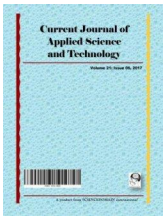

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Effect of Exogenous Trehalose in Protection of Cells and Their Membranes in Wheat (*Triticum aestivum* L.) Genotypes Under Heat Stress

Aparjot Kaur[1*] and S. K. Thind[1]

[1]Department of Botany, Punjab Agricultural University, Ludhiana-141004, Punjab, India.

Authors' contributions

This work was carried out in collaboration between both authors. Author AK performed the experiment, statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author SKT designed and managed the analysis of the study. Both authors read and approved the final manuscript.

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ABSTRACT

Trehalose (Tre) a non-reducing disaccharide, formed of two alpha D-glucose molecules, is found in many organisms and is able to protect against a variety of environmental injuries and nutritional limitations. The information regarding its occurrence and role in higher plants is scanty. In the present study, effect of trehalose (@1 mM and 1.5 mM) under heat stress of 35±2°C (moderate) and 40±2°C (severe) for 4 and 8 hours was studied in wheat (*Triticum aestivum* L.) genotypes viz. HD2967, PBW175, C306, PBW343, PBW621 and PBW590. Membrane thermal stability (MTS) and cell viability were significantly decreased under heat stress of eight hours. Pretreatment with Tre improved MTS and cell viability of selected wheat genotypes under stress and the effect was more pronounced with higher concentration (1.5 mM). Plant growth and vigour were negatively affected by severe heat stress (40±2°C); the reduction was compensated partially by Tre application. Dry matter accumulation and starch content of all studied wheat genotypes decreased under the heat stress, the ameliorative effect was observed with the trehalose application that helped seedlings to sustain growth under heat stress.

**Corresponding author: E-mail: aparjotranu@gmail.com;*

Keywords: Heat stress; Triticum aestivum; Trehalose; MTS; Triphenyl Tetrazolium Chloride (TTC) test; dry matter accumulation.

ABBREVIATIONS

Tre : Trehalose
MTS : Membrane Thermal Stability
TTC : Triphenyl Tetrazolium Chloride
DAS : Days after Sowing

1. INTRODUCTION

The demand for wheat due to an overwhelming increase in the population has been raised than the previous, but its productivity decreased due to abiotic stresses. Heat stress during the grain filling period of the normal as well as late planted wheat is one of the major environmental factors reducing wheat production [1]. Heat stress drastically reduced both yield and quality of wheat [2,3,4]. Crop heat tolerance could be enhanced by preconditioning of plants under different environmental stresses or exogenous application of osmoprotectants such as trehalose, glycinebetaine and proline.

Heat stress affects the wide spectrum of both biochemical and physiological responses within the plant [5]. All plant processes are sensitive to and can be irreversibly damaged by heat. Thermal stability of photosynthetic apparatus differed markedly between species from temperate and tropical environments [6]. High temperature stress accelerates leaf senescence and adversely affects the grain filling; and is one of the most important causes of reduction of dry matter production in cereals [7].

Trehalose (α-D-glucopyranosyl-[1, 1]-α-D-glucopyranoside) is a non-reducing disaccharide, which plays an important physiological role as a compatible solute in a large number of organisms, including bacteria, yeast and invertebrates. In most plants, trehalose is hardly detectable, except in certain specialised resurrection species that accumulate the compound quantitatively. Some previous studies have shown that trehalose can stabilise proteins and biological membranes efficiently in microorganisms under stress [8,9]. Others have found that trehalose accumulation in transgenic plants can increase their abiotic stress tolerance [10,11] and these results have been widely accepted [12].

It has been shown that trehalose accumulation during heat stress protects cells and cellular proteins of *Saccharomyces cerevisiae* from damage by oxygen radicals [8]. However, in higher plants, the function of trehalose is largely unknown. Therefore, a primary aim of this study is to determine whether trehalose helps to stabilise membranes to protect the viability of cells and sustain growth during heat stress conditions.

2. MATERIALS AND METHODS

2.1 Plant Material and Experimental Design

Six genotypes of wheat (*Triticum aestivum* L.) viz. HD 2967, C306, PBW621, PBW590, PBW343 and PBW175 were selected and seed were obtained from Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana.

With a view of evaluate the effect of heat stress, only healthy seeds of six genotypes of wheat were surface sterilised with 0.01 per cent mercuric chloride for 2-3 min to avoid any fungal infection during seed germination. Petri plates were sterilised in oven at 100°C for one hour. Twenty seeds were sown in each Petri-plate (5 replications) lined with circular blotting paper and incubated at 25±2°C temperature. Seven days after sowing trehalose (1mM and 1.5 mM) application was given followed by heat stress treatment at 35°C and 40°C, for 4 and 8 hrs. Trehalose application was given by spraying the 1mM and 1.5mM trehalose with the help of automizer. The aqueous 1.0 and 1.5mM Tre solution containing 0.1% TWEEN 20 was sprayed on seedlings until run off, twice a day. The three types of Tre non sprayed sets i.e control (normal temperature), moderate stress (35±2°C) and severe stress (40±2°C) were sprayed with only water containing 0.1% TWEEN 20 at 7DAS. After one week of Tre application samples were collected from each set from ten random seedlings were taken for biochemical estimations.

2.2 Determination of Membrane Thermal Stability

For each treatment leaves were excised and washed with distilled water to remove adhering electrolytes. The tissue was immersed in test tubes containing 20 ml of de-ionised water and

stirred continuously at 28°C. After five hours the electrolyte leakage was estimated by the conductivity meter. The samples were then boiled for 30 min and conductivity was measured again. MTS was calculated [13].

$$MTS = (1-T_1/T_2 \times 100)$$

Where $T_1$and T_2 were the conductivity readings before and after autoclaving respectively.

