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Original article

Influence of Allelochemicals on Microbial Community in Ginseng Cultivating Soil

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ABSTRACT

Objective To study the influence of allelochemicals such as benzoic acid, diisobutyl phthalate, diisobutyl succinate, palmitic acid, and 2,2-bis-(4-hydroxyphenyl) propane on the microbial community of ginseng cultivating soil. **Methods** Soil samples were sprayed with five allelochemicals (100 mg/L) and their mixture (20 mg/L), respectively. Carbon metabolic ability variances were analyzed by Biolog method and genetic polymorphism variance was analyzed by RAPD method. The Nei's genetic diversity index and Shannon's information index were calculated. **Results** Significant differences in carbon metabolic activity were found between allelochemical-treated soils and control. Further analysis indicated that although the carbon-utilizing ability and genetic polymorphism of soils treated with di-isobutyl phthalate, di-isobutyl succinate, and allelochemical mixtures were lower than those of the other treatments, genetic similarities of soils treated with di-isobutyl phthalate, diisobutyl succinate, and allelochemical mixtures were much higher than those of the other treatments. **Conclusion** Allelochemicals significantly declined the genetic diversity and carbon metabolic activity of microorganisms in newly reclaimed forest soil for ginseng cultivation.

Key words

allelochemicals; Biolog; *Panax ginseng*; RAPD; soil microorganisms

1. Introduction

Commercial *Panax ginseng* C. A. Meyer, one of the most famous Chinese medicinal herbs, is now mainly dependent on artificial cultivation. However the production of ginseng is severely impeded by continuous cropping obstacles. From sowing to harvest, no more than 60% of ginseng plants can be survived (Wu et al, 2008), meaning that the direct economic loss is about 12 billion Yuan per year, according to the mean annual ginseng yield of 20 thousand tons (Cui and Jin, 2013). Therefore, continuous cropping obstacles of ginseng have been intensively studied. Unfortunately the mechanism and action are not yet clear. Some researchers believed that the yield loss of replant crops was closely related to allelopathy (Wang et al, 2007), in which allelochemicals inhibited the normal growth, development, and metabolism of plants when they were released into adjacent soil through eluvial deposits, secretion, decomposition, etc (Lin et al, 2007). Some researchers

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believed that the microorganisms and related enzymes in soil played the important roles in the process of mineral transformation, soil fertility recycling, enzymatic activity, etc, which were significantly influenced by the crops cultivated over ground (Wu, 2010). The relationship of allelochemicals, soil microorganisms, and plants are complicated in continuous cropping obstacles.

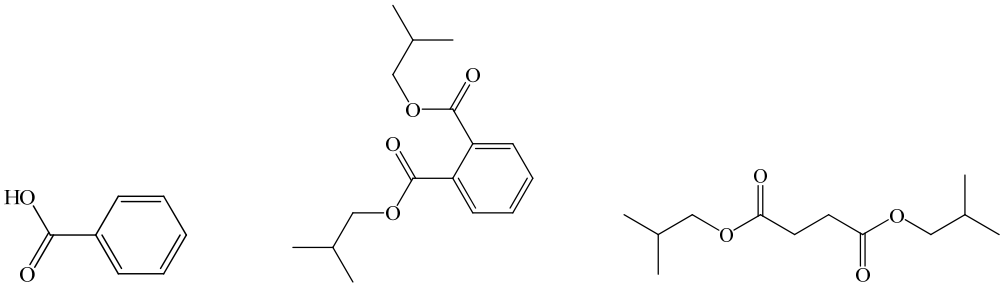
Over the past years, continuous cropping obstacles of *P. ginseng* were investigated in the following aspects, including soil nutrient (Zhang et al, 2010; Li et al, 2010), beneficial microorganisms (Jin et al, 2010), microbial diversity (Guo et al, 1990), etc. Recently, researchers focused on the relationship of allelochemicals and soil microorganisms in ginseng cultivating soil. Five allelochemicals including benzoic acid, di-isobutyl phthalate, di-isobutyl succinate, palmitic acid, and 2,2-bis-(4-hydroxyphenyl) propane were detected from ginseng rhizosphere soil and root exudates simultaneously, which inhibited the germination of ginseng seeds, growth of ginseng plants (Li et al, 2011), enzyme activity of ginseng root (Huang et al, 2010; 2013), total ginseng saponins content (Huang et al, 2009b), and mycelial growth and spore germination of ginseng pathogens (Li et al, 2009a). Therefore, we supposed that the soil microbial community change (Li et al, 2009b) and autotoxicity (Li et al, 2008; Huang et al, 2009a) were the main obstacles of successful continuous cropping of ginseng. Although a few researchers (Fang, 2012) have carried out the investigation on the relationship of soil microbial diversity and plant allelochemicals, they mainly concentrated in the influences of allelochemicals on soil enzyme activity and the metabolic function, and these reports were lack in the comprehensive analysis among the relationship of allelochemical accumulation, microbial community diversity, and carbon metabolic function.

