

Components of salinity tolerance in wheat

**A thesis submitted in fulfilment of the requirements for the degree of
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By

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Table of Contents

List of Tables	viii
List of Figures	x
List of appendices	xvi
List of abbreviations	xvii
List of publications and conference presentation from this dissertation.....	xviii
Abstract	xix
Declaration.....	xxi
Acknowledgements.....	xxii
CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW	1
1.1 Introduction.....	1
1.2 Wheat	3
1.2.1 Species	3
1.2.1.1 Einkorn wheat - <i>T. monococcum</i>	5
1.2.1.2 Bread wheat – <i>T. aestivum</i>	7
1.2.2 Morphology.....	9
1.2.3 Growth and development.....	9
1.2.4 Wheat cultivation in Australia	12
1.2.5 Limitations in wheat productions in Australia.....	13
1.3 Soil salinity	14
1.3.1 Origin, classification and distribution of salt affected soils.....	14
1.3.1.1 Saline soils	15
1.3.1.2 Sodic soils	15
1.3.2 Soil salinity in Australia.....	16
1.3.1.1 Water table induced salinity or Seepage salinity	17
1.3.1.2 Non water- table induced salinity or Transient salinity.....	17

1.4 Effect of salt stress on plant growth.....	19
1.4.1 Osmotic stress	19
1.4.2 Ionic stress	21
1.5 Use of two phase growth model to study the osmotic and ion specific effect of salt stress	23
1.6 Components of salinity tolerance.....	25
1.6.1 Osmotic tolerance	26
1.6.2 Na ⁺ exclusion	27
1.6.3 Tissue tolerance	30
1.7 Use of imaging platform to study changes in morphological and physiological features of various agricultural crops.....	34
1.8 QTL mapping.....	39
1.8.1 Basic requirements for QTL mapping	40
1.8.1.1 Mapping population	40
1.8.1.1.1 F ₂ population.....	40
1.8.1.1.2 Doubled Haploids (DH).....	41
1.8.1.2 Molecular markers	42
1.8.1.2.1 Simple sequence repeats (SSR's).....	42
1.8.1.3 Genetic or linkage maps.....	43
1.8.2 QTL mapping methods	44
1.8.2.1 Single marker analysis	44
1.8.2.2 Simple interval mapping (SIM)	45
1.8.2.3 Composite Interval Mapping (CIM)	45
1.8.3 QTL mapping in <i>T. monococcum</i>	46
1.8.4 QTL mapping in <i>T. aestivum</i>	46
1.9 Rationale for this dissertation	47
CHAPTER 2. GENERAL METHODOLOGY.....	49
2.1 Plant material	49

2.2 Growth conditions.....	49
2.3 Seed germination	49
2.4 Supported hydroponics	49
2.5 NaCl application	51
2.6 Non-destructive 3D plant imaging.....	51
2.7 Measurements of leaf Na ⁺ concentrations	56
CHAPTER 3. QUANTIFYING THE THREE MAIN COMPONENTS OF SALINITY TOLERANCE IN CEREALS	57
3.1 A commentary on quantifying the three main components of salinity tolerance in cereals	58
3.1.1 Overview.....	58
3.1.2 The scanalyser 3D – a new phenotyping tool used to quantify salt damages	59
3.1.3 Screening for the three main components of salinity tolerance in cereals – a perspective	60
3.1.4 Concluding remarks	63
3.2 The published research paper.....	64
CHAPTER 4. GENETIC ANALYSIS OF MAJOR SALINITY TOLERANCE COMPONENTS IN BREAD WHEAT (<i>TRITICUM AESTIVUM</i>).....	78
4.1 Introduction.....	79
4.2 Materials and methods	83
4.2.1 Mapping population.....	83
4.2.2 Experimental setup.....	83
4.2.3 Non-destructive 3D plant imaging.....	84
4.2.4 High throughput salt screening	84
4.2.4.1 Osmotic tolerance screen	85
4.2.4.2 Exclusion screen	85
4.2.4.3 Tissue tolerance screen	86
4.2.5 Phenotypic data analysis	86

4.2.6 The genetic map	87
4.2.7 QTL analysis	87
4.3 Results.....	89
4.3.1 Osmotic tolerance	89
4.3.1.1 Determination of variability for osmotic tolerance in Berkut × Krichauff DH mapping population	89
4.3.1.2 Identification of QTL linked to osmotic tolerance in Berkut × Krichauff DH mapping population	97
4.3.2 Na ⁺ exclusion	102
4.3.2.1 Determination of variability for Na ⁺ exclusion in Berkut × Krichauff DH mapping population.....	102
4.3.2.3 Identification of QTL linked to Na ⁺ exclusion in Berkut × Krichauff DH mapping population.....	107
4.3.3 Tissue tolerance	117
4.3.3.1 Determination of tissue tolerance in Berkut × Krichauff DH mapping population	117
4.3.4 Salinity tolerance of Berkut × Krichauff DH mapping population	120
4.4 Discussion	122
4.4.1 Genetic basis of osmotic tolerance	122
4.4.2 Comparison of Na ⁺ exclusion QTL across different genetic background	125
4.4.3 Limitations in tissue tolerance screening and QTL mapping	127
4.4.4 The combined effect of osmotic tolerance and Na ⁺ exclusion QTL on shoot biomass of mapping lines grown under saline conditions	130
4.5 Future Prospects.....	131
CHAPTER.5 UNDERSTANDING THE GENETIC BASIS OF OSMOTIC AND TISSUE TOLERANCE IN EINKORN WHEAT (<i>TRITICUM MONOCOCCUM</i>) .	132
5.1 Introduction.....	133
5.2 Materials and methods	136
5.2.1 Mapping population.....	136
5.2.2. Experimental setup.....	137

5.2.3 Non-destructive 3D plant imaging.....	137
5.2.4 High throughput salt screening	138
5.2.4.1 Osmotic tolerance screen	138
5.2.4.2 Tissue tolerance screen	138
5.2.5 DNA extraction.....	139
5.2.6 Polymerase Chain Reaction (PCR) master mix	140
5.2.7 Thermocycler programme.....	140
5.2.8 Visualization of molecular markers	140
5.2.9 Statistical analysis.....	141
5.2.9.1 Analysis of variance (ANOVA).....	141
5.2.9.2 Heritability calculations	141
5.3 Results.....	144
5.3.1 Shoot growth of MDR 002 × MDR 043 F ₂ population in salt stress	144
5.3.2 Shoot health of MDR 002 × MDR 043 F ₂ population in saline environments.....	145
5.3.3 Non-destructive phenotyping for osmotic and tissue tolerance in MDR 002 × MDR 043 F ₂ population	146
5.3.3.1 Osmotic tolerance	147
5.3.3.2 Tissue tolerance	150
5.3.3.2.1 In parents.....	150
5.3.3.2.2 In MDR 002 × MDR 043 F ₂ population	154
5.3.4 Genotyping MDR 002 × MDR 043 <i>T. monococcum</i> F ₂ mapping population using SSR markers	160
5.4 Discussion.....	161
5.4.1 Tissue tolerance screening - Challenges and opportunities	161
5.4.2 Constraints in genotyping and construction of molecular linkage map....	164
5.4.3 Difficulties in QTL mapping for osmotic tolerance	165
5.5 Future Prospects.....	166

CHAPTER 6. GENERAL DISCUSSION	167
6.1 Review of thesis aims	167
6.2 Advantages and disadvantages of the high throughput salt screening methodology	168
6.3 Other application of 3D imaging technology.....	173
6.4 Breeding potential for major salinity tolerance components in wheat breeding	174
6.5 Future Directions	177
6.6 Major Conclusions	182
APPENDIX.....	183
REFERENCES	190

List of Tables

Table 1. Classification of wheat species done by Goncharov, (2011).	3
Table 2. Potential sources of genetic variability found in <i>T. monococcum</i> for tolerance to diseases, pest, salt, frost, nutrient uptake and grain qualities.	6
Table 3. Important contribution of RGB, infrared and fluorescence images to study the morphological and physiological characteristics of agricultural crops.....	37
Table 4. Descriptive statistics and broad sense heritability (H^2) of the osmotic tolerance quantified in the parents and Berkut \times Krichauff DH mapping lines.	89
Table 5. Kolmogorov - Smirnov test of normality done for osmotic tolerance quantified in Berkut \times Krichauff DH mapping population.....	91
Table 6. GLM-ANOVA for osmotic tolerance quantified in Berkut \times Krichauff DH mapping population	96
Table 7. Characteristics of osmotic tolerance QTL identified in Berkut \times Krichauff DH mapping population using CIM approach.....	98
Table 8. Descriptive statistics and broad sense heritability (H^2) of the fourth leaf blade [Na^+] (mM) calculated in the parents and Berkut \times Krichauff DH mapping population.	102
Table 9. Kolmogorov – Smirnov test of normality for fourth leaf blade [Na^+] in the Berkut \times Krichauff DH mapping population.	103
Table 10. GLM-ANOVA performed on the fourth leaf blade [Na^+] in Berkut \times Krichauff DH mapping population.....	106
Table 11. Characteristics of Na^+ exclusion QTL identified in Berkut \times Krichauff DH mapping population using CIM approach.....	108
Table 12. Additive \times additive epistatic main effect (<i>aa</i>) and additive \times additive epistasis environment interaction (<i>aae</i>) identified for Na^+ exclusion in Berkut \times Krichauff DH mapping population using mixed composite interval mapping with 2D genome scan by QTL Network 2.0.	109
Table 13. Morphological difference between <i>T. monococcum</i> accessions MDR 002 and MDR 043 at maturity (Jing <i>et al.</i> , 2007).	136
Table 14. The list of 45 polymorphic SSR markers for the MDR 002 \times MDR 043 F_2 mapping population obtained from Dr. Hai-Chun- Jing, *are the markers already have been screened in 3% agarose gels and **are markers with unknown product size. Information about the forward and reverse primers, chromosomes and product size	

were obtained from Graingenes database (<http://wheat.pw.usda.gov/cgi-bin/graingenes/browse.cgi?class=marker>). 142

Table 15. Osmotic tolerance calculations in MDR 043, MDR 002, F₂ individual 29 and F₂ individual 26 in MDR 002 × MDR 043 mapping population. 148

Table 16. Descriptive statistics of the physiological parameters used to estimate tissue tolerance in parents and the MDR 002 × MDR 043 F₂ mapping population. 151

List of Figures

- Figure 1.** Evolution of rye, einkorn wheat, durum wheat, bread wheat and barley. Natural hybridization (black arrows), domestication (green arrows) and selection (red arrows) process are shown as well as the approximate timing of the event, in millions of years (MY) (Reproduced from Feuillet *et al.*, (2008)).8
- Figure 2.** Schematic diagram displaying the growth and developmental stages of wheat. Sw: sowing, Em: emergence, DR: initiation of the first double ridge, TS: terminal spikelet initiation, Hd: heading time, At: anthesis, BGF: beginning of grain filling, PM: physiological maturity and Hv: harvest (Reproduced from Slafer,(2003)). 11
- Figure 3.** Wheat growing areas in Australia (Adapted from Sott.net, (2009)). 12
- Figure 4.** Distribution of salt affected soils over the seven continents in the world (Adapted from FAO, (2000)). 14
- Figure 5.** Map showing areas of dry land seepage salinity regions (red) with potential transient salinity and subsoil constraints (yellow) and the area of grain production in Australia (blue line) (Reproduced from Rengasamy,(2002)). 18
- Figure 6.** Hypothetical diagram displaying the two phase growth response of salt sensitive (S), moderately salt tolerant (M) and tolerant (T) cultivars of a particular plant species grown under saline environment. The salinity tolerance of the cultivars varies in terms of rate of leaf senescence that usually occurs once the salt becomes toxic in the leaf. Phase 1 indicates the effect of osmotic stress on plant growth immediately after NaCl application and the Phase 2 shows the effect of increased accumulation of Na⁺ in the leaves, on plant growth. During Phase 1, all of these cultivars have shown similar response but with decreased plant growth. At Phase 2, the increased Na⁺ accumulation in the leaves further decreased the growth of salt sensitive cultivar than moderately tolerant and tolerant cultivars (Reproduced from Munns, (1993)).23
- Figure 7.** Diagram showing the function of ion transporters, channels and pumps involved in Na⁺ exclusion and tissue tolerance mechanisms in the plant cell. Influx of Na⁺ ions is occur through cyclic nucleotide gated channels (CNGCs), glutamate receptors (GLRs), non-selective cation channels (NSCCs) and HKT transporters (AtHKT1:1, OsHKT1:4, OsHKT1:5 and OsHKT2:1), whereas the efflux of the Na⁺ ions from the cells are occur through Na⁺/H⁺ antiporter (SOS1) that interacts with the serine/threonine protein kinase (SOS2) and the calcium binding protein (SOS3), vacuolar storage of Na⁺ is mediated by a vacuolar Na⁺/H⁺ antiporter (NHX) and the electrochemical potential is provided by the vacuolar H⁺ pyrophosphatase (AVP1)

and the vacuolar H⁺-ATPase (V-ATPase) (Reproduced from Plett and Moller, (2010)).....28

Figure 8. The electromagnetic spectrum with radio waves, microwaves, infrared radiation, visible light, ultraviolet radiation, x-rays and gamma rays (From left to right, in the order of increasing frequency and decreasing wavelength). (Adapted from Google images <http://zebu.uoregon.edu/~imamura/122/lecture-2/em.html>).36

Figure 9. The supported hydroponics setup used to grow plant material for high throughput salt screening. Plants were grown in PVC tubes, filled with polycarbonate pellets. These tubes were arranged in the 25 litre black containers as determined by a randomized block design (RBD). Modified Hoagland’s nutrient solution (see main text) was pumped from the 80 litre blue reservoir tank below into the black containers in a 20 minutes fill/drain cycle.....50

Figure 10. Layout of the LemnaTec Scanalyser (a) Layout of the imaging cabinet and computer workstation, showing the sample loading bay. (b) A wheat plant in the imaging cabinet (c) The top digital camera and fluorescent lights used for image acquisition.....52

Figure 11. Image acquisition schedule for high throughput NaCl screening in wheat. To screen for osmotic stress images of plants were acquired every day (the continuous line) 5 days before and 5 days immediately after NaCl application. Images were then captured three times a week to monitor both osmotic stress and Na⁺ toxicity symptoms in the plant’s shoot (the broken line), the final image was taken on day 31. NaCl application began at the time of fourth leaf blade emergence (approximately day 11), which is indicated by the black arrow.53

Figure 12. Side view reader. A model generated to visualise the functions used in the processing grid of a side view image of 31 days old *T. monococcum* accession, MDR 043 grown in 75 mM saline environment.....54

Figure 13. The lemna launcher software window displaying the false colour image (right) of *T.monococcum* plant in 75 mM NaCl. The different shades of green, yellow and brown region in plant parts were identified through visual selection with the help of few randomly selected plant images.....55

Figure 14. (a) Histogram showing variation for the mean osmotic tolerance of 152 Berkut × Krichauff DH mapping lines grown in winter, early spring and late spring 2008. (Curve: Normal distribution). Osmotic tolerance was determined for each line by dividing the mean relative growth rate 5 days immediately after 150 mM NaCl application by the mean relative growth rate 5 days immediately before NaCl application. The variation between the mean osmotic tolerance of the parents is indicated by arrows. (b) Q-Q chart plotted with the observed quantiles of osmotic tolerance (○) against the expected normal quantiles (Straight line indicates the normal distribution).....90

Figure 15. Growth of HW-893*A086, an osmotic stress tolerant line in (a) winter (b) early spring and (c) late spring. Plants were grown without NaCl until fourth leaf stage (approximately day 14) before 150 mM NaCl (arrow). The total projected shoot areas were calculated from images obtained from the LemnaTec Scanalyser as shown in Chapter 2. The mean relative growth rate of HW-893*A086 before NaCl application was 0.12 day⁻¹, 0.21 day⁻¹ and 0.17 day⁻¹, which was reduced to 0.08 day⁻¹, 0.14 day⁻¹ and 0.15 day⁻¹ after the addition of 150 mM NaCl in winter, early spring and late spring respectively.....92

Figure 16. Growth of HW-893*A008, an osmotic sensitive line (a) winter (b) early spring and (c) late spring. Plants were grown without NaCl until fourth leaf stage (approximately day 14) before 150 mM NaCl (arrow). The total projected shoot areas were calculated from images obtained from the LemnaTec Scanalyser as shown in Chapter 2. The mean relative growth rate of HW-893*A008 before NaCl application was 0.17 day⁻¹, 0.21 day⁻¹ and 0.22 day⁻¹ which was reduced to 0.06 day⁻¹, 0.10 day⁻¹ and 0.09 day⁻¹ after the addition of 150 mM NaCl in winter, early spring and late spring respectively.93

Figure 17. Relationships between the mean relative growth rates of the Berkut × Krichauff DH mapping population measured over the 5 days before NaCl application to the mean relative growth rates measured 5 days immediately after 150 mM NaCl application in (a) winter, (b) early spring and (c) late spring 2008.94

Figure 18. LRS plots of osmotic tolerance QTL identified on (a) 1D, (b) 2D and (c) 5B chromosomes in Berkut × Krichauff DH mapping population of bread wheat with the data obtained from winter (red), early spring (blue) late spring (green) and mean over three experimental time of the year (black). QTL with LRS score >13.8, is considered as highly significant. The positive additive effect indicates the inheritance of the QTL from the osmotic tolerant parent Berkut; the negative additive effect indicates the inheritance of QTL from the osmotic sensitive parent Krichauff.....99

Figure 19. (a) Histogram showing variation for the mean [Na⁺] in the fourth leaf blade of 152 Berkut × Krichauff DH mapping lines grown under 150 mM NaCl for three weeks during winter, early spring and late spring 2008 (Curve: Normal distribution). The variation in mean fourth leaf blade [Na⁺] of parents is indicated by arrows. (b) Q-Q chart plotted with the observed quantiles of [Na⁺] (○) against the expected normal quantiles (Straight line indicates the normal distribution). 104

Figure 20. (a) Histogram showing variation for the log₁₀ of mean [Na⁺] in the fourth leaf blade of 152 Berkut × Krichauff DH mapping lines grown under 150 mM NaCl for three weeks during winter, early spring and late spring 2008 (Curve: Normal distribution). The log₁₀ mean fourth leaf blade [Na⁺] of parents is indicated by arrows (b) Q-Q chart plotted with the observed quantiles of [Na⁺] (○) against the expected normal quantiles (Straight line indicates the normal distribution)..... 105

Figure 21. LRS plots of Na⁺ exclusion QTL identified on chromosomes (a) 1B, (b) 2A, (c) 2D, (d) 5A (e) 5B, (f) 6B and (g) 7A in the Berkut × Krichauff DH mapping population of bread wheat. Data obtained from winter (red), early spring (blue) and late spring (green), as well as the results from the mean of the three seasons (black). QTL with LRS score >13.8, is considered as highly significant QTL. The positive additive effect indicates the inheritance of the QTL from the Na⁺ accumulating parent Berkut; the negative effect indicates the inheritance of QTL from the Na⁺ excluding parent Krichauff. 110

Figure 22. Relationship between (a) projected shoot area and fourth leaf blade [Na⁺] (Y= - 36.79x+16114, R²= -0.14), (b) proportion of green area and fourth leaf blade [Na⁺] (Y= - 0.0072x + 98.22, R²= 0.06) for the mapping lines grown in winter, early spring and late spring 2008. Measurements were taken three weeks after 150 mM NaCl application. 117

Figure 23. The histogram of proportion of green area in the shoot of the Berkut × Krichauff DH mapping population’s health after three weeks of growth in 150 mM NaCl in (■) winter, (■) early spring and (■) late spring 2008. Values closer to 1 indicate the plant is in good health with little senescence of leaf material. Arrows indicate the proportion of green area of the parents measured at the same time. 118

Figure 24. The detected chromosomal locations of QTL linked to osmotic tolerance and Na⁺ exclusion in Berkut × Krichauff DH mapping population. Dashed lines show the epistatic interaction between QTL. 119

Figure 25. The significant association between the markers linked to osmotic tolerance and Na⁺ exclusion to the plant biomass of the Berkut × Krichauff DH mapping population grown in 150 mM NaCl. The mean total projected shoot area which was quantified three weeks after 150 mM NaCl application for the mapping population parents Berkut and Krichauff, as well as the mapping population lines, characterised into four genotypic classes depending on their genotype at salt tolerance QTL: BB (with Berkut alleles at markers *wmc216-1D* and *gwm186-5A*), BK (Berkut at *wmc216-1D*; Krichauff at *gwm186-5A*), KB (Krichauff at *wmc216-1D*; Berkut at *gwm186-5A*) and KK (Krichauff alleles at markers *wmc216-1D* and *gwm186-5A*). Error bars indicate the standard error of mean projected shoot area. 120

Figure 26. Growth curves of (●) MDR 043, (○) MDR 002, (▼) F₂ individual 29, and the (△) F₂ individual 26 were measured using the projected shoot area of the F₂ individuals over time. They were calculated using the three images for each plant captured at 17 time points, 7 before and 10 after 75 mM NaCl application. The final 75 mM NaCl was achieved by three 25 mM NaCl applications, two doses applied on day 16 (arrow). The significance of difference in total projected shoot area was estimated at the last time point through “t” test at P =0.09 level. 144




Figure 27. The proportion of () healthy, () senescing (chlorotic) and senesced () (necrotic) tissue of the, MDR 043, MDR 002, F₂ line 29 and the F₂ line 26 at 19 days after NaCl application. The significance of difference between the proportion of salt induced senesced shoot area (sum of proportion of senescing and senesced tissue) was revealed by “t” test at P ≤ 0.04 levels. 145

Figure 28. The phenotypic variation found for osmotic tolerance in the MDR 002 × MDR 043 *T. monococcum* F₂ mapping population (177 F₂ individuals) grown between July-September 2009. Osmotic tolerance was calculated by dividing the mean relative growth rate 5 days after NaCl application by the mean relative growth rates 5 days immediately prior to NaCl application for every single line. The osmotic tolerance of the parents was marked by arrows. 148

Figure 29. Growth of (a) MDR 043 (b) MDR 002, (c) F₂ line 29 and (d) F₂ line 26 before (●) and after NaCl application (○) in MDR002 × MDR043 mapping population. Arrow indicates time of NaCl application. The final 75 mM NaCl was achieved by three 25 mM NaCl applications, two doses applied on 16th day. 149

Figure 30. The relationship between mean relative growth rates calculated five days before and five days after NaCl application ($R^2 = 0.04$) for all plants in the MDR002 × MDR043 mapping population, which was significant at p<0.05 level. . 150

Figure 31. The phenotypic variation observed for fourth leaf blade tissue [Na⁺] in 177 F₂ progenies of MDR 002 × MDR 043 *T. monococcum*, which was sampled after 19 days of growth in 75mM NaCl. The arrow indicates the position of parents (MDR 043 & MDR 002)..... 152

Figure 32. Development of senescence in MDR 043 (a), (b) and (c) and MDR 002 (d), (e) and (f) accessions, which was quantified 7 days before 75 mM NaCl application (a & d), at 5 days immediately after salt application (during osmotic stress) (b & e) and, after osmotic stress (c & f) respectively. 153

Figure 33. Phenotypic variation observed for proportion of senesced shoot area measured three weeks after 75 mM NaCl application in MDR 002× MDR 043 F₂ mapping population. The senescence in the parents was marked by arrows..... 154

Figure 34. Development of senescence in MDR 002×MDR 043 *T. monococcum* F₂ mapping population, which was quantified 7 days before 75 mM NaCl application (a), at 1-5 days (during osmotic stress) after 75 mM NaCl application (b, c, d, e & f) and 10th, 12th, 14th,17th and 19th days after 75mM NaCl application (g, h, i, j & k) respectively. Except a, each diagram has 177 data points, whereas, diagram a, has 1239 (177 × 7=1239) data points. 155

Figure 35. The relationship between (a) proportion of total senesced shoot area and fourth leaf blade [Na⁺] of the mapping population, 19 days after NaCl application, $R^2= 0.23$ significant at p ≤ 0.05, level..... 159

Figure 36. Use of SSR markers to identify the polymorphism in MDR 002 × MDR043 *T. monococcum* F₂ mapping population. An example of an ethidium bromide stained 3% gel agarose gel demonstrating the polymorphism of the SSR marker Xbarc174 in F₂ progenies with MDR 002 and MDR 043. The first lane was loaded with pUC19 DNA/*MspI*(*HpaII*). 160

Figure 37. The morphological differences between the Berkut× Krichauff DH mapping lines a, HW-893*A008 and b, HW-893*A086 of bread wheat grown three days after 150 mM NaCl application. 170

Figure 38. Two different types of senescence observed in bread wheat Berkut × Krichauff DH mapping lines grown three weeks after 150 mM NaCl application. a) Marginal chlorosis and necrosis followed by burning of entire leaf blade due to ionic toxicity b) dull appearance of leaf blade, loss of turgor, grey discoloration and shrinking of leaf blade due to osmotic stress. 172

List of appendices

Appendix 1. Supporting information with tables and figures for Rajendran <i>et al.</i> ,(2009), presented in Chapter 3.	183
Appendix 2. Generation of F ₃ progenies for future studies	189

List of abbreviations

ABA : Abscisic acid	kPa : Kilopascal
ACS : Australian Commodity Statistics	LOD : Log of odds ration
ANOVA : Analysis of variance	LRS : Likelihood Ratio Statistics
APW : Australian Premium White	M : Molar
CCD : Coupled Charge Device	MCIM : Mixed linear Composite Interval Mapping
CIM : Composite Interval Mapping	mM : millimolar
CIMMYT : International Maize and Wheat Improvement Centre	NILs : Near Isogenic Lines
DArT : Diversity Array Technology™	NLWRA : National Land and Water Resources Audit
DH : Doubled Haploid	P : Probability
dS/m : deci-Siemens/meter	PCR : Polymerase Chain Reaction
Ec _e : Electrical conductivity of saturation extract	PEG : Polyethylene glycol
ESP : Exchangeable sodium percentage	PVC : Polyvinyl chloride
F ₂ : Second filial generation	QTL : Quantitative Trait Loci
FAO : Food and Agriculture Organization of the United Nations	R ² : Regression co-efficient
GLM : General Linear Model	RILs : Recombinant Inbred Lines
H ² : Broad sense heritability	ROS : Reactive Oxygen Species
IBLS : Image Based Leaf Sum	rpm : Revolutions per minutei
IDRC : International Development Research Centre	SARDI : South Australian Research and Development Institute
	SD : Standard deviation
	SIM : Simple Interval Mapping
	SSR : Simple Sequence Repeats

List of publications and conference presentation from this dissertation

Journal publications

Rajendran, K, Tester, M and Roy.S. Quantifying three major components of salinity tolerance mechanisms in cereals. *Plant Cell and Environment*.32: 237-249, 2009.

Golzarian.MR, Frick.RA, Rajendran.K, Berger.B, Roy.S, Tester.M and Lun,D.S. Accurate inference of shoot biomass from high-throughput images of cereal plants. *Plant Methods*.7:2, 2011.

Publication in progress

Rajendran, K, Tester, M and Roy.S. Identification of new source of genetic variability and QTL for osmotic component of salt stress in bread wheat (*T. aestivum*) through non-destructive image analysis.

Rajendran, K, Hudson, I, Tester, M and Roy.S. Use of EM algorithm to evaluate genotype \times seasonal interaction on growth and health of genotypes with diverse combinations of three major salinity tolerance components – a case study with bread wheat (*T. aestivum*).

Oral presentations

Rajendran, K, Tester, M and Roy.S. Imaging growth and senescence through time to separate components of salinity tolerance. *The Genomics of Salinity*. ACPFG Symposium -2009, Adelaide Australia.

Abstract

Soil salinity causes osmotic and ion specific stresses and significantly affects growth, yield and productivity of wheat. The visual symptoms of salinity stressed wheat include stunted shoot growth, dark green leaves with thicker laminar surfaces, wilting and premature leaf senescence. There are three major components of salinity tolerance that contribute to plant adaptation to saline soils: osmotic tolerance, Na⁺ exclusion and tissue tolerance. However, to date, research into improving the salinity tolerance of wheat cultivars has focused primarily on Na⁺ exclusion and little work has been carried out on osmotic or tissue tolerance. This was partly due to the subjective nature of scoring for plant health using the human eye.

In this project, commercially available imaging equipment has been used to monitor and record the growth and health of salt stressed plants in a quantitative, non-biased and non-destructive way in order to dissect out the components of salinity tolerance. Using imaging technology, a high throughput salt screening protocol was developed to screen osmotic tolerance, Na⁺ exclusion and tissue tolerance of 12 different accessions of einkorn wheat (*T. monococcum*), including parents of the existing mapping populations. Three indices were used to measure the tolerance level of each of the three major components of salinity tolerance. It was identified that different lines used different combinations of the three major salinity tolerance components as a means of increasing their overall salinity tolerance. A positive correlation was observed between a plant's overall salinity tolerance and its proficiency in Na⁺ exclusion, osmotic tolerance and tissue tolerance. It was also revealed that MDR 043 as the best osmotic and tissue tolerant parent and MDR 002 as a salt sensitive parent for further mapping work. Accordingly, the F₂ population of MDR 002 × MDR 043 was screened to understand the genetic basis of osmotic tolerance and tissue tolerance in *T. monococcum*. Wide variation in osmotic tolerance and tissue tolerance was observed amongst the progenies. The broad sense heritability for osmotic tolerance was identified as 0.82.

Similar, salinity tolerance screening assays were used to quantify and identify QTL for major components of salinity tolerance in Berkut × Krichauff DH mapping population of bread wheat (*T. aestivum*). Phenotyping and QTL mapping for Na⁺ exclusion and osmotic tolerance has been successfully done in this mapping population. There existed a potential genetic variability for osmotic tolerance and Na⁺ exclusion in this mapping population. The broad sense heritability of osmotic tolerance was 0.70; whereas, it was 0.67 for Na⁺ exclusion. The composite interval mapping (CIM) identified a total of four QTL for osmotic tolerance on 1D, 2D and 5B chromosomes. For Na⁺ exclusion, CIM identified a total of eight QTL with additive effects for Na⁺ exclusion on chromosomes 1B, 2A, 2D, 5A, 5B, 6B and 7A. However, there were QTL inconsistencies observed for both osmotic tolerance and Na⁺ exclusion across the three different experimental time of the year. It necessitates re-estimating the QTL effect and validating the QTL positions either in the same or different mapping population.

Declaration

I, Karthika Rajendran certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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*Rajendran, K, Tester, M and Roy.S. Quantifying three major components of salinity tolerance mechanisms in cereals. *Plant Cell and Environment*.32: 237-249, 2009.

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CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Wheat is commonly known as the king of cereals (Kotal *et al.*, 2010). It is the most predominant food for 40% of the world's population, particularly for people living in Europe, North America and the Western and Northern parts of Asia. It ranks first in global grain production and makes up more than 20% of the total food calories in human nutrition (Peng *et al.*, 2011). The global wheat production in 2011 was 651 million metric tons and it is expected to increase to 880 million metric tons by 2050 (IGC, 2011; Weigand, 2011). In fact, the demand for wheat cultivation is growing faster than any other cereal crop and wheat grain production must increase an annual rate of 2% to meet out human demand by 2050 (Bhalla, 2006). However, a large proportion of the best quality land has already been used for agriculture and it is not currently feasible to further expand the wheat cultivation area for future farming. The aim should now be focused on increasing the productivity on available land on which wheat may have been grown in the past but have been lost to farmers due to degradation of the land (Wild, 2003; Rengasamy, 2006; Rajaram and Braun, 2008).

Soil salinization is one of the most devastating forms of land degradation processes that severely reduce quality of farmlands, with respect to its productivity (McWilliam, 1986). Wheat crop grown under both irrigated and rain-fed environments are affected by soil salinity (Ghassemi *et al.*, 1995; Mujeeb-Kazi and De Leon, 2002). About 8-10% of spring bread wheat cultivated area in the world is already salt affected and it is predicted to increase in the future. Australia, the largest exporter of wheat, undergoes major issues with rising soil salinity in all states of the country (CSIRO, 2008; Hemphill, 2012). Wheat is an important grain crop of Australia; it exports majority of the wheat crop produced (70 per cent) to the global wheat market and contributes approximately 12 per cent of the world's wheat trade (PC, 2010). However, salinity affects the quality and yield of even the most productive farms, especially in the wheat belt regions of Australia (GRDC, 2012b). It is estimated that 70 per cent of the wheat crop cultivated in Australia, has reductions of at least 10 per

cent in yield due to salinity (GRDC, 2011; GRDC, 2012b). It is one of the major concerns to the Australian grain industry, which cause billions of dollars loss to the farming economy every year (GRDC, 2012b).

It is very difficult to control the soil salinization process by itself and salt affected farmlands require a huge effort in both time and cost to become viable again. The development of salt tolerant wheat cultivar is one solution to grow crops on salt affected farmlands and produce high yield. Enormous efforts has been made in recent years to develop salinity tolerant wheat cultivars with high yield potential through conventional (Ashraf and Oleary, 1996) marker assisted (Lindsay *et al.*, 2004) breeding, as well as using genetic engineering (Sawahel and Hassan, 2002; Abebe *et al.*, 2003) techniques. However, the successful release of salinity tolerant wheat cultivars for commercial use has been very limited, due to the physiological and genetic complexity of the salt tolerance trait (Winicov, 1998). New experimental strategies using state of the art techniques would improve the understanding of physiological and genetic limits that restrained the development of salt tolerant cultivars in the early days and help to accelerate breeding processes to generate new salt tolerant cultivars in the near future (Finkel, 2009).

Recent advances in imaging technology allows capturing images of the same plant, from a variety of different angles non-destructively, and can use to phenotype the growth health and morphological features of various genotypes over its growth cycle. This would be a useful tool to quantify the response of the plants growing under saline environment. When combined with tools, QTL mapping and marker assisted selection; it could be used to evolve wheat varieties suitable for saline soils of Australia in a rapid manner (Furbank, 2009; Tester and Langridge, 2010). This literature review will focus on species of wheat, soil salinity, effect of salt stress on plant growth, the major components of salinity tolerance, uses of imaging platform in agricultural science and QTL mapping for salinity tolerance in wheat.

1.2 Wheat

1.2.1 Species

Wheat is the crop of old world Agriculture (Zohary and Hopf, 2000). It was the first cultivated crop in the world followed by rice and maize (Feldman, 1995). Most of the wheat species were originated in South Western Asia, the region called Fertile Crescent that includes areas of North Syria, South east Turkey, Northern Iraq and Western Iran (Feldman and Sears, 1981). Wheat belongs to the phylum *Angiospermatophyta*, class *Monocotyledonopsida*, order *Poales*, family *Poaceae*, subfamily *Pooideae*, tribe *Triticeae*, subtribe *Triticinae* and genus *Triticum* (Balint *et al.*, 2000). Wheat is the common name used to identify the member of species belongs to the genus *Triticum*. Based on the somatic chromosome number the genus *Triticum*, can be divided in to diploid ($2n= 14$), tetraploid ($2n=28$) and hexaploid ($2n=42$). Examples of octoploid and decaploid wheat species can also be found in the literature (Goncharov, 2011). In general, the classification of *Triticum* species is complicated and often controversial because of specific adaption of various wheat species to particular regions of the world (Morrison, 1993). In the past the classifications by Mac Key (Mac Key, 1966; Mac Key, 1977) and Dorofeev (Dorofeev *et al.*, 1979) have been used by wheat researchers around the world. More recently, Goncharov studied the differences between the MacKey and Dorofeev classifications and proposed new classification of wheat species (Goncharov, 2011). The proposed classification of wheat species by Goncharov, (2011) is presented in Table 1.

Table 1. Classification of wheat species done by Goncharov, (2011).

Ploidy level	Species	2n	Genomes	Status
Diploid	<i>T. urartu</i>	14	A ^u A ^u	Wild
	<i>T. boeoticum</i>	14	A ^b A ^b	Wild
	<i>T. monococcum</i>	14	A ^b A ^b	Domesticated
	<i>T. sinskajae</i>	14	A ^b A ^b	Domesticated

Table 1. Continued.

Tetraploid	<i>T. dicoccoides</i>	28	A ^u A ^u BB	Wild
	<i>T. dicoccum</i>	28	A ^u A ^u BB	Domesticated
	<i>T. karamyshevii</i>	28	A ^u A ^u BB	Wild
	<i>T. ispahanicum</i>	28	A ^u A ^u BB	Domesticated
	<i>T. turgidum</i>	28	A ^u A ^u BB	Domesticated
	<i>T. durum</i>	28	A ^u A ^u BB	Domesticated
	<i>T. turanicum</i>	28	A ^u A ^u BB	Domesticated
	<i>T. polonicum</i>	28	A ^u A ^u BB	Domesticated
	<i>T. aethiopicum</i>	28	A ^u A ^u BB	Domesticated
	<i>T. carthlicum</i>	28	A ^u A ^u BB	Domesticated
	<i>T. araraticum</i>	28	A ^u A ^u GG	Wild
	<i>T. timopheevii</i>	28	A ^u A ^u GG	Domesticated
	<i>T. palmovae</i>	28	A ^u A ^u DD/ A ^b A ^b DD	Wild
Hexaploid	<i>T. aestivum</i>	42	A ^u A ^u BBDD	Domesticated
	<i>T. macha</i>	42	A ^u A ^u BBDD	Domesticated
	<i>T. spelta</i>	42	A ^u A ^u BBDD	Domesticated
	<i>T. sphaerococcum</i>	42	A ^u A ^u BBDD	Domesticated
	<i>T. compactum</i>	42	A ^u A ^u BBDD	Domesticated
	<i>T. kiharae</i>	42	A ^u A ^u BBGG	Wild
	<i>T. vavilovii</i>	42	A ^u A ^u BBDD	Domesticated
	<i>T. zhukovskyi</i>	42	A ^u A ^u A ^b A ^b GG	Domesticated
	<i>T. dimococcum</i>	42	A ^u A ^u A ^b A ^b BB	-----
Ocataploid	<i>T. flaksbergeri</i>	56	GGA ^u A ^u BBA ^u A ^u	-----
	<i>T. soveticum</i>	56	BBA ^u A ^u GGA ^u A ^u	-----
Decaploid	<i>T. borisii</i>	70	BBA ^u A ^u DDGGA ^u A ^u	-----

Of the 29 species listed in Table 1, only two wheat species such as *T. monococcum* and *T. aestivum* are used in this thesis.

1.2.1.1 Einkorn wheat - *T. monococcum*

T. monococcum is the domesticated form of diploid wheat (A^bA^b ; $2n=14$) and has a genome size of 5751 Mbp (Arumuganathan and Earle, 1991). It has three sub species; a wild *T. monococcum* subsp. *boeoticum*, domesticated *T. monococcum* subsp. *monococcum* and a weedy *T. monococcum* subsp. *aegilopoides* (Brandolini *et al.*, 2006). *T. monococcum* is believed to be the closest relative of *T. urartu* that donated A^uA^u genome to the major cultivated form of wheat species such as *T. durum* and *T. aestivum* (Dvorak *et al.*, 1988; Dvořák *et al.*, 1989; Dvořák *et al.*, 1993). Archaeological studies identified the northern and eastern parts of Fertile Crescent is the main centre of origin of *T. monococcum* (Harlan and Zohary, 1966; Zohary and Hopf, 1993) and it was domesticated around 7500 BC near Karaca Dag in southeast Turkey (Heun *et al.*, 1997). Although it is still cultivated in France, Italy, Spain, Morocco, the former Yugoslavia and Turkey for animal feed (<http://en.wikipedia.org/wiki/Einkorn>; Heun *et al.*, (1997)), it was forgotten by modern plant breeders as it was replaced with tetraploid and hexaploid wheat varieties (Kimber and Feldman, 1987; Kilian *et al.*, 2007). This untouched novel source of genetic variability in *T. monococcum* could easily be transferred and utilized for genetic improvement of other cultivated wheat species (Kilian *et al.*, 2007; Jing *et al.*, 2007). *T. monococcum* has already contributed genes involved in Na^+ exclusion, the *Nax1* and *Nax2* genes, to the field of salinity tolerance research which have been used to improve the salinity tolerance of commercial wheat cultivars (James *et al.*, 2006a; Byrt *et al.*, 2007; James *et al.*, 2011). This has led to the recently developed salinity tolerant durum wheat cultivar containing *Nax2* gene which is now producing 25% more yield in Australian saline soils (CSIRO, 2012). The potential sources of genetic variability found in *T. monococcum* for tolerance to various biotic, abiotic stresses, nutrient uptake and grain qualities are listed in Table 2.

Table 2. Potential sources of genetic variability found in *T. monococcum* for tolerance to diseases, pest, salt, frost, nutrient uptake and grain qualities.

Traits	References
Leaf rust resistance	Kerber and Dyck,(1990), Hussien <i>et al.</i> , (1997), Anker <i>et al.</i> , (2001), Sodkiewicz and Strzembicka,(2004).
Stripe rust resistance	Mihova,(1988)
Stem rust resistance	Soshnikova, (1990), Kerber and Dyck,(1990)
Blotch disease resistance	Ma and Hughes, (1993), Jing <i>et al.</i> , (2008)
Powdery mildew resistance	Shi <i>et al.</i> , (1998), Yao <i>et al.</i> , (2007a)
Scab resistance	Saur, (1991)
Hessian fly resistance	Bouhssini <i>et al.</i> , (1997)
Aphids resistance	Caillaud <i>et al.</i> , (1994), Deol <i>et al.</i> , (1995), Caillaud and Niemeyer, (1996), Di Pietro <i>et al.</i> , (1998)
Salinity tolerance	Munns <i>et al.</i> ,(2000b), James <i>et al.</i> , (2006a) James <i>et al.</i> , (2011)
Frost tolerance	Knox <i>et al.</i> , (2008)
Grain softness	See <i>et al.</i> , (2004)
Low molecular weight glutenin	An <i>et al.</i> , (2006)
Seed dormancy	Sodkiewicz,(2002)
Increased efficiency of Zn uptake	Cakmak <i>et al.</i> ,(1999)

1.2.1.2 Bread wheat – *T. aestivum*

T.aestivum is the major cultivated form of wheat that contributes to 95% of total wheat production in the world (Shewry, 2009). It is highly preferred by consumers for its nutritious flour which is mainly used to prepare different varieties of bread and other baked products (Bushuk, 1998). The wheat flour contains starch (65-75%), proteins (12-14%), most of the essential amino acids, fats (1.5-2%), minerals (1.5-2%), vitamin B complex, Vitamin E, Vitamin K and crude fibers (2.2%) (Izanloo, 2008; Shewry, 2009).

T.aestivum is a hexaploid (A^uA^uBBDD; 2n=42) with a genome size of 15966 Mbp (Arumuganathan and Earle, 1991). *T.aestivum* is thought to have originated through natural polyploidization process which occurred about 7000 years ago (Feuillet *et al.*, 2008). It is often used as an example to demonstrate allopolyploid speciation in plants (Dvořák *et al.*, 1993; Gustafson *et al.*, 2009). The evolution of *T.aestivum* is presented in Figure 1. *T. uratu* is believed to be the AA genome donor of *T. aestivum* (Huang *et al.*, 2002), however, the source of BB genome is still unknown but it could be a species belonging to *Sitopsis* and a close relative of *Aegilops speltoides*. The DD genome donor of *T. aestivum* is *Aegilops tauschii* (Dvořák *et al.*, 1993; Feuillet *et al.*, 2008). The F₁s derived from all of these three diploid species, after chromosome doubling, resulted the fertile hexaploid wheat, *T. aestivum*. Because of the DD genome, *T.aestivum* has obtained more adaptability to grow under various geographical regions of the world than any other cultivated tetraploid wheat species (Feuillet *et al.*, 2008).

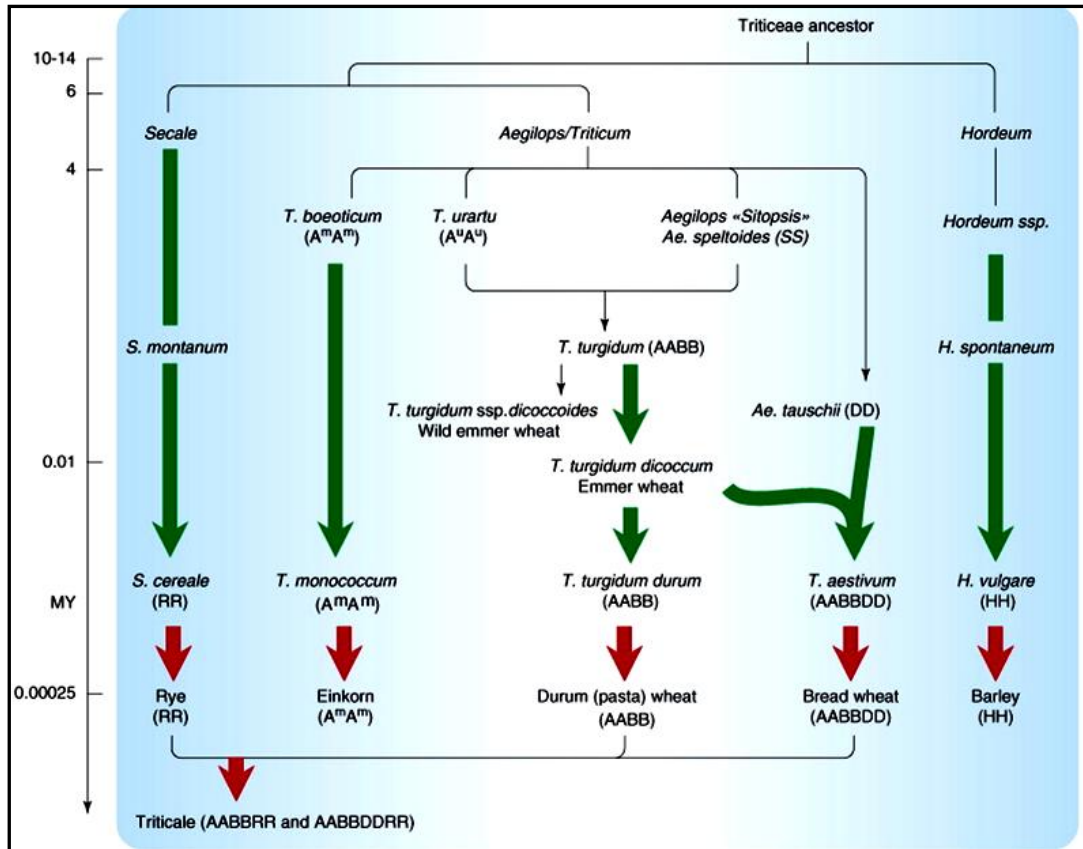


Figure 1. Evolution of rye, einkorn wheat, durum wheat, bread wheat and barley. Natural hybridization (black arrows), domestication (green arrows) and selection (red arrows) process are shown as well as the approximate timing of the event, in millions of years (MY) (Reproduced from Feuillet *et al.*, (2008)).

In addition, *T.aestivum* has undergone crop domestication imposed on it by humans which involves an artificial selection process that helps to modify undesirable characters in the wild forms into a desirable form for human purposes. During the process of domestication it has obtained desirable characters such as reduced plant height (Simons *et al.*, 2006; Hedden, 2003), higher yield (Pozzi and Salamini, 2007), non-shattering, reduced dormancy, soft textured and large size grains (Tanno and Willcox, 2006; Purugganan and Fuller, 2009; Eckardt, 2010) with less glumes and awns (Jantasuriyarat *et al.*, 2004; Simons *et al.*, 2006).

Another useful trait that was domesticated into *T.aestivum* was a life cycle that allowed it to grow and produce seed during the most favourable time of the year. In

temperate countries in the northern hemisphere, winter wheat is sown in autumn and harvested in summer; whereas spring wheat is sown in spring and harvested in autumn. Spring wheat completes its life cycle more quickly than winter wheat, whereas winter wheat takes longer to grow due to needing a long vegetative phase under a cool temperature treatment (a process called vernalization) which is required for flowering.

1.2.2 Morphology

An understanding of wheat morphology is important for this project because that helps to know the phenotypic changes in different genotypes of wheat under salt stress. In brief, wheat is annual plant with determinate growth habit. Plant height usually varies between 30-120 cm with both an adventitious and fibrous root system. It has cylindrical shoots with distinct nodes and internodes, with the internodes either being hollow in some cultivars or solid and filled with pith. Leaves are arranged on both right and left side of the shoot in a single plane (distichous alternate leaves) and every leaf comprises of leaf sheath and lamina. The leaf sheath is usually thick at its base with the margins of the sheath being thin and transparent. At the junction of leaf sheath and lamina membranous ligule and a pair of hairy auricles can be found. Tillers usually originate from the axils of the basal leaves and end up with the inflorescence that turned in to ear head at maturity stage. It has a terminal distichous spike type inflorescence, with a tough central rachis. The arrangement of spikelets in the inflorescence varies between different species or varieties within the same species (Kirby, 1974; Curtis, 2002). In general, wheat descriptors are widely used by plant researchers to characterize different genotypes of the same species and different species of *Triticum* (http://genbank.vurv.cz/ewdb/asp/IPGRI_descr_1985.pdf).

1.2.3 Growth and development

Physiologically, growth stages of wheat could be separated in to germination, emergence, initiation of first double ridge, terminal spikelet initiation, heading time, anthesis, grain filling, maturity and harvest (Slafer, 2003). Nevertheless these growth

stages can be grouped in to three main growth phases: vegetative phase, reproductive phase and the grain filling phase (Miralles and Slafer, 1999). The simplified schematic diagram of wheat development is shown in Figure 2. Vegetative phase begins with germination and ends up with the initiation of double ridges. During seed germination, seminal roots develop first, then the coleoptile. After the complete emergence of coleoptile, the first leaf blade unfolds. Subsequently, leaves are produced one in every 4-5 days. A total of 8-9 leaves are produced in most of the genotypes. At the time of fourth leaf emergence, a primary tiller starts to develop at the coleoptilar node. Subsequent primary tillers appear at regular intervals with fifth and sixth leaf emergence. These entire primary tillers share the common root mass with the main stem. From the auxiliary buds of primary tiller, secondary and tertiary tillers develop. Tillering is one of the most important agronomic characters because the number of tillers per plant usually determines the photosynthetic area and hence the single plant yield (Kasperbauer and Karlen, 1986). As it is the one of the critical stage of wheat development farmers usually apply fertilizers and nutrients at this stage to aid growth. In general, winter wheat produce more tillers than the spring wheat. However, most of the tillers won't produce spikes; they abort before anthesis. The development of increased number of productive tiller per plant is largely influenced by genotype \times environment interaction and the planting density. On the whole, the length of the vegetative stage may vary between 60 to 150 days. It depends on the occurrence of floral differentiation (double ridges) which is also largely influenced by major environmental factors such as photoperiod and vernalisation (Slafer and Rawson, 1994). In fact, there are two types of genes, the photoperiod responsive genes (*Ppd*) and vernalization responsive genes (*Vrn*), which are known to control floral development in wheat (Stelmakh, 1992; Dubcovsky *et al.*, 2006). Wheat is a self pollinated crop. Anthesis in the wheat inflorescence usually begins in the central part of the spike and continues towards the basal and apical part of it. After pollination, fertilisation of the ovule occurs, allowing the development of the seed, the grain filling stage. During this period starch deposition occurs in the endosperm and the embryo develops in the grain (Simmons *et al.*, 1995; Miralles and Slafer, 1999; Curtis, 2002). In this thesis most of experiments were done in the vegetative phase and hence the knowledge about the developmental changes occur in the vegetative phase is the important for this project.

