

Responses of Flavonoids, Phenolics, and Antioxidant Activity in Rice Seedlings between Japonica and Indica Subtypes to Chilling Stress

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Abstract. Chilling is one of the major abiotic stress which limit yield and quality of many crops. The seedlings of rice varieties namely Koshihikari (Japonica subtype) chilling tolerant, and the susceptible NMR2 (Indica subtype) were treated at 25/15 °C and 5/4 °C day/night to determine the growth parameters, phenolic contents, and antioxidant activity. It was found that in all treatments, the growth of MNR2 including root and shoot lengths, and leaf and root weights were inhibited at greater levels than Koshihikari. There were seven phenolic acids identified in leaves of Koshihikari including caffeic acid, vanillin, ferulic acid, sinapic acid, benzoic acid, ellagic acid, and cinnamic acid, but only benzoic acid and ellagic acid were found in leaves of MNR2. In contrast, only vanillic acid and ellagic acid were observed in roots of Koshihikari, whilst ellagic acid and cinnamic acid were found in roots of MNR2. It was found that rice reduced amount of phenolic acids but promoted quantity of total phenolic content (TPC) and total flavonoid content (TFC) and level of antioxidant activity in chilling stress, although the level of responses varied between Japonica and Indica subtypes. Tolerant rice possessed greater bound flavonoids, phenolics and phenolic acids, but susceptible rice accumulated greater free TPC and TFC in reduced temperature. Findings of this study highlighted that phenolic constituents in bound forms of phenols, polyphenols, and flavonoids may play an active role in rice plants than phenolic acids under chilling stress but need further elaboration.

Introduction

Chilling stress is one among the major harmful abiotic stresses which limits growth of temperate plants. Low temperature (chilling) has negative impact on the survival, distribution, growth and reproduction of the plants [1]. The cold stress level is classified into chilling (0-15 °C) and freezing (< 0 °C) [2]. The serious injury by chilling stress to plants is the induction of oxidative damages at cellular levels [3]. Rice is a temperate crop; therefore, low temperature alters its physiological process which leads to low production [4]. Rice is more sensitive to chilling stress than other crops such as wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.), because the origin of this crop is in tropical and sub-tropical regions [2]. Low temperature causes physical damages to rice plants, such as weak germination percentage, low seedling vigor, high death rate and low spike fertility. It also results in the disorder in plant physiological processes, for instance, the changes in chlorophyll contents, lipid peroxidation, proline and other metabolites [5].

Cold stress increases phenolic contents and their constituents in plants cell wall such as suberin or lignin [6]. Flavonoids and lignin are the strong and potent antioxidants to function in rice as plants defenders [7]. The antioxidant activity of phenolic compounds plays a crucial role in neutralizing free radicals, triplet oxygen or decomposing peroxides [8]. However, to date, roles of phenolic compounds in rice against chilling stress have not been much known. The long-term shift and changes in environmental stresses and continued deterioration of cultivation lands are the serious threats to rice cultivation worldwide [9,10]. It is reported that exposing rice in the reproductive stage male sterility and severe yield loss [11]. Cold stress has been reported to cause severe reduction on growth and productivity of rice and affected rice distribution worldwide [12]. In 24 rice producing countries, major cultivating areas in China, Japan, and Korea which included about 15 million ha were under threat of low temperature stress which results in the loss of about 3.5 million tons annually [13-15]. Many rice varieties cannot be grown in approximately 7 million ha in Southeast and South Asia, due to the chilling stress [2, 16]. Therefore, the breeding of rice tolerant to chilling stress is required for sustainable agriculture and food security in rice growing countries [13-17,18]. There were several quantitative trait loci (QTLs) involved in chilling stress have been isolated and cloned [19-21], however introduction of these genes into commercial rice has not been yet approached. The search for compounds in rice which involve in the mechanism of chilling mechanism might be more potential, because the application of these constituents on rice plants during either the vegetative or productive stages might be more promising.

