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Effects of syringic acid on *Pseudomonas* and *Bacillus* spp. communities in rhizosphere of cucumber

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ABSTRACT

Soil microbial communities are critical for the productivity of agricultural systems. The effects of phenolic compounds on crops have been extensively studied recently, but less attention has been paid to how these compounds can affect the *Pseudomonas* and *Bacillus* communities in the soil, which are potential antagonistic pathogens and promote plant growth. In our microcosm experiment, soils were treated with different concentrations of syringic acid (a phenolic compound toxic to cucumber). Community structures and abundances of *Pseudomonas* and *Bacillus* species were estimated by PCR-denaturing gradient gel electrophoresis and quantitative PCR. Results showed that the number of DGGE bands, the Shannon-Wiener and Evenness indices of the *Pseudomonas* spp. community in all concentrations of syringic acid treatment were significantly higher than control, suggesting that the addition of syringic acid increased the *Pseudomonas* spp. community diversity. However, syringic acid increased the abundance of *Bacillus* spp. at 0.02 μmol g^{-1} and decreased the diversity and abundance of *Bacillus* spp. community at 0.05-0.2μmol g^{-1}. Overall results showed that *Pseudomonas* and *Bacillus* spp. responded differently to SA and their structure and abundance varied with the concentration of SA in soil.

Keywords: Autotoxicity, *Bacillus* spp., community structure, cucumber, *Cucumis sativus*, quantitative PCR, *Pseudomonas* spp., rhizosphere, syringic acid.

INTRODUCTION

With the progress of society, there is more and more demand for healthy, green and pollution-free vegetables and fruits (10). To meet people's needs, facility/Greenhouse agriculture has become increasingly popular in China in recent years (22). The planting area has been decreasing, which has improved soil utilization efficiency and aggravated the occurrence of continuous cropping problems for facility vegetables (8,12). Allelopathy is a process where a plant inhibits the growth of another plant by releasing toxic compounds (allelopathic substances) into the environment (4), even under normal cultivation and management conditions, which leads to reduced yield (24), poor quality and poor growth and development of crops such as cucumber (18,19). Cucumber is a horticultural crop widely cultivated in protected horticulture (18). During its growth, the root system produces some compounds that affects its own yield, which are called phenolic compounds (14). Previous studies have shown that syringic acid may have some negative effects on

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#Co-first authors, *Correspondence author cucumbers and is a potential autotoxin of cucumbers in hydroponic solutions and soil conditions, affecting the composition and abundance of soil microbial population (14). Soil microorganisms are closely related to plant health and soil fertility (5). The syringic acid can affect the physical and chemical properties of soil and the composition of microorganisms, reducing the composition and abundance of bacteria, such as *Pseudomonas* and *Bacillus* spp. (14,23).

Phenolic compounds are important plant secondary metabolites, which are involved in interactions between plant-to-plant and plant-to-microorganisms (4,6,8). At a certain concentration, it has plant autotoxicity, such as inhibiting nutrients ion absorption, enzyme activity, photosynthesis and respiration of itself or other plants (16). Soil microorganisms play a vital role in terrestrial ecosystems and have a profound impact on soil nutrients cycling and organic matter decomposition (6,8,20). For example, *Pseudomonas* spp. inhibit the production of several pathogenic bacteria and promotes plant growth (6). Some studies have shown that syringic acid is harmful to some beneficial bacteria in the soil (21).

Cucumber is one of the main cultivated plants used for continuous cropping in polyhouse facilities (4). Studies have shown that cucumber under continuous planting produces the phenolic acid named syringic acid, which is selective for soil microorganisms and has an inhibitory effect on beneficial microorganisms (14,21). Earlier reports from our laboratory showed that syringic acid changes the community compositions of bacteria and fungi in the cucumber root zone by reducing the diversity of bacterial species and increasing the abundance and diversity of fungal communities (14). The present study deals with the evaluation of negative effects of syringic acid on the beneficial rhizosphere bacteria and detailed analysis of changes of rhizosphere bacteria in cucumber seedlings in microcosm.

MATERIALS AND METHODS

Microcosm experiment

The soil samples were collected from the upper soil (0-15 cm) of an open field in the Experimental Station, Northeast Agricultural University, Harbin, China (45°41′N, 126°37′E), which was covered with grass and remained undisturbed for more than 15 years. Activities at these locations did not require specific permits, and soil sampling does not involve endangered or protected species. The soil was sandy loam containing, organic matter, 3.67 %; available N, 89.02 mg/kg; available P, 63.36 mg/kg; available K, 119.15 mg/ kg; and EC (1:2.5, w/v), 0.33 mS/cm; and pH (1:2.5, w/v), 7.78 (14).

