0971-4693/94 Euro 10.00
Allelopathy Journal 36 (2): 197-212 (2015) *International Allelopathy Foundation* 2015
Tables: 6, Figs : 5

Effects of continuous monoculture of *Achyranthes bidentata* on microbial community structure and functional diversity in soil

T. CHEN[1,#], J. LI[1,#], L.K. WU[1], S. LIN[1], J. H. WANG[1], Z.F. LI[1], Z.Y. ZHANG[1] and W.X. LIN[1*]

School of Life Sciences,
Fujian Agriculture and Forest University, Fuzhou, Fujian 350002, China
E. Mail: wenxiong181@163.com

(Received in revised form: August 18, 2015)

ABSTRACT

We studied the structure and functional diversity of microbial community in soil under long term continuous monoculture of *A.bidentata*, using the Community-level physiological profiles (CLPP), phospholipid fatty acid (PLFA) analysis and terminal restriction fragment length polymorphism (T-RFLP) analysis. Biolog analysis showed that the catabolic diversity of 20-years monoculture was similar to control soil. In PLFA analysis, the Gram (-)/Gram (+) bacterial ratio showed no significant difference among the control and soils of 2-years and 20-years monocultures. The actinomycetes population was higher in 20-years monoculture than control and 2-years monoculture soils. Also, the Cy/Pre ratio (an indicator of physiological stress), was significantly higher in 2-years monoculture soil than control and 20-years monoculture soils. The abundance of probiotic bacteria was higher in 20-years monoculture soil than in 2-years monoculture. These results showed that the soil microbial environment remained in a good state under *A. bidentata* continuous monoculture system for a long time.

Keywords: *Achyranthes bidentata*, Biolog, CLPP, continuous monoculture, microbial community, Phospholipid Fatty Acid, PLFA, Terminal Restriction Fragment Length Polymorphism, T-RFLP

INTRODUCTION

In recent years, traditional Chinese medicine (TCM), owing to its reliable therapeutic efficacy, affordability and local availability has gained global acceptance (24,30,45). Currently, TCMs have become scarce resources because of growing market demand (24). Rising demand and prices have driven farmers to use more land for growing the medicinal plant species. However high-quality CHMs (Chinese herbal medicines) are produced only when cultivated in ideal climatic and soil conditions (26,52,56,57).

Generally, continuous monoculture of medicinal plants in the same fields, year after year, often results in significant reduction in yield and quality (25,26,28,41,50,54). *A. bidentata* is suitable crop for continuous monoculture. Its tubers dry weight and quality under 20-years continuous monoculture 14.29% higher than tubers from 1-year

Correspondence author; # Co-first author; [1]Fujian Provincial Key Laboratory of Agroecological Processing and Safety Monitoring, Fujian Agriculture and Forest University, Fuzhou, Fujian 350002, China.

monoculture (14,23). Li *et al.* (26) found that in a 50 year continuous monoculture system, the soil extracts activated the genes encoding the key enzymes involved in terpene and flavonoid synthesis, which increased the synthesis of stimulators that released the plants nutrients into the soil and thereby promoted the growth of *Achyranthes.* The stimulators in soil may originate not only from *Achyranthes* but also from the products of soil microorganisms (1,15). Earlier studies have shown that continuous monoculture not only alters the physical and chemical properties of the soil but also contributes to the development of diverse microbial flora in the soil (3,29,36,38,44,55). In addition, continuous monoculture can also disrupt the balance between pathogenic microorganisms and other soil microbes (3,7,58,59). Our previous study (51,52) on soil biological properties of continuously monocultured *R. glutinosa* and *P. heterophylla* suggested that continuous monoculture induces changes in the biodiversity of microbes and the function of soil ecosystem. In the soil of 2-years continuous monoculture, population of beneficial bacteria decreases significantly, but the population of pathogenic bacteria increases than control and newly planted soils (52).

This study aimed to examine the effects of *A. bidentata* monoculture for different lengths of time on the shifts in microbial community structure and catabolic diversity using Biolog, PLFA and T-RFLP.

MATERIALS AND METHODS

I. Collection of soil samples

Fields of different years of continuously monocultured *A. bidentata* plants were identified at the Wen Xian Agricultural Institute, Jiaozuo City, Henan Province (34°56′N, 112°58′E). This area is ideal for *A. bidentata* cultivation, has continental monsoon climate, annual average temperature of 14.3 ℃ and mean annual precipitation of 552 mm. The soil was alkaline. *A. bidentata* 'Niuqi', cultivar is planted widely in this region. The planting material is tuberous root, planted in April and harvested in October. Following harvest, the fields are kept fallow (not used to grow any other crop) till next April.

Three fields were selected i.e. (i). 20-years continuous monoculture field, (ii). 2-years continuous field and (iii). field with out *A. bidentata* cultivation and no farming. These three fields were 500-1000 m apart. Three soil samples from each field were collected (500 g, 25 cm depth) at random sites, from the 20-years and 2-years continuous monoculture fields and from the nearby field without *Achyranthes* cultivation as control (CK). Soil samples were mixed well and divided into two parts. One part (250 g) was air-dried, sieved (0.45 mm pore size) and kept at room temperature for determining soil physical-chemical properties. The other was used for biolog, PLFA and T-RFLP analyses.

Ⅱ. Soil physicochemical properties

Organic matter (SOM), pH, nitrogen (N), phosphorus (P) and potassium (K) of soils were determined as per Lu (32). Total N was determined by the Kelvin method (32). Total phosphorus and potassium by the H_2SO_4-$HClO_4$ method and NaOH melt-flame photometry, respectively. Available nitrogen was determined by alkali solution diffusion (32). Nitrate N was determined by using phenol disulfonic acid (32)**.** Available phosphorus was extracted using 0.5 M $NaHCO_3$ solution and determined by the Mo-Sb colorimetry

method (32). Available potassium was extracted using a 1 M NH_4OAC solution and determined by flame photometry (32). The potassium dichromate-sulfuric acid oxidation method was used to determine the organic matter content (32). Soil pH was determined by pH meter with water (soil and water 1:1 ratio) (32). The soil moisture content was determined by the gravimetric method (32). All soil parameters were calculated on dry weight basis. All tests were done in triplicates and expressed as the mean ± standard error (SE). The data of these indexes was analyzed based on soil dry weight and all data were subjected to analysis of variance using the Statistical Analysis System Programme (SPSS).

