Biol Invasions
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ORIGINAL PAPER

Response of soil bacterial communities to secondary compounds released from *Eupatorium adenophorum*

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Abstract Exotic plant species can benefit from altering soil microbial communities; however, the knowledge of the mechanisms through which this occurs is limited. The exotic species, *Eupatorium adenophorum* is a perennial plant that is aggressively invading southern China. Its invasiveness is attributed partially to its ability to release secondary chemicals that affect native plant species. Nevertheless, their effect on soil bacterial communities has rarely been explored. Here, we used natural fresh leaf and root leachate of *E. adenophorum*, and two pure, phytotoxic chemicals amorpha-4,7(11)-dien-8-one, and 6-hydroxy-5-isopropyl-3,8-dimethyl-4a,5,6,7,8,8a-hexahydraphthalen-2(1H)-one to evaluate: (a) short-term (3 days) effects of secondary chemicals on soil bacterial communities in mixed forests and (b) long-term (6 months) effects of leaf leachate on soil bacterial communities in three habitats. Additionally, the composition and diversity of soil bacterial communities were explored with high throughput sequencing using IlluminaMiSeq. In the short-term experiments, all treatments affected soil bacterial communities, but leaf leachate most significantly reduced soil bacterial richness and diversity. In the long-term experiments, leaf leachate altered bacterial communities in all soil samples, and reduced bacterial

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richness and diversity in soils from the forest and wasteland, but increased bacterial richness and diversity in soils from roadside. Redundancy analysis indicated that changes in bacterial communities were associated with soil organic carbon, nitrogen content, and pH. These results indicate that the water-dissoluble secondary chemicals of *E. adenophorum*, especially from its leaves, have an impact on soil bacterial communities, though the degree of the impact depends on soil ecotype.

Keywords Allelochemicals · Allelopathy · Exotic plant invasion · Soil microbes · Community invasibility

Introduction

There is a strong relationship between soil biota and aboveground vegetation (Hooper et al. 2000; van der Heijen et al. 2008). Exotic plant invasions can alter the links between native plants and soil communities (Wolfe and Klironomos 2005) by changing the quantity and quality of litters, leaf leachates, and root exudates, which would, in turn, alter the input of nutrients in the soil. This resource-altered environment could have substantial effects on the composition of soil bacterial communities (Drenovsky et al. 2004; Eilers et al. 2010; Ramirez et al. 2010). Some studies indicate that the performance of exotic plant species' benefits from positive feedback from changing soil microbial communities (Callaway et al. 2004; Suding et al. 2013; Maron et al. 2014; Meisner et al. 2014; Piper et al. 2015). Though the effects of soil biota on invasive plants have been receiving much attention lately, little is known about how exotic invasive species affect soil bacterial communities.

Among the various compounds released into the soil from plants, secondary metabolic chemicals of exotic plants are an important component. The success of exotic species in plant–plant interactions is often explained by the allelopathic potential of secondary compounds (Hierro and Callaway 2003). However, recently, evidence of the altered interactions between native plants and soil communities has been taken into consideration as well. Exotic plants can release secondary compounds into the soil environment from their litter, leaf leachate and root exudate, which negatively affect native soil communities because they lack a co-evolutionary history (Callaway and Aschehoug 2000). For example, invasive plant *Centaurea diffusa* releases the antimicrobial agent 8-hydroxyquinoline from its roots (Vivanco et al. 2004) and the allyl isothiocyanate, an allelochemical of *Alliaria petiolata*, can disrupt legume-rhizobia mutualism (Portales-Reyes et al. 2015). In addition, differences in litter quality and root exudate composition may influence soil bacterial communities differently (Haichar et al. 2008; Eilers et al. 2010; Strickland et al. 2009). Previous studies reveal the significance of these differences on microbial community structures, especially when the species involved have strongly contrasting litter chemistries (Chapman et al. 2006; Strickland et al. 2009; Lorenzo et al. 2013).