To understand the responses of rice in chilling stress apparently helps to reduce harmful effects on rice seedlings occurred in low temperature. Therefore, this research was conducted to investigate the changes in phenolic constituents and antioxidant activity of between tolerant (Japonica subtype) and susceptible (Indica subtype) rice varieties in chilling stress condition.

Materials and Methods

Two rice varieties Koshihikari, and NMR2 were selected in this experiment. Of which Koshihikari (subtype: Japonica) seeds were purchased from Japan Agriculture, Higashi Hiroshima, Japan. NMR2 (subtype: Indica) seeds were kindly provided by Agricultural Genetics Institute, Vietnam. The seeds were soaked in 0.1% sodium hypochlorite (NaOCl) for 30 min. They were then washed with distilled water and sown in Petri dishes covered with the moist filter papers and placed at 30 °C in an incubator for one week. The germinated seeds were sown in pots filled with commercial soil (JA-ZENCHU Co., Hiroshima, Japan) containing 0.16 g nitrogen (N), 0.2 g phosphorous (P) and 0.26 g potassium (K) in a greenhouse under optimal condition (25/15 °C day/night time, 14 h photoperiod) for one week. The experiment conducted based on Randomized Complete Block Design (RCBD) with four replications in a greenhouse and laboratory. Each block (replication) consisted of four treatments (2 rice varieties and 2 temperature treatments). Thus; the experiment had totally 64 pots. The seedlings divided into two groups of control and treatment. The control group was maintained in the green house and the treatment group was chilled at (5/4 °C day/night) as chilling stress in a chamber for one week. The samples were then collected to measure plant height and length. The dry weight of leaves and roots were measured after drying in an oven at 65 °C for 72 hr [22]. The samples then stored at -20 °C to identify and quantify phenolic compounds, and determine antioxidant activity.

Extraction of Free Phenolics

Free phenolic contents were measured following the method reported by Elzaawely et al., [23]. The amount of 0.3 g of leaves and 0.1 g of roots were extracted with 20, and 10 ml Methanol (99.5%), respectively. The mixture was placed on a stirrer machine and shaken for 4 h. Then the mixture centrifuged (HITACHI, CF15RXII, Tokyo, Japan) at 15,000 rpm for 10 min at 4 °C to collect the supernatants. The pH was adjusted to 1-2. The solvent was evaporated by a rotary evaporator (SB-350-EYELA, Tokyo, Japan) at 37 °C. The precipitates were collected and mixed with methanol for further analysis.

Extraction of Bound Phenolics

The residue of free phenolic extraction hydrolyzed with 20 ml of 4M NaOH at 50 °C. Then, it was placed in the stirred machine for 4 hours. Thereupon, the mixture was centrifuged at 5,000 rpm for 10 min in room temperature. The mixture filtrated, and its pH was adjusted to 1.5 using concentrated HCl. Then, it was extracted with ethyl acetate 99.5% three times. The supernatants of solvent were combined and dried by rotary evaporator at 30 °C. The dried extract was reconstituted with methanol 99.8% and kept in a cold room at 4 °C for further measurement.

Determination of Total Phenolic Content

The content of total phenolic was measured following the Folin-Ciocalteu colorimetric method reported by Ti et al. [24]. Firstly, 0.125 ml of the sample extracts and 0.5 ml distilled water with 0.125 ml of Folin-Ciocalteu's reagent (10%) were added to test tube and mixed well. Thereupon, 1.25 ml Na₂CO₃ 7.5% (w/v) with 0.5 ml of distilled water was added to the mixture and kept in dark place for 30 min. Finally, the absorbance was measured at 760 nm using spectrophotometer (DR/4000 U-HACH, Colorado, USA).

