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Oxidative stress induced flag leaf injury in wheat (*Triticum aestivum* L.) under drought

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Abstract
The present study evaluated the physiological and biochemical changes associated with flag leaf cell membrane injury in two wheat varieties *viz.* WH 1105 and WH 1025 under drought stress. A decrease in leaf membrane stability was observed under drought stress with an increase in duration of grain development. Leaf membrane injury was low in WH 1025 than WH 1105. Lipoxygenase (LOX) activity was increased under drought stress and higher increase was noticed in drought sensitive WH 1105 than drought tolerant WH 1025. Malondialdehyde (MDA) content in leaves of WH 1105 and WH 1025 were significantly increased. Higher decrease in leaf lipid content was observed in WH 1105 than WH 1025 indicating higher injury to leaf lipids.

Keywords: Wheat *(Triticum aestivum* L.), drought stress, leaf membrane stability, lipoxygenase, leaf lipids, malondialdehyde

Introduction
Wheat is second major food crop in India and consumed largely for its nutrient complement of seed storage proteins and starch. It is a staple food crop that contributes more calories to the world diet than any other cereal crop (Abd-EI-Haleem *et al.*, 2009) [1]. Bread wheat has unique ability to produce broad range of nutritious and appealing foods (Craig. F. M. 2002) [5]. Wheat prefers cold temperature for growth and development and its cultivation has been increased in the country due to introduction of new high yielding varieties (HYV). Adverse environmental conditions are major constraints for crop productivity which cause low yields. Drought, salinity, heat and high temperatures are important environmental factors occurring in some regions and severely limit crop production. Development of impressive irrigation potentials ensured food security in India, yet wheat cultivation is affected by frequent occurrence of droughts (Anil G. 2003) [2].

Leaf membrane stability can be a significant selection criterion for drought stress tolerance (Mehmet *et al.*, 2009) [16]. Cell membranes are one of the first targets of many plant stresses and their integrity and stability under water stress is a major component of drought tolerance (Mohammed *et al.*, 2001) [18]. Electrical conductivity in drought stressed flag leaf tissues is increased due to damage to the cell membrane lipids and consequent solute leakage.

Lipoxygenase (LOX) is one of the main oxidative biocatalysts in the plant cell and its reaction proceeds via free radical mechanism that is known to have a role in membrane integrity (Venesa *et al.*, 2014) [28]. Water stress results significant decrease in polar lipids of wheat (Mimoun *et al.*, 2006) [17]. Malodialdehyde (MDA) content is an indicator of lipid peroxidation and can reflect the degree of damage at adverse conditions. It can also be regarded as a biomarker for lipid peroxidation, so the decrease in MDA content indicates higher antioxidant ability and higher resistance to drought (Dhanda *et al.*, 2004) [6].

Materials and Methods
Seeds of two varieties of wheat *viz.* WH 1105 (Drought sensitive) and WH 1025 (Drought tolerant) were obtained from Wheat and Barley Section, Department of Genetics and Plant Breeding, College of Agriculture, CCSHAU, Hisar. Seeds of uniform size were sown in micro plots by keeping recommended spacing at research field area of department of crop physiology in the university farm under randomized block design (RBD), with pre- sown irrigation only

for drought condition and recommended irrigation schedules for control. Plants were allowed to grow up to maturity.

Leaf samples were collected at four stages starting from 7th day after anthesis (7, 14, 21 and 28 days). Plants samples were brought to the laboratory by keeping them in liquid nitrogen after cutting from plant. Leaf material was cut into small bits and used for determination of leaf membrane thermo stability (LMS). Leaf extracts were also prepared in suitable extraction medium and were used for estimation lipoxygenase (LOX) activity, leaf lipid and malodialdehyde (MDA) contents.

Leaf membrane stability

Leaf membrane thermo stability (LMS) was measured following the method of Ibrahim & Quick (2001) [12]. Fresh leaf material (100 mg) was taken in test tube, washed twice with deionised water, 10 ml of deionised water was added and incubated in water bath at 49 ºC for 30 min. Then the test tube was kept at 10 ºC for 18 to 24 hours to allow diffusion of electrolytes from leaves. The test tube was brought to 25 ºC and shaken, initial conductance (T_1) of content of test tube was measured with an electrical conductivity meter. The test tube was then placed in an autoclave at 0.10 Mpa pressure for 10 min. to completely kill the tissue and to release all electrolytes, again measured electrical conductance (T_2). The level of LMS was determined from the following formula:

$$\text{LMS } (\%) = \left(1 - \frac{T_1}{T_2}\right) \times 100$$

Where, T1 and T2 refer to conductance value before and after autoclaving, respectively.