Eupatorium adenophorum Spreng. is an aggressive invasive plant in Asia, Oceania, Africa, Europe, and North America (Shrestha et al. 2009; Muniappan et al. 2009). It can invade disturbed habitats such as roadsides, abandoned fields, artificial plantations, and relatively intact forest understories, form monodominant communities, and reduce native species richness (Inderjit et al. 2011). *E. adenophorum* can produce a suite of allelochemicals in its leaves and roots (Yang 2006; Yang et al. 2008; Liao et al. 2014; Zhang et al. 2012). Some of these allelochemicals, such as amorpha-4,7(11)-dien-8-one (DTD) and 6-hydroxy-5-isopropyl-3, 8-dimethyl-4a, 5, 6, 7, 8, 8a-hexahydronaphthalen-2(1H)—one (HHO) can only be found in *E. adenophorum*, and are therefore novel to native species (Yang 2006; Yang et al. 2008, 2013). The allelopathic potential of leaf and root leachate on native species has been well documented (Zheng and Feng 2005; Gui et al. 2011; Jia et al. 2009). Alternatively, *E. adenophorum* could facilitate its invasion by altering soil microbial communities (Niu et al. 2007; Sun et al. 2013a, b). Soil biota in invaded sites improves the growth of *E. adenophorum,* but inhibits that of native species (Niu et al. 2007). However, whether these changes are associated with the chemicals from the plant, which may alter soil bacterial communities, or the effect is general among soil communities in different habitats, is unknown.

To determine the effect of secondary metabolic chemicals of *E. adenophorum* on soil bacterial communities, we compared the bacterial communities before and after the addition of leaf and root leachates, and the allelochemicals DTD and HHO. We also

assessed the effects of leaf leachate on soil samples from forest, grassland, and roadside habitats not inhabited by the invasive species. We predicted that the leaf and root leachate would alter the soil bacterial communities, DTD and HHO may affect some special bacterial taxa, and the effect of leaf leachate on the bacterial community would vary between the soil samples from different habitats.

Materials and methods

Experiment set up

Experiment I

Soil samples were collected from four plots (1 × 1 m) in an evergreen broad-leaved forest (101°08′–102°15′E, 26°05′–27°21′N), which was dominated by *Machilus pingii*, *Cyclobalanopsis plaucoides* and *Lithocarpus dealbatus*, and had a very low density of *E. adenophorum* (less than 10% coverage) in Panzhihua, in Hengduan Mountain range, Southwest China. After removing aboveground vegetation and litter, about 100 g of soil sample was collected from the top 10 cm of the soil horizon of each sampling plots. Roots, residue, and gravel were removed from the soil using a 10-mesh (2 mm) sieve. The soil from each plot was mixed and used immediately after collection.

Fresh leaves and roots of *E. adenophorum* were collected from a natural population in the same site where soil samples were collected. The leaves and roots were rinsed with tap water for 10 min and their surfaces were sterilized with sodium hypochlorite (0.3% v/v) for 15 min, followed by 4–5 washes in distilled water. They were immediately immersed in distilled water (1 g fresh material: 10 g distilled water) for 36 h at 25 °C (improved in Zhu et al. 2011). The leaves and roots were removed and the aqueous leachate was filtered using a 75-mm glass funnel with one sheet of 90 mm folded filter paper. To avoid contamination, one of the following methods was utilized to sterilize all materials, tools, and surfaces used in the experiment: soaking in 0.3% aqueous NaOCl, autoclaving for 60–180 min (121 °C, 0.105 MPa), or spraying with 70% ETOH. In all our experiments, the two allelochemical compounds used were prepared as described in Yang et al. (2008).

Before the treatments, we extracted DNA from bacterial cells in the soil samples from the four plots using the E.Z.N.A.®soil DNA Kit (Omega Bio-tek, Norcross, GA, US), according to the manufacturer's protocols. From each plot (as a replicate), 30 g of fresh soil was used to fill five 50-ml tubes, and then four of these tubes were sprayed evenly with a solutions of 2 ml DTD (1 mM), HHO (1 mM), leaf, and root leachates. The fifth tube was treated with ddH_2O as a control. All 20 tubes (4 treatments × 4 replicates and 1 control × 4 replicates) were transported to the laboratory, and placed in a temperature controlled growth chamber (29 °C), on a 16:8 h light:dark cycle. All soil samples were watered with 2 ml of the allelochemical solutions, leachate, or distilled water, after a 48-h incubation period. After a 3-day incubation period, we extracted DNA from the bacterial cells in each soil sample.

Experiment II

To test the response of soil bacterial communities from different habitats to the leachate of *E. adenophorum*, we collected soil samples from three sites—a forest, wasteland, and roadside habitat—that had never been occupied by the invasive species. Site I, a tropical seasonal forest, was dominated by *Pterospermum acerifolium*, *Gynocardia odorata, Eurya groffii, Elaeocarpus sylvestris,* and *Platea latifolia*. Site II, a wasteland, was dominated by *Eupatolium odoratum*, *Oplismenus patens* var. *yunnanensis* and *Crassocephalum crepidioides*. Site III, was along a roadside and had bare soil. At each site, three 1 m × 1 m plots were randomly established, in which about 500 g of 0–10 cm topsoil was collected after removing aboveground plants and litter. Roots, residue, and gravel were removed from the soil using a 2-mm sieve. The soil from each plot was mixed and maintained at 4 °C in a refrigerator until use.

