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Bacterial and fungal diversity in rhizosphere soil of red raspberry (*Rubus idaeus* L. cv. Heritage)

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ABSTRACT

We investigated the bacterial and fungal diversity in the rhizosphere soil of autumn fruiting red raspberry (*Rubus idaeus* L. cv. Heritage) plants of different plant ages and phenological phases using the PCR-DGGE (denaturing gradient gel electrophoresis) method. The diversity of bacteria and fungi did not show significant variations among the plants of different ages but varied with phenological phases. The bacterial diversity reached maximum at budding phase, while fungal diversity was maximum at leaf expansion phase and fruit maturity phase. The stability of bacterial community was higher than that of fungi.

Key words: Bacteria, diversity, Fungi, PCR-DGGE, phenological phases, plant age, red raspberry, rhizosphere soil, *Rubus idaeus*, season.

INTRODUCTION

Soil microorganisms are important in orchard ecosystems, these promotes the transformation of soil organic matter and nutrients and thereby affects the growth and development of plants (1). They are an important index of soil fertility (16). In recent years, research on orchards soil microbial community structure and diversity has received much attention (4,8). Different fruit tree species, plant age, growth phase and management practices (grass cover and fertilization) affects the community structure of soil microorganisms (7,9,13,22,24,28). Research on soil microorganism of apple (5,20), citrus (23,25), banana (17,19), vitis (29) and strawberry (6,26) showed that the total number of soil microorganisms in different orchards vary with plant age and growth phases.

Red raspberry (*Rubus idaeus* L., family Rosaceae) is fruiting shrub, whose fruit-bearing shoots are pruned after fruiting (27). It is not known whether the changes in the soil microflora similar to other orchards, also occur during the growth and development of raspberry. Hence, this study aimed to determine the diversity of bacteria and fungi in the rhizosphere soil of red raspberry during the various growth phases and plants of different ages.

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MATERIALS AND METHODS

Plant material and growth conditions

The rhizosphere soil was collected from the red raspberry plants growing in Binxian, Harbin, Heilongjiang, China (Location: longitude 127°48 ', Latitude 45°; Altitude: 158 m; Annual rainfall: 500-600 mm; Maximum and Minimum Temp: 35 °C and -33°C) (Fig 1A). The t soil was black loam, pH 6.3. The red raspberry cultivar 'Heritage' with bushes of 2, 4, 5 and 7-year's age, planted in 1 x 0.5 m spaced rows were selected for this study (Figure 1B).

