Acta Biologica Hungarica 68(2), pp. 162–174 (2017)
DOI: 10.1556/018.68.2017.2.4

HYDROGEN PEROXIDE AND NITRIC OXIDE REGULATION OF PHENOLIC METABOLISM UNDER WATER STRESS AND ABA IN WHEAT

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(Received: January 11, 2017; accepted: February 7, 2017)

Wheat cultivar PBW644 (drought tolerant) and PBW343 (drought sensitive) were found as ABA-higher sensitive and ABA-lesser sensitive, respectively, in the screen of six wheat cultivars. Both cultivars were studied for H_2O_2 (ROS)/nitric oxide (NO)-regulation of growth and phenolic metabolism under ABA and water stress (WS) by supplying ROS/NO producers as well as scavengers. Endogenous ROS/NO under ABA/WS increased growth, such effect was higher in PBW644. In PBW343, reduced growth under WS was improved by exogenous ROS/NO. Exogenous ROS/NO under ABA/WS decreased lignin and increased phenolics in PBW343 but such relation was not found in PBW644. Endogenous NO under WS increased flavonoids in both cultivars. Both ROS/NO under ABA/WS increased flavonoids in PBW644, however, in PBW343, only ROS increased these in roots. Under WS, PBW644 showed higher levels of cell wall peroxidase (CW-POX) and lower levels of soluble peroxidase (S-POX) than PBW343. However, under ABA, it showed higher levels of both peroxidases. ROS/NO signals under ABA increased both types of POX in both cultivars while under WS, these signals increased both types in PBW343 but CW-POX only in PBW644. Polyphenol oxidases were ABA-upregulated in PBW644 only. Under WS, these enzymes were maintained higher in PBW343. This study indicated that tolerant cultivar under WS contained sufficient endogenous ROS/NO signalling to which susceptible cultivar lacked but showed improvement on exogenous applications. Secondly, tolerant cultivar was using less phenolic activity under WS which could be due to the presence of sufficient levels of primary antioxidants.

Keywords: Abscisic acid – hydrogen peroxide – nitric oxide – phenolic – wheat

INTRODUCTION

Phenolic compounds play important role in stress tolerance as they provide mechanical strength due to cell-wall hardening due to lignification and other cross-linking reactions and act as antioxidants used directly or through peroxidases/polyphenol oxidases to detoxify ROS [12]. Parameters related to phenolic metabolism have been found altered under different stresses and suggested for hormonal regulation [7, 8] but not well understood.

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In our previous study [8], phenolic metabolism was found to be differentially regulated in two wheat cultivars (C306 and PBW343) under ABA and stresses. Supplying H_2O_2 or nitric oxide (NO) exogenously under different abiotic stresses improved the performance of plant in different crops [reviewed in 4]. ABA-, H_2O_2- and NO-signalling networks and their cross-talks are involved for tolerance under different abiotic stresses but so far not well understood. This study was undertaken to reveal such interactions in wheat through supplying ROS/NO donors and scavengers under ABA supply and under water stress.

MATERIAL AND METHODS

Screening of wheat cultivars for ABA sensitivity

Six wheat (*Triticum aestivum*) cultivars (PBW527, PBW644, PBW660, PBW175, PBW343, C273) were used for this experiment. These cultivars are the released winter wheat varieties where PBW527, PBW644, PBW660, PBW175 (recommended for rainfed conditions) and PBW343 (recommended for irrigated conditions) released by Punjab Agricultural University from time to time. PBW644, PBW527 shared one common parent of PBW175 which was derived from C273. PBW175 and C273 were the old wheat varieties recommended to grow under low fertility and rainfed conditions. Seeds of these cultivars were germinated in Petri-dishes (100 seeds per plate of 100 mm) on filter paper moistened with autoclaved distilled water (control) and 20 µM ABA solution (ABA treatment) in the dark at 25 °C for 5 days. Germination index (*G.I.*) was calculated using the equation

$$G.I. = \sum_{i=1}^{k} n_i / t_i$$

where n_i is % germination on i^{th} day and t_i is the number of days from start of experiment and k is the last day of experiment [11]. Growth was measured as shoot and root length on 5^{th} day with scale of 50 seedlings selected randomly.