Before the treatment of the soil samples, pH, organic matter, total carbon (TC), total nitrogen (TN), total phosphorus (TP), and total potassium (TK) content were determined at the Public Technical Service Center of Xishuangbanna Tropical Botanical Garden (XTBG), Chinese Academy of Sciences. Soil DNA from each site was extracted as in experiment I.

Soil samples from each site were put into six 2 L pots and a 1 kg pre pot, and divided into two groups. To one group we added 100 ml of 10% *E.*

adenophorum leaf leachate every 2 weeks; to the other was added 100 ml of water as a control. After 6 months, the soil samples were collected from all the pots. The soil chemical character and soil DNA of each treatment were measured and extracted respectively. In total, there were 27 soil DNA samples (three soil types × two soil treatments × three replicates and three soil types × one pre-treatment × three replicates).

Measurement of bacterial diversity and community structure

PCR amplification

The V4–V5 region of the bacterial 16S ribosomal RNA gene of all soil samples was amplified using PCR (95 °C for 2 min, followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min). The primers 515F 5′-barcode-GTGCCAGCMGCCGCGG)-3′ and 907R 5′-CCGTCAATTCMTTTRAGTTT-3′, where the barcode was an eight-base sequence unique to each sample, were used for the analysis. PCR reactions were performed in triplicates, with a 20-μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mMdNTPs, 0.4μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of the template DNA.

IlluminaMiSeq sequencing

Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, US), according to the manufacturer's instructions. The extracts were then quantified using QuantiFluor^{TM}-ST (Promega, US). Purified amplicons were pooled in equimolar ratios and paired-end sequenced (2 × 250) on an IlluminaMiSeq platformat (Shanghai Majorbio Biopharm Technology Co., Ltd). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database.

Processing of sequencing data

Raw fastq files were demultiplexed and quality-filtered using QIIME (version 1.17) according to the following criteria: (1) the 250-bp reads were truncated at any site receiving an average quality score <20 over a 10-bp sliding window. Truncated reads that were shorter than 50 bp were discarded; (2) exact barcode matching, two nucleotide mismatches in primer matching, and reads containing ambiguous characters were removed. (3) only sequences that overlapped more than 10 bp were assembled according to their overlap sequence. Reads that could not be assembled were discarded. Operational 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 phylogenetic affiliation of each 16S rRNA gene sequence was analyzed against the silva (SSU115)16SrRNA database, with the RDP Classifier (http://rdp.cme.msu.edu/), using a confidence threshold of 70% (Wang et al. 2007).

Statistical analysis

We used a paired sample *t* test to examine the effect of different addition treatments on soil bacterial OTUs, and the Chao and Shannon indices. To compare bacterial community structures across all treatments based on the OTUs, a coordination analysis (PCoA) was performed using CANOCO software (version 4.5, Microcomputer Power, Inc., Ithaca, NY).

An independent *t* test was used to examine the difference between OTUs, the Chao, and Shannon index and soil chemical characters between before and after water addition treatment, and between before and after adding leaf leachate in the three soil sources, respectively. We used PCoA to compare bacterial community structures across addition treatments and soil sources. A redundancy analysis (RDA) was applied to test the effect of soil chemical traits on bacterial community structures using the CANOCO software.

Results

Short-term effects of *E. adenophorum* chemical compounds on soil bacterial communities

From the 24 soil samples, 406,333 reads clustered to 924-4175 bacterial OTUs. Of the five treated samples, only leaf leachate significantly decreased bacterial

