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ARTICLE

Stability of phenolic acids and the effect on weed control activity

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Abstract Phenolic acid is a very important class of allelochemicals with allelopathic weed control activity. In this study, three benzoic acid derivatives (syringic, 4-hydroxybenzoic, and vanillic acids), three cinnamic acid derivatives (cinnamic, 4-hydroxycinnamic, and ferulic acids) were tested, and high-performance liquid chromatography was used to conduct a dynamic analysis on the changes in the concentration of phenolic acids in a bioassay based on the initial concentration and test time. The results showed that the concentration of individual phenolic acids and a solution of mixed phenolic acids decreased to a certain extent irrespective of environment, i.e., bioassay (4–7 days) or a rice-growing environment, and a significant decrease in concentration was measured after 48 h. Based on the above results, the laboratory bioassay was conducted using a fresh solution of phenolic acids every 48 h. The results showed that the instability of phenolic acid could affect its weed control activity, and this effect was more significant for high concentrations of phenolic acids. On the other hand, changing the solution did not have a significant impact on the weed control activity of phenolic acids in the natural environment (pH 6.50), in which allelopathic rice release phenolic acids. These results reveal the instability of phenolic acids could significantly reduce the inhibition rate on the growth index for receptor plants in an indoor bioassay.

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Introduction

Allelopathy refers to an ecological phenomenon, in which a living plant (donor) affects the growth and development of the surrounding plants (acceptors) through the release of secondary metabolites into the environment. These secondary metabolites are referred to as allelochemicals (Rice 1984; Kohli et al. 1998; Singh et al. 2001). Allelochemicals can be released into the environment through volatilization, leaching, root exudation, and residue decomposition (Rice 1984; Mann 1987; Cecile et al. 2003). Allelochemicals study is an important component in the research and use of plant allelopathy. The use of allelochemicals to control weeds and reduce reliance on chemical herbicides has become a research focus (Duke et al. 2000; Bhadoria 2011). Many crops such as rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and sorghum (*Sorghum bicolor* L.) exhibit allelopathic effects which indicates their potential for weed control in field experiment (Olofsdotter et al. 1999; Oueslati 2003; Cheema and Khaliq 2000).

Since many allelopathic plants inhibit the surrounding weed growth through allelopathic substances in root exudates (Bhadoria, 2011), the most common method for identification of allelopathic plants is the petri dish-based filter paper bioassay. In this method, a certain amount of test solution is added to a petri dish with a filter paper, a certain number of seeds of receptor plants are transferred onto the filter paper. The petri dish is placed in a light incubator for 3–7 days, and then the germination rate, root length, plant height, dry weight and other physiological indicators of the receptor plant are measured, in order to

evaluate the biological activity of the test substance. The method is simple, inexpensive and rapid, and therefore, has been widely used by researchers (Wu et al. 2001).

In one such study of Seigler (1996), many secondary metabolites, such as phenolic acids, terpenoids, flavonoids, coumarins, and alkaloids, and their degraded products, have allelopathic activity and play an important role in the ecosystem. Among these substances, phenolic acids are considered as the most important allelochemicals (Siqueira et al. 1991). The common phenolic acids are benzoic acid derivatives and cinnamic acid derivatives (Francisco et al. 2007), and their stability in the environment is affected by abiotic factors, such as light, temperature, oxygen, and pH, as well as biotic factors, such as microorganisms and other plants, which change the concentrations of phenolic acids and cause changes in biological activity. In laboratory bioassay, biological activity can change constantly due to different original concentrations and test times, even though the abiotic factors are removed using an incubator with constant light and temperature. Therefore, it is necessary to conduct a dynamic analysis on the changes in the concentration of phenolic acids in bioassays based on the original concentration and test time. In order to obtain a correct evaluation of bioassay results, we conducted (1) the stability of 6 individual phenolic acids and their mixtures in a natural and environment conditions from 0 to 7 days, and (2) the comparison of weed control activity of the phenolic acid solutions between a normal treatment and a changed treatment.