2.3 Triphenyl Tetrazolium Chloride Reduction Assay

TTC was estimated spectrophotometrically [14]. The sets of two leaves (3.5 cm each) per cultivar were excised, rinsed in deionised water, and each leaf placed in a test tube with 0.1 ml deionised water. The two sets were then heat-treated as follows: the first set was left at 25°C for 90 min, and the second set was placed in a water bath at 49°C for 90 min. Immediately following the 25°C and 49°C treatments, 10 ml of TTC solution (0.8% TTC in 0.05 M NaPO4 buffer, pH 7.4, and 0.5 ml L^{-1} TWEEN 20) was added per tube and vacuum infiltrated for 10 min. The tissue was incubated in the TTC solution for 24 hr at 25°C in the dark. After incubation, leaves were removed and rinsed with distilled water, placed individually in separate spectro-photometric tubes containing 2 ml of 95% ethanol, and submerged for 24 hours at 25°C in the dark. The level of acquired high temperature tolerance was determined by measuring the percentage reduction of TTC to formazan using the following:

$$TTC = (OD_h / OD_c) X 100$$

Where OD_h refers to the mean optical density (485 nm) values for the heat-stressed set, and OD_c refers to the mean optical density for the control set.

2.4 Vigour and Dry Matter Accumulation

Vigour of seedling was calculated using the formula [15]. The accumulation of dry matter in seedlings (shoot as well as root) was estimated by taking the samples at desired intervals.

2.5 Determination of Starch Content

Starch content was also estimated from the control, heat stressed and trehalose applied six wheat genotypes as suggested by McCready et al. [16].

2.6 Statistical Analysis

Analysis of variance (ANOVA), critical difference at 5% level of significance (P< 0.05%) was used for the data analysis.

3. RESULTS AND DISCUSSION

3.1 Membrane Thermal Stability

Heat stress affects a wide spectrum of both biochemical and physiological responses within the plant cells [5]. Rather all plant processes are sensitive to and can be irreversibly damaged by heat. Plants tend to divert resources to cope with the heat stress to sustain development. Heat stress accelerates leaf senescence and adversely affects dry matter production in cereals [7]. Under abiotic stresses, the accumulation of osmoprptectants (compatible solutes) is a common response observed in plants. The adaptation to heat stress was associated with the metabolic adjustments which lead to the accumulation of organic solutes [17].

Membrane thermal stability, a measure of electrolyte diffusion resulting from heat induced cell membrane leakage, has been used to screen and evaluate different wheat genotypes for thermotolerance [18]. In selected wheat genotypes heat stress affect membrane stability and increased electrolyte leakage due to loss of membrane selectivity. When seedlings were subjected to high temperature, electrical conductivity increased due to damage to the cell membrane and consequent solute leakage. The MTS varied significantly among genotypes in control, being maximum in HD2967 and minimum in PBW175 (Table 1). The exogenous application of Tre significantly improved MTS of all genotypes and higher concentration (1.5mM) was better as more percent increase values were recorded. Both moderate (35±2°C) as well as severe (40±2°C) heat stress significantly reduced MTS though the effect was more pronounced in latter. This negative effect of heat stress on MTS was improved in Tre treated sets and higher concentration (1.5 mM) was more promotary. The genetic variability existed even under severe heat stress and maximum MTS value was recorded in HD2967 and least in PBW 175. The application of Tre @1mM (4.72%) and 1.5mM (9.25%) improved MTS of HD2967. No percent increase in MTS was recorded in PBW590 under severe stress for eight hours with the application of 1 mM but an increase of 0.66% was recorded with 1.5 mM of Tre.

In *Triticum* species heat stress might affect membrane stability, increasing the electrolyte leakage as a result of loss of membrane selectivity [19], which could also be associated with increased levels of lipid peroxidation [20,21]. High temperature might further increase fluidity of membrane lipids promoting functional imparements and even thermal damage to photosystem II and accelerated cell death [3], which result in substantial changes in protein composition as well as in the size and distribution of starch granules.

3.2 Triphenyl Tetrazolium Chloride Reduction Assay

As decrease in cell viability resulting from high temperature treatment was attributed to uncoupling of the electron transport chain through disruption of the inner mitochondrial membrane and for inactivation of enzymes of the respiratory pathway [22]. Severe heat stress leads to cellular damage and cell death, sublethal doses of heat stress induce a cellular response, which protects cells from severe damage, allows resumption of normal cellular physiological activities and leads to thermotolerance [23]. Heat is a complex stress causing damage to a range of cellular components, thus a large number of different protective pathways are required in order to survive [24]. The functional damage inncured during heat stress is the consequence of injury to cellular membranes. Lowering of membrane integrity results in an increased permeability and electrolyte leakage. The cell membrane thermostability has also been correlated with whole plant heat tolerance. Primary injury in plants results from a short exposure to extreme temperature (45 to 65°C). The symptoms of such injury include coagulation of protoplasm, protein denaturation, lipid liquefaction or perturbation of membrane integrity [25]. Cell membrane stability after a prehardening treatment has been suggested for estimating thermotolerance of plants [26,27]. Presently observed that pretreatment exogenously supplied Tre was able to improve membrane thermal stability and sustain cell viability reveal that Tre can be used to preserve biological structural under heat stress. Earlier, it was reported that Tre can stabilise dehydrated biological structures, such as lipid membranes or enzymes, more effectively than other sugars [28].