Lipoxygenase (LOX) (EC 1.13.11.12)

Extraction

Lipoxygenase activity was estimated by the method of Surrey (1964), where 1 g of sample material was homogenized with 5 ml of chilled 0.1 M Tris-HCl buffer (pH 7.5), containing 10% NaCl and centrifuged the homogenate at 10,000 × g for 30 min. in a refrigerated centrifuge at 4 ºC. The supernatant represents the enzyme extract, thus decanted the supernatant so obtained and used it as enzyme extract.

Preparation of enzyme substrate

The substrate linoleic acid for lipoxygenase assay was processed in such a way to avoid formation of turbidity upon its addition to the reaction mixture. Accordingly linoleic acid solution was prepared which consisted of 0.5 ml of Tween-20, 100 ml of 0.1 M borate buffer (pH 9.0), 0.5 ml of linoleic acid, 1.3 ml of 1 N NaOH and 97.7 ml of distilled water.

Procedure

Reaction mixture contained 2.785 ml of 100 mM phosphate buffer (pH 6.8) and 200 µl of enzyme extract. The reaction was initiated by adding 15 µl linoleic acid which caused the increase in absorbance due to formation of conjugated dienes, measured at 243 nm in spectrophotometer. Enzyme activity was calculated using a molar extinction coefficient of 2.74×10^4 M^{-1} cm^{-1}.

Leaf lipids

Extraction

Total lipid in leaf was extracted and estimated by the method described by Sawhney & Singh, (2001) [21], where sample material of 1 g was ground in the presence of 5 g anhydrous sodium sulphate using a mortar and pestle, a small amount of acid washed sand was also used as an abrasive material, to that 20 ml of chloroform-methanol mixture was added, transferred to an air tight glass stoppered iodometric flak, shaken the content of the flask on a mechanical shaker for one hour, filtered through a glass sintered funnel, repeated the extraction of the residue twice and pooled the filtrates. The solvent was removed from the residue by distilling under vacuum and extracted it again with 10 ml of chloroform-methanol mixture which contained 1 ml of 1% sodium chloride. The pooled fractions were taken in a separating funnel, shaken it thoroughly and allowed it to stand for 5 min. where lipids were recovered in the lower chloroform layer. Drained out the lower layer and treated the upper layer again three to four times with 5-10 ml of chloroform-methanol mixture to extract any residual lipid from it.

Procedure

Collected the lipid contained fractions in a pre-weighed beaker, evaporated the solvent when kept the beaker in warm water bath (50 ^{0}C) and blew slow stream of nitrogen gas over the surface. Recorded the weight of the beaker, determined the amount of total lipids in the sample by subtracting the weight of the empty beaker and expressed the results in terms of per cent of total lipid in the sample.

Malondialdehyde

Extraction

Malondialdehyde (MDA) content was estimated according to the method of Heath & Packer (1968) [10], where 1 g of sample material was homogenized in 5 ml of 0.1% trichloro acetic acid (TCA) in mortar and pestle, centrifuged the content at 8000 × g for 15 min. decanted the supernatant which was used for MDA estimation.

Procedure

To 0.5 ml of supernatant, 2.3 ml of 20% (w/v) trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid was added. The mixture was heated in a water bath at 95 °C for 30 min. and quickly cooled in ice bath. Then the absorbance was recorded at 532 nm and the value of non-specific absorption at 600 nm was subtracted from it. The concentration of malondialdehyde was calculated using the extinction coefficient of 155 mM-1 cm -1

Results

Leaf membrane stability

Membrane stability (%) of wheat varieties in flag leaf under irrigated and drought stress conditions is shown in Table 1. Membrane stability in flag leaves of wheat varieties decreased under stress condition with different days after anthesis. The value for membrane stability of WH 1105 was 75.64, 74.94, 73.67 and 72.10 per cent under irrigated condition and 65.62, 62.55, 59.64 and 52.07 per cent under drought stress condition at 7th, 14th, 21st and 28th days after anthesis respectively. In WH 1025, value of membrane stability was 78.91, 78.61, 72.44 and 64.00 per cent under irrigated condition and 70.90, 68.00, 59.00 and 50.80 per cent under drought stress condition at 7th, 14th, 21st and 28th days after anthesis respectively.

