



Plant-Mediated Changes in Soil N-Cycling Genes during Revegetation of Copper Mine Tailings

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Nitrogen limitation represents a major bottleneck during the revegetation of mine tailings. However, controls over key nitrogen-cycling genes in rhizospheric soils under differential vegetation management remain poorly understood. The abundance and transcriptional activity of nitrogen-cycling genes and the enzymatic activity of nitrogen transformation are mediated differentially during revegetation of mine tailings by *Imperata cylindrica* and *Chrysopogon zizanioides* plants. Results showed that the highest total organic carbon (TOC), total nitrogen (TN), and NH_4^+ -N contents were found in the rhizosphere of *I. cylindrica*. The *nifH* gene abundances differed between *I. cylindrica* and *C. zizanioides*, and were higher in *I. cylindrica* which demonstrated by 3.39-fold higher mRNA transcript abundance of the *nifH* gene and a 2.15-fold higher nitrogen fixation rate in the rhizosphere. In addition, *C. zizanioides* exhibited a 4.94-fold higher transcript abundance of the archaeal *amoA* gene and the highest nitrification rate ($1.706 \pm 0.293 \mu\text{g N-NO}_2^- \text{g}^{-1} \text{h}^{-1}$) in the rhizosphere. In conclusion, *I. cylindrica* and *C. zizanioides* stimulated the abundances and activities of *nifH* gene and archaeal *amoA* gene, respectively. In addition, *I. cylindrica* appears to be capable of enhancing nitrogen fixation and exhibited accelerated nitrogen accumulation, which may be particularly useful for the rehabilitation of mine tailings.

Keywords: mine tailings, revegetation, nitrogen cycle, rhizosphere, *nifH* gene

INTRODUCTION

Mine tailings are typical engineered materials (Paerl et al., 2000) that undergo plant colonization followed by ecosystem development in mine wasteland. Revegetation is an effective and practical strategy for mining wasteland management (Mendez and Maier, 2008). To rehabilitate soil biogeochemical functions in mine tailings using the revegetation strategy, it is necessary to concert changes in physical, mineralogical, geochemical, and biological properties to support sustainable plant communities (Huang et al., 2012; Zornoza et al., 2015). Therein, the development of N-cycling microbial communities and their functions is one of the most important aspects of the ecological development of mine tailings (Nelson et al., 2015). However, research on this subject is greatly lacking. Therefore, it is in urgent need to identify the effect of rehabilitation strategies on the nitrogen cycle in mining wastelands.

The selection of plant species is an important factor in rehabilitating the biogeochemical functions of mine tailings, especially when plant species selection is correlated with

N-accumulation (Mendez and Maier, 2008; Parraga-Aguado et al., 2014). Plant species should have a competitive advantage (Hobbie, 2015) to use limiting nutrients more efficiently in the mine tailings, and differences in nitrogen use efficiency of different plant species can lead to positive feedbacks on nitrogen cycling (Knops et al., 2002). It is generally known that cluster roots in environments with low inorganic nutrient availability and nitrogen fixing symbioses in low-N environments increase plant access to nutrients in low supply (Chapman et al., 2006; Lambers et al., 2008). In parallel, plants nutrition is also sensitive to ecosystem nitrogen losses via denitrification and nitrogen assimilation through biological nitrogen fixation (Hai et al., 2009; Töwe et al., 2010) in the mining wastelands with low nitrogen content. Previous research indicated that plants played an important role in shaping the microbial community structure and function in the rhizosphere (Henry et al., 2008; Hai et al., 2009; Hussain et al., 2011). For example, the rhizosphere not only significantly affected the diazotrophs (Zhan and Sun, 2011, 2012) but also influenced nitrate reducers and denitrifiers (Henry et al., 2008). Variation in the abundance and diversity of organic acids or other exudates might explain the influence of plants on the abundance and activity of N-cycling microorganisms (Henry et al., 2008; Weng et al., 2013). Moreover, the rhizosphere can alter the relative abundance and number of functional genes (Hai et al., 2009; Hussain et al., 2011; Zornoza et al., 2015) and influence their transcription (Sharma et al., 2005). The rhizosphere effect on nitrogen cycling genes has been widely investigated in many environments (Yergeau et al., 2014; Concilio et al., 2015; Finzi et al., 2015). However, there is little information concerning the relationship between the rhizosphere and the transcription of functional genes involved in nitrogen cycling in the nitrogen-limited mine tailing environment.

There are many copper mine tailings wastelands in Tongling, China, some of which have been naturally or artificially revegetated. A natural *Imperata cylindrica* community and an artificial *Chrysopogon zizanioides* community have been successfully established on the Shuimuchong wasteland of copper mine tailings in Tongling. Both the *I. cylindrica* community and the *C. zizanioides* community exhibit a simple community structure, and nitrogen in these ecosystems is primarily derived from diazotrophs living in the copper mine tailings; this provides a good opportunity to hypothesize that the rhizosphere could stimulate the abundance and transcription of functional genes involved in nitrogen cycling. And the major aims of this study are to investigate the quantities and transcription of rhizosphere-associated functional genes in a copper tailings pond and to elucidate the specific rhizosphere effects of distinct plants on the abundance and activity of these genes.

MATERIALS AND METHODS

Study Areas and Sample Collection

The Shuimuchong copper mine tailing wasteland (30°55'N, 117°50'E) is located in the Tongling copper mine area, Anhui Province, East China, and covers a total surface area of 44 ha. The average annual rainfall in this study district is 1,346 mm, the rainy season is from May to September, the average annual temperature

is 16.2°C, and the frost-free period ranges from 237 to 258 days (Sun et al., 2004). On this copper mine tailing wasteland, two types of plant communities were established over 10 years ago, a naturally colonized *I. cylindrica* community and an artificial seeded *C. zizanioides* community.