However, the mechanisms of microbial community influenced by allelochemicals and the interrelationship of allelochemicals, soil microbial community, and diversity of microorganisms in ginseng-cultivating soil are still unknown yet. In the present research, five allelochemicals were added artificially into newly reclaimed forest soil and their influence on genetic diversity and carbon source metabolism diversity of the soil microbial community were studied. The results would be helpful for the further interpretation of the relationship among allelochemicals, soil microbial community, and diversity of microorganisms in ginseng-cultivating soil, which would help us to understand the formation mechanism of ginseng continuous cropping obstacle.

2. Materials and methods

2.1 Experimental design

The forest soil which was newly reclaimed for ginseng cultivation was sampled from Fusong county of Jilin province, China. Allelochemicals including benzoic acid (**1**), di-isobutyl phthalate (**2**), di-isobutyl succinate (**3**), palmitic acid (**4**), and 2,2-bis-(4-hydroxyphenyl) propane (**5**) (Figure 1) were used in the experiment and diluted by sterile water to 100 mg/L. This concentration was proposed to be high enough to affect the soil, because it was about the highest content of allelochemicals in rhizosphere soil samples examined in previous experiments. These five allelochemical solutions were mixed equally and taken as the allelochemical mixture (M). Each kilogram of soil was sprayed with 10 mL allelochemicals separately and then mixed thoroughly. Each treatment soil (about 2 kg) was filled into a plastic pot (25 cm × 15 cm) with three replicates which were then put on a shelf in a shaded shed to maintain the moisture for three months until sampling.



benzoic acid (**1**) di-isobutyl phthalate (**2**) di-isobutyl succinate (**3**)

palmitic acid (**4**) 2,2-bis-(4-hydroxyphenyl) propane (**5**)

Figure 1 Chemical structures of allelochemicals tested

2.2 Biolog analysis

Each moist soil (10 mg) sampled from the center of each plastic pot 10 cm under the soil surface was added into 90 mL physiological saline solution (0.85%), vortex vibrated at 120 r/min for 30 min and gradient diluted to 1000 times by physiological saline solution. Then 150 μL of each final diluted solution was added into all holes on Biolog^{TM} Eco plates, which were then cultivated at 25 ^{o}C for 7 d. The light absorption value at 590 nm (A_{590}) in each 24 h interval was read automatically by OmniLog Plus and experimental data were recorded by MicroStation^{TM} systematically.

Average well color development (AWCD) was used to analyze the utilization of carbon sources and the formula was shown as follows.

$$AWCD = \frac{\sum Ai - AA1}{31}$$

where "*Ai*" indicates the absorption value of the "*i*"th hole, "A_{A1}" indicates the absorption value of the "*A1*"th hole.

$$\text{Shannon index } (H') = \sum_{i=1}^{s} pi \cdot \ln pi$$

where "s" indicates the total species of microorganisms, "*pi*" indicates the ratio of the absorption value in the "*i*"th hole and total absorption value, i.e. $pi = (C–R) / \sum(C–R)$.

$$\text{Evenness index } (E) = H' / \ln S$$

where "*S*" indicates the total number of carbon sources utilized by soil microorganisms.

The absorption value (OD) ≥ 0.2 for a single hole was regarded as positive and taken into diversity *S*. *H*′ and *E* were used to indicate the diversity of soil microorganisms. Software SPSS 17.0 and SIMCA-P 11.5 Demo were used for diversity index calculation and principal component analysis (PCA).

