EFFECTS OF REPLACEMENT CONTROL WITH *HUMULUS SCANDENS* ON THE RHIZOSPHERE MICROBIAL COMMUNITY DIVERSITY OF THE INVASIVE PLANT, *ALTERNANTHERA PHILOXEROIDES*

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Abstract. The impacts of invasive plants are known to be mediated by plant-microbial interactions. Many ecologists are interested in the variance of the microbial community structure of invasive plants and native plants in the rhizosphere soils. In order to clarify the interaction mechanism between native plants and invasive plants, we reported the characteristics of soil microbial communities in invasive plants under different degrees of replacement plant. *Alternanthera philoxeroides* (Mart.) Griseb. is a serious invasive weed in many regions. Rhizospheric soils of *A. philoxeroides* with different degrees of substitution by *Humulus scandens* (Lour.) Merr. (unreplacement, low degree, high degree, replacement and CK using its coverage in the invaded ecosystems) were collected. pH, soil electrical conductance, available phosphorous and available potassium were major factors to alter the microbial community structure of *A. philoxeroides* in the rhizosphere. Plant replacement significantly reduced the abundance of *Acidobacteria* and *Verrucomicrobia*, but not *Chloroflexi* and *Actinobacteria*. Low and high degrees of *H. scandens* increased the richness of soil fungal community. Overall, the results suggested that rhizosphere microbes were changed by replacement plant of this invader in the novel environment, which provided a theoretical basis for the control of *A. philoxeroides*.
Keyword: *microbial communities, replacement control, invasive species, native species, rhizosphere microorganisms*

Introduction

Biological invasion has become one of the global environmental problems (Sala et al., 2000), and invasive species can disrupt native mutualism, cause population declines, reduce biodiversity, and alter ecosystem function (Claudia and Florens, 2011). At present, the methods to control invasive plants are focused on mechanical control, chemical control, biological control and ecological control. Among the available weed control methods, mechanical control is considered as a type of physical disturbance, which can be time, energy and financially consuming. Meanwhile it cannot last for a long time (Jia et al., 2009). Due to the increasing health concerns of chemical control (Lin et al., 2016) and the possible non-target effects of biological control (Liu et al., 2015), as the main technology of ecological control methods, replacement control has been raised more and more attention (Li et al., 2015). In the study of the replacement control of invasive plants, previous students found that microorganisms living in the rhizosphere, where soil is surrounded and affected by plant roots, have long been recognized to have significant effect on plant nutrition and health (Moreau et al., 2015). Most organisms are directly or indirectly associated with mutualistic partner, just like plants interact with soil

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microorganisms by positive feedback or negative feedback (Wagg et al., 2011). Meanwhile, the decomposers provide the plants with available nutrients by degrading plant residues which is commonly considered as one of the main drivers of plant-microbe interactions (Yu et al., 2011; Shen et al., 2016). Root-associated organisms and their consumers influence the quality, direction, and flow of energy and nutrients between plants and decomposers (Ehrenfeld and Scott, 2001; Bever, 2002; Wardle et al., 2004). It suggests that the research on replacement control of invasive plants is also more inclined to change the relationship between native plants, invasive plants and soil microorganisms.

Alternanthera philoxeroides (Mart.) Griseb. (Amaranthaceae) originated in the Parana River region of South America and is considered a serious weed in the United States, China, Australia, New Zealand, Indonesia, India and Thailand (Julien et al., 1995). The control of the invasive plant is difficult because mechanical, chemical and biological control methods are not effective on sustained reduction in biomass (Jia et al., 2009; Reeves, 2017). We observed that *Humulus scandens* (Lour.) Merr. (Moraceae) often coexist with *A. philoxeroides* in the same habitats in field and significantly affect the growth of the later species. which is a climbing therophyte vine and vegetative growth, usually cluster together with strong ability to adapt to the cold and drought and found cohabiting with *A. philoxeroides* currently. It has various positive uses. For example, it can be harvested for medicinal use (Chen et al., 2012), material of industry (Gargiullo, 2005) and soil and water conservation (Li et al., 2014).

To test these hypotheses, we conducted field investigations of the microbiome interactions between *H. scandens* and *A. philoxeroides*, addressing three questions: (1) How do soil nutrient contents change? (2) How has the soil microbial community structure changed in the change of substitution degree? (3) What is the relationship between soil nutrients and soil microorganisms?

