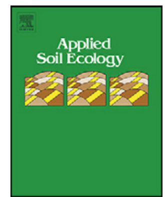
Applied Soil Ecology 67 (2013) 1–9

Contents lists available at SciVerse ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil



Assessment of shifts in microbial community structure and catabolic diversity in response to *Rehmannia glutinosa* monoculture

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ARTICLE INFO

Article history:
Received 22 August 2012
Received in revised form 8 January 2013
Accepted 19 February 2013

Keywords:
Rehmannia glutinosa
Monoculture
Eco microplate
Phospholipid fatty acid
Soil respiration
Microbial ecology

ABSTRACT

Rehmannia glutinosa, a widely used Chinese medicinal herb, has been shown to suffer from serious consecutive monoculture problems that cause significant decline in both yield and quality. The objective of this study was to evaluate the response of the soil microbial community and the effect on catabolic diversity to consecutive monoculture regime using three different techniques: substrate-induced respiration (SIR), phospholipid fatty acid (PLFA) and community-level physiological profiles (CLPP) analyses. We found that basal soil respiration (BSR) was significantly higher in the control and newly planted soils than in the second and third year consecutive monoculture soils. However, no significant difference was observed in SIR among the newly planted, second and third year consecutive monoculture soils. The PLFA signatures indicated that the bacterial biomass was larger than the fungal biomass in all four treatments and both enhanced with the increasing years of monoculture and attained the peak in SM. The ratio of cyclopropyl PLFAs to their metabolic precursors (cy/pre), a measure of physiological stress in microbial communities, in the second and third year consecutive monoculture soils was significantly greater than that in the control and newly planted soils. Biolog analysis results revealed that the consumption of carboxylic acids, phenolic acids and amines, especially acid carbon substrates, in the consecutively monocultured soil was significantly greater than that in the newly planted soil. Both PLFA- and CLPP-based principal component analysis (PCA) and cluster analysis revealed the distinct separation between the control, newly planted plots and the second, third year consecutive monoculture plots. Through our PLFA-based and Biolog analysis, together with microbial respiration determination, we were able to reveal characteristic differences in the microbial community composition and activities in the rhizosphere following *R. glutinosa* monoculture.

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1. Introduction

Rehmannia glutinosa is a perennial medicinal plant and belongs to the family Scrophulariaceae. It is often planted in March and the tuber roots are harvested in the autumn or early winter. The roots are widely used in traditional Chinese medicine and possess various pharmacological actions and chemical compositions. Previous studies showed that *R. glutinosa* and its active components exhibit wide pharmacological actions related to immunity, blood circulation, cardiovascular, endocrine and the nervous system (Zhang et al., 2008). *R. glutinosa* is a medicinal plant mainly produced in Jiaozuo city, Henan Province, the central region of China, which is known as the geo-authentic production zone (34°48′ N to 35°30′ N, 112°02′ E to 113°38′ E). However, this plant has suffered from very serious consecutive monoculture problems (Yang et al., 2011). The local farmers have never consecutively replanted *R. glutinosa* on the same field for two years because consecutively

Abbreviations: AK, available potassium; AN, available nitrogen; AP, available phosphorus; AWCD, average well-color development; BSR, basal soil respiration; CK, control (control plots with no *R. glutinosa* cultivation); CLPP, community-level physiological profiles; cy/pre, cyclopropyl PLFAs to their metabolic precursors; FAME, fatty acid methyl ester; Gram(−), Gram-negative bacteria; Gram(+), Gram-positive bacteria; LMW, low-molecular weight; NP, newly planted soil; PCA, principal component analysis; PLFA, phospholipid fatty acid; SIR, substrate-induced respiration; SM, second year consecutive monoculture soil; SOM, soil organic matter; TK, total potassium; TM, third year consecutive monoculture soil; TN, total nitrogen; TP, total phosphorus.

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http://dx.doi.org/10.1016/j.apsoil.2013.02.008

monocultured *R. glutinosa* leads to severe diseases, significantly reduced biomass and quality of underground tubers, or low harvest rate. In general, fields used for *R. glutinosa* cultivation can be replanted once in every eight years (Wu et al., 2011). The increasing need for *R. glutinosa* has forced farmers to plant them in places outside the Jiaozuo area, but the crop quality could not be assured as these regions are non-authentic production areas with unsuitable environmental conditions (Zhang et al., 2003). Thus it is urgent to understand the underlying mechanism of consecutive cultivation problems that occur in *R. glutinosa*.

Previous studies showed that there are usually three reasons for the consecutive monoculture problems: imbalance of soil nutrients, the autotoxicity of root exudates and the shifts in microbial community (Wu et al., 2011). Li et al. (2012) isolated several autotoxic compounds from the fibrous roots of *R. glutinosa*, nine of which were analyzed for autotoxic effects on the seedling growth. They found that both phenolic acid and aliphatic acid compounds might contribute to the autotoxicity of *R. glutinosa*. However, proper soil ecosystem function is governed largely by rhizospheric microbial dynamics (Nehl and Knox, 2006). The interactions between consecutive monoculture problems and rhizospheric microbial community have been recently highlighted and considered to be an issue worth pursuing (Qi et al., 2009; Qu and Wang, 2008; Wu et al., 2009a, 2011). Qu and Wang (2008) used denaturing gradient gel electrophoresis (DGGE) technique to assess the effect of different types of phenolic acids on soil microbial populations. They further proposed a mechanism by which these phenolic acids could selectively enhance specific microbial populations in the soil which then leads to a shift in microbial communities. Wu et al. (2011) applied soil metaproteomics to analyze the rhizospheric biological properties of consecutively monocultured *R. glutinosa* at the protein level and found that consecutive monoculture can induce the changes in the expression of proteins in the soil by both plants and microbes.

There has been an increase in interest on the rhizospheric biological processes and plant–microbe interactions (Bais et al., 2006; Inderjit et al., 2010). Phospholipid fatty acid (PLFA) analysis can detect shifts in broad groups of soil organisms, such as bacterial, actinomycetic and fungal groups, thereby allowing for soil microbial community fingerprinting (Hebel et al., 2009). The assessment of microbial functional diversity by using Biolog sole carbon (C) substrate utilization tests has been reported to be a rapid, sensitive approach to detect modifications in diversity due to soil management (Gomez et al., 2006) although this method is biased towards the community that can grow rapidly under the conditions in the Biolog plates (Savario and Hoy, 2011). In the study presented here we evaluated the effects on microbial community composition and functional diversity of *R. glutinosa* consecutive monoculture by phospholipid fatty acid (PLFA) profiles and community-level physiological profiling (CLPP) analysis.

