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Impact of applied phenolic acids on the microbes, enzymes and available nutrients in paddy soils

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ABSTRACT

Phenolic acids are important compounds in plant allelopathy, hence the effects of 5-applied phenolic acids (*p*-hydroxybenzoic acid, ferulic acid, salicylic acid, vanillic acid, and cinnamic acid), were determined on the microbial community, enzyme activities and available nutrients in paddy soil. The application of phenolic acids significantly enhanced the soil microbial biomass carbon (MBC), soil microbial respiration rate (MBR), soil microbial populations, improved the soil enzyme activities and proved beneficial to the available nutrients (nitrogen, phosphorus and potassium) in soil. Three days after application of phenolic acids in soil they increased the soil MBC by 23.4% to 110.1% and MBR by 37.6% to 86.1% over the control, but these declined later on. The population of bacteria was increased by 43.3% to 255.0%, 11.1% to 44.4%, for fungi, and 87.8% to 226.8 % for actinomycetes over the control. The activity of urease was 56.6% to 163.3%, protease activity was 75.0% to 162.5%, and sucrase activity was 109.3% to 220.9% higher than control. The available nitrogen, phosphorus and potassium in the soils were significantly increased by 99.0% to 168.5%, 134.6% to 241.7% and 11.5% to 12.9%, respectively. The promoting effects of applied phenolic acids followed the order: *p*-hydroxybenzoic acid > ferulic acid > vanillic acid > salicylic acid > cinnamic acid. Furthermore, all the bacteria, available phosphorus and potassium were significantly positively correlated with the five applied phenolic acids. While the actinomycetes, urease and nitrogen had significantly positive correlation with *p*-hydroxybenzoic acid; the fungi, actinomycetes and protease had significant correlation with ferulic acid, the urease, protease, sucrase and available nitrogen were significantly positively correlated with salicylic acid; the actinomycetes, urease, protease and nitrogen had significantly positive correlation with vanillic acid. The protease and sucrase had significantly positive correlation with cinnamic acid. It suggested that phenolic acids are useful carbon resources to establish soil microbial community, enhance the activities of soil enzyme, and accelerate the nutrients cycling in soil.

Key words: Allelochemicals, cinnamic acid, enzymes, ferulic acid, p-hydroxybenzoic acid, microorganisms, nitrogen, phenolic acids, phosphorus, potassium, salicylic acid, vanillic acid.

INTRODUCTION

Allelopathy is a chemoecological process that one plant exerts a beneficial or harmful influence on its neighbouring species (another plant or microorganisms) through release of

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chemical compounds into environment (4,13,17,18,35). Phenolic acids are potential allelochemicals from plant tissues, root exudates and paddy soil (10,20,21,24,25). The previous study indicated that phenolic acids stimulated the populations of bacteria in soil and were the major carbon source for soil microbes, which activates both bulk soil and rhizosphere bacteria (5,25). The soybean releases many phenolic acids in soil and the microbes in soil could transform and utilize these phenolic acids [these cause the soil sickness problem (22,26,39)]. Accordingly, phenolic acid allelochemicals may be useful or harmful to the soil ecological system, it still remains a controversial issue.

Except the volatiles (released *via* volatilization), all allelochemicals released in leaching, excretion and decomposition are added to the soil, but little is known about their fate and behaviour in soil (15). To understand the mechanism of plant allelopathy in fields, it is necessary to investigate the fate of allelochemicals in soil and to demonstrate how phenolic acids influence the physical, chemical and biological characteristics of soil. Some work has been done (5,6,7,27) on the interactions between the phenolic acids and soil factors (retention and sorption, microbial transformation and utilization, and the effects on microbial populations). But little work has documented the relationships among phenolic acids, microorganisms and nutrients in the soil. Lin *et al* (15) reported that soil microbial biomass is both a labile nutrient pool and an agent for cycling of organic matter and plant nutrients in soil; therefore, is most important microbiological properties. Microbial respiration rate (index of total soil microbial activity) reflects the aerobic catabolic processes in the carbon cycling. Soil enzymes (originate from soil microorganisms), can also indicate the microbial activities in soil environment and plays important role in organic matter decomposition and nutrient cycling. So microbial biomass carbon (MBC), soil microbial respiration rate (MBR), microbial populations and enzyme activities indicate the soil quality. In this study, 5-phenolic acids (*p*-hydroxybenzoic acid, ferulic acid, salicylic acid) were used as applied allelochemicals to determine their impact on soil microbes, nutrients availability and the potential interactions among them.

