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Fate and Impact on Microorganisms of Rice Allelochemicals in Paddy Soil

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Allelopathic rice can release allelochemicals from roots to inhibit neighboring plant species, but little is currently known about their fate and impact on microorganisms in paddy soil. This study showed that allelopathic rice PI312777 released much higher concentrations of allelochemical (5,7,4′-trihydroxy-3′,5′-dimethoxyflavone) than non-allelopathic rice Liaojing-9 in field. When quantitative 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone was added into soil, flavone gave a short half-life of 18.27 ± 2.32 h (r^2 = 0.94) and could easily be degraded into benzoic acid. Benzoic acid with a half-life of 29.99 ± 2.19 h (r^2 = 0.96) was more resistant toward degradation in paddy soil. Furthermore, both the culturable microbial population and the entire microbial community structure of soil incubated with flavone and benzoic acid were evaluated using the soil dilution plate method and phospholipid fatty acid (PLFA) analysis, respectively. It appeared from the results that flavone could reduce microorganisms especially for fungi present in paddy soil, while benzoic acid could induce a higher response for soil microorganisms especially for bacteria. Consequently, flavone would be responsible for the dynamics of soil microorganisms during the early period, and any observed effect during the late period would be very likely due to its degradation product benzoic acid rather than flavone itself. These results suggested that allelopathic rice varieties could modify soil microorganisms to their advantage through the release of allelochemicals. The concentration and fate of discriminating 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone between allelopathic and non-alleloparhic varieties tested in rice soil would result in the different patterns of microbial population and community structure in paddy ecosystems.

KEYWORDS: *Oryza sativa* L.; allelochemical; benzoic acid; degradation dynamics; soil microbial community; 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone

INTRODUCTION

The action of allelochemicals involved in plant−plant allelopathic interactions needs their presence at the phytotoxic level in the soil in the vicinity of the target plant. Thus, the dynamics of allelochemicals and their fate and persistence in soil are crucially important factors for allelochemical interference (*1, 2*). Once allelopathic plant species released allelochemicals from root to soil solution, a series of interactions between allelochemicals with soil abiotic and biotic factors would take place. However, it is complex and poorly understood that allelochemicals endure under soil processes, such as retention, transport, and transformation after release by plant roots into soil (*3, 4*). Particularly, roots lose allelochemicals to the soil at rates of significance to interact with soil microorganisms (*5, 6*). Roots are able to exert an effect on soil microorganisms through the release of allelochemicals. In turn, soil microorganisms consume and decompose allelochemicals and are an important determinant of allelopathic activity (*3, 7*). Microbial degradation of a particular allelochemical depends upon the specific microflora in soil, while certain microbial species may take advantage of allelochmicals in soil. Accordingly, it is extremely difficult to unambiguously demonstrate allelopathy in soil because of the complexity of allelochemical interference and its relationship to soil chemistry and the microbial process (*3, 8*). However, with an increased understanding of soil degradation dynamics occurring in a few typical allelochemicals, such as wheat benzoxazinoids, and the ability to identify allelochemicals and microbial community structure in soil (*1, 9, 10*), an effort should now be directed toward understanding the fate and impact on microorganisms of allelochemicals in the soil environment.

Allelopathic action of several rice (*Oryza sativa* L.) varieties has been established under field situations and controlled conditions (*11, 12*). The use of allelopathic rice cultivars and their allelochemicals can definitely reduce the cost of paddy weed control, particularly by reducing the amount of herbicide

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used (*13, 14*). Thus, rice allelochemicals have been extensively investigated in relation to their effects on the growth of paddy weeds (*4, 15, 16*), but little is currently known about their fate and impact on microorganisms in paddy soil. Soil microorganisms play essential roles in paddy ecosystems. Their dynamics greatly varied with varietial traits, growing periods, and seasons in both pot and field experiments (*17*). Particularly, microbial biomass and population in rice soil are largely controlled by organic substances released from rice roots (*18*). Likewise, the allelochemicals released from allelopathic rice roots could provide carbon to interact with soil microorganisms. Rice allelochemicals would likely have a great impact on soil microorganisms once released. Up to now, rice−microbe interactions mediated by allelochemicals in paddy soil have not been clearly identified yet.

Allelopathic rice varieties released several types of allelochemicals, including phenolic acids (*19*), flavones, and terpenoids (*20–23*). Among these allelochemicals, flavone *O*-glycoside 5,4′-dihydroxy-3′,5′- dimethoxy-7-*O*-β-glucopyranosylflavone seems to be particularly interesting and may be exuded from allelopathic rice roots to the rihzosphere and then transformed rapidly into aglycone 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone with a great diversity of biological activities on soil organisms (*23*). However, the fate and impact on microorganisms of this aglycone in paddy soil remains obscure. In the present study, we investigated the dynamic change of the allelochemical (5,7,4′-trihydroxy-3′,5′-dimethoxyflavone) and its impact on microbial biomass and population in soils planted with allelopathic rice versus non-allelopathic rice varieties during the whole growth stages in paddy fields. Furthermore, the fate and degradation product of flavone interference with microbial population and community structure in soil were evaluated under laboratory experiments, with an attempt to be helpful to our further understanding of how allelopathic rice works in paddy ecosystems.

