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Effects of green manure of wild rocket (*Diplotaxis tenuifolia* L.) on cucumber rhizosphere fungal community

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ABSTRACT

In pot culture, we evaluated the effects of green manure of wild rocket (*Diplotaxis tenuifolia* (L.) DC.) on cucumber (*Cucumis sativus* L.) rhizosphere fungal community composition. Cucumber rhizosphere fungal composition was analyzed by high-throughput amplicon sequencing of fungal ITS regions. Results showed that cucumber seedling rhizosphere fungal community composition was different between the fallow treatment and green manure treatment. However, green manure treatment did not affect the cucumber seedlings fungal community alpha diversity. Compared with the fallow treatment, cucumber grown in green manure of wild rocket had higher relative abundance of phylum *Ascomycota* but lower relative abundance of phylum *Zygomycota*. Moreover, green manure of wild rocket decreased operational taxonomic units (OTUs) classified as *Pseudallescheria* and *Kernia* spp. but increased OTUs classified as *Humicola* and *Fusarium* spp. in cucumber rhizosphere.

Key words: Biofumigation, *Cucumis sativus* L., *Diplotaxis tenuifolia* (L.) DC., fungal community, green manure

INTRODUCTION

In agroecosystems, continuous monocropping on the same land usually negatively affects crop growth and decreases crop yield, and promote soil-borne disease, a phenomenon known as 'soil sickness' (3,21,33,34). Soil-borne plant diseases are difficult to control by conventional strategies, such as the use of resistant host cultivars and synthetic fungicides (2). Green manuring is the practice of incorporating actively growing plant materials into the soil (2,19). For *Brassicaceae* family crops, this is termed as biofumigation (9,11,14). It has been demonstrated that green manures can suppress plant soil-borne diseases through releasing antifungal compounds (2). For example, *Brassicaceae* crops can produce isothiocyanates to inhibit the growths of several plant soil-borne pathogens (10,12).

Plant rhizosphere microorganisms are key determinants of plant health and productivity and are considered a major driver of plant defense to belowground pathogens (11,13,24). Particularly, plant-beneficial microorganisms (*Bacillus*, *Pseudomonas* and *Trichoderma* spp.), effectively protects the plants against pathogen attacks (17). Recent studies showed that green manures of *Brassicaceae* crops can inhibit plant soil-borne pathogens by changing soil microbial communities (5,8). Green manures can promote the indigenous soil plant-beneficial microorganisms and thus can protect plants directly by inhibiting plant pathogens and indirectly by eliciting induced resistance in plants (8,16,27).

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Cucumber (*Cucumis sativus* L.), a vegetable crop commonly monocropped in the greenhouse, is vulnerable to soil sickness (31,32). Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *cucumerinum*, is a serious vascular wilt disease in cucumber production (6,7). Green manures of *Brassicaceae* crops, such as wild rocket (*Diplotaxis tenuifolia* (L.) DC.), were shown to inhibit pathogenic *Fusarium* spp. and control Fusarium wilt of cucumber (*Cucumis sativus* L.), spinach (*Spinacia oleracea* L.) and tomato (*Solanum lycopersicum* L.) (8,9,15,22). We also found that green manure of wild rocket changed the cucumber rhizosphere bacterial community composition. Soil fungi are an important and diverse group of microorganisms in the soil ecosystem, these have multiple functional groups such as decomposers, mutualists and pathogens (13). This study, aimed to determine the effects of wild rocket (*Diplotaxis tenuifolia* (L.) green manure on the cucumber rhizosphere fungal community composition by high-throughput Illumina sequencing of fungal ITS regions.

MATERIALS AND METHODS

Pot experiment

Soil used in this study was collected from the top soil layer (0-15 cms) of greenhouse cultivated with cucumber in Experimental Station, Northeast Agricultural University, Harbin, China (45°41'N, 126°37'E, mean height above sea level: 127.95 m, annual precipitation: 524.5 mm, maximum and minimum temperature: 36.7 °C and -37.7 °C). The soil was sandy loam, 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. Methods used to determine these soil parameters were as described earlier (34). Soils were sieved (2 mm), hence, large stones and plant debris were removed.

