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Detection of haplotypes and QTLs related to tolerance to water deficit and high temperatures in durum wheat: towards the selection of resilient genotypes to climate change

Devant le jury:

Abdelkarim FILALI-MALTOUF	PES	Faculté des Sciences, Université Mohammed V- Rabat	**Président**
Bouchra BELKADI	PES	Faculté des Sciences, Université Mohammed V- Rabat	**Directeur de thèse**
Filippo Maria BASSI	Expert	International Center for Agricultural Research in the Dry Areas (ICARDA), Rabat	**Encadrant**
Samir EL JAAFARI	PES	Faculté des Sciences, Université Moulay Ismail, Meknès	**Rapporteur**
Leila MEDRAOUI	PH	Faculté des Sciences, Université Mohammed V- Rabat	**Rapporteur / Examinateur**
Cherkaoui EL MODAFAR	PES	Faculté des Sciences et Techniques, Université Cadi Ayyad, Marrakech	**Rapporteur / Examinateur**
Miloudi NACHIT	Expert	International Center for Agricultural Research in the Dry Areas (ICARDA)	**Examinateur**

✉ Faculté des Sciences, avenue Ibn Battouta, BP. 1014 RP, Rabat –Maroc
☎ 00212 (05) 37 77 18 76 🖷 00212(05) 37 77 42 61; http://www. fsr.um5.ac.ma

Preface

This thesis has been completed during the course of enrollment in a PhD degree at the University of Mohammed V, Faculty of sciences of Rabat, under the supervision of Prof. **Bouchra Belkadi** of **Microbiological and Molecular Biology Laboratory (LMBM)**. All the work presented in this thesis has been done in the **International Center for Agricultural Research in the Dry Areas (ICARDA)** under the supervision of Dr. **Filippo M. Bassi**. This work is a part of an Australian project funded by the **Grains Research and Development Corporation (GRDC)**.

All the results presented have been already published or are currently been submitted for publication.

Dedication

I would like to dedicate this thesis to my family for its endless support, my beloved parents for providing their moral and emotional support and for their constant prays and encouragement to accomplish the thesis work, my sweet and loving sister and brothers who have been my source of inspiration and gave me strength and to all those who love me, all those who are very close to my heart who shared their words of advice and encouragement to finish this study.

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This thesis has been completed during the course of enrollment in a PhD degree at the Faculty of Sciences, University Mohammed V of Rabat at the Microbiological and Molecular Biology Laboratory (LMBM). All the work presented in this thesis has been done in the International Center for Agricultural Research in the Dry Areas (ICARDA). The study presented in this manuscript was under the supervision of Prof. Bouchra BELKADI and Dr. Filippo Maria BASSI.

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- **El Hassouni K**, Attjioui M, Belkadi B, Filali-Maltouf A, Ghanem M.E, Nachit M and Bassi F. Seeking novel alleles for tolerance to Boron toxicity in a vast collection of durum wheat entries.
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Abstract

Heat and drought are the main abiotic stresses reducing grain yield. In the present study, durum wheat entries were investigated for their root architecture under irrigated and water deficit conditions using the pasta strainer method. Two categories of root types were identified and were tested under field conditions in drought prone sites, to reveal that deep roots generated a 38% yield advantage. A durum wheat panel was exposed to simulated heat stress at the time of flowering. Average yield was reduced by 54 % under heat and grain number per spike found to be the most critical trait for tolerance to warm conditions.

The whole panel was genotyped using 35K Axiom array. The most significant genomic regions related to tolerance to high temperatures were identified on chromosome 1A, 5B and 6B. For yield reduction in case of drought, this trait was controlled by three QTLs on 3A, 3B and 7B. Haplotype analysis confirmed that the class of positive alleles resulted in yield advantage of 20% under the heat-stressed conditions of the Senegal River. Similarly, the positive alleles for the three of QTLs for drought tolerance achieved 12% yield advantage under the extremely dry conditions. Five QTLs were successfully validated into KASP markers and can now be pyramided via MAS to obtain superior cultivars tolerant to two major abiotic stresses.

Keywords: Durum wheat, drought, heat, yield, tolerance, QTLs.

Résumé

La chaleur et le déficit hydrique représentent les principaux stresses abiotiques qui réduisent le rendement du blé dur. Dans la présente étude, des lignées de blé dur ont été examinées pour leur architecture racinaire dans des conditions normales et de stress hydrique en utilisant la méthode de passoire à pâtes. Deux catégories de types de racines ont été identifiés et ont été testés dans les conditions du champ exposé à la sécheresse et ont montré que les racines profondes engendrent un avantage en rendement de 38 %. Nous avons exposé une collection de blé dur au stress thermique simulé au moment de la floraison. Le rendement moyen a été réduit de 54 % en conditions de stress comparant aux conditions témoins et le nombre de grains par épi s'est révélé être le caractère le plus important pour la tolérance de la chaleur.

Toute la collection de blé dur a été génotypée avec 35K Axiom array. Les régions génomiques les plus significatives liées à la tolérance aux fortes chaleurs ont été identifiées sur le chromosome 1A, 5B et 6B aboutissant à un rendement supérieur de 8 %. En cas de déficit hydrique, trois QTLs sur le chromosome 3A, 3B and 7B ont été identifiés liés au rendement entrainant une augmentation en rendement de 12 %. Cinq QTLs ont été validés avec succès en marqueurs KASP et peuvent désormais être rassemblés via la sélection assistée par marqueurs (MAS) pour obtenir de meilleures variétés tolérantes aux deux principaux stresses abiotiques.

Mots-clés : Blé dur, déficit hydrique, chaleur, rendement, tolérance, QTLs.

Résumé détaillé

Le blé dur est l'un des principaux aliments de base dans le monde entier. Toutefois, sa production continue de faire face à de nombreux défis liés aux contraintes environnementales, maladies et ravageurs. La chaleur et la sècheresse représentent les principaux stresses abiotiques qui réduisent le rendement du blé dur. Le stress hydrique au moment de la floraison bloque le mouvement normal des nutriments au niveau de la plante ce qui entraine une réduction significative de la taille des grains et, par conséquent, une réduction du rendement. Dans la présente étude, 100 lignées dérivant d'une collection mondiale de 384 accessions provenant de différents pays ont été examinées pour leur architecture racinaire dans des conditions normales et de sécheresse en utilisant la méthode de passoire à pâtes. Cette étude a permis de constater que l'effet du traitement eau n'est pas significatif pour l'architecture du système racinaire indiquant un fort contrôle génétique. Deux catégories de types de racines ont été identifiés : génotypes avec un système racinaire (i) superficiel et (ii) profond. Les mêmes génotypes ont été testés dans les conditions du champ exposé à la sécheresse et ont montré que l'angle des racines étroit (racines profondes) a engendré un avantage en rendement de 38 %. Le stress thermique terminal affecte l'anthèse et le remplissage des grains entrainant une importante diminution du rendement. C'est la raison pour laquelle nous avons exposé une collection de blé dur au stress thermique simulé au moment de la floraison en couvrant les plantes avec les tunnels plastiques pendant deux saisons. Le rendement moyen a été réduit de 54 % en conditions de stress comparant aux conditions témoins et le nombre de grains par épi s'avère le caractère le plus important pour la tolérance de la chaleur.

Toute la collection de blé dur a été génotypée avec 8,173 marqueurs polymorphiques SNPs via 35K Axiom array. Les régions génomiques les plus significatives liées à la fertilité des épis et aux indices de tolérance à la chaleur ont été identifiées sur le chromosome 1A, 5B et 6B. En cas de sécheresse, trois QTLs sur le chromosome 3A, 3B and 7B ont été identifiés liés au rendement. L'analyse de l'haplotype a confirmé que la combinaison des allèles positifs des trois QTLs liés à la tolérance au stress thermique a abouti à un rendement supérieur de 8 % dans les conditions de chaleur de la vallée du fleuve Sénégal. De même, la combinaison d'allèles positifs des trois QTLs liés à la tolérance au stress hydrique a entrainé une augmentation en rendement

de 12 % dans des conditions de sécheresse extrême de Marchouch au Maroc. Un QTL lié à la sécheresse et trois autres liés au stress thermique ont été validés avec succès en marqueurs KASP et peuvent désormais être rassemblés via la sélection assistée par marqueurs (MAS) pour obtenir de meilleures variétés tolérantes aux deux principaux stresses abiotiques.

Mots-clés : Blé dur, sécheresse, chaleur, architecture racinaire, rendement, tolérance, QTLs.

List of abbreviations

AFLP	Amplified Fragment Length Polymorphism
BIC	Bayesian information criterion
BLUEs	Best linear unbiased estimates
BLUPs	Best linear unbiased predictors
CIMMYT	International Maize and Wheat Improvement Center
CTAB	Cetyl trimethylammonium bromide
CV	Coefficients of variation
DAPC	Discriminant analysis of principal components
DNA	Deoxyribonucleic acid
EDF	Effective degree of freedom
FAO	*Food and Agriculture Organisation*
FIGS	Focused Identification of Germplasm Strategy
GLM	General linear model
GWAS	Genome Wide Association Study
ICARDA	*International Center for Agricultural Research in the Dry Areas*
IGC	International Grain Council
IPCC	Intergovernmental Panel on Climate Change
K	Potassium
KASP	Kompetitive Allele Specific PCR
LD	Linkage disequilibrium
LOD	Likelihood of odd
LSD	*Least significant difference*
MAS	Marker assisted selection
Mbp	Megabase pairs
MLM	Mixed linear model
MTAs	Marker-trait associations
N	Nitrogen
NaOAc	Sodium acetate
NH_4NO_3	Ammonium Nitrate
P	Phosphorus
PCA	Principal component analysis
PCR	polymerase chain reaction
PIC	polymorphic information content
QTL	Quantitative trait loci
RCBD	Randomized complete block design
RFLP	Restriction Fragment Length Polymorphism
RNase	Ribonuclease
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeat

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General introduction

Durum wheat, a major source of calories and proteins for more than 4.5 billion people (Shiferaw et al., 2013) is considered an important cereal crop for human diet. Over decades, breeding efforts have largely contributed to a drastic wheat yield increase in many regions around the world. In the 1960's, the introgression of dwarfing gene underlying the reduced height trait into modern cultivars leaded to the "Green revolution", the extraordinary yield increases in the history of wheat. However, during the three last decades, a stagnation in wheat grain yield has been reported (Brisson et al., 2010) and was attributed to high selection pressure leading to a reduction of genetic diversity in elite breeding germplasm (Reif et al., 2005; Brisson et al., 2010). Climate change was also found to be a putative cause for this yield stagnation since wheat production is highly sensitive to the environmental variations. Moreover, several studies predicted an increase in temperature and drought frequency in the upcoming decades (Dai et al., 2012; Lobell et al., 2013; IPCC, 2007, 2014). According to IPCC (2014), the annual precipitation is predicted to decrease by up to 27%, therefore, severe drought is likely to become more frequent. Extreme heat events are also expected to increase in future (Battisti and Naylor, 2009) causing 6% of wheat yield loss estimated for each global temperature increase of 1°C (Liu et al., 2016). This is worrying given that the expanding wheat growing areas is limited and the food demand is becoming high with rapidly growing population. This crop is grown in many countries in the world and in almost all the important wheat growing regions, water deficit and heat are the two major abiotic stresses that occur the most. Both stresses, drought and heat are threatening food security. They constrain durum wheat productivity and result in yield penalty of 60% and 72%, respectively (Sukumaran et al.,2018). Stress tolerance is then a big challenge and critical in the regions affected by those stresses to increase wheat productivity and thus world food supply. Therefore, developing wheat cultivars with high performance and resilience ready to mitigate such environmental stresses is one of the most important objectives of breeding. Understanding the adaptive morpho-physiological traits underlying drought and heat tolerance should enable breeders to make selection based on the useful traits and loci to maintain yield under targeted stress. The objective of the study is to identify the key traits involved in stress tolerance using high-throughput phenotyping methods and study the genetic control of those traits in various durum wheat genetic backgrounds as well as looking for novel source of tolerance to drought and heat stress to be able to design the varietal ideotype. My PhD research was conducted using a worldwide

durum wheat collection containing cultivars, ICARDA and CIMMYT elite breeding lines and landraces selected based on the algorithm for Focus Identification of Germplasm Sources (FIGS; Mackay et al., 2005; Bari et al., 2012).

The durum wheat global demand is dramatically increasing as a consequence of the rapidly growing population. Climate change is also a threat to food security, particularly in the area with an increasing frequency of severe drought and extreme heat events and where durum wheat is mostly grown. However, a constant genetic gain was observed in the last two decades mainly due to low genetic diversity and most of breeding programs rely on selection for yield per se that is a complex and low heritable trait. Using proxy traits with relatively high heritability is then a good approach to select for drought and heat stresses. Reintroducing genetic diversity from distant germplasm is also a cost-effective approach for breeders that could help on developing resilient cultivars.

In the context of the need to improve the tolerance to drought and heat stress of durum wheat, this PhD was established to study and understand the key adaptive traits to those stresses. The work aimed also to dissect the genetics of drought and heat tolerant traits in durum wheat via GWAS using 35k Affymetrix array. Therefore, suitable parent lines could be selected to design better crosses to transfer a relevant variability into a germplasm and the valuable QTLs identified in this work could be then converted to markers for targeted use in breeding programs. The results and insights obtained should contribute to the improvement of ICARDA and Australian germplasm.

We studied drought and heat stresses separately. For drought, we targeted root system focusing more on root architecture and its impact on grain yield under field conditions with different water regimes, whereas for heat stress, on-field response of above-ground traits to high temperature was evaluated at flowering after exposing a durum wheat subset to simulated heat stress. The key aim of drought experiment was to assess the suitability and high throughput of two methods for root traits evaluation, and to use them to investigate the available genetic diversity for rooting pattern. We examined whether root systems respond differently to water availability. Further, yield trials were conducted in different environments with a range of water regimes to evaluate the potential value of shallow or deep rooting systems, and the possibility of incorporating selection for root traits in breeding programs. Finally, Genome-wide association study using SNP markers was used to identify the genomic regions involved in the control of root traits and the effect of the key genomic regions on yield

performance of the durum wheat collection grown under rainfed conditions was terminal drought was investigated. Regarding heat stress study, the objective was to characterize the impact of short reproductive stage heat stress on yield and yield components, to assess associations between the sensitive traits to high temperature and grain production under each environmental condition and finally to identify some new molecular markers linked to QTLs associated with those traits under each environmental condition and heat tolerance indices using GWAS. The allelic effect of the major QTLs on the performance of a large set of durum genotypes grown under environmental heat conditions was then investigated.

Chapter I: Literature review

El Hassouni Khaoula

Unpublished

Literature review

1. Durum wheat features

1.1. Classification and genetic origin

Durum wheat belongs to the tetraploid group of the *Triticum* genus (Feillet, 2000). The botanical classification is presented in table1. The polyploidization has played an important role in the evolution of the *Poaceae* family (Salse et al., 2008), The tetraploid wheat hybridization occurred 10,000 years ago and believed to have been naturally occurred between *Triticum urartu* (A genome donor) and *Aegilops speltoides* (B genome donor) resulting in *Triticum dicoccoides* (wild emmer). The latter has been domesticated to *T. dicoccum* that is probably the direct ancestor of *T. durum* (Bennici, 1986).

Table 1. Durum wheat classification (FNA Ed. Comm., 2007; Feillet, 2000)

Class	*Equisetopida*
Subclass	*Magnoliidae*
Superorder	*Lilianae*
Order	*Poales*
Family	*Poaceae*
Subfamily	*Pooideae*
Tribe	*Triticeae*
Subtribe	*Triticinae*
Genus	*Triticum*
Species	*Triticum durum*

The assumed center of origin of tetraploid wheat is the Fertile Crescent region extending from south-western Iran, through northern Iraq and south-eastern Turkey to central Syria, Palestine, Israel and Jordan (Feldman, 2001). Since, it has been spreaded worldwide and thus, diversified in terms of adaptation through mutations and many other natural phenomenon (Feldman, 2001).

1.2. Description and growth cycle

Durum wheat, tetraploid wheat ((2n = 4x = 28; AABB) is annual plant that belong to the monocotyledon group. It is characterized by soft loose glumes, a free-threshing grain

(Zohary and Hopf, 2000) compared to ancient wheat varieties with thick husk around the grain. It has usually bearded heads and stems are hollow, relatively tall and solid. The grain is long, translucent, amber in color and hard (Peterson, 1956).

Durum wheat growth can be divided into four phases: Emergence/tillering, stem extension/booting, heading/flowering, and kernel formation/ripening (Figure 1). Plants emergence occurs about one week after planting (Zadoks 11), and leaves begin to develop on the mainstem. The next stage is tillering that starts when the fourth leaf appears (Zadoks 21). The stems extend, and the plant grows taller in the first phase of reproductive growth. The first node starts to appear and as the stem continues to develop, several joints may appear. The flag leaf appears then at the top of the stem (Zadoks 37). The boot stage occurs when the stem containing the grain head swells.

After that, the heading phase of development starts by the emergence of the first spikelet until the grain head fully emerge from the stem (Zadoks 58). One week later the flowering begins.

Durum wheat is an autogamous self-pollinated crop and grain kernels begin to form just after pollination. Dry matter starts accumulating in the kernels during the ripening stage from the milk, the soft dough, hard dough to maturity. When the grain become very hard and the moisture level of the kernels drops to14 %, the grain is harvest ripe (Zadoks et al., 1974; Weisz, 2013).

The establishment and growth of wheat roots include primary and secondary root systems occurring in two successive phases. The roots that appear the first are seminal roots. They grow from the seeds and support the plant until tillering stage when the secondary roots, also known as adventitious or nodal roots appear at the base of tillers (Tottman, 1987). The whole root system of wheat can reach 2m depth in normal field conditions by the end of the growth cycle (King, 2003).

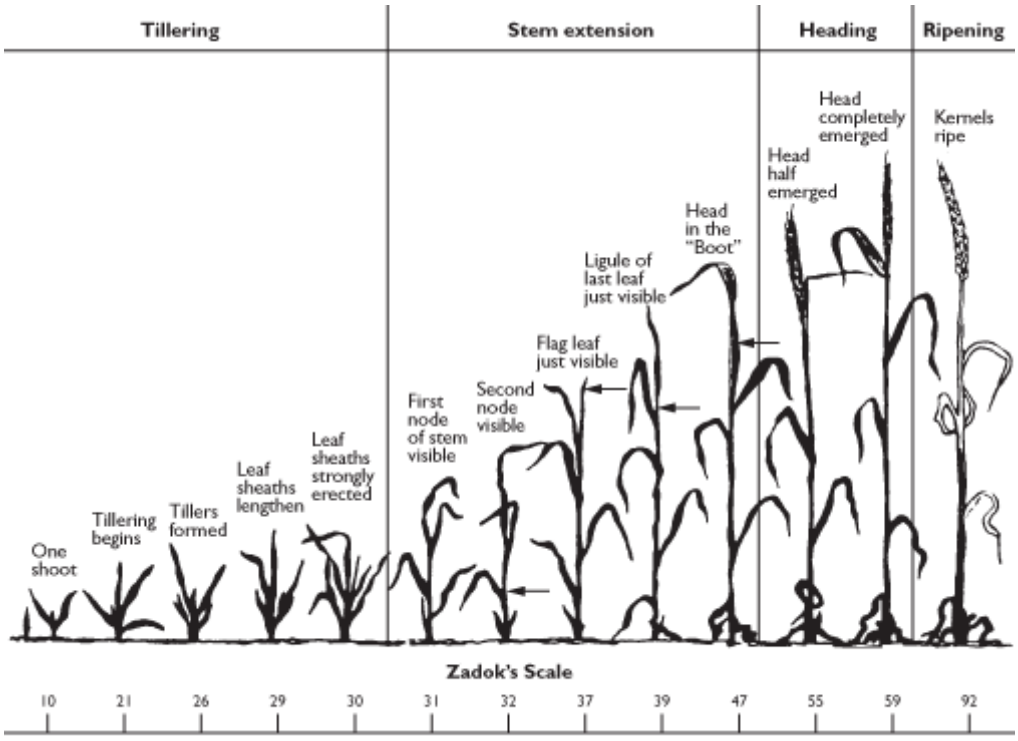

Fig. 1. Illustration of wheat growth and development expressed at Zadoks scale. (Zadoks et al., 1974; Tottman, 1987).

1.3. Importance, production and different uses of durum wheat

Durum wheat (*T. durum*) is one of the oldest and most important wheat species in the world (Royo et al., 2009). It is an important crop for human diet and animal feed particularly in the Mediterranean basin. Durum semolina and flour are used for making pasta and other food products such as couscous, freekeh, burgul, industrial and artisanal bread making, cake making, biscuits and various other uses. In addition, this wheat has a high nutritional value providing calories and proteins (Braun and Payne, 2012). It contains water, carbohydrates, proteins, minerals, fibers … Therefore, durum wheat is considered as the main staple food worldwide and a valuable commodity.

This crop is almost grown under rainfed conditions (Bennacci, 1986; Araus et al., 2002). It is grown about 17 million hectares in the world, equivalent to 5 % of the wheat cultivated acreage. It is more concentrated in the Mediterranean basin (Italy, Spain, Morocco, Algeria, Tunisia, Turkey and the Middle East) where it is considered the most substantial component of nourishment and accounts for about 60 % of the total area cultivated with this species.

The global production of durum wheat has increased recently and reached 39.4 million tonnes in 2017-2018 according to the IGC (International Grain Council, IGC Grain

Market reports) with the E.U., Canada, Turkey, Mexico, the United States, Morocco, Algeria and Kazakhstan being major producers (Figure 2). However, this production has in the past and continues to face many environmental constraints.

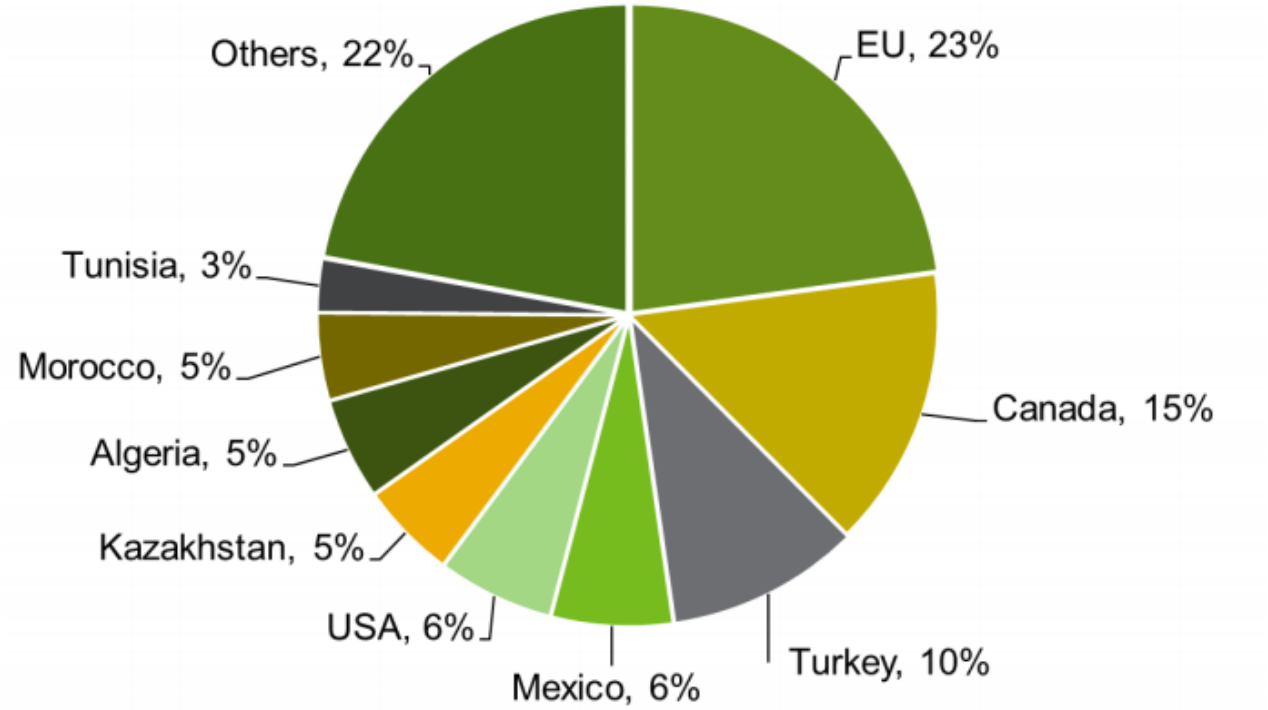

Fig. 2. Durum wheat production by region. (IGC, 2017)

1.4. Challenges associated with durum wheat production

The demand for cereals, including durum wheat is predicted to increase by approximately 50 % in the upcoming years (Borlaug and Dowswell, 2003), due to the expected growing of the world population and thus their needs for food and feed. Therefore, a significant increase in durum wheat production is crucial and will be required to meet the projected demands. Durum wheat crop is mainly affected by many environmental factors limiting its production such as water scarcity and high temperature, two main abiotic stresses found to have the highest negative impact on crop production that lead to a significant reduced yield (Lesk et al., 2016; Cattivelli et al., 2008; Talukder et al., 2014). As a result of climate change, the incidence of extreme environmental conditions namely severe drought and the high temperature are predicted to increase in most durum production regions worldwide (Christensen et al., 2007; Carvalho et al., 2014; Battisti and Naylor, 2009) posing a considerable threat to food security. It has recently been estimated that for each 1°C of temperature increase,

6 % of global wheat production will be reduced (Asseng et al., 2015). Drought was also found to cause a significant yield loss (Prasad et al., 2011; Dodig et al., 2012). To tackle these challenges, improved varieties with stable and high performance and good resilience under a range of environmental conditions including drought and heat stresses is a priority to reduce the negative effect on yield and thus minimizing yield variability and production fluctuations (Cattivelli et al., 2008).

2. Breeding for grain yield improvement in durum wheat

2.1. Characterization of the two major abiotic stresses: drought and heat

Drought stress is considered when a hydrologic imbalance occurs, and the water extracted by the plant is less than the amount of water lost via transpiration (Reddy et al., 2004) mainly due to precipitation shortage affecting crop production, whereas heat stress is defined as an increase of temperatures exceeding the plant optimum temperature for growth causing serious damages (Farooq et al., 2011).

Drought and heat are two major abiotic stresses that occur in the most durum wheat production regions worldwide. In particular, in the Mediterranean basin which is considered one of the future hotspot regions for these climatic events that constraint durum grain productivity. The frequency of severe drought and heat stresses are tended to increase (Lesk et al., 2016). Rainfall patterns are predicted to decrease (Araus et al., 2002; Christensen et al., 2007; Carvalho et al., 2014), while environmental temperatures are projected to increase (Battisti and Naylor, 2009; Hansen et al., 2015) by the end of this century in the course of climate change. The major decrease in durum wheat production in many parts of the world is very likely due to severe drought and or extreme heat events during reproductive development (Wheeler, 2012).

2.2. Impact of water deficit and high temperature stresses on durum wheat production

Drought and heat stresses are globally two major environmental factors that affect negatively the crop development resulting in many physiological changes; i.e. photosynthesis reduction, chlorophyll content, grain number, grain weight, grain yield (Prasad et al., 2011; Pradhan et al., 2012; Suzuki et al., 2014; Perdomo et al., 2017). These alterations depend on the intensity (difference between the stressed and optimum conditions), duration (extent of the stress) and timing (temporal distribution)

of the event, the crop species and the genotype *per se*. To evaluate the impact of those stresses based on the indicators abovementioned, we usually compare the measured values under stress to the values under non-stress conditions. Many studies reported the negative impact of drought and/or heat stress on plant growth and development, yield and yield components (Saini et al., 1983; Kumar et al., 2007; Pinto et al., 2010; Suzuki et al., 2014). Drought was reported to affect negatively the grain yield by up to 77% (Pinto et al., 2010; Prasad et al., 2011; Xue et al., 2014) and 59 % reduction by heat (Pinto et al., 2010). However, the severity and damages are worse when these two abiotic stresses occur simultaneously than when they are taken one by one (Rizhsky et al., 2002; Prasad et al., 2011; Vile et al., 2012).

Both stresses affect the wheat plant from germination to maturity, but the reproductive phase in all these events is referred to be the most critical stage of many cereal crops including durum wheat (Wardlaw and Wrigley, 1994; Loss and Siddique, 1994; Royo et al., 2010; Wheeler, 2012; Slafer, 2012; Bassi and Sanchez-Garcia, 2017; Sukumaran et al., 2018). Heat stress during anthesis and grain filling results in a significant reduction on grain number and weight (Wollenweber et al., 2003; Dias and Lidon, 2010; Dolferus et al., 2011) due to floret sterility (Saini and Aspinall, 1982) and decreasing starch biosynthesis. Saini et al. (1983) reported that High temperature during this stage has a high impact on both male (pollen) and female (embryo sac) fertility. It was also reported that heat shocks have a negative effect on grain quality through changes in protein composition (Corbellini et al., 1998). Drought stress also affects the reproductive organs when it coincides with anthesis and thus reduce the grain size and weight by limiting the remobilization of assimilates (Tardieu, 2006). Drought and heat stresses have also an impact on root development. Water deficit limit root growth by reducing water uptake through modification in the soil physical properties (Lucas et al., 2000), whereas High temperature has a negative effect on root growth by decreasing number and length due to alteration in the source-sink relationship, i.e., competition for assimilates.

2.3. Improvement of tolerance to drought and heat stress

Durum wheat production worldwide and more specifically in Mediterranean region where it is widely grown is mainly affected by water scarcity and high temperature (Loss and Siddique, 1994). It is also threatened by climatic fluctuations and an increasing frequency of extreme drought and heat stresses (Lesk et al., 2016).

Therefore, the urgency to advance durum wheat productivity under these conditions by breeding for tolerant, resilient and stable varieties, has increased attention on understanding adaptive traits affecting yield increase. Selection for yield per se has contributed to significant yield improvement. However, genetic gain has slowed (Fisher and Edmeades, 2010) due to the fact that yield trait is controlled by complexes of genes.

