Analyses of the community compositions of root rot pathogenic fungi in the soybean rhizosphere soil

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ABSTRACT

Soybean (*Glycine max* [L.] Merr.) root rot is an important disease of soybean under continuous cropping, and root rot is widely distributed throughout the world. This disease is extremely harmful, and it is difficult to prevent and control. The study aimed to elucidate the composition of root rot pathogenic fungal communities in the continuous cropping of soybean. In this study, we employed PCR-DGGE technology to analyze the communities of root rot pathogenic fungi in soybean rhizosphere soil subjected to continuous cropping during a season with a high incidence of root rot in Heilongjiang province, China, the main soybean producing area in China. The results of 13 DGGE bands were analyzed by phylogenetic revealed that the predominant root rot pathogenic fungi in rhizosphere soil in the test area were *Pythium ultimum* and *Fusarium* species. The results of cluster analysis showed that the duration of continuous cropping, the soybean variety and the plant growth stage all had significant effects on the diversity of root rot pathogenic fungi in rhizosphere soil.

Key words: Community composition, continuous cropping, *Glycine max*, PCR-DGGE, root rot disease.

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Received: 14 July 2015.
Accepted: 10 November 2015.
doi:10.4067/S0718-58392016000200007

INTRODUCTION

Soybean (*Glycine max* [L.] Merr.) is an important grain and oilseed crop (Qin et al., 2014). The demand for soy products in China is rapidly increasing, and the Chinese soybean import market has strongly grown in recent years (He et al., 2013). As a result, there are large areas of soybean under continuous cropping in China (Zhang et al., 2011; Liu et al., 2012; Zhang et al., 2013). Soybean root rot is an important disease of soybean under continuous cropping. Soybean root rot is widely distributed throughout the world. This disease is extremely harmful, and it is difficult to prevent and control (Bolwerk et al., 2005; Govaerts et al., 2007; Hartman et al., 2011; Aoki et al., 2012; Bienapfl et al., 2013).

Many pathogens cause soybean root rot. *Fusarium* species are the main pathogens that cause this disease in many countries (Cichy et al., 2007; Bienapfl et al., 2010; Hashem et al., 2010). In USA, the main pathogens that cause root rot disease in soybean are *Fusarium* and *Pythium* (Ellis et al., 2013). The main pathogenic fungi in Japan are *Pythium* (Sugimoto et al., 2007), while the main pathogenic fungi in Canada are *F. oxysporum* and *F. graminearum* (Thomas et al., 2007). In China, the main pathogens that cause soybean root rot are *Fusarium* (Ding et al., 2011; Guo et al., 2011; Zhang et al., 2013), among these, *F. solani* is the dominant strain in Shandong (Wu et al., 2008; Pan et al., 2010), *F. episphaeria*, *F. tricinctum* and *F. oxysporum* are the main pathogens in Anhui Province, Huaibei area (Tang et al., 2010), *Phytophthora*, *F. oxysporum*, *F. solani* (Cui et al., 2010) are the dominant pathogens in Fujian Province, and *Fusarium*, *Rhizoctonia*, *Pythium*, and *Phytophthora* are the dominant pathogens in the Xinjiang autonomous region (Qiao et al., 2007). The dominant pathogens in Heilongjiang area include *F. oxysporum*, *Rhizoctonia solani*, *Pythium*, and *Phytophthora sojae* (Zhang et al., 2010). Numerous species of pathogenic fungi cause soybean root rot in China because the demand for soybean in China has been increasing yearly, and the use of continuous cropping has become a major obstacle in the soybean growing regions.

Continuous cropping seriously affects the growth of crops, and this system has recently become a major focus of study. Recent studies have shown that continuous cropping of soybean results in changes in the microflora of rhizosphere soil, which gradually changes from high fertility "bacterial type" soil to low fertility "fungi type" soil. Continuous cropping significantly reduces the pH of the soil, making the soil conducive to fungal growth while inhibiting the reproduction of bacteria and actinomycetes. As a result, fungi eventually become the dominant species

in rhizosphere soil. In addition, continuous cropping of soybean leads to the enrichment of root metabolites (such as phenolic acids) in the soil. After adding phenolic acids to soil, the concentration of phenolic acid and the quantity of fungi significantly increase. Therefore, an increase in the quantity of root metabolites inevitably leads to an increase in the proportion of fungi in the soil. As a direct consequence, the quantity of the soil-borne pathogen *Fusarium* increases (Zhao and Zhao, 2007; Qiu et al., 2010). At the same time, continuous cropping of soybean may also lead to an increase in the levels of organic compounds in the soil (sugars, amino acids, organic acids), thus providing a favorable environment for the multiplication of soybean root rot pathogenic fungi.

In this study, we selected three soybean varieties ('Ken Feng 16', 'Heinong 44' and 'Heinong 48') under continuous cropping in Heilongjiang province in a relatively large soybean planting area that employs standard field management practices. We employed polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) technology to analyze the changes in root rot-inducing fungal populations in soybean rhizosphere soil during a season with a high incidence of this disease. The goal of this study was to identify the predominant pathogens causing root rot during different periods of plant growth in order to clarify the composition and diversity of root rot-causing fungi in rhizosphere soil under continuous cropping of soybean in Heilongjiang and to provide a theoretical basis for disease prevention and control measures, which could significantly improve yield and quality of spring soybean in Northeast China.

