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SHORT COMMUNICATION

Effect of salicylic and abscisic acid administered through detached tillers on antioxidant system in developing wheat grains under heat stress

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Abstract The mechanism imparting thermotolerance by salicylic acid (SA) and abscisic acid (ABA) is still unresolved using either spraying technique or in vitro conditions. Alternative way of studying these effects under near in vivo conditions is through the use of liquid culturing technique. Effects of SA and ABA (100 μM) on antioxidative enzymes, antioxidants and lipid peroxidation were studied in detached tillers of three wheat (*Triticum aestivum* L.) cultivars PBW 343, C 306 (heat tolerant) and WH 542 (heat susceptible) cultured in a liquid medium. Ears were subjected to heat shock treatment (45°C for 2 h) and then maintained at 25°C for 5 days. Heat shock treatment resulted in increased peroxidase (POD) activity, while superoxide dismutase (SOD) and catalase (CAT) activities were reduced compared to control. The decrease in CAT activity was more significant in susceptible cultivar WH 542. Concomitantly, content of α-tocopherol and lipid peroxides increased in heat-treated wheat ears, whereas contents of total ascorbate level were reduced. Following treatment with SA and ABA, activities of all three antioxidative enzymes increased in correspondence with an increase in ascorbate and α-tocopherol content. Apparently, lipid peroxide content was reduced by SA in heat tolerant cultivars (PBW 343 and C 306) whereas in susceptible cultivar it was decreased by ABA. The up-regulation of the antioxidant system by SA and ABA possibly contributes to better tolerance against heat shock-induced oxidative damage in wheat grains.

Keywords Abscisic acid · Antioxidants · Antioxidant enzymes · Heat shock · Salicylic acid · *Triticum aestivum*

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Introduction

With the predicted climatic changes and global warming, terminal heat stress for the wheat crop is likely to increase in the near future (Mitra and Bhatia 2008). Wheat being a temperate cereal is particularly temperature sensitive. The crop yield decreases by 4% for every 1°C rise in temperature above the ambient temperature, i.e. 25°C (Wardlaw and Wrigley 1994). The adverse effects of heat stress can be mitigated by developing crop plants with improved thermotolerance (Wahid et al. 2007).

High temperature leads to an increased production of reactive oxygen species (ROS) that disrupts 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 weight antioxidants (ascorbate, tocopherol, glutathione, etc.) and antioxidative enzymes such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), a H_2O_2 generating and degrading enzymes, respectively (Suzuki and Mittler 2006; Turhan et al. 2008). Thermotolerant plants should possess better antioxidative system for effective removal of ROS. Changes in the activity of antioxidant enzymes in response to salinity were reported as different in tolerant and sensitive cultivars (Tsai et al. 2005).

Salicylic acid (SA) and abscisic acid (ABA) are particularly involved in pathways switched on in response to oxidative stress (Agarwal et al. 2005; Chakraborty and Tongden 2005). In fact, SA is considered as strong candidate for stress amelioration (Szepesi et al. 2008) and has recently been recognized as a plant hormone (Hayat and Ahmad 2007). SA has the ability to enhance the heat shock response by potentiating the heat-induced levels of HSP70 due to modulation of heat shock factors (Snyman and Cronje 2008). Likewise, ABA has also been reported to induce tolerance to different abiotic stresses (Larkindale and Knight 2002). Chandrasekar et al. (2000) have reported a higher accumulation of ABA in drought tolerant wheat cultivar C 306 than susceptible Hira in response to water stress.

Information regarding phytohormone application on antioxidant response in a near in vivo condition remains poorly understood. Alternative way of studying these effects is through the use of liquid culturing technique where detached ears of wheat were cultured in liquid medium for studying differential response of SA and ABA on antioxidative enzymes and antioxidants in heat-treated and non-treated wheat ears. This technique has been successfully employed in our laboratory for cereals (Asthir and Singh 1995). The study was facilitated using three wheat cultivars PBW 343, C 306 (heat tolerant) and WH 542 (heat susceptible).