2,3,5- triphenyl tetrazolium chloride (TTC) cell viability assay has been used to evaluate genotypic variability in acquired thermotolerance of selected genotypes. Presently, a significant difference existed among genotypes tested for TTC assay at seedling growth stage in control conditions. TTC reduction was decreased significantly when exposed to heat stress with more effect under severe (40±2°C) stress conditions (Table 2.). This decline with severe heat stress could due to cellular damage and cell death. The exogenous application of Tre improved the TTC reduction values in control as well as under heat stress conditions. The prolonged severe (eight hours) heat stress (40±2°C) had deleterious effect on viability of cells in seedlings of selected genotypes. Tre application ameliorated this negative effect of heat stress upto 5.35% in PBW175 followed by 4.01% in C306. Evidently, Tre was found to be able to protect the integrity of cells again injuries due to heat stress. The exogenous application if Tre at 1.5 mM remarkably conferred protection to denaturation of membranes to keep cells viable even under stress.

Earlier, it has been observed that results obtained by TTC assay did not change with plant age [26]. Plant re-growth, electrolyte leakage and TTC assay are commonly used procedures for evaluating thermotolerance [29]. Under heat stress, accumulation of Tre markedly increased the viability of the cells, showing that Tre accumulation in stressed cells plays a major role in protecting cellular constituents from oxidative damage by acting as free radical scavenger [30].

3.3 Vigour and Dry Matter Accumulation

In control, the seedlings of selected genotypes (HD2967, PBW175, C306, PBW343, PBW621, PBW590) were raised at optimum temperature (25°C). The vigour recorded at 7 DAS varied significantly among genotypes and were recorded more in HD2967, C306 and PBW621 (Fig. 1). Tre application in control had non significantly affected the vigour of seedlings. Under severe heat stress PBW343 and HD2967 performed better than other genotypes and minimum values were recorded in PBW175.

In the sets exposed to moderate stress (35±2°C) the vigour of seedlings was reduced significantly. Lower concentration (1 mM) of tre improved the growth of seedlings to some extent and the effect was more promotary when higher concentration (1.5 mM) of tre was supplied. The negative effect of severe heat stress (40±2°C) was more pronounced and vigour was almost reduced to

Table 1. Effect of trehalose on membrane thermal stability (%) of wheat genotypes under heat stress of 35±2°C and 40±2°C

Treatments	Genotypes					
	HD2967	**PBW175**	**C306**	**PBW343**	**PBW621**	**PBW590**
T1-Control at 25°C	82.6	62.6	69.7	69.4	78.3	66.4
T2-T1+(tre-1 mM)	83.0(0.481%)	63.2(0.949%)	70.4(0.994%)	70(0.857%)	78.6(0.382%)	66.8(0.598%)
T3-T1+(tre-1.5 mM)	84.2(1.900%)	64.3(2.642%)	71.2(2.106%)	70.8(1.977%)	78.6(0.382%)	70.0(5.143%)
Heat stress for 4 hours						
T4-Control at 35°C	78.9	51.2	68.5	67.3	75.4	59.0
T5-T4+(tre-1 mM)	79.2(0.378%)	52.3(2.103%)	69.3(1.154%)	70.0(3.857%)	76.2(1.049%)	59.6(1.007%)
T6-T4+(tre-1.5 mM)	81.0(2.592%)	54.6(6.227%)	70.0(2.142%)	78.3(14.05%)	77.3(2.457%)	60.2(1.993%)
T7- Control at 40°C	60.8	42.7	60.5	48.5	60.7	45.3
T8-T7+(tre-1 mM)	61.8(1.618%)	42.9(0.466%)	66.6(9.159%)	48.9(0.817%)	61.3(0.978%)	46.0(1.522%)
T9-T7+(tre-1.5 mM)	62.2(2.250%)	44.2(3.393%)	67.8(10.766%)	50.6(4.150%)	62.3(2.568%)	48.3(6.211%)
CD 5%	V=2.533, T=2.068, V×T=6.206					
Heat stress for 8 hours						
T10- at 35°C	72.0	45.0	60.0	58.9	66.0	52.0
T11-T10+(tre-1 mM)	74.0(2.702%)	46.0(2.174%)	63.0(4.762%)	60.0(1.833%)	66.2(0.302%)	58.0(10.34%)
T12-T10+(tre-1.5 mM)	76.0(5.263%)	46.2(2.597%)	65.2(7.975%)	62.5(5.76%)	67.2(1.785%)	60.0(13.33%)
T13- at 40°C	36.3	28.2	32.0	30.6	34.6	30.0
T14-T13+(tre-1 mM)	38.1(4.724%)	28.9(2.422%)	32.6(1.840%)	30.8(0.649%)	35.0(1.143%)	30.0(0%)
T15-T13+(tre-1.5 mM)	40.0(9.25%)	31.0(9.032%)	34.0(5.882%)	32.0(4.375%)	38.0(8.947%)	30.2(0.662%)
CD 5%	V=0.428, T=0.524, V×T=1.284					

Figures in bracket represent percent increase over control.

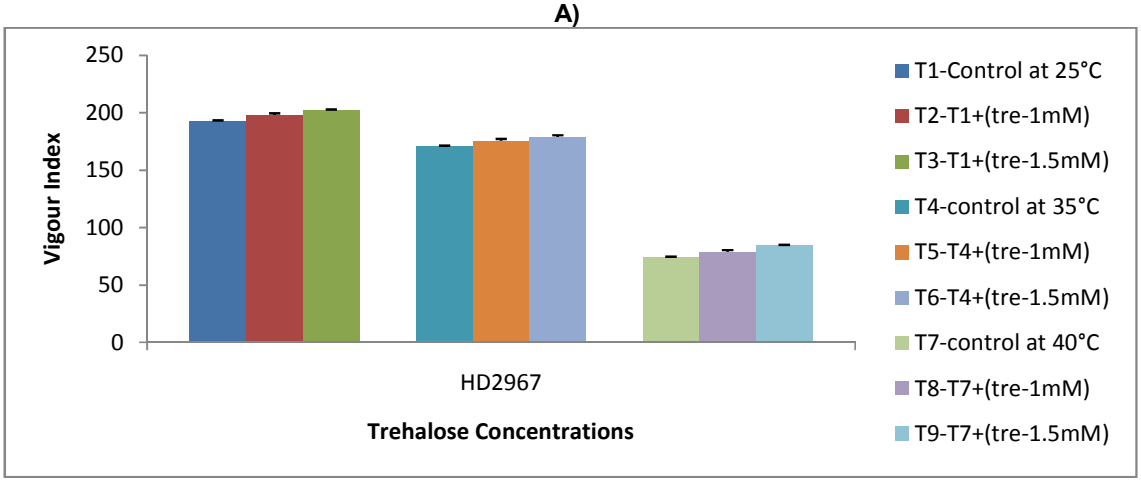
Table 2 Effect of Trehalose on Triphenyl Tetrazolium Chloride reduction assay (%) of wheat genotypes under heat stress of 35±2°C and 40±2°C

