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WHEAT FLAG LEAF MORPHOANATOMICAL CHARACTERISTICS AND GRAIN YIELD COMPONENTS UNDER FIELD CONDITIONS

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Abstract

Drought induces morphological changes in plants, and among these, certain characteristics can be identified as markers of drought tolerance or resistance. The identification of drought resistance morphostructural markers can facilitate the selection of tolerant genotypes with increased productivity. Our aim was to establish a reliable and reproducible set of drought adaptation markers for Izvor and F628 wheat varieties and their DH lines, A1-3, A1-20, A1-65, A1-66, A1- 72, A2-92, A2-255 and B1-16 under field conditions. For this purpose, different leaf morphoanatomical parameters were compared with field productivity parameters. Although other studies associate these characteristics with drought tolerance, in our research most of the studied flag leaf morphological and anatomical traits were not correlated with main spike productivity or the Drought Tolerance Index (DTI). Stomatal density was the only characteristic that showed a significant correlation with the DTI, thus being considered a reliable marker for drought tolerant genotypes with increased productivity.

***Key words**: wheat flag leaf, morphoanatomic characteristic, grain yield, drought resistance markers.*

INTRODUCTION

Drought is an increasingly major challenge due to continuously changing climate conditions, causing worldwide crop yield losses. Plants can undergo morphological, physiological, and biochemical adaptations to drought conditions to overcome the effects of this abiotic stress. More specifically, plants can adjust their growth, reduce resource utilization, decrease the transpiration rate, activate the antioxidant system, or decrease the chlorophyll content (Seleiman et al., 2021). Among the morphological changes in plants, some characteristics could be identified and used as markers for tolerant genotypes discrimination and for developing new varieties with increased productivity under drought stress conditions. The leaves, the main organs involved in transpiration and photosynthesis processes, are the most sensitive to hydric stress and, thus, prone to morphoanatomical changes. The analysis of their morphoanatomical characteristics can directly reflect the response to drought stress (Mehri et al., 2009; Schaller & Paschold 2009; Zhang et al., 2015).

In dicotyledons, the main anatomical features of the leaf structure associated with drought resistance potential mentioned in the literature were the thickness of the epidermal cuticles, a well-developed transport system, small size of the stomata (Ennajeh et al., 2010), leaf thickness, the thickness of the palisade and spongy tissue (Li et al., 2022), the thickness and compaction of the mesophyll tissue, the thickness of the spongy tissue, the thickness of the upper epidermis and especially the palisade tissue/spongy tissue ratio (Hu et al., 2022), the density of stomata and trichomes (Ennajeh et al., 2010). Additionally, stomatal characteristics play a crucial role in drought stress tolerance and resistance. Stomata play a fundamental role in CO_2 uptake and water use, being key factors for efficient water use. Stomata can regulate the intensity of gas exchange through the opening of the ostiole. Besides stomatal opening, size and density are important parameters for hydric deficit tolerance/resistance (Bucher et al., 2017).

Despite the abundance of studies that approaches monocots' drought tolerance, the data on morphoanatomical leaf characteristics and their influence on drought tolerance in this group of plants are scarce, the availability of these type of data being limited (El-Afry et al., 2012; Ouyang et al., 2017; Terletskaya & Kurmanbayeva, 2017; Ghafoor, 2019). This study aimed to investigate some of the flag leaf morphoanatomical parameters as drought tolerance markers for Izvor and F628 wheat varieties and their DH lines under field conditions. For this purpose, different leaf morphoanatomical parameters were compared with field productivity parameters. The correlations between these characteristics and yield components could provide a better insight into the direct effects of morphoanatomical characteristics on crop productivity in variable field drought conditions.

MATERIALS AND METHODS

Plant material

Plant material was represented by wheat plants grown in field conditions. The two wheat genotypes, Izvor and F628 and eight doubled haploid (DH) lines (A1-3, A1-20, A1-65, A1-66, A1- 72, A2-92, A2-255 and B1-16). DH lines were obtained from the cross F628/Izvor, using the protocol described by Giura (2011).

