Mapping and Analysis of QTLs for Tolerance to Salinity in Durum Wheat

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Mapping and Analysis of QTLs for Tolerance to Salinity in Durum Wheat

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Chapter 1: General introduction

1. Domestication of durum wheat

There are two species of wheat: hexaploid bread wheat: *Triticum aestivum* (2n = 6X = 42, AABBDD), and tetraploid, hard or durum-type wheat, *T. durum* (2n = 4X = 28, AABB). Wheat domestication took place 12,000 years ago in the Near East, with the wild ancestor (*T. turgidum* ssp. *dicoccoides*) giving rise to the first domesticated form emmer wheat (*T. turgidum* ssp. *Dicoccum* (Zohary and Hopf, 2000).

Durum wheat (*turgidum durum*) is particularly grown around the Mediterranean Sea and it is the only tetraploid species of wheat that is widely cultivated today. Recently, more than half of the durum wheat is still grown in the Mediterranean basin, mainly in Italy, Spain, France, Greece, West Asian, and North African countries (Maccaferri *et al*., 2003). Durum wheat continued to spread throughout Europe at the end of the 15th century. Thanks to the Columbian Exchange (artificial re-establishment of connections through the commingling of Old and New World plants, animals, and bacteria) durum wheat was spreading throughout America allowed this crop from the old world to the new world (Ren *et al*., 2013). About 2000 years after this event, the spread of agriculture from this region led to the most recent history of durum wheat has been marked by modern genetic improvement, including the substitution of landraces by inbred varieties and the subsequent breeding process for certain desirable characteristics, such as high and stable yields. These historical events are likely to have altered the original genetic structure and genetic diversity pattern of wheat (Ren *et al.*, 2013). In addition, such artificial selection activities may result in significant differentiation at some loci during domestication and the subsequent breeding process, since traits such as grain yield, seed size, plant height, etc., are quantitatively inherited (Peng *et al.*, 2011). These events may also induce the apparition of resistant genotypes to biotic and abiotic stresses especially in those areas where durum wheat was growing.

2. Durum wheat production

World durum production for the 2010/11 marketing year is estimated at 3.0 million metric tons (Fig.1.1), 12 percent lower than 2009/10, largely because of a decline in Canada. A lower price forecast and difficult planting conditions reduced planting areas (USDA, 2010). The decrease in production for 2010-2011 is mainly due to the environmental effects. For instance, durum production in Canada is estimated at 3.0 million tons in 2010/11 compared with 5.4 million tons in 2009/2010. Durum seeded area dropped by 42 percent because of lower anticipated Canadian Wheat Board pool prices during planting and excessive rains at planting time. Flooding and waterlogged conditions in the major durum producing areas affected sowing activities (USDA, 2010). The European Union's (EU) 2010/11 durum wheat production is estimated at 7.9 million tons from 2.7 million hectares. This compares to 8.1 million tons from 2.7 million hectares in 2009/2010 and 8.0 million tons from 2.9 million hectares for the five-year average. The 2010/11 durum crop represents about 6 percent of the total 136.3 million ton EU wheat crop and about 10 percent of its 25.9 million hectares (USDA, 2010). Durum wheat production in Northwest Africa for 2010/11 is estimated at 4.4 million tons from 2.8 million hectares (USDA, 2010). This represents a 25% of reduction in production from 2009/2010 which due basically to unfavorable weather. The biggest reduction in durum wheat in Northwest Africa was in Tunisia, which typically produces about 80% of its wheat as durum wheat. Tunisia's durum wheat production decreased with 50% ranging from 1.4 to 0.7 million tons in 2009/2010 (USDA, 2010). The severe drought in Tunisia significantly reduced its grain production. Morocco's rainfall was excessive from December through March, drastically reducing their grain production.

3. Constraints

In the world, wheat (durum or bread) production and productivity were highly affected by several constraints, among the most important we can cite:

- Drought/heat tolerance

Durum wheat as well as bread wheat, in many regions of the world has been facing water shortages and drought conditions for the last several years due to low rainfall and high temperatures. These conditions affected wheat production both in irrigated and rain-fed areas. To solve this problem it is necessary to breed tolerant varieties through selection and breeding techniques in addition to the relevant use of available irrigation water.

- Diseases

Cereals including wheat are at risk from numerous diseases, especially rusts which induces heavy losses when in epidemic form. For this reason, many efforts were undertaken by breeders in order to develop disease resistant and high yielding varieties.

- Salinity

Salinity is a big constraint to crop production and quality. In the major wheat growing regions of the world, wheat growth, yield and quality are affected by salinity.

4. Salinity problem in the world

4.1 Causes

Primary salinity

Primary salinity is a natural process that affects soils and waters and occurs generally in areas with low rainfall. In these regions, due to high evaporation or transpiration, salinity concentration in the soil will increase (McDowell, 2008). The majority of the globe's saline affected land is influenced by primary salinity caused by natural soil evolution (Hülsebusch *et al*., 2007). Arid tropical areas, in particular, are subjected to potential evaporation that is higher than rainfall, which leads to the rising of water to the topsoil where solutes accumulate

and salinity can occur naturally (Hülsebusch *et al*., 2007). The amount of salinity stored in the soil varies with the soil type, being low for sandy soils and high for soils contain a high percentage of clay minerals. It is also depending on the average of annual rainfall.

Secondary salinity

Secondary salinization results from human activities which affected the hydrological cycle either through the replacement of native vegetation with shallow-rooted vegetation or through the excessive use or inefficient distribution of water in irrigation for agriculture (Beresford *et al*., 2001; Rose, 2004).

4.2 Consequences

Salinity affects many parameters especially the soil structure and plant growth. For instance, when crops are too strongly affected by salinity, surface cover will be affected which will increase the vulnerability of soils to erosion. Further, the suppression of plant growth is caused by different factors such as osmotic potential effect, ion toxicity, which induce nutrient imbalances (Abari *et al*., 2011).

4.3 Solutions

Due to low water quality and the poor drainage systems salinity stress is considered as the greatest problem in many sectors including agriculture. In the world, many areas were affected by salinity (Table1.1) and this constraint is more acute with higher evaporation especially in arid and semi-arid areas where saline soils are widespread and productivity is very low. To minimize these effects many efforts should be provided in order to develop genomic approaches which will be particularly useful in effective engineering of plants for greater salinity tolerance (Cushman and Bohnert, 2000). Enhancing farming and irrigations systems are also important in order to maintain soil quality and avoid its erosion.

5. Mechanisms of growth reduction under salinity stress

Plant growth under salinity stress conditions is regulated by a complex mechanism and the way in which it is affected by the stress is not fully understood. Soil salinity is a big constraint to physiological and metabolic processes in plant life, ultimately reducing growth and yield (Ashraf and Harris, 2004). Halophytes are tolerant to salinity stress compared to glycophytes including wheat. In these plants, salinity induces a significant reduction in their productivity due to specific ion toxicities (e.g. Na^+ and Cl^-) and ionic imbalances acting on biophysical and/or metabolic components of plant growth occurs (Grattan and Grieves, 1999). Increased NaCl concentration has been reported to induce increases in Na and Cl as well as decreases in N, P, Ca, K and Mg level in fennel leading to changes in physiological and metabolic components of plant growth processes (Abd El-Wahab, 2006).

Several mechanisms are responsible for reduction in plant growth under salinity stress such as:

- Osmotic stress
- Ion toxicity
- Nutritional imbalance
- Oxidative stress

5.1 Osmotic stress

According to some researchers, the presence of excessive salt in soil solution reduced the ability of plants to take up water leading to the reduction of plant growth (Munns *et al*., 2006). The primary cause of growth reduction due to the excess of salinity the energy required for growth which is utilized by plants to acquire water from the soil and to make metabolic adjustments. Additionally, water potentials of saline soils are more negative and

water is thus less available for plant uptake due to osmotic forces, even if volumetric soil water contents are as high as field capacity (Oron *et al*., 1999).

5.2 Specific ion toxicity

According to Chinnusamy *et al*. (2005), growth reduction under salinity stress is mainly due to the uptake of certain ions (Na^+ and Cl^-) at supra-optimal level is termed as ion toxicity. Na^+ and Cl^- accumulated in leaves up to toxic levels negatively affect the metabolic processes and delay the growth and development of wheat plants.

Further, salinity alters uptake and absorption rates of all mineral nutrients resulting in deficiency symptoms. Bonilla *et al*. (2004) reported that most toxic effects of NaCl are mainly due to the Na^+ toxicity.

5.3 Nutritional imbalance

Plant growth and development is highly affected by nutritional imbalances. For instance, when the concentration or activity of the essential nutrient element exceeds the optimal range, growth may be inhibited due to either toxicity or to nutrient-induced deficiency. Decreased nitrogen uptake under saline conditions was attributed to interaction between Na^+ and NH_4^+ and/or between Cl^- and NO_3^- (Leidi *et al*., 1991). This reduction of these nutrients leads to decrease the growth and yield of the crop. Many researchers attributed this reduction to Cl^- antagonism of NO_3^- uptake (Bar *et al*., 1997; Feigin, 1990) while others attributed the response to salinity's effect on reduced water uptake (Lea-Cox and Syvertsen, 1993).

Grattan and Grieve (1999) reported that one plant may not exhibit the same response function under saline conditions as it does under non-saline conditions. Indeed, the optimal range may be widened, narrowed, or it may shift in one direction or the other depending on the plant species, the particular nutrient, the salinity level, or environmental conditions. The interaction between salinity and phosphorus (P) is also highly dependent upon many factors

such as plant species, salinity and P concentrations (Grattan and Grieve, 1999). The maintenance of suitable potassic nutrition to support growth of different organs requires a good selectivity, in the aerial organs of K^+ absorption, accumulation and transport compared to Na^+. According to many researchers (Gorham *et al*., 1990; Schactman *et al*., 1991; Yeo *et al*., 1991), a high foliar K^+/Na^+ ratio is a salinity tolerance criterion in halophytes and in some tolerant glycophytes plants.

Moreover, the selectivity of the root system for K^+ over Na^+ must be sufficient to meet the levels of K^+ required for metabolic processes, for the regulation of ion transport, and for osmotic adjustment (Grattan and Grieve, 1999). Cramer *et al*. (1985) reported that the selectivity of K^+/Na^+ is improved by the presence of Ca^{2+}.

5.4 Oxidative Stress

Salinity can induce oxidative stress due to the reduction of CO_2 assimilation. This lead to the accumulation of reactive oxygen species (ROS) which is harmful to plant cells at high concentrations. They cause oxidative damage to membrane lipids, proteins, and nucleic acids (Gómez *et al*., 1999; Hernández *et al*., 2001). Further, the excessive production of ROS under salinity stress caused by impaired electron transport processes in chloroplast and mitochondria as well as pathways such as photorespiration (Fig.1.2). ROS is responsible for the chlorophyll degradation and also for the development of leaf chlorosis and necrosis (Choi *et al*., 2002).

6. Mechanism of tolerance to salinity

Despite the negative effects of salinity on plant functions and metabolisms, many crops could survive in saline conditions. To cope with salinity stress, plants have adapted to several mechanisms (Munns and Tester, 2008):

- The tolerance to osmotic stress

- The Na^+ exclusion from leaf blades
- Tissue tolerance

The tolerance to osmotic stress

The osmotic effect of salinity stress has a strong effect on plant growth and development. In fact, osmotic tolerance involves the plant's ability to tolerate the drought aspect of salinity stress and to maintain leaf expansion and stomatal conductance (Rajendran *et al.*, 2009). According to Munns and Tester (2008), increasing osmotic tolerance in plants is highly related to their ability to continue production and growth of new and greater leaves, and higher stomatal conductance.

The Na^+ exclusion from leaf blades

Salinity can be excluded from entering the plant through its root system, or within the plant, salinity can be restricted from reaching sensitive organs. In the majority of plant species grown under salinity stress, Na^+ appears to reach a toxic concentration before Cl^- does. Therefore, most of researches have concentrated on Na^+ exclusion and the control of Na^+ transport within the plant (Munns and Tester, 2008). Na^+ exclusion by roots ensures that Na^+ does not accumulate to toxic concentrations within leaf blades; however a failure in Na^+ exclusion induces its toxic effect after short or long period, depending on the species, and causes premature death of older leaves (Munns and Tester, 2008).

Tissue tolerance

The third mechanism, tissue tolerance induces an increase of survival of old leaves. It requires compartmentalization of Na^+ and Cl^- at the cellular and intracellular level to avoid toxic concentrations within the cytoplasm, especially in mesophyll cells in the leaf (Munns and Tester, 2008) and synthesis and accumulation of compatible solutes within the cytoplasm. These compatible solutes have a great role in plant osmotic tolerance through different pathways such as protecting enzymes from denaturation, stabilising membrane, macromolecules or maintaining osmotic adjustment (Ashraf and Foolad, 2007).

7. Durum wheat and salinity tolerance

Wheat (*Triticum aestivum*) is a moderately salinity-tolerant crop (Maas and Hoffman, 1977). In the field, where the salinity rises to 100 mM NaCl (about 10 dS m^{-1}), rice (*Oryza sativa*) will die before maturity, while wheat will produce a reduced yield. Even barley (*Hordeum vulgare*), the most-tolerant cereal, dies after extended periods at salinity concentrations higher than 250 mM NaCl (equivalent to 50% seawater). Durum wheat (*Triticum turgidum ssp. durum*) is less salinity tolerant than bread wheat, same as maize (*Zea mays*) and sorghum (*Sorghum bicolor*) (Maas and Hoffman, 1977; USDA-ARS, 2005).

Tolerance to salinity depends on the ability of plants to cope with ion toxicity. For instance, when salinity enters into a plant, the plant responds in two phases. The first phase is a plant response to osmotic stress caused by salinity. In this phase all plants similarly respond to the initial effect of salinity. The second phase is a plant response to ionic stress and the effect is more acute among susceptible species compared to tolerant ones. When ions are accumulated in plant for longer period of time then true difference in salinity tolerance emerges (Munns, 1993). Salinity susceptible genotypes build up ions more rapidly than salinity tolerant genotypes which cause leaf death and eventually plant death (Munns *et al*., 2002).

The response of crops to different phases depends on plant species. For instance, within durum wheat varieties, the osmotic effects of salinity cause rapid and persistent growth inhibition and depression of grain yield and show little genotypic variation (James *et al*., 2002; Munns and James, 2003). In contrast, the ion-specific effects of Na^+ accumulation on growth and leaf senescence start to appear only after several weeks of salinity treatment and show substantial genotypic variation. Therefore, differences in salinity tolerance between durum wheat varieties are generally correlated with Na^+ exclusion from leaves (Husain *et al*., 2003; Munns and James, 2003). Munns (2002) screened 64 modern cultivars and ancient landraces of durum wheat and found that several landraces had leaf blade Na^+ levels

comparable to those of bread wheat, indicating the presence of Na^+ exclusion traits within the A or B genomes. Generally, several efforts have been undertaken in order to improve the salinity tolerance in durum wheat using traditional plant breeding as well as by biotechnological approaches.

8. Breeding for salinity tolerance in durum wheat

Cultivated durum wheat (*Triticum turgidum ssp. durum*) is more sensitive to salinity than bread wheat (*Triticum aestivum*) (Munns and James, 2003), a feature that restricts the production of durum wheat on farms with sodic or saline soils. To increase the salinity tolerance of durum wheat, many researches (Gorham *et al*., 1990; Dvorak *et al*., 1994) have been taken into consideration to improve its sodium exclusion. In durum wheat as well as bread wheat, salinity tolerance is associated with low rates of transport of Na^+ to shoots with high selectivity for K^+ over Na^+ (Gorham *et al*., 1990; Husain *et al*., 2004).