Treatments	Genotypes					
	HD2967	**PBW175**	**C306**	**PBW343**	**PBW621**	**PBW590**
T1-Control at 25°C	77.0	70.8	74.3	72.6	76.0	72.3
T2-T1+(tre-1 mM)	77.2(0.259%)	72.2(1.939%)	74.6(0.402%)	73.2(0.819%)	76.2(0.262%)	73.0(0.959%)
T3-T1+(tre-1.5 mM)	78.0(1.282%)	72.6(2.479%)	74.9(0.801%)	73.2(0.819%)	76.6(0.783%)	73.4(1.499%)
Heat stress for 4 hours						
T4- at 35°C	74.0	68.2	72.0	70.2	72.0	70.0
T5-T4+(tre-1 mM)	74.8(1.069%)	68.4(0.292%)	72.6(0.826%)	70.4(0.284%)	72.6(0.826%)	72.1(2.912%)
T6-T4+(tre-1.5mM)	75.0(1.333%)	68.4(0.292%)	76.2(5.512%)	71.6(1.955%)	72.8(1.098%)	72.6(3.581%)
T7- at 40°C	68.4	60.2	65.8	63.0	66.0	62.3
T8-T7+(tre-1 mM)	68.9(0.725%)	60.6(0.660%)	66.3(0.754%)	63.6(0.947%)	67.2(1.785%)	63.6(2.044%)
T9-T7+(tre-1.5 mM)	70.2(2.564%)	62.0(2.903%)	67.1(1.937%)	65.8(4.255%)	68.9(4.208%)	64.3(3.110%)
CD 5%	V=0.198, T=0.242, V×T=0.595					
Heat stress for 8 hours						
T10- at 35°C	69.0	60.8	67.0	64.0	68.0	62.2
T11-T10+(tre-1 mM)	69.8(1.146%)	65.2(6.748%)	67.8(1.179%)	66.0(3.030%)	68.3(0.439%)	66.1(5.900%)
T12-T10+(tre-1.5 mM)	71.0(2.816%)	66.0(7.879%)	69.0(2.898%)	68.1(6.021%)	69.8(2.579%)	66.3(6.184%)
T13- at 40°C	58.0	46.0	55.0	55.0	56.2	52.0
T14-T13+(tre-1 mM)	58.0(0%)	48.1(4.366%)	56.0(1.786%)	55.2(0.362%)	57.0(1.404%)	52.6(1.141%)
T15-T13+(tre-1.5 mM)	58.4(0.684%)	48.6(5.349%)	57.3(4.014%)	55.6(1.079%)	57.4(2.090%)	52.9(1.701%)
CD 5%	V=0.298, T=0.365, V×T=0.895					

Figures in bracket represent percent increase over control.

half in genotypes. The maximum value of vigour index was recorded in PBW621 and C306, minimum vigour index was recorded in PBW590 and PBW175 genotypes under control conditions (25°C). The genotype PBW621 and HD2967 performed better than other genotypes under moderate (35°C) heat stress. Under severe (40°C) heat stress PBW343 and HD2967 performed better than others. At severe heat stress PBW175 genotype showed minimum vigour index. Tre application promoted growth under stress and this increased with increase in concentration.

A)



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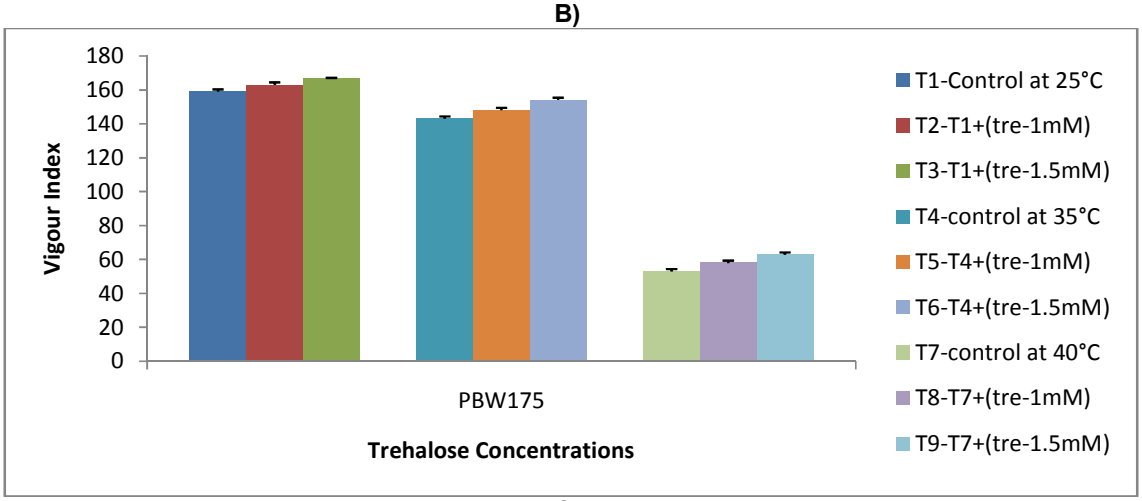