The cultivar Izvor was noticed for its performance in several dry years (Mustăţea et al., 2009; Marinciu et al., 2021), which was attributed mainly to superior osmotic adjustment (Bănică et al., 2008; Ciucă et al., 2009).

All genotypes were planted in the field at the National Research & Development Institute – Fundulea, Romania (44°30’N, 24°10’E) in 2019-2020 and 2020-2021, on chernozem soil, three replicates, in rows spaced at 30 cm.

Morphoanatomical analyses

The plant material used for morphoanatomical analyses was represented by completely developed flag leaves from the 2019-2020 season. The stomata characteristics were determined using the imprinting method. Completely developed flag leaves were detached from three different plants and were used to obtain abaxial leaf imprints. Nail polish was applied to the mid-point between the leaf extremity and the central vein at the middle of longitudinal leaf axis. The imprints were then transferred on glass slides using adhesive tape.

The leaf thickness was measured on five completely developed flag leaf sections made on ethanol-fixed material using the MT.5503 manual microtome. The sections were used as fresh preparations or stained with 0.2% Nil Blue A. The imprints and the sections were observed and imaged under an optical microscope (Imager M2 Axio Zeiss, Germany).

Morphoanatomical parameters

The analysed morphological parameters were stomata arrangement pattern, stomatal density, stomatal size, flag leaf thickness, bulliform cell height (BCH), abaxial (ABE) and adaxial epidermal thickness (ADE).

Stomata density was determined by counting the stomata of eight 1 mm^2 areas from each leaf. Stomata size was determined by measuring the length and the width of five guard cells from each leaf.

The leaf thickness was measured in five leaf areas: at the central vein point (midvein – MvT), on both sides of midvein vicinity areas (MvVT), in the middle of first bulliform cells from the midvein (FBT), at the second stomata row from the midvein (SST), and at the first secondary vein (FSV). All measured data were collected using AxioVision Rel. 4.8 Software.

Grain yield parameters

The registered grain yield parameters were the number of grains/spike (GNS) and the grains weight/spike (GWS) in the dry 2019-2020 season and the favourable 2020-2021 season. For GNS and GWS determination, 30 spikes of each genotype were used.

For the estimation of the abiotic stress response, the Drought Tolerance Index (DTI) was calculated as the ratio between the grain weight per spike measured in 2021 (the season with more favourable conditions for plant development) and in 2020 (the season with lowest average values):

$$DTI = GWS\ 2021/GWS\ 2020$$

The weather data were registered by Fundulea National Agricultural Research and Development Institute’s meteorologic station.

The 2019-2020 season was drier and generally warmer than long-term average for each month. The 42 mm deficit from September, already put soil moisture content on an unfavuorable trend for crops. The rainfall deficit for December 2019 to April 2020 exceeded 114 mm, and this had a negative influence on both winter and spring crops. Even if the reduced rains from May-June (summing up 112 mm together) alleviated temporary the water stress, it increased again in July 2020 when precipitation was 34 mm (37 mm less than long term average) The months November 2019 -March 2020 were (each) with more than +3°C warmer than long-term average. Only May 2020 was as warm as the long-term average (LTA); all other months exceeded their long-term monthly averages. The warmer weather, combined with dry winds (especially in April 2020), significantly increased potential evapotranspiration and created several periods of heat stress conditions, in addition to water stress. During September 2020 - August 2021 period, the weather was rather unstable, the periods with heavy rains and strong winds alternating with prolonged drouthy periods.

During that vegetation season, there were six months (October and November 2020, February, April, May, and July 2021) with precipitation below the long-term average. Rains from June (21 rainy days and high nebulosity) contributed to a lesser extent to the grain filling of winter cereals. April 2021 was the only month cooler than usual (with -1.6°C) during that vegetation season. The period September 2020 - February 2021, was warmer than usual in average +3°C. Even if the cold hardening conditions were unsatisfactory, the winter crops managed to avoid winter kill situations because the minimal temperature didn't drop below 11.9°C (19th of January 2021), and the snow cover provided good thermal insulation.

Statistical analysis

The data were statistically processed, the correlations were calculated, and the significance test available in Excel 2019 was applied.

