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Exogenous application of putrescine at pre-anthesis enhances the thermotolerance of wheat (*Triticum aestivum* L.)

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Antioxidant enzymes, besides being involved in various developmental processes, are known to be important for environmental stress tolerance in plants. In this study, the effect of treatment of 2.5 mM putrescine (Put), heat stress (HS -42°C for 2 h) and their combination on the expression and activity of antioxidant enzymes was studied at pre-anthesis in the leaves of two wheat (*Triticum aestivum* L.) cultivars — HDR77 (thermotolerant) and HD2329 (thermosusceptible). We observed that 2.5 mM Put before HS significantly enhanced the transcript levels of superoxide dismutase (*SOD*), catalase (*CAT*), cytoplasmic and peroxisomal ascorbate peroxidase (*cAPX*, *pAPX*) in both the cultivars. However, the activities of antioxidant enzymes (SOD, CAT, APX and GR), as well as accumulation of antioxidants (ascorbic acid and total thiol content) were higher in HDR77 than in HD2329 in response to the treatment 2.5 mM Put + HS. No significant change was observed in the proline accumulation in response to HS and combined treatment of 2.5 mM Put + HS. A decrease in the H_2O_2 accumulation, lipid peroxidation and increase in cell membrane stability (CMS) were observed in response to 2.5 mM Put + HS treatment, as compared to HS treatment alone in both the cultivars; HDR77 was, however, more responsive to 2.5 mM Put + HS treatment. Put (2.5 mM) treatment at pre-anthesis thus modulated the defense mechanism responsible for the thermotolerance capacity of wheat under the heat stress. Elicitors like Put, therefore, need to be further studied for temporarily manipulating the thermotolerance capacity of wheat grown under the field conditions in view of the impending global climate change.

Keywords: Heat stress, Antioxidants, Putrescine, Wheat, Antioxidant enzymes, Quantitative real-time PCR, Heat shock proteins, *Triticum aestivum* L.

Wheat is the most widely grown cereal crop providing 12% of the carbohydrate in the global human diet. High temperature being increasingly experienced due to global climate change represents a significant threat to the cultivation of wheat crop in large areas of the world. Various approaches are being studied to ameliorate the adverse effects of heat on wheat.

Polyamines (PAs), such as putrescine (Put), spermidine and spermine are ubiquitous polycations involved in different processes of plant growth and development[1]. They are involved in various fundamental cellular processes, such as transcription, RNA modification, protein synthesis and modulation of enzyme activities[2]. PAs are involved in various growth and developmental processes of plant and play very important role as anti-senescence and anti-stress plant growth regulator and also have acid-neutralizing, antioxidant and cell wall-stabilizing properties[3]. They also play an important role in cell division, embryogenesis, root formation, fruit development, ripening and in responses to biotic and abiotic stresses[4,5]. The abundance of PAs has been observed in actively growing tissues under stress condition[6,7]. They are involved in modulating the defense response of plants to diverse environmental stresses, such as metal toxicity, oxidative stress, drought, salinity, chilling and heat stress and also acts as secondary messenger in various signaling pathways[8,9].

Changes in PAs accumulation have been observed when plants are exposed to single or combined

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Abbreviations: ASA, ascorbic acid; APX, ascorbate peroxidase; BSA, bovine serum albumin; CAT, catalase; CMS, cell membrane stability; DTNB, 5, 5-dithiobis 2-nitrobenzoic acid; HS, heat stress; NBT, nitro-blue tetrazolium; PA, polyamine; PGR, plant growth regulator; POX, peroxidase; Put, putrescine; PVP, polyvinyl-polypyrrolidone; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; SA, salicylic acid; SOD, superoxide dismutase; TCA, trichloroacetic acid.

stresses[1,10]. Altered accumulation of free and conjugated PAs has been observed in tissue suspensions of tobacco and alfalfa in response to the heat stress[11]; similar results have also been reported in groundnut and tomato[12,13]. It is suggested that exogenous application of PAs may be effective for enhancing stress tolerance of crops under the abiotic stresses[14,15]. In tomato production, exogenous application of Put at appropriate concentration is found to effectively limit damage from the sub-optimal temperature stress[16].

Plant cells are protected against oxidative stress by various radical-scavenging systems, including antioxidants such as ascorbate, glutathione, α-tocopherol, carotenoids and antioxidant enzymes, such as superoxide dismutases (SOD), peroxidases (POX), ascorbate peroxidases (APX), glutathione reductases (GR)[17]. APX exists as isoenzymes and plays an important role in the metabolism of H_2O_2 in higher plants. Cytoplasmic APX utilizes ascorbic acid (AsA) as its specific electron donor to reduce H_2O_2 to water with the concomitant generation of monodehydroascorbate (MDHA), a univalent oxidant of AsA. Various high and low molecular weight heat shock proteins (HSPs) also play an important role in protecting the nascent proteins from denaturation. Stress-associated proteins are the key factors which determine the thermotolerance level of the plants. Some of the parameters that can be used for assessing the thermotolerance capacity of plants are: cell membrane stability (CMS), lipid peroxidation, antioxidant accumulation, antioxidant enzyme activity and expression of stress associated proteins[18,19].

Lipid peroxidation is a well-established parameter of cellular injury in both plants and animals and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose under stress to form reactive carbonyl compounds like malondialdehyde (MDA) which is used as an indicator of lipid peroxidation. Plant cell protects itself against stresses by synthesizing low molecular mass thiols, such as reduced glutathione (GSH). The most important thiols, cysteine (Cys), glutathione (GSH) and oxidized glutathione (GSSG) constitute the pool of thiols. GSH functions as a reductant of dehydroascorbate *via* dehydroascorbate reductase forming ascorbate and GSSG[20]. GSH, besides the removal of reactive oxygen species (ROS) has been ascribed in several other physiological processes, including stress signaling[17].

