

Full Length Research Paper

Mechanism of action of hydrogen peroxide in wheat thermotolerance - interaction between antioxidant isoenzymes, proline and cell membrane

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Terminal heat stress causes an array of physiological, biochemical and morphological changes in plants, which affect plant growth and development. It has very severe effect on the pollen viability and seed setting in wheat. In the present investigation, an altered expression of H₂O₂ (0.9 µg/g in C-306 and 0.75 µg/g in HD2329) was observed with the highest accumulation at seed hardening stage and against heat shock (HS) of 42°C for 2 h. With the increase in H₂O₂ accumulation, an increase in the number of isoenzymes of superoxide dismutase and catalase were observed with high activities under differential heat shock. A decrease in the proline accumulation was observed under differential heat shock. Exogenous application of H₂O₂ (10 mmole/L) leads to increase in the accumulation of intracellular H₂O₂ and further an increase in the number of isoenzymes of superoxide dismutase (SOD) and catalase (CAT) was observed. The tolerant cultivar was more responsive to exogenous application of H₂O₂ compared to susceptible cultivar. The percentage decrease in cell membrane stability under differential heat shock was low in H₂O₂ treated plants compared to non-treated. The results from this study suggest a potential role for H₂O₂ in regulating the activity of antioxidant enzymes and accumulation of proline inside cells and in turn influence the cell membrane stability under heat stress. All the defense associated genes were observed to be very responsive to intracellular H₂O₂, which gives inference that H₂O₂ has regulatory role to play in controlling the expression and activities of these proteins under abiotic stresses.

Key words: Antioxidant enzymes, wheat, heat stress, H₂O₂, proline, catalase, superoxide dismutase, cell membrane stability, reactive oxygen species.

INTRODUCTION

Plant growth and yield are adversely affected by abiotic stresses such as high or low temperatures, drought, salinity etc. Among abiotic stresses, heat stress influences photosynthesis, cellular and subcellular

membrane components, protein content in cell and antioxidant enzyme activity; thereby significantly limiting crop production (Georgieva, 1999). Heat stress also induces oxidative stress in plants caused by the generation and accumulation of superoxides (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radicals (OH[•]), which are commonly known as reactive oxygen species (ROS) (Breusegem et al., 2001). Although, the daily average temperature for optimal growth conditions of wheat is 22 to 25°C. High temperatures reduce the vegetative growth and seed setting in wheat. Changes in ambient temperature occur within hours, unlike drought

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Abbreviations: SOD, Superoxide dismutase; CAT, catalase; CMS, cell membrane stability; HS, heat stress; ROS, reactive oxygen species.

and salinity stresses. Therefore, plants need to suppress and respond to the adverse effects of heat in a very short time. Gradual temperature increase in a day could cause some alterations in antioxidant metabolism or in other physiological responses. Improving tolerance to heat stress is a major challenge in many C_3 crops given the threat of global warming. Most of the earlier studies on the effects of multitude of abiotic stresses showed changes in the level of several physiological parameters including lipid peroxidation, H_2O_2 production and proline accumulation in wheat (Georgieva, 1999). The mechanism of response of wheat to elevated temperatures would help the development of wheat cultivars that perform better under heat stress. However, there is a limited amount of information available about the mechanism of tolerance in plant against heat stress. The H_2O_2 production is thought to be increased under various abiotic stresses in order to enhance gene expression of active oxygen scavenging (AOS) enzymes (Tanaka et al., 1999). H_2O_2 was also reported to induce small heat shock proteins (HSP26) in tomato and rice (Schoffl et al., 1998). It also plays an important role in a signal transduction for abiotic stress tolerance. Although, H_2O_2 is toxic at high concentrations it was implicated as an elicitor of several genes related to stress tolerance. The dismutation of superoxide radicals into H_2O_2 and oxygen is an important step in protecting the cell and in that conversion superoxide dismutase (SOD) and catalase (CAT) are considered to be key enzymes. Catalase is a common enzyme found in nearly all living organisms that are exposed to oxygen, where it catalyzes the decomposition of hydrogen peroxide to water and oxygen. The levels of ascorbate peroxidase (APX) and catalase (CAT) were observed to be increased in the transgenic rice probably due to generation of H_2O_2 by over expressed superoxide dismutase (Tanaka et al., 1999).

Proline protects membranes and proteins against the adverse effects of high concentrations of inorganic ions and temperature extremes (Santoro et al., 1992). Proline may also function as a protein-compatible hydrotrope and as a hydroxyl radical scavenger. It has a role in the protection of plants by acting as a cellular osmotic regulator between cytoplasm and vacuole (Bohnert and Jensen, 1996). However, it has also been suggested that over-accumulation of proline could be toxic to plant cells (Rizhsky et al., 2004).

Cell membrane stability, as measure of electrolyte diffusion resulting from the heat-induced cell membrane leakage, has been used to screen and evaluate different wheat genotypes for thermotolerance (Saadalla et al., 1990). Electrical conductivity has also been used as index of membrane stability to identify the heat-tolerant genotypes in wheat (Blum and Ebercon, 1981) and for screening of heat-tolerant genotypes in other crops as well. Greater understanding of the mechanism of wheat response to elevated temperature would help the

development of wheat cultivars and technologies for improved performance of this important crop under heat stress condition arising due to global warming and climate change. Of late, investigations have been initiated on the effect of heat stress on wheat but most of it have centered around the adverse impact, and comparatively less on adaptation and mitigation of climate change.

The purpose of this experiment was to study the effect of intracellular as well as exogenous application of H_2O_2 on changes in thermotolerance behavior of tolerant and susceptible cultivars of wheat grown under differential heat stress.

MATERIALS AND METHODS

Wheat (cv. C306 and HD2329) were grown in growth chamber inside National Phytotron facility under controlled conditions with optimum temperature regime of 26/22°C, humidity of 60%, photoperiod of 16 h and light intensity of 350 $\mu\text{mol}/\text{m}^2/\text{s}$. Seeds of both the varieties were sown in 80 pots (40 each) in a group of five using autoclaved vermiculite soil mixed with sand.

Stress treatments

Plants were randomly divided into five groups (3 groups for HS treatment, 1 group for stage specific analysis and 1 group for exogenous H_2O_2 treatment) at seedling stage (10 days old) and heat stress treatments were applied at 30°C (mild heat stress), 35°C (moderate heat stress) and 40°C (extreme heat stress) at different stages of growth. For mild heat stress, the plants in the growth chamber were exposed to a gradual temperature increase from 25 to 30°C with increments of 1°C/10 min. After the temperature reached 30°C, plants were kept at 30°C for 2 h. Same method of stress treatment was followed for moderate and extreme heat stresses plant and the treatment was given for 2 h. After each stress treatment, leaf samples were harvested and immediately frozen in liquid nitrogen for subsequent analyses. One group was grown under 22°C and the samples collected at different stages of growth were used as control.

Changes in cell membrane stability index (CMS)

Samples with similar leaf size were selected at vegetative (8 days old seedlings), pollination, milky dough and seed hardening stages and were used to measure cell membrane stability (CMS) using the method of Fokar et al. (1998) with some modifications.

