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Phenotypic variability for early drought stress resistance in tetraploid wheat accessions correlates with terminal drought performance

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Abstract

Durum wheat is a fundamental staple food for the countries of the Mediterranean basin. Climate change is predicted to cause a trend of increasing drought severity in this region in the near future, necessitating the improvement of durum wheat's resilience to drought stress (DS). Using polyethylene glycol (PEG) to simulate water scarcity, early vigor parameters in germinating seeds are quickly, easily and affordably assessed. Many PEG screenings, however, only consider the seedling stage; consequently, genotypes identified as promising for cultivation in drought scenarios, may not show such features if drought appears in later phenological phases, as happens in Mediterranean climatic areas, generally prone to terminal drought. This is due to the lack of evidence that genotypes that display strong DS resistance during the seedling stage (early vigor) would continue to exhibit significant resistance at a later point in the life cycle. Therefore, it is necessary to confirm the preliminary seedling screening findings for durum wheat. Hence, we used PEG screening to classify fifty-five tetraploid wheat accessions into three clusters (susceptible, medium resistant and highly resistant to DS), based on morpho-physiological traits. These accessions included Durum Wheat Cultivars (DWC) and Landraces (DWL), as well as ancestors like Durum Emmer Wheat (DEW) and Wild Emmer Wheat (WEW). The results of the PEG screenings were combined to subsequent pot experiment using nine randomly selected accessions (three from each cluster), imposing terminal drought, and evaluating their performance in terms of physiological parameters and grain yield (GY). This study provided the first evidence that PEG screenings on germinating seeds could be predictive in evaluating tetraploid wheat accessions drought resistance in terminal drought scenarios, typical of Mediterranean climate zones, thus representing a

cheap, simple and efficient method to carry out large-scale phenotyping. Pot experiments highlighted that genotypes able to extract more water from the soil, sustaining high stomatal conductance and thus photosynthesis and GY, should be preferred in context of moderate to mild terminal drought.

Introduction

Durum wheat (*Triticum turgidum L. ssp. durum*) is the tetraploid wheat species. Although it only accounts for 8% of the world's wheat production (approximately 36 Mt per year), it is a fundamental staple food for the Mediterranean basin, where 60% – 75% of global durum wheat is grown (Giraldo et al., 2016; Igrejas et al., 2020; Sukumaran et al., 2018). The Mediterranean climate is typically characterized by low and unpredictable rainfall patterns, representing a primary source of uncertainty for wheat production, especially considering that wheat is typically cropped as a rainfed cereal in Mediterranean countries (Ahmadizadeh et al., 2012). In addition, a tendency of worsening droughts is anticipated to emerge in this region over the coming decades as a result of climate change. Hence, the need to improve durum wheat drought stress (DS) resistance is evident, since it is a major cereal for semiarid countries of Middle East, Southern Europe and North Africa (Igrejas et al., 2020; Mohammadi et al., 2019). Furthermore, knowing causes and mechanisms regulating durum wheat DS resistance, could represent a precious tool for wheat breeding programs.

Achieve genetic improvement is extremely challenging for breeders, because DS resistance is a complex and quantitative trait, with low heritability and high environmental interactions (Budak et al., 2013; Rampino et al., 2006; Sukumaran et al., 2018). DS can vary greatly in terms of timing, severity and duration in different target environments. Therefore, genotypes, which are suitable for one environment, may be inappropriate for another. For instance, it is not certain that genotypes that exhibit pronounced DS resistance early in development, would also exhibit this resistance to a similar extent in later stages.

Early drought is critical as it compromises seed germination and seedling growth, i.e. a successful crop establishment. Seedling Water Content (SWC), Germination Percentage (GP), Seedling Vigor Index (SVI) are among the main early vigor traits that can be inhibited in water deficit conditions (Acevedo et al., 2002; Ahmad et al., 2018; Almaghrabi, 2012). These parameters are quickly, easily and inexpensively to assess in germinating seeds using high-molecular-weight (> 6000) polyethylene glycol (PEG) (Parmar & Moore, 1966), which simulates drought by inducing a uniform and controlled osmotic stress, with no direct physiological damage as it is inert, non-ionic, and impermeable to cell membranes (Hohl & Schopfer, 1991). Hence, the use of PEG-6000 to simulate water deficit has been sustained by several authors (Almaghrabi, 2012; Peršić et al., 2022; Tuberosa, 2012) and significant differences among wheat genotypes for the aforementioned traits emerged in different studies, suggesting the existence of genetic variability to be exploited for breeding purposes (Almaghrabi, 2012; Dhanda et al., 2004; Moayedi et al., 2009; Pour-Aboughadareh, Etminan, et al., 2020). However, many PEG-6000 screenings consider just the seedling stage; consequently, genotypes identified as promising for cultivation in drought scenarios, may not show such features if drought occurs in subsequent phases of the life cycle.

Even if some information about the correlation between seedling growth parameters in water deficit conditions and adult plant drought resistance is starting to emerge for bread wheat (*Triticum aestivum* L.) (Dodig et al., 2015), further validation of preliminary seedling screening results for durum wheat is required, imposing drought at later phenological stages. In fact, in Mediterranean environments durum wheat is typically affected by the so-called terminal drought, as most precipitation is concentrated in temperate autumn and winter, followed by hot and dry summer, resulting in moderate DS for wheat around anthesis, progressively increasing throughout grain filling stage (Garcıa del Moral et al., 2003). Due to accelerated leaf senescence, reduced flag leaf photosynthesis (Pn), downregulated enzyme activities, altered chlorophyll efficiency parameters and sink limitation, terminal drought has a negative impact on final grain yield (GY), disrupting and shortening the grain filling process (Farooq et al., 2014; Ihsan et al., 2016; Pour-Aboughadareh, Mohammadi, et al., 2020). Genotypes' performance under terminal drought can be accurately assessed conducting pot experiments, measuring several parameters on adult plants, such as gas exchange parameters, e.g. flag leaf stomatal conductance (g_s), transpiration (E), and Pn, chlorophyll performance parameters, e.g. Fv/Fm, i.e. maximum quantum efficiency of Photosystem II (PSII), final GY (Bogale et al., 2011). Pots experiments on adult plants are time- and resource-consuming, but are currently the only reliable method to predict resistance to terminal DS as there is no evidence in the literature regarding the correlation between PEG germination tests and durum wheat DS resistance in terminal drought conditions.

In this study, a screening for the abovementioned seedling traits was conducted, using PEG-6000 to simulate DS on germinating seeds. Fifty-five tetraploid wheat accessions were analyzed, chosen in the attempt to represent as much as possible the biodiversity of tetraploid wheat, with the aim of evaluating the degree of genetic variability for these phenotypic characteristics to be exploited for breeding purposes, and to find promising early vigorous genotypes for contexts of early drought. Moreover, main aim of this study was to couple PEG germination tests results to subsequent pots experiment, imposing terminal drought, with the goal of verifying whether DS resistance data relating to the early growth stages reflect genotypes' potential later in the life cycle.