Maximum membrane stability was observed in WH 1025 (78.91 per cent) at 7th day after anthesis under irrigated condition and minimum (50.80 per cent) at 28th day after anthesis under drought condition. Higher per cent reduction (13.25 to 27.77) in membrane stability was noted in WH 1105

and lower per cent reduction was observed in WH 1025 (10.16 to 19.63) at 7th to 28th days after anthesis. WH 1025 performed better in terms of membrane thermo stability at different days after anthesis compared to WH 1105.

Table 1: Effect of drought stress on leaf membrane stability (%) in flag leaf of wheat during grain development

S. No.	DAA	WH 1105			WH 1025		
		Irrigated	Drought	% Reduction	Irrigated	Drought	% Reduction
1	7	75.64	65.62	13.25	78.91	70.90	10.16
2	14	74.94	62.55	16.54	78.61	68.00	13.50
3	21	73.67	59.64	19.05	72.44	59.00	18.55
4	28	72.10	52.07	27.77	64.00	50.80	19.63
CD at 5%		E= 2.86, DAA = 1.22, E X DAA = 4.72			E= 2.59, DAA= 0.84, E X DAA = 3.22		

Lipoxygenase activity

The data for lipoxygenase activity obtained under irrigated and drought stress conditions are given in Table 2. The lipoxygenase activity of flag leaf was measured between 7th to 28th days after anthesis. The progressive increase in lipoxygenase activity was observed under drought stress condition with a maximum activity recorded at 28th day after anthesis in WH 1105. The lipoxygenase activity ranged from 12.82 to 19.00 units g^{-1} f. wt. under irrigated condition and 16.80 to 29.00 units g^{-1} f. wt. under drought condition in WH 1105, while it was 12.90 to 15.77 units g^{-1} f. wt. under irrigated condition and 15.22 to 20.66 units g^{-1} f. wt. under drought condition in WH 1025 from 7th to 28th days after anthesis. The per cent increase varied from 31.05 to 52.63 in WH 1105 and 17.98 to 31.01 in WH 1025 from 7th to 28th days after anthesis. The per cent increase was found to be more in WH 1105 than WH 1025 indicating that WH 1105 suffered more under drought stress.

Table 2: Effect of drought stress on lipoxygenase activity (unit g^{-1} f. wt.) in flag leaf of wheat during grain development

S. No.	DAA	WH 1105			WH 1025		
		Irrigated	Drought	% Increase	Irrigated	Drought	% Increase
1	7	12.82	16.80	31.05	12.90	15.22	17.98
2	14	15.77	21.20	34.40	13.30	16.40	23.31
3	21	16.40	25.00	52.44	14.00	17.80	27.14
4	28	19.00	29.00	52.63	15.77	20.66	31.01
CD at 5%		E = 0.25, DAA = 0.35, E X DAA = 0.50			E = 0.39, DAA = 0.55, E X DAA = 0.78		

Malondialdehyde

Malondialdehyde content in flag leaf of two wheat varieties under irrigated and drought stress conditions is shown in Table 3. Malondialdehyde content in flag leaf of wheat varieties increased under drought stress condition. The value of malondialdehyde content was 3.70, 3.99, 4.22 and 4.34 µmole g^{-1} f. wt. under irrigated condition and 4.80, 5.05, 5.15 and 5.20 under drought stress condition in WH 1105 at 7th, 14th, 21st and 28th days after anthesis respectively. In WH 1025, the value for malondialdehyde content was 3.80, 4.00, 4.20 and 4.40 under irrigated condition and 4.70, 4.90, 4.93 and 4.99 µmole g^{-1} f. wt. under drought stress condition at 7th, 14th, 21st and 28th days after anthesis respectively. Maximum malondialdehyde content of 5.20 µmole g^{-1} f. wt. was recorded at 28th day after anthesis under drought stress condition and minimum of 3.70 µmole g^{-1} f. wt. was recorded at 7th day after anthesis under irrigated condition in WH 1105. Higher per cent increase (29.73, 26.69, 21.90 and 19.82) in malondialdehyde content was noted in WH 1105 and lower per cent increase (23.68, 22.50, 17.38 and 13.41) in WH 1025 at 7th, 14th, 21st and 28th days after anthesis respectively under drought stress condition over irrigated condition. Considerable difference in malondialdehyde content was observed among both varieties under drought over irrigated condition.

