RESEARCH ARTICLE

Coupling of the chemical niche and microbiome in the rhizosphere: implications from watermelon grafting

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Abstract Grafting is commonly used to overcome soil-borne diseases. However, its effects on the rhizodeposits as well as the linkages between the rhizosphere chemical niche and microbiome remained unknown. In this paper, significant negative correlations between the bacterial alpha diversity and both the disease incidence ($r = -0.832$, $P = 0.005$) and pathogen population ($r = -0.786$, $P = 0.012$) were detected. Moreover, our results showed that the chemical diversity not only predicts bacterial alpha diversity but also can impact on overall microbial community structure (beta diversity) in the rhizosphere. Furthermore, some anti-fungal compounds including heptadecane and hexadecane were identified in the rhizosphere of grafted watermelon. We concluded that grafted watermelon can form a distinct rhizosphere chemical niche and thus recruit microbial communities with high diversity. Furthermore, the diverse bacteria and the antifungal compounds in the rhizosphere can potentially serve as biological and chemical barriers, respectively, to hinder pathogen invasion. These results not only lead us toward broadening the view of disease resistance mechanism of grafting, but also provide clues to control the microbial composition by manipulating the rhizosphere chemical niche.

Keywords rhizodeposits, rhizosphere microbiome, diversity, MiSeq sequencing, watermelon grafting

1 Introduction

Root exudation is an important component of the

rhizodeposition process^[1]. Plant roots release enormous amounts of chemicals, encompassing ions, oxygen, inorganic acids, and water, but mainly consisting of carbon-based compounds^[2,3]. The patterns of root exudation are highly variable among different plant species^[4,5], plant cultivars^[6] and even grafted and un-grafted plants within a cultivar^[7]. Roots can regulate the soil microbial community in their immediate vicinity through the exudation of various compounds^[8]. A small change in root exudates can lead to large alterations in the rhizosphere microbial population^[9]. It has been demonstrated that the microbial communities in the rhizosphere of different plant species growing in the same soil are also often different^[10,11]. Even within species, different genotypes can develop distinct microbial communities in the rhizosphere^[12]. The previous studies revealed a variation in the bacterial community and microbial activity in the rhizosphere of own-root bottle gourd (rootstock), own-root watermelon, and grafted watermelon under field conditions^[13]. However, the linkages between the rhizosphere chemical niche and the microbiome remained unknown.

Grafting has been used to increase tolerance to temperature changes^[14,15], enhance nutrient uptake^[16], improve water-use efficiency^[17], and increase tolerance to salt^[18] and flooding^[19]. Currently, as the use of transgenic crops is still controversial, grafting, an ancient technology, shows great potential for disease resistance. In China, grafting watermelon cultivars onto resistant cucurbit rootstocks is currently being considered as a practical alternative to avoid soil-borne diseases^[13,20]. Moreover, grafting has been a routine technique for resistance to soil-borne disease in continuous mono-cropping systems in many countries and regions all over the world^[21]. The disease resistance-related mechanisms of watermelon grafting have been gradually revealed in recent years. The key factor should be the tolerance of stock plant roots

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to soil borne diseases^[22], but some other multiple non-exclusive explanations for the disease resistance-related mechanism have also been found. Ling et al.^[7] reported that the root exudates of grafted-watermelon discouraged pathogen colonization and were toxic to the pathogens. The higher rhizosphere biodiversity of the grafted watermelon might give a positive feedback to the resistance to soil-born pathogen invasion^[13]. However, knowledge of the disease-resistance of grafting associated with rhizosphere biodiversity still needs to be comprehensively extended by employing more types of rootstocks, including the prevalently used rootstocks, bottle gourd (*Lagenaria siceraria*) and pumpkins (*Cucurbita pepo* L.)^[22].

Based on the increasing awareness of biodiversity in soil and its critical role in maintaining soil health, quality and function^[23], as well as the established knowledge of the relationship between the degree to which soil can suppress plant soil-borne diseases and influence the abundance or diversity of soil microbial communities^[24–26], currently, we hypothesized that the structural variations of a root-associated microbiome after grafting could correspond to the concomitant alteration in rhizodeposits, which would be positively associated with the potential to constrain pathogen invasion. To test this hypothesis, rhizosphere soils of the five plant types grown under field conditions were collected to determine the variations in the rhizodeposits and the microbial community structures by GC-MS and Illumina MiSeq sequencing, respectively. The *Fusarium oxysporum* f. sp. *niveum* (FON) population was quantified by qPCR. We proposed that the coupling of the rhizodeposits and the rhizosphere microbiome may give us further insight to predict and manipulate the microbial community structure and diversity in the soil, as well as to its contribution to soil disease-suppression.