MATERIALS AND METHODS

Rice Varieties, Paddy Soils, and Chemicals. Two rice varieties with similar root systems and agronomic traits, PI312777 and Liaojing-9, were selected for these studies. PI312777 is an allelopathic rice variety originally identify from United States Department of Agriculture−Agricultural Research Service (USDA−ARS) rice germplasm collection (*24*) and has shown the allelopathic action in both U.S. and Chinese paddy fields (*11, 12*). Liaojing-9 is popular commercial cultivar in Northeast China that does not have an allelopathic trait.

Paddy soils were collected from rice field at the Shenyang Experimental Station of Ecology, Chinese Academy of Sciences (Northeast China, N 41°31′, E 123°24′). The soils were air-dried, mixed, and then sieved (2 mm mesh) to remove plant tissues. The soil had a pH of 5.75, organic matter content of 1.31%, and fertility status, total N of 0.97 g kg^{-1}, available N of 108.64 mg kg^{-1}, total P of 0.41 g kg^{-1}, available P of 29.76 mg kg^{-1}, total K of 0.81 g kg^{-1}, and available K of 77.85 mg kg^{-1}.

5,7,4′-Trihydroxy-3′,5′-dimethoxyflavone was obtained from allelopathic rice PI312777 plants by methods developed in our laboratory (*22*). Authentic benzoic acid was purchased from Sigma Co.

Field Trials. The field trials were conducted at the Shenyang Experimental Station of Ecology, Chinese Academy of Sciences, from May to October in 2007. The field had previously been planted with rice for several seasons. The field was divided into numerous plots (2 × 3 m) that were in a completely randomized design with three replicates. The two rice varieties, PI312777 and Liaojing-9, were planted in randomly selected plots by means of direct seeding at a planting density of 3.0×10^5 plants ha^{-1}. Plots not planted with rice served as controls. Hand weeding was employed during the trials. Other management in paddy fields was carried out in accordance with local practices. The soils around rice roots (1 cm in diameter and 0−10 cm in depth) were sampled at seedling (three leaf), tillering, elongation, heading, and mature growth stages, respectively. Soil samples were carried out with a series of chemical and microbiological analyses described below.

Laboratory Experiments. A series of pots (6 × 8 cm) with 100 g of paddy soils described above were incubated with distilled water in the dark at 28 °C for 10 days, and then 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone or benzoic acid at the concentration of 50 μg g^{-1} dry soil was added to the treated pots, respectively. The control pots received distilled water only. The pots were sealed with airtight lids and then were placed in an environmental chamber with 25 ± 1 °C. The pots were taken out from the chamber randomly after various incubation time intervals (day 1, 3, 5, 7, or 10), and the soils were taken for the analyses of their microbial biomass C, population or community structure, and concentration of flavone and benzoic acid as described below.

Chemical Analysis. Soil samples (50 g) were each extracted with 150 mL of MeOH (agitated for 12 h at 25 °C and then centrifuged at 1200 g for 30 min). The extracts were concentrated in a vacuum at 40 °C to give the residues. The residues were, respectively, dissolved in 50% aqueous MeOH (v/v, 2 mL) and loaded onto a reversed-phase C_{18} Sep-Pak cartridges (Waters, Co.), which were eluted with 50% aqueous MeOH (5 × 3 mL) and then MeOH (3 × 3 mL). The MeOH fraction was concentrated with N_2 to obtain a 100 μL concentrate for quantitative analysis of flavone and benzoic acid.

Quantification of flavone and benzoic acid was carried out with a high-performance liquid chromatography (HPLC) HP-1100 equipped with a Zorbax SB-C_{18} reversed column (Hypersil 150 × 4.6 mm, 5 μm) with a diode array detector. HPLC determination conditions: mobile phase was the mixture of MeCN/1% aqueous AcOH (2:3, v/v), eluted at a flow rate of 1.0 mL min^{-1} and detected at 320 nm. The injection volume of samples was 10 μL. Flavone or benzoic acid was quantified by interpolating the peak area on the chromatograms of HPLC to a standard curve constructed by the peak area of authentic benzoic acid or 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone. Triplicate injections of each concentration were made. The average recoveries of known amounts of flavone and benzoic acid added into soil were 83.2 ± 5.1 and 71.7 ± 4.6%, respectively, which were used to correct the concentrations determined. The half-lives ($t_{1/2}$) of flavone and benzoic acid were each calculated on the basis of contents of flavone and benzoic acid at incubation time intervals (*1, 22*).

Microbiological Analysis. The microbial biomass C was determined by the chloroform fumigation−extraction method (*25*) with modifications. Each of the soil samples with various treatment and control as described above were divided into two groups with an equivalent to 15.0 g of dry soil. One group was fumigated with ethanol-free chloroform for 24 h in the dark at 25 °C, and another group was not. Both fumigated and unfumigated soils were extracted with 0.5 M K_2SO_4 solution for 30 min on a shaker [1:4 soil/solution (w/w)]. The supernatant was filtered, and the filtrate was frozen. The amount of extractable carbon was determined with an automated TOC analyzer (Multi N/C 3000, German). The microbial biomass C content (MBC) was calculated by MBC = E_c/K_c, where E_c = (C extracted from fumigated soil) − (C extracted from nonfumigated soil) and K_c with value of 0.38 is a calibration factor (*26*).

