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Effects of cover crops on cucumber growth, soil microbial communities and soil phenolic content

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ABSTRACT

In 2-years pot experiment in greenhouse conditions, we determined the effects of cover crops [Welsh onion (*Allium fistulosum* L.), Mustard (*Brassica campestris* L.) and Wheat (*Triticum aestivum* L.)] on cucumber (*Cucumis sativus* L.) growth, soil microbial communities and phenolics content of soil. The cover crops were grown in winter or summer fallow seasons. Results showed that all cover crops affected the cucumber growth, soil phenolics content and changed the soil microbial communities. Cucumber yield and dry matter in Welsh onion and Mustard cover crop rotations were significantly higher. Mustard crop increased the soil fungal community diversity indices, while the Welsh onion crop rotation decreased it. Further, with an increase in planting seasons, soil microbial biomass carbon (MBC) increased in the cover crop systems. Compared with Welsh onion and mustard cover crop rotations, wheat crop rotation had lower *Fusarium* population for long term and improved the cucumber yield. Welsh onion and mustard cover crop rotaions had short-term effects, while wheat cover crop rotation had a long-term effects on cucumber growth.

Keywords: *Allium fistulosum*, *Brassica campestris*, cover crop, cropping system, *Cucumis sativus*, fungal communities, *Fusarium*, HPLC, mustard, PCR-DGGE, qPCR, soil phenols, *Triticum aestivum,* welsh onion, wheat.

INTRODUCTION

In China, cucumber (*Cucumis sativus* L.) is continuously cropped in green houses, which results in ‘soil sickness’ (15). The cucumber growth is inhibited either from the direct uptake of phenolic compounds or from indirect effects of changes in the soil microflora (40). Application of phenolics in soil shifts the soil microbial biomass and changes the community diversity (22). Cropping systems (crop rotation and inter cropping) and soil management practices influences the soil environment and increases the crop yield by maintaining the diversity and activity of soil microbial communities (1,39).

Cover cropping is common practice in sustainable agriculture (8), as the cover crops reduce the soil nitrogen loss, control weeds and soil-borne plant pathogens (33). They also improves the soil nutrients, organic matter, soil biological activities and soil structure (3), however, their mechanisms to improve the soils are still unclear. The use of summer cover crops restores the degraded soils by changes in soil microbial communities and increase in soil microbial activity (13,17,20,22,29,35,36). Little is known about the

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effects of cover crop on the relationship between the soil microbial communities and crop growth in Northeast China. Brassica crops [oilseed radish (*R. sativus L.* variety *oleiformis*) and fodder radish *(R.sativus* cv *Brutus*)] are always used as cover crops to control weeds and soil-borne diseases (16,19). The corn (*Zea mays* L.), chrysanthemum (*Chrysanthemum segetum* L.) and edible amaranth (*Amaranthus mangostanus* L.) grown as summer cover crops improves the soil microbial biomass carbon and cucumber yield (29). This study aimed to (i). Monitor the dynamics of soil phenolic content in various cover crop systems, (ii). Determine how the soil phenolic contents affects the soil microbial communities and (iii). Know the effects of cover crops on cucumber growth.

MATERIALS AND METHODS

I. Greenhouse experiments and soil sampling

This study was conducted in greenhouse, Northeast Agricultural University, Harbin, China (45°41′N, 126°37′E) from January 2012 to November 2013. We used the the upper soil layer (0-15 cm) from the pots of green house, that was under continuous cucumber cropping for 3- years. It was black soil (Mollisol) with sandy loam texture [organic matter: 2.69%; available N: 53.67 mg kg^{-1}; Olsen P: 283.56 mg kg^{-1}; available K: 354.39 mg kg^{-1}; EC (1:2.5, w/v), 0.88 mS cm^{-1} and pH (1:2.5, w/v): 6.57]. All measurements of soil indices including soil moisture content were based on the method of Bao (2).

Pot culture: The experiment was done in plastic pots (28 cm dia, 35 cm height), each pot contained 10 kg soil and fertilizers (50 g Diammonium Phosphate and 25 g Potassium Sulfate per pot). Two crops of cucumber (March-June and August-November) were grown every year. The experiment was done in randomized block design with three replications. There were three cover crops [Welsh onion (*Allium fistulosum* L.), Mustard (*Brassica campestris* L.) and Wheat (*Triticum aestivum* L.)]. The treatments were : (i). Welsh onion in winter fallow season (OCFC), (ii). Wheat in summer fallow season (FCWC) and (iii). Mustard in winter fallow season (MCFC) (Table 1). Each treatment had three plots, every plots had three pots as 3-replications. To prevent the margin effects, there were 'protect lines' consisting of 28 pots arranged around the plots. There were total 55 plastic pots (3 treatments ×3 plots ×3 replicates and 28 pots for protect line).