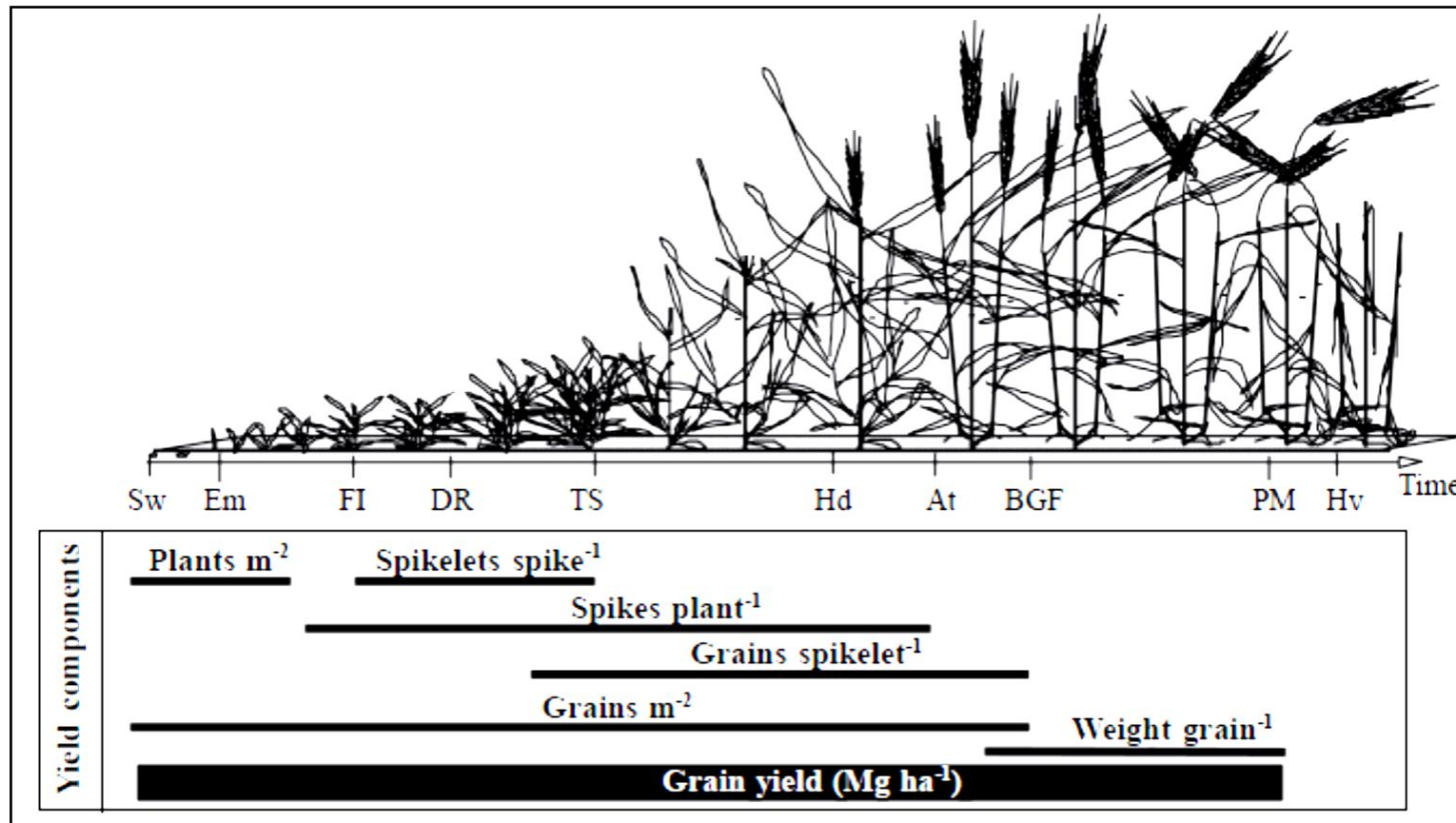


Figure 2. Schematic diagram displaying the growth and developmental stages of wheat. Sw: sowing, Em: emergence, DR: initiation of the first double ridge, TS: terminal spikelet initiation, Hd: heading time, At: anthesis, BGF: beginning of grain filling, PM: physiological maturity and Hv: harvest (Reproduced from Slafer,(2003)).

1.2.4 Wheat cultivation in Australia

Wheat was first introduced in to Australia, at the time of European settlement in 1788, from the ships of the first fleet, which carried varieties of plants and seeds for farming. Further wheat varieties, such as Red Lemmas, White Lemmas, Talavera, Red Tuscan and White Tuscan, were introduced from England and Western Europe between 1800 and 1850 (Simmonds, 1989). However, these introduced wheat cultivars were unable to adapt Australian climatic conditions because of their late maturing nature. Early efforts made by farmers, breeders and growers in the middle of the 18th century lead to the development of early maturing, disease resistant and high milling quality wheat cultivars, which were suitable to grow under Australian climatic conditions (Simmonds, 1989). The scientist, William James Farrer, who is still remembering as the “Father of the Australian Wheat Industry”, had developed wheat cultivars such as Federation, Canberra, Firbank, Cleveland, Pearlie White and Florence, and made a significant contribution to achieve a rapid progress in wheat production in early 19th century (http://en.wikipedia.org/wiki/William_Farrer). From 1950, onwards Australia started to produce surplus quantity of wheat grains and started to export in to world grain markets (Simmonds, 1989). In 2010-2011, wheat was planted on 14 million ha of Australian agricultural area producing 27.9 million tonnes, of which 18.6 million tonnes was exported (ACS, 2011). The wheat growing areas in Australia are shown in Figure 3.

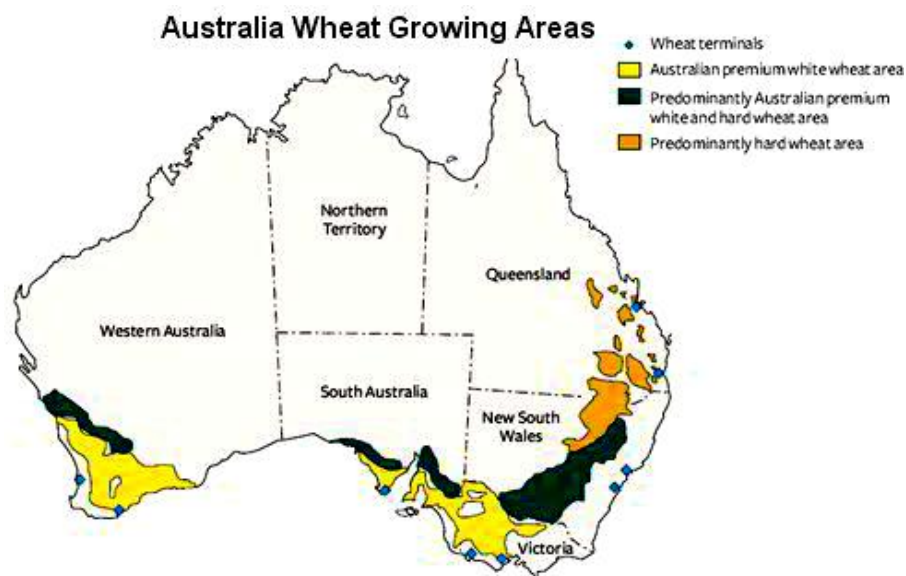


Figure 3. Wheat growing areas in Australia (Adapted from Sott.net, (2009)).

Wheat is cultivated in all of the Australian states except Northern Territory; however, the majority of wheat cultivation is in the Southern half of Australia from Western Australia, through South Australia and Victoria, to New South Wales. Most of the Australian wheat varieties are spring types. They are usually planted in late Autumn and harvested during early or mid summer (Simmonds, 1989).

1.2.5 Limitations in wheat productions in Australia

Drought is the major limiting factor that severely affects wheat production in Australia. Nevertheless, crop production is successful in most of the wheat growing states which have rainfall of 250 and 600 mm per year (Rengasamy, 2002). As the incidence of rainfall is highly unpredictable, drought affects wheat productivity in wheat growing regions to different levels from year to year. It was identified that Australia is free from major droughts only for 20 years in every century (William, 1985; Reynolds *et al.*, 1983). In addition to drought, subsoil constraints including salinity, sodicity, alkalinity, nutrient deficiencies and toxicities due to boron, carbonates and aluminates salinity reduce the yield potential of wheat in Australia (Rengasamy, 2002; Rengasamy *et al.*, 2003). Further, incidence of diseases and poor agronomic practices also limit the productivity of wheat in Australia (Brennan and Murray, 1998; GRDC, 2012a).

Since, drought has a major impact on wheat production; most of the breeding work has been done for drought tolerance and a little work for other stresses (Munns *et al.*, 2006). Particularly, salinity tolerance has had limited study even though it is the second important abiotic stress, after drought, and causes severe production losses in wheat. It is estimated that the dry land salinity in Australia can cost AU\$1.3 billion to the farming economy every year (Rengasamy, 2002). As the farmland affected by soil salinity is expected to increase in the forthcoming years; the loss of revenue of farming economy due to soil salinity is also predicted to increase in the future (NLWRA, 2001). Hence, the study of soil salinity and the development of salinity

tolerance of wheat cultivars are also mandatory to rectify these production losses in the upcoming years.

1.3 Soil salinity

1.3.1 Origin, classification and distribution of salt affected soils

Soil salinization is defined as the process of accumulating water soluble salts in the soil surface to such an extent that it leads to land degradation (Rengasamy, 2006). Approximately 800 million ha of the world area is affected by soil salinity which accounts for more than 6% of the total land area in the world (Martinez-Beltran and Manzur, 2005; Munns and Tester, 2008). Most of the salt affected soils are formed naturally by various pedological, hydrological and geochemical processes. In some areas, human activities such as insufficient irrigation or irrigation with poor quality water, insufficient drainage system or land levelling practices, dry season fallow practices in the presence of a shallow water table, misuse of heavy machinery to break heavy soils particles and chemical contamination can also lead to the development of salt affected soils (FAO, 2005).

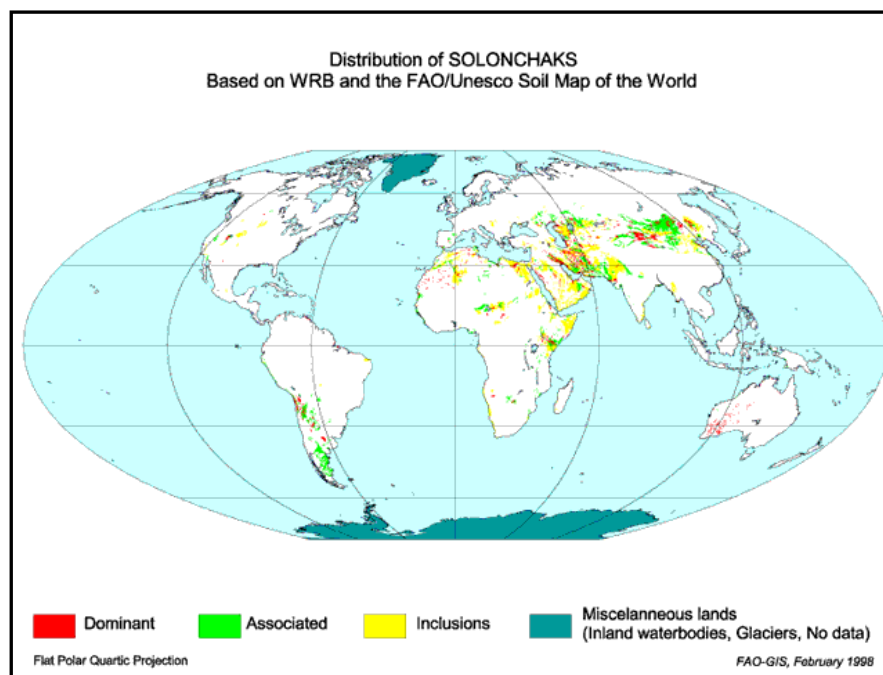


Figure 4. Distribution of salt affected soils over the seven continents in the world (Adapted from FAO, (2000)).

Salt affected soils are spread all throughout the world (Figure 4). Based on the difference in chemical properties, they could be divided in to two main classes such as saline and sodic soils (Szabolcs, 1974).

1.3.1.1 Saline soils

Saline soils possess soluble salts including chlorides (Cl^-) and sulphates (SO_4^{2-}) of sodium (Na^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) in the soil solution. Among them, sodium chloride (NaCl) is the most predominating salt occur in most of the saline soils in the world (Abrol *et al.*, 1988). Generally, saline soils do not possess carbonates and bicarbonates in them. Saline soils can be defined as soils with the electrical conductivity of saturation extract (EC_e) of > 4 dS/m, (approximately 40 mM), exchangeable sodium percentage (ESP) of > 15 % and $\text{pH} < 8.5$. Saline soils usually exhibit good soil structure and tillage characteristics for crop cultivation (Abrol *et al.*, 1988). Wheat is actually a moderately salinity tolerant crop. At the EC_e of 10 dS/m, wheat has shown a decreased yield, while rice died completely before reaching the maturity stage (Maas and Hoffman, 1977).

1.3.1.2 Sodic soils

Na^+ is the predominant cation found in sodic soil. The major anions are Cl^- , SO_4^{2-} , and bicarbonates (HCO_3^-); with little carbonates (CO_3^{2-}). Usually, the surface of the sodic soils are dry, hard and often appear black in colour (Abrol *et al.*, 1988). Sodic soils have high concentrations of exchangeable sodium (ESP > 15), with an electrical conductivity of saturation extract (EC_e) of < 4 dS/m and are extremely alkaline $\text{pH} > 8.5$ (Abrol *et al.*, 1988). Often both soil salinity and sodicity occur together in most of the times as the dominance of NaCl in saline soils favours the adsorption of Na^+ by soil particles resulting in them become sodic, when subject to leaching processes (Rengasamy *et al.*, 2003). Even, the low level of adsorbed Na^+ (6%) in the exchange sites of soil particles can cause severe soil structural degradation (Northcote and Srene, 1972).

Sodic soils have poor structure during dry conditions (Bernstein, 1975). This poor structure increases strength of soil, creates huge mechanical impedance to the growing root tips and limit root elongation and proliferation processes in the soil profile (Masle and Passioura, 1987). The poor soil structure of sodic soil does not allow germination of seeds at dry condition (Abrol *et al.*, 1988). Sodic soils also severely interferes with soil-water and soil-air relation, affect water transport and gas exchange in the rhizosphere (Rengasamy *et al.*, 2003). It reduces the porosity and permeability of soil and results slow water penetration and distribution in the soil profile (Oster and Jayawardane, 1998). Wheat crop grown in sodic soils with ESP>19% have large reductions in root growth and water extraction compared to wheat crop grown in soils with lower sodicity (ESP<19%) levels in the Southern Mallee district, South Australia (DPI, 2009).

Since, the exchangeable Na⁺ displaces exchangeable Ca²⁺ in the soil particle, sodic soils develops Na⁺ induced Ca²⁺ deficiency in wheat (Ehret *et al.*, 1990; Adcock *et al.*, 2001). As has been summarised by Naidu and Rengasamy (1993), sodic soils also have the increase the risk of getting CO₃²⁻ and HCO₃⁻ toxicities and deficiencies of other nutrients such as K, Fe, Mn, Mg, Cu, Zn and P in plants. On the whole, wheat growing in sodic soils does not get sufficient quantity of water, oxygen and nutrients, which is essential to obtain high yield and productivity.

1.3.2 Soil salinity in Australia

Salt affected areas are found in all climatic zones in the world. However the problem is more severe on arid (dry) and semi arid regions (Rengasamy, 2006). Australia is commonly known as the driest continent in world and more than 250 million ha of land is affected by sodicity. However, sodic soils in Australia are defined as soils with ESP ≥ 6; whereas US classification system defines soils with > 15 ESP are sodic (Rengasamy, 2002). The major factors in Australian salinity are seepage (water table induced) salinity and transient (non-water table induced) salinity (Rengasamy, 2002; Rengasamy, 2006). These are discussed below.

1.3.1.1 Water table induced salinity or Seepage salinity

The depth of water table from the land surface depends on the landscape of the area, for instance in valley floors the water table occurs very close to the surface. Under these conditions, natural salts present in these soils leach down and started to accumulate in the ground water. This salted underground water (EC=15 to 150 dS/m) does not affect growth of any natural vegetation, below 4 m from the soil surface (Rengasamy, 2006; Rengasamy, 2002). Importantly, deep rooted vegetations play a major role in keeping this ground water away from the surface of the soil. However, the replacement of deep rooted native vegetation with shallow rooted annual crops and/or pasture grasses at the time of European settlement in Australia, changed the water use pattern, resulting in the raising of waters table bringing salts to the soil surface, which are then concentrated in the topsoil after water evaporation. In general, seepage salinity areas are unproductive and not suitable for agriculture. Replanting of deep rooted perennials could be one of the best options that uses the under-ground water in most efficient way (Rengasamy, 2006; Rengasamy, 2002).

1.3.1.2 Non water- table induced salinity or Transient salinity

In Australia, salt has accumulated in the top soil by wind and rain over many thousands of years. In semi- arid conditions, rainfall has not been sufficient to leach all the salts accumulated in the top soil to deep ground water. Salt bulges form in the top soil, resulting in it becoming very saline and difficult to farm for cultivation. This transient salinity fluctuates with depth and its effect on plant growth varies both with season and rainfall (Rengasamy, 2002).

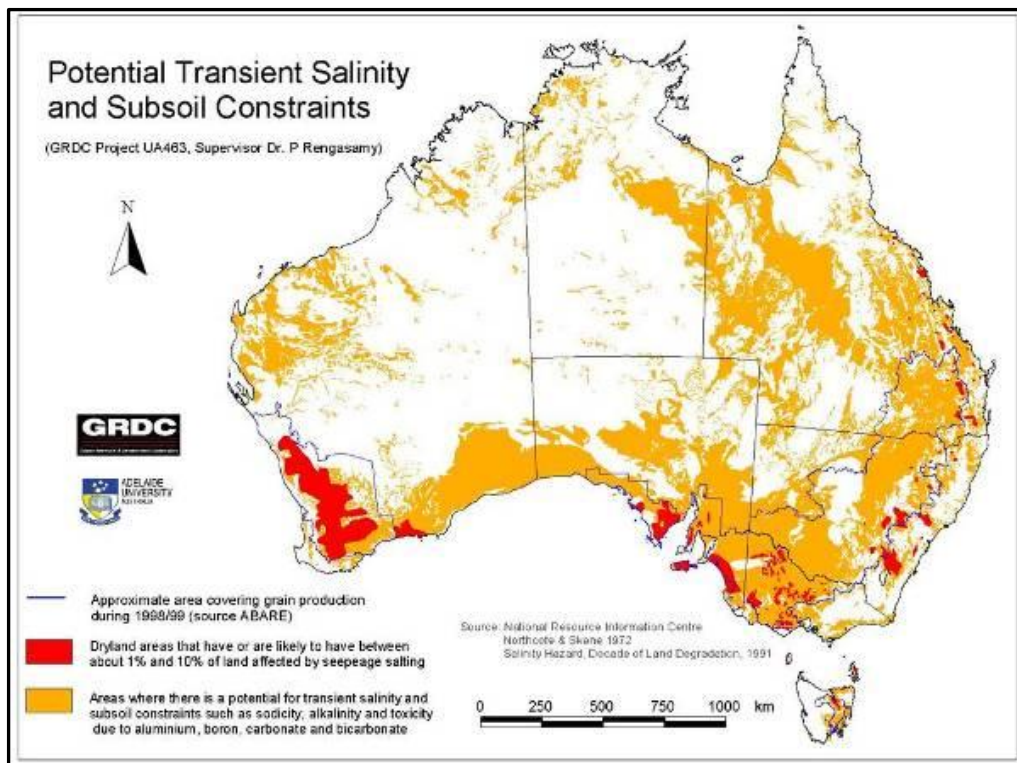


Figure 5. Map showing areas of dry land seepage salinity regions (red) with potential transient salinity and subsoil constraints (yellow) and the area of grain production in Australia (blue line) (Reproduced from Rengasamy,(2002)).

Out of 768 million ha of the total agricultural area, 16% of the cropping area is affected by water- table induced (seepage) salinity, and 67% of the cropping area is subjected to non-water table induced (transient) salinity (Figure 5) (Rengasamy, 2002; Rengasamy, 2006). Unless effective solutions are implemented the salt affected soils are expected to increase significantly in next 40 years, in Australia (NLWRA, 2001).

In general, salt affected areas could be manageable by preventing the influx of salt water through proper farm management practices such as crop rotation, plantation of deep rooted perennials (Munns, 2005), correcting soil toxicities by gypsum application, seed priming and foliar application of growth hormones (Rengasamy, 2002). Of course these high cost farming practices can provide

permanent solutions to soil salinity but they have low benefit to cost ratios and are long term solutions. Moreover, it is estimated that cultivation of crops in Australian saline and sodic soils without any management practices could increase annual profits up to AU\$ 1034.6 million (Hajkowicz and Young, 2005). Accordingly, the development of salt tolerance cultivars is getting more importance rather than other farm based solution to address the problem of soil salinity in Australia. However, the development of salt tolerant cultivar, particularly in wheat crop for this thesis necessitates getting a better understanding about the physiological basis of plant's salinity tolerance in a first hand.

1.4 Effect of salt stress on plant growth

Soil salinity mainly imposes osmotic and ion specific stresses on plants (Bernstein, 1975; Munns *et al.*, 1995). In general, the osmotic effect is caused by non-ionic specific factors, whereas, ionic damages of Na^+ and Cl^- are specific to particular plant species (Munns and Termaat, 1986).

1.4.1 Osmotic stress

The salt in the soil solution reduces water potential and increases the osmotic pressure of the soil solution. This increase in osmotic pressure makes plant much harder to extract the water from the soil. This effect is comparable to conditions a plant would experience in drought, even though water is available (Leon, 1963). When the osmotic pressure of soil solution is about -1000kPa, growth and yield of wheat decrease by more than 50 per cent. If it further increases to -1500kPa, plants will begin to wilt (Rengasamy, 2007; Rengasamy, 2010). In addition to the osmotic effect of soil salinity other environmental factors such as low humidity in the air increases the transpiration rate of plants growing in hot climates and further reduces the water potential of plants growing in saline environment (Munns and Tester, 2008).

During osmotic stress the plant reduces the loss of water through transpiration by closing the stomata, which in turn reduces CO₂ assimilation and photosynthetic rate and hence the growth of the crop (Kingsbury *et al.*, 1984; James *et al.*, 2002; El-Hendawy *et al.*, 2005; James *et al.*, 2008). Retarded shoot growth is the most common symptom of salt stress found in wheat (Nuttall *et al.*, 2006) grown in saline environments. It is widely believed that shoot growth of a crop is more sensitive to salt than root growth in saline conditions (Maas and Hoffman, 1977; Munns and Termaat, 1986; Ray and Khaddar, 1995). It is assumed that plants reduce the leaf area relative to the root growth, thereby conserving soil moisture and preventing further increases in salinity levels (Munns and Tester, 2008), however, with the advent of new imaging technologies this remains to be seen if still true. Since, osmotic stress reduces the cell division and cell elongation process (Yeo *et al.*, 1991; Passioura and Munns, 2000; Fricke and Peters, 2002) the prolonged reduction in cell division and elongation can lead to the development of smaller sized leaves. Moreover, as has been claimed by Munns and Tester (2008), as the area of the cell is more reduced than depth; osmotic stressed leaves appear smaller and thicker than normal leaves.

Osmotic stress has also reported to: affect the rate of seed germination and seedling emergence (Sayar *et al.*, 2010); affect the development of lateral shoot buds and reduces number of tillers; result in reductions leaf area and leaf number in wheat (Maas and Grieve, 1990; Nicolas *et al.*, 1993; Chazen *et al.*, 1995; De Costa *et al.*, 2007; Harris *et al.*, 2010) and other major cereal crops (Yeo *et al.*, 1991; Harris *et al.*, 2010). As has been stated in Munns and Tester (2008), early flowering and the production of fewer flowers is also one of the character of the osmotic stressed plant. However, under the severe saline conditions, osmotic stressed plants will wilt permanently (Munns *et al.*, 1995; Rengasamy, 2006). In wheat, before the premature wilting the shoot will start to develop a dull appearance, particularly on the leaf blade, followed by the greyish discolouration of leaf margins and tips. The grey discolouration slowly spread to the whole leaf laminar surface and the leaf will start to dry and wither. Shoot death will occur completely if the salt stress prolongs for a long time (CIMMYT).

1.4.2 Ionic stress

The salt in the soil solution consists of many ions such as Na^+ , Ca^{2+} , Mg^{2+} , Cl^- , SO_4^{2-} , HCO_3^- , NO_3^- and K^+ and while many of these ions are essential nutrients, when they accumulate to high concentrations in plant cells they can cause ion specific damages in plants (Bernstein, 1975). In plants, sodium (Na^+) and chloride (Cl^-) are the two key ions responsible for ion specific damages during salt stress. Na^+ is toxic to most of the annual crops such as wheat (Munns *et al.*, 2000b), rice (Yeo, 1992), and groundnut (Chavan and Karadge, 1980) and Cl^- toxic to woody perennials (Tester and Davenport, 2003) including avocado (Bingham *et al.*, 1968), grape vine (Downton, 1977; Walker *et al.*, 1981) and citrus (Cole, 1985; Walker *et al.*, 1993). Recent studies in wheat and barley also demonstrated the toxic effect of Cl^- on the plant growth and health (Martin and Koebner, 1995; Dang *et al.*, 2006; Islam *et al.*, 2007; Dang *et al.*, 2008; Tavakkoli *et al.*, 2011) and more research is required in this area to identify the genes and proteins involved in the movement of Cl^- through a crop. Nevertheless, as has been stated by Tester and Davenport (2003), Na^+ causes major ion specific damages to *graminaceae* crops such as wheat and rice, often before symptoms of Cl^- toxicity appear. Therefore, the current focus for this thesis will be only on Na^+ specific damages in wheat.

The symptoms of Na^+ toxicity can initially be observed by marginal chlorosis and necrosis in the leaf blade, which spreads to the leaf blade thus leading to premature leaf senescence (Munns, 2002; Tester and Davenport, 2003; Sheldon *et al.*, 2004). Usually, these symptoms of Na^+ toxicity begin in the older rather than the younger leaves. Because older leaves transpire longer than the younger leaves and accumulate more Na^+ than younger leaves at any given time (Colmer *et al.*, 1995). Since, the premature leaf senescence shortens the life time of individual leaves, the photosynthetic capacity of the plant get reduced, and hence the plant produce lower yield under saline conditions (Munns, 1993; Munns, 2002).

As has been outlined by Tester and Davenport (2003) the accumulated Na^+ ions in the leaf cytoplasm affect various metabolic processes in plant cells. Na^+ can compete with K^+ for binding sites of K^+ transporters leading to K^+ deficiency in plants. High Na^+ accumulation in the cytoplasm disturbs important cellular processes inside the cell (Munns, 1993), such as the activity of enzymes for protein synthesis, which requires K^+ for the binding of t-RNA to ribosome (Bhandal and Malik, 1988; Blaha *et al.*, 2000). High concentrations of Na^+ in a cell have been shown to disturb the function of sub-cellular components such as micro tubules, microfibrils, spherosomes and ribosomes (Mansour *et al.*, 1993). It is therefore essential that a low $\text{Na}^+ : \text{K}^+$ ratio in the cytoplasm is maintained to protect vital cellular processes (Gorham *et al.*, 1990; Dubcovsky *et al.*, 1996b; Maathuis and Amtmann, 1999).

Sometimes, accumulation of Na^+ inside the leaf apoplast also causes osmotic stress that reduces water potential of the cell, resulting in rapid dehydration of the cell. This is the side effect of accumulated Na^+ inside the plant cell, and it will begin once the vacuole stops to accumulate the incoming salt from the xylem stream (Evans and Sorger, 1966; Oertli, 1968; Evans, 1980; Xiong and Zhu, 2002; Munns, 2002). Eventually the plant dies because of building up of Na^+ ions either in the cytoplasm (ion toxicity) or in the cell wall (dehydration). However, salt sensitive genotypes develop these symptoms of ionic stress faster than the genotypes with any one of the ionic tolerance mechanism such as Na^+ exclusion and tissue tolerance. Besides, the degree of Na^+ specific damage also varies with the different levels of soil salinity and changes in the environmental conditions such as temperature and relative humidity. For instance, in hot climates, the low atmospheric air humidity increases the transpiration, favouring high Na^+ uptake and accumulation in plants than at cool climatic conditions (Lauter and Munns, 1987).

1.5 Use of two phase growth model to study the osmotic and ion specific effect of salt stress

The early observations made by Munns and Termaat (1986), lead to the formulation the two phase growth hypothesis, later by Munns (1993). According to the hypothesis, the response of plant growth under saline environment could be separated in two distinct phases; the initial “osmotic phase” and late “ion specific phase”. The osmotic phase begins immediately after an increase in salt concentration around the roots and causes rapid growth reduction in shoots. It stops old leaves expanding and inhibits new leaf emergence (Munns and Tester, 2008). This effect is believed to be due to salt concentration around the root rather than the specific ionic effect of Na^+ in the tissue, as the ions have not had time to build up to high concentrations in the shoot.

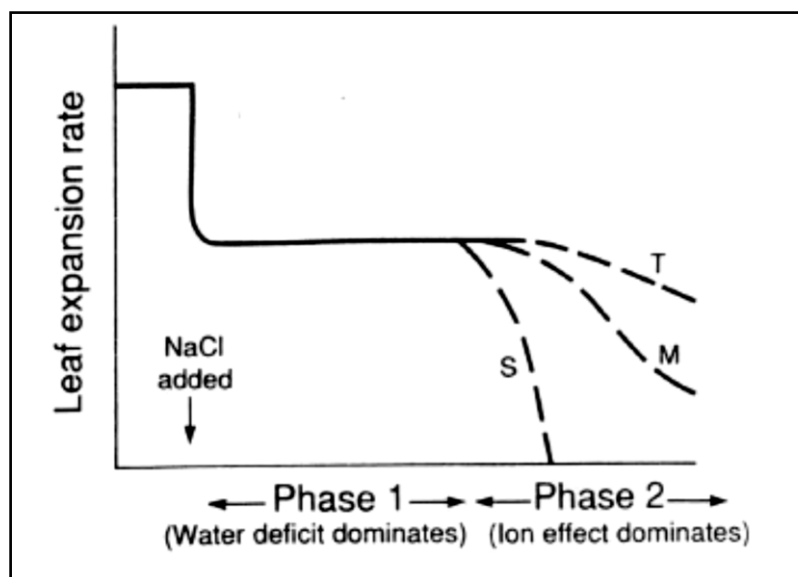


Figure 6. Hypothetical diagram displaying the two phase growth response of salt sensitive (S), moderately salt tolerant (M) and tolerant (T) cultivars of a particular plant species grown under saline environment. The salinity tolerance of the cultivars varies in terms of rate of leaf senescence that usually occurs once the salt becomes toxic in the leaf. Phase 1 indicates the effect of osmotic stress on plant growth immediately after NaCl application and the Phase 2 shows the effect of increased

accumulation of Na^+ in the leaves, on plant growth. During Phase 1, all of these cultivars have shown similar response but with decreased plant growth. At Phase 2, the increased Na^+ accumulation in the leaves further decreased the growth of salt sensitive cultivar than moderately tolerant and tolerant cultivars (Reproduced from Munns, (1993)).

The osmotic effect of NaCl at the initial stage of shoot growth could be similarly explained by other salts of same osmotic pressure (Munns and Termaat, 1986), mannitol and polyethylene glycol (PEG) (Yeo *et al.*, 1991; Sumer *et al.*, 2004). It is not due to ion toxicity because, salt always accumulate in the rapidly expanding tissues than the growing cells and keeps the salt concentrations of shoot apices low to grow as normal (Munns *et al.*, 1995). Moreover the reduction in shoot growth rate is independent of carbohydrate supply (Munns *et al.*, 2000a), water status (Munns *et al.*, 2000a) and nutrient deficiency (Hu *et al.*, 2007). The response of plants to osmotic stress can be seen very quickly and it may vary between minutes to several days (Yeo *et al.*, 1991; Munns, 2002).

Once salt has been transported inside the plant, it is usually sequestered in the vacuoles. However, it can build up to toxic concentrations in the cytoplasm and results in Na^+ specific damage to the plant (Munns, 1993) as discussed above in the section 1.4.2. Accordingly, the second specific ion response phase starts when the salt concentration inside the leaf reaches toxic level. It is a long term phase at this stage that salt sensitive genotypes reduce shoot growth rate and die faster than salt tolerant genotypes (Munns, 1993).

The two-phase growth model was first validated by Munns *et al.*, (1995). The data obtained from this study, strongly supported the two phase growth hypothesis and demonstrated osmotic and ion specific effect of salt stress in wheat and barley cultivars grown under salt stressed condition over time.

Nevertheless, there are currently two different schools of thought which argued the relative importance of osmotic and specific ion damages on plants. The osmotic school of thought (Lutts *et al.*, 1996; Rivelli *et al.*, 2002; Ghoulam *et al.*, 2002) believe that the most of the adverse effects of salinity on grain yield are due to decreased osmotic potential of the saline soil inhibiting growth, whereas the specific ion school of thought (Kingsbury and Epstein, 1986; Montero *et al.*, 1998) suggests that the effects of individual ions, such as Na⁺, have the greatest effect on yield. Attempts have been made in the past to study both main and interactions of osmotic and ion specific effect of salt with the use of glucose, sucrose, D-Mannitol, polyvinyl pyrrolidone (Wiggans and Gardner, 1959) and polyethylene glycol (Yeo *et al.*, 1991; Neumann, 1993), however, the response of plants in organic osmotic agents compared to iso-osmotic salt solutions has given inconclusive results. For example the plant growth inhibition in inert osmotic like Polyethylene glycol is more than salt solution in some studies and less in other cases. In addition, inert osmotic media has failed to provide to information about the specific ion effect where as saline media can provide one or more ions for plant absorption and accumulation thus enhance to understand the specific ion effect along with the osmotic effect. As has been summarized by Munns and Tester (2008), through the use of non-destructive measurements of plant growth and health it is possible to study the osmotic and ion specific effects of salt stress at a same time and determine which stress has a greater effect on plant survival, and whether it is necessary to increase the salt tolerance of a plant at both osmotic and ionic phase is important to develop a complete tolerant plant rather than any one of the phase.

1.6 Components of salinity tolerance

Salinity tolerance is the ability of a plant to grow and complete its life cycle on a substrate that contains high concentrations of soluble salts (Asins *et al.*, 1993). It can also be defined as the inherent ability of plants to withstand the effects of high salt concentrations in the root zone or on the leaves without a significant adverse effect (Yeo, 1983). For cereal crops, such as wheat, salinity tolerance is defined as crops which are able to maintain grain production when grown on saline soils. There are

three major components of salinity tolerance such as osmotic tolerance, Na⁺ exclusion and tissue tolerance to Na⁺, which help plants to cope up with saline environment. They are discussed below in detail.

1.6.1 Osmotic tolerance

Osmotic tolerance helps plant to withstand salt induced osmotic stress condition. Specific mechanisms of osmotic tolerance are yet to be fully understood. It could be chemical and hormonal signals developed from roots may control the growth and development of leaves (Termaat *et al.*, 1985; Munns and Termaat, 1986; Westgate *et al.*, 1996). It could be a function of Abscisic acid (ABA); the levels of ABA increases with high salt concentration in the plants (Kato-Noguchi, 2000). ABA plays an important role in controlling plant growth in saline soils by changing the root: shoot ratio as well as communicating soil conditions to the leaves. ABA is partially responsible for stomatal closure in the transpiration stream of wheat plants under water deficit conditions (Munns, 1992). Other plant hormones such as jasmonates are also found to modulate the expression of numerous genes and influence specific aspects of plant growth, development and response under osmotic stress conditions. But the signal transduction pathway of jasmonates is unknown and it is presumed that jasmonates modify the transcription and translational pathways in plants by interacting with cell receptors (Sairam and Tyagi, 2004).

In addition to hormones, plant antioxidant system is also playing an important role to achieve increased osmotic tolerance in wheat (Sairam *et al.*, 1997b; Sairam *et al.*, 2002). The reduction in photosynthetic rate in the osmotic stressed conditions leads to the formation of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radical inside the plant cell. ROS are partially reduced forms of atmospheric oxygen which can damage DNA, RNA and proteins and do oxidative destruction of plant cell, at excess levels (Asada, 1999; Mittler, 2002). Since, ROS are toxic in nature, plant cells increases the activity of antioxidant enzymes enzymes such as such as catalase, glutathione reductase, superoxide dismutase and glutathione-S-transferase and metabolites including ascorbic acid, glutathione, α -tocopherol,

carotenoids and flavanoids inside the plant cell (Smirnoff, 1995). These enzymes scavenge ROS, effectively regulate the detoxification process and help to keep the concentration of ROS, at the levels required to activate stress responsive signalling pathways (Asada, 1999; Garratt *et al.*, 2002). Under water deficit conditions, higher level of these antioxidant enzymes and metabolites are found in tolerant wheat cultivars than the susceptible ones (Sairam *et al.*, 1997a; Sairam *et al.*, 1997b; Sairam *et al.*, 1998; Sairam *et al.*, 2000; Sairam *et al.*, 2002).

1.6.2 Na⁺ exclusion

It is well documented that a degree of salt tolerance in plants is associated with a more efficient system for the selective uptake of K⁺ over Na⁺ (Tester and Davenport, 2003). Plants usually regulate ionic balance (between Na⁺ and K⁺) inside the cell to maintain normal metabolic functions. Na⁺ excluders are able to exclude more Na⁺ from shoots than salt sensitive plants and in those plants the degree of Na⁺ tolerance is inversely proportional to shoot Na⁺ content (Munns and James, 2003). In many studies low Na⁺ in the leaf blade of wheat is found to be correlated with salinity tolerance (Ashraf and Oleary, 1996; Rashid *et al.*, 1999; Munns *et al.*, 2000b; Poustini and Siosemardeh, 2004). Na⁺ exclusion could be achieved by various ion channels and transporters imbedded across the cell membrane that help to minimize the amount of Na⁺ uptake and regulate Na⁺ transport throughout the plant. The schematic diagram showing the function of ion channels, transporters and pumps involved in Na⁺ exclusion and tissue tolerance mechanisms in the plant cell is adapted from Plett and Moller (2010) and presented in Figure 7.

In general, the electrochemical gradient across the plasma membrane influences the passive entry of Na⁺ through channels (Higinbotham, 1973). The channels, including K⁺ inward rectifying channels, K⁺ outward rectifying channels and non-selective cation channels (NSCC), embedded in the plasma membrane provides a way for Na⁺ influx in to the plant cell (Yamaguchi and Blumwald, 2005). Especially, the NSCC, demonstrated high Na⁺/K⁺ selectivity and facilitated high Na⁺ influx in to the plant cells (Maathuis and Amtmann, 1999). The NSCC usually transport cations such as

Na^+ , K^+ , Ca^{2+} and NH_4^+ in to the plant cell (Demidchik *et al.*, 2002). Na^+ competes with these cations especially with K^+ and enters in to the plant cell. These NSCC are voltage-independent and largely influenced by external Ca^{2+} (Roberts and Tester, 1997; Plett and Moller, 2010). The genetic nature of the NSCC is still unclear, however cyclic nucleotide gated channels and glutamate receptors could be a potential candidates (Demidchik *et al.*, 2002).

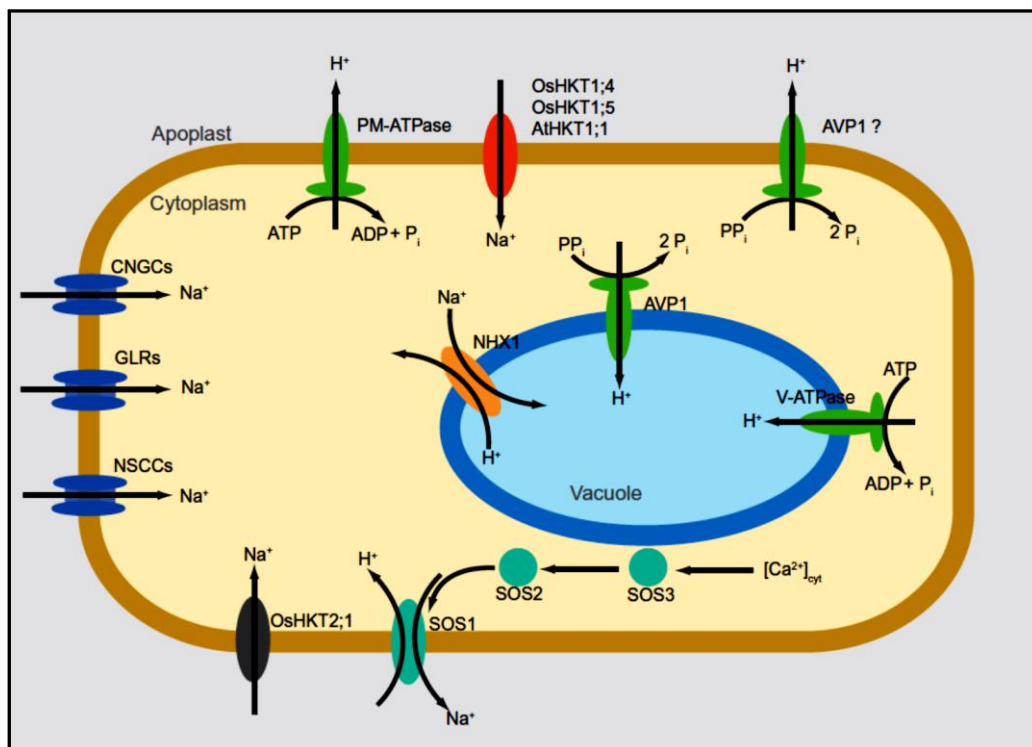


Figure 7. Diagram showing the function of ion transporters, channels and pumps involved in Na^+ exclusion and tissue tolerance mechanisms in the plant cell. Influx of Na^+ ions is occur through cyclic nucleotide gated channels (CNGCs), glutamate receptors (GLRs), non-selective cation channels (NSCCs) and HKT transporters (AtHKT1:1, OsHKT1:4, OsHKT1:5 and OsHKT2:1), whereas the efflux of the Na^+ ions from the cells are occur through Na^+/H^+ antiporter (SOS1) that interacts with the serine/threonine protein kinase (SOS2) and the calcium binding protein (SOS3), vacuolar storage of Na^+ is mediated by a vacuolar Na^+/H^+ antiporter (NHX) and the electrochemical potential is provided by the vacuolar H^+ pyrophosphatase (AVP1)

and the vacuolar H⁺-ATPase (V-ATPase) (Reproduced from Plett and Moller, (2010)).

Likewise, active transport of Na⁺ also enters through the symporters and antiporters located across the plasma membrane. They transport Na⁺ against the electrochemical potential of the plasma membrane and it usually happens with the exchange of one H⁺ (proton) with one Na⁺. As has been summarized by Tester and Davenport (2003), the ion transport mechanisms that minimize the shoot and leaf Na⁺ concentration may include, minimization of initial Na⁺ entry in to the roots from the soil, maximising efflux of Na⁺ from roots back to the soil, minimizing loading of Na⁺ into xylem vessels which transport solutes to shoots, maximising retrieval from xylem vessels in the root and maximising Na⁺ recirculation from shoots via the phloem vessels. In fact, the *HKT* (High affinity potassium transporter) gene family plays a crucial role in exclusion in a number of plant species (Munns *et al.*, 2006; Munns, 2002; Tester and Davenport, 2003).

Importantly, the *HKT* genes from *T. monococcum* have already made a great contribution to the field of salinity tolerance research. There are actually two different *HKT* family genes *Nax1* and *Nax2* from *T. monococcum* were initially identified in the durum wheat line 149, while doing screening for Na⁺ exclusion in the international durum wheat collections (Munns *et al.*, 2003). *Nax1*, is the Na⁺ transporter belongs to *HKT* gene family and identified as *HKT7* (*TmHKT1;4*) in the chromosome 2A (Lindsay *et al.*, 2004; Huang *et al.*, 2006a), whereas, *Nax2* was detected as *HKT8* (*TmHKT1;5*) on the chromosome 5A of durum wheat (Byrt *et al.*, 2007). These *Nax* genes are found helpful to remove Na⁺ from the xylem stream and maintain low [Na⁺] in the leaf blade. However, *Nax1* removes Na⁺ from the xylem in both roots and the leaf sheaths, whereas *Nax2* removes Na⁺ from the xylem only in roots (James *et al.*, 2006a). These *Nax* genes have already been used to improve salinity tolerance of durum wheat and bread wheat. The commercial cultivar of durum wheat cultivar with *Nax2* has produced 25% more yield in saline soils (CSIRO, 2012). Similarly, presence of both *Nax1* and *Nax2* has decreased the leaf blade [Na⁺] by 60% in bread wheat and

demonstrated the potential to increase salinity tolerance of bread wheat in the future (James *et al.*, 2011).

HKT genes expressed in xylem parenchyma cells are found helpful to protect leaves from Na^+ induced salt toxicity by retrieving Na^+ from xylem in both roots and shoot and hence reducing the amount of Na^+ accumulation in shoots (Sunarpi *et al.*, 2005; Horie *et al.*, 2006; Platten *et al.*, 2006; Davenport *et al.*, 2007; Horie *et al.*, 2009). For example, the protein encoded by an *OsHKT* genes retrieving Na^+ from the transpiration stream into the cells around the xylem are shown in Figure 7. The *HKT* genes have now been identified in *T.aestivum* (Rubio *et al.*, 1995), barley (Haro *et al.*, 2005) and durum wheat (Byrt *et al.*, 2007).

Likewise, the Na^+/H^+ antiporter SOS1, on the plasma membrane, effluxes Na^+ from cells and may be important in Na^+ exclusion from roots in to the external medium (Zhu, 2003; Luan, 2009; Weinl and Kudla, 2009). It is expressed in root cells (Munns, 2005; Davenport *et al.*, 2007). *Arabidopsis* undergoing salt stress release Ca^{2+} into the cytoplasm, from intracellular and extracellular stores, which binds in to the plasma membrane bound AtSOS3. AtSOS3 recruits the kinase AtSOS2 to the plasma membrane. AtSOS2 phosphorylates AtSOS1 thereby facilitating the movement of Na^+ out of the cell and helps in salinity tolerance. In addition to *Arabidopsis* these genes could also be found in other crops such as *Thellungiella halophila* (Vera-Estrella *et al.*, 2005), poplar (Wu *et al.*, 2007) and rice (Kolukisaoglu *et al.*, 2004). In general Na^+ exclusion mechanisms are effective at low to moderate levels of salinity.

1.6.3 Tissue tolerance

Even though, the control of Na^+ accumulation in the leaf blade is very important to obtain salinity tolerance in cereal crops, often salinity tolerance demonstrate no or little relationship leaf $[\text{Na}^+]$ and salinity tolerance, for example in some cultivars of

wheat (Ashraf and McNeilly, 1988; Hollington, 2000; Genc *et al.*, 2007). These studies showed that in some cases there are weak relationships between the level of exclusion and salinity tolerance, and also the contribution of other physiological traits such as tissue tolerance which help plants to stay green while accumulating high Na⁺ concentrations in the leaf tissue through vacuolar compartmentation.

Vacuole is commonly known as a storage organ, occupying up to 99% of the cell volume and can be used as a store for inorganic ions which are accumulated in the mature plant cell under saline conditions (Flowers *et al.*, 1977; Karley *et al.*, 2000b; James *et al.*, 2006b). The theory of intercellular compartmentation of inorganic solutes in the vacuole was first postulated by researchers in early 1970's (Jennings, 1968; Pierce and Higinbotham, 1970; Flowers, 1972; Greenway and Osmond, 1972; Shepherd and Bowling, 1973), however, direct evidence for K⁺/Na⁺ exchange across the tonoplast and compartmentation in the vacuole was first reported by Jeschke and Stelter (1976) in barley and *Atriplex* root cells. Subsequently, many other studies have studied intercellular compartmentation of Na⁺ and other ions in other cereal crops (Huang and Van Steveninck, 1989; Leigh and Storey, 1993; Fricke *et al.*, 1996; Colmer *et al.*, 2005; James *et al.*, 2006b).

The excessive ion storage in the vacuole, usually demonstrate differential pattern of Na⁺ accumulation in the leaf cells. Several studies identified huge differences in Na⁺ and Cl⁻ accumulation in the leaf mesophyll and epidermal cells in barley (Leigh and Storey, 1993; Fricke *et al.*, 1994; Fricke *et al.*, 1996) and wheat (James *et al.*, 2006b). Under both low and non-saline conditions Na⁺ is preferentially accumulated at higher concentrations in the epidermal cells than the mesophyll cells (Karley *et al.*, 2000a). However, at high salinity levels, an even distribution of Na⁺ accumulation can be found between mesophyll and epidermal cells (James *et al.*, 2006b).

The movement of ions from xylem apoplast to vacuoles in the leaf cells is controlled by various transporters and ion selective channels located along multiple membranes,

with the vacuole considered to be an important site of ion accumulation (Karley *et al.*, 2000b). The effective compartmentalization of Na^+ inside the vacuole could be achieved by the presence of salt inducible Na^+/H^+ antiporters, such as NHX1, NHX5 and NHX6, as well as V-PPases AVP1, AVP2 which set up proton gradients across the tonoplast, allowing other transporters to move Na^+ into the vacuole. (Blumwald *et al.*, 2000; Gaxiola *et al.*, 2001; Zhu, 2003; Bassil *et al.*, 2011).

NHX1 is a Na^+/H^+ antiporter on the tonoplast membrane. It is expressed in roots and leaves. It selectively transports Na^+ into the vacuole by exchanging one H^+ under saline environment (Blumwald *et al.*, 2000) (Figure 7). It can be found in various plant species including barley (Garbarino and DuPont, 1989), maize (Zörb *et al.*, 2005), sunflower (Ballesteros *et al.*, 1997), tomato (Wilson and Shannon, 1995), cotton (Wu *et al.*, 2004) and *Arabidopsis* (Jha *et al.*, 2010) and helps to increase the Na^+ accumulation and attain higher salinity tolerance.

Likewise, a proton transporter located on the tonoplast membrane (AVP1), uses the energy from the breakdown of pyrophosphate to pump protons into the vacuole, which are likely to help with the sequestration of Na^+ into vacuoles (Davies, 1997; Munns, 2005) (Figure 7). Constitutive expression of AVP1, either alone or in combination with NHX, was used to increase salinity tolerance in *Arabidopsis*, alfalfa, tobacco and bentgrass (Gaxiola *et al.*, 2001; Zhao *et al.*, 2006; Duan *et al.*, 2007; Gao *et al.*, 2006; Brini *et al.*, 2007; Bao *et al.*, 2009).

Usually compartmentation of Na^+ ions within the vacuole will lead to an osmotic imbalance between the vacuole and cytoplasm. Under these conditions, plants decrease the osmotic potential of cell by increasing the accumulation of compatible solutes in the cytoplasm and maintain equilibrium between cytoplasmic and vacuolar osmotic potential inside the cell (Flowers *et al.*, 1977; Wyn Jones *et al.*, 1977; Ludlow and Muchow, 1990). This process is commonly known as osmotic adjustment. Osmotic adjustment is an important cellular process that helps plant not only to

withstand in the water limited environment but also facilitate to obtain high yield in wheat (Morgan, 1977; Morgan, 1995).

Compatible solutes play a major role in osmotic adjustment process and accumulation of these solutes are found to increase the salinity tolerance of many crops. Compatible solutes are low molecular mass compounds and they do not interfere with the normal biochemical reactions in plants (Hasegawa *et al.*, 2000). These compatible solutes include carbohydrates such as sucrose, mannitol, pinnitol, oronitol, sorbitol, glycerol, arabionitol (Munns, 2002; Ghoulam *et al.*, 2002; Sairam and Tyagi, 2004) and nitrogen containing compounds namely, betaine, glutamate, aspartate, proline, glycine, choline and putrescine (Mansour, 2000; Sairam and Tyagi, 2004). Among them, glycine betaine and proline are the compounds commonly synthesized to high concentrations various crops undergoing salinity stress. The differences in the accumulation of these compatible solutes usually vary between different species and between varieties or even between different genotypes.

Halophytes, can synthesize and accumulate higher amounts of proline and glycine betaine than glycophytes in the leaf and protect cells from the osmotic cell damage (Jones and Storey, 1978). The accumulation of glycine betaine has also been associated with salinity tolerance of wheat (Sairam *et al.*, 2002) and other crops such as sorghum (Weimberg *et al.*, 1984), maize (Saneoka *et al.*, 1995). Similarly, accumulation of proline, is found to be associated with salinity tolerance in different crops (Stewart and Lee, 1974; Ahmad *et al.*, 1981; Fougère *et al.*, 1991; Petrusa and Winicov, 1997). However, accumulation of compatible solutes does not always enhance salinity tolerance of crops. For example there was no significant correlation found between glycine betaine accumulation and salinity tolerance in different species of *Triticum*, *Agropyron* and *Elymus* (Wyn Jones *et al.*, 1984). In barley, two fold increased accumulation of proline and glycine betaine was identified in the leaves of salt sensitive cultivar by Chen *et al.*,(2007). Likewise, accumulation of proline does not effectively contribute to the osmotic adjustment of salt stressed rice cultivars (Lutts *et al.*, 1996).

