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ORIGINAL PAPER

Responses of the antioxidative enzymes in Malaysian rice (*Oryza sativa* L.) cultivars under submergence condition

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Abstract The potential involvement of activated oxygen species by submergence stress was studied in two Malaysian rice cultivars, MR219-4 and MR219-9, and cultivar FR13A that is known to be tolerant to submergence. Seedlings of these three rice cultivars were subjected to different submergence periods (4, 8, and 12 days). Under 8 days of complete submergence, FR13A cultivar showed higher lipid peroxidation in terms of malondialdehyde level and activities of antioxidative enzymes, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) when compared to the MR219-4 and MR219-9 cultivars. MR219-9 showed higher SOD, APX, and GR activities after 12 days of submergence. The levels of SOD activity indicated that detoxification of $O_2^{\cdot-}$ to H_2O_2 was maintained at a stable level throughout the submergence stress until up to 8 days and increased rapidly at 12 days of submergence. The results indicated that tolerance to submergence in rice is associated until 8 days submergence for MR219-4 and FR13A cultivars. These findings suggested that tolerance to submergence stress in rice might be proven by increased the capacity of antioxidative system. In addition, CAT activity has much higher affinity for scavenges H_2O_2 than APX. Therefore, ascorbate glutathione cycle might be more efficient to scavenge H_2O_2.

Keywords Antioxidant enzymes · Lipid peroxidase · Cultivars · Periods of stress · Rice · Submergence stress

Abbreviations

ABA	Abscisic acid
APX	Ascorbate peroxidase
CAT	Catalase
DHAR	Dehydroascorbate reductase
EDTA	Ethylenediaminetetraacetic acid
GA	Gibberellic acid
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
LSD	Least significant differences
MDA	Malondialdehyde
MDHA	Monodehydroascorbate reductase
NBT	Nitroblue tetrazolium
NSC	Non-structural carbohydrate
PVPP	Polyvinyl polypyrrolidone
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
QTL	Quantitative trait locus

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Introduction

Flooding due to submergence is among significant natural hazard affecting many countries every year. Most of the plants often suffer from anaerobiosis brought by soil flooding and total submergence (Vartapetian and Jackson 1997). Rice (*Oryza sativa* L.) is the staple food for more than 50% the global population, is a semi aquatic plant, particularly able to survive under the conditions of prolonged oxygen deprivation, at both seedling and adult stages.

The submergence-tolerant rice cultivar is expected to help rice farmers in Asian countries, where 90% of the world's rice production and consumption takes place. By using the submergence-tolerant rice cultivar, the survival rate of rice plants under completely submerged condition is increased. However, the physiology and biochemistry from the use of tolerance cultivars remain applicable limitations in the field. In order to identify the traits required to improve genetic adaptability of rice plants to submergence conditions, it is necessary to properly characterize the floodwater environment and to closely investigate the physiological processes behind the plants. Complete submergence hastens degradation of chlorophyll content in susceptible rice cultivars compared to tolerant ones (Ella et al. 2003; Panda et al. 2006), which also can be used as an indicator of submergence tolerance. Measuring of several valuable parameters could be quantified which can clearly differentiate between sensitive and tolerant cultivars as early as 4–6 days of submergence, when even sensitive cultivars showed no signs of mortality (Ella et al. 2003).

The responses to a specific stress may vary with the genotype; nevertheless, some general reactions occur in all genotypes. Most of rice cultivars are flood sensitive and die within a week when they are completely submerged. Only a few cultivars, such as FR13A, can survive for 10–14 days of complete submergence (Fukao and Bailey-Serres 2008). The submergence tolerance of FR13A is linked to a major quantitative trait locus (QTL), known as *Submergence1* (*Sub1*), on chromosome 9 (Xu and Mackill 1996). The *Sub1A*, is a key regulator of submergence tolerance, effectively expressing ethylene production and responsiveness by shoot elongation and energy consumption (carbohydrates, lipids, and proteins) during and after submergence. The other phytohormones such as gibberellic acid (GA) and abscisic acid (ABA) also are known adapted to take part in survival under submergence conditions via synergism and antagonism actions by regulating shoot elongation and leaf senescence (Setter and laureles 1996; Das et al. 2005; Fukao and Bailey-Serres 2008).