Measurement of growth and phenolic metabolism

These experiments were performed on wheat cultivars PBW644 and PBW343. Seeds were grown over autoclaved sand moistened with autoclaved distilled water for 5.5 days then exposed to treatments, water (CT), 20 µM ABA, 10% PEG 6000, 10 mM H_2O_2, 50 µM SNP, 10 mM DMTU, 50 µM PTIO in specified combinations. DMTU is N,N'-dimethylthiourea (specific scavenger of H_2O_2), PTIO is 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (specific scavenger for nitric oxide) and SNP

is sodium nitroprusside (a donor of nitric oxide). Concentrations of chemicals used in the study were decided from literature as well as by doing preliminary experiments in our laboratory on these two cultivars. Roots and shoots were taken at 36 h after treatment.

Growth was measured as shoot and root length of 50 seedlings selected randomly. Ionic bound fraction of cell wall from tissue was isolated [8] by making extraction in 50 mM potassium phosphate buffer (pH 7.0), centrifuged at 1,000 × *g* for 15 min to get pellet and supernatant. Supernatant was re-centrifuged at high speed for 20 min to get extract as soluble fraction. Pellet was washed four times using the same buffer, dissolved in 1 M NaCl, incubated at 4 °C for 30 min, centrifuged at 1,000 × *g* for 15 min to get supernatant as cell wall fraction. Peroxidase (POX) from both fractions was assayed in 100 mM potassium phosphate buffer (pH 6.5), 50 mM guaiacol and 30 mM H_2O_2 [10] at 470 nm for the formation of tetraguaiacol for 3 min at the interval of 30 sec and calculated using $\epsilon_{tetraguaiacol}$ of 26.6 mM cm^{-1}. Polyphenol oxidase (PPO) from both fractions was assayed [8, 12] in 50 mM potassium phosphate buffer (pH 7.0) with 13.2 mM catechol at 420 nm for appearance of purpurogallin and calculated in PPO unit defined as the change in absorbance of 1 unit.

For lignin [8, 13], tissue was homogenized in 95% ethanol, centrifuged at 10,000 × *g* for 20 min. Pellet was washed three times with 95% ethanol and twice with ethanol: hexane (1:2 ratio) and then dried at 47 °C overnight. Dried pellet was washed with and then dissolved in 25% acetyl bromide in acetic acid and incubated at 70 °C for 30 min, then cooled at room temperature. Then sodium hydroxide, hydroamine-HCl and acetic acid were added to final concentration of 0.19 M, 0.08 M and 13 M, respectively. The mixture was centrifuged for 5 min and read at 280 nm against reagent blank. Lignin amount was determined using standard curve of lignin (5–50 µg). Soluble phenolics and flavonoids were extracted [8, 12] with ice cold 80% methanol. For phenolics estimation, 0.5 ml of appropriately diluted supernatant was reacted to 1 ml of 2% sodium carbonate and 0.1 ml of 0.5 M Folin-Ciocalteu reagent at 45 °C for 1 h. Absorbance was taken at 750 nm against reagent blank. Standard curve of gallic acid (2–15 µg) was used. For flavonoids estimation, 1 ml appropriately diluted supernatant was reacted to 2 ml of 2% aluminium chloride in methanol for 1 h at room temperature. Absorbance was taken at 420 nm against reagent blank. Standard curve of rutin (25–100 µg) was used.

Statistical analysis

Experiments were performed in three biological replicates. Mean ± SD was calculated. Data was analyzed by Duncan's multiple test at $P \leq 0.05$ for statistical differences using DSAASTAT software version 1.101.

RESULTS

Screening of wheat cultivars for ABA sensitivity

Drought tolerance of PBW644 and drought susceptibility of PBW343 was known. Germination and growth inhibition by ABA was taken as measure of ABA-sensitivity in screening experiment (Table 1). PBW644 and PBW660 showed maximum inhibition for germination and shoot growth while PBW343 showed a minimum. This experiment indicated PBW644 and PBW660 as ABA-higher sensitive while PBW343 as ABA lesser sensitive.

Table 1
Screening of wheat cultivars for ABA sensitivity during seed germination and seedling growth where seeds were imbibed in water (CT) and ABA solution for 5 days

Cultivar	% of CT	
	Germination index (GI)	Shoot length
PBW644	46.8±4.5	9±3
PBW660	46.0±7.5	10±3
C273	52.9±3.6	20±10
PBW527	58.1±5.4	18±6
PBW175	58.3±0.4	16±6
PBW343	66.8±0.5	21±6

Further studies

These were done on PBW644 and PBW343. Treatments were grouped in to three groups. The first group of comparison was comparing exogenous supply of PEG (WS), ABA, H_2O_2 (ROS), SNP (NO), DMTU (H_2O_2 scavenger), PTIO (NO scavenger) to water control (CT) to yield the information of regulation by these signals in control plant. The second group of comparison was comparing ABA supply with ABA plus H_2O_2/SNP and ABA plus DMTU/PTIO to yield the information of exogenous as well as endogenous ROS/NO-signalling under high concentration of ABA. The third group of comparison was comparing PEG supply with PEG plus H_2O_2/SNP and PEG plus DMTU/PTIO to yield the information of exogenous as well as endogenous ROS/NO-signalling under water stress.

