**Improvement of salt and waterlogging tolerance in wheat: comparative
physiology of *Hordeum marinum*-*Triticum aestivum* amphiploids with
their *H. marinum* and wheat parents**

SAUD ALI ALAMRI

**This thesis is presented for the degree of
Doctor of Philosophy
of The University of Western Australia**

**School of Plant Biology
Faculty of Science
The University of Western Australia
May 2014**

ABSTRACT

Wheat (*Triticum aestivum* L.) is one of the most important cereal grains, yet is sensitive to the combined salinity and waterlogging that often occurs on saline land. One way to improve the growth of crops on saline land might be to introduce genes from relevant adapted wild germplasm. Sea barleygrass (*Hordeum marinum* Huds.), a wild relative of wheat, can withstand the combined effects of salinity and waterlogging and might therefore be used to improve the tolerance of wheat via wide hybridisations. This PhD project aimed to identify physiological traits contributing to salt and waterlogging tolerance in *H. marinum*-wheat amphiploids in comparison to the parents, and for some of these traits examine the expression patterns of two candidate genes (*HKT1;5*-like and *NHX1*-like transcripts) involved in Na⁺ (and for one also K⁺) transport.

The physiology of salt and waterlogging tolerance was evaluated in 8 accessions of *H. marinum*. Overall, two of the *H. marinum* accessions (H109 and WA9) were identified as being relatively tolerant of the combined salinity and stagnant treatments, whereas H546 was identified as being the most sensitive accession. With the combination of salinity (400 mM NaCl) and stagnant treatments the most tolerant accessions had higher RGRs (46–65% of control compared to 33% of control RGR for the sensitive accession), less accumulation of Na⁺ and Cl[−] in the shoots (concentrations 56% and 20% lower than the sensitive accession, for Na⁺ and Cl[−] respectively) and a lower proportion of dead leaves (5–14% of shoot DM, compared with 58% in the sensitive accession). In addition, the higher RGR of the tolerant accessions was associated with up to 52% higher K⁺ concentration in shoot, and therefore a 67% higher shoot K⁺/Na⁺ ratio than in the sensitive accession. Further studies on two of the *H. marinum* accessions suggested that decreased stomatal conductance was a major cause of the reduction in net photosynthesis of young leaves under saline or stagnant saline treatments.

Amphiploids of wheat and *H. marinum* showed increased tolerance to salinity and hypoxia because of the contribution of the *H. marinum* chromosomes. The amphiploids were more salt and waterlogging tolerant than wheat; this was associated with better leaf ion regulation and improved root aeration traits. Growth (RGR) in the stagnant saline treatment was better maintained in the amphiploids (58–71% of controls) than in wheat (56% of control), and leaf Na⁺ concentrations were 30–41% lower in the

amphiploids than in wheat. The porosity of adventitious roots increased with the stagnant treatments; values were 24–38% in *H. maritimum*, 16–27% in the amphiploids and 16% in wheat. A strong barrier to radial oxygen loss formed in basal root-zones under stagnant conditions in two *H. maritimum* accessions; this barrier was moderate in the amphiploids, absent in wheat, and was weaker with the stagnant saline treatment than in the non-saline stagnant treatment.

Expression (i.e. transcript abundances) of *HKT1;5*-like and *NHX1*-like genes was determined, as the products of these influence Na^+ - K^+ regulation, and in other studies have been associated with salt tolerance. The level of *HKT1;5*-like transcripts across treatments in all genotypes (wheat cv. Chinese Spring, *H. maritimum* (H21), their amphiploid) was higher in roots (~470-fold higher) than in leaf and sheaths. The similar abundance patterns of *HKT1;5*-like transcripts across the three genotypes, and together with the similar concentrations of K^+ in various tissues of the three genotypes suggests that K^+ homeostasis was similar in the genotypes. Under stagnant saline conditions, the level of *NHX1*-like transcripts in all tissues were higher in *H. maritimum* and the amphiploid (~3.6-fold compared with those in control) than wheat. Moreover, the Na^+ concentrations were higher in sheaths of *H. maritimum* (4.4-fold higher) and the amphiploid (38% higher) than leaf or roots. These two results suggest that *H. maritimum* and the amphiploid have a higher capacity to sequester Na^+ in the vacuoles of the sheath compared with wheat. The greater transcript levels of *NHX1*-like sequences in the amphiploid, appears to be contributed by the wild parent *H. maritimum*.

In conclusion, tolerant accessions of *H. maritimum* were able to better maintain their growth and Na^+ and Cl^- ‘exclusion’ from the leaves, and showed better maintenance of leaf K^+ concentrations, at high external NaCl and even when in an oxygen-deficient saline root-medium, than the less tolerant accessions. The presence of the chromosomes from the *H. maritimum* accessions in the amphiploids improved growth, root aeration and ionic balance in the shoots when in combined stagnant (hypoxic) and salinity stresses. This study adds to the knowledge of the combined effects of salinity and waterlogging on plants, and demonstrates the potential for transfer of some tolerance from *H. maritimum* into wheat.

ACKNOWLEDGMENTS

First and foremost, I thank my supervisors Tim Colmer, Ed Barrett-Lennard and Tash Teakle for their support and advice over the period of my candidature. Thank you for encouraging me and providing critical advice on my experiments during these last years and carefully editing this thesis.

Considering her inputs into this thesis, Dr. Ricarda Jost deserved to be my co-supervisor, not only in the direct supervision during the processes of molecular part of my PhD work (experiments in Chapter 6), but also helping in calculation issues and editing of Chapter 6. I am deeply indebted for all your help.

Special thanks to Dr. Islam (University of Adelaide) for kindly raising and providing seeds of the amphiploids for my experiments; as part of a GRDC funded project within the Future Farm Industries CRC. Thanks to R. von Bothmer (Swedish Agricultural University, Alnarp) for providing accessions of *H. marinum* from the Nordic Seed Bank; and K. A. Shepherd for collecting accessions in Western Australia. Thanks to Dr. J. Damien Platten (International Rice Research Institute, Los Baños, Philippines) for kindly providing the sequence information on the *Hordeum marinum* *HKT* gene.

Thank you all the past and present members of Tim Colmer's lab and the molecular lab in Plant Biology for assisting me to learn the new equipment and techniques, and for making the two labs enjoyable places to work.

I am grateful to King Saud University for providing my PhD Scholarship. I would also like to thank Western Australian State Centre of Excellence in Ecohydrology and the School of Plant Biology for providing the project funds that made this project possible. Additionally, I acknowledge the facilities provided for this research by the School of Plant Biology, UWA.

Finally, and most importantly, I dedicate this thesis to all of my family members, as they have given me their unwavering encouragement and support over my many years of study, and without their endless love, support and encouragement, it would not have been possible for me to finish my PhD.

TABLE OF CONTENTS

Contents

<u>ABSTRACT</u>	iii
<u>ACKNOWLEDGMENTS</u>	v
<u>TABLE OF CONTENTS</u>	vi
<u>THESIS DECLARATION</u>	ix
<u>CHAPTER 1: GENERAL INTRODUCTION</u>	1
<u>CHAPTER 2: REVIEW OF LITERATURE</u>	5
<u>2.1 INTRODUCTION</u>	6
<u>2.2 PLANT RESPONSES TO SALINITY</u>	7
<u>2.2.1 Salinity impacts on plants</u>	7
<u>2.2.2 Physiological mechanisms associated with salinity tolerance</u>	8
<u>2.2.3 Regulation of ion transport under salinity</u>	11
<u>2.3 PLANT RESPONSES TO WATERLOGGING</u>	14
<u>2.3.1 Impact of waterlogging on plants</u>	14
<u>2.3.2 Mechanisms of tolerance to waterlogging</u>	14
<u>2.4 THE EFFECT OF THE INTERACTION BETWEEN SALINITY AND WATERLOGGING ON PLANTS</u>	16
<u>2.5 TOLERANCE OF WHEAT AND <i>HORDEUM MARINUM</i> TO SALINITY AND WATERLOGGING</u>	17
<u>2.5.1 Tolerance to individual stresses of salinity and waterlogging</u>	17
<u>2.5.2 Tolerance to the interaction of salinity and waterlogging</u>	22
<u>2.6 CONCLUSION</u>	23

TABLE OF CONTENTS

Contents

CHAPTER 3: Variation in salt and waterlogging tolerances amongst eight *Hordeum*

<u><i>marinum</i> accessions</u>	25
<u>3.1 ABSTRACT</u>	26
<u>3.2 INTRODUCTION</u>	26
<u>3.3 MATERIALS AND METHODS</u>	28
<u>3.4 RESULTS</u>	32
<u>3.5 DISCUSSION</u>	45
<u>3.6 SUPPLEMENTARY MATERIALS</u>	51

CHAPTER 4: An evaluation of the growth, photosynthetic rate, stomatal conductance and leaf damage of two *Hordeum marinum* accessions with NaCl and stagnant treatments

<u>4.1 ABSTRACT</u>	56
<u>4.2 INTRODUCTION</u>	56
<u>4.3 MATERIALS AND METHODS</u>	58
<u>4.4 RESULTS</u>	62
<u>4.5 DISCUSSION</u>	76
<u>4.6 SUPPLEMENTARY MATERIALS</u>	82

CHAPTER 5: Improvement of salt and waterlogging tolerance in wheat: comparative physiology of *Hordeum marinum*-*Triticum aestivum* amphiploids with their *H. marinum*

<u>and wheat parents</u>	89
<u>5.1 ABSTRACT</u>	90
<u>5.2 INTRODUCTION</u>	90
<u>5.3 MATERIALS AND METHODS</u>	92
<u>5.4 RESULTS</u>	96
<u>5.5 DISCUSSION</u>	106
<u>5.6 SUPPLEMENTARY MATERIALS</u>	111

TABLE OF CONTENTS

Contents

<u>CHAPTER 6: <i>Hordeum marinum</i> (H21)-<i>Triticum aestivum</i> amphiploid tolerates combined salinity and waterlogging: Na⁺ concentrations and levels of <i>HKT1;5</i>-like and <i>NHX1</i>-like transcripts in tissues of the amphiploid and its parents.....</u>	115
6.1 ABSTRACT	116
6.2 INTRODUCTION	117
6.3 MATERIALS AND METHODS	119
6.4 RESULTS	125
6.5 DISCUSSION	137
6.6 SUPPLEMENTARY MATERIALS	142
 <u>CHAPTER 7: GENERAL DISCUSSION</u>	153
 <u>LITERATURE CITED</u>	163

The work carried out in this thesis is my own work; to the best of my knowledge all sources have been acknowledged. The thesis was completed during the course of my enrolment in a PhD degree at UWA and has not been previously accepted for a degree at this or any other institution.

One section of this thesis (Chapter 5 – bibliographical details listed below) has been published elsewhere. All experimental work and writing was conducted by S. Alamri under the supervision of TD Colmer, EG Barrett-Lennard and NL Teakle. For Chapter 6 permission was obtained from all contributing authors to include the work in this thesis.

Saud Alamri

Coordinating supervisor

Journal Publication:

Alamri SA, Barrett-Lennard EG, Teakle NL, Colmer TD. (2013) Improvement of salt and waterlogging tolerance in wheat: comparative physiology of *Hordeum marinum*-*Triticum aestivum* amphiploids with their *H. marinum* and wheat parents. *Functional Plant Biology*, **40**, 1168–1178.

Conference Abstract:

Alamri SA, Barrett-Lennard EG, Teakle NL, Colmer TD. Improving salt and waterlogging tolerance in wheat (*Triticum aestivum*) using the wild relative *Hordeum marinum*. Rhizosphere 3 Conference, Burswood Convention Centre, Perth, Western Australia, 25–30 September 2011.

CHAPTER 1
GENERAL INTRODUCTION

Ever-increasing salinisation is occurring over large parts of the world's arable land. Salinity means the presence of high concentration ($EC_e = 4$ dS/m or more) of soluble salts in the soil and water (Richards 1954). Salinity affects more than 900 Mha of arable land (OECD/FAO 2012) and decreased crop yields occur in many areas (Greenway and Munns 1980; Szabolcs 1994). In Australia, 5.7 Mha of cultivated land is affected by secondary (human induced) salinity (NLWRA 2001), caused by the removal of native vegetation for dryland or irrigated agriculture, which leads to the rise of water-tables and brings dissolved salts into the root-zone (Barrett-Lennard *et al.* 2003; Pannell and Ewing 2006). By the year 2050, the area in Australia affected by secondary salinity could rise to 17 Mha (NLWRA 2001).

Salinity is often accompanied by waterlogging – causing a very hostile environment for roots of most plants. Waterlogging decreases the availability of O_2 in the root-zone (Armstrong 1979). Excess soil water diminishes the rate of gas movement into soils resulting in low O_2 concentrations (hypoxia) and the excess water can result from over-irrigation, presence of a permanent or temporary high water-table associated with high seasonal rainfall, poor soil structure impeding drainage, and/or flooding (Smedema and Shiati 2002; Rengasamy *et al.* 2003; Rengasamy 2006). The interaction between salinity and waterlogging can typically have adverse impacts on crops (Barrett-Lennard 2003) as very few plants can tolerate the combination of salinity and waterlogging. Tolerance to both salinity and waterlogging is important for crops such as wheat in areas affected by salinity caused by rising water-tables (Colmer *et al.* 2005b).

Wheat (*Triticum aestivum* L.) is one of the most important cereal grains, with an annual world production of over 657.5 million tonnes of wheat grain in 2012 (OECD/FAO 2012). 'Wild', or undomesticated, species from within the same tribe (Triticeae) as wheat are potential sources of genes for abiotic stress tolerance that may be introduced into wheat to substantially improve its stress tolerance (Omielan *et al.* 1991; Taeb *et al.* 1993; Colmer *et al.* 1995; Colmer *et al.* 2006b). Sea barleygrass (*Hordeum marinum* Huds.) is a wild relative of wheat which grows in salt marshes (von Bothmer *et al.* 1995) and has possibly the highest tolerance to salinity and waterlogging within the Triticeae (Colmer *et al.* 2006b). The salt tolerance of *H. marinum* is attributed to a combination of: (i) a high ability to 'exclude' (i.e. regulate entry of) Na^+ and Cl^- from the shoot; and (ii) a better maintenance of tissue K^+ concentrations (Garthwaite *et al.*

2005). The waterlogging tolerance of *H. marinum* is associated with a combination of root aeration characters, including the formation of aerenchyma and a barrier to radial O₂ loss (ROL) in adventitious roots (McDonald *et al.* 2001a; Garthwaite *et al.* 2003; Malik *et al.* 2011). Therefore, *H. marinum* might be used in wide hybridisations to improve the salt and waterlogging tolerance of wheat (Colmer *et al.* 2006b; Islam *et al.* 2007).

An amphiploid is a synthetic hybrid containing all the chromosomes (and therefore genes) from two plant species. Amphiploids of two wild salt-tolerant species (i.e. tall wheatgrasses and sea barleygrass) with wheat had been made in an attempt to improve salt tolerance in wheat (Colmer *et al.* 2006b). *H. marinum*–wheat amphiploids have been produced by AKMR Islam and some of these have been studied to assess their tolerances to salt (Islam *et al.* 2007; Munns *et al.* 2011) and waterlogging (Malik *et al.* 2011). The amphiploids express salt tolerance, or at least key traits associated with tolerance, from *H. marinum* (Islam *et al.* 2007; Munns *et al.* 2011). In terms of waterlogging tolerance, the amphiploids were found in some cases to form a ‘partial’ barrier to ROL in adventitious roots and possess higher root porosity (Malik *et al.* 2011). *H. marinum*–wheat amphiploids were also reported to maintain higher leaf K⁺/Na⁺ ratio when subjected to combination of salinity and hypoxia, compared with their wheat parents (Munns *et al.* 2011). Despite some previous research (described above) aimed at evaluating the responses to salinity and waterlogging stresses of *H. marinum*–wheat amphiploids and their parents, understanding of the physiological mechanisms of salt and waterlogging tolerance of *H. marinum*–wheat amphiploids, in contrast with their parents, is still very limited. Furthermore, knowledge of the underlying genes responsible for salt or waterlogging tolerance of *H. marinum*–wheat amphiploids and the *H. marinum* parents is lacking.

This thesis tested the hypothesis that amphiploids derived from *H. marinum* parents well-adapted to salinity and waterlogging would also be tolerant to these stresses. By comparing the responses to salinity and waterlogging of *H. marinum*–wheat amphiploids with their parents, the aim was to identify physiological traits and in the case of regulation of leaf ion concentrations assess expression of two possible underlying candidate genes contributing to the phenotype of higher leaf K⁺/Na⁺ ratio and potentially also to salt tolerance.

More specifically, the thesis aims were to:

1. Review the literature to summarise physiological traits in *H. maritimum*, wheat and their amphiploids that contribute to the tolerance of salinity and/or waterlogging stress (Chapter 2).
2. Determine how growth, ion relations, root aeration, stomatal conductance and leaf photosynthesis are affected when eight accessions of *H. maritimum* were subjected to a range of salinity and waterlogging treatments (Chapters 3 and 4).
3. Identify the extent that physiological traits associated with salt and waterlogging tolerance in *H. maritimum* are also expressed in *H. maritimum*–wheat amphiploids, the four *H. maritimum* accessions (i.e. H21, H87, H109 and H155), all hybridised individually into a common wheat genetic background (cv. Chinese Spring) (Chapter 5).
4. Determine the effect of combined salinity and waterlogging stress on the expression of two candidate genes (namely: *HKT1;5*-like and *NHX1*-like) for salt Na^+ and K^+ transport of *H. maritimum*–wheat amphiploid (i.e. H21-CS amphiploid) in comparison with its parents (Chapter 6).

This thesis presents new information concerning the interactive effects of salinity and root-zone hypoxia on *H. maritimum*–wheat amphiploids and the involvement of key traits associated with salinity and waterlogging tolerance, in comparisons with their parents. Identification of underlying candidate genes contributing to salinity tolerance (i.e. *HKT* and *NHX* genes) provides important information on the relationships between physiological mechanisms and genetic regulation for salinity tolerance. Furthermore, the identification of a salt and waterlogging-tolerant accession of *H. maritimum* and its amphiploid with wheat provides key information for future development of wheat for saline land, and for improved knowledge on salt and waterlogging tolerance in wheat and *H. maritimum*, and as expressed in the *H. maritimum*-wheat amphiploids.

CHAPTER 2
REVIEW OF LITERATURE

2.1 INTRODUCTION

Salinity threatens the cultivation of wheat in some areas (Szabolcs 1994; Dunne *et al.* 2002), reducing plant growth and crop productivity in different parts of the world (Flowers and Yeo 1995; Richards 1995; Munns *et al.* 2006). In addition to the direct problems caused by high salt concentrations in the soil, many areas of saline land are also prone to waterlogging (Barrett-Lennard *et al.* 1999; Barrett-Lennard 2003). These problems also occur in some areas of Western Australia (Malcolm 1980). Waterlogging decreases the availability of O₂ (Armstrong 1979), increases the accumulation of CO₂ to potentially toxic levels in the root-zone (Greenway *et al.* 2006) and also increases concentrations of ethylene, a hormone in plants (Shiono *et al.* 2008). As a consequence of these changes, waterlogging decreases root growth and affects the viability of roots (Huck 1970; Webb and Armstrong 1983; Barrett-Lennard 1986; Bennett *et al.* 2009). The interaction between salinity and waterlogging has an adverse impact on growth and productivity for most crops (Bernstein and Hayward 1958; Aceves-N *et al.* 1975; Kriedemann and Sands 1984; Gorham *et al.* 1985; Barrett-Lennard 1986; McDonald *et al.* 2001a; Barrett-Lennard 2003; Setter and Waters 2003; Colmer *et al.* 2005b) due to the large increase in Na⁺ and Cl⁻ transport to the shoot, in comparison with salinity alone (Galloway and Davidson 1993; Rogers and West 1993; Barrett-Lennard *et al.* 1999; Barrett-Lennard 2003).

Wheat is one of the world's major cereals. Although wheat can tolerate moderate salinity (Maas 1986), it is relatively sensitive to waterlogging (Setter and Waters 2003). Furthermore, the growth of wheat is severely reduced when salinity is combined with waterlogging (Barrett-Lennard *et al.* 1999; Barrett-Lennard 2003). Therefore, tolerance to both stresses is important for crops such as wheat in areas affected by salinity and waterlogging (Colmer *et al.* 2005b). *Hordeum marinum*, a 'wild relative' of wheat, can withstand the interactive effects of salinity and waterlogging (Malik *et al.* 2009a,b) and might therefore be used to improve the salt and waterlogging tolerance of wheat via wide hybridisations (Colmer *et al.* 2006b; Islam *et al.* 2007; Malik *et al.* 2011).

Evaluating the physiological and genetic basis of tolerance to salinity and waterlogging (separately and simultaneously) is critical to understanding the interaction between these complex traits and the adaptations that plants need to withstand the stresses. This review aims to identify possible physiological or molecular mechanisms of tolerance to

combined salinity and waterlogging stress. It focuses on mechanisms of salinity and waterlogging tolerance and identifies key physiological and molecular traits that could be transferred from wild *H. maritimum* parents into wheat-*H. maritimum* amphiploids for their future use on saline land.

2.2 PLANT RESPONSES TO SALINITY

2.2.1 Salinity impacts on plants

Salinity stress causes a rapid decline in growth for sensitive plants (Flowers and Yeo 1995; Richards 1995; Munns *et al.* 2006). Reductions in growth due to salinity occur in two main phases: (1) decrease in soil water potential caused by a rapid osmotic effect, restricting the availability of water to the plants; and (2) longer-term ion toxicity effects caused by the accumulation of Na^+ and/or Cl^- in the shoot tissues (cf. Munns *et al.* 1995). In addition, growth may also be affected by lower K^+ concentrations in leaves (Greenway and Munns 1980; James *et al.* 2006; Barrett-Lennard and Shabala 2013).