Much of the injury to plants caused by abiotic stresses is associated with oxidative damage at cellular level. Damage to membrane integrity is a common effect of stress, especially in the case of low oxygen. Under anoxia, a decrease in membrane integrity is a symptom of injury, measured as changes in lipid content and composition, and also activation of lipid peroxidation (Blokhina et al. 2003). Plants have active oxygen-scavenging systems consisting of several antioxidant enzymes, and some low molecules of nonenzyme antioxidants, which can neutralize the free radicals and thus retard the progress of many injuries associated with oxidative stress and reactive oxygen species (ROS), such as superoxide radical $\left(O_2^{\cdot-}\right)$, hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($OH^{\cdot}$) (Bowler et al. 1992). ROS are common components of biochemical changes in the chloroplasts, mitochondria or in peroxisomes, when plants are subjected to harmful stress conditions. Among all the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) play key roles in ROS detoxification in cells. SOD, the first enzyme in the detoxifying process, converts superoxide anion radicals $\left({}^{\cdot}O_2^{-}\right)$ to hydrogen peroxide (H_2O_2), CAT converts H_2O_2 to water and oxygen, APX is the most important peroxidase in H_2O_2 detoxification, catalyzing the reduction of H_2O_2 to water using the reducing power of ascorbate, and other endogenous antioxidants, such as glutathione (GSH), and the associated glutathione metabolism enzyme play an important role and an ideal biochemical in protecting plants from oxidative stress damage (Noctor and Foyer 1998; Asada 1999).

This work is investigated to determine the effects of submergence stress periods on antioxidative enzymes (SOD, CAT, APX, and GR) of two Malaysian rice cultivars (MR219-4 and MR219-9), and comparing it with submergence tolerant FR13A in order to understand the tolerant mechanisms to submergence stress, and also to clarify the contribution of activities of antioxidant enzymes to resistant submergent development.

Materials and methods

Plant materials and stress conditions

The study was conducted using two Malaysian rice (*Oryza sativa* L.) cultivars, MR219-4 and MR219-9, and FR13A, which is known to be tolerant to submergence. All those three cultivars were provided by Malaysian Agricultural Research and Development Institute (MARDI) Seberang Perai, Malaysia. MR219-4 and MR219-9 cultivars are two potential rice mutants that were generated from MR219 because of laboratory and glass house selection. Both of these cultivars were used to carry out study on sustainable production of high-yielding irrigated rice under minimal water input. The seeds were surface sterilized in

an aqueous solution of 25 mM sodium hypochlorite for 25 min, rinsed four times in distilled water, and germinated on two sheets of moist filter paper (No 1; Macherey–Nagel, Germany) in darkness at 25°C for 4 days. The germinated seeds were cultured in porcelain tray in a greenhouse with natural environmental conditions with temperature (35°C/25°C) and photoperiod (16 h light/8 h dark). For all experiments, 14-day-old seedlings were completely submerged in tap water for 4, 8, and 12 days in concrete plastic tanks (dimension 60 cm × 60 cm × 1 m). Non-stressed seedlings were cultured under the same conditions.

Chlorophyll analysis

The total chlorophyll content estimation was measured spectrophotometrically following Arnon (1949). Fresh leaves (0.1 g) were powdered in liquid-nitrogen using mortar and pestle. The powder was homogenized with 80% acetone, and centrifuged at 5,000×*g* for 10 min at 4°C. The supernatants were measured the absorbance at A_{645} and A_{663}. Data are calculated using the formula: total chlorophyll content (μg/ml) = 20.2 (A_{645}) + 8.02 (A_{663}).

Preparation of crude enzyme extracts

Three replicated fresh plant parts (roots and shoots) were collected from the greenhouse. The collected plants were cleaned with distilled water, powdered using liquid nitrogen and the samples were homogenized with ice cold 0.05 M sodium phosphate buffer (pH 7.8) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA). Na_2 and 2% (w/v) insoluble polyvinyl polypyrrolidone (PVPP). The homogenates were then centrifuged at 10,000×*g* for 25 min at 4°C and supernatants were used for the determination of protein and antioxidant enzyme activities. Protein concentration of the enzyme extract was determined according to Bradford (1976), using bovine serum albumin as the standard.

