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# Involvement of Phenolics, Flavonoids, and Phenolic Acids in High Yield Characteristics of Rice (*Oryza sativa* L.)

Tran Dang Xuan[1,a*] Do Tan Bach[2,b] Tran Dang Dat[3,c]

[1]Graduate School for International Development and Cooperation, Hiroshima University, Japan

[2]Institute of Life Science - Thai Nguyen University, Vietnam.

[3]Khai Xuan International Co., Ltd, Hanoi, Vietnam

[a]tdxuan@hiroshima-u.ac.jp, [b]tuanbachno.2704@gmail.com, [c]khaixuan.study@gmail.com

*Corresponding author: tdxuan@hiroshima-u.ac.jp; Tel/Fax: +81-82-424-424-6927

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**Abstract.** The present study examined the correlation between phenolic acids and flavonoids with high and low yield traits of rice. It was observed that the difference of phenolic contents among the tested rice lines occurred only at the vegetative stage. The concentrations of phenolic acids were higher in the high yield cultivars than low yield varieties at the vegetative stage, but they either decreased dramatically or disappeared during the development stage. Caffeic acid was found only in high yield, whereas chlorogenic acid was detected only in low yield rice. Sinapic acid was the dominant phenolic acid in high yield cultivars at vegetative stage (3.7 mg/g), followed by ferulic acid (1.2 mg/g). These findings suggested that caffeic acid, ferulic acid, sinapic acid and chlorogenic acid may play a particular role in forming yield components in rice. The cultivar B3 contained high amount of sinapic acid may be used as a natural source for pharmaceutical use.

## Introduction

Rice is the staple food for billions of people in many countries. According to the United Nations Food Agency, among 147.5 million ha of land used for growing rice around the world, 90 % of that belongs to Asia and produces 92 % of the total world's rice. The rapid growth of population in developing countries demand increasing food production. There will be about 8 billion people by the year 2020, of which 4.8 billion will need to be fed with 760 million tons of rice [1]. Meanwhile climate change and the shifting to manufacture in agricultural countries caused reduction of area of cropland cultivation, resulted in lack of food crop production. Currently, in developing country, there are 300 million children under five years old dies of hunger and malnutrition [2]. Breeding to develop high yield rice varieties is necessary to overcome the food shortage.

To increase rice yield, scientists in IRRI (International Rice Research Institute) proposed rice should have short plant height, higher grains per panicle and lower growth duration. Their objective was to improve 15 to 20% rice yield and achieve potential yield of rice from 10 to 12 tons/ha [3]. It has been known that secondary metabolites play important roles in disease resistance, antioxidant, antibiotic in germination stage or production of rice [4-6]. Phenolic and flavonoid compounds are the most widely distributed secondary metabolites in plants. They play as growth inhibitors against weeds, pests as increased the resistance of plants against pathogenic stresses [1, 7]. Phenolics are also important in growth and reproduction of plants and provide protection against predators [8]. Besides, many phenolics are reported to correlate with food quality such as appearance, flavor and health-promoting properties [9]. However, it has not been well understood about the involvement of phenolics and flavonoids to high rice yield traits of rice.

The plant breeding techniques have assisted easy for cultivation, harvest and processing [10]. The breeding new rice cultivars with high yield from mutant lines is promising [11]. In the early 1990s, the initial effort was made by crossing tropical Japonica varieties from Indonesia with semi-

dwarf japonica from China. The resulted plant lines were sunk sized, lodging resistant and no unproductive tillers. But grain yield of these lines was even lower than elite varieties, due to very poor grain filling. These lines also prone to various diseases and insects [12]. However, several lines of those had been used as the parent in hybrid breeding, contribute to breeding programmers in the tropic countries [13].

There are many secondary metabolites have been identified in rice. They are classified to alkaloids, terpenoids, flavonoids, steroids, tannins, and phenolic compounds [14]. These compounds might be employed successfully against pathogens, weed reduction and enhancement of yield in crops [15]. The investigation of these compounds on the defense of plants against pathogens has been extensively carried out [14-16], but the examination on the involvement of secondary metabolites on rice yield has not been known. Hence, this study was conducted to investigate the correlation between high yield characteristics of rice with total phenols and flavonoids, and contents of individual phenolic acids.

