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Alteration in Physiological, Biochemical and Yield Traits by Combined Salinity and Heat Stress and Mechanism of Stress Tolerance of Wheat Genotype

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Abstract
Heat and salinity stress, both individually and combined, significantly reduce wheat yield in tropical and subtropical areas. The flag leaf of wheat, during its terminal growth phase, plays a crucial role in assimilate partitioning and stress tolerance. However, little is known about their combined effects on the crop. This study aimed to explore the effects of individual and combined heat and salt stress on water relations, membrane stability index, photosynthetic pigments, antioxidant defense mechanism, osmolytes accumulation, and yield attributes in flag leaf of bread wheat. Two independent experiments were conducted at the seedling stage. The second experiment was conducted under natural environmental condition (in pots). Experimental treatments comprised of control (timely sowing on the soil of EC 0.25), salt stress alone (timely sowing on soil of EC 7.4), terminal heat stress alone (late sowing on the soil of EC 0.25) and combined salt stress + terminal heat stress (late sowing on soil of EC 7.4). Salt and heat stress posed significant reduction (p0.05) in physiological parameters, along with activating SOD and proline accumulation thus ultimately reducing yield. Here, the combined stress appeared more detrimental then the individual stress due to the hypo-additive interaction between the two stresses. A unique tolerance mechanism was revealed by some wheat genotypes by their ability to maintain comparatively high WUE, chlorophyll a and b content, MSI and higher induction of antioxidant defence system (SOD, APX), osmolyte concentration (proline) and ultimately yield (harvest index and yield per plant) then the susceptible genotypes. These findings will be critical in future studies aimed at improving wheat stress tolerance by fully understanding the genetic pathways behind the physiological and biochemical effects related to combined heat and salt exposure on the wheat flag leaf

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INTRODUCTION

Field crops are frequently subjected to stressful conditions. Many species may be forced to adapt to unexpected, possibly difficult environmental situations that are outside of their regular range of reactions as a result of these stressors. High salinity is predicted to impact 20% of cultivable and 33% of irrigated land globally, and salty regions are growing at a 10% yearly pace due to little precipitation, high evaporation, weathering of local rocks, irrigation with saline water, and poor cultural practices. Furthermore, climate change caused by greenhouse gas emissions is expected to raise the average global temperature by 1.0 to 2.7 degrees Celsius during the next century (Houghton, 2005). Some research discovered that global warming may have a detrimental influence on wheat grain yields, thereby contributing to food shortages and poverty; nevertheless, the degree and direction of climatic impacts on yields of crops will vary by location (Tubiello, 2000).

Salinity stress has a significant impact on wheat seed germination (Abo-Kassem, 2007), either causing dormancy at low levels or entirely inhibiting germination at higher ones (Iqbal et al., 2006). Soil salinity and extreme temperatures are important variables that affect crop yield. Both these stresses affect morphological development, reduce flag leaf gas exchange, and assimilate partitioning, activities of antioxidant enzymes and disruption of membrane functions (Sairam & Tyagi, 2004; Taiz & Zeiger, 2006). High temperatures during reproduction and filling of grain stages are one of the leading causes of wheat yield loss, since they shorten the crop's reproductive cycle and impair both the nutritional value and quantity of the resulting grain (Farooq et al., 2011). Anthesis is a particularly sensitive stage in wheat blooming that is affected by both salt and high temperatures. As a result, stress at this stage is mostly associated with yield reduction. Under high temperature, salinity, or combined stress, plants face water deficit, which results in reduced photosynthesis in the leaves and stomatal conductance, causing thylakoid membrane damage, thus reducing seed set, spikelet size numbers per spike, grain counts per plant, harvest index, and the ultimate yield per plant (Poustin *et al.,* 2007; Sultan *et al*., 2012; Ghafiyehsanj *et al*., 2013; Sabbagh *et al*., 2014; Yu *et al*., 2015; Tavakoli *et al*., 2016). Temperatures exceeding 30 °C during floret production produce total infertility in wheat (Saini et al. 1983). For example, terminal heat stress caused by late planting resulted in decreased grain production, grain number per ear, 1000 weight of grain, number of ears per plant, and so on when compared to optimal sowing (Sikder et al., 2001; Gill, 2009). Terminal heat and salt stress are so typical problems in many wheat-growing areas across the world. Abiotic stress lowers photosynthesis by disrupting chloroplast structure and function, as well as decreasing chlorophyll levels (Xu et al. 1995). By boosting chlorophyllase activity and lowering photosynthetic pigments, the plant's photosynthesis and respiratory activity is significantly reduced (Sharkey and Zhang, 2010). A significant drop in chlorophyll concentration was seen at high temperatures, salinity, and combined stress, with the decline being more severe at combined stress. Wahid et al. (2007); Djanaguiraman et al. (2014); Sabbagh et al. (2014). Terminal heat, salt, and stress, both alone and together, increase enzyme activity (antioxidant defense system) and protect crops by scavenging reactive oxygen species (ROS). H2O2 produced during stress causes oxidative injury in plants (Ozden et al., 2009). It is well recognized that when plants are stressed by drought, temperature, salt, or other environmental factors, osmolytes such as proline accumulate at high concentrations (Ashraf and Foolad, 2007; Afzal et al., 2014). Proline protects plants from stress and is hypothesized to operate as a cellular osmotic regulator, as well as in ROS detoxification. Hare and Cress (1997); Ashraf and Foolad (2007); Tatar and Gevrek (2008); Yu et al. (2015). Furthermore, proline contributes significantly to membrane integrity, enzyme or protein stability, and stress tolerance (Mittler, 2002). Intolerant crop varieties the induction of an antioxidant defence system, accumulation of osmitytes, maintenance of water balance, chlorophyll pigments etc., are quite higher compared to susceptible one. Thus plant adaptation to different physiological, metabolic, and molecular mechanisms to govern

