Doctoral Dissertation

Application of Exogenous Phenolics for Drought Tolerance Improvement in Rice (*Oryza sativa* L.)

NGUYEN THANH QUAN

Graduate School for International Development and Cooperation

Hiroshima University

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NGUYEN THANH QUAN

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Committee on Final Examination:

XUAN Tran Dang, Associate Professor
Chairperson

ZHANG Junyi, Professor

ICHIHASHI Masaru, Professor

MAHARJAN Keshav Lall, Professor

THANH Khuat Huu, Associate Professor
Hanoi University of Science and Technology

Date: July 23, 2018

Approved:

Baba Takuya, Professor
Dean

Date: August 31 2018

Graduate School for International Development and Cooperation
Hiroshima University

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ABSTRACT

Water stress in a climate change scenario is one of the major threats for sustainable rice productivity. A certain level of drought can cause considerable rice yield losses. Drought stress often obstructs rice growth mainly by oxidative damage that reduces leaf photosynthesis and evapotranspiration processes in biological cells. Deeply understanding of rice self-defense mechanism through plant secondary metabolites activity will be useful and necessary to help improving drought tolerance of rice. Under stress conditions, plants usually produce a large of secondary compounds containing a phenol group. These phenolics have been known with various physiological functions such as stressful response, protective properties, and allelopathic interaction during the growth of plants. Besides, these compounds also possess many different biological activities, including anti-artherogenic, anti-inflammatory, anti-micobial, anti-allergenic, anti-thrombotic, antioxidation, and cardioprotective and vasodilative effects. Therefore, this research was conducted to externally apply potential drought-related phenolics for enhancing water-stress tolerance property of rice plant.

In this study, responses of rice under drought stress correlating with changes in chemical compositions were examined. Among 20 studied rice cultivars, Q8 was the most tolerant, whereas Q2 was the most susceptible to drought. Total phenols, total flavonoids, and antioxidant activities, and their accumulation in water deficit condition were proportional to drought resistance levels of rice. In detail, total phenols and total flavonoids in Q8 [65.3 mg GAE (gallic acid equivalent) and 37.8 mg RE (rutin equivalent)] were significantly higher than Q2 [33.9 mg GAE/g and 27.4 mg RE/g, respectively] in both control and drought stress groups. Similarly, the antioxidant activities including DPPH radical scavenging, *ß*-carotene bleaching, and lipid peroxidation inhibition in Q8 were also higher than in Q2, and markedly increased in drought stress. In general, contents of individual phenolic acids in Q8 were higher than Q2, and they were significantly increased

in drought stress to much greater extents than Q2. However, *p*-hydroxybenzoic acid was found uniquely in Q8 cultivars. In addition, only vanillic acid was found in water deficit stress in both drought resistant and susceptible rice, suggesting that this phenolic acid, together with *p*-hydroxybenzoic acid may play a key role in drought-tolerance mechanisms of rice. The use of vanillic acid (VA) and *p*-hyroxybenzoic acid (PHBA) may be useful to protect rice production against water shortage stress.

In the next experiment, two rice cultivars including a drought tolerant (Q8) and a drought susceptible (Q2) were foliar applied with exogenous vanillic acid (VA) and *p*-hydroxybenzoic acid (PHBA) to examine their effectiveness on drought-tolerant levels and induction of pigments, antioxidants, phenolics, flavonoids, and phytoalexin momilactones A (MA) and B (MB). Generally, the tolerant level of Q2 was more accelerated than those of Q8. Total contents of phenolics, flavonoids, pigments, and antioxidant activity were positively promoted, although the difference between Q8 and Q2 was negligible. In the quantitative induction of phenolic acids, VA, PHBA, and VA+PHBA showed variable effects and dose-dependent, of which Q2 was much influenced than Q8. In all treatments, PHBA appeared to have a more significant role toward drought tolerance than VA. Although MB was found only in non-treated Q8, treatments of VA+PHBA caused formation of both MA and MB, however the induced quantities of MA and MB varied among applied doses and rice cultivars. This research is the first to show that, besides increasing antioxidant activity and total pigments, phenolics, and flavonoids, application of VA and PHBA induced phytoalexins MA and MB to enhance rice drought tolerance, of which MB may play a greater role than MA.

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CHAPTER I. INTRODUCTION

1.1. Background

1.1.1. Importance and problems of rice production

Rice (*Oryza sativa*) is a crucial food crop for most of the people in the world, especially in developing countries such as Asia, where agriculture is a main source of livelihood (Redfern et al., 2012). It is estimated about 90% of the world's rice is grown in Asia, with around 640 million tones of rice are produced annually (FAOSTAT, 2016). Among countries with the most rice area in South and Southeast Asia, China and India are two top rice producers, following by Indonesia, Bangladesh, and Vietnam (Figure 1). According to the report of Food and Agriculture Organization (FAO, 2017), in normal growing condtions, global paddy production would imply a 0.9 percent annual expansion, suggesting that if the forecast of global rice production in 2017 was set at 758.9 million tones (503.8 million tones, milled basis), the prediction in 2018 would be 765.7 million tones (508.3 million tones, milled basis).

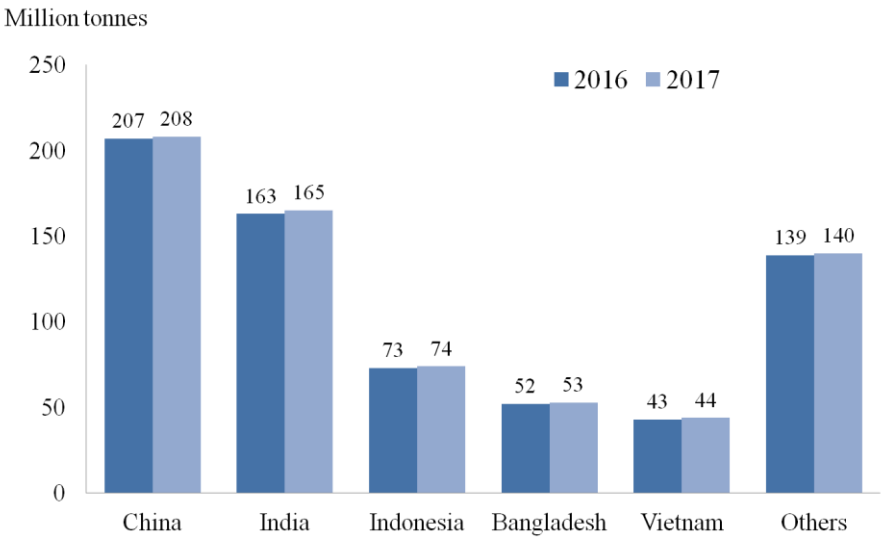


Figure 1. Paddy production in Asia (Source: FAO, 2017).

Rice is rich in nutrients and contains a certain number of vitamins (B vitamins) and mineral, therefore it is an excellent source of complex carbohydrates and the best source of energy. In developing countries, rice accounts for 715 kcal/caput/day, 27% of dietary energy supply, 20% of dietary protein, and 3% of dietary fat (Kennedy et al., 2003). These nutrient composition of rice in reality also can differ significantly between varieties, such as the protein content can ranges from 4 to 14% in *Oryza sativa* (Asian rice variety) and from 9 to 14% in *Oryza glaberrima* (African rice variety) (Juliano and Villareal, 1993). Studies show that the nutrient contents in rice can be influenced by agricultural practices, including soil quality, fertilizer, and environmental conditions, such as temperature, water or light (Kennedy et al., 2003). Besides, not only quality of rice is affected, but also the quantity or rice yield is also decreased and negatively impacted if rice products are exposed to natural environmental stresses, including biotic and abiotic stresses.

In recent years, under the severe circumstance of climate change have been increasingly growing, the rice products of the world in general and Asia in particular have been tremendously affected, leading to the decline of economy and effect to national food security in many countries. The anticipated changes in global climate in the form of rising temperature, increasing amount of carbon dioxide in the atmosphere, greater frequency of extreme weather events (e.g., floods, hot, and storms), and greater incidence of pests and diseases are likely to make things more complicated for rice production. A large share of rice production has been already lost due to various abiotic stresses (flood, drought, and salinity) and biotic stresses (pests, bacteria and virus infection) (Van Alfen, 2014).

1.1.2. Effect of drought on rice plant

Among the abiotic stresses, drought is the strongest constraint, affected nearly a third of the total rice area in the world and causing significant economic losses to poor rice producers in the region. According to the Intergovernmental Panel on Climate Change (IPCC, 2007), drought is considered to be one of the most costly natural hazards, because it

can lead to reduced water supply and consequently have substantial effects on agricultural and socioeconomic activities. The severity of drought depends on its duration, intensity, spatial extent, and local socioeconomic conditions. Singh et al. (2012) estimated global rice yield lost due to drought is 18 million tons annually or 4% of total rice production, which was valued conservatively at 3.6 billion dollars. Furthermore, approximately 34 million ha of shallow rain-fed lowland rice farms and 8 million ha of upland rice farms in Asia, or one-third of the total Asian rice area are subjected to occasional or frequent drought stress (Mohanty et al., 2013).

Drought is a complex natural phenomenon, and its impacts on agriculture and livelihood are enormous, including those that have resulted in major famines in different parts of Asia. For example, in China, the average annual drought-affected area during 1978 to 2003 is estimated to be 14 million ha and the direct economic cost of drought is estimated to be 0.5 to 3.3 % of the agricultural sector GDP (Gross Domestic Product) (Time, 2005). Also, Ding et al. (2008) estimated yield loss of rice due to drought was around 143 to 250 kg/ha in central and southern China, respectively, and value of loss including direct and indirect loss could be 880 million USD. In India, the 2002 drought could be described as a catastrophic event, as it affected 55% of the country's area and 300 million people. Rice production declined by 20% from the inter-annual baseline trend (Pandey et al., 2007). Similarly, the 2003-2005 droughts reportedly affected at least 10,000 ha of winter rice in the Vietnamese Mekong Delta at a cost of 60 million dollars, 650,000 ha in Thailand with the farm production costs increased by 40%, and 500,000 ha of rice area in Cambodia leading to more than 8 million people living in starvation and impoverishment (Son et al., 2012).

Water stress in rice production arises from the higher frequency of El Niño events and reductions in the number of rainy days (Tao et al., 2010), but is also coupled with increasing temperatures and higher evapotranspiration. Because of its semiaquatic

phylogenetic origins, ecosystem diversity, and growing conditions, current rice production systems rely on an ample water supply and thus are more sensitive to drought stress than other crops (Sabar and Arif, 2014). At the whole-plant level, soil water deficit is an important environmental constraint influencing all the physiological processes involved in plant growth and development. Drought is conceptually defined in terms of rainfall shortage vis-à-vis a normal average value in the target region. However, drought occurrence and effects on rice productivity depend more on rainfall distribution than on total seasonal rainfall. Therefore, beyond the search for global solutions to a generic "drought," the precise characterization of drought in the target population of environments is a prerequisite for better understanding their consequences for crop production (Mohanty et al., 2013).

With the onset of climate change, the intensity and frequency of droughts are predicted to increase in rain-fed rice growing areas and droughts could extend further into water-short irrigated areas. According to the International Water Management Institute (IWMI, 2007), by 2025, 15 to 20 million of the world's 79 million hectares of irrigated rice lowlands which provide three-quarters of the global rice will likely experience some degrees of water scarcity as a common result of greenhouse effect and global rainfall changing. Addressing the issue of rice productivity reduction due to drought is becoming a big challenge and it will not be done with one solution. One of the most effective approaches is to develop varieties than can withstand extreme drought conditions to enable them to perform better than current modern high-yielding varieties cultivating in arid and semi-arid regions.

Recently, many studies have been carried out to evaluate the drought tolerance of various rice genotypes with a wide range of genetic variation through designation of field experiments. The scientists used a breeding method known as marker-assisted breeding (MAB) or molecular breeding to incorporate drought tolerant traits into new varieties with

more accuracy and speed (Lang and Buu, 2010). Several key regions of the rice genome called quantitative trait loci (QTLs) related drought tolerance were detected and applied to improve rice grain yield under such conditions. For example, rice QTL/gene clusters for drought-related traits such as root-shoot ratio, deep root mass, relative water content, plant height, root thickness, and number of tiller were identified including nine QTLs in the marker interval between R2417 and RG331 on chromosome 1, three QTLs in the region between RM451 and RM255 on chromosome 4, and six QTLs in the marker interval RZ228-RZ404 on chromosome 9 (Zeng et al., 2006). However, these drought-tolerant QTLs/genes inserted varieties usually give the low-yielding and less quality varieties due to genetic mutation as well as other important traits losing from molecular breeding. In fact, rice plant responses to water stress are very complex and different, relating much to morphological trait expressions, physiological processes and drought-resistant gene activation as well (Anjum et al., 2011). Hence, understanding of biochemical metabolism and stress response mechanism in plants might be one of the key approaches, contributing better to development of water stress tolerant rice varieties.

1.1.3. Plant secondary metabolites

Over the last 50 years, thanks to the development of biochemical technology and the rise of molecular biology, the scientists clearly demonstrated that plant secondary metabolites play a major role in the adaptation of plants to their environment (Bourgaud et al., 2001). These molecules have been described as being antibiotic, antifungal, antiviral and antibacterial to protect plants from pathogens (phytoalexins) and also anti-germinative or toxic for other plants (allelopathy). Moreover, they constitute necessary UV absorbing compounds for preventing severe leaf damage from the sun's light. They also interact with animals, such as insects (anti-feeding properties) or even cattle for avoiding their feeding forage by creating smelly compounds or toxic substances (Akula and Ravishankar, 2011).

Secondary metabolites are chemicals produced by plants that their role does not

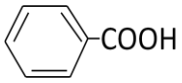
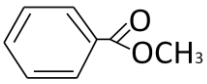
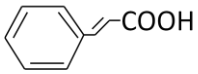
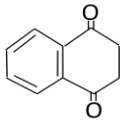
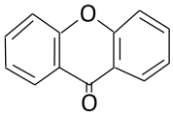
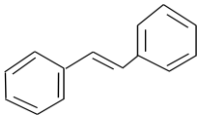
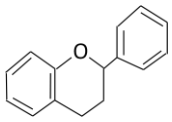
contribute to the growth, photosynthesis, reproduction, or other "primary" functions. It is estimated that there are more than 200,000 known secondary metabolites in plant, representing a vast reservoir of diverse “secondary” functions (Fraire and Balderas, 2013). Due to variable potential biological activities, humans usually use many of these compounds to produce medicines, flavorings, or recreational drugs etc. Base on similar structures and biosynthetic origins, plant secondary metabolites can be grouped into three main classes: (i) flavonoids and allied phenolic and polyphenolic compounds, (ii) terpenoids (made from mevalonic acid, composed almost entirely of carbon and hydrogen), and (iii) nitrogen-containing alkaloids and sulphur-containing compounds.

Plants produce a large variety of secondary compounds containing a phenol group. These phenolic compounds are synthesized via two different routes: the shikimate pathway and the acetate-malonate pathway, and thus represent a heterogeneous group. The shikimate pathway participates in the synthesis of most plant phenolics, whereas the malonate pathway is of less significance in higher plants, although it is an important source of phenolic products in fungi and bacteria (Taiz and Zeiger, 2002). Phenolic compounds are classified into several groups, including anthocyanins, the pigment that attracts animals; flavonoids, the compounds to serve as ultraviolet light protectants; isoflavonoids (phytoalexins), the compounds that act as antifungal and antibacterial defenses; lignin, the phenolic macromolecule which is involved in mechanical support and protection; and tannins, polymeric phenolic compounds that function as feeding deterrents to herbivores.

Phenolics are characterized by having at least one aromatic ring with one or more hydroxyl groups attached. In reality, there arer more than 8,000 phenolic structures have been reported and they are widely dispersed throughout the plant kingdom. Phenolics range from simple, low molecular-weight, single aromatic-ringed compounds to large and complex tannins and derived polyphenols. They can be classified basing on the number and arrangement of their carbon atoms (Table 1) and are commonly found conjugated to

sugars and organic acids (Crozier et al., 2008). Phenolics have been known with various physiological functions such as stressful response, protective properties, and allelopathic interaction during the growth of plants. Besides, these compounds also possess many different biological activities, including anti-artherogenic, anti-inflammatory, anti-micobial, anti-allergenic, anti-thrombotic, antioxidation, and cardioprotective and vasodilative effects (Balasundram et al., 2006).

Table 1. Structural skeletons of phenolic and polyphenolic compounds.

No. of Carbons	Skeleton	Classification	Examples	Basic structures
7	C_6-C_1	Phenolic acids	Gallic acid	
8	C_6-C_2	Acetophenones	Gallacetophenone	
9	C_6-C_3	Hydroxycinnamic acid	*p*-Coumaric acid	
10	C_6-C_4	Naphthoquinones	Juglone	
13	C_6-C_1-C_6	Xanthones	Mangiferin	
14	C_6-C_2-C_6	Stilbenes	Resveratol	
15	C_6-C_3-C_6	Flavonoids	Naringenin	

1.1.4. Drought-tolerant mechanism of plants

Through the history of evolution, plants have developed a wide variety of highly sophisticated and efficient mechanisms to sense, respond, and adapt to a wide range of

environmental changes. When the environment is adverse and plant growth is affected, metabolism is profoundly involved in signaling, physiological regulation, and defense responses (Akula and Ravishankar, 2011). At the same time, in feedback, abiotic stresses affect the biosynthesis, concentration, transport, and storage of primary and secondary metabolites. Metabolic adjustments in response to abiotic stressors involve fine adjustments in amino acid, carbohydrate, and amine metabolic pathways. Proper activation of early metabolic responses helps cells restore chemical and energetic imbalances imposed by the stress and is crucial to acclimation and survival (Fraire and Balderas, 2013).

At the molecular level, many genes are induced or repressed by abiotic stress, involving a precise regulation of extensive stress-gene networks. Products of those genes may function in stress response and tolerance at the cellular level (Ansari and Lin, 2010). Proteins involved in biosynthesis of protectant compounds, detoxification enzyme systems, proteases, transporters, and chaperones are among the multiple protein functions triggered as a first line of direct protection from stress. In addition, activation of regulatory proteins (e.g., transcription factors, protein phosphatases, and kinases) and signaling molecules are essential in the concomitant regulation of signal transduction and stress-responsive gene expression. The response mechanisms of plant early prevent or alleviate cellular damage caused by the stress and re-establish homeostatic conditions and allow continuation of growth. Equilibrium recovery of the energetic, osmotic, and redox imbalances imposed by the stressor are the first targets of plant immediate responses (Fraire and Balderas, 2013).

When rice plants are exposed to drought or commonly water scarcity situation, they respond to the stress by various adaptive mechanisms at the morphological, physiological, and molecular levels, but different varieties often have specific variations in the utilization of these mechanisms. At the early stage of drought, rice usually enhances absorbing water from the underground efficiently via root system, close partially stomata

to reduce water evaporation from the leaf, and alter the endogenous metabolism to match with available carbon resource in plant (Fang and Xiong, 2015). Several osmolytes such as prolines, spermines, betaine, and soluble sugars are also accumulated in plant cells to maintain the cell turgor pressure (Seki et al., 2007). Variations of oxidation-protective enzymes ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR) are also frequently observed in drought-stressed plants (Reddy et al., 2004; .Goswami et al., 2013). A deposition of flavonoids or a flavonoid-DNA complex in nuclei of certain tree species provides a mutual protection against oxidative damage from drought stress (Lattanzio et al., 2006). By analytical method of high-performance liquid chromatography (HPLC), Torras et al. (2012) identified 20 phenolic compounds presented in both watered and water-stressed tobacco, included seven hydroxycinnamoylquinic acids, seven hydroxycinnamic acid glucosides, one salicylic acid glucoside, two conjugated flavonols with disaccharides, and three hydroxycinnamic acid amides (HCAA) of putrescine. However, the levels of these phenylpropanoid compounds were detected increasing higher in water-stressed plant, and it was assumed that propably due to accumulation much of glycosylated and quinylated phenylpropanoid derivatives leading to a potential water-stress response of tobacco plants. Similarly, Hura et al. (2009) carried out an experiment with two genotypes of winter triticale: Lamberto and Ticino, at the propagation phase. The results presented that Lamberto exhibited high content of cell-wall-bound ferulic acid under drought and rehydration. Besides, the photosynthetic apparatus of Lamberto, in relation to Ticino, proved to be the more efficient after four weeks of drought treatment. Evidently, ferulic acid covalently bound to carbohydrates of the cell wall may act as a light filter limiting mesophyll penetration under water deficit conditions and can also support drought adaptation by down-regulation of leaf growth.

In general, the influence of water stress on various secondary metabolites is given in many different plants. Drought-tolerant mechanism controlled by endogenous phenolic

compounds is seen in all plants but its response varies from species to species. Drought often causes oxidative stress and it is reported to show raise in the amounts of flavonoids and phenolic acids in leaves and roots. The importance of phenolic compounds in remaining the survival or enhancing the growth and development of plant has been proved in many previous investigations. However, very few researches have been developed to utilize these compounds (phenolics, flavonoids and theirs derivatives) to improve drought tolerance of crop plants as rice. Therefore, this dissertation is performed to show research works about practical application of exogenous phenolics in help rice production to adapt with water scarcity conditions.

1.2. Research objectives

To search for potential phytochemicals which play as the key roles in resistance to water deficit stress, and then may apply to enhance survival property of rice under drought stress conditions, the study have three main objectives: (1) screening of drought tolerance of collected rice varieties, (2) identifying crucial phenolics involved in water-stress tolerance of rice, and (3) testing the effects of exogenous phenolics on endogenous phytochemical and physiological variations in rice plant under water stress conditions.

CHAPTER II.