ROS scavenging enzyme assays

All spectrophotometric analyses were conducted at 25°C on a UV:visible light spectrophotometer (UV-2602, Labomed, Inc. USA). The assay for superoxide dismutase (SOD, EC 1.15.1.1) activity was estimated spectrophotometrically as the inhibition of photochemical reduction of nitroblue-tetrazolium (NBT) at 560 nm (Stewart and Bewley 1980). The reaction mixture contained enzyme extract, 390 mM L-Methionine, 2.25 mM NBT, 1 mM EDTA, 7% Na_2CO_3, 0.05 M sodium phosphate buffer (pH 7.8), and 60 μM riboflavin. The riboflavin was added last. The reaction mixture was placed 30 cm from light source (about 15 W fluorescent lamps) for 10 min, and the decrease in the absorbance was recorded at 560 nm. The nonirradiated reaction mixture served as control and the value was deducted from that of A_{560}. One unit of SOD activity was defined as the amount of enzyme which caused 50% inhibition of the initial rate of reaction in the absence of enzyme, expressed in units mg protein^{-1}.

Catalase (CAT, EC 1.11.1.6) activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Bergmeyer 1970). Extraction mixture contained 300 μl of enzyme extract, 0.5 ml of 10 mM H_2O_2 and 600 μl of 30 mM potassium phosphate buffer (pH 7.0) and the decrease in absorbance was recorded at 240 nm for 30 s. The enzyme activity was calculated as μmol H_2O_2 decomposed min^{-1} mg protein^{-1} by using extinction coefficient ($\epsilon = 36\ \mu M^{-1}\ cm^{-1}$).

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured by following the method of Nakano and Asada (1981). The assay mixture contained 100 μl of enzyme extract, 600 μl 0.1 mM EDTA in 0.05 M sodium phosphate (pH 7.0), and 400 μl 0.5 mM ascorbic acid. The reaction started with addition of 400 μl of 3% H_2O_2 and the absorbance decreased was recorded after 1 min at 290 nm. The concentration of APX was calculated using extinction coefficient ($\epsilon = 2.8\ mM^{-1}\ cm^{-1}$). One unit of APX was defined as 1 nmol ml^{-1} ascorbate oxidized per minute.

Glutathione reductase (GR, EC 1.6.4.2) activity was estimated by following the method of Goldberg and Spooner (1983). The assay mixture contained of 100 μl enzyme extract, 2.5 ml of 0.1 mM EDTA in 0.05 M sodium phosphate (pH 7.0), and 100 μl 0.5 mM oxidized glutathione (GSSG). After 5 min, 50 μl of 9.6 mM NADH was added and mixed thoroughly. The absorbance decreased was recorded at 290 nm at an interval 1 min. The expression of 1 unit of GR activity is nmol glutathione reduced per minute, calculated using extinction coefficient ($\epsilon = 6.22\ mM^{-1}\ cm^{-1}$).

Lipid peroxidation

The crude extracts were also measured for lipid peroxidation capacity. Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) formation using the thiobarbituric acid method described by Dionisio-Sese and Tobita (1998). For every 1 ml of the crude extract, 4 ml of 20% (w/v) trichloroacetic acid (TCA) containing 0.5% (w/v) thiobarbituric acid (TBA) was added. The mixture was heated at 95°C for 30 min and then cooled quickly on an ice bath. The mixture was centrifuged for 15 min at 10,000×*g* and the absorbance of the supernatant was measured at 532 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA

was calculated by using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Statistical analysis

All the data obtained were subjected to a two-way analysis of variance (ANOVA), and the mean differences were compared by least significant differences (LSD) post hoc analysis using SPSS 11.5 for windows software. Comparisons with $P < 0.05$ were considered significantly different. In all the figures, the spread of values is shown as error bars representing standard deviation of the mean.

