RESEARCH

Membrane stability and antioxidant enzyme activity of rice seedlings in response to short-term high temperature treatments

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ABSTRACT

The impact of temperature on plants varies depending on the temperature level and the time of exposure. The aim of this study was evaluating different short-term temperatures to stimulate rice (*Oryza sativa* L.) seedling growth indicated by some physiological functions. Thus, oxidative stress, membrane stability and antioxidant activity in 'Dular', 'IR64' and 'KDML105' rice seedlings were evaluated in response to 25, 35 and 45 °C for 30 min. At 25 °C, 'IR64' showed higher electrolyte leakage (EL) percentages, protein contents and peroxidase (POX) activity but lower hydrogen peroxide (H_2O_2) content. At 35 °C, the highest H_2O_2 content was found in 'KDML105' followed by 'IR64' and 'Dular'. In all rice seedlings the lowest EL was after 35 °C exposure and activities of catalase (CAT) in 'IR64' and 'KDML105', ascorbate peroxidase (APX) in 'Dular', 'IR64' and 'KDML105' and glutathione reductase (GR) in 'Dular', 'IR64' and 'KDML105', and superoxide dismutase (SOD) in 'Dular' and 'KDML105' were stimulated by higher H_2O_2 production. At 45 °C, all rice seedlings showed pronounced EL increase but reduced H_2O_2 production, protein content and all antioxidant enzyme activities. These results suggested that short-term temperature at 35 °C was an optimum condition for all rice seedlings as indicated by the least membrane damage (EL ≈ 45.04%) and induction of most antioxidant enzymes (CAT, APX, GR and SOD ≈ 48.29 nmol min^{-1} µg^{-1} protein, 247.49 ng ascorbic acid min^{-1} µg^{-1} protein, 0.16 µM min^{-1} µg^{-1} protein and 0.078 unit min^{-1} µg^{-1} protein, respectively). This temperature induced higher H_2O_2 production which plays a significant role in signaling molecules to stimulate protein function and antioxidant enzyme activities. While short-term 45 °C proved to be damaging temperature for all rice seedlings because of membrane damage and decreased reactive oxygen species defense mechanism.

Key words: Antioxidant enzyme, electrolyte leakage, heat stress, hydrogen peroxide, *Oryza sativa*.

INTRODUCTION

Temperature is one of the most important environmental factors affecting plant growth and yield production. At the present, global warming is a serious threat to agricultural productivity. High temperature adversely affects plant growth and development, limits plant biomass production and reduces yield quality in tropical and subtropical areas (Hasanuzzaman et al., 2013b). In addition, it causes an alteration of the physiological processes, biochemistry, and gene regulation (Bita and Gerats, 2013). Temperature changes may also induce cellular responses by perceiving and signaling transduction into cells (Hemantaranjan et al., 2014). Under high temperature, plants demonstrate various survival mechanisms such as avoidance, acclimation or tolerance through molecular, biochemical and physiological processes.

Rice (*Oryza sativa* L.) is the main food crop and plays a vital role in food security in many countries including Thailand. The optimum temperature of rice growth was reported at approximately 33 °C, and temperatures over 33 °C could impose negative effects on rice yield (Sukkeo et al., 2017). Plants exposed to temperature above 5 °C of optimum growth

temperature responded at the cellular level with the production and accumulation of reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$). When plants are exposed to stress, ROS plays an important role in signal transduction. High temperature leads to an increase in membrane fluidity (Asthir, 2015) and oxidative stress (Hasanuzzaman et al., 2013a) resulting in cell membrane damage and electrolyte leakage by lipid peroxidation. Ali et al. (2013) reported that an increased in H_2O_2 in rice was found after the plants were treated with high temperature at 42 ± 3 °C for 24 and 48 h. When ROS burst occurs under high temperature, plants respond by increasing the production and activity of antioxidant enzymes. The antioxidant enzymes such as peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and glutathione reductase (GR) protect cell damage due to ROS in plant cells and subcellular system (Hasanuzzaman et al., 2013b). Timabud and Pongdontri (2015) reported that after 8 h at 40 °C, POX activity in rice 'IR64' was higher than that of 'N22', and 'KDML105' showed similar level of heat sensitivity as 'IR64'. For 'KDML105', an economically important aromatic rice cultivar, limited information exists on its physiological responses to high temperature treatments. Impact of temperature on plants varies depending on the level of temperature and the time of exposure which are divided into short- (up to 1 h), moderate short- (1 h up to 24 h) and long-term temperature (longer than 24 h). There was less knowledge in effect of short-term temperature on physiology in various plants including rice plants. Therefore, this research investigated the oxidative stress, membrane stability and antioxidant enzyme activity (POX, CAT, APX, SOD and GR) in Thai aromatic rice 'KDML105' compared to standard heat tolerance rice 'Dular' (Lang et al., 2015) and standard heat sensitive rice 'IR64' (Coast et al., 2016) exposed to short-term temperature treatments (25, 35 and 45 °C for 30 min).

