

INDUCING THERMO-TOLERANCE IN LATE SOWN WHEAT (*Triticum aestivum* L.) THROUGH PRE-CONDITIONING WITH H₂O₂

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Late sown wheat covers 56% of total cultivated area of wheat in Pakistan which makes it vulnerable to sub and supra optimal temperatures. Although there is no substitutes to timely sowing, techniques are available that could mitigate the harmful effects of late sowing. Therefore; a field study was conducted to evaluate the role of H₂O₂ in mitigation of negative effects of high temperature at reproductive phase, during 2011-12 and 2012-13. Treatments comprised of two sowing dates (20th Nov and 20th Dec) and foliar application of H₂O₂ (75 μ M) and distilled water at different growth stages (tillering, booting and heading) along with a control (no spray). Crop growth rate and net assimilation rate substantially improved under both sowing dates in response to H₂O₂. Better performance of wheat regarding growth attributes indicated the positive role of H₂O₂ in protecting the total soluble proteins. The findings further revealed that H₂O₂ triggered up-regulation of different antioxidants (SOD, POD, and CAT) which offset the negative impacts of supra optimal temperatures that persisted in late planted wheat. Collectively, we concluded that application of H₂O₂ at heading stage improved growth and heat tolerance in late sown wheat.

Keywords: Late sowing, antioxidants, H₂O₂ foliar, wheat productivity

INTRODUCTION

Wheat is sown on 40% of the total cultivated area in Pakistan. More than half of which is sown late due to delayed harvest of cotton and rice thus making it vulnerable to sub and supra optimal temperatures (Govt. of Pakistan, 2013). Quantity and quality of late sown wheat is affected due to low temperature at earlier growth stages (Feng *et al.*, 2008) and to high temperature at later growth stages (Farooq *et al.*, 2011). Late sowing has become a major limiting factor for wheat productivity as temperature is rising globally (Ortiz *et al.*, 2008; Lobell *et al.*, 2011). It is reported that every single day's delay after 15th Nov would bring diminution of 36 kg ha⁻¹ in economical yield (Subhan *et al.*, 2004), thus reducing the wheat production up to two million tons in Pakistan (Rehman *et al.*, 2011). The elevated temperature hampers the vegetative attributes like stem growth, number of leaves, leaf area and biomass production and also upset the photosynthesis process and enzymes activation in crops (Tahir *et al.*, 2009; Mahboob *et al.*, 2005). Low temperature stress causes damages to plasma membrane integrity, protein assembling, photosynthesis (Mahajan and Tuteja, 2005), physiology, allometry and yield attributes (Yu *et al.*, 2003). Although there is no substitute to timely sowing, but techniques that can ensure fair seed emergence and stand establishment under late sown conditions could mitigate the harmful effects of late sowing.

Different roles of H₂O₂ have been documented in crosstalk of acclimation processes in stressed crops (Mittler *et al.*, 2004). Plants under stress always tend to produce free radicals and other reactive oxygen species (Taiz and Zeiger, 2010; Arora *et al.*, 2007). These active oxygenated radicals predominantly H₂O₂ cause oxidative injury, hampered the plant defense system, denature the proteins and organelles of the cells at higher concentration. The seed treated with H₂O₂ proved to be more responsive to different stresses as in water deficit conditions maize leaves showed a marked increment regarding scavenging molecules (Terzi *et al.*, 2014). The dismutation of superoxides (SOD) and catalytic activities of peroxidases and catalase (POD and CAT) enable the plant to cope with devastatingly impact of high temperatures. The pretreated tobacco plants acquired improved resistant against high temperatures as SOD and CAT actives were enhanced (Wi *et al.*, 2010). H₂O₂ is renowned in acquisition of stress tolerance by procuring systematic acquired resistance and hypersensitive response (Torres *et al.*, 2006). Till now, a little work has been reported regarding H₂O₂ under natural environmental conditions. So, we envisaged this study based on the hypothesis that H₂O₂-mediated response would markedly improve the bio-physiological attributes to induce thermo-tolerance in late sown wheat. Therefore, objective of experiment was to assess changes in growth and various antioxidants activities due to H₂O₂ and distilled water pre-conditioning in optimal and late sown wheat.