Alteration in H_2O_2 concentration

The amount of H_2O_2 was quantified as described by Loreto and Velikova (2001). Briefly, leaf samples (0.3 g) were homogenized in 3 ml of 1% (w/v) trichloroacetic acid (TCA). The homogenates were centrifuged at 10,000 \times g (4°C) for 10 min. Subsequently, 0.75 ml of the supernatants was added to 0.75 ml of 10 mM Potassium phosphate buffer (pH 7.0) and 1.5 ml of 1 M potassium iodide (KI). After that H_2O_2 content of the supernatant was evaluated by comparison of the absorbance values at 390 nm to a standard calibration curve standard in the range from 10 to 200 $\text{nmol}/3$ ml cuvette. H_2O_2 concentration was expressed as $\mu\text{mol}/\text{g}$ fresh

weight (FW).

Estimation of proline accumulation

Proline contents were determined according to the modified method of Bates et al. (1973). The reaction mixtures were extracted with 5 ml of toluene and the absorbance values were measured at 520 nm with spectrophotometer. The concentration of proline was calculated using a standard curve.

Superoxide dismutase and catalase activity assay

Leaf materials (1 mg) were ground in 6 ml of ice cold 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium-EDTA and 1% (w/v) polyvinyl-polypyrrolidone (PVP). The homogenates were centrifuged at 10,000 × g (4°C) for 10 min. The tissue extracts were either stored at -78°C or immediately used for subsequent analyses of superoxide dismutase and catalase. For the quantification of soluble protein content, coomassie blue dye-binding assay was used (Bradford, 1976). Bovine serum albumin (BSA) was used for the preparation of the standard curve.

Superoxide dismutase activity was determined by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) in the presence of riboflavin in light (Giannopolitis and Ries, 1977). One unit of enzyme activity was determined as the amount of the enzyme needed for the inhibition of 50% NBT reduction rate by monitoring absorbance at 560 nm with spectrophotometer. Activities of catalase enzyme were measured as described by Chance and Maehly (1955). For assaying catalase activity, the decomposition of H₂O₂ was followed by decline in the absorbance at 240 nm. Catalase activity was determined by following the consumption of H₂O₂ (extinction coefficient, 39.4 mM/cm) at 240 nm over a 3 min interval.

Electrophoretic mobility profiling of superoxide dismutase and catalase isoenzymes

The crude extracts of collected samples were prepared in phosphate buffer (pH 7.5) and 20 µg of proteins (estimated by Bradford method) were loaded on to each well of polyacrylamide gel (10%) for native PAGE. The protocol of Roychaudhari et al. (2003) was followed for isoenzymic study of superoxide dismutase. Illumination was discontinued when maximum contrast between achromatic zones and the general blue color was achieved. The gel was maintained in distilled water till photographed. Isoenzymic pattern of catalase was detected by the procedure as described by Rucinska et al. (1999). 20µg of proteins isolated from collected samples were loaded on to native PAGE gel (10%). White color bands was appeared against blue/greenish background.

Effect of exogenous H₂O₂ on various biochemical parameters

One group of plant was sprayed with H₂O₂ (10 mmole/L; Que et al. 2009a) before differential heat shock treatment at vegetative stage and the samples collected were analyzed for SOD and CAT activities, proline accumulation and CMS index as per the protocols mentioned previously.

Statistical analysis

The experiment was conducted in a completely randomized design. The uppermost fully expanded leaves were collected randomly as three replicates for each treatment. Data were analyzed using one-

way analysis of variance (one-way ANOVA). The standard errors were given in histograms.

RESULTS

Hydrogen peroxide accumulation

A gradual increase in the accumulation of H₂O₂ (0.5, 0.58, 0.78 and 1.1 µmole/g FW) was observed in C-306 against heat shock of 22, 30, 35 and 40°C for 2 h (Figure 1a). The highest accumulation of H₂O₂ was observed when heat shock of 40°C was given for 2 h (Figure 1b). The H₂O₂ concentration was 0.45, 0.72, 0.8 and 0.9 µmole/g FW at vegetative, pollination, milkydough and seed hardening stages. The highest H₂O₂ concentration was observed at seed hardening stage which is also considered as one of the most critical stage for wheat grain development.

An H₂O₂ content of 0.28, 0.52, 0.58 and 0.75 µmole/g FW was observed in HD2329 against heat shock of 22, 30, 35 and 40°C for 2 h (Figure 1c). A continuous increase in the accumulation of H₂O₂ was observed against differential HS. H₂O₂ content was 0.48, 0.65, 0.71 and 0.75 µmole/g FW at vegetative, pollination, milkydough and seed hardening stages (Figure 1d). A significant increase in the accumulation of H₂O₂ was observed at different stages of growth as well as under differential HS.

Electrophoretic mobility of superoxide dismutase

Isoenzymic profiling of C-306 under differential heat shock revealed four different isoenzymes of SOD on the gel against heat shock of 35 and 40°C for 2h (Figure 2a). The number of isoenzymes was observed more after heat shock of 35°C for 2 h and this may be the reason for the high activity of SOD in lane no 3, 4 and 5. No changes in the isoenzyme pattern of SOD were observed in C-306 at different stages of growth (Figure 2b). The number of isoenzyme were observed more in response to differential HS compared to the plant grown under normal condition (22°C).

Two different isoenzymes of SOD were observed in HD2329 against differential HS (30, 35 and 40°C for 2 h). The maximum number of isoenzymes was observed in response to HS of 40°C for 2 h (Figure 2c). Isoenzyme profile remains the same in both the cultivars at different stages of growth (Figure 2d). The activity was observed very high at milkydough and seed hardening stages (Lane no 4 and 5).

Superoxide dismutase activity assay

The specific activity of SOD was 21, 36 and 31 U/mg protein in response to heat shock of 30, 35 and 40°C for

