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Biochemical and physiological constituents and their correlation in wheat (*Triticum aestivum* L.) genotypes under high temperature at different development stages

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Wheat is the most popular and staple food for millions of people. It is severely affected by heat stress in many countries. Vegetative growth and reproductive phases in wheat differ in their sensitivity to temperature. Heat tolerant (GW-190) and Heat susceptible (J-2010-11) genotypes grown up to tillering and grain filling stages and Heat treatments (40°C and 45°C for 2h and 4h) were given using Heating House. After heat treatment samples were collected, the biochemical and physiological analysis such as Protein, Proline, Glycine betaine, Membrane stability, Relative water content, Germination percent, Seed vigour and Heat tolerant index were performed. Protein, proline and glycine betaine were found significantly highest 34.06 mg/g Fr. Wt., 13.70 mg% and 902.24 µg/F. wt respectively in heat tolerant genotype GW-190 at 45°C for 4 h at tillering stage. Membrane stability and relative water content were found significantly highest 56.83% and 86.15 % respectively in heat tolerant genotype GW-190 at tillering stage. Germination percent, Seed vigor and Heat tolerance index were found higher in control group of Heat tolerant GW-190 genotypes. Where, all the biochemical and physiological contents were found lower in heat susceptible J-2010-11 genotype. From the above results it was concluded that GW-190 was heat tolerant genotypes which is suitable for grown in area of high temperature and J-2010-11 was found heat susceptible.

Key words: Relative water content, membrane stability, seed index, heat tolerant index, correlation

INTRODUCTION

Wheat is one of the world's most popular crops. It is the staple food for millions of people and forms an important part of many people's daily diet (Curtis et al., 2002). The yield and quality of cereals are severely affected by heat

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stress in many countries. Wheat genotypes express a differential response to chronic heat as well as a heat shock (Kamal et al., 2010). Temperature requirements and temperature extremes varies widely for different cultivars of the same species, and among species. The reproductive phase of many crop species is relatively more sensitive to heat stress than the vegetative phase (Hall, 1992; Martiniello et al., 2011).

Vegetative growth and the reproductive phase in wheat, differ in their sensitivity to temperature (Almeselmani et al., 2011; Chakrabarti et al., 2011; Karmanenko et al., 2011; Khakwani et al., 2011; Al-Karaki, 2012; Hakim et al.,2012; Hossain et al., 2012; Hossain et al., 2012; Noorka and Teixeira, 2012; Noorka et al., 2013). The total area of wheat production affected by some or other form of heat stress is estimated to be 65 to 70 million ha. Of these, 7 million ha are grown under continual heat stress (Reynolds, et al., 2001). High temperature stress is a major growth restraining factor for most crop plants. Long term or even a temporary exposure to high temperature can change different metabolic functions, thereby affecting various plant parts including leaves, flower buds and roots (Tsukaguchi et al., 2003; Iwaya-Inoue et al., 2004). Most of the changes are noticeable in the cellular membranes. The metabolic changes include changes in the activities or structures of enzymes and biochemical contents. Heat stress affects the enzymes from metabolic compartments such as mitochondria (Nash et al.,1982), cytoplasm (Laurie et al.,1990) or chloroplasts (Dionisio et al., 1999). Thus, high temperature is believed to produce an array of changes in plants. Under heat stress, biochemical and physiological constitutes changed in heat tolerant and heat susceptible genotypes.

MATERIALS AND METHODS

Heat tolerant (GW-190) and heat susceptible (J-2010-11) wheat genotypes were selected from fourteen genotypes for experiment (Ramani et al., 2015). One set was grown up to tillering stage (35 days) and divided into two groups, control and heat treatments. Other set was grown up to Grain filling stage (around 100 days) and also divided into two sets, control and heat treatments. Heat treatments (40°C and 45°C for 2 h and 4 h) were given using Heating House at tillering and grain filling stages and samples were collected under ice condition. After heat treatments the leaf samples were taken out and leaves were weighed and then transferred immediately to the respective extracting medium for biochemical and physiological analysis.