2.3 RAPD analysis of soil microorganisms

Total genomic DNA was extracted by E.Z.N.A. kit (Omega) and 0.8% agarose gel in 1 × TAE buffer (2 mol/L Tris, 50 mmol/L EDTA, 1 mol/L acetic acid, pH 8.0) was used to determine the quality of the extracted DNA.

Based on the screening results, 15 high polymorphism and reliability RAPD primers including OPA15, OPB15, OPB20, OPQ8, OPQ19, OPK3, OPK12, OPK13, OPR3, OPR4, OPR10, OPR13, OPR19, OPS4, and OPT2 were used for the genetic diversity analysis of soil microorganisms. DNA amplification was performed on T gradient 96 thermal cycler (Biometra); Reaction volume and amplifying programs were performed according to Li et al (2012). The amplicons were electrophoresed in 1.5% agraose gel. After being dyed by EB, the gels were photoed by Bio-Rad UV Gel Imager.

2.4 Data analysis

Software NTSYSpc 2.10e and unweighted pair-group method with arithmetic means (UPGMA) were used to construct the dendrogram. Software Popgene version 1.32 (32-bit) was used to analyze the Nei's genetic diversity indexes (*H*) and Shannon's information indexes (*I*). Data were statistically analyzed using software SPSS 15.0 and the analysis of variance (ANOVA) followed by the Fisher's protected LSD test was used to evaluate the significance of changes at $P < 0.05$ level. Excel 2003 was used to calculate the statistical data, and draw histograms and line charts.

3. Results

3.1 Biolog analysis

Biolog results showed that the treated soils were lower than the control in the AWCD value, indicating that the allelochemicals inhibited the reproduction of microorganisms in soil and decreased the total biomass of microorganisms (Figure 2). Except soil D showed higher carbon metabolic activity at 96 to 168 h, AWCD values of others were lower, and those of soils B and C were the lowest.

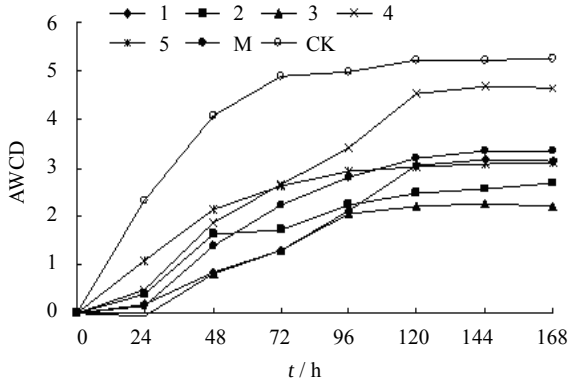


Figure 2 Changes of AWCD values of soil samples during cultivation on Biolog Eco plates

1: benzoic acid 2: di-isobutyl phthalate 3: di-isobutyl succinate 4: palmitic acid 5: 2,2-bis-(4-hydroxyphenyl) propane M: allelochemicals mixture CK: allelochemical-free sterile water same as below

Statistical results showed that the treatments were lower than the control in Shannon's index and Evenness index, indicated that the diversity and balance of different species in soils sprayed by allelochemicals were lower than those in the control. Except for benzoic acid (**1**) and allelochemical mixture (M) treated soils, Shannon's index and Evenness index of the other soils have no significant variance compared with the control, and significant variance were also present between benzoic acid and M^{-} treated soils (Table 1), indicating that benzoic acid significantly decreased microbial diversity and damaged the intrinsic and mutual restrictive balance of the microbial community in the soil environment.

