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DIFFERENTIAL EXPRESSION OF MOLECULAR CHAPERON (HSP70) AND ANTIOXIDANT ENZYMES: INDUCING THERMOTOLERANCE IN RICE (*ORYZA SATIVA* L.).

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Abstract

Hyperthermia adversely affects cell structure and function, leading to necrosis and death of the plant that ultimately result in yield loss. Induced expression of the molecular chaperone protein HSP70 and antioxidant enzymes (SOD, CAT, APX and POD), can prevent heat induced cell death, by protecting structural and functional proteins conformation and detoxification of reactive oxygen species (ROS) respectively. In this study, temporal expression of molecular chaperones (HSP70) and antioxidant enzymes were determined during grain filling stage of eight local rice (IR-6, IR-8, DR-82, DR-83, DR-92, K-95, Sada Hayat, Shahkar) cultivars under different temperature regimes (control, 42±2 ^{0}C and recovery treatments) for different time episodes (24, 48 and 72h). Results revealed heat stress induced expression of HSP70 with increasing duration of heat stress and this temporal expression was strongly correlated with thermotolerance in rice cultivars. Superoxide dismutase (SOD) and peroxidase (POD) gel-based study showed, elevated temperature, in general, enhanced expression and activity; however, it was cultivar specific. Among all cultivars, "DR-82", "DR-83" and "Sada Hayat", showed comparatively low expression of HSP70 as well as compromised and decline activity of SOD and POD under stress condition. Cultivar, "K-95" had strong expression of HSP70, more than one heat responsive bands of SOD and POD, provoking thermotolerance. It is concluded that heat shock proteins specifically expression of HSP70 can minimize heat induced damages and cell death by maintaining structural and functional protein integrity and play key role in development of thermotolerance, therefore can be used as potential biomarker of heat stress tolerance in rice.

Keywords: Hyperthermia, HSP70, antioxidants, screening, thermotolerance

Introduction

Whenever plant face harsh environmental conditions, their cells and tissues are at the high risk for death which culminate in retarded development and loss of yield due to metabolically altered functions (Wahid *et al*., 2007). Number of genes have been reported to express during different stress conditions in plants (Narusaka *et al*., 2003). Among other stresses low and high temperature had multifarious effects on crop productivity but their response varies at different growth stages (Halford, 2009; Narusaka *et al*., 1999). However, oxidative stress due to enhanced expression and accumulation of free radical cause degradation of cell membrane, making the lipids more permeable and leakage of cell sap which is a common and direct response to thermal stress in all plant including rice. In cells and tissues, subjected to heat stress, aggregation of damaged and misfolded cellular proteins are also observed within minutes (Sarkar *et al*., 2013).

Over production of ROS are considered as deleterious and lethal to biological systems because they cause the oxidation of carbohydrates, lipids, proteins and deoxyribonucleic acid. In addition to the oxidative stress related production, toxic levels of ROS cause also a chain reaction of cellular oxidation leading to cell death. (Mittler *et al*., 2006). Excess accumulation of reactive oxygen species can destroy cell homeostasis through induction of oxidative damage to lipids, proteins and DNA (Rabinowitch & Fridovich, 1983; Fridovich, 1986). Plants have developed an antioxidant defence system comprising of both non-enzymatic and enzymatic, that play key role in balancing and detoxifying ROS (Baysal Furtana & Tıpırdamaz, 2010). Enzymatic antioxidants including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POX) (Bowler *et al*., 1992; Çelik & Atak, 2012) are most important. Among all, superoxide dismutase (SOD) are considered as first defence line mechanism (Halliwell, 1974).

To assist newly synthesized proteins to fold into their native form and protect sensitive organelles for assurance of cellular metabolic activities plants express different types of stress induced proteins under stress conditions (Masood *et al*., 2005; Allagulova *et al*., 2003; Wahid *et al*., 2007; Ingram & Bartels, 1996). Among these heat responsive proteins, heat shock proteins (HSPs) are well known to induce and accumulate in stressed cells (Kang *et al*., 2005). Enhanced expression and accumulation of various HSPs has been considered an important and tolerance mechanism manifested to cope with harsh environments (Wahid & Close, 2007).

Identification of heat shock protein (HSP70) using functional molecular biological approach manifested that expression level of HSP70 found strong positive correlation with heat acclimation of cells and tissues and adaptation in changing environmental conditions (Abreu *et al*., 2013, Gurley, 2000). HSP70 is found responsible in various cellular functions including folding of newly synthesized proteins, translation, translocation, proteolysis and transportation of aggregated proteins and specifically refolding of unfolded proteins (Zhang *et al*., 2010; Gorantla *et al*., 2007). In addition, heat responsive polypeptides assist in biosynthesis, energy and carbohydrate metabolisms or in redox homeostasis (Gorantla *et al*., 2007). Heat stress response in many crops including rice is a complicated process, which involves up- or down-regulation of many proteins and genes linked to multiple metabolic pathways (Zhang *et al*., 2010).

Despite consideration of a model crop, rice response towards thermal stress is enigmatic (Kang *et al*., 2010; Shah *et al*., 2011). Detoxification of ROSs and maintenance of proteins conformation is detrimental for survival under unfavorable environmental conditions. Identification of proteins potentially linked to thermotolerance, is an important and practical step. It helps to elucidate the mechanism involved in thermo-tolerance at molecular level and thereby leading to production of plants having enhanced tolerance to stress (Zou & Liu, 2011). Here, we present the significance of HSP70 in heat stress tolerance of rice seedlings and discuss the recovery potential of different rice cultivars from stress condition and their difference is in stress induced damages (Gorantla *et al*., 2007; Lin *et al*., 2014).

Materials and Methods

Growth and stress conditions: Rice seedlings established under natural condition in the green house of Dr. A. Q. Khan Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi. Twenty days old seedlings were transplanted in pots filled with 60-70% sandy loam soil. Each pot had approximately 10 seedlings and later thinned to 3-5 plants per pot. After 10-15 days of transplantation, fertilizer DAP nearly 3-5 g/ pot, were used. Plants were also nourished with full and half strength Hoagland nutrient solution (Hoagland & Arnon, 1950) after every second week up to maturity. The plants were maintained in standing condition approximately 2-4 mm. Flowering started nearly after 3 months while grain filling took 4-5 months from the date of sowing.

Plants at maturity stage were subjected to thermal stress (42±2 °C) by gradually increasing temperature. Humidity was adjusted at 45±2 % and 58±2 % during day and night respectively. Samples were collected after 24, 48 and 72 h of stress treatment and stressed plants transferred back to normal condition (28±2 °C) for recovery treatments. During recovery condition data was recorded after 24, 48, and 72 h. After initial morphological and physiological analysis, remaining samples were stored at -80 ºC for protein and enzyme studies.