Pot experiment was conducted from August to October 2019. Cucumber seedlings (cv. Jinlv 3) with two cotyledons were transplanted into pots contained 150 g soil and maintained in a greenhouse (32 °C Day/22 °C night, relative humidity of 60-80 %, 16 h light/8 h dark) (14). No chemical fertilizer was applied during the experiment. As mentioned above, cucumber seedlings were treated with different concentrations of syringic acid (0, 0.02, 0.05, 0.1, 0.2 $\mu mol \cdot g^{-1}$ soil) every two days successively five times at the single-leaf stage (21). Cucumber seedlings treated with distilled water served as the control. The solution was adjusted to pH 7.0 with 0.1 M NaOH solution. There were five pots for each treatment, in triplicate. The soil moisture content was adjusted with distilled water after every two days to keep the soil moisture at about 60 % of its water holding capacity.

Soil sampling and DNA extraction

Ten days after the first syringic acid treatment, cucumber rhizosphere soil was collected from 5 seedlings in each replicate and mixed to make the composite sample as described above. There were three composite samples for each treatment, a total of six composite samples. According to the manufacturer's instructions, the total soil DNA was extracted from 0.25 g soil by using a soil DNA isolation kit (Carlsbadmore Biolabs, USA). Each composite soil sample was extracted three times, the extracted DNA solutions were combined, and there were three composite DNA samples for each treatment (21).

PCR-DGGE analysis

The community structure of rhizosphere bacteria was analyzed by PCR-DGGE method. The 16S rDNA fragments of *Pseudomonas* and *Bacillus* spp. were amplified by the semi-nested polymerase chain reaction. As mentioned earlier, the primer sets used for the first and second rounds of PCR amplification of *Pseudomonas* spp. were PsF/PsR and GC-338F/518R (2); while for *Bacillus* spp., BacF/BacR and GC-338F/518R (4) were used. The PCR protocol was 95 °C for 5 min, followed by 28 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 90 s, and finally at 72 °C for 10 min. DGGE analysis was performed on a 6 % (w/v) acrylamide gel with a 45-65 % denaturation gradient (3,13). The gel was run, stained and photographed as described previously (3).

Quantitative PCR assay

As mentioned earlier (3,4), the abundance of *Pseudomonas* and *Bacillus* spp. was estimated by quantitative PCR analysis using the PsF/PsR (2) and BacF/BacR (4) primer sets, respectively. A 10-fold dilution series (10^2-10^8) of plasmids containing 16S rRNA genes of *Pseudomonas* and *Bacillus* spp. from soil samples were used to prepare a standard curve (1). Sterile water was used as a negative control to replace the template. All amplifications were made in triplicate. The specificity of the product was confirmed by dissolution curve analysis and agarose gel electrophoresis. The threshold cycle (Ct) value obtained for each sample was compared with the standard curve and expressed as a percentage of the abundance in the soil of control.

Statistical analysis

The banding patterns of the DGGE profiles were analyzed using Quantity One V4.5 (14). Principal component analysis (PCA) is used to compare band patterns and standardized data between samples of different concentrations, using Canoco for Windows 4.5 software. The rhizosphere bacterial community diversity index was calculated as described above, including the number of bands, Shannon-Wiener index, and evenness index. The data was analyzed using the analysis of variance, and there were significant differences between the treatments.

RESULTS AND DISCUSSION

***Bacillus* and *Pseudomonas* spp. Community Structures**

The DGGE map of *Pseudomonas* spp. showed that the three bands of each treatment were roughly similar (Fig. 1a). The principal component analysis diagram of the *Pseudomonas* spp. DGGE diagram clearly separated the different treatments (Fig. 1b). The Shannon-wiener index also confirmed that the *Pseudomonas* spp. communities had significant differences among different treatment concentrations (R= 0.999, P= 0.001). The principal component analysis diagram of the *Pseudomonas* spp. DGGE diagram showed that PC1 and PC2 together accounted for 61.1% of diversity. In the principal component analysis figure, the scatterings of syringic acid treatment at 0.02, 0.05 and 0.1 μmol·g^{-1} clustered together, indicating that syringic acid treatment at 0.02, 0.05 and 0.1 μmol·g^{-1} had similar effects on *Pseudomonas* spp. in the soil rhizosphere (Fig. 1).