The *I. cylindrica* and *C. zizanioides* rhizosphere and bulk samples in the top 20 cm of the mine tailings were collected using the method described by Riley and Barber (1970). In brief, bulk tailings and roots were obtained using a soil corer. The roots plus adhering soil were carefully collected, and the rhizosphere tailings adhering to the roots were removed by shaking. In addition, the remainder portions (mine tailings without roots or adhering soil) in the soil corer were set as the bulk tailings. Twelve samples, including three rhizospheric tailings samples (IC-R) and three bulk tailings samples (IC-B) from *I. cylindrica*, and three rhizospheric tailings samples (CZ-R) and three bulk tailings samples (CZ-B) from *C. zizanioides*, were obtained in October of 2013. Each of the 12 samples represented a combination of 3 field tailings samples, and each sample was divided into three portions. The first portion was used to analyze chemical properties, the second was used to extract DNA to measure the abundance of functional genes involved in nitrogen cycling, the third was used to detect the transcription of functional genes involved in nitrogen cycling (i.e., RNA analysis). The first two portions were placed into sterilized self-sealing plastic bags, and the third was stored in RNAlater (1 g of soils: 1 mL of RNAlater) (Ambion, Austin, TX). All samples were stored in a box at a temperature ranging from 0 to 4°C in the field. After returning to the laboratory, the second and third portions were stored at -20°C and the first was dried at room temperature, with the exception of the fractions used to analyze ammonium (NH₄⁺-N), nitrate (NO₃⁻-N) and nitrite (NO₂⁻-N) contents.

Analysis of Chemical Properties

The pH values were measured using a pH meter (soil:water = 1 g:5 mL). The total organic carbon (TOC) was measured as the mass loss of ignition in a muffle furnace at 550 ± 5°C for 6 h to determine total organic matter in mine wastelands. Total nitrogen (TN) was measured using the Kjeldahl method. Ammonium (NH₄⁺-N), nitrate (NO₃⁻-N) and nitrite (NO₂⁻-N) were extracted from fresh samples in 2 mol·L⁻¹ KCl and measured by UV spectrophotometry (Nanjing Institute of Soil Science, 1978).

Nitrogen Fixation Rate, Nitrification Rate and Denitrification Rate

Nitrification rates were measured by culturing fresh tailing samples in 250-mL sterile bottles without any N-amendments. A control (cultured without C₂H₂) and C₂H₂ amended bottles were incubated for each sample. C₂H₂ was added to the headspace at a final partial pressure of 10 Pa (0.01%). Bottles were incubated at 25°C in the dark, and sample analysis was carried out after 2 weeks of incubation. During incubation, bottles were opened and vented every 2 days to maintain aerobic conditions, and C₂H₂ partial pressure was re-established after resealing. The nitrification rate was calculated as the difference

between the nitrite concentration in the control and C₂H₂-treated samples.

The nitrogen fixation rate was measured in a C₂H₂ reduction assay in which nitrogenase reduction of C₂H₂-C₂H₄ was measured. Denitrification was simultaneously measured by accumulation of N₂O because C₂H₂ inhibits N₂O reduction and nitrification (Klemmedtsson et al., 1988). Fresh tailings were cultured in 250-mL sterile bottles, and 10% of the headspace was evacuated and replaced with C₂H₂, and sterile water was used to keep the soil moist. After sealing the bottles for culturing at 25°C, the gas was measured at 4, 8 h, 1, 3, 7, and 14 d (Figure S1). To measure nitrogen fixation, the evolution of C₂H₂-C₂H₄ was measured with a gas chromatograph, equipped with a thermal conductivity detector (TCD) and a GDX-501 packed column using N₂ as a carrier gas. The temperatures of the detector, injector, and oven were 107, 110, and 60°C, respectively. For denitrification, the N₂O emissions were analyzed with an electron capture detector (ECD) connected to a Porapak Q packed column (length: 5 m; O.D.: 3 mm) using N₂ as a carrier gas. The temperatures of the detector, injector, and oven were 360, 90, and 50°C, respectively. The denitrification rate was calculated as the difference in N₂O produced during the incubation of the control and C₂H₂-treated samples.

DNA Extraction, RNA Extraction and Inverse Transcription

Total DNA was extracted with a PowerSoil DNA Isolation Kit (MoBio, Carlsbad, USA) according to the manufacturer's instructions. Total RNA was extracted employing the mRNA extraction method described by Mettel et al. (2010). RNA-containing samples in RNAlater were thawed on ice, and supernatants were removed by centrifugation at 20,000 × *g* for 1 min. Pelleted RNA was shocked at 6 m·s⁻¹ for 35 s with 0.5 g of beads and 700 μL of TPM [50 mmol·L⁻¹ Tris-HCl (pH 8.0); 20 mmol·L⁻¹ MgCl₂·6H₂O; 2% polyvinylpyrrolidone (PVP)] and then centrifuged at 20,000 × *g* for 2 min to collect the supernatant. Excess pellet was shocked at 6 m·s⁻¹ for 35 s with 700 μL of PBL (5 mmol·L⁻¹ Tris-HCl (pH 8.0); 50 mmol·L⁻¹ EDTA; 1% SDS; 6% Phenol Water), and centrifuged at 20,000 × *g* for 2 min to collect the supernatant to mix with the previously obtained supernatant. RNA was extracted with 500 μL of phenol water (pH 4.5), followed by successive treatments with 500 μL of phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1). RNA was precipitated from the supernatant by adding 1 mL of PEG-NaCl (30% PEG-6000; 1.6 mol·L⁻¹ NaCl) and the samples were maintained at room temperature for 2 h. The precipitated RNA was cleaned via the sequential addition of 70% (v/v) ethanol and 40% (v/v) ethanol, and the supernatant was removed following centrifugation at 20,000 × *g* for 5 min. RNA samples were dissolved in 42.5 μL of water. The DNA was removed with DNase I (TransGen Biotech, Beijing, PRC) and then the total RNA was purified with an RNeasy MinElute cleanup kit (QIAGEN, Hilden, Germany). RNA samples were reverse transcribed into cDNA with TransScript II All-in-one first-strand cDNA supermix for qPCR (TransGen Biotech, Beijing, PRC).