PAs have been reported to enhance the tolerance mechanism of various plants under different abiotic stresses[21]. Exogenous application of Put has been used successfully for enhancing the tolerance level of plants against heat stress[22], drought[23], cold[24], salinity[25] etc. Plants over-expressing genes associated with Put biosynthesis pathways have been reported to tolerate multiple stresses[26]. Exogenous application of PAs has been reported to enhance the thermotolerance of wheat; however, the mechanism behind the modulation is not well understood[27].

In this study, we have investigated the effect of exogenous application of putrescine (Put) at pre-anthesis stage on heat stress (HS) tolerance of two contrasting wheat (*Triticum aestivum* L.) cultivars — thermotolerant (HDR77) and thermosusceptible (HD2329).

Materials and Methods

Plant material, sample collection and heat stress treatments

Wheat (*Triticum aestivum* L.) cultivars — HDR77 (thermotolerant) and HD2329 (thermosusceptible) seeds were procured from the Division of Genetics, Indian Agricultural Research Institute (IARI), New Delhi. Seeds of both the varieties were sown in vermiculite/sand mixture in 54 pots (10 cm) inside the Phytotron chamber under the regulated conditions (mean photosynthetically active radiation of 1050 µmole m^{-2} s^{-1}, average temperature of 22°C and 80% relative humidity). The mean day and night temperatures were 28 ± 3 and 20 ± 2°C, respectively. The 54 pots were divided into two groups comprising of 30 and 24 pots and 15 pots each of both cultivars were used for the pilot experiment with the plants at vegetative stage (30 days after sowing) to determine the optimum concentration of Put for the experiment (Fig. 1).

Both the cultivars at pre anthesis were treated (leaf spray), in triplicates with five concentrations of Put (0, 2.5, 5, 7.5 and 10 mM) and then subjected to HS of 42°C for 2 h for 4 days. Samples were taken on 5th day after treatment to find out the growth and biochemical parameters under HS. Based on the observations of pilot experiment, 2.5 mM Put was found to be most effective. The other groups (12 pots of HDR77 and 12 pots of HD2329) at pre-anthesis stage were subjected to three different treatments in triplicate: T_1, 2.5 mM Put ($Put_{2.5}$), T_2, HS of 42°C for 2 h (HS - $Put_{2.5}$) and T_3, 2.5 mM Put, followed by HS

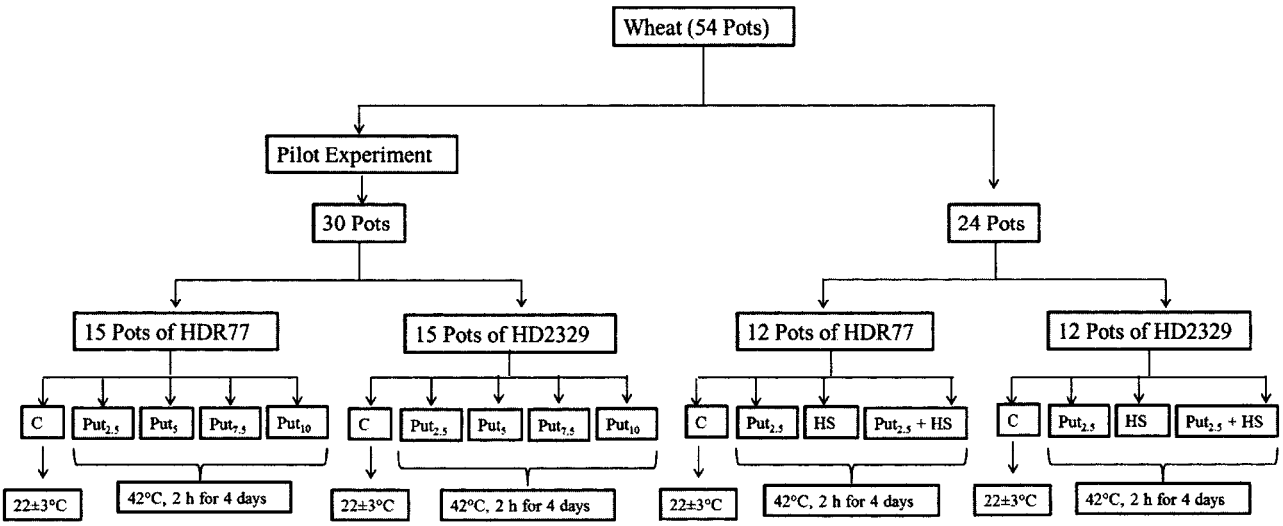

Fig. 1—Diagrammatical representation of planning for the sowing of thermotolerant (HDR77) and thermosusceptible (HD2329) wheat cultivars in pots under regulated condition for executing the pilot experiment and further investigation to study the effect of Put and HS on thermotolerance

of 42°C for 2 h (HS + $Put_{2.5}$). One set was used as the control (without any treatment). The flag leaves from both the cultivars were collected in triplicate on 5th day after treatment and frozen in liquid nitrogen for biochemical and molecular characterization.

Protein estimation and total RNA isolation

Protein estimation was carried by the Bradford method using bovine serum albumin (BSA) as a standard[28]. 100 mg tissues were used for the total RNA isolation by Trizol method and the quantification was done using Qubit^{TM} 2.0 fluorometer (Invitrogen, UK).

Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR was carried out on CFX96 platform (Bio Rad, UK) using the protocol previously described[9]. Primers for quantitative RT-PCR reactions were designed from the deduced sequence corresponding to the wheat *SOD*, *CAT*, *pAPX* and *cAPX* genes using OligoPerfect designer software (Invitrogen, UK) (Table 1). For each stress condition as well as for control, expression measurements were performed using two biological and three technical replicates. The expression levels of the wheat tubulin gene (Gen Bank accession no TAU76544) were used as internal standards for normalization of data. The comparative C_t ($2^{-\Delta\Delta Ct}$) method was used to calculate the relative fold expression[29].

Assay of antioxidant enzymes activities

Fresh leaf material (1 g) was crushed 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 used for the quantification of soluble protein content and analysis of SOD, CAT, APX and GR. Coomassie blue dye-binding assay was used for protein estimation[28] and BSA for standard curve preparation.

SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) in the presence of riboflavin in light[30]. One unit of enzyme activity was defined as the amount of enzyme needed for inhibition of 50% NBT reduction rate by monitoring absorbance at 560 nm with spectrophotometer.

Table 1—Primers used for the transcript profiling of antioxidant enzymes in HDR77 (thermotolerant) and HD2329 (thermosusceptible) cultivars of wheat by quantitative real-time PCR (qRT-PCR)

Primers	Sequence (5'-3')	T_m value (°C)
cAPX-F	GATCCTCAAAACCACCTACTGC	63.6°C
cAPX-R	CCATGACTCAACTCAGGATCAA	64.0°C
pAPX-F	GCTCCCTACTGATAAGGCATTG	63.6°C
pAPX-R	TTTGTGTGATTCAGCGTAGTCC	63.9°C
CAT-F	CAAGAGCGATTCATCAACAGAT	63.0°C
CAT-R	AGACCAGTAGGAGAGCCAGATG	63.6°C
SOD-F	TCCTTTGACTGGCCCTAATG	63.8°C
SOD-R	CTTCCACCAGCATTTCCAGT	64.0°C
Tub-F	TCGATGATCTCCAACTCCACCAGT	62.7°C
Tub-R	TCGTCGAACTCAGCACCAACTTCT	62.7°C

*c - cytoplasmic, p - peroxisomal, F - forward primer, R - reverse primer, Tub - Tubulin

For assaying CAT activity, decomposition of H_2O_2 was followed by decline in the absorbance at 240 nm[31]. CAT activity was determined by following the consumption of H_2O_2 (extinction coefficient, 39.4 mM^{-1} cm^{-1}) at 240 nm over a 3 min interval.

APX was assayed by recording the decrease in optical density (OD) due to ascorbic acid (AsA) at 290 nm[32] and the enzyme activity was calculated as concentration of AsA oxidized/min/mg protein. GR activity was determined from the rate of NADPH oxidation by the decrease in A_{340}[33].

K_m for GSSG and NADPH was determined by varying the GSSG concentration from 0 to 500 µM or the NADPH concentration from 0 to 150 µM, respectively.

Estimation of non-enzymatic antioxidants

The collected leaf samples (fresh weight) were crushed in liquid nitrogen and homogenized in 2 ml of 6% trichloroacetic acid (TCA). After centrifugation at 20,000 *g* (4°C) for 20 min, the supernatant was collected in separate tube and used for the estimation of AsA[34]. Total thiol content was assayed in acid-soluble extracts (0.2 g fresh weight mL^{-1})[35]. The homogenate was prepared in 3% TCA (w/v) and after a brief centrifugation (12,000 *g* for 3 min); the supernatant was diluted 10-fold in 100 mM phosphate buffer (pH 7.5). Thiol content was determined measuring absorbance at 412 nm, in the presence of 5, 5-dithiobis 2-nitrobenzoic acid (DTNB), 0.5 U mL^{-1} GR and 0.2 mM NADPH.

Measurement of H_2O_2 and proline accumulation

Leaf samples (0.3 g) were homogenized in 3 ml of 1% (w/v) TCA and H_2O_2 was quantified as described previously[36] and concentration was expressed as µmol g^{-1} fresh weight (FW). Proline content was determined according to method of Bates *et al*.[37].

Evaluation of membrane lipid peroxidation and cell membrane stability (CMS)

200 mg fresh weight leaf samples was homogenized in 5.0 ml of TCA (0.1%) and oxidative damage to lipids was determined as lipid peroxidation in terms of malondialdehyde (MDA)[38]. MDA content was calculated using an extinction coefficient (ε) of 155 mM^{-1} cm^{-1} and expressed as µmol g^{-1} FW. Flag leaf of uniform size was collected and used to measure cell membrane stability (CMS)[39].

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. For each parameter, differences among treatment were analyzed using one-way analysis of variance (ANOVA). Data points in each histogram represent the mean ± SE of three independent replications.

Results and Discussion

Pilot experiment

A significant increase in the fresh weight of flag leaf was observed under Put treatment before HS in HDR77; maximum fresh weight was, however, observed in response to 2.5 mM Put + HS and no significant difference was observed in HD2329 (Fig. 2a). Increase in total protein content was also observed in both the cultivars under different Put treatments before HS; the maximum being in response to HS + 2.5 mM Put (Fig. 2b). Non-significant difference was observed in the root weight of both the cultivars in response to the treatments (Fig. 2c).

An increase in total RNA content was observed in both the cultivars under different Put treatments, compared to the samples exposed to HS; maximum RNA content was observed in response to HS + 2.5 mM Put in both the cultivars (Fig. 2d). Accumulation of total RNA content was higher in the thermotolerant cultivar, compared to the susceptible one. As all the four parameters selected for the pilot experiment showed better performance in the treatment of HS + 2.5 mM Put in both the cultivars, we selected this treatment for further investigation.

Transcript profiling of antioxidant enzymes

Results of qRT-PCR expression profiling of *SOD*, *CAT*, peroxisomal and cytoplasmic *APX* (*pAPX* and *cAPX*) genes carried out in response to 2.5 mM Put + HS (42°C for 2 h) treatment are depicted in Fig. 3. A slight, but significant increase in *SOD* transcripts was observed in HDR77 in response to 2.5 mM Put, but abundant transcripts were observed against HS + 2.5 mM Put; maximum transcript was, however, observed in response to HS + 2.5 mM Put. The transcript of *SOD* in HD2329 was low, compared to HDR77 under different Put treatments (Fig. 3a). The 2.5 mM Put treatment alone was not effective, compared to heat stress (HS – Put) and HS + 2.5 mM Put, wherein significant increases in transcript were observed. A down-regulation in the transcript of *SOD* was observed in HD2329 under treatments of 2.5 mM Put, heat stress and HS + 2.5 mM Put. The increase in