Determination of DPPH Radical Scavenging Activity

DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity was measured following the method reported by Elzaawely et al. [23]. A 0.5 ml of sample extract was mixed with 0.25 ml of DPPH methanol solution and 0.5 ml of 0.1 M sodium acetate buffer (pH 5.5) and kept in a dark place for 30 min. The absorbance was measured at 517 nm using a spectrophotometer (DR/4000 U-HACH, Colorado, USA). Radical scavenging activity was shown as the inhibition was calculated following the below formula:

$$DPPH \text{ Radical Scavenging Activity (\%)} = [(A_{control} - A_{sample})] \times 100$$

Where A_{control} was absorbance of reaction without a sample. A_{sample} was the absorbance of the sample.

Identification of Phenolic Compounds by HPLC

The total phenolic contents were analyzed with HPLC machine according to Xuan et al. [25]. The HPLC (UV-2075-plus-JASCO, Tokyo, Japan) supplied with 2998 photodiode PDA, quaternary pump detectors, and a J-Pak Symphonica C18 column with dimensions of 4.6 × 250 mm, 5 µm (silica). The purified samples were filtered using a 0.22 µm membrane filter and 5 µl of the sample was injected into the HPLC machine. The mobile phase had two solvents, one (solution B) 0.1 % of acetic acid and the other one (solution A) 100% methanol. The phenolic contents were discovered at 254 nm with a flow rate of 1 ml min⁻¹ using the following time gradient: 5% A (0-5 minute); 20% A (5-10 minute); 50% A (10-20 minute); 80% A (20-30 minute); 100% A (30-40 minute); 100% A (40-50 minute); 5% A (50-60 minute). The amount of 5 µl the sample was used and replicated three times. The total phenolic contents were detected by comparing their retention times and quantified as the sample's peak areas compared with those of standards.

Statistical Analysis

The results are presented as (means ± standard deviation). The data were analyzed with Minitab Minitab® 16.2.3 (2012 Minitab Inc., Philadelphia, USA) software using ANOVA. The significant differences were defined at 5% level.

Results

Effects of Chilling Stress on Rice Growth

Results in Table 1 indicated that the two varieties Koshihikari and MNR2 responded differently against temperature treatments. At 25/15 °C (day/night), Koshihikari even obtained better elongation of shoots and roots, and dried weight of leaves and roots, the growth of MNR2 was less than that of

Koshihikari. At 5/4 °C (day/night), the growth of both Koshihikari and MNR2 was stunt, however Koshihikari was less inhibited than MNR2 (Table 1). Significant difference was observed among treatments, and varieties (except for the root length). Findings of this experiment showed that Koshihikari (Japonica subtype) was more tolerant to chilling stress than MNR2 (Indica subtype).

Table 1. Shoot and roots length, leaves and roots dry weight of seedlings subjected to chilling stress

Variety	Temperature Treatment (°C)	Shoot Height (cm)	Root Length (cm)	Leaf Dry Weight (g)	Root Dry Weight (g)
Koshihikari	25/15	40.0±0.9 ^a	16.2±0.9 ^a	2.3±0.1 ^a	0.9±0.02 ^a
	5/4	34.3±1.1 ^b	12.9±1.0 ^b	1.8±0.11 ^b	0.8±0.02 ^a
MNR2	25/15	29.3±0.5 ^c	14.1±0.8 ^{ab}	1.7±0.22 ^{bc}	0.7±0.04 ^b
	5/4	25.6±0.6 ^d	12.9±1.0 ^b	1.4±0.10 ^c	0.5±0.06 ^c
ANOVA					
Variety		*	NS	*	*
Treatment		*	*	*	*
V × T		NS	NS	NS	NS

Means followed by different letters in a column are significantly different at ($p < 0.05$). * indicates significant and NS, non-significant. Number

Total Phenolic Contents (TPC)

Table 2 showed that at the 25/15 °C treatment, the tolerant Koshihikari principally had higher TPC than MNR2 in both leaves and roots. However, at the lower temperature 5/4 °C, the susceptible MNR2 accumulated much higher TPC than the tolerant Koshihikari. Significant difference was observed among treatments and variety x treatment ($p < 0.05$). It was observed that in chilled condition, susceptible rice accumulated greater amount of TFC than the tolerant cultivar (Table 2).

