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Full Length Research Paper

Lauric acid and 2, 6-ditertbutyl phenol, two major allelochemicals from *Rehmannia glutinosa* inhibiting the germination of succeeding crop, *Sesamum indicum*

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Rehmannia glutinosa **Libosch, a well known herb for Chinese traditional medicine, and sesame are two main crops in Northwestern Henan, China. It has been known however that, a rotation of these two crops, for example, cultivation of sesame preceeded with *R. glutinosa* Libosch, leads to decreased yield and poor quality of sesame. In order to identify the compound or allelochemicals which inhibit the growth of sesame, the effect of the crude tissue extractions of *R. glutinosa* on the germination rate, seedling height, root length and fresh weight of sesame was analysized. We found that most allelochemicals exist in the ethyl acetate extraction of the leaves. Further fractionation of the leaf extraction on silica gel column chromatography was followed by GC-MS (gas chromatography mass spectrometry). We for the first time identified lauric acid and 2,6-ditertbutyl phenol as two main alllelochemicals, which inhibit the seed germination of sesame. The identification of the allelochemicals in this study paves the way for further characterization of the molecular interaction of allelochemicals from the preceeding crop *R. glutinosa*, the soil and the succeeding crop sesame, which could be beneficial for improvement of the crop rotation between *R. glutinosa* and sesame.**

Key words: *Rehmannia glutinosa* Libosch, sesame, allelopathy, allelochemicals.

INTRODUCTION

Allelopathic phenomenon is know as any process involving secondary metabolites produced by plants, algae, bacteria, coral and fungi that influences the growth and development of agricultural and biological systems (Rice, 1971, 1984). The biomolecules are called allelochemicals and are produced by some plants as secondary metabolites. When the allelochemicals are released into the environment, they inhibit or stimulate the development of neighbouring plants. It is reported that allelochemicals were present in virtually all plant tissue, for example, leaves, fruits, stems, as well as roots. These allelochemicals, which affect the growth of other plants and succeeding crops, were released by processes such as volatilization, root exudation, leaching and decomposition of plant residues (Rice, 1984; Kong and Hu, 2001).

Rehmannia glutinosa Libosch. is a perennial herb with reddish-violet flowers, native to China. It is one of the most often used herbs for traditional Chinese medicine. It has been cultivated as one of the major economic crops in Northwest Henan for about 2000 years. The root of *R. glutinosa* is used as an ingredient for Chinese herb medicine to replenish vitality, strengthen the liver, kidney and heart and for the treatment of a variety of ailments like diabetes, constipation, anemia, urinary tract problems, dizziness and regulation of menstrual flow (Ding, 2001). There are many studies on the chemical constituents *of R. glutinosa* (Wang et al., 2001; Wang et al., 2007; Zeng et al., 2006), pharmacological activities (Yu et al., 2001; Wu et al., 2006) and clinical application (Lu and Wang, 2004). Sesame was another important economic crop in Northwest Henan as well as the rest of the Province (Yang and Huang, 2009). It has been found out that for a long time the yield of sesame crops succeeding

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Abbreviations: GC-MS, Gas chromatography mass spectrometry; **SE,** standard error.

the cultivation of *R. glutinosa* drops severely for unknown reason. In order to understand the molecular mechanism of the allelopathic effect of *R. glutinosa* Libosch on succeeding sesame production, tissue extractions from *R. glutinosa* were fractioned by silica gel column chromatography directed by bioassay. The fractions with allelopathic activity were analysized with GC-MS (gas chromatography-mass spectrometry) method. We for the first time identified lauric acid and 2,6-ditertbutyl phenol as two main alllelochemicals from *R. glutinosa*. The identification of the allelochemicals in this study paves the way for further characterization of the molecular interaction of allelochemicals from *R. glutinosa*, the soil and the succeeding crop sesame, which could be beneficial for improvement of the crop rotation between *R. glutinosa* and sesame.

