Full Length Article

Use of Imaging as Non-Destructive Tool for Water Stress Tolerance in Spring Barley (*Hordeum vulgare*)

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Abstract

Climate change poses a major threat on agriculture, thus on food security. Drought stress, a factor in climate change, is a major problem for barley production, since it simultaneously affects morphological, physiological and biochemical traits. The present work was conducted to provide comprehensive information regarding barley genotypes response and adaptation to drought stress by using a high throughput phenotyping approach. Different barley genotypes were grown in a controlled environment greenhouse. Control plants were kept fully irrigated at 100% field capacity (FC), while the treated plants were stressed by reducing irrigation to 50% of FC. The effects of water deficit on barley genotypes development in terms of early detection of plant response to stress. Morpho-physiological parameters were recorded using Scanalyzer 3D High Throughput Phenotyping platform together with more conventional phenotyping methods to identify and select a set of putative drought tolerant genotypes. The results showed significant differences among genotypes in drought stress response based on digital and traditional indices. Among the selected tolerant genotypes, the best performer was a doubled haploid line derived by a cross Roho×Ardhaoui. © 2022 Friends Science Publishers

Keywords: Doubled haploid; High throughput phenotyping; Water stress; Biovolume; Green index; Barley

Introduction

Different approaches can be deployed to cope with climate change and the need for a sustainable agricultural productivity (Malhi *et al*. 2021). One of the main objectives for a sustainable agriculture is protecting and managing water resources for optimal use. In fact, agriculture alone devours ~70% of the world's fresh water supply on the planet; therefore, the observed reduction in precipitation and increasing costs of irrigation water can seriously hamper future food security (Danzi *et al*. 2019; Malhi *et al*. 2021).

The Mediterranean region has been indicated as one of the most prominent hot spots where the oncoming climate change will strike harder, with unpredictable impact on crop production in this area (Araus and Crains 2014). Agriculture is often described as one of the most vulnerable sectors to future impact of climate change. Since 1990, the intergovernmental panel on climate change (IPCC) has issued five assessment reports featuring agriculture, intended as the source of food for humans via crop production and livestock rearing. The main conclusions based on impact and adaptation models are that crop yields will decline in the upcoming decades. Thus, global agriculture is facing major challenges to ensure global food security, such as the need to breed high yielding crops adapted to future climate (Malhi *et al*. 2021) and the urgency for more sustainable agricultural systems based on reduced inputs including water use (Pignone and Hammer 2013).

The complexity of drought tolerance mechanisms explains the slow progress in improving yields in drought-affected environments. Recent insights into the physiology and genomics of crops led to understanding of novel drought tolerance mechanisms, providing breeders with new knowledge and tools for plants improvement (Buschmann *et al*. 2000; Sanchez *et al*. 2002; Jones *et al*. 2003; Tuberosa and Salvi 2006).

The development of drought stress is a dynamic process in nature and can occur at different times of the crop cycle and at different intensities. Therefore, plants have developed various adaptative strategies, which may differ according to species, genotypes, nature of the drought and combination with other stresses. Stress response is based on a series of different traits that interact in the response in a variable way. In wheat, for instance, several quantitative trait loci have been identified in response to water stress (Gupta *et al*. 2017). Within this framework, classical phenotyping has become a major operational bottleneck

limiting the power of genetic analysis (Hartmann *et al.* 2011; Cabrera-Bosquet *et al.* 2012).

Lack of quantitative, highly productive plant phenotyping methods has become evident in recent years due to increasing demand for the development of higher yielding, resource efficient and stress tolerant crops (Finkel 2009; Houle *et al.* 2010; Furbank and Tester 2011; Cobb *et al.* 2013; Fiorani and Schurr 2013). Recently, an innovative approach to study crop drought stress response has been phenotyped in automated platforms allocated in glasshouses and fitted with conveyor systems and controlled irrigation to automatically analyze by imaging methods in a large number of individuals (Tuberosa 2012; Honsdorf *et al.* 2014; Danzi *et al.* 2019, 2022).