MATERIALS AND METHODS

The paddy soil was collected from rice field of our Experimental Farm. The soil samples were brought to the laboratory and sieved with 2 mm mesh sieve at field moisture to remove the plant and animal residues. A portion of soil was air-dried and chemically analyzed. The soil pH 5.6 and contained organic carbon (1.35%), total nitrogen (1.76 $g \cdot kg^{-1}$), available nitrogen (29.4 $mg \cdot kg^{-1}$), total phosphorus (1.23 $g \cdot kg^{-1}$), available phosphorus (31.3 $mg \cdot kg^{-1}$), available potassium (256.4 $mg \cdot kg^{-1}$).

In this experiment, 5-phenolic acids (*p*-hydroxybenzoic acid, ferulic acid, salicylic acid, vanillic acid, and cinnamic acid) were applied to soil to evaluate their effects on soil microorganisms and nutritients availability. Each 200 g air-dried soil was placed in a glass cylinder, then 100 mg phenolic acid was dissolved in 1 mL of ethanol and complemented to 80 mL by double distilled water, respectively, and then added to the soil in glass cylinder. The soil moisture was adjusted to about 40% and the content of phenolic acid in each treated soil sample was 0.5 mg per g (22,36). Simultaneously, 80 mL solution without phenolic acid was also added to soil as control. Each treatment was replicated 6-times. During the experiment, the soil moisture was maintained at 40% water-holding capacity

and the soils were placed in incubator at 30°C (22)]. The samples were incubated for 3, 6 and 9 days at 30°C; the treated soils were sampled for bio-chemical analysis. In soil samples following parameters were determined (i). Microbial biomass carbon (MBC), (ii). Microbial respiration (MBR), (iii). Microbial populations (bacteria, fungi and actinomycetes), (iv). Soil enzyme activities (urease, proteases, and sucrase), (v). contents of available nitrogen, phosphorus, potassium and (vi). phenolic acids.

I. Microbial biomass carbon (MBC) and Microbial respiration (MBR)

The MBC and MBR are important indices for soil microbial community (3).

(i). MBC: Two portions equivalent to 20 g soil were collected from the glass cylinders, respectively. One was fumigated for 24 h at 25°C with chloroform. After removing the fumigant, the soil was extracted with 80 mL 0.5 M K_2SO_4 by horizontal shaking at 200 rpm for 30 min. Simultaneously, the non-fumigated portion was extracted under the same conditions. Organic C in the extracts was measured by potassium dichromate oxidation method (2). MBC was calculated as under:

$$MBC = E_c/K_c,$$

Where E_c =(organic C extracted from fumigated soil) – (organic C extracted from unfumigated soil) and $K_c = 0.38$ (2).

(ii). MBR: Forty g soil of each treatments were collected in the glass cylinders, respectively, and put in a airtight jars containing 5 mL of 0.5 M sodium hydroxide (NaOH) solution to trap the evolved CO_2, adding 2 mL of 1.5 M barium chloride solution corresponding to the NaOH equivalents for the precipitation of CO_2, and titration with 0.5 M hydrochloric acid (HCl) using phenolphthalein indicator (pH 8.3). The results were expressed in mg CO_2 kg^{-1} soil h^{-1} (22).

II. Soil microbial population

The numbers of soil microbial populations were determined with a serial dilution and a pour plate method (31). 10 g soil from each sample was suspended in 100 mL sterile water and 10-fold serially diluted. The Colony-Forming Units (CFU)of bacteria were detected on agar plate with beef extract-peptone medium. The total CFU of actinomycetes and fungi were recorded on Ken Knight's and Martin's Rose Bengal agar medium, respectively. Serial soil dilutions were prepared according to the desired selective microorganisms. The inoculated agar plates were incubated at 28±2°C 3 days for bacteria and fungi, and 7 days for actinomycetes, then the colonies were counted. Results were calculated as CFU per g of dry soil.