INVOLVEMENT OF SECONDARY METABOLITES IN RESPONSE TO DROUGHT STRESS OF RICE (*Oryza sativa* L.)

2.1. Introduction

Drought is one among the most serious problems confronting rice production (Saikumar et al., 2014). A significant decline of rice productivity can be caused by water shortage (Sabar and Arif, 2014). Hence, enhancing survival ability of rice under long-day drought conditions is a crucial issue for rice scientists. Recent studies in plant physiological mechanism responses to abiotic stresses have offered new insights in improving drought tolerance of crops by searching QTLs (quantitative trait locus) or candidate genes relevant to drought, however sucessful results have been limited and unstabilized (Shukla et al., 2012). Thus, it has led to failure among crop breeders in using molecular breeding tools for breeding new crop cultivars adapted to water deficient conditions (Mir et al., 2012).

Studies on the influence of stress signals on secondary metabolites in plants have been increasing since the middle of the 20th century (Bourgaud et al., 2001). Phenolic compounds such as phenolic acids and flavonoids have been found to be the most widespread substantial groups of plant secondary metabolites produced from the shikimate-phenylpropanoid biosynthetic pathway (Torras-Claveria et al., 2012; Ma et al., 2014). These molecules have been described as markers of biotic and abiotic stress tolerance in plants (Balasundram et al., 2006; Lattanzio et al., 2006). Various studies have searched out differences among plant species in the morpho-physiological response to adapt to adverse environmental changes. Plants exposed to salinity stress led to the decrease of shoot dry weight, root ratio and leaf area. Meanwhile, water deficit stress has been found to cause a reduction of leaf photosynthesis and evapotranspiration processes from stomatal closure, and at mild drought levels, increase root depth in the soil (Fayez and Bazaid, 2014;

Fernández-García et al., 2014). Moreover, abiotic stress induces oxidative damage in plant cells due to increased generation of noxious reactive oxygen species (ROS) in chloroplasts (Yildiz-Aktas et al., 2009). Plants possess a number of phenolic compounds and they have been proclaimed to be involved in oxidative stress caused by ROS (Wahid and Ghazanfar, 2006; Tian et al., 2004). On the other hand, plants under certain stress conditions often produce a higher degree of phenolic compounds compared to non-stressed plants (Selmar, 2008). Markham et al. (1998) reported that in different UV-B light levels, C-glycosylflavones contents increasingly appeared in a UV-tolerant rice cultivar, but non-existent in a susceptible cultivar. Torras-Claveria et al. (2012) identified 20 phenolic compounds in both watered and water deficit stressed tobacco plants, and most of these compounds were detected to increase higher in water-stressed plants. Similarly, the observed enhancement of total contents of phenolics, flavonoids, and anthocyanins, and schaftosides, in response to drought in wheat leaves, were demonstrated by Ma et al. (2014). In addition, the use of exogenous phenolic compounds to strengthen drought tolerance in plants was also proved in some previous reports. Typically, rice seeds soaked in 50, 100 and 150 mg l^{-1} of salicylic acid (SA) solution for 48 h expressed better drought resistance than SA untreated seeds at five-leaf stage (Farooq et al., 2009). Spraying of 50 μM SA or 10 mM KNO_3 on barley plants displayed an ability of good cultivation in salt (150 mM NaCl) and water deficit soil (50% SWC) conditions (Fayez and Bazaid, 2014).

In general, secondary metabolic products are ubiquitous in the plant kingdom, particularly their intensity often presents in stress situations. The drought tolerance mechanism controlled by endogenous phenolic compounds is observed in many plants, but it differs among species (Akula and Ravishankar, 2011). In rice, some compounds, mainly phenolic acids and anthocyanins have been detected and examined for their bioactivities in germinated stages and under normal growth status (Tian et al., 2004; Walter and Marchesan, 2011; Walter and Marchesan, 2013).

In this study, changes in chemical compositions including total phenols, total flavonoids, antioxidants and individual phenolic acids in response to drought stress in rice were investigated. It also aims at searching for chemicals which play a crucial role in water deficit stress of rice, which in turn may help to develop bioactive reagents to help ensure rice production against drought.

2.2. Materials and Methods

2.2.1. Chemicals

Fifteen standard phenolic compounds (ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, syringic acid, benzoic acid, protocatechuic acid, vanillin, vanillic acid, catechol, gallic acid, cinnamic acid, caffeic acid, ellagic acid, sinapic acid and chlorogenic acid) and other chemicals were at analytical grades and were purchased from Kanto Chemical Co. Inc., Japan.

2.2.2. Plant materials and treatment

Twenty rice *(Oryza sativa* L.) varieties (Table 2) were grown in a greenhouse. Firstly, the sterilized rice seeds were immersed in 54 ^{o}C water for 15 min, and soaked in 30 ^{o}C water for 48 h with 6-time washing (8 h each) with distilled water. These seeds were then germinated and cultivated in Petri dishes at room temperature (25-27 ^{o}C). After 10 d, they were transplanted to Wagner pots (height: 30 cm; diameter: 20 cm) filled with sterilized soil (JA-ZENCHU Co., Hiroshima, Japan) in the greenhouse for four weeks under optimal conditions (28/20 ^{o}C day/night cycle, 14-h photoperiod and 80% soil moisture). During the whole period, the plantlets were irrigated daily to maintain a level of 85% soil moisture. A moisture meter SM150-HH2 (Delta-T Devices Ltd, UK) was used to monitor soil moisture. The seedlings were divided into two groups: control and test. In the test plants, drought stress was treated in three stages: 5 d, 10 d, and 15 d without watering. At the finish of each stage, the leaf drying, rolling, withering, and recovering were examined. In the first 5 d, the moisture level was decreased from 85% to 65%, and was then maintained at 85%. In the next

10 d, the soil moisture was reduced from 85% to 45% and was moistened again to 85%. In the last 15 d, the moisture capacity was reduced from 85% to 25%, and then the plants were recovered by watering for 2 d before sampling. The leaf samples were kept at -80 ^{o}C for further analysis.

Table 2. Rice cultivars and their origins.

No.	Rice cultivars	Codes	Origins
1	IRRI-C22	C22	PRC, Vietnam
2	Nep vang ong Hoa Binh	Q1	PRC, Vietnam
3	Re nuoc	Q2	PRC, Vietnam
4	Bau quai	Q3	PRC, Vietnam
5	Nep chuoi Hoa Binh	Q4	PRC, Vietnam
6	Nep re	Q5	PRC, Vietnam
7	Lua rac	Q6	PRC, Vietnam
8	Nep lai hoa vang	Q7	PRC, Vietnam
9	Nep nanh ngua Hai Phong	Q8	PRC, Vietnam
10	QTN-1	T1	AGI, Vietnam
11	QTN-2	T2	AGI, Vietnam
12	QTN-3	T3	AGI, Vietnam
13	QTN-4	T4	AGI, Vietnam
14	QTN-5	T5	AGI, Vietnam
15	QTN-6	T6	AGI, Vietnam
16	QTN-7	T7	AGI, Vietnam
17	QTN-BV5	B5	AGI, Vietnam
18	QTN-HTS1	H1	AGI, Vietnam
19	Khang dan 18	K18	AGI, Vietnam
20	Koshihikari	KO	Hiroshima, Japan

PRC: Plant Resource Center, Hanoi, Vietnam; AGI: Agricultural Genetics Institute, Hanoi, Vietnam.

2.2.3. Drought screening procedure

The evaluation of dought resistance was following a Standard Evaluation Scale (SES) developed by International Rice Research Institute (IRRI, 1980) with several modifications (Table 3).

Table 3. Standard evaluation scale of drought tolerant rice.

Scales	Description
Leaf rolling	
0	No symptoms (normal leaves)
1	Leaves starts folding (light V-shaped)
3	Leaves folding (deep V-shaped)
5	Leaves cupped fully (U-shaped)
7	Two leaf margins touching (O-shaped)
9	Leaves rolled tightly
Leaf drying	
0	No symptoms (normal leaves)
1	Slight leaf tip drying (extended to less than 1/4 length of leaves)
3	Tip drying extended to 1/4 length in 25% of all leaves
5	Tip drying extended from 1/4 to 1/2 length in at most 50% of all leaves
7	Tip drying extended to 2/3 length or more in at most 70% of all leaves
9	All plants dryly died
Leaf withering	
1	Leaves had naturally green colour (account for 95% all of the leaves)
5	The backside of all leaves transferred to yellow accounted for 70%
9	Leaves totally transferred to yellow colour
Recovery	
1	90-100% of plants were recovered
3	70-89% of plants were recovered
5	40-69 of plants were recovered
7	20-39% of plants were recovered
9	0-19% of plants were recovered

Source: International Rice Research Institute (IRRI, 1980).

2.2.4. Extraction of phenolic acids

Phenolic acids were extracted using a method reported previously (Ti et al., 2014), with some modifications. Briefly, the residue from the free phenolic extraction was hydrolyzed with 100 mL of 4 M NaOH at 60 ^{o}C with continuous stirring for 4 h. The mixture was centrifuged at 5000 rpm for 10 min and was filtrated by filter papers. Afterwards, the solution was adjusted to pH 2.0 using a 37% HCl solution, and the supernatant was then extracted 3 times with ethyl acetate 99.5% in a separating funnel. After filtration, it was evaporated by a rotary evaporator at 35 ^{o}C to dryness. The dried extract was reconstituted with 99.8% methanol to a final volume of 10 mL at 1000 ppm and then stored at 4 ^{o}C prior to HPLC analysis.

2.2.5. Determination of total phenolic contents

The amount of total phenolics was analyzed using the Folin-Ciocalteu (FC) colorimetric method described previously by Elzaawely and Tawata (2012a), with some modifications. An aliquot of 0.4 mL 7.5% (w/v) Na_2CO_3 and 0.5 mL Folin-Ciocalteu's reagent (10%) were added to 1 mL (1000 ppm) of methanol solution of different extracts. After shaking, the mixture was incubated at room temperature for 30 min. Absorption was measured at 765 nm using a spectrometer (DR/4000U-HACH, Colorado, USA). Total phenolic contents were expressed as gallic acid equivalents (GAE) in milligrams per gram dry weight (DW).

2.2.6. Determination of total flavonoids contents

The total flavonoid contents were determined by a method described in Elzaawely and Tawata (2012b), with some modifications. Briefly, 1 mL aluminum chloride (2% in methanol) was mixed with 1 mL of methanolic solution of different extracts (1000 ppm). After shaking, the mixture was incubated at room temperature for 15 min and then the absorption was measured at 430 nm using a spectrometer (DR/4000U-HACH, Colorado,

USA). Total flavonoid contents were expressed as rutin equivalents (RE) in milligrams per gram dry weight (DW).

2.2.7. Antioxidant assay using the DPPH radical scavenging system

The DPPH radical scavenging activity was evaluated following a method described in Elzaawely et al. (2005). One mL of each methanol solution of extract sample (25, 50, 100, and 1000 ppm) was mixed with 0.5 mL of 0.5 mM DPPH methanol solution and 1 mL of 0.1 M sodium acetate buffer (pH 5.5). After shaking, the mixture was incubated at room temperature in the dark for 30 min, and then the absorption was measured at 517 nm using a spectrometer (DR/4000U-HACH, Colorado, USA). In this method, methanol was used as the negative control. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the formula of Son and Lewis (2002).

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

$A_{control}$ is the absorbance of the control (test sample was replaced by methanol mixed with DPPH solution and sodium acetate buffer) and A_{test} is the absorbance of the test sample (DPPH solution plus antioxidant). The IC_{50} value is identified as the concentration of each sample required giving 50% DPPH radical scavenging activity. Therefore, lower IC_{50} value indicates stronger antioxidant activity.

2.2.8. Antioxidant test using β-carotene bleaching method

The *β*-carotene bleaching for evaluating antioxidant activity followed a method described in Elzaawely and Tawata (2012b). Two mg of *β*-carotene were dissolved in 10 mL chloroform and then 1 mL of this solution was mixed with 20 µL linoleic acid and 200 mg Tween-40. This mixture was evaporated under vacuum conditions for 15 min, at 40 ^{o}C. Afterward, the dry extract was added with 50 mL oxygenated water and strongly shaken until obtaining an emulsion solution. One mL of the *β*-carotene-linoleic acid emulsion was mixed with 0.12 mL of each ethyl acetate fraction sample. Similar to the samples, an equal amount of methanol (0.12 mL) was also used for the negative controls. The tests were

incubated at 50 ^{o}C, and the absorbance was measured using a HACH DR/4000 U spectrophotometer at 492 nm. The samples were measured at zero time at every 15 min up to 180 min. Percentage of lipid peroxidation inhibition (LPI) was calculated relying on the equation of Soares et al. (2009).

$$\% \text{ LPI} = A_1/A_0 \times 100$$

A_0 is the absorbance value measured at zero time for the test sample, while A_1 is the absorbance value measured at 180 min after incubation. A higher LPI % value results in better antioxidant capacity.

2.2.9. Identification of phenolic compounds

The free and bound phenolic fractions were subjected to HPLC analysis with the conditions according to Xuan et al. (2003). The HPLC (UV-2075-plus-JASCO) equipped with a 2998 photodiode PDA, quaternary pump detectors, and a J-pak Symphonia C18 column with dimensions of 4.6 × 250 mm, and 5 µm (silica). These purified extracts were pre-filtered using a 0.22 µm membrane filter and an aliquot of 5 µl of sample was injected into the HPLC system. The mobile phase consisting of two solvents was 0.1% of acetic acid (solution B) and 100% methanol (solution A). The process was established as follows: gradient B v/v solvent A: 0 to 5 min, 0 to 5 %; 5 to 10 min, 5 to 20%; 10 to 20 min, 20 to 50%; 20 to 30 min, 50 to 80%; 30 to 40 min, 80 to 100%; 40 to 50 min, 100%; 50 to 60 min, 100 to 5%. The flow rate was 1 mL per min. The wave length of ultraviolet absorption of defector (absorbance) was at 254 nm. The phenolic constituents of each sample were identified by comparing their retention times and the quantification was calculated by comparing samples' peak areas with those of the standards.

2.2.10. Statistical analysis

The experiments were conducted in a completely randomized design with 5 replicates in each laboratory and greenhouse trials, and 3 replicates in chemical analysis. The samples were analyzed with Minitab® 16.2.3 (copyright © 2012 Minitab Inc.) software

using ANNOVA (analysis of variance) with the significant difference identified at a confidence level of $P = 0.05$.

2.3. Results

2.3.1. Influence of drought stress on rice leaf

The resistance levels of the twenty studied rice cultivars were evaluated through four categories, including leaf rolling, leaf dying, and leaf withering in water deficient stress and the recovery of rice after water was provided, which consisted of different levels of 1 to 9 (Tables 2 and 3). From the grades of drought tolerance indicated in Table 4, the resistant levels were divided as follows: 1-3: strongly tolerant, 3-5: medium tolerant, 5-7: weakly tolerant, and 7-9: drought susceptible. For leaf rolling, drying, and withering, there were six rice cultivars at the medium tolerant level, including C22, Q1, Q4, Q8, T1, and H1, whereas only Q2 was the susceptible variety. The other fourteen rice cultivars were observed at the weakly tolerant level. However, most cultivars of the studied rice exhibited a stronger recovery of 3.0 to 5.7 grades, of which Q8 was the highest, and T7 was the lowest (Table 4). In combination between the response of the rice cultivars under water deficient stress (leaf rolling, drying, and withering) and their recovery, Q8 was selected as the most tolerant variety, whereas Q2 was the most susceptible to drought (Figure 2).

Table 4. Resistant categories and levels of rice under water deficiency stress.

No.	Rice variety	Leaf rolling	Leaf drying	Leaf withering	Recovering
1	C22	5.3 ± 2.3	4.0 ± 2.1	5.0 ± 2.3	3.5 ± 2.0
2	Q1	4.7 ± 2.6	3.9 ± 2.7	5.0 ± 2.3	4.3 ± 2.4
3	Q2	7.9 ± 1.1	7.1 ± 1.0	7.1 ± 1.9	5.5 ± 1.6
4	Q3	5.9 ± 2.5	5.4 ± 2.3	5.3 ± 2.3	3.4 ± 2.0
5	Q4	4.9 ± 2.6	4.0 ± 2.6	5.0 ± 2.3	4.3 ± 2.4
6	Q5	5.3 ± 2.6	4.4 ± 2.6	5.0 ± 2.3	4.5 ± 2.2
7	Q6	6.6 ± 2.2	6.1 ± 1.9	5.5 ± 2.4	5.0 ± 2.3
8	Q7	6.7 ± 1.9	5.7 ± 2.0	5.5 ± 2.1	4.5 ± 2.0
9	Q8	3.7 ± 2.7	3.1 ± 2.0	4.2 ± 2.4	3.0 ± 2.0
10	T1	4.7 ± 2.4	4.2 ± 2.1	4.5 ± 2.4	3.3 ± 1.9
11	T2	6.4 ± 1.3	5.9 ± 1.6	5.0 ± 2.3	4.9 ± 2.3
12	T3	5.4 ± 2.2	4.9 ± 2.1	5.0 ± 2.3	4.3 ± 2.1
13	T4	5.8 ± 2.2	5.0 ± 1.8	5.5 ± 2.1	4.3 ± 1.9
14	T5	6.1 ± 2.2	5.7 ± 2.2	5.3 ± 2.3	4.9 ± 2.2
15	T6	5.9 ± 2.5	5.4 ± 2.5	5.3 ± 2.3	5.0 ± 2.3
16	T7	6.8 ± 1.6	6.2 ± 2.1	5.5 ± 2.4	5.7 ± 1.8
17	B5	5.7 ± 2.6	5.5 ± 2.4	5.0 ± 2.3	3.7 ± 2.1
18	H1	4.7 ± 2.3	4.1 ± 1.9	4.7 ± 2.3	3.7 ±1.9
19	K18	6.1 ± 2.4	5.3 ± 2.3	5.0 ± 2.3	4.3 ± 2.1
20	KO	5.5 ± 2.0	5.1 ± 2.0	5.3 ± 2.1	4.1 ± 2.3

Values are means ± standard errors (SE) (n=5). Grades of drought tolerance: (1) leaf rolling: 0 - normal leaves, 1 - light V-shaped leaves, 3 - deep V-shaped leaves, 5 - U-shaped leaves, 7 - O-shaped leaves, 9 - tight rolled leaves; (2) leaf drying: 0 - normal leaves, 1 - top of leaves are dried lightly, 3 - leaves are dried up to ¼ of leaves length, 5 - ¼ - ½ of leaves are dried, 7 - more ⅔ of leaves are dried, 9 - leaves are dryly died; (3) leaf withering: 1 - leaves are natural green, 5 - backside of leaves transfer to yellow colour, 9 - leaves totally transfer to yellow colour; (4) recovering: 1 - plants are covered from 90% to 100%, 3 - plants are covered from 70% to 89%, 5 - plants are covered from 40% to 69%, 7 - plants are covered from 20% to 39%, 9 - plants are covered from 0% to 19%.

Figure 2. Q2 and Q8 cultivars after 10 days without watering.

2.3.2. Effect of water deficit stress on total phenolic and flavonoid contents

Changes of total phenolic contents (TPC) and total flavonoid contents (TFC) under drought stress and controls (watering condition) are shown in Figures 3 and 4. It was found that even in the watering condition, the capacities of TPC and TFC were proportional to the drought tolerance strength of each rice cultivar. Of them, Q8 obtained significantly higher amounts of TPC and TFC than Q2 (Figures 3 and 4). In the water deficit stress, the quantities of TPC and TFC increased as compared to the watered condition. However, only the change of TPC in Q2 was markedly different. Findings of this experiment suggested that TPC and TFC were closely associated with the strength of rice against drought stress.

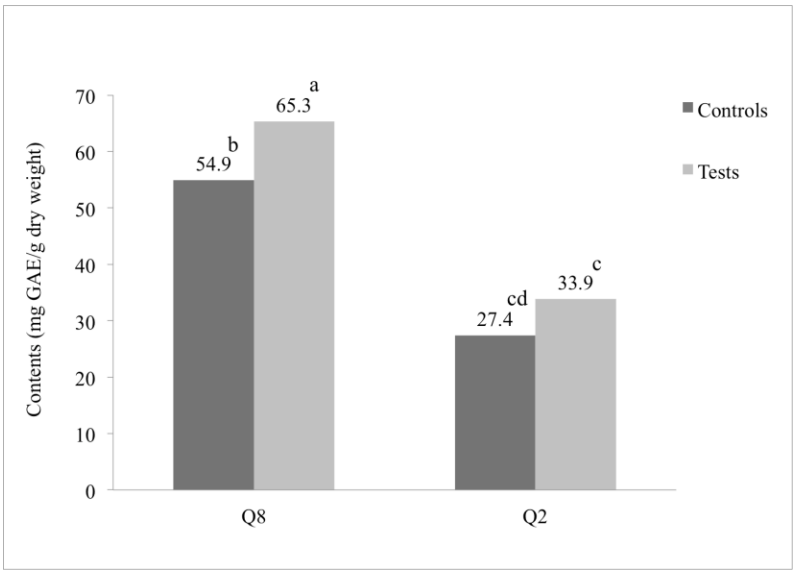


Figure 3. Total phenolic contents of Q8 and Q2 in comparison with the controls. Values are means ± standard errors (SE) (n=3). Means with the same letters are not significantly different (P = 0.05). GAE: gallic acid equivalent.

