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Characteristics of soil microflora of *Panax notoginseng* in different continuous cropping years

H.J. Liu, X.Y. Yang, Z.Q. Miao[1], S.D. Li[1], Y.H. Chen, G. Liu, L.Y.Hao and Z.L. Zhang*
College of Chinese Pharmacy, Beijing University of Chinese Medicine,
Beijing 102400, China
E. Mail: 15832256149@163.com,zhangzilong76@163.com

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ABSTRACT

We examined the soil microbial composition, total number, diversities and the characteristics of soil microflora of *P. notoginseng* in relation to increase in the continuous cropping years. The results showed that bacteria and fungi were the dominant groups in *P. notoginseng* microbial communities. In April (emergence period of *P. notoginseng*), the total number of soil microorganisms decreased initially but then increased. While, in August (flowering period), a reverse trend was seen. The soil microbial community changed from "fungal type" in April to a "bacterial type" in August. Microbial community composition in the new and 1-year cropped soil was similar; both had high microbial diversity "bacterial type". whereas, the 2-years and 3-years continuous cropped soils were similar and had low microbial diversity "fungal type". The soil microflora changed significantly with the seasons and cropping year and these changes may be the cause of *P. notoginseng* replant failure.

Key words: Bacterial type, continuous cropping, fungal type, fungi, microbial composition, *Panax notoginseng*, soil microflora, soil microorganisms.

INTRODUCTION

Panax notoginseng (Burk.) F.H. Chen, Araliaceae, is one of the most important Chinese traditional medicinal plants containing many saponins and steroid glycosides (13). It is mainly cultivated in Yunnan province, China (4). Limitations of land availability lead to its continuous cropping, in which soil sickness is common. This results in replant failures, reduction in seedling growth and susceptibility to various diseases leading to plant death (26). The land used for its cultivation cannot be used for its replanting, unless kept fallow for 8-10 years. Thus the replanting is a serious problem in *P. notoginseng* production.

It is believed that soil microbial change is an important factor in replant failure of medicinal plants (5,21). Deciphering the replant failure mechanisms of *P. notoginseng* mediated by soil microbial changes remains a challenge. Microorganisms are the main driving force in nutrients circulation in the soil and play a critical role in plant growth (3,32). Therefore in recent years, the study of *P. notoginseng* soil microorganisms has drawn more attention to understand the differences in the soil microorganisms (5,7,19) of healthy or diseased plants (17,26,28). This investigation aimed to determine the soil

*Correspondence author, [1]Institute of Plant Protection, Chinese Academy of Agricultural Science, Beijing 100081, China.

microbial characteristics of *P. notoginseng* under continuous cropping and to understand the role of microorganisms in *P. notoginseng* replant failure.

MATERIALS AND METHODS

Soil samples and experimental design

Soil samples were collected in December, 2009 from Tuozhibai Village, Panlongyizu town, Yanshan County, Wenshan District, Yunnan province [Latitude: 23º18'06″, longitude: 104º 23'45″, altitude: 1487 m, mean annual temperature: 16.3°C and annual rainfall: 996 mm]. It is the farmers' *P. notoginseng* growing region. The soil was red and top soil samples were collected from soil surface to 20 cm depth from the region that was planted with *P. notoginseng*. Composite samples were prepared using five point samples and which were evenly mixed. In total, there were four samples viz., (i). New soil with no *P. notoginseng* grown, (ii). 1-year old, (iii). 2 years old and (iv). 3 years old continuously cropped soils.

The chemical characteristics of soil samples are shown in Table 1. The pH was determined by the potentimetric method (37), soil organic matter content by the$K_2Cr_2O_7$ volumetric method (8), total N by Kjeldahl method (37), total P by sodium carbonate fusion method (37), total K by sodium hydrate fusion method (37), alkaline N by the alkali diffusion method (37), available P by the Sodium bicarbonate method (37) and available K by the ammonium acetate extraction method (37).