Wheat synthesizes glycine betaine and proline, under water stressed condition in a natural way however, further enhancement of glycine betaine and proline synthesis could also be achieved through wide hybridization (Colmer *et al.*, 1995) and genetic transformation to get high accumulation improved salinity tolerance in wheat (Colmer *et al.*, 2005). In wheat, a gene *P5CS* from *Vigna aconitifolia* was transferred to increase the accumulation of proline. The transformed wheat plants have shown higher proline accumulation and increased salt tolerance either in the presence or absence of salt (Sawahel and Hassan, 2002). High salt tolerance was also achieved with the expression of *mtLD*, a gene for mannitol synthesis in the *T.aestivum* (Abebe *et al.*, 2003). Moreover, genetic engineering of compatible solutes biosynthesis and high salt tolerance was achieved in tobacco (Lilius *et al.*, 1996; Holmstrom *et al.*, 2000; Huang *et al.*, 2000a; Nuccio *et al.*, 1998; Nuccio *et al.*, 2000; McNeil *et al.*, 2000) *Arabidopsis* (Hayashi *et al.*, 1997; Sakamoto and Murata, 2001) rice (Sakamoto and Murata, 2001) and canola (Huang *et al.*, 2000a) in an artificial way.

Apart from the osmotic adjustment, compatible solutes can also act as ROS scavengers (Wang *et al.*, 2003) and help to protect cells from oxidative stress. However, synthesis of compatible solutes is an energy consuming process and hence some plants use the accumulated Na^+ in the leaves to maintain turgor particularly under dry land conditions (Raven, 1985). The accumulation of Na^+ in leaves requires less energy than the synthesis of any other osmolytes such as glycine betaine or proline to maintain turgidity under salt stressed environment (Raven, 1985).

1.7 Use of imaging platform to study changes in morphological and physiological features of various agricultural crops

One of the main objectives of this thesis is to use the recent advances in imaging technology and quantify growth and health measurement of plants non-destructively for salinity tolerance studies. Hence, it is necessary to review the importance of imaging technology to study the morphological and physiological features of agricultural crops. Images contain large amounts of information on the object being

photographed (Jaffe *et al.*, 1985). They often use ultrasonic (Schätzer, 1967), radiographic (Bell *et al.*, 1994), electromagnetic (Chong *et al.*, 2001; Prasad *et al.*, 2009; Mewes *et al.*, 2009) and optic waves (Kanner and Schilder, 1930) for imaging. Among them, images acquired from electromagnetic waves are more helpful to do research in biological sciences for various purposes (Tillett, 1991).

Electromagnetic waves naturally exist with a continuous range of frequencies or wavelengths, which is commonly known as electromagnetic spectrum. The electromagnetic spectrum is often divided into regions of radio waves, microwaves, infrared radiation, visible light, ultraviolet radiation, X-rays and gamma rays. These regions differs each other in terms of frequencies or wavelength, which is arranged in the order of increasing frequency and decreasing wavelength from left to right in the electromagnetic spectrum (http://en.wikipedia.org/wiki/Electromagnetic_spectrum). For instance, radio wave with the longest wavelength and lowest frequency is located on the far left of the spectrum whereas; gamma ray with the shortest wavelength and highest frequency is located on the far right (Figure 8). In fact, the frequency of electromagnetic waves in the electromagnetic spectrum is directly proportional to the energy of the particular wave that it carries (http://en.wikipedia.org/wiki/Electromagnetic_radiation). So, the energy of the electromagnetic wave increases with the decreasing wavelength and increasing frequency. For instance, gamma rays, the highest frequency wave in the electromagnetic spectrum possess highest energy and become the more dangerous radiation than other waves in the far left of the electromagnetic spectrum.

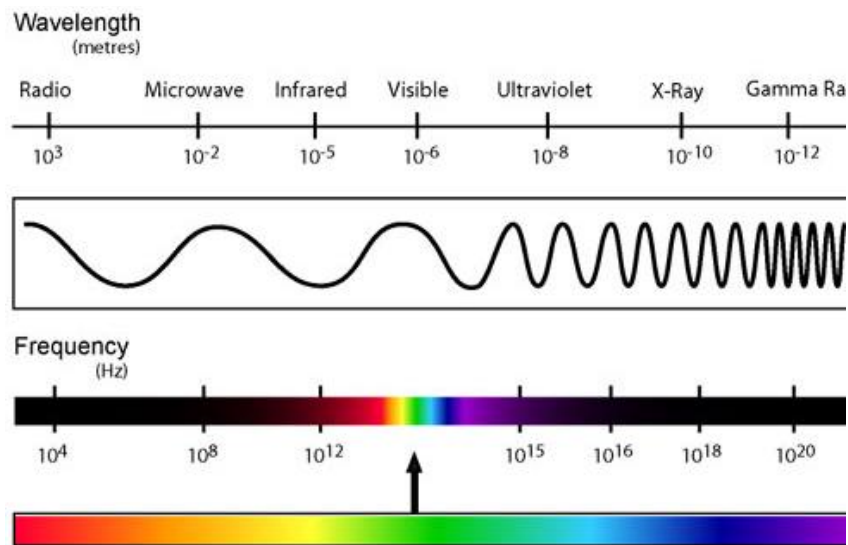


Figure 8. The electromagnetic spectrum with radio waves, microwaves, infrared radiation, visible light, ultraviolet radiation, x-rays and gamma rays (From left to right, in the order of increasing frequency and decreasing wavelength). (Adapted from Google images <http://zebu.uoregon.edu/~imamura/122/lecture-2/em.html>).

Electromagnetic waves have several practical uses in medical and other research areas (Dhawan, 2003; Umbaugh, 2005; Rangayyan, 2005; Oerke *et al.*, 2011). One of the important uses of these electromagnetic waves is capturing images of biological objects, including things which are not visible to human eye. Particularly, images acquired through visible light and infra-red radiation are helpful in plant biology. Images taken using infra red rays and visible light (RGB) can be used to detect changes in the morphological and physiological responses of stressed plants in various crops in a meaningful manner and they have already made remarkable contributions in the agricultural research in the past decades. In addition to this, fluorescence images acquired from blue, green, red and far-red region of the electromagnetic spectrum can also be very useful to study the photosynthetic efficiency and disease assessment in different agricultural crops. Some of the important uses of the infrared, visible light and fluorescence images are listed in Table 3.

Table 3. Important contribution of RGB, infrared and fluorescence images to study the morphological and physiological characteristics of agricultural crops.

Type of images	Uses	References
RGB images (Visual light)	Plant growth	Jaffe <i>et al.</i> , (1985), Meyer and Davison, (1987), Tackenberg, (2007), Rajendran <i>et al.</i> , (2009)
	Plant morphology	Casady <i>et al.</i> ,(1996), Soille, (2000), Foucher <i>et al.</i> , (2004)
	Grading fruits and vegetables	Tao <i>et al.</i> ,(1995), Blasco <i>et al.</i> , (2003), Changyong <i>et al.</i> , (2009), Arefi <i>et al.</i> , (2011)
	Identification of varieties and screening plant genetic resources	Cooke,(1999), Brindza <i>et al.</i> , (1999)
	Seed dimensions	Churchill <i>et al.</i> , (1992)
	Disease assessment	Lindow and Webb, (1983)
	Onset of senescence	Adamsen <i>et al.</i> ,(1999) , Rajendran <i>et al.</i> , (2009)
	Weed interference	Cobber and Morrison,(2011), Piron <i>et al.</i> ,(2011), Golzarian and Frick, (2011)
	Kernel counting in maize	Severini <i>et al.</i> ,(2011)
	Photosynthetic CO ₂ uptake	Migliavacca <i>et al.</i> ,(2011)

Table 3. Continued.

Infrared imaging	Leaf area index	Shibayama <i>et al.</i> ,(2011)
	Disease assessment	Oerke <i>et al.</i> , (2011)
	Leaf temperature, plant water status and drought	Blum <i>et al.</i> , (1982), Kaukoranta <i>et al.</i> ,(2005), Leinonen <i>et al.</i> ,(2006), Sirault <i>et al.</i> , (2009), Jones <i>et al.</i> ,(2009), Qiu <i>et al.</i> ,(2009), Berger <i>et al.</i> , (2010), Jimen-Bello <i>et al.</i> ,(2011), Liu <i>et al.</i> ,(2011)
	Roots	Dokken and Davis (2011)
Fluorescence imaging	Plant growth and maturity	Barbagallo <i>et al.</i> , (2003), Ooms and Destain (2011), Krupenina <i>et al.</i> ,(2011).
	Disease assessment	Chaerle <i>et al.</i> , (2007a), Scholes and Rolfe, (2009), Rolfe and Scholes,(2010), Bauriegel <i>et al.</i> , (2011)
	Photosynthetic efficiency	Genty <i>et al.</i> , (1989), Flexas <i>et al.</i> , (2002), Yao <i>et al.</i> ,(2007b), Baker,(2008), Woo <i>et al.</i> ,(2008), Zhang <i>et al.</i> ,(2010)
	Water stress	Lang <i>et al.</i> , (1996), Lichtenthaler and Miehé (1997).
Combination of thermal and fluorescence imaging	Plant- pathogen interactions	Chaerle <i>et al.</i> ,(2004), Chaerle <i>et al.</i> , (2007b)

Even-though, the use of imaging platform in agricultural sciences are many, in this project, particularly, RGB images are used to study the growth and health status of the wheat plants grown both under control and saline environment.

1.8 QTL mapping

Development of salinity tolerant wheat cultivars has been successful achieved with various approaches such as conventional breeding (Ashraf and Oleary, 1996), QTL mapping (Lindsay *et al.*, 2004) and genetic engineering (Sawahel and Hassan, 2002; Abebe *et al.*, 2003). The use of conventional plant breeding techniques like selection, hybridization and the use of resistant root stocks for breeding of salinity tolerant cultivars are unwieldy and time-consuming, however, they are the main techniques still used today by breeders around the world. Often a direct selection of superior salt tolerant genotypes under field conditions is hindered by the significant influence of environmental factors and various genetic barriers such as low heritability and linkage drag. On the other hand, development of salinity tolerance cultivars through reverse genetics approach is highly risky (Roy *et al.*, 2011). Accordingly, QTL mapping has been suggested to be a powerful approach for the improvement of complex polygenic trait like salinity tolerance in various crops (Ortiz, 1998; Ruttan, 1999; Collard and Mackill, 2008; Roy *et al.*, 2011). QTL mapping is the major application of molecular marker technology that helps breeders to understand the inheritance of polygenic traits and identify marker(s) or gene(s) which are closely linked to the traits having complex mode of inheritance. While using this, breeders do indirect selection of marker(s) or gene(s) linked to the trait of interest in a rapid manner (Sax, 1923; Thoday, 1961). Moreover, it offers excellent opportunity to dissect out and study the genetic and physiological components of complex traits such as salinity tolerance where direct selection is difficult and influenced by various environmental factors (Prioul *et al.*, 1997; Prioul *et al.*, 1999).

1.8.1 Basic requirements for QTL mapping

1.8.1.1 Mapping population

Mapping populations are usually generated from crosses between two parent plants with two extremes of variability for trait of interest. Different types of mapping populations are used in QTL mapping, including B₁F₁ populations, F₂ populations (this study), recombinant inbred lines (RILs), doubled haploids (DHs; this study) and near isogenic lines (NILs) are useful for a variety of studies. Among them, RILs, DHs and NILs are homozygous which allows doing more replicated trials in different environments. The choice of mapping population depends on the objective the research programme. For instance, F₂ population are highly useful to develop a primary linkage map, while RILs are useful for the high resolution linkage map and genetic analysis of complex genetic traits such as salinity tolerance, however, it take a long time to produce. As would be expected, the development of a mapping population for self-pollinated crops is much easier than for an out crossing crop (Collard *et al.*, 2005a; Semagn *et al.*, 2006) for QTL mapping. Two different mapping populations, F₂ and DHs are used for QTL mapping in this thesis and they are described below.

1.8.1.1.1 F₂ population

F₂ populations are usually developed by selfing of the F₁ offsprings which are generated from the original cross between two parents which show wide variation for the desired trait (Collard *et al.*, 2005a; Semagn *et al.*, 2006). F₂ individuals possess all possible recombination of parental alleles in the early segregating generation itself and they are found to be very useful mapping populations for the detection of QTL with additive effects (Semagn *et al.*, 2010). F₂ populations are primarily used for the construction of linkage maps in various crop species (Dubcovsky *et al.*, 1996a; Dubcovsky *et al.*, 1998; Yao *et al.*, 2007a; Jing *et al.*, 2008; Taenzler *et al.*, 2002; Bullrich *et al.*, 2002). It is really easy to develop F₂ population and it consumes less time for the development when compared to other populations such as RILs and DHs (Collard *et al.*, 2005a; Semagn *et al.*, 2006). However, it is hard to analyse the G×E

interaction in F₂ mapping population because each plant is a unique individual, which makes replication of experiments over different environment and time, impossible (<http://www.scribd.com/doc/6229849/Mapping-Population>). Moreover, they are heterozygous in nature and cannot be used for further fine mapping (Semagn *et al.*, 2006).

1.8.1.1.2 Doubled Haploids (DH)

Doubled haploids are usually produced through regenerating plants by artificial chromosome doubling from pollen grains (Collard *et al.*, 2005a). Since, DH is the product of single meiotic cycle, the amount of recombination information present in DH population is comparable to the F₂ population (<http://www.scribd.com/doc/6229849/Mapping-Population>). Nevertheless, the homozygosity of this mapping population is fixed, and hence permits replicated trails to do phenotyping which minimizes the effect of QTL × environment interactions (Martinez *et al.*, 2002). They are the populations highly preferred by most of the scientists, to carry out experiments throughout the world (Gong *et al.*, 1999; Ellis *et al.*, 2002; Huynh *et al.*, 2008; Siahsar and Narouei, 2010; Genc *et al.*, 2010a). Hence, it is useful to precisely map both quantitative and quantitative traits in different crop species. The development of DH populations takes less time than the development of RILs and NILs, however, they do take longer to produce than an F₂ population. It is also only possible to develop a DH population in species having standardised protocol for tissue culturing method (Collard *et al.*, 2005a; Semagn *et al.*, 2006). In fact, individual crops respond differently to the tissue culture technique and the standardised procedure for haploid production is not available for all the crops. Moreover, the development of DH mapping population requires more technical skills and high cost than with the development of other mapping population (Collard *et al.*, 2005a; Semagn *et al.*, 2006).

1.8.1.2 Molecular markers

Molecular markers are used to detect genetic differences between individuals of the same or different plant species. These markers can be located closely to the gene of interest and so can be called as gene tags. It is important, however, that they do not affect the expression of phenotype of the trait of interest (Collard *et al.*, 2005a). Molecular markers are also not influenced by environmental factors or the developmental stage of the plant (Winter and Kahl, 1995). Based on the principle methods used for the analysis of the genomic DNA, molecular markers are classified into hybridization based, such as Restriction Fragment Length Polymorphism (RFLP) markers; PCR based, for example Cleaved Amplified Polymorphic Sites (CAPS), Sequence Tagged Sites (STS), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR) markers; and DNA chip and sequence based markers e.g. Single Nucleotide Polymorphism (SNP) (Khlestkina and Salina, 2006; Collard *et al.*, 2005a). Of these many types of markers, mainly SSR markers are used in this project.

1.8.1.2.1 Simple sequence repeats (SSR's)

Microsatellite markers are highly abundant in plants and they are highly polymorphic in nature (Mrázek *et al.*, 2007; Mohan *et al.*, 1997; Morgante and Olivieri, 1993). They are co-dominant markers (Hayden and Sharp, 2001) and occurs in all eukaryotic genomes, especially in crops such as wheat and barley (Gupta *et al.*, 1999). Microsatellite markers have around 10-60 copies of nucleotide repeat sequences. The frequency of such repeats longer than 20bp is predicted to occur once, every 33kb in a plant's genome (Mohan *et al.*, 1997; Litt and Luty, 1989; Cregan, 1992). In plants, AT is the most common repeat unit followed by AG or TC (Powell *et al.*, 1996; Mohan *et al.*, 1997). Scientists use the nucleotide sequence flanking these repeats and design primers to amplify the different number of repeat units in various individuals (Mohan *et al.*, 1997). These markers are widely used in genetic diversity studies, germplasm screening and finger printing due to their simplicity and ease of screening (Wang *et al.*, 2005; Kottapalli *et al.*, 2007; Marti *et al.*, 2009; Tangolar *et al.*, 2009). These markers have been found very useful in the construction of linkage map for

QTL mapping (Ramsay *et al.*, 2000; Song *et al.*, 2004; Somers *et al.*, 2004; Song *et al.*, 2005).

1.8.1.3 Genetic or linkage maps

Genetic maps are important to find out the location of genes of interest on the specific chromosomes in any specific crop species. Usually genetic maps are constructed based on the linkage and recombination frequency of markers or genes in the region of interest in the genome. The chromosomes with linked genes and recombination frequencies between them are known as linkage map or genetic map or chromosome map (Paterson *et al.*, 1988). The construction of genetic linkage map needs two main items of information; calculation of recombination frequencies between linked markers and the order of the genes (or) markers on the chromosomes. The occurrence of a recombination between homologous chromosomes often decreases the chances of another recombination event occurring on the same chromosome in an adjacent region; this is commonly known as interference. The calculation of interference is a very useful tool in calculating recombination frequency and hence the spread and distance between markers on the chromosomes (King and Mortimer, 1991). There are two main mapping function identified to study the recombination frequencies and the recombination event interference such as Haldane's mapping function and the Kosambi's mapping function. There are several computer programmes available (<http://linkage.rockefeller.edu/soft/>&<http://linkage.rockefeller.edu/soft/list3.html>), which make use of either Kosambi or Haldane mapping functions to calculate the recombination frequencies of markers and to construct the genetic linkage map in an precise manner (Stam, 1993; Manly *et al.*, 2001; Harushima *et al.*, 1998).

Linkage maps for a variety of different crop species have been constructed using various molecular markers such as RFLP, SSR and AFLP (Paterson *et al.*, 1988; Kurata *et al.*, 1994; Pereira and Lee, 1995; Blanco *et al.*, 1998; Varshney *et al.*, 2006). Linkage maps are very useful for the investigation of various major and minor genes (quantitative trait) controlling phenotypic traits of the plants on the chromosomes

(Mesfin *et al.*, 2003; Szalma *et al.*, 2007; Huynh *et al.*, 2008; Bovill *et al.*, 2010). It is these maps that are also helpful for “Marker Assisted Selection” (Graner and Bauer, 1993; Huynh *et al.*, 2008; Genc *et al.*, 2010a) and for “Map based cloning” of gene of interest (Jander *et al.*, 2002; Huang *et al.*, 2003; Lei *et al.*, 2007). Among these various uses this project mainly uses QTL mapping approaches to identify QTL for components of salinity tolerance in *T. monococcum* and *T. aestivum*.

1.8.2 QTL mapping methods

The use of the appropriate QTL mapping method determines the preciseness of the identified QTL for the trait of interest. There are three methods widely used for QTL analysis: single marker analysis, simple interval mapping and composite interval mapping.

1.8.2.1 Single marker analysis

Single marker regression analysis studies the relationship between the genotypes of and the phenotypic value of the trait of interest (Sax, 1923). It considers one single marker at a time and checks the relationship between the marker and the phenotypic trait. While a useful method to use in initial analysis of data, single marker analysis will not detect genes might influence the plant’s phenotype but are situated far from the nearest marker. Moreover, this approach does not also take into account the effect of other significant markers associated with the trait of interest (McMillan and Robertso.A, 1974; Tanksley, 1993). However, it does not require genetic a map of the population or require any prior knowledge of the order of the genes on the chromosome (Jansen, 1994). The level of significance is usually determined by a F- statistics or ANOVA (Edwards *et al.*, 1987; Hackett, 2002) or student “t” test (Tanksley and Hewitt, 1988; Collard *et al.*, 2005a) or likelihood ratio (Weller, 1986; Weller, 1987; Lander and Botstein, 1989; Doerge *et al.*, 1997). However, it was replaced by other mapping methods that have been described in the sections below.

1.8.2.2 Simple interval mapping (SIM)

Simple interval mapping was first used by Lander and Botstein, (1989). It needs a genetic map for analysis. It uses the interval between two flanking markers for the identification of QTL through likelihood ratio statistics. The interval with the highest statistical significance identifies the position of the QTL in the linkage groups. It has mainly two advantages over single marker analysis, firstly the location and the effect of QTL could be assessed more accurately and assigned to a region of the chromosome and, secondly it use LOD (Log of odds ratio) score to test the significance of the QTL (Carbonell and Gerig, 1991), which increases the preciseness of detecting QTL than any other independent statistical tests like ANOVA. The disadvantages of SIM are that, it fails to take into account the effect of other QTL on different linkage groups on the plant's phenotype for the segregating trait in the mapping population and it cannot position two or more QTL on the same chromosome (Jansen, 1994).

1.8.2.3 Composite Interval Mapping (CIM)

Composite interval mapping has the advantages of both interval mapping and single marker multiple regression analysis and is able to overcome the disadvantage of simple interval mapping described above (Jansen, 1993; Jansen and Stam, 1994; Zeng, 1994). It fits QTL based on interval mapping methods; however, it does the analysis for fitting the partial regression coefficients for the other background markers outside the interval. It is considered as the most powerful statistical mapping method for QTL mapping (Hackett, 2002; Hackett and Broadfoot, 2003). Thus it removes the bias of the other QTL which are linked to the interval being identified. However, it is not good enough to predict the QTL in the genetic linkage map where there is an uneven distribution of markers and it does not count the G×E interactions. Moreover, it is also not useful to detect the epistatic interaction in the QTL analysis (Jansen, 1993; Jansen and Stam, 1994; Zeng, 1994).

1.8.3 QTL mapping in *T. monococcum*

A genetic map of diploid wheat, *T. monococcum*, involving 335 markers was reported by Dubcovsky *et al.*, (1996a). They found that the order of the markers in the inverted segments in the *T. monococcum* genome is same as in the B and D genomes of *T. aestivum* and the homology of chromosomes between *T. monococcum* and *T. aestivum* (Dubcovsky *et al.*, 1995). In general, *T. monococcum* has high level of polymorphism and smaller genome than *T. aestivum* and hence it can be used to produce high density genetic maps in various studies that could complement genetic maps of *T. aestivum* (Dubcovsky *et al.*, 1995).

In *T. monococcum*, the earliness per se gene *EPS-A^m1* was identified on chromosome 1A by using a mapping population of a cross between cultivated (DV92) and wild (G3116) accessions (Bullrich *et al.*, 2002). A vernalization sensitivity gene *Vm-Aml* was located on the fifth chromosome of *T.monococcum* which is orthologous to *Vm-A1* on *T. aestivum* (Dubcovsky *et al.*, 1998). Very recently, in a MDR002 × MDR043 mapping population QTL for *septoria* disease resistance was identified on chromosome 7A (Jing *et al.*, 2008).

1.8.4 QTL mapping in *T. aestivum*

QTL mapping techniques has already been used to detect markers linked to both major and minor genes for various biotic and abiotic stresses respectively (Ayala *et al.*, 2002; del Blanco *et al.*, 2003; Schnurbusch *et al.*, 2004; Raman *et al.*, 2005; Bovill *et al.*, 2006; Santra *et al.*, 2008; Ma *et al.*, 2010) in *T. aestivum*. However, the literature about the use of QTL mapping for salinity tolerance in *T. aestivum* is important for this project.

In general, *T. aestivum* is a hexaploid and possess three different (A, B and D) genomes. It is a moderately salt tolerant crop whereas durum wheat, containing only

the A and B genomes, is more salt sensitive than *T.aestivum* (Munns *et al.*, 2006). It could be due to the presence of the locus, *Kna1* on chromosome 4D in *T.aestivum*, which brings low Na⁺ accumulation and enhanced K⁺/ Na⁺ discrimination characters in *T.aestivum* (Dubcovsky *et al.*, 1996b). In addition to this, there are several QTL have been identified by various researchers for salinity tolerance, particularly for the Na⁺ exclusion component in *T.aestivum* on chromosomes 1B, 1D, 4B, 5D and 7D (Ma *et al.*, 2007) , 7A (Edwards *et al.*, 2008; Genc *et al.*, 2010a), 2A (Lindsay *et al.*, 2004; Genc *et al.*, 2010a), 2B and 6A (Genc *et al.*, 2010a) and all the seven groups of chromosomes (Quarrie *et al.*, 2005).

1.9 Rationale for this dissertation

It has already been professed that the successful salinity tolerance wheat cultivars use more than one physiological component to adapt soil salinity (Rivelli *et al.*, 2002; Munns and Tester, 2008; Cuin *et al.*, 2009). However, most of the early salt tolerance has been done only for Na⁺ exclusion (Munns and James, 2003; Byrt *et al.*, 2007; Shavrukov *et al.*, 2009) because it is easy to screen. Little work has been done for osmotic tolerance (Slavík, 1963; James *et al.*, 2002) and tissue tolerance (Genc *et al.*, 2007) because of the limitation in screening techniques. Accordingly, this study aims to develop and contribute a new experimental strategy that helps to improve the understanding of three major salinity tolerance components (Na⁺ exclusion, osmotic tolerance and tissue tolerance) in different two different wheat species (*T. monococcum* and *T. aestivum*). Importantly, development of such experimental technique could be helpful to screen the already existing mapping population of *T. monococcum* and *T. aestivum* and exploit the variability for these major components of salinity tolerance for further forward genetic research approaches such as QTL mapping.

The major objectives of this dissertation are,

- ❖ To develop high throughput salt screening protocol and quantify three major components of salinity tolerance in *T.monococcum* accessions, including parents of the existing mapping population.

- ❖ To use the high throughput salt screening protocol, screen Berkut × Krichauff DH mapping population of *T. aestivum* and identify potential QTL for major salinity tolerance components in *T. aestivum*.

- ❖ To use the high throughput salt screening protocol to screen suitable F₂ mapping populations of *T.monococcum* showing variability for osmotic tolerance and tissue tolerance and study the genetic basis of osmotic tolerance and tissue tolerance for future QTL analysis.

In this thesis, Chapter 2 outlines the general materials and methods used to develop high throughput salt screening protocol, Chapter 3, describes the assay to quantify three major salinity tolerance components in cereals (published paper), Chapter 4, investigates the genetic basis of salinity tolerance components in *T.aestivum* (*T. aestivum*), Chapter 5, examines the osmotic and tissue tolerance component in *T. monococcum* for QTL mapping and Chapter 6 discusses the findings of the research for further applications.

CHAPTER 2. GENERAL METHODOLOGY

2.1 Plant material

In this thesis, the three major components of salinity tolerance were studied in the genotypes and mapping population of two different wheat species; *Triticum monococcum* and *Triticum aestivum*. The detailed information about the genotypes and the mapping population are given in the individual chapters.

2.2 Growth conditions

Plants were grown throughout the year in greenhouse at the facilities of the South Australian Research and Development Institute (SARDI), Adelaide, Australia. The temperature of the greenhouse was maintained at 25°C during the day and 15°C during the night. Humidity and light levels were not controlled within the greenhouse.

2.3 Seed germination

To achieve uniform germination, seeds of approximately same size were selected and placed on moist double layered Whatman 90 mm filter paper (Whatman International Ltd, Maidstone, England) inside 90 mm sterile petri dishes (Techno Plas Pty Ltd, South Australia). Petri dishes were double wrapped with polythene bags (20 petri dishes in each polythene bag) to maintain high humidity. Seeds were germinated for 5 to 7 days at room temperature until the plumule was approximately 2 cm long. The filter paper was moistened with deionised water every second day to prevent seedlings from drying out.

2.4 Supported hydroponics

When the plumule's growth was approximately 2 cm long, seedlings were transplanted into a supported hydroponics setup (Figure 9). Individual seedlings were

picked up one by one and placed in a 280 mm × 45 mm size PVC tubes, filled with black polycarbonate plastic pellets (Plastics Granulated Services, Adelaide, Australia), which acted as an inert soil-like substrate. To facilitate image analysis the rim of the tubes were painted blue to enhance the separation of tube from the plant during analysis. These tubes were transferred to a supported hydroponics system, which comprised of a trolley with two 25 litre black containers sitting above a single 80 litre blue reservoir tank. Each individual 25 litre black container had spacing for 84 tubes (plants). Plants were grown for approximately 10 days in modified Hoagland's nutrient solution (0.2 mM NH₄NO₃, 5 mM KNO₃, 2 mM Ca(NO₃)₂, 0.1 mM KH₂PO₄, 2 mM MgSO₄, 0.5 mM Na₂SiO₃, 10 μM H₃BO₃, 5 μM MnCl₂, 10 μM ZnSO₄, 0.1 μM Na₂MoO₄·2H₂O (0.1 μM), 0.5 μM CuSO₄ and 50 μM FeEDTA-Na₂). All chemicals were obtained from Sigma Aldrich (Castle Hill, Australia). Nutrient solution was pumped from the reservoir tank into the 25 litre black containers in a 20 minutes fill and 20 minutes drain cycle (Shavrukov *et al.*, 2012).

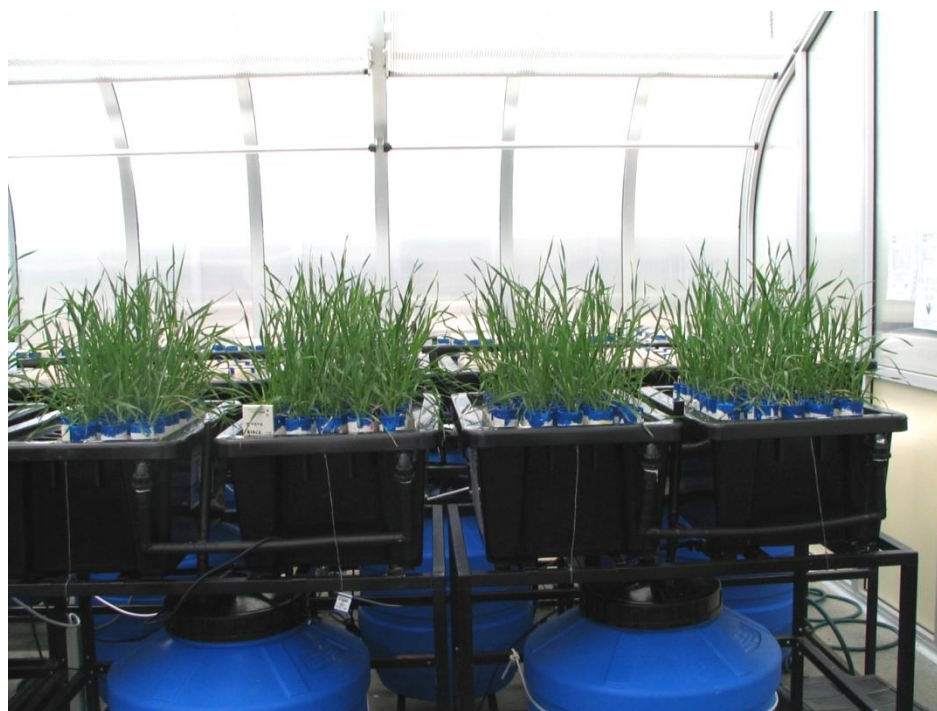


Figure 9. The supported hydroponics setup used to grow plant material for high throughput salt screening. Plants were grown in PVC tubes, filled with polycarbonate pellets. These tubes were arranged in the 25 litre black containers as determined by a randomized block design (RBD). Modified Hoagland's nutrient solution (see main text) was pumped from the 80 litre blue reservoir tank below into the black containers in a 20 minutes fill/drain cycle.

2.5 NaCl application

Seedlings were allowed to grow for approximately 10 days in the control nutrient solution to acclimatise and develop a well established root system. After 10 days (at the time of fourth leaf blade emergence) NaCl was applied in 25 mM increments twice daily, along with the nutrient solution until it reached final concentrations of 75 mM for einkorn wheat (*T. monococcum*) and 150 mM for *T.aestivum* (*T. aestivum*) experiments. Calcium activity was maintained approximately to the $\text{Na}^+:\text{Ca}^{2+}$ molar concentration ratio of 15:1 in all the experiments. It was achieved by the addition of 1.71 mM of supplementary $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ with every 25 mM addition of NaCl to the nutrient solution (Cramer *et al.*, 1985; Cramer *et al.*, 1987; Schachtman *et al.*, 1992; Genc *et al.*, 2010b). To avoid nutrient deficiency, the nutrient solution was changed once in a week, pH was monitored, adjusted with Hydro chloric acid (HCl) and kept as neutral (pH=7). Plants were grown till they were 31-35 days old. Specific information is given in individual chapters.

2.6 Non-destructive 3D plant imaging

Non-destructive imaging was carried out to quantify the effect of Na^+ stress on plant growth and health by a LemnaTec non-destructive 3D plant scanalyser (LemnaTec co, Würselen, Germany, <http://www.lemnatec.com>) (Figure 10). The imaging station consisted of 20 cool white fluorescent lighting tubes (10 on the top and 10 on the side) and two Coupled Charge Device (CCD) cameras. One CCD camera was fixed on the wall for taking side view images and the other one on the ceiling for views from the top. The resolution of the camera was high, allowing objects as small as 2×2 mm in size to be detected and differentiated from other objects in the image. The wall and floor of the imaging station were painted blue, again to help with separating the plant from the background during image analysis. There was a rotating sample holder fitted on the imaging cabinet to record images of plants from different angles. Individual plants were removed, along with their PVC tubes, from the supported hydroponics set-up and placed inside the imaging cabinet. The two CCD cameras were used to obtain three high resolution images of each plant, one from the top and two from the

side at a 90° horizontal rotation, which were stored on a computer as a RAW image file for future analysis. The imaging cabinet door was closed at the time of imaging to prevent varying environmental lighting conditions that affect the quality and uniformity of the taken images.

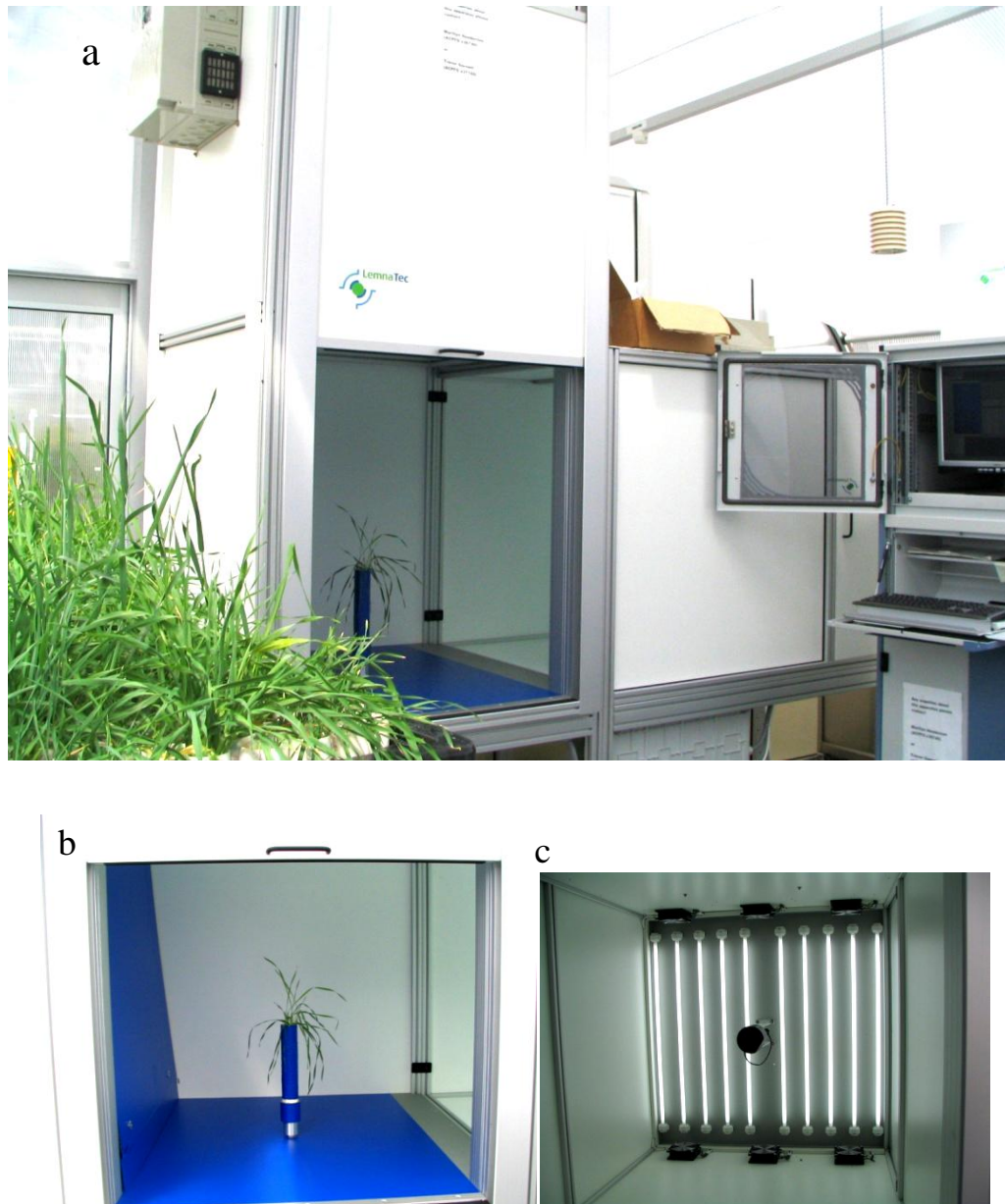


Figure 10. Layout of the LemnaTec Scanalyser (a) Layout of the imaging cabinet and computer workstation, showing the sample loading bay. (b) A wheat plant in the imaging cabinet (c) The top digital camera and fluorescent lights used for image acquisition.

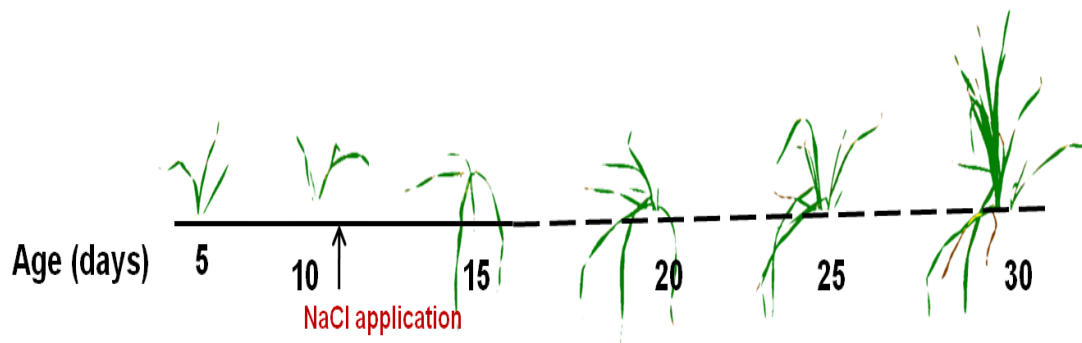


Figure 11. Image acquisition schedule for high throughput NaCl screening in wheat. To screen for osmotic stress images of plants were acquired every day (the continuous line) 5 days before and 5 days immediately after NaCl application. Images were then captured three times a week to monitor both osmotic stress and Na⁺ toxicity symptoms in the plant's shoot (the broken line), the final image was taken on day 31. NaCl application began at the time of fourth leaf blade emergence (approximately day 11), which is indicated by the black arrow.

Initially, images of plants were taken daily from 5 days before to 5 days after NaCl application. These images were used to quantify the changes in plant growth rates due to osmotic stress. Thereafter, images were obtained every second day until the plants were 31 days old, to determine the effects of both osmotic and ionic stress on plant growth (Figure 11). More details are given in individual chapters.

An image processing routine was created in a computer programme designed to analyse images acquired by the imaging station and to extract phenomic information from the images. These program routines are called 'image processing grids' in the associated LemnaTec image analysis software. The grid was designed with three image readers to analyse three views of an image at a time. The image reader was aligned with various image processing operators, filters and devices to process and derive useful information such as plant colour and some morphological parameters (Figure 12 & <http://www.lemnatec.com>).

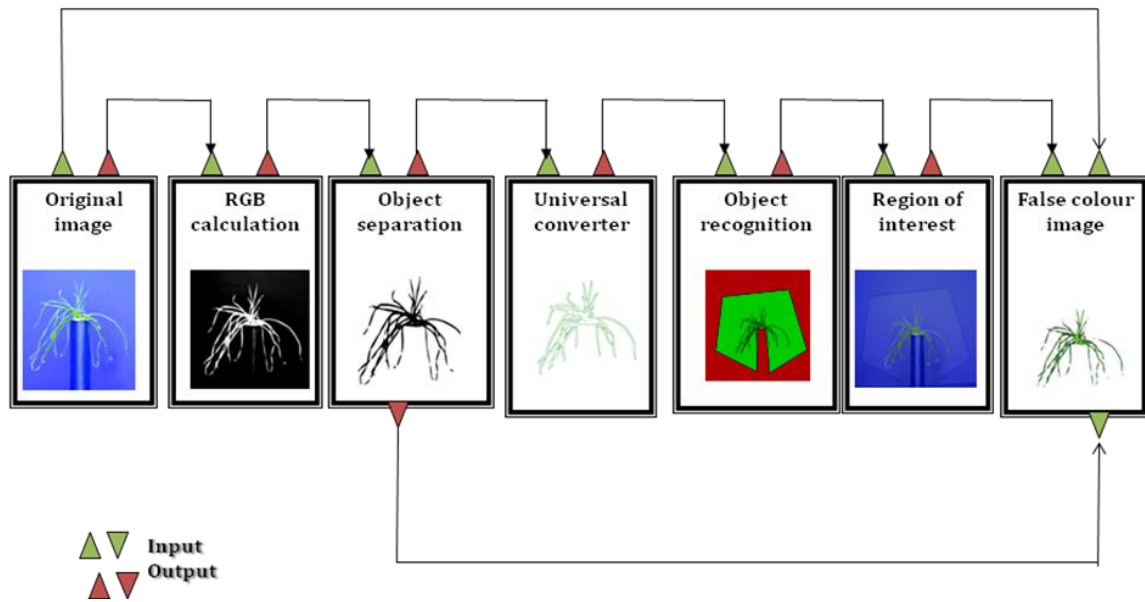


Figure 12.Side view reader. A model generated to visualise the functions used in the processing grid of a side view image of 31 days old *T. monococcum* accession, MDR 043 grown in 75 mM saline environment.

Briefly, by adjusting the image thresholds, the imaging grid converted the red, green and blue (RGB) image taken by the digital camera to a black and white image (grey image), so that the plant appears as white and everything else as black. This image was then inverted, so the plant became black, allowing the region occupied by the plant to be determined (Figure 12). Thus, when the image was converted back to a RGB image, the plant region was separated from the background. Once separated the various colours of the plant were classified into three colour, green (healthy), yellow (chlorotic) and brown (necrotic), by applying the nearest neighbourhood distance-based classification algorithm. The three colour classes were defined through an user-based colour selection and colour anchoring process was performed over a few selected plant images. After colour classification, the plant image was displayed as three false colour regions; green, yellow and brown (Figure 13). The colour classified image was helpful to study the symptoms of Na^+ specific toxicity in plants growing under salt stressed environment. Further, total projected shoot area was calculated using an image-based leaf sum (IBLS) model, where the number of pixels corresponding to the total plant regions in the three image views were summed

together. A calibration to convert pixel number to mm^2 was done using an object with a known area, if necessary.

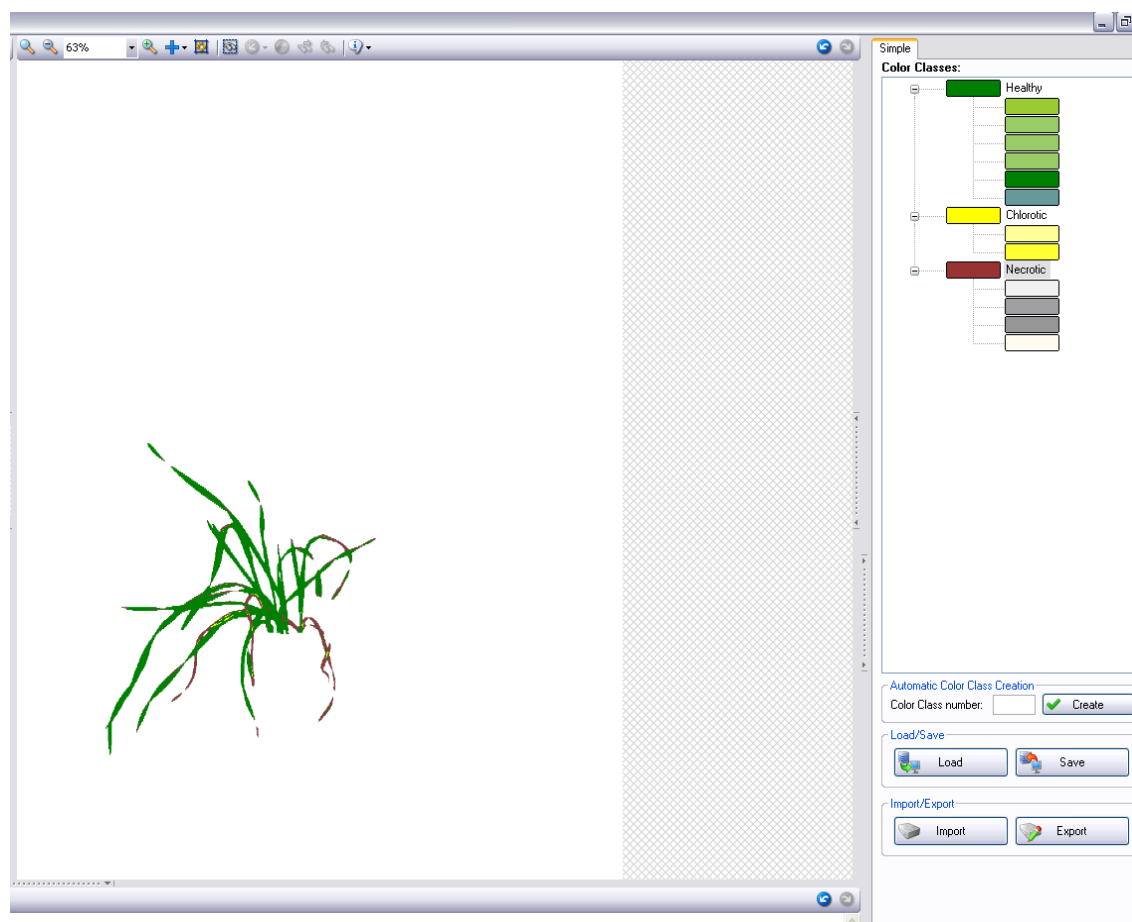


Figure 13. The lemna launcher software window displaying the false colour image (right) of *T.monococcum* plant in 75 mM NaCl. The different shades of green, yellow and brown region in plant parts were identified through visual selection with the help of few randomly selected plant images.

The LemnaTec image processing software enabled the recording of 63 different parameters of each plant studied such as eccentricity, centre of mass and helpful to study changes in the morphological features of plants growing under saline environment. As the main aim of this project was to quantify the plant growth and health status in both Na^+ stressed and control environments, only total plant area and areas of healthy, chlorotic and necrotic tissue were recorded. Future experimentation will likely explore which of the other parameters observed are useful for salinity studies.

2.7 Measurements of leaf Na⁺ concentrations

Approximately three weeks after NaCl application, the fourth fully expanded leaf blade was harvested and the fresh weight of the leaf blade was determined (Specific information is given in individual chapters). Samples were then dried for 2 days at 65°C and the dry weight was recorded. Leaf samples were then digested in 10 ml of 1% HNO₃ at 95°C 4 hours in a 54-well environmental express hot block (Adelab Scientific, Thebarton, Australia). Samples were periodically shaken once an hour to facilitate the digestion process. The concentration of Na⁺ and K⁺ in the leaf digest was measured by flame photometry (Model 420, Sherwood Scientific, Cambridge, U.K).

CHAPTER 3. QUANTIFYING THE THREE MAIN COMPONENTS OF SALINITY TOLERANCE IN CEREALS

This chapter contains the commentary and the copy of the published research paper “Quantifying the three main components of salinity tolerance in cereals” by Rajendran, K., Tester, M. and Roy, S.J, in *Plant, Cell and Environment* 32: 237-249, 2009.

3.1 A commentary on quantifying the three main components of salinity tolerance in cereals

3.1.1 Overview

Extensive research, has been carried out over the past three decades on modelling unicellular organisms (Csonka and Hanson, 1991; Gustin *et al.*, 1998), halophytes (Casas *et al.*, 1991; Niu *et al.*, 1993; Vera-Estrella *et al.*, 2005; Amtmann, 2009) and model species, like *Arabidopsis thaliana* (Knight *et al.*, 1997; Apse *et al.*, 1999; Shi *et al.*, 2003) to acquire knowledge about the physiological and biochemical pathways which help plants to maintain osmotic - ionic homeostasis under saline environment. Since, the response of a plant under saline environment is always the integrated effect of genes inside the plant cells and the growing environment; the understanding derived from the previous studies does not provide comprehensive knowledge about mechanisms of salinity tolerance in various agricultural crops (Munns and Tester, 2008). As most of the commercial cultivars of agricultural crops are glycophytes they now receive a lot of major research attention to develop cultivars with increased salinity tolerance, thereby providing future food sustainability in many countries of the world (Glenn *et al.*, 1999b; Munns *et al.*, 2006; Glenn *et al.*, 1999a). It is therefore crucial to develop new research approaches which can accurately examine the plant and environmental interactions and facilitate easy understanding of physiological mechanisms producing salinity tolerant genotypes in different crop species (Ashraf and Wu, 1994; Genc *et al.*, 2007). In a first hand, researchers need a reliable salt screening method that helps to obtain meaningful information about the responses of plant under saline environment in an efficient manner.

Evaluating salinity tolerance through the calculation of either relative shoot biomass or relative grain yield of salt stressed plants over the control grown plants (Shannon, 1997; Munns and Rawson, 1999), is a destructive, expensive, laborious and time consuming approach. Notably, the same plant cannot be further examined once it has been destroyed. On the other hand, the study of changes in the physiological traits such as chlorophyll fluorescence (Belkhodja *et al.*, 1994; Shabala *et al.*, 1998; Sayed,

2003; Mehta *et al.*, 2010), K^+ uptake (Chen *et al.*, 2005), net CO_2 assimilation (James *et al.*, 2002; Jiang *et al.*, 2006), Na^+ uptake (Munns *et al.*, 2003), K^+ uptake (Chen *et al.*, 2005; Cuin *et al.*, 2010) and K^+/Na^+ ratio (Asch *et al.*, 2000) only help to identify specific salt tolerance mechanisms and not the overall salt tolerance of the plant, since crop salinity tolerance usually arises from a combination of various physiological processes and no single physiological observation can account for variation in whole plant response to salt stress (Hasegawa *et al.*, 2000). In the research paper presented in this chapter, “Quantifying the three main components of salinity tolerance in cereals” by Rajendran *et al.*,(2009) a non-destructive phenotyping protocol was proposed to study and understand the three different components of salinity tolerance in cereals: osmotic tolerance, Na^+ exclusion and tissue tolerance. It has the advantage of using non-destructive 3D imaging technology to monitor changes in the growth and health status of individual plants in a saline condition over a long period of time. This is a commentary of this research paper that illustrates the usefulness of the non-destructive imaging technique that helps to assess salt damages in plants and examines the effectiveness of this new salinity tolerance screening methodology which facilitates to identify mechanisms of salinity tolerance in salinity tolerant genotypes.