Growth

PEG decreased growth in PBW343 while increased shoot growth in PBW644 (Fig. 1). ROS/NO supply under PEG increased growth in PBW343 while in PBW644, DMTU supply decreased growth. Secondly, PTIO under CT, DMTU/PTIO under

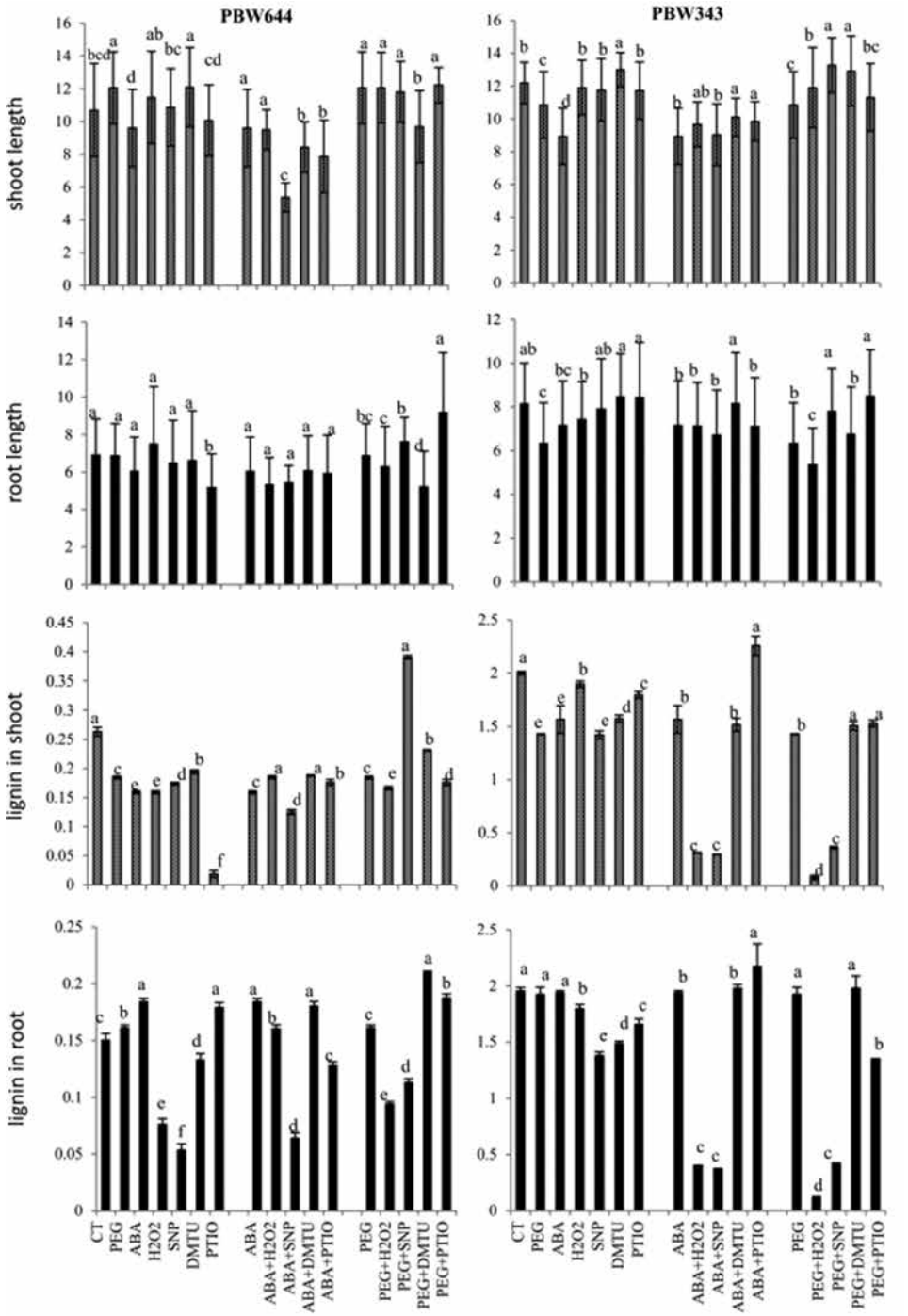

Fig. 1. Effect of different treatments on growth (cm per plant) and lignin (mg per g of fresh weight) of shoot and root of two wheat cultivars PBW644 (left panel) and PBW343 (right panel) at 36 h of treatment given to 5.5-day-old seedlings. Data is analysed by Duncan multiple test ($p \leq 0.05$) for statistical differences in three groups, comparing treatments and CT (group 1), ABA plus treatments and ABA (group 2), PEG plus treatments and PEG (group 3). Different alphabets represent significant difference

