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Effects of Long-term Application of Fertilizers on Microbial Community Structure and Diversity of Tomato Rhizosphere Soil

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Excessive use of chemical fertilizer, unbalanced application of nitrogen, phosphorus and potassium, usually lead to lower utilization of soil fertilizer, decline in the quality of the ecological environment, serious pests and diseases, decrease yield and quality of vegetables. Soil microbiological characteristic is an important characterization of soil quality and can reflect the soil fertility condition. To investigate the effect of fertilizer on soil microorganism, tomato (*Lycopersicon esculentum* L. Liaoyuanduoli) was used as material, an experimental vegetable field which had 20 years of cultivation history and had been under long-term localized fertilization was used as a platform to analyse the soil microbiological characteristics after different fertilization treatments using PCR-DGGE technology. The results showed that the organic fertilizer application could improve the soil microbial quantity, increase the number of dominant species, increase the dominant population number, stable community structure. Excessive use of nitrogen fertilizer could change the species of soil bacteria dominant populations. Different kinds of chemical fertilizer or fertilizer combinations had no effects on soil microorganism community structure.

Key words: Tomato; Fertilizer; Microbial community; Structure and diversity; Rhizosphere soil.

Fertilization is one of the most profound agricultural practices which affect the quality and sustainability of soils. It has notable impacts on soil structure, organic fertility and productivity (Agbede *et al*., 2010; Hartley *et al*., 2013). Excessive fertilization and the use of straight fertilizers are common issues in greenhouse vegetable cultivation, resulting in problems such as a deteriorated soil condition in greenhouses and decreased product quality. Therefore, studying the changes in the soil condition and the physiological function and nutritional properties of soils under different fertilization regimes are important.

Accumulating evidence suggests that the biological properties of soil be more sensitive to changes in the soil matrix caused by human disturbances, such as fertilization (John *et al*., 2000; Yamaguchi *et al*., 2009). In recent years, using the composition of soil microbial community structure,

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soil microbial biomass, soil enzyme activity, and other features as biological indicators of soil health to guide the soil ecosystem management has received much attention (Bossio *et al*., 2005; Aghababaei *et al*., 2014). Lower than 1% of microbial species in nature can be separated, and most of the microorganisms that "can survive but cannot be cultured" cannot be divided and identified (Amann *et al*., 1995). Therefore, the conventional plate culture method has its limitations in the study of soil microbial diversity and will result in the loss of microbial diversity information, affecting the accuracy of the results. The development of molecular biology techniques enables soil ecology researchers to understand the soil microbial community structure and diversity that reflect the soil quality at the molecular level and to analyze their changes under different conditions. Tipayno *et al*. (2012) applied terminal restriction fragment length polymorphism (T-RFLP) and RT-PCR to study the impact of red soil slope utilization on soil bacterial community structure. Zhan *et al*. (2011) studied the effects of different climate, soil, and growing conditions on the diversity of nitrogen-fixing microorganisms using denaturing gradient gel electrophoresis (DGGE) and achieved their desired results. In recent years, there have been many studies on the effects of long-term localized fertilization on soil microbial activities. However, most of these studies have focused on soils in open fields. Studies on the effects of long-term localized fertilization on the activities of soil microorganisms are rarely reported. In particular, no study has used molecular biology techniques to study the effects of long-term localized fertilization on soil microbial characteristics in greenhouse vegetable cultivation.

Therefore, in this study, an experimental vegetable field which had 20 years of cultivation history and had been under long-term localized fertilization was used to study the effects of different fertilization treatments on soil microbial community structure and diversity using PCR-DGGE. The results can provide a scientific basis for establishing rational fertilization regimes, improving the biological and chemical environment of soils, and achieving sustainable utilization of soils.

MATERIALS AND METHODS

Experiment design and materials

The experiment soils were taken from the experimental vegetable field, which had 20 years of cultivation history and had been under long-term localized fertilization. The basic physical and chemical properties of these soils before the experiment were as follows: organic matter content, 24.30 g/kg, total nitrogen content, 1.164 g/kg, total phosphorous content 1.374 g/kg, alkaline hydrolysis nitrogen content, 86.41 g/kg, available phosphorous content, 70.8 g/kg, available potassium content, 56.14 mg/kg. The pH of soil is 6.75.