Plant High Throughput Phenotyping (HTP), based on nondestructive and continuous imaging techniques, implying the possibility to study one and the same individual over its entire life span, addresses the interaction of genotypes with their environment. This interaction is displayed in multiple plant morphological parameters and ultimately in their accumulated biomass and yield (Junker *et al.* 2015). In recent years, automation, imaging and software solutions have paved the way for numerous high throughput phenotyping studies (Munns *et al.* 2010; Busemeyer *et al.* 2013; Chen *et al.* 2014; Paulus *et al.* 2014; Danzi *et al.* 2019, 2022).

Barley is one of the earliest cultivated grain crops that rapidly spread to a wide range of climatic areas across many geographical regions of the Mediterranean (Newman and Newman 2006). It had an enormous importance for the Egyptians, the Greeks and the Romans. Nowadays, barley is generally considered a crop suitable to dry climate agriculture, and it has a regional importance in North Africa, West Asia, and Latin America (Grando and Macpherson 2005). Unfortunately, net barley production is predicted to fall due to temperature and water stresses associate to the climatic crisis.

Barley's tremendous potential for drought tolerance has been extensively and practically identified and tested (Sallam *et al.* 2019). Currently, plant genomics, morphology, physiology and biochemistry are providing new insights, and new tools are being developed to identify and investigate drought tolerance traits (Rosero *et al.* 2020). Here we report the use of a Scanalyzer 3D HTP platform to analyze twelve barley genotypes under well-watered and drought conditions aiming at an early detection of phenotypic plant stress response.

Materials and Methods

Experimental details and treatments

Experimental site description: The experiment was carried on at the Italian National Research Council (CNR) Research Unit allocated at the ALSIA Research Center "Metapontun Agrobios" in Bernalda (Italy, 40°23'31.7"N –

16°47'14.2"E, 16 masl), which hosts the Italian High Throughput Phenotyping (HTP) platform PhenItaly (Costa *et al.* 2019), based on a LemnaTec Scanalyser 3D, that enables to analyze nondestructively and dynamically plant morphological traits through the acquisition and processing of digital images in the visible (RGB) and near-infrared (NIR) spectra, in a three-dimensional manner. In fact, each observation is the result of different images taken along the three main spatial axes X, Y and Z projections (Marko *et al.* 2018). The process is fully automated, following a standardized policy, and in the absence of operational interferences.

Plant material and growing conditions: A set of twelve barley genotypes, produced from the barley breeding program of the National Institute of Agronomic Research of Tunisia and described in Table 1, was used in this work. Plants were grown in a greenhouse hosting the HTP platform under natural ambient light conditions. The greenhouse is equipped with a multipoint sensor that monitors environmental parameters every 30 min (Watchdog Model 450, Spectrum Technologies, Inc., Aurora, IL, USA) and regulates ventilation to avoid the rise of local micro-climatic conditions. Seeds were germinated at room temperature for a maximum of 4 days on wet filter paper in Petri dishes, then transplanted into polystyrene cellular containers filled with a 50:50 mixture of peat and washed river sand. The trays were then stored at 4°C for two weeks to synchronize seedlings growth. Individual plants were then transferred to pots for the actual experiment. The pots, (4 L volume) were filled with 3.5 L of a 50:50 mixtures of peat and river sand, for a total weight of 1200 g. Six replicates for each plant for both treated and control per each genotype were randomized in the greenhouse to avoid the onset of local microclimatic variation while waiting for being loaded in the automated conveyor for 3D scanning at appropriate timing. To allow the automatic individual plants identification in the platform, a barcode was applied at convenient position on the pots. All plants were manually kept fully irrigated up to the booting stage, 45 days after sowing (DAS), while for the duration of the experiment, that is from 45 DAS to 90 DAS, the control plants were kept fully irrigated (100% field capacity), while the treated plants were stressed by reducing irrigation to 50% of the field capacity (FC) through manual irrigation following pot weighting. After 90 DAS irrigation was stopped for all plants until complete maturity.

