0971-4693/94 US $ 5.00
Allelopathy Journal 33 (2): 151-162 (2014) *International Allelopathy Foundation* 2014
Tables: 5, Figs : 2

Allelopathic potential and identification of allelochemicals in *Pseudostellariae heterophylla* rhizosphere soil in different crop rotations

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(Received in revised form: January 8, 2014)

ABSTRACT

Pseudostellariae heterophylla is major medicinal plant in China, it has the problem of soil sickness in continuous cropping, but little is known about the allelochemicals causing this problem. We evaluated the autotoxicity potential of 4-crop rotation in bioassays of lettuce and *P. heterophylla* plantlets, and identified the allelochemicals in different rhizosphere soil samples. The autotoxicity potential of 2-years continuous monoculture was stronger than other crop rotation. The autotoxicity of rice- *P. heterophylla* rotation soil was minimum. The identification of autotoxic substances showed that benzoic acid, myristic acid, cinnamic acid and 2-butenoic acid *etc.* were present in rhizosphere soil of *P. heterophylla*. The benzoic acid, myristic acid and cinnamic acid inhibited the growth of *P. heterophylla* plantlet. In rice -*P. heterophylla* and soybean -*P. heterophylla* rotation, the content of some allelochemicals was decreased. The content of benzoic acid, myristic acid, cinnamic acid and 2-butenoic acid were lower in rice -*P. heterophylla* rotation than 2-years continuous monoculture. The cinnamic acid and 2-butenoic acid were not found in soybean -*P. heterophylla* rotation. These results indicated that crop rotation reduced the accumulation of autotoxic substances and decreased the autotoxicity of *P. heterophylla* continuous monoculture.

Keywords: Allelochemicals, allelopathic potential, monoculture, crop rotation, rhizosphere soil, soil sickness.

INTRODUCTION

The *Pseudostellariae heterophylla* (Caryophyllaceae family) is major traditional Chinese medicine and grown in Fujian, Guizhou, Shandong, Jiangsu and Anhui provinces and is main source of income to farmers. However, its productivity and quality of *P. heterophylla* tuberous products substantially decline after continuous monoculture (8,17). This phenomenon is worldwide and is known as continuous cropping obstacle or soil sickness or replanting disease (23) or continuous monoculture problem (29).

The plant autotoxicity is one major cause of continuous cropping problem (10,12,13,19). The autotoxicity is a plant intraspecific competition and mutual influence. Schreiner proposed that some crop secretions contained some substances which inhibited the growth of their own seedlings (10,19). Later many studies indicated that some

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metabolites (terpenoids, phenolics, steroids, alkaloids and cyanogenic glycosides) secreted by plant roots induces the crop autotoxicity (1,2,8,18,10,24). It has been reported in many crops, such as rice (6), wheat (30), *Zea mays* L. (7), strawberry (22), potato (4), peanut (33), soybean (32), vegetables (25), alfalfa, *Lolium rigidum* Gaud. (3,5), apple (21,28), *Rehmanniae* Libosch. (16), ginseng (35), *Salvia miltiorrhiza* Bge. (15), *Radix Notoginseng* Burk. (36) and *Angelicasinensis* Oliv. (31). Autotoxicity also occurs in *P. heterophylla* continuous monoculture (8,17). However, the mechanism of continuous cropping obstacle is not yet clear in *P. heterophylla* cultivation.

To study allelopathic potential of *P. heterophylla*, we designed four crop rotation in experimental field, viz., (i) 1-year monoculture, (ii) 2-years continuous monoculture, (iii) Rice -*P. heterophylla* and (iv) Soybean -*P. heterophylla* rotations. Soil samples were collected to evaluate the allelopathic potential of different crop rotations. The autotoxic substances were isolated and identified by different polar resins and GC/MS from aqueous extracts of different soil samples. The study aimed to analyze the effects of different rotations on develoment of *P. heterophylla* autotoxicity and provide technology to solve the soil sickness problem of *P. heterophylla* in continuous monoculture.