Table 1. Physico-chemical properties of soil samples

Ginseng planting years	pH	Organic Matter (%)	Total N (g/kg)	Total P (g/kg)	Total K (g/kg)	Alkaline N (mg/kg)	Available P (mg/kg)	Available K (mg/kg)
New Soil	4.39	1.46	1.22	0.47	6.94	172.76	3.50	83.16
1 year	5.14	2.24	1.54	0.72	9.53	107.27	17.18	188.51
2 years	4.70	2.27	1.96	0.70	7.86	152.17	16.18	223.63
3 years	6.93	2.48	2.05	1.10	8.64	130.35	9.50	223.63

The four soil samples were brought to the Wenshan Research Institute of *P. notoginseng*, Yanshan, Yunnan Province. These were cleaned, powdered and sieved (< 2 mm) to remove the stones and organic debris. Then a pot experiment was done to investigate the characteristics of the soil microorganisms in these soils during the following cropping season.

Ten kg soil was placed in each plastic pot (32 cms × 28 cms × 12 cms.). Healthy seedlings were chosen (from the Wenshan *Panax notoginseng* Research Institute) and for surface sterilization, the roots were soaked for 15 min in a solution containing 64 g kg^{-1} Oxadixyl mancozeb + 50 g kg^{-1}Carbendazim x 500 liquid. These were then transplanted in pots (9 seedlings per pot) at row spacing of 10×10 cm. The treatments were replicated thrice in a completely randomized design. The pots were placed in the greenhouse and other management practices were the same as per the local practices**.**

Soil samples were collected from experimental pots in April and August, 2010. The plants were removed carefully and the soil was collected from surface to 20 cm depth around the plant. The soils were sieved (< 2 mm) to remove the visible un-decomposed and semi-decomposed leaves, plant residues and large gravels and stored in closed containers at 4^{o}C for further analyses.

Microbial numbers

Dilution plate method was used to determine the number of different culturable microorganisms (15,30**).** For this, 1 g soil samples were weighed into Erlenmeyer flasks and mixed with 99 mL of sterile water. The flasks were shaken for 15 min at 150 rpm and then allowed to stand for 1 min. The supernatant was diluted upto 10^{-7} and plated.

NA (Nutrient Agar) medium was used to determine the total microbial numbers, KMB (Kings Medium B Agar) was used to determine total bacteria (2,30), PDA (Potato dextrose agar) was used to determine the fungi and Gauze's medium No. 1 was used to determine the actinomyctes. After the media were sterilized and 15 ml of each was poured into sterile plates. When the plates got dry, 0.2 ml diluted soil suspension was spread uniformly. The plates were incubated for 2-7 days at appropriate temperatures (for total microorganisms and fungi: 25℃, for bacteria: 28 ℃ and for actinomycetes 30℃). The number of colonies was determined and the results were expressed in cfu / g of fresh soil.

Soil microbial diversity

Following 9-indices were used to investigate soil microbial diversity.

a. Richness indices

1. Patrick richness index (29) S = total number of species within the community,

2. Margalef richness index (10) D = (S-1) / ln N;

b. Diversity indices

3. Simpson index(30) $D' = 1 - \sum_{i=1}^{S}\left[\frac{N_i(N_i-1)}{N(N-1)}\right]$

4. Shannon-Wiener index (31) $H' = -\sum_{i=1}^{S} p_i lb(p_i)$

5. Brillouin index(31) $H = \frac{1}{N} lb\left(\frac{N!}{N_1!N_2!N_3!\cdots}\right)$

6. McIntosh index (31) $D_{Mc} = \dfrac{N - \sqrt{\sum_{i=1}^{S} N_i}}{N - \sqrt{N}}$

7. Hill diversity index (10,1,25) $N1 = e^H$,

$N2 = 1 / (1- D')$;

c. Evenness indices

8. Pielou index (31) $J' = \dfrac{H'}{H'_{max}} = \dfrac{H'}{lbS}$,

9. Alatalo evenness index (1,10) $E = \dfrac{[1/(1-D')-1]}{e^{H}-1} = \dfrac{N_2 - 1}{N_1 - 1}$.

Where, S : Total number of species; N : Total number of individuals of all species communities; Ni : Individual number of i species in community; lb : Logarithm and its base is 2; pi : Proportion of individual number of species accounted for the total number of all species in the community; pi = Ni / N; D' : Simpson index; H'max : Maximum value of H's.

Analysis of similarity between communities

Bray-Curtis and Morisita distance coefficient methods were used to analyse the similarity between communities (6,9,23)

Statistical analysis

The data were statistically analysed by analysis of variance. Significant differences among treatments were identified using the LSD test. Statistical analysis was done using Excel, SPSS11.5 and DPS7.05.