It was conducted an open-field cultivation experiment to simulate the experimental design of two nitrogen-dominated fertilizers (organic fertilizer and inorganic nitrogen) at three levels twenty years ago. The three levels at which organic fertilizers (manure) were applied were 0 $kg \cdot hm^{-2} \cdot a^{-1}$, 37.5 $kg \cdot hm^{-2} \cdot a^{-1}$, and 75 $kg \cdot hm^{-2} \cdot a^{-1}$. The three levels at which inorganic nitrogen (urea) was applied were 300 $kg \cdot hm^{-2} \cdot a^{-1}$, 600 $kg \cdot hm^{-2} \cdot a^{-1}$, and 900 $kg \cdot hm^{-2} \cdot a^{-1}$. After five experimental runs, the application of inorganic nitrogen (urea) was reduced to 0 $kg \cdot hm^{-2} \cdot a^{-1}$, 300 $kg \cdot hm^{-2} \cdot a^{-1}$, and 600 $kg \cdot hm^{-2} \cdot a^{-1}$. There were nine treatments, the plot area was 16 m^2, and there were three replicates arrayed randomly. There were two growing seasons each year for the field experiment, rotating between eight types of vegetables. The order of cultivation was Chinese cabbage, beans, carrots, onions, cucumbers, potatoes, leafy mustard, and sweet pepper. Since ten years ago, the experimental design was adjusted. The experiment was divided into two parts. The first part retained the six treatments in the open field. It included three inorganic nitrogen treatments with a large amount of organic fertilizer (75 $kg \cdot hm^{-2} \cdot a^{-1}$, labeled M), and three inorganic nitrogen treatments without applying organic fertilizer. It was named M (only organic fertilizer), MN1 (organic fertilizer + a small amount of inorganic nitrogen), MN2 (organic fertilizer + a large amount of inorganic nitrogen), CK (no fertilizer), N1 (a small amount of inorganic nitrogen fertilizer applied) and N2 (a large amount of inorganic nitrogen fertilizer applied). The amounts of organic manure and inorganic nitrogen applied to the plot were calculated from the

application rate used for fields and the area of the plot. The samples of the 12 treatments in the second part were prepared by applying phosphate fertilizer (superphosphate) and potassium fertilizer (potassium sulfate), respectively, to samples collected from the four treatment groups of field experiments: M, CK, MN1, and N1. The fertilization combinations were represented by the following symbols: MP, MK, MPK, P, K, PK, MNP, MNK, MNPK, NP, NK and NPK. All treatments had the same amount of fertilizer. The specific application rates of the various fertilizers are as shown in Table 1. There were a total of 18 treatments and three replicates for each treatment. There were 54 plots, arranged randomly. To limite infiltration of fertilizers between different treatments, a bottomless cement tank with 1.5m long, 1m wide and 0.8m deep was built in each plot. The entire experiment was conducted inside greenhouses that were always covered with plastic film. Organic fertilizer and phosphorous/potassium fertilizers were applied at one time before transplanting. Inorganic nitrogen (urea) fertilizer dressing was conducted twice during the growing season. There was only one long growing season per year inside the greenhouse. The vegetables grew inside the greenhouse since 1997 were eggplant (two growths), tomato (five growths), cucumber (one growth), sweet pepper (two growths), and tomato (one growth). Tomato seedlings were transplanted onto the plot with a plant spacing of 50 × 35 cm, and eight plants were planted in each plot and followed with regular pruning control.

The soil samples were collected after harvesting tomatoes (*Lycopersicon esculentum* L. Liaoyuanduoli). Topsoils were collected at 0 to 20 cm using the five-point sampling method. Gravels and plant residues were removed and then mixed to prepare samples. Soil samples were passed through a 2-mm sieve, placed into a sterile bag, quick-frozen in liquid nitrogen, and stored in a freezer at -80°C for later microbial DNA extraction.