Antioxidant enzyme analysis: Fresh leaf tissue was used to extract antioxidant enzymes according to Springer protocol (Elavarthi & Martin, 2010). 0.1gm leaf tissue homogenized in 1.2 ml enzyme extraction buffer [K.P buffer (0.2 M, pH7.8), with EDTA (1 mM), PVP (1%), PMSF (1 mM)] followed by centrifugation at 13000 rpm at 4°C. Pellet resuspended in K.P. buffer and centrifuged again. Both the supernatant mixed and stored at 4°C for enzyme quantification and Native-PAGE studies.

Quantitative analysis of SOD, CAT, APX and POD were performed according to combined protocol of Springer (2010), Beauchamp & Fridovich (1971), Giannopolitis & Ries, 1977, Rao *et al*., (1990) and Polle *et al*., (1994).

Quantification of SOD was carried out in a reaction mixture (3ml) containing K.P buffer with EDTA, l-methionine, 50 µM NBT and Triton-X100 (0.025%). 20 µl of riboflavin and 20 µl sample were mixed with the reaction mixture and the reaction initiated via illuminating the mixture under a 15 W light source. Four tubes containing similar reaction mixture except enzyme extract were prepared and two of them kept in the light without enzyme while other two with enzyme extract were kept in dark and used as blanks. All the tubes placed in a box covered with aluminum foil during the 15-min light exposure. The reaction was stopped by turning off light and after 1-2 min absorbance was measured at 560 nm. 1 unit was considered at 50% inhibition of the reaction mixture and expressed as U/g FW.

For APX quantification, decrease in absorbance of ascorbate were measured at 290 nm after every 20 sec. Reaction mixture (3 ml) were prepared having 10 µl enzyme extract with K.P. buffer (pH 7.0), ascorbic acid and H_2O_2. The enzyme unit calculated and expressed as U/min/ml/ g FW. CAT was assayed as a decrease in absorbance of H_2O_2 at 240 nm after every 20 se0.01 change in absorbance per min was considered as 1 U of enzyme and expressed in terms of units per minute per ml per gram fresh weight.

For POX activity potassium phosphate buffer (50 mM, pH, 7.0) prepared and Guaiacol (2.7 mM) and enzyme substrate were added. Finally, 50 microliter enzyme extract added and mixed with the reaction mixture. Oxidation of guaiacol was monitored after 20 sec., till 3 min at 470 nm.

Native-PAGE analysis: For qualitative analysis of antioxidant enzymes, superoxide dismutase (SOD), and guaiacol type peroxidase (POD), non-denaturing polyacrylamide gel electrophoresis (Native-PAGE) was performed as described by Davis (1964). Tris-HCl (4mM) and glycine (38 mM) having pH, 8.8 was used as running buffer. For POD 7.5% and 3.5% while for SOD 10 % and 5 %, resolving and stacking gel were prepared respectively. Enzyme extract was mixed with sample dilution buffer (SDB), 0.0625M tris-buffer (pH 6.8), 10% glycerol, 2-mercaptoethanol and bromophenol blue (0.025%), at 1:1 ratio. Electrophoresis performed at 80 V for SOD while at 70 V for POD at 4°C.

Staining of SOD were carried out as described by Beauchamp & Fridovich (1971), Shah & Nahakpam (2012), Lee *et al*., (2001) and Kim *et al*., (2005). Superoxide dismutase (SOD) enzyme showed colorless bands after incubation in 0.25 mmol L^{-1} NBT and K.P. buffer (pH, 7.0) for 30 min having 0.026 mM riboflavin and 0.2% TEMED Qualitative analysis of POD was carried out while treating the gel with K.P buffer (pH 6.0) in which guaiacol (20 mM) was used as substrate along with H_2O_2 (0.01%). The gels were kept in dark for 15-20 min as described by Woodbury *et al*., (1971) and then visualized in light. Orange-brown bands of POD were observed and the gel was preserved for image analysis.

HSP70 expression analysis: Total soluble protein isolated by pulverizing leaf tissue with protein extraction (QB, pH 7.2) buffer followed by centrifugation at13,000 rpm. Protein quantification was carried out using Bradford, (1976) method

Initially extracted proteins are allowed to resolve on ID SDS-PAGE gel under denaturing condition. Two SDS-PAGE gel electrophoresis were performed parallel for each sample, one for staining with Coomassie Brilliant

Blue G-250 (CBB) dye, while second gel was used for blotting to a nitrocellulose membrane (0.45µm). Transfer buffer (0.025 mmol/L Tris-HCl, 125 mmol.L^{-1} glycine, pH 8.3) were used for protein blotting at 4 °C through continuous supply of current (100 V) for an hour.

Detection of HSP70 expression in control and stressed samples was carried out on the basis of antigen-antibody reaction. For this immunological detection, monoclonal antibody (1° Ab, Mouse) to HSP70, purchased from Abcam, (ab69561) was used in Tris-buffered saline (TBS, 10 %) and followed by overnight incubation. TBS buffer (1X) were used for washes three times and anti-rabbit IgG, (2° Ab, goat) alkaline phosphatase (AP) attached was used to treat the membrane for 2 h. Immuno-complex detection were carried out using alkaline phosphatase (ALPH) buffer containing NBT and BCIP (Towbin *et al*., 1979).

Statistical analysis

For statistical analysis results of physiological and biochemical attributes were analyzed by ANOVA test using SPSS17 software and $p<0.05$ level was considered for significant difference. LSD was applied to normality test and Pearson's method for correlation.

Results

Quantitative analysis of superoxide dismutase (SOD) exhibited induction of SOD enzyme under heat stress and declined upon recovery treatments (Fig. 1). Cultivar showed different pattern of enzyme expression thermal stress. Statistical analysis revealed, treatments (T) and their interaction with cultivars (C x T) showed significant ($p<0.05$) differences but cultivars (C) had no significant difference ($p>0.05$) for this attribute.

Among cultivars, "IR-6", "K-95" and "Sada Hayat" showed minimum while "DR-83", DR-92" showed maximum SOD activity under normal growth condition. Although heat stress induced SOD expression initially but declined with increase in heat stress duration. After 24h of thermal stress (T24), highest activity exhibited by "K-95" and "DR-92" (700U/g FW), while lowest observed in "Shahkar" (300U/g FW). At T48, highest and lowest activities of SOD exhibited by "Sada Hayat" and "DR-83", respectively. After 72 h of heat stress (T72), "Shahkar" exhibited maximum while "DR-92" had lowest SOD enzyme units. Quantitative analysis of catalase (CAT) exhibited minimum amount in non-stressed leaf tissues of "Sada Hayat" (233U) while maximum indicated in "DR-82" (500U) followed by "IR-8", "DR-83" and "DR-92" (Fig. 2). At T24, "IR-6" exhibited166U of CAT while "K-95" showed 316U as compared to the other cultivars. After T48 "K-95" had 233U while "IR-6" had 111U, among the group. At T72, maximum and minimum CAT activity observed in "K-95" and "DR-83" with 150U and 100U, respectively. Activity pattern of ascorbate peroxidase (APX) enzyme at maturity stage was similar at flowering but cultivars showed low APX units at this stage (Fig. 3). Under control condition, lowest activity of APX enzyme indicated by "DR-82", followed by "DR-92" while highest APX observed in "Sada Hayat". After T24, the highest APX was 3277U exhibited by "IR-6" while "IR-8" had lowest with 1944U/g FW. After T48 cultivar "DR-83" showed maximum (2277U) followed by "DR-92" while "IR-8" showed lowest (1111U) APX activity. At T72, "IR-6" had highest while "IR-8" had lowest APX among the group. Peroxidase (POD) enzyme showed maximum activity in "DR-83" (3222U/gFW) while lowest in "IR-8" (1277U/gFW) at control condition (Fig. 4). After T24, maximum POD was observed in "Shahkar" (4750U/gFW) and minimum was in "DR-82" (2388U/gFW). At T48, maximum and minimum POD activity exhibited by cultivar "DR-83" and "Sada Hayat", respectively. After T72, "DR-83" had maximum while "DR-82" showed minimum activity among the group.