The pot experiment was conducted from July to September 2016. Wild rocket cv. 'Shuangji' was directly sown into plastic pots (20 cm dia, 17 cm depth) containing 2.5 kg soil. Thirty seeds were planted per pot. After emergence, wild rocket seedlings were thinned to 10 plants per pot. A fallow treatment i.e. pots without green manure crop served as control. All pots were maintained in greenhouse (32°C day/22°C night, relative humidity: 60-80 %, 16 h light/8 h dark). There were 20 pots per treatment and the treatments were replicated thrice in Complete Randomised Design The pots were irrigated every two days with distilled water to maintain the soil moisture at about 65 % water holding capacity.

Forty days after sowing, whole plants of each green manure crop were harvested. The fresh plant materials were chopped to about 3-cm size and thoroughly incorporated by hand into the soil of the same pot, where it was grown. In the fallow treatment, soil was mixed similarly. Then, all pots were covered with black polyethylene films and incubated for 30 days with soil water content maintained at about 50 % of water holding capacity. After incubation, one cucumber seedling (cv. Jinyan 4) with two cotyledons was transplanted per pot (with and without green manure).

***Rhizosphere soil sampling and* soil DNA extraction**

Thirty days after transplanting, cucumber rhizosphere soil samples were collected from five plants in each replicate as described earlier (8). Total soil DNA was extracted with the Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, 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 earlier (31). Both the forward and reverse primers also contained a unique 6-bp barcode for each sample. Each soil sample was independently amplified. Three technically replicated PCR reactions were performed for each soil DNA as previously suggested (20,25). The products of the triplicate PCR reactions were pooled and purified using the agarose Gel DNA purification kit (TaKaRa). Then, purified amplicons were quantified by a TBS-380 micro fluorometer with Picogreen reagent (Invitrogen, USA) and mixed accordingly to achieve the equal concentration in the final mixture. The mixture was then 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 (31). Chimeric sequences were identified and removed using USEARCH 6.1 in QIIME. 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 30,784 ITS gene sequences per sample was performed. For alpha diversity analysis, Chao1, Shannon and inverse Simpson indices were calculated with the 'phyloseq' package in 'R' (Version 3.3.1). For beta diversity, principal coordinates analysis (PCoA) was performed to determine differences in microbial community structures based on Bray-Curtis distances with the 'vegan' package in 'R' (Version 3.3.1). Differences in alpha diversity indices and relative abundances of microbial taxa between treatments were analyzed using Welch's *t* test. Differences in the relative abundance of OTUs (as measured in units of $\log_2$ fold change) between treatments were measured with the 'edgeR' package in 'R' (Version 3.3.1). Similarity percentage analysis (SIMPER) was used to disentangle the most important OTUs that were responsible for the observed differences with the 'vegan' package.

RESULTS AND DISCUSSION

Illumina Miseq sequencing data:

In total, 210,966 quality fungal sequences, with 30,788-42,117 sequences per sample, were obtained. The average read length of the ITS1 regions was 260 bp. A total of 504 OTUs were identified at 97 % sequence similarity. The Good's coverage of each sample, which

reflects the captured diversity, was 99.80±0.03 %. Therefore, this level of sequencing effort was sufficient to estimate the diversity of these fungal communities.

Alpha and beta diversities of fungal communities

For the alpha diversity of cucumber rhizosphere fungal community, the number of OTUs, Chao1, Shannon and inverse Simpson indices did not significantly differ between the fallow treatment and the treatment of wild rocket green manure (Fig. 1a). For fungal community beta diversity, PCoA analysis, based on the Bray-Curtis distance dissimilarity, clearly separated the two treatments from each other along the first axis (Fig. 1b). The first two axes together accounted for 76.77 % of the variation in fungal community composition. Our results are consistent with previous studies showing green manures could alter soil fungal community composition (4,12,29,30). In some cases, biofumigation decreases the alpha diversity of soil fungal communities (30), because *Brassicaceae* crops produces isothiocyanates to inhibit fungal growth (10,18). Differences between control and green manuring were not significant.