Materials and Methods

Early-stage DS screening with PEG-6000

Plant material

Fifty-five tetraploid wheat accessions were selected from the Tetraploid Core Collection (TCC), in order to create a subgroup that could represent the variability herein contained (Table 1). TCC represents 95% of GDP (Global Durum wheat Panel) (Mazzucotelli et al., 2020) and TGC (Tetraploid wheat Germplasm Collection) (Maccaferri et al., 2019) biodiversity (Marone et al., 2021). The chosen fifty-five accessions comprise Durum Wheat Cultivars (DWC), Landraces (DWL), as well as exponents of *turanicum* and *carthlicum* subspecies, and ancestors such as Durum Emmer Wheat (DEW) and Wild Emmer Wheat (WEW).

Tab 1: list of Tetraploid Wheat accessions used in this study. For each accession are indicated the category, the country of origin and the mega-environment. DWC = Durum Wheat Cultivar, DWL = Durum Wheat Landrace (including *turanicum*, *carthlicum* and *aethiopicum* exponents), DEW = Durum Emmer Wheat, WEW = Wild Emmer Wheat.

	Name	Category	Country of origin	Mega-environment
1	Aureo	DWC	Italy	Southern Europe
2	Creso	DWC	Italy	Southern Europe
3	Daurur	DWC	France	Western Europe
4	Haby	DWC	ICARDA	Western Asia
5	Iride	DWC	Italy	Southern Europe
6	Kronos	DWC	DESERT	Northern America
7	Marco Aurelio	DWC	Italy	Southern Europe
8	Margherita	DWC	ICARDA	Western Asia
9	Mohawk	DWC	DESERT	Northern America
10	Monastir	DWC	France	Southern Europe
11	Neodur	DWC	Italy	Southern Europe
12	Nobilis	DWC	France	Western Europe
13	Odisseo	DWC	Italy	Southern Europe
14	Rascon/Tarro	DWC	CIMMYT	Central America
15	Saragolla	DWC	Italy	Southern Europe
16	Simeto	DWC	Italy	Southern Europe
17	Strongfield	DWC	Canada	Northern America
18	Svevo	DWC	Italy	Southern Europe
19	LD068-Beloturka	DWL	Russian Federation	Eastern Europe
20	LD093	DWL	Greece	Southern Europe
21	LD186	DWL	Morocco	Northern Africa
22	LD207	DWL	Turkey	Western Asia
23	TDS219	WEW	Fertile Crescent	Western Asia
24	TDS234	WEW	Fertile Crescent	Western Asia
25	TDS235	WEW	Israel	Western Asia

26	Tetra-ipk_251	DWL-*aethiopicum*	Ethiopia	Eastern Africa
27	Tetra-ipk_814	DWL-*turanicum*	Iraq	Western Asia
28	tetra-ipk_815	DWL-*turanicum*	Russian Federation	Eastern Europe
29	DIC-IPK420	DEW	Turkey	Western Asia
30	EP_084	DWL	Ethiopia	Eastern Africa
31	EP_115	DWC	Ethiopia	Eastern Africa
31	EP_140	DWL	Ethiopia	Eastern Africa
33	EP_193	DWL	Ethiopia	Eastern Africa
34	Cappelli	DWL	Italy	Southern Europe
35	Haurani	DWL	Syria	Western Asia
36	Kubanka-LD127	DWL	Kazakhstan	Central Asia
37	Russello_SG7	DWL	Italy	Southern Europe
38	Trinakria	DWL	ICARDA	West Asia, North Africa
39	SSD-502	DWL	NA	NA
40	TC4	DWL	Daghestan	Western Asia
41	TC8	DWL	Turkey	Western Asia
42	AG061	DWL	Cyprus	Western Asia
43	AG189	DWL-*carthlicum*	Georgia	Western Asia
44	Aus26423	DWL	Greece	Southern Europe
45	Aus26460	DWL	Egypt	Northern Africa
46	Aus26628	DWL	Russian Federation	Eastern Europe
47	Molise Colli	DEW	Italy	Southern Europe
48	L28-Kyperunda	DWL	Cyprus	Western Asia
49	Dic-unibo-008	DEW	Italy	Southern Europe
50	Dic-unibo-012	DEW	Italy	Southern Europe
51	Dic-unibo-022	DEW	UK	Northern Europe
52	Dic-unibo-080	DEW	NA	NA
53	Dic-unibo-100	DEW	Serbia	Southern Europe
54	Dic-unibo-110	DEW	India	Southern Asia
55	Dic-unibo-126B	DEW	Iran	Southern Asia

Drought simulation

PEG-6000-induced osmotic stress, simulating DS conditions, was induced according well-established protocols with minor modifications (Almaghrabi, 2012; van den Berg & Zeng, 2006). Seeds were subjected to 0.0 g/L, 100 g/L, 200 g/L PEG-6000, respectively simulating stress absence, medium and severe DS. These three treatments will hereafter be referred to as Cntr (control), T10 (10% (w/v) PEG treatment) and T20 (20% (w/v) PEG treatment). PEG-6000 solutions were prepared by dissolving PEG-6000 in distilled water, for 16 h under shaking (Van Der Berg and Zeng, 2006). Ninety seeds for each accession were surface sterilized with 10% (w/v) sodium hypochlorite for 5 min, and then rinsed 5 times with distilled water. Thirty seeds for each treatment (distilled water as control, 10% (w/v) PEG-6000 solution, 20% (w/v) PEG-6000 solution) were placed in a Petri dish (140 mm Ø) on two layers of filter paper, and treated with 12 ml of the corresponding solution. Petri dishes were covered to prevent moisture loss, and placed in a growth chamber for 7 days in controlled conditions (Temperature 25 °C, Relative Humidity 70%), with a photoperiod of 8 hours light/16 hours dark. Dishes were recovered 7 days after sowing to carry out measurements on germination and seedlings growth parameters. Each treatment, for each accession, was conducted in three replicates.

Germination and seedlings growth parameters

For each Petri dish, GP, SVI, and SWC were calculated as follows:

GP = (number of normally germinated seeds/total seeds number) x 100

(Ali & Idris, 2015; Caruso et al., 2018; Ilori et al., 2012)

Seeds were counted as normally germinated when the radicle extrusion was at least 3 mm (Almaghrabi, 2012).

SVI = (seedling shoot length (cm) x germination percentage)/100

(Ali & Idris, 2015; Caruso et al., 2018)

SWC (%) = [(fresh weight - dry weight)/fresh weight] x 100

(Ge et al., 2016)

For the determination of SWC, all germinated seedlings (shoots + roots) of the same accession were weighed together, after being taken from the Petri dish (fresh weight). After drying in oven at 70 ° C for 24h, seedlings were weighed again, for dry weight determination (Coskun et al., 2014; Ge et al., 2016).

