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Pol. J. Environ. Stud. Vol. 30, No. 2 (2021), 1-6
DOI: *10.15244/pjoes/120021* ONLINE PUBLICATION DATE:

Original Research

Changes in Cucumber Rhizosphere *Bacillus* spp. Abundance and Community Structure During Long-Term Continuous Monocropping

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Received: 12 January 2020
Accepted: 2 April 2020

Abstract

Continuous monocropping has been a traditional way of cropping system in most parts of the world. It is important to study its effects on soil microbial communities, which are important element of any agroecosystem functioning and for both plant and soil health. In this study, we used real-time PCR and PCR-denaturing gel electrophoresis (PCR-DGGE) techniques to analyze changes in *Bacillus* spp. abundance, diversity and community structure in continuously monocropped cucumber (*Cucumis sativus* L.) rhizosphere during nine cropping seasons (after every 2 seasons i.e. 1, 3, 5, 7, 9). Results showed that continuous monocropping significantly changed the *Bacillus* spp. abundance, diversity and community structure, and the effect was in course of time. The abundance of *Bacillus* spp. increased up to 5th cropping season and then decreased in following cropping seasons. The number of DGGE bands, Shannon-Wiener Index and Evenness indexes decreased during 3rd cropping season and, after that, increasing trend was observed up to 9th cropping season. These results suggest that the diversity of crop specific *Bacillus* spp. might start increasing but overall abundance of those keep decreasing after a specific period of time during long-term cucumber continuous monocropping.

Keywords: allelopathy, bacterial community, plant-microbe interaction, soil sickness

Introduction

To meet the increasing demand of food for the growing world population, it is necessary to take timely measures through sustainable improvement of agricultural productivity [1]. Sustainable agriculture is not all about producing high output crops in short period of time, but rather developing and maintaining a healthy environment for handsome production [2]. For that, land management practices disproportionately impact on soil functioning and agricultural productivity [3]. Studies have shown that selection of proper cropping system (i.e. intercropping, crop rotation, cover crops) could maintain soil organic C, facilitate biological functioning and improve overall soil physical properties [4-6]. In contrary, when we the grow the same crop on the same piece of land repeatedly, it causes accumulation

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of autotoxins in soil, mostly termed as autotoxicity [7]. Autotoxicity lead to soil sickness by alteration in soil microbial community, increase in soil-born pests and disturbance in soil physical and chemical properties [8, 9]. According to Cesarano et al. [10], soil sickness is induced by plant itself which rises unfavorable conditions for plant vegetative and reproductive growth, ultimately resulting in lower production.

Soil microorganisms are one of the largest biodiversity reservoirs and are an important element of any agroecosystem [11, 12]. They play critical roles in different plant-soil biochemical processes such as mediation of carbon and nutrient cycles in ecosystem [13, 14]. The lost in soil microbial diversity could alter whole functioning and stability of any agroecosystem which has been recently a threat for sustainable agriculture [15, 16]. *Bacillius*, a group of mostly plant beneficial bacterial species, is ubiquitous in agricultural systems and population of this genera occur abundantly in most of the rhizosphere soils [17]. Multiple species of *Bacillus* have been found promoting plant growth by affecting directly i.e. by synthesizing plant hormones, increasing mineral nutrient uptake by fixing atmospheric nitrogen (N) and soil phosphorus (P) mineralization, while other species can produce antibiotic metabolites to suppress plant pathogen [18]. In many areas, the overall yield of cucumber (*Cucumis sativus* L.) has been decreased because of continuous monocropping system [25]. One of the explanations for this could be that continuous monocropping have found to be affecting negatively on rhizosphere *Bacillus* spp. in several crops [8, 9, 19]. However, long-term effects of continuous monocropped cucumber have yet to be disclose.