Materials and methods

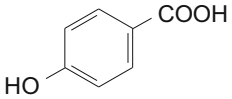
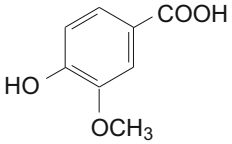
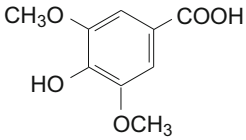
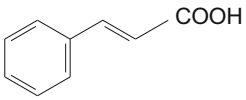
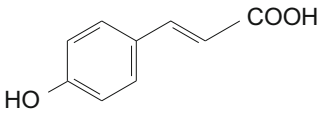
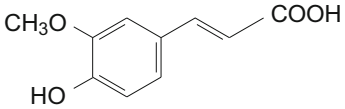
Reagents and solution preparation

The selected compounds were the phenolic acids that are widely recognized as allelochemicals, i.e., syringic, 4-hydroxybenzoic, vanillic, cinnamic, 4-hydroxycinnamic, and ferulic acids (all purchased from Sigma, USA), the chemical characteristics of six phenolic acids used are shown in Table 1. The above phenolic acids and their equimolar phenolic acid mixture were dissolved in methanol (final methanol concentration less than 0.1 %), and distilled water was used to prepare the stock solutions to a constant volume of 10^{-2} mol L^{-1}. Three concentration gradients (10^{-3} mol L^{-1}, 10^{-4} mol L^{-1}, 10^{-5} mol L^{-1}) that were required for the subsequent experiments in the bioassay were prepared using serial dilution with distilled water.

Stability of individual acid–concentration and time factors

Three concentration gradients (10^{-3}, 10^{-4}, 10^{-5} mol L^{-1}) of six phenolic acids were placed in an incubator

Table 1 Chemical characteristics of phenolic acids used

Compound	Chemical formula	pK_a
p-Hydroxybenzoic acid		4.54
Vanillic acid		4.51
Syringic acid		4.34
Cinnamic acid		4.37
p-Coumaric acid		4.00
Ferulic acid		4.58

(28 ± 2 °C, 12-h light (6:00–18:00)). A 1-ml solution was collected every 24 h and filtered through a 0.45-μm filter, pH of individual phenolic acid was studied and high-performance liquid chromatography (HPLC) was used to determine the phenolic acid concentrations based on that of Zheng et al. (2013). Quantitative analysis was carried out with an HPLC instrument Agilent 1206 HPLC (Agilent Technologies, USA),equipped with a C_{18} reversed column (ZORBAX SB-C18, 150 mm × 4.6 mm, 5 μm). Mobile phase was the mixture of methanol (A) and 1 % phosphoric acid (B),the gradient elution program were as follows: A:B = 27:73 (9 min), A:B = 30:70 (2 min), and A:B = 50:50 (4 min). The mobile phase were eluted at a flow rate of 1.6 mL min^{-1} and detected at 280 nm. The injection volume of samples was 5 μL. All experiments were repeated three times.

Stability of the mixture of phenolic acids–concentration, pH and time factors

The equimolar mixture of phenolic acids was prepared by mixing six phenolic acids with same molar concentration. Three concentration gradients (10^{-2}, 10^{-3}, 10^{-4} mol L^{-1}) were used for total of 6 phenolic acids. The mixture solutions (pH 3.35–4.75) were then adjusted to pH 5.50 and

6.50 using a 0.2 mol L^{-1} phosphate buffer solution. Three kinds of mixture solutions (at natural condition, pH 5.50, pH 6.50) were placed in an incubator (28 ± 2 °C, 12-h light (6:00–18:00)), and were sampled every 24 h to measure the concentrations as described above. All experiments were repeated three times.

Bioassay

The weed control activity of phenolic acids was determined using the filter paper method described by Chung et al. (2002). The bioassay procedure was as follows: the barnyardgrass seeds were sterilized with 5–10 % NaClO for 10 min, and were washed with distilled water. Subsequently, distilled water was added to soak the seeds and all the seeds eventually sank to the bottom. Filter paper was placed at the bottom of a tissue culture flask, five germinating barnyardgrass seeds were sown on the filter paper, and 5 ml of equimolar mixture of six phenolic acids at different concentrations (10^{-3}, 5×10^{-4}, 10^{-4}, and 5×10^{-5} mol L^{-1}) were added separately, with distilled water as a control. According to the described results above, degradation appeared after 2 days for the phenolic acid mixture. Therefore, for the phenolic acid bioassay, a normal and treatment group was set up where the solution was not changed and where the solution was changed, respectively. That is, the solution of phenolic acids in the treatment group was changed every 48 h with a fresh solution, and the distilled water in the control group was changed at the same time. The tissue culture flask was placed in an incubator (28 ± 2 °C, 12-h light (6:00–18:00)). All experiments were repeated four times, and the root length and plant height of barnyardgrass were measured after 6 days.