2. Methods

2.1. Field experiment

R. glutinosa 'Wen 85-5′, a cultivar planted widely in the main production region, was used as crop material. *R. glutinosa* was planted on April 15 and harvested on October 30. Following harvest, the field was kept fallow from October 31 to April 15 of next year. The experiment was conducted at the Wen Xian Agricultural Institute, Jiaozuo City, Henan Province (34°56′ N, 112°58′ E). This area is known as the geo-authentic zone for *R. glutinosa* cultivation. The area has a continental monsoon climate, with an annual average temperature of 14.3 °C and an average annual precipitation is 552 mm. The following treatment conditions were organized within a single field site: i.e. (1) control plots (CK); (2) newly planted plots (NP); (3) second year monoculture plots (SM); and (4) third year monoculture plots (TM). Each treatment has three replicates (20 m^2) and the study plots were completely randomized. The growth period and fallow time are listed in Table 1. Prior to planting, each replicate was fertilized by four fertilizers, including 1.6 kg N-P-K complex fertilizer, 1.25 kg $(NH_4)_2HPO_4$, 1.6 kg $Ca(H_2PO_4)_2$ and 0.8 kg K_2SO_4. All treatments were subjected to the same fertilization and field management.

The above ground or below ground biomass between newly planted and consecutively monocultured *R. glutinosa* become significantly different after 70 days of planting (Wu et al., 2011). Therefore, the rhizospheric soils of *R. glutinosa* were collected on June 25, 2011 which is 70 days after planting. Soil sampling of all four treatments was carried out at the same time. Fresh plants were carefully uprooted from the soil with a forked spade from five random locations of each replicate. Their roots and tubers were slightly shaken to remove loosely attached soil. The rhizospheric soils tightly attached to roots and rhizomes were collected and then sieved through 2 mm mesh to remove plant roots, leaf remains and insects. The plot samples were mixed to generate composite samples for further analyses.

2.2. Soil physical–chemical properties determination

All soil samples were air-dried and sieved through 2 mm mesh and then used to determine soil pH, organic matter (SOM), nitrogen (N), phosphorus (P) and potassium (K) (Lu, 2000). Available N (AN) was calculated as the sum of ammonium nitrogen (NH_4-N) and nitrate nitrogen (NO_3-N). Available P (AP) was extracted using 0.5 mol/L $NaHCO_3$ solution and determined by Mo–Sb colorimetry method. Available K (AK) was extracted in ammonium acetate and measured by flame atomic absorption spectrometry. Total N (TN) was measured using the Kjeldahl method. The total P and K (TP and TK) was calculated by first digesting the soil using the H_2SO_4–$HClO_4$ method and then measuring the level as described for AP and AK. We determined the SOM by potassium dichromate oxidation-ferrous sulphate titrimetry and the pH by using a pH meter with water.

2.3. Soil respiration measure

Basal soil respiration (BSR) of the samples was determined by alkali absorption method (Dutta et al., 2010) with slight modifications. Briefly, triplicate soil subsamples (20 g) were placed in separate incubation flasks containing 20 ml 0.1 M NaOH solution to trap the evolved CO_2. The same procedure was followed for blank (in triplicate) in which soil was omitted. The soil and the blank were incubated at 28 °C for 24 h. The residual NaOH was then back-titrated with 0.1 M HCl. BSR was calculated as $\mu g\ CO_2$–$C\ g^{-1}$ d.m. soil h^{-1}. After BSR determination, the soil was used to measure substrate-induced respiration (SIR) following glucose addition (1 ml, 25 mg g^{-1}). Then the soil samples were incubated at 28 °C for 24 h and CO_2 evolved was measured by the same approach used for BSR.

2.4. Phospholipid fatty acid (PLFA) analysis

PFLAs were extracted and derivatized as described by Denef et al. (2007). Phospholipids were methylated by mild alkaline methanolysis (using methanolic KOH) to form fatty acid methyl esters (FAMEs). Then, FAMEs were analyzed using a 450GC/240MS system (Varian, Inc., USA) equipped with a capillary column CP8944 (30 m, 0.25 mm i.d., 0.25 m film thickness; Varian, Inc., USA). The column temperature was programmed to start at 70 °C for 1 min, then ramp up at a rate of 20 °C min^{-1} to 170 °C which was held for 2 min,

Table 1
Growth periods and fallow time of four different treatments.

Treatment	Site code	Apr. 15, 2009–Oct. 30, 2009	Oct. 31, 2009–Apr. 14, 2010	Apr. 15, 2010–Oct. 30, 2010	Oct. 31, 2010–Apr. 14, 2011	Apr. 15, 2011–Oct. 30, 2011
Control (unplanted) soil	CK	Fallow	Fallow	Fallow	Fallow	Fallow
Newly planted soil	NP	Fallow	Fallow	Fallow	Fallow	Planted
Second year monoculture soil	SM	Fallow	Fallow	Planted	Fallow	Planted
Third year monoculture soil	TM	Planted	Fallow	Planted	Fallow	Planted

and followed by a ramp of 5 °C min^{-1} to 280 °C which was held for 5 min. Finally, the oven temperature was increased to 300 °C at 40 °C min^{-1} and held for 1.5 min. The peaks were identified based on relative retention times vs. several external standards: a mixture of 37-Component FAME Mix (47885-U, Supelco Inc., USA), a mixture of 26 Bacterial Acid Methyl Esters (47080-U, Supelco Inc., USA) and several individual FAMEs (Larodan Inc., Sweden). Individual fatty acids were quantified by comparing peak areas from the sample with peak areas of the internal standard 19:0 (nonadeconoic methyl ester) of known concentration.

In total, 25 PLFAs were isolated and detected. The rules of fatty acid nomenclature used were described by Wilkinson et al. (2002). The sum of group-specific PLFAs was used as broad taxonomic microbial groupings. Branched, saturated PLFAs a15:0, a17:0, i14:0, i15:0 and i16:0 represented the 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, 10Me17:0 and 10Me18:0 were regarded as biomarkers for actinomycetes. PLFAs 18:1omega9c and 18:2omega6,9 were ascribed to fungal indicators and PLFA 20:4omega6 represented the protozoan (Brockett et al., 2012; Huygens et al., 2011; Joergensena and Potthoff, 2005; McKinley et al., 2005). 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 PLFAs (cy17:0 + cy19:0) to their metabolic precursors (16:1omega7c + 18:1omega7c) ratio (cy/pre) which is an indicator of physiological stress in microbial communities (Aliasgharzad et al., 2010).

2.5. Biolog method

Community level physiological profiles (CLPP) were assessed by the Biolog Eco Microplate^{TM} system (Biolog Inc., CA, USA). Each 96-well plate consists of three replicates of 31 sole carbon substrates and a water blank. The procedures were carried out according to the method described by Lin et al. (2007). The plates were incubated at 25 °C for 168 h, and the color development in each well was recorded as optical density (OD) at 590 nm with a plate reader (Thermo Scientific Multiskan MK3, Shanghai, China) at regular 24 h intervals.

Microbial activity in each microplate, expressed as average well-color development (AWCD) was determined as follows:

$$\text{AWCD} = \sum \frac{C - R}{31}$$

where C is the optical density within each well and R is the absorbance value of the control well. The 31 carbon substrates in ECO microplates were subdivided into six categories: polymers, carbohydrates, carboxylic acids, amino acids, amines and phenolic compounds, as described by Choi and Dobbs's (1999). The optical density at 96 h incubation time was used for principal component analysis (PCA) and cluster analysis (Han et al., 2007), since it was the shortest incubation time that provided the best resolution among treatments (Gomez et al., 2006).