Materials and Methods

Site description

Samples were obtained from Anhui agricultural university in Hefei, China (31°86'N, 117°25'E). The study area has a Subtropical monsoon humid climate, with an annual mean temperature of approximately 15.7 °C and an annual precipitation of approximately 1000 mm. The type of vegetation of these samples are: *H. scandens*(Humulus), *A. philoxeroides* (Amaranthaceae), *Digitaria sanguinalis* (Poaceae), *Echinochloa crusgalli* (Poaceae), *Setaria viridis* (Poaceae).

Experimental design

In August 2016, rhizospheric soil samples with different degrees of *A. philoxeroides* and *H. scandens* invasion were collected from the chosen area, i.e., unreplacement (0%, A), low degree (<35%, A+H), high degree (>75%, H+A) , replacement(100%, H), and CK (no *A. philoxeroides* and *H. scandens*) using the coverage of *H. scandens* in the replacement ecosystem in spring 2015. Three soil samples within an approximately 20 cm radius of *H. scandens* and *A. philoxeroides* rhizosphere from each replacement degree in each site were collected. The rhizosphere soil collection was used to refer to the Inderjit method (Inderjit, 1997) and the soil of 1cm around the root of the plant was interrhizosphere soil. A total of fifteen treatment combinations were obtained: 3 sample areas × 5 replacement degrees. The soil samples were passed through a 2 mm sieve to

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remove leaves, plant roots, and gravel. All soil samples from one site were homogenized by thorough mixing and then stored for further processing. Soil samples can be divided into two parts, the part is used for the determination of soil physicochemical properties, the other part into sealed sterile bags, put in ice packs back to lab, to -80 °C cryopreservation for DNA extraction.

Determination of soil physicochemical properties

Soil pH values were measured using a glass electrode (1:2.5 soil–water ratios) after shaking the samples for approximately 30 min to equilibrate. Soil organic matter was analyzed using the method of $K_2Cr_2O_7$–H_2SO_4 oxidation. Soil nitrogen (N) concentration was determined by the Semimicro-Kjeldahl method. Soil phosphorus (P) concentration was determined using the Mo-Sb antispetrophotography method. Soil potassium (K) concentration was determined with the NaOH-melt method.

Determination of structure and diversity in soil microbial communities

Genomic DNA was isolated from the 15 samples using a PowerSoil DNA isolation kit (MO Bio Laboratories, Inc. Carlsbad, CA) following the manufacturer's instructions. The internal transcribed spacer (ITS) egion was amplified using fungal-specific primers: ITS3F(5'-GCATCGATGAA GAACGCAGC-3'), ITS4R(5'-TCCTCCGCTTATTGATATGC-3'). Bacterial 16s rRNA gene amplicons were amplified using primers 515F(5'-GTGCCAGCMGCCGCGGTAA-3'), 907R (5'-CCGTCAATTCMTTTRAGTTT-3'). DNA regions were amplified using the HotStarTaq Plus Master Kit (Qiagen, Valencia, CA). Amplicons from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, USA). Paired-end 2×250 bp sequencing was performed on an Illumina MiSeq instrument (Illumina Inc., San Diego, CA, USA).

Statistical analyses

OTU richness and diversity indices (richness, Shannon, inverse Simpson and Pielou's evenness), together with accumulation curves were calculated using the QIIME (http://qiime.org/index.html) (Vishnu et al., 2021). Cluster analysis was performed using the UPGMA method. Canoco 4.5 was used to perform the RDA (Abbas et al., 2021). R statistical software 3.2.5 was used to perform OUT and heatmap.

All data were checked for deviations from normality and homogeneity of variance before analysis. The effects of the degree of *H. scandens* on soil microbial communities of *A. philoxeroides* in the rhizosphere and Shannon–Wiener diversity and evenness indices of soil microorganisms were determined by analysis of variances (ANOVA) with site considered as a block effect using IBM SPSS 20.0. LSD was used at 0.05 of probability for comparing the differences between different treatment means.

