

REGULAR ARTICLE

# Changes in soil microbial diversity and control of *Fusarium oxysporum* in continuous cropping cucumber greenhouses following biofumigation

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## ABSTRACT

Soil biofumigation with Brassica plant parts has been demonstrated to suppress soil-borne pathogens. This effect has been attributed to the bactericidal effect of their glucosinolate degradation products. However, little is known regarding the effect of biofumigation on the microbial community structure and control of *Fusarium oxysporum* in continuous cropping cucumber systems. Field and pot experiments were performed to study the effect of biofumigation with brassica crops of different glucosinolate contents on the microbial diversity and control of *Fusarium oxysporum* during the cucumber growth period in a continuous cropping culture system. Samples collected from biofumigation and control treatments differed in the number of soil bacterial bands observed by denaturing gradient gel electrophoresis (DGGE) profiling, suggesting that biofumigation changed the soil microbial diversity. Compared with control and chemically-fumigated (HY) soils, the biofumigated soil exhibited significantly higher Shannon-Weaver diversity indices for bacteria and fungi. Cluster analysis and principal component analysis revealed that soil bacteria communities were different in the hymexazol (HY)-treated soils compared with the communities before biofumigation (BB) and those in the no fumigation (CK) groups. This was true for the mustard containing high glucosinolates (MHG), mustard containing low glucosinolates (MLG), mustard (MU), and turnip (TU) groups. Pot experiments demonstrated that the amounts of *Fusarium oxysporum* in MHG- and HY-treated soils were significantly lower than those in other biofumigation treatments. These results demonstrate the potential utility of *Brassica* crops in the control of *Fusarium* wilt in continuous cucumber cropping systems, and the control effect of MGH was the best compared with other treatments, which could effectively reduce *Fusarium oxysporum* in soil by more than 60%.

**Keywords:** Biofumigation; PCR-DGGE; Soil microbial community; *Fusarium oxysporum*; Cucumber

## INTRODUCTION

The cucumber is an important species cultivated in greenhouses in Heilongjiang, China. Owing to the limited availability of agricultural land and high market demand, continuous cucumber cropping has been widely adopted. Continuous cropping affects cucumber yield and quality. Soil-borne disease pathogens and secondary salinization are commonly observed in continuously cropped soil.

Currently, the use of methyl bromide for soil fumigation has been banned because repetitive chemical fumigation raises air pollution concerns. Methyl bromide has been known to contribute to the depletion of the atmospheric ozone layer, thus increasing UV-B irradiation and the risks of skin

cancer and cataract in humans (Loenen et al., 2003). Because fumigants are volatile, atmospheric emission is one of the main pathways for offsite transport (Wang et al., 2009). The recent increasing demand for environment-friendly fumigants has led to a renewed interest in finding alternative methods for controlling soil-borne pathogens (Giotis et al., 2009).

Many soil treatments have been suggested as alternatives to chemical soil disinfection (Noble and Coventry, 2005; Van Bruggen et al., 2015). Biofumigation is the suppression of soil-borne pests and pathogens resulting from biocidal compounds released from brassicaceous crops following the hydrolysis of glucosinolates (GSLs) present in their tissues. The disease-suppressive effect of brassica green manures,

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crop residues, and/or processing waste incorporated into soil has been attributed to the glucosinolate content (Kirkegaard, 2006). In a pioneering work on biofumigation, brassica tissue incorporated into wheat fields reduced the abundance of wheat take-all pathogens (*Gaeumannomyces graminis* var. *tritici*) (Angus et al., 1994). This soil treatment increased the soil organic matter content and enhanced soil fertility and has been extensively studied in recent years (Li et al., 2007). Two plants, including the mustard and turnips, which are high in glucosinolate, also inhibit the growth of weeds (Vaughn et al., 2005).

A previous study reported that the main biocidal substances, isothiocyanates, released into brassica plant-fumigated soil as a result of GSL degradation, have similar effects as that of the chemical fumigants Metham sodium (MS) and Dazomet (Sarwar and Kirkegaard, 1998). The inhibitory effects of biofumigation on fungal (*ascomycetes*) and bacterial (*ammonia-oxidizing bacteria*) guilds are superior to that of Metham sodium (Omirou et al., 2011). The biofumigant effect of pepper crop residues could effectively suppress the populations of *Meloidogyne incognita* in laboratory conditions (Buena et al., 2007). In a field experiment, three different green manures (Austrian winter pea, *Pisum sativum* L.; broccoli *Brassica oleracea* L.; or Sudan grass, *Sorghum vulgare*) were incorporated into soil at three different rates, which resulted in similar suppressive effects on *Verticillium dahlia* (Ochiai et al., 2008). *Fusarium* wilt on flax was reduced to a greater extent by complex than by simple amendments (Senechkin et al., 2014), and the suppression was related to microbial activity. Brassica species are increasingly being used as cover crops for the suppression of soil-borne diseases in potato cropping systems (Snapp et al., 2007). Additionally, many studies have reported that fresh brassica tissues significantly reduce the severity of soil-borne diseases (Giotis et al., 2009).

The density of *Fusarium* spp. and the abundance of *Trichoderma* increased significantly in soil after biofumigation with the seed meals of *B. juncea* and *B. carinata* (Paudel et al., 2016). Additionally, the inhibitory effect of biofumigation on *Fusarium oxysporum* f. sp. *lycopersici* is superior to those of soil solarization and soil disinfection methods (Li et al., 2006). Several plants, especially mustard and turnip, have been extensively studied as green manure crops (when grown or incorporated in the soil) because of their ability to suppress weeds or soilborne pests (Vaughn et al., 2005).