Determination methods

Extraction and purification of microbial genomic DNA

Soils (0.5 g) and sterilized quartz sands (0.5 g) were weighed and placed in the nucleic acid extraction tube, and then 850 µl of extraction buffer (100 mmol/L Tris-HCl, pH 8.0; 100 mmol/L sodium EDTA, pH 8.0; 100 mmol/L sodium phosphate, pH 8.0; 1.5 mol/L NaCl; 1% CTAB) was added. The solution was vortexed for 1 min and then shaken for 30 s in a nucleic acid extractor. Later, 50 µl of lysozyme (100 mg/ml), 50 µl of snail enzyme (120 mg/ml), and 50 µl of cellulase (120 mg/ml) were added. The samples were immersed in water at 37°C and shaken for 1 h. 100 µl of 20% SDS was added and mixed, then the tube was kept at 65°C for 30 min with shaking every 10 min, then centrifuged at 12,000 r/min for 10 min. The supernatant was taken and added to 0.2 volume of KAc (8 mol/L) was added, and after 20 min in an ice bath, the content was centrifuged at 14,000 r/min for 20 min. The supernatant was taken and added to 0.5 volume of PEG (20%) and 0.1 volume of NaCl (5 mol/L). It was mixed well and incubated at room temperature for 1 h, then centrifuged for 1 h. The supernatant was discarded, and the precipitate was thoroughly washed with 500 µl of precooled 70% ethanol and centrifuged at 14,000 r/min for 10 min. After the ethanol was completely evaporated at room temperature, 700 µl of TE buffer and an equal volume of phenol/chloroform/ isoamyl alcohol (25:24:1) was added, mixed well, and centrifuged at 12,000 r/min for 10 min. The aqueous phase was taken, and an equal volume of chloroform/isoamyl alcohol (24:1) was added to extract one more time by centrifugation at 12,000 r/min for 10 min. The aqueous supernatant was taken and added to 0.1 volume of NaAc (3 mol/L) and 0.06 volume of pre-cooled isopropanol. These were mixed well and incubated at room temperature for 1 h, then centrifuged at 14,000 r/min for 10 min. The supernatant was discarded. The precipitate was thoroughly washed with 500 µl of pre-cooled 70% ethanol and then dissolved in 50 µl of TE to obtain a crude DNA extract. The crude DNA extracts were purified with the B-type small DNA fragment rapid purification kit from BioDev and then stored at -20°C. The integrity of DNA was determined using 0.8% agarose gel, with Hind III digest (bought from Takara) as the marker.

PCR amplification of DNA samples

The universal bacterial primer 341f-GC/ U758 (Phillips *et al*., 2008) was used for the PCR amplification of total microbial DNA. The sequence of the primer is 341f (5'-3') CCTACGGGAGGCAGCAG and U758 (5'-3') CTACCAGGGTATCTA ATCC. The amplified fragment was 417 bp. A GC clamp was added to the

5' end of primer 341f to prevent premature melting of the DNA fragments during denaturing gradient gel electrophoresis. The PCR conditions were pre-heating at 94°C for 4 min, followed by 35 cycles of melting at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 45 s. Each cycle was extended for one second compared with the previous cycle. The final extension was at 72°C for 10 minutes.

The total PCR mixture consisted of 1 µl of DNA template, 5 µl of PCR buffer (10×), 4 µl of dNTP mixture (2.5 mmol/L), 1 µl of each primer (20 pmol/L), 0.4 µl of *Taq* DNA polymerase (5 U/µl), and sterile deionized water to a total volume of 50 µl. The amplification product was examined by electrophoresis in 1.5% agarose gel with a DL2000 DNA Marker (bought from Takara).

DGEE analysis of PCR product

The D-Code system (Bio-Rad Laboratories Inc., Hercules, CA, USA), 6% polyacrylamide gels and a denaturing gradient of 30-60% (100% is equivalent to 7 mol/L urea and 40% deionized formamide) were used in DGGE. PCR products (40 µl) were added to 1× TAE buffer (40 mmol/L Tris, 20 mmol/L acetic acid, and 1 mmol/L Na_2-EDTA) and kept at 60°C, then electrophoresed at 200 V for 5 h. After the electrophoresis, the gel was stained with Genefinder dye (10,000-fold dilution with 1× TAE) for 40 min and then photographed using a Bio-Rad gel imaging system.

Data analysis

The DGGE profiles were analyzed using image analysis software Quantity One (4.3.1). The number of electrophoretic bands was used to represent the richness of bacterial communities (S); digital DGGE profiles can be used to calculate the diversity index (H) and evenness (E) of bacterial communities in soil samples. The Shannon-Wiener diversity index is H =-£ (Pi) ln (Pi), where Pi = ni / N, ni is the peak intensity of the band, and N is the sum of the peak intensities of all the bands. The Pielou index is E = H / Hmax, where H is the diversity index, Hmax is the largest bacterial diversity index, Hmax = lnS, and S is the total number of bacteria in the community. Cluster analysis was conducted on soil samples using a non-weighted pair group method with arithmetic mean (UPGMA) to form a dendrogram.

