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Mitigating effects of 24-epibrassinolide on heat stress damage by shifting biochemical and antioxidant defense mechanisms in wheat (*Triticum aestivum* L.) at pre-flowering stage and post- flowering stage

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Abstract
High temperature is major environmental factor that limits productivity of cereal crops all over the world. Wheat (*Triticum aestivum* L.), one of the main staple cereal crop, is highly sensitive to heat stress. Two screened wheat genotypes, C-306 (heat tolerant) and HUW-468 (heat susceptible), were selected to evaluate the effect of 24-epibrassinolide @1µM along with its combinations through pre-seed soaking, foliar spray and both combinations together at pre-flowering and post -flowering stage. The heat stress shock was imposed by late sowing in the field. 24-epibrassinolide treatments significantly enhanced chlorophyll, MSI, NRA, APX, and SOD in both C-306 and HUW-468 genotypes under heat stress. Among all the treatments, EBR @1µM seed treatment + 1µM foliar spray revealed higher chlorophyll (37.56 and 42.00 mg g^{-1} FW) at pre-flowering stage whereas (42.43 and 44.13 mg g^{-1} FW) at post-flowering stage, MSI (38.13% and 38.56%) at pre-flowering stage whereas (40.67% and 41.00%) at post-flowering stage, NRA(0.760 and0.666 µ Mol NO_2 h^{-1} g^{-1} FW**)** at pre-flowering stage whereas (0.750 and0.640 µ Mol NO_2 h^{-1} g^{-1} FW) at post-flowering stage, APX(49.03 and 51.50 Unitsmg^{-1}proteinmin^{-1}) at pre-flowering stage whereas(51..67and52.90 Units mg^{-1} protein min^{-1}) at post-flowering stage, SOD (2.600 and 3.477 Units mg^{-1} protein min^{-1}) at pre-flowering stage whereas(2.767 and 3.550 Units mg^{-1} protein min^{-1}) at post-flowering stage in both susceptible and tolerant genotype, respectively, compared to control. 24-epibrassinolide treatments, as evident, minimized the heat stress damage in tolerant genotype as compared to susceptible genotype by enhancing chlorophyll, MSI, NRA and antioxidant enzymes activity (APX and SOD).

Keywords: Antioxidant, chlorophyll, heat stress, nitrate reductase activity, membrane stability index, wheat, 24-epibrassinolide

Introduction
The average global temperature is reported to be increasing at a rate of 0.18°C every decade (Hansen *et al*., 2012; Annual Climate Summary, 2010) [9, 2]. Future temperature and increased frequency of hot days (Pittock, 2003). To adapt new crop varieties to the future climate, we need to understand how crops respond to elevated temperatures and how tolerance to heat can be improved (Halford, 2009) [9, 18]. Wheat (*Triticum aestivum* L.) is very sensitive to high temperature and trends in increasing growing season temperatures have already been reported for the major wheat-producing regions (Alexander *et al*. 2006; Hennessy *et al*., 2008) [1, 11]. Though, heat stress affects the metabolic pathways at every stage of life of wheat finally leading to yield reduction, the effect of high temperature is particularly severe during grain filling; these losses may be up to 40% under severe stress (Wollenweber *et al.*, 2003, Hays *et al*., 2007) [20, 10]. Other effects of high temperatures are decreased grain weight, early senescence, shrivelled grains, reduced starch accumulation, altered starch-lipid composition in grains, lower seed germination and loss of vigor (Balla *et al.* 2012) [4]. End-of-season or 'terminal' heat stress is also likely to increase for wheat in the near future (Mitra and Bhatia, 2008; Semenov and Halford, 2009) [15, 8, 18]. HT has strong deleterious effects on photosynthesis which are attributed to reduced photosynthetic capacity, photosynthetic efficiency of PS-II and photochemical activity associated with PS-I. Pre-treatment with EBR (0.1 mg L-1) remarkably alleviated HT-induced inhibition of photosynthesis which was accompanied with increased activity of antioxidant enzymes and reduced lipid peroxidation in tomato. Exogenous application of EBR was found effective for both HT tolerant and HT-sensitive ecotypes of melon (Zhang *et al*., 2013). EBR pre-treatment (1.0 mg L-1) significantly improved net photosynthetic rate, stomatal conductance, stomatal limitation and water-use