RESULTS AND DISCUSSIONS

Flag leaf anatomical characteristics

The flag leaf transverse sections exhibit the monocot cell organization pattern. The adaxial and abaxial epidermis of the leaf enclose the mesophyll, which is crossed by vascular tissue, resulting in areas of mesophyll alternating with areas of vascular tissue, parallel to each other.

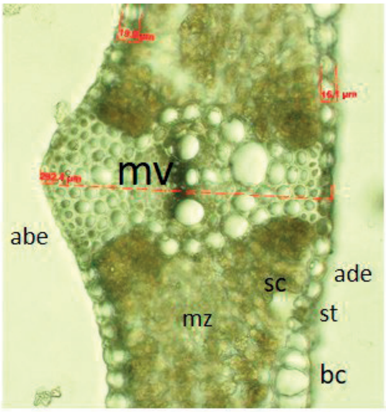

Figure 1. Anatomical structure of F628 genotype flag leaf: **ade** - adaxial epidermis, **abe** - abaxial epidermis, **mv** - midvein, **mz** - mesophyll tissue, **bc** - bulliform cells, **st** - stomata, **sc** - substomatal chamber

At the adaxial epidermis (Figure 2A) level, different cell types can be observed. The largest adaxial epidermis cells are the bulliforms, with globular-oval shape, generally being observed in groups of 3-4 cells (Figures 1 and 2 A). These are positioned in the areas between the vascular tissues. The adaxial epidermis (Figure 2 A) is composed mainly by small, flat cylindrical cells, regularly alternating with stomata. Stomata usually appear flanking the group of bulliform cells. Above the veins are cylindrical cells with thickened walls.

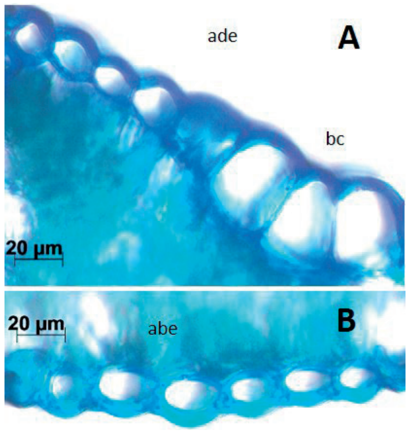

Figure 2. Details of the adaxial and abaxial epidermis of A2-255 genotype flag leaves (A, B): **ade** - adaxial epidermis, **abe** - abxial epidermis, **bc** - bulliform cells

In the less complex abaxial epidermis (Figures 2 B and 3 A) predominate flat cells with thickened walls, smaller than the epidermal cells observed on the adaxial side. Between the vascular tissue areas, stomata were observed. As in case of adaxial epidermis, the abaxial epidermis presents trichomes.

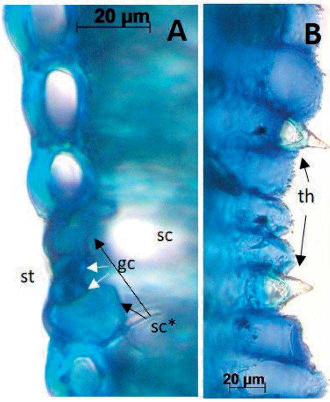

Figure 3. Details of the adaxial and abaxial epidermis of A2-255 (A) and B1-16 (B) genotype flag leaves: **ade** - adaxial epidermis, **abe** - abaxial epidermis, **bc** - bulliform cells, **th** - trichome, **st** - stomata, **gc** - guard cells, **sc*** – subsidiary cells, **sc** - substomatal chamber

Stomatal arrangement pattern

In grasses stomata consist of two dumbbell shaped guard cells positioned in front of each other, which are flanked by two subsidiary cells, together forming the stomatal complex (Figure 3 A).

Our observations show that stomata are usually arranged in parallel simple rows (Figure 4 A) and sometimes in double, intercalary rows (Figure 4 B) along the veins. Stomata alternate in the same row, usually with one interstomatal cell of different sizes (Figure 4 A). All the variants observed in this study presented spaced stomata, being separated by at least one epidermal cell of different sizes. The stomatal arrangement is essential for its proper functioning, being considered that a stomata should be separated by at least one epidermal cell from all neighbouring stomata (Dow et al., 2014). Rapid ionic exchange is needed for guard cells' proper function, and this condition is provided by the epidermal cells surrounding stomata (Dow et al., 2014).

