., 2016). The roles of AMF as biofertilizer in several biochemical and physiological processes are as follows:

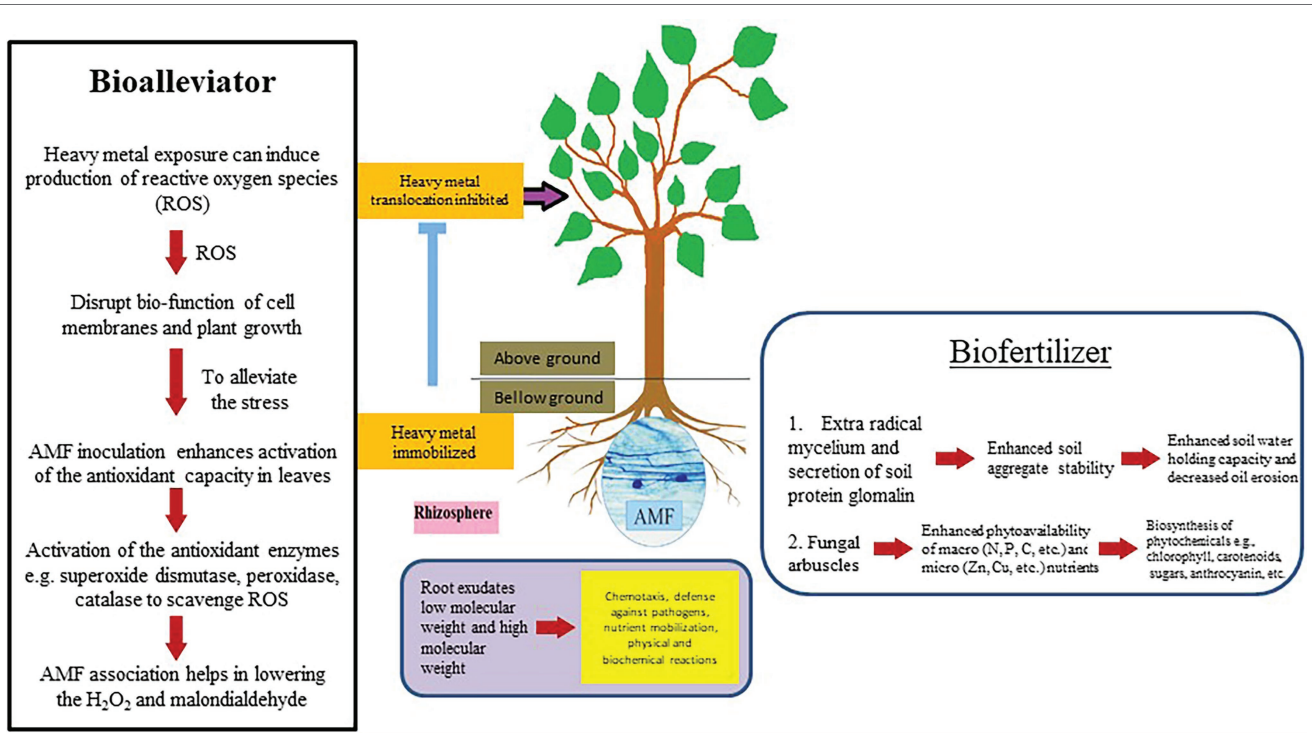

FIGURE 1 | Plant-arbuscular mycorrhizal fungi (AMF) mutualistic performance in the rhizosphere.

Phosphorus Acquisition

The phosphorus (P) absorption in the mutualistic relationship formed between the host plant and AMF offers significant advantages, such as, providing an efficient pathway through which P is retrieved from the soils and directly transferred to the roots. The exchange of nutrients between host plants and microorganisms is a regulated process facilitated by membrane transporting proteins such as, phosphate transport and the P-type H^+-ATPase (Bucher, 2007; Basu et al., 2018).

Nitrogen Acquisition

Plant growth is often hampered by the loss of nitrogen from the soils. The mycorrhiza can facilitate nitrogen absorption from the soils to plants, and increase various types of nitrogen (Smith and Read, 2008; Veresoglou et al., 2011; Makarov, 2019). For instance, many studies demonstrated that plants associated with AMF have five times more affinity for NH_4^+ uptake from the soils (Pérez-Tienda et al., 2012). In addition, many mycorrhizal plants can facilitate nitrogen uptake from the rhizosphere soil through nitrate and ammonium transporters (Breuillin-Sessoms et al., 2015). Recently, few studies have also reported the increased content of ^{15}N in host plants grown on AMF inoculated organic patches of soil (Hodge and Fitter, 2010; Tian et al., 2010; Nath et al., 2018). When the hyphae are supplied with nitrate and ammonium ions, the nitrates are absorbed by active transport coupled with a protonated-symport system, whereas, NH_4^+ is taken up through an antiport mechanism with an H^+ efflux. If ammonium is the only source of N, its assimilation can lead to a deficient supply of carbon for the fungus because of its enhanced consumption in the roots (Basu et al., 2018).

Phytohormones

The fungal colonization develops in the host plant through a complex process that includes well-structured alterations at the morphological and genetic level, thus eventually leading to changes in series of signals (Morrison et al., 2015). Several studies have reported that AMF can produce phytohormones, e.g., auxins, cytokinins, and abscisic acid (ABA), which accelerated plant growth and development. Just as other plants, mycorrhizal fungi also follow the mevalonate pathway and use different precursors of ABA for their production (Nambara and Marion-Poll, 2005; Morrison et al., 2015). The role of ABA in the production and growth of mycelium has been documented in the literature (Ludwig-Müller, 2010; Spence and Bais, 2015; Xu et al., 2018). For instance, the exogenous application of ABA showed an insignificant increase in the growth of *Ceratocystis fimbriata*, while in *Magnaporthe oryzae*, ABA stimulated the production of appressoria and increased germination (Chanclud and Morel, 2016).

MECHANISMS UNDERLYING PLANT-AMF INTERACTIONS IN PHYTOREMEDIATION

Phytoremediation has been considered a more sustainable, cost-effective, and eco-friendly approach for the remediation of contaminated soils, due to its less expenditure and no unfavorable impact on soil fertility or structure (Jadia and Fulekar, 2009). However, phytoremediation cannot be performed alone by the plant itself, because plants and microorganisms in the rhizosphere always interact very closely so that ultimately leads to an enhanced activity associated with soil remediation (Compant et al., 2010). Use of hyperaccumulators associated with efficient endophytic or rhizosphere microbial communities has been proposed as a promising low-cost cleaning technique for the removal of metals from several contaminated sites (Karimi et al., 2011). In this context, AMF may be a good candidate because they reside inside the roots of a large number (approximately 80%) of terrestrial plants from bryophytes and tracheophytes (Smith and Read, 2008). AMF can form a mutual symbiotic association with most terrestrial plants establishing a direct physical link between plant roots and soils (Bothe et al., 2010).

AMF may promote plant metal extraction when metal concentrations are low in soils and also help plants to accumulate a major chunk of toxic metals in plant roots to prevent their transport to aerial parts when there is a high concentration of metals in soils. Singh et al. (2019) studied the impact of the inoculation of four species of AMF (e.g., *Rhizophagus fasciculatus*, *R. intraradices*, *Funneliformis mosseae*, and *Glomus aggregatum*) with *Zea mays* on the removal of Cr, Cd, Ni, and Pb from the tannery sludge. They discovered that all four AMF species enhanced metal accumulation in the roots but decreased shoot metal accumulation. The metal translocation factor was significantly lower as compared to the non-inoculated control plants. These discoveries are important evidence of the capability of AMF to enhance metal phytostabilization. Similarly, Yang et al. (2015a) evaluated the impact of two AMF *F. mosseae* and *R. intraradices* on plant growth-related parameters, Pb accumulation, photosynthesis, and antioxidant enzymatic activity in *Robinia pseudoacacia*. The increased biomass, photosynthetic pigment, gas exchange capacity, and various enzymatic activities in inoculated plants suggests that both AMF species were capable of protecting plants against cellular damage by eliminating ROS under Pb stress. The decreased Pb concentration in the leaves of AMF-inoculated *R. pseudoacica* indicates that these two AMF species have the potential to increase metal phytostabilization.

AMF root colonization helps in increasing the volume and surface area of available soil that in turn helps in better metal translocation from roots to shoots. Similarly, Zimmer et al. (2009) studied the impact of dual inoculation of ectomycorrhiza-associated bacteria (EMAB; *Sphingomonas* sp. and *Micrococcus luteus*) and ectomycorrhizal fungi (*Laccaria laccata* and *Hebeloma crustuliniforme*) on the growth and metal accumulation in *Salix viminalis* cultivated in metal contaminated soils. Total Zn and Cd accumulations in shoots were increased up to 53% post-inoculation with *H. crustuliniforme* in association with *M. luteus*, whereas up to 62% for *Sphingomonas* sp. They found that EMAB enhanced ectomycorrhiza formation, plant growth, and accumulation of Zn and Cd. The findings indicate that the bacterial community facilitates root colonization of plant growth-promoting ectomycorrhizal fungi, which may serve as a potential approach to increase the efficiency of phytoextraction.

Moreover, a field study conducted by Wu et al. (2011) assessed the effect of AMF on Zn and Pb accumulation in *C. zizanioides* grown in mine tailings. They found that the P and N concentrations in plant aerial parts were remarkably higher in mycorrhizal plants as compared to non-AMF treatments. The inoculation of AMF also resulted in a decrease in Zn and Pb concentrations in roots.

The majority of studies available in the literature on AMF-assisted phytoremediation were performed in pot experiments using artificial GCs-polluted soils. Aged-contaminated soils are more complex than spiked soils, as they frequently contain different nature and concentrations of pollutants and their availability is generally lower than that in spiked soils. However, some studies have directly been performed at the contaminated site. It is a well-known fact that the nature of spiked soil used for pot experiments is different from those of naturally contaminated sites. Knowing about the behavior of plant species associated with AMF and the capability of such plants to grow in GC soils is imperative to phytoremediation (Schneider et al., 2016). It can thus be inferred that field studies depict the situation more closely. Therefore, there is a need to perform more and more field-based studies.

For instance, in a field study, a total of 23 species belonging to the genus *Acaulospora, Scutellospora*, *Racocetra*, *Glomus*, *Gigaspora*, and *Paraglomus* were identified in As contaminated areas in Brazil. The most frequently occurring species in all areas were *Paraglomus occultum*, *Acaulospora morrowiae*, and *Glomus clarum*. The relatively high presence of these species demonstrates their tolerance to excess As. In spite of the fact that contamination owing to As decreased AMF species richness, the presence of host plants has the tendency to make up for the reduction (Schneider et al., 2013a). In another field study, 39 species of AMF belonging to 10 genera were identified in Pb contaminated sites in Brazil. The *Acaulospora* and *Glomus* genera had a high occurrence in the rhizosphere and bulk soil. The highest concentration of Pb was found in root and shoot. AMF diversity seems to be correlated with the heterogeneity of area; AMF structure community was related to Pb concentration in soils, and the diversity of plants was significantly related to the diversity of AMF in soils with high Pb concentration. A clearer understanding of AMF communities in the presence of Pb stress may throw some more light on metal-fungal interactions in contaminated sites (Schneider et al., 2016). In a different field study, a total of six species of AMF belonging to two genera *Glomus* and *Scutellospora* were studied. The richness of AMF species was more in the non-contaminated site as compared to sites with contamination of metals. Results are suggestive of the fact that continuously exposing the plant and AMF to GC may result in the tolerant species that may be used for the purpose of phytoremediation (Khade and Adholeya, 2009). Metal transport followed by its distribution is imperative. Metal translocation from below ground to aerial parts is contingent upon the involved metals and plant species (Sarwar et al., 2017). The mobility of different metals differs even inside a plant. For instance, the mobility of Zn and Cd is higher than Pb and Cu. During transportation *via* plant, metals are largely bound to the root cell wall, which leads to enhanced metal concentration in the plant roots. Chelation of metals with the ligands (e.g., thiols, amino acids, and organic acids) facilitates the metals to transport from roots to shoots (Zacchini et al., 2009). Because of the high cation exchange capacity of xylem cells, the movement of metal is significantly retarded when metals are not chelated by ligands. There is an involvement of organic acids for Cd translocation in *Brassica juncea* (Salt et al., 1995), while histidine is involved in long-distance translocation of Ni in hyperaccumulator *Alyssum lesbiacum* (Solanki and Dhankhar, 2011). Since a larger number of GCs may be transported by forming organic compounds-metal complexes (Maser et al., 2001), various types of organic ligands secreted by AMF may alter the existing forms of metal distribution by combining with different metals present in plants, thereby assisting metal translocation from subsurface to aerial parts and hence improving the phytoextraction efficiency (Sheng et al., 2008). According to Ma et al. (2013), the inoculation of metal resistant plant growth-promoting bacterium *P. myrsinacearum* RC6b may effectively mobilize metals such as Pb, Cd, and Zn in soils and notably increased Cd and Zn accumulation in the shoots of *Sedum plumbizincicola*. De Maria et al. (2011) also observed that after inoculating rhizobacteria *Agromyces* sp. and *Streptomyces* sp., and fungus *Cadophora finlandica* with *Salix caprea*, the shoot concentration of Cd and Zn increased, denoting increased translocation of metals from roots to shoots.

There are a number of mechanisms through which plant-AMF interacts during the process of phytoremediation; some of them have been discussed below.

AMF-Induced GC Detoxification

Accumulation of GCs in the plants is a critical problem in the environment, high mobility of GCs has made them an extended component of food chain that affects the health of humans. Vesicles present in mycorrhizal fungi are comparable to fungal vacuoles and they accumulate huge amount of GC in them (Dhalaria et al., 2020). Immobilization of GC occurs in the fungal hyphae residing in symbiotic association with plants that decrease their availability to plants by retaining the GCs in the cytoplasm or vacuole, cell wall by chelation, thereby reducing metal toxicity in the plants (Punamiya et al., 2010). Metal detoxification induced by AMF has been considered as the key mechanism to help plants to alleviate metal toxicity (**Table 1**). By using scanning electron microscope equipped with energy dispersive spectroscopy (SEM-EDS), extended X-ray absorption fine structure (EXAFS), linear combination fitting results of X-ray absorption near-edge spectroscopy (LC-XANES), it has been demonstrated that Cr may be immobilized by AMF *via* reduction of Cr (VI) to Cr (III), forming analogues of Cr (III)-phosphate, probably on the surface of fungi. Apart from this, it has also been unraveled that extra radical mycelium may take up Cr actively and transport it to mycorrhizal roots, but the majority of Cr is immobilized in fungal structures (Wu et al., 2015). Ultra-structural changes were observed in roots and leaves of *Leucaena leucocephala* through a scanning

TABLE 1 | GC detoxification induced by AMF.

Possible mechanisms	References
Immobilizing geogenic contaminants (GCs) by secreting chelating substances, such as, siderophores (ferrichrome and ferricrocin) into the soil.	Ernst et al., 1992; Manoj et al., 2020
Metal-binding to several biopolymers present in cell walls such as glomalin and chitin. Glomalins are amphiphilic peptides that act as a surfactant.	Gonzalez-Chavez et al., 2004; Rillig and Mummey, 2006
Superficial immobilization of GCs in the plasma membrane upon crossing the cell wall.	Ernst et al., 1992
Intracellular chelation by metallothionein, organic acids, and amino acids.	Clemens, 2001
Arresting metals inside the vacuoles.	Gonzalez-Guerrero et al., 2008; Dhalaria et al., 2020
An exclusive mechanism of AMF involves metal transport with the help of fungal coenocytic hyphe.	Gonzalez-Chavez et al., 2002; Gupta et al., 2019
Membrane transporters present in arbuscules of AMF may transport metals to interfacial matrix and their incorporation in the plant.	Ebbs and Kochian, 1998
There is also a possibility that fungi may store metals in some assigned structures (such as vesicles, hyphe, etc.).	Ferrol et al., 2009

electron microscope (SEM), transmission electron microscope (TEM), and light microscopy (LS). Results revealed that plant tissues were colonized by AMF and damage was observed in all treatments of As (Schneider et al., 2013b).