its responses to heat and salinity stress. Thus it can be said that the responses of plants to abiotic stress combinations are highly complex as the horde of multiple interactions (may be hypo-additive or counter-active in nature) involves different dimension of expression depending on many factors like crop type, stage of crop growth, stress type etc., (Kissoudis et al., 2014). These processes are linked and carefully regulated, resulting in unique responses that allow the plant to adapt to changing conditions. The maintenance of biological membrane potential, relative water content, pigments content, the activities of several enzymes, appropriate osmolyte concentration and maintenance of ion homeostasis, specifically Na^+ and K^+ homeostasis are some mechanisms plants adapt to cope with abiotic stresses (Conde et al., 2011).

MATERIALS AND METHODS

Plant materials and experimental treatments

This study consisted of two separate experiments. The first experiment was a lab experiment in which 46 wheat genotypes were collected and screened at the seedling stage (10th day) to evaluate their performance against individual and paired salt stress (EC 4 and 8 dS m-1 and salt composition of NaCl: CaCl2:Na2SO4 in a ratio of 7:2:1) and heat stress (temperature 37 ± 2°C in an incubator). Three resistant (KRL-1-4, KRL-19, and HD-2733) and three susceptible (HI-1563, HT-8, and HD-2987) wheat genotypes were identified based on physiological features detected in a 10-day-old seedling.

The second study was a pot experiment conducted in natural environmental conditions with the goal of studying physiological and biochemical mechanisms of tolerance as well as quantifying changes in yield and yield characteristics in six contrasting sets of wheat genotypes (three tolerant, KRL-1-4, KRL-19, and HD-2733, and three susceptible, HI-1563, HT-8, and HD-2987) selected in the first phase.
The experimental treatments were control (timely planting on EC 0.25 soil), salt stress (timely sowing on EC 7.4), terminal heat stress alone (late sowing on EC 0.25 soil), and combination salt stress + terminal heat stress (late sowing on EC 7.4).

Timely sowing: on 15th November
Late sowing: on 5th January

Table 1: Time, date and temperature of sample collection (flag leaf) at the flowering stage of wheat genotypes in year 2017 and 2018.

Year	Date of sowing	Genotypes	Date of sample collection for physiological and biochemical analysis (flowering stage)	Temperature ^{0}C	Increase in temperature under late sowing
				Max	Min
2016-2017	Timely sown (15th Nov. 2016)	HD-2987	10th February	25.5	11.5
		HI-1563	17th February	26.2	10.0
		HT-8	17th February	26.2	10.0
		HD-2733	18th February	27.2	11.5
		KRL-19	19th February	27.6	12.5
		KRL-1-4	19th February	27.6	12.5
	Late sown (5th Jan. 2017)	HD-2987	6th March	32.2	13.5
		HI-1563	25th March	32.5	19.6
		HT-8	26th March	32.8	20.0

		HD-2733	25th March	32.5	19.6
		KRL-19	26th March	32.8	20.0
		KRL-1-4	27th March	32.6	22.5
2017-2018	Timely sown (15th Nov. 2017)	HD-2987	11th February	23.8	8.6
		HI-1563	17th February	25.5	9.2
		HT-8	19th February	26.8	12.2
		HD-2733	20th February	28.2	10.5
		KRL-19	22th February	27.0	12.0
		KRL-1-4	22th February	27.0	12.0
	Late sown (5th Jan. 2018)	HD-2987	6th March	32.6	17.1
		HI-1563	22th March	35.2	14.3
		HT-8	23th March	35.6	15.2
		HD-2733	24th March	34.7	17.0
		KRL-19	26th March	35.5	15.8
		KRL-1-4	26th March	35.5	15.8

Non saline soil: The soil collected from Mohammadpur, Samastipur, Bihar. The soil is clayey in texture and slightly alkaline in reaction, having low electrical conductivity (EC 0.25) and containing high amount of organic carbon, available phosphorus and available potash)

Saline soil: The soil collected from, Motipur block, Muzaffarpur, Bihar, Indi. The soil is sandy in texture and highly alkaline in reaction having high electrical conductivity (EC 7.4) and was low in organic carbon and available phosphorus and high in available potash.

Table 2: Physio-chemical properties of control and saline soil sample collected from Mohammadpur Pusa Bihar and Motipur, Muzaffarpur, Bihar respectively.

Particular (control soil)	0-15 cm soil depth	Particular (saline soil)	0-15 cm soil depth
Mechanical determination		**Mechanical determination**	
Sand (%)	18.09	Sand (%)	48.09
Silt (%)	41.71	Silt (%)	32.71
Clay (%)	38.53	Clay (%)	19.20
Texture	Clay	Texture	Sandy
Chemical determination		**Chemical determination**	
Soil pH	8.87	Soil pH	8.27
Electrical conductivity (dS m^{-1} at 25 ^{0} C)	0.25	Electrical conductivity (dS m^{-1} at 25 ˚C)	4.30
Electrical conductivity of saturation extract (dS m^{-1})	1. 3	Electrical conductivity of saturation extract (dS m^{-1})	7.4
Organic Carbon (%)	1.18	Organic Carbon (%)	0.25
Available Nitrogen (kg ha^{-1})	225	Available Nitrogen (kg ha^{-1})	95
Available P_2O_5 (kg ha^{-1})	63.98	`Available P_2O_5 (kg ha^{-1})	31.58
Available K_2O (kg ha^{-1})	226	Available K_2O (kg ha^{-1})	305
Zn (ppm)	0.98	Zn (ppm)	0.60
Cu (ppm)	0.80	Cu (ppm)	0.81