Leaf injury from salinity (necrosis) typically occurs after weeks or longer from the accumulation of ions to toxic levels (Munns and Tester 2008). Damage to leaves is generally first observed in the older leaves, which have had the longest exposure to Na^+ and Cl^- in the xylem stream (Colmer *et al.* 1995). At the cellular level, the toxic effects of Na^+ and Cl^- entering the leaf tissues are minimised by compartmentalising these ions into vacuoles (Munns 2002; Cuin *et al.* 2003; Munns and James 2003; Tester and Davenport 2003; Colmer *et al.* 2006b; Munns and Tester 2008). This sequestering of Na^+ and Cl^- in vacuoles protects sensitive enzymes located in the cytoplasm, while providing intracellular solutes that can assist with the maintenance of turgor (Greenway and Munns 1980). Complementing this, organic solutes accumulate in the cytosol to osmotically ‘balance’ this component of the cell (Flowers *et al.* 1977). However, after exceeding a threshold concentration in the vacuole, toxic ions start to accumulate in the cytoplasm or the cell walls (Munns 2002). Accumulating ions in the cytoplasm can inhibit enzymes involved in carbohydrate metabolism, while ion accumulation in the water of the cell walls can decrease cell turgor (Greenway and Munns 1980). Disrupting metabolism reduces available ATP to transport ions across cell membranes, resulting in poor Na^+ ‘exclusion’ from the roots and a decline in K^+ uptake (Munns and Tester 2008). As a consequence, adverse water relations, ion toxicity and mineral deficiency

can occur, contributing to lower rates of growth in plants exposed to high levels of salt. It is clear therefore, that ionic balance is an important mechanism of salt tolerance in plants. The major cause of ionic imbalance due to combined salinity and waterlogging is the increase in Na^+ and Cl^- transport to the shoot (Barrett-Lennard 2003), which will be an important focus in this review.

Salinity has complex effects on photosynthesis, as photosynthesis in salt-stressed plants is limited by stomatal and by non-stomatal factors (Rahnama *et al.* 2010). Stomatal conductance is a sensitive indicator of osmotic stress, as stomata actively close as an initial response to salt stress (James *et al.* 2008). Stomatal factors limit CO_2 absorption of salt-stressed plants, resulting in limited plant growth and photosynthesis (Rahnama *et al.* 2010). For example, in *Atriplex portulacoides* grown with 700 mM NaCl, initial decreases in growth rate (RGR reduced to 25% of control) were associated with decreases in stomatal conductance (40% of control) and hence photosynthesis (reduced by 47% of control; Redondo-Gómez *et al.* 2007). As the salt builds up in the leaves over time, photosynthesis becomes increasingly limited by non-stomatal factors (James *et al.* 2002). High concentrations of Na^+ and Cl^- in leaves is the main cause for the non-stomatal limitation of photosynthesis, i.e. the toxic ions in the leaves begin to degrade the enzymatic processes involved in photosynthesis (Huang and van Steveninck 1989). Monitoring stomatal conductance in salt-stressed plants has been reported to be one of the most sensitive indicators of salinity tolerance for durum wheat (James *et al.* 2002) and sorghum (Netondo *et al.* 2004). However, the combination of salinity and waterlogging is expected to decrease stomatal conductance and photosynthesis, in comparison to salinity alone (Barrett-Lennard 2003). Therefore, measuring stomatal conductance in salt-stressed plants could explain the relationship between stomatal conductance and growth rates under combined salinity and waterlogging (James *et al.* 2008; Rahnama *et al.* 2010).

2.2.2 Physiological mechanisms associated with salinity tolerance

Plants can be divided into three general salinity rating categories: halophytes (plants which grow at high salt concentrations), salt-resistant non-halophytes (plants which can tolerate or grow at moderate salt concentrations), and salt-sensitive non-halophytes (plants which are sensitive to even low concentrations of salt) (Greenway and Munns 1980; Qureshi and Barrett-Lennard 1998). Thus, considerable diversity exists in plant

salinity tolerance. The physiological mechanisms associated with salt resistance involve: tolerance to osmotic stress, Na^+ and Cl^- 'exclusion' from leaf blades, and tissue tolerance (Munns and Tester 2008). These mechanisms will be discussed in the context of the salt tolerance of cereals.

Osmotic stress is caused by the high concentrations of Na^+ and Cl^- in the soil solution reducing the availability of water to roots (Munns *et al.* 1995). Plants restore their osmotic balance by taking up ions and producing organic solutes to osmotically balance the water potential in the cytosol and vacuole (Greenway and Munns 1980). The synthesis of compatible solutes consumes significant amounts of assimilated carbon and energy, and therefore involves a potential growth reduction (Munns and Tester 2008). However, the use of ions for osmotic adjustment has a far lower energy cost. It has been calculated that the accumulation of 1 mole of NaCl as an osmoticum would require approximately 7 moles of ATP, whereas synthesis of 1 mole of a compatible organic solute would require between 30 and 80 moles of ATP (Raven 1985). Thus plants that cannot use ions for osmotic adjustment may have decreases in growth because of the need (or their inability) to produce large amounts of organic solutes (Munns and Tester 2008). Non-halophytes and monocotyledonous halophytes rely on the production of organic solutes to osmoregulate (Greenway and Munns 1980); this is the method employed by *H. maritimum* (Islam *et al.* 2007). On the other hand, dicotyledonous halophytes rely on the accumulation of Na^+ and Cl^- in vacuoles mostly for their osmoregulation, and where salt is in excess, the excretion of ions by leaf glands or into bladder cells (Thomson *et al.* 1988).

The ability to minimise the rate of Na^+ and Cl^- transport to the shoots is termed "ion exclusion" (Greenway 1968; Greenway and Munns 1980; Munns 2002; Munns and James 2003; Tester and Davenport 2003). Ion exclusion involves restricting the entry of Na^+ and Cl^- into the roots and subsequently the shoots, and therefore minimises the toxic effects of these ions on plant growth (Munns 2002). Halophytes and salt resistant non-halophytes typically have the ability to maintain ion exclusion at higher salinities (Munns and Tester 2008). These plants can restrict the influx of Na^+ and Cl^- into roots and then the shoots, by regulating symplastic transport through channels and transporters or by preventing the apoplastic ion flow (Blumwald *et al.* 2000). Restricting the rates of entry of Na^+ and Cl^- into roots is considered the first defence

mechanism against salinity (Tester and Davenport 2003; Møller and Tester 2007; Munns and Tester 2008). However, the ability to exclude Na^+ and Cl^- becomes impaired after certain thresholds are exceeded. For example, *H. maritimum* has an effective capacity to exclude most Na^+ and Cl^- until NaCl concentrations exceed about 450 mM (Garthwaite *et al.* 2005), while the exclusion threshold for wheat is ~200 mM (Colmer *et al.* 2005b).

The ability to preferentially accumulate K^+ over Na^+ is termed “ K^+/Na^+ discrimination” (Gorham *et al.* 1991; Gorham 1993). Several studies have shown that having a high K^+/Na^+ ratio is a salt tolerance criterion (Gorham *et al.* 1990; Schachtman *et al.* 1991; Wolf *et al.* 1991; Yeo 1998; Asch *et al.* 2000; Yao *et al.* 2010). For durum wheat and bread wheat, maintaining a high ratio of K^+/Na^+ is more important for salt tolerance than only maintaining a low concentration of Na^+ (Cuin *et al.* 2009). Under high salt stress, the cytosolic K^+/Na^+ can drop significantly as a result of intense Na^+ accumulation in the cytosol and K^+ leakage from the cells (Maathuis and Amtmann 1999; Shabala 2000; Zhu 2000; Leigh 2001; Shabala *et al.* 2003). This loss of K^+ from cells can cause a NaCl-induced membrane depolarisation (Cakirlar and Bowling 1981; Zhu 2000; Leigh 2001; Shabala *et al.* 2003; 2006; Chen *et al.* 2005; Cuin and Shabala 2005) which activates the depolarisation-activated outward rectifying K^+ channels (Shabala *et al.* 2006). The ability to maintain cytosolic K^+ homeostasis can correlate with salt tolerance (Chen *et al.* 2005; Chen *et al.* 2007a,b; Shabala and Cuin 2008; Smethurst *et al.* 2008; Barrett-Lennard and Shabala 2013); therefore, the NaCl-induced efflux of K^+ from the roots is suggested to be a reliable screening indicator for salinity tolerance in barley (Chen *et al.* 2005; 2007) and wheat (Chen *et al.* 2008).

Some salt tolerant plants can accumulate Na^+ and Cl^- in their shoots, presumably in vacuoles of older leaves, thereby improving their osmotic adjustment; this ability is termed “tissue tolerance” (Yeo and Flowers 1986). Tissue tolerance is the ability to compartmentalise Na^+ at the cellular and intracellular level preventing it from building up in the cytosol (Greenway and Munns 1980) – especially in leaf mesophyll cells (Munns and Tester 2008). Tissue tolerance is considered the second defence mechanism to salinity; however, the ability to compartmentalise Na^+ becomes impaired after the capacity of the vacuoles to compartmentalise salt is reached and then toxicity can occur. Tissue tolerance, however, is difficult to measure directly as there are several factors to

be assessed to quantify tissue tolerance; namely Na^+ concentrations in the vacuoles and the cytoplasm, leaf injury and the expression of NHX transporters (Yeo and Flowers 1983) (characterisation of the *NHX* gene is illustrated in Figure 2.1). In addition, Na^+ concentration in dead leaves might indicate tissue tolerance (Munns and Tester 2008), as some plants can accumulate Na^+ in old leaves to reduce the build-up in photosynthetically active leaves and support continued growth (cf. Yeo and Flowers 1983; Munns *et al.* 2003). Therefore, an approach to the estimate tissue tolerance needs to account for all these factors mentioned above. Tissue tolerance is an adaptive mechanism exemplified by most halophytes (Flowers *et al.* 1986) and some moderately salt-tolerant non-halophytes such as barley, which can accumulate high concentrations of Na^+ in leaf blades (James *et al.* 2006).

2.2.3 Regulation of ion transport under salinity

The regulation of ions (mainly Na^+ , Cl^- , and K^+) is an important mechanism to maintain tolerable concentrations of ions in plant tissues (Watson *et al.* 2001). Thus, salt tolerance of non-halophytes and monocotyledonous halophytes depends on a number of traits, including Na^+ and Cl^- exclusion from sensitive sites, accumulation of Na^+ and Cl^- in the vacuoles of the cells and K^+/Na^+ discrimination (Flowers *et al.* 1977; Greenway and Munns 1980; Munns *et al.* 1995; Serrano 1996; Asch *et al.* 2000; Colmer *et al.* 2005b; Houshmand *et al.* 2005; Munns and Tester 2008). The combination of salinity and waterlogging has adverse impacts on ion uptake and transport, with anoxic conditions often causing impaired ion transport function (Colmer and Greenway 2011). This section focuses on the regulation of Na^+ , Cl^- and K^+ movement in cells. Mechanisms for ion transport into cells are summarised in Figure 2.1.

Under saline conditions, Na^+ enters roots mainly via non-selective cation channels ‘uniporters’ (Davenport and Tester 2000; Demidchik and Tester 2002; Demidchik *et al.* 2002) or via high affinity K^+ ‘symporters’ (Mäser *et al.* 2002). Na^+ can be actively pumped back out again from the roots via Na^+/H^+ antiporters, driven by the electrochemical H^+ difference across the plasmalemma (Tester and Davenport 2003). The net uptake of Na^+ into roots depends on rates of influx and efflux from roots. Inside the cell, Na^+ is segregated into the vacuole by Na^+/H^+ antiporters, driven by the electrochemical H^+ difference across the tonoplast. Cl^- transport processes includes active Cl^- influx into root cells via $2\text{H}^+/\text{Cl}^-$ symporters and the passive influx of Cl^- into

roots via anion channels (White and Broadley 2001). In vacuoles, Cl^- can accumulate through a Cl^-/H^+ antiport across the tonoplast, and also via anion channels driven by the electrochemical H^+ difference (White and Broadley 2001).

Several genes are likely to be involved in ion transport mechanisms associated with salt tolerance. *HKT* (High-affinity K^+ Transporter) genes may play a key role in controlling Na^+/K^+ transport and the regulation of Na^+ homeostasis (Rodriguez-Navarro and Rubio 2006; Munns and Tester 2008). On the basis of amino acid sequence similarity and the differences in Na^+ and K^+ selectivity, *HKT* genes have been classified into two broad subfamilies (Horie *et al.* 2001; Maser *et al.* 2002; Garciadeblás *et al.* 2003; Platten *et al.* 2006). All members of *HKT* subfamily 1 are Na^+ -specific transporters, and some of them are expressed in the stele instead of the root cortex. These regulate Na^+ transport from root to shoot by excluding and/or retrieval of Na^+ from the xylem to prevent flux to the shoot (Huang *et al.* 2008). The presence of *HKT* genes has also been shown to enhance the removal of Na^+ from the xylem into the cells of the leaf sheath, preventing Na^+ entry into the leaf blade, and has been shown to enhance K^+ accumulation in leaf blades and sheaths (James *et al.* 2006). Recently, the incorporation of one gene from the *HKT* family (*TmHKT1;5-A* within the *Nax2* locus) into durum wheat has been reported to increase its capacity to exclude Na^+ from leaves, and therefore reduce leaf Na^+ to 27% of durum wheat without the *Nax2* locus (Munns *et al.* 2012); this gene has also been reported to enhance discrimination of K^+ over Na^+ (Benderradji *et al.* 2011). *Nax1* was reported to remove Na^+ from xylem in of roots and the leaf sheaths, while *Nax2* removes Na^+ from the root xylem only (James *et al.* 2006). *Nax2* was shown to be homologous to *Kna1* in *Triticum aestivum*, namely *TaHKT1;5-D* (Byrt *et al.* 2007). The *Kna1* locus on the long arm of chromosome 4D contains a candidate gene for K^+/Na^+ discrimination (James *et al.* 2011). Despite the roles that *HKT1;5* transporter may play in salt tolerance of wheat by regulating Na^+ and K^+ homeostasis, the exact mechanisms of action of these genes are not yet fully understood (Munns and Tester 2008).

The compartmentalisation of Na^+ into vacuoles is essential for plants to prevent toxic Na^+ concentrations in the cytoplasm. The *NHX* (vacuolar Na^+/H^+ antiporter) genes compartmentalise Na^+ into vacuoles and catalyse the exchange of Na^+ for H^+ across the tonoplast (Nass *et al.* 1997; Blumwald *et al.* 2000; Hasegawa *et al.* 2000; Aharon *et al.* 2003; Pardo *et al.* 2006). *NHX* can catalyse the movement of both K^+ and Na^+ , and

transport them into the vacuoles (Ohnishi *et al.* 2005). In addition, it is reported that the NHX-like protein, SOS1, is expressed at the ectoplast where it contributes to cytosolic Na^+ exclusion (Zhu 2002). The over-expression of the *NHX* genes was found to improve tolerance to salt stress in transgenic *Arabidopsis* (Apse *et al.* 1999), tomato (Zhang and Blumwald 2001), *Brassica napus* (Zhang *et al.* 2001), rice (Fukuda *et al.* 2004) and wheat (Xue *et al.* 2004). Low O_2 in roots can disturb the function of NHX transporters resulting from an ATP deficit, and hence affecting the trans-tonoplast H^+ difference and thus accumulation of Na^+ into vacuoles under high salt concentrations (Teakle *et al.* 2010). Despite the role that *NHX1* genes may play in salt tolerance by accumulating Na^+ into vacuoles under salt stress (Fukuda *et al.* 2004), very little research has been done on the combined effect of salinity and waterlogging stresses on *NHX* genes (Teakle *et al.* 2010).

Fig. 2.1. The ion (Na^+ , K^+ and Cl^-) transporters and channels in cells of higher plants which have been characterised as being involved in salinity tolerance. All proteins (Na^+ , K^+ and Cl^- transporters and channels) have been localised in one cell to illustrate their putative functions and the membrane on which they are believed to be located. Na^+ influx into cells across the plasma membrane is regulated largely by HKT transporters and non-selective cation channels, with efflux via SOS1 the Na^+/H^+ antiporter. NHX, a tonoplast Na^+/H^+ antiporter, is responsible for mediating Na^+ and K^+ in the vacuole. K^+ is also mediated by HAK and AKT symporters localised in the plasma membrane and other symporters of the KT/HAK/KUP family in the tonoplast. $\text{Cl}^-/2\text{H}^+$ symporters mediate Cl^- influx across the plasma membrane while H^+/Cl^- antiporters on the tonoplast accumulate Cl^- into the vacuoles. Anion channels with the direction depending upon the electrochemical difference are also responsible for Cl^- transport across tonoplast and plasma membrane (Amtmann and Sanders 1998; White and Broadley 2001; Colmer *et al.* 2005a; Rodríguez-Navarro and Rubio 2006; Plett and Møller 2010).

Studies on the expression of genes likely to be involved in ion transport mechanisms associated with salt tolerance such as *HKT1;5* and *NHX1* under combined salinity and waterlogging treatments would provide information on the role of each gene in salinity tolerance. Despite the roles that *HKT1;5* and *NHX1* transporters play in salt tolerance by regulating Na^+ and K^+ , the regulation of these transporters in response to different stimuli or their expression in various tissues needs to be explored further to confirm the function of these genes in ion regulation, and their role in salinity tolerance (Maathuis 2007; Munns and Tester 2008). At present, our understanding of the significance of *HKT1;5*-like and *NHX1*-like transporters in the salt tolerance of wheat is limited, and for *H. marinum* is completely lacking. Comparing expression of these transporters in various tissues of wheat, *H. marinum* and their amphiploids could improve the understanding of the genetic basis of salt tolerance.

2.3 PLANT RESPONSES TO WATERLOGGING

2.3.1 Impact of waterlogging on plants

The low concentrations of O_2 (hypoxia) associated with soil waterlogging can be a major stress to some plant species (Grable 1966; Ghassemi *et al.* 1995; Qureshi and Barrett-Lennard 1998). Plants face a range of challenges when the soil becomes saturated with water, owing to a series of physiochemical and biological changes in the soil (Armstrong 1975; Ponnampereuma 1984; Barrett-Lennard 1986; 2003). Low O_2 concentration in soil can increase ethylene accumulation, as well as the products of anaerobic metabolism (e.g. lactate, acetate, formate, ethanol, and carbon dioxide) that are produced by roots and micro-organisms (Romick *et al.* 1996; Barrett-Lennard 2003; Geigenberger 2003). Oxygen is vital for respiration to provide energy to cells; therefore O_2 concentrations can influence the metabolic activities and energy production of plant cells (reviewed by Geigenberger 2003). ATP production can be reduced by 95% in O_2 -deficient roots (Barrett-Lennard 2003) and the subsequent energy deficit can rapidly reduce growth and nutrient uptake (Trought and Drew 1980; Drew 1983; Barrett-Lennard 1986) and will eventually lead to cell death (Greenway and Gibbs 2003).

2.3.2 Mechanisms of tolerance to waterlogging

The O_2 deficiency that occurs in waterlogged soils causes anaerobic metabolism in anoxic tissues, and therefore reduces ATP production from 38 ATP per mole of glucose to 2 ATP (Barrett-Lennard 2003). This decrease in ATP production means there is

insufficient energy to control ion influx and efflux, and hence there is a build-up of ions to toxic concentrations (Barrett- Lennard *et al.* 1999). Plants have different adaptations to overcome O₂ deficiency. Formation of aerenchyma (tissue containing large gas-filled intercellular spaces) is important for waterlogging tolerance (Garthwaite *et al.* 2003; 2005; Evans 2004). The aerenchyma forms an internal pathway for O₂ movement in roots to help avoid O₂ deficiency. Plants can also respond to waterlogging by forming additional adventitious roots, a barrier to radial O₂ loss (ROL) and/or the induction of additional anaerobic metabolism in anoxic tissues (Armstrong 1979; Jackson and Drew 1984; Setter and Waters 2003; Greenway and Gibbs 2003). Many wetland species form a barrier to ROL in the external cell layers of the subapical region of adventitious roots, as this barrier can reduce the loss of O₂ from the aerenchyma to the soil (Armstrong 1979; Armstrong *et al.* 2000; Colmer 2003). Combined with aerenchyma, a barrier to ROL enhances the longitudinal movement of O₂ towards the root tip, which helps the roots to grow into waterlogged soils (Armstrong 1979; Armstrong and Beckett 1987).

The barrier to ROL is inducible in several species inhabiting waterlogging-prone regions, such as in several of the wild *Hordeum* species (Garthwaite *et al.* 2003). Compared with the wild species, cultivated species in the tribe Triticeae, such as barley and wheat, do not develop a barrier to ROL and are less tolerant of waterlogging than several wild *Hordeum* species (McDonald *et al.* 2001a; Garthwaite *et al.* 2003). In comparison, *H. marinum* accessions form a barrier to ROL but show variation in ROL (Malik *et al.* 2009a). Although there are many indications suggesting the barrier to ROL is important for waterlogging tolerance, there is a lack of detailed studies of differences in the barrier thickness from one species to another (Colmer 2003).

Ethylene plays a regulatory role as the signalling molecule for the induction of aerenchyma in the roots of several species (Drew *et al.* 2000; Shiono *et al.* 2008). Hypoxia promoted the synthesis of ethylene by roots and micro-organisms in waterlogged soils (Romick *et al.* 1996). Moreover, exogenous ethylene promotes the formation of aerenchyma in maize (Drew *et al.* 1979), rice (Justin and Armstrong 1991) and wheat (Huang *et al.* 1997) when grown in aerated nutrient solutions. There appear to be different factors regulating the formation of aerenchyma and the induction of the barrier to ROL (Justin and Armstrong 1991; Colmer *et al.* 2006a; Shiono *et al.* 2008). The exact mechanism responsible for the induction of the barrier to ROL is not yet

known (Shiono *et al.* 2008). Exogenous ethylene does not induce the barrier to ROL in rice (Colmer *et al.* 2006a). Given this, it is not surprising that there is also a lack of knowledge on barrier formation under salt stress. To my knowledge, *Hordeum* is the only genus in Triticeae that forms the barrier to ROL (Thomson *et al.* 1992; McDonald *et al.* 2001a,b). Furthermore, the barrier to ROL and aerenchyma are inducible in several *Hordeum* species under stagnant conditions (e.g. *H. marinum* Garthwaite *et al.* 2003; Malik *et al.* 2009a,b). Therefore, *H. marinum* is a useful plant to assess the role of root aeration on ion regulation under combined salinity and waterlogging treatments. For species adapted to marshes and wet saltland, better root aeration is likely to be correlated with salt tolerance (e.g. *Lotus tenuis*, Teakle *et al.* 2007; *H. marinum*, Malik *et al.* 2009a), yet very little is known about this correlation and further work is needed to evaluate the contribution of the barrier to ROL and aerenchyma to salt tolerance.