Materials and methods

Three wheat (*Triticum aestivum* L.) cultivars namely, PBW 343, C 306 and WH 542 were raised in fields of Punjab Agricultural University, Ludhiana (Punjab), India, under recommended agronomic practices. Ears of uniformly developing plants were tagged at anthesis. All the chemicals used were of analytical grade.

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 and 17 mM, respectively. SA and ABA (100 μM each) were added in the culture media. Culture solution devoid of hormones was kept as control. 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 length from the cut end were placed (one ear-head per tube) in culture tube containing 35 ml cold-sterilized liquid medium. These cultured ear-heads were then transferred to water bath maintained at 2–4°C. In the growth chamber, a photosynthetic active radiation (400 μmol m^{-2} s^{-1}) measured with Licor 188B quantum meter (Li-cor Inc., Lincoln, NE, USA) was provided with cool white fluorescent tubes (TL 40W/54, Philips India) and incandescent lamps (60W, Sylvania, India) for a day length of 14 h. Ear-heads were treated at 45°C for 2 h daily for 5 days and non-treated ears were kept as control. After required culturing for 5 days, the grains were separated and used for analysis.

For extraction of enzymes (0.5 g), grains were weighed and ground with a pestle in an ice-cold mortar with 0.1 M Na_2HPO_4/NaH_2PO_4 buffer (pH 6.5) containing 2 mM β-mercaptoethanol. The homogenate was centrifuged at 10,000*g* for 20 min at 4°C and clear supernatant was used for assaying activities of POD, SOD and CAT.

POD (EC, 1.11.1.7) activity was measured using method of Claiborne and Fridovich (1979). The POD reaction mixture contained (3.5 ml) of 50 mM Na–phosphate buffer (pH 6.5), 0.1 ml of *o*-dianisidine (1 mg/1 ml methanol), 0.1 ml of 15 mM H_2O_2 and 0.1 ml of enzyme extract. Change in absorbance of the reaction solution at 460 nm was determined for 2 min at an interval of 15 s. Enzyme activity was expressed as change in absorbance (ΔA 460 min^{-1} g^{-1} FW).

SOD (EC, 1.15.1.1) activity was determined by measuring its ability to inhibit the auto-oxidation of pyrogallol by the method of Marklund and Marklund (1974). The reaction mixture contained 1 ml 0.6 mM pyrogallol, 1.5 ml 100 mM Tris–HCl buffer (pH 8.2), 0.5 ml 6 mM EDTA and 0.1 ml of enzyme extract. The rate of auto-oxidation of pyrogallol was taken from the increase in absorbance at 420 nm in a spectrophotometer 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 auto-oxidation.

CAT (EC, 1.11.1.6) activity was measured according to the method described by Chakraborty and Tongden (2005) with modification. The CAT reaction mixture (3 ml) contained 50 mM Na–phosphate buffer pH 7.0, 15 mM H_2O_2 and 0.1 ml enzyme extract. Change in absorbance of the reaction solution at 240 nm was read after every 20 s in a UV–vis spectrophotometer. Enzyme activity was expressed as ΔA 240 min^{-1} g^{-1} FW.

Total ascorbate content was measured according to the method of Law et al. (1983). After homogenization of 1 g of frozen grains in 10 ml of 5% *m*-phosphoric acid, the sample was centrifuged at 10,000*g* for 30 min. Ascorbate content was determined in a reaction mixture consisting of 200 μl supernatant, 500 μl 150 mM KPO_4 buffer (pH 7.4) containing 5 mM EDTA and 100 μl 10 mM dithiothreitol (DTT) to reduce oxidized-ascorbate to ascorbate. Excess DTT was removed by the addition of 100 μl of 0.5 M *N*-ethylmaleimide in water after 10 min at room

temperature. Colour was developed in reaction mixture by the addition of 400 µl 10% TCA, 400 µl 44% *o*-phosphoric acid, 400 µl 4% α,α-dipyridyl in 700 ml l^{-1} ethanol and 200 µl 30 g l^{-1} $FeCl_3$. After incubation at 40°C for 40 min, the absorbance was read at 525 nm.

