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Effects of crop rotation with wild rocket on cucumber seedling rhizosphere fungal community composition

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ABSTRACT

In pot culture, we evaluated the effects of wild rocket (*Diplotaxis tenuifolia* (L.) DC. Brassicaceae) rotation on cucumber (*Cucumis sativus* L.) rhizosphere fungal community composition. Cucumber rhizosphere fungal composition was analyzed by high-throughput sequencing of the ITS1 regions of the fungal rRNA gene. Results showed that rotation with wild rocket changed the cucumber seedling rhizosphere fungal community composition but did not influence the fungal community alpha diversity. Crop rotation increased the relative abundances of class *Dothideomycetes* but decreased the class *Zygomycetes* in cucumber rhizosphere. In cucumber rhizosphere, the crop rotation also increased the relative abundances of *Gibellulopsis*, *Myrothecium* but decreased *Mortierella*, *Chaetomium*, *Ilyonectria*, *Thielavia* and *Arachnomyces* spp.

Key words: Crop rotation, *Cucumis sativus*, *Diplotaxis tenuifolia* (L.) DC, fungal community, rhizosphere.

INTRODUCTION

In agriculture, long-term monocropping leads to crop yield decline and several soil-borne diseases due to soil sickness (7,12,38,41). The accumulation of autotoxic compounds, build-up of soil-borne pathogens, changes in soil microbial communities are major possible factors contributing to soil sickness (2,11,13,34,36). Plant soil-borne diseases are difficult to control with conventional methods (use of resistant host cultivars and synthetic fumigants) (4). Recent studies have shown that by increasing the spatial and temporal plant diversity in agricultural fields (e.g. intercropping, crop rotation and use of cover crop or green manure) the build-up of soil-borne pathogens can be prevented, improves soil fertility and promotes the plant growth (1,18,25,28,31,36,40).

Fusarium wilt is vascular soil-borne disease worldwide, causing severe losses in many important crops including the cucumber (*Cucumis sativus* L.) (23,37). The green manures of Brassicaceae crops suppresses the Fusarium wilt in several crops (4). For example, green manures of wild rocket (*Diplotaxis tenuifolia* (L.) DC.), (Fig. 1a), controls the Fusarium wilt of cucumber (16). Wild rocket is rich in ascorbic acid, carotenoids, polyphenols and glucosinolates and is usually used as salad (16). Crop rotation and green manure suppresses the plant soil-borne diseases through several

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mechanisms, [releasing antifungal chemicals and changing the soil microbial communities (1,4,26,36)].

Microorganisms, especially these in plant rhizosphere, are key determinants of plant health and productivity and are considered as major drivers of plant defence to below ground pathogens (9,22). In preliminary studies, we found that rotation of wild rocket suppressed the cucumber Fusarium wilt disease severity (14). However, the responses of cucumber rhizosphere fungal communities to the rotation with wild rocket was unclear. This study aimed to determine the effects of rotation with wild rocket on the cucumber rhizosphere fungal community composition by high-throughput Illumina sequencing of fungal ITS regions.

MATERIALS AND METHODS

Pot culture

Pot soil used in this study was collected from the upper soil layer (0-15 cm) of a greenhouse in the experimental station of Northeast Agricultural University, Harbin, China (45°41'N, 126°37'E). The greenhouse has been used for cultivating cucumber continuously since 2006. Two crops of cucumber were raised each year, one in spring (April to July) and the other in autumn (July to October). Soil sampling was done in July 2016 after the harvest of first cucumber crop. Soil samples were sieved (2 mm) to remove the large stones and plant derbies. The soil was sandy loam, contained organic matter, 3.51%; inorganic N (NH_4^+-N and NO_3^--N), 146.60 mg/kg; Olsen P, 284.20 mg/kg; available K, 341.80 mg/kg; EC (1:2.5, w/v), 0.43 mS/cm; and pH (1:2.5, w/v), 7.64 as per standard methods (32,33).

