EFFECT OF GRAPE REPLANT ON THE SOIL MICROBIAL COMMUNITY STRUCTURE AND DIVERSITY

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ABSTRACT
At present, replant obstacle has become an important problem in grape production, and it has seriously restricted the development of the grape industry. This study compared the soil from a replant vineyard and a newly planted vineyard where vine had been grown for 30 years and 3 years respectively. A pot experiment was conducted to investigate the changes of plant growth when grape was grown on root zone soil, sterilized root zone soil and fallow soil from the corresponding vineyards. The variation of the microbial population of the rhizosphere soil, non-rhizosphere soil and fallow soil from different vineyards was studied based on PCR-DGGE approach. The amplified bands of the dominant population were sequenced. The results showed that the plants growth was suppressed by vine replanting, and the plants grew significantly stronger after replant soil sterilization. The bacterial and fungal diversity increased as the period of the grape planting extended. The diversity of the microbial population in the rhizosphere soil was greater than that in non-rhizosphere soil. Cluster analysis showed that the microbial population structure of the rhizosphere soil had the closest association with non-rhizosphere soil after vine replanting and they were different from the population structure of fallow soil. However, the population structure of the rhizosphere soil was different from that of non-rhizosphere and fallow soil in the newly planted vineyard. Comparing to the newly planted vineyard, grape replanting caused a great change in the microbial population of the rhizosphere soil. The relative abundance of Flavobacterium sp. (DQ339585) and Bacillus sp. (AY039821) decreased, while Pedobacter sp. (AJ871084) increased in number. Omphalina farinolens (EF413029) appeared, the relative abundance of Pestalotiopsis sp. (DQ657877, DQ657875, DQ657871), Phacidium lacerum (DQ470976) and Lecythophora decumbens (AF353597) decreased, while that of the fungus Pilidium acerinum voucher (AY48709) increased. Among them, Bacillus sp., Flavobacterium sp. and Pestalotiopsis sp. in the rhizosphere soil of the replant vineyard had antagonism for pathogens, and the decrease in their relative amount reduced the ability to resist pathogens. The increasing number of Pilidium acerinum voucher might relate to a serious disease after vine replanting.

Keywords: vine, replanting, growth, microbial community

Introduction
The grape replant problem is expressed as stunted growth, low productivity and a decline in tree vigor leading to shortened economic life. The problem is common in all vine growing regions. The theories to explain replant obstacle include an imbalance in the physicochemical properties of the soil, the allelopathy effects of root exudates and residues, and the build-up of pests and pathogens (18). Among them, soil microbes are one of the important factors.

Microbial diversity is related to soil functions and ecosystem sustainability (11, 16). According to Brussaard et al. (5), the block of high disease resistance is found with maximum soil microbial diversity. Recent studies have revealed that replant obstacle is associated with an altered microbial community structure and diversity in sick soil (3, 10, 25). The microbial equilibrium, possibly beneficial to plants, is thus disturbed after replant and the selected rhizosphere has a detrimental effect on new crops (6). The build-up of pathogens in replant soil is harmful for plants. Hoestra (9) considered Thielaviopsis basicola to be at least partially responsible for cherry replant problem. Similarly, several authors (4, 13, 14) have reported that Rhizoctonia solani, Pythium intermedium and Fusarium solani have played a causal role in the development of apple replant disease.

However, little is known about the influence of grape replant on soil microbial community. Our purpose was to gain a better understanding of soil obstacle under replant vineyard by: (i) comparing growth changes of grape seedling after soil sterilization from a replant vineyard and a newly planted vineyard by pot experiment; (ii) monitoring how soil bacterial and fungal communities changed after grape replanting.

Materials and Methods
Site description and experimental design
The study was conducted in two vineyards in Shenyang Agriculture University, Liaoning Province, People’s Republic of China. The replant vineyard was established in 1978, and has been renewed twice in situ since 1978. Its soil is aquic brown soil. The newly planted vineyard was established in
2004, and slag was used to develop clayed soil before grape planting. There was long-term application of organic fertilizers (chicken manure and pig manure) in the two vineyards every Spring. For the physical and chemical properties of the soil samples see Table 1.

Soil sampling
Six treatments were established in four replicates each. The treatments were: rhizosphere soil in the replant vineyard (RR); non-rhizosphere soil in the replant vineyard (NR); fallow soil around the replant vineyard (FR); rhizosphere soil in the newly planted vineyard (RN); non-rhizosphere soil in the newly planted vineyard (NN); fallow soil around the newly planted vineyard (FN).