Ⅲ. Biolog method

The Biolog Eco Microplate^{TM} system (Biolog Inc., Hayward, CA, USA) was used to assess the community level physiological profiles (CLPP). Each plate had 96 wells consisting of three replicates of 31 sole carbon substrates and a water blank. (51). Five g of fresh soil was soaked in 100 mL distilled water containing 0.85% NaCl and rotated at 120 rpm for 30 min, then put in an ice bath for 2 min. The soil solution was centrifuged at 2000 rpm for 5 min. The supernatant was removed into a 100 mL sterile triangular bottle and then diluted into 1000-fold. The diluted solution (150 μL), was then added in each slot in the Biolog plate, preheated to 28°C. The plates were incubated at 25°C for 168 h. The colour in each well was recorded at 24 h intervals as optical density (OD) at 590 nm using a plate reader (Thermo Scientific Multiskan MK3, Shanghai, China). Average well-color development (AWCD) was used to indicate the microbial activity in each microplate and determined as under:

$$AWCD = [\sum(C–R)]/N,$$

Where, *C*: OD590 value of each well, *R*: OD value of control well, and *N*: 31 carbon substrates subdivided into 6-categories [polymers, carbohydrates, carboxylic acids, amino acids, amines and phenolic compounds (8)].

The OD value at 96 h was used for principal component analysis (PCA) and cluster analysis as described by Gomez *et al*.'s (12) and Han *et al.*'s (13).

Ⅳ. PLFA analysis

PLFA were extracted from the soil and derivatized as per Kourtev *et al.* (21) and Denef *et al.* (9). Phospholipids were methylated by methanolic KOH to form fatty acid methyl esters (FAME). The FAME solution was filtered through a 0.45 μm filter before injecting into a GC-MS system equipped with phase multi-purpose GC column chromatograph (Varian, Palo Alto, California, U.S.A., VF-5 ms, 30 m × 0.25 μm). The injection volume was 1 μL with the injector temperature was 280°C. Helium was the carrier gas with the flow rate adjusted to 1 mL min^{-1}. The oven temperature was initially programmed at 70°C for 1 min, then increased to 170°C at a rate of 20°C min^{-1} and stabilized at 170°C for 2 min, increased from 170°C to 280°C at a rate of 5°C min^{-1} and remained at 280°C for 5 min, and then increased from 280 to 300°C at a rate of 20°C min^{-1} and remained at 300°C for 1.5 min. MS data were acquired in the negative ionization mode. The full scan mass covered a range from 50 to 1000 m/z. The peaks were identified based on relative retention time vs. several external standards: a mixture of 26 Bacterial

Acid Methyl Esters (47080-U, Supelco Inc., Bellefonte, PA, USA), a mixture of 37-component FAME Mix (47885-U, Supelco Inc., USA) and several individual FAMEs (Larodan Inc., Malmö, Sweden). Individual PLFA was quantified by comparing peak area with that of the internal standard 19:0 (nonadecanoic methyl eater) of known concentration.

Twenty-five PLFAs were detected and named by the rules of fatty acid nomenclature described by Wilkinson *et al.* (49). PLFAs were classified into several broad taxonomic microbial groupings, according to group-specific characteristic of PLFAs. Branched and saturated PLFAs (a15:0, a17:0, i14:0, i15:0 and i16:0) were used to indicated Gram-positive bacteria (Gram (+)), while monoenoic, unsaturated and cyclopropyl PLFAs (16:1omega7c, 16:1omega9t, cy17:0, 18:1omega7c and cy19:0) were used as indicators of Gram-negative bacteria (Gram (–)). The methyl-substituted PLFAs, 10Mel 17:0 and 10Mel 18:0 represented the actinomycetes. PLFAs 18:1omega9c and 18:2omega6, 9 were regarded as biomarkers for fungi and PLFA 20:4omega6 represented protozoans (4,17,18,35). Straight-chain PLFAs (12:0, 13:0, 16:0, 18:0, 20:0, 23:0 and 24:0), 20:5omega3 and 22:1omega9t were used as biomarkers for non-specific PLFAs. The following ratios were also calculated: Gram (+)/Gram (−) PLFA ratio, fungal/bacterial PLFA ratio and cyclopropyl PLFA (cy17:0+cy19:0) to their metabolic precursors (16:1omega7c+18:1omega7c) ratio (cy/pre) which are indicators of physiological stress in microbial communities (2). The resulting levels of individual PLFAs were subjected to PCA and cluster analysis using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) through SPSS software Version 11.5 and DPS software Version 7.05, respectively.

Ⅴ. Terminal restriction fragment length polymorphism (T-RFLP) method

Total microbial DNA was extracted from soil samples using the high salt/SDS method (27). Bacterial 16S rRNA genes were amplified by PCR using the general bacterial primers 8F-FAM 5-AGAGTTTGATCCTGGCTCAG-3 and 1492R 5-GGTTACCTTGTTACGACTT-3. PCR reactions and purification of PCR products followed the method of Wang et al. (48). Purified bacterial 16S rRNA fragments were digested with restriction endonucleases *Rsa* I, *Hae* III, *Msp* I and *Alu* I, for 5 h at 37°C. The 2 μl aliquot of each restriction digest reaction was mixed with 12 μl of formamide and 0.5 μl of standard (GeneScan-1000ROX, Applied Biosystems, Foster City, CA, U.S.A.). Samples were denatured at 96°C for 4 min, chilled on ice, and then run on an automated ABI DNA sequencer (Model 3130, Applied Biosystems) to determine fragment sizes.

T-RFs were identified and quantified using GeneMarker Version 1.2 software. T-RFs that differed within ± 1 bp were considered to be identical. T-RFs ranged in size from 50 to 600 bp. The relative abundance of each T-RF was calculated as the individual T-RF peak area divided by the total area of all T-RF peaks. The fragments were classified using online T-RFLP analysis at the Ribosomal Database Project II website (47).

Statistical analysis

Statistical analysis of T-RFLP profiles was performed based on complete sample profiles. The T-RFLP profile matrix analysis program from the RDP II website was used to determine the similarity among T-RFLP profiles (34). The proportion of shared terminal fragments from T-RFLP analysis was used to calculate similarity coefficients (39). Subsequently, three commonly used indices (Shannon's diversity index, Margalef index, and evenness index) were calculated using the Bio-Dap software package following

standard procedures (33). The resulting levels of individual T-RF were subjected to PCA and cluster analysis using an UPGMA clustering algorithm through SPSS software version 11.5 and DPS software version 7.05, respectively.

RESULTS AND DISCUSSION

Soil chemical properties

The cultivation of *A. bidentata* soils improved the most physico-chemical properties [Total K (TK), available K (AK), nitrate N (N-N) and pH than control] of soil (Table 1). Total N (TN), total P (TP), total K (TK), ammonium N (A-N) and available P (AP) were significantly higher in 20-years monoculture soil than in the 2-years monoculture soil. However, the 2-years monoculture plots had significantly higher content of nitrate N and available K than the 20-years monoculture plots. There was no significant difference in organic matter (OM) and pH between 2-years and 20-years monoculture plots.