Numerous techniques have been utilized to improve the salinity tolerance of durum wheat. These included screening of international germplasm, field evaluation of selected material, conventional breeding, and wide crosses. The major aim was to study genetic variations for salinity tolerance within wheat and its ancestors and also to develop salinity tolerant wheat cultivars. Hollington (1998) suggested the use of relative yield which allows the comparisons between genotypes. This finding was confirmed by Jafari-Shabestari *et al*. (1995), who screened 400 Iranian wheat on one site in California over two seasons, irrigated with water at three salinity levels (0, 1.5 and 8 dS m^{-1}). They measured final biomass and yield, and calculated a 'stress susceptibility index' that relates to grain yield in saline versus non-saline soils. They found weak correlation between grain yield at high salinity with biomass, harvest index, or stress susceptibility index, and noted that some susceptible varieties had low yield potential. Other researchers such as Kingsbury and Epstein (1984) and Meneguzzo *et al*. (2000) suggested that dry matter can be utilized as selection criteria for

evaluating crop salinity tolerance in controlled conditions. In the same concept, Richards *et al*. (1987) and Kelman and Qualset (1991) suggested that to assess the effectiveness of screening criteria reassessment should be done in saline field conditions for improving crop salinity tolerance. However, Houshmand *et al*. (2005) suggested that *in vitro* screening method compared to the field screening was comparably successful in recognizing salinity-tolerant genotypes in durum wheat. Further, Munns and James (2003) reported that salinity tolerance in durum wheat is highly depending on Na^+ exclusion process.

Genetic analysis in durum wheat showed that the low leaf blade Na^+ phenotype was associated with two dominant alleles of major effect and that these alleles were interactive (epistatic) rather than additive (Munns *et al*., 2003). These are *Nax1* and *Nax2* (Na^+ exclusion loci). Recently, a molecular marker linked to *Nax1* was identified and proven to facilitate the rapid transfer of this trait into commercial varieties of durum wheat (Lindsay *et al*., 2004).

9. QTL mapping and genetic basis for salinity tolerance

QTL mapping has been a key tool to study the genetic inheritance of complex traits in plants (Kearsey, 1998). Most agronomically important traits such as yield, grain quality and resistance/tolerance to biotic and/or abiotic stresses are complex traits. QTL mapping of traits is the best method to understand the genetic basis between important traits. This has potential to facilitate a more efficient improvement of target traits.

In salinity tolerance, many studies were undertaken using segregating population. This traditional method which derived from two homozygous parental genotypes has been the common approach for genetic analysis of salinity tolerance in rice (Koyama *et al*., 2001; Lin *et al*., 2004; Lee *et al*., 2006), wheat (Dubcovsky *et al*., 1996; Genc *et al*., 2010) and barley (Mano and Takeda 1997; Ellis *et al*., 2002; Xue *et al*., 2009; Witzel *et al*., 2010).

Several loci were found to encode members of the *HKT*-family of ion transporters which have a key role in enhancing salinity tolerance such as the *Salinityol* locus (Bonilla *et al*.,

2002) and *SKC1* locus (Ren *et al*., 2005) in rice; *Kna1* locus in bread wheat (Dubcovsky *et al*., 1996), and *Nax1* and *Nax2* in durum wheat (Byrt *et al*., 2007; Munns *et al*., 2012).

Additional studies were undertaken on bread wheat using physiological and agronomic traits. For instance, QTLs for chlorophyll content were mapped on homeologous groups 3 and 7 using solution culture (Ma *et al.*, 2007). Similarly, QTLs for dry matter were mapped on homeologous groups 1 and 3 after assessment of salinity for 25 days at 25 dS m^{-1}(Ma *et al.*, 2007). Quarrie *et al*. (2005) assessed salinity tolerance in the field (8.2 and 16.4 dS m^{-1}) and detected QTLs for grain yield on homologous groups 1, 2, 3, 4, 5, 6 and 7.

Since QTL analysis need a large sample size and can only map those differences that are captured between the initial parental lines, these methods had some limitation. Because these lines are unlikely to contain segregating alleles of large effect at every locus contributing to variation in natural populations, some loci will remain undetected (Miles and Wayne, 2008). Furthermore, biparental QTL mapping detects genomic regions associated with traits with an accuracy ranging on average from 10-30 centimorgans (cM) (Salvi and Tuberosa, 2005; Bernardo, 2008) such chromosomal regions could contain a few hundred up to several thousand genes (Ingvarsson *et al*., 2010). Thus, additional fine-mapping or other methods to improve the mapping accuracy are needed to efficiently exploit the genetic variation for salinity tolerance in durum wheat.

10. Association mapping

Association analysis of germplasm collections was recently performed to discover new useful allelic variation through genome-wide scans and/or to validate the effect of previously discovered QTLs by mapping populations (Flint-Garcia *et al*., 2005; Gupta *et al*., 2005). Compared to the traditional method, association mapping allows for a wider range of variation than analysis using segregating population (Gaut and Long 2003; Remington *et al*., 2001; Skøt *et al*., 2007; Tommasini *et al*., 2007; Genc *et al*., 2010). Genome-wide

association studies are becoming increasingly popular in genetic research, and they are an excellent complement to QTL mapping. This method target to identify markers associated with the phenotypes of interest found in a set of unrelated individuals (Pritchard *et al*., 2000b). Association mapping or linkage disequilibrium approach has recently been introduced in plant genetic research as well (Flint-Garcia *et al*., 2003; Kraakman *et al*., 2006; Cockram *et al*., 2010; Zhao *et al*., 2007; Atwell *et al*., 2010; Kloth *et al*., 2012) and they have been demonstrated to be promising to exploit the full potential of novel molecular marker and sequencing technologies (Zhu *et al*., 2008).

In association mapping, linkage disequilibrium (LD) plays a central role. LD is a population statistic for non-random association between alleles of different polymorphic loci. The decay in LD among neighbouring markers determines the marker density and experimental design needed to perform association mapping successfully. Linkage, selection, mutation and admixture all affect the level of LD. LD also depends on the mating system and therefore varies from species to species as well as between populations within species (Flint-Garcia *et al*., 2003; Rostoks *et al*., 2006). In durum wheat, association mapping has also recently been described for different traits related to biotic and abiotic stress such as the resistance to leaf rust (Maccaferri *et al*., 2010), tolerance to drought (Maccaferri *et al*., 2011), resistance to Fusarium (Ghavami *et al*., 2011). However, no comprehensive study for tolerance to salinity was carried out using association analysis. Using QTL mapping, two Na+ exclusion genes were detected in durum wheat as follows: *Nax1* on chromosome 2AL (Lindsay *et al*., 2004) and *Nax2* on chromosome 5AL (James *et al*., 2006; Byrt *et al*., 2007; Munns *et al*., 2012).

Complementary to biparental QTL mapping, association mapping studies for salinity tolerance in durum wheat would greatly help to simplify the genetic architecture of various traits and help to optimally exploit the genetic variation for crop salinity tolerance improvement.

11. The objectives of this thesis

The research presented in this thesis aims to identify traits and QTLs that underlie salinity tolerance in durum wheat. The objectives are:

1. To determine key traits associated with salinity tolerance and parameters for the assessment of salinity tolerance;
2. To evaluate variation of salinity tolerance in a worldwide durum wheat collections.
3. To identify QTLs associated with salinity tolerance at the seedling and maturity stages.

In Chapter 2 the response of 119 varieties of durum wheat to salinity stress was evaluated at seedling and maturity stages. At seedling stage, ten agronomic traits were recorded and four traits at maturity stage were measured. The wide variation of several traits under salinity stress showed the importance of these traits in salinity tolerance. Genetic correlation was calculated in term to understand the relationship between each trait. This study allowed the choice of key trait as a parameter for the assessment of salinity tolerance in durum wheat.

Chapter 3 describes the evaluation of the variation in salinity tolerance in worldwide durum varieties. An association mapping approach is utilized to identify promising alleles contributing to salinity tolerance to facilitate future breeding for salinity tolerance.

In Chapter 4, genetic analysis of the variation observed in salinity tolerance using Saragolla×Razzak ($F_{2:3}$) mapping population is described. Saragolla and Razzek were chosen as parents based on the differences in salinity tolerance between these cultivars.

The General Discussion in Chapter 5 discusses the findings presented in this thesis in relation to the current status and the prospects of breeding for salinity tolerance in durum wheat. The impact of our results on major issues related to trait discovery strategies for salinity tolerance and salinity tolerance mechanisms in durum wheat and other related crops are addressed.

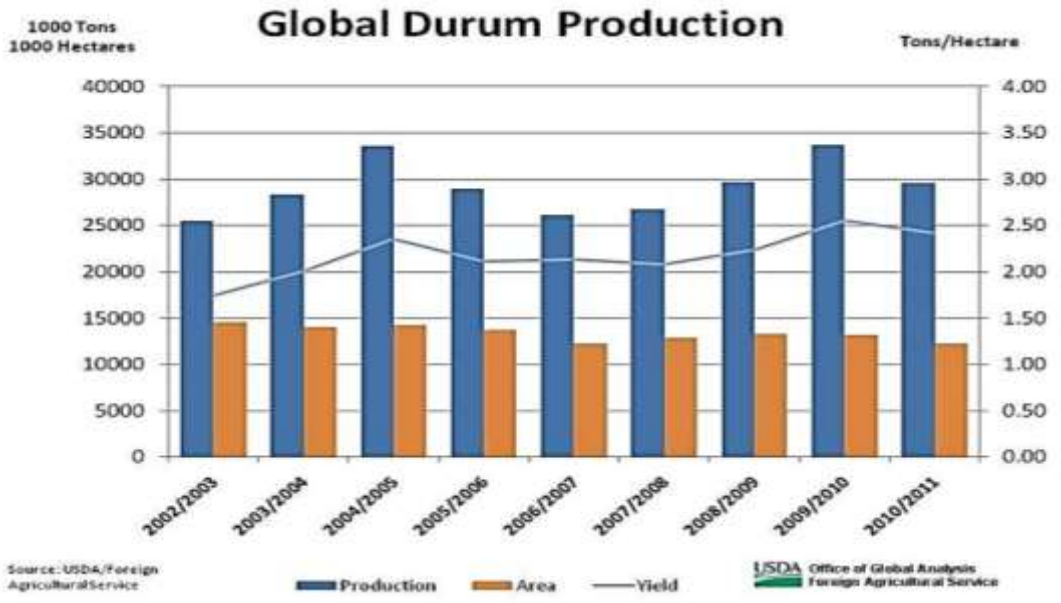


Fig. 1.1 Global durum wheat production (USDA, 2010).

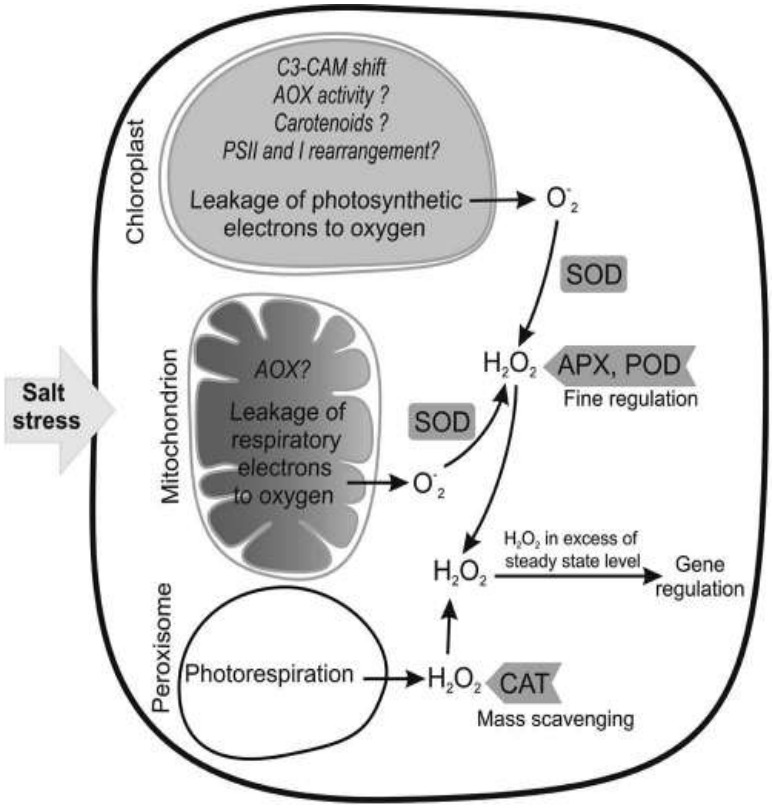


Fig. 1.2 Main intracellular sites of ROS generation under salinity stress (Abogadallah, 2010).

Table 1.1 Salinised areas in the world.

Region	Total area (M ha)
Africa	39
Asia, the Pacific and Australia	195
Europe	7
Latin America	61
Near East	92
North America	5
Total	397

Source: FAO (2008)

Chapter 2: Assessment of salinity tolerance

1. Introduction

Wheat is the most widely grown cereal crop, providing 20% of humanity's dietary energy supply and serving as the main source of protein in developing nations (Braun *et al*., 2010). Salinity stress appears to be one of the constraints for wheat production: more than 800 million hectares of land worldwide, or more than 6% of the world's total land area (FAO, 2008), is salinity affected, severely impairing the agricultural production of many countries. Durum wheat was more sensitive to salinity stress than bread wheat (Munns and James, 2003). Screening wheat for salinity tolerance at the seedling and maturity stages has been performed by a number of different researchers (Akram *et al*., 2002; Khayatnezhad and Gholamine, 2011); however, achieving genetic increases in yield under salinity stress has consistently proven a difficult challenge for plant breeders (Khayatnezhad *et al*., 2010). Moreover, selecting salinity tolerant genotypes is a relatively difficult task, due to the quantitative nature of salinity stress tolerance and the problems associated with developing appropriate and replicable testing environments (Arzani, 2008).

Salinity is defined as a soil condition characterized by a high concentration of soluble salts. Soils are classified as saline when the electrical conductivity (ECe) is 4 dS m^{-1} or more, which is equivalent to approximately 40 mM NaCl and generates an osmotic pressure of approximately 0.2 MPa. This definition of salinity is based on an ECe value which significantly reduces the yield of most crops (USDA-ARS, 2008). Indeed, ionic stress results in premature senescence of older leaves and in toxicity symptoms in mature leaves due to high Na^+ which affects plants by disrupting protein synthesis and interfering with enzyme activity (Munns, 2002). The first response to salinity stress is reduction in the rate of leaf surface expansion, increasing with increased salinity concentration, and leading finally to cessation of expansion (Wang and Nil, 2000). Salinity stress also results in a considerable

decrease in the fresh and dry weights of leaves and stems, and the detrimental effects are observed at the whole-plant level as a decrease in productivity, or finally plant death. The suppression of growth occurs in all plants; however, the tolerance level and rate of growth reduction at lethal salinity concentrations varies widely among different plant species (Parida and Das, 2005).

The present study was conducted with the objective to determine key traits as a parameter for the assessment of salinity tolerance in durum wheat.

2. Materials and methods

2.1 Plant materials

In order to study the effects of salinity stress at the seedling and maturity stages in durum wheat genotypes, two types of experiment were conducted using a randomized complete block design with two replications. In these experiments, a total of 119 varieties of durum wheat (*Triticum durum* L.) from throughout the world were used. These varieties were chosen from 10 different geographic areas (Table 2.1): East Asia, South Asia, the Middle East, North America, South America, Western Europe, Eastern Europe, South Africa, East Africa and North Africa.

2.2 Seedling stage experiment

The seedling stage experiment was conducted using a hydroponic setup. The seeds were sterilized by soaking in a solution of 5% hypochlorite sodium for 5 min. After the treatment, the seeds were washed several times with distilled water, and 10 seeds were placed in each petri dish (9 cm diameter), on moistened filter paper. The petri dishes were placed in a growth chamber at 22°C and 65% relative humidity for 8 days. After this period, selected homogenous seedlings were transplanted to a nutrient solution (a mixture of two solutions). The hydroponic experiment was conducted in a glass house under natural conditions. The

composition of the nutrient solution is shown in Table 2.2. This solution was renewed each week and the pH of the solution was maintained between 6.5 and 7. Ten days after transplantation, NaCl was added to the solution at a concentration of 25 mM, twice a day, until the final salinity concentration reached 100 mM (approximately 10 dS m^{-1}). After thirty days, all plants were harvested and ten parameters were measured as follows: (1) chlorophyll content of the youngest fully expanded leaf blade was measured using a SPAD meter (six measurements per leaf), (2) the number of tillers per plant, (3) the number of leaves per tiller, (4) the proportion of dead leaves was determined by calculating the number of dead leaves divided by the total number of leaves, (5) leaf length, (6) total fresh weight, (7) shoot length, (8) root length, (9) shoot dry weight and (10) root dry weight .The number of tillers, number of total leaves and dead leaves, shoot length and leaf length were measured 25 days after treatment. Other parameters such as root length, shoot fresh weight, shoot dry weight and root dry weight were recorded after harvest. Shoots and roots were weighed after drying in an oven at 70°C for 72 hours. The salinity tolerance index (STI) was calculated for each trait recorded at seedling stage (excepting %DL) using the following formula (1) (Goudarzi and Pakniyat, 2008; Bauci *et al*., 2003; Ahmad *et al*., 2013):

STI = (Value of trait under salinity stress / Value of trait under control) *100 (1)

2.3 Maturity stage experiment

The experiment at the maturity stage was conducted in a vinyl house under natural day-length conditions (13/11h) in 2012 (the average of temperature ranged from 20 to 25°C) at University of Tsukuba in Japan. Six seeds per accession were sown in plastic pots (20 cm diameter * 25 cm tall) and at two-leaf stage; seedlings were thinned to a density of four per pot. Plants were subjected to salinity stress (100 mM NaCl) during boot stage of development (Z41). At maturity stage when grains were ripe (Z92), all plants were harvested. Four parameters were recorded during these experiments as follows: (1) number of fertile

spikes, (2) plant height, (3) biomass production and (4) number of seeds per spike. STI for these traits were calculated using the same formula above. Both experiments used a randomized complete block design with two replicates.