Tocopherol extraction involved xylene that reduces ferric ions to ferrous ions to form red colour with α,α-dipyridyl which can be measured at 520 nm (Hira et al. 2001). 1 g of tissue was extracted in 5 ml of ethanol and after centrifugation at 10,000*g* for 15 min, 3 ml of supernatant was taken and 1.5 ml of xylene was added to all tubes, stoppered and vortexed for 25 min. After centrifugation, 1 ml of xylene layer was pipetted out, 1 ml of 3 mM α,α-dipyridyl reagent was added in all tubes and absorbance was read at 460 nm. Then to the reaction mixture, 0.33 ml of 4.43 mM $FeCl_3$ in absolute ethanol was added and mixed and absorbance was read at 520 nm after 2 min of addition of $FeCl_3$.

The lipid peroxidation products were determined from the contents of thiobarbituric acid reactive substances (TBARS) resulting from the thiobarbituric acid (TBA) reaction as described by Larkindale and Knight (2002). Briefly, 0.5 g of grains was homogenized 3 ml 20% (w/v) trichloroacetic acid (TCA) and 0.5% (v/v) TBA (2:1) and incubated at 95°C for 30 min. The reaction was stopped by placing the reaction tubes in an ice bucket. The lipid peroxidation contents (as MDA) were determined by its extinction coefficient of 155 mM^{-1} cm^{-1}.

The experiment was conducted in three replicates. Statistical analysis was carried out for all the measured parameters by one-way analysis of variance (ANOVA) using SAS software. The differences were considered significant at $P \leq 0.05$.

Results and discussion

Culturing of detached tillers for 5 days in the presence of SA and ABA showed variable response on antioxidant system in grains of heat tolerant (PBW 343, C 306) and susceptible (WH 542) cultivars of wheat. Heat shock treatment increased POD activity, while SOD and CAT activities reduced in comparison with control (Fig. 1). Higher POD activity in PBW 343 and C 306 under elevated temperature demonstrates its superior tolerance mechanism in terms of H_2O_2 scavenging over WH 542. Significance of POD activity in temperature stress tolerance has also been reported by other workers (Chakraborty and Tongden 2005; Almeselmani et al. 2006). Though it is generally known that heat stress tolerant genotypes showed higher level and activity of antioxidant enzymes (Larkindale et al. 2005), decreases in SOD and CAT activities indicated that the scavenging ability in the cells of grains was lowered under high temperature conditions. The response of CAT activity was more significant in susceptible cultivar WH 542. CAT decreased by 29% in the WH 542 and by 22 and 5% in PBW 343 and C 306, respectively. A similar decrease in CAT activity was also observed by Sairam and Srivastava (2001) in grains of C 306 when cultured under water stress. A reduction in CAT activity also occurred during short-time heat shock treatment in mustard seedlings (Dat et al. 1998). Low SOD and CAT activities could be attributed to photoinactivation (Polle 1997) or inhibition of their de novo synthesis in the dark (Dat et al. 1998), which may favour the accumulation of active oxygen species and cause damage to cell membranes (Dhindsa et al. 1981). It may thus be inferred that genotypes tolerant to a particular stress does not necessarily increase the activities of all antioxidant enzymes (Sairam et al. 2005). In some cultivars, a major quantity of ROS are scavenged by CAT route while in others POD pathway operates as observed in the present study.