2.6. Statistical analysis

One way analysis of variance (ANOVA) followed by Tukey's tests ($P < 0.05$) was used for statistical analysis when a significant F value was detected through DPS software version 7.05. The resulting levels of individual PLFA and 96 h AWCD value per well in Biolog ECO microplates were subjected to principal component analysis (PCA) and cluster analysis using UPGMA (unweighted pair group method

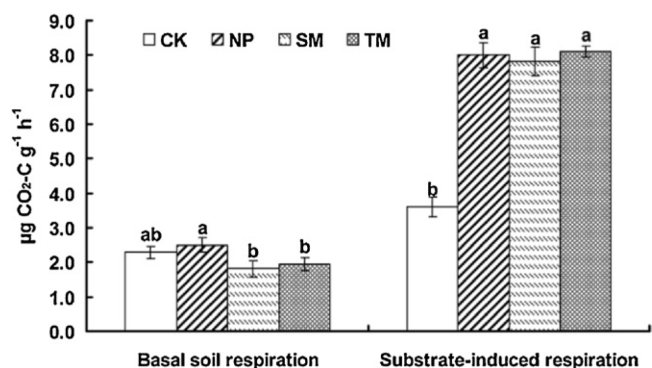

Fig. 1. Production of CO_2–C in rhizospheric soil under different monoculture years before and after addition of substrate. Bars represent SD. Site codes for each sample are given in Table 1.

with average linkage) clustering algorithm through SPSS software version 11.5 and DPS software version 7.05, respectively.

3. Results

3.1. Soil physico-chemical properties

Most physico-chemical properties were lowest in the control soil except for organic matter, total K and pH. Organic matter, total N, available N and available K were significantly higher in the second and third year monoculture soils than in the newly planted soil. However, the newly planted plots had significantly higher available P and total K than the second and third year monocultured plots. pH value significantly decreased with the increasing years of monoculture. There was no significant difference in total P between the newly planted and third year cultivated plots (Table 2).

3.2. Microbial respiration activity

The basal soil respiration (BSR) value was significantly lower in the second year monoculture and the third year monoculture soils than in the newly planted and control soils. The substrate-induced respiration (SIR) in the control soil was lowest. However, there was no significant difference in SIR among the newly planted, second and third year consecutive monoculture soils (Fig. 1).

3.3. Phospholipid fatty acid profiles

The specific, non-specific and group-specific PLFA concentrations in the 12 soil samples (three replicates per soil sample) are listed in Table 3. The levels of bacterial PLFAs were significantly greater than those of fungal and actinomycetic PLFAs in all four treatments. Except for actinomycetes, almost all PLFAs were lowest in the control soil. Total PLFAs, bacteria, Gram(+), Gram(−), actinomycetes and Gram(+)/Gram(−) ratio in the second and third year monoculture soils were significantly higher than that in the control and newly planted soils. The level of these PLFAs was highest in the second year monoculture soil and declined in the third year monoculture soil, but remained remarkably higher than that in the control and newly planted soils. Many bacterial-specific PLFAs also showed the same trend, including i14:0, i15:0, i16:0, a17:0, 16:1omega7c, 16:1omega9t and cy19:0. Similar to certain specific PLFAs, the sum of fungal PLFAs significantly increased with the increasing years of monoculture, but decreased in the third year monoculture soil. The second and third year monoculture soils had a significantly higher level of PLFA 18:1omega9c, a biomarker of fungi, than the control and newly planted soils. However, another fungal PLFA 18:2omega6,9 significantly declined in the third year

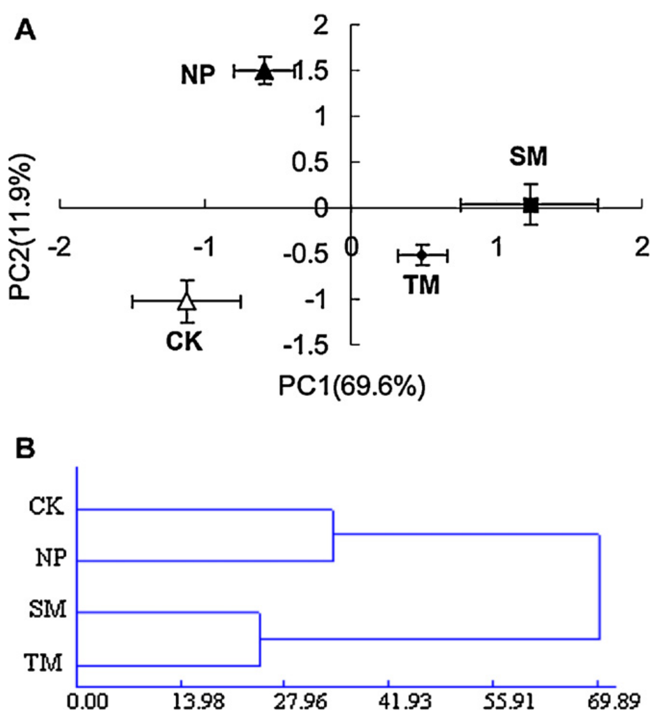

Fig. 2. Classification of treatments by principal component (A) and UPGMA cluster analysis (B) with PLFA data. Site codes for each sample in Table 1.

monoculture soil as compared to the newly planted and the second year monoculture soils. Two cyclopropyl PLFAs cy17:0 and cy19:0 significantly increased in the second and third year monoculture soils. The ratio of cyclopropyl fatty acids to their metabolic precursors (16:1omega7c, 18:1omega7c), referred to as an indicator of physiological stress in microbial communities (Aliasgharzad et al., 2010), was significantly greater in the second and third year monoculture soils than in the control and newly planted soils. This indicates depletion of resources and decreased metabolic activity and bacterial cell growth relative to communities in rhizospheric soil after *R. glutinosa* consecutive monoculture.

The PLFA data from all soil samples (four treatments, three replicates) were subjected to principal component analysis (PCA) (Fig. 2). The first two principal components PC1 and PC2 accounted for 69.64% and 11.88% of total variation respectively, and their eigenvalues were 25.07 and 4.28, respectively. Total PLFAs, 16:1omega9t and 18:2omega6, fungal:bacterial PLFA ratio were discriminated most positively by PC1 and PC2 scores, respectively. However, i:18, fungal:bacterial PLFA ratio and 10Me19:0, Gram(+):Gram(−) PLFA ratio were discriminated most negatively by PC1 and PC2 scores, respectively. PCA results showed a good separation among the four treatments (CK, NP, SM and TM). However, characteristics of second and third year consecutive monoculture soils were closer to each other, implying that they might share similar soil microbial community structure. This result was confirmed by the cluster analysis. The dendrogram of PLFA profiles showed that the control and newly planted soils linked together at a relatively lower Euclidean distance of 24.4 and the second and third year monoculture soils recorded at a higher Euclidean distance of 34.2. These two clusters then linked together at a high Euclidean distance of 69.9.