Results

Effect of submergence condition on total chlorophyll

The total chlorophyll content during submergence periods showed different profiles depending on cultivars (Fig. 1). Cultivar MR219-4 maintained higher total chlorophyll content than cultivar MR219-9 and tolerant cultivar FR13A. A marked decrease of 66 and 79% in chlorophyll content was observed in FR13A and MR219-9 cultivars leaves, respectively, after 12 days of submergence treatment compared to control. In contrast, a slight increase (62%) in chlorophyll content was observed in MR219-4 cultivar leaves after 4 days of submergence, followed by decrease of 25 and 30% compared to control after 8 until 12 days submergence treatment, respectively (Fig. 1). As the rice plants treated nonsubmergence conditions appeared a deeper green and levels of chloroplast pigment was expected. While plants began to display visible symptoms after 4 days of exposure to submergence, and increasing duration of submergence periods progressively

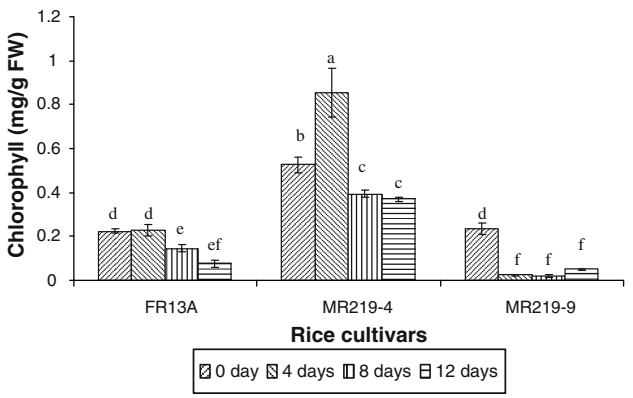

Fig. 1 Changes of chlorophyll content in leaf extracts of rice seedlings cultivars at different days of submergence. Values are mean ± standard deviations based on three replicates of each cultivars. Different letters indicate significant differences at ($P < 0.05$) according to LSD post hoc analysis

increased their weakened state, but still not evident during 8 until 12 days being submerged. The main cause of variance found to be the cultivar with 69%, followed by the interaction between cultivar and treatment (15%) and the treatment (14%) (Table 1).

Effect of submergence condition on antioxidant enzymes activities

Antioxidant activity reflects the role active of O_2-scavenging enzymes in conferring tolerance for submergence. There was a significant difference in SOD activities among cultivars as shown in Fig. 2a. Analysis of variance showed that the interaction of the cultivar × treatment describe the main effect of submergence condition on antioxidant enzymes activities except for SOD (Table 1). At 4, 8, and 12 days exposed to submergence stress the SOD activity in all varieties were slightly higher than the control. SOD activity increased at 8 days after submergence treatment, and after that, the activity was decreased at 12 days after submergence treatment for FR13A and MR219-4 cultivars. In addition, MR219-4 cultivar proved to have similar pattern to submergence-tolerant FR13A in their SOD activity to 4, 8, or 12 days of complete submergence under natural environmental conditions (Fig. 2a).

There was a significant increase in CAT activity detected for flood-tolerant FR13A cultivar about ninefold compared to control at 8 days after submergence treatment, and 1.6-fold for MR219-9 at 4 days submerged compared to control (Fig. 2b). However, catalase which is located mostly in peroxisomes and participates in the breakdown of the photo respiratory H_2O_2, has no significant alterations in neither MR219-4 cultivar.

APX activity of FR13A cultivar showed a significant decrease by 88% under 4 days submergence, although APX activity still lower about 64 and 83% under 8 and 12 days submergence, respectively, compared with the control plants (Fig. 2c). The results showed no significant decrease for MR219-4 and MR219-9 cultivars.

GR activity in submerged seedlings had shown significant differences between submergence treatments, except for MR219-9 cultivar. FR13A and MR219-4 cultivars had increased GR activity in 8 days submergence treatment by almost 10- and 13-fold, respectively, relative to nonsubmerged control (Fig. 2d).

Effect of submergence condition on lipid peroxidation

The main effect of variance showed to be the interaction of cultivar × treatment (41%), followed by the cultivar and treatment for 34 and 25%, respectively (Table 1). Lipid peroxidation levels of the three rice cultivars, measured as the content of MDA, are given in Fig. 3. There was

Table 1 Type III sum of squares value and percentage of corrected total (in bracket) of the effects parameter from univariate analysis of variance

Parameter	Source of variations		
	Cultivar ($df = 2$)	Treatment ($df = 3$)	Cultivar × treatment ($df = 6$)
Chlorophyll	1.394 (69)	0.280 (14)	0.312 (15)
SOD	9,752.234 (5)	86,917.172 (48)	79,053.579 (44)
CAT	2.263 (19)	1.817 (15)	7.448 (62)
APX	1,099.708 (22)	1,220.688 (25)	2,190.471 (44)
GR	10.049 (13)	25.462 (34)	28.338 (38)
MDA	1,145,593.500 (34)	844,730.889 (25)	1,374,657.611 (41)

