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Allelopathic effects of *Salix matsudana* (Koidz) root exudates on soil bacterial diversity

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ABSTRACT

Allelopathy can seriously affect the growth and vitality of plants and microorganisms. We analyzed the effects of Willow (*Salix matsudana* Koidz) root exudates on soil bacterial diversity to explore the interactions between plants and soil microorganisms. A total of 18 compounds were identified from the root exudates of *S. matsudana* variety Yanjiang by GCMS in which the butylacetate content was maximum (72.20 %). Soil was treated with *S. matsudana* root exudates and genomic DNA of microorganisms was extracted and analyzed by 16s-rDNA sequencing. A total of 374 bacterial species were identified, 91 % of which were uncultured. *Uncultured_bacterium_c_subgroup_6* was the dominant bacteria in both treated and control samples. Two species of *uncultured_bacterium_f_Acetobacterales_incertae_sedis* and *uncultured_bacterium_f_Lachnospiraceae* were found only in the treated sample, whereas, two species of *Kofleria_flava* and *uncultured_bacterium_f_Enterobacteriaceae* were present only in the control sample. The abundance of most species differed between the treated and control bacterial communities, e.g., the abundance of *uncultured_bacterium_f_Amb-16S-1034* was 7.22 times higher in the control than in the treated sample and *uncultured_bacterium_g_Bacteroides* was 6.65 times higher in the treated community than in the control. Functional analysis showed that bacteria in the treated sample were enriched for some disease-resistant and cellulose-decomposing strains. The results demonstrated that willow root exudates can change the soil microbial community structure, which might correspondingly affect the plant growth.

Key words: Allelopathy, bacterial diversity, DNA, GCMS, root exudates, *Salix matsudana*, soil, willow.

INTRODUCTION

Plants actively interact with soil microorganisms and soil microbial composition can seriously affect the plant growth. Microorganisms growing in proximity of plant roots convert organic compounds into inorganic components and provide important nutrients for plants (11,14). Additionally, some microorganisms secrete vitamins and growth hormones that promote plant growth (5). For example, *Trichoderma longibrachiatum* has positive effects on maize growth, increases seedling height, root length, shoot dry weight, underground dry weight, shoot fresh weight and underground fresh weight by 44.65 %, 75.74 %, 83.33 %, 300 %, 101.85 % and 356.67 %, respectively (30). The inoculation of *Arbuscular mycorrhizal* fungi on trifoliate orange seedlings increases the dry weight of shoot and underground plant tissues by 251.28 % and 205.40 %, respectively (19) and inoculation of *Rhizobacteria* increases the root length of rice by 60.14 % and the seedling vigour index by 53 % (1).

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Further, plant genotypes and planting methods have an impact on the soil microbial community. For example, the culturable microorganisms in Kiwi fruit orchard soil are mainly bacteria, including *Actinomycetes* and various fungi (29). With respect to planting time, the abundance of *Actinomycetes* decreases, whereas that of fungi increases significantly. In three alpine grassland species in northern Tibet, *Acidobacteria* was dominant bacteria in the rhizosphere, accounting for 25 % of the soil bacterial community (4) and *Azotobacter paspali* was dominant only in the rhizosphere of a specific *Paspalum* variety 'crowngrass' (26). *Azospirillas* sp. has low nitrogen fixation activity in the rhizosphere of maize UR-1 but has high activity in the rhizosphere of hybrid S1 (9). Moreover, the intercropping of *Litsea cubeba* with tea tree significantly changes the population structure of soil microorganisms (7). The abundance of functional bacteria involved in the transformation of N, P and Mn, increases the abundance of plant pathogenic bacteria and decreases fungi significantly (6).

Figure 1. *S. matsudana* variety Yanjiang

Figure 2. Hydroponic culture of *S. matsudana* variety Yanjiang

Plants secrete multiple allelochemicals that promote or inhibit the activity of other plants or microorganisms (20,27). *Salix matsudana* is an important widely planted afforestation tree species (Fig.1). This study aimed (a) to identify the allelochemical components of *S. matsudana* root exudates (willow) and (b) their effects on soil bacterial community structure particularly the plant growth promoting rhizobacteria.