Fe (ppm)	2.72	Fe (ppm)	4.49
Mn (ppm)	3.58	Mn (ppm)	2.77
B (ppm)	0.31	B (ppm)	0.31
S (ppm)	72.02	S (ppm)	70.02

Relative water Content: Leaf relative water content (RWC) was estimated by first recording the fresh weight of a flag leaf sample, then the turgid weight by immersing the same leaf sample in water for 4 hours, and finally the dry weight by drying in a hot air oven until a constant weight was obtained (Weatherley, 1950). It was recorded according to the formula provided below.

RWC=[((Fresh weight-Dry weight))/((Turgid weight-Dry weight))] X 100

Membrane stability index: The approach developed by Sairam et al. was used to assess MSI. Two sets of flag leaves were inserted in test tubes with double-distilled water. The first set was heated to 40°C for 30 minutes, while the second set had been cooked at 100°C for 10 minutes. The solution's electrical conductivity was tested with a conductivity bridge. The MSI was calculated as:

MSI=[1-(C1/C2)] X 100

Photosynthetic pigments: The chlorophyll and carotenoid content of flag leaf material were measured by extraction 0.05 g of leaf material in 10 mL of dimethyl sulfoxide (DMSO) using a technique based on Hiscox and Israelstam's (1979) and Lichtenthaler and Welburn's (1983) procedures. The method is based on chlorophyll extracts absorbing light, which produces pigment leaching owing to DMSO's permeability to the plasma membrane. The solution's absorbance was tested at two wavelengths: 663 nm and 645 nm for chlorophyll concentration and 470 nm for total carotenoid content. The Arnon (1949) formula was used to determine chlorophyll content. To extract the leaves, 50 mg of fresh leaves were placed in test tubes with 10 mL of DMSO and left at 65°C for 4 hours.

Chlorophyll a = (12.7 x A663 - 2.69 x A645) x V / W x 1000

Chlorophyll b = (22.9 x A645 - 4.68 x A663) x V / W x 1000

a) Estimation of superoxide dismutase (SOD) activity

The assay is based on the creation of blue-coloured formazone by nitro-blue tetrazolium and O2.- radical, that absorbs at 560 nm, and the enzyme (SOD) reduces this absorbance by reducing the formation of O2.- radical by the enzyme (Dhindsa et al.,1981).

Reagents: Methionine (200 mM), NBT (2.25 mM), EDTA (3.0 mM), Riboflavin (60 uM), sodium carbonate (1.5 M), and phosphate buffer (100 mM, pH 7.8).

Solution A: Potassium dihydrogen phosphate (6.80 g) was dissolved in water, and the amount was increased to 500 mL using double distilled water.

Solution B: Di-potassium hydrogen phosphate 8.71 g was dissolved in water, and the volume was increased to 500 mL using double distilled water. 8.5 mL of solution A and 91.5 mL of solution B were combined, and the final pH was determined using a pH meter. Grinding medium (0.1 M phosphate buffer, pH 7.5, 0.5 mM EDTA): it was made by combining 16 ml of solution A and 84 ml of solution B, and the final pH was calibrated with a pH meter. To estimate enzyme activity (SOD and POX), 0.0186 g EDTA was dissolved in a grinder medium and the volume was brought up to 100 ml with buffer.

Preparation of enzyme extract

To make the enzyme extract for superoxide dismutase, the weighted amount of flag leaf samples (1 g) was first frozen in liquid nitrogen to avoid proteolytic activity, followed by grinding with 10 ml of extraction buffer. The homogenate was passed through four layers of cheesecloth, and the filtrate was centrifuged for 20 minutes at 15,000 g before being utilized as an enzyme.

a) Enzyme assay

Superoxide dismutase activity was determined by measuring the reduction in optical density of

formazone caused by superoxide radical and nitro-blue tetrazolium dye by the enzyme. Three milliliters of the reaction mixture were made by combining the following reagents:

A. 13.33 mM methionine (0.2 ml of 200 mM)
B. 75 uM nitrobluetetrazolium chloride (0.1 ml of 2.25 mM)
C. 0.1 mM EDTA (0.1 ml of 3 mM)
D. 50 mM phosphate buffer, pH 7.8, (1.5 ml of 100 mM)
E. 50 mM sodium carbonate (0.1 ml of 1.5 M)
F. 0.1 ml enzyme
G. 0.9 to 0.95 ml of water (to make a final volume of 3.0 ml)

The reaction began by adding 2 uM riboflavin (0.1 ml) and putting the tubes under two 15 W fluorescent lights for 15 minutes. The maximum color was produced by a full reaction mixture without enzyme, which served as the control. Turning off the light and placing the tubes in the dark halted the response. The absorbance was measured at 560 nm, and one unit of enzyme activity was defined as the quantity of enzyme that lowered the absorbance value by 50% when compared to tubes lacking enzyme. The enzyme activity was calculated as units per mg protein per minute.

B) Proline content

Proline content was estimated as per the method described by Bates *et al. (1973).*

Reagents: Sulphosalicylic acid (3%), toluene, ninhydrin, glacial acetic acid. The orthophosphoric acid, acid ninhydrin 2.48 g of ninhydrin was dissolved in 60 mL of glacial acetic acid and 40 mL of 6N phosphoric acid.