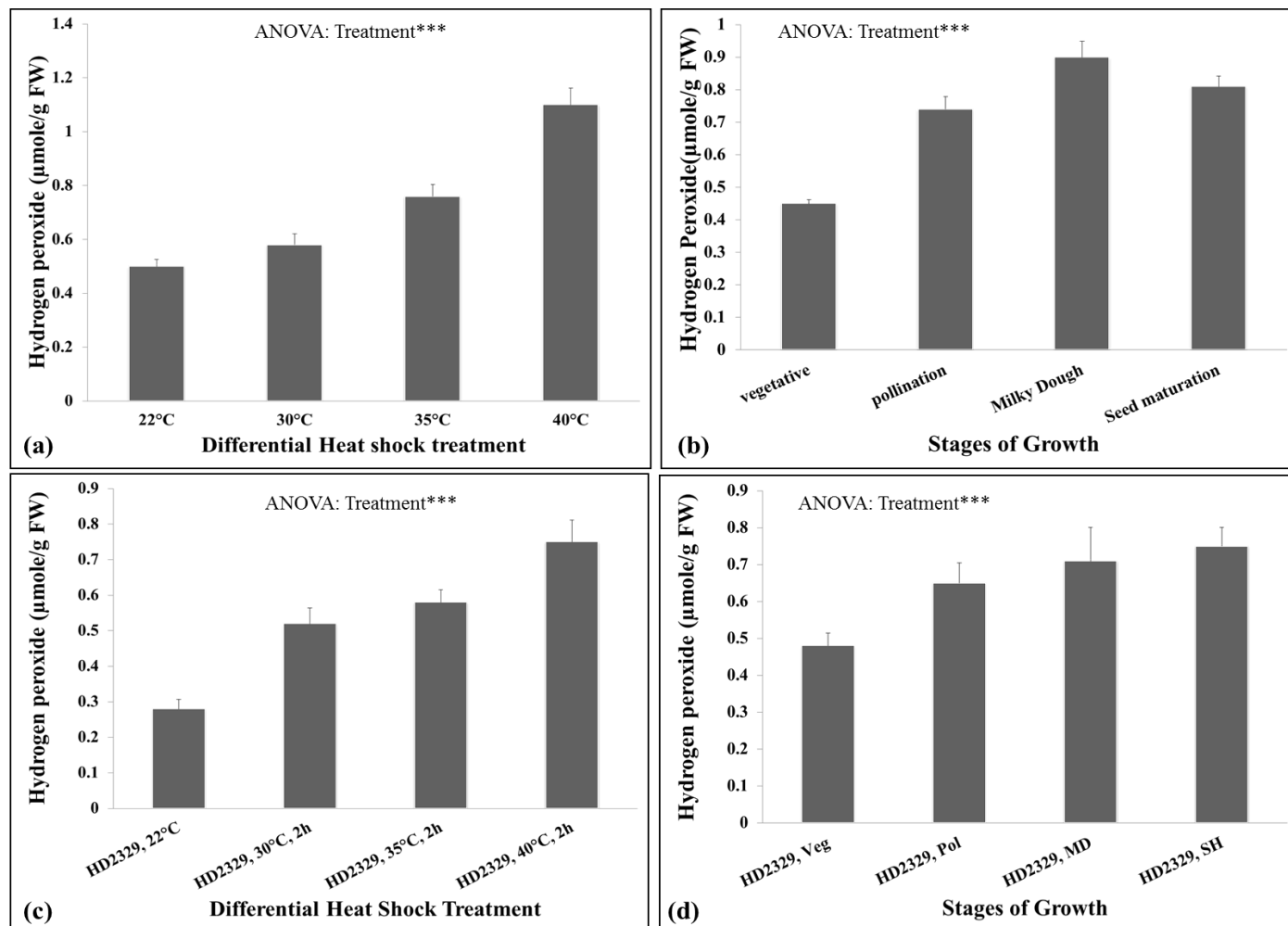


Figure 1. Hydrogen peroxide accumulation in C-306 (thermotolerant) and HD2329 (thermosusceptible) cultivars of wheat against elevated temperature, (a) Hydrogen peroxide accumulation in C-306 against differential heat shock, (b) Hydrogen peroxide accumulation in C-306 at different stages of growth, (c) Hydrogen peroxide accumulation in HD2329 against differential heat shock, (d) Hydrogen peroxide accumulation in HD2329 at different stages of growth, ANOVA significance levels: *, $P < 0.05$; **, $P < 0.001$; ***, vertical bars indicate s.e. ($n=3$).

2 h in C-306 (Figure 3a). The activity of SOD was very low in C-306 at different stages of growth (Figure 3b). The maximum activity was observed during milkydough stage (0.1 U/mg/h). In case of HD2329, the specific activity of SOD was 3.1, 3.4 and 5 U/mg in response to differential heat shock of 30, 35 and 40°C for 2h (Figure 3c). A significant increase in the specific activity was observed with elevation in temperature. Specific activity estimated at different stages of growth showed 4.0, 4.4 and 4.9 U/mg at pollination, milky dough and seed hardening stages (Figure 3d). The specific activity was observed very low in both the cultivars at different stages of growth compared to differential HS.

Electrophoretic mobility of catalase

Isoenzymic pattern profiling of catalase revealed the

expression of 5 (C-306) and 4 (HD2329) different isoenzymes of catalase in response to HS of 30, 35 and 40°C (Lane no 3, 4 and 5; Figure 4a and c). A very high activity of CAT isoenzymes was observed during pollination and milky dough stage in C-306 (Figure 4b). The maximum number of isoenzymes in case of HD2329 was observed at milky dough stages (Figure 4d).

Catalase activity assay

In the present investigation, a significant increase in catalase activity was observed in C-306 under differential heat shock (HS) treatment (Figure 5a). The activity of catalase was 21.35 (22°C), 23.57 (30°C), 31.53 (35°C), and 40.92 U/mg proteins (40°C). The highest activity of catalase was observed in response to heat shock treatment of 40°C for 2 h. Maximum CAT activity was

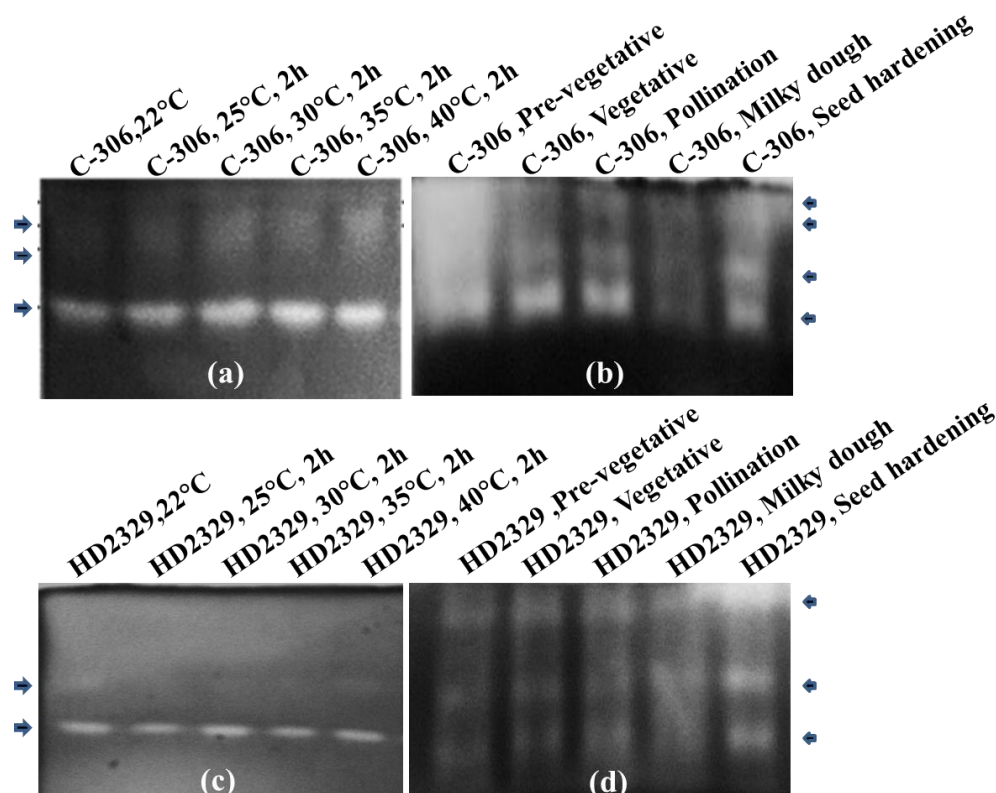


Figure 2. Electrophoretic mobility profiling of superoxide dismutase isoenzymes in thermotolerant (C-306) and HD2329 (thermosusceptible) cultivars of wheat, (a) In gel activity assay in C-306 against differential heat shock, (b) In gel activity assay in C-306 at different stages of growth, (d) In gel activity assay in HD2329 against differential heat shock, (d) In gel activity assay in HD2329 at different stages of growth, Arrows depicting the isoenzymes activity.

observed at milky dough stage in C-306 (Figure 5b). Catalase activity assay in HD2329 (thermosusceptible) showed 2.0, 3.34, 4.1 and 4.4 U/mg protein against differential HS of 22, 30, 35 and 40°C for 2 h (Figure 5c). The samples collected at different stages of growth showed specific activity of 1.8, 3.3, 3.5 and 3.75 U/mg at vegetative, pollination, milky dough and seed hardening stages. The maximum change in catalase activity was observed during pollination stage (Figure 5d).

