UNIVERSITÀ
DEGLI STUDI DELLA
TUSCIA

Università degli Studi della Tuscia di Viterbo

Dipartimento di Scienze Agrarie e Forestali (DAFNE)

Corso di Dottorato di Ricerca in

Scienze delle Produzioni Vegetali e Animali XXXVI ciclo

Durum wheat assessment for organic agriculture and for tolerance to drought and salinity

Settore scientifico-disciplinare
s.s.d. AGR/07

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A.A. 2022/23

Acknowledgments

This research was funded by the European Union's Horizon 2020 research and innovation program under grant agreement No 771367 (ECOBREED project).

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Summary

The agriculture is one of the sectors with major impact on climate change. Organic farming is considered a sustainable approach for overcoming the issues posed by intensive agricultural practices in the past years. The organic practices would also increase the economic benefits due the prohibition of agrochemicals. To maintain high yield and grain quality, the organic farming needs cultivars directly selected with organic breeding programs.

The first part of this study aims to identify genotypes suitable for organic agriculture. It is focused on the screening of durum wheat genotypes in organic farming. Twenty-four genotypes with origin between Mediterranean Basin and central Europe were screened in field experiments in multi-year trials in Italy, Austria, and Hungary. In total ten environments have been evaluated. Data on morphological traits were collected and analysed to compare yield, grain quality and their stability across years and locations. Statistical analysis revealed different performance of the genotypes from the Mediterranean Basin and Central Europe. The genotypes developed in Austria and Hungary are a good resource of grain quality traits but resulted not widely adapted in the warmer environment of Italy. The analysis of stability through Weighted Average of Absolute Score (WAAS) index, which allows weighting between the performance and stability of the variety, was able to discriminate among the studied varieties.

The second part of the research aims to study the tolerance mechanisms under drought and salt stresses. The durum wheat genotypes which resulted to be the best after preliminary analysis of data of the first two years of field experiments, were stressed with water deficit (drought) or salt stress in a controlled environment. The experiments were performed with plants in sand-filled pots, and destructive analyses were carried out to investigate the epigeal part and the hypogeal part with a high-throughput phenotyping system. Moreover, molecular markers were used for a genotypic characterization. The experiments revealed that landraces could represent a great resource of desirable traits for low input environments where drought and salinization of soils can occur. In addition, it was determined that the main capability of genotypes to tolerate the drought and salt stress is related to traits such as root angle, as well as root and shoot biomass.

Riassunto

L'agricoltura è uno dei settori con un impatto significativo sul cambiamento climatico. L'agricoltura biologica è considerata un approccio sostenibile per superare le problematiche poste dalle pratiche agricole intensive degli ultimi anni. Le pratiche biologiche aumenterebbero anche i benefici economici grazie al non impiego di agrochimici. Per mantenere un alto rendimento e una buona qualità della granella, l'agricoltura biologica ha bisogno di cultivar selezionate direttamente in programmi di miglioramento genetico sotto regime biologico.

Lo scopo della prima parte di questo studio era quello di identificare genotipi di frumento duro adatti all'agricoltura biologica. Ventiquattro genotipi di origine tra il bacino del Mediterraneo e l'Europa centrale sono stati esaminati in esperimenti in pieno campo in prove pluriennali in Italia, Austria e Ungheria. In totale sono stati esaminati dieci ambienti. I dati sui tratti morfologici sono stati raccolti ed analizzati per confrontare il rendimento, la qualità della granella e la loro stabilità nel corso degli anni e tra le diverse località. L'analisi statistica ha rivelato differenti performance dei genotipi tra il bacino del Mediterraneo e l'Europa centrale. I genotipi sviluppati in Austria e Ungheria rappresentano delle buone risorse genetiche per i tratti di qualità della granella ma non risultano ampiamente adattati all'ambiente più caldo dell'Italia. L'analisi di stabilità attraverso l'indice WAAS (Weighted Average of Absolute Score), che consente di ponderare tra la performance e la stabilità della varietà, è stata in grado di discriminare tra le varietà studiate.

Lo scopo della seconda parte della ricerca è quello di studiare i meccanismi di tolleranza a stress idrico (siccità e salinità). L'esperimento è stato condotto in condizioni controllate in Italia. I genotipi che hanno mostrato i migliori risultati dopo un’analisi preliminare dei dati dei primi due anni degli esperimenti in pieno campo sono stati sottoposti a stress idrico e salino in un ambiente controllato. Gli esperimenti sono stati condotti con piante poste in vasi riempiti con sabbia e sono state eseguite analisi distruttive per valutare la parte epigea e ipogea con un sistema di fenotipizzazione ad alta risoluzione. Inoltre, sono stati utilizzati marcatori molecolari per una caratterizzazione genotipica. Gli esperimenti hanno rivelato che gli ecotipi antichi potrebbero rappresentare ancora oggi una grande risorsa di tratti desiderabili per ambienti con scarsità di elementi nutritivi in cui possono verificarsi siccità e salinizzazione del suolo. Inoltre, è stato rilevato che la capacità dei genotipi di tollerare gli stress da siccità e salinità è legata a tratti come l'angolo delle radici, e la biomassa radicale e aerea.

Table of Contents

Chapter 1. Introduction 7
1.2 Aim and objectives of the study 7
1.3 Organization of the study 8
1.4 References 9
Chapter 2. European multi-year field screening of durum wheat (*Triticum turgidum* L. var *durum* Desf.) for organic farming 11
2.1 Introduction 12
2.2 Materials and methods 14
2.2.1 Agro-environmental conditions 14
2.2.2 Plant material and experimental design 14
2.2.3 Data recording and analysis 15
2.3 Results 17
2.3.1 Performance of genotypes 23
2.4 Discussion 25
2.5 Conclusion 27
2.6 References 28
Chapter 3. One hundred candidate genes and their roles in drought and salt tolerance in wheat 32
3.1 Global situation 32
3.2 Germplasm 35
3.2.1 Sources of Resistances in Cultivated Species 36
3.2.2 Source of resistance in alien species 36
3.3 Morphological and physiological response 39
3.4 Mechanisms of tolerance 40
3.4.1 Osmotic adjustment 41
3.4.2 Ionic homeostasis 42
3.4.3 Redox homeostasis 43
3.5 Genes and transcription factors involved 44
3.5.1 Genes involved in hydric stress tolerance 44
3.5.2 Stress tolerance-related genes and functional proteins 49
3.5.3 The specific genes involved in ionic homeostasis 50

3.5.4 The genes associated with reduce reactive oxygen species (ROS) 51
3.6 Conclusion 53
3.7 References 53
Chapter 4. Diversity in root architecture of durum wheat at stem elongation under drought stress 66
4.1 Introduction 67
4.2 Materials and methods 68
4.2.1 Plant material 68
4.2.2 Greenhouse experiment design and conditions 69
4.2.3 Phenotyping 70
4.2.4 Field experiment 71
4.2.5 Statistical analysis 72
4.3 Results 72
4.3.1 Drought effect on shoot growth and development in greenhouse experiment 72
4.3.2 Drought effect on whole root system in greenhouse experiment 73
4.3.3 Drought effect on root parameters at topsoil in greenhouse experiment 76
4.3.4 The comparison of root system between greenhouse and field 77
4.3.5 Correlation among traits 79
4.3.6 PCA in response to drought 80
4.4 Discussion 83
4.5 Conclusion and prerspective 84
4.6 References 85
Chapter 5. Phenotypic and genotypic diversity of roots response to salt in durum wheat seedlings 89
5.1 Introduction 89
5.2 Materials and methos 91
5.2.1 Plant material 91
5.2.2 Pot experiment condition and design 91
5.2.3 Roots phenotyping 92
5.2.4 DNA extraction, PCR amplifications, and gel electrophoresis 93
5.2.5 Statistical analysis 93
5.3 Results 94
5.3.1 Salt effect on durum wheat shoots traits 94
5.3.2 Salt effect on the root system 94

5.3.3 Correlation among traits 97
5.3.4 Principal component analysis (PCA) 98
5.3.5 Genetic diversity based on SSR markers 99
5.3.6 More detailed analysis of genomic regions linked to selected SSRs markers and in silico candidate gene expression analysis 101
5.4 Discussion 102
5.6 Conclusion 106
5.7 References 106
Chapter 6. Durum wheat (*Triticum turgidum* L. var. *durum*) root system response to abiotic stresses and genetic characterization for root related traits 112
6.1 Introduction 112
6.2 Materials and methods 114
6.2.1 Plant material and experiment design 114
6.2.2 Plant phenotyping 115
6.2.3 Plant genotyping 115
6.2.4 Statistical analysis 116
6.3 Results 117
6.3.1 Effect of cyclic drought and salt stress on shoot traits 117
6.3.2 Effects of cyclic drought and salt stress on root traits 119
6.3.3 Genotypes response to drought and salt 124
6.3.4 SSR marker analysis 127
6.4 Discussion 129
6.5 Conclusion 131
6.6 References 131
Chapter 7. Conclusion 136

Chapter 1. Introduction

Climate change is increasing significantly the intensity of abiotic stress events such as drought and soil salinization (Mukherjee *et al.,* 2018; FAO, 2022). The Mediterranean basin, in particular, is considered a "hot spot" of climate change on a global scale (Giorgi, 2006), being particularly sensitive and prone to major increases in temperature and decreases in precipitation (Giorgi and Lionello, 2008). Agricultural practices, such as the cultivation of crops, livestock, and deforestation, correspond to the second largest sector responsible for Greenhouse gas (GHG) emissions in Europe, with a rate of 11% (IPCC, 2023; Pendolovska *et al.*, 2023). In the latest Conference of Parties (COP28) ('United Nations Climate Change Conference (COP28)', 2023) the urgency to utilize sustainable practices for the food growing systems and for a reduced environmental footprint has been confirmed. Organic farming resulted to be a more sustainable agricultural system, with a lower impact in terms of GHG emissions than the conventional system due to the lower inputs needed (Lynch *et al.,* 2012). Moreover, organic farming represents a means for improving biodiversity and organic soil matter, with lower pollution due to agrochemicals (Franzluebbers, 2005; Hole *et al.*, 2005; Bux *et al.*, 2022). However, most of the genotypes cultivated under organic conditions are selected with breeding program under conventional conditions (Kirk *et al.,* 2012). Varieties selected under organic or low-input conditions have been shown to perform better in various tests in organic environments (Mikó *et al.*, 2017). A selection under stress may result in more competitive lines adapted to conditions with lower levels of available nutrients and high weed competitiveness, which is often the case of organic farming systems (Kirk *et al.,* 2012; Jimenez-Berni *et al.,* 2018). Thus, direct selection under organic conditions of varieties suitable for organic systems is the way to go. The consequences of climate change are already affecting the production chain of durum wheat (*Triticum turgidum* L. var *durum* Desf.), one of the most important crops for the Mediterranean basin (Xynias *et al.*, 2020). Its productivity is menaced by the more and more frequent drought events and the increasing of salt-affected land, with consequent yield loss (Habash *et al.,* 2009; FAO, 2022). Considering climate change and the need for a more sustainable agriculture, breeding programs are needed which focus on development of new cultivars suitable for organic farming and particularly adapted to warm environments where drought and salt stress occur. Root system phenotyping is a major strategy for identifying stress tolerant genotypes. Nevertheless, due to difficulties in studying the root apparatus, studies on root system are very limited. The recent setup high-throughput methods enable detailed analyses of the root system plasticity.

1.2 Aim and objectives of the study

The first aim of the present research was to compensate the lack of cultivars developed through breeding programs conducted under organic farming. In this perspective, a screening of durum wheat genotypes with different areas of origin (from the Mediterranean Basin to Central Europe) in field experiments under organic practices was performed. The genotypes were characterized at phenotypic and genotypic levels by morphological analysis

and molecular analysis using SSR markers highly associated with traits of interest for organic farming.

The objective of the field experiments was to identify the best and most stable genotypes across years and across locations in terms of yield and grain quality. Through the analysis of the G x E interaction, the most adapted genotypes to the three environments where the field experiments took place, i.e. Italy, Austria, and Hungary, were identified.

Additional aims of present study were to fulfil the gap in root analyses and to identify the principal components for the tolerance to drought and salt stresses. From the screening conducted in the first years of field trials, the best genotypes for yield and grain quality, with balance between the two different geographical origins were selected, and they were tested for drought and salt tolerance in a greenhouse experiment under controlled conditions. The modern cultivars were compared with two well established drought and salt tolerant genotypes, a modern cultivar, and a landrace. Through the high-throughput phenotyping software Win-RHIZO, the plasticity and the architecture of the root systems under drought and salt stress were highlighted. Major traits for drought and salt tolerance breeding were identified. Moreover, a genotypic characterization with SSR molecular markers was performed with the aim to identify the alleles tightly associated with drought and salt tolerance traits.

1.3 Organization of the study

The dissertation consists of an introduction (Chapter 1), two manuscripts under submission (Chapter 2, Chapter 6), three published articles (Chapters 3-5), and conclusion (Chapter 7).

Bonfiglioli, L.; Urbanavičiūtė, I.; Pagnotta, M.A. European multi-year field screening of durum wheat (*Triticum turgidum* L. var *durum* Desf.) for organic farming **(Chapter 2)**

Urbanavičiūtė, I.; Bonfiglioli, L.; Pagnotta, M.A. One Hundred Candidate Genes and Their Roles in Drought and Salt Tolerance in Wheat. Int. J. Mol. Sci. 2021, 22, 6378. https://doi.org/10.3390/ ijms22126378 **(Chapter 3)**

Urbanavičiūtė, I.; Bonfiglioli, L.; Pagnotta, M.A. Diversity in root architecture of durum wheat at stem elongation under drought Stress. Agronomy 2022, 12, 1329. https://doi.org/10.3390/agronomy12061329 **(Chapter 4)**

Urbanavičiūtė, I.; Bonfiglioli, L.; Pagnotta, M.A. "Phenotypic and genotypic diversity of roots response to salt in durum wheat seedlings." Plants 12.2 (2023): 412. https://doi.org/10.3390/plants12020412 **(Chapter 5)**

Bonfiglioli, L.; Urbanavičiūtė, I.; Pagnotta, M.A. Durum wheat (*Triticum turgidum* L. var. *durum*) root system response to drought and salt stress and genetic characterization for root related traits **(Chapter 6)**

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Chapter 2. European multi-year field screening of durum wheat (*Triticum turgidum* L. var *durum* Desf.) for organic farming

Luca Bonfiglioli, Ieva Urbanavičiūtė, and Mario A. Pagnotta

Abstract

Durum wheat is one of the main crops in the Mediterranean Basin and its production is facing major challenges from climate change with negative impacts on its productivity and quality of the product made of it. New varieties, bred under organic conditions and for organic agriculture, are needed to contrast the pollution from agrochemicals, and to reduce the production and profit discrepancy between conventional and organic farming. The aim of the present work was to improve the availability of seeds and varieties widely adapted for organic farming through a direct selection. Field trials conducted over multiple years across Austria, Hungary, and Italy were performed with twenty-four durum wheat genotypes originated in the Mediterranean Basin and Central Europe. The genotypes were screened for grain yield, grain quality and stability across the years. The trials offered a comprehensive understanding how the different factors, including genotype's different origin, varied environmental conditions, and geographical locations, impact the performance of crops. The correlation between heading date and grain yield showed several effects across different locations. The genotype origin and the environment of cultivation significantly influenced the outcomes. Typically, the highest and most consistent yield were observed in the locations where the genotypes were developed. Interestingly, despite this trend, a limited number of genotypes proved to be high performing even in regions that contrasted with their area of origin. This suggests their potential as valuable resources beyond their primary adaptation zones. The most explanatory G×E interaction was represented characterizing the genotypes as "widely adapted" or "specifically adapted" to one environment or a group of environments.

Key words: Durum wheat, organic farming, stability, Central Europe, Mediterranean Basin, sustainability, direct selection.

2.1 Introduction

The organic food production and consumption had been showing a significant growth during the 2010-2020. In 2020, with 14.8 million ha, the organic farming fields were the 9.1% of the total EU agricultural land. Cereals have the 16% of the total surfaces dedicate to organic production (European Commission, 2021). Compared to the conventional one, the organic cereal production can save up to 100% on plant protection costs, and up to 90% on fertiliser cost per hectare, with a lower environment impact due to the restrictions on synthetic chemical products. (Bux et al., 2022). In general, the conventional crop production is characterized by higher yields and profits, but negative effects are implicated, such as soil depletion, groundwater pollution, atmospheric contamination, extensive use of agrochemicals, and loss of biodiversity (Froehlich, et al., 2018; Ozturk and Gul, 2020). Previous studies had shown how the organic farming consists in a sustainable alternative to conventional farming, since lower Greenhouse Gas (GHG) emissions were registered, and for the ecological perspective, its practices help to maintain the integrity of the soil structure, and to avoid the release of polluting molecules in the surrounding environment (Knudsen et al., 2006; Halberg, 2012; Lynch et al., 2012). The need for policies and practices that enable sustainable growth of food systems was one of the main focuses also of the latest Conference of Parties (COP28, 2023), where the member states declared to be a fundamental element now to reduce the environmental footprint of the production systems, and the impact of climate on food security. In the durum wheat (*Triticum turgidum* L. var *durum* Desf.) chain production the major environmental impacts were registered during the cultivation phase, and it highlights the need to adopt sustainable agronomic practices (Todorović et al., 2018). Latest studies reports that the global durum wheat cultivation covers about 17.7 M hectares, representing the 5% of total wheat production (International Grains Council [IGC], 2020; Al-Sayaydeh et al., 2023). Representing globally a minor crop; nevertheless, durum wheat can be considered the main crop in small geographical area, where contribute significantly for food production and agricultural income (Martínez-Moreno et al., 2022). It is mostly cultivated in the Mediterranean Basin due to its ability to generate greater yield with better price than bread wheat (*Triticum aestivum* L.) in hotter and low-rainfall areas, but its cultivation area is shifting to higher latitudes s results of rising temperatures (Ceoloni et al., 2014; ISTAT, 2023). Global analysis across years reports a negative response of wheat yields with the rising temperature, mostly at lower latitude (Lobell and Field, 2007; Ainsworth and Ort, 2010). Moreover. future climate models predicted that the durum wheat production will be highly affected by climate change, mostly due to drought, heat, and saline conditions (Lopes et al., 2015; Urbanavičiūtė et al., 2022). The displacements for new cultivation area with different environmental conditions, brings the cultivars to encounter unfamiliar diseases (Chakraborty and Newton, 2011; Luck et al., 2011). It confirms the need of breeding programs for winter type of durum wheat widely adapted at cultivation areas at higher latitudes. Durum wheat is already facing major challenges from climate change and the associated conditions, with negative impacts on its productivity and quality of the product (De Vita and Taranto, 2019). The desertification phenomenon can cause about 72% of yield loss, and one of its consequences, land salinization, can reach a rate of 50% of the total cultivated land by 2050 (FAO, 2021;

Urbanavičiūtė et al., 2023). The Mediterranean Basin, where the main producers of durum wheat are located, has experienced and will likely experience a reduction in annual rainfall and a widespread warming with a consequent decline of groundwater recharge and soil water content (Noto et al., 2023). The European production fails to meet the demand and durum wheat imports are constantly increasing in EU (European Commission, 2023). Canada has recently expanded the land dedicated to the durum wheat production, becoming the biggest producer in the world, with an average of 4.1 million tons of grain produced, mostly destined for export. Italy, after Algeria, is the second largest purchaser of the Canadian's durum wheat (Statistics Canada, 2018) even if part of the imports were cut off due to the expressed concerns over Maximum Residue Limits (MRLs) for glyphosate (Beres et al., 2020). Considering the increase in cereal imports and the relative quality of imported grain, in addition to the increasingly frequent extreme weather events that threaten production, it is important to improve the organic plant material available for European farmers, screening and developing genotypes with successful biotic and abiotic threat mitigation and with a superior Genotype (G) × Environment (E) × Management (M) interaction for a sustainable production combined with high yield, high grain quality, and their high stability over the years. An estimated 95% of the cultivars currently used in organic farming are developed from the selections under conventional conditions (Kirk et al., 2012). As a conclusion, crop performance in organic could be dramatically increased if the varieties were instead bred under organic conditions, therefore it would be beneficial for cultivars to be adapted at the area and conditions of cultivation (Lammerts, 1999).

Instead of indirect selection, where the breeder doesn't select for a specific environments and management condition, organic farming needs a direct selection of varieties tested under organic conditions and in the geographical area where are to be grown in the future (Ceccarelli, 1994). The Ecobreed project, financed by the Horizon 2020 EU research program, aims to improve by direct selection the availability of seeds and varieties suitable for organic and low-input production. The paper objectives are to perform a detailed phenotypic analysis of a durum wheat core collection for traits and combination of traits suited for the organic production for an efficient use of resources, but also having high grain production, and quality. Twenty-four durum wheat genotypes were evaluated across 4 seasons in Austria, Hungary, and Italy, and they were assessed for their performance in yield, yield stability, grain quality, grain quality stability, phenological trend, and morphology. Several approaches were proposed to dissect the Genotype × Environment interaction (G×E) to better understand it.

2.2 Materials and methods

2.2.1 Agro-environmental conditions

The experiments were carried out in three countries, two with continental climate Raasdorf, Austria (48°14'07.0" N, 16°35'42.0"E, 156 m ASL) and Martonvásár, Hungary (47°18'00.0"N, 18°48'00.0"E, 117 m ASL); and one with Mediterranean climate Viterbo, Italy (42°25'12.0" N, 12°04'48.0"E, 326 m ASL). Four years of trials were performed in Austria and in Italy (seasons 2018/2019, 2019/2020, 2020/2021, and 2021/2022) while three years of trials were performed in Hungary (seasons 2019/2020, 2020/2021, and 2021/2022). The Austrian season 2019/2020 was not object of analysis because the field trial was strongly affected by *Wheat Dwarf Virus* (WDV) which resulted in the total loss of grain production. The Austrian and the Hungarian environments had very similar climate conditions (Table S1); they were characterised by minimum mean temperatures of 0°C for all the winters and around 15°C in the summer, while the maximum mean temperature was rising from 5°C of the winter to 30°C in the summer. Except for the season 2019/2020 for Hungary and season 2021/2022 for Austria, which had heavy winter rainfall, for all the other seasons in the two countries continuous periods of rainfall were registered across all seasons. The Italian environment, being Mediterranean, was warmer than that of the Central European countries (Table S.1). Minimum temperatures below 0°C were registered only in the first season of trial for a short period in January, and maximum temperatures never fell below 10°C. Except for the season 2019/2020, where the rainfall were equally spread across the season with more intensity in autumn/winter period, the other growing seasons were characterized by concentrated rainfall in late autumn-winter period with dry springs and summers.

2.2.2 Plant material and experimental design

Twenty-four durum wheat genotypes with different origin were selected for the organic field trials (Table 2.1). Some of them were developed in the Mediterranean Basin while others were developed in the Central European area. The trials were performed strictly under organic practices in plots of 2.5 m^2 for the 2018/2019 season, while in the other seasons the plots were of 7.8 m^2 in Italy, of 6.2 m^2 in Austria, and of 6 m^2 in Hungary. All the experiments were conducted following a RCBD (Randomized Complete Block Design) with 2 replications in Austria and 3 replications in Hungary and Italy. Each country used a sowing density equal to the sowing density used by the local farmers, resulted in 430 germinable seeds per m^2 in Austria, 450 germinable seeds per m^2 in Hungary, and 500 germinable seeds per m^2 in Italy. The ten environments analysed in the present study were named according to the country (i.e. Austria AT, Italy IT, and Hungary HU) plus the year of harvest (i.e. 2019, 2020, 2021, and 2022).

2.2.3 Data recording and analysis

During the growing season, heading date (HD, number of days from sowing to date of BBCH growth stage 55 (Lancashire *et al.*, 1991), plant height (PH, the height (cm) of plants at maturity measured from ground to top of the spike excluding awns) were recorded. Traits, such as grain yield at 13% of moisture (GY, dt/ha), test weight (TW, kg/hL), one-thousand kernel weight (TKW, g), and grain protein content (PROT, %) were measured after harvest. The protein content was measured with NIR analysis for the environment AT2019 (ICC Standard Method No. 159, DA 7200, Perten Instruments), and IT2019 (IM 9500, Perten Instruments), while for all the other environments, the values were calculated with Dumas method, AACC 46-30.01, AOAC 992.23, and ICC 167 methods (AOAC International, 1998; ICC, 2000; AACC International, 2015). Statistical analysis on the data recorded were performed using the R packages "metan" (Olivoto and Lúcio, 2020) and "hclust" (Nowakowski, 2023) on R version 4.3.1 (Team R, 2010). Analysis of variance (ANOVA) was computed both with genotypes, environments and their interaction as fixed factors, and with genotypes as only one fixed factor while environments and blocks as random effect, to check singularly the genotypes performance against environments and replication effects. WAAS indexes (Weighted Average of Absolute Scores) (Olivoto *et al.*, 2019) were calculated to quantify the genotype's yield and protein content stability across the time in the different locations. The broad-sense heritability (h_b^2) was estimated together for the Austrian and Hungarian environments, and separately for Italian environment with the following formula:

$$h_b^2 = \frac{\hat{\sigma}_g^2}{[\hat{\sigma}_g^2 + \hat{\sigma}_i^2/e + \hat{\sigma}_e^2/(eb)]}$$

Where $\hat{\sigma}_g^2$ is the genotypic variance; $\hat{\sigma}_i^2$ is the genotype-by-environment interaction variance; $\hat{\sigma}_e^2$ is the residual variance; while e and b are the number of environments and blocks, respectively.

Tab. 2.1. Name and origin of 24 durum wheat genotypes tested in multi-year organic trial in Italy, Austria and Hungary between 2019-2022.

Genotype	**Origin**
Aghram (47)	Icarda, SYR
Azeghar2-1(56)	Icarda, SYR
Gammary (55)	Icarda, SYR
HFN 94n	Unknown origin
Icajin 38(64)	Icarda, SYR
Iride	Syngenta, ITA
Levante	Syngenta, ITA
Lunadur	Saatzucht Donau, AT
MVTD16-19	HUN-REN ATK, HUN
MVTD12-23	HUN-REN ATK, HUN
MVTD20-19	HUN-REN ATK, HUN
Mamoor (53)	Icarda, SYR
Miradoux	Florimond Desprez, FRA
Mv Magnadur	HUN-REN ATK, HUN
Mv Makaroni	HUN-REN ATK, HUN
Mv Masnidur	HUN-REN ATK, HUN
Mv Pelsodur	HUN-REN ATK, HUN
Mv Vékadur	HUN-REN ATK, HUN
Ousloukos	ICARDA, SYR
Sambadur	Saatzucht Donau, AT
Saragolla	Syngenta, ITA
Sebatel2-45	Icarda, SYR
Senatore Cappelli	Nazareno Strampelli, ITA
Simeto	SIS, ITA

2.3 Results

The hierarchical analysis was used to consider the results for all the traits into one over-all score using the correlation values as distance matrix. The analysis revealed two major agroecological groups (Figure 2.1). Cluster 1 is constituted by Italian environments, while cluster 2 is constituted by the Hungarian and Austrian environments together. The environmental conditions of the two countries in Central Europe were similar, and that caused a similar response from the genotypes, different from their response in Italy where warmer and drier environments were registered. For this reason, from the identification of the two groups, the analysis proceeded by comparing the Italian environments with the Hungarian and Austrian environments together.

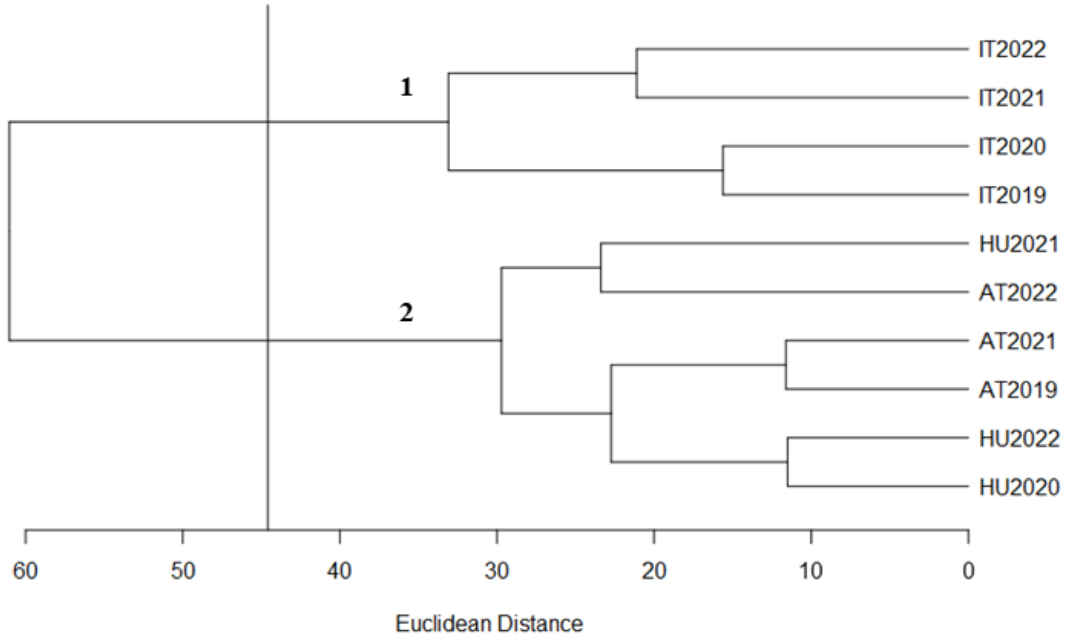

Figure 2.1. Multiple-trait analysis of similarities among organic growing environments based on their reciprocal correlation for grain yield, protein content, heading date, plant height, test weight, and thousand kernel weight. Environments were named according to the country (Austria AT, Italy IT, and Hungary HU) plus the year of the harvest.

The correlation between the values registered in Italy showed a statistically significant negative correlation between heading date (HD) and grain yield (GY), test weight (TW), and also thousand kernel weight (TKW), while the correlation was significantly positive with grain protein content (PROT). Moreover, the quality trait of TW showed a significant positive correlation with GY (Table 2.2). The strong significant correlation between plant height (PH) and PROT may be falsified by the genotype Senatore Cappelli which is a tall old variety with a high protein content (data not shown).

Table 2.2. Correlation analysis among protein content (PROT), heading day (HD), plant height (PH), grain yield (GY), test weight (TW), and thousand kernel weight (TKW) recorded on 24 durum genotypes grown in Italian organic field between 2019-2022. Significance level: * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

Trait	PROT	HD	PH	GY	TW
HD	0.28***				
PH	0.39***	0.31***			
GY	0.07	-0.34***	0.05		
HLW	-0.08	-0.46***	0.16*	0.39*	
TKW	-0.41***	-0.66***	-0.1	0.06	0.5**

In the Austrian and Hungarian environments, compared to the Italian ones, opposite significant correlation was observed between HD and GY, while the correlation between HD and PROT was similarly significant positive, and the correlation between HD and TW, so as HD and TKW were significantly negative, as well (Table 2.3). The effect of Senatore Cappelli on the correlation between PH and PROT was noticeable also in this case (data not shown).

Table 2.3. Correlation analysis among protein content (PROT), heading day (HD), plant height (PH), grain yield (GY), test weight (TW), and thousand kernel weight recorded on 24 durum genotypes grown in Austrian and Hungarian organic field between 2019-2022. Significance level: * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

Trait	PROT	HD	PH	GY	TW
HD	0.61***				
PH	0.13*	0.26***			
GY	-0.33***	0.17**	0.06		
HLW	-0.66***	-0.57***	-0.08	0.33***	
TKW	-0.05	-0.48***	-0.04	-0.23***	0.32

The broad sense heritability showed similar values between the two groups of environments for HD, PH, TW and TKW (Table 2.4). Higher values were detected for HD and PH, while lower for TW and TKW, however, for these traits the genetic components have a major effect for the phenotypic expression. Interestingly, the grain yield (GY) and the protein content (PROT) showed higher values in Austrian and Hungarian environments than in the Italian's, indicating that the environmental effects in the Mediterranean Basin have a stronger influence on these traits.

Tab 2.4. Broad-sense heritability (h_b^2) of protein content (PROT), grain yield (GY), heading day (HD), plant height (PH), test weight (TW), and thousand kernel weight (TKW) in the organic field of Central Europe (Austria and Hungary) and of the Mediterranean Basin (Italy).

Country	h_b^2					
	PROT	**GY**	**HD**	**PH**	**TW**	**TKW**
AUT + HUN	0.78	0.82	0.90	0.98	0.76	0.89
ITA	0.22	0.50	0.93	0.96	0.76	0.84

GY in the Italian trials was significantly higher for the genotypes Iride, Mamoor (53), Ousloukos, and Sebatel2-45 (Table 2.5), all developed in the Mediterranean Basin, while among the genotypes with the higher PROT there are also genotypes developed in Central Europe. TW and TKW were significantly lower for the genotypes developed in Central Europe. HD revealed a clear distinction between the Central European genotypes and the Mediterranean genotypes, with respectively the longest and the shortest vegetation period. All the traits, in Italy, were significantly different among genotypes, environments, and their interactions, except for GY, which was different among genotypes, but not different among environments and their interaction. In the Austrian and Hungarian trials, the best yielding genotypes were all the ones developed in the regions of Central Europe (Table 2.6). Nevertheless, a significant higher PROT was recorded in Sebatel2-45, and Senatore Cappelli, genotypes developed in the Mediterranean Basin. The difference in HD between genotypes of different origin was wider and clearer in the trials conducted in Austria and Hungary. The genotypes developed in the Mediterranean area were significantly earlier than the ones developed in Central Europe. TKW was higher for the Mediterranean genotypes, while they had lower values for TW. From the trials in Central Europe, all the traits showed significant statistical differences among genotypes, and their interaction with the environments. Only TKW and TW showed significant differences also between environments, while GY, PROT, PH, and HD, didn’t show differences between the environments (Table 2.6)

Table 2.5. ANOVA t-values for each genotype (GEN) with significance level of the relative trait. Environments (ENV) and replications were set as nested random effect while genotypes were set as fixed effect. Below means squares of the fixed terms of the model across environments for the different traits. Data calculated on multi-year organic trial of 24 durum genotypes grown in Italy between 2019-2022.

Source of Variation	GY	PROT	PH	HD	TW	TKW
Aghram (47)	-0.37	0.03	0.89	0.50	0.21	0.09
Azeghar2-1(56)	1.37	-0.03	-5.46***	-3.50***	-0.71	-3.27**
Gammary (55)	1.12	0.27	1.58	-0.47	0.63	0.04
HFN 94n	1.7	-0.4	-3.64***	0.00	-0.4	-1.85
Icajin 38(64)	-0.59	2.18*	-3.07**	-3.79***	1.59	4.20***
Iride	2.22*	2.04*	-6.76***	-3.38***	1.22	-1.47
Levante	2.35*	1.48	-4.28***	-1.63	-0.23	-1.6
Lunadur	1.1	2.07*	-5.58***	5.16***	0.7	-0.46
Mamoor (53)	2.25*	2.06*	-2.59*	-4.38***	0	1.41
Miradoux	1.84	0.56	-5.81***	3.85***	-1.47	-2.04*
Mv-Magnadur	1.61	0.71	-4.77***	5.37***	-1.19	-2.31*
Mv-Makaroni	-0.34	0.52	-10.50***	8.34***	-3.79***	-1.07
Mv-Masnidur	0.24	1.59	-2.54*	5.25***	-3.02**	-2.66**
Mv-Pelsodur	1.86	0.63	-4.16***	5.83***	-0.73	-1.09
Mv-Vekadur	1.58	0.46	-3.90***	5.19***	0.08	-2.27*
MVTD16-19	0.99	2.00*	-4.01***	0.58	-0.81	-0.17
MVTD12-23	1.91	2.74**	-4.83***	6.36***	-2.59*	-4.01***
MVTD20-19	-0.11	1.75	-6.51***	4.78***	-4.56***	-1.47
Ousloukos	2.04*	0.25	-3.98***	-0.70	-2.65**	-3.60***
Sambadur	0.76	2.10*	-7.95***	5.01***	-3.89***	-3.35***
Saragolla	1.6	0.41	-8.24***	-0.76	-2.35*	-3.43***
Sebatel2-45	2.04*	2.44*	-6.31***	-3.38***	-1.72	-0.29
Senatore Cappelli	-1.47	3.06**	16.33***	3.09**	2.58*	3.24**
Simeto	1.45	3.11**	-6.17***	-0.35	-1.82	1.41
GEN	146.34**	2.62***	1658.38***	363.9***	16.42***	87.76***
ENV	107.47	84.87***	504.49***	17316***	26.16**	1502.46***
GENxENV	73.2	2.15***	54.46***	25.7***	3.97***	17.68**

Significance level: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Data calculated for grain yield (GY), protein content (PROT), plant height (PH), heading day (HD), test weight (TW), and thousand kernel weight (TKW). Negative values are reported in red.

Table 2.6. ANOVA t-values with significance level for each genotype (GEN) of the relative trait. Environments (ENV) and replications were set as nested random effect while genotypes were set as fixed effect. Below means squares of the fixed terms of the model across environments for the different traits. Data calculated on multi-year organic trial of 24 durum genotypes grown in Austria and in Hungary between 2019-2022.

Source of Variation	GY	PROT	PH	HD	TW	TKW
Aghram (47)	-0.94	-0.68	-0.41	1.8	3.09**	3.14***
Azeghar2-1(56)	-1.48	-2.54*	-6.33***	-4.65***	-0.59	-3.42***
Gammary (55)	-0.37	-0.78	-0.32	-0.9	1.07	-0.73
HFN 94n	0.2	-1.35	-5.26***	-1.8	2.98**	-3.46***
Icajin 38(64)	-0.04	-1.5	-3.18**	-5.27***	2.28*	0.51
Iride	0.83	-2.83**	-8.47***	-3.12**	1.22	-4.78***
Levante	-1.54	-1.15	-7.26***	-2.15*	3.11**	-4.25***
Lunadur	2.09*	-1.91	-5.57***	2.08*	2.32*	-2.70**
Mamoor (53)	0.36	-1.27	-5.01***	-4.37***	0.59	-0.47
Miradoux	2.10*	-2.35*	-6.36***	2.64**	0.39	-0.79
Mv-Magnadur	2.18*	-1.61	-4.51***	0.14	2.94**	-3.16**
Mv-Makaroni	0.18	1.09	-8.62***	9.78***	-4.39***	-6.11***
Mv-Masnidur	2.40*	-2.44*	-0.46	1.98*	2.64**	-3.64***
Mv-Pelsodur	3.27**	-2.50*	-5.47***	1.53	0.18	0.11
Mv-Vekadur	4.11***	-2.68**	-3.11**	0.75	3.36***	-4.04***
MVTD16-19	1.36	0.49	-4.97***	1.8	3.05**	-1.85
MVTD12-23	3.30**	-1.55	-2.52*	3.35***	2.32*	-1.98*
MVTD20-19	1.12	-0.65	-6.44***	2.85**	1.75	-2.37*
Ousloukos	0.62	-0.83	-7.29***	-2.36*	-1.71	-6.14***
Sambadur	5.39***	-2.61**	-7.56***	3.28**	1.09	-4.89***
Saragolla	0.59	-3.13**	-9.76***	-3.61***	-0.79	-6.18***
Sebatel2-45	-0.92	2.19*	-5.97***	-5.00***	-0.39	-0.41
Senatore Cappelli	-2.96**	6.46***	16.45***	1.53	3.20**	0.55
Simeto	-0.55	1.63	-8.94***	-2.78**	-1.02	0.23
GEN	646.4***	3.93***	1348***	185***	14.59***	170.3***
ENV	166.2	0.14	159	6.65	2.883*	355.7***
GENxENV	140.3*	0.86***	32.7*	16.5***	3.704***	22.64*

Significance level: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Data calculated for grain yield (GY), protein content (PROT), plant height (PH), heading day (HD), test weight (TW), and thousand kernel weight (TKW). Negative values are reported in red.

