

Chemical Compositions and Biological Activities of Pyroligneous Acids from Walnut Shell

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Seven kinds of pyroligneous acids were collected at different temperature ranges (K₇₋₁: 90 to 140 °C; K₇₋₂: 140 to 190 °C; K₇₋₃: 190 to 240 °C; K₇₋₄: 240 to 290 °C; K₇₋₅: 290 to 340 °C; K₇₋₆: 340 to 440 °C; and K₇₋₇: 440 to 480 °C). The chemical compositions of the pyroligneous acids were determined by GC-MS. Moreover, the biological activities of pyroligneous acids were investigated by a foliar spray experiment of rape using the pyroligneous acids collected at 90 to 480 °C. The physiological and chemical responses of pyroligneous acids, including the content of soluble protein and the superoxide dismutase (SOD) activity, were detected. The results of GC-MS analysis showed that the chemical profiles of pyroligneous acids varied with pyrolysis temperature. About 62 kinds of compounds belonging to 8 groups were identified, including ketones, phenols, organic acids, ester, benzene and its derivatives, aldehydes, alcohols, and sugar derivatives. Finally, the pyroligneous acids from walnut shell were applied in foliar spray for rape (*Brassica campestris* L.). The results demonstrated that pyroligneous acid from walnut shell with a low concentration significantly enhanced the content of soluble protein and the activity of SOD in rape leaves.

Keywords: Pyroligneous acid; Walnut shell; Chemical composition; Biological activity

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INTRODUCTION

Pyroligneous acid is a kind of crude reddish-brown liquid produced from dry distillation of biomass during the pyrolysis process. It is a complex mixture of water and many organic compounds, such as ketones, organic acids, aldehydes, alcohols, benzene and its derivatives, heterocycle compounds, phenols and their derivatives, alkyl phenyl ether, carbohydrate derivatives, and nitrogen compounds (Wei *et al.* 2010a; Wang *et al.* 2011; Ma *et al.* 2011; Ma *et al.* 2013).

Pyroligneous acid has been widely applied in the areas of medicine, food, and agriculture. In a STAT3 (signal transducers and activators of transcription 3) inactivation experiment, oak wood vinegar showed anti-inflammatory effects on DNCB-induced dermatitis in mice models. The reason was most likely that the wood vinegar from oak inhibited the epithelial proliferation in mice (Lee *et al.* 2011). In agriculture, pyroligneous acid has been used to promote rooting and germination of seed (Mu *et al.* 2003). Additionally, adding the pyroligneous acid into pig manure composting was shown to be an effective way to reduce the total nitrogen loss and control the mobility of Cu and Zn (Chen *et al.* 2010). Pyroligneous acids from coconut shell and bamboo have been applied as coagulator and antifungal agents in the production of natural rubber sheets (Baimark and Niamsa 2009). More importantly, it has been found that

pyroligneous acid exhibits many biological activities, such as antimicrobial and antioxidant activities. Wei *et al.* (2008a) focused on the antimicrobial activities of pyroligneous acids from walnut shell collected at three temperature ranges (90 to 150, 150 to 310, and 310 to 550 °C) using different fungi or bacteria. The antimicrobial activities of pyroligneous acids at 150 to 310 and 310 to 550 °C were higher than those of pyroligneous acid at 90 to 150 °C. However, the determinations of chemical compositions and antimicrobial activities of pyroligneous acids were limited at a wide variety of temperature ranges. To give clearer details, it is necessary to collect the pyroligneous acids at narrow temperature ranges. In addition, pyroligneous acid has been used as a sterilizing agent, deodorizer, and fertilizer, as in studies by Souza *et al.* (2012) and Loo *et al.* (2007). It is suggested that these strong biological activities are correlated with the high contents of organic acids and phenolic compounds in pyroligneous acid (Mun and Ku 2010; Wei *et al.* 2010a).

Generally, many materials can be used to produce pyroligneous acid, such as oak, bamboo, corn, and wood. Walnut shell has also been found to be useful for the production of pyroligneous acid. In recent years, more and more attention has been directed toward pyroligneous acid from walnut shell. Walnut (*Juglans regia* L.) is one of the most important non-timber forest products. It has been reported that the yield of walnut reached 830,000 tons in 2008 in China (Hou 2008). Walnut shell is a kind of agro-forest waste generated during the process of walnut harvesting. The high-value utilization of walnut shell can be extended by pyrolysis of walnut shell for the production of pyroligneous acid.

Although some researchers have reported the compounds and bioactivities of pyroligneous acids from walnut shell, information about the effect of pyrolysis temperatures on the compounds of pyroligneous acids from walnut shell is limited. The objective of this research was to make a systematic and detailed study to evaluate the relationship between the pyrolysis temperature and the chemical constituents of pyroligneous acids during the pyrolysis process of walnut shell. In addition, to enable the wide exploitation of pyroligneous acids, the bioactivities of pyroligneous acids including the soluble protein contents and superoxide dismutase (SOD) for rape were studied in a foliar spray experiment. The soluble protein contents and SOD activities are important physiological and biochemical responses that reflect the quality and growth of a plant, in which the soluble proteins as part of enzymes were contributed to the physiological and biochemical metabolism regulation of fruits and vegetables (Wang *et al.* 2014a). Meanwhile, the soluble proteins were closely related with the growth and development, maturity and aging, stress and disease resistance of plants (Li *et al.* 2008). SOD is an important antioxidant enzyme, and it plays an extremely important role in preventing the overproduction of reactive oxygen species (ROS). On the other hand, SOD enhanced the protection of membrane structures and was thus considered to be the main anti-oxidizing agent in plants (Wang *et al.* 2014b). Finally, this study may provide a potential way to exploit walnut shell for production of pyroligneous acid by pyrolysis technology.