HTP and traditional phenotyping

Images in the visible spectrum were used for automated phenotyping. These images were composed of three layers, each corresponding to the three primary colors red, green, and blue (RGB). In the platform three RGB images were taken, one from above the plant and two laterally at an orthogonal angle. Starting from 45 DAS and up to 90 DAS, 3D RGB images, involving three mutually orthogonal

vantage points, were captured at intervals of 45. 60, 75, 80, 87 and 90 DAS according to Petrozza *et al*. (2014). Closer interval between 87 and 90 DAS was used to better monitor plant senescence. The RGB images were used to produce HTP digital indices value, that is plant height (PH), digital biovolume (DB), green index (GI) and health index (HI) (Petrozza *et al*. 2014). Image analysis was performed by using specific pipelines aimed at measuring specific digital characters (Petrozza *et al*. 2014; Danzi *et al*. 2019). A complete list of the traditional and digital characters analyzed is given in Table 2.

DB was calculated from three orthogonal images of the same plant according to Eberius and Lima-Guerra (2009) and Petrozza *et al*. (2014). GI was calculated starting from the RGB images by applying the function $(R - B)/(R + B)$, where R and B are the red and the blue image component, respectively (Kawashima and Nakatani 1998). HI was calculated by transforming images from RGB color space into Hue, Saturation and Intensity (HSI) color space, and deriving from these data an index using an appropriate procedure described by Pydipati *et al*. (2006).

For traditional phenotyping analyses, the following traits were scored at complete plant maturity, when plants were ready for harvest: spike length (SL), number of spikes per plant (NSPP), number of spikelets per spike (NSPS), number of kernels per spike (NKPS), kernel yield per spike (KYPS), thousand kernel weight (TKW) and kernel yield per plant (KY). All counts were conducted on each single plant and then classified per treatment and genotype.

Statistical analysis of data

Mean data are presented for each trait. Statistical analysis was performed using SPSS 20 and differences between genotypes were tested by using *t*-student test. In addition, to analyze the differences between thesis were assessed by means of multifactor analysis of variance (ANOVA) was carried out. For the sake of readability, some data are not shown in the present article, but may be provided upon request of interested audience.

Results

Traditional phenotyping assessment

Results showed that drought stress significantly affected all productivity traits by reducing their value, even though not all to the same extent. PH was the least affected character by drought stress, showing a significant reduction of 8.49% of this character in the drought lot (Table 3). All the remaining traits were dramatically affected by drought. KYPS, NSPS, and, consequently, KY reductions were significantly greater in the stressed lots than in the well-watered ones, with an average reduction of 47.68, 37.96 and 30.77%, respectively (Table 3). The reduction in thousand kernel weight (TKW), although significant, is not of the same order as the

reduction of KYPS, resulting in a loss of 14. 37% of the weight (Table 3).

When considering the performance of each genotype under the two treatments, the differences among the genotypes emerged (data not shown). In this analysis, Ardhaoui (G9), Safra (G10), DH1 Roho/Ardhaoui (G11) and DH3 Roho/Ardhaoui (G12) showed the best KYPS under drought stress. Genotypes Tej (G6), DH1 Momtez/Roho (G4), and Manel (G2) showed the greatest differences in the number of the kernel KYPS between stressed and well-watered conditions. Differences in the level of reduction of KY were observed among the individual genotypes. Genotype DH3 Roho/Ardhaoui (G12) was the best performing together with Ardhaoui (G9), showing the least reduction in KY, while the worst performing genotype was Manel (G2) with a reduction of 63.5% (Fig. 1).