Since high stability can sometimes be present in the genotypes with average low yield, the interaction between genotype's stability and productivity, and therefore the ability to perform steadily at all environments, is showed in Figure 2.2 through the representation of the WAAS index by grain yield (GY) and grain protein content (PROT). Lower WAAS scores are attributed to the most widely adapted genotypes, therefore the genotypes collocated in the bottom of the graphs are more stable than the genotypes at the top. Moreover, the genotypes collocated at the right side of the graph are the most productive, vice versa are less productive the genotypes at the left side. The elite genotypes, with high stability and with a production over the average, are the ones belonging to the quadrant IV at the right-bottom side. This analysis conducted on Austrian and Hungarian environments showed five elite genotypes belonging to quadrant IV for GY, and seven elite genotypes for

PROT. For GY all the genotypes were developed in Central Europe, while also Mediterranean genotypes resulted among the elite genotypes for PROT. In the Italian environments twelve elite genotypes were detected for GY, of which four were developed in Central Europe. For PROT, the elite genotypes were eight, of which two were developed in the Mediterranean Basin. Lower values of WAAS index for environments, instead, are indicating high homogeneity among the genotypes, therefore the more the environment is placed at the bottom of the graph, the greater the homogeneity between the genotypes. The Austrian and Hungarian environments were slightly more productive across the years in terms of GY than Italian ones, while for PROT, the Austrian environments were resulted to be more productive than that of Hungary, but both locations were registered with a high instability, at the same time. Three seasons (2018/20019, 2020/2021 and 2021/2022) in Austria were homogeneous between the genotypes for values of GY, but low in productivity. The Hungarian environments of seasons 2019/2020 and 2021/2022 were more productive but within less homogeneity than that of Austria, except for the season 2020/2021 which resulted to be consistent between genotypes but with a grain production below the average. The season 2019/2020 in Italy resulted to be the most productive and uniform for the grain production. In this season, rainfall was distributed all over the months with higher intensity in autumn/winter period. This made an optimal environmental condition which led to a high and stable performance of the genotypes. In contrary, the season 2020/2021 has brought a strong rainfall event right after sowing, which have compromised the grain production and quality. The season 2021/2022 has experienced a strong drought event from late winter to harvest time with a total seasonal rainfall below the half of the average of the values recorded annually. However, it resulted to be in terms of grain production less homogeneous but with a general production over the mean due the potential of the genotypes developed in the Mediterranean Basin. For PROT, none of the Italian environments was under the threshold of the uniformity.

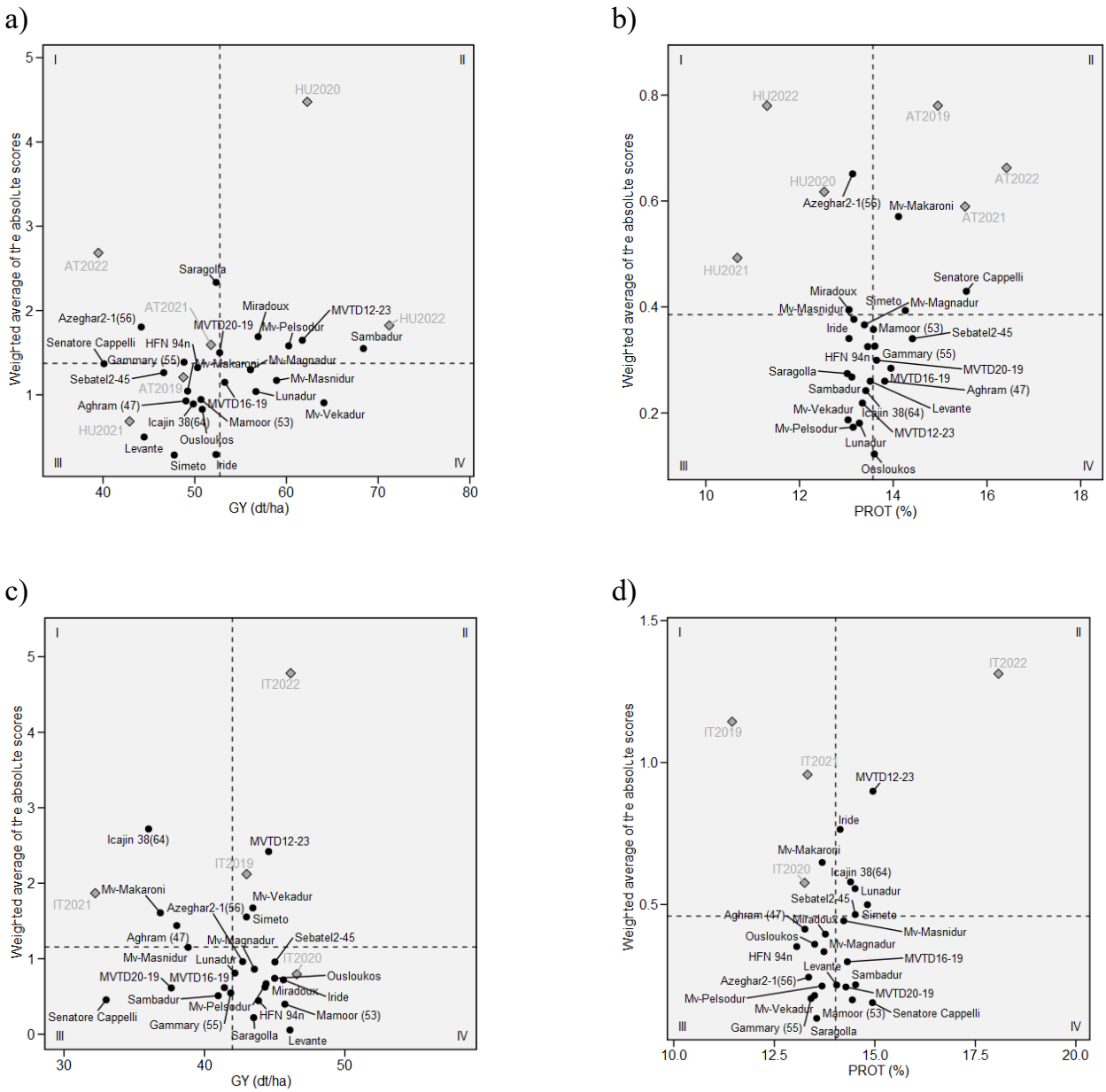

Figure 2.2. Stability analysis of 24 durum genotypes computed with the Weighted Average of Absolute Score (WAAS) of the grain yield (GY) and protein content (PROT) of Austrian and Hungarian environments together (a, b) and Italian environments (c, d). Genotypes are represented in black, while environments are represented in grey. Environments were named according to the country (Austria AT, Italy IT, and Hungary HU) plus the year of the harvest. The entries in the bottom right corner (quadrant IV) can be considered both stable and top yielding for the traits of interest in organic farming.

2.3.1 Performance of genotypes

With the average of 46.1 dt/ha, the highest-yielding genotype across Italian locations was Levate, followed by Iride, Sebatel2-45, and Ousloukos with productions over 45 dt/ha. All of them resulted to be also highly stable (Table 2.7). Although the genotype MVTD12-23, developed in Central Europe, has registered in Italy higher values of GY and PROT

(resulting also the best genotype in one year), its stability resulted to be low across the years. However, the same genotype in Austria and in Hungary, resulted to be highly stable with high production and grain protein content (Table 2.8). Generally, higher protein content was registered in Italy for the genotypes developed in Central Europe, although with a corresponding lower stability than Mediterranean genotypes. In Austria and Hungary, the protein content seemed to be a trait not influenced by the genotype origin. HD in Italy ranged from 136 days from sowing of Sebatel2-45 to 163 days of Mv Makaróni, while a longer period (over 200 days) was necessary to reach the heading phase in higher latitudes, with the Mediterranean genotypes earlier than the Central European ones. In terms of plant height (PH), Senatore Cappelli was consintently the tallest genotype in all locations (Tables 2.7, 2.8), whereas Mv Makaróni was the shortest in Italy, and Saragolla in Austria and Hungary. The two quality traits, TW and TKW were generally higher in the Central European environments, within higher values registred from genotypes developed in the respective area.

Table 2.7. Means, coefficient of variation (CV) and stability performance of 24 durum wheat genotypes examined in Italian organic fields between 2019-2022 for protein content (PROT, %), heading date (HD, days after sowing), plant height (PH, cm), grain yield (GY, dt/ha), test weight (TW, kg/hL), and thousand kernel weight (TKW, g).

Genotype	PROT		HD		PH		GY		TW		TKW		▼ GY quadrant	▼PROT quadrant
	mean	CV	mean	CV	mean	CV	mean	CV	mean	CV	mean	CV		
Aghram (47)	13	17.1	151	9.5	99.3	12.4	38	32.1	80.3	1.8	39.6	9.8	I	III
Azeghar2-1(56)	13	19.8	146	9.6	86.5	14.2	42.7	30.7	79.9	0.5	34.4	9.2	IV	III
Gammary (55)	13.2	19.5	151	10.0	103	11.9	41.9	35.8	80.7	2.0	39.6	13.2	III	III
HFN 94n	12.8	20.2	151	9.1	90.8	13.3	43.9	26.4	80	2.7	36.6	16.0	IV	III
Icajin 38(64)	14.2	12.0	146	10.4	92.1	13.5	36	39.7	81.3	2.3	46.2	13.9	I	II
Iride	14.1	22.7	146	9.5	83.4	13.2	45.6	28.3	81.1	2.6	37.2	12.8	IV	II
Levante	13.8	16.2	149	9.7	89.2	10.5	46.1	26.0	80.2	1.4	37	14.3	IV	IV
Lunadur	15.4	21.7	162	10.6	89.9	4.9	41.8	28.7	80.6	1.3	38	18.3	IV	II
Mamoor (53)	14.1	19.4	145	9.6	93.2	15.3	45.7	41.8	80.3	2.2	41.8	18.8	IV	IV
Miradoux	13.3	24.5	157	9.7	85.6	11.4	44.3	18.1	79.4	1.6	36.3	11.0	IV	III
Mv-Magnadur	13.4	22.2	159	9.4	88.1	8.7	43.6	23.6	79.5	2.2	35.9	15.4	IV	III
Mv-Makaroni	13.3	28.2	163	8.3	74.6	18.9	36.9	30.4	77.8	1.7	37.9	18.6	I	I
Mv-Masnidur	13.9	24.3	159	8.6	93.3	12.2	38.8	27.3	78.3	2.8	35.4	15.5	III	IV
Mv-Pelsodur	13.4	20.9	160	9.3	89.5	12.1	44.4	26.8	79.8	2.0	37.8	14.6	IV	III
Mv-Vekadur	13.3	18.3	159	9.5	90.1	15.1	43.4	38.7	80.4	2.5	36	18.8	II	III
MVTD16-19	14.1	17.7	152	8.2	89.9	12.8	41.4	27.3	79.8	2.6	39.3	16.0	III	IV
MVTD12-23	14.5	31.0	160	9.1	87.9	16.0	44.6	36.1	78.6	3.0	33.2	18.8	II	II
MVTD20-19	13.9	21.8	158	9.7	84	8.8	37.6	24.3	77.3	1.8	37.2	14.0	III	IV
Ousloukos	13.2	21.4	150	9.9	89.9	9.6	45	36.9	78.6	3.4	33.9	16.3	IV	III
Sambadur	15.4	19.7	162	10.3	83.9	8.5	40.5	28.4	77.4	5.3	32.9	26.4	III	IV
Saragolla	13.3	19.2	150	9.2	79.9	12.1	43.5	25.1	78.8	2.2	34.1	18.5	IV	III
Sebatel2-45	14.3	12.9	146	10.5	84.5	11.8	45	27.1	79.2	2.5	39.1	17.6	IV	IV
Senatore Cappelli	14.6	18.5	156	9.2	138	12.8	33	35.5	82	1.2	44.7	11.6	III	IV
Simeto	14.7	11.8	151	9.7	84.8	13.1	43	27.2	79.1	1.6	41.8	17.3	II	II

▼ Genotype performance at grain yield (GY), yield stability, grain protein content (PROT), and protein content stability expressed with the number of the quadrant belonging referring to Fig 2.

Tab 2.8. Means, coefficient of variation (CV) and stability performance of 24 durum wheat genotypes examined in Austrian and Hungarian organic fields between 2019-2022 for protein content (PROT, %), heading date (HD, days after sowing), plant height (PH, cm), grain yield (GY, dt/ha), test weight (TW, kg/hL), and thousand kernel weight (TKW, g).

Genotype	PROT		HD		PH		GY		TW		TKW		▼ GY quadrant	▼PROT quadrant
	mean	CV	mean	CV	mean	CV	mean	CV	mean	CV	mean	CV		
Aghram (47)	13.8	18	206	4.32	89.2	12.5	50.5	27.5	79.8	2.39	51.3	6.26	III	IV
Azeghar2-1(56)	13.1	19.2	201	3.84	77.4	10.6	45.3	31	79.5	3.16	46.1	9.04	I	I
Gammary (55)	13.6	18.1	205	4	88.6	8.09	49.2	27.4	80.4	2.54	50.2	5.37	I	IV
HFN 94n	13.5	18.9	204	4.3	79.4	14.2	51.2	31.6	81.3	3.12	46.1	13.2	III	III
Icajin 38(64)	13.4	14.8	201	4.26	83.3	7.64	50.4	24.7	81	3.77	52	10.3	III	III
Iride	13.1	19.2	203	4.35	73.4	9.94	53.4	21.7	80.6	2.54	44.1	7.77	III	III
Levante	13.5	18.6	204	3.94	75.7	10.7	45.1	29.9	81.4	3.36	44.9	9.24	III	III
Lunadur	13	20.4	206	4.08	76.5	11.1	59	31.9	81.7	2.29	48	21	IV	III
Mamoor (53)	13.6	15.1	202	3.78	79.9	10.6	51.8	30.7	80.1	3.51	50.6	11.3	III	IV
Miradoux	13	20.9	208	4.76	77.3	12.8	57.9	36.5	80	2.7	50.1	11	II	I
Mv-Magnadur	13.4	15.6	206	4.72	80.8	13.3	58.2	28.1	81.3	3.53	46.5	12.2	IV	III
Mv-Makaroni	14.1	18.4	215	5.16	73.1	13.2	51.2	32.6	77.6	4.27	42.1	15.6	III	II
Mv-Masnidur	13.2	18.3	208	4.31	87.7	14.6	58.3	27.5	81.1	1.92	46.1	11.5	IV	III
Mv-Pelsodur	13.1	16	207	4.87	79	14.6	62	29.7	79.9	2.7	51.4	7.9	II	III
Mv-Vekadur	12.7	15.4	206	5.05	81.9	13	65.9	22.7	81.7	1.46	45.2	9.73	IV	III
MVTD16-19	13.9	15	208	4.71	79.9	15.7	55.3	35.5	81.7	3.38	48.5	13	IV	IV
MVTD12-23	14.6	13.7	215	2.26	86	12.9	62	30.6	80	1.63	47.7	13.1	II	III
MVTD20-19	13.6	20.2	209	4.77	77.2	13	54.5	28.9	80.7	5.08	47.7	12.3	II	IV
Ousloukos	13.6	17	204	4.05	75.6	11.9	52.7	26.8	79	5.67	42.1	13.1	III	IV
Sambadur	13.1	15.3	210	4.31	75.1	12.9	67	26.3	80.2	2.9	44.4	12.3	II	III
Saragolla	12.8	17.7	202	4.04	71	9.25	52.6	28	79.4	2.67	42	10.5	I	III
Sebatel2-45	14.4	14.6	201	3.41	78.1	10.2	47.3	34.7	79.6	5.18	50.7	11.6	III	IV
Senatore Cappelli	15.6	17.7	207	4.12	120	9.24	40.1	27.4	81.4	3.2	52.1	3.72	III	II
Simeto	14.3	17.6	203	4.08	72.5	14.1	48.6	24.2	79.3	4.05	51.6	22.1	III	II

▼ Genotype performance at yield (GY), yield stability, protein content (PROT), and protein content stability expressed with the number of quadrant belonging referring to Fig 2.

2.4 Discussion

The environmental component of the variance explained only a small portion of the total source of variation for traits such as GY for Italy, and GY, PROT, PH, and HD for Austria and Hungary, indicating that, in Central Europe, for these traits the differences among genotypes were higher than the differences among environments. In Italy the differences among environments were detected for all traits except for GY, while in Central Europe only for TW and TKW were detected. In both locations, the G×E interaction showed a low amount of variance, with a bigger genotypic component indicating that in both Central Europe and Mediterranean Basin, the genotypes are not all very well adapted. Previous studies have already shown the high values of heritability detected in Central European regions in durum wheat field experiments (González-Ribot et al., 2017; Zaïm et al., 2017; Taneva et al., 2019; Dragov et al., 2022). In our study the heritability showed values for GY of 0.82 for Austrian and Hungary, and only 0.50 for Italy, indicating that the multiple genes control over this trait was strongly affected by the interaction between these genes and the

warmer and more variables environments of the Mediterranean Basin (Gahlaut et al., 2019). The low heritability detected for PROT can be explained, as for the low hereditability of GY, with the effect of high temperatures and water deficit that occur at the end of the agricultural cycle associated with high climate variability in the Mediterranean-type climates. In agreement with present results, low heritability values were detected also in other studies conducted in the warmer environments than the ones of Central Europe (Yagdiet al., 2007; Yagdi and Sozen, 2009; Kahrizi et al., 2010, González-Ribot et al., 2017). Of course, high heritability of traits is an advantage for the selection of new desirable cultivars for a specific environment (Xynias et al., 2020). In these terms, the selection of widely adapted genotypes in Central Europe can count on a higher genetic component, therefore to the innates capabilities of the genotypes for traits such as GY and PROT.

The results of the present study showed that there was not an absolute winner genotype, instead, the large environmental differences between the two groups (Fig. 1) would favour to different performance strategies for the selection. In contrast with Marti and Slafer (2014), whose results showed the importance to breed durum wheat for the thousand kernel weight because strictly associated with grain yield, the results of this study show between TKW and GY a negative correlation and no correlation respectively in Central Europe and in Italy. In contrary, the positive correlation between HD and GY in Austria and in Hungary, and their negative correlation in Italy, suggest that HD can be a good indicator for high production in the two areas, mostly for dry areas where early heading plays a major role for the tolerance to terminal drought events (Schuhwerk et al., 2011). A negative correlation between GY and HD was also found in other studies conducted in warmer environments with less rainfall registered (Tsegaye et al., 2012; Kheiralla and Abdelrhman, 2016). The genotypes developed in Central European regions, with a longer biological cycle, resulted to be less productive in Italy and with lower grain quality except for the protein content. Vice versa, the genotypes developed in the Mediterranean Basin were more performant in terms of production and quality. In the agricultural area of Central Europe, the genotypes developed around the Mediterranean Basin, considered “early genotypes” with a short biological cycle, had a lower production and grain protein content but higher test weight and weight of thousand grains than the genotypes developed in Austria and Hungary. In fact, the elite genotypes belonging to the quadrant IV (Fig. 2a), are all late genotypes for Austria and Hungary; vice versa, the elite genotypes are detected mostly among the early ones for Italy. Interestingly, Azeghar2-1(56) which is one of the elite genotypes in Italy, resulted to be the worst genotype for Central European environments, with low protein content, low grain production and low stability of both of them. Conversely, in the Austrian and Hungarian environments, the genotype Mv Vékadur resulted to be one of the elite genotypes for the interaction stability/productivity, while in Italy wasn’t among the most stable genotypes even if high grain production was registered. Instead, the genotype Levante had the best performance in Italy, while was also one of the most stable in Austria and Hungary, but with a low grain production. The most productive and stable genotypes for protein content was Sebatel2-45 for Austria and Hungary, while for Italy it was Senatore Cappelli, an old durum variety. However, also in Italy, Sebatel2-45 was registered among the most productive and stable genotypes. In a previous study (Grausgruber et al., 2000), the genotypes particularly

adapted to dry and less productive conditions are not able to maintain high quantity and quality standards in different and varying conditions. In this study, it was shown that the same thing does not apply to genotypes developed in Central Europe and tested in drier lands, which may be an optimal resource of GY and PROT components for breeding programs. The climate conditions that characterized the studied environments have significantly affected the productivity of genotypes. Among the ten environments analysed, only one (Italy, season 2019/2020) has been found to belong to quadrant IV (Fig. 2) for the trait GY, hence with a high grain production and a high homogeneity among genotypes. All the other environments resulted to be low productive, less uniform, or both. Previous studies conducted in the Mediterranean Basin have highlighted the higher production and grain quality of irrigated durum wheat than rainfed (Rharrabtiet al., 2003; Elhani et al., 2007; Rezzouk et al., 2022). The continuous rainfall registered in the trial area during the season 2019/2020 may have simulated the regular irrigation, resulting in high yields homogeneous among the genotypes. For PROT, none of the environments was registered in quadrant IV, contrary, all of them were found over the stability threshold. This high instability within the environments represents the high divergence among the capacity of the genotypes, hence is the indicator of the strict association among the productivity, the geographical area of the genotype's origin, and the geographical area of cultivation. Although numerous studies with field experiments conducted over the years have shown that in the conventional farming the grain qualitative traits are higher than in the organic production, resulting in higher yields (Quaranta et al., 2010; Campiglia et al., 2015; Iannucci and Codianni, 2016; Rakszegi et al., 2016), the results of Rempelos et al. (2020) showed how the greater yield potential of varieties bred for conventional farming may only be realised carrying out the agronomic practices with high inputs allowed in conventional agriculture.

From an economic perspective, although the organic farming represents a more sustainable agricultural practice with lower production costs and higher selling price of organic products, the gross income for conventional durum wheat production is still 55% higher (Bux et al., 2022). Previous studies have shown that the direct selection for organic farming can result in yields higher up to 31% than indirect selection (Murphy et al., 2007; Kirket al., 2012), indicating that the economic gap between conventional and organic systems could be partially covered with the preliminary strategy of using varieties selected under organic conditions.

2.5 Conclusion

In this study an analysis of data was performed based on the examination of 24 durum wheat genotypes screened in Italy, Austria and Hungary in multi-year field trials under organic growing conditions. The collaboration between the countries, in the frame of the Ecobreed project, represented a major international effort for the methodology of assessing durum wheat for organic farming, and for distributing useful durum wheat germplasm suitable for organic farming in two geographical area with different climate conditions. Genotypes such as Mamoor (53) and Sebatel2-45 resulted to be particulary adapted for

organic farming in central Italy, showing great potential for grain yield and protein content and their stability across years. For the regions in Central Europe, the genotype widely adapted with high productivity and high stability was a Hungarian breeding line MVTD16-19. The present study confirmed the importance of a direct selection of genotypes for organic farming and revealed the importance to practice the direct selection with local organic breeding programs strictly associated with the area of interest. The results has shown not a consistent trend among the genotypes in the three locations, therefore the origin of the material play an important role for the final performance under organic farming conditions. Local multi-year field trials are essential for the selection of the elite genotypes highly productive and stable.

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Chapter 3. One hundred candidate genes and their roles in drought and salt tolerance in wheat

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Abstract

Drought and salinity are major constraints to agriculture. In this review, we present an overview of the global situation and the consequences of drought and salt stress connected to climatic changes. We provide a list of possible genetic resources as sources of resistance or tolerant traits, together with the previous studies that focused on transferring genes from the germplasm to cultivated varieties. We explained the morphological and physiological aspects connected to hydric stresses, described the mechanisms that induce tolerance, and discussed the results of the main studies. Finally, we described more than 100 genes associated with tolerance to hydric stresses in the *Triticeae*. These were divided in agreement with their main function into osmotic adjustment and ionic and redox homeostasis. The understanding of a given gene function and expression pattern according to hydric stress is particularly important for the efficient selection of new tolerant genotypes in classical breeding. For this reason, the current review provides a crucial reference for future studies on the mechanism involved in hydric stress tolerance and the use of these genes in mark assistance selection (MAS) to select the wheat germplasm to face the climatic changes.

Keywords: osmotic adjustment; ionic; redox homeostasis; transcription factors; salt-responsive genes; genetic diversity; germplasm; cross-transferability

3.1 Global situation

Water and consequently an adequate hydric balance are essential for life, determining crop production in terms of quantity and quality. In the present century, the main problems are related to hydric stress, including dwindling water resources and excessive salinity of irrigation water and soil. Moreover, it is expected that global warming and climate change would increase the frequency of drought events, leading to a severe threat to food security [1–3]. In nature, plants are exposed to various abiotic factors and exhibit complex adaptation responses that depend on their degree of plasticity. In addition, they are facing environmental changes that increase the risk factors, such as rainfall distribution, loss of soil fertility and organic carbon, fluctuation in temperature and light, evaporative intensity, biological stresses, increasing pollution, and declining biodiversity [4]. Water is the most critical factor for the growth and development of living organisms, determining 82% of the variation in grain yield in areas receiving less than 400 mm of annual rainfall [5]. Land plants are anchored in the soil and rely on their root system to ensure water availability and nutrient uptake. About 30% of the Earth's land is arid or semi-arid, and this percentage is

expected to increase due to climate change [6]. The prevision indicates an erratic rainfall distribution, an overall decrease in total annual rainfall, and a more pronounced occurrence of dry periods [7], in addition to an increase in temperatures with a consequent reduction in ecosystem health through species extinction or a reduction in biodiversity, due to migration and changes in behavioural patterns [8]. Among all stresses, drought and salinity (soil and water) are the main abiotic factors that reduce a crop's productivity, causing the most damage for grain production all over the world [9]. Other than grain production, water scarcity also affects the growth rate, leaf size, stem extension, root proliferation, susceptibility to disease, plant colour, etc. [10,11]. Particularly in conventional farming, the rainfall water effectively decreases due to increased surface runoff, soil evaporation through unplanted and bare soil surfaces, and evaporation caused by the aeration of soil during intensive tillage [3]. Factors such as rainfall intensity, duration, exact time of rain events, and the soil properties affect the soil moisture status, especially the plant-available water (PAW), which can differ significantly with the same total amount of precipitation under different conditions. In addition, the water availability for plants decreases due to the high soluble salt concentrations in the soil, which causes absorption difficulties; i.e., water deficit or hydric stress. The main causes of soil salinization can be classified as natural or caused by human activity, named primary and secondary salinization, respectively (Figure 3.1) [12].

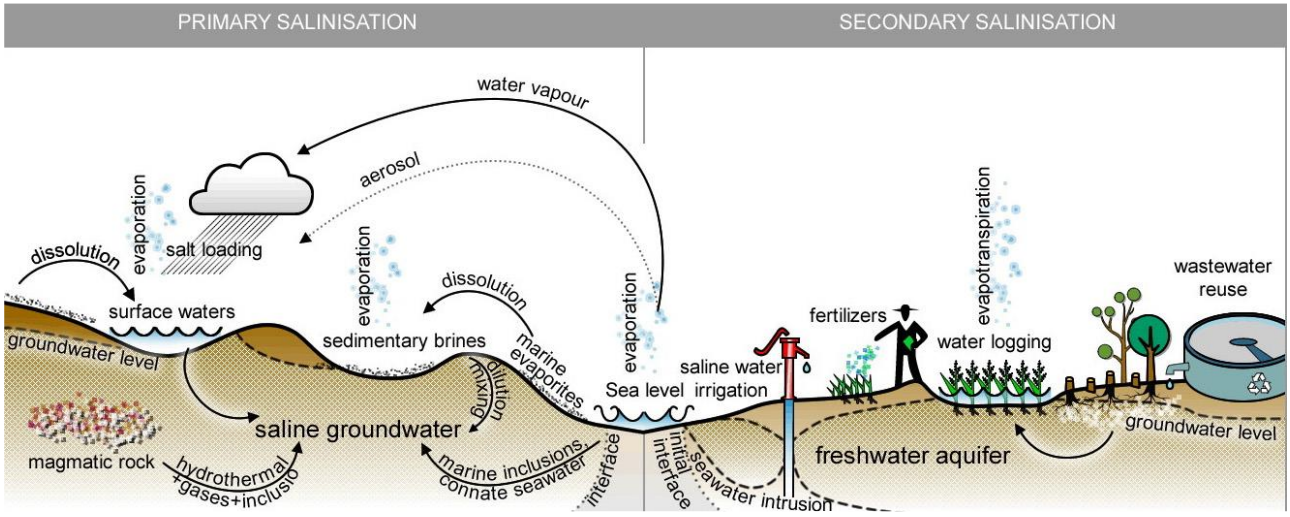

Figure 3.1. Primary and secondary soil salinity mechanisms (Reprinted with permission from ref. [12]. 2016, Elsevier).

Current estimations indicate that salt issues affect about 20% of global lands and almost 40% of irrigated lands. This phenomenon is increasing and 50% of total cultivated land worldwide will be salinized by 2050 [13–15]. Global warming is the increase in temperatures often associated with rainfall decrease, leading to more extreme and unpredictable events. As a conclusion, the need to have a system protecting agriculture from an erratic climate becomes important worldwide. The global grain production is an important indicator of food security, but unfortunately it grows slower than the human population. This unfavourable situation is exacerbated by abiotic factors such as drought, high salinity, extreme temperatures, flooding, and heavy metals [16,17]. There is an urgent need to increase food, which cannot be done by simply raising the agricultural lands, which

causes deforestation and consequently climate change problem. Increasing the production per unit area (i.e., yields) represents a potential solution. Yields can be improved by using genetics methods, appropriate crop rotation, adequate fertilization, and early planting. For example, a six-week delay in planting time resulted in yield reductions of about 42% in barley and 22–32% in wheat [18]. While early planting, proper fertilization, and appropriate crop rotation were found to increase the water-use efficiency (WUE) [19]. Useful and adequate breeding programs, addressed to reduce crop yield lost under stress conditions, are necessary to face the consequences of climate change. Attention should be focused on the drought-related traits, which include leaf canopy temperature, cell osmotic adjustment, cell membrane stability, leaf water potential, stomatal resistance, leaf rolling index, and leaf waxiness [18]. In wheat, for example, the total aboveground part of the plant (biological yield) in a water-limited production area is directly related to the water supply [20]. Although biological yield offers little hope of modification through breeding, the harvest index, which reflects the ratio of grain to total biological yield, appears to be amenable to genetic improvement for enhanced drought tolerance. For this reason, hereafter is reported an overview of the sources of genes presents in wheat and in its wild relatives, and the morphological and physiological mechanisms involved in their tolerance. Moreover, this review provides updated information on more than one hundred candidate genes that provide plasticity in wheat tolerance to hydric stresses, providing tools that open possibilities for breeding new tolerant wheat genotypes.

3.2 Germplasm

Significant differences in drought and salinity stresses were reported between plant species [21]. Their growth rates were highly different under stress, indicating that germplasm is the main reserve of useful genes that could be used to improve cultivated crops. Figure 3.2 shows the differences in salt sensitivity among some plant species. Tall wheat grass (*Thinopyrum ponticum* (Podp.) Z.-W. Liu and R.-C. Wang; (synonymous *Agropyron elongatum* (Host) P. Beauv.) and saltbush (*Atriplex anicola* Paul G. Wilson) are extremely tolerant to salinity. *Arabidopsis thaliana* (L.) is more sensitive to salinity while alfalfa (*Medicago sativa* L.) is much more tolerant, compared to cereals. Among the cereals, rice (*Oryza sativa* L.) is the most sensitive while barley (*Hordeum vulgare* L.) is the most tolerant. Within the intermediate tolerant species, durum wheat (*Triticum turgidum* subsp. *durum* (Desf.) Husn) is more sensitive than bread wheat (*Triticum aestivum* L.) [21].

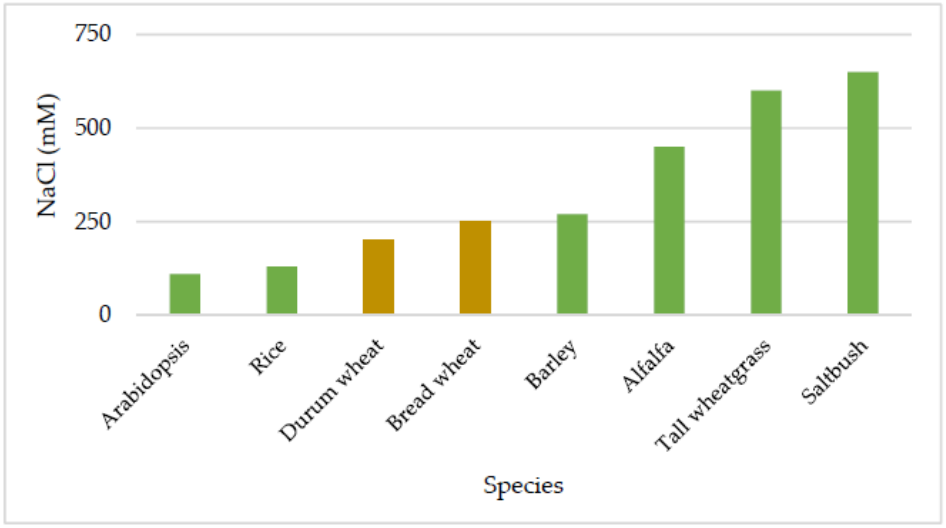

Figure 3.2. Salt tolerance differences among various species, expressed as NaCl concentration to inhibit dry matter production [21].

3.2.1 Sources of Resistances in Cultivated Species

Genes to improve tolerance to drought and salt stresses could be available in accessions, especially landraces of durum and bread wheat, but also in their wild relatives. Landraces have been successfully used to obtain salt-tolerant varieties by specific breeding programs [22,23]. The tolerance to these stresses could be measured with diverse methodology and observations. Some of these methods focus on the tolerance mechanisms, such as the number and dimension of stomata, or on the evaluation of the Na^+ and K^+ ion concentrations in leaves, or on looking at the root system and architecture. A more empirical tolerance evaluation looks at the differences in yield under stress levels. This latter approach is more useful for direct utilization and study of the effect on agricultural production. In turn, the former give information about single genes, information useful for gene pyramiding and to understand the physiological mechanism involved in the tolerance. The homology among genomes is also different: the A, B, and D genomes contain similar loci that in some cases are difficult to be discriminated or to identify the specific locus for a specific function. In addition, the variability in terms of allele number depends of the species population. For example, the *Dreb* genes, one of the most important gene families conferring drought tolerance, are distributed in the different genomes. However, few alleles have been found related to abiotic stresses, and some allele-specific markers are developed for marker-assisted selection [24–26]. Within durum wheat, some alleles due to SNPs and/or INDEL mutations have been identified among varieties for *DREB1*, *HKT1*, and *WRKY1* genes [27,28]. In spite of the homology among the A, B, and D genome, some higher resistance to salt stress is present in the D genome. Bread wheat, *Triticum aestivum* L. (genomes AABBDD), accumulates less sodium and more potassium in expanding and young leaves than durum wheat, T. *turgidum* subsp. *durum* (Desf.) Husn (genomes AABB) [29].

3.2.2 Source of resistance in alien species

Wild relatives are useful sources of genes for stress tolerance. They could be used in breeding programs to transfer a single gene to reduce crop yield loss under stress (see below). The genes conferring resistance were transferred to the amphiploid, which often has very low quality and productivity. This is because the wild relatives, even if having genes able to confer resistance, are lacking in all the other genes selected during 10,000 years of agriculture and breeding, such as to have high yield and high-quality production. Several works [30–33] focus on the source or resistance available in several genomes of the *Triticeae* family, from the cultivated species to the donors of the A, B, and D genomes arriving to more distant species having genomes S, C, G, M, N, U, E, and J, up to the *Hordeum* genera (genomes I, H, and X). Table 3.1 reports a synthesis of the source of resistance found. The transferability from the wild relatives to the cultivated ones is certainly different and depends on the genetic distance among species. Therefore, wild emmer, *Triticum dicoccoides* (Körn. ex Asch. and Graebn.) Schweinf (synonymous *Triticum turgidum* subsp. *dicoccoides*), is a useful potential donor for salt-tolerant genes, which can be transferred to cultivated cultivars by classical and/or modern techniques; while, to transfer genes from other more distant species, it would require passing through the amphidiploids. As reported in Table 3.1, the positive aspect is that there exists variability in salt tolerance amongst members of the *Triticeae*, with the tribe even containing a number of halophytes. Unfortunately, the amphiploid combines in the same

genotypes the donors' characteristics, which, together with useful tolerance genes, have also several unproductive characteristics. For this reason, few salt-tolerant varieties have been released from previous attempts at employing this approach. Modern technologies for assisted evolution give the possibility to transfer only the useful genes without transferring the whole background of the donor accession. Gorham and co-workers analyzed several species and, looking at the tolerance of *Aegilops tauschii* (Coss.) Schmal (genome DD), which store less Na^+, postulated the hypothesis that the D genome has the highest involvement in conferring tolerance [34,35]. Nevertheless, Na^+ exclusion should not be the only mechanism since some tolerant individuals were found with a high level of sodium stored [36]. The *Kna1* locus on chromosome 4D regulated the ratio of accumulated K^+/Na^+ to leaves under salt stress, for this reason bread wheat is generally a better Na^+ 'excluder' than durum wheat. To improve durum wheat, the *Kna1* locus was transferred from the D genome of hexaploid wheat into tetraploid wheat [29,37]. In the background of the durum cultivar Cappelli, the distal part of the long arm of chromosome 4B was substituted with chromosome 4D, creating some new germplasm with an enhanced K^+/Na^+ ratio but with not significantly different Na^+ concentrations [37,38]; also, the A genome should have greater Na^+ exclusion and K^+/Na^+ discrimination than the B genome, since *Triticum urartu* Thumanjan ex Gandilyan (AA) is more tolerant than durum wheat (AABB) [30]. This could be because the B genome negatively interact with the A genome, or because the B genome carry genes increasing Na^+ entry [30]. Furthermore, within the tetraploid species there are accessions more tolerant to salinity than durum wheat; for example, wild emmer wheat (*T. turgidum* L. ssp. *dicoccoides* (Körn. Ex Asch. and Graebn.) Thell.) has lower rates of Na^+ [39,40]. In durum wheat, the locus *Nax1* mapped on chromosome 2A has considerable variation in capacity to 'exclude' Na^+ [41].

Table 3.1. Summary of the studies on a source of genes for tolerance to drought and salt, modified from [30,31].