Total df = 35. The α of the ANOVA for cultivar, treatment and cultivar × treatment interaction for one parameter was $*\alpha < 0.05$

df degree of freedom

a highly significant difference in MDA production at 4 days submergence for MR219-4 and MR219-9 cultivars. Submergence intolerant MR219-9 had much more MDA production than FR13A and MR219-4 cultivars, but at 8 days of submergence, there was a significant increase in MDA production in tolerant FR13A cultivar compared to MR219-4 and MR219-9 cultivars (Fig. 3). Moreover, at 12 days of submergence treatment, no significant difference was observed for MDA production between FR13A and MR219-9 cultivars.

Discussion

The rice cultivar MR219 is more than 70% of the population rice planted and suited to minimal water requirement in Malaysia. Submergence is an important factor affecting rice plants production during September until December every year. In this study, we evaluated submergence stress by measuring decrease in total chlorophyll content, induction of oxidative stress and MDA content in the rice plants using two cultivars originally from Malaysia (MR219-4 and MR219-9) and FR13A cultivar which is found to be tolerant to submergence.

The present experiment indicated that the physiological reactions to submergence were associated with decreased in total chlorophyll content and stimulation of the activity of antioxidant enzymes, such as SOD, CAT, APX, GR, and MDA in rice during submergence treatment. Superoxide radicals are generated during normal physiological process mainly in mitochondria. Although the superoxide anion is by itself a weak oxidant, it gives rise to the powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress (Meyer and Isaksen 1995; Mittler 2002). Therefore, superoxide radical scavenging by antioxidants has physiological implications.

In order to understand the effects of submergence condition on photosynthesis, total chlorophyll content was determined. The tolerant submergence cultivar (FR13A) and MR219-4 cultivar were maintained a level of chlorophyll during 4 days submergence treatment compared with control (Fig. 1). However, the chlorophyll content in MR219-9 cultivar decreased during 4 days submergence, and remained constant until 12 days of submergence. The reduction being more prominent and sharper in cultivar MR219-4, particularly after 8 days, compared with the other two cultivars. Based on Sarkar et al. (2004), susceptible cultivar, such as IR42 maintained higher chlorophyll content than tolerant cultivar FR13A up to 8 days after submergence. Significant differences were noticed between susceptible and tolerant cultivars only after 10 days of submergence. We found that the degradation of chlorophyll by submergence was specifically because of the cultivar (Table 1). Similarly, in the comparison of three rice cultivars (FR13A, Kalaputia, and IR42) were found in chlorophyll content of 21-day-old rice seedlings subjected to completely submerged for 8 days (Panda et al. 2008). It is important to notice that plant responses to stress (submergence) may use in common ways, and mostly depending on the plant genera or families (Gaspar et al. 2002).

The correlation between chlorophyll content and antioxidant activity, such as SOD, CAT, APX, and GR were negative (Table 2). The possibility of chlorophyll content degradation during submergence, especially for intolerant cultivars related to the structural damage by the photosynthetic apparatus as evident of decreased in the value of F_o, F_m and F_v/F_m ratio, and also related to nonstructural carbohydrate (NSC) status of rice cultivars (Macek et al. 2006; Panda et al. 2008). Our result showed that the correlation between chlorophyll content and MDA production was stronger negatively ($R = -0.48^{**}$). There is a possibility that chlorophyll content decreased because of MDA accumulation. MDA accumulation is likely to further aggravate and dismantle the PS2 complex (Mishra et al. 2008).