MATERIALS AND METHODS

Samples preparation and collection

This study was conducted in May-October 2013 at the Sugar Industry Research Institute Experiment Station of Harbin Institute of Technology. Three varieties of soybean commonly cultivated in Heilongjiang Province were selected as test materials: 'Ken Feng 16' (KF16, a short, intermediate-type variety with an average protein content of 40.17% and an average oil content of 20%), 'Heinong 44' (HN44, a high oil variety with an average protein content of 36.06% and an average oil content of 23.01%), and 'Heinong 48' (HN48, a high-protein variety with an average protein content of 45.23% and an average oil content of 19.50%).

Sample collection was performed twice: at 30 d after the emergence of seedlings (seedling stage) and at 60 d after the emergence of seedlings (branching stage). When sampled, the surface debris near the aboveground parts of the plants was removed. Rhizosphere samples were then collected randomly using a punch, and the plant material was removed using scissors to clip the root samples. Three root samples and soil samples under the same duration of continuous cropping were mixed, producing a mixed sample. The experiments were repeated in triplicate. While three groups of samples under different durations (years) of continuous cropping were denoted as CK(R), RS1, and RS2; the soil samples were denoted as CK(S), SS1, and SS2, respectively. Soybean test plots at year zero (CK), 1 yr of continuous cropping and 2 yr of continuous cropping were selected. The main initial physical and chemical indicators of three soil types were as follows: 26.13 g organic matter kg^{-1}, 1.69 g full N kg^{-1}, 25.4 g·full K kg^{-1}, 5.5 g·whole P kg^{-1}, 133.1 mg·alkaline hydrolysis N kg^{-1}, 13.14 mg quick-acting P kg^{-1}, 206 mg·quick-acting K kg^{-1}, and pH 7.0.

Statistics of disease incidence of the soybean root rot

Root rot were observed on the basal stem. The root of each soybean cultivar was collected, washed under running tap water, and assessed for the presence and severity of root rot symptoms on a 0-4 scale (Zhou et al., 2014), where: 0: no symptoms, 1: mild symptoms (discoloration but no visible lesions), 2: obvious lesions, 3: severe lesions on the stem and diminished plant vigor, and 4: stem rotten, plant dead. The soybean plants of 1-4 scale are pathogenic plant. Disease Incidence Statistics were dealed with 50 plants of each soybean cultivar.

Extraction of genomic DNA and PCR

A soil DNA kit E.Z.N.A Soil DNA Kit (Omega Bio-Tek, Norcross, Georgia, USA) was used to extract total genomic DNA in rhizosphere soil. The total genomic DNA was examined by 1% agarose gel electrophoresis. The total genomic DNA was stored in centrifuge tubes at -20 °C until analysis.

The primers used for PCR were as follows: NS1: 5'-GTAGTCATATGCTTGTCTC-3' and Fung GC: 5'-CG CCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCG CCCCATTCCCCGTTACCCGTTG-3'. These primers are suitable for amplifying the 18S rRNA gene region (V1+V2) of most fungi (May et al., 2001), producing a fragment size of approximately 430 bp after amplification.

The reaction system (20 µL) included the following: 2.0 µL 10× Ex Taq Buffer (Mg^{2+}), 1.6 µL 2.5 mM dNTP Mixture, 0.2 µL 5 U Taq enzyme and 2 µL 1 µM primers, respectively. The reaction conditions were as follows: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 57 °C for 1 min and 72 °C for 1 min; 72 °C for 10 min.

DGGE and cluster analysis

The PCR products of the 18S rRNA (V1+V2) variable region amplified from the extracted total DNA genomic soybean (as a template) were subjected to denaturing gradient gel electrophoreses. The trial conditions for DGGE were as follows: 8.0% acrylamide (acrylamide: bis acrylamide = 37.5:1), 40%-65% denaturant (100% denaturant was 40% formamide and 7 mol L^{-1} urea), electrophoresis temperature 60 °C, electrophoresis voltage 130 V, electrophoresis time 9 h and silver staining for 15 min. After the band unicity test,

the PCR products were recovered from the gel, cloned into the pGM-T Easy vector and transformed into *E. coli* DH5α competent cells. Positive clones were selected and cultured in liquid; 1 mL samples were placed into 1.5 mL centrifuge tubes and sent to Shanghai Invitrogen Biological Engineering Co Ltd. for sequencing. The resulting sequences were BLASTED against sequences in the GenBank database, and phylogenetic analysis was performed based on this analysis. Cluster analysis of the DGGE bands were performed using Quantity One 4.62 software (Bio-Rad, Hercules, California, USA).