Species	Genome	Common Name	Reference
Triticum monococcum L. ssp. *aegilopoides* (Link) Thell.	AA	Wild einkorn	[22,42]
Triticum monococcum L. ssp. *monococcum*	AA	Einkorn	[43]
Triticum urartu Thumanjan ex Gandilyan	AA		[42,44,45]
Triticum turgidum L. ssp. *dicoccoides* (Körn. Ex Asch. and Graebn.) Thell.	AABB	Wild emmer	[33,40,44–46]
Triticum turgidum ssp. *durum* L. (Desf.) Husn	AABB	Durum wheat	[22,47]
Triticum aestivum L. ssp. *aestivum*	AABBDD	Bread wheat	[48–50]
Aegilops markgrafii (Greuter) K. Hammer	CC		[34]
Aegilops cylindrica Host	CCDD	Jointed goat grass	[34,51,52]
Aegilops triuncialis L.	C^uC^uCC	Barb goat grass	[51]
Aegilops tauschii Coss.	DD	Goat grass	[34,36,45,53]
Elytrigia elongata Host Nevski	E^bE^b	Tall wheatgrass	[54–57]
Thinopyrum ponticum (Podp.) Barkworth and DR Dewey	EEEEEEEEEE		[55,58,59]
Thinopyrum bessarabicum (Savul and Rayss) Á. Löve	E^jE^j	Tall wheatgrass	[60]
Triticum timopheevii (Zhuk.) Zhuk. ssp. *armeniacum* (Jakubz.) Slageren	GGAA		[35]
Triticum timopheevii (Zhuk.) Zhuk. ssp. *timopheevii*	GGAA		
Aegilops bicornis (Forssk.) Jaub. and Spach	S^bS^b		[34,51]
Aegilops sharonensis Eig	S^jS^j		[34,51]
Aegilops longissima Schweinf. and Muschl.	S^jS^j		[34,51]
Aegilops speltoides Tausch var. *speltoides*	SS		[45]
Aegilops searsii Feldman and Kislev ex K. Hammer	S^SS^S		[34]
Aegilops umbellulata Zhuk.	UU	Jointed goat grass	[34,51]
Aegilops biuncialis Vis.	UUMM		[34]
Aegilops ovata auct.	UUMM	Ovate goat grass	[34,51]
Aegilops variabilis Eig	UUSS		[34,51]
Thinopyrum junceiforme (Á. Löve and D. Löve) Á. Löve	$J_1J_1J_2J_2$		[61]
Thinopyrum scirpeum (K Presl) DR Dewey	JJJJ		[61]
Thinopyrum junceum L. (Á. Löve)	JJJJEE	Sand couch Sea wheatgrass	[62]
Aegilops comosa Sm.	MM		[34]

Farooq and coworkers analysed tolerance in *Triticeae* relatives. They specifically assessed the *Aegilopes* diversity working in Pakistan, which has about 6.8 million hectares of salty land [32,52,63]. Leaf ion concentrations was detected in several *Aegilops* species exposed to 50 mM of NaCl and the most tolerant and promising sources of tolerance were found in *Aegilops cylindrica* Host and in *Ae. geniculata* Roth. [60,61]. Although Cl did not differ significantly in any of the studied *Aegilops* species, they have differences in the Na^+ and K^+ concentrations in leaves. *Ae. comosa* Sm. and *Ae. umbellulata* Zhuk. (M and U genomes, respectively) had accessions with low Na^+ concentrations. Several accessions of *Ae. tauschii* (DD), *Ae. cylindrica* (CCDD), and Ae. ovata auct. (UUMM) survived under severe stress [64]. The *DRF1* (Dehydration Responsive Factor 1) was isolated and characterized in *Ae. speltoides*. The *DRF1* belongs to the *DREB* gene family and encodes transcription factors

playing a key role in water-stress responses. Studying the variation in *DRF1*, Thiyagarajan et al. [65] found a high similarity between the B and S genome, but also suggested that the two genomes have evolved independently. *Thinopyrum bessarabicum* (Savul and Rayss) Á. Löve (JJ syn, EbEb; 2*n* = 14) could bemuch more convenient than polyploids for wheat cytogenetic manipulations. *Elytrigia. elongata* Host Nevski (EE; syn. *Lophopyrum elongatum* (Host) D.R. Dewey), the diploid tall wheatgrass, grows in salt marshes around the Mediterranean and survived exposure to 500 mM NaCl [55,66]. The hydric stresses tolerance is often different regarding the type of stress and the mechanisms of resistance. For example, *Ae. tauschii* is more drought-tolerant, while E. elongata is more salt-tolerant than other species in the *Triticeae* [54,67]. In addition, *Dasypyrum villosum* (L.) Borbás could be used as a source of resistance carrying, on chromosomes5 and 6, loci with significant positive effects on salt tolerance [66]. To transfer tolerance from wild relatives to the cultivated species several interspecific crosses have been performed. *T. timopheevii* (Zhuk.) Zhuk. ssp. *timopheevii* (GGAA) has been hybridized with *Ae. tauschii* (DD) to make the synthetic hexaploid (GGAADD) [68,69]. Synthetic hexaploids have been made crossing durum wheat (AABB) and *Triticum monococcum* L. ssp. *monococcum, T. urartu*, and *T. monococcum* ssp. *aegilopoides* (Link) Thell. [68,69]. The aim was to have lines able to exclude Na^+ and a higher K^+/Na^+ ratio.

3.3 Morphological and physiological response

When plants suffer from hydric stress, significant changes in their morphology can be observed. Usually, hydric stresses affect plant size, which becomes smaller due to a decreasing number of leaves and decreasing area. However, the roots are lengthened searching for water, which results in an increase in the root-to-shoot ratio. This adverse effect of water scarcity on crop plants causes fresh and dry biomass losses. Early maturity or early flowering is another adaptation strategy where a shorter vegetative phase in wheat can help to avoid stress in further very sensitive flowering and post-anthesis grain filling stages [70]. Drought induces a plethora of negative physiological alterations, such as cell turgor loss, reduction in CO2 assimilation, oxidative stress, and nutritional imbalance [63]. Nutritional imbalances due to drought stress occur by decreasing the water uptake and leaf transpiration, combined with alterations in nutrient uptake. Plants try to counteract these effects by activating drought resistance mechanisms, such as accumulation of salts and water, to improve the cell osmotic adjustment and stomata function. K^+ and Cl^- ion regulation, as well water transport, have been important mechanism for plants under drought. K^+ and Cl^- are involved in leaf cells osmotic adjustment, with the consequent water retention in cells; stomatal closure prevents water loss [71]. The presence of high ionic concentrations in soil, especially NaCl, is a great challenge for the plant's physiology. To balance the high sodium concentrations in the soil, plants cells must maintain high concentration of potassium and low concentration of sodium inside the cells. The root system has been studied less than the aboveground morphology. During the juvenile phases, the root architecture is plastic and can adapt to several environmental conditions. It has been detected that roots can escape a high salinity zone, moving to less salty soil. Galvan-Ampudia et al. [72] identified halotropism as the plant's possibility to reduce their exposure to salinity by moving their root system to an adequate saline environment. The auxin distribution in the root tip affects the root response to salty environments, as summarized in Figure 3.3 [72]. Furthermore, changes in the PIN subcellular localization affect root auxin

transport and can cause various deformities in root architecture, including the size, less lateral roots, root meristem collapse, and others [73].

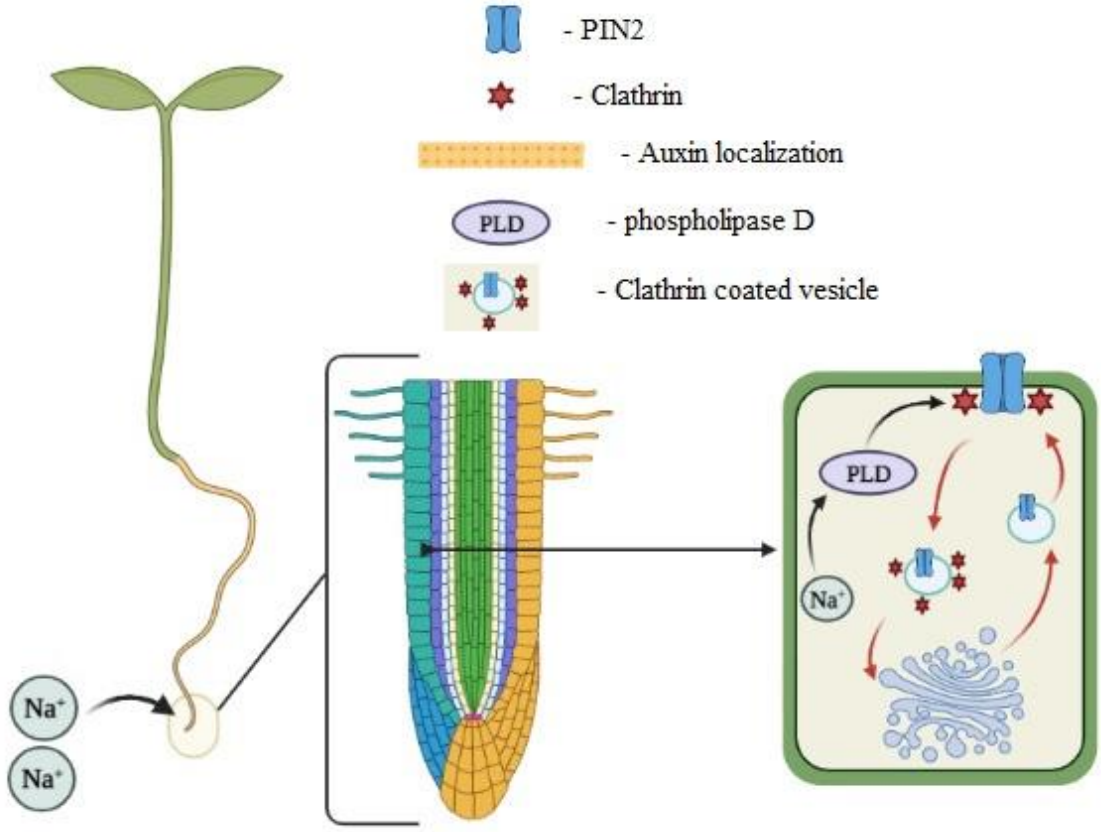

Figure 3.3. Salt-activated phospholipase D (PLD) induces clathrin-mediated endocytosis of PIN2, which allows reorganizing the auxin and roots' growing direction (modified from Galvan- Ampudia et al. [72]).

Salinity inhibits the root length and reduces the number of roots, but also influences the root growth direction. Sun et al. [74] found that the root direction could change with NaCl increasing; the curved roots could be visible already with a concentration of 100 mM of NaCl in the media. Salt stress reduces gravitropism of root growth, altering the PIN-FORMED2 (PIN2) protein abundance and polar distribution and overly salt-sensitive (sos1 and sos2) genes. Julkowska et al. [75] found that more than 100 genetic loci activated under salt stress are associated with root system architecture changes. Among these, CYP79B2 is correlated with lateral root development under salt stress while HKT1 reduce the lateral root development.

3.4 Mechanisms of tolerance

Since plants cannot easily change habitats where conditions are more favourable, to survive and multiply, they evolutionarily adapted, gaining plasticity and specific responses. Generally, plant reactions to abiotic factors include morphological, physiological, biochemical responses, and various adaptation scenarios. In addition, a plant's response can be either common to many kinds of stresses or specific to each one. Stress-tolerance mechanisms are divided in two macro groups: mechanisms involved in osmotic-stress

tolerance and mechanisms involved in ion-stress tolerance [76]. The stomatal adjustment belongs to the first class of mechanisms. Plants under drought and osmotic stresses in non-irrigated systems tend to close their stomata to minimize transpiration, which is reflected in a reduction in growth and production. Exclusion and compartmentalization of toxic ions, avoiding their high concentration in plant cells, are mechanisms of ion-stress tolerance [21]. Each type of physical stress is translated by the plant into a biochemical response, and then into a pathway of interconnected signals with the expression of specific genes that are involved in the stress-tolerance mechanisms. Table 3.2 lists some of the genes that are candidates for the pathway process and their defence mechanisms for osmotic and ion stresses [21].

Table 3.2. Plant salinity-tolerance mechanisms, ordered by processes and their relevance for the three components of salinity tolerance [21].

		Osmotic Stress	Ionic Stress		
Process	**Candidate Genes**	**Osmotic Tolerance**	**Na^+ Excluding**	**Tissue Tolerance**	**References**
Signaling	*SOS3, SnRKs*	Signaling regulation	Activation of ion antiporter	Regulation of vacuolar loading	[77]
Photosynthesis	*ERA1, PP2C, AAPK, PKS3*	Stomatal closure regulation	Protection of chloroplast from ion toxicity	Delay Na^+ toxicity effect in chloroplast	[78,79]
Accumulation of Na^+ in shoots	*HKT, SOS1*	-	Decreasing long distance transport of Na^+	Decreasing energy used on Na^+ exclusion	[80–82]
Accumulation of Na^+ in vacuoles	*NHX, AVP*	-	Increased sequestration of Na^+ into root vacuoles	Increased sequestration of Na^+ into leaf vacuoles	[80,83]
Accumulation of organic solutes	*P5CS, OTS, MT1D, M6PR, S6PDH, IMT1*	Increasing osmotic adjustment	Reduction of Na^+ accumulation	Accumulation of organic solutes in cytoplasm	[82,84]

3.4.1 Osmotic adjustment

Plants have an initial general response to stress by abscisic acid (ABA) biosynthesis in roots as a signalling molecule due to interactions with different receptors; it may activate the general or very specific response to a particular type of stress [85]. After ABA, the first plant-specific response to hydric stresses is to maintain the cell water content, by increasing osmotic force, named osmotic adjustment (OA), using various mechanisms described by Blum [86]. Molecules involved in osmotic adjustment signalling pathways regulate tolerance against osmotic stress via control of stomatal conduction, with the consequent accumulation of organic solutes in the cytoplasm, in order to decrease the osmotic potential of the cytosol. The main mechanisms of defence against osmotic stress predict the accumulation of useful solutes and the use of polyamines. Among the first are proline, glycine betaine, sugars, and polyols.

- The proline content increases under salt stress at the intracellular level and acts as a reserve of organic nitrogen during the stress period. Deivanai et al. [87] highlighted how rice treated with proline improves its response under salt stress.
- Glycine betaine, known also as trimethyl glycine (TMG), is a quaternary ammonium compound with three methyl groups derived from glycine found in many plants and microbes. The TMG is electrically neutral on a wide range of pH and highly water-

soluble, but it also contains groups of non-polar methylins. Due to its unique structural characteristics, it interacts with both hydrophobic domains and hydrophilic macromolecules, such as enzymes and protein complexes. Glycine betaine increases the osmolarity of the cell during the period of stress [88], stabilizes the proteins [89], protects the photosynthetic apparatus from stress damage [90], and then plays an important role in stress mitigation [91].

- Sugars: Plants under saline stress tend to accumulate carbohydrates that play a role in osmo-protection and energy reserves during the stress phases [92].
- Polyols are chemical compounds composed of multiple oxydrilic groups available for organic reactions. They are classified into two types: cyclical (e.g., pinitol) and acyclic (e.g., mannitol). Polyols acts as protectors or enzyme stabilizers when stress related to dehydration occurs [93].

The polyamines have a low molecular weight and are widely spread in the plant kingdom, playing various roles such as the regulation of somatic embryogenesis, cell differentiation, morphogenesis, seed germination, the development of flowers, and fruits and their senescence. Polyamines are associated with gene regulation processes, which are involved in the synthesis of solutes capable to maintain cell membrane integrity and related to the accumulation of Na^+ and Cl^- ions [94].

3.4.2 Ionic homeostasis

The response reaction as ionic homeostasis mainly focuses on the exclusion of the Na^+ ion, avoiding toxic concentrations in plant tissue [21]. Plants can avoid the harmful effect of ions by reducing their accumulation in shoots or by transferring them into vacuoles [95]. One of the responses to ion stress was identified in the SOS (Salt Overly Sensitive) stress signalling and tolerance pathway. It has been shown that *Arabidopsis thaliana* respond to salt mainly through the SOS signal path, which consists of three components involved in ionic homeostasis. SOS1 encodes a plasma membrane Na^+/H^+ "antiporter" protein (i.e., a protein involved in the secondary active transport of different molecules through the membrane), which plays a critical role in sodium extrusion [96]. SOS2 encodes a kinase protein [97], while SOS3 encodes a Ca^{2+}-binding protein that acts as a calcium sensor for salt tolerance [98]. Salinity produces two independent types of secondary responses in plants. The limited plant growth could depend on the osmotic effect of salt in the soil or to the accumulation of sodium ions within plant cells. Generally, osmotic stress has an immediate and great effect with a decrease in the growth of new shoots. Ionic stress acts secondly with milder effects on plants and only in environments with a high salinity rate, or on species highly sensitive to it, increasing the senescence of older leaves [21]. Munns [99] explained how hydric stress acts over time. As reported in Table 3.3, after a few minutes and up to a few hours after NaCl as imposed, plants undergo osmotic stress that slows down the growth rate of the leaves and roots, as the salt tends to seize the available Ca^{2+}, seriously compromising root growth.

Table 3.3. Timing of the plant's response to salinity after the stress was imposed. The effects on a salt-tolerant plants are fundamentally identical to those due to soil water deficit (Reprinted with permission from ref. [99]. 2002, John Wiley and Sons).

Time	Water Stress Effect (Salt-Tolerant Plants)	Salt-Specific Effects Salt-Sensitive Plants
Minutes	Immediate reduction in leaf and root elongation rate and then rapid partial recovery	
Hours	Constant but reduced rate of leaf and root elongation	
Days	Leaf growth more affected than root growth; Reduced rate of leaf emergence	Visible injury in the oldest leaf
Weeks	Reduced the final size of the leaves and/or the number of side shoots	Death of older leaves
Months	Altered flowering time, reduced seed production	Younger leaves dead, plants may die before the seed matures

Rodriguez et al. [100] revealed that root growth was not compromised if Ca2+ was also administered along with NaCl. After a few days under saline stress conditions, the plants encounter ion stress, due to the accumulation of sodium ions and/or chlorine ions in the leaves' cells, thus compromising mainly the epigeal part of the plants. After weeks or months under saline stress, plants reduce the number and size of the new shoots, and the older ones start to yellow until death. It seems that only plants with an inner capacity to produce more shoots have a better chance to be maintained under adverse conditions, as they still have sufficient photosynthesis tissues. However, after weeks and months under stress, plants may also face death before the maturation of the seed. In cereals, it reduces the number of florets per ear, and alters the time of flowering and hence maturity [99].

3.4.3 Redox homeostasis

Water shortages also cause an increase in the reactive oxygen species (ROS) levels and induce cellular redox homeostasis. Therefore, to avoid the harmful effects of these molecules, as a defense mechanism, plants increase production of enzymatic and nonenzymatic antioxidants. The genes responsible for antioxidant activity are very important as candidates for breeding programs to increase abiotic stress tolerance. In plants under drought and/or saline stresses, deregulation or even disruption of the electron transport chain in chloroplasts and mitochondria can occur. This involves the formation of oxidizing molecules because oxygen acts as an electron acceptor, creating compounds such as hydrogen peroxide, radical oxydrile, or radical superoxide, which can damage cellular integrity [101]. Plants can defend against the ion stress with antioxidant molecules, including among the most important nitric oxide (NO) [96]. NO not only is involved in the regulation of various processes of plant growth and development, but it also reacts with free lipid radicals, protecting the oxidation of lipids. Furthermore, the NO helps in the activation of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX), and glutathione reductase (GR) [102].

3.5 Genes and transcription factors involved

State-of-the-art molecular genetic approaches have provided valuable insights about the plant's response to environmental change, and signalling pathways to activate mechanisms of adaptation. Moreover, identification of several genes associated with stress adaptation has led to rational breeding programs. Hereafter, more than 100 genes associated with tolerance to hydric stresses in the *Triticeae* sp. are described. They are presented in the Supplementary Tables S1–S3, together with characteristics such as Accession Nr., dimension (bp), Annotation, Function, Primers, and reference. These genes belong to several groups of responses with different biological functions, and for easier discussion, we grouped them into osmotic adjustment (Table S1), ionic (Table S2), and redox homeostasis (Table S3). In plant cells, the adaptation to adverse conditions begins from signal perception to the production of functional proteins through activation of the target genes. Understanding of a given gene function, and expression pattern under hydric stress conditions, is particularly important for efficient selection of new tolerant genotypes in classical breeding.

3.5.1 Genes involved in hydric stress tolerance

Sixty-nine genes involved in osmotic adjustment (OA) are reported in Table S1. These genes are responsible for defence mechanisms against osmotic stress via control of stomatal conductance; accumulation of organic solutes in the cytoplasm to decrease the osmotic potential of the cytosol; and ionic balance, i.e., the Na^+/K^+ ratio (the accumulation of sodium can be used for OA, especially if it is balanced by the accumulation of potassium). The initial general plant response to hydric stress is abscisic acid (ABA) biosynthesis in roots, which, as a signalling molecule due to interactions with different receptors, may activate the different mechanisms of tolerance [85]. The ABA phytohormone accumulation is one of the main and most common ways to inform plant tissues and cells about changes in environmental conditions. Therefore, genes involved in ABA synthesis play an essential role in the perception and transformation of information about adverse conditions. For example, the accumulation of ABA, and therefore increased expression of the *TaNCED1* gene isolated from *Triticum aestivum*, significantly improved drought tolerance in transgenic tobacco plants [103]. In addition, a previous study has shown that oxidative cleavage of cis-epoxycarotenoids catalysed by NCED (9-cis-epoxycarotenoid dioxygenase) is the critical step in the ABA biosynthesis [104]. Phylogenetic data showed that *TaNCED1* has the highest (95%) identity with the barley *HvNCED1* gene [103]. The genes involved in the ABA biosynthetic pathway are potential candidates for breeding to improve plants tolerance to various stresses. Furthermore, plants can transmit information about stress conditions though the lipid-dependent signalling pathway (Figure 3.4). Three phospholipases (A, C, and D) involved in this pathway produce secondary signalling messengers, which play important roles in plant response and tolerance under hydric stress [105]. Phospholipases D catalyse the hydrolysis of phospholipids (PC—phosphatidylcholine, and PE—phosphatidylethanolamine) to produce phosphatidic acid (PA), which is a very important second messenger that modulate many cellular processes, including the signalling pathways related to plant defence, and tolerance [106].

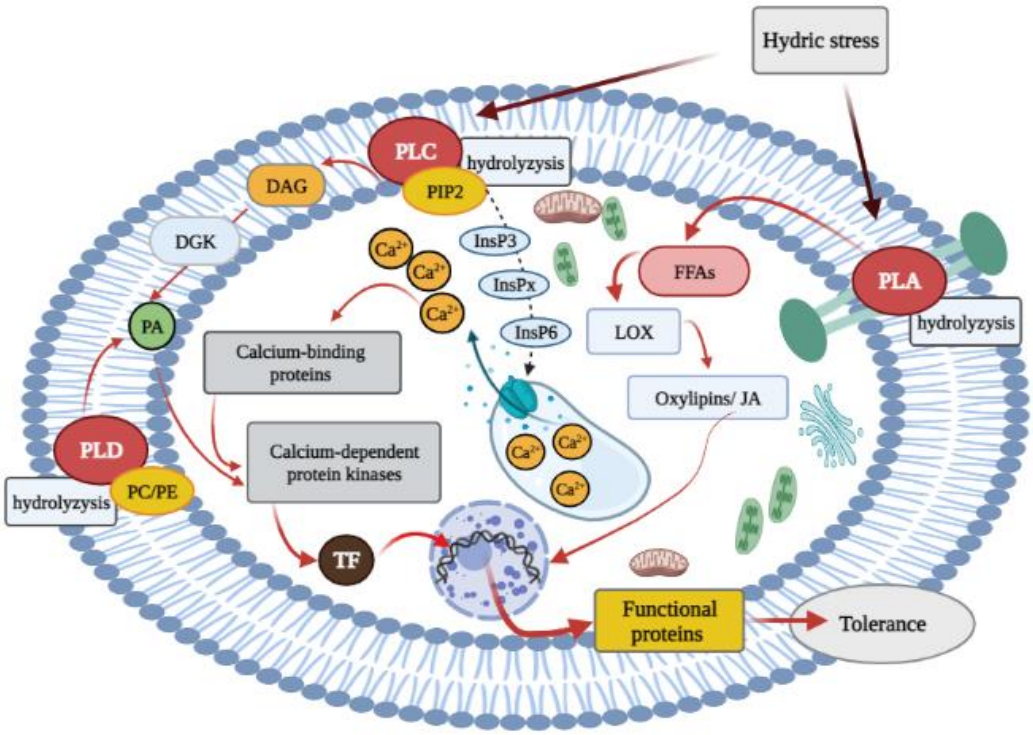

Figure 3.4. The general scheme of plant cell response to hydric stress in the lipid-dependent pathway: signal perception, transduction, and activation of the target genes. ABA—abscisic acid, PLD—phospholipases D, PC—phosphatidylcholine, PE—phosphatidylethanolamine, PA—phosphatidic acid, PLC—phospholipase C, PIP2—phosphatidylinositol bisphosphate, IP3—inositol triphosphate, DAG—diacylglycerol, DGK—diacylglycerol kinase, PLA—phospholipase A, FFAs—free fatty acids, LOX—lipoxygenase pathway, JA—jasmonic acid, TF—transcription factors.

Another way to produce phosphatidic acid (PA) and transmit information about the environmental changes is through the regulation of the calcium ion concentration in the cytosol, via the phospholipid pathway [107]. The hydrolysis of phosphatidylinositol bisphosphate (PIP2) by phospholipase C produces diacylglycerol (DAG) and inositol triphosphate (InsP3). In animals, IP3 is responsible to release calcium, while in plants this function is related to IP3 and his derivative IP6 (Figure 3.4) [108,109]. Several studies reported that the *TaPLC1* gene is involved in plant response to cold, salt, and drought stresses. It encoded phospholipase C (PLC), a specific enzyme that mediates abscisic acid signal transduction in guard cells via increasing the cytoplasmic Ca^{2+} [110–112]. Moreover, it was found that a reduced level of phosphoinositide-specific phospholipase C in guard cells is associated with the inhibition of stomatal opening by ABA [112]. The importance of this gene for plant tolerance is demonstrated by its prevalence in the plant kingdom from GenBank: Arabidopsis *AtPLC1* (AT5G58670) common tobacco *NtPLC1* (AF223351.1), rice *OsPLC1* (AJ276277.2), cowpea (U85250.1), and *Lilium davidii LdPLC1* (AY735314.1). Another pathway is the lipoxygenase-related (LOX) pathway and its metabolites, oxylipins and jasmonates, produced from free fatty acids (FFAs) that is released free by phospholipase A (PLA) from the membrane phospholipids (Figure 3.4). The LOX pathway based on polyunsaturated fatty acids oxidation, such as _-linolenic acid, is particularly important since it represents a substrate in the synthesis of the phytohormone jasmonic acid (JA). Moreover, it has been demonstrated that biotic and abiotic stresses induce the genes involved in _-linolenic acid metabolism [105,113]. The first allene oxide cyclase (AOC) gene was isolated from *T. aestivum* cultivar SR3 (cross between Cv. JN177 and tall

wheatgrass *Thinopyrum ponticum*). *TaAOC1* copy was 720 bp in length and was transcribed in all tissues even with different degrees. Zhao et al. [113] detected the highest levels of this gene at the booting stage, particularly in roots, but also in other aerial organs. Interestingly, during the anthesis, a high level of *TaAOC1* transcription was detected in the awn and ears [113]. Moreover, superoxide dismutase (SOD) activity was increased in transgenic plants constitutively expressing *TaAOC1*, indicating that the regulation of ROS homeostasis is activated by jasmonic acid (JA) to increase salinity tolerance. As mentioned above, phospholipase C (PLC) activity releases calcium ions into the cytosol (Figure 3.4) and increases the amount of these ions, raising the interaction with "Ca^{2+} sensors families" such as calmodulins (CaM), CaM-like proteins, calcineurin B-like proteins (CBLs) and their interacting kinases (CIPKs), and Ca^{2+}-dependent protein kinases (CDPKs) [114]. The Ca^{2+} ions bind with calcium-binding EF-hand proteins, which consist of a helix–loop–helix structure, and an interhelical loop of 12–14 amino acids that bind calcium ions. One of these calcium-binding EF-hand proteins—*TaCab1*—was isolated and characterized from wheat leaves, and is upregulated by biotic and abiotic stresses [115]. Moreover, high affinity calcium-binding proteins CRT (calreticulin protein), which was found in several plant species, is responsible for the hydric stress tolerance in crops [116]. In *T. aestivum*, a full-length cDNA 1446 bp was isolated, encoding calreticulin protein namely *TaCRT* [117], which has high homology with other plants' CRT. Jia et al. [117] underline the role of *TaCRT* in drought tolerance, and found a higher water-use efficiency (WUE), water retention ability (WRA), relative water content (RWC), and lower membrane damaging ratio (MDR) under water-stress conditions. Besides, three *TaCRT* genes, named *TaCRT1, TaCRT2* and *TaCRT3-1*, have been identified in wheat. The all three genes were strongly induced under salt stress, but exhibited different expression patterns in different tissues. Their localizations were on 2 L, 5 L, and 3 L, respectively, with additional homologous copies in the three genomes A, B, and D [116]. *TaCRT1* with an open reading frame of 1287 bp is involved in defence responses and stresses tolerance in wheat. Moreover, Xiang et al. [116] detected an association between *TaCRT1* and the superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities, which suggest that they may be included in redox homeostasis pathways. Calcium-binding proteins change their conformation after binding calcium ions, and this transformation leads to activation of various calcium-dependent protein kinases [114]. The SNF1-related protein kinase SnRK2 subfamily, belonging to Ser/Thr protein kinase class, has shown to be responsible for signal transductions in various stresses. Overexpression of the SnRK2 subfamily genes, such as *TaSnRK2.4*, *TaSnRK2.8*, and *W55a*, enhanced osmotic potential in transgenic Arabidopsis, and they can be used in breeding to improve osmotic adjustment in wheat species [118–120]. Subcellular localization showed that both *TaSnRK2.4* and *TaSnRK2.8* are presented in the cell membrane, cytoplasm, and nucleus, proving that they are responsible for signal transmission in plant cell. The most highly homologous in the GenBank library for these genes are members of the rice *SnRK2* subfamily: *W55a-OsSAPK1* (90.38%), *TaSnRK2.8-OsSAPK8* (94.8%), and *TaSnRK2.4-OsSPAK4* (92.5%). Other protein kinases, such as *TaSK5* and *TaGSK1*, belong to the glycogen synthase kinase–shaggy kinases family, and they are involved in signal transmission in a stressful condition. The structure of these protein kinases contains both a Ser/Thr protein kinase catalytic domain and phosphorylation site, and they are signal transmitters in plant response to salt stress [121,122]. In common wheat, the Leucine-rich repeat receptor-like serine/threonine-protein kinase *TaER-B1* was strongly expressed, and upregulated by numerous environmental stresses, including drought

and salinity [123]. Among the protein kinases *TaABC1* (Table S1), a member of the ABC1 family was identified in *T. aestivum* with an activity on the BC1 complex. Members of the kinase family have in common a conserved domain, but they have differences that lead to different localizations of ABC1 proteins and probably cause differences in their functions. Wang et al. [124] studied the effects of *TaABC1* protein on transgenic Arabidopsis, underling its role in reducing water loss and increasing osmotic potential, photochemistry efficiency, and chlorophyll content. Moreover, *TaABC1* is related to the expression of *DREB1A, DREB2A, RD29A, ABF3, KIN1, CBF1,* LEA, and *P5CS*, which are well-known stress-responsive genes. The plants require the expression of a large number of genes and specific transcription factor families (TFs) to activate their tolerance mechanisms. Among these, an important role is played by protein kinases, which use adenosine triphosphate (ATP) as a donor of the phosphate group for transcription factor phosphorylation (Figure 3.5). This mechanism has been identified as a plant response to abiotic stresses, which allows a fast plant response via fast switching transcription factors from the dephosphorylated state to phosphorylated state and back [125]. Together with protein kinases in TF activation phosphatases, ATP and/or ADP are involved.

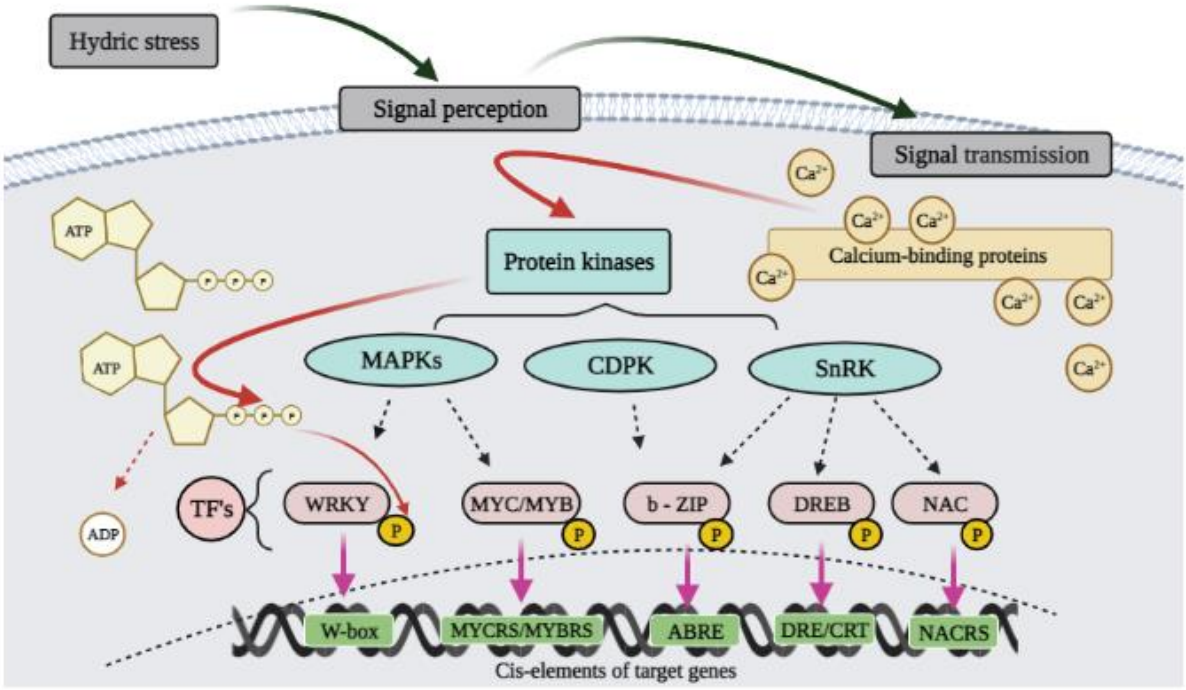

Figure 3.5. The general scheme for the activation of transcription factors by different protein kinase families in response to hydric stress. TFs—transcription factors, MAPKs—mitogen-activated protein kinases, CDPK—calcium-dependent protein kinases, SnRK2—serine-threonine kinases, WRKY—DNA binding proteins, MYC/MYB—myeloblastosis viral oncogene homolog b – ZIP—leucine zipper, , DREB—dehydration-responsive element-binding protein, NAC—(i) NAM (No Apical Meristem), (ii) ATAF1/2 (Arabidopsis thaliana Transcription Activator Factor 1/2) and (iii) CUC2 (Cup-shaped Cotyledon2), W-box—DNA cis-regulatory element sequence, MYCRS/MYBRS—mycorrhiza – responsive sequence), ABRE—ABA responsive element, DRE/CRT—*cis*-acting element ,NACRS—NAC recognition sequence.

According to the TFs' DNA-binding domain characteristics, they were divided into multi-gene families (AP2/EREBP, bZIP, MYB/MYC, NAC, and WRKY) [125]. Moreover, these families are phosphorylated by various protein kinases families (Figure 3.5), for example MYB/MYC and WRKY by mitogen-activated protein kinases (MAPKs), while NAC and DREB by serine-threonine kinases (SnRK2), and bZIP also by SnRK2 and some calcium-dependent protein kinases (CDPKs) [125]. The AP2/EREBP family has a domain structure divided into four subfamilies: AP2, RAV, ERF, and DREB. The AP2 transcriptional factor subfamily has two AP2 domains (RAV one AP2) and one B3 domain, only one AP2 domain characterizing DREB and the ethylene-response transcription factor (ERF) subfamilies [126]. The overexpression genes, such as *TaDREB1, TaDREB6*, and *WDREB2*, in transgenic plants enhanced the tolerance to abiotic stresses, such as drought, high salinity, and freezing, via the activation of stress-inducible genes (LEA/COR/DHN) with DRE/CRT cis-acting element in their promoter region [126–128]. The bZIP proteins belongs to the most large and diverse TF superfamily, classified into 14 subgroups, and carrying a highly conserved bZIP domain, which include a basic region and a leucine zipper. Members of bZIP family that carry ABA response element binding factors (AREBs) belong to subgroup-A, and are involved in the abscisic acid signalling response. These transcription factors regulate the expression of the genes responsible for drought tolerance and have the ABA-responsive cis-element (ABRE), as does numerous of the LEA family genes, such as the cold regulated (COR), responsive to dehydration (RD), early responsive to dehydration (ERD), and responsive to ABA (RAB) genes [129]. For example, in wheat bZIP, the gene WABI5, from group A, regulated the COR/LEA genes in stress responses such as freezing, osmotic, and salt stresses [130]. The other bZIP-type transcription factors from group S, such as *Wlip19* and *TaOBF1*, and their direct protein–protein interaction, also act as a transcriptional regulator of the COR/LEA genes for stress tolerance [131]. However, the bZIP transcription factor *TaABL1* regulates the expression of other LEA family genes, such as the osmotic adjustment-related function genes *RD29B* (responsive to dehydration), *RAB18* (responsive to ABA), and other stress-related genes, which control stomatal closure in transgenic Arabidopsis [132]. TFs from the subfamily of basic leucine zipper (bZIP) might be useful genetic resources for breeding tolerant genotypes. One important and large subfamily of TFs MYB/MYC involved in response and tolerance to various stresses, and characterized by a DNA-binding domain, consist of helix– turn–helix (HTH) or basic helix–loop–helix (bHLH) domains [125]. The overexpression of MYB TFs, such as *TaMYB19-B, TaMYB56-B*, and *TaMYBsdu1*, cause the expression of a number of abiotic stress-related genes, and are important regulators involved in wheat adaptation to water scarcity [133–135]. A group of MYB genes respond to hydric stress (*TaMYB1, TaMYB29, TaMYB34, TaMYB57*, and *TaMYB72*) were isolated and identified in 60 wheat MYB genes during abiotic stress expression analyses [136]. In addition, the overexpression of this family members, such as *TaMYB32, TaMYB33*, and *TaMYB73*, which were specific salt-inducible genes, enhanced tolerance to salt stress in transgenic Arabidopsis [136–138]. Moreover, *TabHLH39*, as an MYC transcription factor, consists of the basic helix–loop–helix (bHLH) domain, and overexpression of this TF improved tolerance to drought, salt, and freezing in transgenic Arabidopsis [139]. MYB/MYC family proteins are crucial in plant stress responses and may improve crop tolerance to hydric stress. A further large superfamily belongs to plant-specific WRKY transcription factors; its specific binding domain consists of a conserved WRKYGQK sequence followed by a zinc-finger motif. The importance of WRKY in abiotic stresses tolerance is shown via the regulation of some stress-responsive genes with specific

cis-acting element W-box in their promoters, specific for the WRKY binding domain [140]. For example, *TaWRKY2* binds the promoter of gene *RD29B* (dehydration inducible gene), and *TaWRKY19* binds a few more, *RD29A* and *COR6.6*, which led to increased tolerance to water deficit in transgenic Arabidopsis [141]. Moreover, the overexpression of TFs *TaWRKY10* and *TaWRKY44* in transgenic tobacco plants significantly activated the genes involved in osmotic adjustment and ROS scavenging, improved tolerance to hydric stresses via proline and other soluble sugar accumulation, and enhanced the antioxidant system [142,143]. Besides that, WRKY TFs impart tolerance to abiotic stresses, they also control plant development and growth, such as *TaWRKY13* and *TaWRKY80*, which improved the root development characteristics together with an increased proline level in transgenic Arabidopsis [144,145]. Another plant-specific TF superfamily proteins feature is the NAC domain (for NAM, ATAF1/2, and CUC2) in the N-terminal region and various C-terminal sequences [125]. Multiple abiotic stresses in transgenic Arabidopsis cause overexpression of NAC TFs, such as *TaNAC2, TaNAC29, TaNAC47, TaNAC67*, and *TaNAC69*, which, in turn, improve dehydration tolerance via controlling expression of some abiotic stress-response genes [146–150]. Moreover, the NAC family members *TaNAC4* and *TaNAC8* overexpression in wheat were induced not only by abiotic stimuli but also by stripe rust pathogen infection. Both of them contained a plant-specific NAC domain in the N-terminus and transcriptional activity in the C-terminal region [151,152].