Statistical analysis

Statistical analysis was performed in R statistical environment (*R Development Core Team*, 2021). A widely used approach when dealing with data expressed as percentage, is to use the arcsine transformation, in order to normalize the data and use ANOVA and related post hoc tests to separate the means, according to the procedure reference in Gomez & Gomez, 1984. However, more recent literature highlights how the arcsine transformation may often not be effectively able to solve the problems related to data normality and heteroskedasticity. In these cases, a better approach appears to be the use of Generalized Linear Models (GLM) for germination data, as exhaustively exposed by Sileshi, 2012. In our study, the efficacy of the arcsine transformation in guaranteeing the necessary assumptions for ANOVA was verified for all the data using Shapiro-Wilk and Levene test, respectively. Where these assumptions were not met, specific non-parametric tests or GLM were employed, as specified in the results. Analysis of variance and pair wise comparison on GLM were conducted as described in Hastie & Pregibon, 1992 and Hothorn et al., 2008. Furthermore, all collected data were used to perform cluster analysis, average method. The elbow method verified that three was the proper number of clusters to be adopted. Thus, cluster analysis was used to divide all fifty-five genotypes in susceptible, medium resistant and highly resistant to DS, based on GP, SVI and SWC data taken together.

Performance assessment towards terminal drought conditions in pot experiment

Plant material and cropping method

For the validation of PEG-6000 screening results, three genotypes were randomly selected from each cluster to perform pot experiment, as reported in Table 2. Seeds were surfaced sterilized as described above and pre-germinated for ten days at 4°C in dark conditions. Then, seeds were sown in plastic pots (20 cm diameter and 20 cm length) filled with professional topsoil (Tappeti erbosi, Vigorplant Italia Srl) with 64 % Field capacity (FC) and 25% Permanent Wilting Point (PWP). Plant phenological development was assessed according to the internationally recognized BBCH scale (Biologische Bundesanstalt, Bundessortenamt, and CHemicalindustry) (Meier, 1997). For each accession, six pots were assigned to the stress treatment (DS), and six pots to the well-watered control (Cntr), arranged in Randomized Complete Block Design. Twelve seeds per pot were planted, and then seedlings per pot were thinned to six at 2 leaves stage (BBCH 12), so that all plants were uniform in size and vigor. From sowing until the reaching of 50% heading (BBCH 55), all pots were kept at 100% FC. At the reaching of BBCH 55, continuously till full maturity (BBCH 89), Cntr pots were kept at 100% FC, while DS pots were kept at 50% FC, thus imposing terminal DS, simulating typical Mediterranean type climate conditions. Pots' water content was assessed gravimetrically: the amount of water depleted from each pot was obtained by weighing them every two days, and the loss in weight was restored by re-watering until the weight of every pot became equal to the weight of the predetermined water level. Pots were fertilized at first tillering (BBCH 21) and first booting (BBCH 40) with 2 g of granular N:P:K (14% - 8% - 13%). Pots were kept in a greenhouse, with day/night temperature 24/15 °C (12 hours photoperiod). For each accession, at BBCH stage 73-83 (between milky ripe and soft dough, corresponding to the grain filling stage), gas exchange and chlorophyll fluorescence parameters were assessed. At full maturity, GY was assessed.

Table 2: Randomly selected accession to perform pot experiment

Accession	Species	Candidate
Marco Aurelio	DWC	Susceptible
Haurani	DWL	Susceptible
Kubanka	DWL	Susceptible
Odisseo	DWC	Medium resistant
Tetra IPK 814	DWL	Medium resistant
SSD 502	DWL	Medium resistant
Strongfield	DWC	Highly resistant
AG 189	DWL	Highly resistant
EP 193	DWL	Highly resistant

Measurements of gas exchange parameters

Gas exchange parameters were assessed using a Portable Photosynthesis System (Li-Cor 6400, LI-COR Inc., Lincoln, NE, USA) on the flag leaf of nine randomly selected plants for each thesis of each accession. Specifically, Pn, g_s and E were measured, imposing 500 μmol PAR (Photosynthetically Active Radiation) level and a constant 400 ppm CO_2 flux. Gas exchange parameters measurements were performed between 10:00 a.m. and 14:00 p.m., when temperature, humidity and radiation level in the greenhouse were uniform. The selected plants were properly marked to perform on the same sample chlorophyll fluorescence and GY measurements.

Measurement of chlorophyll fluorescence parameter

Among leaf photochemistry parameters, Fv/Fm is indicated by several authors as an easy to assess selection criterion for wheat drought resistance (Almeselmani et al., 2011; Araus et al., 1998; Farshadfar et al., 2014), based on evidence that a decrease in Fv/Fm, measured on dark-adapted leaves, is related to down-regulation of photosynthesis and photoinhibition. Thus, Fv/Fm was measured on the flag leaf of nine randomly selected plants for each thesis of each accession (same selected plants for gas exchange parameters assessment), after 30 minutes of dark adaptation, using a fluorimeter (Handy PEA+, Advanced continuous excitation chlorophyll fluorimeter, Hansatech Instruments, Kings Lynn, UK). Fv/Fm was assessed on dark adapted leaves by exposing them to a single strong 1 s-light pulse (3500 μmol/m2/s, peak 650 nm).

Grain yield assessment

At full maturity, on the selected plants, GY of the main spike was assessed. Moreover, GY was assessed for each spike in each pot.

Statistical analysis

Statistical analysis was performed in R statistical environment (R Development Core Team, 2021). To assess DS effect on the considered traits, control and stressed plants were compared using t-test for each accession. ANOVA was used in order to verify if different accessions had significantly different values for physiological, GY and chlorophyll fluorescence parameters in control conditions. For both ANOVA and t-test, data normal distribution and homoscedasticity were verified before use, by applying Shapiro-Wilk and Levene test, respectively. Where data normality or homoscedasticity were not met, Kruskal-Wallis and Wilcoxon rank Sum test were used, respectively. PCA (Principal Component Analysis) was performed on stressed individuals combining Pn, gs, E, GY data (expressed as percentage of the respective control) and Fv/Fm data. R packages used: ade4, FactoMineR, factoextra, corrplot.

Results

Simulated water stress at early stages of germination and phenotypic variability

Arcsine transformation did not provide normal and homoscedastic structure to the GP dataset, as verified by Shapiro - Wilk and Levene tests, respectively ($P < 0.05$); therefore, a GLM with logistic regression was used to analyze GP data. Analysis of variance on GLM with logistic regression highlighted that PEG treatment had a significant effect on GP, as well as significant differences emerged among accessions (Fig. 1, Supplementary Figure 1). Pair wise comparison showed that GP was significantly reduced, in a dose-dependent way by PEG. No difference among accessions in control conditions were highlighted. T10 treatment produced modest differences among accessions, which did not lead to a relevant distinction in terms of groups. T20 treatment, on the other hand, produced significant differences, allowing a distinction into susceptible, medium and highly resistant groups.

For example, the highest values were observed in TDS 219 and TDS 234 (WEW) as well as Dic-unibo-022 and Dic-unibo-080 (DEW) and LD093 (DWL), maintaining 100% GP in severe stress conditions, and the lowest in Aureo and Creso (DWC), for which GP dropped to 32% and 34%, respectively. On average, in T20 conditions, DWC, DWL, DEW and WEW showed a GP percentage of respectively 69.5% ± 20.2, 77.3% ± 17.4, 95.2% ± 4.7 and 87.4% ± 21.8, giving indication of a better performance for local varieties and wild relatives compared to modern cultivars.