EXPERIMENTAL

Materials

Walnut shells were provided by the walnut kernel processing factory of Shanyang county (Shaanxi province, China). About 25.16% cellulose, 21.32% hemicelluloses, and 53.52% lignin were reported in the walnut shell (Wei *et al.* 2010b).

The chemical reagents, including ether, potassium hydrogen phthalate, and sodium hydroxide, were all of analytical grade. Activated carbon (chemically pure) was purchased from Xilong Chemical Co., Ltd. (Guangdong, China). The biochemical reagents Coomassie brilliant blue G-250 and thiobarbituric acid, as well as liquid amino acid fertilizer, were also used. The seeds of rape (*Brassica campestris* L.) were Shaanyou 6 variety.

Methods

Preparation and refinement of pyroligneous acids from walnut shell

Pyroligneous acids from walnut shell were produced by a pyrolytic retort (made by Nanjing Forestry University) with dimensions of 130 mm (diameter) × 270 mm (height) equipped with a water-cooled condenser. Approximately 500 g of walnut shell was loaded into the retort, which was heated with a gradient temperature elevation of 1 °C/min until it reached 480 °C. Pyroligneous acids were collected at seven temperature ranges: 90 to 140 °C, 140 to 190 °C, 190 to 240 °C, 240 to 290 °C, 290 to 340 °C, 340 to 440 °C, and 440 to 480 °C, based on a preliminary test. This process was repeated six times; then, the raw pyroligneous acids obtained at the same temperature were combined. The raw pyroligneous acids were weighed to calculate the yields. The collected pyroligneous acids were stored for one month at room temperature. Three layers appeared in pyroligneous acids: a thin layer of oil on the top, a thick and clarified layer in the middle, and a thin layer of wood tar at the bottom. The middle layer was siphoned off, and then 5% (w/w) activated carbon was added. The mixtures were stirred for 10 min and then stood for 72 h to adsorb wood tar residues sufficiently. Supernatant fluids (pyroligneous acids) were obtained by filtration according to a previous report (Wei *et al.* 2008). The refined pyroligneous acids from walnut shell were labeled as K₇₋₁ for that collected at 90 to 140 °C, K₇₋₂ for 140 to 190 °C, K₇₋₃ for 190 to 240 °C, K₇₋₄ for 240 to 290 °C, K₇₋₅ for 290 to 340 °C, K₇₋₆ for 340 to 440 °C, and K₇₋₇ for 440 to 480 °C.

The pyroligneous acids from walnut shell prepared for the foliar spray experiment were collected at 90 to 480 °C, stood for one month at room temperature, were refined with the above method, and labeled as K.

Physicochemical properties of pyroligneous acids

The pH values of pyroligneous acids were measured by a pH meter (model PHS-3CHS). Organic acids were determined by the method of acid-base titration with 0.1 M NaOH and expressed as acetic acid. Densities of pyroligneous acids were detected by the pycnometer method (Dutta *et al.* 2012). Refractive indexes were measured by an Abbe refractometer.

Analysis of pyroligneous acids by GC-MS

Refined pyroligneous acids were subjected to extraction by ether six times, in which 30% (v/v) of ether were used each time. The combined solutions were dried and

distilled to remove ether. Concentrated pyroligneous acids were obtained and used for gas chromatograph-mass spectroscopy (GC-MS) analysis.

The GC-MS analysis was carried out with a gas chromatography-mass spectrometer (Trace GC-Trace DSQ, Finnigan). Pure helium was used as carrier gas with a constant flow rate of 1.0 mL/min. Separation was performed using a DB-WAX capillary column (30 m × 0.25 mm × 0.25 mm film thickness). The injection port temperature was 220 °C. The column temperature was maintained at 60 °C for 2 min and then increased to 240 °C at a rate of 6.0 °C/min, which was held for 8 min. The split injection was conducted at a split rate of 80:1. Additionally, the mass spectroscopic conditions were electron energy of 70 eV, ion source temperature of 250 °C, and mass scanning range of 35 to 400 m/z. Compounds were identified by comparison of the retention time and mass spectra with library data of mass spectra (NIST) according to the procedure described previously (Li *et al.* 2014). The contents of compounds were measured by their relative peak areas.

Foliar spray experiment of rape

Treatment agent K was the pyroligneous acid collected at 90 to 480 °C from walnut shell. Agent K was then diluted 400 and 800 times with distilled water for treatment 1 (T1) and treatment 2 (T2), respectively. Distilled water was used as a blank control (CK) treatment, and foliar fertilizer was used as a positive control treatment. The seed of rape (*Brassica campestris* L.) was used as a receptor.