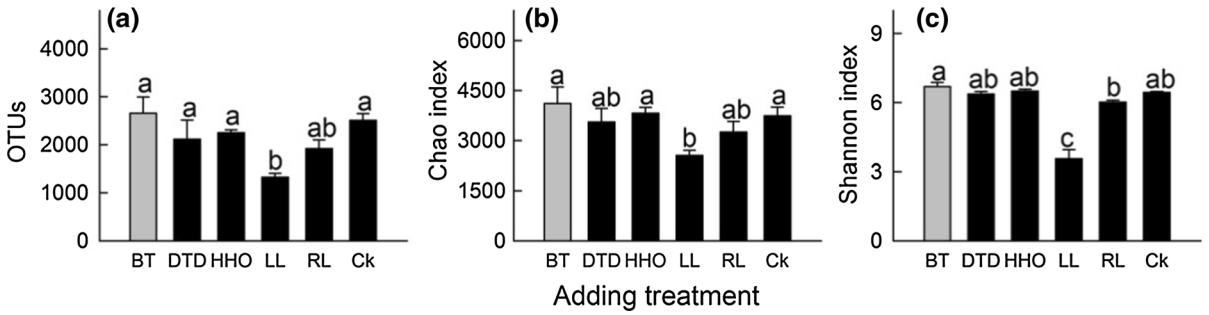

Fig. 1 Bacterial OTUs (**a**), Chao index (**b**) and Shannon index (**c**) of soil with pre-treatment (BT), DTD, HHO, leaf leachate (LL), root leachate (RL) and control (CK) treatments. *Bars* are mean ± SE, n = 4. *Different letters* indicate significant differences among treatments after Duncan's test ($P < 0.05$)

diversity (Shannon index) and richness (OTUs and Chao index) (Fig. 1).

The 16S rDNA homologous sequences consisted of 41 identified phyla, with 189 orders. *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* were the dominant phyla (Fig. S1). Of all the addition treatments, leaf leachate alone increased the relative abundance of *Proteobacteria* and decreased the presence of *Acidobacte*ria and *Actinobacteria* (Fig. S1). At the order level, *Rhizobiales*, *Planctomycetales*, and *Acidobacteriales* were the dominant orders (Fig. 2). Compared with the control treatment, only leaf leachate

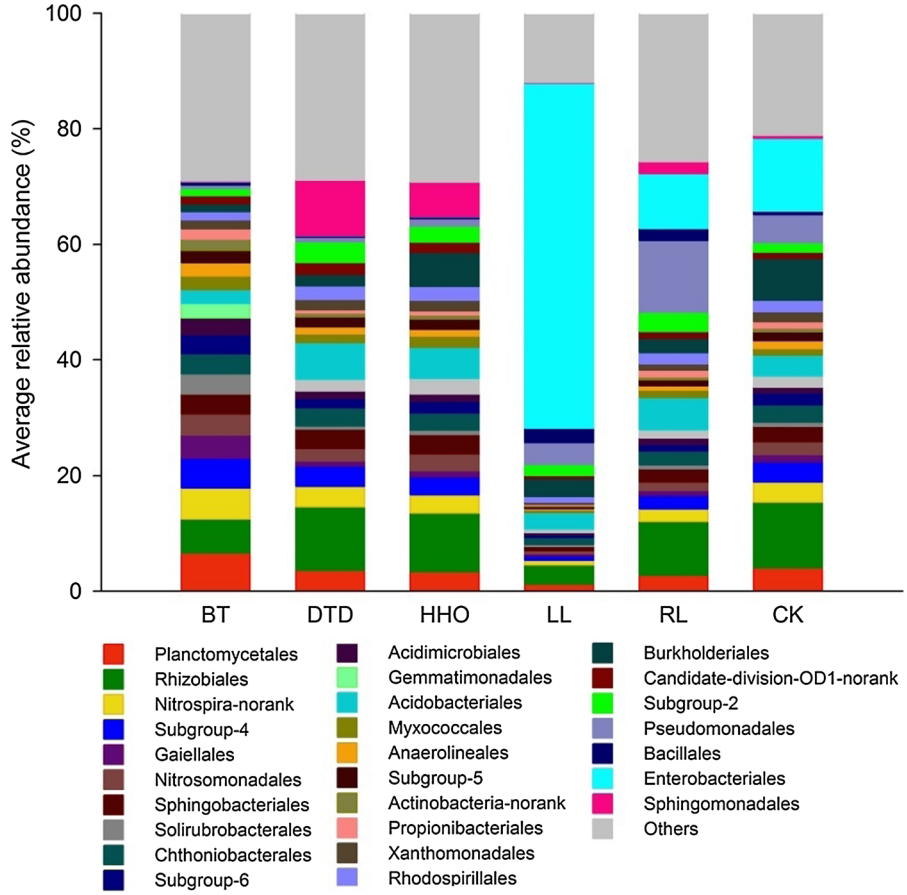

Fig. 2 Average relative abundance of bacterial order in soils with pre-treatment (BT), the addition of DTD, HHO, leaf leachate (LL), root leachate (RL) and control (CK) treatments. *Bars* represent mean ± SE, n = 3