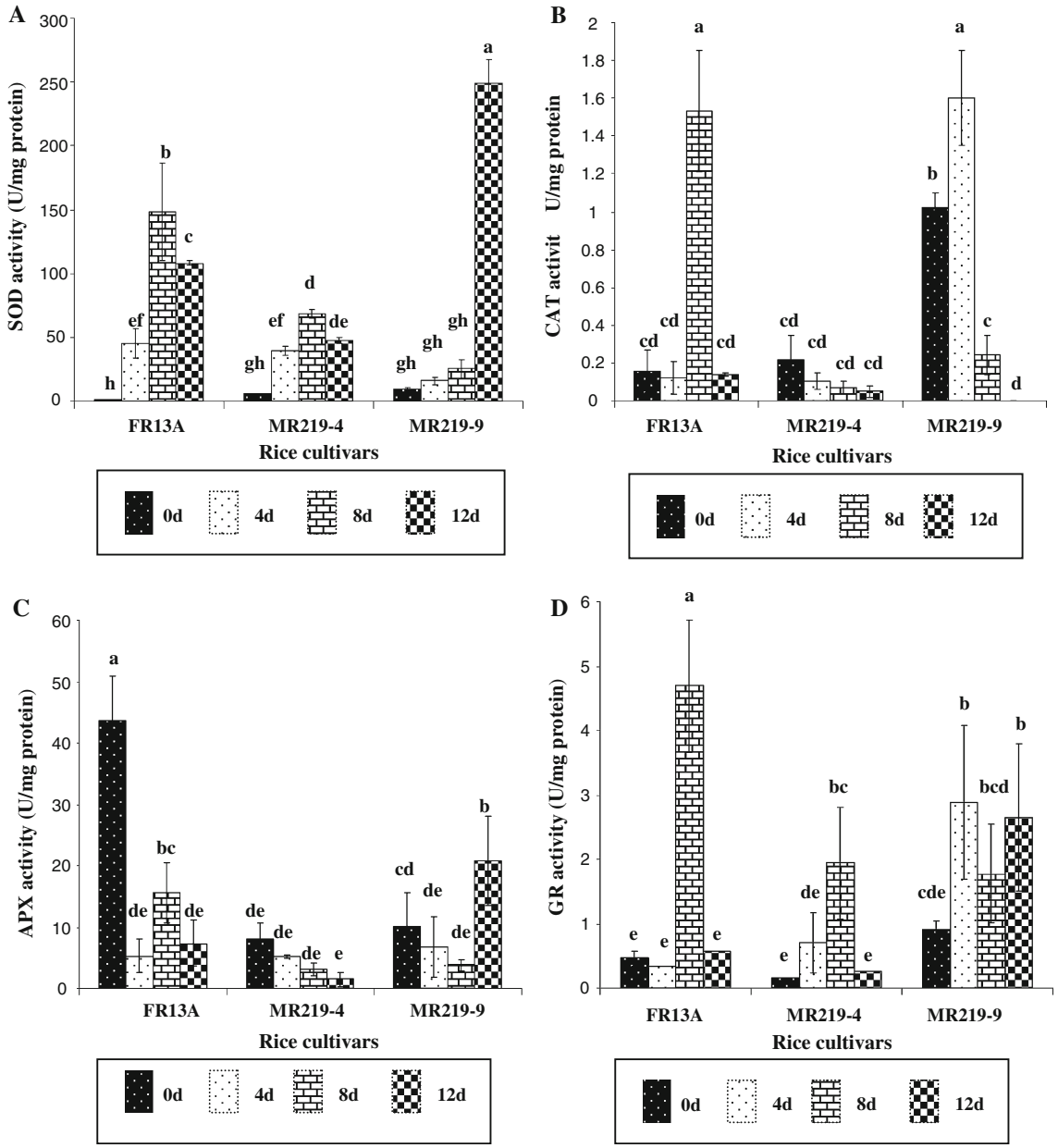

Fig. 2 SOD activity (**a**), CAT activity (**b**), APX activity (**c**) and GR activity (**d**) in rice seedlings cultivars at different days of submergence. Values are mean ± standard deviations based on three independent assays for each determination. Different letters indicate significant differences at ($P < 0.05$) according to LSD post hoc analysis

Under stress, ROS production is high resulting in oxidative damage. In the present study, it was seen that SOD and CAT did not participate in active H_2O_2 reduction at the same period of submergence treatment, except for flood-tolerant FR13A at 8 days submergence treatment. Regarding the data obtained, there appears to be a correlation between SOD and CAT activity in rice seedlings and the period of submergence treatment in their sensitivity (Fig. 2a, b), such as, MR219-9 cultivar, SOD activity is activated more than 230-fold higher and lower CAT activity compared to control at 12 days submerged. CAT activity is more than 50% higher and lower SOD activity compared to control at 4 days submerged. This implies that MR219-9 cultivar is having the steady state between SOD and CAT activation by submergence stress due to the prevention plants from oxidative stress damage under 4 days submerged. The combined action of SOD and CAT is critical in mitigating the effects of oxidative stress, since the former merely acts on the superoxide anion converting it to another reactive intermediate (H_2O_2) and the latter acts on H_2O_2 converting it to water and oxygen (Matés 2000). Our result also showed similar trends for SOD activity