2.4 THE EFFECT OF THE INTERACTION BETWEEN SALINITY AND WATERLOGGING ON PLANTS

Wherever salinity and waterlogging occur simultaneously, the adverse effects on plant growth are usually severe for most species (Drew and Läuchli 1985; Barrett-Lennard 1986). The major impact of waterlogging on plants is the low concentration of O₂ (hypoxia) in the soil (Grable 1966; Ghassemi *et al.* 1995; Qureshi and Barrett-Lennard 1998). The absence of O₂ inhibits ATP production in roots by approximately 95%, and this long-term decline in energy adversely affects plant growth (Drew 1983; Barrett-Lennard 1986; 2003). Furthermore, waterlogging can kill root tissues and enhance senescence of leaves (Webb and Armstrong 1983; Barrett-Lennard *et al.* 1988; Marcar 1993). Oxygen deficiency also adversely affects solute transport processes as well as cytoplasmic pH regulation (Trought and Drew 1980; Buwalda *et al.* 1988; Barrett-Lennard *et al.* 1999; Greenway and Gibbs 2003). Furthermore, membrane selectivity can be disrupted; under these conditions the movement of ions becomes much more strongly influenced by the mass flow of water (Drew 1983; Buwalda *et al.* 1988; Morard and Silvestre 1996). Disrupting membrane selectivity may lead to an increase in the rate of entry of Na⁺ and Cl⁻, to the detriment of other ions such as K⁺; therefore ionic toxicity will occur more rapidly (Barrett-Lennard 2003; Barrett-Lennard and Shabala 2013). Another impact of waterlogging is that the transfer of water through roots can decline because of decreases in the hydraulic conductivity of O₂ deficient roots (Bramley *et al.* 2010); therefore stomatal closure of plants can occur under both non-

saline (Bradford and Hsiao 1982; Jackson and Hall 1987) and saline conditions (Kriedemann and Sands 1984; Huang *et al.* 1995). The combined effect of salinity and waterlogging increases the rate of accumulation Na^+ and Cl^- and reduces K^+ uptake to shoots, causing growth reduction of poorly-adapted species (Barrett-Lennard 2003; Barrett-Lennard and Shabala 2013). Therefore, the regulation of ion transport is a key mechanism to maintain tolerable concentrations of ions in plant tissues (Watson *et al.* 2001). Better root aeration enables tolerant species to control ion transport (Galloway and Davidson 1993).

Various studies have evaluated the individual impacts of salinity (e.g. Maas and Hoffman 1977; Gorham *et al.* 1985; Maas 1986) and waterlogging (e.g. Trought and Drew 1980; Thomson *et al.* 1992) in wheat (*Triticum aestivum* L.). However, few studies have evaluated the combined effect of salinity and waterlogging (Aceves-N *et al.* 1975; Barrett-Lennard 1986; Musgrave 1994; Malik *et al.* 2009a,b; Munns *et al.* 2011; Zhang *et al.* 2011; Saqib *et al.* 2013). A study by Barrett-Lennard *et al.* (1999) concluded that the combination of stresses can adversely affect wheat growth and survival, as the combined effect of both factors is greater than the product of the individual effects.

2.5 TOLERANCE OF WHEAT AND *HORDEUM MARINUM* TO SALINITY AND WATERLOGGING

2.5.1 Tolerance to individual stresses of salinity and waterlogging

Wheat (*Triticum aestivum* L. 2n=42=AABBDD) is considered to be moderately salt-tolerant as it can survive at up to ~250 mM NaCl, equal to ~50% of the salinity of seawater (Maas and Hoffman 1977; Maas 1986). However, wheat is relatively sensitive to waterlogging (Setter and Waters 2003), decreasing the yield, number and weight of spikes and seeds, protein content, and chlorophyll a and b (Olgun *et al.* 2008). Sea barleygrass (*Hordeum marinum* Huds. 2n=14=XX) and wheat are from the same tribe (Triticeae), but *H. marinum* is more salt and waterlogging tolerant than wheat (Malik *et al.* 2009a,b). *H. marinum* has possibly the highest tolerance to salinity and waterlogging within the Triticeae (Colmer *et al.* 2006b), as it inhabits salt marshes (von Bothmer *et al.* 1995). The salinity tolerance of *H. marinum* is attributed to its superior capacity to ‘exclude’ Na^+ and Cl^- from the shoot and better maintain tissue K^+ concentrations,

compared with wheat (Garthwaite *et al.* 2005). Waterlogging tolerance is associated with the ability to develop aerenchyma and a barrier to radial O₂ loss (ROL) in adventitious roots when exposed to waterlogging (McDonald *et al.* 2001a; Garthwaite *et al.* 2003; Malik *et al.* 2011). The barrier to ROL is a key waterlogging tolerance trait (Armstrong 1979; Jackson and Armstrong 1999; Colmer 2003), which could be transferred to wheat through the development of amphiploids (Colmer and Islam 2002; Malik *et al.* 2011). Variation within *H. maritimum* at the level of subspecies (ssp. *maritimum* and ssp. *gussoneanum*) has been reported by Malik *et al.* (2009a). *H. maritimum* ssp. *maritimum* tended to maintain lower leaf Na⁺ and Cl⁻ than ssp. *gussoneanum*, although the two subspecies did not differ in their mean reductions in RGR in response to stagnant saline treatment (Malik *et al.* 2009a). Compared to wheat, *H. maritimum* has a superior capacity to maintain adequate root aeration and better leaf ion regulation – so it is not surprising that *H. maritimum* can withstand the combined effects of salinity and waterlogging (Malik *et al.* 2009a). Therefore, *H. maritimum* might be used to improve the salt and waterlogging tolerance of wheat via wide hybridisations (Table 2.1; Gorham *et al.* 1986; Colmer *et al.* 2006b; Islam *et al.* 2007).

Table 2.1. A summary of published data on the effects of salinity, waterlogging and the combination of these two stresses on selected *H. maritimum* accessions, wheat (*Triticum aestivum*) ‘Chinese Spring’ (CS) and *H. maritimum*-wheat amphiploids.

Genotype	Accession / Amphiploid	RGR (% of control)	Leaf Na ⁺ (μmol g ⁻¹ DM)	Root porosity %	Barrier to ROL	Treatment details	Source
<i>H. maritimum</i>	H90	97	-*	18	present	~27 d, stagnant non-saline solution	Garthwaite <i>et al.</i> (2003)
	H21	74	150	-	-	30 d, aerated plus 300 mM NaCl	Islam <i>et al.</i> (2007)
	H21	42	358	17	strong	~29 d, stagnant plus 200 mM NaCl	Malik <i>et al.</i> (2009a)
	H87	50	250	14	slight		
	H90	53	256	19	partial		
	H109	52	255	27	strong		
	H155	39	231	21	partial		
	H546	28	472	-	strong		
	H563	61	462	31	strong		
	H826	53	725	17	partial		
	WA9	50	357	29	partial	~29 d, stagnant plus 200 mM NaCl	Malik <i>et al.</i> (2009b)
	WA29	58	378	25	strong		
	H21	104	-	21	strong	21 d for RGR and ~27 d for other measurements, stagnant non-saline	Malik <i>et al.</i> (2011)
	H87	-	-	24	strong		
	H109	-	-	25	strong		
	H155	-	-	25	strong		
<i>T. aestivum</i> (CS)		24	2210	-	-	28 d, aerated plus 300 mM NaCl	Islam <i>et al.</i> (2007)
		39	2260	17	slight	~29 d, stagnant plus 200 mM NaCl	Malik <i>et al.</i> (2009a)
		21	4172	13	slight	~29 d, stagnant plus 200 mM NaCl	Malik <i>et al.</i> (2009b)
Amphiploid	H21-CS	51	870	-	-	28 d, aerated plus 300 mM NaCl	Islam <i>et al.</i> (2007)
	H21-CS	96.5	-	24	partial	21 d for RGR and ~27 d for other measurements, stagnant non-saline	Malik <i>et al.</i> (2011)
	H87-CS	78	-	20	partial		
	H109-CS	89	-	21	partial		
	H155-CS	77	-	20	partial		

RGR is the whole plant relative growth rate relative to controls, Na⁺ concentration is in the youngest fully expanded leaf, and porosity refers to adventitious roots. *- indicates data not available.

An amphiploid is a synthetic hybrid containing all the genes from two plant species. Several amphiploids of wild salt-tolerant species with wheat had been made in an attempt to improve salt-tolerance in wheat (Colmer *et al.* 2006b). Recent work on sea barleygrass (*Hordeum marinum*) and their wheat amphiploids (AABBDDXX) has assessed their tolerance to salt (Islam *et al.* 2007), waterlogging (Malik *et al.* 2011) and the interactive effects of salinity and waterlogging (Munns *et al.* 2011). The amphiploids that have been produced by hybridisation of *H. marinum* with wheat (as reported by Colmer *et al.* 2005b) are more salt and waterlogging tolerant than wheat. The amphiploids showed intermediate tolerance to salt and waterlogging compared to the wild relative and wheat parent (Fig. 2.2 and Table 2.1; Islam *et al.* 2007; Malik *et al.* 2009a; Munns *et al.* 2011). *H. marinum* has the ability to ‘exclude’ Na⁺ from leaves (153 $\mu\text{mol g}^{-1}$ DM; Table 2.1); while the amphiploid demonstrated a significant improvement in Na⁺ exclusion as compared to wheat (Na⁺ concentration in the amphiploid was 39% of that in wheat; Table 2.1). Wheat has been identified as an ion excluder (Watson *et al.* 2001), and is not a species with high tissue tolerance (Munns *et al.* 2011). Tissue tolerance has not been assessed in *H. marinum*; although it has been reported that *H. marinum* had the lowest shoot Na⁺ concentrations of the eight *Hordeum* species grown at 450 mM (Garthwaite *et al.* 2005). It is self-evident that the improvement in *H. marinum*-wheat amphiploids is most likely to have come from the genetic contribution of the *H. marinum* chromosomes (Islam *et al.* 2007). In a test of the relative salt tolerance of the first amphiploid between *H. marinum* (H21) and wheat (cv Chinese Spring) at a range of salinities, there was a general negative relationship between relative growth rates and the proportion of the shoot that had died. At the highest salinity tested (300 mM NaCl) the wheat had lowest rates of growth ($\sim 30 \text{ mg g}^{-1} \text{ day}^{-1}$) and the highest percentage of dead leaf mass ($\sim 15\%$). However, under the same conditions the amphiploid had more than twice the growth rate and a third of the dead leaves compared to wheat (Fig. 2.2).

Fig. 2.2. Whole plant relative growth rate (RGR) versus dead leaf mass of *H. marinum* (H21), wheat (Chinese Spring) and their amphiploid when grown under varying conditions of salinity. Measurements were taken 28 d after reaching the final concentration of NaCl (0.2, 100, 200 and 300 mM NaCl; concentrations are not distinguished in the graph). These data have been re-drawn from Islam *et al.* (2007).

H. marinum and *H. marinum*-wheat amphiploids have been reported to form barriers to ROL which were absent in wheat (summarised in Table 2.1 and illustrated in Fig. 2.3). When grown in stagnant solution, the *H. marinum* accession H87 and its amphiploid with Chinese Spring showed a decline in ROL from near the root tip towards the root base, indicating the formation of at least a partial barrier to ROL (Fig. 2.3). The *H. marinum* accession formed a strong barrier to ROL when grown in stagnant non-saline conditions (i.e. large declines in ROL in basal zones relative to near the root tip; Fig. 2.3a), while the amphiploid formed a partial barrier to ROL (i.e. only modest declines, or similar ROL in basal zones relative to the root tip – Fig. 2.3b). By contrast, for wheat grown in the stagnant non-saline treatment, the rate of ROL was greatest in basal zones and declined towards the root tip, indicating only a weak (or absent) barrier to ROL (Fig. 2.3c). It should be noted however, that the higher levels of ROL from the root tips of the *H. marinum* accession and the amphiploid, compared to ROL in wheat, could also be due to higher root porosity (porosity was: 21% in *H. marinum*, 16% in wheat and 24% in the amphiploid).

Fig. 2.3. Rates of radial O_2 loss (ROL) along adventitious roots of *Hordeum marinum* accession (H87; (a)), its amphiploid (b) and wheat (Chinese Spring (CS); (c)) when in an O_2 -free root medium and with shoots in air. Plants were grown for 18-25 d in aerated non-saline nutrient solution (open symbols) and stagnant non-saline nutrient solution (closed symbols), and measurements were conducted in non-saline medium. Treatments were imposed when plants were 14-d-old for *H. marinum*, and 11-d-old for wheat and the amphiploid. Values are the mean of three replicates $\pm$ standard errors, and each replicate represents a single plant grown in different pots. Note the different scale on the y-axis for (a) compared to (b) and (c). Data from Malik *et al.* (2011).

2.5.2 Tolerance to the interaction of salinity and waterlogging

The presence of a damaging interaction between salinity and waterlogging has been suggested to be a key reason for the lack of success of wheat breeding programs aimed at saline lands (Barrett-Lennard 2003). The question therefore arises: could the introduction of genes from a wild parent with combined salt and waterlogging tolerance overcome these problems to create a wheat for saline waterlogged land? Despite reports of *H. marinum*–wheat (Chinese Spring) amphiploids expressing salt tolerance (Islam *et al.* 2007) and moderate waterlogging tolerance (Malik *et al.* 2011), the interaction of these stresses has not been tested on any *H. marinum*–wheat (Chinese Spring) amphiploids. Tolerance to the combination of these stresses is critical if the amphiploid is to become a viable cropping option for saltland. The range of studies on the responses of *H. marinum*–wheat (Chinese Spring) amphiploids (produced by AKMR Islam) to the individual stresses of salinity and waterlogging have been summarised in Table 2.1. Two *H. marinum* (H90)-wheat amphiploids (wheat varieties other than Chinese Spring) were reported to tolerate the combination of stresses better than their respective wheat parents (Munns *et al.* 2011). However, diversity in salt and waterlogging tolerance has

been observed amongst *H. marinum* accessions (Malik *et al.* 2009a,b). My analysis reveals that *H. marinum* H90 is not the most suitable wild parent for wide hybridisation to improve salt and waterlogging tolerance of wheat, as H90 developed only a partial barrier to ROL and had low adventitious root porosity in comparison to other *H. marinum* accessions (Table 2.1). Root aeration traits are essential to withstand the interactive effects of salinity and waterlogging (Malik *et al.* 2009a). Therefore, it is expected that the incorporation of better adapted *H. marinum* accessions into amphiploids would produce more tolerant amphiploids.

The effects of salinity and/or waterlogging (individually) on eight *H. marinum* accessions, wheat (*Triticum aestivum*) ‘Chinese Spring’ (CS) and four *H. marinum*-wheat amphiploids are given in Table 2.1. Stagnant conditions alone did not affect growth for *H. marinum*, but salinity decreased growth (~60% - 90% of control) and the combination of stresses caused even greater decreases in growth (~30% - 60% of control). Not surprisingly, wheat was sensitive to the combination of salinity and waterlogging and suffered up to ~80% decrease in growth. By comparison, growth in the stagnant or saline conditions was better maintained in the amphiploids than in wheat, however the effect of the interaction between salinity and waterlogging on these amphiploids has not been previously assessed.

2.6 CONCLUSION

If breeders are to incorporate (through wide hybridisation) traits from wild species like *H. marinum* to improve the salt and waterlogging tolerance of wheat, it is important to understand the genetic and physiological basis of salt and waterlogging tolerance in *H. marinum* (Colmer *et al.* 2006b).

This review has:

1. Highlighted the importance of evaluating the physiological and genetic basis of salinity and waterlogging tolerance (separately and simultaneously) in an attempt to understand their complex interaction.
2. Focused on possible physiological and molecular mechanisms of salinity and waterlogging tolerance of *H. marinum*-wheat amphiploids, in contrast with their parents.

3. Highlighted the key physiological and molecular traits that could be expressed in the wild parent.

Tolerance to salinity and waterlogging from *H. maritimum* has been reported to be expressed in *H. maritimum*–wheat amphiploids (Munns *et al.* 2011). However, further research is needed to: (i) elucidate the mechanisms responsible for these tolerances both separately and in combination, (ii) determine levels of salt or waterlogging tolerance occurring within selected *H. maritimum* accessions and the available *H. maritimum*–wheat amphiploids, (iii) determine levels of salt or waterlogging tolerance expressed in the *H. maritimum*–wheat amphiploids, (iv) identify candidate genes contributing to tolerance to these stresses, and (v) determine the effect that the individual stresses of salinity and waterlogging, as well as these stresses combined, have on the expression of candidate gene(s) for salt tolerance of *H. maritimum*–wheat amphiploids in comparison with its parents. This thesis will present new information concerning the physiological traits contributing to tolerance in *H. maritimum*–wheat amphiploids in comparison to the parents to combined salinity and waterlogging stress. Expression levels of two candidate genes involved in Na⁺ transport are also assessed for various tissues. Such information is needed for future development of wheat for saline land, and for improved knowledge on salt and waterlogging tolerance in wheat and *H. maritimum*, and as expressed in the *H. maritimum*–wheat amphiploids.

CHAPTER 3

Variation in salt and waterlogging tolerances amongst eight *Hordeum marinum* accessions

3.1 ABSTRACT

Eight accessions of *Hordeum marinum*, a wild species in the Triticeae, were evaluated for tolerance to salinity, waterlogging, and these two stresses combined. Interest in *H. marinum* has grown from the potential to use it as a source of genes for resistance to abiotic stress tolerance in wheat. This chapter evaluated the variation amongst eight *H. marinum* accessions for physiological responses to salt (NaCl) and hypoxic root-zone treatments. It was hypothesised that the differential tolerance and responses to salinity and waterlogging stresses among the *H. marinum* accessions might be associated with key physiological traits and thus reveal the mechanisms contributing to tolerance of these stresses combined. Plants were subjected to aerated solutions or deoxygenated stagnant agar (to simulate the changes in root-zone gas composition that occur during waterlogging) and either 0.2, 200 or 400 mM NaCl for 26 d. Growth responses, shoot ion concentrations (Na^+ , Cl^- and K^+) and leaf tissue injury were evaluated. Two of the *H. marinum* accessions (H109 and WA9) were identified as being relatively tolerant of combined salinity and stagnant treatments, whereas H546 was identified as being the most sensitive accession. Low RGRs were correlated with higher shoot Na^+ ($r^2 = 0.66$ in aerated and 0.89 in stagnant solutions) and Cl^- concentrations ($r^2 = 0.70$ in aerated and 0.89 in stagnant solutions) and greater percentages of dead leaf material ($r^2 = 0.86$ in aerated and 0.83 in stagnant solutions), while high RGRs were correlated with higher shoot K^+ concentrations ($r^2 = 0.47$ in aerated and 0.75 in stagnant solutions) and K^+/Na^+ in the tissues ($r^2 = 0.64$ in aerated and 0.81 in stagnant solutions) at 400 mM NaCl. Similarly, the leaf extension rate was up to 68% higher in tolerant accessions compared to H546. This study successfully identified two salt and waterlogging tolerant *H. marinum* accessions and demonstrated that tolerance was associated with a better capacity for shoot ion regulation, reduced leaf death and higher rates of leaf growth.

3.2 INTRODUCTION

Salinity adversely affects more than 900 Mha of arable land (OECD/FAO 2012), reducing the growth of sensitive species (Flowers and Yeo 1995; Munns *et al.* 2006) and threatening the cultivation of crops in many areas (Greenway and Munns 1980; Szabolcs 1994; Dunne *et al.* 2002). In highly affected soils, salinity not only inhibits growth but can even cause plant death (Flowers and Yeo 1995; Munns *et al.* 2006). Salinity exerts its adverse impact by causing water deficits and ion toxicities in cells

(Greenway and Munns 1980; Flowers and Dalmond 1992; Munns 2005). Salinity is often accompanied by waterlogging (Barrett-Lennard 2003; Bennett *et al.* 2009; Barrett-Lennard and Shabala 2013), and in saturated soils roots can suffer hypoxia or even anoxia (Armstrong 1979). Waterlogging decreases root growth and affects the viability of roots (Huck 1970; Webb and Armstrong 1983; Barrett-Lennard 1986). The interaction between salinity and waterlogging can have an adverse impact on crop growth and productivity (Barrett-Lennard 2003; Barrett-Lennard and Shabala 2013). Moreover, the combination of salinity and waterlogging decreases the capacity of roots to 'exclude' Na^+ and Cl^- from shoots (e.g. wheat, Barrett-Lennard *et al.* 1999), but some halophytes can tolerate combined waterlogging and salinity (e.g. *Puccinellia ciliata*; Teakle *et al.* 2013).

Wheat improvement has previously relied on genes sourced from wild species from within the tribe Triticeae to improve resistance to biotic stress; more recently attempts have also been made to introduce tolerances to abiotic stress from such sources (Colmer *et al.* 2006b). Sea barleygrass (*Hordeum marinum*) is from the same tribe (Triticeae) as wheat. *H. marinum* grows naturally in salt marshes (von Bothmer *et al.* 1995) and it is more salt and waterlogging tolerant than wheat (Colmer *et al.* 2005b). The tolerance of *H. marinum* to salinity is associated with good regulation of ions to maintain low concentrations of Na^+ and Cl^- , but high K^+ in leaves, and tolerance to waterlogging by forming adventitious roots with aerenchyma and a barrier to radial O_2 loss (ROL) (McDonald *et al.* 2001a; Garthwaite *et al.* 2003; 2005; Malik *et al.* 2009a).