Proline accumulation

A gradual decrease in the accumulation of proline (0.9, 0.79 and 0.66 $\mu\text{mole/g FW}$) was observed in response to HS of 30, 35 and 40°C for 2 h in C-306 (Figure 6a). A very high proline concentration (1.0 $\mu\text{mole/g FW}$) was observed in C-306 at vegetative stage (Figure 6b) which further decreases at different stages of growth. In response to differential HS of 25, 30, 35 and 40°C for 2 h, the proline accumulation in HD2329 was 0.75, 1.1, 1.25 and 1.29 $\mu\text{mole/g FW}$ (Figure 6c). Proline profiling in HD2329 showed 0.69, 0.91, 0.99 and 0.75 $\mu\text{mole/g FW}$ at vegetative, pollination, milky dough and seed hardening stages (Figure 6d).

Cell membrane stability index

The cell membrane stability (CMS) in C-306 was 70% (vegetative), 68 (pollination), 62 (milky dough) and 68 % (seed hardening; Figure 7a). A direct correlation between the H_2O_2 accumulation and CMS has been observed in both the cultivars. In case of HD2329, the cell membrane stability at vegetative, pollination, milky dough and seed hardening stages were 50, 54, 47 and 42% respectively (Figure 7b). The highest CMS was observed at vegetative (C-306) and pollination (HD2329) stages.

Effect of exogenous H_2O_2 on various biochemical parameters

Samples collected from both varieties sprayed with H_2O_2 (10 mmole/L) were analyzed for change in various biochemical parameters like intracellular H_2O_2 and proline accumulation, SOD and CAT activity and overall effect on cell membrane stability index (Que et al., 2009a). The intracellular H_2O_2 concentration was 0.95, 1.13, 1.23 and 1.30 (C-306) and 0.81, 1.01, 0.95 and 0.96 (HD2329) in response to differential HS of 22, 30, 35 and 40°C for 2 h (Figure 8a and b). A continuous increase in the

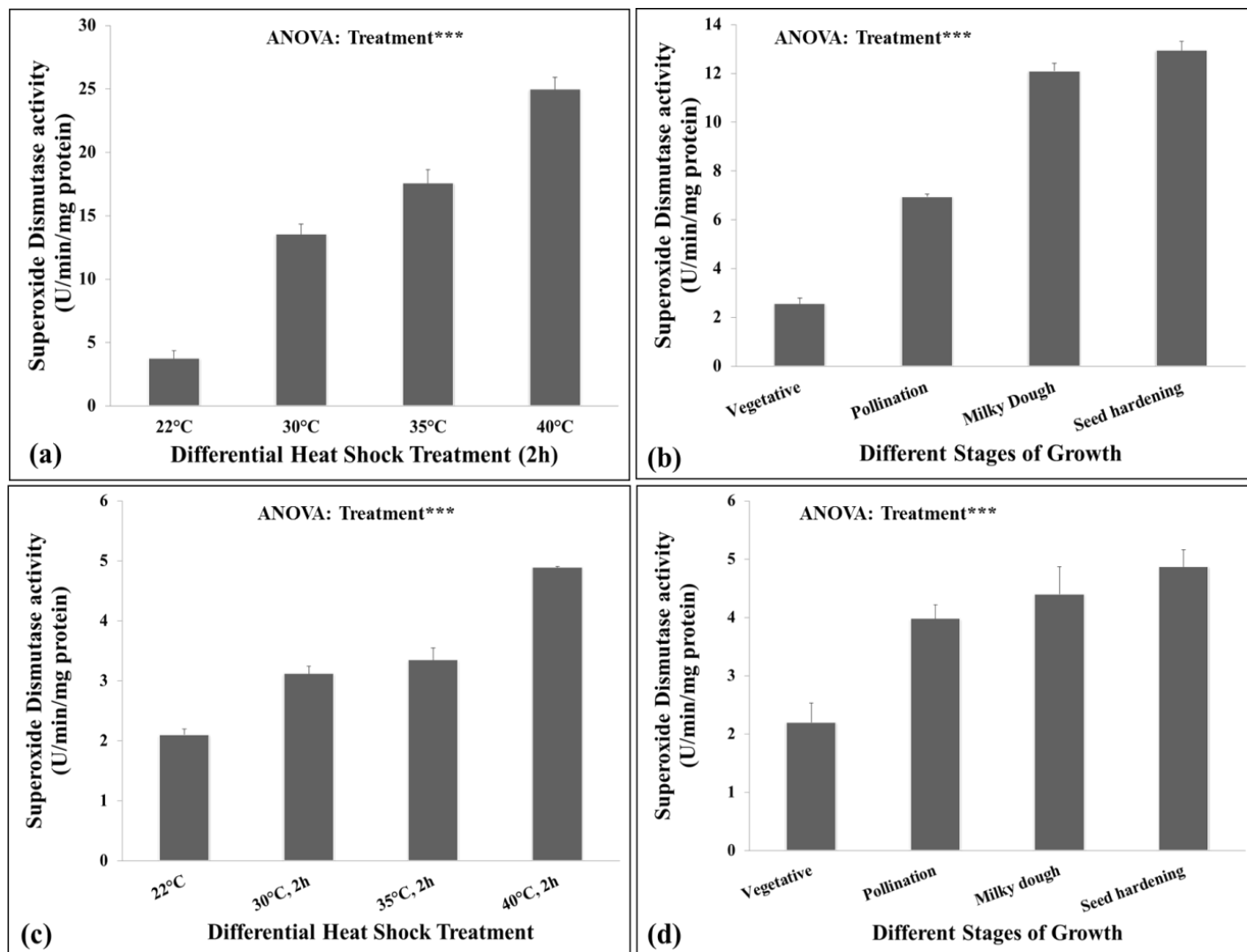


Figure 3. Specific activity of superoxide dismutase enzymes in thermotolerant (C-306) and susceptible (HD2329) cultivars of wheat, (a) SOD specific activity in C-306 against differential heat shock, (b) SOD specific activity in C-306 at different stages of growth, (c) SOD specific activity in HD2329 against differential heat shock, (d) SOD specific activity in HD2329 at different stages of growth, ANOVA significance levels: *, $P < 0.05$; **, $P < 0.001$; ***, vertical bars indicate s.e. ($n=3$).

accumulation of intracellular H_2O_2 was observed in thermotolerant wheat cultivar whereas, in case of susceptible, maximum accumulation was observed in response to HS of 30°C for 2 h.

H_2O_2 act as signaling molecules against abiotic stresses and influence the expression of stress associated genes like HSPs, antioxidant enzymes etc. SOD activity analysis showed 2.9, 4.8, 4.5 and 4.6 U/mg (C-306) and 2.46, 4.1, 4.06 and 4.2 U/mg (HD2329) under differential HS of 22, 30, 35 and 40°C for 2 h (Figure 8e and f). Same samples were used for the observation of catalase activity. Catalase activity showed 2.96, 4.9, 4.4 and 4.63 U/mg (C-306) and 2.73, 3.7, 3.66 and 3.73 U/mg (HD2329) in response to HS of 22, 30, 35 and 40°C for 2 h (Figure 8g and h). An increase in the

activity of both the antioxidant enzymes was observed against differential HS. The activity of SOD and CAT was comparatively high in samples collected after spraying of H_2O_2 under differential HS.