MATERIALS AND METHODS

Plant material and growth condition

Three rice (*Oryza sativa* L.) cultivars were evaluated in this study: heat tolerant 'Dular' (Lang et al., 2015), heat sensitive 'IR64' (Coast et al., 2016) and 'KDML105'. The seeds were sterilized with 70% ethanol for 1 min, washed with distilled water three times, soaked in 10% sodium hypochlorite for 15 min and washed with distilled water three times. Sterilized seeds were imbided in distilled water for 1 d. After that, seeds were germinated on wet seed germination paper until root length was approximately 3 cm. Twenty germinated seeds were transplanted in 36 pots (pot size approximately 12.5 L) containing 8 kg paddy soils per pot. The paddy soil in this experiment had a texture of sandy soil with pH 5.18, organic matter 0.487%, total N 0.0235%, available P 5.64 mg kg^{-1}, exchangeable K 37.28 mg kg^{-1} and electrical conductivity (EC) 0.05 dS m^{-1}. The seedlings were grown for 21 d. Then, seedlings were subjected to different temperature regimes in a temperature chamber (VRV Corporation, Bangkok, Thailand). The temperature regimes used for rice seedlings are shown in Figure 1. The temperature in the chamber started at 25 °C and increased up to 25, 35 or 45 °C for 30 min (Figure 1). Time courses of increasing temperature from 25 °C reached to 35 or 45 °C were 50 min and 1 h 36 min, respectively and then decreasing temperature from 35 or 45 °C reached to 25 °C were 50 min and 1 h 36 min, respectively. The relative humidity and light intensity in the chamber were controlled at 70% and 400 μmol photons m^{-2} s^{-1}, respectively. After target temperature was reached, it was maintained for 30 min and then cooled down to 25 °C (Dongsansuk et al., 2017). Immediately after removal of pots from the chamber, fully expanded leaves of the treated plants were collected for the determination of H_2O_2, electrolyte leakage (EL) and antioxidant enzymes activity including peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and glutathione reductase (GR).

Figure 1. Model of temperature control in the temperature chamber.
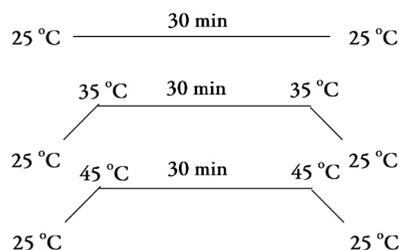
Determination of electrolyte leakage
Electrolyte leakage (EL) was determined according to Bajji et al. (2002). Fresh leaves (0.1 g) were placed in a 15 mL test tube containing 10 mL deionized water (DI water) and incubated at room temperature for 24 h. The EC of the suspension (EC_1) was measured by using EC meter (Tomy Model ES-315, Tomy Seiko, Japan), and the tube was then heated to 100 °C for 15 min. The samples were then cooled to 25 °C and the final EC (EC_2) was measured. The EL was calculated according to the following equation:

$$EL (\%) = (EC_1/EC_2) \times 100$$

Determination of protein content and antioxidant enzyme activity
Protein was extracted by the modified method of Velikova et al. (2000). A sample of 2 g fresh leaves was ground with liquid nitrogen. After that, 6 mL 100 mM potassium phosphate buffer pH 7.0 containing 1% (w/v) polyvinylpyrrolidone (PVP) was added. The crude extract was centrifuged at 4500×g for 1 h at 4 °C, and supernatant was used as enzyme extracts. Protein content was quantified according to Bradford (1976).

Hydrogen peroxide (H_2O_2) content was determined using a colorimetric assay based on the reaction producing a yellow complex of ammonium molybdate which absorbs light at 405 nm according to the method of Góth (1991). Measurement of H_2O_2 content was extracted from protein extract, containing 1 mL crude protein (dilute 10X) with 20 μL 0.5% (w/v) trichloroacetic acid (TCA). The reaction mixture (100 μL protein extract and 100 μL 32.4 mM ammonium molybdate) was prepared in a 96-well microplate and set aside for 10 min, and the absorbance at 405 nm was measured in an auto microplate reader (UT-2100C, MRC Ltd., Holon, Israel) and expressed as mmol g^{-1} FW.

Peroxidase (POX) activity was determined according to Chance and Maehly (1955). The assay of POX activity was measured as the rate of increase in the reaction product. The 25 μL of 3 μg protein extract was added to the reaction mixture (75 μL 100 μM potassium phosphate buffer pH 6.0, 50 μL 120 μM guaiacol solution and 50 μL 20 μM H_2O_2). The absorbance was measured with fluorescence microplate reader (SpectraMax M5, Molecular Devices LLC, Sunnyvale, California, USA) at wavelength 415 nm. The activity was expressed as mOD min^{-1} $μg^{-1}$ protein.

Catalase (CAT) activity was determined according to Aebi (1984). Analysis of CAT activity was based on the measurement of remaining H_2O_2 content in the reaction mixture, of which absorbance was measured at 240 nm. The 25 μL 10 μg protein extract was added to reaction mixture (150 μL 50 μM potassium phosphate buffer pH 7.0 and 25 μL 100 μM H_2O_2). The absorbance was measured with fluorescence microplate reader (SpectraMax M5) at wavelength 240 nm. An enzyme unit was calculated by standard curve of H_2O_2 and the activity was expressed as nmol min^{-1} $μg^{-1}$ protein.

Ascorbate peroxidase (APX) activity was determined according to Nakano and Asada (1987). The APX activity was measured based on the decrease in absorbance of ascorbic acid at 290 nm. The 25 μL 10 μg protein extraction was added to the reaction mixture (100 μL 100 μM potassium phosphate buffer pH 7.0, 40 μL 5 mM ascorbic acid and 35 μL 20 μM H_2O_2). The absorbance was measured with fluorescence microplate reader (SpectraMax M5) at wavelength 290 nm. An enzyme unit was calculated by standard curve of ascorbic acid and the activity was expressed as ng ascorbic acid min^{-1} $μg^{-1}$ protein.