Thirty seeds of wild rocket (cv. Shuangji) were directly sown per plastic pot (20 cms dia, 17 cms depth) containing 2.5 kg fresh soil. After emergence, wild rocket seedlings were thinned to 10-plants per pot. A fallow treatment, pots with no wild rocket plants served as control. No fertilizer was used during the experiment. All pots were maintained in greenhouse (32°C day/22°C night, relative humidity of 60-80%, 16 h light/8 h dark). Each treatment included three replicates (i.e. three blocks) with 10 pots per replicate (30 pots per treatment in total). Soil water content was maintained at 65% of its water holding capacity (WHC).

Forty days after sowing, above ground shoots were harvested, while roots were left in the soil. Then, all pots were covered with black polyethylene films and the soil water content was maintained at about 65% of its WHC. After incubation for 30 days, cucumber seedlings (cv. Jinyan 4) with two cotyledons were planted in these pots. Each pot contained one seedling. All pots were maintained in greenhouse (32°C day/22°C night, relative humidity of 60-80%, 16 h light/8 h dark). Soil water content was maintained at about 65% of its WHC.

Rhizosphere soil sampling and soil DNA extraction

Thirty days after transplanting, cucumber rhizosphere soil samples were collected as described before (35,39,42). Samples from 10-plants in each replicate of the individual treatment were combined to make a composite sample. After sieving (2 mm

mesh), these rhizosphere soils were stored at -80°C for DNA extraction. Total soil DNA was extracted from 0.25 g rhizosphere soil sample of each pot with the PowerSoil DNA Isolation Kit (MO BIO Laboratories, CA, USA) as per the manufacturer's instructions.

Illumina Miseq sequencing and data processing

The ITS1 regions of the fungal rRNA gene were amplified with primer sets of ITS1F/ITS2 as described before (8,29,37). Both the forward and reverse primers also had a 6-bp barcode unique to each sample. Each soil sample was independently amplified, the products of the triplicate PCR reactions were pooled, purified and paired-end sequenced (2×300) on an Illumina Miseq platform at Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China. Raw sequence reads were de-multiplexed, quality-filtered and processed using FLASH as described before (21,36). Chimeric sequences were identified and removed using USEARCH 6.1 in QIIME (6). Sequences were binned to Operational taxonomic units (OTUs) at 97% sequence similarity with USEARCH using an agglomerative clustering algorithm. Then, a representative sequence of each OTU was taxonomically classified through BLAST against the Unite database.

Statistical analysis

To avoid potential bias caused by sequencing depth, a random subsampling effort of 30,785 sequences per sample was done. The defined OTUs were used to calculate taxon accumulation curves. For alpha diversity analysis, Shannon and inverse Simpson indices were calculated (42). Beta diversity analysis was used to evaluate differences in fungal community structures with UPGMA hierarchical clustering analyses based on Bray-Curtis distances. The shared and unique OTUs between treatments were counted and their distributions were shown in a Venn diagram. Differences in alpha diversity indices and relative abundances of microbial taxa between treatments were analyzed using Student's *t* test. All these analyses were done in 'R' (Version 3.3.1).

RESULTS AND DISCUSSION

Illumina Miseq sequencing data

In total, Illumina Miseq sequencing yielded 219,809 quality fungal sequences, with 30,785-43,927 sequences per sample. The average read length of the ITS1 regions was 260 bp. A total of 448 OTUs were identified at 97 % sequence similarity. Rarefaction curves of OTUs at 97 % sequence similarity of all samples tended to approach the saturation plateau (Fig. 1b). The Good's coverage of each sample, which reflects the captured diversity, was above 99.70 %. Therefore, the Illumina Miseq sequencing data were enough for community analysis in this study.