3.5.2 Stress tolerance-related genes and functional proteins

The LEA3 proteins belong to the big “late embryogenesis abundant” proteins family, whose function is usually associated with maintaining the water content in plant cells under abiotic stresses [153]. These proteins, according to their structure, are characterized by an 11-mer amino acid motif (TAQAAKEKAGE), which are important in formatting protein _-helical during dehydration [154]. It was shown that transgenic *Phellodendron amurense* overexpressing wheat *TaLEA3* gene were tolerant to water deficit by fast stomatal closure [155]. Moreover, overexpressing LEA3 family genes, *WZY3-1* and *TaLEA3*, effectively enhanced the drought and salinity tolerance in transgenic plants due to protecting the cell membrane from damage, enhancing photosynthetic efficiency, and reducing ROS [156–158]. LEA3 family genes *Wrab18* and *Wrab19* encoded cold-responsive LEA/RAB-related COR proteins, which are induced under salt and drought stresses, too [159]. Both genes contain core ACGT motifs in the promoter regions and are direct target genes for b-ZIP type transcription factor *WLIP19* [131]. The late embryogenesis abundant proteins (LEA2) family, also named dehydrins, according to a combination of conserved segments, were subdivided into seven groups: KS, SK3, YSK2, Y2SK2, Kn, Y2SK3, and YSK3 [160]. SK3 dehydrin type genes *TaDHN2.1*, *TaDHN2.2*, and *TaDHN2.3* encode cold acclimation proteins, which protect the plasma membrane against freezing and dehydration stress via drought or salinity. Genes belonging to the YSK2 group encode dehydrins TaDHN6, *TaDHN11*, and *TaDHN17*, which their transcript levels were relatively higher only under dehydration stress. Moreover, dehydrins from the same group, *TaDHN9* and *TaDHN17*, induced expression under salt stress. Dehydration also induced expression of dehydrins from group Kn, such as *TaDHN18* and *TaDHN23*. Furthermore, expression of dehydrin *TaDHN7*, from the Y2SK2 group, was induced by both dehydration and salt [160]. Dehydrins genes are valuable for breeding programs; for example, overexpression of *DHN5* in transgenic plants increases tolerance to drought and salinity stress via osmotic adjustment

[161]. Aquaporins belongs to another important group of functional proteins, and it is responsible for regulating fast transmembrane water flow in plants. According to the amino acid sequence, homology, and protein subcellular localization, water-selective channel proteins are grouped into subfamilies, such as the nodulin 26-like intrinsic proteins (NIPs), the tonoplast intrinsic proteins (TIPs), the plasma membrane intrinsic proteins (PIPs), and the small basic intrinsic proteins [162]. The overexpression of the gene *TaNIP* (*Triticum aestivum* L. nodulin 26-like intrinsic protein) resulted in higher salt tolerance of transgenic Arabidopsis. Gao et al. [163] considered that the *TaNIP* gene induces plant salt tolerance mostly due to controlling the K^+/Na^+ ratio and Ca2+ concentrations, but not by proline accumulation. However, the *TaTIP2;*2 (tonoplast intrinsic protein) overexpression reduced *P5CS1* expression and decreased the osmotic tolerance of transgenic Arabidopsis, due to the suppression of proline synthesis [164]. It has also been suggested that this gene may be a negative regulator of stress, due to the downregulation of *P5CS1* and other stress-tolerance-related genes [164]. Thus, aquaporin may be involved not only in fast water transport but also as a secondary function–intermediate regulator in processes related to the osmotic adjustment. For example, the PIP2 subgroup genes *TaAQP7* and *TaAQP8* provide drought and salinity tolerance in transgenic tobacco not only by controlling a better water state but also by inducing expression of antioxidant enzymes, which reduces the ROS levels and membrane damage [165,166]. Such a contrary response of the PIP subfamily genes to stress may be related to their subcellular localization. Osmotic adjustment is positively related to the *TaAQP7* and *TaAQP8* genes products, which are localized on the cell plasma membrane, while it is negatively related to *TaTIP2*; two products which are localized on the endomembrane system. Among the many different mechanisms of defence against osmotic stress, there is the accumulation of useful solutes such as proline, glycine betaine, sugars, and others. The plants can regulate the amount of amino acid L-proline in two ways, either by increasing its synthesis or by inhibiting its degradation. Proline acts not only like an osmoprotectant, but it is also involved in stabilizing membranes, enzymes, cellular structures, and reducing reactive oxygen species (ROS) [167]. Therefore, genes involved in the proline synthesis, such as *P5CS* (pyrroline-5-carboxylate synthetase), *P5CR* (pyrroline-5-carboxylate reductase), and degradation *PDH* (pyrroline-5-carboxylate dehydrogenase), are very useful for drought tolerance and could help to enhance the yield of wheat under salinity stress [84].

3.5.3 The specific genes involved in ionic homeostasis

Twenty-five genes involved in the Salt-Overly-Sensitive (SOS) signal transduction pathway are reported in Table S2. These genes are responsible for the regulation of the ion transport system that facilitates ion homeostasis [168]. Calcineurin B-like (CBL) proteins act as a Ca^{2+} ion sensor and play an important role in signal transduction in response to various environmental stimuli. Some CBL family proteins (Table S2) are responsible for very specific signalling pathways that ensure ionic homeostasis under salt stress. Moreover, a complex of CBL proteins with their protein-interacting protein kinases, CIPKs, have a crucial regulatory function in ionic homeostasis via the SOS salt stress-signalling pathway. At the beginning of this pathway, calcium-binding protein SOS3, activated by Ca^{2+} ions, formulate a complex with SOS2 (sucrose non-fermenting-related serine/threonine protein kinase), ready to activate the SOS1 gene. The SOS1 gene encodes a putative Na^+/H^+ anti-porter [169], which regulate the circulation of the Na^+ ion from shoots to roots and

accumulate it into the vacuoles [170,171]. Such CBL/CIPK complexes were identified in wheat, and the candidate gene for the calcium-binding protein SOS3 is TaCBL4, the orthologue of AtSOS3, which strongly interacted with six candidate genes for SOS2, TaCIPKs (*TaCIPK3, 5, 14, 15, 26*, and *31*) [77]. It was also found that other calcium-binding proteins, *TaCBL1* and *TaCBL9*, strongly interacted with five (*TaCIPK3, 5, 14, 15*, and *25*) and two (*TaCIPK11* and *31*) CBL-interacting protein kinases, respectively [77]. In wheat, these complexes can activate H^+/Na^+ (*SOS1*) anti-porters, as plasma membrane *TaSOS1*, or vacuole *TNHX1* and *TaNHX2* [80,81,83]. In order to keep active the Na^+/H^+ anti-porters (*SOS1*) and remove the sodium ions into the vacuole in exchange for H^+, the H+ gradient generated by the vacuole H+-ATPase and H+-pyrophosphatase (H+-PPiase) is crucial. In turn, it provides energy for successful exchanges. Among the three classes of H+-ATPase's in plants, the vacuolar H^+-ATPase (V-ATPase) is the most complex one, consisting of two sub-complexes: the peripheral V1, responsible for ATP hydrolysis, and the membrane-integral V0 complex, responsible for proton translocation. In wheat, each sub-complex consists of a number of subunits, whose overexpression evokes tolerance for salinity in transgenic Arabidopsis [172]; for example, subunits A, D, and G from the sub-complex V1, are responsible for ATP hydrolysis and H^+ transport, and sub-units C, F, and H are responsible for the stabilization of sub-complex V1 and its connection with V0 [173]. As mentioned earlier, vacuole H^+-pyrophosphatase (H+-PPiase) generates a proton gradient and controls Na^+/H^+ transport but uses the energy for hydrolysis of pyrophosphate (PPi) molecules [80]. High-affinity K^+ transporters (HKT) belong to another gene family that helps regulate potassium transportation, but that are not involved in the SOS pathway. They are common in many plant species, including Arabidopsis [174], rice [175], barley [176], and wheat [28,177]. HKT proteins transport monovalent cations through the electrophysiological gradient, and they have been divided into two subfamilies. The first subfamily in the first pore loop of the protein have a serine (S-G-G-G) and make them more selective for the Na^+ ion than subfamily 2 members, which have glycine (G-G-G-G) in the first loop of the protein [178]. Laurie et al. [179] have shown that downregulation of *TaHKT2;1* (*TaHKT1*) in wheat increased the shoot fresh weight from 50% to 100% under 200 mM NaCl treatment and K^+ deficiency. *TaHKT2;1* expressed in the root, including root hairs, perform as Na^+/K^+ symporters at low Na+ concentrations, and a Na+ uniporter at high Na^+ concentrations [178,179]. One more gene induced by salt stress, and not involved in the SOS pathway, *TaSOS4*, encodes two pyridoxal kinases (PL), and their function is to convert vitamin B6 to pyridoxal 50-phosphate (PLP) [180]. PLP in plants is the pivotal cofactor for various enzymes that are involved in biosynthesis of chlorophyll, ethylene, de novo sphingolipid, and metabolism of amino acids and carbohydrates [180]. In wheat, the gene encoding cytoplasmic pyridoxal kinase *TaSOS4* was identified with the amino acid sequence that is 78% identical to Arabidopsis *AtSOS4* [180].

3.5.4 The genes associated with reduce reactive oxygen species (ROS)

The plant response to oxidative stress starts with producing antioxidant enzymes and a non-enzymatic antioxidant, which reduces the harmful effect of reactive oxygen species (ROS) [181]. Ten genes involved in the ROS are reported in Table S3, with their relative reference, accession number, dimension (bp), annotation, function, and primers usable to amplify. The main enzymatic antioxidants involved in the scavenging of ROS are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR),

glutathione peroxidase (GPX), and peroxidase (POX). Among the enzymes with antioxidant activity, the superoxide dismutase's (SODs) serve as the first line of defense against abiotic stress and ROS [182]. Moreover, drought and salinity in *T. aestivum* affect differently mitochondrial TaMn-SOD (manganese superoxide dismutase) and cytosolic *TaCu/Zn-SOD* (Cu/Zn-superoxide dismutase). After the longterm effects of salt stress on *Triticum aestivum*, the total SOD activity was the highest in the salt-tolerant line chloroplastic fraction followed by mitochondrial, and the lowest in the cytosolic fraction [183,184]. Ascorbate and glutathione are two essential non-enzymatic compounds, reducing the harmful effect of oxidative stress via detoxification of hydrogen peroxide. Enzymes such as ascorbate peroxidase *TaAPX* and glutathione peroxidases *W69* and *W106* play key roles in maintaining the ascorbate and glutathione contents in plants [185]. Sairam and Saxena [186] determined that water-stress-tolerant wheat genotype PBW 175 had the highest ascorbate peroxidase, glutathione reductase, and peroxidase activity, as well had the lowest lipid peroxidation and highest membrane stability under water stress. Glutathione peroxidase (GPX) genes from wheat *W69* and *W106*, localized in chloroplasts, showed their peroxidase activity in vitro, and their overexpression, enhanced tolerance to salt, and H2O2 in transgenic Arabidopsis [187]. To decrease the harmful effects of H2O2, organic hydroperoxide, and lipid hydroperoxide, the GPX uses glutathione (GSH) or thioredoxin (Trx) as the reducing agents. Moreover, the overexpression of *Ta-sro1* (poly (ADP ribose) polymerase) in wheat promotes the activity of these ascorbate-GSH cycle enzymes and are involved in maintaining the genomic integrity, which allows plants to regulate redox homeostasis under salinity stress [188]. Peroxidases (PRXs) are also involved in various responses to abiotic stresses. *TaPRX-2A* gene, which is a member of the wheat class III peroxidase gene family, was recently cloned and characterized by Su et al. [189]. They detected an improved salt tolerance in wheat, with its main expression level located in the root tissues, with 1026 bp an open reading frame (ORF); furthermore, they showed that transgenic wheat plants with *TaPRX-2A* overexpressed have also higher activities of other genes involved in the redox mechanisms. Moreover, wheat catalase *TaCAT* activities in leaves retain their water status, associated with resistance to drought, and preventing the grain yield components from being compromised [190]. Non-enzymatic antioxidant flavonoids belong to a large family of phenolic compounds, protecting plants against various environmental stresses [191]. The flavonoids' biosynthesis is regulated genetically and controlled by key enzymes, one of them flavanone 3-hydroxylase, which is encoded by gene *F3H1*. Moreover, the *F3H1* belongs to the 'early' flavonoid synthesis gene family; its expression patterns under stress conditions can regulate the flavonoid biosynthesis pathway and produce antioxidants with high efficiency [192]. Plant 12-oxo-phytodienoic acid reductases (OPRs) that catalyse the reduction of double bonds in α, β-unsaturated aldehydes, and ketones, do not interact with jasmonate synthesis nor the jasmonate signalling pathway, but are dependent on the abscisic acid (ABA) regulation signalling pathway [193]. In *T. aestivum*, *TaOPR1* (oxophytodienoate reductase), enhanced salinity and drought tolerance, via promoting activity in an ABAdependent pathway and Reactive Oxygen Species Scavenging [194]. Out of the 1347 bp full-length *TaOPR1* includes two untranslated regions at the 50 and 30 ends of 60-bp and 69-bp, respectively, while the central bp open reading frame is of 1110-bp. The analyses with aneuploid stocks revealed its location on chromosome 2BS [193].

3.6 Conclusion

Wheat, like many other plants, reacts to hydric stress, activating several mechanisms, and these mechanisms involve several genes. The response could vary in accord with (i) the hydric stress typology (drought versus salinity); (ii) the plant stage; and (iii) the stress intensity. Knowledge and the understanding of the mechanisms involved in stresses tolerance, together with the genes activated by the stress, help the selection and use of plants suitable for cultivation in location with water scarcity (quantity and quality). This review aims to be a reference to understand these mechanisms and to have a clear list of the most important genes involved in conferring plant tolerance to hydric stresses. All the information here present are useful to properly run breeding programs to face climatic change either using classical breeding, helped by the specific molecular markers, or by using advanced biotechnological tools, able to transfer specific genes from a species to another.

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Chapter 4. Diversity in root architecture of durum wheat at stem elongation under drought stress

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Abstract

Durum wheat is a major crop in the Mediterranean basin, where water deficit is the most important factor affecting its production. Under drought conditions, the root system has a crucial role in crop productivity as a water and nutrition supplier. The aim of the study was to analyze root system diversity in six contrasting durum wheat accessions, including two hydric stresstolerant genotypes, and to evaluate root traits using the high-throughput phenotyping scanner Win-RHIZO in order to determine the main traits to be used in breeding programs. Six durum wheat accessions were subjected to two drought events under greenhouse conditions from the seedlings stage (BBCH12) for 49 days. Root phenotyping data were validated with results from plants grown in the rainfed field. This study highlighted a great variability among the analyzed genotypes in terms of development, distribution, and architecture of the root system under difficult environments, un-derlining a good resilience to climate change. Interestingly, the two hydric stress-tolerant geno-types, Cham1 and J. Khetifa, showed different root system ideotypes and rooting patterns under drought conditions. The late flowering landrace J. Khetifa (as also genotypes; Pelsodur and Vulci) showed a steep and long root system ideotype that led to the maintaining of the highest root bio-mass, length, and volume under drought conditions, while the early flowering genotype Cham1 (as also genotype; Sebatel) was distinguished by a wider root system ideotype, and by increasing the root volume in the topsoil as a strategy to tolerate drought. Moreover, a significant positive correlation was obtained between the root angle of plants grown under greenhouse conditions and plants from the field. Our results demonstrated that screening plant roots in early stages grown under greenhouse conditions using high-throughput phenotyping systems can speed up the se-lection for crop improvement and future drought stress breeding programs.

Keywords: *Triticum durum*; *Triticum turgidum*; abiotic stress; phenotyping; root architecture; Win-RHIZO

Abbreviations

CR5	number of crossings in the first 5 cm below the ground
CRF	number of crossings in the first 5 cm below the ground of plants from field
CRW	total number of crossings
FR5	number of forks in the first 5 cm below the ground
FRF	number of forks in the first 5 cm below the ground of plants from field
FRW	total number of forks
NL	number of leave
NT	number of tillers
RAC	root angle of plants grown in greenhouse as control
RAD	root angle of plants grown in greenhouse under drought condition
RAF	root angle of plants grown in the field
RDW	root dry weight
RL5	sum of roots lengths in the first 5 cm below the ground
RLD	root length density
RLF	sum of roots lengths in the first 5 cm below the ground of plants from field
RLW	sum of all root lengths
RS	root shoot ratio
RV5	root volume in the first 5 cm below the ground
RVF	root volume in the first 5 cm below the ground of plants from field
RVW	total root volume
SA5	root surface area in the first 5 cm below the ground
SAF	root surface area in the first 5 cm below the ground of plants from field
SAW	total root surface area
SDW	shoot dry weight
TI5	numbers of tips in the first 5 cm below the ground
TIF	number of tips in the first 5 cm below the ground of plants from field
TIW	total number of tips

4.1 Introduction

As a result of climate changes, drought is probably the most severe and unpredictable abiotic stress, significantly affecting crop production due to decreased water and fertilizer availability [1,2]. Drought negatively affects the grain yield and crop production, with a variation reaching 82%, as plants experience both water deficit and the ability for nitrogen uptake and assimilation, which reduce plant vigour [3,4]. Moreover, drought reduces grain yield depending on its intensity, duration, and timing [5,6]. In the Mediterranean basin, water deficit is the most important factor affecting grain yield. Durum wheat (*Triticum turgidum* L. subsp. *durum* (Desf) Husn.) is one of the major cultivated crops according to the importance and cultivated area [7,8]. Furthermore, the Mediterranean basin also has high volatility in rainfall distribution, so water scarcity, with a negative effect on productivity, can also occur in the early growth stages. Several studies revealed that drought affects grain yield more during the vegetative stage (tillering and stem elongation) than in reproductive stages, and can cause about 72% of yield loss [9–11]. Since spikelet initiation starts at the seedling stage to tillering, and floret initiation begins from tillering to stem elongation, both these stages determine spike and spikelet numbers per plant, and in turn grain yield [12]. Therefore, studies on tillering and stem elongation stages should get more attention, as they have a significant impact on grain yield; moreover, selection in early stages can save labour and time. Root system architecture (RSA) plays a crucial role in crop productivity, especially in drought conditions as a water and nutrition supplier [13,14]. Previous studies have shown that early vigour is important for crop development in dry areas [15,16], and that RSA is directly involved in the resilience of wheat in drought-prone environments [17,18]. The root system cannot be considered only as a whole, as the roots could be divided into seminal, lateral, crown and primary roots, and different root typology could react differently to different external stimuli across different genotypes [19].

However, roots are not easily studied; they are not accessible by non-destructive analyses as opposed to aboveground plant organs, and different methods to study root systems have not always given consistent results. Difficulties in studying root systems increase with plant growth, since a wider root system has a higher percentage of damage during the measurement. Researchers try to avoid these problems by (i) analysing plants at an earlier stage (seedlings) and (ii) using artificial systems or an easy-to-clean substrate. Moreover, it was reported that RSA traits detected on the seedling stage can be used to predict the RSA of adult plants in the field-grown [20] or in an artificial system [21] in order to evaluate the crop adaptation under water stress conditions, but these reports were validated only for what concerned the roots angle and seminal root apparatus. The studies of root system characteristics have recently received more attention since several high-throughputs, multifunctional root phenotyping platforms have been developed, and the studies of root system characteristics have recently received more attention. Consequently, some root system ideotypes and their growth patterns under different environments have been identified [22]. For example, the root angle is considered a very important feature to select wheat accessions for drought tolerance, since it has high heritability, and is able to give some indications about root ideotypes' capacity for soil water extraction under a drought regime [23,24]. In early stages, wheat genotypes with a narrow root angle have grown deeper compared with varieties that have wider root angles [21,25]. Such findings suggest that wheat with longer roots and with a narrower root angle will be more drought tolerant, as it can reach water from deeper soil layers. In addition, it has been observed that drought-tolerant genotypes have a great number of nodal and seminal roots concentrated in the crown region and located near the surface, while in susceptible genotypes, roots are located far from the top of the soil [26]. Root phenotyping using the high-throughput technique could help to determine root system architectures, traits or ideotypes and rooting patterns, which can be used to select durum wheat for conditions similar to the Mediterranean basin. The present study was conducted to better understand the genotypic diversity of root architecture in six contrasting durum wheat accessions by evaluating root traits using a high-throughput scanner Win-RHIZO. High-throughput analysis can accelerate the determination of root traits for crop improvement under drought conditions and enable the selection of tolerant and susceptible durum wheat genotypes for breeding programs at the early stages.

4.2 Materials and methods

4.2.1 Plant material

The plant materials consisted of six durum wheat accessions with contrasting morphological and stress resistance characteristics, including two hydric stress-tolerant genotypes Cham1 and Jennah Khetifa (J. Khetifa). J. Khetifa is a landrace grown in the dry areas of Algeria and Tunisia that shows specific adaptation to the North African continental dry land; it is tall and resistant to abiotic and biotic stresses [27,28]. The semi-dwarf variety Cham1, selected at ICARDA (the International Center for Agricultural Research in the Dry Areas) and released for commercial production in several countries of the Mediterranean basin, is characterized by both salt and drought tolerance and yield stability [29,30]. Several studies report that both of these genotypes are hydric stress-tolerant [31–34], and only one mentioned abiotic stress effect on roots [27]. The other four genotypes were chosen

according to the flowering time, as Mv-Pelsodur (from now on just Pelsodur) and Vulci were chosen for late, and Azeghar 2-1 (56) (from now on just Azeghar), and Sebatel2 (45) (from now on just Sebatel) were chosen for early [35].

4.2.2 Greenhouse experiment design and conditions

The six genotypes were sown, with one seed per pot placed with the embryo facing down, in the greenhouse at the Tuscia University experimental farm (Viterbo, Italy), on 20 January, 2021. The experimental design was a randomized complete block design with three replicates. The air temperature ranged from 22 to 28 °C during the day and from 14 to 17 °C during the night. The pots (17 cm diameter, 16 cm high) were filled with 2.5 L of sand. Pots were irrigated three times per week to keep them at 50% soil field capacity (FC), and 80 mL/per pot were filled with a water nutrition solution composed of nitric acid (0.286 mL/L), calcium nitrate (0.432 g/L), potassium nitrate (0.436 g/L), dihydrogen phosphate potassium (0.13 g/l), potassium sulphate (0.04 g/L), magnesium nitrate (0.244 g/L) and Mikron (0.23 g/L) (Prof. Giuseppe Colla, personal communication). The experiment lasted 49 days, and drought treatment was applied two times by discontinuing irrigation; treated plants were re-watered to prevent death. The first drought treatment started when all plants reached early seedling stages (two leaves unfolded—BBCH12) and continued for 14 days. The first drought treatment plants were re-watered (80 mL/per pot) three times per week for two weeks. The second drought treatment, which continued for seven days, started after re-watering (Figure 4.1).

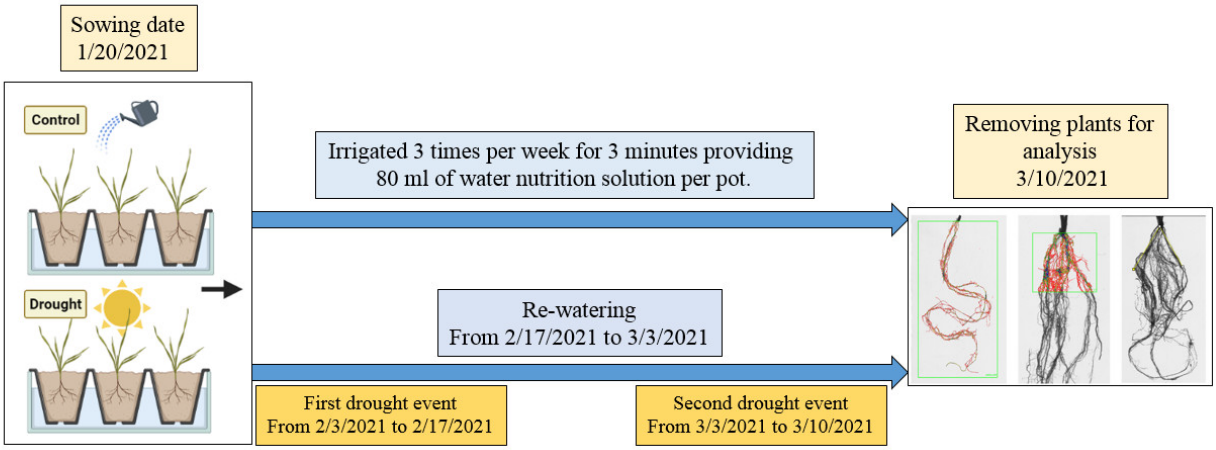


Figure 4.1. Experimental model.

Drought conditions were managed by discontinuing watering, and the field capacity (FC, %) of the soil in the pots was determined during the drought period by weighing the pots regularly according to Grewal et al. [36] (Figure 4.2).

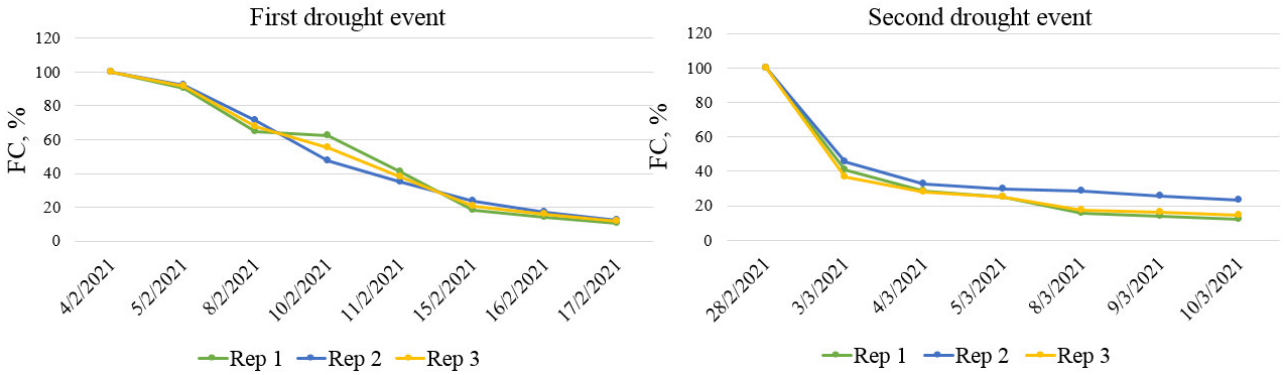


Figure 4.2. Pots' water content in terms of percentage of field capacity (FC, %) for first and second drought treatments.

4.2.3 Phenotyping

The stress effects on plant growth and development in durum wheat genotypes were evaluated by several morphological characters above and below ground. All plants from both treatments were collected 49 days after sowing; roots were carefully washed to remove the sand using a soft spray watering head. The morphological traits of the roots were recorded both for the whole root system (W) and separately in the first 5 cm (5) below ground (Figure 4.3A,B), such as root length (RLW, RL5 sum of all roots lengths, cm), surface area (SAW, SA5 total root surface area, cm2), root volume (RVW, RV5 total root volume, cm3), number of tips (TIW, TI5), forks (FRW, FR5), and crossings (CRW, CR5) using Win-RHIZO Pro software v2009 (version 4.0b; Regent Instruments, Montreal, QC, Canada). The root angle (RA°) was measured using the software *ImageJ* when the angle between the two extreme sides of the roots with the center set in the middle of the crown, as shown in Figure 4.3C. After measurements, the plants were separated into roots and shoots, dried in an oven (80 °C for 12 h), and weighed for shoot and root dry weight (SDW, RDW). In addition, the number of leaves (NL), and number of tillers (NT) were assessed. Moreover, some ratios were calculated, such as the root/shoot (RS) ratio, to determine which above-ground or below-ground part of the plant is dominant. The ratio of total root length and number of tips (RL/TI) was also evaluated, since it could show root system pattern, i.e., the capacity to generate a longer root system or to increase the number of new roots under stress conditions. To determine root system distribution at different soil layers, the ratio of some root traits between the whole system and topsoil was evaluated, such as RL5/RLW, SA5/SAW, and RV5/RVW. Root length density (RLD) was calculated using the following equation: RLW/Soil volume (cm/cm^{-3}). The recorded raw data are public available (after an access request) at the Zenodo web site with DOI: 10.5281/zenodo.5883299.

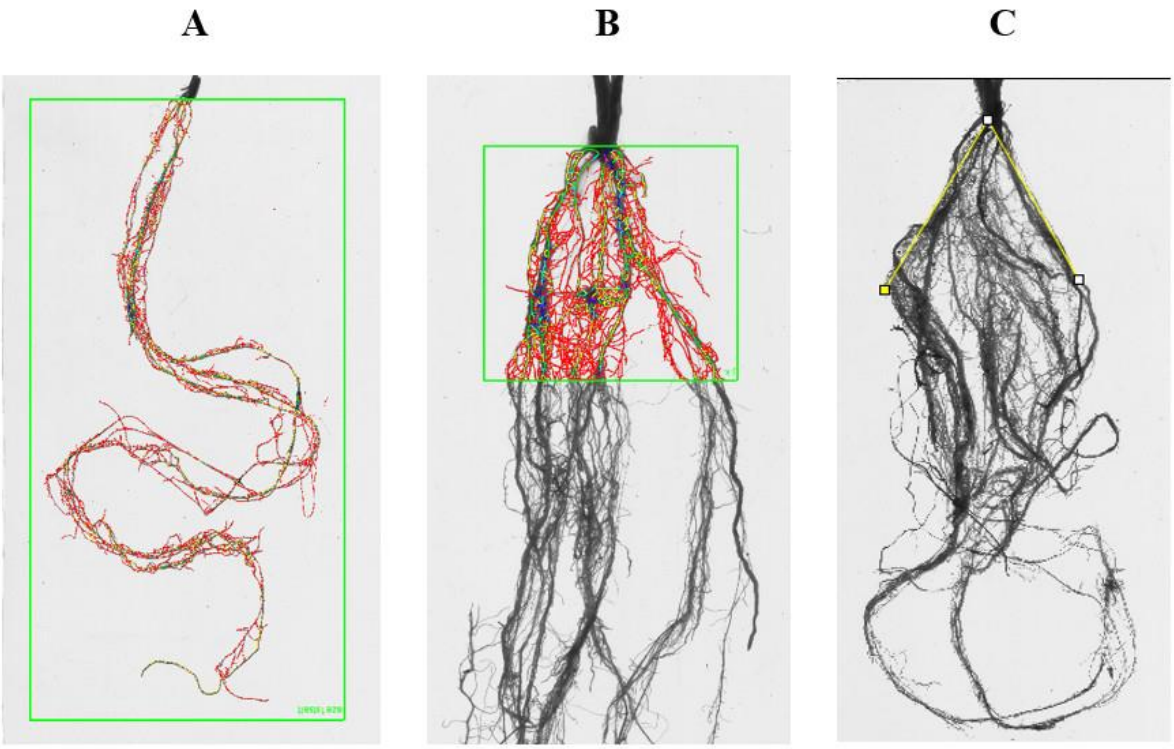


Figure 4.3. The measurements of morphological traits for whole roots (A), topsoil (B) using Win-RHIZO system, and root angle (C) using ImageJ (°).

4.2.4 Field experiment

To validate the results obtained in greenhouse conditions, the same genotypes were sown in rain fed field conditions without any specific treatment (i.e., natural agronomic conditions). A field experiment was performed at Tuscia University experimental farm (Viterbo, Italy). The experimental design was a randomized complete block design with three replicates. Plot size was 1.5 × 5.2 m (7.8 m^2) and sowed with 234.7 g seeds per plot on 11 November 2021. The soil was sandy loam, the pH was 6.9 and organic matter was 14.8 g kg^{-1}. Plants were collected on 17 March 2022, when all genotypes reached the end of tillering and the beginning of stem elongation stages. Plant samples from the field were collected very carefully so as not to damage the root system of the topsoil, at 5 cm deep. Roots were carefully washed to remove soil and analysed for root angle (°) using *ImageJ* software. The morphological traits of the roots of plants from field such as root length (RLF), surface area (SAF), root volume (RVF), number of tips (TIF), forks (FRF), and crossings (CRF) were recorded using Win-RHIZO Pro software v2009 (version 4.0b; Regent Instruments, Montreal, QC, Canada).

4.2.5 Statistical analysis

All of the statistical analyses were performed using R Studio (Version R-4.1.0). Two-way analysis of variance (ANOVA) was conducted at a significance level of 5% using the aov() function, while, one-way ANOVA was used to test the variance component of each trait under each treatment, with genotype as a factor. Fischer's least significant difference (LSD) test was used for mean comparisons. The correlation matrix between all traits was constructed and Pearson correlation coefficients were calculated using the corrplot function R package [37]. Principal component analysis was performed using the prcomp() function and then biplot was generated with the ggbiplot function R package [38].

4.3 Results

4.3.1 Drought effect on shoot growth and development in greenhouse experiment

Recurrent short events of drought at the vegetative stage, from two fully expanded leaves to stem elongation, had adverse effects and growth reductions both below and aboveground in all genotypes. Compared with the control condition, water deficit sig nificantly reduced shoot dry weight (SDW) in the six investigated durum wheat genotypes (Table 4.1). The ANOVA revealed the presence of highly significant differences among genotypes and treatments for shoot traits, such as number of leaves (NL) and number of tillers (NT) per plant, while there were no statistical differences among genotypes for shoot dry weight (in control condition) (Table 4.1). Under control conditions, the studied genotypes were divided into two groups in accord with the number of leaves produced: J. Khetifa, Pelsodur, and Vulci, with a higher number of leaves (on average 32 leaves), and Cham1, Azeghar, and Sebatel with a lower number of leaves (on average 19 leaves) (Table 1). Furthermore, J. Khetifa and Pelsodur also produced a higher number of tillers (on average 10 tillers) in comparison with the other genotypes (on average four tillers). Not surprisingly, in general, NL and NT are reduced by the drought stress (Table 4.1). Even so, these decreases under drought conditions have different degrees in the different genotypes. Vulci and Cham1 have a stronger drought effect on NL (−47.2% and −62.3% respectively) than on NT (−23.5% and −42.9% respectively), while Azeghar loses more tillers than leaves (−66.7% and −55.3% respectively) (Table 4.1).

Table 4.1. Drought effect on shoot growth and development in greenhouse experiment. D/C—drought vs. control.

Genotype	Shoot Dry Weight (g)			Number of Leaves			Number of Tillers		
	Control	Drought	D/C, %	Control	Drought	D/C, %	Control	Drought	D/C, %
Azeghar	2.1 ± 0.1a	0.51 ± 0.1c	−75.7 ***	16 ± 3d	7b	−55.3 *	3d	1c	−66.7 *
Cham1	2.1 ± 0.1a	0.50 ± 0.1bc	−76.3 ***	23 ± 2c	9 ± 1b	−62.3 ***	5 ± 1bc	3 ± 1b	−42.9 *
J. Khetifa	2.6 ± 0.2a	0.63 ± 0.2a	−75.9 ***	37 ± 1a	16 ± 1a	−56.4 ***	9a	4 ± 1a	−51.9 ***
Pelsodur	1.6 ± 0.1b	0.58 ± 0.1a	−63.7 *	31 ± 1ab	15 ± 2a	−51.1 ***	10 ± 1a	5 ± 1a	−50.0 ***
Sebatel	2.1 ± 0.1a	0.66 ± 0.1a	−68.4 **	17 ± 4d	10 ± 1b	−43.1ns	4 ± 1cd	2 ± 1b	−36.4ns
Vulci	2.0 ± 0.1a	0.55 ± 0.1ab	−72.1 **	30 ± 6b	16 ± 3a	−47.2 ***	6 ± 1b	4 ± 1a	−23.5ns
Genotype		ns			***			***	
Treatments		***			***			***	
G × T		ns			**			***	

Values are means ± standard deviations (n = 3). Means with same letter in each column are not significantly different between genotypes ($p < 0.05$) (LSD test). ns—Not significant; *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

Interestingly, J. Khetifa and Pelsodur have similar degrees of reduction in both the number of leaves and number of tillers, while the main effect of treatment was not significant for the Sebatel genotype. Results confirm that the development dynamics of the number of tillers and number of leaves highly depend not only on environment conditions but also by genotype inner capacity. This is also highlighted by the significant G × T interaction (Table 4.1). Moreover, regarding shoot biomass (SDW), Sebatel, J. Khetifa, Pelsodur and Vulci maintained significantly higher SDW than Cham1 and Azeghar under drought conditions. That means that these genotypes are more tolerant to drought according to their successful development of shoots under stress conditions.

4.3.2 Drought effect on whole root system in greenhouse experiment

Under control conditions, no significant differences between genotypes for some root traits, such as total root length (RLW), root surface area (SAW), root tips (TIW), and root length density (RLD), were found. However, the effect of recurrent short events of drought was significant and varied between genotypes (Table 4.2).

Table 4.2. Drought effect on the whole root growth and plants development.