## Materials and Methods

*Materials and Experimental Design*

There were four rice cultivars were grown and examined (Table 1). Of which, B1 and B2 cultivars were received from the Agricultural Genetics Institute (AGI, Hanoi, Vietnam), and B3 and B4 were received from Khai Xuan International Co., Ltd (Hanoi, Vietnam). Of which, DT84DB was a cultivar bred from the mutated DT84 by a method as described in Tu Anh et al. [11]. The experiment was conducted following a randomized complete block design (RCBD) with three replications on a paddy field at Hiroshima University, Japan, in 2015. The area of each plots was 60 $m^2$, with a plant spacing of 15 × 20 cm with one seedling per hill. The rice seeds were soaked in 0.1% NaOCl for 30 min and then thoroughly washed in distilled water, repeated twice within 48 h. They were then placed on petri plates between two layers of moist filter paper, and then placed in a plant growth chamber for germination set at 30 °C for 2 days. The germinated seedlings were transferred on to trays with a uniform layer of moist soil on May 10$^{th}$, transplanted in May 26$^{th}$ and harvested in October 2015.

**Table 1.** List of rice materials.

| Cultivar codes | Cultivar names | Rice origin |
|---|---|---|
| B1 | Bao thai | Indica |
| B2 | DT84 | Indica |
| B3 | DT84DB x Bao thai | Indica |
| B4 | DT84DB | Indica |

DT84DB: a cultivar bred from mutated DT84

*Rice Yield Calculation*

At the mature stage, there were 30 rice plants selected at random (except border plants) to measure the plant height and number of panicles per hill. The number of plants per $m^2$ was fixed to 33, and then, ten panicles were selected to measure the lengths of panicle, the numbers of filled grains, and percentages of unfilled grains. Growth durations were counted from the day of seedling to mature stage (when 80% panicles have 80% ripened spikelet). After harvesting, rice grains were sun dried for 5 d, then placed into a convection oven to adjust moisture approximately to 14%.

Rice yield was calculated by the following formula:

Yields (t/ha) = number of panicles/$m^2$ × number of filled grains × weight of 1000 grains

**Extraction procedure**

Two rice varieties (the highest and lowest yield) were selected from the mentioned above four cultivars, and their leaves were used for chemical analysis. The leaf samples of each rice lines that were collected in 1, 2, and 3 months after transplanting, corresponded with vegetative, reproductive and ripening phases of rice. They were dried in a convection oven in 2 weeks, then grinded in a blender, and kept in -4 $^{o}$C for further experiment. An amount 1 g of the leaves was extracted with 100 mL of 50% ethanol stirred for 24 h. The extracts were then filtered through filter papers. The supernatant was evaporated using a rotary evaporator set at 30 $^{o}$C. The weight of the precipitate was recorded and dissolved in methanol and preserved in the dark at 4 $^{o}$C for further analysis.

**Phenolic standards and reagents**

There were fifteen phenolic standards included benzoic acid, caffeic acid, catechol, cinnamic acid, chlorogenic acid, ellagic acid, ferulic acid, gallic acid, protocatechuic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, sinapic acid, syringic acid, vanillic acid, rutin, and vanillin were used. All of them were at analytical grade and purchased from Wako company, Japan.

**Determination of total phenolic content (TPC)**

In total, phenolic content of fresh and boiled samples were estimated using Folin–Ciocalteu reagent following a method described in Singh et al. [17] with some modifications. An aliquot of 0.2 ml sample extract was mixed into test tubes with 1 ml of 10% Folin–Ciocalteu's reagent and stand for 3 min of reaction, then 0.8 ml of $Na_2CO_3$ at 7.5% was added into mixture. The solution was shaken for 30 secs at 2000 rpm, and incubated for 30 min at room temperature. Measurement of absorbance was performed using a spectrophotometer (HACH DR/4000U-Japan) and measured at 765 nm. Total phenolic content was calculated as mg of gallic acid equivalent (GAE) per g dry weight (DW).

**Determination of total flavonoid content (TFC)**

Total flavonoid content was measured following a method described in Djeridane et al. [18] with minor modifications. An aliquot of 0.5 mL of the diluted sample (0.1 mg/mL) was mixed with 0.5 ml of 2% aluminum chloride-methanol ($AlCl_3.6H_2O$). The solution was then shaken for 30 s at 2000 rpm and incubated at room temperature for 15 min. The absorbance was measured by the mentioned above spectrophotometer and measure at 430 nm, whereas methanol was used as a blank sample.