3.4. Community level physiological profiles (CLPP) analysis

Except for polymers, the 96 h AWCD data of the other five substrate groups (carbohydrates, carboxylic acids, amino acids, amines and phenolic compounds) were all highest in the third year

Table 2
Physical and chemical properties of soils (5–25 cm) from the treatment plots 70 days after planting *R. glutinosa*.

Treatment	SOM (g/kg)	TN (g/kg)	AN (mg/kg)	TP (g/kg)	AP (mg/kg)	TK (g/kg)	AK (mg/kg)	pH
Control (unplanted) soil	9.37c	0.34c	9.47d	0.48c	17.40d	1.71b	106.89d	7.82a
Newly planted soil	7.94d	0.49b	27.57c	0.60b	56.27a	1.98a	168.43c	7.62b
Second year monoculture soil	14.40a	0.63a	37.09a	0.92a	50.93b	1.67b	246.09a	7.46c
Third year monoculture soil	12.06b	0.63a	34.62b	0.61b	36.24c	1.70b	204.88b	7.26d

Data are means ± SD. SOM, soil organic matter; TN, total nitrogen; AN, available nitrogen; TP, total phosphorus; AP, available phosphorus; TK, total potassium; and AK, available potassium. Site codes for each sample are given in Table 1. Different letters in columns show significant differences determined by Tukey's test ($P \leq 0.05$, $n = 3$).

Table 3
Concentrations (nmol g^{-1} d.m. soil) of the different specific PLFAs, sum of total, non-specific and group-specific PLFAs in the four different treatments.

PLFA		Control	Newly planted	Second year monoculture	Third year monoculture	Comment
1	15:0	0.62b	0.76b	1.37a	0.94b	Bacteria[a]
2	a15:0	2.83c	4.45b	6.28a	5.20b	Gram(+)[b]
3	a17:0	7.49b	7.53b	15.95a	15.41a	Gram(+)[b]
4	i14:0	2.52b	3.02b	4.75a	4.05a	Gram(+)[b]
5	i15:0	4.25b	5.07b	8.34a	7.97a	Gram(+)[b]
6	i16:0	3.23b	3.84b	5.76a	4.96a	Gram(+)[b]
7	16:1omega7c	10.01b	12.02b	16.60a	17.17a	Gram(−)[b]
8	16:1omega9t	2.90b	3.63b	6.05a	5.00a	Gram(−)[c]
9	18:1omega7c	14.52c	20.47b	24.29a	19.79b	Gram(−)[b]
10	cy17:0	2.05c	2.24bc	3.01a	2.85ab	Gram(−)[b]
11	cy19:0	4.51c	6.12b	10.79a	9.82a	Gram(−)[b]
12	10Me17:0	3.93bc	3.37c	6.13a	5.14ab	Actinomycetes[b]
13	10Me18:0	3.66a	2.26b	3.71a	3.42ab	Actinomycetes[d]
14	18:1omega9c	6.96c	8.74b	13.37a	12.45a	Fungi[b]
15	18:2omega6,9	6.57c	14.29a	15.96a	11.27b	Fungi[b]
16	20:4omega6	1.18b	1.98a	1.60ab	2.07a	Protozoan[b]
17	Non-specific	33.81c	35.78bc	51.69a	42.49b	–[e]
Total PLFA		111.04b	135.58b	195.63a	169.99a	1–17
Bacteria		54.94b	69.14b	103.18a	93.17a	1–11
Gram(+)		20.33b	23.90b	41.07a	37.59a	2–6
Gram(−)		33.99c	44.47b	60.74a	54.64a	7–11
Actinomycetes		7.59ab	5.64b	9.84a	8.56a	12–13
Fungi		13.52c	23.04b	29.33a	23.71b	14–15
Gram(+)/Gram(−)(%)		59.86b	53.72c	67.61a	68.82a	
Fungi/bacteria(%)		24.59c	33.39a	28.44b	25.47c	
Cy/pre(%)		26.68b	25.75b	33.79a	34.26a	(10 + 11)/(7 + 9)[f]

Different letters within a line indicate different levels of significance ($P \leq 0.05$, Tukey's test, $n = 3$). Site codes for each sample in Table 1.
[a] Joergensena and Potthoff (2005).
[b] McKinley et al. (2005).
[c] Huygens et al. (2011).
[d] Brockett et al. (2012).
[e] 12:0, 13:0, 16:0, 18:0, 20:0, 20:5omega3, 22:1omega9t, 23:0 and 24:0.
[f] Wu et al. (2009b), cy = (cy17:0 + cy19:0), pre = (16:1omega7c + 18:1omega7c).

monoculture (Fig. 3). The microbial communities from the second and third year monoculture soils exhibited a higher level of carboxylic acids, phenolics and amines than those from the newly planted and control soils. The AWCD values of amino acids and polymers showed no significant differences between the second year monoculture and the newly planted soils. However, the sum of AWCD values of carboxylic acids, phenolic compounds and amino acids increased significantly with the increasing years of monoculture (Fig. 4), suggesting that the microflora feeding on acids gradually became predominant in the consecutive monoculture soil.

Principal component analysis (PCA) with Biolog data (four treatments, three replicates) indicated that 96 h AWCD data successfully distinguished among the four soil community's responses

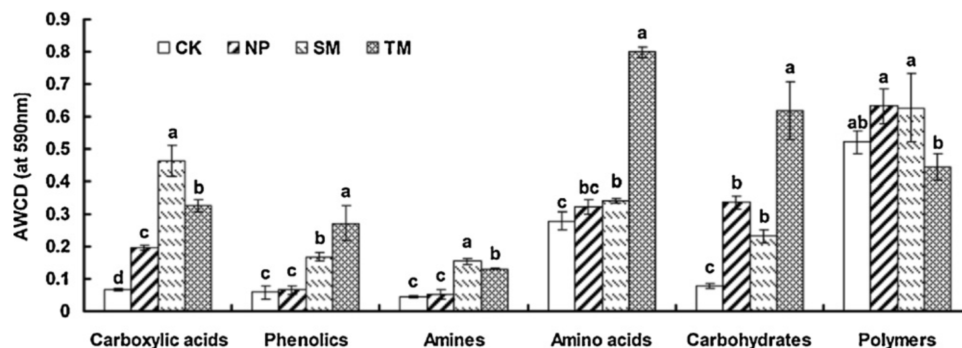

Fig. 3. Mean catabolic activity per well for each substrate group at 96 h in four treatments. Site codes for each sample are given in Table 1. Bars represent SD. Different letters represent significant differences among soil samples as determined by Tukey's test ($P \leq 0.05$, $n = 3$).

Table 4
Most discriminant five carbon substrates as determined by PCA on the data of community level carbon source utilization using Biolog Eco microplates by different soil communities.

