

Higher activity of monodehydroascorbate reductase and lower activities of leaf and spike vacuolar invertase and glutathione S-transferase reveals higher number of grains per spike in spring wheat genotypes grown under well-watered and drought conditions

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Abstract

Background: To improve our understanding about the physiological mechanism of grain yield reduction at anthesis, three spring wheat genotypes (L1, L2 and L3) having contrasting yield potential under drought in field were investigated under controlled greenhouse conditions., drought stress was imposed at anthesis stage by withholding irrigation until all plant available water was depleted, while well-watered control plants were kept at 95% pot water holding capacity.

Results: Compared to genotype L1 and L2, pronounced decrease in grain number (NGS), grain yield (GY) and harvest index (HI) were found in genotype L3, mainly due to its greater kernel abortion (KA) under drought. A significant positive correlation of leaf monodehydroascorbate reductase (MDHAR) with both NGS and HI was observed. In contrast, significant negative correlations of glutathione S-transferase (GST) and vacuolar invertase (vacInv) both within source and sink with NGS and HI were found. Likewise, a significant negative correlation of leaf abscisic acid (ABA) with NGS was noticed. Moreover, leaf aldolase and cell wall peroxidase (cwPOX) activities were significantly and positively associated with TKW.

Conclusion: Collectively, distinct physiological markers were correlating with yield traits and higher activity of leaf aldolase and cwPOX may be chosen as predictive bio-markers for higher TKW. Also, higher activity of MDHAR within the leaf can be selected as a predictive bio-marker for higher NGS in wheat under drought. Whereas, lower activity of vacInv and GST both within leaf and spike can be selected as bio-markers for higher NGS and HI. The results highlighted the role of antioxidant and carbohydrate-metabolic enzymes in the modulation of source-sink balance in wheat crops, which could be used as bio-signatures for breeding and selection of drought-resilient wheat genotypes for a future drier climate.

1. Background

Wheat is a staple food in many countries and grown at 215 million ha area globally. However, cultivation of this crop in an ever-decreasing water scenario due to climate changes has caused enormous challenges to meet the global food security. Water is vital throughout wheat growth phases and, anthesis and post-anthesis stages are considered more sensitive to drought (Öztürk and Aydin, 2004; Farooq et al., 2014). Limited water availability at these stages directly affects grain number and grain weight and ultimately yield potential is severely reduced [1].

In the past, this crop has been improved through breeding for its drought tolerance [3]. However, there is a need to understand the in-depth physiological mechanism to breed drought-resilience wheat. Specially the understanding of physiological changes under drought stress and variation in the activity of key carbohydrate and antioxidants enzymes can be helpful to devise a strategy of plant improvement. Primarily, plant drought avoidance is achieved through closure of stomata. This closure of stomata is induced through root-to-shoot signaling mainly abscisic acid (ABA) which is transported from roots to leaves via xylem vessels to induce stomatal closure [4]. Plant genotypes having high yield potential under drought often regulate their stomata to maintain higher photosynthetic rate and/or lower transpiration rate thus a less reduction of biomass and grain yield [5]. Limited supply of photosynthates (carbohydrates) from source to sink could be due to both a lowered source activity and limited sink strength, while reduction of sink strength is the main reason of lower grain yield [6].

It is well-known that reduced photosynthesis under drought is associated with modified carbohydrate metabolism of leaves [7, 8]. On the other hand, reduction of sink strength due to impaired carbohydrate metabolism has been reported in reproductive tissues under drought stress [9]. Under drought, limited supply of carbon creates a competition between sink organs, which resultants in reduced sink strength and yield [10]. Several key metabolic enzymes have been reported to perform a vital role for sugar conversions within source and sink, which have been demonstrated to have striking roles in regulating abiotic stress tolerance. Albacete et al. [11] found that plant adaptation to drought stress can be improved by the over-expression a cell wall invertase gene (CIN1). Likewise, carbohydrates are stored in sub-cellular organelles, and vacuoles are considered an important energy reservoir of cells. Under severe drought stress conditions, when no photosynthates are being synthesized/produced or translocated to the developing sinks, a decrease in the activity of cell wall invertase (cwInv) enzyme could happen [11], and plants may turn to use the stored carbohydrates to fulfill their energy demands [12, 13]. This response may be associated with an increased activity of other invertases like cytoplasmic and vacuolar invertase which facilitates the remobilization of photo-assimilates from source to sink [14]. Roitsch and González [15] reported higher activity of vacuolar invertase (vacInv) which stimulates the remobilization of stored carbohydrates into reproductive organs is required for normal plant growth. Likewise, Goetz et al. [16] reported that both vacInv and cwInv play a role in tobacco pollination. Cytoplasmic invertase (cytInv), are also called acid/neutral-Invertases (A/N invertase) due to alkaline/neutral pH of this invertase. Barratt et al. [17] reported that the growth of *Arabidopsis thaliana* was not affected by deficiency in sucrose synthase (SuSy) but was severely reduced due to deficiency of acid/neutral cytoplasmic invertase (cytInv). Vargas and Salerno [18] depicted that cytInv has two central signaling pathways and may have a role in cross talk regulation of novel physiological functions. CytInv is located at multiple places within cell and may have a more important role than SuSy in coordination of metabolism [18]. VacInv plays a role in sugar storage and regulation of sweetening [15]. Wang et al. [19] studied the role of vacInv in the development of cotton fibers. They reported the contribution of this enzyme to cell expansion and that fibers having higher activity of vacuolar invertase developed fast. Furthermore, Ni [20] observed an increase of stomatal opening in transgenic cotton over-expressing a vacuolar invertase gene, indicating that this enzyme may be involved in the regulation of stomatal opening. Yamada et al. [21] detected that osmotic potential in *Arabidopsis thaliana* was regulated through an increase in the activity of vacuolar invertase both under drought and salinity stress. Consistent with this, Trouverie et al. [22] reported that ABA-induced increase in the activity of acid vacuolar invertase contributes hexose accumulation hence osmotic adjustment in maize leaves. Aldolase is an important enzyme of glycolysis playing an important role in plant development, regulation and biotic and abiotic stresses tolerance [23]. Khanna et al. [24] reported a decrease in the activity of this enzyme under drought stress. Likewise, Uematsu et al. [25] found that overexpression of aldolase increased photosynthetic rate, growth and biomass in tobacco plant.

Partitioning of carbohydrates from source to sink depends upon the sink strength which is altered under water deficit [26]. This export of carbohydrates from source to sink is required for grains development. Prioul and Schwebel-Dugué [27] in their experiment on maize revealed a massive kernel abortion and limited

grain filling due to slight decrease of soluble sugars within source. Likewise, Yelle et al. [28] reported differences in carbohydrate metabolism within sink of two tomato species and radioactive sucrose used to study their conversion from source to sink. They reported a higher level of radioactive sucrose and lower level of invertase activity within fruit of *Lycopersicum chmielewskii* while lower level of radioactive sucrose in the fruits of *Lycopersicum esculantum* indicating both source and sink metabolism is involved. Likewise the activity of adenosine diphosphate-glucose pyrophosphorylase (AGPase) is associated with grain filling [29] and under drought conditions an increased activity of AGPase within the grains of wheat and rice was reported by Yang et al. [14] and Zhang et al. [30]. Under drought stress, translocation of limited carbohydrates from source tissues to grains [31] and; from sink tissues to grains is important to determine the grain yield under stress [32]. Li et al. [33] reported an increase in the activity of spike phosphoglucomutase and aldolase under high CO₂ indicating the role carbohydrate metabolism within spike in response to an enhanced source activity.

Thus, the modulation of the carbohydrate-catalyzing enzymes activity in response to drought in both source and sink organs of crop plants would play a crucial role in determining reproductive development and yields. In the past, the role of individual enzymes had been investigated by many researchers both under well-watered and drought conditions [11, 21, 34]. However, the global correlations between the yield components and carbohydrate metabolism in source and sink of wheat as affected by drought stress remains largely unknown and merits further investigations.

In addition to affecting carbohydrate-metabolic enzyme activity, drought stress often induces accumulation of reactive oxygen species (ROS) within plant cells [35]. Plants detoxify ROS through enzymatic and non-enzymatic antioxidants. First line of abiotic stress tolerance includes superoxide dismutase (SOD), catalase (CAT), peroxidases (POX), cell wall peroxidases (cwPOX), and different studies reported a steady-level of CAT [36] while enhanced activity of SOD, POX and cwPOX [37, 38, 39] under stress conditions. Likewise, other antioxidant enzymes of the ascorbate-glutathione cycle, such as monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) are important to maintain the redox homeostasis under abiotic stress [35, 40]. Studies reported an increase in the activity of MDHAR in rice under drought stress and; enhanced activity of DHAR and GR under drought stress in wheat [37, 39, 40]. Moreover, glutathione-S-transferase (GST) also plays an important role to reduce the oxidative damage within plants [41, 42] to improve the tolerance to different stresses [43]. Plant genotypes having higher activity of these antioxidants are expected to produce more yield under stress conditions.