The rape seeds were sown on May 4, 2013. About 30 days later, the seedlings of rape grew to 6-7 leaves, and then the foliar spray experiments were carried out. There were three replicates of the treatments with a randomized complete block design. The experiments were carried out four times every 15 days using the treatment agents. There were 32 seedlings of rape in each treatment. Approximately 250 mL of treatment agent was sprayed onto the seedlings using an aerosol sprayer to ensure that the constant amounts of treatment agent were used. In each experiment, from top to bottom of rape seedlings, the third and the fourth fully expanded leaves were chosen and collected to determine their physiological and biochemical responses. The same process was repeated for the second, the third, and the fourth spray once a week.

Determination of physiological and biochemical responses of rape leaves

The rape leaves were homogenized in a cooled mortar with quartz and 5 mL of 0.1 M phosphate-buffered solution (PBS) (pH=7.5) containing 1% (w/v) polyvinylpyrrolidone (PVPP). The homogenate was centrifuged at 8000 g for 20 min at 4 °C. The supernatant was collected to determine the soluble protein content and SOD activity (Liu *et al.* 2012).

The content of soluble protein in the leaves of rape was measured according to the Coomassie brilliant blue (CBB) protein dye-binding assay. (Bradford 1976).

SOD activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The modified method from Giannopolitis and Ries (1977) was used. The assay was performed with illumination for 20 min at 25±1 °C in a 5 mL cuvette containing 0.3 mL of 130 mM methionine, 0.3 mL of 750 μM NBT, 0.3 mL of 500 μM EDTA-2Na, 0.3 mL of 100 μM vitamin B₂, 0.05 mL of supernatant, and 1.5 mL of 50 mM phosphate buffer (pH 7.8) against a blank with no protein

suspension. One unit of SOD activity was defined as the amount of enzyme that was required to cause a 50% inhibition of the reduction of NBT at 560 nm.

Statistical analysis

Microsoft Excel, SPSS, and Origin 8.1 were used to analyze the data. Comparisons among all groups were performed using Duncan's test, and a p-value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Yields of Raw Pyroligneous Acids

The yields of raw pyroligneous acids collected at seven temperature ranges are given in Table 1. As can be seen, the pyrolysis at 90 to 140 °C (K₇₋₁), 140 to 190 °C (K₇₋₂), 190 to 240 °C (K₇₋₃), 240 to 290 °C (K₇₋₄), 290 to 340 °C (K₇₋₅), 340 to 440 °C (K₇₋₆), and 440 to 480 °C (K₇₋₇) yielded 3.88%, 3.46%, 10.05%, 17.66%, 4.13%, 2.20%, and 0.82% (% dry matter, w/w) of walnut shell, respectively. The total yields of raw pyroligneous acids were 42.20% of walnut shell. The yield of raw pyroligneous acid increased from 3.46% to 17.66% with a temperature increase from 140 to 290 °C, while a decreasing yield, from 17.66% to 0.82%, was observed with a further increase in temperature from 290 to 480 °C. The yield of K₇₋₄ was the highest, followed by K₇₋₃, which indicated that the pyroligneous acids were mostly produced at temperatures of 190 to 290 °C.

Table 1. Yields and Physicochemical Properties of Pyroligneous Acids Obtained at Seven Temperature Ranges

Pyroligneous acids	Yields (wt. %)	pH	Refractive index (%)	Total organic acids (%)	Color	Specific gravity
K	42.20±0.58 f	2.65±0.07 b	2.20±0.03 e	14.63±0.06 f	Wine red	1.037±0.005 d
K ₇₋₁	3.88±0.16 c	4.44±0.04 f	0.20±0.01 a	0.17±0.02 a	Colorless	1.001±0.001 ab
K ₇₋₂	3.46±0.02 c	3.01±0.06 f	0.50±0.02 b	4.10±0.03 c	Pale yellow	1.005±0.002 a
K ₇₋₃	10.05±0.03 d	2.47±0.03 a	3.28±0.02 f	24.48±0.09 h	Bronzing	1.054±0.001 e
K ₇₋₄	17.66±0.09 e	2.51±0.01 ab	4.10±0.03 h	17.17±0.20 g	Black brown	1.064±0.002 f
K ₇₋₅	4.13±0.06 c	2.81±0.08 c	3.83±0.04 g	12.96±0.16 e	Reddish brown	1.056±0.002 e
K ₇₋₆	2.20±0.07 b	3.79±0.01 e	1.96±0.01 d	5.76±0.06 d	Scarlet	1.018±0.002 c
K ₇₋₇	0.82±0.01 a	3.92±0.01 e	0.95±0.02 c	1.19±0.01 b	Yellow	1.010±0.005 b

Note: Values are the mean of three replicates ± standard error. Significance levels ($p \leq 0.05$) among different PA are presented with letters (a-h).