Results

Soil physicochemical properties

Soil pH value increased by 1.1% to 6.5% as the replacement degree of increased (*Table 1*, P < 0.05) whereas high degrees had no significant effect on soil pH value (*Table 1*, P > 0.05). High degrees of increased soil electrical conductance; the difference between the effects of low, high and none degrees of replacement on soil electrical

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conductance was not significant (*Table 1*, P > 0.05). Soil available phosphorous concentration under low and high degrees of replacement was significantly higher than that under none degrees (*Table 1*, P < 0.05), which is the same as soil available potassium (*Table 1*, P < 0.05). Both none, low and high degrees did not significantly change soil organic matter, total nitrogen, total phosphorous, total potassium concentrations (*Table 1*, P > 0.05).

Table 1. *Physicochemical properties of the soil samples in five replacement situation*

Treatment	Soil pH	EC×10³us/cm	TN g/kg	TK g/kg	TP g/kg	AP mg/kg	AK mg/kg	Organic matter g/kg
H	7.68±0.26ab	0.11±0.017ab	2.81±0.39a	12.73±1.64a	0.27±0.071a	5.4±1.25a	195.67±15.044a	11.56±2.67a
H+A	7.98±0.20a	0.16±0.02a	2.62±0.12a	12.42±0.86a	0.41±0.19a	6.35±1.37a	196.33±25.58a	11.22±3.72a
A+H	7.57±0.168b	0.14±0.026ab	2.31±1.18a	12.84±0.94a	0.42±0.11a	5.01±0.59a	197.67±18.56a	8.67±1.46a
A	7.49±0.07b	0.11±0.00b	2.28±0.46a	14.11±1.75a	0.25±0.03a	0.68±0.49b	148.33±3.79b	9.4±0.75a
CK	7.75±0.05ab	0.14±0.058ab	3±0.42a	11.9±1.07a	0.39±0.16a	6.89±1.29a	212.33±10.50a	8.92±1.92a

The values in the table represent means of the values of the three replicates with the same replacement degree of *H. scandens*. Data with different superscript letters in a vertical row indicate significant difference (P < 0.05).H: replacement (100%), H+A: high degree of replacement (>75%), A+H: low degree of replacement (<35%), A: none degrees of replacement (0%), CK (no A. philoxeroides and *H. scandens*

Structure of soil microbial communities

Analyzing the microbial communities associated with the rhizospheres of *A. philoxeroides*, we obtained 151,757 and 648,340 total seqs, which resulted in 20,184 and 9,411 OTUs for bacteria and fungi, respectively (*Table 2*). The levels of bacteria diversity (chao1, Shannon, Simpson,) of *A. philoxeroides* in the rhizosphere tended to be higher in the high degrees than in the other samples (*Table 2*). However, the levels of fungi diversity of *A. philoxeroides* in the rhizosphere tended to be higher in the low degrees than in the other samples (*Table 2*).

Table 2. *Shannon–Wiener diversity(H'), Simpson, chao1 index and average genetic diversity of bacteria and fungi in five replacement situation*

	Replacement situation	No. of seqs	No. of OTUs	chao1	Observed species	Simpson	Shannon
Bacteria	CK	10289	1232	1713.026	1000	0.9786	7.6742
	H+A	8180	1394	2004.943	1260	0.9917	8.6321
	H	11296	1319	1676.083	1035	0.9821	7.9177
	A	9512	1457	1995.713	1223	0.9832	8.2654
	A+H	11308	1326	1711.178	1034	0.9831	7.9260
Fungi	CK	41204	682	836.716	593	0.7212	3.7890
	H+A	49296	514	672.494	416	0.6697	2.9415
	H	48606	673	796.176	547	0.9106	4.8395
	A	33288	512	699.756	470	0.4695	2.4016
	A+H	43720	756	900.246	631	0.9165	5.0244

Statistics of No. of seqs for each sample: sum the values of each column, that is, the total number of sequences for each sample. Statistics of No. of OTUs for each sample: in each column of values, all values greater than 0 are recorded as 1 and summed, that is, the total number of OTUs for each sample