Table 1. Chemical properties of 3-soil samples

Samples	TN (g/kg)	TP (g/kg)	TK (g/kg)	A-N (mg/kg)	N-N (mg/kg)	AP (mg/kg)	AK (mg/kg)	OM (mg/kg)	pH
Control	0.48c	0.72c	7.01b	0.11c	17.68b	23.17c	228.67a	11.77b	8.12a
2-years monoculture	0.64b	1.02b	6.57c	0.13b	28.16a	31.69b	182.25b	12.40a	7.86c
20-years monoculture	0.77a	1.43a	7.34a	0.20a	12.48c	43.58a	114.87c	12.99a	7.98b

The values are on air dry weight basis. Different letters in columns indicate significant differences determined by Tucky's test ($P \leq 0.05$, n=3). TN: Total nitrogen, TP: Total phosphorus, TK: Total potassium, A-N: Ammonium nitrogen, N-N: Nitrate nitrogen, AP: Available phosphorus, AK: Available potassium and OM: Organic matter.

Biolog analysis

Except for amino acids and polymers, the 96 h AWCD data 4-substrates groups (amines, phenolic compounds, carboxylic acids and carbohydrates) were highest in 2-years monoculture soil (Fig. 1). The microbial communities from the control soil exhibited the lowest levels of amino acids, amines, polymers, phenolic compounds and carbohydrates utilization. The AWCD values of polymers showed no significant differences between the soils of 2-years and 20-years monocultures. Also, no significant difference was seen in the AWCD values (carboxylic acids) of the 20-years monoculture and control soil. The total of AWCD values of phenolic compounds, carboxylic acids and amino acids (Fig. 2) was the highest in the 2-years monoculture soil and lowest in the control soil.

The microbial community in the 2-years monoculture soil showed a greater potential for use of carboxylic acids, phenolic acids and amino acids. However, these communities declined in the 20-years monoculture soil. This may be because of the accumulation of acidic-compounds from root exudates in the 20-years monoculture soil was less than that in 2-years monoculture soil. This however needs further investigation. Singh and Mukerji (43) reported that the root exudates may eliminate soil bacteriostasis

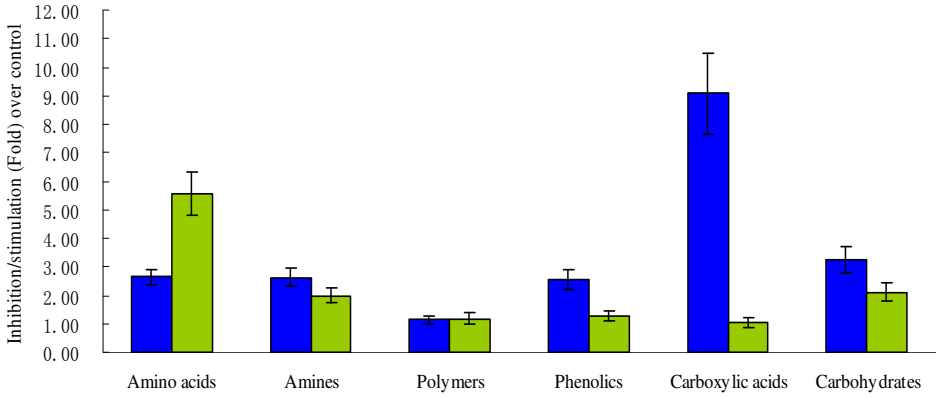

Figure 1. Inhibitory/stimulatory effects of monocultures on the physiological profiles of soils
2 Years monoculture, 20 Years monoculture

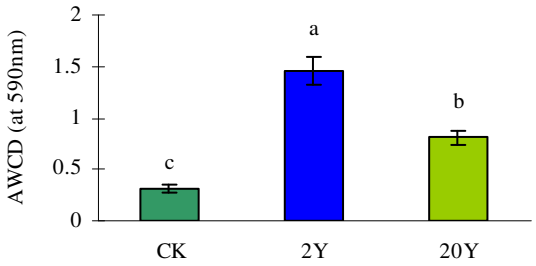

Figure 2. Sum of AWCD values of carboxylic acids, phenolic compounds and amino acids at 96 h.
2 Years monoculture, 20 Years monoculture

and act as chemo-attractants that selectively promote the growth of specific microbes. Wu *et al.*, (52) reported that under continuous monoculture, *R. glutinosa* releases a large number of low molecular weight components in the root exudates including sugars, carboxylic acids, amino acids and phenolics. Wu *et al.* (51) also reported that the microbial community showed a greater potential for use of carboxylic acids, phenolic acids and amino acids in the second and third year of *R. glutinosa* continuous monoculture. In these soils, some microbes gradually became dominant, suggesting that the soil microbial community structure in the second and third year of monoculture soils are similar compared with the soil community in the newly planted plants and control soils. However, the cluster results based on Biolog data in our present study revealed that the control and 20-years monoculture soils of *A. bidentata* share a similar soil microbial community structure.

Principal component analysis with Biolog data showed that 96 h AWCD data distinguished very well between the community responses of 3-soil samples to the carbon

substrates (Fig. 3). The first two principal components (PC), PC1 and PC2, accounted for 62.71% and 22.68% of the total variation in ECO microplate data, respectively. The PCs could clearly separate the catabolic diversity of the microbial community in three treatments (CK, 2-years and 20-years). Table 2 lists the five carbon substrates with the most negative and positive scores on PC1 and PC2. Cluster analysis (Fig. 3) showed the Euclidean distance of different soil samples, implying a shift in soil microbial community structure and catabolic diversity after *A. bidentata* continuous monoculture. The cluster results showed that the catabolic diversity of 20-years monoculture was close to that of control soil.

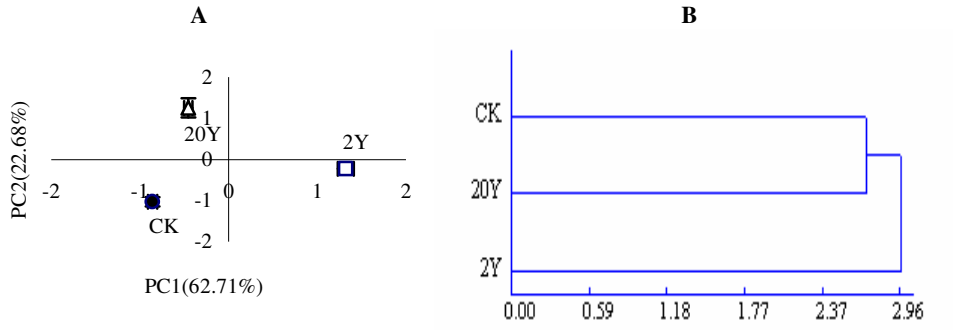

Figure 3. Classification of treatments by principal component (A) and UPGMA cluster analysis (B) with Biolog data.