Physicochemical Properties of Pyroligneous Acids

The physicochemical properties of pyroligneous acids are shown in Table 1. The relative parameters of the 7 kinds of pyroligneous acids showed significant difference. The pH values decreased from 4.44 (K₇₋₁) to 2.47 (K₇₋₃) and then increased from 2.51 (K₇₋₄) to 3.92 (K₇₋₇) as the collection temperature increased. However, the total acids

showed an opposing trend. Among the 7 kinds of pyroligneous acids, the minimum pH value was 2.47 from K₇₋₃, which corresponded to the highest content of total acids (up to 24.48%). While K₇₋₁ showed the maximum pH value (4.44), its content of total acids was only 0.17%. As the pyrolysis temperature increased, the water content in the pyroligneous acids decreased (Jiang *et al.* 2014). Therefore, it was considered that the proton concentration in K₇₋₆ and K₇₋₇ were relative high, though the total organic acid in these products was low. Table 1 also indicates that the values of relative refractive index and specific gravity were related to the colors of pyroligneous acids. The values of relative refractive index and specific gravity increased as the pyroligneous acids became darker. Interestingly, the color of pyroligneous acid became darker with increasing pyrolysis temperature in this study. The pyroligneous acid (K₇₋₄) with black brown color collected at 240 to 290 °C had the highest values of relative refractive index (4.10%) and specific gravity (1.064).

GC-MS Analysis

The chemical compositions of pyroligneous acids from walnut shell were analyzed by GC-MS. NIST database and manual-assisted elucidation were also applied to determine the types of compounds. A typical example for identification of the compounds in pyroligneous acids using GC-MS was previously carried out by Souza *et al.* (2012). Meanwhile, the normalization method of peak area was applied to determine the relative content of compounds in pyroligneous acids (Lopes *et al.* 2011). After the library retrieving and manual assisted elucidation, 62 constituents with match percentage $\geq 90\%$ were identified. The results are shown in Table 2. In total, 62 kinds of organic compounds belonging to 8 groups were identified in 7 kinds of pyroligneous acids. The GC-MS analysis indicated that there were 18 kinds of ketones, 17 kinds of phenols, 9 kinds of organic acids, 7 kinds of ester, 4 kinds of benzene and its derivatives, 5 kinds of aldehydes, 1 kind of alcohol, and 1 kind of sugar derivative in pyroligneous acids from walnut shell.

Table 2. Results of GC-MS Analysis of Pyroligneous Acids

NO.	RT (min)	Compounds	Relative Percentage (%)						
			K ₇₋₁	K ₇₋₂	K ₇₋₃	K ₇₋₄	K ₇₋₅	K ₇₋₆	K ₇₋₇
1	5.35	Cyclopentanone	-	-	-	-	0.27	1.46	0.53
2	6.95	Dihydro-2-methyl-3(2H)-furanone	-	0.26	-	-	-	-	-
3	7.38	3-Hydroxy-2-butanone	0.24	-	-	-	-	-	-
4	7.74	1-Hydroxy-2-propanone	-	0.28	0.58	1.31	0.83	-	-
5	8.87	2-Cyclopenten-1-one	-	0.21	0.21	0.76	0.93	1.13	0.67
6	9.05	4-Hydroxy-4-methyl-2-pentanone	-	-	-	-	-	-	0.62
7	9.12	2-Methyl-2-cyclopenten-1-one	-	-	0.14	0.40	0.58	1.63	0.69
8	9.28	1-Hydroxy-2-butanone	-	0.59	1.98	0.77	0.45	-	-
9	11.48	3,4-Dimethyl-2-cyclopenten-1-one	-	-	-	-	-	0.32	-
10	12.04	1-(2-Furanyl)-ethanone	-	-	0.18	-	0.58	-	-
11	12.26	3-Methyl-2-cyclopenten-1-one	0.51	0.19	-	0.45	0.68	1.61	0.86
12	14.14	Dihydro-5-methyl-2(3H)-furanone	-	-	-	-	-	0.16	-