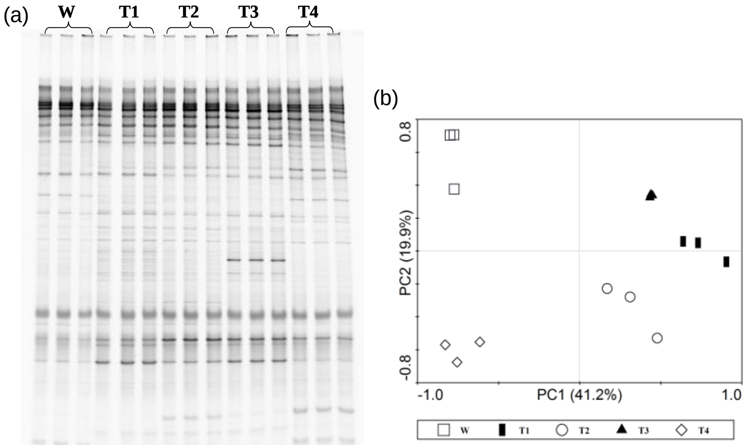

Figure. 1. DGGE map of *Pseudomonas* spp. community treated with syringic acid (a) and principal component analysis map (b). Different concentrations of syringic acid: T1: 0.02μmol·g^{-1}, T2: 0.05μmol·g^{-1}, T3:0.1μmol·g^{-1}, T4:0.2μmol·g^{-1}, W: Water

After application of syringic acid, the DGGE chart of *Bacillus* spp. showed that there were significant differences in the bands between different treatments (Fig. 2). All treatments showed disappearance of certain bands (Fig. 2a). Principal component analysis also could not clearly separate the five treatments from each other (Fig. 2b). Quantitative PCR analysis showed that T1 at 0.02 μmol·g^{-1} concentration increased the abundance of *Bacillus* spp., while T2, T3 and T4 were successively lower than the control, indicating that the abundance of *Bacillus* increased at 0.02 μmol·g^{-1}, and gradually decreased with the increase in concentration. Therefore, SA at concentration ≥ 0.05 μmol g^{-1} soil inhibited the abundance of *Bacillus* spp. in rhizosphere of cucumber seedling (Fig. 2).

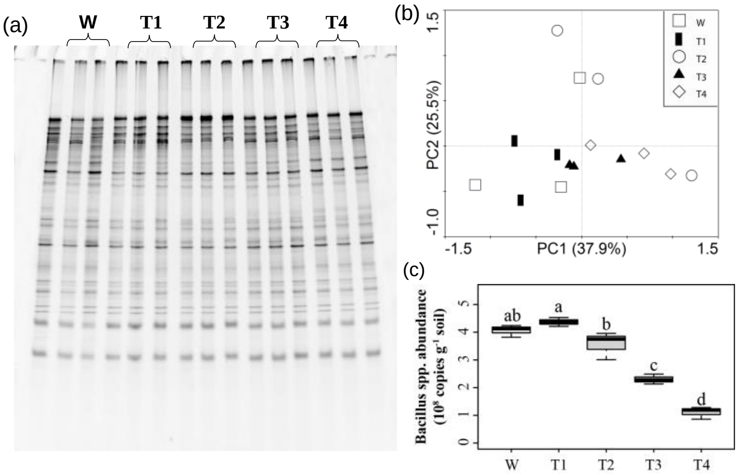

Figure. 2. DGGE map of Bacillus community treated with syringic acid (a) and its principal component analysis diagram (b), quantitative PCR analysis (c).

Table 1. The number of DGGE spectrum bands (S), Shannon-Wiener index (H) and Evenness index (E)

Syringic acid concentration (μmol·g^{-1} soil)	*Pseudomonas* spp.			*Bacillus* spp.		
	S	*H*	*E*	*S*	*H*	*E*
0	21.67±0.58 c	2.87±0.04 c	0.79±0.01 c	23.33±0.58 a	3.00±0.01 a	0.90±0.01 a
0.02	27.67±0.58 a	3.14±0.02 a	0.87±0.01 a	22.00±1.00 a	2.87±0.08 a	0.86±0.03 a
0.05	25.00±0.00 b	3.01±0.01 b	0.83±0.00 b	22.00±1.00 a	2.87±0.05 a	0.86±0.02 a
0.1	24.33±1.15 b	3.00±0.05 b	0.83±0.01 b	23.00±1.00 a	2.99±0.05 a	0.90±0.01 a
0.2	23.67±1.53 bc	2.92±0.06 bc	0.81±0.02 bc	22.33±0.58 a	2.95±0.02 a	0.88±0.01 a