Enumeration of culturable bacteria, actinomycetes, and fungi was performed with the soil dilution plate method (*27*). Briefly, samples (10 g soils) were suspended in 10 mL of sterile water and 10-fold serially diluted. The colony-forming unit (CFU) of bacteria was determined by spreading 100 mL of diluted samples on agar plates with beef extract−peptone medium. The medium for actinomycetes contained 1% soluble starch, 0.2% NH_4SO_4, 0.1% K_2HPO_4, 0.2% $MgSO_4$, 0.7% NaCl, 0.3% $CaCO_3$, and 2% agar (w/v). The CFU of actinomycetes were determined by probing colonies that developed with a dissecting needle: if the colony remained as a discrete, small mass, it was considered to be an actinomycete, whereas if the colony smeared and lost its periphery, it was considered to be a bacterium other than an actinomycete. These empirical observations were confirmed by microscopic examination of the colonies. The CFU of fungi were estimated on Rose Bengal (33 μg mL^{-1}) and streptomycin (30 μg mL^{-1}) agar, on which 100 mL of 10-fold serially diluted soil samples

Table 1. Dynamics of Soil Microorganisms and Concentration of 5,7,4′-Trihydroxy-3′,5′- dimethoxyflavone and Benzoic Acid at Different Growth Stages of Allelopathic PI312777 and Non-allelopathic Liaojing-9 Varieties Tested in Paddy Fields[a]

		growth stages				
soil parameter	variety	seedling	tillering	elongation	heading	mature
microbial biomass C	PI312777	213.5 ± 19.5 b	296.0 ± 29.4 b	284.0 ± 36.5 a	176.4 ± 28.1 a	243.0 ± 40.3 a
(mg of C kg^{-1} of soil)	Liaojing-9	272.4 ± 32.5 a	350.5 ± 19.2 a	231.5 ± 29.1 b	119.8 ± 30.5 b	200.6 ± 23.2 b
	control	138.0 ± 12.0 c	110.6 ± 25.3 c	134.2 ± 32.0 c	70.4 ± 10.3 c	123.5 ± 9.2 c
microbial population						
bacteria	PI312777	83.3 ± 4.6 a	135.2 ± 20.5 a	132.6 ± 18.7 a	83.2 ± 7.0 b	90.3 ± 5.4 b
(10^6 CFU g^{-1} of soil)	Liaojing-9	49.8 ± 21.5 b	116.4 ± 26.8 b	113.2 ± 8.9 b	92.3 ± 11.5 a	109.3 ± 12.8 a
	control	43.3 ± 8.6 b	53.5 ± 11.8 c	42.5 ± 10.9 c	51.0 ± 9.9 c	46.9 ± 7.3 c
actinomycetes	PI312777	80.7 ± 20.5 b	100.3 ± 9.9 b	91.3 ± 22.4 b	72.7 ± 8.9 b	109.4 ± 11.5 b
(10^5 CFU g^{-1} of soil)	Liaojing-9	91.2 ± 6.4 a	140.5 ± 11.5 a	126.5 ± 18.9 a	101.6 ± 25.4 a	112.6 ± 10.7 a
	control	58.6 ± 11.2 c	60.3 ± 6.3 c	64.1 ± 12.0 c	59.4 ± 14.1 c	66.7 ± 16.8 c
fungi	PI312777	33.8 ± 9.6 b	36.3 ± 5.9 a	35.3 ± 2.9 a	27.5 ± 7.4 a	31.2 ± 6.8 a
(10^3 CFU g^{-1} of soil)	Liaojing-9	41.3 ± 12.5 a	40.4 ± 1.6 a	38.6 ± 6.4 a	26.4 ± 5.6 a	26.3 ± 5.6 a
	control	27.5 ± 3.8 c	23.1 ± 8.4 b	25.9 ± 14.8 b	22.0 ± 3.8 a	22.6 ± 7.3 ab
concentration						
flavone	PI312777	47.5 ± 7.2 a	25.2 ± 4.1 a	9.3 ± 1.2 a	4.9 ± 0.8 a	2.8 ± 0.6 a
(μg g^{-1} of soil)	Liaojing-9	8.6 ± 1.1 b	4.6 ± 0.8 b	2.2 ± 0.6 b	1.0 ± 0.2 b	0.5 ± 0.1 b
	control	ND	ND	ND	ND	ND
benzoic acid	PI312777	5.3 ± 0.8 a	6.4 ± 1.2 a	7.2 ± 1.6 a	7.1 ± 1.2 a	6.5 ± 1.0 a
(μg g^{-1} of soil)	Liaojing-9	5.6 ± 1.0 a	6.2 ± 0.8 a	7.5 ± 1.2 a	6.9 ± 1.2 a	6.8 ± 1.1 a
	control	3.9 ± 0.6 b	4..6 ± 0.8 b	5.8 ± 1.0 b	6.2 ± 0.2 b	5.5 ± 0.8 b

[a] ND = not detected. Means ± standard error (SE) from three independent experiments for each determination were shown. Data in a column followed by the same letter are not significantly different at $p = 0.05$, analysis of variation (ANOVA) with Duncan's multiple-range test.

was spread. The CFU was counted after incubation for 5 days for bacteria and 7 days for actinobacteria and fungi at 28 ± 1 °C.