3.1.2 The scanalyser 3D – a new phenotyping tool used to quantify salt damages

To obtain a complete understanding about the three salinity tolerance mechanisms used by a plant to survive salt stress, it is necessary to use a robust and efficient screening technology that can detect the phenotypic changes in a plant throughout its growth and development. In the past such observations had to be done by eye, however, human observation is very much biased and not accurate enough to estimate visual symptoms of salt damages. In the paper below, alterations in the morphology, and growth rates of plants were non-destructively quantified using LemnaTec’s image capturing equipment, a non-destructive 3D plant scanalyser (LemnaTec co, Würselen, Germany, <http://www.lemnatec.com>) (More details are given in Chapter 2). This proved to be a powerful and sensitive tool to detect changes in plant growth and health under stressed environment, utilizing less time and effort for measuring hundreds of plants when compare to other classical methods of salinity tolerance

evaluation. Repeated measurements of the same plant with the scanalyser showed it was very accurate, less than 1% of the SEM and proved to be precise in quantifying subtle changes in the growth of plant over time. It was particularly helpful to obtain a colour classified image of the growing plant, providing the ability to assess the growth and health of the plant based on measures of shoot area with different shades of green (healthy plant productive area) or yellow and brown (dead plant area). In addition, morphological features of shoots, such as compactness, eccentricity, centre of mass and calliper length, could be quantified under both normal and saline conditions (More details are given in Chapter 2). Since, the main aim of this paper was to quantify the plant growth and health status in both Na⁺ stressed and the controlled environment, only the changes in the growth and health of the shoot area were taken into account for the development of high throughput salt screening method. Future experimentation will likely explore which of the other morphological parameters observed are useful for salinity studies.

3.1.3 Screening for the three main components of salinity tolerance in cereals – a perspective

Cereals growing under saline environment mainly undergo osmotic and ionic stresses and reduce its growth and productivity (Munns and Tester, 2008). There are three major components of salinity tolerance that contribute to plant adaptation to saline soils: osmotic tolerance, which enables the plant to withstand salt induced water stress; Na⁺ exclusion, which involves the control of sodium uptake into roots and transfer to shoots; and tissue tolerance, which enables leaves to remain green while accumulating high Na⁺ in the tissue through compartmentation of the Na⁺ in vacuole. To date, research into improving the salinity tolerance of cereals has focused primarily on exclusion of Na⁺ (Munns and James, 2003; Byrt *et al.*, 2007; Shavrukov *et al.*, 2009), and little work has been carried out on osmotic or tissue tolerance (Slavík, 1963; James *et al.*, 2002; James *et al.*, 2006b; Genc *et al.*, 2007). However, in this research with the help of imaging technology a high throughput salinity tolerance screening method was developed to quantify genotypic differences for all the three main components of salinity tolerance in cereals. The important findings obtained through this high throughput salinity tolerance screening methodology are described here as follows.

The experimental results reported below strongly supported the two phase growth hypothesis with one major exception. Munns and Tester (2008) hypothesized that the shoot growth reduction of plants at osmotic stress would be maintain throughout the experimental period, with any further reduction in the shoot growth throughout the experimental period due to ionic effects of salt stress. However, in this study, shoot growth rate recovery was observed in some of the *T. monococcum* accessions, 5-7 days after NaCl application while some *T. monococcum* accessions were continued to maintain the same growth rate throughout the experiment (Supplementary Table I in Appendix 1). This suggests after an initial reduction of growth due to the osmotic stress, some plants can recover growth rates, and are therefore not as sensitive to osmotic stress as they first appear. Usually, in ample water conditions, osmotic stress is recoverable because root cell walls can easily change its plasticity to the external environment (Neumann, 1993; Allakhverdiev *et al.*, 2000). The recovery could also be the function of stress shock proteins which were usually synthesized when the plant undergoes a mild or non-lethal level of osmotic stress (Uma *et al.*, 1995).

Osmotic tolerance screening method used in this paper, revealed a substantial shoot growth reduction among different *T. monococcum* accessions immediately after first application of NaCl. This is one of the interesting observations made in this study as this was one of the first reports for describing variability for osmotic tolerance (Munns *et al.*, 1995; James *et al.*, 2002). The alterations in growth rate could be due to the function of aquaporins which not only controls water uptake but also sense osmotic pressure differences and give signals to synthesize a phytohormone ABA in the plant cells. The synthesized ABA in the plant cell closes the stomata, reduces the net photosynthetic rate and further reduce shoot growth rate and helps to maintain turgidity of plants under low or non-transpiring state (Hose *et al.*, 2000; Hill *et al.*, 2004; Wan *et al.*, 2004). However, what maintains this reduction in growth rate over several days still remains a mystery, since the ABA signalling is transient (Zhu, 2002; Fricke *et al.*, 2004; Davies *et al.*, 2005). Experiments carried out by James *et al.*, (2008) has also revealed a positive relationship between the stomatal conductance of fourth leaf blade and relative growth rate of shoot between 2-12 days in saline condition.

Similarly, screening for Na⁺ exclusion identified a huge variability for [Na⁺] in the fourth leaf blade of the *T. monococcum* accessions, sampled after three weeks of exposure to 75 mM NaCl. *T.monococcum* accessions such as MDR 308 and AUS 90436 had the ability to maintain a reduce shoot [Na⁺] possibly by using one or more of the mechanisms described by Tester and Davenport (2003): minimization of initial Na⁺ entry in to the roots from the soil, maximising efflux of Na⁺ from roots back to the soil, minimizing loading of Na⁺ into xylem vessels which transport solutes to shoots, maximising retrieval from xylem vessels in the root and maximising Na⁺ recirculation from shoots via the phloem. It is important to note that genetic variability for Na⁺ exclusion in *T. monococcum* has already contributed significantly to the field of salinity tolerance by providing Na⁺ exclusion genes namely *Nax1* and *Nax2* (James *et al.*, 2006a; Munns and James, 2003; Byrt *et al.*, 2007).

In general, screening for tissue tolerance is a difficult task and there was no proper screening method available in the early studies to screen tissue tolerance because of the subjective nature of the human observation. However, through the use of non-destructive imaging platform a quantitative assay has been developed to screen tissue tolerance in this study. The accessions MDR 043 and AUS 18755-4 accumulated high amount of Na⁺ in their leaf blade while maintaining good plant health compared to other *T. monococcum* accessions suggesting they have good tissue tolerance mechanisms. It remains to be seen if MDR 043 and AUS 18755-4 have higher expression of gene homologues to the *Arabidopsis NHX* (Apse *et al.*, 1999; Gaxiola *et al.*, 1999) and *AVP* (Davies, 1997) genes which have been shown to be important in compartmentalising Na⁺ away from where it can do damage. NHX is a Na⁺/H⁺ antiporter located on the tonoplast membrane. It is expressed in roots and leaves and selectively transports Na⁺ into the vacuole during salt stress, as well as K⁺ in non saline conditions (Munns, 2005). AVP is a vacuolar pyrophosphatase located on the tonoplast membrane that is likely to help with the sequestration of Na⁺ in to vacuoles as it establishes an proton electrochemical gradient that is used by proteins such as NHX (Gaxiola *et al.*, 1999).

Finally, the indices developed in this research paper were useful to assess the tolerance level of individual genotype for three main components of salinity tolerance. In the future, this could be used to identify the best combinations of salinity tolerance components producing salt tolerant phenotypes for breeding (Rajendran *et al.*, 2009). Ranking of genotypes and development of salinity tolerance indices based on their germination rate, survival rate, leaf or root elongation rate, leaf injury, shoot weight, root weight, shoot number, days to 50% flowering, yield and develop indices for salinity tolerance is a customary practice and it was widely followed by various crop physiologists on different crops (Caro *et al.*, 1991; Shannon, 1997; El-Hendawy *et al.*, 2007; Singh *et al.*, 2010). The indices developed in this paper, identified that salt tolerant *T. monococcum* accessions used various combinations of salinity tolerance components to achieve the total plant salinity tolerance. *T. monococcum* accessions either with the combination of tissue tolerance and osmotic tolerance or with the combination of Na⁺ exclusion and osmotic tolerance were identified as salt tolerant than the *T. monococcum* accessions with any single component of salinity tolerance. Interestingly, it did not identify any of the accessions with Na⁺ exclusion and tissue tolerance. Doing experiments with different NaCl concentrations such as 0, 75, 100 & 150 mM would be helpful to see if an excluding accession swaps over to being a tissue tolerator in future.

3.1.4 Concluding remarks

This research paper has shown the potential use of non-destructive high throughput salt screening methodology to quantify three major components of salinity tolerance in cereals. It has identified that combinations of these three components are important for whole plant salinity tolerance. The paper identified two new source of variability for osmotic tolerance and tissue tolerance in *T. monococcum* which could be for further QTL mapping. It also advises to select accessions either with combinations of osmotic tolerance and Na⁺ exclusion or with the combinations of osmotic tolerance and tissue tolerance to generate successful salinity tolerance genotypes.

3.2 The published research paper

Rajendran, K., Tester, M. and Roy, S.J. (2009). Quantifying the three main components of salinity tolerance in cereals. *Plant, Cell and Environment* 32: 237-249.

Statement of contributions:

Rajendran performed the necessary experimentation, analysis and interpretation of the results.

Tester and Roy helped conceive and design the experiments.

All authors contributed to the discussion of the results.

I, Mark Tester, hereby give written permission for the Rajendran *et al.*, (2009) paper to be included in this thesis.

Signature:

Date: 16/10/2012

I, Stuart Roy, hereby give written permission for the Rajendran *et al.*, (2009) paper to be included in this thesis.

Signature

Date 16/10/2012

Rajendran, K., Tester, M. & Roy, S.J. (2009) Quantifying the three main components of salinity tolerance in cereals.

Plant, Cell and Environment, v. 32(3), pp. 237-249

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This publication is included on pages 65-77 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

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CHAPTER 4. GENETIC ANALYSIS OF MAJOR SALINITY TOLERANCE COMPONENTS IN BREAD WHEAT (*TRITICUM AESTIVUM*)

Overview

In the previous chapter a high throughput image based salt screening protocol was developed and used to screen twelve accessions of *T. monococcum*. It was possible not only to identify which plants were salinity tolerant but it was also possible to identify which of the three tolerance mechanisms the accessions used. To date however, it has not been possible to measure such traits easily in bread wheat, to an extent where it would be possible to screen a large mapping population, enabling the identification of QTL linked to both osmotic tolerance and ionic tolerance. It also remains to be investigated whether bread wheat has sufficient osmotic tolerant and/or tissue tolerant mechanisms which it can employ during salt stress and, if it does, which tolerance mechanism, if any, predominates.

In this chapter a bread wheat double haploid (DH) mapping population, Berkut × Krichauff, is screened to explore the potential variability and identify QTL for the major salinity tolerance components. This chapter examines the use of high throughput salt screening protocols, as has been published in the previous chapter (Rajendran *et al.*, 2009), to screen a Berkut × Krichauff doubled haploid mapping population of bread wheat. The genetic variability for the three salinity tolerance mechanisms will be assessed and potential QTL for major components of salinity tolerance identified. It is believed that this is the very first study to use non-destructive imaging techniques to identify potential QTL for both osmotic and ionic tolerance in bread wheat.

4.1 Introduction

Bread wheat (*Triticum aestivum*) is the most widely cultivated form of wheat in the world (IDRC, 2010). Currently, it occupies approximately 95% of world's total wheat cultivated area (Shewry, 2009). In many countries bread wheat is grown in regions which are affected by soil salinity, significantly reducing the yield of various commercial cultivars (Rengasamy, 2002). For example, the Australian bread wheat cultivar Baart46 has a fivefold yield reduction at an EC_e of 10 dS/m than when grown in non-saline environment (Genc *et al.*, 2007). In order to increase the yield potential of bread wheat in saline soils, scientists are aiming to develop salt tolerant cultivars that possess genes responsible for physiological functions such as prevention and alleviation of salt injury, maintenance of growth rate and the capacity to re-establish homeostatic conditions in a salt stressed environment (Epstein *et al.*, 1980; Flowers and Yeo, 1995; Flowers *et al.*, 1997; Shannon, 1997). After decades of research only a few salinity tolerance genes, such as *Kna1* (*HKT 1:5*) (Gorham *et al.*, 1990), *MTID* (Abebe *et al.*, 2003), *HKT2;1* (Schachtman and Schroeder, 1994; Laurie *et al.*, 2002) have been identified in bread wheat. There are still many unknowns about the physiological and genetic basis of salinity tolerance mechanisms in bread wheat (Ashraf and Wu, 1994; Flowers *et al.*, 1997; Colmer *et al.*, 2005). Therefore, we need to design new approaches that will help to provide a better understanding of whole plant salinity tolerance, in a first hand before the development of salt tolerant cultivar in bread wheat.

Bread wheat is a moderately salt tolerant crop (Maas and Hoffman, 1977). Many bread wheat cultivars use Na^+ exclusion as a primary strategy to survive under salt stress by maintaining low shoot $[Na^+]$ but high $[K^+]$ (Gorham *et al.*, 1990; Munns and James, 2003). There are quite a number of early studies reporting a positive relationship between salinity tolerance and Na^+ exclusion in bread wheat (Rashid *et al.*, 1999; Poustini and Siosemardeh, 2004; Cuin *et al.*, 2009; Cuin *et al.*, 2010; Genc *et al.*, 2010a). However, more recent studies indicate that salinity tolerance of bread wheat was also found to be associated with traits such as osmotic tolerance (Rivelli *et al.*, 2002; Rahnama *et al.*, 2011) and tissue tolerance (Genc *et al.*, 2007). The major

contributing components of salinity tolerance have already identified in bread wheat, however to date most of the salinity tolerance research has been focused mainly on Na⁺ exclusion; there are few studies that have focused on characterizing bread wheat for osmotic and tissue tolerance. Little is known about the osmotic and tissue tolerance components in bread wheat due in part to complex and often destructive screening methods which make screening for QTL difficult. However, it is predicted that the most successful salinity tolerant wheat cultivars use several salinity tolerance mechanisms to adapt soil salinity (Rivelli *et al.*, 2002; Rajendran *et al.*, 2009; Cuin *et al.*, 2009). Hence, selection of genotypes and/or lines (breeding material) with different combinations of these three major components of salinity tolerance could be employed as a reliable alternative strategy rather than the selection of single physiological component (Na⁺ exclusion) for the salinity tolerance breeding of bread wheat in near future. Accordingly, this current study was designed to the study the physiological and genetic basis of the three major components of salinity tolerance in bread wheat.

The success of salinity tolerance breeding mainly depends on the availability of a reliable salt screening protocol that helps to identify potential genotypes/lines in the breeding material for further selection and hybridization processes (Munns and James, 2003; Genc *et al.*, 2010a). There are many field based (Richards *et al.*, 1987; Isla *et al.*, 1997; Takehisa *et al.*, 2004) and lab based (Ayers, 1948; Niazi *et al.*, 1992; Al-Karaki, 2001) screening procedures available for salinity tolerance screening. Early salt screening methodologies had some limitations, particularly those for tissue tolerance and osmotic tolerance due to the subjective nature of scoring for salt toxicity symptoms by human eye and the heterogeneity of the field environment affecting the consistency of salt screening/experimental results (Greenway and Munns, 1980; Richards, 1983; Shannon, 1985).

The recent advances in the imaging technology open up new avenues to quantify growth and health of individual plants throughout their growth cycle under controlled environment. In the previous chapter, such imaging techniques were used as a tool to develop a high throughput salt screening protocol to screen for the three components

of salinity tolerance, Na⁺ exclusion, osmotic tolerance and tissue tolerance, in *T. monococcum*. It was very useful, not only to identify which *T. monococcum* accessions were salt tolerant but also to identify which of the three tolerance components the accessions used (Rajendran *et al.*, 2009). Hence, it is now possible to measure and reveal the inherent variability for the three major component of salinity tolerance in the breeding material of *T. monococcum* and other cereal crops such as wheat and barley (Chapter 3). Accordingly, in this chapter, a study was conducted to apply similar image-based salt screening protocol to quantify the three major components of salinity tolerance in a doubled haploid (DH) mapping population of bread wheat for further genetic analysis.

The development of salinity tolerant bread wheat cultivar has been successful with various approaches such as conventional breeding (Ashraf and Oleary, 1996), QTL mapping (Lindsay *et al.*, 2004) and genetic engineering (Sawahel and Hassan, 2002; Abebe *et al.*, 2003). QTL mapping has been suggested to be a powerful approach for the improvement of complex polygenic trait like salinity tolerance in various crops (Ortiz, 1998; Ruttan, 1999; Collard and Mackill, 2008; Roy *et al.*, 2011). QTL for salinity tolerance have already identified in bread wheat, include QTL for Na⁺ exclusion (Gorham *et al.*, 1987; Dubcovsky *et al.*, 1996b; Ogbonnaya *et al.*, 2008; Edwards *et al.*, 2008; Genc *et al.*, 2010a) as well as for various morphological traits associated with salinity tolerance (Ma *et al.*, 2007). To date, however, none of them were studied QTL for both osmotic and ionic tolerance components and their effect on increase in shoot biomass in bread wheat. Accordingly, this study was formulated to quantify and identify the QTL linked to the three major physiological components of salinity tolerance such as osmotic tolerance, Na⁺ exclusion and tissue tolerance in the Berkut × Krichauff DH mapping population of bread wheat.

The objectives this chapter are,

- To use high throughput salt screening protocol and screen for the components of salinity tolerance in bread wheat.

- To study the phenotypic and genetic variability for these three major components of salinity tolerance in bread wheat.
- To map QTL linked to the three major components of salinity tolerance for further marker assisted breeding and other candidate gene(s) approaches.

4.2 Materials and methods

4.2.1 Mapping population

Seeds of 152 Berkut × Krichauff DH mapping lines and the parents were kindly supplied by Drs. Hugh Wallwork, Yusuf Genc, and Klaus Oldach (South Australian Research and Development Institute (SARDI), Adelaide, Australia) for this study. Krichauff (Pedigree: Wariquam/Kloka/Pitic62/3/Warimek/Halberd/4/3Ag3Aroona), the male parent of this population, is an Australian premium white (APW) quality, yellow alkaline noodle, winter bread wheat variety, developed at SARDI in 1997. Krichauff has been shown to be a good Na⁺ excluder, excluding more Na⁺ than other bread wheat varieties grown in saline conditions (Genc *et al.*, 2007). Moreover, it is moderately resistant to the wheat lesion nematode *Pratylenchus thornei* (Smiley, 2009). On the contrary, Berkut (Pedigree: IRENA/BAVIACORAM 92//PASTOR, 2002), the female parent in this cross, was developed at International Maize and Wheat improvement Centre, (CIMMYT), Mexico. Berkut accumulates more shoot Na⁺ than Krichauff and it is hypothesised to use tissue tolerance mechanisms to tolerate saline environments (Genc *et al.*, 2007). It has also been shown to be a high yielding and drought tolerant variety. The Berkut × Krichauff DH mapping population had already been screened for QTL linked to shoot Na⁺ exclusion however, the tools were not available at the time to identify QTL linked to osmotic tolerance and/or tissue tolerance (Genc *et al.*, 2010a).

4.2.2 Experimental setup

DH mapping populations are homogenous in nature and hence they are highly replicable over time (Gong *et al.*, 1999; Ellis *et al.*, 2002; Huynh *et al.*, 2008; Siahsar and Narouei, 2010; Genc *et al.*, 2010a). Accordingly, a total of three repeated, experiments with 152 DH mapping lines and their parents were grown in a greenhouse during winter, early spring and late spring of 2008. Because of the constraint in image capturing, which requires manual feeding of plants to the imaging station, each mapping lines was only replicated once in every experiment. However, the parents Berkut and Krichauff were replicated six times and randomized along with

the mapping lines in every experiment. The mapping population was grown in a supported hydroponics setup by following the methodology described in Chapter 2. Comprehensive information about seed germination, transplantation, supported hydroponics system and growth conditions are given in Chapter 2.

4.2.3 Non-destructive 3D plant imaging

The detailed information about the LemnaTec non-destructive 3D plant imaging technology has already been elucidated in Chapter 2. In brief, RGB images of Berkut × Krichauff DH mapping population was captured, by a LemnaTec scanalyser, Würselen, Germany. A total of 23,829 RGB images of this mapping population was acquired over 13 time points which include 5 time points before and 8 time points after NaCl application. Plants were imaged from three different angles every day from 5 days before NaCl application to 5 days immediately after NaCl application. Thereafter images were obtained every second or third day until the plants were 31 days old. The images were analysed and the plant shoot size (Total projected shoot area) as well as plant health were determined as follows in section 4.2.4.

4.2.4 High throughput salt screening

A similar high throughput salt screening protocol, as has been described in the previous chapter, was used to quantify the three major components of salinity tolerance in the mapping population. In order to measure the variability for three major salinity tolerance components mapping lines were subjected to 150 mM NaCl at the time of fourth leaf emergence which is approximately 14 days after germination. The final concentration of NaCl (150 mM) and CaCl₂ (7.02 mM) was reached by six consecutive doses of 25 mM NaCl, along with 1.17 mM CaCl₂, which was applied twice everyday to the nutrition solution in the supported hydroponics tank. The detailed information of NaCl application is given in Chapter 2.

4.2.4.1 Osmotic tolerance screen

The initial growth reduction rate, immediately after NaCl application can mainly be attributed to osmotic stress and is independent of the accumulation of Na^+ in the shoot tissues (Munns & Tester 2008). Screening for osmotic tolerance of the DH lines was carried out as described in Chapter 3, with one major exception, images were only obtained of plants undergoing salt stress since there was no control grown plants raised in the experiment. Therefore, osmotic tolerance was calculated by measuring changes in each individual plant's relative growth rate 5 days after the addition of NaCl and comparing that to the relative growth rate 5 days before NaCl application. The mean relative growth rate 5 days before and 5 days immediately after NaCl application was calculated using macros in excel the spread sheet (<http://www.ozgrid.com/forum/showthread.php?t=94519>). As shown in Chapter 3, osmotic tolerance was calculated by dividing the mean relative growth rate of a line 5 days immediately after NaCl application with the mean relative growth rate of the same line 5 days before NaCl application. Thus the osmotic tolerance of 152 DH mapping population and their parents were calculated individually.

4.2.4.2 Exclusion screen

Excluders were identified through the analysis of $[\text{Na}^+]$ in the fourth leaf blade of individual mapping lines. The fourth leaf blade was sampled after three weeks of growth in 150 mM NaCl. The $[\text{Na}^+]$ and $[\text{K}^+]$ in the fourth leaf blade was measured by flame photometry (Model 420, Sherwood scientific, Cambridge, U.K). Subsequently lines with low $[\text{Na}^+]$ and high $[\text{Na}^+]$ were identified as good Na^+ excluders and Na^+ accumulators respectively. The further details of measuring $[\text{Na}^+]$ are given in Chapter 2 and 3.

4.2.4.3 Tissue tolerance screen

The calculation of tissue tolerance needs two parameters, the non-destructive quantification of the proportion of salt induced senescence in the shoot and the destructive measurement of $[\text{Na}^+]$ in the leaf blade. The total senesced shoot area was calculated from the image of lines captured at the last time point, which was three weeks after 150 mM NaCl application. Immediately after the acquisition of the last image the fourth leaf blade of each mapping line was sampled for $[\text{Na}^+]$ analysis as has been described in the previous section. More details are given in Chapter 3, Section 4.3.3 and 4.4.3.

4.2.5 Phenotypic data analysis

After quantification of salinity tolerance components, descriptive statistics were carried out to study the mean, standard deviation (SD) and the range of the phenotypic data. Initially, the data was subjected to Kolmogorov-Smirnov test of normality before the phenotypic distributions of the data were examined through Q-Q charts. The Kolmogorov-Smirnov test of normality was used to examine the normality of the data set and the Q-Q chart was used to compare the probability distributions. If the residuals were found to be normal, the data was left untransformed and the heterogeneous data set was log transformed for further analysis. In order to determine the significance of the genotypic differences among the mapping lines and among the experiments, analysis of variance (ANOVA) was carried out using General Linear Model (GLM) procedure. The broad sense heritability (H^2) was calculated by using the formula $H^2 = 1 - (M_2 / M_1)$ (Knapp *et al.*, 1985). Where M_1 is the mean square of mapping lines and M_2 is the error mean square. Since this study was conducted in an incomplete block design with single replication and repeated in three distinct seasons, it was not possible to estimate genotype \times environment interaction. In this case the error mean square was used as M_2 instead of the mean square of genotype \times environment for broad sense heritability calculation (Huang *et al.*, 2006b). The confidence interval (CI) of the H^2 was calculated to determine the precision of heritability calculations. The lower 90% CI was estimated as $1 - [(M_1/M_2)$

$\times F_{1-\alpha/2;df2,df1}^{-1}$ and the upper 90% CI was calculated as $1 - [(M_1/M_2) \times F_{\alpha/2;df2,df1}]^{-1}$ (Knapp *et al.*, 1985). The H^2 was classified as low, medium and high by following the method of Johnson *et al.*, (1955). All of the statistical analyses were done in SPSS statistics version 17.0 (SPSS, Inc., Chicago, IL, USA).

4.2.6 The genetic map

The genetic map of Berkut \times Krichauff DH mapping population was obtained from Dr. Klaus Oldach (SARDI, Waite campus, The University of Adelaide (Genc *et al.*, 2010a). Briefly, it possessed 216 polymorphic SSRs, *vrn* genes and 311 DArT markers. Genotyping of SSR markers was done using standard PCR protocols, with primers spanning the region containing the SSR, and subsequent gel electrophoresis using either using 8% polyacrylamide gels or a ABI3730 capillary sequencer (Applied Biosystems, Warrington, U.K) (Hayden *et al.*, 2008b). DArT markers were mapped by Triticarte Pty Ltd. (<http://www.triticarte.com.au/>) using the method described by Akbari *et al.*, (2006). The genetic linkage map was constructed using Map Manager QTX version QTXb20 (Manly *et al.*, 2001), using the Kosambi mapping function at $p=0.01$ level. The marker order was cross checked through the use of RECORD computer software, linkage groups were arranged and used for QTL analysis (Van Os *et al.*, 2005).

4.2.7 QTL analysis

The position and effect of QTL were studied through composite interval mapping (CIM) by WINQTL CART v.2.5 (Wang *et al.*, 2010). For CIM, Model 6 (standard model) with 10 control background markers and a window size of 10cM was used for QTL analysis. Forward and backward regression method was used to select for CIM analysis. The significant threshold of the QTL was determined through likelihood ratio statistic (LRS) analysis with 1000 permutation combinations at 2cM walk speed ($p < 0.05$ level). The epistatic interaction between two different loci and the interaction between the QTL \times environment were analysed through mixed linear composite interval mapping (MCIM) approach in QTL Network 2.0 (Yang *et al.*, 2007; Yang *et*

al., 2008; Wang *et al.*, 2011) with 1cM walk speed and 2D genome scan. The critical F-value was estimated at 1000 permutation to find out the significant threshold for the presence of QTL and QTL \times environment interactions. Candidate interval selection and putative QTL detection were done with an error of 0.05 and 0.01 respectively using Henderson method III. Consistency of QTL was examined in all the three individual seasons and also in the mean values across the seasons. QTL with LRS score >13.8 and R^2 values $>10\%$ either in any one of the three seasons as well as in the mean over three seasons were declared as major QTL in this study. For a highly significant QTL, 95% confidence interval of QTL position was calculated using 1-LOD support interval from CIM analysis. The nomenclature of the QTL was performed as per the International Rule of Genetic Nomenclature (<http://wheat.pw.usda.gov/ggpages/wgc/98/Intro.htm>). The graphical representation of detected QTL were done through Map Chart 2.2, Plant Research International, Wageningen, The Netherlands (Voorrips, 2002).

4.3 Results

4.3.1 Osmotic tolerance

4.3.1.1 Determination of variability for osmotic tolerance in Berkut × Krichauff DH mapping population

In order to investigate the variability for osmotic tolerance in the Berkut × Krichauff DH mapping population, 150 mM NaCl was applied when the plants were 14 days old and the growth reduction of seedlings, 5 days immediately after NaCl application, was quantified. The osmotic tolerance of the plants was calculated by dividing the mean relative growth rate of seedlings 5 days immediately after NaCl application by the mean relative growth rate of seedlings 5 days before NaCl application. The mean osmotic tolerance of the parents and mapping population are presented in Table 4. It was observed that there was a considerable difference in osmotic tolerance between the parents, with Berkut exhibiting greater osmotic tolerance than Krichauff (Table 4).

Table 4. Descriptive statistics and broad sense heritability (H^2) of the osmotic tolerance quantified in the parents and Berkut × Krichauff DH mapping lines.

Component of salinity tolerance	Parents			
	Berkut		Krichauff	
	Mean ± S.E	Range	Mean ± S.E	Range
Osmotic tolerance	0.59 ± 0.05	0.50 - 0.66	0.31 ± 0.07	0.18 - 0.42
	DH mapping population			
	Range		H^2	CI for H^2
	0.15 - 0.79		0.70	0.60 - 0.76

There existed a wide range of variation for osmotic tolerance among the mapping lines. The range of mean osmotic tolerance within the whole mapping population varied from 0.15 (highly sensitive) to 0.79 (highly tolerant) (Table 4 & Figure 14).

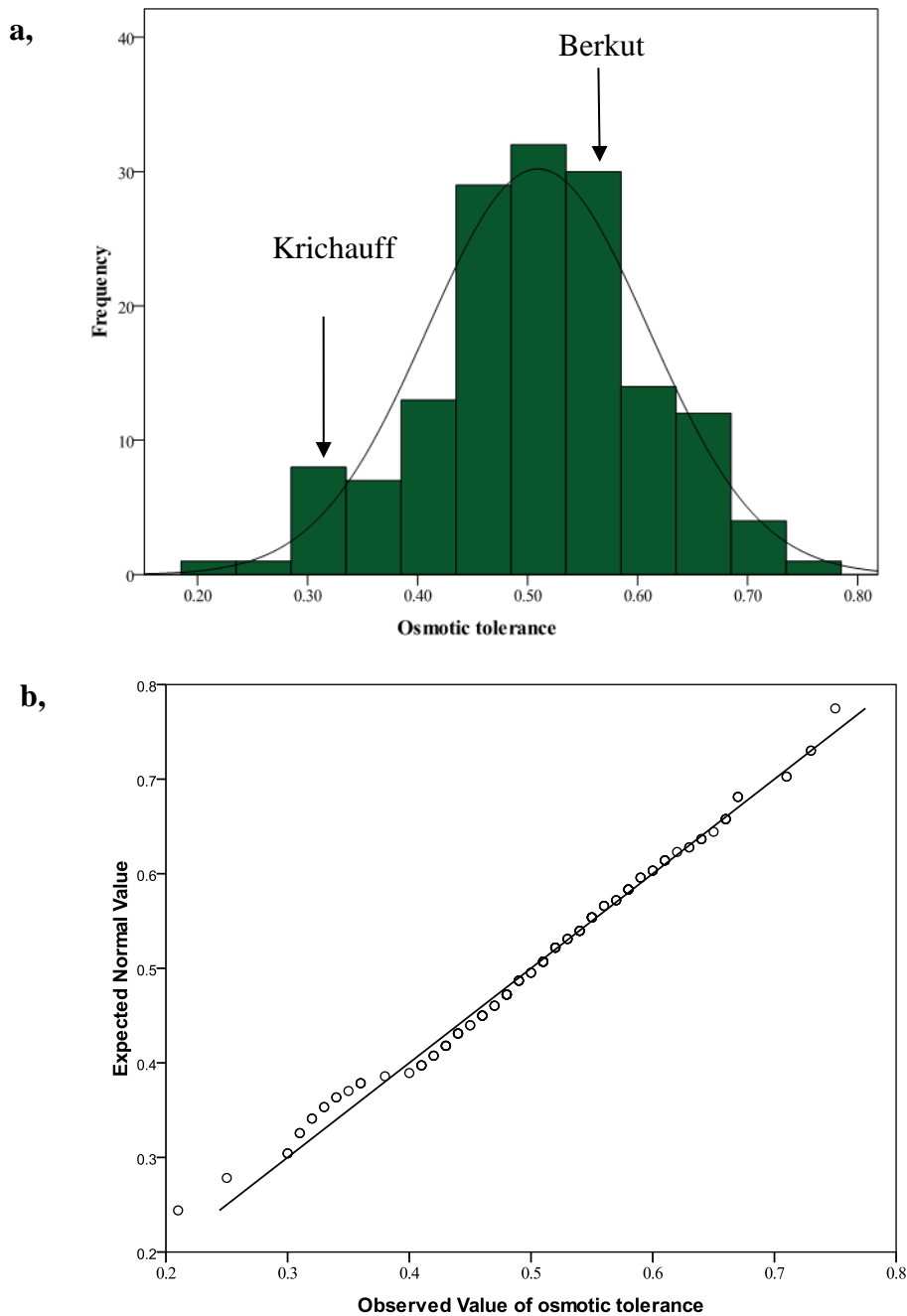


Figure 14. (a) Histogram showing variation for the mean osmotic tolerance of 152 Berkut \times Krichauff DH mapping lines grown in winter, early spring and late spring 2008. (Curve: Normal distribution). Osmotic tolerance was determined for each line by dividing the mean relative growth rate 5 days immediately after 150 mM NaCl application by the mean relative growth rate 5 days immediately before NaCl application. The variation between the mean osmotic tolerance of the parents is indicated by arrows. (b) Q-Q chart plotted with the observed quantiles of osmotic tolerance (\circ) against the expected normal quantiles (Straight line indicates the normal distribution).

The result obtained from the Kolmogorov-Smirnov test of normality has identified that the osmotic tolerance in the mapping population was normally distributed ($P>0.02$) (Table 5).

Table 5. Kolmogorov - Smirnov test of normality done for osmotic tolerance quantified in Berkut × Krichauff DH mapping population.

Salinity tolerance component	Kolmogorov-Smirnov		
	Statistic	df	Sig
Osmotic tolerance	.062	152	.200*

Further data analysis with Q-Q chart demonstrated that both observed value of osmotic tolerance and expected normal value were in the defined range (0.2-0.8) and clustered against the line of normal distribution. The results also indicated that they showed continuous variation, suggesting the trait was polygenic. Transgressive segregation was noticed in this population, with the presence of progenies which exhibited either more or less osmotic tolerance than the parents (Figure 14).

In the mapping population, osmotic stress tolerant plants, such as line HW-893*A086, show minimal growth reduction after salt application, while others that are osmotically sensitive, such as HW-893*A008, showed considerable growth reduction after NaCl application (Figure 15 & 16). It is also important to note that there was no relationship found between mean relative growth rate of the mapping lines 5 days before NaCl application and the mean relative growth rate of lines 5 days after 150 mM NaCl application, suggesting that osmotic tolerance was independent of growth rate and could be observed in both slow and fast growing lines (Figure 17).

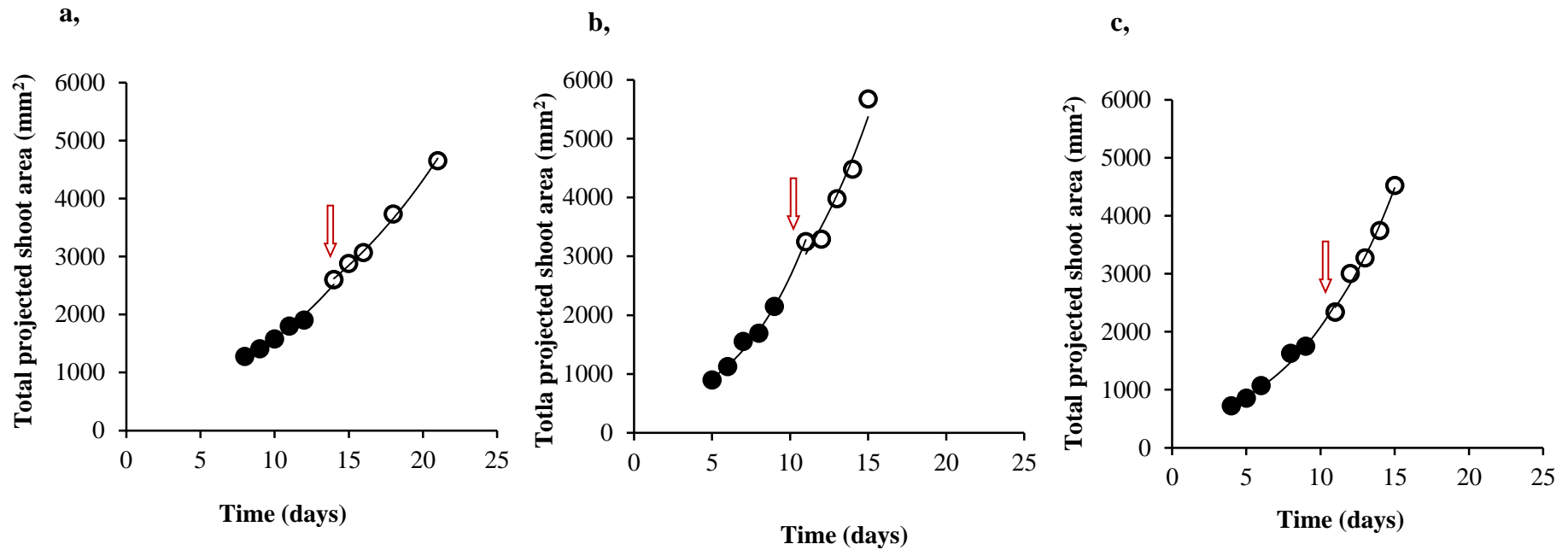


Figure 15. Growth of HW-893*A086, an osmotic stress tolerant line in (a) winter (b) early spring and (c) late spring. Plants were grown without NaCl until fourth leaf stage (approximately day 14) before 150 mM NaCl (arrow). The total projected shoot areas were calculated from images obtained from the LemnaTec Scanalyser as shown in Chapter 2. The mean relative growth rate of HW-893*A086 before NaCl application was 0.12 day⁻¹, 0.21 day⁻¹ and 0.17 day⁻¹, which was reduced to 0.08 day⁻¹, 0.14 day⁻¹ and 0.15 day⁻¹ after the addition of 150 mM NaCl in winter, early spring and late spring respectively.

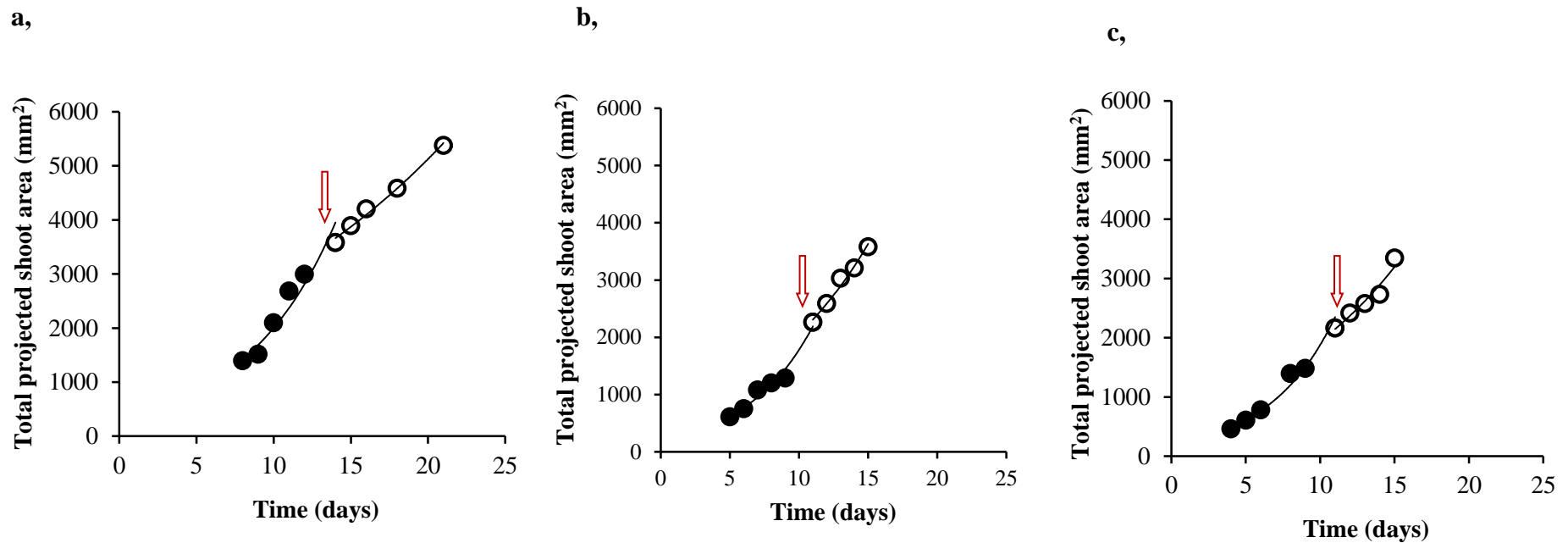


Figure 16. Growth of HW-893*A008, an osmotic sensitive line (a) winter (b) early spring and (c) late spring. Plants were grown without NaCl until fourth leaf stage (approximately day 14) before 150 mM NaCl (arrow). The total projected shoot areas were calculated from images obtained from the LemnaTec Scanalyser as shown in Chapter 2. The mean relative growth rate of HW-893*A008 before NaCl application was 0.17 day^{-1} , 0.21 day^{-1} and 0.22 day^{-1} which was reduced to 0.06 day^{-1} , 0.10 day^{-1} and 0.09 day^{-1} after the addition of 150 mM NaCl in winter, early spring and late spring respectively.

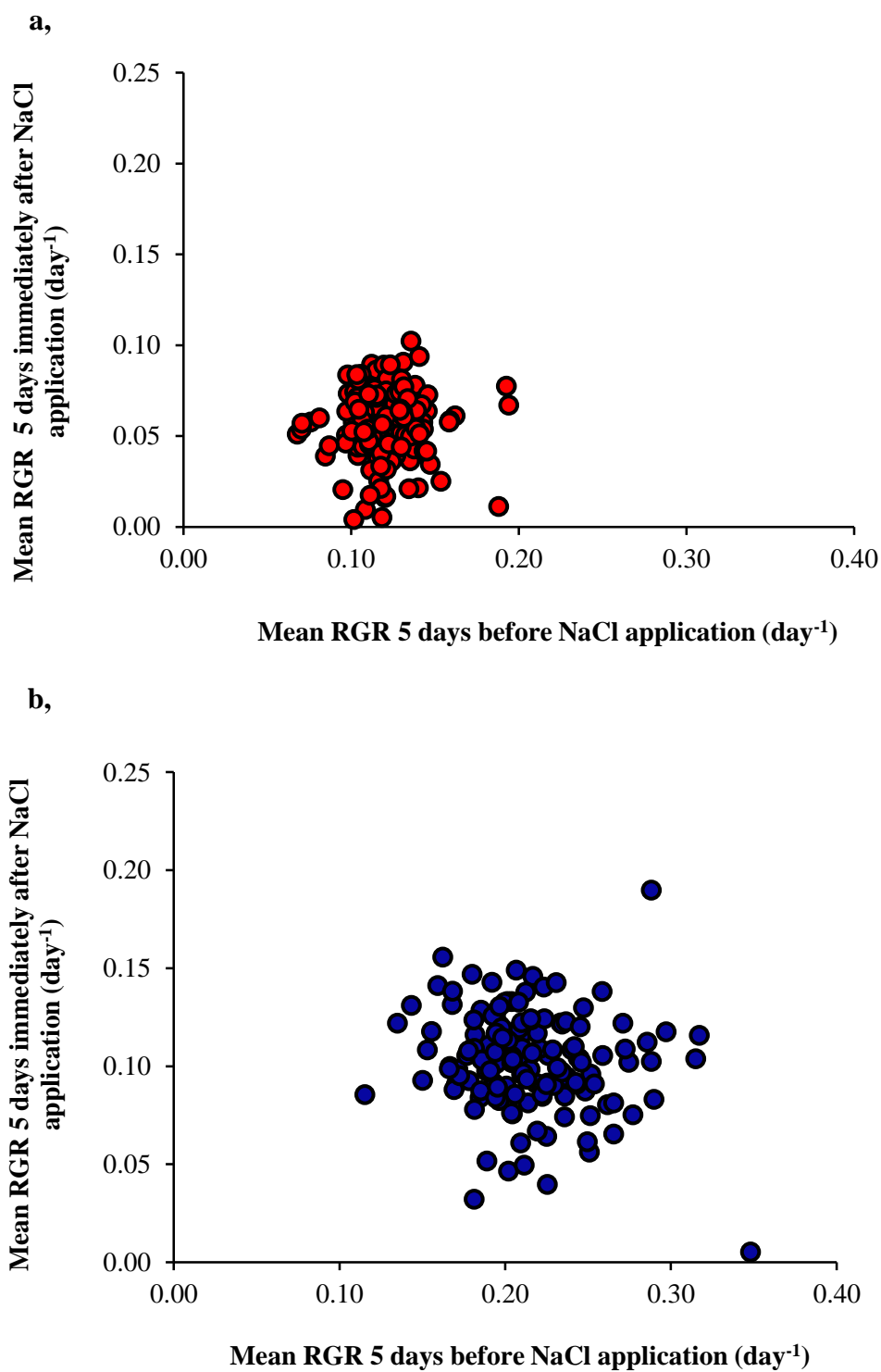


Figure 17. Relationships between the mean relative growth rates of the Berkut × Krichauff DH mapping population measured over the 5 days before NaCl application to the mean relative growth rates measured 5 days immediately after 150 mM NaCl application in (a) winter, (b) early spring and (c) late spring 2008.

c,

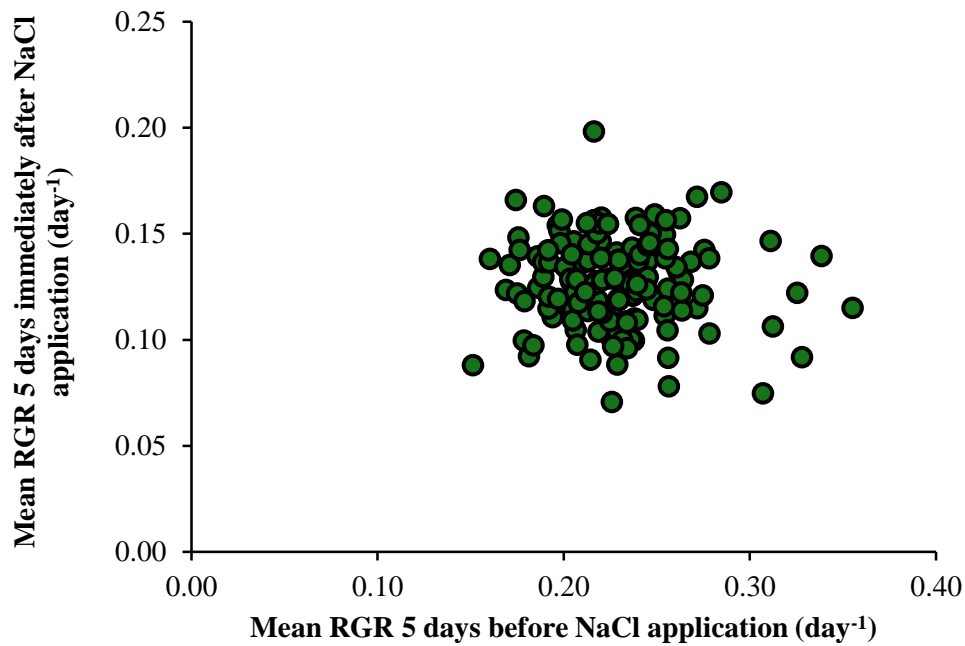


Figure 17. Continued.

Nonetheless, a GLM-ANOVA was used to analyse the results for the osmotic tolerance of mapping population. Significant genotypic differences were found between the mapping lines for osmotic tolerance ($P = 0.05$). In addition, significant difference was also noticed for osmotic tolerance ($P=0.05$) across three different experimental time of the year (Table 6). The H^2 of osmotic tolerance was 0.70 and the 90% confidence interval of H^2 was 0.60- 0.76 (Table 4 & 6).

Table 6. GLM-ANOVA for osmotic tolerance quantified in Berkut × Krichauff DH mapping population

Source	Type III Sum of Squares	Degrees of freedom	Mean Square	F value	Significance
Corrected Model	5.143	153	0.034	3.283	0.05
Intercept	75.571	1	75.571	7380.789	0.000
Genotypes (M ₁)	4.962	151	0.033	3.209	0.05
Seasons	0.077	2	0.039	3.772	0.05
Error (M ₂)	2.130	208	0.010		
Total	99.248	362			
Corrected Total	7.273	361			

4.3.1.2 Identification of QTL linked to osmotic tolerance in Berkut × Krichauff DH mapping population

As it was now possible to assign a quantitative value to a plant's osmotic tolerance, and a molecular map had previously been generated for this population, QTL linked to osmotic tolerance could now be determined. CIM identified a total of four QTL for osmotic tolerance on 1D (*QSot.aww-1D*), 2D (*QSot.aww-2D*) and 5B (*QSot.aww-5B.1* and *QSot.aww-5B.2*) chromosomes (Table 7, Figure 18 & 24). They collectively contributed up to 28.4% of the phenotypic variability for osmotic tolerance in this mapping population. With the exception of *QSot.aww-5B.1*, the alleles inherited from the Berkut parent contributed a positive effect on osmotic tolerance.

The QTL at *QSot.aww-1D* made a large contribution for osmotic tolerance in the early spring, explaining 13.7% of the phenotypic variability in the mapping population. It was found between the SSR markers *wPt8960* and *wPt2897*. Even though the QTL at *QSot.aww-1D* was small in winter, it did occur at higher levels in both the early spring and late spring, as well as at high levels if the mean over three experimental time of year is taken into account. A second QTL, *QSot.aww-2D*, accounted for 1.5 to 8.7% of the phenotypic variability in the mapping population and was identified between the SSR markers *ksm073* and *cfD044*. It was found to be at low levels in winter and late spring but at higher levels in early spring as well as in the mean calculated over three experimental time of year. The other two QTL, *QSot.aww-5B.1* and *QSot.aww-5B.2* have explained only minor phenotypic variability for osmotic tolerance in this mapping population (Table 7, Figure 18 & 24). Further, QTL analysis with MCIM approach did not reveal any epistatic interaction for osmotic tolerance in this mapping population.

Table 7. Characteristics of osmotic tolerance QTL identified in Berkut × Krichauff DH mapping population using CIM approach.