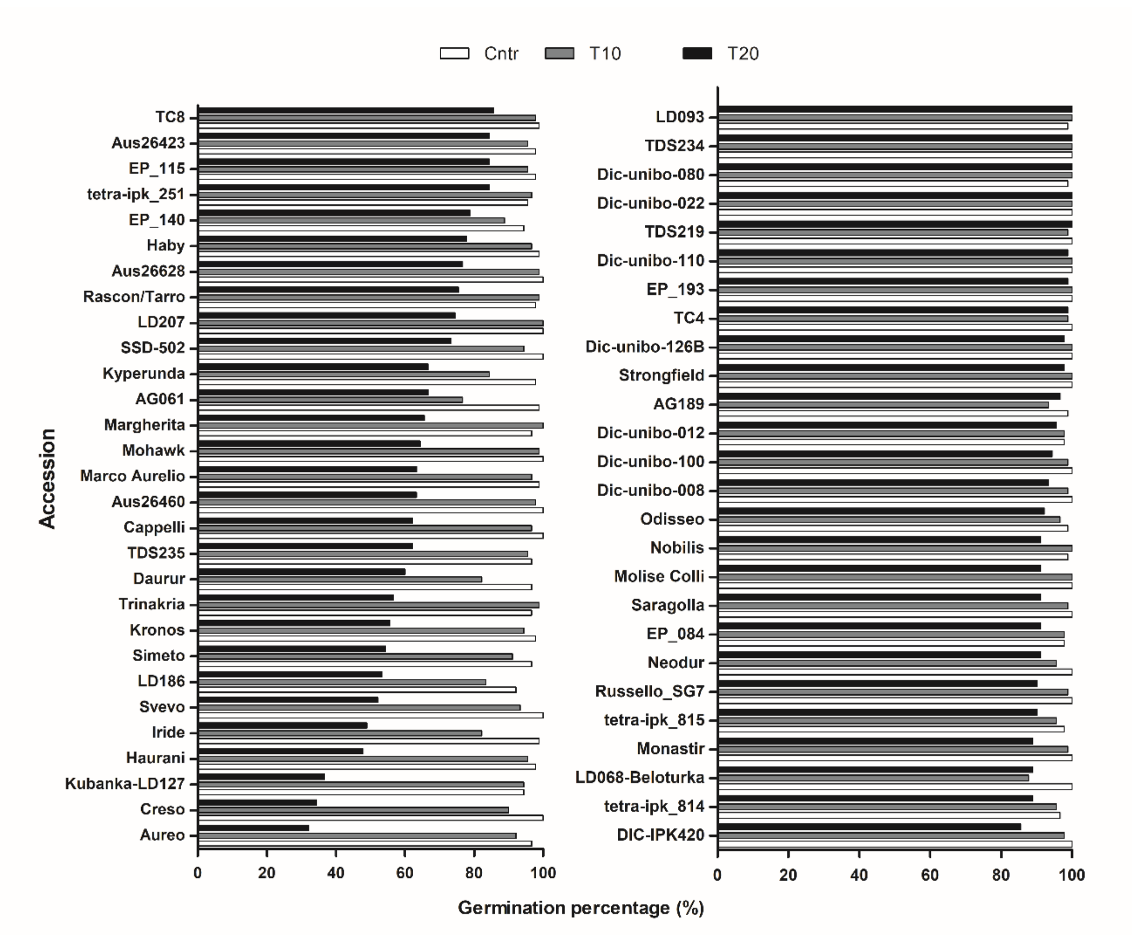


Fig. 1: Germination rate, expressed as percentage, of fifty-five wheat accessions, in control conditions (Cntr) and in the presence of PEG-6000 10% (T10) and PEG-6000 20% (T20) (w/v). Means derive from 30 seeds/treatment and the experiment was repeated in triplicate.

For the majority of the accessions under evaluation, T20 treatment reduced the average shoot length to zero. Thus, T20 was found to be too aggressive, totally preventing shoot development for most of the accessions analyzed. On the contrary, T10 highlighted significant differences among accessions, proving informative for phenotyping. In fact, for T10, SVI ranged from 1.75 to 11.1, showing a broad variability. SVI data, both for Cntr and T10, showed a non-normal but homoscedastic structure, as verified with Shapiro-Wilk ($P < 0.05$) and Levene tests ($P > 0.05$), respectively. Non-parametric Kruskal-Wallis test showed that T10 treatment produced a significant reduction in SVI compared to Cntr ($P < 0.05$). The same test highlighted significant differences in SVI among accessions in control conditions. In order to compare different accessions, with different shoot length in the Cntr group, SVI at T10 and T20 was expressed as percentage with respect to the untreated control, for each replicate of each accession (Fig. 2). Data expressed as percentage of the respective Cntr showed great variability. Lower values were found for DWC Creso and Marco Aurelio (25.6% and 27.6%, respectively), while DWL tetra-ipk 251 and TC8 showed the highest ones (78.3% and 78.6%, respectively).

T10 SVI percentage values with respect to their control for each accession (3 replicates), were compared to highlight significant differences. By treating data expressed as a percentage, the arcsine transformation was applied, which in this case produced satisfactory results, guaranteeing data normality of distribution and homoskedasticity, as verified with Shapiro-Wilk and Levene test, respectively ($P > 0.05$). ANOVA highlighted a significant difference ($P < 0.05$), confirming that T10 was informative to discriminate accessions for SVI.