Due to the sensitivity of soil microbes to isothiocyanates, these compounds could significantly change the structure of the soil microbial community. The main GSL found in Turnip is 2-hydroxy-3-butenyl glucosinolate. Although the hydrolysis products of this compound exert little inhibitory effect on *Rhizoctonia* or *Pratylenchus*, these products increase the abundance of *Streptomyces* spp. and a nitric oxide

synthase-containing holophytic *Nitrobacterium* in the soil (Cohen et al., 2005).

*Fusarium oxysporum* is the main disease that affects cucumber yield and quality in continuously cropped cucumber soil. The changes in soil microbial diversity resulting from biofumigation in continuous cucumber soil are not clear. Denaturing gradient gel electrophoresis (PCR-DGGE) of PCR-amplified 16S rRNA gene fragments has been widely used to analyze the composition and diversity of microbial communities present in natural environments (Omirou et al., 2011; van Diepeningen et al., 2005). In the present work, brassica crops with different GSL contents were used to fumigate continuously cropped cucumber soil. The objectives of the present work were (1) to use the PCR-DGGE method to evaluate microbial community structures and their diversity in continuously cropped cucumber soil treated with different brassica crops for biofumigation and (2) to determine the effect of biofumigation materials on *Fusarium* wilt in soil continuously cropped with cucumber.

## MATERIALS AND METHODS

### Soil microbial diversity and glucosinolate concentration analysis

#### Experiment design

This experiment was conducted at the horticultural experimental station of Northeast Agricultural University, China (45°45'N, 126°38'E). The soil used in this experiment was used for cropping cucumber soil for ten years and was infected with *Fusarium oxysporum* f. sp. *cucumerinum*. Before the soil was biofumigated, properties of soil at depths from 0 to 20 cm were determined following Shidan Bao's (2005) method and the following results were obtained: total C, 31.3 g kg<sup>-1</sup>; total N, 3.94 g kg<sup>-1</sup>; available N, 269.7 mg kg<sup>-1</sup>; available P, 203.6 mg kg<sup>-1</sup>; available K, 184.4 mg kg<sup>-1</sup>; pH 7.20.

The experimental design consisted of six treatments (three replicates each). Each plot was 7.0 × 3.0 m in size. The following biofumigation materials were used in experiments: (1) mustard containing medial glucosinolates (MU), (2) mustard containing high glucosinolates (MHG), (3) mustard containing low glucosinolates (MLG), (4) turnip (TU). These materials were cut into small pieces of less than 2 cm and were incorporated into the 0-20 cm depth soil by a rotary machine (garden equipment; 3.5 kg m<sup>-2</sup>). Then, the soil was irrigated to ensure that sufficient moisture was present for glucosinolate hydrolysis. Subsequently, the soil was covered with plastic film mulch. Ten days later, the mulch was removed and the soil was thoroughly ploughed. Chemical fumigation (hymexazol (HY), 3g m<sup>-2</sup>)

and no fumigation (CK) were used as control treatments. Soil samples were collected before the transplantation of cucumber seedlings in June of 2011.

Cucumber seeds under examination were wrapped in gauze and placed in a water bath at 50°C for 30 min. The seeds were then incubated at a constant temperature incubator (28°C) until the radicle length reached 1 cm. The radicles were placed in bowl containing nutrients in horticultural experiment station. When the cucumber seedlings had 3 leaves, they were transplanted into soil and cultured in a greenhouse at 25 °C in day time and 20 °C at night.

#### ***Glucosinolate concentration analysis in plant roots***

Six roots of 7 cm in diameter for each biofumigation material (MLG, MU, MHG, and TU) were randomly selected, freeze-dried and then grounded for GSL extraction.

The freeze-dried roots were crushed (100 mesh) and mixed. Dust materials were weighed accurately (0.50 g), transferred into centrifuge tube A and B, and extracted with 4 mL of 70% methanol (75°C). Tube B had another 200 µl of 5 mmol L<sup>-1</sup> sinigrin as an inter-standard. The extracts were heated for 10 min at 75°C in a water bath, followed by adding 1 mL of 0.4 M acetic barium and centrifuged at 4000 r/m for 10 min. The supernatants were decanted and pour into 10 ml flask, pellets were re-extracted twice with 3 mL of 70% methanol. Supernatants were combined and made up to a total volume of 10 ml. 5 mL extract was loaded onto acetic acid type anion exchange column of DEAE-SephadexA-25, and allowed to enzymatic 12 h with 250 µl sulfatase. Rinse with 5 ml pure water, mix up eluent and use 0.45 µm microporous membrane filtration, sealing, liquid chromatography to be analyzed.

HPLC analysis and quantification: The 10 µl sample volume was analyzed in a Merck HPLC system consisting of L7100 gradient pump, L7200 type autosampler, L7455 UV detector set at 229 nm and a Novapak C18 column (at 30°C). The mobile phase was ultra-pure water and acetonitrile. The flow rate was 1.3 ml•min<sup>-1</sup> in a linear gradient from 0 to 20% acetonitrile during 2 to 34 min (A pump) and constant 20% acetonitrile for 6 min (B pump), finally eluted with 100% acetonitrile (C pump) 10 min and equilibrated with 0 B prior to the injection of the next sample.

