Research Article

Antioxidant defence system and oxidative damages in Rice seedlings under heat stress

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Citation

Muhammad Kazim Ali, Abid Azhar, Haneef Ur Rehman and Saddia Galani. Antioxidant defence system and oxidative damages in Rice seedlings under heat stress. Pure and Applied Biology. Vol. 5, Issue 4, pp1131-1141.
<http://dx.doi.org/10.19045/bspab.2016.50136>

Received: 04/10/2016

Revised: 13/10/2016

Accepted: 18/10/2016

Online First: 22/10/2016

Abstract

Impact of climate change showed heat stress most severely affecting crop growth and yield worldwide. In current study, oxidative damages and response of antioxidant enzymes were compared in 4 rice cultivars (IR-6, IR-8, K-95 and Sada Hayat). These varieties were exposed to heat (42 ± 2 °C) stress at seedling stage. After 72 h, stressed plants transferred to normal temperature for recovery treatments. Oxidative stress damages were quantified through analysis of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content. Effect of heat stress on antioxidant enzymes activities were determined by quantitative analysis of ascorbate peroxidase (APX) and catalase (CAT) enzymes. With increasing oxidative stress significant proline accumulation was detected in rice cultivars and K-95 had maximum proline among the group. Significant increment of MDA and hydrogen peroxide (H_2O_2) content was found in as compared to others. CAT and APX showed significantly declined activity in Sada Hayat and IR-8 under heat stress condition however K-95 showed maximum activity. These findings suggesting that (i) K-95 is comparatively thermotolerant cultivar (ii) thermo-tolerance in rice is closely related to efficient antioxidant enzyme defence system.

Keywords: Oxidative stress; Antioxidants; Lipid peroxidation; Thermotolerance Rice; Enzymes

Introduction

Abiotic and biotic stresses are major challenges for sustainable agriculture that posing severe threat to maximum yield through limiting plant growth and development [1]. Specifically, abiotic stress is the primary cause of crop yield loss worldwide [2]. Yield of major economically important crops like wheat, maize and rice

are affected by heat stress [3]. It is reported that for a 1 °C increase in daily mean temperature from threshold nearly 10% rice yield decreased [4]. Some specific effects are reported for each stress factor on plants but oxidative stress is common in all stress conditions due to over production and accumulation of reactive oxygen species (ROS). Over production of ROS are

considered as deleterious and lethal to living systems because these are responsible of carbohydrates, lipids, proteins and deoxyribonucleic acid peroxidation leading cell necrosis. Consequently, higher level of free radicals causes a cellular oxidation through a chain reaction. In addition to the oxidative stress related production [5]. Plants have developed an antioxidant defence system and they play key role in detoxifying and balancing ROS in cell [6]. Enzymatic antioxidants including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POX) [7, 8]. Among these antioxidant enzymes, SOD is considered as first defence line because it detoxifies superoxide which is the first reactive oxygen species, generate in cell during stress condition [9]. DNA, Protein and lipids are at high risk due to increased production and accumulation of free radicals under oxidative stress [10, 11]. Prolonged exposure to high temperature during sensitive growth stage of plant may lead to leaf senescence, wilting, damages to leaf margins, desiccation, stunted growth, interrupted reproductive development and death of plant [12]. Accumulation of osmolytes (proline) in addition to antioxidant enzymatic scavenging system is another important adaptive strategies of plant to cope with unfavorable environmental conditions. Free proline accumulation is closely related with plant thermal stress tolerance significantly in crops and other plants under stress condition specifically under oxidative stress [13, 14, 15, 16]. High concentrations of proline can accumulate in plant cells as an osmoprotectant without disrupting cell metabolism and cellular structure in plants subjected to heat stress conditions. Hence, under thermal stress condition, osmotic adjustment, membrane integrity and detoxification of ROS is significantly

correlated with proline accumulation in plants [17, 18, 19].