MATERIALS AND METHODS

The experiment was conducted in experimental field of *P. heterophylla*, Zherong County, Ninde Municipality, Fujian Province, China (26°39'N, 119°31'E, 660 m of mean height above sea level, acidic red loam) in 2009-2011. The *P. heterophylla* cultivar, 'Zheseng-2', was planted in December and harvested in July next year. The experiment treatments consisted of 5-crop rotations: (i) 1-year monoculture (M1), (ii) 2-years continuous monoculture (M2), (iii) Rice -*P. heterophylla* rotation (RP), (iv) soybean (*Glycine max* (L.) Merr.) -*P. heterophylla* rotation (BP) and (v) fallow (kept uncultivated, F1). These experimental treatments were replicated thrice. The experimental plots were 5×5 m (25 m^2). Individual *P. heterophylla* root was planted in plots at 5 × 10 cm spacing (5 cm plant to plant and 10 cm row spacing). One-year monoculture was started in December, 2010. The planting of 2-years continuous monoculture, rice -*P. heterophylla* and soybean -*P. heterophylla* rotation was started in December, 2009 and in December, 2010, respectively. The rice and soybean were planted in July, 2010 and harvested in November, 2010, respectively. The different crop rotations of *P. heterophylla* were harvested in July, 2011. Top soil samples (20 cm depth) were collected from all crop rotations, except fallow at harvest. The soil sample from adjacent uncultivated field was collected as control. The design of crop rotations was shown in Table 1.

II. Aqueous extraction of autotoxic compounds and bioassays

Soil Extract bioassay: The air-dried soils (250 g), passed through 2 mm sieve, were soaked in 750 mL distilled water and rotated at 150 r min^{-1} for 2 h. The extract was filtered and the extraction was done thrice. The aqueous extracts from three extractions were combined and concentrated to 50 mL under vacuum at 45^{o}C i.e. 1 mL of aqueous extracts contained substances of 5 g soil. The aqueous extract was diluted with distilled water into 1 mL solution, respectively, contained substances of 0.2, 0.5, 1, 2 and 5 g soil. The 5 mL

aqueous extract of every concentration was added petri dish, while distilled water was used as control. Ten lettuce seeds were placed on the filter paper in the petri dishes and cultured at 25 ^{o}C for 5 d. Each treatment was repeated thrice. The radicle lengths of treated seedlings in comparison to controls were used as index of inhibition rate (IR). IR was calculated as under:

$$IR= Treatment - Control/Control \times 100\%.$$

When, IR < 0, indicates inhibition; when IR > 0, indicates promotion. All data were statistically analysed by variance using the Statistical Analysis System Program (SPSS). Each value was mean of three replicates.

***P. heterophylla* plantlet bioassay:** About 2 g plantlets of *P. heterophylla* were planted in a culture flask containing 25 mL solid medium. The basic recipe of solid medium was MS which contained 1.5 mg L^{-1} 6-BA and 0.1 mg L^{-1} NAA. The bioassay method was same as described above. After 20 days, the plantlets of different treatments were weighed. The inhibition rate (IR) was calculated as under:

$$IR= Treatment\ weight - Control\ weight/Control\ weight \times 100\%.$$

III. Methanol extraction through different polar resins and bioassays

The aqueous extract was diluted into 1 mL solution, contained substances of 2 g soil. Five different type of polar resins ADS-7, ADS-8, ADS-17, ADS-21 and ADS-F8 were respectively used to absorb the substances from diluted aqueous extract. The 5 g resin and 50 mL aqueous extract (2 g mL^{-1}) were added into 250 mL triangular flask and rotated at 150 r min^{-1} for 24 h. After keeping for 30 min, the liquid supernatant was removed and 50 mL methanol was added into triangular flask and was rotated at 150 r min^{-1} for 12 h. The methanol eluent was collected and used for bioassay. The 5 mL methanol eluent was transferred into the petri dish containing double-layered filter paper. The dishes were placed in fume cupboard and methanol was volatilized at room temperature. Then 5 mL distilled water was added into the dish. The bioassays of methanol extract were done in same way as water extract.