2.4 Statistical analysis

The data for the control and salinity stress was analyzed separately using ANOVA (JMP software). Correlation analyses were conducted using Spearman correlation coefficient (r) to determine the relationship between all traits.

3. Results

3.1 Effect of salinity at seedling stage

Salinity stress induced significant variation in plant growth during the experimental period. After the stress for 30 days, considerable reductions in various agronomic traits were observed in plants grown under 100 mM NaCl conditions.

Salinity stress resulted in significant ($p < 0.05$) decreases in chlorophyll content (SPAD value) (Table 2.3), with the STI for this trait ranging from 17.7% (Durum 852 from Egypt) to 97.4% (Algeria 113-2 from Algeria). The number of tillers per plant was also significantly ($p < 0.05$) affected by 100 mM NaCl (Table 2.3) , with the STI for tiller number ranging from 16.7% (Aktiubinskaja 74 from Kazakhstan) to 100 % (Durum 1P2 from Egypt; Veneny 39/2 from Hungary; Durum 30 from Iraq; Malta 2 from Malta; Saratovskaja 53 from the Russian Federation; Durum 65 from Ukraine; Cuzco from Peru; Durum 6685 from Uzbekistan; Kubanka Karakolskaya from Kyrgystan; Ethiopia 199 from Ethiopia; Ethiopia 258 from Ethiopia; and 87-9-28-2-5D from Pakistan. In addition, increased proportion of dead leaves was observed in all the tested genotypes. The symptoms of leaf necrosis and wilting began to appear in basal leaves after only 4 to 5 days of exposure to salinity. The proportion of dead

leaves ranged from 0 to 100% with an average of 43% (Fig. 2.1). Significant variation in the proportion of dead leaves was observed among different genotypes at 100 mM NaCl. Based on the salinity tolerance/susceptibility scale, the variety 87-10-8-3-5D from Ethiopia was highly susceptible to salinity stress, exhibiting severe symptoms of stress compared to the variety IWA8608450 from Iran, which exhibited extreme tolerance to salinity stress. The number of leaves per tiller was significantly ($p < 0.05$) diminished by salinity stress (Table 2.3), but this trait was comparatively less affected than other agronomic traits. The STI for the number of leaves ranged from 61.5% (IWA8608908 from Iran) to 166.6% (87-10-8-3-5D from Ethiopia). In addition, statistical analysis showed that leaf elongation was significantly ($p < 0.05$) affected by 100 mM NaCl (Table 2.3); the STI for leaf length ranged from 37.5% (for genotype 23: Roumania from Japan) to 88% (for genotype durum 36-6 from Ethiopia). Total fresh weight was highly affected by salinity (Table 2.3), with the STI for total fresh weight ranging from 7.3% (Durum 852 from Egypt) to 99.5% (IWA8608450 from Iran). Shoot length for all genotypes significantly ($p < 0.05$) diminished at 100 mM NaCl (Table 2.3), with the STI for shoot length ranging from 33.5% (87-10-8-3-5D from Ethiopia) to 98.8% (ELS6404-126-3 from Eritrea). At the same time, we noticed a significant reduction in root length within different landraces and improved varieties of durum wheat (Table 2.3), with the STI for root length ranging from 23.1% for the variety 87-10-8-3-5D from Ethiopia, to 100% for the variety Durum 9935 from Lebanon. Both shoot and root dry weight significantly diminished under salinity stress (Table 2.3), and the STI for these traits varied within different genotypes. The STI for the shoot dry weight ranged from 7.2% (87-10-8-3-5D from Ethiopia) to 100% (durum 36-6 from Ethiopia). The STI for the root dry weight ranged from 5.3% (Morocco 204 from Morocco) to 100% (IWA8609455 from Iran). Significantly positive correlations ($p<0.01$) were observed between the shoot dry weight and chlorophyll content, between the shoot dry weight and the number of tillers, between the shoot dry weight and the total fresh weight, between the shoot dry weight and the shoot

length and between finally the shoot dry weight and the root dry weight (Table 2.4). The proportion of dead leaves exhibited a negative correlation with most of the traits, such as chlorophyll content, the number of tillers, total fresh weight, shoot length, root length, shoot dry weight and root dry weight (Table 2.4).

3.2 Effect of salinity on matured plants

At the maturity stage, biomass production, plant height, number of fertile spikes, and number of seeds per spike were significantly ($P < 0.05$) affected by both genotype and salinity treatment (Table 2.3). The STI for biomass exhibited a wide range of variation among genotypes, from 11% (IWA8606401 from Iran) to 99% (Saragolla from Italy). There was a reduction in the number of fertile spikes due to salinity in all genotypes, but the effect was highly pronounced in a large number of genotypes, where we observed 100% of sterility of spikes. The STI for fertile spikes ranged from 0% (Hordeiforme 27 from Kyrgyzstan; Muriciense from Poland; Akmamenka from Hungary; FAO 29917 from Cyprus; Durum 75 from Ukraine; Wakooma from Canada; Durum 9935 from Lebanon; Beloturka from Kyrgyzstan; IWA8608450 from Iran; IWA8606401 from Iran; durum 9848 from Iraq; IWA8610979 from Iran; durum 2725 from India; Razzek from Tunisia; Sculptur from Italy; Karim from Mexico; Nasr from Tunisia; Turkey 32 from Turkey; Morocco 204 from Morocco; Algeria 88 from Algeria; Algeria 113-2 from Algeria; and 87-10-8-3-5D from Ethiopia, to 100% (Ramsey from United States; Saratovskaja 53 from the Russian Federation; MG17978 from Algeria; Malta 2 from Malta; Durum 6905 from Uzbekistan; Cocorit from Mexico; D68-5-18A-1A from Tunisia; D8016 from the US; Criollo from Bolivia; MG17970 from Algeria; Arandani from Bulgaria; ND574 from the US; Durum 2797 from Egypt; MG18260 from Algeria; Durum 36-6 from Ethiopia; Karaki Hamra from Jordan; Giza 56 from Egypt; Palestinka 7 from Ukraine; Morocco 130 from Morocco; Morocco 201 from Morocco; and Ethiopia 258 from Ethiopia. A reduction in the number of

seeds per spike under stress was observed in all the genotypes (Fig. 2.4), with the STI for the number of seeds per spike ranging from 0% (Hordeiforme 27 from Kyrgyzstan; Muriciense from Poland; Akmamenka from Hungary; FAO 29917 from Cyprus; Durum 75 from Ukraine; Wakooma from Canada; Durum 9935 from Lebanon; Beloturka from Kyrgyzstan; IWA8608450 from Iran; IWA8606401 from Iran; durum 9848 from Iraq; IWA8610979 from Iran; durum 2725 from India; Razzek from Tunisia; Sculptur from Italy; Karim from Mexico; Nasr from Tunisia; Turkey 32 from Turkey; Morocco 204 from Morocco; Algeria 88 from Algeria. Algeria 113-2 from Algeria and 87-10-8-3-5D from Ethiopia to 90.6% (Morocco C10895 from Morocco).

There was a positive correlation at the maturity stage between the number of fertile spikes and the number of seeds per spike; and we observed a significant ($p < 0.01$) correlation between biomass production and all the yield parameters: plant height, number of fertile spikes, and number of seeds per spike (Table 2.4). The level of variation in salinity tolerance is reflected by the shifts in distributions for the highly affected traits at seedling and maturity stages (Fig. 2.1).

3.3 Choice of key trait to assess salinity tolerance in durum wheat

A wide variation was observed among traits recorded at seedling and maturity stages, the selection of one trait as a parameter is a good way to simplify the assessment. Two criteria should be provided in this trait as follows: the broader range of variation among varieties under treated conditions and a narrower range of variation within the same variety. The proportion of dead leaves exhibited the narrowest range of variation within the same variety because of the lowest CV recorded between replications (Table 2.5) and the broadest range of variation among varieties under treated conditions (Fig. 2.1, Fig. 2.2). Thus, the proportion

of dead leaves (%DL) is a suitable parameter for assessment of salinity tolerance in durum wheat.

4. Discussion

The current study evaluated 119 varieties of durum wheat under salinity stress. Many traits were used in this study in order to select the suitable parameter for the assessment of salinity tolerance in durum wheat. We also notified a wide variation in salinity tolerance among all varieties. For instance, tolerant varieties have the ability to cope with salinity stress through several mechanisms. In this concept, Munns (2002) reported that different plant species have developed different mechanisms to cope with the effects of salinity. Furthermore, Chen *et al*. (2007) showed in his study on beans that different cultivars showed differential response to salinity stress. In the present study, based on the STI, we observed a wide variation among traits recorded at seedling and maturity stages (Fig. 2.3, Fig. 2.4, and Table 2.6). Moreover, a significant reduction was recorded among all genotypes however; the decrease is more severe within susceptible genotypes compared to tolerant ones. For instance, the chlorophyll content of leaves typically decreased under salinity stress. High accumulation of sodium in plant tissues has been reported as an influential factor in the reduction of photosynthetic pigments and the rate of photosynthesis (Sairam *et al*., 2002; Ashraf, 2004). In addition, the oldest leaves develop chlorosis, and finally fall, as a result of a prolonged period of salinity stress (Hernandez *et al*., 1995; Gadallah, 1999; Agastian *et al*., 2000), which has the effect of decreasing chlorophyll content in leaves. Salinity stress also affected leaf elongation. It has been reported that a common cause of reduction in leaf expansion is a decrease in cell turgor, rather than any salinity-specific effects. However, Ball (1988) indicated that such reduction is not related to a loss in turgor pressure, but is due to the change in hormonal signaling from roots to leaves. In the salinity-sensitive genotypes, where salinity is not effectively excluded from the transpiration stream, salt may accumulate

to toxic levels in the leaves, resulting in the death of old leaves, and injury to new leaves that may become succulent in order to dilute the salts (Munns and James, 2003). Thus, an increase in the proportion of dead leaves was observed among different varieties under salinity stress.

The number of tillers per plant was significantly affected by salinity stress; however, we observed a lesser decrease in the number of tillers in tolerant genotypes than in susceptible genotypes. Our results were in agreement with Goudarzia and Pakniyat (2008), who reported significant reduction in the tillers per plant, of various wheat cultivars, due to salinity. In this regard, El-Hendawy *et al*. (2005) conducted a hydroponic experiment, evaluating 13 wheat genotypes, and found significant reduction in the number of tillers under salinity stress. In addition, salinity stress was found, in the present study, to significantly affect both shoot and root length, and this was in agreement with Jamal *et al*. (2011), who showed that salinity application had an overall substantially negative effect on shoot length and root length. This decrease in root and shoot length might be due to a decrease in the water potential of the rooting medium due to higher ion concentration (Munns *et al*., 1995). Total fresh weigh was significantly affected by salinity; however, we observed that a higher STI for fresh weight was maintained by genotypes IWA8608450 and IWA8609455, in comparison with the salinity-sensitive genotype Durum852. The sizeable inter-genotype variation in this trait showed that total fresh weight may offer a useful criterion for evaluating salinity tolerance at the seedling stage. Such fresh weight reduction has been attributed to the effect of salinity stress in reducing leaf area and durability, which in turn reduces both photosynthesis and dry matter accumulation (Singh *et al*., 1994).

In the present study, salinity stress also resulted in a considerable reduction in both the fresh and dry weights of stems and roots, and these findings were in agreement with numerous studies (Hernandez *et al*., 1995; Ali-Dinar *et al*., 1999; Chartzoulakis and Klapaki, 2000). The STI for shoot dry weight of genotypes Durum 36-6, ELS 6404-124-3 and Karaki

Hamra indicated maximum biomass production at the seedling stage, so these may be considered for cultivation as tolerant landraces in saline areas. Genotypes Durum 18, Durum 852, and 87-10-8-3-5D were clearly salinity-sensitive, because they produced minimum biomass at the seedling stage; and these results were in accordance with Khan *et al*. (1995), who showed that tolerant accessions produced more biomass than sensitive ones, because the former have a lower uptake of Na^+ than the latter (Ashraf *et al*., 2008). Roots, the first organ to develop, are sensitive to increased levels of salinity (Akram *et al*., 2007) and the lesser availability of O_2 under saline conditions deprives plants of their primary energy source, while the accumulation of high levels of ethylene inhibits root growth (Akram *et al*., 2007). The STI for root dry weight suggested that root growth was reduced by salinity stress.

Numerous studies have evaluated salinity tolerance at the maturity stage only in genotypes that showed tolerance at the seedling stage; however, this method is not always reliable, because some genotypes that are susceptible at the seedling stage can be recovered at maturity and produce spikes, and some genotypes that show tolerance at the seedling stage may yet reveal weakness in producing yield at maturity. For this reason, evaluation of salinity tolerance at different stages is important for selecting genotypes that can produce a significant yield in saline areas, since improving the grain yield of wheat is consistently the main target in plant breeding. Therefore, the evaluation of final grain yield and of growth parameters determining grain yield is critical to breeding programs. The final yield of wheat is determined by the number of spikes per plant and various yield components, such as the fertile spikelet number, grain number, and grain weight. In this regard, the STI for various yield-related traits under salinity stress were studied, and a significant reduction in plant height for different varieties exposed to salinity was observed, which is in agreement with Khan *et al*. (1995). The STI for biomass production at the maturity stage was higher in tolerant than in susceptible genotypes. This trait showed a positive correlation with plant height, the number of fertile spikes, and the number of seeds per spike, which indicated its

significant role in salinity tolerance at the maturity stage. Salinity stress tended to shorten the duration of spikelet differentiation, resulting in fewer spikelets per spike, and these results are supported by the findings of several studies (Maas and Grieve, 1990; Grieve *et al*., 1993; Francois *et al*., 1994), which concluded that salinity significantly reduced the number of spikelet primordia on the main spike. In addition, the florets in the basal spikelets appear to be significantly less viable than those in the apical spikelets under saline conditions (Grieve *et al*., 1992). A reduction in floret viability seriously affects the total number of kernels per spike (Francois *et al.*, 1994). Our results are in conformity with the above finding, that the number of seeds per spikelet decreases under salinity stress, and this effect was more pronounced in sensitive than in tolerant genotypes.

In terms of the relation between traits at the seedling and maturity stages, a negative correlation was found between the proportion of dead leaves at the seedling stage, and yield parameters at maturity stage showed that tolerance at the early stage could induce tolerance at the maturity stage for some genotypes; and this hypothesis was in accordance with several studies done on rice, which suggested that screening for salinity tolerance in rice at the seedling stage could well correlate with yield and yield components under saline conditions (Aslam *et al*., 1993; Gregorio *et al*., 1997; Ali *et al*., 2004). This correlation between early and late stage suggest that the choice of one parameter for salinity tolerance is a good way to simplify this study and understand the genetic variation of this character. In this study, the proportion of dead leaves was suggested to be a selective parameter for salinity tolerance because of the broader range of variation among varieties under treated condition and the narrower range of variation within the same variety. This parameter will be used in association analysis (Chapter 3) to identify QTLs for salinity tolerance. STI will be used as additional traits in this study.