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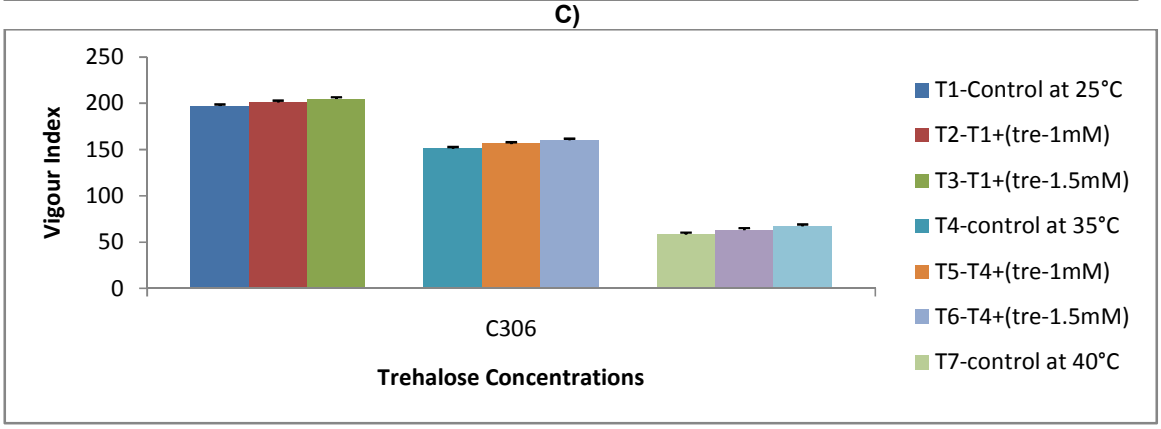


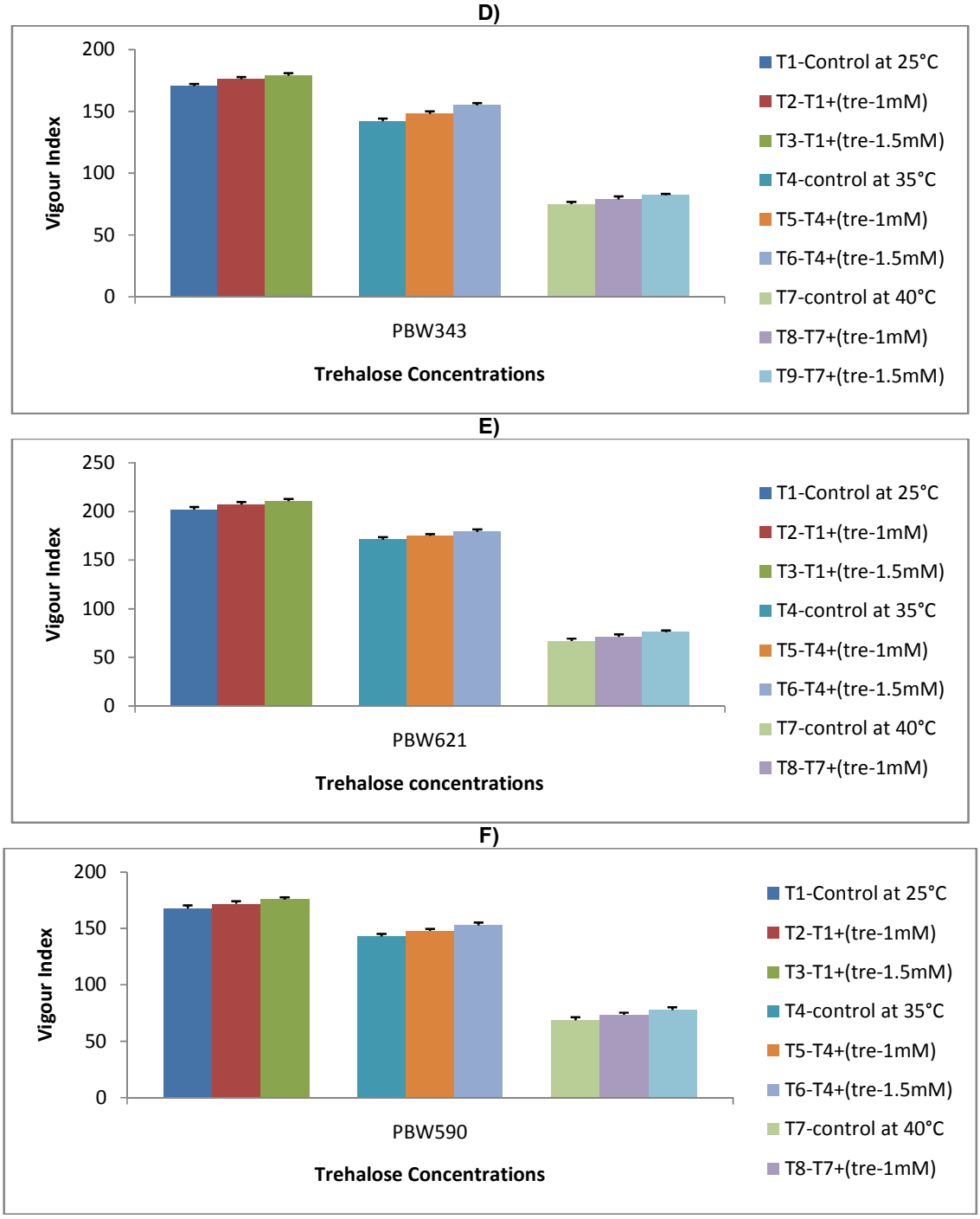
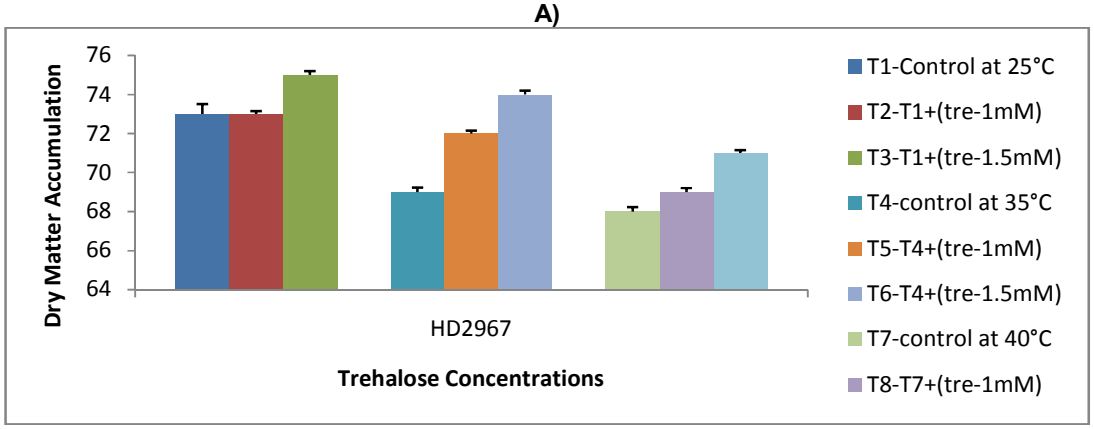