Substrate no.	PC1	Score	PC2	Score
1	D-galactonic acid γ-lactone	0.9661	α-Ketobutyric acid	0.9789
2	4-Hydroxy benzoic acid	0.9588	i-Erythritol	0.9725
3	β-Methyl-D-glucoside	0.9249	D-glucosaminic acid	0.9619
4	L-phenylalanine	0.9111	D,L-α-glycerol phosphate	0.8897
5	α-Cyclodextrin	0.9075	D-galacturonic acid	0.8724
1	2-Hydroxy benzoic acid	−0.9808	D-cellobiose	−0.7266
2	Tween 40	−0.9398	*N*-acetyl-D-glucosamine	−0.6855
3	Glycogen	−0.8857	L-arginine	−0.6683
4	Glycyl-L-glutamic acid	−0.7583	α-D-lactose	−0.5880
5	D-xylose	−0.7122	D-xylose	−0.5431

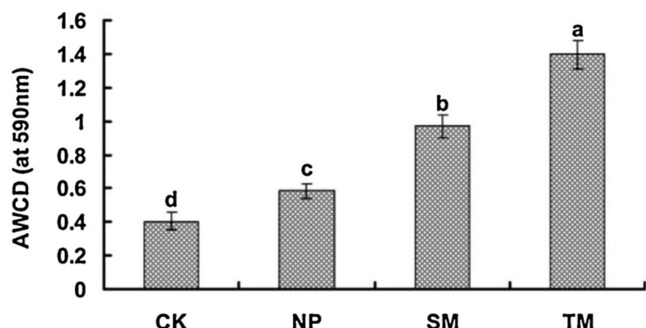

Fig. 4. Sum of AWCD values of carboxylic acids, phenolic compounds and amino acids at 96 h. Site codes for each sample and abbreviation of chemical properties are given in Table 1.

to the carbon substrates (Fig. 5A). The first two PCs, PC1 and PC2 accounted for 46.2% and 28.5%, respectively, of the total variation in ECO microplate data. The catabolic diversity of microbial community in the four treatments (CK, NP, SM and TM) could be clearly separated by the first two principal components. The five carbon substrates with the most positive and most negative scores (i.e., contributing most strongly to a separation of samples) on PC1 and PC2 are listed in Table 4. 4-hydroxy benzoic acid, D-galactonic acid γ-lactone and α-ketobutyric acid, D-glucosaminic acid were

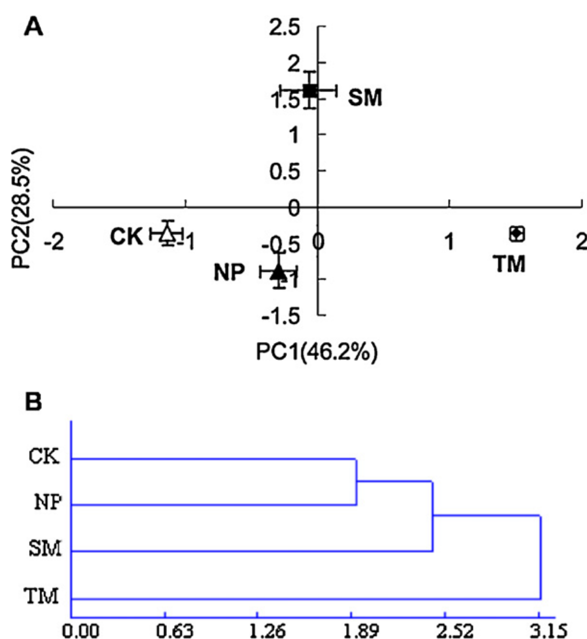

Fig. 5. Classification of treatments by principal component (A) and UPGMA cluster analysis (B) with Biolog data. Site codes for each sample in Table 1.

discriminated most positively by PC1 and PC2 scores, respectively. However, 2-hydroxy benzoic acid, tween 40 and L-arginine, D-cellobiose were discriminated most negatively by PC1 and PC2 scores, respectively. Cluster analysis showed that Euclidean distance of the control (CK) and the treated plots (NP, SM and TM) increased with the increasing years of monoculture, implying a shift in soil microbial community structure and catabolic diversity after *R. glutinosa* cropping (Fig. 5B). The results combining the PLFA and Biolog data, as determined by PCA and cluster analysis, reveal that soil microbial community structure and catabolic diversity in the second and third year consecutive monoculture soils are considerably different from that in the control and newly planted soils.

4. Discussion

Soil microbial community structure and function diversity are commonly considered as indicators of soil fertility and quality. Recent studies have focused on the complexity and biodiversity of the underground world (Bartelt-Ryser et al., 2005; Kapoor and Mukerji, 2006). Plant–bacterial interactions play crucial roles in soil quality, crop health and yield (Marschner, 2007). Soil nutrient deficiency is a major limiting factor for plant growth. However, there is no consensus on the relationship between soil nutrient and consecutive monoculture problem. Some researchers suggested that the nutritional decline in soil is not the fundamental reason for consecutive monoculture problems (Liang et al., 2004). However, there was no decline in the soil physical–chemical properties detected in this study. Organic matter, total N, available N and available K were significantly higher in the consecutively monocultured soil than in the newly planted soil, while available P and total K showed the opposite trend (Table 2). However, *R. glutinosa* monoculture resulted in rhizosphere acidification (Table 2). Meriles et al. (2009) also found that pH is more acidic in the soybean monocultured soil than in the crop rotation soil. In Australia, yield decline under long-term sugarcane monoculture appears to correlate with soil acidification (Bramley et al., 1996).

Assessment of soil respiration provides a measure of the overall potential microbial activity and is considered as a bio-indicator of soil quality (Dutta et al., 2010). Significantly lower basal soil respiration (BSR) values were observed in the second and third year monoculture soil, indicating monoculture-induced stress on soil microbial flora at the sampling time, although the levels of total and group-specific PLFAs were greater in the SM and TM (Table 3). Qi et al. (2009) also found that the respiration rate and metabolic quotient in newly planted *R. glutinosa* plots were significantly higher than that in the second year monocultured plots and crop rotation plots. Furthermore, the PLFA analyses in this study revealed that the cyclopropyl fatty acids:monoenoic precursors ratio (cy/pre ratio), an indicator of physiological stress in

microbial communities, was significantly greater in the second and third year consecutive monoculture plots than in the control and newly planted plots, implying a decrease in bacterial growth rates and metabolic activity (Macdonald et al., 2004). However, when carbon substrates were added into the soil, the respiration was significantly enhanced in the second and third year consecutive monoculture soil, which can be attributed to the increase in metabolic activity of a growing microbial community in response to more available carbon resource in the consecutively monocultured soil. The substrate-induced respiration (SIR) showed no significant differences among the newly planted, second and third year consecutive monoculture soils (Fig. 1). The Biolog data also revealed that when group-specific carbon substrates were used, the AWCD of carboxylic acids, phenolics, amines, amino acids and carbohydrates were higher in the second and third year consecutive monoculture soils than in the control and newly planted soils (Fig. 3).

