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Effects of Rice Blast Fungus (*Pyricularia grisea*) on Phenolics, Flavonoids, Antioxidant Capacity in Rice (*Oryza sativa* L.)

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Abtract. Rice blast fungus (*Pyricularia grisea*) is one of the most problematic pathogen to significantly reduce rice production worldwide. In this study, after being inoculated with *P. grisea*, changes in phenolic components and antioxidant capacity and correlation with the resistant level against rice blast fungus were investigated. Among screened rice cultivars, AV-3 was the strongest resistant, whereas BII-3 was the most susceptible. It was found that although total contents of phenolics and flavonoids, and antioxidant capacities varied among studied varieties, no significant coefficient with the resistance against *P. grisea* was observed. After rice was affected by rice blast fungus, total phenolics and flavonoids were markedly reduced, but in contrast, the DPPH scavenging activities of only the susceptible rice cultivars was reduced. Among the 11 phenolic acids detected, catechol was found only in the tolerant cultivar AV-3, whereas the amount of cinnamic acid was increased after infection. Quantity of vanillin was also promoted, except in the susceptible cultivar BII-3 that was significantly reduced. Findings of this study showed that the resistant level against *P. grisea* was proportionally correlated to the antioxidant capacity. Catechol, cinnamic acid, and vanillin may play a role but it needs further elaboration. Observations of this study suggested that the infection of blast disease by reducing amount of phenolics and flavonoids that may weaken the resistance of rice against this detrimental fungus.

Introduction

Rice is an important staple food for more than half of the world's population, especially in developing countries such as Asia [1]. The world population is rapidly growing to require an increase in demand for rice [2]. However, majority from the loss of rice yield is the occurrence of pests and diseases, particularly blast disease that caused by a fungus (*Pyricularia grisea*), is recognized as the most explosive and harmful to the rice crop. *P. grisea* has been reported to affect rice production in over 85 countries worldwide [3]. This blast fungus can affect almost growth stages of rice, as it affects leaves, leaf collars, necks, panicles and seeds. This problem happens annually to cause rice productivity reduction up to 85% [4]. As a result, the decrease in rice yield due to blast fungus, especially in Southeast Asia, to eliminate rice that can feed approximately 60 million people per year [5].

Phenolic compounds are in one of the most important groups of secondary metabolites, which are produced when plants are in biotic or abiotic stresses. Phenolic compounds play a role as defense molecules to protect plants from various adverse conditions or agents, especially fungus and other pathogens [6]. In addition, many studies reported that stress conditions affect to the accumulation of reactive oxygen species (ROS), which harm plant's cell [7-13]. ROS accumulation can be prevented by antioxidant activity of plants [13,14]. However, the biosynthesis of antioxidant compounds can be obstructed by blast fungus due to penetration of it into rice cell and production of its toxic compounds [15]. Hyogo et al. 2010 [16] reported that antioxidant activity can be determined by the occurrence of phenolic compounds, for example the increase of antioxidant enzymes and induction of the synthesis of antioxidant proteins are related to the existence of phenolics.

To data, major studies on blast fungus have conducted to find out measures to reduce the destruction of this fungus against crops. However, the correlation of important secondary metabolites of rice in response to the blast disease has not been well understood. In this study, changes in phenolics, flavonoids, and antioxidant activities of rice inoculated with *P. grisea* were investigated. The identification and quantification of individual phenolic acids relevant to infection of the blast disease was also conducted.

Materials and Methods

Plant materials

Four rice varieties [OM8150, BII-3, AV-3, AI-1] were obtained from Cuu Long Delta Rice Research Institute, Vietnam. All experiments were conducted from March to October 2015 in Hiroshima University.

Isolation and preparation of P. grisea

The spore of *P. grisea* was isolated from infected rice leaves from a rice cultivar Co39. The spore was inoculated and transferred to petri dishes (9 cm in diameter) containing Potato Dextrose Agar (PDA) (200 g potato, 20 g glucose, 17 g agar). The petri dishes were placed at room temperature of 25 °C for 12 days. After that, the fungus were collected by scraping the surface of PDA and put under the light for 3-4 days for sporulation. Finally, the concentration of 10^5 conidia per milliliter was prepared for infecting rice leaves.