QTL	Flanking markers	Support interval (cM)	Winter- 2008			Early spring- 2008			Late spring -2008			Mean over three different experimental time of the year		
			LRS Scores*	R ² (%)	Add**	LRS Scores*	R ² (%)	Add**	LRS Scores*	R ² (%)	Add**	LRS Scores*	R ² (%)	Add**
<i>QSot.aww-1D</i>	<i>wPt8960-wPt2897</i>	55.40 – 104.60	0.24	0.17	0.01	22.15	13.7	0.06	3.15	2.4	0.01	7.75	5.4	0.03
<i>QSot.aww-2D</i>	<i>ksm073 – cfd044</i>	89.60 - 143.10	2.32	1.5	0.03	12.08	8.7	0.04	0.23	0.33	0.01	11.80	5.9	0.03
<i>QSot.aww-5B.1</i>	<i>barc340b-barc028b</i>	3.20-17.70	12.34	7.6	-0.05	1.45	2.6	-0.01	0.48	1.3	-0.01	12.30	4.4	-0.03
<i>QSot.aww-5B.2</i>	<i>wPt2707-wPt9504</i>	89.0-129.20	9.35	5.1	0.05	4.78	3.4	0.02	4.86	2.3	0.02	13.38	5.8	0.04

Main characters of osmotic tolerance QTL are listed in descending order according to their presence from 1A to 7D chromosomes. R² = Phenotypic variance explained by individual QTL. * Identification of QTL with LRS scores >13.8 were denoted as highly significant for all the three experiments and mean over three experimental time of the year. **Positive values of additive regression co-efficient (Add) are intended for increasing effect from Berkut alleles and negative values are meant for increasing effect from Krichauff alleles.

a,

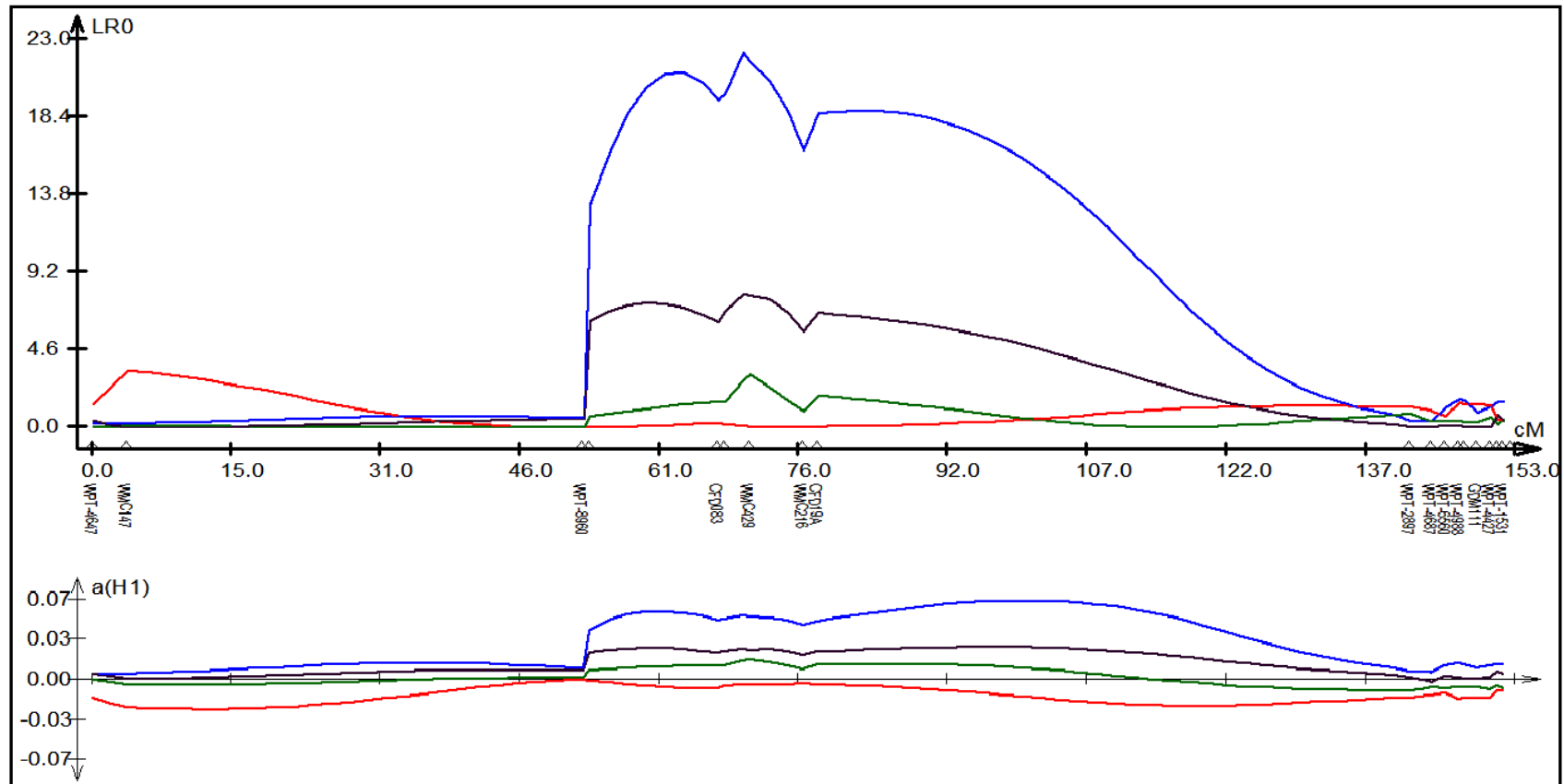


Figure 18. LRS plots of osmotic tolerance QTL identified on (a) 1D, (b) 2D and (c) 5B chromosomes in Berkut \times Krichauff DH mapping population of bread wheat with the data obtained from winter (red), early spring (blue) late spring (green) and mean over three experimental time of the year (black). QTL with LRS score >13.8 , is considered as highly significant. The positive additive effect indicates the inheritance of the QTL from the osmotic tolerant parent Berkut; the negative additive effect indicates the inheritance of QTL from the osmotic sensitive parent Krichauff.

b,

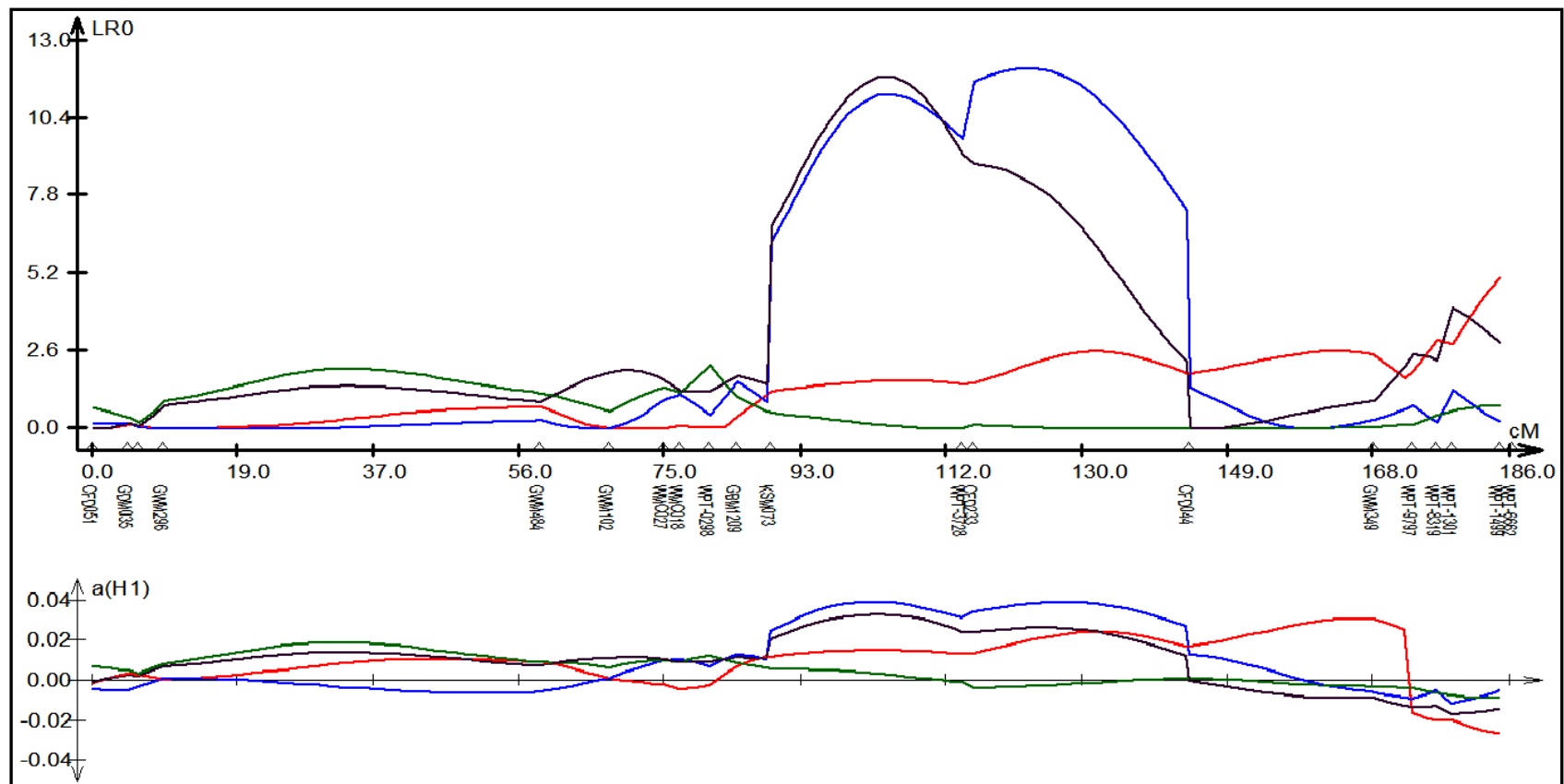


Figure 18. Continued.

c,

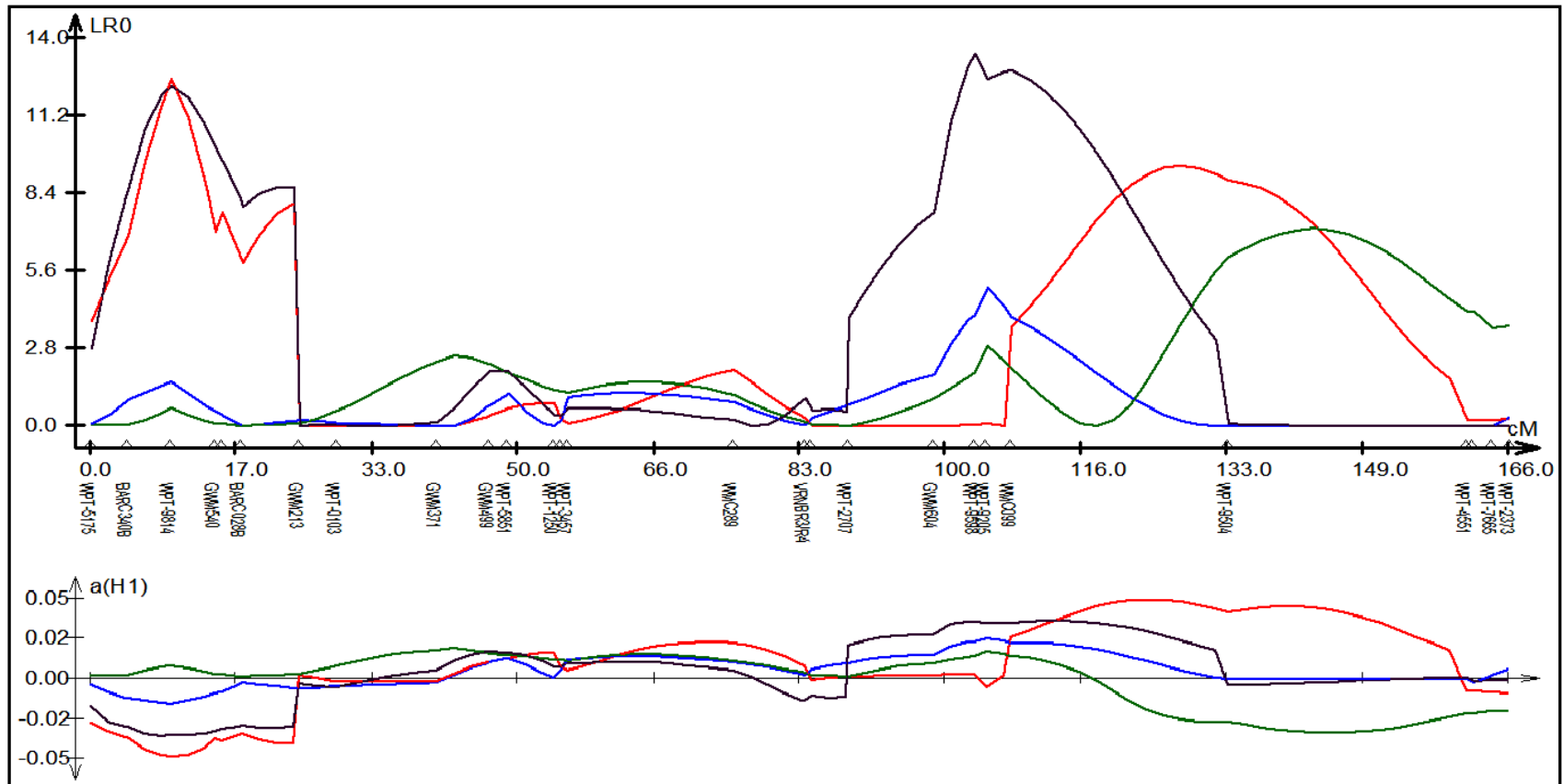


Figure 18. Continued.

4.3.2 Na⁺ exclusion

4.3.2.1 Determination of variability for Na⁺ exclusion in Berkut × Krichauff DH mapping population

In order to measure Na⁺ exclusion, the fourth leaf blade of individual mapping lines were sampled three weeks after 150 mM NaCl application. The [Na⁺] and [K⁺] of fourth leaf blade was analysed to identify both excluders and accumulators. It was found that Krichauff accumulated lower amounts of Na⁺ than Berkut in all the three experiments. The mean fourth leaf blade [Na⁺] of Berkut was 49.09 mM whereas it was 15.21 mM in Krichauff. The mean fourth leaf blade [Na⁺] of the mapping population was ranged from 5.7 to 235.02 mM. The population demonstrated a large phenotypic variation for fourth leaf blade [Na⁺] (Table 8).

Table 8. Descriptive statistics and broad sense heritability (H²) of the fourth leaf blade [Na⁺] (mM) calculated in the parents and Berkut × Krichauff DH mapping population.

Component of salinity tolerance	Parents			
	Berkut (mM)		Krichauff (mM)	
Na ⁺ exclusion	Mean ± S.E	Range	Mean ± S.E	Range
	49.09 ± 8.27	38.34-65.34	15.21 ± 2.52	11.75 - 20.11
	DH mapping population			
	Range (mM)		H ²	CI for H ²
	5.7 – 235.02		0.67	0.57-0.74

A Kolmogorov-Smirnov test of normality and Q-Q chart plotting both the observed and the expected normal value of the fourth leaf blade [Na⁺] revealed that the variables were significantly deviated from normal distribution and positively skewed towards Na⁺ exclusion (P<0.2) (Table 9 & Figure 19).

Table 9. Kolmogorov – Smirnov test of normality for fourth leaf blade [Na⁺] in the Berkut × Krichauff DH mapping population.

Salinity tolerance component	Kolmogorov-Smirnov test		
	Statistic	df	Sig
Na ⁺ exclusion	.176	150	.000
Log Na ⁺ exclusion	.041	150	.200*

Accordingly, a log transformation was made on the data set in order to satisfy the statistical assumption of normality for further analysis. After log transformation, the frequency distribution of the mean fourth leaf blade [Na⁺] has shown a normal distribution (Table 9 & Figure 20).

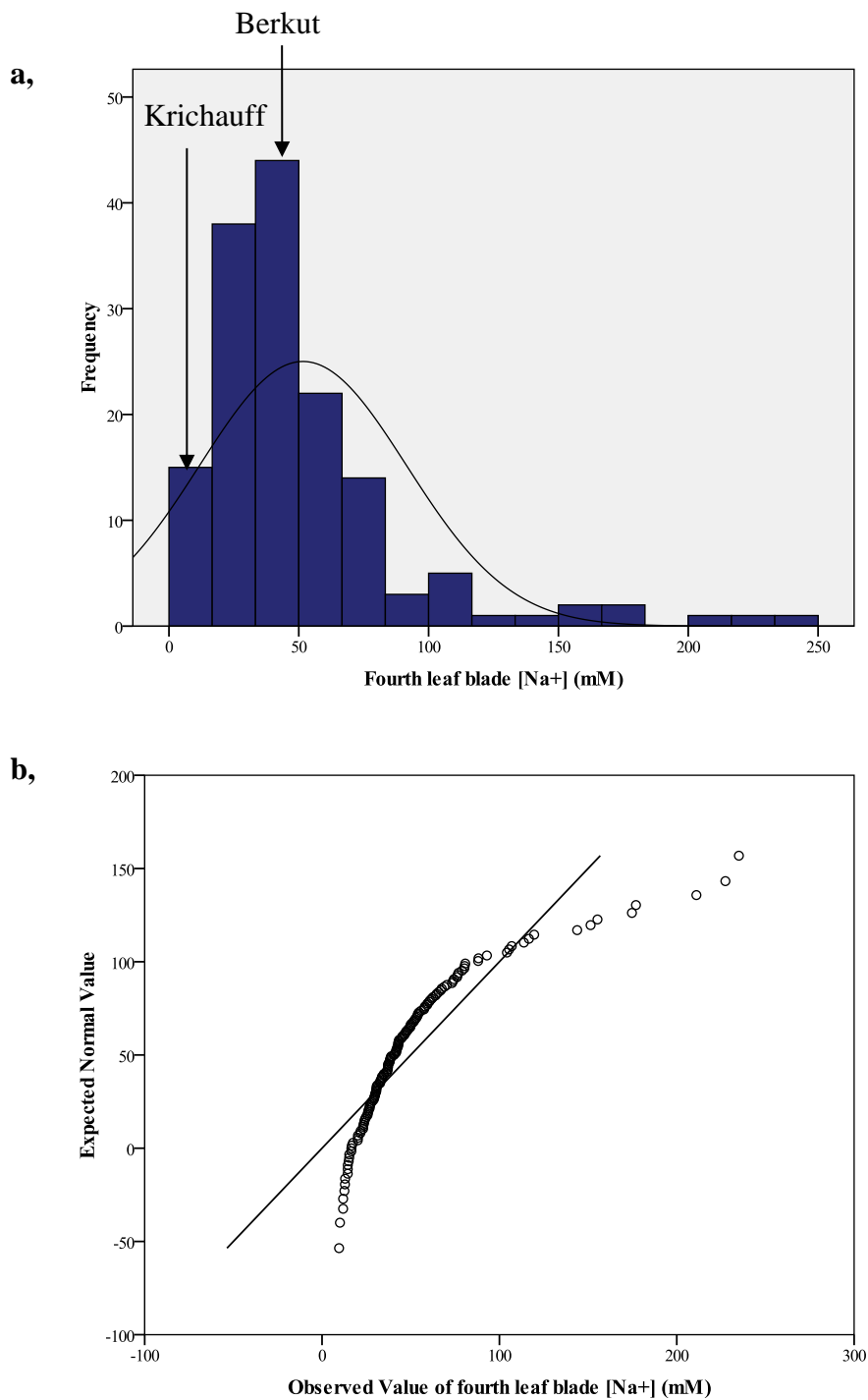


Figure 19. (a) Histogram showing variation for the mean [Na⁺] in the fourth leaf blade of 152 Berkut × Krichauff DH mapping lines grown under 150 mM NaCl for three weeks during winter, early spring and late spring 2008 (Curve: Normal distribution). The variation in mean fourth leaf blade [Na⁺] of parents is indicated by arrows. (b) Q-Q chart plotted with the observed quantiles of [Na⁺] (○) against the expected normal quantiles (Straight line indicates the normal distribution).

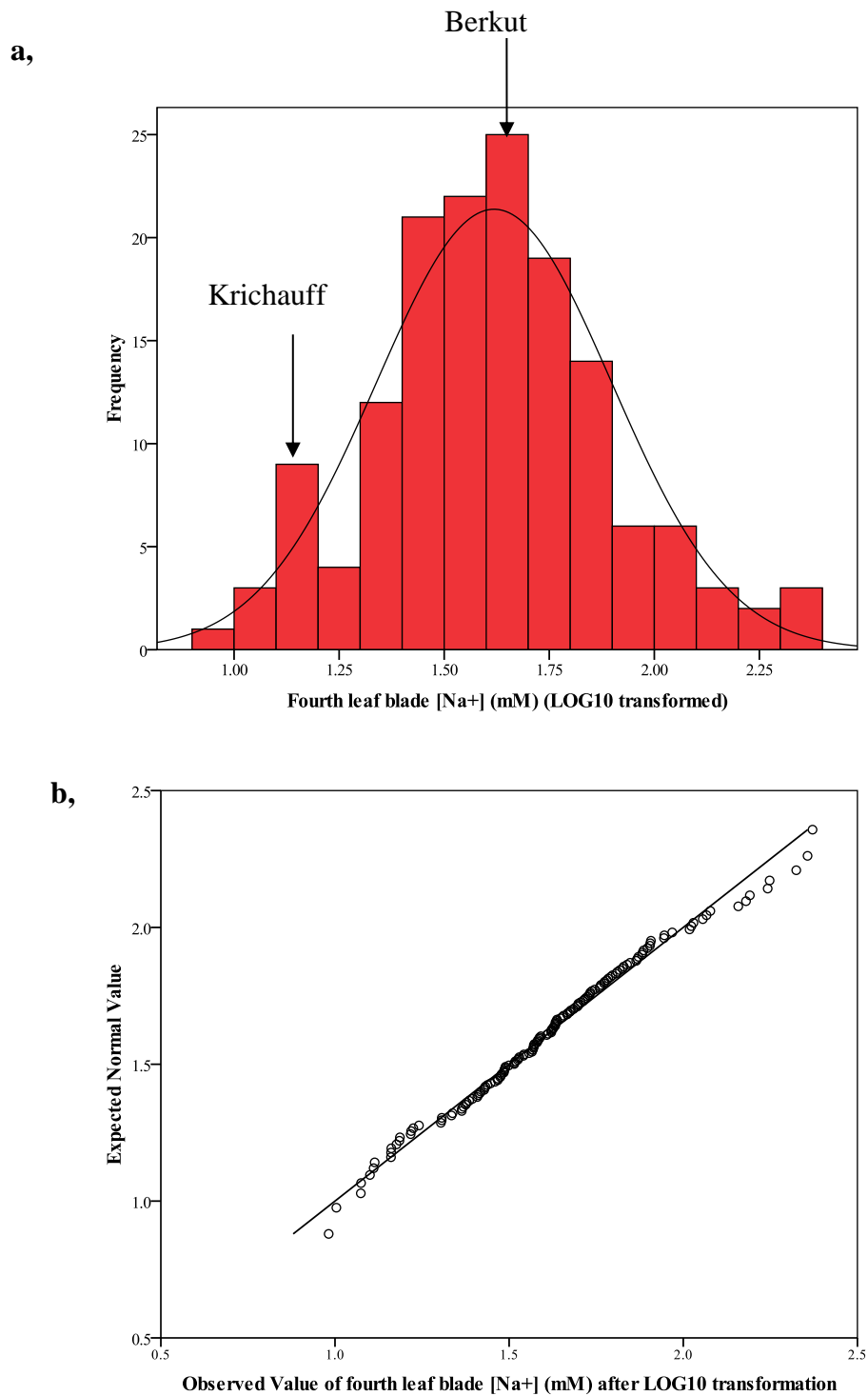


Figure 20. (a) Histogram showing variation for the \log_{10} of mean $[\text{Na}^+]$ in the fourth leaf blade of 152 Berkut \times Krichauff DH mapping lines grown under 150 mM NaCl for three weeks during winter, early spring and late spring 2008 (Curve: Normal distribution). The \log_{10} mean fourth leaf blade $[\text{Na}^+]$ of parents is indicated by arrows (b) Q-Q chart plotted with the observed quantiles of $[\text{Na}^+]$ (\circ) against the expected normal quantiles (Straight line indicates the normal distribution).

The GLM- ANOVA has revealed a significant difference for fourth leaf blade [Na⁺] between both the genotypes (P =0.10) and the experimental season (P =0.001). The variability within mapping lines grown over three different time of the year was more than the variability between mapping lines (Table 10). The H² of Na⁺ exclusion was high (0.67) and suggesting that phenotypic selection of progenies could be done at the early generation (Table 8 &10).

Table 10. GLM-ANOVA performed on the fourth leaf blade [Na⁺] in Berkut × Krichauff DH mapping population

Source	Type III Sum of Squares	Degrees of freedom	Mean Square	F value	Significance
Corrected Model	31.374	151	0.208	3.065	0.05
Intercept	922.486	1	922.486	13606.79	0.000
Genotypes (M ₁)	30.350	149	0.204	3.004	0.10
Seasons	1.042	2	0.521	7.681	0.001
Error (M ₂)	17.424	257	0.068		
Total	1056.403	409			
Corrected Total	48.798	408			

4.3.2.3 Identification of QTL linked to Na⁺ exclusion in Berkut × Krichauff DH mapping population

For Na⁺ exclusion, CIM identified a total of eight QTL with additive effects on chromosomes 1B (*QSel.aww-1B*), 2A, (*QSel.aww-2A.1*), 2D (*QSel.aww-2D.1*), 5A (*QSel.aww-5A.1* and *QSel.aww-5A.2*), 5B (*QSel.aww-5B*), 6B (*QSel.aww-6B.1*) and 7A (*QSel.aww-7A*) (Table 11, Figure 21 & 24). They were consistently found in at least two of the experiments and collectively contributed to 35% of the phenotypic variability for Na⁺ exclusion in this mapping population. QTL contributing to Na⁺ exclusion could be observed from both parents, *QSel.aww-5A.1* and *QSel.aww-6B* were inherited from Berkut, whereas, *QSel.aww-1B*, *QSel.aww-2A*, *QSel.aww-2D.1*, *QSel.aww-5A.2*, *QSel.aww-5B* and *QSel.aww-7A* were inherited from Krichauff. A major QTL, *QSel.aww-5A.2* was identified for Na⁺ exclusion in this mapping population. It was flanked by the SSR marker *barc193A* and *cfa2155*, and explained much of the phenotypic variation, with R² values from 7.5 to 12.1%. A second QTL, *QSel.aww-5A.1*, was observed in early spring and late spring and was also significant when the mean was taken across the three different experimental time of the year. It has explained up to 7% of the phenotypic variability for Na⁺ exclusion in the population. Other QTL observed in the population showed smaller additive effect and explained 1.1-6.7% of the phenotypic variability in the population (Table 11 & Figure 21).

Further MCIM analysis detected three pairs of epistatic interactions for Na⁺ exclusion in this mapping population (Table 12). They collectively explained about 13.8% of the phenotypic variability in the population. Among them *QSel.aww-2A.1* has shown both additive main and epistatic interaction effects. But, the other five QTL, *QSel.aww-2A.2*, *QSel.aww-2D.2*, *QSel.aww-6B*, *QSel.aww-7D.1* *QSel.aww-7D.2* have demonstrated only epistatic interactions. All the three pairs were shown to be significant additive × additive epistatic main effect at p<0.001 level. However, the additive × additive epistasis environment interaction was not significant with any of these QTL pairs (Table 12).

Table 11. Characteristics of Na⁺ exclusion QTL identified in Berkut × Krichauff DH mapping population using CIM approach.

QTL	Support Interval (cM)	Nearest marker	Winter - 2008			Early spring - 2008			Late spring - 2008			Mean over three experimental time of the year		
			LRS ¹	R ² %	Add	LRS ¹	R ² %	Add	LRS ¹	R ² %	Add	LRS ¹	R ² %	Add
<i>QSel.aww-1B</i>	22.50 – 46.10	<i>wPt3753 – gwm413</i>	0.89	3.4	-0.01	6.20	3.1	-0.08	8.39	4.4	-0.08	1.61	5.5	-0.03
<i>QSel.aww-2A.1</i>	87.40-103.10	<i>wPt3114 – wmc170</i>	0.26	1.5	-0.01	6.76	2.9	-0.09	11.26	4.7	-0.07	7.61	2.6	-0.07
<i>QSel.aww-2D.1</i>	129.30 - 167.80	<i>wPt3728 – gwm349</i>	8.34	2.9	-0.08	4.94	1.3	-0.06	4.87	1.2	-0.05	17.54	4.2	-0.14
<i>QSel.aww-5A.1</i>	26.10-83.3	<i>wPt1165- barc193A</i>	2.04	1.1	0.04	12.29	6.7	0.11	13.08	7.0	0.12	14.22	4.9	0.11
<i>QSel.aww-5A.2</i>	130.8-173.40	<i>barc193A – cfa2155</i>	16.01	11.3	-0.14	16.90	7.8	-0.17	10.46	7.5	-0.12	19.57	12.1	-0.17
<i>QSel.aww-5B</i>	4.40- 24.30	<i>barc340b – gwm213</i>	6.55	4.4	-0.07	3.64	1.6	-0.05	3.79	1.1	-0.04	2.31	0.93	-0.03
<i>QSel.aww-6B.1</i>	70.90 – 97.10	<i>cf001A- wPt3733</i>	0.98	2.1	0.02	10.63	6.7	0.09	5.25	4.7	0.09	2.13	2.2	0.03
<i>QSel.aww-7A</i>	19.60 – 55.10	<i>wPt4835- gwm060</i>	5.83	8.3	-0.08	5.10	2.3	-0.07	2.51	2.4	-0.05	3.82	3.1	-0.06

Main characters of Na⁺ exclusion QTL are listed in ascending order according to their presence from 1A to 7D chromosomes. R²- Phenotypic variance explained by individual QTL. * Identification of QTL with LRS scores >13.8 were denoted as highly significant for all the three experiments and mean over three experimental time of the year. **Positive values of additive regression co-efficient (Add) are intended for increasing effect from Berkut alleles and negative values are meant for increasing effect from Krichauff alleles.

Table 12. Additive \times additive epistatic main effect (*aa*) and additive \times additive epistasis environment interaction (*aae*) identified for Na⁺ exclusion in Berkut \times Krichauff DH mapping population using mixed composite interval mapping with 2D genome scan by QTL Network 2.0.

Epistatic interaction	Flanking markers	Support Interval (cM)	Position (cM)	<i>aa</i> effect	<i>R</i>² (%)	<i>aae</i>₁	<i>aae</i>₂	<i>aae</i>₃
<i>QSel.aww-2A.1</i> and <i>QSel.aww-7D.1</i>	<i>wPt3114-wmc170</i> and <i>gdm145-wmc436B</i>	90.5-114.6 and 53.5-68.8	98.5 and 57.8	-0.0787*	4.9	-0.0033	-0.0178	0.0216
<i>QSel.aww-2A.2</i> and <i>QSel.aww-7D.2</i>	<i>gwm294-gdm093</i> and <i>wmc436B-barc214</i>	115.6-142.8 and 117.8-126.8	131.6 and 126.8	-0.0821*	3.7	0.0000	-0.0001	0.0001
<i>QSel.aww-2D.2</i> and <i>QSel.aww-6B.2</i>	<i>wmc018-wPt-0298</i> and <i>gwm626-wPt-3581</i>	73.8-80.1 and 116.6-135.7	77.1 and 118.8	0.0794*	5.2	0.0001	-0.0000	-0.0001

* $p < 0.001$ level. The positive value in *aa* and *aae* indicate that the effect of parental alleles are greater than the recombinant alleles whereas the negative value in *aa* and *aae* indicate that the effect of recombinant alleles are greater than the parental alleles.

a,

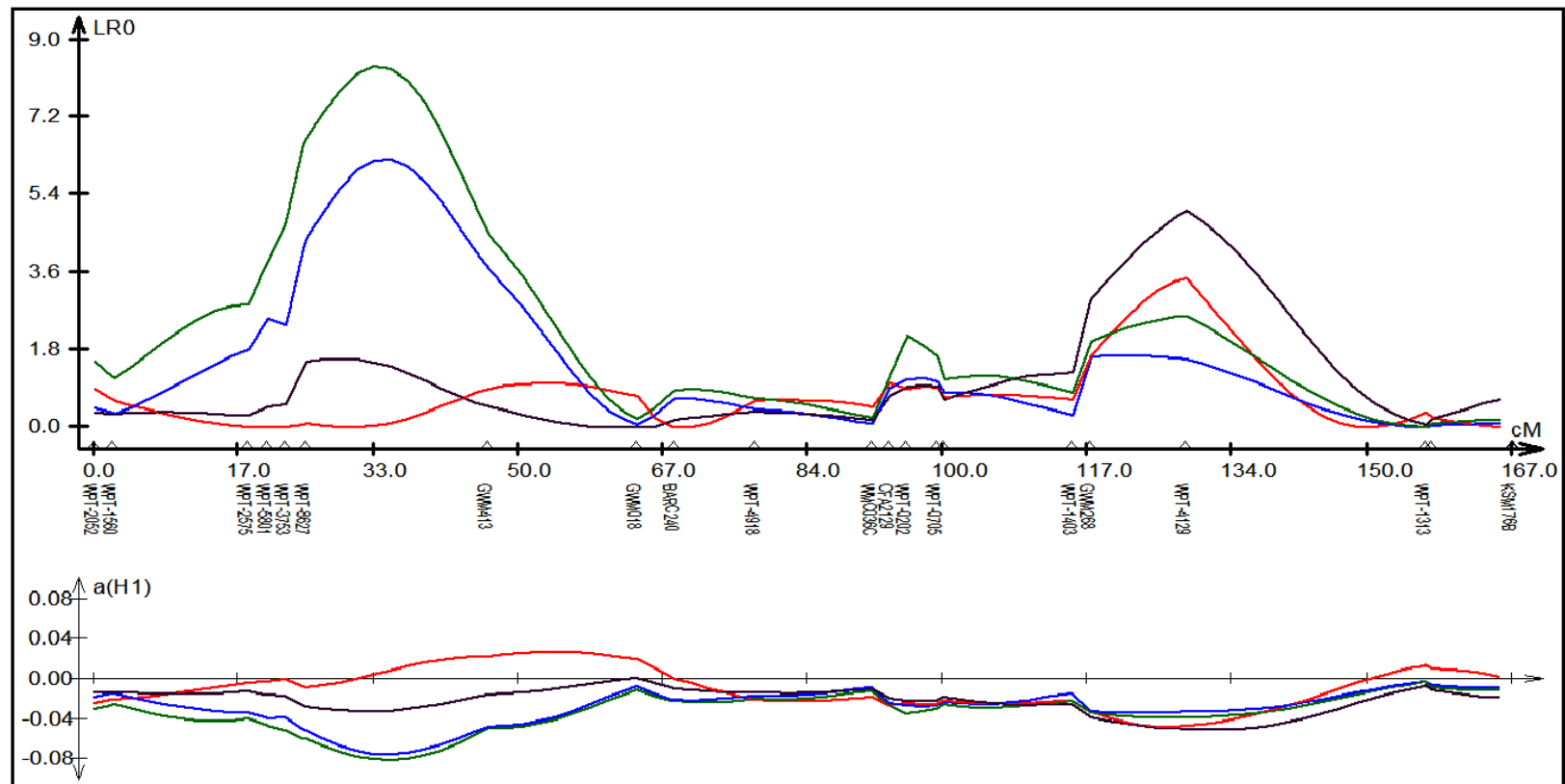


Figure 21. LRS plots of Na^+ exclusion QTL identified on chromosomes (a) 1B, (b) 2A, (c) 2D, (d) 5A (e) 5B, (f) 6B and (g) 7A in the Berkut \times Krichauff DH mapping population of bread wheat. Data obtained from winter (red), early spring (blue) and late spring (green), as well as the results from the mean of the three seasons (black). QTL with LRS score >13.8 , is considered as highly significant QTL. The positive additive effect indicates the inheritance of the QTL from the Na^+ accumulating parent Berkut; the negative effect indicates the inheritance of QTL from the Na^+ excluding parent Krichauff.

b,

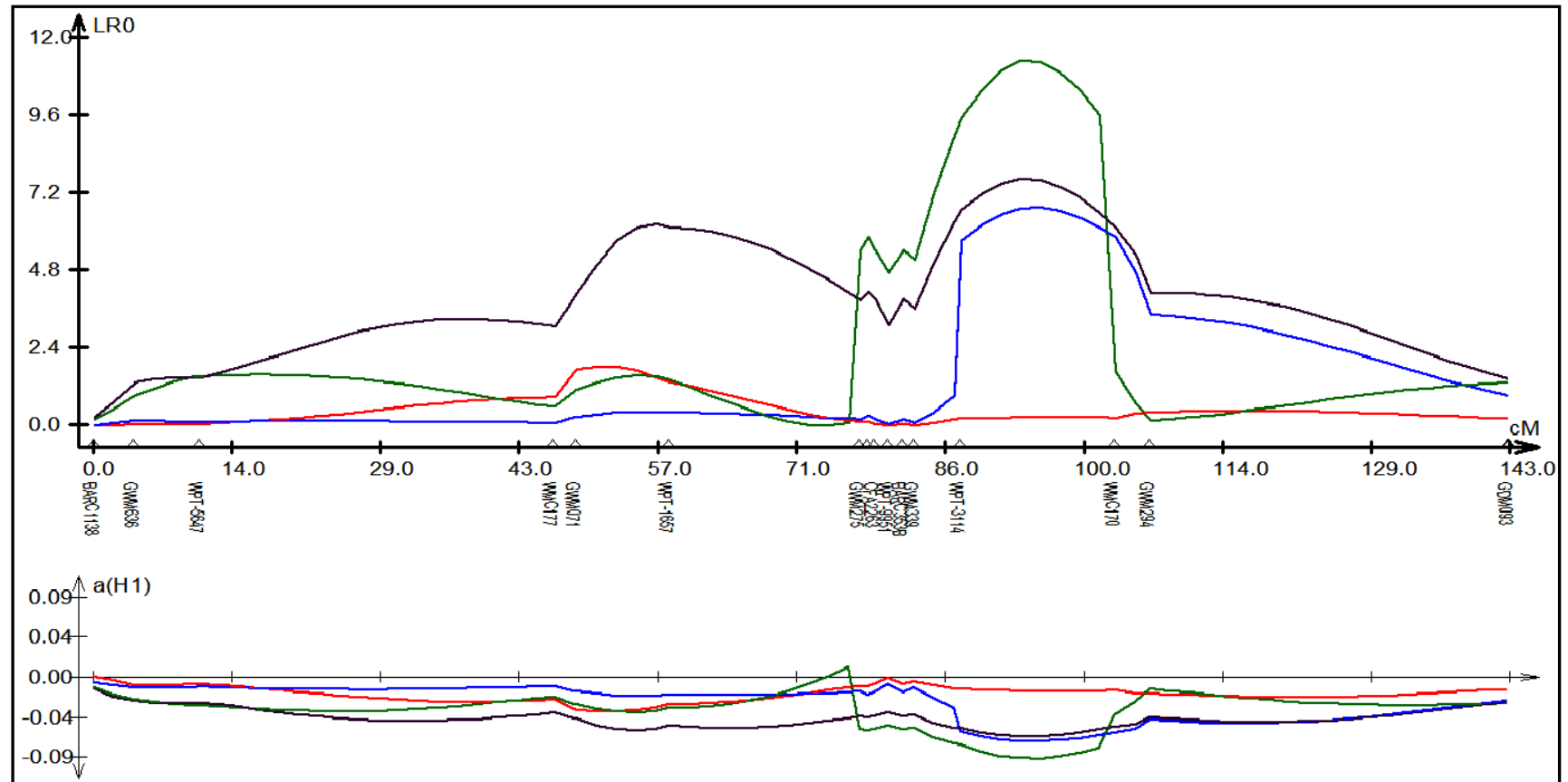


Figure 21. Continued.

c,

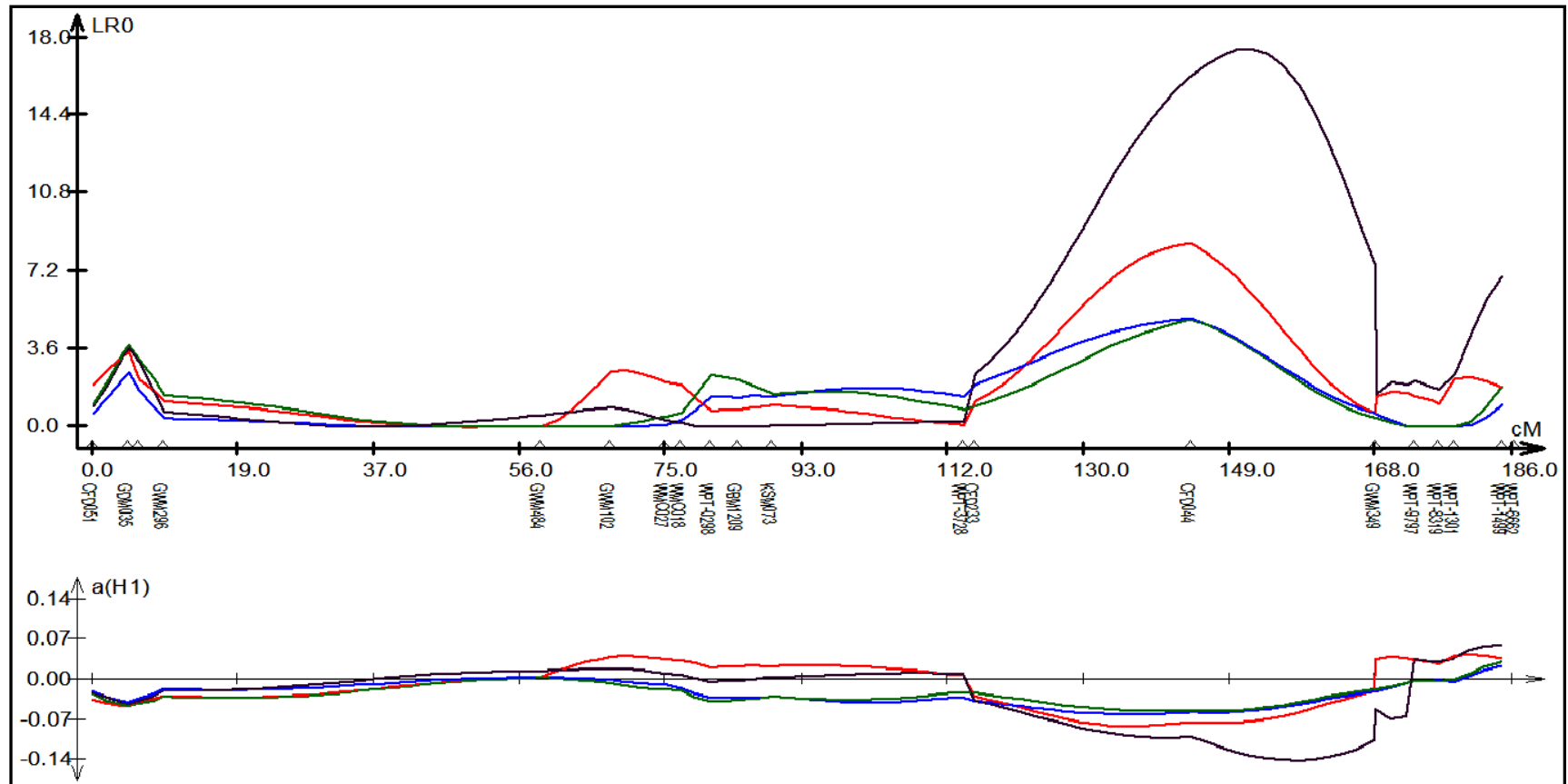


Figure 21. Continued.

d,

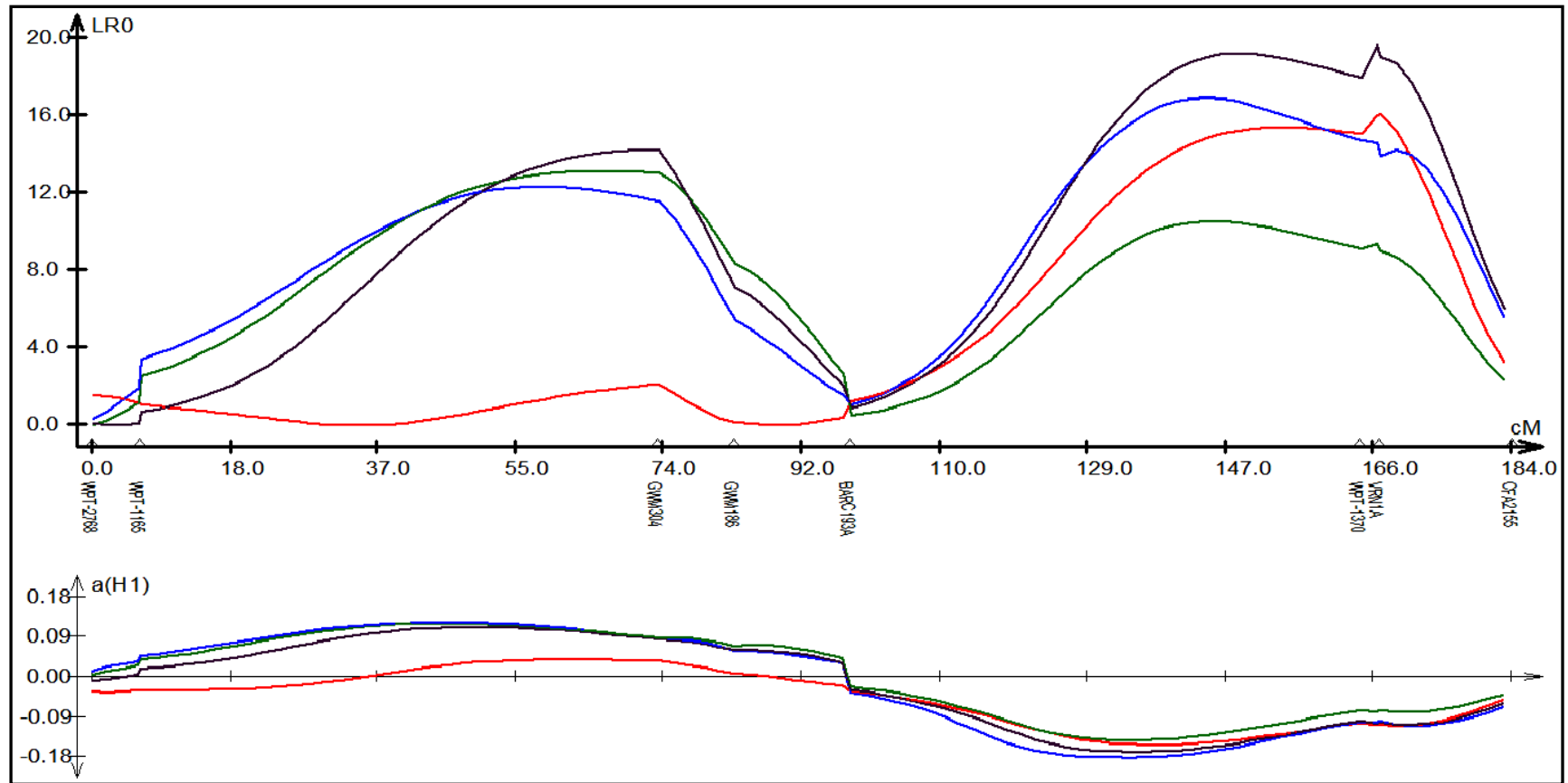


Figure 21. Continued.

e,

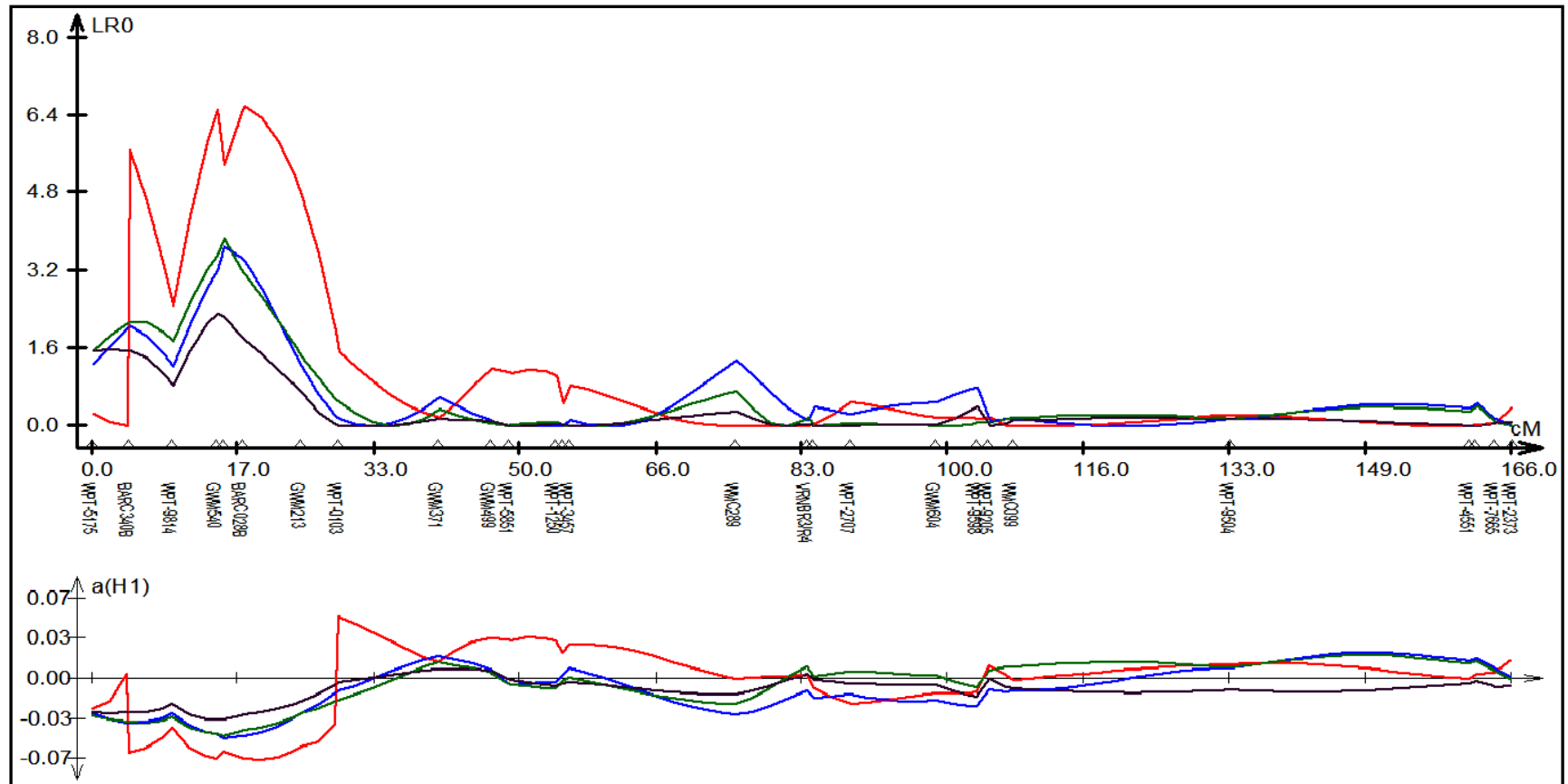


Figure 21. Continued.

f,

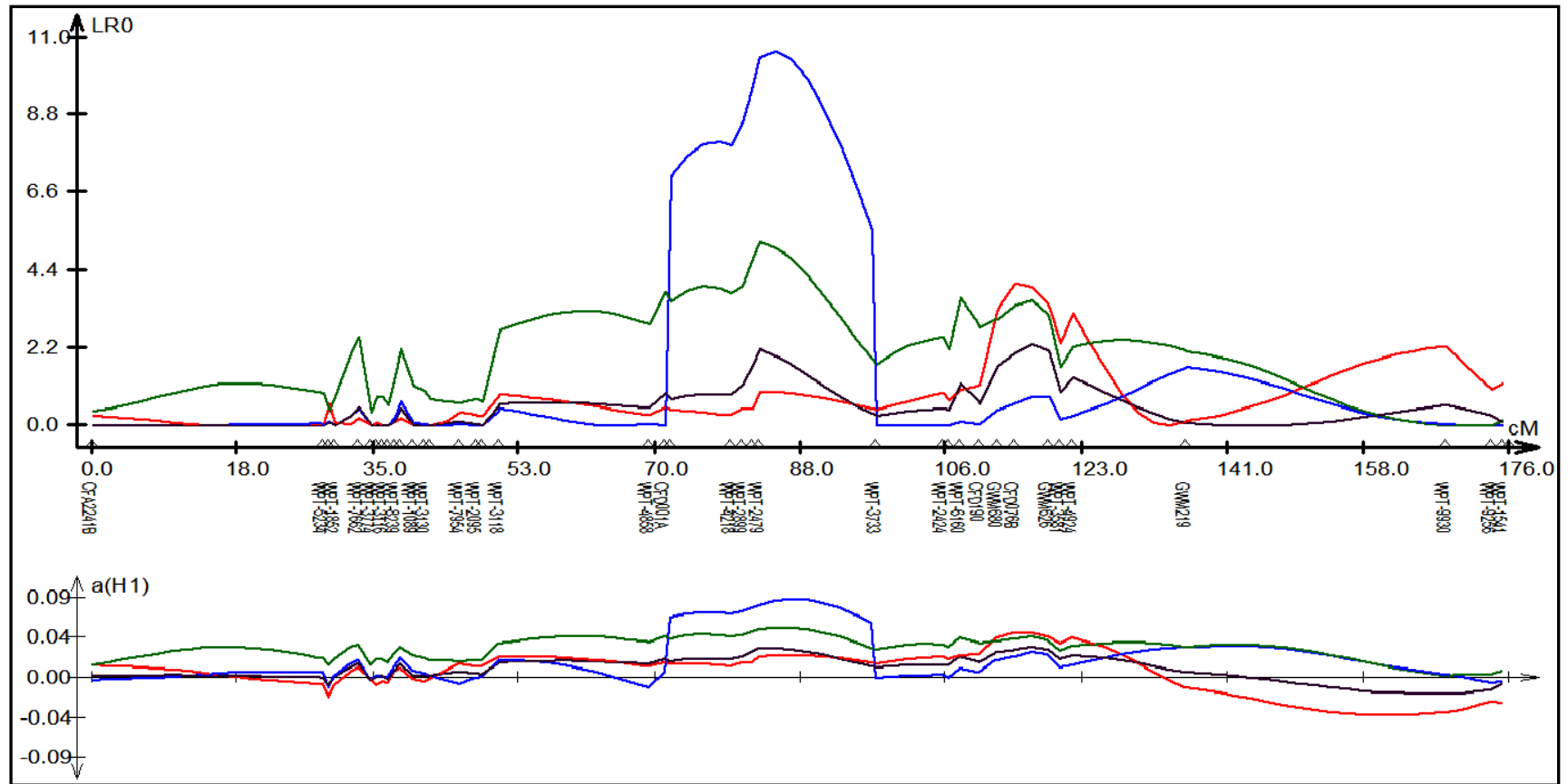


Figure 21. Continued.