HTP to study plants response to stress

Based on previous reported experiments, some HTP indices have been selected in these analyses: the DB, GI, and HI. HTP was applied at a vegetative phase of the plant life corresponding to spring conditions in the field. In the Southern Mediterranean regions and particularly in Tunisia, April is the month in which precipitations become lower and temperatures rise, and when the onset of water stress produces the worst effect. In all genotypes analyzed a negative variation in the DB was observed in drought stressed plants for the entire length of the treatment, even though at different levels in the different genotypes (Fig. 1). Some genotypes were more affected by water deficit, while others were more tolerant. In particular, genotypes Safra (G10), DH2 Roho/Ardhaoui (G11), DH3 Roho/Ardhaoui (G12), Lamsi (G8) and Kounouz (G7) showed a smaller reduction in DB during the entire length of the experiment (Figure 2). In all genotypes the differences between control and stressed plants were significant at 60 DAS (15 days after the beginning of the treatment). From that point on, in some genotypes the differential between treated and control continued to grow, e.g., in Rihane (G1), Manel (G2), Lamsi (G8), Ardhaoui (G9), while in others it remained constant after a period of apparent adaptation, e.g., in DH1 Momtez/Roho (G4), Safra (G10) and DH3 Roho/Ardhaoui (G12) (Fig. 1).

GI, which expresses the leaf chlorophyll content, is obtained from RGB images. The evaluation of GI in control and drought-subjected plants showed at 45 DAS up to 80 DAS fairly stable values in both treatments, then dropped significantly reaching the minimum at 90 DAS (Fig. 2). The GI did not vary significantly between fully irrigated, and drought treated plants in Rihane (G1), Manel (G2), Ardhaoui (G9), Safra (G10), DH2 Roho/Ardhaoui (G11) and DH3 Roho/Ardhaoui (G12). Other genotypes, such as Manel (G2), Roho (G5) or Lamsi (G8) showed a rapid drop in the GI reaching a maximum at 90 DAS (Fig. 2).

Table 1: Origin and pedigree of the twelve barley genotypes used in the present study

Genotype	Code	Origin	Pedigree
Rihane	G1	INRAT (Tunisia) / ICARDA (Syria)	Atlas 46/Arrivat//Athenais
Manel	G2	INRAT (Tunisia) / ICARDA (Syria)	L572/5/As54/Tra//2*Cer/Toll/3/Avt/Toll//Bz/4/Vt/Pro/Toll
Momtez	G3	ICARDA Alep (Syria)	M126/CM67/As/Pro/3/Arizona 5908/ths//Lignée 640
DH1	G4	INRAT (Tunisia)	Momtez/roho
Roho	G5	INRAT (Tunisia) / Laboratoire Riso (Denmark)	Roho 03573
Tej	G6	INRAT (Tunisia) / ICARDA (Syria)	Bonus/C13576 (W12198-Australia)
Kounouz	G7	INRAT (Tunisia) / ICARDA (Syria)	Alanda/5/Aths/4/Pro/Toll//Cer*2/Toll/3/5106/6/24569
Lamsi	G9	USA	Rapidan, USA
Ardhaoui	G9	Tunisia	Local landrace
Safra	G10	Tunisia	Local landrace
DH2	G11	INRAT (Tunisia)	Roho/Ardhaoui
DH3	G12	INRAT (Tunisia)	Roho/Ardhaoui
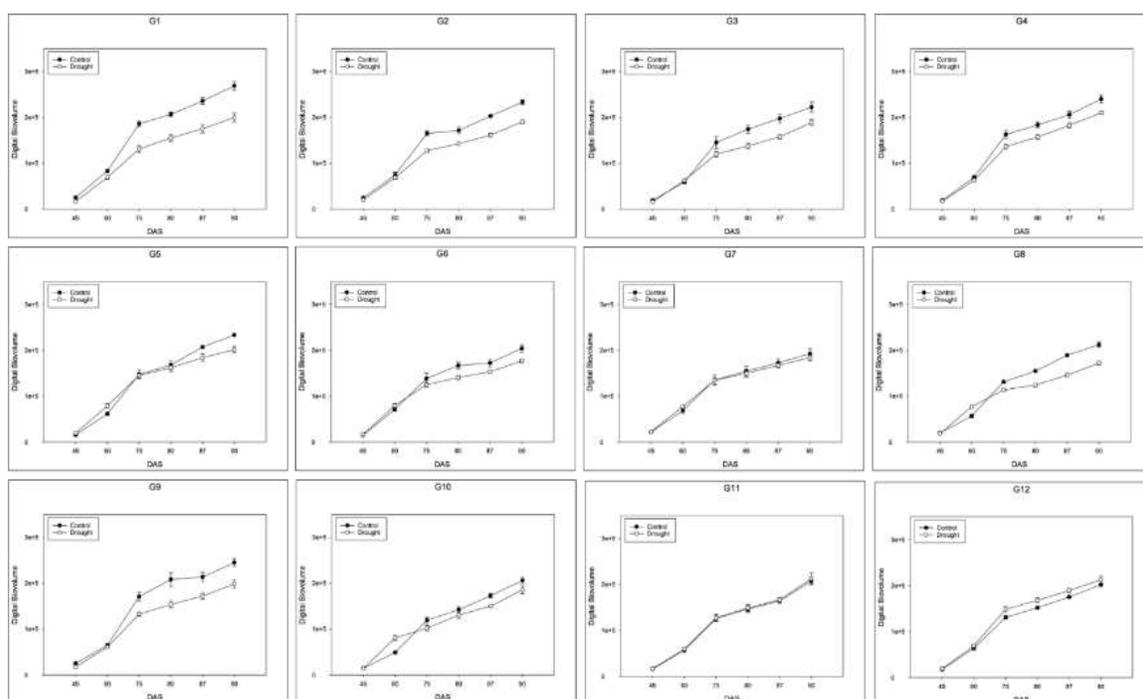
Fig. 1: Evolution of DB on twelve barley genotypes under normal and stressed growth conditions in the period 45 to 90 DAS (days after sowing)