In this study, the response of three wheat genotypes (L₁, L₂ and L₃) having contrasting yield potential under drought in field were investigated in pot experiment under controlled greenhouse condition. Drought stress was imposed at anthesis stage as it is the most sensitive stage of wheat crops to water deficits. Our aim was to explore the changes in yield parameters and variation in leaf and spike carbohydrate metabolic and antioxidant enzyme activity signatures and their associations in the three wheat genotypes under both well-watered and drought stress conditions. The results will help to find discriminating biomarkers in order to devise future strategies for breeding drought resilient wheat cultivars.

2. Results

2.1. Leaf gas exchange and water relations

No significant differences in stomatal conductance (Gs), photosynthetic rate (An), relative water content (RWC) and osmotic potential (Ψ_{π}) were observed between the three genotypes. Compared to the well-watered controls, drought stress significantly decreased Gs, An, RWC and Ψ_{π} conditions in all genotypes (Table 2). Genotypes were significantly different for osmotic adjustment (OA) and highest value of osmotic adjustment (OA) was recorded in genotype L₂ and lowest in L₁ (Table 3).

Table 1
Parentage and pedigree of the analyzed wheat genotypes

Genotype	Status	Parentage	Pedigree	Centre of Origin
L ₁	Advanced Line	BCN//SORA/AE.SUARROSA (323)/4/WBL1/KUKUNA//TACUPETO F2001/3/BAJ #1/5/SERI.1B//KAUZ/HEVO/3/AMAD*2/4/KIRITATI	SDSS12B00908T-0Y-0B-0B-2Y-0B-0MXI	CIMMYT, Mexico [96]
L ₂	Vorobey (Approved variety)	CROC-1/AE.TA(WX-224)//OPATA-M-85/3/PASTOR[3692]	CMSS-96-Y-02555-S[3692]; CMSS-96-Y-02555-S-040Y-020M-050SY-020SY-27M-0Y	CIMMYT, Mexico [96]
L ₃	Punjab-11 (Approved variety)	AMSEL/ATTILA//INQ.91/PEW'S'	Pb.30196-1A-0A-2A-0A	AARI, Pakistan [97]

Table 2

Analysis of variance, mean and standard errors of the three genotypes for photosynthesis, stomatal conductance and transpiration rate under well-watered and drought conditions

Genotype	Photosynthesis (A_n , $\mu\text{mol m}^{-2} \text{s}^{-1}$)		Stomatal conductance (G_s , $\text{mol m}^{-2} \text{s}^{-1}$)		Transpiration rate (E , $\text{mmol m}^{-2} \text{s}^{-1}$)	
	WW	D	WW	D	WW	D
L ₁	17.4 ± 1	-1.63 ± 0.18	0.32 ± 0.06	0.03 ± 0.01	4.32 ± 0.55	0.39 ± 0.05
L ₂	18.88 ± 1.61	0.63 ± 0.16	0.32 ± 0.08	0.07 ± 0.03	2.992 ± 0.20	0.3206 ± 0.08
L ₃	16.84 ± 1.69	-1.17 ± 0.09	0.39 ± 0.05	0.02 ± 0.01	5.06 ± 0.48	0.4352 ± 0.07
P _G	0.13		0.83		0.002	
P _W	< 0.001		< 0.001		< 0.001	
P _{W×G}	0.87		0.45		0.005	

WW = well-watered; D = drought; P_W = P value of drought effect; P_G = P value of genotype effect and P_{W×G} = P value of the interaction of drought by genotype

Table 3

Analysis of variance, mean and standard errors for plant water relations of the three genotypes of wheat under well-watered and drought conditions

Genotypes	L ₂ LL ₁		L ₂		L ₃		P value
	WW	D	WW	D	WW	D	
Relative water content (%)	91.78 ± 1.54	53.35 ± 3.89	92.71 ± 1.79	61.28 ± 5.36	94.56 ± 1.33	47.09 ± 4.91	P _G = 0.39 P _W < 0.001 P _{W×G} = 0.06
Leaf osmotic potential (MPa)	-1.04 ± 0.08	-2.31 ± 0.11	-0.9 ± 0.12	-2.85 ± 0.44	-0.86 ± 0.07	-2.9 ± 0.05	P _G = 0.15 P _W < 0.001 P _{W×G} = 0.06
Osmotic adjustment (OA)		218.06 ± 2.76		268.53 ± 15.48		223.44 ± 6.05	P _G < 0.01

WW = well-watered; D = drought; P_W = P value of drought effect; P_G = P value of genotype effect and P_{W×G} = P value of the interaction of drought by genotype

2.2. Activity of carbohydrate metabolic enzymes in leaf

Activity of leaf vacuolar invertase (vacInv) was significantly different between the three genotypes under control conditions, and the highest activity was recorded in L₃ and lowest in L₁. Compared to well-watered controls, no significant differences were recorded for the activity of vacInv under drought conditions. All genotypes exhibited similar cytoplasmic invertase (cytInv) activity under control conditions, while drought caused a non-significant increase of cytInv activity. The activity of cell wall invertase (cwlInv) was statistically similar among the genotypes, though L₃ showed a lower activity than L₁ and L₂ under control conditions. Drought significantly enhanced the activity of this enzyme in comparison to the well-watered controls. (Table 4).

Table 4

Analysis of variance, mean and standard errors for the activity of carbohydrate metabolic enzymes (nkat/g FW) within leaf and spike tissue under well-watered and drought conditions

Enzymes activity in leaf							
Genotypes	L ₁		L ₂		L ₃		P-value
	WW	D	WW	D	WW	D	
Cell wall invertase (cwlInv)	41.61 ± 11.8	79.73 ± 13.3	43.65 ± 14.3	59.27 ± 14.1	18.57 ± 4.83	64.48 ± 21.16	P _G = 0.41 P _W = 0.009 P _{W×G} = 0.54
Vacuolar invertase (vacInv)	18.12 ± 0.97	38.88 ± 6.25	36.86 ± 9.19	47.67 ± 8.49	63.61 ± 12.3	144.87 ± 67.1	P _G = 0.04 P _W = 0.12 P _{W×G} = 0.42
Cytoplasmic invertase (cylInv)	13.54 ± 1.96	25.96 ± 9.56	13.69 ± 3.45	22.83 ± 7.48	13.94 ± 1.52	31.07 ± 17.02	P _G = 0.88 P _W = 0.08 P _{W×G} = 0.89
Adenosine diphosphate phosphorylase (AGPase)	2.82 ± 0.20	2.36 ± 0.30	1.75 ± 0.17	1.34 ± 0.31	2.76 ± 0.27	2.06 ± 0.38	P _G = 0.003 P _W = 0.03 P _{W×G} = 0.87
Uridine diphosphate glucose phosphorylase (UGPase)	25.10 ± 1.35	22.75 ± 3.32	20.08 ± 1.44	13.65 ± 1.13	22.58 ± 2.34	22.14 ± 1.89	P _G = 0.01 P _W = 0.13 P _{W×G} = 0.3
Hexokinase (HXK)	0.05 ± 0.01	0.11 ± 0.02	0.20 ± 0.4	0.07 ± 0.03	0.08 ± 0.004	0.15 ± 0.02	P _G = 0.08 P _W = 0.99 P _{W×G} < 0.001
Fructokinase (FK)	1.46 ± 0.17	0.81 ± 0.15	1.38 ± 0.28	0.97 ± 0.29	2.66 ± 0.20	1.46 ± 0.50	P _G = 0.02 P _W = 0.2 P _{W×G} = 0.75
Phosphoglucomutase (PGM)	13.14 ± 1.90	12.39 ± 2.97	11.87 ± 2.39	8.84 ± 1.93	8.27 ± 0.51	8.40 ± 1.81	P _G = 0.11 P _W = 0.47 P _{W×G} = 0.72
Phosphoglucosomerase (PGI)	9.55 ± 0.49	13.34 ± 0.91	15.04 ± 1.93	11.43 ± 1.78	17.62 ± 0.65	21.02 ± 1.69	P _G < 0.001 P _W = 0.31 P _{W×G} = 0.03
Phosphofructokinase (PFK)	0.41 ± 0.06	0.39 ± 0.09	0.31 ± 0.06	0.19 ± 0.02	0.53 ± 0.3	0.43 ± 0.13	P _G = 0.02 P _W = 0.20 P _{W×G} = 0.76
Fuctose-1,6-bisphosphate aldolase (Aldolase)	0.30 ± 0.08	0.23 ± 0.15	0.54 ± 0.09	0.11 ± 0.05	0.56 ± 0.03	0.33 ± 0.07	P _G = 0.21 P _W < 0.001 P _{W×G} = 0.07
Enzymes activity in spike							
Genotypes	L ₁		L ₂		L ₃		P-value
	WW	D	WW	D	WW	D	
Cell wall invertase (cwlInv)	28.73 ± 9.45	21.67 ± 2.52	49.52 ± 19.4	58.19 ± 15.7	49.68 ± 28.4	63.50 ± 20.44	P _G = 0.18 P _W = 0.73 P _{W×G} = 0.83
Vacuolar invertase (vacInv)	18.12 ± 0.97	47.72 ± 14.2	36.86 ± 9.2	47.67 ± 8.49	92.56 ± 46.7	128.22 ± 40.4	P _G = 0.02 P _W = 0.25 P _{W×G} = 0.88