A total of 30 distinct bacterial phyla (relative abundance>1%) were detected across all samples. The most abundant sequences of every degree of *H. scandens* were affiliated with the phylum *Acidobacteria* (33.4-18.7% of total relative abundance), followed by *Proteobacteria* (23.5-16.7%), *Chloroflexi* (30.3-12.9%), *Actinobacteria* (12.7-3.4%), *Verrucomicrobia* (7.5-5.3%), *Gemmatimonadetes* (5.8-2.6%), *Bacteroidetes* (2.8%-1.1%), *Nitrospirae* (2.5-1.2%) (*Figure 1a*). The abundant sequences of *Acidobacteria* and *Verrucomicrobia* decreased with the increase of substitution, but increasing degrees of *H. scandens* increased the abundance of *Chloroflexi* and *Actinobacteria* of soil bacterial community in rhizosphere of *A. philoxeroides*.
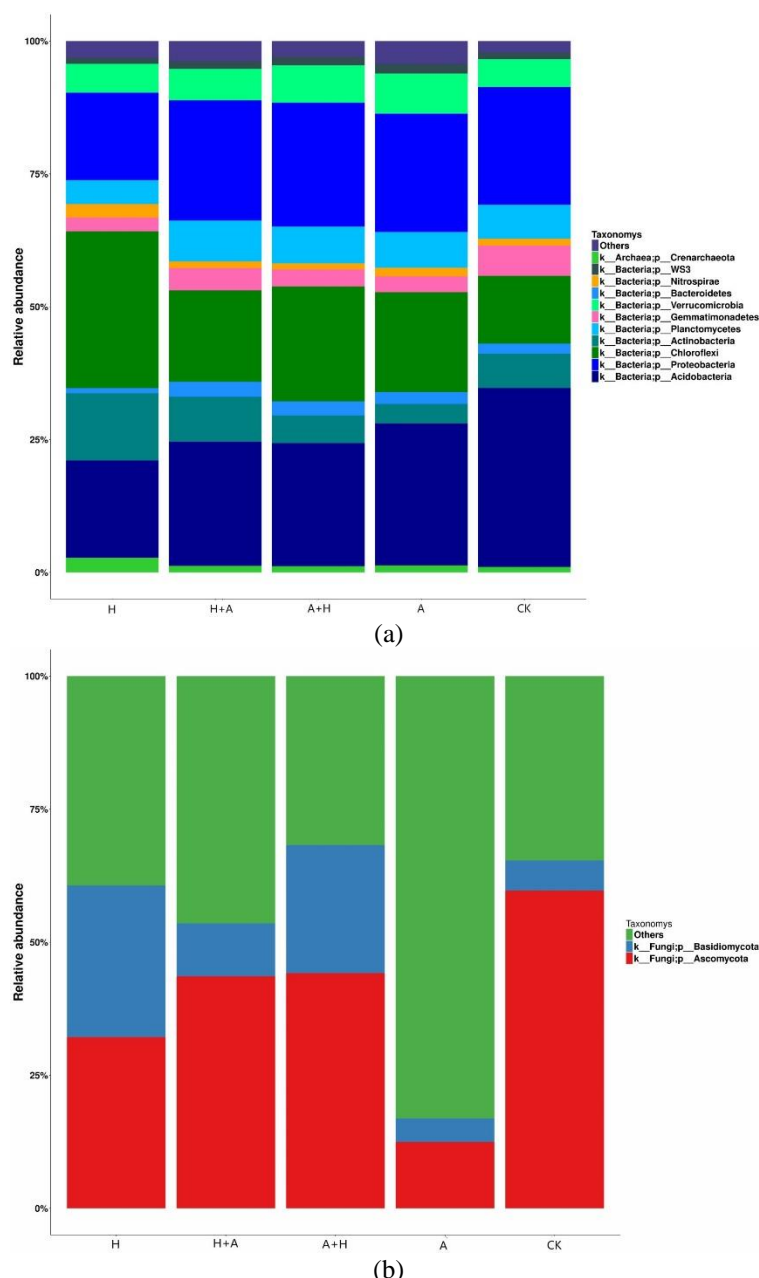

(a)

(b)

Figure 1. *Mean relative abundances of taxa (phylum levels) (a) bacterial and (b) fungal communities within each degree. The group 'Other' encompasses unclassified sequences together with phylums representing ≤0.5% of total sequences*

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Fungal communities were dominated by the phylum *Ascomycota* (59.7-12.6%) and *Basidiomycota* (57.3-10.1%). Compared with the rhizosphere microorganism in the unsubstituted region, *Ascomycota* in other samples were significantly reduced (*Figure 1b*).