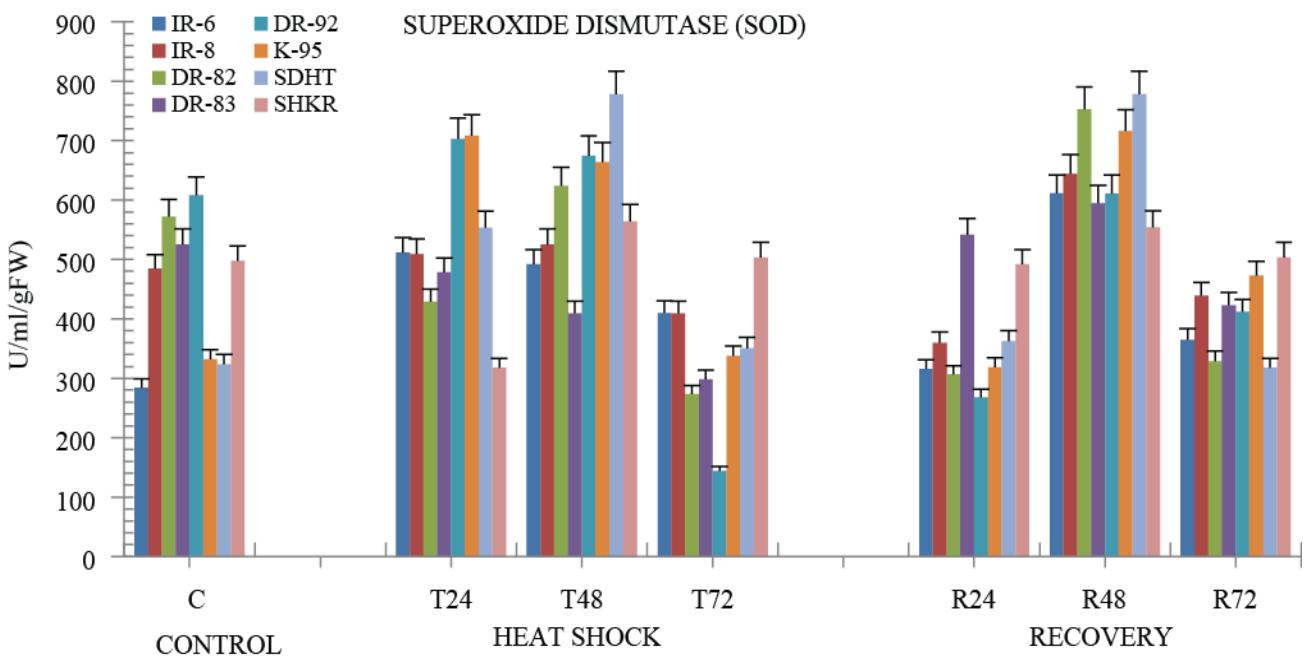

Fig. 1. Spectrophotometric quantification of Superoxide dismutase (SOD) enzyme activity of eight rice cultivars, IR-6, IR-8, DR-82, DR-83, DR-92, K-95, Sada Hayat and Shahkar, at grain filling stage, under normal (28±2 °C) (C), heat shock (42±2 °C) for 24h (T24), 48h (T48), 72h (T72) and recovery (28±2 °C) after 24h (R24), 48h (R48) and 72h (R72).

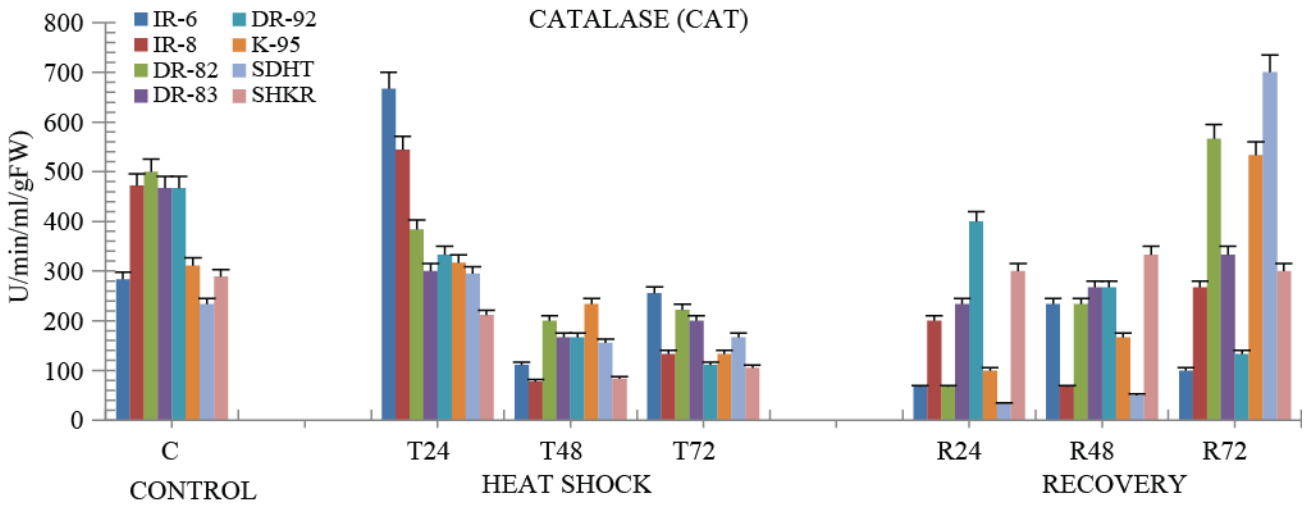

Fig. 2. Spectrophotometric quantification of Catalase (CAT) enzyme activity of eight rice cultivars, IR-6, IR-8, DR-82, DR-83, DR-92, K-95, Sada Hayat and Shahkar, at maturity stage, under normal (28±2 °C) (C), heat shock (42±2 °C) for 24h (T24), 48h (T48), 72h (T72) and recovery (28±2 °C) after 24h (R24), 48h (R48) and 72h (R72).