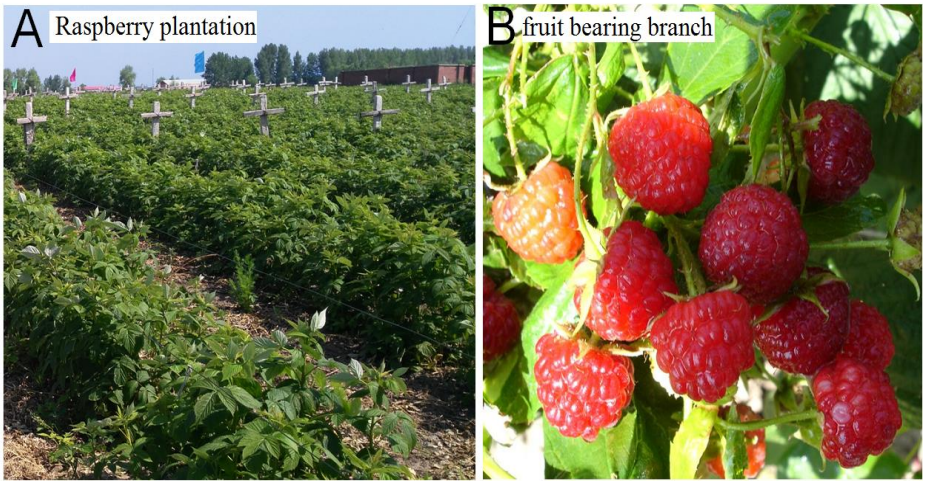

Figure 1. Red raspberry of *Rubus idaeus* L. cv. Heritage

Soil sampling

The rhizosphere soils of 2, 4, 5 and 7-year's age plants were collected at leaf expansion phase (Early May, 2017), budding phase (Early Aug, 2017), fruit maturity phase (Mid-September, 2017) and defoliation phase (Mid-October, 2017), respectively. Five points were selected for each sampling and 2 plants were selected for each point, using the S-type multi-point sampling method, so that rhizosphere soils of 10 plants were collected for each phenological phase and each plant age (Fig 2). After digging out the plants with shovel, the soil near the root surface was gently shaken off and collected as the rhizosphere soil. The soil from 10-plants was mixed evenly and 100 g soil was weighted and stored at 4℃ for PCR-DGGE analysis. Soil from the corresponding inter-row locations of the collected plants was used as control. The laboratory study was conducted from May 2017 to May 2018.

Total soil DNA extraction

The Omega Soil DNA extraction kit (E.Z.N.A.®soil DNA Kit, Specification: D5625) was used to extract and analyze the soil DNA, using 1% agarose gel electrophoresis and then stored at -20 °C in refrigerator (3,11).

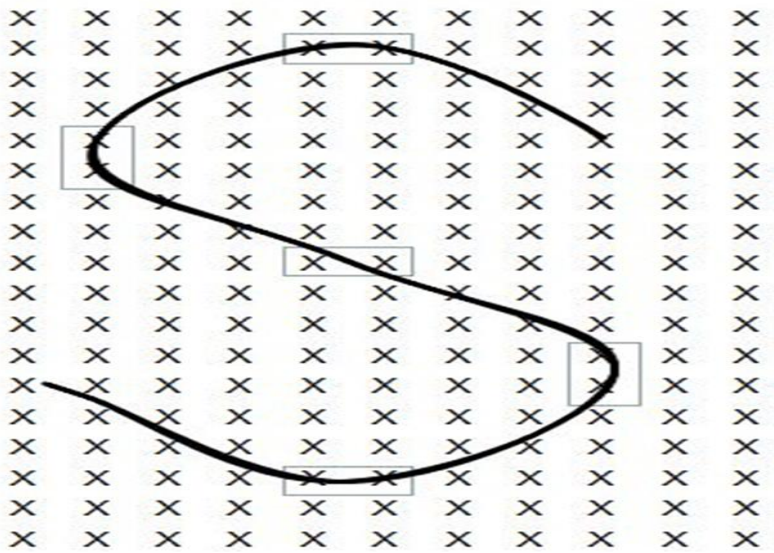

Figure 2. The S-type multi-point sampling method
The "X" in the box represents the sample plant

Analysis of Bacterial and Fungilal diversity by PCR-DGGE

For bacteria, the specific primer of 16SrDNA V3 region, upstream primer F357GC sequence:
5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCCTACGGGAGGCAGCAG-3', downstream primer R518 Sequence: 5'-ATTACCGCGGCTGCTGG-3' were used. For fungi, ITS1 sequence of upstream Primers 5'-TCCGTAGGTGAACCTGCGG-3' and Downstream primer ITS2 sequence:
5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCCCCGCCCGGCTGCGTTCTTCATCGATGC-3', were used. The underlined part is the GC clip structure.

Reaction system for PCR (50 μL) contained 10× PCR Buffer 5 μL, dNTP 4 μL, 10 mM primer 1 μL, template 1 μL, Taq enzyme 0.5 μL, ddH_2O 37.5 μL. The reaction procedure was: 94 ^oC 5 min, (94 ^oC 45 s, 65 ^oC 35 s, 72 ^oC 45 s), 35 Loops, 72 ^oC 10 min, termination reaction at 4 ^oC, PCR products were analyzed and detected by 1% agarose electrophoresis.