A mixture of phenolic acids was tested with pH 6.50 in Hoagland's solution, which was adjusted with 0.1 mol L^{-1} HCl and the final concentrations were 10^{-3}, 5×10^{-4}, 10^{-4}, and 5×10^{-5} mol L^{-1}. In this experiment, the normal and treatment group were set up to conduct the bioassay using the above method, with Hoagland's solution (pH 6.50) as a control.

Statistical analysis

The significant change of Ct/C_0 at specific time point compared to those at time 0 h was tested by one-way analysis of variance (ANOVA), followed by Dunnett' t test. Two independent-sample T test (Welch's t test) was used to determine the significant difference of the weed control activity between the normal group and treatment group in bioassay. ANOVA and T tests were carried out with SPSS 19.0 program. Statistical significance for all tests was set as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data were tested for normality, and non-normal data were log-transformed to meet assumptions of normality and homoscedasticity. However, all values presented in figures have been back-transformed.

Results

Stability of individual phenolic acids

A considerable degree of degradation was found in the syringic, 4-hydroxybenzoic, vanillic, cinnamic, 4-hydroxycinnamic, and ferulic acids during the study period (Fig. 1), where shifts in solution pH occurred slowly over time (Table 2). During the first 48 h, the concentrations of six phenolic acids were 10 % lower than the original concentration, showing good stability. At 72 h, the concentration of each phenolic acid was reduced by 30–40 % ($P < 0.01$), and then decreased further ($P < 0.001$). On day 7 (144 h), the concentrations of benzoic acid derivatives (syringic, 4-hydroxybenzoic and vanillic acids) were approximately 40–45 % of their initial concentrations (Fig. 1A, B, C), while the concentrations of cinnamic acid derivatives (cinnamic, 4-hydroxycinnamic and ferulic acids) were approximately 25–30 % of their initial concentrations (Fig. 1D, E, F).

Stability of the phenolic acid mixture

In a natural condition, the pH values of the mixture of phenolic acids range between 3.50 and 5.00, respectively (Table 2), while the pH value suitable for the growth of rice seedlings in soil ranges from 5.50 to 6.50. Therefore, we explored the stability of the mixed phenolic acid solution in a natural condition and in an environment with rice growth pH (Fig. 2). Similar to the individual phenolic substances, the phenolic acid mixture showed small changes in concentration during the first 48 h, and then exhibited a significant downward trend ($P < 0.001$). On day 7 (144 h), the concentration decreased by 70 %. When the initial concentration of the mixed phenolic acids was 10^{-4} mol L^{-1}, that is, the concentration of each phenolic acid was 2×10^{-5} mol L^{-1}, the concentration after 144 h became lower than the detection limit of the HPLC ($<2 \times 10^{-5}$ mol L^{-1}), and therefore the concentration of the mixture could not be determined (Fig. 2C). Under different pH conditions (pH 5.50, pH 6.50 and natural pH state), the concentration of phenolic acids also decreased over time. This was consistent with the trend in the concentration change in a natural state, but the percent degradation was not linearly related to the pH value or the concentration of the solution. These results indicate that the concentration of phenolic acid decreased to a certain extent for an individual phenolic acid and the mixed phenolic acid