MATERIALS AND METHODS

Preparation of extractions from *R. glutinosa*

Roots, stems, leaves of *R. glutinosa* were dried at 50°C for 48 h. 20 g of each part was weighed and soaked in distilled water for 24 h at 25°C, respectively. Then they were filtered and diluted with distilled water to 200 ml, respectively. So the concentration of crude extraction is 100 mg ml^{-1}. Extraction with concentrations of 50 and 10 mg ml^{-1} was got by dilution.

Separating allelochemicals method

Leaves of *R. glutinosa* were dried at 50°C for 48 h and extracted with distilled water. The solution was extracted with petroleum ether, dichloromethane, ethyl acetate, 1-butanol in turns. Each organic phase was condensed with rotatory evaporator and dried at 50°C for 24 h. Each solid specimen was prepared to appropriate concentration for bioassay.

The ethyl acetate extract was separated using column chromategraphy performed on silica gel (chloroform /MeOH gradient elution). Fractions which have the same profile by thin layer chromatography on silica gel plates were bulked together. Fractions 1 ~ 21 were got by eluting.

Bioassay

Sesame was chosen for bioassay as test plants. Each of the extractions from roots, stems and leaves of *R. glutinosa* was added to a sheet of filter paper in a 9-cm Petri dish and dried. Same volume of distilled water was added to ck group. The operation adding organic phase and fractions eluted from column chromatography to Petri dish was the same. But the ck Petri dish was added to corresponding organic solvent. Seeds of the test species were sterilized in a 2% (wt/V) solution of sodium hypochlorite for 15 min and rinsed in distilled water four times. 5 ml distilled water was added to each Petri dish. Thirty seeds were sown on filter paper in Petri dishes and allowed to germinate in the dark at 28°C for 3 days. Then the germinated seeds were counted and the germination percentage was calculated by reference to that of control seeds which had been treated with distilled water. The shoot and root lengths of the seedlings were then measured with a ruler and the mass of 10 plants of sesame was weighed. Growth rate (%) = (germinated seed number in 3 days/ test seed number) × 100%

GG-MS analysis

Silylation

The specimen was silylated by tsilylating agent (BSTFA) before GC-MS analysis in order to determinate nonvolatile components. The specimen was freeze-dry and 250 µl tsilylating agents were added (BSTFA:Pyridine = 5:1); and then kept in 80°C for 2 h for derivation.

Chromatographic conditions were: Capillary column SE-54 (30 mm × 0.25 mm × 0.25 mm). The column temperature was set at 120°C and elevated to 250°C at 15°C/min, keeping 3 min. The flow rate was 0.70 mL/min. The temperature of sample vent was 285°C; inject volume, 1 µL. MS condition, the ionization mode was electron ionization. The ionization source temperature was 250°C. The electron energy was 70 eV. The carrier gas was He. The components were separated and identified by GC-MS and elucidated on the standard mass spectral data.

Data analysis

All data were analyzed by Excel2000 software. Duncan's tests of Data Processing System (DPS, Version 3.11) were used to multiple comparisons of means.

RESULTS

Effect of extraction from different parts of *R. glutinosa* on sesame

Bioassay, using extraction from roots, stems and leaves of *R. glutinosa* was performed with sesame seed as test plant. The results are shown in Table 1.

The results of Table 1 showed that extractions from different parts of *R. glutinosa* inhibited seed germination rate, seedling height, root length and fresh weight of sesame, except that extraction with concentration 10 mg ml^{-1} from root promotes indexes of sesame. Inhibitory effect on sesame was enhanced with rising extraction concentration. Inhibition effect of root length of sesame by extraction was more serious than seed germination rate, seedling height and fresh weight of sesame. Allelopathic activity of leaves extraction was stronger than extractions from roots and stems. Germination rate, seedling height, root length and fresh weight were all inhibited by leaves extraction at concentration of 10 mg ml^{-1}. But the difference of germination rate and seedling height was not significant comparing with control group. The difference of root length and fresh weight was significant. At concentration of 50 mg ml^{-1} of leaves extraction, the difference of germination rate was not significant comparing with control group. The differences of other indexes were extremely significant comparing with control group. At concentration of 100 mg ml^{-1} of leaves extraction, the differences of all indexes were extremely significant comparing with control group.