efficiency of both ecotypes of melon under HT stress. EBR-induced improvement in photosynthesis is also associated with up-regulation of photosynthetic pigment contents and phototchemical activity of PS-I. In eggplant, exogenous application of EBR (0.05-0.2 µM) significantly minimizes HT-induced harmful levels of ROS and increases reduced ascorbate (AsA), reduced glutathione (GSH), proline, soluble sugar and soluble protein content under HT stress (Wu *et al*., 2014) [21]. Therfore, keeping EBR's role into active consideration in different crop plants,it appeared worthwhile to mitigate and to observe the degree of intensity of negative effects of heat stress for optimum biochemical and anti-oxidant gain in two genotypes of wheat through seed hardening as well as pre and post foliar application for effective physiological parameters.

Materials and Methods

The experiment was conducted in the Agronomy Research farm, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi. Wheat had been raised in field and spacing between plants to plant is 25×10cm and all physical precautions will be kept in view in order to protect the crop from the external damage.. A small screening experiment was conducted in view of identifying the effective concentration of 24 epi brassinolide among the optimum (i.e., 0.5, 1.0, 2.5, 5.0 µM) concentrations on growth response of wheat. Finally, 1µM was taken as effective concentration based on morpho-physiological studies.The two different genotypes of wheat viz.,: HUW-468 (susceptible variety) and C-306 (tolerant variety) and the individual and combined treatments were taken as T_1 = Control (at normal temperature and without brassinolide), T_2 = Hardened seeds(S) T_3 = Foliar spray (F),T_4 = Hardened seeds and foliar application both at early and late sown stage. The treatments were replicated three times. Long term studies were made at 65 and 85 days after germination (DAG) in order to study biochemical and antioxidant enzyme analysis.

Biochemical and antioxidant enzyme assessment:

Biochemical and antioxidant enzymes activity, viz., total chlorophyll content (Yoshida *et al*. 1972) [22]; nitrate reductase activity (Srivastava, 1974) in leaves were recorded at pre-flowering and post-flowering stage. Membrane stability index was determined in terms of percentage of electrolyte leakage (Cekic *et al*., 2001) [5]. The ascorbate peroxidase (APX) activity was measured as the decrease in absorbance at 290 nm due to ascorbate oxidation (Nakano and Asada, 1981) [16] while superoxide dismutase (SOD) activity was assayed by the method of (Dhindsa *et al*., 1981) [6] through measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium.

Statistical analysis: Experimental data were recorded with average mean values for three replicates of each treatment and data were subjected to ANOVA for split plot design. Critical difference was taken at $p \leq 0.05$. Standard error mean was calculated as described by (Gomez and Gomez 1984) [7].

Result and Discussion

Present research was carried out to deal with effect of heat stress on biochemical and antioxidant enzyme analysis of wheat genotypes (HUW- 468, C-306), their tolerance level and favourable effect of 24-epi brassinolide for alleviating the deleterious effect of heat stress on total chlorophyll content, nitrate reductase activity, membrane stability index, ascorbate peroxidase activity, superoxide dismutase activity.