Analysis of community diversity

The community diversity was expressed based on the abundance (*S*) and Shannon-Wiener index (*H*). The abundance of each PCR product in a gel was deduced using Gel-Pro Analyzer 4.5; the Shannon-Weiner index (*H*) reflecting the richness and evenness of a species, it was calculated using the formula:

$$H = -\sum_{i=1}^{s} (P_i)(\log_2 P_i)$$

where *S* is the number of bands on the sample DGGE fingerprint image and P_i is the dominance of the i^{th} band.

According to the UPGMA (unweighted pair group method with arithmetic averages) calculation method, in the DGGE atlas, if a band appeared, it was recorded as 1, while no band was recorded as 0; then get twice matrix paired with DGGE band, according to the twice matrix. SPSS16.0 software was used for statistic analysis, and the phylogenetic tree was constructed by DNAMAN 7.0 (Lynnon Biosoft, San Ramon, California, USA) based on the DGGE band atlas.

RESULTS

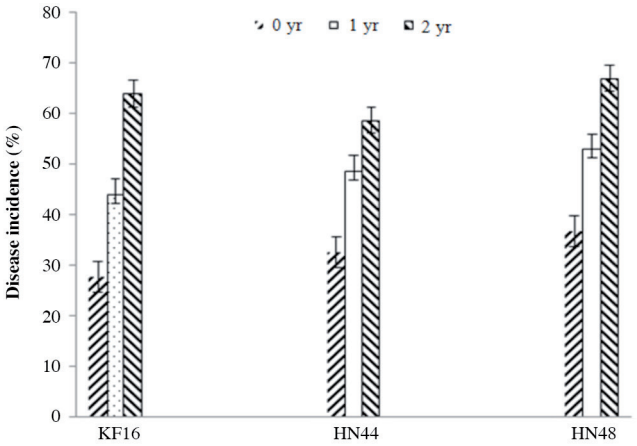
The effect of continuous cropping on the disease incidences of the soybean root rot

The disease incidences in different soybean cultivars were analyzed statistically (Figure 1). There were significant differences among the zero year, continuously cropped for 1 yr and 2 yr. With the increase of continuous cropping years, the disease incidence was significantly higher. Three soybean cultivars also had different disease incidences, the disease incidences of HN48 were higher than KF16 and HN44. Obviously, continuous cropping and soybean cultivar could influence the fungal community structure and disease incidence.

Total DNA extraction and PCR amplification in soybean rhizosphere

Total DNA extraction and PCR amplification of 18S rRNA (V1+V2) region in soybean rhizosphere soil from different periods and under different durations of continuous cropping.

Figure 1. The effect of continuous cropping on the disease incidences of the root rot for different soybean cultivars.



When total genomic DNA extracted from root samples was subjected to 1% agarose gel electrophoresis, no band was detected. However, genomic DNA extracted from soil produced bands following 1% agarose gel electrophoresis; the genomic DNA samples from rhizosphere soil were approximately 15 000 bp in size.

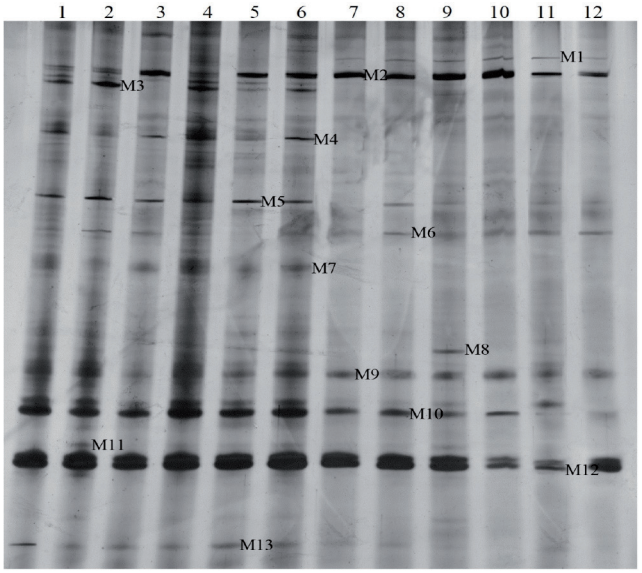
Using genomic DNA extracted from soil samples and root samples as a template and NS1 and FungGC as primers, PCR was conducted, followed by 1% agarose gel electrophoresis detection. The PCR product in the target band was approximately 430 bp.

DGGE band sequencing

The DGGE map was analyzed for root rot pathogenic fungi from rhizosphere soil after 3 yr of continuous cropping. The 18S rRNA (V1+V2) product was examined by DGGE; the position and quantity of the bands from each sample differed (Figures 2, 3, and 4). The DNA from 13 bands was extracted and sequenced; the sequencing results are shown in Table 1. Analysis of the 13 bands revealed the presence of *Pythium ultimum*, *Fusarium*, Ascomycete, Pleosporales, *Alicorhagia*, *Rhizoctonia solani*, Mucor, *Penicillium* and five uncultured fungi. From the sequencing results, we determined that in the rhizosphere soil microflora structure, the dominant soybean root rot fungal pathogens mainly comprised *Pythium ultimum* and *Fusarium*.