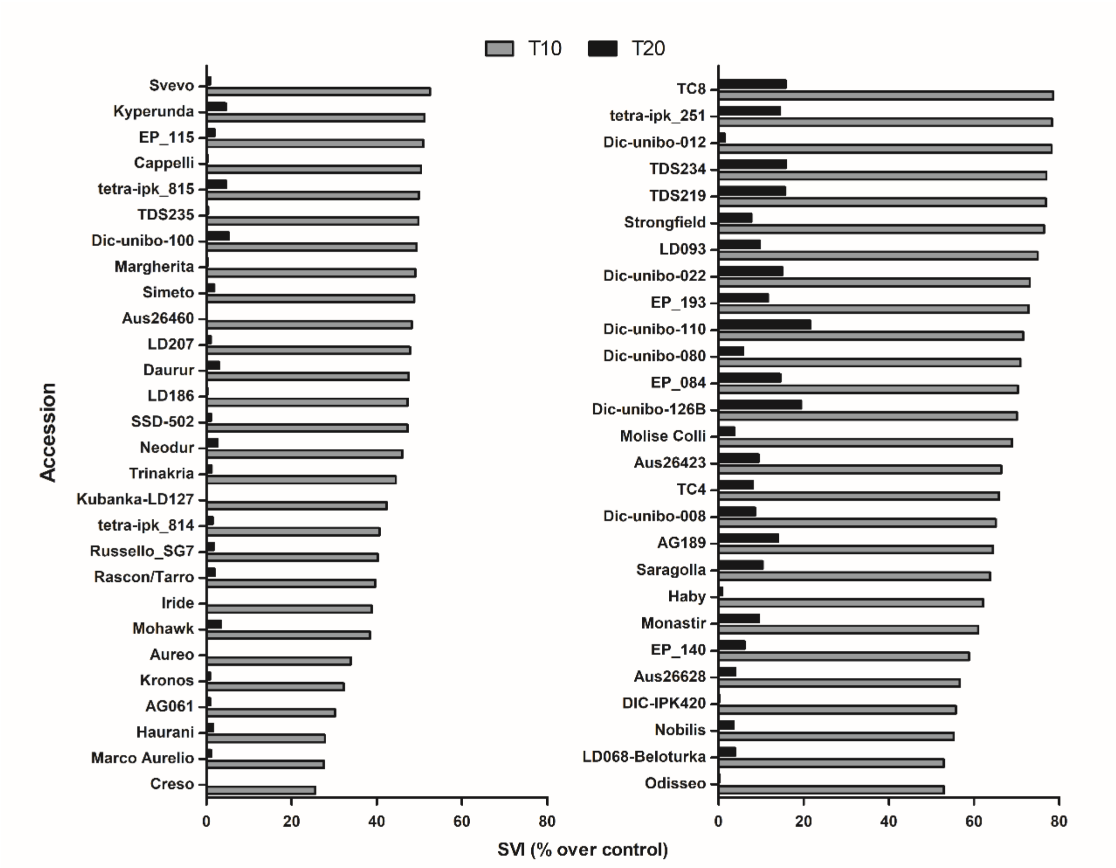


Figure 2: SVI, expressed as percentage of the relative untreated control, of fifty-five wheat accessions. Means derive from 30 seedling/treatment and the experiment was repeated in triplicate.

Phenotyping was performed also taking into account the SWC trait at T10. Figure 3 shows SWC data for T10. Being SWC data expressed as a percentage, the arcsine transformation was applied to cope with non-normal-distribution and heteroskedasticity, as verified by Shapiro-Wilk and Levene test, respectively ($P > 0.05$). ANOVA showed a significant difference among accessions ($P < 0.05$), confirming that it was informative to analyze the accessions' ability to extract water from the culture medium under stress conditions. On average, SWC was 69.5% in the fifty-five investigated accessions. Lower values were observed in Creso and Marco Aurelio DWC (59.5% and 60.4%, respectively), as well as in Kubanka –LD127 and Haurani DWL (60% and 60.9%, respectively). Highest SWC were found in WEW TDS 219 and TDS 234 (78% and 78.2%, respectively) and DWL TC8 (77.5%).

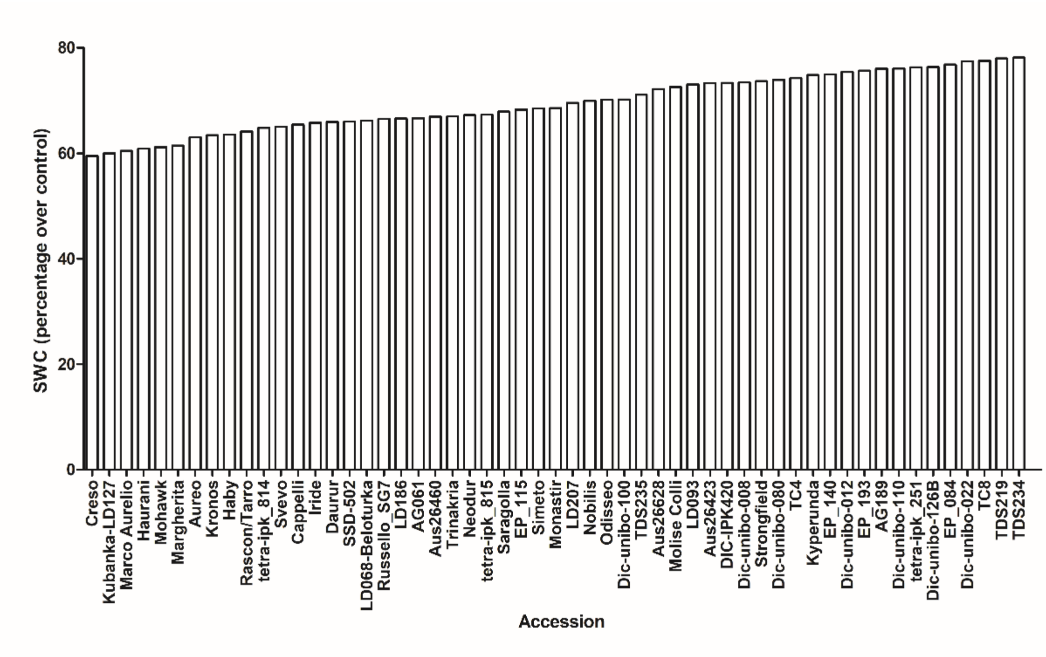


Figure 3: T10 SWC, expressed as percentage of the relative untreated control, of fifty-five wheat accessions. Means derive from 30 seedling/treatment and the experiment was repeated in triplicate.

T20 GP, T10 SVI and T10 SWC values were significantly informative in discriminating the accessions under investigation for early DS resistance, and thus used to perform cluster analysis. Cluster analysis, by average hierarchical method, grouped accessions for similar susceptibility to early DS into three clusters: highly resistant, moderately resistant and susceptible. The choice to create three clusters was confirmed through the elbow method. DWC were mostly represented in the susceptible cluster, while only one DWC was present in the highly resistant one (cv. Strongfield). DWL were ubiquitarious in the three clusters, while DEW and WEW were mainly distributed in highly resistant cluster (Fig. 4 and Table 3).