Quantification of DNA and cDNA by Real-Time Quantitative PCR (qPCR)

The PCR primers used to amplify regions of functional genes from nitrogen fixers, nitrifiers, and denitrifiers are listed in **Table 1**. Amplification was carried out in a total volume of 20 μL containing 2 μL of diluted template DNA, 0.4 μL of each primer (concentration of 10 μM), 0.4 μL of passive reference dye(50×), 6.8 μL of ddH₂O, and 10 μL of KAPA SYBR® FAST qPCR Kit Master Mix (2×) Universal (KAPA Biosystems, Boston, US). All samples and standard reactions were performed in triplicate using a StepOne Real-Time PCR System (ABI, New York, US). Standard curve templates were constructed with 10-fold serial dilutions of linearized recombinant plasmids harboring amplicons amplified from mine tailings samples. Standard curves for the real-time PCR were developed as follows. The genes were amplified from the total genomic DNA using the same primers as in the qPCR. The PCR products were purified by Easypure Quick Gel extraction kit (TransGen Biotech, China) and cloned into a pEASY-T3 clone vector (TransGen Biotech, China). The resulting ligation mixes were transformed into a Trans1-T1 phageresistant chemically competent cell (TransGen Biotech, China). Plasmids were used as the standard from the positive clone, and the standard sequences were deposited in the GenBank Data Library under accession numbers MG256653-MG256661. The recombinant plasmids containing one copy of a gene fragment were diluted from 1:10² to 1:10⁷ copy·μl⁻¹ (list in **Table 1**) and used as targets for qPCR standard curves. The PCR program for total bacterial quantification was as follows, according to the manufacturer's instructions: 3 min at 95°C, followed by 40 cycles of 3 s at 95°C, and 25 s at 60°C. After each qPCR assay, the amplification specificity was verified by generating melting curves and performing agarose gel electrophoresis. Standard curves were constructed based on triplicate 10-fold dilutions of a DNA standard containing a known copy number. Negative controls without template DNA were included in all experiments to monitor for contamination. The real-time PCR standard curves for all functional genes were repeated three times. Gene copy numbers were calculated by comparing threshold cycles obtained in each PCR run with those of known standard DNA concentrations. Each R²-value of each standard curve for each replicate was determined to be above 0.99.

Data Analysis

Multivariate analysis of variance (MANOVA) was used to analyze the mixed effects of plant species, rhizosphere, and their interaction on chemical properties and the abundances and transcripts of functional genes. One-way analysis of variance (ANOVA, LSD *post-hoc* test with a significance at *P* < 0.05) was used to analyze the chemical properties, quantities and transcript abundances of the functional genes in different samples. A Pearson correlation analysis was carried out using SPSS 19.0 to test the relationships between functional gene quantities and chemical properties, the level of significance was set at *P* < 0.05 (two-tailed), and the false discovery rate (*q*) was calculated to establish significance of correlations. Regression analysis was performed to structure the linear relationships between

TABLE 1 | Primer pairs used in real-time qPCR analysis for amplification of nitrogenase (*nifH*), bacterial and archaeal ammonia monooxygenase (*amoA*), nitrite oxidoreductase (*nxr*), nitrate reductase (*narG* and *napA*), nitrite reductase (*nirK* and *nirS*), nitric oxide reductase (*cnorB*), and nitrous oxide reductase (*nosZ*) genes.

Target group	Primer name	Sequence(5'-3')	Length of amplicon(bp)	Detection thresholds	Efficiency and R^2	References
<i>nifH</i>	PolF	TGCGAYCCSAARGCBGACTC	342	98.1–9.81 × 10 ⁷ copies	90.05–91.43%	Poly et al., 2001
	PolR	ATSGCCATCATYTCCCGGA			$R^2 = 0.996–0.998$	
Archaeal- <i>amoA</i>	arch-amoAF	STAATGGTCTGGCTTAGACG	635	56.5–5.65 × 10 ⁷ copies	93.91–95.12%	Francis et al., 2005
	arch-amoAR	GCGGCCATCCATCTGTATGT			$R^2 = 0.995–0.999$	
Bacterial- <i>amoA</i>	amoA1F	GGGGTTTCTACTGGTGGT	491	25.8–2.58 × 10 ⁷ copies	90.55–91.21%	Rotthauwe et al., 1997
	amoA2R	CCCCTCKGSAAAGCCTTCTTC			$R^2 = 0.996–0.999$	
<i>nxr</i>	F1norA	CAGACCGACGTGTGCGAAAG	322	76.1–7.61 × 10 ⁷ copies	94.76–96.01%	Attard et al., 2010
	R2norA	TCCACAAGGAACGGAAGGTC			$R^2 = 0.998–0.999$	
<i>narG</i>	narG1960m2f	TAYGTSGGGCAGGARAACCTG	109	25.1–2.51 × 10 ⁷ copies	94.80–95.91%	López-Gutiérrez et al., 2004
	narG2050m2r	CGTAGAAGAAGCTGGTGTGTT			$R^2 = 0.998–0.999$	
<i>napA</i>	napAV66cf	TAYTTYTNHNSAARATHATGTAYGG	385	24.3–2.43 × 10 ⁷ copies	99.01–99.83%	Flanagan et al., 1999
	napAV67CR	DATNGGRTGCATYTCNGCCATRTT			$R^2 = 0.997–0.999$	
<i>nirK</i>	F1aCu	ATCATGGTCTGCCGCG	473	49.0–4.90 × 10 ⁷ copies	94.46–94.88%	Hallin and Lindgren, 1999
	R3Cu	GCCTCGATCAGRTTGTGGTT			$R^2 = 0.998–0.999$	
<i>nirS</i>	Cd3aF	G TSAACG TSAAGGARACSGG	425	40.4–4.04 × 10 ⁷ copies	94.71–5.11%	Throback et al., 2004
	R3Cd	GASTTCGGRTGSGTCTTGA			$R^2 = 0.996–0.999$	
<i>cnorB</i>	cnorB2F	GACAAGNNNACTGGTGGT	398	21.3–2.13 × 10 ⁷ copies	96.94–97.05%	Baker and Banfield, 2003
	cnorB6R	GAANCCCCANACNCCNGC			$R^2 = 0.996–0.998$	
<i>nosZ</i>	nosZ1F	WCSYGTTCMTCGACAGCCAG	259	15.1–1.51 × 10 ⁷ copies	98.25–99.02%	Henry et al., 2006
	nosZ1R	ATGTGCATCARCTGVKCRTTYTC			$R^2 = 0.999$	

Y, C/T; R, A/G; B, C/G/T; S, C/G; K, G/T; N, A/T/C/G; H, A/T/C; D, G/A/T; W, A/T; M, A/C; V, G/A/C.

the nitrogen transformation rate and the gene and transcript abundances of functional genes. A redundancy analysis (RDA) was performed in the R environment and explanatory variables were chosen with 999 permutations using the “permutest” function of the vegan package, with RDA axes plotted with solid arrows representing chemical variables significantly associated ($P < 0.05$) with the variation. Variation partitioning was carried out using the “varpart” function in R.