Use benzyl GSs as an internal standard, according to the retention times and peak areas to determine glucosinolates component qualitative and quantitative. GSs concentration is expressed by containing in several GSs micromoles per gram sample of dry powder (µmol/g DW).

#### ***DNA extraction and purification***

Soil from each plot was sampled at 9 random locations in each plot two hours before the biofumigation (BB) and ten days after the fumigation (AB). Soil samples from plots were also collected when the cucumber (*Cucumis sativus* L. Changchunmici) was at seedling (SS), flowering (FS), and maturity (MS) stages. Soil samples were collected from 0 to 20 cm depth in each plot treatment according to a staggered grid method using a 3.0-cm diameter gauge. All soil samples were stored at -80°C for DNA analysis.

DNA was extracted from 1 g of frozen soil according to Zhou et al. (1996) method with minor modifications. Soil samples were pretreated with EDTA and PVPP. Because this treatment efficiently removed humic substances, EDTA was used in the extraction of crude DNA. To 1 g of each soil sample, 3 ml TENP buffer [50 mmol L<sup>-1</sup> Tris (pH 8.0), 100 mmol L<sup>-1</sup> NaCl, 20 mmol L<sup>-1</sup> EDTA, and 0.01 g ml<sup>-1</sup> PVPP, pH 10.0] was added, the mixture was shaken, and allowed to stand for 2 min, and repetition of 3 times. Centrifugation at 9,000 rpm for 5 min, 3 ml PBS buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, 0.24 g KH<sub>2</sub>PO<sub>4</sub>, soluble in 1 L water, pH 7.4) was added. And the mixture was repeatedly shaken, and allowed to stand for 2 min, and repetition of 3 times before centrifugation at 9,000 rpm for 5 min.

After pretreating with EDTA and PVPP, the soil sample was mixed with 3 ml of DNA extraction buffer (100 mmol L<sup>-1</sup> Tris-HCl, 100 mmol L<sup>-1</sup> EDTA, 100 mmol L<sup>-1</sup> Na<sub>3</sub>PO<sub>4</sub>, 1.5 mmol L<sup>-1</sup> NaCl, 1% CTAB, pH 8.0), 20 µl of proteinase K (20 mg ml<sup>-1</sup>), and 30 µl lysozyme (100 mg ml<sup>-1</sup>) in centrifuge tubes. To this mixture, 0.5 ml of a 20% solution of SDS was added and the samples were incubated in a water bath at 65°C for 2 h with gentle end-over-end inversions every 15 to 20 min. After centrifuging these samples at 9,000 rpm for 15 min at room temperature, the supernatants were collected and transferred into new centrifuge tubes. Supernatants from multiple extractions performed for the same sample were combined, mixed with an equal volume of phenol-chloroform-isopentyl alcohol (25:24:1, vol/vol) mixture, and centrifuged at 9,000 rpm for 15 min. The supernatants were collected into new centrifuge tubes, mixed with an equal volume of chloroform-isopentyl alcohol (24:1, vol/vol) mixture, and centrifuged at 9,000 rpm for 15 min. The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1 h. The pellet of crude nucleic acids was collected by centrifuging this mixture at 12,000 rpm for 20 min at room temperature. The nucleic acid pellet was subsequently washed with cold 70% ethanol, centrifuged at 12,000 rpm for 2 min, and resuspended in sterile deionized water to a final volume of 50 µl.

The crude DNA thus extracted was diluted in 100 µl of TE buffer (100 mmol L<sup>-1</sup> Tris-HCl, 100 mmol L<sup>-1</sup> EDTA, PH 8.0) and purified with the help of the DNA gel Kit from haigene according to the manufacturer's instructions. The purified DNA was stored at - 20°C until use.

### Polymerase chain reaction

The V3 region of the bacterial 16S rRNA gene was amplified according to the method described by Muyzer et al. (1993) using the primer pair F388-GC/R518 [Table.1, (HuaDa Genetic technology, Beijing, China)]. The PCR reaction system contained 0.5 µl each of forward and reverse primers (10 pmol µl<sup>-1</sup>), 1 µl of DNA template, 2 µl of dNTP, 0.25 µl of Taq polymerase, 2.5 µl 10×PCR buffer, and 18.25 µl sterile deionized water. PCR amplification was performed on a PCR System 9700 (Foster, CA, USA) instrument. The thermal cycling conditions were the following: 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min, and extension at 72°C for 1 min with a final extension at 72°C for 10 min. The presence of PCR products was confirmed by electrophoresis on 1.5% agarose gels, which were stained with EB buffer and photographed.

When analyzing the samples for fungi, the primer set NS7-GC/NS8 was used according to the method described by White et al. (1990) and an annealing temperature of 61°C was applied [Table.1, (HuaDa Genetic technology, Beijing, China)]. Each 25 µl of PCR mixture contained 0.5 µl each primer, 1 µl of DNA template, 2 µl of dNTP, 0.25 µl of Taq polymerase, 2.5 µl 10×PCR buffer, and 18.25 µl sterile deionized water. PCR amplification was performed under the following conditions: 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min, and extension at 72°C for 1 min, with a single final step of elongation at 72°C for 10 min. The

PCR products were separated by electrophoresis on 1.5% agarose gel, stained with EB buffer, and photographed using a digital camera.

### Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was employed to resolve the PCR products on 8% polyacrylamide gels with a denaturing gradient ranging of 40%-65% and 25%-55% for bacteria and fungi, respectively, where 100% denaturant was defined as a mixture of 7 M urea and 40% deionized formamide (Liang et al., 2008). A maker lane containing standard DNA mixtures were used. DGGE analysis was used to perform by a DCode Universal Mutation Detection System (Bio-Rad, USA), and run in a 1 × TAE buffer for 14 h under constant conditions of 60°C and 70 V. DNA sample volume per well was 30ul. After the electrophoresis, the gel was silver-stained using the method described by Bassam et al. (1991). DGGE profiles were immediately photographed on a white light box.

### Analysis of the DGGE banding patterns

The bands in DGGE profile were analyzed using the Quantity One software (version 4.6.2, Bio-Rad), which automatically determined the position and intensity of each band. The density value of each band was divided by the average band density of the lane to minimize the effect of variations in the amount of DNA loaded (Garland and Mills, 1991). Bands were considered common if they migrated to the same distance in the gel (Wallis et al., 2010). Each band was considered to represent a single operational taxonomic unit (OTU). Normalized data were used for cluster analysis. These analyses were performed using the SPSS 19.0 software. The diversity of soil microbial community was evaluated by the Shannon-Weaver index of general diversity, H (Shannon and Weaver, 1963). H was calculated from the peak height for the different bacterial groups using the following equation:  $H = -\sum (n_i/N) \log (n_i/N)$ , where  $n_i$  is the height of the peak and N

**Table 1: Total glucosinolate and various types of glucosinolates concentrations(umol g<sup>-1</sup> DW)in different dry biofumigation materials roots**

Glucosinolate	MLG	MU	MHG	TU
Progoitrin	-	-	-	6.41±0.014 <sup>a</sup>
Gluconapoleiferin	0.07±0.003 <sup>b</sup>	0.07±0.001 <sup>b</sup>	0.06±0.003 <sup>b</sup>	1.76±0.115 <sup>a</sup>
Glucoalyssin	0.04±0.003 <sup>c</sup>	0.12±0.008 <sup>b</sup>	0.05±0.002 <sup>c</sup>	0.29±0.041 <sup>a</sup>
Gluconapin	21.05±0.355 <sup>c</sup>	26.05±0.561 <sup>b</sup>	32.7±0.000 <sup>a</sup>	1.78±0.036 <sup>d</sup>
Glucobrassicinapin	4.47±0.174 <sup>b</sup>	4.18±0.083 <sup>b</sup>	5.19±0.000 <sup>a</sup>	4.03±0.195 <sup>b</sup>
Glucoruciferin	-	-	-	0.15±0.087 <sup>a</sup>
Glucobrassicin	0.13±0.009 <sup>b</sup>	0.54±0.025 <sup>a</sup>	0.17±0.000 <sup>b</sup>	0.17±0.100 <sup>b</sup>
4-methoxyglucobrassicin	0.14±0.003 <sup>c</sup>	0.17±0.004 <sup>a</sup>	0.16±0.000 <sup>b</sup>	0.12±0.001 <sup>d</sup>
4-hydroxyglucobrassicin	-	-	-	0.26±0.035 <sup>a</sup>
Neoglucobrassicin	0.25±0.004 <sup>bc</sup>	0.33±0.015 <sup>b</sup>	0.15±0.003 <sup>c</sup>	0.54±0.065 <sup>a</sup>
Gluconasturtiin	-	-	-	6.56±0.080 <sup>a</sup>
Ttotal	26.15±0.328 <sup>c</sup>	31.1±0.701 <sup>b</sup>	38.49±0.003 <sup>a</sup>	22.09±0.000 <sup>d</sup>

Values in the same column, followed by the same letter, do not significantly differ ( $P \leq 0.05$ , SPSS).



is the sum of all peak heights in the densitometric curve. Statistical analysis was performed using the general linear model (GLM) procedure of the SPSS 19.0 software (SPSS Institute Inc., IBM, USA).

The Quantity one software (version 4.6.2, Bio-Rad) was used in the Cluster analysis of bacterial and fungal communities, which assessed the similarity between the different soil microbial species and classified the edaphon to different clusters.

### Inhibition of cucumber *Fusarium* wilt

#### Experiment design

A pot experiment was carried out to study the effect of biofumigation on cucumber *Fusarium* wilt. The soil used in this study was collected from a continuously cropped cucumber greenhouse in which the temperature was 18°C - 32°C, and the relative humidity was 60% - 90%. The soil was filtered through a 2-mm sieve and sterilized prior to use. Each pot was filled with 2 kg soil. Fresh mustard plants containing high glucosinolate, mustard plants containing low glucosinolate, and spinach without glucosinolate were cut into small pieces and incorporated into the soil at a rate of 3.5 kg m<sup>-2</sup>. Chemically fumigated (hymexazol) soil and non-fumigated continuously cropped soil served as control treatments. One cucumber seedling was inoculated with *Fusarium oxysporum* and transplanted into each pot. There were five treatments in total, and each treatment had eight replicates.