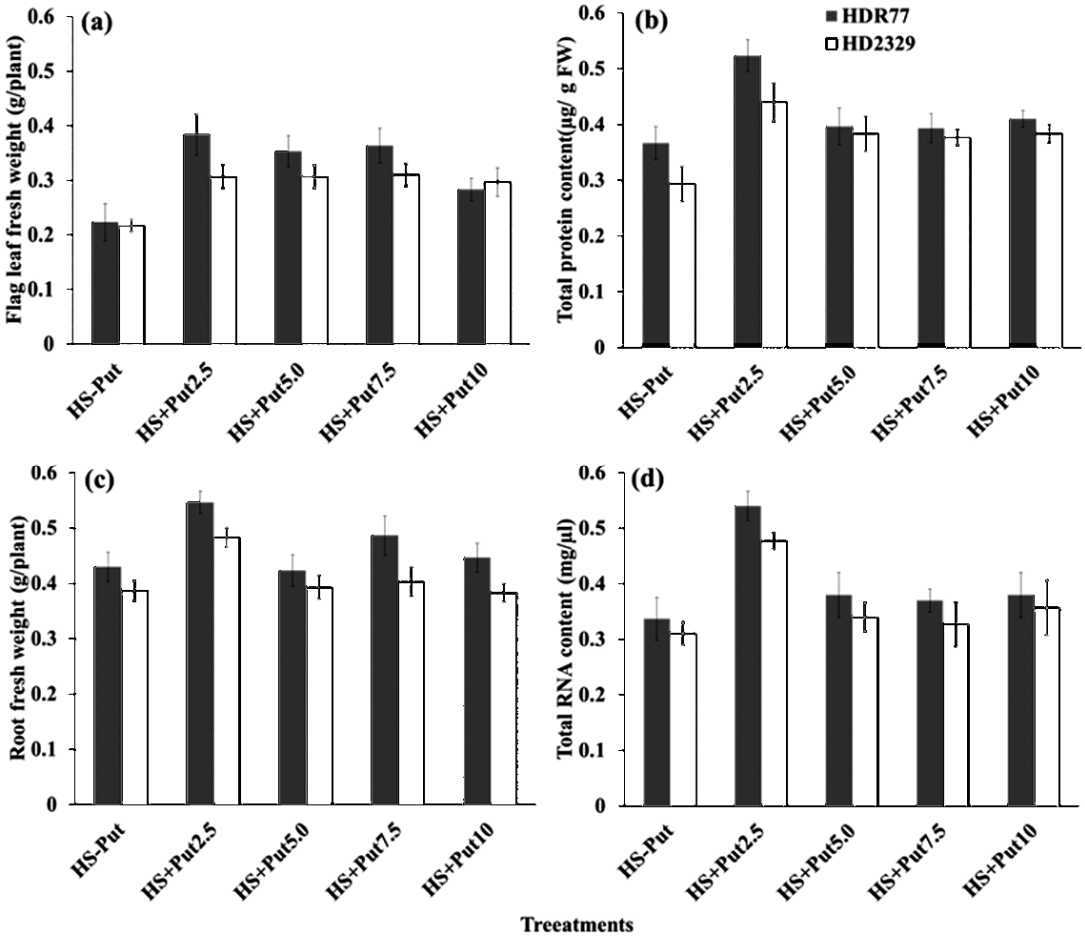

Fig. 2—Pilot experiment to study the effect of Put and HS on physiological and biochemical parameters in HDR77 and HD2329 [(a) Flag leaf fresh weight, (b) total protein content, (c) fresh weight of root, and (d) total RNA content, putrescine treatment - 2.5, 5.0, 7.5 and 10 mM, H - 42°C for 2 h). Put, Putrescine, HS, heat stress. Significant differences among treatments were observed at $P<0.05$ based on ANOVA analysis. Vertical bars indicate s.e. (n = 3)]

the transcript was non-significant in response to combined treatment of HS + 2.5 mM Put, compared to heat stress (HS – Put). Although not much difference in the fold change expression of *SOD* was observed in both the cultivars, but even a slight change in expression of SOD has been found to have significant impact on thermotolerance levels of the plant[40]. Semi-qRT-PCR analysis of *HbCCH1* (copper chaperone) showed constitutive expression of the gene in all the tested tissues[41]. Manipulation of arginase gene (involved in Put biosynthesis) expression has been found to modulate the abiotic stress tolerance level of *Arabidopsis* by controlling ROS accumulation[42]. The expression of cytCuZnSODs has been reported in various parts of *Nelumbo nucifera* like leaf stalks, young leaves and roots[43].

The transcript levels of *CAT* in HDR77 and HD2329 were maximum in response to the combined effect of HS + 2.5 mM Put (Fig. 3b). The *CAT* was more responsive to 2.5 mM Put treatments before HS, compared to other antioxidant enzymes. The fold change in expression was higher in case of HDR77, compared to HD2329 under HS + 2.5 mM Put. Treatment of 2.5 mM Put caused decrease in the expression of *pAPX* and *cAPX* in both the cultivars (Fig. 3c & d), whereas an increase in both the transcripts were observed under HS. Combined treatment of Put and HS resulted in up-regulation of *cAPX* and *pAPX* in HDR77, while no significant change in the transcript was observed in HD2329. Increased levels of Put have been found to inhibit DNA methylation, permitting the expression of specific genes[44], or affect gene expression by altering sequence-specific DNA-protein interactions[45], or activate/modulate translocation of protein kinases, such as CK2 in signal transduction[46].