Since most of the past studies were conducted on single or few monocropping seasons, it would be interesting to investigate long-term effects of cucumber monocropping on rhizosphere *Bacillus* spp. community. To understand the mechanism of changes in certain microbial community, we monitored *Bacillus* spp. community in rhizosphere of continuously monocropped cucumber for nine cropping seasons with the aim to analyze how its abundance, diversity and community structure affected by long-term continuous monocropping. We hypothesized that (1) long-term continuous monocropping will negatively affect *Bacillus* spp. abundance, diversity and community structure, and (2) this effect will be duration dependent.

Materials and Methods

Plant Material and Greenhouse Experiment

Cucumber Jinlv 3, which is a famous cultivar in northeast China, was used in a pot experiment conducted from April 2005 to April 2009 in a greenhouse located in the Horticulture Experimental Station in Northeast Agricultural University, Harbin, China (45º41'N, 126º37'E). Each year, cucumber was cultivated twice (April-July, July-October). Each pot contained 8 kg of soil, which was collected (for first cropping season) from the upper layer of grass covered open field in the same Horticulture Experimental Station, undisturbed for ≥15 years. The soil used in experiment was black soil with sandy loam texture, containing available N: 89.02 mg kg^{-1}; available P: 63.36 mg kg^{-1}; available K: 119.15 mg kg^{-1}; organic matter: 3.67%, and with EC (1:2.5, w/v), 0.33mS cm^{-1}; and pH (1:2.5, w/v), 7.78.

Before germinated in dark at 28ºC, cucumber seeds were sterilized with 2.5% NaClO for 10 minutes and washed thoroughly with distilled water. Germinated seedlings were then transplanted at four true leaves stage to experimental pots (one seedling per pot) on April 25 and July 25 of each year for each cropping seasons. After completion of first experiment, soil was kept in pots to use for following season experiment, up to 9^{th} cropping. Each cropping season contained a total of 60 pots which were placed randomly in the greenhouse. Fertilizers were applied according to traditional recommendation with decomposed swine manure (organic matter: 15%; N: 0.5%; P: 0.5%; K: 0.4%). For top-dressing, urea fertilizer (N: 46%) was used at the rate of 25 g per pot after 30 days of transplantation. Ground water was applied twice a week to maintain proper suitable moisture of 55±2 of water holding capacity and weeds were removed manually.

Sample Collection and DNA Extraction

The soil samples were collected from five plants for each replicate as previously described by Zhou et al. [20] three times (on 30, 40 and 50 days of transplanting). Collected samples were promptly stored at -70ºC for soil microbial DNA extraction. DNA was extracted using the PowerSoil Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). The amount and purity of extracted DNA was estimated spectrophotometrically by measuring the optical density at 260 and 280 nm, and DNA was stored at -20ºC for further analysis.

PCR-DGGE Analysis

The community structure of *Bacillus* spp. was estimated through PCR-DGGE analysis. PCR amplification for *Bacillus* spp. was carried out using nested primer sets BacF/BacR and F338GC/R518 as previously described by Zhou et al. [9]. The PCR conditions were: 95ºC for 5 min followed by 28 cycles of 95ºC for 30s, 56 ºC for 45s, 72ºC for 90s, and final extension at 72ºC for 10 min. For DGGE analysis, 6% (w/v) acrylamide gel with 56-60% denaturing gradient was used. A 1xTAE (Tris-acetate-EDTA) buffer was used for running gel for 14 h under conditions of 60ºC and 80 V with a Dcode universal mutation detection

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system (Bio-Rad Lab, LA, USA). Gel was stained in 1:3300 (v/v) GelRed (Biotium, USA) nuclic acid staining solution promptly after electrophoresis. Finally, an AlphaImager HP imaging system (Alpha Innotech Corp., CA, USA) was used to photograph the DGGE profiles under UV light.