In our experiments, we used 4-crops [Cucumber cv 'Zhende F1', Welsh onion (*Alliumfistulosum* L.) cv 'Chicken Onions', wheat (*Triticumaestivum* Linn.) cv 'Pinzi II-5' and mustard (*Brassica campestris* L.) cv 'Huashupakchoi']. Cucumber seeds (cv. Zhende F1) were washed for few minutes with sterilized water, then first soaked in water at 55^0 C for 15 min and then at 28^0 C for 6 h, to soften the seed coat, accelerate the activity of enzymes, and promote the hydrolysis of storage substances, to create conditions for the germination of seeds. After rinsing the seeds several times with sterilized water, these were germinated in dark at 28^0 C for 14 h. After emergence, seedlings were first planted in cups (10 cm dia, 10 cm high) containing 150 g soil. When the seedlings had 3 leaves, they were transplanted into larger plastic pots in early March (Spring season) and in end August (Autumn season). There was one cucumber plant per pot.

Summer cover crop wheat was sown at 120 seeds per pot in early July and cut in mid August (Table 1). As wheat was cover crop, hence, havested twice when plants were 20 cms tall, these were cut twice (2 cm above the ground) and the residues were added back into the pots. Winter cover crops (Welsh onion and mustard), were sown in separate pots in early December and harvested in end January. The cover crop-cucumber treatments and dates of sowing and harvest are listed in Table 1.

Before the growth-decline period of cucumber (50 days old crop), the rhizosphere soil samples were collected by uprooting the plants gently and shaking off the soil from the roots. The rhizosphere soil samples were collected from 3-plots one from each replication (8). Soils of same treatment were mixed, sieved through a 2 mm mesh sieve, put into sterile plastic bags, stored in lab. at -80°C for soil microbial community analysis.

II. Cucumber yield

All cucumber plants (except the protected lines) fruits were harvested and cucumber yield (g per plant) was determined. The total yield of each treatment [nine plants (nine pots)] was calculated and converted.

III. Soil microbial biomass (MB) estimation

Soil microbial biomass carbon and microbial biomass nitrogen content was determined by chloroform fumigation-extraction method (5,30). The extractability factors of 0.38 (30) and 0.54 (5) were used to calculate the MBC and MBN, respectively.

IV. Soil phenolics extraction and determination

The rhizosphere soil samples (7) were collected from the harvested and uprooted cucumber plants, these were sieved through 2 mm mesh sieve to remove roots etc. Soil phenolics were extracted and estimated by the methods described previously (6,40). Briefly, 25 g soil was added to 150 ml 2M NaOH and shaken for 24 h on shaker at 25℃. The suspension was centrifuged at 6000 *g* for 15 min and the supernatant was filtered through filter paper No GB/T1914-2007. The pH of filtrate was adjusted to 2.5 with 5 M HCl and extracted five times with 5 ml ethyl acetate. The resulting extracts were pooled and evaporated to dryness at 40℃ by rotary evaporation. The residue was dissolved in 5 ml of 80% methanol and kept in dark at 4℃.

For HPLC analysis, the methanol solution of soil extracts was filtered through 0.22 µm filter membrane and used for soil phenolics analysis using Waters HPLC system (Waters, Milford, MA). The mobile phase was a mixture of 20% methanol and 80% water. The flow rate was kept constant at 0.8 ml minute^{-1}. Detection was at 280 nm using a UV detector. The injection volume was 15 µl and the column temperature was maintained at 25 °C. Identification and quantification of phenolic compounds was done by comparing the retention times and areas with that of pure standards. The content of each identified phenolic compound in soil was expressed as µg per g of dry soil . Soil dry weight was determined as described previously (2).

The standard phenolics were purchased from Dalian China ittrich Co. Ltd.

Table 1. Details of various crop rotations and crops durations

Treatment Code	Winter fallow (Dec- January)			Spring (March to June)			Summer fallow (July to August)			Autumn (August to November)		
	Crop	Dates of		Crop	Dates of		Crop	Dates of		Crop	Dates of	
		Sowing	Harvest		Sowing	Harvest		Sowing	Harvest		Sowing	Harvest
OCFC	Welsh onion	Jan. 1	Feb. 15	Cucumber	March 1	June 18	Fallow	-	-	Cucumber	Sept. 1	Dec. 20
FCWC	Fallow	-	-	Cucumber	March 1	June 18	Wheat	July 15	Aug. 30	Cucumber	Sept. 1	Dec. 20
MCFC	Mustard	Jan. 1	Feb. 15	Cucumber	March 1	June 18	Fallow	-	-	Cucumber	Sept. 1	Dec. 20