IV. Identification of autotoxic chemicals

Preliminary bioassay study indicated that the methanol extract of ADS-7 exhibited the greatest inhibitory activity on radicle growth of lettuce. Thus, the extract of ADS-7 was further analyzed by GC/MS (Autospec-240 MS Ion Trap mass spectroscopy, Varian, California, U.S.A.). The 5 mL of ADS-7 extract was freeze-dried under liquid N_2 at -180 ℃ and derivatized by silane. The solution was filtered through a 0.45 μm filter before injected into the GC-MS system.

The injection volume was 1 μL. Helium was used as the carrier gas, and the flow rate was adjusted to 1 mL min^{-1}. The oven temperature was initially programmed at 60 ℃, ramped to 140 ℃ at a rate of 15 ℃ min^{-1} and remained at 140 ℃ for 2 min, ramped from 140 to 200 ℃ at a rate of 15 ℃ min^{-1} and remained at 200 ℃ for 5 min, ramped from 200 to 250 ℃ at a rate of 4 ℃ per minute and remained at 250 ℃ for 10 min. The injector

temperature was 280 ℃. The substances were separated by reversed phase multi-purpose GC column chromatography (Varian California, U.S.A., VF-5 ms, 30 m×0.25 μm). MS data were acquired in the negative ionization mode. The full scan mass covered a range from m/z 50 to 1000. The m/z values of compounds obtained in the spectra were analyzed according to the spectrogram in the literature. The percentage of chemicals in different soil samples was calculated as: quantity of one chemical/quantity of total chemical×100%. All data were subject to an analysis of variance using the Statistical Analysis System Program (SPSS). Each value was expressed as the mean of three replicates.

V. Bioassays of allelochemicals

The allelochemicals benzoic acid, myristic acid, cinnamic acid (identified by GC/MS) and ferulic acid were purchased from Sigma Chemicals Co., U.S.A. These 4-allelochemicals were respectively diluted into 1 L solution contained 50, 100, 200, 300 and 400 μmol. The 2 mL solution of different concentrations including 0, 50, 100, 200, 300 and 400 μmol L^{-1} were respectively added in 25 mL solid medium of a culture flask. The bioassay material was *P. heterophylla* plantlet and bioassay method was same as above.

VI. Protective enzyme activity and malondialdehyde content determination in *P. heterophylla* leaf

The *P. heterophylla* plantlets planted on solid medium containing 200 μmol L^{-1} of different allelochemicals were used to analyse the protective enzyme activity and malondialdehyde (MAD) content. The 0.5 g of *P. heterophylla* plantlets was weighed and grinded into homogenate using 2 mL phosphate buffer (50 mM phosphate, pH 7.0, 1% w/v PVP). The homogenate was moved into 10 mL centrifuge tube and centrifuged at 1,000 g for 10 min. The supernatant was used to detect superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) activity, MAD and protein content (18).

RESULTS AND DISCUSSION

Soil aqueous extract bioassay

Plant autotoxicity caused by allelochemicals from plant's own secretion, leaching, volatilization and decomposition are main causes of continuous cropping soil sickness problem (14,19,24). The aqueous extract of 2-years monoculture soil was more inhibitory effect to growth and biomass of lettuce and *P. heterophylla* plantlets than other soil samples (Table 1, 2). The magnitude of inhibition increased with higher concentrations of soil extract. At the same concentration, the IR of 2-years monoculture soil was higher than 1-year monoculture and fallow soil.