The PCR products were separated by Denatured gradient gel electrophoresis (DGGE) apparatus of Bio-rad Company. Electrophoresis buffer was 1×TAE. The loading sample was 20 μL DNA + 5 μL 6 × bromophenol blue dimethyl cyanide solution. Electrophoresis system was: electrophoresis voltage 75 V, temperature 60 ^oC, and time 12 h. The concentration of bacterial denaturing agent was 30~60 %, but fungal degeneration gradient was 20~50 %. The results were recorded by photographing the gels using a Gel Imaging System. The PCR-DGGE map of soil microbial DNA was analyzed with Quantity One software, and the brightness of the peaks and the number of electrophoresis bands

were determined. Similarly, clustering analysis was done based on % content of brightness peak value. The Shannon-Weiner index, Evenness and richness were also calculated. Similarity clustering analysis was based on % content of luminance peak value. The Shannon-Weiner index, Evenness and richness of community were also calculated (11,18).

Shannon index (18) was determined using the equation :

$$\mathbf{H} = \sum_{i=1}^{s} PiIn, \qquad Pi\,\mathbf{I}\,\frac{ni}{N}$$

Evenness index (18) by E=H/lnR.

Where R: Maximum value of Bands' Number.

The Richness index (18) is represented by the total number of bands in R-lane.

Data analysis

Excel 2010 and DPS v7.05 software were used to analyze the data.

RESULTS AND DISCUSSION

Diversity Analysis of Bacteria in rhizosphere soil

PCR-DGGE analysis of soil bacteria in raspberry rhizosphere soils of different aged plants and at various times during the season, within a age group showed that all samples (including control) varied in the total number. However, there was no significant difference in number of bands with different ages at the same phenological phase. The number of bands reached a maximum at 'budding phase' (6-9 lanes) (Fig 3A-3B) for all the samples of different ages.

Table 1. Bacterial diversity at various phenological phases determined by PCR-DGGE

Phenological phase		Raspberry rhizosphere soil				Control soil between the rows
		2 years old	4 years old	5 years old	7 years old	
Leaf expansion phase **(1-5 lanes**)	R	9	8	11	11	14
	H	0.8318	0.7128	0.9809	0.9748	1.1550
	E	0.2255	0.1932	0.2660	0.2643	0.3131
Budding phase **(6-10 lanes)**	R	13	13	13	15	13
	H	1.2443	1.0772	1.0540	1.0603	0.9789
	E	0.3373	0.2920	0.2858	0.2874	0.2654
Fruit maturity phase **(11-15 lanes)**	R	9	9	10	10	8
	H	0.6834	0.6921	0.9507	0.7746	0.5485
	E	0.1852	0.1876	0.2577	0.2100	0.1487
Defoliation phase **(16-20 lanes)**	R	10	11	10	11	8
	H	0.7517	0.8678	0.7790	0.9406	0.6585
	E	0.2038	0.2352	0.2112	0.2550	0.1785

R: Richness index, H: Shannon-Weiner index, E : Evenness index

The results of Richness index, Shannon index and Evenness index showed that the diversity of bacteria in raspberry rhizosphere soils depended on the growth phases. The diversity in 'leaf expansion phase' (the phase in which plant buds are curled or lobed by leaf veins from the buds, with one or two leaves are flattened) was relatively high, which reached maximum in 'budding phase' (the phase in which the plants budding started and was lowest in 'fruit maturity phase' and slightly increased in 'defoliation phase'. These results indicate that the bacterial diversity of raspberry rhizosphere soil at the budding phase was higher than other phases (Table 1).