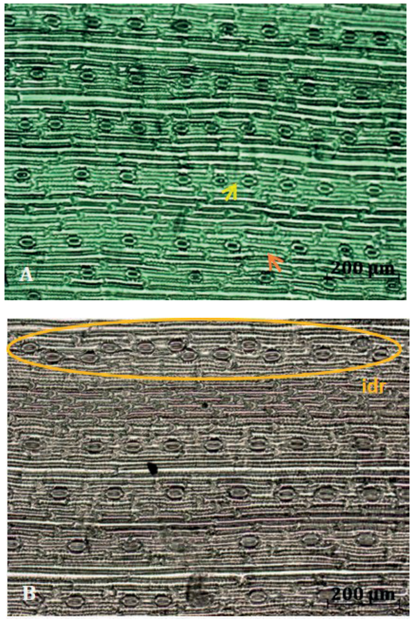

Figure 4. Stomatal arrangement patterns in Izvor (A) and F628 (B) wheat genotypes: **idr** - intercalary double rows; **yellow arrow** - short interstomatal cell; **brown arrow** - long interstomatal cell

Stomatal density and size

The stomatal density was relatively uniform among the studied genotypes and varies between 41.68 and 57.43/mm^2 (Table 1). However, the genotype with the highest stomatal density, B1-16, had significantly higher stomatal density comparing with its genitor F628 and A1-3 ($p < 0.05$). The A1-3 variant had the lowest value, being significantly different comparing to A1-66, A1-72, A2-92, A2-255 and B1-16 ($p < 0.05$).

Table 1. Stomatal density and stomatal area in F628 and Izvor parental lines and their DH descendants

Genotype	Stomatal density/ mm^2	Stomatal area µm^2
F628	47.26±3.60	346.08±2.01
Izvor	50.50±4.60	251.06±1.31
A1-20	48.76±4.73	276.91±1.22
A1-3	41.68±5.04	340.43±2.40
A1-65	50.37±3.8	408.61±0.97
A1-66	56.71±5.45	182.87±0.77
A1-72	55.28±4.78	199.32±2.18
A2-255	54.14±4.12	212.93±1.19
A2-92	55.65±4.09	266.41±2.01
B1-16	57.43±4.50	187.71±1.02

Data from scientific literature showed that generally lower stomatal density was associated with drought tolerance/resistance (Hepworth et al., 2015, Hughes et al., 2017, Cain et al., 2019, Dun et al., 2019), due to total pore surface decrease.

Our results showed that the area of stomata varies between 182.87 and 408.61 μm^2. The highest area of stomata was registered in A1-65, F628 and A1-3 genotypes, while A1-66, B1-16, A1-72 had the lowest stomata area (Table 1). Also, the resistant genotype Izvor had smaller stomata than the F628 genotype. It was advanced the idea that having smaller stomata is a desirable characteristic for hydric stress tolerant/resistant lines. Smaller stomata leave more space for other epidermal structures like trichomes, subsidiary cells (Franks & Beerling, 2009), and bulliform cells, which also have important roles in water stress resistance. Additionally, in genotypes with smaller stomata, a larger area of epidermal cells is available for the secretion of waxes and cutin, which together form the outer protective layer, the plant cuticle. Higher total cell surface area of smaller stomata compared with its volume allows faster ion fluxes across the plasma membrane and tonoplast, which results in more rapid guard cells movement and, implicitly, a faster ostiole closure (Lawson & Vialet-Chabrand, 2019).

In our study, a strong negative correlation was found between stomata density and stomata area (Figure 5). Studies on different plant species also evidenced that often there is a negative correlation between stomatal density and stomatal size, in case of genotypes with a high number of stomata their size being smaller (Hetherington & Woodward, 2003, Camargo & Marenco, 2011, Faralli et al., 2019) and vice versa. This negative correlation represents an adjustment mechanism of total stomata opening. The variation of these characteristics is not always correlated, the modifications occurring sometimes independently. In the study of Doheny-Adams et al. (2012), Arabidopsis plants grown in hydric deficiency conditions did not change their stomatal density but were observed modifications like stomatal size, guard cells area, ostiole area, and implicit gas exchange rate.