2 Materials and methods

2.1 Plant materials

The commercial watermelon [*Citrullus lanatus* (Thunb.) Matsum. and Nakai var. Zaojia 8424, Nanjing Institute of Vegetable Science, China] was used as the scion. The bottle gourd [*Lagenaria siceraria* (Molina) Standl. var. dayehuizi, Nanjing Institute of Vegetable Science, China] and pumpkin (*Cucurbita pepo* L. var. Jiuzhan No. 1, Nanjing Institute of Vegetable Science, China) were selected as the rootstocks. Therefore, five types of plants were employed in this study: own-root watermelon (W), watermelon grafted onto bottle gourd (WB), watermelon grafted onto pumpkin (WP), own-root bottle gourd (B) and own-root pumpkin (P). Seedlings were grafted by hand in a glasshouse with the insertion grafting method described by Lee^[22].

2.2 Field design and soil sampling

The plants were grown in a plastic greenhouse in the Nanjing Institute of Vegetable Science, China. The soil in the greenhouse was used to plant own-root watermelon in the previous year. The greenhouse covered a field area of 200 m² which was divided into 15 plots (13 m² per plot). The five treatments (W, WB, WP, B and P) were organized in a completely randomized block design. Each treatment contained three plots as three replicates, and each plot had 10 seedlings. The plants were transplanted to the field when the seedlings formed true leaves. After transplanting, the seedlings were grown at temperatures ranging from 24 to 38°C, and managed according to the typical farming practice (270 kg N by urea, 140 kg P₂O₅ by superphosphate, and 300 kg K₂O by muriate of potash per hectare applied with water 12 times during the entire growth stage).

The disease incidence was calculated at the flowering stage. Then triplicate rhizosphere soils were destructively sampled from three plots of each treatment. The rhizosphere soil of healthy plants was sampled as previously described^[27] with some modifications. Briefly, four plants from each plot were uprooted, pooled together and gently shaken to remove all the soil loosely adhering on the roots. The roots with adhering soil (the soil could not be shaken off) were collected and cut into 0.5 cm segments. These segments were immersed in sterile distilled water and sonicated for 10 min to separate the adhering soil. The soil suspension was collected and freeze-dried to obtain the rhizosphere soil. The soil from each replicate was then divided into two sub-samples for DNA extraction and rhizosphere chemical deposition assay.

2.3 Extraction and identification of chemical compositions from rhizosphere

Two grams of soil for each replicate were placed in a 50 mL centrifuge tube, and 20 mL ethyl acetate was added at a ratio of 1:10. After shaking at 30°C for 1 h, the suspension was filtered (0.45 µm) into 100 mL Erlenmeyer flask and concentrated to 0.5 mL at 35°C using a vacuum rotary evaporator, then, the sample was stored in a sample bottle for GC-MS determination.

The extracts of the rhizosphere soil were identified by GC-MS. The mass spectrometer was operated in the electron ionization mode at 70 eV, with a source temperature of 230°C. The initial oven temperature of 60°C was held for 3 min, increased at a rate of 5°C·min⁻¹ to 150°C, further increased at a rate of 3°C·min⁻¹ to 240°C, and held for 10 min. A continuous scan from 40 m/z to 500 m/z was used. Helium trickled as the carrier gas at a linear velocity of 1.0 mL·min⁻¹. The mass spectra of compositions were compared with those in the National Institute of Standards and Technology (NIST) database (Version 2.0).

2.4 DNA extraction

Total genomic DNA was extracted using a PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and quantity of the DNA samples was checked by a spectrophotometer (NanoDrop, ND2000, Thermo Scientific, Wilmington, DE, USA) after extraction.

2.5 Determination of FON by quantitative PCR

The abundance of FON in the rhizosphere soil was determined by quantitative PCR (qPCR). The specific primers (Fn-1/Fn-2) used for quantification of FON were described by Zhang et al.^[28]. Plasmid standard for the quantification of FON was generated from a cloned target gene from FON genomic DNA. The real-time qPCR amplifications were conducted by a 7500 Fast Real-Time qPCR System (Applied Biosystems) using the SYBR® Premix Ex Taq™ kit (Takara). The standard curve was generated according to a previous report^[29]. The abundance of FON was expressed as copy concentration, as described previously^[30].

2.6 Illumina MiSeq sequencing and data analysis

The composition and diversity of bacterial and fungal communities were assessed by Illumina MiSeq sequencing analysis of the 16S rRNA gene and ITS region, respectively. The universal primers 520F (5'-AYTGG-GYDTAAAGNG-3') and 802R (5'-TACNVGGGTATC-TAATCC-3') were selected for the PCR amplification of the V4 region. Fungi-specific primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') were selected for the PCR amplification of the fungal ITS1 region. The reverse primer contains a 6 bp error-correcting bar code unique to each sample. Illumina MiSeq sequencing was conducted by the Personal Biotechnology Co., Ltd. (Shanghai, China) using an Illumina MiSeq platform. Sequences were submitted to the NCBI database under the accession number SRR2141199 for bacteria and SRR2141200 for fungi.

