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Effects of long term continuous monocropping on community structure and abundance of *Pseudomonas* spp. in cucumber rhizosphere

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ABSTRACT

We grew cucumber in pots in greenhouse for 9-successive cropping cycles and analyzed the rhizosphere *Pseudomonas* spp. community structure and abundance by PCR-denaturing gradient gel electrophoresis and quantitative PCR. Results showed that continuous monocropping changed the cucumber rhizosphere *Pseudomonas* spp. community. The number of DGGE bands, Shannon-Wiener index and Evenness index decreased during the 3rd cropping and thereafter, increased up to the 7th cropping, however, however, afterwards they decreased again. The abundance of *Pseudomonas* spp. increased up to the 5th successive cropping and then decreased gradually. These findings indicated that the structure and abundance of *Pseudomonas* spp. community changed with long-term cucumber monocropping, which might be linked to soil sickness caused by its continuous monocropping.

Key words: Abundance, community structure, Continuous monocropping, Cucumber, *Cucumis sativus* L., DGGE bands, Evenness index, PCR, *Pseudomonas* spp., Rhizosphere Shannon-Wiener index, Soil sickness

INTRODUCTION

Crop monoculture is the cultivation of same crop or plant continuously in the same field year after year (21). It is not sustainable in the long term, as it decreases the crop yield and increases the soil-borne plant pathogens in rhizoshphere soil (3,30,35,39) and this phenomenon is called ‘Soil Sickness’ (39). Many factors induces the soil sickness viz., autotoxicity of root exudates, imbalance of plant nutrients availability and deterioration of soil physico-chemical properties (24,43). Recently, changes in the soil microbial populations have also been proposed to contribute to the soil sickness (18,30). Soil microorganisms maintains the soil health and are affected by the land management practices (7,9,13,22,23,26,29,37). The continuous monocropping leads to disruption of soil microbial community composition and structure (18,19,25). It decreases the bacteria/fungi ratio and thereby imbalances the soil microflora (4,36).

The *Pseudomonas* spp. is ubiquitous in terrestrial ecosystems (3) and is most studied bacterial taxa in soil (8). These bacteria work as xenobiotic degraders (5), plant growth promoters (31) and plant pathogens (34). Besides, they Induces the systemic resistance and directly inhibits the *Fusarium oxysporum* f.sp. *cucumerinum* (20). Recent studies have shown that agricultural management practices could change the community of *Pseudomonas* spp. in plant rhizosphere (8,17,33). However, how the continuous monoculture affects the *Pseudomonas* spp. community is not understood.

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Cucumber (*Cucumis sativus* L), a major vegetable crop world over, is vulnerable to soil sickness (12,41). The continuous monoculture of cucumber is commonly practised in protected cultivation (15). In an earlier study, we found that continuous cropping of cucumber significantly changed the soil fungal communities in cucumber rhizosphere (39). However, the changes in *Pseudomonas* spp. community during continuous cropping of cucumber is still not clear. Therefore, this study aimed to determine the effects of continuous mono-cropping of cucumber on rhizosphere *Pseudomonas* spp. community structure and abundance using PCR-DGGE and qPCR techniques.

MATERIALS AND METHODS

Greenhouse experiment

A greenhouse experiment in plastic pots (30 cm diameter, 25 cm height) was done in the Experiment Station, Northeast Agricultural University, Harbin, China (45°41'N, 126°37'E). Harbin is situated in very cold region; hence, crops can be grown only during the summer season (July-October). Thus, two crops of cucumber were grown i.e., in April-July and July-October (minimum and maximum temperature 15°C and 32°C, respectively) in 2015-2019. The experiment treatments are shown in Table 1.

Table 1. Details of Sowing and sampling dates of 9- crops of cucumber monocultures

Year	Crop	Crop Duration	Sowing Date	Sampling Date
2005	1	April-July	April 25	July 25
	2	July-October	July 25	October 15
2006	3	April-July	April 25	July 25
	4	July-October	July 25	October 15
2007	5	April-July	April 25	July 25
	6	July-October	July 25	October 15
2008	7	April-July	April 25	July 25
	8	July-October	July 25	October 15
2009	9	April-July	April 25	July 25

* New pots were filled with fresh soil upper soil layer (0-15 cm) of fallow field undisturbed for > 15 years).

In the first experiment (i.e., April 25-July 15, 2005), pots were filled with new fresh soil [collected from the upper soil layer (0-15 cm) of fallow field (covered with grass and undisturbed for > 15 years)]. Later on, soils from the previous cropping cycles in the pots was used (no new soil was added), and cucumber was sown again in these pots for all the following cropping cycles. Each pot contained 8 kg soil. One cucumber (cv. ‘Jinlv 3’) seedling was planted per pot. In each cropping cycle, there were 20 pots x 3 Replications = 60 pots, these were kept in the glasshouse in completely randomized design. Fertilizers were added as per local recommendations with decomposed swine manure (15% organic matter, 0.5 % N, 0.5 % P, 0.4 % K) at 750 g per pot. Urea fertilizer was top-dressed at 25 g per pot in 31 days old cucumber plants. The pots were irrigated twice per week with ground water, so that plants did not experience drought stress and there was no standing water in the pots. The soil samples from the 1st, 3rd, 5th, 7th and 9th cropping cycles were collected for the DNA extraction and PCR-DGGE studies.