H_2O_2 accumulation also influences the osmolyte accumulation in the cells by altering the expression of pathway associated genes. Accumulation of proline in cell was estimated to be 0.95, 1.52, 1.61 and 1.57 $\mu\text{mole/g}$ FW (C-306) and 0.85, 1.39, 1.38 and 1.42 $\mu\text{mole/g}$ FW (HD2329) in response to HS of 22, 30, 35 and 40°C for 2 h (Figure 8c and d). With increase in the accumulation of H_2O_2 inside cell, an increase in proline was also observed under differential HS. It was observed in the present investigation that spraying of H_2O_2 (10 mmole/L) enhances the build-up of different proteins

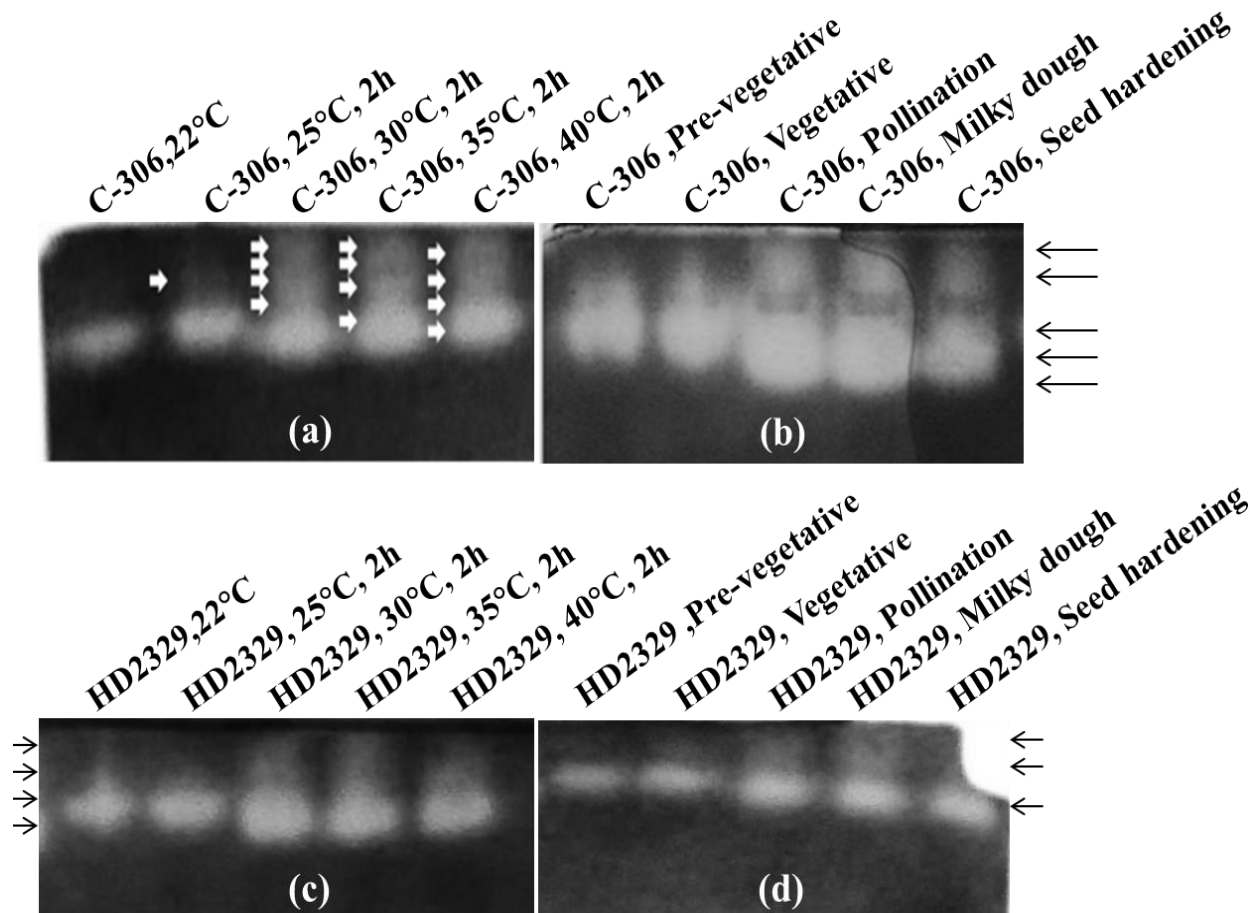


Figure 4. Isoenzymic pattern profiling of catalase in C-306 (thermotolerant) and HD2329 (susceptible) cultivars of wheat, (a) In gel activity assay in C-306 against differential heat shock, (b) In gel activity assay in C-306 at different stages of growth, (c) In gel activity assay in HD2329 against differential heat shock, (d) In gel activity assay in HD2329 at different stages of growth, Arrows depicting the isoenzymes activity.

(SOD, CAT) and osmolytes (proline) inside cell.

The tolerance capacity of the cells was also estimated in term of biochemical parameter like cell membrane stability (CMS). CMS calculated was 72.3, 68, 67.3 and 68% (C-306; Figure 9a) and 56.3, 49.6, 47.6 and 45.6% (HD2329; Figure 9b) under differential HS of 22, 30, 35 and 40°C for 2 h. The percentage decrease in CMS was very low in both tolerant and susceptible cultivars sprayed with H_2O_2 (10 mmole/L) compared to non-treated samples (tolerant and susceptible) under differential HS.

DISCUSSION

A change in the level of cell membrane stability, H_2O_2 production, proline accumulation and antioxidant isoenzymes activities in plant cells is an indicator of oxidative stress. Hydrogen peroxide acts as a signaling molecule inside the plant system and it is considered as the second line of defense in response to heat stress. An

increase in the amount of H_2O_2 is associated with oxidative damage (Ozden et al., 2009). In this study, there was an increase in hydrogen peroxide accumulation at high temperatures, which acts as a signaling molecule for the expression of stress induced genes which provide tolerance to the plants by either scavenging the free oxygen radicals or by protecting the innate protein from denaturation. The concentrations of H_2O_2 in normal tissues like leaves lies between 0.1 and 5 $\mu\text{mol/g}$ FW (Cheeseman, 2006) and its turnover is rapid. An increase in the intracellular accumulation of hydrogen peroxide was observed with exogenous application of H_2O_2 (10 mmole/L). The percentage increase in accumulation was more in thermotolerant (C-306) cultivar compared to susceptible (HD2329; Figure 8).

Population difference in heat tolerance is associated with tolerance to oxidative stress and the difference in sensitivity is due to the accumulation of H_2O_2 rather than tolerance to H_2O_2 (Soliman et al., 2011) as observed in the present investigation (Figure 1). With the increase in