Phospholipid fatty acid (PLFA) analysis was performed by a combination of two methods (*28, 29*). Triplicate 5 g (dry weight) subsamples of soil were extracted for 2 h in 19 mL of a one-phase extraction mixture containing $CHCl_3$/MeOH/phosphate buffer (1:2:0.8, v/v/v). The amount of phosphate buffer was corrected to account for soil−water content. After centrifugation, the supernatant was decanted to a separatory funnel. The soil was then vortexed and re-extracted with an additional 19 mL of extractant. Supernatant from the second extraction was added to the first. A total of 10 mL of phosphate buffer and 10 mL of $CHCI_3$ were added. Samples were shaken and separated. The $CHCl_3$ layer was decanted and dried under N_2 at 30 °C. Phospholipids were separated from neutral and glycolipids on solid-phase extraction columns, 0.50 g of Si (Supelco, Inc.). The column was conditioned with 3 mL of $CHCl_3$, and then lipids were transferred to the column with 4 × 250 μL aliquots of $CHCl_3$. Neutral lipids and glycolipids were eluted with 5 mL of $CHCl_3$, followed by 10 mL of acetone. Polar lipids were eluted with 5 mL of MeOH and dried under N_2 at 30 °C. Samples were then subjected to mild alkaline methanolysis by dissolving in 1 mL of MeOH/toluene (1:1, v/v) and 1 mL of 0.2 M KOH and heating at 37 °C for 15 min. A total of 2 mL of H_2O and 0.3 mL of 1.0 M acetic acid were added. The resultant fatty acid methyl esters (FAMEs) were separated and then quantified and identified by gas chromatography mass spectrometry (GC−MS).

PolarisQ ion-trap GC−MS (Thermo Fisher Scientific, Inc.) was used with a 30 m DB5-MS column. One μL injection with a 1:50 split was analyzed. Helium was used as a carrier gas (1.0 mL/s). The oven temperature was programmed from 140 to 190 °C at 4 °C/min, held for 1 min, from 190 to 230 °C at 3 °C/min, held for 1 min, and then from 230 to 250 °C at 2 °C/min. Thereafter, the temperature was increased again at 10 °C/min to 290 °C and held for 2 min. Identification of FAMEs was based on retention time comparisons with FAME controls (Supelco, Inc.). Quantification was carried out by calibration against standard solutions of nonadecanoate methyl ester (C19:0), which were also used as the internal standard. A total of 37 PLFAs were identified in the different soil samples. Among them, fatty acids present in proportions >0.5% were used in the analyses. The prefixes "a" and "i" indicate antiso- and isobranching; "br" indicates unknown branching; and "cy" indicates a cyclopropane fatty acid. The sum of 18 fatty acids was used to assess bacterial biomass (14:0, 2OH14:0, i15:0, a15:0, 15:0, i16:0, 16:0, 10Me16:0, 16:1ω9, 16:ω7c, 2OH16:0, i17:0, 17:0, cy17:0, 18:1ω7, 18:0, cy19:0, and 20:0). The ratio between the PLFAs i15:0, i16:0, 10Me16:0, and a15:0 to PLFAs 16:1ω7c, cy17:0, 18:1ω7c, and cy19:0 was used as an index of Gram-positive and Gram-negative bacteria, respectively. Fungal biomass was assessed by quantifying 18:2ω6,9c. In addition, the ratios of fungal to bacterial (fungi/bacteria) and Gram (−) to Gram (+) bacterial content (GN/GP) were calculated. Before being subjected to principal component analyses (PCAs), the results were expressed by a percentage of the total PLFA.

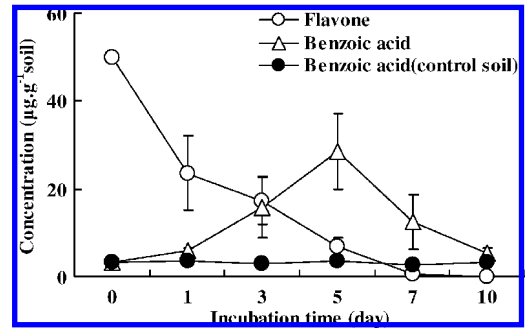

Figure 1. Soil degradation dynamics of 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone. Means ± SE from three independent experiments for each determination were shown.

PCA was performed using the STATISTICA software package version 6.0 (Statsoft, Inc., Tulsa, OK) and was applied separately to PLFA proportions as well as showed relationships among different samples that contain multiple variables. PCA is useful for discerning patterns within the PLFA data itself and describes the axes of maximum variability in the multivariate data set (*29*).