RESULTS

Soils microbial composition

The results of soil analysis for the microbial number and composition done in April (seedling emergence period of *P. notoginseng*) are shown in Table 2. There was decrease in the total number of soil microorganisms in the first year but as the cropping years increased, the total numbers also increased**.** The highest number of microorganisms was in the 3-years continuous cropped soil. Comparing the number and proportion between the three groups, bacteria, fungi and actinomycetes, the bacteria and fungi were the dominant groups, while the number of actinomycetes was relatively small. Soil bacteria, fungi and actinomycetes population declined with increased planting years, especially bacteria. The number of bacteria in the new soil was 72.57×10^3cfu/g, while the number in 1-year old soil was 37.52×10^3cfu/g, and non significant number of bacteria were found in the 2-year and 3-year old soils**.**

Table 2. Variations in main microorganisms in replant soils during April and August

Ginseng planting years	Total (×10³cfu/g)		Bacteria (×10³cfu/g)		Fungi (×10³cfu/g)		Actinomycetes (×10³cfu/g)	
	Soil Sampling Months from pots							
	April	August	April	August	April	August	April	August
0 year*	386.67 ab	188.57 c	72.57 a	59.62 b	11.05 a	2.21ab	12.76 a	0.02 a
1 year	287.62b	533.33 a	37.52 b	17.33 c	1.71 b	1.77b	3.62 b	0.00 a
2 years	245.71 b	348.57 b	0.00 c	94.10 a	6.67 ab	1.89 ab	2.67 b	0.00 a
3 years	436.19 a	379.05 b	0.00 c	61.33b	2.86 b	2.59a	1.14 b	0.04 a
F value	3.833*	12.973*	22.207*	59.62 b	3.469	2.428	6.78*	1.833

*: New soil, Note：The * denotes significant at 0.05 level, the small letters denote the differential level at 0. 05. The same as below.

The fungi, accounted for > 70% of the total microorganisms in 2-years and 3-years cropped soils. This resulted in the microbial community changing from the “bacterial type” to the “fungal type”.

The microbial number and composition in soil samples collected in August (flowering period of *P. notoginseng*), are also shown in Table 2. The results showed that continuous cropping had a significant effect on the total number of soil microorganisms. In August, the numbers of microorganisms in the 1-year and 3-years continuous cropped soils were more than double than in new soil and the 2-years old soil was nearly 2 times than new soil. The largest number was in the 1-year cropped soil. The total number of microorganisms first increased and then decreased. The number of bacteria first decreased and then increased and there was a slight decline in the 3-years cropped soil. However, there was no significant change in the number of fungi and actinomycetes. Bacteria became the dominant community in soil and the number of fungi and actinomycetes were < 10% of the total number. Compared to the samples collected in April, the soil microbial community in August changed from a "fungal type" to a "bacterial type".

Soil microorganisms are an important component of soil microbial ecosystem which directly or indirectly reflects the changes in the soil health (14,33,22). Investigations on soil microbial changes continuously cropped for 2-years with *Angelica sinensis* (Oliv.) Diels showed that the soil microbial composition and structure had changed and the bacterial diversity was decreased. This change in soil microbial composition and structure was considered one of the reasons for Angelica replant failure (34). Long-term monoculture of Vanilla is also reported to significantly change the soil microbial population (27). The soil fungal diversity index increased with the increase in continuous cropping years, while bacterial diversity was relatively stable. Continuous cropping of cucumber is also reported to change the total soil microbial number (38).

The dominant microbial groups change with the growth of *P. notoginseng*. Fungi dominated in the 2-years and 3-years soils in the emergence period (in April), while bacteria dominated in the florescence period (in August). One possible reason for this may be the change in temperature. In April, the temperature was relatively low with the average maximum temperature was 23 ^{o}C and the average minimum temperature was 13℃, and this suited fungal growth. The temperature and humidity in August were relatively high, with the average maximum temperature was 27 ^{o}C and the average minimum temperature was 17 ^{o}C and this suited the multiplication of bacteria. Thus the soil microbial population was “fungal type” in April and “bacteria type” in August. This is consistent with the results of Huilin Guan (11). In our studies reported herein, we also found that the number of bacteria, fungi and actinomycetes in the soil decreased with the increase in planting years, This is consistent with previous studies on vegetables, Angelica and Ginseng (14,16,18,35,36).