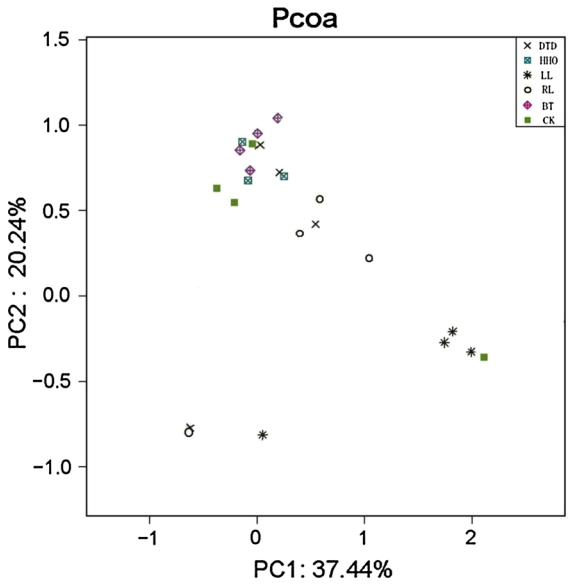

Fig. 3 PCoA plots based on the OTUs of bacterial community in soils with pre-treatment (BT), the addition of DTD, HHO, leaf leachate (LL), root leachate (RL), and control (CK) treatments

obviously reduced the relative abundance of the three dominant orders, and enhanced that of *Enterobacteriales*. DTD and HHO increased *Sphingomonadales* and root leachate increased *Pseudomonadales* (Fig. 2). The PCoA results also showed that the composition of bacterial communities in soils treated with leaf leachate distinctively differed from that of the pre-treated soil (Fig. 3).

Long-term effects of *E. adenophorum* on chemical compounds on soil bacterial communities from different habitats

Leaf leachate interacted strongly with habitat to affect bacterial diversity and richness (Fig. 4). The leaf leachate of *E. adenophorum* significantly decreased bacterial diversity (lower Shannon index) and richness (lower OTUs, Chao index) in the soils from the forest, but significantly increased in soils from the roadside (Fig. 4). Moreover, albeit Chao and Shannon indices increased after the addition of the leaf leachate in the soil samples from the roadside habitat, the OTUs and Chao index did not change in the control treatment, whereas there was a decline in the Shannon index (Fig. 4). In soil samples from the wasteland habitat, the addition of the leaf leachate and water in the control treatment increased bacterial richness and diversity (Fig. 4; Table 1).

In this study, 460,722 high-quality sequences were generated from 27 samples, and were homologized to produce 36 phyla and 193 orders. The bacterial composition differed among the three habitats, and showed different responses to chemicals from the leaf leachate of *E. adenophorum* (Fig. S2). For example, *Proteobacteria* was the dominant phylum in the soil samples from all three habitats, and its relative abundance increased after the addition the leaf leachate in soil samples from the forest and roadside, but decreased in samples from the wasteland (Fig. S2). At the order level, some changes in the relative abundance of the functional bacterial taxa were observed. The relative abundance of *Rhizobiale* increased in all soil samples in the leaf leachate treatment, most notably in those from the roadside habitat (Fig. 5). The relative abundance of *Burkholderiales* and *Xanthomonadales* increased in soil samples from the forest and roadside, but decreased in samples from the wasteland (Fig. 5). There was a 4% increment in the relative abundance of *Nitrosomonadales* and a considerable reduction in the relative abundance of *Pseudomonadales* and *Lactobacillales* in the leaf leachate treatments of samples from the roadside habitat (Fig. 5).

The PcoA analyses showed that the bacterial communities of all soil samples in the leaf leachate treatments differed from those in the pre-treatment, especially in samples from the roadside habitat (Fig. 6). Except for soil samples from the forest habitat, soil bacterial communities in the control were separated from those of the pre-treatment (Fig. 6).

The leaf leachate of *E. adenophorum* also affected soil chemical characters (see Table S1). The Monte Carlo permutation test showed that soil pH and SOC were important factors influencing the bacterial composition of soil communities (Table 1).

Discussion

The secondary chemicals released by invasive plants not only have allelopathic effects on native species, but may also play an important role in influencing soil microbial community. Our results indicated that the secondary chemicals of *E. adenophorum* influence soil bacterial community. Obvious changes in the relative