Addition of 100 µM to each of SA and ABA recorded a significant increase in POD, SOD and CAT activities in grains of all three cultivars (Fig. 1) over heat-treated ears indicating that phytohormones alleviated the adverse effects of heat stress and are involved in the induction of thermotolerance. Interestingly, for SOD, the response with SA or ABA in both relative and absolute term was greatest in the susceptible cultivar WH 542. However, higher activity of SOD alone in WH 542 is not sufficient for providing heat tolerance, as the enzymes for H_2O_2 scavenging (POD and CAT) are less active in this cultivar. A similar situation seems to prevail under water stress in wheat where variation in SA and ABA responsiveness in tolerant and susceptible genotypes was observed (Agarwal et al. 2005; Almeselmani et al. 2006).

Total ascorbate level decreased in heat-treated wheat ears compared with control (without SA, ABA), while α-tocopherol and lipid peroxidation levels increased in all three cultivars (Fig. 2). Therefore, total ascorbate and α-tocopherol contents respond differently to experimental treatments. It is generally assumed that increase in tocopherol content contributes to plant stress tolerance while its decrease is associated with stress susceptibility (Gossett et al. 1994). The decrease in ascorbic acid content could be attributed to the decrease in ascorbic acid oxidase activity (Abdel-Kader 2001). Similar decrease in ascorbic acid content induced by severe water stress was reported by Bartoli et al. (1999). Addition of SA increased the levels of total ascorbate and α-tocopherol over heat-treated ears in all the cultivars, whereas lipid peroxidation level increased only with ABA in tolerant cultivars (PBW 343, C 306). However, administration of phytohormones (SA and ABA) were effective in slightly negating the negative effects of heat stress in wheat grains by reducing lipid peroxide content compared to heat-treated ears.