Table 2. Responses of total phenolics to chilling stress in rice leaves and roots

Variety	Temperature Treatment (°C)	Total Phenolics (mg GAE/g dry weight)			
		Leaf		Root	
		Free	Bound	Free	Bound
Koshihikari	25/15	19.7±2.1 ^c	64.7±12.7 ^b	12.9±1.8 ^c	53.5±5.3 ^b
	5/4	29.1±1.9 ^b	95.8±1.5 ^a	14.1±1.5 ^b	101.1±6.2 ^a
MNR2	25/15	19.6±0.8 ^c	45.6±5.9 ^c	2.6±0.5 ^d	31.1±2.1 ^c
	5/4	50.4±1.5 ^a	100.3±2.3 ^a	18.2±0.9 ^a	100.6±3.4 ^a
ANOVA					
Variety		*	NS	NS	*
Treatment		*	*	*	*
V × T		*	*	*	*

GAE: Gallic acid equivalent. Means followed by different letters in a column are significantly different at ($p < 0.05$). * indicates significant and NS, non-significant.

Total Flavonoid Content (TFC)

The value of TFC of leaves and roots of the two rice cultivars was illustrated in Table 3. It was found that the amount of TFC in rice leaves was greater than roots. In the 25/15 °C, TFC between the two cultivars Koshihikari and MNR2 was differed in bound form, but marked difference was observed between the two treatments. Significant difference was also found in the treatment x variety correlation (Table 2).

Table 3. Responses of total flavonoid contents to chilling stress in leaves and roots

Variety	Temperature Treatment (°C)	Total Flavonoid Content (mg RE/g dry weight)			
		Leaves		Roots	
		Free	Bound	Free	Bound
Koshihikari	25/15	49.9±3.1 ^b	3.4±0.4 ^d	0.70±0.1 ^b	1.00±0.2 ^b
	5/4	90.4±1.6 ^a	6.3±0.6 ^c	1.07±0.3 ^{ab}	1.53±0.10 ^a
MNR2	25/15	54.7±10 ^b	4.3±1.0 ^d	0.79±0.1 ^b	0.80±0.2 ^b
	5/4	76.7±3.5 ^a	9.7±0.6 ^c	1.24±0.1 ^{ab}	3.14±0.2 ^a
ANOVA					
Variety		*	NS	NS	*
Treatment		*	*	*	*
V × T		*	*	*	*

RE: Rutin equivalent. Means followed by different letters in a column are significantly different at ($p < 0.05$). * indicates significant and NS, non-significant.

Changes of Phenolic Acids in Rice Leaves and Roots

Thirteen standard phenolic acids were examined to explore their presence in seedlings of Koshihikari and MNR2 varieties under control and chilling stress. The appearance and retention time of these compounds in the HPLC profile are shown in Figure 1.