Identification of resistant level to blast fungus

A total of 10 seeds of each rice variety were sown in a row in a density of 2x2 cm with 3 replications. After 7 days of infection, the resistance to the blast fungus were recorded according to infective levels (0-2: resistance; 3-5: susceptibility) following the Standard Evaluation System for rice (SES) of International Rice Research Institute (IRRI).

Extraction of samples

Rice leaves were collected after 7 days of infection for chemical analysis. An amount of 0.5 g of dried powdered rice leaves was extracted in 10 mL solution (8 mL methanol: 1.9 mL water: 0.1 mL of 1M HCl). The samples were stirred for 2 hours and the mixtures were centrifuged at 5000 rpm for 10 min followed by filtration and repeated. The supernatant was collected, evaporated to dryness and weight, dissolved in methanol and kept in the dark at 4 °C for further analysis.

Phenolic contents

The phenolic contents were measured using the Folin-Ciocalteu method described by Ti et al 2014 [17]. The amount of 62.5 µL of each sample (0.5 mg/L) was mixed with 62.5 µL of Folin-Ciocalteu's reagent (10%) and after 6 min, an aliquot of 0.625 mL Na_2CO_3 and 0.5 mL distilled was added. The solutions were mixed and allowed to stand for 90 min. The absorbance was measured at 765 nm using a HACH DR/4000U spectrophotometer. The total phenolic content was reported as mg gallic acid equivalents (GAE) per gram dry weight (DW).

Flavonoids content

The amount of total flavonoids was determined according to a method described by Djeridane et al. 2006 [18]. One mL of extract (0.5 mg/mL) was mixed with 1 mL aluminium chloride 2%. The mixture was stirred and kept at room temperature for 15 min. The absorbance was measured at 430 nm using a HACH DR/4000U spectrophotometer. Total flavonoids were expressed as mg rutin equivalents (RE) per gram dry weight (DW).

Antioxidant activity by DPPH scavenging assay

The DPPH free radical scavenging assay described by Elzaawely et al. 2005 [19] was used to determine the antioxidant capacity of the extracts. The mixture consisted of 0.5 mL sample extracts, 0.25 mL of 0.5 mM DPPH, and 0.5 mL of 0.1 M acetate buffer (pH 5.5). The mixture was kept in the dark at room temperature for 30 min. BHT (benzo-thiadiazole-7-carbothioic acid S-methyl ester) was used as a positive reference, while methanol was used as a control. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the formula,

$$\% \text{ radical scavenging activity} = [(A_{control} - A_{test})/A_{control}] \times 100$$

where $A_{control}$ corresponds to the absorbance of the control and A_{test} corresponds to the absorbance of the test extract. The IC_{50} value was also calculated using % radical scavenging activity. Lower IC_{50} values indicate higher antioxidant activity.

Estimation of antioxidant activity by reducing power method

The reducing power of different extracts was determined following a method described previously by Yildirim et al. 2003 [20] with some modifications. Two hundred µL of each extract and 200 µL BHT at concentrations of 0.1, 0.5, 1.0 and 2 mg/mL in methanol was mixed with 0.5mL phosphate buffer (0.2 M, pH 6.6) and 0.5mL potassium ferricyanide [$K_3Fe(CN)_6$] (10 g/L). The mixture was incubated at 50 °C for 30 min. Then an aliquot of 0.5 mL trichloroacetic acid (100 g/L) was added to the mixture, which was subsequently centrifuged at 4000 rpm for 10 min. Finally, 0.5 mL of the supernatant solution was mixed with 0.5 mL distilled water and 0.5mL $FeCl_3$ (1 g/L) and the absorbance was measured at 700 nm. By this method, the increased absorbance of the reaction mixture indicated the strength of reducing power. The IC_{50} values were calculated following a method described previously [21]. Lower IC_{50} value indicates higher reducing power.

Quantification by HPLC

The HPLC (High Performance Liquid Chromatography) was used to identify and quantify phenolic acids as described by Xuan et al. 2003 [22]. The extracts were filtered separately using 0.45 µm filter (KANTO chemical, Tokyo Japan) then injected into the HPLC [JASCO PU-2089 Plus, column: J-Pak Symphonia C18 110A (4.6mmØx15mm), solvent system: (solution A) 0.1% of acetic acid, (Solution B) 100% methanol, gradient program: 5-10 min, 5-20% (A); 10-30 min, 20-80% (A); 30-40 min, 80-100% (A), wavelength: 254 nm and flow rate: 1.0 mL/min]. Concentrations of phenolic compounds in the samples were calculated by comparing peak areas of samples with those of the standards.