Correlation analysis between microorganisms and soil physicochemical

Results showed that the different classes of bacteria and fungi were significantly correlated with these soil parameters (*Table 3*). Factors such as pH and nutrient status are the main drivers controlling composition and diversity of soil microbial communities. *Acidobacteria* had significant negative (r=-0.592, p=0.02) relationships with soil electrical conductance. *Proteobacteria* and *Nitrospirae* had significant positive relationships with pH (r=0.545, p=0.027) (r=0.692, p=0.004) and soil electrical conductance (r=0.607, p=0.006) (r=0.682, p=0.005). *Gemmatimonadetes* had positive relationship with available phosphorous (r=0.532, p=0.034), available potassium (r=0675, p=0.002) and total phosphorous (r=0.515, p=0.035). *Ascomycota* had positive relationship with available phosphorous (r=0.662, p=0.002) and available potassium (r=0.672, p=0.002). *Basidiomycota* and *Glomeromycota* had significant positive relationships with pH (r=0.683, p=0.002) (r=0.815, p=0.000053), soil electrical conductance (r=0.684, p=0.005) (r=0.604, p=0.006) and available phosphorous (r=0.655, p=0.005) (r=0.701, p=0.003). *Chytridiomycota* had positive (r=0.775, p=0.001) relationship with soil organic matter.

Table 3. Correlation between soil properties and the different bacterial and fungal phylums

	Phyla	Soil pH	EC	TN	AP	AK	TK	TP	Organic matter
Bacteria	Acidobacteria	-0.283	-0.592*	0.244	0.067	0.131	-0.063	-0.328	0.136
	Proteobacteria	0.545*	0.607*	0.258	0.278	0.073	-0.359	0.254	-0.385
	Chloroflexi	-0.275	0.038	-0.366	-0.371	-0.257	0.417	0.24	0.059
	Actinobacteria	-0.216	0.161	-0.027	-0.138	-0.169	0.067	-0.092	-0.497
	Planctomycetes	-0.216	-0.097	0.214	-0.148	-0.07	0.037	0.053	-0.165
	Verrucomicrobia	-0.49	-0.162	0.278	0.349	0.347	-0.318	-0.169	-0.341
	Gemmatimonadetes	-0.09	0.211	0.114	0.532*	0.675**	-0.377	0.515*	-0.069
	Bacteroidetes	-0.222	-0.241	-0.101	0.044	0.18	0.066	0.256	0.08
	Nitrospirae	0.692**	0.682**	0.235	0.401	0.269	-0.193	0.293	0.279
Fungi	Ascomycota	0.181	-0.039	0.307	0.662**	0.672**	-0.384	0.122	0.164
	Basidiomycota	0.683**	0.684**	0.252	0.655**	0.495	-0.426	0.402	0.032
	Zygomycota	0.094	-0.299	0.411	0.372	0.26	-0.143	-0.212	0.262
	Glomeromycota	0.815**	0.604*	0.258	0.701**	0.513	-0.235	0.446	0.42
	Chytridiomycota	-0.17	-0.124	0.093	-0.105	0.079	0.201	0.03	0.775**

* indicates significant differences at the 0.05 probability level. ** indicates significant differences at the 0.01 probability level

Redundancy analysis was conducted to quantify the relative influence of the selected variables on microbial community composition (*Figure 2a*), which showed the relationship between the bacteria and soil chemistry parameters (*Figure 2b*). The first axi and the second axi explained 36.32% and 11.53%. Notably, the first axi had relationship with soil electrical conductance (r=-0.6278) and available potassium (r= 0.62059); the