Table 2. Most discriminant 5-carbon substrates as determined by PCA on the data of community level carbon source utilization

Substrate No.	PC1	Score	PC2	Score
1	Tween 40	-0.66	Tween 40	-0.75
2	Phenylethylamine	-0.60	i-Erythritol	-0.34
3	D-Malic Acid	-0.42	Tween 80	-0.29
4	D-Mannitol	-0.33	D-Xylose	-0.27
5	Glucose-1-Phosphate	-0.32	D, L-α-Glycerol Phosphate	-0.19
1	α-Cyclodextrin	1.00	L-Threonine	1.00
2	Glycogen	1.00	Glycyl-L-Glutamic Acid	1.00
3	Pyruvic Acid Methyl Ester	1.00	2-Hydroxy Benzoic Acid	0.99
4	γ-Hydroxybutyric Acid	1.00	L-Asparagine	0.98
5	α-D-Lactose	0.99	Glucose-1-Phosphate	0.95

A. bidentata and *R. glutinosa* are two well-known TCM plants grown in China and are predominantly planted in Henan Province. These two tuberous medicinal plants exhibit an opposite phenomenon during continuous monoculture. *A. bidentata is* monocultured continuously for more than 50-years at the same site (26), while *R. glutinosa* needs to be replanted once every 15-20 years (51,52). Recent studies have reported that soil health has a close relationship with soil microbial dynamics (3,29,37,53); Earlier, an imbalance of nutrients or the autotoxicity of root exudates were considered to be related to soil health (37,38,55). Studies with *R. glutinosa* continuous monoculture by Wu *et al.* (51)

suggested that there was no consensus on the relationship between soil nutrients and the problems experienced. Our studies with *A. bidentata* monocultured continuously confirm these results.

Phospholipid fatty acid profiles

PLFA could be used for fingerprinting soil microbial community by detecting shifts in broad groups of soil organisms, including bacteria, fungi and actinomycetes groups (16). Table 3 lists the concentrations of specific, non-specific and group-specific PLFAs in different soil samples. In all soil samples, the amounts of bacterial PLFAs were significantly higher than fungal and actinomycetes PLFAs. The levels of total PLFA, Gram(+) and Gram(−) bacteria, fungi, actinomycetes and the fungal/bacterial ratio were lowest in the control soil, except for the Gram(−)/Gram(+) ratio and Cy/Pre ratio (Fig. 4 and Fig. 5). The level of these PLFAs was highest in 2-years monoculture soil, except for actinomycetes and the fungal/bacterial ratio (Fig. 5). Many bacterial-specific PLFAs, including 15:0, a15:0, i14:0, i15:0, i16:0, 16:1omega7c, 16:1omega9t, cy17:0 and cy19:0, showed the same trend, and were highest in the 2-years monoculture soil.

Table 3. Concentrations of various specific PLFAs, sum of total, Non-specific and Group-specific PLFAs

PLFA	Control	2-years monoculture	20-years monoculture	Comment
1 15:00	5.60±0.29 c	8.90±0.14a	6.19±0.06b	Bacteria
2 a15:0	3.28±0.36b	5.13±0.04a	3.68±0.07b	Gram(+)
3 a17:0	8.28±0.39c	17.05±0.79a	9.50±0.20b	Gram(+)
4 i14:0	2.71±0.20b	3.24±0.08a	2.52±0.06b	Gram(+)
5 i15:0	3.96±0.73b	4.95±0.12a	3.96±0.13b	Gram(+)
6 i16:0	16.75±0.91c	22.10±0.56a	19.74±0.57b	Gram(+)
7 16:1omega7c	12.09±0.97c	20.61±0.31a	17.53±0.52b	Gram(−)
8 16:1omega9t	8.31±0.63b	9.06±0.38a	7.16±0.41c	Gram(−)
9 18:1omega7c	1.40±0.25a	1.36±0.11a	0.92±0.19b	Gram(−)
10 cy17:0	1.46±0.20c	2.90±0.03a	2.25±0.11b	Gram(−)
11 cy19:0	7.38±0.39b	12.29±0.93a	7.40±0.29b	Gram(−)
12 10Me17:0	0.89±0.02c	1.49±0.15b	1.87±0.08a	Actinomycete
13 10Me18:0	2.22±0.17c	2.99±0.12b	3.42±0.12a	Actinomycete
14 18:1omega9c	25.46±2.15c	35.63±3.65a	29.56±1.80b	Fungi
15 18:2omega6,9	7.52±0.71c	17.06±2.18a	14.54±0.56b	Fungi
16 20:4omega6	2.55±0.16b	3.02±0.13a	2.95±0.10a	Protozoan
17 Non-specific	23.07±0.88b	30.39±1.22a	24.12±1.08b	Bacteria
Total PLFA	133.27±8.05c	198.17±2.90a	156.33±3.97b	1-17
Gram(+)	34.98±1.79c	52.47±0.82a	39.40±1.85b	2-6
Gram(−)	30.65±1.87c	46.22±0.66a	35.28±0.93b	7-11
Bacteria	71.57±4.44c	107.59±1.11a	79.87±1.76b	1-11
Actinomycete	3.11±0.19c	4.48±0.24b	5.29±0.55a	12-13
Fungi	32.97±2.86c	52.69±0.83a	44.10±1.23b	14-15
Gram(−)/Gram(+) (%)	88.49±2.81a	88.10±1.93a	89.85±0.84a	
Fungi/Bacteria (%)	46.03±1.33c	48.98±0.75b	55.21±0.69a	
Cy/Pre (%)	65.79±4.39b	69.13±1.17a	52.32±1.67c	(10+11)/(7+9)

Concentration expressed in (nmol g^{-1} d.m. soil)