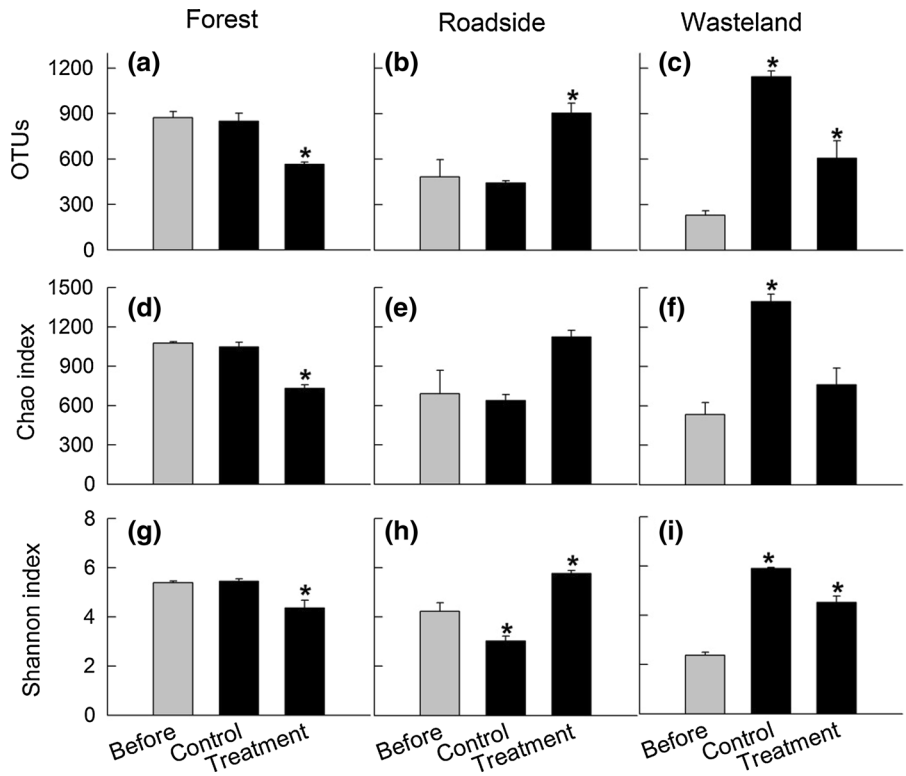

Fig. 4 Bacteria OTUs (**a**), Chao index (**b**) and Shannon index (**c**) of soil samples from forest, roadside, and wasteland before adding leaf leachate, after adding water (control), and leaf leachate. *Bars* are mean ± SE, n = 3. *Asterisk* indicate significant differences from that before adding leaf leachate treatments according to *t* test ($P < 0.05$)

Table 1 Monte Carlo permutation test

Soil traits	*F* value	*P* value	Variance explain (%)
pH value	5.276	0.006	42.6
Soil organic carbon	3.492	0.003	25.6
Soil total nitrogen	2.634	0.064	16.6
Soil total phosphorous	2.400	0.080	10.9
Soil total potassium	1.594	0.210	4.3

abundance of some taxa, such as *Sphingomonadales*, were observed after the addition of two pure allelopathic chemicals, DTD and HHO, from the invasive species (Fig. 2). The secondary chemical compounds from the root, and more significantly, the leaf leachate, further altered the composition and diversity of the soil bacterial community (Fig. 2, Fig. S1). Consistently, the diversity and composition of the soil bacterial community significantly changed after the addition of the leaf leachate for 6 months (Fig. 4). Similar results have been found in other studies. For instance, Lorenzo et al. (2013) found that the leachate from the invasive plant, *Acacia dealbata,* led to a different soil microbial composition in soil samples from a pine forest.

Novel chemicals of invasive plants may have a negative impact on native microbial community in the soil. Native species cannot utilize these chemicals as carbon sources or develop a tolerance to them because they lack a co-evolutionary history. In this study, short-term experiment displayed that soil bacterial diversity significantly decreased after the addition of the leaf leachate of *E. adenophorum* in the short-term experiment (Fig. 1c). In the long-term experiment, leaf leachate reduced bacterial diversity of one of the three soil sample sites (Fig. 4 g). Bacterial diversity did not decrease after the addition of water in the control treatments in these two experiments (Figs. 1c, 4g), suggesting that reductions in nutrient input did not cause these changes. Therefore, the observed effects