O: Welsh onion, C: Cucumber, F: Fallow, W: Wheat, M: Mustard

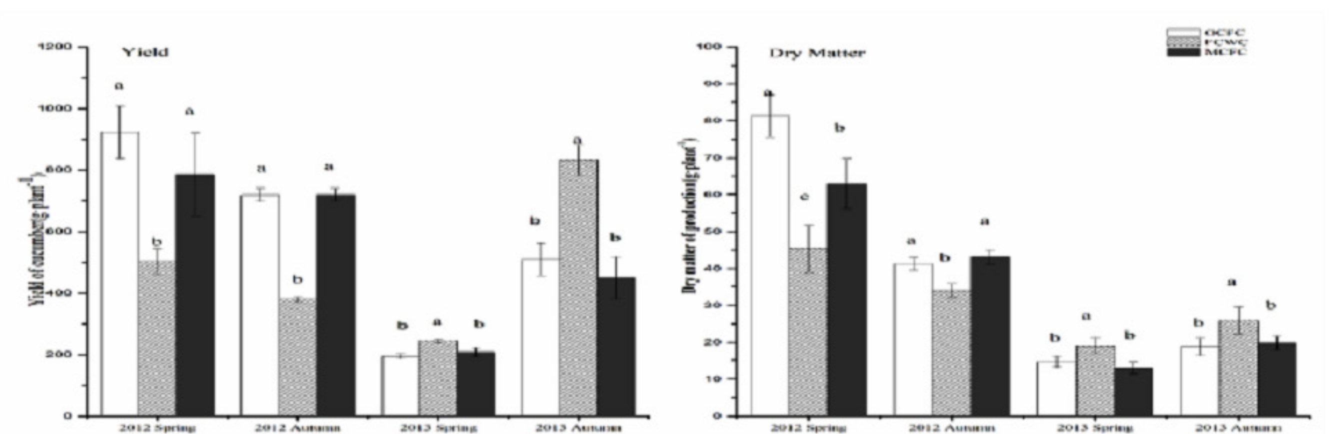

Figure 1. Effects of cover crop on cucumber yield and dry matter production. Bars indicate the standard errors of the means from three replicates. Columns with different letters are statistically different between treatments ($P< 0.05$, Tukey's HSDtest). OCFC: Welsh onion in winter fallow season, FCWC: Wheat in summer fallow season, and MCFC : Mustard in winter fallow season.

V. DNA extraction, denaturing gradient gel electrophoresis(PCR-DGGE) and quantitative polymerase chain reaction (qPCR)

Soil fungal community structures were analyzed by the PCR-DGGE method (40). Total soil DNA was extracted with a Power Soil®DNA isolation Kit (MO BIO Laboratories, CA, USA). A nested PCR protocol was used to amplify the fungal internal transcribed spacer (ITS) region of the rRNA gene (10) with primer sets of ITS1F/ITS4 (10) and GC-ITS1F/ITS2 (12) for the first and second round of PCR amplifications, respectively (28). DGGE was performed using an 8% (w/v) acrylamide gel with 20-55% denaturant gradient and run in a 1×TAE (Tris-acetate-EDTA) buffer for 10 h under conditions of 60℃ and 75 V with a DCode universal mutation detection system (Bio-Rad Lab, LA, USA). After the electrophoresis, the gel was stained with 1:3300 (v/v) GelRed (Biotium, USA) nucleic acid staining solution for 20 min. DGGE profiles were photographed with an Alpha Imager HP imaging system (Alpha Innotech Crop., CA, USA) under UV light.

qPCR assays targeting the fungal ITS gene and *Fusarium* Ef1αgene, were used to estimate fungal and *Fusarium* community sizes using the IQ5 real-time PCR system (Bio-Rad Lab). The primer pair ITS1F/ITS4 (10) was used to quantify the fungal communities with the total soil DNA as template. For *Fusarium* communities, 2 μl of 100-fold diluted first-round PCR products was amplified with primer pair Alfie1/Alfie2 (37). Care was taken to ensure that the first-round PCR products were all in exponential amplification phase of the PCR (31). Standard curves were created with 10-fold dilution series of plasmids containing the ITS region or Ef1αgene from soil samples. The relative *Fusarium* community size was calculated as described by Wakelin et al. (31). Sterile water was used as a negative control to replace template. All amplifications were performed in triplicate. The specificity of the products was confirmed by melting curve analysis and agarose gel electrophoresis.

VI. Statistical analyses

Data were analyzed using the analysis of variance (ANOVA) and mean comparison between treatments was performed based on the Tukey's honestly significant difference (HSD) test at the 0.05 probability level with SAS 9.1 software. Banding patterns of the DGGE profiles and principal component analysis (PCA) were analyzed by the Quantity One software (version 4.5) and Canoco for Windows 4.5 software respectively. The diversity of fungal communities was estimated by using the Richness (S), evenness (E) and diversity (H) indices were calculated as described previously (34).

RESULTS AND DISCUSSION

Dry matter and Cucumber yield

Cucumber yield and dry matter in Welsh onion and mustard cover crop rotations were significantly higher than in wheat cover crop rotation in spring and autumn seasons of 2012 ($P < 0.05$), while the results were opposite in spring and autumn seasons of 2013 (Fig. 1). Compared with Welsh onion and mustard rotations, wheat cover crop rotation showed different tendency in 2-year experiment. It may due to the interactions of different kinds of phenolics, root exudates or plant secondary metabolites (18). This implies that

wheat cover crop rotation has better effect on cucumber growth under long-term cultivation.