Changes in soil microbial diversity

Soil microbial diversities in April are shown in Table 3. It was found that the most indices showed a "V" shape with increase in continuous cropping years. Compared with the new soil, the species diversity indices (D', H', H, D_{Mc}, N1, N2) and evenness indices (J', E) of 1-year cropped soil were reduced to a different degree, indicating that continuous cropping decreases the microbial diversity. With further increase in continuous cropping years, the Patrick richness index S and Margalef richness index D gradually decreased, and the species diversity index and evenness index showed an increasing trend. This phenomenon may be due to the dominance of only fungi and actinomycetes with few bacteria in 2-years soil and 3-years soil, resulting in a decline in the richness index. Thereafter, the number of fungi and actinomycetes were similar, eventually causing the evenness indices (J', E) to increase.

The species diversity index reflected both the number of species and individuals of the community, and also reflected the degree of evenness to the species distribution. Accordingly, the increase in evenness indices (J', E) in 2-years and 3-years soils, inevitably led to an increase of species diversity indices (D', H', H, DMc, N1, N2).

Soil microbial diversities in August are shown in Table 4. It is seen that the soil microbial richness index changed little but the species diversity index and evenness index increased with the increase in continuous cropping years. Bacteria were the main group of soil microorganisms; hence, the relationship between the number of bacteria and the soil microbial diversity index was very close. When the number of bacteria decreased, the gap between them and the number of fungi and actinomycetes decreased. Correspondingly, the evenness index also increased. Thus it could be seen that the change in the trend of the bacterial number in the soil was opposite to that of the evenness index. The number of bacteria in the soil was “И” type, while on the contrary, the variation of soil microbial evenness was "N" type. The species diversity index and evenness index were closely related and the species diversity index of soil microbes showed "N" type change.

Table 3. Diversities of microorganismsin *P. notoginseng* replant soils in April

Ginseng planting years	Richness index		Diversity index						Evenness index	
	S	D	D´	H´	H	DMc	N1	N2	J´	E
New Soil	3.000a	0.174ab	0.392b	1.003a	0.943a	0.245bc	2.775a	1.681b	0.633bc	0.376c
1 year	3.000a	0.189a	0.267b	0.714a	0.604b	0.171c	2.086a	1.388b	0.450c	0.342c
2 years	2.000a	0.110bc	0.455ab	0.841a	0.647ab	0.350b	2.338a	1.925b	0.841ab	0.656b
3 years	1.700a	0.082c	0.627a	0.918a	0.532b	0.554a	2.505a	2.684a	0.918a	1.119a
F value	—	6.134*	5.559*	1.262	3.296*	9.571*	1.271	8.572*	6.892*	31.412*
Trends	↓	∧	∨	∨	↓	∨	∨	∨	∨	∨

Table 4. Diversities of microorganisms in *P. notoginseng* replant soils in August

Ginseng planting years	Richness index		Diversity index						Evenness index	
	S	D	D´	H´	H	DMc	N1	N2	J´	E
New Soil	2.333a	0.100aa	0.070b	0.226b	0.225b	0.035b	1.253b	1.075b	0.199b	0.294b
1 year	2.000a	0.082a	0.175a	0.454a	0.454a	0.093a	1.585a	1.219aa	0.454a	0.364a
2 years	2.000a	0.073a	0.038b	0.138b	0.138b	0.019b	1.149b	1.040b	0.138b	0.267b
3 years	2.667a	0.125a	0.079b	0.251b	0.251b	0.040b	1.286b	1.086b	0.189b	0.299b
F value	1.833	1.656	7.331*	9.585*	9.585*	6.803*	7.23*	5.47*	8.738*	10.338*
Trends	∨	∨	N	N	N	N	N	N	N	N

Soil microbial community similarity

Six soil samples were taken from the experimental pots to analyse the microbial community similarity. Three were from the new soil to ensure uniformity, named as New Soil a (A), New Soil b (B) and New Soil c (C). The others were from continuous cropped soil of 1 year (D), 2 years (E) and 3 years (F). The Bray-Curtis distance coefficient and Morisita distance coefficient of microbial communities among different soil samples were calculated according to the composition and quantity of microbial communities. The results are shown in Table 5.