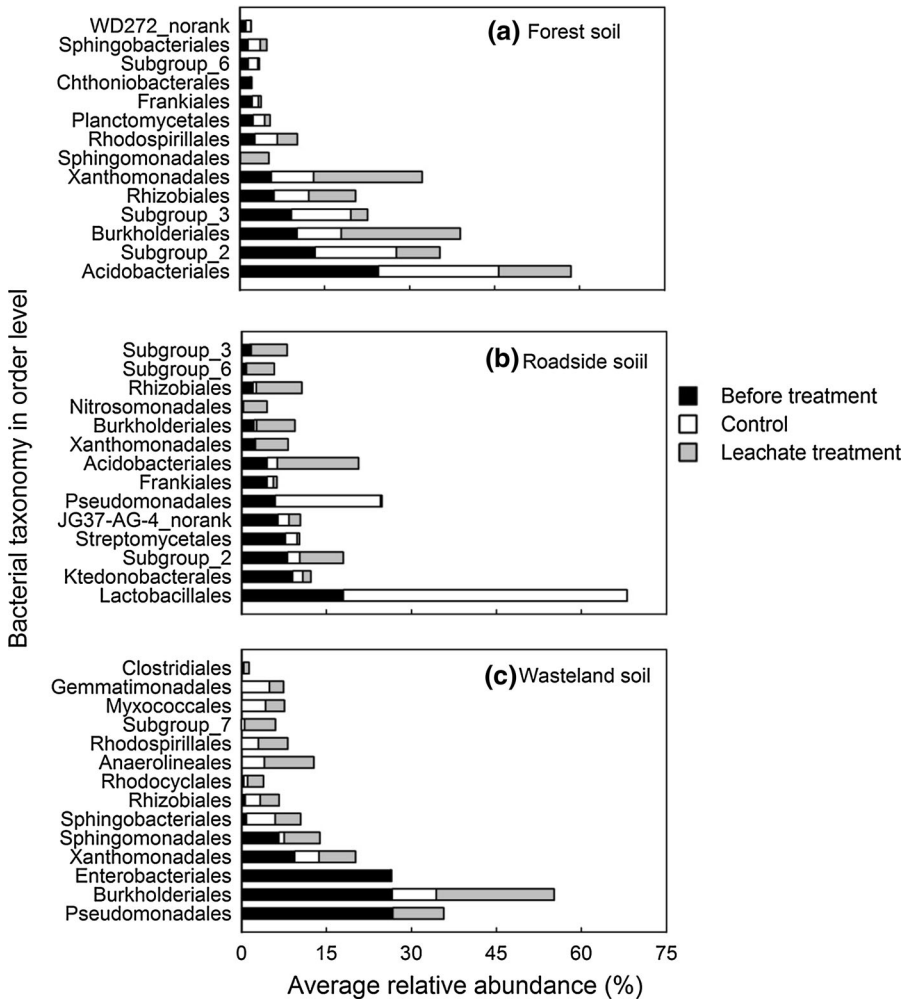

Fig. 5 Average relative abundance of most abundance 14 bacterial orders in before and after the addition of water (control) and the addition of leaf leachate of *E. adenophorum* in soils from the forest (**a**), roadside (**b**) and wasteland (**c**)

were attributed to the inability of soil bacteria to tolerate the secondary chemicals of the invasive species.

Nevertheless, secondary chemicals of invasive plants may be beneficial to some bacteria, because some native bacteria may acquire the ability to use these novel chemicals by cross acclimatizing them to similarly structured chemicals. In this study, significant increases in the relative abundance of *Sphingomonadales*, which can degrade a variety of chemicals, such as aromatic compounds, in the environment (Fujii et al. 2003; Sohn et al. 2004), were observed after the addition of DTD or HHO (Fig. 2). In another study, we isolated a *Sphingobium* strain (order *Sphingomonadales*) that could degrade DTD and HHO in soils invaded by *E. adenophorum* (unpublished data). This may also be one of the causes of an increasing bacterial diversity in soils from roadside habitats after the addition of the leaf leachate of *E. adenophorum* for 6 months. Our results indicate that the addition of secondary chemicals of the invasive plant species causes an aggregation of bacteria that degrade these chemicals.

The input of various chemical compounds from plants might be a primary nutrient resource of soil bacteria and changes in their quality and quantity may influence soil bacterial community. Thus, secondary chemicals of invasive plants may influence soil bacterial community by altering soil nutrient level. For soil samples from new roadsides without much vegetation cover, the addition of the *E. adenophorum* leachate increased nutrient inputs. However, for soil samples from forests, only leaf leachate of one species decreased nutrient inputs. This might be why *E.*