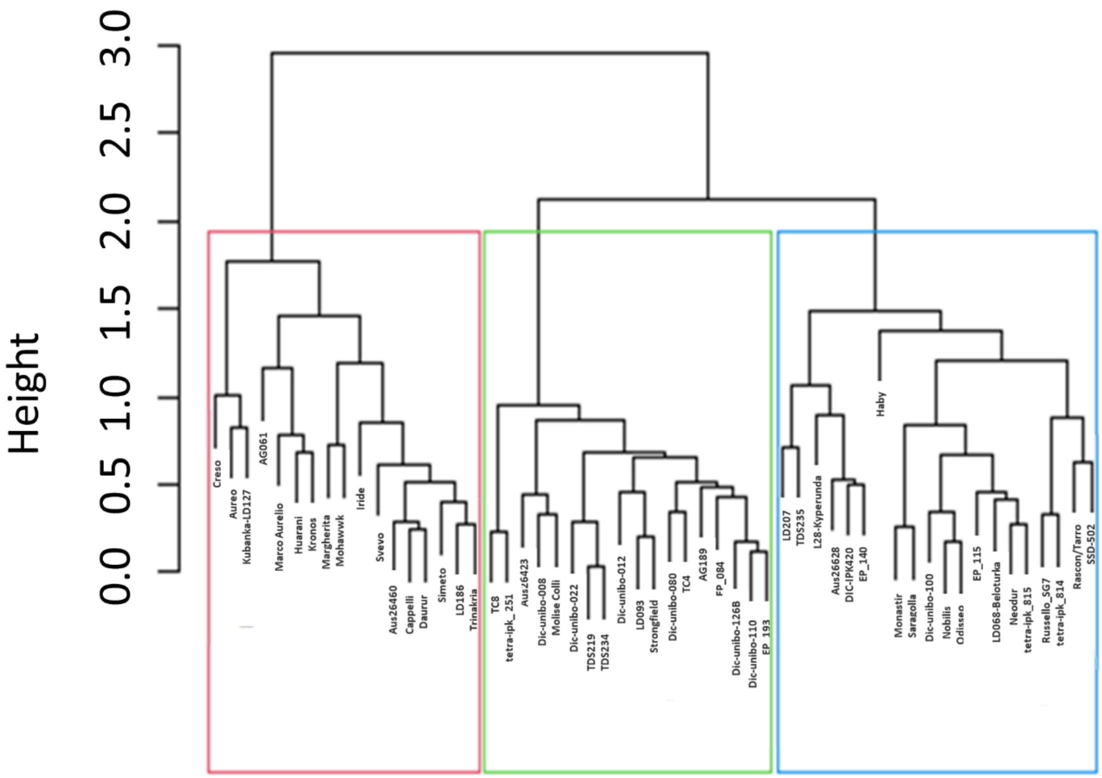


Figure 4: Cluster dendrogram for early DS resistance of the fifty-five accessions under investigation. Accessions that fell within the same cluster had an overall similar susceptibility to early DS: susceptible accessions (red cluster), highly resistant accessions (green cluster), and moderate resistant accessions (blue cluster).

Table 3: Number of accessions of DWC, DWL, DEW and WEW in each cluster

Species	Highly resistant	Moderately resistant	Susceptible
DWC	1	8	10
DWL	8	9	7
DEW	7	2	0
WEW	2	1	0

Simulated water stress in early germination stages is a reliable predictor for assessing water stress resistance in terminal drought stage

Three genotypes for each cluster were randomly selected to perform terminal DS in pot experiments. Terminal DS reduced all physiological parameters for all accessions under investigation and the reduction was significant to t-test only for some of them, as reported in Figure 5. On the contrary, Fv/Fm ratio did not show any significant drop in drought stressed pots ($P > 0.05$).

DS significantly reduced gs for all susceptible candidates (Marco Aurelio, Kubanka, Haurani), Tetra ipk 814 (medium resistant candidate) and Strongfield (Highly resistant candidate) ($P < 0.05$). The same treatment significantly reduced E for all susceptible candidates (Marco Aurelio, Kubanka, Haurani), Tetra ipk 814 and Odisseo (medium resistant candidates) and Pn for Marco Aurelio, Haurani and Odisseo ($P < 0.05$). All others reductions were not significant.

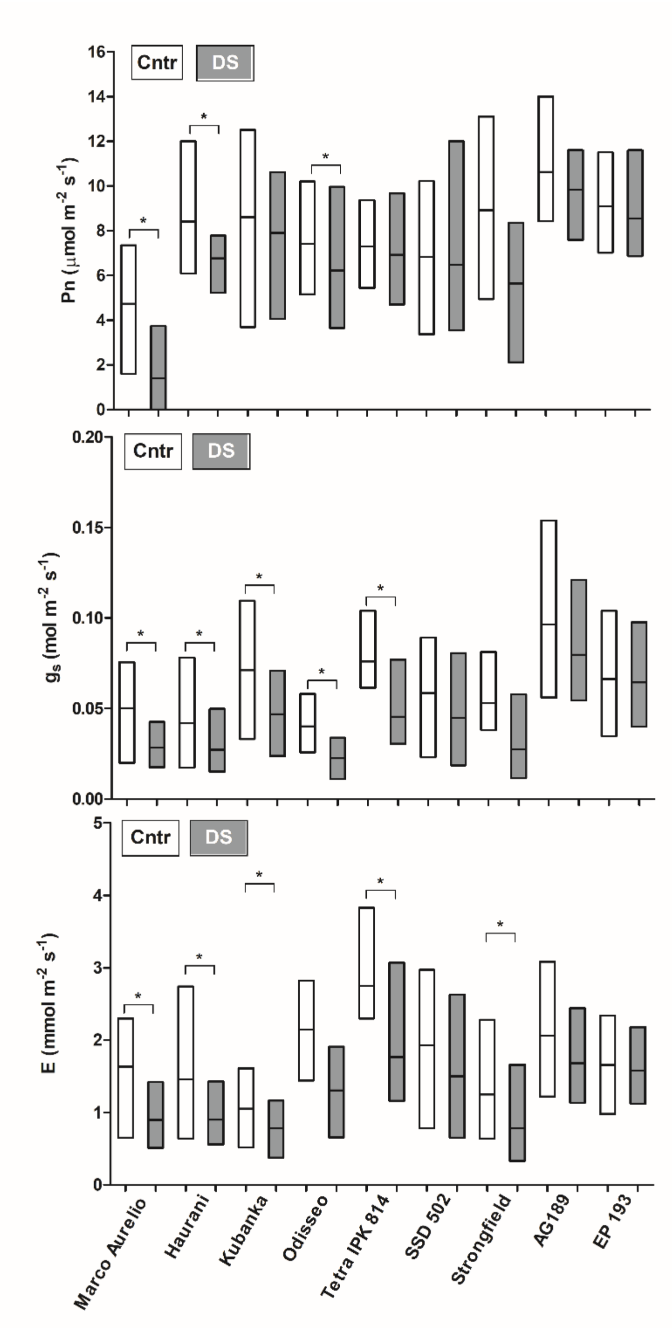


Figure 5: Flag leaf physiological parameters, measured during grain filling in Control (Cntr) and Stressed pots (DS). Net Photosynthesis (Pn), Stomatal Conductance (g_s) and Transpiration (E). Mean is highlighted as horizontal line. Bars marked with an asterisk indicate a significant difference between Cntr and DS pots submitted to t-test for each accession ($P < 0.05$).

DS reduced GY for all accessions under investigation. The reduction was significant for all susceptible candidates ($P < 0.05$). Highly resistant candidates had the lowest GY reduction (Table 4).

Table 4: average GY per spike in control and stressed conditions for each accession. Different letters indicate significant difference between control and stressed conditions, for each accession. Average yield loss in stressed conditions, expressed as percentage, is also reported.

	GY/spike (g)		
Genotype	**Control**	**Stress**	**Average yield loss (%)**
Strongfield	0.66 ± 0.17 a	0.64 ± 0.17 a	1.9
AG 189	0.32 ± 0.15 a	0.31 ± 0.09 a	2.9
EP 193	0.78 ± 0.21 a	0.76 ± 0.14 a	2
Odisseo	0.46 ± 0.18 a	0.45 ± 0.21 a	4.1
Tetra IPK 814	0.33 ± 0.13 a	0.30 ± 0.13 a	9
SSD 502	0.44 ± 0.04 a	0.39 ± 0.03 b	10.7
Haurani	0.58 ± 0.11 a	0.41 ± 0.11 b	29.3
Kubanka	0.32 ± 0.11 a	0.27 ± 0.05 b	16.7
Marco Aurelio	0.39 ± 0.20 a	0.27 ± 0.23 b	30.4

ANOVA showed that different accessions had significantly different physiological parameters and GY values in control conditions. Thus, in order to compare the stress response of different accessions, Pn, gs, E and GY data were expressed as percentage with respect to the untreated control, for each individual of each accession, to perform PCA. ANOVA did not show any significant difference among accessions for Fv/Fm in control conditions, allowing the use of pure data.