Statistical analysis

Data were analysed using one way ANOVA (analysis of variance) with the significant difference determined at a confidence level of $P < 0.05$.

Results and Discussion

Effect of blast fungus on rice varieties

The influence of blast fungus on the studied rice varieties was recorded and presented in Table 1. The variety BII -3 had 10 to 12 lesions per leaf with the biggest size of disease spots (0.2 – 1.5 mm in width and 1.0 – 3.0 mm in length) as compared with other cultivars. The varieties OM8105 and AI-1 had relatively lower number of disease lesions than the cultivar BII-3. In cultivar OM8105, there were 3 – 4 small disease lesions with 1 mm smaller in width and length. It is found that there was no lesion exposed on leaves of variety AV-3 (Table 1). It is concluded that the most susceptible cultivar was BII-3 and the most resistant cultivar was AV-3.

Table 1. Blast resistant levels of rice cultivars

Rice variety	OM8105	BII-3	AV-3	AI- 1
Levels	1	3	0	2
Phenotype	3 – 4 small lesions/leaf	10 – 12 large lesions/leaf	No lesion	7 – 8 medium lesions/leaf
	(< 1mm)	(1.5 – 3 mm)		(0.5 – 1 mm)

Influence of blast infection on total phenolic and flavonoid contents

Data in Table 2 indicated that in the controls (non-infection of the blast fungus), the total phenolics in the resistant rice cultivars (AV-3 and AI-1) was significantly lower than in the susceptible rice (OM8105 and BII-3). However, after being infected, the total phenolics was proportional decreased in all rice cultivars. But the total phenolic contents in the susceptible rice OM8105 was markedly higher than the other rice cultivars. In total flavonoids, there was no significant difference among non-infected rice, but in the treatments, the total flavonoids of AV-3 was the most reduced. It is proposed that total phenolic and flavonoid contents had no correlation with the resistant strength of rice against the blast fungus.

Table 2. Influence of blast infection on total phenolic and flavonoid contents of rice varieties

Rice sample	Total phenolics (mg GAE/g DW)	Total flavonoids (mg RE/g DW)
Controls (non-infection)		
OM8105	15.78 ± 1.19 a	9.16 ± 0.15 abc
BII-3	15.82 ± 0.49 a	9.90 ± 0.13 a
AV-3	13.27 ± 0.75 bc	9.20 ± 0.16 abc
AI-1	13.58 ± 0.68 bc	9.07 ± 0.10 bc
Treatments (infected)		
OM8105	14.50 ± 0.31 ab	9.50 ± 0.34 ab
BII-3	11.90 ± 1.01 cd	9.09 ± 0.27 bc
AV-3	10.77 ± 0.06 d	8.25 ± 0.55 d
AI-1	12.30 ± 0.66 cd	8.52 ± 0.16 cd

Values are means of three replications ± SD (standard deviation).
Means with the same letter in each column are significantly different ($P < 0.05$).

The DPPH radical scavenging activity and reducing power activity of difference rice varieties after infection of the blast fungus were showed in Table 3. It is found that the reducing power capacity was not different among studied rice, but the susceptible cultivars showed higher DPPH scavenging capacity than the resistant varieties. However, after being infected, no significant difference in resistant rice (AV-3 and AI-1) was observed, whereas that of the susceptible rice OM8105 and BII-3 was found. In case of the reducing power capacity, no marked difference compared with the controls were revealed. It was suggested that the DPPH scavenging activity may have a positive correlation to the resistance of rice against the infection of blast fungus.