Soil microbial biomass Carbon (MBC) and microbial biomass Nitrogen (MBN)

Soil MBC was much higher under Welsh onion and mustard rotations than wheat cover crop rotation in all cropping seasons except in 2013 autumn season (Fig. 2). The Welsh onion and mustard rotation had the highest MBN content during the 4-crop seasons, while the wheat cover crop rotation showed the lowest content during the two crop seasons of 2013 (Fig. 2). Soil microorganisms are one of the important players in the rhizosphere of plants, as they can provide plants with nitrogen, phosphorus and other minerals through decomposition of soil organic matter, and changes in the soil microbial communities may affect the plant growth (1,40). So changes in soil MBC and MBN may be both cause and a reflection of poor plant performance in different crop rotations.

Phenolics in soil

Seven phenolic compounds were detected by HPLC in both soils collected, before the sowing soil and during the crop growth. In soil samples, 7-Hydroxycoumarin was most abundant, followed by vanillic acid > *p*-hydroxybenzoicacid > ferulic acid > syringic acid > vanillin > *p*-coumaric acid. The content of these 7-phenolic compounds was significantly improved with increase in crop culture time (Fig. 8). In spring season of 2012, the content of 7-Hydroxycoumain, vanillic acid (11), *p*-hydroxybenzoic (14), ferulic acid (14) and syringic acid (26) in mustard cover crop rotation were significantly lower than that in wheat and Welsh onion cover crop rotations ($P< 0.05$). Only the content of syringic acid was significantly higher in the wheat cover crop rotation than in other cover crop rotations ($P< 0.05$) in autumn season of 2012. In spring of 2013, the content of 7-Hydroxycoumain and vanillic acid of Welsh onion cover crop rotation were significantly higher than in wheat and mustard cover crop rotations ($P< 0.05$); however, the content of 7-Hydroxycoumarin and vanillic acid as well as syringic acid, vanillin and *p*-coumaric acid were significantly higher in wheat cover crop rotation than in Welsh onion and mustard cover crop rotations ($P< 0.05$) in the autumn season of 2013. Generally, the soil phenolic content increased with the increasing cropping time (Fig. 8). Researchers have found that cover crop not only affects the soil nutrients availability and cycling, but also influences the soil phenolics uptake and use by plant, root exudates and plant secondary metabolites (25,28). Phenolics are detrimental to plant growth, by influencing the nutrients uptake, enzyme activities, water relations and photosynthesis and respiration of plants (13). The processes such as transport, retention and transformation may influence the quantitative and qualitative availability of phenolics in the soil.

Soil fungal community structures

DGGE profiles revealed that the compositions of soil fungal communities differed among the various cover crop rotations, as there was variability in DGGE band number, position and density in different cover crop rotations (Figs. 3a, 4a, 5a and 6a). The PCA analysis showed clear separation of 3-cover crop rotation soils under different cropping periods (Figs. 3b, 4b, 5b and 6b), indicating that different cover crop rotations had variable influences on the fungal community structures.

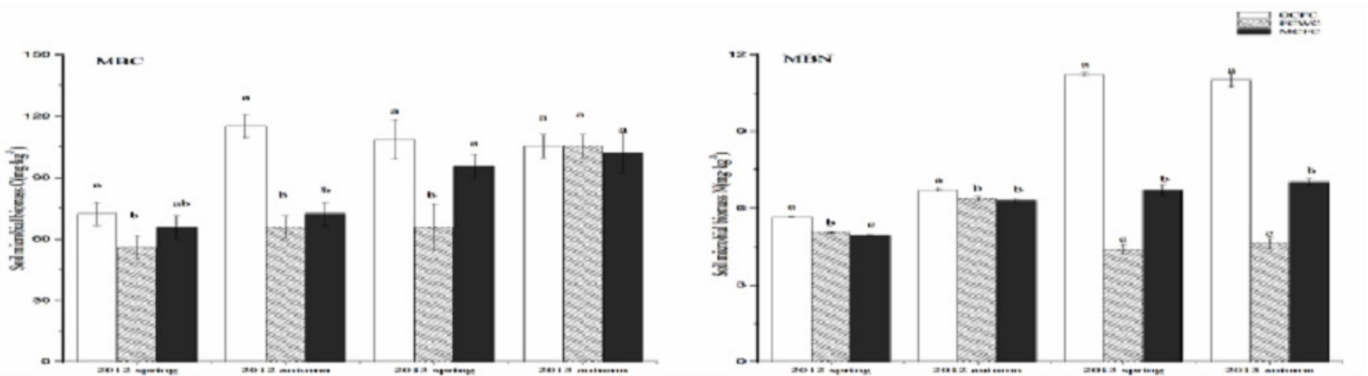

Figure 2. Effects of cover crop on soil microbial biomass C and microbial biomass N content. Values with different letters are significantly different between treatments ($P < 0.05$, Tukey's HSD test). OCFC: Welsh onion in the winter fallow season, FCWC: Wheat in the summer fallow season, and MCFC : Mustard in the winter fallow season. MBC: Microbial biomass carbon and MBN: Microbial biomass nitrogen