Superoxide dismutase (SOD) activity was determined according to Beauchamp and Fridovich (1971). Analysis of SOD activity was based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium salt (NBT) by superoxide radicals. The 25 μL 20 μg protein extract was added to the reaction mixture (100 μL 50 mM potassium phosphate buffer pH 7.8, 20 μL 2.5 mM xanthine, 42 μL distilled water, 5 μL 20 mM NBT solution and 8 μL 0.05 μg xanthine oxidase). The absorbance of reduced product of NBT was measured with fluorescence microplate reader (SpectraMax M5) at wavelength 550 nm. One unit of SOD enzyme activity is defined as the amount of enzyme causing 50% NBT reaction. The SOD enzyme activity was expressed as unit min^{-1} $μg^{-1}$ protein.

Glutathione reductase (GR) activity was determined according to modified method from Carlberg and Mannervik (1985). The unit of GR was calculated from the extinction coefficient of NADPH at 6.2 mM cm^{-1}. The 25 μL 30 μg protein extract was added to the reaction mixture (145 μL 50 mM potassium phosphate buffer pH 7.4, 10 μL 20 mM EDTA, 10 μL 10 mM NADPH and 10 μL 20 mM GSSG). The absorbance was measured with fluorescence microplate reader (SpectraMax M5) at wavelength 340 nm. The GR activity was determined by the extinction coefficient of NADPH and expressed as μM min^{-1} $μg^{-1}$ protein.

Experimental design and data analysis

The experimental design was Factorial in completely randomized design (CRD) with four replicates, each of one pot with two factors: (1) three rice cultivars (Dular, IR64 and KDML105) and (2) three temperatures of 25, 35 and 45 °C. Data were analyzed by ANOVA. Significant differences among various treatments were determined by Duncan's multiple range tests (DMRT) using SPSS for Windows version 17.0 (IBM, Armonk, New York, USA).

RESULTS

Effect of short-term temperature exposure on oxidative stress

The oxidative stress affected from short-term temperature was indicated by hydrogen peroxide (H_2O_2) contents (Figure 2). The highest H_2O_2 content was observed in rice 'KDML105' (approximately 0.27 mmol g^{-1} FW) after short-term temperature at 35 °C. Seedlings of all three rice cultivars exhibited the highest H_2O_2 contents with increasing temperature at 35 °C for 30 min. This result related to the interaction between the cultivars and temperature (CV × Temp) showed significant difference for H_2O_2 contents (Table 1). After short-heat exposure at 45 °C, rice 'Dular' and 'KDML105' showed a gradual decreased H_2O_2 contents, whereas 'IR64' exhibited nonsignificant slightly decreased H_2O_2 contents (Figure 2).

Effect of short-term temperature exposure on membrane stability

The effect of short-term temperature exposure on membrane stability was measured by electrolyte leakage (EL) (Figure 3); CV × Temp interaction was significant for EL (Table 1). The significant differences were mainly due to low and high temperature stimulating EL of all the rice cultivars (Figure 3, Table 1). According to the higher EL was found in seedlings of all rice cultivars after treated with short-term temperature at 25 and 45 °C. The lowest EL levels were found at 35 °C in 'IR64', followed by 'KDML105' and 'Dular', respectively (Figure 3).

Effect of short-term temperature exposure on protein content and antioxidant enzyme activity

The effect of short-term temperature on protein content in three rice seedling cultivars is shown in Figure 4A. At 25 °C, seedlings of 'IR64' had the highest protein content among the three cultivars. After an exposure to 35 °C, protein content significantly increased in 'Dular' and 'KDML105' (CV × Temp interaction was significant for protein content; Table 1). In contrast, protein content in 'IR64' significantly decreased. And at this temperature all three cultivars showed comparable amount of proteins in their tissues. When temperature was raised to 45 °C, only 'Dular' showed a significant reduction in protein content. The 'IR64' showed a slight reduction in protein content, while no reduction was observed in 'KDML105'.

Figure 2. Effect of short-term exposure of different temperatures on hydrogen peroxide (H_2O_2) contents in three rice cultivars.
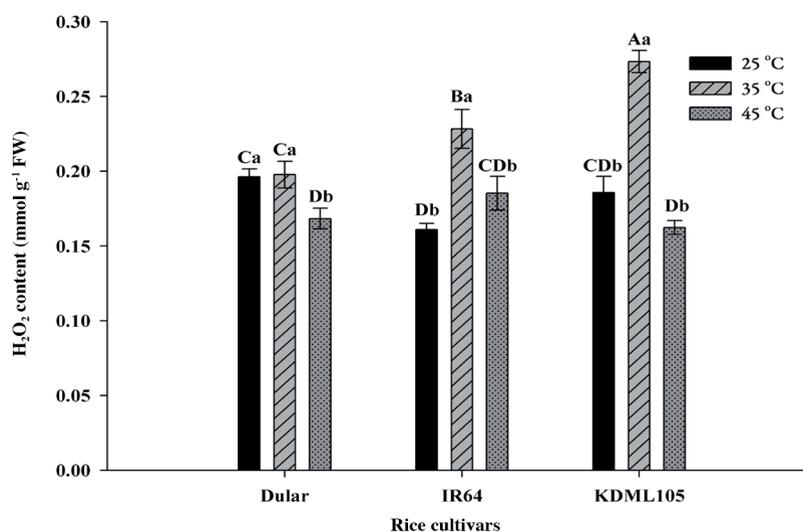
Values were means ± SE (n = 3-4). Different uppercase letters indicated significant differences among different temperatures and cultivars according to Duncan's multiple range tests (DMRT) at P ≤ 0.05. Different lowercase letters indicated significant differences among different temperatures in each cultivar by DMRT at P ≤ 0.05.