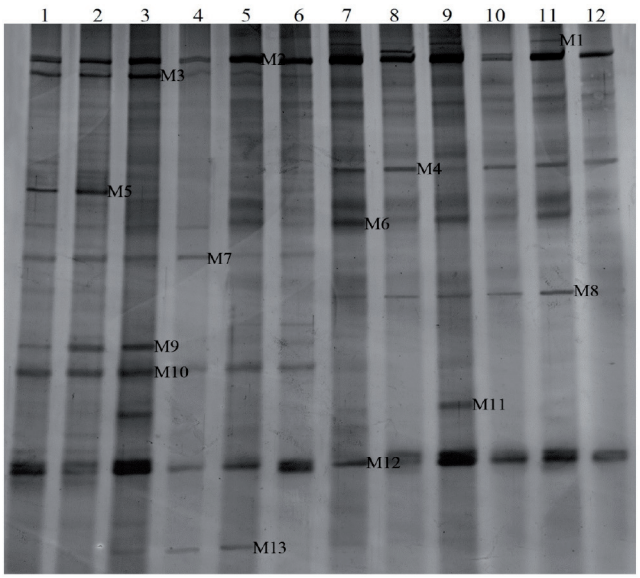
The sequencing result revealed that most fragments were approximately 430 bp in size, and the sequences were highly similar to the sequences of known species in the database, with most sequence similarities greater than 97%; we were therefore able to initially determine the genus of each dominant fungus. The flora represented by each band were as follows: M1, *Pythium ultimum* (sequence similarity 97%); M2, *Fusarium* (sequence similarity up to 99%); M3, Ascomycete (sequence similarity up to 99%); M4, Pleosporales (sequence similarity 98%); M5, *Alicorhagia* (99%); M6, *Rhizoctonia solani* (100%); M8, Mucor (98%); and M11, *Penicillium* (99%). The remaining five bands

Figure 2. DGGE profiles of soil samples and root samples in the control group for three soybean varieties at the seedling stage and in soil subjected to 1 yr of continuous cropping.



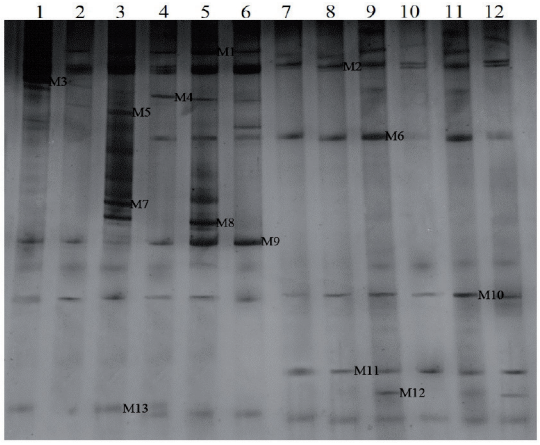
1: KF16 control root sample; 2: HN44 control root sample; 3: HN48 control root sample; 4: KF16 control soil sample; 5: HN44 control soil sample; 6: HN48 control soil sample; 7: KF16 1 yr of continuous cropping root sample; 8: HN44 1 yr of continuous cropping root sample; 9: HN48 1 yr of continuous cropping root sample; 10: KF16 1 yr of continuous cropping soil sample; 11: HN44 1 yr of continuous cropping soil sample; 12: HN48 1 yr of continuous cropping soil sample.

Figure 3. DGGE profiles of soil samples and root samples in the control group for three soybean varieties at the branching stage and in soil subjected to 1 yr of continuous cropping.



1: KF16 control root sample; 2: HN44 control root sample; 3: HN48 control root sample; 4: KF16 control soil sample; 5:HN44 control soil sample; 6: HN48 control soil sample; 7: KF16 1 yr of continuous cropping root samples; 8: HN44 1 yr of continuous cropping root sample; 9: HN48 1 yr of continuous cropping root sample; 10: KF16 1 yr of continuous cropping soil sample; 11: HN44 1 yr of continuous cropping soil sample; 12: HN48 1 yr of continuous cropping soil sample.

Figure 4. DGGE atlas of soil subjected to 2 yr of continuous cropping and root samples and soil samples harboring plants at the seedling and branching stages.



1: KF16 seedling stage root sample; 2: HN44 seedling stage root sample; 3: HN48 seedling stage root sample; 4: KF16 seedling stage root sample; 5: HN44 seedling stage soil sample; 6: HN48 seedling stage soil sample; 7: KF16 branching stage root sample; 8: HN44 branching stage root sample; 9: HN48 branching stage root sample; 10: KF16 branching stage soil sample; 11: HN44 branching stage soil sample; 12: HN48 branching stage soil sample.

belonged to uncultured fungi; the sequence similarity reached approximately 97%. Among the selected samples, the bands that represented the predominant pathogenic fungi causing root rot in rhizosphere soil could be initially classified as 12 different species. Moreover, the sequencing results revealed that in the rhizosphere soil microflora structure, the dominant pathogenic fungi of soybean root rot were primarily *Pythium ultimum* and *Fusarium*.