Although the genotypes with low values of stomatal conductance determined by the low stomata density and/or small stomata opening would be appropriate candidates for drought tolerance/resistance, it should be considered that the photosynthesis rate decreases with stomatal conductance increase. A low photosynthesis rate can negatively influence plant development and implicitly crop productivity.

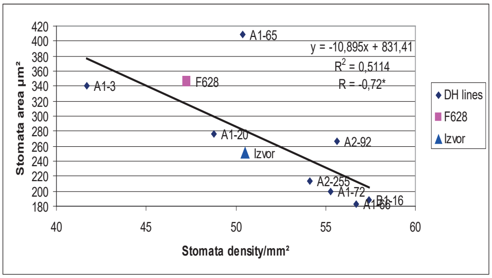

Figure 5. Correlation between stomata density/mm^2 and area (μm^2)

Bulliform cells size

Considering the longitudinal size of the bulliform cells in the transverse section, the genotypes B1-16, A1-66, A1-65, and A2-255 overcame the parental lines. Additionally, in the resistant genotype Izvor, higher values of bulliform cell size were observed compared to the F628 genotype (Table 2).

Table 2. Bulliform cell height (BCH), adaxial epidermal thickness (ADE), abaxial epidermal thickness (ABE), ratio between ADE and ABE (ADE/ABE) and difference between ADE and ABE (ADE-ABE) in F628 and Izvor parental lines and their DH descendants

Genotype	BCH (μm)	ADE (μm)	ABE (μm)	ADE/ ABE	ADE- ABE
F628	37.26 ±1.48	20.75 ±1.94	17.65 ±1.46	1,18	3,10
Izvor	41.38 ±2.42	20.13 ±1.85	18.85 ±2.57	1,07	1,28
A1-20	59.46 ±2.24	18.47 ±2.37	16.80 ±1.79	1,10	1,67
A1-3	37.47 ±2.36	22.07 ±0.88	18.5 ±0.93	0,99	-0,16
A1-65	47.47 ±2.47	20.46 ±1.64	21.88 ±2.59	1,11	1,96
A1-66	46.33 ±6.58	21.40 ±2.07	17.57 ±1.71	0,98	-0,48
A1-72	35.59 ±2.53	19.74 ±2.45	17.64 ±2.32	1,12	2,16
A2-255	48.73 ±4.44	23.43 ±1.58	22.23 ±1.34	1,33	5,79
A2-92	35.76 ±2.93	17.69 ±1.70	17.05 ±1.43	1,04	0,64
B1-16	42.56 ±4.02	19.40 ±1.36	21.20 ±2.12	0,92	-1,80

The bulliform cells, also called “motor cells” are involved in leaf movements, and play an important role in leaf curling, which is related to

resistance of plants in adverse conditions (Zheng et al. 2002, Kirkham, 2014). In drought conditions, the bulliform cells dehydrate, causing the leaf to adaxially fold and thus reducing the water loss (Kirkham, 2014; Roodt, 2021).
The size of bulliform cells can influence the speed of leaf rolling/folding, the reduction of exposed foliar area and implicitly the water loss limitation (Willick et al., 2018).

Epidermal thickness
The thickness of the epidermal layer varied between 17.62-23.34 μm for the adaxial epidermis and 16.8-22.23 μm for the abaxial epidermis (Table 2). In general, the thickness of the adaxial epidermal layer was higher than the abaxial one, with the exception of the B1-16 line. The two parental lines had very close values of adaxial epidermis thickness, and the descendants A1-66, A1-3 and A2-255 presented an increase of this parameter comparing with Izvor and F628. The variants B1-16, A1-65, and A2-255 had thicker abaxial epidermis compared with the two parental lines. Previous studies showed that anatomical characteristics like adaxial and abaxial epidermis thickness represent features that can indicate the potential resistance of different varieties to stress conditions like drought, salinity and high temperature (Terletskaya and Kurmanbayeva, 2017, Erezhetova et al., 2021, Hu et al., 2022).

