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Physiological changes and study of comparative proteome of wheat (*Triticum aestivum* L.) by 2D electrophoresis under temperature stress

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Abstract
Two selected wheat (*Triticum durum* L.) genotypes consisted of one heat tolerant genotype GW-190 and one heat susceptible genotype J-2010-11 were used to investigate physiological character like relative water content, membrane stability and proteomics changes by comparative proteomics approach at grain filling stages. Relative water content and membrane stability were marker for comparison between heat tolerant and heat susceptible wheat genotypes. Relative water content and membrane stability in wheat leaves were higher in heat tolerant compare to susceptible genotype. Protein was extracted for 2D electrophoresis analysis from the leaves of heat tolerant and heat susceptible wheat genotypes at grain filling stages after heat stress at 40 °C and 45 °C for 2 h and 4 h of treatment duration. These protein extract were subjected to 2D electrophoresis for comparative analysis between heat tolerant and heat susceptible genotype under heat stress condition. Spots were analyzed by Platinum Master software. At grain filling stage, Heat tolerant genotype GW-190 displayed 48 protein spots between molecular weight ranging with 3.5 to 205 kDa. Out of 48 spots, 31 spots were matched with control group. Four spots of high intensity present in the control of heat tolerant genotype might be genotype specific or heat shock specific spots. A small heat shock proteins (sHSP) like Putative low molecular weight heat shock protein (24.1 kDa/6.88), Putative chaperonin 21 precursor (23.2kDa/6.88), 16.9 kDa class I heat shock protein 2 (16.9/6.18).

Keywords: Heat tolerant, Heat susceptible, 2D Electrophoresis

Introduction
Proteins are compounds of elementary substance for all functions in the cell. It is well known that alteration of gene expression is always involved in preparing plants for an existence under stress. Exposure of plants to elevated temperature for short term (heat shock) results in a complex set of gene expression and selective translation of mRNA encoding heat shock proteins (HSPs), thereby, enhancing thermotolerance and improving cellular survival under subsequent heat stress (Gong *et al*., 2001) [1]. In higher plants, these HSPs function as molecular chaperons that promote the degradation of misfolded proteins, aid in refolding of denatured proteins and prevent them from aggregation and resolubilize the proteins that have already aggregated (Boston *et al*., 1996) [2]. A large number of studies reveal a positive correlation between induction of HSPs and acquisition of thermotolerance. However, the definite mechanism that imparts heat tolerance to plants still remains to be elucidated and there is pressing need to understand the precise physiological, biochemical and molecular mechanisms of heat tolerance in plants so that these can be introduced in the plant of interest through genetic engineering. Heat stress leads to drastic change in relative water content, membrane stability, biochemical contents and protein expression (Hernandez *et al*., 2004) [3]. The response mechanism of wheat to elevated temperature would help the development of wheat cultivars that perform better under heat stress. Therefore, present study was undertaken to analyze the changes in relative water content, membrane stability and protein expression in wheat at tillering and grain filling stages under heat stress.

Material and Methods
Plant growth condition, sampling description and heat treatments
One heat tolerant GW-190 and heat susceptible J-2010-11 wheat genotypes were selected. They were grown in germination bag filled with soil. Set was grown up to grain filling stage (100 days) and divided into two groups, control and heat treatments. Heat treatments (40°C and 45°C for 2h and 4h) were given using handmade Heating House 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 various analysis. An experiment was carried at Department of Biochemistry, Junagadh Agriculture University, Junagadh.

Relative Water content and Membrane stability (%)

Relative water content was estimated as per formula and expressed as RWC = [FW-DW] X 100/ [TW–DW] by standard method Turner 1986 [4]. Membrane stability was estimated from leaf as per method described by Martineau *et al.*, 1979 [5].

Protein Profiling By 2D Electrophoresis

Extraction of Protein for 2D electrophoresis

TCA precipitation was based on the method adopted with some modification (Damerval *et al.*, 1986 and Jiang *et al.*, 2008)[6-7] and GE health care guideline was used when ever needed. Brief, fresh wheat leaf tissue (500 mg) was finely powdered in liquid nitrogen with a precooled ceramic mortar and pestle. The resulting powder was suspended in 500 µl cool rehydration buffer (8M Urea, 2% CHAPS, 7 mg of DTT/2.5ml buffer) Once it is finely homogenized, the volume is made up to 1.5 ml. The mixture was incubated at Room temperature for 10 minute. Centrifuge the mixture at 12000rpm for 30 minute. Take supernatant 500 µl and add 10 % TCA in acetone allow precipitation of protein at -20 °C for overnight. Next day centrifugation at 12000 rpm for 15 min at 4°C, the protein pellet was washed four-five times with chilled 90% acetone. After centrifugation at 12000 rpm for 15 min between rinses, the supernatant was discarded and the pellet was subjected to air dry. The dried powder was solubilised in lysis buffer (8M urea, 2% CHAPS, 2% ampholyte pH 4–7(GE Healthcare Bio-Science, Little Chalfont, UK), 0.2 % DTT, 10 µl/ml Protease inhibitor mix). This is then stored overnight at 4°C for protein extraction. Then protein was loaded onto isoelectrofocusing (IEF) polyacrylamide gels (IGP Strip) with rehydration method.