Phylogenetic analysis

To better elucidate the genetic relationship and system status between the target sequence and known fungus, we used the bacterium that had the closest genetic relationship with each sequence in the GenBank database and its closest relatives to construct a phylogenetic tree (Figure 5). As shown in the phylogenetic tree, the 13 DGGE bands were mainly derived from three types of fungus.

Effect of continuous cropping of soybean varieties on root rot pathogens

Effect of the duration of continuous cropping of different soybean varieties on the diversity of root rot pathogens in the rhizosphere soil environment. Based on the abundance values of the three main species, we determined that regardless of the root or soil sample, the abundance value of each variety was higher under 2 yr of continuous cropping than under 1 yr of continuous cropping, suggesting that increasing the duration of continuous cropping increases the abundance of root rot pathogens. The abundance value of root rot pathogenic fungi under the same duration of continuous cropping at the same

Table 1. Results of BLAST analysis of 13 sequences.

Clone number	Sequence size (bp)	Accession number	The strains which have the highest identity from NCBI (accession)	Similarity
M1	391	KF650045	*Pythium ultimum* (AB370108)	(97%)
M2	416	KF650046	*Fusarium* sp. (EU862185)	(99%)
M3	402	KF650047	*Ascomycete* sp. (DQ310778)	(99%)
M4	387	KF650048	*Pleosporales* sp. (DQ310782)	(98%)
M5	434	KF650049	*Alicorhagia* sp. (EU675633)	(99%)
M6	384	KF650050	*Rhizoctonia solani* (JF499071)	(100%)
M7	439	KF650051	Uncultured eukaryote (JN846872)	(94%)
M8	401	KF650052	*Mucor* sp. (JX537955)	(98%)
M9	398	KF650053	Uncultured eukaryote (KF357480)	(97%)
M10	400	KF650054	Uncultured fungus (HQ190217)	(93%)
M11	393	KF650055	*Penicillium* sp. (FJ025151)	(99%)
M12	430	KF650056	Uncultured fungus (JN583491)	(98%)
M13	396	KF650057	Uncultured eukaryote (FN393193)	(93%)

growth period varied among different soybean varieties; ANOVA results revealed that the differences were significant (Table 2). For example, at seedling stage RS2, there was no significant difference between KF16 and HN44, but these varieties were significantly different from HN48. In rhizosphere soil of soybean at the seedling stage grown in soil subjected to 2 yr of continuous cropping, the abundance values of root rot pathogenic fungi were relatively large in HN48 rhizosphere soil.

Statistical analysis of the diversity indices of three soybean varieties revealed that regardless of whether root samples or soil samples were examined, the diversity indices in soil subjected to 2 yr of continuous cropping for different varieties and different treatments were higher than those in soil subjected to 1 yr of continuous cropping. These results demonstrate that continuous cropping is conducive to increasing the diversity index of root rot pathogens. In soil subjected to the same duration of continuous cropping harboring different soybean cultivars at the same growth stage, the diversity index of root rot pathogenic fungi in the rhizosphere soil environment differed; ANOVA revealed that these values were significantly different (Table 3). For

Figure 5. Phylogenetic tree of 13 DGGE sequences and the sequences of closely related species.