Fungal community alpha and beta diversities

For fungal community alpha diversity in cucumber rhizosphere, the number of OTUs, Shannon and inverse Simpson indices (42) were similar between the treatments

of monocropping and crop rotation (Fig. 1c). For fungal community beta diversity, hierarchical clustering revealed that monocropping and rotation samples separated from each other (Fig. 1d).

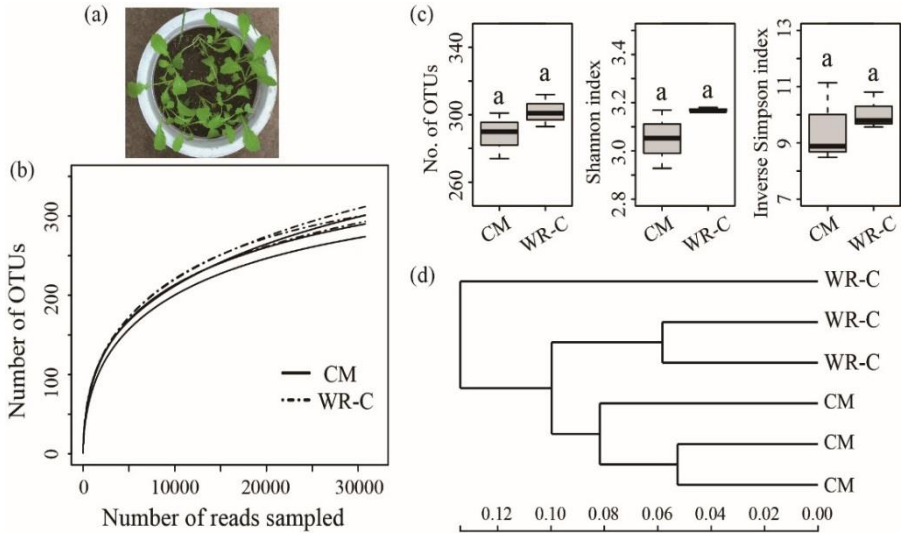

Figure 1. Photograph of wild rocket (a), Rarefaction curves of the number of OTUs (b), alpha diversities (c) and hierarchical clustering (d) of rhizosphere fungal community.
CM: Cucumber monocropping, WR-C: Wild rocket rotation with cucumber. Different letters indicate significant different (P<0.05).

Taxonomic characteristics of fungal communities

In total, 1.16 % sequences were unclassified at the phylum level (Fig. 2a). *Ascomycota* and *Zygomycota* were the dominant phyla, which accounted for 81.13 % and 16.49 % of the total fungal sequences, respectively. Compared with the monocropped cucumber, rotation with wild rocket increased the relative abundance of *Unclassified Fungi* but decreased that of *Zygomycota* in cucumber rhizosphere (P <0.05).

At the class level, *Sordariomycetes*, *Pezizomycetes* and *Zygomycetes* were the dominant classes (average relative abundance >10 %) (Fig. 2b). These four classes accounted for > 92.83 % of the total fungal sequences. Rotation with wild rocket increased the relative abundances of *Dothideomycetes* and *Unclassified Fungi* but decreased that of *Zygomycetes* in cucumber rhizosphere (P <0.05).

At the order level, *Microascales*, *Pezizales*, *Mortierellales*, *Sordariales* and *Hypocreales* were the dominant classes (average relative abundance >10 %) (Fig. 2c). Crop rotation of wild rocket increased the relative abundances of *Pleosporales* and *Unclassified Fungi* but decreased those of *Mortierellales* and *Arachnomycetales* in cucumber rhizosphere (P <0.05).