The present experiment evaluated the interactive effects of salinity and waterlogging on eight *Hordeum marinum* accessions for key traits associated with salinity and waterlogging tolerance, viz: growth responses, shoot ion concentrations (i.e. Na^+ , Cl^- and K^+), and leaf tissue injury.

Variation in tolerances has been previously observed within *Hordeum marinum* accessions for salinity and low root-zone O_2 stress (Malik *et al.* 2009a,b). Examination of this previous dataset showed that some accessions had excellent adaptation to aerated saline conditions, but poor growth under stagnant saline conditions, and *vice versa*. The objective of the present study was to evaluate the variation among eight *H. marinum*

accessions for physiological responses to various NaCl and stagnant treatments. The study identified two *H. marinum* accessions that demonstrated highly efficient Na⁺ and Cl⁻ 'exclusion' from the shoots and better maintenance of K⁺ concentrations, at high external NaCl, even when in an O₂-deficient root-medium. The findings of the present study are discussed in the context of understanding salt and waterlogging tolerance in *H. marinum*, a wild relative to wheat in the Triticeae, and the complex effects of the interaction between salinity and waterlogging on plants.

3.3 MATERIALS AND METHODS

Plant material and growth conditions

The experiment was conducted with eight diploid accessions of *Hordeum marinum* Huds.; three of these were *H. marinum* subsp. *marinum* and three were *H. marinum* subsp. *gussoneanum* from different parts of the world, and two were *H. marinum* (sub-species not known) from Western Australia (WA). Seeds of accessions from outside WA were originally provided by the Nordic Gene Bank (R. von Bothmer, Swedish Agricultural University, Alnarp) and were raised in a glasshouse. WA accessions were collected by K. A. Shepherd (previously at UWA, now at WA Herbarium), and raised in a glasshouse to provide those seeds used here. The codes of the accessions used are listed in Table 3.1.

Experimental design

The eight accessions (Table 3.1) were subjected to 2 aeration treatments × 3 salinity treatments × 4 replications in a completely randomised design. Each pot was either aerated or stagnant and had one of three salinity treatments (0.2, 200 or 400 mM NaCl). The experiments were conducted in a naturally-lit phytotron (20/15°C day/night with an average PAR at midday during the experimental period of 1250 μmol m⁻² s⁻¹). Experimental units (pots) were randomised weekly to minimise possible effects on plants of any environmental variation within the phytotron. The experiment was conducted between 29-Sep-2009 and 14-Nov-2009 at the School of Plant Biology, UWA, Perth Western Australia.

Seeds were surface-sterilised with 0.04% NaHClO in de-ionised (DI) water for 45 s, and then rinsed thoroughly with DI water prior to being imbibed overnight in aerated 0.5 mM CaSO₄. Seeds were then transferred onto mesh floating on 10% strength aerated nutrient solution in darkness (pots completely covered with Al-foil) in a 20/15 °C day/night phytotron for the first 4 d. Seedlings were exposed to light after 4 d and nutrient solutions were changed to 25% strength.

Eight seedlings per pot were transplanted into 4.5 L pots containing full-strength nutrient solution on day 7. Seedlings were held in the pot lids using polystyrene foam in holes (2 cm diameter). The full-strength nutrient solution contained (mM): K⁺, 3.95; Ca²⁺, 4; Mg²⁺, 0.4; NH₄⁺, 0.625; Na⁺, 0.2; NO₃⁻, 4.375; SO₄²⁻, 4.4; H₂PO₄⁻, 0.2; H₄SiO₄⁻, 0.1; with micronutrients (μM): Cl⁻, 50; B, 25; Mn, 2; Zn, 2; Ni, 1; Cu, 0.5; Mo, 0.5; Fe-EDTA, 50. *H. maritimum* can be prone to Fe-deficiency under these conditions; therefore additional 5 μM FeSO₄ was supplied routinely (on 3 occasions to the young plants) to prevent any risk of such deficiency, but always to all pots. The solution was also buffered with 2.5 mM 2-[N-Morpholino]ethanesulfonic acid (MES) and the pH was adjusted to 6.5 using KOH, increasing the final K⁺ concentration to ~6 mM.

Treatments and harvests

Treatments were imposed when plants had 2–2.5 leaves (14-d-old plants). Prior to the treatments being imposed, an initial harvest of 4 replicate plants for each accession was taken to measure initial dry mass (DM). After this, NaCl was stepped up in pots allocated to saline treatments by 50 mM every 12 h until the final concentrations were reached (36 h for 200 mM and 84 h for 400 mM NaCl). After reaching the final NaCl concentration, pots allocated to the stagnant treatment were given a ‘hypoxic pre-treatment’ in which the solution in these pots was bubbled with N₂ for ~2 h; previous experience has shown that this would have decreased the dissolved O₂ concentration to less than ~0.03 mM (Wiengweera *et al.* 1997); these pots were then left overnight without bubbling. The following day, the nutrient solution in the stagnant pots was replaced with deoxygenated (i.e. flushed with N₂ overnight) stagnant nutrient solution containing 0.1% (w/v) agar. The agar inhibits convective movements in the nutrient solution, impeding the re-entry of O₂ and promoting the accumulation of ethylene, both

important changes in the gas composition of waterlogged soils (Wiengweera *et al.* 1997). The nutrient solutions of pots allocated for aerated treatments were also renewed at the same time as the stagnant treatment, but these solutions were without agar and continued to be bubbled with air. A second harvest of 4 replicate plants for each accession was taken 24 h after stagnant agar treatments were imposed to measure root and shoot DM to enable calculations of RGR for plants during the treatment proper. Solutions in all pots were renewed every 7 d and aerated pots were topped up with DI water as required.

The plants were harvested 26 d after the stagnant agar treatment commenced. On day 26, plants were harvested for measurements of DM and tissue ion analyses. At each harvest, roots and the stem base were washed 3 times for 30 s in mannitol solutions iso-osmotic with the Na^+ plus Cl^- concentration of the nutrient solution; these wash solutions also contained 4 mM CaSO_4 . The plants were then separated into four tissue classes: the youngest fully expanded leaf (YFEL), other green shoot tissues (i.e. stems, sheaths and all other green and turgid leaf blades), dead leaves and roots.

Growth measurements

The rate of extension of the youngest expanding leaf on the main stem was measured by recording leaf length from the top of the foam holder to the leaf tip over ten consecutive days, starting from when the stagnant agar treatment was imposed. The leaf extension linear phase was estimated for each replicate by plotting leaf length against days, points out of extension phase were excluded, and therefore the linear phase of each replicate consisted of four to five points of each replicate. At the final harvest, the number of tillers was counted before the plants were separated into the components described above. Fresh mass was recorded and DM of all samples was measured after being oven dried for 72 h at 65°C. The relative growth rate (RGR) was calculated from the DM of the whole-plant samples at the start and end of the treatment period (day 1 and 26 after stagnant agar treatment), using the formula described by Hunt (1978):

$$\text{RGR} = (\ln DM_2 - \ln DM_1) / (t_2 - t_1)$$

Where DM_1 is the dry mass (g) at time one (initial harvest), DM_2 is the dry mass (g) at time two (final harvest), t_1 and t_2 are times one and two in d, and $\ln$ is the natural logarithm.

Tissue ion analyses

Concentrations of Na^+ , Cl^- and K^+ were determined in various shoot tissues. Oven dried samples were ground and extracted in 0.5 M HNO_3 by shaking for 48 h at 20–25°C. Na^+ and K^+ concentrations were determined in dilutions of the extracts using a flame photometer (Jenway PFP7, Essex UK). Cl^- concentration was determined in the extracts using guanidine protocol of a chloridometer (Slamed CHLO 50 cl, Frankfurt, Germany). Blanks and a reference plant material with known ion concentrations were taken through the same procedures. The reference sample was from the commercial supplier IUPAC. Recoveries of Na^+ , Cl^- and K^+ from the reference tissue were 102, 101 and 111%, respectively. The data presented have therefore not been adjusted.

Data analyses

Statistical analyses were conducted using GENSTAT 12th Edition (VSN International Ltd., Hemel Hempstead, UK). Residuals were checked for normality and no transformations were needed. A two-way ANOVA was used to identify overall significant differences between genotypes and between treatments. When significant differences were found, mean-separations were calculated using the Fisher unprotected LSD test. Unless otherwise stated, the significance level was $P \leq 0.05$. For the P -values from the ANOVA on the data of the parameters described below, see Supplementary Materials Table 3.S2.

3.4 RESULTS

Growth

Whole plant RGR varied among the accessions when subjected to separate NaCl and stagnant root-zone stresses, and also when these two stresses were combined. Under control conditions, the RGR was relatively similar for all accessions, ranging from 0.19 to 0.22 g g⁻¹ d⁻¹ (Fig. 3.1A). For the stagnant non-saline treatment, RGR was reduced (compared to aerated conditions) by ~15% (Fig. 3.1A). Increasing salinity reduced the RGR of all accessions relative to the aerated non-saline controls. In aerated saline solutions, H546 and H155 were the least tolerant, with growth reduced (relative to control) by 30–40% with 200 mM NaCl and by 50–70% with 400 mM NaCl. By contrast, RGR reductions were similar for the other accessions with an average reduction of ~17% in aerated-plus 200 mM NaCl and ~38% in aerated-plus 400 mM NaCl, relative to control. Compared with controls, the combined stagnant and NaCl treatments reduced RGR for all accessions by an average of ~40% with 200 mM NaCl and by ~57% with 400 mM NaCl. However, there was variation among accessions. In the stagnant-plus 200 mM NaCl treatment, growth was reduced by 51% for the sensitive accession (H546) but only 29% for the tolerant accession (H563; Fig. 3.1B and 3.1C). In the stagnant-plus 400 mM NaCl treatment, growth was reduced by 69% for the sensitive accessions H546 and H155, compared with 53% for the other accessions. Although, stagnant saline treatments reduced RGR by an average of 23% relative to aerated saline treatments, there were no significant differences among accessions in those treatments (Fig. 3.1B and 3.1C). The treatment effects on total plant DM at the final harvest were consistent with these general trends in RGR (see Supplementary Materials, Fig. 3.S1).

Fig. 3.1. Whole plant relative growth rates (RGR) for eight accessions of *Hordeum marinum*. Treatments were imposed when plants were 14-d-old. Plants were treated for 26 d with aerated (control) or stagnant non-saline nutrient solution (A), aerated or stagnant nutrient solution plus 200 mM NaCl (B), or aerated or stagnant nutrient solution plus 400 mM NaCl (C). Note the different y-axes of (A) and (B and C). The data and errors for the 200 and 400 mM treatments (B and C) are reported as % of control. Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The L.S.D. represents significant differences between genotypes at $P = 0.05$.

Percentage of dead leaf material

The percentage of dead leaf material varied among accessions when subjected to separate or combined stagnant and NaCl root-zone stresses. In the aerated non-saline controls, the percentage of dead leaf material was low in all accessions, ranging between 0 and 0.9% of shoot DM (Fig. 3.2A). Relative to controls, the stagnant non-saline treatment increased dead leaf material by an average of ~19-fold across all accessions; the smallest increase (1% of shoot DM) occurred with H563, whereas the greatest increase (17% of shoot DM) was with H546 (Fig. 3.2A). Increasing salinity also increased the amount of dead leaves across all accessions, by averages of ~12-fold in aerated-plus 200 mM NaCl and ~27-fold in aerated-plus 400 mM NaCl (relative to control). H563 had the smallest increases in response to NaCl with dead leaves accounting for only 2% and 5% of shoot DM when grown in aerated-plus 200 or 400 mM NaCl respectively. In contrast with the more sensitive accession H546, dead leaves accounted for 18% (at 200 mM NaCl) and 32% (at 400 mM NaCl) of shoot DM. Compared with controls, the combined stagnant and NaCl treatments increased leaf tissue death in all accessions by averages of ~38-fold in the stagnant-plus 200 mM NaCl treatment and ~54-fold in the stagnant-plus 400 mM NaCl treatment. In the stagnant-plus 200 mM NaCl treatment, the smallest increase in leaf tissue death (7% of shoot DM) was with WA9, whereas the greatest increase was with H546 (32% of shoot DM). In the stagnant-plus 400 mM NaCl treatment, the smallest increase (10% of shoot DM) was in H563, while the greatest increase in leaf tissue death was in H546 (58% of shoot DM). Stagnant saline treatments, relative to aerated saline treatments, increased leaf tissue death by an average of 66% (200 mM NaCl) and 49% (400 mM NaCl), however, there was no difference among the accessions in their responses to this change in root medium O₂ supply (Fig. 3.2B and 3.2C).

Fig. 3.2. Dead leaf material (as a percentage of shoot DM, wt/wt) for eight accessions of *Hordeum marinum*. Treatments were imposed when plants were 14-d-old. Plants were treated for 26 d with aerated (control) or stagnant non-saline nutrient solution (A), aerated or stagnant nutrient solution plus 200 mM NaCl (B), or aerated or stagnant nutrient solution plus 400 mM NaCl (C). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between genotypes at $P = 0.05$; ns = not significant.

Na⁺ concentrations in green shoot tissues

Shoot Na⁺ concentrations were similar among accessions when grown in aerated non-saline conditions (control), ranging from 179 to 207 $\mu\text{mol g}^{-1}$ DM (Fig. 3.3A). Across all accessions salinity increased Na⁺ concentration by an average of ~3-fold in the aerated-plus 200 mM NaCl treatment and by ~3.5-fold in the aerated-plus 400 mM NaCl treatment (relative to controls). The smallest increases in response to 200 mM NaCl were in H155 and H546 (2.2-fold relative to control), whereas the greatest increase was 3.9-fold in H826. Contrasting results were found in the aerated-plus 400 mM NaCl treatment; H826 actually had the smallest relative increase in shoot Na⁺ concentration (2.6-fold) and H546 had the greatest relative increase (6-fold), in comparison with controls (Fig. 3.3B and 3.3C). In the stagnant-plus 200 mM NaCl treatment, shoot Na⁺ concentrations increased by 3-fold (relative to control) in all accessions; H109 had the smallest increase (2.1-fold) whereas H546 had the largest relative increase (4.3-fold). Similarly, in the stagnant-plus 400 mM NaCl treatment, Na⁺ concentrations increased by an average of ~4.2-fold (relative to control) across all accessions; H826 recorded the smallest relative increase (3.1-fold) and H546 had the largest relative increase (7.4-fold) (Fig. 3.3B and 3.3C). There were no systematic effects of stagnant conditions on shoot Na⁺ concentrations in plants grown in solutions containing 200 mM NaCl; the only exception was for H546, which had a 1.6-fold increase in Na⁺ concentration when grown under stagnant saline condition. By contrast, at 400 mM stagnant conditions increased Na⁺ concentrations across all accessions by an average of ~14%; however, there were no significant differences among the accessions (Fig. 3.3B and 3.3C).

Cl⁻ concentrations in green shoot tissues

Shoot Cl⁻ concentrations were similar for all accessions under aerated non-saline conditions (control), ranging from 213 to 262 $\mu\text{mol g}^{-1}$ DM (Fig. 3.3D). The stagnant non-saline treatment increased Cl⁻ concentrations (relative to controls) by an average of ~38% (Fig. 3.3D). Salinity treatments increased Cl⁻ concentration (relative to controls) by an average of ~71% and ~77% in the aerated-plus 200 and aerated-plus 400 mM NaCl treatments respectively. The smallest increase in Cl⁻ concentration in response to the aerated 400 mM NaCl treatment was in H109 (70%) and the greatest increase was in H546 (83%) (Fig. 3.3E and 3.3F). Relative to the aerated saline treatment, the combined stagnant saline treatment increased Cl⁻ concentrations in all accessions by an average of

~11% in the stagnant-plus 200 mM NaCl treatment and by ~17% in the stagnant-plus 400 mM NaCl treatment; there were no significant differences among the accessions (Fig. 3.3E and 3.3F).

K⁺ concentrations in green shoot tissues

For plants grown in aerated non-saline conditions (control), the K⁺ concentration in the shoot varied among accessions, ranging from 1204 $\mu\text{mol g}^{-1}$ DM in WA9 to 1501 $\mu\text{mol g}^{-1}$ DM in H563 (Fig. 3.3G). In general, all saline and stagnant treatments substantially decreased K⁺ concentrations in shoots of all accessions in comparison with controls (Fig. 3.3G, 3.3H and 3.3I). Under non-saline conditions, the stagnant treatment reduced K⁺ concentrations across accessions by an average of ~30% (Fig. 3.3G). WA9 was least affected by stagnant treatment (9% reduction), and H826 and H563 were most affected (45% reduction). Salinity treatments, reduced shoot K⁺ concentrations by an average of ~22% (200 mM NaCl) and ~24% (400 mM NaCl) relative to control; the only exception was for WA29, which had similar K⁺ levels across all aerated treatments. The greatest salinity effect was in H546, with 200 and 400 mM NaCl decreasing K⁺ concentrations by 37% and 53% respectively (Fig. 3.3H and 3.3I). The combined stagnant plus saline treatments reduced shoot K⁺ by an average of ~46% at both 200 and 400 mM NaCl, in comparison with controls. The smallest treatment effect was in WA29, with shoot K⁺ being decreased to 20-35% of controls, and the greatest treatment was in H563 and H546, with shoot K⁺ being decreased to 58-68% of controls (Fig. 3.3H and 3.3I). Relative to the aerated saline treatment, the combined stagnant saline treatment decreased K⁺ concentrations across all accessions by an average of ~36% and ~28% at 200 and 400 mM NaCl respectively. In the stagnant-plus 200 mM NaCl treatment, relative to aerated-plus 200 mM NaCl treatment, H546 had the least K⁺ concentration change (19% decrease) and H90 had the greatest K⁺ concentration change (48% decrease). There were no significant variations in K⁺ concentrations among accessions grown in the stagnant-plus 400 mM NaCl and aerated-plus 400 mM NaCl treatments (Fig. 3.3H and 3.3I).

Concentrations of Na⁺, Cl⁻ and K⁺ were also measured in the youngest fully-expanded leaf (YFEL) and the results showed the same general trends as those described here for the green shoot tissues. However, for the 400 mM NaCl treatments, there was insufficient leaf material to accurately measure the concentrations of ions in the YFEL (see Supplementary Materials, Fig. 3.S2).

Fig. 3.3. Green shoot tissue concentrations of Na^+ (A, B and C), Cl^- (D, E and F) and K^+ (G, H and I) for eight accessions of *Hordeum marinum*. Treatments were imposed when plants were 14-d-old. Plants were treated for 26 d with aerated or stagnant non-saline nutrient solution (A, D and G), aerated or stagnant nutrient solution plus 200 mM NaCl (B, E and H), or aerated or stagnant nutrient solution plus 400 mM NaCl (C, F and I). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The L.S.D. represents significant differences between genotypes at $P = 0.05$; ns = not significant.

K⁺/Na⁺ ratio in green shoot tissues

Compared with aerated non-saline controls, the stagnant saline treatments decreased the ratio of K⁺/Na⁺ in shoots of nearly all accessions (Table 3.1). In the controls, the shoot K⁺/Na⁺ ratio ranged from 6.3 (in WA29) to 8.4 (in H536) and the stagnant non-saline treatment reduced the K⁺/Na⁺ ratio by an average of ~14% across all accessions; the two exceptions were WA29, which increased K⁺/Na⁺ by 19% (relative to control) and H155, in which there was no change. The response to stagnant treatment was greatest for H563, with the K⁺/Na⁺ ratio decreasing by 26% (relative to control). Across all accessions, increasing salinity decreased the K⁺/Na⁺ ratio by averages of ~77% (at 200 mM NaCl) and ~82% (at 400 mM NaCl), relative to controls. In the aerated-plus 400 mM NaCl treatment, the smallest treatment effect was in H109 (77% decrease in K⁺/Na⁺), and the greatest treatment effect was in H546 (93% decrease), in comparison with controls. The combined NaCl and stagnant treatments decreased the K⁺/Na⁺ ratio across all accessions by averages of ~86% (at 200 mM NaCl) and ~89% (at 400 mM NaCl), relative to controls. In the stagnant-plus 200 mM NaCl treatment, H109 had the smallest treatment effect (77% decrease in K⁺/Na⁺) and H546 had the greatest treatment effect (90% decrease in K⁺/Na⁺), relative to controls. By contrast, in the stagnant-plus 400 mM NaCl treatment, WA29 had the smallest treatment effect (83% decrease in K⁺/Na⁺) and H546 had the greatest treatment effect (95% decrease in K⁺/Na⁺), relative to controls. Comparing the effects of waterlogging with aeration at the same level of salinity, at 200 and 400 mM NaCl, stagnant conditions decreased the ratio of K⁺/Na⁺ across all accessions by an average of ~38%. In the stagnant-plus 400 mM NaCl treatment, WA29 had the least change (19% decrease in K⁺/Na⁺) and H563 and H826 had greatest change (52% decrease), relative to the aerated-plus 400 mM NaCl treatment.

Table 3.1. Ratio of K^+/Na^+ in green shoot tissues of eight accessions of *Hordeum marinum* grown in aerated or stagnant nutrient solution, also containing 0.2, 200 or 400 mM NaCl. Accession codes, subspecies and country of origin are also listed (Malik *et al.* 2009a,b).