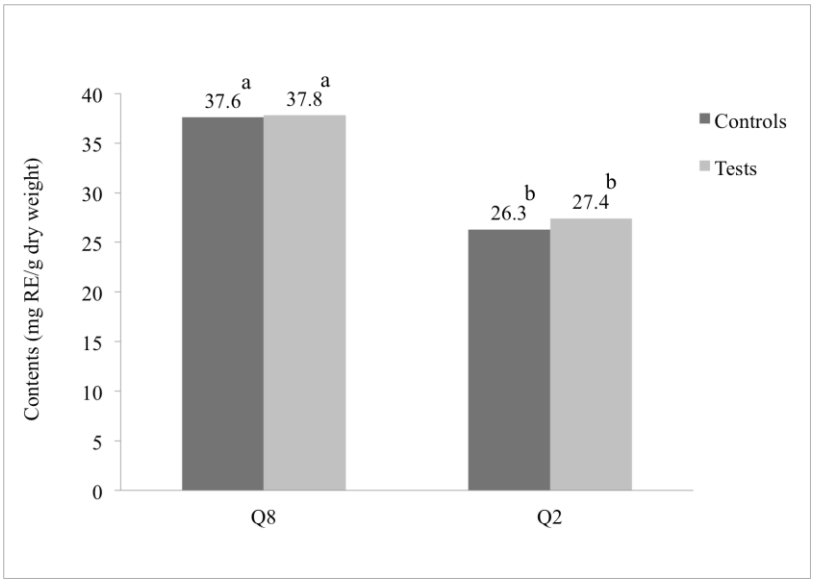


Figure 4. Total flavonoid contents of Q8 and Q2 in comparison with the controls. Values are means ± standard errors (SE) (n=3). Means with the same letters are not significantly different (P = 0.05). RE: rutin equivalent.

2.3.3. Effects of water deficient stress on antioxidant capacity

The DPPH radical scavenging activity of the Q8 and Q2 cultivars are shown in Figure 5 and are exhibited in IC_{50}, that the lower values indicate the stronger activity. As a

result, Q8 showed stronger DPPH radical scavenging activity than Q2. Similarly, the antioxidant activities of β-carotene bleaching method and lipid peroxidation inhibition of Q8 were also stronger than Q2, and were significantly different from those of the controls (Figure 6). Observations of this experiment indicate that the antioxidant activities of rice were promoted in water deficient stress, and the antioxidative strength was proportional to the drought resistance levels of rice cultivars.

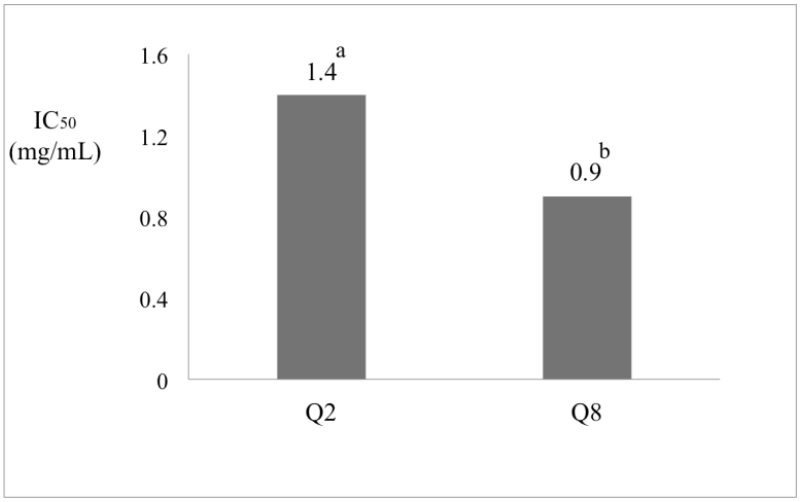


Figure 5. Comparison of DPPH radical scavenging activity between Q2 and Q8 (IC_{50}). Values are means ± standard error (SE) (n=3). Means that do not share a letter are significantly different ($P = 0.05$).

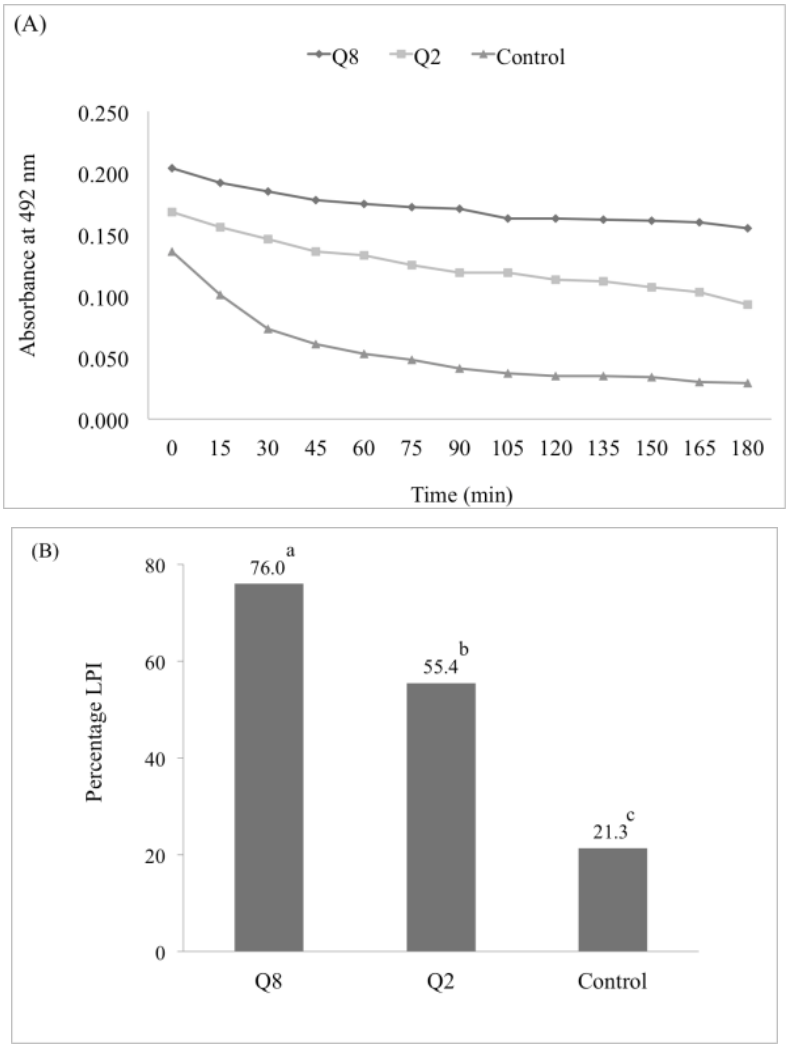


Figure 6. Antioxidant activities of phenolic extracts of Q8 and Q2 measured by *ß*-carotene bleaching method (A) and lipid peroxidation inhibition (% LPI) (B). Means that do not share a letter are significantly different ($P = 0.05$).

2.3.4. Changes of phenolic components under drought stress

Fifteen standard phenolic acids were used to examine their presence in Q2 and Q8 cultivars in controls (watering condition) and water deficit stress. The identification and quantification of these compounds were determined by a HPLC (Figure 7). However, only eight constituents were detected (Table 5). In the controls, six phenolic acids were found, whereas in Q2 cultivar, only three phenolics were presented. In general, ferulic acid (FA), *p*-coumaric acid (PCA), and benzoic acid (BA) which were found in both Q8 and Q2, presented in Q8 in much greater quantities than Q2. Under water deficit stress, the amounts

of these compounds extensively increased, however the extents in Q8 was also much greater than Q2 (Table 5). Vanillin and cinnamic acid were available in Q8 but they were only found in the drought susceptible Q2 cultivar in drought stress. Interestingly, vanillic acid was not detected in the controls of Q8 and Q2, but they were both found in drought stress. In addition, *p*-hydroxybenzoic acid was found only in Q8 in water deficit stress, suggesting that this compound may play a critical role in the defense mechanism of rice against drought (Table 5).

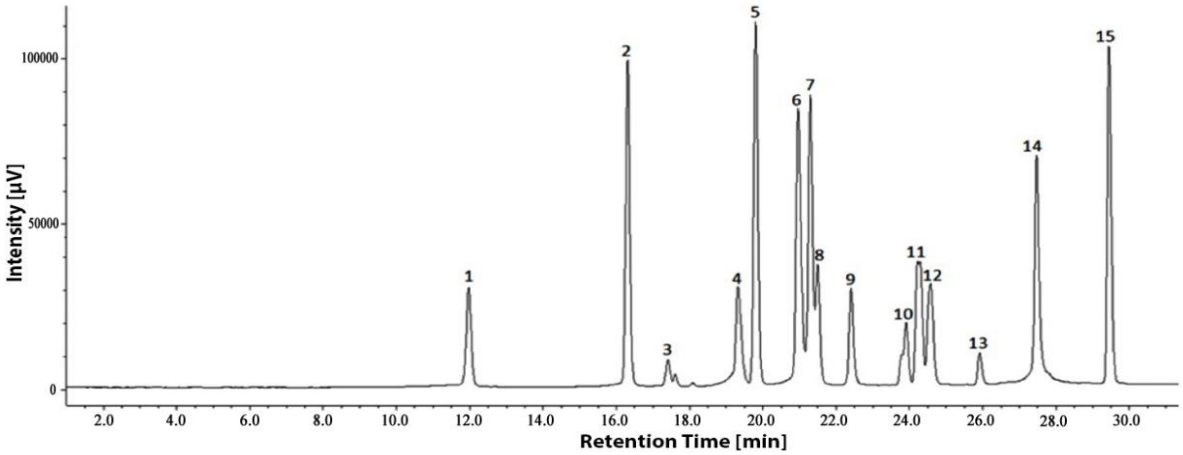


Figure 7. HPLC chromatogram (at 254 nm) shows the separation of standard phenolic acids, 1: gallic acid (GA); 2: protocatechuic acid (PA); 3: catechol (C); 4: chlorogenic acid (CHA); 5: *p*-hydroxybenzoic acid (PHBA); 6: vanillic acid (VA); 7: caffeic acid (CFA); 8: syringic acid (SYA); 9: vanillin (V); 10: ferulic acid (FA); 11: sinapic acid (SIA); 12: *p*-coumaric acid (PCA); 13: benzoic acid (BA); 14: ellagic acid (EA); 15: cinnamic acid (CA).

Table 5. Contents of phenolic acids in Q8 and Q2 determined by HPLC.

No.	Phenolic acids	Retention times (min)	Q8		Q2	
			Controls (mg/g DW)	Drought stress (mg/g DW)	Controls (mg/g DW)	Drought stress (mg/g DW)
1	PHBA	19.82	-	0.161 ± 0.08	-	-
2	VA	20.99	-	0.029 ± 0.02ns	-	0.009 ± 0.01ns
3	SYA	21.56	0.746 ± 0.37a	0.157 ± 0.08b	-	-
4	V	22.40	0.298 ± 0.03ns	0.447 ± 0.22ns	-	0.152 ± 0.08ns
5	FA	23.97	0.304 ± 0.15ns	0.638 ± 0.02ns	0.219 ± 0.11ns	0.299 ± 0.10ns
6	PCA	24.38	0.998 ± 0.12ab	1.295 ± 0.65a	0.598 ± 0.01bc	0.576 ± 0.29bc
7	BA	26.02	0.332 ± 0.17b	1.016 ± 0.40a	0.274 ± 0.01b	0.407 ± 0.20b
8	CA	29.65	0.025 ± 0.01ns	0.053 ± 0.01ns	-	0.056 ± 0.004ns

DW: dry weight. (-): Not detected, ns: not significantly different. Values are means ± standard errors (SE) (n=3). Means in each row with the same letters are not significantly different ($P = 0.05$). PHBA: *p*-hydroxybenzoic acid; VA: vanillic acid; SYA: syringic acid; V: vanillin; FA: ferulic acid; PCA: *p*-coumaric acid; BA: benzoic acid; CA: cinnamic acid.

2.4. Discussion

The processes of delayed leaf rolling and reduced leaf drying are often expressed in drought stress tolerance of rice plants under non-watered condition (Hu et al., 2006). Besides, leaf rolling and leaf withering have been also known as responding mechanisms of plants to avoid water loss caused by stomatal transpiration on the leaf surface (Hura et al., 2012). The bioactivity of leaf phenolic molecules is considered as the signal triggers which lead to the protective mechanisms against drought stress (Akula and Ravishankar, 2011). Previous studies highlighted that the accumulation of phenolic acids and flavonoids as antioxidants and sunshields involved in responses of plants to drought stress and ultraviolet radiation (Nichols et al., 2015). Water stress was reported to generate cell-damaging oxidants, but it also resulted in synthesizing a large amount of flavonoids and phenolic acids in wheat leaves (Ma et al., 2014). Sánchez-Rodríguez et al. (2011) found a high increase of kaempferol and

quercetin in drought-resistant tomato cultivars, while these flavonoids were reduced in drought sensitive cultivars. Some phenylpropanoid compounds were identified in maize under drought, in which *p*-coumaric acid and caffeic acid contents showed a building up, whereas ferulic acid quantity trended towards a lower decrease in water-stressed plants (Alvarez et al., 2008).

In this research, Q8 and Q2 were used to examine the difference in chemical composition and their changes in drought stress. Total phenols, total flavonoids, and antioxidant capacity of Q8 were found to be markedly higher than Q2 in watering condition (Figures 3-6). Their amounts and antioxidative levels were increased in drought stress. The findings indicate that total phenols, flavonoids, and antioxidant activities were closely associated with the drought resistance strength of rice. Their increases in drought stress were found proportional to drought resistance levels. However, the identification of what constituents in the flavonoid group relevant to the drought stress should be identified, as a number of individual phenolic acids of the phenol group have already been found in this study. Some investigations have also presented that there is a positive correlation between antioxidant activity and the contents of total phenolics in plants. For example, Oki et al. (2002) observed that radical-scavenging ability in red-hulled and black-hulled rice depended on the concentrations of proanthocyanidins and anthocyanins, respectively. During the process of finger millet malting, the contents of phenolic acids were changed, reflecting their antioxidant capacity (Subba-Rao et al., 2002). The high levels of flavonols, quercetin and kaempferol contents were associated with enhanced stress tolerance capacity of white clover under UV-B radiation and drought conditions (Nichols et al. (2015). In rice, kaempferol and quercetin components were also identified in rice seeds (Chatterjee et al.,1976). In addition, Karimi et al. (karimi et al., 2014) detected a large amount of kaempferol in rice straw that were able to scavenge free radicals. In this study, we did not find any involvement of the two compounds in response to water deficit stress, Molecular

weights of kaempferol and quercetin are 286 and 302, respectively, and they both greater than those of the detected phenolics (*p*-hydroxybenzoic acid: 138; cinnamic acid: 148; ferulic acid: 194; *p*-coumaric acid: 164, benzoic acid: 122, syringic acid: 198). In the HPLC profile, basically, kaempferol and quercetin should have retentions greater than these phenolic acids (19.82 to 29.65 min; Table 5). However, we could not find any trace of other compounds appeared in the HPLC profile at retention times >30 min, suggesting that kaempferol and quercetin did not involve in the drought stress of rice. It is proposed that the two compounds may be involved in other defense mechanisms of rice against abiotic stresses that need further critical elaboration.

Among fifteen phenolic acids, eight compounds were found to correlate with the drought resistance levels of Q8 and Q2 rice cultivars. However, their presence should also be examined in other rice varieties with different origins and cultivated conditions to elaborate the actual roles of these compounds in rice. The significant increase in quantities of the phenolic acids detected in Q8 and Q2 proposed that these compounds involved in the response of rice against water deficit stress.

Plants often synthesize a series of chemicals with various bioactivities in response to specific stresses (Callaway and Vivanco, 2006). In the shikimate pathway, a biosynthetic pathway for aromatic L-amino acid (AAs), one of the common secondary metabolic precursors is activated early in stress-induced plants (Park et al., 2013; Tzin and Galili, 2010; Parker et al., 2009; Cho and Lee, 2015). Many phenolic compound synthesis-related genes in the shikimate pathway are also induced immediately by stresses (Cho and Lee, 2015). The expression of PAL genes (*OsPALs*) were proved prior to the accumulation of sakuranetin and phenylamide phytoalexins in UV-irradiated rice leaves (Park et al., 2013; Cho and Ly, 2015). The *in vivo* biosynthesis of kaempferol and quercetin glucosides were decided by three functional genes, UGT706C1, UGT706D1 and UGT707A3 (Ko et al., 2008). Furthermore, in higher plants, the formation of C_6-C_1 acids

by removal of a 2-carbon fragment from C_6-C_3 acids was verified to be a common route for biosynthesis of *p*-hydroxybenzoic acid and vanillic acid (Terashima et al., 1975; El-Basyouni et al., 1964).

The ability to tolerate the drought stress of phenolic acids was reported to differ greatly among plant genotypes (Sabar and Arif, 2014). If further extracting protocols are applied, the existence of further phenolics and other secondary metabolites in Q8 and Q2 can be detected and understood. According to the response of their presence and quantities, cinnamic acid, vanillin, and vanillic acid showed a potent involvement in the drought tolerance of rice. In addition, *p*-hydroxybenzoic acid was found only in drought stress of the drought tolerant Q8 cultivar, suggesting that this phenolic may also possess a critical role. Further assays to examine how much *p*-hydroxybenzoic acid, cinnamic acid, vanillin, and vanillic acid involved in the drought resistance of rice should be conducted. On the other hand, the examination of derivatives including enzymes and proteins synthesized these phenolic acids, especially *p*-hydroxybenzoic acid and vanillic acid, needs to be explored and their functions clarified. The data of phenolic acids biosynthesis and metabolism may provide useful evidence for developing bioactive reagents to protect rice production against drought.

2.5. Conclusions

Among twenty studied rice varieties, Q8 was the most tolerant, whereas Q2 was the most susceptible to drought stress. The increase of total phenolic and flavonoid contents, individual phenolic acids, and antioxidant acitivities in water-stressed cultivars probably contributed to drought-resistant property of rice. Importantly, both of vanillic and *p*-hydroxybenzoic acids were identified as the potent phenolic compounds mainly involived in drought tolerance mechanism of rice; and these phenolic acids would be continued to use as exogenous reagents for improvement of water-stress resistance in rice plant.

CHAPTER III.

FOLIAR APPLICATION OF VANILLIC AND *P*-HYDROXYBENZOIC ACIDS ENHANCED DROUGHT TOLERANCE AND FORMATION OF PHYTOALEXIN MOMILACTONES IN RICE

3.1. Introduction

Water stress obstructs rice growth mainly by oxidative damage in biological cells to cause a reduction of leaf photosynthesis and evapotranspiration processes (Farooq et al., 2009; Fayez and Bazaid, 2014). A loss of turgor maintenance and osmotic adjustment (OA) caused by water deficit leads to a decrease of cell expansibility and cell wall thickness, resulting in the leaf rolling and ageing of plant (Shao et al., 2008; Hura et al., 2012; Lanari et al., 2015; Le Gall et al., 2015). In water scarcity status, an accumulation of cell wall-bound phenolics is often observed as a natural physiological mechanism of plant to adapt to stress (Reuber et al., 1996; Schultheiss et al., 2002; Hura et al., 2011; Sánchez-Rodríguez et al., 2011). These phenolics are mostly derived from shikimic acid or aromatic amino acids, many of which play important roles in defense against biotic and abiotic stresses (Rehman et al., 2012; Neelam et al., 2014). Previous studies have shown that process of solute accumulation, such as inorganic cations, organic acids, carbohydrates, and free amino acids helps to maintain the turgor and OA, and thereby adapts to water stress (Chaves and Oliveria, 2004; Basu et al., 2016). Ashraf and Foolad (2007) reported that proline and glycine betaine were important organic osmolytes in response to drought stress by OA and detoxification of reactive oxygen species (ROS), protection of membrane integrity and photosynthetic apparatus. Possible drought tolerance level differs greatly among plant species, reflecting their synthesized secondary compound doses (Sabar and Arif, 2014; Al-Gabbiesh et al., 2015). It was reported that flavonoids and anthocyanins increased by 45% and 80%, respectively, in drought-stressed *Pisum sativum* plants

(Nogués et al., 1998). Similarly, *Hypericum brasiliese* accumulated a fourfold content of rutin and quercetin comparing with non-drought plants (de Abreu and Mazzafera, 2005). However, there was also a reallocation of assimilated carbon, with water-stressed plants presenting a significant increase in phenolic compounds, while their growth progressively reduced (de Abreu and Mazzafera, 2005). A remarkable rise in the content of cell wall-bound ferulic acid improved the productivity of triticale under water stress condition (Hura et al., 2012). However, in contrary, salicylic acid at high concentrations inhibited the growth of wheat and tomato plants (Khandaker et al., 2011). Generally, the appearance of secondary metabolites, including phenolics, flavonoids or phytoalexins has important roles such as growth regulators or allelopathic compounds, offering a survival advantage to the plants in environmental stress conditions (Du Fall and Solomon, 2011). Therefore, a great potential for metabolic engineering and crop breeding in agriculture may be aided by the application of these metabolites.