Real-Time PCR Assay

To estimate total abundance of *Bacillus* spp., a real-time PCR assay was carried out using primer set of BacF/BacR as described earlier [9]. The protocol set in the assay was: 95ºC for 5 min; followed by 30 cycles of 95ºC for 30s, 65ºC for 30s and 72ºC for 90s; and finally, an extension at 72ºC for 10 min. A dilution series (10^2-10^8) of plasmid DNA containing *Bacillus* spp. 16S rRNA genes was used to develop a standard curve. For negative control, sterilized water was used to replace the template. Amplification of each replicate was done in triplicate. To confirm the specificity of the product, melting curve analysis and agarose gel electrophoresis were used. The initial copy number of target gene were determined by comparing obtained threshold cycle (*Ct*) value of each sample with standard curve.

Statistical Analysis

For the analysis of DGGE profiles banding patterns, the Quantity One v4.5 (Bio-Rad Lab, LA, USA) was used. To compare the banding pattern between the samples, principle component analysis (PCA) was used with Canoco for Windows 4.5 software (Plant Research International, Wageningen, the Netherlands). The *Bacillus* spp. community diversity parameter including number of bands, Shannon-Wiener and evenness indexes were calculated as described previously by Zhou et al. [20]. Data was analyzed by analysis of variance (ANOVA), and the mean values between treatments for *Bacillus* spp. diversity parameters were compared using Tukey's honestly significant difference (HSD) test at probability level of <0.05.

Results and Discussion

The soil functioning carried out by microbial communities mainly decides the productivity and sustainability of agriculture system [21]. It is well understood now that agricultural practices such as tillage, fertilization and cropping system could

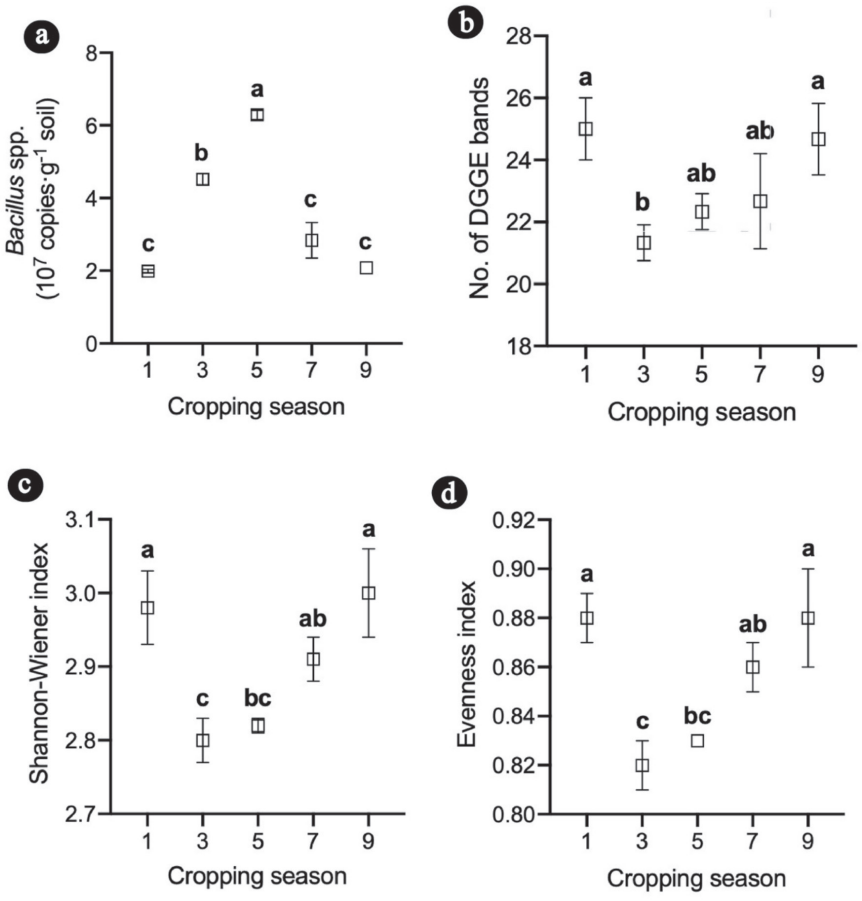

Fig. 1. Changes in Bacillus spp. community abundance a), and diversity parameters including no. of DGGE bands b), Shannon-Wiener c) and evenness d) indexes during different cropping seasons. The values of each season in particular figure labeled with different letter are significantly different (P<0.05; Tukey's HSD test).