RESULTS

Soil Biogeochemical Properties

The multivariate analysis of variance (MANOVA) of mixed effects of plant species (P), rhizosphere (R) and their interaction on the chemical properties and nitrogen transformation rates (Table 2) showed that plant species significantly influenced the soil chemical properties including TOC, TN, C:N ratio, NH_4^+ -N, NO_3^- -N, and NO_2^- -N contents, as well as the nitrogen fixation rate and nitrification rate. The rhizosphere significantly impacted the inorganic nitrogen (NH_4^+ -N, NO_3^- -N, and NO_2^- -N) contents and nitrogen fixation rate. In addition, the interaction effect of plant species and rhizosphere on the chemical properties and nitrogen transformation rates was pointed out in TOC, NH_4^+ -N and NO_3^- -N contents as well as the nitrogen fixation rate and nitrification rate.

Variance analysis (ANOVA) showed that TOC was significantly higher under IC than CZ in both the rhizosphere and bulk tailings (Table 3). TN and NH_4^+ -N contents were

TABLE 2 | Multivariate analysis of variance (MANOVA) (F -value, P -value) of mixed effects of plant species (P), rhizosphere (R) and their interaction on the chemical properties and nitrogen transformation rates.

	Plant species(P)	Rhizosphere(R)	P × R
pH	(0.058, 0.816)	(2.846, 0.130)	(0.091, 0.771)
TOC	(67.615, <0.001)	(4.564, 0.065)	(6.993, 0.030)
TN	(12.210, 0.008)	(1.899, 0.206)	(1.584, 0.244)
C:N ratio	(33.700, <0.001)	(0.552, 0.479)	(1.178, 0.309)
NH_4^+ -N	(15.816, 0.004)	(34.149, <0.001)	(26.992, <0.001)
NO_3^- -N	(6.297, 0.036)	(5.740, 0.043)	(6.245, 0.037)
NO_2^- -N	(5.377, 0.049)	(6.128, 0.038)	(1.17, 0.311)
NFR	(65.540, <0.001)	(95.604, <0.001)	(83.691, <0.001)
NR	(24.738, 0.001)	(4.029, 0.080)	(6.131, 0.038)
DNR	(0.395, 0.547)	(0.200, 0.667)	(0.667, 0.438)

Index in bold shows the significance at $P < 0.05$. NFR, Nitrogen fixation rate; NR, nitrification rate; DNR, denitrification rate.

significantly higher in IC than CZ for the rhizospheric samples (Table 3). In addition, the rhizospheric sample of *I. cylindrica* has the highest nitrogen fixation rate (Table 4). Thus, *I. cylindrica* was more effective in the formation and accumulation of soil organic matter and nitrogen in copper mine tailings than *C. zizanioides*.

When the rhizosphere and bulk tailings of *I. cylindrica* were compared, the former exhibited higher TOC and NH_4^+ -N contents than the latter (Table 3). In contrast to *I. cylindrica*, the

TABLE 3 | Soil basic chemical properties of bulk tailings and rhizospheric samples of *Imperata cylindrica* and *Chrysopogon zizanioides*; lowercase letters indicate significant differences in each column (LSD, $P < 0.05$).

	pH	TOC (g kg ⁻¹)	TN (g kg ⁻¹)	C:N ratio	NH ₄ ⁺ -N (mg kg ⁻¹)	NO ₃ ⁻ -N (mg kg ⁻¹)	NO ₂ ⁻ -N (μg kg ⁻¹)
IC-R	7.18 ± 0.06a	41.31 ± 6.15c	0.25 ± 0.03b	167.31 ± 37.22b	50.00 ± 4.30b	9.29 ± 2.78a	3.85 ± 1.35a
IC-B	7.29 ± 0.06a	26.21 ± 6.65b	0.19 ± 0.01ab	104.92 ± 36.01b	16.88 ± 3.15a	9.54 ± 0.80a	1.18 ± 0.51a
CZ-R	7.18 ± 0.05a	6.98 ± 2.46a	0.15 ± 0.04a	48.92 ± 21.31a	22.48 ± 2.71a	20.99 ± 6.70b	10.35 ± 5.69b
CZ-B	7.26 ± 0.17a	8.59 ± 5.62a	0.15 ± 0.05a	54.80 ± 20.04a	20.54 ± 8.50a	9.56 ± 3.48a	3.55 ± 3.07a

Values are means (n = 3) ± Std.

TOC, Total organic carbon; TN, total nitrogen; Samples were bulk tailings of *Imperata cylindrica* (IC-B), rhizospheric samples of *I. cylindrica* (IC-R), bulk tailings of *Chrysopogon zizanioides* (CZ-B), and rhizospheric samples of *C. zizanioides* (CZ-R).

TABLE 4 | The rates of nitrogen fixation (NFR), nitrification (NR) and denitrification (DNR) in bulk tailings and rhizospheric samples of plant species; lowercase letters indicate significant differences in each column (LSD, $P < 0.05$).

	NFR (μg C ₂ H ₄ g ⁻¹ h ⁻¹)	NR (μg N- NO ₂ ⁻ g ⁻¹ h ⁻¹)	DNR (μg N ₂ O g ⁻¹ h ⁻¹)
IC-R	0.088 ± 0.005b	0.537 ± 0.150a	0.005 ± 0.004a
IC-B	0.041 ± 0.003a	0.610 ± 0.320a	0.001 ± 0.001a
CZ-R	0.045 ± 0.003a	1.706 ± 0.293b	0.005 ± 0.003a
CZ-B	0.044 ± 0.006a	1.003 ± 0.291a	0.005 ± 0.008a

rhizospheric tailings of *C. zizanioides* contained higher NO₃⁻-N and NO₂⁻-N contents than the bulk tailings. And the the rhizospheric sample of *I. cylindrica* has the highest nitrification rate (Table 4).

The effects of plant species on the pH values of the rhizosphere and bulk tailings were not significant (Table 3). However, plant species affected the TOC and TN content, and both the rhizosphere and bulk tailings of *I. cylindrica* exhibited higher C:N ratios than those of *C. zizanioides*.