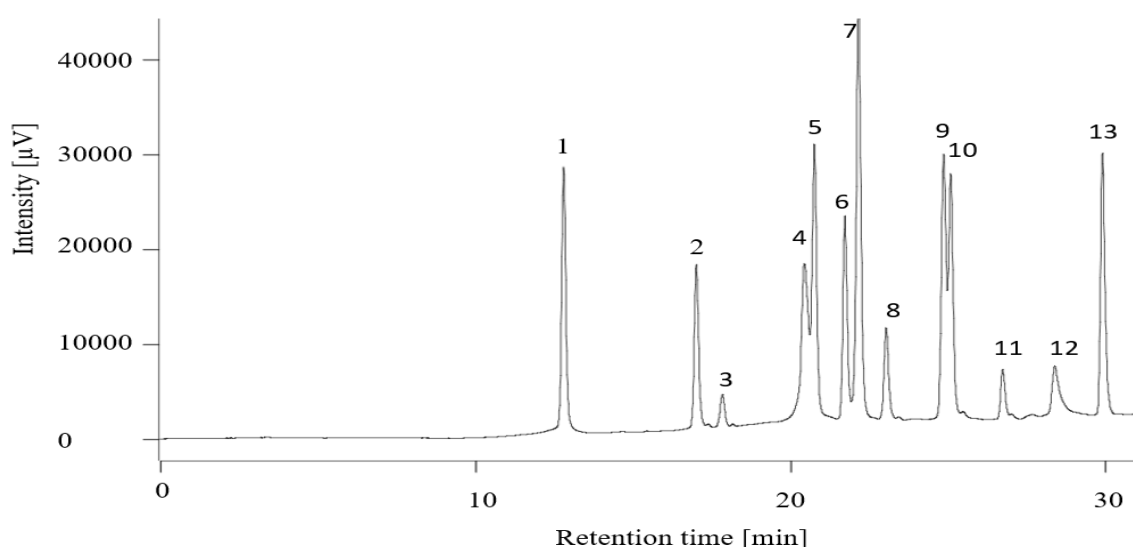


Figure 1. Phenolic acids detected by HPLC (at 254 nm). 1. Gallic acid; 2. Protocatechuic acid; 3. Catechol; 4. Chlorogenic acid; 5. *p*-hydroxyl benzoic acid; 6. Vanillic acid; 7. Caffeic acid; 8. Vanillin; 9. Ferulic acid; 10. Sinapic acid; 11. Benzoic acid; 12. Ellagic acid; 13. Cinnamic acid

However, only seven phenolic constituents were detected in rice leaves (Table 3) whilst three compounds were observed in roots (Table 4). It was found that ellagic acid was present in both leaves and roots of Koshihikari and MNR2, whilst benzoic acid was found only in their leaves (Tables 3 and 4).

Table 4. Phenolic constituents in rice leaves detected by HPLC

Phenolic Acids	Retention Time (min)	Variety			
		Koshihikari		MNR2	
		25/15	5/4	25/15	5/4
Caffeic acid	24.48	4.02±1.19 ^a	-	-	-
Vanillin	25.59	2.73±0.06 ^b	0.82±0.14 ^d	-	-
Ferulic acid	25.76	0.62±0.03 ^d	0.31±0.05 ^d	-	-
Sinapic acid	26.08	2.42±0.33 ^b	1.56±0.05 ^c	-	-
Benzoic acid	27.60	2.60±8.71 ^b	1.23±0.01 ^c	1.40±0.05 ^a	1.45±0.04 ^a
Ellagic acid	29.14	0.69±0.03 ^d	0.83±0.10 ^d	0.46±0.02 ^b	0.51±0.08 ^b
Cinnamic acid	30.12	6.42±6.87 ^a	2.68±0.62 ^b	-	0.45±0.02 ^b

Means followed by different letters in columns of a variety are significantly different at ($p < 0.05$); 25/5 and 5/4 are the treated day/night temperature; -: not detected

Table 5. Phenolic constituents in rice roots detected by HPLC

Phenolic Acids	Retention Time (min)	Variety			
		Koshihikari		MNR2	
		25/15	5/4	25/15	5/4
Caffeic acid	24.48	-	-	-	-
Vanillin	25.59	2.15±0.83 ^a	-	-	-
Ferulic acid	25.76	-	-	-	-
Sinapic acid	26.08	-	-	-	-
Benzoic acid	27.60	-	-	-	-
Ellagic acid	29.14	0.88±0.04 ^b	0.50±0.03 ^c	0.82±0.02 ^a	0.26±0.01 ^b
Cinnamic acid	30.12	-	0.24±0.00 ^d	0.76±0.01 ^a	0.21±0.01 ^b

Means followed by different letters in columns of a variety are significantly different at ($p < 0.05$); 25/5 and 5/4 are the treated day/night temperature.

