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BRIEF COMMUNICATION

Putrescine modulates antioxidant defense response in wheat under high temperature stress

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Abstract

Effects of putrescine (Put) on responses of wheat (*Triticum aestivum*) seedlings or detached tillers at mid-milky stage to high temperature (HT) stress were investigated. The heat tolerant cv. PBW 343 exhibited higher content of antioxidants and activities of antioxidative enzymes, while lower content of lipid peroxides as compared to the heat-sensitive cv. HD 2329. HT elevated peroxidase (POX) and superoxide dismutase (SOD) activities, while diamine oxidase (DAO) and polyamine oxidase (PAO) activities were reduced in roots, shoots and developing grains. Application of Put under HT further enhanced POX and SOD activities along with increased content of ascorbate and tocophereol in grains. Invariably POX and SOD revealed higher activities in roots while CAT, DAO and PAO activities were higher in shoots. The content of lipid peroxides was increased in roots and shoots of HT stressed seedlings but less in Put-treated cv. PBW 343.

Additional key words: ascorbate, diamine oxidase, peroxidase, polyamine oxidase, superoxide dismutase, tocopherol, *Triticum aestivum.*

High temperature (HT) induces numerous biochemical responses including increased production of reactive oxygen species (ROS) that disrupt normal metabolism of plants causing lipid peroxidation, protein denaturation and DNA damage (Almeselmani *et al*. 2006). ROS content is controlled by an antioxidant system including low molecular antioxidants (ascorbate, tocopherol) and antioxidative enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POX; EC1.11.1.7) and catalase (CAT; EC 1.11.1.6) (Suzuki and Mittler 2006, Turhan *et al*. 2008). Number of studies have demonstrated that ROS scavenging mechanisms play an important role in protecting plants against HT stress (Larkindale and Knight 2002, Asada 2006, Suzuki and Mittler 2006, Ding *et al*. 2010, Hu *et al*. 2010). Similarly, changes in the activity of antioxidant enzymes in response to salinity were reported as different in tolerant and sensitive cultivars (Tsai *et al*. 2005).

Polyamines (PAs) regulate the enzyme activities of plants. These regulations may be attributed to the potential effect of putrescine (Put) which acts as radical scavenger (Todorov *et al*. 1998, Asthir *et al*. 2010a,b). Put treatment decreased POX activity of sorghum (Choudhary *et al*. 1993) but foliar application of Put on wheat plant increased CAT and SOD activities (Bekheta and El-Bassiouny 2005). Despite numerous studies on PAs acting as second messengers modulating various anatomical, biochemical and physiological processes under environmental stresses (Cavusoglu *et al*. 2008), there are no studies, to our knowledge, elucidating the effects of exogenous PAs treatments on antioxidant system in plants exposed to HT stress. The present study was planned to investigate the mechanism of Put action during heat stress in tolerant and susceptible wheat cultivars.

Seeds of two wheat cultivars (*Triticum aestivum* L.) namely PBW 343 (resistant) and HD 2329 (susceptible) were obtained from Department of Plant Breeding and Genetic, Punjab Agricultural University, Ludhiana, Punjab, India. Seeds were surface sterilized with 0.1 %

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Abbreviations: CAT - catalase; DAO - diamine oxidase; PAO - polyamineoxidase; POX - peroxidase; Put - putrescine; ROS - reactive oxygen species; SOD - superoxide dismutase; Spd - spermidine; Spm - spermine; TBARS - thiobarbituric acid reactive substances.
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$HgCl_2$ for 1 min, rinsed thoroughly with distilled water and germinated in dark for 24 h at 25 °C in Petri dishes (9 cm) on filter paper moistened with 5 cm^3 of distilled water. Heat shock treatment (45 °C for 2 h) was applied to germinated seeds (radicle at least 5 mm) followed by recovery growth at 25 °C for 5 d in dark. Seedlings grown at 25 °C without any treatment were taken as control. Twenty seedlings were used in each experiment and each experiment was repeated in triplicates.