According to previous studies, the microbial biomass and community structure are affected by the rhizodeposition and availability of substrate (McKinley et al., 2005; Semenov et al., 1999; Wu et al., 2011). Plants are able to shape their rhizosphere microbiome, as evidenced by the fact that different plant species host specific microbial communities when grown on the same soil (Berendsen et al., 2012). Consecutive monoculture has a negative impact on soil microbial diversity, which in turn will affect the plant production (Chen et al., 2008). The balance between the rhizosphere microflora and plant pathogens is crucial for plant health (Berendsen et al., 2012). The quantities of exudates released may be substantial, with fast-growing bacteria, actinomycetes and fungi stimulated to grow by the carbon pulses provided as exudates (Gregory, 2006). Griffiths et al. (1999) devised an experimental system to study the effects of substrate quantity on microbial community structure using synthetic root exudates and reported that microbial community structure changed consistently as substrate loading increased. Many studies have shown that increased rhizodeposition occurs in response to physical, chemical and biological components of the environment (Crowley, 2000; Gregory, 2006; Okubara and Paulitz, 2005). Recent advances have shown that low-molecular weight (LMW) organic compounds from root exudates in the rhizosphere have a specific role in plant–microbe interactions (Mukerji and Manoharachary, 2006). Results from PLFA analyses showed that the soil microbial communities of the consecutively monocultured soil differed from the control and newly planted soils (Fig. 2, Table 3). As evident in the soil physical–chemical properties in Table 2, more plant litter and organic substrates (SOM, C and N) and presumably more root exudates were present in the consecutively monocultured soil (SM, TM) than in the newly planted and control soils. Thus the levels of total PLFAs, bacteria, Gram(+), Gram(−), actinomycetes and fungi were much higher in the second and third year consecutive monoculture soils than in the control and newly planted soils. Moreover, Gram(−) bacterial biomarkers dominated the PLFA biomass in all treatments (CK, NP, SM and TM). Gram(−) bacteria tend to rely particularly on LMW carbon compounds that are exuded from plants and roots (Kramer and Gleixner, 2006). Many fungi and Gram(−) have been shown to be pathogenic towards plants (Termorshuizen and Jeger, 2008; Valkonen et al., 1993). However, the exact relationship between these two microbial groups and plant production under *R. glutinosa* consecutive monoculture regime need to be studied further. Interestingly, the levels of these PLFAs reached the highest point in the second year consecutive monoculture soil and gradually declined in the third year consecutive monoculture soil, but remained higher than that in the control and newly planted soils. This observation may be attributed to the relatively lower soil organic matter in the third year consecutive monoculture soil than in the second year consecutive monoculture soil (Table 2). Murphy et al. (2011) found that the microbial biomass C (MB-C) positively ($P < 0.05$) correlated with total soil C, particularly correlated with light fraction organic matter C (LFOM-C) and dissolved organic C (DOC).

The proportion of PLFA cy17:0 has been found to increase with addition of simulated root exudates, which was attributed to root exudates to some extent (Griffiths et al., 1999). However, many researchers have found that the proportion of cyclopropyl fatty acids, especially the cyclopropyl fatty acids:monoenoic precursors ratio, could be affected by nutrient status and physiological state of the microbial community (Aliasgharzad et al., 2010; Macdonald et al., 2004; McKinley et al., 2005). Cyclopropyl PLFAs (cy17:0, cy19:0) are formed by transmethylation of *cis*-monounsaturated precursors (16:1omega7c, 18:1omega7c) when the cell enters the stationary phase. This modification of *cis*-monounsaturated precursors to the more stable cyclopropyl fatty acids is favorable for maintaining a functional living membrane during stress (Aliasgharzad et al., 2010). A higher cyclopropyl fatty acids to metabolic precursor ratio has been related to a decrease in bacterial growth rates and an increase in carbon limitation (Fierer et al., 2003). The cyclopropyl fatty acids:monoenoic precursors ratio was significantly greater in the second and third year consecutive monoculture plots than in the control and newly planted plots (Table 3). This suggests that in the *R. glutinosa*-monocultured plots, the microbial communities are more nutrition-limited, with greater microflora feeding on relatively limited resource, or suffering from physical–chemical disturbance (i.e. acidic pH).

The consumption of carbon substrates present in Biolog system is a sensitive indicator of short-term changes in the microbial functional diversity in response to various soil management such as tillage, fertilization and amendment applications (Gomez et al., 2006; Govaerts et al., 2007) and cultivation modes such as rotation, intercropping and monoculture (Ma et al., 2012; Pankhurst et al., 2005). In the current work, the Biolog analyses revealed differences between soil microbial functional diversity from the *R. glutinosa* monocultured plots and the control or newly planted plots (Fig. 5). For the second and third year consecutive monoculture soils, the microbial community showed a greater potential for utilization of carboxylic acids, phenolic acids and amino acids, which may indicate that a greater proportion of the community is able to utilize acid-compounds (Fig. 4). Many previous studies have shown that under consecutive cultivation regime, *R. glutinosa* releases a large number of LMW root exudates including sugars, carboxylic acids, amino acids and phenolics (Wu et al., 2011), which serve as carbon substrates for certain specific microbial communities. The number of microbes feeding on phenolic acids gradually increased with the extended monoculture (Fig. 3). More evidences have demonstrated the roles of phenolic compounds in the establishment of plant–microbe interactions (Badri et al., 2009; Bais et al., 2006; Mandal et al., 2010). Microbial attenuation or alteration of root exudates, like phenylpropanoid signals is an important aspect of rhizosphere ecology (Carmona et al., 2009; Mandal et al., 2010). The root exudates might cancel out soil bacteriostasis, and act as chemoattractants to selectively promote the growth of specific microbes (Singh and Mukerji, 2006). Both PLFA- and CLPP-based principal component analysis (PCA) and cluster analysis showed distinct separation between the control, newly planted plots and the second, third year consecutive monoculture plots, suggesting an alteration of rhizospheric microbial community composition and activity following *R. glutinosa* cropping. Based on the results as mentioned above, improved management practices, such as microbial fertilizer application, organic matter amendment and enhanced plant diversity (Latz et al., 2012), could be used to relieve the consecutive monoculture problems of *R. glutinosa* in future.

5. Conclusions

We have demonstrated that the structure and functional diversity of soil microbial communities varies between the control, newly planted plots and the consecutively monocultured plots. Moreover the variation became more pronounced with the increasing years of monoculture. The PLFA levels for five main microbial groups exhibited the same trend in all treatments (CK, NP, SM and TM) in the following order: Gram(−) > Gram(+) > fungi > actinomycete > protozoan. Total PLFAs, bacterial, Gram(−), Gram(+) and actinomycete PLFAs were significantly higher in the consecutively monocultured soils than in the control and newly planted soils. The microbial communities in consecutively monocultured soil are more nutrition-limited or suffer from physical–chemical disturbance. They exhibited a decline in basal soil respiration and a promotion in the consumption of acid carbon substrates. Further work is required at the species and genus level to isolate, determine (using qRT-PCR) and characterize certain specific microflora, such as microbes with a preference for acid compounds, beneficial microbes that produce antibiotics, Gram(−) or Gram(+) bacterial or fungal pathogens, to be able to fully assess impact of these microflora on the growth of *R. glutinosa* and the rhizospheric biological processes between plant–microbe interactions.