#### Inoculating cucumber *Fusarium oxysporum* f. sp. *cucumerinum* Owen

The radicle method was used for the inoculation of cucumber with the *Fusarium* wilt pathogen. *Fusarium oxysporum* f. sp. *cucumerinum* Owen was obtained from the Pathology Laboratory of Northeast Agricultural University. The isolated *Fusarium oxysporum* f. sp. *cucumerinum* Owen had been previously tested for pathogenicity. Potato dextrose agar medium inoculated with *Fusarium* wilt pathogen was incubated for 10 d at 30°C and then filtered with double gauze to remove the hypha. The filtrate was centrifuged at 4,000 rpm for 1 min at a relative centrifugal force value of 5366 g. The supernatant was discarded, and the centrifugate was diluted with sterile distilled water to a concentration of 2×10<sup>6</sup> spores ml<sup>-1</sup>. The cucumber seeds were wrapped in gauze, incubated in a water bath at 50°C for 30 min, and germination was accelerated at 28°C in a constant temperature incubator to a radicle length of approximately 1 cm. The radicles were soaked in the spore suspension for 30 min and transferred into pots with different fumigation treatments. The cucumber seedlings were cultivated for 30 d in a greenhouse at a temperature of 18°C - 32°C and a relative humidity of 60% - 90%.

#### *Fusarium oxysporum* density in soil

Colony forming units (CFU) were determined to analyze the *Fusarium* wilt pathogen density in the soil. Soil from each treatment group was collected and stored refrigerated at 4°C for less than one week before counting the colonies.

We weighed 1.00 g soil, added it into 100 ml sterilized water and shaken for 20 s. From this suspension, 1 ml was drawn and added to 9 ml sterile water. An aliquot of 0.4 ml of this soil suspension was plated evenly on the surface of pentachloronitrobenzene selective culture-medium (1g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 5g L<sup>-1</sup> peptone, 0.5g L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 20.0g L<sup>-1</sup> agar, 300.0mg L<sup>-1</sup> streptomycin, 1.0g L<sup>-1</sup> 75% pentachloronitrobenzene wettable powder) and cultured for seven days at 28°C. Following this, the colonies were counted. Each treatment was performed in quadruplicates.

#### Growth index

A ruler was used to measure the height of cucumber seedlings. Vernier calipers were used to measure the stem diameter. The cucumber seedlings were harvested and dried at 70 °C to obtain a constant weight.

## STATISTICAL ANALYSIS

All statistical analyses were performed using SPSS statistical software (IBM SPSS Statistics 19). Differences at the P ≤ 0.05 level were considered to be statistically significant.

## RESULTS

### Glucosinolate concentrations in different fumigation materials

Seven glucosinolates (gluconapoleiferin, glucoalyssin, gluconapin, glucobrassicinapin, glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin) were identified in three mustard fumigation materials of which gluconapin was the main glucosinolate and accounted for 80%-85% of the total (Table.2). In addition to the seven glucosinolates contained in mustard, turnip also contained four glucosinolates, of which progoitrin and gluconasturtiin were the main glucosinolates and accounted for 29% and 28% of the total, respectively.

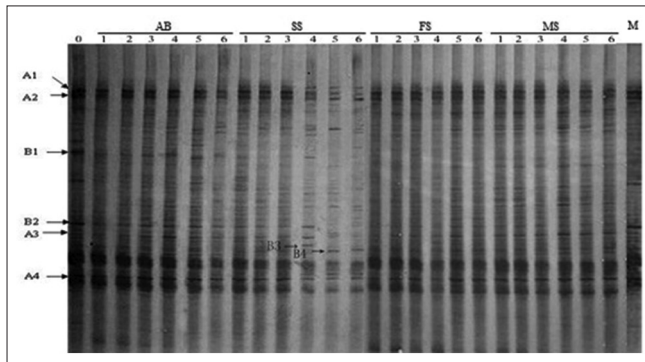
### Dynamic change in soil bacterial diversity assessed by PCR-DGGE

The bacterial diversity in the soil samples collected at different cucumber developmental stages was evaluated by DGGE analysis of the amplified partial 16S rRNA genes. The number of bands in the DGGE profiles indicated number of OTUs detected, and the band intensity indicated the population density of each OTU. In the DGGE profile, the bands A1, A2, and A4 were common and were of high density at all sampling times and treatments (Fig. 1).

**Table 2: Primer sequences used for amplifying nucleic acids isolated from bacteria and fungi**

	Primer	Sequence
Bacteria	F338-GC	5'-CGCCCGCCGCGCGCGGGCGGGCGGGG GCCGGGGGGCTACGGGAGGCAGCAG-3'
	R518	5'-ATTACCGCGGCTGCTGG-3'
Fungi	NS7-GC	5'-CGCCCGCCGCGCCCCGCGCCCGGCCGCGG CCCCCGCCCCGAGGCAATAACAGGTCTG-3'
	NS8	5'-TCCGACAGTTACCTACGGA-3'

When analyzing the samples for bacterial, the primer set F338-GC/R518 was used according to the method described by Muyzer et al. (1993). When analyzing the samples for fungi, the primer set NS7-GC/NS8 was used according to the method described by White et al. (1990)



**Fig 1.** DGGE profiles of bacterial communities in cucumber soil. The numbers 0, 1, 2, 3, 4, 5, and 6 above the line indicate the following experimental treatments: Before biofumigation (BB); control (CK); hymexazol (HY); mustard (MU); mustard containing low glucosinolates (MLG); mustard containing high glucosinolates (MHG); and turnip (TU). AB = between the end of biofumigation material incorporation and before the transplantation of cucumber seedlings; SS = seedling stage; FS = flowering stage; FB = maturity stage.

There were significant differences in the positions or the intensities of some bands. The B1 and B2 bands from samples collected from the soil that were not fumigated were of high intensity. These bands became weak after fumigation and at the flowering and maturity stages. In contrast, band A3 was weak before fumigation and was relatively strong at the flowering stage. In MLG treatments, band B3 showed higher intensity at the seedling stage. In MHG and TU treatments, the B4 band was of a higher intensity at the seedling stage.