WW = well-watered; D = drought; P_W = P value of drought effect; P_G = P value of genotype effect and P_{W×G} = P value of the interaction of drought by genotype

Enzymes activity in leaf							
Cytoplasmic invertase (cytInv)	64.66 ± 8.79	87.68 ± 11.6	7.73 ± 4.01	34.53 ± 7.85	30.68 ± 6.28	57.22 ± 15.81	P _G < 0.001 P _W = 0.005 P _{W*G} = 0.98
Adenosine diphosphate phosphorylase (AGPase)	0.43 ± 0.04	0.58 ± 0.05	0.77 ± 0.02	0.87 ± 0.04	0.60 ± 0.11	0.48 ± 0.05	P _G < 0.001 P _W = 0.37 P _{W*G} = 0.08
Uridine diphosphate glucose Phosphorylase (UGPase)	10.63 ± 0.63	11.49 ± 0.94	9.42 ± 1.09	10.16 ± 1	9.59 ± 0.27	10.46 ± 0.29	P _G = 0.23 P _W = 0.09 P _{W*G} = 0.93
Hexokinase (HXK)	0.04 ± 0.01	0.09 ± 0.02	0.11 ± 0.01	0.12 ± 0.02	0.08 ± 0.01	0.09 ± 0.01	P _G = 0.003 P _W = 0.03 P _{W*G} = 0.23
Fructokinase (FK)	0.41 ± 0.08	0.47 ± 0.04	0.56 ± 0.18	0.84 ± 0.1	0.41 ± 0.1	0.56 ± 0.06	P _G = 0.04 P _W = 0.06 P _{W*G} = 0.56
Phosphoglucomutase (PGM)	5.38 ± 0.74	5.80 ± 0.47	4.32 ± 0.69	5.09 ± 1.03	3.17 ± 0.19	3.71 ± 0.35	P _G = 0.01 P _W = 0.23 P _{W*G} = 0.96
Phosphoglucoisomerase (PGI)	4.63 ± 0.31	5.99 ± 0.46	7.69 ± 0.64	7.44 ± 0.89	6.05 ± 0.85	6.01 ± 0.99	P _G = 0.02 P _W = 0.56 P _{W*G} = 0.56
Phosphofructokinase (PFK)	0.22 ± 0.02	0.34 ± 0.03	0.10 ± 0.01	0.25 ± 0.04	0.11 ± 0.04	0.18 ± 0.01	P _G < 0.001 P _W < 0.001 P _{W*G} = 0.30
Fuctose-1,6-bisphosphate aldolase (Aldolase)	0.05 ± 0.01	0.05 ± 0.03	0.13 ± 0.07	0.07 ± 0.02	0.09 ± 0.03	0.05 ± 0.01	P _G = 0.08 P _W = 0.00 P _{W*G} = 0.56
WW = well-watered; D = drought; P _W = P value of drought effect; P _G = P value of genotype effect and P _{W*G} = P value of the interaction of drought by genotype							

The activities of AGPase and UGPase were significantly different among the three genotypes where, the lowest activities of both enzymes were noticed in L₂ in comparison to the other two genotypes. Compared to the well-watered controls, significant reduction of leaf AGPase activity by drought was observed. Drought did not affect the activity of UGPase. Also, the activity of fructokinase (FK) was significantly different among genotypes where, higher activity was recorded in genotype L₃ in relation to the other two genotypes. Drought significantly reduced the activity of FK in comparison to well-watered controls. The activity of hexokinase (HXK) was neither affected by genotype nor by drought; whereas, interaction between water*genotype which was 0.2 to 0.07 nkat g⁻¹ Fw was significant in genotype L₂ (Table 4).

Phosphoglucomutase (PGM) activity was statistically similar among the three genotypes. Activities of phosphoglucoisomerase (PGI) phosphofructokinase (PFK) varied significantly among three genotypes and highest were recorded in L₃ in comparison to other two genotypes. Drought did not affect the activity of PGM, PGI and PFK. Non-significant differences were recorded among genotypes for the activity of aldolase. Compared to well-watered controls, activity of aldolase was reduced significantly under drought (Table 4).

2.3. Activity of carbohydrate metabolic enzymes in spike

The activity of vaclnv was significantly different among genotypes and the highest activity was recorded in L₃ in relation to L₁ and L₂. Compared to the well-watered controls, the activity of vaclnv was not significantly affected by drought. The activity of cytlnv enzymes was significantly different among three genotypes where, higher activity was recorded in L₁ in comparison to other two genotypes. Compared to well-watered controls the activity of cytlnv was significantly increased under drought. The activity of cwlcv was identical among the three genotypes and it was unaffected by drought (Table 4).

Significant differences in the activity of AGPase were found between genotypes and it was highest for L₂ in comparison to other two genotypes. No significant effect of drought on the activity of AGPase was noticed. Neither genotype nor drought affected the activity of UGPase significantly. The activities of FK and HXK was significantly varied between the genotypes, where higher activities of these enzyme were found in L₂ compared to other two genotypes, and FK activity was not significantly affected by drought (Table 4). In contrast, the activity of HXK was significantly increased under drought as compared to well-watered controls (Table 4).

Differences were significant among genotypes for the activities of PGM and PFK and higher activities were recorded in L₁ in comparison to other two genotypes (Table 4). Significant differences for the activity of PGI were noticed among the genotypes and higher activity was recorded in L₂. However, activities of PGM and PGI were not significantly affected by drought. Compared to well-watered controls, drought significantly enhanced the activity PFK. Aldolase activity was neither affected by genotypes nor by drought (Table 4).

2.4. Abscisic acid concentration and antioxidants activity in leaf

Leaf ABA concentrations differed significantly among the three genotypes where highest ABA concentration was recorded in L₂ compared to other two genotypes. Compared to the well-watered controls, leaf ABA concentration was significantly higher under drought conditions. A significant interaction between water*genotype was also notice for leaf ABA concentration where pronounced effect was recorded in genotype L₃ (Table 5).

Table 5

Analysis of variance, mean and standard errors for the activity of antioxidant enzymes and abscisic acid within leaf and spike under well-watered and drought conditions

Leaf Antioxidants (nkat/g FW)							
Genotypes	L1		L2		L3		P value
	WW	D	WW	D	WW	D	
Dehydroascorbate reductase (DHAR)	0.32 ± 0.04	0.75 ± 0.17	0.64 ± 0.22	0.91 ± 0.15	0.59 ± 0.09	0.55 ± 0.12	P _G = 0.018 P _W = 0.06 P _{W*G} = 0.23
Glutathione reductase (GR)	12.69 ± 1.98	5.28 ± 0.21	4.97 ± 1.41	8.16 ± 5.48	9.03 ± 2.81	9.23 ± 2.01	P _G = 0.44 P _W < 0.46 P _{W*G} = 0.07
Glutathione-S-transferase (GST)	2.28 ± 0.68	13.47 ± 0.26	3.19 ± 0.26	11.08 ± 0.21	1.49 ± 0.29	10.27 ± 0.2	P _G < 0.001 P _W < 0.001 P _{W*G} < 0.001
Peroxidase (POX)	9.65 ± 0.49	8.76 ± 2.98	3.08 ± 0.49	2.90 ± 0.42	4.75 ± 1.07	2.91 ± 0.82	P _G < 0.001 P _W = 0.4 P _{W*G} = 0.83
Cell wall peroxidase (cwPOX)	4.11 ± 0.71	4.28 ± 0.21	5.6 ± 0.32	4.14 ± 0.29	6.46 ± 0.50	4.76 ± 0.19	P _G = 0.009 P _W = 0.007 P _{W*G} = 0.06
Monodehydroascorbate reductase (MDHAR)	0.28 ± 0.10	0.13 ± 0.04	0.38 ± 0.13	0.22 ± 0.16	0.08 ± 0.03	0.12 ± 0.02	P _G = 0.056 P _W = 0.17 P _{W*G} = 0.36
Abscisic acid (ng/g FW)	1045 ± 263.8	4776 ± 595	1868 ± 685.5	2062 ± 362.4	428 ± 132.4	3672 ± 933.1	P _G < 0.001 P _W < 0.001 P _{W*G} < 0.001
Spike Antioxidants (nkat/g FW)							
Genotypes	L ₁		L ₂		L ₃		P value
	WW	D	WW	D	WW	D	
Dehydroascorbate reductase (DHAR)	0.84 ± 0.03	0.96 ± 0.04	2.12 ± 0.19	1.52 ± 0.12	1.02 ± 0.12	0.91 ± 0.24	P _G < 0.001 P _W = 0.11 P _{W*G} = 0.06
Glutathione reductase (GR)	3.90 ± 0.68	1.24 ± 0.36	1.14 ± 0.48	2.34 ± 0.67	4.21 ± 0.71	4.09 ± 0.43	P _G = 0.002 P _W = 0.27 P _{W*G} = 0.01
Glutathione-S-transferase (GST)	2.63 ± 0.67	13.47 ± 0.20	2.24 ± 0.51	11.09 ± 0.34	1.39 ± 0.68	10.28 ± 0.16	P _G < 0.001 P _W < 0.001 P _{W*G} = 0.07
Peroxidase (POX)	1.87 ± 0.07	1.88 ± 0.09	1.27 ± 0.11	0.91 ± 0.14	3.27 ± 0.38	2.35 ± 0.28	P _G < 0.001 P _W = 0.67 P _{W*G} = 0.052
Cell wall peroxidase (cwPOX)	3.55 ± 0.17	2.82 ± 0.08	2.60 ± 0.40	2.47 ± 0.31	2.92 ± 0.34	3.31 ± 0.19	P _G = 0.053 P _W = 0.45 P _{W*G} = 0.15
Monodehydroascorbate reductase (MDHAR)	0.37 ± 0.08	0.26 ± 0.13	0.05 ± 0.02	0.08 ± 0.04	0.47 ± 0.07	0.26 ± 0.04	P _G < 0.001 P _W = 0.11 P _{W*G} = 0.27