MATERIALS AND METHODS

This study was conducted from March 2021 to March 2022 in Beijing Forestry University, Beijing (Longitude, 116° 23′ 17″ N; latitude, 39° 54′ 27″ E; Altitude, 60 m; Weather conditions: temperate monsoon climate; Annual precipitation: 448 mm).

I. Soil samples

The soil samples taken from the farmland contained 0.67 % moisture, pH : 8.34 (measured by mixing soil and H_2O in 1:2.5) and the electrical conductivity: 178.7 µS/cm

(measured by mixing soil and H_2O in 1:5). The soil was crushed and sieved to remove the plant residues.

II. *Salix* root exudates

Ten cuttings of one-year old *S. matsudana* variety Yanjiang were cultured in hydroponic solution for 15 d (Fig. 2). The willow roots were collected, dried in oven (105 ℃) overnight and weighed as dry weight (DW). The root hydroponic solution was diluted to 1.5 mg/ml (root dry weight/solution volume) as treatment solution. The treatment solution was partly used for gas chromatographic mass spectroscopy (GC-MS) test and the rest was used for soil treatment.

III. Chromatography-mass spectrometric (GC-MS) analysis

The exudate solution of willow roots was filtered with gauze and 0.22 μm microporous filter. Five ml of filtrate were mixed well with 2 mL ethyl acetate. One μl ethyl acetate extract was analyzed by GCMS-QP2020 Ultra GC-MS (manufactured by Shimadzu Corporation). The GC conditions were followings: inlet temperature 250 ℃, helium (99.99 %) carrier gas flow at constant 1 $ml \cdot min^{-1}$, injector split ratio 10:1. The temperature programme for GC started at 50 ℃ and increased at the rate of 15 $℃ \cdot min^{-1}$ to 180 ℃ for 5 min and then at the rate of 10 $℃ \cdot min^{-1}$ until it reached 250 ℃ for 15 min. MS analysis was conducted in positive electron ionization mode. The source temperature was held at 230 ℃, with an electron ionization potential of 70 eV and ions were scanned over the molecular weight range of 33-400 atomic mass units. Each metabolite was identified via the standard mass spectrum database of NIST14 lib and the relative content of each component was counted by area normalization.

IV. Soil treatment with *Salix* root exudates and analysis of soil bacterium

Soil sample (2.0 g) was transferred to a 7 ml centrifuge tube, mixed well with 150 μL root treatment solution (or distilled H_2O as control) and incubated at 28 ℃ for 3 d. The total microorganisms DNA in soil was extracted by TianGen DP812 extraction kit. The 16S rDNA library of soil microorganisms was constructed by amplification with the specific primers (V3V4F: 5'-ACTCCTACGGGAGGCAGCA-3'; V3V4R: 5'-GGACTACHVGGG-TWTCTAAT-3'). The amplified products were sequenced by Illumina Novaseq 6000 (BMK). Raw reads were filtered by Trimmomatic v 0.33, removed the primer sequences by cutadapt 1.9.1 and finally generated high-quality reads. Chimeric sequences were identified and removed by UCHIME v4.2 and generating effective reads. Usearch was applied to cluster reads with similarity above 97.0 %, generating OTUs. The community structure map and species clustering heat map of each sample at the level of phylum, class, order, family, genus and species taxonomy were obtained by TBtools.

V. Heatmap of species abundance

The heatmaps were generated by R package according to species composition and relative abundance at level of phylum, class, order, family, genus and species classification. The values shown on heatmap are z-scores generated by z-normalization of relative species abundance. Species with high abundance and low abundance were clustered. The similarity and difference in community composition among samples were reflected by colour gradient.

VI. Encyclopaedia of Genes and Genomes (KEGG) analysis of the bacterial community

PICRUSt2 was applied to perform species annotation on feature sequences based on reference phylogenetic tree. Potential functions and functional genes in samples were predicted based on Integrated Microbial Genomes (IMG) database，which further revealed the difference in functions between samples. The significance of difference in function abundance between samples was evaluated by G-TEST (Number of annotated functional genes > 20) and Fisher (Number of annotated functional genes < 20) in STAMP. Threshold for significant difference was set as P-value smaller than 0.05.