GCs may be immobilized in the fungal hyphae (Ouziad et al., 2004) that can fix GCs in the cell wall and store them in the vacuole or make a complex with other substances like glycoprotein-metal complex (Dhalaria et al., 2020) in the cytoplasm to decrease plants metal toxicity (Punamiya et al., 2010). AMF can enhance plant biomass by changing plant physiological and morphological properties (e.g., enhanced secondary metabolite levels, increased leaf area, increased seedling weight, etc.) under severely stressful conditions and uptake of important immovable nutrients (such as phosphorus, copper, and zinc; Miransari, 2017).

Increased plant biomass in rhizosphere soil is the primary cause of metal dilution in plant tissues (Audet, 2014). AMF may restrict Zn and Cd uptake in the cell wall of mental hyphae and cortical cells, thereby improving plant yield and health (Garg and Chandel, 2012). Metal detoxification mediated by *Rhizophagus phaseolina* in *Glycine max* was studied by Spagnoletti et al. (2016), where AMF boosted a defensive response by decreasing oxidative damage even in the presence of *M. phaseolina* and As.

Mycorrhizae may influence plant metal uptake from the rhizosphere and their translocation from the root zone to aerial parts (Li et al., 2015). The mycelium of several AMF has a high cation exchange capacity, and it helps in metal uptake (Takács and Vörös, 2003). For instance, Hammer et al. (2011) found an increase in the uptake of silicon in the hyphae and spores of *Rhizophagus irregularis* and its subsequent translocation to host roots. Cd toxicity and mobility can also be alleviated through AMF by enhancing the soil pH (Shen et al., 2006). AMF can restore Cd in the extraradical mycelium and bind Cd to glomalin (Janouškova and Pavlíková, 2010).

AMF colonization has been shown to reduce metal stress in a convincing way (Hall, 2002). For instance, AMF colonization considerably increased the glutamine synthetase activity, therefore enhancing Ni tolerance in *Berkheya coddii* (Sessitsch et al., 2013). To reduce metal toxicity, AMF resort to processes such as adsorption of GCs to the cell wall, immobilization of metallic compounds, chelation of GCs inside fungus, and precipitation of polyphosphate granules in soils (Meier et al., 2012; Wu et al., 2015). Janousková et al. (2006) reported that inoculation of *Glomus intraradices* with *Nicotiana tabacum* cultivated in Cd contaminated soil decreased Cd toxicity to the plants due to Cd immobilization in soil. A study conducted by Wu et al. (2015) investigated the mechanisms involved in Cr immobilization in *Daucus carota* inoculated with AMF and found that AMF can immobilize Cr *via* reduction of Cr(VI) to Cr(III) by forming Cr(III)–phosphate analogs.

Molecular Regulation of Genes

Molecular regulation of genes plays a crucial role in accumulating GCs and fungal cell detoxification, subsequently leading to the prevention of translocation of these GCs toward the host plant (Emamverdian et al., 2015). Efflux of GCs is a strategy used by AMF to protect plants from metal toxicity (Latef et al., 2016; Shi et al., 2019). Several transcriptional genes take part in the efflux of GCs and the involved genes get activated by metal exposure (Dhalaria et al., 2020). To provide plant tolerance against Cd and Cu, *GmarMT1* that is a cDNA-encoding metallothionein-like functional polypeptide has been discovered from germinated *Gigaspora margarita* spores (Lanfranco et al., 2002). Also, GC exposure upregulates *GmarMT1* expression in the symbiotic mycelium (Lanfranco et al., 2002). *GintABC1* identified as a putative ATP-binding cassette (ABC) transporter from extra radical mycelium of *Glomus intraradices* is believed to be involved in Cd and Cu mitigation (González-Guerrero et al., 2010). A number of genes are responsible for maintaining cellular homeostasis against GCs, such as *GintABC1*, *GmarMT1*, *RintZn*, and *GrosMT1* (Azcón et al., 2013). To maintain the redox potential and safeguard the fungus from oxidative stress, *GmarMT1* codes for metallothioneins have been found in *G. margarita* BEG 34 (González-Guerrero et al., 2007). *GintABC1* assists in detoxifying Zn and Cu (González-Guerrero et al., 2007; **Table 2**). *RintZnT1* isolated from *Rhizophagus intraradices*, helps in vacuolar sequestration of Zn (Gonzalez-Guerrero et al., 2005). *GintGRX1*, the first characterized glomeromycotan glutaredoxin, is a multifunctional enzyme that expresses in response to oxidative stress (Benabdellah et al., 2009).

AMF resorts to several molecular mechanisms to protect them from GC stress. One of mechanisms is the upregulation of several transcriptional factors that activate glutathione-S-transferase and Zn transporter in intra- and extra- mycelia of AMF *Glomus intraradices* against metal stress (Hildebrandt

TABLE 2 | The function of some receptor genes.

Receptor gene with their signaling component	Function	References
BEG34/GintZnT1	Enhanced transcription levels of putative Zn transporter gene and protection against Zn stress.	Gonzalez-Guerrero et al., 2005
Sy167	Alleviation of oxidative stress due to GCs.	Hildebrandt et al., 2007
GintABC1	Cd and Cu detoxification in the extra radical mycelium of *Glomus intraradices*.	Gonzalez-Guerrero et al., 2005
MtCbf1 and *MtCbf2*	Root tissue reprogramming during the establishment of AM symbiosis.	Hogekamp et al., 2011
Kinase SymRK	Involved in transduction of signals to the cytoplasm after perception of signals from Nod and Myc factors.	Gherbi et al., 2008; Genre and Russo, 2016
NUP 85/NUP133	Involved in transporting macromolecules through nuclear envelope.	Parniske, 2008
CYCLOPS	Serves as phosphorylation target of calcium/calmodulin-dependent protein kinase (CCaMK) gene and is supposed to be the diverging point of common symbiosis (SYM) pathway.	Kistner et al., 2005
CASTOR/POLLUX	Specific channel of cations important for perinuclear Ca spiking right after reception of Myc or Nod factors.	Kistner et al., 2005
CCaMK	Calmodulin and Ca dependent protein kinase, which acts as a sensor of Ca and is supposed to be involved in phosphorylation of *CYCLOPS*.	Kistner et al., 2005

et al., 2007). GC stress also leads to expression of numerous genes. These genes encoding proteins are involved in detoxification/tolerance against GCs (Rivera-Becerril et al., 2005).

Based on molecular understanding, scientists have reported an upregulation in metallothionein gene expression of *Gigaspora margarita* BEG34 in the symbiotic mycelia due to Cu (Lanfranco et al., 2002) and also an enhanced level of transcription of a putative transporter gene for Zn (*GintZnT1*) that belongs to cation diffusion facilitator family. These genes have been found in the G. intraradices mycelium under short and long-term Zn exposure indicating that this enzyme protects plants against Zn stress (Gonzalez-Guerrero et al., 2005). The role of some AMF in phytoremediation of some GCs (Bundschuh et al., 2017) has been discussed in **Table 3**.

Metal-binding proteins called metallothioneins are generated in a diverse range of organisms when they are exposed to toxic metals (e.g., Cd, Zn, and Cu). Cu predominantly induces the production of metallothionein in non-AMF (Kumar et al., 2005). Cu-induced stress distinctly upregulates the metallothionein gene *BI451899* in extraradical mycelium of *G. intraradices*. However, a certain concentration of Zn can also upregulate the metallothionein gene, but such a response is not observed due to Cd. This upregulation establishes and supports the primary function of fungal metallothioneins in detoxifying Cu (Lanfranco et al., 2002).

Metal Mobilization

Strong binding of metals to soil particles or precipitation results in insolubilization of the significant fraction of metals in soils ultimately causing their unavailability for plant uptake. Metal solubilization and mobility have been considered as critical factors that affect phytoextraction efficiency (Ma et al., 2013). In this regard, microbes that can mobilize metals may be used to amend the habitat of rhizosphere in soils affecting metal element speciation as well as mobility inside soil by way of biogeochemical cycling processes of GCs that primarily include protonation, chelation, and acidification (Ma et al., 2011; Rajkumar et al., 2012; Sessitsch et al., 2013).

Protonation

AMF may also acidify their environment through exporting protons to replace GC cations at the site of sorption (Rajkumar et al., 2012). Extensive studies have been performed to characterize them using attenuated total reflection-Fourier transforms infrared (ATR-FTIR) spectroscopy and thereafter to understand the interaction between fungal cells, protons, and metal ions. Results suggest that the carboxylate moieties present on the bacterial surface play a vital role in the extracellular biosorption of Ni^{2+}, which establishes a comparatively weaker bond with the metal ion.

Chelation

Natural organic chelators are metal-binding compounds that comprise siderophores, organic acid anions, metallophores, and biosurfactants (Sessitsch et al., 2013). Both fungi and plants can release these compounds that scavenge metal ions from sorption sites (Gadd, 2004) and ROS (Leitenmaier and Küpper, 2013). Metal chelation through metal-binding peptides such as metallothioneins and phytochelatins (PC) may eliminate the harmful effect of free metallic ions, thereby facilitating metal uptake and their sequestration, followed by compartmentation, loading in xylem tissues, and finally their transport (Cai and Ma, 2002). Phytochelatins are the GC binding peptides that are produced by tripeptide glutathione and/or by an enzyme-catalyzed reaction through PC synthase (Solanki and Dhankhar, 2011). Metallothioneins may also be found in AMF and genes that encode numerous enzymes for PC synthesis may be activated in the root of mycorrhiza. These enzymes assist in enhancing photosynthesis in mycorrhizal plants subjected to stress caused by metals (Rivera-Becerril et al., 2005).

Acidification

Soil pH is one of the most important factors that affect metal content and its bioavailability. For several metals (e.g., Cu and Zn), a rise in soil pH caused a fall in their mobility (Richards et al., 2000). Generally, soil pH is affected by the action of both microorganisms and plants. Rhizosphere gets acidified due to H^+ ions excreted by roots that may displace GC cations adsorbed on soil particles. Root exudates may decrease the

TABLE 3 | Role of AMF in phytoremediation of geogenic contaminated soils.

Plant	Types of mycorrhizae	GCs	Remarks	References
Helianthus annus	*Claroideoglomus claroideum* (BEG210)	Ni	AMF *Claroideoglomus claroideum* (BEG210) enhanced Ni accumulation in *H. annus* by 38%.	Ma et al., 2019
Solanum nigrum	*Rhizophagus irregularis*	Cd	*Rhizophagus irregularis* increased Cd accumulation in roots.	Wang et al., 2020
Zea mays	*Glomus aggregatum*	Pb, Cd, and Zn	AMF along with moderate amount of phosphorous may decrease GC uptake and increase plant growth.	Nafady and Elgharably, 2018
Medicago sativa	*Rhizophagus irregularis*	Cd and Ni	AMF inoculation enhanced the uptake of both metals.	Mnasri et al., 2017
Taraxacum platypecidum	*Rhizophagus irregularis*	Cr	Immobilized Cr in roots and prevents Cr phytotoxicity.	Wu et al., 2016
Zea mays	*Funneliformis mosseae* and *Diversispora spurcum*	Cd, Zn, Pb, and As	The transfer of GC was restricted by both fungi.	Zhan et al., 2018
Solanum nigrum	*Glomus intraradices*	Cd	Inoculation with AMF resulted in decreased Cd uptake in roots and shoots, thereby facilitating metal phytostabilization.	Khan et al., 2017
Triticum aestivum	*Rhizoglomus intraradices*	As	AMF inoculation assisted the host plant to ameliorate As-induced phosphorous deficiency and also strengthened thiol metabolism and antioxidant defence mechanism.	Sharma et al., 2017
Cynodon dactylon	*Funneliformis mosseae*	Sb	AMF inoculation inhibited Sb (V) to Sb (III) reduction, thereby decreasing Sb toxicity.	Wei et al., 2016
Oryza sativa	*Rhizophagus intraradices*	Cd	AMF decreased Cd uptake in *O. sativa* by altering the expression of Cd transporters.	Chen et al., 2019
Zea mays	*Glomus intraradices*	Hg	AMF increased Hg uptake in roots.	Debeljak et al., 2018
Lactuca sativa	*Funneliformis mosseae* and *Rhizophagus intraradices*	Zn	AMF inoculation at increased Zn concentrations AMF has the capability of decreasing Zn uptake.	Konieczny and Kowalska, 2017
Cynodon dactylon	*Diversispora spurcum*	Pb, Zn	AMF inoculation increased the uptake of Pb and Zn.	Zhan et al., 2019
Sorghum bicolor	*Claroideoglomus etunicat*	Mo	AMF inoculated plants accumulated up to four times higher Mo than non-mycorrhizal plants.	Shi et al., 2020

pH of the rhizosphere (Sheoran et al., 2011), causing increased metal mobility and bioavailability in soil solution (Kim et al., 2010).

Metal Immobilization

Phytostabilization is GC immobilization in the plant root system by precipitation, reduction, and absorption without its accumulation in the shoot (Radziemska et al., 2017). There is an extensive root system for immobilizing metals in hyperaccumulators (Mendez and Maier, 2008). In addition to some common mechanisms of tolerance, increase biosynthesis of the cell wall, metal inactivation in the rhizosphere and its accumulation in roots are very specific to phytostabilizers (Janeeshma and Puthur, 2020). An association with AMF increases the properties of metal stabilization of plants (Zhang et al., 2019). For instance, the association of *Trifolium pratense* with mycorrhizae enhanced Zn retention in the roots, thereby preventing its translocation in the aerial plant parts (Chen et al., 2003).

The glomalins released by AMF enhance toxic metal immobilization. Metallothionein protein, released by some AMF, is also known to reduce the toxicity caused by GCs. Besides, the synthesis of a 90 kD heat shock protein and glutathione-S-transferase as a response to GC stress suggest that these proteins are involved in immobilizing GCs in the rhizosphere of *Lycopersicon esculentum* plant (Bano and Ashfaq, 2013). Glomalins are known to sequester several metals such as Zn, Pb, Fe, Cd, Cr, and Cu and decrease their bioavailability (Gil-Cardeza et al., 2014). Glomalins may extract Pb, Cd, and Cu from polluted soil.

Several GCs get immobilized because of the binding capacity of fungal hyphae to metals. As a result of this binding capacity, there is a decreased translocation of GCs to plant tissues (Wasserman et al., 1987). A slight increase in the mycorrhizosphere pH may also cause immobilization of some GCs (e.g., Zn) due to mycorrhizal association (Bano and Ashfaq, 2013). Inoculation of *Glomus* species resulted in reduced mobility of metals in *Zea mays* (Kaldorf et al., 1999). Other studies demonstrated a notable absorption of Zn in the mycelium of AMF by using different glomus species in association with *Lolium perenne* or *Trifolium* sp. (Joner et al., 2000).

Metal immobilized in fungal hyphae that are symbiotically associated with the plants decreases their availability to host plants by holding the metals in the cytoplasm, vacuole, or cell wall, thereby reducing metal toxicity in plants (Punamiya et al., 2010). They also immobilize the GCs in the root cortical region by binding with them and prevent the translocation of metals to shoot, thus preventing leaf tissue damage (Schubler, 2001). Some AMF may decrease plant metal uptake or its

translocation factor by reducing metal bioavailability in soils through several processes such as alkalinization, precipitation, and complexation (Ma et al., 2016).