The raw sequences obtained were then processed using the Quantitative Insights Into Microbial Ecology (QIIME) toolkit^[31] and UPARSE pipeline^[32]. The standard primer sets and barcodes were excluded. Sequences with a quality score lower than 25 were removed. Sequences that were shorter than 200 bp or contained any unresolved nucleotides were trimmed. Illumina MiSeq sequencing data were de-noised, and chimeras were identified and eliminated from the data sets. We picked operational taxonomic units (OTUs) to obtain an OTU table using the UPARSE pipeline. Sequences with 97% similarity were assigned to OTUs, and 7617 bacterial OTUs and 2918 fungal OTUs were obtained. We picked a representative sequence from

each OTU and assign taxonomic data to each of these representative sequences using the Ribosomal Database Project (RDP) classifier^[33]. The UniFrac matrix^[34] of samples was generated by QIIME.

2.7 Network analysis

For network inference, we constructed a correlation matrix by calculating all possible pairwise Spearman's rank correlations between the microbial genera and the chemicals in the present study (306 bacterial genera, 338 fungal general and 58 chemicals). A co-occurrence event was considered statistically robust if the *P* was < 0.01 and the Spearman's correlation coefficient (ρ) ranged from 0.60 to 0.93^[35]. The nodes in the reconstructed network represent the microbial genera (nodes labeled by black) and the chemical compounds (nodes labeled by red), whereas the edges (connections) correspond to a robust correlation between nodes. To describe the topology of the resulting network, the average node connectivity and modularity index were calculated^[36]. As described by Newman^[37], the modularity index > 0.4 suggests that the network has a modular structure. All statistical analyses were carried out in the R environment using vegan packages^[38]. Networks were explored and visualized by the interactive platform Gephi^[39].

2.8 Statistical analysis

The disease incidence was calculated as the percentage of the diseased plants in each plot. High throughput sequencing data was transformed to the relative abundance of the OTUs of each sample. The relative abundance of each OTU was expressed as the number of sequences affiliated with that group divided by the total number of sequences per sample. The GC-MS data was also transformed by dividing each peak area by the sum of all peak areas per sample. One-way ANOVA was used to compare the relative abundance among treatments at the significance level of 0.05 (SPSS software, version 16.0).

Shannon diversity was used as the measure of both chemical and microbial alpha diversities. A linear regression analysis was used to evaluate if the microbial alpha diversities at an individual sample-level were significantly related to the corresponding chemical alpha diversity (i.e., bacterial and fungal Shannon diversities with chemical Shannon diversity).

The transformed data were then transformed into three distance matrices (i.e., one for chemicals, one for bacteria and one for fungi) using the Bray-Curtis index of community dissimilarity^[40]. For the data visualization, non-metric multidimensional scaling (NMDS) was also applied to all samples, but only the centroids and standard deviations (SDs) of the different treatment groups were displayed on the plots.

To examine if the beta diversity (pairwise dissimilarity in community composition) for the rhizosphere deposition

predicts beta diversity of soil microbial communities, we compared Bray-Curtis dissimilarity matrices (pairwise comparisons between all samples) for chemicals with those of bacteria and fungi using Mantel tests and Pearson correlations (using R version 3.0.2). For the avoidance of the pseudo-replication, we then averaged the dissimilarities by treatment. Thus, the final pairwise comparisons are between treatments, averaged from sample-level dissimilarities. Then, we tested whether chemical beta diversity was a significant predictor of bacterial and fungal beta diversity.

3 Results

3.1 Variation in the rhizodeposits and microbial composition

To evaluate the rhizodeposits in the rhizosphere, the ethyl acetate extracts from rhizosphere soils of the W, WB, WP, B and P treatments were analyzed by GC-MS (Appendix A, Table S1, Fig. S1). The GC-MS identified 48 compounds, including 6 esters, 22 alkanes, 4 phenols and 10 aromatic hydrocarbons. Twenty-five of the compounds were shared by W, WB and WP treatments, while 12 components appeared only in grafting treatments (Appendix A, Table S1). Identified chemicals with significant difference ($P < 0.05$) in relative abundance among treatments are shown in Table 1. The concentration of heptadecane, 2,6,10-Trimethyltetradecane, 1,8-Dimethylnaphthalene, etc. was significantly higher in the rhizodeposits of WB, WP, B and P than that of the W (Table 1), while the relative abundance of 2,2'-Methylenbis(6-tert-butyl-4-methylphenol) and 1,2-Benzenedicarboxylic acid, 1,2-bis(2-propylhexyl) ester was higher in the rhizosphere of un-grafted watermelon than that of other treatments (Table 1).