RESULTS AND DISCUSSION

WILLOW ROOT EXUDATES

I. Chemical Components

To evaluate the effects of willow plants on soil microorganisms, the root exudates of the willow tree were analyzed by gas chromatography/mass spectrophotometric (GC-MS) analysis. A total of 18 compounds were identified (Table 1). For all of them, the similarity index with known compounds was over 80 %, indicating that the identifications were reliable. Isobutyl acetate was the major component, representing 72.20 % of all compounds identified. The next common compound was n-hexadecanoic acid (5.56 %), undecane,3,8-dimethyl (3.05 %) and octadecanoic acid 2-(2-hydroxyethoxy) ethyl ester (2.79 %). Other detected compounds concentrations were below 2 % of the total fractions.

Table 1. The contents of allelopathic chemical components in root exudates of *S. matsudana* variety Yanjiang.

Peak #	Name	Concentration (%)
1	Sulfurous acid, pentyl 2-propyl ester	0.95
2	Isobutyl acetate	72.20
3	Benzene, 1,3-dimethyl	0.96
4	Butane, 2,2-dimethyl	0.62
5	Dodecane, 1-iodo	0.57
6	Hexane, 3,3-dimethyl	1.04
7	Heptane, 3,3-dimethyl	1.58
8	Oxalic acid, isobutyl pentyl ester	1.54
9	Nonane, 1-iodo	0.85
10	Butane, 2,2-dimethyl	1.02
11	Pentane, 2,2-dimethyl	1.75
12	Octane, 3,3-dimethyl	1.12
13	Heptane, 3,3-dimethyl	0.99
14	Decane, 3,7-dimethyl	1.55
15	Undecane, 3,8-dimethyl	3.05
16	Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters	1.87
17	n-Hexadecanoic acid	5.56
18	Octadecanoic acid, 2-(2-hydroxyethoxy) ethyl ester	2.79

It had been reported that isobutyl acetate is important allelopathic compound released by *Acorus tatarinowii* involved in the growth inhibition of *Microcystis aeruginosa.* When the stems and leaves of *Acorus tatarinowii* are cut, the content of isobutyl acetate in the soil increases by 9.1 % than with no mowing (17). Further, isobutyl acetate is abundant in melon and banana volatile organic compounds, indicating the allelopathic effects of these mature fruits (15,18). Therefore, the abundant isobutyl acetate found in *S. matsudana* root exudates could impact the soil microbial community.

II. Soil bacterial diversity

To determine the allelopathic effects of willow root exudates on soil bacterial diversity, the total microorganisms DNA in soil was isolated. According to the 16S rDNA sequencing results, 1514 operational taxonomic units (OTUs) were identified between the soil samples treated by the root exudates and control (CK) and 97.42 % of them were unculturable bacteria. Comparably, 1511 OTUs were identified in the control and 1509 OTUs in the treated sample.

The two samples shared 1506 OTUs, with 5-OTUs specific to the control sample: *s_Kofleria_flava, s_uncultured_ bacterium_g_ Solirubrobacter, s_uncultured _bacterium_g_Candidatus_alysiosphaera, s_uncultured_bacterium_f_ Enterobacteriaceae and s_ uncultured_bacterium_c_Gammaproteobacteria*.

Additionally, 3-OTUs were specific in the treated sample:

s_uncultured_bacterium_o_Rokubacteriales, s_uncultured_bacterium_f_ Acetobacterales_ incertae_sedis and s_uncultured_bacterium_f_Lachnospiraceae.

Further classification showed that the bacterial community in the treated and control soil samples shared the same 23 phyla and 68 classes. However, one order, one family and one genus were missing in the treated sample, whereas, two families and two genera were missing in control (Table 2). In total, of the 374 bacterial species, 341 (91.18 %) were uncultured and were identified in the treated and CK samples. Compared to CK, the treatment sample included two more species (*uncultured_bacterium_f_ Acetobacterales_ incertae_sedis, uncultured_bacterium_f_Lachnospiraceae*) and two missing species (*Kofleria_flava, uncultured_bacterium_f_Enterobacteriaceae*). *Proteobacteria* accounting for 40.94 % and 40.23 % and was the dominant phylum in both the treated and control (CK) microbial communities respectively, this was followed by the *Acidobacteria* phylum, accounting for 27.05 % and 28.93 %, respectively. At the species level, *uncultured_ bacterium_c_subgroup_6* was the dominant bacterium in both treated and CK samples, accounting for 17.14 % and 17.91 %, respectively.