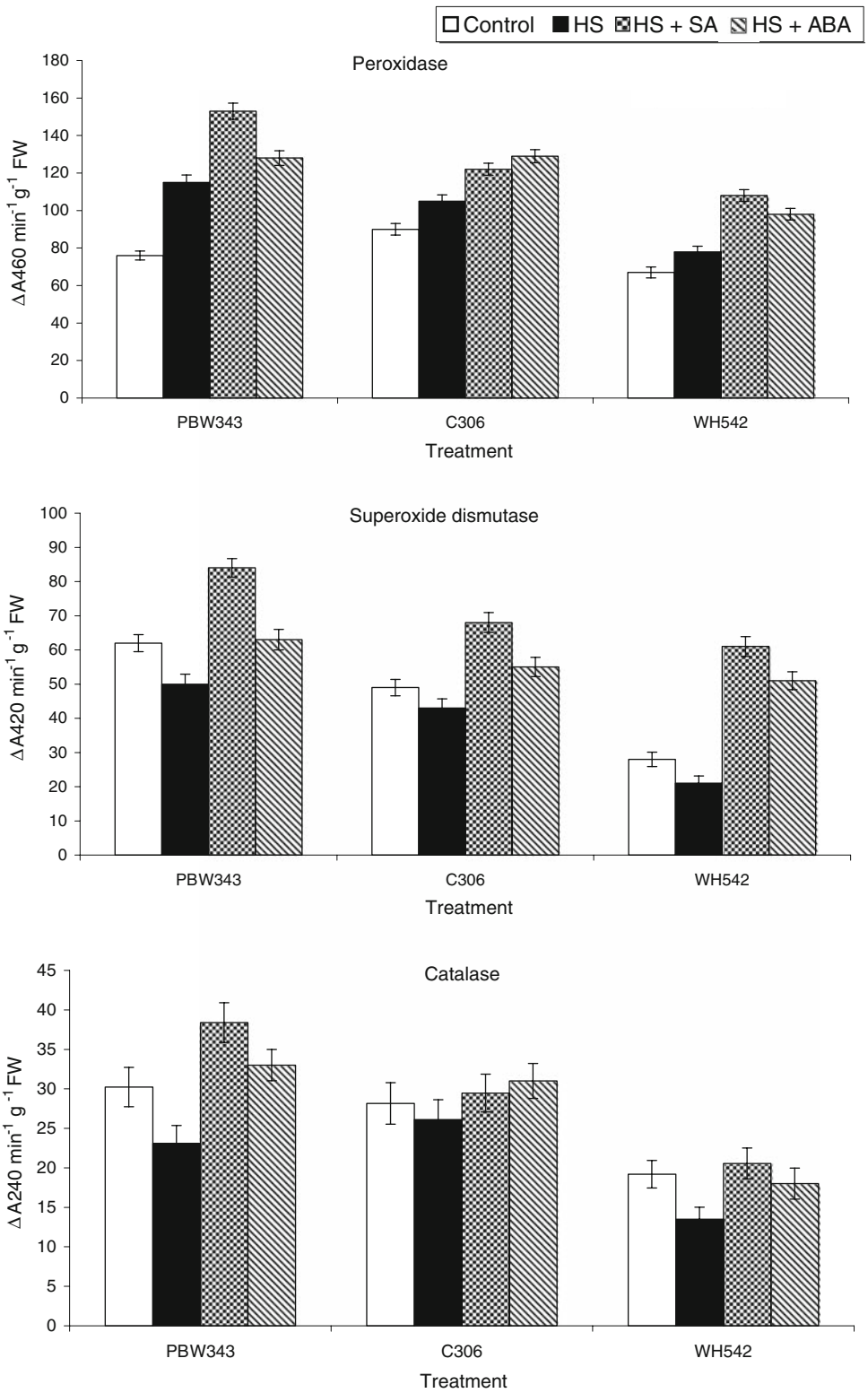

Fig. 1 Effects of salicylic acid (SA) and abscisic acid (ABA) on peroxidase, superoxide dismutase and catalase activities in heat-treated tillers of three wheat cultivars PBW 343, C 306 and WH 542. Activities after 5 days of culturing without heat treatment and phytohormones referred to as control, whereas heat treatment without phytohormone is referred as HS. Values are represented as mean ± SE ($n = 3$). All values are significant at $P < 0.05$

The accumulation of MDA, which is a product of lipid peroxidation, is often used as an indicator of lipid peroxidation. Heat stress increased MDA content in all the cultivars (Fig. 2), similar to what has been found in other species (Gong et al. 1997). The increase in MDA content was related to reductions in SOD and CAT activities. Thus, membrane lipid peroxidation occurred from the malfunction of the scavenging system, which could lead to damage to main cellular components. Lipid peroxidation was greatest in heat susceptible cultivar even under control