The chemical alpha diversities (measured by the chemical Shannon diversity) varied in the rhizosphere of the different plants, as did the bacterial and fungal alpha diversities (measured by the microbial Shannon diversity) (Fig. 1). The chemical alpha diversity of the WP was the highest of the five treatments with a mean of 3.28 and significantly higher than that of the W (2.57) and B (2.56) (Fig. 1a). The bacterial alpha diversities among the five treatments showed a similar variation trend with the chemical alpha diversities (Fig. 1b). Moreover, the fungal alpha diversity of W was 3.03 on average, significantly lower than that of the other four treatments (ranging between 3.46 and 3.80) (Fig. 1c). Taken together, the grafted plants (WB and WP) were arresting in alpha diversities comparison, in which all the chemical, bacterial or fungal alpha diversities were the highest. These results indicated that the grafted watermelon (WB and WP) rhizosphere had a richer variation of components which can accommodate a more diverse bacterial community

when compared with that of the un-grafted watermelon (W).

Two-dimensional non-metric multidimensional scaling (NMDS) plots revealed how the different plant types related to changes in the soil chemical composition and microbial communities (Fig. 2). The stress values were below 0.2 and R^2 values were greater than 0.9. For the chemicals in the rhizosphere, the pairwise contrasts indicated that the W was separated from the other four treatments by axis 1, while the WP and WB were quite similar in both axes (Fig. 2a). This result showed that the grafted watermelon (WB and WP) shared relatively more similar assemblages in their rhizospheres than the un-grafted ones. However, the WB and WP can also be detached by different root-stocks. Moreover, both bacterial and fungal community compositions showed significant variations between the plant types (Fig. 2b, Fig. 2c).

3.2 Coupling the rhizodeposits and rhizosphere microbiome in alpha and beta diversity

The chemical alpha diversity was significantly influenced by the corresponding microbial alpha diversity. Positive and significant ($r = 0.91$, $P < 0.001$) correlations between the bacterial alpha diversities and the corresponding chemical alpha diversities were observed (Fig. 3). The chemical alpha diversity explained 83% ($R^2 = 0.83$) of the variation of the bacterial alpha diversity, although no significant relationships between the fungal and chemical alpha diversities were detected ($r = 0.506$, $P = 0.054$). These results indicated that the alpha diversity of soil bacteria would be positively associated with that of the chemical composition in the rhizosphere, which was in agreement with our hypothesis, but the chemical alpha diversity could not predict the fungal alpha diversity in the rhizosphere.

In addition to the alpha diversity, our hypothesis predicting significant positive correlations between soil microbiome and the rhizodeposits across plots was also supported by the beta diversity, i.e., the sites that were more distinct in the composition of their chemical niches also harbored more distinct soil microbial communities. This was consistent for fungi ($r = 0.780$, $P = 0.001$) and, to a lesser extent, bacteria ($r = 0.684$, $P = 0.005$, Fig. 4). The chemical niches explained 61% ($R^2 = 0.61$) of the variation of fungal community structures and 47% ($R^2 = 0.47$) of the variation of bacterial community structures in terms of beta diversity.

3.3 Correlations between the disease incidence/pathogen population and the bacterial/fungal alpha diversities

The highest disease incidence was observed in the W treatment, 65%, while the disease incidence in WB and WP treatments was 30% and 12%, respectively (Appendix

Table 1 Identified chemicals with significant difference in relative abundance between treatments