In each treatment replicate, soil cores were sectioned into a depth of 15-20 cm in an S-shaped pattern using a digging knife. Only the soil adhering to the roots was considered as rhizosphere soil (15). Non-rhizosphere soil was collected in the root zone at 1 m distance from the tree trunk. The unplanted grape soil around the corresponding vineyard was considered fallow soil. The soil samples were mixed to form a composite sample, ground, and passed through a 2 mm sieve and divided into two parts. One part was immediately stored at -20°C for subsequent DGGE analysis, and another part of the soil was used for pot experiments.

Pot experiments
Six treatments were established: rhizosphere soil and non-rhizosphere soil of replant vineyard were mixed, which was referred to as root zone soil (ZR); sterilized root zone soil of replant vineyard, which was sterilized by electrothermal steam sterilizing pot for 2 h under 126°C, 0.11~0.12Mpa (SR); the fallow soil around replant vineyard (FR); rhizosphere soil and non-rhizosphere soil of the newly planted vineyard were mixed (ZN); sterilized root zone soil from the newly planted vineyard (SN); fallow soil around the new-planting vineyard (FN).

Grape seedlings (V. vulpina × V. labrusca Beta) were planted in circular-section pots (20 cm diameter, 15 cm height) under shelter cultivation for avoiding rain. The pots received similar amounts of dry soil (1.6 kg), and the mean weight of the pots after draining gave the amount of water corresponding to 100% soil water holding capacity. Throughout the cultivation, the pots were buried in soil, with daily water adjustments to 80% soil water holding capacity. After 3 months cultivation, 30 grape plants grown in individual pots were harvested for analysis. Plant height, stem diameter, fresh weight of shoot and fresh weight of root were measured.

Physical and chemical properties of soil samples

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Organic matter/ Total N/Total P/Total K (g/kg)</th>
<th>Available_N/Available_P/Available_K (mg/kg)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replant vineyard</td>
<td>18.2356/1.2798/1.6070/5.5961</td>
<td>142.70/142.27/136.71</td>
<td>6.97</td>
</tr>
<tr>
<td>Newly planted vineyard</td>
<td>11.4089/1.0313/1.0072/5.3540</td>
<td>103.90/96.94/135.34</td>
<td>6.45</td>
</tr>
</tbody>
</table>

PCR amplification of 16S rRNA V3 region from soil bacteria

PCR amplifications were performed with GC-341f

(5′-CGCCCGCGCCGCGCCGCCGCGCGCGCGCGCGGCCGG GCCACGGGGGGCCTACGGGAGGCAGCAG-3′) and 518 r (5′-GTATTACCGCGCTGCTGG-3′) universal primers, located at the V3 region of the 16S rRNA genes of bacteria. A GC-rich clamp attached to the forward primer prevented the complete melting of the PCR products during subsequent separation in DGGE.

The reaction mixture (50 µl) contained 2 µl of each primer, 5 µl of 10 × Taq Buffer, 4.0 µl deoxyribonucleotide triphosphate mixture (dNTP), 1.0 µl DNA template (10 ng), and 2.5 U Taq DNA polymerase, sterile filtered milli-Q water to a final volume of 50 µl PCR amplification. PCR amplification was performed by using a PTC-200 (Bio-Rad, United Kingdom) under the following conditions: 94°C for 5 min followed by 9 cycles of 94°C for 1 min, 65°C for 30 s and 72°C for 1 min, and followed by 9 cycles of 94°C for 30 s, 60°C for 30 s, cooled at -0.5°C per cycle and 72°C for 1 min, then 9 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and a final elongation step at 72°C for 10 min (2).

PCR amplification of 28S rRNA fragment from soil fungi

PCR amplifications were performed with GC-U1 (5′-CGCC CGCGCCGCGCGCCGCCGCGCGCGCGCGCGGCCGG GCCACGGGGGGCCTACGGGAGGCAGCAG and U2 (5′-GAC TCC TGG GTC GTT-3′) fungus-specific primers. The reaction mixture (50 µl) contained 2 µl of each primer, 5 µl of 10 × Taq Buffer, 4.0 µl deoxyribonucleotide triphosphate mixture (dNTP), 1.0 µl DNA template (10 ng), and 2.5 U Taq DNA polymerase, sterile filtered milli-Q water to a final volume of 50 µl PCR amplification. PCR amplification was performed by using a PTC-200 (Bio-Rad, United Kingdom) under the following conditions: 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 50°C for 40 s and 72°C for 1 min, and a final elongation step at 72°C for 10 min (19).