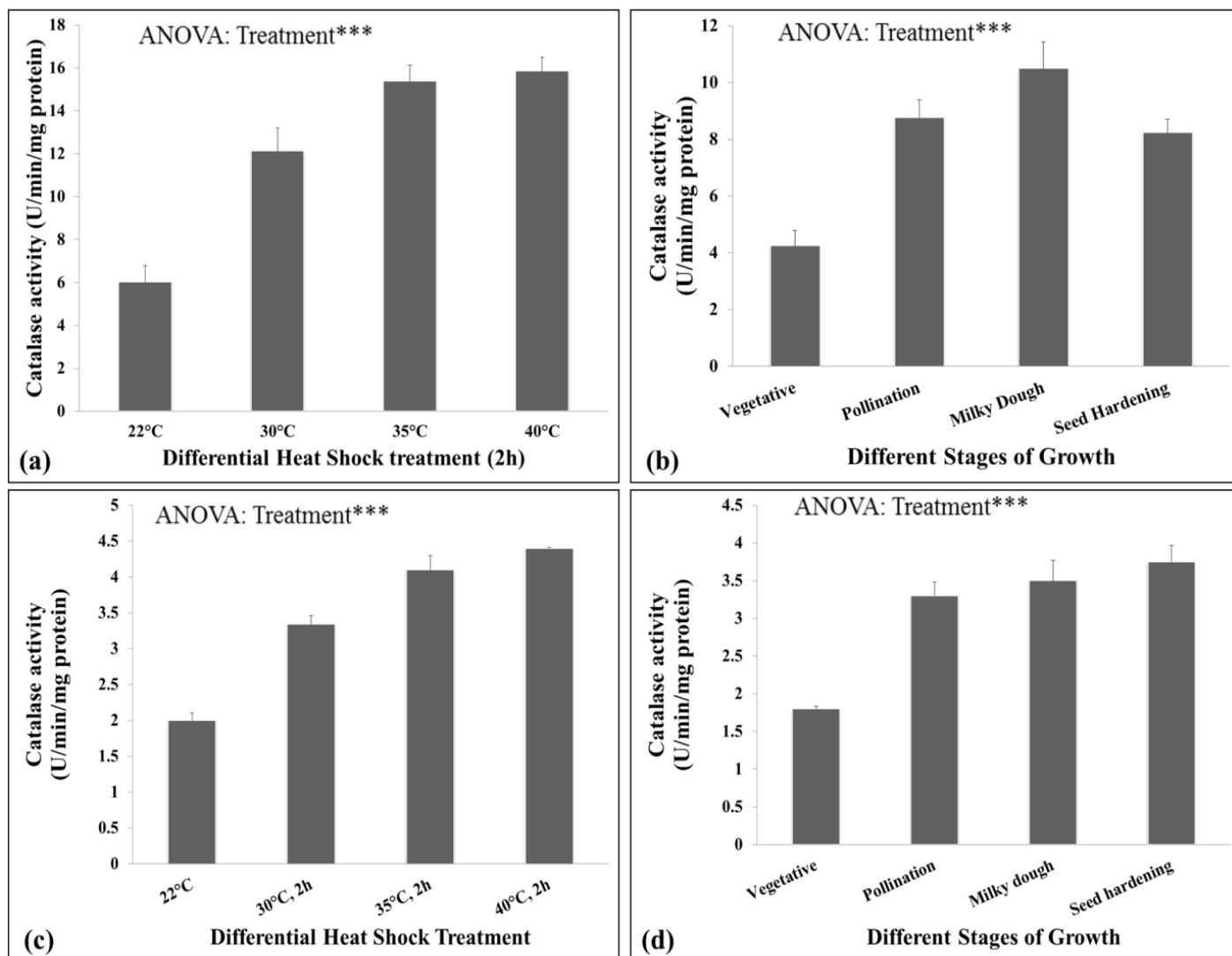


Figure 5. Specific activity of catalase enzymes in thermotolerant (C-306) and susceptible (HD2329) cultivars of wheat, (a) CAT specific activity in C-306 against differential heat shock, (b) CAT specific activity in C-306 at different stages of growth, (c) CAT specific activity in HD2329 against differential heat shock, (d) CAT specific activity in HD2329 at different stages of growth, ANOVA significance levels: *, $P < 0.05$; **, $P < 0.001$; ***, vertical bars indicate s.e. ($n=3$).

H_2O_2 accumulation inside cell because of either HS or by exogenous spraying of H_2O_2 , the activity of antioxidant enzymes (SOD and CAT) was observed to increase proportionately. Similar observation was made by Xuejun et al. (2011) where he reported an increase in the activity of all antioxidant enzymes (SOD, CAT, APX except GR) in all lines after heat treatment and the increase was more significant in proline overproducing seedlings. Superoxide dismutase activity observed in the present investigation conforms to the observation made by He et al. (2005), who reported that the activity of SOD in wheat significantly increased after 2 h of heat stress application, but decreased to the control levels after 6 h of heat stress application. An increase in SOD activity was observed after 12 days of drought, heat or combined stress of both

in cool-season turf grasses (Jiang and Huang, 2001). Here, the observed diminish activity of superoxide dismutase in response to 40°C heat shock could be explained by either enzyme denaturation or low transcript abundance of SOD (Figure 3). The observations made by Rizhsky et al. (2002) in tobacco plant are in accordance with our results. The high activity of SOD at pollination and milky dough stage may be due to the peroxide scavenging nature of SOD which helps in protecting important enzymes involved in various metabolic pathways from denaturation or damage because of ROS. It was also observed that SOD respond very well to exogenous spraying of H_2O_2 compared to normal plants (Figure 8). Similar observation was notice in case of catalase. A significant increase in CAT activity was