WW = well-watered; D = drought; P_W = P value of drought effect; P_G = P value of genotype effect and P_{W*G} = P value of the interaction of drought by genotype

Leaf Antioxidants (nkat/g FW)							
Abscisic acid (ng/g FW)	818 ± 166.4	3403 ± 884.4	1751 ± 461.1	1057 ± 64.3	487 ± 101.6	1554 ± 399.6	P _G = 0.014 P _W = 0.002 P _{W×G} <0.001
WW = well-watered; D = drought; P _W = P value of drought effect; P _G = P value of genotype effect and P _{W×G} = P value of the interaction of drought by genotype							

Neither genotypes nor drought changed the activities of DHAR, MDHAR and GR statistically. Difference were significant among genotypes for GST where, highest activity was recorded in genotype L₁ as compared to other two genotypes. Compared to the well-watered controls, drought significantly increased the activity of GST. Likewise, the interaction of water*genotype was also significant and pronounced increase which was 1.49 to 10.27 nkat g⁻¹ FW recorded in genotypes L₃. Differences were significant for the activity of POX among genotypes, where greater activity was observed in genotype L₁ compared to other two. However, non-significant differences for the activity of POX were recorded between the well-watered and drought-stressed plants. Similarly, differences were also significant among genotypes where, highest activity for cwPOX was observed in genotype L₃ compared to other two genotypes, Moreover, compared to well-watered controls, cwPOX was significantly affected by drought. (Table 5).

2.5. Abscisic acid and antioxidants activity within spike

The ABA concentration was significant among genotypes where, highest ABA was recorded in L₁ compared to other two genotypes. As expected, ABA concentration was significantly increased by drought in comparison to well-watered controls. There was also a significant interactive effect of water*genotype on spike ABA concentration where pronounced increase of ABA by drought was recorded in genotype L₃ in relation to L₁ and L₃ (Table 5).

Differences were significant among genotypes for GST activity and the highest value was recorded in genotype L₁ and lowest in L₃. Drought significantly increased the activity of GST in comparison to well-watered controls. Likewise, differences were significant among genotypes for the activity of DHAR where, highest activity was recorded in L₂ in comparison to the other two genotypes. The activity of GR was significantly different between the genotypes and the highest value was recorded in L₁. No significant differences were observed for the activities of DHAR and GR between the well-watered and the drought stressed plants. Significant interaction of water*genotype was observed for GR where, more pronounced decrease in the activity of GR by drought was observed in L₁. Differences were significant among genotypes for the activity of POX and the highest value was observed in L₃. Again, compared to well-watered controls, non-significant effect of drought was recorded for the activity of POX (Table 5). Moreover, neither genotype nor drought significantly affected the activity of cwPOX. Differences were significant among genotypes for the activity of MDHAR where the lowest value was observed for L₂ as compared to other genotypes. In relation to the well-watered controls, drought did not affect activity of MDHAR (Table 5).

2.6. Agronomic parameters

Shoot biomass was identical among the three genotypes, while it was significantly reduced by drought in relation to the well-watered controls. There was significant interaction between water*genotype on shoot biomass, where more pronounced reduction in plant biomass by drought was recorded in L₂ in comparison to the other two genotypes. Grain yield pot⁻¹ (GY) and harvest index (HI) were significantly different between the three genotypes, L₃ had the lowest GY and HI in comparison to the other two genotypes. In comparison to the well-watered controls, GY and HI were significantly reduced under drought (Table 6).

Table 6

Analysis of variance, mean and standard errors among three genotypes for yield and yield related traits grown under well-watered and drought conditions

Genotypes	L1		L2		L3		P value
	WW	D	WW	D	WW	D	
Biomass (g)	65.06 ± 3.32	53.51 ± 2.34	76.08 ± 5.93	44.82 ± 3.62	67.48 ± 5.13	44.22 ± 2.11	P _G = 0.42 P _W < 0.001 P _{W*G} = 0.03
Grain yield (g)	64.93 ± 5.06	37.78 ± 2.01	77.26 ± 7.79	36.40 ± 3.87	52.52 ± 4.30	23.62 ± 2.14	P _G < 0.001 P _W < 0.001 P _{W*G} = 0.21
Harvest index (%)	49.81 ± 1.17	41.40 ± 2.04	50.25 ± 1.42	44.71 ± 3.2	43.72 ± 1.18	34.77 ± 2.8	P _G < 0.001 P _W < 0.001 P _{W*G} = 0.63
Number of grains spike ⁻¹	52.86 ± 4.64	27.88 ± 2.32	50.76 ± 2.64	43.60 ± 1.32	34.48 ± 6.92	21.35 ± 3.46	P _G < 0.001 P _W < 0.001 P _{W*G} = 0.006
Kernel abortion (%)	15.18 ± 2.87	51.30 ± 4.96	14.63 ± 1.93	21.19 ± 5.51	40.77 ± 7.84	59.08 ± 5.06	P _G < 0.001 P _W < 0.001 P _{W*G} = 0.025
Thousand kernel weight (g)	42.76 ± 1.11	37.34 ± 1.94	54.23 ± 3.23	38.51 ± 4.15	52.52 ± 2.76	48.44 ± 3.48	P _G = 0.002 P _W < 0.001 P _{W*G} = 0.07
WW = well-watered; D = drought; P _W = P value of drought effect; P _G = P value of genotype effect and P _{W*G} = P value of the interaction of drought by genotype							

Number of grains spike⁻¹ (NGS) were significantly different among three genotypes with the highest NGS recorded for L₂ and lowest for L₃. Moreover, in comparison to well-watered controls, drought significantly reduced NGS. Additionally, significant interaction of water*genotype was recorded for NGS, where pronounced grain reduction due to drought was found in genotype L₁ as compared to L₂ and L₃. Kernel abortion (KA) was significantly different among all genotypes. Highest KA was recorded in genotype L₃ and lowest in L₁ (Table 6). As compared to well-watered controls, drought significantly increased KA. Moreover, interaction between water*genotype was also significant and pronounced reduction was noticed in L₁. Differences were also significant among genotypes for TKW with the highest value recorded for L₃. Drought significantly reduced the TKW in comparison to well-watered controls.

2.7. Principal component analysis and combined correlations between yield traits and enzymatic activities

Separated PCA analyses for plants grown under well-watered and drought-stressed conditions were performed visualizing the associations between the yield traits and the enzymatic activities. Principal component 1 (PC₁) and principal component 2 (PC₂) described 67.41% and 28.9% variability among the variables for the well-watered treatment, respectively. Biplot analysis of PC₁ and PC₂ showed that cluster of NGS, GY and HI was closer to An, activity of L-cwlnv and L-MDHAR, and these variables were in opposite direction of the cluster for L-vaclnv, S-vaclnv, and L-cwPOX. The activities of S- and L-aldolase were clustered closer to BM and in opposite direction to S-cytlnv (Fig. 1A). Under drought, 52.05% and 45.17% of variability was described by PC₁ and PC₂, respectively (Fig. 1B). Biplot of these PC's showed that NGS, RWC, HI and GY were clustered closer to An, Gs, S-aldolase and L-MDHAR and were in opposite direction of L-vaclnv and S-vaclnv, KA, TKW and L-cwPOX.

Significant negative correlations between leaf RWC and leaf cwlnv and leaf cytlnv were evident (Table 7). Also, leaf ABA concentration was significantly positively correlated with leaf cwlnv (Table 7). Leaf aldolase showed a significant negative correlation with Ψ_{π} (Table 7). Plant biomass was significantly positively correlated with leaf aldolase, cwPOX, An, Gs and E. In contrast, plant biomass was negatively correlated with leaf cytlnv, GST, spike GST and Ψ_{π} (Tables 7 & 8). NGS were significantly positively correlated with leaf MDHAR, An, Gs and E. However, NGS was significantly negatively correlated with leaf ABA, leaf and spike vaclnv, leaf GST and Ψ_{π} (Tables 7 & 8). Additionally, TKW was significantly positively correlated with leaf aldolase, leaf cwPOX, An, Gs and E (Table 7) TKW was significantly negatively correlated with leaf cwlnv, spike cytlnv, UGPase, PGM, PFK, leaf and spike GST and Ψ_{π} (Tables 7 & 8). Significant positive correlations of HI were recorded with leaf MDHAR, An, Gs and E. Moreover, negative significant correlations of HI with leaf and spike vaclnv, leaf and spike GST and Ψ_{π} were noticed (Tables 7 & 8).