Estimation of total protein

True protein was estimated by the standard method of Lowry et al., 1951. The samples (0.1 ml) and standard BSA (0.5 - 3 ml from 0.1 mg/ml BSA stock) were taken in a series of test tubes and the volume was made up to 3.0 ml with distilled water. Then 5 ml solution-C was added, mixed well and incubated at room temperature for 10 mins. Then 0.5 ml Folin - Ciocalteau reagent was also added mixed immediately and incubated in dark for 30 mins. A reagent blank was prepared by taking only reagents and volume made up with distilled water. Light blue color was observed after incubation which measured at 660 nm in a spectrophotometer. The protein content was calculated by taking Bovine serum albumin as standard.

Estimation of free proline

Proline was estimated by using acid ninhydrin method of Bates et al., 1973. The samples (0.3 ml) and standard proline (0.1 to 0.6 ml from 0.05 mg/ml proline stock) were taken in a series of test tubes and the volume was made up to 1.0 ml with distilled water. Then 2 ml gacial acetic acid and 2 ml acid ninhydrin reagent were added. Then tubes were kept in boiling water bath for 1 hour. The tubes were cooled in running water at room temperature. After that 4 ml toluene was added. The absorbance was recorded from toluene phase at 520 nm in spectrophotometer. The free proline was calculated by taking Proline as standard.

Estimation of glycine betaine

Glycine betaine was estimated by Grieve and Grantta, 1983, 0.5g leaf powder was shaken in 20 ml distilled water for 24h at 25 °C. The samples were filtered and supernatant was used for analysis. 0.5 ml of supernatant and equal volume of 2N H_2SO_4 were kept in ice water for 1 h. 0.2 ml of cold KI-I_2 was added and reactants were gently vortex. The tubes were kept at 0 to 4°C for 16 h and centrifuge at 10,000 rpm for 15 minute at 0°C. Supernatant were discarded. A periodical crystal were dissolved in 9 ml of 1, 2-dichromoethane. After 2h, the absorbance measured at 365nm using glycine betaine as standard.

Estimation of membrane stability

0.2 g of leaves of uniform size from control and treated plants were taken in tubes containing 30 ml of distilled water. The treatment tubes were then covered with plastic wrap and incubated in a thermostatically controlled water bath for 15 min at 53°C. The control tubes were maintained at 25 °C for the same period. After the elevated temperature treatment, the treatment tubes were quickly cooled down to 25°C. Both tubes were then stored in an incubator overnight for 18 h at 10°C to allow the diffusion of electrolytes from the discs. The tubes were then brought to 25 °C, inverted several times to mix the contents and initial conductance was measured using an electrical conductivity meter. After completing the measurement, tubes were covered and kept at 95 °C for 10 min to completely kill the leaf tissue. Then tubes were cooled down to 25 °C, mixed thoroughly, and the final conductance was measured. Membrane stability was calculated as described in Martineau et al., 1979 method. Membrane Stability Index= (1-T1/T2) X 100, Where, T1 = Initial conductance of sample kept at 53°C, T2 = Final conductance of sample kept at 95°C.

Estimation of relative water content

0.2 g fresh leaves were weighed to record fresh weight (FW), followed by dipping half of their portion in Petri dish containing 30 ml distilled water for 12 h. The leaves were blotted to wipe off excess water, weighed to record fully turgid weight (TW), and Subjected to oven drying at 70°C for 8 h to record the dry weight (DW). Relative water content (RWC) was calculated as per formula and expressed as: RWC = [FW-DW] × 100/ [TW-DW] of standard method of Turner, 1986.

Table 1. Biochemical and physiological constitutes of heat tolerant and heat susceptible wheat genotypes at tillering and grain filling stages.