		Country of origin	K ⁺ / Na ⁺					
Accession code	Subspecies		0.2 mM NaCl		200 mM NaCl		400 mM NaCl	
			Aerated	Stagnant	Aerated	Stagnant	Aerated	Stagnant
H90	<i>marinum</i>	Greece	6.9 ± 0.12	6.4 ± 0.01	1.4 ± 0.07	0.8 ± 0.04	1.3 ± 0.06	0.9 ± 0.02
H109	<i>marinum</i>	Greece	7.0 ± 0.07	6.5 ± 0.12	1.8 ± 0.08	1.5 ± 0.11	1.6 ± 0.14	0.9 ± 0.15
H546	<i>marinum</i>	Spain	6.8 ± 0.25	5.8 ± 0.18	1.3 ± 0.14	0.7 ± 0.08	0.5 ± 0.06	0.3 ± 0.11
H155	<i>gussoneanum</i>	Greece	6.5 ± 0.03	6.5 ± 0.09	2.6 ± 0.81	1.0 ± 0.04	1.3 ± 0.08	0.8 ± 0.07
H563	<i>gussoneanum</i>	Spain	8.4 ± 1.14	6.2 ± 0.09	1.3 ± 0.07	0.9 ± 0.02	1.3 ± 0.04	0.6 ± 0.04
H826	<i>gussoneanum</i>	Turkey	7.3 ± 0.18	5.9 ± 0.25	1.5 ± 0.01	0.9 ± 0.09	1.0 ± 0.08	0.7 ± 0.05
WA9	- -	Australia	7.9 ± 0.39	6.4 ± 0.09	1.8 ± 0.05	1.2 ± 0.04	1.5 ± 0.10	1.1 ± 0.15
WA29	- -	Australia	6.3 ± 0.10	7.8 ± 0.98	1.6 ± 0.08	1.1 ± 0.08	1.3 ± 0.14	1.1 ± 0.01
l.s.d.			1.3	1.1	ns	0.2	0.3	0.3

Treatments were imposed when plants were 14-d-old. The plants were treated for 26 d after the stagnant agar treatment commenced. Values are the mean of four replicates ± standard errors, and each replicate represents a single plant grown in a different pot. The l.s.d. values are for comparisons of accessions at $P = 0.05$; ns = not significant.

Leaf extension rate

Leaf extension rate varied significantly among accessions and across treatments. Under aerated non-saline conditions (control), the leaf extension rate ranged from 1.8 mm d⁻¹ (H546) to 2.7 mm d⁻¹ (WA29 and H109; Fig. 3.4A). On average, the stagnant non-saline treatment reduced leaf extension rate by an average of ~20% compared to controls. H155 was least affected by the stagnant treatment (5% decrease) and H546 was most affected (45% decrease) (Fig. 3.4A). Increasing salinity decreased the leaf extension rate of all accessions by averages of ~40% (at 200 mM NaCl) by ~65% (at 400 mM NaCl), relative to controls (Fig. 3.4B and 3.4C). In the aerated-plus 200 mM NaCl treatment, H563 had the smallest treatment effect (25% decrease in leaf extension rate) and H155 had the greatest treatment effect (53% decrease in leaf extension rate), in comparison with controls (Fig. 3.4B). In the aerated-plus 400 mM NaCl treatment, H826 had the smallest treatment effect (58% reduction in leaf extension rate) and H546 had the greatest treatment effect (77% reduction in leaf extension rate), in comparison with controls (Fig. 3.4C). The combined stagnant saline treatments decreased the leaf extension rate of all accessions by averages of ~51% (at 200 mM NaCl) and ~71% (at 400 mM NaCl), relative to controls (Fig. 3.4B and 3.4C). In the stagnant-plus 200 mM NaCl treatment, H563 had the smallest effect on leaf extension rate (27% decrease) and H546 had greatest effect on leaf extension rate (66% decrease), in comparison with controls (Fig. 3.4B). In stagnant-plus 400 mM NaCl treatment, H563 had the smallest effect on leaf extension rate (61% decrease) and H546 had greatest effect (88% decrease), in comparison with controls (Fig. 3.4C). However, initial leaf length among accessions and across treatments did not differ on the commence of leaf extension rate measurements (see Supplementary Materials Table 3.S1).

Fig. 3.4. Leaf extension rate for eight accessions of *Hordeum marinum*. Treatments were imposed when plants were 14-d-old. Plants were treated for 26 d with aerated (control) or stagnant non-saline nutrient solution (A), aerated or stagnant nutrient solution plus 200 mM NaCl (B), or aerated or stagnant nutrient solution plus 400 mM NaCl (C). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between genotypes at $P = 0.05$.

Relationships between some key traits (growth, ion concentrations in green shoot tissues and dead leaf material) related to NaCl and waterlogging tolerance

Potential relationships between some key traits of NaCl and waterlogging tolerance were investigated using linear regression analyses (Fig. 3.5 and 3.6). For plants grown at 400 mM NaCl, there were strong negative relationships ($r^2 = 0.66$ in aerated and 0.89 in stagnant solutions) between whole plant RGR and shoot Na^+ concentration for plants in aerated or stagnant-plus 400 mM NaCl solution ($P \leq 0.001$) (Fig. 3.5A). With Na^+ concentrations of less than 800 $\mu\text{mol g}^{-1}$ DM, RGRs were between 0.10 and 0.13 $\text{g g}^{-1} \text{d}^{-1}$, but as Na^+ concentrations increased to more than 1200 $\mu\text{mol g}^{-1}$ DM, RGRs decreased to values less than 0.07 $\text{g g}^{-1} \text{d}^{-1}$. Shoot Cl^- concentration showed a similar negative trend ($r^2 = 0.70$ in aerated and 0.89 in stagnant solutions) with RGR to that of Na^+ , with $P \leq 0.01$ in aerated saline solution and $P \leq 0.001$ in stagnant saline solution (Fig. 3.5B). At Cl^- concentrations less than 1000 $\mu\text{mol g}^{-1}$ DM, RGRs were between 0.12 and 0.13 $\text{g g}^{-1} \text{d}^{-1}$, but at more than 1200 $\mu\text{mol g}^{-1}$ DM of Cl^- concentrations, RGRs decreased to values less than 0.09 $\text{g g}^{-1} \text{d}^{-1}$. By contrast, strong positive relationships ($r^2 = 0.47$ in aerated and 0.75 in stagnant solutions) were found between RGR and concentration of K^+ in shoots of plants in saline aerated ($P \leq 0.05$) and stagnant ($P \leq 0.001$) solutions (Fig. 3.5C). With K^+ concentrations of more than 1100 $\mu\text{mol g}^{-1}$ DM, RGRs were 0.13 $\text{g g}^{-1} \text{d}^{-1}$, but as K^+ concentrations decreased to less than 1000 $\mu\text{mol g}^{-1}$ DM, RGRs decreased gradually (although there were two exceptions) to reach the lowest value of 0.05 $\text{g g}^{-1} \text{d}^{-1}$ at the lowest K^+ concentration.

Given the trends for Na^+ and K^+ described above, not surprisingly the ratio of K^+/Na^+ also showed a positive trend with RGR, with $P \leq 0.001$ in both aerated and stagnant saline (400 mM NaCl) solution (Fig. 3.5D). With 1.3 K^+/Na^+ ratio and more, RGRs were between 0.11 and 0.13 $\text{g g}^{-1} \text{d}^{-1}$, but as the ratios K^+/Na^+ decreased to less than 1, RGRs decreased to values less than 0.09 $\text{g g}^{-1} \text{d}^{-1}$. RGR was strongly negatively ($r^2 = 0.86$ in aerated and 0.83 in stagnant solutions) related to the percentage of dead leaf material in both aerated and stagnant saline solutions ($P \leq 0.001$) (Fig. 3.5E). With dead leaf percentages of less than 20% of shoot DM, RGRs were between 0.10 and 0.13 $\text{g g}^{-1} \text{d}^{-1}$, but as the percentage dead leaf increased to more than 20% of shoot DM, RGRs decreased gradually to reach the lowest value of 0.05 $\text{g g}^{-1} \text{d}^{-1}$ at the highest dead leaf percentages; with one exception only.

Again using the plants grown at 400 mM NaCl, Figure 3.6 shows the relationships between the percentage of dead leaf on the plants and concentrations of Na^+ and K^+ in the green leaves or the ratio of K^+/Na^+ . With plants grown in aerated or stagnant solutions, high percentages of dead leaf material were correlated with increased shoot Na^+ concentrations ($P \leq 0.05$ for aerated plants; $P \leq 0.001$ for stagnant plants) and decreased shoot K^+ concentrations ($P \leq 0.001$ for aerated plants; $P \leq 0.05$ for stagnant plants) (Fig. 3.6A and 3.6B). Unsurprisingly, relationships between dead leaf material and the ratio of K^+/Na^+ were significant at $P \leq 0.01$ in both aerated and stagnant saline solutions (Fig. 3.6C).

Fig. 3.5. Relationships between whole plant RGR and: (A) Na^+ concentrations in shoots, (B) Cl^- concentrations in shoots, (C) K^+ concentrations in shoots, (D) the ratio of K^+/Na^+ , and (E) the percentage of dead leaf material. *H. maritimum* accessions were grown for 26 d in aerated (open symbols with dashed line) or stagnant (closed symbols with solid line) solutions with 400 mM NaCl. Treatments were imposed when plants were 14-d-old. Each value is the mean of four replicates, and each replicate is a single plant grown in a different pot. Regression lines were calculated using all the raw data (32 points). The least tolerant accession, H546, is indicated in each part of the figure (considered in Discussion).

Fig. 3.6. Relationships between the percentage of dead leaf material and: (A) Na^+ concentrations in shoots, (B) K^+ concentrations in shoots, and (C) ratio of K^+/Na^+ . *H. maritimum* accessions were grown for 26 d in aerated (open symbols with dashed line) or stagnant (closed symbols with solid line) conditions with 400 mM NaCl. Treatments were imposed when plants were 14-d-old. Each value is the mean of four replicates, and each replicate is a single plant grown in a different pot. Regression lines were calculated using all the raw data (32 points). The least tolerant accession, H546, is indicated in each part of the figure (considered in Discussion).

3.5 DISCUSSION

This study evaluated the tolerance of eight *H. maritimum* accessions to combinations of aerated/stagnant and non-saline/salinised nutrient solutions, comparing these accessions for effects on leaf extension rate, whole plant RGR, tissue concentrations of Na^+ , Cl^- and K^+ , and leaf tissue injury. The study identified two *H. maritimum* accessions (H109 and WA9) as being relatively tolerant to combined salinity and stagnant treatments; these had higher RGR and better regulation of ion concentrations in leaves, when grown under stagnant saline conditions). Interestingly there was also one relatively sensitive accession (H546), which had a lower RGR and poorer regulation of ion concentrations in leaves when grown under stagnant saline conditions. With the 400 mM NaCl treatment (aerated or stagnant), tolerant *H. maritimum* accessions had highly effective Na^+ and Cl^- ‘exclusion’ to keep these ions at relatively low concentrations in leaves, and better maintenance of leaf K^+ concentrations. Furthermore, when grown under stagnant saline conditions, these tolerant accessions had lower leaf death, higher RGR, higher leaf extension rates and better regulation of ion concentrations in leaves. In tolerant accessions, there could be a causal link between lower leaf Na^+ and Cl^- concentrations and a higher ratio of K^+/Na^+ , decreased leaf injury and the better growth in stagnant saline solutions, as compared with sensitive accessions.

This evaluation of *H. maritimum* accessions showed significant variation of NaCl and waterlogging tolerance and confirmed the importance of ion regulation for minimising leaf death and maintaining high rates of leaf growth and thus tolerance to these stresses. In the present study, two *H. maritimum* accessions (H109 and WA9) had higher tolerance to salinity and combined stagnant-saline treatments than H546, the most sensitive accession, while the remaining accessions (H90, H155, H563, H826 and WA29) had intermediate tolerances. For example, under the most stressful conditions (stagnant-plus 400 mM NaCl) the RGR of H109 and WA9 (the most tolerant *H. maritimum* accessions) was ~48% of control; by contrast, H546 (the most sensitive accession) had a RGR of only 24% of control (Fig. 3.1). The lower RGR of the most sensitive accession was clearly associated with higher shoot Na^+ and Cl^- concentrations, a failure to maintain K^+ concentrations (Fig. 3.3) and a far higher percentage of dead leaves (viz. 58% versus ~13%; Fig. 3.2). The lower growth of the most sensitive accession is likely not only caused by low capacity of the roots to ‘exclude’ Na^+ and Cl^- but also less ‘dilution’ by growth of the salt arriving to the shoot.

Minimal leaf injury and the maintenance of a high rate of leaf growth are important indications of plant health during salinity stress (Munns 1993; Munns 2002). Here I discuss the evidence for these as likely contributing factors to the tolerance of *H. maritimum* accessions to combined salinity and stagnant treatments. In comparison with salinity alone, the combination of hypoxia and salinity often increases leaf injury (necrosis) (Barrett-Lennard 2003; Munns *et al.* 2011). This increased leaf injury is considered to be a result of higher Na^+ and Cl^- and decreased K^+ transport to the shoots (Barrett-Lennard 2003; Barrett-Lennard and Shabala 2013), which in turn results in higher concentrations of Na^+ and Cl^- , and lower concentrations of K^+ in tissues/compartments, which causes leaf damage and consequently reduced plant growth (Munns and Tester 2008). The present study found that for *H. maritimum*, exposure to salinity or stagnant treatment with salinity caused relatively low amounts of dead leaf material (ranging between 3% and 13% of shoot DM) in tolerant accessions, but much greater leaf death (up to 58% of shoot DM) in the sensitive accession (H546; Fig. 3.2B). The greater leaf damage in H546 was due to older leaves dying faster than in the tolerant accessions. In the long run, when dead leaf material becomes a significant percentage (more than 20%) of the whole shoot, this reduces photosynthetic capacity,

decreases plant growth and can ultimately threaten survival (Munns 2002). Thus, the greater than 20% leaf death for the sensitive accession in aerated saline or stagnant saline solution (Fig. 3.2), as well as the slowed leaf extension rate (to ~12% of control; Fig. 3.4) suggests that this accession would be unlikely to survive to maturity. By contrast, the relatively low leaf death and high leaf extension rate in the two tolerant *H. marinum* accessions suggests that these would likely have tolerated the combination of salinity and waterlogging to reach the reproductive phase.

In monocotyledonous plants, salinity tolerance is achieved through a combination of quantitative traits, which are: (i) the ability to minimise the rate of Na^+ and Cl^- transport into shoots, (ii) the ability to maintain the ratio of K^+/Na^+ in leaves, (iii) the restoration of osmotic balance, and (iv) the compartmentalisation of toxic ions into vacuoles (Munns 2002; Cuin *et al.* 2003; Munns and James 2003; Colmer *et al.* 2006b; Munns and Tester 2008). The present data show that the tolerant *H. marinum* accessions were better able to ‘exclude’ Na^+ and Cl^- from the shoot, compared with the sensitive accession (H546). For example, when grown in saline or stagnant-saline solutions, green shoot tissues Na^+ concentration was up to 44% higher in H546 than in the tolerant accessions (H109 and WA9, Fig. 3.3B and 3.3C). Moreover, Malik *et al.* (2009a) reported that *H. marinum* accessions not only differ in capacity for ion ‘exclusion’ from leaves, but also speculated that accessions could also differ in ‘tissue tolerance’, as indicated for other species in the Triticeae. Tissue tolerance is considered as the ability to improve osmotic adjustment by accumulating Na^+ and Cl^- in the vacuoles of leaves (Greenway and Munns 1980; Munns and Tester 2008) whilst maintaining leaf function and plant growth. Concentrations of internal Na^+ above 100 mM will likely start to reduce the function of most enzymes (Munns *et al.* 1983), so when Na^+ concentrations exceeded 100 mM in tissue, salt tolerant plants have a highly effective ability to sequester Na^+ in vacuoles (Munns and James 2003). In the present study, two *H. marinum* accessions (H90 and WA29) maintained growth with less leaf injury (Fig. 3.5E), but had higher Na^+ concentrations in their shoots than other intermediate tolerant accessions (Fig. 3.5A); these accessions with high tissue Na^+ concentrations and little leaf damage and reasonable growth thus might have had better compartmentalisation of Na^+ and Cl^- in the vacuoles.

The ratio of K^+/Na^+ is an important trait for salinity tolerance (Maathuis and Amtmann 1999; Rubio *et al.* 1999; Cuin *et al.* 2003; Munns and Tester 2008; Barrett-Lennard and Shabala 2013). Under salt stress, the cytosolic ratio of K^+/Na^+ can drop significantly as a result of Na^+ accumulation in the cytosol and K^+ leakage from the cells (Maathuis and Amtmann 1999; Zhu 2000; Shabala 2000; Leigh 2001; Shabala *et al.* 2003). Ratios of K^+/Na^+ less than 1 have been reported to reduce the function of enzymes (e.g. case of wheat germ; Greenway and Munns 1980). The ability to maintain cytosolic K^+ homeostasis was reported to correlate with salinity tolerance (Chen *et al.* 2005; Chen *et al.* 2007a,b; Shabala and Cuin 2008; Smethurst *et al.* 2008; Barrett-Lennard and Shabala 2013). The contribution of K^+/Na^+ to salinity tolerance varies between species (Munns and Tester 2008). For example, at 100 mM NaCl the ratio of K^+/Na^+ for bread wheat is 4.8-fold higher than for barley (Gorham *et al.* 1990). Two tolerant *H. maritimum* accessions (i.e. H109 and WA9) maintained lower shoot Na^+ (10-23% lower than the other accessions) and higher K^+ concentrations (17-26% higher than the other accessions), resulting in higher ratios of K^+/Na^+ compared to the other accessions.

Compared to the aerated saline treatment, the combined stagnant and saline treatment mostly had only slight additional impacts on growth and shoot ion concentrations in *H. maritimum* accessions. This is consistent with *H. maritimum* being tolerant to waterlogging (McDonald *et al.* 2001a; Garthwaite *et al.* 2003), salt (Garthwaite *et al.* 2005; Islam *et al.* 2007) and the two stresses combined (Malik *et al.* 2009a,b). The present study extended this knowledge by demonstrating the direct impact of the combined stagnant and saline treatments on ion regulations and the subsequent impacts on leaf extension rate and leaf tissue injury of *H. maritimum*, and even at the much higher NaCl treatment level of 400 mM (present study) than the 200 mM NaCl used in the previous work by Malik *et al.* (2009a,b).

Salinity stress causes a rapid decline in the leaf growth rate (Fig. 3.4 present study; Munns 1993) and consequently reduces plant growth (Fig. 3.1 present study; Richards 1995; Flowers and Yeo 1995; Munns *et al.* 2006). Growth reductions due to salinity have been suggested to occur in two phases; the rapid, osmotic effect of salt outside the roots (which persists) and the longer-term ionic phase during which the toxic effects of Na^+ and/or Cl^- in the shoot tissues occur when threshold tissue/compartments concentrations are exceeded (cf. Munns *et al.* 1995). With the maintenance of higher K^+

concentrations (above 800 $\mu\text{mol g}^{-1}$ DM) and lower Na^+ concentrations (less 1000 $\mu\text{mol g}^{-1}$ DM) in the shoot, tolerant *H. marinum* accessions were able to sustain a ratio of K^+/Na^+ of 0.9 in the shoot tissues, which presumably was still above 1 in the cytoplasm, regarded as the lowest value of this ratio for enzymes to function (Greenway and Munns 1980). In addition, the tolerant *H. marinum* accessions were able to avoid leaf damage (less than 20% of shoot DM; Fig. 3.6) and maintain higher growth (0.09 $\text{g g}^{-1} \text{d}^{-1}$; Fig. 3.5). Salinity inhibits leaf growth as one of the main effects of salt stress (Munns 1993). However, the underlying mechanisms of leaf growth inhibition by salinity are still not fully understood (Munns 1993; Lazof and Bernstein 1998; Zhu 2001; Kravchik and Bernstein 2013). In the present study, the higher leaf growth of the tolerant accessions, compared to the sensitive accession, was presumably a result of the higher capacity in the tolerant accessions to maintain higher ratio of K^+/Na^+ in the shoot and therefore sustain enzyme functions, as well as possible differences in osmotic adjustment.

Quantities of Na^+ and Cl^- can reach toxic levels in older leaves if the external NaCl is high, or even at moderate external NaCl when combined with waterlogging (Barrett-Lennard 2003). Accumulation of ions to toxic levels causes leaf injury, which becomes visible as chlorosis and then necrosis (Barrett-Lennard *et al.* 1999; Munns and Tester 2008), which adds an extra restriction on plant growth to osmotic effect of salt outside the roots (Munns *et al.* 1995). However, ion toxicity occurs after a given threshold when the tolerable concentration in the tissue is exceeded which can occur when the ability to exclude ions becomes impaired. This external concentration for which the exclusion ability of the roots and tissue tolerance determine the external threshold concentration is lower in wheat (200 mM NaCl; Colmer *et al.* 1995) and barley (300 mM NaCl) than wild *Hordeum* species (450 mM; Garthwaite *et al.* 2005). The relationships between these key traits (i.e. growth, internal tissue ion concentrations and dead leaf materials) of salt and waterlogging tolerance were investigated using regression analyses for all *H. marinum* accessions grown in aerated and/or stagnant plus 400 mM NaCl solutions. The regression analysis showed that higher shoot Na^+ and Cl^- concentrations and dead leaf material were significantly related to reduced plant growth. By contrast, the greater plant growth rates were significantly related to higher shoot K^+ and K^+/Na^+ ratio (Fig. 3.5). On the contrary, the greater percentage of dead leaf material was significantly positively related to shoot Na^+ and negatively related to shoot K^+ and K^+/Na^+ ratio (Fig. 3.6). *H. marinum* accessions have superior leaf ion regulation than wheat, which make

them more tolerant to combined salinity and waterlogging (Garthwaite *et al.* 2003; 2005; Malik *et al.* 2009a,b). This evaluation of the relationships between key traits associated with salt and waterlogging tolerance has confirmed the importance of ion regulation for minimising leaf death and maintaining high rate of leaf growth and thus tolerance to these stresses.