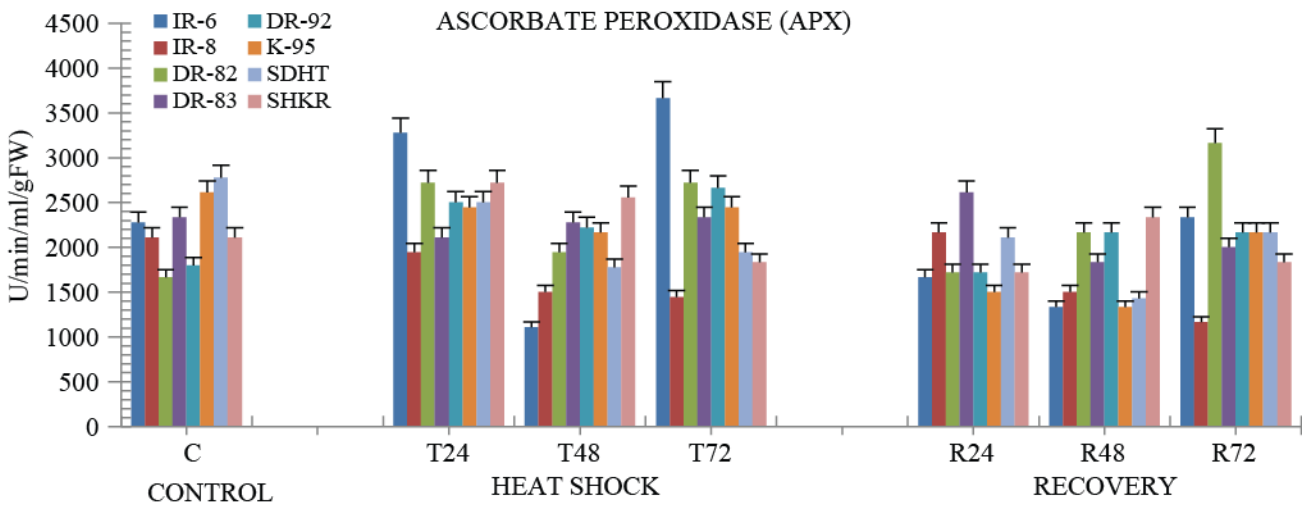

Fig. 3. Spectrophotometric quantification of Ascorbate Peroxidase (APX) enzyme activity of eight rice cultivars, IR-6, IR-8, DR-82, DR-83, DR-92, K-95, Sada Hayat and Shahkar, at grain filling stage, under normal (28±2 °C) (C), heat shock (42±2 °C) for 24h (T24), 48h (T48), 72h (T72) and recovery (28±2 °C) after 24h (R24), 48h (R48) and 72h (R72).

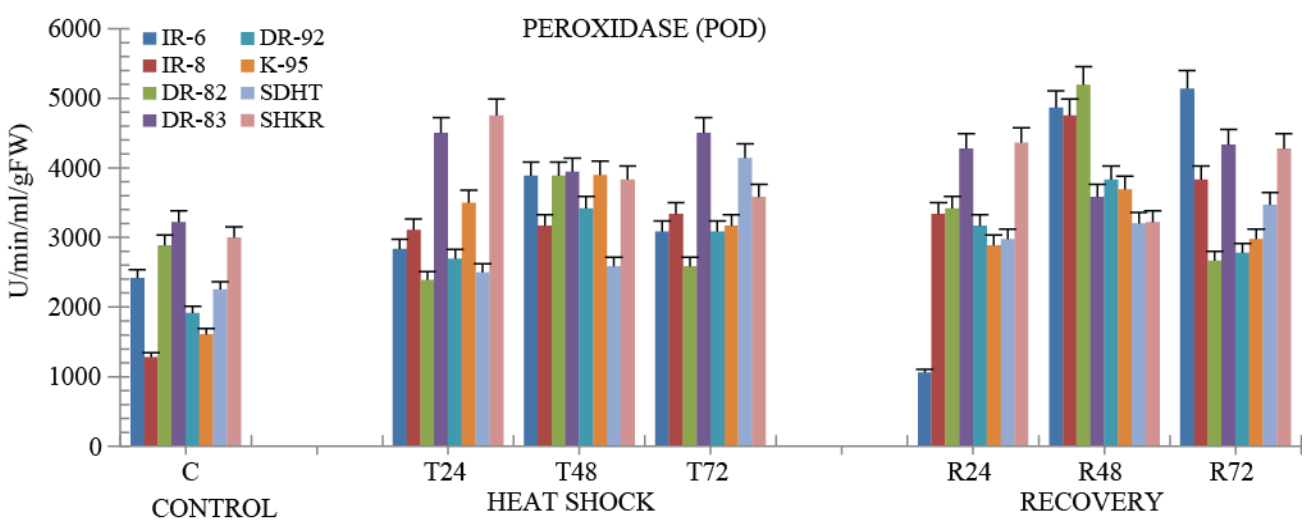

Fig. 4. Spectrophotometric quantification of Peroxidase (POD) enzyme activity of eight rice cultivars, IR-6, IR-8, DR-82, DR-83, DR-92, K-95, Sada Hayat and Shahkar, at maturity, under normal (28±2 °C) (C), heat shock (42±2 °C) for 24h (T24), 48h (T48), 72h (T72) and recovery (28±2 °C) after 24h (R24), 48h (R48) and 72h (R72).