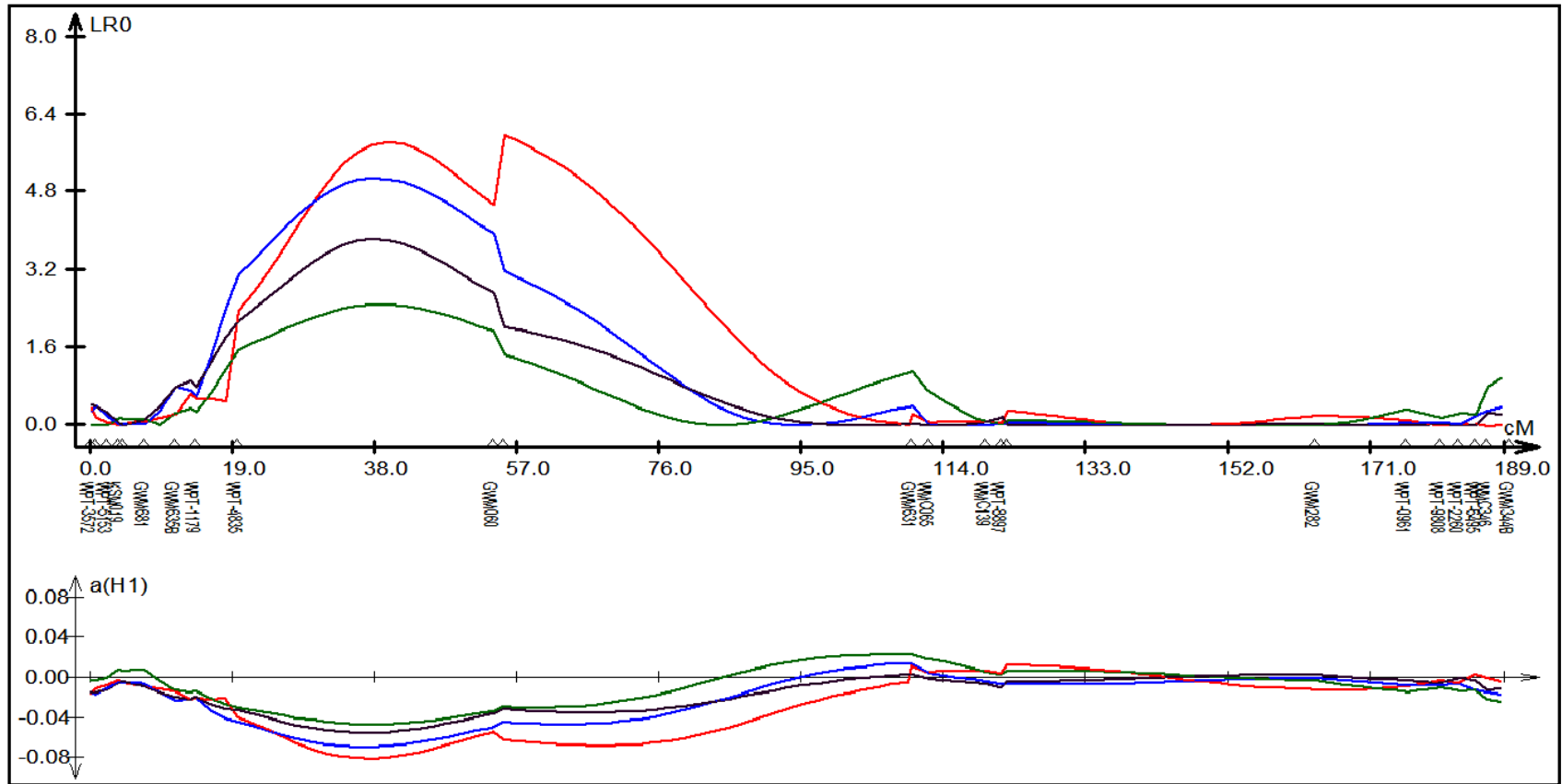


Figure 21. Continued.

4.3.3 Tissue tolerance

4.3.3.1 Determination of tissue tolerance in Berkut × Krichauff DH mapping population

There was no or little negative relationship ($R^2 = -0.14$) found between the projected shoot area and the $[Na^+]$ of the population grown over three different experimental time of the year. Further, there was no linear relationship found between the proportion of green area and the fourth leaf blade $[Na^+]$ of the mapping population grown over three different experimental time of the year (Figure 22). These results are suggesting that some lines accumulate $[Na^+]$ however, are able to maintain their growth and health comparable to those lines excluding Na^+ from the shoot. These $[Na^+]$ accumulating mapping lines must have mechanisms of tissue tolerance to protect themselves from the accumulated Na^+ toxicity in the leaf blade.

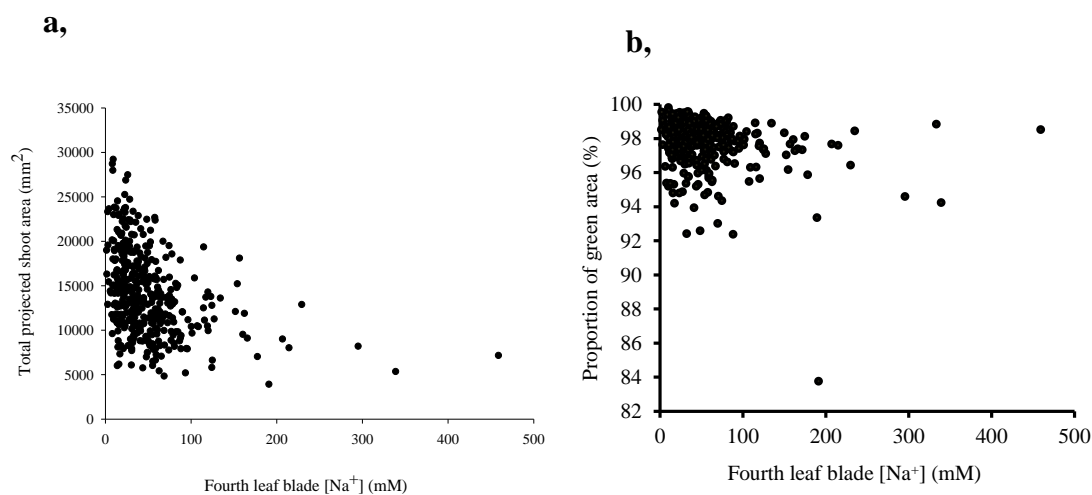


Figure 22. Relationship between (a) projected shoot area and fourth leaf blade $[Na^+]$ ($Y = -36.79x + 16114$, $R^2 = -0.14$), (b) proportion of green area and fourth leaf blade $[Na^+]$ ($Y = -0.0072x + 98.22$, $R^2 = 0.06$) for the mapping lines grown in winter, early spring and late spring 2008. Measurements were taken three weeks after 150 mM NaCl application.

To quantify tissue tolerance, however, in addition to the leaf $[Na^+]$ a measurement of the senescent shoot area in salt stressed condition is required. While this population demonstrated good phenotypic variation for fourth leaf blade $[Na^+]$, unlike the *T. monococcum* in Chapter 3, it exhibited low variability for the proportion of senesced shoot area. The mapping lines in this population demonstrated good health after salt treatment with the proportion of green area between 0.83 to 1 (Figure 23). This meant it was not possible to quantify tissue tolerance using the same procedure as in Chapter 3. More details are given in section 4.4.3 and Chapter 6.

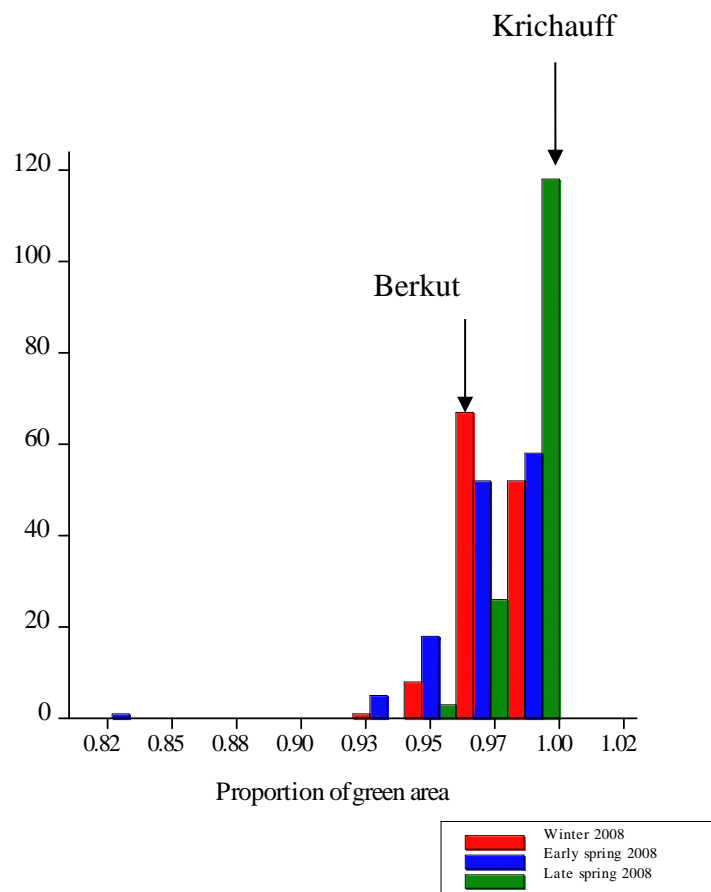


Figure 23. The histogram of proportion of green area in the shoot of the Berkut \times Krichauff DH mapping population’s health after three weeks of growth in 150 mM NaCl in (■) winter, (■) early spring and (■) late spring 2008. Values closer to 1 indicate the plant is in good health with little senescence of leaf material. Arrows indicate the proportion of green area of the parents measured at the same time.

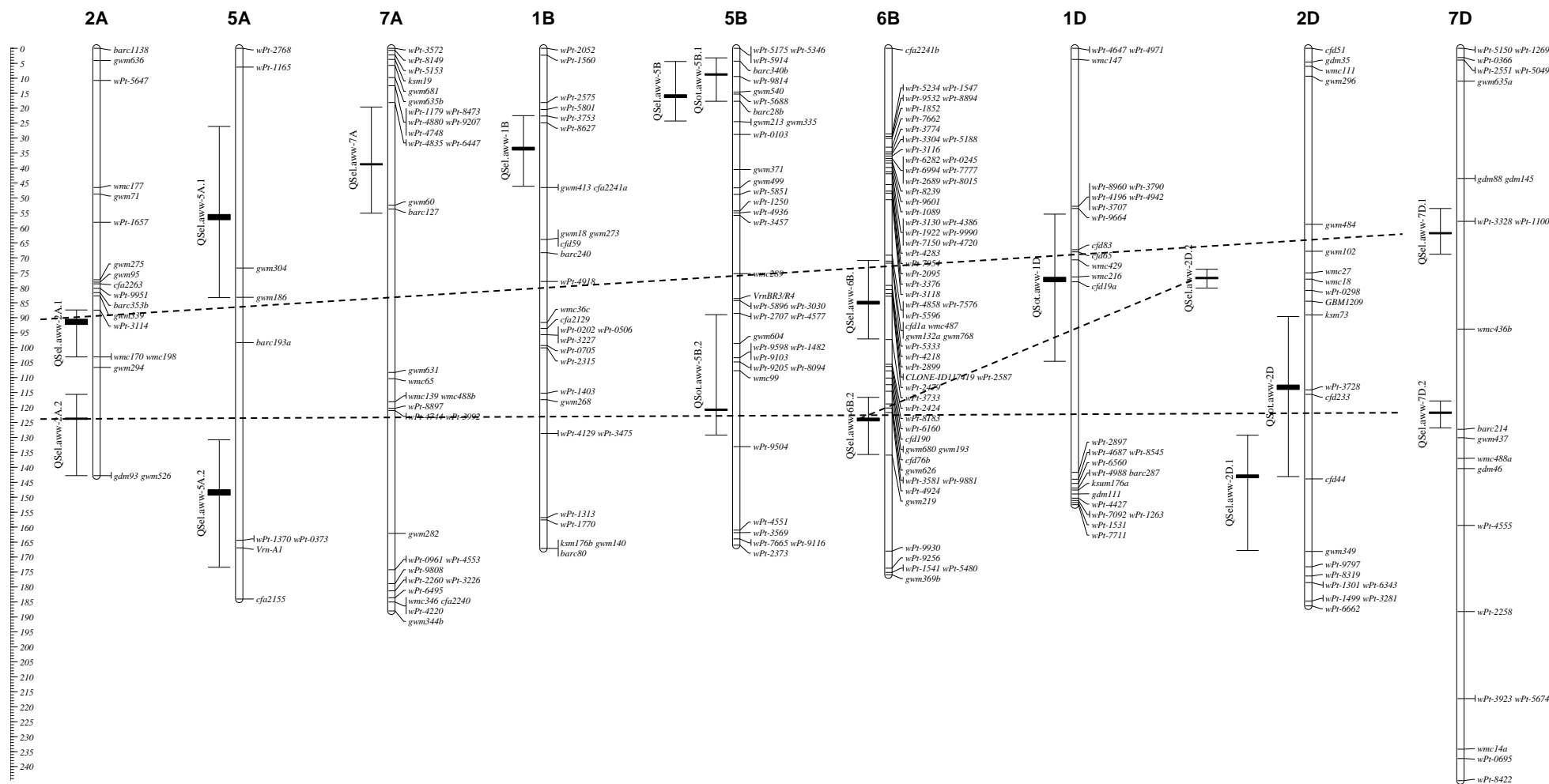


Figure 24. The detected chromosomal locations of QTL linked to osmotic tolerance and Na⁺ exclusion in Berkut × Krichauff DH mapping population. Dashed lines show the epistatic interaction between QTL.

4.3.4 Salinity tolerance of Berkut × Krichauff DH mapping population

The total projected shoot area of the mapping lines grown three weeks after 150 mM NaCl application depends on the salinity tolerance individual mapping lines. It would be, therefore, interesting to investigate the contribution of identified osmotic tolerance and Na⁺ exclusion QTL on the total projected shoot area of mapping lines. Among the four osmotic tolerance QTL, the *Q_{sot.aww.1D}* has contributed considerable phenotypic variability in two of three experiments. On the other hand, the QTL, *Q_{sel.aww.5A.2}* has demonstrated major phenotypic variability for Na⁺ exclusion across three different experimental time of the year. But, it was closely linked to the vernalization (*vrn1*) gene, and hence the second major QTL, *Q_{sel.aww.5A.1}* was taken in to account for this investigation.

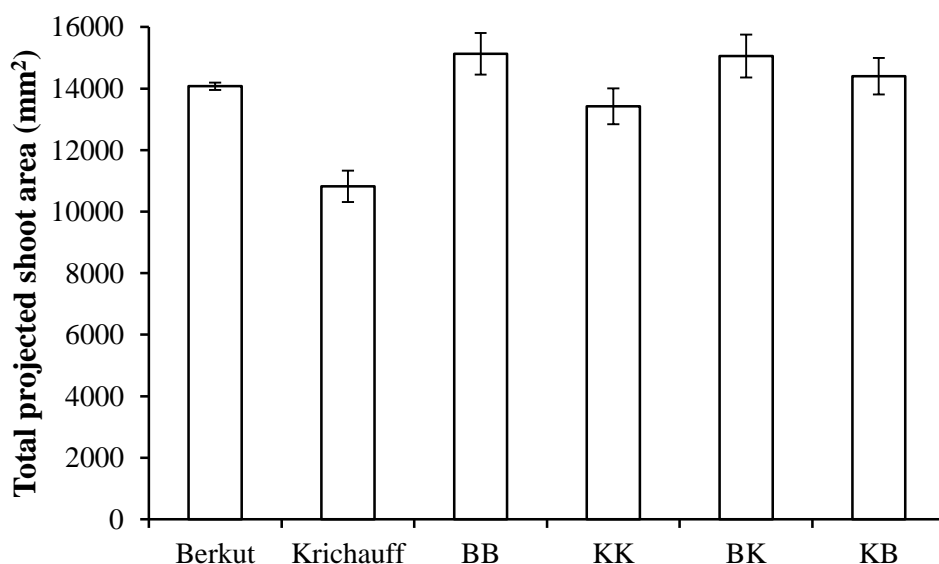


Figure 25. The significant association between the markers linked to osmotic tolerance and Na⁺ exclusion to the plant biomass of the Berkut × Krichauff DH mapping population grown in 150 mM NaCl. The mean total projected shoot area which was quantified three weeks after 150 mM NaCl application for the mapping population parents Berkut and Krichauff, as well as the mapping population lines, characterised into four genotypic classes depending on their genotype at salt tolerance QTL: BB (with Berkut alleles at markers *wmc216-1D* and *gwm186-5A*), BK (Berkut at *wmc216-1D*; Krichauff at *gwm186-5A*), KB (Krichauff at *wmc216-1D*; Berkut at *gwm186-5A*) and KK (Krichauff alleles at markers *wmc216-1D* and *gwm186-5A*). Error bars indicate the standard error of mean projected shoot area.

The markers nearest to the *Q_{sot.aww.1D}* and *Q_{sel.aww.5A.1}* QTL are *wmc216* and *gwm186* respectively. The combined effect of markers *wmc216-1D* and *gwm186-5A* on the total projected shoot area of mapping lines is shown in Figure 25. Mapping lines which are homozygous for Berkut alleles at both loci exhibited a greater projected shoot area than lines containing the Krichauff alleles (Figure 25). Mapping lines which had Krichauff allele at *wmc216* and Berkut allele at *gwm186* demonstrated an intermediate phenotype; however, the total projected shoot area of lines with the combination of Berkut allele at both loci was not significantly different to those with the Berkut alleles at *wmc216* and Krichauff allele at *gwm186*.

4.4 Discussion

This study aimed to identify QTL for the three major components of salinity tolerance in bread wheat and determine their effect on overall plant salt tolerance. The knowledge gained would be used to develop bread wheat cultivars with improved salinity tolerance. A DH mapping population of Berkut × Krichauff was selected and subjected to image based high throughput salt screening, due to the parents having visible differences in their salinity tolerance. However, because of constraints in developing a quantitative tissue tolerance screen (Detailed information is given in Section 4.4.3), only osmotic tolerance and Na⁺ exclusion were quantified and used for further genetic analysis in this mapping population.

4.4.1 Genetic basis of osmotic tolerance

Osmotic tolerance in Berkut × Krichauff DH mapping population was screened non-destructively through the use of imaging techniques. Imaging platform was helpful to measure the relative changes in the shoot growth rate of plants before and immediately after NaCl application, in a non-destructive manner. This is believed to be the first study to use such techniques to identify osmotic tolerance in bread wheat. As shown in Figure 17, a large phenotypic variation for osmotic tolerance was found in this mapping population. It shows continuous variation which indicates the quantitative nature of the trait (East, 1913). The osmotic tolerance observed in the mapping population demonstrated transgressive segregation, with lines having better or worse osmotic tolerance than the parents. Understanding transgressive segregation is important in plant breeding (DeVicente and Tanksley, 1993; Rieseberg *et al.*, 1999) and it can provide a source for new alleles for the development of novel bread wheat cultivars with improved osmotic tolerance in future.

The broad sense heritability (H^2) of osmotic tolerance was high in this mapping population. It suggests the phenotypic selection would be effective at the moderately saline environments. Of course, heritability calculation is not only useful to identify

the response of a mapping population but also to identify the optimum environments for selection (Allen *et al.*, 1978; Ceccarelli, 1989). Moreover, the high broad sense heritability reflects the accuracy and the reproducibility of the screening methodology used to evaluate osmotic tolerance in a green house environment. However, in this study the different replicates of the mapping lines were grown in different time of the year and the experiments were done in an incomplete block design. Hence, the calculated H^2 could be overestimated because the incomplete block design does not allow a proper estimate of experimental error (Maccaferri *et al.*, 2008). In addition, the single observation for each line for each season is not sufficient enough to characterize mapping lines for osmotic tolerance and hence repeated measurements are necessary in every experiment for the accurate estimation of experimental error. A replicated trial will greatly reduce the environmental error in the data set of homogeneous experimental population and helpful to study genotype \times environmental interactions (Hurlbert, 1984).

In this study, CIM identified a total of four QTL on 1D, 2D and 5B chromosomes, which could explain genotypic differences for osmotic tolerance in this mapping population. The favourable alleles came from both parents, demonstrating the genetic basis of the observed transgressive segregants, which can have the best alleles from both parents. Among the four observed QTL, only *QSot.aww-1D* has demonstrated considerable amount of phenotypic variation (13.7%) for osmotic tolerance in two of three experiments. The other three QTL, *QSot.aww-2D*, *QSot.aww-5B.1* and *QSot.aww-5B.2* have demonstrated minor phenotypic variances, so further studies are required to confirm the relationship of these QTL with osmotic tolerance. There were inconsistencies found in QTL identified for osmotic tolerance across three different experimental time of the year. As has been shown in Table 6, presence of the significant influence of environment on osmotic tolerance of plants grown across three different time of the year could be the reason for this inconsistency. However, as discussed earlier, the use of single replication in each experiment restricted the precise estimation of $G \times E$ interaction of the detected QTL. In order to find out the stable QTL for osmotic tolerance, replicated experiments should be done over different time of the year. Moreover, inconsistencies of QTL indicate the adaptive nature of the

QTL identified in this study. The adaptive QTL could be found only in the specific environmental conditions (Collins *et al.*, 2008).

The scarcity of major QTL for osmotic tolerance, indicates the complex genetic nature of this physiological component (Kearsey and Farquhar, 1998). It could be the result of poor marker coverage in the genetic map that may obstruct the identification of one or many QTL yet to be detected in this study. It is possible to obtain polymorphic SSR markers from Roder *et al.*, (1998), Pestova *et al.*, (2000) and Somers *et al.*, (2004) and develop a high density map for future studies. Construction of such high density map would also help to narrow down the marker interval. For instance, the marker interval at *QSot.aww-ID* on chromosome 1D is large (44.1cM) and would contain several thousand genes residing in this region. Reducing the confidence interval of QTL region to <10cM that would be more useful to precisely tag the QTL for marker assisted selection. It has been already identified that, the SSR marker *cf19* located within the confidence interval of *QSot.aww-ID* is closely linked to the major gene controlling crown rot and powdery mildew disease resistance of bread wheat (Huang *et al.*, 2000b; Collard *et al.*, 2005b; Bovill *et al.*, 2010). It may be due to overlapping of physiological pathways and gene networks that control common physiological mechanisms of plants under stressed environment (Shinozaki and Yamaguchi-Shinozaki, 2007). But, it also suggests, the chance of getting unrelated phenotypes is quite high in this region, hence fine mapping is important to narrow down and identify the marker tagged to the osmotic tolerance trait. Fine mapping in future would facilitate the candidate gene identification for osmotic tolerance through map based cloning, such as how the Boron transporter was identified in barley (Sutton *et al.*, 2007). Once the osmotic tolerance QTL has been cloned, it could be expressed in to the model plant such as rice or *Arabidopsis* to test the function.

However, there has been little research conducted to study the genetic basis of osmotic tolerance under saline environment. On the other hand, QTL controlling osmotic tolerance in drought conditions have been identified in barley (Teulat *et al.*, 1998), rice (Lilley *et al.*, 1996) and maize (Lebreton *et al.*, 1995). Genomic region for osmotic adjustments have been found on chromosomes 7A, 5A and 5D of bread

wheat (Morgan, 1991; Quarrie *et al.*, 1994; Morgan and Tan, 1996), but, the results presented here did not reveal any QTL for osmotic tolerance in these regions.

4.4.2 Comparison of Na⁺ exclusion QTL across different genetic background

The fourth leaf blade [Na⁺] demonstrated a wide range of variability among mapping lines (Figure 19 & 20). A total of eight QTL were detected for Na⁺ exclusion in this mapping population. Among them, the Na⁺ exclusion QTL *QSel.aww-2A.1* lying between the marker interval *wPt3114* and *wmc170* on chromosome 2A, has been repeatedly observed in either this (Genc *et al.*, 2010a) or different mapping population of bread wheat (Roder *et al.*, 1998; Harker *et al.*, 2001). The marker *wmc170* is closely linked to the *Nax1* gene (Lindsay *et al.*, 2004) *HKT1; 4* is the candidate gene for the *Nax1* locus, which encodes a protein that retrieves Na⁺ from the xylem in roots and leaf sheaths, preventing it from reaching the leaf blade (Byrt *et al.*, 2007). Recently, James *et al.*, (2011) used this marker and introgressed *Nax1* genes in to the commercial cultivars of wheat. It is therefore likely that the QTL observed in this study may also be linked to *Nax1*, however it should be noted that the *Nax1* gene was introgressed in to durum wheat from *T. monococcum* and so the version of it here could be quite different. The QTL, *QSel.aww-2A.1* in this study, however, explained a lower phenotypic variability (1.5-2.9%) and obtained minor importance than other QTL.

Two other major QTL (*QSel.aww-5A.1* and *QSel.aww-5A.2*) were found to be associated with Na⁺ exclusion in the mapping population. They were both located on chromosome 5A and possess a large confidence interval. The QTL *QSel.aww-5A.1* was identified on the short arm of the 5A chromosome. This was a novel QTL discovered in this experimentation as the previous study by Genc *et al.*,(2010a) did not identify the same QTL in this population. However, our lab result has shown that the *QSel.aww-5A.1* was consistently found in the different mapping population of bread wheat. The consistency of QTL across either in same or different mapping population suggest the constitutive nature of the QTL (Collins *et al.*, 2008).

The other QTL on chromosome 5A, *QSel.aww-5A.2*, was co-located with the *VRNI* gene on distal end of chromosome 5A. Again this was not found to be associated with Na⁺ exclusion in previous studies, however, it was found to be linked to leaf [K⁺] in the same mapping population (Genc *et al.*, 2010a). The co-location of *QSel.aww-5A.2* with *VRNI* gene could be due to (a) the pleiotrophic nature of *VRNI* gene (Hollington *et al.*, 2002; Mahar *et al.*, 2003) (b) the tight linkage between the *VRNI* gene and a gene important in Na⁺ transport. It is important to note that, the 5A chromosome in durum wheat has *Nax2* gene which is homeologous to the *Knal* region on 4D chromosome. However, poor marker coverage of 5A chromosome should be increased to obtain clear results.

The minor QTL, *QSel.aww-5B* and *QSel.aww-6B.1* and *QSel.aww-7A* indentified in this current study, were not identified in any previous research.

However, the utility of QTL for marker assisted selection depends of the magnitude of phenotypic variability explained by the individual QTL identified for the trait of interest (Collard *et al.*, 2005a). On the whole, QTL identified for Na⁺ exclusion, collectively contributed to 35% of the phenotypic variability for in this mapping population. Such low percentage of total phenotypic variance explained QTL observed for Na⁺ exclusion again confirmed the polygenic nature of the trait. It could be also due to the influence of size of the mapping population on the proportion of phenotypic variance observed for the particular trait of interest (Collard *et al.*, 2005a; Genc *et al.*, 2010a). In theory, the proportion of additive genetic variance explained by the detected QTL is inversely related to h^2N (where h^2 is the narrow sense heritability of the trait and the N is the population size). Hence, to identify major QTL with large effect, a big mapping population is required (Lande and Thompson, 1990). Vales *et al.*, (2005), demonstrated the effect of population size on QTL number and QTL effect on DH mapping population of barley for stripe rust resistance. They identified the low power of QTL detection and large bias in QTL effects in small populations. They suggested that population size of N = 300 DH lines would be very effective to reduce bias in QTL effects.

Finally, QTL analysis done by MCIM approach in this study identified three pairs of epistatic interaction for exclusion in this mapping population. Epistasis was used as an important source of variation for the genetic improvement of various crops (Lark *et al.*, 1994; Li *et al.*, 1997; Cao *et al.*, 2001; Zhang *et al.*, 2008). It usually makes the selection of complex genetic traits difficult and it is often neglected in most of the studies (Carlborg and Haley, 2004). However, in this study MCIM analysis detected three pairs of epistatic interactions for exclusion in this mapping population. These epistatic QTL have not been reported in any other previous studies. They have collectively contributed to 13.8 % of phenotypic variability for exclusion in this mapping population. It is actually lower than the phenotypic variance explained by QTL with additive main effects. However, before selection further investigations are needed to confirm their association with Na⁺ exclusion in the same or different mapping population.

4.4.3 Limitations in tissue tolerance screening and QTL mapping

The study of QTL mapping requires variation for the trait of interest. As shown in Figure 17 & 20 this mapping population has shown variability for osmotic tolerance and Na⁺ exclusion respectively. But it was not suitable to study the segregation of tissue tolerance because, only little variation is observed in shoot senescence of the mapping lines. In fact, the mapping progenies demonstrated huge genotypic differences in the Na⁺ accumulation in the fourth leaf blade. The range of fourth leaf blade [Na⁺] varied from 5.7 to 235.02 mM (Figure 19 & 20). It seems some of the mapping progenies might have mechanisms for tight control for Na⁺ uptake and transport than the Na⁺ accumulating ones. The control of Na⁺ uptake could be achieved by minimizing the initial entry of Na⁺ to the roots from the soil, maximising efflux of Na⁺ from roots back to the soil, minimizing loading of Na⁺ into xylem vessels which transport solutes to shoots, maximising retrieval from xylem vessels in the root, maximising Na⁺ recirculation from shoots via the phloem vessels (Tester and Davenport, 2003; Munns and Tester, 2008) and by retention of transported Na⁺ in the leaf sheath (James *et al.*, 2006b).

On the other hand, mapping lines, which do not have any of these Na⁺ exclusion mechanism as described above, might have accumulated the transported Na⁺ in the leaf blade. They have accumulated up to 235.02 mM of Na⁺ in the leaf blade. In fact, the amount of cytosolic [Na⁺] that can cause leaf damage is not certain (Cheeseman, 1988), however, it should be kept less than 100mM to avoid Na⁺ toxicity in the leaves (Greenway and Osmond, 1972; Wyn Jones and Gorham, 2004). It seems, the accumulated [Na⁺] in the leaf blade was sufficient enough to cause Na⁺ specific damage, this mapping population have shown only low amount of variability for senescence in shoots (Figure 23). In order to avoid, Na⁺ toxicity, the accumulating lines in these mapping may effectively compartmentalize the accumulated Na⁺ in to the vacuole and keep the [Na⁺] below toxic level. In fact, transported Na⁺ from xylem first enter in to the leaf vacuole, once the vacuole has exceeded the loading capacity, the Na⁺ starts to accumulate in the cytoplasm of the leaf and cause ion specific damages in plants (Rausch *et al.*, 1996). Moreover, as shown in Figure 22, there was no linear relationship found between the fourth leaf blade Na⁺ concentration and the proportion of green area found among the progenies of the mapping population grown over three experimental time of the year. These results also strongly suggests that the accumulating mapping lines must have mechanisms of tissue tolerance to protect themselves from the accumulated Na⁺ in the leaf blade and stay healthy like the excluding mapping lines grown under saline environments.

Nonetheless, still there is a problem with tissue tolerance screening in this mapping population; as the equation for tissue tolerance used in this study takes in to account the whole shoot senescence and the fourth leaf blade [Na⁺], if there is not enough shoot senescence then the value for the [Na⁺] has a large disproportionate effect on the final value. In fact, male parent of the mapping population, Krichauff excludes and always keeps low Na⁺ in the leaf blade, whereas, the female parent Berkut is a tissue tolerant one that stays green while accumulating Na⁺ than Krichauff in the leaf tissue (Genc *et al.*, 2007). Accordingly, the progenies get high proportion of green shoot area which was ranged from 0.83 to 1; when it was multiplied with the fourth leaf blade Na⁺ that gives the data which was much more similar to the Na⁺ value itself. Hence it was hard to quantify variability for tissue tolerance in this population using the methods established in Chapter 3.

Increasing the dose of NaCl application and/or increasing the time of exposure to NaCl may induce senescence in leaf blades and help to obtain the genotypic differences for tissue tolerance in Berkut × Krichauff DH mapping population. It is already identified that, four weeks of growth at 150 mM NaCl is not found as a lesser dose to distinguish the genotypic differences in salinity tolerance of bread and durum wheat cultivars (Rivelli *et al.*, 2002). Further, the use of transgenic or near-isogenic lines with difference in vacuolar sequestration would also be useful to address this issue. Otherwise, selection of a different mapping population with a cross between a tissue tolerance and salt (ionic) sensitive parent, or development of a new method to quantify tissue tolerance would be more helpful to identify tissue tolerance QTL in bread wheat in future.

While developing a new method to assess tissue tolerance in bread wheat, efficacy of the two parameters: such as proportion of salt induced senesced shoot area and fourth leaf blade Na⁺ are need to be considered in a first hand. For instance, the proportion of senesced shoot area calculated in this study may have an influence from both salt induced ionic stress and osmotic stress (More details are given in Chapter 6). So, while developing new method for tissue tolerance screen, future experiments must consider and calculate salt induced osmotic stress and ionic stress in a separate manner. It would also be better to measure these two types of accelerated senescence on individual leaves on a plant, rather than the whole shoot. On the other hand, measuring the concentration in the leaf blade (Upper part of the leaf) of the plant sample is widely used method by various researchers to evaluate the genetic differences for Na⁺ accumulation in different crops (Schachtman *et al.*, 1991; Genc *et al.*, 2007; Shavrukov *et al.*, 2009) and it can be used as it is for tissue tolerance estimation in the future. For instance, the use of [Na⁺] in the whole leaves (including leaf blades and sheaths) may be misleading because, it is evident that genes controlling Na⁺ exclusion such as *Nax1* preferentially accumulate Na⁺ in leaf sheaths (lower part of the leaf) and always help to maintain low Na⁺ concentrations in the leaf blades for where bulk of photosynthesis and transpiration happens (Munns, 2005; James *et al.*, 2006a). Moreover, analysis of shoot Na⁺ does not provide accurate results because it would have contained dead leaves, stems and sheaths which would

be highly likely to provide wrong information about $[Na^+]$ for tissue tolerance calculation (Schachtman and Munns, 1992; Colmer *et al.*, 1995).

4.4.4 The combined effect of osmotic tolerance and Na^+ exclusion QTL on shoot biomass of mapping lines grown under saline conditions

As shown in Figure 25, at homozygous condition Berkut alleles at markers *wmc216-1D* and *gwm186-5A* (BB) increased the total projected shoot area of mapping lines than Krichauff alleles at markers *wmc216-1D* and *gwm186-5A* (KK) by 11.2 %. It indicates that Berkut, the osmotic and tissue tolerant parent can potentially grow bigger than Krichauff which is an osmotic sensitive and excluding parent under moderately saline environment. Moreover, mapping lines with BB alleles has produced 4.8 % of increased total projected shoot area over KB alleles. Mapping lines with BB alleles did not show any significant difference in the total projected shoot area over BK alleles. They suggested that increase in osmotic tolerance of mapping population would be helpful to obtain genotypes with increased total projected shoot area and hence higher yield.

The QTL analysis done in this current study could be providing markers to the breeders that help to develop high salt tolerance genotypes. Through the use of marker-assisted backcross breeding Berkut alleles could be introgressed into Krichauff or other susceptible lines, for higher osmotic tolerance. However, QTL inconsistencies, observed for both osmotic tolerance and Na^+ exclusion, from this study strongly recommends re-estimating the QTL effect and validating QTL positions either in the same or different mapping population, before marker assisted selection and other forward genetic studies.

4.5 Future Prospects

This study demonstrated the significance contribution of osmotic tolerance to increase the total projected shoot area of Berkut× Krichauff DH mapping population of bread wheat under a moderately saline environment. Berkut alleles could potentially be selected in future breeding programmes to incorporate osmotic tolerance into sensitive genotypes to improve their salinity tolerance. Accordingly, it is possible to go for phenotypic selection through conventional breeding for the genetic improvement of the trait. However, in practice conventional breeding requires quite long time to finish off this process; therefore it will be necessary to develop closely linked markers to the osmotic tolerance QTL for marker assisted selection to be a viable option for the genetic improvement of this trait. However, in this Chapter, a very first genetic analysis has been made to study the inheritance of osmotic tolerance in bread wheat. So, QTL identified for osmotic tolerance need to be validated either in the same or different mapping population to strengthen the QTL results. Once it has been validated, fine mapping should be done for further marker assisted selection and map based cloning approaches.

CHAPTER.5 UNDERSTANDING THE GENETIC BASIS OF OSMOTIC AND TISSUE TOLERANCE IN EINKORN WHEAT (*TRITICUM MONOCOCCUM*)

Overview

T. monococcum has already demonstrated variability for three of the major components of salinity tolerance: Na⁺ exclusion, osmotic tolerance and tissue tolerance. Since *T. monococcum* has already contributed valuable genes involved in Na⁺ exclusion to improve salinity tolerance of commercial wheat cultivars (James *et al.*, 2006a; Byrt *et al.*, 2007; James *et al.*, 2011), this study was formulated to explore the natural genetic variability for other key components of salinity tolerance such as osmotic and tissue tolerance in *T. monococcum* for further forward genetic approaches such as QTL mapping in the future.

5.1 Introduction

Natural variation provides a basic resource for doing genetic investigation in any plant species (Koornneef *et al.*, 2004). From the results as has been demonstrated in Chapter 3 and Rajendran *et al.*, (2009), it was understood that *T. monococcum* was found to exhibit promising untouched natural variability for both osmotic and tissue tolerance. However, estimation of genetic parameters such as genetic variances and heritability (Falconer and Mackay, 1996) is needed to get the knowledge about the ability of the *T.monococcum* in response to artificial selection. Understanding the genetic basis of the natural variation is also an important step that helps to identify potential candidate gene(s) controlling osmotic tolerance and tissue tolerance through forward genetic studies such as QTL mapping in the future.

To begin with this, the MDR 002 \times MDR 043 F₂ mapping population of *T.monococcum* is chosen for this study. It is used not only to estimate genetic parameters of osmotic tolerance and tissue tolerance but also simultaneously construct the primary linkage map for QTL mapping in the near future. In Chapter 4, genetic studies were carried out in a DH mapping population; however, the choice of mapping population varies with the objective of the research programme as well as time constraints of the research project. Among various types of mapping population, F₂ mapping populations are highly preferred by many researchers for construction of primary linkage maps in a short time due to their ease of production (Dubcovsky *et al.*, 1996a; Dubcovsky *et al.*, 1998; Bullrich *et al.*, 2002; Taenzler *et al.*, 2002; Yao *et al.*, 2007a; Jing *et al.*, 2008). Since all possible recombination of parental alleles (AA, Aa, aa) are available in F₂ progenies, these populations can be used to detect the linkage between markers and segregation of trait of interest in early generation itself (Collard and Mackill, 2008). It is also important to note that the parents of the mapping population, MDR 002 and MDR 043 had shown high salt tolerance (good osmotic and tissue tolerance) and salt sensitivity respectively, in Chapter 3 and Rajendran *et al.*, (2009).

The MDR 002 × MDR 043 F₂ mapping population was originally developed by Dr. Hai-Chun- Jing, Rothamsted Research Station, Harpenden, and U.K and seeds of this population were obtained for this current study (Additional information is given in Section 5.2.1). Studies with this population to date have focused primarily on the genetics and cellular basics of fungus (*Mycosphaerella graminicola*) and host (*T. monococcum*) interactions which cause *septoria tritici* blotch disease of wheat. MDR 002 was found to be susceptible and MDR 043 was resistant to the fungal pathogen (Jing *et al.*, 2008). Within this population a potential QTL for *septoria* disease resistance on chromosome 7A was identified and it was associated with the SSR marker *Xbarc174* (Jing *et al.*, 2008).

In addition to the seeds of the MDR 002 MDR 043 F₂ mapping population, a list of 45 polymorphic SSR markers, which have already identified in the same (MDR 002 × MDR 043) F₂ mapping population, was obtained from Dr. Hai-Chun-Jing, for the construction of primary linkage map (Table 14). Molecular markers, such as RAPD (Kojima *et al.*, 1998), RFLP (Taenzler *et al.*, 2002), AFLP (Taenzler *et al.*, 2002) SSRs (Jing *et al.*, 2008), SNPs (An *et al.*, 2006) and daRT (Jing *et al.*, 2009), have been successfully used to map the *T. monococcum* genome (More details are given in Chapter 1). The best markers to use appear to be SSR markers which are found frequently in plant genomes (Mrázek *et al.*, 2007; Morgante and Olivieri, 1993). They are co-dominant by nature (Hayden and Sharp, 2001) and provide highly reproducible results over time (Jones *et al.*, 1997).

In the previous Chapter, genetic basis of major salinity tolerance components were studied in *T. aestivum*. However, it would be more interesting to know the genetic basis of osmotic tolerance and tissue tolerance in *T. monococcum* because, *T. aestivum* possess narrow genetic base and possess lower polymorphism than *T. monococcum* (Gale *et al.*, 1990). It would be good to see if these genes are either eroded during the evolutionary process or survived through the natural selection in *T. monococcum* (Reif *et al.*, 2005). Moreover, the diploid nature of *T. monococcum* (2n=14) will reduce the complexity in detecting QTL when compared to the

hexaploid, *T. aestivum* ($2n=42$) (Singh *et al.*, 2007). With this background, the present study was formulated with the following objectives,

1. To phenotype the MDR 002× MDR 043 F₂ mapping population of *T. monococcum* for osmotic and tissue tolerance using non-destructive high throughput salt screening assays.
2. To study the genetic variability and heritability for osmotic and tissue tolerance.
3. To use SSR markers, genotype the mapping population and construct the primary linkage map in *T. monococcum*
4. If possible, identify the QTL controlling osmotic and tissue tolerance for further candidate gene(s) approaches.

5.2 Materials and methods

5.2.1 Mapping population

About 500 seeds harvested from F₁ plants of a cross between MDR 002 and MDR 043 accessions were received from Dr. Hai-Chun- Jing, The Rothamsted Research Station, Harpenden, U.K. The parents exhibited significant differences for various morphological characters, as listed in Jing *et al.*, (2007) and Table 13.

Table 13. Morphological difference between *T. monococcum* accessions MDR 002 and MDR 043 at maturity (Jing *et al.*, 2007).

Characters	MDR 002 (<i>T. monococcum ssp.</i> <i>triaristatum</i>)	MDR 043 (<i>T. monococcum ssp.</i> <i>vulgare</i>)
Origin	Balkans	Greece
Tiller number	41.80 ± 6.38	56.60 ± 10.26
Height (cm)	132.30 ± 4.09	145.40 ± 4.04
Awn length (cm)	5.50 ± 0.58	7.00 ± 0.82
Peduncle length (cm)	45.00 ± 5.76	49.23 ± 2.88
Ear to flag leaf length (cm)	25.53 ± 6.02	30.37 ± 3.22
Spikelet number	28.40 ± 0.60	33.60 ± 1.46
Ear length	17.23 ± 0.35	15.80 ± 1.03
100 seed weight (g)	26.86 ± 2.11	30.40 ± 3.05
1000- seed volume (ml)	34.10 ± 3.45	45.57 ± 3.13
Awn colour	Black	Yellow
Grain texture	Hard	Soft

5.2.2. Experimental setup

Seeds were germinated in two batches following the protocol as described in Chapter 2. The germination percentage was found to be poor with only 220 out of 500 individuals germinating. The germinated seeds were transplanted into supported hydroponics in the greenhouse when they were 5 days old. Experiments were conducted during July-September 2009 at the Waite Campus, The University of Adelaide. Of the 220 germinated individuals, only 177 individuals survived after transplantation. More comprehensive information about seed germination, supported hydroponics system and growth conditions is explained in Chapter 2.

5.2.3 Non-destructive 3D plant imaging

The detailed information about the non-destructive 3D plant imaging technology has already been described in Chapter 2. RGB images of the MDR 002 × MDR 043 F₂ population were captured using a LemnaTec scanalyser, (LemnaTec, Würselen, Germany). A total of 3277 RGB images of the mapping population was acquired over 17 time points, which includes 7 time points before and 10 time points after NaCl application. Plants were imaged every day from 7 days before NaCl application to 5 days immediately after 75 mM NaCl application. Thereafter images were obtained every second or third day until the plants were 35 days old. All of these images were analysed and the total projected shoot area, as well as shoot health were determined allowing the dissection of osmotic and tissue tolerance from each other in the MDR 002 × MDR 043 F₂ population as shown in section 5.2.4. Once imaging was completed, the surviving plants were transferred to soil to collect F₃ seed for future experiments, as shown in Appendix 2.

5.2.4 High throughput salt screening

A similar salt screening protocol, as has been described in previous chapters, was used to screen osmotic and tissue tolerance in the MDR 002 × MDR 043 F₂ population. Plants were subjected to 75 mM NaCl stress at the time of fourth leaf emergence which is approximately 16 days old. The final concentration of NaCl (75 mM) and CaCl₂ (3.51 mM) was reached by three consecutive doses of 25 mM NaCl, along with 1.17 mM CaCl₂, which was applied twice every day to the nutrition solution in the supported hydroponics tank (Additional information of NaCl application is described in Chapter 2). Osmotic and tissue tolerance screening were performed as shown in Section 5.2.4.1 & 5.2.4.2.

5.2.4.1 Osmotic tolerance screen

As shown in previous chapters, the total projected shoot area of F₂ individuals capture immediately prior to and after salinization can be used to calculate growth rates non-destructively, thereby allowing the determination of osmotic tolerance for each individual. The mean relative growth rate of seedlings at 5 days before and 5 days immediately after NaCl application was calculated using macros in Microsoft Excel (<http://www.ozgrid.com/forum/showthread.php?t=94519>). Osmotic tolerance was calculated by dividing the mean relative growth rate of a F₂ individual 5 days immediately after NaCl application with the mean relative growth rate of the same F₂ individual 5 days before NaCl application. Accordingly, osmotic tolerance of all 177 F₂ individuals was calculated.

5.2.4.2 Tissue tolerance screen

The estimation of tissue tolerance requires two parameters, the non-destructive quantification of the proportion of salt induced senescence in the shoot which can be obtained from the RGB images, and the destructive measurement of [Na⁺] in the leaf

blade. The total senesced shoot area was calculated from images of F₂ individuals which were captured at the last time point, 19 days after 75 mM NaCl application (Chapter 2, 3 & section 5.3.3.2). Immediately after the acquisition of the last image, the fourth leaf blade of each mapping line was sampled for [Na⁺] analysis using flame photometry (Model 420, Sherwood scientific, Cambridge, U.K). Additional information of Na⁺ measurements are given in given in Chapter 2.

5.2.5 DNA extraction

Once image analysis was completed, young leaf blades of all the 177 F₂ individuals were collected for DNA extraction. The leaf blades were cut into three pieces and put in 96 well micro tubes (National scientific, Quakertown, USA). The samples were incubated in a vacuum freeze drier (Christ Alpha 1-2 LD, Germany) at -60⁰C overnight before DNA extraction were performed using the protocol published in Shavrukov *et al.*, (2010). Briefly, after vacuum freezing, 14-mm stainless steel ball bearings were added to each 96 well plates and the tissue was ground to a powder in a mixer mill (Model MM 300, Retsch Mill, Germany) for 5 minutes. The ball bearings were removed, and 600 µl of extraction buffer (0.1 M Tris-HCl, pH 7.5; 0.05 M EDTA, pH 8.0; 1.25% sodium dodecyl sulfate) was added in to each tube. The 96 well plates were shaken thoroughly, with their lids on, to facilitate extraction. Samples were incubated at 65⁰C for 30 minutes and then at the room temperature for 15 minutes. Once at room temperature, 300 µl of 6 M ammonium acetate buffer was added in to each tube. Tubes were shaken vigorously, incubated again for 15 minutes at 4⁰C and centrifuged for 15 minutes at 4,000 rpm (Centrifuge Model 2-5, Sigma, USA). After centrifugation, 600 µl of the supernatant was transferred to the new micro tubes. Subsequently, 360 µl of iso-propanol was added in each well. They were gently mixed thoroughly, kept at room temperature for 15 minutes and centrifuged for 15 minutes at 4,000 rpm to precipitate DNA. The supernatant was discarded and the tube was inverted on top a paper towel, in order to remove any excess supernatant. After draining, DNA pellets were washed in 400 µl of 70% ethanol, followed by the centrifugation at 4000 rpm for 15 minutes. DNA pellets were resuspended in 400 µl of milli-Q water and kept at 4⁰ C for overnight in the fridge. The next day samples were

centrifuged for 20 minutes at 4000 rpm and 300 µl of the supernatant was transferred to fresh 96 well plates and stored at -20°C for long term usage. These samples were used directly for PCR reactions as described below.

5.2.6 Polymerase Chain Reaction (PCR) master mix

Polymerase Chain Reactions (PCRs) were performed using Invitrogen's Platinum *Taq* DNA polymerase enzyme and primers designed to amplify specific SSRs (Table 15). A reaction mixture consisted of the following ingredients: 2 µl of DNA extracted through freeze- Dry method, 1.5 µl of 2 mM dNTPs (Fisher Biotec, Perth, Australia), 1.5 µl of 10X PCR buffer, 0.60 µl of 50 mM MgCl₂, 0.10 µl of 1.25 units Platinum *Taq* polymerase, 0.75 µl of 5 µM forward and reverse primers, 0.75 µl of DMSO (VMR International Ltd, England, U.K) and 7.05 µl of milliQ water.

5.2.7 Thermocycler programme

All PCR reactions were carried out in Programmable Thermal Controller PCR-PTC-100™ (MJ Research Inc, Waltham, USA). The Platinum *Taq* enzyme was activated by incubating at 94°C for 1 minute before a 15 cycle repeat of DNA denaturing, primer annealing and product extension using the following conditions: 94°C for 30 seconds DNA denaturing, 50-60°C depending on primer annealing temperature for 30 seconds and 72°C for 30 seconds for product extension. After initial amplification a further 38 cycles of 94°C for 15 seconds, 55°C for 30 seconds and 72°C for 45 seconds was done with the same reaction mixture. A final extension of 72°C for 5 minutes was made, incubated at 15°C and stored at -20°C, if necessary for a long term use.

5.2.8 Visualization of molecular markers

All the 177 F₂ progenies of MDR 002× MDR 043 mapping population and their parents were genotyped using polymorphic 45 SSR markers (Table 14) using 3 %

agarose (Bioline, New South Wales, Australia) gel electrophoresis. However, such a low number of polymorphic markers available for this study do not help to produce a linkage map with good marker coverage. The number of markers would be useful, however, to construct a primary linkage map and also facilitate to do a single marker analysis that helps to find out the chromosome or region of interest at the early stage (More details of single marker analysis are given in Chapter 1).

5.2.9 Statistical analysis

5.2.9.1 Analysis of variance (ANOVA)

Experiments were done in a complete randomized block design. ANOVA was used to separate the components of variability for osmotic tolerance in MDR 002 × MDR 043 F₂ mapping population and their parents (Microsoft Office Excel 2007).