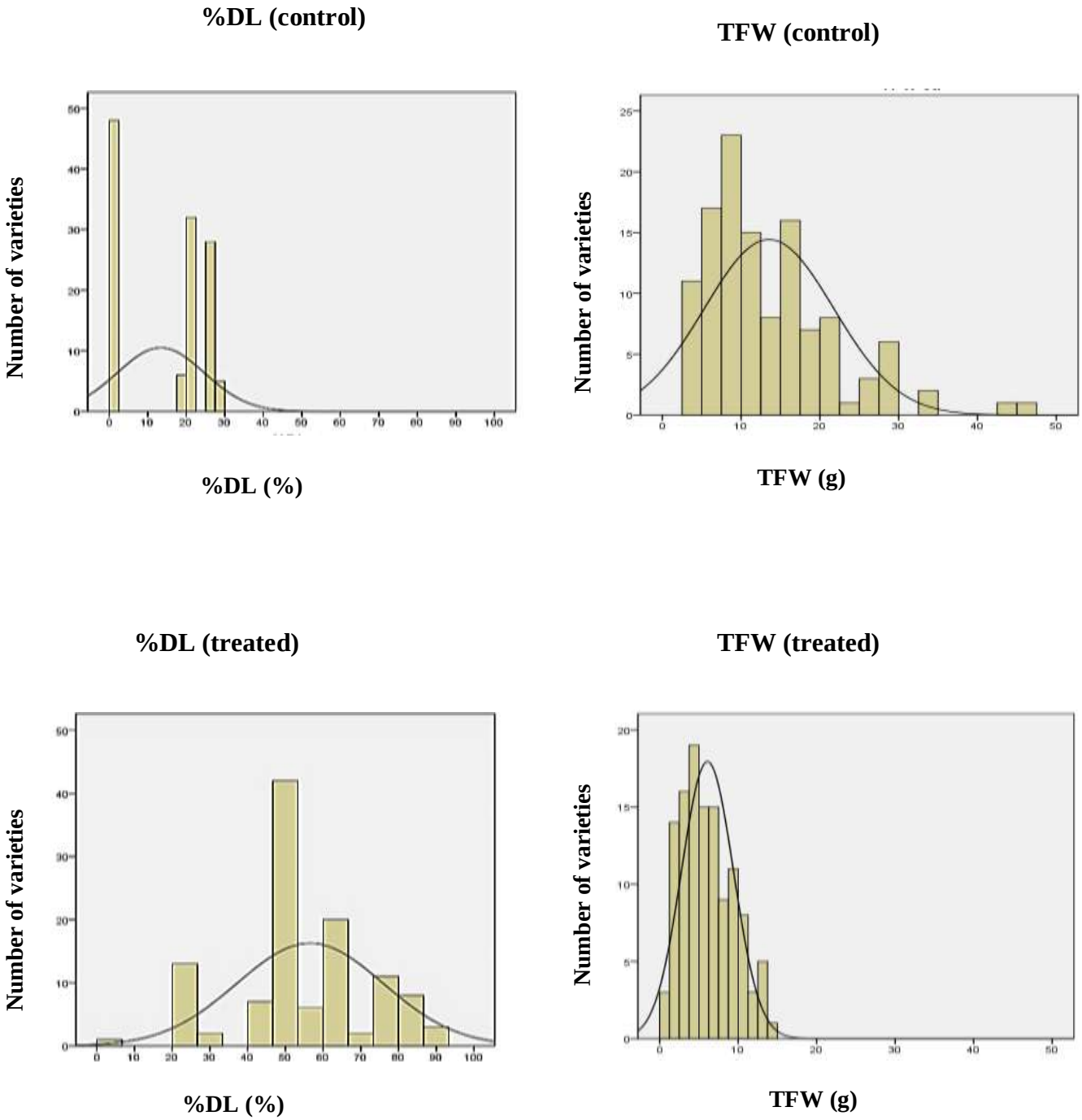


Fig. 2.1 Frequency distribution of traits highly affected by salinity under control and treated conditions.

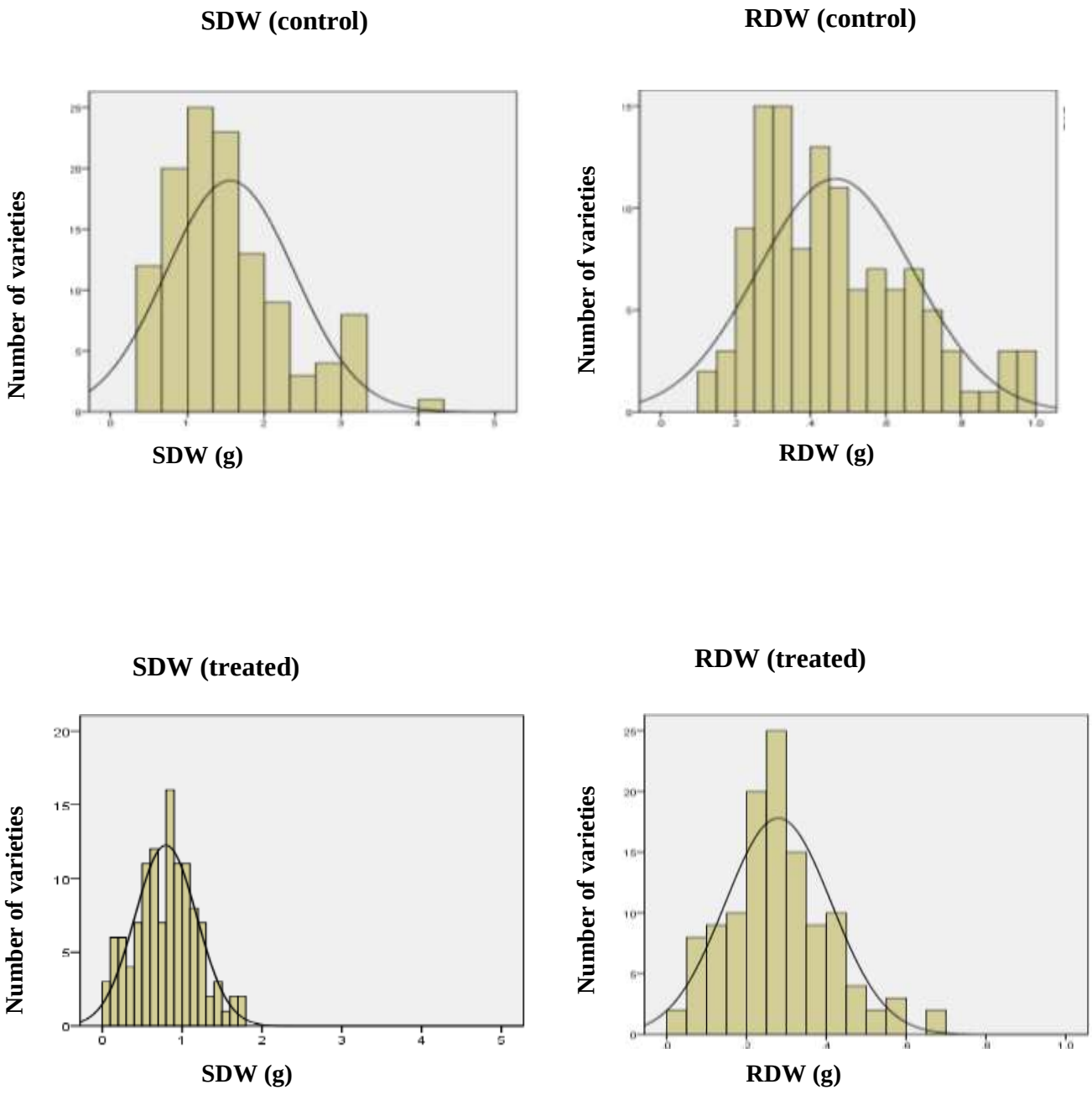


Fig. 2.1 Continued.

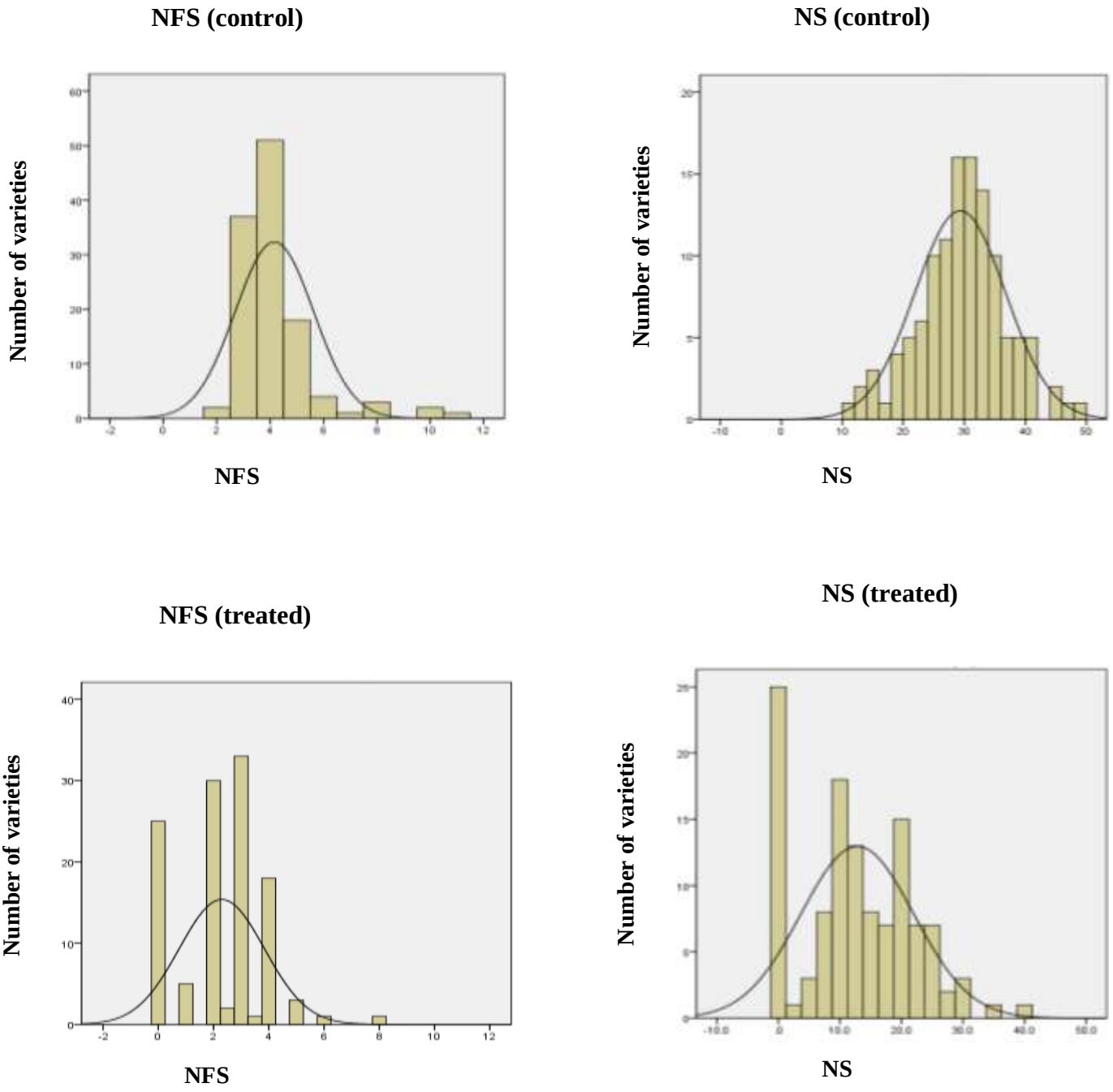
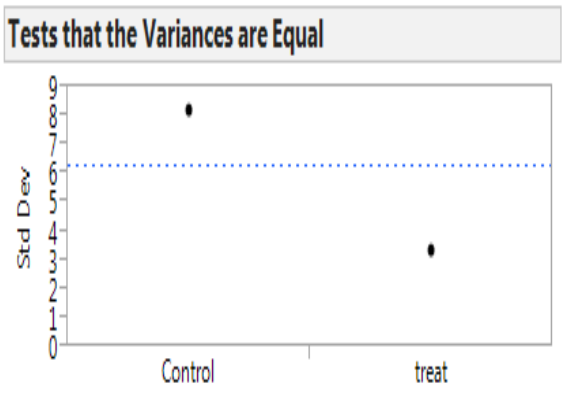
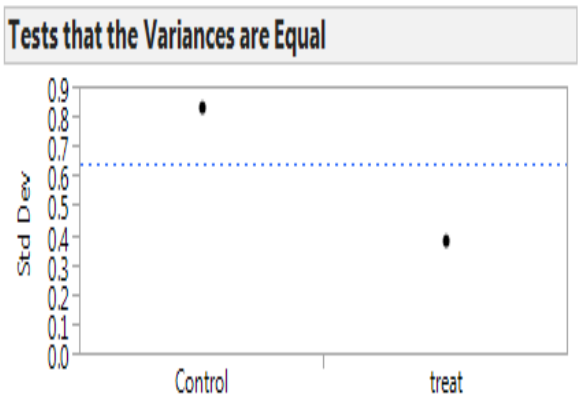


Fig. 2.1 Continued.

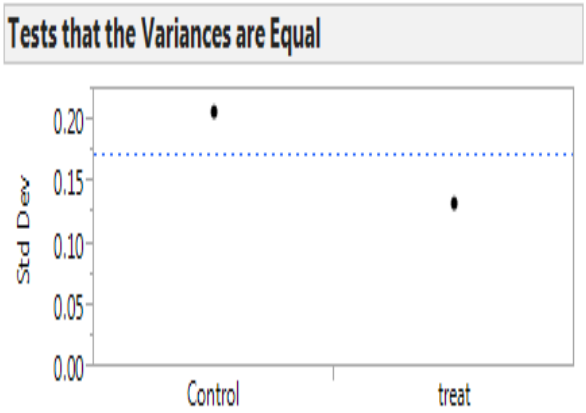
TFW



SDW



RDW



%DL

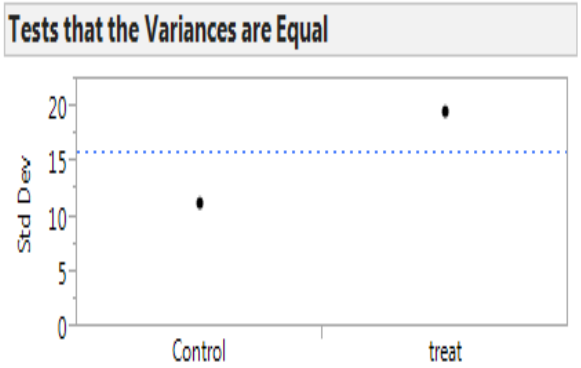


Fig. 2.2 Evaluation of variation under treated and control conditions using equal variance test.

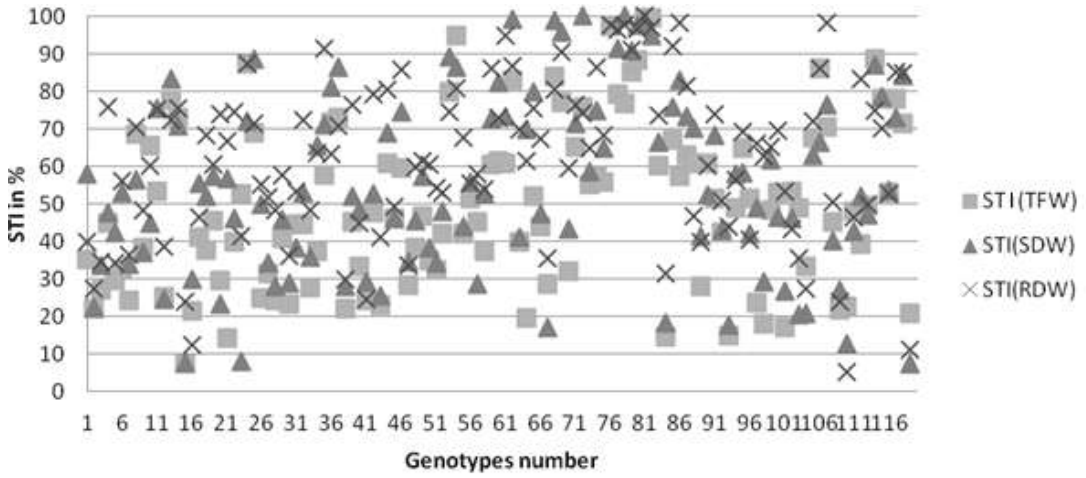


Fig. 2.3 Variation of STI for traits highly affected by salinity at seedling stage.