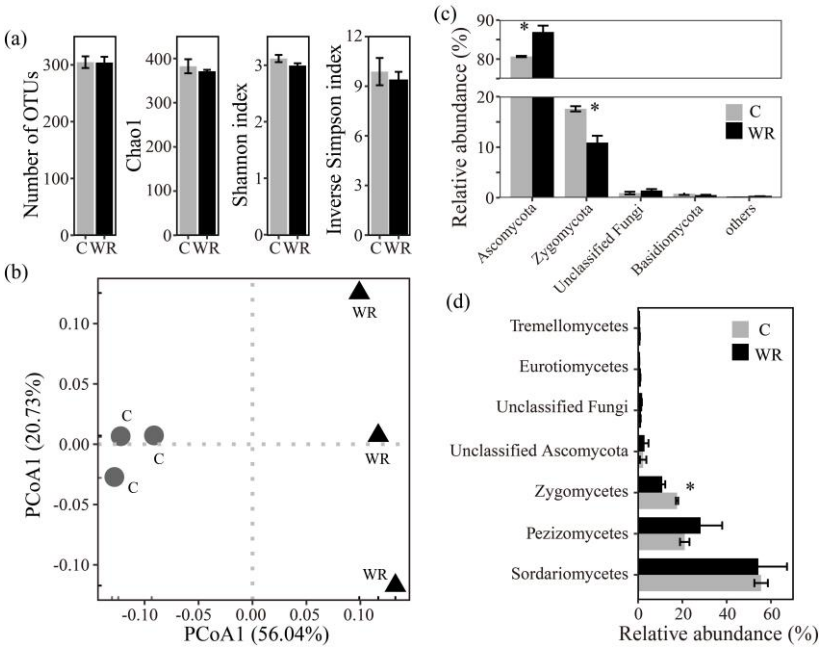

Figure 1. Fungal community alpha (a) and beta diversities (b) and relative abundances of fungal phyla (c) and main fungal classes (d).

Taxonomic characteristics of fungal communities

Across all samples, 1.17 % sequences were unclassified at the phylum level (Fig. 1c). Six fungal phyla detected were *Ascomycota*, *Basidiomycota*, *Zygomycota*, *Chytridiomycota*, *Blastocladiomycota* and *Rozellomycota*. The *Ascomycota* and *Zygomycota* were the

dominant phyla, which accounted for 83.80 % and 14.25 % of the total fungal sequences, respectively. Compared with the fallow treatment, cucumber grown in green manure of wild rocket had higher relative abundance of *Ascomycota* but lower relative abundance of *Zygomycota* ($P < 0.05$). Yim (30) also reported that biofumigation with *Brassica juncea* and *Raphanus sativus* increased the relative abundance of *Ascomycota* in some orchards with replant disease.

At the class level, *Sordariomycetes*, *Pezizomycetes* and *Zygomycetes* were the dominant classes in all samples (average relative abundance >10 %) (Fig. 1d). Other detected minor fungal classes were *Tremellomycetes*, *Eurotiomycetes*, *Dothideomycetes*, *Chytridiomycetes*, *Agaricomycetes*, *Leotiomycetes*, *Orbiliomycetes*, *Wallemiomycetes* and *Ustilaginomycetes*. Compared with the fallow treatment, cucumber grown in green manure of wild rocket had lower relative abundance of *Zygomycota* ($P < 0.05$).

At the OTU level, differential abundance analysis with 'edgeR' detected 39 and 33 OTUs enriched in the fallow treatment and green manure treatment, respectively (Fig. 2a). OTUs enriched in the fallow treatment mainly belonged to the fungal phylum *Ascomycota*, while, OTUs enriched in wild rocket green manure treatment mainly belonged to *Ascomycota* and unclassified Fungi (Fig. 2b). SIMPER analysis revealed that OTU550 (class *Pezizomycetes*), OTU255 (class *Sordariomycetes*, genus *Fusarium*), OTU150 (class *Sordariomycetes*, genus *Pseudallescheria*), OTU573 (class *Sordariomycetes*, genus *Humicola*) and OTU67 (class *Mortierellomycetes*, genus *Mortierella*) accounted for much of the difference between the fallow treatment and wild rocket green manure treatment (Fig. 2c).