The aqueous extract of rotation soil was less inhibitory to growth of lettuce and *P. heterophylla* plantlets than 2-years monoculture soil (Fig.1). The rotations decreased the autotoxic potential of soil caused by continuous cropping of *P. heterophylla*. The rice - *P. heterophylla* rotation had greater decreasing effects on soil autotoxity than soybean - *P. heterophylla* rotation. The rotation is effective method to overcome the problem of continuous cropping soil sickness. The rotation of different crops could decreases the

Table 1. Sowing and harvest dates of crops in various crop rotations

Crop Rotation	First crop		Second Crop	
	Date sown	Date harvested	Date sown	Date harvested
P. heterophylla cultivation of	None	None	December,	July, 2011
P. heterophylla cultivation of	December,	July, 2010	December,	July, 2011
P. heterophylla cultivation of	December,	July, 2010	December,	July, 2011
P. heterophylla cultivation of	December,	July, 2010	December,	July, 2011
Rice cultivation of RP	July, 2010	November,	None	None
Soybean cultivation of BP	July, 2010	November,	None	None

F; Fallow soil, M1: I- Year Monoculture, M2: 2- Years Monoculture, RP: Rice- *P. heterophylla*, BP: Bean- *P. heterophylla*

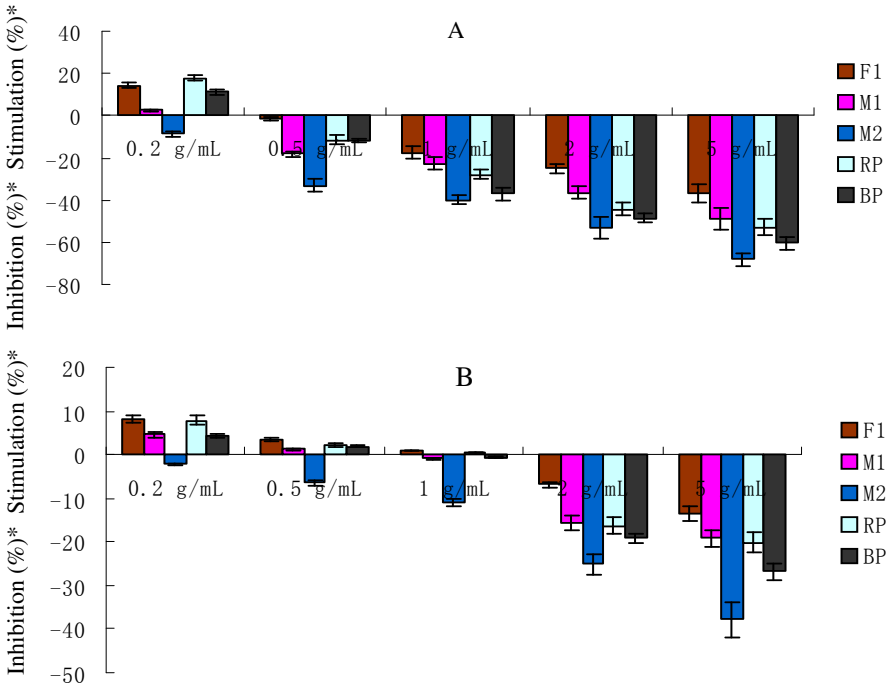

Figure 1. Inhibitory effects of aqueous extracts from rhizosphere soil on radicle growth of lettuce and *P. heterophylla* plantlets. F; Fallow soil, M1: I- Year Monoculture, M2: 2- Years Monoculture, RP: Rice- *P. heterophylla*, BP: Bean- *P. heterophylla;* A, inhibitory effects on radicle growth of lettuce; B, inhibitory effects on radicle growth of *P. heterophylla* plantlets

autotoxicity and adjust the soil microbial community composition by decreasing the population of harmful microbes (14). For example, rotation of American ginseng - *Perilla frutescens* reduces the continuous cropping problem of American ginseng (39). Ginseng

Table 2. Compounds in methanol extract of ADS-7 from *P. heterophylla* rhizosphere soil as identified by GC/MS analysis