Effect of organic phase extracted from *R. Glutinosa*

Leaves extraction with distilled water from *R. glutinosa*

Table 1. Effects of extraction from different parts of *R. glutinosa* Libosch. on germination rate, seedling height, root length and fresh weigh of sesame (mean ± SE).

Part	Conc. (mg ml^{-1})	Germination rate (%)	Seedling height (cm)	Root length (cm)	Fresh weight (g)
Control		92.0 ± 3.6aA	2.74 ± 0.26 aA	5.28 ± 0.28 aA	0.277 ± 0.018aA
Root	10	93.3 ± 3.1aA	2.78 ± 0.13 aA	5.46 ± 0.29 aA	0.283 ± 0.005aA
	50	90.7 ± 3.1abA	2.64 ± 0.21 aA	4.98 ± 0.34 bA	0.242 ± 0.013bAB
	100	84.0 ± 4.6bA	2.22 ± 0.24 bB	4.24 ± 0.27 cB	0.215 ± 0.017cB
Stem	10	90.3 ± 3.5aA	2.61 ± 0.25aA	5.16 ± 0.27aA	0.266 ± 0.019abA
	50	86.7 ± 3.1abA	2.41 ± 0.24aAB	4.66 ± 0.23bB	0.251 ± 0.022abA
	100	83.7 ± 2.1bA	2.05 ± 0.22bB	4.09 ± 0.18cC	0.232 ± 0.01bA
Leaf	10	88.0 ± 6.6aA	2.46 ± 0.17bAB	4.16 ± 0.23bB	0.195 ± 0.018bB
	50	82.0 ± 3.6aAB	2.14 ± 0.17cB	2.79 ± 0.17cC	0.158 ± 0.016cBC
	100	69.0 ± 6.1bB	1.64 ± 0.16dC	2.05 ± 0.15dD	0.114 ± 0.016dC

The small and capital English letters in the same line indicate significant differences at 5% and 1% level, respectively, among the handled and control of the same crop.

Table 2. Effects of different concentration extraction of *R. glutinosa* leaf on germination rate, seedling height, root length and fresh weigh of sesame (mean ± SE).

Phase	Conc. (mg ml^{-1})	Germination rate (%)	Seedling height (cm)	Root length (cm)	Fresh weight (g)
Control		91.7 ± 3.2aA	2.71 ± 0.21aA	5.30 ± 0.17aA	0.27 ± 0.015aA
Petroleum ether	100	90.0 ± 5.66aA	2.66 ± 0.24aA	5.28 ± 0.13aA	0.27 ± 0.022aA
Dichloromethane	5	89.0 ± 2.6abA	2.67 ± 0.11aAB	5.12 ± 0.22abAB	0.27 ± 0.014aA
	10	86.0 ± 3.0abA	2.60 ± 0.21aAB	4.82 ± 0.28bB	0.25 ± 0.013aAB
	20	83.7 ± 3.2bA	2.32 ± 0.20bB	4.16 ± 0.25cC	0.21 ± 0.022bB
Ethyl acetate	5	83.3 ± 3.8bA	2.45 ± 0.16bB	4.24 ± 0.12bB	0.219 ± 0.023bB
	10	74.0 ± 2.6cB	2.04 ± 0.12cC	3.16 ± 0.29cC	0.154 ± 0.014cC
	20	64.0 ± 3.6dC	1.66 ± 0.13dD	1.87 ± 0.20dD	0.109 ± 0.02dC
1-butanol	5	86.7 ± 3.2ab	2.70 ± 0.13aA	5.21 ± 0.11aA	0.26 ± 0.016aA
	10	83.3 ± 2.1b	2.56 ± 0.19aAB	4.94 ± 0.24bA	0.24 ± 0.013aAB
	20	76.7 ± 4.2c	2.29 ± 0.16bB	4.28 ± 0.22cB	0.21 ± 0.017bB

The small and capital English letters in the same line indicate significant difference at 5% and 1% level, respectively, among the handled and control of the same crop.

leaves was condensed with rotatory evaporator 50. Next extracted is the solution with petroleum ether, dichloromethane, ethyl acetate 1-butanol in turns. Bioassay was performed using organic phases and results are shown in Table 2.