Under heat stress conditions response of rice investigation at biochemical basis are rare and antioxidative defence mechanisms performance are hardly studied specifically indica rice. Previous investigations on rice response to environmental harsh conditions are mostly focus on the drought stress and salt stress effects on rice and other species of the family. Effect of elevated temperature is rarely studied and some consequences of heat stress at field condition was reported only in rice yield. Although there are many rice cultivars currently sowing in Pakistan but in Sindh province non-aromatic rice mostly used because it experiences warmer summer as compare to other regions. There are no such studies reported before to screen local rice cultivars for thermotolerance. This research is designed in order to determine antioxidant enzyme activities, lipid membrane peroxidation and proline accumulation heat stressed rice seedlings. Molecular mechanisms of thermotolerance is still enigmatic in rice therefore to help in identify thermo-tolerance mechanisms, comparison of different rice cultivars responses towards heat stress will allowing further insights into the heat induced oxidative stress.

Materials and methods

Plant materials, growth conditions, and stress treatments

The seeds of rice (*Oryza sativa* L.) cultivars (IR-6, IR-8, K-95 and Sada Hayat) were collected from Rice Research Institute, Dokri, (Pakistan). After surface sterilization, rice seeds were allowed to germinate in pots. Seedlings grown in a controlled growth chamber (28 ± 2 °C) with 16 h light ($400 \mu\text{mol m}^{-2} \text{s}^{-1}$) photoperiods and 8 h dark. For irrigation Hoagland's solution (half strength) was used [20]. Then plants (20-day-old seedlings), growing under normal temperature was considered as control (C),

were subjected to heat stress (HS) by gradually increasing the temperature and maintained at 42 ± 2 °C. Sampling were carried out after 24, 48 and 72 h of heat stress. After 72 h, stressed plants transferred to normal (28 ± 2 °C) temperature for recovery treatments (RC). For control treatment, rice seedlings allowed to grow in the same way and watered with properly in addition of Hoagland's solution supplement. Before investigation of physiological attributes, seedlings were harvested, shoot lengths, fresh and dry weights of shoots were observed. Analysis of MDA and hydrogen peroxide content were determined followed by total quantification of catalase (CAT) and ascorbate peroxidase (APX) enzymes.

Growth parameters

To show that heat stress effects on rice growth visually, total shoot lengths were recorded followed by photography of samples. Fresh shoot and dry weights were measured as weight of shoot tissue. Shoot and root samples were taken from rice seedlings treated with control, heat stress and recovery conditions, for analysis of fresh weight (FW) at 30 days of rice growth. For dry weights (DW) analysis rice seedling samples were kept in oven at 60 °C for 24 h.

Hydrogen peroxide (H₂O₂) content determination

The production level of H₂O₂ was measured spectrophotometrically according to Jessup [21]. H₂O₂ was extracted by homogenizing 0.1g fresh leaves in 1ml of 0.1% TCA. The homogenate was centrifuged at 12000 rpm for 15 minutes. To quantify H₂O₂ concentration, 0.5 ml of potassium phosphate (10 mM) and 1 ml of potassium iodide (1M) was mixed with supernatant. Supernatant yellow color intensity was measured 390 nm using UV-spectrophotometer. Extinction coefficient ($0.28 \mu\text{mole}^{-1}\text{cm}^{-1}$) was used to calculate concentration of H₂O₂ in controlled and heat

stressed rice seedlings and expressed as $\mu\text{mole g}^{-1}\text{FW}$.

Determination of malondialdehyde (MDA) content

Strength of lipid peroxidation was measured by quantification of total malondialdehyde (MDA) content generated by heat stress treatments for the evaluation of cell membrane damages, according to Ohkawa [22]. Leaf tissues (0.1 g) was homogenized in 1 ml of 5% trichloroacetic acid (TCA). The homogenates subjected was then centrifugation for 15 at 12,000 rpm. Supernatant was mixed with freshly prepared thiobarbituric acid (TBA, 0.5%) in trichloroacetic acid (TCA, 20%) and incubated for 30 min at 95 °C into new eppendorf tube followed by centrifugation at 10,000 rpm. Correction for un-specific turbidity during measurement of MDA content was performed by subtracting the absorbance of 600 nm and absorbance of 532 nm. Results were expressed in terms of nmol MDA g⁻¹ FW after calculation of MDA contents using extinction coefficient ($155 \text{ mM}^{-1}\text{cm}^{-1}$).