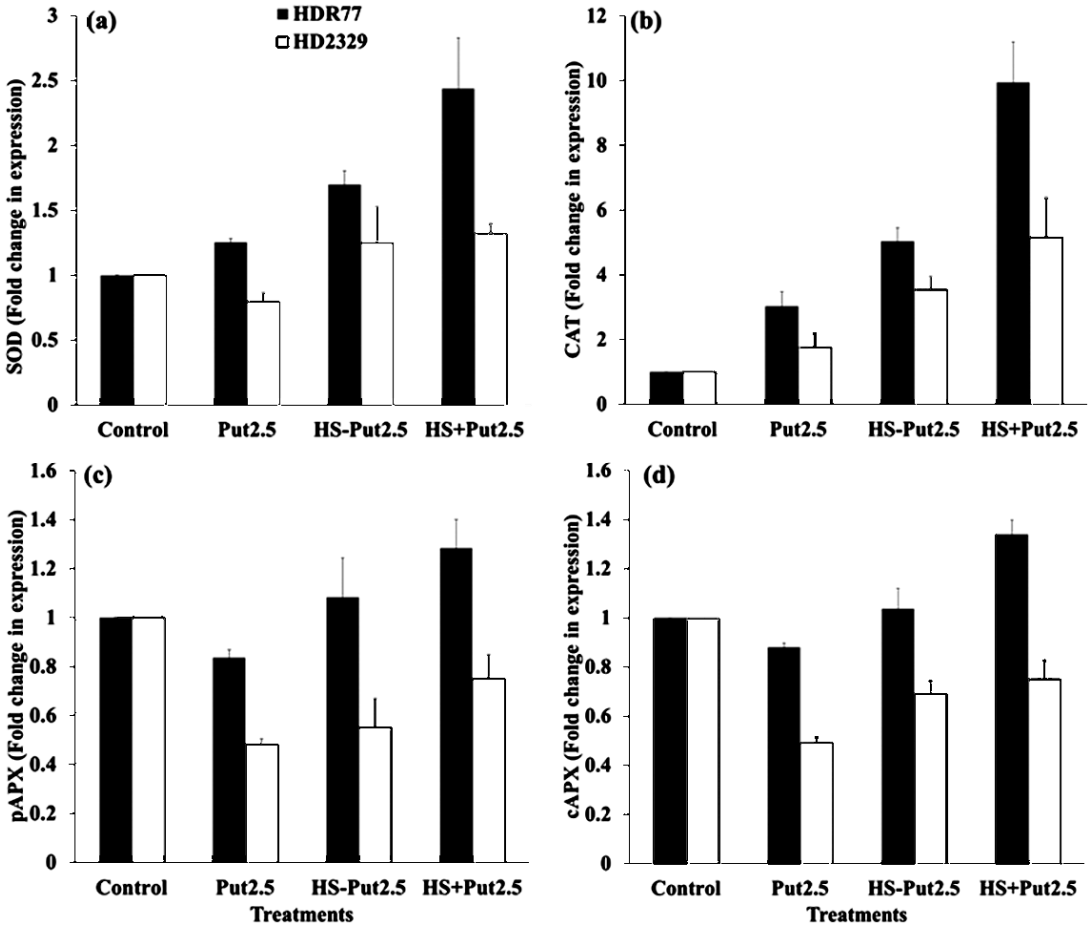

Fig. 3—Transcript profiling of antioxidant enzymes in HDR77 and HD2329 in response to Put and HS [(a) *SOD*, (b) *CAT*, (c) *pAPX* and (d) *cAPX*. Put - 2.5 mM ($Put_{2.5mM}$), HS - 42°C for 2 h ($HS\text{-}Put_{2.5mM}$) and combined effect of both (HS + $Put_{2.5mM}$). Expression levels of wheat tubulin gene were used as internal standard for normalizing the data. Significant differences among treatments were observed at $P<0.05$ based on ANOVA analysis]

Changes in antioxidant enzymes activity

The SOD activity was significantly higher in response to HS alone and combined treatment of HS and Put (HS + 2.5 mM Put) in both the cultivars (Fig. 4a). A significant increase in the SOD activity was noted in HDR77 and HD2329 against HS, compared to control. Under 2.5 mM Put treatment, no significant change in the activity was observed. This might be due to fact that SOD was more responsive to HS than Put. Earlier, an increase in the SOD activity is reported with over-expression of genes invovled in Put biosynthesis pathway[42]. In case of CAT, a continuous increase in the activity was observed under combined HS and HS + 2.5 mM Put treatment in both the cultivars (Fig. 4b); the maximum activity was observed in response to HS + 2.5 mM Put. We found that CAT was very responsive to Put treatment and CAT isoenyme in HDR77 was more heat-stable than in HD2329. PAs have been shown to promote the activity of intracellular CAT[47]. Put at 0.1 and 1 mM has been shown to increase the CAT activity, while 10 mM Put decreases its activity[48].

No significant difference in the GR activity was observed in both the cultivars under 2.5 mM Put treatment, while HS caused significant increase in the GR activity. But, the maximum activity was observed in response to combined treatment (HS + 2.5 mM Put) in both the cultivars (Fig. 4c). Non-significant increase in the GR activity was observed under HS and HS + 2.5 mM Put in HD2329 and under combined effect of HS + 2.5 mM Put in HDR77.

Non-significant increase in the APX activity was observed under 2.5 mM Put treatment, while a sudden increase in the activity was observed under HS with the activity being very high in HDR77 compared to HD2329 (Fig. 4d). Combined treatment (2.5 mM Put + HS) had no marked difference in the APX activity in HD2329, whereas in HDR77, an increase in the