Table 2. Classification of the bacterium in control and treated soil samples

Sample	Kindom	Phylum	Class	Order	Family	Genus	Species
Control	1	23	68	145	221	344	372
Treatment	1	23	68	144	222	345	372

III. Soil bacterial abundance

Even the treated and CK samples shared most of the bacterial species, the abundance differences in the community might result in their relationship and their interactions changed and further influence the plant growth. Several phyla increased under salt treatment such as *FCPU426*, the abundance in the treated sample was 1.75 times greater than in CK. Some phyla decreased under salt treatment such as *GAL* 15, 0.75 times less abundant in the treated sample than in CK. Also, several phyla performed similarly in both samples such as *Planctomycetes* and *Nitrospirae* (Fig. 3). Similar cases were also revealed at species level.

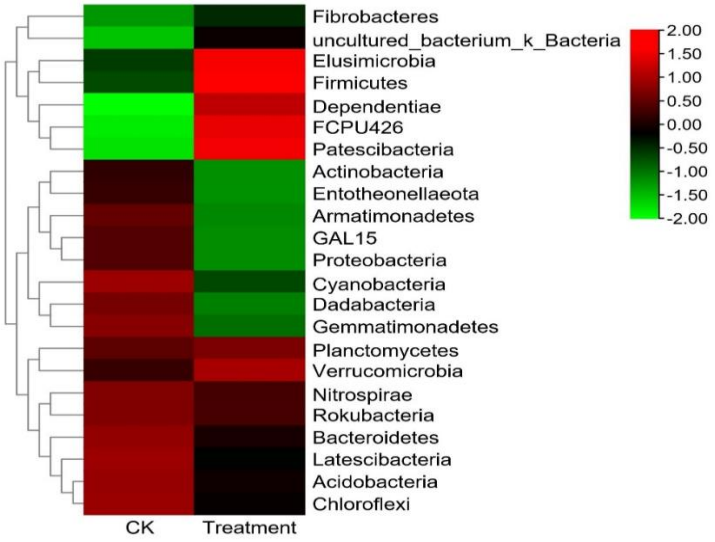

Figure 3. Abundance heat map of 23 phylum in treatment and CK samples.
Clustering on left side represents similarity of species among samples. The colour gradient from blue to red represents relative abundance richness (low to high).

A proportion of a species in a community was calculated (Fig. 4). Some species proportion increased under salt treatment such as *uncultured_bacterium_f_Amb-16S-1034* showed 7.22 times abundance in the treatment sample than in CK. Some species proportion decreased in treatments such as *uncultured_bacterium_g_Bacteroides*, which in CK was 6.65 times than treated sample Several species such as *uncultured_bacterium-_o_Acidobacteria_bacterium _IGE-011*, *uncultured_bacterium_f_Burkholderiaceae* and *uncultured_bacterium_o_ Azospirillales* have similar abundances in treated and CK samples.

Obviously, willow root exudates could seriously affect the soil bacterial community through allelopathic reactions. This was supported by previous reports. For example, the dominant bacterial phyla in the rhizosphere of *Halostachys capsica* are *Actinobacteria, Proteobacteria* and *Gemmatimonadetes* (3). *Acidobacteria* and *Proteobacteria* are more abundant in the rhizosphere and non-rhizosphere soils of *Solidago canadensis* L., accounting for 42.92 % and 34.23 %, respectively, of the microbial community (21). The relative

abundances of *Candidatus solibacter*, *Ellin6067*, *Burkholderia-Caballeronia-Paraburkholderia* and *Bradyrhizobium* were significantly higher in the rhizosphere soil of *Solidago canadensis* L. than in non-rhizosphere soil, whereas, the abundance of *Acinetobacter* was significantly lower. Thus, the impacts of different plants on microbial communities were specific.