As a general trend, a constant increase of the HI was observed in all plants till 80 DAS, followed by a light decrease at the end of the treatment (Fig. 3). Also in the case of HI differences among the genotypes are evidenced. Some genotypes kept a high HI throughout the experiment, as Ardhaoui (G9), Safra (G10), DH2 Roho/Ardhaoui (G11) and DH3 Roho/Ardhaoui (G12), while others showed a clear decrease in this value under water stress, such as in Rihane (G1), Manel (G2), and Momtez (G3) (Fig. 3).

Discussion

Water stress before anthesis can reduce wheat plant fertility defined as the number and weight of grains per spike (Dancic *et al*. 2000; Mary *et al*. 2001). Apart from specific differences, wheat and barley tend to respond to water stress in comparable manners (Zeeshan *et al*. 2020). Moreover, some of the spike traits are reported to be associated to the total crop production in cereals (Sial 2007; Xue *et al*. 2010), while drought during grain filling can lead to differences in individual grains weight (Giunta *et al*. 1993; Lopez-Castaneda and Richards 1994; Voltas *et al*. 1998). Here we observed that some of these traits were more intensely affected by water stress. The NSPS and KYPS were significantly reduced in the drought stressed samples (31.85 and 52.68%, respectively) and total KY evidenced a loss in grain yield per plant averaging 35.99%. This observation implied that under water stress conditions, the loss in barley yield under field conditions may reach one third of the potential yield (Table 3). Some of the genotypes tested in our experiment proved to be less affected by the drought treatment, thus suggesting that they could

Table 2: List of the traditional and HTP/Digital traits used in the present study, and of their scoring time