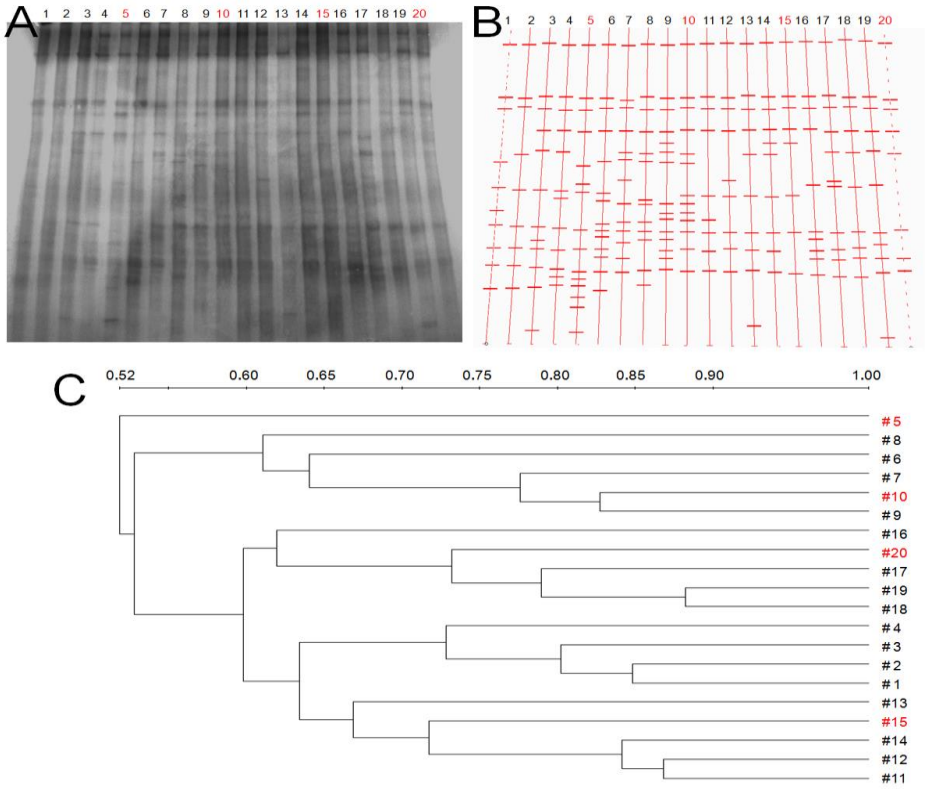

Figure 3. DGGE profile and cluster analysis of V3 region of bacteria 16SrDNA gene
(A) DGGE profiles of V3 region of the 16SrDNA gene amplified by PCR from DNA extracts with raspberry soil of different growing phases; (B) DGGE patterns of bacteria; (C) Cluster analysis (UPGMA, Dice coefficient of similarity) generated by 16SrDNA V3-DGGE profile; 1-5 lanes: The rhizosphere soil and control soil at leaf expansion phase, 6-10 lanes| at budding phase, 11-15 lanes at fruit maturity phase and 16-20 lanes, at defoliation phase of raspberry plants of 2, 4, 5, 7 year and the control.
Each phenological phase had a control for itself. The 5th lane is the control of leaf expansion phase; the 10th lane is the control of budding phase; the 15th lane is the control of fruit maturity phase; the 20th lane is the control of defoliation phase.

The relationship between the DGGE bands of raspberry rhizosphere soil bacteria in different phases was also analyzed by cluster analysis. The results showed that the bands can be divided into 4 categories except in the 5th lane.

In the 1stclass (leaf expansion phase: 1-4 lanes) the similarity coefficient was 70-75%.

In the 2nd class (budding phase: 6-10 lanes) it was 60-65 %.

In the 3rd class (fruit maturity phase: 11-15 lanes) it was 65-70 %,

In the 4th class (defoliation phase: 16-20 lanes), it was 60-65% (Fig 3C).

The higher similarity indicated that the bacterial community structure in the rhizosphere soil was stable. The DGGE bands of raspberry rhizosphere soil bacteria were similar in same phenological phase of different plants ages (Table 1**)**, which suggested that the variation in bacterial community structure were greatly influenced by the phenological phase of raspberry plants. Our result was similar to previous studies (2,7,21). It had been reported that the community structure of soil microorganisms in orchards was temporal and spatial with the changes of time and space (2). Seasonal changes could affect the soil microbial structure through changes in environmental temperature, light, moisture and root exudates.