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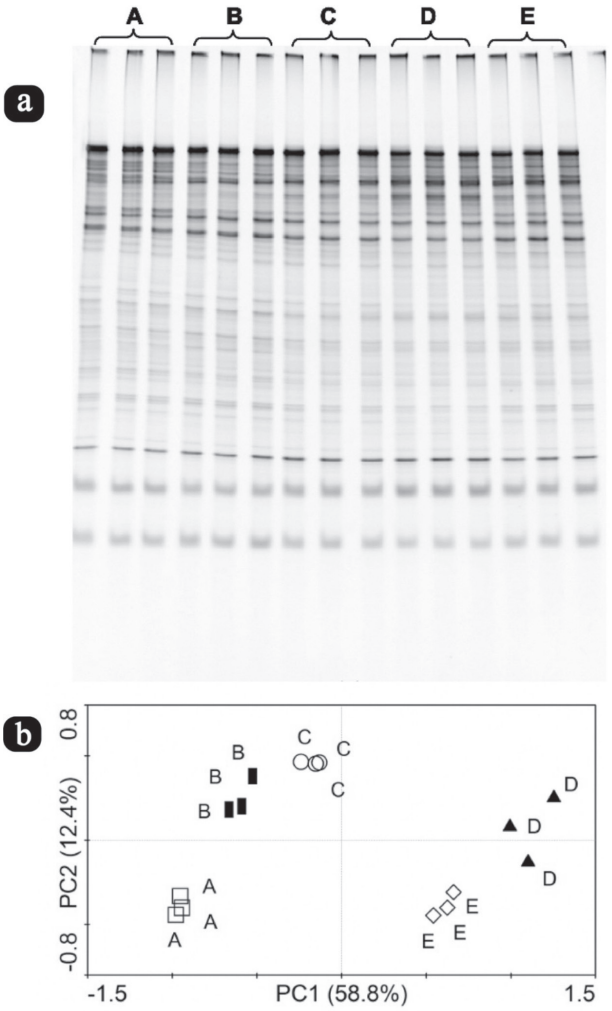

Fig. 2. The DGGE map a) and PCA diagram b) of *Bacillus* spp. community during different cropping seasons. Capital letters in diagrams indicate the no of cropping season as A: 1, B: 3, C: 5, D: 7, E: 9.

significantly influence changes in soil microbial community [20, 22]. In this study, we used real-time PCR and PCR-denaturing gel electrophoresis (PCR-DGGE) techniques to monitor the changes in *Bacillus* spp. community abundance, diversity and structure in cucumber rhizosphere during pot experiments for 9 cropping seasons.

Changes in *Bacillus* spp. Community Abundance and Diversity

Real-time PCR analysis showed a significant difference in *Bacillus* spp. abundance between 1st, 3rd and 5th cropping seasons, while there was no difference found between abundance during season 1, 7 and 9 of continuous monocropping (Fig. 1a). The highest abundance was examined during 5th cropping season followed by 3rd cropping season, while the *Bacillus* spp. community was lowest during 1st, 7th and 9th seasons among all 9 monitored cropping seasons. Our results showed an increasing trend in *Bacillus* spp. abundance in the beginning up to 5th cropping season, however, it decreased gradually to 1st cropping season at the end of experiment. The abundance of *Bacillus* spp. was lowest during first cropping season and reached to the highest value during 5th cropping season among all examined 9 cropping seasons. In contrary, DGGE analysis revealed that continuously monocropping did significantly affect number of bands (Fig. 1b), Shannon-Wiener (Fig. 1c) and evenness (Fig. 1d) indexes of samples from 1st, 3rd and rest of cropping seasons ($P<0.05$). The highest number of bands were found during 1st and 9th followed by 7th and 5th cropping season, respectively, while least were during 3rd cropping season. Almost the same trend was seen in Shannon-Wiener and evenness indexes of *Bacillus* spp. diversity parameters. These results of diversity parameters showed that the diversity of *Bacillus* spp. decreased until 3rd cropping season but increased afterward to 1st cropping season at 9th cropping season.