III. Soil enzyme activities

The soil enzyme activities were determined as per slightly modified methods of Research Centre of Microbiology (19) as under:

(i). Urease activity: 1 g of soil was collected in glass-cylinder containing 1 mL of toluene, 10 mL of 10% urea, 20 mL of 0.5 M citric acid buffer solution (pH 6.7) and put in

a greenhouse for 24 h at 37 ^{o}C. After the mixture solution was filtered, 3 mL of filtrate was collected in a new glass-cylinder and the distilled water was added until 20 mL. Afterwards, 4 mL of sodium phenylate and 3 mL of 0.9% sodium hypochlorite solution were added immediately. After 20 min, the urease activity was measured at 578 nm during 1 h and presented as mg NH_3-N g^{-1} soil·h^{-1}.

(ii). Protease activity: One g of soil was collected in glass-cylinder containing 1 mL of 0.05 M Tris-HCl buffer solution (pH 8.0), 0.5 mL of toluene, 2 mL of 1% casein solution and put in a incubator for 24 h in 37 ^{o}C. After 24 h treatment, 3 mL 15% trichloroacetic acid was added into the mixture solution and filtered. One mL of filtrate were collected in a new glass-cylinder containing 5mL of Na_2CO_3 (0.4 mol·L^{-1}) and 1 mL of Folin-Ciocalteu reagent, and put in a incubator at 37 ^{o}C. After 15 min later, protease activity was measured at 680 nm and presented as mg NH_3-N g^{-1} soil·min^{-1}

(iii). Sucrase activity: Five g of soil was collected in glass-cylinder containing 15 mL of 8 % sucrose solution, 5 ml of 0.1 M phosphatic buffer solution (pH 5.5), 0.25 mL of toluene and put in a incubator for 24 h at 37 ^{o}C. After the mixture solution was filtered, 1 mL of filtrate were collected in a new glass-cylinder, containing 3 mL of 0.02 M 3,5-dinitronaphthol salicylic acids and put in boiling water for 5 min. After the mixture solution was cooled, the distilled water was added until 25 mL. Finally, the sucrase activity was detected at 508 nm and expressed as mg glucose g^{-1} soil·day^{-1}.

IV. Available nitrogen, phosphorus and potassium

The available nitrogen was assayed by alkali N-proliferation method. The available phosphorus was determined at 700 nm by spectrophotometry of ammonium molybdate. The available potassium was analyzed by flame spectrophotometer (2).

V. Determination of phenolic allelochemicals by HPLC

After the soils were treated with five phenolic acid at 3, 6, and 9 days, respectively, 2 g of soils were collected and dipped in 5 mL of 100% methanol (LC grade and adjusted to pH 2.6 with 2 N HCl), shaken at 37^{o}C, 225 rpm/min for 2 h (28). Thereafter the supernatant was collected, and the work was repeated three times. Before analysis by HPLC, all supernatants were further purified by microfiltering through a 0.45 μm glass fibre. The model of HPLC is Waters 1525, dual λ absorbance detector is Waters 2487 and a 300 mm × 3.9 mm ID column, filled with Bondapak 18^{o}C was placed. Linear gradient elution was carried out at a flow rate of 1.0 ml/min. Solvent A was 5% acetic acid in distilled water, and solvent B was methanol. The detected time of HPLC was 20 min, and the solvent A : solvent B was 2 : 3.

The standard curves of five phenolic acids were prepared, respectively. Fifty μg of each phenolic acid were dissolved in 1 mL ethanol and diluted in 20, 40, 60, 80 and 100-folds by double distilled water, respectively. The solutions were used to detect the phenolic acid by HPLC and make standard curve of each phenolic acid. The condition of HPLC was as described above. Identification of the phenolic compounds was done by comparing their retention times with those of standard compounds. Simultaneously, peak area of each phenolic compound was registered to analyze the different contents of phenolic compounds.

VI. Statistical analysis

All experiments were replicated thrice with completely randomized design. Data in the tables are expressed as means± SD of three separate experiments performed in duplicate. All data were analyzed using DPS Data processing system (32). The means of the data obtained from different experiments were considered significant at LSD = 0.05. The correlation analysis among index of soil microbial populations and the associated allelopathic potentials were calculated by one-way ANOVA with Pearson's test.