Rehydration of IGP Strip

Slide the protective lid completely off the Immobiline dry strips reswelling tray. Ensure that the tray is clean and dry. Level the tray by turning the levelling feet until the bubble in the spirit level is cantered. 24 cm IGP strips were rehydrated in rehydration buffer (8M Urea, 2% CHAPS, 1% Bromophenol Blue, 0.5 % IGP buffer (pH4-7), Add 7 mg DTT per 2.5 ml rehydration solution at time of use) for at least 20 h in 400 µl rehydration buffer based on length of strips. Remove the protective cover foil from the Immobiline dry strips gel. Be careful not to trap bubbles under the Immobiline dry strips. Overlay each immobiline dry strips with Mineral oil to cover dry strips to prevent evaporation and urea crystallization. Slide the lid onto the immobiline dries strips reswelling tray and allow the immobiline dry strips to rehydrate at room temperature. A minimum of 10-20 h is required for rehydration; overnight is recommended. Transfer the immobiline drystrips from the reswelling tray to the manifold by using a pair of forceps. Place the immobiline drystrips with gel side up and with the acidic end of the strips oriented toward the anodic side of the instrument. Place a pre moist paper wick electrode onto the acidic and basic ends of the gel. Clip down the electrodes firmly onto the electrode papers. Ensure that there is good contact between the paper electrodes and the metal (Han *et al*., 2009) [8].

First dimensional software running protocol

Start the Ettan IPGphor 3 control software and turn on the Ettan IPGphor 3 instrument. Connect the software with the IPGphor 3 instrument on which the run is to be made.Set protocol using the advanced mode protocol selection as mention below:

Step	Mode	Voltage (V)	Time (Hour) Hourhouhour
1	Step	200	1:00
2	Step	500	7:00
3	Step	1000	1:00
4	Gradient	8000	8:00
5	Step	8000	5:00
6	Step	500	4:00

Note: Do not programme the Ettan IPGphor IEF unit to deliver more than 75 µA per Immobiline dry strips.

Start run by clicking the start button in the Ettan IPGphor 3 Control software. The selected protocol is now downloaded to the selected instrument and the run is started. After completion of the run equilibrate Immobiline dry strips prior to carried out the second dimension separation.

Equilibration of focused Immobiline IGP strip: SDS equilibrium Buffer

Reagent	Final Concentration	Amount
Urea	6M	72.1 g
Tris- HCl pH 8.8	75 mM	10.0 ml
Glycerol (87% w/w)	29.3 % (v/v)	69 ml (84.2 g)
SDS	2% (w/v)	4.0 g
1% Bromophenol blue	0.002%(w/v)	400 µl
Double Distilled water	-	to 200ml

Add Dithiothritol (DTT: 100mg/10ml) and Iodoacetamide (250mg/ 10ml) into working SDS equilibrium for first and second equilibration respectively for 15 minute each, just prior to second dimension run.

First equilibrium

Add 10 ml of the DTT-containing equilibration solutionto to each tube. Incubate the strips for 15 min with gentle agitation. Do not over equilibrate, as proteins can diffuse out of the strip during this step.

Second equilibrium

Add 10 ml of the Iodoacetoamide-containing equilibration solution to each tube. Incubate the strips for 15 min with gentle agitation. Do not over equilibrate, as proteins can diffuse out of the strip during this step.

Second dimension Separation (SDS PAGE)

The separation of protein in this method is based on the size of the protein molecules by 12% SDS PAGE by Sambrook *et al*., 1998 [9].

Placement of IGP strip for second dimensional running

Fill the glass plant with 12% gel and place in the Ettan DALT cassette rack. There was no need of stacking gel. Holding one end of the Immobiline drystrip with forceps, carefully place the Immobiline Dry Strip in-between the two glass plates of the gel. Using a thin plastic spacer, push against the plastic backing of the immobiline drystrip (not the gel itself) and slide the strip between the two glass plates until it comes into contact with the surface of separating gel.

Sealing of IPG strip with Agarose: 0.5 % Agarose overlay Solution

Reagent	Quality	Final Concentration
SDS Electrophoresis running buffer	100 ml	-
Low melting point agarose	0.5 g	0.5 %
1% Bromophenol blue stock	200 µl	0.002 %

Before loading the IPG strip on gel, electrode buffer was poured into the electrophoresis chamber. Formation of bubbles was avoided during sealing the IPG strip on the separating gel. The electrophoresis unit was connected with the power supply. The current was turned on allowing 80 V for initial 20 minutes until the sample travels through the IPG strip into separating gel. Then current was increased up to 150 V till the tracking dye reach to the bottom.

Removal of the gel and gel analysis

When the tracking dye reached the end of the running gel after complete separation of molecules, power supply was turned off. The gel was gently removed from the space between the plates, immersed in staining solution contained in a tray. After sufficient incubation period, the gel was distained by adding the detaining solution followed by Scanning of gel by Typhoon FLA Scanner. Analysis of gels was done by Platinum Master software provided by GE healthcare.