ABA decreased root growth and shoot growth, respectively, in PBW644 only. NO under ABA decreased shoot growth in PBW644.

Lignin

Lignin levels (Fig. 1) were much higher in PBW343 than in PBW644 in CT plant. ROS/NO supplies decreased lignin but DMTU/PTIO also decreased it in both cultivars except in root of PBW644. PEG and ABA decreased lignin in shoot of both cultivars while increased it in roots of PBW644. Under ABA/PEG, ROS/NO decreased lignin in PBW343 while in PBW644, such decreases were more pronounced in roots.

Soluble phenolics

Both cultivars had almost same level of phenolics (Fig. 2) in shoot but in root, PBW343 had higher level under CT. PEG/ROS/NO supply increased phenolics in PBW644 only. Under ABA/PEG, ROS/NO supplies increased while DMTU/PTIO decreased phenolics and such changes were more pronounced in PBW343, higher under ABA than PEG.

Flavonoids

Only PEG increased flavonoids (Fig. 2) in shoot of both cultivars while other supplies ABA/ROS/NO did not increase flavonoids in both cultivars. Under ABA/PEG, ROS/NO increased flavonoids and such increases were more under PEG than under ABA and more in PBW644 than PBW343. Moreover, PTIO under PEG decreased flavonoids in both cultivars.

Peroxidases

Exogenous supplies of ABA/ROS/NO/PEG increased cell wall associated peroxidases (CW-POX) (Fig. 3) mainly in PBW644, however, in PBW343, such increases were not seen. For soluble peroxidase (S-POX) (Fig. 3), ABA supply increased it in shoots of PBW644 and NO supply increased it in shoots of PBW343. Under PEG, PBW644 showed rather decreases of S-POX while PBW343 showed maintained levels of it equivalent to CT. Under ABA, in PBW644, NO supply increased both types of peroxidase by small amount but PTIO supply decreased these by large amount while in PBW343, these enzymes were improved on supplying ROS/NO exogenously. Under PEG, exogenous NO application increased both types of enzymes in PBW343 while in PBW644, this was seen for CW-POX only but not for S-POX.