RESULTS AND DISCUSSION

Table 1 showed that soil microbial biomass and culturable microorganisms were affected greatly with rice varietal characteristics and growth stages in paddy fields. There are significant differences in soils microbial biomass and culturable microorganisms between planted and unplanted rice, either allelopathic PI312777 or non-allelopathic Liaojing-9 varieties tested. Both rice varieties significantly increased soils microbial biomass C and culturable populations during all growth stages. This may be due to the fact that metabolites released from rice plants may stimulate soil microbial biomass and populations in the rhizosphere (*17, 18*). However, these dynamic changes could

Table 2. Impact of 5,7,4′-Trihydroxy-3′,5′-dimethoxyflavone and Benzoic Acid on Microbial Biomass C and Culturable Populations of Paddy Soil[a]

		incubation time (day)				
microorganism	allelochemical	1	3	5	7	10
microbial biomass C	flavone	58.3 ± 30.2 c	61.7 ± 36.1 c	129.5 ± 28.2 c	146.2 ± 31.9 b	173.5 ± 20.3 a
(mg of C kg^{-1} of soil)	benzoic acid	146.7 ± 20.4 a	264.1 ± 31.4 a	237.5 ± 26.4 a	177.4 ± 21.2 a	149.7 ± 29.2 a
	water (CK)	105.1 ± 10.5 b	125.7 ± 16.2 b	159.6 ± 19.6 b	146.6 ± 10.2 b	144.7 ± 12.3 a
microbial population						
bacteria	flavone	31.8 ± 8.8 c	44.1 ± 7.8 c	58.2 ± 9.1 b	67.2 ± 5.4 a	44.4 ± 6.3 a
(10^6 CFU g^{-1} of soil)	benzoic acid	106.9 ± 9.4 a	86.4 ± 12.1 a	72.9 ± 11.4 a	64.6 ± 8.2 a	49.9 ± 10.8 a
	water (CK)	42.5 ± 10.2 b	60.7 ± 14.5 b	54.6 ± 10.6 b	59.9 ± 4.6 a	42.7 ± 8.7 a
actinomycetes	flavone	22.4 ± 8.8 b	20.8 ± 9.9 c	35.7 ± 5.3 a	39.3 ± 10.8 a	22.4 ± 14.7 a
(10^5 CFU g^{-1} of soil)	benzoic acid	42.2 ± 3.6 a	79.1 ± 10.0 a	55.8 ± 9.1 c	41.0 ± 10.6 a	33.8 ± 5.4 a
	water (CK)	18.7 ± 4.8 c	47.6 ± 13.7 c	43.9 ± 12.4 b	32.1 ± 11.5 a	29.2 ± 7.0 a
fungi	flavone	0.6 ± 0.1 c	4.9 ± 0.7 c	6.3 ± 0.2 b	4.7 ± 0.3 b	5.1 ± 0.5 a
(10^3 CFU g^{-1} of soil)	benzoic acid	5.9 ± 0.4 a	10.9 ± 1.0 a	8.6 ± 0.9 a	8.2 ± 0.7 a	6.3 ± 0.6 a
	water (CK)	3.0 ± 0.3 b	6.1 ± 0.3 b	7.5 ± 0.5 b	5.0 ± 0.3 b	5.6 ± 0.2 a

[a] Means ± SE from three independent experiments for each determination were shown. Data in a column followed by the same letter are not significantly different at $p = 0.05$, ANOVA with Duncan's multiple-range test.

be distinguished by the allelopathic and non-allelopathic rice varieties tested. An increase of soil microbial biomass C was more significant in non-allelopathic Liaojing-9 than in allelopathic PI312777 during the early growing periods (from seedling to tillering), but such a trend was reversed during the late growing periods (from elongation to mature). Paddy soil grown PI312777 had a higher bacteria population from seedling to elongation stages when compared to soil grown Liaojing-9, but there were lower actinomycetes and fungi populations in PI312777 than Liaojing-9 (**Table 1**). Allelopathic PI312777 released allelochemical 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone in sufficient quantities into soils at their early growth stages (maximal level of 47.5 ± 7.2 μg g^{-1} soil at the seedling stage) and then decreased dramatically at elongation stages. While non-allelopathic Liaojing-9 released flavone of deficiency into paddy soils at all growth stages. Benzoic acid was also found in soil grown both PI312777 and Liaojing-9 at all growth stages, but there were not any quantitative differences in the release of benzoic acid between allelopathic PI312777 and non-allelopathic Liaojing-9 (**Table 1**). Benzoic acid and several phenolic acids are ubiquitous compounds in almost all higher plants, and thus, they usually act as model allelopathic agents (*30, 31*). Paddy soils always contain these phenolic acids resulting from lignin of rice residue decomposition (*16*). Phenolic acids released from living rice roots are unlikely to explain the rice allelopathy because their concentrations in the paddy never reached phytotoxic levels (*32*). Accordingly, it is postulated that the presence of discriminating 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone in paddy soil would result in different patterns on soil microorganisms between allelopathic and non-alleloparhic varieties tested. To confirm this postulation, a series of experiments were carried out with the laboratory condition.

First of all, quantitative 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone was added into paddy soil, and then, their amounts were analyzed at various time intervals. Initially, the soil contained flavone, but flavone amounts declined rapidly with incubation time and were not detected after 7 days of incubation. Flavone given a short half-life of 18.27 ± 2.32 h ($r^2 = 0.94$) indicated that flavone could easily be degraded by soil interactions once released or incubation. Several unknown intermediates were observed during early incubation time (days 1 and 3) of flavone. Particularly, benzoic acid amounts in soil increased dramatically during days 3 and 5 of incubation, and the concentrations were 5−10-fold compared to control soil (**Figure 1**). Flavone is a phenolic compound and could be easily degraded into benzoic acid by soil biotic and abiotic factors. Although detail degradation process of flavone remained obscure, it is a fact that benzoic acid is an important degradation product of flavone in paddy soil. Furthermore, benzoic acid with the half-life of 29.99 ± 2.19 h ($r^2 = 0.96$) was more resistant toward degradation in paddy soil. These results implied that flavone would be responsible for the dynamics of soil microorganisms during early incubation time, and any observed effect during late incubation time would be very likely because of its degradation products, particularly benzoic acid, rather than flavone itself.