Data in Table 2 showed that petroleum ether phase had no inhibitory effect on sesame at any set of concentration. Dichloromethane, ethyl acetate and 1-butanol phase all had inhibitory effect on sesame. But ethyl acetate phase had the strongest inhibitory effect. The differences of all indexes of sesame were extremely significant comparing with control group at any set of concentration of ethyl acetate phase. In a word, ethyl acetate phase had the highest content of allelochemicals inhibiting sesame growth. Ethyl acetate phase was used as material for follow-up separation of allelochemicals.

The solid condensed from ethyl acetate phase was black, 5.13 g, and was mixed with silica gel to perform silica gel column chromatography. Fractions 1-21 were got by gradient elution with chloroform/methyl alcohol. Effect of fractions 1-5; 8 - 21 was not obvious comparing with control group. So Table 3 only listed the bioassay results of fractions 6 - 8. Results in Table 3 showed that germination rate, seedling height, root length and fresh weight of sesame were all suppressed by fractions 6 - 8 at any set of concentration. Fraction 7 had the strongest inhibitory effect. The differences of germination rate, seedling height, root length and fresh weight of sesame were extremely significant comparing with control group. So fraction 7 contained more allelochemicals than the other fractions.

GC-MS results of Fraction 7

According to the results, fraction components eluted from silica gel column chromatographic were analyzed by GC-

Table 3. Effects of elution of Fractions 6 - 8 on germination rate, seedling height, root length and fresh weigh of sesame (mean ± SE).

Elution	Conc. (mg ml^{-1})	Germination rate (%)	Seedling height (cm)	Root length (cm)	Fresh weight (g)
Control		91.3 ± 3.5aA	2.70 ± 0.20aA	5.30 ± 0.15aA	0.272 ± 0.016aA
Fraction 6	5	88.0 ± 3.0abA	2.65 ± 0.09aA	5.12 ± 0.21aAB	0.265 ± 0.014abAB
	10	85.0 ± 3.0abA	2.54 ± 0.18abA	4.81 ± 0.18bBC	0.245 ± 0.010bcAB
	20	83.0 ± 4.4bA	2.39 ± 0.15bA	4.58 ± 0.22bC	0.232 ± 0.008cB
Fraction 7	5	82.3 ± 2.5bB	2.46 ± 0.17bA	4.58 ± 0.22bB	0.234 ± 0.022bA
	10	72.7 ± 3.1cC	2.06 ± 0.15cB	3.20 ± 0.20cB	0.177 ± 0.021cB
	20	64.7 ± 2.3dD	1.72 ± 0.10dC	1.62 ± 0.22dD	0.093 ± 0.006dC
Fraction 8	5	86.7 ± 3.2abA	2.5 1 ± 0.16abA	5.10 ± 0.19aA	0.264 ± 0.015aA
	10	84.3 ± 2.5bAB	2.42 ± 0.16bAB	4.39 ± 0.27bB	0.226 ± 0.024bAB
	20	76.0 ± 3.6cB	2.12 ± 0.18cB	3.74 ± 0.22cC	0.197 ± 0.021bB

The small and capital English letters in the same line indicate significant difference at 5% and 1% level, respectively, among the handled and control of the same crop.