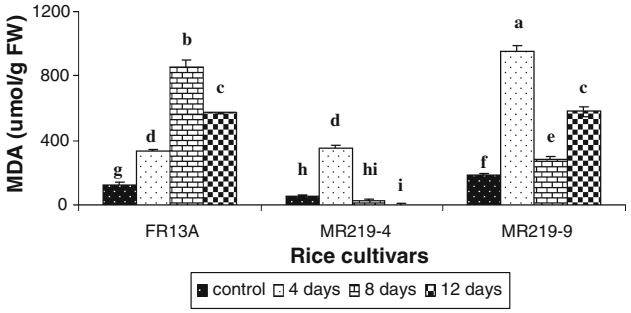

Fig. 3 MDA content in rice seedlings cultivars at different days of submergence. Values are mean ± standard deviations based on three independent assays for each determination. Different letters indicate significant differences at ($P < 0.05$) according to LSD post hoc analysis

Table 2 Correlation coefficients for the associations among the parameters

Parameter	SOD	CAT	APX	GR	MDA
Chlorophyll	−0.31*	−0.34*	-0.19^{ns}	−0.40**	−0.48**
SOD	–	-0.05^{ns}	0.10^{ns}	0.49**	0.43**
CAT	–	–	-0.03^{ns}	0.64**	0.66**
APX	–	–	–	0.02^{ns}	0.03^{ns}
GR	–	–	–	–	0.67**

ns nonsignificant (based on Pearson correlation)

*,** Correlation is significant at 0.05 and 0.01 levels

between flood-tolerant FR13A and MR219-4 cultivars suggest a possibility that the mechanism in detoxification of signaling oxidative stress is similar.

Peroxidase, such as APX and CAT are the major H_2O_2 detoxifying enzymes in plants related to stress. In our study under submergence stress, APX activity was significantly decreased at 4 days submergence condition compared to control for FR13A tolerant cultivar and maintained lower until 12 days after being submerged. However, CAT activity was ninefold higher compared to control at 8 days submergence. The other cultivars showed APX activity that was stable lower until 12 days of submergence, except for MR219-9 cultivar, the activity become higher at 12 days submerged (Fig. 2b, c). Although CAT has low affinity toward H_2O_2 (Willekens et al. 1997), in tolerant-submergence cultivar (FR13A) and MR219-9 cultivar, CAT activity was more efficient in destroying H_2O_2 than was APX under submergence stress. CAT is activated and APX is deactivated in response for rice plants. The decrease in APX activity is likely to contribute to an accumulation of H_2O_2 and eventual death of aleuron cell of barley (Fath et al. 2002). CAT, which is present only in the peroxisome, has low substrate affinities since it requires simultaneous access of two molecules of H_2O_2. APX is primarily located in both the chloroplasts and the cytosol, and as the key enzyme of the glutathione ascorbate pathway, it eliminates peroxides by converting ascorbic acid to dehydroascorbate (Izzo et al. 1997; Blokhina et al. 2003).

CAT is considered as a key enzyme removing toxic hydrogen peroxide, and APX have a complementary duty (Malecka et al. 2009). However, our results disagree with Malecka et al. (2009), the activities of CAT and APX were not significant at the rice plants under submergence treatment compared to control for MR219-4 cultivar (Fig. 2b, c). The results indicated that CAT and APX activities are differentially activated by submergence stress in rice plants at different stages of growth. It is possible that glutathione (GSH) and GR are important factors participating in redox state regulation of plant cells and are needed at a sufficiently high concentration for protection against submergence stress (Fig. 2d). Combining APX with GR can be removed H_2O_2 via recurrent oxidation–reduction reactions promoted by GSH, hence preventing cell damage (Hung et al. 2005; Kocsy et al. 2001).