Table 7

Combined correlations of leaf carbohydrate metabolic and antioxidants activity with leaf water relations, gaseous exchange, abscisic acid, and

RWC	-0.71***	-0.65***	-0.35	-0.43*	0.66***	0.39*	0.30	-0.89***	-0.89***	0.54*	0.91***	0.78***	0.79***	0.74*
L-ABA	0.56**	0.57**	0.096	-0.44*	-0.42*	-0.17	0.77***	0.59***	-0.39	-0.71***	-0.68***	-0.71***	-0.40*	
	L-cwInv	0.078	0.46**	-0.46*	-0.24	-0.079	0.57**	0.55**	-0.24	-0.55**	-0.52**	-0.55**	-0.40*	
		L-CytIn	0.03	-0.068	-0.21	-0.19	0.42*	0.42*	0.12	-0.42*	-0.33	-0.37*	-0.31	
			L-vacInv	-0.11	0.19	-0.31	0.19	0.39*	-0.20	-0.33	-0.28	-0.24	-0.33	
				L-aldo	0.63***	0.16	-0.63***	-0.68***	-0.26	0.66***	0.64***	0.59***	0.56*	
					L-cwPOx	0.12	-0.53**	-0.48**	-0.56**	0.44*	0.50**	0.46**	0.45*	
						L-MDHAR	-0.26	-0.28	0.62**	0.35	0.22	0.11	0.31	
							L-GST	0.87***	-0.28	-0.94***	-0.84***	-0.87***	-0.74*	
								Ψ_{π}	0.51	-0.91***	-0.79***	-0.80***	-0.78*	
									OA	0.80***	0.30	0.17	0.019	
										An	0.91***	0.87***	0.80*	
											Gs	0.92***	0.72*	
												E	0.63*	
														BM

P* < 0.05, P** < 0.01 and P*** < 0.001, RWC = relative water content, L-ABA = leaf abscisic acid L-cwInv = Leaf cell wall invertase, L-cytIn = Leaf cytoplasmic i aldolase, L-cwPOx = Leaf cell wall peroxidase, L-MDHAR = Leaf monodehydroascorbate reductase, L-GST = Leaf glutathione- S-transferase, Ψ_{π} = Leaf osmoti Gs = Stomatal conductance, E = Transpiration rate, BM = Plant biomass, GY = Grain yield, NGS = Number of grains spike⁻¹, KA = Kernel Abortion, TKW = Thous

Table 8

Combined correlations of spike carbohydrate metabolic and antioxidants activity with gaseous exchange, abscisic acid, and with yield and yield

S-ABA	-0.21	0.34	0.032	0.11	0.21	0.47**	-0.16	0.52***	0.42*	-0.40*	-0.47**	-0.19	-0.21	-0.29
S-cwlnv	0.03	0.63***	-0.078	-0.39*	-0.15	0.33	-0.032	0.006	0.061	-0.012	-0.14	-0.096	-0.064	
S-cytlnv	0.21	0.53***	0.39*	0.76***	-0.39*	0.48**	-0.45*	-0.34	-0.19	-0.36*	-0.36*	-0.30		
S-vaclnv	-0.095	-0.37*	0.013	0.13	0.10	-0.20	-0.12	-0.054	-0.36	-0.42*	-0.45*			
S-UGPase	0.25	0.48**	-0.12	0.41*	-0.43*	-0.30	-0.32	-0.31	-0.33	-0.35				
S-PGM	0.66***	-0.064	0.28	-0.17	-0.17	-0.18	-0.073	-0.009	0.26					
S-PFK	-0.27	0.71***	-0.58**	-0.49**	-0.42*	-0.49**	-0.43*	-0.16						
S-aldo	-0.41*	0.41*	0.35	0.19	0.31	0.35	0.28							
S-GST	-0.94***	-0.84***	-0.85***	-0.71***	-0.72***	-0.55**								
An	0.91***	0.87***	0.80***	0.85***	0.65***									
Gs	0.92***	0.72***	0.72***	0.50**										
E	0.63***	0.60***	0.40*											
BM	0.87**	0.45*												
GY	0.75***													
NGS														

P* < 0.05, P** < 0.01 and P*** < 0.001, S-ABA = leaf abscisic acid S-cwlnv = Spike cell wall invertase, S-cytlnv = Spike cytoplasmic invertase, S-vaclnv = Spike v spike UDP glucose phosphorylase, S-PGM = Phosphoglucomutase, S-PFK = Phosphofruktokinase, S-aldo = Spike aldolase, S-GST = Spike glutathione S-trans Gs = Stomatal conductance, E = Transpiration rate, BM = Plant biomass, GY = Grain yield, NGS = Number of grains spike⁻¹, KA = Kernel Abortion, TKW = Thous Harvest index

3. Discussion

A better understanding of the physiological and biochemical mechanisms attributing to grain yield losses can be tracked by studying the diverse genotypes having varying yield potential under both well-watered and drought-stressed conditions. In this study, three contrasting genotypes (L₁, L₂ and L₃) having different yield potential in field were selected. Genotype L₁ and L₃ were selected as drought tolerant and drought sensitive, respectively, while L₂ was of intermediate yield potential based upon their performance in the field.

Drought stress at anthesis causes limited availability of photosynthates which modifies sink capacity [44] and reduces plant biomass, yield and ultimately the harvest index [45]. The reduction of grain yield in wheat caused by "anthesis drought" was attributed to both reduced grain number and individual grain weight [46]. Besides, modification of key carbohydrates metabolism enzymes both in leaf and spike could have been associated with the reduced source activity and sink strength which resulted in increased kernel abortion and lowered thousand kernel weight [33]. The limited supply of carbohydrates and alteration in the activity of key carbohydrates metabolic enzymes may induce further modifications within the plants. Moreover, the production of reactive oxygen species (ROS) and detoxification of ROS through antioxidants is one of the most prominent mechanisms of plants response to drought stress [37].

3.1. The correlation of An and OA with HI

It has been well established that drought reduces the carbon assimilation and photosynthate supply in crop plants [47]. This limited supply of concurrent photosynthate could also modify dynamics of key carbohydrate metabolism in both source to sink organs [48]. In this study, severe reduction in photosynthesis, stomatal conductance and transpiration rate were recorded under drought condition. The lowered An might have contributed to the decreased grain yield of the wheat genotypes due to source limitation. Osmotic adjustment (OA) is an important mechanism of plant adaptation under drought stress [49]. Moinuddin et al. [50] reported a positive association of OA with grain yield in wheat. Notwithstanding this, here the highest OA in L₂ did not associated a least yield reduction by drought; nonetheless the higher NGS and HI in this genotype could have been associated with the higher OA. A high ability of plants to adjust osmotically under drought may help the plants survival during the stress which however, may reduce the yield as OA is often causing a metabolic cost [51]. HI describes the partitioning of photosynthates into reproductive parts in terms of dry mass. Higher An and HI was recorded in L₂ in comparison to

other two genotypes (Tables 2 & 6 respectively) indicating an important role of photosynthates contribution towards the HI. Consistent with this, Earl and Davis [52] reported a reduced HI due to limited supply of photosynthetic active radiations in maize under drought conditions.

3.2. Correlation of HI with leaf ABA concentration and the activity of carbohydrate metabolic enzymes

Primarily, decrease in the activity of carbohydrate metabolic enzymes was recorded in the leaf and increased activity was recorded in the spike except aldolase and invertases under drought conditions. Positive correlation of ABA was recorded with *cwlnv* (Table 7) and similar results were reported by Ji et al. [53] in rice peduncle where higher concentration of ABA and higher activity *vaclnv* was recorded under water stress conditions, indicating that higher concentration of ABA may have a role in regulation of *cwlnv* activity. Several studies reported that subcellular metabolism of carbohydrates within plastids, cytosol and vacuole are involved in stress related responses [71, 72]. A higher activity of *vaclnv* under drought conditions was reported by Yamada et al. [21] and in line with this, we also recorded higher activity of *vaclnv* under drought. On the other hand, the increased activity of invertases in the leaf could result in accumulation of hexoses, which would contribute to more negative Ψ_{π} as the case for L_3 in the present study (Tables 4 & 3 respectively; Table 7). Similar correlation of Ψ_{π} with cytoplasmic invertase was also recorded in genotype L_1 indicating the role of stored sugars in production of osmolytes under drought conditions (Table 7). Low HI and high *vaclnv* activity in leaf and; high activity of *vaclnv* in spike were recorded in genotype L_3 (Tables 6 & 4 respectively). Likewise, highest leaf *cytlmv* and lowest HI was recorded in the same genotype while vice versa for the others. Roitsch and González [15] reported that the activity of vacuolar invertase regulates sugars translocation into reproductive parts under drought stress conditions. It is further indicating that sucrose was being hydrolyzed in the source but reduced translocation into the sink causing a lowered HI. Moreover, our experiment explains that correlations of invertase isoenzymes with HI (Table 7) were negative which are in good agreement with earlier findings [56]. In conclusion, our results show that higher ABA accumulation correlates with the increased activity of invertase, which could be indicative for a function of ABA in regulating invertase activity. However, the increased invertase activities may not facilitate an increased HI in the studied genotypes. Instead, the liberated sugars are being utilized to increase Ψ_{π} contributing to OA in the plants.