Estimation

Leaves were homogenized in 3% sulphosalicylic acid and filtered using Whatman filter paper No. 1. The mixture was cooked in a water bath at 100°C for an hour before cooling and being put to a test tube. Toluene was added, then vortexed for 15-20 seconds. The chromophore was extracted from the phase of water, and the toluene phase's absorbance was measured at 520nm. Toluene's absorbance was utilized as a blank and subtracted from the sample. The proline content was calculated using a standard curve with L-Proline as a standard and given in mg proline/g fresh weight.

A) Harvest index

Harvest index was recorded at full maturity of wheat plant. It was recorded as per the formula given below:

$$\text{Harvest Index} = \frac{\text{Total grain weight ofall plants present in the pot}}{\text{Total weight of above ground part of all plants in the pot}}$$

B) Yield/plant

Yield/plant was recorded at full maturity of wheat plant. The weight of all grain obtained from all spike of wheat plant in a pot was recorded on electronic balance and the average of grain weight of 2 pot was calculated.

Statistical analysis:

The statistical analysis was carried out using Panse and Sukhatme's (1985) methodology for the Factorial Completely Randomised Design. Data was analyzed using the statistical program "DSAASTAT" (version 1.101). Duncan's Multiple Range Test (DMRT) was used at the 5% level to determine mean separations.

EXPERIMENTAL FINDINGS

Relative water content (RWC)

The study found that the root water content (RWC) of wheat genotypes significantly decreased under various stress treatments. The maximum RWC was found in KRL-1-4 under control conditions, while the minimum was in HI-1563. The maximum reduction was observed in susceptible genotypes under salinity stress, with the highest reduction in susceptible genotypes. Terminal heat stress also significantly reduced RWC in all wheat genotypes, with the highest reduction in susceptible genotypes and the lowest in tolerant genotypes. The combined stress significantly reduced RWC in all wheat genotypes, with the maximum decrease observed in susceptible genotypes and the minimum in tolerant genotypes. The reduction in RWC under combined stress was more pronounced than individual stresses. Similar trends were observed in all wheat genotypes during 2017-18, with the decrease being more pronounced in susceptible genotypes under combined stress. [Table 1].

Membrane stability index (MSI)

During 2016-17, the maximum and minimum mean stress intensity (MSI) in wheat genotypes were observed under various stress treatments. The maximum reduction was observed in HD-2987 (84.8%) under control conditions, while the minimum was in HI-1563 (77.2%). The maximum reduction was observed for susceptible genotypes (HT-8 (24.9%), HI-1563 (21.4%), and HD-2987 (30.6%), while the minimum reduction was observed in tolerant genotypes (KRL-1-4 (14.6%), KRL-19 (13.9%), and HD-2733 (15.5%). High temperature also resulted in similar reductions in all wheat genotypes, with the maximum decrease observed in HT-8 (20.2%) and HD-2987 (19.9%). The combined stress treatment significantly reduced MSI in all wheat genotypes, with the maximum decrease observed in susceptible genotypes (39.5%) and the minimum decrease in tolerant genotypes (18.6%). Similar decreasing trends were observed in all genotypes during 2017-18.

Chlorophyll-a content

Chlorophyll-a content significantly decreased in wheat genotypes during 2016-17, with maximum content in HD-2733 and minimum in HI-1563. Salinity stress caused a significant reduction in chlorophyll-a content, with susceptible genotypes experiencing the greatest reduction. Terminal heat also significantly decreased chlorophyll-a content, with the highest decrease in genotypes HT-8 and HD-2987. The combined stress reduced chlorophyll-a content more than individual stress. Chlorophyll-a content also showed a decreasing trend under similar conditions in 2017-18. The study highlights the importance of understanding the effects of stress on wheat genotypes and their effects on chlorophyll-a content.

Chlorophyll-b content

In 2016-17, chlorophyll-b content in wheat flag leaf decreased significantly in both tolerant and susceptible genotypes under different stress treatments. The maximum chlorophyll-b content was found in HD-2733 under control conditions, while the minimum was in HD-2987High temperature also led to a significant decrease in chlorophyll-b content, with major decreases in genotypes HT-8 and HD-2987, and minor decreases in genotypes KRL-1-4, KRL-19, HI-1563, and HD-2733. A significant decrease in chlorophyll-b content was observed under combined stress treatment, with higher decreases in susceptible genotypes and lower reductions in tolerant genotypes. The reduction in chlorophyll-b content under combined stress was more pronounced than individual stresses. The chlorophyll-b content decreased similarly in flag leaf of both tolerant and susceptible wheat genotypes during 2017-18 under similar treatments.

Superoxide dismutase (SOD) activity

During 2016-17, SOD activity in wheat genotypes increased significantly under all stress treatments. KRL-19 showed the highest SOD activity, while HI-1563 had the lowest. Under salinity stress, all wheat genotypes showed a significant increase in SOD activity, with the least increase in susceptible genotypes and the most in tolerant genotypes. High temperature conditions also increased SOD activity, with the least increase in susceptible genotypes and the most in tolerant genotypes. The combined stress treatment also increased SOD activity in all wheat genotypes, with the least increase in susceptible genotypes and the most in tolerant genotypes. The increase in SOD activity under combined stress was more pronounced than individual stresses. The SOD activity showed a similar pattern of change in data collected during 2017-18 in response to all stress treatments.