13	14.9	2,5-Dihydro-3,5-dimethyl-2-furanone	-	-	0.18	0.21	0.23	0.21	
14	16.91	2(5H)-furanone	-	-	0.09	0.38	0.37	-	-
15	18.18	3-Methyl-1,2-cyclopentanedione	-	-	0.51	2.59	3.48	2.68	-
16	19.33	3-Ethyl-2-hydroxy-2-cyclopenten-1-one	-	-	0.16	0.93	1.36	1.14	0.19
17	30.73	1-(4-Hydroxy-3-methoxyphenyl)-2-propanone	-	-	1.49	1.31	1.39	1.10	-
18	35.55	1-(4-Hydroxy-3,5-dimethoxyphenyl)-ethanone	-	-	0.53	0.56	0.45	-	-
	Total	Ketones	0.75	1.53	6.05	9.67	11.60	11.44	3.56
19	10.73	Acetic acid	21.15	55.81	49.50	18.37	14.30	11.20	4.59
20	12.02	Formic acid	-	1.34	-	-	-	-	-
21	12.65	Propanoic acid	0.87	3.11	4.23	2.42	2.68	6.07	2.25
22	13.28	2-Methyl-propanoic acid	-	-	-	-	-	0.49	0.45
23	14.46	Butanoic acid	0.42	0.34	-	-	-	4.11	1.78
24	16.72	Pentanoic acid	-	-	-	-	-	0.55	0.21
25	31.32	Tetradecanoic acid	-	-	-	-	-	-	1.19
26	34.12	n-Hexadecanoic acid	-	-	-	-	-	-	8.41
27	38.23	Octadecanoic acid	-	-	-	-	-	-	0.36
	Total	Organic acids	22.44	60.60	53.73	20.79	16.98	22.42	19.24
28	11.21	Furfural	-	3.77	9.64	1.72	-	-	-
29	13.44	5-Methyl-2-furancarboxaldehyde	-	0.51	1.07	0.20	-	-	-
30	21.56	1H-pyrrole-2-carboxaldehyde	-	0.31	0.12	-	-	-	-
31	34.68	4-hydroxy-3,5-dimethoxy-benzaldehyde	-	-	0.50	0.42	0.25	-	-
32	38.84	4-hydroxy-2-methoxycinnamaldehyde	-	-	0.41	-	-	-	-
	Total	Aldehydes	0	4.59	11.74	2.34	0.25	0	0
33	5.67	Ethyl acetate	0.42	0.22	-	-	-	-	-
34	13.34	4-Oxo-pentanoic acid methyl ester	-	-	0.11	0.25	0.35	0.32	-
35	13.88	1,2-Ethanediol-dipropanoate	-	-	0.09	-	-	-	-
36	14.51	Butyrolactone	-	-	0.31	-	-	-	-
37	17.17	Methyl 4-hydroxybutanoate	-	-	-	-	-	0.20	-
38	27.74	Pentanedioic acid monomethyl ester	-	-	-	-	-	0.40	-
39	30.06	4-Hydroxy-3-methoxy-benzoic acid methyl ester	-	-	0.21	-	-	-	-
	Total	Esters	0.42	0.22	0.72	0.25	0.35	0.92	0
40	15.15	2-Furanmethanol	0.33	1.20	0.53	1.41	2.04	2.30	1.45
	Total	Alcohols	0.33	1.20	0.53	1.41	2.04	2.30	1.45
41	18.77	2-Methoxy-phenol	0.28	0.38	4.33	8.47	8.45	9.10	5.75
42	20.19	2-Methoxy-5-methyl-phenol	-	-	-	0.24	0.27	0.42	0.29
43	20.42	2-Methoxy-4-methyl-Phenol	-	-	0.72	4.27	3.97	1.96	1.45
44	21.24	Phenol	2.70	2.77	1.63	1.91	2.95	11.30	23.78
45	21.64	4-Ethyl-2-methoxy-phenol	-	-	0.71	3.20	2.40	0.46	0.24

46	22.49	4-Methyl-phenol	0.20	0.25	-	0.85	1.08	2.00	4.18
47	22.9	2-Methoxy-4-propyl-phenol	-	-	0.18	0.25	0.35	2.75	6.26
48	25.32	2,6-Dimethoxy-phenol	-	-	5.81	15.69	18.3	10.00	3.00
49	27.38	5-Tert-butylpyrogallol	-	-	1.18	4.75	4.72	0.96	-
50	28.83	3-Methoxy-1,2-benzenediol	-	-	-	-	-	-	0.22
51	28.98	3-Methoxy-5-methyl-phenol	-	-	-	-	0.20	-	-
52	29.19	2,6-Dimethoxy-4-(2-propenyl)-phenol	-	-	1.08	1.65	0.41	-	-
53	31.44	1,2-Benzenediol	-	-	-	-	-	-	8.77
54	33.25	2-Methoxy-4-(methoxymethyl)-phenol	-	-	-	-	0.24	-	-
55	35.67	2-Methyl-1,4-benzenediol	-	-	-	0.49	-	1.48	-
56	35.91	Desaspidinol	-	-	1.27	1.68	1.11	0.9	-
57	36.25	Hydroquinone	-	-	0.13	0.74	1.20	3.79	1.33
Total		Phenol and derivatives	3.18	3.40	17.04	44.19	45.65	45.12	55.27
58	27.25	1,4:3,6-Dianhydro- α -D-glucopyranose	-	-	-	0.32	0.50	0.77	-
Total		Sugar derivations	0	0	0	0.32	0.50	0.77	0
59	21.96	1,2,3-Trimethoxy-5-methyl-benzene	-	-	-	0.19	0.19	-	-
60	26.56	1,2,4-Trimethoxybenzene	-	-	-	7.47	8.02	2.7	0.61
61	35.08	4-Hydroxy-3,5-dimethoxy-benzoic acid hydrazide	-	-	0.21	0.37	0.24	-	-
62	35.43	Methyl-(2-hydroxy-3-ethoxy-benzyl)ether	-	-	-	0.24	0.23	-	-
Total		Benzene derivations	0	0	0.21	8.27	8.68	2.7	0.61