	No.	RT	Name	Area%				
				W	WB	WP	B	P
Alkane	GC11	12.17	2,6,10-Trimethyldecane	0 (0) b	0.19 (0.12) a	0.18 (0.12) a	0.18 (0.09) a	0 (0) b
	GC22	17.34	Heptadecane	0 (0) b	0.46 (0.13) a	0.54 (0.13) a	0.56 (0.28) a	0.50 (0.20) a
	GC28	19.18	10-methyl nonadecane	0 (0) b	0 (0) b	0.19 (0.14) a	0 (0) b	0 (0) b
	GC37	23.22	2,6,10-Trimethyl tetradecane	1.19 (0.60) b	2.23 (0.96) a	2.66 (0.88) a	2.62 (0.74) a	2.27 (0.85) a
	GC38	23.42	Heptacosane	0 (0) c	0 (0) c	0.79 (0.10) a	0 (0) c	0.40 (0.18) b
	GC47	25.08	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	0 (0) b	0.76 (0.45) a	0.73 (0.17) a	0 (0) b	0 (0) b
	GC50	25.71	9-Hexyl heptadecane	0 (0) b	0.51 (0.36) a	0 (0) b	0 (0) b	0 (0) b
	GC64	36.18	Pentacosane	0 (0) b	0.39 (0.24) ab	0.57 (0.10) a	0.65 (0.57) a	0 (0) b
	GC67	40.61	Tetratetracontane	0 (0) b	0.74 (0.38) a	0 (0) b	0.25 (0.08) b	0.32 (0.09) b
	GC9	11.50	3,5-Dimethyl cumene	0 (0) b	0.29 (0.10) b	0.36 (0.16) b	1.07 (0.77) a	0 (0) b
Arene	GC10	11.87	Pentamethyl benzene	0 (0) b	0.27 (0.21) ab	0.26 (0.12) ab	0.7 (0.55) a	0 (0) b
	GC21	17.14	1,8-Dimethylnaphthalene	0 (0) b	0.74 (0.49) a	0.62 (0.18) ab	0.71 (0.33) a	0.75 (0.53) a
Alcohol	GC3	8.45	2-Methyloctanol	0 (0) b	0.29 (0.23) a	0 (0) b	0 (0) b	0 (0) b
Phenol	GC18	14.58	3-tert-Butylphenol	0 (0) b	2.41 (1.69) a	3.78 (1.94) a	2.65 (2.30) a	0 (0) b
Ester	GC68	42.25	2,2'-Methylenebis(6-tert-butyl-4-methylphenol)	5.73 (1.82) a	1.19 (0.78) b	0.34 (0.31) b	0 (0) b	0.54 (0.34) b
	GC26	18.65	Dimethyl phthalate	0 (0) b	0.75 (0.44) a	0.59 (0.07) a	1.88 (1.64) a	0.65 (0.16) a
	GC54	28.17	1,2-Benzenedicarboxylic acid, dihexyl ester	0 (0) b	6.72 (5.23) ab	10.24 (6.09) a	4.96 (4.11) ab	6.68 (6.72) ab
	GC58	30.05	Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate	0 (0) b	0.37 (0.24) a	0.53 (0.16) a	0 (0) b	0.35 (0.25) a
	GC65	38.31	3-Methoxy 4-(methoxycarbonyl)-5-methylphenyl 4-[(2,4-dimethoxy-6-methylbenzoyl)oxy]-2-methoxy-6-methylbenzoate	0 (0) b	0.35 (0.02) a	0.35 (0.06) a	0.2 (0.16) ab	0.22 (0.18) ab
Acid	GC69	45.61	1,2-Benzenedicarboxylic acid, 1,2-bis(2-propylhexyl) ester	28.74 (23.76) a	2.55 (1.83) b	2.40 (1.77) b	0.43 (0.10) b	2.50 (1.06) b
	GC56	29.38	2-Propenoic acid, 3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-	0 (0) b	0 (0) b	0 (0) b	0.32 (0.21) a	0.50 (0.15) a
Others	GC2	7.00	Hydroxylamine, O-decyl-	0 (0) b	0.20 (0.12) a	0 (0) b	0 (0) b	0.20 (0.09) a

Note: No., the number of chemical substance identified by GC-MS; RT, retention time; Area%, the relative abundance mean value of three replicates, the color of each cell from green to red represents of the value from low to high. The values in parentheses are SDs. The values followed by different letters are significant difference ($P < 0.05$, one-way ANOVA). W, un-grafted watermelon; WB, watermelon grafted onto bottle gourd; WP, watermelon grafted onto pumpkin; B, bottle gourd rootstock; P, pumpkin rootstock.

Fig. 1 Shannon diversities for the chemical composition (a), bacteria (b) and fungi (c) in the rhizosphere of all the treatments. Each column represents the mean value of three replicates. Bars represent the SEs of the mean. The means in a column with the same letter are not significantly different at $P < 0.05$. Different colors represent different treatments. W, un-grafted watermelon; WB, watermelon grafted onto bottle gourd; WP, watermelon grafted onto pumpkin; B, bottle gourd rootstock; P, pumpkin rootstock.

A, Table S2). According to the results of qPCR, the copy number of FON in the W rhizosphere was up to 10^7 , while in the WP and WB treatments it was 10^5 and 10^6 , respectively (Appendix A, Table S2). The correlation analysis results showed that the bacterial alpha diversity was negatively and significantly correlated with both the corresponding disease incidence ($r = -0.832$, $P = 0.005$) and the FON population ($r = -0.786$, $P = 0.012$) (Fig. 5a, Fig. 5b). Intriguingly, the fungal alpha diversity did not significantly affect the corresponding disease incidence ($r = -0.645$, $P = 0.061$) and FON population ($r = -0.569$, $P = 0.110$) (Fig. 5c, Fig. 5d). These results indicated that the alpha diversity of soil bacteria would be positively related with the potential to constrain pathogen invasions.

3.4 Co-occurrence between the chemicals and bacterial genera in the rhizosphere

The co-occurrence patterns between the chemicals and

Fig. 2 First two non-metric multidimensional scaling (NMDS) axes of community structures of chemical composition (a), bacteria (b) and fungi (c). The points represent the means (both axes) of three replicates and are colored by treatments. The bars show the SE along each NMDS axis. W, un-grafted watermelon; WB, watermelon grafted onto bottle gourd; WP, watermelon grafted onto pumpkin; B, bottle gourd rootstock; P, pumpkin rootstock.

bacterial genera in the rhizosphere were visualized using network inference based on strong ($\rho > 0.6$) and significant ($P < 0.01$) correlation (Fig. 6). The resulting network (Fig. 6) consisted of 136 nodes (including 95 genera and 41 chemicals) and 202 edges (average degree or node connectivity 1.49). The modularity index was 0.711 suggesting that the network had a modular structure. Based on the modularity class, the entire network could be