Category	Retention time (min)	Compound name	NIST match	M^+/Z for major fragmentation peaks	GC/MS intensity (k counts)	CAS
Acids	21.094	9,12,15-Octadecatrienoic acid	87	137	57.73	463-40-1
	21.656	Benzoic acid	90	105	105.03	65-85-0
	27.089	Myristic acid	93	83	189.03	544-63-8
	28.694	Palmitic acid	89	43	213.09	57-10-3
	35.526	2-Butenoic acid	83	86	93.37	3724-65-0
	37.869	Cinnamic acid	92	147	296.47	140-10-3
Alcohols	25.673	Propatriol	93	45	87.55	57-55-6
	30.732	Ergost-8(14)-en-3β-ol	87	44	77.53	632-32-6
Esters	29.329	Dibutyl phthalate	89	149	113.47	84-74-2
	38.755	BIS(2-Ethylhexyl)phthalate	91	149	77.83	117-81-7
Benzene	26.312	Danthron	90	180	69.58	84-65-1
compounds	36.647	2,2'-Methylenebis(6-tert-butyl-4-methylphenol)	89	176	89.64	119-47-1

Table 5. Effects of four allelochemicals on SOD, POD, CAT activities and MDA content in *P. hererophyll* plantlet

	SOD (U min^{-1} g^{-1} FW)		POD (U min^{-1} g^{-1} FW)		CAT (H_2O_2 mg g^{-1} FW)		MAD (nmol g^{-1} FW)	
Treatments	200 μmol L^{-1}	400 μmol L^{-1}	200 μmol L^{-1}	400 μmol L^{-1}	200 μmol*L^{-1}	400 μmol L^{-1}	200 μmol L^{-1}	400 μmol L^{-1}
Distilled water	0.011d	0.011d	0.22d	0.22d	40.55c	40.55d	1.22e	1.22d
Benzoic acid	0.025a	0.031a	0.33b	0.41b	55.67a	61.65a	1.46a	1.52a
Myristic acid	0.018b	0.027b	0.26c	0.32c	46.71b	55.54b	1.42b	1.54a
Cinnamic acid	0.019b	0.028b	0.41a	0.45a	42.54c	50.38c	1.37c	1.42b
Ferulic acid	0.016c	0.020c	0.35b	0.40b	58.09a	60.43a	1.31d	1.35c

SOD: Superoxide dismutase, POD: Peroxidase, CAT: Catalase, MAD: Malonyl dialdehyde, FW: Fresh weight. The lowercase letters behind data represented significant differences（$P<0.05$）

rotation with other crops reduces the continuous cropping problem, improves the soil texture and composition of soil microorganisms (39). The rotation of pepper with rice not only increased the yield, but also reduced the blight incidence (34).

Bioassay of methanol extracts

The substances absorbed by different types of polar resin showed variable inhibitory effects on growth of lettuce and *P. heterophylla* plantlets (Fig.2). The fraction absorbed by polar resin ADS-7 was most inhibitory. The IR of ADS-7 fraction's on lettuce radicles in different crop rotations followed the inhibition order: Fallow (-33.97%), rice- *P. heterophylla* rotation (37.79%), 1-year monoculture (-38.39%), soybean -*P. heterophylla* rotation (-47.12%) and 2-years continuous monoculture (-49.67%). The inhibitory effects of ADS-7 fraction on growth of *P. heterophylla* plantlets followed the order: -12.49% (Fallow), -15.38% (1-year monoculture), -13.69% (Rice -*P. heterophylla* rotation) and -19.27% (Soybean -*P. heterophylla* rotation), -27.23% (2-years continuous monoculture). The fraction of ADS-7 in different soil samples were further identified by GC/MS.