Results and Discussion

Effect of heat stress on membrane stability

Membrane stabiltiy was found significantly highest 53.54% in heat tolerant genotype GW-190 at grain filling stage. The heat susceptible genotype J-2010-11 recorded the lowest (29.08%) at grain filling stage. When plants were kept for 2 h, membrane stability was found higher in both genotypes 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 compared to treated plants. As the temperature and duration of temperature treatment increased the membrane stability was also decreased in both genotypes at 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 as moderately tolerant and the rest three varieties took the shortest heat killing time and considered as heat sensitive. Thermo tolerant wheat genotypes showed higher MSI compared to thermo sensitive wheat genotypes at different development stages (Yildirim *et al.*, 2009) [10]. The Membrane thermostabilty was significantly affected by different growth stages.

Effect of heat stress on relative water content

Relative water content was found significantly highest 56.53% in heat tolerant genotype GW-190 at grain filling stage. The heat susceptible genotype J-2010-11 recorded the lowest (23.45%) at grain filling stage. When plants were kept for 2 h, relative water content was found higher in both genotypes 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. As the temperature and duration of temperature treatment increased the relative water content was also decreased in both genotypes at 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 (Bhesaniya, 2005) [11].

Protein profiling of heat tolerant and heat susceptible genotypes at grain filling stage

Proteomic study makes essential path between transcriptome and metabolome (Wang *et al*., 2004 and Gray and Health, 2005) [12-13]. Proteomics is useful to understand the biochemical pathways and their responses against stress condition. At grain filling stage, total 972 protein spots were present in control and treated genotypes. Out of 972 protein spots, 231 protein spots were present in heat tolerant and heat susceptible genotypes under control condition. Out of 231 spots, 48 spots were present in control of heat tolerant genotype GW-190 while 184 spots present in control of heat susceptible genotype J-2010-11. Between control of both the genotypes, out of 231 spots, 31 spots were matched with each other. Out of 972 spots, 740 spots were present under heat stress condition in heat tolerant and heat susceptible genotypes. Out of 740 spots, 436 spots were present in treated group of heat tolerant genotype GW-190 while 304 spots were present in treated group of heat susceptible J-2010-11. Out of 740 spots, 106 spots were matched with treated group of heat tolerant and heat susceptible genotypes. Out of 972 spots, 137 spots were matched between control and treated group of heat tolerant and susceptible genotypes (Table 2).

Protein profiling of heat tolerant genotype GW-190 at grain filling stage

Control

The 2-D electrophoresis result of proteins was given in fig.1a. Heat tolerant genotype GW-190 displayed 48 protein spots between molecular weight ranging with 3.5 to 205 kDa. Out of 48 spots, 31 spots were matched with control group. Out of 48 spots, 21 spots were with the molecular masses ranging with 43.0 to 97. 4 kDa, while 27 protein spots with molecular weight ranging from the 3.5 to 29.0 kDa were present under control condition. A small heat shock proteins (sHSP) like Putative low molecular weight heat shock protein (24.1 kDa/6.88), Putative chaperonin 21 precursor (23.2kDa/6.88), 16.9 kDa class I heat shock protein 2 (16.9/6.18) were reported by Han *et al*., 2009 [8].Similar ranges of spots were found in control of heat tolerant genotype.

Heat treatment of 40 °C for 2 hour

Protein profiling of heat tolerant genotype GW-190 at 40 °C for 2 h was presented in fig.1b. Heat tolerant genotype GW-190 had expressed total 148 protein spots between molecular weight ranging from 3.5 to 205 kDa and 106 spots were matched with treated group. Out of 148 spots, 64 spots were present with molecular masses ranging from 43.0 to 97.4 kDa. Five spots of high intensity were found in same ranges which were not found in control of heat tolerant genotype. Out of 148 spots, 84 spots were present with molecular weight ranging from the 3.5 to 29.0 kDa. Twelve spots of high intensity were found in lower molecular weight region. Compared to control, in heat stress condition expression of spots were increased. Higher molecular weight protein spots showed the differential expression pattern in treated genotype which might be responsible for the stronger heat tolerance.

Heat treatment of 40 °C for 4 hour

Protein profiling of heat tolerant genotype GW-190 at 40 °C for 4 h was presented in fig.2a. Heat tolerant genotype GW-190 showed total 69 spots with molecular weight ranging from 3.5 to 205 kDa. Out of 69 spots, 36 spots were found with molecular weight ranging from 43.0 to 205 kDa that was high molecular weight heat stress responsive protein and 33

spots were presented with molecular weight from 3.5 to 29.0 kDa.

Heat treatment of 45 °C for 2 hour

Protein expression of heat tolerant genotype GW-190 at 45 °C for 2 h was given in fig.2b. Total 94 spots were detected with molecular weight ranging from 3.5 to 205 kDa. Out of 94 spots, 10 spots were matched with other treated group. Out of 94 spots, 31 spots were detected in range of 43.0 to 97.4 kDa and around 63 spots were found between 3.5 to 29.0 kDa. Two spots of high intensity of lower molecular weight were found between pH 6 to 7 while the expression of higher molecular weight proteins were decreased as duration of heat treatment increased. Under high temperature, expression of small molecular weight sHSP increased which might be responsive for heat tolerance.