Sr. No	Genotypes	Heat treatment (ċ)	Protein(mg/g.f.wt)				Proline (mg %)				Glycine betaine (µg/f.wt)				Membrane stability (%)				Relative water content (%)			
			Tillering		Grain filling		Tillering		Grain filling		Tillering		Grain filling		Tillering		Grain filling		Tillering		Grain filling	
			Time (h)		Time (h)		Time (h)		Time (h)		Time (h)		Time (h)		Time (h)		Time (h)		Time (h)		Time (h)	
			2	4	2	4	2	4	2	4	2	4	2	4	2	4	2	4	2	4	2	4
1	GW-190	Control	19.49	19.57	11.86	11.66	1.88	1.93	1.59	1.61	185.55	185.93	156.35	156.12	56.83	55.65	53.49	53.23	86.15	86.07	56.53	56.42
		40	24.91	27.07	13.74	18.44	3.58	6.17	3.07	5.22	335.49	644.93	267.09	449.49	52.68	48.84	43.29	37.26	80.01	74.79	46.82	44.61
		45	29.01	34.06	19.65	20.84	11.07	13.70	8.32	11.00	439.72	902.24	342.01	771.96	43.55	31.58	35.80	33.04	67.37	58.41	37.51	29.29
2	J-2010-11	Control	17.88	18.06	11.23	11.66	1.66	1.73	1.30	1.38	150.22	149.83	117.32	117.26	51.23	51.37	45.29	46.72	79.19	79.37	51.73	51.67
		40	20.77	25.68	12.60	13.31	2.86	4.92	2.55	4.52	211.72	556.98	188.92	361.55	42.53	41.22	35.26	31.43	66.79	62.91	45.00	31.24
		45	28.03	29.05	15.14	16.92	8.59	10.19	7.24	9.29	315.95	765.44	227.23	635.16	37.92	37.32	36.96	35.33	53.45	46.14	28.21	23.45
CD@ 5%			0.78		N.S.		N.S.		0.16		N.S.		N.S.		N.S.		N.S.		N.S.		3.31	
CV %			1.91		1.90		0.98		0.21		5.15		6.45		3.95		4.6		2.21		4.69	

*table show the mean values of three replication, N.S. = none significant.

Estimation of germination percent

Seed were surface sterilized with 0.1% HgCl solution for 2 min and then washed with distilled water. Twenty five seeds each were sown on Whatman filter paper No. 1 bed kept in petri dishes and watered with distilled water. These petri dishes were kept for germination at 22°C (Control), 40°C and 45°C in seed germinator for 7 days to germinate. The filter paper beds were moistened whenever necessary. The germination was recorded after 48 hours and after 7 days. The germination percentage and rate of germination was calculated as per formula given by Almaghrabi, (2012).

Germination Percentage (GP) = Ng/Nt × 100,

Where, Ng = Total number of germinated seeds, Nt = Total number of seeds evaluated.

Estimation of seed vigor

Seeds were allowed to germinate for 7 days after 7 days, the seedling length (shoot and root length) was recorded. The seedling vigor was calculated as per the formula described by Buriro et al., 2011.

Seedling Vigor = Germination percentage × Seedling length at 7 days.

Estimation of heat tolerant index

Heat tolerance index (HTI) was calculated as per the formula given by Fernandez (1992). Heat tolerance index (HTI) = $(G_{si} \times G_{pi})/Gp^2$, Where, G_{si} = germination of each genotype under stress condition, G_{pi} = Germination of each genotype under control condition, Gp^2= Average of germination in all genotypes under control condition.

Correlation of biochemical and physiological constitutes

Mean values of biochemical and physiological constitutes were used for correlation analysis using SPSS 16.0 version statistical software.

RESULTS AND DISCUSSION

Results shown in Table 1 to 3 revealed different levels of the biochemical and physiological constitutes and their correlation between heat tolerant and heat susceptible genotypes under high temperature stress at different development stages.

Protein content

Protein was found significantly highest 34.06 mg/g in heat tolerant genotype GW-190 at 45°C for 4 h at tillering stage. The heat susceptible genotype J-2010-11 recorded the lowest (11.23 mg/g. wt.) at grain filling stage. When plants were kept for 2 h, protein content was found lower in both genotypes at both stages as compared to 4 h of heat treatment. All the control plants of heat tolerant GW-190 and susceptible genotypes J-2010-11 showed lower protein as compared to treated plants at both stages. Compared to grain filling stage the protein content was found higher at tillering stage in heat tolerant and heat susceptible

genotypes. As the temperature and duration of temperature treatment increased, the protein was increased in both genotypes at tillering and grain filling stages (Table 1). Plants cope with heat stress in a complex manner, where heat shock proteins (HSPs) which might play a central role in the complex cellular network (Baniwal et al., 2004).