5.2.9.2 Heritability calculations

The broad sense heritability of osmotic tolerance in MDR 002 × MDR 043 F₂ mapping population was calculated using the formula given below (Mahmud and Kramer, 1951).

$$\text{Heritability (h}^2\text{)} = \frac{\sigma_{F_2}^2 - \sqrt{\sigma_{P_1}^2 \cdot \sigma_{P_2}^2}}{\sigma_{F_2}^2}$$

Whereas,

$\sigma_{F_2}^2$ - Variance of F₂ progenies

$\sigma_{P_1}^2$ - Variance of parent1 (MDR 002)

$\sigma_{P_2}^2$ - Variance of parent 2 (MDR 043)

Table 14. The list of 45 polymorphic SSR markers for the MDR 002 × MDR 043 F₂ mapping population obtained from Dr. Hai-Chun- Jing, *are the markers already have been screened in 3% agarose gels and **are markers with unknown product size. Information about the forward and reverse primers, chromosomes and product size were obtained from Graingenes database (<http://wheat.pw.usda.gov/cgi-bin/graingenes/browse.cgi?class=marker>).

Markers	Forward primers	Reverse primers	Chromosomes	Product size (bp)
Xbarc83	5' AAGCAAGGAACGAGCAAGAGCAGTAG 3'	5' TGGATTTACGACGACGATGAAGATGA 3'	1A	305*
Xbarc108	5' GCGGGTCGTTTCCTGGAAATTCATCTAA 3'	5' GCGAAATGATTGGCGTTACACCTGTTG 3'	7A	198*
Xbarc119	5' CACCCGATGATGAAAAT 3'	5' GATGGCACAAGAAATGAT 3'	1A	260*
Xbarc146	5' AAGGCGATGCTGCAGCTAAT 3'	5' GGCAATATGGAAACTGGAGAGAAAT 3'	6A	203*
Xbarc174	5' TGGCATTTTCTAGCACCAATACAT 3'	5' GCGAACTGGACCAGCCTTCTATCTGTTC 3'	7A	238*
Xbarc213	5' GCGTAGATTCTCGGTTTGTGGCTTGC 3'	5' CCGTCCCTCCTTCCTGGTCT 3'	1A	222*
Xcfa2040	5' TCAAATGATTCAGGTAACCACTA 3'	5' TTCCTGATCCACCAAACAT 3'	7A	286
Xcfa2049	5' TAATTTGATTGGGTCGGAGC 3'	5' CGTGTCGATGGTCTCCTTG 3'	7A	164
Xcfa2141	5' GAATGGAAGGCGGACATAGA 3'	5' GCCTCCACAACAGCCATAAT 3'	5A	229
Xcfa2153	5' TTGTGCATGATGGCTTCAAT 3'	5' CCAATCCTAATGATCCGCTG 3'	1A	200
Xcfa2193	5' ACATGTGATGTGCGGTCATT 3'	5' TCCTCAGAACCCATTCTTG 3'	3A	195
Xcfd039	5' CCACAGCTACATCATCTTTCCTT 3'	5' CAAAGTTTGAACAGCAGCCA 3'	5A	175
XdupW004	5'GGTCTGGTCGGAGAAGAAGC 3'	5'TGGGAGCGTACGTTGTATCC3'	4A	335*
Xpsp3001	5'GCAGAGAGATGAGGGCACCC3'	5'CTCTGCTCCCTTAACCTTCTG3'	7A	207
Xwmc048	5'GAGGGTTCTGAAATGTTTTGCC3'	5'ACGTGCTAGGGAGGTATCTTGC3'	4B	123
Xwmc161	5'ACCTTCTTTGGGATGGAAGTAA3'	5'GTAAGTGAACCACTTGTAAACGCA3'	4A	250*
Xwmc201	5'CATGCTCTTTCACCTGGGTTTCG3'	5'GCGCTTGCAGGAATTCAACACT3'	6A	249
Xwmc278	5'AAACGATAGTAAAATTACCTCGGAT3'	5'TCAAAAAATAGCAACTTGAAGACAT3'	1A	165
Xwmc296	5'GAATCTCATCTTCCCTTGCCAC3'	5'ATGGAGGGGTATAAAGACAGCG3'	2A	155
Xwmc420	5'ATCGTCAACAAAATCTGAAGTG3'	5'TTACTTTTGGCTGAGAAAACCT3'	5A	125

Xwmc468	5'AGCTGGGTAAATAACAGAGGAT3'	5'CACATAACTGTCCACTCCTTTC3'	4A	150
Xwmc469	5'AGGTGGCTGCCAACG3'	5'CAATTTTATCAGATGCCCGA3'	1A	148
Xwmc488	5'AAAGCACAACCAGTTATGCCAC3'	5'GAACCATAGTCACATATCACGAGG3'	7A	136
Xwmc580	5'AAGGCGCACAAACACAATGAC3'	5'GGTCTTTTGTGCAGTGAAGTGAAG3'	6A	329
Xwmc596	5'TCAGCAACAAACATGCTCGG3'	5'CCCGTGTAGGCGGTAGCTCTT3'	7A	143
Xwmc603	5'ACAAACGGTGACAATGCAAGGA3'	5'CGCCTCTCTCGTAAGCCTCAAC3'	7A	120
Gwm639	5'AGGCAACTCACAGGAACT3'	5'ATCTTCGGTTCCGTCGCA3'	3A	420*
Xwmc680	5'TGAGTGTTCAGGCCGCACTATG3'	5'ATCCTTGTTCAGGAATCCCCGT3'	4A	214
Xwmc705	5'GGTTGGGCTCCTGTCTGTGAA3'	5'TCTTGCACCTTCCCATGCTCT3'	5A	172
Xwmc753	5'AAGGTGAAGATGATGCTCGC3'	5'TGACTGATCATGGATTGCC3'	6A	269
Xwmc795	5'GGCTCGATTCCGTTACCTCA3'	5'GGCGATTGCGCCACACCT3'	5A	183
Xwms005	5'GCCAGCTACCTCGATACTCAACTC3'	5'GCCAGCTACCTCGATACTCAACTC3'	3A	172
Xwms122	5' GGGTGGGAGAAAGGAGATG 3'	5' AAACCATCCTCCATCCTGG 3'	2A	-----**
Xwms129	5' TCAGTGGGCAAGCTACACAG 3'	5' AAAACTTAGTAGCCGCGT 3'	5A	-----**
Xwms135	5' TGTCAACATCGTTTTGAAAAGG 3'	5' ACACTGTCAACCTGGCAATG 3'	1A	-----**
Xwms136	5' GACAGCACCTTGCCCTTTG 3'	5' CATCGGCAACATGCTCATC 3'	1A	-----**
Xwms186	5' GCAGAGCCTGGTTCAAAAAG 3'	5' CGCCTCTAGCGAGAGCTATG 3'	5A	-----**
Xwms311	5' TCACGTGGAAGACGCTCC 3'	5' CTACGTGCACCACCATTTTG 3'	2A	-----**
Xwms397	5' TGTCATGGATTATTTGGTCGG 3'	5' CTGCACTCTCGGTATACCAGC 3'	7B	-----**
Xwms415	5' GATCTCCCATGTCCGCC 3'	5' CGACAGTCGTCACCTGCCTA 3'	5A	-----**
Xwms443	5' GGGTCTTCATCCGGAACCTCT 3'	5' CCATGATTTATAAATCCACC 3'	5B	-----**
Xwms698	-----	-----	7A	-----**
Xwms715	-----	-----	-----	-----**

5.3 Results

5.3.1 Shoot growth of MDR 002 × MDR 043 F₂ population in salt stress

RGB images of the MDR 002 × MDR 043 F₂ population and their parents, captured at 17 time points during their growth period were used to do a plant growth analysis and study the response of shoot growth under saline environment, non-destructively. All F₂ progenies exhibited significant difference in total projected shoot area, which was quantified 19 days after 75 mM NaCl application, at the last time point (P=0.09 level) with F₂ individuals such as 29 and 26 identified as one of the larger and smaller F₂ individuals in the mapping population, respectively. The F₂ line 29 was found to be substantially larger and exhibited more non-linear growth than the parents MDR 043, MDR 002 and the F₂ line 26, at 19 days after 75 mM NaCl application (Figure 26).

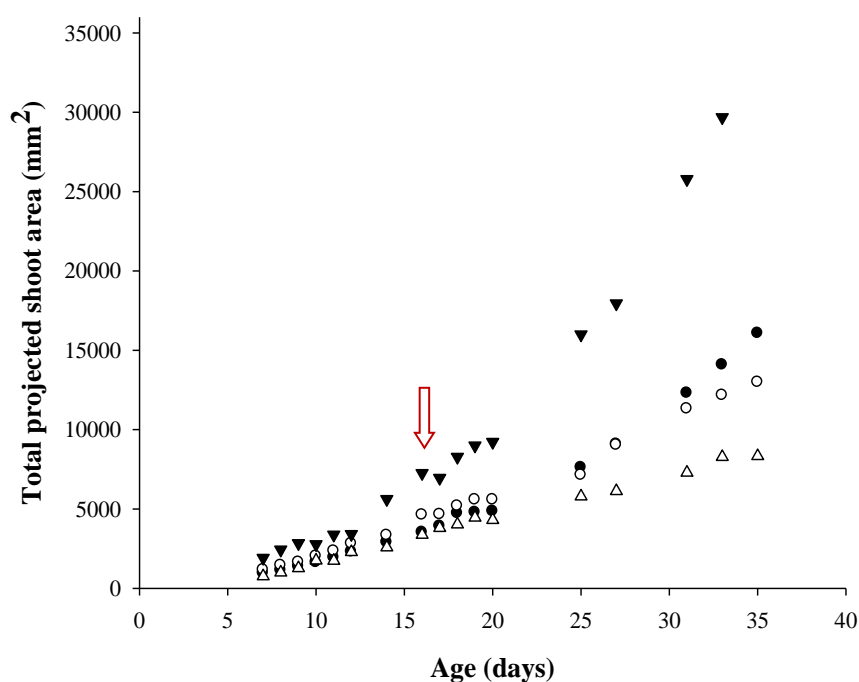


Figure 26. Growth curves of (●) MDR 043, (○) MDR 002, (▼) F₂ individual 29, and the (△) F₂ individual 26 were measured using the projected shoot area of the F₂ individuals over time. They were calculated using the three images for each plant captured at 17 time points, 7 before and 10 after 75 mM NaCl application. The final 75 mM NaCl was achieved by three 25 mM NaCl applications, two doses applied on day 16 (arrow). The significance of difference in total projected shoot area was estimated at the last time point through “t” test at P =0.09 level.

5.3.2 Shoot health of MDR 002 × MDR 043 F₂ population in saline environments

The details of plant health analysis are explained in Chapter 2. In brief, to study shoot health of MDR 002 × MDR 043 F₂ population in saline environments, the colour areas in the RGB images of plant shoot identified as green (healthy), yellow (senescing) and brown (senesced) were calculated over time. However, for this study the total senesced shoot area was calculated by adding the senescing and senesced shoot area of every single line 19 days after 75 mM NaCl application. For example, F₂ line 26 had more proportion of senesced shoot area (41%) than MDR 002 (35%) than MDR 043 (18%) and an F₂ line 29 (11%) at 19 days after 75 mM NaCl application (Figure 27), which was used for tissue tolerance screen as shown in section 5.3.3.2.

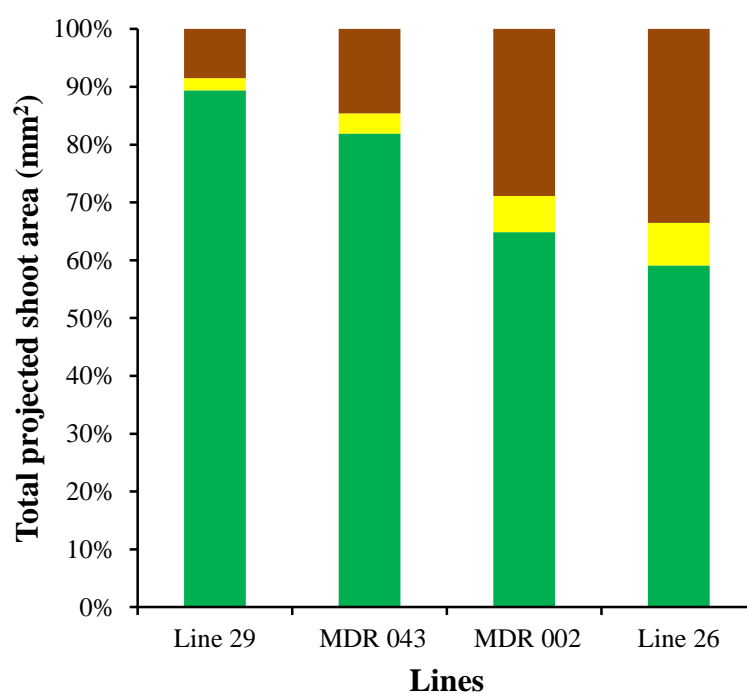


Figure 27. The proportion of (■) healthy, (■) senescing (chlorotic) and senesced (■) (necrotic) tissue of the, MDR 043, MDR 002, F₂ line 29 and the F₂ line 26 at 19 days after NaCl application. The significance of difference between the proportion of salt induced senesced shoot area (sum of proportion of senescing and senesced tissue) was revealed by “t” test at $P \leq 0.04$ levels.

5.3.3 Non-destructive phenotyping for osmotic and tissue tolerance in MDR 002 × MDR 043 F₂ population

With the growth and health characteristics of the mapping population documented in above Section 5.3.1 & 5.3.2, it is now possible to determine the salinity tolerance of each mapping line and the salinity tolerance mechanism used by each line. Accordingly, the growth and health records of all 177 F₂ individuals in the mapping population were used further to screen and study the segregation of osmotic tolerance (section 5.3.3.1) and tissue tolerance (section 5.3.3.2) in MDR 002 × MDR 043 F₂ mapping population as described below.

5.3.3.1 Osmotic tolerance

The osmotic tolerance of MDR 002 × MDR043 F₂ mapping population was calculated by dividing the mean relative growth rate of an individual seedlings 5 days immediately after NaCl application to the mean relative growth rate of the same individual seedling 5 days before NaCl application (Detailed information are given in section 5.2.4.1). There was a greater difference in osmotic tolerance found among the parents, with MDR 043 demonstrating greater osmotic tolerance than MDR 002 (Table 15). A wide range of phenotypic variability for osmotic tolerance was identified in this mapping population. The range of osmotic tolerance of this population was varied from 0.07 to 0.99. The trait was continuously distributed and the heritability of the trait was 0.82. Transgressive segregants with better osmotic tolerance than MDR 043 and progenies with lower osmotic tolerance than MDR002 were found in this population (Figure 28).

Interestingly, both the biggest and the smallest F₂ individual 29 and 26 respectively, in terms of biomass, had same level of osmotic tolerance (Table 15, Figure 26 & 29). On the other hand, the mean values with MDR 043 and MDR 002 had similar growth rates before NaCl application (0.14), but the growth rate has been reduced to 36 % and 64% respectively due to osmotic stress (Figure 29 & Table 15). Further, there was no relationship found between the mean relative growth rates which were calculated 5 days before and immediately after NaCl application in all 177 F₂ individuals in the population (Figure 30). It seems that osmotic tolerance could be found in both slow and fast growing F₂ individuals in the MDR 002 × MDR043 mapping population.

Table 15. Osmotic tolerance calculations in MDR 043, MDR 002, F₂ individual 29 and F₂ individual 26 in MDR 002 × MDR 043 mapping population.

Genotypes	Mean relative growth rates five days before NaCl application (day ⁻¹)	Mean relative growth rates five days after NaCl application (day ⁻¹)	Osmotic tolerance
MDR 043	0.14	0.09	0.64
MDR 002	0.14	0.05	0.36
F ₂ individual 29	0.15	0.07	0.47
F ₂ individual 26	0.13	0.06	0.46

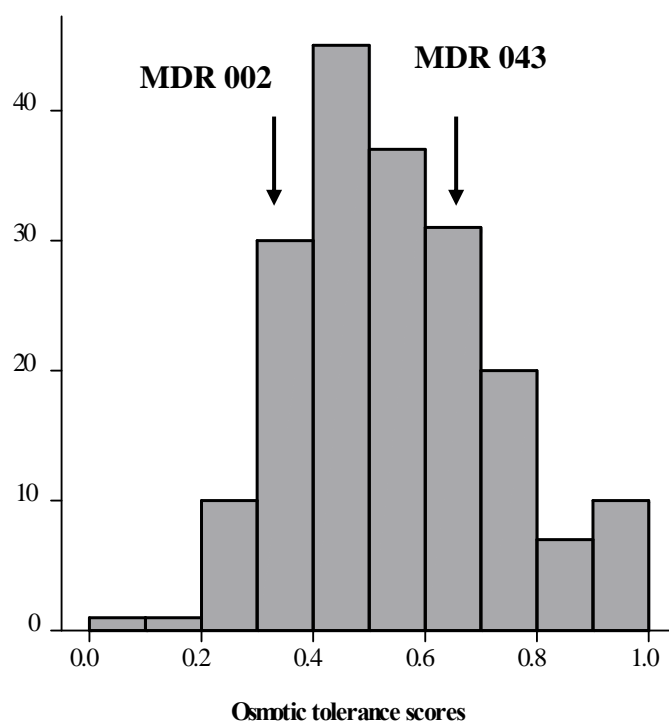


Figure 28. The phenotypic variation found for osmotic tolerance in the MDR 002 × MDR 043 *T. monococcum* F₂ mapping population (177 F₂ individuals) grown between July-September 2009. Osmotic tolerance was calculated by dividing the mean relative growth rate 5 days after NaCl application by the mean relative growth rates 5 days immediately prior to NaCl application for every single line. The osmotic tolerance of the parents was marked by arrows.

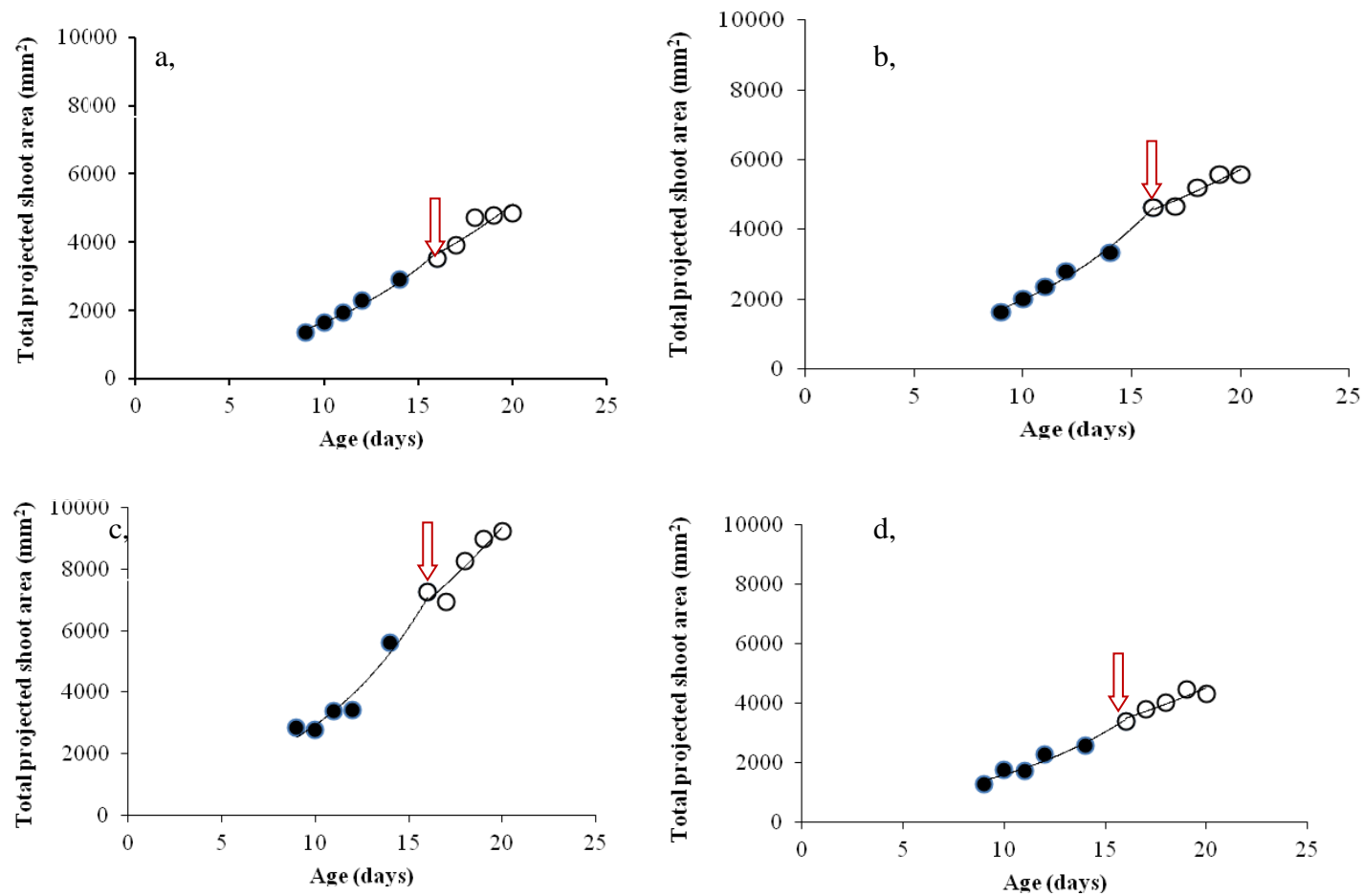


Figure 29. Growth of (a) MDR 043 (b) MDR 002, (c) F₂ line 29 and (d) F₂ line 26 before (●) and after NaCl application (○) in MDR002 × MDR043 mapping population. Arrow indicates time of NaCl application. The final 75 mM NaCl was achieved by three 25 mM NaCl applications, two doses applied on 16th day.

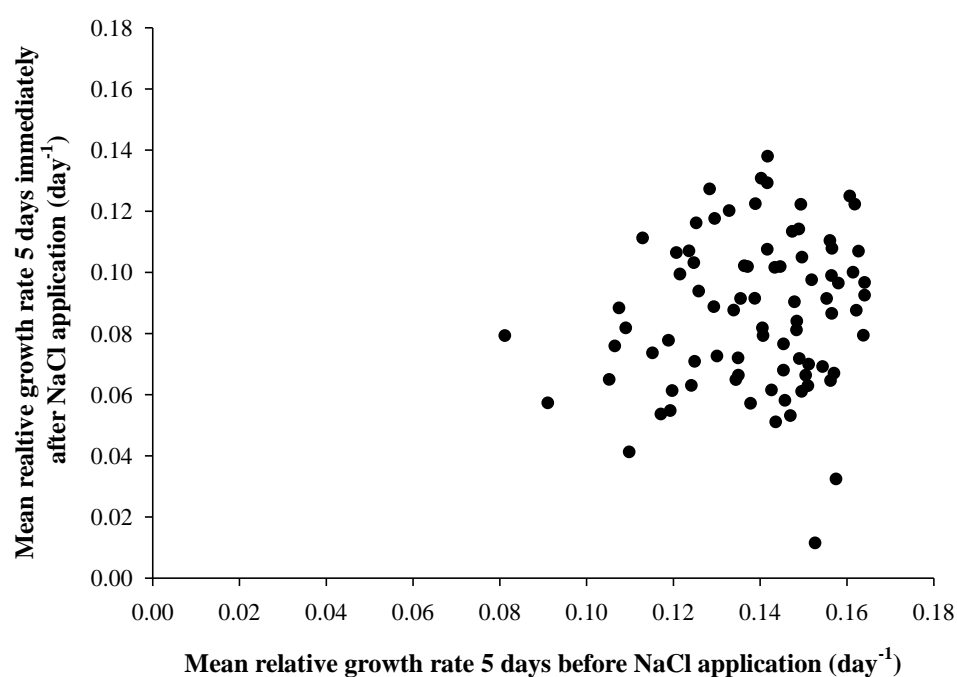


Figure 30. The relationship between mean relative growth rates calculated five days before and five days after NaCl application ($R^2 = 0.04$) for all plants in the MDR002 \times MDR043 mapping population, which was significant at $p < 0.05$ level.

5.3.3.2 Tissue tolerance

As has been elucidated in Section 5.1, MDR 002 \times MDR 043 mapping population was obtained, not only to study the genetic variability for osmotic tolerance but also tissue tolerance. The occurrence of tissue tolerance in parents and mapping population are discussed below in following subsection 5.3.3.2.1 & 5.3.3.2.2, respectively.

5.3.3.2.1 In parents

In this present study, both MDR 002 and MDR 043 parents accumulated approximately the same amount of $[Na^+]$ (199 and 188 mM, respectively) in the fourth leaf blade, which was sampled 19 days after 75 mM NaCl application

(Table 16 & Figure 31). At the same time, however, they have shown a difference in the proportion of senesced shoot area, which was 35% in MDR 002 and 18 % in MDR 043 (Table 16 & Figure 27). This again suggests that the MDR 043 parent has better tissue tolerance than MDR 002 parent.

Table 16. Descriptive statistics of the physiological parameters used to estimate tissue tolerance in parents and the MDR 002 × MDR 043 F₂ mapping population.

Physiological parameters	Parents				MDR 002 × MDR 043 F ₂ mapping population
	MDR 002		MDR 043		
	Mean ± S.E	Range	Mean ± S.E	Range	Range
Fourth leaf blade [Na ⁺] (mM)	199 ± 30	114 - 313	188 ± 18	124 - 273	49 - 348
Proportion of senesced shoot area (%)	35 ± 9	30 - 84	18 ± 0.7	17 - 19	3 - 47

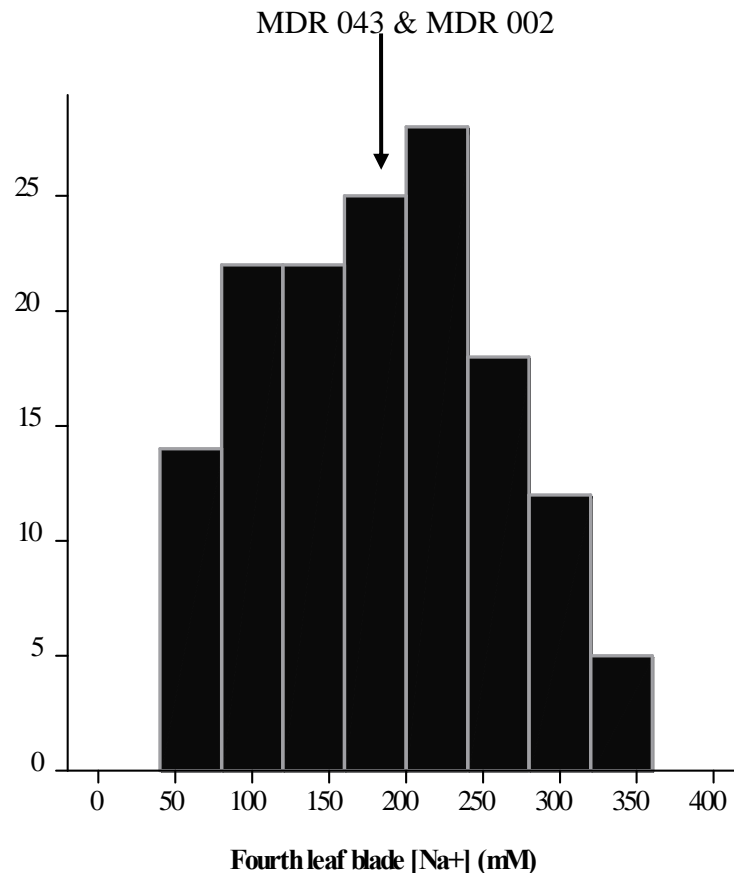


Figure 31. The phenotypic variation observed for fourth leaf blade tissue [Na⁺] in 177 F₂ progenies of MDR 002 × MDR 043 *T. monococcum*, which was sampled after 19 days of growth in 75mM NaCl. The arrow indicates the position of parents (MDR 043 & MDR 002).

The development of senescence in both MDR 043 and MDR 002 was exponential up to 5 days immediately after NaCl application which includes from time before NaCl application to the period of osmotic stress (Figure 32, a, b, d and e). After osmotic stress, the development of senescence was slow and linear in MDR 043 but it was fast, exponential in MDR 002 (Figure 32, c and f).

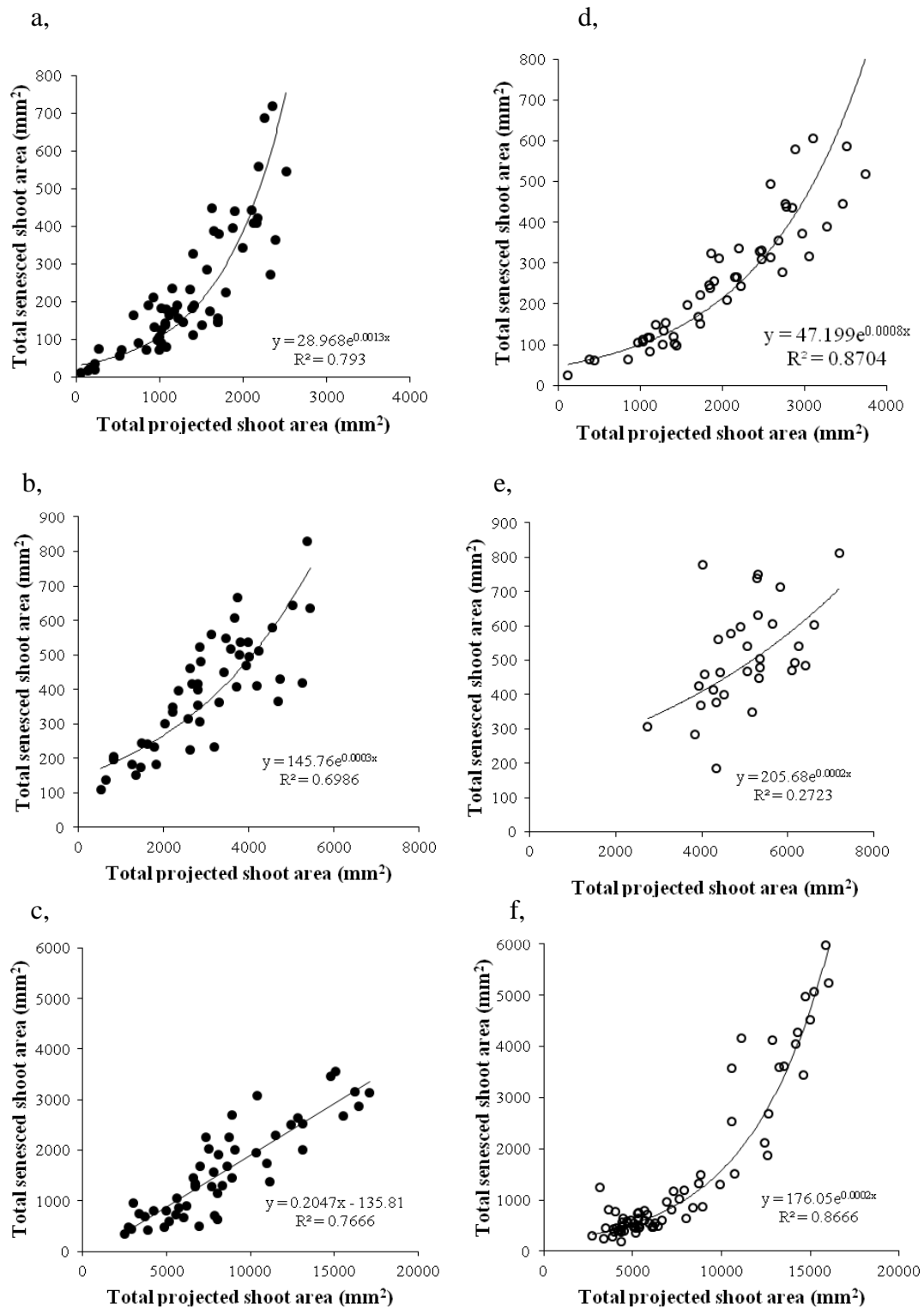


Figure 32. Development of senescence in MDR 043 (a), (b) and (c) and MDR 002 (d), (e) and (f) accessions, which was quantified 7 days before 75 mM NaCl application (a & d), at 5 days immediately after salt application (during osmotic stress) (b & e) and, after osmotic stress (c & f) respectively.

5.3.3.2.2 In MDR 002 × MDR 043 F₂ population

The range of the fourth leaf blade [Na⁺] in the MDR 002 × MDR 043 F₂ mapping population was from 49 to 348 mM on a tissue water basis (Table 17 & Figure 31). The proportion of total senesced shoot area of F₂ individuals grown under saline conditions was ranged from 3% to 47% (Table 17 & Figure 33). The development of senescence in F₂ individuals was linear before NaCl application and during osmotic stress period (Figure 34. a, b, c, d, e and f), however, the relationship was progressively lost from 11th day and there was no relationship found between the projected shoot area and the senesced shoot area of the progenies at 19th day after 75 mM NaCl application (Figure 34. g, h, i, j and k).

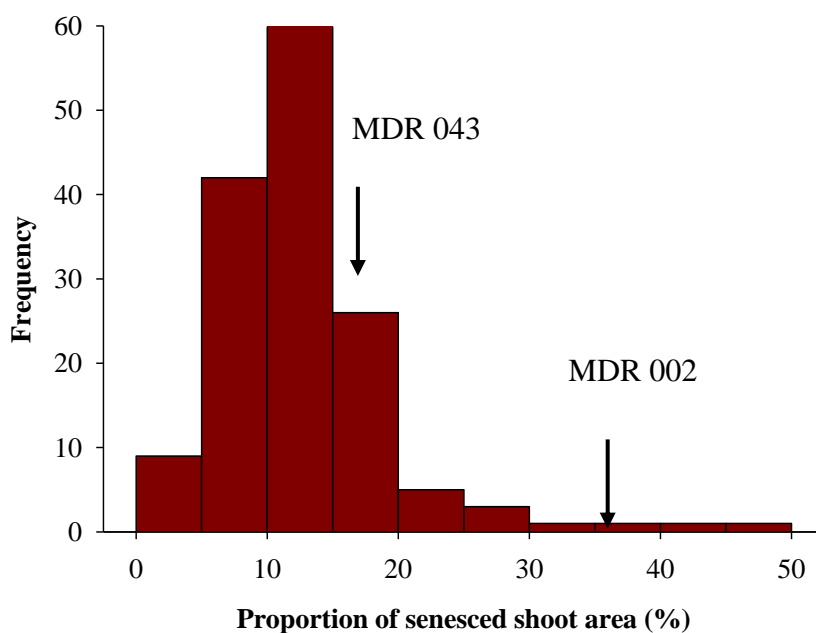
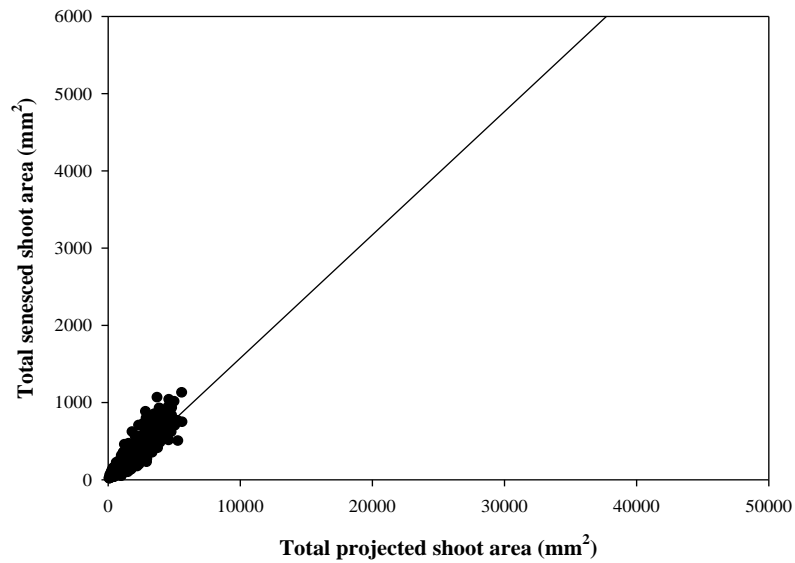


Figure 33. Phenotypic variation observed for proportion of senesced shoot area measured three weeks after 75 mM NaCl application in MDR 002× MDR 043 F₂ mapping population. The senescence in the parents was marked by arrows.

a,



b,

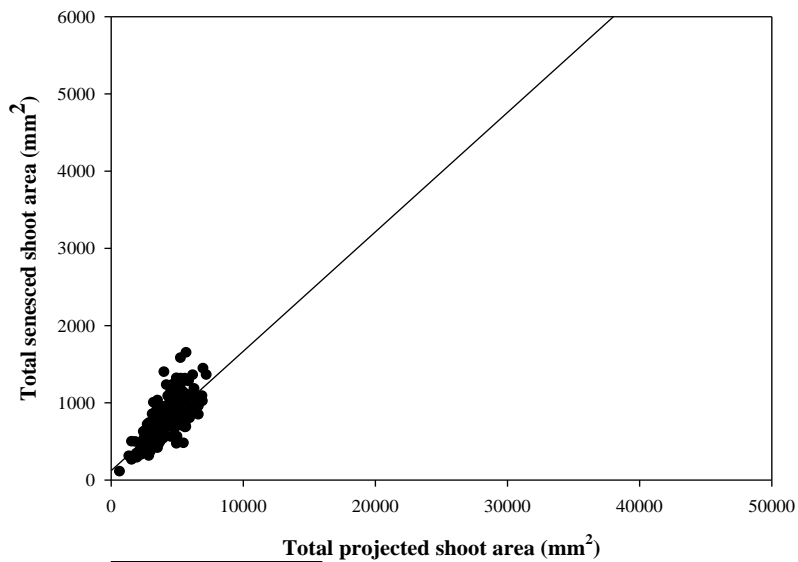
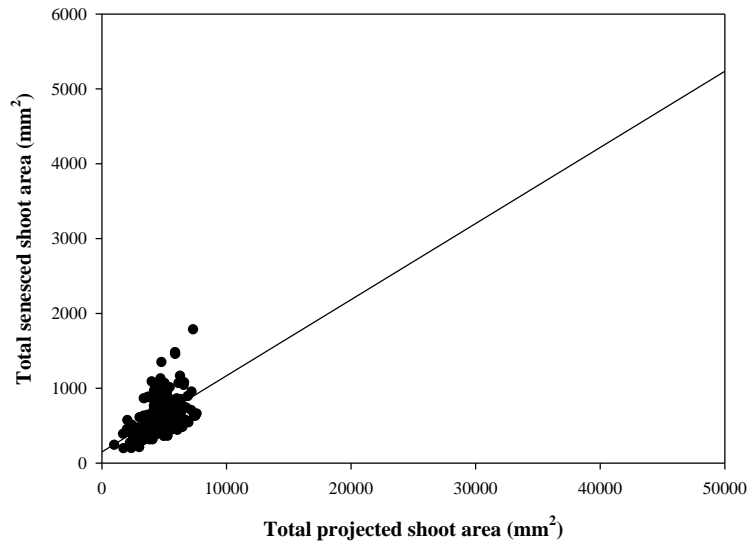
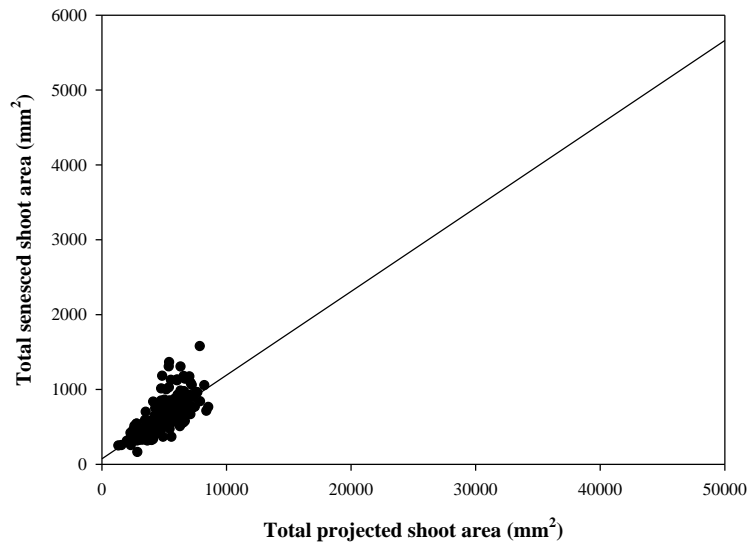


Figure 34. Development of senescence in MDR 002×MDR 043 *T. monococcum* F₂ mapping population, which was quantified 7 days before 75 mM NaCl application (a), at 1-5 days (during osmotic stress) after 75 mM NaCl application (b, c, d, e & f) and 10th, 12th, 14th, 17th and 19th days after 75mM NaCl application (g, h, i, j & k) respectively. Except a, each diagram has 177 data points, whereas, diagram a, has 1239 (177 × 7=1239) data points.

c,



d,



e,

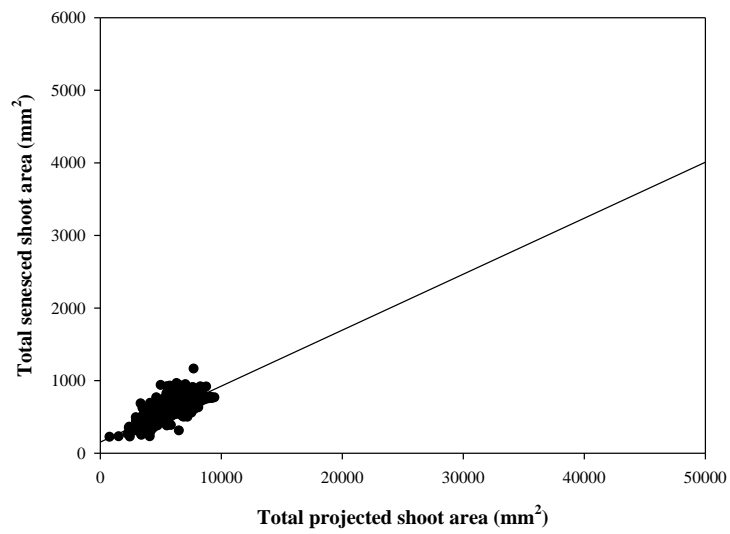
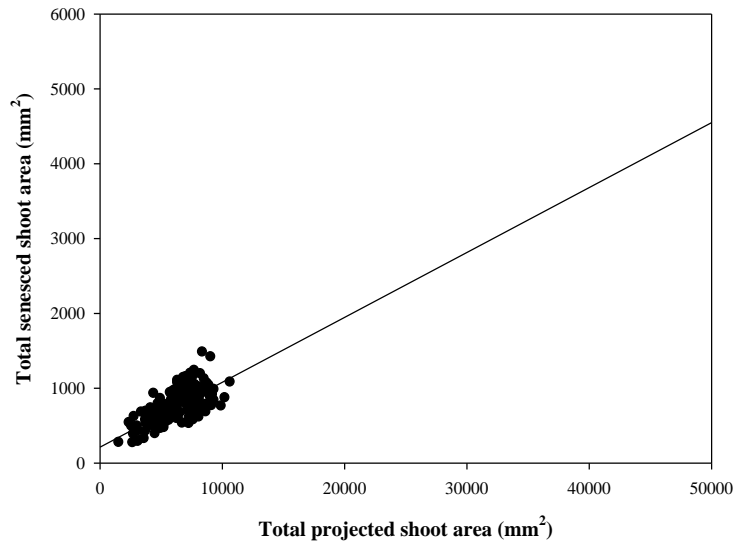
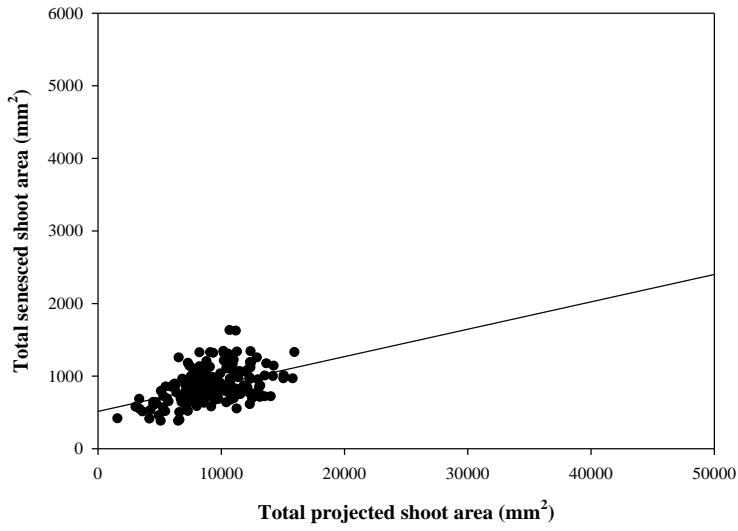


Figure 34. Continued.

f,



g,



h,

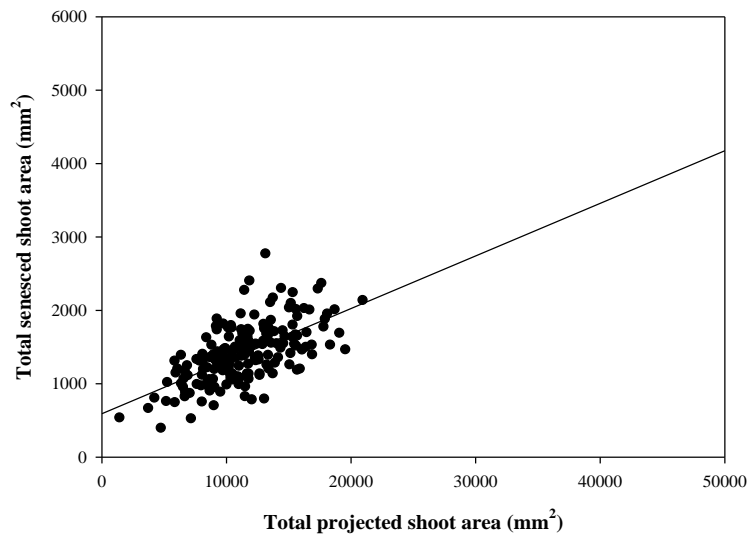
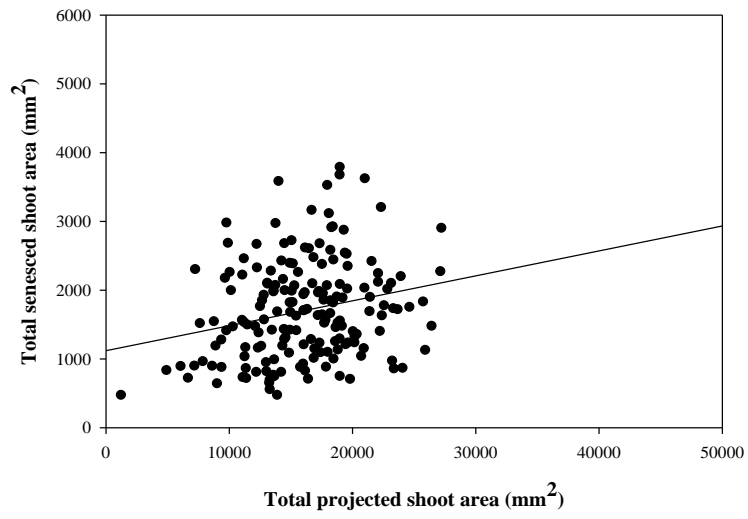
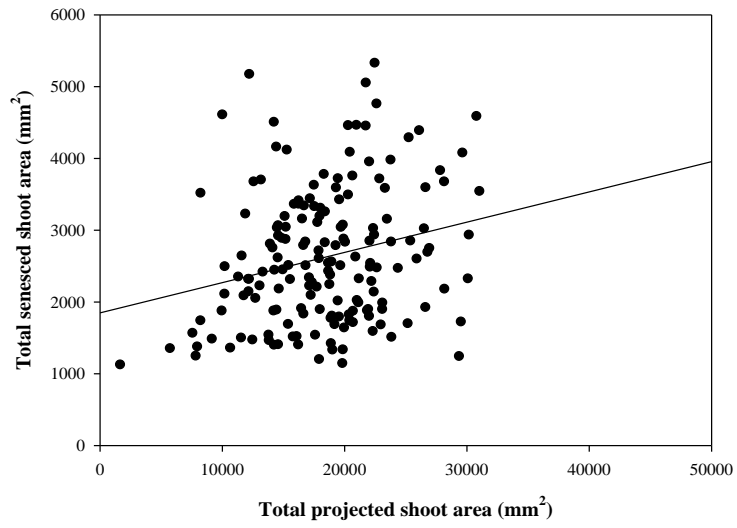


Figure 34. Continued.

i,



j,



k,

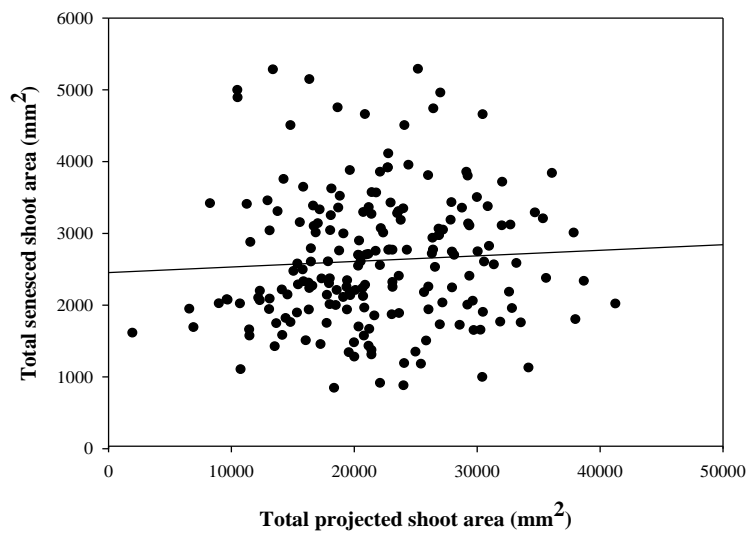


Figure 34. Continued.

At the end of the experiment, there was a weak relationship found between the proportion of senesced shoot area and the fourth leaf blade $[Na^+]$ (Figure 35), suggesting some of the F_2 individuals stayed healthier than others with the same level of $[Na^+]$ in the fourth leaf blade; the healthy ones would have tissue tolerance mechanisms to protect themselves from the accumulated Na^+ toxicity inside the leaf blade.

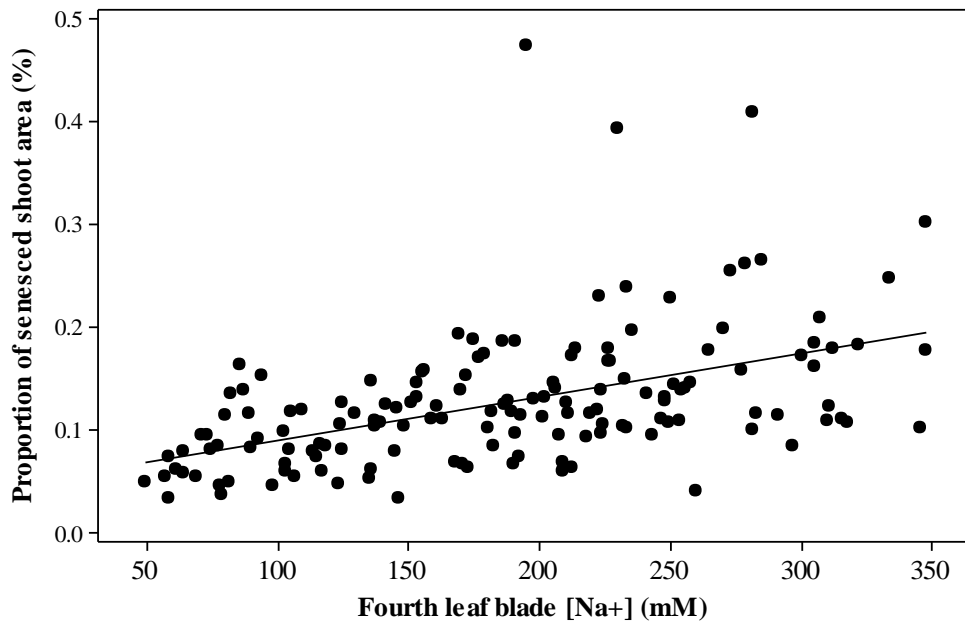


Figure 35. The relationship between (a) proportion of total senesced shoot area and fourth leaf blade $[Na^+]$ of the mapping population, 19 days after NaCl application, $R^2 = 0.23$ significant at $p \leq 0.05$, level.