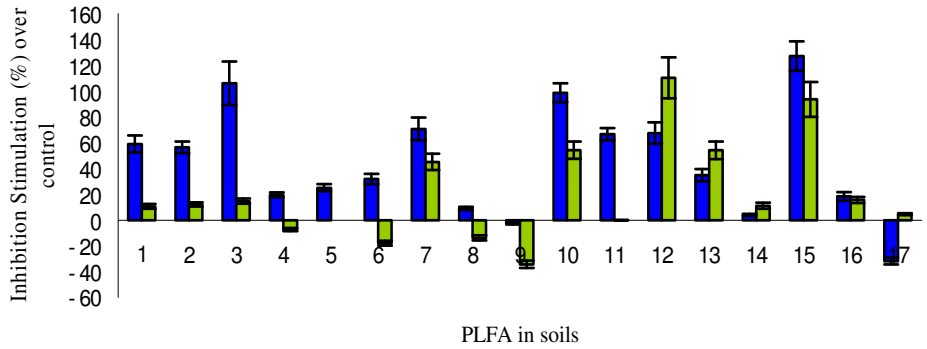

Figure 4. Inhibitory/stimulatory effects of monocultures on PLFA of soils. 2 Years monoculture, 20 Years monoculture

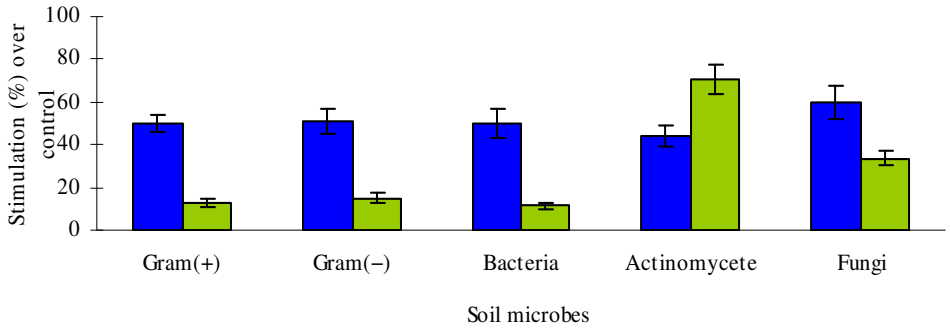

Figure 5. Inhibitory/stimulatory effects of monocultures on Gram (+), Gram (-), Bacteria, Actinomycetes and Fungi. 2 Years monoculture, 20 Years monoculture

Also the levels of total PLFAs, Gram positive and Gram negative bacteria, actinomycetes and fungi were much higher in the second and third year of *R. glutinosa* continuous monoculture soils than in the control and in newly planted soils (51). Gram negative bacteria in particular, are prone to feed on low molecular weights carbon compounds exuded by the plants roots (22). Termorshuizen and Jeger (46) found that many fungi and Gram negative bacteria were shown to be pathogenic towards plants. A High Gram(−)/Gram(+) and fungal/bacterial ratio of PLFA could reflect that the soil is unhealthy (2,51). However, the Gram(−)/Gram(+) ratio showed no significant difference between the control and soils of the 2-years and 20-years monocultures. Also, the fungal/bacterial ratio was lower in the control and 2-years monoculture soils than in the 20-years monoculture soil.

In addition, the Cy/Pre ratio (cyclopropyl fatty acids/monoenoic precursors ratio) of soils could be grouped as: 2-years monoculture > control > 20-years monoculture. This finding is different from that observed in *R. glutinosa* continuous monoculture. Wu *et al.* (51) found that the Cy/Pre ratio significantly increased with the increasing years of

monoculture for *R. glutinosa*. Several studies (2,10,35) have reported that when microbial communities suffered physiological stress, the *cis*-monounsaturated precursors (16:1omega7c, 18:1omega7c) could modify into the more stable cyclopropyl fatty (cy17:0, cy19:0) for maintaining a functional living membrane during stress.

The level of actinomycetes was highest in 20-years monoculture than in the other treatments analyzed here. Most actinomycetes function as antagonistic microorganisms against to soil-borne pathogens (42). The results of PLFA analysis indicated that continuous monoculture had no negative effect on the structure of the microbial community in soils of a monoculture system of *A. bidentata*.

Principal component analysis was applied to analyze the PLFA data from different soil samples. PC1 and PC2 accounted for 60.25% and 20.59%, of the total variation in the PLFA data, respectively. The Gram(−), Cy/Pre ratio and 18:1omega7c, 10Me17:0 were discriminated most positively with PC1 and PC2 scores, respectively, while 18:1omega7c, fungal/bacterial ratio and Cy/Pre ratio, actinomycetes were discriminated most negatively with PC1 and PC2 scores, respectively. The control and 20-years monoculture soils had similar characteristics of, indicating that they might share similar soil microbial community structures.

T-RFLP analysis of bacterial communities

T-RFLP was used to further study the shift occurring in bacterial communities in the soil of *A. bidentata* under continuous monoculture. Bacteria account for 70% of the microorganisms in the soil and constitute the microbial fraction that is most active in promoting energy flow and nutrient cycling (47). Changes in bacterial populations and activities could serve as excellent indicators of changes in soil health (3,11,19,20,31,44). In the present study, based on the *Rsa1*'s T-RF data, diversity index (Table 4) and principal component analysis were applied to analyze the shift in the bacterial communities of the three soil samples. The diversity indices showed no significant difference between the soils of the 2-years and 20-years monocultures (Table 4). The diversity indices of the control soil were the lowest of the three soil samples, while the diversity indices showed no significant difference between soils of the 2-years and 20-years monocultures, except for the Shannon Index.

Table 4. Diversity index of bacterial community in the *A. bidentata* soil under continuous monoculture

Cropping System	Simpson	Shannon	Pielou	Brillouin	McIntosh
Control	0.9529b	5.4248c	0.8709b	4.6086b	0.8437b
2-Years Monocolture	0.9850a	6.0837a	0.9118a	5.1387a	0.9301a
20Y- Monocolture	0.9782a	5.9990b	0.9069a	5.0521a	0.9100a

PC1 and PC2 accounted for 53.55% and 46.45%, respectively, of the total variation in the *Rsa1*'s T-RF data. Eight and seven T-RF digested by *Rsa1* were discriminated most positively with PC1 and PC2 scores (Table 5 and Table 6), respectively; also, six and three T-RF digested by *Rsa1* were discriminated most negatively with PC1 and PC2 scores (Table 5 and Table 6), respectively. These bacteria identified from the T-RF with the most positive and negative with PC1 and PC2 scores had functions related

Table 5. The predominant T-RF fragments of microorganisms related to principal component 1