Alkalinization

A few AMF exhibit the capability to adsorb metals by substratum alkalinization activity, hence affecting the stability of metals in soils (Büdel et al., 2004). The effect of alkalinization induced by AMF *via* release of OH^-, may result in active uptake of nitrate by microbes and reduction in metal bioavailability in the rhizosphere by secreting glomalin (Giasson et al., 2008). AMF may act as a sink of metals to reduce the available and mobile metal cations in soils, resulting in the creation of a more conducive environment for plants growing in metal contaminated soils (Gohre and Paszkowski, 2006). Inoculation of *G. mosseae* and *G. caledonium* with *Lolium perenne* and *Sedum alfredi* notably reduced soil DTPA-extractable Cd by 21%–38% through the alkalinization process, hence facilitating in stabilization and extraction of Cd *in-situ* from Cd infected soils (Hu et al., 2013).

Precipitation

Some plant-associated microorganisms can promote enzyme-catalyzed precipitation of toxic metals [e.g., chromium (Cr) and selenium] and radionuclides (e.g., technetium and uranium) *via* microbial reduction process, which is promising for phytoremediation of metal-polluted soils (Payne and DiChristina, 2006). Some studies suggest that fungi can protect the host plant from the inhibitory effects of an excess concentration of Cr^{6+} by reducing toxic and mobile Cr^{6+} to immobile and non-toxic Cr^{3+} in soils. Besides, some insoluble forms of minerals, metals, and radionuclides may also be immobilized either indirectly through bacterial oxidation of Fe (Zhou et al., 2013) or directly *via* enzymatic actions (such as microbial reduction process; Pagnanelli et al., 2010).

Complexation

Extracellular polymeric substances (EPS) excreted by AMF are of immense importance, making a protective hindrance against the adverse effects of metal biosorption (Hou et al., 2013). The mechanisms involved in metal biosorption onto EPS include the complexation with negatively charged functional groups, precipitation, metal ion exchange, and adsorption (Zhang et al., 2006). In this regard, AMF may produce insoluble metal-absorbing glycoprotein named glomalin that decreases the metal mobility or sequesters them, which may be taken into account for metal stabilization in soils (Vodnik et al., 2008). In an *in-situ* field experiment, the glomalin-related soil protein was used to estimate AMF derived from glomalin in soils in sequestrating Pb and Cd (Wu et al., 2014).

CONCLUSION

In this review, the interactions between plant and mycorrhizal fungi in metal phytoremediation were unraveled through (1) an in-depth establishment of mutualistic symbiosis; (2) gaining insight into the role of AMF in phytoremediation; and (3) understanding the mechanisms including alleviation of metal toxicity by AMF, plant-AMF signaling and perception, metal bioaccumulation of plant-AMF association, metal mobilization and immobilization, metal transport, and distribution, which could add to the existing application knowledge of phytoremediation technologies. Associations with mycorrhiza increase the available surface area for absorption beyond the zone of root hair that in turn increases the uptake of water and minerals. It results in the high production of biomass that is imperative for successful phytoremediation. This review combined all the existing information available on AMF in a coherent way for better understanding. The primary focus of upcoming research should be on (1) identification of new genes as well as gene products that are crucial in plant-mycorrhizae fungal interactions and (2) optimizing applied theory, including mobilization, immobilization, and perfecting the gene control mechanisms. The application of mycorrhizal techniques has fewer disadvantages and more advantages. Various factors such as redox potential, pH, inorganic and organic ligands (e.g., root exudates, fulvic acid, and humic acid) can regulate metal sorption or desorption and its bioavailability. The impact of the dynamics of these factors on phytostabilization, phytotransformation, or phytoextraction in association with AMF are still unclear and require more attention and detailed studies for additional application of phytoremediation processes. The review also advocates more and more field-based studies for further exploring the potential of AMF. Furthermore, applying it to practice, to enhance the utility and efficiency of mycorrhizal remediation of GCs are some practical problems that needs to be solved on an urgent basis.

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

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REVIEW
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Influences of Biochar on Bioremediation/Phytoremediation Potential of Metal-Contaminated Soils

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A number of anthropogenic and weathering activities accumulate heavy metals in soils, causing adverse effects on soil characteristics, microbial activity (diversity), agricultural practices, and underground aquifers. Controlling soil heavy metal pollution is difficult due to its persistence in soils, resulting in the deposition and transmission into the food web *via* agricultural food products, ultimately affecting human health. This review critically explores the potential for remediation of metal-contaminated soils using a biochar-based responsible approach. Plant-based biochar is an auspicious bio-based residue substance that can be used for metal-polluted soil remediation and soil improvement as a sustainable approach. Plants with rapid growth and increased biomass can meet the requirements for phytoremediation in large quantities. Recent research indicates significant progress in understanding the mechanisms of metal accumulation and contaminant movement in plants used for phytoremediation of metal-contaminated soil. Excessive contamination reduces plant biomass and growth, which has substantial hyperaccumulating possibilities and is detrimental to the phytoremediation process. Biochar derived from various plant sources can promote the growth and phytoremediation competence of native or wild plants grown in metal-polluted soil. Carbon-enriched biochar encourages native microbial growth by neutralizing pH and providing nutritional support. Thus, this review critically discusses the influence of plant and agricultural waste-based biochar on plant phytoremediation potential in metal-contaminated soils.

Keywords: heavy metals, biochar, plants, bioremediation, phytoremediation

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INTRODUCTION

Land degradation and soil contamination are a persistent threat to humans' and the environment's wellbeing (Azam, 2016). Heavy metal and metalloid intensification in soil have increased rapidly in terms of natural phenomena and anthropogenic activities, including mining, agricultural activities, and industrial and municipal discharge, which all pose severe threats to environmental protection and public health (Sharma et al., 2022). Because they are non-biodegradable, they might remain in the soil, enter into the food chain *via* agricultural crops, and even accumulate in humans

via biomagnification/bioaccumulation (Gogoi et al., 2021). Heavy metals are a class of elements distinguished by their high atomic weight and mass, with a specific density of greater than 5 g/cm^3 (Buha et al., 2014). There are 21 non-metals, 16 light metals, and 53 heavy metals among the 90 naturally occurring elements (Gholizadeh and Hu, 2021). Such elements are divided into two categories: those that could be needed in trace amounts (Cu, Zn, Ni, Fe, V, Mn, Co, and Mo) by certain organisms and others such as Pb, As, Cd, and Hg, which are entirely considered as dangerous (Naja and Volesky, 2017). Heavy metals in their natural state are not available for root uptake or are not accessible to living beings. Anthropogenic sources, such as battery manufacturing, mineral extraction (mining), explosives, pesticides, herbicides, chemical fertilizers, and effluent irrigation, cause an excessive increase in such elements, contributing to their deposition and distribution (Dutta and Sharma, 2019). When these activities exceed acceptable levels, they endanger all living beings and have disastrous effects on their concentration. These elements are tolerant in various ways depending on the life form with which they are confronted (Villa-Achupallas et al., 2018). Metal pollution has posed a significant risk to human health as well as the environment due to its toxic nature. Hence, remediation of metal pollution from soils is critical (Rathour et al., 2022). Numerous remediation strategies depending on their mobilization or immobilization mechanisms have indeed been established to address these issues (Akcil et al., 2015; Wang et al., 2021). However, they are typically very expensive, and planned remediation is often delayed due to the absence of sufficient funds (Yrjälä and Lopez-Echartea, 2021). Unique nature-based substances are emerging that ought to be cost-effective in remedial work but necessitate further development because they require useful insights into the structure–function relationships (Byrne et al., 2018). Biochar, a carbon-rich component, is thought to play an important role in the bioavailability of heavy metal-polluted soil, resulting in biotransformation and bioremediation (Yu et al., 2019). However, biochar is frequently produced from various feedstocks using different pyrolysis processes; hence, the surface characteristics may vary significantly (Yu et al., 2019). Plants and biochar blending can be used to enhance the *in situ* or on-site bioremediation. Nevertheless, this is critical to address a few essential lines of study to ensure the safe and long-term use of biochar. Biochar is being developed for use in the environmental cleanup of both inorganic and organic contaminants, and their integration with phytoremediation is an excellent option (Rodriguez-Franco and Page-Dumroese, 2021). Since then, biochar-blended phytoremediation has grown in popularity as a groundbreaking technology for enhancing the phytoremediation potency in metal-polluted soils (Muthusaravanan et al., 2020). Various biochar properties demonstrate their influence on heavy metal transport, mobilization, and precipitation, improving soil structure, the release of nutrients, and microbial diversity, thus supporting plant growth (Yuan et al., 2019). This review presents the environmental influence and applications of biochar-blended phytoremediation of heavy metal-polluted soils and their interaction with plants during remediation.

AGRICULTURAL WASTES FOR BIOCHAR FABRICATION

The primary ingredients for biochar production are agricultural, forestry, household, and livestock waste (**Figure 1**), which are all abundant across the world. Agricultural waste has previously been used in a limited number of applications, including as a renewable source and animal feed (Spiertz and Ewert, 2009). Another report stated that the nationwide possibilities for producing biochar from agricultural biomass have been calculated and predicted to be around 3.1 million tons of biochar from around 10.7 million tons of biomass (Awasthi et al., 2021). The highest biomass is derived primarily from rice husk, which has a yield of 6.8 million t y^{-1} and can produce biochar up to 1.77 million t y^{-1}, accounting for approximately 56.48% of the total nationwide biochar fabrication potential (Susilawati et al., 2020).

BIOCHAR PROPERTIES

Physicochemical Properties

Biochar is being used as a soil conditioner and is acquired at low temperature pyrolysis—ranging from 400 to 700°C—of numerous biomasses including manure (cow dung), agriculture waste (wastes of maize, sugarcane, weeds, and so on), and biosolids in the absence of oxygen. It is thus differentiated from charcoal (Khiari et al., 2019). The physical and chemical properties of biochar obtained from wood, agricultural residues, poultry manure, or sludge at various pyrolysis temperatures are summarized in **Table 1**. Although the physicochemical characteristics of biochar diversified substantially due to the fabrication from a wide range of feedstocks using varying pyrolysis processes, biochar is usually basic in nature with such a large specific surface area, huge porosity, changeable charges, and different functional groups, as mentioned in **Table 1**. Such properties can also have an impact on pH, conductivity (CEC), and surface adsorption capacities. Biochar particle size is determined by the standard particle size of the feedstock; nevertheless, it is usually much smaller due to shrinking and attrition during the pyrolysis process. Due to the improved tensile strength of raw materials at higher pyrolysis temperatures, it may yield smaller-sized biochar particles (Albalasmeh et al., 2020). The functional groups on the surface of biochar, porous structure, and ionic charges can aid in the physical adsorption (Zhang et al., 2020), co-precipitation (Chen et al., 2022), complexation, mobilization/immobilization (Hu et al., 2020), and detoxification (Alrashidi et al., 2020) of metal pollutants and support the hyperaccumulator's phytoremediation potential.

Nutritional Property

Biochar consists of a variety of nutrients, including K, Mg, K, Ca, and P, which are derived from the pyrolysis raw material. During pyrolysis, the dissolved organic material is also formed (de Figueiredo et al., 2021). Hence, the biochar amalgamation could provide plants and microorganisms with bioavailable nutrients. The quantity and the type of the bioavailable nutrient content

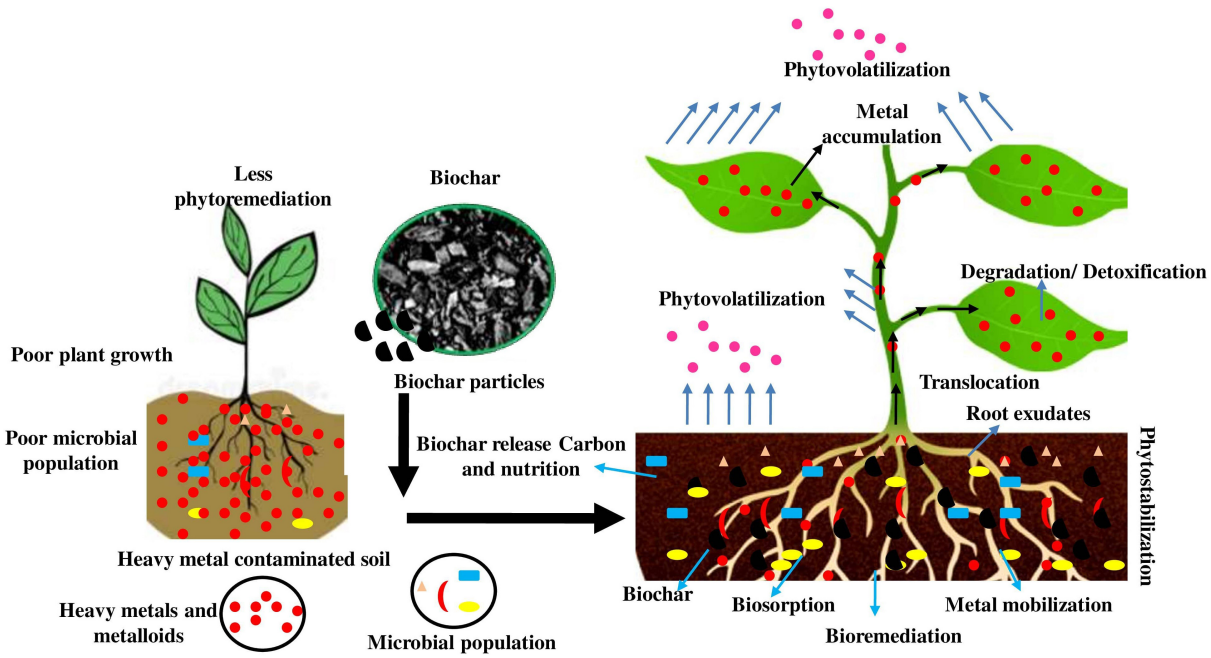

FIGURE 1 | Biochar fabrication from various agricultural wastes.

in biochar, on the other hand, are highly dependent on the raw material (feedstock) and pyrolysis conditions (Yang et al., 2019).

The elements such as C and N in biochar differed significantly while obtained from pine trees, poultry manure, and peanut husk at 400 and 500°C, respectively (El-Bassi et al., 2021). Furthermore, transferable phosphate, potassium, calcium, and magnesium were significantly higher in biochar produced at 500°C than in biochar produced at 400°C (Ferjani et al., 2019). The deviation was primarily associated with the high pyrolysis temperature, which enhanced raw material mineralization besides reduced CEC. From this standpoint, obtaining nutrient-enriched biochar from a nutrient-enriched raw material under appropriate pyrolysis conditions is critical (Ma et al., 2015). In fact, plant-derived biochar appear to have a reduced nutrient composition than biochar derived from manure (Embrandiri et al., 2012).

Constancy Property

When biochar is applied to the soil, it appears as a separate particulate matter that differs from many other kinds of solid organic materials, which are either encapsulated in soil pore spaces or adsorbed on the mineral surfaces and obscured in aggregate particles (Kumar et al., 2018). Biochar with much more aromatic black carbons on the exterior seems to be more consistent in soil than other forms of organic carbons, thus improving carbon storage potential in soil properties (Lian and Xing, 2017). A previous study reported that the biochar mineralization rates are very low, with carbon half-lives up to 100 years (Williams et al., 2019). According to another investigation, perfect biochar particles were found in soils in wet tropical climates including the Amazon for millennia (Agegnehu et al., 2017).