DGGE

DGGE was performed with a Dcode™ Universal Mutation Detection System (Bio-Rad Laboratories Inc. Hercules, CA, USA). PCR products were loaded onto 8% polyacrylamide gel (80 g/l acrylamide in 1 × TAE buffer) with a 30% to 60% denaturant gradient (where 100% denaturant contains 7.0 mol/l urea and 400 g/kg formamide). Samples (40 µl) of the PCR products were loaded onto gels and then run in 1× TAE (Tris-acetate-EDTA) buffer for 5 h at constant conditions of

TABLE 1
60°C and 200 voltage. After the runs, gels were removed from the setup, stained for 30 min with Gene Finder, studied under ultraviolet light and photographed.

**Sequence analysis of DGGE bands**

DNA fragments to be nucleotide sequenced were excised with sterile blade and transferred to 30 µl TE. DNA was allowed to diffuse into the TE at 4°C overnight. 3 µl of the DNA solution was used as a template and re-amplified using PCR as described above. Single and correctly- positioned products were cleaned by Wizard PCR Prepkit (Promega, Madison, Wisconsin, USA). Sequencing was performed by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd., China. Searches in BLAST from GenBank were used to find the closest known relatives to the sequences.

**Statistical analysis**

Variance analysis of the data was performed by SPSS 14.0 professional software (SPSS Inc.). The DGGE banding patterns were digitized with Quantity One software (Bio-Rad Inc., Carlsbad, CA, USA). Based on band intensity and position, the diversity of the microbial communities was analyzed by Shannon-Weaver index \( H = -\sum p_i \ln p_i \). The evenness was calculated as \( E = H / \ln S \), where \( p_i \) is the ratio between specific band intensity and total intensity of all bands in a lane sample, and \( S \) is the number of bands.

**Results and Discussion**

In the replant vineyard the growth of grape growing on sterilized root zone soil was best in comparison to other treatments. Whereas, in the newly planted vineyard there was no significant difference of plant growth, except for plant height, between the grape growing on root zone soil and sterilized root zone soil (Fig. 1).

Plant growth was greatly reduced for the grape growing on the root zone soil of the replant vineyard compared to that growing on the root zone soil of the newly planted vineyard (Fig. 1). Plant height, stem diameter, fresh weight of shoot and root decreased by 39.80%, 6.35%, 49.97% and 27.19% respectively. After sterilization of the root zone soil from the replant vineyard, the plants exhibited better growth condition in comparison to the plants growing on the root zone soil of the newly planted vineyard. These results indicated that soil microbes were an important factor of grape replant obstacle.

**Bacterial community structure and diversity studied by 16S rRNA DGGE**

DNA was successfully extracted from all the soil samples and adequately purified for subsequent PCR-DGGE analysis. PCR amplification of 16S rRNA fragments successfully generated 177-260-bp products that were visible as strong bands in the gel after electrophoresis.

To determine the community diversity, the Shannon-Weaver index of diversity \( H \) and evenness \( E \) were calculated on...
the basis of the number and relative intensities of bands on a gel (Table 2). There were differences in the Shannon-Weaver index obtained from the DGGE analysis of 16S rRNA gene fragments relating to different treatments. The soil of the replant vineyard had higher diversity of bacteria than that of the newly planted vineyard. Compared to NR and FR, RR showed higher community diversity. The Shannon-Weaver index in the newly planted vineyard was in order FN>NN>RN. The results clearly demonstrated that there was low bacterial community diversity in short-term planted soil and high diversity in long-term replant soil, especially in the rhizosphere soil.

The intensity of the bands may represent numerically dominant bacteria. For the identification of major bacterial populations, prominent DGGE bands derived from different soils were excised and used for nucleotide sequence analysis (numbered DGGE bands in Fig. 2). The dominant bands (B1-B5) that appeared in the vineyard samples were analyzed for general information regarding the dominant species, as shown in Table 3. B2, B3 and B4 were dominant bands in the newly planted vineyard. They were closely related to *Flavobacterium* sp. (DQ339585), *Bacillus* sp. (AY039821) and *Burkholderia* sp. (FM212275), respectively.