Functions	Phylum	RsaI	HaeIII	MspI	AluI	Organism name	CK	2Y	20Y	Correlation coefficient
N metabolism	Proteobacteria	110	195	152	210	*Bradyrhizobium japonicum*	0	0	1.85	0.99**
S cycling	Proteobacteria	116	191	161	141	*Rhodomicrobium vannielii*	1.22b	1.12b	2.40a	1.00**
N metabolism	Proteobacteria	153	77	82	235	*Thauera*	0	0	0.93	0.99**
Probiotics	Phylum acilicutes Nitrospira	167	39	494	72	*Pseudomonas mendocina*	0	0	0.94	0.99**
Probiotics	Firmicutes	454	307	143	73	*Bacillus subtilis.*	2.21b	1.32c	5.64a	0.96*
Probiotics	Proteobacteria	485	231	554	73	*Bacillus*	5.58b	3.32c	15.47a	1.00**
N metabolism	Proteobacteria	105	189	399	204	*Rhizobium*	0.72b	1.12a	0	-0.97*
C metabolism	Bacteroidetes	112	255	487	201	*Cytophaga*	1.19a	1.20a	1.03b	-1.00**
S cycling	Proteobacteria	487	199	164	213	*Desulfovibrio alcoholovorans str.*	2.22b	2.78a	0	-1.00**
C metabolism	Firmicutes	490	205	173	436	*Selenomonas sputigena*	2.46b	2.61a	2.23c	-0.96*

Y: Years

Table 6. The predominant T-RF fragments of microorganisms related to principal component 2

Functions	Phylum	RsaI	HaeIII	MspI	AluI	Organism name	CK	2Y	20Y	Correlation coefficient
C metabolism	Clostridiales	65	286	235	87	*Eubacterium ruminantium*	0	1.42a	0.67b	0.99*
Saprophytes	Actinobacteria	80	67	142	220	*Nocardioides*	1.00c	1.82a	1.61b	0.99*
Facultative nutrition	Proteobacteria	107	39	438	207	*Rhodospirillum rubrum*	0	1.07a	0.69b	1.00**
S cycling	Proteobacteria	243	205	164	218	*Desulfobulbus*	0	1.33a	0.57b	0.98*
C metabolism	Spirochaetes	300	208	284	71	*Spirochaeta zuelzerae*	1.11a	0	0.72b	-0.96*
C metabolism	Bacteroidetes	308	39	87	200	*Caprocytophaga sputigena*	1.47a	0.93c	1.04b	-0.98*
Probiotics	Firmicutes	476	223	139	151	*Bacillus edaphicus*	3.92a	1.85c	2.41b	-0.97*

to C metabolism, N metabolism, S cycling, and as probiotics, pathogen and saprophytes. The abundance of potential pathogens (*Clostridium paraputrificum* and *Clostridium* spp.) in 20-years soil was significantly higher than that in 2-years monoculture soils.

The abundance of probiotic organisms (*Pseudomonas mendocina*, *Bacillus subtilis* and *Bacillus edaphicus*) were higher in 20-years monoculture soil than those in 2-years monoculture. However, Chen *et al.* (6) found that the abundance of beneficial decreased while and detrimental microbes increased, in the soil of *R. glutinosa* during continuous monoculture. The denitrifying bacteria and anaerobic cellulose decomposing bacteria increased, while the aerobic cellulose decomposing bacteria and probiotic bacteria decreased. *Pseudomonas mendocina*, *Bacillus subtilis* and *Bacillus edaphicus* have been reported to be antagonistic bacteria to soil-borne pathogens (42). Burdrnan *et al.* (5) isolated a strain of *Bacillus subtilis* A-13, which could effectively inhibit fungal diseases and promote the growth of many plants.

CONCLUSIONS

Our results of Biolog, PLFA and T-RFLP analyses revealed that the microbial community structure and functional diversity in the soil of *A. bidentata* remains in good condition over time; this observation is significantly different from *R. glutinosa* under long-term continuous monoculture. Berendsen *et al.* (3) have pointed out that a complex plant-associated microbial community contains thousands of species of enormous diversity. They referred to this condition as the second genome of the plant crucial for plant health. The high yield and good quality of *A. bidentata* depends on a benign microbial community during a long-term continuous monoculture. Based on this study, we believe that under the continuous monoculture conditions, *A. bidentata* may secrete many substances that act as positive stimulators to promote the development of plant growth-promoting microorganisms and these maintain the soil health. In our future work, we plan to extract these substances that act as positive stimulators from *A. bidentata*, and also isolate the beneficial microorganisms from *A. bidentata* soil, for use for in soil improvement at sites where other medicinal plants are cultivated under continuous monoculture.

ACKNOWLEDGEMENTS

We would like to thank National Natural Science Foundation of China for providing funds (Grant no. U1205021, 31401950 and 81303170).

REFERENCES

1. Akiyama, K. (2005). Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* **435**: 824-827.
2. Aliasgharzad, N., Mårtensson, L.M. and Olsson, P.A. (2010). Acidification of a sandy grassland favours bacteria and disfavours fungal saprotrophs as estimated by fatty acid profiling. *Soil Biology & Biochemistry* **42**: 1058-1064.
3. Berendsen, R.L., Pieterse, C.M. and Bakker, P.A. (2012). The rhizosphere microbiome and plant health. *Trends in Plant Sciences* **17**: 478-486.