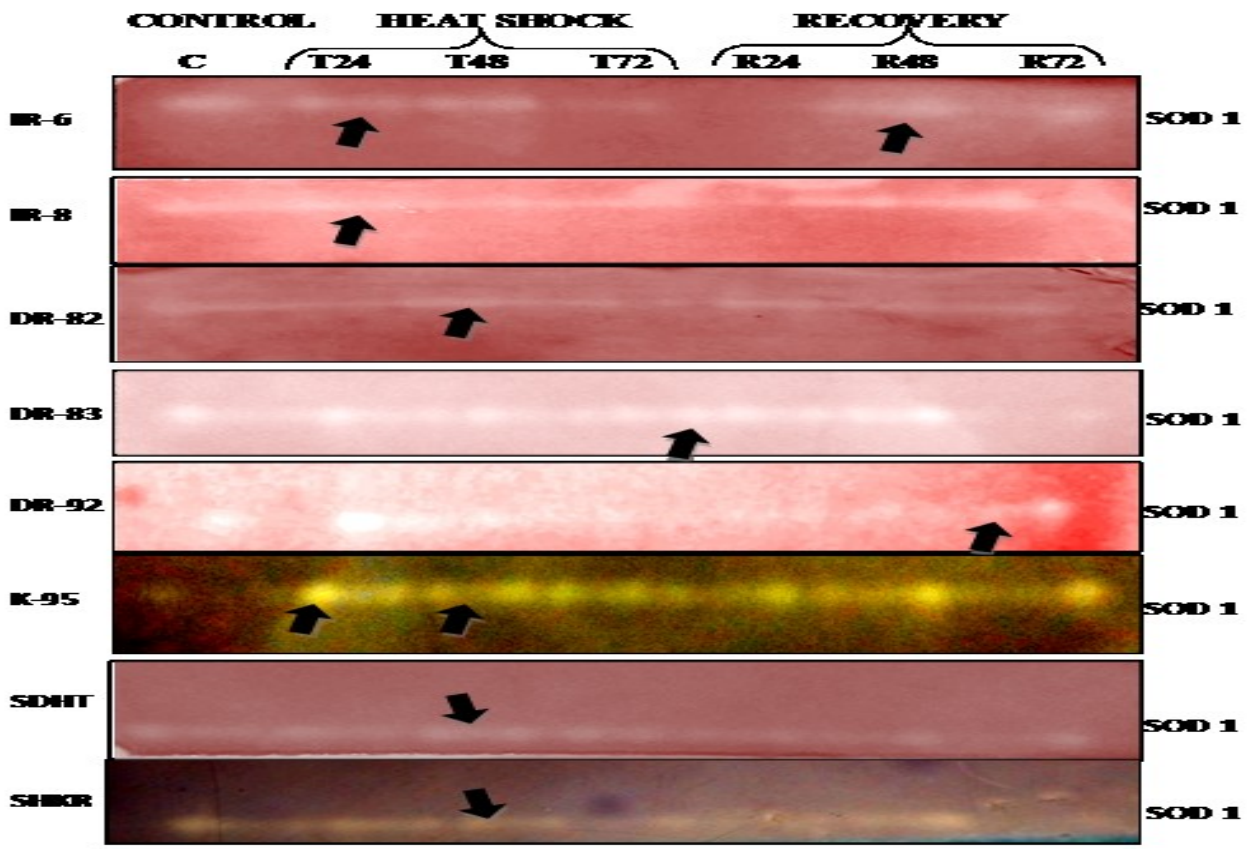

Fig. 5. Native-PAGE analysis of SOD of eight rice cultivars subjected to control (C), Heat Shock (42±1 °C) for 24 (T24), 48 (T48) and 72 (T72) h and recovery after 24 (R24), 48 (R48) and 72 (R72) h of heat stress at maturity stage.

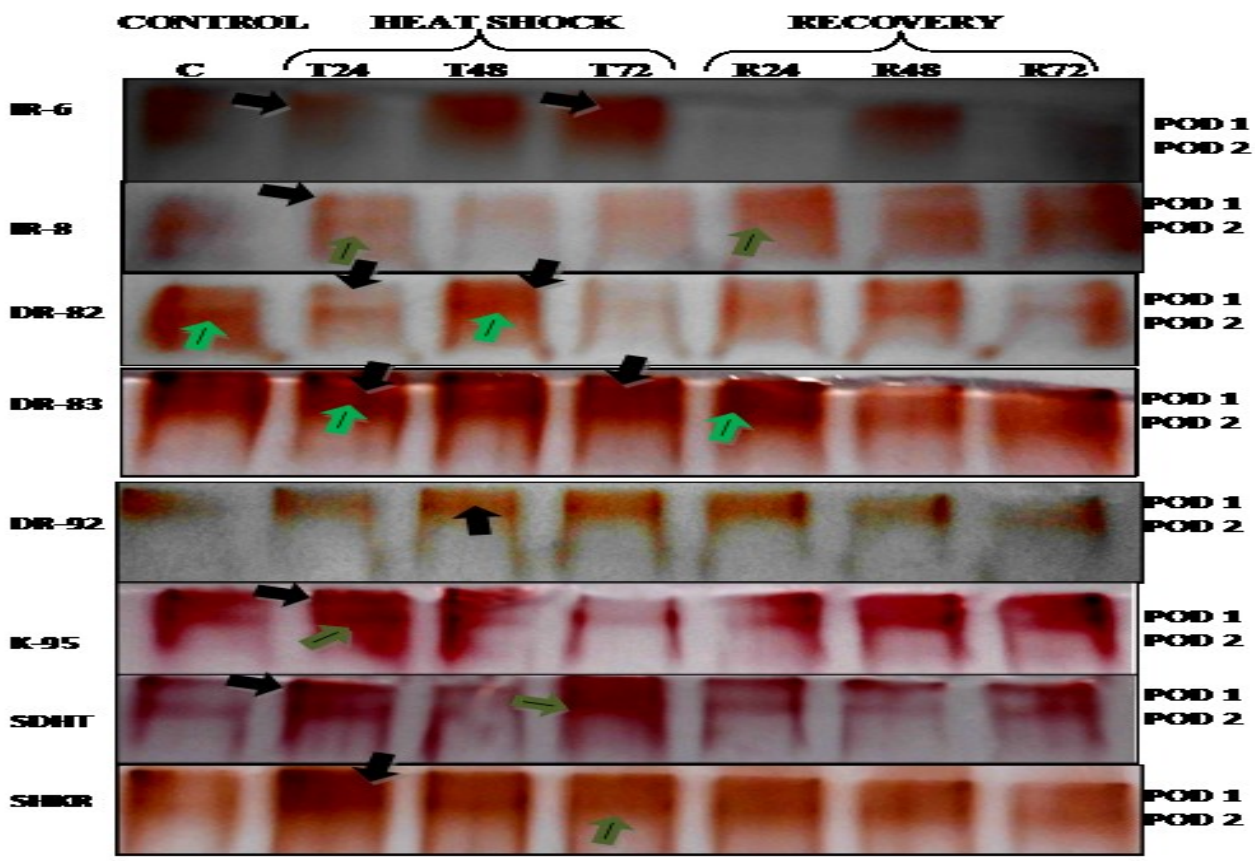

Fig. 6. Native-PAGE analysis of POD of eight rice cultivars subjected to control (C), Heat Shock (42±1 °C) for 24 (T24), 48 (T48) and 72 (T72) h and recovery after 24 (R24), 48 (R48) and 72 (R72) h of heat stress at maturity stage.

Isozyme analysis of SOD exhibited differential expression pattern (Fig. 5). Among rice cultivar, IR-6 showed single band of SOD (SOD1) however the band intensity of SOD1 exhibited a declined expression under heat stress while recovered after recovery treatment. Expression of SOD was very low as compared to seedling and flowering growth stage in IR-8. DR-83 and exhibited very low band intensity of SOD although up regulated after T48 but declined at T72, during heat stress. DR-83 also showed minimum SOD expression however, unlike DR-82 the band intensity of DR-83 had no significant variation under heat stress and recovery condition. At maturity stage, DR-92 had single type of SOD (SOD1) at control, slightly increased during stress while decreased upon recovery. At this stage, SOD expression analysis in K-95 revealed that heat stress increased its expression as the band thickness of T24, T48 and T72 were quite greater than control. During recovery SOD expression showed similar response as stress condition. Cultivar Sada Hayat and Shahkar showed no significant change in SOD1 expression observed during and after heat stress application.