### Diversity index of soil microbial community of different vineyards

<table>
<thead>
<tr>
<th>Soil samples</th>
<th>Soil bacteria</th>
<th>Soil fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>S</td>
</tr>
<tr>
<td>FN</td>
<td>2.40</td>
<td>11</td>
</tr>
<tr>
<td>NN</td>
<td>2.30</td>
<td>10</td>
</tr>
<tr>
<td>RN</td>
<td>2.19</td>
<td>9</td>
</tr>
<tr>
<td>RR</td>
<td>2.71</td>
<td>15</td>
</tr>
<tr>
<td>NR</td>
<td>2.48</td>
<td>12</td>
</tr>
<tr>
<td>FR</td>
<td>2.48</td>
<td>12</td>
</tr>
</tbody>
</table>

### Sequence analysis of DGGE bands in grape plant soil

<table>
<thead>
<tr>
<th>Bands</th>
<th>Accession no.</th>
<th>Homologous species</th>
<th>Maximum identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>FJ416630</td>
<td><em>Pedobacter</em> sp. (AJ871084)</td>
<td>100%</td>
</tr>
<tr>
<td>B2</td>
<td>FJ416631</td>
<td><em>Flavobacterium</em> sp. (DQ339585)</td>
<td>100%</td>
</tr>
<tr>
<td>B3</td>
<td>FJ416629</td>
<td><em>Bacillus</em> sp. (AY039821)</td>
<td>100%</td>
</tr>
<tr>
<td>B4</td>
<td>FJ416632</td>
<td><em>Burkholderia</em> sp. (FM212275)</td>
<td>100%</td>
</tr>
<tr>
<td>B5</td>
<td>FJ416633</td>
<td><em>Frateuria</em> sp. (EU170476)</td>
<td>100%</td>
</tr>
<tr>
<td>F1</td>
<td>FJ416634</td>
<td>Uncultured basidiomycete (DQ341936)</td>
<td>98%</td>
</tr>
<tr>
<td>F2</td>
<td>FJ416635</td>
<td><em>Omphalina farinolens</em> (EF413029)</td>
<td>98%</td>
</tr>
<tr>
<td>F3</td>
<td>FJ416636</td>
<td><em>Pestalotiopsis</em> sp. (DQ657877, DQ657875, DQ657871)</td>
<td>100%</td>
</tr>
<tr>
<td>F4</td>
<td>FJ416637</td>
<td>Uncultured soil fungus clone (EU691990)</td>
<td>100%</td>
</tr>
<tr>
<td>F5</td>
<td>FJ416638</td>
<td><em>Phacidium lacerum</em> (DQ470976)</td>
<td>98%</td>
</tr>
<tr>
<td>F6</td>
<td>FJ416639</td>
<td><em>Pilidium acerinum</em> voucher (AY48709)</td>
<td>100%</td>
</tr>
<tr>
<td>F7</td>
<td>FJ416640</td>
<td><em>Lecythophora decumbens</em> (AF353597)</td>
<td>100%</td>
</tr>
</tbody>
</table>
The intensities of B2 (Flavobacterium sp.) and B3 (Bacillus sp.) bands decreased, and that of B4 (Burkholderia sp.) kept invariant in the replant vineyard samples. The fragment designated B1 represented one of the dominant species in RR, and it was faint in other treatments. Its sequence closely matched with that of Pedobacter sp. The results suggested that the bacterial community structure changed after grape replanting.

Cluster analysis (Fig. 3) showed that the community structure of RR had the closest association with NR, and they were different from FR. However, the community structure of NN was similar to FN, which was different from RN. The results clearly demonstrated that long-term replanting affected the bacterial community of the rhizosphere and non-rhizosphere soil, while short-term planting only affected that of the rhizosphere soil.

### Fungal community structure and diversity studied by 28S rRNA DGGE

The Shannon-Weaver index of fungi in the replant vineyard was higher than that in the newly planted vineyard (Table 2). There was more community diversity in the rhizosphere soil than in non-rhizosphere soil, but lower than in the fallow soil around the corresponding vineyard. The results showed that the fungal community diversity decreased after grape planting.

The dominant bands (numbered DGGE bands in Fig. 4) that appeared in the vineyard samples were analyzed for general information regarding the dominant species, as shown in Table 3. The appearance of F5 band and disappearance of F1 and F4 bands in RN was an indication of change of fungal community diversity in comparison with FN, and these bands related closely to Phacidium lacerum, uncultured basidiomycete and uncultured soil fungus clone. Compared to FN, the intensity of F7 increased and F3 was faint in RN, and their sequences closely matched that of Lecythophora decumbens and Pestalotiopsis sp., respectively.