MATERIALS AND METHODS

Proposed study was conducted at Agronomic Research Area, University of Agriculture, Faisalabad (30°31 N, 73°74 E and 184 meters above the sea level) to evaluate pre-conditioning of H_2O_2 at various growth stages of late sown wheat in Pakistan. Wheat (Millat-2011) seeds were taken from Wheat Research Institute, Ayub Agricultural Research Institute Faisalabad, Pakistan. Treatments comprised of two sowing dates (20th Nov and 20th Dec) and foliar application of H_2O_2 at the rate of 75 μM and distilled water at different growth stages (tillering, booting and heading) along with a control (no spray). Experiment was conducted in randomized complete block design (RCBD) with split plot arrangement. Sowing dates were kept in main plots and foliar applications in sub-plots having net size of 6 m \times 1.8 m. A pre-soaking irrigation (rouni) was applied before seedbed preparation. Seedbed was prepared by two cultivations with tractor mounted cultivator each followed by a planking. Crop was sown with the help of a single row hand drill using 125 kg seed per hectare. Nitrogen, phosphorus and potash were applied at rate of 110, 88 and 60 kg ha⁻¹. Urea, DAP and SOP were the sources of nitrogen, phosphorus and potassium fertilizers, respectively. Phosphorus and potassium fertilizers were applied as basal dose. However, Urea was applied in three equal splits viz. at sowing, first irrigation and second irrigation. Agro-meteorological Observatory is located about 500 m away from the experimental site. Data regarding climatic condition has been presented in Table 1. Details about methods and procedures for recording parameters has been given in following paragraphs.

Growth: Plant samples for recording leaf area, fresh weight and dry weight were taken from a randomly selected unit area of 1 m². Crop growth rate (CGR) was computed through dry weight increase per unit of dry weight per unit

area per unit time. Leaf area index was calculated by using Watson (1947) method. Digital leaf area meter (JVC TK-5310) was used to measure the leaf area. Net assimilation rate was computed using formula given by Hunt (1978).

$NAR = TDM / LAD$

Total soluble proteins and antioxidants: Antioxidants (SOD, POD and CAT) activity was measured on the basis of total soluble proteins (TSP). For this purpose flag leaf of crop after 10 days of last spray was used for the analysis. Extraction was done by using Sambrook and Russell (2001) method. 0.5 g fresh flag leaves were grounded in a chilled pastor mortar. Phosphate buffer saline (PBS) with pH 7.2 was used. It was made by using 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 2.7 mM KCl and 1.37 mM NaCl which was dissolved in distilled water and cocktail protease inhibitors (1 μM) were added to buffer solution just before extraction of leaves and then it was centrifuged at 12000 \times g for 5 min. The contents were determined followed by prescribed method of Bradford (1976) by using spectrophotometer (UV 4000 UV-VIS spectrophotometer) at 595 nm. Standard curve method was plotted to determine the concentration (mg mL⁻¹) of total soluble or heat stable fractions of proteins. Standard curve was prepared from bovine serum albumin (BSA) absorbance. Linear regression equation was used to calculate TSP.

Enzymatic antioxidants computed by Giannopolitis and Ries (1977) for SOD and Chance and Maehly (1955) methods for CAT and POD. Spectrophotometer (UV 4000 UV-VIS spectrophotometer) was used for recording absorbance. The extraction samples (5 mL) were made by using 50 mM phosphate buffer (pH 7.8). These were centrifuged at 15000 \times g for 20 min. the supernatant was preserved and used further in analysis of SOD, CAT and POD activity. Their activities were measured at absorbance of 560, 240 and 470 nm, respectively.

Statistical analysis: Data was analyzed statistically using

Table 1. Metrological data during 2011-12 and 2012-13.