4. Brockett, B.F.T., Prescott, C.E. and Grayston, S.J. (2012). Soil moisture is the major factor influencing microbial community structure and enzyme activities across seven biogeoclimatic zones in western Canada. *Soil Biology & Biochemistry* **44**: 9-20.
5. Burdrnan, S., Jurkevitch, E. and Okon, Y. (2000). Recent advances in the use of plant growth promoting Rhizobacteria (PGPR) in agriculture. In: *Microbial Interactions in Agriculture and Forestry*, Vol. II. (N.S. Subba Rao, Y.R. Dommergues, eds.), pp. 229-250. Science Publishers, Enfield, NH, USA.
6. Chen, H., Hao, H.R., Xiong, J., Qi, X.H., Zhang, Z.Y. and Lin, W.X. (2007). Effects of successive cropping Rehmannia glutinosa on rhizosphere soil microbial flora and enzyme activities. *Chinese Journal of Applied Ecology* **18**: 2755-2759. (Chinese)
7. Chen, M.M., Chen, B.D. and Marschner, P. (2008). Plant growth and soil microbial community structure of legumes and grasses grown in monoculture or mixture. *Journal of Environment Science* **20**: 1231-1237.
8. Choi, K.H. and Dobbs, F.C. (1999). Comparison of two kinds of biolog microplates (GN and ECO) in their ability to distinguish among aquatic microbial communities. *Journal of Microbiology Methods* **36**: 203-213.
9. Denef, K., Bubenheim, H., Lenhart, K., Vermeulen, J., Van Cleemput, O., Boeckx, P. and Müller, C. (2007). Community shifts and carbon translocation within metabolically-active rhizosphere microorganisms in grasslands under elevated CO_2. *Biogeoscience Discussion* **4**: 1437-1460.
10. Fierer, N., Schimel, J.P. and Holden, P.A. (2003). Variations in microbial community composition through two soil depth profiles. *Soil Biology & Biochemistry* **35**: 167-176.
11. Giller, K.E., Witter, E. and Steve, P.M. (2009). Heavy metals and soil microbes. *Soil Biology & Biochemistry* **41**: 2031-2037.
12. Gomez, E., Ferreras, L. and Toresani, S. (2006). Soil bacterial functional diversity as influenced by organic amendment application. *Bioresource Technology* **97**: 1484-1489.
13. Han, X.M., Wang, R.Q., Liu, J., Wang, M.C., Zhou, J. and Guo, W.H. (2007). Effects of vegetation type on soil microbial community structure and catabolic diversity assessed by polyphasic methods in North China. *Journal of Environment Science* **19**: 1228-1234.
14. Hao, H.R., Li, Z.F., Xiong, J. and Lin, W.X. (2008). Variation of microbial flora and enzyme activity in rhizospheric soil under continuous cropping of Achyranthes bidentata. *Chinese Journal of Eco-Agriculture* **2**: 307-311. (Chinese)
15. Hartwing, U.A. (1990). Chrysoeriol and luteolin released from alfalfa seeds induce nod genes in rhizobium meliloti. *Plant Physiology* **92**: 116-122.
16. Hebel, C.L., Smith, J.E. and Cromack, Jr. K. (2009). Invasive plant species and soil microbial response in the cascade range of Oregon. *Applied Soil Ecology* **42**: 150-159.
17. Huygens, D., Schouppe, J., Roobroeck, D., Alvarez, M., Balocchi, O., Valenzuelaf, E., Pinocheta, D. and Boeckxb, P. (2011). Drying-rewetting effects on N cycling in grassland soils of varying microbial community composition and management intensity in south central Chile. *Applied Soil Ecology* **48**: 270-279.
18. Joergensena, R.G. and Potthoff, M. (2005). Microbial reaction in activity, biomass and community structure after long-term continuous mixing of a grassland soil. *Soil Biology & Biochemistry* **37**: 1249-1258.
19. Jonassona, S., Castroa, J. and Michelsen, A. (2006). Interactions between plants, litter and microbes in cycling of nitrogen and phosphorus in the arctic. *Soil Biology & Biochemistry* **38**: 526-532.
20. Koga, K., Suehiro, Y., Matsuoka, S.T. and Takahashi, K. (2003). Evaluation of growth activity of microbes in tea field soil using microbial calorimetry. *Journal of Bioscience and Bioengineering* **95**: 429-34.
21. Kourtev, P.S., Ehrenfeld, J.G. and Haggelom, M. (2002). Exotic plant species alter the microbial community structure and function in the soil. *Ecology* **83**: 3152- 3166.
22. Kramer, C. and Gleixner, G. (2006). Variable use of plant- and soil-derived carbon by microorganisms in agricultural soils. *Soil Biology & Biochemistry* **38**: 3267-3278.
23. Li, J., Huang, J. and Zhang, Z.Y. (2010). Effect of consecutive monoculture on content of phytosterone and triterpenoids in achyranthes. *Lishizhen Medicine and Materia Medica Research* **21**: 2433-2434.
24. Li, P., Qi, L.W., Liu, E.H., Zhou, J.L. and Wen, X.D. (2008). Analysis of Chinese herbal medicines with holistic approaches and integrated evaluation models. *Trac-trends in Analytical Chemistry* **27**: 66-77.
25. Li, Z.F., Yang, Y.Q., Xie, D.F., Zhu, L.F., Zhang, Z.G., Huang, M.J., Liu, Z.Q., Zhang, Z.Y. and Lin, W.X. (2012). Effects of continuous cropping on the quality of *Rehmannia glutinosa* L. and soil micro-ecology. *Chinese Journal of Eco-Agriculture* **20**: 217-224. (Chinese)