Heat treatment of 45 °C for 4 hour

Protein expression of heat tolerant genotype GW-190 at 45 °C for 4 h was presented in fig. 3a. Total 125 spots were identified with molecular weight ranging from 3.5 to 205 kDa. Out of 125 spots, 42 spots were matched with treated group and 54 spots were found in higher molecular weight range that 43.0 to 97.4 kDa. and 71 spots were found with molecular weight ranging from 3.5 to 29.0 kDa. Higher molecular weight and lower molecular weight protein spots were presented in 5.5 to 7 pH range. For qualitative and quantitative study, genotype showed the significant results of protein expression under stress condition showing the ability of genotype to heat tolerance.

Protein profiling of heat susceptible genotype J-2010-11 at grain filling stage

Control

Protein profiling of heat susceptible genotype J-2010-11 were presented in fig.3b. Heat susceptible genotype showed 184 spots present between molecular weight ranging from 3.5 to 205 kDa. Out of 184 spots, 31 spots were matched with control of heat tolerant genotype GW-190. Out of 184 spots, 76 spots were present with molecular weight ranging from 43.0 to 97.4 kDa while 108 spots were present with molecular weight ranging from 3.5 to 29.0 kDa. Compared to control of heat tolerant genotype GW-190, heat susceptible genotype J-2010-11 showed increase no. of spots that might be genotype specific spots.

Heat treatment of 40 °C for 2 hour

Protein expression of heat susceptible genotype J-2010-11 at 40 °C for 2 h was presented in fig. 4a. Total 74 spots were found in treatment at 40 °C for 2 h of heat susceptible genotype J-2010-11. Out of 74 spots, 38 spots were matched with treated group. Out of 74 spots, 12 spots were found with molecular weight ranging from 43.0 to 97.4 kDa while 62 spots were present with molecular weight ranging from 3.5 to 29.0 kDa.

Heat treatment of 40 °C for 4 hour

Protein profiling of heat susceptible genotype J-2010-11 at 40 °C for 4 h was presented in fig. 4b. Total 109 spots were detected with molecular weight ranging from 3.5 to 205 kDa. Out of 109 spots, 11 spots were matched with treated group and 30 spots were found in range of 43 to 97.4 kDa molecular weight proteins, while 79 spots were present with molecular weight ranging from 3.5 to 29.0 kDa.

Heat treatment of 45°C for 2 hour

Protein expression profile of heat susceptible genotype J-2010-11 was given in fig. 5a. Total 45 spots were found with molecular weight ranging from 3.5 to 205 kDa. Out of 45 spots, 23 spots were present with molecular weight ranging from 3.5 to 29.0 kDa. and 22 spots with molecular weight ranging from 43 to 205 kDa.

Heat treatment of 45°C for 4 hour

Protein profiling of heat susceptible genotype J-2010-11 was presented in fig. 5b. Total 76 spots were found with molecular weight ranging from 3.5 to 205kDa. Out of 76 spots, 32 spots were matched with the treated group. Out of 76 spots, 28 spots were found with molecular weight ranging from 43 to 97.4 kDa while 48 spots were found with molecular weight ranging 3.5 to 29.0 kDa.

Heat stress is associated with enhanced risk of protein folding and denaturation of several intracellular and membrane complexes. Heat stress leads to increased expression of several proteins with chaperon function and heat shock proteins (HSP110, HSP90, HSP70, HSP60 and sHSPs (Khalil *et al.,* 2009) [14]. Present finding were in accordance with earlier work published by Han *et al* who reported that small heat shock proteins (sHSP) like Putative low molecular weight heat shock protein (24.1 kDa/6.88), Putative chaperonin 21 precursor (23.2kDa/6.88), 16.9 kDa class I heat shock protein 2 (16.9/6.18) were found to be expressed under heat stress condition (Baniwal *et al.,* 2004) [15]. Khalil and his colleague carried out two successive seasons to alleviate the harmful effects of high temperature stress (35°C ± 2) on wheat cultivar (Giza 168) by the application of arginine or putrescine (0.0, 1.25 and 2.5 mM). 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 (Khalil *et al.,* 2009) [14].

Proteins such as RuBisCO activase and proteins related to lignification were affected by high temperature in the range from 35 °C to 45 °C. Different strategies were adopted when exposed to the different high temperatures; the higher temperature, the more protection machineries were involved. Slight high-temperature stresses at 35 °C, some protective mechanisms are activated to maintain the photosynthetic capability. Certain antioxidative pathways are also active if rice seedlings suffer a high-temperature stress at or above 40 °C. Once rice seedlings suffer an intense high-temperature stress at 45 °C, besides the antioxidative pathways and photosynthesis protection processes, HSP-related protection mechanisms are induced (Han *et al*., 2009) [8].

Conclusion

Relative water content and membrane stability is a good indicator against stress condition. Relative water content and membrane stability were higher in heat tolerant genotype GW-190 compared to heat susceptible genotype J-2010-11. At grain filling stage, heat tolerant genotype GW-190 showed total 148 protein spots at 40°C for 2 h duration while spots were decreased (94 spots) at 45°C for 2h in heat tolerant genotype GW-190. As per increase in temperature the number of protein spots decreased in heat tolerant genotype GW-190. As longer the duration of period, spots were higher in heat susceptible genotype J-20101-11 (109 spots) at 40°C for 4h compared to heat tolerant genotype GW-190,while this was true for heat tolerant genotype GW-190 at 45°C.