F2 as one novel dominant band appeared in the replant vineyard sample, the sequence of which closely matched that of Omphalina farinolens. The intensities of F3, F5 and F7 bands of RR were fainter than RN, but the intensity of F6 increased in RR, and the sequence of F6 related closely to Pilidium acerinum voucher. The results suggested that the fungal community structure changed after grape replanting.

Cluster analysis (Fig. 5) showed that there existed a huge difference in soil fungal community structure between grape planting soil and un-planted grape soil. RR, NR and RN formed one cluster, and NN trended to another cluster. FN and FR were grouped into one cluster.

In this study we aimed to mimic the effects of replant soil sterilization on grape growth. Our results showed that shoot and root growth of plant seedlings were suppressed by grape replanting, and after soil sterilization the plants grew significantly better. Therefore, we concluded that the soil microbes were one of the important factors of grape replanting.

In our study, significant differences in bacterial and fungal community structure of the two vineyards were observed. Bacteria are dominant components of soil microflora. Benizri et al. (3) considered that changes in the composition of individual microbial groups in the rhizosphere of peach trees excavated from healthy or sick soils indicate the role of rhizobacteria in the etiology of the replant sickness of peach soil. It appears that in sick soil, there was a shift in the structure of bacterial communities in favor of phytotoxic microorganisms which can produce compounds such as HCN.

In our study, compared to the newly planted vineyard, the relative abundances of Bacillus sp. and Flavobacterium sp. in the rhizosphere soil decreased over years after grape replanting. Bacillus sp. is an important antagonistic microorganism of pathogens, which exists widely in the soil and on plant surface. On the one hand, it can promote defensive enzyme activity and hormone content in plants, and on the other hand, it induces lethal effects on pathogens by means of extracellular products (26). For example, inoculation with an antagonistic Bacillus subtilis strain was reported to protect young apple trees from Phytophthora cactorum crown and root rot (21). Flavobacterium sp. can secrete chitinase, which is antagonism towards fungal pathogens (7). It was not beneficial to the antagonism towards pathogens that the numbers of Bacillus sp. and Flavobacterium sp. decreased relatively in the rhizosphere soil of the replant vineyard.

Fungi are also important in the soil. Pilidium acerinum is a pathogenic fungi (17), and the increasing relative abundances of Pilidium acerinum voucher in the rhizosphere soils of the replant vineyard might relate to disease aggravation after grape replanting. In addition, Pestalotiopsis sp. can secrete organic acid which is antagonism towards fungal pathogens (12). However, there are few reports on the functions of other microbial population, and pathogenicity tests need to be performed to indicate their role in grape replant development.

The variation of microbial community diversity in the rhizosphere was greater than that in non-rhizosphere soil (10).
It showed that root exudates could affect rhizosphere microbial communities. Our data further supported this observation in the replant vineyard. A rise in the number of self-induced root diseases by root exudates has been observed for many crops (23). It has been shown that root exudates and antagonistic relations in the rhizosphere of apple monoculture may lead to the dominance of saprophytic phytotoxic microorganisms which negatively affect the microbial equilibrium of the soil, and thus also the development and the health of plants (6). Some studies have also shown that the resistance of the roots to soil-borne pathogens is related to the constitution of the root exudates (20, 24). On root exudates of certain peanut cultivars resistant to root rot disease, Abd El-Moneem et al. (1) showed that root exudates of the tested susceptible cultivar contain a greater number of sugars as well as amino acids than those of the tested resistant cultivar. In these cases, exudates might provide nutrition or suitable physical environment for the survival of the pathogen, thus indirectly increasing the incidence of soil-borne diseases. Therefore, root exudates could be treated as core factor of rhizosphere microecology, and studies on the mechanism between root exudates and rhizosphere microorganisms would be helpful to reveal reasons of grape replant obstacle.
Conclusions
Soil microbes were considered as an important factor of grape replant obstacle. The decrease in the relative amount of Bacillus sp., Flavobacterium sp. and Pestalotiopsis sp., which have antagonism towards pathogens in the rhizosphere soil of the replant vineyard, reduced the ability to resist pathogens. The increased number of Pilidium acerinum voucher might relate to the serious disease after grape replanting.

Acknowledgements
Financial support was provided by the Science and Technology Key Project Fund from the Dept. of Science and Technology of Liaoning Provincial Government, China (No.2008204003), and Special Fundation of Modern Agricultural Industry Technology System Construction of China (CARS-30-yz-6).

REFERENCES