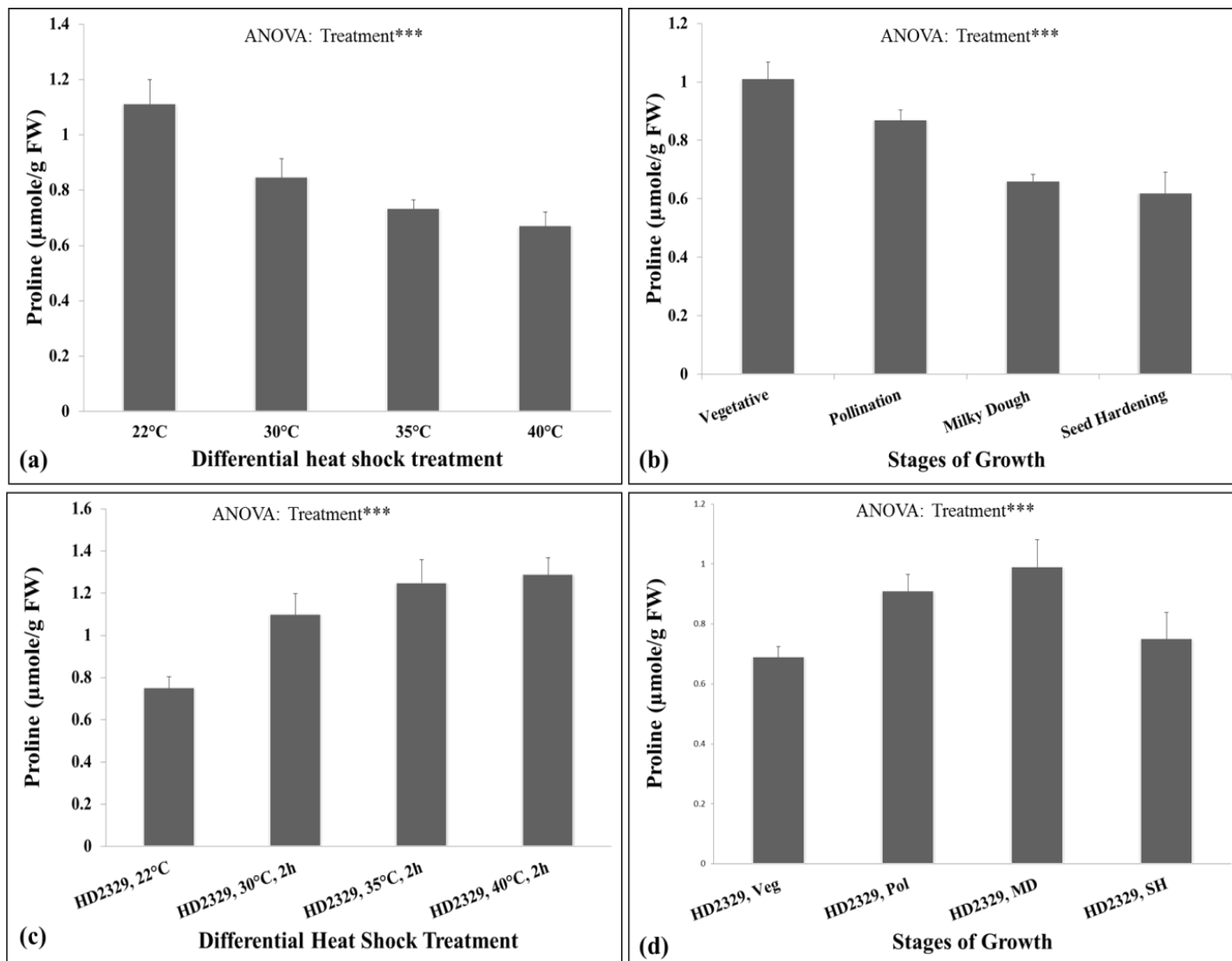


Figure 6. Proline accumulation in C-306 (thermotolerant) and HD2329 (thermosusceptible) cultivars of wheat against elevated temperature, (a) Proline accumulation in C-306 against differential heat shock, (b) Proline accumulation in C-306 at different stages of growth, (c) Proline accumulation in HD2329 against differential heat shock, (d) Proline accumulation in HD2329 at different stages of growth, ANOVA significance levels: *, $P < 0.05$; **, $P < 0.001$; ***, vertical bars indicate s.e. ($n=3$).

observed with differential heat shock treatment. Kele and Oncel (2002) reported that heat treatment increased CAT activities in *T. aestivum* genotypes, but decreased in *T. durum* genotypes. CAT activity reached up to the maximum level after a $\frac{1}{2}$ and 1 h of heat treatments and thereafter decreased in Kentucky bluegrass (He et al., 2005) as observed in the present investigation (Figure 5). SOD leads to increase in the accumulation of intracellular H_2O_2 inside cells. The balance in the concentration of accumulated H_2O_2 is very important for the cells otherwise it may leads to death of the cells. This is particularly taken care of by CAT. CAT scavenges H_2O_2 to nontoxic levels or catalyzes the formation of water and oxygen and hence, an increase in CAT activity could play

a role in the protection of the plants from damaging effect of H_2O_2 (in higher concentration) in wheat leaves in response to heat shock. An increase in the CAT activity was observed with exogenous application of H_2O_2 which gives us inference that CAT also respond very well to the intracellular H_2O_2 concentration (Figure 8).

Under the different abiotic stress conditions and H_2O_2 treatments, proline is accumulated in cells as osmoprotectant (Ozden et al., 2009) which is contradictory to that of our observations. However, the present results clearly showed that heat stress caused a decline in the level of proline accumulation in wheat (Figure 6). Xuejun et al. (2011) reported that proline accumulation under heat stress decreases the thermotolerance, probably

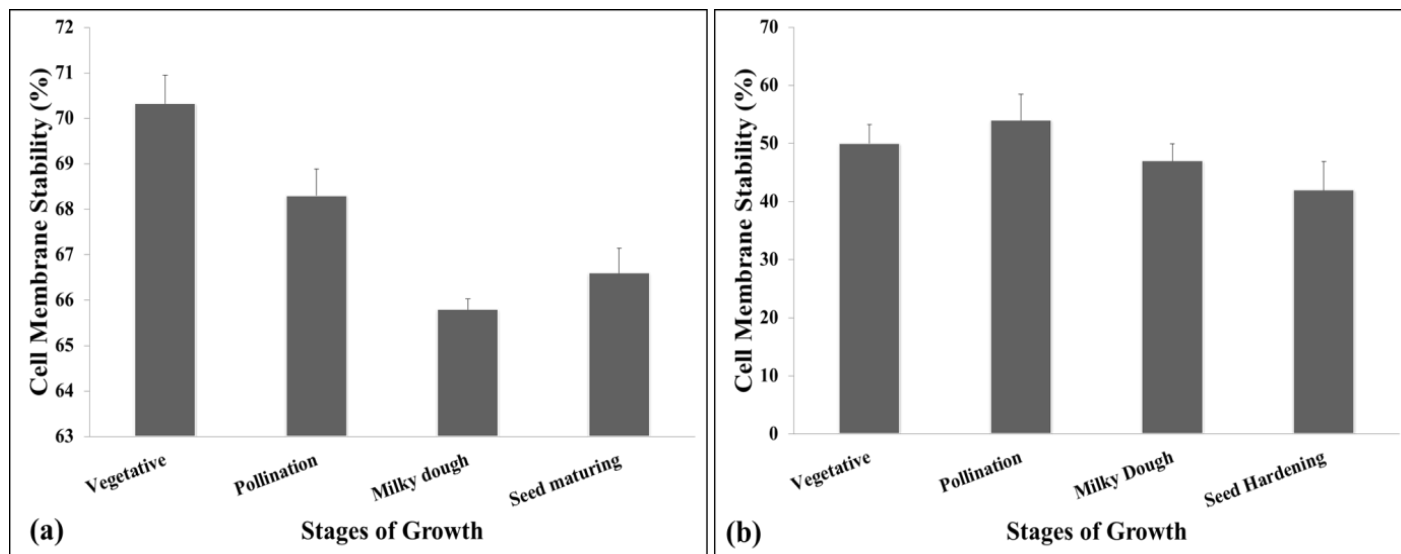


Figure 7. Cell membrane stability index of (a) thermotolerant (C306) and, (b) thermosusceptible (HD2329) cultivars of wheat at different stages of growth and development, Fokar's method was used for the estimation.

by increased ROS production via Pro/P5C cycle which is in conformity with present observation. Stress-related alterations in proline metabolism may impinge on systems of redox control of plant gene expression (Hare and Cress, 1997). The level of proline, thought to be important for plant protection during drought stress, is strongly suppressed in *Arabidopsis* after heat stress treatments and a combination of drought and heat stresses (Rizhsky et al., 2004). Exogenous application of H_2O_2 increase the accumulation of proline inside cells which makes us to conclude that H_2O_2 concentration inside cells has some correlation with genes involved in proline biosynthesis pathways which leads to alteration in concentration under differential HS (Figure 8).

The thermotolerance capacity of the varieties used in this experiment was interpreted in terms of biochemical parameter like CMS. Cell membrane stability was very high in tolerant (C-306) compared to susceptible (HD2329) wheat cultivars (Figure 7). With elevation in temperature, a decrease in the CMS was observed in both cultivars. The percentage decrease in CMS was observed to be very low when the plants were treated with H_2O_2 (10 mmole/L). This gives us inference that on pre-treatment with H_2O_2 , the accumulation of intracellular H_2O_2 increases and it leads to alteration in the expression of various stress associated proteins which are involved in defense mechanism against abiotic stresses. Membrane stability may be only a small part of the numerous properties and processes that interact to provide tolerance to stress during maturation (Assad and Paulsen, 2002). Since, membrane is the first line of defense having many heat responsive sensors which helps plant to activate its defense mechanism well in

advance against heat shock, so the integrity of the membrane is one of the important parameter. A change in the CMS in response to heat shock at pollination stage is the major issue of concern which needs to be addressed in future.