The role of GR and glutathione in the H_2O_2 scavenging in plant cells has been well established in Halliwell–Asada pathway (Bray et al. 2000). GR are important factors involved in the recycling of reduced glutathione (GSH), providing a constant intracellular level of GSH, therefore, elevating the total quantity of GSH within a plant becomes the critical step in reducing stress injury (Kocsy et al. 2001; Hung et al. 2005). In the present study, submergence treatment led to a significant increase in GR activity for FR13A and MR219-4 cultivars at 8 days after being submerged, but for MR219-9 cultivar increased at 4 days submerged and still maintained until 12 days submerged (Fig. 2d). Higher GR activities observed under the periods of submergence stress indicates that mechanism of antioxidant defense was by enhanced oxidation of GSH to GSSG (oxidized glutathione) by dehydroascorbate reductase (DHAR) yielding ascorbic acid (AA). This AA produced by nonenzymatic dispropotionation of MDHA (monodehydroascorbate reductase) was used by APX to directly detoxify H_2O_2 (Shanker et al. 2004). Increase in GR activity with decreasing APX activity was shown at 8 days submergence treatment for FR13A and MR219-4 cultivars (Fig. 2c, d). The findings indicated that, under submerged periods (8 days), rice plants experienced stress to induce GR activity, and suggest that enhanced levels of submerged periods may be necessary for the observed protection from submergence stress. In addition, it could be explained that the ascorbate–glutathione cycle might be more efficient enzymatic way and operating at a high rate in order to scavenges cytotoxic H_2O_2 than CAT and APX in these plants (Table 2), and reacts nonenzymatically with other ROS: singlet oxygen, superoxide radical, and hydroxyl radical (Larson 1988).

It is already known that free radical-induced peroxidation of lipid membranes is a reflection of stress-induced damage at the cellular level (Jain et al. 2001). Under anoxia, a decrease in membrane integrity is a symptom of injury, and it can be measured as changes in the lipid content and composition (Blokhina et al. 2003). Therefore, the level of MDA, produced during peroxidation of membrane lipids, is often used as an indicator of oxidative damage. In our studies, with the increase of submergence treatment (8 days) there appeared to be an increase MDA and then decreased at 12 days submergence treatment for FR13A cultivar (Fig. 3). These results suggest that submergence is associated with the induction of oxidative stress in rice plants, as peroxidation of membrane lipids as well as loss of thiol groups are indicators of oxidative stress. Lower levels of MDA indicate better submergence tolerance (Ella et al. 2003; Mishra et al. 2008; Yordanova and Popova 2007). However, MR219-4 and MR219-9 cultivars had significantly lower MDA level than nonsubmerged control at 8 days submergence, and still maintained until 12 days submerged for MR219-4 cultivar. It can be suggested that GR activity was significant higher at 8 days submergence for MR219-4 cultivar, and the H_2O_2 was not sufficient available for lipid peroxidation (Figs. 2d, 3). Boscolo et al. (2003) and Hodges et al. (1999) observed that carbohydrates and even some protein oxidation rather than lipid peroxidation are known may undergo decomposition and produce MDA as an end-product and suggested that the target of oxidative stress varies depending on the plant species, type and intensity of stress factors. Our results suggested that efficiency of ROS-detoxifying enzymes (SOD, CAT, APX, and GR) and level of MDA, because of effects submergence conditions varied with treatments at various submergence conditions and interaction between cultivars and submergence conditions (Table 1).

In conclusion, activities of antioxidative enzymes by submergence stress in rice cultivars were associated until 8 days submergence as already shown in other research (Ella et al. 2003; Blokhina et al. 2003; Matés 2000; Mittler 2002; Sing et al. 2001). In this work, attention to MR219-4 cultivar was found to be promising for tolerance to submergence, since it proved to be similar pattern to submergence-tolerant FR13A cultivar in their chlorophyll content, SOD, and GR activities. Submergence treatments may enhance the level of GR activity in MR219-4 cultivar. This indicated that GR activity could be served as criteria for evaluating the submergence tolerance of rice cultivar. However, further biochemicals evaluation need to be carried out such as analyzing the submergence-responsiveness of GR in co-operation with other antioxidants related to the AsA-GSH cycle regarding participate in the redox state regulation as a submergence stress signal in rice plants.

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