Compared with the control, the allelochemical-treated soils were also lower than the control in carbon sources utilization efficiency of microorganisms. The polymers and carbohydrates utilization efficiency of 2,2-bis-(4-hydroxyphenyl) propane (**5**) treated soil, the carbohydrates utilization efficiency of di-isobutyl phthalate (**2**), palmitic acid (**4**), and 2,2-bis-(4-hydroxyphenyl) propane (**5**) treated soils, the carbohydrates utilization efficiency of palmitic acid

Table 1 *H′* and *E* of soil samples treated with allelochemicals

Soil treatments	*H'*	*E*
1	2.17 ± 0.32 a	0.70 ± 0.12 a
2	2.59 ± 0.23 bc	0.83 ± 0.10 bc
3	2.54 ± 0.17 bc	0.82 ± 0.03 bc
4	2.59 ± 0.10 bc	0.83 ± 0.06 bc
5	2.57 ± 0.05 bc	0.83 ± 0.03 bc
M	2.48 ± 0.03 b	0.80 ± 0.04 b
CK	2.76 ± 0.05 c	0.89 ± 0.03 c

Different lowercase letters indicate significance at $P < 0.05$ level

(**4**) treated soil, and the carboxylic acids utilization efficiency of di-isobutyl phthalate (**2**) treated soil were close to those of the control and showed significant difference from the other allelochemical-treated soils. The amine and phenolic acids utilization efficiency of soils sprayed with each allelochemical or allelochamical mixture were significantly lower than those of the control (Figure 3).

By principal component analysis (PCA), principal component 1 (PC1) and principal component 2 (PC2) were generated and their contribution rates on total variable were 26.78% and 22.71%, respectively. From the distribution on four quadrants based on their carbon metabolic characteristics, the soil samples tested were separated into four parts, part i including benzoic acid (**1**), 2,2-bis-(4-hydroxyphenyl) propane (**5**), and M treated soils, part ii including di-isobutyl

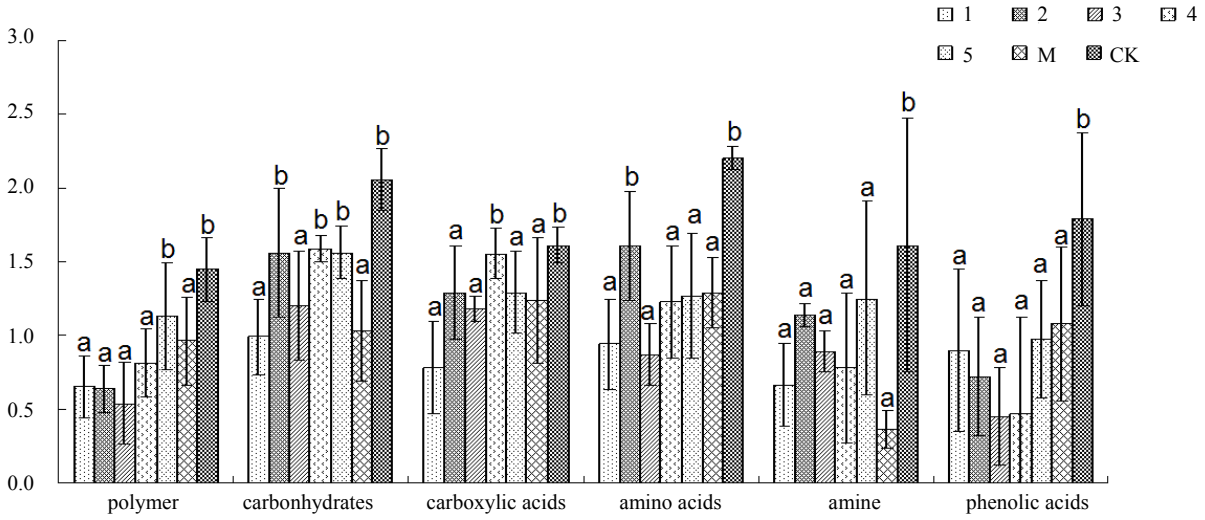


Figure 3 Utilization of carbon sources on Biolog Eco plate by microorganisms

Different lowercase letters indicate significance at $P < 0.05$ level; same as below

phthalate (**2**) and palmitic acid (**4**) treated soils, part iii and part iv only including di-isobutyl succinate (**3**) treated soil and CK, respectively (Figure 4). PCA results indirectly showed that, though soils sprayed with each allelochemical or M separated into three quadrants, they all tended to cluster around the origin of coordinates, and far away with the control, which indicated that the carbon utilization ability of soil microorganisms changed significantly when treated by alllelochemicals, and small differences were also present among soils sprayed allelochemicals (Figure 4).