Genotype	RVW (cm³)		RLW (cm)		TIW		RDW (g)	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought
Azeghar	5.3 ± 1.1b	0.97 ± 0.3c	989 ± 84	569 ± 186b	1989 ± 217	1314 ± 329b	0.53 ± 0.1c	0.17 ± 0.1c
Cham1	7.0 ± 0.3b	1.5 ± 0.5bc	1056 ± 182	816 ± 125ab	2009 ± 312	2299 ± 524a	0.83 ± 0.1a	0.26 ± 0.1ab
J. Khetifa	9.7 ± 1.9a	2.2 ± 0.2ab	973 ± 124	887 ± 107a	2218 ± 309	1970 ± 168ab	0.86 ± 0.1a	0.30 ± 0.1a
Pelsodur	6.4 ± 0.9b	2.7 ± 0.4a	835 ± 187	912 ± 190a	1775 ± 556	1812 ± 438ab	0.68 ± 0.1b	0.36 ± 0.1a
Sebatel	6.1 ± 1.6b	1.5 ± 0.1abc	921 ± 231	806 ± 168ab	1704 ± 385	1799 ± 489ab	0.62 ± 0.1b	0.25 ± 0.1ab
Vulci	9.4 ± 0.5a	2.4 ± 1.5ab	992 ± 93	768 ± 36ab	2024 ± 68	1742 ± 316ab	0.72 ± 0.1b	0.29 ± 0.1a
Genotype	***		ns		ns		*	
Treatments	***		**		ns		***	
G × T	**		ns		ns		ns	

Genotype	Root/shoot ratio		SAW (cm²)		Root angle (°)		RLD (cm cm⁻³)	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought
Azeghar	0.25 ± 0.1c	0.33 ± 0.1c	256 ± 33	83 ± 26c	125 ± 4a	108 ± 5b	0.39 ± 0.03	0.23 ± 0.07b
Cham1	0.42 ± 0.2ab	0.51 ± 0.0b	304 ± 28	123 ± 24bc	115 ± 8ab	121 ± 1a	0.42 ± 0.07	0.33 ± 0.05ab
J. Khetifa	0.33 ± 0.1abc	0.48 ± 0.1b	345 ± 56	156 ± 15ab	102 ± 2c	105 ± 4b	0.39 ± 0.05	0.35 ± 0.04a
Pelsodur	0.46 ± 0.1a	0.63 ± 0.1a	281 ± 72	175 ± 15a	94 ± 2c	98 ± 3c	0.33 ± 0.07	0.36 ± 0.08a
Sebatel	0.3 ± 0.02bc	0.38 ± 0.0c	265 ± 68	124 ± 14bc	114 ± 9b	121 ± 1a	0.37 ± 0.09	0.32 ± 0.07ab
Vulci	0.43 ± 0.02ab	0.67 ± 0.1a	315 ± 36	147 ± 50ab	114 ± 6b	106 ± 1b	0.31 ± 0.04	0.31 ± 0.01ab
Genotype	***		*		***		ns	
Treatments	***		***		ns		**	
G × T	ns		ns		***		ns	

Values are means ± standard deviations (n = 3). Means with same letter in each column are not significantly different between genotypes ($p < 0.05$) (LSD test). RVW—total root volume; RLW—sum of all root lengths; TIW—total number of tips; RDW—root dry weight; SAW—total root surface area; RLD—root length density. ns—Not significant; *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

The RLW of Azeghar shortened by about 40% in drought compared with control (Table 4.2), while Pelsodur, which had the lowest RLW under control conditions, grew slightly during the drought and had the longest roots. RLW decreased by about 23% in Vulci and Cham1, while in J. Khetifa it was most stable, having the smallest difference between drought and control (Table 4.2). The root surface area (SAW) appeared to be a quite important characteristic, as it has a crucial role for water and nutrition uptake efficiency. Interestingly, no significant differences were detected between genotypes for SAW under control conditions (Table 4.2). Contrarily, drought affected the root surface area in the different genotypes differently. Pelsodur maintained the highest value of SAW, while Azeghar the lowest. Significant differences clustered genotypes into those with higher (Pelsodur, Vulci, and J. Khetifa) and those with lower (Azeghar, Cham1, and Sebatel) root surface area under drought, where two putative hydric stress-tolerant genotypes J. Khetifa and Cham 1 were separated. In addition, it was observed that genotypes were allocated into the same groups according to phenological development under control conditions. Cham1 was assigned to the early flowering group together with Sebatel and Azeghar. Tolerant landrace J. Khetifa showed a late flowering habit and was grouped with late Pelsodur and Vulci. No significant differences were detected between genotypes for RLD (Table 4.2). However, root length density was affected by drought. The trends indicated that J. Khetifa and Pelsodur were affected the least, and maintained the highest RLD. Meanwhile, the RLD of Azeghar was above 40 percent, and it had the lowest RLD in drought conditions. Highly significant differences among genotypes and treatments were obtained for total root volume (RVW). Under control conditions, J. Khetifa and Vulci had significantly larger RVW compared to other genotypes (Table 2). However, under drought conditions, Pelsodur

maintained the highest RVW, while Azeghar experienced great losses and had the lowest RVW. Genotypes were divided by root volume values under drought treatment into three groups; (i) Pelsodur, Vulci and J. Khetifa, with a root volume over 2 cm^3; (ii) Cham1 and Sebatel, with RVW less than 2 cm^3; while (iii) Azeghar was less than 1 cm^3 (Table 4.2). Drought effects also varied between genotypes in terms of number of tips (TIW). Although Sebatel and Pelsodur had the lowest number of tips under control conditions, under drought TIW remained the same. Azeghar had highest TIW losses (around 33.9%), followed by Vulci (−14.4%) and J. Khetifa (−9%), while only Cham1 increased the number of TIW (+14%) under drought conditions. According to the ratio of root length by the number of tips (RLW/TIW), J. Khetifa and Pelsodur had the smallest drought effect on both, growing new roots and maintaining the root length (Figure 4.4). However, Cham1 and Sebatel increased the number of new roots under drought conditions, since the RLW/TIW ratio decreased from 33% to 17% (Figure 4).

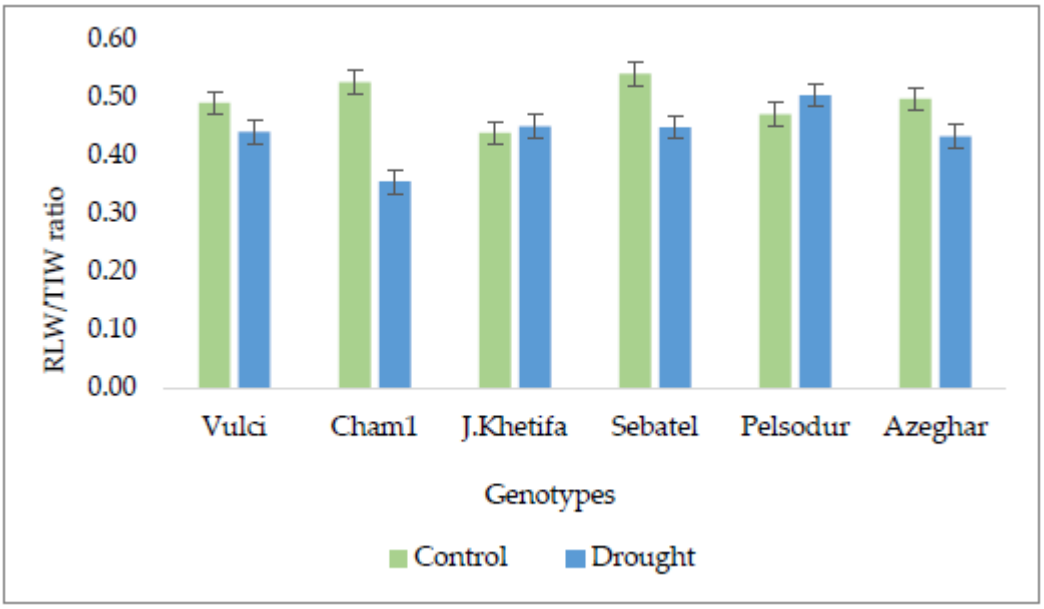


Figure 4.4. The ratio of sum of all root lengths and total number of tips (RLW/TIW) under greenhouse conditions.

The short drought events had a significant negative effect on root biomass (RDW), even if the effect was different among genotypes. Under control conditions, J. Khetifa and Cham1 produced the highest root biomass, but Pelsodur, Vulci, and J. Khetifa had the highest root mass under drought. Azeghar had the lowest root biomass under both conditions. Genotypes could be distinguished by different root system angles, where Azeghar had the widest root angle, J. Khetifa, and Pelsodur the steepest, and Vulci, Sebatel, and Cham1 were of medium root angle. However, there were highly significant differences between genotypes and their response, as indicated by the significant interaction (G × T) (Table 4.2). The RA of Azeghar and Vulci narrowed, passing from control to drought, while in all other genotypes it flattened (Table 4.2). According to RDW and root system angle, six investigated genotypes showed five root ideotypes under control (C) conditions. These are: (iC) widest angle and lowest root biomass (i.e., Azeghar); (iiC) moderate root angle moderate biomass (i.e., Sebatel and Vulci); (iiiC) moderate root angle and high biomass (i.e., Cham1); (ivC) narrow root angle moderate biomass (i.e., Pelsodur); and (vC) narrow root angle high biomass (i.e.,

J. Khetifa). However, under drought (D) conditions, four root ideotypes were highlighted according to the same traits, root angle and biomass. These are: (iD) widest root angle and retained moderate root biomass (i.e., Sebatel and Cham1); (iiD) moderate root angle and low root biomass (i.e., Azeghar); (iiiD) moderate root angle and high root biomass (i.e., J. Khetifa, and Vulci); and (ivD), narrowest root angle and high root biomass (i.e., Pelsodur). In terms of overall plant development, as shown by the proportion of shoots and roots, three groups were highlighted under drought stress. (i) Pelsodur with Vulci had the highest root shoot ratio, (ii) Sebatel with Azeghar had the lowest, and (iii) two tolerant genotypes, J. Khetifa with Cham1, fell in between.

4.3.3 Drought effect on root parameters at topsoil in greenhouse experiment

At a depth of the first 5 cm, the total root length (RL5) had no significant differences between genotypes under control conditions (Table 4.3), just like the total length of the whole root (Table 4.2).

Table 4.3. Root characteristics at a depth of the first 5 cm below ground in greenhouse experiment

Genotype	RL5 Root Length (cm)		SA5 Root Surface Area (cm²)		TI5 Tips		RV5 Root Volume (cm³)	
	Control	drought	control	drought	control	drought	control	drought
Azeghar	144 ± 35	96 ± 12c	40 ± 12b	17. ± 1.5b	341 ± 60	181 ± 43b	0.9 ± 0.3	0.24 ± 0.03b
Cham1	149 ± 33	124 ± 7bc	46 ± 9ab	21 ± 3b	311 ± 82	375 ± 38a	1.14 ± 0.2	0.28 ± 0.07b
J. Khetifa.	102 ± 27	181 ± 28ab	57 ± 15ab	34 ± 3a	231 ± 79	451 ± 60a	2.88 ± 0.9	0.52 ± 0.07ab
Pelsodur	133 ± 4	217 ± 49a	51 ± 3ab	36 ± 4a	283 ± 63	396 ± 89a	1.58 ± 0.2	0.49 ± 0.01ab
Sebatel	120 ± 42	202 ± 58a	43 ± 15b	36 ± 7a	246 ± 97	435 ± 137a	1.26 ± 0.4	0.52 ± 0.05ab
Vulci	138 ± 71	140 ± 16bc	63 ± 3a	33 ± 13a	261 ± 97	326 ± 57a	3.04 ± 2.3	0.65 ± 0.44a
Genotype	ns		**		ns		ns	
Treatments	***		***		**		***	
G × T	ns		ns		ns		ns	
	(RL5/RLW) * 100		(SA5/SAW) * 100		RL5/TI5		(RV5/RVW) * 100	
Genotype	control	drought	control	drought	control	drought	control	drought
Azeghar	14.7	17.8bc	15.6ab	21.4bc	0.4	0.5	16.6	26.5
Cham1	14.2	15.4c	15.1b	17.2c	0.5	0.3	16.3	19.4
J. Khetifa	10.6	20.5ab	16.5ab	22.0bc	0.4	0.4	28.3	23.7
Pelsodur	16.5	23.7a	18.9ab	20.7bc	0.5	0.6	21.7	18.1
Sebatel	12.8	24.8a	16.2ab	29.1a	0.5	0.5	20.6	34.3
Vulci	13.6	18.2bc	20.1a	22.5b	0.5	0.4	37.2	28.3
Genotype	ns		**		ns		ns	
Treatments	***		***		ns		ns	
G × T	ns		*		ns		ns	

Values are means ± standard deviations (n = 3). Means with same letter in each column are not significantly different between genotypes ($p < 0.05$) (LSD test). RL5—sum of root lengths in the first 5 cm below the ground; SA5—root surface area in the first 5 cm below the ground; TI5—number of tips in the first 5 cm below the ground; RV5—root volume in the first 5 cm below the ground. Descriptions added i.e. "ns—Not significant; *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively".

In order to determine the peculiarities of rooting in different soil layers, the ratio between the root traits was also recorded on the whole root and the ones recorded on the topsoil layer (first 5 cm) were calculated and expressed as a percentage. Under drought, the root reduction in the different layers varied among genotypes, while at 5 cm topsoil the total root length significantly increased for J. Khetifa, Pelsodur, and Sebatel. The largest decrease of

RL5 was found in Azeghar (about 30%), then in Cham1 (about 17%). Vulci remained almost the same RL5 under drought as under control conditions. According to the RL5/RLW ratio, in the first 5 cm below ground, the root length ranged from 10 to 16 percent of the whole root length under control conditions, while under drought it increased (from 15 to 25%); indicating a higher root concentration in the first soil layer. Nevertheless, the effect of drought on root length distribution in different layers varied among genotypes, even if not statistically. According to the RL5/RLW ratio, J. Khetifa, Sebatel, and Pelsodur showed an increase of root length at topsoil under drought conditions, which almost doubled, while Cham1 maintained almost the same root distribution between layers under both conditions. Although the effect of drought on the roots length at different depths varied, all genotypes under drought conditions have increased concentration of the roots at topsoil (Table 4.3). The number of TI5 at a depth of first 5 cm (TI5, Table 4.3) had no significant differences between genotypes under control conditions, but the response to drought showed Azeghar to have the lowest tip number. Under stress conditions, Azeghar lost about 50% of TI5, while other genotypes increased it, especially J. Khetifa and Sebatel, where TI5 doubled. Regarding the ratio between root length and number of TI5 at the first 5 cm below ground (RL5/TI5), some genotypes maintained the same pattern under drought as the ratio of the whole plant (RLW/TIW) (Figure 4.4). J. Khetifa had the smallest drought effect on developing new roots and maintaining the length in both the upper and deeper layers of the soil (Table 4.3). Sebatel, in contrast, showed smaller changes at the topsoil level. Interestingly, Azeghar applied different strategies at different layers; when the whole plant RLW/TIW ratio decreased, at the topsoil level this ratio (RL5/TI5) increased. According to ratio SAW/SA5, at a depth of five centimeters under control conditions, the surface area (SA5) accounted for 15 to 20 percent of the whole root SAW, where Vulci had the highest SA5 and Azeghar with Sebatel had the lowest SA5. Under drought conditions the ratio SAW/SA5 increased (about 17 to 30%) and varied between genotypes (Table 4.3). Pelsodur, Sebatel, J. Khetifa, and Vulci maintained significantly higher SA5 compared with Azeghar and Cham1. The smallest changes in SA5 between control and drought conditions were observed for Sebatel. Comparing the distribution of SAW under drought conditions among soil layers, the smallest changes in the SA5/SAW ratio were found for Pelsodur, Cham1, and Vulci. Furthermore, drought root volume at a depth of the first 5 cm (RV5) significantly decreased. Also, the distribution of root volume among layers varied between genotypes. Sebatel had the most concentrated root volume at 5 cm depth under drought conditions (Table 4.3). Moreover, Azeghar and Cham1 also increased RV5 concentration at topsoil under drought, while J. Khetifa, Vulci, and Pelsodur maintained a higher volume of the whole root systems.

4.3.4 The comparison of root system between greenhouse and field

The total root volume (RVF) and the number of crossings (CRF) of plants collected from field experiments, at a depth of first 5 cm (Figure 4.5) had no significant differences between genotypes (Table 4.4). However, the two tolerant genotypes, Cham1 and J. Khetifa, had significantly higher root length (RLF) and root surface area (SAF). J. Khetifa also had a significantly higher number of root tips (TIF) and forks (FRF).

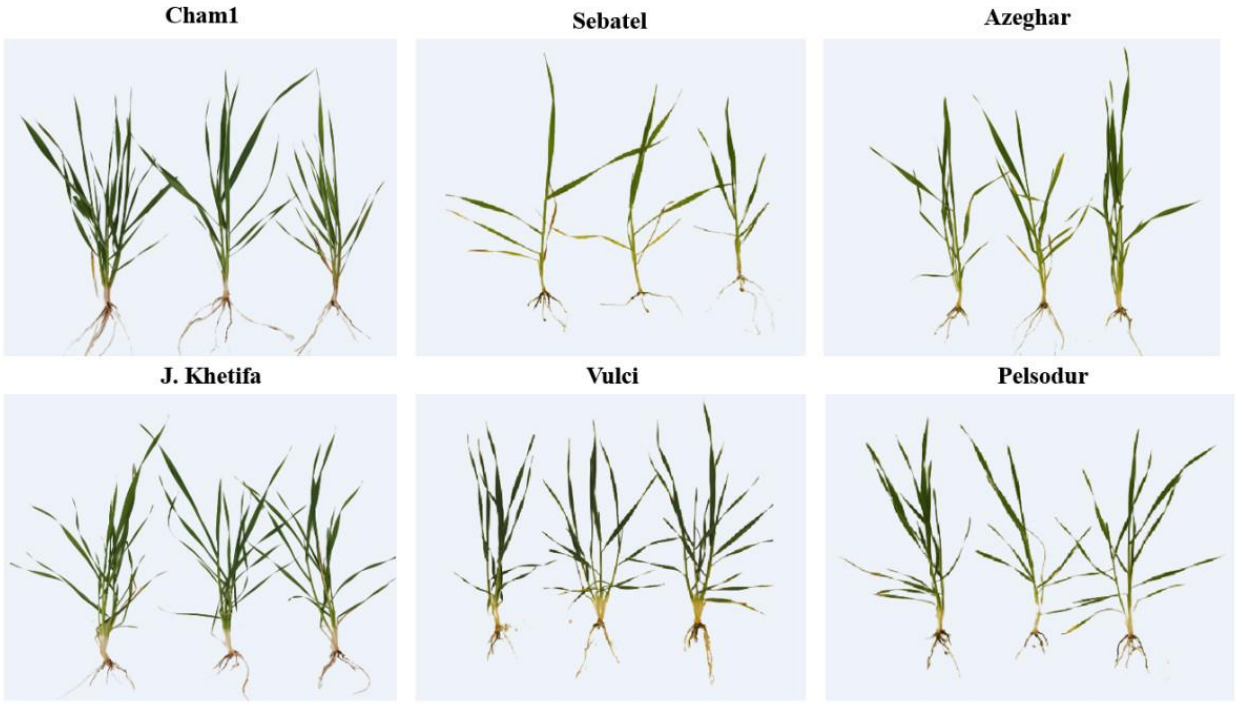


Figure 4.5. Pictures of six durum wheat genotypes grown in field.

The comparison between genotype root angles, measured using *ImageJ* software in the greenhouse under control condition and in the field, showed the same trend, where Azeghar had the widest root angle, J. Khetifa and Pelsodur the steepest, and Sebatel and Cham1 were between wide and narrow (Tables 4.2 and 4.4).

Table 4.4. Root characteristics of plants collected from field at a depth of the first 5 cm below ground.

	Azeghar	Cham1	J. Khetifa	Pelsodur	Sebatel	Vulci	ANOVA
RVF, cm³	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.0	ns
RLF, cm	59 ± 4cd	89 ± 6a	84 ± 15ab	49 ± 6de	38 ± 3e	69 ± 11bc	***
SAF, cm²	11.8 ± 2.1bc	19.1 ± 3.4a	14.5 ± 1.3b	11.3 ± 2.7bc	8.2 ± 2.9c	15.0 ± 2.2ab	**
TIF	224 ± 48bc	266 ± 44b	390 ± 65a	218 ± 83bc	142 ± 30c	261 ± 20b	**
FRF	223 ± 5bc	345 ± 34ab	449 ± 88a	248 ± 84bc	114 ± 50c	446 ± 155a	**
CRF	22 ± 8	28 ± 7	50 ± 29	15 ± 3	8 ± 2	44 ± 29	ns
RAF, °	112 ± 6a	102 ± 5ab	93 ± 6bc	84 ± 7cd	105 ± 5ab	76 ± 13d	***

Values are means ± standard deviations (n = 3). Means with same letter in the line are not significantly different between genotypes ($p < 0.05$) (LSD test). RVF—root volume in the first 5 cm below the ground of plants from field; RLF—sum of all root lengths in the first 5 cm below the ground of plants from field of plants from field; SAF—root surface area in the first 5 cm below the ground of plants from field of plants from field; TIF—number of tips in the first 5 cm below the ground of plants from field of plants from field; FRF—number of forks in the first 5 cm below the ground of plants from field of plants from field; CRF—number of crossings in the first 5 cm below the ground of plants from field of plants from field; RAF—root angle of plants grown in field. ns—Not significant; *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

Although all genotypes showed lower values of root angle in the field than in the greenhouse, Vulci had the greatest differences compared with control conditions in the greenhouse, where the root angle was wider. This could be explained by the fact that the plants were grown by a single plant per pot in a greenhouse, without any competition, unlike in the field, where the plants were sown in dense conditions.

4.3.5 Correlation among traits

The correlation matrix among traits under the control condition (Figure 4.6) shows that the number of leaves (NL) and number of tillers (NT) had a highly significant positive correlation between themselves and the root volume at topsoil (RV5). Moreover, a significant positive correlation was found for traits such as root shoot ratio (RS), root dry weight (RDW), root volume (RVW), and root surface area (SAW; SA5).

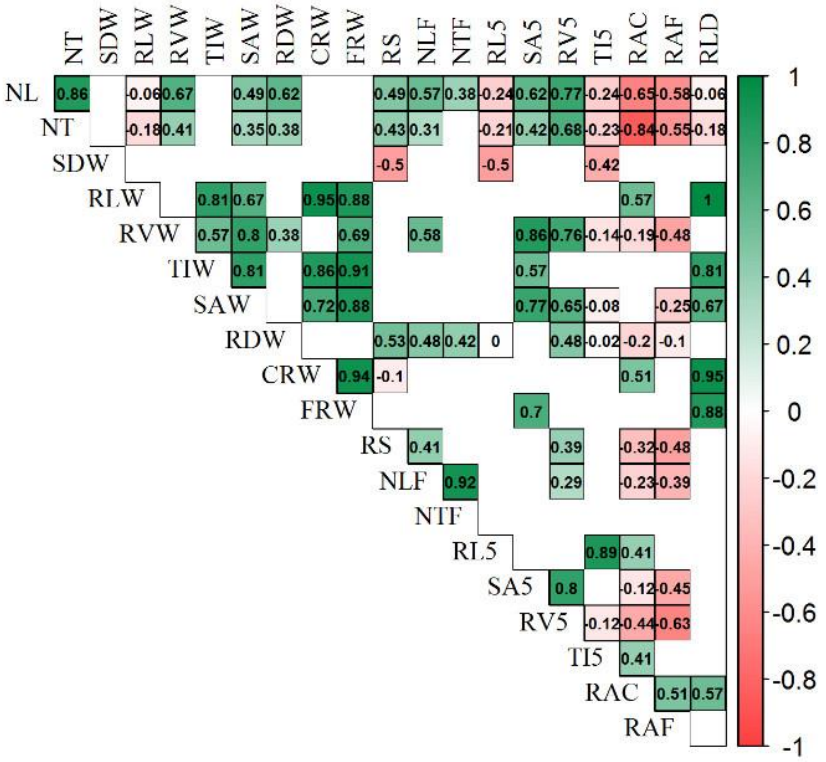


Figure 4.6. Positive significant correlation (in green) and negative significant correlation (in red) among all traits under control conditions. NL—number of leaves; NT—number of tillers; SDW—shoot dry weight; RVW—total root volume; RLW—sum of all root lengths; TIW—total number of tips; RDW—root dry weight; SAW—total root surface area, RLD—root length density, CRW—total number of crossings; FRW—total number of forks; RS—root shoot ratio; RL5—sum of root lengths in the first 5 cm below the ground; SA5—root surface area in the first 5 cm below the ground; TI5—number of tips in the first 5 cm below the ground; RV5—root volume in the first 5 cm below the ground; RAC—root angle of plants grown in greenhouse as control; RAD—root angle of plants grown in greenhouse under drought conditions; RAF—root angle of plants grown in the field.

Furthermore, both NL and NT had highly significant negative correlation with root angle under both greenhouse (RAC) and field conditions (RAF). Root length (RLW) under control conditions had a highly significant positive correlation with root angle (RA), number of leaves (NL), and number of root elements such as tips (TIW), forks (FRW), and crosses (CRW). Root volume (RVW) under control conditions had a highly significant positive correlation with RV5, SAW, SA5, and FRW. Interesting results were found in the relation between traits under drought conditions and root system angle under all conditions: control (RAC), drought (RAD), and field (RAF) (Figure 4.7). For example, between almost all traits, statistically positive significant correlations were obtained, except shoot biomass (SDW), and root angle under all conditions, which had a significant negative correlation. However, root angles from all conditions (RAC, RAD, and RAF), had a significant positive correlation between each other and showed a similar trend in the relation with other traits such as a significant negative correlation with almost all traits under drought conditions

(Figure 4.7). Furthermore, a significant negative correlation (−0.57) between root angle under control (RAC) and root length under drought stress (RLW) was obtained, which means that genotypes with a narrower root angle in control conditions retained longer roots in water deficit environments. Moreover, a significant negative correlation was obtained between the root angle of plants under all conditions (control, drought, and field) and plants root biomass under drought conditions (RDW), which means that genotypes with a narrower root angle can grow higher root biomass. Highly statistically ($p < 0.001$) significant positive correlations were detected between the main traits of the whole root system and topsoil, such as RL and RL5 (0.8), RV and RV5 (0.8), SA and SA5 (0.8), TI and TI5 (0.7).

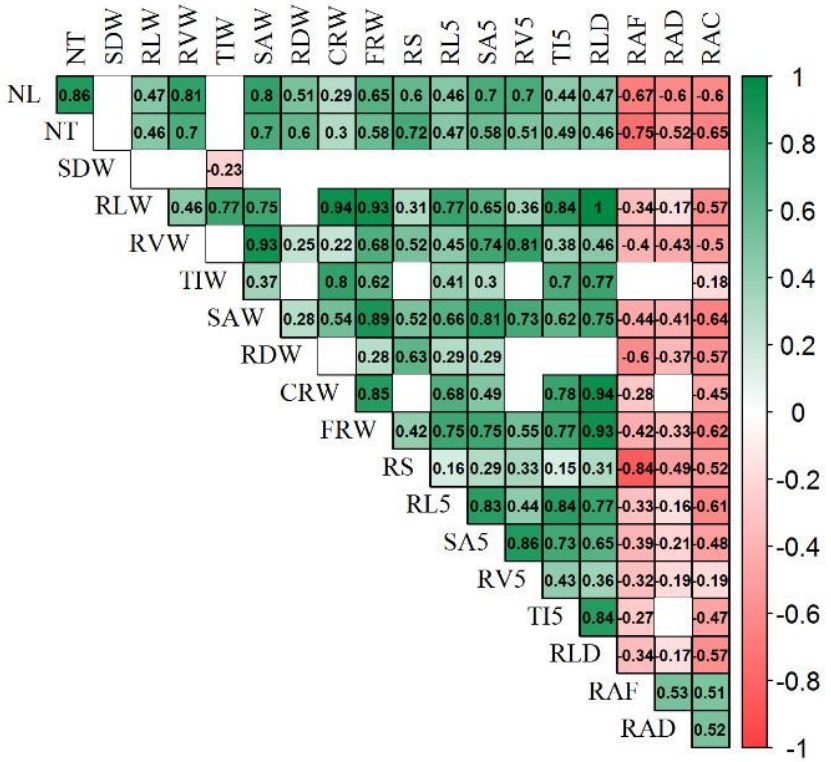


Figure 4.7. Positive significant correlation (in green) and negative significant correlation (in red) among all traits under drought conditions and root angle under different conditions.
NL—number of leaves; NT—number of tillers; SDW—shoot dry weight; RVW—total root volume; RLW—sum of all root lengths; TIW—total number of tips; RDW—root dry weight; SAW—total root surface area, RLD—root length density, CRW—total number of crossings; FRW—total number of forks; RS—root shoot ratio; RL5—sum of root lengths in the first 5 cm below the ground; SA5—root surface area in the first 5 cm below the ground; TI5—number of tips in the first 5 cm below the ground; RV5—root volume in the first 5 cm below the ground; RAC—root angle of plants grown in greenhouse as control ; RAD—root angle of plants grown in greenhouse under drought conditions; RAF—root angle of plants grown in the field.

4.3.6 PCA in response to drought

The three top PCA account for 93.9% of the total variation of shoot root traits under drought stress and root angle from field plants (RAF) (Table S1). PC1 accounted for 66.0% of the total variation and was strongly influenced by several traits (i.e., RLW, RVW, SAW, SA5, NL, NT, and RDW). PC2 accounted for 16.8% of the total variation and was mainly associated with RS, -RL5, -TI5, -RAD, AND -RAF, while PC3 accounted for 11.1% and

was strongly associated with TIW, -SDW, and -RV5. The first two principal components, explaining 82.8% of the total variation, divided genotypes into two groups (I) and (II) (Figure 4.8).

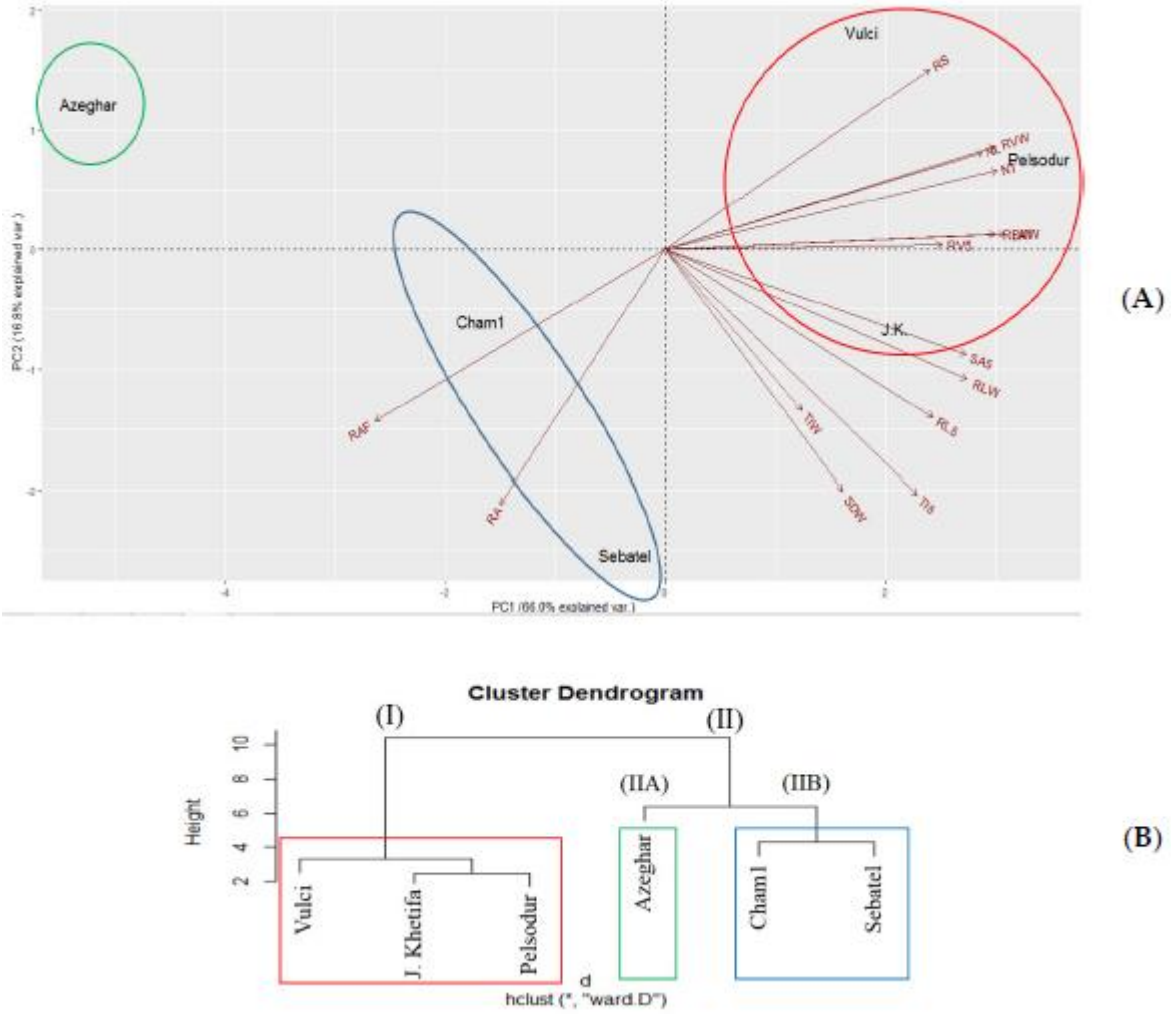
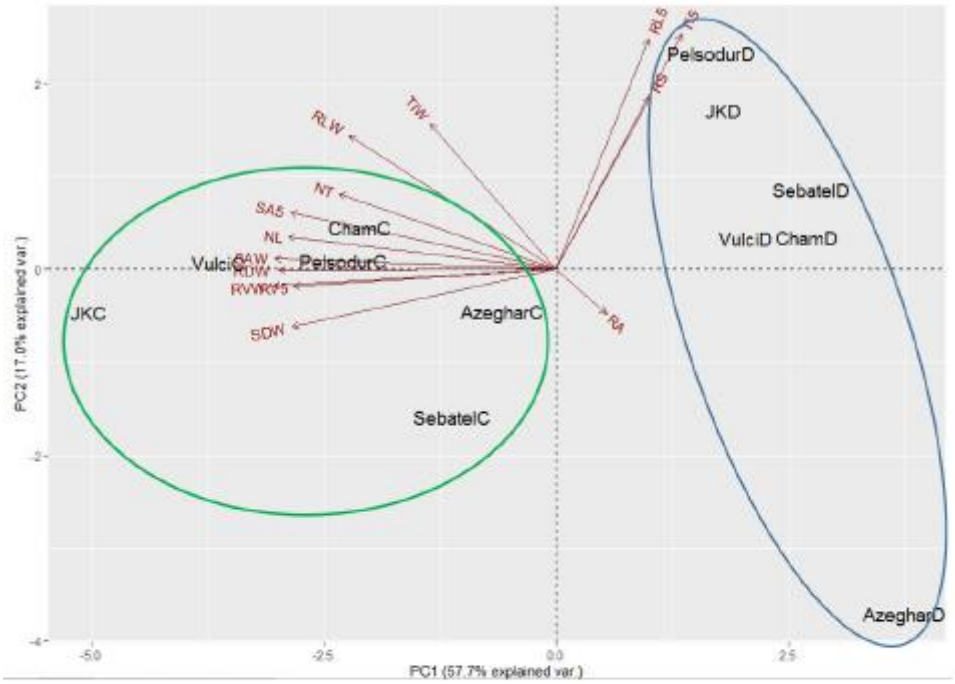


Figure 4.8. Principal component analysis (A) and hierarchical cluster dendrogram applying Ward's method (B) of the six investigated durum wheat genotypes under drought conditions. NL—number of leaves; NT—number of tillers; SDW—shoot dry weight; RVW—total root volume; RLW—sum of all root lengths; TIW—total number of tips; RDW—root dry weight; SAW—total root surface area; RS—root shoot ratio; RL5—sum of root lengths in the first 5 cm below the ground; SA5—root surface area in the first 5 cm below the ground; TI5—number of tips in the first 5 cm below the ground; RV5—root volume in the first 5 cm below the ground; RA—root angle of plants grown in greenhouse under drought conditions; RAF—root angle of plants grown in the field.

Pelsodur and Vulci were assigned to cluster (I) with the drought tolerant landrace J. Khetifa, due to their positive association with traits on the positive side of PC1, while the Sebatel and Azeghar with tolerant Cham1 under drought conditions are on the negative side of PC1, associated with root angle under drought (RAD) and field (RAF), and assigned to cluster (II). Interestingly, these two groups overlapped with genotype shoot development either early or late flowering. Moreover, the same groups were highlighted regarding RV changes at different soil layers; early flowering growth genotypes had the lowest RV, but maintained higher root volume at 5 cm depth compared with a group of late genotypes. However, cluster (II) was divided into two subgroups, where Azeghar (IIA) was separated from the Cham1 and Sebatel cluster (IIB) due to the largest losses in the aboveground part of the plant under drought conditions. The first three components of PCA considering all traits

under both drought and control conditions accounted for 89.9% of total variation: 57.7% to PC1, and 17.0% to PC2, and 14.4% to PC3 (Table S2). Interestingly, all the genotypes under drought stress are grouped on the positive side of PC1 with more or less the same values (ranging from about 2 to 4). While the same genotypes under control conditions are on the negative side of PC1 with different values, AzegarC is close to zero, while J. Khetifa (JKC) is almost at −5 of PC1. Considering that PC1 is mainly determined by –RVW, -RV5, -RDW, -SAW, SA5, -NL, and -SDW, these traits are different among the genotypes under control (ending with C in Figure 4.9) and drought (ending with D in Figure 4.9) conditions, and in particularly are negatively associated (decreased) in the case of drought. On the other hand, PC2, which is mainly determined by RLW, RS, RL5, TIW, and TI5, is very wide (ranging from 2 to −4) for the genotypes under drought while it is more or less constant (ranging from about 1 to −1) for the same genotypes under controlled conditions.



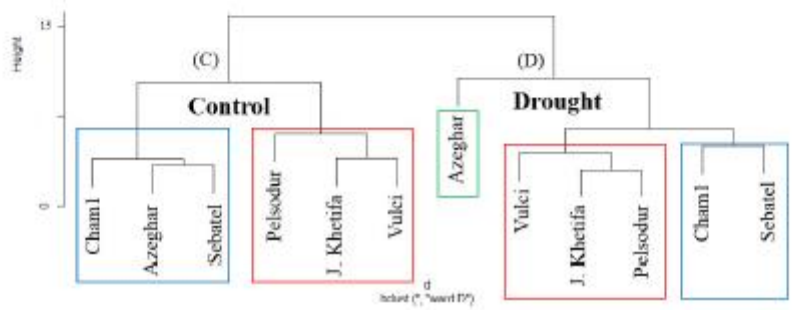


Figure 4.9. Principal component analysis and cluster dendrogram of 6 investigated durum wheat genotypes under control (C) and drought (D) conditions. NL—number of leaves; NT—number of tillers; SDW—shoot dry weight; RVW—total root volume; RLW—sum of all root lengths; TIW—total number of tips; RDW—root dry weight; SAW—total root surface area; RS—root shoot ratio; RL5—sum of root lengths in the first 5 cm below the ground; SA5—root surface area in the first 5 cm below the ground; TI5—number of tips in the first 5 cm below the ground; RV5—root volume in the first 5 cm below the ground; RA—root angle of plants grown in greenhouse under drought conditions; RAF—root angle of plants grown in the field.

The tolerant landrace J. Khetifa was assigned together with Pelsodur and Vulci to the same cluster under both conditions. However, under control conditions J. Khetifa was closer to the Vulci, while under drought conditions it was closer to the late flowering time Pelsodur. Early flowering genotypes, such as Cham1, Sebatel, and Azeghar formed one cluster under control conditions, while the drought separate Azeghar, which is probably a susceptible genotype to hydric stress.