Tillers at mid-milky stage, *i.e.* 12 - 15 days post anthesis (DPA) were cut under water below penultimate node and cultured according to the method of Asthir and Singh (1995), keeping three replications for each treatment. Ten such tillers for each treatment were used. The concentrations in the culture medium of sucrose and L-glutamine were 117 mM and 17 mM, respectively. Put (10 μM each) was added in the culture media. After adjusting the pH of the culture solution to 5.5, the medium was ultra-filtered through 0.22 μM *Millipore* membrane. Before culturing, the flag leaf and its sheath were removed and stems were surface sterilized with 40 % EtOH followed by quick washing with distilled water. Ear-heads carrying 20-cm peduncle were placed in culture tube (one ear-head per tube) containing 35 cm^3 cold-sterilized liquid medium. These cultured ear-heads were then transferred to water bath maintained at 2 - 4 °C as described by Asthir *et al*. (2009).

Seedlings or grains (1 g) were homogenized in 3 cm^3 of ice-cold 50 mM phosphate buffer pH 6.5 (for POX, SOD), pH 7.5 (CAT) and pH 7.0 (DAO, PAO). The homogenate was centrifuged at 10 000 *g* for 20 min at 4 °C and clear supernatant was used for assaying activities of POX, SOD and CAT. POX (EC 1.11.1.7) activity was measured using method of Claiborne and Fridoric (1979). The reaction mixture contained (3.5 cm^3) of 50 mM Na-phosphate buffer (pH 6.5), 0.1 cm^3 of *o*-dianisidine (1 mg cm^{-3} methanol), 0.1 cm^3 of 15 mM H_2O_2 and 0.1 cm^3 of enzyme extract. Change in absorbance of the reaction solution at 460 nm was determined for 2 min at an interval of 15 s. SOD activity was determined by measuring its ability to inhibit the autooxidation of pyrogallol by the method of Marklund and Marklund (1974). The reaction mixture contained 1 cm^3 0.6 mM pyrogallol, 1.5 cm^3 100 mM Tris HCl buffer (pH 8.2), 0.5 cm^3 6 mM EDTA and 0.1 cm^3 of enzyme extract. The rate of auto-oxidation of pyrogallol was taken from the increase in absorbance at 420 nm after an interval of 15 s up to 2 min. One unit of SOD activity was defined as the amount of enzyme that would inhibit 50 % of pyrogallol autooxidation. CAT activity was measured according to the method of Chakraborty and Tongden (2005) with modification. The reaction mixture (3 cm^3) contained 50 mM Na-phosphate buffer, pH 7.0, 15 mM H_2O_2 and 0.1 cm^3 enzyme extract. Change in absorbance of the reaction solution at 240 nm was read every 20 s in a UV-VIS spectrophotometer (*Eliso SL-159*, India). DAO and PAO were extracted in 100 mM K-phosphate buffer (pH 6.5) containing 5 mM dithiothreitol (DTT) and the extract was centrifuged at 16 000 *g* for 20 min at 4 °C. The supernatant was used for assay as per Asthir *et al*. (2002) by using Put (for DAO) and spermidine (Spd for PAO) as substrates. The reaction mixture of 2.0 cm^3 consisted of 0.1 cm^3 of enzyme extract, 50 units of catalase, 0.1 % 2-aminobenzaldehyde and the reaction started with one of the two different buffer and substrate combinations, *i.e.