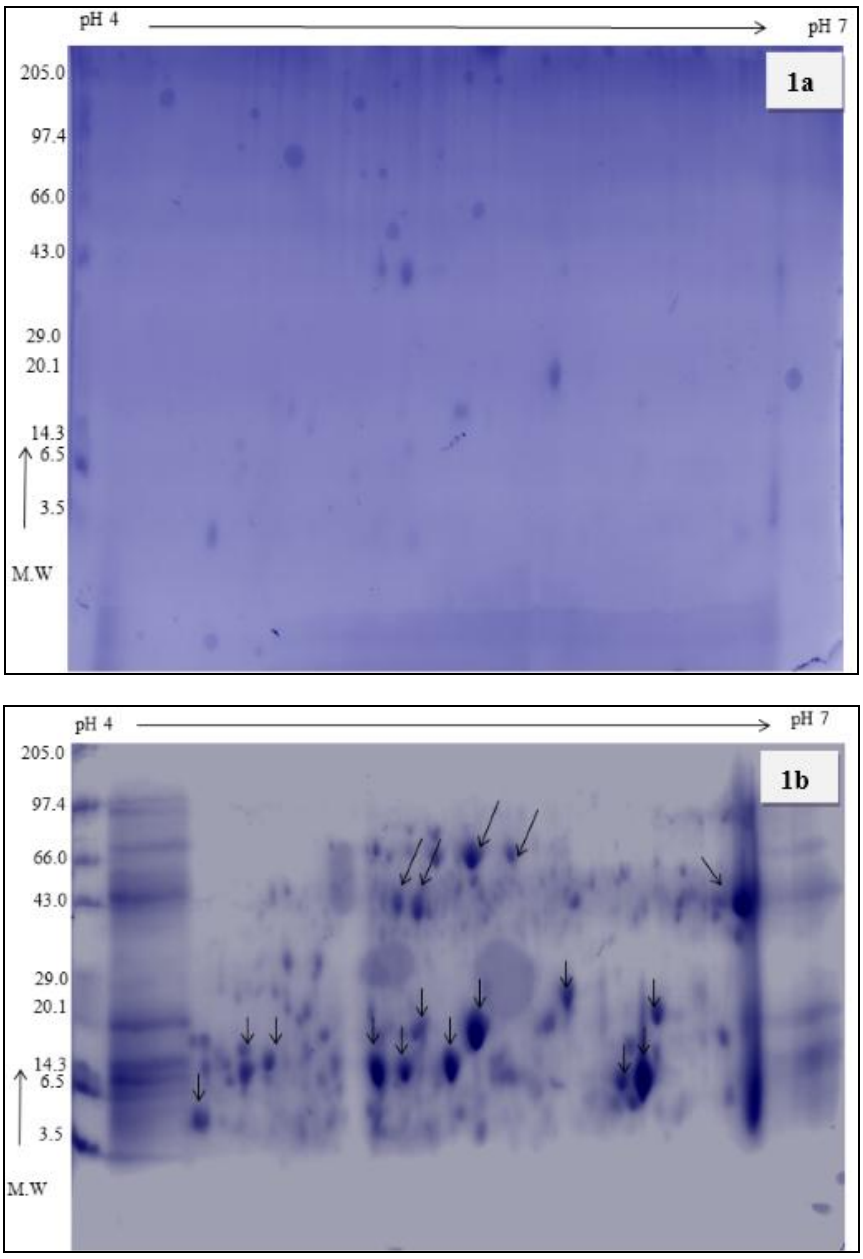
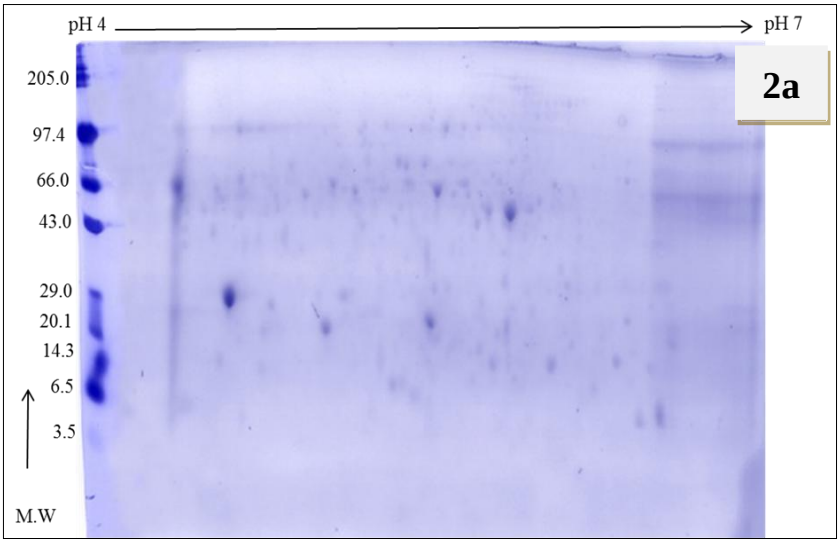


Fig 1: Protein profiling of heat tolerant genotype GW-190 under heat stress at grain filling stage by 2D electrophoresis (a) Control (b) 40°C for 2h