Table 1. ANOVA of effects of different rice cultivars and different temperatures on hydrogen peroxide (H_2O_2), electrolyte leakage (EL), protein and antioxidant enzyme activities (n = 3-4).

Sources	Mean square							
	H_2O_2	EL	Protein	POX	CAT	APX	SOD	GR
Cultivars (CV)	0.001[*]	223.73[*]	0.815[*]	40698.25[*]	5.511[*]	5034.62[*]	0.000[*]	0.005[*]
Temperature (Temp)	0.010[*]	3815.16[*]	0.368[*]	18555.40[*]	38.60[*]	64362.13[*]	0.000[*]	0.006[*]
CV × Temp	0.002[*]	221.85[*]	0.618[*]	5788.72[*]	204.97[*]	19305.44[*]	0.000[*]	0.001[*]
Error	0.000	42.05	0.025	25.66	0.741	60.73	0.000	0.000

[*]Indicated significantly different at P ≤ 0.05 by ANOVA.
POX: Peroxidase; CAT: catalase; APX: ascorbate peroxidase; SOD: superoxide dismutase; GR: glutathione reductase.

Figure 3. Effect of short-term exposure of different temperatures on electrolyte leakage in three rice cultivars.
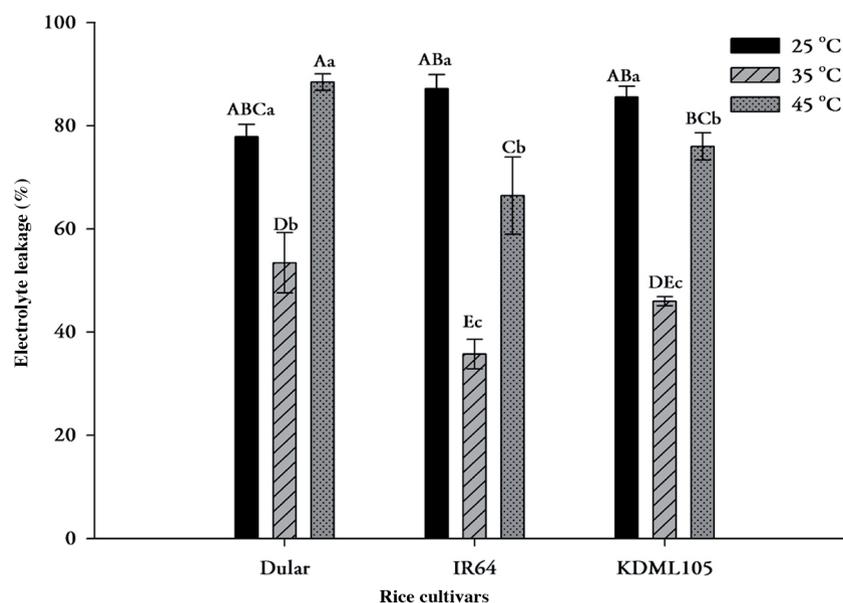
The values were means ± SE (n = 3-4). Different uppercase letters indicated significant differences among different temperatures and cultivars according to Duncan's multiple range tests (DMRT) at P ≤ 0.05. Different lowercase letters indicated significant differences among different temperatures in each cultivar by DMRT at P ≤ 0.05.

The activities of peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and glutathione reductase (GR) in different rice seedling cultivars are shown in Figures 4B-4F.

The effect of short-term temperature exposure on POX activity is shown in Figure 4B. At 25 °C, the highest POX activity was found in 'IR64' followed by 'KDML105' and 'Dular'. After an exposure to 35 °C, only 'Dular' responded with a significant increase in POX activity. In contrast, the enzyme activity was significantly reduced in 'IR64' and 'KDML105'. A further increase in temperature to 45 °C resulted in significant reductions in POX activity in all cultivars. This result was related to CV × Temp interaction which showed significant difference (Table 1). At this high temperature 'Dular' showed the most dramatic reduction leading to this cultivar having the lowest POX activity. At all three temperature regimes, 'IR64' demonstrated the highest POX activity. Among all treatment and cultivar combinations, the highest POX activity was found in 'IR64' at 25 °C, followed by 'IR64' at 35 °C and 'KDML105' at 25 °C. The lowest POX activity was found in 'Dular' at 45 °C.

The effect of short-term temperature exposure on CAT activity is shown in Figure 4C. At 25 °C, 'Dular' presented the highest CAT activity, which was significantly higher than those of 'IR64' and 'KDML105' (CV × Temp interaction was significant for CAT activity; Table 1). Rice 'Dular' showed decreased trend of CAT activity with increasing temperature from 25 to 45 °C. In contrast, CAT activity of 'IR64' increased with increasing temperatures, producing the highest activity among genotypes at 45 °C. 'KDML105' showed a significant increase in CAT activity at 35 °C and then the activity

significantly reduced at 45 °C to the level slightly higher than that at 25 °C. Among all treatment and cultivar combinations, the highest CAT activity was found in 'IR64' at 45 °C, followed by 'KDML105' at 35 °C and 'Dular' at 25 °C.