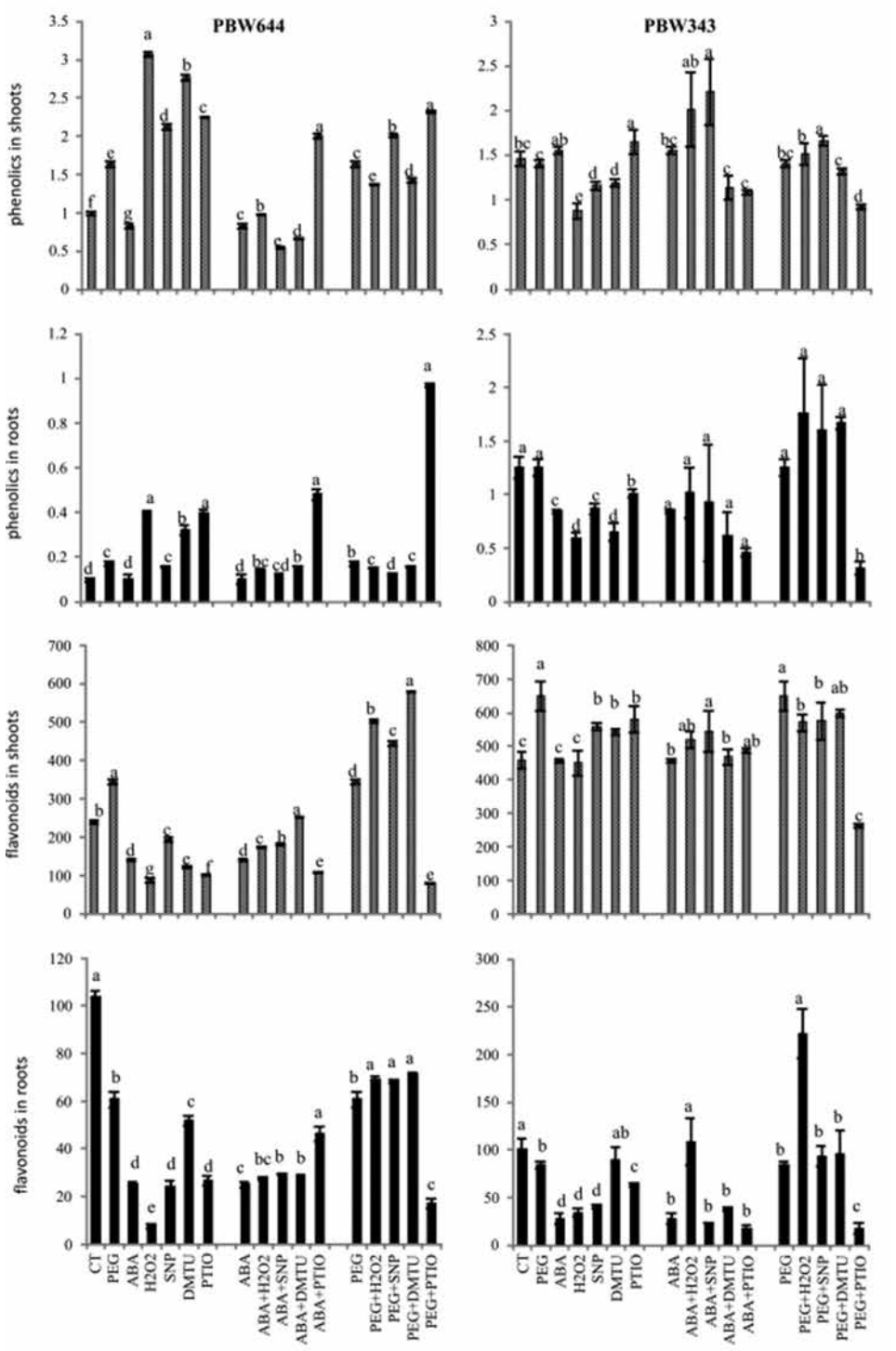

Fig. 2. Effect of different treatments on phenolics (mg gallic acid equivalent per g of fresh weight) and flavonoids (mg rutin equivalents per g of fresh weight) in shoot and root of two wheat cultivars PBW644 (left panel) and PBW343 (right panel) at 36 h of treatment given to 5.5-day-old seedlings. Statistics is same as in Fig. 1