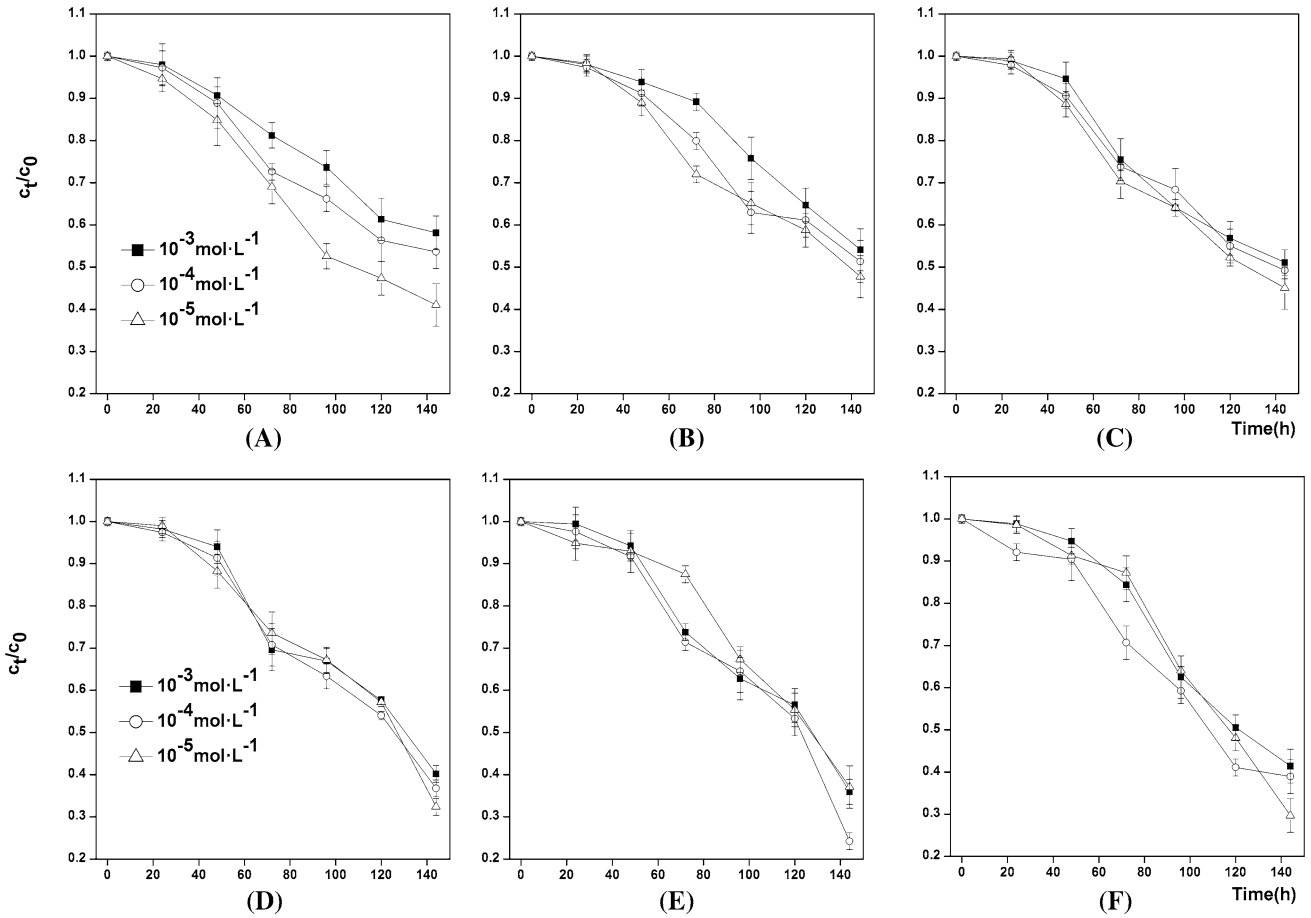


Fig. 1 Concentration of syringic (**A**), *p*-hydroxybenzoic (**B**), vanillic (**C**), ferulic (**D**), *p*-coumaric (**E**), and cinnamic acids (**F**) determined over time using HPLC, relative to the initial concentration (10^{-3}, 10^{-4}, and 10^{-5} mol L^{-1}) under natural conditions. c_t-the concentration of phenolic acids over time, c_0-the initial concentration of each phenolic acid. Mean ± SE from three replications for each determination are shown

solution during the routine experimental observations in the bioassay (4–7 days) and the rice-growing environment. We speculated that the instability of phenolic acids would affect their weed control activity. This was verified in the following experiments.