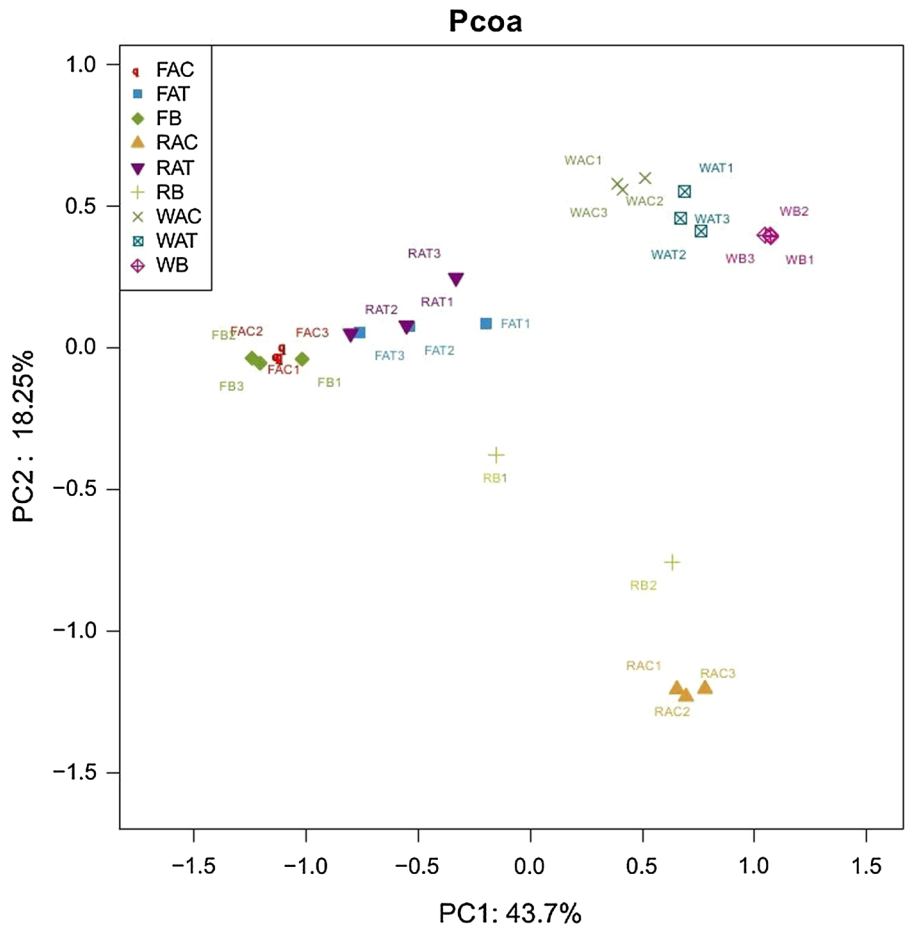

Fig. 6 PCoA plots based on the OTUs of bacterial community in soils from forest, roadside, and wasteland habitats. *Letters* "*F*," "*R*," and "*W*" indicate soils from forest, roadside and wasteland habitats respectively. *Letters* "*B*," "*AC*," and "*T*" indicate before, control, and leaf leachate of *E. Adenophorum* treatments

adenophorum leachate increased bacterial richness and diversity in soil samples from the roadside habitats, but reduce those in soil samples from the forest habitats. Similarly, Lorenzo et al. (2013) found that the leachate of the invasive plant *Acacia dealbata* reduced bacterial richness and diversity of soils from pine forest, but did not affect those in mixed oak forests.

The changes in the soil function of bacterial groups, following the input of the leachate, are likely to affect soil nutrient levels. *E. adenophorum* leachate increased the relative abundance of *Rhizobiale*, an order containing nitrogen-fixing bacteria, especially in soil samples from the roadside. The relative abundance of *Nitrosomonadales*, an order associated with nitrogen cycling, also increased in soil samples from roadside habitats. This may be one of reasons which TN increases in the soil samples from roadside habitats. In other studies, increases in available nitrogen and TN, with the invasion of *E. adenophorum,* have been observed (Dai et al. 2012; Xu et al. 2012). It is helpful for invasive plants to increase the efficiency of their resource-use in order to compete with native species (Funk and Vitousek 2007).

Soil pH is important in structuring the composition of bacterial communities (Fierer and Jackson 2006; Lauber et al. 2009; Tripathi et al. 2012). The addition of the *E. adenophorum* leaf leachate, for 6 months, significantly increased soil pH, which may have influenced the soil bacterial community. The Monte Carlo permutation tests showed that pH and SOC were important factors influencing the composition of bacterial communities.

Our results indicate that the allelochemicals of *E. adenohporum* significantly influence soil bacterial community diversity and structure. The magnitude and direction of this effect depend on soil sources. There is still a knowledge gap between soil bacterial communities and the invasiveness of *E. adenophorum*.

This study is just the first step towards understanding the link between plant invasion and soil bacterial communities. Further studies need to focus on comparing the effects of secondary compounds on native and invasive plants, and examining the effects of changes in bacterial communities on species coexistence.

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