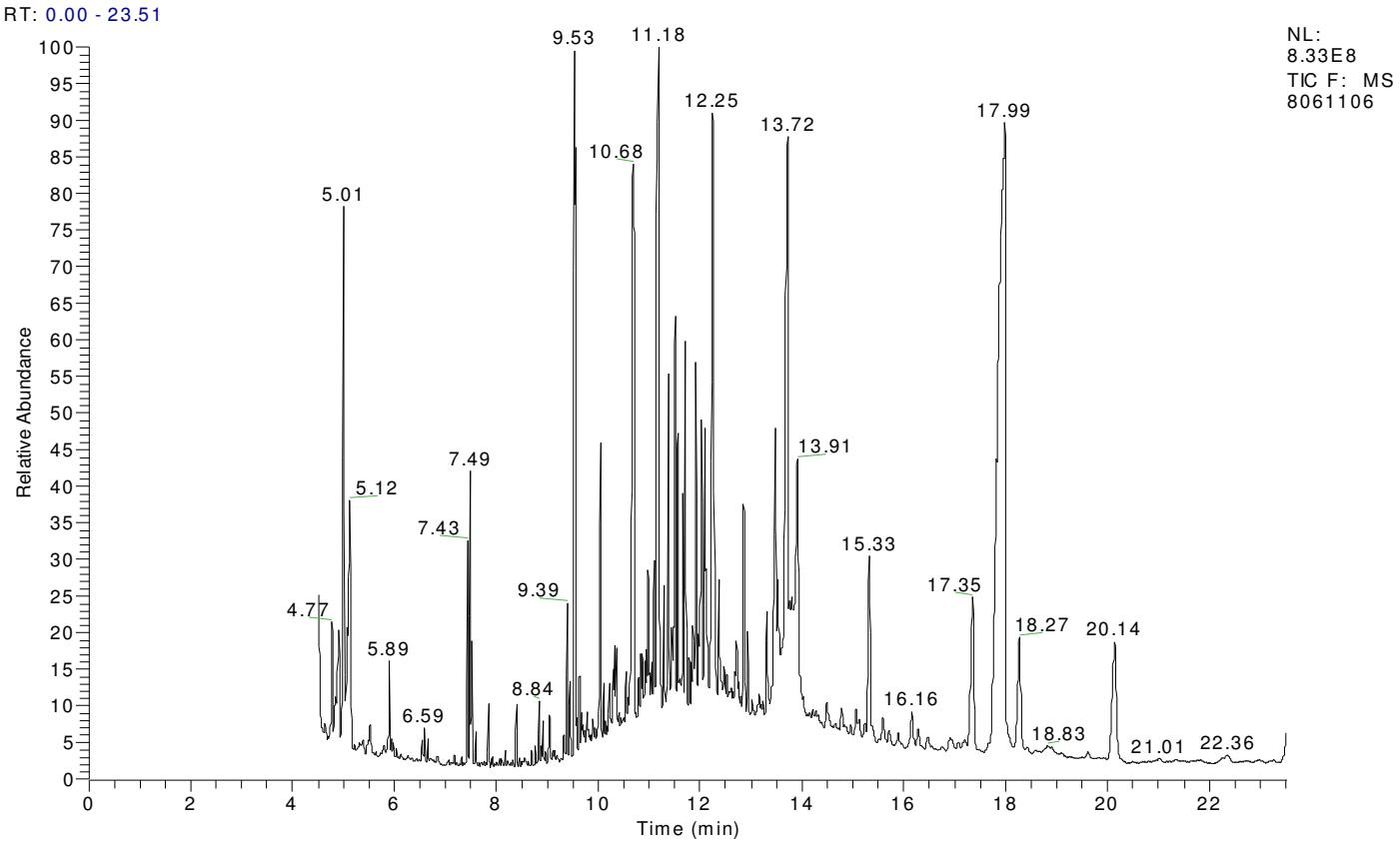

Figure 1. Separation total ion flow graph of Fraction 7.

MS after silylation processing. The separation total ion flow graph was obtained as shown in Figure 1. Component mass spectrum chart was obtained too. Some chemicals were identified by checking with the standard spectrogram collection (NIST05 version) and artificial spectrogram analyzes. Appraisals of the organic compound confirmed mainly included the long chain organic acid, mellow, the ester and so on listed in Table 4.