Differential expression of carbohydrate biosynthesis and metabolic pathway enzymes was also inferred in plants that need high energy to cope with heat stress. The responses of rice seedlings to different high-temperature stresses at 35, 40 and 45°C for 48 h. At 35°C, some protective mechanisms were activated to maintain the photosynthetic capability. At 40°C, antioxidative pathways were also active so, the overall protein content increased with temperature (Han et al., 2009). When seven day old rice seedlings encountered high-temperature stress at 45°C, in addition to those induced at 35°C and 40°C, heat shock proteins were effectively induced so the protein content also increased as temperature increased. The appearance of new proteins in wheat shoots subjected to the high temperature stress are heat shock proteins of molecular weights 111, 90, 70, 45, 32, 24 and 8 KDa and they were accumulated under high temperature(Khalid and Devaraj, 2011).

Proline content

Proline was found significantly highest 13.70 mg% in heat tolerant genotype GW-190 at 45°C for 4 h at tillering stage. The heat susceptible genotype J-2010-11 recorded the lowest (1.30 mg%) at grain filling stage. When plants were kept for 2 h, proline content was found lower in both genotypes at both stages as compared to 4 h of heat treatment. All the control plants of heat tolerant GW-190 and susceptible genotypes J-2010-11 showed lower proline as compared to treated plants at both stages. Compared to grain filling stage, the proline content was found higher at tillering stage in heat tolerant and heat susceptible genotypes. As the temperature and duration of temperature treatment increased the proline content was also increased in both genotypes at tillering and grain filling stages (Table 1). Accumulation of certain low molecular mass organic compounds, generally called compatible solutes or osmoprotectants, is a key adaptive mechanism in many plants grown under abiotic stress including extreme temperature (Hare, et al., 1998). A variety of osmolytes may accumulate in different plant species under stress. These include sugars and sugar alcohols (polyols), proline, tertiary and quaternary ammonium compounds and tertiary sulphonium compounds (Sairam and Tyagi, 2004). At high temperature (35°C) the heat tolerant genotypes produced more than double (> 200%) proline than that of 25^{o}C but the heat susceptible genotypes produced less quantity of proline at 35°C when compared to that in heat susceptible genotypes at 25°C (Ahmed and Hasan, 2011). Proline content was remarkably declined at different stages of growth with lowest accumulation at seed hardening stage (Kumar et al., 2012). Compared to heat sensitive genotypes, proline content were higher in tolerant genotypes under late sown conditions (Dhyani et al., 2013).

Glycine betaine

Glycine betaine was found significantly highest 902.24 µg/F. wt in heat tolerant genotype GW-190 at 45°C for 4 h at tillering stage. The heat susceptible genotype J-2010-11 recorded the lowest (117.32 µg/F. wt) at grain filling stage. When plants were kept for 2 h glycine betaine was found lower in both genotypes at both stages as compared to 4 h of heat treatment. All the control plants of heat tolerant GW-190 and susceptible genotypes J-2010-11 showed lower glycine betaine as compared to treated plants at both stages. Compared to grain filling stage the glycine betaine content was found higher at tillering stage in heat tolerant and heat susceptible genotypes. As the temperature and duration of temperature treatment increased the glycine betaine content was also increased in both genotypes at tillering and grain filling stages (Table 1). Glycine betaine is an amphoteric quaternary amine that plays an important role as osmoprotectant in wheat plants and accumulated under a range of abiotic stresses including high temperature. Stress, caused a significant increase in Glycine betaine content of wheat genotypes at tillering and anthesis stages. The responses of wheat genotypes to drought, heat stress condition and analyzed mechanism of glycine betaine were involved in an improvement of wheat tolerance (Wang et al., 2010).