The top two principal components (PC1 and PC2) accounted for 83% of the total variation present in the dataset (60.9% and 22.1%, respectively). PC1 was mainly influenced by GY, Pn, gs and E. PC2 was mainly associated with Fv/Fm. Correlation values between variables and principal components, and variables contribution values to principal components are reported in Table 5.

Pn, gs, E, and GY showed a strong correlation with PC1, the principal dimension, explaining most of the total variation in the dataset. Moreover, Pn, gs, E, and GY similarly contributed in the definition of PC1. Fv/Fm had a very low correlation with PC1, and a high correlation with PC2, contributing almost exclusively in defining this dimension.

Table 5: Correlation values between variables and principal components, and variables contribution values to principal components

	Correlation values			**Variables contribution (%)**	
	PC1	PC2		PC1	PC2
Pn	-0.86	-0.13		24.3	1.58
g_s	-0.9	0.18		26.5	3.02
E	-0.93	0.22		28.42	4.25
GY	-0.79	-0.32		20.77	9.5
Fv/Fm	0.01	-0.95		0.001	81.64

Observing the correlation values among these variables (Fig. 6), globally, physiological parameters (Pn, gs, E) showed good positive correlation with each other, and GY showed a good positive correlation with Pn. On the contrary, Fv/Fm did not show any significant correlation with physiological and performance parameters.

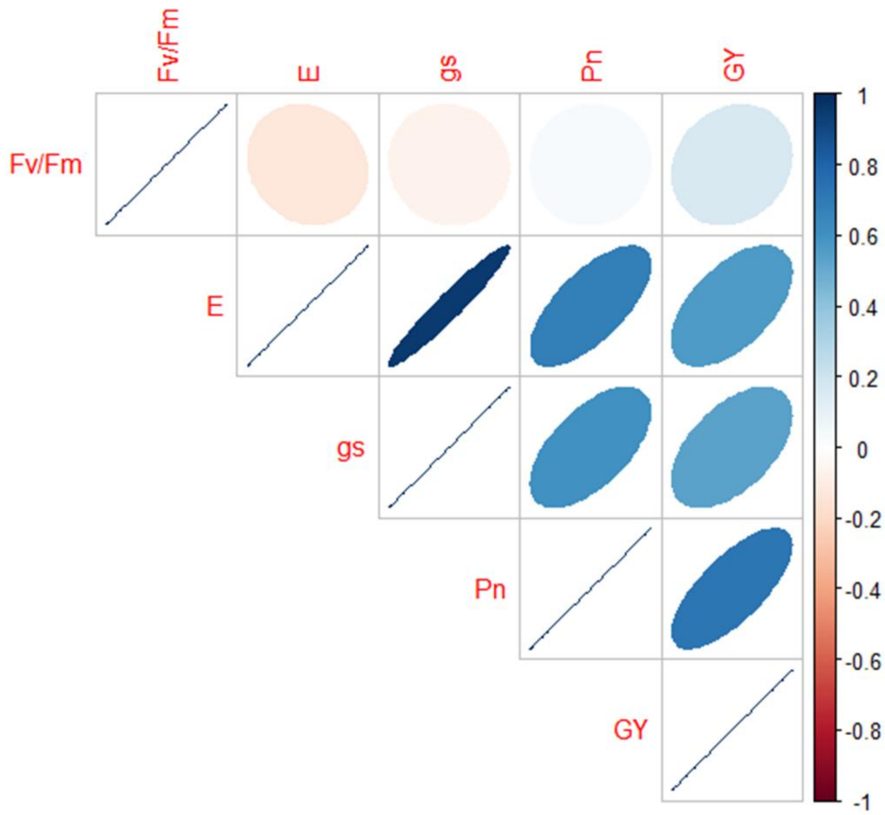


Figure 6: correlation values among physiological (Pn, gs, E), chlorophyll (Fv/Fm) and performance (GY) parameters in DS conditions. Physiological and performance parameters are expressed as percentage of the respective control.

Considering the biplot from the PCA analysis, it was possible to identify the performance of single individuals under drought stress. According to this analysis, superior resistant individuals should be selected based on low PC1 values. On average, highly resistant candidates showed lower PC1 values, corresponding to better physiological and performance parameters, thus distributing mainly in the two left quadrants. Susceptible individuals, on the contrary, were mainly distributed in the two right quadrants, indicating altogether lower Pn, gs, E and GY for these individuals. Medium resistant candidates individuals uniformly distributed in left and right quadrants, indicating an intermediate overall performance in DS conditions.

PCA biplot of individuals (susceptible, medium resistant and highly resistant candidates) and variables is reported in Figure 7.