Diversity analysis of fungi in Raspberry Rhizosphere soil

The PCR-DGGEanalysis of 18S rDNA and 5.8S rDNA interval sequences showed that there were differences in the number, position and brightness of DGGE bands**,** especially the bands which appeared after the 1-5 Lanes leaf expansion phase, which did not appear in later phases (Fig 4A-4B**).**

The number of bands in the leaf expansion phase (1-3 Lanes) and the fruit maturity phase (11-13 Lanes) in the plants of 2, 4, and 5 years old were higher than in defoliation phase (16-18 Lanes) and the budding phase (6-8 Lanes). They were highest in 7-year's old plants at fruit maturity phase (14 Lane); while, in the inter-row soil, the number of bands reached highest in budding phase (Figure 4A-4B).

The cluster analysis of relationship between the DGGE bands of rhizosphere soil fungi of raspberry plants at different phases showed that the similarity in 1-4 Lanes and 11-14 Lanes was high, except in the 17th lane. In the 1st class (leaf expansion phase: 1-4 lanes) the similarity coefficient was 40-50%, in the 3rd class (fruit maturity phase: 11-14 lanes) it was 50-60%. Lanes 6-9 and 16-19 had no clustering. This indicated that the structure of rhizosphere soil fungi in leaf expansion phase of each raspberry bush age was similar to fruit maturity phase. The low similarity showed that the change in fungal community structure in budding phase and defoliation phase of each raspberry bush age was larger than in other phases (Fig 4C).

The results of richness index, Shannon Index and evenness Index showed that, the fungal diversity of raspberry rhizosphere soil in the plants of 2, 4 and 5 year old were highest during the leaf expansion phase. In 7-year's old plants, these were highest in the fruit maturity phase, while, in inter-row soil, the fungal diversity was highest in the budding phase (Fig 4A-4B, Table 2).