Vanillic acid (VA) and *p*-hydroxybenzoic acid (PHBA) are phytochemicals found in many plants showing various biological activities, such as antioxidation, antimicrobial, antifungal, and antimutagen (Hegab and Ghareib, 2010; Khadem and Marles, 2010). However, at low doses, the two phenolic acids played as plant growth regulators (Yoshioka et al., 2004; Hegab and Ghareib, 2010). In rice, both VA and PHBA are derivatives from hydroxybenzoic acid group (Setyaningsih et al., 2016). The free radical scavenging and antioxidant activities of phenolics depend on the number and position of H-donating hydroxyl groups (Soobrattee et al., 2005). A high content of VA in *Rhizophora mangle* and *Krameria erecta* was recorded contributing to strong antioxidant capacity due to the arrangement of hydroxyl groups in its nuclear structure for donating hydrogen to stabilize phenoxyl radical (Moran-Palacio et al., 2014). However, the antioxidative effect of VA at low concentrations induced an allelopathic potential by stimulating germination and growth of tomato (Ghareib et al., 2010). Otherwise, VA was also found quantitatively

increased water stressed berry skins (Villango et al., 2016) and fungus attacked tomato epicarp (Ruelas et al., 2006). Meanwhile, PHBA is well-known as a phytoalexin that its existence in plant is associated with anti-pathogenic activities. It is assumed that extracellular accumulation of PHBA may involve in defense mechanism of date palm (*Phoenix dactylifera* L.) plant against the brittle leaf disease (Latreche and Rahmania, 2011). PHBA was reported as a hormone-like substance, relating to either stimulation or inhibition activities on plant growth. In the range of 20–100 μg ml^{-1}, PHBA promoted a significant growth of *Avena* coleoptiles; however, it expressed an intensive growth inhibition at higher contents of 200 μg ml^{-1} (Vieitez et al., 1966). PHBA showed growth activity in freshwater green alga *Pseudokirchneriella subcapitata* at 0.1 to 1.0 mM doses, while it restrained remarkably root growth of lettuce seedlings at 1 mM (Yoshioka et al., 2004; Kamaya et al., 2006).

In general, the bioactive potentials of VA and PHBA are evidently proved in physiological processes, growth regulation, and plant protection from biotic and abiotic stresses. Furthermore, our study previously suggested that VA and PHBA may be among key phenolic acids involving in drought tolerance mechanisms of rice (Quan et al., 2016). Thus exogenous application of VA and PHBA could be expected to improve the water stress tolerance in rice. This study was therefore conducted to investigate the influence of foliar spraying at different concentrations of VA and PHBA and their combinations on morphological criteria, correlations to the drought tolerant capacity as well as changes of some phytochemicals possibly involved in water stress tolerance of rice.

3.2. Materials and Methods

3.2.1. Plant materials and chemicals

Seeds of two rice (*Oryza sativa* L.) varieties, Nep nanh ngua Hai phong (Q8) and Re nuoc (Q2), were obtained from the Plant Resource Center (PRC), Hanoi, Vietnam. In our previous study, Q8 was selected as the most tolerant, whilst Q2 was the most

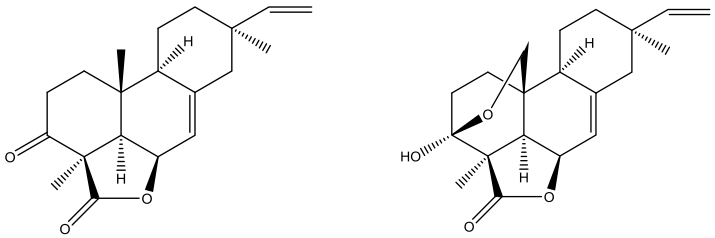
susceptible against drought among examined rice cultivars (Quan et al., 2016). All authentic chemicals used for treatments, extract preparation, and HPLC analysis, were in analytical grades and procured from Junsei Chemical Co., Ltd., Japan. The standard momilactones A and B were isolated in our laboratory from rice husk. Of which, a total of approximately 150 mg MA and 100 mg MB were purified from 10 kg rice husks (*Oryza sativa* L. var. Koshihikari) in November 2016, Hiroshima University, Japan, following a method described in Fukuta et al. (2007) and Xuan et al. (2016). The chemical structures of MA and MB were determined by EI-MS, ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) and compared with literatures (Chung et al., 2005; Kato et al., 1973). Chemical structures of MA and MB are showed in Figure 8.

3.2.2. Experimental treatments

The seeds of Q8 and Q2 cultivars were sterilized by 0.2% $HgCl_2$ solution for 5 min and washed thoroughly by tap water. These seeds were then immersed in 30 °C water for 48 h with 6-time rinsing (8 h each) by distilled water. Afterwards, they were continuously germinated in Petri dishes at 25 °C for 7 d. Q8 and Q2 seedlings were subsequently grown in Wagner pots (height: 30 cm; diameter: 20 cm) contained 4 kg of sterilized soil (JA-ZENCHU Co., Hiroshima, Japan) placed in a greenhouse (27/20 °C day/night cycle, 14-h photoperiod, and 85% soil moisture). Soil moisture was monitored using a moisture meter SM150-HH2 (Delta-T Devices Ltd., UK). After 30 d, the dilutions of vanillic acid (VA), *p*-hydroxybenzoic acid (PHBA), and their mixtures were applied on the foliage of rice plants at different concentrations, as described in Table 6.

Foliar application of VA and PHBA was conducted for two consecutive days with 6-time spraying on all plants (150 mL of solution per plant). A nylon sheet was used to cover rice leaves to optimize spraying process and chemical osmotic permeability as well (Figure 9). The drought treatment was applied immediately after spraying finish with three stress stages: 5 d, 10 d, and 15 d without watering. At the end of each stage, the soil

moisture was maintained again at 85% to recover plants before moving to the next stage. Leaf samples were collected after recovering at 15-d drought stage, and stored at -80 °C for chemical analysis.



Momilactone A (MA) Momilactone B (MB)

Figure 8. Chemical structures of momilactones A and B.

Table 6. Different foliar applications of VA and PHBA on Q8 and Q2 cultivars.

Treatments	
1.	Control plants were sprayed with water + 85% soil water content (SWC)
2.	Q8 and Q2 plants were sprayed with 25 µM VA + 85% SWC
3.	Q8 and Q2 plants were sprayed with 50 µM VA + 85% SWC
4.	Q8 and Q2 plants were sprayed with 25 µM PHBA + 85% SWC
5.	Q8 and Q2 plants were sprayed with 50 µM PHBA + 85% SWC
6.	Q8 and Q2 plants were sprayed with 25 µM VA + 25 µM PHBA + 85% SWC
7.	Q8 and Q2 plants were sprayed with 50 µM VA + 50 µM PHBA + 85% SWC

Figure 9. Foliar spray of VA and PHBA.

3.2.3. Drought tolerance screening process

The drought tolerance was assessed based on four main indicators included leaf rolling, leaf drying, leaf withering, and recovering, describing in the method of International Rice Research Institute (IRRI, 1996) with some modifications (Table 7).

Table 7. Standard evaluation scale of drought tolerance rice.

Scales	Description
Leaf rolling	
0	No effects (healthy leaves)
1	Shallow V-shaped leaves
3	Deep V-shaped leaves
5	Leaves totally cupped forming U-shape
7	Leaf margins touched forming O-shape
9	Leaves tightly rolled
Leaf drying	
0	No effects (normal leaves)
1	Leaf tips slightly dried (drying less than 1/4 length of leaves)
3	Dried tips extended up to 1/4 length in most leaves
5	Tips dried extending from 1/4 to 1/2 length of all leaves
7	Dried tips extended more than 2/3 length of all leaves
9	All leaves fully died
Leaf withering	
1	Natural green leaves accounted for 95% approximately
5	Leaf backsides changed to yellow accounted for 70%
9	All leaves completely transferred to yellow color
Recovery	
1	Plants recovered 90-100%
3	Plants recovered 70-89%
5	Plants recovered 40-69%
7	Plants recovered 20-39%
9	Plants recovered 0-19%

Source: International Rice Research Institute (IRRI, 1996).

3.2.4. Identification of photosynthetic pigment contents

Contents of chlorophyll a (chl a), chlorophyll b (chl b) and total carotenoids were determined by a method described in Lichtenthaler and Wellburn (1983), with several modifications. Briefly, a given weight of fresh rice leaves was placed in an aliquot of 80% (v/v) aqueous acetone. After shaking, the extract was centrifuged at 5,009 ×*g* (5000 rpm) for 10 min, and then the supernatant was spectrophotometrically measured the absorbance (A) at three wavelengths of each 440, 645, and 663 nm. The contents of photosynthetic pigment were expressed as mg g^{-1} fresh weight (FW) and calculated using the following equations:

- Chl a (μg mL^{-1}) = $12.7 \times A_{663} - 2.69 \times A_{645}$
- Chl b (μg mL^{-1}) = $22.9 \times A_{645} - 4.68 \times A_{663}$
- Carotenoid (μg mL^{-1}) = $4.7 \times A_{440} - (1.38 \times A_{663} + 5.48 \times A_{645})$

3.2.5. Preparation of phenolic extracts

Phenolic extracts and chemical analysis were prepared and carried out following a protocol described previously by Quan et al. (2016), with some modifications. Briefly, 1 g of ground dried rice leaves was shaken with 50 mL 99.5% ethanol (EtOH) for 24 h at room temperature (RT). After centrifugation (10 min, 5,009 ×*g*), the supernatant ethanol solution, considered as free phenolic extracts, was retained to filtrate (by filter papers), evaporate to dryness (SB-350-EYELA, Rikakikai Co., Ltd., Tokyo, Japan), and then dissolve with 99.8% MeOH (adjusted at 1000 μg mL^{-1}).

Bound phenolic extracts were made by using 0.1 L of 4 M NaOH to hydrolyze the residue of free phenolic extraction at 60 °C with 4-h stirring. Afterwards, this mixture was centrifuged (10 min, 5,009 ×*g*,), filtrated (by filter papers), adjusted to pH 2.0 (by 37% HCl), and extracted by 99.5% EtOAc (3 times). It was continuously evaporated (SB-350-EYELA, Rikakikai Co., Ltd., Tokyo, Japan) to dryness at 35 °C, and finally dissolved with 99.8% MeOH (adjusted at 1000 μg mL^{-1}). These phenolic extracts were

subsequently kept in 4 °C for further chemical analysis.

3.2.6. Identification of total phenolic contents

A mixture including 1000 µL (adjusted at 1000 µg mL^{-1}) of methanol solution of phenolic extracts, 400 µL 7.5% (w/v) Na_2CO_3, and 500 µL 10% Folin-Ciocalteu's reagent was shaken and kept at 25 °C for 30 min. The content of total phenolics defined as gallic acid equivalents (GAE) in mg/g dry weight (DW) was measured at 765 nm absorption, using a spectrometer (DR/4000U-HACH, Colorado, USA) (Quan et al., 2016).

3.2.7. Identification of total flavonoids contents

A mixture including 1000 µL 2% (w/v) $AlCl_3$ (in methanol) and 1000 µL of methanol solution of phenolic extracts (adjusted at 1000 µg mL^{-1}) was shaken and incubated at 25 °C for 15 min. Total flavonoid contents expressed as rutin equivalents (RE) in mg/g DW were determined at 430 nm absorption by a spectrometer (DR/4000U-HACH, Colorado, USA) (Quan et al., 2016).

3.2.8. Antioxidant activity determined by DPPH radical scavenging assay

One thousand µL of each methanol solution of phenolic extract (25, 50, 100, and 1000 µg mL^{-1}) were mixed with 500 µL of 0.5 mM DPPH methanol solution and 1000 µL of 0.1 M sodium acetate buffer (pH 5.5). After incubating at 25 °C in the dark for 30 min, the mixture was measured at 517 nm absorption by a spectrometer (DR/4000U-HACH, Colorado, USA) (Quan et al., 2016). In this protocol, MeOH was replaced for test extracts to mix with DPPH solution and sodium acetate buffer, using as the negative control. The percentage of DPPH radical inhibition was calculated as follows:

$$\% \text{ DPPH Radical inhibition} = [(A_{control} - A_{test})/A_{control}] \times 100$$

Where, $A_{control}$ and A_{test} are the absorbance of the control and test extract sample, respectively. The IC_{50} value is defined as the required concentration of each extract sample to inhibit 50% DPPH radicals. Accordingly, a lower value of IC_{50} shows stronger antioxidant activity (Quan et al., 2016).

3.2.9. Identification and quantification of phenolic acids and momilactones

** Phenolic acids*

The content of endogenous phenolic acids in both free and bound extracts was analyzed using a HPLC (UV-2075-plus-JASCO, Tokyo, Japan), equipped with a 2998 photo diode array (PDA) detector, a PU-2089 Plus quaternary pump, and a J-pak Symphonia C18 column (250 × 4.6 mm, 5 μm (silica gel)). The purified phenolic extracts were pre-filtered using a 0.45 μm membrane filter and an aliquot of 5 μL of these samples was injected into the HPLC system. The mobile phases including two solvents were 100% methanol (solvent A) and 0.1% of acetic acid (solvent B). The process was programmed as follows: gradient B v/v solvent A: 0 to 5 min, 0 to 5%; 5 to 10 min, 5 to 20%; 10 to 20 min, 20 to 50%; 20 to 30 min, 50 to 80%; 30 to 40 min, 80 to 100%; 40 to 50 min, 100%; 50 to 60 min, 100 to 5%. The flow rate was 1000 μL per min. The wave length of ultraviolet absorption was detected at 254 nm. The identification and quantification of phenolic acids in each sample were determined by comparing the retention times and peak areas with those of the phenolic standards, respectively.

** Momilactones (MA and MB)*

The methanol extraction was separately evaporated (SB-350-EYELA, Rikakikai Co., Ltd., Tokyo, Japan) at 35 °C to dryness and extracted with an aliquot of methanol: hexane (1:1) in a separating funnel. After filtration, it was again made dryly and reconstituted with MeOH (99.8%) to a final volume of 5 mL at 4000 μg mL^{-1}. The estimation of momilactones was subjected to HPLC (UV-2075-plus-JASCO, Tokyo, Japan) analysis with the equippment a Waters Spherisorb S10-ODS2 column (250 × 4.6 mm, I.D., 10 microm). After purification (by 0.22 μm filter), an injection of 5 μL momilactone extracts (4000 μg mL^{-1}) into the HPLC was carried out. The system was established used a mobile phase consisting of 0.1% TFA (trifluoroacetic acid) in H_2O: acetonitrile (30:70, v/v). The flow rate was adjusted at 400 μL per min and the wave length was set at 210 nm. The

identification and quantification of MA and MB in each extract sample were determined by comparing their retention times and peak areas with those of the standards MA and MB, respectively.

3.2.10. Statistical analysis

All measurements were carried out in triplicate for each trial and obtained data were showed as means ± standard errors (SE). Minitab software (version 16.2.3, copyright © 2012, Minitab Inc., Pennsylvania, USA) was used for two-way analysis of variance (ANOVA) and SPSS software (version 2.0, copyright © 2015, SPSS Inc., Chicago, USA) was subjected to correlation analysis of samples with $P \leq 0.05$. The coefficient correlations of VA, PHBA, and combination of VA + PHBA against phenotypes (leaf rolling, leaf drying, leaf withering, and recovery), with quantities of phenolic acids and momilactones A and B were analyzed in similar software at $P \leq 0.05$ and 0.01.

3.3. Results

3.3.1. Screening of water stress tolerance

The drought tolerance ability of Q8 and Q2 after foliar application of VA and PHBA were assessed following different grades of 1 to 9 through four indicators, including leaf rolling, leaf drying, leaf withering, and recovery of rice plant (Table 8). The tolerant levels of Q8 and Q2 ranged from 4.1 to 5.9 and from 5.4 to 8.1, respectively. The mixture of 50 μM VA + 50 μM PHBA resulted in maximum tolerant levels observed in both Q8 and Q2 (Figure 10), ranging from 4.1 to 4.8 and from 5.4 to 6.6, respectively (Figure 10; Table 8). These results indicated that the tolerance and drought adaptive potential of Q8 are better than Q2 both untreated (control) and treatments of VA and PHBA.

Table 8. Influence to drought tolerance by foliar treatments of VA and PHBA.

Q8

Treatments	Leaf rolling	Leaf drying	Leaf withering	Recovering
1. Control (spraying water)	5.7 ± 3.3	5.9 ± 3.4	5.0 ± 2.9	4.8 ± 2.8
2. 25 μM VA	5.9 ± 3.4	5.7 ± 3.3	5.0 ± 2.9	5.0 ± 2.9
3. 50 μM VA	5.7 ± 3.3	5.0 ± 2.9	5.0 ± 2.9	4.3 ± 2.5
4. 25 μM PHBA	5.5 ± 3.1	5.2 ± 3.0	5.0 ± 2.9	4.3 ± 2.5
5. 50 μM PHBA	5.4 ± 3.1	5.4 ± 3.1	5.0 ± 2.9	4.3 ± 2.5
6. 25 μM VA + 25 μM PHBA	5.0 ± 2.9	5.0 ± 2.9	4.1 ± 2.4	4.1 ± 2.4
7. 50 μM VA + 50 μM PHBA	4.8 ± 2.8	4.6 ± 2.6	4.1 ± 2.4	4.1 ± 2.4

Q2

Treatments	Leaf rolling	Leaf drying	Leaf withering	Recovering
1. Control (spraying water)	8.1 ± 4.7	7.2 ± 4.2	6.8 ± 3.9	7.2 ± 4.2
2. 25 μM VA	6.1 ± 3.5	5.9 ± 3.4	6.3 ± 3.7	6.3 ± 3.7
3. 50 μM VA	7.5 ± 4.3	7.0 ± 4.0	7.2 ± 4.2	6.6 ± 3.8
4. 25 μM PHBA	7.2 ± 4.2	7.5 ± 4.3	6.3 ± 3.7	6.1 ± 3.5
5. 50 μM PHBA	7.5 ± 4.3	6.8 ± 3.9	6.3 ± 3.7	6.1 ± 3.5
6. 25 μM VA + 25 μM PHBA	7.0 ± 4.0	6.8 ± 3.9	5.4 ± 3.1	5.9 ± 3.4
7. 50 μM VA + 50 μM PHBA	6.6 ± 3.8	6.3 ± 3.7	5.4 ± 3.1	5.5 ± 3.2

Values are means of 3 replicates ± standard errors (SE). Levels of water stress tolerance: (1) leaf rolling: 0 - no effects, 1 - shallow V-shaped leaves, 3 - deep V-shaped leaves, 5 - U-shaped leaves, 7 - O-shaped rolling leaves, 9 - tightly rolling leaves; (2) leaf drying: 0 - no effects, 1 - slight dried leaf tips, 3 - ¼ of leaf length become dried, 5 - ¼ - ½ of leaf length become dried, 7 - ⅔ of leaf length become dried, 9 - all leaves are fully dried; (3) leaf withering: 1 - natural green leaves, 5 - 70% of leaf backsides changed to yellow color, 9 - leaves completely changed to yellow color; (4) recovery: 1 - plants cover 90-100%, 3 - plants cover 70-89%, 5 - plants cover 40-69%, 7 - plants cover 20-39%, 9 - plants cover 0-19%.

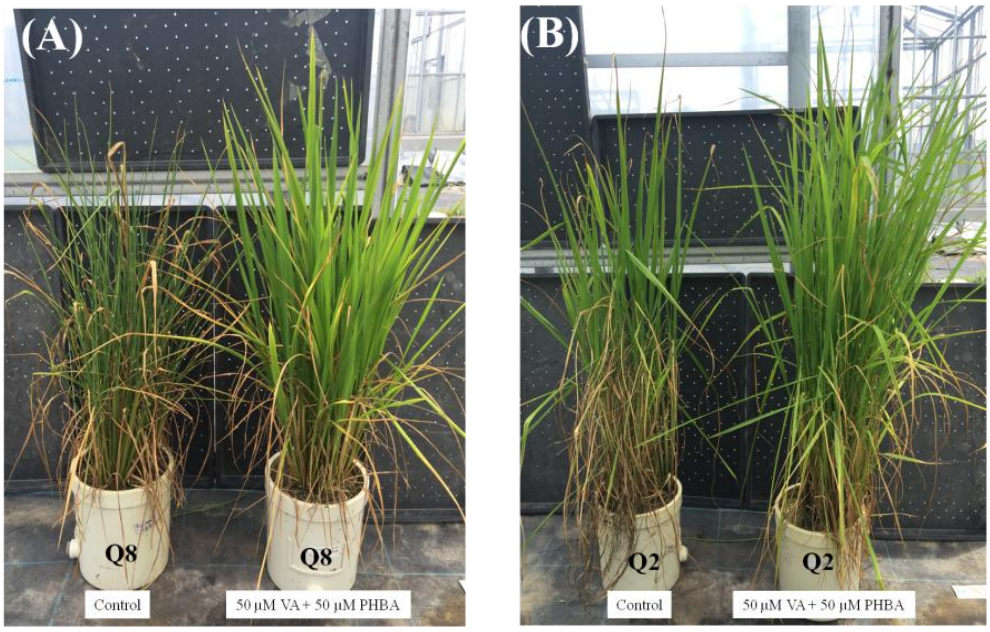


Figure 10. Comparison between exogenous application of 50 µM VA + 50 µM PHBA and water spraying treatments (controls) in Q8 (A) and Q2 (B) cultivars after 10-day drought stress.

3.3.2. Content of total pigments

In response to drought, the pigment content of Q8 was higher than Q2 in comparison in non-treated plants (Table 9). However, the foliar application of 25 µM VA on Q2 was more effectively than Q8, by increasing total pigments of Q2 leaves by 15% (0.124 mg/g FW), whereas it reduced by 3.6% (0.108 mg g^{-1} FW) in Q8. In general, most of foliar applications of VA and PHBA resulted in significant increases of chl a, chl b, and carotenoid contents relative to the non-treated controls (Table 9). The treatment of VA and PHBA apparently improve the drought tolerance by promoting total pigment production in both Q8 and Q2 cultivars, as compared to the respective controls.

Table 9. Effect of different treatments on pigment contents (mg g^{-1} FW) of Q8 and Q2 cultivars relative to controls.