Table 3: Effect of drought stress on malondialdehyde (µmole g^{-1} f. wt.) in flag leaf of wheat during grain development

S. No.	DAA	WH 1105			WH 1025		
		Irrigated	Drought	% Increase	Irrigated	Drought	% Increase
1	7	3.70	4.80	29.73	3.80	4.70	23.68
2	14	3.99	5.05	26.69	4.00	4.90	22.50
3	21	4.22	5.15	21.90	4.20	4.93	17.38
4	28	4.34	5.20	19.82	4.40	4.99	13.41
CD at 5%		E = 0.69, DAA = NS, E X DAA = NS			E = 0.09, DAA = 0.13, E X DAA = NS		

Leaf lipids

Table 4 shows the lipid content (%) in flag leaf under irrigated and drought stress conditions at different days after anthesis. Reduction in lipid content was observed under drought condition at different days after anthesis. WH 1025 showed less reduction (15.09, 10.86, 10.81 and 11.42 per cent) and WH 1105 showed more reduction (28.07, 27.90, 20.58 and 16.66 per cent) at 7th, 14th, 21st and 28th days after anthesis respectively under drought stress condition compared to irrigated condition. The lipid content was higher at 7th day after anthesis in both the varieties. The lipid content decreased from 0.57 to 0.30 per cent under irrigated condition from 7th to 28th DAA in WH 1105 and from 0.41 to 0.25 per cent under drought condition. Similarly, it decreased from 0.53 to 0.35 per cent under irrigated condition and from 0.45 to 0.31 per cent under drought condition in WH1025.

Table 4: Effect of drought stress on leaf lipid content (%) in flag leaf of wheat during grain development

S. No.	DAA	WH 1105			WH 1025		
		Irrigated	Drought	% Reduction	Irrigated	Drought	% Reduction
1	7	0.57	0.41	28.07	0.53	0.45	15.09
2	14	0.43	0.31	27.90	0.46	0.41	10.86
3	21	0.34	0.27	20.58	0.37	0.33	10.81
4	28	0.30	0.25	16.66	0.35	0.31	11.42
CD at 5%		E = 0.46, DAA = 0.65, E X DAA = NS			E = 0.19, DAA = 0.01, E X DAA = 0.02		

Discussion

Leaf membrane stability can be a significant selection criterion for drought stress tolerance (Mehmet *et al.* 2009) [16]. When tissues are subjected to high temperature, electrical conductivity (EC) increases due to damage to cell membranes and consequent solute leakage (Ranjeet *et al.* 2012) [26]. In present investigation, a decrease in leaf membrane stability was observed under drought stress with increase in duration of grain developmental stages. The lowest value was observed at 28th day after anthesis (DAA) in WH 1025 under drought stress (Table 1). Leaf membrane stability was found to be high in WH 1025 as compared to WH 1105. With a prolonged drought stress a decrease in leaf membrane stability was observed in both varieties. Reactive oxygen species produced during drought stress can damage cell membrane lipids which cause loss of membrane integrity and thereby release cellular components which could raise the EC. The enhancement of EC in terms of cell membrane damage was more pronounced under drought stress in both the varieties. WH 1025 showed less leaf membrane damage under drought stress condition. The results observed in present study strongly support the earlier observations by Lyudmila *et al.* (2009) [14] who reported that membrane stability was lower in drought-sensitive than in tolerant varieties and did not diminish during grain filling. Our results also show that membrane intactness was better maintained in WH 1025 variety as compared to WH 1105. This is in agreement with earlier reports which show that tolerant wheat genotypes showed higher cell membrane stability (Simova-Stoilova *et al.* 2008; Hojjat *et al.* 2012) [22, 11]. A decrease in membrane stability as reflected by the extent of lipid peroxidation is caused by reactive oxygen species (Sairam & Srivasthava, 2001; Anjum *et al.* 2011) [20, 3]. Gomathi & Rakkiyappan, (2011) [8] reported higher membrane stability under water stress condition. Constantina *et al.* (2008) [4] reported that osmotic adjustment had significantly slower water stress induced senescence of leaves, lower drought induced membrane injury.