Flag leaf thickness
The flag leaf thickness, measured in five different leaf areas, varied among the samples and according to the measured area (Table 3). Our results did not show significant differences in flag leaf or epidermal thickness between the Izvor drought tolerant genotype and the F628 genotype, the measured values being very close. However, Al-Maskri et al. (2013) demonstrated that modifications in leaf structural features related to drought tolerance included a thick epidermis and highly developed bulliform cells, which prevent water loss in semi-arid wheat varieties. Also, highly tolerant ecotypes of *Cenchrus ciliaris* L. showed increased epidermal thickness in leaves with well developed bulliform and decreased stomatal density on both leaf surfaces (Mansoor et al. 2019).

Table 3. Flag leaf thickness in different leaf areas: midvein - MvT, midvein vicinity areas - MvVT, first bulliform cells from the midvein - FBT, second stomata row from the midvein - SST, and first secondary vein - FSV

Genotype	MvT (μm)	MvVT (μm)	FBT (μm)	SST (μm)	FSV (μm)
F628	299.10 ±6.81	236.92 ±12.52	215.53 ±8.00	196.00 ±8.60	191.45 ±4.53
Izvor	301.57 ±38.11	255.98 ±27.62	237.21 ±21.30	201.66 ±13.70	191.26 ±10.54
A1-20	276.66 ±4.07	250.53 ±6.07	255.33 ±5.70	241.72 ±11.20	216.47 ±11.43
A1-3	314.06 ±5.07	249.23 ±8.10	249.57 ±4.00	230.73 ±8.60	223.93 ±11.16
A1-65	342.94 ±3.15	283.93 ±7.02	274.86 ±10.10	261.37 ±4.50	251.23 ±3.47
A1-66	375.30 ±5.62	287.48 ±6.26	273.73 ±9.80	243.82 ±15.00	224.50 ±3.12
A1-72	307.88 ±2.76	244.65 ±17.85	242.57 ±22.10	235.49 ±16.10	202.84 ±16.75
A2-255	392.02 ±2.13	287.84 ±9.38	269.81 ±9.60	257.86 ±12.00	218.59 ±9.27
A2-92	292.52 ±4.99	252.68 ±6.20	242.87 ±8.80	228.88 ±12.40	208.88 ±17.56
B1-16	397.00 ±8.83	322.91 ±17.39	321.74 ±10.00	294.37 ±19.50	239.54 ±10.80

In case of DH lines we observed some differences comparing with the parental lines. Regarding the MvT parameter, only two of the descendants registered lower values than the parental lines. The second measured foliar parameter MvVT showed a difference between the two parental lines F628 being outclassed by all the analysed descendants while the Izvor line only by four of the descendants. For all the other foliar parameters FBT, SST, and FSV, the parental lines registered the lowest values. The observation that for most traits, the values recorded for the DH lines were different (higher in majority) than the values of both parents, suggests the possibility of obtaining transgressions.

Correlation between morphoanatomical traits and grain yield parameters
Correlation coefficients between the studied leaf traits showed a very strong association between the thicknesses measured at different leaf parts (Table 4). This indicates that an isometric growth occurred, the proportions of the analysed leaf anatomical characteristics being maintained among the genotypes studied.
However, lower correlations were observed with epidermis thickness, suggesting a relatively independent genetic determination.

Table 4. Correlations between leaf characteristics of F628 and Izvor parental lines and their DH descendants

Analyse of traits	MvT	MvVT	FBT	SST	FSV	BCH	ADE	ABE
MvT	1							
MvVT	**0.90**	1						
FBT	**0.79**	**0.95**	1					
SST	**0.73**	**0.86**	**0.95**	1				
FSV	0.58	**0.72**	**0.80**	**0.84**	1			
BCH	**0.82**	**0.72**	0.53	0.46	0.53	1		
ADE	0.53	0.19	0.05	0.03	0.11	0.62	1	
ABE	0.49	0.45	0.47	0.29	0.41	0.31	0.38	1

Least significant correlation coefficient (P< 5%) = 0.63
Least significant correlation coefficient (5%<P< 1%) = 0.79