Result of POD isozyme analysis through Native-PAGE, revealed differential expression pattern as compared to SOD. Maximum 2 bands of POD were observed in most of the cultivars at grain filling stage. The isoform, POD1 (black) showed increased sometimes while POD2 (green) declined upon application of heat stress (Fig. 6). Cultivar IR-6 revealed no clear bands of POD but the intensity was significantly different among the treatments. Heat stress after 24h showed declined in POD expression but at T48 and T72, it showed increased in expression while during recovery only R48 had POD bands. Similar pattern of POD bands also found in IR-8 however cultivar DR-82 showed slightly higher POD activity at control and T48 as compared to other treatments. In DR-92 although the bands were not clear but overall expression showed induced expression of POD upon exposure to thermal stress. K-95 explored two types of POD (POD1 and POD2). Control treatment had maximum POD expression while heat stress declined its activity but increased upon recovery. POD2 showed increased expression after 24h of heat stress but later its expression decreased while recovery treatments increased POD expression. Shahkar showed relatively better POD intensity but isoforms of POD were not clear.

Cultivar IR-6 at maturity stage revealed newly induced protein, approximately, 70 kDa was observed under heat stress and remained expressed during recovery condition (Fig. 7). Protein profiling showed, T24 had maximum expression of HSP70 (Black), during stress condition and R48 during recovery. Western blotting also showed highest protein expression at T24, declined to some extant at T48 but T72 showed increased expression of HSP70. R24 had no band but R48 and R72 had this newly induced stressed protein during recovery. While protein profiling of IR-8 indicated heat induced protein (70 kDa) at T48 and T72 and during recovery only R48 had maximum expression (Fig. 8). Another heat induced protein approximately 90kDa was observed only at T72. The expression of HSP70 observed in T24, T48 and R48 only. Third type of heat induced, 40kDa (green), protein band observed at T24 and maximum expression showed at T48 during heat stress while upon recovery R72 had maximum expression. Western blotting analysis revealed expression of HSP70 only at T48 while T24 showed very little expression and there were no band HSP70 at T72 during heat stress application. Proliferation of protein extracted from DR-82 showed very low amount of protein (Fig. 9). Heat induced protein having approximately 70 kDa observed only at T48 and R72 while rest of the treatments had no band of HSP70. It is confirmed by immunoblotting analysis (B) which showed very low expression of HSP70 at T48 and R72 (blue arrow). Cultivar DR-83 protein profiling showed induction of 90 kDa protein after 48h of heat stress (T48) while it was absent in rest of the treatments. Comparatively 70 kDa protein bands (black) were quite prominent and clearly visible at T48, R24 and R72 and similarly 40 kDa protein band were also observed at these three treatments. Identification of HSP70 through antigen-antibody reaction manifested expression and accumulation of HSP70 at T48, T72 and R48 (Fig. 10). While in DR-92, 70 kDa proteins was up regulated during stress condition as the seedling and flowering stage. Blotting results also showed that at T24 and T72, HSP70 were over expressed under high temperature stress (Fig. 11). Induction of 90 kDa protein (HSP90) was not clear but protein band of nearly 70kDa (black) found over expression after 72h of heat stress (T72) in K-95 (Fig. 12a). It also showed up regulation of 40kDa (green) protein band. Immunoblotting detection of HSP70 manifested that induction and accumulation of heat induced HSP70 upon heat exposure was most prominent after 72h (T72) only (Fig. 12b). Sada Hayat exhibited induction of HSP90 only at T72 at which 70 kDa was absent while 70 kDa protein induced in T24, T48 and R24 in which 90 kDa protein was absent. Interestingly low molecular weight 40 kDa (green) protein was also induced only at T24 and T48 while it was absent in T72. Western blot showed that HSP70 expressed and accumulated in T24 and T48 while no band was observed in T72 as there were no bands observed in protein profiling (Fig. 13). In Shahkar, 90 kDa protein was absent under heat stress and recovery also but 70 kDa protein bands were observed during heat stress conditions most prominently at T24 (Fig. 14). 40kDa protein was also induced under heat stress. Western blot analysis of "Shahkar" manifested up regulation of 70kDa protein upon heat stress after 24h (T24) while only R48 showed very low concentration of HSP70.

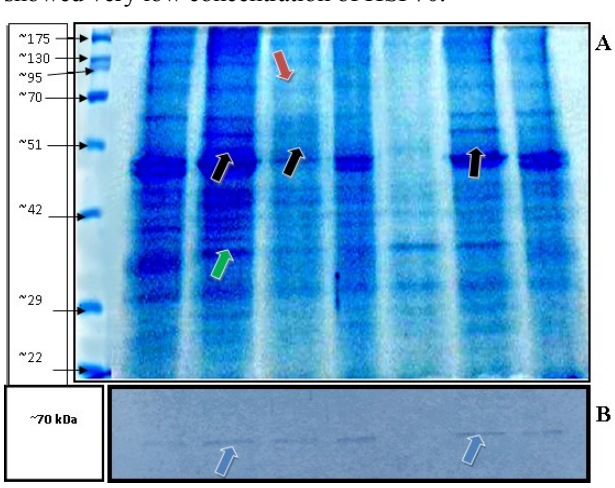

Fig. 7. SDS-PAGE protein profiling (A) and immunoblotting (B) of "IR-6" at maturity, under control (28±2 °C) (C), heat shock (42±2 °C) for 24h (T24), 48h (T48), 72h (T72) and recovery (28±2 °C) treatments for 24h (R24), 48h (R48) and 72h (R72).

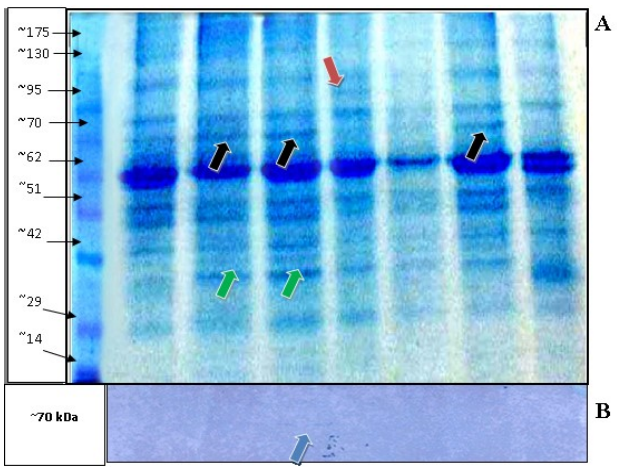

Fig. 8. SDS-PAGE protein profiling (A) and immunoblotting (B) of "IR-8" at maturity, under control (28±2 °C) (C), heat shock (42±2 °C) for 24h (T24), 48h (T48), 72h (T72) and recovery (28±2 °C) treatments for 24h (R24), 48h (R48) and 72h (R72).