There are two types of methodology to gain an understanding of soil microorganisms, which are for culturable population or whole community. In general, the former is performed on the culturable microbial population with the soil dilution plate method (*27*), while the latter is used to assess changes in the entire microbial community structure by determination of phospholipid fatty acid (PLFA) (*28, 29*). Thus, culturable microbial population and the entire microbial community structure of soil incubated with flavone and benzoic acid were investigated using the soil dilution plate method and PLFA analysis, respectively. Subsequently, 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone reduced soil culturable bacteria greatly during early incubation time. Although the data in **Table 2** showed that flavone increased soil culturable bacteria slightly during late incubation time, the effect resulted from degradation products rather than flavone itself. The populations of culturable actinomycetes and fungi were always inhibited by flavone, particularly during early incubation time, but the inhibition disappeared smoothly after incubation for 5 days. On the contrary, application of benzoic acid led to a distinct increase in populations of bacteria, actinomycetes, and fungi during the whole incubation time (**Table 2**). These results indicated that flavone and benzoic acid had substantially different impacts on culturable microorganisms once incubated or released in paddy soil, but it was not clear whether such a result could be generalized to the entire microbial community.

PLFA profiling showed that total PLFA concentrations varied greatly with the incubation time of flavone and benzoic acid. Of the 23 PLFAs used to assess microbial community composition, 18 displayed significant differences among the soil samples. The relative abundance of Gram-positive bacteria PLFAs (i15:0, i16:0, 10Me16:0, and a15:0) was significantly greater in the samples of the first day sampling than those of the other days. Two PLFAs (16:1ω7c and 18:1ω7c) belonging to Gram-negative bacteria separated the first and third day of sampling from the other days. However, the trend of fungal PLFAs (18:1ω9c, 18:2ω6, 18:3ω9, and 18:1ω5c) was not the same as the Gram-positive bacteria. The concentration of total fungal PLFAs