The effect of short-term temperature exposure on APX activity is shown in Figure 4D. At 25 °C, the highest APX activity was found in 'KDML105', which increased when exposed to 35 °C, but decreased at 45 °C to levels slightly higher than that at 25 °C. Both, 'Dular' and 'IR64', showed slightly APX activity at 25 °C but significantly increased when exposed to 35 and 45 °C (CV × Temp interaction was significant for APX activity; Table 1). In 'Dular' and 'IR64' the highest activity was found at 45 °C.

Figure 4. Effect of different temperatures on protein contents (A) POX (B), CAT (C), APX (D), SOD (E) and GR (F) activities in three rice cultivars.
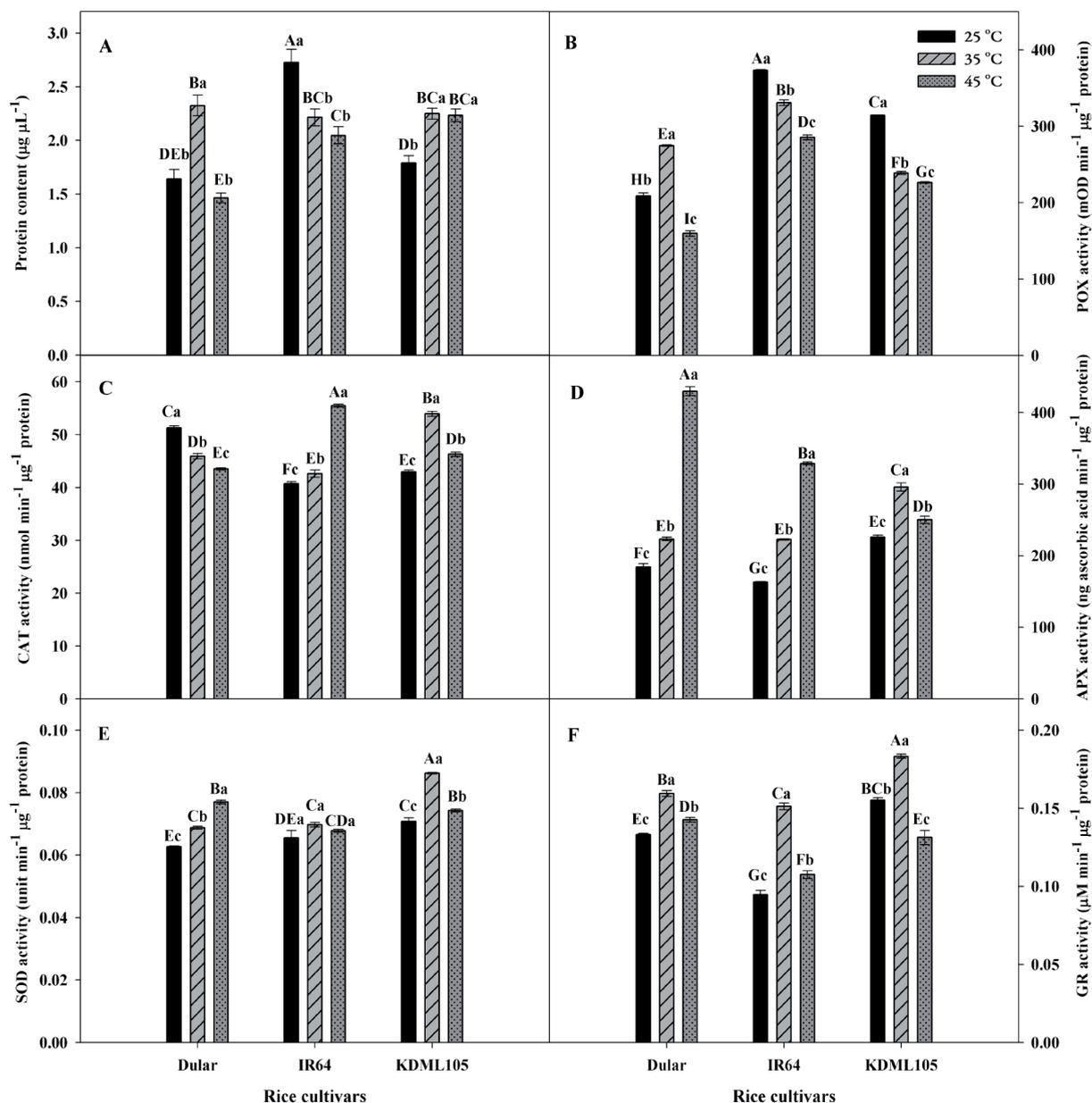
The values were means ± SE (n = 3-4). Different uppercase letters indicated significant differences among different temperatures and cultivars according to Duncan's multiple range tests (DMRT) at P ≤ 0.05. Different lowercase letters indicated significant differences among different temperatures in each cultivar by DMRT at P ≤ 0.05.
POX: Peroxidase; CAT: catalase; APX: ascorbate peroxidase; SOD: superoxide dismutase; GR: glutathione reductase.