Table 5. Bray-Curtis and Morisita dissimilarities between microbe communities in *P. notoginseng* replant soils

Ginseng planting years	New soil a(A)	New soil b(B)	New soil c(C)	Continuous cropping soil for 1 year(D)	Continuous cropping soil for 2 years(E)	Continuous cropping soil for 3 years(F)
New Soil a(A)		0.428	0.543	0.200	0.769	0.825
New Soil b(B)	0.356		0.432	0.491	0.759	0.889
New Soil c(C)	0.004	0.295		0.384	0.823	0.920
1 year(D)	0.009	0.397	0.010		0.832	0.878
2 years(E)	0.882	0.402	0.789	0.920		0.400
3 years(F)	0.873	0.338	0.772	0.914	0.185	

Note: Data in the top right and down left are Bray-Curtis and Morisita dissimilarities, respectively. Three soils were from the new soil to ensure uniformity, named as New Soil a (A), New Soil b (B) and New Soil c (C). The others were continuous cropped soil for 1 year (D), for 2 years (E) and for 3 years (F). The same below.

Polar ordination analysis on microbial communities, according to the Bray-Curtis distance coefficient and Morisita distance coefficient matrix was performed. The results are shown in Table 6. The Euclidean distance between six communities was obtained by ordering coordinates and then correlation analysis was done with the Euclidean distance between the original community. The correlation coefficient of Bray-Curtis measure and Morisita measure were 0.9214 and 0.9436 between two Euclidean distance coefficient matrices, indicating that the sorting results were good. Thus, the pole ranking results based on Bray-Curtis and Morisita two distance coefficients, could well reflect the dissimilarity between the original communities.

Table 6. Taxis of extremity for different communities

Community Number	Bray-Curtis Measure （R=0.9214）			Morisita Measure （R=0.9436）		
	X	Y	Deviation	X	Y	Deviation
New Soil a(A)	0.251	0.000	0.481	0.037	0.106	0.036
New Soil b(B)	0.132	0.130	0.411	0.458	0.000	0.228
New Soil c(C)	0.000	0.160	0.000	0.121	0.168	0.121
1 year(D)	0.122	0.063	0.365	0.000	0.086	0.000
2 years(E)	0.742	0.694	0.358	0.920	0.920	0.000
3 years(F)	0.920	0.920	0.000	0.895	0.503	0.184

An X-Y scatter diagram on pole sorting results is shown in Fig 1. From the position of each community in the coordinate plane, A, B, C and D were closer to each other and they could be seen as a community group; E, F could be seen as another community group. The two communities were far from each other. Thus it can be seen that, the community structure of soil microorganisms varied greatly with continuous cropping years. The microbial community structure of new soil and 1-year old soil was similar. The 2-years and 3-years soils were also similar. It further indicated that the microbial community composition was not significantly altered after 1-year-planting of *P. notoginseng*, but it changed significantly with increase in the number of cropping years. The results of Bray-Curtis Measure and Morisita Measure therefore have good consistency.

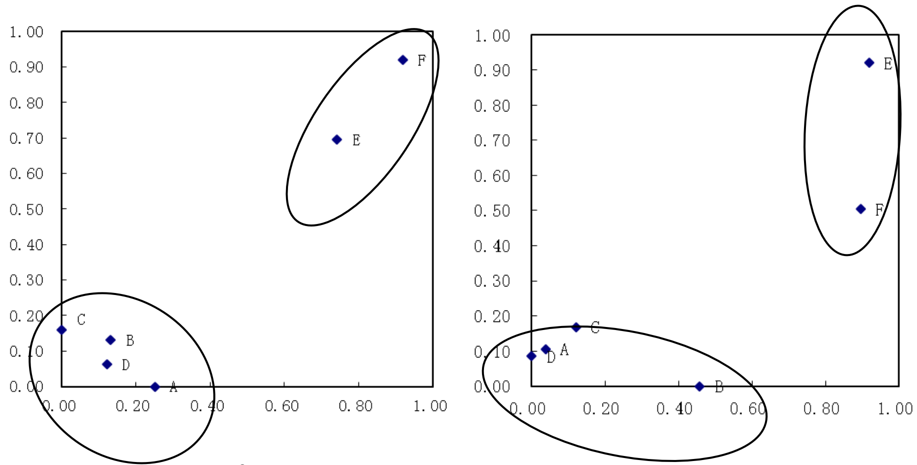

Figure 1. X-Y scatter plot of pole sorting results.
The left plot was according to Bray-Curtis Measure and the right plot was according to Morisita Measure.