In general, more regarding to the contents of phenolic acids, in Koshihikari leaves, all compounds significantly reduced their quantities in the 5/4 °C as compared to those of the 25/15 °C by two folds, except the amount of ellagic acid was increased, whilst caffeic acid was detected only in the 25/15 °C treatment (Table 4). However, MNR2 possessed only ellagic acid and cinnamic acid which were detectable in the leaves, however their contents were not significantly different between the two treatments. In roots, ellagic acid was solely found in both Koshihikari and MNR2, whereas cinnamic acid was detected only in MNR2. Their quantities were also significantly reduced in the chilled condition as compared to that of the 25/15 °C condition (Table 4). Findings in Table 3 and 4 highlighted that phenolic acids in leaves responded to the chilling stress were in a greater number than roots. Although the quantity of TFC in leaves and roots were almost similar, it was proposed that phenolic compounds other than phenolic acids may involve in the tolerant to chilling stress in

Antioxidant Activity

Figure 2 showed that the DPPH radical scavenging activity increased remarkably in leaves and roots of both Koshihikari and NMR in chilled treatment. At the control 25/5 °C, the antioxidant activity of Koshihikari was significantly higher than NMR2, whereas insignificant difference was observed between their roots. In the treated 5/4 °C, the DPPH radical scavenging activity in both leaves and roots of the two cultivars were markedly increased. The level of antioxidant activity in leaves was insignificant, whilst MNR2 exhibited remarkable greater capacity of the DPPH radical scavenging activity than that of Koshihikari (Figure 2).

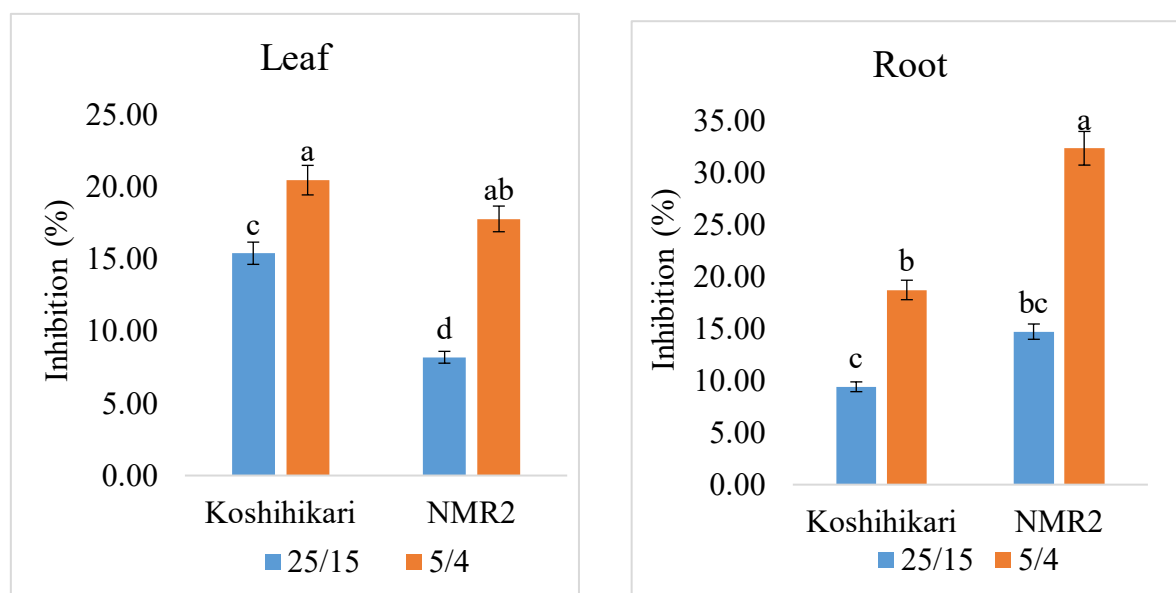


Figure 2. DPPH radical scavenging activity of Koshihikari and NMR2 in chilled stress. Values with similar letters are not significantly different ($p < 0.05$)

Discussion

The findings of our study indicated that cold stress affected differentially the morphological traits, dry matter, phenolic contents and antioxidant activity of rice seedlings. Since the population is rapidly growing and urbanization is increasing, the expanding of rice production areas is facing difficulties. The spreading of rice cultivation is limited in colder areas because the high sensitivity of rice plant during vegetative and development stages against chilling stress. The results of this study revealed that chilled treatments inhibited growth of rice seedlings and declined rice biomass (Table 1). This observation has consisted with a previous study that cold stress might result in biomass reduction through limiting the water uptake by roots [1]. The tolerant Koshihikari revealed greater plant height, root length and dry matter in chilled treatment rather than NMR2 (Table 1).