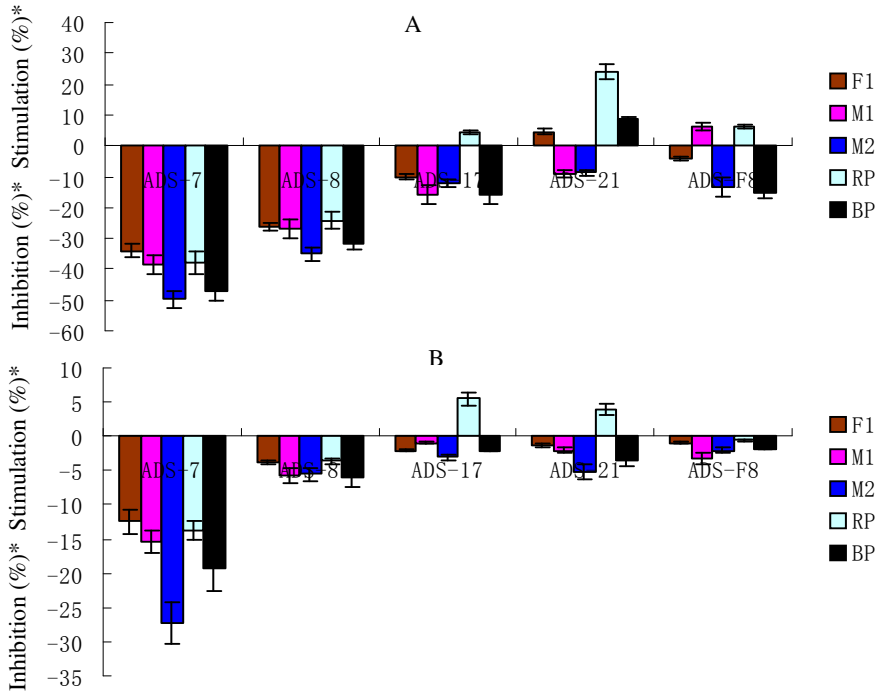

Figure 2. Inhibitory effects of methanol extracts by different polar resin on radicle growth of lettuce and *P. heterophylla* plantlets. F; Fallow soil, M1: I- Year Monoculture, M2: 2- Years Monoculture, RP: Rice- *P. heterophylla*, BP: Bean- *P. heterophylla;* A, inhibitory effects on radicle growth of lettuce; B, inhibitory effects on radicle growth of *P. heterophylla* plantlets

Compounds identified in ADS-7 fraction

The GC/MS analysis of ADS-7 fractions identified 83 compounds (m/z range from 40 to 300) identified with GC/MS user-library spectrum of pure reference compounds. The identified substances were: alkanes, esters, alcohols, acids, amines, sugar, aldehydes, phenols and others. The researchers found that phenol and terpenoid compounds were the main allelochemicals in higher plants caused autotoxicity in continuous cropping (37,38).

Twelve substances in different soil samples which had great variability in contents were further analyzed (Table 2,3). Among them, the benzoic acid, myristic acid, 2-Butenoic acid and cinnamic acid identified in *P. heterophylla* rhizosphere soil were autotoxic substances (19,24). These substances were also present in rhizosphere soil of other crops soil sickness problem in continuous cropping. For example, more than 10 autotoxic substances (including benzoic acid, cinnamic acid and ferulic acid) were identified in root exudates of tomato, cucumber, pepper and other horticultural crops (20,27). When the accumulation of these autotoxic substances in soil reached a certain concentration, they could the change the soil properties, microbial community structure, nutrients cycling in soil and finally damages the crop protective enzymes and growth regulator system, resulting in poor crop growth and death of plants (19). In fallow soil only three substances (9,12,15-Octadecatrienoic acid, benzoic acid and BIS(2-Ethylhexyl) phthalate) were detected. The benzoic acid content in soils of 1-year monoculture and 2-years continuous monoculture was 10 and 13.5 times more than fallow soil. The contents of octadecatrienoic acid, myristic acid, 2-butenoic acid, cinnamic acid, ergost-8(14)-en-3β-ol, BIS (2-Ethylhexyl) phthalate and danthron in 2-years continuous monoculture soil were higher than fallow soil and 1-year monoculture.

