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Enhanced phytoremdiation of *Robinia pseudoacacia* in heavy metal-contaminated soils with rhizobia and the associated bacterial community structure and function

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HIGHLIGHTS

- Bacterial composition in *Robinia pseudoacacia* rhizosphere soils was analyzed.
- Soil contamination level was the main factor affecting the bacterial communities.
- Rhizobial inoculation improved phytoremediation efficiency of *R. pseudoacacia.*
- Rhizobial inoculation increased biomarkers related to plant growth promotion.
- Rhizobial inoculation upregulated genes encoding ATP-binding cassette transporters.

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ABSTRACT

Heavy metals can cause serious contamination of soils, especially in mining regions. A detailed understanding of the effects of heavy metals on plants and root-associated microbial communities could help to improve phytoremediation systems. In this study, black locust (*Robinia pseudoacacia*) seedlings with or without rhizobial inoculation were planted in soils contaminated with different levels of heavy metals. Bacterial communities in rhizosphere and bulk soil samples were analyzed using 16S rRNA gene sequencing on the Illumina MiSeq platform and shotgun metagenome sequencing on the Illumina HiSeq platform. Soil bacterial communities varied significantly depending on the level of soil contamination, and planting also had some influence. Although inoculation of *Mesorhizobium loti* HZ76 (a natural microsymbiont of *R. pseudoacacia*) was a relatively minor factor, it did influence the soil bacterial community. Under the selective pressure, plant growth promotion-related biomarkers in the rhizosphere increased after inoculation compared with non-inoculated controls, especially those associated with *Mesorhizobium*, *Variovorax*, *Streptomyces*, and *Rhodococcus* genera. Genes encoding ATP-binding cassette transporters were up-regulated in the rhizosphere after inoculation compared with genes related to sulfur/nitrogen metabolism. These results provide insight into soil bacterial communities and their functions in the *R. pseudoacacia* rhizosphere in response to rhizobial inoculation and heavy metal contamination. This knowledge may prove useful for improving phytoremediation of metal-contaminated soils.

1. Introduction

Microorganisms are essential for global biogeochemical cycling (Falkowski et al., 2008; Wagg et al., 2014). They play a key role in plant growth and soil properties, especially in the rhizosphere niche (Philippot et al., 2013). The rhizosphere is a critical interface

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supporting the exchange of resources between plants and the surrounding soil environment. Microbial communities in the rhizosphere utilize root exudates (e.g., flavonoids, antimicrobials, organic acids, and amino acids) released by their plant host, and plants therefore provide a selective niche for particular microbial species (Mendes et al., 2013). In return, microbes present in the rhizosphere influence numerous plant and soil processes, including nutrient acquisition, elemental cycling, and soil formation (Nannipieri et al., 2003; Fitter et al., 2005; Cardon and Whitbeck, 2011). Studying the structural and functional traits of rhizosphere microbial communities is therefore important for optimizing plant growth and crop productivity.

Microbial community structure in the rhizosphere varies with soil type, plant species, plant genotype, and growth stage (Peiffer et al., 2013; Turner et al., 2013; Chaparro et al., 2014; Edwards et al., 2015). Microbial diversity, abundance, and composition in soils are also strongly influenced by heavy-metal contamination (Wang et al., 2007; Sun et al., 2015; Fan et al., 2016). Extensive studies on soil microbial communities and functions in the rhizosphere have focused on normal ecosystems (Mendes et al., 2014; Yan et al., 2017), but less attention has been paid to the rhizosphere of plants growing in soils contaminated with heavy metals. Moreover, the microbial communities can respond to phytoremediation, a low-cost, environmentally friendly method to remove contaminants from the environments (Yergeau et al., 2014; Bourceret et al., 2016). Although some studies have investigated the impact of heavy-metal contamination on rhizosphere microbial communities (Gremion et al., 2003; Sipilä et al., 2008; Deng et al., 2015; Bourceret et al., 2016), few have reported on variations in the structure and function of microbial communities during phytoremediation of heavy-metal contamination (Yergeau et al., 2014).