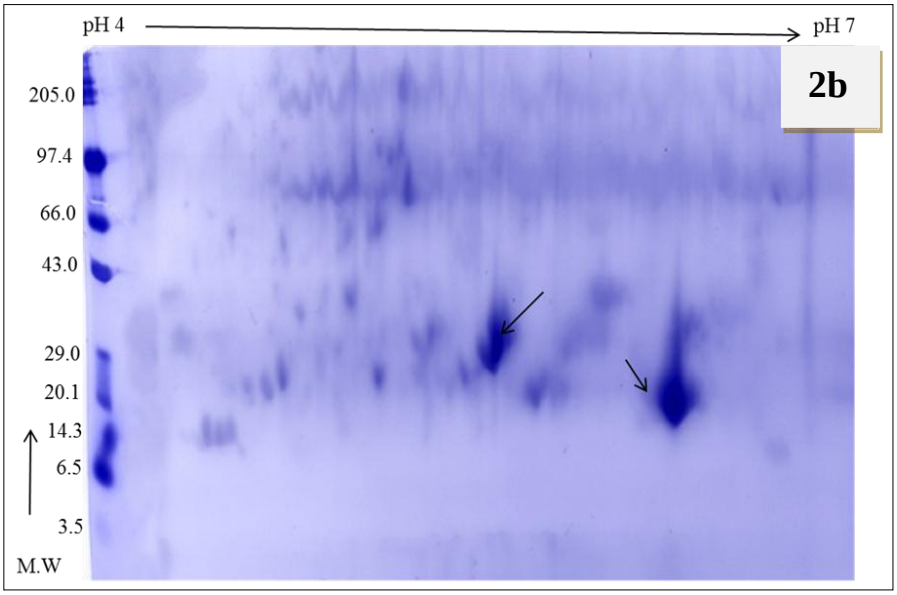
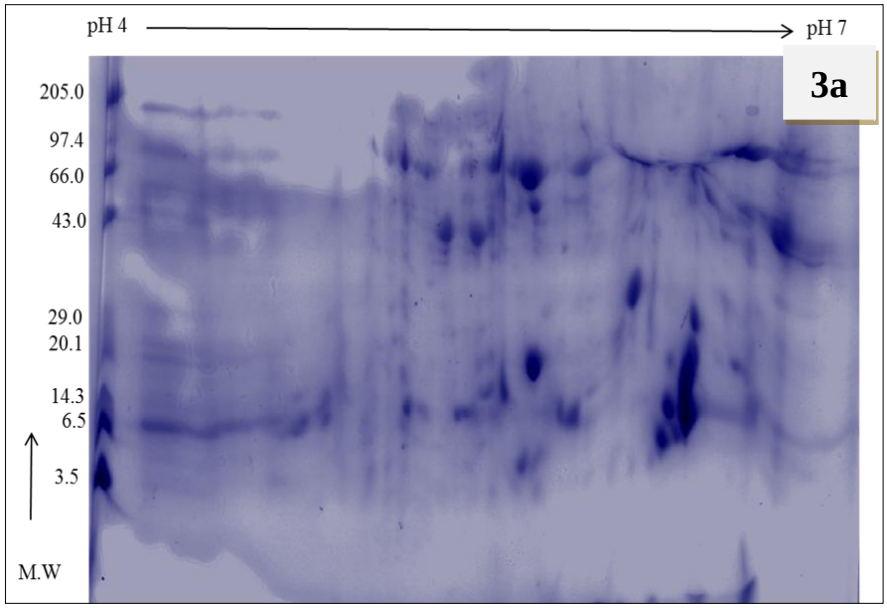


Fig 2: Protein profiling of heat tolerant genotype GW-190 under heat stress at grain filling stage by 2D electrophoresis (a) 40°C for 4h (b) 45°C for 2h.



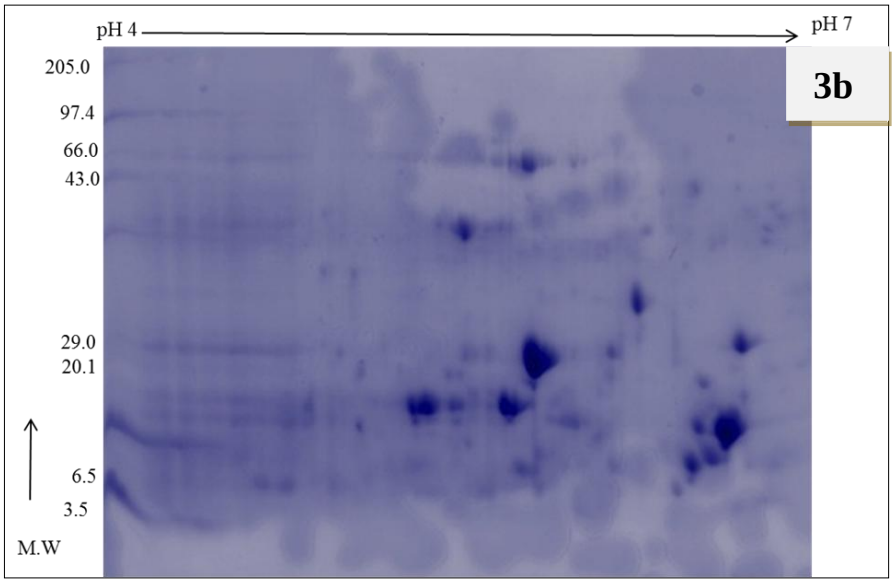


Fig 3: Protein profiling of heat tolerant genotype GW-190 (a) 45°C for 4h and Heat susceptible J-2010 for (b) Control at grain filling stage by 2D electrophoresis.

