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

Nguyen Phu Toan¹,a, Pham Thi Thu Ha¹,b, Tran Dang Xuan¹,c*

¹Graduate School for International Development and Cooperation (IDEC), Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8529, Japan

a nguyenphutoan1983@gmail.com, b phamthithuhabt@gmail.com, c tdxuan@hiroshima-u.ac.jp

**Keywords:** total phenolics, total flavonoids, rice, blast disease, phenolic acids, resistant levels

**Abstract.** 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

---

This paper is an open access paper published under the terms and conditions of the Creative Commons Attribution license (CC BY) ([https://creativecommons.org/licenses/by/4.0](https://creativecommons.org/licenses/by/4.0))
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 10³ 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 Na₂CO₃ and 0.5 mL distilled water 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,

\[
\% \text{ radical scavenging activity} = \left( \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \right) \times 100
\]

where \(A_{\text{control}}\) corresponds to the absorbance of the control and \(A_{\text{test}}\) 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 \(\mu\)L of each extract and 200 \(\mu\)L BHT at concentrations of 0.1, 0.5, 1.0 and 2 \(mg/mL\) in methanol was mixed with 0.5\(mL\) phosphate buffer (0.2 M, pH 6.6) and 0.5\(mL\) 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 \(\mu\)m 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 |
| (< 1 mm)     | (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 |
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.

Acknowledgements
The authors thank to Taoyaka program, Hiroshima University for financial support. Thanks are also due to La Tuan Nghia, Nobukazu Nakagoshi, Do Tan Khang, Phung Thi Tuyen, La Hoang Anh, Do Tan Khang, Truong Ngoc Minh, Nguyen Van Quan, and other lab members for their assistance to this research.

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

| Compounds               | OM8105 Controls | OM8105 Infected | BII-3 Controls | BII-3 Infected |
|-------------------------|-----------------|-----------------|----------------|----------------|
| Gallic acid             | 0.8±0.006a      | 0.7±0.012ab     | 0.7±0.021ab    | 0.6±0.015c     |
| Protocatechuic 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   |
| Compounds                | AV-3 Control     | AV-3 Infected    | AV-1 Controls | AV-1 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

References

[1] L. Banos, Standard evaluation system for rice (SES), International Rice Research Institute, Philippines, 2002. (retrieved: June 25th, 2015).
[2] P.B. Tinker et al., Report of the fifth external programme and management review of International Rice Research Institute (IRRI), Brasilia: Food and Agriculture Organization of the United Nation, 1998.
[3] W.A.D. Jayawardana et al., Evaluation of DNA markers linked to blast resistant genes, pikh, pit(p), and pita, for parental selection in Sri Lankan rice breeding, Trop. Agric. Res. 26 (2014) 82-93.
[4] X. Wang et al., Current advances on genetic resistance to rice blast disease, Agric. Biol. Sci. (2014) 195-208.
[5] N.J. Talbot, Fungal genomics goes industrial, Nat. Biotech. 25 (2007) 542-543.
[6] B. Patra et al., Transcriptional regulation of secondary metabolite biosynthesis in plants, Bochim. Biophys. Acta. 1829(11) (2013) 1236-1247.
[7] R. Mittler, Oxidative stress, antioxidant and stress tolerance, Trends Plant Sci. 7 (2002) 405-410.
[8] K. Apel, H. Hirt, Reactive oxygen species: metabolism, oxidative stress, and signal transduction, Annu. Rev. Plant Biol. 55 (2004) 373-399.
[9] S. Mahajan, N. Tuteja, Cold, salinity and drought stresses: an overview, Arch. Biochem. Biophys. 444 (2005) 139-158.
[10] N. Tuteja, Chapter Twenty-Four - Mechanisms of high salinity tolerance in plants, Methods in Enzymology. 428 (2007) 419-438.
[11] N. Tuteja, Cold, salt and drought stress, in: H. Hirt (Ed.), Plant Stress Biology: From Genomics towards System Biology, Wiley-Blackwell, Weinheim, Germany, 2010, pp. 137-159.
[12] N.A. Khan, S. Singh, Abiotic stress and plant responses, I K Pub, New Delhi, 2008.
[13] S.S. Gill et al., Amelioration of cadmium stress in crop plants by nutrients management: Morphological, physiological and biochemical aspects, Plant Stress. 5(1) (2011) 1-23.

[14] R. Mittler et al., Reactive oxygen gene network of plants, Trends Plant Sci. 9 (2004) 490-498.

[15] M. Walter, E. Marchesan, Phenolic compounds and antioxidant activity of rice, Braz. Arch. Boil. Technol. 54 (2011) 371-377.

[16] A. Hyogo et al., Antioxidant effects of protocatechuic acid, ferulic acid, and caffeic acid in human neutrophils using a fluorescent substance, Int. J. Morphol. 28 (2010) 911-920.

[17] H. Ti et al., Free and bound phenolic profiles and antioxidant activity of milled fractions of different indica rice varieties cultivated in southern China, Food Chem. 159 (2014) 166–174.

[18] A. Djeridane et al., Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds, Food Chem. 97(4) (2006) 654–660.

[19] A.A. Elzaawely, T.D. Xuan, S. Tawata, Antioxidant and antibacterial activities of Rumex japonicus HOUTT. Aerial parts, Biol. Pharm. Bull. 28(12) (2005) 2225–2230.

[20] A. Yildirim, A. Mavi, A.A. Kara, Antioxidant and antimicrobial activities of Polygonum cognatum Meissn extracts, J. Sci. Food Agric. 83(1) (2003) 64-69.

[21] Z. Zhang et al., Antioxidant phenolic compounds from walnut kernels (Juglans regia L), Food Chem. 113 (2009) 160-165.

[22] T.D. Xuan et al., Correlation between growth inhibitory exhibition and suspected allelochemicals (phenolic compounds) in the extract of alfalfa (Medicago sativa L.), Plant Prod. Sci. 6(3) (2003) 165–171.