RESULTS

Microbial biomass carbon (MBC) and Microbial respiration (MBR)

The application of allelochemicals enhanced both the MBC and MBR in paddy soils (Figure 1). The highest MBC and MBR were found on third day and thereafter declined significantly in next 6-days. On third day, the soil MBC was 23.4% to 110.1% higher than control and the MBR was 37.6% to 86.1% higher over control. The MBC and MBR responded differently to applied allelochemicals and followed the order: *p*-hydroxybenzoic acid > ferulic acid > vanillic acid > salicylic acid > cinnamic acid.

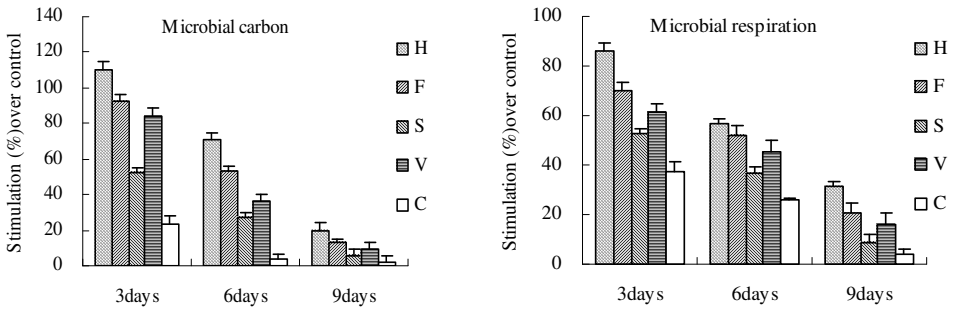

Figure 1. Effects of phenolic acids on MBC and MBR at 3, 6 and 9 days after application. ▨: Hydroxybonzoic acid (H), ▨:Ferulic acid (F), ▨:Salicylic acid (S),▨ Vanillic (V) , □: Cinnamic acid (C).

Soil microbial population

The applied allelochemicals stimulated the soil microbial populations (Figure 2). The highest population of bacteria, fungi and actinomycetes were found on day 3. The population of bacteria was 43.3% to 255.0%, fungi was 11.1% to 44.4%, and actinomycetes was 87.8% to 226.8 % higher than control. The microbial population declined dramatically after 3 days. At the end of experiment, the population of bacteria was 0.0% to 31.7%, fungi was 0.0% to 5.6%, and actinomycetes were 4.8% to 54.8% higher than control. The applied phenolic acids improved the soil microbes population in order : *p*-hydroxybenzoic acid > ferulic acid > vanillic acid > salicylic acid > cinnamic acid. The trends was same in bacteria, fungi and actinomycetes. Thus application of phenolic acids could stimulate the activities of soil microorganisms in paddy soil.

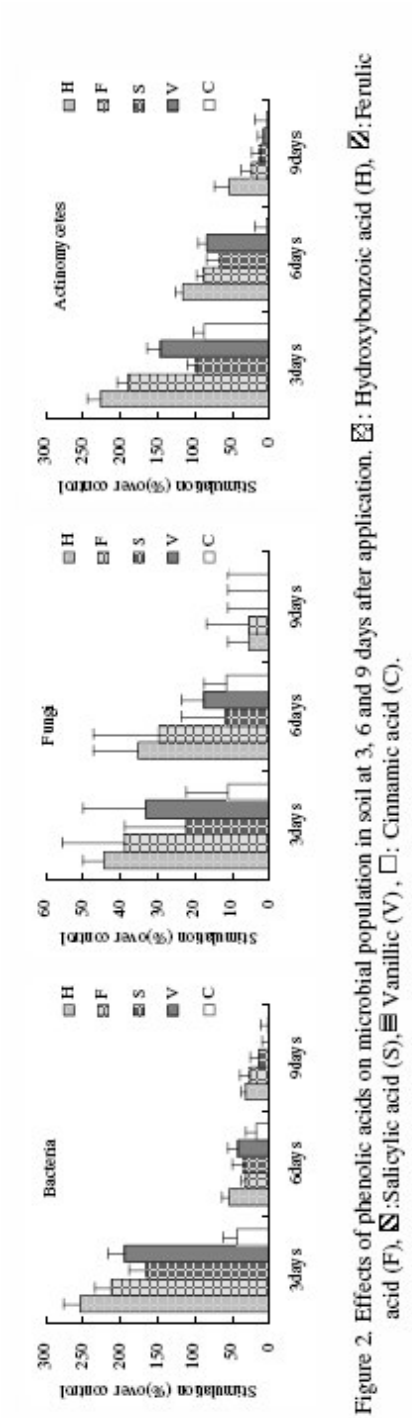

Figure 2. Effects of phenolic acids on microbial population in soil at 3, 6 and 9 days after application. ☒: Hydroxybonzoic acid (H), ☒: Ferulic acid (F), ☒ :Salicylic acid (S),☰ Vanillic (V) , □: Cinnamic acid (C).