Growing season		Avg. Temp.	Rainfall	Avg. RH	Max Temp.	Min Temp.
November	2011	20.5	00.0	61.2	27.3	13.3
	2012	17.8	00.0	73.0	24.8	10.8
December	2011	20.9	00.0	59.1	20.9	4.2
	2012	12.4	17.2	81.5	18.8	6.1
January	2012	10.2	3.8	69.6	17.2	3.2
	2013	10.2	1.5	77.6	16.8	3.5
February	2012	11.5	08.0	62.1	18.4	4.6
	2013	13.8	55.0	81.0	19.2	8.3
March	2012	18.8	01.5	58.2	25.9	11.7
	2013	20.0	01.3	61.2	27.0	13.0
April	2012	25.3	10.5	59.1	32.7	17.9
	2013	26.6	21.6	36.7	33.5	19.7
May	2012	32.7	00.0	43.2	38.9	26.9
	2013	32.0	04.6	32.0	39.7	24.4

statistix 8.1 software. The significant interactions were compared by implying least significant difference test (Steel *et al.*, 1997).

RESULTS

Growth attributes: Foliar application of H_2O_2 and even only distilled water (DW) affected the crop growth rate (CGR), leaf area index (LAI) and net assimilation rate (NAR), irrespective of the growth stage and sowing date of wheat. Crop growth rate varied in response to sowing dates (Fig. 1) during both years. November sown crop experienced better CGR over late sown crop. Crop growth rate achieved its maximum value at 70-80 DAS in November sowing crop as compared to late sowing (60-70 DAS) during both years. Impact of H_2O_2 was prominent than DW applications both at booting and heading stages during both years. Linear increment in LAI occurred up to 80 DAS in November sown crop (Fig. 2). However, LAI in case of

December sown crop increased only up till 70 DAS. Crop followed similar trend during next years. Foliar application of H_2O_2 especially at heading and booting helped in improving LAI. Effect was more pronounced in late sown crop. Although DW also helped the crop to gain more LAI over control but positive effect was far less as compared to H_2O_2 .

Crop response to NAR was variable to foliar application of H_2O_2 and sowing dates as more value was recorded in normal sowing (20th Nov) (Fig. 3). Interactive effects of sowing dates and foliar treatments were found to be significant during both years. Effect of H_2O_2 foliar applications on rate of dry matter accumulation was highest at heading stage during first year while same stages of crop showed with non-significant difference with booting for NAR values during second year. The differences among control and DW treatments were non-significant.

Total soluble proteins: Total soluble proteins (TSP) expressed variable response to sowing dates. Maximum

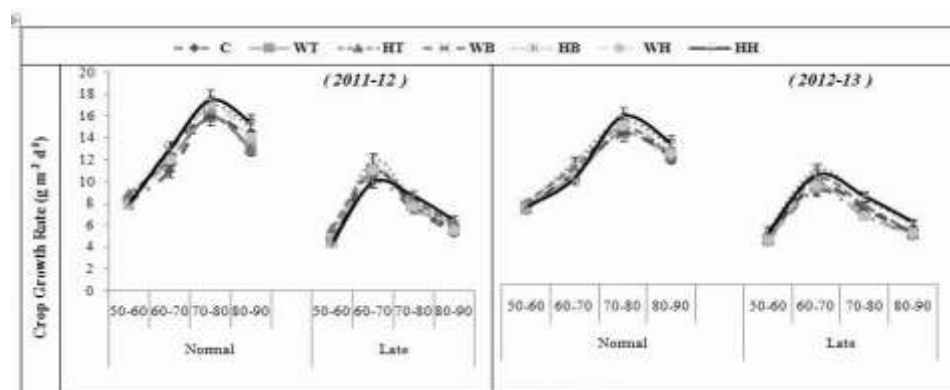


Figure 1. Effect of foliar applied H_2O_2 at different growth stages of wheat on crop growth rate (CGR) ($g\ m^{-2}d^{-1}$) (C = Control, WT = Water spray at tillering stage, HT = Foliar spray of H_2O_2 at heading stage) under normal and late sown conditions.