3.3. Correlation between activity of carbohydrate metabolic enzymes and NGS

The association of NGS and TKW with the activity of key carbohydrate metabolic enzymes was further studied to elaborate the HI response. NGS were severely reduced under drought stress and highest KA was recorded in the drought sensitive genotype L_3 and lowest in the intermediate drought responsive genotype L_2 . Reduction in grain number under drought stress has frequently been reported in earlier studies [74, 75]. Cattivelli et al. [59] reported that drought severely affects meiosis at anthesis, which directly impacts grain number and ultimately the grain yield. Simkin et al. [60] reported that grain yield can be improved significantly by increasing the photosynthesis. Here, significant correlation of An was found with NGS and higher An and NGS were recorded in genotype L_2 in comparison to other genotypes (Table 7). Semenov et al. [61] also reported fewer grains due to decreased photosynthesis. The correlation of NGS with the activity of key carbohydrate metabolic enzymes was studied and negative correlation was recorded with *vaclnv* of sink (Table 8). Higher activity of spike *vaclnv* was recorded in genotype L_3 and this genotype was also having the lowest NGS. Yamada et al. [21] reported abiotic stress-inducible transporter for monosaccharides in *Arabidopsis thaliana* termed as ESL1 might function coordinately with the activity of vacuolar invertase to regulate osmotic pressure by affecting the accumulation of sugar in plant cells under drought conditions. Likewise, Roitsch and González [15] reported that the activity of *vaclnv* exhibits the regulation of carbohydrates in plant metabolism under drought stress. It is further indicating that limited photosynthetic rates may force plants to utilize stored carbohydrates under severe drought conditions but in our study these carbohydrates are not being utilized to enhance grain number. During glycolysis, sucrose is converted into glucose and fructose by invertases. The hexoses are further phosphorylated with the help of HXK and FK respectively [62]. In the present study, a decreased activity of HXK and FK was recorded in the leaf. No supporting literature is available to explain our findings. However, Whittaker et al. [63] reported that higher activity of HXK in the leaves of *Sporobolus stapfianus* could be responsible for drought tolerance. Likewise, Fulda et al. [64] reported that SIFRK3, a protein responsible for the activity of FK was upregulated in drought tolerant plants of sunflower under water deficit conditions. Karni and Aloni [65] also reported a decreased activity of FK in anthers under heat stress. These studies although reported in different plant species and tissues yet our studies and previous literature indicate the limited transport of sugars under drought conditions. This limited availability of sugars could induce seed abortion resulting in lower grain numbers.

3.4. Correlation of carbohydrate catalyzing enzymes with TKW

Maintenance of higher TKW is necessary to produce higher grain yield under drought conditions in wheat. In the current experiment highest TKW and lowest HI was recorded in the drought sensitive genotype L_3 under drought conditions. Biplot analysis indicates a close association of TKW with leaf aldolase and leaf *cwPOX*; Individually, significant and positive correlations of leaf aldolase, leaf *cwPOX* activity with TKW were noticed (Table 7). Aldolase has been reported to play a key role in physiochemical processes regulating plant development [31] and responses to abiotic stresses [83, 84, 85, 86]. A successive decline in the specific activities of aldolase was reported under drought [24]. While, an overexpression of gene encoding leaf aldolase increased photosynthetic rate, enhanced growth and biomass production in tobacco plants [25]. In agreement with previous findings, here a higher activity of leaf aldolase and higher TKW was observed in L_3 (Tables 4 & 6 respectively). Additionally, Simkin et al. [70] reported that stimulation of sedoheptulose 1,7-bisphosphatase, fructose 1,6-bisphosphate aldolase has improved photosynthetic efficiency as well as seed yield in *Arabidopsis*. In contrast, negative correlations of TKW with spike UGPase, PGM and PFK were also found under drought conditions (Table 8). UGPase is key enzymes for sucrose synthesis/breakdown [71], PGM provides intermediate products of glycolysis while PFK can regulate the glycolysis process through allosteric inhibition [72]. No supporting literature is available to confirm the results of the present study however, Kato et al. [29] found that AGPase has positive correlation with grain fillings and Kang et al. [73] reported that overexpression of the TaLSU I gene has significantly increased AGPase activity, which positively correlated with endosperm starch weight, grain number per spike and single grain weight, implying that the modification of the activities of these enzymes are associated with the grain filling process hereby influencing the TKW.

3.5. Correlation of antioxidant enzymes with HI, NGS and TKW

In current experiment the correlation between the activity of antioxidants in leaf and spike and HI was analyzed. Among the antioxidant enzymes, an increase in the activity of GST was recorded under drought conditions both in leaf and spike (Table 6). Cummins et al. [41] and Roxas et al. [42] also reported an increase in the activity of GST under oxidative stress in transgenic tobacco. Diverging from previous reports, a decrease in the activity of GST was recorded with increasing NGS and significant negative correlation of leaf and spike GST with grain yield traits (Tables 7 & 8). In contrast, a positive correlation of leaf MDHAR activity with the HI was noticed (Table 7), implying that plants possessing a higher activity of MDHAR in source tissue would maintain redox homeostasis, which may enhance the resistance of photosynthesis to drought stress thus sustain the HI. Melandri et al. [74] also reported higher DHAR activity could reduce drought-induced grain yield losses in rice. In addition, here the activity of leaf MDHAR was positively associated with NGS indicating that higher activity of this antioxidant in the source could have enhanced the drought tolerance of the wheat plants in terms of sustaining the grain number (Table 7), though the underlying mechanisms remain unknown. In line with our results, Sudan et al. [75] reported an increased MDHAR expression and enzyme activity under drought stress. Likewise, Sultana et al. [76] reported that overexpression MDHAR contributes to salt stress tolerance in rice. Eltayeb et al. [77] reported overexpression of MDHAR gene in tobacco is involved in osmotic stress tolerance under drought conditions.

In addition, a positive correlation between activity of leaf cwPOX and TKW was noticed in the present study (Table 7). A higher activity of POX under drought was reported by Veljovic-Jovanovic et al. [78] while work of Devi et al. [37] on wheat genotypes suggested a higher POX activity under drought helps plant to sustain grain yield. Our results are in-line with previous findings supporting that higher activity of leaf cwPOX (Table 7) which may be the reason of less drop in TKW.

4. Conclusion

Results of this study showed that drought stress at anthesis depressed photosynthesis hereby reduced the source activity and photosynthate supply to the sink. This limited photosynthates supply could have caused reductions in NGS as well as TKW in wheat genotypes. Genotype L₁ maintained higher grain yield both under well-watered and controlled conditions mainly due maintenance of higher NGS, RWC and Ψ_{π} while genotype L₃ showed less grain yield mainly due to less RWC, Ψ_{π} and higher KA under drought conditions. A high activity of aldolase, MDHAR, An, Gs and E in the source leaf might contribute towards sustaining carbohydrates remobilization from source to sink hence sustained NGS as well as HI (Fig. 2) while higher activity of aldolase and cwPOX enabled the plants to maintain a higher TKW. Under drought, a high activity of vacuole and GST in both source and sink may have contributed to osmolytes production which is indicated by less Ψ_{π} and limited carbohydrate translocation from source and sink and higher utilization of incoming sugars by the sink may have negatively affected NGS and TKW. The findings of this study provided some insights into the biochemical mechanisms regulating grain yield of wheat in response to drought stress and distinct tolerance to drought was predicted by physiological markers which could be used as important biomarkers for breeding drought resilience wheat cultivars for a future drier climate.

5. Methods

5.1. Plant material and growth conditions

Three genotypes (L₁, L₂ and L₃) of contrasting drought tolerance under field conditions, developed at the International Maize and Wheat Improvement Centre (CIMMYT), Mexico and the Ayub Agricultural Research Institute (AARI) Pakistan, respectively, were selected. Genotype L₁ and L₃ were drought tolerant and drought sensitive, respectively, while L₂ was of intermediate drought response (Table 1). Four seeds were sown in 4 liters pots (filled with peat material, Sphagnum, 32% organic matter, pH = 5.6–6.4 and EC = 0.45 mS cm⁻¹) and only two seedlings were remained after one week of emergence by thinning. Twenty-four replications for each genotype were grown under well-watered conditions.

4.1. Growth conditions

After few days of emergence automatic fertigation (irrigation + mixture of essential nutrients) was applied to the plants. Furthermore, weight of each pot was kept at the same water level by manual weighing of the pots. At flowering time, 4 replications of each genotype were harvested to study different agro-physiological parameters before applying drought stress. Remaining 20 replications of each genotype were divided into two sets: irrigation was withdrawn during anthesis for one set (10 pots) and water status of the other set (10 pots) was kept at 95% pot water holding capacity. Daily evapotranspiration (ET) of each pot was recorded by weighing. Total transpirable soil water was the change of between the pot weight at 95% water holding capacity (about 3.2 kg pot weight) and when evapotranspiration of the drought plants decreased to 10% of the well-watered plants (when pot weight was ca. 1.6 kg).