BIOCHAR – METAL(LOID) INTERACTION

Biogeochemical interactions in the ecosystem have a significant impact on the destiny, transfer, and conversion or modifications of metals and metalloids (Breda et al., 2018). Because ionic metals and metalloids can occur in both anionic and cationic aspects, their behavior will be influenced by interactions with anionic and cationic charges of the biochar surface (Fijałkowska et al., 2021). When combined with topsoil, biochar with negative charges can strongly adsorb positive components (e.g., Cd^{2+} and Pb^{2+}), whereas biochar with cationic charges can maintain anionic metal(loid)s (e.g., arsenite and arsenate) (Gupta et al., 2021). Adsorption mechanism, surface (co)precipitation, and surface complexation with active functional moieties are the major mechanisms for the immobilization of cationic metals (including Pb^{2+}) and metalloids through biochar (Gupta et al., 2021). Thus, the biochar-stimulated improvements in soils, including increased soil pH, can reduce the bioavailability of cationic metals and metalloids even further. Since the physical and chemical attributes of biochar depend on the raw material type and pyrolysis circumstances (e.g., temperature and frequency of temperature rise), it is essential to recognize appropriate raw materials for biochar fabrication that have the efficiency to remediate various metals and metalloids in specific soils (Akhil et al., 2021). Anionic metalloids, including Cr, Se, and As, are frequently found as dominant species in soils with alkaline pH compared to cationic metalloids that are poorly adsorbed by negatively charged soil (Gupta et al., 2021).

The redox potential of metals and metalloids can influence their mobility in soils. For instance, the reduced redox potential of As (As^{3+} and As^{5+}) has much higher permeability in soils

TABLE 1 | Physical and chemical properties of biochar obtained from various plant residues and manure.

Biochar materials	pH	Temperature: °C (pyrolysis)	CEC (mmol kg^{-1})	Carbon (%)	Carbon/ Nitrogen ratio	Total phosphate (mg kg^{-1})	Elements (%)						Surface area (m^2 g^{-1})	Volatiles (%)	Ash (%)	References
							Ca	Fe	Mg	N	P	K				
Rice husk	8.9	300–400	37.3	23.4	–	–	0.21	0.26	0.18	0.73	0.48	0.54	–	–	44.35	Susilawati et al., 2020
Oak wood	3.7–6.4	60–600	75.7–182	47.1–87.5	444–489	5–29							450–642	27.5–88.6	0.3–1.3	Lehmann et al., 2011; Sun et al., 2018
Palm bunches	9.39	350–450	9.9	42.33	–		0.4	0.5	0.67	0.99	0.49	8.65	–	–	27.09	Susilawati et al., 2020
Pine needles	6.4–10.6	300–700	–	84.2–93.7	22–26	–	–	–	–	–	–	–	4.1–391	6.2–38.6	7.2–18.7	Sun et al., 2018
Bamboo	9.30	350–450	9.30	50	–	–	0.16	0.16	0.13	1	0.45	3.18	–	–	11.26	Sun et al., 2018; Susilawati et al., 2020
Corn stover	6.7–9.4	60–600	252–459	42.6–70.6	51–83	526–2,114	–	–	–	–	–	–	293–527	23.5–85.2	8.8–16.7	Susilawati et al., 2020
Coconut shell	9.61	250–350	9.61	29.69	–	–	0.29	0.29	4.43	1.28	0.52	2.96	–	–	48.96	Sun et al., 2018
Chicken litters	8.2–10.3	60–700	58.7–363	7.9–38	10–25	493–16,685	–	–	–	–	–	–	1–94	18.3–60.5	16.9–72.5	Susilawati et al., 2020
Sludge	4.9–12	400–700	–	20–20.4	8.4–17	528–740	–	–	–	–	–	–	–	15.8–25.7	63.3–72.5	Sun et al., 2018
Palm cake	8.30	350–500	8.30	23.73	–	–	0.09	0.04	0.30	0.87	0.44	0.72	–	–	59.32	Susilawati et al., 2020
Branch legume	9.4	–	7.05	18.11	–	–	–	–	–	0.58	0.1	1.11	–	–	–	Sun et al., 2018

than the increased redox potential of Cr (Cr^{6+} and Cr^{3+}) (Wang et al., 2020). Furthermore, the oxidation state of soils can influence the redox potential of metals and metalloids. For instance, it has been revealed that biochar converts Cr^{6+} to the less mobile Cr^{3+} through consistently transferring electrons, which may have been connected with oxygen-containing active functional groups on that biochar surface (Dong et al., 2017). Furthermore, microbial metabolism utilizing biochar-derived organic carbon material can reduce Cr^{6+}. The poor Cr solubility led to the reduction process, and Cr immobilization in soil has been enhanced. The adsorption and desorption mechanisms of metalloids and metals in soils are also significantly influenced by pH and organic matter because the adsorption of positively charged metals in biochar is high in acidic soil. In acidic soils (pH 3.5–6.0), Cr occurs predominantly in the positively charged forms $Cr_3(OH)_4{}^{5+}$ and $Cr(OH)^{2+}$ (Wang et al., 2020). The biochar amendment to soil could perhaps alter the dissolved organic content (DOC) and pH, thus resulting in the mobility of metals and metalloids.

According to some research findings, biochar-amended soil could improve the mobility of metals and metalloids such as Sb, As, and Cu (Beesley et al., 2013; Sun et al., 2018). For example, increased pH in biochar-amended soils led to increased As mobility (Beesley et al., 2013). Electrostatic interaction between anionic As and Sb elements and negatively charged biochar substrates may enhance effective desorption of As and Sb by increasing mobilization. In the case of Cu, mobility is strongly correlated with the DOC content in biochar. Cu can be immobilized by the adsorption process in the biochar (prepared at 600°C) surface with an elevated DOC content (Sun et al., 2018).

BIO-/PHYTOREMEDIATION WITH BIOCHAR

Biochar aids in the bioremediation of organic and inorganic pollutants. The primary mechanism is an upsurge of microbial diversity that degrades hydrocarbons (petroleum) in biochar-amended polluted soils (Karppinen et al., 2017). Heavy metals and metalloids cannot be deteriorated or completely eradicated from the ecosystem, but they can be transformed from one form to another, from higher concentration to lower concentration. Heavy metals and metalloids can also accumulate in organisms (Verma et al., 2021). Hence, most frequently, two strategies are used for the heavy metal and metalloid bioremediation process (Li et al., 2019). Absorption and accumulation of metals and metalloids in timber plants and crops with bioenergy potential in metal-polluted farmlands, and their deduction by harvesting the biomass containing/accumulated with metals and metalloids, and the transformation of toxic metals and metalloids into lesser toxic products (complex form), which can be adsorbed by native microorganisms and further reduce their toxicity and migration (Sun et al., 2018).

Cd^{2+} denotes cationic metal ion (A) physiological adsorption of cationic metals and metalloids of water from soil pores; (B) biochar co-precipitation with chloride, carbonates, silicate, and phosphate with metals; (C) complex formation with biochar surface functional groups; and (D) gradual nutritional discharge of DOC, N, Ca, P, and K for growth of plants and microbes in the root region (**Figure 2**). The mechanisms (A), (B), and (C) can minimize the bioavailable metal content in pore water, lowering phytotoxicity even further.

The negatively charged outer layer of biochar and its alkaline character can adsorb and sustain toxic metals through various mechanisms. Biochar, *via* gradually discharging nutrients and maintaining healthy soil structures and properties, also generates much more favorable soil conditions for the growth of beneficial microbes and plants (Das et al., 2021).

The existence of biochar significantly increased the lowering precipitation of Cr^{6+} to Cr^{3+} in the contaminated soils due to remarkably improved microbial activities encouraged by releasing carbon and other nutrients from biochar (Choppala et al., 2012). Furthermore, a decrease in the concentration may aid in the immobilization of metals and metalloids, including Cr^{6+} and U^{6+}, but no evidence to date has demonstrated the role of biochar in bioremediation/phytoremediation. In addition to the effectively improved bioremediation, the existence of biochar does provide an indirect mechanism for metal and metalloid bioremediation (Sun et al., 2018; Gong et al., 2019).

Calcite precipitation caused by microbes can firmly adsorb and co-precipitate metals and metalloids on the surfaces. The metal ions along with an ionic radius similar to that of Ca^{2+}, including Cu^{2+}, Cd^{2+}, and Pb^{2+}, may be integrated into calcite crystal particles through alternative reactions during calcite precipitation (Achal et al., 2011). Biochar aided this strategy by making microbe-friendly soil conditions and potentially increasing bioremediation efficiency (Arif et al., 2020). *Bambusa vulgaris* biochar with an O_2-releasing bead has been recently demonstrated as a promising O_2-releasing substance used in soils and groundwater bioremediation (Wu et al., 2015). This kind of biochar does have the potential to enhance the oxidation level (from As^{3+} to a less mobile form) of metals and metalloids.

Influence of Biochar in Bio-/Phytoremediation

A few research studies have investigated on biochar-augmented phytostabilization of metals and metalloids (e.g., Zn, As, Ni, Cd, Sb, Cu, and Cr) in contaminated soils (Uchimiya et al., 2012). **Figure 3** represents the possible influence of biochar on bioremediation/phytoremediation of metal-contaminated soil. Arsenic (As) is well-recognized to react differently from some other metals and metalloids since the mobility of As can be diminished in acidic soils, owing to the enhanced sorption process on ferric oxide under an acidic environment. Hartley et al. (2009) demonstrated that biochar can also be applied for phytostabilization with *Miscanthus* species. Moreover, the analysis revealed that adding biochar derived from hardwood to soil samples did not improve As transport to *Miscanthus* plants, whereas alkaline biochar can mobilize As in metal-contaminated soils (Hartley et al., 2009). Cu and Pb were relatively straightforward to be stabilized in biochar-administered soils, whereas Cd and Ni differed widely depending on the nature

FIGURE 2 | Possible benefits of biochar amendment in bio- and phytoremediation.

of biochar used (Uchimiya et al., 2012; Sun et al., 2018). The stabilization mechanism is often probably due to increases in soil pH. The detailed research has shown that soil alterations (addition of lime) can be merged with phytoremediators to considerably reduce the bioavailability of metals and metalloids. Furthermore, biochar is also more effective at governing the accessibility of toxic compounds, as well as enhancing plant biomass fabrication and restoration performance (Břendová et al., 2015; Maddalwar et al., 2021).

Plant yield increases with biochar supplementation (Ambika et al., 2022) are connected to water and nutrient retainment, enhanced biological activities, and neutralized soil pH. Hence, biochar has the efficiency to be used as an amendment to reduce metal bioaccumulation in plants. Moreover, alterations in soil pH in the rhizosphere can feasibly influence the metal and metalloid mobilization efficiency of biochar in soils, while rhizosphere acidification should be avoided (Houben and Sonnet, 2015). Biochar is thought to interact with soils and balance their properties for an extended period of time. Thus, the redox mechanisms may cause biochar to change, a process called aging (Gul et al., 2015). The immobilization of heavy metals and metalloids in biochar has been associated with the lability of metals (e.g., Pb^{2+} is more mobile than Cd^{2+}). A wide range of functional groups, including hydroxyl, carboxylic, and phenolic groups could be established during the aging process, and biochar aging had no effect on the immobilization of positively charged metals and metalloids in soils containing aged biochar (Heitkötter and Marschner, 2015; Fan et al., 2018).

Biochar-Assisted Phytoremediation

Phytoremediation is a multidisciplinary field with the goal of mobilizing and/or immobilizing pollutants from different environmental conditions (Shah and Daverey, 2020). Phytoremediation encompasses phytostabilization, rhizoremediation, phytoextraction, phytodegradation, and phytovolatilization in general (Shah and Daverey, 2020). In comparison to certain other remediation practices for heavy metals and metalloids, including chemical immobilization, digging, and dumping, phytoremediation is gaining popularity due to its efficiency and lower cost (Wu et al., 2015). Other advantages, including erosion control and pollutant leaching prevention, are critical for future soil management and development. **Table 2** summarizes some biochar-assisted phytoremediation plants for metal- and metalloid-polluted soils (Sun et al., 2018).

Biochar-Assisted Phytoextraction

The primary method for remediating soil contamination is the phytoextraction process, which is typically associated with the ability of hyperaccumulators and energy plants to bioaccumulate metals and metalloids (Rezania et al., 2016). Numerous plant species were also used to extract various metals (e.g., Cr, Cd, Pb, As, Co, Cu, Zn, and Ni) from soils (Cameselle and Pena, 2016). Plant species preferably being used for phytoextraction should not just accumulate significant concentrations of the target metals and metalloids; nevertheless, they also have an increased biomass

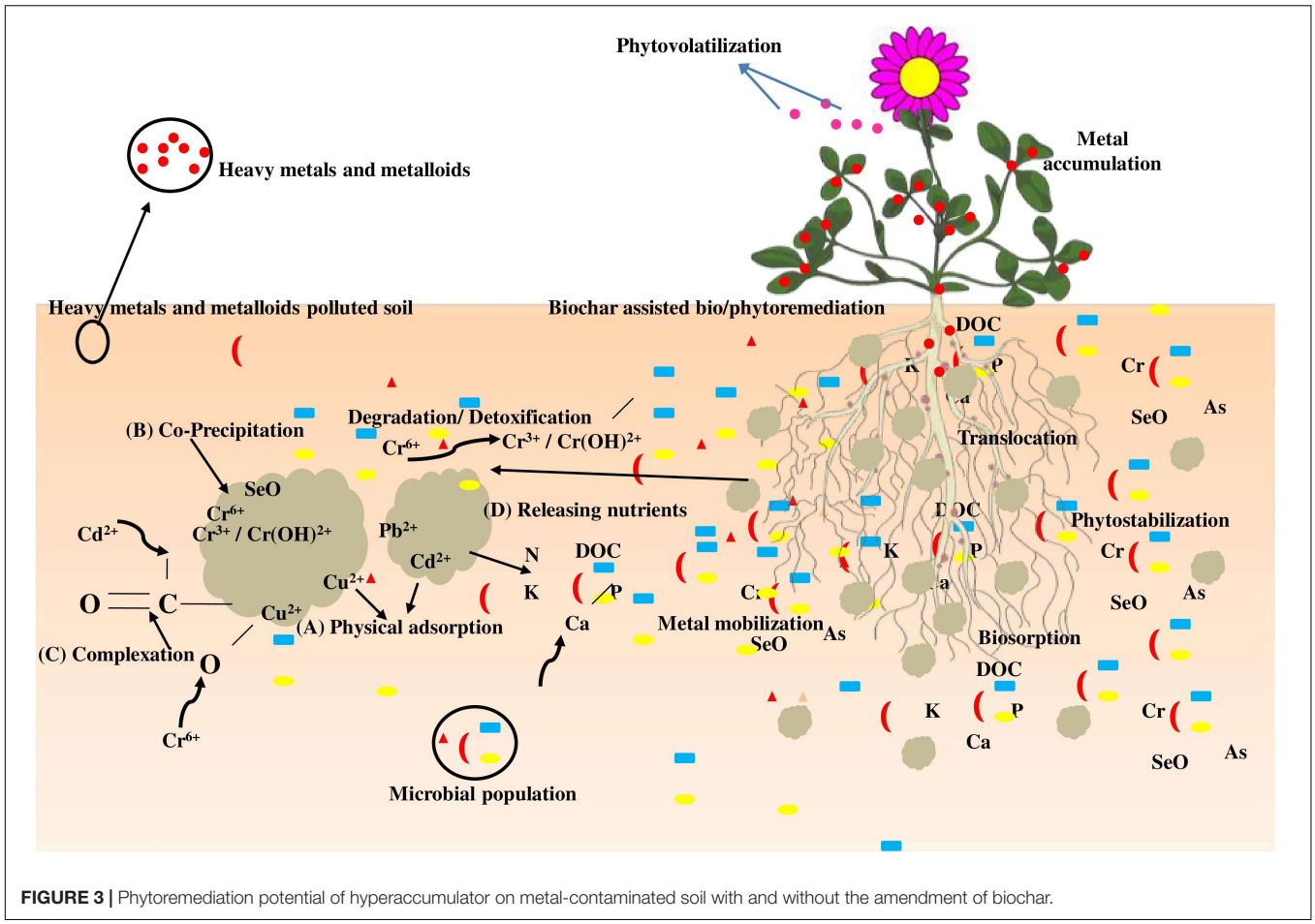

FIGURE 3 | Phytoremediation potential of hyperaccumulator on metal-contaminated soil with and without the amendment of biochar.