Physiological and morphological traits that confer drought tolerance can be related to two major processes: i) an increase in water absorption which is controlled by root growth, osmotic adjustment and related solutes and membrane stability; ii) a decrease in transpiration, which depends on atmosphere, stomatal conductance and transpiration efficiency (FAO, 2002). Several studies underline the importance of below-ground traits (root architectural traits) in drought environment as roots represent the first interface for nutrient and water uptake (Waines and Ehdaie, 2007; Mace et al., 2012). The access to the available water at depth where the top-soil profile is dry is of paramount importance in such environment (Reynolds et al., 2007; Manschadi et al., 2008). Root length and root depth are significantly correlated to yield increase. The ability to extract and use water during post-anthesis phase through deeper root system lead to an increase in grain yield as was reported in wheat (Manschadi et al., 2006) and sorghum (Borrel et al., 2014). Root angle has been associated with root depth in different cereal crops such as rice (Kato et al., 2006; Uga et al., 2013), sorghum (Mace et al., 2012) and wheat (Manschadi et al., 2008). Such root characteristic is one of the most important component of drought tolerance and thus highly desirable to better perform in water limited environments. Above ground traits have been largely investigated and the three yield components: number of spikes per unit of growing area, the number of grains per spike and the grain weight per spike were found to be the drivers that can be used to improve tolerance of wheat to different abiotic stresses, particularly heat stress (Moragues et al., 2006). In wheat during grain filling, the carbohydrates accumulated in the stem and leaves are remobilized into the grains. It was reported that 40% of the grain filling depend on this translocation when the plant is under heat stress and water limited environment (Royo et al., 1999). Genetic variability for all the adaptive traits related to improvement of tolerance to drought and heat stress is at the base of the breeding to increase durum wheat productivity. During the last two decades, a drastic reduction of genetic variability was observed within wheat breeding gene pools (Longin et al., 2012) due to selection pressure in breeding

and intensive germplasm exchange between breeding programs (Reif et al., 2005). A potential approach for genetic enrichment of breeding pools could be crosses from completely different environment (Whitford et al., 2013) to bring advantageous characteristics from many lines that are adapted to specific target environments (Lopes et al., 2015). Another strategy could be exploiting wild relatives and primitive wheats to restore genetic diversity to modern breeding lines (Longin and Reif, 2014). Some wild species of wheat have been already identified to have adaptive traits to several abiotic stresses including drought (Feldman and Millet, 1993; Trethowan and Mujeeb-Kazi, 2008; Trethowan, 2014; Nachit et al., 2015). To exploit the diversity, a genotyping of a highly diverse and large germplasm is required (Massawe et al., 2016) combined with precise phenotyping to better choose a targeted accession for pre-breeding populations (Longin and Reif, 2014). Today, genotyping is getting more developed, while phenotyping tools remained the bottleneck for traits characterization in breeding programs (Yang et al., 2013). Despite the sensitivity to environmental conditions, high-throughput phenotyping methods are of great help in physiological breeding to identify precisely different mechanisms for each climatic scenario. During the last three decades genotyping tools have seen significant advances enabling a rapid genetic characterization (Mir et al., 2013) using high-throughput sequence variation Single Nucleotide Polymorphism (SNP) markers that became the most used markers in genomics replacing the initial and classical markers like Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR). Different genotyping platforms are used in wheat plant breeding (Bassi et al., 2016) and they are summarized in Table 2. The genotypic data generated can be used for various purposes such as genetic diversity analysis, identifying genetic variation associated with a trait of interest and genomic selection. To identify the genomic regions with important effect on desirable traits, genome -wide association study is one of the best approaches allowing the association between phenotype and genotype of large collection of unrelated genotypes. This approach has received increasing attention and recently become a powerful approach to map genes or quantitative trait loci (QTL) in plants mainly due to its high resolution, broader allele coverage and cost effectiveness (Edae, 2013). This method is suitable for plant breeders to incorporate the valuable QTLs responsible of the trait of interest into their breeding programs to improve crop production.

Table 2. Comparison of genotyping platforms in wheat. (source: Bassi et al., 2016)

Platform	Number of markers[a]	Cost per individual (US $)[b]	Advantage	Disadvantages	Provider
KASPAr	90	1	No missing data Co-dominant Scalable number of markers	Bi-allelic	LGC Genomics
Illumina 15K	10,000	35	No missing data Co-dominant	Bi-allelic	Various
Genotyping by Sequencing	10,000	12	Multi-allelic Co-dominant	Lots of missing data Massive data	Universities
DArT Seq	10,000	25	Multi-allelic Co-dominant	Missing data	Triticarte
Illumina 90K	15,000	50	No missing data Co-dominant	Bi-allelic	Various
Axiom 35K	20,000	50	No missing data Co-dominant	Bi-allelic	Affymetrix
Axiom 850K	300,000	250	No missing data Co-dominant	Bi-allelic Massive data	Affymetrix

a: Expected number of polymorphic markers based on literature. b: Derived from quotes received in the past 12 months; each provider might change the price based on the population size or established collaborations. These estimates do not include the cost of DNA etraction nor of shipping plates to providers.

3. Molecular markers and genotyping

3.1. Single nucleotide polymorphisms (SNP) markers

SNPs (single nucleotide polymorphisms) belong to the last-generation molecular markers and exist all over the genome. SNP is a bi-allelic marker and defined as single base pair variation among individual samples in a population or group of genotypes (Wang et al., 1998; Brookes, 1999). Thousands of SNPs can be analyzed simultaneously by application of DNA microarrays. Therefore, using modern technologies, the effectiveness of SNP analysis can be many times higher than that of other methods of DNA analysis (Khlestkina and Salina, 2006).

3.2. Genome Wide Association Study (GWAS)

The progress of a breeding program relies on the use of polygenes because useful agronomical and physiological traits are generally controlled by multiple genes. In crop breeding, the detection of useful genes is challenging (Pasam et al., 2012). A quantitative trait locus (QTL) is a region in the genome that is associated with a quantitative trait. A variety of molecular markers are widely used to tag genes or to identify genomic regions associated with desirable traits. After developing high density single nucleotide polymorphisms (SNPs), genome wide association studies (GWAS) became a major tool for QTL detection over linkage mapping.

GWAS is a powerful approach for explaining associations between genotype and phenotype based on LD (Reimer *et al.* 2008). This method, offers the opportunities for fine mapping that are difficult to achieve through linkage analysis (Mackay and Powell

2007). Therefore, it facilitates the gene discovery and lead to an efficient marker assisted selection in the breeding programs. The advantage of GWAS is that marker trait associations can be studied in a collection of unrelated individuals for several traits and multiples alleles per locus can be evaluated. One of the major drawbacks of the method is that when the diversity panels and populations exhibit high levels of population substructure and diverse levels of familial relatedness among individuals, spurious associations can occur (Atwell et al., 2010; Varshney et al., 2012).

3.3. KASP (Kompetitive Allele Specific PCR)

Kompetitive Allele Specific PCR is a fluorescence-based genotyping assay of polymerase chain reaction. This method enables bi-allelic scoring of SNPs at specific loci. It consists of two competitive allele-specific primers and one common reverse primer. The KASP technology is suitable for use on several equipment platforms and provides flexibility in terms of the number of SNPs and allow to analyze a large number of samples. The KASP chemistry functions has been used over many years in genetics in large and small laboratories. The KASP genotyping follows six steps starting by preparing the array DNA samples into the reaction plate, preparing the KASP genotyping mix, dispense genotyping mix onto the reaction plate, then seal and centrifuge the plate to run the thermal cycle and finally read the plate and analyse the data (He et al. 2014)

The KASP assay has many advantages including: accuracy and performance, tremendous flexibility where this technic supports from low to high-throughput studies and individual repeat assay and needs only the fluorescence reader and qPCR. Another advantage is the breakthrough cost saving where KASP doesn’t need the expensive labelled assay-specific primers or probes, requires small quantity of DNA sample per each SNP and low reagent volumes and cost. The KASP genotyping technology is based on the simple PCR equipement.

Chapter II: Materials and methods

El Hassouni Khaoula

Unpublished

Materials and methods

1. Core collection design

A large collection of 1,500 durum wheat accessions was assembled at the field station of the International Center for Agricultural Research in the Dry Areas (ICARDA) in Terbol, Lebanon. The collection was characterized with 10 single nucleotide polymorphisms (SNPs) associated to known genes and was assessed for similarity in flowering time, response to toxic level of boron, disease response, lodging and visual selection to define a core subset. Genotypes including elites, cultivars and landraces. The subset of 384 was selected to be similar in phenology and diverse for all other traits. It includes 96 durum wheat landraces from 24 countries and 288 modern lines from nine countries and two International research centers CIMMYT and ICARDA. The landraces were selected on the basis of the algorithm for Focus Identification of Germplasm Sources (FIGS; Mackay et al., 2005; Bari et al., 2012; Anglin et al., 2018) targeting the model to identify sources of resistance to different diseases and tolerance to major abiotic stresses such us drought and heat. The FIGS approach considers the presence of a potential relationship between the environment under which landrace grows and specific adaptive traits (Anglin et al., 2018).

The set used to convert Axiom markers into KASP and validate them included 94 ICARDA's elite lines that constituted the 2017 international nurseries 40th International Durum Yield Trial (IDYT) and 40th International Durum Observation Nurseries (IDON). This set was tested at the experimental station of Marchouch for drought experiment and at the station of Kaedi along the Senegal River for heat study.

2. Phenotypic evaluation for tolerance to drought and heat stress

2.1. Measurement of root traits

- ***Clear pot method***

Durum wheat genotypes were phenotyped for seminal root angle (SRA) using Clear pot method which is suitable for screening small grain crops (Richard et al., 2015; Robinson et al., 2016; Alahmad et al., 2018) (Figure 1). Clear plastic pots (ANOVApot ® 200-mm diam., 190-mm height) were filled with peat moss soil known for its high water and nutrient-holding capacity. Seeds were sown according to the Richard et al. (2015) method. A randomized complete block design (RCBD) was adopted, where each 4L pot containing 24 seeds was considered a block. Pots were placed on the

bench in a distinct column/row grid according to the design. Using forceps, seeds were sown carefully with the embryo facing downward the wall of the pot to allow enhanced visibility of the seminal roots following germination. Plants were grown in the glasshouse under diurnal natural light conditions and constant temperature (17 ± 2 °C). Images were captured 5 days after sowing (seminal roots 3–5 cm in length) using a digital camera. The images were analyzed for SRA, where the angle between the first pair of seminal roots emerging from the seed was measured from the images using online free software ImageJ (http://imagej.nih.gov/ij/).

Fig. 1. Durum wheat seedlings phenotyped for seminal root angle using clear pots. **(a)** seedlings sown in clear pots and grown under controlled conditions. **(b)** clear pots inside black pots to avoid the light. **(c)** image of plant root in clear pot taken using digital camera.

- ***Pasta strainer method***

'Pasta Strainer' method was used to evaluate mature root system traits of durum wheat (Figure 2). The space-planted field conditions experiment was performed in the field, in sandy soil (silt loam 0–20 cm, sandy loam 20–40 cm) under the protection of a net house. Plastic pasta strainers (height = 11 cm, diameter = 20 cm) were filled with sandy soil and buried at 11-cm depth. Strainers were placed with 20 cm distance between each other to ensure 40-cm spacing between plants, to avoid barriers to the growth of the root system in all three dimensions. Three seeds were placed in the middle of each strainer representing one genotype. At the four-leaf stage (growth stage 14, according to the Zadoks decimal growth scale; Zadoks et al., 1974), the three seedlings were thinned, retaining the most vigorous plant. During the growing season, standard cultural practices were used, with 150 kg ha−1 of N, P, and K incorporated in the soil before planting, followed by 50 kg ha−1 of NH4NO3. Cherokee fungicide (1.5 L ha−1, Syngenta, chlorothalonil-cyproconazolepropiconazole) was applied to prevent development of fungal diseases, and Pirimor (500 g ha−1, Syngenta, pyrimicarbe) was applied to control aphid infestation. Weeds were controlled by two applications of tank mixture of Mustang (0.6 L ha−1, DOW Agrosciences, 2,4-D florasulame) and Pallas

(0.5 L ha−1, DOW Agrosciences, pyroxulame), with additional mechanic weeding. The experimental design was an α lattice. Before maturity, the number of fertile spikes (SN) and tillers (TN), as well as the number of spikelets on each spike (SPN), was recorded for each plant, together with the plant height (PLH) excluding the awns. In addition, a relative surrogate for the chlorophyll content was measured using a chlorophyll meter (Konica Minolta SPAD 502). At maturity, the shoot was cut 3 mm above the soil and weighed to determine the dry shoot biomass (SB). The spikes were then threshed to determine the grain weight (GW), and the weight of 1000 kernels (TKW) was determined using a precision balance. The stay-green trait was calculated as the days elapsing between the heading and maturity dates. The belowground traits were recorded by first removing the strainer from the soil using a shovel. The strainer was then divided into three sections: an upper Layer 1 (2–8 cm), a middle Layer 2 (8–10 cm), and a lower Layer 3 (10–13 cm). The three layers marked on the strainer corresponded to the angles from the horizontal ground level of 0 to 30, 30 to 60, and 60 to 90°, respectively. The number of roots protruding from the holes in the sides of the plastic container were counted for each level, and the number of roots for each layer was then expressed as a ratio of the total root number (TRN), resulting in three root ratios (RR 2–8 cm, RR 8–10 cm, and RR 10–13 cm). The root sections protruding from the plastic container were cut off to leave an exact volume of soil and roots corresponding to the volume contained inside the pasta strainer, equivalent to 2100 cm3. The sandy soil was then gently removed from the container, avoiding damaging the roots in the process. The type of soil used was ideal for this task, as its loam content prevented loss through the holes of the strainer until a small pressure was imposed, whereas its sandy nature facilitated the task of removing the soil without damaging the roots. Roots were then rinsed in water to remove any remaining sand and allowed to dry for 10 d. The dry roots were first weighed to obtain root biomass (RB) and then scanned using an Epson Perfection V700 scanner. This image was analyzed with ImageJ to measure the root angle (RA) (Figure 3), setting the center of the angle in the middle of the crown and the two extreme sides of roots as the final point of the angle. All roots angles were visually controlled to prevent errors.

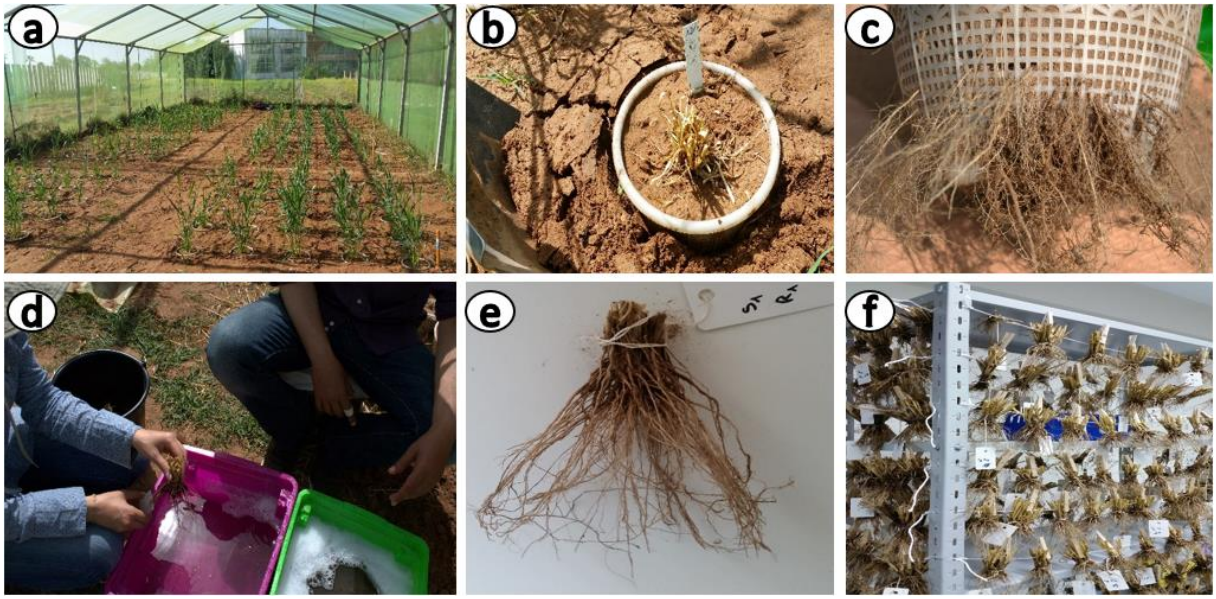

Fig. 2. Root phenotyping using 'pasta strainer' method. (a) Durum wheat plants grown in pasta strainers buried in the soil. (b) strainer removed from the soil using a shovel. (c) Roots emerging from different sections of the plastic basket. (d) rinsing roots in water and soap to remove the remaining soil. (e) Clean root crown (f) novel system allowing roots to dry with conservation of the root shape.

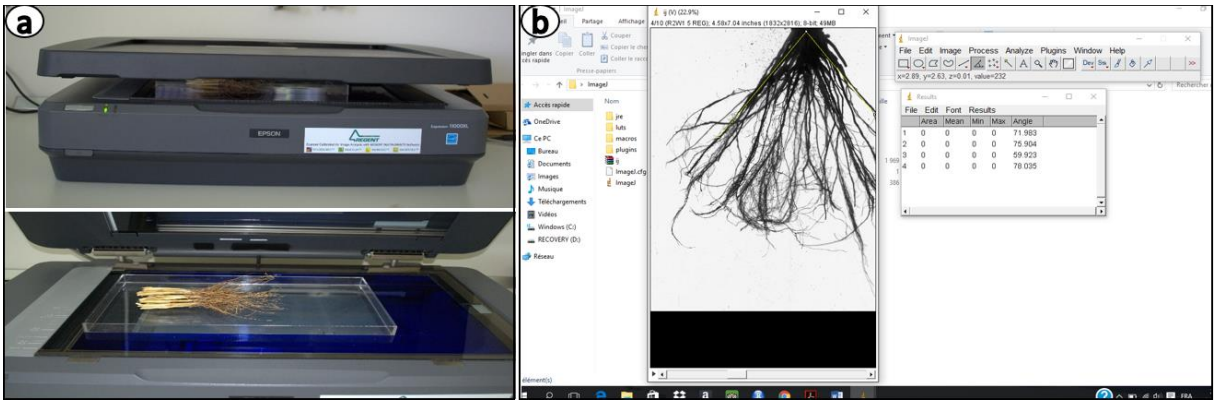

Fig. 3. Measuring mature root angle. **(a)** scanning roots using Epson Perfection V700 scanner. **(b)** measuring images for root angle using ImageJ software.

2.2. Screening for heat tolerance

Each entry is sown on a plot surface of 1.5 m^2 per genotype at a sowing density of 300 plants per m^2. The experiment was an alpha lattice with two replications, block size of six, and two treatments arranged in split-plot. Each six genotypes were arranged in close proximity to maximize competition between the genotypes, and compose one block of 9 m^2. Each block was surrounded by a border of barley to avoid border effect. Each block was spaced 1 m apart to allow the application of the plastic tunnel (figure 4). The two treatments were normal rainfed conditions and plastic tunnel-mediated

heat stress. The normal treatment followed standard agronomic practices with a base pre-sowing application of 50 Kg ha^{-1} of N, P, and K. At stage 15 of Zadok's (Z) scale herbicide was applied in a tank mixture (Pallas + Mustang at 0.5 L ha^{-1}) to provide protection against both monocots and dicots. At Z17 ammonium nitrate was provided to add 36 kg ha^{-1} of N and a final application of urea was used to add 44 kg ha^{-1} of N before booting (Z39). Weeds were also controlled mechanically to ensure clean plots. The heat-stress treatment followed the same agronomic practices, with the difference that at the time of booting (Z45) a 10 m^2 and 1.5 m high plastic tunnel was placed over each block and left there until early dough stage (Z83) (figure 4). An electronic thermometer (temperature data logger) was placed in the middle of each block (normal and heat stressed) to reveal that the temperatures were up to 16 °C higher inside the plastic tunnels, to reach a maximum of 49 °C.

The following traits were recorded: days to heading (DTH) measured at the moment when the awns became visible, plant height (PH) measured from the ground to the top of the highest spike excluding the awns, and the number of fertile spikes per meter square (Spkm^2) was counted in a 0.25 m^2 area. The whole plot was harvested by hand and the dry biomass (Biom) was weighed before threshing. Grain yield (GY) was weighed for each plot and expressed as kg ha^{-1}. The weight of a thousand kernels (TKW) was expressed in grams. The harvest index (HI) was calculated as the ratio between GY and Biom. The grain number per spike (GNSpk) was derived from dividing grain number per meter square by Spkm^2.

Fig. 4. Heat stress experiment. **(a)** normal conditions. **(b)** Plastic tunnel-mediated heat stress.

2.3. Field yield trials

For drought study, field yield trials containing the whole collection of 384 genotypes were conducted in five locations. The trials were carried out using an augmented design of 19 blocks of 24 plots, each block with four commercial checks. Sown plot

size was 3.6 m^2 (six rows), and 2.4 m^2 (four rows) were harvested for assessing yield performances. The five environments included two rainfed environments with strong terminal droughts (Marchouch in Morocco and Kfardan in Lebanon) and three irrigated environments. The irrigated environments were Terbol in Lebanon, where supplemental moisture was provided via three sprinkler irrigations; Melk Zehr in Morocco, where drip irrigation was used to provide the majority of the moisture; and Tessaout in Morocco, where nearly all in-season water was provided via gravity-fed irrigation. Optimal agronomic management practices were applied in all environments. For the heat study, two sets were field tested in Kaedi, Mauritania where the temperature reached a maximum of 41 °C and an average maximum daily temperature of 34 °C throughout the season. The trial was carried out under augmented design with a plot surface of 4.5 m^2. Standard agronomic management practices were adopted.

3. Genotyping

3.1. DNA extraction and quantification

Extraction of DNA from the durum wheat lines was done at ICARDA-Egypt. DNA was extracted from leaf samples using a standard cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987) as described below.

Leaf material were collected and put in the 2 ml tubes with stainless steel beads. The material was put into a Retsch Mill (Retsch, Haan, Germany) for grinding to fine powder for ten minutes. 1000-1200 µl of CTAB buffer was then added to each tube following incubation for 60 to 90 minutes in a warm water bath at 65°C with gentle swirling and then allowed to cool down at room temperature. 600 µl of chloroform:isoamylalcohol (24:1) were added to each tube with gentle shaking by inversion for about five minutes. The tubes were then centrifuged for ten minutes at 10000 rcf to form two distinct phases. 800 µl of the uppermost aqueous phase were pipetted into new 1.2 ml tubes. After adding of 300µl of chloroform:isoamylalcohol (24:1) the samples were centrifuged for ten minutes at 3500 rcf. Again, 400µl of the top aqueous phase were pipetted into new tubes containing 10 µl RNase A (1.2mg/ml), incubated at room temperature for eight minutes following mixing by gentle inversion and another incubation at room temperature for 30 minutes. 400 µl of isopropyl alcohol were then added, mixed by gentle inversion and centrifuged for eight minutes at about 600 rcf. After discarding the supernatant, 100 µl of a 76% ethanol/10 mM NaOAc solution were added, gently mixed for 5 minutes and centrifuged for eight minutes at about 600 rcf. After discarding the

supernatant, the DNA pellet was let to dry overnight and dissolved in 100 µl ddH2O or 0,1 x TE buffer. Lastly, for complete dissolution of the DNA pellet the samples were mixed for a few hours following determination of DNA concentration. The DNA quantification (NanoDrop® ND-1000 Spectrophotometer, NanoDrop Technologies Inc., USA) was conducted using 2 µl of DNA sample. The ratio of absorbance at 260nm and 280nm was used to assess the purity of DNA. A ratio of ~1.8 was accepted as pur.

3.2. Single nucleotide polymorphisms (SNP) markers

The collection of 384 accessions was genotyped by 35K Affymetrix Axiom wheat breeders array at Trait Genetics (Gatersleben, Germany) following the manufacturer instructions. This array was developed by choosing tags of proven high polymorphism when tested on modern bread wheat elites, among the 817k SNP Axiom HD platform. The quality filtering was applied to all 35K co-dominant SNP markers based on several quality metrics included in the genotyping report. The marker data was re-formatted to a commonly used format where −1 and 1 denote homozygous marker alleles whereas 0 denotes heterozygous allele calls. 7652 high-fidelity polymorphic SNPs were obtained, showing less than 1% missing data, minor allele frequency (MAF), i.e. the frequency of the least common allele present in the collection, higher than 5%, and heterozygosity less than 5%. The sequences of these markers were aligned with a cut-off of 98% identity to the durum wheat reference genome (Maccaferri et al., 2019) (available at: http://www.interomics.eu/durum-wheat-genome), to reveal their physical position.

4. Statistical analysis

4.1. Phenotypic analysis

Many different methods have been used to analyze data. One of the most powerful statistical methodologies used to capture the variation in the field experiments was the general linear mixed model using the residual maximal likelihood (REML) approach. It provides a powerful method to analyze any linear model with or without covariates (Gilmour et al., 1995; Smith et al., 2001, 2005; Gilmour et al., 2009).

For the first root experiment, a mixed linear model was formulated in accordance with the experimental design to obtain best linear unbiased estimates (BLUEs) for all traits, where genotype, treatment, and year were considered as fixed effects and replication and block as random effects nested in treatment and year. The model was fit using the

lmer function of the lme4 package (Bates et al., 2015).

For the second root experiment with larger number of genotypes spatial analysis were conducted. For the ‘clear pot’ method, a two-dimensional autoregressive (AR1×AR1) model was fitted to the experimental data in ASReml-R library (Butler et al., 2009) to account for the spatial variation in the glasshouse. To obtain BLUPs the genotype, replicate, pot, and position were fitted as random terms. For the ‘pasta strainer’ experiment, a mixed model with a two-dimensional P-spline basis was fitted to the data to account for the spatial trend in the space-planted field conditions. The best linear unbiased predictors (BLUPs) were obtained considering genotype and block within replicate as random effects and the replicate as fixed term. The model was fitted in R using SpATS library (Rodriguez-Alvarez et al., 2018).

The relationships between the different traits were evaluated using Pearson correlation coefficients using the corrplot package (Wei and Simko, 2017).

Principal component analysis (PCA), Fisher LSD and hierarchical clustering of genotypes using the average method were used for root classification. To assess the differences between the average performances of the different root classes, a Tukey test was performed for all pairwise comparisons.

For the heat experiment, a mixed linear model was run using the lme4 package in R to obtain best linear unbiased estimates (BLUEs) of the normally distributed traits. For count traits (DTH, Spkm2, GNSpk), the generalized mixed linear model was used to get the BLUEs by Proc GLIMMIX in SAS. In both models, genotype, treatment, year, and replication were considered as fixed effects and block as random effect Broad-sense heritability was estimated based on a random model as the ratio between the genotypic and phenotypic variance (Falconer and Mackay, 1996). Phenotypic variance was calculated using the method suggested by DeLacy et al. (1996).

The relationship between the target trait grain yield and yield components was studied using the Pearson correlation coefficient and the additive regression model. The additive model incorporates flexible forms (i.e., splines) of the functions to account for non-linear relationship contrary to linear regression model estimated via ordinary least squares (Wood, 2017). For the additive model, the effective degree of freedom term determines the nature of the relationship between the predictor and the response variables where EDF = 1 indicates linearity and EDF > 1 the non-linearity. The additive regression analysis was performed using the mgcv package (Wood et al., 2016).

4.2. Genetic analysis

4.2.1. Structure and linkage disequilibrium

Population structure analysis was used to assign individuals to subpopulations. Details of Structure analysis have been discussed in Kabbaj et al. 2017. Briefly, to derive separate structures for genotypes based on phenotypic and marker data, polymorphic information content (PIC) was calculated and the discriminant analysis of principal components (DAPC) was performed. The value of k was tested from 2 to 50 and the number of clusters was determined as the value of k above which Bayesian information criterion (BIC) values decreased. Analysis of admixture by kinship was performed using software STRUCTURE v2.3.4 (Pritchard et al., 2000) using 50,000 burning periods and 10,000 replicates.

Linkage disequilibrium (LD), the non-random association of alleles at different loci, plays a vital role in association mapping and determines the resolution of association study (Flint-Garcia *et al.* 2003). LD was calculated as squared allele frequency correlations (r^2) in TASSEL V 5.0 software (Bradbury et al., 2007), using the Mb position of the markers along the bread wheat reference genome. Linkage disequilibrium (LD) decay was estimated and plotted using the “Neanderthal” method. The LD decay was measured at 51.3 Mb for $r^2 < 0.2$ as presented in Bassi et al. (2019).

4.2.2. Genome Wide Association Study (GWAS)

The GWAS was performed for all the significant traits. For the two studies, two models were fitted and compared using two covariate parameters, Q (population structure) and K (Kinship). Q model was performed using a general linear model (GLM), and Q + K model using a mixed linear model (MLM). The best model for each trait was selected based on the quantile-quantile (Q-Q) plots (Sukumaran et al., 2012). Flowering time (DTH) was used as covariate in all analyses to remove the strong effects of flowering genes from the study. The value calculated for the LD decay of 51.3 Mb indicated that this association panel interrogated the 12,000 Mb of the durum wheat genome via 248 “loci hypothesis,” and hence the Bonferroni correction for this panel was set to 3.1 LOD for $p < 0.05$ as suggested by Duggal et al. (2008). Local LD decay for $r^2 < 0.2$ was calculated for a 100 Mbp window around the marker with highest LOD for all marker-trait associations (MTAs) identified at a distance inferior to 104 Mbp (twice the LD decay). The MTAs that occurred at a distance inferior to twice the local LD were

considered to belong to the same QTL. All the MTAs analyses were performed using Tassel 5 software (Bradbury et al., 2007).

4.2.3. Validation: Markers Conversion to KASP (Kompetitive Allele Specific PCR)

The array sequences of the markers associated to desirable traits (MTA) were submitted to LGC Genomics for in-silico design of KASP primers using their proprietary software. Those that passed the in-silico criteria were purchased and used to genotype the independent validation set. For each marker that amplified and showed polymorphism, the regression cut off between phenotype and haplotype was imposed at r = 0.105 following Pearson's critical value (Pearson, 1985). Each KASP marker was tested for association with target trait. The top 20 and worst 20 lines were considered as the true positive and true negative for heat tolerance in the case of heat study and for drought tolerance in the case of root study. Hence, the accuracy was calculated as the ratio of the correct allelic call among all, sensitivity as the ratio of the correct positive allelic among the top 20 yielding lines, and specificity as the ratio of the correct negative allelic calls among the 20 worst yielding lines.

Chapter III: Root system architecture and its association with yield under different water regimes in durum wheat

El Hassouni K., S. Alahmad, B. Belkadi, A. Filali-Maltouf, L.T. Hickey, F.M. Bassi,

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Two high-throughput methods for root phenotyping were used to explore the genetic variability in a subset of tetraploid wheat. Mutli-environmental yield trials were also conducted to evaluate the impact of each rooting type on grain yield performance.

Root System Architecture and Its Association with Yield under Different Water Regimes in Durum Wheat

Abstract

Durum wheat (*Triticum durum* Desf.) is a major cereal crop grown globally, but its production is often hindered by droughts. Breeding for adapted root system architecture should provide a strategic solution for better capturing moisture. The aim of this research was to adapt low-cost and high-throughput methods for phenotyping root architecture and exploring the genetic variability among 25 durum genotypes. Two protocols were used: the “clear pot” for seminal root and the “pasta strainer” to evaluate mature roots. Analysis of variance revealed significant segregation for all measured traits with strong genetic control. Shallow and deep root classes were determined with different methods and then tested in yield trials at five locations with different water regimes. Simple trait measurements did not correlate to any of the traits consistently across field sites. Multitrait classification instead identified significant superiority of deep-rooted genotypes with 16 to 35% larger grains in environments with limited moisture, but 9 to 24% inferior in the drip irrigated site. Combined multitrait classification identified a 28 to 42% advantage in grain yield for the class with deeper roots at two environments where moisture was limited. Further discrimination revealed that yield advantage of 37 to 38% under low moisture could be achieved by the deepest root types, but that it also caused a 20 to 40% yield penalty in moisture-rich environments compared with the shallowest root types. In conclusion, the proposed methodologies enable low-cost and quick characterization of root behavior in durum wheat with significant distinction of agronomic performance.

1. Introduction

Durum wheat (*Triticum durum* Desf.) is one of the main sources of daily caloric intake and a major staple crop in the Mediterranean region. It is known for its unique quality characteristics, in particular high protein content and hard kernels that make it ideal for pasta, couscous, and bourghul manufacturing (Able and Atienza, 2014; Habash et al., 2014; Kezih et al., 2014; Stuknytė et al., 2014). Unlike common wheat (*Triticum aestivum* L.), durum wheat is primarily grown in marginal environments of the Mediterranean and semiarid regions of the world, where moisture is mostly provided through rain (Able and Atienza, 2014; Habash et al., 2014). Annual variation in rainfall

is common in the Mediterranean environment, with late-season droughts happening frequently. When droughts coincide with the flowering or grain-filling phase, it can dramatically affect yield and grain quality (Loss and Siddique, 1994; Belaid, 2000; Mohammadi et al., 2011; Bassi and Sanchez-Garcia, 2017). Furthermore, the Mediterranean region is predicted to lose 30% of its in-season rainfall due to the warming climate (Christensen et al., 2007). However, winter rainfall is typically abundant in Mediterranean climates, which leads to percolation of moisture into the deeper layers of the cultivated soils. According to modeling studies, wheat yield would increase by 55 kg ha^{-1} on average for each millimeter of water extracted from the soil after anthesis (Manschadi et al., 2006; Christopher et al., 2013). This highlights the importance of identifying root systems that provide better exploration of soil layers to capture rainfall early in the season or with the capacity to reach the residual moisture deep in the soil profile toward the end of the season.