No.	Treatments	Chl a	Chl b	Carotenoids	a/b ratio	Total	%
			Q8				
1	Control	0.053 ± 0.001	0.036 ± 0.001	0.023 ± 0.002	1.472	0.112	100
2	25 µM VA	0.047*** ± 0.001	0.028*** ± 0.001	0.033* ± 0.005	1.679	0.108	96.4
3	50 µM VA	0.054 ± 0.001	0.036 ± 0.001	0.034** ± 0.001	1.500	0.124	111
4	25 µM PHBA	0.056** ± 0.001	0.045** ± 0.002	0.014* ± 0.006	1.244	0.115	103
5	50 µM PHBA	0.057** ± 0.000	0.048** ± 0.003	0.018* ± 0.004	1.188	0.123	110
6	25 µM VA+25 µM PHBA	0.057** ± 0.001	0.047** ± 0.001	0.020 ± 0.001	1.213	0.124	111
7	50 µM VA+50 µM PHBA	0.056** ± 0.001	0.044* ± 0.004	0.024 ± 0.004	1.273	0.124	111
			Q2				
1	Control	0.046 ± 0.000	0.035 ± 0.002	0.027 ± 0.004	1.314	0.108	100
2	25 µM VA	0.054** ± 0.001	0.032** ± 0.002	0.038** ± 0.002	1.688	0.124	115
3	50 µM VA	0.053** ± 0.002	0.032** ± 0.002	0.033* ± 0.006	1.656	0.118	109
4	25 µM PHBA	0.054** ± 0.001	0.033* ± 0.001	0.038** ± 0.002	1.636	0.125	116
5	50 µM PHBA	0.056** ± 0.001	0.037** ± 0.003	0.032*** ± 0.004	1.514	0.125	116
6	25 µM VA+25 µM PHBA	0.054** ± 0.001	0.048** ± 0.002	0.021 ± 0.005	1.125	0.123	114
7	50 µM VA+50 µM PHBA	0.056** ± 0.001	0.039* ± 0.001	0.029 ± 0.001	1.436	0.124	115

*, **, *** Significant at $P \leqslant 0.05$, 0.01, and 0.001, respectively. Values are means of 3 replicates ± standard errors (SE). Statistically significant differences of exogenous phenolic treatments were compared to the values of control at similar column. FW: fresh weight.

3.3.3. Total phenolic and flavonoid contents

Changes of total phenolic content (TPC) and total flavonoid content (TFC) in Q8 and Q2 under water stress are showed in Figures 11 and 12. It was indicated that the spray dose of 50 µM VA + 50 µM PHBA increased significantly both TPC and TFC (Figures 11 and 12). In details, the TPC in Q8 and Q2 were promoted by 29.3% and 28.5%, respectively. Similarly, the TFC was increased by 21.3% and 25.2%, in Q8 and Q2, respectively (Figure 12). The remarkable increase of both TPC and TFC in Q8 and Q2

provided evidence that the foliar application promoted phenolic and flavonoids compounds in rice.

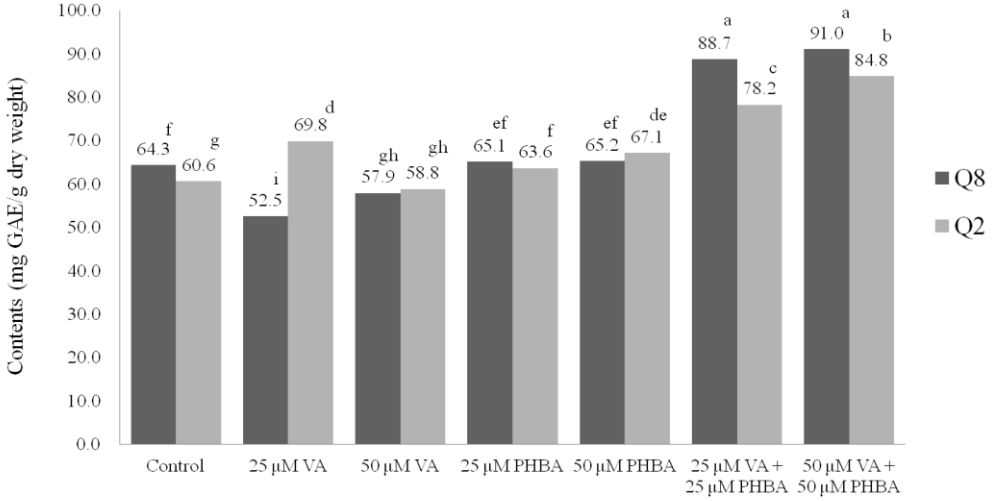


Figure 11. Changes of total phenolic content in Q8 and Q2 in different foliar treatments of vanillic acid and *p*-hydroxybenzoic acid. Values are means ± standard errors (SE) (n=3). Means subjected to same letters are not significantly different ($P \leq 0.05$). GAE: gallic acid equivalent.

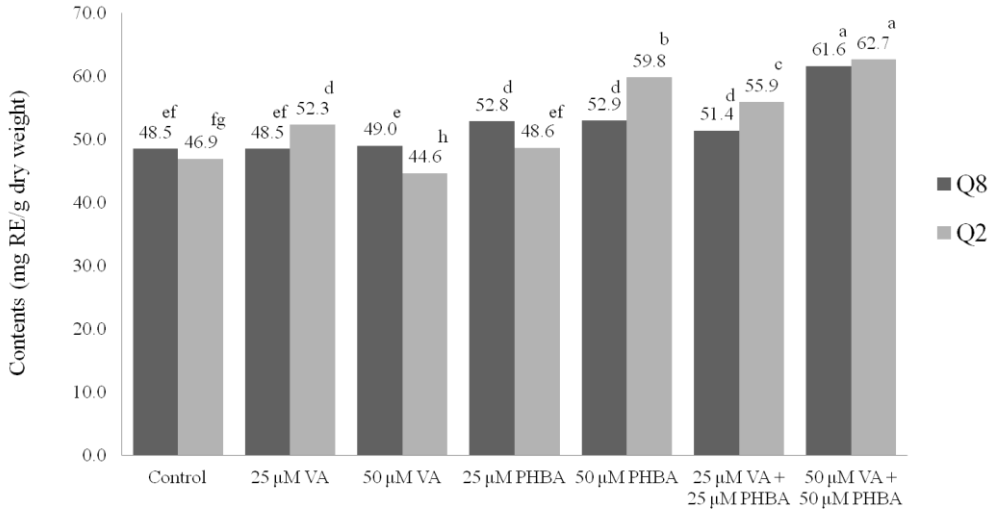


Figure 12. Changes of total flavonoid content in Q8 and Q2 in different foliar treatments of vanillic acid and *p*-hydroxybenzoic acid. Values are means ± standard errors (SE) (n=3). Means subjected to same letters are not significantly different ($P \leq 0.05$). RE: rutin equivalent.

3.3.4. Antioxidant activity

The changes of DPPH scavenging activity of Q8 and Q2 cultivars under drought stress are showed in Figure 13 by IC_{50} values. As a result, the leaves of Q8 and Q2 at 25 µM VA + 25 µM PHBA and 50 µM VA + 50 µM PHBA doses exhibited maximum antioxidant activity, in which the antioxidative level of 50 µM VA + 50 µM PHBA treatment was stronger than 25 µM VA + 25 µM PHBA. It is found that the application of VA and PHBA promoted antioxidant capacity of rice in drought stress, and the level of antioxidation was proportional to the applied doses, although there was no significant different between Q8 and Q2 was observed (Figure 13).

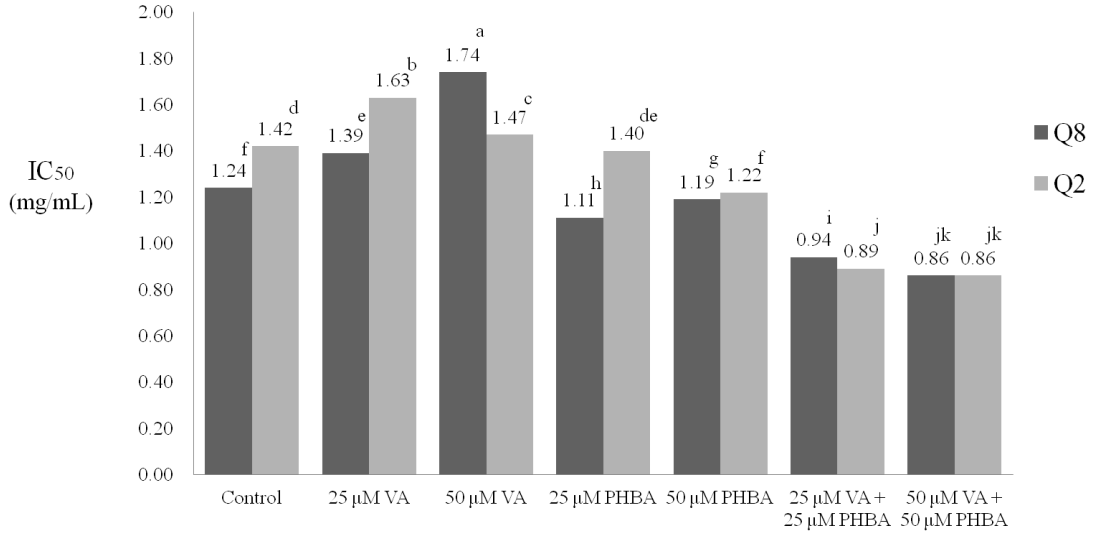


Figure 13. DPPH radical scavenging activity (IC_{50}) of the Q8 and Q2 varieties in different foliar treatments of vanillic acid and *p*-hydroxybenzoic acid. Values are means ± standard errors (SE) (n=3). Means that share a letter are not significantly different ($P \leq 0.05$).

3.3.5. Changes of endogenous phenolic acids and momilactones A and B

** Phenolic acids:*

In controls (non-treated plants), there were six and five phenolic acids were found in each Q8 and Q2, respectively, although the phenolic profiles and contents varied among cultivars (Table 10). The foliar treatments caused different influence on phenolic components between Q8 and Q2. At lower doses of 25 and 50 µM VA, there were only two phenolic acids including vanillin and *p*-coumaric acid were found in Q8. However, in Q2, at the 25 µM VA, similar 5 phenolic acids were observed, but the 50 µM VA promoted the appearance of vanillin and *p*-hydroxybenzoic acid in Q2, whereas no trace of vanillic acid and sinapic acid were detected (Table 10). Application of 25 and 50 µM PHBA either inhibited or promoted the appearances of some phenolic acids in both Q8 and Q2. However, at the doses of 25 µM VA + 25 µM PHBA and 50 µM VA + 50 µM PHBA, there were seven to eight in Q8 and seven phenolic acids Q2 were detected (Table 10). Amounts of detected phenolic acids were fluctuated, some compounds were greater whether some phenolic acids were reduced in quantity, as compared with the controls.

** Momilactones A and B:*

Only MB was found in the drought tolerant Q8 cultivars. However, there were no trace of neither MA nor MB in applied doses of 25-50 µM of each VA and PHBA, indicating that these doses inhibited appearance of both MA and MB (Table 10). However, both MA and MB were detected in the applied combined doses of 25-50 µM VA and PHBA, of which the quantity of both MA and MB in Q2 were greater in Q8, except MB at the maximum application of 50 µM VA + 50 µM PHBA, it was 8.989 µg g^{-1} DW in Q8 (> 2 folds than the control, and > 5 folds than Q2). Findings of this experiments indicated that foliar treatments of VA and PHBA promoted the induction of MA and MB in rice. Both MA and MB may correlate to the drought tolerant mechanism in rice, of which MB may play a greater role than MA.

Table 10. Effect of exogenous treatments of VA and PHBA on endogenous phenolics (mg g^{-1} DW) and momilactones (mg g^{-1} DW) in Q8 and Q2.

Compounds		Retention times (min)	Q8						
			Controls	**25 µM VA**	**50 µM VA**	**25 µM PHBA**	**50 µM PHBA**	**25 µM VA + 25 µM PHBA**	**50 µM VA + 50 µM PHBA**
Phenolic acids	PHBA	19.78	-	-	-	-	0.154 ± 0.00b	0.153 ± 0.00b	0.217 ± 0.00a
	VA	20.94	-	-	-	-	0.045 ± 0.00ns	0.039 ± 0.00ns	-
	SYA	21.48	0.384 ± 0.01b	-	-	0.417 ± 0.01a	-	0.135 ± 0.00d	0.203 ± 0.00c
	V	22.38	0.590 ± 0.27ns	0.193 ± 0.00ns	0.147 ± 0.00ns	0.443 ± 0.00ns	0.270 ± 0.02ns	0.194 ± 0.12ns	0.206 ± 0.08ns
	FA	23.88	0.622 ± 0.12ns	-	-	-	1.099 ± 1.05ns	0.402 ± 0.03ns	0.528 ± 0.00ns
	SIA	24.23	6.581 ± 2.48ns	-	-	3.158 ± 0.11ns	-	-	2.812 ± 0.04ns
	PCA	24.55	0.780 ± 0.14ns	1.056 ± 0.09ns	0.866 ± 0.02ns	1.557 ± 0.01ns	0.631 ± 0.12ns	1.066 ± 0.72ns	0.751 ± 0.02ns
	BA	25.89	0.546 ± 0.43ns	-	-	-	-	-	0.952 ± 0.01ns
	EA	27.45	-	-	-	1.320 ± 0.01a	0.491 ± 0.00c	0.456 ± 0.01d	0.653 ± 0.00b
Momilactones	A	18.19						0.491 ± 0.14b	2.834 ± 0.07a
	B	15.03	3.689 ± 0.40b					1.002 ± 0.15c	8.989 ± 0.24a
			Q2						
Phenolic acids	PHBA	19.78	-	-	0.184 ± 0.00c	-	-	0.705 ± 0.00b	1.406 ± 0.00a
	VA	20.94	-	-	0.003 ± 0.02	-	-	-	-
	SYA	21.48	-	-	-	-	1.145 ± 0.01b	0.717 ± 0.01c	1.418 ± 0.00a
	V	22.38	0.604 ± 0.00d	0.670 ± 0.005c	-	-	0.931 ± 0.02b	0.661 ± 0.00c	1.301 ± 0.02a
	FA	23.88	2.036 ± 0.08bc	2.077 ± 0.002b	1.106 ± 0.57c	-	2.681 ± 0.00b	2.192 ± 0.05b	4.023 ± 0.12a
	SIA	24.23	3.178 ± 0.04c	3.298 ± 0.295c	-	-	6.003 ± 0.26b	5.368 ± 0.34b	13.680 ± 0.30a
	PCA	24.55	2.453 ± 0.03d	2.915 ± 0.004c	0.666 ± 0.03e	-	3.622 ± 0.05b	2.380 ± 0.01d	4.771 ± 0.01a
	BA	25.89	-	-	-	0.383 ± 0.013	-	-	-
	EA	27.45	2.249 ± 0.00d	2.782 ± 0.00c	0.574 ± 0.04f	0.969 ± 0.008e	3.448 ± 0.01b	2.236 ± 0.00d	4.364 ± 0.02a
Momilactones	A	18.19						0.931 ± 0.04ns	2.815 ± 1.85ns
	B	15.03						1.435 ± 0.31ns	1.641 ± 0.05ns

DW: dry weight. (-): Not detected, ns: not significantly different. Values are means ± standard errors (SE) (n=3). Means in each row with the same letters are not significantly different ($P \leq 0.05$). PHBA: *p*-hydroxybenzoic acid; VA: vanillic acid; SYA: syringic acid; V: vanillin; FA: ferulic acid; SIA: sinapic acid; PCA: *p*-coumaric acid; BA: benzoic acid; EA: ellagic acid.

3.3.6. Coefficient correlation of VA, PHBA, and VA + PHBA treatments with phenotypes, and contents of phenolic acids and momilactones A and B

Table 11 indicated that in the treatments of either VA or PHBA resulted in less effectiveness than the combination of both VA and PHBA on phenotypes of both Q8 and Q2 cultivars. Of which, the VA application was effective only on leaf drying, whereas PHBA increased tolerance of leaf rolling and recovery of Q8, but no significant difference was observed. However, on the drought susceptible Q2, except the leaf withering, VA and PHBA promoted drought tolerance, although no remarkable difference was found (Table 11). In the combination of VA + PHBA, all phenotype indicators were affected, but the significant difference was found only in leaf rolling (Table 11).

Regarding to the appearance and quantity of phenolic acids and MA and MB, the application of either VA or PHBA and combination VA + PHBA was varied between Q8 and Q2. Treatment of VA showed no effectiveness on VA and PHBA themselves and ellagic acid in Q8 and ellagic acid in Q2. The sorely application of PHBA also was ineffective against VA and PHBA themselves and benzoic acid in Q2. Quantities of other phenolic acids were either increased or decreased between Q8 and Q2, and varied among phenolic acids (Table 11). The combination of VA and PHBA in Q8 increased significantly amounts of PHBA itself and ellagic acid, and ineffective against VA, whether they reduced quantity of other compounds. However, in Q2, except for VA and benzoic acid, amounts of other phenolic acids were significantly promoted (Table 11).

For MA and MB, the applications of either only VA or PHBA were ineffective in Q2, and they reversely correlated to MB in Q8. The quantity of both MA and MB were increased in combined treatments of VA + PHBA, however the coefficient correlation of in Q8 was MA and Q2 was MB were significant at $P \leq 0.01$ level (Table 11).

Table 11. Correlation of exogenous phenolic treatment at different increasing concentrations (0, 25, 50 μM) to leaf phenotypes, endogenous phenolics, and momilactones (A, B) contents in Q8 and Q2 cultivars.

	Q8			Q2		
	VA	**PHBA**	**VA+PHBA**	**VA**	**PHBA**	**VA+PHBA**
Phenotypes						
Leaf rolling	0	-0.327	-0.650	-0.577	-0.548	-0.866**
Leaf drying	-0.327	0	-0.433	-0.433	-0.327	-0.480
Leaf withering	0	0	-0.327	0	-0.274	-0.655
Recovery	0	-0.577	-0.548	-0.433	-0.480	-0.612
Phenolics						
p-Hydroxybenzoic acid	0	0.866**	0.974**	0.866**	0	1.000**
Vanillic acid	0	0.865**	0	0.389	0	0
Syringic acid	-0.866**	-0.828**	-0.706*	0	0.866**	1.000**
Vanillin	-0.781*	-0.698*	-0.681*	-0.816**	0.343	0.900**
Ferulic acid	-0.854**	0.196	-0.291	-0.787*	0.223	0.892**
Sinapic acid	-0.833**	-0.948**	-0.575	-0.839**	0.463	0.949**
p-Coumaric acid	0.377	-0.138	-0.001	-0.754*	0.317	0.851**
Benzoic acid	-0.677*	-0.677*	0.490	0	0	0
Ellagic acid	0	0.369	0.974**	-0.728*	0.483	0.863**
Momilactone A	0	0	0.939**	0	0	0.663
Momilactone B	-0.860**	-0.860**	0.636	0	0	0.918**

*, ** Significantly different at $P \leq 0.05$ and 0.01, respectively.

PHBA: *p*-hydroxybenzoic acid; VA: vanillic acid.

3.4. Discussion

Plants often overcome water stress through a series of physiological and biochemical mechanisms, ranging from swift responses to low soil water content to main recovery mechanisms (Basu et al., 2016). Drought-related traits, such as foliar morphology, root length, OA capability, cell membrane stability, and endogenous phenolic content are obviously realized in water stress responding mechanisms; therefore, they are usually used as visual

indicators to assess the drought tolerance of plants (Fang and Xiong, 2015). Hura et al. (2012) reported that a considerable increase in cell wall-bound phenolics correlated with improvement in water content within plant and delaying in the leaf dehydration and leaf rolling. Moreover, these phenolic compounds, such as hydroxycinnamic acid derivatives (ferulic acid and *p*-coumaric acid) and flavonoids (kaempferol and quercetin), localized in the cell walls and vacuoles of the epidermis may function as photoprotectors of the photosynthetic apparatus, preventing the potential damage of leaf cell structures from UV radiation (Morales et al., 1996; Bilger et al., 1997; Hura et al., 2012). A previous report of Nichols et al. (2015) indicated that glycosylflavones of kaempferol and quercetin were enhanced by exposure to UV-B light and water stress, and their higher contents were detected at increased stress resistance.

Foliar application of exogenous chemicals to improve crop performance under adverse growing conditions and develop pharmaceutical chemistry technology has been performed in many early investigations. It was revealed that content of total phenols and several identified phenolic acids, such as vanillic, salicylic, ferulic and trans-ferulic acids in the leaves and stems significantly increased after exogenous treatment with 2% of chlorocholine chloride (CCC) on buckwheat seeds (Sytar et al., 2014). Besides, water stress resistance expression of rice, maize, wheat and rapeseed plants were also ameliorated when they were externally applied with 20 or 30 mM acetic acid (Kim et al., 2017). Under 10^{-5} M concentration of salicylic acid (SA) spraying, the growth ability and accumulation of total polyphenol, betacyanin, chlorophyll and antioxidants in red amaranth (*Amaranthus tricolor* L.) were promoted and greatly increased, comparing with untreated plants (Khandaker et al. 2011). The spray of 50 μM SA solution also contributed to the enhancement of photosynthetic pigment contents and drought tolerance in 50% SWC treated barley (*Hordeum vulgare* L.) cultivars (Fayez and Bazaid, 2014). Similarly, root application of 8 mM SA detected high

contents of phytoalexins including momilactone A, oryzalexins A, C and F, suggesting that these compounds may play an important role in resistance to blast fungus *Magnaporthe grisea* in rice plants (Daw et al., 2008). In the correlation between total phenolic contents and anti-oxidation property in plants, Moran-Palacio et al. (2014) also found the high abundances of vanillic acid and ferulic acid contents present in the strongest antioxidant fractions of *Rhizophora mangle* and *Krameria erecta* plants (Moran-Palacio et al., 2014). The contents of anthocyanins and proanthocyanidins decided antioxidant activity of black-hulled and red-hulled rice, respectively (Oki et al., 2002). VA and PHBA acted as the growth-promoting substances that caused to a reduction of leaf shrinkage and senescence under heat stress condition (Ahmad et al., 2016). Therefore, the foliar spraying of exogenous VA and PHBA together probably played as the trigger in drought-resistant phytoalexin production, strengthened the survival ability of rice in soil water scarcity situation.