Acknowledgements

This work was supported by grants from the National Science Foundation of China (Grant nos. 30772729 and 30973875), the National Key Basic Research Program of China (Grant nos. 2012CB126309 and U1205021) and the Natural Science Foundation of Fujian province (Grant no. 2008J0051). The authors would like to thank Wen Xian Agricultural Institute, Jiaozuo City, Henan Province, China for providing the field used in this study and for field management during the study.

References

Aliasgharzad, N., Mårtensson, L.M., Olsson, P.A., 2010. Acidification of a sandy grassland favours bacteria and disfavours fungal saprotrophs as estimated by fatty acid profiling. Soil Biol. Biochem. 42, 1058–1064.

Badri, D.V., Weir, T.L., van der Lelie, D., Vivanco, J.M., 2009. Rhizosphere chemical dialogues: plant–microbe interactions. Curr. Opin. Biotechnol 20, 642–650.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S., Vivanco, J.M., 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Annu. Rev. Plant Biol. 57, 233–266.

Bartelt-Ryser, J., Joshi, J., Schmid, B., Brandl, H., Balser, T., 2005. Soil feedbacks of plant diversity on soil microbial communities and subsequent plant growth. Perspect. Plant Ecol. 7, 27–49.

Berendsen, R.L., Pieterse, C.M.J., Bakker, P.A.H.M., 2012. The rhizosphere microbiome and plant health. Trends Plant Sci. 17, 478–486.

Bramley, R.G.V., Ellis, N., Nable, R.O., Garside, A.L., 1996. Changes in soil chemical properties under long-term sugar cane monoculture and their possible role in sugar yield decline. Aust. J. Soil Res 34, 967–984.

Brockett, B.F.T., Prescott, C.E., 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 Biol. Biochem. 44, 9–20.

Carmona, M., Zamarro, M.T., Blázquez, B., Durante-Rodríguez, G., Juárez, J.F., Valderrama, J.A., Barragán, M.J., García, J.L., Díaz, E., 2009. Anaerobic catabolism of aromatic compounds: a genetic and genomic view. Microbiol. Mol. Biol. R. 73, 71–133.

Chen, M.M., Chen, B.D., Marschner, P., 2008. Plant growth and soil microbial community structure of legumes and grasses grown in monoculture or mixture. J. Environ. Sci. 20, 1231–1237.

Choi, K.H., Dobbs, F.C., 1999. Comparison of two kinds of biolog microplates (GN and ECO) in their ability to distinguish among aquatic microbial communities. J. Microbiol. Methods 36, 203–213.

Crowley, D., 2000. Function of siderophores in the plant rhizosphere. In: Pinton, R., Varanini, Z., Nannipieri, Z. (Eds.), The Rhizosphere: Biochemistry and Organic Substances at the Soil–Plant Interface. Marcel Dekker, New York, pp. 223–261.

Denef, K., Bubenheim, H., Lenhart, K., Vermeulen, J., Van Cleemput, O., Boeckx, P., Müller, C., 2007. Community shifts and carbon translocation within metabolically-active rhizosphere microorganisms in grasslands under elevated CO_2. Biogeosci. Discuss. 4, 1437–1460.

Dutta, M., Sardar, D., Pal, R., Kole, R.K., 2010. Effect of chlorpyrifos on microbial biomass and activities in tropical clay loam soil. Environ. Monit. Assess. 160, 385–391.

Fierer, N., Schimel, J.P., Holden, P.A., 2003. Variations in microbial community composition through two soil depth profiles. Soil Biol. Biochem. 35, 167–176.

Gomez, E., Ferreras, L., Toresani, S., 2006. Soil bacterial functional diversity as influenced by organic amendment application. Bioresour. Technol. 97, 1484–1489.

Govaerts, B., Mezzalama, M., Unno, Y., Sayre, K.D., Luna-Guido, M., Vanherck, K., Dendoouen, L., Deckers, J., 2007. Influence of tillage, residue management, and crop rotation on soil microbial biomass and catabolic diversity. Appl. Soil Ecol. 37, 18–30.

Gregory, P.J., 2006. Plant Roots: Growth, Activity and Interaction with Soils. Blackwell Publishing, Oxford.

Griffiths, B.S., Ritz, K., Ebblewhite, N., Dobson, G., 1999. Soil microbial community structure: effects of substrate loading rates. Soil Biol. Biochem 31, 145–153.

Han, X.M., Wang, R.Q., Liu, J., Wang, M.C., Zhou, J., Guo, W.H., 2007. Effects of vegetation type on soil microbial community structure and catabolic diversity assessed by polyphasic methods in North China. J. Environ. Sci. 19, 1228–1234.

Hebel, C.L., Smith, J.E., Cromack Jr., K., 2009. Invasive plant species and soil microbial response in the cascade range of Oregon. Appl. Soil Ecol. 42, 150–159.

Huygens, D., Schouppe, J., Roobroeck, D., Alvarez, M., Balocchi, O., Valenzuelaf, E., Pinocheta, D., Boeckxb, P., 2011. Drying-rewetting effects on N cycling in grassland soils of varying microbial community composition and management intensity in south central Chile. Appl. Soil Ecol. 48, 270–279.

Inderjit, ajpai, D., Rajeswari, M.S., 2010. Interaction of 8-hydroxyquinoline with soil environment mediates its ecological function. PLoS ONE 5, e12852.

Joergensena, R.G., Potthoff, M., 2005. Microbial reaction in activity, biomass, and community structure after long-term continuous mixing of a grassland soil. Soil Biol. Biochem. 37, 1249–1258.

Kapoor, R., Mukerji, K.G., 2006. Rhizosphere microbial community dynamics. In: Mukerji, K.G., Manoharachary, C., Singh, J. (Eds.), Microbial Activity in the Rhizosphere. Springer-Verlag, Heidelberg, pp. 89–120.

Kramer, C., Gleixner, G., 2006. Variable use of plant- and soil-derived carbon by microorganisms in agricultural soils. Soil Biol. Biochem 38, 3267–3278.

Latz, E., Eisenhauer, N., Rall, B.C., Allan, E., Roscher, C., Scheu, S., Jousset, A., 2012. Plant diversity improves protection against soil-borne pathogens by fostering antagonistic bacterial communities. J. Ecol. 100, 597–604.

Li, Z.F., Yang, Y.Q., Xie, D.F., Zhu, L.F., Zhang, Z.G., Lin, W.X., 2012. Identification of autotoxic compounds in fibrous roots of Rehmannia (*Rehmannia glutinosa* Libosch.). PLoS ONE 7, e28806.