These results show that the MDR 002 \times MDR 043 mapping population demonstrates evidence for segregation of tissue tolerance in their progenies. Since, it has shown natural senescence even before NaCl application (Figure 34, a) it is necessary to determine the extent of natural senescence in this population before tissue tolerance quantification (Chapter 3 & Rajendran *et al.*, (2009)). The continuation of tissue tolerance screen is discussed in section 5.4.1.

5.3.4 Genotyping MDR 002 × MDR 043 *T. monococcum* F₂ mapping population using SSR markers

Genomic DNA was extracted from young seedlings of the complete mapping population (Section 5.2.8), PCR reaction was carried out and attempts have been made to genotype them mapping population with 45 polymorphic SSR markers, as has been listed in Table 14.

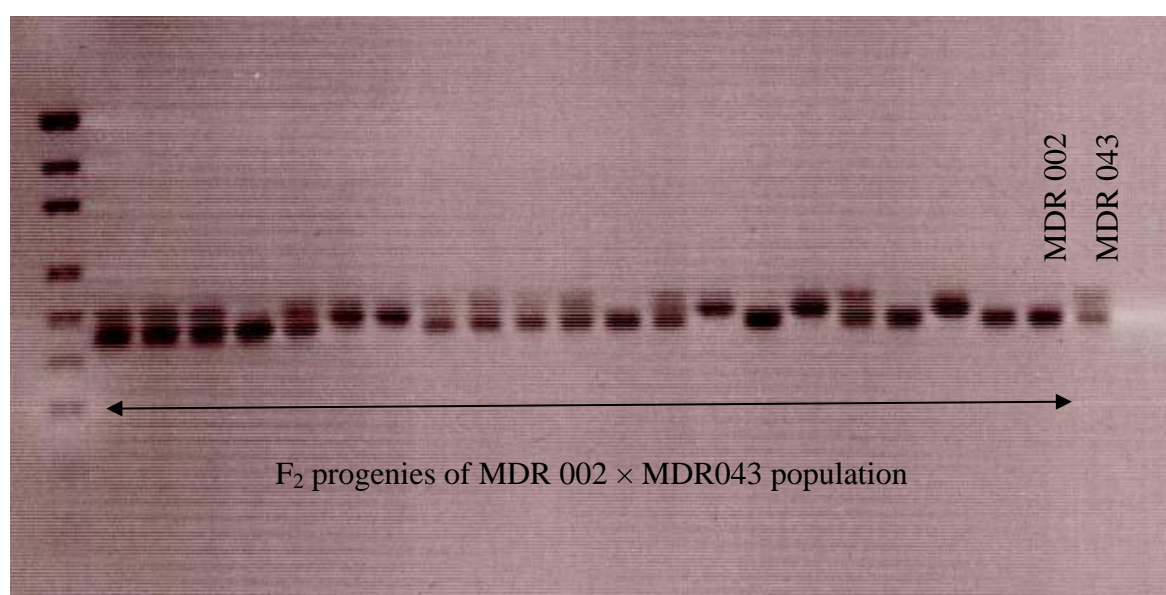


Figure 36. Use of SSR markers to identify the polymorphism in MDR 002 × MDR043 *T. monococcum* F₂ mapping population. An example of an ethidium bromide stained 3% gel agarose gel demonstrating the polymorphism of the SSR marker Xbarc174 in F₂ progenies with MDR 002 and MDR 043. The first lane was loaded with pUC19 DNA/*MspI*(*HpaII*).

From the list of 45 available polymorphic SSR markers unfortunately there was only time to genotype the 177 F₂ individuals with nine SSR markers, an example is shown in Figure 36. The single marker analysis was done to find out the relationship with the already screened nine SSR marker and osmotic tolerance quantified in the mapping population. Results from single marker analysis do not identify any significant relationship between the SSR markers screened so-far with osmotic tolerance. It was

difficult to screen the low molecular weight markers in 3% agarose gel. Genotyping work is still in progress to screen those remaining markers in 7% poly acrylamide gels.

5.4 Discussion

The MDR 002 × MDR 043 F₂ mapping population was obtained from Rothamsted Research Station, Harpenden, (U.K) for this study and a high throughput salt screening protocol was used to screen and study the genetic variability for osmotic and tissue tolerance in this population. As described in section 5.3.3.1 screening for osmotic tolerance has been successfully completed for this project. However, as described below in section 5.4.1, 5.4.2 and 5.4.3, tissue tolerance screening, linkage map construction and QTL mapping for osmotic tolerance respectively, are still in progress and were not completed due to time constraints.

5.4.1 Tissue tolerance screening - Challenges and opportunities

As shown in Figure 31 and Table 16, the parents of this mapping population, MDR 002 and MDR 043, have each accumulated approximately 200 mM Na⁺ in the fourth leaf blade. Nevertheless, MDR 043 has half as much senesced shoot area than MDR 002 under saline conditions (Table 16 and Figure 33). MDR 043 must therefore effectively compartmentalize accumulated Na⁺ in the vacuole, keeping the [Na⁺] below toxic levels in the cytosol and maintaining healthier growth. The high Na⁺ accumulation and hypothesised poor compartmentation in the salt sensitive genotype MDR 002, results in high leaf damage and reduced photosynthetic capacity (Greenway and Munns, 1980; Yeo *et al.*, 1985; Seemann and Critchley, 1985; Fricke *et al.*, 1996; Sibole *et al.*, 2003; James *et al.*, 2006b).

As shown in section 5.3.5, the MDR 002 × MDR 043 *T. monococcum* F₂ mapping population demonstrated evidence for the presence of tissue tolerance in their

progenies. There was a weak relationship found between the proportion of senesced shoot area and the fourth leaf blade $[Na^+]$ (Figure 35), indicating this population has some F_2 individuals which can stay healthier than others with the same level of $[Na^+]$ in the fourth leaf blade. The healthy plants must have tissue tolerance mechanisms to protect themselves from the accumulated Na^+ toxicity inside the leaf blade. However, there are two pieces of information required for the quantification of tissue tolerance for further QTL mapping; the proportion of salt induced senesced shoot area (as opposed to the total senesced area measured here) and fourth leaf blade $[Na^+]$ (Chapter 3 & Rajendran *et al.*, (2009)). Interpretation of analysed $[Na^+]$ in the fourth leaf blade, for tissue tolerance calculation is very straightforward, however, the total senesced shoot area measured in *T. monococcum* F_2 individuals, may have an influence from natural senescence. A considerable amount of natural senescence was found in the mapping progenies grown in the nutrient solution before 75mM NaCl application (Figure 34, a). Hence, the estimation of natural senescence is more important in *T. monococcum* and it needs to be subtracted from the measured total senesced shoot area to obtain total salt induced senesced shoot area for precise tissue tolerance calculations (Chapter 3 & Rajendran *et al.*, (2009)).

Usually, various biotic and abiotic stresses such as salt, drought and incidence of diseases induces premature leaf senescence in plants (Morris *et al.*, 2000). However, development of premature leaf senescence under controlled environment, for example in glasshouses could be associated with the problems in the nutrient solution used for the particular experiment. The MDR 002 \times MDR 043 population was grown in a modified Hoagland's nutrient solution that provided complete nutrients for plant growth and health. Moreover, the same concentration of nutrient solution was used by various researchers to assess salinity tolerance of major cereal crops such as wheat and barley (Shavrukov *et al.*, 2006; Genc *et al.*, 2007). It is more likely that this wild species of wheat has high natural levels of senescence, due to its different growth form to cultivated wheats. Nevertheless it is still necessary to be able to distinguish between natural senescence and salt induced senescence.

In Chapter 3, *T. monococcum* accessions were grown in both control and saline environments. Accordingly, the salt induced senesced shoot area was predicted by subtracting the natural senesced shoot area of the control grown plants with their total measured senesced shoot area in saline conditions. But, it is impossible to grow control plants when using an F₂ mapping population, due to each plant being a unique individual, which makes replication of experiments and hence growing a plant genotype in both control and salt stress condition is impossible. If timer permitted, the production of a double haploid population would allow genetically identical plants from the same line to be grown in both control and saline conditions, allowing the amount of natural senescence to be determined empirically.

As a double haploid population was not available, a solution to the problem was to use the slope of the line drawn between the total projected shoot area and total senesced shoot area for 35 days old MDR 002 and MDR 043 parents grown in control conditions to produce a standard curve to calculate the natural rate of senescence in the population. This is not ideal, however, as this mapping population was a cross between a tissue tolerance and a salt sensitive parent, therefore the mapping progenies had different pattern of senescence development under saline environments, potentially resulting an inaccurate estimation of the natural senescence in F₂ individuals. For example, the development of senescence in MDR 043, it was exponential before NaCl application and during the period of osmotic stress, however, it started to grow more linearly 11 days after NaCl application. On the other hand, natural senescence was always exponential for the MDR 002 parent (Figure 32). However, the development of senescence in F₂ progenies was more linear until the period of osmotic stress and the linear relationship did not hold up later in the salt stress. This suggests that, the senesced shoot area of the F₂ individuals was depending on plant size before NaCl application until the period of osmotic stress, for example big plants die more than the small ones, however, 11 days after NaCl application, some F₂ individuals showed greater senescence than others, which exhibited either reduced or minimal senescence (Figure 34). In this scenario, the use of measured natural senescence in their parents in control condition would not be helpful to predict the natural senescence in their progenies grown in saline environment. Hence, the natural senescence in their F₂ progenies needs to be determined separately.

Use of natural senescence calculations in control grown F_2 derived F_3 families every 177 progenies, is one of the alternative, for this issue. Because F_3 progenies are replicable (Agrama and Moussa, 1996; Asghari *et al.*, 2007) and it is possible to raise the same families in both control and saline environments. The F_3 families growing in control environment would be useful to calculate the natural senescence of every individual family and hence the calculation of salt induced senesced area, as has been done in Chapter 3, & Rajendran *et al.*, (2009).

5.4.2 Constraints in genotyping and construction of molecular linkage map

The study of QTL analysis requires a genetic linkage map with good marker coverage. To begin with this, 45 polymorphic SSR markers, obtained from Rothamsted Research Station was used in study (Table 15). However, among those 45 SSR only 9 markers were able to be scored for 177 F_2 individuals and their parents in 3% agarose gels. The remaining low molecular weight markers should be scored in 7% acrylamide gels using a Gelscan500 for faster screening (Hayden *et al.*, 2008a). Moreover, genotyping F_2 individuals could also make use of the AA genome SSR readymade marker kit and multiplex ready PCR to detect and genotype the new polymorphic markers in *T. monococcum* MDR002 × MDR 043 mapping population (Hayden *et al.*, 2008a). It helps to get more polymorphic markers and hence to increase the marker density in the linkage map. Once the genotyping work has been completed, the scores of every 177 F_2 progenies for example AA, Aa, or aa nature of F_2 individuals needed to be recorded as scores to study the genetic nature of progenies (parental types and recombinants). However, because of time constraint genotyping and hence the linkage map construction work is still in progress.

The construction of genetic linkage map requires a calculation of recombination frequencies between linked markers and the order the genes (or) markers on the chromosomes. The occurrence of a recombination between homologous chromosomes often decreases the chances of another recombination event occurring

on the same chromosome in an adjacent region; this is commonly known as interference. The calculation of interference is a very useful tool in calculating recombination frequency and hence the spread and distance between markers on the chromosomes (King and Mortimer, 1991). It should be made clear that the distance measured along a chromosome is not a physical distance but rather a calculation of recombination frequency: 1cM equals to 1% recombination frequency between two linked markers (Conneally, 1991). There are several computers softwares available, now days to construct the genetic linkage map in an efficient way (<http://linkage.rockefeller.edu/soft/>&<http://linkage.rockefeller.edu/soft/list3.html>). It uses either Kosambi or Haldane mapping functions to calculate the recombination frequencies of markers and construct the genetic linkage map accordingly (Stam, 1993; Manly *et al.*, 2001).

The diploid genome size of *T. monococcum* is 12201Mbp (<http://data.kew.org/cvalues/CvalServlet?querytype=2> (Bennett, 1982). It is the rule that the number of linked groups in a species is equal to its gametic chromosome number (n). *T. monococcum* has seven chromosomes (or) linkage groups. The recombination frequencies are used as map units for the construction of linkage map and could be estimated through the use of F₂ population. An even spread molecular marker at a genetic distance of 1 to 5 cM is considered as a good map. However, it again depends on cross over frequencies, near centromeric and telomeric regions in every individual chromosomes. Such maps were already done for rice (Harushima *et al.*, 1998), wheat (Somers *et al.*, 2004), maize (Mano *et al.*, 2008), sorghum (Bowers *et al.*, 2003), barley (Wenzl *et al.*, 2006) soybean (Xia *et al.*, 2007) and *T. monococcum* (Kojima *et al.*, 1998).

5.4.3 Difficulties in QTL mapping for osmotic tolerance

As demonstrated in Section 5.3.3.1 & Figure 30, the MDR 002 × MDR 043 *T. monococcum* F₂ mapping population have shown potential genetic variability and high heritability for osmotic tolerance, which would be exploited for further QTL mapping and candidate gene(s) identification. In general, genetic variation, which

occurs due to differential expression of genes among plant populations, is considered as one of the most important requirements for QTL mapping in any crop species (Ashikari and Matsuoka, 2006). Because of difficulties in linkage map construction, as has been explained in previous Section, 5.4.2, QTL analysis for osmotic tolerance has not been completed yet. However, single marker regression analysis, was attempted to marker trait associations for osmotic tolerance in this population. Single marker regression analysis does not require any map for QTL analysis but none of the 9 screened SSR markers were found to be associated with osmotic tolerance.

5.5 Future Prospects

Once QTL for osmotic and tissue tolerance are identified they would be narrowed down and fine mapped for candidate gene(s) selection. Once candidate gene(s) for both osmotic and tissue tolerance has been identified, work would begin to confirm, whether the gene (s) are responsible to get an osmotic and tissue tolerant phenotype. It would involve sequencing of osmotic and tissue tolerance gene(s) and its mRNA product from both parent lines, as well as confirming that the mRNA is expressed in both parents. In addition the effect these gene(s) has on the plant's phenotype will be investigated by over-expressing or down-regulating the candidate gene in both rice and *Arabidopsis*, and if possible wheat. If any Na⁺ transporters are identified, the properties of the protein will be further examined in heterologous expression systems such as yeast. At the end, the identified candidate gene(s) for both osmotic tissue tolerance in *T. monococcum* would be either introgressed or transferred in to commercial wheat varieties, as has already been done for *Nax1* and *Nax2*.

CHAPTER 6. GENERAL DISCUSSION

6.1 Review of thesis aims

In this project, salinity tolerance mechanisms of two different wheat species, einkorn wheat (*T. monococcum*) and bread wheat (*T. aestivum*), were dissected into their three separate components, osmotic tolerance, Na⁺ exclusion and tissue tolerance, using non-destructively 3-D imaging technology. This project had three aims to: 1) develop salt screening protocol that facilitates high throughput quantification of the three major components of salinity tolerance in cereals; 2) use these protocols to screen the Berkut × Krichauff DH mapping population of *T. aestivum* for QTL analysis; and 3) use the high throughput screening protocol to screen F₂ mapping population of *T. monococcum* and explore the genetic variability for osmotic and tissue tolerance for future QTL analysis.

In the Chapter 3, a high throughput salt screening protocol was developed to quantify the three major components of salinity tolerance in twelve different *T.monococcum* accessions. Three indices were used to assess the tolerance level of each plant for the three major components of salinity tolerance. The whole plant salinity tolerance was predicted through a multivariate analysis and compared with the reduction in shoot growth of *T. monococcum* accessions grown under saline conditions with those grown in non-saline conditions. Results suggest that lines growing in saline environment use at least two different tolerance mechanisms.

In the Chapter 4, a high throughput salinity tolerance screening method was used to identify QTL for three major salinity tolerance components in bread wheat Berkut × Krichauff DH mapping population. It successfully identified potential source of variability for osmotic tolerance and Na⁺ exclusion in Berkut × Krichauff DH mapping population. From this study, QTL for Na⁺ exclusion and osmotic tolerance were also identified in this mapping population. However, this mapping population

was not useful to study tissue tolerance because of the lack of variation observed for shoot senescence in bread wheat.

In the Chapter 5, MDR043× MDR002 *T.monococcoum* F₂ mapping population was screened to explore the genetic variability for osmotic and tissue tolerance. Wide variation in osmotic tolerance was observed amongst the progenies. There was also strong evidence for the presence of tissue tolerance; however, it was not possible to quantify tissue tolerance in their progenies because of the difficulties in natural senescence calculation (Chapter 5). Construction of linkage map for future QTL analysis in this population is still in progress.

The significant outcomes of this project have already been discussed in the individual chapters and in this chapter the possibility of improving high throughput salt screening methodology, breeding potential for components salinity tolerance and future directions are discussed in the light of past and current research as follows.

6.2 Advantages and disadvantages of the high throughput salt screening methodology

The new high throughput 3D imaging salinity tolerance screening protocol developed in this study was used to detect changes in the growth and health status of plants grown under salt stress, allowing characterisation of both the osmotic and ionic phase (Chapter 2 & 3). Previously, there was no such high throughput screening method available to quantify both osmotic and ionic tolerance of cereal crops. Much of the early salinity tolerance screening methods either quantified osmotic component of salt stress by using parameters such as leaf elongation rate (Munns and James, 2003), leaf water potential (Marcum and Murdoch, 1990; Lutts *et al.*, 1996; Moghaieb *et al.*, 2004), relative water content (Ghoulam *et al.*, 2002), stomatal conductance (James *et al.*, 2008) and transpiration efficiency (Richards *et al.*, 2010) or used parameters such as leaf health and leaf longevity and assessed the ionic tolerance of various crops

(Munns *et al.*, 2002; Munns and James, 2003). These screening methods required a huge amount of labour, time, and frequently, were imprecise, unreliable and unrepeatable (Sirault *et al.*, 2009). Moreover, they mainly focused on changes in the physiological processes occurring in the leaf, such as the rates of photosynthesis, respiration and transpiration, and few measurements were made of whole plant responses over time. The whole plant response is important to consider as there are multiple mechanisms for salt tolerant and no single physiological observation can account for variation in whole plant response to salt stress (Hasegawa *et al.*, 2000).

In general, plants growing under osmotic stress conditions close the stomata, reduce the CO₂ assimilation and photosynthetic rate and hence develop plants with reduced shoot growth (Munns and Tester, 2008). Eventually osmotic sensitive genotypes produce less plant biomass than osmotic tolerant genotypes, immediately after NaCl application. This is the basic premise which has been used to develop osmotic tolerance assay in this study; the, osmotic tolerance screen was done by comparing the changes in the relative shoot growth rate of plants growing under saline versus control conditions. If there were no control grown plants in the experiment, osmotic tolerance was calculated by measuring changes in each individual plant's mean relative growth rate 5 days after the addition of NaCl and comparing that to the relative growth rate 5 days before NaCl application (Chapter 4 & 5). However, in addition to retarded shoot growth, development of wilt is also a common phenomenon in plants growing under salt induced osmotic stressed conditions. In this study, some of the wheat genotypes were observed with temporary wilt symptoms during the period of osmotic phase; they lose its turgidity and drooped down the leaves through the wall of the growing tubes (Figure 37,a). Quantification of this change in plant morphology may be another way of screening for osmotic tolerance in salt stressed plants in the future. From images, such as Figure 37, a, it is possible to characterization the shape of a plant's shoot using various mathematical descriptors such as compactness and centre of mass. These measurements, in addition to alterations in plant growth these measurements of plant form would be useful to screen different genotypes for osmotic stress tolerance in future.

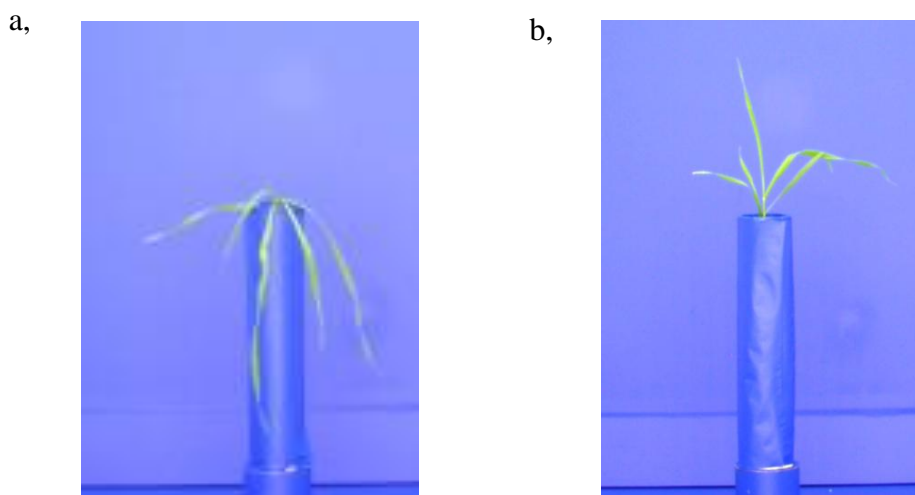


Figure 37. The morphological differences between the Berkut× Krichauff DH mapping lines a, HW-893*A008 and b, HW-893*A086 of bread wheat grown three days after 150 mM NaCl application.

Alternatively, another approach for an osmotic tolerance screen is through the use of infra-red thermography. In many aspects, plants growing under both drought and saline environment demonstrate similar phenotypic responses: closure of stomata and reduction in photosynthetic rates are quite common in plants growing under drought and salt induced osmotic stressed conditions. It is possible to utilize the benefits of infra-red thermography to the screen the osmotic component of salt stress (Sirault *et al.*, 2009). Screening by infra-red thermography, measures the canopy temperature of a plant and screens the plant based on the leaf water content. It identifies an osmotic tolerant genotype with cooler leaves and osmotic sensitive genotype with hotter leaves. The cooling nature of osmotic tolerant genotypes (Martin and Ruiz-Torres, 1992; Medrano *et al.*, 2002; Berger *et al.*, 2010) is believed be due to the occurrence of higher stomatal conductance and a higher rate of photosynthesis than the osmotic sensitive genotypes. Infra-red cameras and infra-red thermometers have been successfully used to detect the canopy temperature of plants growing in field conditions. However, these measurements are often subject to changes in the environmental conditions, therefore conducting experiments in more sophisticated glasshouse conditions with automated and high throughput phenotyping with infrared thermal imaging could minimize the environmental error (Berger *et al.*, 2010;

Furbank and Tester, 2011). A fusion of colour images with infrared images was found to be more powerful to study the leaf orientation, canopy structure and canopy temperature of plants growing under stressed environment over time (Leinonen and Jones, 2004; Möller *et al.*, 2007; Berger *et al.*, 2010).

In this study, the screening for tissue tolerance calculation takes an account of proportion of salt induced senescence in the whole shoot at the end of the experiment as one of the important parameters. The symptoms of Na⁺ toxicity in plants begins with marginal chlorosis and necrosis of leaf tips and margins followed by the complete senescence of entire leaf blade (Tester and Davenport, 2003) (Figure 38, a). However, senescence under saline environment could occur also due to salt induced Ca²⁺ deficiency (Greenway and Munns, 1980) and effect of losing water from the cell because of osmotic stress caused by the accumulated Na⁺ outside the plant cells (Oertli, 1968; Ghoulam *et al.*, 2002).

The high Na⁺ concentrations of saline solutions replaces the Ca²⁺ from the membrane of root cells and cause salt induced Ca²⁺ deficiency in plants (Cramer *et al.*, 1985). Ca²⁺ is a very weak mobile nutrient and hence the deficiency symptoms are first developed in the new leaf blades whereas the old leaves keeps Ca²⁺ in a favourable status. Ca²⁺ deficient young leaf blade will appear green, withered and begins to roll up from leaf tip. In the experiments carried out for this dissertation, supplemental calcium was added to increase the activity of Ca²⁺ in the nutrient solution for plant uptake (Cramer, 2004). Hence, there were no symptoms of Ca²⁺ deficiency observed in plants growing under saline conditions.

a,



b,



Figure 38. Two different types of senescence observed in bread wheat Berkut × Krichauff DH mapping lines grown three weeks after 150 mM NaCl application. a) Marginal chlorosis and necrosis followed by burning of entire leaf blade due to ionic toxicity b) dull appearance of leaf blade, loss of turgor, grey discoloration and shrinking of leaf blade due to osmotic stress.

Nevertheless, symptoms of senescence due to the osmotic effect of accumulated Na^+ inside the plant cells were found in most of the experiments done for this dissertation. The accumulated Na^+ in the leaf apoplast and vacuoles, reduce water potential of individual cells and also causes senescence of plant leaves. Visually this can be seen as a dull appearance of the old leaf blade followed by grey discoloration, loss of turgor and shrunken, senesced leaf blade (Figure 38 b). This type of senescence usually occurs in the old leaf blade, which have more time to accumulate Na^+ in the apoplast when the water evaporates in the xylem stream. This osmotically driven removal of water affects cell membranes, normal cellular activities and results in leaf death (Evans and Sorger, 1966; Evans, 1980; Xiong and Zhu, 2002; Munns *et al.*, 2002; Munns and James, 2003).

In addition to colour images, it is possible to utilize the benefits of near infra red imaging and chlorophyll fluorescence imaging platform available in the plant accelerator facility. In the future these could be used to get meaningful information about the two different types of senescence. Near infra-red images are usually helpful to detect the difference in the water status of individual leaves in a plant where as the fluorescence images facilitate to know the photosynthetic capacity and the health status of plants (Berger *et al.*, 2010). While combining all these information it could be possible to differentiate the development of two types of senescence in the early stage, which is before visible to human eyes and separate the effect of osmotic stress and Na⁺ toxicity developed by the accumulated Na⁺ inside the leaf blade during the late osmo-ionic phase of plant growth. The differentiation of these two types of senescence would be helpful to further refine the osmotic and tissue tolerance assays developed in this study.

6.3 Other application of 3D imaging technology

The digital imaging technique could also be used to quantify senescence due to nutrient deficiencies, toxicities and pathogen deficiencies. It was successfully used to pick up the symptoms of boron damage in leaves, derive the quantitative data of germanium toxicity in barley mapping population and used to identify QTL for boron tolerance (Schnurbusch *et al.*, 2010). The digital imaging platform has also been used to predict the shoot dry matter of the experimental plants non-destructively over time to calculate transpiration efficiency of various wheat genotypes grown under salt stressed conditions (Harris *et al.*, 2010). It was used to estimate the leaf lesions caused by fungal pathogens such as *Alternaria solani* and *Ascochyta pteridium* and assess the disease severity on various crops (Lindow and Webb, 1983; Kampmann and Hansen, 1994; Pydipati *et al.*, 2006). Likewise, it was also used for detecting pest damage in various agricultural crops (Sena Jr *et al.*, 2003; Koumpouros *et al.*, 2004; Murakami *et al.*, 2005). However, it was found that it was hard to use solely RGB digital images to assess symptoms of disease infection and pest damages in plants because of difficulties in image acquisition and development of colour class to analyse and follow the development of lesions in diseased plants over time (Furbank and Tester,

2011). The digital images combined with chlorophyll fluorescence images are quite useful now-a-days to quantify symptoms of disease and pest damages in plants (Berger *et al.*, 2010; Bauriegel *et al.*, 2011; Leinonen and Jones, 2004; Möller *et al.*, 2007).

6.4 Breeding potential for major salinity tolerance components in wheat breeding

Even though existence of genetic variation for salinity tolerance has been reported in many crop species plant breeders have often been unable to incorporate these genetic variation in to commercial cultivars of various agricultural crops. There have been only a few varieties that have been developed with improved salinity tolerance in various cereal crops in the past two decades. This is mainly due to the lack of proper screening methodology that helps to select potential sources of genetic variability present in the breeding materials. It is one of the main reasons for the limited success of salinity tolerance breeding programmes in the past two decades. However, in this study a new high throughput salinity tolerance screening methodology was used to select the genetic variability for both osmotic and ionic tolerance components of salt stress in two different wheat species.

In Chapter 3, a high throughput non-destructive screening was done to identify potential sources of genetic variability for three major components of salinity tolerance components such as Na⁺ exclusion, osmotic tolerance and tissue tolerance in *T. monococcum*. It was found that *T. monococcum* has a potential source for natural genetic variability identified for the three major components of salinity tolerance. This potential source of variability for three major components of salinity tolerance in *T.monococcum* could be used to improve salinity tolerance in wheat breeding programme. Genetic variation for Na⁺ uptake and Na⁺ transport has already been identified in the close relatives of commercial wheat cultivars possessing AA genome (*T. urartu*, *T. monococcum* and *T. boeoticum*) (Gorham *et al.*, 1991) and DD genome (*Aegilops tauschii*) (Schachtman *et al.*, 1992). Synthetic hexaploids have already been developed to improve salinity tolerance of bread wheat through the use of

genetic variation in *Aegilops tauschii* (Schachtman *et al.*, 1992). However, there are so many factors limit the use of wheat relatives in the wheat breeding programme, including barriers which limit the transfer of genes from wild species to wheat. One of the key barriers is occurrence of limited recombination between homeologous chromosomes of cultivated and wild species in wheat. The frequency of this homeologous recombination is low thereby limiting the ability to reduce the DNA fragment introduced to the commercial cultivar from the wild relative (Islam and Shepherd, 1991; Jiang *et al.*, 1993). This effect is genetically commonly known as linkage drag and prevents the removal of unwanted characteristics that can be introduced at the same time as the beneficial characteristics are introduced, even if tight molecular markers to the beneficial trait can be produced (Paterson *et al.*, 1991; Fedak, 1999).

The genetic variability present in *T. monococcum* offers a new opportunity to utilize association mapping and bi-parental mapping techniques to identify novel QTL and genes for salinity tolerance which could then be transferred into wheat. The efficiency of association mapping is much higher in *T. monococcum* than any other plant species (Jing *et al.*, 2007). The association mapping technique takes in to account of linkage disequilibrium between markers and the large polymorphism available across a wide range of germplasm, making it an ideal technique for detecting QTL for candidate gene identification approaches. When compared to the generation of linkage maps for bi-parental mapping it requires less time for construction and can provide high resolution of map for QTL mapping (Zhu *et al.*, 2008). It complements linkage mapping and it is currently being widely used by many researchers (Bradbury *et al.*, 2007; Ahmadi *et al.*, 2011; Gurung *et al.*, 2011; Wuerschum *et al.*, 2011). However, in Chapter 5, MDR 002 × MDR 043 F₂ mapping population was used to exploit the potential variability for osmotic tolerance and tissue tolerance, because the population had already been developed by Dr. Hai Chun Jing, Rothamsted Research Station (WGIN, 2007; Jing *et al.*, 2008) and was readily available for genetic analysis.

In Chapter 4, the Berkut × Krichauff DH mapping population of bread wheat has shown potential genetic variability for osmotic tolerance and Na⁺ exclusion.

Histograms for osmotic tolerance and Na⁺ exclusion of Berkut × Krichauff DH mapping population showed a continuous distribution and confirmed the quantitative nature of the trait. A strong environmental interaction was found for osmotic tolerance and Na⁺ exclusion in this mapping population. The presence of environmental interaction for osmotic tolerance and Na⁺ exclusion emphasizes the difficulty of phenotypic selection through conventional breeding, particularly growing plants over three different experimental time of the year. Promisingly, however, the results from this chapter can be used to suggest molecular markers linked to QTL for salinity tolerance, which can be used in breeding programmes.

Identification of molecular markers such as *wmc216* and *gwm186*, which are linked to osmotic tolerance and Na⁺ exclusion QTL on chromosome 1D and 5A, have shown potential to be used in marker assisted breeding. However, selection of these QTL for MAS would only be successful if they consistently explained a large amount of phenotypic variance for the trait of interest consistently across multiple environments (Tanksley, 1993; Ribaut and Betrán, 1999; Hittalmani *et al.*, 2002). For instance, it would be better to use MAS on QTL explaining >10% phenotypic variance for osmotic tolerance and/or Na⁺ exclusion identified in different environments and at different experimental times (Collard *et al.*, 2005a). Hence, the results from this study can only be described as preliminary and there needs to be further re-evaluation of the QTL and their positions in this mapping population. Such validation tests are very important because use of QTL with small effect may reduce the effect of marker assisted selection, even lower than the traditional phenotypic selection (Bernardo, 2001). Moreover, the success of the marker assisted selection largely depends on the unbiased assessment of QTL effects (Melchinger *et al.*, 1998). Nevertheless, marker assisted selection has already been successfully used to improve Na⁺ exclusion of bread wheat and durum wheat cultivars. As a result, salinity tolerant durum wheat cultivars have been recently developed which yield 25% more in saline soils of Australia (James *et al.*, 2011).

6.5 Future Directions

Based on the outcome of the study, future research on the three major components of salinity tolerance should focus on the following criteria:

1, Select genotypes combination of salinity tolerance components rather than the single component to increase total salinity tolerance of wheat

The results from Chapter 3, study strongly recommends the selection of genotypes either with combinations of osmotic tolerance and Na^+ exclusion or with the combinations of osmotic tolerance and tissue tolerance rather than with a single salinity tolerance component to generate successful salinity tolerant wheat cultivar in the future. Combinations of osmotic tolerance and tissue tolerance components would help plants to tolerate the other type of osmotic stress that develops due to increased Na^+ and Cl^- accumulation in the vacuoles. The high Na^+ accumulation in the vacuole leads to osmotic imbalance between cytoplasm and vacuole, decreasing the water potential of the vacuole and can cause water to leave the cytosol. Hence, in addition to tissue tolerance, if the plant possesses any of the osmotic tolerance mechanisms, for example synthesis of compatible solutes, this would help plants to achieve increased salinity tolerance in these conditions. Moreover, selection of genotypes with combination of osmotic tolerance and tissue tolerance would be more appropriate to wheat cultivars growing in salt affected farmlands, particularly under rain-fed conditions, because, synthesis of compatible solute is an energy consuming process. Eventually, the accumulation of $[\text{Na}^+]$ in the vacuole help plants to retrieve water from the soil, maintain turgor and withstand under dry land conditions.

The selection for only low Na^+ accumulation in wheat has been beneficial to improving crop yield under salt stress (Rivelli *et al.*, 2002; Munns, 2002), however, by selecting in favour of a combination of osmotic tolerance and Na^+ exclusion still would enable plants to maintain growth rates during the early stages of salt stress, in addition to the later stages and again would be an advantage in both irrigated and dry-land conditions.

While the plants with the best salinity tolerance had either osmotic tolerance and Na⁺ exclusion or osmotic tolerance and tissue tolerance, no genotypes were identified with both Na⁺ exclusion and tissue tolerance. This could be because Na⁺ exclusion and tissue tolerance are mutually exclusive or, as discussed in Rajendran *et al.*, (2009), it could be due to the issues in the method that was used to estimate tissue tolerance component, and the dosage of NaCl applied in these study, which does not allow the Na⁺ excluding genotypes to accumulate enough Na⁺ in the leaf tissue to assess its tissue damage. Doing experiments in future with higher levels of salinity would be useful to know the reason for this issue.

2, Select suitable mapping populations to screen for the three major components of salinity tolerance by QTL mapping

In the Chapter 4, the Berkut × Krichauff DH mapping population was used to study the segregation of osmotic tolerance and Na⁺ exclusion. However, it was not suitable to study the segregation of tissue tolerance perhaps because the parents of this population use two different ionic tolerance mechanisms. Krichauff has been found to exclude Na⁺ and always has low Na⁺ concentrations in the leaf blade, whereas, Berkut is a tissue tolerant one that always stays green while accumulating high Na⁺ in the leaf tissue (Genc *et al.*, 2007). Accordingly, the progenies show a very high proportion of green shoot area which ranged from 0.83 to 1 when phenotyped using the LemnaTec system. This low variation in green shoot area when multiplied with the fourth leaf blade [Na⁺] meant that for the calculations used in this study, the concentration of Na⁺ in the shoot had a dominant effect on the final tissue tolerance value. An alternative approach would be to select for parents that show differences in one of the ionic tolerance component, rather than selecting parents which are either good excluders or good tissue tolerators (Chapter 4).

Moreover, in Chapter 5, because of difficulties doing replication with the F₂ mapping population, it was not possible to get information about the rates of the natural senescence within this population and hence the tissue tolerance assessment in

MDR 002 × MDR 043 F₂ mapping population. As, each plant is a unique individual in a F₂ mapping population, this makes designing experiments with replication impossible and prohibits growing a plant of a specific genotype in both control and salt stress conditions. Hence, doing replicated trials with DH, RILs, NILs or F₃ mapping population rather than screening F₂ mapping population would be allowing replicated trials and help to quantify tissue tolerance for QTL mapping in the future.

3, Study the genotype × environmental interaction of genotypes with diverse combinations of salinity tolerance over different environments or seasons

In this study, combination of salinity tolerance components was found to increase the shoot biomass of two different wheat species, *T. monococcum* and *T. aestivum* (Chapter 3). The combination of osmotic tolerance and Na⁺ exclusion was positively associated with the increase in shoot biomass of Berkut × Krichauff DH mapping lines grown in saline conditions (Chapter 4). In the future, it seems selection for salinity tolerance should be in favour of genotypes with combinations of osmotic tolerance and Na⁺ exclusion or with the combinations of osmotic tolerance and tissue tolerance to develop successful salinity tolerance cultivars in an efficient manner. But, at this stage, it is essential to study the genotype × environmental interaction of genotypes with diverse combinations of salinity tolerance components across different environment or seasons. Indeed, the knowledge about the suitability of genotypes with specific combination of salinity tolerance components either for a particular season or for wide range of climatic conditions is important for breeders (Finlay and Wilkinson, 1963). Sometimes, genotypes reflect differences in adaptation which could be exploited for specific environmental conditions (with favourable interactions) or wide adaptation (minimizing interactions) (Allard and Bradshaw, 1964). The knowledge of interaction between genotypes with diverse combination of salinity tolerance components over different seasons would be helpful to establish future breeding goals, identify experimental season, and formulate recommendations for future research and wheat farming.

4, Use gene pyramiding approach to develop salt tolerant cultivar for the near future

One of the usefulness of marker assisted selection would be pyramiding genes linked to all three major salinity tolerance components and developing a cultivar which is suitable to grow under different saline conditions. A gene pyramiding approach has been suggested by various researchers to develop salinity tolerance cultivars in major cereal crops, particularly in rice (Yeo *et al.*, 1990; Gregorio *et al.*, 2002; Lin *et al.*, 2004). It involves selection of physiological traits which can correlate well with the salinity tolerance and combine the alleles with similar effects from different loci (Xu, 2010). A gene pyramiding approach would be helpful to develop cultivars with more salinity tolerance and it can better survive than existing commercial cultivars under saline environments. However, pyramiding would be more efficient if we use QTL with large effects on phenotype (Kearsey and Farquhar, 1998), especially for the complex genetic traits like salinity tolerance. For instance, Lin *et al.*, (2004), pyramided QTL for seedling survival, shoot $[Na^+]$, shoot $[K^+]$ and Root $[Na^+]$, explaining a proportion of total phenotypic variation from 13.9 to 48% to achieve salinity tolerance in rice. Nevertheless, in the study reported here, QTL for osmotic tolerance and Na^+ exclusion explained only small amount of phenotypic variance in these populations and at this stage would not help gene pyramiding approach. However, identification of QTL with larger effects for osmotic tolerance and Na^+ exclusion in the same or different mapping population would be used for QTL pyramiding approach in future.

5, Screen roots for salinity tolerance studies

In this study, recovery in shoot growth was noticed 5-7 days after NaCl application in all the experiments. Sudden decrease in shoot growth rate and rapid recovery are common phenomenon of osmotic stress which is induced immediately after NaCl application (Munns, 2002). Similar to shoots, roots can also show rapid reductions in

growth rate immediately after the application of NaCl to the growth solution (Rodríguez *et al.*, 1997; Munns, 2002). This is also due to the osmotic effect of salt stress and similar effects can be observed with other osmoticum such as KCl and mannitol (Frensch and Hsiao, 1994; Munns, 2002). Generally, shoot growth is more affected than root growth by soil salinity and root growth can recover faster than shoot growth from osmotic shock (Hsiao and Xu, 2000), however, the recovery of root growth depends on the level of osmotic stress imposed on plants (Munns, 2002; Frensch and Hsiao, 1995). The ion specific effect of salt stress on root growth occur as salt induced Ca^{2+} deficiency and affect root elongation (Munns, 2002).

Very little work has been done so far, however, to study the effect of salt on plant root growth because it is hard to characterize *in vivo* plant root responses under stressed environments over time. Moreover, it is difficult to separate root from soil and cleaning of roots is a labour intensive process. A rhizotron system oriented with an imaging device is widely preferred by various researchers to study the response of wheat under stressed conditions (Andrén *et al.*, 1996; Pan *et al.*, 1998). Similarly, a rhizotron attached with digital and near infra-red spectroscopy could be useful to do characterize the changes in the biological processes of root growth, development, activity and longevity under salt stressed environments (Vamerali *et al.*, 2012; French *et al.*, 2012).

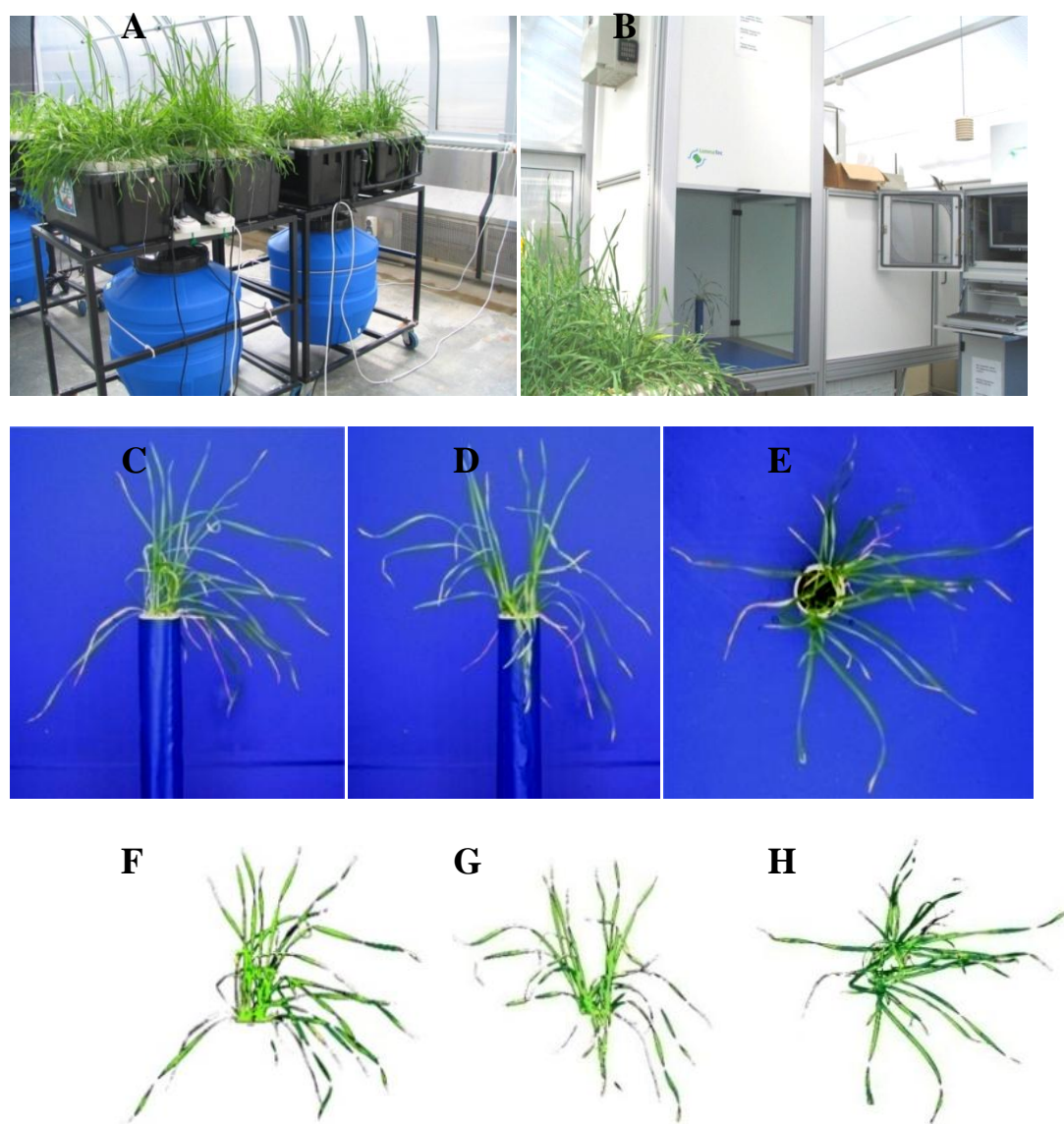
6.6 Major Conclusions

The major conclusions of this dissertation are as follows.

- 1) A new high throughput salt tolerance screening methodology developed in this study has the potential to screen the three major components of salinity tolerance in breeding materials of various cereal crops.
- 2) Different *T. monococcum* accessions use various combinations of the three components of salinity tolerance to increase their total salinity tolerance. Accessions with the combinations of osmotic tolerance and Na⁺ exclusion or with the combinations of osmotic tolerance and tissue tolerance have greater whole plant tolerance than the accessions with single salinity tolerance components.
- 3) Two new sources of genetic variability were found for osmotic tolerance and tissue tolerance in *T. monococcum* and it could be utilized for further breeding and QTL mapping.
- 4) Genetic control of variation for osmotic tolerance is probably very complex. A total of four QTL were identified for osmotic tolerance in Berkut × Krichauff DH mapping population of bread wheat on chromosomes 1D, 2D and 5B. Among them, 1D was identified to contribute large phenotypic variability for osmotic tolerance in two of three experiments. There were a total of eight QTL identified for Na⁺ exclusion in Berkut × Krichauff DH mapping population of bread wheat on chromosomes 2A, 2D, 5A, 5B, 6B and 7A. Both osmotic tolerance and Na⁺ exclusion QTL inherited favourable alleles from both parents, Berkut and Krichauff.
- 5) QTL inconsistencies were found for both osmotic tolerance and Na⁺ exclusion across the three different experimental time of the year. It necessitates re-estimation of QTL effect and validation of QTL positions either in the same or different mapping population.

APPENDIX

Appendix 1. Supporting information with tables and figures for Rajendran *et al.*,(2009), presented in Chapter 3.



Supplementary Figure 1. Non-destructive growth analysis techniques. A, Plants were grown in a supported hydroponics system in 25 L tanks above an 80 L storage reservoir. B, Plants were placed in a LemnaTec Scanalyzer for image acquisition. C-H Snapshots of original and false colour images of 31 d old MDR 043 accession in 75mM NaCl.

Supplementary Table I. *Two phase growth response of T. monococcum accessions.*

Mean relative plant growth rates are calculated between the d indicated, for both 0 mM and 75 mM NaCl conditions, and differences in growth rate ratios (G.R.) calculated (n = 7 to 10) . Plants grown in winter¹; plants grown in spring²

Accession		Mean relative growth rate in osmotic phase (d ⁻¹)				Mean relative growth rate in recovery phase (d ⁻¹)						
		0-3	0-4	0-5	0-7	0-11	0-12	0-13	0-14	0-15	0-16	0-19
AUS 18758 ²	Control	0.13	0.15	0.15			0.13		0.13		0.13	0.12
	Salt	0.08	0.08	0.09			0.09		0.09		0.09	0.09
	G.R.	0.62	0.53	0.60			0.69		0.69		0.69	0.75
AUS 90423 ¹	Control		0.05		0.08	0.12		0.13		0.14		0.13
	Salt		0.05		0.04	0.12		0.13		0.13		0.12
	G.R.		1.00		0.50	1.00		1.00		0.93		0.92
MDR 002 ¹	Control		0.07		0.08	0.13		0.14		0.15		0.15
	Salt		0.07		0.04	0.08		0.09		0.10		0.10
	G.R.		1.00		0.50	0.62		0.64		0.67		0.67
MDR 044-1 ¹	Control		0.08		0.14	0.11		0.13		0.14		0.13
	Salt		0.07		0.05	0.11		0.12		0.12		0.11
	G.R.		0.86		0.36	1.00		0.92		0.86		0.85

AUS 18763 ²	Control	0.16	0.15	0.13	0.13	0.13	0.12	0.12
	Salt	0.07	0.09	0.09	0.09	0.08	0.09	0.08
	G.R.	0.44	0.60	0.69	0.69	0.62	0.75	0.67
MDR 044-2 ¹	Control	0.13	0.14	0.14	0.13	0.12	0.12	0.11
	Salt	0.04	0.06	0.06	0.09	0.09	0.09	0.09
	G.R.	0.31	0.43	0.43	0.69	0.75	0.75	0.81
AUS 16273 ¹	Control		0.05	0.11	0.12	0.13	0.13	0.13
	Salt		0.03	0.04	0.11	0.12	0.13	0.12
	G.R.		0.60	0.36	0.92	0.92	1.00	0.92
MDR 037 ²	Control	0.14	0.14	0.13	0.12	0.12	0.12	0.11
	Salt	0.06	0.06	0.06	0.09	0.09	0.09	0.09
	G.R.	0.43	0.43	0.46	0.75	0.75	0.75	0.82
MDR 043 ²	Control	0.12	0.13	0.14	0.13	0.13	0.12	0.12
	Salt	0.06	0.10	0.11	0.10	0.10	0.10	0.10
	G.R.	0.50	0.77	0.79	0.77	0.77	0.83	0.83
AUS 90436 ¹	Control		0.08	0.06	0.12	0.14	0.14	0.14
	Salt		0.07	0.05	0.12	0.13	0.13	0.12
	G.R.		0.88	0.83	1.00	0.93	0.93	0.86

MDR 308 ¹	Control	0.09	0.10	0.13	0.14	0.15	0.14	
	Salt	0.08	0.06	0.12	0.12	0.13	0.12	
	G.R.	0.89	0.60	0.92	0.86	0.87	0.86	
AUS 18755-4 ²	Control	0.13	0.14	0.14	0.13	0.13	0.12	0.12
	Salt	0.09	0.11	0.11	0.10	0.10	0.10	0.10
	G.R.	0.69	0.79	0.79	0.77	0.77	0.83	0.83

Supplementary Table II. *Comparison of the two methods for generating an osmotic tolerance index.*

Mean relative growth rates were calculated for 0 mM and 75 mM grown plants, before and after salt application, as described in Fig 3A, to generate two osmotic tolerance indices for comparison.

Accessions	Osmotic tolerance	Osmotic tolerance
	$Z \div X$	$Z \div [(W+Y)/2]$
MDR 037	0.46	0.47
MDR 043	0.79	0.63
MDR 044-2	0.43	0.36
AUS 18758	0.60	0.62
AUS 18755-4	0.79	0.71
AUS 18763	0.69	0.64

Supplementary Table III. *The sources of accessions used in the study*

Accessions	Origin	Source
MDR 308	Italy	Hai-Chun Jing, Rothamsted Research, UK.
MDR 002	Balkan	
MDR 044	Turkey	
MDR 037	Armenia	
MDR 043	Greece	
AUS 16273	Unknown	Australian Winter Cereal Collection
AUS 90436	USA	
AUS 90423	USA	
AUS 18755-4	Unknown	
AUS 18758	Turkey	
AUS 18763	Turkey	

Appendix 2. Generation of F₃ progenies for future studies

The surviving plants were transferred to soil to collect F₃ seed for future experiments. The F₂ population was grown 3 weeks in NaCl for salt screening. After that, NaCl solution was removed and fresh ACPFG nutrient solution (see chapter 2 for protocol) was refilled in hydroponics tank. The plants were remained to be grown in nutrient solution for 5 days to remove NaCl debris in roots. Then plants were transferred from hydroponics tubes to pots filled with coco peat (2/3rd) and topped up with UC soil (1/3rd) for moisture conservation. Plants were watered thrice a week for 2 months then twice a week for another two months. *T. monococcum* is a winter wheat; require vernalisation for flowering in controlled environment. Vernalisation has not done for this population, because it was suspected to have co-segregation with Na⁺ exclusion QTL from our lab results. Hence, 50ppm of gibberellic acid (GA₃), sigma Aldrich, Germany was applied to all the plants twice a week at tillering stage using hand 2 litres garden sprayer. Selfing has been done to most of the plants to generate F₃ progenies.

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