Fig. 1. Effect of trehalose on Vigour of six wheat genotype (A, B, C, D, E, F) seedlings under control and heat stress of 35±2°C and 40±2°C for eight hours. Critical difference at 5% level of significance analysed by ANOVA. Variety (V) =2.533, Treatment (T) = 2.068, V×T =6.206

The dry matter accumulation of selected genotypes was found to differ with duration of heat stress and significantly more values of dry matter accumulation were recorded in HD2967 and PBW621under control as well as varying levels of heat stress (Fig 2.). Dry matter

accumulation decreased with increased level of heat stress. Induced moderate and severe heat stress significantly decreased dry matter accumulation of all genotypes, but more severe effect was recorded in PBW175 and PBW590. Genotypes PBW621, HD2967 and C306 performed better under moderate and severe heat stress. These showed increment in accumulation of dry matter in seedlings after the application of higher concentration (1.5 mM) as compared with lower concentration of Tre tested presently. Thus Tre @ 1.5 mM had more ameliorative effect on the accumulation of dry matter in all selected genotypes.

A)



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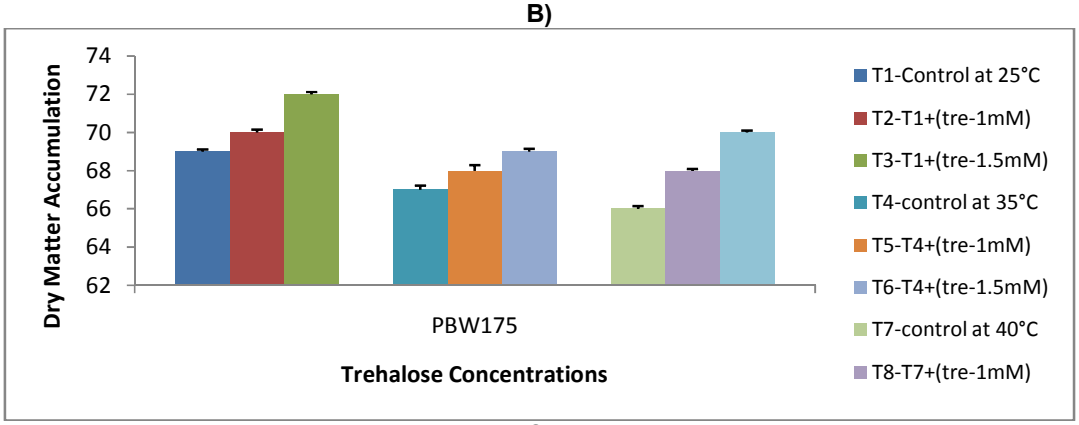

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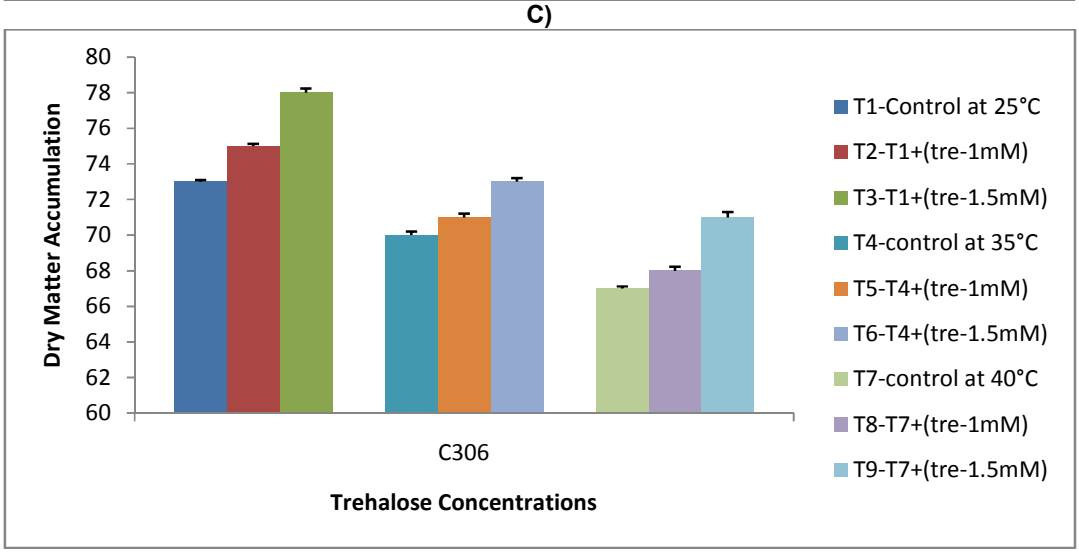