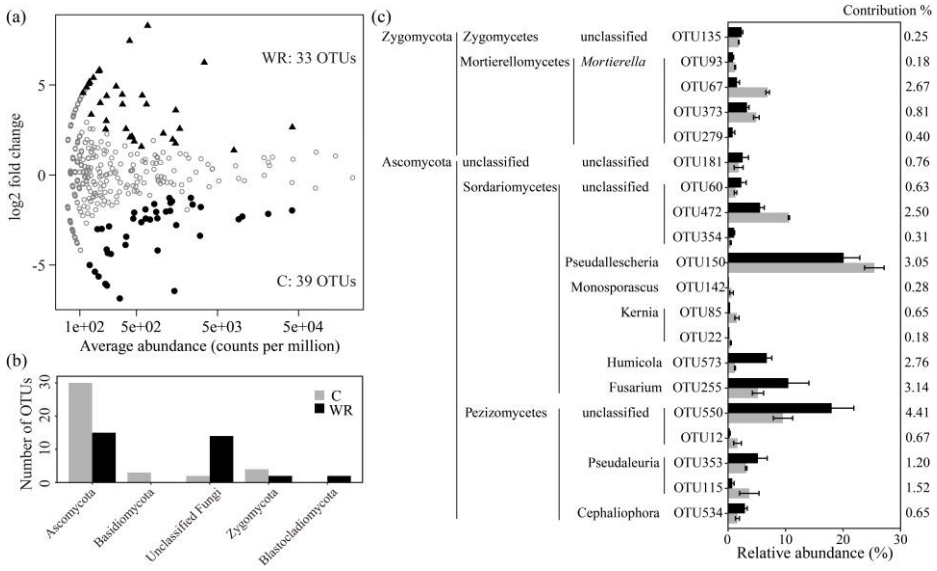

Figure 2. Log_2-fold change in relative abundance of OTUs (a) and the taxonomic classification of these differential OTUs (b) and the SIMPER statistical calculation of the 20 highest-ranking OTUs (c).

Pseudaleuria spp. are more in healthy soil than in diseased soil of pea (*Pisum sativum* L.) field (28). Two OTUs (OTU115 and OTU353) though classified as the same genus *Pseudaleuria*, had contrast responses to the green manure treatment. *Mortierella* spp. is ubiquitous in terrestrial ecosystem and can decompose lignocellulose (23). In the present study, fungal OTU belonged to *Mortierella* spp. responded differently to the green manure treatment. These results indicated that these taxa in the same genus might have different niches and play different roles in the soil. Therefore, caution should be taken, when inferring the function of fungal taxa based on its taxonomic information.

In this study, wild rocket green manure stimulated the relative abundances of OTU255 (class *Sordariomycetes*, genus *Fusarium*) and OTU573 (class *Sordariomycetes*, genus *Humicola*) in cucumber rhizosphere (Fig. 2c). Similarly, Oilseed meals of camelina (*Camelina sativa*) increased the relative abundances of *Fusarium* and *Humicola* spp. (4). Previously, we found that wild rocket green manure decreased the abundance of pathogenic *Fusarium oxysporum* f.sp. *cucumerinum* in cucumber rhizosphere (8). Beside these phytopathogenic ones, *Fusarium* spp. contains many species of agricultural importance. For example, some *Fusarium* spp. play important roles in organic matter decomposition (26), while others non-pathogenic strains of *Fusarium* spp. are able to control *Fusarium* diseases (1). Therefore, it is possible that wild rocket green manure stimulated the non-pathogenic but not pathogenic strains of *Fusarium* spp. This study laid the ground for further studies to isolate representative strains of *Fusarium* spp. and test their interaction and effects on cucumber and pathogenic *Fusarium oxysporum* f.sp. *cucumerinum*.

CONCLUSIONS

Green manure of wild rocket (*Diplotaxis tenuifolia* (L.) DC.) altered the cucumber seedling rhizosphere fungal community composition but had no significant effects on cucumber seedlings fungal community richness and diversity indices. Green manure of wild rocket increased the relative abundance of phylum *Ascomycota* but decreased that of phylum *Zygomycota*. Moreover, green manure of wild rocket decreased the OTUs classified as *Pseudallescheria* and *Kernia* spp. but increased OTUs classified as *Humicola* and *Fusarium* spp.

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