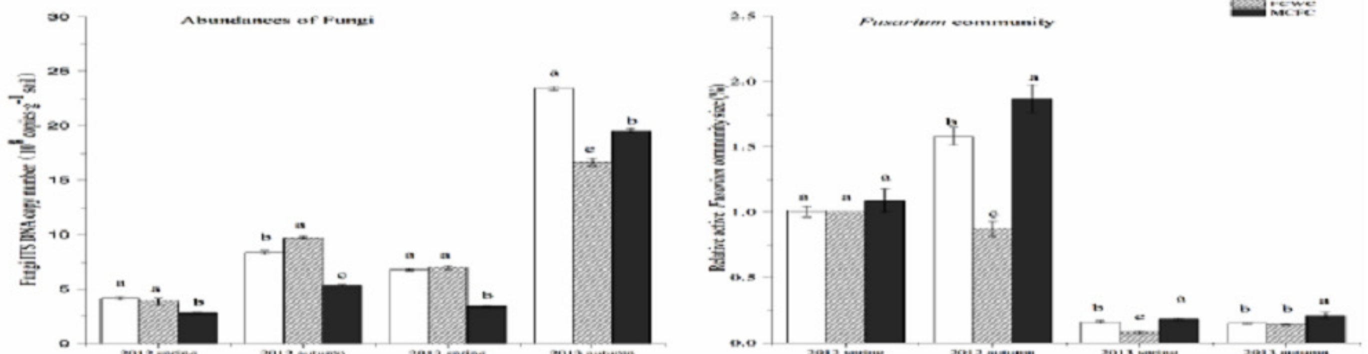

Figure 7. Abundances of fungi and *Fusarium* community in the cover crop rotation systems. Values with different letters were significantly different between treatments ($P < 0.05$, Tukey's HSD test). OCFC: Welsh onion in the winter fallow season, FCWC: Wheat in the summer fallow season, and MCFC : Mustard in the winter fallow season.

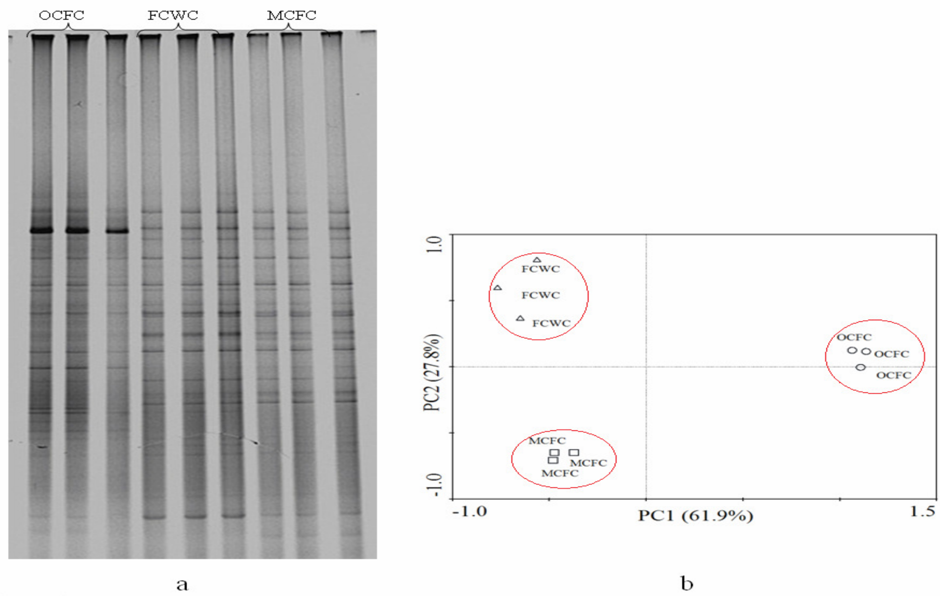

Figure 3. DGGE profile (a) and PCA analysis (b) of soil fungal communities from different cover modes in the spring of 2012. OCFC: Welsh onion in the winter fallow season, FCWC: Wheat in the summer fallow season, and MCFC : Mustard in the winter fallow season.