Effect of the stability of phenolic acids on weed control activity

The results presented in Fig. 2 show a significant decrease in the concentration of the mixed phenolic acids after 48 h. Therefore, we considered replacing the original phenolic acid solution with a fresh solution every 48 h and this group was the treatment group. The group where the phenolic acid solution was not changed was the normal group. The weed control activities of different concentrations of the phenolic acid mixture on root length or plant height of barnyardgrass in a natural state are shown in Fig. 3. When the concentration of mixed phenolic acids was 10^{-3} mol L^{-1}, the inhibition rates of the normal group on the root length and plant height of barnyardgrass were 37.63 and 30.17 %, respectively. In contrast, the inhibition rates of the treatment group on the root length and plant height of barnyardgrass were 77.29 and 72.78 %, respectively ($P < 0.001$, Fig. 3B). When the concentration of the mixed phenolic acids was 5×10^{-4} or 10^{-4} mol L^{-1}, there were significant differences in the relative inhibition rate on the root length and plant height of barnyardgrass between the treatment and control group ($P < 0.05$, Fig. 3A, B). This indicated that the instability of phenolic acid could significantly affect the results of biological activity measured in a bioassay. When the solution pH in the bioassay was 6.50, the differences in the inhibition rate on the root length and plant height of barnyardgrass between the treatment and normal group only appeared at a phenolic acid concentration of 10^{-3} mol L^{-1}, while there was no significant difference at a low phenolic acid concentration ($P < 0.05$, Fig. 4A, B). However, irrespective of the natural state or an environment with a pH of 6.50, the relative inhibition rate

Table 2 The pH of individual phenolic acid and an equimolar mixture of six compounds every 24 h used for the stability test

Compound	Concentration (M)	pH						
		0 h	24 h	48 h	72 h	96 h	120 h	144 h
p-Hydroxybenzoic acid	10^{-3}	3.84	3.75	3.78	3.89	3.80	3.90	3.75
	10^{-4}	4.65	4.80	4.66	4.64	4.69	4.63	4.60
	10^{-5}	6.15	6.02	6.12	6.13	6.44	6.47	6.10
Vanillic acid	10^{-3}	3.78	3.84	3.81	3.76	3.73	3.79	3.75
	10^{-4}	4.49	4.60	4.63	4.53	4.60	4.66	4.57
	10^{-5}	6.13	6.04	6.14	5.92	5.87	6.01	5.97
Syringic acid	10^{-3}	3.82	3.83	3.81	3.99	3.67	3.71	3.68
	10^{-4}	4.57	4.80	4.73	4.83	4.72	4.88	4.90
	10^{-5}	6.02	6.21	6.09	5.88	5.74	5.78	5.70
Cinnamic acid	10^{-3}	4.20	3.87	3.92	3.80	3.79	3.75	3.69
	10^{-4}	4.66	4.9	4.99	4.79	4.87	4.87	4.82
	10^{-5}	6.10	6.48	6.56	5.97	6.09	6.17	6.17
p-Coumaric acid	10^{-3}	3.94	3.88	3.86	3.89	3.85	3.82	3.92
	10^{-4}	4.64	4.66	4.68	4.73	4.74	4.68	4.79
	10^{-5}	6.05	6.00	5.97	6.09	6.00	6.08	6.28
Ferulic acid	10^{-3}	3.90	3.91	3.89	3.92	3.87	3.96	3.88
	10^{-4}	4.69	4.98	5.07	5.49	5.97	6.25	6.29
	10^{-5}	6.22	6.13	6.08	6.06	6.03	6.36	6.25
Equimolar mixture[a]	10^{-2}	3.35	3.23	3.26	3.29	3.23	3.31	3.21
	10^{-3}	3.85	3.91	3.91	3.91	3.93	4.03	3.87
	10^{-4}	4.75	4.84	5.04	5.17	5.35	5.61	5.89

[a] Equimolar mixture, the concentrations used (10^{-2}, 10^{-3} and 10^{-4} mol L^{-1}) were for the total of six phenolic acids