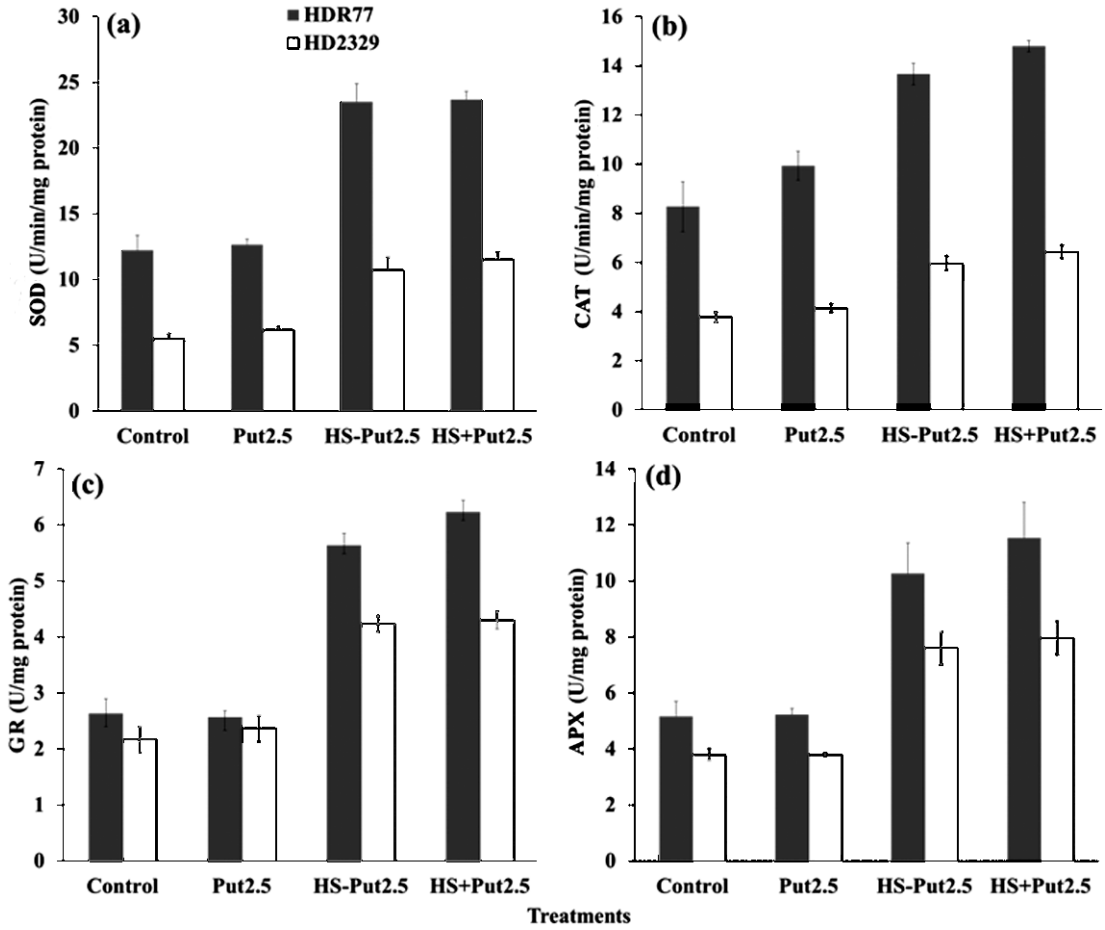

Fig. 4—Changes in antioxidant enzymes activity under Put and HS treatments in HDR77 and HD2329 [(a) SOD, (b) CAT, (c) GR, and (d) APX. Put – 2.5 mM ($Put_{2.5mM}$), HS - 42°C for 2 h (HS - $Put_{2.5mM}$) and combined effect of both (HS + $Put_{2.5mM}$). APX activity was calculated as concentration of ascorbic acid (AsA) oxidized/min/mg protein. Significant differences among treatments were observed at $P<0.05$ based on ANOVA analysis]

activity was observed. Increase in the activity and stability of the enzymes might be due to the HS and Put, respectively. Individually, 2.5 mM Put had no effect on the activity of different antioxidant enzymes, whereas combined treatment (HS + 2.5 mM) Put caused significant increase in the activity. Foliar spray with spermidine has been shown to improve the APX and CAT activities in cucmber seedling grown under the HS[49].

Putrescine binds to antioxidant enzymes and allows them to permeate to the sites of oxidative stress within the cells. A covalent Put-SOD complex is found to be 20-fold more membrane permeable than SOD alone, facilitating oxidant protection in a mammalian system[50]. High concentration of Put treatment decreases the activity of various antioxidant enzymes in wheat[48]. Earlier study has reported that exogenous application of either arginine or Put has been shown to significantly decrease the peroxidases (POX) and potato polyphenol oxidase (PPO) activities and increase the SOD and CAT activities in wheat, as compared to control plant or plants that are exposed to high HS without foliar treatment[51].

Change in antioxidant compound accumulation under exogenous stresses

Ascorbic acid (AsA) content of control, Put, HS - Put and HS + Put was 15.6, 16.0, 16.4 and 13.2 µmole g^{-1} DW, respectively in HDR77 (Fig. 5). A drastic decrease in AsA content (10.4 µmole g^{-1} DW) was observed in response to HS treatment (Fig. 5a), while 2.5 mM Put treatment before HS decreased AsA content, but the decrease was less compared to that of HS. Similarily, AsA content of control, Put, HS - Put, HS + Put was 13.5, 14.4, 7.3 and 8.9 µmole g^{-1} DW, respectively in HD2329. AsA content which was reduced due to HS was restored to some extent by

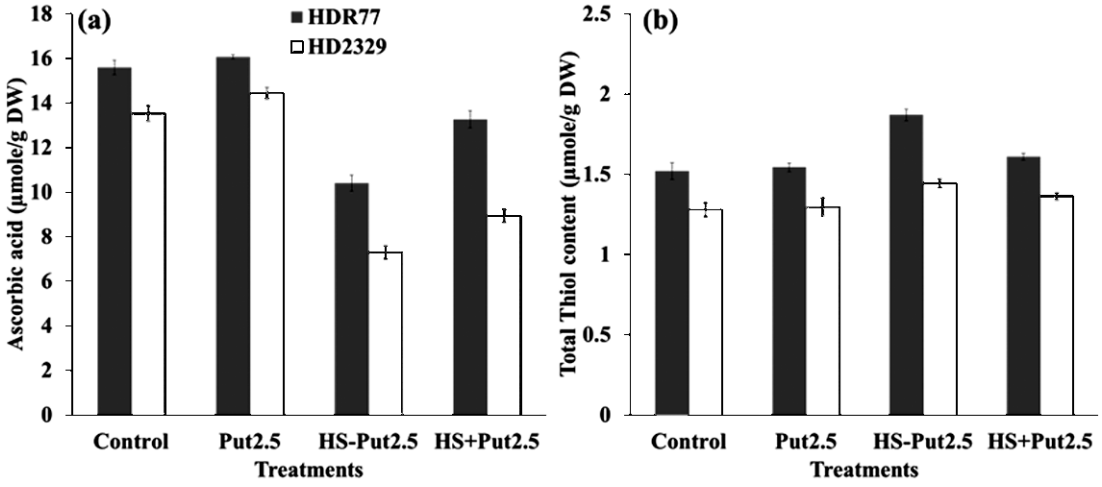

Fig. 5—Changes in accumulation of antioxidant compounds in leaves under Put and HS treatments in HDR77 and HD2329 [(a) Reduced ascorbic acid, and (b) total thiol content. Put - 2.5 mM ($Put_{2.5mM}$), HS - 42°C for 2 h (H - $Put_{2.5mM}$) and combined effect of both (HS + $Put_{2.5mM}$). Data are mean ± SE of three replications. Significant differences among treatments were observed at $P<0.05$ based on ANOVA analysis]