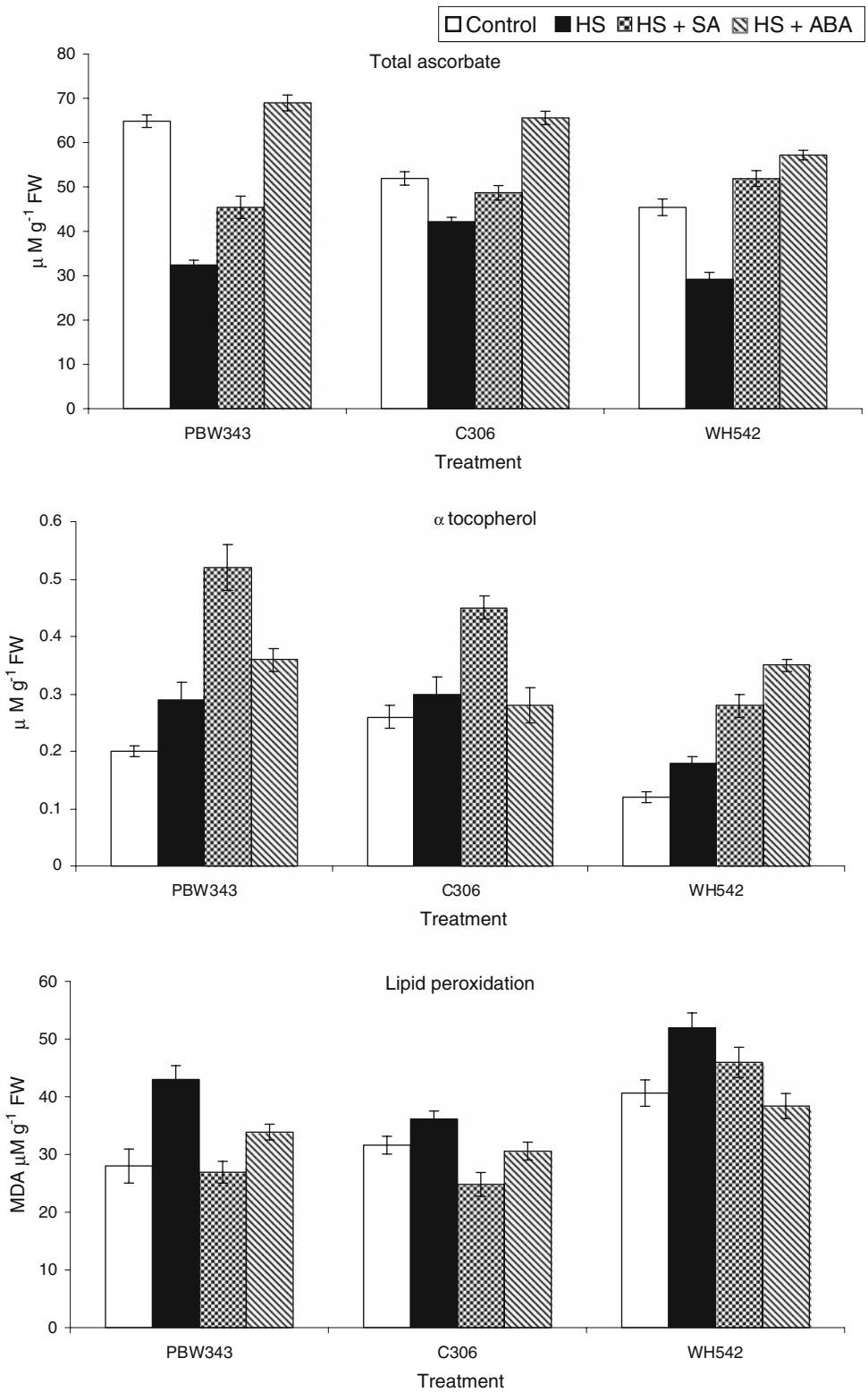

Fig. 2 Effects of salicylic acid (SA) and abscisic acid (ABA) on total ascorbate, α-tocopherol and lipid peroxidation contents in heat-treated tillers of three wheat cultivars PBW 343, C 306 and WH 542. Contents of antioxidants and MDA after 5 days of culturing without heat treatment and phytohormones referred to as control, whereas heat treatment without phytohormone is referred as HS. Values are represented as mean ± SE ($n = 3$). All values are significant at $P < 0.05$

conditions, and relative increase in peroxidation due to heat was actually greatest in one of the tolerant cultivar (PBW 343). It seems that heat tolerance had little to do with response of lipids to heat. However, SA application was effective in reducing MDA content in heat tolerant cultivars (PBW 343 and C 306), whereas in susceptible cultivar (WH 542) ABA treatment was found to be more effective. Less response of ABA in tolerant cultivars in reducing MDA content may be due to higher endogenous ABA concentration in C 306 as reported by Chandrasekar

et al. (2000). Reports suggest that endogenous level of ABA increased when plants are subjected to drought stress (Cao et al. 2000) and higher ABA concentration can cause increase in lipid peroxidation in plant cells (Bueno et al. 1998).

Overall, it appears that up-regulation of the antioxidant system by SA and ABA possibly contributes to better tolerance against heat shock in wheat genotypes, though all the enzymes involved in amelioration of oxidative stress may not increase in activity in a given tolerant genotype. The high POD activity, tocopherol and low lipid peroxide contents could be directly linked with enhanced tolerance to heat-induced oxidative damage.

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