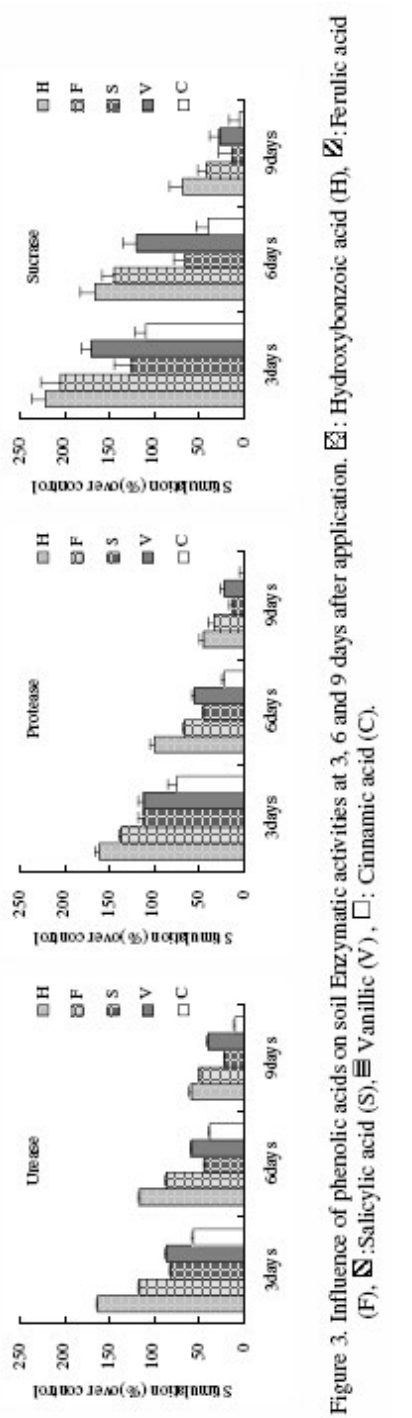

Figure 3. Influence of phenolic acids on soil Enzymatic activities at 3, 6 and 9 days after application. ☒: Hydroxybonzoic acid (H), ☒: Ferulic acid (F), ☒ :Salicylic acid (S),☰ Vanillic (V) , □: Cinnamic acid (C).

Soil enzyme activities

Similar to soil microbial population, MBC and MBR the application of phenolic acids also increased the activity of urease, protease, and sucrase in soil (Figure 3). The highest activities of urease, protease, and sucrase in the soil were found on day 3 in all treatments. The activities of urease was 56.6% to 163.3%, protease was 75.0% to 162.5% and sucrase was 109.3% to 220.9% higher than control. Thereafter, activities of soil enzymes declined on 9th day, the activity of urease was 9.9% to 59.2%, protease was 0.0% to 44.4% and sucrase was 4.5% to 68.2% higher than control. The applied phenolics acids have variable effects on soil enzymes and followed the order : *p*-hydroxybenzoic acid > ferulic acid > vanillic acid > salicylic acid > cinnamic acid. Thus the addition of phenolic acids was beneficial for the biochemical processes in paddy soil, which was in accord with the flourishing soil microbial population.

Soil nutrients availability

Three days after the soil application of phenolic acids, the contents of available nitrogen in the soils increased significantly by 99.0% to 168.5% and phosphorus by 134.6% to 241.7% over the control (Figure 4), and declined afterwards i.e. from 3-9 days. The change in available potassium was different from nitrogen and phosphorus. Phenolics application enhanced the available potassium by 11.5 to 12.9%, on day 3, but no significant difference was found among all the applied phenolic acids at each sampling stage. Furthermore, the effects of phenolic acids on the enhancement of available nitrogen, phosphorus and potassium were similar to soil microorganisms and enzyme activities and followed the order: *p*-hydroxybenzoic acid > ferulic acid > vanillic acid > salicylic acid > cinnamic acid.