Fig. 3 Relationships between the chemical Shannon diversity and Shannon diversities for bacteria (a) and fungi (b) in the rhizosphere of all treatments. The relationship between the Shannon diversities for chemical composition with bacteria ($r = 0.910$, $P < 0.001$) was stronger than with fungi ($r = 0.506$, $P = 0.054$). Different colors represent the different treatments, and each treatment includes three replicates. W, un-grafted watermelon; WB, watermelon grafted onto bottle gourd; WP, watermelon grafted onto pumpkin; B, bottle gourd rootstock; P, pumpkin rootstock.

parsed into 11 major modules. The two largest modules: Module 2 and 3 contained 31 and 30 of the vertices respectively. Eleven from a total of 41 chemicals occupied Module 2, including 9 kinds of alkanes, 1 hydroxylamine and 1 alcohol. The alkane vertices were enriched from 19 in 41 (in the whole network) to 9 in 11 in this module.

As described by Li et al.^[41], the most densely connected node in each module was defined as the ‘hub’ in the following statements. The ‘9-Hexylheptadecane’ was the hub of Module 2, which was co-occurrent with 18 genera including *Azoarcus* ($r = 0.677$, $P = 0.006$) and *Brevibacillus* ($r = 0.663$, $P = 0.007$). The ‘3-tert-Butylphenol’ was the hub for Module 3 (Fig. 6) which was co-occurrent with 16 genera including *Bacillus* ($r = 0.732$, $P = 0.002$) and *Pseudomonas* ($r = 0.772$, $P = 0.001$). *Geothrix*, *Asanoa* and *Dokdonella* were the top 3 genera with the most connections (9, 8 and 8, respectively) with the chemicals in the network. Six of the 9 chemicals, including 2,6,10,14-tetramethylpentadecane, 2-methyloctanol, 2-methyltriacetane, 9-hexylheptadecane, nonacosane and octadecane could significantly affect all the three genera.

Fig. 4 Relationships between the beta diversity for the chemical composition and bacteria (a) ($r = 0.684$, $P = 0.005$) and fungi (b) ($r = 0.780$, $P = 0.001$). Each point represents the dissimilarity in the taxonomic composition between a pair of treatments, calculated as the average of the Bray-Curtis dissimilarities for all the comparisons (typically 9) between the replicates from those two treatments. The different colors represent the comparisons between two different treatments or within a treatment. W, un-grafted watermelon; WB, watermelon grafted onto bottle gourd; WP, watermelon grafted onto pumpkin; B, bottle gourd rootstock; P, pumpkin rootstock.

3.5 Co-occurrence between the chemicals and fungal genera in the rhizosphere

The co-occurrence patterns between the chemicals and fungal genera in the rhizosphere were also investigated using a network analysis approach based on the same qualification as described before (Fig. 7). The resulting network (Fig. 7) consisted of 188 nodes (including 144 genera and 44 chemicals) and 332 edges (average degree of node connectivity 1.77). The modularity index was 0.766. In view of the modularity class, the entire network could be parsed into 12 major modules. 68 of 188 total vertices fell into the two largest modules: Modules 9 and 6, which had obviously different topology structures compared with other modules. 42 of 188 total vertices and 128 of 332 edges belonged to Module 9. 26 of 188 total vertices and 72 of 332 edges were in Module 6. The node connectivities were 3.05 and 2.77 in Module 9 and 6, respectively, which were much higher than the whole network node connectivity of 1.77. *Inonotus* and *Khuskia* in Module 9 owned the most interactions (12 and 11,

Fig. 5 Relationships between the bacterial Shannon diversity and the disease incidence (a), the bacterial Shannon diversity and the number of FON (b), the fungal Shannon diversity and the disease incidence (c), the fungal Shannon diversity and the number of FON (d). Different colors represent the different treatments, and each treatment includes three replicates. W, un-grafted watermelon; WB, watermelon grafted onto bottle gourd; WP, watermelon grafted onto pumpkin.

respectively) with the chemicals in the network. Ten of the 12 chemicals could significantly affect both the two genera, including 2,6,10,14-tetramethylpentadecane, 2-methyloctanol, 2-methyltriacontane, 9-hexylheptadecane, nonacosane, octadecane, 1,8-Dimethylnaphthalene, etc.

4 Discussion

The patterns of root exudation strongly vary between plant species and cultivars^[4], even between grafted and un-grafted plants^[7]. In this study, considerable differentiation of the chemical deposition, diversity and structure in the rhizosphere was observed between the five treatments (W, WB, WP, B and P) (Fig. 1 and Fig. 2). Thus, the rhizosphere of each treatment formed a distinct resources niche. It is important to mention that, compared with the substances identified in previous studies of plants grown under hydroponic conditions^[7,42], the chemicals identified by GC-MS in this study (e.g., esters, alkanes, phenols and aromatic hydrocarbons) were different and much more complex. This difference might have occurred because *in situ* soil has a much more complex background nature than

hydroponic culture and many substrate components, such as soil organic matter, can easily interact with the root exudates^[43]. Moreover, some organic substance secreted by the rhizosphere microbiomes may inevitably be extracted. Future experiments should be designed to clarify the exact origin of the rhizosphere chemicals. However, our method still presents a simple untargeted metabolomics workflow for broadly analyzing a rhizosphere chemical niche and provides a way for the analysis of the relationship between rhizospheric chemicals and microbial compositions *in situ*.