*, 10 mM Put in 50 mM K-phosphate buffer (pH 7.5) for DAO; 10 mM Spd in 50 mM K-phosphate buffer (pH 6.0) for PAO. The reaction mixture was incubated at 30 °C for 3 h, and then stopped with 2 cm^3 of 10 % (v/v) perchloric acid and the tubes centrifuged at 6 500 *g* for 15 min. Formation of the Δ-pyrroline product was determined by reading the absorbance at 430 nm. Control reactions were carried out with inactivated enzyme prepared by heating for 20 min in a boiling water bath. Total ascorbate content was measured according to the method of Law *et al.* (1983). After homogenization of 1 g of frozen grains in 10 cm^3 of 5 % *m*-phosphoric acid, the sample was centrifuged at 10 000 *g* for 30 min. Reaction mixture consisted of 200 cm^3 supernatant, 500 cm^3 150 mM KPO_4 buffer (pH 7.4) containing 5 mM EDTA and 100 cm^3 10 mM DTT to reduce oxidized-ascorbate to ascorbate. Excess DTT was removed by the addition of 100 cm^3 of 0.5 M N-ethylmaleimide. Colour was developed by the addition of 400 cm^3 10 % trichloroacetic acid, 400 cm^3 44 % *o*-phosphoric acid, 400 cm^3 of 4 % α,α-dipyridyl in 700 cm^3 ethanol and 200 cm^3 $FeCl_3$. After incubation at 40 °C for 40 min, the absorbance was read at 525 nm. Tocopherol extraction involved xylene that reduce ferric ions to ferrous ions to form red colour with α,α -dipyridyl which can be measured at 520 nm as described by Asthir *et al*. (2010a,b). 1 g of tissue was extracted in 5 cm^3 of ethanol and after centrifugation at 10 000 *g* for 15 min, 3 cm^3 of supernatant was taken and 1.5 cm^3 of xylene was added to all tubes, stoppered and vortexed for 25 min. After centrifugation, 1 cm^3 xylene layer was pipetted out, 1 cm^3 of 3 mM α,α-dipyridyl reagent was added and absorbance was read at 460 nm. Then 0.33 cm^3 of 4.43 mM $FeCl_3$ in absolute ethanol was added to the reaction mixture and absorbance was read at 520 nm. The lipid peroxidation was determined as content of thiobarbituric acid reactive substance (TBARS) as described by Larkindale and Knight (2002). Briefly, 0.5 g of grains were homogenized 3 cm^3 20 % (m/v) trichloroacetic acid and 0.5 % (v/v) thiobarbituric acid (2:1 ratio) and incubated at 95 °C for 30 min. The reaction was stopped by placing the reaction tubes in an ice bucket. The lipid peroxidation was determined using coefficient of absorbance of 155 mM^{-1} cm^{-1}.