Table 3. DPPH radical scavenging activity and reducing power activity of rice

Rice samples	DPPH IC_{50} (mg/mL)	Reducing power IC_{50} (mg/mL)
Controls (non-infection)		
OM8105	0.370 ± 0.020 c	1.867 ± 0.034 b
BII-3	0.364 ± 0.011 c	1.804 ± 0.079 b
AV-3	0.547 ± 0.019 a	2.291 ± 0.151 ab
AI-1	0.544 ± 0.017 ab	2.274 ± 0.157 ab

Treatments (infected)		
OM8105	0.496 ± 0.004 b	2.168 ± 0.047 ab
BII-3	0.532 ± 0.023 ab	2.207 ± 0.138 ab
AV-3	0.576 ± 0.020 a	2.475 ± 0.156 a
AI-1	0.550 ± 0.016 a	2.434 ± 0.168 a

Means with same letters in each column are not significantly different ($P < 0.05$).
Values are means of three replications ± SD (standard deviation)

There were total 11 phenolic acids were detected by HPLC (Table 4). There were 5 compounds including gallic acid, protocatechuic acid, vanillin, benzoic acid, and cinnamic acid. Benzoic acid in the cultivar BII-3 was an exception, it was strongly increased in the cultivar OM8105 but in contrary, its amount was decreased in the cultivar BII-3. Quantities of cinnamic acid were increased after infection. Similarly, the amount of vanillin was promoted, except in the susceptible cultivar BII-3 it was significantly reduced (Table 4). Ferulic acid, syringic acid, and vanillic acid did not show any involvement. However, catechol was found only in the most tolerant variety AV-3.

Conclusions

By this research, it was found that the cultivar BII-3 was the most susceptible, whereas the variety AV-3 was the most tolerant against the blast disease. Total phenolics and flavonoids and the reducing power capacity of rice did not show any role to *P. grisea*, but the DPPH scavenging capacity may play a role in the resistance of rice against this harmful fungus. Among 11 detected phenolic acid, catechol, cinnamic acid, and vanillin may play a role, but it needs further elaboration.

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Table 4. Phenolic compositions and concentrations (μg/g DW) in rice leaves

Rice cultivars				
	OM8105		BII-3	
Compounds	Controls	Infected	Controls	Infected
Gallic acid	0.8±0.006a	0.7±0.012ab	0.7±0.021ab	0.6±0.015c
Procatechuic acid	6.8±0.009b	6.8±0.003b	6.8±0.016a	6.8±0.0014ab
Catechol	nd	nd	nd	nd
p-Hydroxybenzoic acid	nd	nd	3.6±0.002a	nd
Vanillic acid	nd	0.2±0.001b	1.1±0.002a	nd
Syringic acid	nd	3.9±0.005d	3.4±0.013e	nd
Vanillin	2.3±0.007h	3.4±0.002f	7.0±0.014a	5.5±0.008d
Ferulic acid	14.0±0.021a	nd	9.3±0.015b	nd
p-Coumaric acid	14.5±0.025e	14.4±0.005a	nd	13.3±0.018b
Benzoic acid	0.5±0.002g	13.3±0.014d	17.1±0.002a	14.5±0.008c
Cinnamic acid	0.3±0.001b	0.4±0.002a	0.1±0.010d	0.9±0.0016bc

	AV-3		AV-1	
Compounds	Control	Infected	Controls	Infected
Gallic acid	0.3±0.001d	0.3±0.022d	0.3±0.007d	0.2±0.004e
Procatechuic acid	6.8±0.015b	6.8±0.014ab	6.8±0.005bc	6.8±0.007c
Catechol	0.2±0.024b	0.3±0.020a	nd	nd
p-Hydroxybenzoic acid	nd	3.5±0.016b	nd	nd
Vanillic acid	0.001±0.001d	nd	0.2±0.002c	0.1±0.001c
Syringic acid	4.5±0.032b	4.7±0.028a	4.0±0.042c	3.9±0.013d
Vanillin	6.5±0.002c	6.9±0.021b	3.1±0.001g	3.6±0.016e
Ferulic acid	nd	8.228±0.031c	nd	nd
p-Coumaric acid	12.7±0.035d	13.1±0.026c	11.7±0.030d	11.7±0.006d
Benzoic acid	13.3±0.001d	13.2±0.002e	14.7±0.001b	11.8±0.001f
Cinnamic acid	0.1±0.002e	0.2±0.001c	0.3±0.011b	0.3±0.023c

Values are means of three replications ± SD (standard deviation).
Values with similar letters in each column are not significantly different ($P < 0.05$).
nd: not detected

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