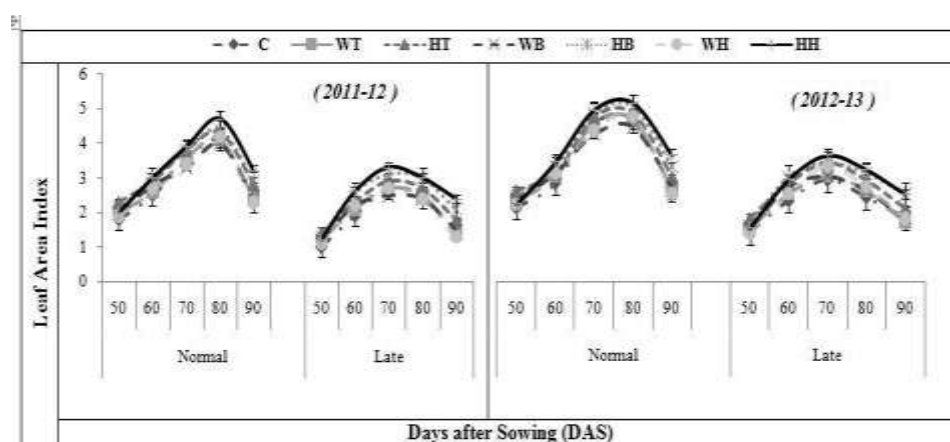


Figure 2. Effect of foliar applied H_2O_2 at different growth stages of wheat on leaf area index (C = Control, WT = Water spray at tillering stage, HT = Foliar spray of H_2O_2 at heading stage) under normal and late sown conditions.

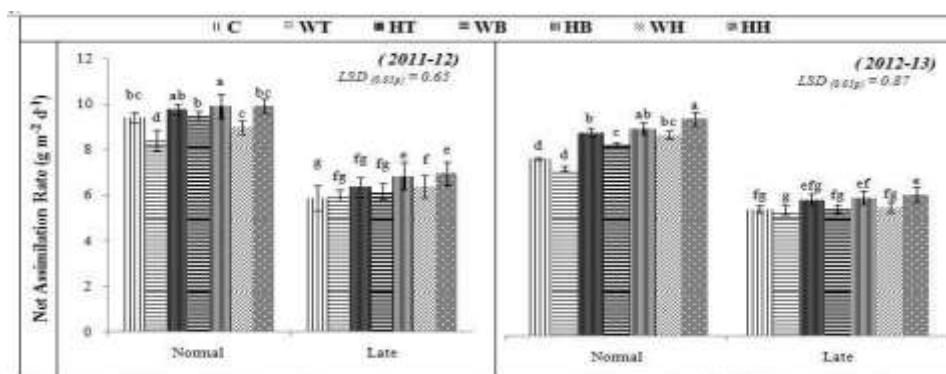


Figure 3. Effect of foliar applied H₂O₂ at different growth stages of wheat on net assimilation rate (gm⁻²d⁻¹) (C = Control, WT = Water spray at tillering stage, HT = Foliar spray of H₂O₂ at heading stage) under normal and late sown conditions.

amount of TSP were produced in late sown wheat. Early sown wheat produced fewer amounts of proteins. Foliage sprays of DW and H₂O₂ at various growth stages articulated a well-marked impact on the TSP assessed from flag leaf. Significant interaction was observed only during first year while it remained non-significant during second year of study. Wheat sown at optimum time (20th Nov) showed invariable response to foliar application of H₂O₂ and DW. However; the late sown wheat exhibited a significant impact of different foliar treatments in improving the TSP. Maximum amount was noted with H₂O₂ at heading and booting stages in late sown crop.

Antioxidants: Foliar application of H₂O₂ improved the plant growth through increasing activity of SOD, POD and CAT

(Table 2). Interactive effect of foliar applied H₂O₂ and sowing dates was significant regarding the activity of SOD, POD and CAT.

Maximum activity of SOD was observed at heading (26.6 and 31.1%) and booting stages (20 and 19.3%) for normal sown whereas in late sown maximum was noted at heading (21.6 and 24.7%) and booting stage (16.6 and 18.3%) during 2012 and 2013 respectively in response to H₂O₂. (Parenthesis values show the percent increment from control)

Crop sown at optimum time (20th Nov) expressed low activity of POD than late planted crop (20th Dec). The highest POD activity was judged in the plants which were sprayed with H₂O₂ at heading stage (43.3 and 22.2% for

Table 2. Effect of foliar applied H₂O₂ at different growth stages of wheat on biochemical attributes under normal and late sown conditions.