Yield per Plant

Data from 2016-17 indicated that yield per plant declined considerably in both tolerant and sensitive wheat genotypes across all stress regimens. Under controlled conditions, HD-2733 had the highest yield per plant (12.0 g), whereas KRL-19 had the lowest (8.9 g). Under salinity stress, significant decreases were found, with the highest reductions in genotypes HT-8 (42.6%), HI-1563 (46.6%), and HD-2987 (47.0%), and the lowest in genotypes KRL-1-4 (25.6%), KRL-19 (23.6%), and HD-2733 (20.6%). Under high temperatures, yield per plant dropped dramatically in all wheat genotypes, with the

biggest loss reported in genotypes HT-8 (32.0%) and HD-2987 (32.9%) and the lowest in genotypes KRL-1-4 (23.5%), KRL-19 (20.6%), HI-1563 (17.4%), and HD-2733 (12.2%). The yield per plant dramatically fell with the combined stress treatment. [Table 2].

DISCUSSION

A cell's relative water content reflects the plant's water status and is connected with unavoidable environmental circumstances such as salty stress and final heat stress (Akhilesh et al., 2000). Salinity stress causes alterations in a variety of physiological and metabolic processes, including physiological drought and ion toxicity, depending on the degree and duration of the stress (Morales et al. 2003). Raised leaf and canopy temperatures reduced RWC, ψw, and transpiration rate (Farooq et al., 2011). Reduced leaf RWC causes a decrease in water potential, which disrupts numerous physiological processes (Tsukaguchi et al., 2003), including photosynthesis, transpiration rate, protein synthesis, enzyme activity, ion uptake and transport, and many others (Khalil et al., 2009). Similar alterations in photosynthesis (lower pigment content) and enzyme activity (higher SOD concentration) were identified in the current study. The current study found that salt, high temperature, and combined stress significantly decreased leaf relative water content (LRWC) in all genotypes studied, with the effect being more deleterious under combined stress. Moshe et al. (2017) observed a similar finding for tomato plants subjected to both salt and extreme temperature stress. Thus, it may be inferred that combined salt and heat stress provided a greater threat to water shortage in plants than individual stress, reflecting the hypo-additive response of both stressors. It was also shown that sensitive genotypes, such as HT-8, HI-1563, and HD-2987, showed a greater decline in LRWC, but tolerant genotypes, such as KRL-1-4, KRL-19, and HD-2733, maintained higher RWC throughout all stress treatments. Thus, genotypes that can resist various sorts of stressors by keeping leaves turgid in stressful settings would have physiological benefits and can sustain larger yields.

Membrane stability index

Cell membrane stability index (MSI) is widely used to express stress tolerance in plants and higher membrane stability is correlated with stress tolerance (Premchandra *et al*., 1990). Mahla *et al.* (2012) reported that accumulation of H_2O_2, which is a strong reactive oxygen species (ROS) under stress, led to disruption of cellular membrane integrity. In the present investigation also it was observed that salinity and high temperature stress individually and in combination, significantly decreased membrane stability index (MSI) and the effect was more detrimental under combined individual stress (Savchenko *et al*., 2002). Heat and salinity stresses generate excessive reactive oxygen species (ROS) than optimal level resulting in enhanced membrane damage in leaf tissue (Kipp and Boyle, 2013; Dhindsa, *et al*., 1981; Bhattacharjee and Mukherjee, 1997; Shim *et al*., 1999). In the present study also greater decrease in MSI was observed in susceptible genotypes, *viz*., HT-8, HI-1563 and HD-2987 under all stress treatments. However, tolerant genotypes KRL-1-4, KRL-19 and HD-2733 were found to reduced membrane damage by inducing higher level of antioxidant defense system (in terms of increased SOD) under all stress treatment that counteract the damaging effect of ROS thereby maintain comparatively high MSI. The results are in accordance with the findings of Sairam and Srivastava, (2002). Therefore, it may be a strategy of tolerance to decrease membrane fluidity by increasing the content of saturated and monounsaturated fatty acids by modulating their metabolism and increase the production of antioxidant defense system in response to increasing stress (Zhang *et al*., 2005a).

Chlorophyll contents

Photosynthesis is a crucial indicator of stress in plants, as growth and yield are linked to it. In this study, leaf chlorophyll content of wheat genotypes showed a significant positive correlation with yield per plant (Kocal et al. 2008; Tomaz et al. 2010). (Tables). Salinity and heat affect photosynthesis differently. Oxidative stress decreases chlorophyll levels in salt-stressed plants, inhibiting chlorophyll synthesis and activating its degrading enzyme chlorophyllase (Smirnoff, 1996; Santos, 2004). Stomatal closure in salt-treated plants is also a

cause of photosynthesis inhibition. Heat stress mainly affects the biochemical reactions of photosynthesis, causing plants to produce more reactive oxygen species (ROS), decreasing chlorophyll pigments and indirectly reflecting stress levels. (Poljakoff-Mayber & Lerner 1994; Camejo et al. 2005). Combined salt and heat stress posed greater threats to photosynthetic pigments than individual stress, and the response of these combined stresses is hypo-additive in behavior (Havaux 1993; Allakhverdiev et al., 2003; Wang et al., 2009; Chen et al., 2012). The tolerate mechanism adopted by some genotypes may be due to maintaining higher turgidity of cells, inducing a higher level of the antioxidant defence system, and successfully compartmentalizing salts in the vacuole (Sabbagh et al., 2014). This approach counteracts the damaging effect of ROS, resulting in less damage to photosynthetic pigments and maintaining higher yield.