RESULTS

Soil microbial genomic DNA extraction and PCR amplification

The DNA fragments extracted from the soil samples had lengths of approximately 23 kb (Fig. 1). The purity and integrity of the bands were

Table 1. Amount of fertilization in different treatments

Treatment	Manure ($kg \cdot plot^{-1}$)	Urea ($g \cdot plot^{-1}$)	Calcium supper phosphate ($g \cdot plot^{-1}$)	Potassium sulphate ($g \cdot plot^{-1}$)
CK	0	0	0	0
N1	0	97.8	0	0
N2	0	195.6	0	0
P	0	0	720	0
K	0	0	0	53.79
PK	0	0	720	53.79
NP	0	97.8	720	0
NK	0	97.8	0	53.79
NPK	0	97.8	720	53.79
M	11.25	0	0	0
MN1	11.25	97.8	0	0
MN2	11.25	195.6	0	0
MP	11.25	0	720	0
MK	11.25	0	0	53.79
MPK	11.25	0	720	53.79
MNP	11.25	97.8	720	0
MNK	11.25	97.8	0	53.79
MNPK	11.25	97.8	720	53.79

Table 2. Diversity of soil bacterial community of fertilizer treatments

Treatment	Richness index	Shannon-wiener index	Pielou evenness index
CK	20	2.574	0.859
N1	22	2.535	0.820
N2	13	1.993	0.777
K	19	2.31	0.785
P	17	2.095	0.740
PK	15	2.211	0.817
NK	19	2.467	0.838
NP	16	2.264	0.816
NPK	21	2.671	0.877

Table 3. Diversity of soil bacterial community of organic fertilizer treatments

Treatment	Richness index	Shannon-wiener index	Pielou evenness index
M	19	2.938	0.998
MN1	21	3.035	0.997
MN2	19	2.941	0.999
MK	16	2.772	1.000
MP	19	2.940	0.999
MPK	13	2.565	1.000
MNK	19	2.933	0.996
MNP	20	2.990	0.998
MNPK	23	3.174	1.012

good. However, the microbial DNA concentrations differed among different treatments. This variance may have been due to the difference in the number of microorganisms in the samples, which only had a small impact on the PCR amplifications. In the following step, these DNAs were used as templates.

The gel electrophoresis picture of the amplification products of the bacterial V3-V4 regions after the PCR amplification of the microbial DNAs from soils under different fertilization treatments was showed in Fig. 2. The amplification product bands of various treatments were sharp and bright and showed no non-specific amplification. The size of the amplified product fragment was approximately 400 bp, similar to the length of target fragment. Therefore, these products were used for the DGGE experiment.

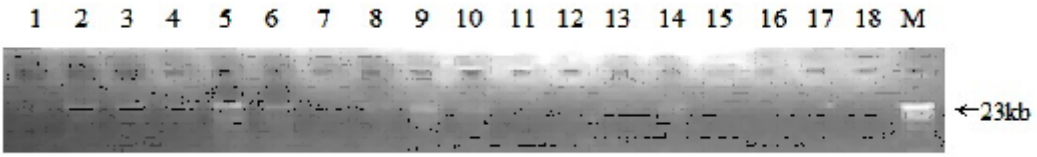

Fig. 1. Agarose gel (0.8%) electrophoresis of microbial DNA extracted from soil

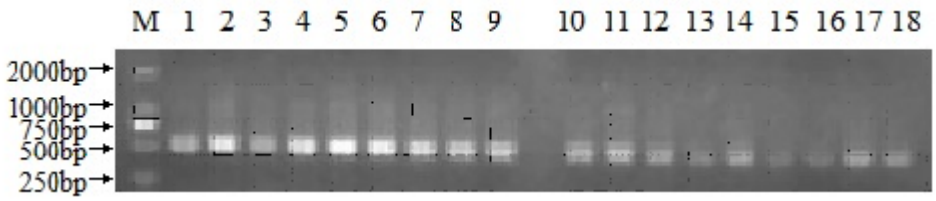

Fig. 2. Agarose gel (1.5%) electrophoresis of PCR amplification products

Analysis of bacterial community structure and diversity in soils under long-term chemical fertilizer application

The PCR-DGGE profile of bacterial 16S rDNA of the control soil and soils under long-term chemical fertilizer-only treatment was showed in Fig. 3. The figure shows little difference in the number of bands or the migration position of each band between lanes. However, there were some brightness differences between some bands within the lanes. All lanes had bands b, c, and d, and band c was brighter than band b and d in each treatment. It indicated that the bacterial community described by band c was the dominant group. Band a was present in lanes N2, NK, and NPK at the same site and was brightest in the NPK lane. Band e was only present in the lines of control and N2 treatment and was brighter in the control lane. Band f was only present in lanes N1 and N2, indicating that the bacteria represented by bands b and f were only present when applying straight nitrogen alone.