4.4 Discussion

According to Reynolds and Langridge [39], several crucial steps are required to improve crop tolerance for abiotic stress; among them, trait-based breeding and a "crop ideotype design" are the first to be considered. Breeding programs have focused largely on above ground traits, leaving the root system importance overlooked [40]. Some of the previous studies reported that RSA traits on the seedling stage can be used to predict the RSA of adult plants in field-grown [20] or in an artificial system [21] to evaluate the crop adaptation under water stress conditions; unfortunately, these were validated only for what concerned the root angle and seminal root apparatus. Conversely, in the present study, the analysis was performed on a more advanced stage than seedling and on several root traits since different root types could perform differently under different conditions [23], as also highlighted by the present results. For what concerns the substrate, in the present study we used sand that allows roots to grow around the 360° rather than rhizotrons (*GrowScreen-Rhizo 1*) [41], growth pouch, or clear pot [21] which fundamentally allow a root development in only two dimensions. Moreover, Gregory et al. [42] recorded a significant soil by genotype interaction, when wheat root length among accessions grown on agar plates showed positive differences of about 40%, while in a sandy loam soil the differences were negative and at about 30%. However, in our study, we obtained a positive correlation between the root angle of genotypes grown under greenhouse and field conditions, which are good traits for selection for drought tolerance [23,24]. In addition, the highly statistically significant correlations between the main traits of the whole root system and topsoil indicate that it would be possible to extrapolate the root phenotyping of the whole root system by analysing only the topsoil root system. After simulations of several root system traits and ideotypes for drought conditions, it was proposed that for cereals a "deep, steep, and cheap" root system is the most proper for water scarcity environments [43,44]. A deep root system is the most important characteristic for the drought tolerant crop ideotype, as it allows access to residual water resources and N from deeper soil layers [45]. Moreover, recent studies observed an association between steep root angle and the depth of RSA, when narrower angles allow for an increase in deep rooting [14,46,47]. The same relationship was found in our study, as genotypes from the same cluster with the narrowest root angle in control conditions, such as J. Khetifa and Pelsodur, retained longer roots in water deficit environments and vice versa, while Azeghar with the widest angle in control conditions had the shortest RLW under drought conditions. Furthermore, a significant negative correlation between root angle under control (RAC) conditions and root length under drought stress (RLD) support the methods proposing to analyse the root angle at a seedling stage and select the genotypes adapted to drought on the basis of that data. These root system characteristics are often associated with the germplasm origin and the relative climate conditions. In general, a narrow root angle and deep root system are more common for rain-fed crops, which depend on stored water in the soil. However, the analysis of durum wheat landraces from the Mediterranean basin showed large variability in root system architecture

[48–50]. Cultivars originating from the western Mediterranean showed a narrow root angle, the same as genotype J. Khetifa, which originated from Tunisia [34,48]. In agreement with Ober et al. [51], we demonstrated that a narrow root angle was associated with higher root biomass; for example, J. Khetifa and Pelsodur had a narrow root angle under control conditions (RAC) and maintained higher root biomass (RDW) under water scarcity. Manschadi et al. [14] demonstrated how the yield increases of 55 kg/ha for each millimetre of water extracted from the soil after anthesis (i.e., in grain filling stage); hence a deep root system, but also wide root system, are the most desirable in environments with terminal drought [40]. In addition, genotypes from cluster (II) Cham1 and Sebatel showed different root ideotypes than genotypes from cluster (I). They had a wider root angle under control conditions, and under drought stress maintained moderate root biomass, length, surface area, and volume. However, regarding shoot biomass (SDW), Sebatel maintained significantly higher SDW than Cham1 under drought conditions. Interestingly, most of the differences between the Sebatel and Cham1 genotypes were found in topsoil root traits. Sebatel maintained significantly longer root length, volume, and surface area at the topsoil compared to Cham1, which helps to develop a significantly higher shoot biomass. Similar results were obtained in other studies, where the late flowering genotypes (in our case J. Khetifa, Pelsodur and Vulci) had a more uniform distribution of the root system compared to the early flowering genotypes (in our case Cham1 and Sebatel), which kept a higher root volume at 5 cm depth under drought [52]. The deep root allocation for durum wheat was determined to be a good strategy for drought avoidance in Mediterranean-type environments, which facilitates access to subsoil water resources [43]. In agreement with the “deep, steep, and cheap” root system ideotype, the genotypes Pelsodur, J. Khetifa, and Vulci can be considered drought tolerant; confirming the suitability for drought-tolerant breeding, as it was reported that this root system had significant functional relation to water absorption and drought avoidance in rice [53]. Also, the genotypes Sebatel and Cham1 with compact topsoil rooting patterns under drought condition could be good candidates for breeding, as it was demonstrated that higher root mass and root length density in subsoil layers contribute to the grain yield of winter wheat under drought conditions [54]. Finally, Azeghar with the lowest root biomass, surface area, length, and volume under drought stress and the widest root angle under control conditions shows susceptible root ideotype for hydric stress.

4.5 Conclusion and prerspective

This study highlighted huge variability among a small number of genotypes in terms of the development, distribution, and architecture of the root system in order to tolerate difficult environments and to increase resilience to climate change. Even two hydric stress-tolerant genotypes showed different root system ideotypes and rooting patterns under drought. Moreover, it was demonstrated that screening plants’ roots in the early stage grown under control conditions using a high-throughput scanner can expedite the selection of novel traits for crop improvement in plant breeding.

4.6 References

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Chapter 5. Phenotypic and genotypic diversity of roots response to salt in durum wheat seedlings

Ieva Urbanavičiūtė, Luca Bonfiglioli, and Mario A. Pagnotta

Abstract

Soil salinity is a serious threat to food production now and in the near future. In this study, the root system of six durum wheat genotypes, including one highly salt-tolerant (J. Khetifa) used as a check genotype, was evaluated, by a high-throughput phenotyping system, under control and salt conditions at the seedling stage. Genotyping was performed using 11 SSR markers closely linked with genome regions associated with root traits. Based on phenotypic cluster analysis, genotypes were grouped differently under control and salt conditions. Under control conditions, genotypes were clustered mainly due to a root angle, while under salt stress, genotypes were grouped according to their capacity to maintain higher roots length, volume, and surface area, as J. Khetifa, Sebatel, and Azeghar. SSR analysis identified a total of 42 alleles, with an average of about three alleles per marker. Moreover, quite a high number of Private alleles in total, 18 were obtained. The UPGMA phenogram of the Nei (1972) genetic distance clusters for 11 SSR markers and all phenotypic data under control conditions discriminate genotypes almost into the same groups. The study revealed as the combination of high-throughput systems for phenotyping with SSR markers for genotyping it's a useful tool to provide important data for the selection of suitable parental lines for salt-tolerance breeding. Nevertheless, the narrow root angle, which is an important trait in drought tolerance, is not a good indicator of salt tolerance. Instated salt tolerance is more important than the number of roots.

Keywords: durum wheat; salt stress; roots; high-throughput phenotyping; genotyping; SSR; QTL

5.1 Introduction

According to the FAO 2021 global map of salt-affected soils [1], around 400 million hectares of topsoil and more than 800 million hectares of subsoil are affected by salt. Moreover, FAO reported that soil salinity per year reduces production potential by taking up to 1.5 million ha of farmland out of production and more than 30 million USD as an annual loss in agricultural productivity [1]. Unfortunately, at such rates, 50% of total cultivated land is expected to be salinized by 2050 [2] as a consequence of the phenomenon of "desertification," and land degradation due to climate change and human action by poor land management [3–5]. Projections up to 2099 assume an extreme increase in drought and the subsequent soil salt accumulation in Eurasia, Africa, and Australia [6]. Soil salinization may occur through a natural process (primary salinization) or by human management (secondary salinization), through irrigation with salty water, excessive use of fertilizers, and intensive agriculture practices [7–9]. Salt, mainly NaCl, in the soil slows down plant

development due to two stresses. At first, the plant experiences a physiological drought (osmotic stress), which reduces leaves and root growth due to the inhibition of cell expansion, cell wall synthesis, and stomata conductance [10,11]. Later the plant response is related to the toxic effect of salt, where plants accumulate toxic Na^+ and Cl^- in old leaves over a long period. Due to the harmful effects of salt on germination, water uptake, transpiration, photosynthesis, enzyme activities, and metabolism of protein and lipids, a significant reduction in yield of up to 40% in agricultural lands has been reported [12,13]. Since the three most widely consumed kinds of cereal (maize, rice, and wheat) are susceptible to saline soil conditions [14], it is essential to improve studies about mechanisms to induce salt stress tolerance to secure our future on food storage. Plants could tolerate salt stress by several strategies: (i) salt exclusion, retaining the Na^+ in woody stems or roots; (ii) regulating toxic ions balance through the maintenance of a high K^+/Na^+ ratio with a mechanism of translocation of K^+ over Na^+, to reduce the Na^+ concentration in the shoot cytoplasm; and (iii) by salt compartmentalization in vacuoles, protecting the cytoplasm from the ion toxicity [15,16]. The root system under an excessive salt concentration in the soil due to toxic ions accumulation suffers from inhibition of nutrition supply and elements adsorption [17]. Salinity, as drought, hurts the whole root system due to reduced cell activity in the root meristem [18]; however, growth rate reduction was determined more in the main root than in lateral roots [19]. As a response to stress, plants can remodel root system architecture (RSA) to adapt to adverse conditions [20]. However, the direction of root growth as a response to external stimuli differs in salt and drought conditions. Under drought conditions, roots use positive hydrotropism, growing towards the water, but in the presence of salt, roots remodelling due to negative halotropism directs the growth away from salinity. Moreover, it was reported that the roots of Arabidopsis exhibited reduced gravitropism under salt conditions to grow against the gravity vector [21]. In addition, the identified gene in cereal crops responsible for gravitropism versus anti-gravitropism regulatory mechanisms also controls the root angle [22]. The narrow root angle was determined as a very important feature in selecting genotypes for drought tolerance since genotypes with a narrow root angle can grow deeper compared with varieties with a wider root angle [23–25]. However, root system remodelling strategies to tolerate drought and salt stress differentiate in terms of tropism type, and it does not mean that a narrow root system able to adapt to drought is also capable of growing in saline soil. In traditional breeding programs, phenotyping the above-ground plant parts was the main aspect of determining resistance to abiotic stress. However, the development of several high-throughput methods and techniques for root phenotyping in recent years has finally made it possible to determine which root systems and remodelling strategies are associated with the most successful plant development under stress conditions [26–29]. Phenotypic root traits could be used to determine genetic diversity; however, they are often influenced by environmental conditions. According to Soriano et al. [30], the stable quantitative trait loci (QTL) and closely linked molecular markers can improve durum wheat genetically through marker-assisted selection, cloning, and pyramiding in breeding programs to create new cultivars with desired quantitative traits. Molecular markers such as simple sequence repeat (SSR) and its based association mapping analysis may allow us to determine the chromosomal regions involved in root architecture features [30–33]. Moreover, SSRs are chromosome-specific and highly effective molecular tools due to their large level of polymorphism, distributed over all of the genomes [34,35]. Simple sequence repeat (SSR) molecular markers closely linked with genome regions associated with root architecture features could

be helpful in selecting genotypes for salt tolerance breeding programs. Moreover, the identification of closely linked candidate genes can give a better understanding of the genetic response to specific stimuli. n this study, six durum wheat genotypes, including one high salt-tolerant J. Kethifa as check genotype, were used to evaluate differences in shoot and root traits under control and salt conditions at the seedling stage and to determine their involvement in salt tolerance. The root system was evaluated using a high-throughput phenotyping system. The genotyping was performed using 11 SSR markers closely linked with genome regions associated with root traits, and in silico, candidate gene expression analysis was performed. The study aimed to evaluate genetic diversity among genotypes based on phenotypic and genotypic variations to identify candidate parental genotypes for salt-tolerance breeding.

5.2 Materials and methos

5.2.1 Plant material

For phenotyping and genotyping was used six *Triticum turgidum* L. subsp. *Durum* (Desf.) Husn. accessions, including one high salt-tolerant genotype Jennah Khetifa as check [78,79]. Other genotypes characterized by different root angles, Cham1, Azeghar 2-1 (56) (Azeghar), and Sebatel2 (45) (Sebatel) with wide root angle, and two genotypes Mv-Pelsodur (Pelsodur) and Vulci with narrow root angle [25].

5.2.2 Pot experiment condition and design

A pot experiment was performed in a greenhouse at Tuscia University's experimental farm (Viterbo, Italy) from January to February 2021. A Randomized Complete Block Design (RCBD) with three replications, six genotypes, and two salinity levels was used. The six genotypes were sown with one seed per pot placed with the embryo facing down on 20 January 2021. Each pot was 17 cm (diameter) by 16 cm (high) and filled with 2.5 L of sand (Figure S1). The temperature in the greenhouse was constantly maintained at around 25 °C during the day and around 15 °C during the night to have optimum growing conditions. Before starting the treatments, all pots (control and salinity treatment) were irrigated, using a dropping pipe, three times per week (80 mL/per pot) with a water nutrition solution composed of dihydrogen phosphate potassium (0.13 g/L), potassium sulphate (0.04 g/L), nitric acid (0.286 mL/L), calcium nitrate (0.432 g/L), potassium nitrate (0.436 g/L), magnesium nitrate (0.244 g/L) and Micron (0.23 g/L) [25]. Two weeks after sowing, when all seedlings reached two fully expanded leaves stage, plants under salinity treatment were started treated with a salt solution (250 mM NaCl) 3 times per week (80 mL/per pot) for 14 days, while the ones under control treatment were irrigated with the same solution but without NaCl. After two weeks of salt treatment, pots were watered with a water nutrition solution without salt for 14 days. After two weeks (no NaCl), for a week, the salt solution was applied again, and after that, the plants were removed for analysis (Figure 5.1).

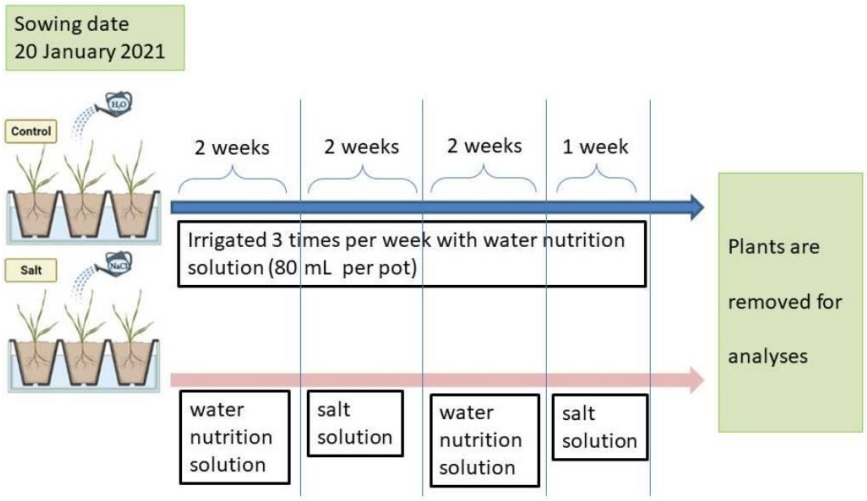

Figure 5.1. Experimental model, indicating when salt stress was applied by adding NaCl to the water nutrition solution.

5.2.3 Roots phenotyping

Plants for analysis were collected after 7 weeks after sowing in total. Roots were carefully washed to remove the sand, using a soft spray watering head, and analysed using Win-RHIZO Pro software v2009 (version 4.0b; Regent Instruments, Montreal, QC, Canada). The morphological traits of the roots were recorded for the whole root system (Figure 5.2A), such as total root length (RL), the sum of all roots lengths, cm, surface area (SA), total root surface area, cm2, root volume (RV), total root volume, cm3, number of tips (TI), forks (FR), and crossings (CR). The root angle (RA, degree on the vertical _) between the two extreme sides of the roots with the center set in the middle of the crown was measured using the software ImageJ, which could be freely download at https://imagej.nih.gov/ij/download.html; accessed on 13 January 2023 (Figure 5.2B). Morphological traits for shoots, such as plant height, number of leaves, and number of tillers, were also recorded.

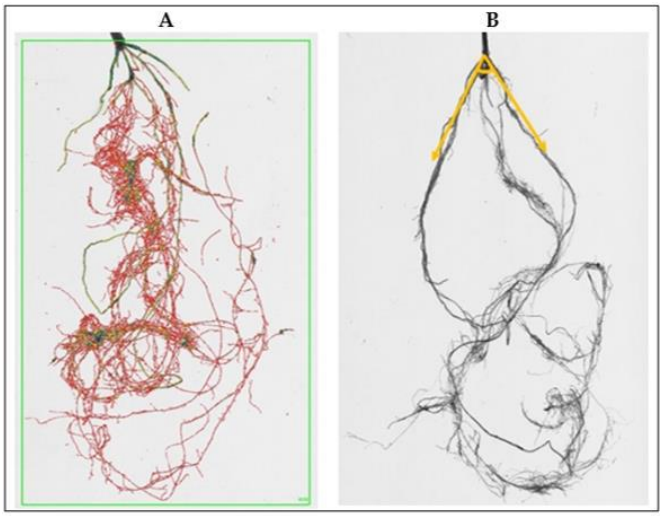

Figure 5.2. The measurements of morphological root traits (A) using Win-RHIZO system and root angle (B) using ImageJ (°).

5.2.4 DNA extraction, PCR amplifications, and gel electrophoresis

For genotypic diversity analysis, 11 microsatellites associated or linked to genomic regions related to root traits from previously reported studies (Table S2) were chosen. The genomic DNA was extracted from fresh leaves of each genotype using the extraction kit PureLink Plant Total DNA Purification kit (Invitrogen; Thermo Fisher Scientific,Waltham, Massachusetts USA). The PCR amplifications were carried out using 0.125 µL of GoTaq G2 DNA Polymerase (Promega, Madison,Wisconsin, USA), 5 µL of 5X Colorless GoTaq® Reaction Buffer, 0.5 µL of 10 µM dNTP mix, 2 µL of 10 µM/µL forward and reverse primer, 100 ng of gDNA, and sterile water for a total reaction amount of 25 µL. The amplifications were performed in SwiftMaxi Thermal Cyclers (Esco Technologies, St. Louis,Missouri, USA) with an initial denaturation of 5 min at 94 °C followed by 35 cycles with 15 s at 94 °C and 20 s at different temperatures, according to the annealing temperature of each marker (see Table 5.1), and 20 s at 72 °C. As a final step, it was performed an extension of 10 min at 72 °C. The electrophoresis was carried out through the capillary electrophoresis device QIAxcel Advanced Instrument (Qiagen, Hilden, Germania) using the QIAxcel DNA High-Resolution Kit, and allele analyses were performed with QIAxcel ScreenGel Software (Qiagen, Hilden, Germania). Each allele was named for its band size in the base pair (Table S5).

Table 5.1. Primers used for PCR amplification.

Marker	Primer Forward (5′-3′)	Primer Reverse (5′-3′)	Chr.	AT (°C)
cfa2086	TCTACTTTCAGGGCACCTCG	TCTCTCCAAACCTCCCTGTAA	2A	56
gwm573.2	AAGAGATAACATGCAAGAAA	TTCAAATATGTGGGAACTAC	7B	45
wmc727	CATAATCAGGACAGCCGCAC	TAGTGGCCTGATGTATCTAGTTGG	5A	55
wms205	CGACCCGGTTCACTTCAG	AGTCGCCGTTGTATAGTGCC	5A	56
wms5	GCCAGCTACCTCGATACAACTC	AGAAAGGGCCAGGCTAGTAGT	3A	56
gmw459	ATGGAGTGGTCACACTTTGAA	AGCTTCTCTGACCAACTTCTCG	6A	54
gwm234	GAGTCCTGATGTGAAGCTGTTG	CTCATTGGGGTGTGTACGTG	5B	55
gwm427	AAACTTAGAACTGTAATTTCAGA	AGTGTGTTCATTTGACAGTT	6A	45
gwm499	ACTTGTATGCTCCATTGATTGG	GGGAGTGGAAACTGCATAA	5B	52
gwm637	AAAGAGGTCTGCCGCTAACA	TATACGGTTTTGTGAGGGGG	4A	55
cfa2257	GATACAATAGGTGCCTCCGC	CCATTATGTAAATGCTTCTGTTTGA	7A	49

Gene models were identified in the NCBI database https://www.ncbi.nlm.nih.gov/ data-hub/genome/GCA_900231445.1/, accessed on 13 January 2023) based on the positions of SSR markers in the durum wheat reference sequence (https://wheat.pw.usda.gov/GG3/jbrowse_Durum_Svevo, accessed on 13 January 2023). Identification of upregulated gene models under abiotic stress at the seedling stage was carried out using the RNAseq data at http://www.wheat-expression.com/ (accessed on 13 January 2023), using gene models from the NCBI database.

5.2.5 Statistical analysis

The statistical analyses of phenotypic data were performed using R Studio (Version R-4.1.0, R Foundation for Statistical Computing, Vienna, Austria). Two-way analysis of variance (ANOVA) was conducted at a significance level of 5% using the aov() function. At the same time, one-way ANOVA was used to test the variance component of each trait under each treatment, with genotype as a factor. Fisher's least significant difference (LSD) test was used for means comparisons. Pearson correlation coefficients were calculated using the

corrplot function R package [80]. Principal component analysis was performed using the prcomp() function, and then a biplot was generated with the ggbiplot function R package [80]. The genetic distance [36] matrix was utilized to construct, using GDA software [81]. The Mantel test to compare genetic distance and Euclidean matrices was computed by GenAlEx, an Excel macro realized by Peakall and Smouse [82].

5.3 Results

5.3.1 Salt effect on durum wheat shoots traits

Highly statistically significant differences were detected between genotypes and both treatments (salt vs. control) for seedling length (PH), the number of leaves (NL), and the number of tillers (NT) (Table 5.2). Moreover, significant genotype by treatment interaction highlighted that the different genotypes used different strategies in terms of shoot development under salt stress. For example, the genotypes Azeghar and Sebatel had not shown a significant salt effect on any shoot traits and maintained one tall main stem with 5–7 leaves. In the genotypes, Cham1 and Pelsodur seedling length and the number of leaves were not significantly affected by salt; however, there was a significant negative reduction in the number of tillers. The salt-tolerant genotype J. Khetifa as a check plant, under salt stress, reduced seedling length and significantly increased the number of leaves (Table 5.2).

Table 5.2. Salt versus control effect on shoot traits of six durum wheat genotypes.

Genotype	Seedling Length, cm			Number of Leaves			Number of Tillers		
	Control	Salt	Diff. (%)	Control	Salt	Diff. (%)	Control	Salt	Diff. (%)
Azeghar	34.6 ± 3.2 b	40.5 ± 1.6 a	16.96 ns	5.0 ± 0.0 c	6.7 ± 0.6 c	33.3 ns	1.0 ± 0.0 c	1.0 ± 0.0 b	0.0 ns
Cham1	37.1 ± 0.6 ab	31.3 ± 0.8 bc	−15.71 ns	6.0 ± 1.7 c	6.3 ± 1.2 c	5.6 ns	2.3 ± 0.6 b	1.0 ± 0.0 b	−57.1 **
J. Khetifa	42.8 ± 1.1 a	33.1 ± 2.6 b	−22.74 *	9.3 ± 0.6 a	14.0 ±0.0 a	50.0 ***	3.0 ± 0.0 ab	3.3 ± 0.6 a	11.1 ns
Pelsodur	34.0 ± 1.5 b	29.2 ± 2.2 c	−14.18 ns	8.0 ± 0.0 ab	7.3 ± 1.5 c	−8.3 ns	3.3 ± 0.6 a	1.3 ± 0.6 b	−60.0 ***
Sebatel	36.4 ± 2.8 b	34.8 ± 0.8 b	−4.40 ns	5.7 ± 0.6 c	7.3 ± 0.6 c	29.4 ns	1.0 ± 0.0 c	1.0 ± 0.0 b	0.0 ns
Vulci	35.2 ± 7.0 b	27.6 ± 2.4 c	−21.76 ns	6.7 ± 1.5 bc	11.3 ± 1.2 b	70.0 ***	2.3 ± 0.6 b	2.7 ± 0.6 a	14.3 ns
Genotype		**			***			***	
Treatment		***			***			**	
G × T		**			***			***	

Values are means _ standard deviations (n = 3). Means with the same letter in the column of each treatment are not significantly different between genotypes ($p < 0.05$) (LSD test). Diff.% reports the differences between Control (column) and Salt (column). It is computed as [Diff.% = ((salt/control) - 1) _ 100], and its statistic is indicated as ns—Not significant; *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

5.3.2 Salt effect on the root system

The analysis of variance revealed the presence of significant differences between genotypes and treatments for all root traits except average root diameter (AG) (Table 5.3). Significant interaction effects were detected for root angle (RA) and the number of tips (TI).

Table 5.3. Analysis of variance (ANOVA) for root traits.

ANOVA	Root Volume	Root Length	Tips	Root Surface Area
Genotype	*	*	*	**
Treatments	***	***	***	***
G × T	ns	ns	*	ns
ANOVA	**Root Angle**	**Crossings**	**Forks**	**Average Root Diameter**
Genotype	***	*	**	ns
Treatments	***	*	**	ns
G × T	***	ns	ns	ns

A description is added, i.e., ns—Not significant; *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

Azeghar, which maintained a wide root angle under both conditions, had a significant positive effect of salt only on root volume and surface area (Table 5.4). While Cham1, with wide and not affected by salt root angle as Azeghar, had a significant positive effect on root length (RL) that increased significantly by more than 80%, and the number of tips (TI) by more than double, and negative effect on root mean diameter (Table 5.4). Sebatel, Vulci, and Pelsodur showed a significant effect of salt only on root angle, which expanded in the presence of salt in the soil; this was particularly evident for Pelsodur, where the differences were around 40%. The highest positive significant effect on the root system under salt stress was determined for the salt-tolerant genotype J. Khetifa. Features such as root length (RL), root volume (RV), root surface area (SA), and the number of tips, forks, and crossings doubled under salt stress in seedlings of J. Khetifa. Under control conditions, Azeghar had a higher root length (RL) than J. Khetifa and Cham1, while under salt stress J. Khetifa maintained the longest roots, Sebatel and Azeghar had medium root length, and Vulci, Pelsodur, and Cham1 had the shortest. Under control conditions, Azeghar, Sebatel, and Cham1 had a wide root angle (RA), while J. Khetifa, Pelsodur, and Vulci had a narrow RA. However, RA widened significantly only for Pelsodur, Sebatel, and Vulci under stress. Under salt conditions, the mean root diameter (AG) significantly decreased only for Cham1 by about 24%.

Table 5.4. Salt versus control effect on root traits of six durum wheat genotypes.

Trait	Treat	Genotype					
		Azeghar	Cham1	J. Khetifa	Pelsodur	Sebatel	Vulci
Root volume (cm^3)	control	0.80 ± 0.28 a	0.57 ± 0.15 a	0.57 ± 0.04 a	0.61 ± 0.19 a	0.68 ± 0.18 a	0.48 ± 0.18 a
	salt	1.28 ± 0.24 a	0.57 ± 0.02 c	1.07 ± 0.34 ab	0.68 ± 0.06 bc	0.99 ± 0.15 abc	0.77 ± 0.29 bc
Diff. (%)		59.6 *	0.0 ns	88.8 **	12.4 ns	45.1 ns	61.5 ns
Root Length (cm)	control	684 ± 106 a	333 ± 73 c	475 ± 104 bc	521 ± 80 abc	578 ± 55 ab	530 ± 120 ab
	salt	826 ± 52 ab	603 ± 23 b	890 ± 192 a	590 ± 7 b	737 ± 200 ab	627 ± 155 b
Diff. (%)		20.6 ns	81.2 *	87.3 **	13.4 ns	27.6 ns	18.2 ns
Number of tips	control	1243 ± 287 a	633 ± 94 a	1188 ± 406 a	1214 ± 225 a	1326 ± 507 a	1272 ± 353 a
	salt	1510 ± 119 b	1362 ± 21 b	2399 ± 143 a	1401 ± 265 b	1480 ± 352 b	1311 ± 309 b
Diff. (%)		21.5 ns	115.3 *	101.9 ***	15.4 ns	11.6 ns	3.0 ns
Root surface area (cm^2)	control	81.4 ± 7.8 a	48.5 ± 11.8 b	57.7 ± 4.2 b	62.8 ± 14.5 ab	69.9 ± 11.6 ab	56.4 ± 16.5 b
	salt	114.9 ± 14 a	65.5 ± 0.0 c	109 ± 28 ab	71.1 ± 2.6 bc	95.3 ± 20.3 abc	77.2 ± 21.7 bc
Diff. (%)		41.2 *	35.1 ns	89.0 **	13.2 ns	36.3 ns	37.0 ns
Root angle (°)	control	110.1 ± 4.4 a	109.4 ± 7.4 a	90.1 ± 3.3 b	79.7 ± 1.3 c	106.1 ± 2.3 a	84.9 ± 2.8 bc
	salt	117.0 ± 7.9 a	104.5 ± 3.2 bc	95.4 ± 4.3 d	109.5 ± 6.6 ab	115.3 ± 2.7 a	96.7 ± 2.9 cd
Diff. (%)		6.2 ns	−4.5 ns	6.0 ns	37.3 ***	8.7 *	13.9 *
Crossings	control	956 ± 487 a	421 ± 162 a	942 ± 204 a	887 ± 62 a	857 ± 136 a	859 ± 218 a
	salt	1056 ± 97 abc	512 ± 315 c	1543 ± 423 a	981 ± 241 abc	1122 ± 503 ab	916 ± 266 bc
Diff. (%)		10.5 ns	21.8 ns	63.9 *	10.6 ns	30.9 ns	6.6 ns
Forks	control	4990 ± 914 a	2774 ± 542 b	4651 ± 511 a	4736 ± 531 a	4587 ± 897 a	4152 ± 931 ab
	salt	6606 ± 143 ab	3547 ± 242 c	8180 ± 1987 a	4801 ± 183 bc	6100 ± 2120 abc	5111 ± 1201 bc
		32.4 ns	27.9 ns	75.9 *	1.4 ns	33.0 ns	23.1 ns
Mean diameter (mm)	control	0.39 ± 0.1 ab	0.46 ± 0.01 a	0.39 ± 0.06 ab	0.38 ± 0.03 ab	0.38 ± 0.05 ab	0.33 ± 0.3 b
	salt	0.44 ± 0.03 a	0.35 ± 0.01 b	0.39 ± 0.03 ab	0.39 ± 0.02 ab	0.42 ± 0.03 ab	0.39 ± 0.06 ab
Diff. (%)		12.8 ns	−23.9 *	−1.7 ns	1.3 ns	8.7 ns	17.0 ns

Values are means _ standard deviations (n = 3). Means with the same letter in each row are not significantly different between genotypes ($p < 0.05$) (LSD test). The differences between Control and Salt [Diff.% = ((salt/control) - 1)_100] and its statistic is indicated as ns—Not significant; *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

5.3.3 Correlation among traits

The correlation matrix among traits (Figure 5.3) shows that the root angle under both conditions (RAC and RAS) had a strong significant ($p < 0.05$) negative correlation with shoot traits such as the number of leaves and tillers under control (NLC, NTC) and salt (NLS and NTS) conditions. It could be suggested that genotypes with steeper root angles tend to produce more tillers and leaves. Moreover, root angle under both conditions (RAC and RAS) had a highly significant positive correlation with root volume and surface area under control conditions. An interesting association was found between the root length (RL) and root traits that show the formation of new roots, such as the number of tips (TI), forks (FR), and crossings (CR). Root length under control (RLC) had a significant ($p < 0.05$) positive correlation with these traits these traits (TIC, FRC, and CRC) only under control conditions and RLS with the same traits only under salt conditions. Moreover, root length under salt conditions (RLS) had a strong positive correlation with root volume (RVS) and root surface area (SAS) under salt conditions. In general, a significant positive correlation was found between root volume (RV) and surface area (SA) under both conditions, while both of them had a positive correlation with root angle (RAC) only under control conditions.

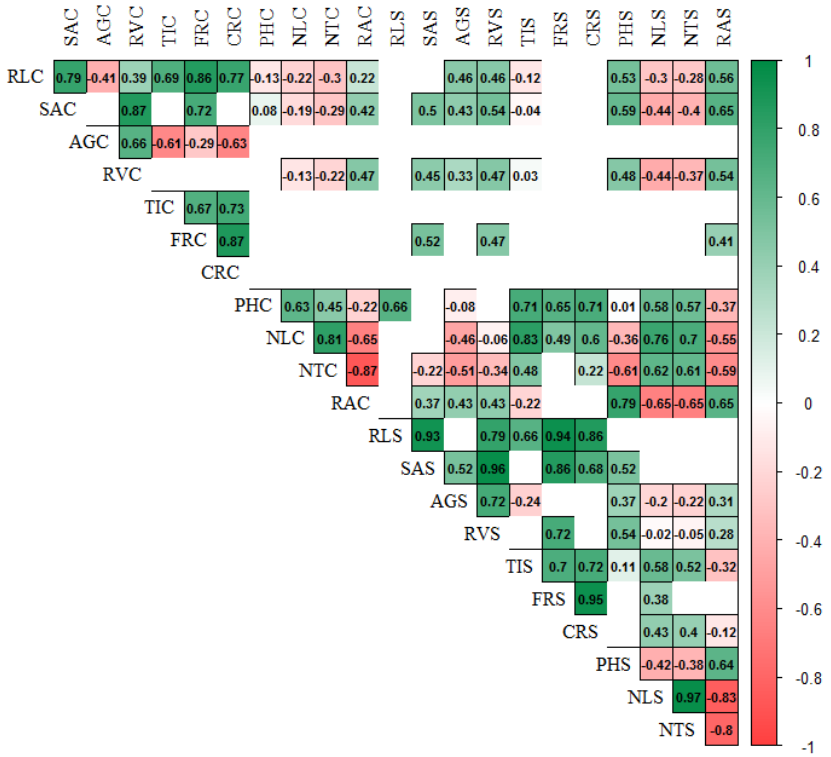

Figure 5.3. Correlation matrix among all the recorded traits in pots experiment under C-control and S-salt conditions. Positive (in green) and negative (in red) indicate significant ($p < 0.05$) correlations. For abbreviations, please see the abbreviation list at the end of the paper.

5.3.4 Principal component analysis (PCA)

The top two principal components of PCA account for 82.6% of the total phenotypic variation under control conditions (Figure 5.4A). PC1 explained 49.1% and was mainly associated with root traits such as -RL, -SA, -TI, -FR, -CR (Table S1). At the same time, PC2 explained 33.5% of the total variation and was strongly associated with shoot trait and root angle (Figure 5.4A). Genotype J. Khetifa was grouped with Vulci and Pelsodur on the positive side, while Azeghar, Sebatel, and Cham1 were on the negative side of PC2. However, J. Khetifa with Cham1 was assigned on the PC1 positive side, Azeghar and Sebatel on the negative, and Vulci with Pelsodur were close to zero value. Three clusters of genotypes were identified based on hierarchical classification under control conditions (Figure 5.4B). One cluster grouped J. Khetifa, Vulci, and Pelsodur, the second group Sebatel and Azeghar; while Cham1 separated from the rest genotypes quite far as a third group. Under salt conditions, the top two principal components of PCA account for 92.3% of the total variation of root and shoot traits (Figure 5.4A). PC1 explained 57.5% and divided genotypes Vulci, Pelsodur, and Cham1 on the positive side of PC1, while J. Khetifa, Azeghar, and Sebatel were on the negative side.

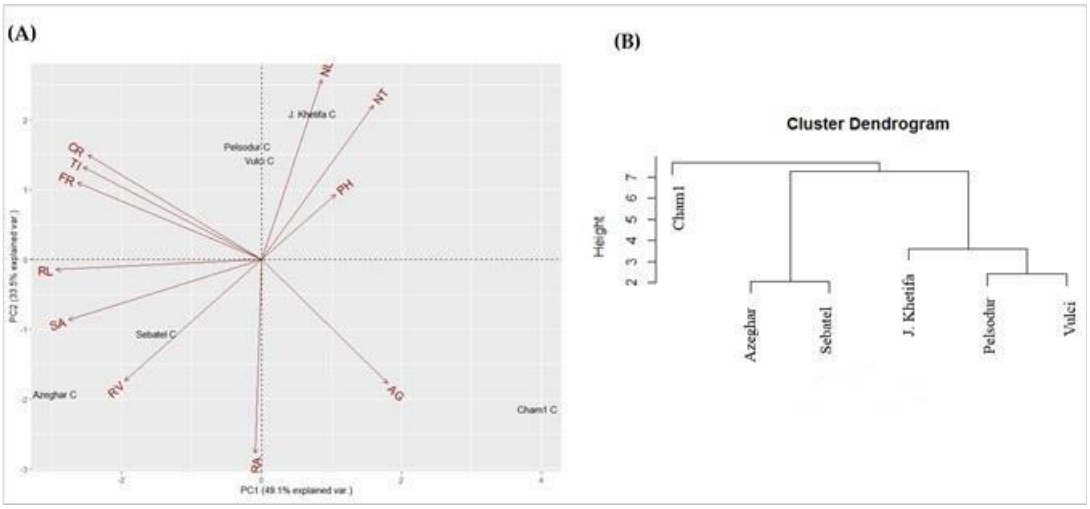

Figure 5.4. Biplot of the principal component analysis (A) and hierarchical analysis (B) of the shoot and root traits under control conditions. For abbreviations, please see the abbreviation list at the end of the paper.

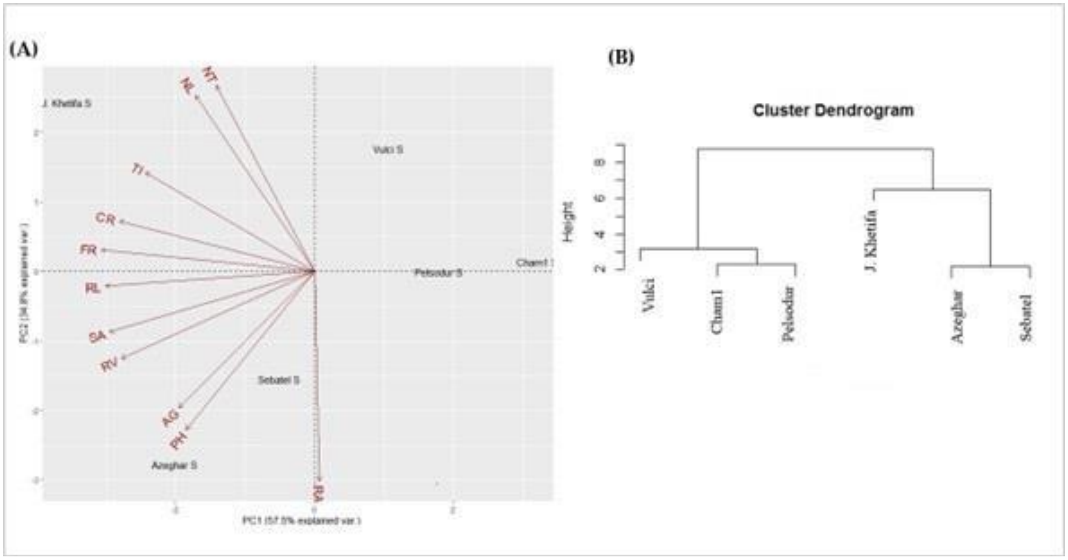

Figure 5.5. Biplot of the principal component analysis (A) and hierarchical analysis (B) of the shoot and root traits under salt conditions. For abbreviations, please see the abbreviation list at the end of the paper.

PC2 explained 34.8% and was associated with shoot trait and root angle the same as under control conditions (Figure 5.5A). J. Khetifa with Vulci was assigned on the PC2 positive side, Azeghar and Sebatel on the negative, and Cham1 with Pelsodur were close to zero value. According to hierarchical analysis, all genotypes were distinguished into two major clusters, where Sebatel and Azeghar were assigned together with the salt-tolerant J. Khetifa to one cluster, and Vulci, Pelsodur, Cham1 were grouped to the second one (Figure 5.5B).

5.3.5 Genetic diversity based on SSR markers

Eleven co-dominant SSR markers associated with specific root morphological traits were used to determine the genetic characteristics of the six genotypes. The number of alleles detected for each marker was very variable, ranging from 2 for wms205 to 5 for cfa2257, with an average of about three alleles per marker (Table 5.5). Some markers, i.e., gwm234 and wmc727, identified two loci labeled with a and b after the SSR name.

Table 5.5. Genetic diversity in durum wheat based on 11 SSR markers identifying 13 loci.

Locus	Na	I	Ho	He	uHe	PIC	Size, bp
cfa2086	5	1.561	0.000	0.778	0.848	0.744	223–299
wms5	3	1.011	0.000	0.611	0.667	0.536	180–190
gwm234a	2	0.451	0.000	0.278	0.303	0.240	214–218
gwm234b	2	0.693	0.000	0.500	0.545	0.375	242–256
wmc727a	2	0.679	0.833	0.486	0.530	0.368	88–96
wmc727b	2	0.451	0.000	0.278	0.303	0.240	null-231
cfa2257	5	1.424	0.333	0.722	0.788	0.680	104–156
wms205	2	0.693	1.000	0.500	0.545	0.375	157–170
gwm427	4	1.330	0.000	0.722	0.788	0.672	201–256
gwm573.2	5	1.424	1.000	0.722	0.788	0.680	181–240
gwm636	3	1.011	0.000	0.611	0.667	0.536	110–131
gwm459a	3	1.011	0.000	0.611	0.667	0.536	131–153
gwm499b	4	1.330	0.000	0.722	0.788	0.672	147–197
mean	3.2	1.005	0.244	0.580	0.633		

Na—No. of Alleles, I—Shannon's Information Index = □1 _ Sum (pi _ Ln (pi)), Ho—Observed Heterozygosity, He—Expected Heterozygosity, uHe—Unbiased Expected Heterozygosity, PIC—polymorphism information content. Different locus of same marker are highlighted as "a" and "b".