Effect of allelochemicals from *R. glutinosa* on sesame growth

The chemical substances identified by GC-MS were used to do bioassay using sesame as test plants. The results showed that lauric acid and 2,6-ditertbutyl phenol had strong allelopathy on sesame which is listed in Table 5. Other chemicals had no obvious allelopathy on sesame

Table 4. Compounds in elution Fraction 7 identified by GC-MS.

Ordinal apex	Retention time (min)	Name	Molecular formula	Molecular weight	Relative content (%)
1	7.43	Glycerine	$C_3H_8O_3$	92	0.66
2	7.49	Phosphoric acid	H_3PO_4	98	1.48
3	8.18	Nonanoic acid	$C_9H_{18}O_2$	158	0.1
4	8.45	Glutaric acid	$C_5H_8O_4$	132	0.03
5	8.84	Decanoic acid	$C_{10}H_{20}O_2$	172	0.23
6	9.39	2,6-Ditertbutyl phenol	$C_{14}H_{22}O$	206	0.5
7	9.53	Dodecanol	$C_{12}H_{26}O$	186	5.65
8	9.49	Methyl Paraaben	$C_7H_6O_3$	138	0.01
9	10.05	Lauric acid	$C_{12}H_{24}O_2$	200	1.28
10	10.30	Diisobutyl adipate	$C_{14}H_{26}O_4$	258	0.95
11	11.18	Myristic acid	$C_{14}H_{28}O_2$	228	5.43
12	11.51	N,N-dimethyl-1-hexadecanamine	$C_{18}H_{39}N$	270	2.12
13	11.66	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	0.86
14	11.72	Hexadecanol	$C_{16}H_{34}O$	242	3.02
15	12.25	Palmitic acid	$C_{16}H_{32}O_2$	256	5.79
16	13.48	Oleic acid	$C_{18}H_{34}O_2$	282	2.62
17	13.72	Octadecanoic acid	$C_{18}H_{36}O_2$	204	5
18	17.99	Dioctyl ester	$C_{24}H_{38}O_4$	390	15.74

Table 5. Bioassay results of lauric acid and 2,6-ditertbutyl phenol (mean ± SE).

Elution	Conc. (mg ml^{-1})	Germination rate (%)	Seedling height (cm)	Root length (cm)	Fresh weight (g)
Control		92.0 ± 2.4aA	2.71 ± 0.14aA	5.29 ± 0.17aA	0.273 ± 0.009aA
Lauric acid	0.05	89.3 ± 2.6aA	2.63 ± 0.14aA	5.11 ± 0.20aA	0.263 ± 0.015aA
	0.1	75.3 ± 4.1bB	2.18 ± 0.24bB	4.27 ± 0.29bB	0.218 ± 0.006bB
	0.2	66.7 ± 4.1cC	1.78 ± 0.23cC	3.16 ± 0.22cC	0.140 ± 0.019cC
	0.3	57.3 ± 6.2dD	1.32 ± 0.20dD	1.62 ± 0.22dD	0.068 ± 0.001dD
2,6-ditertbutyl phenol	0.05	88.0 ± 3.3aA	2.52 ± 0.19aA	5.01 ± 0.17aA	0.251 ± 0.008aA
	0.1	72.3 ± 3.3bB	2.14 ± 0.24aB	4.15 ± 0.23bB	0.202 ± 0.011bB
	0.2	63.7 ± 4.6cC	1.70 ± 0.19aC	3.29 ± 0.23cC	0.144 ± 0.005cC
	0.3	55.3 ± 4.6dD	1.19 ± 0.17aD	1.43 ± 0.25dD	0.050 ± 0.004dD

he small and capital English letters in the same line indicate significant difference at 5% and 1% level, respectively, among the handled and control of the same crop.

which would not be listed. Results in Table 5 showed that germination rate, seedling height, root length and fresh weight of sesame were suppressed by lauric acid and 2, 6-ditertbutyl phenol. But the differences were not significant when concentration of two chemicals was 0.05 mg ml^{-1}. The differences were extremely significant when concentrations of two chemicals were 0.1, 0.2 and 0.3 mg ml^{-1}. So we could draw the conclusion that allelochemicals that suppressed sesame growth were lauric acid and 2, 6-ditertbutyl phenol.