Membrane stability

Membrane stability was found significantly highest 56.83% in heat tolerant genotype GW-190 at tillering stage. The heat susceptible genotype J-2010-11 recorded the lowest (31.43%) at grain filling stage. When plants were kept for 2 h, membrane stability was found higher in both genotypes at both stages as compared to 4 h of heat treatment. All the control plants of heat tolerant GW-190 and susceptible genotypes J-2010-11 showed higher membrane stability when compared to treated plants at both stage. Compared to grain filling stage the membrane stability was found higher at tillering stage in heat tolerant and heat susceptible genotypes. As the temperature and duration of temperature treatment increased the membrane stability was also decreased in both genotypes

Table 2. Germination per cent, Seed vigor and heat tolerant index of heat tolerant and heat susceptible wheat genotypes under high temperature.

Sr. No	Genotypes	Heat treatment (ċ)	Germination (%)	Seed vigor	Heat tolerant index
		Control	100	1945	1.00
1	GW-190	40	92.67	1190	0.97
		45	90.67	980	0.89
		Control	100	1871	1.00
2	J-2010-11	40	70.33	820	0.87
		45	62.67	658	0.67

at tillering and grain filling stages (Table 1). Based on membrane thermostability (MT) test, varieties took maximum heat killing time and were classified as heat tolerant, three varieties are moderately tolerant and the rest took the shortest heat killing time and are considered as heat sensitive (Skider, et al., 2001). Thermo tolerant wheat genotypes showed higher Membrane stability index when compared to thermo sensitive wheat genotypes at different development stages (Mohammadi, et al., 2007). The Membrane thermostabilty was significantly affected by different growth stages. Membrane stability of wheat genotypes decreased during the later developmental stages. Membrane stability of flag leaf at the early milk stage was significantly correlated with grain yield (Yildirim et al., 2009).

Relative water content

Relative water content was found significantly highest 86.15% in heat tolerant genotype GW-190 at tillering stage. The heat susceptible genotype J-2010-11 records the lowest (23.45%) at grain filling stage. When plants were kept for 2 h, relative water content was found higher in both genotypes at both stages as compared to 4 h of heat treatment. All the control plants of heat tolerant GW-190 and susceptible genotypes J-2010-11 showed higher relative water content compared to treated plants at both stage. Compared to grain filling stage the relative water content was found higher at tillering stage in heat tolerant and heat susceptible genotypes. As the temperature and duration of temperature treatment increased the relative water content was also decreased in both genotypes at tillering and grain filling stages (Table 1). The rise in temperature during late sowing significantly decreased leaf relative content at 8 and 23 days after anthesis in wheat (Sairam et al., 2000). This is in accordance with present results. Wheat genotypes had lower relative water content values at early sowing and late sowing date compared to normal sowing date (Bhesaniya, 2005). Relative water content in root was not affected by higher temperature at all stages of wheat seedling. While coleoptiles relative water content decreased at all stages of seedling development (Savicka and Skute, 2012).

Germination

The mean germination percentage recorded significantly higher 100% germination in control of GW-190 followed by (92.67%) and (90.67%) at 40°C and 45°C respectively while significantly lowest value was observed with genotype J-2010-11 (62.67%) at 45°C (Table 2). As the temperature increased the germination of seeds decreased in both genotypes but the ratio of decreased in heat susceptible genotype J-2010-11 was more. Germination characters increased with increasing temperature from 15 to 35°C in all wheat genotypes but heat tolerant ability was not based on speed of germination (Hasan et al., 2004). Germination characteristics in heat tolerant cultivars increased with increasing temperature as compared to heat sensitive cultivars (Sikder and Paul, 2010). However, high temperature treatment at 30 to 40°C, germination was reduced in rice (Prasanth et al., 2012) and at 27°C and 30°C temperature treatments, germination was reduced in barley (Ghazi, et al., 2007).

Seed vigor

The mean seed vigor was recorded significantly higher (1945) in control of GW-190 followed by (1190) and (980) at 40°C and 45°C respectively while significantly lowest value was observed with genotype J-2010-11 (658) at 45°C. As the temperature increased the seed vigor decreased in both genotypes but the ratio of decreased in heat susceptible genotype J-2010-11 was more (Table 2). Seedling vigor index was increased with increasing temperature (10 to 30°C) and maximum was recorded at 20°C and 30°C (Buriro et al., 2011). The seedling vigor is adversely affected by heat stress (36°C) which resulted in reduced shoot and root length (Grass and Burris, 1995).