Effects of the Rhizosphere on Functional Genes Involved in the Nitrogen Cycle

The gene and transcript abundances of ten genes involved in the nitrogen cycle, specifically *nifH*, *arch-amoA*, *bact-amoA*, *nxr*, *narG*, *napA*, *nirK*, *nirS*, *norB*, and *nosZ*, were measured by qPCR (Table 1). The primers used to amplify the functional genes (such as *nifH* gene) for qPCR do not target all genes, so only a subset would be quantified in the assay. Even so, the plant species merely impacted the abundance of *nifH* and *nxr* genes and the transcripts of *nifH* and *amoA* (including archaeal *amoA* and bacterial *amoA*) genes, and the plant rhizosphere and the interaction of plant species and rhizosphere influenced the transcripts of *nifH* and archaeal *amoA* genes (Table 5). In parallel, the abundances of these ten functional genes were not significantly different between the rhizosphere and bulk tailings (Figure 1). However, there were significant differences in transcriptional activities of the *nifH* gene between the rhizosphere and bulk tailings for *I. cylindrica*, and for the *arch-amoA* gene for *C. zizanioides* (Figure 2). The rhizosphere of *I. cylindrica* demonstrated a 3.39-fold higher transcript abundance of the *nifH* gene than in bulk tailings, while the transcript

TABLE 5 | Multivariate analysis of variance (MANOVA) (F -value, P -value) of mixed effects of plant species (P), rhizosphere (R) and their interaction on the abundances and transcripts of functional genes.

	Plant species(P)	Rhizosphere(R)	P × R
DNA	<i>nifH</i>	(51.142, <0.001)	(2.238, 0.173)
	Archaeal <i>amoA</i>	(0.708, 0.425)	(0.076, 0.79)
	Bacterial <i>amoA</i>	(1.527, 0.252)	(0.526, 0.489)
	<i>nxr</i>	(4.304, 0.072)	(9.092, 0.017)
	<i>narG</i>	(1.154, 0.314)	(0.507, 0.497)
	<i>nirK</i>	(1.022, 0.342)	(1.876, 0.208)
	<i>nirS</i>	(3.18, 0.112)	(0.383, 0.553)
	<i>norB</i>	(0.542, 0.483)	(3.19, 0.112)
	<i>nosZ</i>	(0.211, 0.658)	(0.872, 0.378)
	RNA	<i>nifH</i>	(12.499, 0.008)
Archaeal <i>amoA</i>		(9.716, 0.014)	(7.751, 0.024)
Bacterial <i>amoA</i>		(8.012, 0.022)	(1.115, 0.322)
<i>nxr</i>		(0.072, 0.795)	(1.469, 0.260)
<i>narG</i>		(4.007, 0.080)	(0.137, 0.721)
<i>nirS</i>		(0.017, 0.899)	(1.305, 0.286)

Index in bold shows the significance at $P < 0.05$.

abundance of the archaeal *amoA* gene in the rhizospheric samples of *C. zizanioides* were 4.94-fold higher than those in bulk tailings.

Moreover, *nifH* gene abundance differed significantly between *I. cylindrica* and *C. zizanioides*. The *nifH* gene abundances in both the rhizosphere and bulk tailings of *I. cylindrica* were significantly higher than those of *C. zizanioides* (Figure 1).

The quantities of the *napA* genes, which participate in nitrate reduction, were lower than the detection limit (data not shown). Although the abundance of the *nirK* gene was $>2.0 \times 10^6$ copies·g⁻¹ dw soil, its gene transcript abundances were below the detection limit. Similar to the *nirK* gene, the gene transcript abundance of *norB* and *nosZ* were also below the detection limit (data not shown).

Correlations between Gene Abundance, Transcript Abundance and the Chemical Properties of Copper Mine Tailings

The nitrogen fixation rate was significantly enhanced by the increased *nifH* gene abundance and transcription (Table S1; for