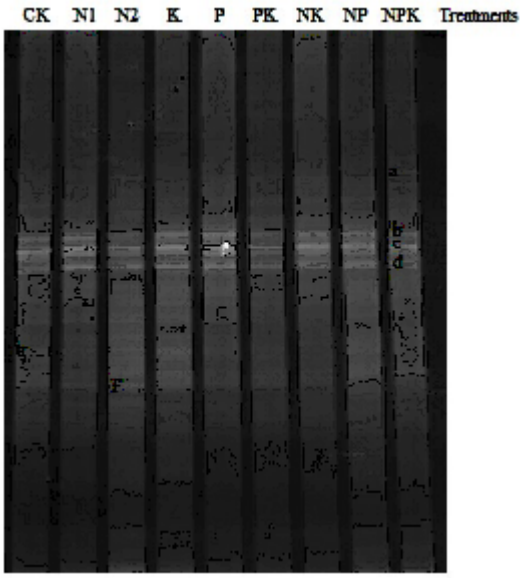

Fig. 3. Denaturing gradient gel electrophoresis of bacterial 16S rDNA PCR amplification products of fertilizer treatments

A total of 26 band patterns existed in the entire DGGE profile, and the number of bands in each lane was 13 to 22. Lane N1 had the greatest number of bands, 22; lane N2 had the smallest number of bands, 13 (Table 2). This table lists the diversity indices and Pielou evenness indices of the bacterial communities in the control and different fertilizer-treated soils, calculated based on the digitized DGGE profile. The bacterial diversity index and evenness index were slightly different between treatments. The order of the diversity indices was NPK, CK, N1, NK, K, NP, PK, P and N2. The order of the evenness indices was NPK, CK, NK, N1, PK, NP, K, N2 and P. The bacterial diversity and evenness of NPK treatment were similar to the control and were the highest among all chemical fertilizer treatments. It indicated that there were more soil bacterial species, more evenly distributed, and the bacterial communities were relatively stable.

Cluster analysis was performed to form a dendrogram using UPGMA (Fig. 4). NPK treatment was the closest to the control in terms of bacterial

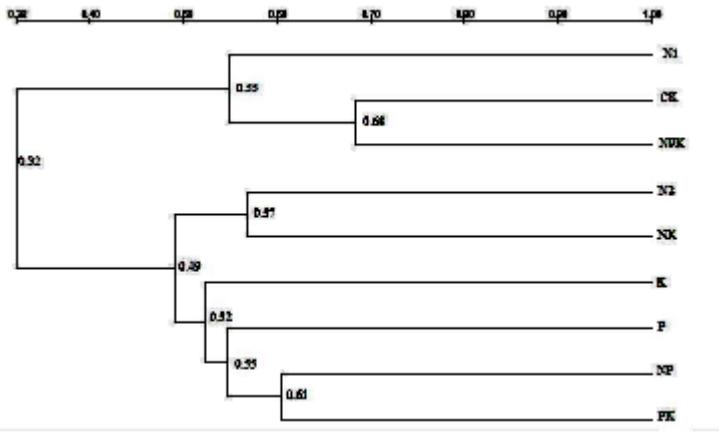

Fig. 4. UPGMA cluster analysis of bacterial 16S rDNA PCR amplification products of fertilizer treatments

population composition, with a similarity of 0.68. The DGGE fingerprints of the straight nitrogen treatment and control were the most similar, so these two treatments clustered into one class while other fertilizer treatments clustered into another class. The similarity coefficient between the two classes was 0.32. Among treatments to which nitrogen, phosphorus, and potassium were applied, the bacterial kinship was closest between the PK and NP treatments, followed by NK and N2, which were clustered into the same cluster. Compared with the NK/NPK branch, the soil bacterial community composition of treatments that received straight phosphorus or potassium fertilizer alone was more similar to that of Pk/NP branch.