Roots also play an essential role in plant health, as they enable not only access to water, but also nutrients vital for high productivity (Sharma et al., 2009). Hence, targeted breeding for specific root system architecture should ultimately result in more resilient durum wheat cultivars under water-limited environments (Sanguineti et al., 2007; Manschadi et al., 2008). For example, in rainfed cropping systems, it was shown that a narrow and deep root architecture with more branching at depth provided greater access to soil nutrient and moisture in environments experiencing terminal drought (Manschadi et al., 2006; Kirkegaard et al., 2007; Christopher et al., 2008, 2013). In addition, deeper and more efficient root systems were demonstrated to be significantly correlated to yield increases in wheat (Kirkegaard et al., 2007; Fang et al., 2017), rice (*Oryza sativa* L.; Arai-Sanoh et al., 2014), and sorghum [*Sorghum bicolor* (L.) Moench; Mace et al., 2012]. Roots length density (also known as the length of roots per unit of soil volume) and root depth are key components for enhanced deep soil water extraction (Asif and Kamran, 2011; Borrell et al., 2014). Seminal root angle (SRA), also called gravitropic set-point angle (Digby and Firn, 1995), has been shown to be a good proxy to determine the depth of roots in the field across different cereal crops such as wheat (Manschadi et al., 2008), rice (Kato et al., 2006) and sorghum (Mace et al., 2012). This variation in root traits is mostly regulated by multiple adaptive genes with minor additive effects often combined with epistasis, resulting in a degree of genotype × environment interaction (Price et al., 2002; Tuberosa et al., 2002; Giuliani et al., 2005;

MacMillan et al., 2006; de Dorlodot et al., 2007; Cooper et al., 2009; Ren et al., 2012; Christopher et al., 2013).

Despite its complexity, breeding for beneficial root system architecture holds great potential to enhance drought adaptation and offers a great opportunity for rapid genetic gain for grain yield (GY) in marginal land (Hammer and Jordan, 2007). The significance of root traits contributing to yield under water-limited environments has long been recognized (Richards, 1991), and roots have likely been subjected to indirect selection in breeding programs as a result of selection for high yield in the target environment (Wasson et al., 2012). However, incorporating selection for root traits directly in a breeding program has been met with many challenges, foremost the difficulty of phenotyping large numbers of genotypes in a cost- and time-efficient manner (Mace et al., 2012). Several wheat studies have evaluated roots using different phenotyping methods including rhizotrons (Nagel et al., 2012; Lobet and Draye, 2013; Clarke et al., 2017), soil coring (Trachsel et al., 2011; Wasson et al., 2012; Wasson et al., 2014), lysimeters (Ehdaie et al., 2014; Elazab et al., 2016), hydroponics (Liu et al., 2015), paper roll culture and Petri dishes for seedling (Tomar et al., 2016), rhizoboxes (Fang et al., 2017), and X-ray-computed tomography (Gregory et al., 2003; Mairhofer et al., 2013; Colombi and Walter, 2017; Flavel et al., 2017). However, most of these techniques are either expensive or not precise enough and reproducible. This has encouraged researchers to develop high-throughput strategies that focus on key proxy traits linked to root system architecture displayed in the field (Petrarulo et al., 2015; Richard et al., 2015). One such example is the “clear pot” method first developed by Richard et al. (2015) in hexaploid wheat, and later adapted to barley (*Hordeum vulgare* L.; Robinson et al., 2016). It involves growing genotypes in plastic transparent pots under semicontrolled conditions in the glasshouse, whereby the SRA can be measured without removing the seedlings from the soil. Some degree of infrastructure is still required to perform the assay, but some success in breeding has already been shown (Hickey et al., 2017). Another method for characterization of mature roots is the “basket” or “pasta strainer” method. It involves sowing isolated genotypes directly in the field inside a plastic container with holes on all its sides. The baskets are then removed from the soil to assess the behavior of the roots in proximity of the crown. This inexpensive and fast phenotyping technique was originally developed for wheat (Oyanagi et al., 1993) to assess the growth angle of seminal roots in greenhouse pots.

It was then adapted to field conditions to study rice roots 6 wk after sowing (Uga et al., 2009; Uga, 2012).

In this study, the clear pot method was deployed in combination with an adapted space-planted field pasta strainer (basket) method to explore the seminal and mature root traits for a set of durum wheat genotypes under two different water regimes. The objective was to assess the suitability and high throughput of these methods, and to use them to investigate the available genetic diversity for rooting pattern. Further, we examined whether root systems respond differently to water availability. Finally, yield trials were conducted in different environments with a range of water regimes to evaluate the potential value of shallow or deep rooting systems, and the possibility of incorporating selection for root traits in breeding programs.

2. Material and methods

2.1. Plant Material

A subset of 25 durum wheat (2*n* = 4*x* = 28, AABB) genotypes derived from a collection of 384 accessions originating from different countries were evaluated for root growth pattern using the clear pot and pasta strainer methods. The panel comprised four landraces, six cultivars, and 15 ICARDA accessions. Details for the different genotypes are provided in Table 1.

Table 1. Name, origin, and genetic background of the 25 durum wheat genotypes evaluated in this study.

Accession name	Origin	Pedigree
IG:86075	India	Landrace
IG:79509	Ethiopia	Landrace
IG:85026	Spain	Landrace
IG:85620	Afghanistan	Landrace
Jabal2	ICARDA	Korifla/AegSpeltoidesSyr//Mrb5
Amina	ICARDA	Korifla/AegSpeltoidesSyr//Loukos
Heirum	ICARDA	Heider/TAraticumMA//Mrb5
Icamator	ICARDA	IcamorTA041/4/Aghrass1/3/HFN94N8/Mrb5//Zna1/5/Malmuk1/Serrator1
Ouassara1	ICARDA	Ouasloukos1/5/Azn1/4/BEZAIZSHF//SD19539/Waha/3/Gdr2
Margherita 2	ICARDA	Terbol975/Geruftel2
Icadezful	ICARDA	Geromtel1/IRANYT053//Mgnl3/Ainzen1
Icarasha2	ICARDA	Stj3//Bcr/Lks4/3/Ter3
Icamoram7	ICARDA	ICAMORTA0472/Ammar7
Maci115†	ICARDA	Maamouri2/CI115/5/F413J.S/3/Arthur71/Lahn//Blk2/Lahn/4/Quar
Miki3	ICARDA	Stj3//Bcr/Lks4
Bezaghras	ICARDA	Ossl1/Stj5/5/Bicrederaa1/4/BEZAIZSHF//SD19539/Waha/3/Stj/Mrb3/6/Mgnl3/Aghrass2
Secondroue	ICARDA	Stj3//Bcr/Lks4/3/Ter3/4/Bcr/Gro1//Mgnl1
Bezater	ICARDA	Ossl1/Stj5/5/Bicrederaa1/4/BEZAIZSHF//SD19539/Waha/3/Stj/Mrb3/6/Stj3//Bcr/Lks4/3/Ter3
Omrabi17	ICARDA	Jori c69/Hau
Bellaroi	Australia	920405/920274
Jupare C2003	CIMMYT	STOT//ALTAR84/ALD
Yavaros79	CIMMYT	JORI69/ANHINGA//FLAMINGO
Creso	Italy	Yaktana-54/Norin 10-B//2*Cappelli-63/3/3*Tehuacan-60/4/Capelli-B144
Marzak	Morocco	BD113
Kofa	USA	dicoccum alpha pop-85 S-1

† Genotype was not evaluated using the clear pot method.

2.2. Evaluating Seminal Root Traits Using the Clear Pot Method

Clear plastic pots (ANOVApot, 200-mm diam., 190-mm height) were filled with peat moss soil known for its high water- and nutrient-holding capacity. Seed sowing was performed according to Richard et al. (2015). A randomized complete block design was used, where each pot containing 24 seeds was considered a block, and a total of 20 blocks were used. Genotype Maci115 was omitted from this experiment to facilitate a balanced experimental design, since only 24 entries could be accommodated in each clear pot. Using forceps, seeds were positioned vertically between the pot wall and soil with the embryo facing downward at 3-cm depth, ensuring the easy visualization of the seed embryo through the transparent pot. Plants were grown in the glasshouse under diurnal natural light conditions. Images were captured 5 d after sowing (seminal roots 3–5 cm in length) using a digital camera. The images were analyzed for SRA, where

the angle between the first pair of seminal roots emerging from the seed was measured using online free software ImageJ (http://imagej.nih.gov/ij/).

2.3. Evaluating Mature Root System Traits Using the Pasta Strainer Method

The method of Uga et al. (2009), initially developed for assessing rice roots in plastic baskets, was adapted to durum wheat as follows. The space-planted field conditions experiment was performed in Guich Station (33°59′ N, 6°50′ W; Rabat, Morocco) in the field, in sandy soil (silt loam 0–20 cm, sandy loam 20–40 cm) under the protection of a net house. Plastic pasta strainers (height = 11 cm, diameter = 20 cm) were filled with sandy soil and buried at 11-cm depth (Fig. 1A). Strainers were placed with 20 cm distance between each other to ensure 40-cm spacing between plants, to avoid barriers to the growth of the root system in all three dimensions. Three seeds were placed in the middle of each strainer representing one genotype. At the four-leaf stage (growth stage 14, according to the Zadoks decimal growth scale; Zadoks et al., 1974), the three seedlings were thinned, retaining the most vigorous plant.

During the growing season, standard cultural practices were used, with 150 kg ha^{-1} of N, P, and K incorporated in the soil before planting, followed by 50 kg ha^{-1} of NH_4NO_3. Cherokee fungicide (1.5 L ha^{-1}, Syngenta, chlorothalonil-cyproconazole-propiconazole) was applied to prevent development of fungal diseases, and Pirimor (500 g ha^{-1}, Syngenta, pyrimicarbe) was applied to control aphid infestation. Weeds were controlled by two applications of tank mixture of Mustang (0.6 L ha^{-1}, DOW Agrosciences, 2,4-D-florasulame) and Pallas (0.5 L ha^{-1}, DOW Agrosciences, pyroxulame), with additional mechanic weeding to ensure pristine plot.

The experimental design was an α lattice with two replications and five incomplete blocks of size five. Two independent trials were conducted over two seasons, each with independent randomization based only on the genotype factor. Each trial received a different amount of moisture as follows: after flowering (growth stage 55 on the Zadoks scale), a plastic tarp was placed over the roof and sides of the net house to prevent rainfall from reaching the experimental setup. The well-watered trial then received four irrigation events, one every 10 d (total amount of 40 mm), whereas the deficit trial did not receive any additional moisture for 40 d. At this point, one final irrigation of 20 mm was provided to both. These experiments were conducted during the seasons 2014–2015 and 2015–2016 from December to May.

Before maturity, the number of fertile spikes (SN) and tillers (TN), as well as the number of spikelets on each spike (SPN), was recorded for each plant, together with the plant height (PLH) excluding the awns. In addition, a relative surrogate for the chlorophyll content was measured using a chlorophyll meter (Konica Minolta SPAD 502) during the grain-filling stage to confirm the treatment effects. At maturity, the shoot was cut 3 mm above the soil and weighed to determine the dry shoot biomass (SB). The spikes were then threshed to determine the grain weight (GW), and the weight of 1000 kernels (TKW) was determined using a precision balance. The stay-green trait was calculated as the days elapsing between the heading and maturity dates.

The belowground traits were recorded by first removing the strainer from the soil using a shovel. The strainer was then divided into three sections (Fig. 1B): an upper Layer 1 (2–8 cm), a middle Layer 2 (8–10 cm), and a lower Layer 3 (10–13 cm). The three layers marked on the strainer corresponded to the angles from the horizontal ground level of 0 to 30, 30 to 60, and 30 to 90°, respectively. The number of roots protruding from the holes in the sides of the plastic container were counted for each level, and the number of roots for each layer was then expressed as a ratio of the total root number (TRN), resulting in three root ratios (RR 2–8 cm, RR 8–10 cm, and RR 10–13 cm). The root sections protruding from the plastic container were cut off to leave an exact volume of soil and roots corresponding to the volume contained inside the pasta strainer, equivalent to 2100 cm^3. The sandy soil was then gently removed from the container, avoiding damaging the roots in the process. The type of soil used was ideal for this task, as its loam content prevented loss through the holes of the strainer until a small pressure was imposed, whereas its sandy nature facilitated the task of removing the soil without damaging the roots. Roots were then rinsed in water to remove any remaining sand and allowed to dry for 10 d. The dry roots were first weighed to obtain root biomass (RB) and then scanned using an Epson Perfection V700 scanner. This image was analyzed with ImageJ to measure the root angle (RA), setting the center of the angle in the middle of the crown and the two extreme sides of roots as the final point of the angle (Fig. 1C). All roots angles were visually controlled to prevent errors. Image scanning and ImageJ was performed only in the second season.

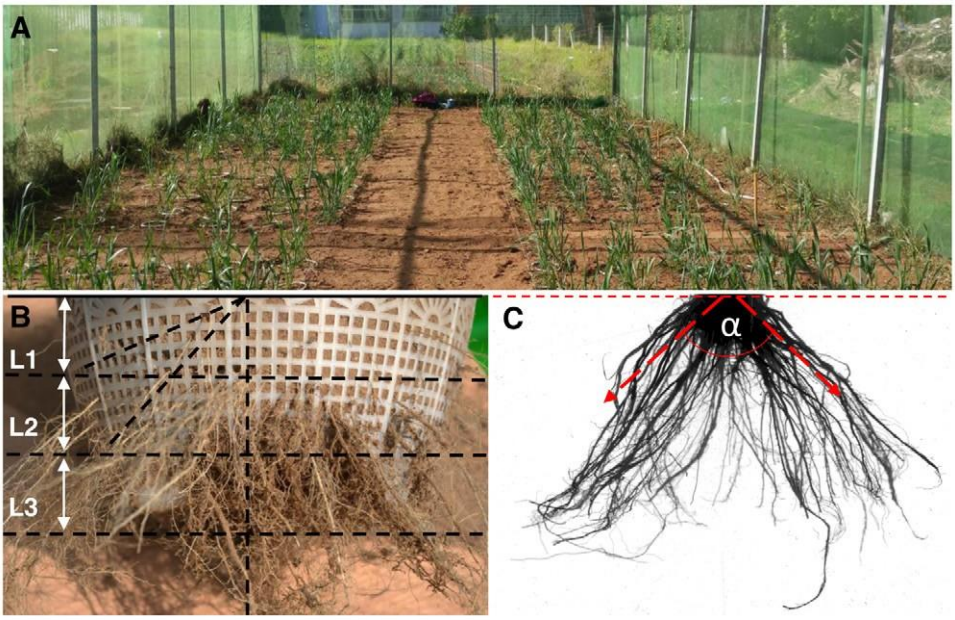

Fig. 1. Root phenotyping using the "pasta strainer" method. Panel A shows durum wheat plants grown in pasta strainers buried in the soil. The left-hand side represents the well-watered treatment, and the right-hand side is where drought was imposed at flowering. Panel B shows a diagram of measuring mature root angle by counting root ratio per level (Layer 1 [L1], Layer 2 [L2], and Layer 3 [L3]) with the pasta strainer method. Panel C shows a diagram for measuring root growth angle by ImageJ. α, the angle between the two extreme sides of the roots with the center set in the middle of the crown.

2.4. Field Trials

The 25 entries used in this study were included in yield trials containing a larger set of 384 genotypes. Each trial was conducted using an augmented design of 19 blocks of size 24 plots, each block with four commercial checks. Sown plot size was 3.6 m^2 (six rows), and 2.4 m^2 (four rows) were harvested for assessing yield performances. In addition, 1000 randomly selected kernels were weighted to determine TKW. The five environments listed in Table 2 included two rainfed environments with strong terminal droughts (Marchouch in Morocco [MCH16] and Kfardan in Lebanon [KFD16]) and three irrigated environments. The irrigated environments were Terbol (TER16), where supplemental moisture was provided via three sprinkler irrigations; Melk Zehr (MKZ16), where drip irrigation was used to provide the majority of the moisture; and Tessaout (TES16), where nearly all in-season water was provided via gravity-fed irrigation. Optimal agronomic management practices were applied in all environments. The total rainfall and the amount of irrigation water applied are presented in Table 2.

Table 2. Site characteristics and irrigation practices at five experimental stations used to determine field performances of different root types.

Code	Site	Country	Year	Climate	Irrigation method	Moisture†	Coordinates	Soil type
						mm		
MKZ15	Melk Zhar	Morocco	2014–2015	Mediterranean, hot and temperate	Drip irrigation	297 + 324	30°2′33″ N, 9°33′4″ W	Sandy limestone
TER16	Terbol	Lebanon	2015–2016	Mediterranean, temperate	Sprinkler	356 + 80	33°48′29″ N, 35°59′22″ W	Chromic vertisols
TES16	Tessaout	Morocco	2015–2016	Hot steppe	Gravity irrigation	132 + 360	29°49′48″ N, 8°34′48″ W	Calcic xerosols
MCH16	Marchouch	Morocco	2015–2016	Mediterranean, warm temperate	–	183	33°34′3.1″ N, 6°38′0.1″ W	Clay vertisol
KFD16	Kfardan	Lebanon	2015–2016	Mediterranean, temperate	–	236	33°32′54″ N, 35°51′18″ W	Sandy clay

† Determined by rainfall + irrigation.

2.5. Data Analysis

All statistical analyses for root trials were computed in R (R Development Core Team 2016). A mixed linear model was formulated in accordance with the experimental design to obtain best linear unbiased estimates (BLUEs) for all traits, where genotype, treatment, and year were considered as fixed effects and replication and block as random effects nested in treatment and year. The model was fit using the *lmer* function of the lme4 package (Bates et al., 2015). Broad-sense heritability was estimated based on a random model as the ratio between the genotypic (σ^2_g) and phenotypic (σ^2_p) variance (Falconer and Mackay, 1996):

$$H^2 = \sigma^2_g/\sigma^2_p$$

Phenotypic variance was calculated using the method suggested by DeLacy et al. (1996):

$$s^2_p = s^2_g + \frac{s^2_{G'T}}{t} + \frac{s^2_{G'Y}}{y} + \frac{s^2_{G'T'Y}}{ty} + \frac{s^2_e}{tyr}$$

where: $\sigma^2_{G\times T}$ =genotype x treatment variance, $\sigma^2_{G\times Y}$ =genotype x year variance, $\sigma^2_{G\times T\times Y}$ =genotype x treatment x year variance, σ^2_e =residual variance, *r* is the number of replications per treatment, *t* is the number of sites, and *y* is the number of years.

The adjusted means of the field trials were calculated using ACBD-R. The adjusted means of the tested genotypes were extracted from the full field experiment and then used for a simple two-way ANOVA to test the main effects of root type (class), environment, and class × environment interaction using car (Fox and Weisberg, 2011) and FSA (Ogle, 2016) packages. To assess the differences between the average performances of the different root classes, a Tukey test was performed for all pairwise comparisons.

Pearson correlation coefficients were estimated for single plant studies for all traits that displayed a significant genotype effect, rather than using the BLUEs across treatments or the single treatment mean, when the treatment effect was significant. The critical value of the correlation significance was determined at 0.505 for $p < 0.01$ and 0.617 for $p < 0.001$ (df = 23) using the corrplot package (Wei and Simko, 2017).

2.6. Multitrait Analyses to Define Root Architecture Behavior

The first multitrait root classification method was performed using multivariate statistical analysis with principal component analysis (PCA) for RR per level. The packages MASS (Venables and Ripley, 2002) and ggplot2 (Wickham, 2009) were used for this scope. The graphical biplot was then divided into three root classes by splitting in two each angle of the main vectors. The second method for multitrait classification was generated by calculating the Fisher LSD for each relative root number for the three layers at a significance level of 0.05 using the agricolae package. Each class was then graphically represented with a different color for values nonsignificantly different than twice the LSD from the maximum value. Through graphical representation, four classes of genotypes could be determined. The third multitrait method compared the SRA against the mature RA, where averages for each axis on the biplot were used to separate the genotypes into four root classes.

2.7. Combined Multitrait Analyses to Define Root Architecture Behavior

The results obtained from the three multitrait methods were used for hierarchical clustering of genotypes using the average method (ggdendro and ggplot2 packages) as was used for functional classification based on rooting types in the study of Bodner et al. (2013). “Main” root classes were determined by separating hierarchical dendrogram once at 90% of the total variation, and then at 50% of the total variation to determine “extreme” classes. Two extreme classes were identified, and three

genotypes from each were selected: Jabal2, 79509, and Bellaroi representing the shallowest, and Icamator, Margherita2, and Omrabi17 representing the deepest rooted. The yield performance of these extreme classes was graphically represented using box-and-whisker plots (ggplot2 package).

3. Results

3.1. Mixed-Model ANOVA for Individual Traits

An ANOVA for seminal root traits measured using the clear pot method revealed highly significant differences among the tested genotypes (Table 3). Average SRA was 48.6°, and it ranged from 8.5 (Jupare C2003) to 115.7° (Miki3). Space-planted field testing for adult plant root behavior revealed significant ($p < 0.001$) genotype differences for all root and shoot traits (Table 3). The treatment effect was significant for RB at $p < 0.01$ and for RA at $p < 0.001$. There was no genotype × treatment interaction for RR per level, but all other root traits did show this interaction. The genotypes presented a large diversity in terms of rooting pattern. The RR ranged from 0.05 to 0.51 in the upper section, followed by 0.13 to 0.54 in the middle section and from 0.18 to 0.77 in the deepest section. The broad-sense heritability was relatively high for all the morphological and architectural root traits, and the highest values were for RB (broad-sense heritability = 0.90) and RR 2 to 8 cm, RR 8 to 10 cm, and RR 10 to 13 cm (0.82, 0.52, and 0.87, respectively). The SRA and adult plant RA also showed high heritability at 0.64 and 0.82, respectively (Table 3).

Table 3. Descriptive statistics of all measured traits among 25 durum wheat genotypes (G) tested for 2 yr (Y) under two different moisture treatments (T) (drought stress vs. well-watered).

Trait	Abbreviation	Mean	LSD	Min.	Max.	CV†	H^2‡	G	T	Y	G × T	T × Y	G × Y	G × T × Y
Root ratio at 2–8 cm	RR 2–8 cm	0.20	0.07	0.05	0.51	23	0.82	***	ns§	*	ns	**	ns	**
Root ratio at 8–10 cm	RR 8–10 cm	0.34	0.08	0.13	0.54	15	0.52	***	ns	ns	ns	ns	**	ns
Root ratio at 10–13 cm	RR 10–13 cm	0.46	0.08	0.18	0.77	12	0.87	***	*	*	ns	*	*	*
Total root no.	TRN	40	12	15	83	20	0.56	***	ns	ns	***	*	***	**
Root biomass (g)	RB	3	0.8	0.6	14	27	0.90	***	**	**	***	ns	***	***
Root angle	RA	62	8	37	106	10	0.82	***	***	–	***	–	–	–
Days to maturity (d)	DTM	142	10	117	158	4	0.66	***	ns	–	**	–	–	–
Flag leaf chlorophyll content (SPAD)	CC	55	7	33	66	7	0.10	***	ns	–	***	–	–	–
Stay-green	SG	52	23	10	67	15	0.61	*	ns	–	ns	–	–	–
Days to heading (d)	DTH	97	4	76	125	4	0.97	***	ns	***	ns	ns	ns	ns
Plant height (cm)	PLH	70	4	35	175	6	0.93	***	**	*	ns	ns	***	***
Tiller no. per plant	TN	7	1	2	16	21	0.65	***	**	***	ns	ns	**	*
Spikes no. per plant	SN	6	1	2	14	20	0.63	***	**	***	ns	ns	**	ns
Avg. spikelet no. per plant	SPN	17	2	8	29	12	0.83	***	*	**	ns	ns	ns	ns
Shoot biomass (g)	SB	36	12	4	179	33	0.89	***	*	***	ns	ns	*	ns
Grain weight (g)	GW	14	4	2	41	27	0.60	***	**	***	*	ns	*	**
Harvest index	HI	0.4	0.1	0.1	0.9	22	0.72	***	ns	ns	*	ns	*	**
1000-kernel weight	TKW	41	7	19	67	17	0.46	**	ns	**	ns	ns	ns	ns
Seminal root angle (°)	SRA	49	13	9	116	12	0.64	***	–	–	–	–	–	–

*,**,*** Significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

† CV, coefficient of variation.

‡ H^2, broad-sense heritability.

§ ns, no significant effects.

3.2. Correlations among All Traits

The matrix in Fig. 2 shows only the significant (p < 0.001) positive and negative correlations among traits. Traits for which the water treatment had no significant effect were presented as combined values, whereas when the treatment was significant, a letter D for simulated drought or a letter W for well-watered were added to separate

the two values. The adult RA under water deficit was strongly associated (r = 0.68) with RA under well-watered conditions, and both were positively correlated with RR 2 to 8 cm and RR 8 to 10 cm and negatively associated with RR 10 to 13 cm, whereas RR 2 to 8 cm and RR 10 to 13 cm were in repulsion under both water scarce (r = −0.85) and well-watered (r = −0.81) treatments. Total root number exhibited a significant correlation with RB and RA in both treatments. Adult plant RA was not directly correlated to SRA. Strong correlations also existed between above- and belowground traits. Root angle in the well-watered treatment was positively associated with GW and its components TN, SN, and SB under the same treatment. The opposite trend was observed for well-watered RR 10 to 13 cm that was negatively correlated to GW (well-watered), TN, SN, SB, and PLH as well. Deficit RR 10 to 13 cm was negatively associated with only TN. Total root number and RB were positively correlated to almost all shoot traits, with the exception of chlorophyll content, TKW, and harvest index, which showed a negative correlation. The estimated stay-green was negatively correlated to SB, SPN, PLH, and TRN and positively correlated to chlorophyll content and SRA.

To determine if the above- and belowground traits measured under space-planted conditions had a significant effect in determining agronomic performances, five field trials were conducted in two drought-prone environments and under three types of irrigation (drip, flood, and supplemented via sprinklers). All below- and aboveground traits measured on single-plant studies were tested via correlation against the values measured in the field (Supplemental Table S1) and did not provide significant associations, with the following exceptions. Thousand-kernel weight in Kfardan was positively affected (r = 0.55) by deeper roots (RR 10–13 cm) and positively correlated to TKW measured in well-watered trial in the space-planted experiment. Grain yield in MKZ15 was negatively correlated to TRN and RB for belowground traits and negatively associated with TN, SN, SPN, and SB for aboveground traits. However, the correlation results were not consistent among dry or irrigated environments and thus were of limited use overall. Hence, these correlations and their meaning are not discussed further.

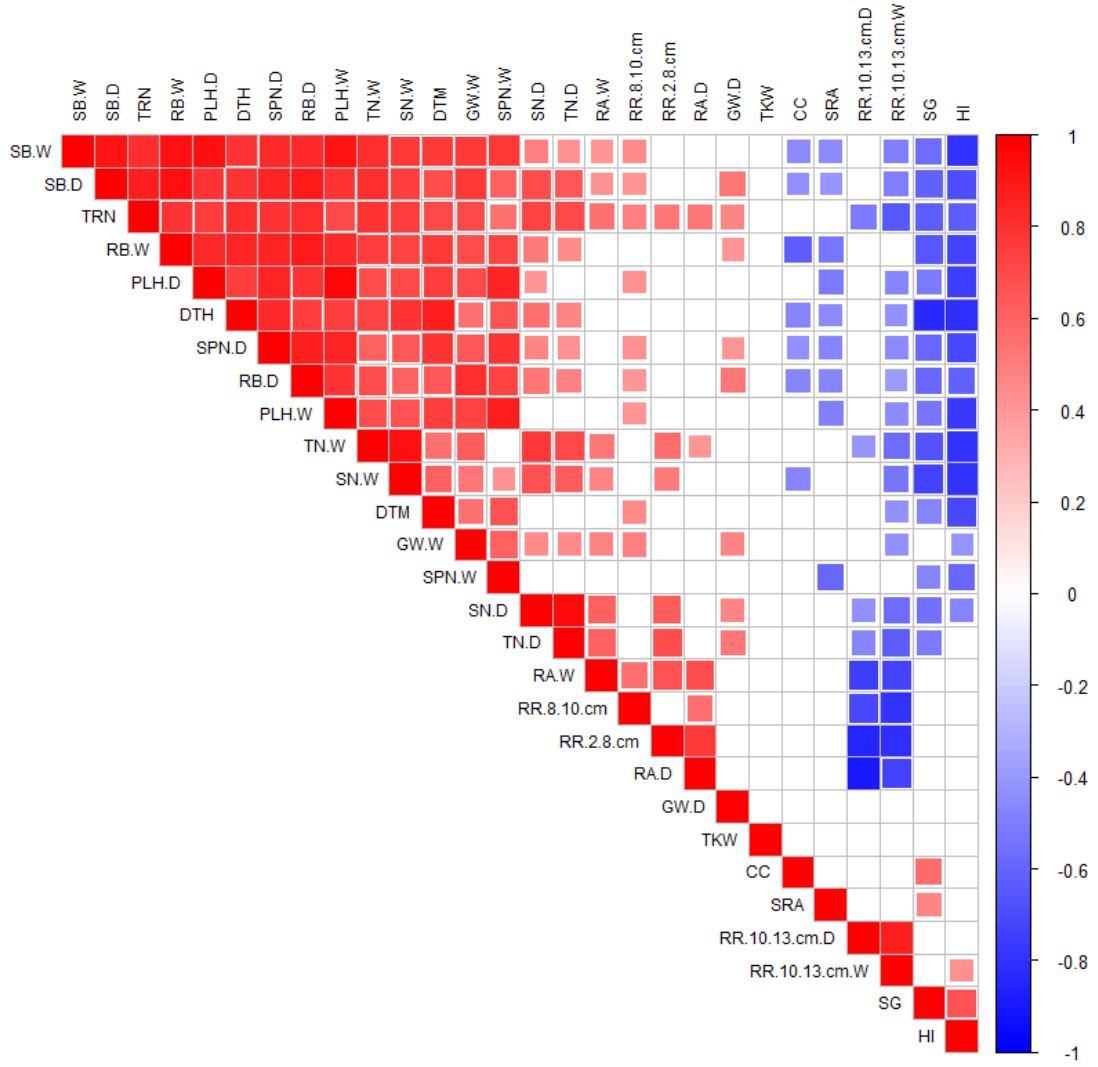

Fig. 2. Correlation matrix for above- and belowground traits in durum wheat genotypes tested in two treatments: drought (D) vs. irrigated (W) conditions. Correlations were tested for individual treatments only for those traits that showed a significant treatment effect based on ANOVA and for best linear unbiased estimates across treatments when the treatment effect was not significant. SB, shoot biomass; TRN, total root number; RB, root biomass; PLH, plant height; DTH, days to heading; SPN, average spikelet number per plant; TN, tiller number; SN, spikes number; DTM, days to maturity; GW, grain weight; RA, root angle; TWK, 1000-kernel weight; CC, chlorophyll content; SRA, seminal root angle; RR.2.8 cm, root ratio at 2 to 8 cm; RR.8.10 cm, root ratio at 8 to 10 cm; RR.10.13 cm, root ratio at 10 to 13 cm; SG, stay-green; HI, harvest index.