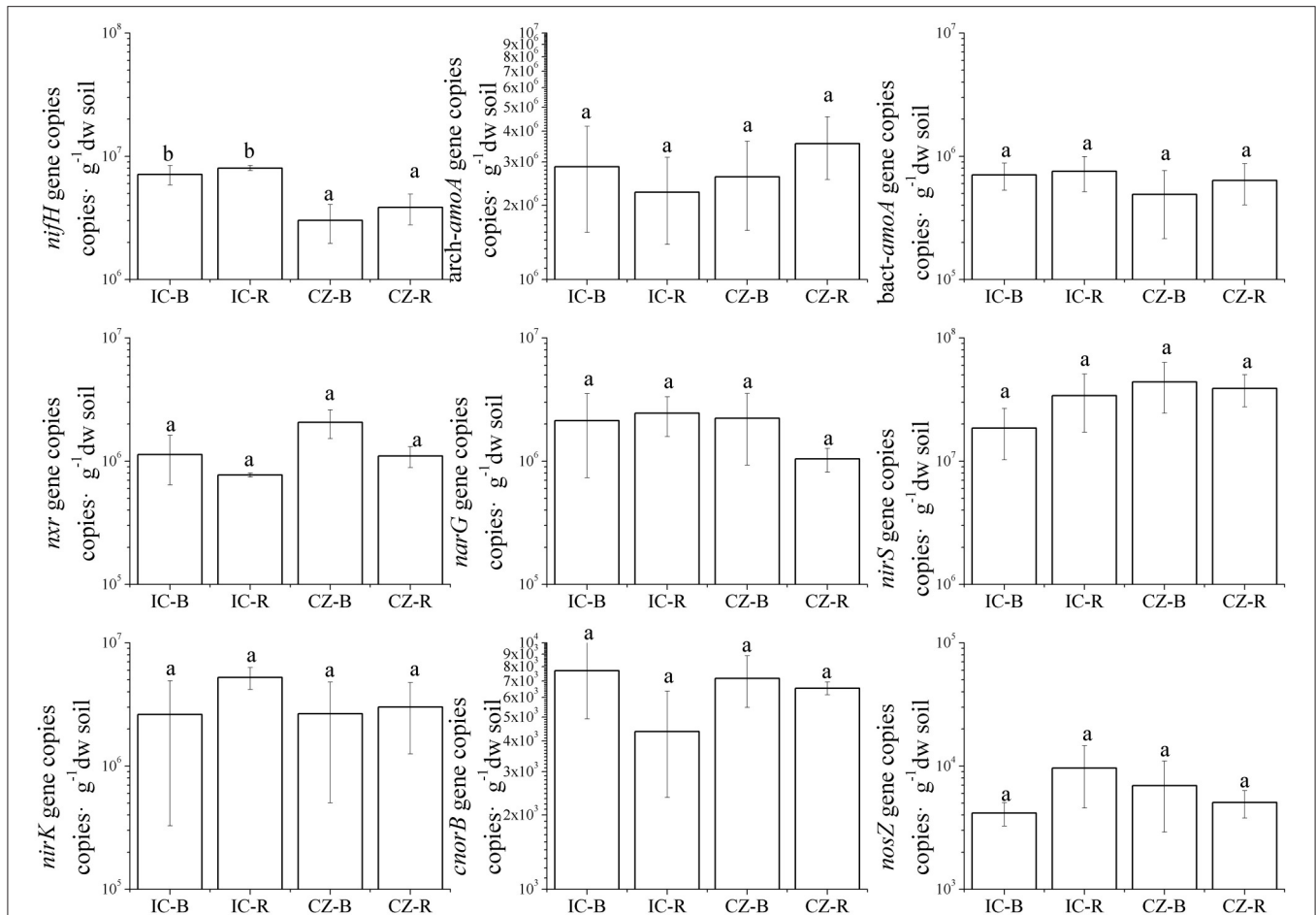


FIGURE 1 | Abundances of genes involved in nitrogen cycling in the rhizosphere and bulk soils of *Imperata cylindrica* and *Chrysopogon zizanioides*. Different letters indicate significant differences in samples (LSD, $P < 0.05$). Samples were bulk tailings of *I. cylindrica* (IC-B), rhizosphere samples of *I. cylindrica* (IC-R), bulk tailings of *C. zizanioides* (CZ-B), and rhizosphere samples of *C. zizanioides* (CZ-R).

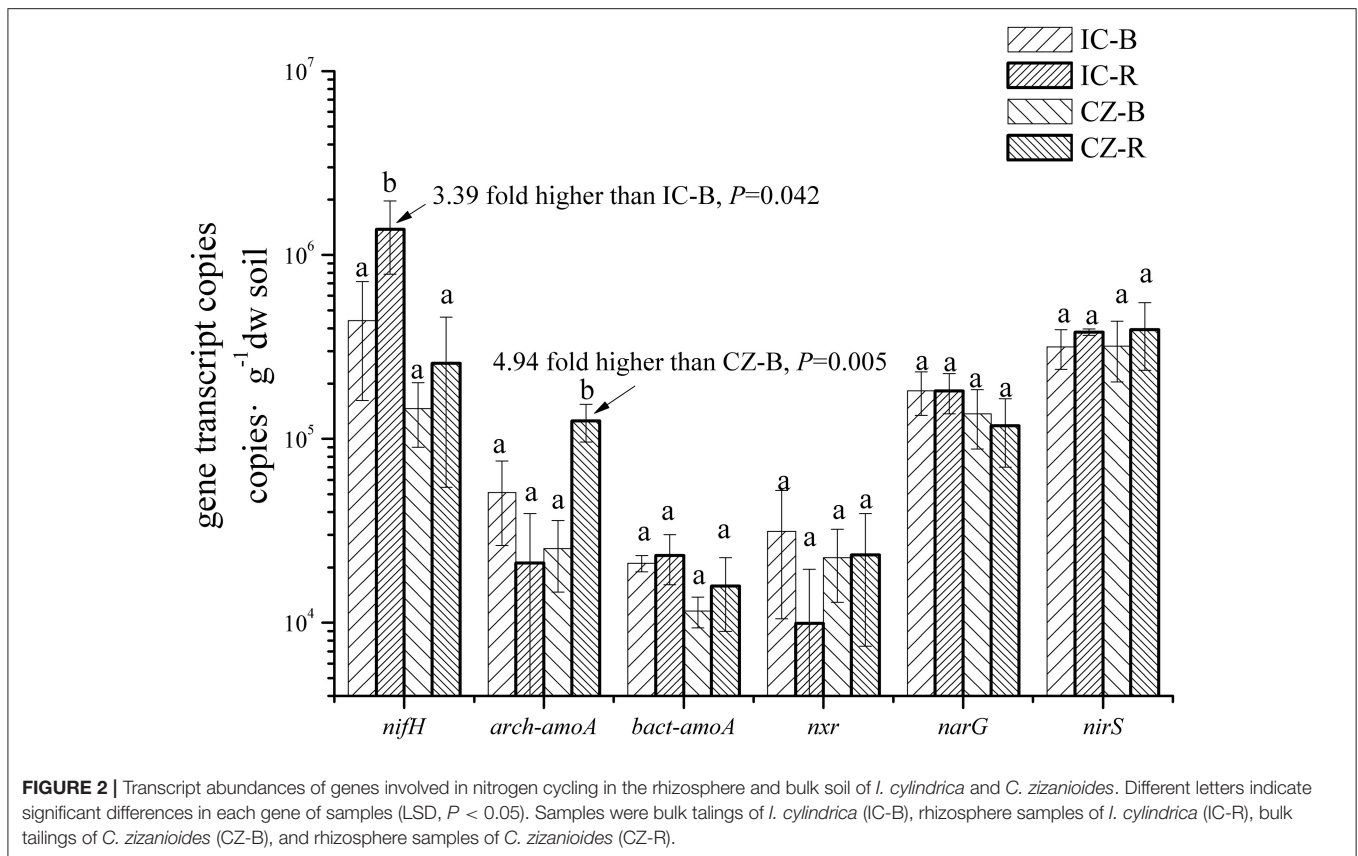
gene abundance: $Y_{\text{NFR}} = 0.027 + 4.987 \times 10^{-9} X_{\text{nifHcopy}}$, $F = 5.042$, $P = 0.049$; for transcript abundance: $Y_{\text{NFR}} = 0.038 + 2.921 \times 10^{-8} X_{\text{nifHtranscript}}$, $F = 23.704$, $P = 0.001$. Finally, the nitrification rate was enhanced by the increased transcript abundance of the *arch-amoA* gene (Table S1; $Y_{\text{DNR}} = 0.031 + 4.475 \times 10^{-7} X_{\text{arch-amoAtranscript}}$, $F = 8.736$, $P = 0.014$).