DNA extraction and PCR-DGGE

Total soil DNA was extracted with an E.Z.N.A. Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA). The community structure of the *Pseudomonas* spp. in soil was determined by PCR-DGGE with the primers as previously described (3).

In DGGE analysis, 6% (w/v) acrylamide gel with 45-65 % denaturant gradient was used for *Pseudomonas* spp. community. The gel was run in 1×TAE (Tris-acetate-EDTA) buffer for 14 h under conditions of 60 °C and 80 V with a DCode universal mutation detection system (Bio-Rad Lab, LA, USA). After the electrophoresis, the gel was stained in 1:3300 (v/v) GelRed (Biotium, USA) nucleic acid staining solution for 20 min. DGGE profiles were photographed with an AlphaImager HP imaging system (Alpha Innotech Crop., CA, USA) under UV light.

Quantitative PCR Assay

Abundance of *Pseudomonas* spp. community was determined by quantitative PCR assays with primer sets of PsF/PsR, as described before (32). The PCR protocol was: 95 °C for 5 min; followed by 30 cycles of 95 °C for 30 s, 65 °C for 30 s, 72 °C for 90 s; and a final extension at 72 °C for 10 min. Standard curves were made with 10-folds dilution series (10^2-10^8) of plasmids containing 16S rRNA genes of *Pseudomonas* spp. from soil samples. Sterile water was used as a negative control to replace the template. All amplifications were done in triplicate. The specificity of the products was confirmed by melting curve analysis and agarose gel electrophoresis. The threshold cycle (*Ct*) values obtained for each sample were compared with standard curve to determine the initial copy number of the target gene.

Statistical Analysis

Data were statistically analyzed by ANOVA using SAS 8.1 software and an average comparison between the treatments was performed based on Tukey's True Significant Difference (HSD) test ($P < 0.05$) at a 0.05 probability level. DGGE bands were analyzed with Bio-Rad Quantity One software (version 4.5). Principal component analysis (PCA) was conducted by using Canoco for Windows 4.5 software (42), and calculated the number of visible bands (S), Shannon-Wiener index (H) and evenness index (E) of DGGE (11).

RESULTS AND DISCUSSION

Pseudomonas spp. community structure

PCR-DGGE analysis showed that the *Pseudomonas* spp. community structures varied in different cropping cycles (Figure 1a). However, no significant differences were found in the number of visible bands (S) in DGGE between the 1st and 3rd cropping cycles. The number of visible bands in the 3rd cropping cycles were less than in the 5th, 7th and 9th cropping cycles. There were new bands in the 7th cropping cycle. The number of visible bands in the 7th cropping cycle were higher than in other cropping cycles (Table 2).

Table 2. The number of visible bands (S), Shannon-Wiener index (H) and Evenness index (E) of *Pseudomonas* spp. in continuously mono-cropped cucumber system

Number of crops	S	H	E
1	16.67±0.58 bc	2.70±0.04 b	0.84±0.01 b
3	15.00±0.00 c	2.47±0.04 c	0.77±0.01 c
5	17.33±0.58 b	2.68±0.06 b	0.83±0.02 b
7	21.33±1.15 a	2.90±0.09 a	0.90±0.03 a
9	18.33±0.58 b	2.74±0.02 b	0.85±0.01 b

S: Visible bands, H: Shannon-Wiener index (H), E: Evenness index

PCA analyses separated the samples of different cropping cycles from each other. The distances between the 7th cropping cycle and the other cropping cycles were greater than the distances among other cropping cycles (Figure 1b).