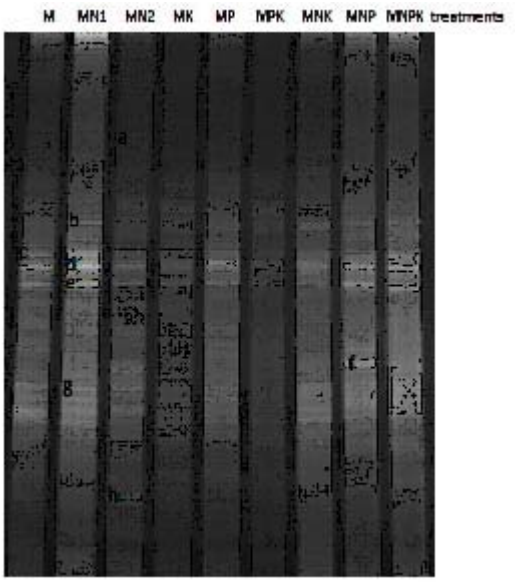

Fig. 5. Denaturing gradient gel electrophoresis of bacterial 16S rDNA PCR amplification products of organic fertilizer treatments

Analysis of bacterial community structure and diversity in soils under long-term application of organic manures

Compared with applying chemical fertilizers alone, the DGGE profiles of bacteria in soils treated with organic fertilizers had more bands and higher band light, and the migration positions and brightness levels were somewhat different between lanes (Fig. 5). As the DGGE profiles of bacteria in soils treated with chemical fertilizers, three bands were common to all lanes: bands c, d, and e. Band d was brighter than bands c and e, suggesting that the bacterial communities represented by bands c, d, and e were the inherent dominant populations and that the communities represented by band d was the largest. Bands a and h were only present in the third lane, indicating that the bacterial community was a unique dominant community only present in the treatments of long-term application of organic fertilizers and a large amount of nitrogen fertilizers. Band f in the eighth lane was clear and bright, representing a different bacterial community in the soils under MNP treatment. Bands in the same migration position as band b were also present in the MNK, MNP, and MNPK lanes but with different brightness, indicating that MN1, MNK, MNP, and MNPK treatments had common dominant bacterial community, but the community sizes were different.

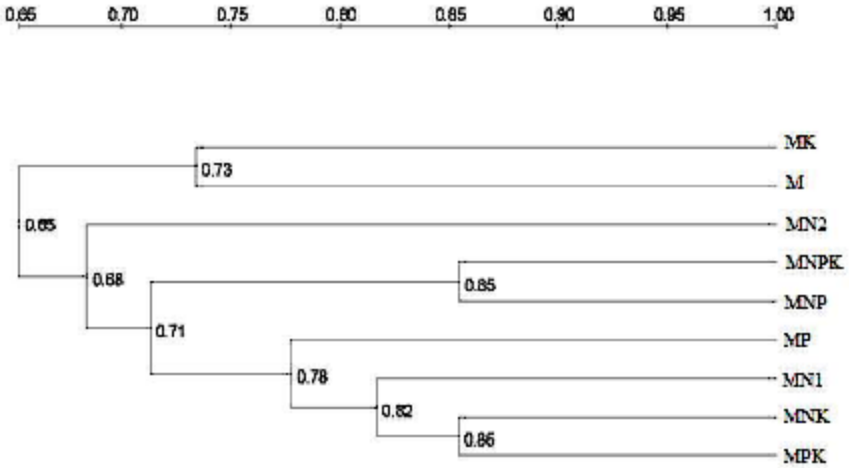

Fig. 6. UPGMA cluster analysis of bacterial 16S rDNA PCR amplification products of organic fertilizer treatments

Band g was present in lanes 1-5, indicating that the same class of dominant bacterial community existed in M, MN1, MN2, MNK, and MP treatment, but the community sizes were different, like band b.

The UPGMA dendrogram of bacteria in soils under long-term application of organic manure was showed in Fig. 6. Compared to treatments with only chemical fertilizer application, the soil bacterial community composition had a higher degree of similarity among various organic fertilizer treatments. The complete clustering dendrogram was divided into two large branches. One was the cluster of treatments M and MK, with a similarity coefficient of 0.73; the other large branch was formed by other treatments whose bacterial community composition was mostly different from that of the above two treatments. The similarity coefficient between the two branches was 0.65. Among all treatments in which organic fertilizers were applied, the community structures were most similar between MNPK and MNP treatments, as well as between MNK and MPK treatments, which had a similarity coefficient of 0.85. Thus, they were separated into two clusters. The structures of MN1- or MP-treated soil bacterial community were similar to the MNK/MPK-treated cluster, while the structure of MN2-treated bacterial community was mostly different from the others.