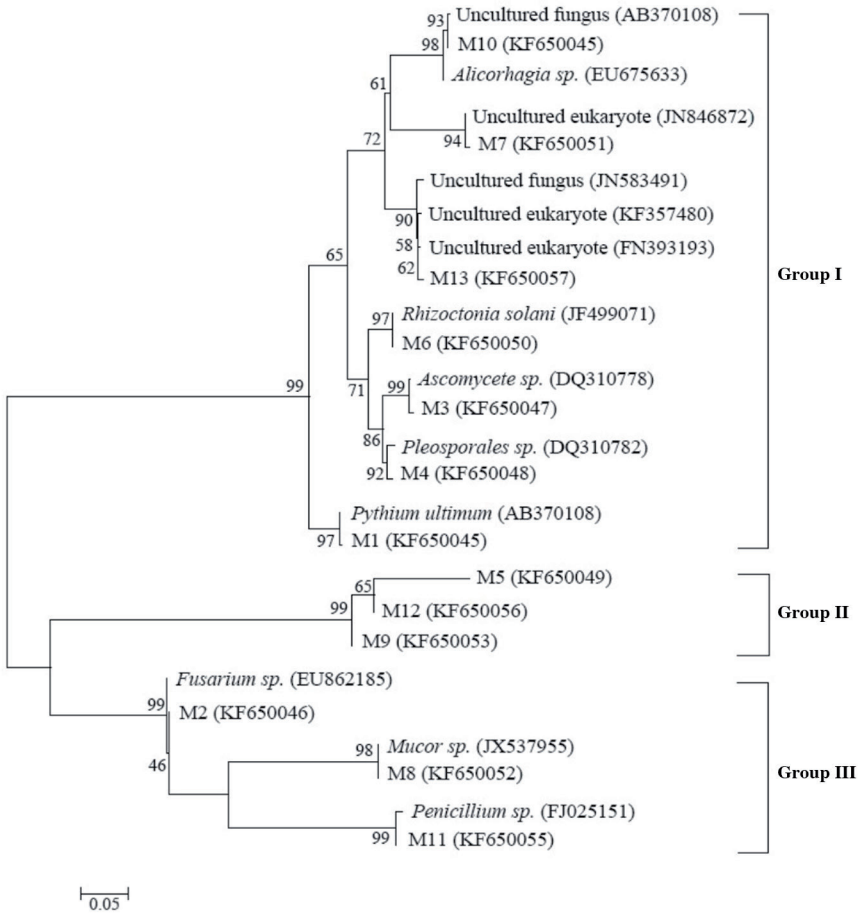


Table 2. Variance analysis of the effects of different continuous cropping durations and plant growth stages on the abundance of root rot pathogenic fungi among various soybean varieties.

Growth period	Sample number	Varieties		
		KF16	HN44	HN48
Seedling stage	CK(R)	7a	7a	7a
	RS1	8b	8b	7a
	RS2	9a	9a	13b
	CK(S)	9b	9b	7a
Branching stage	SS1	11c	10b	9a
	SS2	11b	9a	11b
	CK(R)	7a	8b	7a
	RS1	7a	8b	8b
	RS2	9a	11b	12c
	CK(S)	8a	8a	9b
	SS1	9a	9a	9a
	SS2	11a	11a	11a

Note: Lower-case letters denote significant differences at $p < 0.05$.

Table 3. Variance analysis of the effect of the duration of continuous cropping and plant growth stages on the diversity index of root rot pathogenic fungi among three soybean varieties.

Growth period	Sample number	Varieties		
		KF16	HN44	HN48
Seedling stage	CK(R)	1.51 ± 0.01a	1.98 ± 0.02c	1.63 ± 0.02b
	RS1	1.92 ± 0.02b	2.02 ± 0.02c	1.72 ± 0.02a
	RS2	2.63 ± 0.02a	2.61 ± 0.03a	2.96 ± 0.03b
	CK(S)	1.99 ± 0.01c	1.71 ± 0.01b	1.63 ± 0.02a
	SS1	2.10 ± 0.02a	2.48 ± 0.02c	2.29 ± 0.03b
	SS2	2.52 ± 0.03b	2.01 ± 0.03a	2.73 ± 0.03c
Branching stage	CK(R)	1.79 ± 0.02a	2.36 ± 0.02c	2.11 ± 0.02b
	RS1	2.37 ± 0.02b	2.51 ± 0.02c	2.21 ± 0.02a
	RS2	3.05 ± 0.02a	3.32 ± 0.02b	3.08 ± 0.03a
	CK(S)	2.16 ± 0.01a	2.44 ± 0.02b	2.56 ± 0.02c
	SS1	2.27 ± 0.01a	2.49 ± 0.02b	2.61 ± 0.02c
	SS2	2.70 ± 0.01a	2.76 ± 0.02a	3.08 ± 0.02b

Note: Lower-case letters denote significant differences at $p < 0.05$.

example, at seedling stage RS2, there was no significant difference between KF16 and HN44, while these varieties were significantly different from HN48. The results demonstrate that in soil subjected to 2 yr of continuous cropping, the diversity index of root rot pathogenic fungi in HN48 rhizosphere soil was higher than that of the others during the seedling stage; the structure of root rot pathogenic fungi is complex.

Analysis of the DGGE map and the abundance and diversity indices of the three soybean varieties at the seedling and branching stages revealed that the duration of continuous cropping has a significant influence on the structure of root rot fungal pathogens in rhizosphere soil for three varieties of soybean during different growth periods. We also found that the abundance values and diversity indices of root rot pathogenic fungi were higher after 2 yr of continuous cropping than after 1 yr continuous cropping; the latter values were higher than those of the control group. These results suggest that increasing the duration of continuous cropping is conducive to the growth and infection of root rot pathogenic fungi. Root rot pathogenic fungi existed in the rhizosphere soils of three varieties of soybean in three different years of continuous cropping during different growth stages. The infection was stable, and pathogenic fungi in the rhizosphere soils of each infected soybean variety were not identical. The root rot pathogenic fungi that infected intermediate-type soybean KF16 were *Pythium ultimum*, Pleosporales and three uncultured fungi. Those that infected fatty-type soybean HN44 were Ascomycete, Pleosporales, *P. ultimum*, Pleosporales and two uncultured fungi. Finally, those that infected the high protein-type soybean HN48 were *P. ultimum*, Ascomycete, *Rhizoctonia solani*, and three uncultured fungi. The predominant root rot pathogenic fungi that infected the three varieties of soybean in soil subjected to three different durations of continuous cropping during different growth periods differed: *P. ultimum* were the dominant fungus in KF16 in all three planting years; *Pleosporales* were the dominant fungus in HN44 in all three planting years; *P. ultimum* were the dominant fungus in HN48 in all three planting years.