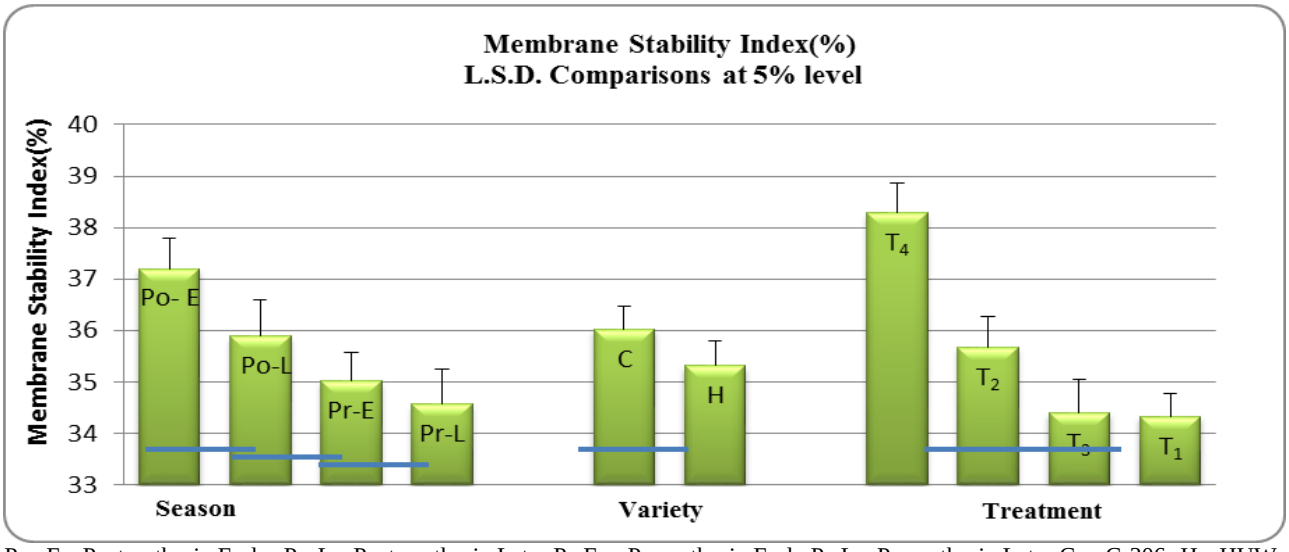
Under late sown condition decline in chlorophyll content in both tolerant (C-306) and susceptible (HUW-468) genotypes was more as compared to normal plants, while per cent reduction in chlorophyll content was more prominent in susceptible genotype (Table 1). However, in absolute quantitative terms, genotype C-306 revealed relatively higher chlorophyll content than the HUW-468 under heat stress which is duly supported by (Kumar *et al*., 2013) who reported that tolerant mung bean genotypes maintained relatively higher level of total chlorophylls. Photosystem I (PS-I) is stimulated by heat (as measured by the rate of P700+ reduction) due to greater reduction of the plastoquinone (PQ) pool by ferredoxin (Fd) at high temperatures (Tóth *et al*., 2007). In contrast, photosystem II (PS-II), particularly the oxygen-evolving complex, is deactivated even at slightly elevated temperatures (Yamane *et al*., 1998), demonstrating that this process is especially sensitive to temperature stress (Pushpalatha *et al*., 2008). 24-epibrassinolide treatment @1µM seed treatment +1µM foliar spray revealed maximum ((37.56 and 42.00 mg g^{-1} FW) at pre-flowering stage whereas (42.43 and 44.13 mg g^{-1} FW) at post-flowering stage chlorophyll content as compared to early condition, though the amount was lesser in HUW-468 (Table 1). These results indicate that tolerant genotype might produce higher photo-assimilates via maximizing photosynthetic rate due to greater chlorophyll content under heat stress.

Table 1: Effect of 24-epibrassinolide on Total chlorophyll content (mg g^{-1} fresh weight) of two wheat genotypes (*Triticum aestivum* L.) at pre-flowering (65 DAS) and post – flowering (85 DAS) under heat stress

Treatments	Pre-anthesis				Post –anthesis			
	Early		Late		Early		Late	
	HUW-468	C-306	HUW-468	C-306	HUW-468	C-306	HUW-468	C-306
T_1	36.00	37.67	35.10	36.60	40.56	40.86	40.47	40.53
T_2	40.03	42.80	34.63	39.00	42.86	43.76	41.20	43.36
T_3	37.10	40.43	34.06	38.33	40.96	41.30	40.10	41.00
T_4	42.00	43.03	37.56	42.00	43.93	44.76	42.43	44.13
Mean	38.78	40.98	35.34	39.26	42.07	42.67	41.05	42.15
Interactions	SEm±				CD 5%			
G	0.508				1.436			
S	0.718				2.031			
T	0.718				2.031			
G X S	1.016				2.873			
GXT	1.016				2.873			
SXT	1.437				4.063			
GXSXT	2.033				5.747			

G =Genotype, T = Treatment, S= Season, DAS= Days after sowing ; T_1 = Control,T_2 = Seed hardened (1 µM BL), T_3 = Foliar application(1 µM BL), T_4 = Seed & foliar both application (1 µM BL)