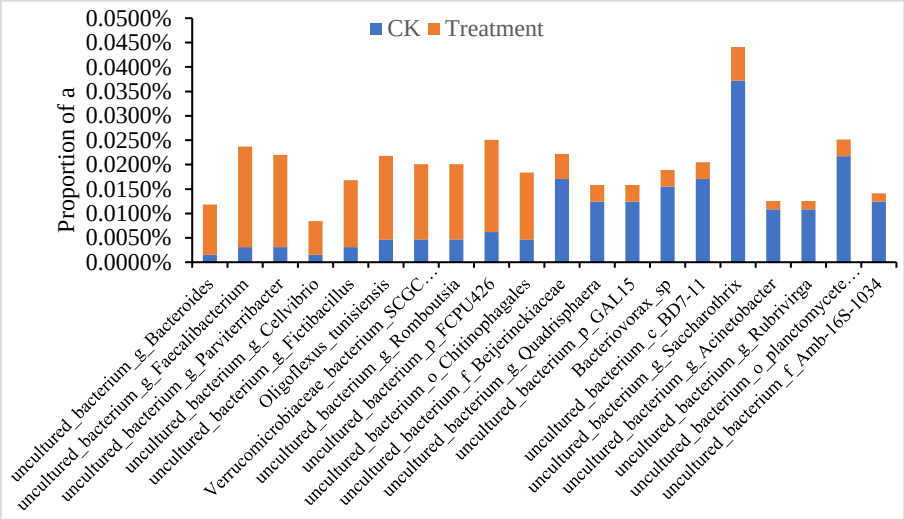

Figure 4. Proportion of the partial bacterial species in the community of two soil samples.
Proportion: Reads number of species, total reads of a community (CK or Treatment). CK : Bacterial community in regular condition. Treatment : Community treated by willow root exudates.

IV. Functional properties of soil bacteria

Each bacteria specie plays its specific role in the community and plant growth. To understand the contribution of each bacteria specie to the community and plant growth, the function properties of soil bacteria were analyzed. In comparison to previous reports, the soil samples treated by *S. mastudana* root exudates were rich in some disease-resistant microbial strains (*uncultured_bacterium_g_Fictibacillus*) and these were increased 4.43 times than in CK. A study had shown that *Fictibacillus* efficiently controls the soil-borne diseases via root irrigation and significantly reduces the bacterial and fungal diseases in plant leaves (24). *Uncultured_bacterium_g_Aeromicrobium* was abundant in the treatment sample, 2.44 times than in CK. The *Aeromicrobium* can produce antibiotics, these potentially protects plants from pests and diseases (23). Additionally, *uncultured_bacterium_g_ Panacagrimonas* was 2.44 times higher in treatment than in CK and can advance flowering time in plants (10). Several *Acidobacterium_sp* can degrade the plant residues and cellulosethereby, providing carbon sources for plant growth (12,16). Therefore, *S. matsudana* root exudates might be beneficial to plants by controlling the bacterial community.