TABLE 2 | Biochar-assisted phytoremediation for metal- and metalloid-polluted soils.

Name of plant	Phytoremediation on metal- and metalloid-contaminated soil	Biochar and dose	Effects on phytoremediation	References
Anthyllis vulneraria, Noccaea rotundifolium and Poa alpina	Ni, Cd, Ti, Zn, Cr, Pb, Cu, and Fe	Pruning residues and manure: 1.5–3%	Reduced water-extractable Zn, Cu, Cd, and Cr. Increased pH	Fellet et al., 2014
Lolium perenne L. var. Cadix	Pb and Cu	Oka, Ash, and Birch: 20% v/v	Reduced pore water-mediated Pb and Cu doses in shoots	Sun et al., 2018
Solanum lycopersicum	As, Cd, Zn, and Cu	Hardwood	Raised pore water with Cu and As. Immobilize Zn and Cd owing to elevated DOC and pH	Beesley and Marmiroli, 2011
Oryza sativa	As, Zn, Cd, Ni, Cr, Co, Pb, and Cu	Sewage sludge: 5 and 10%	Reduced pore water Pb, As, Ni, Cr, and Co owing to elevated soil pH. Mobilize Cd, Cu, and Zn	Khan et al., 2013
Brassica juncea	Cd, Pb, and Cu	Poultry manure and green waste	Increased (353%) plant shoot dry biomass. Decreased Pb, Cd, and Cu accumulation in plants	Park et al., 2011
Brassica napus	Cd, Zn, and Pb	Miscanthus: 5 and 10%	Reduced metals bioavailability in shoot biomass	Bandara et al., 2017
Miscanthus × *giganteus*	As	Hardwoods: 20%	Improved pore water with As	Sun et al., 2018
Lycopersicon esculentum	Cr, Mn, and Ni	Wood: 2.5–5%	Reduce exchangeable Cr, Ni, and Mn. Enhanced plant growth	Bandara et al., 2017

yield, tolerate the toxic effects of metals and metalloids, should be adaptable to soil and climatic conditions, are resistant to insects and pathogens, and will also be suitable for cultivation (Ranieri et al., 2020). The effectiveness of phytoextraction is determined by two factors: yield and metal and metalloid concentrations (Cameselle and Pena, 2016). Thus, the uptake of metals and metalloids, which is the outcome of the two factors, can sometimes be positive or negative (Coumar et al., 2016).

Based on this, we identify that neither research findings fulfill all of the aforementioned criteria. Nevertheless, one study found that, while biochar-amended metal-polluted soil enhanced the willow plant biomass, the concentrations of Cd and Zn in willow were constant. Nonetheless, phytoextraction is improved (Břendová et al., 2015). In practice, phytoextraction is frequently used in farmland soils to reduce hazardous metal and metalloid concentrations below soil quality standards, thereby improving soil environmental quality and ensuring food security (Sun et al., 2018).

Phytoextraction of heavy metal-polluted soils, including mine sludge, could take centuries. Hence, the pollutant limits of the target agricultural fields should be kept to a minimum for phytoextraction (Mani and Kumar, 2014). However, a hyperaccumulator has the potential to acquire elevated metal and metalloid concentrations, but its slow growth rate frequently limits its application (Ranieri et al., 2020). Energy and economical plants, including sunflower and rapeseed, are often used to retrieve Cd from farmland soils. Recently, biochar-assisted phytoextraction has been emerging rapidly and used in practice. Accordingly, biochar-assisted *Brassica napus* was used to retrieve Cd metal-polluted agricultural soil (Houben and Sonnet, 2015). Various plant species and biochar are often used in multi-metal-contaminated soils. Nevertheless, limited research has focused on the combined effect of biochar on phytoextraction of heavy metal-contaminated soils (Houben and Sonnet, 2015; Sun et al., 2018). Correspondingly, *Amaranthus tricolor* was subjected to biochar-assisted phytoextraction to treat Cd-polluted agricultural soils (Lu et al., 2015). So far, many research findings showed that adding biochar to plants considerably reduces heavy metal and metalloid bioavailability. Nevertheless, some plants necessitate elevated doses of bioavailable metals and metalloids to accumulate them. The advantages of biochar include improved contaminated soil physicochemical properties, increased microbial population and activities, and increased ability to enhance agriculture production (Lu et al., 2015; Ye et al., 2016). Hence, using biochar to remediate metal- and metalloid-polluted soils not only immobilizes them but also increases microbial population, lowering the environmental threat of heavy metals and metalloids in soils even further (Frankel et al., 2016).

Biochar-Assisted Phytostabilization

Phytostabilization is another phytoremediation method that is widely used for the stabilization of metals and metalloids in mine sludges (Barbosa and Fernando, 2018). The revegetation approach reduces dispersion and erosion because plant roots stop leaching, which contributes significantly to the immobilization of metals and metalloids (Sarkar and Sadhukhan, 2022). Precipitation, complexation, metal electron reduction, and root adsorption are the potential phytostabilization mechanisms (Ma et al., 2016). Phytostabilization, as opposed to phytoextraction, is more concerned with metal and metalloid sequestration in the rhizosphere than in other plant tissues (Yan et al., 2020). Metals and metalloids are typically stabilized by applying soil amendments (such as biochar and compost) and microbes *in situ*, which improve metal immobilization and plant growth (**Figure 3**; Kumpiene et al., 2019).

APPLICATION OF BIOCHAR AIDED PHYTOREMEDIATION OF MINE SITES

Mining (e.g., coal, gold, copper, magnesite, bauxite, and iron mining) activities can degrade soil quality and structure and disturb biological systems and vegetation, thus leading to widespread soil pollution (Gabarrón et al., 2019). Heavy metal toxicity and elevated acidity of soil contaminated by mining activity reduce the revegetation possibilities of metal-polluted soils. Remediation of such metal-polluted soils can be accomplished through phytoremediation, a long-term and cost-effective rehabilitation approach that promotes revegetation to minimize the chances of contaminant transfer and land reclamation. However, these are difficult to accomplish in the absence of appropriate soil amendments (e.g., biochar) (Fellet et al., 2014). The biochar amalgamation with heavy metal-polluted soil may improve pH fertility and water-holding capacity, minimize the mobility of pollutants, and encourage revegetation (Kelly et al., 2014). Phytoremediation of mine sludge soil with biochar obtained from residues of orchard prune and organic manure at four distinct concentration levels (0, 1, 5, and 10%) demonstrated substantial benefits of biochar in revegetating plant species in metal- and metalloid-contaminated soils. Also, the bioavailability of Zn, Cd, and Pb reduced proportionally as the biochar content increased (Fellet et al., 2014).

CONCLUSION

One of the most important remedial technologies for heavy metal- and metalloid-polluted soils is biochar-blended bioremediation/phytoremediation. Biochar-stimulated phytoremediation has a significant potential for immobilizing cationic heavy metals and metalloids in mine sludge soils and other metal-contaminated soils, especially those under high acidic conditions. Biochar can significantly decrease the bioavailability and leachability of cationic metals and metalloids in soils; enhance soil structure, physicochemical properties, fertility, and revegetation; and foster soil microbial populations. Nevertheless, since biochar appears to become less efficient in stabilizing highly harmful cationic metals and metalloids, which provide their mobility in soils, the implementation of biochar-aided phytoremediation is competent in attempting to resolve multi-metal-polluted soils. Furthermore, it is essential to select suitable biochar in order to develop a successful strategy for immobilizing anionic metals and metalloids initially through an *in vitro* approach. Moreover, more extensive research is required to assess the efficacy of biochar-amended bioremediation/phytoremediation of heavy metal-polluted soils. Scientific investigations should concentrate on the following important themes: (A) demonstrating the interrelations between raw materials used in pyrolysis, biochar physicochemical properties, and soil bioremediation/phytoremediation; (B) assessing the biochar consistency and its impacts on the transfer of metals and metalloids in mine sludge and metal-polluted soils in a field-level study; (C) knowing the mechanisms of biochar-influenced bioremediation/phytoremediation,

particularly the interactions between biochar, microbial populations, plant roots, and soil particles.

AUTHOR CONTRIBUTIONS

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

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Plant growth-promoting bacteria in metal-contaminated soil: Current perspectives on remediation mechanisms

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Heavy metal contamination in soils endangers humans and the biosphere by reducing agricultural yield and negatively impacting ecosystem health. In recent decades, this issue has been addressed and partially remedied through the use of "green technology," which employs metal-tolerant plants to clean up polluted soils. Furthermore, the global climate change enhances the negative effects of climatic stressors (particularly drought, salinity, and extreme temperatures), thus reducing the growth and metal accumulation capacity of remediating plants. Plant growth-promoting bacteria (PGPB) have been widely introduced into plants to improve agricultural productivity or the efficiency of phytoremediation of metal-contaminated soils *via* various mechanisms, including nitrogen fixation, phosphate solubilization, phytohormone production, and biological control. The use of metal-tolerant plants, as well as PGPB inoculants, should hasten the process of moving this technology from the laboratory to the field. Hence, it is critical to understand how PGPB ameliorate environmental stress and metal toxicity while also inducing plant tolerance, as well as the mechanisms involved in such actions. This review attempts to compile the scientific evidence on this topic, with a special emphasis on the mechanism of PGPB involved in the metal bioremediation process [plant growth promotion and metal detoxification/(im)mobilization/bioaccumulation/transformation/translocation] and deciphering combined stress (metal and climatic stresses) tolerance.

KEYWORDS

plant growth-promoting bacteria, metal bioavailability, metal detoxification, climatic stresses, bioremediation

Introduction

Soil contaminated with heavy metals has become a serious worldwide problem due to geologic and anthropogenic activities, such as mining, fossil fuel combustion, application of agrochemicals, and so on. As heavy metals are non-biodegradable and extremely persistent in the environment, they can easily accumulate in different foods. Metal contamination of various foods, such as crops, meat, fish, milk, and eggs, threatens food safety. Metals contaminate agricultural soils, irrigation water, plants, and animals, resulting in their incorporation into the food chain and posing a significant threat to human health and ecosystems (Abdel-Rahman, 2022). The major sources of heavy metals and their harmful effects are summarized in Table 1. There are currently numerous methods for controlling heavy metal pollution. The advantages and disadvantages of different techniques are summarized in Table 2. Traditional remediation technologies for contaminated soil, such as cleaning, heat treatment, electrochemistry, and amendment application, often have complex processes that easily destroy soil structure and fertility. They are ineffective for treating both low concentration and large-scale heavy metal contamination in soils.

TABLE 1 The sources and harmful effects of metals.

Metal	Sources	Harmful effects	Reference
Cd	Electroplate, mine, smelt, fuel, battery, and chemical wastewater discharge	Carcinogenic, bone injury, kidney stone, failure, coughing, emphysema, and headache	Sarwar et al., 2017
Pb	Paint, coating, smelt, hardware storage battery, puffed food, hair dye, and fire coal	Renal failure, cardiovascular disease, mental decline, high blood pressure, and anorexia	Guo et al., 2020
Cu	Metal processing, machinery manufacturing, iron and steel production, and copper-zinc mining	Brain and kidney damage, severe anemia, abdominal pain, and diarrhea	Vardhan et al., 2019
Zn	Zinc mining, smelt, and machinery manufacturing	Carcinogenic, ataxia, depression, and gastrointestinal irritation	Li et al., 2019
Hg	Instrument and meter plant, salt electrolysis, precious metal smelting, cosmetics, lighting lamp, and dental material	Depression, fatigue, hair loss, visual and hearing impairment, ulcer, and kidney damage	Kim et al., 2016
As	Mine, smelt, chemical pharmacy, insecticide, chemical fertilizer, and arsenate drug	Anorexia, gastrointestinal disorders, corneal sclerosis, skin darkening cardiovascular, and respiratory disorder	Balali-Mood et al., 2021
Cr	Steel industry, tanneries, sludge, and solid waste	Bronchopneumonia, chronic bronchitis, diarrhea, emphysema, liver diseases, and renal failure	Sarwar et al., 2017
Ni	Kitchen appliances, surgical instruments, steel alloys, and automobile batteries	Dermatitis, hepatotoxic, lungs, dry cough, and shortness of breath	Rajendran et al., 2021

TABLE 2 Advantages and disadvantages of the available remediation techniques for metal-contaminated soils.

Method	Remediation technique	Advantages	Disadvantages	Reference
Physical remediation	Soil washing	Simple technology	High cost, installing solutions, collection wells, or underground drains may be difficult	Rajendran et al., 2021
	Surface covering	Easy to install, low cost, and high security	Limited to a small area, the soil loses its natural environmental function	Liu et al., 2018
	Soil replacement	Fast to implement and high efficiency	High cost, limited to seriously polluted small-scale soil	Rajendran et al., 2021
	Encapsulation	High security and fast install	High cost, limited to small and shallow contamination areas	Li et al., 2019
Chemical remediation	Thermal remediation	Simple process and thorough treatment	Large energy consumption and secondary pollution	Gong et al., 2018
	Vitrification technique	High efficiency	High cost, limited to small soil area, treated land and soil losing environmental functions	Dhaliwal et al., 2020
	Chemical fixation	Fast to implement, high efficiency	High cost and limited application site	Nejad et al., 2018
	Electrokinetic remediation	Economical and efficient	Limited to low permeability soils	Singh and Prasad, 2015
Bioremediation	Phytoremediation	Low cost, eco-friendly, almost no side effects	Slow process, low efficiency, and long cycle	Liu et al., 2020
	Microbial bioremediation	Remove the contaminants, soils retain their properties and could be replaced on the reclaimed site	Microbes are easily affected by soil's physical and chemical properties	Grover et al., 2021

Phytoremediation is a potential solution for remediating metal-polluted soils since it is a cost-effective plant-based approach (Ma et al., 2016a). During global climate change scenarios, plants are more severely and frequently subjected to episodes of climatic stress, such as high temperature, drought, and salinity, limiting their growth and performance. Furthermore, the direct (e.g., competition of ions) or indirect (e.g., alteration of soil physicochemical-biological properties) impact of climate change on metal bioavailability in soils may impede plant adaptation, making them more susceptible to stress and thus limiting the widespread application of phytoremediation (Rajkumar et al., 2013). Plant beneficial microorganisms (PBM), particularly plant growth-promoting bacteria (PGPB) create symbiotic relationships with plants, alleviating the toxicity of heavy metals, promoting multimodal tolerance of plants to metals and climatic stresses, and affecting the bioavailability of metals in soils (Ma et al., 2016b). For instance, PGPB can alleviate metal toxicity and alter metal bioavailability in soils through metal biosorption, bioaccumulation, redox reaction, mobilization, precipitation, and transformation (Ma et al., 2016a). They can also provide plants with multiplex tolerance to a variety of climatic stresses (such as drought and high salinity) by producing 1-aminocyclopropane-1-carboxylate deaminase (ACCD), siderophore, and phytohormone, and dissolving insoluble mineral nutrients (such as nitrogen, phosphorus, and potassium). These PGPB strains could also protect plants from phytopathogens by producing antibiotics and inducing induced systemic resistance (Grover et al., 2021). Understanding the interaction between plants and PBM has lots of potential for accelerating metal phytoremediation under various environmental stressors (e.g., salinity, drought, and extreme temperature). There have been very few investigations on plant-microbe associations for bioremediation of metal-polluted soils under climatic stresses.