A redundancy analysis (RDA, **Figures 3A,C**) revealed that TOC, TN and the ratio of C:N were the primary environmental factors (according to forwards with a $P < 0.05$) defining the abundance of microbial communities involved in the nitrogen cycle. TOC, TN, and $\text{NH}_4^+\text{-N}$ were the primary dimensions affecting the transcript abundance of the microbial communities involved in the nitrogen cycle. Furthermore, variation partitioning indicated that the total N and $\text{NH}_4^+\text{-N}$ contents explained the most variance in gene abundance (**Figure 3B**) and transcript abundance (**Figure 3D**), respectively. In addition, the gene and transcript abundances of the *nifH* gene were positively correlated with the TOC and $\text{NH}_4^+\text{-N}$ content, respectively (Table S1). In particular, the *nifH* transcript was significantly enhanced by increasing the $\text{NH}_4^+\text{-N}$ content

($Y_{\text{nifHtranscript}} = -2.82 \times 10^5 + 3.02 \times 10^4 X_{\text{ammonia}}$, $F = 13.403$, $P = 0.004$).

DISCUSSION

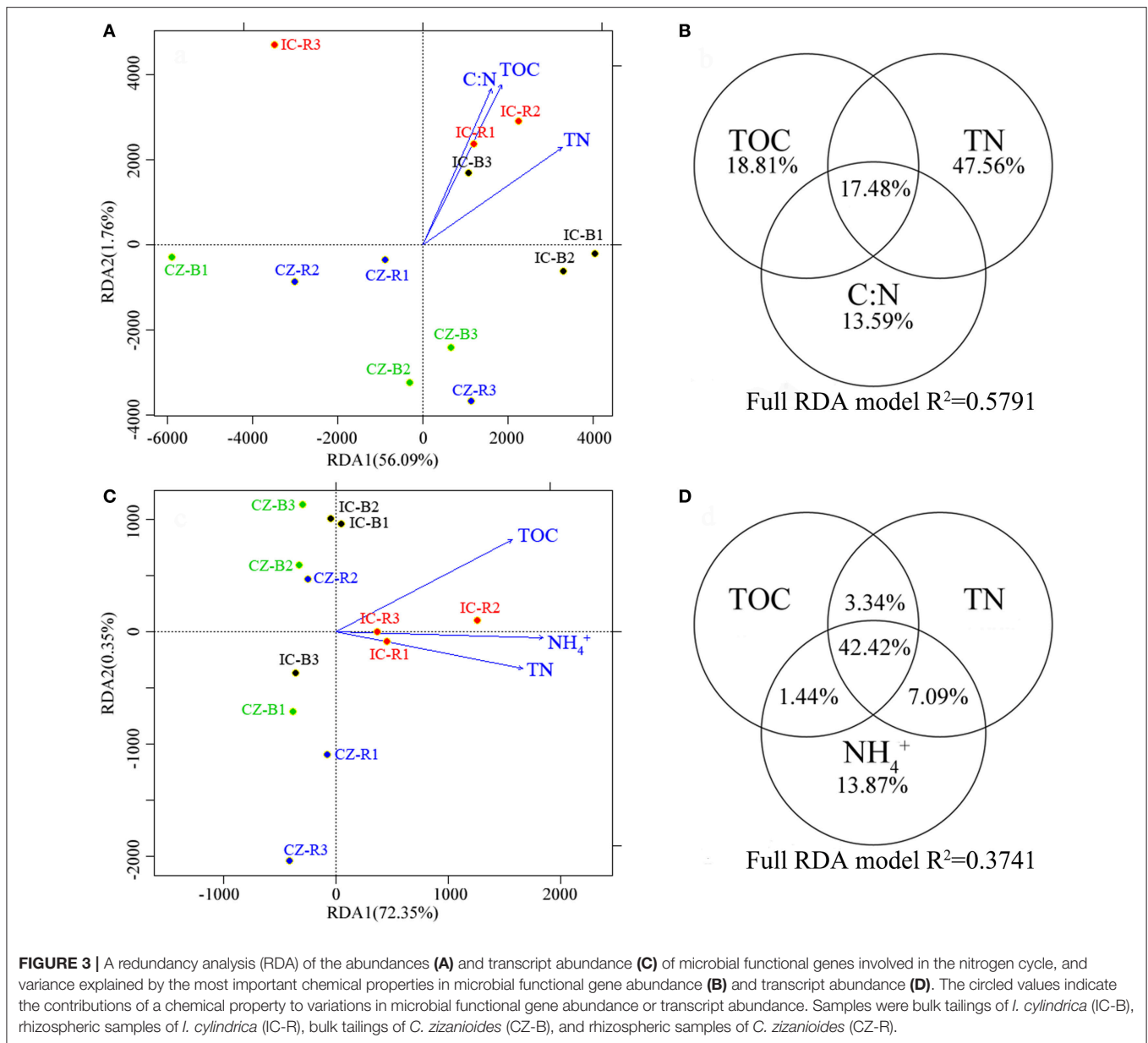
The major objective was to assess whether different plant communities influenced the N-cycling of functional microbes in the rhizosphere, which were reflected by the abundances and transcripts of N-cycling genes. The comparison between rhizosphere and bulk tailings next to the plant roots in this study could reflect that which genes were most heavily affected. The microbes harboring N-cycling genes in this study included nitrogen-fixing bacteria (with *nifH* gene), nitrifiers (including ammonia-oxidizing bacteria with the bacterial *amoA* gene, ammonia-oxidizing archaea with the archaeal *amoA* gene and nitrate bacteria with the *nxr* gene) and denitrifiers (microbes participating in one or more reduction reaction with the genes *narG*, *napA*, *nirS*, *nirK*, *norB*, and *nosZ*). In this study, the gene transcript abundance of *nirK*, *norB*, and *nosZ* were below the detection limit, maybe nitrite reduction was not the primary