Antioxidant defense system

The plants can induce the expression of antioxidant enzymes under stress condition to counteract oxidative damage. In the present study also, the exposure to salinity and high-temperature stress individually and in combination, up regulated the enzyme activity in terms of increased activity of superoxide dismutase in all tested wheat genotypes. However, the enzyme activities were higher under combined stress conditions (Tables), indicating that the response of two stress hypo-additively triggered antioxidative defence system to scavenge ROS (Tsukaguchi et al., 2003; Sairam & Tyagi, 2004). In addition, these enzymes exhibit an oxidase activity, mediating the reduction of oxygen to superoxide (O_2^-) and H_2O_2 radical into either ordinary molecular oxygen or hydrogen peroxide (Lin and Kao, 2002). The result of the present study also revealed that susceptible genotypes showed less increase in antioxidative enzymes activity (SOD) under all stress treatments, while the tolerant genotypes showed a higher increase in POX and SOD activity under similar stress treatments (Mittler, 2002). It indicates that the tolerant genotypes are more efficient in scavenging ROS by triggering the up-regulation of antioxidative defence machinery under given stress treatments to prevent the damage of impotent cell components i.e., protein, enzymes, pigments etc.) Thereby improving growth and yield (Zhao *et al.*, 2007).

Osmoprotectants

The accumulation of osmoprotectants, such as proline, is a crucial adaptive mechanism in plants subjected to various stresses (Sakamoto and Murata, 2000). These primary metabolites directly participate in the osmotic adjustment. The study found that salinity and high temperature stress individually and in combination induce the accumulation of proline in all wheat genotypes. However, the intensity of proline content increase was hypo-additive and more prevalent under combined stress (Ashraf and Foolad, 2007; Afzal et al., 2014; Hare and Cress, 1997; Ashraf and Foolad, 2007; Tatar and Gevrek, 2008; Yu et al., 2015). Proline accumulates to a high level in plants under stress such as drought, temperature, salinity, and other environmental stresses, providing protection of membrane integrity, stabilization of enzymes or proteins, and tolerance to stress (Yang et al., 2009; Sabbagh et al., 2014; Dadkhah and Rassam, 2016). The study also showed that tolerant genotypes accumulated a high amount of proline under individual and combined salinity and heat stresses (Mittler, 2002). The increase in proline content was more prominent in tolerant genotypes compared to the control and lesser in susceptible genotypes (Misra and Gupta, 2005, Zhu et al., 2004). Additionally, during the grain filling stage, moderate heat stress activated the accumulation of proline in higher amounts in wheat flag leaves, indicating that proline's role is related to protective action under moderate stress.

Yield components

The study found that excessive abiotic stress can cause irreversible damage to plant growth and yield. All wheat genotypes experienced a significant decrease in harvest index and yield under salinity, high temperature, and combined stress treatments (Tables) (Giaveno and Ferrero, 2003). Such reduction in yield and its associated parameters may be because under heat stress conditions the stomata open to cool the leaves

by transpiration, but when heat was combined with drought or salinity (physiological drought), stomata remained closed, resulting in a higher leaf temperature and a lower photosynthetic rate causing yield loss (Rizhsky *et al*., 2004). Heat stress shortened the duration of grain filling in spring wheat with a reduction in kernel growth leading to a reduction in kernel density and grain weight by up to 7% and thus resulting in yield loss in wheat (Guilioni *et al*., 1997). The study revealed that tolerant genotypes shower higher harvest index and grain yield per plant then susceptible genotypes. Similar results were reported from other studies also (El-Hendawy *et al*., 2005; Asgari *et al*., 2012, Prasad *et al*., 2008; Narayanan *et al*., 2015). The better performance of the tolerant genotype under all stress conditions was due to better physiological and biochemical responses in terms of increased levels of RWC, MSI, and chlorophyll content, high antioxidative enzyme activity (SOD), more accumulation of osmolyte (proline), in the flag leaves of wheat. Antioxidants protect the plants from oxidative damage thus overall maintaining higher yield (hervest index and yield per plant). Therefore, some indices like high LRWC, photosynthetic pigments, antioxidant capacity and osmolyte production, grain number per plant, floret sterility, harvest index and yield per plant etc. may further be studied in detail by the researchers to understand the mechanism of stress tolerance and to improve yield in wheat genotypes grown under high temperature and salinity-prone areas of India and world.

Table 3: Effect of salt, high temperature, and combination stress on wheat genotypes' leaf relative water content (%) during the flowering stage.

Genotypes (G)	**Treatments (T)**							
	Control (T_0)	**Salinity (T_1)**	**% Change**	**High Temperature (T_2)**	**% Change**	**Salinity + High Temperature (T_3)**	**% Change**	**Mean**
KRL-1-4 (G_1)	92.4	72.8	-21.2	77.5	-16.1	69.1	-25.3	77.9
KRL-19 (G_2)	89.9	72.8	-19.1	74.2	-17.4	66.6	-25.9	75.9
HD-2733 (G_3)	91.2	70.3	-22.9	79.3	-13.0	71.6	-21.5	78.1
HT-8 (G_4)	89.2	58.7	-34.2	69.0	-22.6	52.2	-41.4	67.3
HI-1563 (G_5)	87.9	59.9	-31.8	73.9	-15.9	60.0	-31.7	70.4
HD-2987 (G_6)	90.3	60.8	-32.6	72.6	-19.6	54.4	-39.8	69.5
Mean	90.1	65.9		74.4		62.3		73.2
Factor	**CD at 5%**	**CD at 1%**	**SEM**	**G_1, G_2 and G_3 indicate tolerant group G_4, G_5 and G_6 indicate susceptible group**				
Treatments	0.67	0.89	0.24					
Genotypes	0.82	1.09	0.29	**% Change indicate decrease over control**				
T x G	1.64	2.18	0.58					

Table 4: The effect of salt, high temperature, and combination stress on the membrane stability index (%) of wheat genotypes during flowering.