The current review has discussed the underlying mechanisms of PGPB involved in the heavy metal bioremediation in response to metal alone or in combination with climatic conditions (e.g., drought, salt, and heat) have been discussed. The main objective is to provide an overview of recent advances in developing PGPB-assisted phytoremediation under various climatic stresses, including the strategies to improve remediating plants tolerance and biomass, metal detoxification, bioaccumulation, transformation, and translocation activities. This review also emphasizes the commercial application of PGPB to improve phytoremediation efficiency.

Metal-resistant plant growth-promoting bacteria

Plant growth-promoting bacteria are a type of bacteria that may colonize rhizosphere soils and plant tissues and stimulate plant growth through various plant growth-promoting (PGP) activities under different conditions (Hashem et al., 2019). PGPB can improve plant abiotic and biotic stress tolerance by directly modulating phytohormone levels and facilitating resource acquisition, and/or indirectly by protecting plants against phytopathogens through the production of antibiotics and siderophores (Backer et al., 2018). They may act as free-living or rhizosphere bacteria (that form specific symbiotic relationships with roots), endophytic bacteria (that can colonize plant interior tissues), *Rhizobia* spp., and cyanobacteria (Glick et al., 2012). Figure 1 shows the type of plant growth-promoting bacteria. They all use the same PGP methods (direct and indirect); however, there are distinctions among these bacteria. Endophytic bacteria are more valuable in real-world applications than rhizobacteria because of their stable living environment and closer contact with plants for nutrient supply (Afzal et al., 2019). It has been reported that a group of metal-resistant PGPB, such as *Pseudomonas*, *Arthrobacter*, *Agrobacterium*, *Bacillus*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Klebsiella*, *Alcaligenes*, *Serratia*, *Rhizobium,* and *Enterobacter* species have great potential to promote the growth of various plants in the metal-contaminated environments (Enebe and Babalola, 2018). These metal-resistant PGPB were found to enhance plant metal tolerance by improving detoxification rates of plants, enzymes secreted by plant roots, and soil pH modification (Guo et al., 2020). Moreover, certain metal-resistant PGPB can also alter metal mobility and bioavailability, and consequently plant usage rate by releasing chelating agents, acidification, and redox changes (Verma and Kuila, 2019). Therefore, these metal-resistant PGPB strains can be used as a suitable candidate for metal phytoremediation to minimize the adverse impact of metals and enhance metal accumulation capacity of plants. A number of metal-resistant PGPB have been reported to improve plant bioaccumulation/phytoextraction capacity through the secretion of siderophores and organic acids, which improve metal bioavailability by reducing soil pH (Manoj et al., 2020). In contrast, some metal-resistant PGPB can release polymeric substances (such as glomalin and polysaccharides) that speed up metal phytostabilization by limiting their mobility (Ma et al., 2016a).

However, microbes have a strong dependence on the environment, and changes in environmental conditions can modulate the diversity, abundance, and functioning of bacteria (Afzal et al., 2019). Sánchez-Marañón et al. (2017) studied bacterial communities in eight soils selected along a soil-forming gradient and found that distinct bacterial distributions were positively connected to organic carbon, water-stable aggregates, porosity, water, and acidity. Furthermore, endophytic microbiota can be influenced by the age, genotype, nutritional status, and geographical location of the host plants (Ahmed et al., 2020). Carvalhais et al. (2013) confirmed that the transcriptional changes of *B. amyloliquefaciens* caused by the nutrient-deficient corn exudates were significantly correlated with the concentrations of amino acids, aspartic acid, valine, and glutamic acid in the root exudates. Furthermore, differences in microbial communities can result from host plant preferences for stress conditions. Wu et al. (2020a) reported the growth of *B. subtilis* at high salinity reduces

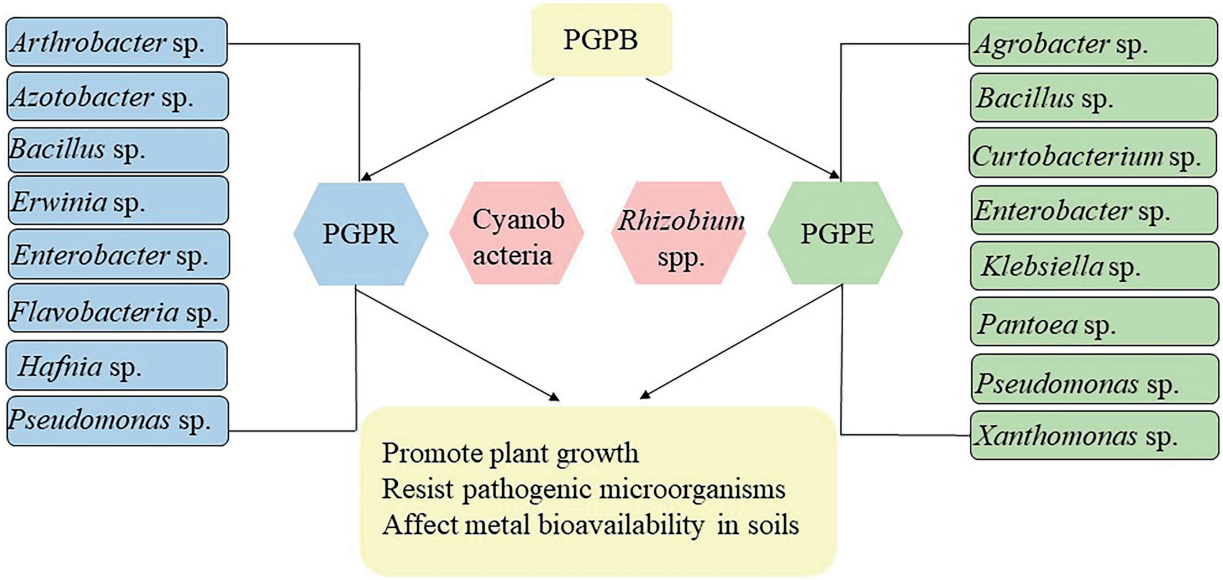

FIGURE 1
Type of plant growth-promoting bacteria (Glick et al., 2012).

the cell expansion pressure due to the passage of water through an osmotic gradient. Similarly, Xu et al. (2018) also demonstrated that drought delayed the development of early root microbiota in *Sorghum bicolor*. Nevertheless, inoculation of climatic stress-resistant PGPB has great potential to increase plant resistance/tolerance to such environmental stresses. Bruno et al. (2020) pointed out that *Bacillus cereus* improved the growth of *S. bicolor* and its phytoremediation potential in Cr-contaminated soil at elevated atmospheric temperature by producing siderophores and indole-3-acetic acid (IAA).

Mechanisms of PGPB in remediation of metal-contaminated soils under various climatic stresses

The microbiome is essential for plant growth and function, particularly PGPB play a key role in plant growth regulation *via* phytohormone production, plant nutrient acquisition, and abiotic and biotic stress alleviation, which enable plants to tolerate high concentrations of heavy metals and thus better survive in challenging conditions. Figure 2 depicts the mechanism of the synergistic effect of PGPB on the phytoremediation of metal-contaminated soils. PGPB could promote plant growth directly and/or indirectly under metal stress. The direct plant growth promotion by PGPB involves producing phytohormones (e.g., auxin, cytokinin, gibberellin, abscisic acid, and ethylene), or facilitating plant nutrient uptake (e.g., nitrogen, phosphorus, potassium, etc.; Figure 2A). Since the interaction between the antibacterial activity of PGPB and nutrient competition inhibits the growth of pathogenic bacteria, the production of antibacterial compounds and the coexistence of pathogens enhance ISR and indirectly promote plant growth (Figure 2B). One or more of these mechanisms can be used by specific PGPB to enhance plant resistance to environmental stresses. PGPB also can adsorb metals through coordinate, chelate, and ion exchange. Under climatic stresses, PGPB can also effectively change metal bioavailability through mobilization, stabilization, and transformation, thereby improving bioremediation efficiency and reducing the climatic stress effect by regulating the antioxidant enzyme activity and ion balance in plants (Figure 2C).

Resistance mechanism of PGPB in alleviating stress in plants

The biomass of remediating plants and soil metal bioavailability are key factors influencing phytoremediation efficiency. Nitrogen, phosphorus, potassium, and other minerals are essential nutrients for plant growth. Although there are many phosphorus and potassium elements present in soils, most of them are insoluble and not bioavailable for plants. The existence of metals can aggravate the loss of nutrients in soils, making them unable to be effectively absorbed and utilized by plants (Ashraf et al., 2017; Ahemad, 2019). Metals can also have a significant impact on plant growth and development. Many studies have shown that when plants are grown under metal stress conditions, the membrane system of plants is damaged, and then the structure and function of organelles are affected, and various physiological and biochemical processes (such as chlorophyll content,

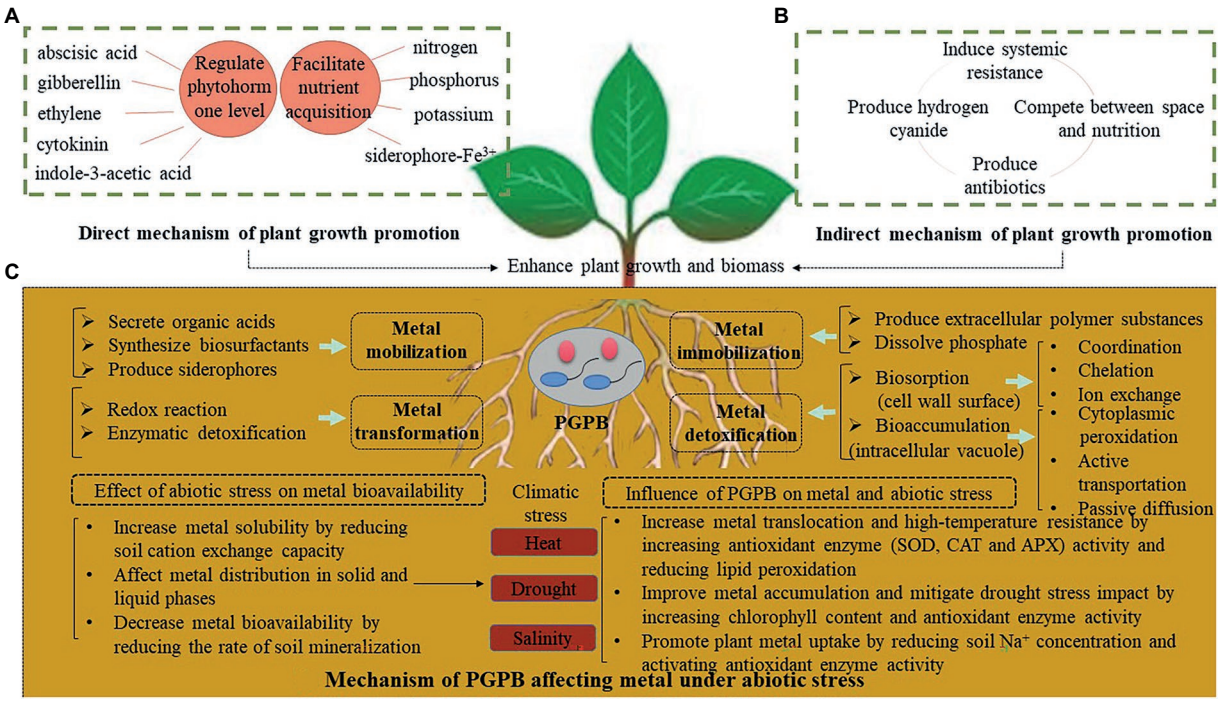

FIGURE 2
Mechanism of the synergistic effects of PGPB on the bioremediation of metal-contaminated soils. **(A)** Direct mechanism of plant growth promotion; **(B)** Indirect mechanism of plant growth promotion; **(C)** Mechanism of PGPB affecting metal under abiotic stress.

photosynthesis rate, biomass reduction, and so on) in their tissues are impaired (Zhu et al., 2020). In metal-contaminated soil, PGPB can improve plant tolerance to such stresses (metal and other climatic stresses) and stimulate plant growth by maintaining nutrient status and adjusting phytohormonal balance through the production of plant growth regulators. Several studies have indicated that PGPB contributes significantly to phytohormone production, which can not only regulate plant growth, regular development, and physiological processes and but also control biological and non-biological stress responses (Afzal et al., 2019; Hewage et al., 2020). Chen et al. (2017) reported that under the stress of Zn and Cd, *Pseudomonas fluorescens* can promote the growth and physiological indicators (above ground chlorophyll and enzyme activity) of *Sedum alfredii* by producing IAA, and improve plant Cd absorption by regulating the expression and transport genes of Cd. Furthermore, some IAA-producing PGPB could increase plant uptake of nutrients and water and reduce the stress effects of salt and drought on plants by changing the roots system architecture (Etesami and Maheshwari, 2018). Pan et al. (2019) demonstrated the potential of abscisic acid (ABA)-producing *B. subtilis* to minimize Cd accumulation in *Arabidopsis thaliana*. A number of bacteria with gibberellin acid production capacity alleviate metal toxicity by reducing Cd uptake and lipid peroxidation, altering hormonal balance, and regulating activities of proteases, catalase, and peroxidase (Etesami, 2018). Moreover, PGPB containing ACCD can help plants to cleave the synthesis ethylene precursor 1-aminocyclopropane-1-carboxylate by metabolizing it into α-butanone acid and ammonia, thereby alleviating the ethylene level in plants and improving their climatic stress tolerance (Vejan et al., 2016).

Microorganisms can also improve plant growth directly *via* nitrogen fixation, phosphorus dissolution, and potassium dissolution (Ashraf et al., 2017). *Stenotrophomonas rhizophila* and *B. amyloliquefaciens* were able to fix nitrogen, thus providing abundant nitrogen to *Brassica napus* (Liu et al., 2021). Besides, certain PGPB like *Klebsiella variicola* can convert insoluble phosphate to soluble forms through the secretion of enzymes (phosphonates and C–P lyases) and organic acids (citric acid oxalic, fumaric, and malic; Manoj et al., 2020), thereby improving the phosphorus availability in the rhizosphere under metal stress. Potassium-solubilizing bacteria can form biofilms on the surface of rhizosphere minerals by producing capsular polysaccharides, hydroxyl anions, iron carriers, and extracellular enzymes, as well as dissolve K-containing minerals in soils and effectively release K by synthesizing organic and inorganic acids (Etesami and Maheshwari, 2018). Many recent investigations have revealed that a number of PGPB, namely *Pseudomonas*, *Bacillus*, *Klebsiella*, and *Pantoea*, can release K from insoluble minerals such as mica and illite through a variety of mechanisms (including acidolysis, chelation, exchange reactions, and complexolysis), including the production of organic acids (Bakhshandeh et al., 2017). Some microorganisms can absorb iron from the siderophore-Fe complex through chelation degradation and release of iron, direct uptake of siderophore-Fe complex and ligand exchange (Ma et al., 2016b). Agarwal et al. (2020) found that *Staphylococcus warneri* GL1, *B. velezensis* GL3, GL5, and GMC2, isolated from *Gnetum gnemon*,

are able to secrete various siderophores with high affinity for Fe^{3+}, effectively inhibiting the growth of *Ralstonia solanacearum* in the rhizosphere.