The increase of higher radical scavenging activity in both Koshihikari and NMR2 might associate with the accelerated level of total phenolic contents (Table 2, Figure 2). Total phenolic content (TFC) were strongly accelerated in both rice cultivars (Table 2). In this study, the TFC in rice leaves and roots under chilling stress was higher as compared to the TFC in a previous study under water deficit stress [26]. The TFC included both free and bound form in leaves and roots was associated with the reduced temperature (Table 2). Findings of this study were similar with previous reports which noticed that abiotic stress induced the synthesis of phenolic compounds in plant tissue [27-29]. In contrast, Posmyk et al. [30] concluded that phenolic contents decreased dramatically in soybean seedlings during exposure to cold stress. The determination of phenolic contents in response to stress may help to explore the tolerant mechanism of crops, thus helped to prevent from loss of crop production. In this study, seven phenolic constituents were found in rice leaves, whilst only three phenolic acids were detected in rice roots to exhibit that phenolic acids in rice leaves responded to chilled stress than roots (Tables 3,4). Phenolic compounds play the role as antioxidants and induce the enzymatic antioxidant system in the cell during the stress [31,32]. In our findings, the presence and amounts of phenolic acids responded differentially under chilling stress. However, whilst the DPPH radical scavenging activity and TFC were significantly promoted, the quantity and presence of phenolic acids were reduced to indicate that phenolic compounds other than phenolic acids might be involved in the tolerance to chilling stress in rice. Further studies are needed to explore the presence and involvement of other phenolic compounds other than phenolic acids such as phenols, polyphenols, and flavonoids in cold treatments.

Reactive oxygen species (ROS) including superoxide (O_2^-), the hydroxyl radical ($HO\cdot$) which are produced at low levels as normal byproducts of plant cellular metabolism, mainly in organelles

such as chloroplast, mitochondria. However the abiotic stresses lead to excessive production of ROS that then react with DNA, lipid and protein to cause cellular oxidative damage [33, 34,]. It is already known that (ROS) is induced through chilling stress to cause a series of deleterious process, such degradation of protein, lipid peroxidation and nucleic acids in the cell [35]. The antioxidant activity of rice leaves and roots in this study were measured using the method of DPPH assay (Figure 2), thus the changes of other antioxidant capacities such as ABTS and reducing power should also be surveyed, as they were reported to be associated with abiotic stresses from submergence [36] and drought [37]. Secondary metabolites such as momilactones A and B were reported to be involved in drought and salinity tolerance in rice [38], but their roles against chilling stress should be further elaborate.

Conclusion

Findings of this study revealed that the responses of secondary metabolites including flavonoids and phenolics, and antioxidant activity between tolerant and susceptible rice in chilling stress were differed between Japonica and Indica subtypes. The antioxidant levels were strongly accelerated in 5/4 °C as compared to that of the 25/15 °C treatment, however roots of the susceptible rice increased the highest DPPH scavenging activity. Although there was greater number of phenolic acids in leaves involved in the tolerant potential than roots, rice significantly reduced quantity of phenolic acids in chilled condition, whereas total phenolic contents and total flavonoid content were in contrast strongly promoted. This study showed that the DPPH radical scavenging and bound forms of phenols, polyphenols, and flavonoids may associate with the tolerance of rice to chilling stress rather than their free form, although phenolic acids may not be much involved.

Conflict of Interest

The authors declare no conflict of interests.

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