The experiment was conducted in three replicates. Statistical analysis was carried out for all the measured parameters by three-factorial *ANOVA*.

The activities of antioxidant enzymes (POX, SOD, CAT, DAO and PAO) in roots, shoots and developing grains of two wheat genotypes under the influence of high temperature (HT) stress were assayed (Tables 1,2).

Table 1. Activities of POX [ΔA_{460} g^{-1}(f.m.) min^{-1}], SOD [U g^{-1}(f.m.)], CAT [ΔA_{240} g^{-1}(f.m.) min^{-1}], DAO and PAO [nmol(Δ-pyrroline) g^{-1}(f.m.) min^{-1}] and TBARS [μmol g^{-1}(f.m.)] content in roots and shoots of two wheat cultivars PBW 343 and HD 2329 under HT stress in the presence or absence of Put. Means ± SE (n = 3). Significant effects ($P \leq 0.01$) of heat stress ([a]), putrescine ([b]) and cultivar ([c]).

Parameter	Temperature [°C]	Roots				Shoots			
		PBW 343		HD 2329		PBW 343		HD 2329	
		-Put	+Put	-Put	+Put	-Put	+Put	-Put	+Put
POX	25	146 ±5	170 ±6[b]	124 ±5[c]	161 ±7[bc]	115 ±5	140 ±7[b]	95 ±4[c]	130 ±7[bc]
	45	238 ±7[a]	260 ±8[ab]	208 ±7[ac]	241 ±8[abc]	215 ±9[a]	240 ±7[ab]	188 ±6[ac]	221 ±8[abc]
SOD	25	105 ±5	135 ±3[b]	71 ±4[c]	115 ±5[bc]	95 ±3	123 ±4[b]	66 ±2[c]	103 ±4[bc]
	45	209 ±7[a]	231 ±6[ab]	165 ±6[ac]	204 ±7[abc]	190 ±5[a]	215 ±7[ab]	162 ±5[ac]	201 ±6[abc]
CAT	25	8.3±0.4	19.0±1.3[b]	6.4±0.85[c]	25.4±2.0[bc]	12.3±1.0	26.0±2.4	9.2±0.63[c]	36.0±3.0[c]
	45	14.2±0.5[a]	20.0±0.8[ab]	14.2±0.5[ac]	35.3±2.0[abc]	15.0±0.6[a]	23.0±1.6[a]	11.0±0.7[ac]	32.0±3.4[ac]
DAO	25	72.3±4.1	94.1±5.2[b]	64.3±4.2[c]	87.5±8.5[bc]	81.2±4.3	105.2±5.4[b]	72.3±4.4[c]	93.2±5.1[bc]
	45	61.5±3.8[a]	83.4±4.0[ab]	51.4±3.8[ac]	70.1±7.2[abc]	73.3±4.5[a]	95.4±5.3[ab]	59.6±3.4[ac]	82.3±5.5[abc]
PAO	25	58.1±4.2	80.4±5.1	49.1±0.2[c]	70.5±4.7[bc]	63.4±3.7	86.3±4.2[b]	57.3±2.6[c]	79.4±3.8[bc]
	45	47.3±5.2[a]	70.0±5.8[ab]	39.3±2[ac]	42.4±1.4[abc]	57.2±3.8[a]	80.2±4.2[ab]	48.1±2.7[ac]	70.5±4.3[abc]
TBARS	25	27.3±2.5	21.5±2.2[b]	46.1±2.7[c]	32.3±2.1[bc]	18.2±1.7	15.4±1.1[b]	27.3±2.0[c]	22.5±1.8[bc]
	45	32.4±2.0[a]	30.0±2.1[ab]	50.5±2.1[ac]	43.2±2.7[abc]	20.1±1.1[a]	17.5±1.3[ab]	30.1±2.5[a]	24.4±2.3[abc]

Table 2. Activities of POX [ΔA_{460} g^{-1}(f.m.) min^{-1}], SOD [U g^{-1}(f.m.) min^{-1}], DAO and PAO as [nmol(Δ-pyrroline) g^{-1}(f.m.) min^{-1}] and content of total ascorbate and α-tocopherol [μmol g^{-1}(f.m.)] in grains of wheat cultivars PBW 343 and HD 2329 under HT stress in the presence or absence of Put. Means ± SE (n = 3). Significant effects ($P \leq 0.01$) of heat stress ([a]), putrescine ([b]) and cultivar ([c]).

Parameter	Temperature [°C]	PBW 343		HD 2329	
		-Put	+Put	-Put	+Put
POX	25	390 ±7	404 ±9[b]	346 ±7[c]	399 ±8[bc]
	45	409 ±9[a]	471 ±11[ab]	366 ±6[ac]	407 ±10[abc]
SOD	25	253 ±11	353 ±14[b]	201 ±13[c]	347 ±14[bc]
	45	271 ±8	304 ±9[b]	300 ±9[c]	340 ±11[bc]
DAO	25	76.3 ±4.8	89.1 ±5.5	61.4 ±3.5[c]	72.3 ±4.2[c]
	45	69.5 ±2.4[a]	98.2 ±5.8[a]	58.1 ±2.4[ac]	85.5 ±4.8[ac]
PAO	25	62.1 ±2.4	80.0 ±4.2[b]	56.5 ±2.5[c]	71.3 ±4.8[bc]
	45	54.5 ±2.8[a]	76.3 ±5.5[ab]	47.5 ±2.5[ac]	69.7 ±4.8[abc]
Ascorbate	25	46.4 ±2.3	65.2 ±3.5[b]	38.5 ±2.8[c]	52.6 ±3.7[bc]
	45	72.7 ±5.2[a]	90.1 ±2.5[ab]	65.4 ±3.4[ac]	82.1 ±5.2[abc]
α-Tocopherol	25	0.22±0.02	0.30±0.05[b]	0.15±0.03[c]	0.27±0.04[bc]
	45	0.30±0.03	0.41±0.08[b]	0.29±0.04[c]	0.38±0.07[bc]