Over the past 2 decades, there has been a better understanding of antibiotics as the basis of the biological control mechanism of PGPB and a variety of antibiotics have been identified such as amphibious steroids, 2,4-diacetylphloroglucinol, hydrogen cyanide (HCN), oomycin A, phenazine, pyoluteorin, pyrrolidin tensin, and troponin (Compant et al., 2005). *Pseudomonas fluorescens* can inhibit the root rot of *Nicotiana tabacum* caused by *Thielaviopsis basicola* by synthesizing pyocyanin and 2,4-diacetyl fluorescein (Morales-Cedeño et al., 2021). Competition is one of the important mechanisms of PGPB's resistance, including nutrition competition and locus competition. Through high-density colonization in plant rhizosphere or tissues, PGPB competes with indigenous microorganisms in the same micro-environment for oxygen, nutrition, and space (Compant et al., 2005). Induced systemic resistance (ISR) is the term being used for microbe-mediated induce plant resistance to infection by pathogenic fungi, bacteria, viruses, nematodes, and pests (Manoj et al., 2020). ISR is a central mechanism for *Pseudomonas*, *Trichoderma*, and *Bacillus* to protect plant against various pathogens (Saeed et al., 2021). In addition, lipopolysaccharide, flagellum and siderophores produced by PGPB can also cause ISR in plants (Ahmed et al., 2020). Inoculation with hydrogen cyanide-producing *Brevibacterium casei* MH8a significantly increased the biomass and accumulation of Cd, Zn, and Cu in *Sinapis alba* (Plociniczak et al., 2016). This is probably due to the potential of bacterial HCN to enhance plant growth and metal mobilization (Manoj et al., 2020). Recently, the release of stress-related volatile compounds was also found to increase plant biomass, yield, and survival under water stress (Etesami and Glick, 2020).

Mechanism of action of PGPB on heavy metals

Climatic stresses have a significant impact on metal bioavailability. For instance, extreme temperatures can disrupt the nutrient and metal pathway by dissolving organic matter, decomposing microbial cells and destroying soil aggregates, and altering metal bioavailability, absorption, and distribution in plant tissues (Rajkumar et al., 2013). Li et al. (2011) found that higher temperature increased Cd accumulation in roots while decreased root elongation of *Triticum aestivum* in Cd-contaminated soils. This is because higher temperatures increased Cd toxicity to plant roots by increasing Cd accumulation and changing the subcellular distribution of Cd. Furthermore, changes in soil moisture can affect soil pH, EH, calcium carbonate, soluble organic content, and the electrochemical characteristics of the soil surface, all of which have an indirect impact on metal distribution in the soil solid–liquid phase and thus metal bioavailability. Pascual et al. (2004) noticed lower bioavailability of Zn, Cu, Mn, and Ni concentrations in soils when water was scarce. This was ascribed to a lower rate of soil mineralization as a result of drought stress. In response, increased metal deposition in the leaves has the ability to improve drought stress resistance and delay the negative consequences by reducing stratum corneum transpiration or increasing osmotic pressure in cells (Rajkumar et al., 2013).

Metal mobilization

Heavy metals are commonly present in both bioavailable and non-bioavailable forms in soils. The mobility and solubility of metals in soils are considered to be important factors influencing plant extraction efficiency (Ma et al., 2016b). Metal-resistant PGPB can mobilize metals and thus increase metal availability in the soil environment by secreting various organic acids (such as oxalic acid, acetic acid, and citric acid) and biosurfactants. Wu et al. (2018) noted that the inoculation of *Buttiauxella* sp. SaSR13 increased the content of root exudates (especially malic and oxalic acids) of *S. alfredii*, resulting in significant increases in the bioavailability and plant uptake of Cd. Additionally, biosurfactants can promote the entry of hydrophobic contaminants into the aqueous phase by solubilizing and micellizing the contaminants. These micelles and dissolved contaminants allow metal removal *via* soil washing or make them easily absorbed by plants (Akbari et al., 2018). Therefore, biosurfactant-producing microorganisms present in contaminated soils can effectively enhance metal mobility (Lal et al., 2018). San Martín et al. (2021) demonstrated that rhamnolipid-producing *Pseudomonas* Y3-B1A achieved a maximum vanadium removal efficiency of 85.5%.

Metal-resistant PGPB can also mobilize metal through biomethylation, leading to their volatilization. Certain PGPB can transfer methyl groups to metals (Pb and Se, etc.) to form methylated metal compounds with altered volatility, solubility, and toxicity (Ahemad, 2019). Microbial methylation of arsenic (As) raises trace levels of As species like monomethylarsinic acid and dimethylarsinic acid (DMA) in soils. Methylated As is absorbed by plant roots more slowly than inorganic As. DMA does not form compounds with plant chelating agents and is not quenched in vacuoles, allowing for efficient DMA transport inside the plants (Bali and Sidhu, 2021). Zhang et al. (2015) also proposed that As trioxide S-adenosylmethionine methyltransferase of *Pseudomonas alcaligenes* play a major role in the methylation and detoxification of As (III), which can be used in the bioremediation of As-contaminated environment.

Metal stabilization

The combination of metals with extracellular substances (e.g., anionic functional groups and extracellular polymers) can reduce metal bioavailability in soils, therefore reducing metal absorption or migration to aboveground plant parts. For instance, many metals can be efficiently immobilized in soils by combining with anionic functional groups on the cell surface (e.g., mercapto, carboxyl, hydroxyl, sulfonate, amine, and amide groups; Jacob et al., 2018). These substances reduce

metal toxicity by forming complexes or effective barriers around cells (Ahemad, 2019). Some PGPB can also reduce metal bioavailability in soils through precipitation, alkalization, and complexation processes. The inorganic acids secreted by PGPB (e.g., hydrogen sulfide, bicarbonate, and phosphate) can also react rapidly with certain dissolved metals (Cu, Fe, Zn, and Pb) to form insoluble precipitates (Ma et al., 2016b).

Extracellular polymer substances (EPS) produced by PGPB are biosynthetic polymers composed mainly of polysaccharides, proteins, uronic acid, hummus, lipids, and other compounds. A previous study noted that bacterial EPS play a variety of biological activities in microbes and plants (Manoj et al., 2020). EPS generated by PGPB was shown to bind firmly to potentially harmful trace elements and capture precipitated metal sulfides and oxides to form organic metal complexes, enhancing resistance to toxic trace elements (Etesami and Maheshwari, 2018). The role of PGPB in the biosorption of Cs^{+} was confirmed by the production of EPS and biofilm formation of *Nocardiopsis* sp. (Sivaperumal et al., 2018). Silambarasan et al. (2019b) discovered that *Rhodotorula* sp. CAH2 could survive up to 6 mmol L^{-1} Al and produce EPS consisting of glucose, mannose, and galactose even under multiple stress conditions (salt and drought), along with the yield increasing as the stress level increased.

Phosphate solubilized by PGPB can also precipitate metals as metal accumulation in bacterial biomass is mediated by phosphatases that release inorganic phosphates from supplied organophosphate donor molecules (e.g., glycerol 2-phosphate) and metal cations precipitate on the biomass as phosphates (Gadd, 2004). In addition, microbial-induced carbonate precipitation (MICP) has been proposed as a viable bioremediation approach for metal immobilization. In MICP, carbonates can bind to the metals (e.g., Pb^{2+} and Cu^{2+}) on the surface, after which these metal elements change from soluble forms to insoluble forms, thus reducing their toxicity (Tamayo-Figueroa et al., 2019). The MICP caused by *B. pasteurii* ATCC 11859 maintained the microbial growth while reducing the available Pb content in the soil, resulting in a decrease in Pb extraction and available Pb content by 76.34 and 41.65%, respectively (Chen et al., 2021).

Metal transformation

The valence state of the metal determines its toxicity. The oxidation–reduction process of metal by bacteria results in various chemical transformations of metal, affecting their shape and mobility in soils, which is regarded as an essential detoxifying mechanism (Ma et al., 2016b). Metal ions' redox reactions can be regulated by PGPB *via* cell metabolism, and metals could be converted into non-bioavailable states in the rhizosphere to reduce their toxic effects on plants (Sharma, 2021). It has been found that variation in metal-reducing bacteria can catalyze the reduction reaction and use metal to replace Fe^{3+} and S^{0} as terminal electron receptors in anaerobic respiration (Yin et al., 2019).

Enzyme-mediated reduction in toxic metals to less destructive forms is another popular strategy for reducing metal toxicity, which helps to improve microbial resistance to metal ions. These enzymes cleave bonds and use the energy generated by biochemical reactions to assist in the transfer of electrons from one compound to another (Voica et al., 2016). Harmful pollutants are eventually oxidized to innocuous molecules as a result of these processes. Furthermore, these enzymes aid in the humification of various phenolic compounds produced by lignin degradation in the soil environment, as well as the detoxification of various xenobiotics, such as aniline or phenolic compounds *via* chemical interactions (Jacob et al., 2018). Giovanella et al. (2016) found that *Pseudomonas* B50A removed 86% of Hg in the medium. This is probably due to the fact that Hg (II) reductase produced by strain B50A could effectively reduce Hg (II) to Hg (0) to reduce its toxicity.

Metal detoxification

The metal detoxification processes induced by PGPB have a significant influence on phytoremediation efficiency. Metal biosorption and bioaccumulation by bacteria, as well as the synthesis of plant hormones, ACCD, and other secretions, have all been proven to improve plant resistance to metals (Etesami and Maheshwari, 2018; Yaashikaa et al., 2021). Biosorption and bioaccumulation of inorganic and organic pollutants are determined by interaction traits of biomass and concentration of pollutants. In biosorption, the contaminants adhere to the surface of the cell wall, whereas in bioaccumulation, the contaminants accumulate within the cells (Ma et al., 2016b; Priyadarshanee and Das, 2021). Huang et al. (2020a) found that *B. subtilis* had significant biosorption potential, since it adsorbed about 10–20 mg L^{-1} concentration of Cd^{2+}. This type of biosorption phenomenon can reduce the pollutants (especially metals) toxicity to plants. In another study, Sedlakova-Kadukova et al. (2019) proved that *Streptomyces* K11 isolated from alkaline brown mud disposal site considerably reduced the Zn toxicity through extracellular accumulation and chelation, which are related to its Zn tolerance and high bioaccumulation efficiency.

Genetically modified organisms for bioremediation

The use of genetically engineered microorganisms (GEMs) is also a promising strategy to clean up metal-polluted soils. Transgenic methods are not only used to convert functional genes but also to elevate particularly recognized promoters to existing gene functions connected with metal accumulation/translocation/detoxification mechanisms and introduce them to target bacteria (Pratush et al., 2018). As a low molecular weight, cysteine-rich protein, metallothioneins (MTs) found in many bacteria have the ability to bind metals and form complex biochemical structures (Venegas-Rioseco et al., 2022). Some gene transformation experiments have convincingly demonstrated that MT_S produced by PGPB (e.g., *P. putida* and *Mycobacterium tuberculosis*) can improve plant tolerance to metals (Mierek-Adamska et al., 2017; Nanda et al., 2019). Li et al. (2021) proved that *E. coli* cells

expressing SUMO-*Sh*MT3 bioaccumulated Cd^{2+}, Cu^{2+}, and Zn^{2+}. The biofilm-forming marine bacterium *P. aeruginosa* N6P6 possessing the *bmtA* gene resisted a variety of metals (e.g., Pb, Cd, Hg, Cr, and Zn; Kumari and Das, 2019).

Since several elements in the promoter region of the metals responsive gene can be activated by plant hormones and growth regulators, the relationship between these regulator compounds and metal chelator phytochelatins is very important, which are in the first line of heavy metal defense mechanism is critical (Pal et al., 2018). Phytochelatins are enzymatically synthesized from glutathione by phytochelatin synthase activity in the presence of metal and their synthesis also initiates/transforms the entities of metal anions (Ag, Au, Cd, Cu, Hg, Pb, and Zn) and cations (As; Ozyigit et al., 2020). de Souza and Vicente (2020) proved that recombinant *E. coli* clones expressing the synthetic phytochelatin EC20 have higher Cd^{2+} biosorption capacity and tolerance than that without EC20. Heavy-metal ATPases (HMA), a subfamily of P-type HMA transporters, are found in a wide range of microorganisms. The energy released by ATP hydrolysis is mostly used to power the transmembrane transport of some metal ions, such as Ag^{+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, and Ni^{2+} (Yang et al., 2022). Begum et al. (2019) showed that *HSP70* and *HMA3* genes (a member of the HMA family) were highly expressed in *Panicum virgatum* inoculated with *P grimontii* and *P. vagans* under Cd stress, resulting in an increase in the biomass and IAA yield in inoculated plants, but a decrease in Cd accumulation. Both the natural resistance-associated macrophage protein family and the yellow streak-like transporters are also responsible for the absorption, transport, and detoxification of transition metals (Chowdhury et al., 2021; Yang et al., 2022).

The application of these GEMs is a very effective method to remove pollutants from the environment. However, the application of GEMs can affect the natural ecosystem, posing risks to the environment (Hussain et al., 2018). GEMs are considered a competitive alien species to the ecosystem and their introduction may reduce microbial biodiversity in the ecosystem. Besides, they may have adverse effects on human health, causing cancer and other genetic diseases (Saravanan et al., 2022). Undoubtedly, GEMs have potential ecological risks, but it is possible to find an efficient way to implement GEMs in the bioremediation of metal-polluted soils in the future through technical safeguards and innovation. For instance, some countries have issued necessary guidelines for assessing and monitoring the risks of GEMs in the environment, emphasizing risk stratification when applying GEMs for bioremediation (Wu et al., 2021). There have been recent attempts to design and track GEMs including the development of a set of criteria for the utilization of GEMs (Ezezika and Singer, 2010). In addition, another containment approach mainly involves designing "suicidal GEMs," when the pollutants are degraded, the killer gene is activated and the GEMs are then eradicated (Rebello et al., 2021).

Application and commercialization of PGPB in phytoremediation under environmental stress

The use of PGPB in bioremediation has become more and more popular due to their abilities to detoxify and degrade toxins and promote plant growth. Silambarasan et al. (2020) demonstrated that the inoculation with *Pseudomonas citronellolis* SLP6 improved bud and root growth (length, fresh, and dried biomass), chlorophyll content, antioxidant enzyme activity, and Cu uptake in roots and shoots under Cu and Cu+ salt stress. Interestingly, they concluded that the *P. citronellolis* SLP6 amalgamation could be an effective approach for phytostabilization in Cu-contaminated saline soils. Accordingly, Bruno et al. (2021) reported that the inoculation of multi-metal (MM) and increased atmospheric temperature (IAT) tolerant *B. cereus* TCU11 significantly improved the growth and phytoextraction (Pb, Zn, Ni, Cu, and Cd) potential of *Zea mays* in metal-contaminated soils. However, most PGPB applications in metal bioremediation are done in pot or greenhouse experiments, with *in situ* investigations in field conditions being rare. The field experiment conducted by Ren et al. (2019) showed that *B. cepacia* J62 increased the contents of ascorbic acid and glutathione in *B. napus* and reduced the oxidative stress caused by metals. The successful colonization contributed to increasing the biomass and the total Cu absorption (67.91%). Prapagdee and Khonsue (2015) found that the Cd accumulation in the root, ground tissue, and whole plant increased by 1.2-, 1.4-, and 1.1-fold, respectively, after inoculation of *Arthrobacter* sp. with *Ocimum gratissimum* for 2 months in the field conditions. The applications of PGPB in metal phytoremediation in the past 5 years are summarized in Table 3.

Furthermore, the use of microbial agents in soil bioremediation *via* bioaugmentation techniques would be extremely beneficial to the industrialization of microbial inoculum. Surprisingly, it also boosted the commercialization and market demand for various microbial inoculations. Microbial agents are abundant in high-activity beneficial PGPB, such as N-fixing bacteria and K-dissolving bacteria. These microorganisms' metabolic activities can effectively reduce metal concentrations and toxicity in the environment (Ma et al., 2016a). Notably, *B. subtilis* has been produced and sold under the trade names RhizoVital® and FZB24® TB for use in alleviating environmental stress and promoting plant growth (Ngalimat et al., 2021). *Pseudomonas fluorescens* has also been used to produce commercial inoculants under the trade names Conquer and Victus (Suyal et al., 2016). Moreover, some inoculant products currently on the market contain several different microorganisms (O'Callaghan et al., 2020). Wang et al. (2020) noted that the combination of *Enterobacter* sp. and *Comamonas* sp. can efficiently fix Cd. In addition, various inorganic materials (mainly clay and talc), organic

TABLE 3 Application of PGPB in bioremediation under environmental stress.