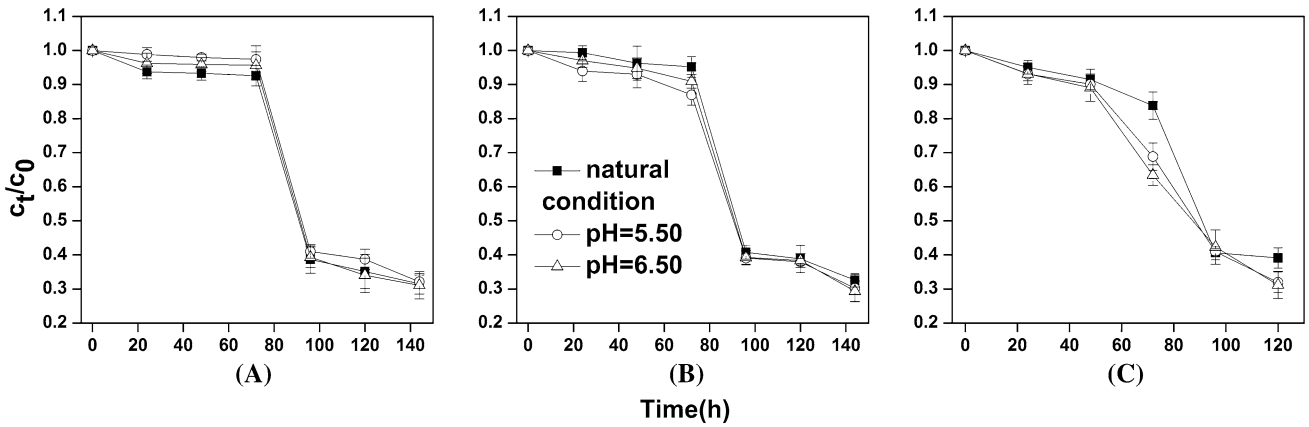


Fig. 2 Concentration of mixed phenolic acids at 10^{-2} mol L^{-1} (**A**), 10^{-3} mol L^{-1} (**B**), and 10^{-4} mol L^{-1} (**C**) determined using HPLC under natural conditions, pH 5.50 and pH 6.50, relative to the initial concentration. c_t-the concentration of mixed phenolic acids over time, c_0-the initial concentration of mixed phenolic acids. Mean ± SE from three replications for each determination are shown

of phenolic acids from the treatment group on the root length and plant height of barnyardgrass was lower than that of the normal group, suggesting that instability can affect the weed control activity of phenolic acids, especially for those at high concentrations. However, in the natural environment (pH 6.50) where allelopathic rice releases phenolic acids, the solution-change treatment had no significant impact on the weed control activity of phenolic acids. This is largely because phenolic acids exist mainly in a deprotonated form rather than a protonated form, resulting in very low inhibitory activity against barnyardgrass.