3.3. Determination of Root Classes based on Multiple Traits and their Field Responses

Single-trait characterization could not be used to identify meaningful classes of agronomically different genotypes when tested in the field. Therefore, other methods that combine multiple traits were tested. The first method assessed for multitrait prediction of classes used PCA among RR 2 to 8 cm, RR 8 to 10 cm, and RR 10 to 13

cm values (Fig. 3). The first two axes together explained 99% of the total variation. The angle of the trait vectors indicated that the two traits RR 2 to 8 cm and RR 10 to 13 cm were negatively correlated with one another and perpendicular to the vector of RR 8 to 10 cm. These associations among the three classifiers were consistent with the Pearson correlations (Fig. 2). The three root categories derived using this method indicated that the ICARDA Genebank (IG) landrace IG:79509, as well as the modern lines Bellaroi and Jabal2, were the closest to the RR 2 to 8 cm vector and hence constitute a group of genotypes that produce a larger portion of the roots in the upper soil layer. In contrast, genotypes including IG:85620, IG:85026, Amina, Bezaghras, Ouassara, Icadezful, and Icamoram7 formed another group that concentrate their roots in the medium layer of the soil. All the remaining genotypes were more associated with RR 10 to 13 cm developing a deep root system.

Fig. 3. Biplot showing trait vectors (root ratios at 2–8 [RR 2–8 cm], 8–10 [RR 8–10 cm], and 10–13 cm [RR 10–13 cm]) and position of the genotypes tested. PC1, Principal Component 1; PC2, Principal Component 2.

The second method of multitrait classification was done via LSD test (Fig. 4), and it revealed four major classes. The first class contained genotypes colonizing the

superficial soils; the second class occupies the first and second layers; the third class is semideep, and its roots colonize the second and third layers, and the fourth class primarily develops roots in the third layer, exploring the deeper part of the soil.

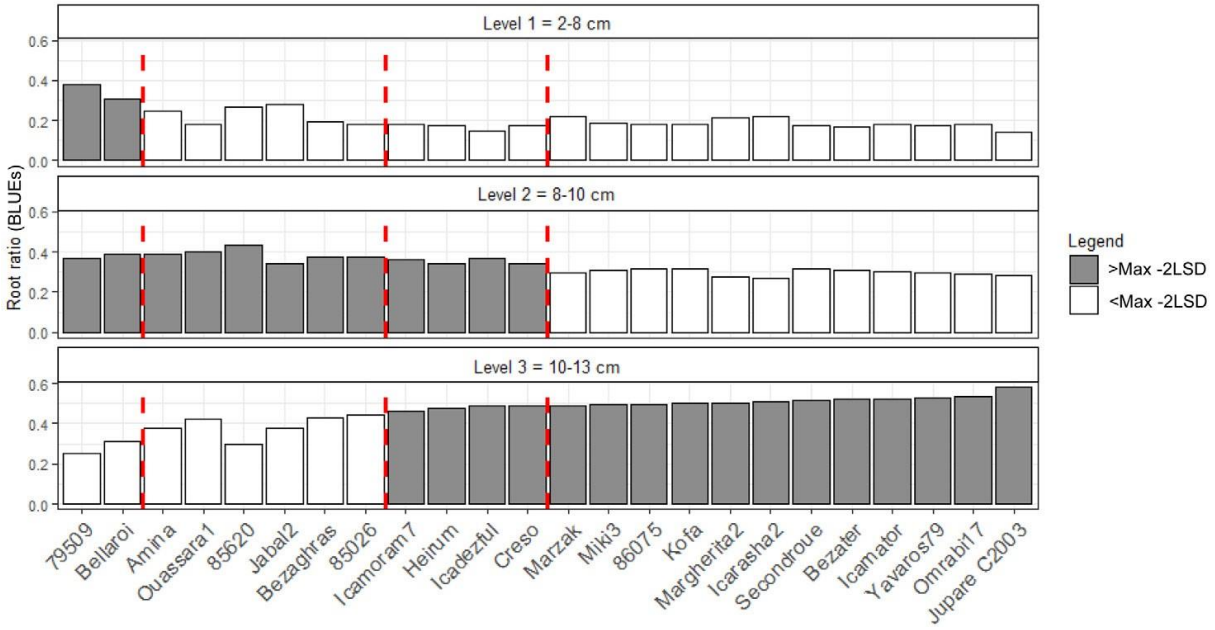

Fig. 4. Exploration of three different soil levels by the roots of the 25 genotypes tested, expressed as root ratio of the total. The values are best linear unbiased estimates (BLUEs) calculated over two replications, two treatments, and 2 yr. Gray bars indicate genotypes with values within two LSD levels from the maximum of that soil level. Vertical dashed red lines guide the visual distinction into four root categories.

A third multitrait methodology was based on the comparison of the SRA and adult RA. Since these two traits are not correlated, their combined study is of interest to determine root behavior (Fig. 5). Four classes could be determined, with the first and the third group showing wide and narrow angles in both seedling and adult plants, respectively. The second group comprised genotypes that start their growth with a narrow SRA but then expand the exploration of superficial soils as the plant ages, whereas the fourth group comprised genotypes with the RA changing from wide at the seedling stage to narrow at maturity. The two methods identified statistically significant differences (Table 4) for grain size among the dry environments, with PCA and LSD root classes explaining 34.3 and 25.9% of the TKW variation, respectively. In case of PCA, the medium and deep classes reached 36, 37, 29, and 30 grams in Kfardan and Marchouch, respectively, which was 20, 23, 16, and 20% above the TKW recorded for the shallow class in the same environments (Supplemental Table S2). In case of LSD, the deep class reached 38 and 31 grams in Kfardan and Marchouch, respectively,

which was 35, and 29% above the TKW recorded for the shallow class in the same environment (Supplemental Table S2). Instead, the third classification method based on SRA vs. RA identified significant differences (Table 4) between classes of TKW combining the three irrigated environments and explained 16.4% of the variation. Considering individual environments, this difference was evident only in Melk Zehr under drip irrigation, where the shallow-rooted (shallow to shallow) plants reached 61 grams of TKW, 24, 13, and 9% higher than the deep to shallow, deep to deep, and shallow to deep classes, respectively.

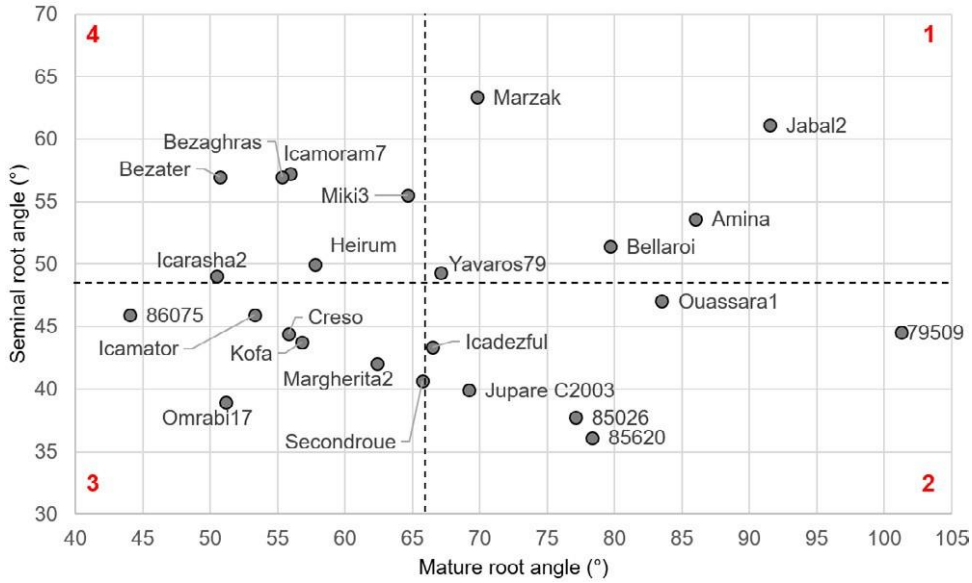

Fig. 5. Variation in genetic control of root angle at seedling and mature stages. The dashed line denotes the average value of root angle on each axis.

Table 4. Statistics of different root types (classes) for grain yield and thousand-kernel weight (TKW) in dry and favorable environments using different classification methods.

Classification method†	Source of variation	Rainfed environments				Irrigated environments			
		Grain yield		TKW		Grain yield		TKW	
		Variation	*p* value	Variation	*p* value	Variation	*p* value	Variation	*p* value
		%		%		%		%	
Multitrait PCA classification	Class	62.3	0.58	25.9	0.01*	7.3	0.62	3.2	0.38
	Env‡	9.7	0.68	72.9	0.00***	84.7	0.00**	93.3	0.00***
	Class × Env	28.0	0.78	1.3	0.78	7.9	0.90	3.5	0.70
LSD classification	Class	4.9	0.99	34.3	0.00**	7.9	0.73	5.9	0.30
	Env	9.5	0.69	64.3	0.00***	67.0	0.00**	90.2	0.00***
	Class × Env	85.6	0.69	1.4	0.89	25.1	0.65	3.8	0.87
Biplot of SRA vs. RA	Class	20.3	0.96	6.8	0.66	18.1	0.41	16.7	0.00**
	Env	10.7	0.69	92.8	0.00***	68.8	0.00**	79.3	0.00***
	Class × Env	69.1	0.79	0.4	0.99	13.2	0.91	4.0	0.76
Combined multitrait classification	Class	90.9	0.00**	15.2	0.12	1.8	0.60	0.6	0.66
	Env	2.0	0.59	81.6	0.00**	51.7	0.04*	94.3	0.00***
	Class × Env	7.1	0.32	3.2	0.45	46.6	0.05	5.2	0.45

*,**,*** Significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

† PCA, principal component analysis; SRA, seminal root angle; RA, root angle.

‡ Env, environment.

3.4. Determination of Root Classes by Hierarchical Combination of Multiple Traits

The three multitrait methodologies were combined to derive a single score of root behavior via hierarchical clustering (Fig. 6). Two and five classes were defined to explain 90 and 60% of the variation, respectively. The two main classes incorporated 11 genotypes with preference for exploring the superficial soil layers, and 13 genotypes with deeper rooting patterns. This classification method identified significant differences between classes ($p < 0.05$) for GY when the genotypes were field tested in environments exposed to terminal droughts, but not for TKW or irrigated environments. The highest variance of 90.9% was explained by the class effect, followed by 1.9% for environment and 7.1% for class × environment interaction effect (Table 4). Genotypes belonging to the deep-rooting class had a mean of 2883 and 2475 kg ha^{-1} for KFD16

and MCH16, respectively, which was 42 and 28% higher than the mean of the shallow-rooted class in the same environments (Table 5). These two mega-classes were further divided into two subclasses each to determine differences between extreme types (Fig. 6). The shallowest group included Jabal2, 79509, and Bellaroi, whereas the deepest included Miki3, Icarasha2, Bezater, Icamator, 86075, Margherita2, Secondroue, Omrabi17, Kofa, and Creso. To reduce bias, three genotypes (Icamator, Margherita2, and Omrabi17) were selected as the most representative of the deepest root class and were compared with the same number of entries of the shallowest class. Figure 7 shows the GY performances of these two groups over different environments (rainfed and irrigated), with the deep-rooting types achieving a yield advantage of +1194 and +1225 kg ha^{-1} in MCH16 and KFD16, respectively. However, the shallow-rooting types were superior (+2000 kg ha^{-1}) in MKZ16 under drip irrigation in sandy soils and in Tessaout (+1095 kg ha^{-1}) under gravity irrigation.

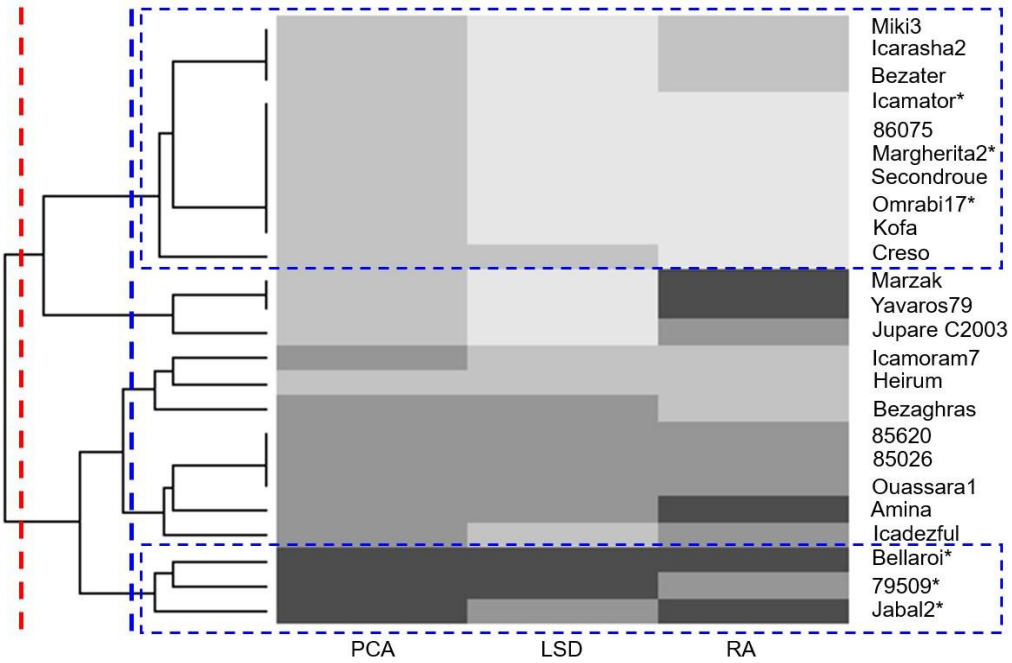

Fig. 6. Combined multitrait method via dendrogram to distinguish root behavior of 24 genotypes (Maci115 excluded from the analysis). The *x* axis lists the three different multitrait methods that were combined in the analyses: first method is principal component analysis (PCA) of root ratios, the second method is based on two significant (LSD) differences for root ratio at each level, and the third method is based on root angle (RA) measured at maturity and seedling. For each method, the different classes identified are color coded with different shades of gray. A vertical red dash line indicates the position that explains 90% of variation and splits the genotypes into two classes. The blue dashed line indicates the five subclusters of the two main classes. Asterisks (*) indicate the selected genotypes exhibiting extremely narrow and wide root types.

Table 5. Field response of two root architecture classes defined via combined multitrait method. Significance difference were tested at a 0.05 level of confidence.

Environment	Class	Grain yield†	1000-kernel weight†
		kg ha^{-1}	g
Rainfed			
KFD16	Shallow	1658 ± 439a	38 ± 5a
	Deep	2883 ± 332b	39 ± 4a
MCH16	Shallow	1785 ± 588a	28 ± 1a
	Deep	2475 ± 331b	33 ± 2a
Irrigated			
MKZ15	Shallow	6432 ± 1615a	56 ± 3a
	Deep	5382 ± 677a	56 ± 4a
TER16	Shallow	7546 ± 635a	46 ± 4a
	Deep	7287 ± 1067a	45 ± 3a
TES16	Shallow	6330 ± 734a	39 ± 2a
	Deep	8417 ± 1013a	44 ± 8a

† Mean ± SD.

Means in the same column within the same environment followed by the same letter are not significantly different at the 0.05 probability level

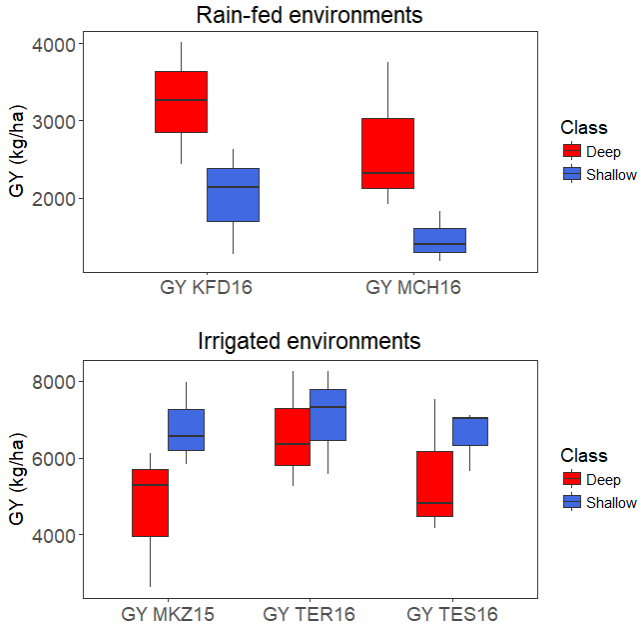

Fig. 7. Boxplot of grain yield (GY) of the two extreme root types over different environments (rainfed and irrigated). The ends of the whiskers indicate the minimum and maximum values of GY, the bottom and the top of the boxes indicate the second and third quartile values, and the band inside the box indicates the median.

4. Discussion

Plant behavior belowground is deemed of fundamental importance for water uptake and nutrient acquisition, particularly in water- and resource-limited environments (de Dorlodot et al., 2007; Lynch and Wojciechowski, 2015). Here, an attempt was made to adapt low-cost and scalable methodologies to reliably characterize root behavior in durum wheat. The clear pot method has already been used to study other crops and was most recently adapted to durum wheat (Alahmad et al., 2018). Its scalability and reliability have been discussed in depth elsewhere (Richard et al., 2015). The pasta strainer method under upland-field conditions was first presented in rice (Uga et al., 2009; Uga, 2012), demonstrating a variation in root morphology among different rice genotypes. Some minor modifications were necessary to adapt it to the characterization of mature roots of durum wheat and asses its reliability.

4.1. Interaction between Root Traits and Water Regimes

Significant genotypic variation was observed for all assessed traits, which demonstrated the usefulness of this panel for root genetic research. The heritability for root fractions measured at different soil depths—RR 2 to 8 cm (0.82), RR 8 to 10 cm (0.52), and RR 10 to 13 cm (0.87)—was extremely high, and the water treatment imposed after flowering had no significant effect. These results support the hypothesis that root preference for shallow or deep soil layers is under strong genetic control, and hence that traits measured under space-planted controlled conditions could well represent the root behavior under true field conditions in different soil and moisture types. This hypothesis is further tested here.

Instead, in the case of RB, which was also revealed to be a highly heritable trait (Broad-sense heritability = 0.9), the amount of moisture did have a significant effect on the root behavior. Thus, this trait is more affected by the environment and hence it would be harder to predict its behavior under true field conditions using data from space-planted experiments. However, strong correlation was found between RB and SB, suggesting that above- and belowground behavior have shared genetic controls (López-Castaneda and Richards, 1994). Further, this above- and belowground connection provides a simple proxy for breeders to select for high RB, without physically measuring the belowground component of the plant. A major achievement of the Green Revolution was to maximize harvest index by converting biomass into

grains (Manschadi et al., 2006, 2008; Hammer, 2006; Kirkegaard et al., 2007), so it does create a challenge for breeders, as a targeted increase in RB might ultimately result in a reduced harvest index and a detrimental loss in yield (Richards et al., 2007; Rebetzke et al., 2012). Perhaps selection for higher root number may be better suited for breeding. This trait is strongly correlated to the TN and also to GW per plant. Both these characteristics are sought after by breeders, hence targeting an increase in root number does not appear to be linked to any negative effect in terms of productivity. Therefore, aboveground selection for TN could represent an ideal proxy to also achieve rapid genetic gain for root number.

4.2. Field Variation for Grain Size based on Differences in Root Behavior

It has been observed in several crop species that large diversity exists for root characteristics through the use of different root phenotyping methods (Nakamoto et al., 1991; Manschadi et al., 2006, 2008; Chen et al., 2016). These authors came to the conclusions that the plasticity of root architecture increases adaptability, which in turn should improve productivity when the best fit is deployed for specific soils and moisture conditions. In cereals, the root growth angle is useful for predicting root distribution in the soil layers (Nakamoto et al., 1991; Oyanagi, 1994); therefore, this a convenient proxy to predict the mature root system architecture without digging to the lower soil layers.

The ability of roots to penetrate deep into the soil or to fully occupy the superficial layer is an adaptive mechanism to maximize the amount of moisture absorbed by the plant to be then converted into biomass and grains. As mentioned, extending water availability after anthesis is normally linked to a prolonged grain-filling period before drying out. In turn, this extra time and moisture should normally be used by the plant to better fill its grains (Kirkegaard et al., 2007; Vadez et al., 2013; Vadez, 2014). All traits connected to RA show very high heritability and were not affected by the simulated water scarcity after anthesis, which as indicated above, supports the case for strong genetic control. However, it cannot be concluded that other water treatments not tested here could have a more significant effect on rooting behavior, especially if water scarcity was to occur before flowering. Still, it was already demonstrated that the presence of the gene *DEEPER ROOTING 1* (*DRO1*) in rice cultivars affords the crop yield stability under drought, and it does not cause any penalty under irrigated

conditions (Uga et al., 2011, 2013). This finding is also in agreement with Voss-Fels et al. (2017), who found that *VERNALIZATION 1*, a key gene influencing flowering time and aboveground development in wheat, also has an important pleiotropic role on root system architecture and alone controls 8% of RA total variation.

In the experiment described here, RA traits were correlated with each other but did not show any strong and direct dependence on any aboveground trait. Hence, no simple proxy could be identified to replace the hard job of physically measuring the belowground feature of the plant. However, none of the single-trait classifications measured in space-planted trials could be correlated to the actual field performances. Therefore, to determine the field-level response of root behavior in terms of preferred depth of colonization, multitrait and combined multitrait methods were assessed and used to identify difference in agronomic response in the field. Two of the multitrait methods (LSD and PCA classifications) were capable of capturing part of the variation for grain size (TKW) in field yield trials with an increase of 16 to 35% for the deep-rooting classes under rainfed conditions, but they could not predict any change in GY (Supplemental Table S2). Among the multitrait analyses, the PCA method relies on the simplest trait to measure, as it does not need imaging software or even removing the roots from inside the basket. The second method used an LSD determination of differences using the same trait as Method 1. Method 3 was the most time consuming, as it required conducting a separate seminal root experiment by clear pots, as well as imaging each root crown to determine the angle at maturity. Interestingly, the clear pot and pasta strainer methods reached good agreement of RA for 11 of the tested genotypes, whereas the results were different for the remaining entries. The observed differences between the two stages could be explained by trait adjustment during the life cycle of the plant. A previous study in rice showed that RA changed from early to mature stages to follow moisture in water-scarce conditions (Uga et al., 2013). Hence, it is of interest to conduct analysis using both methods. In fact, there could be a specific interest in these more plastic genotypes that change root behavior over their lifespan, but more detailed studies are required to better understand these types. Still, the SRA vs. mature RA method could only determine significant differences for TKW under drip-irrigated trials, with the shallowest types (shallow to shallow) outperforming the other classes by 9 to 24%.

Considering that two of the three multitrait methods could distinguish differences in TKW under water-scarce field trials, it could be advisable to use Methods 1 or 2, as these are less time consuming and do not demand imaging software for processing. Instead, Method 3 becomes advantageous only when aiming for adaptation to drip irrigation.

4.3. Deeper Rooting Types Have Higher Yields under Terminal Droughts

The three multitrait methodologies were combined via hierarchical clustering to identify two mega-classes, one of shallower rooting genotypes, and one of deeper rooting types. This type of grouping reduces the amount of detailed classification of the single methodologies but allows combining of all measured traits. The two root mega-classes showed significant increases for GY under drought conditions, where water access is critical for wheat production. The genotypes allocating more roots at depth had on average 28 to 42% higher yield, probably due to a better capacity to capture deep soil moisture during grain filling (Lynch and Wojciechowski, 2015; Yu et al., 2015).

Since breeders are normally more interested by extreme types, a second distinction was made to identify five subclasses, two of which represent the shallowest and deepest rooting genotypes, and the remaining three the intermediate ones. Within the deepest rooting group are included the genotypes Margherita 2 and Omrabi 17, two elites that have been identified by several countries as most suited for environments prone to terminal drought, a type of condition most suitable for deep-rooting genotypes. Instead, the shallowest group includes Bellaroi and Jabal 2. The first is an Australian cultivar that performs particularly well in the southern region, where topsoil rarely exceed 60 cm in depth and the amount of rainfall received in short periodic intervals during crop growth period is higher than in northern and western regions. Also, Jabal2 finds its most appreciation in the Atlas Mountains of Morocco, where the impenetrable rock layers are located just 50 cm below the surface. In both cases, these appear as ideal conditions for shallow-rooted genotypes. Together, these considerations provide good support for the correct distinction into classes. Considering only the lines displaying extremely narrow and wide root phenotypes, the deepest types had a 37 to 38% yield increase under low moisture. However, under drip irrigation in sandy soils (MKZ16) and gravity irrigation in heavier calcic xerosols (TES16), the deepest rooted types had a significant yield disadvantage of 20 to 40%. Hence, there might be a cost

associated with seeking deeper soil layers when moisture is abundant. Therefore, breeders could make important yield gains by selecting for the root architecture that better fits the specific environment. For instance, Uga et al. (2013) showed yield gains in rice of 44 and 63% when genotypes carrying the narrow angle allele at the *DRO1* quantitative trait locus (QTL) were field tested under moderate and severe drought, respectively. Also, in wheat, many QTLs have been detected for RA that colocated with QTLs for GY and TKW in multienvironment studies (Canè et al., 2014; Maccaferri et al., 2016). Similarly, Richard et al. (2018) showed that it was possible to shift the allele frequency for RA in wheat, with good benefit for the lines combining favorable alleles for each root ideotype.

5. Conclusion

In this study, GY advantages of up to 40% could be obtained in both moisture-rich and -poor environment by selecting for the ideal root pattern. Further, grain size could be increased by up to 34% under moisture stress by selecting for deep-rooting types. Hence, it can be said that each root architecture is suitable to particular environmental scenarios and selecting and breeding for root system architecture is a cost-effective strategy to increase crop productivity and adaptation (Siddique et al., 2001).

The combination of methods presented here was confirmed as a suitable practice to identify these differences in durum wheat genotypes and can be used for breeding selection. The pasta strainer method is low cost, as it requires only the purchase of extremely affordable punctured plastic containers. In addition, the maintenance of the experiment occurs directly in the field, which reduces the investment in greenhouse maintenance. The most time-consuming aspect is certainly the removal of the plastic containers from the ground to study the fully developed mature root system of durum wheat. However, even this step is relatively simple when only the extruding roots are measured (PCA and LSD method), without the need for angle imaging. In addition, the RA showed strong heritability and therefore appeared as a trait of choice for rapid genetic gain through breeding. Together, the affordability, scalability, strong genetic control, and high effect on GY make the suggested methodology a protocol of choice for further use in breeding new cultivars that are well adapted to different moisture conditions.

Conflict of Interest

The authors declare that there is no conflict of interest.

Supplemental Material Available

Supplemental material for this article is available online.

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Chapter IV: Molecular dissection of root architectural traits and their association with drought adaptation in durum wheat.

El Hassouni K., S. Alahmad, A. AL-Abdallat, L.T. Hickey, M. Nachit, A. Filali-Maltouf, B. Belkadi, F.M. Bassi,

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We studied the genetic control of root system architecture in two developmental stages. Haplotype analysis confirmed a yield advantage under terminal drought stress of the genotypes carrying the major QTLs identified using GWAS.

Molecular dissection of root architectural traits and their association with drought adaptation in durum wheat.

Abstract

Roots play a key role for adaptation to environmental stresses. Hence, understanding the genetic control of root system architecture is of great importance to help durum wheat breeders deliver cultivars better adapted to climatic constrains. In this study, a panel of 100 durum wheat genotypes, including landraces and modern lines, was evaluated at two different developmental stages for seven of root traits using 'clear pot' and 'pasta strainer' methods. Large phenotypic variation was observed, with relatively high heritability and strong genetic effect. The germplasm was genotyped using 7,652 polymorphic SNPs markers via 35K Axiom array. A genome wide association study (GWAS) identified four major loci on chromosomes 3AS, 3BS, 5BL and 7BL associated with one or more root traits. Haplotype analysis on a larger set of 370 entries confirmed that the presence of the positive alleles at all the three QTLs associated with root angle provided 12% yield advantage when field tested under terminal drought stress. The QTL located on chromosome 3B was converted to KASP and validated on an independent set of elites to show that the positive allele explained >10% of the phenotypic variation for grain yield under terminal drought. This study provides new information on the genetic control of mature root system in durum wheat and it provides new tools for its exploitation in improving crop performance against terminal moisture stress.

Keywords:

Root system architecture, durum wheat, QTL, drought

1. Introduction

Durum wheat (Triticum durum Desf., 2n = 4x =28) is one of the most important cereal crops in the world. It is mainly cultivated in semi-arid regions where drought is a major limiting factor to its production, particularly when it occurs at anthesis (Loss and Siddique, 1994; Royo et al., 2010, Mohammadi et al., 2011; Bassi and Sanchez-Garcia, 2017). Moreover, climate change models for the Southern Mediterranean regions predict a severe reduction in moisture availability at the end of the cycle and increasing probability of severe droughts (Carvalho et al., 2014; ESCWA et al., 2017). Therefore, developing better adapted cultivars is of paramount importance.

Plant breeders have historically focused their selection on aboveground plant traits to increase grain yield, whereas the below-ground traits have been somewhat neglected, and their level of understanding is well-behind that of plant's aerial components (Zhang et al., 2009). Roots play an important role in capturing soil resources (Smith and De Smet, 2012; Lynch et al., 2013) and hence, influence the plant growth development and productivity (Shen et al., 2013; Palta and Yang, 2014; Lynch and Wojciechowski, 2015; Paez-Garcia et al., 2015). Examining and understanding the parameters defining root system architecture is a promising strategy to cope with drought and other edaphic stresses (Manschadi et al., 2008). However, the multiple parameters related to drought adaptation such as root length, branching, and root depth are difficult to measure (Tuberosa et al., 2002). Still, these traits have been often associated with the crown root angle (Nakamoto et al. 1991; Oyanagi et al., 1993; Oyanagi, 1994; Borell et al., 2014), and this is an easier trait to measure and a good proxy for root distribution in the soil layers (Manschadi et al., 2006; Lilley and Kirkegaard, 2011). For example, genotypes with narrow root angle have been associated with deeper root systems that can extract water stored in the deep soil layers (Ehdaie et al., 2003; Manschadi et al., 2006; Hammer et al., 2009; Wasson et al. 2012; Uga et al., 2013). Especially late in the season, this becomes particularly advantageous since most of the superficial moisture is rapidly consumed or it evaporates, while water can still be found in the lower layers (Kashiwagi et al., 2005). In other circumstances, a shallow root system derived from a wider root angle can be more advantageous, especially when high evaporation, the soil type, or the strategy for providing moisture do not allow the water to percolate to deeper soil layers. This was shown to be the case in agro-ecologies with shallow soils combined with short intermittent rainfalls, or when using drip irrigation, or when daily temperatures exceed 30° C (Robinson et al., 2018; El Hassouni et al., 2018; Alahmad et al., 2019). The relationship between root angle and yield is context dependent. Therefore, "one size does not fit all", and it is critical to deploy the root system that better fit each target to improve crop adaptation and maximize yield (Siddique et al., 2001; El Hassouni et al., 2018).

In that optic, it becomes essential to dissect the molecular basis of the different root traits to better understand how these interact with the environment and what combinations are the most suitable. Association mapping has been already used to find the genomic regions controlling some of the root related traits (Canè et al., 2014; Maccaferri et al., 2016; Robinson et al., 2016; Alahmad et al., 2019), and a major QTL

controlling seminal root angle has been reported for chromosome 6A of durum wheat (Alahmad et al., 2019).

In this study, a panel of durum wheat genotypes was evaluated at two different developmental stages for a seven of root traits using 'clear pot' and 'pasta strainer' methods to identify the variation among genotypes. Genome-wide association study using SNP markers was used to identify the genomic regions involved in their control.

2. Materials and methods

2.1. Germplasm

Three set of germplasm were used in this study. A discovery set of 100 durum wheat (2n = 4x = 28, AABB) genotypes (table S1) was selected from a larger panel of 384 landraces, cultivars and elites originating from different countries. The subset is representative of the global genetic diversity and it consists of elite breeding lines from ICARDA (49) and CIMMYT (13), cultivars (29) and landraces (9). To reduce the bias of phenology in the experiment, the accessions were pre-selected for having similar flowering time.