The variety of soil microbial community under different treatments with long-term organic fertilizer application is shown in Table 3. There were 30 types of DGGE profile patterns. The MNPK lane showed the greatest number of bands, 23; the MPK lane showed the smallest number of bands, 13; the M, MN2, MP, and MNK lanes all had 19 bands. As shown in Table 3, there were some differences in the diversity indices of the soil bacterial community between treatments, while the evenness indices were similar. Both the diversity index and evenness index of the MNPK treatment were the highest among all treatments. The MN1 and MNP treatments had high bacterial community diversity indices but low evenness indices; in contrast, the MPK and MK treatments had low diversity indices but high evenness indices.

DISCUSSION

DGGE was invented as an electrophoresis method to detect DNA mutations (Fisher *et al*., 1983). Ever since Muzyer *et al*. (1983) applied the DGGE technique to study microbial ecology, an increasing number of scholars have used this technique to study the genetic diversity and population differences of microbial communities because it has the advantages of good repeatability, simple operation, and conduciveness to subsequent study (Sheridan *et al*., 2003; Yagi *et al*., 2011; CycoD *et al*., 2013). In this study, DGGE was used to investigate the impacts of long-term localized fertilization on the bacterial community structure and diversity in the planting soils of greenhouse tomato. It was found that long-term fertilizer application changed the structural composition of soil bacterial communities, consistent with the results of previous studies (Anthony *et al*., 2001; Marschner *et al*., 2003). Long-term application of organic fertilizers significantly increased the diversity and evenness of soil bacterial communities, while treatments that only applied chemical fertilizers decreased the microbial diversity index and evenness index. Thus, organic fertilizer was superior to chemical fertilizers in terms of improving soil microbial evenness and diversity. The reason that organic fertilizer can increase the diversity of soil bacteria may be because applying organic fertilizer can improve the physical and chemical environment of soils and provide many nutrients for microbial growth and reproduction (Dick *et al*., 1992). The prolonged use of fertilizer alone significantly reduced soil pH, resulting in severe soil acidification, soil environment damage to the environment. It finally reduced soil microbial diversity. O'Donnell *et al*. (2001) believed that the fertilization measures affected the microbial community structure mainly because fertilization changed the pH value of soils and, thereby, changed the microbial community structure. The fact that the diversity index and evenness index of bacterial communities under N2 treatment were lowest also supports this statement; soil microbial diversity is always high if nitrogen, phosphorus, and potassium fertilizers are all applied, i.e., balanced fertilization. It is most likely because balanced fertilization can maintain the soil pH at a level which does not cause serious soil acidification, and large amounts of nutrients can promote the crop growth, improve the reproduction of soil microorganisms and the development of

microbial communities.

In this study, the UPGMA cluster analysis dendrograms of soil bacteria showed that the treatments with organic fertilizers and treatments with only chemical fertilizers did not display similar classification trends. It was indicated that different chemical fertilizers or different combinations of fertilizers had no effect on soil bacterial community composition. The effects of inorganic fertilizers or combinations of fertilizers on soil microbial diversity showed no obvious pattern, except for the high microbial diversity and evenness of the NPK treatment. Sarathchandra *et al*. (2001) reported that an inorganic nitrogen and phosphorus fertilizers had no significant effect on soil microbial diversity. This lack of effect have been observed because soils have the greatest number of bacteria, and their community structures are relatively stable and can adapt to changes in the environment. For these reasons, adding or eliminating certain types of elements does not affect the community composition of soil bacteria. The NPK-treated community structure had the greatest similarity with the control, corroborating the findings of O'Donnell *et al*. (2001) and Kinura *et al*. (2002).

In summary, compared with applying chemical fertilizer alone, applying organic fertilizer can increase the total number of soil bacteria and the numbers of dominant species, increase community diversity, and stabilize the community structure. All fertilization treatments and controls had common dominant bacterial strains, and a few had their own individual dominant strains. The differences in chemical fertilizers and manure combinations did not affect the soil bacterial community structure.

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