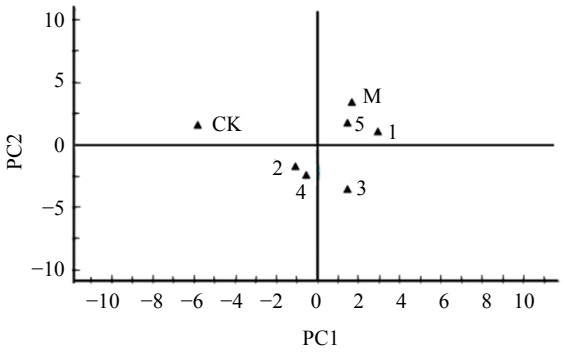


Figure 4 PCA of soils based on carbon sources utilization

3.2 Genetic diversity analysis of soil microorganisms

After amplified by RAPD primers, products were separated in agorase gel, distinctive and stable DNA bands from 100 to 1200 bp were recorded (Figure 5). In general, 205 distinct reliable DNA bands were amplified, 198 of them present polymorphism among soil samples with the average polymorphism rate of 96.59%. On average, 13.67 bands and 13.2 polymorphic DNA bands were amplified by one primer. Genetic analysis showed that the *H* and *I* of control were the highest (0.4998 and 0.6930, respectively). Indices of soils sprayed benzoic acid (**1**), palmitic acid (**4**), and 2,2-bis-(4-hydroxyphenyl) propane (**5**) were close to the control, and showing significant variance to soils sprayed di-isobutyl phthalate (**2**), di-isobutyl succinate (**3**), and M at $P < 0.05$ level (Table 2).

Clustering results from NTSYS and Popgene analysis were coincidental. The genetic similarity index of soils sprayed with palmitic acid (**4**) and 2,2-bis-(4-hydroxyphenyl) propane (**5**) was the highest (0.6765), those of the soils sprayed with di-isobutyl phthalate (**2**) and M were higher (0.6373 and 0.6569, respectively), and those of the soils sprayed with di-isobutyl succinate (**3**) and palmitic acid (**4**)

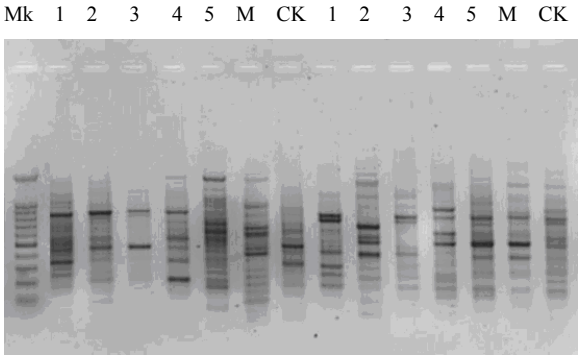


Figure 5 Electrophoresis of RAPD amplifying products on agarose gel

Mk: 100−1200 bp DNA ladder 1−5: treated with benzoic acid, di-isobutyl phthalate, di-isobutyl succinate, palmitic acid, and 2,2-bis-(4-hydroxyphenyl) propane, respectively M: allelochemicals mixture CK: allelochemical-free sterile water. The group left and the group right were amplified by different RAPD primers.

Table 2 Genetic polymorphism of soil microorganisms

Soil treatments	*H*	*I*
1	0.4983 ± 0.027 a	0.6914 ± 0.062 a
2	0.3938 ± 0.043 b	0.5829 ± 0.053 b
3	0.4444 ± 0.070 b	0.6365 ± 0.027 b
4	0.4998 ± 0.100 a	0.6930 ± 0.046 a
5	0.4969 ± 0.054 a	0.6901 ± 0.032 a
M	0.4723 ± 0.03 b	0.6652 ± 0.024 b
CK	0.4998 ± 0.05 a	0.6930 ± 0.035 a

were the lowest (0.4902). Taken 0.61 as the threshold value, seven kinds of soils were separated into three parts, part i including soils sprayed benzoic acid (**1**), palmitic acid (**4**), 2,2-bis-(4-hydroxyphenyl) propane (**5**), and CK; part ii including soils sprayed with di-isobutyl phthalate (**2**) and M; and part iii only including soil sprayed with di-isobutyl succinate (**3**) (Figure 6). Clustering results showed that the influence on soils treated by allelochemicals di-isobutyl phthalate (**2**), di-isobutyl succinate (**3**), and M were the highest, soils spayed with palmitic acid (**4**) and 2,2-bis-(4-hydroxyphenyl) propane (**5**) were influenced by allelochemicals palmitic acid (**4**) and 2,2-bis-(4-hydroxyphenyl) propane (**5**) moderately, and the influence of soil treated by benzoic acid (**1**) was the lowest.