Character code	Traditional (T) or digital (D)	Period	Description
SL	T	Complete maturity	Spike length including awns (mm)
NSPP	T	Complete maturity	Number of spikes per plant
NSPS	T	Complete maturity	Number of spikelets per spike
NKPS	T	Complete maturity	Number of kernels per spike
KYPS	T	Complete maturity	Kernel yield per spike (g)
TKW	T	Complete maturity	One thousand kernels weight (g)
KY	T	Complete maturity	Total kernel yield per plant (g)
PH	D	90 DAS	Plant height (mm)
DB	D	45, 60, 75, 80, 87, 90 DAS	Digital biovolume based on 3D imaging
Green index	D	45, 60, 75, 80, 87, 90 DAS	Color index based on 3D imaging indicating leaf greenness
Health index	D	45, 60, 75, 80, 87, 90 DAS	Color index based on 3D imaging indicating plant health and senescence status
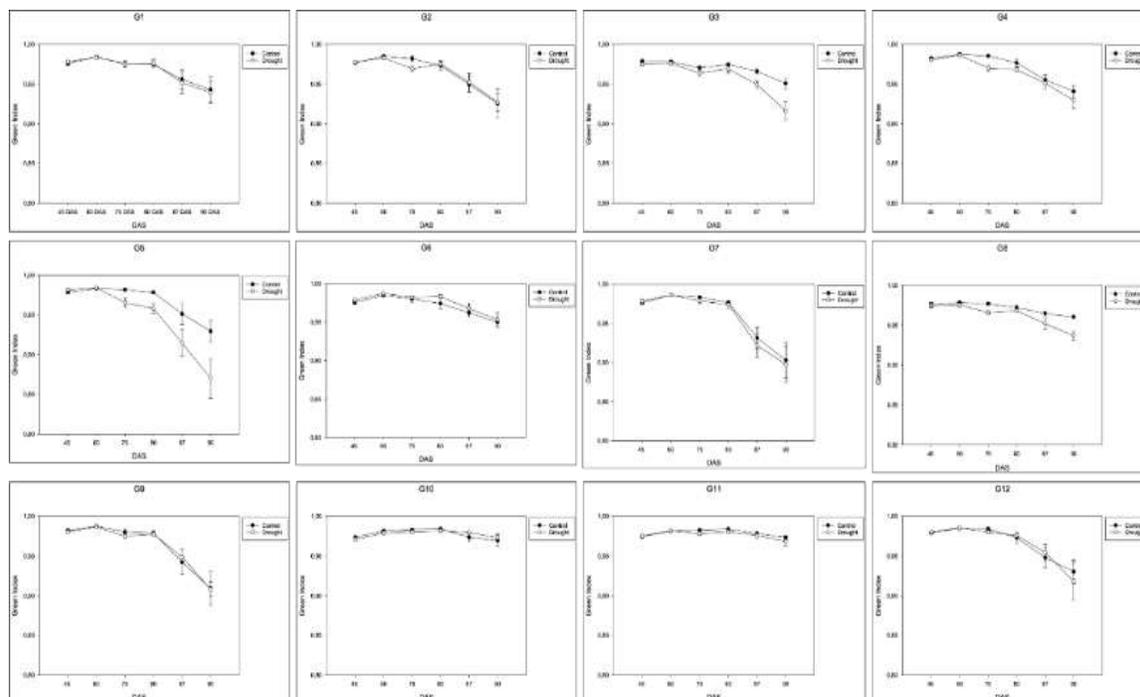
Fig. 2: Evolution of the GI on twelve barley genotypes under normal and stressed growth conditions in the period 45 to 90 DAS (days after sowing)

possibly bear traits for adaptation to drought stress. For instance, G12 (DH3 Roho/Ardhaoui) proved to be much less affected by water stress conditions in comparison to G7 (Kounouz) and G2 (Manel), consequently they might be preferred in water deficit environments as far as the above three traits are considered.

Some literature data lead to the conclusion that a reduction in yield is mostly due to lower grain weight and only minimally to lower grain number (Sofield *et al*. 1977; Tashiro and Wardlaw 1990). Our data show that KY was the trait most affected by drought, showing a reduction of about 47.86% (Table 3), while the reduction of TKW was much lower. This occurrence might be possibly due to the reduction of other spike traits, such as a lower number of seeds per spike (KYPS). It can be hypothesized that this response might correspond to an evolutionary strategy favoring the production of higher quality seeds, even though

with a lesser abundance. In fact, seed morphology has been reported to have a strong influence on a seed germination and vigor (Ambika *et al*. 2014). Moreover, NSPP although showing an appreciable reduction of 27,40% did not appear as much as significant as NKPS, a trait that was significantly reduced in the stressed plants (37, 9%, Table 3). On average among all the genotypes, G12 is the genotype least affected by water stress.