The results also demonstrated that the varieties and relative contents of those compounds in the pyrolygneous acids were affected by pyrolysis temperatures. In total, 10, 17, 34, 36, 37, 33, and 27 kinds of compounds were identified for K₇₋₁, K₇₋₂, K₇₋₃, K₇₋₄, K₇₋₅, K₇₋₆, and K₇₋₇, respectively. From Table 2, it can be seen that benzene and its derivatives were generated at 190 °C, and sugar derivatives were generated at 240 to 440 °C, while aldehydes were generated at 140 to 340 °C. Interestingly, ester disappeared when the temperature was higher than 440 °C. Meanwhile, the chemical compositions of pyrolygneous acids are shown in Fig. 1. It was found that organic acids were the primary components of K₇₋₁ and K₇₋₂. Organic acids, phenolic compounds, ketones, aldehydes, and benzene and its derivatives were the primary components obtained at the middle temperature range (190 to 290 °C). At a high temperature range (> 290 °C), organic acids, phenols, ketones, and benzene and its derivatives were the primary components of pyrolygneous acids, with the contents of phenol and its derivatives the highest, which was the highest content for phenol in pyrolygneous acids. In comparison, the content of phenol at higher temperature (K₇₋₆ and K₇₋₇) was significantly higher than that obtained at lower temperatures. In addition, as the collected temperature increased, the relative content of organic acids increased from 22.44% (K₇₋₁) to 60.6% (K₇₋₂), and then decreased to 19.24% (K₇₋₇).

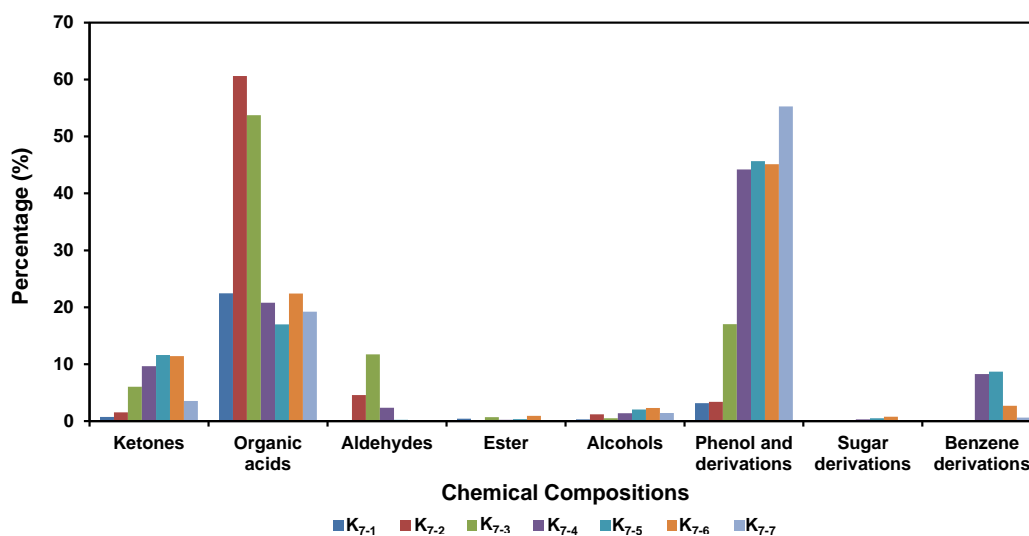


Fig. 1. Chemical compositions of pyrolygneous acids from walnut shell

Following phenol and its derivatives, organic acids (16.98 to 60.60%) were the primary compounds in pyrolygneous acids, which were produced at a relatively low temperature. Among the organic acids, acetic acid (4.59 to 55.81%) was identified as the major compound. Moreover, ketones and aldehydes also accounted for 0.75 to 11.6% and 0 to 11.74% of all compounds, respectively, in which the content of furfural from hemicelluloses could reach up to 9.64% in K₇₋₃. In addition, benzene and sugar derivations were minor constituents.

In fact, thermal cracking of wood is the degradation of hemicelluloses, cellulose, and lignin (Yang *et al.* 2007). The micellar-like fragments produced by pyrolysis were degraded to smaller compounds by dehydration, dehydrogenation, deoxygenation, and decarboxylation. Meanwhile, the reactions of rearrangement among those smaller compounds occurred *via* condensation, cyclization, and polymerization. In these reactions, some new compounds were produced (Nakamura *et al.* 2007). The drying of walnut shell happened when the temperature was lower than 140 °C. In this study, the content of water was the highest in the pyrolygneous acids, while acetic acid was the main compound and its content was 21.15%. In thermal analysis, the rates of pyrolysis of hemicelluloses and cellulose were fast. The weight loss of hemicelluloses happened at 220 to 315 °C, while that of cellulose occurred at 315 to 400 °C. However, lignin was more difficult to decompose, since its weight loss happened in a wide temperature range (from 160 to 900 °C). From the viewpoint of energy consumption, the pyrolysis of the cellulose was an endothermic process, while the others were exothermic processes (Yang *et al.* 2007). In the cracking process of the three compounds, the destructive reaction of cellulose was controlled by the decreasing of polymerization degree. However, lignin was converted into phenol, guaiacol, syringol, pyrocatechol, and their derivatives. It was suggested that the aromatics and phenols and their substituted fractions of alkyl, which were the preliminary degradation products of lignin, were formed by recombination and cyclization reactions *via* aldol condensation from C₂, C₃, and C₄ fragments (Liu *et al.* 2012). Further reaction may produce furans, aldehyde, and ketones (Demirbas 2002). Moreover, the extraction by supercritical water and partial reactions of liquefaction occurred during the pyrolysis process. Acetic acid was formed during the thermal

decomposition of hemicelluloses and lignin. In the pyrolysis reactions of biomass, water was formed by dehydration; acetic acid came from the elimination of acetyl groups originally linked to the xylose units and lignin, while furfural was formed by dehydration of the xylose units (Gaugler and Grigsby 2009). To sum up, in all the temperature ranges, organic acids, phenols, ketones, and alcohols were formed from the cracking process of the three main compounds of walnut shell (cellulose, hemicelluloses, and lignin). These results suggested that the chemical compositions of pyroligneous acids were correlated with the pyrolysis temperature. Thus, investigating the chemical compositions of its pyroligneous acids is an effective way to understand the pyrolysis process of walnut shell.