4. Discussion

All results elucidate that the allelochemicals severely decrease the diversity of microorganisms in ginseng fresh soil. RAPD analysis has shown that the allelochemicals have evident influence on the genetic construction of microbial communities, and the Shannon's index of control was significantly higher than those of the soils sprayed with allelochemicals, probably resulting in an imbalance of the microbial ecological system of soils. Biolog analysis also showed that the characteristics of carbon sources utilization of control have significant differences from soils sprayed with

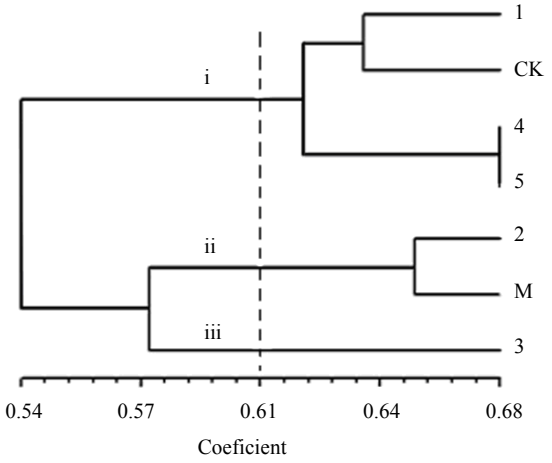


Figure 6 UPGMA dendrogram of soil samples

allelochemicals. In the present research, we also found that the clustering results calculated by RAPD results in Figure 6 and Biolog results in Figure 4 were not identical, which indicated that the ribosomal DNA based genetic polymorphism can only reveal the change of microbial composition, and carbon metabolism based polymorphism can only reveal the change of carbon utilization by soil microorganisms. For soil microorganisms, the genetic variance was not simply equal to the ecological function change. In general, if there is only a minor change in microbial community, the metabolic function may be replaced by microorganisms that have similar ecological function, and the evident changes on the total metabolic profile will not happen (Marshall et al, 2011; Nannipieri et al, 2003).

The results of carbon sources metabolic analysis indicated that although there were evident standard deviations among replicates from the mean of different treatments and overall variation tendency, we could conclude that the allelochemicals used in the present research also significantly influenced the intrinsic carbon metabolic function of microorganisms in the soil. The similar results were also reported by Ma et al (2005). Furthermore, we found that the influence on the carbon metabolic function of micro-organisms was correlated with the chemical structures of allelochemicals. For example, benzoic acid (**1**), 2,2-bis-(4-hydroxyphenyl) propane (**5**), and M separated into a quadrant have benzene ring and oxhydryl, and di-isobutyl phthalate (**2**) and palmitic acid (**4**) separated into a quadrant have ester group. Though carboxyl-containing di-isobutyl succinate (**3**) was separated into another quadrant, it was adjacent to di-isobutyl phthalate (**2**) and palmitic acid (**4**). So we deduced that the allelochemicals played a directional selection role to microorganisms living in the soil. In addition, the different clustering results were found between RAPD and Biolog analysis. Therefore, high throughput sequencing technique is suggested to establish the relationship between the changes in microbial community treated with allelochemicals and the changes of carbon metabolic function that generated by Biolog analysis.

References

Cui ZR, Jin LX, 2013. Status and development strategy of ginseng industry in China. *Rural Econ Sci Technol* 24(3): 51-52.

Fang SW, 2012. Ginseng allelochemicals' impact on soil enzyme activities and microbial diversity, as well as ginseng allelochemicals' mobility in soil. Jilin Agricultural University, Changchun.