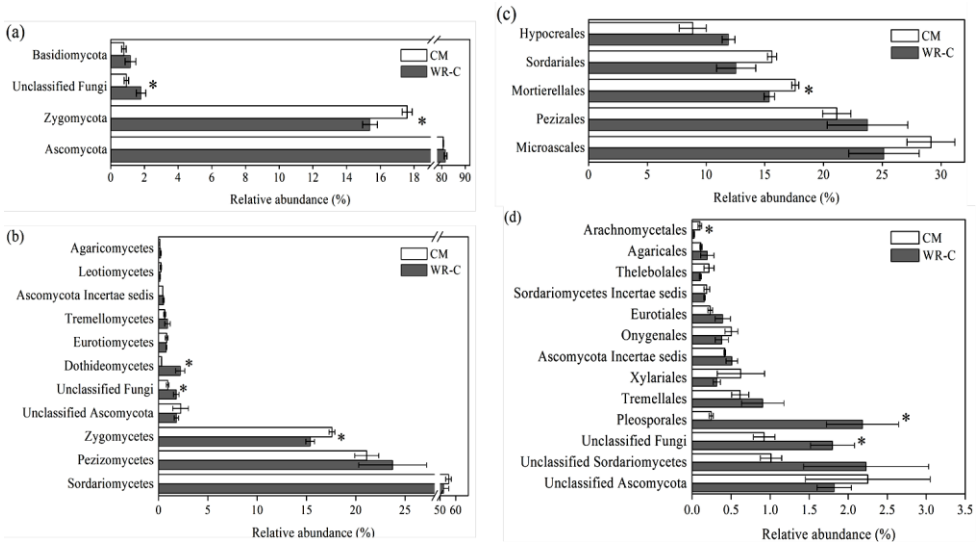

Figure 2. Relative abundances of main fungal phyla (a), classes (b) and orders (c, d).
CM: Cucumber monocropping, WR-C: Wild rocket rotation with cucumber. Asterisks indicate significant different (P<0.05).

At the order level, *Microascales*, *Pezizales*, *Mortierellales*, *Sordariales* and *Hypocreales* were the dominant classes (average relative abundance >10 %) (Fig. 2c). Crop rotation with wild rocket increased the relative abundances of *Pleosporales* and *Unclassified Fungi* but decreased those of *Mortierellales* and *Arachnomycetales* in cucumber rhizosphere (P<0.05) (Fig. 2c, d).

More than 140 fungal genera were detected across all samples analyzed (data not shown). Among the classified genera, *Pseudallescheria*, *Mortierella*, *Chaetomium*, *Fusarium*, *Pseudaleuria*, *Humicola*, *Acremonium* and *Kernia* spp. had mean relative abundances > 1.0 % (Fig. 3a). Rotation with wild rocket increased the relative abundances of *Gibellulopsis*, *Myrothecium* but decreased those of *Mortierella*, *Chaetomium*, *Ilyonectria*, *Thielavia* and *Arachnomyces* spp. in cucumber rhizosphere (P < 0.05) (Fig. 3a, b).

Shared and unique OTUs

In total, about 74.78 % of the total OTUs were shared by all treatments (Fig. 3c). OTUs unique to the treatment of cucumber monocropping were mainly composed of sequences belonging to classes of *Sordariomycetes*, *Agaricomycetes* and *Unclassified Fungi*, while those unique to rotation with wild rocket with cucumber were dominated by sequences belonging to classes of *Dothideomycetes*, *Unclassified Fungi* and *Agaricomycetes*.

Soil microorganisms rely on carbon and nutrient resources from plant rhizodeposition and litter and the chemistry of compositions of plant rhizodeposition and litter differ among the plant species (3,30). Therefore, plants can exert species-specific

effects on the soil microbial communities, which may contribute to the observed changes in soil microbial communities in crop rotation systems (20). *Gibellulopsis* spp. has been found in the rhizosphere of several plants (15). *Gibellulopsis nigrescens* is a pathogen of Brassicaceous plants (10). It is possible that specific compounds from wild rocket had stimulating effects on *Gibellulopsis* spp. However, the effects of *Gibellulopsis* spp. on cucumber growth is unclear.