The compounds detected in the pyroligneous acids from walnut shell were similar to those found in the other parts of walnut. Wei *et al.* (2010b) reported that the chemical compositions of the pyroligneous acids from walnut branches were mainly phenols and organic acids which accounted for 32.68% and 30.78%, respectively. In their study, the dominating acid of the organic acids was identified as acetic acid accounting for 22.62% (Wei *et al.* 2010b). Additionally, there were small amounts of alcohols, esters, sugar derivatives, and nitrogen compounds (Wei *et al.* 2010b). In fact, apart from temperature, the chemical compositions of the pyroligneous acids were affected by many factors, such as catalyst, reaction time, and materials. For example, hydroxyacetone and carboxylic acids (acetic and propionic acids) in the pyroligneous acid were converted into aliphatic ketones (acetone and 2-butanone) *via* a ketonization reaction over the ZrO_2-FeO_x catalyst (Mansur *et al.* 2013). The materials and the reaction time also had effects on the chemical compositions of the pyroligneous acids. Zhai *et al.* (2010) reported the chemical compositions of pyroligneous acids from corncob, pinecone, and walnut branch under 90 to 550 °C by GC-MS. The results showed that 66, 55, and 59 kinds of compounds were identified for the pyroligneous acids from corncob, pinecone, and walnut branch, respectively. This was because the compositions of cellulose, hemicelluloses, and lignin differed among the substances.

Physiological and Biochemical Responses of Rape after Foliar Spray

Content of soluble protein

The soluble protein contents of rape after foliar spray are shown in Fig. 2. A decreasing trend from the second spray to the third spray and then an increasing trend to the fourth spray can be seen in both treatments. However, the soluble protein content of rape was almost the same and remained at a low level for CK. Compared with CK, both T1, T2, and the positive control could promote the increasing of the soluble protein contents. In T1 (K was diluted 400 times), the content of soluble protein was lower than that of CK after the second and the third sprays. However, the content was then significantly increased above that of CK; after the fourth spray ($p < 0.05$), the content of soluble protein was 19.03% higher than that of CK. On the other hand, in T2 (K was diluted 800 times), the content of soluble protein was significantly ($p < 0.05$) higher than those of CK and other treatments. After the second spray, the content of soluble protein reached the highest point that was 47.95% higher than that of CK. These results demonstrated that pyroligneous acid with a high concentration resulted in a reduction of the soluble protein content of rape, while pyroligneous acid with a low concentration could increase the soluble protein content of rape. One possible explanation may be that if the dilution time was low, some phenols in pyroligneous acid were at a high concentration, which could inhibit the growth of the plant. However, if the dilution time

was high, and the concentration of these phenols was low, these phenols could enhance the soluble protein content (Loo *et al.* 2008).

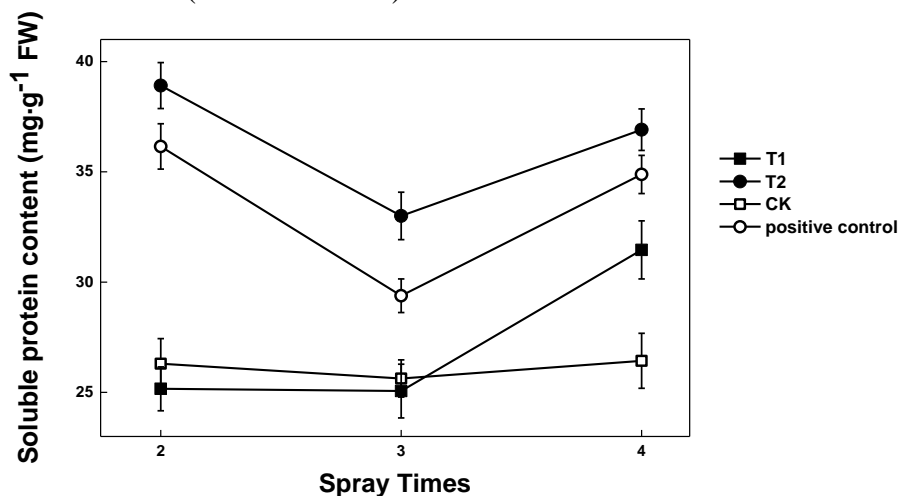


Fig. 2. Effect of different treatments of pyroligneous acids on soluble protein content in rape leaves by foliar spray. Values are the mean of three replicates \pm standard deviation