In heat stress condition, a decrease in the MSI was found in both the genotypes (tolerant and susceptible) genotypes under late sown condition at pre-flowering stage and post-flowering stage as compared to normal condition. MSI was found in 24-epibrassinolide treatment @ 1µM seed treatment + 1µM foliar spray maximum, MSI (38.13% and 38.56%) at pre-flowering stage whereas (40.67% and 41.00%) at post-flowering stage in susceptible and tolerant genotype, respectively (Figure 1). The enhanced resistance was attributed to BR-induced effects on membrane stability and osmoregulation (Wang *et al*., 1993) [19]. Tolerant genotype registered greater MSI than the susceptible in both normal and stressed plants.



Po- E= Post-anthesis Early, Po-L= Post- anthesis Late, Pr-E = Pre-anthesis Early,Pr-L= Pre-anthesis Late, C = C-306, H= HUW-468, T_1 = Control,T_2 = Seed hardened (1 µM BL), T_3 = Foliar application(1 µM BL), T_4 = Seed & foliar both application (1 µM BL)

Fig 1: Effect of 24-epibrassinolide on Membrane stability index (%) of two wheat genotypes (*Triticum aestivum* L.) at pre-anthesis (65 DAS) and post –anthesis (85 DAS) under heat stress

Under late sown condition decline in nitrate reductase activity in both tolerant (C-306) and susceptible (HUW-468) genotypes was more as compared to normal plants, while per cent reduction in nitrate reductase activity was more prominent in tolerant genotype. NRA was found in 24-epibrassinolide treatment @ 1µM seed treatment + 1µM foliar spray maximum (0.760 and0.666 µ Mol NO_2 h^{-1} g^{-1} FW**)** at pre-flowering stage whereas (0.750 and0.640 µ Mol NO_2 h^{-1} g^{-1} FW) at post-flowering stage, in susceptible and tolerant genotype, respectively (Table. 2). The influence of 24-epiBL on the activities of the enzymes catalase, peroxidase, carbonic anhydrase and nitrate reductase also exhibited a significant enhancement in mustard plants grown under nickel stress. (Bajguz. A *et al*., 2008).

Table 2: Effect of 24-epibrassinolide on Nitrate reductase activity (µ Mol NO_2 h^{-1} g^{-1} FW) of two wheat genotypes (*Triticum aestivum* L.) at pre-anthesis (65 DAS) and post -anthesis (85 DAS) under heat stress

Treatments	Pre-anthesis				Post –anthesis			
	Early		Late		Early		Late	
	HUW-468	C-306	HUW-468	C-306	HUW-468	C-306	HUW-468	C-306
T_1	0.750	0.640	0.723	0.620	0.720	0.615	0.690	0.600
T_2	0.754	0.657	0.748	0.636	0.730	0.630	0.720	0.626
T_3	0.753	0.653	0.750	0.632	0.710	0.629	0.700	0.620
T_4	0.765	0.699	0.760	0.666	0.760	0.655	0.750	0.640
Mean	0.756	0.662	0.745	0.639	0.730	0.632	0.715	0.622
Interactions	SEm±				CD 5%			
G	0.0015				0.0044			
S	0.0022				0.0063			
T	0.0022				0.0063			
G X S	0.0031				0.0089			
GXT	0.0031				0.0089			
SXT	0.0044				0.0126			
GXSXT	0.0063				0.0178			

G =Genotype, T = Treatment, S= Season, DAS= Days after sowing; T_1 = Control,T_2 = Seed hardened (1 µM BL), T_3 = Foliar application(1 µM BL), T_4 = Seed & foliar both application (1 µM BL)

24-epibrassinolide significantly enhanced APX activity as compared to control plants under heat stress. As compared to susceptible genotype (HUW-468), tolerant genotype (C-306) showed higher i.e., 51.50 (Unitsmg^{-1}proteinmin^{-1}) at pre-flowering stage whereas (52.90 Units mg^{-1} protein min^{-1}) at post-flowering stage APX activity with 24-epibrassinolide treatment @ 1µM seed treatment + 1 µM foliar spray and minimum i.e., 42.76 (Units mg-1 protein min-1) at pre-flowering stage and 52.90 (Units mg-1 protein min-1) with control under heat stress (Table 3). These results put forward that tolerant genotype has better H_2O_2 scavenging ability than the susceptible genotype resulting in to low lipid peroxidation and membrane damage under heat stress. The influence of 24-epiBL on some enzymatic antioxidants in tomato leaf disc