The shortest distance method was used to cluster the Bray-Curtis and Morisita distance coefficient matrices and the results are shown in Fig 2. From the Bray-Curtis Measure cluster analysis, it was suggested that the community A and D were the closest, the two communities initially clustered together at a class spacing of 0.200 and then clustered with community C at 0.384. Community E and F gathered at 0.400 and then community A, D, C and B jointly clustered together at 0.428. When the class spacing was 0.759, six communities could be clustered into 2 groups, including community A, B, C, D as one group, community E and F as another group. From Morisita Measure cluster analysis, it is suggested that the community A and C were the closest, the two communities first clustered together at a class spacing of 0.004 and then clustered with community D at 0.009. Community E and F gathered at 0.185 and then community A, C, D and B jointly clustered together at 0.295. When the class spacing was 0.338, 6 communities could be clustered into 2 groups, including community A, B, C, D as one group, community E, F as another group. The results of cluster analysis of Bray-Curtis and

Morisita distance coefficients were essentially in agreement and showed the same trend with the pole sorting method.

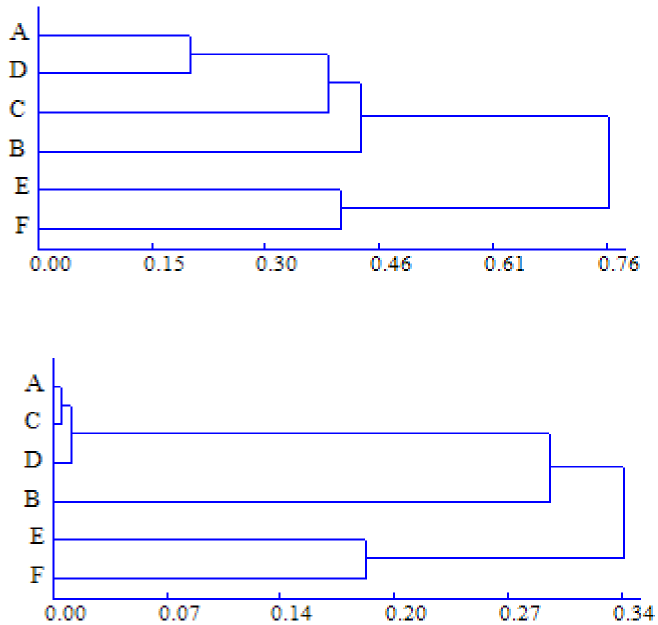

Figure 2. Hierarchical clustering analysis diagram of soils with different replant years. The above figure was according to Bray-Curtis distance coefficient and the below figure was according to Morisita distance coefficient.

Earlier studies have shown that soil microbial community function was different in continuous cropping years (12). According to the results of pole sorting and clustering analysis combined with the composition and diversity of microbial communities, 6 soil microbial communities could be divided into two large soil groups: the new soil and the 1-year soil were similar and it was high microbial diversity-bacteria type soil and the 2-years and 3-years soils were also similar, being low soil microbial diversity-fungal type soil. It was concluded that successive planting of *P. notoginseng* will reduce the diversity of soil microbes and continuous cropping for more than two years, significantly changes the soil microbial community structure. These results are similar to those obtained by Wu (24), in continuous cropped cucumber soil microbial communities.

We recognize that the method used to determine the soil microorganism is very restrictive as dilution plating does not allow one to identify a very large number of microorganisms that are not culturable (20). Also, our results which show the number of bacteria to be absent are perhaps only a reflection of the method and may not reflect the true situation. Our analysis and conclusions are primarily based on the small number of culturable soil organisms. Despite these deficiencies, analysis of the results by using various indices suggests that significant changes occur in the soil microbial status both during April and August and in the continuously *P. notoginseng* planted soils. The changes seen herein, however, need further examination by using modern methodologies to understand the dynamics of microorganism in the root zones of *P. notoginseng.* That may establish that the present findings are a true reflection and that soil microorganism have role in replant problem**.**

CONCLUSIONS

The soil microbial change is an important phenomenon in the continuous cropping of *P. notoginseng* with different period and years. In the emergence period, the main soil microbial community was "fungal type", while in the flowering period, the main soil microbial community was "bacterial type". Continuous cropping for > 2 years changed the soil microbial community, the 2-years continuous cropping soil belong to low soil microbial diversity-fungal type, while the new soil and the 1-year cropping soil belong to high microbial diversity-bacterial type. The above changes are perhaps one of the core factors for *P. notoginseng* soil sickness.

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