energy source for these denitrifiers in mine tailings. The general knowledge that plant roots can stimulate rhizosphere heterotrophs and activate root-derived N in a soil environment (Thion et al., 2016); formed the basis of our hypothesis that the rhizosphere stimulates the abundances and activities of N-cycling functional microorganisms. Although The primers in the study used to amplify the functional genes for qPCR do not target all genes, the two assayed plants *I. cylindrica* and *C. zizanioides* stimulated the abundances and activities of some *nifH* gene and archaeal *amoA* gene, respectively. The rhizosphere in this study hardly influenced the abundances of the denitrifiers and the activities of denitrification rate activities. In the paddy and forest soil, the denitrification was unapparent under neutral aerobic (Lan et al., 2015; Pajares and Bohannan, 2016) and/or nitrogen deficiency conditions (Pajares and Bohannan, 2016). In addition, root exudate carbon was previously observed to be effective at decreasing the risk of N loss by increasing the potential for heterotrophic microbial immobilization (Fisk et al., 2015) and plant roots might not provide an appropriate survival environment for denitrifiers (Fisk et al., 2015). Under aerobic conditions, the nitrogen loss was mainly attributable to ammonia oxidizers (Florio et al., 2016), and in this study the stimulating effects of plant roots on ammonia oxidizers still did not aid the accumulation of nitrogen. In the revegetation area, plant litter and root exudate contributed a large amount of organic nitrogen (Baxendale et al., 2014; Thion et al., 2016), and the inorganic nitrogen mineralized by this organic nitrogen was provided to

plants and microbes (Thion et al., 2016). In the area revegetated by *C. zizanioides*, the phytocommunity obtained the inorganic nitrogen in the process of nitrogen transformation and grew well, but the TN in the tailings experienced no improvement compared to the bare wastelands assayed in our previous study (Li et al., 2016a). In contrast, plant-derived C from *I. cylindrica* stimulated the activity of nitrogen fixing bacteria. The organic carbon released by the root exudation and litter deposition of *I. cylindrica* increased significantly, causing a higher C:N ratio in the study field. Although no significant correlation between the C:N ratio and *nifH* gene and transcript abundance or nitrogen fixation rate was exhibited in this study, it has been confirmed that nitrogen-fixing bacteria could increase in abundance with the C:N ratio (Bürmann et al., 2003).

Plant species selection is a critical issue for the long-term success of phytoremediation in mine tailings (Mendez and Maier, 2008; Parraga-Aguado et al., 2014). Apart from showing tolerance to extreme pH values, high metal concentrations, drought, or salinity, plants must also address nitrogen limitation (Mendez and Maier, 2008; Zornoza et al., 2015). The two selected plant species in this field study exerted significant differential effects on N transformation under the nitrogen-limiting conditions. Plant biomass does not explain this difference, because the below-ground biomass of *C. zizanioides* was similar to that of *I. cylindrica* (Li et al., 2016b), whereas the aboveground biomass of *C. zizanioides* was higher (Shu et al., 2002). There are many causes of this rhizosphere effect, including different



types and contents of organic acids or other exudates produced by roots (Henry et al., 2008; Weng et al., 2013). The rhizosphere processes are an important component of the terrestrial nitrogen cycle (Finzi et al., 2015), and that the nitrogen increase by plants such as *I. cylindrica* exceeds their utilization, particularly with respect to nutrient uptake (Finzi et al., 2015). The TN was the most important factor influencing nitrogen cycle functional genes under neutral conditions in this field study. Plant responses to increased TN content may be critical for microbial adaptation (Huang et al., 2015b), and increased N may lead to higher vegetation density and enhance the competition between plants and microbes (Huang et al., 2015a). In addition, microbial biomass and community structure also influences soil nitrogen cycling by interacting with plants, modulating soil

properties (Waring et al., 2013), and immobilizing nutrients (Deng et al., 2016). Therefore, plant-derived C inputs by *I. cylindrica* stimulated nitrogen fixation and accelerated the nitrogen accumulation, which may be particularly useful for the rehabilitation of mine tailings, and might explain the extensive distribution of this plant in many different habitats (Tabor, 1949; Macdonald, 2004).

Changes in transcription of the *nifH* and *arch-amoA* genes were detected, although only a subset of some genes would be quantified in the qPCR assays. And few of the other genes demonstrated a significant response to plant species or rhizosphere, suggesting that nitrogen fixers and archaeal ammonia oxidizers are sensitive to the effects of plant species and the rhizosphere. This sensitivity may be associated with the

presence of nitrogen-poor substrates. Nitrogen fixers are a vital source of nitrogen in nitrogen-poor environments, which are widely found in different types of soils (Bürmann et al., 2003; Coelho et al., 2009; Huang et al., 2011; Chinnadurai et al., 2014) and rhizospheres (Meng et al., 2012). In our previous studies, the application and establishment of certain types of vegetation in mine tailings promotes the presence of important free-living nitrogen fixers in these ecosystems (Zhan and Sun, 2011, 2012). And some studies (Huang et al., 2011; Chinnadurai et al., 2014; Collavino et al., 2014) also indicated that NH_4^+ -N improved the abundance and diversity of nitrogen-fixing bacteria. Moreover, stimulation of nitrification in the rhizosphere of *C. zizanioides* was associated with increases in archaeal ammonium monooxygenase (arch-*amoA*) transcript abundance. In contrast, no significant differences between the rhizospheres and bulk tailings were identified for bacterial genes involved in nitrification (*bact-amoA* and *nxr*) (Figures 1, 2). Archaeal *amoA* and bacterial *amoA* genes are known to dominate nitrification activity in different soil niches based on NH_4^+ -N availability (Taylor et al., 2012; Sun et al., 2015). Ammonia-oxidizing archaea seem to prefer the low- NH_3 substrate conditions in mine tailings, and are more sensitive to changes in soil properties (Di et al., 2010), although no statistical correlation between the gene and transcript abundances of archaeal *amoA* and NH_4^+ -N content was observed (Pearson correlation: $r = -0.169$, $P_{\text{adjust}} = 0.829$; $r = -0.311$, $P_{\text{adjust}} = 0.648$, respectively).

In conclusion, our data did not support the hypothesis that the rhizosphere stimulates the abundances and activities of

N-cycling functional microorganisms. The nitrogen increased by *C. zizanioides* was used by plants and microbes as well as reduced by ammonia-oxidizing archaea, which caused no improvement in the TN in mine tailings. In contrast, plant-derived C from *I. cylindrica* stimulated the nitrogen fixation process in the rhizosphere and further accelerated the nitrogen accumulation in mine tailings. Therefore, we suggest that *I. cylindrica* exerts much stronger effects on nitrogen accumulation than *C. zizanioides*, the two selected plant species in this field study.

AUTHOR CONTRIBUTIONS

YL, ZJ, and QS designed the study; YL, JC, YY, JZ, and DW targeted the research question; YL contributed to writing the manuscript; QS found the funding to carry out the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fenvs.2017.00079/full#supplementary-material>

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

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