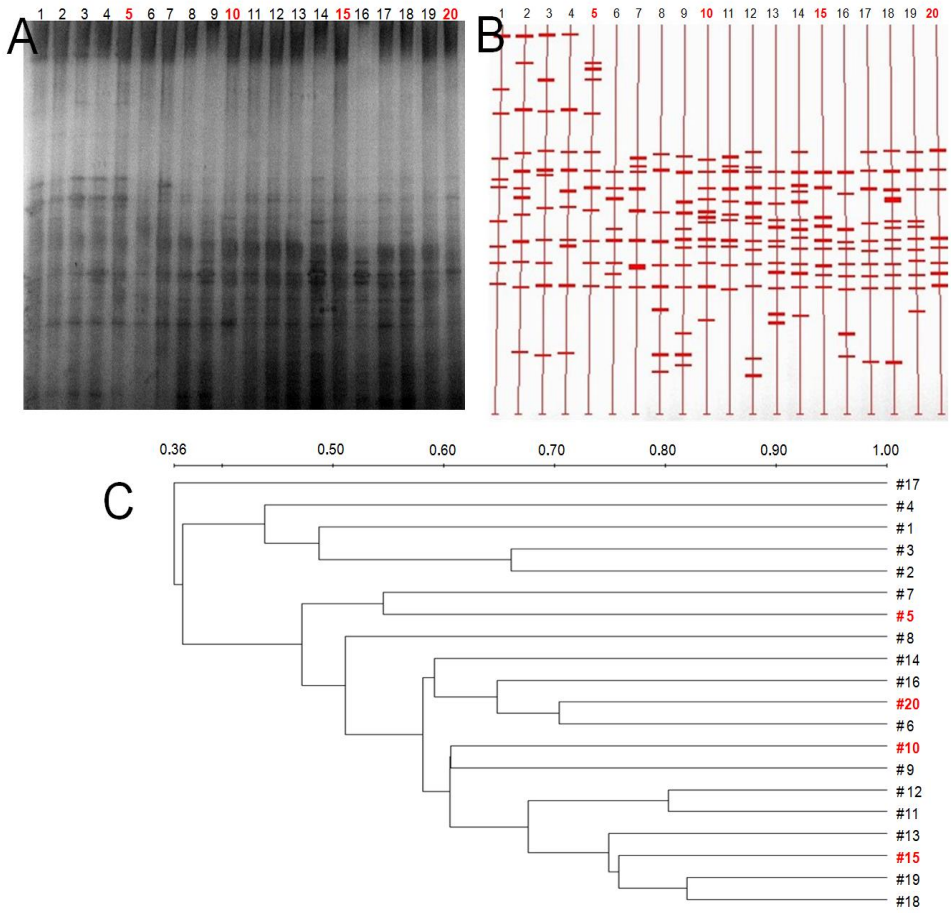

Figure 4. DGGE profiles and cluster analysis of fungi 18Sr DNA-5.8 SrDNA spacer sequence.
(*A*) DGGE profiles of fungi 18Sr DNA-5.8Sr DNA spacer sequence; (*B*) DGGE patterns of fungi; (*C*) Cluster analysis (UPGMA, Dice coefficient of similarity) generated by 18Sr DNA-5.8Sr DNA DGGE profile.; 1-5 lanes: The rhizosphere soil and control soil at leaf expansion phase, 6-10 lanes| at budding phase, 11-15 lanes at fruit maturity phase and 16-20 lanes, at defoliation phase of raspberry plants of 2, 4, 5, 7 year's and the control.
Each phenological phase had a control for itself. The 5th lane is the control of leaf expansion phase; the 10th lane is the control of budding phase; the 15th lane is the control of fruit maturity phase; the 20th lane is the control of defoliation phase.

The results of PCR-DGGE analysis of fungi also showed that the diversity of fungi in rhizosphere soil of raspberry was similar to bacteria and was greatly affected by phenological phase and not by plant age. In addition, there were 5-common bands of soil bacteria (Fig 3A-B) and only 2 common bands of soil fungi (Fig 4A-B). It indicated that the stability of bacterial communities in the rhizosphere soil of raspberry plants was higher than that of fungi.

Table 2. Fungal diversity at various phenological phases determined by PCR-DGGE

Phenological phase	Index	Raspberry rhizosphere soil				Control soil between the rows
		2 years old	4 years old	5 years old	7 years old	
Leaf expansion phase **(1-5 lanes)**	R	11	10	12	11	10
	H	0.8623	0.8871	0.9969	0.7644	0.7945
	E	0.2535	0.2608	0.2931	0.2248	0.2336
Budding phase **(6-10 lanes)**	R	8	9	9	12	12
	H	0.5372	0.6041	0.6081	0.7734	0.7967
	E	0.1579	0.1776	0.1788	0.2274	0.2343
Fruit maturity phase **(11-15 lanse**)	R	11	13	12	13	9
	H	0.7172	0.8338	0.7893	1.1325	0.5910
	E	0.2109	0.2451	0.2321	0.3330	0.1737
Defoliation phase **(16-20 lanes)**	R	10	9	13	11	9
	H	0.6766	0.7959	0.8522	0.7180	0.5452
	E	0.1989	0.2340	0.2506	0.2111	0.1603

R: Richness index, H: Shannon-Weiner index, E : Evenness index

Regarding the variability trend in soil microorganisms with plant age, previous studies have reported that the structure of bacteria and fungi in soil microorganisms in apple orchard changed with the planting age (5,10). Other studies have also reported that with the increase in apple tree age, the DGGE bands of bacterial population in soil decreased and the stripe brightness decreased markedly, while the trend of fungi number was opposite and increased (12). However, our results suggested that that the diversity of fungi (Fig 4A-B) and bacteria (Fig 3A-B) in the raspberry soil do not show specific trend with the change in plant age. The reason for this may be related to the growth habit and management of raspberry plants as well as the age of the apple plants which may be 7 years. Raspberry is a small fruiting shrub which is heavily pruned before the budding phase in spring and the fresh shoots become the fruit bearing branches in the next year. The pruning method reduces the effects of plant age on rhizosphere soil microorganisms (27). In addition, the effects of fertilization (9,24,28), weeding (14,16) and irrigation (22) on rhizosphere microorganisms exceeds the influence of planting age. Hence, there is no direct correlation between the changes in soil microorganism and plant age.

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