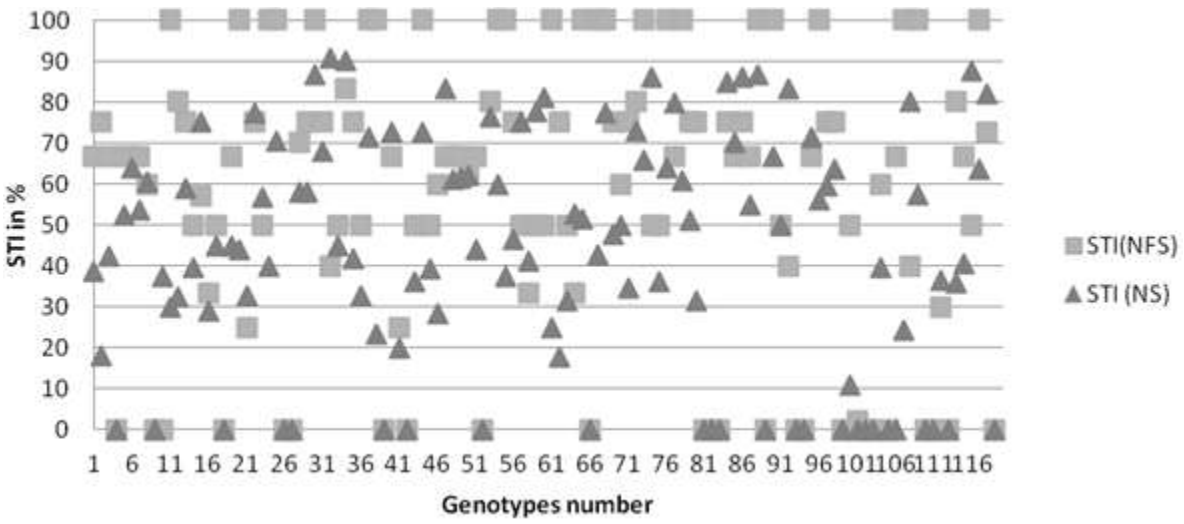


Fig. 2.4 Variation of STI for traits highly affected by salinity at maturity stage.

Table 2.1 Origin and geographical regions of different varieties of durum wheat.

Varieties code	Country	Geographical region of origin	Identifier
V1	Greece	West Europe	Chryssodur
A28	Morocco	North Africa	Morocco 201
V2	Italy	West Europe	Saragolla
A29	Morocco	North Africa	Morocco 204
V7	Tunisia	North Africa	Razzek
V9	Italy	West Europe	Dakter
V10	Italy	West Europe	Sculptur
A40	Algeria	North Africa	Algeria88
A41	Algeria	North Africa	Algeria113-1
V11	Mexico	North America	Karim
A42	Algeria	North Africa	Algeria 113-2
V13	Tunisia	North Africa	Nasr
A20	Italy	West Europe	Produra
A22	Turkey	Middle East	Turkey 32
A24	Turkey	Middle East	Morocco 130
A46	Ethiopia	Middle East	Ethiopia 199
A47	Ethiopia	East Africa	Ethiopia 200
A52	Ethiopia	East Africa	Ethiopia 228
A60	Ethiopia	East Africa	Ethiopia 226
A25	Morocco	North Africa	Morocco 130
A62	Pakistan	South Asia	87-9-28-2-5D
A63	Ethiopia	East Africa	87-10-8-3-5D
A26	Morocco	North Africa	Morocco 131
44	Morocco	North Africa	durum Marrocos
28	Egypt	North Africa	durum 1P2
32	Turkey	Middle East	durum Sensat
63	Hungang	East Asia	durum Veneny 39/2
91	Bosnia and Herzegovina	East Europe	durum 52
69	Chile	South America	durum Candealfen 4
112	Egypt	North Africa	durum 852
131	Iran	Middle East	durum IWA8609089
50	Russian federation	East Europe	durum Hordeiforme 27
26	China	East Asia	durum 2912
129	Iran	South Asia	durum IWA8607861
62	Hungary, pest	East Europe	durum Akmamenka
64	Hungary, pest	East Europe	durum Arnaut de Studina

Table 2.1 Continued.

Varieties code	Country	Geographical region of origin	Identifier
94	Algeria	North Africa	durum MG 17970
20	Uzbekistan	Central Asia	durum 6685
55	Hungary, pest	East Europe	durum I-1-2440
65	Bulgaria	East Europe	durum Arandani
59	Malta	West Europe	durum Malta Yellow
120	Bolivia	South America	durum Criollo
100	US	North America	durum D8016
111	US	North America	durum ND 574
103	Chile	South America	durum Chorlito 'S'
114	Egypt	North Africa	durum 2797
53	Italy	West Europe	durum Duro SG3
46	Cyprus	Middle East	durum Akathiotico Naurotheri
74	Serbia	East Europe	durum T-840
52	Greece	West Europe	durum 374
2	Peru	South America	durum Cuzco
11	Tunisia	North Africa	durum Ajili
13	Pakistan	south asia	durum Type No. 2
8	Eritrea	East africa	durum ELS 6404-126-3
116	Algeria	North Africa	durum MG 18260
138	Iran	Middle East	durum IWA8607524
54	Turkey	Middle East	durum 073/44
105	Mexico	North America	durum D 27676-10M-4Y-1M-OY
86	Mexico	North America	durum Chapala 67
38	Iraq	Middle East	durum 9848
87	Egypt	North Africa	durum Giza 56
88	Montenegro	East Europe	durum 43
41	Bosnia and Herzegovina	East Europe	durum Brkulja
80	Ethiopia	East Africa	durum 36-6
48	Jordan	Middle East	durum Karaki Hamra
123	Ukraine	East Europe	durum Palestinka 7

Table 2.1 Continued.

Varieties code	Country	Geographical region of origin	Identifier
144	Iran	Middle East	durum IWA860845
92	China	East Asia	durum Bian Sui
125	Kazakhstan	East Asia	durum Aktiubinskaja 74
57	Poland	East Europe	durum Provinciale
127	Iran	Middle East	durum IWA8606236
45	Portugal	West Europe	durum Candeal de Grao Escuro
121	Bolivia	South America	durum 111
56	Poland	East Europe	durum Muriciense
40	Saudi Arabia	Middle East	durum 2
109	Tunisia	North Africa	durum RL 7075
4	US	North America	durum Ramsey
14	India	South Asia	durum 18
95	Algeria	North Africa	durum MG 17978
97	Peru	South America	durum Trigo
25	Iraq	Middle East	durum 30
58	Malta	West Europe	durum Malta 2
84	Mexico	North America	durum Cocorit
78	Cyprus	Middle East	durum FAO 29917
67	France	West Europe	durum D 172
17	Ukraine	East Europe	durum 75
23	Japan	East Asia	durum Roumania
12	Canada	North America	durum Wakooma
16	Ukraine	East Europe	durum 65
3	US	North America	durum Kubanka 75-3-15
27	Georgia	North America	durum Cltr 10108

Table 2.1 Continued.

Varieties code	Country	Geographical region of origin	Identifier
39	Lebanon	Middle East	durum 9935
99	Ethiopia	East Africa	durum MG 31497
115	Oman	Middle East	durum Musane
110	Tunisia	North Africa	durum D 68-5-18A-1A
66	Bulgaria	East Europe	durum Katschulka
75	Spain	West Europe	durum Lucana 45
6	Canada	North America	durum Hercules
29	Yemen	Middle East	durum Aden
34	Turkey	Middle East	durum Sert
42	Spain	West Europe	durum Blancal de Nules
22	Japan	East Asia	durum Medea
122	Russian federation	East Europe	durum Saratovskaja 53
7	Eritrea	East Africa	durum ELS 6404-124-2
83	Macedonia	East Europe	durum II/1
36	Turkey	Middle East	durum 3987
19	Kygystan	East Asia	durum Beloturka
51	Kyrgystan	Central Asia	durum Kubanka Karakolskaya
148	Iran	Middle East	durum IWA8610979
96	Algeria	North Africa	durum MG 18026
70	India	South Asia	Unknown
132	Iran	Middle East	durum IWA8609455
128	Iran	Middle East	durum IWA8606401
37	India	South Asia	durum 9725a

Table 2.2 Chemical composition of nutrient solution.

	TN(AN/NN)	P_2O_5	K_2O	MgO	MnO	B_2O_3	CaO (Ca)	Fe	Cu	Zn	Mo
Otsuka 1	10.0	8.0	27.0	4.0	0.10	0.10	-	0.18	0.002	0.006	0.002
Otsuka 2	11.0 (NN)	-	-	-	-	-	23.0 (16.4)	-	-	-	-

TN: Total nitrogen, AN: Ammonia nitrogen, NN: Nitrate nitrogen.

Table 2.3 Analysis of variance (single factor) for different traits recorded at seedling and maturity stages.

Source of Variation	SS	df	MS	P-value
CHL	4576.05	1	4576.05	8.2E-45
NT	188.84	1	188.84	2.3E-17
NL	2.84	1	2.84	0.01376
LL	4534.49	1	4534.49	2.8E-50
TFW	3310.33	1	3310.34	2.2E-17
SL	7976.18	1	7976.19	3.8E-29
RL	6385.69	1	6385.70	4.4E-15
SDW	34.37	1	34.37	6.5E-17
RDW	2.11	1	2.11	4.2E-15
%DL	89659.10	1	89659.10	5.1E-46
NFS	206.14	1	206.14	1.9E-18
PH	20470.38	1	20470.40	1.1E-12
Bio	2672.61	1	2672.62	6.7E-39
NS	16198.87	1	16198.90	5.1E-37

Table 2.4 Phenotypic correlation coefficients (r) between each pair of parameters.

	STI (CHL)	%DL	STI (NL)	STI (LL)	STI (TFW)	STI (SL)	STI (RL)	STI (SDW)	STI (RDW)	STI (NFS)	STI (PH)	STI (BIO)	STI (NS)
%DL	-0.184*												
STI (NL)	-0.172	0.170											
STI (LL)	0.252**	-0.197*	-0.267**										
STI (TFW)	0.243**	-0.437**	-0.162	0.441**									
STI (SL)	0.356**	-0.318**	-0.339**	0.618**	0.493**								
STI (RL)	0.262**	-0.166	-0.245**	0.200*	0.149	0.132							
STI (SDW)	0.288**	-0.566**	-0.216*	0.425**	0.847**	0.491**	0.212*						
STI (RDW)	0.298**	-0.547**	-0.179	0.378**	0.755**	0.321**	0.161	0.826**					
STI (NFS)	0.164	-0.196*	-0.048	0.027	0.197*	0.135	0.057	0.257**	0.186*				
STI (PH)	-0.104	0.227*	-0.054	-0.113	-0.283**	-0.141	0.075	-0.218*	-0.208*	0.164			
STI (BIO)	0.055	-0.062	-0.085	-0.068	-0.090	0.051	0.094	-0.039	-0.011	0.291**	0.285**		
STI (NS)	0.112	-0.187*	-0.007	0.010	0.074	0.143	-0.032	0.187*	0.117	0.706**	0.075	0.290**	
STI (TN)	0.165	-0.340**	0.277**	0.460**	0.329**	0.269**	0.615**	0.555**	0.225*	-0.074	0.16	0.219*	-0.375**

** Correlation is significant at the 0.01 level.

* Correlation is significant at the 0.05 level.

Table 2.5 Coefficient of variation (CV) within variety and level of significance on F test for highly affected traits under control (C) and treated conditions (T).

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Parameter name		CV (%)	F test (2 sides)
Proportion of dead leaves (%DL)	C	1.7	0 **
	T	2.4	
Total fresh weight (TFW)	C	7.1	0**
	T	8.6	
Shoot dry weight (SDW)	C	5.4	0**
	T	8.2	
Root dry weight (RDW)	C	12.06	0**
	T	8.8	
Number of fertile spikes (NFS)	C	10.5	0.73 NS
	C	9.5	
Number of seeds/spike (NS)	C	12.5	0.02 *
	T	10.2	

** Significant at the 0.01 level.
* Significant at the 0.05 level.

Table 2.6 Maximum (Max), minimum (min), mean, standard deviation (SD) and coefficient of variation (CV) within variety for each parameter under control (C) and treated conditions (T).

Parameter name		Max	Min	Mean	SD	CV (%)	STI (%)
Proportion of dead leaves (%)	C	30.0	0.0	15.0	11.0	1.7	43.0*
	T	100.0	0.0	58.0	19.5	2.4	
Number of tillers	C	11.0	3.0	5.6	1.7	6.6	69.6
	T	8.0	1.0	3.9	1.0	6.1	
Number of leaves/tiller	C	7.0	3.0	4.3	0.6	3.5	95.3
	T	6.5	3.0	4.1	0.7	3.8	
Leaf length (cm)	C	35.5	15.5	26.0	3.0	4.5	66.6
	T	24.0	6.5	17.3	8.2	5.6	
Total fresh weight (g)	C	45.1	3.0	13.5	3.3	7.1	45.1
	T	14.4	0.5	6.1	1.0	8.6	
Shoot length (g)	C	69.0	28.2	41.0	5.9	8.1	74.2
	T	57.5	11.7	30.4	4.9	8.9	
Root length (cm)	C	88.5	22.0	48.2	9.4	4.1	82.7
	T	59.5	8.8	39.8	7.5	3.1	
Shoot dry weight (g)	C	5.2	0.3	1.6	0.8	5.4	50.0
	T	1.7	0.0	0.8	0.3	8.2	
Root dry weight (g)	C	1.0	0.1	0.4	0.2	12.06	58.7
	T	0.6	0.0	0.2	0.1	8.8	
Number of fertile spikes	C	11.0	2.0	4.1	1.4	10.5	55.9
	T	8.0	0.0	2.3	1.5	9.5	
Plant height (cm)	C	140.0	50.0	90.4	12.9	3.9	79.4
	T	103.0	43.5	71.8	10.7	3.1	
Biomass production (g)	C	31.2	5.9	13.1	3.2	13.5	64.1
	T	13.6	3.4	8.4	1.1	11.7	
Number of seeds/spike	C	51.0	11.0	29.3	7.4	12.5	43.7
	T	38.0	0.0	12.8	9.1	10.2	
Chlorophyll content (%)	C	35.9	19.1	26.5	3.5	6.5	66.9
	T	27.8	4.5	17.7	3.1	9.5	

*: The increased proportion of dead leaves: calculated based on the difference between treated and control condition.

Chapter 3: Variation in salinity tolerance and association analysis

1. Introduction

Durum wheat *(Triticum durum* L.) is one of the main cultivated species of wheat, and approximately 75% of the durum wheat produced worldwide is consumed in the Mediterranean region (Morancho, 1995; Belaid, 2000). Durum wheat is used to produce several end products such as pasta, couscous (local) and noodles. However, the production of durum wheat is limited by biotic and abiotic stresses such as water scarcity, high temperature, flooding and salinity. Climate changes associated with increased exposure to these abiotic stresses have major effects on crop yields. Salinity is considered to be one of the severe constraints among abiotic stresses (Tuteja, 2007). Approximately 7% of the world's total land area is affected by salinity (Flowers *et al*., 1997). This problem is most severe in arid and semi-arid areas where the evaporation rate is high and this exerts a significant effect on durum wheat production. Increases in soil salinity may have three effects: reducing water potential, causing an ion imbalance and disturbing ion homeostasis, and inducing toxicity (Munns, 1993). This altered water status leads to an initial growth reduction and limits plant productivity (Hagemann and Erdmann, 1997; Hayashi and Murata, 1998). While the growth of all plants is suppressed, the tolerance levels and rates of growth reduction at lethal salinity concentration varied widely among different plant species (Parida and Das, 2005). Bread wheat is more tolerant to salinity than durum wheat (Noori and McNeilly, 2000; Munns and James, 2003), creating a major challenge for breeders to incorporate tolerance genes into high yielding varieties of durum wheat.