Adverse interactions between waterlogging and salinity on non-halophytes, such as wheat, result in increased leaf damage due to increased rates of Na⁺ and Cl⁻ transport to the shoot (Barrett-Lennard 2003). However, wetland halophytes, such as *H. maritimum*, have the ability to minimise the interaction due to better ion regulation and high root porosity (Colmer and Flowers 2008). *H. maritimum* has the ability to exclude Na⁺ and Cl⁻ at high salinity (450 mM NaCl in aerated solution; Garthwaite *et al.* 2005), also when combined with stagnant conditions (Fig. 3.3 this study for 400 mM NaCl; extending the work at 200 mM NaCl by Malik *et al.* 2009a,b). The ability to minimise leaf death and maintain growth (Fig. 3.2 and 3.4), makes *H. maritimum* a potential candidate to improve the salt and waterlogging tolerance of wheat via wide hybridisations (Colmer *et al.* 2005b; 2006b; Islam *et al.* 2007; Malik *et al.* 2009a,b; Chapter 5).

3.6 SUPPLEMENTARY MATERIALS

Fig. 3.S1. Total dry mass for eight accessions of *Hordeum marinum*. Treatments were imposed when plants were 14-d-old. Plants were treated for 26 d with aerated (control) or stagnant non-saline nutrient solution (A), aerated or stagnant nutrient solution plus 200 mM NaCl (B), or aerated or stagnant nutrient solution plus 400 mM NaCl (C). The data and errors for the 200 and 400 mM treatments (B and C) are reported as % of control. Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between genotypes at $P = 0.05$; ns = not significant.

Fig. 3.S2. Concentrations of Cl^- (A and B), Na^+ (C and D) and K^+ (E and F) in the youngest fully expanded leaves (YFEL) for eight accessions of *Hordeum marinum*. Treatments were imposed when plants were 14-d-old. Plants were treated for 26 d with aerated or stagnant non-saline nutrient solution (A, C and E), or aerated or stagnant nutrient solution plus 200 mM NaCl (B, D and F). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between genotypes at $P = 0.05$; ns = not significant.

Table 3.S1. Initial leaf length on the commencement of leaf extension rate measurements of eight accessions of *Hordeum marinum* grown in aerated or stagnant nutrient solution, also containing 0.2, 200 or 400 mM NaCl.

Accession	Subspecies	Initial leaf length					
		0.2 mM NaCl		200 mM NaCl		400 mM NaCl	
		Aerated	Stagnant	Aerated	Stagnant	Aerated	Stagnant
H90	<i>marinum</i>	5.8 ± 0.3	4.6 ± 0.7	3.9 ± 0.6	4.7 ± 0.4	4.0 ± 1.0	2.0 ± 0.9
H109	<i>marinum</i>	3.2 ± 0.5	5.0 ± 1.2	2.8 ± 0.4	5.7 ± 1.2	5.3 ± 0.5	3.5 ± 0.6
H546	<i>marinum</i>	3.7 ± 1.3	4.9 ± 0.2	2.7 ± 0.7	4.7 ± 0.7	2.6 ± 0.8	2.1 ± 0.6
H155	<i>gussoneanum</i>	3.3 ± 0.9	2.8 ± 0.7	5.1 ± 1.5	4.6 ± 0.7	4.3 ± 0.4	3.3 ± 0.3
H563	<i>gussoneanum</i>	4.2 ± 0.3	3.5 ± 0.7	4.2 ± 1.3	2.5 ± 0.5	4.3 ± 0.9	3.6 ± 0.9
H826	<i>gussoneanum</i>	3.3 ± 1.5	3.0 ± 0.6	4.4 ± 0.9	4.1 ± 0.7	4.1 ± 0.9	3.2 ± 0.9
WA9	- -	4.7 ± 1.5	5.9 ± 0.8	3.7 ± 0.2	3.8 ± 0.5	3.1 ± 0.7	2.2 ± 0.3
WA29	- -	4.9 ± 0.8	4.5 ± 0.6	4.7 ± 0.8	2.8 ± 0.7	4.3 ± 1.3	4.4 ± 0.6
l.s.d.		ns	ns	ns	ns	ns	ns

Treatments were imposed when plants were 14-d-old. The measurements were started from when the stagnant agar treatment was imposed. Values are the mean of four replicates ± standard errors, and each replicate represents a single plant grown in a different pot. ns = not significant at $P = 0.05$.

Table 3.S2. *P* values from the ANOVA of all parameters fitted to the entire data set (all treatments).

Parameter	S	A	Acc	S×A	A×Acc	S×Acc	A×S×Acc	Fig. and Table
RGR	<0.001	<0.001	<0.001	0.147	0.667	0.001	0.689	Fig. 3.1
at 0.2 mM NaCl	-	<0.001	<0.001	-	0.161	-	-	3.1A
at 200 mM NaCl	-	<0.001	<0.001	-	0.362	-	-	3.1B
at 400 mM NaCl	-	<0.001	<0.001	-	0.98	-	-	3.1C
Percentage of dead leaf material	<0.001	<0.001	<0.001	0.311	0.004	<0.001	0.391	Fig. 3.2
at 0.2 mM NaCl	-	<0.001	<0.001	-	<0.001	-	-	3.2A
at 200 mM NaCl	-	<0.001	<0.001	-	0.132	-	-	3.2B
at 400 mM NaCl	-	<0.001	<0.001	-	0.267	-	-	3.2C
Green shoot Na ⁺ concentration	<0.001	<0.001	<0.001	0.223	0.775	0.227	0.908	Fig. 3.3A, 3.3B and 3.3C
at 0.2 mM NaCl	-	<0.001	<0.001	-	<0.001	-	-	3.3A
at 200 mM NaCl	-	0.004	0.078	-	0.624	-	-	3.3B
at 400 mM NaCl	-	0.006	0.092	-	0.917	-	-	3.3C
Green shoot Cl ⁻ concentration	<0.001	0.061	<0.001	<0.001	0.01	<0.001	0.115	Fig. 3.3D, 3.3E and 3.3F
at 0.2 mM NaCl	-	<0.001	0.277	-	0.2	-	-	3.3D
at 200 mM NaCl	-	0.774	<0.001	-	<0.001	-	-	3.3E
at 400 mM NaCl	-	0.006	<0.001	-	0.807	-	-	3.3F
Green shoot K ⁺ concentration	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	Fig. 3.3G, 3.3H and 3.3I
at 0.2 mM NaCl	-	<0.001	0.062	-	<0.001	-	-	3.3G
at 200 mM NaCl	-	<0.001	<0.001	-	<0.001	-	-	3.3H
at 400 mM NaCl	-	<0.001	<0.001	-	0.782	-	-	3.3I
Green shoot K ⁺ /Na ⁺ ratio	<0.001	<0.001	<0.001	0.449	0.006	0.076	<0.001	Table 3.1
at 0.2 mM NaCl	-	0.001	0.21	-	0.002	-	-	Table 3.1
at 200 mM NaCl	-	<0.001	0.002	-	0.143	-	-	Table 3.1
at 400 mM NaCl	-	<0.001	<0.001	-	0.025	-	-	Table 3.1
LER	<0.001	<0.001	<0.001	0.002	0.192	<0.001	0.061	Fig. 3.4
at 0.2 mM NaCl	-	<0.001	<0.001	-	0.214	-	-	3.4A
at 200 mM NaCl	-	<0.001	<0.001	-	0.024	-	-	3.4B
at 400 mM NaCl	-	0.007	<0.001	-	0.896	-	-	3.4C
Dry mass	<0.001	<0.001	<0.001	<0.001	0.373	0.276	0.003	Fig. 3.S1
at 0.2 mM NaCl	-	<0.001	0.019	-	0.025	-	-	3.S1A
at 200 mM NaCl	-	<0.001	<0.001	-	0.149	-	-	3.S1B
at 400 mM NaCl	-	<0.001	<0.001	-	0.632	-	-	3.S1C
YFEL Na ⁺ concentration	<0.001	<0.001	<0.001	0.006	0.303	0.2	0.005	Fig. 3.S2A and 3.S2B
at 0.2 mM NaCl	-	<0.001	<0.001	-	<0.001	-	-	3.S2A
at 200 mM NaCl	-	<0.001	0.002	-	0.255	-	-	3.S2B
YFEL Cl ⁻ concentration	<0.001	<0.001	<0.001	0.027	0.373	<0.001	0.345	Fig. 3.S2C and 3.S2D
at 0.2 mM NaCl	-	<0.001	0.076	-	0.344	-	-	3.S2C
at 200 mM NaCl	-	<0.001	<0.001	-	0.545	-	-	3.S2C
YFEL K ⁺ concentration	<0.001	<0.001	0.354	0.232	0.029	0.175	0.246	Fig. 3.S2E and 3.S2F
at 0.2 mM NaCl	-	<0.001	0.552	-	0.142	-	-	3.S2E
at 200 mM NaCl	-	<0.001	0.1	-	0.057	-	-	3.S2F

Treatments were imposed when plants were 14-d-old. Plants were treated for 26 d. Four replicates each one represents a single plant grown in a different pot. S = salinity; A = aeration; Acc = accession; LER = leaf extension rate; YFEL = youngest fully expanded leaf.

CHAPTER 4

An evaluation of the growth, photosynthetic rate, stomatal conductance and leaf damage of two *Hordeum marinum* accessions with NaCl and stagnant treatments

4.1 ABSTRACT

Hordeum marinum, a wild species in the Triticeae, is a relatively salinity and waterlogging tolerant species as compared with wheat. The hypothesis tested was that a well-adapted *H. marinum* accession would maintain low concentrations of toxic ions (Na^+ and Cl^-) in young leaves and have a greater capacity to tolerate high ion concentrations prior to tissue death in older leaves. This study investigated the variation between two *H. marinum* accessions with respect to growth parameters, leaf gas exchange, leaf ion concentrations and tissue damage when subjected to stagnant and 400 mM NaCl root-zone treatments. Accession H109 displayed higher tolerance to salinity and stagnant treatment than H155. Under combined stagnant and salinity treatment H109 had higher DM (1.3-fold) than H155. In addition to the lower proportion of dead leaf material in H109 of 16% of shoot DM under saline treatment compared to 27% of shoot DM in H155, the amounts of Na^+ and Cl^- in dead leaf material was higher (up to 18% higher) in H109 than H155. The greater tolerance to salinity and/or stagnant treatment in H109 was also evident by the maintenance of higher gas exchange rates in young leaves. Both accessions suffered large reductions in net photosynthesis under aerated saline or stagnant saline treatments, which was caused by low stomatal conductance. This study identified *H. marinum* accession H109 as salt and stagnant-tolerant, and demonstrated that the better maintenance of growth was associated with less leaf tissue death, lower tissue Na^+ and Cl^- concentrations in young leaves but higher in the old leaves and higher rates of leaf gas exchange, than in the less tolerant accession H155.

4.2 INTRODUCTION

Saline environments affect plant growth due to the osmotic stress of salt in the root-zone, as well as the toxicity effects caused by accumulating Na^+ and/or Cl^- in the shoot tissues (Munns *et al.* 1995; James *et al.* 2008; Munns and Tester 2008). Furthermore, salinity is often accompanied by waterlogging (Barrett-Lennard 2003; Bennett *et al.* 2009; Barrett-Lennard and Shabala 2013). Waterlogging decreases root growth and affects the viability of roots (Barrett-Lennard 1986; Bennett *et al.* 2009). Consequently, the resulting root hypoxia can reduce growth for sensitive species (Setter and Waters 2003). In addition, the combination of salinity and waterlogging decreases the capacity of roots to ‘exclude’ Na^+ and Cl^- and maintain K^+ in shoots (e.g. wheat, Barrett-Lennard *et al.* 1999) and increases leaf injury (necrosis) (Barrett-Lennard 2003; Munns *et al.*

2011; Barrett-Lennard and Shabala 2013) which decreases the area for whole plant photosynthesis.

The amount of dead leaf material is an important indicator of plant health during salinity stress (Munns 2002). Increased leaf injury is a result of higher Na^+ and Cl^- and decreased K^+ concentrations in the shoots, which causes leaf damage and consequently reduces growth rate (Barrett-Lennard 2003; Munns *et al.* 2011). The combination of salinity and waterlogging (and also when each stress is alone) can also cause stomatal closure (Kriedemann and Sands 1984; van der Moezel *et al.* 1989; Galloway and Davidson 1993; Barrett-Lennard 2003). Stomatal conductance is a sensitive indicator of osmotic stress, as stomata actively close as an initial response to salt stress (James *et al.* 2008) or waterlogging (Folzer *et al.* 2006). Stomatal factors (i.e. low conductance) limit CO_2 absorption, resulting in reduced photosynthesis and plant growth (Rahnama *et al.* 2010). Photosynthesis can also be limited by non-stomatal factors occurring over time as the salt builds-up in the leaves (James *et al.* 2002) and damages the photosynthetic machinery or enzyme functioning more generally. Thus, ion toxicity in leaves also seems to be a main cause for the non-stomatal limitation of photosynthesis (Huang and van Steveninck 1989; Cramer *et al.* 1993). Therefore, assessments of stomatal and/or non-stomatal limitations to photosynthesis in plants could explain the decreased growth rate of plants with combined salinity and waterlogging.

Hordeum marinum Huds. is a wild Triticeae species that grows in salt marshes (von Bothmer *et al.* 1995) and shows tolerance to waterlogging (McDonald *et al.* 2001a; Garthwaite *et al.* 2003), salinity (Garthwaite *et al.* 2005), and these stresses combined (Malik *et al.* 2009a,b; Chapter 3); it has possibly the highest tolerance to salinity and waterlogging within the Triticeae (Colmer *et al.* 2006b). Diversity in *Hordeum marinum* accessions for salinity and waterlogging tolerance has been previously reported (Malik *et al.* 2009a,b; Chapter 3). Tissue tolerance has not been assessed in previous studies of *H. marinum*, although it has been reported that *H. marinum* had low shoot Na^+ concentrations compared to some other *Hordeum* species (Garthwaite *et al.* 2005). The objective of the present study was to compare two *H. marinum* accessions (H109 and H155) for physiological responses to salt and stagnant treatments. The key traits evaluated were growth responses, leaf ion concentrations (Na^+ , Cl^- and K^+), leaf tissue injury and parameters of leaf gas exchange. The findings are discussed in the context of

understanding salt and waterlogging tolerance in the Triticeae and the interactive effects of salinity and root hypoxia on plants.

The hypotheses tested were that:

- (a) the combination of salinity (400 mM NaCl) and stagnant treatments would result in a more detrimental response than the product of the individual factors, for both *H. marinum* accessions
- (b) the more sensitive accession (H155) would reach a toxic level of leaf Na⁺ and Cl⁻ faster than the more tolerant accession (H109), as indicated by relationships between the increased concentrations of these ions in the leaves and: (i) a high rate of leaf death, (ii) restrictions in the growth of new tillers, and (iii) restrict to photosynthetic machinery; and
- (c) the tolerant accession would have higher Na⁺ and Cl⁻ concentrations in the dead leaves, possibly indicative of a capacity to tolerate higher ion concentrations prior to tissue death.

4.3 MATERIALS AND METHODS

Plant material and growth conditions

Three diploid accessions of *Hordeum marinum* Huds. (H109, H155 and H546) were initially used, based on results from the first experiment (Chapter 3), which showed significant variation in salinity and waterlogging tolerance. Unfortunately, seeds of accession H546 did not germinate, so only H109 and H155 were used in the experiment. Seeds were originally provided by the Nordic Gene Bank (R. von Bothmer, Swedish Agricultural University, Alnarp) and were raised in a glasshouse to provide those seeds used here.

Experimental design

The experiment consisted of 2 accessions (H109, H155) subjected to 2 salinity treatments (0.2 or 400 mM NaCl) × 2 aeration treatments (aerated or stagnant solutions) × 4 replicates, in a completely randomised design. Salt concentration (400 mM NaCl) was chosen based on results from the first experiment (Chapter 3), as *H. marinum*

accessions showed significant variation in salinity and stagnant treatment tolerance when grown at 400 mM NaCl.

The experiments were conducted in a naturally-lit phytotron (20/15°C) day/night with an average PAR of 1,160 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at midday during the experimental period. The experiment was conducted during the period between 24-Mar-2011 and 29-May-2011 at the School of Plant Biology, UWA, Perth, Western Australia. After imposition of treatments (see below) experimental units (pots) were rotated weekly to minimise any possible environmental variation within the phytotron. Each pot was either aerated or stagnant and had one of two salinity levels (0.2 or 400 mM NaCl).

Seeds were surface-sterilised with 0.04% NaHClO in de-ionised (DI) water for 45 s, and then rinsed thoroughly with DI water prior to being imbibed overnight in aerated 0.5 mM CaSO_4 . Seeds were then transferred onto mesh floating on 10% strength aerated nutrient solution in darkness (pots completely covered with Al-foil) in a 20/15 °C day/night phytotron for the first 4 d. Seedlings were exposed to light after 4 d and nutrient solutions were changed to 25% strength.

Eight seedlings per pot were transplanted into 4.5 L pots containing full-strength nutrient solution on day 7. Seedlings were held in the pot lids using polystyrene foam in holes (2 cm diameter). The full-strength nutrient solution contained (mM): K^+ , 3.95; Ca^{2+} , 4; Mg^{2+} , 0.4; NH_4^+ , 0.625; Na^+ , 0.2; NO_3^- , 4.375; SO_4^{2-} , 4.4; H_2PO_4^- , 0.2; H_4SiO_4^- , 0.1; with micronutrients (μM): Cl^- , 50; B, 25; Mn, 2; Zn, 2; Ni, 1; Cu, 0.5; Mo, 0.5; Fe-EDTA, 50. *H. marinum* can be prone to Fe-deficiency under these conditions; therefore additional 5 μM FeSO_4 was supplied routinely (on 5 occasions to the young plants) to prevent any risk of such deficiency, but always to all pots. The solution was also buffered with 2.5 mM 2-[N-Morpholino]ethanesulfonic acid (MES) and the pH was adjusted to 6.5 using KOH, increasing the final K^+ concentration to ~6 mM.

Treatments and harvests

Treatments were imposed when plants had 2–2.5 leaves (14-d-old plants). Prior to the treatments being imposed, an initial harvest of 4 replicate plants for each accession was taken to measure initial dry mass (DM).

After this, NaCl was stepped up in pots allocated to saline treatments by 50 mM every 12 h until the final concentrations were reached (84 h). After reaching the final NaCl concentration, pots allocated to the stagnant treatment were given a ‘hypoxic pre-treatment’ in which the solution in these pots was bubbled with N₂ for ~2 h until the dissolved O₂ concentration was less than ~0.03 mM; these pots were then left overnight without bubbling. The following day, the nutrient solution in the stagnant pots was replaced with deoxygenated (i.e. pre-flushed with N₂ overnight) stagnant nutrient solution containing 0.1% (w/v) agar (non-saline or saline, as appropriate). The agar inhibits convective movements in the nutrient solution, impeding the re-entry of O₂ and promoting the accumulation of ethylene, both important changes in the gas composition of waterlogged soils (Wiengweera *et al.* 1997). The nutrient solutions of pots allocated for aerated treatments (non-saline and saline) were also renewed at the same time as the stagnant treatment, but these solutions were without agar and continued to be bubbled with air. A second harvest of 4 replicate plants for each accession was taken 24 h after the imposition of stagnant treatments to measure root and shoot DM. Solutions in all pots were renewed every 7 d and aerated pots were topped up with DI water as required.

The treatments were imposed for 38 d after the stagnant treatment commenced. During the treatment period dead leaves (approx. 90% of tissue dead on an individual leaf blade) were collected from each plant on the day that this extent of individual leaf damage occurred and the dead parts were placed into separate paper bags and then oven-dried (for 72 h at 65°C) for measurements of DM and tissue ion analyses from all dead leaves. In addition, six harvests of one plant from each pot, providing 4 replicate plants of each accession, were taken on days 3, 10, 17, 24, 31 and 38 after treatments commenced. At each harvest, roots and the stem base were washed 3 times for 30 s in mannitol solutions iso-osmotic with the Na⁺ plus Cl[−] concentration of the nutrient solution; these wash solutions also contained 4 mM CaSO₄. For harvests on days 3 and 24, 4 replicate plants for each accession were separated into five tissue classes: the youngest fully expanded leaf (YFEL), second youngest fully expanded leaf (2nd YFEL),

60

other green shoot tissues (i.e. stems, sheaths and all other green and turgid leaf blades), dead leaves and roots. Additional plants were harvested for measurements of whole shoot and root DM and tissue ion analyses.

Growth measurements

The final harvest was taken 38 d after the imposition of the stagnant treatment. For all harvests, shoot and root fresh mass (FM) and number of tillers were recorded before plants were separated into the components described above. DM of all samples was measured after being oven dried for 72 h at 65°C. The relative growth rate (RGR) was calculated from the DM of the whole-plant samples at the start and end of the treatment period, using the formula described by Hunt (1978):

$$\text{RGR} = (\ln DM_2 - \ln DM_1) / (t_2 - t_1)$$

Where DM_1 is the dry mass (g) at time one (initial harvest), DM_2 is the dry mass (g) at time two (final harvest), t_1 and t_2 are times one and two in d, and $\ln$ is the natural logarithm.

Leaf gas exchange

Leaf gas exchange measurements were taken of the intact YFEL and 2nd YFEL on days 2 and 21 of treatments. Measurements were taken between 10:00 and 14:00 hours using a LI-COR 6400 Photosynthesis System at ambient relative humidity (50–60%), CO_2 concentrations of 380, 800 and 1200 $\mu\text{mol mol}^{-1}$, flow rate of 200 $\mu\text{mol s}^{-1}$ and PAR of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For salt treated plants (aerated and stagnant), the measurements with CO_2 concentrations above ambient were used to assess whether photosynthesis was restricted by stomatal or non-stomatal limitations (Pérez-López *et al.* 2012).

Tissue ion analyses

Concentrations of Na^+ , Cl^- and K^+ were determined in the different shoot tissues. Oven dried samples were ground and extracted in 0.5 M HNO_3 by shaking for 48 h at 20–25°C. Na^+ and K^+ concentrations were determined in dilutions of the extracts using a flame photometer (Jenway PFP7, Essex UK). Cl^- concentration was determined in the extracts using guanidine protocol of a chloridometer (Slamed CHLO 50 cl, Frankfurt,

Germany). Blanks and a reference plant material with known ion concentrations were taken through the same procedures. Recoveries of Na^+ , Cl^- and K^+ from the reference sample were 104, 101 and 95%, respectively. The data presented have therefore not been adjusted.