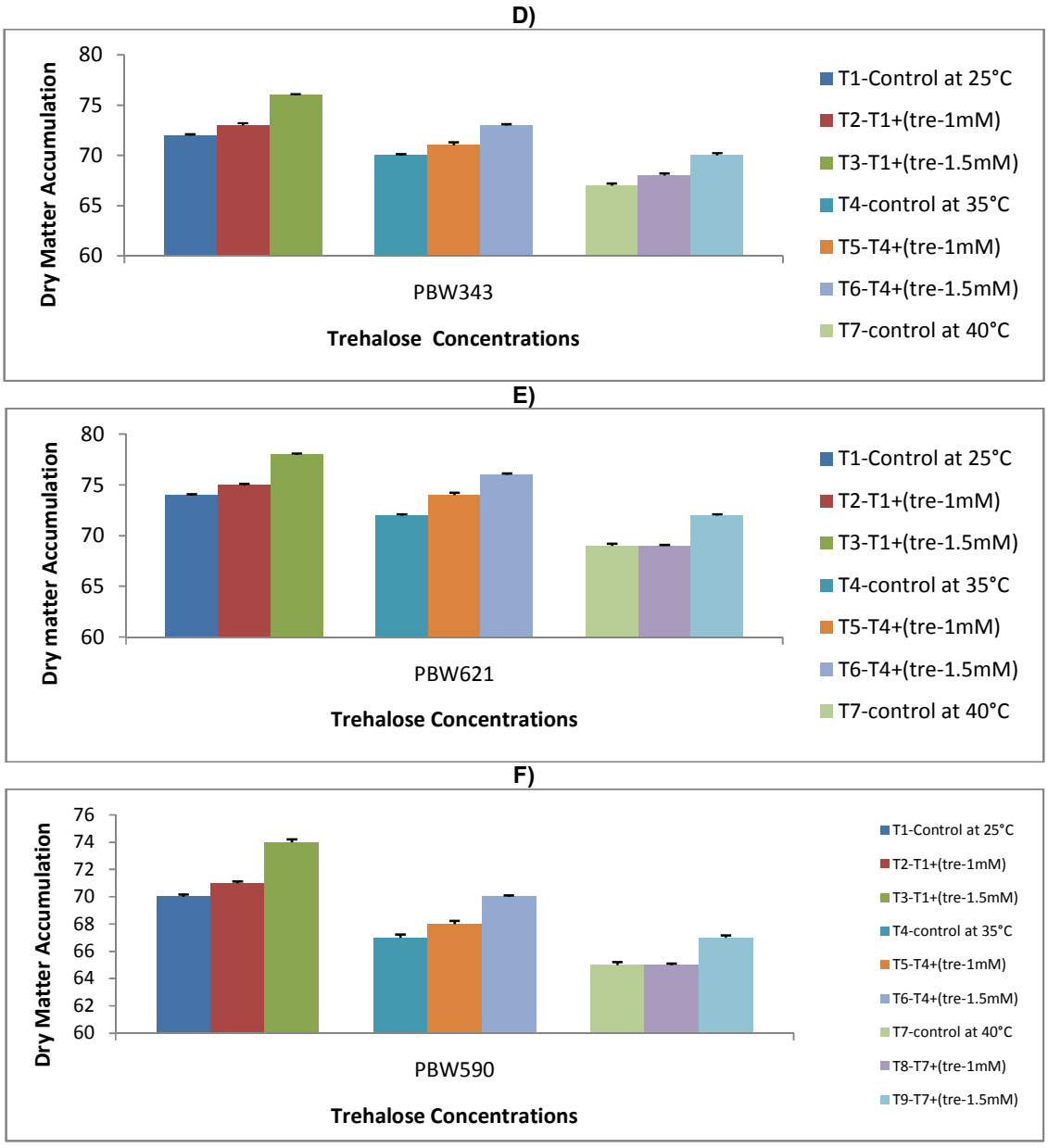

Fig 2. Effect of trehalose on dry matter accumulation of six wheat genotype (A, B, C, D, E, F) seedlings under heat stress of 35±2°C and 40±2°C for eight hours. Critical difference at 5% level of significance analysed by ANOVA. Variety (V) =0.0735, Treatment (T) = 0.0654, V×T =1.0369

In selected wheat genotypes Tre application results in an increase in vigour and dry matter accumulation under control as well as moderate and severe stress conditions. It is probably because Tre function at the molecular level by forming molecular complex with biomolecules. Tre accumulation in transgenics could increase abiotic stress tolerance [11-12]. Carbohydrate changes are of particular importance because of their direct relationship with physiological processes and dry matter accumulation. The enzymes involved in starch biosynthesis are sensitive to heat stress [31,32]. A reduction in starch content account for most of the reduction in dry matter above 22°C [33].