The diversity indices calculated from the DGGE band patterns are presented in Table. 3. The Shannon–Weaver index of diversity for bacterial communities in MHG-treated soil (3.49, the highest diversity) was significantly higher than ( $P < 0.05$ ) that found in other treatment conditions. There was no significant difference ( $P > 0.05$ ) between the MU and MLG treatments. The Shannon diversity indices were lower in MU (3.44), TU (3.24), and HY (3.12) treatments.

The uniqueness of the DGGE patterns indicated that each sample had a bacterial community. Cluster analysis of PCR amplicons demonstrated that the profiles of these bacterial communities were clustered into three groups (Fig. 2). Group I contained only the HY treatment. Group II

**Table 3: Shannon–Weaver index of soil bacteria and fungi at cucumber flower stage following various fumigation treatments**

Treatments	Shannon–Weaver index of diversity (H)	
	Bacteria	Fungi
BB	3.08	2.35
CK	3.08	2.38
HY	3.12	2.44
MU	3.44	2.42
MLG	3.45	2.42
MHG	3.49	2.74
TU	3.24	2.29

Values in the same column, followed by the same letter, do not significantly differ ( $P \leq 0.05$ , SPSS).

included the BB and CK treatments. The MHG, MLG, TU, and MU treatments were clustered into group III.

#### Dynamic change in soil fungal diversity assessed by PCR-DGGE

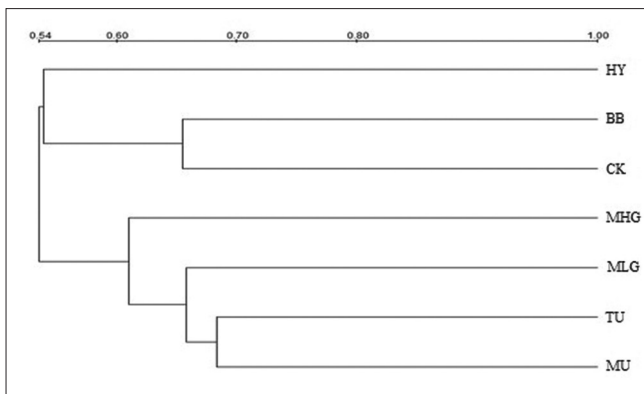
The analysis of DGGE profiles showed variations in the intensity and number of bands among the soil samples (Fig. 3). The DGGE fingerprints were complex, with the number of bands detected in the samples varying between 15 and 19. The bands A1 and A2, which were of high intensity during cucumber growth periods, were present in all samples. When fungi from soil samples under various biofumigation treatments were analyzed, the number of bands and their position were found to be different. For example, in the MU, MHG, and TU treatments, the band B1, which was not present before fumigation, showed a higher intensity at the seedling stage. The band B2 was stronger at the maturity stage than at other cucumber growth periods. In MHG treatment, the band C1 showed a higher intensity at the seedling stage. At the flowering stage, the intensity of the band C2 was higher in the TU treatment than in the other biofumigation treatments.

The Shannon–Weaver index of diversity for fungal communities was determined from the DGGE banding patterns. The Shannon diversity indices thus determined that the different soil treatment conditions were significantly different (Table. 3). Among all of treatments, the diversity index of the communities in the MHG-treated soil (2.74) was the highest ( $P < 0.05$ ) and that of the communities in TU-treated soil was the lowest. There was no significant

**Table 4: Influence of fumigation materials on cucumber seedling growth indicators and *Fusarium* wilt disease index at seeding stage**

Treatments	Dry matter plant (g)	Plant height (cm)	Stem diameter (cm)	Numbers of <i>fusarium oxysporum</i> ( $\times 10^3$ cfu g <sup>-1</sup> DW)
CK	1.01 <sup>c</sup> ±0.02	18.11 <sup>d</sup> ±0.14	0.40 <sup>c</sup> ±0.03	69.04 <sup>a</sup> ±2.77
HY	3.44 <sup>a</sup> ±0.24	49.06 <sup>a</sup> ±0.11	0.68 <sup>a</sup> ±0.04	16.33 <sup>d</sup> ±0.88
SP	2.46 <sup>b</sup> ±0.13	34.78 <sup>c</sup> ±0.23	0.51 <sup>b</sup> ±0.02	58.98 <sup>b</sup> ±2.79
MLG	3.42 <sup>a</sup> ±0.04	49.25 <sup>a</sup> ±0.13	0.55 <sup>b</sup> ±0.02	31.15 <sup>c</sup> ±1.65
MHG	3.07 <sup>a</sup> ±0.02	40.61 <sup>b</sup> ±0.09	0.59 <sup>ab</sup> ±0.01	25.91 <sup>c</sup> ±0.97

Values in the same column, followed by the same letter, do not significantly differ ( $P \leq 0.05$ , SPSS)



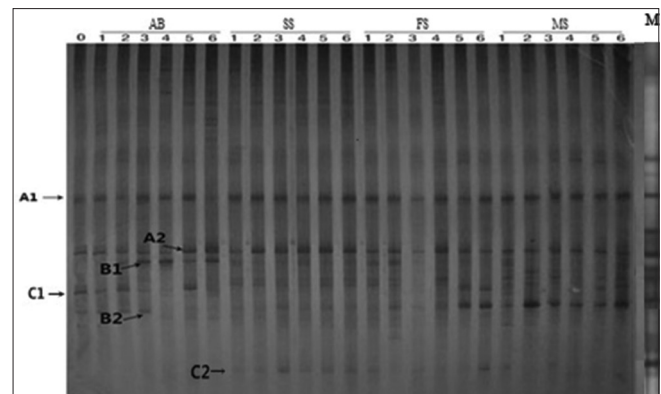
**Fig 2.** Cluster analysis of bacterial DGGE profiles at cucumber flower stage. BB = before biofumigation, CK = control, HY = hymexazol, MU = mustard, MLG = mustard containing low glucosinolates, MHG = mustard containing high glucosinolates, TU = turnip.

difference ( $P > 0.05$ ) between the indices obtained for HY, MU, and MLG treatments.