Liang, Y.L., Chen, Z.J., Xu, F.L., Zhang, C.G., Du, S.N., Yan, Y.G., 2004. Soil continuous cropping obstacles in facility agriculture on Loess Plateau. J. Soil Water Conserv. 18, 134–136 (in Chinese with English abstract).

Lin, R.Y., Rong, H., Zhou, J.J., Yu, C.P., Ye, C.Y., Chen, L.S., Lin, W.X., 2007. Impact of allelopathic rice seedlings on rhizospheric microbial populations and their functional diversity. Acta Ecol. Sin. 27, 3644–3654.

Lu, R.K., 2000. Methods of Soil and Agricultural Chemistry Analysis. China Agricultural Science and Technology Press, Beijing.

Ma, T.T., Luo, Y.M., Christie, P., Teng, Y., Liu, W.X., 2012. Removal of phthalic esters from contaminated soil using different cropping systems: a field study. Eur. J. Soil Biol. 50, 76–82.

Macdonald, L.M., Paterson, E., Dawson, L.A., McDonald, A.J.S., 2004. Short-term effects of defoliation on the soil microbial community associated with two contrasting *Lolium perenne* cultivars. Soil Biol. Biochem 36, 489–498.

Mandal, S.M., Chakraborty, D., Dey, S., 2010. Phenolic acids act as signaling molecules in plant–microbe symbioses. Plant Signal. Behav. 5, 359–368.

Marschner, P., 2007. Plant–microbe interactions in the rhizosphere and nutrient cycling. In: Marschner, P., Rengel, Z. (Eds.), Nutrient Cycling in Terrestrial Ecosystems. Springer-Verlag, Heidelberg, pp. 159–182.

McKinley, V.L., Peacock, A.D., White, D.C., 2005. Microbial community PLFA and PHB responses to ecosystem restoration in tallgrass prairie soils. Soil Biol. Biochem. 37, 1946–1958.

Meriles, J.M., Vargas Gil, S., Conforto, C., Figoni, G., Lovera, E., March, G.J., Guzmán, C.A., 2009. Soil microbial communities under different soybean cropping systems: characterization of microbial population dynamics, soil microbial activity, microbial biomass, and fatty acid profiles. Soil Till. Res. 103, 271–281.

Mukerji, K.G., Manoharachary, C., 2006. Rhizosphere biology-an overview. In: Mukerji, K.G., Manoharachary, C., Singh, J. (Eds.), Microbial Activity in the Rhizosphere. Springer-Verlag, Heidelberg, pp. 1–39.

Murphy, D.V., Cookson, W.R., Braimbridge, M., Marschner, P., Jones, D.L., Stockdale, E.A., Abbott, L.K., 2011. Relationships between soil organic matter and the soil microbial biomass (size, functional diversity, and community structure) in crop and pasture systems in a semi-arid environment. Soil Res. 49, 582–594.

Nehl, D.B., Knox, O.G.G., 2006. Significance of bacteria in the rhizosphere. In: Mukerji, K.G., Manoharachary, C., Singh, J. (Eds.), Microbial Activity in the Rhizosphere. Springer-Verlag, Heidelberg, pp. 89–120.

Okubara, P.A., Paulitz, T.C., 2005. Root defense responses to fungal pathogens: a molecular perspective. Plant Soil 274, 215–226.

Pankhurst, C.E., Stirling, G.R., Magarey, R.C., Blair, B.L., Holt, J.A., Bellf, M.J., Garside, A.L., 2005. Quantification of the effects of rotation breaks on soil biological properties and their impact on yield decline in sugarcane. Soil Biol. Biochem. 37, 1121–1130.

Qi, J.J., Yao, H.Y., Ma, X.J., Zhou, L.L., Li, X.N., 2009. Soil microbial community composition and diversity in the rhizosphere of a Chinese medicinal plant. Commun. Soil Sci. Plant Anal. 40, 1462–1482.

Qu, X.H., Wang, J.G., 2008. Effect of amendments with different phenolic acids on soil microbial biomass, activity, and community diversity. Appl. Soil Ecol. 39, 172–179.
Savario, C.F., Hoy, J.W., 2011. Microbial communities in sugarcane field soils with and without a sugarcane cropping history. Plant Soil 341, 63–73.
Semenov, A.W., van Bruggen, A.H.C., Zelnev, V.V., 1999. Moving waves of bacterial populations and total organic carbon along roots of wheat. Microb. Ecol. 37, 116–128.
Singh, G., Mukerji, K.G., 2006. Root exudates as determinant of rhizospheric microbial biodiversity. In: Mukerji, K.G., Manoharachary, C., Singh, J. (Eds.), Microbial Activity in the Rhizosphere. Springer-Verlag, Heidelberg, pp. 39–53.
Termorshuizen, A.J., Jeger, M.J., 2008. Strategies of soilborne plant pathogenic fungi in relation to disease suppression. Fungal Ecol. 1, 108–114.
Valkonen, J.P.T., Heiroth, W.von, Savela, M.L., 1993. Fungi and Gram-negative bacteria as soilborne minor pathogens of goat's rue (*Galega orientalis* Lam.). Ann. Appl. Biol. 123, 257–269.
Wilkinson, S.C., Anderson, J.M., Scardelis, S.P., Tisiafouli, M., Taylor, A., Wolters, V., 2002. PLFA profiles of microbial communities in decomposing conifer litters subject to moisture stress. Soil Biol. Biochem. 34, 189–200.
Wu, F.Z., Wang, X.Z., Xue, C.Y., 2009a. Effect of cinnamic acid on soil microbial characteristics in the cucumber rhizosphere. Eur. J. Soil Biol. 45, 356–362.
Wu, L.K., Wang, H.B., Zhang, Z.X., Lin, R., Zhong, Z.Y., Lin, W.X., 2011. Comparative metaproteomic analysis on consecutively *Rehmannia glutinosa*–monocultured rhizosphere soil. PLoS ONE 6, e20611.
Wu, Y.P., Ma, B., Zhou, L., Wang, H.Z., Xu, J.M., Kemmitt, S., Brookes, P.C., 2009b. Changes in the soil microbial community structure with latitude in eastern China, based on phospholipid fatty acid analysis. Appl. Soil Ecol. 43, 234–240.
Yang, Y.H., Chen, X.J., Chen, J.Y., Xu, H.X., Li, J., Zhang, Z.Y., 2011. Differential miRNA expression in *Rehmannia glutinosa* plants subjected to continuous cropping. BMC Plant Biol. 11, 53.
Zhang, R.X., Li, M.X., Jia, Z.P., 2008. *Rehmannia glutinosa*: review of botany, chemistry and pharmacology. J. Ethnopharmacol. 117, 199–214.
Zhang, Z.Y., Li, P., Chen, J., Liu, Y.S., Xing, J.B., 2003. Analyses on the trace elements of soils in geo-authentic and non-authentic production areas of Flos Lonicearae. Chin. Mater. Med. 28, 207–213 (in Chinese with English abstract).