The observed heterozygosity was zero for most of the markers. Nevertheless, despite working with a self-pollinated species, the observed heterozygosity was not always equal to zero. Some markers detected high observed heterozygosity (i.e., wmc727a, wms205, and gwm573.2). Regarding the heterozygosity of wms205, it should be noted that all six genotypes have the same allelic situation leaving a suspect of a non-perfect allelic situation among the two detected bands common for all the genotypes. The gene diversity, computed as expected heterozygosity (He), and the polymorphism information content (PIC) provides information on a marker's ability to determine polymorphism. In the present study, the gene diversity is quite high, ranging from 0.278 (gwm234a and wmc727b, which are second loci detected by an SSR marker) to 0.778 (cfa2086) (Table 5.5). The PIC values had a similar but not equal ranking among markers compared with the gene diversity parameter. PIC ranged from 0.24 for gwm234a and wmc727b to 0.74 for cfa2086 (Table 5.5). The Shannon Information Index (Table 5.5) indicates richness, and the evenness is always quite high ranging from 0.45 to 1.56, with an average of 1.01. The polymorphism among the different genotypes was not dramatically different; it ranged from about 13% in Pelsodur to about 27% in Azeghar and Sebatel, with an average of 21% (data not shown). Interestingly the number of Private alleles, i.e., alleles present only for a single genotype, is quite high (Table 5.6). All the genotypes have at least one allele present only in those genotypes. This goes from Vulci with one private allele to Azeghar and Pelsodur with four private alleles. Not surprisingly, cfa2086, with the higher PIC, also has a higher number of private alleles detected.

Table 5.6. Private alleles (defined by a set of populations currently active).

Locus	Allele	Frequency	Found in
cfa2086	223	1	Sebatel
cfa2086	299	1	Pelsodur
cfa2086	269	1	Cham
cfa2086	277	1	J. Khetifa
wms5	185	1	Cham
gwm234a	214	1	J. Khetifa
wmc727a	1	1	Pelsodur
cfa2257	121	1	Cham
cfa2257	142	0.5	Azeghar
cfa2257	104	0.5	Azeghar
gwm427	237	1	Sebatel
gwm427	201	1	Azeghar
gwm573.2	226	0.5	Pelsodur
gwm573.2	181	0.5	Azeghar
gwm636	131	1	Pelsodur
gwm459a	131	1	Vulci
gwm499b	147	1	Sebatel
gwm499b	154	1	J. Khetifa

Different locus of same marker are highlighted as "a" and "b".

The UPGMA phenogram of the Nei (1972) [36] genetic distance cluster for 11 SSR markers (Figure 5.6) and all phenotypic data under control conditions (Figure 2B) discriminate the tested genotypes almost into similar clusters. Genotypes with wide root angles, such as Azeghar and Sebatel, were grouped morphologically and in terms of genetic distance. Another cluster, according to genetic distance, consisted of genotypes with narrow root angles, such as J. Khetifa, Pelsodur, and Vulci (Figure 4). Cham1, mainly in terms of phenotypic, was far from other genotypes, while genetically is far from the other genotypes but closer to Azeghar and Sebatel.

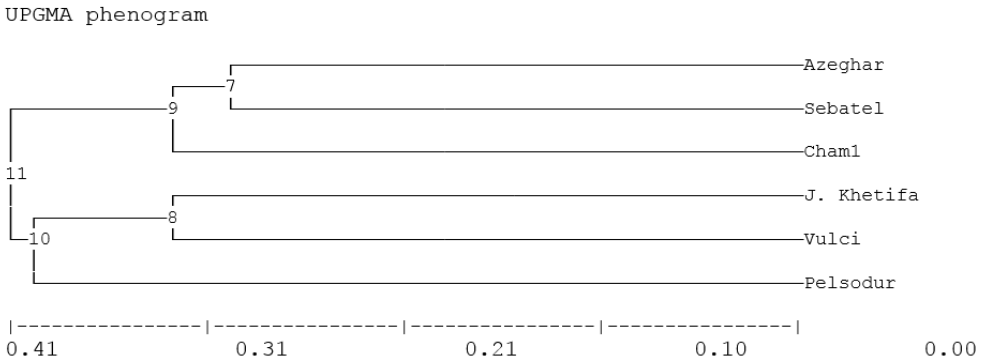

Figure 5.6. UPGMA phenogram of the Nei genetic distance among six durum wheat genotypes based on 11 SSRs markers.

5.3.6 More detailed analysis of genomic regions linked to selected SSRs markers and in silico candidate gene expression analysis

The SSRs markers used in this study are distributed in seven chromosomes and overlap with 32 root-related QTLs (Table S2). For example, wms5, located on chromosome 3A in the durum wheat genome, overlapped with seven root-related QTLs for the number of root tips, primary root diameter, primary root length, lateral root diameter, total lateral root surface and volume, and total root number. Markers gwm427 and gwm459 located on chromosome 6A overlapped in total with eleven QTLs associated with root traits such as total root length, root angle, average root length, primary root length, root surface area, root tips, and total lateral root surface and volume, primary root volume. Markers gwm499 and gwm234, located on chromosome 5B, overlapped with three QTLs related to total root length, total lateral root length, and primary root volume. Gwm636 and cfa2086 markers on chromosome 2A overlapped with QTLs related to lateral root number per primary root, total root number, and root growth angle. Markers wmc727 and wms205 markers located on chromosome 5A overlapped with QTLs related to average root length and primary root length. Cfa2257 marker located on chromosome 7A overlapped with Meta QTL associated with total root number and primary root length. Marker, gwm573.2, located on chromosome 7B, overlapped with root traits related to Meta QTL. Candidate genes for investigating and estimating gene expression levels were identified according to SSRs location on the durum wheat 'Svevo' genome (Table S3). A total of 57 gene models were detected and analyzed at http://www.wheat-expression.com/ (accessed on 13 January 2023), using their RNAseq to obtain the expression of genes involved in root response to abiotic stress at the seedling stage (Table S4). In the durum wheat genome, (RefSeq v1.0 Chr 2A region), the mapped marker gwm636 was close to nine genes models associated with growth and tolerance to abiotic and biotic stresses, such as cell differentiation protein RCD1, sterol 3-beta-glucosyltransferase, cytochrome P450, Sec24-like transport protein, AAR2 family protein, leucine-rich repeat receptor-like protein kinases. All genes were up-regulated in seedlings' roots under abiotic stress (Table S4). The marker wms5 was close to seven genes models associated with response to stress or plant development under stress conditions, including thioredoxinlike protein, homeobox leucine-zipper protein, citrate-binding protein, DUF1677 domaincontaining protein, AGAMOUS-like MADS-box protein, kinase superfamily protein, and core-2/I-branching beta-1, 6-N-acetylglucosaminyltransferase family protein.

Five of these were up-regulated in seedlings' roots under abiotic stress. Wmc727 on chromosome 5A was linked to 10 genes models mostly involved in the regulation of secondary metabolites related to antioxidant activity, metabolic pathways, transcription, and signaling, such as membrane protein insertase YidC, O-methyltransferase family protein, nuclease S1, short-chain dehydrogenase/reductase family protein, root phototropism protein, Chalcone, and Curcuminoid synthases, F-box-like protein, and Palmitoyl protein thioesterase containing protein. Eight from 10 genes linked to marker gwm499 on chromosome 5B were close to genes associated with response to abiotic stress, such as receptor kinase, basic helix-loop-helix transcription factor, and ADP-ribosylation factor GTPase-activating protein involved in the root growth direction, and was most expressed gene near gwm499 marker. The marker gwm427 on chromosome 6A was linked to seven genes models related to plant's adaptation and tolerance to abiotic stress, such as actin-related protein 2/3 complex subunit, plastid movement impaired 1-related 1 G protein, cytochrome P450, inosine-5'-monophosphate dehydrogenase, CBS domain-containing protein. All genes linked to marker gwm427 were expressed under abiotic stress in seedling roots. The marker gwm573.2 was close to 21 gene models associated with plant growth and development under abiotic stress such as trehalose 6-phosphate phosphatase, a protein of the DNA-directed RNA polymerase the ABC transporter, phosphatidylinositol: ceramide inositol phosphotransferase, shikimate kinase, G-protein, ATP-dependent zinc metalloprotease FtsH1, Aldo/keto reductase family oxidoreductase, Sec14p-like phosphatidylinositol transfer family protein, Pentetratricopeptide repeat proteins, a bHLH transcription factor, the regulator of chromosome condensation (RCC1) family with FYVE zinc finger domain-containing protein, HVA22-like protein, TCP transcription factor, Photosystem (PS) II CP43 reaction center protein, BTB/POZ domain-containing protein, Sphingoid base hydroxylase 2, and two F-box-like protein. Almost all of them were up-regulated in wheat seedlings' roots under abiotic stress conditions (Table S4).

5.4 Discussion

The root system remodeling strategies under adverse conditions is a great opportunity for adaptation and optimal development of the plant, as the first organ that senses and responds to abiotic stress, and then through efficient uptake of water and nutrients. Latest studies demonstrated that root system features, especially root angle in crop plants, play a main role in adapting to adverse conditions [20,22,37]. According to Pariyar et al. [38], together with root traits, leaf-related traits should be included for more efficient selection in breeding programs for whole plant establishment. At the same time, the ideotype of the root system plays a key role in ensuring sufficient leaf area, which in turn is responsible for efficient photosynthesis, the crucial process for plant development. Genotypes that produce new leaves faster than the die of old ones and maintain a high number of leaves or shoot biomass under stress conditions could be considered tolerant. Moreover, previous studies have shown that salt-tolerant genotypes of durum wheat accumulated higher shoot biomass and maintained a bigger leaf area under salt stress compared with susceptible varieties [39–41]. In our study, some shoot traits were associated with root angle. For example, the salt-tolerant genotype, J. Khetifa, under salt stress, maintained a narrower root angle and the highest number of leaves. Moreover, Vulci, genetically close to J. Khetifa, was characterized by a narrow root angle and a large number of leaves under salt conditions. However, in our previous drought study [25], Pelsodur, under control and drought

conditions, maintained a narrow root angle, which was associated with good shoot development and drought tolerance. While in this study, under salt stress, the Pelsodur root angle changed significantly from narrow to wide, and this caused a significant negative effect on his shoot development, especially tillers that decreased by 60%. Moreover, in genotypes with wider root angles, such as Sebatel, Cham1, and Azeghar, the number of leaves was not significantly affected by salinity. It is well known that a narrow root angle is one of the main traits for drought tolerance, but it was demonstrated that a shallower root growth angle could enhance rice yields in saline environments [20]. Phenotypic data from our pot experiment obtained using the WinRHIZO software (version 4.0b) highlighted a few trends in plant response to salt stress at the seedling stage. Genotypes under control conditions were distinguished regarding root angle into two groups, genotypes Vulci and Pelsodur with high salt-tolerant J. Khetifa had a narrower root angle than genotypes Sebatel, Azeghar, and Cham1. It was reported that root angle depends on plant root ability to control gravitropic versus anti-gravitropic mechanisms, which is the main factor in avoiding salty soil [20–22]. In this regard, root tips could be a good indicator for selection as they are involved in root gravity perception and regulation. In addition, the salt-tolerant genotype J. Khetifa under salt stress doubled the number of root tips and had a significantly higher number compared with the other genotypes. However, under salt conditions, the salt-tolerant J. Khetifa, with a narrow root angle, was grouped with genotypes Azeghar and Sebatel, which had a wider root angle. These results suggest that a narrow root angle is not a good indicator of salt stress tolerance. in general, the check genotype J. Khetifa under salt stress at the seedling stage improved the number of leaves, which is a good salt tolerance indicator in terms of accumulation of toxic ions and efficient photosynthesis. Moreover, J. Khetifa improved almost all root traits under salt conditions, except the root angle, which stayed narrow, and the mean root diameter. The other two genotypes, Azeghar and Sebatel, from the same cluster of J. Khetifa under salt stress, had wide root angles, and salt did not affect shoot traits significantly. Moreover, genotype Azeghar under salt increased root volume and surface area, and the roots of Sebatel were not affected significantly. Based on present results, it can be concluded that a narrow root angle is not a good indicator of salt tolerance, moreover, exist more root system adaptation strategies that depend on the inner capacity of genotypes to maintain an efficient amount of roots. Dendrograms based on the cluster analysis of the genetic similarity coefficients and phenotypic data under control conditions grouped genotypes mainly due to root angle. In this regard, the SSR marker wms5 can be quite informative in selecting genotypes for narrow-angle root system ideotype. It was reported that the wms5 marker was significantly associated with root angle [42], and it was located on chromosome 3A in the durum wheat genome. In our study, genotypes grouped in the same clusters according to root angle, such as J. Khetifa, Vulci, and Pelsodur, had narrow root angles (wms5 band of 190 bp), and Sebatel and Azeghar (wms5 band of 180 bp), Cham1 (wms5 band of 185 bp) had wide root angles. Moreover, in the durum wheat genome, the location of wms5 was close to gene models representing proteins involved in plant development and response to stress. Such as thioredoxin, which is a small ubiquitous protein that reduces protein disulfides, protects cells from oxidative damage, and protects plants from the oxidative deactivation of photosynthesis-related enzymes [43]. Moreover, homeobox leucine-zipper transcription factors are responsible for plant growth adaptation in water deficit conditions [44,45]. Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein is involved in cell elongation during the seedling growth stage and senescence of the leaf process [46]. Citrate-binding protein

family is involved in plant development and growth, also in response to stress related to the accumulation of toxic ions in the vacuoles [47]. DUF1677 family proteins are DUF domain-containing proteins that play a vital role in plant stress responses, including salinity [48,49]. Kinases superfamily proteins are well known as signal transmitters in various stresses [2]. Members of the MADS-box gene family are involved in many processes of plant development and morphogenesis, but it was also reported to modulate root architecture [50,51]. Our results revealed that the root system under the salt of genotypes Azeghar and Sebatel were close to the one of salt-tolerant J. Khetifa, regardless of root angle differences, making them good candidates as parent lines in breeding programs. In this respect, some rare alleles of SSR markers could help to select salt-tolerant candidates in the segregant populations. For example, the salt-tolerant J. Khetifa and Sebatel had rare alleles near marker cfa2086, 277 bp, and 223 bp, respectively. Moreover, salt-tolerant J. Khetifa had rare alleles by SSR marker gwm234 (214 bp) and near marker gwm499 (154 bp). Moreover, genotype Sebatel had a rare allele also near marker gwm499. In the durum wheat genome, the mapped marker gwm499 was close to gene models for kinases superfamily protein, which mostly regulates transcription factors. Moreover, it was near to basic helix-loop-helix transcription factors, which are involved in a range of abiotic stress responses and adaptive responses to drought [52]. ADP-ribosylation factor GTPase-activating protein was shown that is crucial for determining directional root hair growth in Arabidopsis [53]. Genotypes Azeghar and Sebatel had rare alleles near marker gwm427, 201 bp, and 237 bp, respectively. The marker gwm427 position was found to overlap with previously reported durum wheat major QTL for root growth angle (qSRA-6A), where candidate gene analysis showed loci related to gravitropism, polar growth, and hormonal signalling [54]. In the durum wheat genome (RefSeq v1.0 Chr 6A region), the mapped marker gwm427 was close to genes models for actin-related protein and plastid movement-impaired related protein. Marker gwm427's location in the durum wheat genome was close to two related genes such as (i) the actin-related protein and (ii) the plastid movement impaired 1-related 1 G protein that regulates organelle movement [55]. In general, the movement and positioning of the organelles play an important role in the adaptation of plants to environmental stress. Moreover, actin-related protein regulates developmental processes such as cell division and development and cell polarity [56]. Marker gwm427 location in the durum wheat genome was close to other five gene annotations (Table S4); (iii) cytochrome P450 from the oxidoreductases enzymes class has been reported to be involved in plant defense against various abiotic and biotic stresses [57]. (iv) Inosine-5'-monophosphate dehydrogenase catalyzes the biosynthesis of the guanine nucleotide, which is important for DNA and RNA synthesis, signal transduction, energy transfer, glycoprotein synthesis, and cellular proliferation [58]. (v) Proline-tRNA ligase is an enzyme involved in proline metabolism and KEGG and aminoacyl-tRNA biosynthesis pathways (BRENDA:EC 6.1.1.15). L-Proline in a stressful environment is crucial for plants due to its protective role as an osmoprotectant. and stabilizes cellular structures and enzymes, and scavenging reactive oxygen species [59]. (vi) Potassium-transporting ATPase could help plant cells to maintain a high concentration of potassium and a low concentration of sodium inside in case of high sodium concentrations in the soil. (vii) Gene containing cystathionine _-synthase (CBS) domain was found to be upregulated under high salinity, and overexpressing this gene showed higher abiotic stress tolerance in transgenic plants [60]. Genotype Azeghar had three more rare alleles, two near marker cfa2257 (104 bp and 142 bp) and one near gwm573.2 (181 bp) (Table S4). The marker gwm573.2 was close to gene models translated to trehalose 6-

phosphate phosphatase, which regulates the activity of protein kinase, and this has a large impact on cell division, development, and function [61]. The protein of the DNA-directed RNA polymerase belongs to complex molecular machines for the essential function of the synthesis of RNA from DNA templates, otherwise transcription [62]. Abscisic acid is a substrate of the ABC transporter that is involved in the active transport of various molecules across biological membranes, such as heavy metals, lipids, glucosinolates, and phytohormones [63] allowing to coordinate of physiological and developmental processes at the whole-plant level and being involved in response to several induced by biotic stresses. Other related genes (Table S4) are (i) Phosphatidylinositol: ceramide inositol phosphotransferase, which is essential for sphingolipid biosynthesis and plays an important role in dehydration stress tolerance [64]. (ii) Shikimate kinase catalyses the reaction of the shikimate pathway that redirects carbon from the central metabolism pool to a wide range of secondary metabolites responsible for plant development, growth, and stress responses [65]. (iii) G-proteins are universal signal transducers involved in many cellular responses of plants [66]. (iv) ATP-dependent zinc metalloprotease FtsH 1 is involved in the formation of thylakoid membranes during early chloroplast development and in the proteolysis of damaged photosystem II (PSII) protein complex repair cycle, which is essential to avoid photoinhibition due to the accumulation of photodamaged PSII [67]. (v) Aldo/keto reductase family oxidoreductase is involved in many plant metabolites reactions, such as the biosynthesis of osmolytes, reactive aldehyde detoxification, secondary metabolism, and membrane transport [68]. (vi) Sec14p-like phosphatidylinositol transfer family protein is a plasma membrane-associated protein that has a crucial role in rooting patterning due to auxin signalling and PIN distribution in Arabidopsis [69]. (vii) Pentatricopeptide repeat proteins regulate chloroplast gene expression and RNA metabolism in higher plants [70]. (viii) Transcription factor bHLH involved in response and tolerance to various plant stresses, including soil salinity [71]. (ix) The regulator of chromosome condensation (RCC1) family with FYVE zinc finger domain-containing protein is essential for regulating auxin flows toward the direction of gravity and root branch angle in Arabidopsis [72]. (x) Transcriptomic analysis in roots revealed that the ABA-related gene encoding HVA22-like proteins was up-regulated, and considering that ABA biosynthesis is correlated with auxin transport to the root tips, their co-expression can be beneficial for the development and morphology of the roots [73]. (xi) TCP transcription factor may activate OsNHX1 gene expression, which response to salt and PEG-induced drought stress in rice, and may be associated with abiotic stress tolerance [74]. (xii) Photosystem (PS) II CP43 reaction center protein is the main antenna of the PS II pigment-protein complex in the thylakoid membrane and plays an essential role in the photosynthetic responses to stresses in higher plants [75]. (xiii) BTB/POZ domain-containing proteins are associated with plant growth and development, also involved in plant responses and defense against biotic and abiotic stresses [76]. (xiv) The enzyme Sphingoid base hydroxylase II is essential for growth and viability in Arabidopsis [77]. The marker gwm573.2 was also close to (xv) two F-box-like protein genes models, which were described earlier as genes involved in salt tolerance, and rooting abilities.

5.6 Conclusion

The identification of molecular markers linked to root system traits is very important for marker-assisted selection in breeding programs. The SSR markers linked to genetic regions associated with root traits in this study were effective in discriminating the genotypes and can be used to screen candidate lines for salt tolerance. Nevertheless, the narrow root angle is not a good indicator of salt tolerance since there are more root system adaptation strategies that depend on the inner capacity of genotypes to maintain an efficient amount of roots. Moreover, present results of phenotypic response to salt stress using Win-RHIZO Pro software together with molecular analysis can provide useful highlights to increase the efficiency of plant breeding programs.

5.7 References

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Chapter 6. Durum wheat (*Triticum turgidum* L. var. *durum*) root system response to drought and salt stress and genetic characterization for root related traits

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Abstract

Abiotic stresses such as drought and salt are significant threats to crop productivity. The root system adaptation and tolerance to abiotic stresses are regulated by many biochemical reactions, which create a complex and multigenic response. The present study aims to evaluate the diversity of root responses to cyclic abiotic stress in three modern durum wheat varieties and one hydric stress-tolerant landrace in a pot experiment from seedling to more advanced plant development stages The genotypes had responded to abiotic stress during the whole experiment very differently, and at the end of the experiment, nine out of the thirteen traits for the landrace J. Khetifa were significantly higher than other genotypes. Moreover, SSR genetic analysis revealed high polymorphism among the genotypes screened, and interesting private alleles associated with root system architecture traits. We propose that the markers used in this study could be a resource as material for durum wheat breeding programs based on marker assisted selection to increase the vegetal material with high drought and salt stress tolerance and to identify candidates with strong early vigor and efficient root systems. This study provides appropriate genetic materials for marker-assisted breeding programs as well as a basic study for the genetic diversity of root traits of durum wheat crop.

Keywords: durum wheat, abiotic stress, high throughput root phenotyping, SSR molecular markers, genetic diversity.

6.1 Introduction

Drought and salt are the most harmful abiotic stresses for crop productivity all over the world (Mondini and Pagnotta, 2015; Urbanavičiūtė et al., 2021). Particularly in the Mediterranean region, water shortage and salty water irrigation represent critical issues affecting crop yields, specifically for durum wheat, *Triticum turgidum* L. subsp. *durum* (Desf.), one of the most cultivated crops in the region (Carvalho et al., 2014; Rezzouk et al., 2022). Although both stresses have negative impacts on plant growth and development, each one affects plants differently (Oliveira et al., 2013; Golldack et al., 2014). The consequence of water deficit, in addition to osmotic stress, is that plants also suffer the toxic effects of accumulated Na^+ and Cl^- ions in the presence of salt (Munns and Gilliham, 2015; Liang et al., 2018). Overall, complex factors are involved in response to stresses which are mainly determined by the environment and the genetic capacity of a plant to recognize and respond

to adverse conditions. Moreover, the impacts of abiotic stresses on plants depend on their timing, severity, and dose. It was reported that a major loss of production in wheat occur when drought events affect the plants in early stages, such as tillering and stem elongation stage (Blum et al., 1990; Saeidi et al., 2015). It was also demonstrated that salinity applied before terminal spikelet differentiation (TSD) caused a reduction in number of spikelets per spike and number of tillers per plant, whereas, salinity imposed after TSD, significantly reduced only kernels number and weight (Francois et al., 1994). Moreover, it was shown that severe drought stress reduced wheat grain quality while moderate stress has improved its quality (Ozturk et al., 2022). Furthermore, grain yield reduction varied among durum wheat genotypes under different salt concentrations (Husain et al., 2003). The harmful effects on crop yield also depend on stress frequency, such as a single severe drought or mild cyclic drought events (Izanloo et al., 2008; Ding et al., 2018).

Plant roots are essential to detect and respond to abiotic stresses. However, the plasticity of their growth and development in response to changing soil conditions is crucial and remains a challenge (Shelden and Munns, 2023). Recently, several studies have highlighted relevant root adaptation strategies as a reaction to abiotic stresses and have shown the root system importance for plant performance (Chen et al., 2018; El Hassouni et al., 2018; Dwivedi et al., 2020; Zou et al., 2021). The root angle for example was defined as a principal trait for the selection of drought tolerance genotypes (Wasson et al., 2012; Urbanavičiūtė et al., 2022). It was found that genotypes with a narrow root angle had longer roots under drought conditions and had better water availability from deeper soil layers (Richard et al., 2015; Chen et al., 2018; El Hassouni et al., 2018). However, it was demonstrated that shallower root growth could enhance rice yields in a salty environment (Kitomi et al., 2020). The impact of abiotic stress on root angle is associated with gravitropism versus anti-gravitropism regulatory mechanism. Under drought, roots grow toward water in the direction of the gravity vector, while they direct their growth away from salt against the gravity vector through negative halotropism (Sun et al., 2008). Moreover, the gene responsible for gravitropism regulatory mechanisms and root angle was identified (Fusi et al., 2022). The root response to abiotic stress and consequently the root angle is mainly regulated in root tips, where the auxin distribution determines the reorientation of the root along or against gravity, as adaptation strategies to avoid unfavourable environments (Waite et al., 2020; Ober et al., 2021). Although several genes responsible for root angle have been identified (Wang et al., 2018; Furutani and Morita, 2021; Fusi et al., 2022), incorporating them into breeding programs remains challenging.

Both the root system plasticity and tolerance to abiotic stress are multigenic traits regulated by several biochemical reactions. However, plants have several adaptation systems that aid in tolerating or resisting the negative impact of abiotic stresses. Although numerous QTLs were identified to be involved in abiotic stress tolerance (Colasuonno et al., 2015), breeding for drought and salt stress affected environments, is extremely challenging due the complexity and the multitudes of mechanisms adopted by plants to mitigate the stress effect (Reynolds et al., 2005). The marker-assisted selection with molecular markers such as single sequence repeats (SSRs) is still a solid solution for the study of the root architecture of

durum wheat cultivars better adapted to water-limited and salinity conditions (Maccaferri et al., 2016; Alahmad et al., 2019). The identification and the introgression of alleles that contribute to drought and salt stress tolerance, is a desirable approach for the improvement of drought susceptible cultivars (Canè et al., 2014). The SSRs represents an effective tool for the allelic identification due to their large level of polymorphism and distribution in the whole genome (Maccaferri et al., 2007; Mondini et al., 2009). The analysis of SSR may allow to determine the principal chromosomal region involved in root architecture features (Soriano and Alvaro, 2019).

Furthermore, a good and time-tested breeding strategy for abiotic stress tolerance is the evaluation and exploration of natural genetic diversity including wild genotypes, landraces, and modern varieties (Nazco et al., 2012; Soriano et al., 2016). At the same time, the high-throughput root analysis technologies allow us to see more detailed information on root system plasticity, and it can help determine the target root traits for crop improvement under abiotic stress (Nagel et al., 2012; Richard et al., 2015; Urbanavičiūtė et al., 2022).

The present study aims (i) to evaluate the phenotypic and genotypic diversity in root traits for three modern varieties and one landrace of durum wheat, (ii) to assess the responses to multiple abiotic stresses such as drought and salt after several cyclic stress events, (iii) to assess the genetic diversity among the modern varieties and the landraces through the analysis based on SSR markers highly linked to genome regions associated with root traits of interest for drought and salt tolerance breeding programs.

6.2 Materials and methods

6.2.1 Plant material and experiment design

Four genotypes were used in the study: one hydric stress-tolerant landrace J. Khetifa (Mondini, et al., 2015), and three modern varieties Cham1, Azeghar 2-1 (56) (abbreviated as Azeghar), and Sebatel2 (45) (abbreviated as Sebatel) developed in the Mediterranean Basin. In total 144 seeds, 36 per genotype with one seed per pot placed with the embryo facing down were sown on the 20th of January 2021 in the greenhouse at Tuscia University experimental farm (Viterbo, Italy; 42°25'29N 12°04'47"E). Some extra-seeds sown in the same condition to have the possibility to replace the not germinated plants. Pots in size 17 cm diameter by 16 cm high were filled with 2.5 L clean sand. Pots for each treatment (control, drought, and salt) were placed on separate tables with automatic watering. Each table consisted of four sets of twelve plants each disposed in a Randomized Complete Block Design for the four genotypes in three replications (Figure 6.1A). During the experiment, stress (drought and salt) was applied four times. After each treatment event, one set from each table (control, drought, and salt) was removed for analysis, while the rest treated plants were re-watered to prevent death and treated again (Figure 6.1B). The first drought and salt stress treatments were started when all plants reached two leaves unfolded stage.

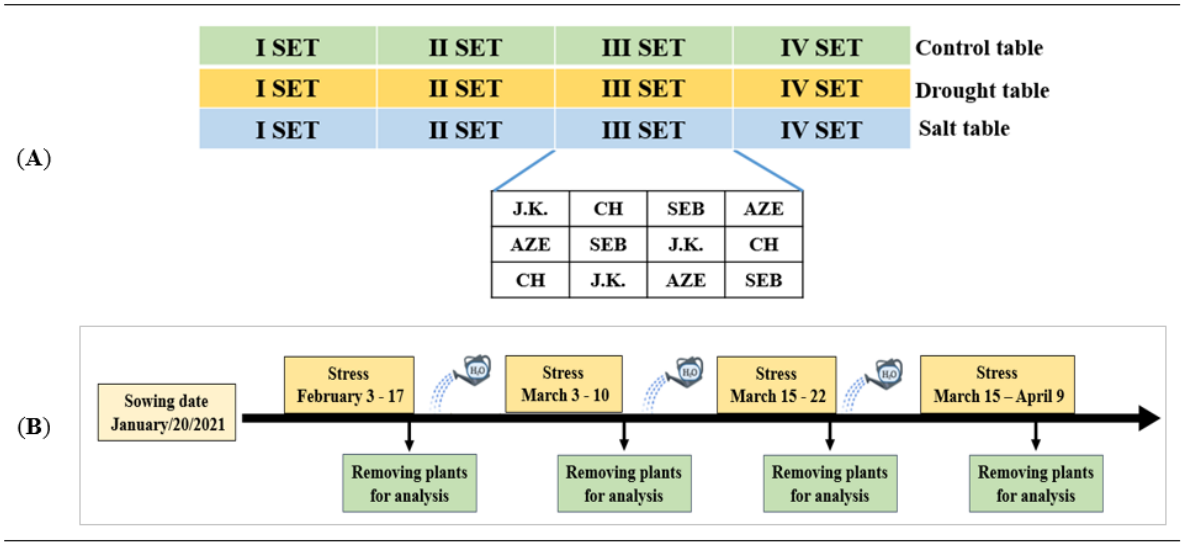

Figure 6.1. The experimental design. A) genotype allocation, and B) treatment management.

The control treatment was irrigated using a dropping pipe three times per week (80 mL/per pot) with a water nutrition solution (Supplementary Table 1S). Drought treatment was managed by discontinuing watering, while salt treatment received solution irrigation as the control but added NaCl to have 250 mM of NaCl.

6.2.2 Plant phenotyping

Roots were analysed using Win-RHIZO Pro software v2009 (version 4.0b; Regent Instruments, Montreal, QC, Canada) to obtain root traits such as total root length (RL, cm), surface area (SA, cm2), total root volume (RV, cm3), number of tips (TI), forks (FR), and crossings (CR). The root angle (RA, degree on the vertical °) was measured using the software ImageJ. After measurements using Win-RHIZO, roots were separated, dried in an oven (at 80 °C for 12 h), and weighed for root dry weight (RDW). Plant length (PH) was measured during whole experiments, while the number of leaves (NL) and tillers (NT) were measured only from the second stress event due to the phenological state of plants. After each stress event, plants were carefully removed from pots and washed from sand using a soft spray watering head.

6.2.3 Plant genotyping

The genetic diversity analysis was conducted using 11 SSRs already identified by Urbanavičiūtė et al. (2023). The markers were already demonstrated as highly associated with chromosomal regions related to root traits (Supplementary Table S2). The genomic DNA of each accession was extracted from fresh leaves with PureLink Plant Total DNA Purification kit (Invitrogen; Thermo Fisher Scientific,Waltham, Massachusetts USA). The PCR reactions were carried out following the manufactor instruction of GoTaq G2 DNA Polymerase (Promega, Madison,Wisconsin, USA) for mixture protocols and cycling conditions. The amplifications were run in in SwiftMaxi Thermal Cyclers (Esco Technologies, St. Louis,Missouri, USA) with different annealing temperature for each

primer (Supplementary Table S3). The amplicons were further analysed through capillary electrophoresis with the device QIAxcel Advanced Instrument (Qiagen, Hilden, Germania) using the QIAxcel DNA High-Resolution Kit, and the amplifications were validated with QIAxcel ScreenGel Software (Qiagen, Hilden, Germany). Each amplicon was named as its molecular weight, the raw data are reported in the Supplementary Table S4.

6.2.4 Statistical analysis

Morphological data were analysed using R Studio (Version R-4.1.0), and a Two-way analysis of variance (ANOVA) was conducted at a significance level of 5% using the aov() function. A one-way ANOVA was used to test the variance component of each trait under each treatment, with genotype as a factor. Fisher's least significant difference (LSD) test was used to compare means. Regression and correlation analyses between phenotypic and genotypic traits were performed by SPSS (version 14.0 for Windows) statistical software. The genetic analysis was performed though the software GDA (Lewis and Zaykin, 2001) and GenAlEx (Peakall and Smouse, 2012).

6.3 Results

6.3.1 Effect of cyclic drought and salt stress on shoot traits

After the first stress treatment, the abiotic stress-tolerant genotype J. Khetifa had a significantly longer seedling length (PH) under drought and salt stress, although under the control conditions, genotypes did not differ from each other (Table 6.1).

Table 6.1. Plant length (PH) (cm) after repeated drought and salt stresses (C – control, D – drought, S – salt).

T	Genotype	1st	2nd	3rd	4th
C	Azeghar	30.3±1a	34.6±3.2b	56.7±3a	76.2±4.4a
C	Cham1	30.1±3.7a	37.5±0.2b	56.7±3.7a	68.2±5.3a
C	JK	37.8±2.8a	42.8±1.1a	48.6±6.2b	55.6±8.9b
C	Sebatel	30±7a	36.4±2.8b	51.1±3.3ab	72.7±5.9a
D	Azeghar	25.2±3.6c	36.7±1.5a	41.3±5.6a	58.4±6.5a
D	Cham1	30±2.7bc	30.5±2.9a	41.7±7a	53.9±2.6ab
D	JK	37.7±3.7a	33.5±3.6a	44.3±3.9a	39.5±1.1c
D	Sebatel	32.3±0.6ab	34.1±5.6a	48.4±1a	51.2±0.8b
S	Azeghar	27.6±1.3bc	40.5±1.6a	46.4±7.9a	54.3±4.2a
S	Cham1	24.4±1.8c	31.3±0.8b	43.2±2.9a	52.8±2.2a
S	JK	38.8±0.3a	33.1±2.6b	38±3.8a	50.4±0.9a
S	Sebatel	30.5±3.1b	34.8±0.8b	47.5±15.1a	48.2±4.5a
	Genotype (G)	***	*	ns	***
	Treatments (T)	ns	**	***	***
	G × T	ns	**	ns	*

Values are means ± standard deviations (n = 3). Means with the same letter in each column, within treatment are not significantly different between genotypes ($p < 0.05$) (LSD test). ns – Not significant; *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

Overall, a significant effect of treatments was not detected. However, genotype Cham1 at early stages was more sensitive to salt than to drought stress. After the second stress treatment, significant differences between genotypes, treatments and their interaction (G×T) were detected in terms of PH (Table 6.1). Both drought and salt stress had undesirable effects on the PH of Cham1 and J. Khetifa; conversely, the PH of Sebatel was not affected at all. Furthermore, Azeghar PH increases slightly under salt stress compared with the control conditions. After the third stress treatment, PH was affected negatively for all the genotypes. The PH of Sebatel had no significant differences between control and stress conditions, while in J. Khetifa a major negative effect was detected from salt treatment than drought. Cham1 reduced PH under drought and salt conditions, while Azeghar reduced PH only under drought. After the fourth drought and salt stress event, a significant reduction of PH was recorded; the reduction was similar for Azeghar, Cham1, and Sebatel under drought or salt conditions. However, the PH of J. Khetifa was more affected by drought than by salt stress.

After two stress events, the number of leaves (NL) significantly increased for Azeghar and J. Khetifa, while Sebatel and Cham1 had no significant differences between control and stress conditions (Table 6.2).

The third stress event had a negative impact on NL of all genotypes for both treatments. After the fourth stress event, a major effect of salt stress was detected on NL than drought. It was recorded for all genotypes except for Azeghar, which had no significant difference between NL under control and drought conditions. Although abiotic stress decreased the number of leaves, J. Khetifa was characterized by many leaves during all experiments and under all conditions: control, drought, and salt.

After the second stress event, the number of tillers (NT) of all genotypes was not significantly affected by drought or salt conditions, except Cham1, which decreased NT under both stresses (Table 6.2). After the third stress event, the NT of J. Khetifa and Azeghar was reduced equally by drought and salt stress.

Table 6.2. Number of leaves (NL) and number of tillers after repeated drought and salt stresses (C – control, D – drought, S – salt).

		Number of leaves			**Number of tillers**		
T	**Genotype**	**2nd**	**3rd**	**4th**	**2nd**	**3rd**	**4th**
C	Azeghar	5±0b	15.7±2.9c	19.7±3.1b	1±0c	3±0c	3.7±0.6b
C	Cham1	5±0b	23±2b	25±1b	2±0b	4.7±0.6b	4.3±0.6b
C	JK	9.3±0.6a	36.7±1.2a	96.3±6a	3±0a	9±0a	24.3±1.2a
C	Sebatel	5.7±0.6b	17±3.6c	25.7±4.5b	1±0c	3.7±0.6c	4.7±0.6b
D	Azeghar	7±0b	7±0c	14±4.6b	1±0b	1±0c	3±1b
D	Cham1	8±1.7b	8.7±1.5bc	18±3b	1±0b	2.7±1.2b	3.3±0.6b
D	JK	11.7±0.6a	16±1a	36.3±8.5a	3±0a	4.3±0.6a	6±1.7a
D	Sebatel	7.3±2.1b	9.7±0.6b	13.7±1.2b	1.3±0.6b	2.3±0.6b	3±0b
S	Azeghar	6.7±0.6b	6±1b	6.7±0.6b	1±0b	1±0b	1±0b
S	Cham1	7±0b	7.3±0.6b	5.7±2.3b	1±0b	1±0b	1±0b
S	JK	14±0a	16.7±3.5a	23±1a	3.3±0.6a	4±0a	3.7±0.6a
S	Sebatel	7.3±0.6b	8.5±0.7b	6.7±0.6b	1±0b	1.5±0.7b	1±0b
	Genotype (G)	***	***	***	***	***	***
	Treatments (T)	***	***	***	ns	***	***
	G × T	*	***	***	**	***	***

Values are means ± standard deviations (n = 3). Means with the same letter in each column, within treatment are not significantly different between genotypes ($p < 0.05$) (LSD test). ns – Not significant; *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

However, in Cham1 and Sebatel were recorded lower values of NT under salt stress than under drought. After the fourth stress event, salt had a more negative effect on NT than drought. However, the reduction of NT varied among genotypes. J. Khetifa had the greatest NT loss, but its number of tillers during the whole experiment under all conditions was significantly higher than the rest of the genotypes. Moreover, the number of tillers of Azeghar and Cham1 after the fourth drought stress was not significantly different from the control conditions.

6.3.2 Effects of cyclic drought and salt stress on root traits

The total root length (RL) was significantly affected by treatments. Nevertheless, no significant difference among genotypes was detected after the first stress treatment (Table 6.3). At the early seedling stage, Cham1 and J. Khetifa significantly increased the RL under drought, compared with control and salt stress conditions. After the second stress event, Cham1 and J. Khetifa increased RL under drought and salt stress, while Sebatel and Azeghar had no significant differences between control and stress conditions. After the third stress event, Azeghar decreased RL under drought and salt stress, while Cham1 and J. Khetifa significantly decreased RL only under salt conditions. Sebatel continues to have no significant differences between control and stress conditions. Also, a statistically significant interaction after the fourth stress event in terms of the RL was found (Table 6.3). The RL of Azeghar was significantly negatively affected only by salt stress. While Cham1 decreased RL under both types of stress, under salt stress the effect was statistically significant higher. J. Khetifa showed the ability to grow roots after repeated stress treatment and had no differences between RL under stress and control conditions. The fourth drought and salt stresses negatively affected the RL of Sebatel at the same level.