**Identification and quantification of phenolic acids**

The high-performance liquid chromatography (HPLC) analysis was performed [Jasco system (LC-Net II/ ADC, UV-2075 Plus and PU-2089 Plus)] equipped with a column [Jasco RPC18 (250 mm × 4.6 mm × 5 μm)]. The mobile phase contained 99.8% methanol in water (v/v) (solvent A) and 0.1% acid acetic (v/v) (solvent B). The mobile phase A with gradient concentration was 5% to 10% in the first 5 min, then increased from 10% to 90% for next 45 min, and 100% for the last 10 min; wavelength: 254 nm; flow rate: 1.0 mL/min. Each sample was measured in triplicate, and each phenolic compound was identified and quantified in comparison with retention times and peak areas, and compared with those of their respective standards.

**Statistical analysis**

A statistical analysis was performed using a Minitab 16 software. The results are given as means ± standard error (SE). An analysis of variance of one factor ANOVA using the Tukey test was performed, and the value of $p < 0.05$ was considered as statistically significant.

## Results

### Yield and yield components

The grain yields and yield components of the four rice lines are shown in Table 2. All rice cultivars are medium growth duration as shown 125-130 days to heading. The plant height was 112.20 to 119.30 cm, and the panicle length ranged from 22.62 to 23.95 cm. The number of panicle per hill was 6.70 to 9.93. The unproductive tillers were practically non-existent. The highest grain yield and number of filled grain were recorded in B3 cultivar (11.70 t/ha and 189.73, respectively). The lowest grain yield was obtained in B1 and B4 cultivars, (6.48 t/ha and 7.10 t/ha, respectively). The lowest 1000-grain weight was found in B4 cultivar (17.30 g). Statistically, the B1 cultivar was chosen as the lowest yield rice, while the B3 cultivar was selected as the highest yield rice. Yields and yield components of the B2 and B4 cultivars were not significantly different. Therefore, they were not selected for further analysis

**Table 2**. Yield and yield components of examined rice cultivars.

| Rice cultivars | GD | PH | PL | NPH | GW | NFG | UG | GY |
|---|---|---|---|---|---|---|---|---|
| B1 | 125 | 117.70a | 22.62b | 9.17a | 21.32b | 99.51c | 60.28a | 6.48b |
| B2 | 130 | 119.30a | 23.66ab | 6.70a | 23.16a | 156.55b | 27.58c | 8.05ab |
| B3 | 125 | 112.20b | 23.40ab | 9.93a | 18.77c | 189.73a | 32.93c | 11.70a |
| B4 | 130 | 114.20ab | 23.95a | 8.07a | 17.30d | 154.37b | 47.70b | 7.10b |

Different letters in a column indicate significant difference ($p < 0.05$)
GD: growth duration (days); PH: Plant height (cm); PL: Panicle length (cm); NPH: Number of panicle per hill; GW: 1000-grain weight (g); NFG: Number of filled grain; UG: Unfilled grain (%); GY: Grain yield (t/ha)

### Total phenolic and flavonoid contents

The changes of total phenolic content (TPC) and total flavonoid content (TFC) during growth duration of the two cultivars B1 and B3 are shown in (Table 3). The highest values of TPC and TFC in the leaf extracts were observed in the first month after transplanted. During the growth of rice at the vegetative stage, in general, there was an apparent decline in TPC and TFC. The TPC in the first month of B3 was significantly higher than B1, but in contrast, the TFC was lower. The lowest TPC and TFC values were found in the third month (2.34-2.44 mg GAE/g DW and 7.74-7.96 mg RE/g, respectively). It is observed that both TPC and TFC were remarkably reduced when rice cultivars are in the development stage, however the values were not significantly different among the two cultivars (Table 3). Findings of this experiment proposed that the value of TPC was positively correlated to high rice yield, whereas the value of TFC against high yield of rice was negative.

**Table 3**. Total phenolic and flavonoid contents.

| Rice lines | Month after transplanting | Phenolic content (mg GAE/g DW) | Flavonoid content (mg RE/g DW) |
|---|---|---|---|
| B1 | First | 8.00±0.18b | 30.98±0.93a |
| | Second | 4.99±0.25c | 22.33±0.83b |
| | Third | 2.44±0.05d | 7.74±0.10c |
| B3 | First | 10.43±0.29a | 25.51±2.04b |
| | Second | 5.01±0.24c | 23.51±052b |
| | Third | 2.34±0.07d | 7.96±0.21c |

GAE: gallic acid equivalent; RE: rutin equivalent; Values represent means ± SE (standard errors); DW: dry weight; Different letters in similar column indicate significant differences at $p < 0.05$

**Contents of individual phenolic acids**

Table 4 shows the identified phenolic acids and their amounts of the two rice B1 and B3 from first to third months after transplanting. There was a wide variation among the presence and amounts of these compounds. In B1 cultivar, there are some constituents found in both varieties but they disappeared after either the first or second months after transplanting (MAT), including chlorogenic acid, vanillic acid, and vanillin. In contrast, some phenolic acids were not detected at the first month, but were found at either the second and third MAT (protocatechuic acid, ferulic acid, and sinapic acid).