Plants known as phytoremediators can extract, stabilize, filter, volatilize, or degrade organic and inorganic contaminants by capitalizing on the 'rhizosphere effect' (Salt et al., 1998; Pilon-Smits, 2005). Unlike organic contaminants, heavy metals are not degraded but rather extracted or stabilized by plants (Grandlic et al., 2009; Wood et al., 2016). Inoculation of plant growth-promoting rhizobacteria (PGPRs) into the plant rhizosphere can enhance the phytoremediation efficiency in heavy metal-contaminated environments (Dary et al., 2010; Wood et al., 2016). Heavy metal-resistant PGPRs have the potential to increase plant yield and withstand environmental stresses through fixing N, producing siderophores, auxin, and 1-aminocyclopropane-1-carboxylate deaminase activities, and synthesizing antimicrobial compounds (Bulgarelli et al., 2013). Although thousands of PGPR strains have been isolated over recent decades, the influence of inoculating PGPRs on the indigenous microbial communities and their functions in plant rhizosphere soils contaminated with heavy metals remains unclear. Specifically, whether PGPRs inoculated into the rhizosphere are able to remain in balance with indigenous microbial communities, or whether they detrimentally influence the indigenous microbial ecology and cause ecological imbalances in the soil remains unknown.

As a pioneer species, black locust (*Robinia pseudoacacia*) is a woody legume characterized by fast growth, deep root system, heavy metal tolerance, and symbiosis with rhizobia. Thus, *R. pseudoacacia* holds promise for use in the phytoremediation of soils contaminated with heavy metals (Uselman et al., 2000; Vlachodimos et al., 2013; Yang et al., 2015). Legume-rhizobium symbiosis is believed to be crucial for phytoremediation of heavy metal-contaminated soils due to its influence on symbiotic N fixation (Pajuelo et al., 2008; Dary et al., 2010). Rhizobia possessing heavy metal-resistant and plant growth-promoting traits have been isolated from *R. pseudoacacia* nodules and found to enhance the efficiency of phytoremediation by stimulating the uptake of heavy metals and increasing the biomass and N content in plant tissues (Hao et al., 2015). Thus, *R. pseudoacacia* and its N-fixing symbionts (rhizobia) can serve as a model to investigate the influence of inoculating PGPRs on rhizosphere microbial communities in heavy metal-contaminated environments.

In this microcosm study, our main objectives were to: (1) assess the influence of *R. pseudoacacia* planting and contamination level on bacterial diversity and community structure in heavy metal-contaminated soils, and (2) identify taxa and functions in the rhizosphere that differ after inoculation of PGPR compared with non-inoculated controls. Bacterial community diversity and function were analyzed using 16S rRNA gene sequencing on the Illumina MiSeq platform and shotgun metagenome sequencing on the Illumina HiSeq platform. Our results provide novel insight into the response of bacterial communities and their functions to inoculation with PGPR in the rhizosphere soils contaminated with heavy metals, which may be of profound significance for improving phytoremediation.

2. Materials and methods

2.1. Experimental design

In June 2014, soils contaminated with different levels of heavy metals were collected from two sites surrounding a Pb-Zn smelter in southeast Mianxian County (33°07′03.4″N, 106°47′49.1″E), Shaanxi Province, China. Light and heavy contamination sites were chosen based on grade III of the environmental quality standard (GB15618-1995) for soils in China. The study area has been contaminated for almost 10 years by dust (lightly contaminated) and mine drainage (heavily contaminated), and has a soil pollution index (Kabata-Pendias, 2010) of 1.16 and 4.32, respectively (Supplementary Table S1). The main vegetation was weeds. The soil type was yellow-brown soil, and the soil texture was clay.

The study used a randomized controlled experimental design. There were 16 treatments, including two levels of heavy-metal contamination (lightly and heavily), two levels of planting (unplanted and planted with *R. pseudoacacia*), and four levels of rhizobial inoculation (uninoculated [WJ], and inoculated separately with PGPR strain *Mesorhizobium loti* HZ76 [J76], *Ensifer adhaerens* HZ14 [J14], or *Rhizobium radiobacter* HZ6 [J6]). Details of the greenhouse experiment are included in Supplementary Information S1 and Table S2.

2.2. Sampling and chemical analysis

After 90 days of growth, plants were completely removed from pots and shaken vigorously to remove excess soil. Soil still adhering to the roots (i.e., rhizosphere soil) was collected as described previously with some modifications (Bulgarelli et al., 2012). Meanwhile, bulk soil from unplanted pots was taken at a depth of 0–5 cm and at least three subsamples per pot were collected and pooled. At least three replicates were performed for each treatment to give 48 samples in total.

For molecular analyses, the collected soil samples were frozen in liquid N_2 and immediately stored at −80 °C until needed for nucleic acid extraction. For chemical analysis, soil samples were air-dried for 2 weeks before determination of nutrient and metal contents (Supplementary S2). For *R. pseudoacacia* plants, the number of nodules was counted, and the dry weight of shoots and roots was measured for each plant according to the treatment. Details of plant chemical analysis (metal and N contents) are included in Supplementary S2.