Different concentrations of syringic acid changed cucumber seedling rhizosphere *Pseudomonas* and *Bacillus* spp. community diversity to varying degrees (Table1). All concentrations of syringic acid (0.02, 0.05, 0.1, 0.2 μmol g^{-1} soil) significantly increased the number of DGGE spectrum bands, Shannon-Wiener index and evenness index of the *Pseudomonas* spp. community (P <0.05). When the concentration of syringic acid was 0.2 μmol g^{-1} soil, the number of DGGE spectrum bands and Shannon-Wiener index increased most significantly, but with increasing SA concentration the changes in DDGE banding pattern were less significant. This indicated that syringic acid concentration at 0.02 μmol g^{-1} soil significantly increased the diversity of cucumber rhizosphere *Pseudomonas*, and the increase was less with the increase of concentration between 0.05 and 0.2 μmol g^{-1} soil. However, the number of DGGE spectrum bands, Shannon-Wiener index of the *Bacillus* spp. were decreased by different concentrations of syringic acid (P <0.05).

***Bacillus* spp. Community Abundance**

Quantitative PCR analysis showed that T1 at 0.02 μmol·g^{-1} concentration increased the abundance of *Bacillus* spp., while T2, T3 and T4 were successively lower than the control, indicating that the abundance of *Bacillus* increased at 0.02 μmol·g^{-1}, and gradually decreased with the increase in concentration. Therefore, SA at concentration ≥ 0.05 μmol g^{-1} soil inhibited the abundance of *Bacillus* spp. in rhizosphere of cucumber seedling (Fig. 2).

This study aimed to determine the effects of syringic acid on the cucumber seedling rhizosphere bacteria, *Pseudomonas* and *Bacillus* spp. Syringic acid is a self-toxic phenolic compound (21). As mentioned earlier, we found that the concentration of soil syringic acid in the cucumber monoculture system ranged from 0.1- 0.15 μmol·g^{-1} (21). In both natural and agricultural ecosystems, the concentration of soil phenolic acids ranged from 0.01 to 0.5 μmol g^{-1} soil (14). Therefore, concentrations of syringic acid used in this study (0.02 to 0.2 μmol g^{-1} soil) might be within the realistic range of concentrations in the soil reported before.

Different genera of bacteria responded differently to various concentrations of syringic acid. Studies have shown that agricultural intensification will have a negative impact on *Pseudomonas* and *Bacillus* spp. in the soil. For example, continuous cropping of *P. sylvestris* changed the community structure and reduced the abundance of soil *Pseudomonas* spp. (2), and continuous cropping of Jerusalem artichoke changed the diversity of *Pseudomonas* and *Bacillus* spp. in the soil (3).

After continuous cultivation of the same crop (cucumber) in the same field, the crop usually shows decreased growth and vitality and an increase in disease index (11,15). PCR-denaturing gradient gel electrophoresis found that the diversity of *Pseudomonas* spp. increased, but for *Bacillus* spp. a decrease was observed after application of SA. Quantitative PCR showed that the abundance of cucumber rhizosphere *Bacillus* spp. decreased when SA concentration of ≥ 0.05 μmol. g^{-1}. In this study, we found that low concentrations (0.02 to 0.1 μmol g^{-1} soil) of SA increased the diversity of *Pseudomonas* spp., but at higher SA concentrations, there was less increase in diversity of *Pseudomonas* spp. This may indicate that low concentration of SA promoted the diversity of *Pseudomonas*, while high concentration inhibited it. Wang *et al.* (14) found that SA inhibited *Pseudomonas* spp., which are inhibitory to plant pathogens and soil-borne diseases. Hence, the application of SA may increase the species of *Pseudomonas* but inhibit its population. *Pseudomonas* spp. promotes plant growth through phosphate solubilization, production of phytohormones and volatile growth stimulants (7,9,16). *Bacillus* spp. secretes antibacterial substances to produce antagonistic effects, induces host resistance, and help in dissolving organophosphorus in soil and promote plant growth (6,17,25). Therefore, SA application at low concentration may indirectly affect plant growth by altering the composition and abundance of rhizosphere *Pseudomonas* and *Bacillus* spp. in the continuous cropping system.

CONCLUSIONS

The different concentrations of syringic acid changed the composition and abundance of cucumber rhizosphere *Pseudomonas* and *Bacillus* spp. to varying degrees. The results showed that SA at concentration of 0.02- 0.2 μmol g^{-1} soil increased the diversity of *Pseudomonas* spp. and decreased that of *Bacillus* spp. Quantitative PCR analysis showed that SA increased the abundance of *Bacillus* spp. community when used at 0.02 μmol g^{-1} concentration soil but decreased as the concentrations of SA increased from 0.05 to 0.2 μmol g^{-1} soil.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. All authors agree to publish it.

DECLARATION

We declare that all authors of this Ms. have made substantial contributions. We did not exclude any author who substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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PUBLISHER NOTE