The effect of short-term temperature exposure on SOD activity is shown in Figure 4E. From Table 1, CV × Temp interaction was significant for SOD activity. The significant difference was mainly due to different temperatures affecting SOD activity in all the rice cultivars (Figure 4E and Table 1). The three cultivars exhibited different patterns of responses to temperatures. At 25 °C, the highest SOD activity was found in 'KDML105' which was slightly higher than that of 'Dular' and 'IR64'. In response to increasing temperatures, only 'Dular' showed significantly increasing SOD activities from 25 to 35 and 45 °C. 'IR64' showed a slight increase in SOD activity when temperature increased from 25 to 35 °C. The SOD activity of 'KDML105' significantly increased when temperature was raised from 25 to 35 °C, but as the temperature increased to 45 °C, SOD activity reduced to the level slightly higher than the original level at 25 °C. Among all treatment and cultivar combinations, the highest SOD activity was found in 'KDML105' at 35 °C.

The effect of short-term temperature exposure on GR activity is shown in Figure 4F. At 25 °C, the GR activity was highest in 'KDML105' followed by 'Dular' and 'IR64'. The patterns of responses to various temperatures were similar for all cultivars in which enzyme activity significantly increased at 35 °C but then decreased when exposed to 45 °C. This result related to CV × Temp interaction showed significant difference (Table 1). The GR activities for 'Dular' and 'IR64' at 45 °C were slightly higher than those at 25 °C. But the GR activity for 'KDML105' at 45 °C reduced to the level significantly lower than that at 25 °C. Among all treatment and cultivar combinations, the highest GR activity was found in 'KDML105' at 35 °C followed by 'Dular' at 35 °C, 'KDML105' at 25 °C and 'IR64' at 35 °C.

Interaction of rice cultivars and temperatures on H_2O_2, EL, protein, POX, CAT, APX, SOD and GR
The interaction of various rice cultivars and temperatures influenced H_2O_2 contents, EL, protein content, POX, CAT, APX, SOD and GR (Table 1). We further determined that this interaction of rice cultivars and temperatures significantly (P ≤ 0.05) affected oxidative stress, membrane stability, protein content and antioxidant enzyme activities.

The correlation between H_2O_2 contents, EL, protein content, POX, CAT, APX, SOD and GR in three rice cultivars exposed to short-term temperature is shown in Table 2. At 25 °C, H_2O_2 contents showed negative correlations with EL, protein content and POX but it showed positive correlations with CAT, APX and GR. Within the same temperature

Table 2. Correlation coefficient between hydrogen peroxide (H_2O_2) electrolyte leakage (EL), protein, and antioxidant enzyme activities in rice seedling 'Dular', 'IR64' and 'KDML105' after treated with 25, 35 and 45 °C for 30 min (n = 3-4).

Sources	°C	H_2O_2	EL	Protein	POX	CAT	APX	SOD	GR
H_2O_2	25	1	-0.706*	-0.664	-0.763*	0.735*	0.426	0.060	0.616
	35	1	-0.250	0.017	-0.407	0.694*	0.809**	0.876**	0.684*
	45	1	-0.465	0.004	0.485	0.555	0.083	-0.625	-0.497
EL	25	-0.706*	1	0.564	0.756*	-0.736*	-0.024	0.077	-0.226
	35	-0.250	1	-0.410	-0.541	0.402	0.073	0.067	0.208
	45	-0.465	1	-0.678*	-0.782*	-0.718*	0.484	0.725*	0.624
Protein	25	-0.664	0.564	1	0.796**	-0.692*	-0.660*	0.047	-0.854**
	35	0.017	-0.410	1	-0.112	-0.115	-0.063	-0.130	0.029
	45	0.004	-0.678*	1	0.701*	0.441	-0.923**	-0.488	-0.521
POX	25	-0.763*	0.756*	0.796**	1	-0.969**	-0.165	0.378	-0.494
	35	-0.407	-0.541	-0.112	1	-0.914**	-0.779**	-0.748**	-0.893**
	45	0.485	-0.782*	0.701*	1	0.935**	-0.589*	-0.954**	-0.905**
CAT	25	0.735*	-0.736*	-0.692*	-0.969**	1	0.029	-0.496	0.343
	35	0.694*	0.402	-0.115	-0.914**	1	0.924**	0.932**	0.951**
	45	0.555	-0.718*	0.441	0.935**	1	-0.280	-0.970**	-0.919**
APX	25	0.426	-0.024	-0.660*	-0.165	0.029	1	0.587*	0.921**
	35	0.809**	0.073	-0.063	-0.779**	0.924**	1	0.975**	0.939**
	45	0.083	0.484	-0.923**	-0.589*	-0.280	1	0.357	0.373
SOD	25	0.060	0.077	0.047	0.378	-0.496	0.587*	1	0.352
	35	0.876**	0.067	-0.130	-0.748**	0.932**	0.975**	1	0.917**
	45	-0.625	0.725*	-0.488	-0.954**	-0.970**	0.357	1	0.902**
GR	25	0.616	-0.226	-0.854**	-0.494	0.343	0.921**	0.352	1
	35	0.684*	0.208	0.029	-0.893**	0.951**	0.939**	0.917**	1
	45	-0.497	0.624	-0.521	-0.905**	-0.919**	0.373	0.902**	1

*, **Significantly different by ANOVA at P ≤ 0.05 and 0.01, respectively.
POX: Peroxidase; CAT: catalase; APX: ascorbate peroxidase; SOD: superoxide dismutase; GR: glutathione reductase.

condition, a positive correlation existed between protein content and POX (Table 2). At 35 °C, we found positive correlations between H_2O_2 content and CAT, APX, SOD and GR. While, POX correlated negatively with CAT, APX, SOD and GR. At 45 °C, we noted a negative correlation between H_2O_2 content and EL and between protein contents and EL, APX, SOD and GR.