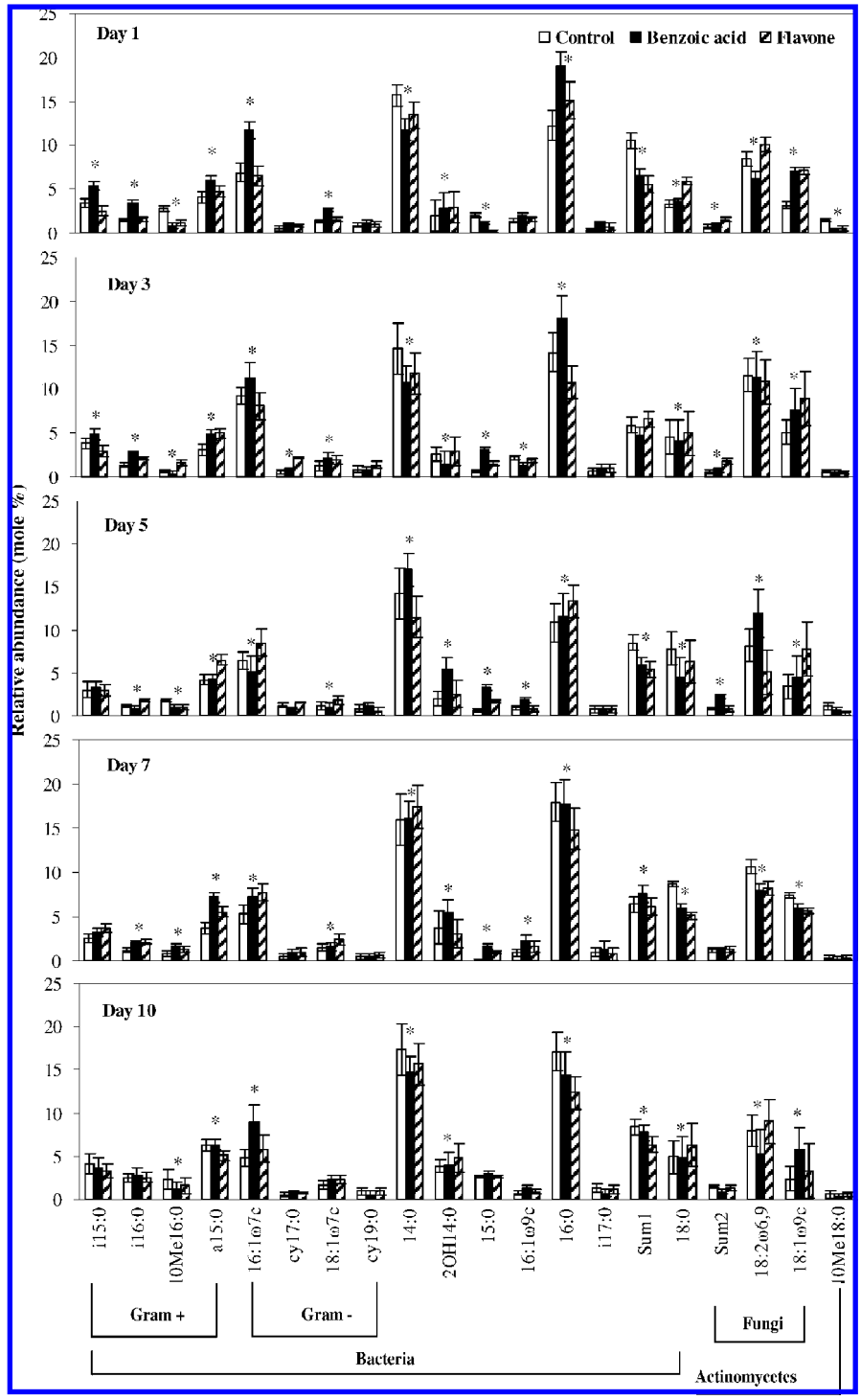

Figure 2. Relative abundance of bacterial, actinomycetal, and fungal PLFA in soil incubated with benzoic acid and flavone. Sum1 = 17:0 + 20:0 + 2OH16:0, and Sum2 = 8:3ω9,12,15c + 18:1ω5c. (*) $p < 0.05$.

was much lower in soil incubation with flavone than in soil incubation with benzoic acid (**Figure 2**).

PLFA profiles may indicate the presence of different groups of soil microorganisms, and thus, diagnostic fatty acids were used to classify different microbial groups (*33, 34*). Flavone resulted in a reduction of actinomycete and fungal communities compared to benzoic acid, but both flavone and benzoic acid had an effect on aerobic (16:1ω7c and 18:1ω7c) and anaerobic (cy19:0) bacteria with different incubation times. The concentration of the sulfate-reducer indicator (10Me16:0) decreased clearly in the sample incubation with flavone or benzoic acid compared to the control (**Table 3**). Benzoic acid increased the Gram (−) to Gram (+) bacterial content (GN/GP) ratio on the first day and then decreased rapidly with the incubation time. Flavone was degraded to benzoic acid that led to a significant increase in GN/GP on the seventh day (**Table 3**). A significantly greater fungal/bacterial PLFA ratio occurred in soil incubation with benzoic acid, indicating that fungi were more abundant in soil incubated with benzoic acid. Nevertheless, the evidence of relatively low fungal/bacterial ratios in all soil samples clearly indicated that bacterial PLFAs surpassed fungal PLFAs (**Table 3**).

Table 3. Concentration of Specific Fatty Acids and the Main Ratio Indices of Paddy Soil Incubated with 5,7,4′-Trihydroxy-3′,5′-dimethoxyflavone and Benzoic Acid[a]

		fatty acid concentration (nmol g^{-1} soil)						
time	treatment	anaerobe	aerobe	sulfate reducers	actinomycetes	fungi	GN/GP	fungi/bacteria
day 1	flavone	0.93 ± 0.13 c	0.10 ± 0.02 b	1.16 ± 0.31 b	0.21 ± 0.08 b	3.53 ± 0.76 b	0.94 ± 0.16 b	0.33 ± 0.03 a
	benzoic acid	2.42 ± 0.66 a	0.19 ± 0.07 a	0.82 ± 0.12 c	0.34 ± 0.03 a	4.98 ± 1.01 a	1.34 ± 0.28 a	0.26 ± 0.02 b
	control	1.59 ± 0.36 b	0.17 ± 0.06 a	1.74 ± 0.56 a	0.28 ± 0.09 ab	4.39 ± 0.96 a	0.81 ± 0.11 c	0.26 ± 0.02 b
day 3	flavone	4.29 ± 0.79 a	0.66 ± 0.06 a	1.66 ± 0.26 a	0.25 ± 0.04 a	6.75 ± 0.56 c	1.14 ± 0.36 a	0.19 ± 0.02 b
	benzoic acid	4.05 ± 0.81 a	0.49 ± 0.04 b	1.16 ± 0.26 b	0.28 ± 0.03 a	10.96 ± 1.48 a	0.98 ± 0.21 b	0.27 ± 0.02 a
	control	2.19 ± 0.69 b	0.40 ± 0.05 b	1.61 ± 0.38 a	0.23 ± 0.05 a	7.80 ± 1.32 b	0.85 ± 0.09 c	0.23 ± 0.02 ab
day 5	flavone	2.15 ± 0.42 b	0.19 ± 0.02 b	1.03 ± 0.17 b	0.24 ± 0.01 a	4.12 ± 0.66 c	0.55 ± 0.08 b	0.15 ± 0.02 b
	benzoic acid	2.44 ± 0.44 a	0.27 ± 0.02 b	1.04 ± 0.11 b	0.31 ± 0.01 a	10.05 ± 1.88 a	0.98 ± 0.13 a	0.29 ± 0.02 a
	control	2.39 ± 0.31 a	0.46 ± 0.03 a	1.84 ± 0.19 a	0.26 ± 0.03 a	8.35 ± 1.06 b	0.46 ± 0.07 b	0.23 ± 0.02 ab
day 7	flavone	2.28 ± 0.31 a	0.22 ± 0.01 b	1.28 ± 0.18 c	0.22 ± 0.01 a	5.78 ± 0.86 b	1.14 ± 0.16 a	0.16 ± 0.01 c
	benzoic acid	2.57 ± 0.49 a	0.22 ± 0.01 b	1.65 ± 0.22 b	0.21 ± 0.01 a	12.12 ± 2.76 a	0.73 ± 0.09 b	0.35 ± 0.04 a
	control	2.39 ± 0.55 a	0.40 ± 0.03 a	1.85 ± 0.39 a	0.26 ± 0.02 a	11.35 ± 2.02 a	0.46 ± 0.05 c	0.29 ± 0.02 b
day 10	flavone	2.77 ± 0.41 a	0.20 ± 0.01 b	1.25 ± 0.17 b	0.19 ± 0.01 a	3.46 ± 0.32 b	0.56 ± 0.06 b	0.13 ± 0.01 b
	benzoic acid	2.93 ± 0.33 a	0.25 ± 0.03 b	1.19 ± 0.34 c	0.21 ± 0.01 a	5.00 ± 0.79 a	0.63 ± 0.08 a	0.15 ± 0.01 a
	control	2.77 ± 0.29 a	0.41 ± 0.04 a	1.33 ± 0.17 a	0.20 ± 0.01 a	4.92 ± 0.72 a	0.28 ± 0.02 c	0.19 ± 0.01 a