Findings of this study observed that the foliar application of VA and PHBA and their combination caused positive effects on drought tolerance and phytochemical induction of rice, although the effectiveness varied between rice cultivars and applied doses. They have more positive effect to enhance the phenotypes related to drought tolerance (leaf rolling, leaf drying, leaf withering, and plant recovery) on Q2 (drought susceptible) than Q8 (drought tolerance) (Table 8). In general, VA and PHBA promoted total pigments, including chlorophylls a and b, and carotenoids, and the effects were dose-dependent (Table 9). Total phenolic and flavonoid contents, and the DPPH scavenging activity were also therefore promoted even though the difference among treatments were negligible (Figures 11, 12, and 13). It is found that the induction of pigments, phenolics, flavonoids, and antioxidant activity strongly correlated to the drought tolerance in rice, and it was remarkable enhanced by the foliar treatments of VA and PHBA in this study.

Regarding to the induction of phenolic acids in number and quantity, both VA and

PHBA showed variable effects, but the combination of both VA and PHBA showed greater influence (Table 10). In general, treatments of VA and PHBA were more effective on the induction of PHBA than VA themselves in treated rice plants (Table 10). Except for PHBA and ellagic acid which were significantly promoted, other phenolic acids were reduced in Q8 cultivar, in contrast, except for the sorely treatment of VA, application of PHBA and combination of VA + PHBA generally remarkably promoted quantity and number of individual phenolic acids in drought susceptible Q2 cultivar (Table 10).

Momilactones A (MA) and B (MB) have been known to play an important role in rice allelopathy (Kato-Noguchi and Peter, 2013a; Xuan et al., 2016). At concentrations higher than 100 μM of MB and 300 μM of MA, MA and MB showed strong inhibition on paddy weeds (Kong et al., 2004; Kong et al., 2006; Kato-Noguchi and Ota, 2013b). Moreover, MA and MB have been documented as phytoalexins involved in the defense mechanism of rice against weeds and plant pathogens (Xuan et al., 2016). Although MA presented in greater quantity than MB, but its phytotoxic levels of MB were greater than those of MA (Fukuta et al., 2007). Our previous study also found that MA and MB correlated to salinity and drought tolerance of rice than weed resistance (Xuan et al., 2016). In this work, contents of MA and MB in 30 rice cultivars of various origins, including hybrid, foreign sticky, local, upland sticky, and upland rice of the two subtypes Indica and Japonica were determined. Quantities of MA and MB were then analyzed with salinity tolerance, drought tolerance, weed resistance, total flavonoids, total phenols, and antioxidant capacity. It was observed that MA and MB had very low correlation with weed resistance, with the r^2 coefficients of 0.001 and 0.09, respectively. The correlation was higher with drought tolerance, of 0.65 for MA and 0.27 for MB. However, the changes of MA and MB in drought stress as well as the correlation with other phytochemicals in rice have not been investigated.

In this study, MB was detected only in the drought tolerance Q8 cultivar, indicating

that this phytoalexin may play a role in drought tolerance mechanism in rice. The foliar application of only either VA or PHBA did not result in induction of both MA and MB, however the combined treatments of both VA and PHBA produced both MA and MB (Table 10). The coefficient correlations were maximum and significant for MA in Q8 and MB in Q2 proposed that the two phytoalexins MA and MB may play different roles, depending on the drought tolerance levels in rice cultivars in drought stress, however the mechanism needs further elucidation.

Besides allelopathic, antibacterial, and antifungal activities (Kato et al., 1973; Kato-Noguchi and Ota, 2013b; Fukuta et al., 2007), this study is the first to show that the exogenous application of VA and PHBA induced MA and MB in drought stress. In our recent report, quantities of MA and MB were examined in many cultivars with different origins showed higher coefficient correlation in drought and salinity tolerance than weed resistance and concluded that both MA and MB may much involve in drought and salinity tolerance than rice allelopathy (Xuan et al., 2016). Since the presence and induction of MA and MB have been known to be inherited (Kato-Noguchi and Peters, 2013a), the introduction of rice cultivars with high quantity MA and MB, especially the use of molecular markers relevant to MA and MB synthesis genes, including the dehydrogenase gene (AK103462) and two P-450 genes (CYP99A2 and CYP99A3) form a chitin oligosaccharide elicitor- and UV-inducible gene cluster, together with OsKS4 and OsCyc1, the diterpene cyclase genes, on the chromosome 4 (Shimura et al., 2007), may be effective to enhance the drought tolerance by rice breeding. The root treatment of protocatechuic acid and vanillic acid was effective to enhance submergence tolerance of rice (Xuan and Khang, 2018), highlighting that either the accumulation of phenolic acids may be useful to promote tolerance of rice against environmental stresses.

Although VA and PHBA were potent to enhance the drought tolerance in rice, the

direct foliar application of the two compounds in fields is still costly for farmers. Thus, further examination on VA and PHBA should be carried out to clarify what functional groups in these two compounds are responsible for the drought tolerance enhancement. Then, derivatives of VA and PHBA should be synthesized to have products with acceptable price and even much more effective than VA and PHBA. These derivatives will be carefully examined whether they can be developed to be potent agrochemicals used in agricultural practices to enhance drought tolerance in rice.

3.5. Conclusions

The pretreatment of exogenous VA and PHBA caused a positive effect on rice plants exposed to water deficit stress. VA and PHBA promoted total contents of phenolics, flavonoids, pigments (chlorophylls a and b, and carotenoids), and DPPH scavenging activity, resulting in drought tolerance improvement of Q8 and Q2 cultivars. Treatments of VA and PHBA, and VA + PHBA showed variable effects and dose-dependent in the quantitative induction of phenolic acids, of which the drought susceptible Q2 was much influenced than the drought tolerance Q8. This study is the first to reveal that foliar application of exogenous VA and PHBA induced formation of phytoalexins MA and MB to increase the tolerance level against water stress in rice, of which MB may play a greater role than MA.

CHAPTER IV. GENERAL DISCUSSION

4.1. Discussion

Secondary metabolism refers to phenolic compounds present in specialized cells that are not directly essential for basic photosynthetic or respiratory metabolism but are through to be required for plants survival in the environment (Lattanzio et al., 2006). Phenolic compounds apparently act as defense against biotic stresses and signal compounds to protect the plant from abiotic stresses including water stress (Akula and Ravishankar, 2011). The contents of various secondary plant products are strongly dependent on the growing conditions and the metabolic pathways responsible for the accumulation of the related natural phenolic compounds (Rao and Ravishankar, 2002). Many evidences indicated that phenolic compounds have potent antioxidant properties and free radical scavenging capabilities (Tian et al., 2004). Drought often causes oxidative stress and was reported to show increase in the amounts of flavonoids and phenolic acids in wheat leaves (Ma et al., 2013). At the molecular genetic level, Yuan et al. (2012) reported that water deficit increased the expression of several flavonoids biosynthesis genes in *Scutellaria baicalensis* Georigi roots. Also, Dramé et al. (2007) proved that water shortage induced variation in expression of stress-responsive phenolics-coded genes in *Arachis hypogaea* L. leaves with different tolerance to drought. In recent years, HPLC has been widely used for the analysis of phenolic compounds under drought conditions. Torras-Claveria et al. (2012) identified eighteen phenolic acids and two conjugated flavonols with disaccharides to present in water-stressed tobacco with the higher concentration compared with non-stressed plants. Similarly, in this research, Q8 is expressed as the strongest drought-tolerant variety, of which total phenolic and flavonoid contents are greater than controls and other drought-stressed rice varieties, especially as comparing with Q2, the most susceptible variety under water-stress condition. In addition, the antioxidant

activity of Q8 is also stronger than Q2, suggesting that there is a proportional correlation between total phenolic and flavonoid contents with antioxidant activity in rice. Other researches also found the linear correlation between total phenolic contents and free radical scavenging activities (Oki et al., 2002). In fact, the antioxidant ability of phenolic compounds or polyphenols originates from their properties of chelate formation, proton loss, and dismutation of radicals.

Exogenous application of phytochemicals to promote crop performance under adverse environmental conditions has been conducted in many recent researches. For example, Fayez and Bazaid (2014) developed the drought and salinity tolerance barley by using combination of salicylic acid and potassium nitrate. Farooq et al. (2009) improved the drought tolerance in rice (*Oryza sativa* L.) by exogenous application of different salicylic acid contents. Hura et al. (2009) reported that possible contribution of ferulic acid resulted in water-stress resistance and recovery in triticale seedlings. Likewise, abscisic acid (ABA) was proved having a very helpful role in stomatal aperture regulation, which helps plants to adapt and survive stress conditions, such as drought, salinity, and cold stresses. Thus, external application of ABA to enhance adaptive responses to abiotic stresses was performed in cucumber, alfalfa, bean and potato (Sah et al., 2016).

In the present study, vanillic acid (VA) and *p*-hydroxybenzoic acid (PHBA) have been played a major role involving in drought tolerance of rice plant. The combined spraying treatment of exogenous VA and PHBA at suitable concentrations (25 μM VA + 25 μM PHBA and 50 μM VA + 50 μM PHBA) on the leaf significantly improved water-stress tolerance of rice varieties (Q2 and Q8). The pretreatment of external VA and PHBA caused the modifications of internal phytochemicals, including endogenous VA and PHBA revealed that these phenolic acids possibly involved in cell-membrane-passed absorbing mechanisms, relating to transformation or their translocation in rice leaf tissues. The progressive

application of exogenous VA and PHBA mostly resulted in direct proportion to increasing accumulation of endogenous phenolic acids, except some decrease-varied internal compounds probably due to the osmotic feature or absorption capacity of every rice variety. As Tagliavini and Toselli (2005) also reported that the genetic factors such as leaf anatomy and leaf surface's chemical constituents in different plants affected to cuticular penetration of nutrient droplets.

Regarding to the formation of phytoalexin momilactones A (MA) and B (MB), it could be explained by the biosynthesis of other unknown mediate compounds from VA and PHBA triggers, in which their stress responding role and function had been not fully discovered. In this respect, we also suspect that probably there is an unclear bioactive association between VA and PHBA, or on either these compounds severs as a signal substance, stimulating the other one's biosynthesis. Latreche and Rahmania (2011) showed that both *p*-hydroxybenzoic acid (PHBA) and *p*-coumaric acid (PCA) involved in defense mechanism of date palm (*Phoenix dactylifera* L.) against Brittle leaf disease, however the biosynthetic pathway of PHBA was proved formation from PCA. Meanwhile, VA is produced from an oxidized form of vanillin, observed at great contents in vanilla beans and *Angelica sinensis* plants (Gitzinger et al., 2011). Both VA and PHBA were reported to be the plant growth regulators contributing to limitation of the leaf shrinkage and senescence under heat-stress conditions (Ahmad et al., 2016). Hence, the combined treatment of VA and PHBA probably acted as a trigger producing drought-resistant phytoalexins to cope with soil water deficit situation. A same observation in the moss *Hypnum plumaeforme*, jasmonic acid activated the biosynthesis of MA and MB, showing that the appearance of these momilactones correlated to abiotic and biotic stressors, to which the moss may be exposed (Kato-Noguchi and Kobayashi, 2009). Also, jasmonic acid functioned as a signal transduction molecule involving in production of MA by treatment with an elicitor (N-acetylchitoheptaose) in

suspension-cultured rice cells (Nojiri et al., 1996). Otherwise, jasmonic acid not only participated in momilactone biosynthesis, but also stimulated producing plant secondary metabolites against abiotic stresses, such as salinity and water stresses (Akula and Ravishankar, 2011). The findings of this study suggested that besides allelopathic, antibacterial, and antifungal activities (Kato-Noguchi and Ota, 2013b; Fukuta et al., 2007), rice momilactones A and B induced by exogenous VA and PHBA combination might be relevant to drought tolerance ability. As the report of Xuan et al. (2016) also shown that water-stress tolerance activity of MA and MB was higher than weed resistance (allelopathic activity against weeds) in rice plants. There was no linkage between allelopathy and momilactone biosynthetic gene cluster by QTL (quantitative trait locus) mapping (Jensen et al., 2001). The important role of momilactones has recently been highlighted by the appearance of a specific gene cluster in the genome of rice, which has been associated with plant defense mechanism (Xu et al., 2012). Shimura et al. (2007) also reported that the presence of two functional genes, *CYP99A2* and *CYP99A3* on chromosome 4 involved in rice momilactone biosynthesis. However, the activation of momilactone biosynthesis genes could vary among rice varieties that possibly induce the difference of MA and MB concentrations (Xuan et al., 2016). This also explained why a significant amount of MB was only detected in Q8 control and the progressive change of MA and MB contents in Q8 tended to increment greater than Q2 after conjugated treatments of VA and PHBA (Table 10). Evidently, Q8 performed its drought tolerance attribution better than Q2 in absorbance of VA and PHBA and biosynthesis of momilactones A and B.

4.2. Conclusions

In summary, our results corroborate that conjugated pretreatment of exogenous VA and PHBA caused a positive effect on rice plant exposed to water stress. The capacity of tolerance and recovery of rice plant under water stress conditions depended on the relevant

spraying concentration of VA and PHBA. Interestingly, this study is first to prove that VA and PHBA correlated to the production of MA and MB and these momilactones may also involve in drought tolerance mechanism of rice plants. Therefore, the bioactive of VA and PHBA related to rice diterpenoid momilactone biosynthesis can serve as a molecular evidence for further investigations of genetic engineering or plant breeding, contributing to development of new crops against environmental stresses.

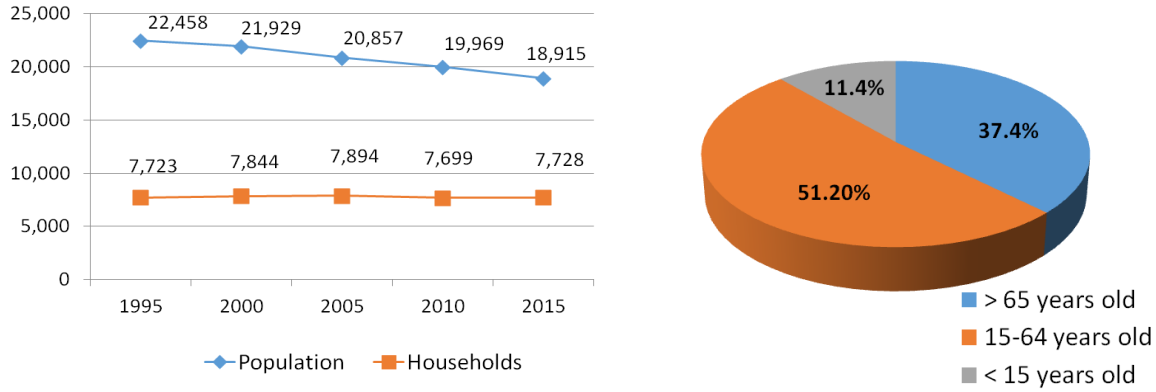
In the relationship to common objectives of Taoyaka program to create a flexible, enduring, and peaceful society, the aim of this study partly contributed to the development of agriculture sector in general and rice crop in particularly in developing countries. In fact, the use of exogenous phenolic compounds as bio-reagents to enhance stress tolerance of plants is not only treated on rice product, but also applied on other food crops with adaptation to various severe environmental changes, such as hot, cold, submergence, salinity and drought stresses. The phytoalexins momilatones (A and B) and phenolic acids, vanillic and *p*-hydroxybenzoic acids will continue to be further investigated to clarify their function groups and search for novel compounds with lower cost and higher bioactivities to be able to produce potent agrochemicals using for drought tolerance improvement in rice. On the other hand, in some measure, developing bioactive reagents to protect rice production from drought-prone conditions also contributes to the emissive reduction of atmospheric methane (CH_4), one of the most serious greenhouse gases produced by consuming and oxidizing bacterial processes, which organic carbon sources for methanogenic substrates exudated from rice roots in flooded paddy fields.

CHAPTER V.

ONSITE TEAM PROJECT – TAOYAKA PROGRAM

5.1. Background and key issues

Japan's population is decreasing rapidly (Matanle and Rausch, 2011). Many small rural municipalities are facing to the depopulation problem caused by aging society and rural out-migration (Thompson et al., 2003; Coulmas et al., 2008). Kita-Hiroshima is one of the typical towns seriously affected by depopulation issue, resulting in the existence of many empty houses and abandoned agricultural lands. Kita-Hiroshima town is located in the north western part of Hiroshima prefecture belong to the Chugoku mountainous area, where has a population of 18,915 people are living and working. The population density in the town is quite sparse, only 31 people/km^2, while Hiroshima prefecture's average is 337.4 people/km^2 (Japan Statistics Bureau, 2016). The total population of Kita-Hiroshima has in fact decreased in the 20 years from 22,458 people in 2006 to 18,915 in 2015 (Figure 14a). Whereas, the number of households remain unchanged remarkably over the last 20 years, from 7,723 households in 1995 to 7,728 in 2015 (Figure 14a). Furthermore, the number of children under 15 years old accounts for 11.4% much less than the number of people over 65 years old, accounting for 37.4% (Figure 14b). Due to the existence of old population and people's livelihood mostly depends on husbandry and social insurance benefits from the government, obviously the socio-economy in Kita-Hiroshima is being decreased in the a beforehand scenario.



(a) (b)

Figure 14. Depopulation issue (a) and age profile (b) in Kita-Hiroshima (Kitahiroshima, 2016)

To solve and slow down the depopulation problem in Kita-Hiroshima town, the Onsite team project with the theme: "Revitalization of Kita-Hiroshima by Eco-tourism" was conducted under the support and supervision of TAOYAKA program, Hiroshima University. The main objective of this project is to introduce new local business of eco-tourism to attract more visitors or outside people coming to Kita-Hiroshima for traveling, residing, and working. Besides, this project also offer good chances to local community to earn side income by utilizing local available resources, such as human, cultural, natural, and agricultural resources.

In this project, agriculture sector was considered as one of the economic activities of local people, thus the practical investigations on agricultural development in order to support for tourism activities were implemented. Futhermore, environmental aspect was considered as an un-separable sector to evaluate the impacts of agricultural developing activities, therefore it also included in the project. In this plan, basing on personal background knowledge, besides supporting for tourism activities, I was mainly in charge of experimental researches on agricultural crop improvement, with the title "Application of rice-husk in paddy-weed suppression". The aim of this study is to find out new alternative solutions to replace commercial herbicide in paddy-weed control, contributing to reduction of soil environment

pollution from chemical residues of herbicide.

In a net investigation in rice fields of Kita-Hiroshima, paddy-weed was determined as one of the major threats leading to the decrease of rice yield and quality. Among harmful weeds, monochoria and barnyard grasses are the common paddy-weeds presented in many rice fields in Kita-Hiroshima (Figure 15). These weeds often inhibit the growth and development of rice plants by exuding endogenous toxic chemicals from roots (Cheng and Cheng, 2015). However, in the previous studies, rice-husk was also showed containing some specific chemical components such as phenolics and phytoalexin momilactones, which may suppress the invasion of paddy-weeds (Khanh et al., 2007). Therefore, the role of rice-husk using as a helpful residue material to replace chemical herbicide was researched and performed in this project.

In addition, the survey on agricultural sector in Kita-Hiroshima suggested that one of three common rice-husk varieties including Koshihikari, Akiroman and Akitakomachi probably accumulated much allelochemicals against the growth of monochoria and barnyard grasses. Hence, this research also supports for chemical analysis and extraction from above collected rice-husk samples, and then these compounds might be developed to produce bio-herbicide in future.

Figure 15. Monochoria and barnyard grasses were popularly observed in a rice field in Kita-Hiroshima.

5.2. Objectives

The main objectives of this study included: (1) evaluation of paddy-weed suppression property of three varieties of rice-husks (Koshihikari, Akiroman and Akitakomachi), (2) Identification of total phenolic contents of three collected rice-husk varieties, (3) extraction of potent allelochemicals (phytoalexin momilactones) involved in allelopathic activity of rice-husk against paddy-weeds.

5.3. Materials and methods

** Materials:*

The rice-husk samples of Koshihikari, Akiroman, and Akitakomachi varieties were collected in Kita-Hiroshima area, with 3 kg per each rice variety (Figure 16a). The seeds of two common paddy-weeds included barnyard grass (*Echinochloa crus-galli*) and monochoria (*Monochoria vaginalis* Presl var.) were also collected from the rice fields of Kita-Hiroshima

(Figures 16b and 16c). Besides, soil and herbicide reagent (KAI) were bought from JA Co., Hiroshima, Japan. All chemicals used for chemical analysis and extraction, including standards of phenolics and phytoalexin momilactones were purchased from Kanto Chemical Co. Inc., Japan.

(a) (b) (c)

Figure 16. Rice-husks of Koshihikari, Akiroman, and Akitakomachi (a) and paddy-weed seeds of barnyard grass (b) and monochoria (c) collected from Kita-Hiroshima area.

** Methods:*

The experiments were conducted in Plant Physiology laboratory of the Graduate School for International Development and Cooperation (IDEC), Hiroshima University for three months, from October to December, 2016.