The root volume (RV) had no significant differences between control and stress conditions for all genotypes after the first stress event, except J. Khetifa, which significantly increased RV under drought stress (Table 6.3). After the second stress event, genotypes respond in terms of RV depending on the type of stress. This was highlighted by a significant G x T interaction. Azeghar increased RV under salt while Cham1 under drought. J. Khetifa significantly increased RV under both stresses, and Sebatel had no significant difference between control and stress conditions. The root volume was mostly affected negatively after the third stress event, even if the genotype response varied among drought and salt conditions. The RV of J. Khetifa, Azeghar, and Sebatel were similarly affected by both drought and salt stresses. Cham1 was more affected by salt than drought. After the fourth stress event, the RV of J. Khetifa and Azeghar was affected similarly by both drought and salt stresses, while Cham1 and Sebatel were more affected by salt than drought.

Table 6.3. Total root length (RL) (cm) and root volume (RV) (cm3) after repeated drought and salt stresses (C – control, D – drought, S – salt).

		Total root length				Root volume			
T	**Genotype**	**1st**	**2nd**	**3rd**	**4th**	**1st**	**2nd**	**3rd**	**4th**
C	Azeghar	286±58a	685±107a	989±84a	1298±309a	0.74±0.19a	0.8±0.28a	5.31±1.14b	5.44±0.72b
C	Cham1	285±86a	333±73c	1056±182a	1457±29a	0.82±0.28a	0.57±0.15a	7.01±0.26b	5.24±0.8b
C	JK	360±9a	475±105bc	973±124a	756±161b	0.47±0.09a	0.57±0.04a	9.73±1.92a	21.61±6.33a
C	Sebatel	382±32a	578±55ab	921±231a	1353±299a	0.65±0.14a	0.68±0.18a	6.05±1.6b	5.21±0.09b
D	Azeghar	408±145a	898±175a	569±186b	895±94ab	0.57±0.13a	0.63±0.06b	0.97±0.31b	1.39±0.38b
D	Cham1	547±22a	784±93a	816±125ab	806±174b	0.67±0.09a	0.86±0.13ab	1.49±0.47b	1.77±0.29b
D	JK	526±84a	948±143a	887±107a	1141±223a	0.74±0.16a	1.03±0.09a	2.18±0.21a	4.51±0.26a
D	Sebatel	402±7a	820±243a	806±168ab	746±111b	0.83±0.07a	0.61±0.24b	1.53±0.07b	1.71±0.54b
S	Azeghar	330±44a	826±52a	406±238b	728±150ab	0.49±0.16a	1.28±0.24a	0.59±0.37b	0.55±0.11b
S	Cham1	268±41a	603±23a	391±29b	461±235b	0.45±0.12a	0.57±0.02b	0.56±0.06b	0.46±0.2b
S	JK	324±41a	890±192a	641±100b	875±209a	0.55±0.04a	1.07±0.33ab	1.2±0.35ab	1.24±0.31a
S	Sebatel	348±53a	737±200a	1051±104a	828±13a	0.55±0.05a	0.98±0.15ab	1.85±1.02a	0.67±0.14b
	Genotype (G)	ns	ns	*	ns	ns	ns	***	***
	Treatments (T)	***	***	***	***	*	**	***	***
	G × T	ns	ns	**	***	ns	**	**	***

Values are means ± standard deviations (n = 3). Means with the same letter in each column, within treatment are not significantly different between genotypes ($p < 0.05$) (LSD test). Ns – Not significant; *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

The root angle (RA) (degree) varied significantly among genotypes in response to stress and over time (Table 6.4).

After the first stress event, Azeghar significantly widened RA under drought and salt conditions. Cham1 narrowed RA more under drought, and J. Khetifa narrowed RA more under salt conditions. The RA of Sebatel had no significant difference between control and stress conditions. After the second treatment, the RA of Azeghar and Cham1 was not significantly affected by drought and salt stresses compared with control conditions. The RA of J. Khetifa significantly widened under drought stress. Sebatel has shown a wider RA under both stresses compared with the control, but the RA detected under drought was significantly wider than under salt stress. After the third treatment, the RA of J. Khetifa, Sebatel, and Cham1 under both stresses had no significant differences compared with the control. Azeghar had a significantly narrower RA under drought compared to salt and control conditions. After the fourth treatment, the RA of Cham1 and Sebatel had no differences between control and stress conditions. Azeghar had a significantly narrower RA under drought stress. J. Khetifa under both stresses widened RA, but it was significantly widener under salt stress than under drought.

Azeghar and Sebatel root surface area (SA) was not significantly affected after the first drought and salt stress. The SA of J. Khetifa increased significantly only under drought conditions, and the SA of Cham1 significantly decreased only under salt conditions (Table 6.4). After the second treatment, Azeghar increased SA under salt stress, and Cham1

significantly increased SA under drought stress. The SA of J. Khetifa significantly increased under drought and salt treatments at the same level, and the SA of Sebatel had no significant differences between treatments and control. After the third stress event, J. Khetifa and Azeghar had a significant negative effect under drought and salt conditions on the SA. The SA of Cham1 also significantly decreased under both treatments, but salt applications had a stronger effect than drought. The SA of Sebatel significantly decreases only under drought conditions. After the fourth stress event, all genotypes had a significant negative impact of drought and salt on the SA. However, the salt treatment decreased the SA of Cham1 and J. Khetifa much more compared to drought.

Table 6.4. The root angle (RA) (°) and the root surface area (SA) (cm2) after repeated drought and salt stresses (C – control, D – drought, S – salt).

		Root angle				Total root surface area			
T	**Genotype**	**1st**	**2nd**	**3rd**	**4th**	**1st**	**2nd**	**3rd**	**4th**
C	Azeghar	75.4±1.2b	110.1±4.4a	124.8±3.5a	118.4±3.7a	51.7±11.9a	81.4±7.8a	256±33a	297±53b
C	Cham1	107.2±8.3a	105.9±5.9a	114.9±8.1a	120.5±9.2a	54±17.4a	48.5±11.8c	304±28a	309±23b
C	JK	82.3±3.8b	90.1±3.3b	101.8±1.6b	93.3±5.1b	45.8±4.4a	57.7±4.2bc	345±56a	444±37a
C	Sebatel	109.1±12.1a	106.1±2.3a	113.9±8.7a	117.2±1.8a	55.6±8.4a	69.9±11.6ab	264±68a	296±32b
D	Azeghar	102±8.6ab	121.2±9.1ab	108.4±5.3b	111.7±3.7b	53.7±15.7a	83.7±8ab	83±26b	124±23b
D	Cham1	85.5±9.3bc	115.5±9b	121.1±0.6a	125.4±2.7a	68±3.6a	92.2±12ab	122±24a	133±13b
D	JK	88±2.9c	119.9±4.2ab	105.4±4.1b	110.6±2.7b	69.5±9.3a	110.5±5.6a	156±15a	254±32a
D	Sebatel	111.7±7.4a	132.1±4.9a	121.1±1.4a	109.6±6.7b	64.8±2.2a	79.1±27b	124±14a	125±19b
S	Azeghar	99.2±3.4a	117±7.9a	127.7±1.4a	125.4±6.8a	45±9a	115±14.1a	55±33b	71±12b
S	Cham1	93±3.3a	102.9±1.9b	120.9±3.9ab	122.6±5.3a	39.1±8.3a	65.5±0b	52±5b	52±24b
S	JK	67.2±1.8b	95.4±4.3b	104±3.1c	118.8±3.5a	47.1±1.2a	109.1±28.5a	98±22ab	116±26a
S	Sebatel	90.6±7.6a	115.3±2.7a	116.1±11.6b	121.9±6.3a	48.8±6.1a	95.3±20.3ab	154±52a	83±9ab
	Genotype (G)	***	***	***	***	ns	*	**	***
	Treatments (T)	*	***	***	***	***	***	***	***
	G × T	***	*	**	***	ns	*	ns	*

Values are means ± standard deviations (n = 3). Means with the same letter in each column, within treatment are not significantly different between genotypes ($p < 0.05$) (LSD test). Ns – Not significant; *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

The root average diameter (AG) varied significantly among genotypes in response to stress and over time (Table 6.5). The first stress event significantly decreased the AG for Azeghar and Cham1 under both stresses. The AG of Cham1 has shown a stronger negative effect by drought than salt stress. The AG of J. Khetifa and Sebatel was not significantly affected after the first and the second stress treatments, while for Cham1 AG significantly decreased under both stresses. The AG of Azeghar significantly decreased only under drought conditions. After the third stress event, the AG of Azeghar, Sebatel, and Cham1 significantly decreased under both stresses equally, while the AG of J. Khetifa was more affected by salt stress. After the fourth stress event, salt negatively affected the AG of all genotypes more than drought stress, except the AG of J. Khetifa, which was affected by both types of stress equally. The number of root tips (TI) varied significantly in response to drought and salt stresses among genotypes,

and over time (Table 6.5). Though J. Khetifa had the lowest TI under control condition, after the first stress event significantly increased TI only under drought condition. However, the TI of Cham1 and Sebatel decreased significantly only under salt stress. The TI of Azeghar had no significant differences between control and stress conditions. After the second stress event, the response to different stress varied among all genotypes. The TI of J. Khetifa and Cham1 increased significantly under drought and salt stress, but Cham1 increased TI more under drought compared with salt. The TI of Azeghar increased significantly only under drought condition, and the TI of Sebatel had no significant differences between control and stress conditions. After the third stress event, the TI of J. Khetifa and Sebatel had no significant differences between control and stress treatments. However, the TI of Azeghar and Cham1 decreased significantly only under salt stress. After the fourth stress event, the TI of Azeghar had no significant differences between control and stress conditions. The TI of Cham1 decreased similarly under both stresses, and Sebatel decreased significantly only under drought. The TI of J. Khetifa significantly increased only under salt treatment.

Table 6.5. The root average diameter (mm) and the number of root tips (TI) after repeated drought and salt stresses (C – control, D – drought, S – salt).

		Root average diameter				Number of root tips			
T	**Genotype**	**1st**	**2nd**	**3rd**	**4th**	**1st**	**2nd**	**3rd**	**4th**
C	Azeghar	0.57±0.03a	0.39±0.1a	0.82±0.08b	0.74±0.07b	455±27c	1243±287a	1989±217a	2723±850ab
C	Cham1	0.6±0.02a	0.46±0.01a	0.93±0.08b	0.67±0.06b	896±110a	633±94a	2009±312a	3614±423a
C	JK	0.41±0.05b	0.39±0.06a	1.13±0.04a	1.93±0.45a	538±42c	1188±406a	2218±309a	1726±194b
C	Sebatel	0.46±0.03b	0.38±0.05a	0.91±0.01b	0.71±0.09b	733±37b	1326±507a	1704±385a	2870±730a
D	Azeghar	0.43±0.04b	0.3±0.04b	0.47±0.05a	0.44±0.04b	666±231b	2493±913a	1314±329b	2044±106a
D	Cham1	0.4±0.03b	0.37±0.01a	0.48±0.08a	0.53±0.08b	802±39ab	2195±210a	2299±524a	1915±411a
D	JK	0.42±0.06b	0.37±0.04a	0.56±0.03a	0.71±0.05a	973±78a	2897±885a	1970±168ab	2359±509a
D	Sebatel	0.52±0.02a	0.3±0.02b	0.5±0.05a	0.54±0.1b	819±6ab	2116±430a	1799±489ab	1714±560a
S	Azeghar	0.43±0.06a	0.44±0.03a	0.43±0.02a	0.31±0.03b	473±26ab	1510±119b	921±558b	1909±606bc
S	Cham1	0.46±0.03a	0.35±0.01b	0.43±0.02a	0.37±0.03ab	367±74b	1362±21b	866±97b	1139±592c
S	JK	0.46±0.05a	0.39±0.03ab	0.48±0.04a	0.42±0.04a	515±10a	2399±143a	1805±316a	3073±541a
S	Sebatel	0.45±0.01a	0.42±0.03a	0.46±0.11a	0.32±0.03b	592±97a	1480±352b	2492±13a	2580±190ab
	Genotype (G)	*	ns	***	***	**	ns	**	ns
	Treatments (T)	***	**	***	***	***	***	**	**
	G × T	***	*	*	***	***	ns	***	***

Values are means ± standard deviations (n = 3). Means with the same letter in each column, within treatment are not significantly different between genotypes ($p < 0.05$) (LSD test). Ns – Not significant; *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

After the first stress event, the number of root forks (FR) of J. Khetifa and Cham1 increased significantly only under drought conditions, while for Azeghar and Sebatel wasn't registered significant differences between the treatments (Table 6.6). After the second stress event, the FR of J. Khetifa and Azeghar increased similarly both under drought and salt, while Cham1 significantly increased FR only under drought condition. The FR of Sebatel had no significant differences between control and stress conditions. Third drought and salt stress events

significantly reduced the FR for Azeghar, Cham1, and J. Khetifa, however at different levels. The FR of Azeghar decreased similarly under both stresses, while the FR of Cham1 and J. Khetifa decreased significantly more under salt than drought. After the third stress event, the FR of Sebatel still had no significant differences between control and both stress conditions. After the fourth stress event, the FR of Azeghar, Cham1, and Sebatel decreased under the two treatments, while J. Khetifa had no significant differences between control and stress conditions.

After the first stress event, the number of root crossings (CR) of J. Khetifa, Azeghar, and Cham1 significantly increased only under drought, while Sebatel had no significant differences between control and stress conditions (Table 6.6). After the second stress event, the CR of Khetifa and Azeghar significantly increased only under drought, while the CR of Cham1 significantly increased under drought and salt stress. The CR of Sebatel still had no significant differences between control and both stresses. After the third stress event, the CR of Azeghar and Cham1 significantly decreased only under salt stress, while the CR of J. Khetifa and Sebatel had no significant differences between control and stress conditions. After the fourth stress event, the CR of Cham1 and Sebatel significantly decreased under drought and salt, while Azeghar significantly decreased only under salt conditions. The CR of J. Khetifa had no significant differences among the treatments.

Table 6.6. The number of root forks (FR) and the number of root crossings (CR) after repeated drought and salt stresses (C – control, D – drought, S – salt).

		Number of root forks				Number of root crossings			
T	**Genotype**	**1st**	**2nd**	**3rd**	**4th**	**1st**	**2nd**	**3rd**	**4th**
C	Azeghar	2318±472a	4990±914a	12865±2083a	18389±5170ab	225±54b	956±487a	1313±159a	2579±846a
C	Cham1	2090±1290a	2774±542b	13726±1967a	21535±1692a	188±168b	421±162a	1349±265a	3634±649a
C	JK	3027±508a	4651±511a	15185±3056a	12982±2057b	511±165a	942±204a	1355±318a	1081±381b
C	Sebatel	2924±370a	4587±897a	11618±3490a	19550±4570ab	401±52ab	857±136a	1033±381a	2782±962a
D	Azeghar	3026±795b	7069±955b	5757±2285b	9494±2480b	465±175b	1854±470ab	969±459a	1760±554a
D	Cham1	4005±567ab	6204±562b	8520±1651ab	10006±2240b	720±178ab	1111±121b	1451±264a	1832±610a
D	JK	4677±602a	9924±1236a	10519±1930a	15941±3782a	772±154a	2052±564a	1579±353a	2195±669a
D	Sebatel	3350±351ab	6256±2456b	8299±1758ab	9274±1659b	455±62b	1361±639ab	1328±500a	1590±437a
S	Azeghar	2274±468ab	6606±143ab	3332±2065c	5410±1180b	300±79ab	1056±97ab	511±338c	1257±330ab
S	Cham1	1627±200b	3547±242b	3457±598c	3834±2005b	189±11b	694±26b	562±152c	828±416b
S	JK	2706±252a	8180±1987a	6432±1397b	9741±3287a	340±45a	1543±423a	1007±158b	1970±770a
S	Sebatel	2597±551a	6100±2120ab	10827±1684a	6623±1228ab	331±93a	1122±503ab	1934±206a	1505±200ab
	Genotype (G)	**	**	*	ns	**	*	*	ns
	Treatments (T)	***	***	***	***	***	***	*	***
	G × T	ns	ns	*	**	*	ns	**	**

Values are means ± standard deviations (n = 3). Means with the same letter in each column, within treatment are not significantly different between genotypes ($p < 0.05$) (LSD test). Ns – Not significant; *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

The root dry weight (RDW) significantly decreased under drought and salt for all genotypes. Despite significant losses in RDW among all genotypes, at the end of the experiment, J. Khetifa had significantly bigger RDW under control, drought, and salt conditions (Table 6.7).

After the third stress event, the root-shoot ratio (RS) of Azeghar and Cham1 significantly decreased only under salt stress. The RS of J. Khetifa also significantly decreased under salt stress but significantly increased under drought (Table 6.7). The RS of Sebatel had no significant differences between control and stress conditions. After the fourth stress event, the root-shoot ratio (RS) of Azeghar significantly decreased under salt stress but significantly increased under drought. The RS of J. Khetifa and Cham1 significantly increased only under drought stress, while the RS of Sebatel significantly decreased only under salt stress.

Table 6.7. The root dry weight (RDW) (g) and the Root/Shoot ratio (RS) after repeated drought and salt stresses (C – control, D – drought, S – salt).

		Root dry weight		Root/Shoot ratio	
T	**Genotype**	**3rd**	**4th**	**3rd**	**4th**
C	Azeghar	0.53±0.12b	0.82±0.08b	0.25±0.06a	0.11±0.01b
C	Cham1	0.83±0.11a	1.09±0.14b	0.42±0.16a	0.15±0.02b
C	JK	0.86±0.2a	2.1±0.22a	0.33±0.08a	0.28±0.05a
C	Sebatel	0.62±0.15ab	1.09±0.28b	0.3±0.02a	0.15±0.04b
D	Azeghar	0.17±0.06b	0.29±0.11b	0.33±0.05b	0.16±0.02b
D	Cham1	0.26±0.06ab	0.42±0.04b	0.51±0.04a	0.21±0.03b
D	JK	0.3±0.07a	0.98±0.07a	0.48±0.06a	0.42±0.05a
D	Sebatel	0.25±0.04ab	0.34±0.1b	0.38±0.03b	0.16±0.02b
S	Azeghar	0.1±0.06b	0.15±0.02b	0.11±0.04a	0.07±0.01d
S	Cham1	0.11±0.02b	0.12±0.06b	0.15±0.04a	0.12±0.01b
S	JK	0.21±0.06ab	0.32±0.05a	0.2±0.03a	0.21±0.01a
S	Sebatel	0.31±0.11a	0.19±0.04b	0.2±0.09a	0.09±0c
	Genotype (G)	**	***	**	***
	Treatments (T)	***	***	***	***
	G × T	*	***	ns	**

Values are means ± standard deviations (n = 3). Means with the same letter in each column, within treatment are not significantly different between genotypes ($p < 0.05$) (LSD test). Ns – Not significant; *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

6.3.3 Genotypes response to drought and salt

At the early seedling stage after the first drought and salt stress application, root and shoot traits of Sebatel have no significant differences between control and stress conditions, except for the number of root tips (TI), which decreased under salt stress (Table 6.8). In turn, J. Khetifa significantly increased some traits, including RL, RV, SA, TI, FR, and CR, but only under drought conditions.

Moreover, at the early seedling stage, J. Khetifa had a significantly narrower root angle under salt stress. Azeghar, after the first (drought and salt) stress application, widened the RA and decreased AG under both conditions, and increased CR, but only under drought.

Table 6.8. Genotypic response after the first drought and salt application.

Genotype	PH	RV	AG	TI	CR	FR	RL	SA	RA
Azeghar	no	no	∨DS	no	∧D	no	no	no	∧DS
Cham1	∨S	no	∨DS	∨S	∧D	∧D	∧D	∨S	∨D
J. Khetifa	no	∧D	no	∧D	∧D	∧D	∧D	∧D	∨S
Sebatel	no	no	no	∨S	no	no	no	no	no

(no) = no significant differences between stress and control conditions; (∧) = significantly increased; (∨) = significantly decreased; (D) = under drought; (S) = under salt; (DS) = under drought and salt. . Plant Height (PH); Root Volume (RV); Root Average Diameter (AG); Number of Tips (TI); Number of Crossings (CR); Number of Forks (FR); Total Root Length (RL); Root Surface Area (SA); Root Angle (RA).

Cham1 seedlings had both positive and negative effects on most of the traits. Salt stress negatively affected PH, SA, and TI. Under drought conditions, Cham1 significantly narrowed the RA, and increased parameters such as RL, FR, and CR. The AG of Cham1 was reduced by both stresses, but significantly more under drought. After the second stress event, Sebatel had no significant differences between control and stress or all traits except the RA, which widened under drought and salt conditions. J. Khetifa significantly increased some traits under both types of stress, such as RL, RV, SA, NL, TI, and FR (Table 6.9). However, the RA and CR increased only under drought stress. After the second stress event, only the PH of J. Khetifa was affected negatively by drought and salt stress. Azeghar response to the different types of stress was very variable. Traits such as PH, RV, and SA significantly increased only under salt stress, while TI and CR significantly increased only under drought conditions.

Table 6.9. Genotypic response after the second drought and salt application.

Genotype	NL	NT	PH	RV	AG	TI	CR	FR	RL	SA	RA
Azeghar	∧DS	no	∧S	∧S	∨D	∧D	∧D	∧DS	no	∧S	no
Cham1	no	∨DS	∨DS	∧D	∨DS	∧DS	∧DS	∧D	∧DS	∧D	no
J. Khetifa	∧DS	no	∨DS	∧DS	no	∧DS	∧D	∧DS	∧DS	∧DS	∧D
Sebatel	no	no	no	no	no	no	no	no	no	no	∧DS

(no) = no significant differences between stress and control conditions; (∧) = significantly increased; (∨) = significantly decreased; (D) = under drought; (S) = under salt; (DS) = under drought and salt. Number of Leaves (NL); Number of Tillers (NT); Plant Height (PH); Root Volume (RV); Root Average Diameter (AG); Number of Tips (TI); Number of Crossings (CR); Number of Forks (FR); Total Root Length (RL); Root Surface Area (SA); Root Angle (RA).

The NL and FR significantly increased under both stresses, while AG under drought significantly decreased. After the second stress event, Cham1 had the largest number of negatively affected traits compared to other genotypes, including PH, NT, and AG. However, under both types of stresses Cham1 significantly increased RL, TI, and CR, and only under drought significantly increased RV, SA, and FR.

After the third stress event, Sebatel had no significant differences between control and stress conditions for half of the evaluated traits, except for NL, NT, RV, and AG which significantly decreased under drought and salt (Table 6.10).

Table 6.10. Genotypic response after the third drought and salt application.

Genotype	NL	NT	PH	RV	AG	TI	CR	FR	RL	SA	RA	RDW	RS
Azeghar	∨DS	∨DS	∨D	∨DS	∨DS	∨S	∨S	∨DS	∨DS	∨DS	∨D	∨DS	∨S
Cham1	∨DS	∨DS	∨DS	∨DS	∨DS	∨S	∨S	∨DS	∨S	∨DS	no	∨DS	∨S
J. Khetifa	∨DS	∨DS	∨S	∨DS	∨DS	no	no	∨DS	∨S	∨DS	no	∨DS	∧D∨S
Sebatel	∨DS	∨DS	no	∨DS	∨DS	no	no	no	no	∨D	no	∨DS	no

(no) = no significant differences between stress and control conditions; (∧) = significantly increased; (∨) = significantly decreased; (D) = under drought; (S) = under salt; (DS) = under drought and salt. Number of Leaves (NL); Number of Tillers (NT); Plant Height (PH); Root Volume (RV); Root Average Diameter (AG); Number of Tips (TI); Number of Crossings (CR); Number of Forks (FR); Total Root Length (RL); Root Surface Area (SA); Root Angle (RA); Root Dry Weight (RDW); Root/Shoot Ratio (RS).

However, the SA and SDW of Sebatel significantly decreased only under drought conditions. After the third stress event, J. Khetifa had no significant differences between control and stress for traits such as TI, CR, and RA. The PH and RL of J. Khetifa were more sensitive to salt than to drought. Interestingly, the RS of J. Khetifa significantly increased under drought but significantly decreased under salt stress. After the third stress event, Cham1 had no significant differences between control and stress for the RA. All the other traits significantly decreased after both types of stresses, except TI, CR, and RS, which were more sensitive to salt than drought. All traits of Azeghar were significantly affected but varied regarding types of stress. The PH and RA of Azeghar were more sensitive to drought than salt, and TI, CR, and RS were more sensitive to salt than drought.

After the fourth stress event, all traits in all genotypes were significantly affected by both or just one of the stresses. However, J. Khetifa had no significant differences between control and stress conditions for traits such as RL, CR, and FR (Table 6.11). Moreover, under salt, J. Khetifa significantly increased TI. After the fourth stress event, RA and RS varied the most between genotypes in response to stress. The root angle of Cham1 and Sebatel had no statistical differences between control and stress conditions.

Table 6.11. Genotypic response after the fourth drought and salt application.

Genotype	NL	NT	PH	RV	AG	TI	CR	FR	RL	SA	RA	RDW	RS
Azeghar	∨S	∨S	∨DS	∨DS	∨DS	no	∨S	∨DS	∨S	∨DS	∨D	∨DS	∧D∨S
Cham1	∨DS	∨DS	∨DS	∨DS	∨DS	∨DS	∨DS	∨DS	∨DS	∨DS	no	∨DS	∧D
J. Khetifa	∨DS	∨DS	∨D	∨DS	∨DS	∧S	no	no	no	∨DS	∧DS	∨DS	∧D
Sebatel	∨DS	∨DS	∨DS	∨DS	∨DS	∨D	∨DS	∨DS	∨DS	∨DS	no	∨DS	∨S

(no) = no significant differences between stress and control conditions; (∧) = significantly increased; (∨) = significantly decreased; (D) = under drought; (S) = under salt; (DS) = under drought and salt. Number of Leaves (NL); Number of Tillers (NT); Plant Height (PH); Root Volume (RV); Root Average Diameter (AG); Number of Tips (TI); Number of Crossings (CR); Number of Forks (FR); Total Root Length (RL); Root Surface Area (SA); Root Angle (RA); Root Dry Weight (RDW); Root/Shoot Ratio (RS).

However, the RA of J. Khetifa widened significantly under both stresses, and the RA of Azeghar narrowed only under drought. Regarding root shoot ratio (RS), Cham1 and J. Khetifa significantly increased RS under drought and the RS of Sebatel decreased significantly only under salt. Interestingly, the RS of Azeghar significantly increased under drought and significantly decreased under salt stress.

6.3.4 SSR marker analysis

The genetic analysis with the eleven SSR markers has showed a total of 38 alleles across the four genotypes, four SSRs (i.e. gwm234, wmc727, gwm459, and gwm499) identified two loci which were named ‘a’ and ‘b’. The number of alleles per locus ranged from 1 of wmc727b, gwm459b, and gwm499a, to 5 of cfa2257 (Table 6.12). The expected heterozygosis (He), excluding for the three markers with one allele per locus which was 0, ranged from 0.38 of gwm234a and gwm636, to 0.75 of cfa2086, cfa2257, and gwm427. PIC was observed to differ significantly, ranging from 0.43 (gwm234a, gwm636) to 0.86 (cfa2086, cfa2257, gwm427), revealing that these markers have the required properties to be used in diversity studies for root traits. The high Shannon Information Index (I) detected showed values ranging from 0.56 to 1.49, reflecting the heterozygous nature of the genotypes studied for these markers. The percentage of polymorphic bands were respectively 20% for J. Khetifa and Cham1, and 27% for Azeghar and Sebatel (data not shown).

Table 6.12 Genetic diversity based on the 11 SSR markers with 15 different loci.

Locus	Na	I	Ho	He	PIC	Size, bp
cfa2086	4	1.39	0	0.75	0.86	223-277
wms5	3	1.04	0	0.63	0.71	180-190
gwm234a	2	0.56	0	0.38	0.43	214-218
gwm234b	2	0.69	0	0.50	0.57	242-256
wmc727a	2	0.69	1	0.50	0.57	88-96
wmc727b	1	0.00	0	0.00	0.00	231
cfa2257	5	1.49	1	0.75	0.86	104-156
wms205	2	0.69	1	0.50	0.57	157-170
gwm427	4	1.39	0	0.75	0.86	201-256
gwm573.2	4	1.26	1	0.69	0.79	181-240
gwm636	2	0.56	0	0.38	0.43	110-125
gwm459a	2	0.69	0	0.50	0.57	137-153
gwm459b	1	0.00	0	0.00	0.00	292
gwm499a	1	0.00	0	0.00	0.00	91
gwm499b	3	1.04	0	0.63	0.71	147-197

Na—No. of Alleles, I—Shannon's Information Index = -1 * Sum (pi * Ln (pi)), Ho—Observed Heterozygosity, He—Expected Heterozygosity, PIC—polymorphism information content. Different locus of same marker are highlighted as "a" and "b".

Interestingly, all the genotypes have at least on private allele, and the three markers with the highest PIC registered (cfa2086, cfa2257, gwm427) have also the highest number of private alleles. The number of private alleles detected were similar for all the 4 genotypes, although the landraces J Khetifa had the highest number of private alleles.

Table 6.13. Markers private alleles.

Locus	Allele	Frequency	Found in
cfa2086	223	1	Sebatel
cfa2086	269	1	Cham,1
cfa2086	277	1	J. Khetifa
cfa2086	234	1	Azeghar
wms5	185	1	Cham,1
wms5	190	1	J. Khetifa
gwm234	214	1	J. Khetifa
cfa2257	150	0.5	Sebatel
cfa2257	121	1	Cham,1
cfa2257	142	0.5	Azeghar
cfa2257	104	0.5	Azeghar
gwm427	237	1	Sebatel
gwm427	214	1	Cham,1
gwm427	256	1	J. Khetifa
gwm427	201	1	Azeghar
gwm573.2	236	0.5	J. Khetifa
gwm573.2	181	0.5	Azeghar
gwm637	125	1	Sebatel
gwm499	147	1	Sebatel
gwm499	154	1	J. Khetifa

The cluster analysis of Nei's genetic distance (Nei, 1972) has shown J. Khetifa (JK) grouped alone, while the other three genotypes are grouped together (Figure 6.2). This marks the clear differences between landraces and modern varieties which was also clear for the root angle trait, where J Khetifa has shown a significant narrower root angle than other genotypes under all treatments and for each phenological stage.

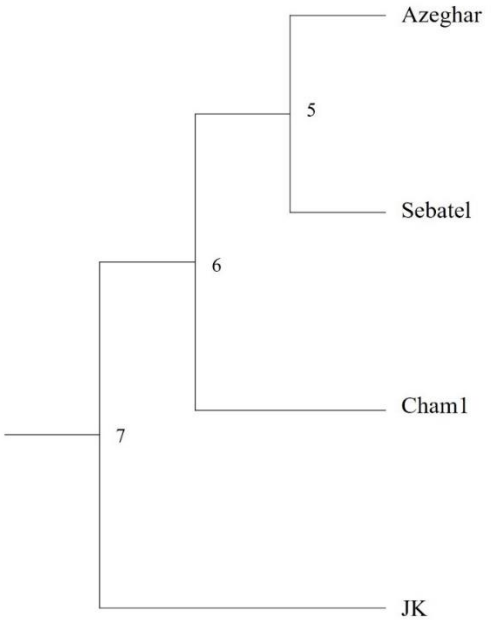

Figure 6.2. UPGMA phenogram of the Nei genetic distance among the four screened genotypes base on the genetic analysis of 11 SSR markers.

6.4 Discussion

Durum wheat production in the Mediterranean environment depends upon rainfall during the growing season, as most precipitation falls in winter. The Mediterranean basin is characterized by high volatility in rainfall distribution; the fluctuation in rainfall during the growing season results in cyclic water availability. Moreover, due to climate changes in this region, the sea level rises, increasing salt wedge and increasing salt concentrations in farmlands near cost, (Corwin, 2021). Salt stress represents a serious threat, it resulted in a negative impact on wheat plants' growth and yield, affecting leaves area, root and shoot biomass, and grain number and size (Talaat and Hanafy, 2022). Root plasticity plays a crucial role in adaptation to abiotic stress, while root diversity among landraces and modern varieties can play a key role in durum wheat breeding programs. Since the understanding of the complex genetic mechanisms behind the stress-escape strategies is a major challenge in breeding program for drought and salt tolerance, accurate phenotyping is the key to develop new useful material (Gilliham et al., 2017). Durum wheat landraces, such as J. Khetifa used in the present study, are an essential group of genetic resources for breeding due to their great genetic diversity, good adaptation to the local environment, and resilience to abiotic and biotic stresses (Nachit et al., 2001; Talaset et al., 2011; Mondini et al., 2012; Mohammadi et al., 2015; Soriano et al., 2016). In addition, Rossellò et al. (2019) showed a

huge variability in the seminal root system among 160 durum wheat landraces from 21 Mediterranean countries. Boudiar et al. (2019) reported the superiority of early drought tolerance of Algerian durum wheat landraces, which are good resources for breeding. In our study, the landrace J. Khetifa after the first application of drought and salt was more sensitive to water deficit, and improved important root parameters, such as total root length (RL), root volume (RV), root surface area (SA), number of root tips (TI) forks (FR), and crossings (CR). In comparison with other genotypes, J. Khetifa had the strongest positive response to stress regarding root traits particularly at early seedling stage. Meanwhile, Cham1 increased RL, CR, and FR, Azeghar only CR, while Sebatel did not respond to any stress. J. Khetifa showed the same significant positive response for RL, RV, SA, TI, NL, and FR after the second stress application too, but under both stresses. After the third stress event, negative effects were observed for all genotypes, but the sensitivity of traits to individual stress differed. Although all genotypes responded differently to both stresses at different development stages during the whole experiment, J. Khetifa had significantly higher NL, NT, RL, RV, SA, AG, RDW, RS, and FR than the other genotypes at the end of the experiment. Therefore, it can be concluded that a positive response at the early seedling stage, and strong early vigour of J. Khetifa helped in adaptation to abiotic stresses in more advanced stages. The root system architecture and its plasticity under stressful environments are complex and controlled by many genes. In addition, root phenotyping is time-consuming and not easy to manage particularly in advanced stages. Therefore, the identification of molecular markers related to QTLs involved in root morphology it's useful for the early marker-assisted selection of genotypes suitable for environments at high risk of drought and salt stress. In the present study 11 root related SSR markers were selected and used to characterize four durum wheat genotypes, and to evidence the differences between one landrace with three modern varieties. The study previously conducted by Urbanavičiūtė et al. (2023) has enhanced the highly association of the SSR marker here used with known QTLs involved in root traits plasticity (Table S2). The private alleles found in the landrace (Table 6.13) may represent a useful resource of molecular material for the selection or breeding for drought and salt affected environments. The selection for cfa2086, wms5 gwm234, gwm427, gwm573, and gwm499 can be useful to detects the introgression in new cultivars or in progeny for the related QTLs for a better adaptation to drought and salt stress. The marker cfa2086 and the marker gwm427 were found strictly associated with root growth angle (RGA) QTLs, respectively QRga.ubo-2A.3 and QRga.ubo-6A.2, which are associated to total root diameter and root angle traits, and co-localized with QTLs related to grain yield and grain size (Maccaferri et al., 2016). The QTLs associated with wms5 are related to the root system architecture (RSA) for traits such as total root length and lateral root length, while the QTLs mtaq-5B.1 and mtaq-5B.2 were found associated with respectively total lateral root length and primary root volume trait for the markers gwm499 and gwm234 (Roselló et al., 2019). The marker gwm573.2 were found associated to the QTL MQTL7B.3 associated with root related traits and yellow pigment content in terms of quality (Soriano et. al., 2021). Moreover, as demonstrated in Urbanavičiūtė et al. (2023), the marker wms5 has given a consistent result, discriminating for the root angle the genotype J. Khetifa which showed a narrower root angle with the private allele of 190 bp. A high

positive correlation between the root angle of plants grown in pots under controlled environments and root angle of plants, at the same phenological stage, grown in the field was already found in Urbanavičiūtė et al. (2022). The markers in this study used for discriminating the root angle are a resource for the selection of genotypes with narrow root angle, hence with improved drought tolerance. Moreover, in Thapa et al. (2017) was found a significant negative effect of salinity on roots parameters of plants grown in field. A major impact, growing with soil depth, was registered for traits such as root surface area, root length, root volume, and root biomass. In terms of marker assisted selection, the alleles of the screened SSR markers could help for the selection of drought and salt tolerant genotypes. However, further studies are needed to state that these markers can be used to transfer stress tolerance alleles from tolerant genotypes to susceptible ones.

6.5 Conclusion

The present study shows how a high capacity of plasticity, with the consequent ability to modify the root architecture from an early stage, is useful, for plants, to better tolerate hydric stress; hence this characteristic is to be considered in breeding programs to develop varieties for drought and/or salty environments. Moreover, such as J. Khetifa and its alleles, the landraces still represent an important resource for the introgression of desirable traits into modern cultivars. Due to the complexity of the genetic regulation of abiotic stress tolerance, MAS is a high-potential tool for the identification of QTLs related to the trait of interest. The combination of high-throughput phenotyping with SSRs screening can provide important highlights to increase the efficiency of stress-related breeding programs.

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Chapter 7. Conclusion

The field experiments highlighted the importance of direct selection in breeding programs under organic conditions, revealing the best adapted genotypes in each location, with high stability and high values of yield and grain quality. The traits screened during the agricultural seasons may be considered as main traits for screening the GxE interaction of durum wheat under organic conditions. Sebatel2-45, particularly suitable for the central Italy environment, was selected and submitted to the Italian Ministry of Agriculture for registration in the national variety list.

The greenhouse experiment with hydric stress has revealed through high-throughput phenotyping the importance of the root angle trait under drought conditions. Azeghar 2-1(56), the genotype with the widest root angle, showed the lowest root biomass, surface area, root length and root volume. On the other hand, Pelsodur, with the narrowest root angle, had higher values of root traits, indicating a good candidate for drought breeding. The greenhouse experiment with salt stress has highlighted, instead, that the root angle is not a good indicator for salt tolerance. The genotype with the narrowest root angle, Pelsodur, had the lowest epigeal biomass, while the genotypes with wider root angle, Sebatel2-45, Cham1, and Azeghar2-1(56) did not show a reduction of epigeal biomass. The shoot and root biomass are good salt tolerance indicators for the capacity of accumulation of toxic ions in tillers and old leaves. The genetic analysis with SSR markers selected in this study, associated with genes involved in root system analysis, was effective in discriminating the genotypes, and can be used to screen new candidate lines with root system plasticity, capable to tolerate drought and salt stress. However, further studies are needed to state the possibility to transfer the stress tolerance alleles associated to the SSR markers from the tolerant genotypes to the susceptible ones. The capacity to modify root architecture and its plasticity from early stages appears as the best characteristic to search for in hydric stress tolerant breeding programs. A variation of the root system architecture of J. Khetifa was found under different environmental conditions, increasing radical biomass under stress conditions. High root system plasticity, that is a high capability to modulate root architecture under different conditions, is a fundamental characteristic for genotype adaptation to stressed environments. This characteristic could be provided with introgression from old landraces, which still represent an important resource for breeding modern cultivars tolerant to abiotic stress and efficient in nutrient up-take also in poor soils.