**Table 4**. Quantities of phenolic acids (µg/g dry weight) between high yield and low yield rice cultivars.

| Phenolic components | B1 (Low yield) | | | B3 (High yield) | | |
|---|---|---|---|---|---|---|
| | First month | Second month | Third month | First month | Second month | Third month |
| Protocatechuic acid | nd | 126.86±0.40$^{a}$ | 31.21±0.34$^{c}$ | nd | 67.36±0.32$^{b}$ | 27.68±0.73$^{d}$ |
| Chlorogenic acid | 855.45±6.47 | nd | nd | nd | nd | nd |
| Vanillic acid | 67.72±0.31$^{a}$ | 44.73±2.40$^{b}$ | nd | 51.59±1.18$^{b}$ | nd | nd |
| Caffeic acid | nd | nd | nd | 187.73±3.47 | nd | nd |
| Vanillin | 961.86±5.21$^{a}$ | 762.77±4.23$^{c}$ | nd | 800.98±4.90$^{b}$ | nd | nd |
| Ferulic acid | nd | 505.26±19.11$^{b}$ | 246±2.76$^{d}$ | 1198.21±6.63$^{a}$ | 423.99±6.45$^{c}$ | 154.50±2.97$^{e}$ |
| Sinapic acid | nd | nd | 350.35±2.49$^{c}$ | 3702.47±14.89$^{a}$ | 678.69±0.01$^{b}$ | 279.31±11.82$^{d}$ |
| *p*-Coumaric acid | 598.85±14.88$^{a}$ | 398.31±10.57$^{b}$ | 81.16±0.26$^{c}$ | 421.06±9.67$^{b}$ | nd | nd |
| Cinnamic acid | 68.88±2.55$^{b}$ | 106.01±7.59$^{a}$ | 45.11±2.80$^{bc}$ | 123.36±2.39$^{a}$ | 45.03±0.01$^{bc}$ | 41.30±3.56$^{c}$ |

nd: not detected
Values represent means ± standard errors
Different letters in similar row indicate significant difference ($p < 0.05$)

In B3 variety, protocatechuic was not found in the first month, but it was detected in the second and third months. Vanillic acid, caffeic acid, and vanillin were identified in the first month, but they were not found in the second and third months (Table 2). In general, the quantities of phenolic acids declined from the first month to third month of MAT in both cultivars (Table 2). Caffeic acid was identified only in B3, whereas chlorogenic cid was found only in B1. *p*-Coumaric acid was found in both B3 and B1 cultivars. Sinapic acid may play a role to high yield trait of rice as it was in the greatest contents (3702.47 and 678.69 µg/g at the first and second month of MAT, respectively) and found only in B3 cultivar.

**Discussion**

This study evaluated the chemical components, including TPC and TFC, and individual phenolic acids between the selected high yield and low yield rice cultivars. It was found that the B3 cultivar has promising growth duration, number of panicles, number of filled grain, and grain yield, that possibly meet with the high yield rice designated by IRRI [13]. Compared with B1, the grain weight of B3 was lower, but number of filled grains was two times higher, that makes the grain yield was nearly double. The plant height of B3 was also the shortest among tested cultivars, that can positively effect to grain number. It was reported that plant height is one of the most important characteristics that directly contribute to rice yield [19]. B1 was the Bao Thai cultivar, a traditional rice variety grown widely in North of Vietnam more than 10 years ago, but the cultivated area of this cultivar was rapidly reduced, regardless to its good quality. The major reason might be the low yield of Bao Thai cultivar. In this study, Bao Thai showed 6.48 tons/ha (Table 2), but the actual yield when growing in North of Vietnam might be 3-4 tons/ha. However, the B3 (or DT84DB x Bao Thai) possessed much higher yield than B1, and showed good characteristics of having rice yield traits as mentioned above. It might be due to the cultivar B3 was a cross between DT84DB (a cultivar from the mutated DT84 variety) x Bao Thai, thus the yield of B3 was increased 44.62%

compared with that of B1 cultivar (Table 2). Further experiments should be conducted to determine whether the mutation applied on B3 cultivar play any role in breeding high yield rice. In addition, if this mutation can be widely applied, it may effectively help increase rice yield and reduce the risk from food shortage.