[a] Means ± SE from three independent experiments for each determination were shown. Data in a column followed by the same letter are not significantly different at $p = 0.05$, ANOVA with Duncan's multiple-range test.

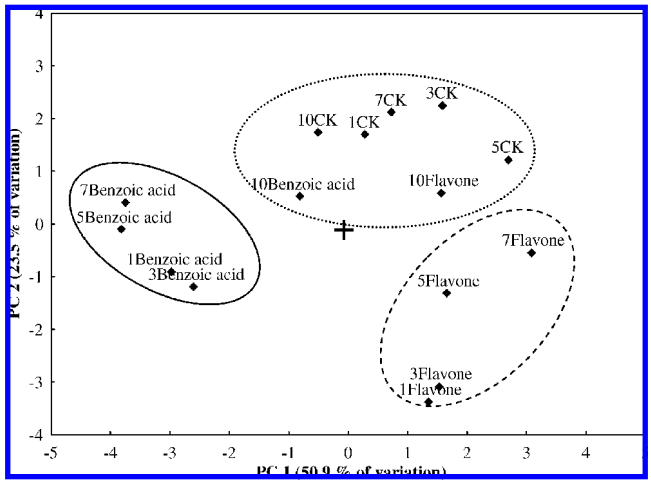

Figure 3. Plots of the microbial community structure of soil incubated with flavone and benzoic acid by PCA. PC indicates a principle component, and (+) indicates the (0, 0) points.

PCA of PLFAs was clearly distinguished in the composition of total PLFAs between incubation of flavone or benzoic acid and control (water), each of which occupied very different ordination space. Principal component axes PC1 and PC2 accounted for 74.4% of the variation among soil samples (**Figure 3**). Composite scores for different incubation times in soils incubated with benzoic acid occupied the negative portion of PC1, on which several bacterial PLFAs (a15:0, 10Me16:0, and 18:1ω7c) received negative weights (from −0.687 to −0.887) on this axis. In contrast, composite scores for flavone treatment resided on the positive portion of PC1, the result of the high positive weights (0.912−0.668) given to several bacterial PLFAs (14:0, 16:1ω7c, and i17:0). On PC2, flavone was separated from the control and benzoic acid because of high weights (0.775−0.823). This analysis revealed a remarkable difference in microbial community composition between flavone and benzoic acid. Particularly, the treatment of flavone, benzoic acid, and control occupied the same ordination space during late incubation time (**Figure 3**), which indicated that the microbial community structure resulted from similar chemical composition in soil. The results agreed with variation of the concentration and fate of flavone in paddy soil.

Although the data generated in this study were not completely consistent between the culturable microbial method and PLFA profile, it is a fact that variation of the soil microbial population and community structure could be distinguished by flavone and benzoic acid. It appears from the results that flavone may reduce microorganisms especially for fungi present in paddy soil, while benzoic acid may induce a higher response for soil microorganisms. It is not strange because flavone has inhibitory activity on *Pyricularia oryzae* and other fungi (*35, 36*), but benzoic acid is a major carbon source for soil microorganisms and would be able to activate both bulk soil and rhizosphere bacteria (*37, 38*). Flavones exuded from roots into rhizosphere seem to be particularly interesting, in that they may act as allelochemicals to inhibit other plant species (*16, 22*) or be a signal to specific microbes (*39*). This study showed that 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone could result in different impacts of microbial population and community structure in rice soil. The impacts might be attributable to the interactions of all present chemicals in paddy soil, but flavone could be an important allelochemical to interfere with soil microorganisms. Actually, the capacity of metabolites exuded from rice roots to affect soil microorganisms could be attributed to some of their individual components (*8, 40*). However, this study did not clarify which soil microbial species were responsible for flavone or benzoic acid. Further clarification of the interactions between rice allelochemicals and soil microorganisms offers many potential implications and applications in paddy ecosystems.

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