Further genomic approaches should be taken into consideration for designing durum wheat ready for climate change with a focus on salinity tolerance. These approaches have

been also undertaken to identify QTL for the tolerance to salinity and incorporate it into the elite varieties. QTLs for tolerance to salinity stress have been detected using segregating population and related methods. Association analysis for germplasm collection was recently performed to discover new useful allelic variation through genome-wide scans and/or to validate the effect of previously discovered QTLs by mapping populations (Flint-Garcia *et al*., 2005; Gupta *et al*., 2005). Compared to the traditional method, association mapping allows for a wider range of variation than analysis using segregating population (Gaut and Long 2003; Remington *et al*., 2001; Skøt *et al*., 2007; Tommasini *et al*., 2007; Genc *et al*., 2010).

A number of QTLs/genes associated with salinity tolerance have already been identified in wheat. Some genes related to Na^+ and K^+ homeostasis were detected in various genomic regions. The *Kna1*, gene for sodium exclusion, was detected on chromosome 4D in bread wheat (Dubcovsky *et al*., 1996). Two genes for sodium exclusion were mapped on chromosomes 2AL (Lindsay *et al*., 2004) and 5AL (James *et al*., 2006; Byrt *et al*., 2007; Munns *et al*., 2012) in durum wheat. Most of QTLs for salinity tolerance in wheat were investigated at either the seedling or maturity growth stage. As salinity tolerance is highly dependent on the plant growth stage, it is important to evaluate tolerance at both stages. Few studies on salinity tolerance have been conducted in durum wheat, and the mapped QTLs/genes are not sufficient to enhance salinity tolerance in this crop. Additional studies are still needed at both growth stages (seedling and maturity) to control the stability of salinity tolerance over the whole life of the plant and then to improve the salinity tolerance among various durum wheat varieties.

This study aimed at evaluating the variation in salinity tolerance among durum varieties and to identify QTLs for salinity tolerance.

2. Materials and methods

2.1 Plant materials and growth conditions

A total of 119 varieties of durum wheat (*Triticum durum* L.) were used in this study (Table 2.1). These varieties were subjected to two different experiments as described in the previous chapters. For instance, seedling stage experiment was conducted in a glasshouse under natural condition using a hydroponic setup.

Seeds were sterilized in a 5% solution of sodium hypochlorite for 5 min. After the treatment, the seeds were washed several times with distilled water. Ten seeds were sown on filter paper moistened in Petri dish (with a 9-cm diameter). The Petri dishes were placed in a growth chamber for 8 days at 22 °C and 65% relative humidity. Four selected homogenous seedlings per treatment were transplanted to a nutrient solution (a mixture of two solutions) with one plant per hole. The nutrient solution was renewed every week, and the pH of the solution was adjusted between 6.5 and 7. Ten days after transplantation, salt was added to the solution at 25 mM twice per day until the final salinity concentration reached to 100 mM (approximately 10 dS m^{-1}). After thirty days, all plants were harvested and ten parameters were measured.The experiment at the maturity stage was conducted in a vinyl house under natural day-length conditions (13/11h) in 2012 (the average of temperature ranged from 20 to 25°C) at Agricultural and Forestry Research Center of the University of Tsukuba in Japan. The soil used in this experiment contains 200 mg/l of N, 1000 mg/l of P and 200 mg/l of K. Six seeds per accession were sown in plastic pots (20 cm diameter * 25 cm tall) and at two-leaf stage; seedlings were thinned to a density of four per pot. Plants were subjected to salinity stress (100 mM NaCl) during boot stage of development (Z41). At maturity stage when grains were ripe (Z92), all plants were harvested. Four parameters were recorded during these experiments. Both experiments used a randomized complete block design with two replicates.

2.2 Evaluation of phenotypes

Phenotypic data described in the previous chapter will be used for association analysis. These involve the proportion of dead leaves (%DL) as a main parameter and additional traits such as chlorophyll content (CHL), the number of tillers per plant (NT), the number of leaves per tiller (NL), leaf length (LL), total fresh weight of shoot and root (TFW), shoot length (SL), root length (RL), shoot dry weight (SDW) and root dry weight (RDW), number of fertile spikes (NFS), plant height (PH), biomass production (BIO) and the number of seeds per spike (NS). The salinity tolerance index (STI) was calculated for all parameters, except for the proportion of dead leaves using the same formula (1) described in chapter 2.

2.3 Genotyping

Out of 196 SSR markers that were screened for 119 varieties, 94 were polymorphic. The following markers were selected from the whole genome and chosen from previous studies: *barc* (Song *et al*., 2002; 2005), *cfa* (Sourdille *et al*., 2003), *cfd* (Guyomarc'h *et al*., 2002), *gdm* (Pestsova *et al*., 2000), *gwm* (Röder *et al*., 1998) and *wmc* (Gupta, 2002).

Modified CTAB was used to extract DNA from leaf samples. The 10 µl polymerase chain reaction (PCR) solutions contained 25 ng of template DNA. The PCR profile was maintained by an initial denaturation at 94°C for 2 min, and then the reaction was subjected to 40 cycles of 94°C for 30 seconds, 1, 3 min at the annealing temperature, and 72°C for 30 seconds, with a final elongation step of 7 min at 72°C. The annealing temperature changed depending on the marker. The amplification products were separated on an 8% acrylamide gel with ethidium bromide in TBE buffer, and visualized under UV illumination.

2.4 Data analyses

The data were subjected to analysis of variance (ANOVA) and other statistical tests performed in JMP software, including the Spearman correlation (r) and the frequency distribution. As an alternative to graphical clustering methods, a model-based Bayesian approach implemented in the software package Structure 2.3.4 (Pritchard *et al.*, 2000a) was used to analyze the population structure of 119 durum wheat varieties. The optimal number of populations (K) was inferred by running an admixture ancestry model with correlated allele frequencies starting from two populations K = 1 to K = 10, with three runs at each K. For each run, 10000 burn-ins were performed followed by 100000 Markov chain Monte Carlo (MCMC) simulations. The number of subpopulations (K) was identified based on the maximum likelihood and delta K (ΔK) values detected based on the rate of change in the log probability of data between successive K values (Evanno *et al*., 2005). Thus, we chose a K value (in this case K=7) and estimated the proportion of the genome in accession i that originated from the j population: q_{ij}. The **Q** matrix with (i, j) elements was incorporated into different models of association mapping for QTL detection. The kinship matrix **K** was calculated using SSR markers and was also used in association mapping.

Genetic diversity was calculated at each locus based on allelic polymorphism information content (PIC), and the PIC values for each SSR marker were estimated by determining the frequency of alleles per locus according to the following formula (2) (Anderson *et al*., 1993): where n is the number of marker alleles for marker i and P_{ij} is the frequency of the j^{th} allele for marker i.

$$PIC = \sum_{I=1}^{n} p^2 ij \qquad (2)$$

The LD among mapped SSR loci was estimated by D and r, where D is the standardized disequilibrium coefficient and r represents the correlation between alleles at two loci. The significance (p values) of the LD for SSR pairs was determined by 1000 permutation tests performed for each pair (Weir 1996). The map position for most of the SSR loci was inferred from Somers *et al*. (2004). Association analysis among the SSR marker alleles was carried out, and the least squares means of 14 agronomic parameters in different stages were determined using the general linear model (GLM) option provided in TASSEL v.4.3.5 software (Bradbury *et al*., 2007). Information about the population structure (i.e., the **Q** matrix) of the selected wheat collection was used to control false positive associations. The *P* value of an SSR marker was used to declare whether it was associated with any agronomic parameter, and R^2 expressed the fraction of the total variance explained by the marker term. Additionally, the mixed linear model (MLM) was run using K and Q+K models. Regression was used to evaluate which model had the lowest error based on expected and observed P values.

3. Results

3.1 Assessment of salinity tolerance and its variation among durum wheat varieties

Salinity stress induced significant differences in plant growth during the experimental period. After a continuous period of stress, considerable decreases were observed in various agronomic parameters. The proportion of dead leaves was significantly increased under salinity compared to control conditions. The mean of the increase proportion of dead leaves (%DL) ranged from 0 to 100%, with an average of 43%. The wide variation in performance under stress conditions among wheat varieties was also revealed by the phenotypic distribution (Fig. 2.1), confirming the importance of this parameter for the

assessment of salinity tolerance. Based on this parameter, all the varieties were divided into three groups: tolerant, moderately tolerant and susceptible (Table 3.1).

STI for additional traits varied within different genotypes (Table 2.6). The mean ranged from 44.9% for TFW to 95.3% for NL. A wide range of variation in the STI was observed within durum wheat varieties at both stages, especially among parameters highly affected by salinity stress. At the seedling stage, the shoot length (SL), root length (RL) and the total number of leaves per tiller (NL) were less affected by salinity stress compared to the other parameters, such as TFW, SDW, RDW, LL, CHL and NT. At the maturity stage, the plant height (PH) showed the lowest reduction under salinity stress by 20.5%, followed by the biomass production (BIO), by 35.8%. The STI of number of fertile spikes varied significantly among durum wheat varieties, ranging from 0 to 100%, with an average of 55.9%. This parameter was also important to evaluate salinity/susceptibility among varieties, as it reflects the ability of the plant to persist after a continuous period of stress. STI (NS) varied widely, ranging from 0 to 90.6% under salinity stress with an average of 43.7%. Only 94 varieties produced seeds, and the other varieties were sterile. The phenotypic correlation coefficient (r) was estimated separately for all parameters.

3.2 Structural analysis and genetic diversity

STRUCTURE gave a maximum population (Pop) number (k) of seven (Fig. 3.1). The assignment of varieties into populations was consistent among the different runs. In this study, the first subpopulation included 23 varieties involving seven improved varieties and 16 landraces originating from Africa and west Europe. The second subpopulation included 21 durum wheat varieties, mostly from Eastern Europe and the Middle East. The third subpopulation comprised 14 varieties from North America and Eastern Europe. The fourth subpopulation included 12 varieties, mostly from the Middle East and Asia. The fifth

subpopulation was composed of 12 varieties, mostly from the Middle East and Europe. The sixth subpopulation included 15 durum wheat varieties, mostly from America and Western Europe. The last subpopulation comprised 22 varieties, mostly from North Africa and the Middle East.

A total of 489 bands (alleles) were detected using 94 SSR markers covering all 14 chromosomes of tetraploid wheat and the genetic diversity of the 119 varieties was characterized. The number of alleles ranged from 2 to 20 and the primer "*wmc*633" detected the highest number of alleles, with an average of 5.14 alleles per locus. The PIC value of each SSR marker can be evaluated based on the number of alleles amplified. This value varied greatly for all the SSR loci tested, ranging from 0.07 for the marker "*wmc118*" to 0.93 for the marker "*wmc633*", with an average of 0.5. Most of the PIC values recorded for the 94 SSR markers were above 0.5, indicating that these markers were informative and useful in this study (Table 3.2).

3.3 Analysis of LD among SSR markers

A total of 4372 pairs generated from 94 polymorphic markers were tested to evaluate their LD (Fig. 3.2). In total, 16.65%, 5.4% and 1% of the intra-chromosomal marker pairs exhibited LD at $p<0.01$, $p<0.001$, and $p<0.0001$, respectively. R^2 ranged from 0 to 0.68 for all pairs, with an average value of 0.0267.

3.4 Association analyses of quantitative parameters

Marker-parameter association was tested using GLM (General Linear Model) and MLM (Mixed Linear Model) models. Based on the -Log10 (*P*) value, 2.1 was set as a threshold for significance for all studied models.

3.5 Detection of QTLs by the GLM model

Two GLM models were used: the Naive model and the Q model. For instance, the Naïve model identified 35 SSR loci associated with 12 parameters, with $-Log_{10}$ (P) values ranging from 2.1 to 3.6 and explaining from 11-20% of the phenotypic variation. The second model (Q model) was useful in reducing type I error due to the population structure. This Q model identified 14 SSR loci associated with 8 parameters with –Log10 (P) values ranging from 2.1 to 3.5 and explained from 10-18% of the phenotypic variation. More than one QTL was detected for most of the parameters. Indeed, six SSR loci were associated with the STI of root length, and the strongest association was recorded by the marker *gwm403* on the chromosome 3A. This marker was also associated with the STI of two more parameters: the number of leaves with a –Log10 (P) of 3.04 and the root dry weight, with a –Log10 (P) of 2.51.

3.6 Detection of QTLs by the MLM model

The MLM model comprised two different models: the K model, which reduces Type II errors by considering familial relatedness, and the Q+K model, which considers both population structure and relatedness. We found that the K model detected almost the same loci detected by the Q+K model. Based on these results, these two models were used here to predict QTLs.

The MLM model identified 12 SSR loci associated with eight parameters with –Log10 (P) values ranging from 2.1 to 3.00 and explained from 11-20% of the phenotypic variation. These QTLs were as follows: a single QTL for %DL was mapped on chromosome 4B and explained 13% of the phenotypic variation. A single QTL for STI (CHL) was mapped on chromosome 3A and explained 12% of the phenotypic variation. Three QTLs for STI (NL) was mapped on chromosome 3A, 5A and 5B and explained

17%, 15% and 13% of the phenotypic variation respectively. A single QTL for STI (LL) was mapped on chromosome 5A and explained 19% of the phenotypic variation. Moreover, a single QTL for STI (NT) was mapped on chromosome 7A and explained 16% of the phenotypic variation. Three QTLs for STI (RL) were detected as the follows: one major QTL (-Log10 (P) =3.0) was detected on chromosome 3A and explained 20% of the phenotypic variation. The second was mapped on chromosome 6A and explained 17% of the phenotypic variation, while the third locus (*gwm*540) was detected on chromosome 5B and explained 15% of the phenotypic variation. A single QTL for STI (SL) was mapped on chromosome 7A and explained 15% of the phenotypic variation. A single QTL for STI (PH) was identified on chromosome 7A and explained 18% of the phenotypic variation (Table 3.3, Fig. 3.3).

3.7 Comparison between GLM and MLM

To evaluate each model regarding the control over statistical errors, we plotted the observed P values (x) against the expected P values (y), as described by Stich *et al*. (2008). The Naïve model showed the highest deviation from y=x, follow by Q model (Fig. 3.4). The results showed that K and Q+K model were more consistent in their predictions for all traits. In comparison with the Naïve and Q models of association analysis, the K and Q+K models detected the same loci, showing that these models had strong control over statistical errors. Because of this result, these two models were used in this study to predict QTLs.

4. Discussion

The effects of salinity on other cereals have been studied at different developmental stages. For example, rice was treated with salinity at the early tillering, late tillering and

heading stages (Pearson and Bernstein, 1959), and sorghum was treated during the vegetative, reproduction and maturation periods (Maas *et al.*, 1986; Azhar and McNeilly, 1989) to determine the inheritance of salinity tolerance. In wheat, numerous studies have evaluated salinity tolerance in either the seedling or maturity stages, but this method is not always reliable because some genotypes that exhibit tolerance at the early stage may be unable to grow under continuous stress and produce yield. It is important to evaluate salinity tolerance at different stages to select genotypes that can produce significant yield in saline areas, as improving the grain yield of wheat is the main goal of breeding. Therefore, this study demonstrated the variation of different parameters recorded at the seedling and maturity stages among durum wheat varieties under salinity stress.

At seedling stage, salinity had a strong effect on the reduction of fresh and dry weights. However, tolerant varieties produced more biomass than susceptible varieties. Our results for these parameters were supported by Singh *et al*. (1994), who reported that a reduction in biomass weight has been attributed to the effects of salinity stress in reducing leaf area, durability and then photosynthesis and dry matter accumulation, which in turn reduces grain yield.

At maturity stage, we observed more than 56% decreases in NFS and NS under salinity stress in comparison with the control conditions. This decrease in yield-related parameters demonstrated the sensitivity of durum wheat to this abiotic stress. In bread wheat, grain yield is reduced to 50% of its potential when the electrical conductivity of the soil saturation extract (EC) reaches to 13 dS/m (Ayers and Westcot, 1985). These results are comparable to our findings because bread wheat has a higher resistance to salinity than durum wheat (Munns and James, 2003). Thus, breeding for abiotic stress has become even more important to improve salinity tolerance among durum wheat varieties.