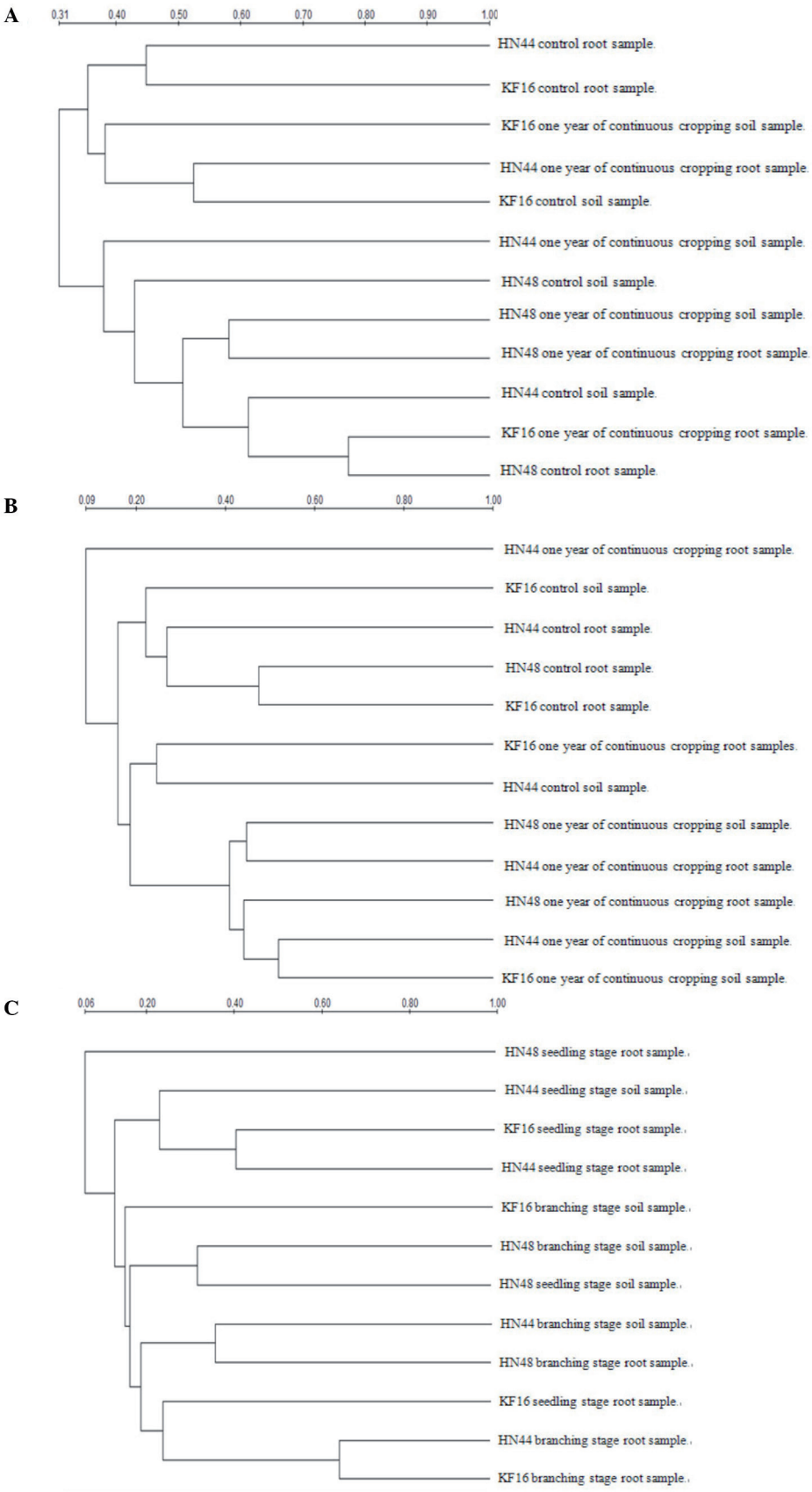
Cluster analysis of the DGGE profiles showed that the pathogens of soybean root rot communities were clustered into eight groups (Figures 6a, 6b, 6c). Group I included the control root samples (HN44 and KF16). Group II included 1 yr of continuous cropping of soil sample (KF16). Group III included 1 yr of continuous cropping root sample (HN44) and control soil sample (KF16). Group IV included 1 yr of continuous cropping soil sample (HN44). Group V included control soil sample (HN48). Group VI included 1 yr of continuous cropping root sample (HN48) and 1 yr of continuous cropping soil sample (HN48). Group VII included control soil sample (HN44). Group VIII included 1 yr of continuous cropping root sample (KF16) and control root sample (HN48) (Figure 5a). Other results were only shown in Figures 5b and 5c, but not explained. The results revealed that the pathogens of soybean root rot community compositions were significantly affected by soybean cultivars, cropping duration and micro-habitats: soil or root. The pathogens of soybean root rot communities are cultivar-specific and dependent on the cropping duration.