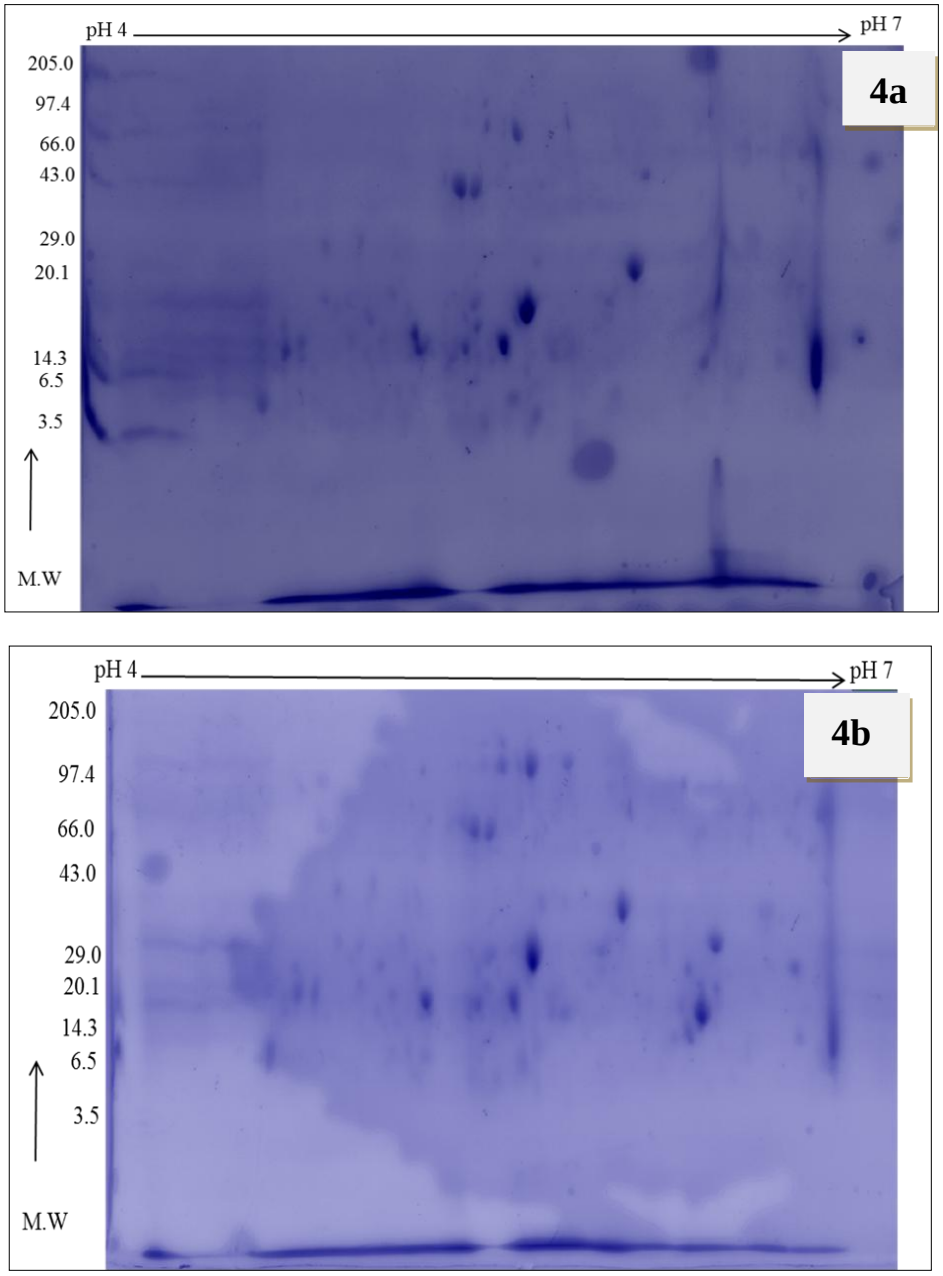
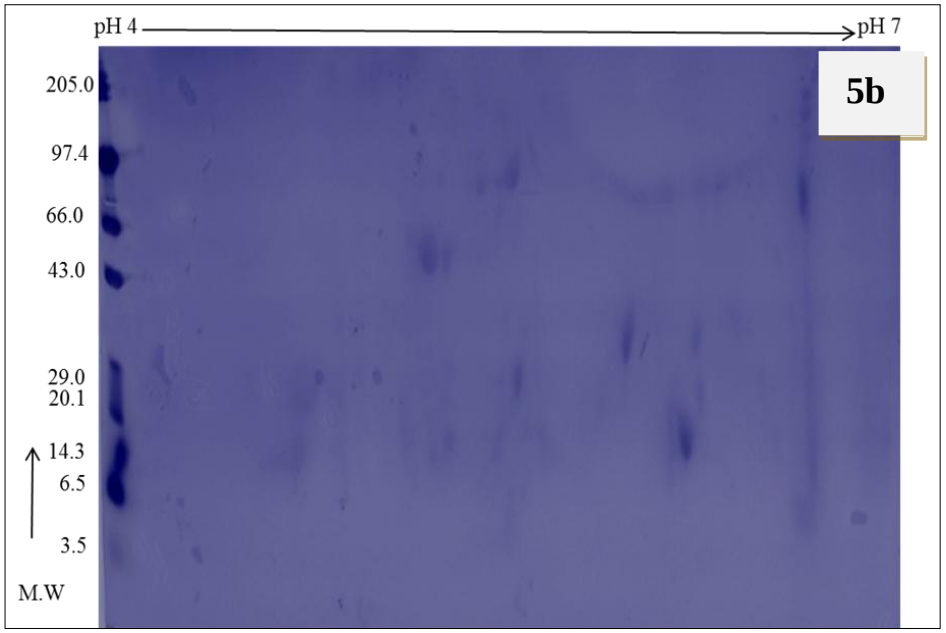