Data analyses

All statistical analyses were conducted using GENSTAT 12th Edition (VSN International Ltd. Hemel Hempstead, UK). Residuals were checked for normality and no transformations were needed. An ANOVA was used to identify overall significant differences between genotypes and between treatments. When significant differences were found, mean-separations were calculated using the Fisher unprotected LSD test. Unless otherwise stated, the significance level was $P \leq 0.05$. For the P -values from the ANOVA on the data of the parameters described below, see Supplementary Materials Table 4.S1.

4.4 RESULTS

Growth

Separate or combined salt and stagnant root-zone stresses significantly reduced total DM with time relative to control. In the control treatment total DM increased ~2.6-fold each 7 days in both accessions; however, by the end of the experiment (day 38) whole plant DM was higher in H155 (6.7 g) than H109 (2.7 g; Fig. 4.1A). Under stagnant non-saline treatment, H109 maintained higher DM (70% of control) than H155 (14% of control) by the end of the stagnant treatment (Fig. 4.1B). Under the aerated saline treatment, H109 also maintained higher DM (46% of control) than H155 (7% of control) until day 24 of treatment, however, by the end of the treatment, both accessions suffered great DM reductions (less than 7% of control; Fig. 4.1C). The combined salt and stagnant treatments further reduced DM of both accessions with time, relative to controls (Fig. 4.1D). Total DM was severely reduced in both accessions, but nevertheless was less affected in the H109 (5% of controls) than in H155 (1% of control) by the end of the stagnant saline treatment. Treatment effects on total plant RGR was consistent with these general trends in DM (see Supplementary Materials, Fig. 4.S1).

Fig. 4.1. Total DM of two *H. maritimum* accessions (H109 and H155) grown in aerated or stagnant nutrient solution with or without 400 mM NaCl. Treatments were imposed when plants were 14-d-old. Plants were treated for 38 d with aerated or stagnant treatments in non-saline or saline (400 mM NaCl) nutrient solutions. Harvests were taken on days 3, 10, 17, 24 31 and 38. Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between accessions at $P = 0.05$. Significant differences between accessions are indicated ($P < 0.05$, *; $P < 0.01$, **). Note the different y-axis for A (g) compared to B, C and D. The data and errors for the stagnant non-saline, aerated saline and stagnant saline treatments (B, C and D) are reported as % of control.

Number of tillers

In the aerated non-saline controls, the number of tillers increased with time but it was relatively similar for the two accessions, increasing from ~4 tillers on day 3 to ~100 tillers by day 38 of treatment (Fig. 4.2A). The number of tillers decreased in both accessions when subjected to separate or combined salt and stagnant root-zone stresses. Exposure of plants to the stagnant non-saline treatment decreased tillers in both accessions although the effects on H155 were more severe than for H109; after 38 days of treatment tiller number had been decreased to 45% of control for H109 but to only 18% of control for H155 (Fig. 4.2B). Exposure of plants to salinity reduced the number of tillers of both accessions relative to control even more severely. In aerated saline solutions, the number of tillers was reduced to ~19% of control for both accessions after by day 38 of treatments (Fig. 4.2C). The combined salt and stagnant treatments further reduced the number of tillers for both accessions relative to control (Fig. 4.2D). The number of tillers was significantly different between the two accessions by day 38 of stagnant saline treatment, with about 12 tillers (13% of control) recorded for H109, compared to about 4 tillers in H155 (5% of control). Relative to the aerated saline treatment, the combined stagnant saline treatment reduced the number of tillers to only 34% in H155, compared to 55% for H109.

Proportion of dead leaf material

Separate or combined salt and stagnant root-zone stresses significantly increased the proportion of dead leaf material relative to control; however, no dead leaf material was recorded in the first 17 days of either non-saline treatment. In the aerated non-saline controls, the proportion of dead leaf material was low in the two accessions, less than ~1.6% of shoot DM between days 3 and 38 of treatment (Fig. 4.3A). Relative to controls, the stagnant non-saline treatment increased dead leaf material by ~3-fold (~7% of shoot DM) for the two accessions between days 17 and 38 of treatment (Fig. 4.3B). In response to salt, H109 recorded less dead leaf (16% of shoot DM) than H155 (27% of shoot DM) by the end of the experiment (day 38) (Fig. 4.3C). The combined salt and stagnant treatments increased leaf death even further to 23% of shoot DM in H109 and to 34% of shoot DM in H155 by the end of the treatment (Fig. 4.3D). The combined salt and stagnant treatment increased leaf tissue death to ~37% relative to aerated saline treatment, with no significant difference between in the two accessions.

Fig. 4.2. The absolute number of tillers of two *H. maritimum* accessions (H109 and H155) grown in aerated or stagnant nutrient solution with or without 400 mM NaCl. Treatments were imposed when plants were 14-d-old. Plants were treated for 38 d with aerated or stagnant treatments in non-saline or saline (400 mM NaCl) nutrient solutions. Harvest were taken on days 3, 10, 17, 24 31 and 38. Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between accessions at $P = 0.05$; ns = not significant. Significant differences between accessions are indicated ($P < 0.05$, *; $P < 0.01$, **). Note the different scale on the y-axis for A compared to B, C and D.

Fig. 4.3. Dead leaf material (as a percentage of shoot DM) of two *H. maritimum* accessions (H109 and H155) grown in aerated or stagnant nutrient solution with or without 400 mM NaCl. Treatments were imposed when plants were 14-d-old. Plants were treated for 38 d with aerated or stagnant treatments in non-saline or saline (400 mM NaCl) nutrient solutions. Harvests were taken on days 3, 10, 17, 24, 31 and 38. Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between accessions at $P = 0.05$; ns = not significant. Significant differences between accessions are indicated ($P < 0.05$, *).

Shoot water content

The shoot water content of both accessions was significantly reduced when plants were in stagnant non-saline, aerated saline or the combination of stagnant saline solutions (Fig. 4.4). In controls, shoot water content was similar in the two accessions ($\sim 6 \text{ mL g}^{-1}$) by the end of the treatment (Fig. 4.4A). Stagnant non-saline treatment, decreased shoot water content, however, H109 had higher shoot water content (83% of control) than H155 (76% of control) by the end of the treatment (Fig. 4.4B). Relative to controls, saline treatment decreased shoot water content ($\sim 50\%$ of control) by the end of the treatment, however, no significant difference between in the two accessions by the end of saline treatments (Fig. 4.4C). The combined salinity and stagnant treatment reduced shoot water content even further to 69% of saline treatment (34% of control) by the end of the treatment (Fig. 4.4D).

Leaf gas exchange parameters

Saline treatments (aerated and stagnant) generally reduced gas exchange and these effects became more severe with time (Fig. 4.5 and 4.6). In the controls net photosynthesis and stomatal conductance in the two leaves were similar for both accessions and ranged ($\sim 475 \text{ mmol m}^{-2} \text{ s}^{-1}$ for stomatal conductance and $\sim 24 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ for net photosynthesis), indicating a relative similarity in instantaneous water use efficiency ($\sim 7 \text{ mmol mol}^{-1}$). Stagnant treatment did not affect the net photosynthesis and stomatal conductance in the first two days of treatments. By contrast, after day 23 of stagnant treatment, stomatal conductance was reduced substantially to an average of $\sim 16\%$ of control, accompanied by reductions in net photosynthesis (up to 54% of control). The one exception was for H109's YFEL, which maintained similar net photosynthesis rate in both control and stagnant non-saline treatments. Salinity treatment caused greater reductions in stomatal conductance (10–34% and 3–17% of control on day 2 or day 23 of treatment), and to net photosynthesis (less than 54% of control). The combined salinity and stagnant treatment reduced the stomatal conductance even further to 48–93% of the saline treatment on day 2 and from almost 0 to 56% of the saline treatment on day 23 of treatment. No differences were found between the two accessions within treatments. On day 23 of treatment, net photosynthesis in 2nd YFEL of H155 was completely inhibited with stagnant non-saline, aerated saline and stagnant saline treatments and stomata were almost closed, while the net photosynthesis in 2nd YFEL of H109 was only inhibited completely by the stagnant

saline treatment, while both leaves of H109 maintained reasonable photosynthetic activity in all other treatments (Fig. 4.5D and 4.6D). In general, treated H109 maintained better gas exchange than H155, however, in both accessions; gas exchange was mostly lower in treated 2nd YFEL than in the YFEL.

Elevated CO₂ would have increased the CO₂ availability in the chloroplasts, and therefore, this approach was used to assess whether the photosynthesis limitations were caused by the low stomatal conductance – if rates are limited by restricted CO₂ supply via stomata then photosynthesis should increase markedly when additional CO₂ is supplied to overcome this diffusion limitation. These time-consuming measurements could only be taken for salt treated plants (aerated and stagnant), owing to time limitations. With elevated CO₂, there was a substantial increase in the net photosynthetic rate in both leaves of plants in aerated saline and stagnant saline treatments (Fig. 4.7). Increasing CO₂ to 800 $\mu\text{mol mol}^{-1}$, increased net rates of photosynthesis by up to 10 and 7-fold on day 2 and day 23, relative to the ambient CO₂ concentration. At even higher concentrations of CO₂ (1200 $\mu\text{mol mol}^{-1}$) the net photosynthetic rate increased even further to 14 and 18-fold on day 2 or day 23, relative to ambient CO₂ concentration. No differences were found between stomatal conductance measurements at different CO₂ levels (data not presented). Therefore, the similarity in stomatal conductance at different CO₂ levels and importantly the ability of tissues to have high rates of net photosynthesis when elevated CO₂ was supplied, indicate that the photosynthesis was limited by stomatal conductance and the rates achieved under these high CO₂ conditions indicated that the photosynthetic machinery was functional.

Fig. 4.4. Shoot water content of two *H. maritimum* accessions (H109 and H155) grown in aerated or stagnant nutrient solution with or without 400 mM NaCl. Treatments were imposed when plants were 14-d-old. Plants were treated for 38 d with aerated or stagnant treatments in non-saline or saline (400 mM NaCl) nutrient solutions. Harvest were taken on days 3, 10, 17, 24 31 and 38. Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between accessions at $P = 0.05$; ns = not significant. Significant differences between accessions are indicated ($P < 0.05$, *; $P < 0.01$, **).

Fig. 4.5. Responses of net photosynthetic rate under ambient CO_2 concentrations ($380 \mu\text{mol mol}^{-1}$) in the youngest fully expanded leaf (YFEL) and 2nd YFEL of *H. maritimum* accessions (H109 and H155) grown in aerated or stagnant nutrient solution with or without 400 mM NaCl. Treatments were imposed when plants were 14-d-old. Plants were grown under aerated non-saline, stagnant non-saline, aerated saline or stagnant saline conditions in nutrient solutions for 2 (A and B) or 23 days (C and D). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between net photosynthetic concentration at various treatments at $P = 0.05$.

Fig. 4.6. Responses of stomatal conductance of the youngest fully expanded leaf (YFEL) and 2nd YFEL of *H. maritimum* accessions (H109 and H155) grown in aerated or stagnant nutrient solution with or without 400 mM NaCl. Treatments were imposed when plants were 14-d-old. Plants were grown under aerated non-saline, stagnant non-saline, aerated saline or stagnant saline conditions in nutrient solutions for 2 (A and B) or 23 days (C and D). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between stomatal conductance at various treatments at $P = 0.05$.

Fig. 4.7. Response to elevated CO_2 concentrations of the net photosynthetic rate of the youngest fully expanded leaf (YFEL) and 2nd YFEL of *H. maritimum* accessions (H109 and H155) grown in aerated or stagnant nutrient solution with 400 mM NaCl. Treatments were imposed when plants were 14-d-old. Plants were grown under aerated saline or stagnant saline conditions in nutrient solutions for 2 (A and B) or 23 days (C and D). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between net photosynthetic rate at different CO_2 concentrations at $P = 0.05$.

K⁺ concentrations in the youngest fully expanded leaf (YFEL) and 2nd YFEL

The highest leaf K⁺ concentration was measured in plants grown in aerated non-saline solutions (~1300 $\mu\text{mol g}^{-1}$ DM). In general, K⁺ concentrations in the YFEL and 2nd YFEL were substantially reduced when subjected to stagnant non-saline, aerated saline or the stagnant saline treatment, in comparison with the aerated non-saline controls (Fig. 4.8A and 4.8B). The stagnant non-saline treatment reduced K⁺ concentrations (to ~80% of aerated control) in both leaves in both accessions. In the aerated saline treatment, K⁺ concentrations were also reduced (to ~63% of the aerated control) for the two accessions. The combined salt and stagnant treatment reduced K⁺ concentrations further to ~78% of aerated saline treatment. In general across treatments, in the YFEL K⁺ concentrations were ~19% higher in H109 than in H155; by contrast, 2nd YFEL there were no differences in K⁺ concentrations 2nd YFEL between the two accessions. However, comparison between the leaves showed that K⁺ concentrations in the YFEL were higher (up to 38% higher) than in 2nd YFEL across treatments (Fig. 4.8A and 4.8B).

Na⁺ concentrations in the youngest fully expanded leaf (YFEL) and 2nd YFEL

The concentration of Na⁺ was low in leaves of plants grown in aerated or stagnant non-saline solutions (~40 $\mu\text{mol g}^{-1}$ DM), but the salinity treatment (in aerated or in stagnant) increased Na⁺ concentrations in the YFEL and 2nd YFEL (to values up to 21 times the control) in the two accessions (Fig. 4.8C and 4.8D). Compared with the controls, exposure of plants to salinity increased Na⁺ concentrations to ~20-times the control values in the two accessions. Interestingly, in comparison to the aerated saline treatment, the combined stagnant plus saline treatment had lower Na⁺ concentrations (values ~55% of those in the aerated saline treatment). Concentrations of Na⁺ declined over time, with the average values on day 24 being about 24% less than on day 3 (cf. Fig. 4.8C and 4.8D with Fig. 4.S4C and 4.S4D). No differences were found between the two accessions or the two tissues within treatments.

K⁺/Na⁺ ratio in the youngest fully expanded leaf (YFEL) and 2nd YFEL

In non-saline treatments, the ratios of K⁺/Na⁺ in the YFEL were similar (~37) in the two accessions. Compared with aerated non-saline controls, salinity treatments caused substantial reductions in the ratio of K⁺/Na⁺ (to ~5.3% of this ratio in the controls) in the

YFEL in the two accessions (Table 4.1). No differences were found in the K^+/Na^+ ratio within treatments due to accessions or tissues.

Cl⁻ concentrations in the youngest fully expanded leaf (YFEL) and 2nd YFEL

In general, leaf Cl^- concentrations showed similar trends to those of Na^+ in the various treatments (Fig. 4.8E and 4.8F). In non-saline solutions, Cl^- concentrations in the YFEL and 2nd YFEL were similar ($\sim 230 \mu\text{mol g}^{-1} \text{DM}$) in the two accessions. The aerated saline treatment increased Cl^- concentrations to ~ 5 -times the control values in the two accessions. In the combined stagnant plus saline treatment, concentrations of Cl^- were $\sim 35\%$ less than in the aerated saline treated plants of the two accessions. No differences were found in Cl^- concentrations within treatments due to accessions, tissues or time factors (cf. Fig. 4.8E and 4.8F with Fig. 4.S4E and 4.S4F).

Na⁺ concentrations in dead leaf material

Dead leaf material of the two accessions contained very high concentrations (up to 50-fold of control) of Na^+ and Cl^- when subjected to saline treatments under aerated or stagnant conditions (Table 4.1). With the aerated saline treatment, H109 had higher Na^+ concentrations ($4930 \mu\text{mol g}^{-1} \text{DM}$) than H155 ($3828 \mu\text{mol g}^{-1} \text{DM}$). In the stagnant saline treatment, H109 also had 12% higher Na^+ concentrations than H155 in the dead leaf material, the concentration of Na^+ these materials was 16% lower than in the same tissues of the aerated saline treatment.

Cl⁻ concentrations in dead leaf

Generally, Cl^- concentrations in dead leaf material of the two accessions showed similar trends to those of Na^+ in the various treatments (Table 4.1). Dead leaf material of the two accessions had ~ 19 -fold higher Cl^- concentrations subjected to salinity under aerated or stagnant conditions (Table 4.1). With the aerated saline treatment concentrations of Cl^- were higher in H109 ($5751 \mu\text{mol g}^{-1} \text{DM}$) than in H155 ($4755 \mu\text{mol g}^{-1} \text{DM}$). With the combined salinity and stagnant treatment, H109 had 10% higher Cl^- concentrations than H155 in the dead leaf material. Again, the concentration of Cl^- in the dead leaf materials was lower ($\sim 15\%$ lower) in plants grown with the combined stagnant saline treatment compared with the aerated saline treatment.

Fig. 4.8. Concentrations of K^+ (A and B), Na^+ (C and D) and Cl^- (E and F) in the youngest fully expanded leaf (YFEL) and 2nd YFEL of *H. maritimum* accessions (H109 and H155) grown in aerated or stagnant nutrient solution with or without 400 mM NaCl. Treatments were imposed when plants were 14-d-old. Plants were grown in aerated, stagnant non-saline, aerated saline or stagnant saline in nutrient solution for 24 days. Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between ion concentrations at various treatments at $P = 0.05$.

Table 4.1. Ratio of K^+/Na^+ in the youngest fully expanded leaf (YEFL; day 24) and Na^+ and Cl^- concentration in dead leaf material (from day 3–38) of *H. marimum* accessions (H109 and H155), when grown in aerated, stagnant non-saline, aerated saline or stagnant saline (saline = 400 mM NaCl). Values are the means of four replicates $\pm$ standard error, and each replicates represents single plants grown in different pots.

Treatment		Accession	K ⁺ /Na ⁺ in the YFEL	Na ⁺ concentration in dead leaves (μmol g ⁻¹ DW)	Cl ⁻ concentration in dead leaves (μmol g ⁻¹ DW)
Non-saline	Aerated	H109	37 ± 7.0	266 ± 132	374 ± 72
		H155	44 ± 8.5	76 ± 27	245 ± 28
	Stagnant	H109	44 ± 5.3	41 ± 9	111 ± 24
		H155	38 ± 9.7	43 ± 17	82 ± 13
Saline	Aerated	H109	2.4 ± 0.7	4930 ± 271	5751 ± 301
		H155	1.4 ± 0.2	3828 ± 242	4755 ± 123
	Stagnant	H109	2.5 ± 0.1	3866 ± 182	4643 ± 198
		H155	2.1 ± 0.4	3444 ± 214	4219 ± 149
l.s.d. A (treatment)			11.4	330	334

^AThe l.s.d. represents significant differences between treatment at $P = 0.05$.

4.5 DISCUSSION

The results of the present chapter show that *Hordeum marimum* accession H109 has higher tolerance to the combination of salinity with stagnant treatment than H155. The greater tolerance to salinity and/or salinity plus stagnant in H109 (i.e. higher DM as % of control as compared with H155), was associated with less leaf injury, with higher concentrations of Na^+ and Cl^- in the dead leaves, and with the maintenance of relatively higher gas exchange rates in the YFEL. Furthermore, the greater growth of H109 than H155 under saline/waterlogged conditions occurred despite the fact that the two accessions had similar concentrations of Na^+ and Cl^- in the YFEL and 2nd YFEL suggesting that accession H109 might have higher tissue tolerance to ions (cf. Yeo and Flowers 1983). In non-halophytes and monocotyledonous halophytes, also, tolerance not only depends on Na^+ and Cl^- exclusion from leaves (reviewed by Munns and Tester 2008). Earlier studies showed several genotypes with a similar degree of salinity tolerance, despite having different Na^+ concentrations in young leaves (e.g. 230–510

$\mu\text{mol g}^{-1}$ DM in a non-durum tetraploid subspecies, Munns *et al.* 2003). *H. marinum* also demonstrated an ability to avoid ion toxicity in the photosynthetically active leaves by restricting entry of Na^+ and Cl^- to the shoot. In young leaves, concentrations of Na^+ , K^+ and Cl^- were similar in H109 and H155 (Fig. 4.8), suggesting the difference in tolerance to salinity between the two accessions was more related to the large difference in both the proportion of dead leaf material, the amount of Na^+ and Cl^- accumulated in the dead tissues (Fig. 4.3C and 4.3D) and possible differences in tissue tolerance.

The amount of dead leaf material is an important indicator of plant health during salinity stress (Munns 2002). The saline treatments resulted in much larger leaf injury than did the non-saline treatments (Fig. 4.3). The osmotic effect of salt in the root-zone is the major initial component of the growth reduction of plants with saline root-zones, and the toxicity effects caused by accumulating Na^+ and/or Cl^- in the shoot tissues also play an inhibitory role with longer times of exposure (Munns *et al.* 1995; James *et al.* 2008; Munns and Tester 2008). Leaf injury from salinity (necrosis) is caused by the accumulation of toxic ions above tolerable levels (Munns and Tester 2008), which occurs first in the older leaves (Colmer *et al.* 1995). Salinity increased the proportion of dead leaves more in H155 (28%) than in H109 (16% after 38 days of treatment; Fig. 4.3C). Munns (2002) suggested that it was not until 20% of wheat leaves had died that the rate of leaf production declined dramatically and then eventually some plants died. Increased leaf injury caused by high tissue ion concentrations is a result of higher Na^+ and Cl^- transport to the shoots, causing injury to older leaves and consequently reducing the growth rate (Barrett-Lennard 2003; Munns *et al.* 2011). In addition to the lower proportion of dead leaf material in H109 compared with H155, the concentrations of Na^+ and Cl^- in these materials was ~18% and ~13% higher (respectively) in H109 than H155 (Table 4.1). These differences in ion concentrations in dead leaves may also be evidence of greater tissue tolerance in H109 than H155: presumably the higher Na^+ and Cl^- concentrations in the dead leaves of H109 than H155 are indicative of the higher threshold Na^+ and Cl^- concentrations that the cells contain at the moment of their death. Barley, a relative of *H. marinum*, has also been reported to accumulate Na^+ in old leaves to reduce the build-up in photosynthetically active leaves, and hence it has been suggested that barley has a high tissue tolerance (Munns *et al.* 2003). *H. marinum* demonstrated a high ability to avoid ion toxicity in the photosynthetically active leaves

by minimising the concentration of these ions in the young leaves accompanied by higher Na^+ and Cl^- concentration in old leaves prior to their death.