5.2. Leaf and spikes sampling

Stress was imposed at anthesis until all the plant available water in the pot was consumed. In genotype L₁ and L₂ the drought treatment lasted 9 days while in genotype L₃ the drought treatment lasted 8 days. At the end of the stress period, samples were taken from both well-watered and stressed plants. Two main tillers of each plant were selected for sampling. Flag leaf and attached spike from each of the tillers were taken and snap frozen in liquid nitrogen after tightly wrapping into aluminum foil. These samples were kept at -80 °C until the further use to analyze antioxidant and carbohydrate metabolic enzyme activities and osmotic potential. Then, 5 replications of each treatment were harvested to study their eco-physiology and dry biomass of the plants (Additional file1).

5.3. Gaseous exchange and plant water relations

Leaf photosynthetic rate (An, $\mu\text{mol m}^{-2} \text{s}^{-1}$) and stomatal conductance (Gs, $\text{mol m}^{-2} \text{s}^{-1}$) were determined from fully expanded flag leaves between 11:00 and 14:00 h with a portable photosynthetic system (LiCor-6400XT, Li-Cor, NE, USA). Measurements were performed at 20°C chamber temperature and 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic active radiation (PAR), and 400 ppm CO₂ concentration in cuvette. Relative water content (RWC) were determined in flag leaves according to the method by Jensen et al. (2000). The RWC was calculated as follow:

$$\text{RWC (\%)} = [(\text{FW}-\text{DW})/(\text{TW}-\text{DW})] \times 100$$

where FW and DW are leaf fresh and dry weights, respectively, and TW is leaf turgid weight.

To measure osmotic potential (Ψ_{π}) of the plant tissue, frozen material wrapped in aluminum foil was thawed, squeezed, and a piece of filter paper was dipped into the obtained sap. Ψ_{π} was determined using psychrometers (C-52 sample chambers, Wescor Inc., Logan, UT, USA) connected to a datalogger (Wescor's Dew Point Microvoltmeter, model HR-33T). Likewise, osmotic adjustment (OA) was recorded using following formula;

$$\text{OA} = \text{RWC (well-watered)} \times \Psi_{\pi} \text{ (well-watered)} - \text{RWC (drought)} \times \Psi_{\pi} \text{ (drought)}$$

5.4. Extraction of samples for enzymes analysis

Samples extraction was done following the protocol by Jammer et al. [34]. Briefly, leaf and 10 spikelets from the middle of the spike excluding rachis homogenized in liquid nitrogen was used. 250 mg leaf and 500 mg spike material, respectively, was extracted with 1 ml of extraction buffer consisting of 40 mM TRIS-HCl pH 7.6, 3 mM MgCl₂, 1 mM EDTA, 0.1 mM PMSF, 1 mM benzamidine, 14.34 mM β-mercaptoethanol, 24 μM NADP and milliQ H₂O was added into plant material to get dialyzed extract. A piece of dialysis tube for each sample (~ 3 -4cm for 1 mL sample) was cut and sealed with a clip having number on it. This setup of dialysis tube was placed in cold water (4 °C) for 15 minutes. Extracted supernatant was Pipette into the dialysis tubes according to the arrangements. Air bubbles from the dialysis tube were removed before sealing the other end of the tube with another clip. Likewise, 1 ml of high salt buffer comprised of 1 M Tris HCl pH 7.6, 500 mM MgCl₂, 250 mM EDTA, 4 M NaCl, ddH₂O was added to obtain cell wall extract.

Eleven carbohydrate metabolic enzymes were selected to check their activity within leaf and spike tissue. Dialyzed extract was used for the estimation of vacInv, cytlv, AGPase, UGPase, HXK, FK, PGM, PGI, PFK, Aldolase, and cell wall extract was used to determine the activity of cwInv.

5.5. Carbohydrate metabolic enzyme assays

Method described by Jammer et al. [34] was used to determine the activity of invertases. Concisely, 5 μL of the extract were added in flat bottom 96-well plates to determine the activity of all invertases. While, 5 μl of 100 mM sucrose and 5 μl of reaction buffer pH 4.5 (454 mM Na₂HPO₄/273 mM citric acid) was added into dialyzed and cell-wall extract to determine the activity of vacuolar invertase (vacInv) and cell wall invertase (cwInv) respectively while reaction buffer with pH 6.8 (772 mM Na₂HPO₄/114 mM citric acid) was added into dialyzed extract to determine the activity of cytoplasmic invertase (cytlv). sucrose was not added into control. Likewise, calibration curve was added by glucose standard (0–50 nmol). These plates were incubated at 37 °C for 30 minutes after adding the distilled water to raise the total reaction volume of 50 μl. Plates were put at room temperature for 20 minutes after removing from incubator. 200 μl of GOD-POD reagent (10 U ml⁻¹ GOD, 0.8 U ml⁻¹ POD, and 0.8 mg ml⁻¹ ABTS in 0.1 M potassium phosphate buffer, pH 7.0 was added in each well. The absorbance was measured at 405 nm of plate reader. Principle of Sung et al. [79] was used to determine the activity of all the invertase enzymes.

All remaining carbohydrate enzyme activities were determined using higher throughput method described by Jammer et al. [34]. For the activity of HXK and FK was determined following the principle of Petreikov et al. [80]. Moreover, 100 mM fructose, 50 mM NAD, 100 mM ATP, 3500 U ml⁻¹ PGI, 1000 U ml⁻¹ G₆PDH (from *Leuconostoc mesenteroides*) and common buffer (composed of 1 M Tris HCl with pH 8.0, 0.25 M EDTA, 0.5 M MgCl₂) was used to determine the activity of FK. TPI was not used and 100 mM fructose was replaced with 100 mM glucose to assay the activity of HXK. For the activity of UGPase and AGPase, principle of Pelleschi et al. [7] and Appeldoorn et al. [81] was used. Again, glucose and fructose were omitted from the control. For the activity of AGPase and UGPase, common buffer, 10% BSA, 100 mM Na-PPI, 10 mM NADP, 50 mM 3-PG, 1.28 U ml⁻¹ G₆PDH from *Saccharomyces cerevisiae*, 1000 U ml⁻¹ PGM, 50 mM ADP-Glucose (for AGPase) and 100 mM UGP-glucose (for UGPase), was used to determine the activity of AGPase and UGPase respectively. However, for the control samples ADP-glucose and UDP-glucose were omitted. Similarly, principle of Manjunath et al., [82] was used to determine the activity of PGM. In continuation, to assay the activity of PGM 1 M Tris-HCl pH 8.0, 0.5 M MgCl₂, 500 mM DTT, 10 mM Glc-1,6-bisP, 100 mM Glc-1-P*, 10 mM NADP, 6000U ml⁻¹ G₆PDH (from *S. cerevisiae*) was used. Activity of PGI was determined following the principle of Zhou and Cheng (2008). However, to determine the action PGI 10 mM glc-1,6-bisP and 100 mM glc-1-P*, were replaced with fruct-6-P* and; glc-1-P* and fruct-6-P*. Mastermix was prepared using common buffer, 25 mM fruct-1,6-bisP*, 25 mM NADH, GPDH 2100 U ml⁻¹, TPI 6000 U ml⁻¹. Additionally, activity of PFK was determined following the principle of Klotz et al. [84]. Similarly, apart from common buffer, 100 mM fruct-6-P*, 25 mM NADH, 100 mM ATP, 372 U ml⁻¹ aldolase, GPDH 2100 U ml⁻¹, TPI 6000 U ml⁻¹ was used for the activity of PFK. Fruct-6-P* was omitted as substrate in the control samples. and activity of aldolase was determined following the principle of Schwab et al. [85]. The absorbance was studied at 340 nm for 30 minutes and deviation of readings/peaks was monitored during this period and calculation of specific enzyme activity in nkat g FW⁻¹. Gen5 v3.04.17 software (Biotek Instruments. Inc) was used to measure the absorbance of different enzymes.