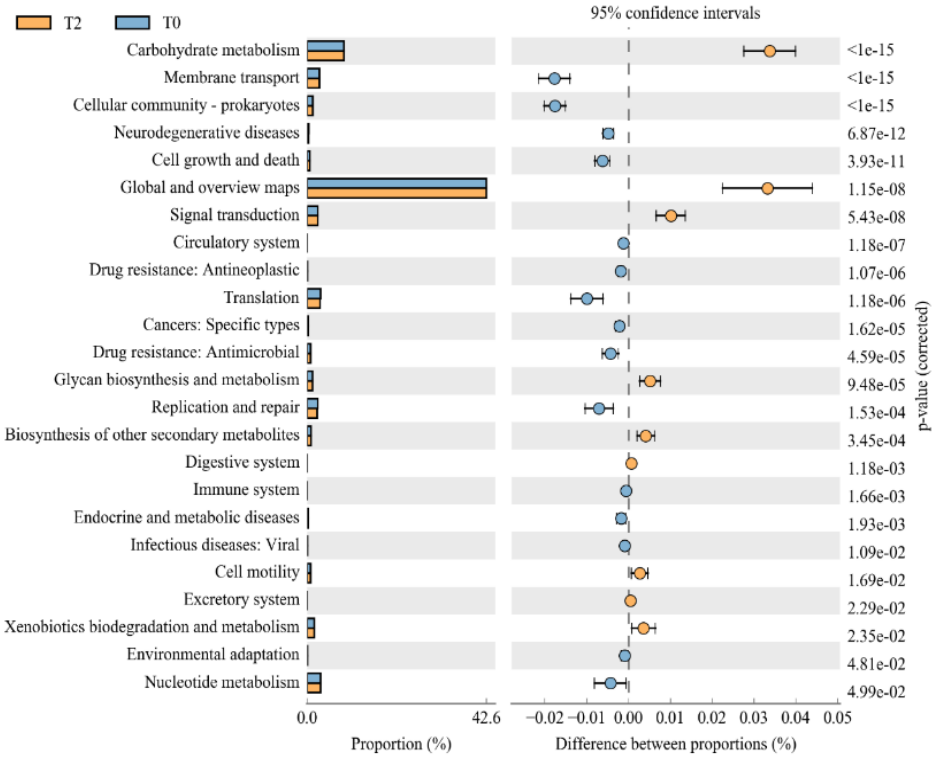

Figure 5. KEGG metabolic pathways mediated by population microorganisms in treatment (yellow) and CK (blue) soil samples. The left part shows the abundance proportion of different functions in two samples, the middle part shows the difference proportion of functional abundance within the 95 % confidence interval and the value on the far right is p value. In the middle part, the yellow ball means the pathway more active in treatment than in CK sample, while the blue ball means the pathway more active in CK than in treatment sample.

Also, the application of *S. matsudana* root exudates could reduce some types of bacteria, such as *uncultured_bacterium_f_Enterobacteriaceae*. A previous report has shown that inoculation of *Enterobacter* on *Polygonum hydropiper* and *Polygonum rumex* significantly increased the plant height, chlorophyll and carotenoid content and cadmium and arsenic content in leaves (8). Further, *Kofleria_flava*, which was missing in samples treated with willow root exudates, could regulate the soil microbial community by stabilizing the beneficial microorganisms and decreasing the pathogens in the soil (22). *Uncultured_bacterium_f_Beijerinckiaceae*, which was also deficient in the treated sample, participates in the nitrogen cycle (28). Therefore, the application of *S. matsudana* root exudates improved the diversity of soil bacterial community.

The bacterial species in a community always interact to control the plant growth (13). To well understand the mechanism, the possible metabolites of each species were entirely characterized by Kyoto Encyclopaedia of Genes and Genomes (KEGG) and analysed to reveal their adaptation to the treatment condition. The significant variations in multiple

metabolic processes between the treated and CK bacterial community samples were determined. Comparably, the items, such as digestive system, excretory system, cell motility, biosynthesis and secondary metabolites, were specific in the treatment sample, whereas, the items of circulatory system, immune system, infectious disease, environmental adaption, endocrine and metabolic system categories were abundant in CK (Fig. 5). Excretory system is an important way for bacteria to interact with the host. Proteins secreted by bacteria could play an important role in nutrition acquisition, environmental adaptation, signal communication and virulence for plants (2). Enhancement of excretory system in treatment sample indicates potential effects on plant growth. Secondary metabolites (antibiotics, pigments and other bioactive compounds) are organic compounds with complex chemical structures and diverse physiological functions. Many of these compounds have important agricultural and medical applications (25). Thus, the pathways in the bacteria community affected by willow root exudates would be of more concern. The related genes, metabolites and possible effects on plant growth and stress resistance would be further explored

CONCLUSIONS

Salix matsudana Koidz root exudates contained multiple allelopathic compounds, which resulted in the soil bacteria diversity. The bacteria community affected the plants growth and resistance to stress conditions via gene interactions. Further study of the interactions mechanisms would contribute to more knowledges about plant growth and apply them in management of plant production.

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DECLARATION

We declare that all authors of this manuscript made a significant contribution, and we have not excluded any author that substantially contributed. We have followed the ethical norms established by our respective institutions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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PUBLISHER NOTE