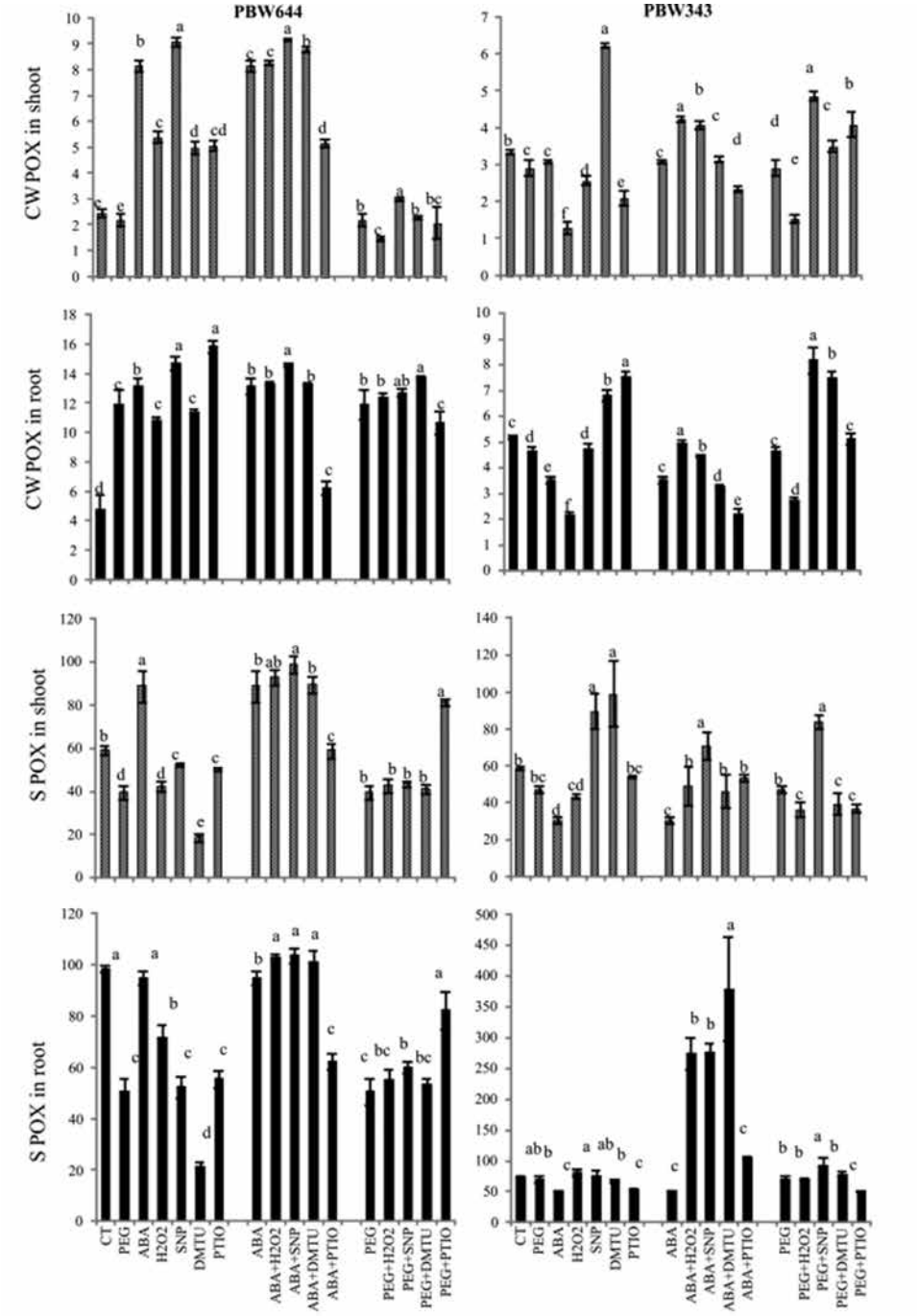

Fig. 3. Effect of different treatments on cell wall peroxidase (CW-POX) and soluble peroxidase (S-POX) (μmole of tetraguaiacol produced per min and g of fresh weight) in shoot and root of two wheat cultivars PBW644 (left panel) and PBW343 (right panel) at 36 h of treatment given to 5.5-day-old seedlings. Statistics is same as in Fig. 1