5.6. Activity of antioxidants enzymes

Methodology described by Garcia-Lemos et al. [86] was used to determine the activities of different antioxidant and 96-well plates format was utilized while, the activities were determined photometrically. Briefly, activities for ascorbate peroxidase (APX) was determined based upon the principle of Yoshimura et al. [87]. For the reactions, dialyzed extract was used. Master mix comprised of 50 mM KPO₄ buffer pH 7.6, 0.25 mM ascorbate and 0.5 mM H₂O₂ was used and absorbance was recorded at 290 nm. Likewise for control H₂O₂ was omitted [86]. For the activities for catalase (CAT) principle of Aebi [88] was followed. Master mixed containing 50 mM KPO₄ buffer pH7, 0.001% antifoam agent 204 and 100 mM H₂O₂ was mixed with dialyzed extract and absorbance was recorded at 240 nm. Likewise, for control reactions H₂O₂ was omitted as mentioned by Garcia-Lemos et al. [86]. To determine the activity of peroxidase (POX) or cell wall peroxidase (cwPOX) principle of Polle et al. [89] was used. For determination of POX activity method described by Garcia-Lemos et al. [86] was used. Again, dialyzed extract was mixed with master-mix containing 100 mM KPO₄ buffer pH 7, 2 mM guaiacol and 0.15 mM H₂O₂ was used. Absorbance was measured at 450 nm and H₂O₂ was omitted for control reactions. However, cell wall extract was used for the activity of cwPOX. The activity of superoxide dismutase (SOD) were determined following the principle of McCord and Fridovich [90]. Similarly, dialyzed extract was used to mix with master mix containing

50 mM KPO₄ buffer pH 7.8, 0.1 mM EDTA, 0.05 mM cytochrome c, 10 mM xanthine and 0.0002 U mg⁻¹ xanthine oxidase. Absorbance was recorded at 550 nm as described by Garcia-Lemos et al. [86]. However, xanthine was omitted in control reactions. Activities of glutathione reductase (GR) principle of Edwards et al. [91] was used. Dialyzed extract was mixed with master mix containing 100 mM buffer of Tris HCl with pH 7.8, 25 mM NADPH and 30 mM glutathione oxidized (GSSG). Absorbance was detected at 340 nm for 40 minutes and GSSG was omitted for control reactions. For the activity of dehydroascorbate reductase (DHAR) principle of Dalton et al. [92] was followed. Again, dialyzed extract was mixed with master mix comprised of 100 mM KPO₄ with pH 6.5, 50 mM glutathione reduced (GSH) and 50 mM dehydroascorbic acid (DHA). The activity was determined at 290 nm for 40 minutes and DHA was not used in control reactions. To determine the activity of monodehydroascorbate reductase (MDHAR) principle described by Arrigoni et al. [93] was followed. Dialyzed extract was mixed with reaction mixture comprised of 50 mM KPO₄ buffer with pH 7.2, 25 mM NADH, 50 μl⁻¹ ascorbic acid oxidase (OAA) and 50 mM ascorbate. Activity was measured at 340 nm for 40 minutes and ascorbate was omitted in control reactions. Additionally, the activities of Glutathione S-transferase (GST) were determined following the principle of Li et al. [94]. Again dialyzed extract was mixed with reaction mixture (100 mM KPO₄ buffer with pH 7.4, 50 mM GSH and 2,4-dinitrochlorobenzene (CDNB)). Absorbance was measured at 334 nm for 30 minutes and CDNB was not used for control reactions.

5.7. Abscisic acid assay

ABA concentration in leaf and spike samples was determined through an enzyme linked immunosorbent assay (ELISA) using a monoclonal antibody for ABA (AFRC MAC252) (Asch, 2000).

5.8. Agronomic traits measurement

At the end of drought treatment pots from each treatment were re-watered until the maturity of the plants. Plant maturity stage was determined as described by Zadoks et al. [95]. Harvesting was done at maturity and following traits were recorded:

- a. number of grains spike⁻¹ (NGS): Five spikes from each replication were taken and their averages were recorded;
- b. thousand grain weight (TKW): Thousand grains were counted from each replication and their weight was recorded in grams (g);
- c. Kernel abortion (KA): KA was recorded using following formula: (number of grains spike⁻¹ / number of florets spike⁻¹) x 100
- d. Plant biomass pot⁻¹ (BM): Both plants from each pot were harvested from soil level and weight of whole plant was expressed in grams (g);
- e. Grain yield pot⁻¹ (GY): Spikes from the pot were threshed into grains and their weight was expressed in grams (g);
- f. harvest index (HI) were recorded using following formula: Grain yield (g)/Biomass (g) x 100

5.9. Statistical analysis

Analysis of variance (two-way ANOVA) was done using RStudio 1.0.153.exe to reveal the significance of the effect of genotype, water and their interaction on the measured variables at P = 0.05 level. Similarly, regression analysis was done in Microsoft excel 2016. PCA and Biplot analysis was done in MetaR-v6.0_BASE_setup.exe software and "L" and "S" are indicating leaf and spike antioxidants or carbohydrate metabolic enzymes or phytohormones in biplot figure like, "L-aldolase" was used for leaf aldolase enzymes and "S-aldolase" was used for spike aldolase enzyme.

Abbreviations

cwInv = Cell wall invertase, cytlv = Cytoplasmic invertase, vacInv = Vacuolar invertase, UGPase = UDP glucose phosphorylase, AGPase = ADP-glucose phosphorylase, HXK = Hexokinase, FK = Fructokinase, PGM = Phosphoglucomutase, PGI = Phosphoglucoisomerase, PFK = Phosphofructokinase, RWC = relative water content, Ψ = Leaf osmotic potential, NGS = Number of grains spike⁻¹, BM = Plant biomass, TKW = Thousand kernel weight, GY = Grain yield, HI = Harvest index, cwPOX = Cell wall peroxidase, MDHAR = monodehydroascorbate reductase, ABA = Abscisic Acid, GST = glutathione S-transferase, An = Photosynthesis, Gs = Stomatal conductance, E = Transpiration rate

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request

Competing interests

There is no financial and non-financial competing interest

Funding

Authors' contributions

SS and FL conceive the idea to plan this study. SS conducted an experiment with the help of FL. Lab work for enzymes and antioxidants was done under the guidance of TR. Data analysis was done by SS, FL and DKG. Manuscript was written by SS and FL, DKG and TR gave their valuable inputs to improve the manuscript.

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Declaration:

The authors declare that they have no conflict of interest.

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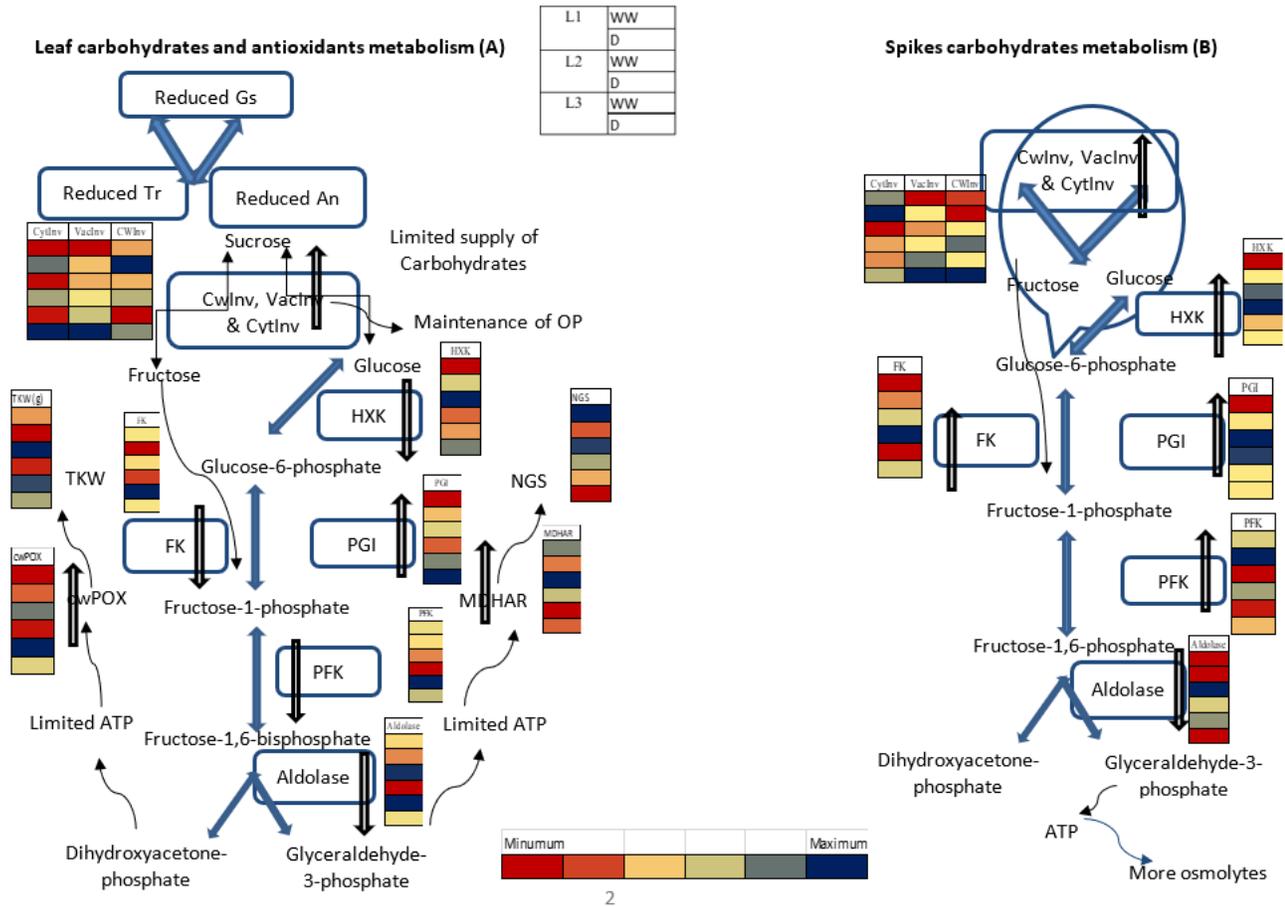


Figure 2

Heat map showing the effect of drought stress on carbohydrate catalyzing and antioxidant enzymes of leaf (A) and spike(B) in three wheat genotypes. Upward arrows indicating rise in the activity of enzymes in comparison to well-watered conditions and downward arrows indicating decrease in the activity.

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