DISCUSSION

We investigated, herein, the physiological responses of three different rice cultivars at seedling stage to various temperatures. Exposure to various temperatures (at 25, 35 and 45 °C) for 30 min induced marked changes in physiological and biochemical processes resulting in different performances of the three rice cultivars.

Such short-term temperature at 25 °C stimulated the highest protein accumulation and the lowest H_2O_2 production in rice seedling 'IR64'. Because this temperature condition may stimulate an accumulation of H_2O_2-scavenging proteins such as POX (according to positive correlation between protein and POX +0.796; Table 2, Figures 4A and 4B) resulting in H_2O_2 reduction. POX is an antioxidant enzyme involving the protection of plant cells. It removes ROS occurring in the cell wall (Jajic et al., 2015) by catalyzing the oxidation of H_2O_2. In plant tissues, POX can oxidize phenolic compounds. As a result, heat-sensitive rice seedling 'IR64' (Coast et al., 2016) exposed to 25 °C for 30 min showed higher POX production, capable of eliminating H_2O_2 more effectively than the heat-tolerant 'Dular' (Lang et al., 2015) and tropical rice 'KDML105'. Although, membrane of 'IR64' was highly damaged under this temperature condition (Figure 2), this suggested that the membrane of 'IR64' may recover quickly due to its physiological performance such as photosynthetic efficiency, Hill reaction and photosynthetic pigment contents which were still high under this temperature condition (data not show). Mackill and Khush (2018) produced a breeding program for 'IR64' in International Rice Research Institute (IRRI) in a quest for a high yield and disease and insect resistance cultivar. Unfortunately, 'IR64' is susceptible to low P, Fe toxicity, drought stress and heat.

Under the short-term temperature at 35 °C, the rice seedlings 'Dular', 'IR64' and 'KDML105' showed the highest H_2O_2 content but produced the lowest EL. Because plant cells were metabolically active under this temperature condition. Therefore, we suggested that the temperature at 35 °C was the optimum for each of the three rice cultivars used in this study according to Hasanuzzaman et al. (2013a) and Pansarakham et al. (2018). As a result, it could modulate physiological mechanism including generation of ROS such as H_2O_2. According to our result, growing evidence in recent years suggested that ROS production is required for a wide range of plant responses to developmental and environmental cues, leading to growth and acclimation (Foyer and Noctor, 2016). Moreover, H_2O_2 is required directly in oxidative cross-linking reactions leading to cell wall strengthening (Smirnoff and Arnaud, 2019). In general, low ROS levels are necessary for the progression of basic biological process including cellular proliferation and differentiation (Huang et al., 2019). Compared with other ROS such as $O_2^{\bullet-}$ and $OH^{\bullet-}$, H_2O_2 is relatively long-lived, accumulates transiently and functions as signaling molecules in signal transduction pathways influencing gene expression in normal plant development including stomatal functions, root hair and pollen tube growth (Smirnoff and Arnaud, 2019), and in programmed cell death in response to pathogen infections and under temperature stress (Awasthi et al., 2015).

In addition to higher H_2O_2 production under short-term temperature at 35 °C, higher protein content was also found at this temperature which may play the important roles in all plant cell processes. In rice seedlings 'Dular' and 'KDML105', especially 'KDML105', higher activity of SOD was induced (Figure 2) hence higher rate of dismutation of $O_2^{\bullet-}$ to H_2O_2 resulting in higher H_2O_2 production (Figure 4E). This accorded with positive correlation between SOD and H_2O_2 (0.876, Table 2). SOD is a metalloenzyme that plays a central role in the defense of oxidative stress. It scavenges free radicals by dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2 (Sharma et al., 2012), thereby decreasing the risk of hydroxyl radical formation in plants. SOD occurs in chloroplast, mitochondria, cytosol, peroxisome and apoplast (Jajic et al., 2015). Within the same temperature condition, CAT activity in 'IR64' and 'KDML105' as well as APX and GR in 'Dular', 'IR64' and 'KDML105' were stimulated by higher H_2O_2 production, indicated by a positive correlation between H_2O_2 and CAT (+0.694), APX (+0.809) and GR (+0.684) (Table 2). Because H_2O_2 also plays a role as signaling molecule to increase antioxidant enzymes. Antioxidant enzymes, such as POX, CAT, APX and GR, and non-enzymatic antioxidants such as ascorbate (AsA), glutathione (GSH) and carotenoid (Hasanuzzaman et al., 2013b), are involved in the protection of plant cells by the elimination of the toxic oxygen intermediates such as $O_2^{\bullet-}$ and H_2O_2; CAT, which predominantly occurs in

peroxisome (Jajic et al., 2015), participates in H_2O_2 elimination by directly breaking down the H_2O_2 structure to produce water and oxygen molecules.