Treatments	Total soluble proteins (mg g ⁻¹)				Superoxide dismutase (SOD) (unit mg protein ⁻¹)			
	2012		2013		2012		2013	
	Normal	Late	Normal	Late	Normal	Late	Normal	Late
C = Control	0.467 d	0.520 c	0.478	0.538	102.5 e	137.1 g	91.1	115.1
WT = H ₂ O spray at Tillering	0.471 d	0.539 c	0.486	0.550	116.4 d	141.9 f	102.2	120.7
HT = H ₂ O ₂ spray at Tillering	0.476 d	0.559 b	0.496	0.568	125.4 b	156.2 c	108.8	135.5
WB = H ₂ O spray at Booting	0.472 d	0.525 c	0.486	0.554	121.9 c	148.3 e	108.1	126.6
HB = H ₂ O ₂ spray at Booting	0.477 d	0.566 ab	0.502	0.580	129.3 a	161.3 b	112.9	140.9
WH = H ₂ O spray at Heading	0.470 d	0.535 c	0.490	0.550	123.7 bc	151.4 d	104.5	130.2
HH = H ₂ O ₂ spray at Heading	0.481 d	0.582 a	0.495	0.586	131.7 a	168.5 a	132.3	152.9
LSD Value	0.0333		ns		2.69		ns	
	Peroxidase (POD) (unit mg protein ⁻¹)				Catalase (CAT) (unit mg protein ⁻¹)			
C = Control	8.3 cd	13.2 b	8.8	12.8	18.1 c	21.1 cd	9.7 e	16.4 e
WT = H ₂ O spray at Tillering	10.1 c	13.5 b	12.8	13.3	17.8 d	19.5 e	11.7 d	17.4 cd
HT = H ₂ O ₂ spray at Tillering	13.8 ab	14.8 ab	14.8	15.9	20.5 b	21.9 c	13.1 c	18.1 c
WB = H ₂ O spray at Booting	11.5 bc	13.5 b	12.6	12.2	18.0 c	20.9 cd	12.1 d	16.4 de
HB = H ₂ O ₂ spray at Booting	14.2 a	15.6 ab	15.4	16.3	21.9 a	24.7 b	15.1 b	19.7 b
WH = H ₂ O spray at Heading	12.9 ab	13.2 b	12.9	13.5	18.1 c	20.5 d	13.1 c	16.3 e
HH = H ₂ O ₂ spray at Heading	14.8 a	16.1 a	15.6	17.4	22.7 a	28.3 a	17.2 a	20.8 a
LSD Value	2.46		ns		1.31		0.99	

Means not sharing the same letter within column differ significantly at $P \leq 0.05$.

normal and late sown, respectively) than control. Minimum activity of POD was computed for control treatment where nothing was sprayed. Similar trend was noted during next growing season (2013).

During 2012, maximum CAT activity was observed from plants treated with H₂O₂ at heading stage (32.1 and 23.4% for normal and late sown, respectively) than control. While minimum values were recorded for DW at all stages and control treatments. Similar response of CAT activity was shown during 2013.

DISCUSSION

Leaf area index, CGR and NAR are dependent on cell expansion and elongation. Positive role of H₂O₂ in current studies under late sown condition is clear because it helped in increasing LAI, CGR and NAR. Reactive oxygen species produced through oxidation of NADPH⁺ are one of the important factors responsible for cell expansion because they regulate Ca⁺² influx (Foreman *et al.*, 2003). This increased Ca⁺² efficiency can be linked to the application of H₂O₂ because it enhances the uptake of Ca⁺² that ultimately help in elongation and expansion of primary meristems (Foreman *et al.*, 2003). Moreover, H₂O₂ regulated the cell morphogenesis (Slesak *et al.*, 2007) and it stabilized internal homeostasis by inducing mitogen-activate protein kinase (MAPK) (Moon *et al.*, 2003).