Put. AsA content in HDR77 cultivar was significantly higher than HD2329. Decrease in AsA accumulation in response to HS + 2.5 mM Put was, however, more in HDR77 than in HD2329. This indicated susceptible cultivar was more responsive to HS + 2.5 mM Put in accumulating antioxidant compounds like AsA.

Putrescine forms complex with antioxidant compounds and enhance their movement to the site of oxidative stress by ameliorating the stress and enhancing the thermotolerance. AsA is reported to be the most abundant antioxidant that protects the plant cells and is involved in a wide range of important functions, such as antioxidant defense, photo-protection, regulation of photosynthesis and growth[52]. PAs are inefficient scavengers of oxidative radicals, such as $HO^{\cdot}$, t-$BuO^{\cdot}$ and O^{2-} when compared with potent antioxidants, such as vitamins C and E[53].

Total thiol content showed a reduction of 13.9% (HDR77) and 5% (HD2329) in response to HS + 2.5 mM Put, compared to HS alone (Fig. 5b), indicting that total thiol content was higher in tolerant cultivar, compared to susceptible. Earlier, decrease in total thiol content is reported in *Amaranthus lividus* seedling under HS, while treatment with Ca^{2+} under HS has shown higher total thiol level[54].

Proline and H_2O_2 accumulation

Non-significant change in proline accumulation was observed in both the cultivars under 2.5 mM Put and HS treatments. Maximum accumulation of proline was observed with HS + 2.5 mM Put in HDR77, whereas in HD2329 it was in response to HS (Fig. 6a). Proline accumulation was not influenced by 2.5 mM Put treatment at pre-anthesis in both the cultivars. Earlier, we have shown that proline accumulation decreases under differential HS in wheat[55]. In other crops also, proline accumulation in response to HS has been reported. However, our findings were at variance with earlier observation that application of PGRs like PAs, gibberellic acid etc. at appropriate concentration promotes an increase in the organic osmolytes (proline and soluble sugars)[16].

H_2O_2 acts as signaling molecule in response to HS and activates various defense mechanisms involved in enhancing the thermotolerance capacity of the plants[56]. Treatment of 2.5 mM Put at pre-anthesis stage alone had no significant effect on the accumulation of intracellular H_2O_2 in both the cultivars. HS caused increase in the intracellular H_2O_2 accumulation in both the cultivars (Fig. 6b). The accumulation was, however, maximum in the HS alone, compared to combined treatment of Put and HS (HS + 2.5 mM Put). Exogenous application of PAs has been shown to reduce the H_2O_2 levels and raise the levels of antioxidants in Chickpea[57]. In HD2329, a decrease in H_2O_2 accumulation was observed under HS + 2.5 mM Put treatment (Fig. 6b). ANOVA analysis showed the results to be significant ($P < 0.01$). HS causes increase in ROS and intracellular H_2O_2, which are injurious to the cells and key enzymes above a certain limit.

In present study, we observed that Put regulated the accumulation of intracellular H_2O_2 by altering the activity of various antioxidant enzymes, such as

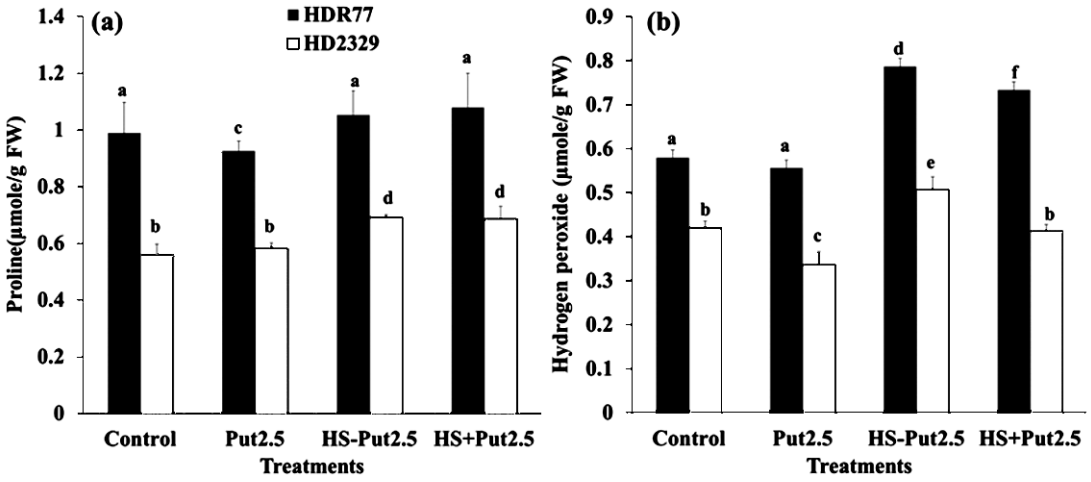

Fig. 6—Effect of Put and HS on accumulation of proline and H_2O_2 in HDR77 and HD2329 [(a) Proline accumulation, and (b) H_2O_2 concentration. Put - 2.5 mM ($Put_{2.5mM}$), HS - 42°C for 2 h (HS - $Put_{2.5mM}$) and combined effect of both (HS + $Put_{2.5mM}$). Data are presented as mean ± SE of three replicates, Different letters above each bar indicate a significant difference between treatments at $p<0.05$ based on ANOVA analysis]

SOD, CAT etc. which are involved in neutralizing the peroxide radicals. Concentration of intracellular H_2O_2 was less in HD2329 than in HDR77. The late response of HD2329 might be due to HS, since H_2O_2 act as signaling molecule. Put on catabolism produces signaling molecules like H_2O_2 which enhance the expression of stress-associated genes[58]. It has been observed that both intracellular Put and H_2O_2 accumulation increases under HS, thus enhancing the overall thermotolerance capacity of the plants against abiotic stresses[1]. Simultaneously, HS enhances the PAs catabolism which has direct effect on the accumulation of H_2O_2, predicted to be one of the mechanisms through which PAs work.