There is a tight relationship between the carbon composition and the soil microbial community composition^[44,45]. Based on the multifariousness of the rhizodeposits, rhizosphere microflora can dramatically vary in structure and species composition^[10–12]. Our data reveal a differentiation in the rhizosphere bacterial and fungal microflora among the watermelon (W), two types of grafted watermelon (WB and WP) and the corresponding rootstocks (B and P). There were obvious variations in both the alpha diversity (Fig. 1) and the beta diversity (Fig. 2) of the rhizosphere microbial communities. In the rhizosphere, the rhizodeposits can serve as food for microbes, and different

Fig. 6 Network analysis revealing the co-occurrence patterns among the bacterial genera and chemicals in the rhizosphere. A connection stands for a strong (Spearman's $\rho > 0.6$) and significant ($P < 0.01$) correlation. The nodes were colored according to modularity class. The size of each node is proportional to the number of connections (the degree). A red label represents an identified chemical and a black label represents a bacterial genus.

food structures can recruit different diners (which here refers to the microorganisms)^[46]. In the present study, the grafted, un-grafted and rootstock plants can form various chemical diversities and structures, as well as distinct microbial diversities and structures in the rhizosphere. These data followed the trends observed in other studies, e.g., Broeckling et al.^[47], which revealed that the root exudates of *Arabidopsis thaliana* and *Medicago truncatula* could regulate the diversity of the soil fungal community.

Furthermore, a significant positive correlation was detected between bacterial alpha diversity and chemical alpha diversity in the present study (Fig. 3), which indicates that the alpha diversity of soil bacteria would be positively correlated with that of the chemical

composition in the rhizosphere. Broeckling et al.^[47] reported that soil fungal biomass significantly increased when resident plants were supplemented with nonresident root exudates, which suggested that the alteration of carbon substrate diversity could change the microbial community. While, according to our results, the effect of chemical alpha diversity on the fungal alpha diversity was not significant. Our results indicate that the bacterial alpha diversity was more responsive to the rhizosphere condition variations than the fungal alpha diversity. Coincidentally, the chemical beta diversity (compositional dissimilarity between plots) was significantly associated with the beta diversity of both the bacterial and fungal communities (Fig. 4). This result is consistent with recent conclusions

Fig. 7 Network analysis revealing the co-occurrence patterns among the fungal genera and chemicals in the rhizosphere. A connection stands for a strong (Spearman's $\rho > 0.6$) and significant ($P < 0.01$) correlation. The nodes were colored according to modularity class. The size of each node is proportional to the number of connections (the degree). A red label represents an identified chemical and a black label represents a fungal genus.

that the beta diversity of soil microorganisms corresponds to the beta diversity of plants^[48]. We concluded that chemical diversity can predict patterns in bacterial alpha diversity and patterns in the structure of soil microflora. More diverse rhizodeposits would be expected to occur with more diverse soil microbial communities, and more distinct rhizodeposits would be expected to occur with more distinct soil microbial communities. It has been reported that the relationship between plant and microbial diversity is stronger for fungal than bacterial groups because fungi are often more directly dependent on plant residuals^[47,49], and mycorrhizal fungi are more dependent on direct symbiotic relationships with plants^[50]. Contrasting

with these reports, Prober et al.^[48] found that plant diversity predicted beta but not alpha diversity of soil microorganisms across grasslands worldwide. According to our data, the relationship between the chemical composition and fungal community was stronger than the bacterial community in beta diversity, while it was much weaker in alpha diversity.

Grafting is widely used to resist soil-borne diseases. The vigorous roots of the rootstock plant can exhibit remarkable tolerance to serious soil borne diseases and the degree of tolerance may vary considerably with the rootstocks^[22]. In the present study, our results revealed that grafting watermelon onto resistant rootstocks can

dramatically reduce pathogenic fungi in the rhizosphere soil (Appendix A, Table S2). In addition, different types of root stocks (B and P) showed various degrees of resistance to *Fusarium* wilt in watermelon. The key factor should be that the roots of resistant cucurbit rootstocks are not susceptible to pathogenic fungi, however, *Fusarium* wilt can occur in grafted plants with non-host root as well^[22]. Therefore, multiple non-exclusive explanations for disease resistance-related mechanism of grafting should exist. As soil-borne plant pathogens must pass across the rhizosphere to invade the plant roots, the pathogens must interact with the local microbial community^[51]. Thus, the pathogen tolerance of the resistant rootstocks should be relevant to both the biological and chemical niches in the rhizosphere.