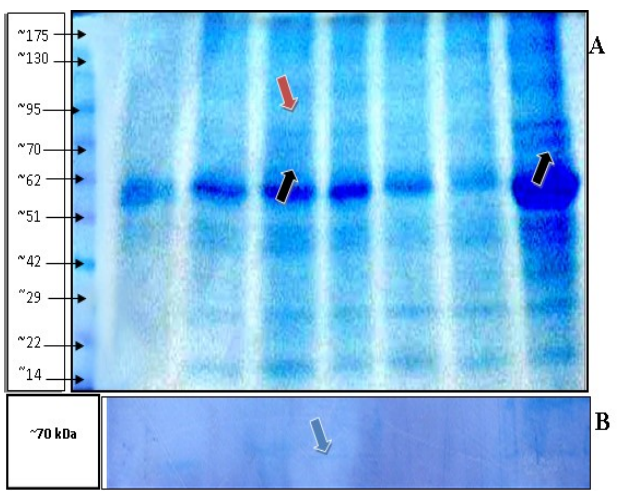

Fig. 9. SDS-PAGE protein profiling (A) and immunoblotting (B) of "DR-82" at maturity stage, under control (28±2 °C) (C), heat shock (42±2 °C) for 24h (T24), 48h (T48), 72h (T72) and recovery (28±2 °C) treatments for 24h (R24), 48h (R48) and 72h (R72).

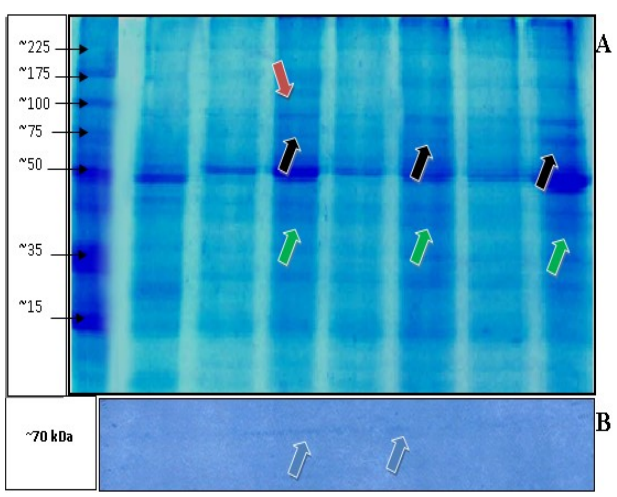

Fig. 10. SDS-PAGE protein profiling (A) and immunoblotting (B) of "DR-83" at maturity stage, under control (28±2 °C) (C), heat shock (42±2 °C) for 24h (T24), 48h (T48), 72h (T72) and recovery (28±2 °C) treatments for 24h (R24), 48h (R48) and 72h (R72).

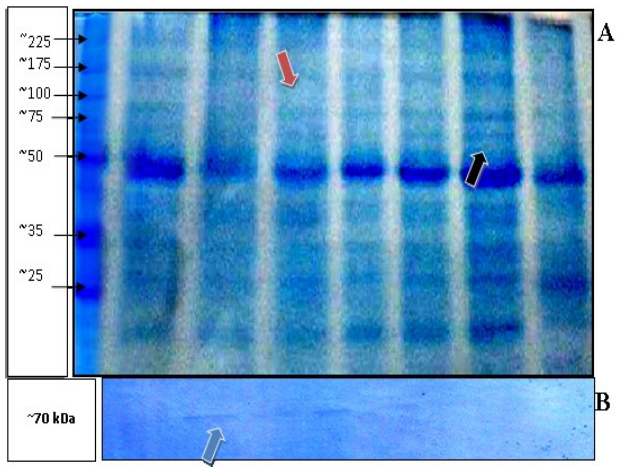

Fig. 11. SDS-PAGE protein profiling (A) and immunoblotting (B) of "DR-92" at maturity stage, under control (28±2 °C) (C), heat shock (42±2 °C) for 24h (T24), 48h (T48), 72h (T72) and recovery (28±2 °C) treatments for 24h (R24), 48h (R48) and 72h (R72).

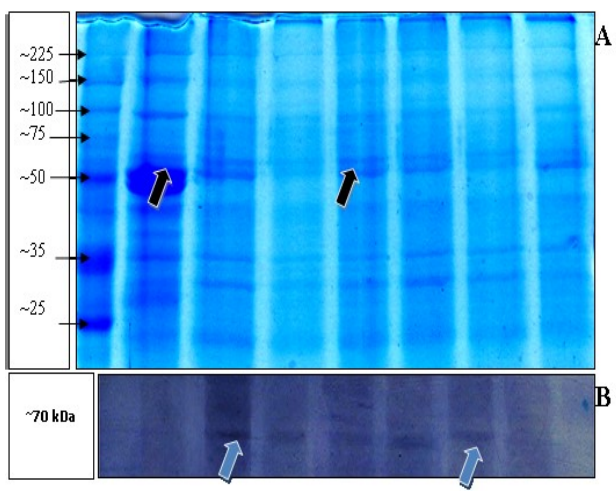

Fig. 12. SDS-PAGE protein profiling (A) and immunoblotting (B) of "K-95" at maturity stage, under control (28±2 °C) (C), heat shock (42±2 °C) for 24h (T24), 48h (T48), 72h (T72) and recovery (28±2 °C) treatments for 24h (R24), 48h (R48) and 72h (R72).

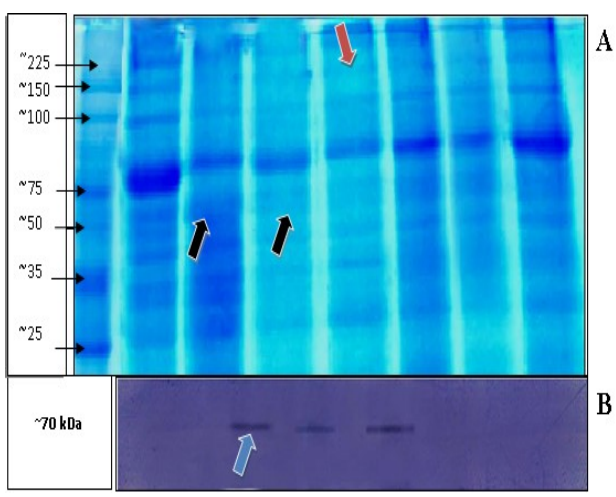

Fig. 13. SDS-PAGE protein profiling (A) and immunoblotting (B) of "Sada Hayat" at maturity stage, under control (28±2 °C) (C), heat shock (42±2 °C) for 24h (T24), 48h (T48), 72h (T72) and recovery (28±2 °C) treatments for 24h (R24), 48h (R48) and 72h (R72).