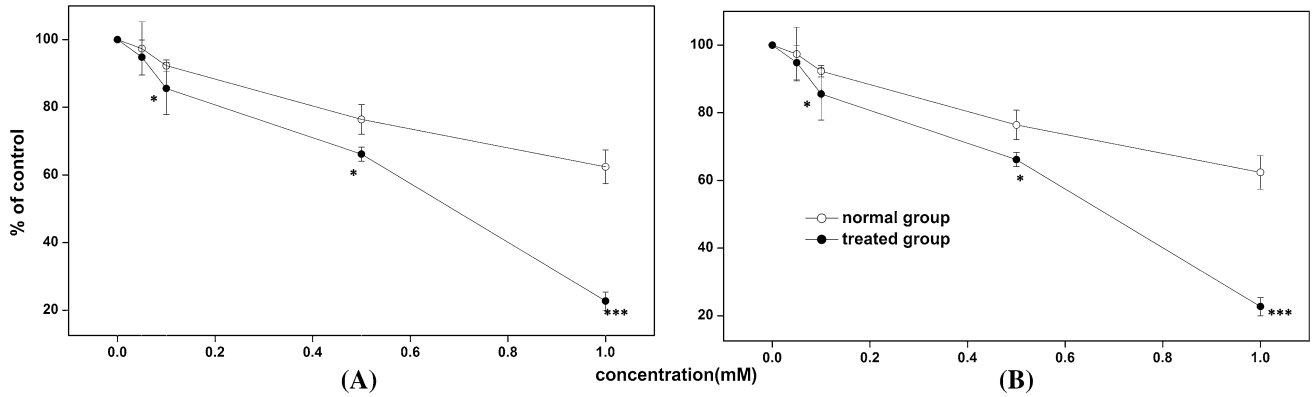


Fig. 3 The inhibitory effect of phenolic acids in the normal and treatment groups on root length (**A**) and plant height (**B**) of barnyardgrass in a natural state. Mean ± SE from four independent experiments on five plants are shown for each determination. Asterisks indicate significant differences between the normal and treatment groups: $*P < 0.05$, $***P < 0.001$ (Welch's *t* test)

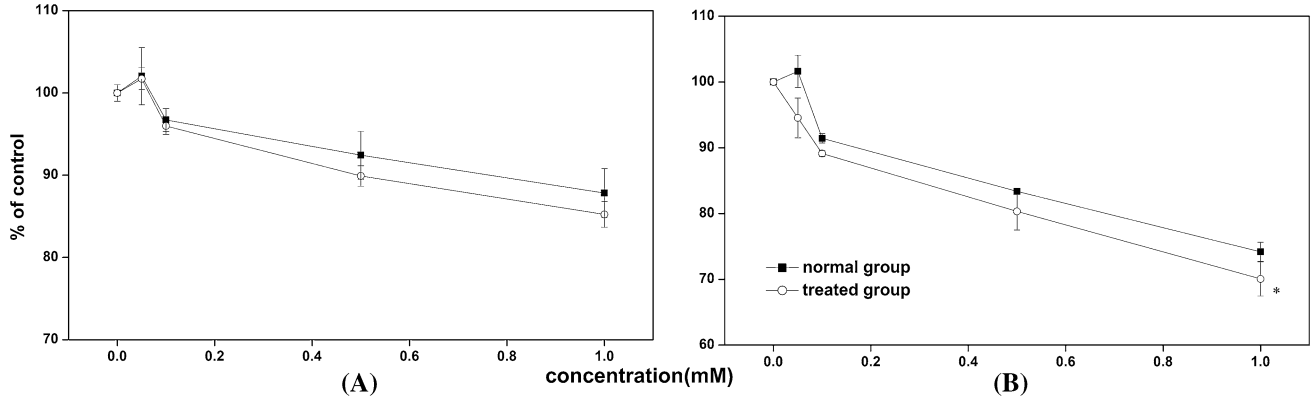


Fig. 4 The inhibitory effect of phenolic acids in the normal and treatment groups on root length (**A**) and plant height (**B**) of barnyardgrass in an environment with a pH of 6.50. Other details as for Fig. 3

Discussion

In this study, three benzoic acid derivatives (syringic, 4-hydroxybenzoic, and vanillic acids) and three cinnamic acid derivatives (cinnamic, 4-hydroxycinnamic, and ferulic acids) were selected. Among these compounds, except for cinnamic acid, the benzene ring contains a hydroxyl (–OH) and a carboxyl (–COOH) group, and the cinnamic acid derivatives also contain an unstable styryl group. Because of these characteristics, the compounds are prone to degradation under light. The results showed that both individual phenolic acids and their mixture were unstable in the time required for conventional bioassays, irrespective of concentration and pH. The concentrations of these phenolic acids were only 60–70 % of the initial concentrations after 3 days (Fig. 1). The trend of the concentration changes in the equimolar mixture of these phenolic acids over time (Fig. 2) was similar to that of individual phenolic acids, except for a sudden drop after 72 h, which may be due to the complex interaction among phenolic acids, such as antagonistic, synergistic, or additive effect.

Phenolic acids are an important substance for allelopathic rice to exert allelopathic effects, and syringic, 4-hydroxybenzoic, vanillic, and salicylic, cinnamic, 4-hydroxycinnamic, caffeic and ferulic acids can be detected in the secretions of allelopathic rice (Mattice et al. 1998; Rimando et al. 2001; Chung et al. 2002; Seal et al. 2004a). Chung et al. (2002) found that the inhibition rate of a mixture of phenolic acids was higher than that of individual phenolic acids. In research on allelopathic rice, the rice is typically grown in a hydroponic solution or soil. The results presented in Fig. 2 show that compared with the natural pH, the decrease in concentration of the phenolic acids did not show significant differences in the buffer solutions with different pH values. This suggests that the concentration of the phenolic substances that are secreted from rice roots will be reduced over time. In addition, abiotic and biotic soil factors affect the phenolic acid concentration in soil, so the phenolic acid concentration in

soil measured using HPLC was considerably lower than the effective concentration in the bioassay.