Phenolic acids residues in soil

The phenolic acids were added to the soil at 0.5 mg·g^{-1} soil. Their concentration was decreased by 45.7% to 73.5% on day 3 and further decreased by 91.0% to 99.7% on day 9 (Table 1). From 3-9 days, the residues of five phenolic acids in soil were drastically decreased from 132.3 to 2.65 μg·g^{-1}, 193.2 to 3.1 μg·g^{-1}, 210.2 to 1.6 μg·g^{-1}, 271.3 to 32.3 μg·g^{-1}, and 242.6 to 45.1 μg·g^{-1} for *p*-hydroxybenzoic acid, ferulic acid, salicylic acid, vanillic acid and cinnamic acid, respectively. There were also significant differences in five allelochemicals at the same incubating stages.

Table 1. The residues of five phenolic acids added in soil at different time courses (μg·g^{-1} soil)

Phenolic acid	Treatment time		
	Third day	Sixth day	Ninth day
p-hydroxybenzoic acid	132.3±10.6d	42.3±12.1c	2.65±0.6b
Ferulic acid	193.2±12.6c	53.6±13.3c	3.1±0.2b
Falicylic acid	210.2±18.4bc	76.4±11.9bc	1.6±0.3b
Vanillic acid	271.3±13.5a	131.1±13.5a	32.2±3.94a
Cinnamic acid	242.6±15.7ab	102.8±12.8ab	45.1±4.58a

Note: H: *p*-hydroxybenzoic acid; F: ferulic acid; S: falicylic acid; V: vanillic acid; C: cinnamic acid. Different letters represent significantly difference at 0.05 levels.

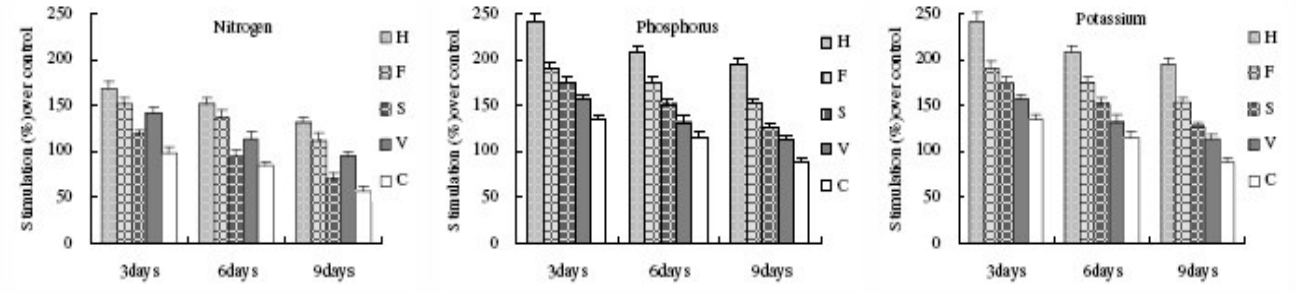

Figure 4. Impact of applied phenolic acids on N, P and K availability at 3, 6 and 9 days after application. ⊠: Hydroxybonzoic acid (H), ▨:Ferulic acid (F), ▧:Salicylic acid (S),▤ Vanillic (V) , □: Cinnamic acid (C).

Table 2. Correlationship among index of soil microbial populations and the associated allelopathic potential

Phenolic acid	Soil microbial organism			Enzyme activities			Nutrition available		
	Bacteria	Fungi	Actinomycetes	Urease	Protease	Sucrase	Nitrogen	Phosphorus	Potassium
p-hydroxybenzoic acid	0.98*	0.95	1.00**	0.96*	0.94	0.90	0.97*	1.00**	1.00**
Ferulic acid	0.99*	0.96*	0.99**	0.95	0.97*	0.89	0.93	0.96*	1.00**
Falicylic acid	0.98*	0.94	0.92	1.00**	0.99**	0.97*	0.98*	0.98*	1.00**
Vanillic acid	0.98*	0.91	0.98*	1.00**	0.99**	0.94	1.00**	1.00**	1.00**
Cinnamic acid	0.99**	0.95	0.95	0.95	0.99**	1.00**	0.94	0.97*	1.00**

Note: H: *p*-hydroxybenzoic acid; F: ferulic acid; S: salicylic acid; V: vanillic acid; C: cinnamic acid. **Correlation was significant at 0.01 level.*Correlation is significant at the 0.05 level.