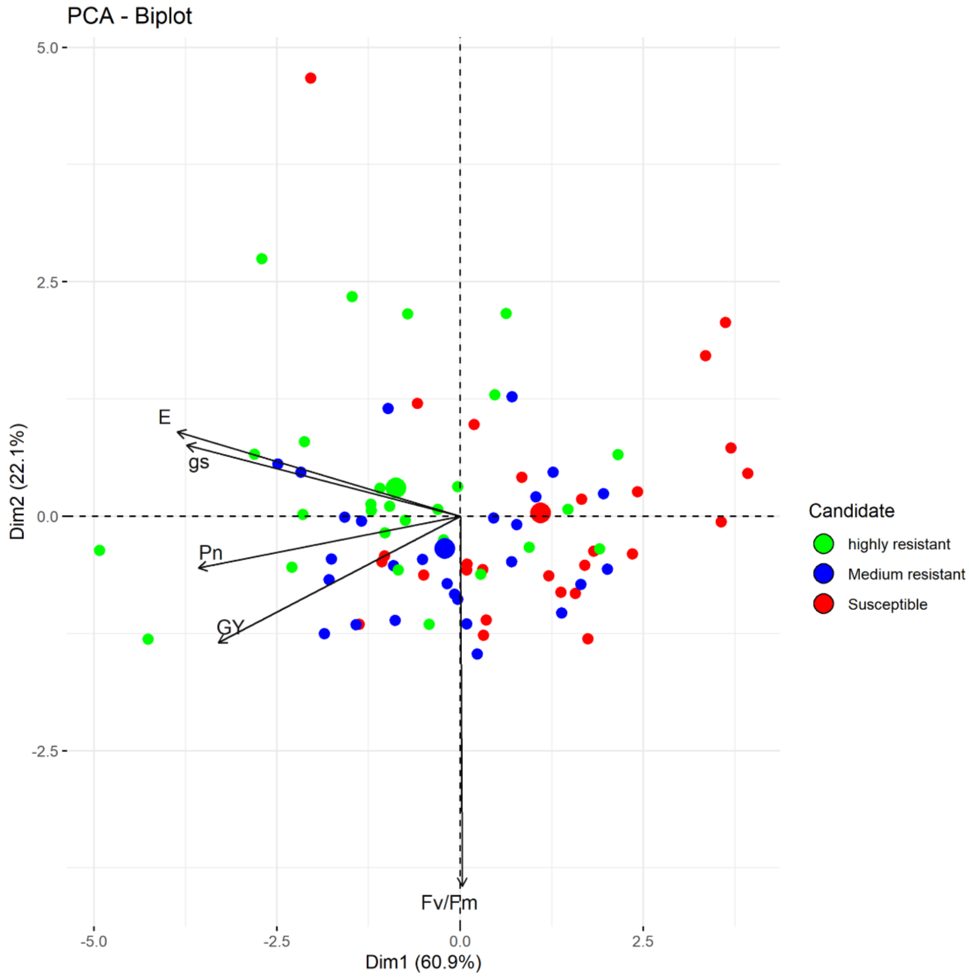


Figure 7: PCA biplot of individuals, and variables correlation circle in DS conditions. Axes represent the principal components: Dim1, explaining 60.9% of the variance, and Dim2, explaining 22.1% of the variance. Colours in the PCA indicate genotypes clustering together for early drought stress resistance.

Discussion

The use of PEG-6000 to mimic DS on germinating seeds and young seedlings proved to be effective in phenotyping for DS resistance in early stages (early vigor), highlighting significant differences among the selected fifty-five tetraploid wheat accessions for morpho-physiological traits.

Specifically, T20 treatment resulted informative in discriminating genotypes based on GP, while T10 in discriminating them based on SVI and SWC.

PEG screenings have proven to be an easy, quick, and affordable tool for assessing early vigor at seedling stage in drought conditions. In fact, combining GP, SVI, and SWC data allowed to group the genotypes into three clusters, identifying candidates for DS who were susceptible, moderately resistant and highly resistant.

The results herein reported suggest a remarkable degree of genetic variability in the TCC for these phenotypic traits, to be potentially exploited for breeding purposes, in order to develop new promising early vigorous cultivars for environmental contexts prone to early drought. In particular, the DWL, DEW and WEW of the TCC represent a source of genetic repertoire to be further investigated for the above-mentioned purpose, as DEW and WEW resulted to be highly resistant to PEG induced DS as herein demonstrated.

This evidence is in agreement with the suite of complex morphological, physiological, and genetic changes undertaken during wheat domestication followed by intense breeding programs, which did not include traits regarding stress resistance, as seed and yield related traits were given priority. Hence, also drought resistance features were lost as a sharp decrease in genetic diversity, generally referred to as 'genetic bottleneck', was imposed on wheat during domestication and modern breeding programs. As result, modern durum wheat varieties characterized by being lodging resistant and highly responsive to nutrient inputs, are grown in all major wheat cropping environments and are characterized by a narrow genetic base (Lopes et al., 2015). Thus, DWL, DEW and WEW represent a source of allelic repertoire to be exploited to transfer drought resistance features to modern DWC (Giraldo et al., 2016; Merchuk-Ovnat et al., 2016) and this study provides indication that TCC includes interesting genetic variability for breeding purposes.

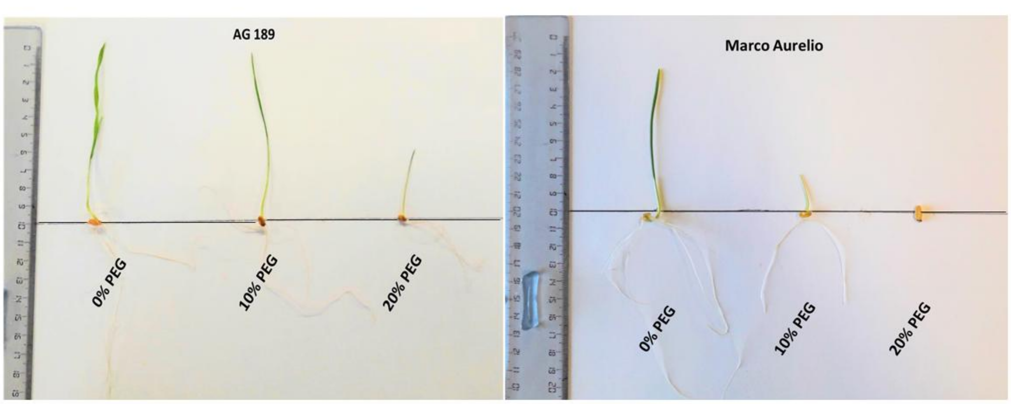
The reliability of PEG screenings on germinating seeds as indicators of tetraploid wheat DS resistance under terminal drought conditions, typical of Mediterranean climate zones, is currently still poorly supported by the literature. Therefore, in order to determine if genotypes' resistance to early drought stress correlates with their performance under terminal drought, validation of preliminary seedling screening results through pot experiments is required. In this study, PEG tests results were coupled to pots experiment, in order to verify if screenings at early stages might be predictive also for terminal drought resistance. Pot experiment was arranged in order to mimic typical Mediterranean conditions, where DS appears around heading, lasting until full maturity, particularly compromising wheat performance during the grain filling stage.

At grain filling stage, DS differentially reduced accessions' performance, and the analyzed traits, i.e. flag leaf gs, E and Pn, resulted informative in discriminating between drought susceptible and resistant ones. On the contrary, Fv/Fm was not informative, as supported literature highlighting how Fv/Fm is insensitive to DS, except in cases of severe dehydration, or concomitant presence of other stress sources, such as high radiation level, as reported in Poggi et al. (2023). Our results confirmed that Fv/Fm does not represent per se a useful breeding tool for DS resistance, especially considering not extremely severe stress conditions, as in this study (50% FC). All physiological parameters showed significant positive correlation with each other, such as Pn and E (r = 0.69) or g_s and E (r = 0.96) and GY showed a significant positive correlation with Pn (r = 0.73).

In particular, significant differences were observed for the physiological performance parameters of the flag leaf, whose major contribution to GY is well documented, mostly during the grain filling stage, when other leaves start to senesce (Loss & Siddique, 1994; Sylvester-Bradley et al., 1990). The positive correlation of flag leaf Pn and GY demonstrate that in context of moderate to mild terminal drought, genotypes able to extract more water from the soil should be preferred, as this will sustain high g_s and thus CO_2 fixation, being finally beneficial for GY. Fv/Fm showed no significant correlation with any physiological parameter and GY. Highly resistant genotypes showed, on average, better performance if compared to susceptible ones, as supported by the PCA biplot. On average, supposed highly resistant individuals were characterized by lower PC1 values, corresponding to higher GY and better flag leaf physiological parameters. On the contrary, supposed susceptible individuals showed higher PC1 coordinates. Medium resistant ones resulted located in an intermediate position.

In conclusion, the assessment of morpho-physiological parameters on tetraploid wheat seedlings in PEG-6000 induced DS proved to be effective in cluster wheat accessions for drought resistance not only for early, but also terminal drought conditions.

Supplementary materials



Supplementary Figure 1: identified resistant (left) and susceptible (right) accessions to PEG-induced drought stress during germination and seedling growth stage

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