The rice-husks of Koshihikari, Akiroman, and Akitakomachi were firstly ground into powder form, and then they were mixed with standard soils, following the scale: 1 gram of rice-husk + 500 gram of soil (one treatment). After that, water was filled up to keep moisture of rice-husk mixed soil for 5 days before planting rice plants and paddy-weeds together. In this experiment, ten rice seeds of Koshihikari variety were used as the test rice plants to germinate together with twenty seeds of barnyard grass or monochoria in different rice-husk mixed soil (Figure 17). After 14 days sowing, the survival rate of paddy-weeds and rice plants (Koshihikari) would be checked and calculated to evaluate the paddy-weed inhibiting property of three kinds of rice-husks (Koshihikari, Akiroman, and Akitakomachi).

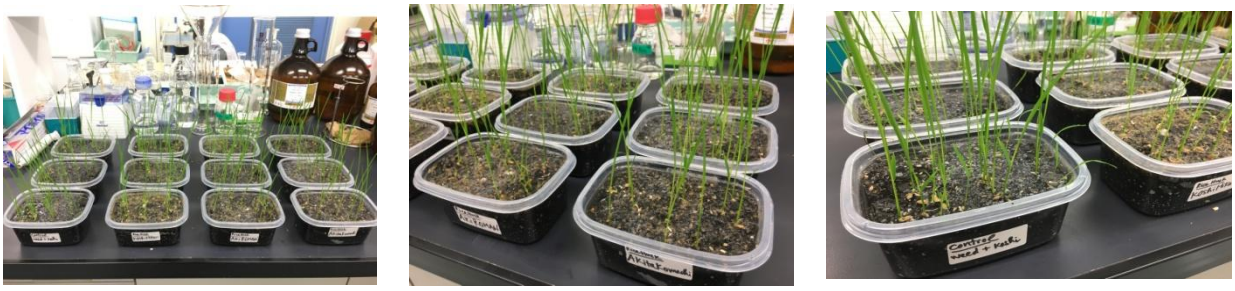


Figure 17. Ten rice seeds and twenty weed seeds were grown together in the different rice-husk mixed soils and each treatment was replicated 3 times

In this experiment, herbicide reagent was used as a separated treatment to evaluate its inhibited effectiveness on the growth of rice plant (Koshihikari) and paddy-weeds (monochoria and barnyard grasses). The control test was treated in the normal soil condition (no herbicide and rice-husks).

5.4. Results and discussion

The survival rate of rice plants (Koshihikari) was quite high and not much affected by allelopathic activity of three rice-husk varieties (Koshihikari, Akiroman, and Akitakomachi). It ranges from 86.7% to 96.7% at all treatments, including herbicide and control tests (Figure 18). The survival rate of monochoria was reduced significantly, comparing with rice plants. Koshihikari and Akitakomachi rice-husks (inhibited 95%) showed stronger allelopathic activity than Akiroman (inhibited 93.3%) in inhibiting monochoria weeds. Meanwhile, herbicide treatment inhibited monochoria weed at 98.3%. It was indicated that there was no much difference between herbicide treatment and Koshihikari and Akitakomachi rice-husk treatments.

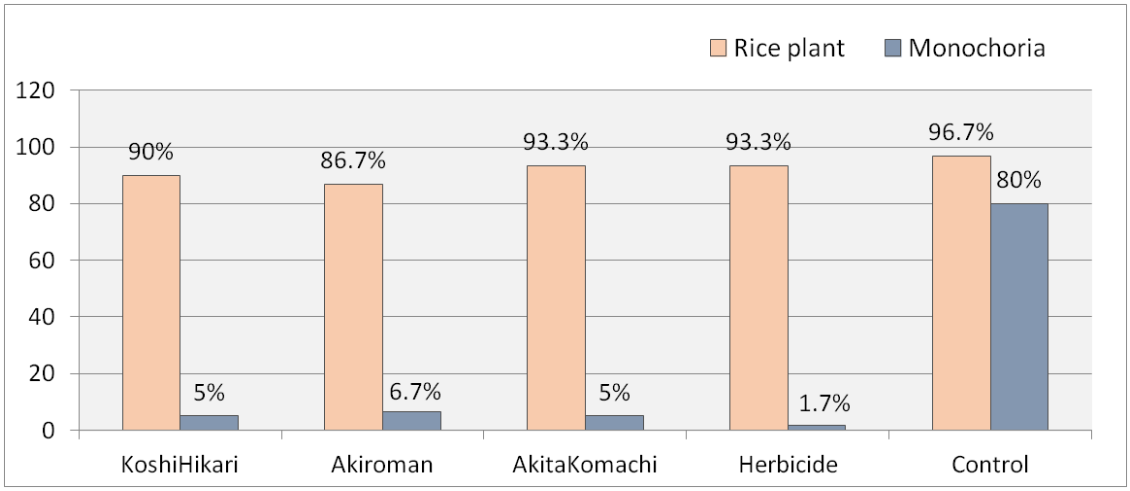


Figure 18. The survival rate of rice and monochoria after 14 days growing in the rice-husk mixed soil.

Similarly, three kinds of rice-husks did not much affect to the growth of Koshihikari rice plants, as compared with control (ranged from 86.7% to 96.7%). While, the growth of barnyard grass was inhibited significantly by treatments of rice-husks, especially barnyard grass was suppressed growing at 90% (10% survival rate) by Koshihikari rice-husk treatment. Also, by treatment of herbicide, barnyard grass was strongly inhibited at 96.7% (3.3% survival rate) (Figure 19).

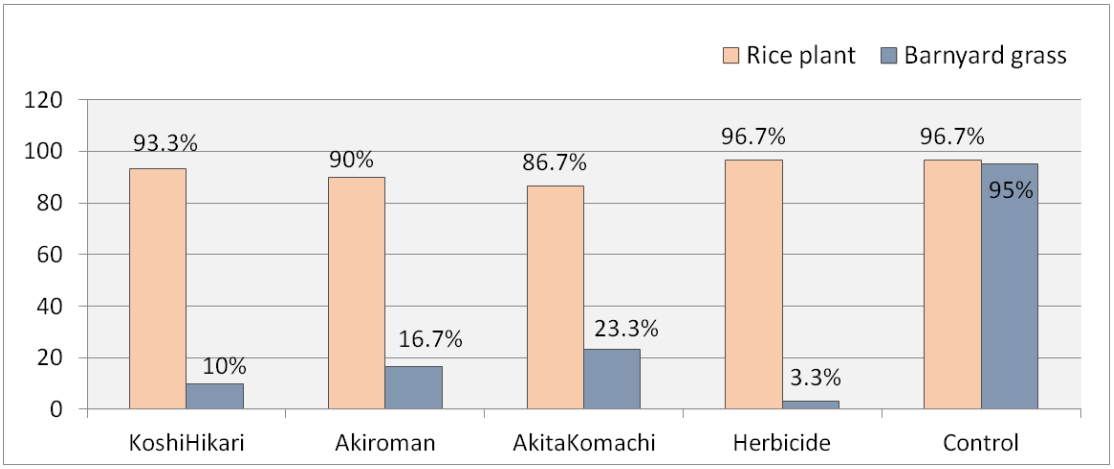


Figure 19. The survival rate of Koshihikari rice plants and barnyard grass after 14 days growing in the different rice-husk mixed soils.

Regarding to total phenolic contents in rice-husk varieties, many previous reports indicated that these phenolic compounds played an important role in plant development, especially their main function was to protect plants from environmental stresses (Bhattacharya et al., 2010). The increased phenolic contents are often closely associated with stress-resistant strength of rice plant (Oki et al., 2002). Therefore, to identify the best potential rice-husk variety, this study also analyzed the total phenolic contents of three different rice-husks (Koshihikari, Akiroman, and Akitakomachi). As a result, Koshihikari rice-husk contained the highest total phenolic content (96.54 mg gallic acid equivalent (GAE)/g dry weight), compared with other rice-husks (Figure 20). Other hand, from Koshihikari rice-husks, we suspected that this variety might contain much potent allelochemicals against the invasion of paddy-weeds. Thus, the extraction of these chemicals from Koshihikari rice-husks may help to develop eco-friendly herbicides in future. By method of Kato-Noguchi and Ota (2013), this research extracted the purified amounts of 130 mg momilactone A and 77 mg momilactone B from 30 kg Koshihikari rice-husks. In fact, momilactones A and B were firstly isolated from rice-husks as plant growth inhibitors (Kato et al., 1973) and it was also reported as phytoalexins against plant disease pathogens or as allelochemicals suppressing the development of paddy-weeds (Xuan et al., 2016).

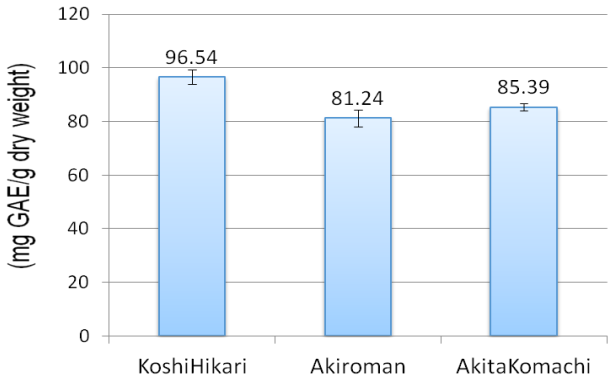


Figure 20. Total phenolic content of three rice-husks Koshihikari, Akiroman, and Akitakomachi.

5.5. Conclusions

- Koshihikari rice-husk with the highest accumulation of total phenolic content showed the strongest allelopathic activity, inhibiting significantly the growth of monochoria and barnyard grasses.
- Koshihikari rice-husk could be utilized as the useful material to replace herbicide in paddy-weed control
- Momilactones A and B are potential chemical components which should be continuously investigated to be able to produce bio-herbicides in future.

5.6. Evaluation of feasibility

Through the achieved results from the research, the effect of rice-husks on paddy-weed suppression was quite potential and effective, and mostly it is equal to the performance of commercial herbicide (Table 12). Furthermore, rice-husk is always available in rice fields after harvesting. Relating to the cost, it is necessary to grind rice-husk into powder form before applying in field, followed by the expense for grinding with a machine 7.5 Kwh is 150 Yen/100 kg/1000m^2. Whereas, using herbicide will be more expensive with the cost is 2,960 Yen/500ml/1000m^2; and this method is harmful to environmental soil as well as natural ecology. Hence, this study recommends that Koshihikari rice-husk is considerd as the useful residues to utilize for paddy-weed control and management (Table 12).

Table 12. Effective comparison between rice-husks and commercial herbicide.

Factors	**Herbicide**		**Rice-husk (Koshi)**	
	Positive	Negative	Positive	Negative
Cost		✓	✓	
Environment impacts		✓	✓	
Weed suppression effects	✓		✓	
Energy consumption	✓			✓

5.7. Recommendations

The positive effect of utilizing Koshihikari rice husks for paddy-weed control has been demonstrated in laboratory experiments. The cost for using rice-husk is also cheeper than commercial herbicide. However, the reality application in rice field needs a given time to implement and evaluate on the feasibility. Therefore, the Onsite project in Kita-Hiroshima should be extended the time and supported more about finance. Especially, this research forwards to development of bio-herbicides after extraction of potential allelochemicals, such as momilactone A and momilactone B in Koshihikari variety. Moreover, it is also necessary to organize a workshop or symposium to introduce new solution of paddy-weed management to local people, and then propably applying widely in Kita-Hiroshima.

REFERENCES

Ahmad, W., Noor, M. A., Afzal, I., Bakhtavar, M. A., Nawaz, M. M., Sun, X., Zhou, B., Ma, W., & Zhao, M. (2016). Improvement of sorghum crop through exogenous application of natural growth-promoting substances under a changing climate. *Sustainability, 8*(12), 1330.

Akula, R., & Ravishankar, G. A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling and Behavior*, *6*(11), 1720-1731.

Al-Gabbiesh, A., Kleinwachter, M., Selmar, D. (2015). Influencing the contents of secondary metabolites in spice and medicinal plants by deliberately applying drought stress during their cultivation. *Jordan J Biol Sci*. *8*(1), 1-10.

Alvarez, S., Marsh, E. L., Schroeder, S. G., & Schachtman, D. P. (2008). Metabolomic and proteomic changes in the xylem sap of maize under drought. *Plant Cell Environ.*, *31*, 325-340.

Anjum, S. A., Xie, X. Y., Wang, L. C., Saleem, M. F., Man, C., & Lei, W. (2011). Morphological, physiological and biochemical responses of plants to drought stress. *African Journal of Agricultural Research*, *6*(9), 2026-2032.

Ansari, M. I., & Lin, T. P. (2010). Molecular analysis of dehydration in plants. *International Research Journal of Plant Science*, *1*, 21-25.

Ashraf, M., & Foolad, M. (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environ Exp Bot., 59*(2), 206-216.

Balasundram, N., Sundram, K., & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food chemistry*, *99*(1), 191-203.

Basu, S., Ramegowda, V., Kumar, A., Pereira, A. (2016). Plant adaptation to drought stress. *F1000Research*, 5.

Bhattacharya, A., Sood, P., & Citovsky, V. (2010). The roles of plant phenolics in defence and communication during agrobacterium and rhizobium infection. *Molecular plant pathology*, *11*(5), 705-719.

Bilger, W., Veit, M., Schreiber, L., & Schreiber, U. (1997). Measurement of leaf epidermal transmittance of UV radiation by chlorophyll fluorescence. *Physiol Plant.*, *101*(4), 754-763.

Bourgaud, F., Gravot, A., Milesi, S., & Gontier, E. (2001). Production of plant secondary metabolites: a historical perspective. *Plant Science, 161*(5), 839-851.

Callaway, R. M., & Vivanco, J. .M. (2006). Can plant biochemistry contribute to understanding of invasion ecology?. *Trends in Plant Science*, *11*(12), 574-580.

Chatterjee, A., Saha, P. K., Gupta, P. D., Ganguly, S. N., & Sircar, S. M. (1976) Chemical examination of viable and non-viable rice seeds. *Physiol. Plant.*, *38,* 307-308.

Chaves, M. M., & Oliveira, M. M. (2004). Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. *J. Exp. Bot., 55*(407), 2365-2384.

Cheng, F., & Cheng, Z. (2015). Research progress on the use of plant allelopathy in agriculture and the physiological and ecological mechanisms of allelopathy. *Frontiers in plant science*, *6*, 1020.

Cho, M. H., & Lee, S. W. (2015). Phenolic phytoalexins in rice: Biological functions and biosynthesis. *Int. J. Mol. Sci*., *16*, 29120-29133.

Chung, I. M., Hahn, S. J., & Ahmad, A. (2005). Confirmation of potential herbicidal agents in hulls of rice, *Oryza sativa*. *J. Chem. Ecol. 31*(6), 1339-1352.

Coulmas, F., Conrad, H., Schad-Seifert, A., & Vogt, G. (Eds.). (2008). The demographic challenge: A handbook about Japan. *Brill*.

Crozier, A., Clifford, M. N., & Ashihara, H. (Eds.). (2008). Plant secondary metabolites: occurrence, structure and role in the human diet. *John Wiley & Sons.* Chapter 1: 1-2.

Daw, B. D., Zhang, L. H., & Wang, Z. Z. (2008). Salicylic acid enhances antifungal resistance to *Magnaporthe grisea* in rice plants. *Australasian Plant Pathology, 37*(6), 637-644.

de Abreu, I. N., & Mazzafera, P. (2005). Effect of water and temperature stress on the content of active constituents of *Hypericum brasiliense* Choisy. *Plant Physiol Biochem.*, *43*(3), 241-248.

Ding, S., Pandey, S., Chen, C., & Bhandari, H. (2008). Drought and farmers' coping strategies in poverty-afflicted rural China. *In SARPN Conference on Social Protection for Chronic Poverty, University of Manchester.* Available at www. sed. Manchester. ac. Uk/research. Accessed on June 25th, 2018.

Dramé, K. N., Clavel, D., Repelin, A., Passaquet, C., & Zuily-Fodil, Y. (2007). Water deficit induces variation in expression of stress-responsive genes in two peanut (*Arachis hypogaea* L.) cultivars with different tolerance to drought. *Plant Physiology and Biochemitry, 45*, 236-243.

Du Fall, L. A., & Solomon, P. S. (2011). Role of cereal secondary metabolites involved in mediating the outcome of plant-pathogen interactions. *Metabolites 1*(1), 64-78.

El-Basyouni, S. Z., Chen, D., Ibrahim, R. K., Neish, A. C., & Towers, G. H. N. (1964). The biosynthesis of hydroxybenzoic acids in higher plants. *Phytochemistry*, *3*, 485-492.

Elzaawely, A. A., Tawata, S. (2012a). Antioxidant capacity and phenolic content of *Rumex dentatus* L. grown in Egypt. *J. Crop Sci. Biotech., 15*, 59–64.

Elzaawely, A.A., Tawata, S. (2012b). Antioxidant activity of phenolic rich fraction obtained from *Convolvulus arvensis* L. leaves grown in Egypt. *Asian J. Crop Sci*., *4*, 32–40.

Elzaawely, A. A., Xuan, T. D., & Tawata, S. (2005). Antioxidant and antibacterial activities of *Rumex japonicus* Houtt. aerial parts. *Biol. Pharm. Bull., 28,* 2225-2230.

Fang, Y., & Xiong, L. (2015). General mechanisms of drought response and their application in drought resistance improvement in plants. *Cell. Mol. life Sci., 72*(4), 673-689.

Farooq, M., Basra, S. M. A., Wahid, A., Ahmad, N., & Saleem, B. A. (2009). Improving the drought tolerance in rice (*Oryza sativa* L.) by exogenous application of salicylic acid. *Journal of Agronomy and Crop Science*, *195*(4), 237-246.

Fayez, K. A., & Bazaid, S. A. (2014). Improving drought and salinity tolerance in barley by application of salicylic acid and potassium nitrate. *Journal of the Saudi Society of Agricultural Sciences*, *13*(1), 45-55.

Fernández-García, N., Olmos, E., Bardisi, E., García-De la Garma, J., López-Berenguer, C., Rubio-Asensio, J. S. (2014). Intrinsic water use efficiency controls the adaptation to high salinity in a semi-arid adapted plant, henna (*Lawsonia inermis* L.). *J. Plant Physiol*., *171*, 64–75.

Food and Agriculture Organization of the United Nations (FAO). (2016). *Statistical database*. [online] Rome: Food and Agriculture Organization of the United Nations. Available at: http://faostat3.fao.org/home/E. [Accessed on 21 May 2018].

Food and Agriculture Organization of the United Nations (FAO), (2017). *Rice Market Monitor, 20(1)*. Available at: http://fao.org/economic/RMM. [Accessed on 21 May 2018].

Fraire-Velázquez, S., & Balderas-Hernández, V. E. (2013). Abiotic stress in plants and metabolic responses. In: Abiotic Stress-Plant Responses and Applications in Agriculture. InTech. Chapter 2: 25-48.

Fukuta, M., Xuan, T. D., Deba, F., Tawata, S., Khanh, T. D., & Chung, I. M. (2007). Comparative efficacies in vitro of antibacterial, fungicidal, antioxidant, and herbicidal activities of momilatones A and B. *Journal of Plant Interactions*, *2*(4), 245-251.

Ghareib H. R. A., Abdelhamed, M. S., & Ibrahim, O. H. (2010). Antioxidative effects of the

acetone fraction and vanillic acid from *Chenopodium murale* on tomato plants. *Weed Biol Manag., 10*(1), 64-72.

Gitzinger, M., Kemmer, C., Fluri, D. A., Daoud El-Baba, M., Weber, W., & Fussenegger, M. (2011). The food additive vanillic acid controls transgene expression in mammalian cells and mice. *Nucleic acids research*, *40*(5), e37-e37.

Goswami, A., Banerjee, R., & Raha, S. (2013). Drought resistance in rice seedlings conferred by seed priming. *Protoplasma*, *250*(5), 1115-1129.

Hegab, M. M., & Ghareib, H. R. (2010). Antioxidative effects of acetone fraction and vanillic acid from *Chenopodium murale* L. on tomato plant. *Weed Biol. Manag., 10*, 64-72.

Hu, H., Dai, M., Yao, J., Xiao, B., Li, X., Zhang, Q. & Xiong, L. (2006). Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proc. Nat. Acad. Sci. USA*, *103*, 12987–12992.

Hura, T., Hura, K., Dziurka, K., Ostrowska, A., Bączek-Kwinta, R., & Grzesiak, M. (2012). An increase in the content of cell wall-bound phenolics correlates with the productivity of triticale under soil drought. *J. Plant Physiol., 169*(17), 1728-1736.

Hura, T., Hura, K., & Grzesiak, M. (2011). Soil drought applied during the vegetative growth of triticale modifies the physiological and biochemical adaptation to drought during the generative development. *J. Agron. Crop Sci. 197*(2), 113-123.

Hura, T., Hura, K., & Grzesiak, S. (2009). Possible contribution of cell-wall-bound ferulic acid in drought resistance and recovery in triticale seedlings. *Journal of Plant Physiology*, *166*, 1720-1733.

International Rice Research Institute [IRRI]. 1996. Standard evaluation system for rice, 4th Edition, IRRI, Manila, Philippines, p. 37.

IRRI (International Rice Research Institute). Standard evaluation system for rice. 2nd Edition, IRRI, Los Banos, Philippines, **1980**; p. 44 (accessed on 25 June 2015).

Japan Statistics Bureau (JSB), Ministry of Internal Affairs and Communication (2016). Japan statistical yearbook, Chapter 2: Population and households. (Retrieved on June 6th, 2018).

Japan Statistics Bureau (JSB), Ministry of Internal Affairs and Communication (2015). Population and number of households in KitaHiroshima-cho. http://www.citypopulation.de/php/japan-hiroshima (Accessed on June 6th, 2018).

Jensen, L. B., Courtois, B., Shen, L., Li, Z., Olofsdotter, M., & Mauleon, R. P. (2001). Locating genes controlling allelopathic effects against barnyardgrass in upland rice.