Historically, low Na^+ concentration has been a surrogate for salinity tolerance (Schachtman *et al*., 1992; Ashraf and O'Leary 1996; Rashid *et al*., 1999; Munns and James, 2003; Poustini and Siosemardeh, 2004). However, previous studies reported a correlation between Na^+ concentration and many others agronomic parameters (Ashraf and O'Leary, 1996; Genc *et al*., 2010). In this study, the variation of the %DL within durum wheat varieties is highly related with their ability to prevent salinity toxicity in the leaves. For instance, the cause of the injury is probably due to the accumulation of salts (Na^+ and Cl^-) which overcomes the toxic concentrations. Thus, the old leaves die (usually old expanded leaves) and the young leaves, no more supported by the export of photosynthates, undergo a reduction of growth and new leaves production (Munns and Tester, 2008). Therefore, tolerant plants could cope with salinity stress and prevent salinity from reaching toxic levels in the transpiring leaves by producing photosynthetically active leaves.

Similarly, the wide range of STI (CHL) recorded in this study showed the importance of this parameter in salinity tolerance. Indeed, tolerant varieties exhibited lower reduction of chlorophyll content compared to susceptible ones which confirm their ability to cope with sodium toxicity. This finding was reported by several researchers (Davenport *et al*., 2005) who noted that Na^+ accumulation in photosynthetic tissues affects photosynthetic components such as enzymes, chlorophylls, and carotenoids. In the same concept, Tester and Davenport (2003) reported that salinity stress is associated with a reduction in chlorophyll content and inhibits photosynthesis, inducing leaf senescence and premature leaf death.

Additionally, the contribution of several others parameters to salinity tolerance reflect their importance as useful selection criteria at either seedling or maturity stages. While different parameters were used to assess salinity tolerance at the seedling and maturity

growth stages, some parameters recorded at the seedling stage were correlated with yield-related parameters. The proportion of dead leaves was suggested to be one of these parameters because of its correlation with many related parameters including yield parameters. This parameter exhibited a broader range of variation among varieties and narrower range of variation within variety compared to other traits (Table 2.5, Fig.2.1 and Fig.2.2). Therefore, this parameter is important to assess salinity/susceptibility tolerance and could be selected as a single selection criterion for breeding for salinity tolerance among durum wheat varieties.

LD-based genetic association studies are one approach to map genes with modest effects (Hirschhorn and Daly 2005). Durum wheat is a self-pollinating crop and is expected to have high levels of LD and homozygosity, both of which facilitate LD mapping (Nordborg *et al*., 2002). Variations in LD levels were observed across chromosomes or genomes. The distribution of the LD across these chromosomes may significantly affect the power of association mapping and the effectiveness of marker-assisted breeding. According to Yu *et al*. (2013), a high level of LD in many chromosomal regions of the population suggests that association mapping can be an effective method for QTL identification and validation in these regions. In this study, variations in LD levels were observed across chromosomes and genomes. Somers *et al*. (2007) reported similar results indicating the variation of LD across chromosomes, and suggested that the changes in LD along chromosomes indicate genome areas under selective pressure.

The results indicated that LD extended up to 41 cM. Previous studies of wheat suggested different LD levels, with Crossa *et al*. (2007) reporting an LD block extending even up to 87. Further, we noted that 16.65% of pairs showed LD at $p<0.01$. Remington *et al*. (2001) calculated the LD among 47 SSR loci distributed across the maize genome and found considerably lower levels of LD (9.7% of SSR pairs showed LD at $p<0.01$ in a

collection of 102 lines).The mean R^2 of all interchromosomal pairs in our study is 0.026. This result was comparable to that of Breseghello and Sorrells (2006) who used 149 cultivars of wheat and found pairwise estimates of R^2 varying from 0.000 to 0.133, with a median of 0.022. Stich *et al*. (2006) reported that LD generated by selection, population structure, relatedness, and genetic drift might be theoretically useful for association mapping in specific situations and population groups as it reduces the number of markers needed for association mapping, but careful attention is required to control factors that affect LD (e.g., population structure and relatedness) to enable unbiased population-based association mapping in plants (Liu and Muse, 2005; Pritchard *et al*., 2000b). Therefore, the MLM model was suitable for these analyses, as it controls most of the factors that can affect the LD and performed well in detecting QTLs with minor errors. Several QTLs associated with salinity tolerance were detected on chromosomes 3A, 4B, 5A, 5B, 6A and 7A. Four QTLs for STI (NL) (2 QTLs), STI (LL) and STI (RL) were detected on chromosomes 5A and 5B. This finding was also in accordance with the report by Koebner *et al*. (1996), who found that the homologous chromosome 5 carries loci involved in the response to salinity stress in hydroponic conditions according to the analysis of wheat cytogenetic stocks. Moreover, QTLs for salinity tolerance were detected for various parameters. A single locus for %DL was detected on chromosome 4B and explained 13% of the phenotypic variation as Genc *et al*. (2010) detected two QTLs for tiller number and seedling biomass on the same chromosome. Three QTLs for STI (NL) were detected on chromosome 3A, 5A and 5B explaining 17%, 15% and 13% of the phenotypic variation respectively. A QTL for STI (NL) was overlapped with the STI (CHL) on the same chromosome (3A) which reflects the contribution of these parameters to the osmotic tolerance by producing new leaves photosynthetically active. The osmotic effect resulting from salinity may cause disturbances in the water balance of the plant and inhibiting

growth as well as provoking stomatal closure and reducing photosynthesis (Hernandez and Almansa, 2002). For this reason increased osmotic tolerance involves an increased ability to continue production and growth of new and greater leaves. Three QTLs for STI (RL) was detected on chromosomes 3A, 5B and 6A explaining 20%, 15% and 17% of the phenotypic variation respectively. Xu *et al*. (2013) reported 2 QTLs for RL in bread wheat on chromosomes 2A and 6A. A single QTL for STI (LL) was detected on chromosome 5A explained 19% of the phenotypic variation. Garcia-Suarez *et al*. (2010) detected a single QTL for leaf length on chromosome 1A. The QTL for STI (LL) detected in this study was overlapped with one QTL for the STI of the number of leaves. Lauchli and Grattan (2007) reported that growth inhibition of leaves is a consequence of inhibition by Na^+ of symplastic xylem loading of Ca^{2+} in the root and the final leaf size depends on both cell division and cell elongation.

The accumulation of the QTLs on chromosome 5A reflected the importance of these genomic regions for salinity tolerance. This chromosome was previously known to be associated with Na^+ exclusion gene *Nax2* (5AL) identified in durum wheat (James *et al*., 2006). *Nax2* confers a reduced rate of transport of Na^+ from root to shoot and has a higher rate of K^+ transport, thus resulting in enhanced K^+ versus Na+ discrimination (known as K^+/Na^+ ratio) in the leaf (James *et al*., 2006). The presence of QTLs for several parameters such as NL and LL near the gene for Na^+ exclusion may reflects the contribution of these parameters to salinity tolerance through sodium exclusion process. Sodium exclusion is one of the essential mechanisms of tolerance involves the ability to reduce the ionic stress on the plant by minimizing the amount of Na^+ that accumulates in the cytosol of cells, particularly those in the transpiring leaves (Munns and Tester, 2008). Na^+ exclusion by roots ensures that Na^+ does not accumulate to toxic concentrations within leaf blades. Thus plants could assimilate calcium to maintain leaf length and production.

In our study, a single QTL for STI (CHL) was detected on chromosome 3A and explained 12% of the phenotypic variation. This agrees with the report by Ma *et al*. (2007), who also found a QTL for chlorophyll content on chromosome 3A. A single locus for STI (NT) was detected on chromosome 7A and contributed with 16% of total the phenotypic variation. Dıaz *et al*. (2011) reported 2 QTLs for NT on chromosomes 2A and 5A. Furthermore, a single QTL for STI (SL) was mapped on chromosome 7A and explained 15% of the phenotypic variation. QTLs for this parameter was reported by different studies such as Xu *et al*. (2013) who mapped one QTL on chromosomes 6A which explained 13% of total the phenotypic variation. The QTL for STI (SL) mapped in this study was overlapped with the QTL for STI (NT) on chromosome 7A.

In this study, no QTLs were detected for STI (TFW), STI (SDW), STI (RDW), STI (NFS), STI (BIO) and STI (NS) above the threshold value of –Log10 (P), and decreasing this value to 2 only resulted in one QTL for STI (NFS). The remaining parameters (STI (TFW), STI (SDW), STI (RDW), STI (BIO) and STI (NS)) were critical for the evaluation of salinity tolerance because of their strong relationship with yield. The absence of these QTLs may be due to the low density of markers and presence of high environmental variation of these parameters. Among maturity stage related-parameters, only a single QTL for STI (PH) were detected. This QTL was located on chromosomes 7A and explain 18% of the phenotypic variation. QTLs for plant height was reported by Diaz *et al*. (2011) on chromosome 5A and 7A, explaining 9% and 12% of the phenotypic variation respectively.

This study demonstrated the importance of several parameters for salinity tolerance through different processes. For instance, some QTLs mapped on chromosome 5A may be associated with sodium exclusion process. Indeed, QTLs for STI (NL) and STI (LL) detected on chromosome 5A were distant from those mapped for *VRN-1A* (Kato *et al*.,

2000). Thus, increasing the number of leaves and leaf length under salinity stress may not be associated with *VRN-1A* locus. In the same concept, Genc *et al*. (2010) mapped 5 QTLs for tiller number, seedling biomass, chlorophyll content, maturity and K^+ concentration on the same position of VRN-1A locus. Similarly, QTLs for STI (RL) and STI (NL) were mapped on chromosome 5B, these latter were also distant from the locus *VRN-1B* (Fu *et al*., 2005). Therefore, increasing the number of leaves and the root length under salinity stress may not be associated with *VRN-1B* locus.

The location of a novel QTL for %DL on chromosome 4B was matching those reported previously (Genc *et al*., 2010) for other parameters under salinity stress. These regions could also be considered in future studies to improve salinity tolerance in durum wheat. The SSR locus gwm403 (on chromosome 3A, 49 cM) associated with STI (CHC), STI (RL) and STI (NL) may have direct effects on maintaining growth under salinity stress. Further, the SSR loci *gwm*4 and cfd6 (on chromosome 7A) associated with STI (SL), STI (NT) and STI (PH) may also contribute to salinity tolerance by enhancing plant vigor. Thus, these markers could be targeted for a marker assisted approach to breeding for improved salinity tolerance in durum wheat.

Finally, this study indicated a novel QTL associated with salinity tolerance in chromosome 4B. It also indicated novel QTLs associated with STI of additional traits on different chromosomes.

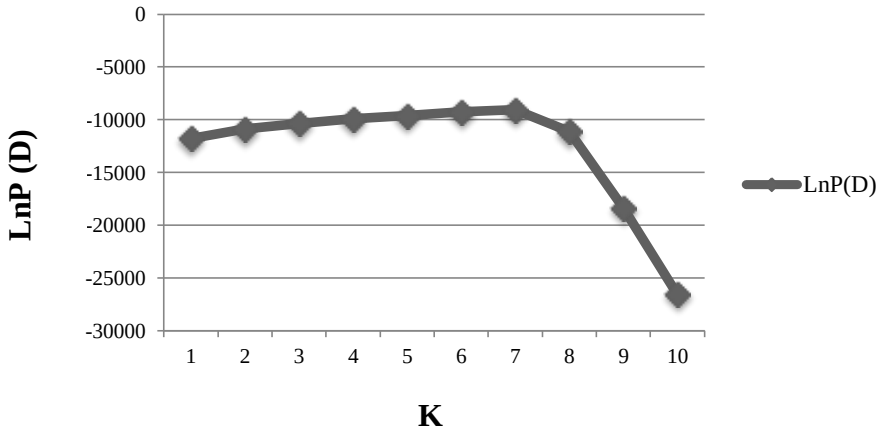


Fig. 3.1 Changes in the natural log probability of the data (LnP (D)) against the number of lines showed error bars.

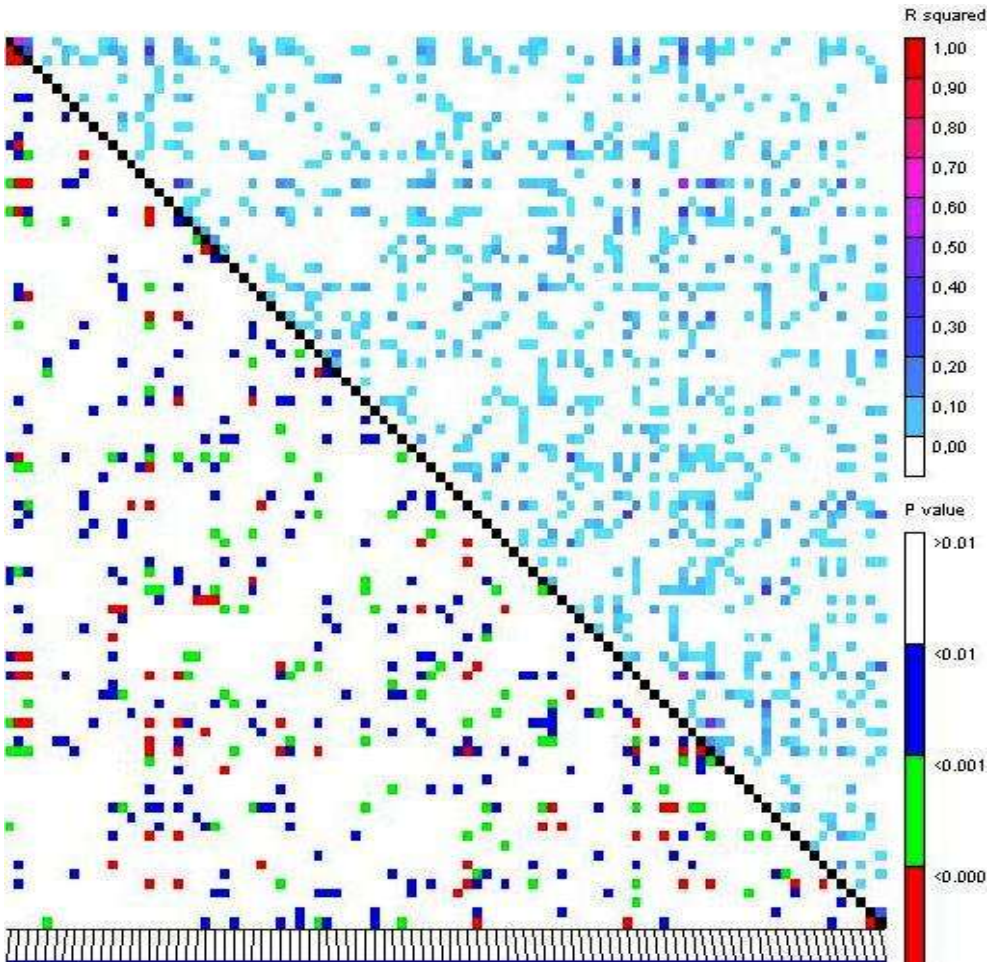


Fig. 3.2 LD measurements (R^2, above the diagonal line) and probability value (P, below the diagonal line) for 94 SSR markers located on 14 chromosomes using 119 varieties. The picture represents all pair-wise comparisons of polymorphic sites.