Fig 4: Protein profiling of heat susceptible genotype J-2010-11 under heat stress at grain filling stage by 2D electrophoresis (a) 40°C for 2h (b) 40°C for 4h.



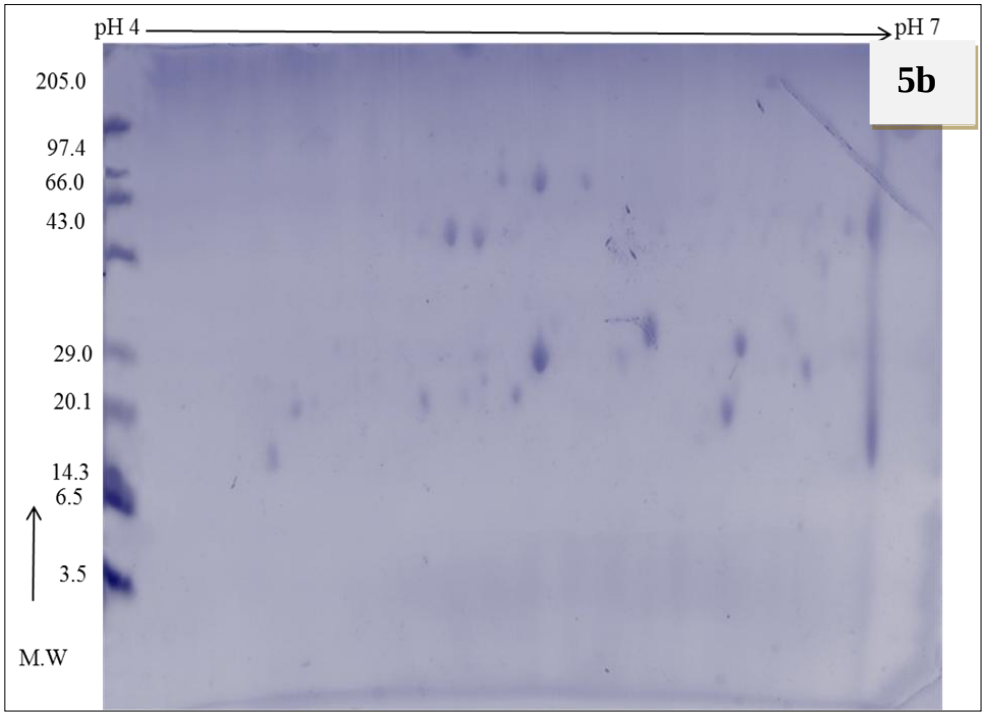


Fig 5: Protein profiling of heat susceptible genotype J-2010-11 under heat stress at grain filling stage by 2D electrophoresis (a) 45°C for 2h (b) 45°C for 4h.

Table 1: Relative water content and Membrane stability of heat tolerant and heat susceptible genotypes under heat stress.

Sr. No.	Genotypes		Relative water content (%)			Membrane stability (%)		
		Heat treatment (HT) (°C)	Time (T) (hour)		MEAN(HT)	Time (T) (hour)		MEAN(HT)
			2 (T1)	4 (T2)		2 (T1)	4 (T2)	
1	GW-190 (G1)	Control	56.53	56.42	56.48	53.49	53.23	53.36
		40 °C	46.82	44.61	45.72	43.29	37.26	40.28
		45°C	37.51	29.29	33.40	35.80	33.04	34.42
		MEAN G1	46.96	43.44	45.20	44.19	41.18	42.69
2	J-2010-11(G2)	Control	51.73	51.67	51.70	45.29	46.72	46.01
		40 °C	45.00	31.24	38.12	35.26	31.43	33.35
		45°C	28.21	23.45	25.83	30.33	27.83	29.08
		MEAN G2	41.65	35.45	38.55	36.96	35.33	36.14
		MEAN T	44.30	39.45		40.58	38.25	
			CD @5%	CV %	SEm±	CD @ 5%	CV %	SEm±
	GXHTXT		3.31	4.69	0.56	N.S	4.6	1.5

Table 2: No. of protein spots in heat tolerant and susceptible genotypes of wheat at tillering stage through 2D at grain filling stage

Genotype	Control	40 °C		45 °C	
		2h	4h	2h	4h
GW-190 (T)	48	148	69	94	125
J-2010-11 (S)	184	74	109	45	76

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