DISCUSSION

Effect of continuous cropping of soybean on changes in root rot pathogenic fungi flora

Continuous crop production leads to changes in soil nutrients, soil microorganisms, soil enzyme activity and so on. For example, continuous cropping may cause significant decreases in pH, making the soil switch from neutral to acidic, which favors the growth of fungi (Urashima et al., 2012), inhibits the breeding of bacteria and actinomycete (Helgason et al., 2009; Page et al., 2013) and leads to the dominance of fungi. At the same time, continuous cropping results in the enrichment of root exudates, which also increases the quantity of soil fungi (Suyker and Verma, 2009). These changes are not conducive to the growth of soybean or other crops, resulting in the phenomenon of the continuous cropping obstacle. The rhizosphere soil environment is also

Figure 6. Cluster analysis of DGGE profiles for root and soil samples from different soybean cultivars at the seedling and branching stage.



a) The samples from 1 yr of continuous cropping at the seedling stage. b) The samples from 1 yr of continuous cropping at the branching stage. c) The samples from 2 yr of continuous cropping at the seedling and branching stage.

conducive to the occurrence of root rot disease due to a series of changes caused by continuous cropping.

In this study, the abundance values in different years of continuous cropping were different. From the control group (zero year) to soil subjected to 2 yr of continuous cropping, the microbial population increased with increasing duration of continuous cropping. Throughout the experimental period, some dominant species remained dominant in all rhizosphere samples subjected to different durations of planting. For example, in band M2, the levels of some species at different times were completely different. Moreover, in M1 and M4, some species were absent in the control, while their numbers increased as the duration of continuous cropping increased. These results suggest that continuous cropping is conducive to the growth of pathogens that cause root rot disease (Hati et al., 2007; Zhang et al., 2011). Continuous cropping causes the rhizosphere soil environment to change from the bacterial type (conducive to plant growth) to the fungal type. Therefore, in the soil microflora structure, the proportion of soil-borne pathogenic fungi increases.

However, the band intensity of some fungi weakened with increasing duration of continuous cropping, and some fungi even disappeared. For example, in the control group, band M12 fungi were the dominant species. This band was quite intense, but after 1 yr of continuous cropping, the band intensity weakened, and it disappeared after 2 yr of continuous cropping. This observation may be attributed to the inhibitory effects of long-term continuous cropping on soybean root rot. Indeed, continuous cropping may provide a coevolutionary environment for pathogens and inhibitory microbes (Zhang et al., 2011). Coevolution is the primary generator of biodiversity on our planet, and it is the main source of this type of organism suppression in the soil (Kinkel et al., 2011). Long-term continuous cropping patterns cause the soil itself to be inhibitory, leading to a decrease in the populations of some root rot-producing fungi.

The infection of different soybean genotypes with root rot

In rhizosphere soil harboring different soybean genotypes at different growth stages, the soybean root rot pathogenic fungi significantly differed. At the same growth stage, more root rot pathogenic fungal species infected KF16 than HN48, while the fewest root rot pathogenic fungi infected NH44. For the same soybean genotype, more soybean root rot pathogenic fungi were present in soil harboring plants at the branching stage than at the seedling stage, and pathogenic fungal species in roots were not completely consistent with those in the rhizosphere soil environment; for the same period and the same variety of soybean, there were more soybean root rot pathogenic fungi in the rhizosphere soil environment than in roots.

Previous studies have shown that in root exudates of different soybean varieties, the levels of sugars, amino acids, vitamins, organic acids and other substance differ (Li et al., 2009); the secretion of these substances increases as the plant grows. The amino acid composition of the root exudates of different soybean varieties plays an important role in the generation of pathogen. The degree of pathogen expression is influenced by the type and concentration of amino acids in the rhizosphere; this effect differs among pathogens (Mjoun et al., 2010). This observation indicates that different soybean varieties have different degrees of susceptibility to some types of soybean root rot pathogenic fungi, which also changes during the growth process. Therefore, in order to minimize the harm of the continuous cropping obstacle, it is critical to understand the differences in soybean root rot pathogenic fungi in different soybean root rhizosphere environments as well as specific variations in soybean root rot infection in soybean roots at different growth stages. Only this type of information will help reduce the damaging effects of the continuous cropping obstacle on the production of soybean.

CONCLUSIONS

The results of 13 DGGE bands included that *Pythium ultimum*, *Fusarium*, *Ascomycete*, *Pleosporales*, *Alicorhagia*, *Rhizoctonia solani*, *Mucor*, *Penicillium* and five uncultured fungi. Phylogenetic analysis revealed that the predominant pathogenic root rot fungi were *Pythium ultimum* and *Fusarium*. Contemporary, the five uncultured fungi also had a close genetic relationship with these two pathogenic fungi. Compared with the communities of different year of continuous cropping, different soybean variety and different plant growth stage, the results showed that the duration (years) of continuous cropping, soybean variety and plant growth stages had a significant effect on the DGGE map of pathogenic fungi in rhizosphere soil. The dominant populations and the least dominant populations in all samples differed, suggesting that different continuous cropping durations also had a significant effect on the population structure of pathogenic fungi causing soybean root rot. Therefore, this study could provide the theoretical basis for tillage mode and disease prevention.

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Natural Science Foundation of China (nr 31170466), High-level Personnel Supported Program of Heilongjiang University (ecological restoration team Hdtd 2010-12).

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