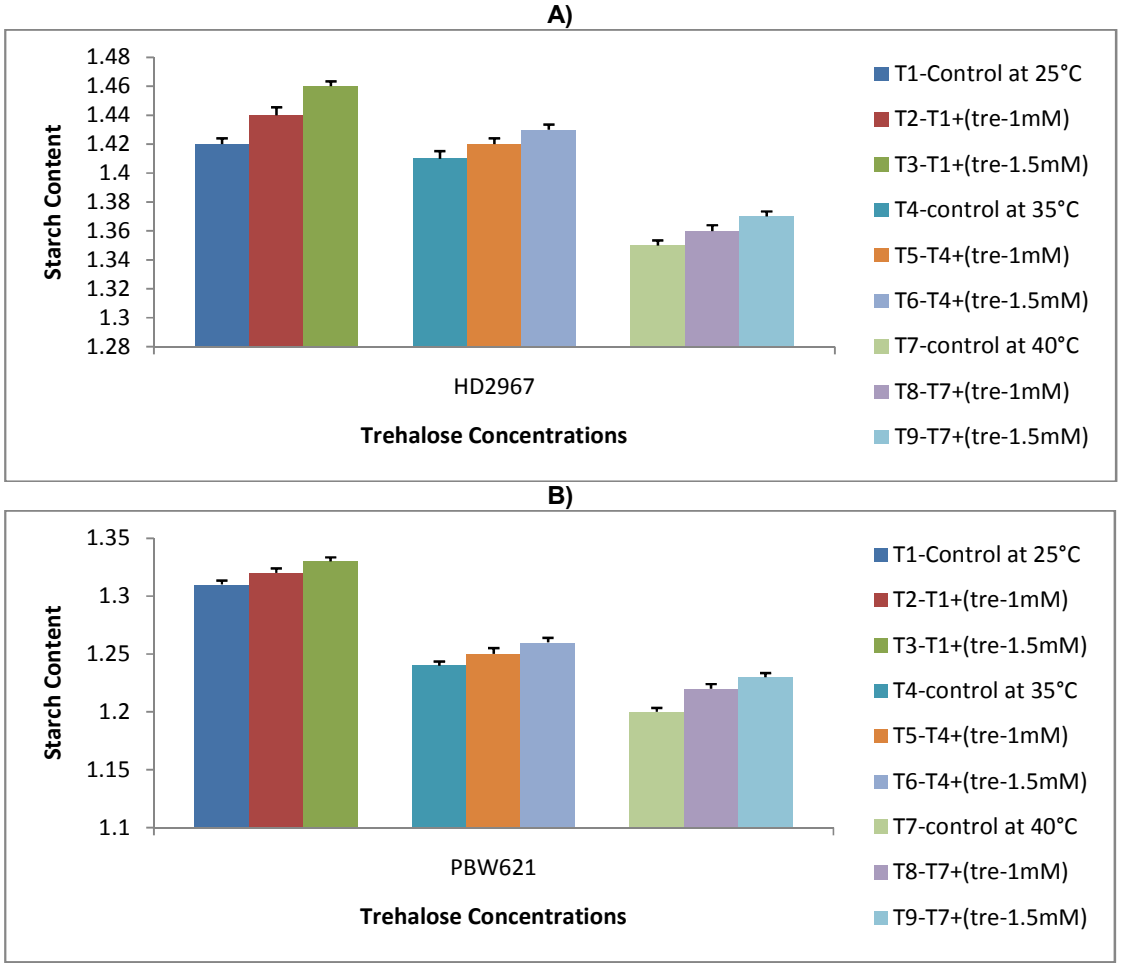

Fig. 3. Effect of trehalose on starch (mg gm^{-1} fresh weight) content of HD2967 (A) and PBW621 (B) seedlings under heat stress of 35±2°C and 40±2°C for eight hours. Critical difference at 5% level of significance analysed by ANOVA. Variety (V) =0.048, Treatment (T) = 0.097, V×T = 0.192.

3.4 Starch Content

The content of starch was decreased in genotypes given heat stress (40±2°C) for eight hours duration (Fig. 3). In control as well as all treated sets, the accumulation of starch was recorded more in HD2967 as compared with PBW621. At normal temperature (25±2°C) the application of Tre had non-significant effect. Moderate heat (35±2°C) did not affect the accumulation of starch in seedlings. Whereas severe heat stress (40±2°C) negatively affected it. This reduction was compensated in Tre (1.5 mM) treated sets.

At the time of germination, starch present in the endosperm was hydrolysed to glucose by amylases, which then converted to sucrose by sucrose phosphate synthase, was transported to the growing embryo axis or seedling.

The changes in carbohydrate levels are of particular importance because of their relationship with important physiological processes such as photosynthesis, respiration and translocation. As respiratory substrate, monosaccharides promote respiration and mitochondrial electron transport which would oppose the onset of quiescence and favour metabolism and production of energy. Generally adaptation to heat stress is associated with the metabolic adjustments that lead to accumulation of organic solutes. From among the soluble carbohydrates, sucrose and fructans have role in adaptation to stress and sucrose can act in water replacement to maintain membrane phos-

pholipids in the liquid-crystalline phase, also prevents structural changes in soluble proteins.

Earlier, it has been observed that accumulation of Tre in wheat plants promote biosynthesis of both sucrose and starch [34]. Further, the accumulation of Tre increased in wheat cultivation under drought stress [35].

4. CONCLUSION

Presently, as the six wheat genotypes were subjected to moderate (35±2°C) and severe (40±2°C) heat stress conditions. It has been observed in all the genotypes that the heat stress resulted in a loss of membrane stability and more electrolyte release that resulted into loss of cell viability, decreased starch content and dry matter accumulation. Adverse occurred under the severe heat stress conditions as compared to control and moderate stress. Now in the treatments with the exogenously applied trehalose, membrane stability, cell viability, starch content and dry matter accumulation increased as the trehalose known to protect the biological membranes from degradation by stabilising them. Thus, the pre-sent findings demonstrated that exogenous application of trehalose to wheat has considerable potential for protecting the cell membranes of seedlings growing under heat stress. Further, increased accumulation of starch that resulted in dry matter accumulation and helped to sustain growth under heat stress.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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