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second axi had relationship with total nitrogen (r= -0.562422) and pH (r= -0.519036). The result of relative between fungus and soil properties showed that the first axi and the second axi explained 25.89% and 13.43% (*Figure 2b*). The first axi had relationship with soil electrical conductance (r=-0.72508); the second axi had relationship with total nitrogen (r=0.7949).
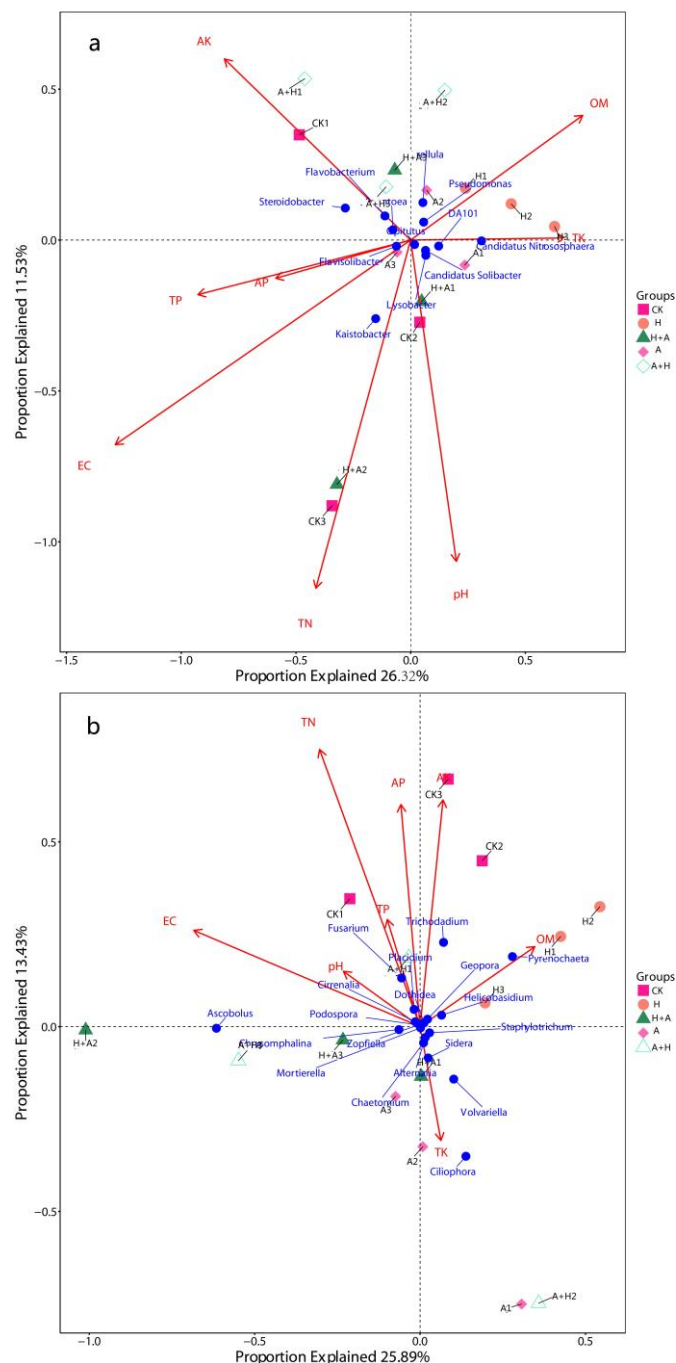
Figure 2. Distance-based redundancy analysis (db-RDA) biplot of (a) bacterial and (b) fungal communities. Only the environmental variables that significantly (P < 0.05) explained variability in microbial community structure are shown (arrows). The direction of the arrows indicates the direction of maximum change of that variable, whereas the length of the arrow is proportional to the rate of change

Surprisingly, environmental variables of pH, soil electrical conductance, available potassium, available phosphorous appeared to exert an important effect on the reference soils community composition.

Discussion

Currently, there are many studies on the impact of alternative control of invasive plants on soil physicochemical properties and microbial community structure (Lankau, 2010). However, few studies have used *A. philoxeroides* with *H. scandens* as alternative controls (Cao et al., 2013). This study suggested that soil physicochemical, including pH, soil electrical conductance, total nitrogen, available phosphorous and available potassium, had positive relationship with different degrees of replacement. Meanwhile, there were significant differences in the microbial diversity of *A. philoxeroides* in the rhizosphere at different degrees. Plant root exudates mostly are inorganic salts, soluble sugar, organic acid and other active substances, which can change the soil physicochemical properties and provide nitrogen source and carbon source for the growth of microorganisms, thereby changing the composition of soil microorganisms (de Vries et al., 2012).