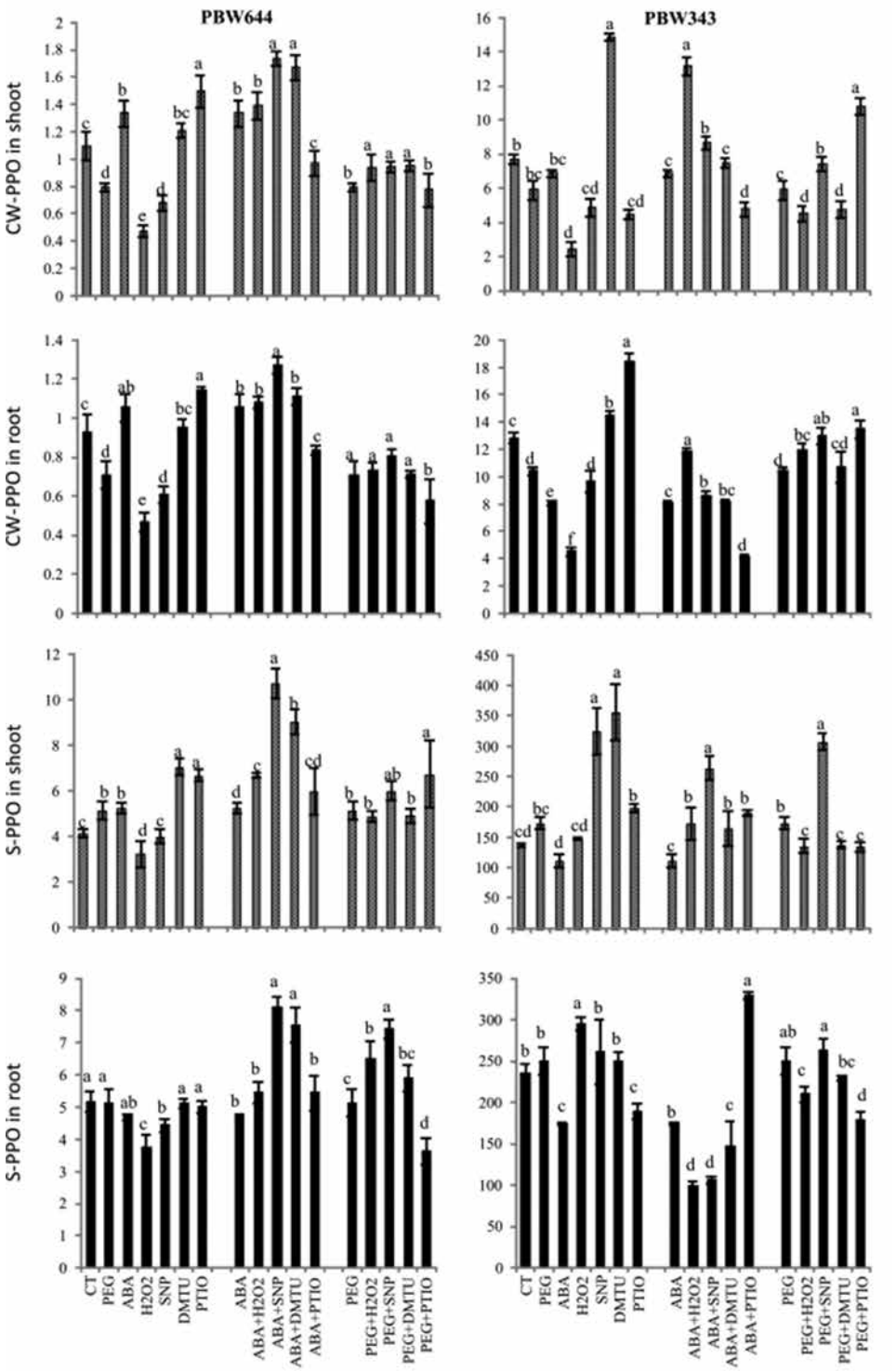

Fig. 4. Effect of different treatments on cell wall polyphenol oxidase (CW-PPO) and soluble polyphenol oxidase (S-PPO) (change of absorbance units per min and g of fresh weight) in shoot and root of two wheat cultivars PBW644 (left panel) and PBW343 (right panel) at 36 h of treatment given to 5.5-day-old seedlings. Statistics is same as in Fig. 1

Polyphenol oxidases

These enzymes were present in much higher amount in PBW343 than PBW644 in control plant (Fig. 4). ROS/NO did not increase these enzymes in both cultivars except in PBW343, ROS and NO supply increased S-PPO in roots and shoots, respectively. ABA up-regulated these enzymes in PBW644 only. ROS/NO signals under ABA increased these enzymes in both cultivars. Under PEG, S-PPO was maintained higher than CW-PPO in both cultivars. ROS/NO signals under PEG increased these enzymes mainly in PBW343 while in PBW644, these increased S-PPO in roots only.

DISCUSSION

Higher growth maintenance by PBW644 under PEG could be related to drought tolerant feature of the cultivar [9, 10, 19]. This cultivar used endogenous ROS signalling for growth maintenance which was lacked in PBW343 and improved on supplying ROS/NO exogenously. PBW644 used endogenous ROS/NO for growth maintenance under ABA and under CT conditions, too. Under drought, both NO and H_2O_2 were found to play crucial role in adventitious rooting in marigold plant [14, 18] where at moderate doses, these molecules protected ultrastructure of mesophyll cells and improved photosynthetic performance of leaves. Inhibitions of growth by exogenous NO under ABA in PBW644 may be related to toxicity produced by high amount of two signals (NO and ABA). ROS/NO at low levels act as signals and could promote growth but at higher levels, these cause toxicity and inhibit growth [2, 3, 18].

Response of lignin in control plant towards ROS/NO may be due to the fact that these molecules at low concentration could increase lignin but at high concentration, decrease lignin. Same observation was reported for lignin and phenylalanine ammonia-lyase (PAL), in root of soybean seedlings where SNP was used as NO-supply [2]. Transcriptomic studies in roots of *Arabidopsis thaliana* under NO supply [1] and sunflower seedlings treated with PTIO [3] also showed that NO may down-regulate the genes of phenylpropanoid/lignin biosynthesis. Exogenous ABA increased lignin in roots of rice seedlings [15], in roots of two wheat cultivars PBW343 and C306 during 12–48 h of duration where increase was higher in PBW343 [8] where C306 was drought tolerant cultivar. However, under water stress (6% mannitol supply), C306 showed decrease of root lignin accompanied with increased root length [8]. Root response of PBW644 may vary from that of C306 in terms of lignification and root extension under water stress or it could be due to different stress level produced by 6% mannitol or 10% PEG. Water stress may increase lignin or decrease lignin depending upon the different regions of root, deposition of lignin was found greater in basal portion of root accompanied with greater reduction in growth than apical region [17]. Aluminium-stress in *Cassia tora* (tolerant to low pH and aluminium toxicity) increased lignin synthesis and cell wall peroxidase which was suppressed by NO but activated by jasmonate [23] where it has also been found that increased NO level under aluminium was reduced by jasmonate. In white clover, lignification was

related to reduced growth and higher oxidative damage during terminal water stress due to higher involvement of peroxidases towards lignin synthesis than in protection against oxidative damage [13].