The second set of germplasm was used for haplotype analysis and it included all the 370 genotyped lines of the original panel.

The third was used to convert Axiom markers into KASP and validate them. It included 94 ICARDA's elites that constituted the 2017 international nurseries 40th IDYT and 40th IDON. This set was also tested at the station of Marchouch.

2.2. Phenotyping

2.2.1. 'Clear pot' and 'pasta strainer' methods for characterization of seminal root and mature root system traits

The detailed phenotyping methods used in this study was described by El Hassouni et al. (2018). The panel was phenotyped for seminal root angle (SRA) using the 'clear pot' method initially developed by Richard et al. (2015). Seeds were sown facing the pot wall with the embryo downward and the angle was measured for the first pair of seminal roots at 3 cm depth. Images were then captured five days after sowing and used for measuring the angle with the free software 'ImageJ'. Plants were grown in a controlled-environment growth room with 12h photoperiod under constant temperature (18°C) and the experiment was conducted using randomized complete block design (RCBD) with 4 replicate seeds for each genotype.

The same panel was evaluated for mature root system using the 'pasta strainer' method (EL Hassouni et al., 2018). Individual plants were space-planted in near field conditions at Guich experimental station (33°59' N, 6°50' W; Rabat, Morocco). Plastic pasta strainers were buried in the soil with 20 cm distance between each other. Three seeds were placed in the middle of each pasta strainer representing one genotype and the three seedlings were thinned to one at the fourth leaf stage (growth stage 14, according to the Zadoks decimal growth scale; Zadoks et al., 1974). Standard cultural practices were adopted. This experiment was conducted during the season 2015-2016 under α lattice design with two replications, and sub-blocks of size 10, following a rows-columns spatial design. At maturity, the pasta strainers were shovel out and belowground traits were recorded. The strainer was divided into three sections to count the root ratios for each section RR 2–8 cm (RL1), RR 8–10 cm (RL2), and RR 10–13 cm (RL3), and the total root number (TRN). After rinsing in water, the roots were dried to obtain root biomass within the strainer (RB), and then scanned to measure the mature root angle (RA) with ImageJ software.

2.2.2. Field yield trial

The 100 lines used in the two experiments mentioned above were included in yield trial containing the whole collection of 384 genotypes. The trial was conducted during two cropping seasons 2015/16 and 2016/17 in Marchouch (MCH) station in Morocco, which represents a rainfed environment with no rainfall occurring after anthesis and severe terminal drought. The rainfall was 234 mm and 280 mm for 2015-16 and 2016-17 respectively during the growing season. The soil type is clay-lime with depths of 0.8-0.9 m. An augmented design was used with 19 blocks of 24 plots, each block included four commercial checks. Sown plot size was 3.6 m2 (six rows), and 2.4 m2 (four rows) were harvested for assessing yield performances. Optimal agronomic management practices were applied during the crop season.

2.3. Statistical analysis

For the 'clear pot' method, a two-dimensional autoregressive (AR1×AR1) model was fitted to the experimental data in ASReml-R library (Butler et al., 2009) to account for the spatial variation in the glasshouse. To obtain BLUPs the genotype, replicate, pot, and position were fitted as random terms.

For the 'pasta strainer' experiment, a mixed model with a two-dimensional P-spline

basis was fitted to the data to account for the spatial trend in the space-planted field conditions. The best linear unbiased predictors (BLUPs) were obtained considering genotype and block within replicate as random effects and the replicate as fixed term. The model was fitted in R using SpATS library (Rodriguez-Alvarez et al., 2018).

The BLUPs of each trait were used to estimate Pearson's correlation coefficients, and the critical value was determined to be 0.20 for $p < 0.05$ ($df = 98$) using the corrplot package (Wei and Simko, 2017).

To determine the root classes (class), multi-trait analyses using the three classification methods and combined multi-trait analysis were performed as described in EL Hassouni et al. (2018).

The field trial was analyzed using a mixed linear model to obtain best linear unbiased estimates (BLUEs) using the lme4 package (Bates et al., 2015) and considering genotypes and environments as fixed terms. The emmeans package (Lenth et al., 2017) based on ANOVA model was used to discriminate among the grain yield means of haplotypes. All statistical analyses were computed in R (R Development Core Team, 2016).

2.4. Genotyping

The panel was genotyped using 35K Affymetrix wheat Breeders array at Trait Genetics (Gatersleben, Germany) following the manufacturer instructions. The details of this genotyping step have been previously discussed in Kabbaj et al. (2017). Briefly, from a total of 35,143 SNP markers 7,652 high-fidelity polymorphic SNPs were obtained, with less than 1% missing data, a minor allele frequency superior to 5% and heterozygosity <10%. The sequences of these markers were aligned with a cut-off of 98% identity to the Durum wheat genome assembly (Maccaferri et al., 2019), to define their physical position. A sub-set of 500 highly polymorphic SNPs were selected on the basis of even distribution across the genome for structure analysis which reveal the existence of 10 main sub-groups (Kabbaj et al. 2017). To avoid bias, these 500 markers were then removed from all downstream analysis.

2.5. Association mapping

Linkage disequilibrium was calculated as squared allele frequency correlations (r2) in TASSEL V 5.0 software (Bradbury et al. 2007), using the Mb position of the markers along the reference genome. Linkage disequilibrium (LD) decay was measured at the

value 51.3 Mb for $r^2<0.2$ as presented in Bassi et al. 2019. The BLUPs obtained for all the root traits were searched for association with the allelic scores of 7,652 Axiom markers and find marker trait associations (MTAs). The LD decay value was used to determine the significant threshold for MTAs based on the Bonferroni correction, and it resulted in LOD = 3.1 for $p<0.01$ (Bassi et al. 2019). All the MTAs analysis were performed using Tassel 5 software (Bradbury et al., 2007). Two models were fitted and compared using two covariate parameters, Q (population structure) and K (Kinship matrix). Q model was performed using a general linear model (GLM), and Q+K model using a mixed linear model (MLM). The kinship matrix calculated by Kabbaj et al. 2017 was used. The best model for each trait was selected based on the quantile-quantile (Q-Q) plots examination (Sukumaran et al., 2012). MTAs that occurred at a distance inferior to 104 Mbp (twice the LD decay) were considered to belong to the same QTL.

2.6. Conversion of markers to Kompetitive Allele Specific PCR (KASP)

The Axiom array sequences of 10 markers associated to root architectural traits were submitted to LGC Genomics for in silico design of KASP primers using their proprietary software. Those that passed the *in silico* criteria were purchased and used to genotype the independent validation set. For each marker that amplified and showed polymorphism, the regression cut-off between phenotype and haplotype was imposed at r = 0.105 following Pearson's critical value (Pearson, 1895).

3. Results

3.1. Phenotypic variation for root traits

The germplasm was evaluated for SRA by 'clear pot' method and for mature root angle, total root number, and root biomass by 'pasta strainer' method. Large variation was observed for all the root traits measured (Table 1). Based on the spatial terms, global variation and trend were more important than local variability except for RL1 (Figure S1). The panel had an average angle of 71.4° and 66.2° for seminal (range 43.7° – 109°) and mature (range 38.6° – 102°) roots, respectively. 'Jupare C2003' had the narrowest and 'ADYT_097' the widest SRA, while 'Artena' displayed the narrowest phenotype and 'unibo024' the widest for mature root angle. Root biomass ranged from 2.3 to 11.4 g, with a mean value of 5.6, and the landrace 'ig:85026' showed the highest root biomass, whereas 'unibo066' had the lowest. Root ratio per level ranged from 0.17 for RR 2-8 cm to 0.55 for RR 8-13 cm. Two main root classes were defined via

hierarchical clustering incorporating 39 genotypes with shallow root system, and 61 genotypes with preference for exploring the deep soil layers (Figure S2). The coefficients of variation (CV) value ranged from 9 % for RR 8-10 cm to 28 % for RB. The broad-sense heritability values ranged from 0. 60 for RR 8-10 cm to 0.93 for RA of adult plants, indicating that all the root traits scored are relatively stable and more controlled by genetic factor than the environment.

The frequency distributions of the traits were approximately normal, with slight skewness for RL3 and RA (Figure 1), but no transformation was deemed necessary for downstream analysis.

Table 1. Descriptive statistics and heritability for seven root traits

Trait	Abbreviation	Mean	Min.	Max.	CV[a]	IQR[b]	Gvar[c]	H^2
Root ratio 2-8 cm	RR 2-8 cm	0.28	0.17	0.45	24.35	0.09	0.01***	0.84
Root ratio 8-10 cm	RR 8-10 cm	0.35	0.27	0.44	9.26	0.03	0.00***	0.60
Root ratio 10-13 cm	RR 10-13 cm	0.38	0.22	0.55	21.27	0.14	0.00**	0.85
Total root no.	TRN	44.00	33	58	12.98	8.65	55.24**	0.66
Root biomass (g)	RB	5.64	2.28	11.4	28.78	1.86	3.23**	0.89
Root angle (°)	RA	66.20	38.6	102	22.99	22.4	255.9**	0.93
Seminal root angle (°)	SRA	71.40	43.7	109	22.04	23.66	263.42**	0.89

a: coefficient of variation; b: interquartile range; c: genetic component of variance

3.2. Correlation among root traits

The phenotypic correlations among the examined root parameters are reported in Figure 1. Correlation analysis showed significant associations amongst most of the traits investigated. RR 10-13 cm was significantly and negatively correlated to RR 2-8 cm (r=-0.83) and RA (r=-0.84) that had a positive association (r=0.77). RR 10-13 cm exhibited also a significant negative correlation with RB (r=-0.34) and TRN (r=-0.49). In addition, RB showed a significant positive association with TRN (r=0.52) and RA (r=0.26) and these two traits were positively correlated (r=0.38). There was no apparent correlation between adult plant RA and SRA.

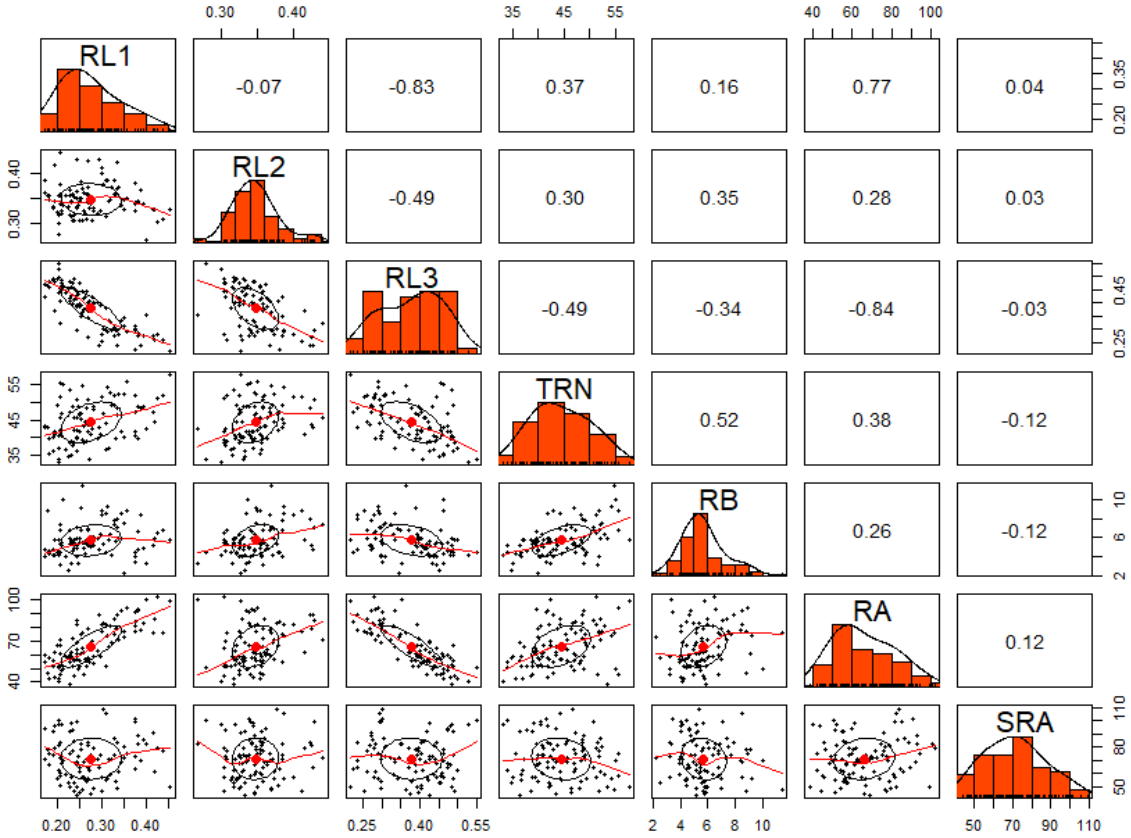

Fig. 1. Pearson's correlation matrix and frequency distribution of the seven root traits measured in this study. Scatter plot with fitted smoother of the seven traits measured (below diagonal). Pearson correlation values (above diagonal). RL1, root ratio in the first soil level (RR 2-8 cm); RL2, root ratio in the second soil level (RR 8-10 cm); RL3, root ratio in the third soil level (RR 10-13 cm); TRN, total root number; RB, root biomass: RA, root angle of adult plants; SRA, seminal root angle.

3.3. Genome wide association mapping of root traits

Genome wide association mapping was carried out for all the root traits. A total of 21 MTAs corresponding to four QTLs were significantly associated with one or more traits (Table 2). Depending on the marker and the trait, the MTAs explained from 10 to 15 % of the phenotypic variation. Two QTLs located on chromosomes 3AS and 3BS (Q.icd.root.01 and Q.icd.root.02) were found associated with RR 10-13 cm, RA and RB, one QTL on chromosome 5BL controlled RB (Q.icd.root.03), and one on chromosome 7BL affected SRA and root class (Q.icd.root.04).

3.4. Effect of different allele combination on grain yield

Haplotype analysis was conducted for the three QTLs controlling more than one trait

(Q.icd.root.01, Q.icd.root.02, Q.icd.root.04) to assess their allelic effect on grain yield under terminal drought (Figure 2). Four haplotype classes could be identified among the tested germplasm when considering only the allele harbored by the marker reaching the highest LOD score. The haplotype class carrying the favorable alleles at all three loci had the highest GY reaching an average of 2,537 kg ha^{-1} with a maximum value of 3,036 kg ha^{-1}. The group of lines with only two and one positive alleles reached average GY of 2,349 kg ha^{-1} and 2,257 kg ha^{-1}, respectively, whereas genotypes that did not carry any positive allele averaged 2,229 kg ha^{-1}. The analysis of variance confirmed that the haplotype group with all three positive alleles was significantly superior to the others, and it provided an average yield gain of 8% compared to the two positive alleles class, 11 % to one positive allele class and finally 12 % to the class without any positive allele.

Table 2. Significant QTLs associated with root traits.

Locus	Chr.	Trait	Main marker	Position	Max LOD	Max r^2
Q.icd.root.01	3A	RL3, RA, RB	AX-94840598	149,265,969	3.42	0.13
Q.icd.root.02[a]	3B	RL3, RA, RB	AX-95176186	220,340,053	3.42	0.13
Q.icd.root.03	5B	RB	AX-94708811	554,341,230	3.45	0.11
Q.icd.root.04	7B	Class, SRA	AX-95175842	641,697,572	3.51	0.15

[a] QTL validated to KASP marker
Chr. – chromosome; RL3 - Root ratio in the third level; RA – Root angle; RB – Root biomass; SRA – seminal root angle

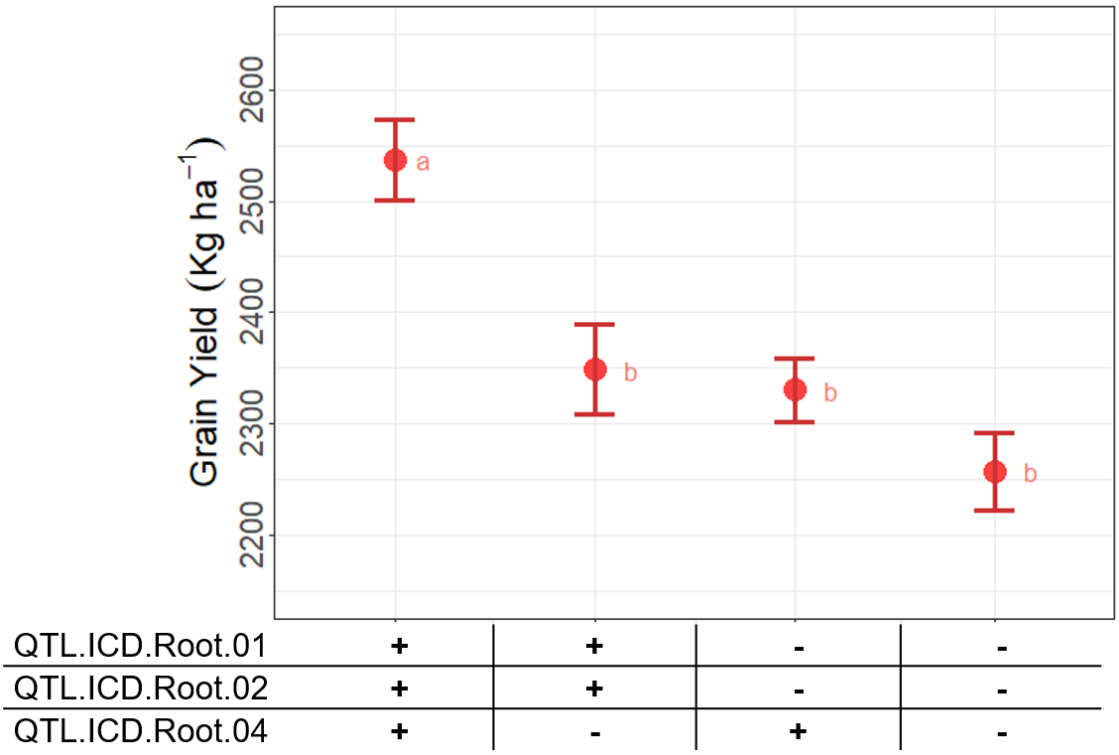

Fig. 2. Effect of different allele combinations of the significant loci associated with root angle on yield performance of 370 accessions tested under drought stressed conditions in Marchouch, Morocco. Average (mean ± SE) of 2 seasons are presented. The genotypes were divided into four clusters based on their allelic content. "+" mark the positive and "-" the unfavorable alleles.

3.5. Validation of marker for MAS

The Axiom sequences of 10 MTAs associated with the four QTLs identified have been submitted to LGC Genomics for designing of KASP primers. For five sequences, the *in silico* approach failed to design reliable primers and where then discarded. The remaining five were used for genotyping an independent set of ICARDA's elites. Three failed the amplification and one did not identify any polymorphism within this germplasm set. Only AX-95176186, tagging Q.icd.root.02, identified a clear polymorphism among the lines. Its allelic score explained >10% of the grain yield variation when the germplasm was field tested at Marchouch station under terminal drought.

4. Discussion

4.1. Variation for root architectural traits

Plant roots play a pivotal role in assuring the uptake of inputs from the soil and converting it to yield (Hirel et al., 2007; Hodge et al., 2009; Blum, 2009). Regardless of

their recognized importance in increasing productivity, the incorporation of root selection as part of breeding is limited by the low throughput of the existing screening methods and their scarce scalability (Canè et al., 2014). Two medium-throughput root phenotyping techniques have been recently adapted to accelerate durum wheat research: the 'clear pot' method (Samir et al., 2018, 2019) and the 'pasta strainer' method (El Hassouni et al., 2018). Here, these methodologies were deployed on a set of 100 durum wheat genotypes, to define a good range of variation for all root traits. These appeared to be under strong genetic control, as demonstrated by the high broad-sense heritability measured. This result was in good agreement with other authors that reported high variation and good heritability for seminal and mature root traits in durum wheat (Maccaferri et al. 2016; El Hassouni et al. 2018; Alahmad et al. 2019), but also in maize (Pace et al., 2015; Cai et al., 2012), sorghum (Mace et al., 2012), and bread wheat (Richard et al., 2015, 2018). These results confirm that there is good potential for genetic gain in below-ground traits, if breeding selection can be scaled.

4.2. Identification of genomic loci associated with the control of root architectural traits

To more readily deploy breeders' selection for root architecture traits, several authors have called for the need to identify the loci underpinning the control of these traits (Varshney et al. 2005; Maccaferri et al. 2016; El Hassouni et al. 2018; Alahmad et al. 2019). Here, four major QTLs were identified on chromosomes 3AS, 3BS, 5BL and 7BL of durum wheat. Two QTLs were detected in somewhat colinear regions on chromosomes 3AS and 3BS at positions of 149 Mbp and 220 Mbp, respectively. These were associated to the same three traits: root growth angle for adult plants characterized using two techniques, the image analysis (RA) and the root ratio (RR 10-13 cm), and root biomass (RB). The genome browser of the durum wheat genome assembly (Maccaferri et al. 2019) was used to scout for overlaps between QTLs presented here and those previously reported in the literature. Maccaferri et al. (2016) reported QTL1530_3A responsible to control the number of seminal root tips in the Colosseo/Lloyd mapping populations in the same 3AS position of Q.icd.root.01. In addition, Iannucci et al. (2017) also reported a QTL in close proximity and associated with seminal root tips number, and (Guo et al., 2012) with total root length.

Maccaferri et al. 2016 also reported QTL1539_3B controlling average root length and

QTL1540_3B associated with total seminal root length in the Meridiano/Claudio mapping population as overlapping with Q.icd.root.02. Ma et al. (2017) and Kabir et al. (2015) also reported for chromosome 3BS QTLs associated with total seminal root length.

In the case of Q.icd.root.03 on chromosome 5BL associated with RB, Maccaferri et al. (2016) reported in the same genomic location QTL0509_TRL, identified by association mapping as controlling total root length. Several authors (Ma et al., 2017; Kabir et al., 2015; Bai et al., 2013; Iannucci et al., 2017) also reported an involvement of this locus with root length, root diameter, and number of root tips.

Finally, for Q.icd.root.04 on chromosome 7BL associated with SRA and root class, Maccaferri et al. (2016) identified via association studies QTL0545_RGA as also responsible for seminal root growth angle and QTL0544_PRL for seminal primary root length. In addition, this region has been previously determined to be involved with the control of root length and root surface area in wheat seedling (Iannucci et al., 2017, Ren et al., 2012, Liu et al., 2013, Kabir et al., 2015, Maccaferri et al., 2016, Ma. et al., 2017).

Hence, all the QTLs identified in this study have been previously reported by other authors in seedling screenings, and their importance for durum wheat is made apparent. Still, this is the first time that they have been associated with adult root traits. For root angle, it was not possible in our study to identify QTLs involved in simultaneously controlling seminal and adult root angle. This seems to suggest that different genetic basis govern root architecture in different stages of the plant growth as suggested by Ehdaie et al. (2014) and by us in a previous work (El Hassouni et al. 2018). However, other authors found that root traits measured in seedling stage were good indicators of mature plants performances (Manschadi et al., 2008; Placido et al., 2013) and predicted the root system in the field (Li et al., 2015; Landi et al. 2016). With the exception of Q.icd.root.04, it was not possible to pinpoint any QTLs associated with SRA in our study, even though other authors reported them for the same locations were our adult root QTLs were identified. Richard et al. (2018) also reported that seedling root architecture of wheat is controlled by QTLs that are different than those for mature root trait as in our case, but they identified a region for SRA on 6A, which has also been identified as critical by other authors (Maccaferri et al. 2016; Alahmad et al. 2019). Our study failed to identify this region. Hence, it can be concluded that the germplasm used here, which included also several landraces, it is not segregating for

this region and it has limited diversity for SRA.

4.3. Markers to be deployed in MAS for drought tolerant genotypes

Four groups with different allelic combination were identified when combining the three significant QTLs associated with root angle of the first developmental stage and adult plants. Two loci were commonly detected for both root angle and root biomass and one major locus for seminal root angle. The effect of these allelic groups on grain yield was then assessed and showed that these traits play significant role in grain yield performance of genotypes grown under terminal drought stress. Root angle, a root system architectural feature of great significance was recently highlighted by several authors (Manshadi et al., 2006; Christopher et al., 2013; Kuijken et al., 2015). It is a valuable indicator of root system distribution and thus a useful proxy for rooting depth (Kato et al., 2006; Wasson et al., 2012). Deeper exploratory roots enable the plant to capture stored water at depth in environment experiencing terminal drought (Manschadi et al., 2008, Lynch, 2013; Voss-Fels et al., 2018, EL Hassouni et al., 2018) contrary to shallow root system that is more advantageous for plants growing in shallow soils with intermittent rainfall or in environments receiving high amount of rainfall in short periodic intervals (Voss-Fels et al., 2018). This implies that root architecture affects the plant performance. Hence, in order to maximize the productivity for a rapid genetic gain, plant breeding programs have to optimize the plant's hidden half characteristics in addition to above-ground traits. Therefore, the combination of the three positive alleles of QTLs identified in this study seemed to be very important and may help in providing molecular markers that will facilitate deployment of the root angle in future breeding programs focused on improving yield under water-limited environments. The genetic control of root architecture could provide an efficient manipulation of root system architecture via marker-assisted selection.

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Chapter V: Loci controlling adaptation to heat stress occurring at the reproductive stage in durum wheat

El Hassouni K., B. Belkadi, A. Filali-Maltouf, A. Tidiane-Sall, AL Abdallat, M. Nachit, F.M. Bassi.

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Loci governing heat stress tolerance were detected using GWAS in a subset of durum wheat genotypes. Significant SNPs were converted into KASP markers for deployment via MAS. Four genotypes were confirmed as tolerant carrying positive alleles for the main genomic regions.

Loci Controlling Adaptation to Heat Stress Occurring at the Reproductive Stage in Durum Wheat

Abstract

Heat stress occurring during the reproductive stage of wheat has a detrimental effect on productivity. A durum wheat core set was exposed to simulated terminal heat stress by applying plastic tunnels at the time of flowering over two seasons. Mean grain yield was reduced by 54% compared to control conditions, and grain number was the most critical trait for tolerance to this stress. The combined use of tolerance indices and grain yield identified five top performing elite lines: Kunmiki, Berghouata1, Margherita2, IDON37-141, and Ourgh. The core set was also subjected to genome wide association study using 7652 polymorphic single nucleotide polymorphism (SNPs) markers. The most significant genomic regions were identified in association with spike fertility and tolerance indices on chromosomes 1A, 5B, and 6B. Haplotype analysis on a set of 208 elite lines confirmed that lines that carried the positive allele at all three quantitative trait loci (QTLs) had a yield advantage of 8% when field tested under daily temperatures above 31° C. Three of the QTLs were successfully validated into Kompetitive Allele Specific PCR (KASP) markers and explained >10% of the phenotypic variation for an independent elite germplasm set. These genomic regions can now be readily deployed via breeding to improve resilience to climate change and increase productivity in heat-stressed areas.

Keywords: heat stress; durum wheat; yield; tolerance; fertility; climate change; resilience

1. Introduction

Heat stress is a major environmental constraint to crop production. Terminal heat stress is defined as a rise in temperature that occurs between heading and maturity. When this stress matches with the reproductive phase of the wheat plant, it affects anthesis and grain filling, resulting in a severe reduction in yield [1]. High temperatures at the time of flowering cause floret sterility via pollen dehiscence [2], decrease photosynthetic capacity by drying the green tissues, and reduce starch biosynthesis [1,3]. These in turn result in a negative effect on grain number and weight [4–7]. The optimum growing temperature for wheat during pollination and grain filling phases is

21 °C [8,9], and for each increase of 1 °C above it is estimated a decline of 4.1% to 6.4% in yield [10]. Environmental temperatures have been increasing over the last century and more frequent heat waves are predicted in the next decades [11–13]. Therefore, breeding for tolerance to chronic as well as short term heat stress is a major objective worldwide [14–19]. Breeding selection would benefit by a better understanding of traits associated with tolerance to high temperatures, as well as the identification of the genomic regions controlling these traits.

In wheat, a large number of quantitative trait loci (QTLs) has been identified under heat stress via linkage analysis and genome-wide association study (GWAS) for yield, yield related traits, and some physiological traits such as chlorophyll content, chlorophyll fluorescence, and canopy temperature [20–27]. Grain number per spike and chlorophyll content were found to be the most critical traits for adaptation to warm conditions [24,25,28]. Heat stress reduces leaf chlorophyll content [29] affecting the amount of carbohydrates transported to the grains and final grain weight and size. High temperatures around anthesis reduce the number of grains per spike due to a decrease in spike growth and development, and an increase in ovules abortion [2,25,29,30]. To the best of our knowledge, molecular markers associated with heat tolerance are not generally used in wheat breeding programs [31–33]. The limited understanding of genes underlying physiological mechanisms and the regulation of yield components in wheat, and the lack of cloned major QTL for traits associated with heat tolerance has restricted the improvement in breeding for tolerance to this stress.

In the current study, a set of durum wheat lines were heat stressed by imposing a > 10 °C raise in maximum daily temperatures via the deployment of plastic tunnels at the time of flowering. GWAS studies allowed the identification of major QTLs controlling the adaptation to this stress and these were validated for marker assisted selection (MAS) in an independent germplasm set for rapid deployment via breeding.

2. Materials and Methods

2.1. Plant Material

A subset of 42 durum wheat inbred lines were selected from a global collection of 384 genotypes based on their similarity in flowering time and identified genetic diversity [34]. Briefly, the complete collection is highly diverse and includes 96 durum

wheat landraces from 24 countries, and 288 modern lines from nine countries and two International research centers CIMMYT and ICARDA. The subset selected for this study includes 34 ICARDA and CIMMYT lines, five cultivars and one landrace. The list of the 42 genotypes and their details are provided in Table S1.

A second subset of 208 modern entries was also obtained from the global collection and field tested under severe high temperatures during 2014–2015 and 2015–2016 seasons along the Senegal River in Kaedi, Mauritania. Full details on this field experiment have been published in Sall et al. [35].

The third and final set was used for Kompetitive Allele Specific PCR (KASP) markers validation and it was composed of 94 ICARDA's elite lines that constituted the 2017 international nurseries 40th International Durum Yield Trial (IDYT) and 40th International Durum Observation Nurseries (IDON). This set was also tested at the station of Kaedi along the Senegal River in season 2015–2016.

2.2. Field Experiment Conditions and Phenotyping

The first subset of 42 entries was grown at Marchouch station (33°34'3.1" N, 6°38'0.1" W) in Morocco during two successive crop seasons (2015–2016 and 2016–2017). Each entry was sown in mid-November on a plot surface of 1.5 m^2 per genotype at a sowing density of 300 plants per m^2. The experiment was an alpha lattice with two replications, block size of six, and two treatments arranged in split-plot. Each six genotypes were arranged in close proximity to maximize competition between the genotypes, and compose one block of 9 m^2. Each block was surrounded by a border of barley to avoid border effect. Each block was spaced 1 m apart to allow the application of the plastic tunnel. The two treatments were normal rainfed conditions and plastic tunnel-mediated heat stress. The normal treatment followed standard agronomic practices with a base pre-sowing application of 50 Kg ha^{-1} of N, P, and K. At stage 15 of Zadok's (Z) scale herbicide was applied in a tank mixture (Pallas + Mustang at 0.5 L ha^{-1}) to provide protection against both monocots and dicots. At Z17 ammonium nitrate was provided to add 36 kg ha^{-1} of N and a final application of urea was used to add 44 kg ha^{-1} of N before booting (Z39). Weeds were also controlled mechanically to ensure clean plots. The soil of the experimental station is clay-vertisol type. The available on season moisture was 234 and 280 mm for 2015–2016 and 2016–2017, respectively, during the growing season, whereas the average daily

temperature was 14.1 ˚C for the first year and 13.5 ˚C for the second year. The heat-stress treatment followed the same agronomic practices, with the difference that at the time of booting (Z45) a 10 m^2 and 1.5 m high plastic tunnel was placed over each block (Figure 1) and left there until early dough stage (Z83). An electronic thermometer (temperature data logger) was placed in the middle of each block (normal and heat stressed) to reveal that the temperatures were up to 16˚ C higher inside the plastic tunnels, to reach a maximum of 49 ˚C (Figure 1). Marchouch is a drought prone site, and no rainfall occurred after Z45 in any of the two field seasons.