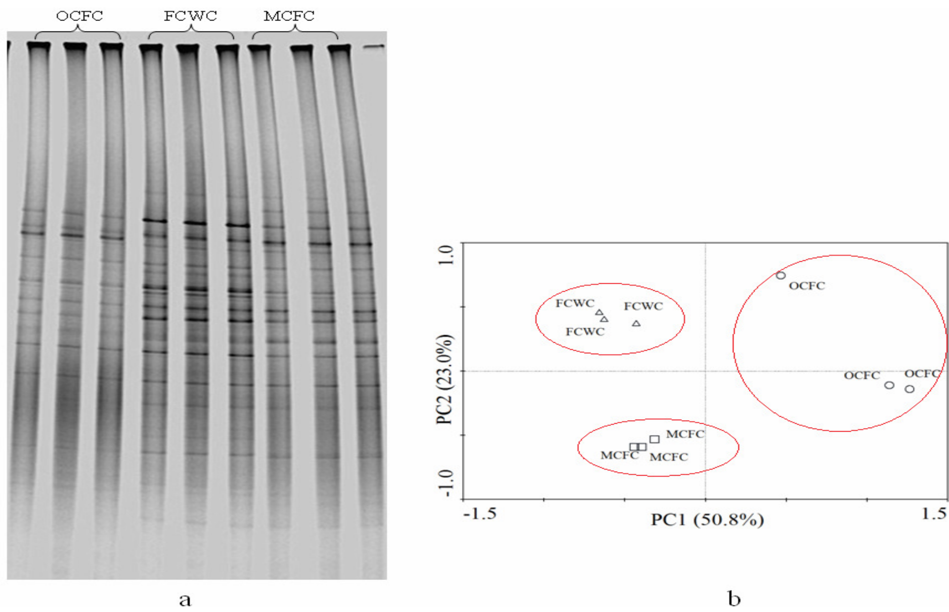

Figure 4. DGGE profile (a) and PCA analysis (b) of soil fungal communities from different modes in the autumn of 2012. OCFC: Welsh onion in the winter fallow season, FCWC: Wheat in the summer fallow season, and MCFC : Mustard in the winter fallow season.

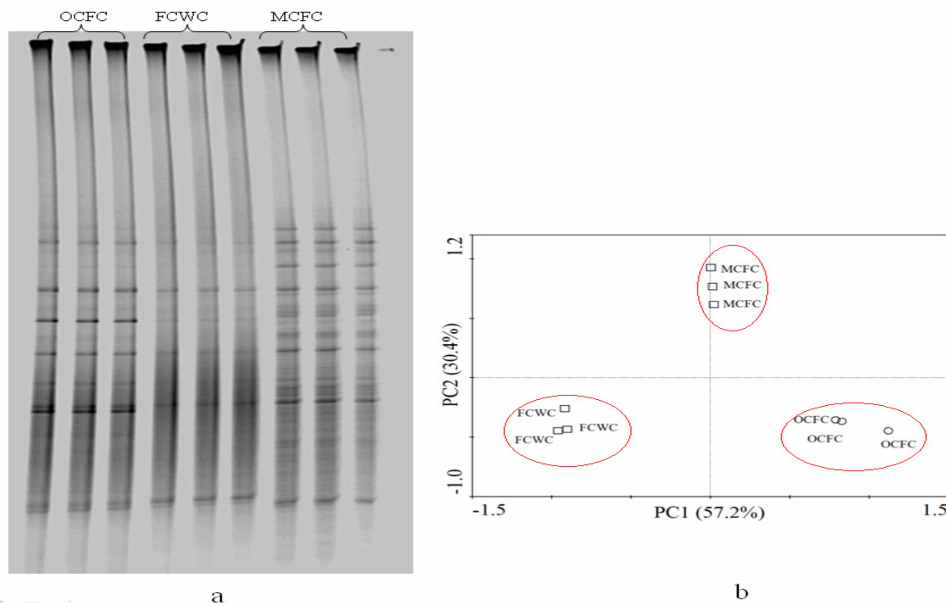

Figure 5. DGGE profile (a) and PCA analysis (b) of soil fungal communities from different cover modes in the spring of 2013. OCFC: Welsh onion in the winter fallow season, FCWC: Wheat in the summer fallow season, and MCFC : Mustard in the winter fallow season.

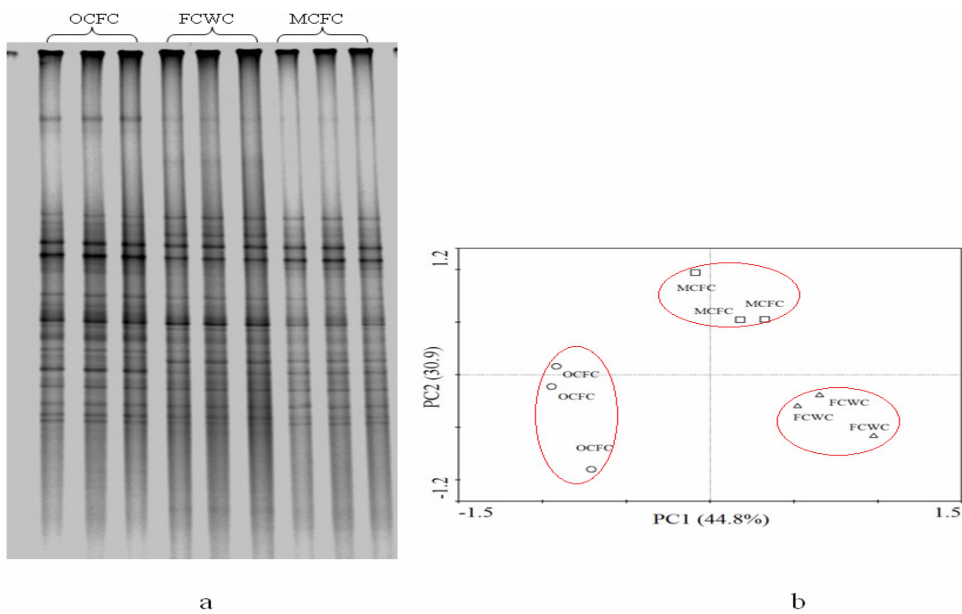

Figure 6. DGGE profile (a) and PCA analysis (b) of soil fungal communities from different cover modes in the autumn of 2013. OCFC:Welsh onion in the winter fallow season, FCWC: wheat in the summer fallow season, and MCFC : mustard in the winter fallow season.