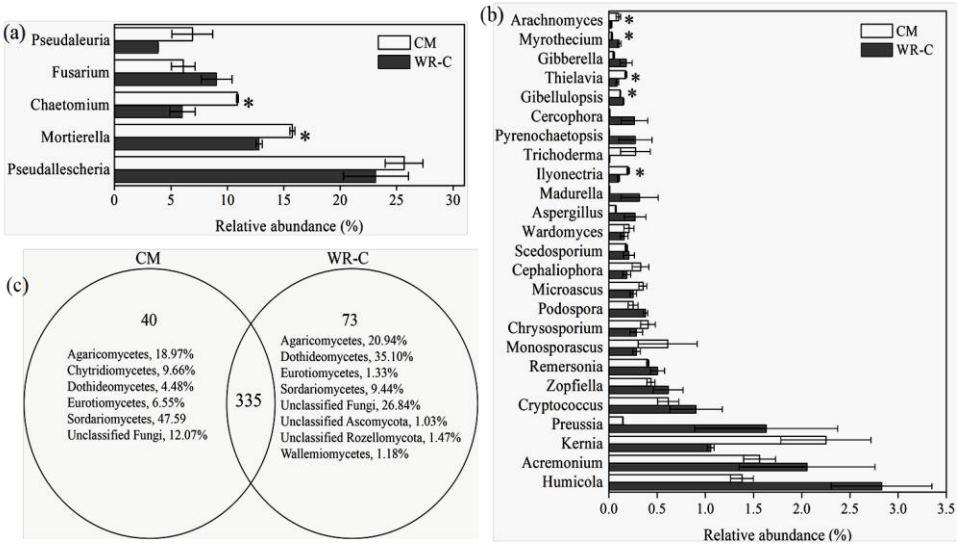

Figure 3. Relative abundances of classified fungal genera (a, b) and Venn diagram analysis (c). For the Venn diagram, frequencies of OTUs unique to each treatment at the class level were shown. CM: Cucumber monocropping, WR-C: Wild rocket rotation with cucumber. Asterisks indicate significant different (P<0.05).

In this study, rotation with wild rocket decreased the relative abundances of *Ilyonectria* (27) and *Thielavia* (24) spp., which contained species that are pathogenic to many plants. Soil microbial community composition and its function are closely linked and changes in soil microbial community composition may alter the functions of the community and thus have feedbacks on plant health and fitness (2,36,37). For example, tomato-celery-cucumber- Chinese cabbage crop rotation changed the soil microbial community composition to stimulate the cucumber growth (36). Our results indicated that rotation with wild rocket may shape a rhizosphere community that is more beneficial to cucumber. However, more work is needed to validate this hypothesis, such as evaluating the effects of crop rotation with wild rocket on the growth of specific pathogens of cucumber and plant beneficial microbes.

The accumulation of autotoxic compounds, such as phenolics, is one of the major possible factors contributing to soil sickness (2,36). In this study, we found that rotation with wild rocket decreased the relative abundances of fungal taxa with phenolic

compound-degrading abilities (such as *Chaetomium* and *Mortierella* spp.) in the cucumber rhizosphere. It was demonstrated that vanillin, syringic acid and *p*-coumaric acid promoted the *Chaetomium* and *Mortierella* spp. in cucumber rhizosphere (29,33,42). Crop rotation is able to change the soil chemical properties, including soil nutrients contents (42). Previous studies (5,17,19,43) have shown that soil nutrients status and interspecific plant interactions can change the plant metabolism, including the root exudation. Therefore, it is possible that rotation with wild rocket altered the cucumber root exudation component and thus, changed the cucumber rhizosphere fungal community.

CONCLUSIONS

Rotation of wild rocket changed the cucumber seedling rhizosphere fungal community composition but had no significant effects on cucumber seedling rhizosphere fungal community alpha diversity. Moreover, rotation of wild rocket decreased the relative abundances of fungal taxa containing potential plant pathogens and taxa with phenolic compound-degrading capability in cucumber rhizosphere.

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