POX, SOD and CAT activities increased significantly in both root and shoot with HT over control values whereas DAO and PAO activities decreased. Invariably, the level of POX and SOD were higher in roots while CAT, DAO and PAO predominated in shoots of both cultivars. It may thus be inferred, that a major quantity of ROS are scavenged by POX route in roots, whereas in shoots the CAT pathway operates. Higher POX, SOD, CAT, DAO and PAO activities in PBW 343 over HD 2329 under normal and HT demonstrates its superior tolerance mechanism in terms of H_2O_2 production and utilization. Furthermore, application of 10 μM Put was effective for increasing the activities of all studied enzymes in the two cultivars under normal and HT conditions indicating that Put alleviated the adverse effects of heat stress and might be involved in induction of thermotolerance. Similar increase in antioxidant enzymes were reported in callus cultures of pine (Tang *et al*. 2005) and in seedlings of *Brassica juncea* (Verma and Mishra 2005) treated with NaCl. DAO and PAO activities decreased with HT which could partly be a consequence of limiting concentration of substrate (Put or Spd). This decrease was ameliorated by addition of Put (Tables 1,2). The beneficial impact of Put was either direct or due to its conversion to Spd or spermine (Spm) as mentioned earlier by Capell *et al*. (2004).

Interestingly, CAT activity increased tremendously with Put in both roots and shoots of susceptible genotype

(HD 2329). Though, heat stress susceptible genotypes usually possess lower content and activities of antioxidant enzymes (Larkindale *et al*. 2005), increase in CAT activity in HD-2329 did not specify the overall scavenging ability efficient enough to combat HT stress as TBARS content was high in this cultivar. In fact, TBARS content was higher in roots than shoots of heat stressed seedlings which led us to suggest that shoots are better protected from oxidative stress under HT than roots as has been reported in other species (Gong *et al*. 1997). Since Put led to a decrease in TBARS content, it is probable that it ameliorates HT stress in wheat seedlings by a mechanism involving membrane repair. Protection of membranes from lipid peroxidation could involve direct binding to membrane phospholipids and thus stabilizing cell walls under stress conditions (Todorova *et al*. 2007).

To study a near *in vivo* effect of Put, the detached tillers were cultured in complete liquid medium for 5 d to estimate the effect of Put on antioxidative enzymes (POX, SOD, CAT, DAO, PAO) and antioxidants (ascorbate, α-tocopherol) in PBW 343 and HD 2329 cultivars. HT increased POX and SOD activities in grains similarly as in roots and shoots while DAO and PAO activities decreased together with increased content of ascorbate and α-tocopherol (Table 2). The contents of both the antioxidants were higher in grains of tolerant cultivar compared with the susceptible one. Seeds in particular contain large amount of α-tocopherol, which acts as very effective antioxidant for plant membranes (Franzen and Haab 1991), effectively reduces lipid peroxidation even at low concentration (Munne-Bosch 2005) and has the ability to quench free radicals. Application of 10 μM Put further enhanced the activities of POX, SOD, DAO and PAO and content of α-tocopherol and ascorbate in both cultivars (Table 2). Hence, Put has been implicated in direct scavenging of free radicals, thereby directly reducing oxidative damage, or may act indirectly by elevating content of antioxidants. Thus exogenous Put application is a convenient and effective approach for enhancing stress tolerance of wheat.

Overall, it appears that up-regulation of the antioxidant system by Put in wheat cultivars contributes to better protection of membrane integrity in root and shoot against HT through reduced TBARS content. Put also increases ascorbate and tocopherol content in developing grains. Furthermore, shoots are better protected against the destructive effects of ROS than roots.

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