Environmental stress can cause either increase or decrease CAT activity depending on severity, duration and type of stresses (Sharma et al., 2012). As for APX, it plays a key role in scavenging ROS, protecting plant cells and eliminating H_2O_2 in water-water cycle and ascorbate-glutathione cycle. The APX activity occurs in chloroplast, mitochondria and cytosol. With regard to GR, an oxidoreductase enzyme, which eliminates H_2O_2 by Halliwell-Asada enzyme pathway, and also plays an important role maintaining the ratio between antioxidant and pro-oxidant in cells. It can be found in chloroplast, mitochondria, cytosol and peroxisomes (Yousuf et al., 2012). Among the roles of antioxidant enzymes (POX, CAT, APX, SOD and GR), it is the elimination of $O_2^{\cdot}$ (Zhu et al., 2020) and H_2O_2 resulting in plant cell membrane and organelle membrane unaffected by ROS toxicity, evidenced by lower EL levels at this temperature condition in all rice seedlings (Figure 3).

Notably, membranes of rice seedlings 'Dular', 'IR64' and 'KDML105' after short-term temperature at 45 °C (short-term heat stress) were damaged as indicated by higher EL (Figure 3). The membrane is the first part of the cell that recognizes stress from the environment and acts as a signal transmission in plants (Krishnan et al., 2011). Increased heat caused acceleration in the movement of lipids leading an increase in membrane fluidity, denaturation of protein components of the membrane, and finally ion leakage, membrane rupture and inhibition of physiological processes. The EL values, which indicate the degree of membrane damage, was estimated by the electrical conductivity of cell liquid leak from leaf tissues. Consequently, the higher EL value resulting from short-term temperature at 45 °C resulted in reduced membrane stability. Plant cells were inactive and led to low H_2O_2 production accorded to negative correlation between H_2O_2 and EL after treated with short-term temperature at 45 °C (-0.465, Table 2). Our results were similar to those of Kaushal et al. (2011), who reported that the highest EL in chickpea was found at 45/40 °C (day/night), as well as those of Timabud (2015) who found the higher EL in rice seedlings 'IR64' (heat susceptible) than those 'N22' (heat tolerant) and 'KDML105'. Borriboon et al. (2018) reported that EL was increased with increasing temperature at 40 °C for 1 and 2 wk in rice abnormal seedlings ('Dular', 'KDML105' and 'Riceberry'). In their research using rice 'Dular', Pansarakham et al. (2018) showed that leaves at dough grain stage exhibited no changes in EL after short-heat stress at 42 °C for 30 min. This indicated that leaves of this cultivar had higher level of membrane tolerance at dough grain stage (Pansarakham et al., 2018) than seedling stage (this study). However, leaves of rice 'IR64' and 'KDML105' exhibited membrane damage at both dough grain stage (Pansarakham et al., 2018) and seedling stage (current study).

Moreover, under short-term 45 °C, protein content in 'Dular' and 'IR64' was reduced. Therefore, short or medium-short exposure to temperature higher than 40 °C had direct effects on plant injury and protein metabolism by interrupting protein synthesis and increasing protein denaturation and aggregation (Waraich et al., 2012). Consequently, the cause of protein reduction after short-term heat stress (at 45 °C in this study) may be related to the decrease in the strength of H-bonds and electrostatic interactions between polar groups of proteins within aqueous phase of the membrane resulted in protein disassociation, and then protein molecules became denatured, aggregated and unfunctional. This was according to negative correlation between protein and EL (-0.678, Table2). Moreover, at this temperature condition (short-term heat stress), POX and GR in 'Dular', 'IR64' and 'KDML105', CAT in 'Dular' and 'KDML105' and APX and SOD in 'KDML105' were found significantly decreased activity. Short-term heat stress (at 45 °C for 30 min) may affect these enzymes by protein degradation leading to loss of functions. Heat stress also causes structural proteins and enzymes to become unfolding or misfolding (Usman et al., 2014). These breakdown and aggregation of cellular proteins or enzymes led to loss of protein structure and activity.

CONCLUSIONS

Among the three temperature treatments, 25, 35 and 45 °C, the greatest membrane stability was observed at 35 °C although the highest H_2O_2 content also occurred at this temperature. The higher membrane stability and the highest protein content indicated that rice seedlings were most metabolically active at this temperature, and the H_2O_2 level was regulated at the levels high enough to act as signaling molecules for normal growth and developmental processes including the activation of antioxidant enzyme activities. At 35 °C, antioxidant enzyme activity such as superoxide dismutase (in 'Dular' and 'KDML105') and catalase, ascorbate peroxidase and glutathione reductase (in 'Dular', 'IR64' and 'KDML105') were

stimulated. However, heat stress following a short exposure at 45 °C resulted in severe membrane damage as indicated by increased electrolyte leakage. At this temperature, the activity of some antioxidant enzymes was reduced compared to the 35 °C treatment while that of others were enhanced, and different cultivars employed different enzymes to varying extent. In addition, it was found that 'Dular' with short-term temperature at 45 °C exhibited membrane damage at seedling stage while no membrane damage was observed at dough grain stage. Thus, 'Dular' was heat sensitive at seedling stage while it was heat tolerant at dough grain stage. Short temperature treatment at 25 °C which was 10 °C lower than the optimum growth temperature also caused membrane damage leading to more electrolyte leakage than treatment at 35 °C. However, at 25 °C, 'IR64' demonstrated remarkably fast recovery from membrane injury.

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