Proline content determination

Bates [23] method was used for analysis of amount of free proline. For this purpose, 0.1 g leaf tissue was homogenized in 3% sulphosalicylic acid followed by centrifugation at 1200 rpm for 10 minutes. Homogenized tissue (1 ml) was incubated with acid ninhydrin (2ml) and glacial acetic acid (1 ml) in a test tube incubated for 1 h at 100 °C. The reaction was stop by place the sample on ice. Toluene (2 ml) was used to extract the reaction mixture by mixed vigorously followed by incubation at room temperature (30 min) until two phase separation was observed. The chromospheres (upper phase) was used to quantify free proline at 520 nm using toluene for a blank.

Determination of enzyme activities

For antioxidant enzyme analysis, leaf samples were homogenized followed by centrifugation at 13,000 rpm. Supernatant of each sample were used for enzyme activity measurements after centrifugation (20 min) of homogenate at 12,000 g at 4 °C. Bradford method [24] was used to determine total protein concentration in leaf extracts. Bovine serum albumin (BSA) was used as standard. Ascorbate peroxidase (APX) enzyme quantification was carried out according to Wang [25] protocol. Suspension solution (1 ml) having Tris-HCl (pH 7.2, 50 mM), PVP (2%), EDTA (1 mM) and 2 mM of ascorbate mixed with samples containing 100 µg of protein. For initiation of the reaction, assay medium was mixed with potassium phosphate buffer (50 mM, pH 6.6), ascorbate (0.25 mM) and H₂O₂ (1 mM). The absorbance of ascorbate was measured at 290 nm for 90s. 1 unit of APX was defined if 0.001 change in absorbance of ascorbate occurred in per minute.

Catalase (CAT) activity was measured by using Chance and Maehly, [26] method. Extracted samples having protein (100 µg) were mixed in Tris-HCl (1 ml, 50 mM) solution at pH 7.8. Potassium phosphate (50 Mm, pH 7.0) buffer and H₂O₂ (10 mM) was

mixed with assay medium. The decline in absorbance of H₂O₂ was monitored at 240 nm for 90 s. 1 unit of CAT was defined as 0.001 change in hydrogen peroxide absorbance per minute.

Data analysis

All analyses were done using a completely randomized design with 4 replicates (n = 4). All data were subjected to one-way analyses of variance (ANOVA). Duncan's multiple range test was used to separate statistically different means at $p < 0.01$ and $p < 0.05$.

Results and discussion

Growth parameters

Result analysis revealed that with increase in intensity of heat stress treatment, fresh weights of shoots of all rice cultivars decreased but difference in degree of decline in shoot weight observed. Similarly, dry weight of heat treated rice seedling's shoots decreased significantly ($p \leq 0.05$) in all cultivars with increase in duration of thermal stress while increment found upon recovery treatments (Table 1). Throughout the experiment no significant effect of thermal stress on the shoot length of rice seedlings were observed although plants showed diverse shoot length within the group may be due to difference in metabolic activities, growth regulators and genetic factors.

Table 1. The effect of heat stress treatments on growth parameters of rice (*Oryza sativa* L.) cultivars

Growth Attributes	Cultivar	Control	Heat Stress			Recovery		
			T24	T48	T72	R24	R48	R72
Shoot Length (cm)	IR-6	15±1.0	15±5.4	15±4.5	14±0.6	16±2.1	15±4.1	16±3.2
	IR-8	16±3.2	15±3.4	15±3.2	16±0.9	15±3.2	16±3.2	16±1.3
	K-95	18±0.2	19±1.2	19±2.8	18±1.2	18±4.3	18±5.2	18±1.2
	SDHT	15±3.2	13±7.6	13±1.6	13±2.5	13±5.4	13±3.5	14±5.3
P > 0.05								
Shoot Fresh Weight (gm)	IR-6	0.24±3.7	0.20±1.3	0.17±2.3	0.14±0.1	0.13±0.3	0.15±2.1	0.16±3.1
	IR-8	0.24±2.3	0.18±0.9	0.15±4.2	0.13±0.4	0.13±0.4	0.14±2.3	0.15±3.2
	K-95	0.26±1.4	0.24±1.2	0.21±1.2	0.19±0.7	0.19±0.6	0.21±2.1	0.22±2.3
	SDHT	0.20±1.2	0.16±2.9	0.13±4.3	0.10±0.9	0.11±0.5	0.11±3.1	0.13±1.2
P < 0.05								
Shoot Dry Weight (gm)	IR-6	0.10±0.2	0.09±2.1	0.08±2.1	0.06±2.1	0.06±0.1	0.06±1.2	0.07±0.1
	IR-8	0.10±0.1	0.08±0.8	0.07±3.1	0.05±3.2	0.05±1.3	0.05±0.7	0.06±0.3
	K-95	0.12±0.4	0.11±1.4	0.10±2.5	0.09±4.2	0.09±0.5	0.10±0.6	0.12±0.3
	SDHT	0.09±0.5	0.07±0.5	0.06±4.2	0.04±1.2	0.05±0.6	0.05±0.5	0.05±0.1
P < 0.05								