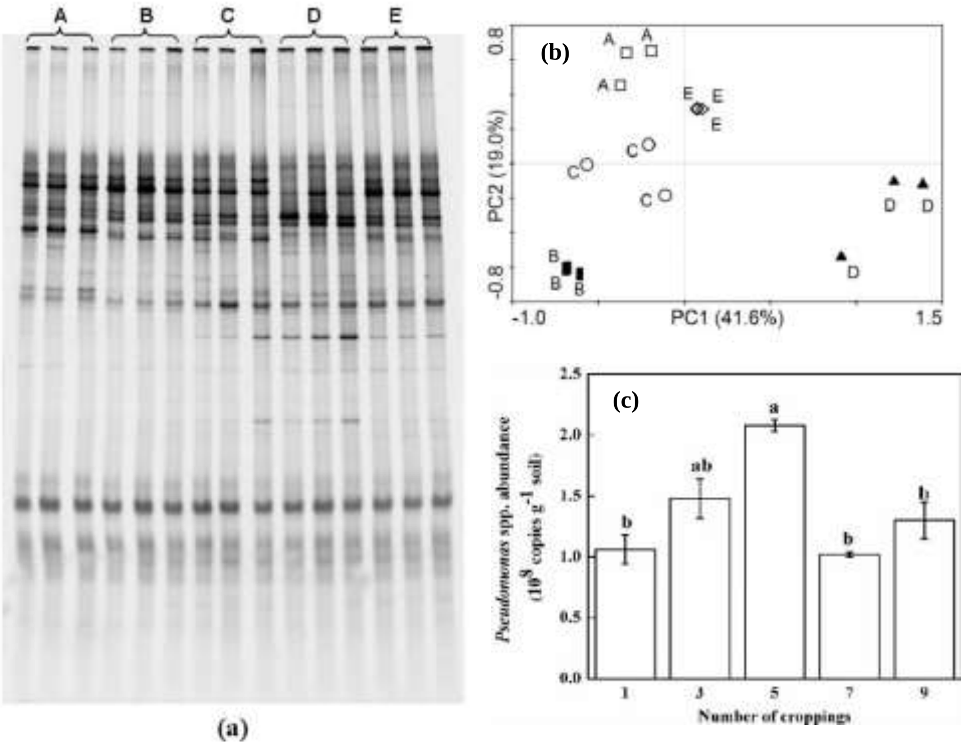

Figure 1. DGGE profile (a), PCA analysis (b) and abundance (c) of *Pseudomonas* spp. community in cucumber continuously monocropped system. A, B, C, D and E represent the first, third, fifth, seventh and ninth cucumber cropping cycle, respectively. Values (mean ± SD) with different letters are significantly different ($P < 0.05$, Tukey's HSD test).

In addition, Shannon-Wiener index (H) and Evenness index (E) of the 7th cropping cycle were higher than other cropping cycles. In contrast, the Shannon-Wiener index (H) and Evenness index (E) of the 3rd cropping cycle were lower than other cropping cycles. There were no significant differences in Shannon-Wiener index (H) and Evenness index (E) in other two treatments (5th and 9th cropping cycles) compared to the 1st cropping cycle (Table 2).

***Pseudomonas* spp. community abundance**

Except in the 3rd cropping cycle, the *Pseudomonas* spp. community abundance in the 5th cropping cycle was higher than other cropping cycles. There was no significant difference in *Pseudomonas* spp. community abundances among the other cropping cycles, except for the *Pseudomonas* spp. community abundance in the 5th cropping cycle (Figure 1c).

Continuous cropping in the same soil, have accumulative effects, which affects the diversity of soil microbe (39). The *Pseudomonas* spp. is major rhizobacteria, that play key role in soil directly by promoting the plant growth and decreasing the plant diseases by inhibiting the plant pathogens and indirectly by inducing the systemic resistance to disease in plants (27,32). In this study, we found that the community structure and abundance of *Pseudomonas* spp. changed in the repeatedly grown mono-cropped cucumber system. PCA analyses separates the samples of different cropping cycles from each other, indicating that the community structures of *Pseudomonas* spp. differed among the cropping cycles. Moreover, our results showed that the Shannon-Wiener index and Evenness index of *Pseudomonas* spp. were lowest in the 3rd cropping and increased in the 7th cropping. However, the abundance of *Pseudomonas* spp. increased with repeated cropping from the 1st to 5th cropping and decreased in 7th cropping. These results agree with the findings of Chen (3), who found that the diversity of *Pseudomonas* spp. significantly declined with the prolonged monoculture. These inconsistencies might be attributed to the differences in the crop investigated and continuous cropping cycles (39).

Cucumber root exudates and plant debris/residues have autotoxicity potential (38). Phenolic compounds have been identified as autotoxins in cucumber exudates and those accumulated in the soil after continuous monocropping of cucumber (2,16,39,40). Phenolic compounds can selectively inhibit or stimulate the soil microorganisms and the stimulatory or inhibitory effects depended on their concentrations (10,14). Therefore, the changes in structure and abundance of cucumber rhizosphere *Pseudomonas* spp. in the continuous cropping system might be due to the accumulation of autotoxins in root zone.

The changes in composition of soil microbial community affects the plant health and growth (28). A previous study showed that cucumber growth was negatively affected in the 7th cropping cycle due to the increased *Fusarium* communities in rhizosphere (39). The *Fusarium* genus is a major pathogen of many plants causing root rot, while, *Pseudomonas* are major antagonists (1,6,20). Therefore, we concluded that the accumulation of autotoxins due to long-term cucumber continuous monocropping decreased the abundance of plant antagonistic *Pseudomonas* spp.

CONCLUSIONS

We found that the community structure and abundance of *Pseudomonas* spp. Were changed in the continuously monocropped cucumber system. PCA analysis showed that the community structure of *Pseudomonas* spp. varied in different cropping cycles. The number of visible bands and Shannon-Weiner and Evenness diversity indices of *Pseudomonas* spp. were lowest in the 3rd cropping, and increased in the 7th cropping. However, the abundance of *Pseudomonas* spp. increased from the 1st to 5th repeated cropping cycles and then decreased gradually. The accumulation of autotoxins due to long-term cucumber continuous monocropping decreased the abundance of plant antagonistic *Pseudomonas* spp. community, which may contribute to the soil sickness associated with continuous cultivation of cucumber.

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