SOD activity

The activities of SOD in the leaves of rape after foliar spray experiments are shown in Fig. 3. T2 (K was diluted 800 times) and fertilizer treatment revealed a decreasing trend of SOD activity, and the CK group showed the same trend as well. The SOD activity in these two treatments were higher than that in CK. The differences were significant after the second and the third spray ($p < 0.05$). After the fourth spray, the SOD activity decreased rapidly for treatment 2, which was just a little higher than that in CK. Moreover, the SOD activity in treatment 1 was higher than that of the fertilizer treatment. The difference was significant ($p < 0.05$), except for the third spray. However, in T1 (K was diluted 400 times), the SOD activity was decreased from the second spray to the third spray and then was increased to the fourth spray. Interestingly, after the second and third spray, the SOD activity in T1 was even lower than that of CK. After the fourth spray, the increment of SOD activity in treatment 1 was significant ($p < 0.05$) in comparison with those in the CK, T2, and fertilizer treatments. The results showed that the SOD activity in T1 was 12.46% higher than that in CK. It was possible that the rape plants suffered some injuries under a high concentration of pyroligneous acid, which contained high amounts of phenols and organic acids. These injuries were beyond the ability of regulation and control of rape. As the spray times increased, the rape plants could adapt these changes. As a result, the pyroligneous acid could induce the appearance of the SOD activity in the leaves of rape.

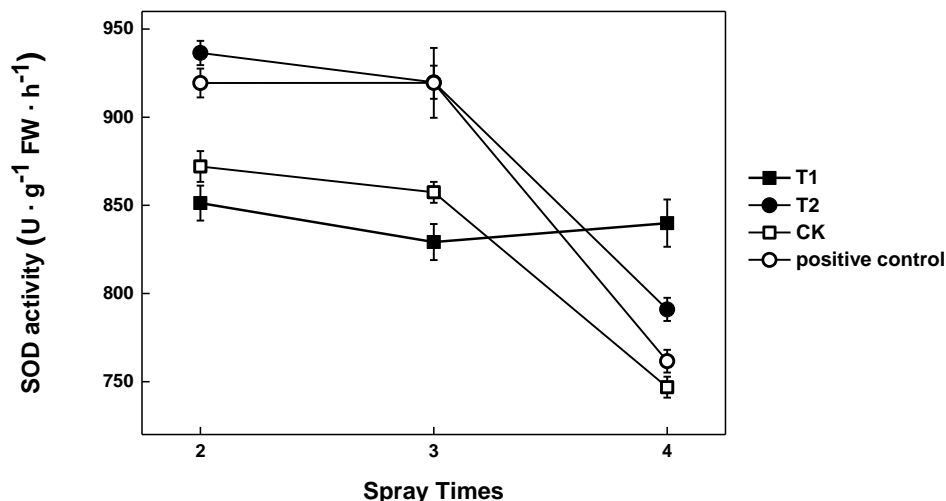


Fig. 3. Effect of different treatments of pyroligneous acids on SOD activities in rape leaves by foliar spray. Values are the mean of three replicates \pm standard deviation

It was found that the soluble protein contents increased significantly in rape leaves, and the SOD activity decreased slower after the foliar spray experiment. The results indicated that the growth of rape plants was affected by the pyroligneous acids. Up to now, many biologically active substances such as terpenoids, phenolics, alkaloids, and fats have been found in the extractives of trees (Tachibana 1995), in which some substances have a regulatory effect on plant growth. Interestingly, the pyroligneous acids from walnut shell contains numerous low-molecular-weight compounds, which also appeared to have some effect on the seedling growth. On the other hand, some hormone-like substances such as karrikins, a series of butenolides that can also enhance plant growth were found in the smoke of burning plant material (Chiwocha *et al.* 2009). It was shown that karrikins had the ability to stimulate the germination of seed (Zhou *et al.* 2014). According to the results of GC-MS analysis, the pyroligneous acids from walnut shell also contains some hormone-like substances such as some furan and pyran derivatives that may enhance plant growth. Besides, pyroligneous acids from walnut shell are rich in organic acids and phenols, which have a high bioactivity and promoted the growth of the plants (Mungkumchao *et al.* 2013).

The result of T2 (K was diluted 800 times) was better than that of T1 (K was diluted 400 times). The promotional effect of pyroligneous acids from walnut shell appeared at a low concentration. This is in accord with hormonal substances, which have an effect at only trace concentration. This was consistent with some earlier findings that suggested that a high concentration of pyroligneous acid could restrain plant growth (Mu *et al.* 2004). Therefore, pyroligneous acids from walnut shell as foliar spray could be applied in the field of agriculture in an attempt to reduce the dependence on the use of chemicals.

CONCLUSIONS

1. Pyroligneous acids were collected at seven temperature ranges and were analyzed by GC-MS. The chemical compositions of pyroligneous acids varied with pyrolysis temperature.

2. The main compositions of pyroligneous acids were ketones, phenols, organic acids, ester, benzene and its derivatives, aldehydes, alcohols, and some sugar derivatives, in which phenols and organic acids were the dominating compounds.
3. The bioactivity of pyroligneous acids from walnut shell collected from 90 to 480 °C was also detected by foliar spray experiment. The results demonstrated that pyroligneous acids with a low concentration had a significant effect on the growth of rape plant by increasing the soluble protein content and SOD.

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