Genotypes (G)	**Treatments (T)**							
	Control (T_0)	**Salinity (T_1)**	**% Change**	**High Temperature (T_2)**	**% Change**	**Salinity + High Temperature (T_3)**	**% Change**	**Mean**
KRL-1-4 (G_1)	83.6	71.4	-14.6	75.3	-9.9	68.1	-18.6	74.6
KRL-19 (G_2)	80.6	69.4	-13.9	72.1	-10.6	64.8	-19.6	71.8

HD-2733 (G_3)	81.1	68.6	-15.5	73.5	-9.4	63.3	-21.9	71.6
HT-8 (G_4)	81.9	59.0	-27.9	65.3	-20.2	49.5	-39.5	63.9
HI-1563 (G_5)	77.2	60.7	-21.4	69.6	-9.9	53.4	-30.8	65.2
HD-2987 (G_6)	84.8	58.8	-30.6	67.9	-19.9	53.5	-36.9	66.2
Mean	81.2	65.4		59.1		55.3		65.2
Factor	**CD at 5%**	**CD at 1%**	**SEM**	**G_1, G_2 and G_3 indicate tolerant group**				
Treatments	0.54	0.72	0.19	**G_4, G_5 and G_6 indicate susceptible group**				
Genotypes	0.66	0.89	0.23	**% Change indicate decrease over control**				
T x G	1.33	1.77	0.47					

Table 5: The effect of salt, extreme temperatures, and combined stress on pigment a concentration (mg g-1 fresh weight) in wheat genotypes at flowering.

	Treatments (T)							
Genotypes (G)	**Control (T_0)**	**Salinity (T_1)**	**% Change**	**High Temperature (T_2)**	**% Change**	**Salinity + High Temperature (T_3)**	**% Change**	**Mean**
KRL-1-4 (G_1)	2.06	1.32	-36.0	1.44	-29.8	1.20	-41.8	1.50
KRL-19 (G_2)	2.11	1.35	-35.9	1.46	-31.0	1.20	-43.3	1.53
HD-2733 (G_3)	2.13	1.33	-37.7	1.54	-27.9	1.30	-39.1	1.58
HT-8 (G_4)	2.13	1.19	-44.1	1.26	-41.0	0.98	-54.2	1.39
HI-1563 (G_5)	1.99	1.08	-45.5	1.44	-27.9	0.95	-52.2	1.37
HD-2987 (G_6)	2.09	1.17	-44.1	1.24	-40.6	0.97	-53.6	1.37
Mean	2.09	1.24		1.40		1.10		1.46
Factor	**CD at 5%**	**CD at 1%**	**SEM**	**G_1, G_2 and G_3 indicate tolerant group**				
Treatments	0.116	0.155	0.041	**G_4, G_5 and G_6 indicate susceptible group**				
Genotypes	0.143	NS	0.050	**% Change indicate decrease over control**				
T x G	NS	NS	0.100	**NS= Non significant**				

Table 6: Effects of salt, high temperature, and mixed stress on chlorophyll b concentration (mg g-1 fresh weight) in wheat genotypes during flowering.

Genotypes (G)	Treatments (T)							
	Control (T_0)	Salinity (T_1)	% Change	High Temperature (T_2)	% Change	Salinity + High Temperature (T_3)	% Change	Mean
KRL-1-4 (G_1)	0.69	0.47	-32.3	0.48	-30.4	0.41	-40.4	0.51
KRL-19 (G_2)	0.60	0.41	-32.5	0.43	-28.2	0.34	-43.1	0.45
HD-2733 (G_3)	0.72	0.47	-35.3	0.56	-22.7	0.43	-39.9	0.54
HT-8 (G_4)	0.63	0.35	-43.9	0.38	-39.8	0.26	-58.5	0.40
HI-1563 (G_5)	0.65	0.37	-44.1	0.51	-22.5	0.33	-49.3	0.46
HD-2987 (G_6)	0.56	0.32	-42.6	0.33	-40.9	0.26	-54.3	0.37
Mean	0.64	0.40		0.45		0.34		0.46
Factor	CD at 5%	CD at 1%	SEM	G_1, G_2 and G_3 indicate tolerant group G_4, G_5 and G_6 indicate susceptible group				
Treatments	0.129	0.172	0.045					
Genotypes	0.158	0.210	0.055	% Change indicate decrease over control NS= Non significant				
T x G	NS	NS	0.111					

Table 7. Effect of salinity, high temperature, and combination stress on superoxide dismutase activity (unit mg-1 protein min-1) during the flowering stage of wheat genotypes.

Genotypes (G)	Treatments (T)							
	Control (T_0)	Salinity (T_1)	% Change	High Temperature (T_2)	% Change	Salinity + High Temperature (T_3)	% Change	Mean
KRL-1-4 (G_1)	4.12	6.72	63.0	6.08	47.5	7.22	75.2	6.04
KRL-19 (G_2)	4.42	6.90	56.2	6.62	49.9	7.29	65.0	6.31
HD-2733 (G_3)	3.96	6.02	52.2	6.04	52.7	7.13	80.1	5.79
HT-8 (G_4)	4.03	5.60	38.7	5.17	28.3	5.89	46.1	5.17
HI-1563 (G_5)	3.94	5.42	37.5	6.12	55.3	6.00	52.2	5.37
HD-2987 (G_6)	4.00	5.51	37.7	5.17	29.0	5.95	48.7	5.16
Mean	4.08	6.03		5.87		6.58		5.64
Factor	CD at 5%	CD at 1%	SEM	G_1, G_2 and G_3 indicate tolerant group G_4, G_5 and G_6 indicate susceptible group				
Treatme	0.55	0.73	0.19					

nts				
Genotypes	0.67	0.90	0.24	**% Change indicate increase over control**
T x G	NS	NS	0.47	**NS= Non significant**

Table 8: Effect of salt, high temperature, and combination stress on proline concentration (mg g-1 fresh weight) in wheat genotypes during flowering.