Phenolics are reported to show positive or negative relation with lignin. Their negative relation under stresses is considered as cellular adaptation to stress so to use phenolics as endogenous antioxidant [17]. In PBW343, under ABA/PEG, ROS/NO-signalling seems to increase phenolics and reduce lignin, so may divert phenolics for ROS scavenging. This type of regulation may not be needed in PBW644 owing to the presence of other antioxidants due to its higher stress tolerant pathways.

Both cultivars appear to use endogenous NO to increase flavonoids under PEG. This can be related to report [16, 20] where under UV-B radiation, endogenous NO up-regulated chalcone synthase gene (*Chs*) (an enzyme involved in flavonoid synthesis) and increased flavonoids. Phenolics and flavonoids were positively related to increased oxidative stress under high application rates of ABA, suggesting that high amount of ABA improved production of such phytochemicals [5].

Higher level of CW-POX in PBW644 under PEG may be related to drought tolerance. This enzyme activity was also found higher in drought tolerant C306 wheat cultivar under ABA/water stress/salt stress [8] as compared to PBW343. Exogenous application ABA increased this enzyme level in rice roots [15]. PBW644 showed endogenous NO signalling under ABA to upregulate both types of peroxidases but under PEG, this signalling upregulated CW-POX but not S-POX. One reason could be that tolerant cultivar may increase S-POX only under higher level of stress, where ABA supply may resemble to higher stress level. PBW343 did not show enough level of endogenous signalling and hence showed improvement on supplying ROS/NO exogenously under ABA as well as under PEG. Regulations of cell wall bound and soluble peroxidases may vary as supported by some reports. For example, exogenous NO supply in the form of SNP increased CW-POX at low concentrations but increased S-POX at high concentrations in roots of soybean seedlings [2]. Two wheat species *Triticum durum* (salt tolerant) and *Triticum aestivum* (salt sensitive) showed increased level of both types of peroxidases under salt stress but tolerant species contained more of CW-POX while sensitive had much more S-POX [6]. In wheat cultivars, drought tolerant cultivar showed enhanced CW-POX and diamine oxidase and decreased S-POX and GR to increase extracellular H_2O_2 over drought sensitive cultivar which showed elevated SOD and diamine oxidase and decreased APX and GR to elevate H_2O_2 under salinity [24].

In the literature polyphenol oxidases have been suggested to have many functions, some are known and some are still unknown. This enzyme has been related to desiccation tolerance [22], as well as antioxidant to scavenge ROS under stresses like biotic and abiotic stresses [21]. Higher levels of these enzymes in control as well as water stressed PBW343 indicated that these enzymes may be utilized by this cultivar to scavenge ROS under controlled as well as stressed conditions. PBW644 being drought tolerant may have other antioxidant machinery to fight with ROS under such conditions, so up-regulating it only under severe stress, like ABA supply, as shown in the present study. ABA up-regulation of these enzymes were also found in C306 [8].

Comparison of ROS signalling with NO signalling in case of phenolic metabolism is difficult. One may compare these two signalling under PEG for peroxidases and polyphenol oxidases only. It appears NO-signalling contributes more than ROS-signalling for the up-regulation/maintainance of these enzymes. However, for other components of phenolic metabolism like lignin, phenolics and flavonoids, such differences were not seen.

In summary, drought tolerant cultivar shows higher growth and cell-wall peroxidases under water stress. However under ABA, this cultivar shows higher up-regulation of all phenolic parameters like peroxidases, polyphenol oxidases, soluble phenolics, lignin. This indicates that these parameters are up-regulated by ABA but the tolerant cultivar may not use these at low stress level due to the presence of other antioxidants and use these only under severe stress condition. ROS/NO signals under ABA and water stress regulate growth and phenolic metabolism in both cultivars. Tolerant cultivar has a high endogenous level of such signals under stress while the sensitive cultivar lacks it and shows improvement upon exogeneous supplying of these signals.

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