The performed yield traits analysis showed that a solid level of variability exists with respect to all the phenotypic traits examined. This has been also supported by the HTP analysis based on some imaging tools.

Plant development was analyzed through the DB, which is a morphometric, non-destructive measurement previously employed in high throughput phenotyping studies (Briglia *et al*. 2019; Danzi *et al*. 2019). The curves of DB in the time domain showed that water stress induced

Table 3: Summary statistics of barley average response to stress and control condition. Only traditional traits are considered

Treatment	PH (cm)	NSPP	SL	NSPS	NKPS	KYPS (g)	TKW (g)	KY (g)
Control	90,635 (1.063)*	13.824 (0.494)	6,836 (0,131)	29.432 (0.639)	19.486 (0.985)	1.173 (0.059)	61.365 (1.830)	4.095 (0.069)
Drought stress	82,944 (0,855)	9.956 (0.455)	5.653 (0.125)	20.367 (0.632)	12.089 (0.752)	0.614 (0.046)	53.35 (3.286)	2.835 (0.039)
P	<0.05	<0.01	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001
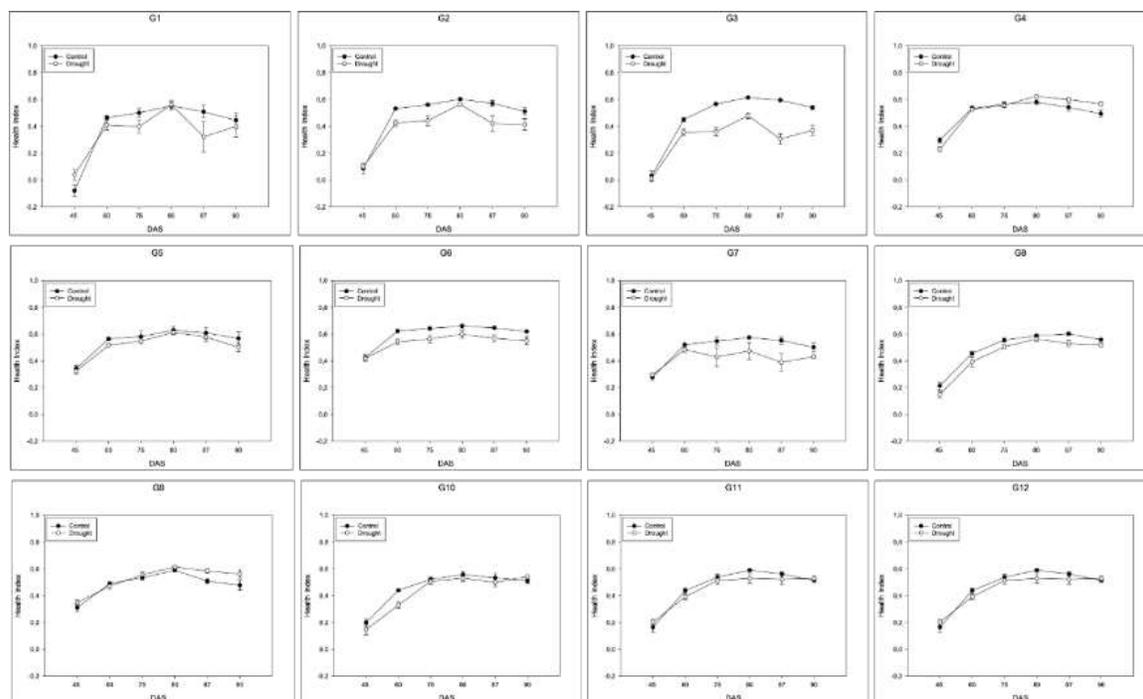
Fig. 3: Evolution of the HI on twelve barley genotypes under normal and stressed growth conditions in the period 45 to 90 DAS (days after sowing)