Table 5. Correlations between leaf anatomical traits, main spike yield components and DTI

Analyze of traits	GNS	GWS	TGW	DTI
MvT	0.31	0.27	0.15	0.29
MvVT	0.27	0.26	0.15	0.28
FBT	0.25	0.32	0.27	0.2
SST	0.32	0.46	0.47	0.27
FSV	0.27	0.33	0.32	0.15
BCH	0.02	-0.08	-0.17	0.33
ADE	0.03	-0.07	-0.14	-0.18
ABE	0.04	-0.07	-0.17	-0.26
ADE/ABE	0.02	0.05	0.07	0.11
ADE-ABE	-0.01	0.01	0.05	0.11
SD	0.05	0.15	0.19	**0.77**
SA	0.15	0.01	-0.1	-0.26
GNS	1	0.87	0.53	-0.08
GWS	0.87	1	0.88	-0.05

None of the studied leaf anatomical traits was correlated with the spike yield components, and only one correlation with DTI was significant (Table 5). These results suggest that although some anatomical characteristics related to drought stress tolerance had higher values in DH lines than in the parental genotypes, this is not necessarily transcribed in the genotype's productivity.

The only significant correlation with DTI was the one with stomata density, the genotypes with higher density showing better drought response (Figure 6). Other studies showed that genotypes with lower stomatal density have better drought tolerance capacity (Hepworth et al., 2015; Hughes et al., 2017; Cain et al., 2019; Dun et al., 2019). However, our results are in agreement with Shahinnia et al. (2016), who showed that a drought-tolerant wheat line had significantly more and smaller stomata compared with a genotype that had a significantly reduced yield under the same conditions.

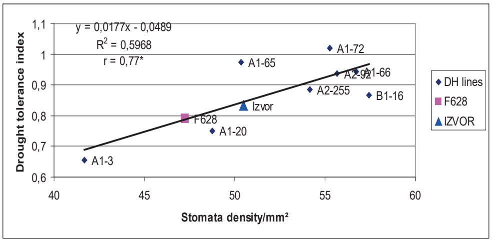

Figure 6. The strong correlation between high stomata density/mm^2 and the DTI (r> 0.5)

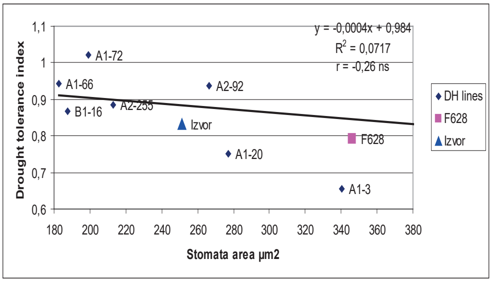

Figure 7. Relationship between stomata area (µm^2) and the DTI

As seen in Figures 6 and 7, our data showed a better association of the DTI with stomata density than with stomata area. This suggests that, in the germplasm represented by the studied genotypes, stomatal density may be a better selection criterion than stomatal area.

Overall, the grain yield performances of the studied genotypes could not be significantly sustained by the analyzed morphoanatomical traits, except for stomatal density. This observation suggests the potential involvement of additional mechanisms for drought tolerance and grain yield productivity in these genotypes.

CONCLUSIONS

The related genotypes included in this study exhibited variability in the morphological and anatomical traits of the flag leaf. For most traits, the DH lines obtained from the cross F628/Izvor had values that were, in the majority, higher than those of the parents, suggesting a high frequency of transgressions.

Thickness measurements at different parts of the leaf were strongly correlated, while the correlations of these characteristics with epidermis thickness were lower and mostly non-significant.

Most of the studied morphological and anatomical traits of the flag leaf were not correlated with main spike productivity or the DTI, suggesting that these foliar parameters play a relatively small role in determining the difference in yield or drought response of the analyzed genotypes.

Stomata density was negatively and significantly correlated with stomata area, but only stomata density showed a significant correlation with the DTI. Thus, stomatal density can be considered a reliable marker for drought tolerance in productive genotypes.

Although these results were obtained with only a small number of genotypes, they may help in the search for efficient selection criteria in breeding for drought tolerant genotypes.

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