The number of visible bands, Shannon diversity index and evenness index were higher in mustard cover crop rotation than in other two treatments in each growing season except in 2013 autumn, and these parameters in wheat cover crop rotation were significantly higher than in Welsh onion cover crop rotation during the spring and autumn seasons of 2012 ($P<0.05$) (Table 2). This suggests that changes in soil fungal populations occur in response to different cover crop rotations and was confirmed by the changes in fungal community (Fig. 7). These results are consistent with those of Zhou *et al*. (39), who found that soil bacterial and fungal communities differed in various intercropping systems. This may be due to the interaction of soil microorganisms and the different kinds of soil phenolic components with the increasing cropping seasons (24,40). In addition, it may also be caused by different plants root exudates which differently affects the soil microbial communities (5).

Table 2. Numbers of visible bands (*S*), Shannon index (*H*) and evenness index (*E*) based on DGGE analysis of fungal communities in DGGE profiles

Crop rotation	Visible Bands (*S*)	Shannon Index (*H*)	Evenness Index (E)
		2012 Spring	
OCFC	17.00±0.00 b	2.39±0.02 b	0.73±0.00 ab
FCWC	20.00±0.00 a	2.80±0.05 a	0.86±0.02 a
MCFC	20.00±0.00 a	2.87±0.02 a	0.87±0.01 a
		2012 Autumn	
OCFC	15.33±0.58 b	2.51±0.04 c	0.79±0.01 b
FCWC	16.67±0.58 b	2.65±0.03 b	0.81±0.01 b
MCFC	20.33±0.58 a	2.82±0.05 a	0.87±0.02 a
		2013 Spring	
OCFC	15.00±0.00 b	2.49±0.07 b	0.78±0.02 b
FCWC	12.00±0.00 c	2.02±0.03 c	0.64±0.01 c
MCFC	23.00±0.00 a	2.98±0.01 a	0.94±0.00 a
		2013 Autumn	
OCFC	22.33±0.58 a	2.77±0.02 ab	0.85±0.01 ab
FCWC	20.00±0.00 b	2.84±0.03 a	0.87±0.01 a
MCFC	20.33±0.58 b	2.73±0.04 b	0.84±0.01 b

Values with different letters were significantly different between treatments ($P< 0.05$, Tukey's HSD test). OCFC: Welsh onion in winter fallow season, FCWC: Wheat in summer fallow season, and MCFC : Mustard in winter fallow season.

Abundance of Fungal and *Fusarium* community

qPCR assays showed that fungal community abundance was significantly increased in cropping seasons except in the autumn season of 2013. Fungal community abundance of Welsh onion and wheat cover crop rotations were significantly higher than in mustard cover crop rotation in all cropping seasons except the autumn season of 2013 ($P < 0.05$). Generally, compared with the first cropping season, different cover crops increased the number of fungal community abundance (Fig. 7). In all cropping seasons, the *Fusarium* community abundance was significantly reduced during the two crop seasons of 2013 than in the two crop season of 2012 ($P< 0.05$) (Fig. 7). The three cover crop rotations reduced the *Fusarium* community abundance through root exudates and the inhibitory