Cluster analysis of PCR amplification results divided the samples into three clusters, namely clusters I, II and III. Cluster I consisted of the TU treatment, Cluster II consisted of CK and BB, and Cluster III consisted of MU, MLG, MHG, and HY treatments (Fig. 4), which suggested that the fungus composition between biofumigation and unbiofumigation is different.

#### Inhibition of cucumber fusarium wilt by fumigation materials at the seedling stage

The incorporation of brassica materials significantly promoted the growth of cucumber seedlings in all fumigation treatments (Table. 4). Compared with samples grown in soil incorporated with spinach, the dry weight of cucumber in soil fumigated with MLG and MHG increased by 40.3% and 26.3%, respectively. The stem diameter in the MHG treatment was significantly greater than that in other treatments. The abundance of *F. oxysporum* CFUs in the MHG and HY treatments was significantly lower than that in other treatments. The *F. oxysporum* CFUs in samples of soil biofumigated by plants were markedly different from that in CK- treated soil sample.



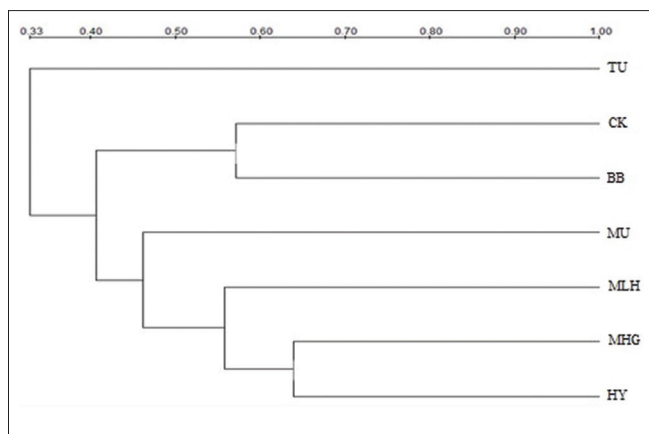
**Fig 3.** DGGE profiles of fungal communities in cucumber soil. The numbers 0, 1, 2, 3, 4, 5, and 6 above the lane indicate the following experimental treatments: Before biofumigation; control; hymexazol; mustard; mustard containing low glucosinolates; mustard containing high glucosinolates; and turnip. AB = between the end of biofumigation material incorporation into soil and before transplantation of cucumber seedlings; SS = seedling stage; FS = flowering stage; FB = maturity stage.

## DISCUSSION

This study investigated the effects of biofumigation (mustard with three levels of glucosinolates and turnip) and chemical fumigation (hymexazol) on cucumber *Fusarium* wilt and soil microbial community in continuously cropped cucumber soil.

#### Disease suppression by brassica plant material

*F. oxysporum* cannot multiply in many non-host plants that are used as rotation crops or for soil fumigation (Leoni et al., 2013). Biofumigation treatment reduced the *Fusarium oxysporum* population in the soil, lowered disease incidence in cucumber, and promoted cucumber growth (Table. 4). These results were consistent with that of earlier studies, which found that brassica plant materials were effective in reducing *Fusarium* wilt (Cohen et al., 2005; Larkin and Griffin, 2007). Additionally, Wang et al. (2014) recently reported that biofumigation successfully controls *phytophthora blight*. Suppression of *Fusarium oxysporum* by biofumigation is likely due to the effect of isothiocyanates released following the hydrolysis of glucosinolate present



**Fig 4.** Cluster analysis of fungal DGGE profiles at flowering stage. BB = before the biofumigation, CK = control, HY = hymexazol, MU = mustard, MLG = mustard containing low glucosinolates, MHG = mustard containing high glucosinolates, TU = turnip.

in brassica crops. Isothiocyanates are toxic for soil-borne pathogens and have been successfully used to reduce soil-borne populations of fungal pathogens (Olivier et al., 1999; Muehlchen et al., 1990). Our results showed that the disease index of cucumber fumigated with different brassica crops is different. This is likely due to the differences in the amounts and forms of isothiocyanates released from different brassica crops.

In our pot experiments, a lower amount of *Fusarium oxysporum* was found in the MHG treatment. Our glucosinolate analysis showed that the Brassica rapa cv hongyuan used in the MHG treatment contained a high amount of gluconapin (Table.1) the hydrolysis product (3-butenyl isothiocyanate) of which effectively kills soil-borne pathogens (Jang et al., 2010). This likely explains the significantly lower abundance of *Fusarium oxysporum* found in the MHG-treated soil. Therefore, it appears that glucosinolate content of the brassica crop influences the biofumigation effect to some extent. Owing to the different types of isothiocyanates, the levels of *Fusarium* wilt suppression were varied. An earlier study by Sarwar and Kirkegaard (1998) reported similar results. The susceptibility of fungi to isothiocyanates contributes to the control of cucumber *Fusarium* wilt. Brown and Morra (1997) have reported differences in the sensitivity of fungal species to isothiocyanates. Hydrolysis products of glucosinolates or other factors likely play important roles in disease control. Therefore, different Brassica plants control cucumber *Fusarium* wilt pathogens to different extents.