Previous studies have shown that plant replacement significantly reduced the abundance of *Acidobacteria* and *Verrucomicrobia*, but not *Chloroflexi* and *Actinobacteria*. This result may be attributed mainly to the fact that *Acidobacteria* and *Verrucomicrobia* belong to the oligotrophic bacteria that are sensitive to soil nutrient content and decrease with the increase of soil nutrient content (Ramirez et al., 2012). Ramirez found that nitrogen application reduced the relative abundance of *Acidobacteria* and *Verrucomicrobia* (Ramirez et al., 2012). *Actinobacteria* are potent plant polysaccharide degrading microbes that play an important role in plant biomass degradation by producing a variety of lignocellulolytic enzymes and amylolytic enzymes in soil and various other environments (Kanokratana et al., 2011). Low and high degrees of invasion increased the richness of the soil fungal community and low degree was elevated significantly, which was the same as the results of *Wedelia trilobata* (Si et al., 2013). That roots of native plant proliferate sufficiently impedes the nutrient uptake of invasive species, which correlate strongly with the mycorrhizal dependence of species encountered in the invaded range (Stinson et al., 2006).

The correlation analysis between dominant population and soil physicochemical properties showed that pH, soil electrical conductance, total nitrogen, available phosphorous and available potassium were the main soil factors affecting microbial community diversity (Saggar et al., 1999; Liu et al., 2015). Redundancy analysis indicated that the bacteria community composition was most strongly affected by total nitrogen content followed by pH, soil electrical conductance and available potassium. The previous study showed that there was a significant negative correlation between *Acidobacteria* and pH (Campbell et al., 2010). In contrast, there was no correlation between *Acidobacteria* and pH in this study. Soil inorganic salts, organic matter and plants are other factors that alter *Acidobacteria* abundance (Liu et al., 2014). *Proteobacteria* and *Nitrospirae* had significant positive correlation with pH and soil electrical conductance, and nitrogen content was reported to be the factor that changed pH (Noah et al., 2012). Meanwhile, both *Proteobacteria* and *Nitrospirae* are active in the nitrogen cycle (Janssen, 2006). *Gemmatimonadetes* had significant correlation with nitrogen and phosphorus content., and recently, one of its few representatives, Gemmatimonas phototrophica, was found to contain purple bacterial photosynthetic

reaction centers, which appear to favour soil and wastewater treatment-associated habitats (Zeng et al., 2016). The results of PE Mortimer also indicated that soil electrical conductance and total nitrogen were the main soil factors of soil fungal community diversity (Mortimer et al., 2008). The main dominant species of *Ascomycota* and *Basidiomycota* are mainly pathogens. Studies have shown that some pathogenic fungi, such as *Fusarium* (Tan et al., 2002), *Nimbya* (Pomella et al., 2007) and *Alternaria* can infect *A. philoxeroides*.

By leaching and secretion activity in the alternative control process of *A. philoxeroides* changed soil physicochemical properties (Coleman et al., 2000), which also indirectly changed the soil microbial community structure (Saggar et al., 1999), making the resources conducive to the growth of *H. scandens*. The previous studies showed that replacement plants can alter soil physicochemical properties by changing the soil microbial community (which is closely related to plant growth and development), thus changing the invasion process (Yan et al., 2011). After the intercropping of *Lolium Perenne* and *Trifolium repens*, *T. repens* also changed the rhizosphere microbial community structure of Triticale (Hiddink et al., 2005), the reason of which may be that plants changed the soil microbial community through rhizosphere exudates and facilitate replacement plant growth (Bending and Lincoln, 1999; Smolinska and Horbowicz, 2010). However, elucidating the effects of single species of soil biota outside of the context of the entire soil community may not accurately describe the interactions that occur in nature (Reinhart and Callaway, 2006). The development of prevention and control of *A. philoxeroides* may be focus on the species interaction mechanism (*Acidobacteria*, *verrucomicrobia Chloroflexi*, *Actinobacteria* and fungi of *Ascomycota* and *Basidiomycota*), and the synergistic effects of the entire below-ground community may be particularly useful in determining the effects of soil biota on plants in their native and nonnative ranges.

Conclusion

In the current study, we provided evidence that pH, soil electrical conductance, available phosphorous and available potassium were major factors to alter the rhizosphere microbial community structure of *A. philoxeroides*. Plant replacement significantly reduced the abundance of *Acidobacteria* and *Verrucomicrobia*, but not *Chloroflexi* and *Actinobacteria*. Low and high degrees of *H. scandens* increased the richness of soil fungal community. Overall, the results suggested that rhizosphere microbes were changed by replacement plant of this invader in the novel environment, which provided a theoretical basis for the control of *A. philoxeroides*.

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Additional Information. The authors declare no competing financial interests.

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