PGPB strain	Metal	Abiotic stress	Host plant	PGP trait	Remarks	Phytoremediation method	Experimental condition	Reference
Pseudomonas fluorescens, *Luteibacter* sp., and *Variovorax* sp.	Pb	-	*Lathyrus sativus*	IAA, siderophores	Improved the photosynthetic pigments biosynthesis, membrane stability, and the accumulation of proline and soluble sugars; Increased Pb tolerance and accumulation in plants	Phytoextraction	Pot experiment	Abdelkrim et al., 2018
Bacillus sp. CIK-516	Ni	-	*Raphanus sativus*	IAA, ACCD, and EPS	Increased plant biomass, chlorophyll and nitrogen contents, and Ni uptake	Phytoextraction	Pot experiment	Akhtar et al., 2018
Streptomyces pactum Act 12	Cd, Cu, Zn, and Pb	-	*Triticum aestivum*	IAA, siderophores, ACCD	Increased plants biomass and the uptake of Cd, Cu, and Zn in shoots and roots; Decreased antioxidant activities and lipid peroxidation	Phytoextraction	Pot experiment	Ali et al., 2021
Bacillus sp. SB1, *Halobacillus* sp. SB2	Zn, Al, Pb	Salinity	*Arachis hypogaea*	N fixation, P solubilization	Promoted plant growth and reduced Zn, Al, Pb toxicity to the seedlings	Phytostabilization	Petri dish experiment	Banik et al., 2018
B. cereus TCU11	Pb, Zn, Ni, Cu, Cd	High temperature	*Zea mays*	IAA, siderophores	Increased plant biomass, chlorophyll, carotenoid and protein contents, and Pb, Zn, Ni, Cu, and Cd accumulations in plant tissues, and their translocation from root to bud	Phytoextraction	Pot experiment	Bruno et al., 2021
B. cereus TCR17, *Providencia rettgeri* TCR21, *Myroides odoratimimus* TCR22	Cr	High temperature	*Sorghum bicolor*	IAA, siderophores	Increased the crown length, root length, plant fresh and dry weight, and antioxidant status (SOD, CAT, and APX); Reduced proline, MDA content, and Cr accumulation in plants	Phytostabilization	Pot experiment	Bruno et al., 2020
Variovorax sp., *Micrococcus* sp., *Microbacterium* sp.	Zn, Cd	-	*Noccaea caerulescens*, *Rumex acetosa*	IAA, ACCD, P solubilization, siderophores	Increased chlorophyll, carotenoid contents, and soil nutrient cycling; Facilitated Zn and Cd translocation in plants	Phytostabilization	Pot experiment	Burges et al., 2017
Sphingomonas sp. C40	Cd	-	*Oryza sativa*	IAA, siderophores	Successfully colonized the rhizosphere soils and root interiors; Increased plant biomass and root polyamine production and their related gene expression; and Reduced Cd accumulation and translocation from roots to grains	Phytostabilization	Pot experiment	Cheng et al., 2021
B. aryabhattai AS6	As	-	*O. Sativa*	N fixation, IAA, P solubilization, siderophores, ACCD, and EPS	Improved plant biomass and SOD and CAT activities; Ameliorated As toxicity in plants; and Exhibited bio-removal and bioaccumulation of As	Phytoextraction	Pot experiment	Ghosh et al., 2018

(Continued)

TABLE 3 Continued

PGPB strain	Metal	Abiotic stress	Host plant	PGP trait	Remarks	Phytoremediation method	Experimental condition	Reference
Bacillus sp. QX8 and QX13	Cd, Pb	-	*Solanum nigrum*	IAA, siderophores, ACCD, P solubilization	Increased plant biomass, enzymatic activity, and Cd and Pb accumulation by plants	Phytoextraction	Pot experiment	He et al., 2020
B. cereus HM5, *B. thuringiensis* HM7	Mn	-	*Broussonetia papyrifera*	IAA, P solubilization, siderophores	Increased plant biomass, total root length, surface area, and Mn bioavailability in soils; Inhibited plant lipid peroxidation; Decreased MDA content, antioxidant enzyme activity in leaves, and the toxic effect of Mn on plants	Phytoextraction	Pot experiment	Huang et al., 2020b
Serratia sp. ZTB	Zn	-	*Z. mays*	IAA, ACCD, siderophores, and P and K solubilization	Decreased Zn phytotoxicity; Improved plant growth and Zn accumulation in host plants	Phytostabilization	Pot experiment	Jain et al., 2020
S. pactum Act12, *B. subtilis, B. licheniformis*	Cd, Zn	-	*Brassica juncea*	P solubilization	Promoted microbial community, enzymes activity, plant biomasses, and accumulation of Cd and Zn	Phytoextraction	Pot experiment	Jeyasundar et al., 2021
Acinetobacter sp. RA1, *Bacillus* sp. EhS7, *Bacillus* sp. RA2	Cu, Cd	-	*Perennial ryegrass*	IAA, siderophores, P solubilization	Increased the shoot and root biomass; Reduced SOD activity, MDA content, and Cu, Cd transfer to the above-ground parts	Phytostabilization	Pot experiment	Ke et al., 2021
P. azotoformans ASS1	Cu, Zn, Ni	Drought	*Trifolium arvense*	ACCD, siderophores, N fixation, P solubilization	Increased chlorophyll content of plants, accumulation of antioxidant enzymes (CAT, POD and SOD), and Cu, Zn, Ni uptake of *T. arvense*; Reduced proline accumulation and oxidative damage of membrane lipids of host plants	Phytostabilization	Pot experiment	Ma et al., 2017
Bacillus sp. TZ5	Cd	-	*Lolium perenne*	IAA, P solubilization	Colonized well in soils and increased plant biomass; Decreased Cd accumulation in ryegrass	Phytostabilization	Pot experiment	Ma et al., 2020
B. atrophaeus GQJK17 S8, *E. asburiae* QB1	Cu, Cd	Salinity	*Chenopodium quinoa willd.*	IAA, siderophores, P solubilization	Improved the germination rate, seedling biomass and growth vigor index, and plant tolerance to Cu and Cd	-	Petri dish experiment	Mahdi et al., 2021
Kocuria flava AB402, *B. vietnamensis* AB403	Cu, Cr, Ni, Zn, Co, Cd	-	*O. sativa*	IAA, siderophores, EPS	Colonized successfully in rice plant root; Enhanced plant growth; Decreased As uptake and accumulation in plants	Phytostabilization	Pot experiment	Mallick et al., 2018

(Continued)

TABLE 3 Continued

PGPB strain	Metal	Abiotic stress	Host plant	PGP trait	Remarks	Phytoremediation method	Experimental condition	Reference
A. baumannii BacI43, *Pseudomonas* sp. BacI7	Hg	-	*Z. mays*	Siderophores, P solubilization	Enhanced total dry biomass; Increased total Hg bioaccumulation and volatilization; Reduced soil Hg content	Phytovolatilization	Pot experiment	Mello et al., 2020
B. safensis FO-036b(T), *P. fluorescens* p.f.169 (along with SiO_2 and zeolite NPs)	Pb, Zn	-	*Helianthus annuus*	IAA, siderophores, ACCD	Promoted plant growth; Reduced the accumulation of Pb and Zn in plant tissues	Phytoextraction	Pot experiment	Mousavi et al., 2018
Rhodobacter sphaeroides	Cd, Zn	-	*T. aestivum*	IAA	Enhanced the wheat cellular homeostasis; Reduced the accumulation of Cd and Zn in plants	Phytostabilization	Pot experiment	Peng et al., 2018
Pseudomonas sp. K32	Cd	-	*O. Sativa*	IAA, N fixation, P solubilization	Increased total chlorophyll content, amylase activity, total sugar content; Decreased MDA content and Cd uptake	Phytostabilization	Hydroponic cultivation	Pramanik et al., 2021
Arthrobacter sp. TISTR 2220	Cd		*Ocimum gratissimum*	IAA	Enhance Cd accumulation and translocation of Cd from plant roots to the shoots during a 2-month harvest period	Phytoextraction	Field trial experiment	Prapagdee and Khonsue, 2015
Proteus sp. DSP1, *Pseudomonas* sp. DSP17, *Ensifer meliloti* RhOL6	Cu, Pb, Zn	High temperatures	*Medicago sativa*	IAA, siderophores, N fixation, P solubilization	Colonized plant root system; Enhanced plant growth, synthesized non-enzymatic metabolites and enzymes; and Decreased metal (Cu, Pb, and Zn) translocation to shoots	Phytostabilization	Pot experiment	Raklami et al., 2019
Microbacterium oxydans JYC17, *P. thivervalensis* Y1-3-9, and *B. cepacia* J62	Cu		*Brassica napus*	IAA, ACCD, siderophores, P solubilization	Colonized plant rhizosphere and endosphere; Enhanced plant biomass and Cu uptake; Decreased POD activity	Phytoextraction	Field trial experiment	Ren et al., 2019
P. aeruginosa CPSB1	Cu, Cr, Cd	-	*T. aestivum*	ACCD, IAA, HCN, siderophore, P solubilization	Enhanced root dry biomass, shoot and spikes; Decreased the levels of proline, antioxidant enzymes, MDA content, and metal (Cu, Cr, and Cd) uptake by plants	Phytostabilization	Pot experiment	Rizvi and Khan, 2017
Curtobacterium herbarum CAH5	Al	Drought	*Lactuca sativa*	IAA, ACCD, siderophores, and P solubilization	Enhanced chlorophyll contents and antioxidant enzymes; Reduced MDA content in leaves and Al accumulation in plants; Exhibited bio-removal of Al	Phytostabilization	Pot experiment	Silambarasan et al., 2019c

(Continued)

TABLE 3 Continued

PGPB strain	Metal	Abiotic stress	Host plant	PGP trait	Remarks	Phytoremediation method	Experimental condition	Reference
Rhodotorula mucilaginosa CAM4	Al	Drought and salinity	*L. sativa*	IAA, siderophores	Improved plant growth, photosynthetic pigment content and accumulation of antioxidant enzymes; Reduced oxidative stress and Al accumulation in plants	Phytostabilization	Pot experiment	Silambarasan et al., 2019a
Serratia sp. CP-13	Cd	-	*Z. mays*	IAA, P solubilization	Increased plant biomass, photosynthetic pigments, antioxidative machinery (SOD, POD, and CAT); Decreased Cd uptake and concomitant lipid peroxidation in plants	Phytostabilization	Petri dish experiment	Tanwir et al., 2021
Providncia sp.	Cr	Drought	*Z. mays*	IAA, ACCD, siderophores	Increased plant growth, pigments, protein, phenolics and relative water content; Decreased the lipid peroxidation, proline, superoxide dismutase activity, and Cr translocation	Phytostabilization	Pot experiment	Vishnupradeep et al., 2022
B. megaterium H3	Cd, Pb	-	*Brassica rapa, Brassica campestris*	IAA, siderophores	Increased plant biomass, the rhizosphere soil organic matter content and invertase activity; Decreased Cd and Pb translocation factors	Phytostabilization	Pot experiment	Wang et al., 2018
P. fluorescens	Cd	-	*Sedum alfredii*	IAA	Increased the formation of lateral roots of its host plants and Cd accumulation in plant roots	Phytoextraction	Pot experiment	Wu et al., 2020b
B. contaminans ZCC	Cd	-	*Soy beans*	ACCD, siderophores, organic and inorganic P solubilization, IAA	Promoted plant dry biomass, nitrogen content in above-ground parts, and plant tolerance to Cd; Reduced membrane lipid peroxidation	Phytoextraction	Pot experiment	You et al., 2021
P. fluorescens 002	Al	Salinity	*Z. mays*	IAA, ACCD, siderophores	Improved root fresh and dry biomass, chlorophyll, carbohydrate content and the tolerance of plants to Al	-	Petri dish experiment	Zerrouk et al., 2016
P. plecoglossicida	Al	Salinity	*Z. mays*	IAA, ACCD	Increased the root length, the number and length of fine roots, the number of lateral roots and the quality of root trunk and the tolerance of plants to Al	-	Pot experiment	Zerrouk et al., 2019

ACCD, 1-aminocyclopropane-1-carboxylate deaminase; APX, ascorbate peroxidase; CAT, catalase; EPS, extracellular polymeric substance; HCN, hydrogen cyanide; IAA, indole-3-acetic acid; MDA, malondialdehyde; N, nitrogen; P, phosphorus; POD, peroxidase; and SOD, superoxide dismutase.

materials (peat, charcoal, and plant waste materials), and polymers (polysaccharides, protein, and synthetic polymers) were used as carriers for PGPB encapsulation (Szopa et al., 2022). Alginate hydrogels seem to be a successful commercial product. Su et al. (2021) noted that the maximum adsorption capacity of Cu on multilayer calcium alginate beads containing diatom bacteria and *B. Subtilis* can reach 141.34 mg g^{-1}. *Bacillus* strains immobilized in alginate macrobeads were also found to enhance drought stress adaptation of *Guinea grass* (Mendoza-Labrador et al., 2021). Tu et al. (2020) used corn stalk biochar and *Pseudomonas* sp. as materials and illustrated the stabilization mechanism of biochar-loaded microbial inoculum on Cd-and Cu-contaminated soils.

Conclusion and future prospects

This review has addressed the mechanism of PGPB promoting plant growth and enhancing plant resistance under biotic and abiotic stress and the function in response to metal bioavailability and toxicity. This has significant scientific and practical implications for the use of PGPB in the phytoremediation of metal-contaminated soils as well as an understanding of the interaction between external pressure factors and biological processes. However, due to the nature of PGPB themselves, there are limitations in their utilization process. First, the genetic stability of PGPB is poor and easy to change, making them difficult to remove all pollutants. Second, there is a competitive survival relationship between PGPB and indigenous microbes, and eventually, these PGPB strains may be eliminated due to competitive failure. Finally, PGPB are easily affected by other factors such as external environment temperature, soil pH, and so on, thereby hampering the bioremediation efficiency. More research is needed to better understand the interaction between major factors namely metal, soil, microorganisms and plants.

We should pay close attention to how PGPB is employed in actual soil rehabilitation. Soil pH, humidity, and other environmental parameters could affect the efficiency of bioremediation. Compare with the studies of PGPB strain under laboratory conditions, the research on the field and *in situ* remediation experiments of PGPB strain under different environmental conditions is still very scarce.

The effects of different inoculation or application methods of PGPB on the phytoremediation efficiency of metal-polluted soil were explored. The appropriate inoculation method not only can change the soil nutritional status and directly affect PGPB survival and colonization efficiency but also can indirectly affect metal bioavailability in soils by changing the quantity and composition of root exudates of host plants.

There is a need to strengthen the utilization and safe disposal of post-remediation materials. Some bioaccumulating plants could produce certain harmful biomass after soil remediation. If these dry materials cannot be effectively treated, the original significance of bioremediation will be compromised. Therefore, the follow-up treatment of bioremediation technology and the recovery and treatment of metal in soils also have great research value.

It is necessary to develop additional research to analyze and anticipate how metals may influence plant development, metal accumulation, and ecophysiological responses in soils as a result of global climate change. The exact repercussions of climate change on plant–metal interactions in the future are difficult to anticipate due to the complicated interactions between various metals. Furthermore, much of the research lacks information on the behavioral dynamics and metabolomics of PGPB under environmental stresses. As a result, we need to improve our understanding of rhizosphere micro-ecological processes at the molecular level and choose the best couple of plants and PGPB to provide theoretical direction for long-term pollution decontamination.

Author contributions

YM developed the ideas and wrote the manuscript, and was the project sponsor. YW wrote the first draft of the manuscript. YM, MN, XS, XC, ZL, and DN revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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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