Lipid peroxidation and cell membrane stability

A decrease in MDA content was observed when cultivars were exposed to 2.5 mM Put alone (Fig. 7a), but HS caused increase in the MDA content in both the cultivars. The combined treatment of HS + 2.5 mM Put showed 42.7% (HDR77) and 24.5% (HD2329) decrease in the MDA content, compared to HS treatment. The result obtained in HDR77 was highly significant ($P < 0.01$), compared to HD2329 ($P < 0.05$) in response to different treatments. A decrease in MDA content is indicator of increase in the thermotolerance capacity of the plant. Decrease in MDA content in response to 2.5 mM Put and HS + 2.5 mM Put and increase in MDA in response to HS (Fig. 7a) were in agreement with previous report[57] which reported that exogenous application of PAs decreases the oxidative damage by reducing the MDA content in response to different stresses in pine and chickpea plants. Pre-anthesis Put treatment, followed by HS reduced the MDA content in the cells and increased the tolerance capacity under HS and was accordance with result observed in *A. thaliana*[59]. But, decrease in the MDA content has been reported in chickpea in response to PA treatment[57]. Pre-anthesis Put treatment showed marked effect on the CMS of both the cultivars (Fig. 7b).

Increase in CMS was 17% (HDR77) and 25% (HD2329) in response to HS + 2.5 mM Put, when compared to HS treatment alone; change in CMS of HDR77 was, however, more significant ($P < 0.01$). CMS is one of the most important parameters currently in vogue for screening germplasm of the wheat for thermotolerance capacity[55].

Putrescine is shown to resist the change in the lipid composition of the cell membrane under HS. Put and other PAs might be interacting with membranes, either by inhibiting trans-bilayer movement of phospholipids[1,60] or by stabilizing molecular complexes of thylakoid membranes[61]. In another study has also reported that PAs maintain membrane integrity during HS by decreasing membrane electrolyte leakage and lipid peroxidation[62]. PAs may serve as membrane surface stabilizers through interaction with phospholipids or other negatively-charged groups of membrane[63]. In addition, they may protect against stress *via* stabilizing protein structure and preventing the proteins from degradation by conjugation to proteins[1,14].

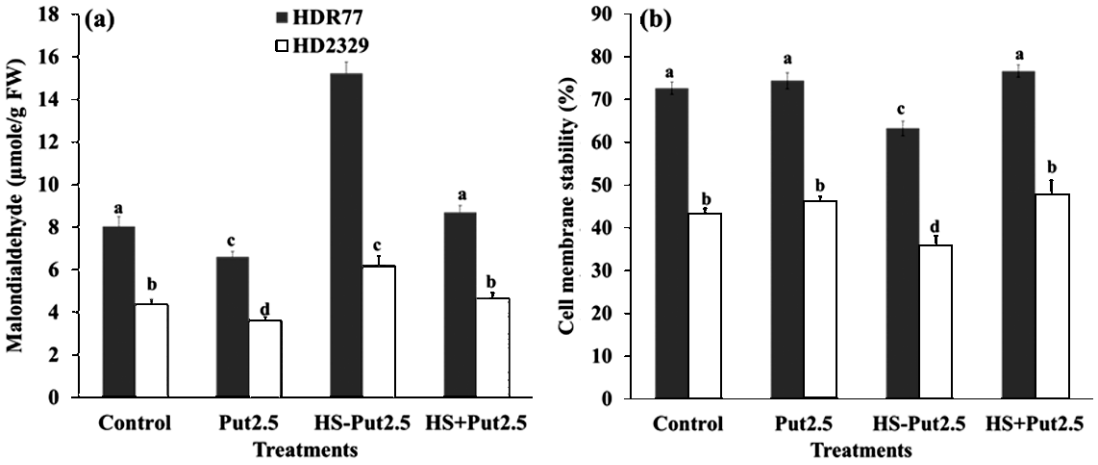

Fig. 7—Effect of Put and HS on lipid peroxidation and cell membrane stability of HDR77 and HD2329 [(a) MDA content, and (b) Cell membrane stability. Put - 2.5 mM ($Put_{2.5mM}$), HS - 42°C for 2 h (HS -$Put_{2.5mM}$) and combined effect of both (HS + $Put_{2.5mM}$). MDA, malondialdehyde, HS, heat stress. Data are presented as mean ± SE of three replicates. Different letters above each bar indicate a significant difference between treatments at $p<0.05$ based on ANOVA analysis]

Conclusions

Polyamines enhance the tolerance capacity of the plants under abiotic stresses. In present study, exogenous treatment of 2.5 mM Put at pre-anthesis before HS (42°C for 2 h) showed increase in the transcripts of *SOD*, *CAT*, *cAPX* and *pAPX*. The abundance of antioxidant enzymes transcripts was observed in thermotolerant (HDR77), compared to thermosusceptible (HD2329). Lipid peroxidation in response to HS + 2.5 mM Put was lower in HDR77 than in HD2329. The other parameters, such as activities of antioxidant enzymes (SOD, CAT, APX, GR) and CMS also increased significantly in response to HS + 2.5 mM Put in HDR77 than in HD2329. Thus, pre-anthesis treatment with 2.5 mM Put before HS enhanced the overall thermotolerance capacity of the wheat; however, field experiments are needed to validate these findings for practical use in protecting the wheat from heat stress.

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