26. Li, Z.F., Zhang, Z.G., Xie, D.F., Dai, L.Q., Zhu, L.F., Li, J., Liu, Z.Q., Yang, Y.Q., Wu, L.K., Huang, M.J., Zhang, Z.Y. and Lin, W.X. (2011). Positive allelopathic stimulation and underlying molecular mechanism of achyranthe under continuous monoculture. *Acta Physiologiae Plantarum* **33**: 2339-2347.
27. Lin, S., Zhang, A.J., Wang, R.Y., Cheng, T., Zhou, M.M., Yuan, Z.N. and Lin, W.X. (2012). Effects of sugarcane ratooning cultivation on the alteration of bacterial communities in the rhizosphere soil. *Sugar Technology* **14**: 275-283.
28. Lin, S., Huangpu, J.J., Chen, T., Zhang, Z.Y. and Lin, W.X. (2014). Evaluation of allelopathic potential and identification of allelochemicals in *Pseudostellariae heterophylla* rhizosphere soil of different cropping patterns. *Allelopathy Journal* **33**: 151-162.
29. Lin, W.X., Xiong, J., Zhou, J.J., Qiu, L., Shen, L.H., Li, Z.F., Chen, H., Hao, H.R., Chen, T., Lin, R.Y., He, H.B. and Liang, Y.Y. (2007). Research status and its perspective on the properties of rhizosphere biology mediated by allelopathic plants. *Chinese Journal of Eco-Agriculture* **15**: 1-8. (Chinese)
30. Liu, C.H., Tang, W.R., Wang, H.M. and Lee, K.C. (2013). How cancer patients build trust in traditional Chinese medicine. *European Journal of Integrative Medicine* **5**: 495-500.
31. Lorenzo, P., Pereira, C.S. and Rodríguez-Echeverría, S. (2013). Differential impact on soil microbes of allelopathic compounds released by the invasive Acacia dealbata Link. *Soil Biology & Biochemistry* **57**: 156-163.
32. Lu, R.K. (2000). *Methods of Soil and Agricultural Chemistry Analysis*. China Agricultural Science and Technology Press, Beijing.
33. Magurran, A.E. (2004). *Measuring Biological Diversity*. Blackwell, Oxford.
34. Maidak, B.L., Cole, J.R., Parker, C.T., Garrity, G.M., Larsen, N., Li, B., Lilburn, T.G., McCaughey, M.J., Olsen, G.J., Overbeek, R., Pramanik, S., Schmidt, T.M., Tiedje, J.M. and Woese, C.R. (1999). A new version of the RDP (Ribosomal Database Project). *Nucleic Acids Research* **27**: 171-173.
35. McKinley, V.L., Peacock, A.D. and White, D.C. (2005). Microbial community PLFA and PHB responses to ecosystem restoration in tallgrass prairie soils. *Soil Biology & Biochemistry* **37**: 1946-1958.
36. Minh, V.D. (2005). Soil-plant nutrients balance of tea crops in the northern mountainous region, Vietnam. *Agriculture, Ecosystems & Environment* **105**: 413-418.
37. Mithufer. (2002). Suppression of plant defence in rhizobia-legume symbiosis. *Trends in Plant Science* **7**: 440-444.
38. Ndabamenye, T., Van Asten, P.J.A., Blomme, G., Vanlauwe, B., Uzayisenga, B., Annandale, J.G. and Barnard, R.O. (2013). Nutrients imbalance and yield limiting factors of low input East African highland banana (*Musa* spp. AAA-EA) cropping systems. *Field Crops Research* **147**: 68-78.
39. Nunan, N., Daniell, T.J., Singh, B.K., Papert, A., McNicol, J.W. and Prosser, J.I. (2005). Links between plant and rhizoplane bacterial communities in grassland soils, characterized using molecular techniques. *Applied and Environmental Microbiology* **71**: 6784-6792.
40. Qu, X.H. and Wang, J.G. (2008). Effects of amendments with different phenolic acids on soil microbial biomass, activity, and community diversity. *Applied Soil Ecolgy* **39**: 172-179.
41. Savario, C.F. and Hoy, J.W. (2011). Microbial communities in sugarcane field soils with and without a sugarcane cropping history. *Plant Soil* **341**: 63-73.
42. Singh, A., Parmar, N., Kuhad, R.C. and Ward, O.P. (2011). Bioaugmentation, biostimulation, and biocontrol in soil biology. In: *Bioaugmentation, Biostimulation and Biocontrol*, (Eds., N.S. Subba Rao, Y.R. Dommergues), pp: 1-26. Springer Berlin, Germany.
43. Singh, G. and Mukerji, K.G. (2006). Root exudates as determinant of rhizospheric microbial biodiversity. In: *Microbial Activity in the Rhizosphere* (Eds., Mukerji, K.G., Manoharachary, C., Singh, J.), pp. 39-53. Springer-Verlag, Heidelberg.
44. Solaiman, Z.M. and Anawar, H.M. (2015). Rhizosphere microbes interactions in medicinal plants. In: *Plant Growth-Promoting Rhizobacteria (PGPR) and Medicinal Plants*, (Eds., D. Egamberdieva, S. Shrivastava, A. Varma), pp: 19-42. Springer Berlin, Germany.
45. Song, X.Y., Li, Y.D., Shi, Y.P., Jin, L. and Chen, J. (2013). Quality control of traditional Chinese medicines: A review. *Chinese Journal of Natural Medicines* **11**: 596-607. (Chinese)
46. Termorshuizen, A.J. and Jeger, M.J. (2008). Strategies of soilborne plant pathogenic fungi in relation to disease suppression. *Fungal Ecology* **1**: 108-114.
47. Wang, H.B., Zhang, Z.X., Li, H., He, H.B., Fang, C.X., Zhang, A.J., Li, Q.S., Chen, R.S., Guo, X.K., Lin, H.F., Wu, L.K., Lin, S., Chen, T., Lin, R.Y., Peng, X.X. and Lin, W.X. (2011). Characterization of metaproteomics in crop rhizospheric soils. *Journal of Proteome Research* **10**: 932-940.

48. Wang, H.Y., Jiang, X.L., Guan, H.S. and Mu, H.J. (2004). An emerging method for characterizing microbial ecology-T-RFLP technique. *Microbiology* **3l**: 90-94.
49. Wilkinson, S.C., Anderson, J.M., Scardelis, S.P., Tisiafouli, M., Taylor, A. and Wolters, V. (2002). PLFA profiles of microbial communities in decomposing conifer litters subject to moisture stress. *Soil Biology & Biochemistry* 34: 189-200.
50. Wu, F.Z., Wang, X.Z. and Xue, C.Y. (2009). Effects of cinnamic acid on soil microbial characteristics in the cucumber rhizosphere. *European Journal of Soil Biology* **45**: 356-362.
51. Wu, L.K., Li, Z.F., Li, J., Muhammad, A.K., Huang, W.M., Zhang, Z.Z. and Lin, W.X. (2013). Assessment of shifts in microbial community structure and catabolic diversity in response to *Rehmannia glutinosa* monoculture. *Applied Soil Ecology* **67**: 1-9.
52. Wu, L.K., Wang, H.B., Zhang, Z.X., Lin, R., Zhang, Z.Y. and Lin, W.X. (2011). Comparative metaproteomic analysis on consecutively *Rehmannia glutinosa*-monocultured rhizosphere soil. *PLoS One* **6**: e20611.
53. Yang, C.H., Crowley, D.E. and Menge, J.A. (2000). 16S rDNA fingerprinting of rhizosphere bacterial communities associated with healthy and phytophora infected avocado toots. *FEMS Microbiology Ecology* **35**: 129-136.
54. Yang, Y.H., Chen, X.J., Chen, J.Y., Xu, H.X., Li, J. and Zhang, Z.Y. (2011). Differential miRNA expression in *Rehmannia glutinosa* plants subjected to continuous cropping. *BMC Plant Biology* **11**: 53-63.
55. Yoneyama, K. and Natsume, M. (2010). Allelochemicals for plant-plant and plant–microbe interactions. *Comprehensive Natural Products II* **4**: 539-561.
56. Zeng, L.J., Lin, M.Z., Li, Z.F., Dai, L.Q., Li, J., Li, J.J., Zhang, Z.Y. and Lin, W.X. (2012). Effects of continuous cropping on photosynthesis and medicinal quality of *Pseudostellariae heterophylla*. *Acta Agronomica Sinica* **38**: 1522-1528. (Chinese)
57. Zhang, H.Y. and Xue, Q.H. (2010). Research progress in control of continuous cropping obstacle in Ginseng. *Acta Agriculturae Jiangxi* **22**: 68-71. (Chinese)
58. Zhang, Z.Y. and Lin, W.X. (2009). Continuous cropping obstacle and allelopathic autotoxicity of medicinal plants. *Chinese Journal of Eco-Agriculture* **17**: 189-196. (Chinese)
59. Zhang, Z.Y., Niu, M.M., Chen, T., Li, J., Lin, R.Y. and Lin, W.X. (2011). Research on medicinal plant autotoxicity and innovation of cultivation technology. *Modern Chinese Medicine* **13**: 4-7. (Chinese).