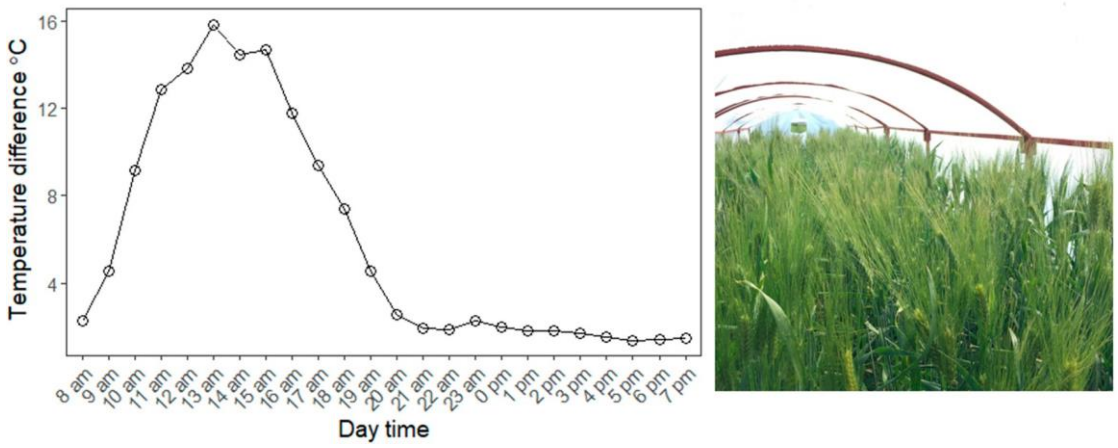

Fig. 1. Mean temperature difference of 18 days over two seasons between the plastic tunnel-mediated heat stress and normal field conditions between 8 a.m. and 8 p.m., and a picture of the plastic tunnel at 9 a.m.

The following traits were recorded: days to heading (DTH) measured at the moment when the awns became visible, plant height (PH) measured from the ground to the top of the highest spike excluding the awns, and the number of fertile spikes per meter square (Spkm^2) was counted in a 0.25 m^2 area. The whole plot was harvested by hand and the dry biomass (Biom) was weighed before threshing. Grain yield (GY) was weighed for each plot and expressed as kg ha^{-1}. The weight of a thousand kernels (TKW) was expressed in grams. The harvest index (HI) was calculated as the ratio between GY and Biom. The grain number per spike (GNSpk) was derived from dividing grain number per meter square by Spkm^2 as follows:

$$\text{Grain number/m}^2 = \frac{\text{Grain weight of the plot}}{1.5m^2 \times {}^{TKW}/_{1000}} \quad (1)$$

$$\text{GNSpk} = \frac{\text{Grain number/m}^2}{Spkm^2} \quad (2)$$

The second and third sets were field tested in Kaedi, Mauritania (16°14” N; 13°46” W) during season 2014–2015 and 2015–2016 where the temperature reached a maximum of 41 °C and an average maximum daily temperature of 34 °C throughout the season. The trial was carried out under augmented design with a plot surface of 4.5 m2. Standard agronomic management practices were adopted. Full details for this experiment are published elsewhere [35].

2.3. Data Analysis

A mixed linear model was run using the lme4 package [36] in R [37] to obtain best linear unbiased estimates (BLUEs) of the normally distributed traits. For count traits (DTH, Spkm^2, GNSpk), the generalized mixed linear model was used to get the BLUEs by Proc GLIMMIX in SAS. In both models, genotype, treatment, year, and replication were considered as fixed effects and block as random effect nested in treatment and year. Broad-sense heritability was calculated based on variance components from random model using the method suggested by DeLacy et al. [38]:

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + \frac{\sigma_{G\times T}^2}{t} + \frac{\sigma_{G\times Y}^2}{y} + \frac{\sigma_{G\times T\times Y}^2}{ty} + \frac{\sigma_e^2}{tyr}} \quad (3)$$

Where: $\sigma_{G\times T}^2$ = genotype × treatment variance, $\sigma_{G\times Y}^2$ = genotype × year variance, $\sigma_{G\times Y\times T}^2$ = genotype × treatment × year variance, σ_e = residual variance, *r* is the number of replications per treatment, *t* is the number of treatments, and *y* is the number of years.

Box-and-whisker plots where constructed by ggplot2 package [39] using the BLUEs combined over year per each treatment. The relationship between the target trait GY and yield components (GNSpk, TKW, Biom, HI) was studied using the Pearson correlation coefficient and the additive regression model. The critical value of the correlation significance was determined at 0.30 for $p < 0.05$ and 0.39 for $p < 0.01$ for 40 df using the corrplot package [40]. The additive model incorporates flexible forms (i.e., splines) of the functions to account for non-linear relationship contrary to linear regression model estimated via ordinary least squares [41]. For the additive model, the effective degree of freedom term determines the nature of the relationship between the

predictor and the response variables where EDF= 1 indicates linearity and EDF > 1 the non-linearity. The additive regression analysis was performed using the mgcv package [42].

Two stress tolerance indices were calculated to identify the heat tolerant genotypes. The stress susceptibility index (SSI) [43,44] was calculated as follows:

$$SSI = \frac{[1 - (Ys)/(Yp)]}{[1 - (\bar{Y}s)/(\bar{Y}p)]} \quad (4)$$

Where Ys and Yp are yield values of the genotypes evaluated under heat stress and normal conditions, respectively, and $\bar{Y}s$ and $\bar{Y}p$ are the mean yields of the lines evaluated under heat stress and normal conditions, respectively.

The stress tolerance (TOL) [45] was calculated as follows:

$$TOL = Yp - Ys. \quad (5)$$

The classInt package [46] was used to identify the possible number of class intervals of the indices for the frequency distribution of the subset.

The cut-off value for tolerant vs. susceptible genotypes for SSI was equal to 1, with lines having SSI < 1 being stress tolerant. Regarding the TOL index, the smaller TOL values indicate the genotypes with low yield depression and hence more tolerant. The experiment-wide TOL mean (1608 kg ha^{-1}) was identified as the cut-off value for tolerant vs. susceptible. The emmeans package [47] based on ANOVA model was used to discriminate among the grain yield means of haplotypes.

2.4. Genotyping and Marker-Trait Associations

Details of the genotyping step of the core set and panel have been previously discussed in Kabbaj et al. [34] and Sall et al. [35]. Briefly, 7652 high-fidelity polymorphic single nucleotide polymorphism (SNPs) were obtained, showing less than 1% missing data, minor allele frequency (MAF) higher than 5%, and heterozygosity less than 5%. The sequences of these markers were aligned with a cut-off of 98% identity to the durum wheat reference genome [48] (available at: http://www.interomics.eu/durum-wheat-genome), to reveal their physical position. The average length of the Axiom probe is of 75 bp, hence the 2% allowed miss-match was set to account for the existence of 1 SNP within each sequence. A sub-set of 500 highly polymorphic SNPs were selected on the basis of even spread along the

genome, and used to identify the existence of population sub-structure, which revealed the existence of 10 main sub-groups [34]. To avoid bias, these 500 markers were then removed from all downstream association analysis. Linkage disequilibrium was calculated as squared allele frequency correlations (r^2) in TASSEL V 5.0 software [49], using the Mb position of the markers along the bread wheat reference genome. Linkage disequilibrium (LD) decay was estimated and plotted using the "Neanderthal" method [50]. The LD decay was measured at 51.3 Mb for $r^2 < 0.2$ as presented in Bassi et al. [51].

The genome wide association study (GWAS) was based on BLUEs of all the traits that displayed a significant treatment effect and the two stress tolerance indices. Two models were fitted and compared using two covariate parameters, Q (population structure) and K (Kinship). Q model was performed using a general linear model (GLM), and Q + K model using a mixed linear model (MLM). The best model for each trait was selected based on the quantile-quantile (Q-Q) plots [52]. Flowering time (DTH) was used as covariate in all analyses to remove the strong effects of flowering genes from the study. The value calculated for the LD decay of 51.3 Mb indicated that this association panel interrogated the 12,000 Mb of the durum wheat genome via 248 "loci hypothesis," and hence the Bonferroni correction for this panel was set to 3.1 LOD for $p < 0.05$ as suggested by Duggal et al. [53]. Local LD decay for $r^2 < 0.2$ was calculated for a 100 Mbp window around the marker with highest LOD for all marker-trait associations (MTAs) identified at a distance inferior to 104 Mbp (twice the LD decay). The MTAs that occurred at a distance inferior to twice the local LD were considered to belong to the same QTL. QTL associated to flowering time were removed from all downstream analyses (Table S2). A regression analysis was performed between the haplotype of the peak marker of each QTL to determine possible duplicate or homeolog loci. In addition, all the MTAs analyses were performed using Tassel 5 software [49].

2.5. Markers Conversion to KASP (Kompetitive Allele Specific PCR)

The array sequences of 20 markers associated to traits (MTA) were submitted to LGC Genomics for in-silico design of KASP primers using their proprietary software. Those that passed the in-silico criteria were purchased and used to genotype the independent validation set. For each marker that amplified and showed

polymorphism, the regression cut-off between phenotype and haplotype was imposed at $r = 0.105$ following Pearson's critical value [54]. KASP markers AX-95260810, AX-94432276, and AX-95182463 were tested for association with grain yield, while AX-94408589 for association with biomass. In addition, the top 20 and worst 20 lines were considered as the true positive and true negative for heat tolerance. Hence, the accuracy was calculated as the ratio of the correct allelic call among all, sensitivity as the ratio of the correct positive allelic among the top 20 yielding lines, and specificity as the ratio of the correct negative (wt) allelic calls among the 20 worst yielding lines. The sequence of the validated KASP markers is provided in Table S3, or the primers can be ordered directly at LGC Genomics indicating the Axiom code used in this article.

3. Results

3.1. *Agronomic Performance of the Genotypes and Sensitivity of Traits to Heat Stress*

The combined analysis of variance across four environments (two different temperature treatments over two crop seasons) revealed significant genotypic differences for all traits measured (Table 1). The yield performance of the genotypes across environments averaged 2171 kg ha^{-1} and ranged from 352 kg ha^{-1} obtained under heat stress conditions for the lowest yielding line DWAyT-0215, to 4658 kg ha^{-1} under normal conditions for the highest yielding line DWAyT-0217. The top yielding line under heat-stress was the ICARDA/Moroccan cultivar 'Faraj' with an average yield of 2249 kg ha^{-1} over the two seasons.

Table 1. Descriptive statistics, component of trait variation and heritability (h^2) among a set of 42 durum genotypes (G) tested under two treatments (T): normal and plastic tunnel-mediated heat stress during seasons 2015-2016 and 2016-2017

Trait	Acronym	Mean	Min	Max	Genetic variance (%)	Treatment variance (%)	GxT (%)	h^2
Days to heading	DTH	92	71	109	34**	1^{ns}	1^{ns}	0.78
Plant height (cm)	PH	81	71	92	60**	1^{ns}	16^{ns}	0.76
Biomass (Kg ha^{-1})	Biom	8,407	4,792	13,108	49**	7**	7**	0.79
Spikes number per m²	Spkm²	524	370	640	14**	1^{ns}	2**	0.50
Grain yield (Kg ha^{-1})	GY	2,171	352	4,658	30**	44**	12*	0.63
Harvest index (%)	HI	26	1	50	15**	34**	13^{ns}	0.20
Thousand kernel weight (g)	TKW	36	27	45	48**	1^{ns}	18**	0.72
Grain number per spike	GNSpk	13	3	24	19*	29**	16**	0.46

*, ** Significant at the 0.05 and 0.01 probability levels, respectively.

The treatment effect was significant only for Biom, GY, HI, and GNSpk, whereas DTH, PH, Spkm^2, and TKW were not significantly affected by treatments (Figure 2). The yield components were all significantly reduced under heat stress except TKW that showed a slight increase for the genotypes exposed to heat. The genotypes tested under plastic-tunnels had 61%, 54%, 42%, and 17% lower average GNSpk, GY, HI and Biom, respectively, compared to control. Relatively high heritability was observed for all the phenological and agronomical traits except for HI that had the lowest heritability (h^2 = 0.20).

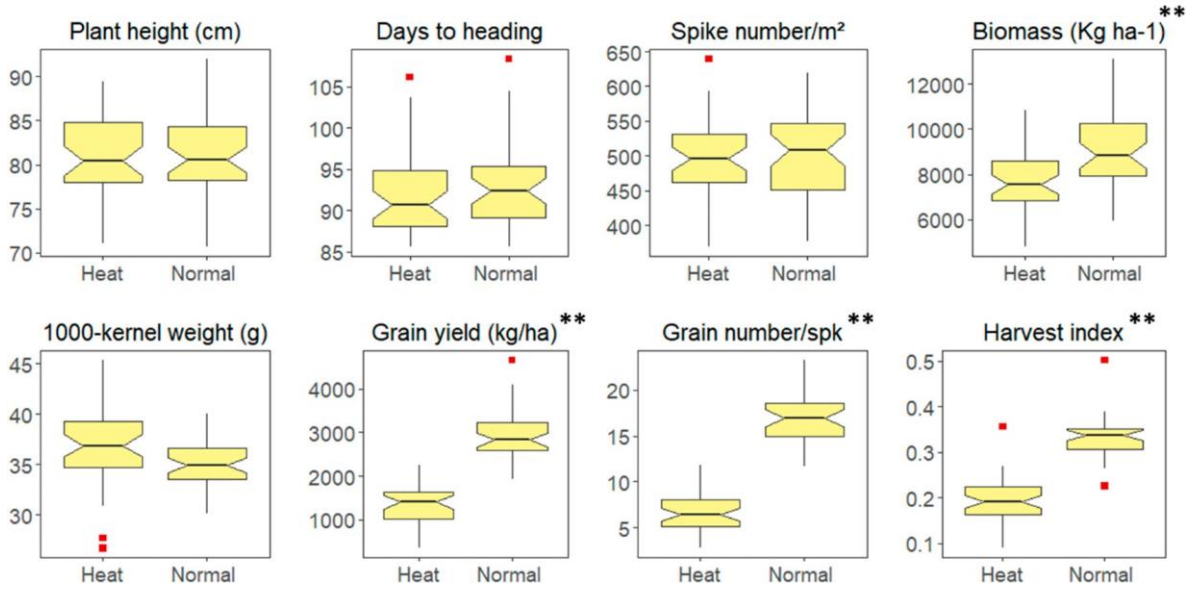

Fig. 2. Boxplot of the best linear unbiased estimates (BLUEs) for various traits under two different environmental conditions (Heat: plastic tunnel-mediated heat stress and Normal) across two years. ** indicate significant difference between the means of control and heat-stressed plants at $p < 0.05$.

3.2. The Traits Interrelationship under Each Environmental Condition

Correlation analysis (Figure 3; Tables S4 and S5) was first conducted to investigate the interrelationship among all agronomic traits. Under both treatments, GNSpk had the highest association with GY (r = 0.81 under heat, r = 0.67 under normal), while Spkm^2 and TKW were the least correlated with GY. Biomass was also correlated with GY with r = 0.61 under heat and r = 0.67 under normal conditions. HI also showed a significant positive correlation with yield under both treatments, but its effect was stronger under heat stress (r = 0.72) than normal conditions (r = 0.54). DTH was not significantly correlated to any trait except HI (r = −0.44) under normal conditions.

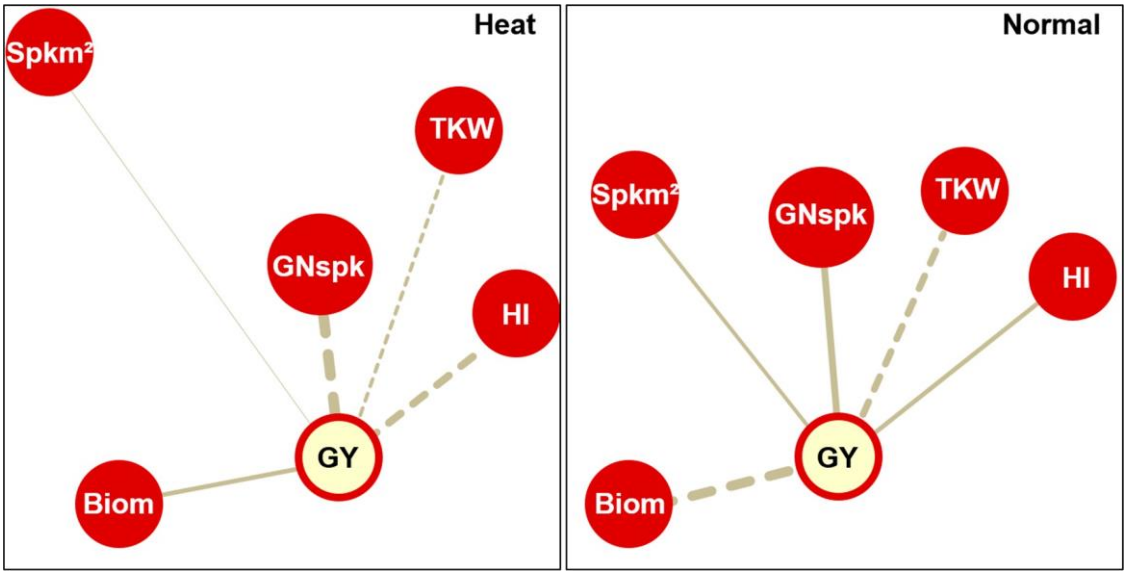

Fig. 3. Relationships between grain yield (GY) and yield components (grain number per spike (GNspk), harvest index (HI), dry biomass (Biom), number of fertile spikes per meter square (Spkm2), weight of a thousand kernels (TKW)) under plastic tunnel-mediated heat stress and normal conditions assessed by Pearson correlation and simple generalized additive model. The continuous grey line represents a linear relationship; the dashed grey line represents a non-linear relationship. The thickness of the line indicates the level of predictivity of the trait for GY. The length of the lines represents the correlation, the shorter the line the more the trait is correlated to GY.

Among yield components, the only significant and positive associations under the two environmental conditions were observed between Spkm^2, TKW, and Biom and between HI and GNSpk. Under heat conditions, a positive and significant correlation was noticed between GNSpk and Biom while under normal conditions HI was positively associated to TKW (Figure 3; Table S4).

The additive model was then used to further determine the nature of the relationship between GY and each predictor variable under normal and heat conditions (Figure 3; Table S5). The similarities observed between the two treatments in terms of the nature of relationship between GY and each of the predictors were the constantly linear and non-linear relationship between Spkm^2, TKW and the response variable GY, respectively.

GNSpk was considered the best predictor (deviance = 0.73%) with a complex relationship (EDF = 2.64) with GY under heat stress, whereas under normal conditions this trait was the second best predictor (deviance = 0.44%) with a linear relationship (EDF = 1). A similar trend was observed for HI in both treatments. Biom was found to be the best predictor (deviance = 0.52%) for GY with a non-linear relationship (EDF = 2.52) under normal conditions (Table S2; Figure S1).

3.3. Stress Tolerance Indices

Two different stress tolerance indices were calculated for GY: SSI and TOL (Figure 4). The genotypes showed wide variation for these indices. Seven SSI groups were identified with four having an SSI lower than 1 and the three remaining groups of genotypes having SSI > 1. The frequency distribution of the panel showed a wide variation and indicated the presence of susceptibility, with 45% of the genotypes falling in the very heat-susceptible class of SSI higher than 1, and only 7% of the lines showing high tolerance at SSI < 1. For TOL index, seven groups were also identified with 48% of the lines showing high yield depression and 5% of the genotypes presenting high stability. The smaller TOL values indicate the genotypes with low yield depression and hence more tolerant. However, good heat tolerance can also be reached by low yielding lines, but their value for breeding would be questionable. Hence, a scatterplot was devised to compare the GY under normal conditions and each of the heat indices (SSI and TOL). Five genotypes (four ICARDA lines, one Moroccan cultivar): Kunmiki, Berghouata1, Margherita2, IDON37-141, and Ourgh were found to have above average yield, low yield depression (low TOL values) and good heat tolerance (SSI < 1).

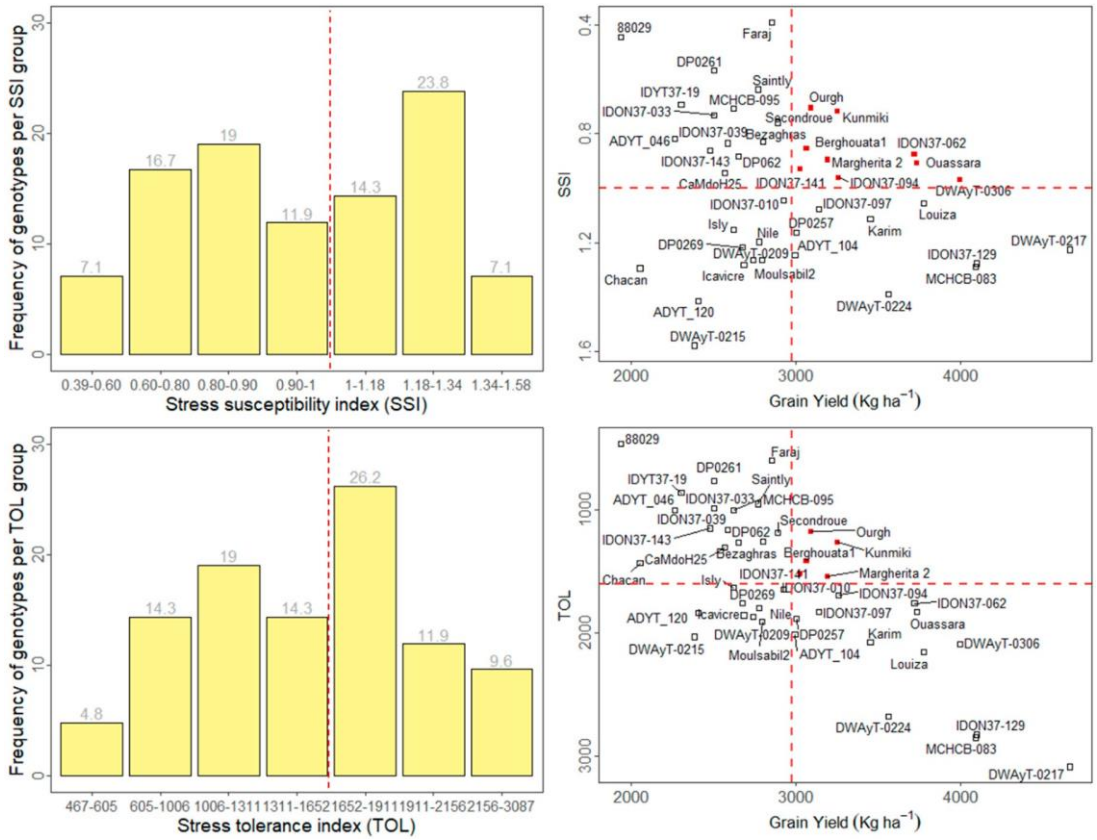

Fig. 4. Two different stress tolerance indices SSI (stress susceptibility index) and TOL (tolerance index) of grain yield, comparing plastic tunnel-mediated heat stress with normal conditions for the 42 durum wheat genotypes. The bars plot shows the frequency distribution of SSI and TOL for the genotypes tested. The dashed red lines mark the separation between tolerant (left) and susceptible (right) genotypes. The scatter plot shows the yield performance of genotypes tested under normal conditions against each of SSI and TOL. The vertical dashed red lines indicate the average GY. The horizontal dashed red lines indicate the cut-off value for tolerant vs. susceptible genotypes for each index. Red dots indicate genotypes that were identified as superior by both bi-plots.

3.4. Markers Associated to Heat Stress Tolerance

A total of 204 MTAs were identified for four traits (GY, GNSpk, HI and Biom) under both stress and normal conditions and 49 MTAs were recorded for the two GY stress tolerance indices. Regression analysis and clustering based on local LD decay confirmed that these associations were distributed over 12 loci (Table 2 and Table S6). Chromosome 1A had the highest number of MTAs (27) while chromosome 4A had the lowest (6).

Under normal conditions, 56 MTAs were detected for three traits GY, GNSpk, and HI, with the third trait having the highest number of MTAs (48). No common region for these traits was identified under the non-stress environment. Under heat stress, a

higher number of associations (148) were identified with trait variation (r^2) ranging from 0.25 to 0.36. The highest number of MTAs were detected for GNSpk distributed over 10 different loci, followed by HI on six loci. A common region for GY, GNSpk, HI, and Biom was identified under the heat condition on chromosome 6BS. Loci associated with both GNSpk and HI were detected on 1AL, 1BL, 2AL, 3AL, and 3BL. For heat tolerance indices (SSI-GY and TOL-GY), 49 MTAs were identified. The common loci associated with the two indices were on chromosomes 2AL, 5AL, and 5BL, while the loci on chromosomes 1AL and 6BS were identified only for TOL-GY and SSI-GY, respectively.

A comparison of the significant loci under each treatment and including the heat tolerance indices indicated a locus on chromosome 2AL, which was consistently identified for the indices, and both treatments for GNSpk and HI. Two loci on chromosomes 3AL and 3BL were associated with GNSpk and HI under both control and stress conditions, but were not associated with any of the indices. Three significant loci on chromosomes 1AL, 5BL, and 6BS were shared among heat stress treatment and stress tolerance indices, but not under normal conditions, making of these the most interesting genomic regions that specifically respond to heat stress. Overall, a total of 12 unique significant loci were identified (numbered QTL.ICD.Heat.01–QTL.ICD.Heat.12) and can be consulted in Table 2. Local LD decay was estimated for the 100 Mbp genomic region surrounding the peak marker. It varied between 31.7 and 108.7 Mbp, or a −38% to 112% variation compared to the average LD decay calculated for the whole panel (51.3 Mbp). This variation was accounted for to determine the correct physical size in each genomic region to assign multiple MTAs to the same QTL.

Table 2. QTLs associated with multiple traits under plastic tunnel-mediated heat stress, normal conditions, and based on stress indices.

Locus	Trait	Chr. †	Main marker	Position‡ (bp)	Local LD (Mbp)	Max LOD	Max r²	Heat stress	Normal	Indices
QTL.ICD.Heat.01	GNspk, HI, TOL-GY	1AL	AX-94863732	570,040,339	31.7	3.38	0.27	*		*
QTL.ICD.Heat.02	GNspk, HI	1BL	AX-94447402	632,403,981	43.1	3.38	0.27	*		
QTL.ICD.Heat.03	GNspk, HI, SSI-GY, TOL-GY	2AL	AX-94538070	748,624,588	36.3	3.06	0.25	*	*	*
QTL.ICD.Heat.04	GY, HI	2BS	AX-95193898	6,012,904	36.0	3.67	0.36		*	
QTL.ICD.Heat.05	GNspk, HI	3AL	AX-95632723	562,421,267	75.4	3.39	0.27	*	*	
QTL.ICD.Heat.06	GNspk, HI	3BL	AX-95174625	788,551,042	85.4	3.38	0.27	*	*	
QTL.ICD.Heat.07	GNspk	5AS	AX-95247611	27,923,949	108.7	3.38	0.27	*		
QTL.ICD.Heat.08§	SSI-GY, TOL-GY	5AS	AX-94631521	421,078,546	41.3	4.93	0.45			*****
QTL.ICD.Heat.09§	GNspk, SSI-GY, TOL-GY	5BS	AX-95182463	427,098,066	50.3	4.17	0.37	*****		*****
QTL.ICD.Heat.10§	GNspk, HI, Biom, SSI-GY	6BS	AX-94408589	157,777,006	56.0	3.20	0.36	*		*
QTL.ICD.Heat.11	GNspk	7AL	AX-95074729	660,833,752	153.6	3.60	0.29	*		
QTL.ICD.Heat.12	GNspk, HI	7AS	AX-94381852	16,943,364	44.8	3.42	0.37		*	

†Chr. – Chromosome, based on alignment to durum wheat genome assembly [48]

***** - Significant QTL

‡ - Based on alignment to durum wheat genome assembly [48]

§ - These QTLs have been converted into KASP markers and validated

GNspk - Grain number per spike; HI – Harvest index; TOL-GY – Tolerance index for grain yield; SSI-GY – Stress susceptibility index for grain yield; GY – Grain yield; Biom – Biomass.

3.5. Effect of Different Allele Combination on Yield Performance

The loci identified on chromosomes 1AL, 5BL, and 6BS appeared as the most critical for heat tolerance and were then tested further. These regions were associated with the control of multiple traits under heat stress: GY, GNspk, HI, Biom and the two indices SSI-GY and TOL-GY. A set of 208 modern lines were investigated for haplotype diversity at these three loci. Five groups with different allelic combinations were identified (Figure 5). Their allelic effect on GY was then assessed when field tested under high temperatures along the Senegal River [35]. The haplotype class with positive alleles at all three loci had the highest GY average reaching 2381 kg ha^{-1} with a maximum value of 3856 kg ha^{-1}. Genotypes of the haplotype classes with only two favorable alleles reached GY of 2199 and 2103 kg ha^{-1}, while lines that only carried one positive allele 2103 and

2023 kg ha^{-1} (Figure 5). ANOVA confirmed that the haplotype group with all three positive alleles was significantly superior to the others.

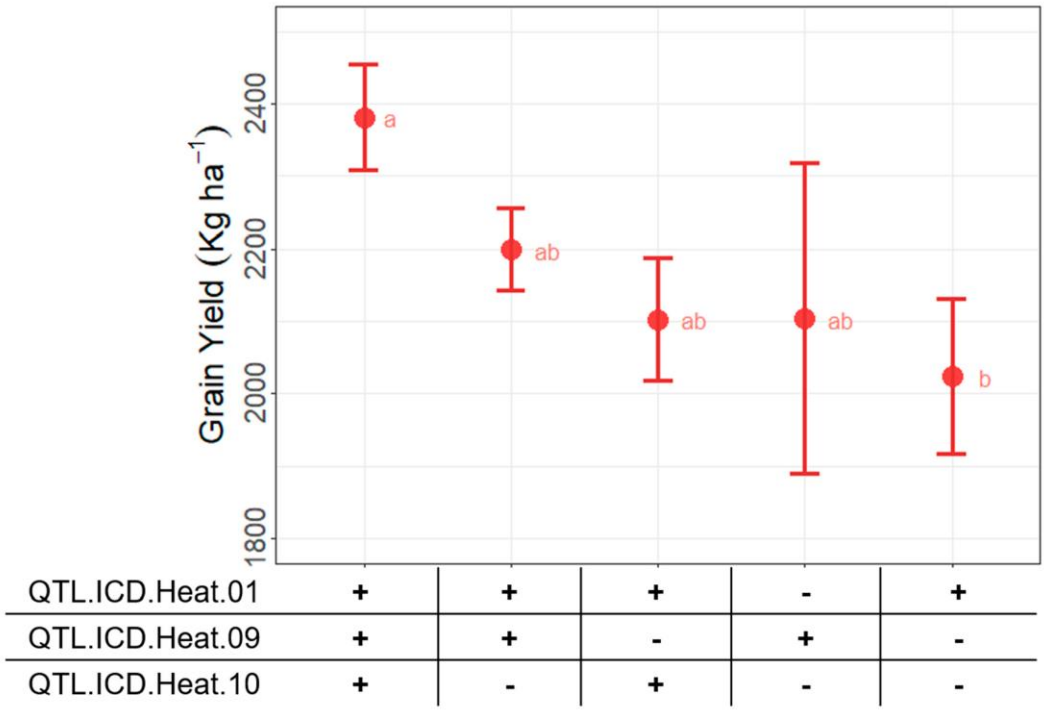

Fig. 5. Effect of different allele combinations of the significant loci on yield performance of 208 accessions tested under heat stressed conditions along the Senegal River. The circle indicates the average of each class over 2 years, and the whiskers show the standard error of the mean. The accessions were divided into five clusters based on their haplotype for three major QTLs: "+" mark the positive and "-" the wild-type alleles. Letters (a, b, ab) indicate significant differences between the clusters.