Biodiversity of microflora is increasingly recognized to constrain invasion by pathogens^[52,53]. Characterizing the rhizosphere microbial community structure is vital to our understanding the mechanism of resistance toward soil borne diseases conferred by grafting. The grafted watermelon (WB and WP) rhizosphere had a richer variation of components, which can accommodate a more abundant and diverse bacterial community compared with the ungrafted watermelons (W). Our data revealed that the disease incidence and FON population decreased with the increase of the bacterial alpha diversity (Fig. 5). This result is in line with previous studies reporting that microbial diversity was inversely related to the invasibility of the wheat rhizosphere by *Pseudomonas aeruginosa*^[54]. Microbial diversity also influenced the ability of *Ralstonia solanacearum* to induce wilting disease in tomatoes and significant negative relationships were observed between the number of wilted plants, the bacterial strains and the carbon sources^[51]. Our results implied that one of the disease resistance-related mechanisms of grafting should be the enriched biodiversity to serve as bio-barrier for excluding the invasion, and thus confirmed that manipulating a chemical niche to regulate rhizosphere microbial diversity may provide a new perspective to control soil-borne diseases.

The volatile organic compounds from plants and bacteria had been demonstrated to have antimicrobial activity and behaved as a signal among the bacterial communities^[55,56]. Yuan et al.^[55] and Raza et al.^[57] reported that volatile organic compounds including alkane, benzene, alcohol, aldehyde, ether, ester and naphthyl compounds can inhibit the growth and spore germination of *Fusarium oxysporum* to various degrees. Volatile compounds emitted from the intact growing roots of Chinese chive can inhibit spore germination of *Fusarium oxysporum* f. sp. *cubense*^[56]. In the present study, certain identified chemicals were enriched in the rhizosphere of at least one type of grafted plant compared to the un-grafted watermelon. Some of those substances were reported as anti-pathogen compounds in previous studies, such as

heptadecane and hexadecane^[57]. Thus, the chemicals with antifungal activity can constitute a chemical barrier in the rhizosphere to suppress *Fusarium oxysporum* invasion.

There was a close relationship between the carbon composition and soil microbial community composition and function^[44]. The co-occurrence patterns between the chemicals and microbial genera in the rhizosphere were explored using network inference (Fig. 6 and Fig. 7). It has been reported that carbon is present in the soil in many different forms and compounds greatly vary in their chemical nature^[58]. The aryl-C content which reflects relatively stable carbon forms can strongly influence the microbial composition. While, carbonyl-C content reflecting relatively labile carbon forms, can strongly influence the microbial activity^[44]. Shi et al.^[59] reported that organic acids caused a significant increase in the richness of the soil bacterial community and large shifts of dominant taxa. In our results, some chemicals can also group together with some genera and form sub-groups (Modules) in the rhizosphere. These chemicals could originate either from plant root secretion or from the metabolites from the rhizospheric microbiome. Therefore, it is reasonable to speculate that the microbial sub-group differentiated from the overall rhizospheric microbiome should be attributed to a certain specific chemical niche derived from the root exudates or microbial metabolism. However, where these chemicals exactly originated still need to be clarified. Moreover, to gain a better understanding of manipulating the soil microbiome, an examination of the relationship between the rhizodeposits and the function and composition of the microbial community following the addition of different chemical predictors to the soil should be conducted in the future.

5 Conclusions

The results of this study broaden our view of the relationship between rhizosphere chemicals and the microbial composition. We conclude that more diverse rhizodeposits would be expected to occur with a more diverse soil microbial community. Moreover, it is not only the biodiversity of microbial communities in rhizosphere that has the potential to constrain invasion by pathogens, but also some anti-pathogen chemicals were detected in the rhizodeposits which may serve as weapons to suppress the pathogens. Thus it is reasonable to propose that, before the FON successfully invades the roots, the pathogen must break through two barriers in the rhizosphere: one is the biological barrier comprised of diverse bacteria, and the other is the chemical barrier consisting of some anti-fungal compounds in rhizodeposits (Fig. 8). These results will not only lead us toward and improved understanding of the mechanism of disease resistance achieved through grafting, but also give clues to shape the microbial composition

Fig. 8 Schematic representation of the disease resistance mechanism achieved through grafting. Before the pathogen successfully invades the roots, it must break through two barriers in the rhizosphere: one is the biological barrier (the blue arc) comprising diverse bacteria, and the other barrier is the chemical barrier (the red arc) consisting of some anti-fungal compounds in rhizodeposits.

by controlling the rhizosphere chemical composition.

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Compliance with ethics guidelines Yang Song, Chen Zhu, Waseem Raza, Dongsheng Wang, Qiwei Huang, Shiwei Guo, Ning Ling, and Qirong Shen declare that they have no conflict of interest or financial conflicts to disclose. This article does not contain any studies with human or animal subjects performed by any of the authors.

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