a reduction of the plant total biomass. Nevertheless, not all the genotypes showed similar response to the stress; some showed a sudden drop when the stress was applied but could recover and continued to grow at a relatively lesser rate than that of control plants. Other genotypes, instead, tended to chronically suffer the stress and reduce their growth rate over time with respect to the controls (Fig. 1). This may be an indicator that the former ones were better able to resist a chronic water deficit. In this study genotypes G4 (DH1 Momtez/Roho), G10 (Safra) and G12 (DH3 Roho/Ardhaoui) appeared to possess this ability (Fig. 2). These results supported the efficacy of the DB as an excellent phenomic proxy of the overall health status of the plant in response to external stimuli. It has the great advantage of being nondestructive, thus allowing to follow each plant for the entire course of its development, so reducing the aleatory effect of comparing different individuals. Nondestructive phenotyping indices are scalable and applicable to many crop plants, an issue that enrich their applicability for both basic and applied research. Therefore, DB can surely be proposed as a tool for germplasm selection aimed at pre-breeding and breeding programs or at evaluating the effect of agricultural practices on plant growth (Danzi *et al.* 2019).

The degradation of chlorophyll during abiotic stress or during senescence leads to a reduction of GI, based on the reflectance of the green component of the visible spectrum (Jiang *et al.* 2020). Of course, the GI tends to be zero because of the yellowing of leaves independently of its cause, stress, senescence or disease. Leaf yellowing at late developmental stages is the result of remobilization of carbohydrates and nitrogen from the older leaves to developing tissues and/or reproductive organs to ensure the reproduction of the plant (Abdelrahman *et al.* 2017). For this reason, a drop of the GI along with maturation of the plants is a physiological event. In the case of water stress, the GI dropped more rapidly in more sensitive genotypes (Fig. 2). A reduced remobilization of nutrients may explain the decrease in grain yield components, which is lower in the resistant genotypes. In fact, in our experiment the genotypes G10 (Safra) and G12 (DH3 Roho/Ardhaoui), which have a lower reduction of NSPS and KYPS, are characterized by a high GI and biovolume at 90 DAS, when plant maturation initiates.

In our experiment, the extent of GI over time is the second most sensitive proxy of plant stress after the

reduction of DB. Nevertheless, this GI an advantage over DB. In fact, DB is the result of a three-dimensional evaluation, implying that each plant has to be analyzed by three different geometrical perspectives, a feature not easy to perform in the field. Conversely, GI is a monodimensional index, and it can be derived by a single image. This occurrence makes this index more easily scorable in the field by both field phenotyping platforms and flying UAVs.

Based on RGB imaging analysis, the plant health status was followed during growth development and stress (Ma *et al*. 2019). Genotypes G9 (Ardhaoui), G10 (Safra), G11 (DH2 Roho/Ardhaui) and G12 (DH3 Roho/Ardhaoui) maintained a high HI compared to the other genotypes (Fig. 3). The complexity of plant response to drought needs of an accurate trait dissection to deepen the understanding of resistance or adaptation to drought. High-throughput phenotyping associated to more traditional indicators provided a significant new opportunity to identify genotypes able to better elucidate the genetic basis of these responses. The tools developed for HTP can be transferred to the field in order to assess the health of crops in response to environmental changes, and to changing agricultural techniques employing lower inputs (Leakey *et al*. 2009; Harfouche *et al*. 2012, 2014; Aitken and Bemmels 2016).

Conclusion

The use of imaging techniques and parameters to accurately provided comprehensive information on the response of barley genotypes to drought and to facilitate selection in crop improvement programs. Genotype G12 (DH3 Roho/Ardhaoui) performed better in stress condition as it had a reasonable NSPS and KYPS as well as it gave higher 1000 kernel weight and GI as compared with other genotypes. This line would be a valuable genetic resource for both breeding more productive cultivars with novel agronomic traits. This work provides a powerful approach for the early and quantitative determination of drought-tolerance among different barley genotypes.

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