3.6. Validation of Markers for Marker Assisted Selection

To effectively deploy in breeding the most interesting QTLs via MAS, it is first required a step of validation using more affordable marker methodologies and in different genetic backgrounds and environments. A total of 20 MTA sequences linked to important agronomical and spike fertility traits were submitted for KASP primers design. Among these, only 14 could be successfully designed, and 11 identified a polymorphism within the validation set. Four showed significant ($p < 0.05$) correlation to the test phenotype (Figure 6). Three QTLs were represented by these four markers, AX-95260810 and AX-94432276 tagged QTL.ICD.Heat.08 on chromosome 5AL, AX-95182463 underlines QTL.ICD.Heat.09 on chromosome 5BL, and AX-94408589 tags QTL.ICD.Heat.10 on chromosome 6BS. The latter two QTLs are among the three main effect regions identified in this study (Figure 5). AX-95260810 reached 15%

correlation to grain yield under heat, 74% accuracy, 43% sensitivity, and 100% specificity. Especially, its ability to identify 100% of non-heat tolerant entries is particularly remarkable. AX-95182463 and AX-94408589 also reached significant correlations of 14% and 32% for grain yield and biomass under severe heat, respectively, with sensitivities of 62% and 40%, accuracies of 30% and 65%, and specificities of 4% and 90%. Overall, AX-9526081 and AX-94408589 appeared as the most suitable for MAS application.

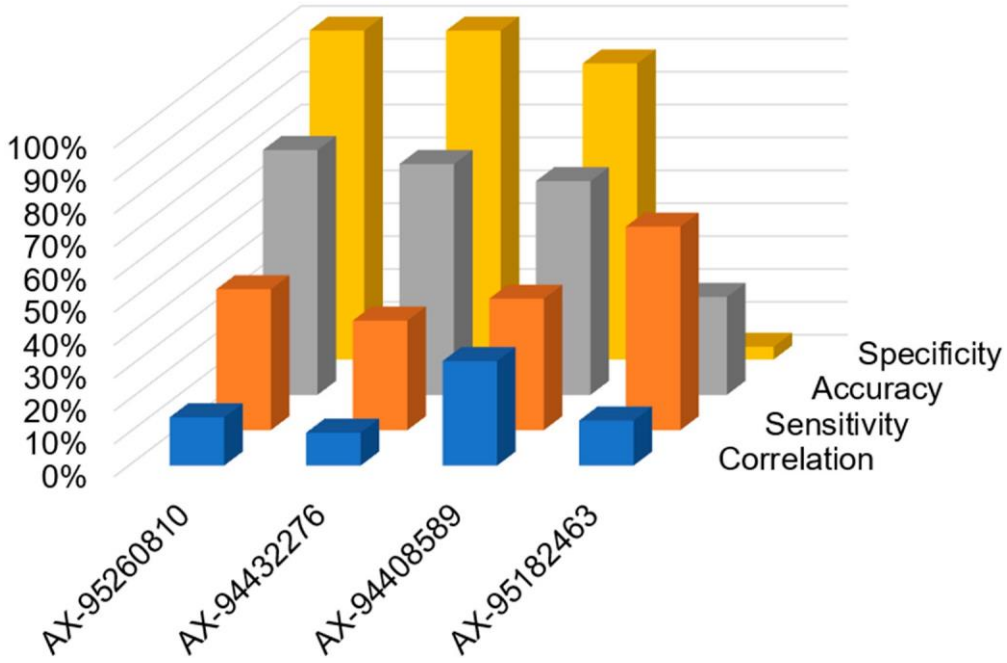

Fig. 6. Kompetitive Allele Specific PCR (KASP) markers validation on an independent set of 94 elite lines of ICARDA tested under severe heat for grain yield and biomass. Correlation was measured between the BLUE for grain yield recorded along the Senegal River and the haplotype score. Accuracy, sensitivity, and specificity where determined using only the top 20 and worst 20 lines. AX-95260810 and AX-94432276 tag QTL.ICD.Heat.08, AX-95182463 tags QTL.ICD.Heat.09, AX-94408589 tags QTL.ICD.Heat.10.

4. Discussion

4.1. *Evaluation of the Phenotypic Performance of Yield and Yield Components under Normal and Heat Stress Conditions*

Several studies reported that wheat plants are very sensitive to elevated temperatures during flowering and grain filling phases [9,55,56], due to a reduction in seed development and fertility [56–58]. This study evaluated a set of durum wheat genotypes derived from a global collection for GY and yield components under heat and normal conditions. The genetic and phenotypic diversity shown by this set

together with its relatively similar flowering time, promote it as an ideal panel to test heat tolerance. Further, the plastic tunnel method deployed here allowed to increase the temperatures well above 21 °C, the value that defines the absence of the stress [9]. A similar methodology was also successfully deployed by Corbellini et al. [54] to study the effect of heat shock proteins on technological quality characteristics. Compared to timely vs. delayed sowing experiments to simulate heat stress, the use of the plastic tunnel method avoids incurring false discovery due to changes in the phenological behavior of plants.

In the present study, a short and severe episode of heat stress was applied from the beginning of heading to the early dough stage, and resulted in 54% reduction in grain yield. This was in agreement with the study conducted by Ugarte et al. [59] that found a reduction of up to 52% when thermal treatment was applied via transparent chambers. Interestingly, our stress treatment caused an average temperature increase of 10 °C, which caused an average GY reduction of 5.4% for each 1 °C raise. This value is well within the 4.1% to 6.4% interval suggested by Liu et al. [10] for 1 °C raise in temperatures. GNSpk was the most affected trait (−61%) with the highest positive correlation to GY. This is in good agreement with previous studies that have shown that seed setting is the most sensitive parameter to heat stress, with a noticeable influence on yield [28,60–62]. Still, its non-linear relationship to yield confirms the complexity of the trait. Biom and HI were also found to have an influence on yield [63,64] with different relationships based on the occurrence of the stress. The presence of dissimilarities of the associations between the two treatments indicates clearly that there is a trade-off among the yield components as previously reported by Sukumaran et al. [65] for grain weight and grain number. Variation of one of the yield components affect the others positively or negatively. Compared to the simple regression, the additive model allowed to reveal the complexity of the relationship between GY and yield related traits.

The stress index SSI was developed by Fisher and Maurer [43] and modified by Nachit and Ouassou [44] as a useful indicator and a good parameter for selection. It measures the severity of the heat stress [66,67] and was also used in earlier studies in wheat to seek heat tolerant genotypes [23,68,69]. The TOL index is instead useful for selecting against yield depression, and it was used in several studies for heat or drought tolerance in wheat [27,44,67,70]. Improving heat tolerance should not be

based on the use of these criterions alone as was suggested by Clarke et al. [71]. It is important to select simultaneously for good yield performance coupled with good adaptability (SSI < 1) and stability (low TOL) [44]. In that sense, the accessions Kunmiki, Berghouata1, Margherita2, and IDON37-141 originated from ICARDA durum wheat program, and Ourgh, a Moroccan cultivar, have been identified as high yielding genotypes that also show good heat stress tolerance based on the two indices.

4.2. Dissection of Heat-Specific QTLs Associated with Yield-Related Traits and Stress Tolerance Indices

The significant correlation identified between yield and its components were not linear in nature, and tend to change their mode of action based on the occurrence of the stress. Therefore, several physiological processes are simultaneously involved in protecting the wheat plant from the heat stress [72], and there is value in dissecting it into its genetic components. In this study GWAS was used to identify the genetic regions controlling the response of the various traits. To prevent the confounding effect that phenology-related loci might have [73], MTAs were identified for DTH and removed from downstream analysis. Additionally, flowering time was used as covariate in all analyses for the other traits. Very few MTAs for DTH were observed either in normal or stressed conditions due to the synchronized flowering of the entries used in this study. This indicated the absence of confounding effects between the two trials. i.e., almost all the accessions were exposed to the same conditions in each developmental phase [74] before imposing the stress.

Out of 12 QTLs identified, three occurred only when the heat stress was imposed, including indices. These three main genomic regions occurred on chromosomes 1AL, 5BL, and 6BS, and were considered as QTLs controlling heat tolerance. These three loci were confirmed by mean of haplotype analysis on a larger panel of modern lines (208 entries) field tested under severe heat along the Senegal River valley [35], to confirm that the presence of the positive alleles at all three loci provided a significant GY advantage of +182 kg ha^{-1} (+8%). The QTL on the long arm of chromosome 1A controlled GNSpk, HI, and TOL-GY, and it explained up to 27% of the phenotypic variation. In a study with double haploid population of bread wheat, Heidari et al. [75] identified a major QTL on the same chromosome (1A), influencing grain number per

spike, grain weight per spike, and spikes/m^2. However, their phenotypic assessment was not performed under heat stress, the marker systems used was different compared to our study and the locus was identified in the short arm of chromosome 1A. Therefore, it is quite difficult to align the results from that study to the current one. Another study had previously reported many MTAs on chromosome 1A detected for yield components under heat stress, but all were found to have a pleiotropic relationship with days to heading and were also located on the short arm of 1A [26], instead of 1AL found here. A heat-specific QTL was also detected on the same chromosome in the short arm for spikelet compactness and leaf rolling in bread wheat [76]. An earlier study identified a QTL on 1AS for yield but associated with different stress conditions [77]. To the best of our knowledge, this is the first time that this region on 1AL is presented as associated to GNSpk, HI, and TOL-GY in durum wheat under heat stress conditions. The second major QTL region was detected on the long arm of chromosome 5B and found to be associated with GNSpk and the two indices SSI-GY and TOL-GY, contributing to 37% of the phenotypic variation. A region in the short arm of the same chromosome has been previously reported to be associated with grain number per square meter in bread wheat [76], and controlling thousand grain weight in durum wheat [27] under combined drought and heat stress. Shirdelmoghanloo et al. [25] and Acuna-Galindo et al. [78] reported loci for grain weight and other important traits on chromosome 5B under heat and non-heat conditions in hexaploid wheat. On the other hand, the same chromosome has been previously suggested to carry heat-specific QTLs for yield per se in bread wheat [26]. Sukumaran et al. [27] identified markers for heat susceptibility (HSI or SSI) and tolerance (TOL) indices for yield and grain number per square meter on the short arm of the chromosome 5B. Mason et al. [64] also detected QTL for HSI for kernel number on 5BL in bread wheat. The genomic region identified in this study on 5BL is likely to be a new QTL since no information has been reported earlier for this locus associated to GNSpk, SSI-GY, and TOL-GY in durum wheat and specific to heat stress, but we cannot exclude that it overlaps with previously reported QTLs. A third heat-responsive locus was identified on the short arm of chromosome 6B related to GY, SSI-GY, GNspk, HI, and Biom accounting for 36% of the phenotypic variance. An earlier study on bread wheat identified a locus on chromosome 6BS underpinning chlorophyll loss rates and heat susceptibility index for grain weight and chlorophyll

loss rates under heat-stress conditions [25]. Under post-anthesis high temperatures stress, Vijayalakshmi et al. [20] reported a QTL on the short arm of chromosome 6B for senescence related traits in hexaploid wheat. McIntyre et al. [79] and Pinto et al. [21] reported QTLs on chromosome 6BL that were associated with many important traits (grain number per square meter and grain yield and water-soluble carbohydrate content) related to drought and heat tolerance. Ogbonnaya et al. [26] found a locus on the short arm of chromosome 6B for grain yield under heat stress in bread wheat. These previously reported QTLs in 6B could overlap with the one identified in this study, but they were either identified not in association with heat tolerance or detected in hexaploidy wheat. Therefore, this region is also assumed to have been reported for the first time here in relationship to heat tolerance for durum wheat. This locus affects multiple traits (GY, GNspk, HI, Biom, and two heat susceptibility indexes) and hence it is of good importance for deployment in breeding. The principal breeding objective is to develop varieties with high grain yield and stability when exposed to different stresses. However, grain yield is a complex trait controlled by many genes and strongly influenced by the environment [80–86]. Therefore, a good understanding of traits and underlying loci associated with tolerance to elevated temperatures is of a great importance for breeding new heat tolerant cultivars [87].

4.3. Pyramiding Heat-Tolerant QTLS via MAS

Three loci on chromosomes 1AL, 5BS, and 6BS showed an additive nature by means of haplotype analysis (Figure 5), revealing that only the combination of all three positive alleles generated a true yield advantage. Among the most heat tolerant elite lines identified here ‘Kunmiki’, ‘Berghouata1’, and ‘Ourgh’ confirmed to harbor the positive alleles for all three loci. This prompts their use in crossing schemes to pyramid the positive alleles, as well as the deployment of simple marker system to conduct MAS.

Axiom to KASP marker conversion and validation was attempted for 20 MTAs. Eleven KASP markers generated polymorphic haplotypes in an independent set of ICARDA elite lines. Four revealed a significant ($p < 0.05$) correlation to GY and biomass assessed under severe heat along the Senegal River Valley (Figure 6). In particular, AX-95182463 tags QTL.ICD.Heat.09 located on chromosome 5B and it revealed good correlation and sensitivity, but lacks in accuracy and specificity, and it is hence protected from Type II errors, but prone to Type I, with several elite lines

wrongly identified as carrying the positive alleles. AX-95260810 tags QTL.ICD.Heat.08, linked to the two stress tolerance indices for GY (SSI-GY and TOL-GY) located on chromosome 5A. AX-94408589 tags QTL.ICD.Heat.10 located on chromosome 6B, and associated to several traits GNspk, HI, Biom, SSI-GY. In these two cases, the KASP markers explained 15% and 33% of the phenotypic variation of an independent validation set, with 100% and 90% specificity, and 74% and 65% accuracy, but medium sensitivity (43% and 40%). As such, these markers are protected against Type I errors (no false positive), but prone to Type II errors, with several elite lines identified as not carrying the positive allele while instead being tolerant to heat. Hence, while all converted KASP markers are prone to different types of errors, these three markers can be considered as validated and ready to be deployed in breeding. The combination of the three might represent a more stringent approach to protect against both types of errors. An additional nine QTLs were identified in this study, and their KASP conversion and validation are still ongoing and will require better targeted efforts to be achieved.

5. Conclusions

Heat stress causes a complex cascade of negative effects on the wheat plant, resulting in drastic reductions in grain yield. The deployment of heat tolerant varieties that will benefit greatly farmers requires first to enhance our understanding of this mechanism and loci governing it. Our study combined a discovery phase with a core set tested over two field seasons in Morocco under artificial heat-treatment with plastic tunnels, followed by a different confirmation set of germplasm grown for two seasons in Kaedi, Mauritania under severe natural heat, and completed with one final validation set tested one season in Kaedi. Our results confirmed that spike fertility (GNSpk) and maintenance of green leaves (Biom) are the most critical traits to drive tolerance to this stress, and hence should be the primary targets of durum wheat breeders. Further, the deployment of plastic tunnels proved to be a strategic methodology to study this stress and reveal its mechanisms without affecting the phenology of the plant. In addition, 12 loci were identified as responsible for controlling the main heat tolerance traits. Among these, three were activated only when the stress occurred and hence represent ideal targets for breeding. Two of these were validated into a KASP marker and are now ready for deployment via MAS, especially if associated with a third, also validated, KASP. Finally, three

ICARDA elite lines and one Moroccan cultivar were confirmed as tolerant to heat, with high grain yield, and carrying positive alleles for three main QTLs. These are freely available and should be incorporated as crossing parents by other breeding programs. Altogether, this study has confirmed the key traits for heat tolerance as well as a new methodology to study it in durum wheat, it has revealed the main loci controlling these traits and proceeded to validate three of them for MAS, and it has also provided freely available elite lines to breed new cultivars better adapted to the stress.

Author Contributions

F.M.B., M.N. and K.E.H. conceived and designed the study. K.E.H. and F.M.B. performed the field experiment. A.T.S. performed the field experiment in the Senegal river. A.A. contributed in the genotyping.
K.E.H. and F.M.B. analyzed the data. K.E.H. Wrote the original draft. K.E.H., B.B., A.F.M., A.A., M.N., and F.M.B. wrote or reviewed the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Conclusions

and

future prospects

Durum wheat is a major staple food source and an important cereal crop for several regions around the world. It is grown primarily in areas that often faces a multitude of abiotic stresses such as drought and heat. These stresses are very likely to reduce the productivity and thus threaten global food security. Therefore, breeding future durum wheat genotypes that are climate-resilient through enhancing drought and heat tolerance is a priority. The genetic improvement can be achieved by selecting for yield per se or physiological traits by using proxy traits. However, it is essential to dissect the genetic basis of the adaptive traits.

This dissertation aimed to study if there is a differential tolerance to drought and heat stress within the lines studied and the genetic control of the adaptive traits responsible of the tolerance to these abiotic stresses in various durum wheat germplasm.

The first study (chapter III and IV) aimed to investigate the drought tolerance in durum wheat looking at root system architecture traits to improve adaptation to water limited environments. Two different phenotyping methods ‘clear pot’ and ‘pasta strainer’ were deployed to evaluate seminal and mature root traits. The objective was to assess the suitability and high throughput of these methods, and to use them to investigate the available genetic diversity for rooting pattern. Further, we examined whether root systems respond differently to water availability. Finally, yield trials were conducted in different environments with a range of water regimes to evaluate the potential value of shallow or deep rooting systems, and the possibility of incorporating selection for root traits in breeding programs.

Using the same phenotyping methods, a lager set of durum wheat genotypes was evaluated at two different developmental stages for a number of root traits. Genome-wide association study using SNP markers was then used to identify the genomic regions involved in their control and haplotype analysis confirmed the positive allelic effect on grain yield performance.

In these chapters, we showed that (i) the root traits measured had significant segregation with strong genetic control and therefore appeared as a trait of choice for rapid genetic gain through breeding. (ii)The combination of methods presented in this study was confirmed as a suitable practice to identify these differences in durum wheat genotypes and can be used for breeding selection. (iii) Grain yield advantages of up to 40% could be obtained in both moisture-rich and -poor environment by selecting for the ideal root pattern. Clear advantage was shown for grain yield for the class with deeper roots at water-limited environments whereas in moisture-rich environments

yield advantage was achieved by the shallowest root types. Therefore, it can be said that each root architecture is suitable to particular environmental scenarios and selecting and breeding for root system architecture is a cost-effective strategy to increase crop productivity and adaptation. To improve the tolerance to water deficit, our work resulted in the identification of (iv) genomic regions underpinning root traits and their positive effect on grain production under drought stress conditions.

The ICARDA/INRA variety 'Nachit' with deep roots and large grains was released in 2017 for drought adaptation. It produced 5.1 t ha-1 during the very dry season 2018-19 at Marchouch (Rommani). This study provides innovative results about the genetic control of mature root system in durum wheat, traits that have been vastly neglected in the past. The genomic regions Q.icd.root.01, Q.icd.root.02 and Q.icd.root.04 on chromosome 3A, 3B and 7B respectively, could help to the establishment of a program using molecular marker assisted selection aiming at improving crop performance under water deficit.

The second study (chapter V) concerned the dissection of the plant response to heat stress conditions. A set of durum wheat lines was exposed to simulated heat stress at the time of flowering using plastic tunnels in the field. The objective was to evaluate the impact of high temperatures on yield and yield components. Moreover, to explore the nature of the relationship between grain yield and yield-related traits and identify the stable genotypes. Finally, the study aimed to identify the important genomic regions involved in the control of agronomic traits under heat stress.

This study found that (i) the grain number per spike was the most critical trait for tolerance to warm conditions when temperature is high during flowering. The heat stress resulted in 54% reduction in grain yield. Additionally, (ii) five lines with good heat tolerance and low yield depression: Kunmiki, Berghouata1, Margherita2, IDON37-141 and Ourgh were identified using stress tolerance indices. To improve the tolerance to heat, GWAS studies allowed (iii) the identification of major QTLs, QTL.ICD.Heat.01, QTL.ICD.Heat.09 and QTL.ICD.Heat.10 on chromosomes 1A, 5B and 6B respectively, controlling the adaptation to this stress and (iv) three were validated for MAS in an independent germplasm set demonstrating the utility of these regions for breeders.

The genetic dissection of the traits involved in the control of the tolerance of each of drought and heat stress resulted in the detection of valuable QTLs highlighting their complex genetic bases and will be then useful for molecular improvement of durum wheat germplasm. Also, five markers for these abiotic stresses were validated and

converted into KASP markers easy for breeders to use.

The valuable genomic regions identified in this study may allow breeders to reinstate genetic variation in their germplasm, to select tolerant lines to each of these stresses and the pyramiding of useful alleles could lead to develop an ideotype resilient to climate change with high productivity. They can be also used to design the breeding crosses for a progeny with maximum positive alleles. The example of Margherita2 which is the potential entry that carries the maximum positive allele for the significant QTLs found in this research project. Also, crosses between Kunmiki, Berghouata1 and Ourgh as Heat tolerant parent and Jupare C2003, Miki3 and Secondroue as drought tolerant parent could be designed to pyramid all positive alleles. These genotypes belong to the haplotype class carrying the favorable alleles at three loci related to tolerance to each of the abiotic stress studied. Therefore, a number of nine combinations of crosses between one drought tolerant parent and one heat tolerant parent would be possible to integrate all major QTLs.

This work proposed new methodologies and protocols enabling low-cost and field characterization of key traits for drought and heat in durum wheat. The work has also generated new scientific insights and valuable results for breeding application to enhance the genetic gain in durum wheat.

Yet, on another level, research on physiology could be of great interest to understand the mechanism of heat and drought tolerance. Also, it will be interesting to investigate other root traits and in more contrasting moisture levels than those explored in the present study. Some interactions such as root x soils may also be considered while investigating root architechture and water relations. The genomic regions that were identified in this study are highly relevant for further investigations, studying the gene itself would provide better insight on the response to heat and water deficit.

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Appendices

Appendices
Appendix I: Supplementary materials from

El Hassouni K., S. Alahmad, B. Belkadi, A. Filali-Maltouf, L.T. Hickey, F.M. Bassi. (2018). Root system architecture and its association with yield under different water regimes in durum wheat. *Crop science*, 58, 1-16. doi: 10.2135/cropsci2018.01.0076.

Supplemental Table S1. Matrix of correlation between below and above ground traits measured in the single plant studies and above ground traits measured in field trials under dry and irrigated conditions.

	DTH MCH16	GY MCH16	TKW MCH16	DTH KFD16	GY KFD16	TKW KFD16	DTH TER16	GY TER16	TKW TER16	DTH TES16	GY TES16	TKW TES16	DTH MKZ15	GY MKZ15	TKW MKZ15
	Dry environments									Irrigated environments					
SRA	-0.13	0.11	0.11	-0.08	-0.13	0.02	0.32	0.04	0.32	0.13	-0.08	0.27	0.36	0.42	0.22
RA D	0.20	-0.02	-0.23	-0.44	0.25	-0.46	-0.34	-0.14	-0.18	-0.25	0.10	-0.20	-0.47	-0.27	-0.26
RR 2-8 cm D	0.08	-0.17	-0.25	-0.63	0.32	-0.56	-0.49	-0.10	-0.03	-0.47	0.24	0.00	-0.25	-0.06	-0.26
RR 8-10 cm D	-0.06	0.05	-0.30	-0.43	0.16	-0.37	0.04	-0.03	0.09	0.04	0.12	-0.25	-0.30	-0.17	-0.24
RR 10-13 cm D	-0.04	0.08	0.33	0.64	-0.28	0.55	0.30	0.07	-0.02	0.27	-0.19	0.15	0.36	0.12	0.29
TRN D	0.08	-0.35	-0.36	-0.33	0.13	-0.12	-0.03	0.19	-0.24	-0.04	0.20	-0.18	-0.35	-0.32	-0.23
RB D	-0.06	-0.21	-0.27	-0.42	0.29	-0.20	-0.03	0.22	-0.13	-0.02	0.38	-0.13	-0.18	-0.55	-0.30
DTH D	0.22	-0.19	-0.39	-0.24	0.27	-0.32	-0.19	0.11	-0.41	-0.23	0.20	-0.53	-0.43	-0.67	-0.56
PLH D	0.11	-0.09	-0.20	-0.38	0.22	-0.08	-0.16	0.22	-0.08	-0.06	0.42	-0.42	-0.39	-0.64	-0.49
TN D	0.21	-0.48	-0.41	-0.38	0.08	-0.27	-0.36	0.38	-0.21	-0.15	0.23	-0.31	-0.11	-0.23	-0.39
SN D	0.28	-0.51	-0.50	-0.31	0.08	-0.24	-0.38	0.33	-0.37	-0.12	0.15	-0.37	-0.26	-0.25	-0.32
SPN D	-0.01	-0.07	-0.17	-0.40	0.14	-0.23	-0.06	0.15	-0.18	-0.05	0.37	-0.32	-0.32	-0.62	-0.45
SB D	0.22	-0.30	-0.37	-0.32	0.17	-0.11	-0.08	0.39	-0.24	0.04	0.38	-0.39	-0.17	-0.58	-0.35
GW D	-0.09	-0.30	-0.21	-0.17	-0.02	-0.01	-0.02	0.09	-0.03	0.03	0.16	-0.01	0.10	-0.01	-0.10
TKW D	0.10	-0.29	-0.04	0.32	-0.29	0.37	0.38	0.10	-0.02	0.42	-0.14	-0.03	-0.04	0.37	0.44
RA W	0.34	-0.14	-0.28	-0.29	0.11	-0.16	-0.24	0.20	-0.20	-0.10	0.00	-0.36	-0.49	-0.02	-0.29
RR 2-8 cm W	0.20	-0.23	-0.37	-0.56	0.19	-0.50	-0.29	0.13	-0.06	-0.28	0.06	-0.25	-0.36	-0.31	-0.44
RR 8-10 cm W	-0.17	0.05	-0.23	-0.32	0.10	-0.34	0.07	0.00	0.04	0.13	0.18	-0.21	-0.14	-0.19	-0.16
RR 10-13 cm W	-0.05	0.12	0.38	0.58	-0.20	0.56	0.17	-0.08	0.03	0.14	-0.16	0.31	0.34	0.33	0.40
TRN W	0.44	-0.22	-0.30	-0.43	0.29	-0.30	-0.35	0.10	-0.17	-0.35	0.27	-0.35	-0.37	-0.66	-0.56
RB W	0.17	-0.16	-0.34	-0.28	0.26	-0.15	-0.07	0.26	-0.25	-0.02	0.29	-0.35	-0.22	-0.74	-0.43
DTH W	0.22	-0.20	-0.37	-0.28	0.25	-0.35	-0.18	0.15	-0.40	-0.22	0.16	-0.47	-0.39	-0.71	-0.55
PLH W	0.07	-0.12	-0.21	-0.35	0.23	-0.10	-0.12	0.23	-0.05	-0.02	0.43	-0.36	-0.29	-0.66	-0.42
TN W	0.42	-0.35	-0.54	-0.37	0.25	-0.18	-0.24	0.38	-0.33	-0.18	0.30	-0.37	-0.29	-0.56	-0.47
SN W	0.41	-0.25	-0.51	-0.39	0.27	-0.21	-0.31	0.35	-0.35	-0.31	0.29	-0.50	-0.34	-0.61	-0.70
SPN W	0.01	0.17	0.05	-0.26	0.38	-0.13	-0.08	0.06	0.06	-0.14	0.39	-0.18	-0.26	-0.52	-0.35
SB W	0.26	-0.13	-0.32	-0.31	0.29	-0.08	-0.08	0.30	-0.18	-0.04	0.32	-0.41	-0.29	-0.69	-0.41
GW W	0.07	-0.19	-0.19	-0.22	0.32	0.12	-0.10	0.29	0.09	0.00	0.28	-0.08	-0.04	-0.40	-0.25
TKW W	0.03	-0.18	0.03	0.00	-0.37	0.56	0.28	0.13	0.16	0.43	0.17	0.11	0.10	-0.02	0.28

Supplemental Table S2. Mean of each class of root system architecture across rain-fed and irrigated environments using multi-trait and combined multi-trait classification methods.

Classification method	Class	Rain-fed environments		Irrigated environments	
		GY (Kg/ha)	TKW (gr)	GY (Kg/ha)	TKW (gr)
PCA classification	S	2032 a	27 A	6073 a	44 a
	M	2386 a	33 B	6361 a	45 a
	D	2111 a	34 B	6701 a	46 a
LSD classification	S	2277 a	26 A	5852 a	42 a
	MS	2205 a	31 Ab	6645 a	44 a
	MD	2195 a	32 Ab	6674 a	46 a
	D	2149 a	34 B	6326 a	47 a
Biplot of SRA vs MRA	S to S	2092 a	34 A	6901 a	50 b
	D to S	2293 a	32 A	6169 a	41 a
	D to D	2187 a	33 A	6060 a	47 ab
	S to D	2137 a	31 A	6705 a	46 ab
Combined multi-trait classification	S	1722 a	33 A	6768 a	47 a
	D	2679 b	36 A	7028 a	48 a

*Means of different classes in the same environment followed by different letters are significantly different at 0.05% level of probability.

S-shallow roots; M-medium; D-deep root.

Appendix II: Supplementary materials from

El Hassouni K., S. Alahmad, A. AL-Abdallat, L.T. Hickey, M. Nachit, A. Filali-Maltouf, B. Belkadi, F.M. Bassi. Molecular dissection of root architectural traits and their association with drought adaptation in durum wheat. (Unpublished)

Table S1. Name, origin, pedigree of the durum wheat genotypes used in this study.