Lipoxygenase (LOX) is one of the main oxidative biocatalysts in the plant cell and its reaction proceeds *via* a free radical mechanism that is known to have a role in membrane integrity (Vanessa *et al.* 2014) [28]. It has been proved that singlet oxygen and superoxide anions can be formed during the LOX catalyzed oxidation of fatty acids and increased LOX activities are interpreted as reason for an increased lipid peroxidation under stress condition. A reduced LOX activity under stress condition, no matter if due to down regulation or a simple inhibition can be considered beneficial to plants as LOXs are oxidative enzymes which can set radicals free while they are working (Lynch & Thompson, 1994) [13]. The results presented in Table 2 indicate the LOX activity increases under drought stress and higher increase was observed in WH 1105 than WH 1025 during different stages of grain development. The damage caused by LOX was less in WH 1025 which was due to its lesser activity in leaves. These results are in accordance with Gong *et al.* (2008) [9] who had also observed an increase in LOX activity under drought stress in wheat at different developmental stages. Markus & Manfred (2002) [15] reported that the specific activity of LOX reduced by 60 per cent in the drought stressed plants. As rewatering caused an increased in LOX activity, the observed reduction can fairly be attributed to drought.

Malondialdehyde (MDA) content is an indicator of lipid peroxidation and can reflect the degree of damage at adverse conditions. It can also be regarded as a biomarker for lipid peroxidation so the decrease in MDA content indicates higher antioxidant ability and higher resistance to drought (Dhanda *et al.* 2004) [6]. As shown in Table 3, MDA content in leaves of WH 1105 and WH 1025 significantly increased under drought stress. At different developmental stages of grain under continuous drought stress, MDA content increased at higher rate in WH 1105 as compared to WH 1025. The results showed that under drought stress, the oxidative damage of the leaves of WH 1105 was greater than WH 1025. Our findings are in accordance with the observations of Usha & Bhumika (2012) [27] who had reported that accumulation of MDA content was three times higher in susceptible varieties than tolerant wheat varieties. The results are also in corroboration with Nikolaeva *et al.* (2010) [19] who observed significant increase in MDA content in wheat leaves grown under water stress indicating the acceleration of lipid peroxidation. Rabiye & Asim (2006) [25] observed that MDA content increased in the early period of drought stress and later decreased. Similarly, Mimoun *et al.* (2006) [17] reported that MDA is augmented under water stress in Moroccan wheat varieties and the rate of increase was 41 and 19 per cent in Nasma and Tigre cultivars respectively.

Much of the injury to plants under abiotic stress is linked to oxidative damage at cellular level. ROS can cause direct damage to membrane lipids (El-Hafid *et al.* 1989; Smirnoff, 1993) [7, 23]. Results in Table 4 shows that leaf lipid content decreased under drought stress in leaves of both wheat varieties at different developmental stages of grain, however, more decrease was observed in WH 1105 than WH 1025 indicating higher injury to the membrane lipids. The results also demonstrated that WH 1025 adapted to drought stress showed a more lipid content than WH 1105. Similar results are reported by Mimoun *et al.* (2006) [17] who showed that water stress leads to significant decrease in polar lipids in Namsa and Tigre wheat varieties.

Conclusions

Leaf membrane stability decreased under drought stress condition. More reduction was observed in WH 1105 (27.77 per cent) than WH 1025 (19.63 per cent).

Lipoxygenase activity and malondialdehyde content increased under drought stress and WH 1105 showed higher increase as compared to WH 1025. The lipoxygenase activity increased from 31.05 to 52.63 per cent in WH 1105 and 17.98 to 31.01 per cent in WH 1025 from 7th to 28th days after anthesis. Higher MDA content in WH 1105 reflected more damage in comparison to WH 1025 under stress condition.

Flag leaf lipid content was decreased under drought stress in both varieties, but more reduction was observed in WH 1105. The per cent decrease in lipid content was found to be more in WH 1105 than WH 1025 with respect to different grain developmental stages.

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