The TPC and TFC in both B1 and B3 cultivars were at maximum levels at the vegetative stage, although their values tended to decrease through the development stage (Table 3). There was a downward trend of TPC found in rice husk and rye grain during grain development [5, 20]. In contrast, the TPC content was found increased in germinated rice [21] indicated that TPC may play a role in initial stage of rice growth. The difference of concentrations between the two rice cultivars B1 and B3 occurred only in early stage. The content of TPC in B3 variety was higher than B1, but in contrast, the quantity of TFC was lower (Table 3). This finding agrees with the previous study which revealed that the grain weight had a positive correlation with TPC, but it was negative against TFC [22]. In the reproductive and ripening phases, there was no difference of either TPC or TFC found between high and low yield rice cultivars (Table 3).

The HPLC analysis demonstrated that there was a variation among the presence of detected phenolic acids and their contents in B1 and B3 cultivars. In general, the quantities of most of phenolic concentrations in B3 were higher than those in B1 cultivar (Table 4). At the vegetative stage, there were seven phenolic compounds detected in high yield rice (B3), whereas only five phenolic acids were found in low yield rice (B1). Findings of this study suggested that caffeic acid, ferulic acid, sinapic acid and chlorogenic acid might play a certain role in the formation of high yield rice. It has been previously reported that caffeic acid, ferulic acid and chlorogenic acid implicated in plant defense against insect herbivores due to their ability to act as pro-oxidants by generating reactive oxygen species [23, 24].

The greatest amount of sinapic acid was found in B3 cultivar. Sinapic acid is one of the four most common derivatives of hydroxycinnamic acid that can be found widespread in the plant kingdom. Table 5 showed the contents of sinapic acid in various edible sources. The content of this constituent in B3 cultivar was three times higher than borage and eight times greater than strawberry (1210 and 450.3 μg/g, respectively). The quantity of sinapic acid in B3 cultivar was much higher than other fruits and vegetable listed in Table 5. Currently, sinapic acid has already been evaluated for its various biological activities, such as antimicrobial [25], antioxidant [26], anti-inflammatory [22], anticancer [27], and anti-anxiety activities [28]. In addition, this compound showed a protective effect over arsenic induced toxicity [29], and prevented lysosomal dysfunction in isoproterenol induced myocardial infarcted rats [30]. It is suggested the cultivar B3 may be promising to exploit as a source for pharmaceutical purpose.

**Table 5**. Presence of sinapic acid in different plants [31].

| Source | Sinapic acid (μg/g) | Source | Sinapic acid (μg/g) |
|---|---|---|---|
| Fruits | | | |
| Pears | 0.96 | Raspberries | 36.89 |
| Mango | 7.55 | Strawberry | 450.30 |
| Pomelo | 22.2 | Blueberry | 73.1 |
| Orange | 9.67-17.28 | American cranberries | 210 |
| Lemon | 72.1 | Apple | 13.42 |
| Avocado | 1.93 | Black currant | 11.7 |
| Vegetables | | | |
| White onion | 2.6 | Tronchuda cabbage | 180.1 |
| Garlic | 0.5 | Kale seeds | 5.037 |
| Broccoli | 25-82 | White cabbage | 1.80 |
| Leaf rape | 10.41 | Kale | 2.42 |
| Herb and Spices | | | |
| Borage | 1210 | Thyme | 260 |
| Dill | 230 | Basil | 150 |
| Anise | 100 | Chilly | 100 |
| Rosemary | 690 | Nutmeg | 348 |
| Sage | 280 | Mage | 940 |

However, further investigation should be conducted to quantify the content of sinapic acid in different rice cultivars and origins to clarify whether it has a critical correlation to the high yield traits of rice. It does not know whether the chemical mutation applied for DT84DB play any effective role on enhancing sinapic quantity in rice, that needs elucidation. The examination of sinapic acid application by either soil incorporation or foliar spray to promote rice yield should also be considered.

## Conclusions

This study revealed that total phenolics and contents of phenolic acids were positive to high yield, whereas that of total flavonoids were negative. Among nine phenolic compounds identified by HPLC, caffeic acid, ferulic acid, sinapic acid and chlorogenic acid may contribute an active role in forming high yield. It should examine whether those compounds, especially sinapic acid, can be used as natural products to increase rice yield. In addition, the variety B3 with rich quantity of sinapic acid may be considered as a source to exploit for pharmaceuticals use. It should be clarified whether the chemical mutation used for breeding B3 cultivar has any role to strongly promote quantity of sinapic acid in rice.

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