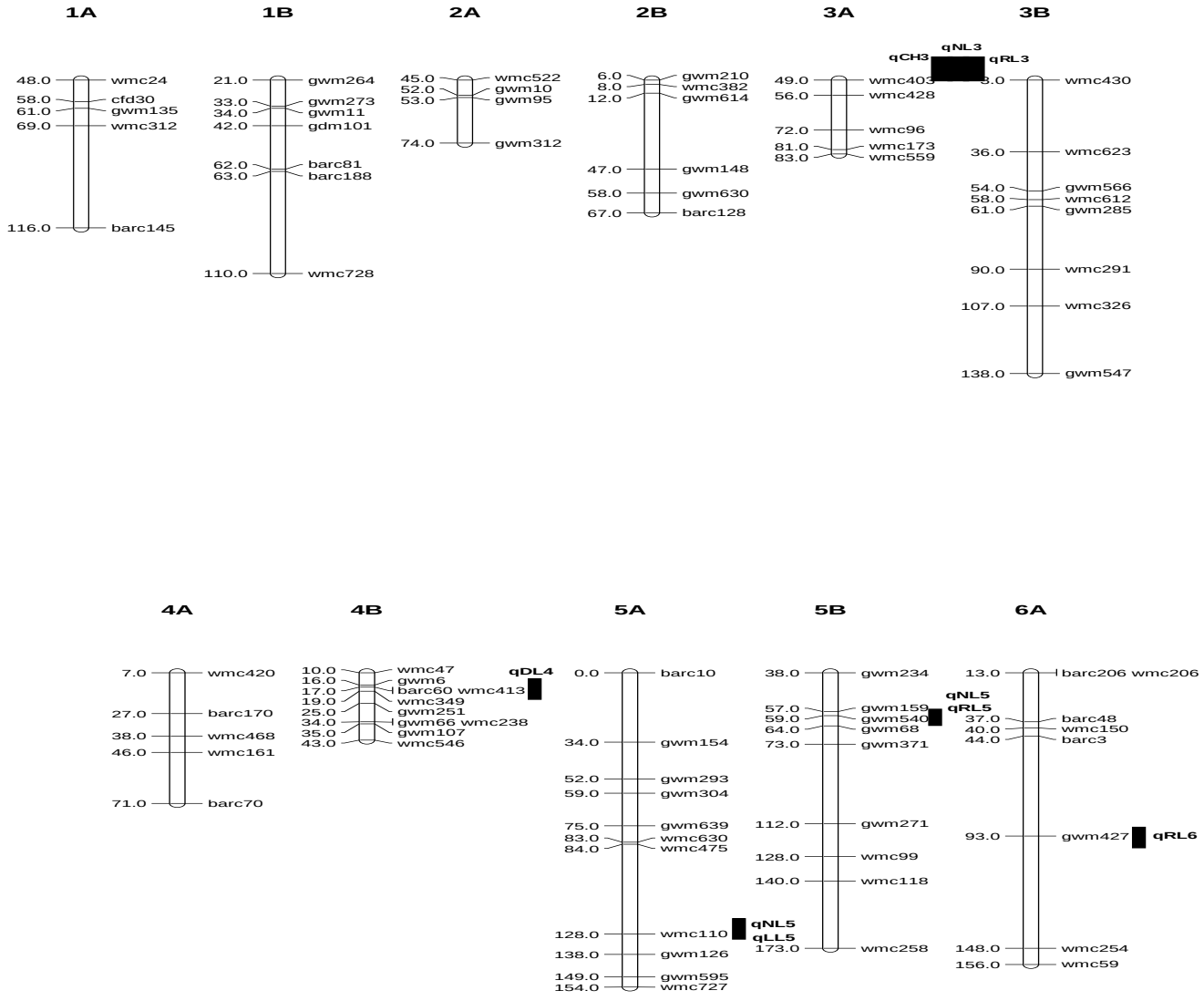


Fig. 3.3 Chromosomal locations of QTLs associated with different parameters related to salinity tolerance.

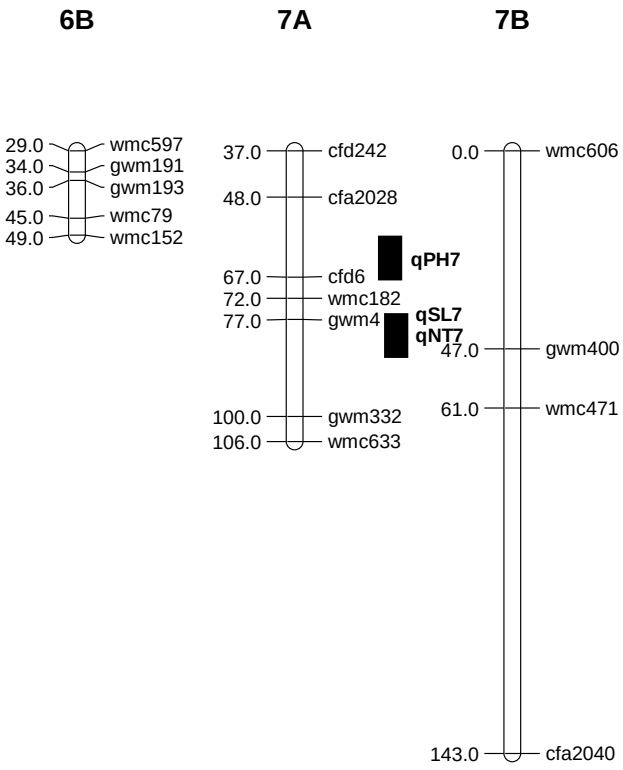
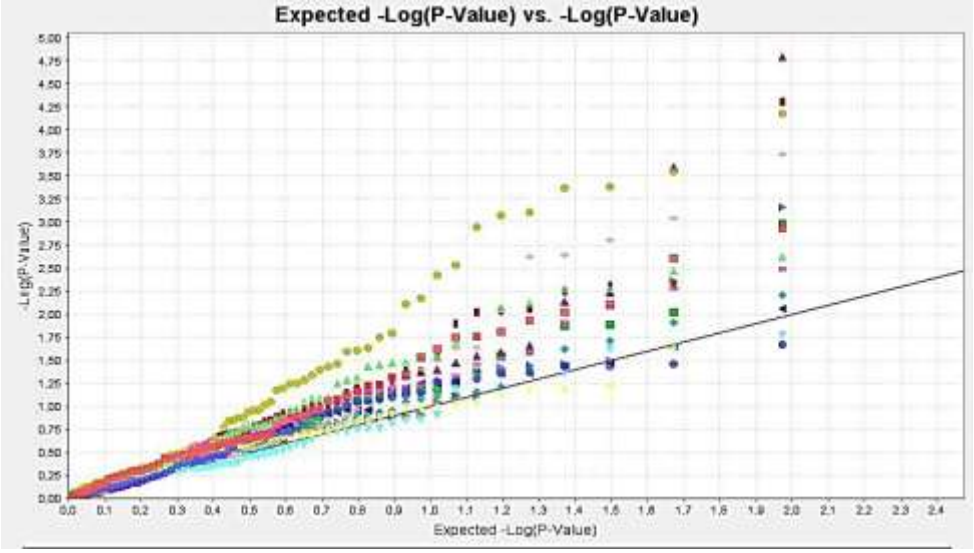
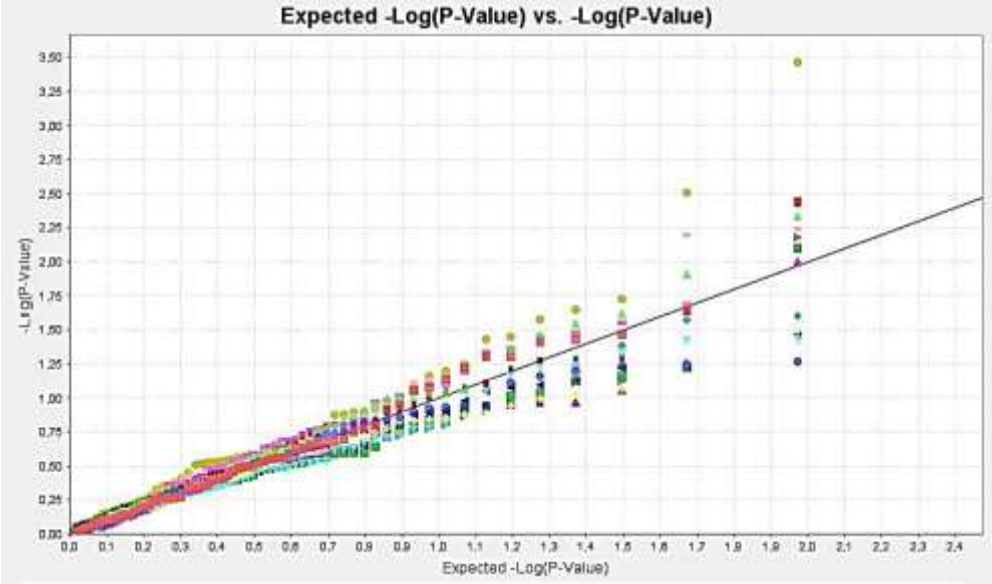


Fig. 3.3 Continued.



(a) GLM model (Naive model) followed by Q model



(b) MLM model (K model) followed by Q+K model

Fig. 3.4 Variation of observed P-value (x) against the expected P-values (y) among all traits using GLM and MLM models.

Table 3.1 Salinity tolerance categories of durum wheat varieties.

Salinity tolerance category	Range of the proportion of dead leaves (%DL)	No. of varieties	Varieties (Country)
Tolerant	0-29.9%	33	144 (Iran),48 (Jordan), V2 (Italy),131 (Iran), 21 (Uzbekistan), 41 (Bosnia and Herzegovina),113 (Egypt), 14 (India), 45 (Portugal), 4 (US), 121 (Bolivia),39 (Lebanon), 42 (Spain), 13 (Pakistan), 20 (Uzbekistan), 114 (Egypt), 46 (Ukraine), 54 (Turkey), 51 (Kyrgystan), 132 (Iran), 138 (Iran), A22 (Turkey), A40 (Algeria), A42 (Algeria), A60 (Ethiopia), 88 (Montenegro), 6 (Canada), 84 (Mexico), 96 (Algeria), 105 (Mexico), A24 (Turkey), A25 (Morocco), 34 (Turkey).
Moderately tolerant	30-59.9%	42	40 (Saudi Arabia),58(Malta), 110 (Tunisia), 127 (Iran), 22 (Japan), 7 (Eritrea), 130 (Iran), A46 (Ethiopia), A47 (Ethiopia),), 83 (Macedonia), 19 (Kyrgystan), 78 (Cyprus), 87 (Egypt), 57 (Poland), A62 (Pakistan), 63 (Hungang), 32 (Turkey), 97 (Peru), 26 (China), 27 (Georgia), 11 (Tunisia), 25 (Iraq), 60 (Morocco), V1 (Greece), , 28 (Egypt), 38 (Iraq), 53 (Italy), 94 (Algeria), 86 (Mexico), 109 (Tunisia), 99 (Ethiopia), A20 (Italy), 112 (Egypt), 12 (Canada), 69 (Chile), 123 (Ukraine), 23 (Japan), 29 (Yemen), A26 (Morocco), 44 (Morocco), 56 (Poland), 100 (US).
Susceptible	60-100%	44	95 (Algeria), 3 (US), 67 (France), 16 (Ukraine),75 (Spain), 65 (Bulgaria), 8 (Eritrea), 80 (Ethiopia), 128 (Iran), V9 (Italy), A41 (Algeria), A52 (Ethiopia), 92 (China), 62 (Hungary, pest), 17 (Ukraine), 2 (Peru), 55 (Hungary, pest), 122 (Russian federation), A28 (Morocco), 129 (Iran), 64 (Hungary), 31 (Italy), , 37 (India), 66 (Bulgaria), 52 (Greece), 103 (Chile), 91 (Bosnia and Herzegovina), 120 (Bolivia),111 (US), V11 (Mexico), 70 (India), 148 (Iran), A29 (Morocco), 50 (Russian federation), 115 (Oman), 59 (Malta), 116 (Algeria), V13 (Tunisia), V10 (Italy), 74 (Serbia), 125 (Kazakhstan), V7 (Tunisia), 36 (Turkey), A63 (Ethiopia).

Table 3.2 Chromosome locations, position in each chromosome (in CM), number of polymorphic alleles and polymorphism information content (PIC) of 94 SSR markers.

Markers	Chromosome locations	Position(CM) in each chromosome	Number of polymorphic alleles	PIC
Gwm614	*2B*	12	4	0.60
Wmc382	*2B*	8	3	0.57
Gwm403	*3A*	49	4	0.24
Wmc728	*1B*	110	10	0.85
Barc81	*1B*	62	4	0.56
Barc188	*1B*	63	3	0.59
Cfd30	*1A*	58	2	0.40
Wmc312	*1A*	69	8	0.82
Cfd6	*7A*	67	3	0.54
Wmc24	*1A*	48	7	0.68
Gwm135	*1A*	61	3	0.10
Gwm11	*1B*	34	4	0.47
Wmc522	*2A*	45	10	0.80
Gwm95	*2A*	53	4	0.51
Gdm101	*1B*	42	6	0.75
Barc145	*1A*	116	3	0.46
Gwm264	*1B*	21	5	0.66
Barc128	*2B*	67	5	0.78
Barc60	*4B*	17	7	0.71
Wmc597	*6B*	29	5	0.73
Gwm148	*2B*	47	4	0.69
Gwm210	*2B*	6	2	0.78
Wmc173	*3A*	81	4	0.48
Wmc215	*3A*	89	5	0.31
Wmc430	*3B*	3	4	0.32
Wmc623	*3B*	36	10	0.87
Wmc559	*3A*	83	5	0.65
Wmc428	*3A*	56	4	0.65
Wmc326	*3B*	107	4	0.69
Wmc182	*7A*	72	3	0.53

Table 3.2 Continued.

Markers	Chromosome locations	Position(CM) in each chromosome	Number of polymorphic alleles	PIC
Wmc471	7B	61	3	0.65
Gwm630	2B	58	3	0.45
Gwm285	3B	61	6	0.67
Gwm4	7A	77	6	0.38
Wmc291	3B	90	5	0.58
Gwm547	3B	138	2	0.55
Gwm312	2A	74	5	0.71
Wmc630	5A	83	5	0.62
Wmc475	5A	84	4	0.15
Gwm159	5B	57	4	0.64
Wmc238	4B	34	3	0.51
Gwm595	5A	149	7	0.80
Gwm371	5B	73	6	0.74
Wmc152	6B	49	2	0.31
Wmc59	6A	156	5	0.69
Wmc150	6A	40	5	0.59
Barc206	6A	13	6	0.62
Barc3	6A	44	6	0.33
Wmc206	6A	115	6	0.22
Wmc99	5B	128	5	0.69
Barc48	6A	37	3	0.59
Wmc79	6B	45	3	0.66
Wmc468	4A	38	4	0.50
Gwm304	5A	59	5	0.84
Gwm234	5B	38	8	0.74
Wmc161	4A	46	6	0.74
Wmc349	4B	19	4	0.66
Gwm251	4B	25	7	0.70
Gwm6	4B	16	9	0.90
Gwm154	5A	34	5	0.60
Gwm273	1B	34	3	0.50

Table 3.2 Continued.

Markers	Chromosome locations	Position(CM) in each chromosomes	Number of polymorphic alleles	PIC
Wmc606	7B	0	7	0.80
Wmc633	7A	106	20	0.93
Gwm107	4B	35	4	0.50
Gwm191	6B	34	5	0.70
Gwm193	6B	36	6	0.50
Wmc727	5A	154	5	0.60
Barc170	4A	27	12	0.85
Gwm639	5A	75	6	0.64
Cfa2040	7B	143	7	0.70
Wmc110	5A	128	4	0.32
Gwm68	5B	64	4	0.52
wmc546	4B	43	4	0.40
Gwm427	6A	93	8	0.77
Wmc254	6A	148	8	0.70
Cfd242	7A	37	3	0.32
Cfa2028	7A	48	4	0.57
Wmc118	5B	140	4	0.07
Gwm400	7B	47	8	0.74
Gwm332	7A	100	8	0.76
Gwm271	5B	65	3	0.61
Wmc258	5B	173	3	0.51
Wmc47	4B	10	2	0.33
Wmc420	4A	7	3	0.59
Wmc96	3A	72	4	0.67
Gwm66	4B	32	3	0.25
Wmc413	4B	17	3	0.50
Gwm293	5A	52	4	0.62
Gwm126	5A	138	8	0.79
Barc10	5A	0	4	0.48
Barc70	4A	71	9	0.82

Table 3.3 Location and phenotypic contribution of QTLs associated with different parameters.

QTLs associated with parameters	chromosomes	Marker	QTL position (cM)	-Log10(P)	R^2%
%DL	4B	*Wmc238*	34	2.40	13
STI (CHL)	3A	*Gwm403*	49	2.15	12
STI (NL)	3A	*Gwm403*	49	2.53	17
	5A	*Wmc110*	128	2.36	15
	5B	*Gwm540*	59	2.25	13
STI (LL)	5A	*Wmc110*	128	2.61	19
STI (NT)	7A	*Gwm4*	78	2.50	16
STI (RL)	3A	*Gwm403*	49	3.00	20
	6A	*Gwm427*	93	2.61	17
	5B	*Gwm540*	59	2.30	15
STI (SL)	7A	*Gwm4*	78	2.42	15
STI(PH)	7A	*Cfd6*	67	2.60	18

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