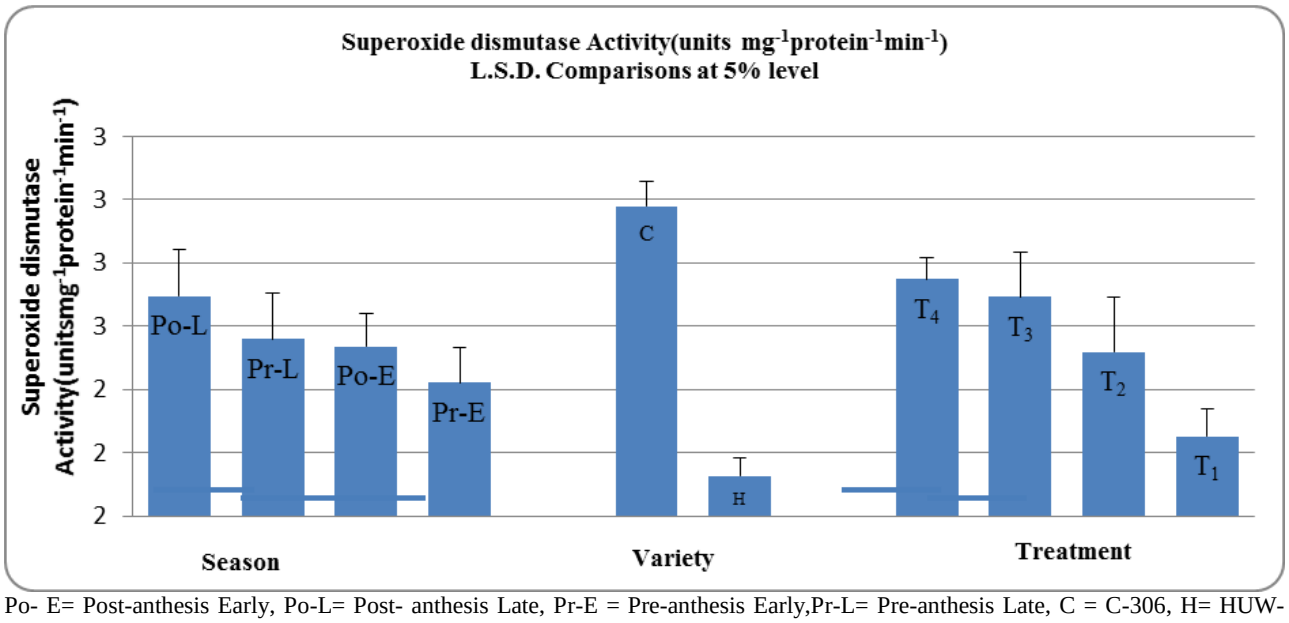
under high (40^0C) temperature was reported (Mazorra L.M. *et al.*, 2002) [14] BR increased the activity of CAT, peroxidase and SOD in respond to high temperature in tomato leaves. Besides these, APX activity plays a vital role in maintaining the balance between generation of free radicals and elimination by converting H_2O_2 into H_2O along with regeneration of NADP+ (Lin *et al*., 2004) [13].

Table 3: Effect of 24 epibrassinolide on ascorbate peroxidise activity(Unitsmg^{-1}protein min^{-1})of two wheat genotypes (*Triticum aestivum* L.) at pre-anthesis (65 DAS) and post –anthesis (85 DAS)under heat stress

Treatments	Pre-anthesis				Post –anthesis			
	Early		Late		Early		Late	
	HUW-468	C-306	HUW-468	C-306	HUW-468	C-306	HUW-468	C-306
T_1	36.27	42.50	39.03	42.76	45.90	48.67	46.33	49.70
T_2	42.40	45.50	43.10	46.93	46.73	49.33	49.13	50.40
T_3	43.53	48.33	43.76	49.17	49.73	51.37	50.83	51.40
T_4	48.43	50.33	49.03	51.50	50.70	52.40	51.67	52.90
Mean	42.65	46.67	43.73	47.59	48.26	50.44	49.49	51.10
Interactions	SEm±				CD 5%			
G	0.399				1.129			
S	0.564				1.597			
T	0.562				1.597			
G X S	0.798				2.258			
GXT	0.798				2.258			
SXT	1.129				3.193			
GXSXT	1.597				4.516			