Each value represents the mean of 3 replicates (n = 3) and standard deviation of mean (±SD). Duncan's multiple range test showed significant differences in means (±SD) between control and heat-treated plants at $P < 0.05$

Hydrogen peroxide, melondialdehyde and proline contents

H₂O₂ is a reactive compound produced due to scavenging of superoxide radicals by superoxide dismutase (SOD) enzyme. Lipid peroxidation and membrane injury is a sign of higher accumulation of H₂O₂ leading death of plants and yield loss [27]. Linear grew in content of H₂O₂ in all cultivars were observed with increasing level of heat stress condition however maximum ($p \leq 0.01$) generation and accumulation of H₂O₂ content were found in Sada Hayat as compared to others (Figure 1). In the present study, K-95 cultivars presented lowest values of H₂O₂ accumulation under thermal stress, indicated that K-95 has efficient free radical quenching system at a cellular level that offered maximum protection against oxidative stress damages.

Oxidative stress leading cytotoxic damage of protein, DNA and lipid observed in plants subjected to environmental stress conditions because reactive oxygen species (ROS) production overcomes antioxidant defence system capacity [28]. MDA quantification in order to figure out level of lipid peroxidation has been considered as a convenient biomarker due to its reaction with thiobarbituric acid (TBA) forming coloured chromogen [29]. Minimal increment in content of MDA was observed in “K-95”, seedlings of “Sada Hayat” (Figure 2). The MDA content was found to be significantly highest in Sada Hayat and lowest in K-95. Minimum values of MDA exhibited by K-95 suggest that it has developed an efficient and effective ROS scavenging system and therefore, showed better performance. Compared to control plants,

heat treated rice seedlings had increase level of MDA which shows that rise in temperature beyond optimum for growth could cause peroxidation of membrane lipids making cell porous consequently cell sap leakage leading cell shrink and death [30].

In many plant species proline accumulations under stressful conditions has been positively correlated with tolerance mechanism [16]. The cultivars showed significant increment patterns for proline content in shoot tissues in relation to severity of high temperature stress (Figure 3). Other researchers have reported that stress-tolerant cultivars exhibited higher accumulation of free proline opposed to stress-sensitive varieties [31]. The accumulation of free proline under harsh environmental condition as an advantageous mechanism of living system to protect membrane lipids, proteins and key cellular components insuring maintenance of cell homeostasis when relative water content (RWC) decreases [32]. Decrease in RWC with minimum MDA suggest that increased accumulation of proline insuring cell membrane integrity. In various plants (cucumber, sunflower, chickpea and tomato), increase in free proline accumulation have been reported under oxidative stress [33-37], which is considered important adaptive and protection mechanism to cope with consequences of heat stress. In addition of proline measurement, analysis of chlorophyll fluorescence can be carried out in order to elucidate effect of thermal stress on photosynthetic apparatus [38].

Figure 1. Hydrogen peroxide (H_2O_2) content of four rice cultivars, IR-6, IR-8, K-95 and Sada Hayat subjected to control (C), heat stress (T24, T48 and T72) and recovery (R24, R48 and R72) conditions at seedling stage

Figure 2. Content of melondialdehyde (MDA) in four rice cultivars, IR-6, IR-8, K-95 and Sada Hayat under control (C), heat stress (T24, T48 and T72) and recovery (R24, R48 and R72) conditions

Figure 3. Accumulation of osmolyte (proline) in four rice cultivars, IR-6, IR-8, K-95 and Sada Hayat subjected with control (C), heat stress (T24, T48 and T72) and recovery (R24, R48 and R72) treatments at early growth stag

Antioxidant system

Elevated temperature from threshold can cause oxidative damage due to over production of ROS. Thus, plant cells need different mechanisms for quenching of excess free radicles and keep them in balance condition. To elucidate differences in response of antioxidant system of rice cultivars under heat stress, response of ascorbate peroxidase (APX) and catalase (CAT) were observed in heat treated seedlings of rice cultivars.