Table 3. The content of substances in various crop rotations

Substance	Crop rotations				
	F1 (%)	M1 (%)	M2 (%)	RP (%)	BP (%)
9,12,15-Octadecatrienoic acid	0.04d	0.16c	0.26b	1.05a	0.29b
Benzoic acid	0.09d	0.85b	1.20a	0.68c	1.16a
Myristic acid	0	0.25a	0.13b	0	0
Palmitic acid	0	6.35a	4.82b	3.63c	4.90b
2-Butenoic acid	0	0.28b	2.50a	0.20c	0
Cinnamic acid	0	0	1.24a	0.57b	0
Propatriol	0	0.7a	0.19c	0	0.43b
Ergost-8(14)-en-3β-ol	0	0.37b	0.51a	0	0
Dibutyl phthalate	0	4.47a	2.19c	3.63b	3.42b
BIS(2-Ethylhexyl)phthalate	6.07d	5.98d	8.47b	8.03c	8.83a
Danthron	0	0.30b	1.19a	0	0
2,2'-Methylenebis(6-tert-butyl-4-methylphenol)	0	4.57c	4.31c	7.92b	12.00a

F; Fallow soil, M1: I- Year Monoculture, M2: 2- Years Monoculture, RP: Rice- *P. heterophylla*, BP: Bean- *P. heterophylla*

The content of these substances changed in soil of rice -*P. heterophylla* and soybean -*P. heterophylla* rotation. Myristic acid, propatriol, ergost-8(14)-en-3β-ol and danthron were not detected in soil of rice -*P. heterophylla* rotation. While in soil of

soybean -*P. heterophylla*, myristic acid, 2-butenoic acid, cinnamic acid, ergost-8(14)-en-3β-ol and danthron were not detected. The content of 2, 2'-methylenebis (6-tert-butyl-4-methylphenol) in soil of rice -*P. heterophylla* and soybean -*P. heterophylla* rotation was higher than that in 2-years continuous monoculture soil.

Allelochemicals Bioassay on *P. heterophylla*

The inhibitory effects of 4-allelochemicals (benzoic acid, myristic acid, cinnamic acid and ferulic acid) were studied on *P. heterophylla*. All these allelochemicals inhibited the growth of *P. heterophylla* plantlet (Table 4). The inhibitory effects become stronger with increasing amount of allelochemicals added in solid medium. There was no significant difference in inhibitory effects of these 4-allelochemicals on growth of *P. heterophylla* plantlet.

Table 4. Inhibitory effects of four allelochemicals on growth of *P. hererophylla* plantlet

	Inhibition rate (%) in growth of *P. heterophylla* plantlets				
Treatments	50 μmol L^{-1}	100 μmol L^{-1}	200 μmol L^{-1}	300 μmol L^{-1}	400 μmol L^{-1}
Benzoic acid	-1.38c	-3.41b	-6.50c	-12.55a	-15.77a
Myristic acid	0.56b	-2.95b	-8.15b	-12.48a	-16.19a
Cinnamic acid	3.44a	-1.43b	-4.42d	-8.55b	-12.76c
Ferulic acid	-0.93d	-4.60a	-10.63a	-12.23a	-14.39b

The lowercase letters behind data represented significant differences（P<0.05）

To determine the protective enzyme activity and MAD content the *P. heterophylla* plantlets were planted on solid medium containing 200 μmol L^{-1} and 400 μmol L^{-1} allelochemicals (Table 5). The application of benzoic acid, myristic acid, cinnamic acid and ferulic acid increased the activities of SOD, POD, CAT and content of MAD in plantlets than treated by distilled water. Thus benzoic acid, myristic acid, cinnamic acid and ferulic acid affected the protective enzyme activity and MAD content in *P. heterophylla*.

CONCLUSIONS

The rice and soybean-*P. heterophylla* rotations reduced the content of autotoxic substances and decrease the *P. heterophylla* continuous cropping soil sickness problem. The change in root exudates' component content significantly influenced the rhizosphere microbial community structure and functional diversity. In different crop rotations, the accumulation of autotoxicity substances in *P. heterophylla* rhizosphere soil had variable influences on microbial groups. Therefore, we need to further study the diversity of microbial structure and functions in *P. heterophylla* rhizosphere soil under different crop rotations and whether the ecosystem in *P. heterophylla* rhizosphere soil was restored.

ACKNOWLEDGEMENTS

We would like to thank National Natural Science Foundation of China for providing funds (Grant no. 2012CB126309 and U1205021), and Prof. Chou C. H. (China Medical University, Taiwan) for support and assistance.

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