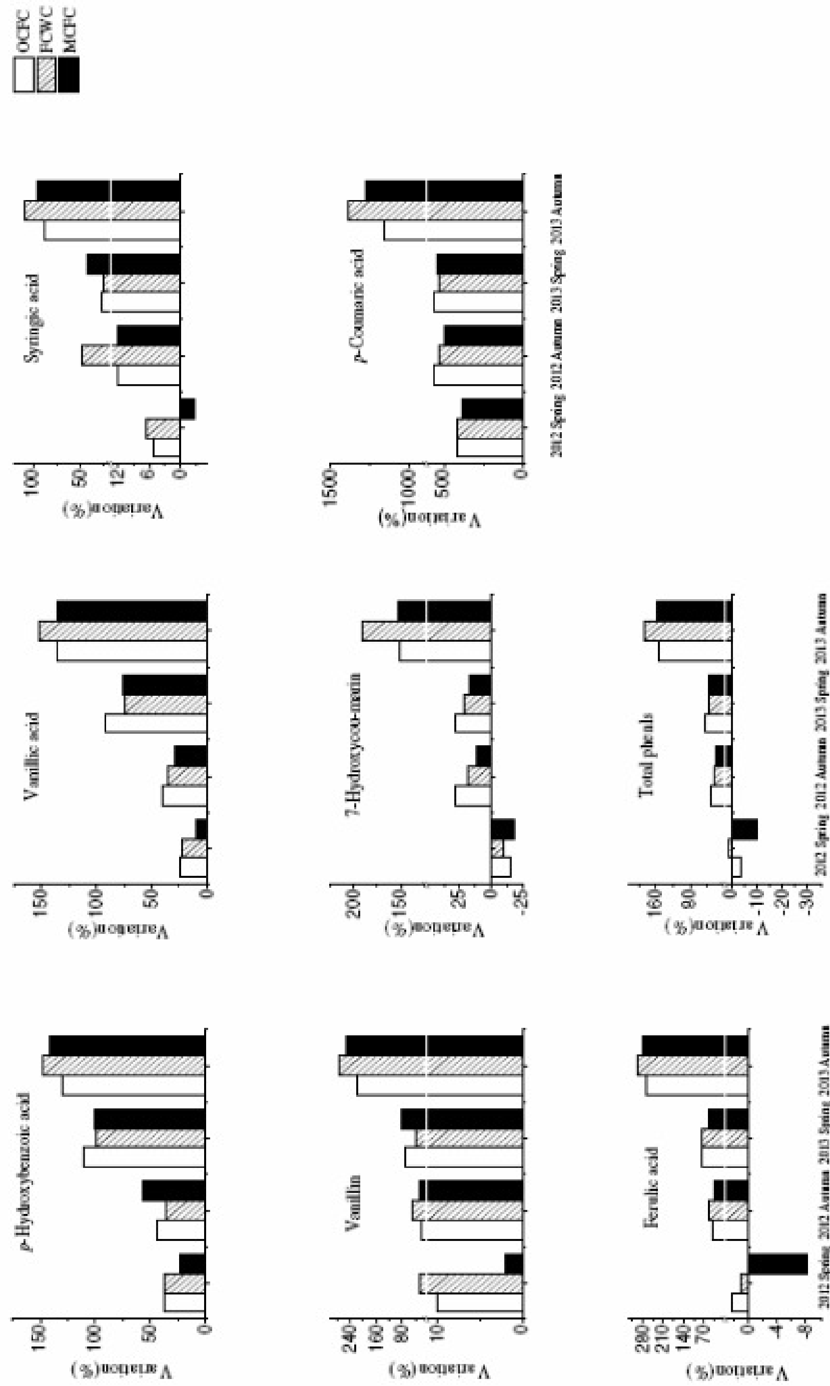

Figure 8. Variation in soil phenolic content in different cover crops systems. OCFC:Welsh onion in the winter fallow season, FCWC: Wheat in the summer fallow season, and MCFC : Mustard in the winter fallow season.

effects increased with the increase in cultivation years. The quantity and quality of crop residues also affected the soil *Fusarium* communities. Cover crops not only affect the soil nutrients availability and nutrient recycling, but also influence the soil phenolic content, which in turn can influence the soil microbial activities and diversities (25,28). Previous studies have shown that the soil phenolic contents increases the soil microbial respiration (28), MBC content (32) and the abundance of bacterial and fungal communities (35) and also changes the microbial community structures (22,34,41). Moreover, low molecular weight phenolics can serve as carbon sources for soil microorganisms (27) and enhances the microbial activity, which in turn increases the microbial biomass (22).

With the increase in cropping periods, soil MBC content as well as the fungal communities in the pot experiment were increased in different covers crop. The cover crops such as sweet corn, chrysanthemum and edible amaranth have variable effects on the growth of cucumber and improves the MBC content (29). Welsh onion and mustard cover had short-term effects on the cucumber growth, while wheat cover crop rotation had a long-term effect.

There are many reports on the soil phenolics in cucumber cultivated soil (38,40) and many phenolics [ferulic, *p*-coumaric, *p*-hydroxybenzoic, vanllic acids etc.] have been identified. Seven phenolics (7-Hydroxycoumarin, vanillic acid, *p*-hydroxybenzoicacid, ferulic acid, syringic acid, vanillin, *p*-coumaric acid) were identified in the present studies. Coumarins present in *Apiaceae*, *Rutaceae*, *Asteraceae* and *Fabaceae* families (21) are involved in defense against pathogens and are produced in response to stress (23). The reports of 7-Hydroxycoumarin presence in cucumber grown soil is rare (23). Further studies are therefore, needed to understand the main function of the 7-Hydroxycoumarin in cucumber grown soil.

CONCLUSIONS

All cover crop rotations affected the soil phenolics contents and thereby changed the soil fungal communities associated with cucumber growth. The mustard crop rotation maintained relatively higher soil fungal community diversity indices, while the Welsh onion crop rotation had the lowest. Compared with Welsh onion and mustard rotations, wheat cover crop rotation increased the cucumber yield in long-term cropping and kept the *Fusarium* population at lower level.

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