### Soil microbial communities

DGGE profile analysis revealed an increase in the number and a change in the position of bands after Brassica plants were incorporated into the soil (Fig. 1). For examples, bands B3 and B4 were unique to other treatments (Fig. 1).

Although some bands were present in the profiles of all treatment groups, their intensities varied according to the treatment. For example, the band B2 was weak after biofumigation, whereas the band A2 was strong in the MHG and TU treatment groups at the seeding stage. Additionally, the cluster analysis results showed that the microbial communities in biofumigation treatments differed from those in the HY and CK treatments (Fig. 2). It has been shown that continuous application of 2-phenylethyl isothiocyanate to soil samples leads to noticeable changes in the soil microbial community structure. Taken together, our results suggest that the incorporation of different types of Brassica changed the soil microbial community structures. This finding is in line with those of earlier reports on the incorporation of organic materials into soil (Omirou et al., 2011). Hanna et al. (2009) found variations in the microbial community structure following the incorporation of mustard. In a recent study employing fatty acid methyl ester (FAME) analysis, Omirou et al. (2011) used broccoli plant residues for biofumigation and found that the treatments temporarily altered the composition of microbial communities in the soil. It is possible that changes in the soil microbial community structure led to the observed disease suppression. Several studies have examined the effect of biofumigation with brassica crop residues on soil bacterial communities (Omirou et al., 2011; Cohen et al., 2005).

The Shannon diversity indices of the bacterial and fungi communities that were present in the soil collected after the biofumigation treatments were higher than those of CK and BB treatments (Table. 3). Thus, compared with chemical fumigation and the control, biofumigation increased the soil microbial diversity. This is in line with the results of previous studies that found a rapid increase in bacterial biomass within 5 to 30 days after the incorporation of fresh plant residues (Zelenev et al., 2006; Hanna et al., 2009). These studies identified organic carbon content as the main factor that affects the microbial community structure. In contrast to biofumigation, chemical fumigation with commercial Metham sodium, which releases isothiocyanate into the soil, was found to cause a dramatic reduction in the activity of the soil microbial community, which persisted for at least 30 days (Omirou et al., 2011). Some studies have concluded that biofumigation does not exert a direct toxic effect on important microbial guilds and that the changes observed reflected a general microbial response to the addition of fresh organic matter into the soil (Omirou et al., 2011). In other words, the brassica materials acted as green manure to alter the physicochemical properties of soil, leading to changes in soil microbial community structures. Wang et al. (2014) reported that biofumigation increased the contents of the total N,  $\text{NO}_3^-$ -N, available P, and available K in the soil. These authors suggested that the control of pepper disease by biofumigation was mainly linked to the increase



in soil bacterial diversity rather than the population of soil-borne pathogens. Our results also showed that the diversity of bacterial communities increased after biofumigation. However, in contrast to earlier reports, our results suggest that the fungal diversity also increased after biofumigation (Hanna et al., 2009).

The results of cluster analysis of bacterial DGGE profiles showed that the MHG, MLG, TU, and MU treatments were clustered into one group that was different from that of HY and control (Fig. 4). This result suggested that the bacterial community structure of the biofumigated soil was different from that of the chemically fumigated and control soils. The results from the cluster analysis of bacterial DGGE showed that biofumigation with various brassica crops had a similar effect on bacterial community structure and resulted in diversity indices that were higher than those in the HY and CK treatments. Several studies have shown that brassica crops themselves exert a selective influence on the soil microbial communities because brassica plant species, together with other environmental factors, could determine which organisms were active and available to reproduce (Marschner et al., 2004; Innes et al., 2004). Furthermore, incorporation of brassica plants used as biofumigant material increased soil carbon content and tended to influence the soil microbial community structure. The results of the cluster analysis of fungal DGGE profiling showed that the fungi in CK and BB treatments belonged to one group, which was in line with the results obtained for bacteria. However, in contrast to that found from the bacterial cluster analysis, the fungi in the MHG and HY treatments appeared in the same group. This result suggested that the influence of different brassica crops on the soil bacterial and fungi structures is different.

In this study, we analyzed the effect of incorporation of brassica plants into soil as biofumigants (LSM, HSM, MU, and TU) on the soil microbial communities without focusing on the changes in specific microbial strains in the treated soil samples. Therefore, additional studies are needed to further elucidate the impacts of biofumigation on the composition of microbial communities.

## CONCLUSIONS

In conclusion, the results of this work indicate that Brassica plants are useful in the control of soil-borne fungal pathogens. Although the pathogen control was not as effective as that by hymexazol, substantial reduction in the abundance of cucumber *Fusarium* wilt pathogens was observed. Furthermore, incorporation of brassica plants as biofumigant materials increased the content of organic matter in the soil and tended to influence the soil microbial

communities, particularly fungi.

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## AUTHOR'S CONTRIBUTIONS

XTY, ZCY and RZZ performed most of the experiments, data analysis and original draft preparation. MLB and XRW, NX, CYZ contributed to investigation and acquisition of data. CYZ and TTL were involved in sample analysis. SML designed the research and reviewed the manuscript.

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