In conclusion, hydrogen peroxide acts as regulatory molecules in altering the various parameters responsible for enhancing the thermotolerance capacity and protecting the plant against terminal heat stress. Accumulation of intracellular hydrogen peroxide increases the activity of SOD and CAT under HS. Even decrease in the proline concentration was observed in the cell under the influence of H_2O_2 . The percentage decrease in CMS was very low in both the cultivars under HS. Exogenous application of H_2O_2 leads to increase in the intracellular H_2O_2 accumulation which in turn increases the activity of antioxidant enzymes as well as accumulation of proline inside cell. A very high CMS was observed in H_2O_2 treated plants at all the stages compared to normal plant under differential HS. Under heat stress conditions, particularly at reproductive stage, the level of expression of genes and their upstream regulatory regions that regulate the stress associated proteins needs to be studied in more detail to better understand the mechanism of heat tolerance in wheat.

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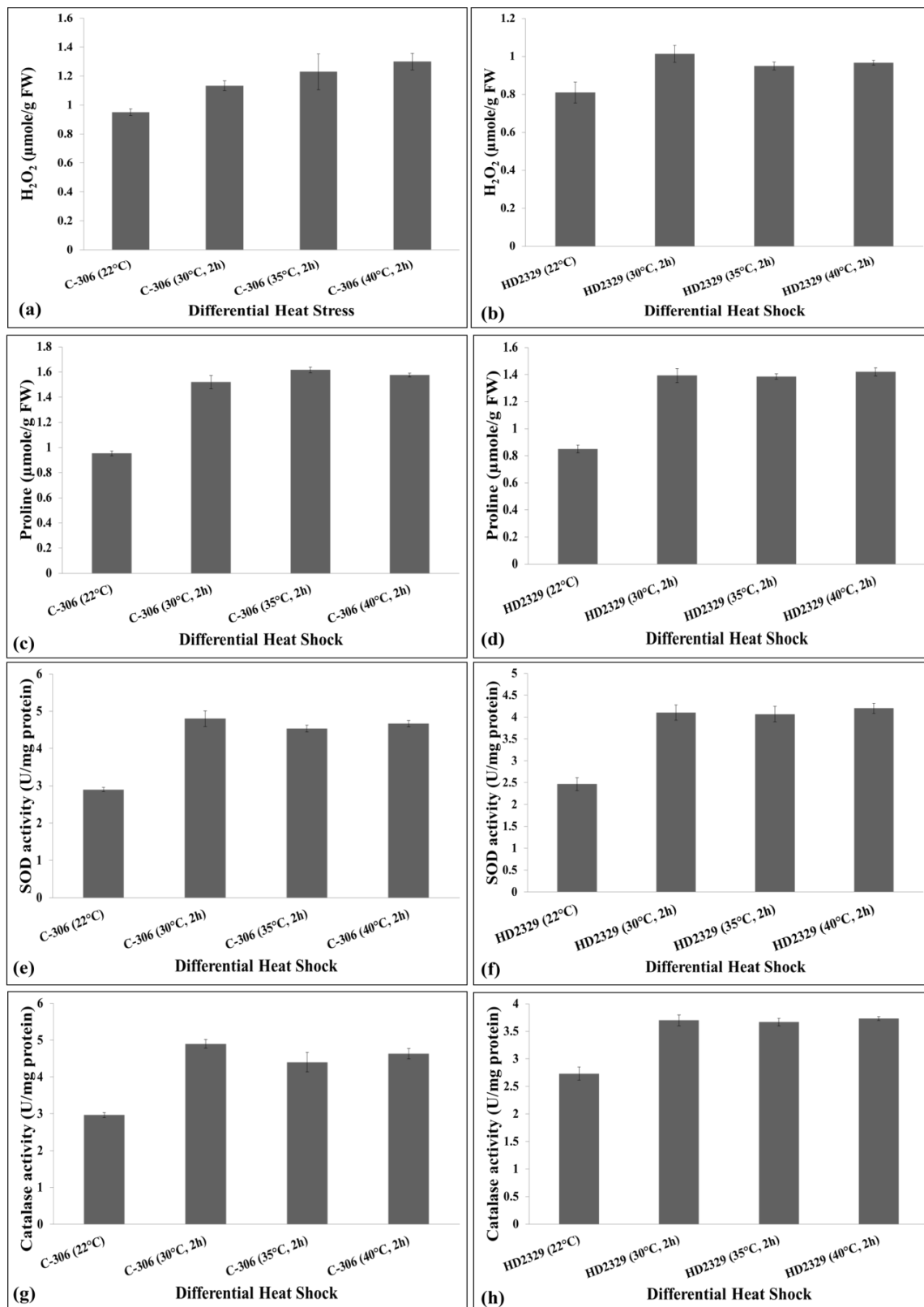


Figure 8. Effect of exogenous application of hydrogen peroxide (10 mmole/L) on various biochemical parameters in C-306 (thermotolerant) and HD2329 (susceptible) cultivars of wheat under differential HS, (a) Intracellular H₂O₂ accumulation in C-306, (b) Intracellular H₂O₂ accumulation in HD2329, (c) Proline accumulation in C-306, (d) Proline accumulation in HD2329, (e) SOD activity in C-306, (f) SOD activity in HD2329, (g) Catalase activity in C-306, (h) catalase activity in HD2329.

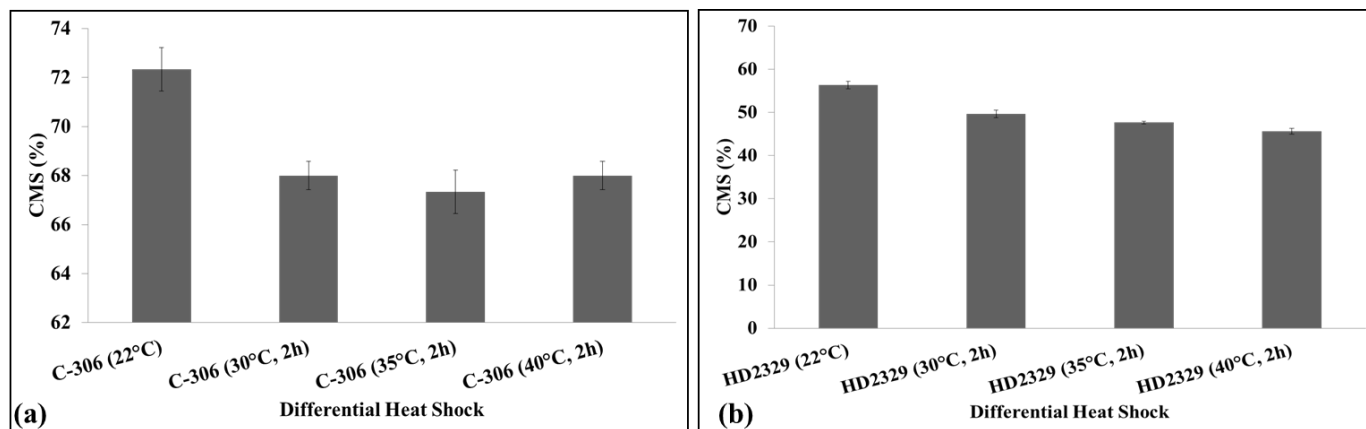


Figure 9. Effect of exogenous hydrogen peroxide (10mmole/L) application on alteration in cell membrane stability index of C-306 (thermotolerant) and HD2329 (Susceptible) wheat cultivars, (a) CMS in C-306, (b) CMS in HD2329.

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