November sown crop exhibited more CGR and LAI at 70-80 DAS whereas, under late sowing higher values were achieved earlier (60-70 DAS). This difference can be articulated to higher temperatures as heat might have hastened the crop growth rate to complete its life cycle earlier (Porter, 2005). Slow growth under late sown conditions is due to high temperature (Table 1) as hot days enhanced the process of leaf senescence and reduce the ground cover (Lim *et al.*, 2007). Leaf aging or fast rate of leaf decaying could also be one of reasons in reducing leaf area or canopy area (Asseng *et al.*, 2011). Timely and late sown crop would differ in prevailing climatic conditions (Table 1) along the crop cycle that ultimately brings up to 20% reduction in growth attributes under late sown conditions (Rahman *et al.*, 2009). Lower accumulation of dry matter per day under late sown condition was due to higher temperatures that hampered assimilate translocation in plants as heat stress at pre-anthesis, anthesis or heading stage adversely influenced assimilate translocation towards sinks (Asseng *et al.*, 2011). Results of our studies show noticeable improvement in LAI, CGR and NAR values under late sown conditions after H₂O₂ application possibly due to its positive role in the process of photosynthesis. Gong *et al.* (2001) observed enhancement in photosynthetic activity in H₂O₂ treated *Arabidopsis* leaves. H₂O₂-induced higher activity of SOD, POD and CAT might have protected proteins of chlorophylls as maize crop treated with H₂O₂

exhibited better activity of antioxidants which conferred higher tolerance to heat and salt stress.

According to Karpinska *et al.* (2000) pre-conditioning with H₂O₂ under late sown conditions permitted the survival of more green leaf area, as 65% of the leaves damaged by the photooxidation did not recover in control. Similarly pre-treatment of H₂O₂ improved the growth attributes of the sugarcane and ameliorated negative impacts of surrounding environments (Srivastava *et al.*, 2012). The acclimatized results of stressed wheat are agreeing with the findings published by Çavusoglu and Kabar (2010) who stated that pre-treated barley crop with H₂O₂ exhibited better growth performance by quenching free radicals of oxygen species through over-expressed antioxidants induced by H₂O₂. Our results exhibited similar trend in lowering CGR, LAI and NAR in late planted crop because more frequency of hot days was experienced by crop at most sensitive growth stages (Table 1). Thus, observed increment in growth attributes under late sown crop conferred positive role of H₂O₂ in offsetting negative impacts of deleterious possessions of temperatures (Gao *et al.*, 2010).

H₂O₂-mediated up-regulation of stress responsive genes is one of possible causes in improving total soluble proteins under late sown conditions. Moreover, H₂O₂ induced antioxidants ensured the complete extinguish of highly reactive free oxygen species and radicals ultimately protecting proteins and organelles of the cells (Cheng and Song, 2006). As we know, heat-induced ROS production impelled the oxidative push in plants which allow the degradation of the proteins (Arora *et al.*, 2007). Our data suggest H₂O₂ induced higher tolerance against these reactive oxygen species. So, H₂O₂ application conferred higher tolerance to heat stress as higher activity of SOD, POD and CAT was recorded under late sown condition.

Application of H₂O₂ increased the activity of CAT and two SODs (MnSOD, Cu/ZnSOD) in potato (Mora-Herrera *et al.*, 2005) and tobacco (Wi *et al.*, 2010). Increased activity of these enzymes can compensate the loss induced by stress. In our study, H₂O₂ aided in getting higher growth and activity of enzymes when it was applied at booting and heading stage because it can induce stress tolerance as shown in pre-treated *Arabidopsis* which recorded lesser damage of activated oxygen species due to up regulated scavengers' molecules (Slesak *et al.*, 2007).

Wang *et al.* (2010) showed that activity of CAT, POD, SOD and AOX (alternative oxidase) was over-expressed at low concentrations of H₂O₂ in grasses. This up-regulation of antioxidants to the applied H₂O₂ is supported by various researchers as in mung bean (Hung *et al.*, 2007) and wheat (Feng *et al.*, 2008). Authors reported that exogenously applied H₂O₂ conferred higher amount of secondary metabolites and primary defense system molecules. It significantly improved the crop performance under various stress conditions and acclimatized it to perform well and

gave maximum yield as compared with control or untreated plots (Mittler *et al.*, 2004). So, results indicated the similar improvement in growth and biochemical attributes in response to H₂O₂ under late sown conditions.

Conclusion: Pre-conditioning of H₂O₂ resulted in better growth of field-sown wheat. The over-expressed enzymatic antioxidants in response to H₂O₂ application show the improvement of plant's defense. Hydrogen peroxide helped in acclimatization of plants to cope with heat-induced oxidative stress that was particularly observed in late sown wheat. These facts proved that H₂O₂ at low concentration plays the signaling molecule role which improves the thermo-tolerance of late sown wheat.

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