When same crop is grown again and again on same piece of land, the autotoxins released through root exudates and from plant debris start accumulate in root zone [23, 24]. An increasing number of studies have shown that the accumulation of plant released phenolic compounds due to continuous monocropping and exogenously applied autotoxins can alter the composition of soil microbial communities [25-29] including plant beneficial soil microbial species such as *Bacillus* spp. [30-32]. According to Zhou and Wu [33], vanillic acid significantly reduced abundance and changed community structure of *Bacillus* spp. during cucumber monocropping. Similarly, another autotoxin compound, vanillin, adversely affected *Bacillus* spp. community composition in cucumber rhizosphere soil [31]. Soil assembled autotoxin compounds can also damage plant roots and increase ion leakage rate and affect rhizosphere microbial communities [34]. In this study, the decreasing trend in *Bacillus* spp. abundance after 5th cropping season could be due to increase in higher accumulation of cucumber autotoxins in soil with increase in number of cropping seasons. For example, Li et al. [35] found that the accumulation of autotoxin i.e. *p*-hydroxybenzoic acid, ferulic acid, cinnamic acid and *p*-coumaric acid increased with increase in number of monocropping seasons of strawberry. Concerning the contrary results of *Bacillus* spp. diversity parameters, one can assume that growing same crop consistently without introduction of new crop might increase abundance of crop-specific bacterial strains resulting an increase in *Bacillus* spp. diversity with increase in number of cropping seasons.

Changes in *Bacillus* spp. Community Structure

The DGGE analysis showed an obvious visual difference in banding pattern of *Bacillus* spp. among 9

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cropping seasons (Fig. 2a). PCA analysis showed that continuously monocropped cucumber totally changed *Bacillus* spp. community structure during 9 consecutive cropping seasons as separated samples can be seen in Fig. 2b. PCA plot of DGGE banding pattern of *Bacillus* spp. explained 58.8% and 12.4% in first and 2nd PCA axis, respectively. In particular, the difference between 3rd and 5th cropping season with the rest of cropping seasons was more obvious. It has examined that several factors, such as crop type, cropping system, tillage management and plant growth stage, could significantly affect rhizosphere microbial communities [27, 36]. Among these factors, cropping system is one of the key factors which could potentially affect soil microbial structure [37]. Similarly, in our study, PCA analysis can distinguish *Bacillus* spp. community structure in rhizosphere samples taken from different cropping seasons (Fig. 2b). This might be due to different root exudation pattern, because root exudates are the main derivers for rhizosphere microbial compositions [38]. Another possible reason could be the accumulation of autotoxin in potting soil after regular cucumber monocropping. The phenolic acids are revealed to greatly affect soil bacterial community structure in previous studies [31, 39]. For example, the community structure of *Bacillus* spp. was comprehensively altered by exogenously application of phenolic compounds [33].

Conclusion

Long-term monocropping of cucumber could decrease rhizosphere *Bacillus* spp. community abundance and changing community structure. From these results, we speculate that the accumulation level of autotoxins with long-term and constantly planting cucumber increased which resulted in lowering the abundance and changing in community structure of *Bacillus* spp. with increase in number of cropping seasons. This study has provided us great insights that how long-term continuously monocropping could affect plant beneficial microbial genre such as *Bacillus* spp.

Acknowledgment

This work was supported by the the Natural Science Foundation of Heilongjiang Province (YQ2019C009), National Natural Science Foundation of China (31772361), China Agricultural Research System (CARS-23-B-10), and Longjiang Scholars Program (Q201920).

Conflict of Interest

The authors declare no conflict of interest to disclose.

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