Generally shoot and root growth of heat susceptible

Table 3. Pearson correlations between biochemical and physiological constitutes in heat tolerant and susceptible wheat genotypes under high temperature stress.

	Protein	Proline	Glycine betaine	Membrane stability	Relative water content	Germination percent	Seed vigor	Heat tolerance index
Protein	1.000							
Proline	0.960**	1.000						
Glycine betaine	0.994**	0.950**	1.000					
Membrane stability	-0.750*	-0.808*	-0.803*	1.000				
Relative water content	-0.721	-0.818*	-0.768*	0.962**	1.000			
Germination percent	-0.368	-0.452	-0.454	0.801*	0.861*	1.000		
Seed vigor	-0.735	-0.732*	-0.804*	0.944**	0.920**	0.868*	1.000	
Heat tolerance index	-0.531	-0.661	-0.580	0.811*	0.934**	0.913**	0.817*	1.000

*correlation is significant at 0.05, ** correlation is significant at 0.01 (1-tailed).

genotype in relation to length is affected more than thoseof heat tolerant genotype at higher temperature-35°C (Hasan et al., 2004).

Heat tolerant index

The mean Heat tolerant index was recorded significantly higher (1.00) in control of GW-190 followed by (0.97) and (0.89) at 40°C and 45°C respectively while significantly lowest value was observed with genotype J-2010-11 (0.67) at 45°C. As the temperature increased the seed vigor decreased in both genotypes but the ratio of decreased in heat susceptible genotype J-2010-11 was more (Table 2). Stress tolerance index (STI) and stress susceptible index (SSI) were more accurate criteria for the selection of heat tolerant and high yielding genotypes by growing under normal and stress condition (Khodarahmpouret et al., 2011).

Correlation analysis

One heat tolerant and one heat susceptible genotypes GW-190 and J-2010-11 respectively were used for correlation analysis (Pearson correlation test) including biochemical and physiological parameters as listed earlier (Table 3). Results of correlation analysis under heat stress condition showed that, protein had highly significant and positive correlation with proline and glycine betaine content whereas negatively and significant correlation with membrane stability. Also protein content was negatively and non-significantly correlated with relative water content, germination percentage, seed vigor and heat tolerant index. Proline content had high significant positive correlation with glycine betaine and membrane stability, while relative water content and seed vigor were negatively and significantly correlated whereas, germination percent and heat tolerant index were negatively and non-significantly correlated. Glycine betaine had significant negative correlation with membrane stability, relative water content and seed vigor, Membrane stability had highly significant positive correlation with relative water content and seed vigor, and positive significant correlation with germination and heat tolerant index. Relative water content was positively and significantly correlated with germination percent, seed vigor and heat tolerant index. Seed vigor was positively correlated with heat tolerant index (Table 3). Result clearly indicated that relative water content and membrane stability was positively correlated and more effective indicator for screening of heat tolerant genotypes. Protein content, proline and glycine betaine content were positively correlated, they were higher in heat tolerant genotypes. Membrane stability index was good indicators of stress tolerance (Sairam and Srivastava, 2001).

Conclusion

Some abiotic constraints such as high temperature, cold, drought, high concentrations of toxic minerals and salinity results in, severe loss of crop yield and quality. At both tillering and grain filling stages, Protein, proline, glycine betaine content were higher in tolerant genotype as compared to susceptible genotypes. Also these content were increased with increase in temperature in both genotypes. But the amount of increase was higher

in tolerant genotype when compared to susceptible genotypes. Membrane stability, relative water content, heat tolerant index, seed vigor and germination were higher in tolerant genotypes when compared to susceptible genotypes but as temperature increased, they were decreased in both the genotypes at both stages. Also biochemical and physiological content was significantly co-related.

Conflict of interest

The authors have not declared any conflict of interest

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