Accession ID	Origin	Pedigree
86075	INDIA	Landrace
79509	ETHIOPIA	Landrace
85026	SPAIN	Landrace
85620	AFGHANISTAN	Landrace
Jabal	ICARDA	Korifla/AegSpeltoidesSyr//Mrb5
Amina	ICARDA	Korifla/AegSpeltoidesSyr//Loukos
Heirum	ICARDA	Heider/TAraticumMA//Mrb5
Icamator	ICARDA	IcamorTA041/4/Aghrass1/3/HFN94N8/Mrb5//Zna1/5/Malmuk1/Serrator1
Ouassara1	ICARDA	Ouasloukos1/5/Azn1/4/BEZAIZSHF//SD19539/Waha/3/Gdr2
Margherita 2	ICARDA	Terbol975/Geruftel2
Icadezful	ICARDA	Geromtel1/IRANYT053//Mgnl3/Ainzen1
Icarasha2	ICARDA	Stj3//Bcr/Lks4/3/Ter3
Icamoram7	ICARDA	ICAMORTA0472/Ammar7
Maci115	ICARDA	Maamouri2/CI115/5/F413J.S/3/Arthur71/Lahn//Blk2/Lahn/4/Quar
Miki3	ICARDA	Stj3//Bcr/Lks4
Bezaghras	ICARDA	Ossl1/Stj5/5/Bicrederaa1/4/BEZAIZSHF//SD19539/Waha/3/Stj/Mrb3/6/Mgnl3/Aghrass2
Secondroue	ICARDA	Stj3//Bcr/Lks4/3/Ter3/4/Bcr/Gro1//Mgnl1
Bezater	ICARDA	Ossl1/Stj5/5/Bicrederaa1/4/BEZAIZSHF//SD19539/Waha/3/Stj/Mrb3/6/Stj3//Bcr/Lks4/3/Ter3
Omrabi17	ICARDA	Joric69/Hau
Bellaroi	AUSTRALIA	920405/920274
Jupare C2003	CIMMYT	STOT//ALTAR84/ALD
Yavaros79	CIMMYT	JORI69(SIB)/(SIB)ANHINGA//(SIB)FLAMINGO
CRESO	ITALY	Yaktana54/Norin10B//2*Cappelli63/3/3*Tehuacan60/4/CapelliB144
MARZAK	MOROCCO-TUNISIA	INRAEII,12SelectioninCIMMYT
KOFA	SOUTH-WESTERN USA	NA
DUREX	SOUTH-WESTERN USA	NA
KRONOS	SOUTH-WESTERN USA	APBMSFRSPOPSel(D0312)
MESSAPIA	ITALY	Mex/Crane//Tito

MEXICALI75	CIMMYT	GerardoVZ469/3/Jori//ND61130/Leeds
MOHAWK	SOUTH-WESTERN USA	NA
REVA	SOUTH-WESTERN USA	NA
GUEROU1	ICARDA	Ato//Ibis/Fg
NILE	ICARDA	Snipe/Fg
Waldamez1	ICARDA	GdoVZ512/Cit//Ruff/Fg/3/DWL5023
Semperdur	AUSTRIA	NA
AC Pathfinder	CANADA	Dt367/WB881
Kamilaroi	AUSTRALIA	NA
Atil C2000	CIMMYT	SOOTY9/RASCON37
Samaypa C2004	CIMMYT	SOMAT4/INTER8
UNIBO-0263	CIMMYT	VANRRIKSE6.2//1A1D2+125/3*WB881
UNIBO-0267	CIMMYT	ROLA5/3/AJAIA12/F3LOCAL(SEL.ETHIO.135.85)//PLATA13/4/MALMUK1/SERRATOR1
Commander	CANADA	NA
Tjilkuri	AUSTRALIA	Brindur///Yallaroi*2//DurA/Yallaroi////RAC875/Kalka//Tamaroi///Lingzhi/Yallaroi
Jandaroi	AUSTRALIA	110780/111587
Hyperno	AUSTRALIA	Kalka/Tamaroi
Saintly	AUSTRALIA	Tamaroi/WLYY9//WLYY96a1773
MCHCB-082	ICARDA	Bicrederaa1/Tavoliere//Gdr1
MCHCB-083	ICARDA	Cham5*4/Ae.speltoides401294/4/ICAMORTA0469/3/Bcr/Gro1//Mgnl1/5/Stj3//Bcr/Lks4/3/Ter3
MCHCB-0161	ICARDA	319ADDO/5/D68193A1A//Ruff/Fg/3/Mtl5/4/Lahn
MCHCB-0213	ICARDA	Icasyr1/4/Assassa//Waha/Brch/3/Bicrederaa1
96203	MOROCCO	Landrace
85403	ETHIOPYA	Landrace
98680	CHINA	Landrace
99214	YEMEN	Landrace
99224	YEMEN	Landrace
DAWRyT-0317	ICARDA	Korifla/AegSpeltoidesSyr//Mrb5
DWAyT-0205	ICARDA	Younes/TdicoAlpCol//Korifla
DWAyT-0214	ICARDA	Korifla/AegSpeltoidesSyr//Amedakul

DWAyT-0215	ICARDA	Korifla/AegSpeltoidesSyr//Amedakul
DWAyT-0224	ICARDA	Korifla/AegSpeltoidesSyr//Waha
DWAyT-0306	ICARDA	Korifla/AegSpeltoidesSyr//Heider
ADYT_008	ICARDA	Ossl1/Stj5/5/Bidra1/4/BezaizSHF//SD19539/ Waha/3/Stj/Mrb3/6/Icajihan1
ADYT_009	ICARDA	Azeghar1/6/Zna1/5/Awl1/4/Ruff//Jo/Cr/3/F9.3/ 7/Azeghar1//Msbl1/Quarmal
ADYT_019	ICARDA	Ter1//Mrf1/Stj2/3/Icasyr1
ADYT_046	ICARDA	IcamorTA041/4/IcamorTA0469/3/Bcr/Gro1//M gnl1/5/MIKI2
ADYT_097	ICARDA	Ossl1/Stj5/5/Bicrederaa1/4/BEZAIZSHF//SD1 9539/Waha/3/Stj/Mrb3/6/Stk/Hau//Heca1
ADYT_104	ICARDA	Bcr/Lks4//Mrf1/Stj2/3/Ouasbar2
ADYT_120	ICARDA	Aghrass1/3/HFN94N8/Mrb5//Zna1/4/IcamorT A0458
Ouassara	ICARDA	Ouasloukos1/5/Azn1/4/BEZAIZSHF//SD1953 9/Waha/3/Gdr2
CaMdoH25	ICARDA	CM829/CandocrossH25
IDON37-010	ICARDA	Marsyr3/3/Gcn//Stj/Mrb3
Icavicre	ICARDA	ICAMORTA0468/6/21563/AA//Fg/3/D68102A 2A1A/4/Vitron/5/Bcr
IDON37-039	ICARDA	Mgnl3/Ainzen1/3/Ter1//Mrf1/Stj2
IDON37-052	ICARDA	Adnan2/Otb4//CM829/CandocrossH25
IDON37-062	ICARDA	Ter1/3/Stj3//Bcr/Lks4/4/Icajihan18
IDON37-105	ICARDA	Azeghar1//Blrn/Mrf2/3/Bicrederaa1/Azeghar2
Icamoram8	ICARDA	ICAMORTA0473/Ammar8
Hessept	ICARDA	IcamorTA0462/4/Gdr2//(SwAlgia/Gdr1)43/3/Ic amorTA0463/5/Ter1//Mrf1/Stj2
MERIDIANO	ITALY	Simeto/WB881/Duilio/F21
QUADRATO	ITALY	Creso/Trinakria
UNIBO-013	CIMMYT	DUKEM/3/RUFF/FGO//YAV79
UNIBO-018	CIMMYT	ROK/FGO//STIL/3/BISU1
UNIBO-021	CIMMYT	FOCHA1/5*ALAS
UNIBO-022	CIMMYT	TOPDY21/RASCON33
UNIBO-024	CIMMYT	GS/CRA//SBA81/3/HO/MEXI1/5/MEMO/6/2* …

UNIBO-025	CIMMYT	RASCON37/2*TARRO2
ARTENA	SPAIN	NA
BOLENGA	SPAIN	NA
AMRIA	MOROCCO	H.Mouline/Saada//Karim
ANOUAR	MOROCCO-TUNISIA	NA
ISLY	MOROCCO-TUNISIA	ERPEL(SIB)/(SIB)RUSO
JAWHAR	MOROCCO-TUNISIA	NA
Tomouh	MOROCCO-TUNISIA	Joric69/Hau
CHACAN	ICARDA	Cham1/5/Cando/4/BY*2/Tace//II27655/3/TME //ZB/W*2
Moulchahba1	ICARDA	H.MOUL(MOR)/CHABA88
UNIBO-066	ICARDA	KRS/HAUCAN
Moulsabil2	ICARDA	H.mouline(Mor)/Sabil2
BRAVADUR	SOUTH-WESTERN USA	NA
CAPEITI8	ITALY	Eiti6/Cappelli
DON PEDRO	ITALY	Carc/Auk

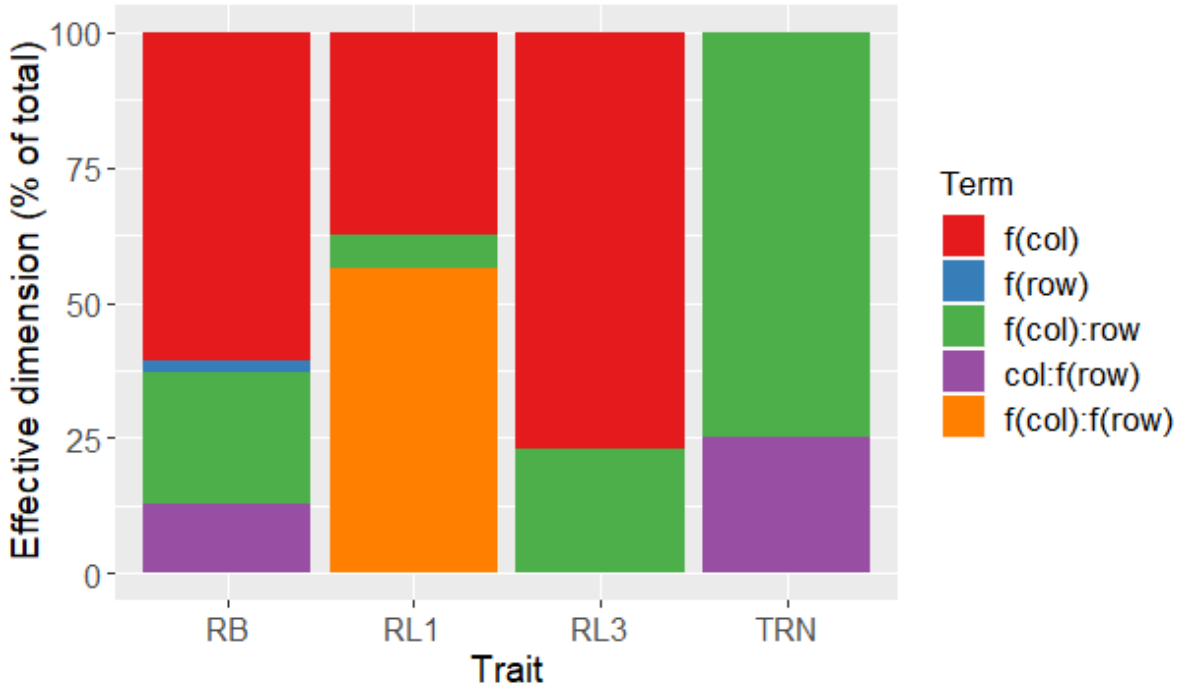


Figure S1. The importance of spatial terms from two-dimensional P-spline for the most affected traits by field variation.

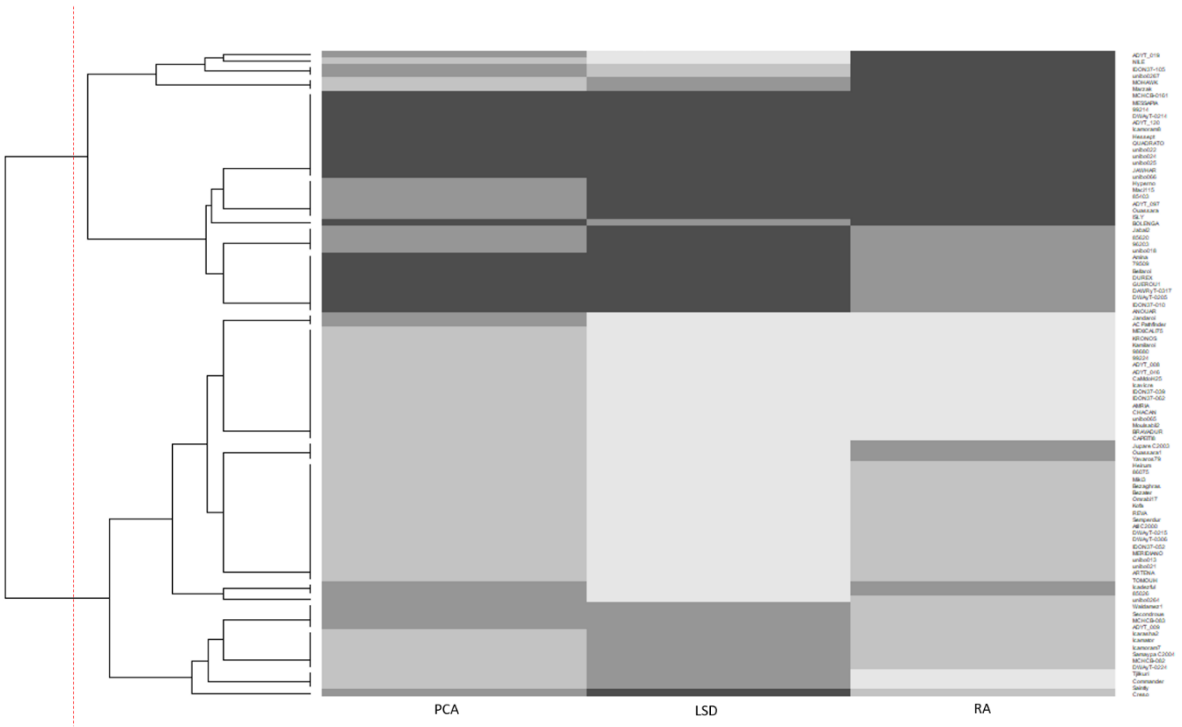


Figure S2. Combined multitrait method via hierarchical clustering to distinguish root behavior of 100 genotypes. The x axis lists the three different multitrait methods that were combined in the analyses: first method is principal component analysis (PCA) of root ratios, the second method is based on two significant (LSD) differences for root ratio at each level, and the third method is based on root angle (RA) measured at maturity and seedling. For each method, the different classes identified are color coded with different shades of gray. A vertical red dash line indicates the position that explains 80% of variation and splits the genotypes into two classes.

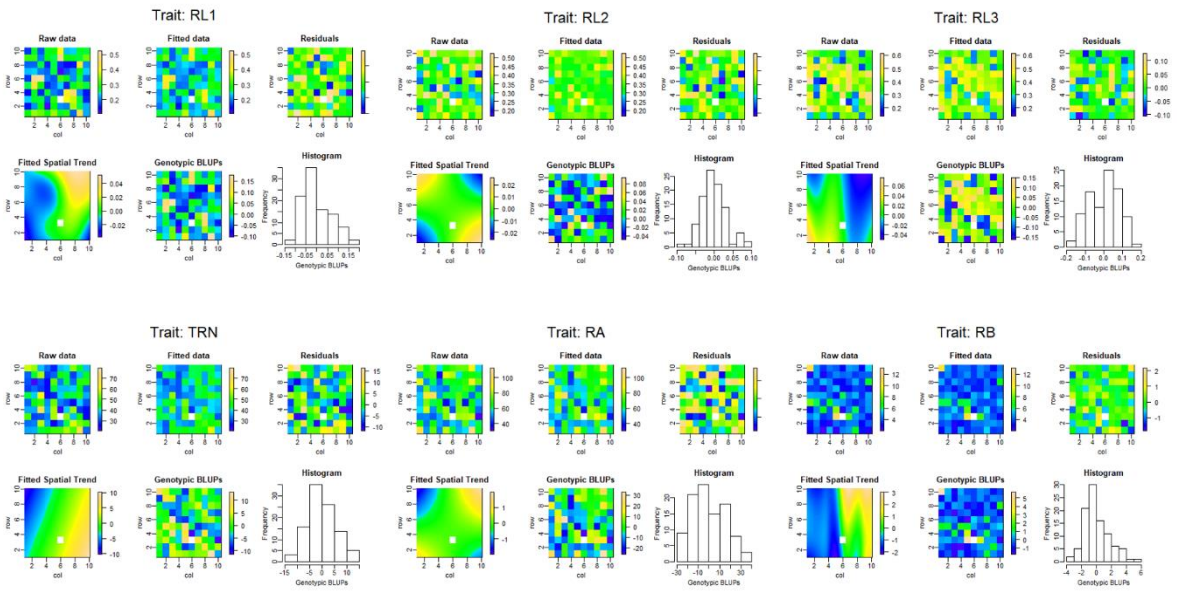


Figure S3. Diagnostic plots from two-dimensional P-spline for RL1, RL2, RL3, TRN, RB and RA.

Appendix III: Supplementary materials from

El Hassouni K., B. Belkadi, A. Filali-Maltouf, A. Tidiane-Sall, AL-Abdallat, M. Nachit, F.M. Bassi. Loci controlling adaptation to heat stress occurring at the reproductive stage in durum wheat. *Agronomy*. DOI: 10.3390/agronomy9080414

Table S1. List of durum wheat genotypes evaluated under plastic tunnel-mediated heat stress in the present study.

Accession name	Origin	Pedigree
ADYT_046	ICARDA	IcamorTA041/4/IcamorTA0469/3/Bcr/Gro1//Mgnl1/5/MIKI2
ADYT_104	ICARDA	Bcr/Lks4//Mrf1/Stj2/3/Ouasbar2
ADYT_120	ICARDA	Aghrass1/3/HFN94N8/Mrb5//Zna1/4/IcamorTA0458
Berghouata1	ICARDA	Ter1//Mrf1/Stj2
Bezaghras	ICARDA	Ossl1/Stj5/5/Bicrederaa1/4/BezaizSHF//SD19539/Waha/3/Stj/Mrb3/6/Mgnl3/Aghrass2
CaMdoH25	ICARDA	CM829/CandocrossH25
Chacan	ICARDA	Cham1/5/Cando/4/BY*2/Tace//II27655/3/Tme//ZB/W*2
DP0257	CIMMYT	1A.1D5+106/2*WB881//1A.1D5+106/3*Mojo/3/Bisu_1/Patka_3
DP0261	CIMMYT	Cndo/Primadur//Haiou_17/3/Snturkmi8384375/NIGRIS_5//TANTLO_1
DP0269	CIMMYT	Somat_3/Phax_1//Tilo_1/Lotus_4
DP062	ICARDA	Chhb88/Deraa
DWAyT-0209	ICARDA	Korifla/Ae.SpeltoidesSyr//Amedakul
DWAyT-0215	ICARDA	Korifla/Ae.SpeltoidesSyr//Amedakul
DWAyT-0217	ICARDA	Korifla/Aeg.SpeltoidesSyr//Loukos
DWAyT-0224	ICARDA	Korifla/Ae.SpeltoidesSyr//Waha
DWAyT-0306	ICARDA	Korifla/Ae.SpeltoidesSyr//Heider
Faraj	ICARDA/ Morocco	F413J.S/3/Arthur71/Lahn//Blk2/Lahn/4/Quarmal
Icavicre	ICARDA	IcamorTA0468/6/21563/AA//Fg/3/D68102A2A1A/4/Vitron/5/Bcr
IDON37-010	ICARDA	Marsyr3/3/Gcn//Stj/Mrb3

IDON37-033	ICARDA	Mgnl3/Ainzen1//Ammar1
IDON37-039	ICARDA	Mgnl3/Ainzen1/3/Ter1//Mrf1/Stj2
IDON37-062	ICARDA	Ter1/3/Stj3//Bcr/Lks4/4/Icajihan18
IDON37-094	ICARDA	Aghrass1//Bezaiz982/Bcrch1/4/IcamorTA0462/3/Quabrach3//Vitron/Bidra1/5/Stj3//Bcr/Lks4/3/Ter3
IDON37-097	ICARDA	Mgnl3/Ainzen1/3/Bcr/Gro1//Mgnl1
IDON37-129	ICARDA	CM829/CandocrossH25//Icajihan10
IDON37-141	ICARDA	IcamorTA0471//IcamorTA0459/Ammar8/4/Stj3//Dra2/Bcr/3/Ter3
IDON37-143	ICARDA	Mrb3/Mna1//Ter1/3/IcamorTA0459/Ammar7/4/Beltagy2
IDYT37-19	ICARDA	Mgnl3/Ainzen1//Maamouri3
IG:88029	Ethiopia	Landrace
Isly	Morocco	Erpel(SIB)/(SIB)Ruso
Karim	CIMMYT	Jori69(SIB)/(SIB)Anhinga//(SIB)Flamingo
Kunmiki	ICARDA	MorlF38//Bcrch1/Kund1149/3/Bicrederaa1/Miki
Louiza	Morocco	na
Margherita 2	ICARDA	Terbol975/Geruftel2
MCHCB-083	ICARDA	Cham5*4/Ae.Speltoides401294/4/IcamorTA0469/3/Bcr/Gro1//Mgnl1/5/Stj3//Bcr/Lks4/3/Ter3
MCHCB-095	ICARDA	Mck2/Tilo2//Bcrch1/Kund1149
Moulsabil2	ICARDA	H.mouline(Mor)/Sabil2
Nile	ICARDA	Snipe/Fg
Ouassara	ICARDA	Ouasloukos1/5/Azn1/4/BezaiSHF//SD19539/Waha/3/Gdr2
Ourgh	Morocco	D67gta/2/Boyero/Bit//Mexicali
Saintly	Australia	Tamaroi/WLYY9//WLYY96a1773
Secondroue	ICARDA	Stj3//Bcr/Lks4/3/Ter3/4/Bcr/Gro1//Mgnl1

Table S2. Markers associated with days to heading (DTH) under heat stress and normal conditions.

Locus	Chr.	Main marker	Position	Max LOD	Max r^2	Heat stress	Normal conditions
MTA.DTH.01	4A	AX-94954115	135455654	3.1	0.29	*	*
MTA.DTH.02	4B	AX-95082485	417259719	3.1	0.29	*	*
MTA.DTH.03	4B	AX-94397040	418863736	3.1	0.29	*	*
MTA.DTH.04	6A	AX-94732269	65926884	3.1	0.27		*

Table S3. Sequence information of the KASP markers.

QTL	Marker	Location	Sequence
QTL.ICD.Heat.08	AX-95260810	5AS	CGGTCAACGCCCTCTCTGGACACCATGGACGACGA[C/G]TCGCTCCCAGGCTCAACCCCAACCATGAAGCACTG
QTL.ICD.Heat.08	AX-94432276	5AS	TATGTCATGGTGAATTCGAATCAGACCGTGATTCT[C/T]GCTGAAAGAGAATTGTTGGCTATATGTATGGTGCT
QTL.ICD.Heat.09	AX-95182463	5BS	CCCTGGGTGCAACTGCAGCAAGACCCTGAAGAGAA[C/T]GAAAGTTTACTTGGGCAGCGATCGGCAATGGTCAA
QTL.ICD.Heat.10	AX-94408589	6BS	AAATCTTCAGGTTCATATAAGTCAGCCAAGTCCAC[C/G]GATAAAATAGACGGTACTTCAGTGCCAAACATGTT

Table S4. Pearson correlation matrix between all the measured traits under heat conditions (upper part) and normal (lower part) conditions. GY – Grain yield; Biom – Biomass; HI – Harvest index; Spkm² - Spikes per square meter: GNspk - Grain number per spike; TKW – Thousand kernel weight; DTH – Days to heading. *, ** Significant at the 0.05 and 0.01 probability levels, respectively.

	GY	Biom	HI	Spkm²	GNspk	TKW	DTH
GY		0.61**	0.73**	0.18 ns	0.81**	0.49**	0.09 ns
Biom	0.67**		-0.02 ns	0.36*	0.30*	0.44**	0.07 ns
HI	0.54**	-0.18 ns		-0.09 ns	0.78**	0.28 ns	0.01 ns
Spkm²	0.45**	0.53**	0.02 ns		-0.12 ns	-0.04 ns	0.03 ns
GNspk	0.67**	0.27 ns	0.51**	-0.27 ns		0.16 ns	-0.18 ns
TKW	0.56**	0.44**	0.35*	0.28 ns	0.06 ns		0.17 ns
DTH	-0.18 ns	0.19 ns	-0.44**	-0.06 ns	-0.17 ns	-0.09 ns	

Table S5. Correlation (r), linear regression estimated via ordinary least squares (OLS) and flexible regression estimated via regression additive model. a; under heat stress. b; under normal conditions.

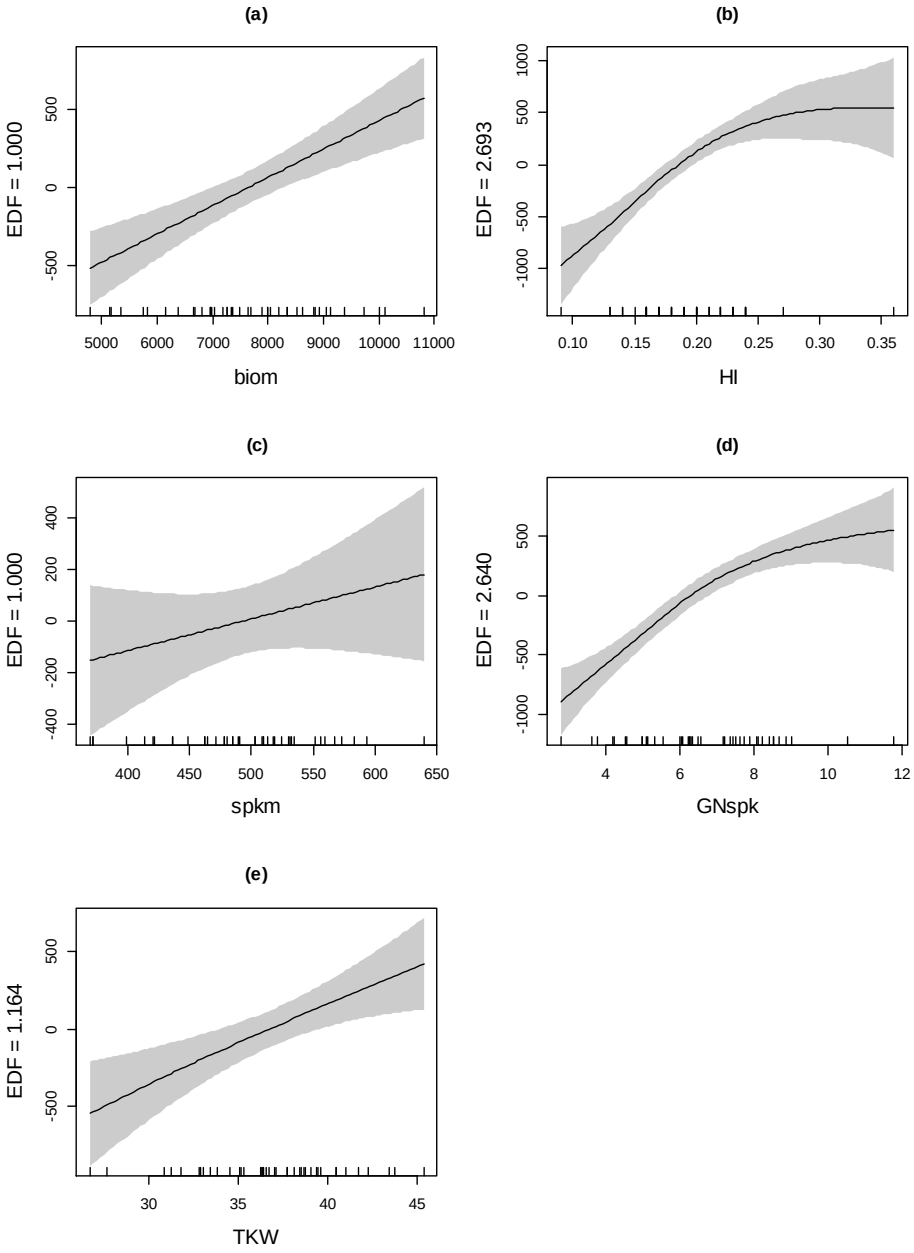
a) Plastic tunnel-mediated heat stress

Trait	*r*	OLS regression			GAM			L-R test
		B	*t*	Deviance	E.D.F.	*F*	Deviance (%)	
Biomass	0.61**	0.18	4.85**	37.10	1.00	23.54**	37.10	**
HI	0.73**	6702.90	6.65**	52.50	2.69	19.75**	64.00	**
Spkm²	0.18^{ns}	1.24	1.16^{ns}	3.29	1.00	1.35^{ns}	3.29	*
GNspk	0.81**	179.95	8.72**	65.50	2.64	30.37**	72.80	*
TKW	0.49**	51.72	3.58**	24.30	1.16	10.48**	25.00	ns

b) Normal conditions

Trait	*r*	OLS regression			GAM			L-R test
		b	*t*	Deviance	E.D.F.	*F*	Deviance (%)	
Biomass	0.67**	0.24	5.71**	44.90	2.52	12.45**	51.80	ns
HI	0.55**	7203.30	4.12**	29.80	1.14	14.24**	30.40	ns
Spkm²	0.45**	4.64	3.19**	20.20	1.00	10.14**	20.20	**
GNspk	0.66**	139.90	5.54**	43.40	1.00	30.76**	43.50	**
TKW	0.56**	151.62	4.32**	31.90	2.76	7.43**	42.00	ns

a)


(a)
EDF = 1.000
biom
(b)
EDF = 2.693
Hl
(c)
EDF = 1.000
spkm
(d)
EDF = 2.640
GNspk
(e)
EDF = 1.164
TKW

b)

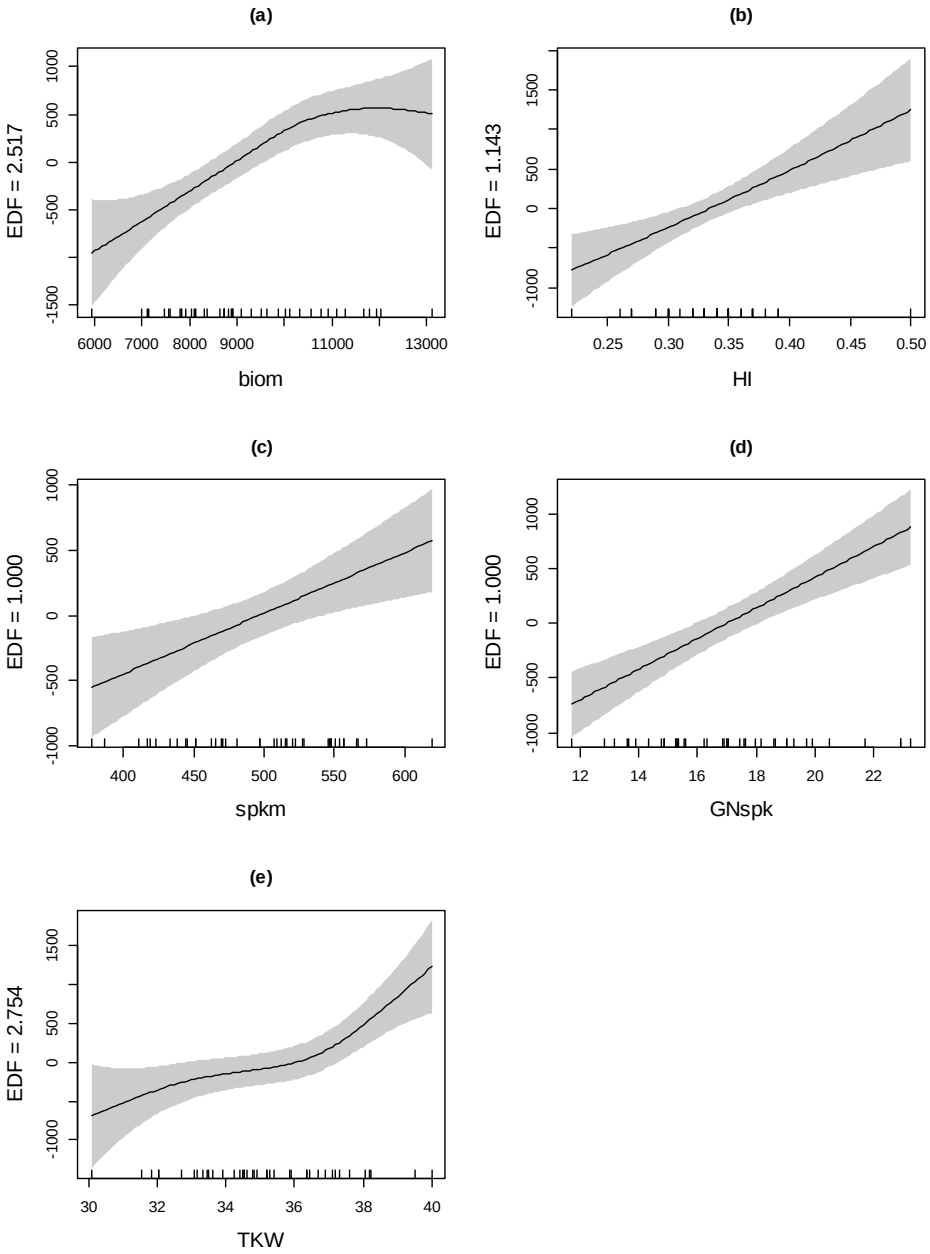


Figure S2. Plots of the additive regression model showing GNspk, biom, TKW, spkm² and HI as the spline function of the target trait grain yield (GY). a; under heat stress. b; under normal conditions