G =Genotype, T = Treatment, S= Season, DAS= Days after sowing; T_1 = Control,T_2 = Seed hardened (1 µM BL), T_3 = Foliar application(1 µM BL), T_4 = Seed & foliar both application (1 µM BL)

Superoxide dismutase (SOD) is considered as first line of defense against oxidative damage through scavenging of reactive oxygen species (ROS). Increased SOD activity was correlated with increased protection from damage associated with oxidative stress (Asada, 1999) [3]. In response to high temperature, tolerant genotype showed significantly higher SOD activity along with percent increment as compared to susceptible genotype. Interestingly under heat stress, 24-epibrassinolide @ 1µM seed treatment + 1 µM foliar spray acquired higher per cent increment on SOD activity i.e., 3.47% and 3.55%, while it was lesser in control i.e., 2.59% and 2.78% in tolerant genotype (Figure 2). When maize (Zea mays) seedlings treated with BL were subjected to water stress, the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), as well as ascorbic acid and carotenoids contents increased. Rice seedlings exposed to saline stress and treated with BR showed a significant increase in the activities of CAT, SOD and glutathione reductase (GR) and a slight increase in APX.



Po- E= Post-anthesis Early, Po-L= Post- anthesis Late, Pr-E = Pre-anthesis Early,Pr-L= Pre-anthesis Late, C = C-306, H= HUW-468, T_1 = Control,T_2 = Seed hardened (1 µM BL), T_3 = Foliar application(1 µM BL), T_4 = Seed & foliar both application (1 µM BL)

Fig 2: Effect of brassinolide on Superoxide dismutase Activity (units mg^{-1} Protein^{-1} min) of two wheat genotypes (*Triticum aestivum* L.) at pre-anthesis (65 DAS) and post - anthesis (85 DAS)under heat stress

Findings reveal that heat stress hindered growth and development of wheat by decreasing the total chlorophyll content, membrane stability index, nitrate reductase activity. However, though APX and SOD activities were quite higher in tolerant genotype. Among all the treatments, 24-epibrassinolide @ 1µM seed treatment + 1 µM foliar spray shows higher chlorophyll, MSI along withAPX and SOD enzymes activity in tolerant (C-306) genotype than the susceptible (HUW-468) under heat stress.

HomoBL also had a stimulatory effect on the growth of drought tolerant (C-306) and drought-susceptible (HD-2329) wheat (*Triticum aestivum* L.) varieties under stress conditions. Application of homoBL resulted in increased relative water content, nitrate reductaseactivity, chlorophyll content and photosynthesis under both conditions. It also improved membrane stability (lower injury). These beneficial effects resulted in higher leaf area, biomass production, grain yield and yield related parameters in the treated plants. However, homoBL showed a higher activity in drought tolerant wheat variety under water stress conditions. Increased water uptake and membrane stability and higher carbon dioxide and nitrogen assimilation rates in BR-treated plants under stress were correlated with BR-induced drought tolerance. (Sairam R.K. *et al*., 1994) [17]

In conclusion, 24-epibrassinolide (EBR) treatments intolerant genotype provide a greater tolerance potential to survive under heat stress by uniform supply of soluble sugars through enhancing chlorophyll content which leads higher photosynthetic rate or through hydrolysis of reserve starch for continuation of ATP energy production under oxidative respiration. Further, NRA contents which are indicators of nitrogen metabolism were more prominent in susceptible genotype due to lesser MSI as compared to tolerant genotype. The level of antioxidant enzymes (APX and SOD) was moderately higher in tolerant genotype than the susceptible one under heat stress. Certainly, EBR, both pre-seed soaking and foliage applied, establishes prospective significance in ameliorating the harmful effects of heat stress in wheat genotypes especially at post-flowering stage.

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