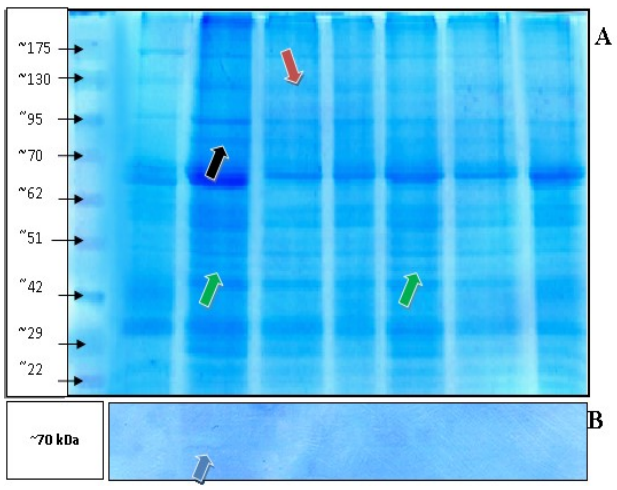

Fig. 14. SDS-PAGE protein profiling (A) and immunoblotting (B) of "SHAHKAR" at grain filling stage, under control (28±2 °C) (C), heat shock (42±2 °C) for 24h (T24), 48h (T48), 72h (T72) and recovery *(28±2 °C) treatments for 24h (R24), 48h (R48) and 72h (R72)*

Discussion

Response of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD) were determined, in order to elucidate and correlate their expression and activity with heat stress tolerance in rice cultivars. The results of both quantitative (Spectrophotometric) and qualitative (Native-PAGE) analysis are discussed.

Quantitative analysis of antioxidants enzymes was performed according the standard protocol and the outcome of the study are discussed. Statistical analysis revealed significant (p<0.05) difference in enzymes activity among rice cultivars at different temperature treatments. The general expression pattern of these enzymes manifested that SOD and POD increased their expression while APX and CAT were declined. Activity of SOD, CAT, APX and POD enzyme were highest in cultivar "K-95" as compared to other cultivars at all three growth stages. Response of individual enzyme towards thermal stress is discussed below.

Quantitative analysis of superoxide dismutase (SOD) exhibited that heat stress induced its expression and declined upon recovery treatment. The over expression and induction of SOD under heat stress condition, might have helped them to cope with heat induced damages because maximum SOD activity is strongly associated with plant heat tolerance (Mohammad & Tarpley, 2009). At grain filling stage cultivars showed upregulation of SOD expression after 24h of heat stress application (T24) but with increased in time duration SOD activity declined. Similarly, recovery treatment after 24h (R24) and 48h (R48) showed slight increment in SOD activity as compared to T72 but after 72h (R72) its activity again declined.

Expression pattern of CAT showed variable response under high temperature in different crops i.e. wheat showed increased CAT activity (Almeselmani *et al*., 2006) but we found that rice had strongly cultivar and stage specific. CAT enzymes showed induction under thermal stress but quantity of enzymes was slightly lower as compared to seedling stage (data not shown). Among cultivars "K-95" had maximum expression of CAT. In case of CAT activity our result was not similar as Nahakpam & Shah (2011), reported in rice treated with heat stress and cadmium stress, possibly due to combination of stresses in their experiment.

APX enzyme indicated stage specific activity as CAT enzymes, may be due to the similar function. Studies showed that ascorbate peroxidase (APX) was more sensitive under high temperature stress in plants (Panchuk *et al*., 2002) therefore for better performance of APX enzyme under heat stresses it must have high temperature inducible APX transcriptional activation genes (Foyer *et al*., 1997). It can be concluded that different response of APX activity at different growth stages and among cultivars was possibly due to the number of genes and their regulation under stress condition.

Increased activity of POD and its role in thermotolerance under high temperature stress have been reported (Ilba, 2002). Although rice has many genes for POD but isoforms showed, most of POD isozymes are same molecular weight and charges therefore difficult to differentiate (Shah & Nahakpam, 2012). Rice shoots had seven groups of POD isofroms, observed constitutively expressed under control condition which showed increased band intensity upon exposure to heat stress (Shah *et al*., 2004) but we observed maximum 4 POD isoforms in leaves of rice cultivars. All cultivars at each growth stages and treatments, showed increased POD activity under heat stress while declined during recovery condition. At grain filling stage heat stress induced expression and activity of POD enzyme, but with increased in exposure time to heat stress, POD declined in some cultivars. The expression pattern was further confirmed by electrophoresis (zymography). Native-PAGE analysis manifested that POD isoforms responded under heat stress differently at different growth stages. Some had thick bands under control while some showed enhanced expression during recovery.

In conclusion, a high temperature stress tolerant cultivar should have increased activity of antioxidant enzymes including APX, CAT, POD and SOD under heat stress condition because maximum expression of these enzymes can detoxify and control over production and accumulation of ROS. Which ultimately protect lipid peroxidation and excess electrolyte leakage, assuring life consistency under stress condition. We observed that all those rice cultivars which showed improved and consistent antioxidant enzyme activity under high temperature stress also had better growth and maximum yield. Qualitative analysis of SOD and POD enzyme explored the differences among cultivars to cope with heat stress.

In this study, protein expression analysis revealed induction of a polypeptide, approximately 70 kDa, in heat stressed rice leaf samples which was identified as HSP70 through antigen-antibody reaction. Stress conditions activates genes to produce enzymes that mitigate stress and promote growth in plants (Khan *et al*., 2011). High temperature generally increases the expression of HSPs and such a response allows organisms to become tolerant to lethal heat waves (Chang *et al*., 2007). Differential expression of HSP70 observed in different rice cultivars. It

may be due to activation of heat shock factor (HSFs) and number of genes responsible for HSP70 synthesis because it enables cells and tissues in stressed conditions to maintain proper folding of proteins hence maintained cellular homeostasis (Sarkar *et al*., 2013). Immediate induction and maximum accumulation of heat-inducible proteins, specifically 70 kD protein (Hsp70), was intimately linked to enhanced cell survival under heat stress. They help in recovery of cellular damages mediated by the ability to catalyse the reassembly of damaged ribosomal proteins (Daugaard *et al*., 2007). It means in these cultivars degraded proteins was accumulated and synthesis of new proteins was retarded due to low expression of HSP70 because HSP70 also involved in protein import and translocation processes (Frydman, 2001; Frank *et al*., 2009; Bita *et al*., 2011). It means that for protein stability and function, favorable tissue internal environment and better cell membrane thermo-stability is required as in grape genotypes, higher level of HSP70 and genes related to stress protection and metabolism were found in heat tolerant genotypes under elevated temperatures but even under normal temperature a tolerant genotype exhibited maximum heat shock gene expression (Wang *et al*., 2004; Zhang *et al*., 2005).

Efficient scavenging of reactive oxygen species like H_2O_2 through antioxidant defense system can prevent membrane lipids degradation, ensures maintenance of cell membrane integrity during stress conditions. HSP70 assist in sustained and improved activity of antioxidant enzymes. Cultivar "K-95" was more thermo-tolerant with efficient expression of HSP70 which may help to overcome oxidative stress damages and ensured plant survival during stress conditions. At this level it can be concluded that this potential biomarker (HSP70) should be considered in future rice breeding programs for development of thermo-tolerant rice varieties because in barley also it is observed that thermo-tolerant cultivars had higher level of HSP70 while heat sensitive showed very low expression (Kausar *et al*., 2013).

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