The experimental time for measuring activity in laboratory bioassay is at least 5 days, which is needed to obtain data that shows differences in the measurement of receptor plant-related indicators, such as the germination rate, root length, plant height, and dry weight. For example, Chung et al. (2002) and Sea et al. (2004b) measured the indoor allelopathic activity of common phenolic allelochemicals using a measurement period of seven days. The results shown in Figs. 1 and 2 indicate that the concentration of phenolic acids had already decreased on day 3, and the concentration on day 7 was only 30–40 % of the initial concentration. This indicates that in the late stage bioassay, the effective concentration of phenolic acids to inhibit the growth of receptor plants was much lower than the actual concentration due to the instability of the phenolic acids. Therefore, we changed the bioassay solution every 48 h to determine whether there was any difference in the two bioassay methods. The results showed that changing the solution did not affect the control group (distilled water was used), and there was no difference in the root length or plant height of the receptor plants. For the group with the phenolic acid mixture, however, the root length and plant height of the receptor plants where the solution was changed (i.e., the treatment group) were significantly lower than those where the solution was not changed (the normal group). However, in an environment with a pH of 6.50, the bioassay method, in which the solution was changed every 48 h, did not have a significant impact on the group with the low concentration of phenolic acids. Although the phenolic substances in the rhizosphere of allelopathic rice are unstable and the concentration can be reduced, the weed control activity of phenolic acids is not reduced because the concentration of phenolic acids in paddy soils is below 10^{-6} mol g^{-1} soil (Blum et al. 1991). Therefore, we believe that if an indoor bioassay is used to screen for the allelopathic activity of phenolic acids, the impact of their instability on the inhibition rate should be considered, which could be reduced by replacing a fresh solution of phenolic acids every 48 h. However, if soil is used as the medium for screening, the impact of the instability of phenolic acids can be ignored.

In acid aqueous solution, two forms of an acid molecule exist: protonated (HA) and deprotonated (A-), which serve as a buffer solution themselves. The concentrations of HA and A- are closely related to pH; for every order of magnitude that pH increases, the HA content decreases tenfold. Therefore, different concentrations of individual phenolic acids and different pH values will change bioassay results (Chung et al. 2002). The pH values of the mixture of multiple phenolic acids generally ranged between 4.00 and 6.00, while the soil pH suitable for the growth of rice seedlings was between 5.50 and 6.50. In a soil environment, the soil solution is a considerable repository of phenolic acids, and can absorb excess phenolic acids or release some to replenish consumed phenolic acids as needed (Amy et al. 2004; Blum 2004). The various buffer systems in soil maintain the relative stability of soil pH, and therefore maintain the relative stability of the phenolic acid content. Although the degradation of phenolic acids in routine indoor bioassays also occurs in soil (Smith and Ley 1999), the phenolic acids can be effectively replenished by the repository of phenolic acids in soil. The bioassay result at pH 6.50 was used because this pH was closer to soil pH, and therefore, the results were more in line with the actual field conditions. The weed control activities of the phenolic acids released by rice in a natural environment should be a result of the long-term effect of phenolic acids with low concentrations at neutral pH. Therefore, we believe that the indoor bioassay results obtained from a short-term (3–7 days) period in an environment with pH conditions generated by a mixture of phenolic acids cannot accurately reflect the actual situation of the weed control effect of phenolic acids in a rice field.

In conclusion, the instability of the phenolic substances with allelopathic effects was due to their structural features. This instability can significantly reduce the inhibition rate on the growth index for receptor plants in an indoor bioassay, and this impact can be reduced by replacing the solution with fresh solution during the bioassay. In subsequent studies, the effects of factors such as the time to change the solution and the receptor plant species will need to be explored in order to find a more suitable filter paper bioassay.

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