DISCUSSION

The continuous cropping barrier is a very serious problem in planting the *R. glutinous*. The plant has the most serious continuous cropping obstacles. It cannot be planted again during the next 8 - 10 years due to no

expansion in tuber root, which is used as medicine (Wen et al., 2002). The small expansion root has no medical use and economic benefits. Root exudates might be the cause of continuous cropping obstacles. And relative research showed that phenolic acid materials such as ferulic acid, and vanillic acid, are possibly the factors that resulted in continuous cropping obstacles (Yin et al., 2009; Du et al., 2009). But this study showed that allelochemicals from *R. glutinous* which supressed sesame growth are lauric acid and 2,6-ditertbutyl phenol which do not inhibit growth of *R. glutinous.* So for the *R. glutinous*, the allelochemicals affecting it and the sesame were different. At present, there was no relative report about the relation of autotoxicity and allelopathy on other plants.

Sesame also had serious continuous cropping barrier. Effect of sesame root exudates on soil and microorganisms was done by Zhang Y. X. (Zhan, 1957). Sesame root exudates could promote production and activity of microorganism. But the microorganisms would do harm to sesame growth. (Li et al. 1989, 1993, Li and Wang, 1991) thought that diseases were serious because of continuous cropping, which would result in production reduction from 10 - 20%. Researches of allelopathy of returning the sesame stalk to the field on sesame growth were conducted by (Li et al. 1990, Li and Luo, 1994). The results showed that rice sheath blight could be inhibited by returning the sesame stalk to the field. He thought that some materials such as phenol, plant alkaloid, and sesame seed toxin, decomposed from sesame stalk could inhibit pathogen rice sheath blight. The cause of sesame continuous cropping barrier was not reported at present. It was not clear whether it was caused by sesame root exudates. And it was not known whether sesame root exudates were similar to materials decomposed from sesame stalk. So lauric acid and 2,6-ditertbutyl phenol could inhibit sesame growth. But research work on whether lauric acid and 2,6-ditertbutyl phenol were secreted from sesame root or decomposed from stalk needs to be further studied.

Allelochemicals were difficult to collect, separate and identify. The modern chemical analysis technique and progress identification technology provided the powerful technical support for process. GC-MS technique was the commonly used identification method. Allelochemicals such as ageratoehromen, ageratoehromen and stigmastra-5, 22-dien-3β-ol from ageratum were separated and identified using GC-MS technique (Wei and Zeng, 1997). Phenolic acid and saponins allelochemicals from alfalfa were separated and identified using GC-MS technique (Zhu et al., 2004). Cyanides and anthraquinones allelochemicals from *Eucalyptus grandis* were separated and identified using GC-MS technique (Wang et al., 2006). Alkenoic esters and aromatic esters allelochemicals from *E. grandis* were separated and identified using GC-MS technique (Hou et al., 2007). Therefore, inhibitory fraction could be separated by silica gel column chromatographic. It was feasible to identify allelochemicals by GC-MS technique. It was known that only 20% organic substance could be identified while 80% organic substance could not be identified by GC-MS (Kong and Xu, 2003). An example of such is the ingredient which is hot in stable or cannot be gasified (Liu et al., 2003; Meng et al., 2006). There-fore, lauric acid and 2,6-ditertbutyl phenol allelochemicals are the factors affecting sesame growth. Further studies might find other allelochemicals from *R. glutinous* on sesame. It is important to explore interaction of allelochemicals with soil and transformation of allelochemicals in soil.

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