Genotypes (G)	Treatments (T)							
	Control (T_0)	Salinity (T_1)	% Change	High Temperature (T_2)	% Change	Salinity + High Temperature (T_3)	% Change	Mean
KRL-1-4 (G_1)	0.033	0.048	45.9	0.043	30.6	0.052	58.2	0.044
KRL-19 (G_2)	0.031	0.046	48.0	0.041	31.8	0.047	50.7	0.041
HD-2733 (G_3)	0.036	0.050	40.6	0.047	32.7	0.058	62.7	0.048
HT-8 (G_4)	0.033	0.039	20.2	0.038	15.5	0.043	32.5	0.038
HI-1563 (G_5)	0.030	0.038	29.1	0.039	33.0	0.041	38.6	0.037
HD-2987 (G_6)	0.028	0.036	27.9	0.034	21.6	0.041	46.7	0.035
Mean	0.032	0.043		0.040		0.047		0.040
Factor	CD at 5%	CD at 1%	SEM	**G_1, G_2 and G_3 indicate tolerant group G_4, G_5 and G_6 indicate susceptible group**				
Treatments	0.0032	0.0042	0.0011					
Genotypes	0.0039	0.0052	0.0014	**% Change indicate increase over control NS= Non significant**				
T x G	NS	NS	0.0027					

Table 9: The effect of salt, high temperature, and combination stress on wheat genotypes' harvest index %).

Genotypes (G)	Treatments (T)							
	Control (T_0)	Salinity (T_1)	% Change	High Temperature (T_2)	% Change	Salinity + High Temperature (T_3)	% Change	Mean
KRL-1-4 (G_1)	41.9	35.0	-16.5	37.3	-10.9	33.1	-21.1	36.8
KRL-19 (G_2)	38.9	33.9	-12.8	35.5	-8.8	31.2	-19.7	34.9
HD-2733 (G_3)	41.1	35.3	-14.0	38.2	-7.1	34.1	-17.0	37.2
HT-8 (G_4)	40.6	30.1	-25.9	33.9	-16.5	28.0	-31.0	33.2
HI-1563 (G_5)	37.5	29.4	-21.7	34.1	-9.2	27.4	-26.9	32.1
HD-2987 (G_6)	38.0	30.5	-19.8	28.8	-24.2	25.3	-33.5	30.7

Mean	39.7	32.4		34.6		29.9		34.1
Factor	**CD at 5%**	**CD at 1%**	**SEM**	**G_1, G_2 and G_3 indicate tolerant group G_4, G_5 and G_6 indicate susceptible group**				
Treatments	NS	NS	3.30					
Genotypes	NS	NS	4.05	**% Change indicate decrease over control**				
T x G	NS	NS	8.10	**NS= Non Significant**				

Table 10: The effects of salt, high temperature, and combination stress on wheat genotype yield plant-1 (g).

Genotypes (G)	**Treatments (T)**							
	Control (T_0)	**Salinity (T_1)**	**% Change**	**High Temperature (T_2)**	**% Change**	**Salinity + High Temperature (T_3)**	**% Change**	**Mean**
KRL-1-4 (G_1)	9.7	7.2	-25.6	7.4	-23.5	6.3	-35.4	7.7
KRL-19 (G_2)	8.9	6.8	-23.6	7.1	-20.6	5.5	-38.1	7.1
HD-2733 (G_3)	11.2	8.9	-20.6	9.8	-12.2	7.8	-30.4	9.4
HT-8 (G_4)	9.3	5.3	-42.6	6.3	-32.0	4.5	-52.0	6.3
HI-1563 (G_5)	9.5	5.1	-46.6	7.8	-17.4	4.7	-50.5	6.8
HD-2987 (G_6)	9.0	4.8	-47.0	6.1	-32.9	4.3	-52.0	6.1
Mean	9.6	6.3		7.4		5.5		7.2
Factor	**CD at 5%**	**CD at 1%**	**SEM**	**G_1, G_2 and G_3 indicate tolerant group G_4, G_5 and G_6 indicate susceptible group**				
Treatments	2.46	NS	0.87					
Genotypes	NS	NS	1.06	**% Change indicate decrease over control**				
T x G	NS	NS	2.12	**NS= Non significant**				

CONCLUSION

A highly significant positive correlation of physiological traits (leaf photosynthetic pigments, LRWC and MSI) and biochemical traits (antioxidative enzyme activity and osmoprotectant) was observed with yield per plant. Furthermore, combined strains caused altered water relations and chlorophyll content, as well as elevated proline content and SOD activities, as compared to solo stress. Meanwhile, tolerant genotypes with lower yield loss (harvest index and yield per plant) had higher RWC, CSI, pigments produced by photosynthesis, antioxidant activity, and proline levels than susceptible plants, resulting in higher harvest index and yield per plant.These results highlight the need to develop new strategies for improvement of stress tolerance in wheat population that target high temperature and salinity-prone areas Traits like high LRWC, photosynthetic pigments, antioxidant capacity, osmolyte production and harvest index and grain yield per plant can be used for breeding programs for development of stress tolerant varieties under combined salinity and high temperature stress condition.

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