Agronomy Journal, *93*(1), 21-26.

Juliano, B. O., & Villareal, C. P. (1993). Grain quality evaluation of world rices. Manila, Philippines, Int. Rice Res. Inst. (IRRI), 205.

Kamaya, Y., Tsuboi, S., Takada, T., & Suzuki, K. (2006). Growth stimulation and inhibition effects of 4-hydroxybenzoic acid and some related compounds on the freshwater green alga *Pseudokirchneriella subcapitata*. *Arch. Environ. Contam. Toxicol., 51*(4), 537-541.

Karimi, E., Mehrabanjoubani, P., Keshavarzian, M., Oskoueian, E., Jaafar, H. Z., & Abdolzadeh, A. (2014). Identification and quantification of phenolic and flavonoid components in straw and seed husk of some rice varieties (*Oryza sativa* L.) and their antioxidant properties. *J. Sci. Food Agric*., *94*, 2324-2330.

Kato-Noguchi, H., & Kobayashi, K. (2009). Jasmonic acid, protein phosphatase inhibitor, metals and UV-irradiation increased momilactone A and B concentrations in the moss *Hypnum plumaeforme*. *Journal of Plant Physiology*, *166*(10), 1118-1122.

Kato-Noguchi, H. (2004). Allelopathic substance in rice root exudates: rediscovery of momilactone B as an allelochemical. *Journal of plant physiology*, *161*(3), 271-276.

Kato-Noguchi, H., & Peters, R. J. (2013a). The role of momilactones in rice allelopathy. *J. Chem. Ecol., 39*(2), 175-185.

Kato-Noguchi, H., & Ota, K. (2013b). Biological activities of rice allelochemicals momilactone A and B. *Rice Research. 1*(2), 108.

Kato, T., Kabuto, C., Sasaki, N., Tsunagawa, M., Aizawa, H., Fujita, K., Kato, Y., Kitahara, Y., & Takahashi, N. (1973). Momilactones, growth inhibitors from rice, *Oryza sativa* L.. *Tetrahedron Lett. 14*(39), 3861-3864.

Kennedy, G., Burlingame, B., & Nguyen, V. N. (2003). Nutritional contribution of rice and impact of biotechnology and biodiversity in rice-consuming countries.

Kim, J. M., To, T. K., Matsui, A., Tanoi, K., Kobayashi, N. I., Matsuda, F., Habu, Y., Ogawa, D., Sakamoto, T., & Matsunaga, S. (2017). Acetate-mediated novel survival strategy against drought in plants. *Nat. Plants 3*(7), 17097.

Kitahiroshima-cho data (2016). http://www.town.kitahiroshima.lg.jp (accessed on 11 June 2018).

Khadem, S., & Marles, R. J. (2010). Monocyclic phenolic acids; hydroxy- and polyhydroxybenzoic acids: occurrence and recent bioactivity studies. *Molecules 15*(11), 7985-8005.

Khandaker, L., Masum-Akond, A. S. M. G., & Oba, S. (2011). Foliar application of salicylic acid improved the growth, yield and leaf's bioactive compounds in red amaranth (*Amaranthus tricolor* L.). *Vegetable Crops Res. Bulletin, 74*, 77-86.

Khanh, T. D., Xuan, T. D., & Chung, I. M. (2007). Rice allelopathy and the possibility for weed management. *Annals of Applied Biology*, *151*(3), 325-339.

Ko, J. H., Kim, B. G., Kim, J. H., Kim, H., Lim, C. E., Lim, J., Lee, C., Lim, Y., & Ahn, J. H. (2008). Four glucosyltransferases from rice: cDNA cloning, expression, and characterization. *J. Plant Physiol*., *165*(4), 435-444.

Kong, C. H., Li, H. B., Hu, F., Xu, X. H., & Wang, P. (2006). Allelochemicals released by rice roots and residues in soil. *Plant Soil*, *288*(1), 47-56.

Kong, C., Liang, W., Xu, X., Hu, F., Wang, P., & Jiang, Y. (2004). Release and activity of allelochemicals from allelopathic rice seedlings. *J. Agric. Food Chem., 52*(10), 2861-2865.

Lanari, V., Silvestroni, O., Palliotti, A., Green, A., & Sabbatini, P. (2015). Plant and leaf physiological responses to water stress in potted 'Vignoles' grapevine. *Hort. Sci. 50*(10), 1492-1497.

Lang, N. T. & Buu, B. C. (2010). Quantitative trait loci influencing drought tolerance in rice (*Oryza sativa* L.). *Omonrice*, *17,* 22-28.

Latreche, K., & Rahmania, F. (2011). High extracellular accumulation of *p*-hydroxybenzoic acid, p-hydroxycinnamic acid and *p*-hydroxybenzaldehyde in leaves of Phoenix dactylifera L. affected by the brittle leaf disease. *Physiological and Molecular Plant Pathology*, *76*(2), 144-151.

Lattanzio, V., Lattanzio, V. M., & Cardinali, A. (2006). Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. *Phytochemistry: Advances in Research*, *661*, 23-67.

Le Gall, H., Philippe, F., Domon, J. M., Gillet. F., Pelloux, J., & Rayon, C. (2015). Cell wall metabolism in response to abiotic stress. *Plants 4*(1), 112-166.

Lichtenthaler, H. K., & Wellburn, A. R. (1983). Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochem. Soc. Trans., 11*, 591-592.

Ma, D., Sun, D., Wang, C., Li, Y., & Guo, T. (2013). Expression of flavonoid biosynthesis genes and accumulation of flavonoid in wheat leaves in response to drought stress. *Plant Physiology and Biochemistry*, *80*, 60-66.

Markham, K. R. Tanner, G. J., Caasi-Lit, M., Whitecross, M. I., Nayudu, M., & Mitchell, K. A. (1998). Possible protective role for 3', 4'-dihydroxyflavones induced by enhanced UV-B in a UV-tolerant rice cultivar. *Phytochemistry*, *49*, 1913–1919.

Matanle, P., & Rausch, A. S. (2011). Japan's shrinking regions in the 21st century: contemporary responses to depopulation and socioeconomic decline. *Cambria Press*. https://papers.ssrn.com/sol3/papers.cfm?abstract_id=1971833 (Accessed on June 6th, 2018).

Mir, R. R., Zaman-Allah, M., Sreenivasulu, N., Trethowan, R., & Varshney, R. K. (2012). Integrated genomics, physiology and breeding approaches for improving drought tolerance in crops. *Theor. Appl. Genet.*, *125*, 625–645.

Mohanty, S., Wassmann, R., Nelson, A., Moya, P., & Jagadish, S. V. K. (2013). Rice and climate change: significance for food security and vulnerability. *Discussion Paper Series No. 49,* International Rice Research Institute, Los Baños, Philippines: 14 p.

Morales, F., Cerovic, Z. G., & Moya, I. (1996). Time-resolved blue-green fluorescence of sugar beet (*Beta vulgaris* L.) leaves. Spectroscopic evidence for the presence of ferulic acid as the main fluorophore of the epidermis. *Biochimica et Biophysica Acta (BBA)-Bioenergetics 1273*(3), 251-262.

Moran-Palacio, E. F., Zamora-Álvarez, L. A., Stephens-Camacho, N. A., Yáñez-Farías, G. A., Virgen-Ortiz, A., Martínez-Cruz, O., & Rosas-Rodríguez, J. A. (2014). Antioxidant capacity, radical scavenging kinetics and phenolic profile of methanol extracts of wild plants of Southern Sonora, Mexico. *Trop. J. Pharm. Res., 13*(9), 1487-1493.

Neelam, M., Rahul, M., Ajiboye, M., Kafayat, Y., & Lateefat, Y. (2014). Salicylic acid alters antioxidant and phenolics metabolism in *Catharanthus roseus* grown under salinity stress. *Afr. J. Tradit. Complement. Altern. Med., 11*(5), 118-125.

Nichols, S. N., Hofmann, R. W., Williams, W. M. (2015). Physiological drought resistance and accumulation of leaf phenolics in white clover interspecific hybrids. *Environ. Exp. Bot. 119*, 40-47.

Nogués, S., Allen, D. J., Morison, J. I., & Baker, N. R. (1998). Ultraviolet-B radiation effects on water relations, leaf development, and photosynthesis in droughted pea plants. *Plant Physiol., 117*(1), 173-181.

Nojiri, H., Sugimori, M., Yamane, H., Nishimura, Y., Yamada, A., Shibuya, N., Kodama, O., Murofushi, N., & Omori, T. (1996). Involvement of jasmonic acid in elicitor-induced phytoalexin production in suspension-cultured rice cells. *Plant*

Physiology, *110*(2), 387-392.

Oki, T., Masuda, M., Kobayashi, M., Nishiba, Y., Furuta, S., Suda, I., & Sato, T. (2002). Polymeric procyanidins as radical-scavenging components in red-hulled rice. *J. Agric. Food Chem., 50*(26), 7524-7529.

Pandey S., Bhandari, H. S., & Hardy, B. (Eds.). (2007). *Economic costs of drought and rice farmers' coping mechanism: a cross-country comparative analysis*. Los Banos, Philippines, Int. Rice Res. Inst. (IRRI).

Park, H. L., Lee, S. W., Jung, K. H., Hahn, T. R., & Cho, M. H. (2013) Transcriptomic analysis of UV-treated rice leaves reveals UV-induced phytoalexin biosynthetic pathways and their regulatory networks in rice. *Phytochemistry*, *96*, 57-71.

Parker, D., Beckmann, M., Zubair, H., Enot, D. P., Caracuel-Rois, Z., Overy, D. P., Snowdon, S., Talbot, N. J., & Draper, J. (2009). Metabolomic analysis reveals a common pattern of metabolic re-programming during invasion of three host plant species by *Magnaporthe grisea*. *Plant J*., *59*, 723-737.

Quan, N. T, Anh, L. H., Khang, D. T., Tuyen, P. T., Toan, N. P., Minh, T. N., Minh, L. T., Bach, D. T., Ha, P. T. T., Elzaawely, A. A., Trung, K. H., Khanh, T. D., & Xuan, T. D. (2016). Involvement of secondary metabolites in response to drought stress of rice (*Oryza sativa* L.). *Agriculture 6*(2):23.

Rao, S. R., & Ravishankar, G. A. (2002). Plant cell cultures: chemical factories of secondary metabolites. *Biotechnology Advances*, *20*, 101-153.

Reddy, A. R., Chaitanya, K. V., & Vivekanandan, M. (2004). Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. *Journal of Plant Physiology*, *161*(11), 1189-1202.

Redfern, S. K., Azzu, N., & Binamira, J. S. (2012). Rice in Southeast Asia: facing risks and vulnerabilities to respond to climate change. *Build Resilience Adapt Climate Change Agri Sector*, *23*, 295.

Rehman, F., Khan, F. A., & Badruddin, S. M. A. (2012). Role of phenolics in plant defense against insect herbivory. Chemistry of phytopotentials: *Health, energy and environmental perspectives.* Springer, Berlin, Heidelberg, 309-313.

Reuber, S., Bornman, J. F., & Weissenböck, G. (1996). Phenylpropanoid compounds in primary leaf tissues of rye (*Secale cereale*). Light response of their metabolism and the possible role in UV-B protection. *Physiol. Plant. 97*(1), 160-168.

Ruelas, C., Tiznado-Hernandez, M. E., Sanchez-Estrada, A., Robles-Burgueno, M. R., &

Troncoso-Rojas, R. (2006). Changes in phenolic acid content during *Alternaria alternate* infection in tomato fruit. *J. Phytopathol., 154*(4), 236-244.

Sabar, M., & Arif, M. (2014). Phenotypic response of rice (*Oryza sativa*) genotypes to variable moisture stress regimes. *Int. J. Agric. Biol. 16*, 32-40.

Sah, S. K., Reddy, K. R., & Li, J. (2016). Abscisic acid and abiotic stress tolerance in crop plants. *Frontiers in Plant Science*, *7*, 571.

Saikumar, S. Gouda, P. K., Saiharini, A., Varma, C. .M. K., Vineesha, O., Padmavathi, G., & Shenoy, V. V. (2014). Major QTL for enhancing rice grain yield under lowland reproductive drought stress identified using an *O*. *sativa*/*O*. *glaberrima* introgression line. *Field Crops Res., 163*, 119-131.

Sánchez-Rodríguez, E., Moreno, D. A., Ferreres, F., del Mar Rubio-Wilhelmi, M., & Ruiz, J. M. (2011). Differential responses of five cherry tomato varieties to water stress: changes on phenolic metabolites and related enzymes. *Phytochemistry, 72*(8), 723-729.

Schultheiss, H., Dechert, C., Kogel, K. H., & Hückelhoven, R. (2002). A small GTP-binding host protein is required for entry of powdery mildew fungus into epidermal cells of barley. *Plant Physiol., 128*(4), 1447-1454.

Seki, M., Umezawa, T., Urano, K., & Shinozaki, K. (2007). Regulatory metabolic networks in drought stress responses. *Current Opinion in Plant Biology*, *10*(3), 296-302.

Selmar, D. (2008). Potential of salt and drought stress to increase pharmaceutical significant secondary compounds in plants. *Landbauforschung Volkenrode*, *58*, 139–144.

Setyaningsih, W., Saputro, I. E., Palma, M., Barroso, C. G. (2016). Pressurized liquid extraction of phenolic compounds from rice (*Oryza sativa*) grains. *Food Chem. 192*, 452-459.

Shao, H. B., Chu, L. Y., Jaleel, C. A., Zhao, C. X. (2008). Water-deficit stress-induced anatomical changes in higher plants. *C. R. Biol. 331*(3), 215-225.

Shimura, K., Okada, A., Okada, K., Jikumaru, Y., Ko, K. W., Tomoyasu, T., Sassa, T., Hasegawa, M., Kodama, O., & Shibuya, N. (2007). Identification of a biosynthetic gene cluster in rice for momilactones. *J. Biol. Chem. 282*(47), 34013-34018.

Shukla, N., Awasthi, R. P., Rawat, L., Kumar, J. (2012). Biochemical and physiological responses of rice (*Oryza sativa* L.) as influenced by *Trichoderma harzianum* under drought stress. *Plant Physiol. Biochem.*, *54*, 78–88.

Singh, K. M., Jha, A. K., Meena, M. S., & Singh, R. K. P. (2012). Constraints of rainfed rice

production in India: An overview. *Innovation in Rice Production, Ed: PK Shetty, MR Hegde and M. Mahadevappa, National Institute of Advance Studies, Indian Institute of Science Campus, Bangalore*, 71-84.

Soares, A. A., de Souza, C. G. M., Daniel, F. M., Ferrari, G. P., da Costa, S. M. G., & Peralta, R. M. (2009). Antioxidant activity and total phenolic content of *Agaricus brasiliensis* (*Agaricus blazei* Murril) in two stages of maturity. *Food Chem.*, *112*, 775–781.

Son, N. T., Chen, C. F., Chen, C. R., Chang, L. Y., & Minh, V. Q. (2012). Monitoring agricultural drought in the lower Mekong basin using MODIS NDVI and land surface temperature data. *International Journal of Applied Earth Observation and Geo-information*, *18*, 417-427.

Son, S., & Lewis, B. A. (2002). Free radical scavenging and antioxidative activity of caffeic acid amide and ester analogues: structure-activity relationship. *J. Agric. Food Chem.*, *50,* 468–472.

Soobrattee, M. A., Neergheen, V. S., Luximon-Ramma, A., Aruoma, O. I., & Bahorun, T. (2005). Phenolics as potential antioxidant therapeutic agents: mechanism and actions. *Mutat. Res. Fund. Mol. Mech. Mut. 579*(1), 200-213.

Subba Rao, M. V. S. S. T., & Muralikrishna, G. (2002). Evaluation of the antioxidant properties of free and bound phenolic acids from native and malted finger millet (Ragi, *Eleusine coracana* Indaf-15). *J. Agric. Food Chem*., *50*(4), 889-892.

Sytar, O., & Borankulova, A., Hemmerich, I., Rauh, C., & Smetanska, I. (2014). Effect of chlorocholine chlorid on phenolic acids accumulation and polyphenols formation of buckwheat plants. *Biol. Res. 47*(1), 19.

Tagliavini, M., & Toselli, M. (2005). Foliar application of nutrients. *Encyclopedia of Soils in the Environment*, *14*, 53-59

Taiz, L., & Zeiger, E. (2002). Plant Physiology. 3rd. *Ed. Pub. Sinauer*.

Tao, F., & Zhang, Z. (2010). Adaptation of maize production to climate change in north China plain: quantify the relative contributions of adaptation options. *European Journal of Agronomy*, *33*, 103-116.

Terashima, N., Mori, I., & Kanda, T. (1975). Biosynthesis of *p*-hydroxybenzoic acid in poplar lignin. *Phytochemistry*, *14*, 1991-1992.

Thompson, C. S., Traphagan, J. W., & Knight, J. (2003). Depopulation in rural Japan: population politics in Tōwa-Chō. *Demographic change and the family in Japan's*

aging society, 89-106.

Tian, S., Nakamura, K., & Kayahara, H. (2004). Analysis of phenolic compounds in white rice, brown rice, and germinated brown rice. *Journal of Agricultural and Food Chemistry*. 52, 4808-4813.

Ti, H., Zhang, R., Zhang, M., Li, Q., Wei, Z., Zhang, Y., & Ma, Y. (2014). Dynamic changes in the free and bound phenolic compounds and antioxidant activity of brown rice at different germination stages. *Food Chem*., *161*, 337-344.

Time, A. (2005). Grim reaping for Thai farmers. A news report on Southeast Asia. Asia Times Co., Ltd, Hong Kong, China. Online data: http://www.atimes.com. Accessed on June 25th, 2018.

Torras-Claveria. L., Jáureguib, O., Codinaa, C., Tiburcioa, A. F., Bastida, J., & Viladomat, F. (2012). Analysis of phenolic compounds by high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry in senescent and water-stressed tobacco. *Plant Science*, *182*, 71-78.

Tzin, V., & Galili, G. (2010). New insights into the shikimate and aromatic amino acids biosynthetic pathways in plants. *Mol. Plant*, *3*, 956-972.

Van Alfen, N. K. (2014). Food security: Development strategies. *Encyclopedia of Agriculture and Food Systems: 5-volume set* (Vol. 1). Elsevier.

Vieitez, E., Seoame, E., Gesto, D. V., Mato, C., Vazquez, A., & Carnicer, A. (1966). *p*-Hydroxybenzoic acid, a growth regulator, isolated from woody cuttings of *Ribes rubrum*. *Physiolg. Plant. 19*(2), 294-307.

Villango, S., Szekeres, A., Bencsik, O., Laposi, R., Palfi, Z., & Zsofi, Z. (2016). The effect of postveraison water deficit on the phenolic composition and concentration of the Kekfrankos (*Vitis vinifera* L.) berry. *Sci. Hort. 209*, 113-116.

Wahid, A., & Ghazanfar, A. (2006). Possible involvement of some secondary metabolites in salt tolerance of sugarcane. *J. Plant Physiol*., *163*, 723–730.

Walter, M., Marchesan, E., Massoni, P. F. S., da Silva, L. P., Sartori, G. M. S., Ferreira, R. B. (2013). Antioxidant properties of rice grains with light brown, red and black pericarp colors and the effect of processing. *Food Res. Int.*, *50,* 698–703.

Walter, M., & Marchesan, E. (2011) Phenolic compounds and antioxidant activity of rice. *Braz. Arch. Biol. Technol. 54*, 371–377.

Xuan, T. D., Khang, D. T. (2018). Effects of exogenous application of protocatechuic acid and vanillic acid to chlorophylls, phenolics and antioxidant enzymes of rice (*Oryza*

sativa L.) in submergence. *Molecules 23*(3), 620.

Xuan, T. D., Minh, T. N., & Khanh, T. D. (2016). Allelopathic momilactones A and B are implied in rice drought and salinity tolerance, not weed resistance. *Agronomy for sustainable development*, 36(3), 52.

Xuan, T.D., Tsuzuki, E., Terao, H., Matsuo, M., & Khanh, T. D. (2003). Correlation between growth inhibitory exhibition and suspected allelochemicals (phenolic compounds) in the extract of alfalfa (*Medicago sativa* L.). *Plant Prod. Sci.*, *6*, 165-171.

Xu, M., Galhano, R., Wiemann, P., Bueno, E., Tiernan, M., Wu, W., Chung, L. M., Gershenzon, J., Tudzynski, B., Sesma, A., & Peters, R. J. (2012). Genetic evidence for natural product-mediated plant–plant allelopathy in rice (*Oryza sativa*). *New Phytologist*, *193*(3), 570-575.

Yildiz-Aktas, L., Dagnon, S., Gurel, A., Gesheva, E., Edreva, A. (2009). Drought tolerance in cotton: Involvement of non-enzymatic ROS-scavenging compounds. *J. Agron. Crop Sci.*, *195*, 247-253.

Yoshioka, T., Inokuchi, T., Fujioka, S., & Kimura, Y. (2004). Phenolic compounds and flavonoids as plant growth regulators from fruit and leaf of *Vitex rotundifolia*. *Zeitschrift für Naturforschung C. 59*(7-8), 509-514.

Yuan, Y., Liu, Y. J., Wu, C., Chen, S. Q., Wang, Z. Y., Yang, Z. C., Qin, S. S., & Huang, L. Q. (2012). Water deficit affected flavonoid accumulation by regulating hormone metabolism in *Scutellaria baicalensis* georgi roots. *Plos One*, 7, 1-9.

Zeng, H., Zhong, Y., & Luo, L. (2006). Drought tolerance genes in rice. *Functional & integrative genomics*, *6*(4), 338-341.