Correlation analysis

The correlation analysis among the added allelochemicals, soil biological and chemical properties showed that the bacteria, phosphorus, and potassium had significant ($p > 5\%$), or extremely significant ($p > 1\%$) positive correlation with five phenolic acids (Table 2). The actinomycetes, urease, and nitrogen had significant, or extremely significant positive correlation with *p*-hydroxybenzoic acid. The same tendency was also found in fungi, actinomycetes and protease with ferulic acid, the urease, protease, sucrase, and nitrogen with salicylic acid, the actinomycetes, urease, protease, and nitrogen with vanillic acid; and the cinnamic acid was had significantly positive correlation with protease and sucrase activities.

DISCUSSION

Using allelopathy to control field weeds is considered as a key technique for sustainable agriculture in the future (11,29,37). Phenolic acids are recognized as the major allelochemicals in crops (8,9,30), and they are also important carbon sources for soil microbes and activates both the bulk soil and rhizosphere bacteria (25). Knowing about how phenolic acids act on the soil biological and biochemical properties will be beneficial to clarify the mechanism of plant allelopathy(33,34). The results of this study showed that MBC and MBR were enhanced by the applied phenolic acids in paddy soils. The application of phenolic acids significantly stimulated the population density of bacteria and actinomycetes, and the results are also true for fungi except the 9^{th} day in this study. This implied that phenolic acids are useful carbon resource for soil microbes and had an unbalanced impacts on soil microbial flora, this may lead to sickness (22,26,39). Furthermore, the strong allelopathic rice releases more amount of phenolic acids into the rhizosphere soil (8), while the rhizosphere soil microbes from strong allelopathic rice can consume the phenolic acids more quickly than non-allelopathic ones (16), the addition of phenolic acids decreased (45.7% to 73.5%) on day 3 and further decreased (91.0% to 99.7%) on day 9. Some rhizospheric microbes can affect the allelopathic effects through change the content level and nature of allelopathic compounds and vice versa, the allelopathic compounds can also affect the soil rhizosperic microbial population (19). Thus the mechanism between production and consumption of phenolic acids may be an underlying strategy for allelopathic rice to suppress the growth of weeds.

Soil enzymes, generally originate from soil microbes and their activities also reflect the intensity of edaphon and activity of biochemical reactions in soil, and they indicate the soil quality (1,38). The results showed that the added phenolic acids played a positive role in enzymes (urease, protease, and sucrase) activities in soil. The enhanced activities of acid phosphatase, alkaline phosphatase, invertase and catalase activity were also found in rhizospheric soils of allelopathic rice at seedling stage, while the activities of hydrogenase, peroxide, polyphenoloxidase, urease and cellulose were inhibited(16). The behavior of total root exduates in rice rhizosphere soil influence the activities of soil enzymes, eg. the activity of urease partly depends on the added phenolic acids, owing to the integrated action of all kind of allelochemicals. In addition, rice allelopathic potential is significantly negatively correlated with the activity of polyphenoloxidase (16), it implies that the lower activity of polyphenoloxidase in soil will be conducive to the retention and

accumulation of phenolic acids, in turn this provides a indirect proof of phenolic acids are closely related to the performance of rice allelopathy.

The increased activities of urease, protease and sucrase were beneficial to the process of N and C cycles (10,16,20,21). The added phenolic acids not only improve the popultion of microorganisms and promote the biochemical reactions, but also accelerates the rate of nutrients cycling in soil and consequently increased the soil nutrients availability. Furthermore, the highest MBC, MBR, microbial populations and enzymes activities, together with the lowest residues of *p*-hydroxybenzoic acid were found in soils, 3-days after treatment. It implies that *p*-hydroxybenzoic acid might be the most active allelochemicals among the phenolic acids.

CONCLUSIONS

The added phenolic acids had positive effects on the microorganisms and enzymes activity in soil, which provides the insight into the relationship between phenolic acids and soil quality. Nevertheless, research is needed more to analyze the diversity of microorganisms in soil mediated by phenolic acids to further understand the impact of added phenolic compounds in soil ecosystem.

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