Catalase (CAT) enzyme is the principal component of antioxidant defence system responsible if scavenging harmful oxygen species specifically H_2O_2 , therefore, better activity of CAT play important role in reducing heat stress-induced damages [39]. In this investigation, catalase enzyme showed markedly declined activities under stress condition and recovered upon recovery treatments (Figure 4) suggesting that thermal stress has negative effect on CAT activity. The increment in CAT

enzyme activity under stress conditions can be explained by the very low affinity of this enzyme for H_2O_2 , as it becomes active at relatively high H_2O_2 concentrations [40]. It is also suggested that higher CAT concentrations may have removed the superoxide ($O_2^{\cdot-}$) radicals induced by heat stress [30].

High temperature treatment showed insignificant rise in ascorbate peroxidase (APX) activity in all cultivars and Sada Hayat seedlings showed significantly ($p \leq 0.05$) declined pattern of APX (Figure 5). According to Celikkol [41], APX enzymes experience oxidation and inactivation upon application of stress treatments leading enhanced accumulation of ROS in cells. Previously decline in activity of APX enzyme has been reported under water stress condition [42].

The findings of the investigation of various biochemical and physiological attributes manifested that under high temperature stress rice cultivars shared common

characteristics, however, cultivar K-95 appeared to be biochemically as well as physiologically effective and strong mechanism, hence, less effected. CAT enzyme activity might have played important and protective role in K-95 and IR-6 which showed thermotolerance under heat stress condition. Despite the over production of H_2O_2 content in K-95 shoots under heat stress conditions, lipid peroxidation (MDA) content did not increase, indicating cell membrane integrity due to lower oxidative damages. In all cultivars, CAT enzyme seems to play key role in maintaining H_2O_2 concentration at normal level, inhibiting chain reaction of reactive oxygen species that may lead to cell death. In K-95 despite the activity of catalase alone, decline in APX activity

seems to cause an increment in level of H_2O_2 concentration, however, in Sada Hayat and IR-8 slight uplift in activity of APX enzyme together with CAT activity achieved the fine modulation of H_2O_2 content. K-95 showed increment in content of hydrogen peroxide at lesser magnitudes of high temperature stress where ascorbate and catalase had insignificant activity; however, MDA amounts were stable as compared to the controls. According to our results, two cultivars (K-95 and IR-6) were thermo-tolerant, whereas K-95 appeared to be less affected biochemically and physiologically, rendering K-95 thermotolerant followed by IR-6 while Sada Hayat and IR-8 were thermo-susceptible. The rice cultivar Sada Hayat had more damages as compared to IR-8.

Figure 4. Catalase (CAT) enzyme activity in four rice cultivars, IR-6, IR-8, K-95 and Sada Hayat at seedling stage grown under control (C), heat stress (T24, T48 and T72) and recovery (R24, R48 and R72) conditions.

Figure 5. Effect of heat stress on Ascorbate peroxidase (APX) enzyme activity in four rice cultivars, IR-6, IR-8, K-95 and Sada Hayat subjected with control (C), heat stress (T24, T48 and T72) and recovery (R24, R48 and R72) conditions

Conclusion

From the findings, it is concluded that rice cultivar “K-95” comparatively performed better under heat stress condition showing thermotolerant ability with minimal oxidative damages and improved antioxidant system and maximum accumulation of osmolyte (Proline) followed by “IR-6,” while “Sada Hayat” showed heat susceptibility with maximum oxidative damages and compromised antioxidant system having weak capability of osmolyte accumulation for maintaining cell homeostasis under harsh condition.

Author's contributions

Conceived and designed the experiments: S Galani, Performed and analyzed the data and paper wrote: MK Ali, Paper Writing: HU Rehman, Contributed reagents /materials/ analysis tools: A Azhar.

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