Guo SH, Wang YX, Geng YQ, Wang YZ, 1990. Effect of the ecological distribution of soil microorganisms by ginseng-cultivating. *Acta Ecol Sin* 10: 286-287.

Huang LL, Cheng WS, Chen YD, Hou F, 2013. Research progress in ginseng fingerprint. *Chin Tradit Herb Drug* 44(2):241-246.

Huang XF, Li Y, Ding WL, 2009a. Autotoxicity effect of ginseng root exudates on seeds germination. *Seed* 28: 4-7.

Huang XF, Li Y, Liu SL, Ding WL, Fu JF, 2009b. Effects of five allelochemicals on ginsenosides in ginseng seedling roots. *World Sci Technol / Mod Tradit Chin Med Mater Medica* 11: 70-74.

Huang XF, Li Y, Yi XX, Ding WL, 2010. Effects of five allelochemicals on enzyme activity in *Panax ginseng* roots. *Chin Tradit Herbal Drugs* 41: 117-121.

Jin JR, Ma QL, Wang XQ, Dou S, Wang QB, 2010. Study on effect of ecology method on *Panax ginseng* continuous cropping ground soil improvement. *Ginseng Res* 1: 35-38.

Li Y, Huang XF, Ding WL, 2011. Autotoxicity of *Panax ginseng* rhizosphere and non-rhizosphere soil extracts on early seedlings growth and identification of chemicals. *Allelopathy J* 28: 145-154.

Li Y, Liu SL, Huang XF, Ding WL, 2009a. Allelopathy of root exudates on pathogens of ginseng. *Acta Ecol Sin* 29: 161-168.

Li Y, Liu SL, Yi QQ, Fu JF, Ding WL, 2009b. Dynamic of microorganisms from root region of ginseng with different growing years. *Agric Sci Tech* 10: 141-143.

Li Y, Ying YX, Zhao DY, Ding WL, 2012. Microbial community diversity analysis of *Panax ginseng* rhizosphere and non-rhizosphere soil using randomly amplified polymorphic DNA method. *Open J Genet* 2: 95-102.

Li Y, Ying YX, Zhao DY, Jin S, Ding WL, 2010. Genetic diversity analysis on rhizosphere soil microbial population of *Panax ginsengand Panax quinquefolium* by RAPD. 41(11):1871-1875.

Li Y, Zhu DL, Huang XF, Ding WL, 2008. Inhibition of different soil extracts on growth of ginseng seeds. *Chin Tradit Herb Drugs* 17: 1070-1074.

Lin WX, Xiong J, Zhou JJ, Qiu L, Shen LH, Li ZF, Chen H, Hao HR, Chen T, Lin RY, He HB, Liang YY, 2007. Research status and its perspective on the properties of rhizosphere biology mediated by allelopathic plants. *Chin J Ecol Agric* 15: 74-81.

Ma YH, Wang XF, Wei M, Qi YF, Li TL, 2005. Accumulation of phenolic acids in continuously cropped cucumber soil and their effects on soil microbes and enzyme activities. *Chin J Appl Ecol* 16: 2149-2153.

Marshall CB, McLaren JR, Turkington R, 2011. Soil microbial communities resistant to changes in plant functional group composition. *Soil Biol Biochem* 43: 78-85.

Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G, 2003. Microbial diversity and soil functions. *Eur J Soil Sci* 54: 655-670.

Wang SQ, Han QZ, Qiao YF, 2007. Allelopathy of root exudates and their effects on rhizosphere microorganism. *Chin J Soil Sci* 38: 1219-1226.

Wu LJ, Zhao YH, Guan YM, Pang SF, 2008, A review on studies of the reason and control methods of succession cropping obstacle of *Panax ginseng* C. A. Mey. *Spec Wild Econ Anim Plant Res* 2: 68-72.

Wu WX, 2010. Study on the autotoxic substances of tobacco and the effects of autotoxic substances on the rhizospheric microbiology. Agricultural and Forestry University, Fuzhou.

Zhang YY, Sun H, Song XX, Sun CW, Hou L, 2010. Correlative study on the activity of enzyme and soil nutrient level in the different years of cultivate ginseng soil in farmland. *Spec Wild Econ Anim Plant Res* 4: 43-46.