Salt tolerant plants have three general methods for decreasing the adverse effects of the entry of Na^+ and Cl^- into shoots: they can block the uptake of ions at the root surface, adsorb ions from the xylem stream on the way to the leaves, and within leaf cells, efficiently compartmentalise the ions in the vacuoles thereby minimising the effect of ion toxicity on the enzymatic processes that occur primarily in the cytoplasm (Flowers *et al.* 1977). Moreover, salt tolerant plants, such as *H. maritimum*, have the ability to ‘exclude’ ions from young leaves (see Garthwaite *et al.* 2005; Islam *et al.* 2007) and presumably improved capacity to sequester ions that do enter into vacuoles (‘tissue tolerance’) before damaging the leaves (Genc *et al.* 2007). In a previous study of *H. maritimum*, its YFEL had concentrations of Na^+ and Cl^- of less than $\sim 700 \mu\text{mol g}^{-1}$ DM at external NaCl concentrations up to 450 mM NaCl (Garthwaite *et al.* 2005); relatively similar concentrations were found in the present study of $890 \mu\text{mol g}^{-1}$ DM in YFEL when plants were grown in 400 mM NaCl for 44 days (Fig. 4.8C and 4.8D). By contrast, dead leaves of *H. maritimum* contained high concentrations of Na^+ (up to $4930 \mu\text{mol g}^{-1}$ DM), which is similar with other species suggested to have high tissue tolerance (e.g. $4080 \mu\text{mol g}^{-1}$ DM were found in barley cv. Skiff; Munns *et al.* 2003). However, concentrations of internal Na^+ above 100 mM (equivalent to $500 \mu\text{mol g}^{-1}$ DM with the shoot tissue water content of *H. maritimum*, Fig. 4.4) will start to reduce the function of most enzymes (Munns *et al.* 1983), so when Na^+ concentrations exceeded $500 \mu\text{mol g}^{-1}$ DM in tissue, salt tolerant plants must have an effective compartmentation of Na^+ in vacuoles (Munns and James 2003).

Salinity tolerance is also associated with the ratio of K^+/Na^+ (Maathuis and Amtmann 1999; Rubio *et al.* 1999; Cuin *et al.* 2003; Munns and Tester 2008; Barrett-Lennard and Shabala 2013). Maintaining a high ratio of K^+/Na^+ in the cytoplasm is critical for enzyme function under salt stress (James *et al.* 2006), and ratios of K^+/Na^+ of less than 1 have been reported to reduce the function of enzymes (e.g. wheat germ; Greenway and Munns 1980). The ratio of K^+/Na^+ varies between species; for example at 100 mM external NaCl in bread wheat the ratio of K^+/Na^+ was 4.8-fold higher than in barley, and barley has higher tissue tolerance to Na^+ than bread wheat (Gorham *et al.* 1990). In the present study, the saline treatments (aerated and stagnant) reduced the ratio of K^+/Na^+ to

3.2–6.8% of that in the control treatment in both accessions, however, both accessions had a ratio of K^+/Na^+ above the proposed critical threshold of 1 (of 1.4–2.5 in the YFEL; Table 4.1).

Stomatal conductance is a sensitive indicator of osmotic stress caused by salinity (James *et al.* 2008) and waterlogging (Folzer *et al.* 2006). Osmotic stress can limit water availability, causing stomatal closure (e.g. rice; Dionisio-Sese and Tobita 2000; barley; Pérez-López *et al.* 2012). However, ionic stress can also cause partial stomatal closure as the concentration of Na^+ in the apoplast around the guard cells begins to rise (e.g. *Aster tripolium*; Perera *et al.* 1994). In the present study, H109 had a greater ability to maintain higher gas exchange rates in the YFEL compared with H155. Furthermore, net photosynthesis in 2nd YFEL of H155 was completely inhibited by 400 mM NaCl, in aerated and stagnant treatments and stomata were almost completely closed at day 23 of treatment, while only the stagnant saline treatment inhibited the net photosynthesis in 2nd YFEL of H109. In both accessions, 2nd YFEL had severely inhibited net photosynthesis by stagnant non-saline, aerated saline and stagnant saline treatments.

Photosynthesis in salt-stressed plants can be limited by two main factors: (i) stomatal factor, a limitation in CO_2 absorption into the leaf caused by reductions in stomatal conductance; and (ii) non-stomatal factor, resulting from toxic effects of high concentrations of Na^+ and Cl^- accumulated in the leaves over time causing chronic photoinhibition or other damage (Tezara *et al.* 1999; Ashraf 2003; Lawlor and Tezara 2009; Pérez-López *et al.* 2012). Elevated CO_2 increases the intercellular CO_2 concentrations, and therefore, enhances the metabolic capacity of the photosynthetic which was limited by reduced stomatal conductance (Pérez-López *et al.* 2012). In the present study, elevation of CO_2 concentrations substantially increased net photosynthesis (up to 18-fold; Fig. 4.7), providing evidence that the photosynthetic machinery remained active in these leaves, and that the decrease in photosynthesis must have largely been due to stomatal closure caused by osmotic effects (Fig. 4.S2 and 4.S3). Stomatal closure, caused by salt stress, resulted in reduced transpiration rates (Fig. 4.6 and 4.S3). Intercellular CO_2 concentrations did not differ between treatments, further supporting that the inhibition in photosynthesis was accompanied with a decrease in transpiration rates (Supplementary Materials, Fig. 4.S3). This result is corroborated by previous studies that salt stress-induced decreases in gas exchange can

also improve water use efficiency (e.g. *Lycopersicon esculentum*; Romero-Aranda *et al.* 2001; *Glycine tomentella*; Kao *et al.* 2003). Although the two accessions (and both tissues, i.e. YFEL and 2nd YFEL) differed in net photosynthesis, there were no statistical differences between ion concentrations (i.e. Na⁺, K⁺ and Cl⁻) in the two accessions and in the various tissues. However, salinity tolerance is a complex mechanisms made up of a number of sub traits involving responses at the cellular, organ, and whole-plant level (reviewed by Munns and Tester 2008).

The interaction between salinity and waterlogging can increase concentrations of Na⁺ and/or Cl⁻ in leaves above those when salinity occurs alone, although the interactive effect is greater in non-halophytes than halophytes (Barrett-Lennard 2003; Colmer and Flowers 2008). Wetland halophytes have the ability to minimise the interaction because the roots have higher porosity and a better ability to regulate ions (Colmer and Flowers 2008). For non-halophytes such as wheat, the interaction between salinity and waterlogging can reduce the capacity of roots to 'exclude' Na⁺ and Cl⁻ from shoots (Barrett-Lennard *et al.* 1999), while some halophytes maintain their ability to exclude Na⁺ and Cl⁻ from shoots (e.g. *Puccinellia ciliata*; Teakle *et al.* 2013). *H. maritimum* is more tolerant than wheat to the interaction of salinity and waterlogging (Malik *et al.* 2009a), and this is attributed to its superior root aeration traits and better ion regulation. In the present study, the combined stagnant saline treatment reduced K⁺ concentrations in the leaves of H109 to 69% of the aerated saline treatment and to 53% of the aerated saline treatment in H155 (Fig. 4.8A and 4.8B). The combined stagnant saline treatment also reduced tiller number, and the interactive effect was more severe for H155 (34% of aerated saline) than for H109 (55% aerated saline; Fig. 4.2C and 4.2D). In addition, the combined stagnant saline treatment increased leaf tissue death in both accessions to 23% of shoot DM in H109 (47% of aerated saline) and to 34% of shoot DM in H155 (47% of aerated saline; Fig. 4.3C and 4.3D). However, the combined treatments had little adverse effect on DM above that already caused by 400 mM NaCl in aerated solution (Fig. 4.1C and 4.1D). Also with combined stagnant and salinity both accessions had lower Na⁺ (values ~55% of those in the aerated saline treatment) and Cl⁻ concentrations (values ~65% of those in the aerated saline treatment) (Fig. 4.8C, 4.8D, 4.8E and 4.8F) and a similar ratio of K⁺/Na⁺ (Table 4.1) relative to the aerated saline treatment. In addition, after 3 days of stagnant saline treatment, *H. maritimum* accumulated higher levels of Na⁺ in photosynthetically active tissues than at a later stage (24 days of treatments; c.f. Fig. 4.8 with Fig. 4.S4). Such a situation could be

attributed to the induction of specific adaptations like aerenchyma formation, which would be expected to improve ion regulation once formed. Based on these key traits, it is not surprising that *H. marinum* survived the harsh combination of both salinity and stagnant treatments, a position consistent with the view that *H. marinum* is one of the most tolerant species to salinity and waterlogging within the Triticeae (Colmer *et al.* 2006b). In comparison with *H. marinum*, wheat is moderately salt-tolerant as it can survive at up to ~250 mM NaCl (Maas and Hoffman 1977; Maas 1986), and relatively sensitive to waterlogging (Setter and Waters 2003) and therefore, the amphiploids produced from *H. marinum* and wheat express salt tolerance and waterlogging tolerance (Munns *et al.* 2011; Chapter 5).

In conclusion, this experiment has shown that the combination of salinity and stagnant treatments resulted in greater reduction in stomatal conductance, number of tillers and K^+ concentrations in leaves for both *H. marinum* accessions than the effect of the individual factor. Furthermore, the combination of salinity and stagnant increased in leaf damage for both *H. marinum* accessions than saline alone (hypothesis a). H109 is more tolerant to salinity with waterlogging than H155, based on the following key traits: the proportion of dead leaves and their Na^+ and Cl^- concentration (hypothesis c), as well gas exchange (hypothesis b). *H. marinum* has an ability to survive the combination of high levels of salt (400 mM NaCl) and stagnant root-zone stresses. The present findings on *H. marinum* accessions tolerance to salinity and stagnant conditions indicate that the leaf injury and gas exchange are also of interest for characteristics associated with salt and waterlogging tolerance. The results provide evidence for the reputed salt and waterlogging tolerance of *H. marinum* accessions, and demonstrate adaptation to salt and stagnant root-zone stresses in *H. marinum* accessions.

4.6 SUPPLEMENTARY MATERIALS

Fig. 4.S1. Whole plant relative growth rates of *H. marinum* accessions (H109 and H155) grown in aerated or stagnant nutrient solution with or without 400 mM NaCl. Treatments were imposed when plants were 14-d-old. Plants were treated for 38 d with aerated or stagnant treatments in non-saline or saline (400 mM NaCl) nutrient solutions. Harvest were taken on days 3, 10, 17, 24 31 and 38. Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between accessions at $P = 0.05$. Significant differences between accessions are indicated ($P < 0.05$, *). Note the different y-axis for A compared to B, C and D. The data and errors for the stagnant non-saline, aerated saline and stagnant saline treatments (B, C and D) are reported as % of control.

Fig. 4.S2. Response to elevated CO₂ concentrations of the stomatal conductance of the youngest fully expanded leaf (YFEL) and 2nd YFEL of *H. maritimum* accessions (H109 and H155) grown in aerated or stagnant nutrient solution with 400 mM NaCl. Treatments were imposed when plants were 14-d-old. Plants were grown under aerated saline or stagnant saline conditions in nutrient solutions for 2 (A and B) or 23 days (C and D). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. ns = no significant differences between stomatal conductance at different CO₂ concentrations at *P* = 0.05.

Fig. 4.S3. Responses of intercellular CO₂ concentration of the youngest fully expanded leaf (YFEL) and 2nd YFEL of *H. maritimum* accessions (H109 and H155) grown in aerated or stagnant nutrient solution with or without 400 mM NaCl. Treatments were imposed when plants were 14-d-old. Plants were grown under aerated non-saline, stagnant non-saline, aerated saline or stagnant saline conditions in nutrient solutions for 2 (A and B) or 23 days (C and D). Dashed lines represent ambient CO₂ concentrations (380 μmol mol⁻¹). Each value is the mean of four replicates ± standard error, and each replicate is a single plant grown in a different pot. ns = no significant differences between intercellular CO₂ concentration at *P* = 0.05.

Fig. 4.S4. Concentrations of K^+ (A and B), Na^+ (C and D) and Cl^- (E and F) in the youngest fully expanded leaf (YFEL) and 2nd YFEL of *H. maritimum* accessions (H109 and H155) grown in aerated or stagnant nutrient solution with or without 400 mM NaCl. Treatments were imposed when plants were 14-d-old. Plants were grown in aerated, stagnant non-saline, aerated saline or stagnant saline nutrient solution for 3 days. (The saline treatment = 400 mM NaCl). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between ion concentrations at various treatments at $P = 0.05$.

Table 4.S1. *P* values from the ANOVA of parameters fitted to the entire data set.

Parameter	Treat.	Acc.	H.	Treat.×Acc.	Treat.×H.	Acc.×H.	Treat.×Acc.×H.	Fig.
Total DM	<0.001	<0.001	<0.001	<0.001	<0.001	0.314	0.405	4.1
Aerated non-saline	-	<0.001	<0.001	-	-	<0.001	-	4.1A
Stagnant non-saline	-	<0.001	<0.001	-	-	0.516	-	4.1B
Aerated saline	-	<0.001	<0.001	-	-	0.078	-	4.1C
Stagnant saline	-	<0.001	<0.001	-	-	0.044	-	4.1D
Number of tillers	<0.001	0.402	<0.001	<0.001	<0.001	0.726	0.122	4.2
Aerated non-saline	-	0.077	<0.001	-	-	0.743	-	4.2A
Stagnant non-saline	-	<0.001	<0.001	-	-	0.516	-	4.2B
Aerated saline	-	0.010	<0.001	-	-	0.125	-	4.2C
Stagnant saline	-	<0.001	<0.001	-	-	0.079	-	4.2D
Dead leaf material	<0.001	<0.001	0.014	<0.001	<0.001	0.049	0.826	4.3
Aerated non-saline	-	0.069	<0.001	-	-	0.626	-	4.3A
Stagnant non-saline	-	0.83	<0.001	-	-	1.000	-	4.3B
Aerated saline	-	<0.001	<0.001	-	-	0.005	-	4.3C
Stagnant saline	-	0.042	<0.001	-	-	0.855	-	4.3D
Shoot water content	<0.001	<0.001	<0.001	0.194	<0.001	0.007	0.353	4.4
Aerated non-saline	-	<0.001	0.012	-	-	0.993	-	4.4A
Stagnant non-saline	-	<0.001	<0.001	-	-	0.012	-	4.4B
Aerated saline	-	0.212	0.214	-	-	0.518	-	4.4C
Stagnant saline	-	0.052	<0.001	-	-	0.014	-	4.4D

Treatments were imposed when plants were 14-d-old. Harvest were taken on days 3, 10, 17, 24 31 and 38. Four replicates each one represents a single plant grown in different pots. Treat. = Treatment; Acc = Accession; H. = Harvest, DM = Dry mass.

Table 4.S2. *P* values from the ANOVA of parameters fitted to the entire data set.

Parameter	Treat.	Acc.	L.	Traet.xAcc.	Traet.xL.	Acc.xL.	Treat.xAcc.xL.	Fig. and Table
Net photosynthetic rate								Fig. 4.5
Day 2	<0.001	0.576	0.510	0.346	0.904	0.570	0.628	4.5A and 4.5B
Day 23	<0.001	0.451	<0.001	0.551	0.003	<0.001	0.076	4.5C and 4.5D
Stomatal conductance								Fig. 4.6
Day 2	<0.001	0.658	0.863	0.645	0.897	0.756	0.972	4.6A and 4.6B
Day 23	<0.001	0.664	0.232	0.216	0.433	0.872	0.958	4.6C and 4.6D
Intercellular CO ₂ concentration								Fig. 4.S3
Day 2	0.418	0.334	0.672	0.483	0.108	0.807	0.990	4.S3A and 4.S3B
Day 23	0.108	0.629	0.221	0.890	0.498	0.101	0.218	4.S3C and 4.S3D
K ⁺ concentration	<0.001	0.007	<0.001	0.205	0.026	0.226	0.792	Fig. 4.8A and 4.8B
YFEL	<0.001	0.022	-	0.459	-	-	-	4.8A
2 nd YFEL	<0.001	0.259	-	0.307	-	-	-	4.8B
Na ⁺ concentration	<0.001	0.124	0.087	0.193	0.185	0.742	0.838	Fig. 4.8C and 4.8D
YFEL	<0.001	0.166	-	0.473	-	-	-	4.8C
2 nd YFEL	<0.001	0.203	-	0.567	-	-	-	4.8D
Cl ⁻ concentration	<0.001	0.732	0.663	0.895	0.827	0.718	0.944	Fig. 4.8E and 4.8F
YFEL	0.021	0.593	-	0.876	-	-	-	4.8E
2 nd YFEL	0.005	0.997	-	0.950	-	-	-	4.8F
K ⁺ /Na ⁺	<0.001	0.928	0.731	0.578	0.667	0.915	0.998	Table 4.1
YFEL	<0.001	0.988	-	0.731	-	-	-	Table 4.1
2 nd YFEL	<0.001	0.904	-	0.833	-	-	-	Table 4.1
K ⁺ concentration	<0.001	0.067	0.162	0.257	0.590	0.560	0.622	Fig. 4.S4A and 4.S4B
YFEL	<0.001	0.098	-	0.478	-	-	-	4.S4A
2 nd YFEL	0.005	0.096	-	0.691	-	-	-	4.S4B
Na ⁺ concentration	<0.001	0.636	0.198	0.971	0.469	0.558	0.922	Fig. 4.S4C and 4.S4D
YFEL	<0.001	0.896	-	0.995	-	-	-	4.S4C
2 nd YFEL	0.002	0.499	-	0.957	-	-	-	4.S4D
Cl ⁻ concentration	<0.001	0.760	0.070	0.800	0.240	0.607	0.926	Fig. 4.S4E and 4.S4F
YFEL	<0.001	0.759	-	0.357	-	-	-	4.S4E
2 nd YFEL	<0.001	0.581	-	0.962	-	-	-	4.S4F

Treatments were imposed when plants were 14-d-old. Ion analysis was done for plants treated for 38 days. Four replicates each one represents a single plant grown in different pots. Treat. = Treatment; Acc = Accession; L. = Leaf, YFEL = Youngest fully Expanded Leaf.

CHAPTER 5

Improvement of salt and waterlogging tolerance in wheat: comparative physiology of *Hordeum marinum*-*Triticum aestivum* amphiploids with their *H. marinum* and wheat parents

5.1 ABSTRACT

Hordeum marinum is a waterlogging-tolerant halophyte that has been hybridised with bread wheat (*Triticum aestivum* L.) to produce an amphiploid containing both genomes. This study tested the hypothesis that traits associated with waterlogging and salinity tolerances would be expressed in *H. marinum*-wheat amphiploids. Four *H. marinum* accessions were used as parents to produce amphiploids with Chinese Spring wheat, and their responses to hypoxic and 200 mM NaCl were evaluated. Relative growth rate (RGR) in the hypoxic-saline treatment was better maintained in the amphiploids (58–71% of controls) than in wheat (56% of control), but the amphiploids were more affected than *H. marinum* (68–97% of controls). In hypoxic-saline conditions, leaf Na⁺ concentrations in the amphiploids were lower than in wheat (30–41% lower) but were 39–47% higher than in the *H. marinum* parents. A strong barrier to radial oxygen loss formed in basal root-zones under hypoxic conditions in two *H. marinum* accessions; this barrier was moderate in the amphiploids, absent in wheat, and was weaker for the hypoxic-saline treatment. Porosity of adventitious roots increased with the hypoxic treatments; values were 24–38% in *H. marinum*, 16–27% in the amphiploids and 16% in wheat. Overall, the amphiploids showed greater salt and waterlogging tolerances than wheat, demonstrating the expression of relevant traits from *H. marinum* in the amphiploids.

5.2 INTRODUCTION

Salinity is increasing over large parts of the world's arable land (Szabolcs 1994). Salinity impacts adversely on crops by reducing water availability and causing ion toxicity (Munns 1993). It is also often accompanied by waterlogging (Barrett-Lennard 2003), which decreases the availability of O₂ in the root-zone (Armstrong 1979). The combination of salinity and waterlogging has adverse effects on the growth of many species (Barrett-Lennard and Shabala 2013). Root-zone O₂ deficiency increases the rate of Na⁺ uptake (Barrett-Lennard *et al.* 1999), reduces K⁺ retention in roots (Pang *et al.* 2006) and impedes K⁺ uptake into the shoot (Wiengweera and Greenway 2004; Colmer and Greenway 2011; Barrett-Lennard and Shabala 2013), and the resulting increased ion imbalances can cause severe leaf damage or even death of plants in combined salinity and hypoxia (Barrett-Lennard 2003). Therefore, tolerance to both salinity and waterlogging could be important for crops such as wheat in areas affected by salinity caused by rising water-tables (Colmer *et al.* 2005b).

Wheat (*Triticum aestivum* L.) tolerates moderate salinity (Maas 1986) but is relatively sensitive to waterlogging (Setter and Waters 2003). Sea barleygrass (*Hordeum marinum* Huds.), a wild relative of wheat, grows naturally in salt marshes (von Bothmer *et al.* 1995) and has possibly the highest tolerance to salinity and waterlogging within the Triticeae (Colmer *et al.* 2006b). The salinity tolerance of *H. marinum* is attributed to its ability to ‘exclude’ Na^+ and Cl^- from the shoot and better maintain tissue K^+ concentrations (Garthwaite *et al.* 2005). Waterlogging tolerance is associated with the formation of aerenchyma and a barrier to radial O_2 loss (ROL) in adventitious roots (McDonald *et al.* 2001a; Garthwaite *et al.* 2003; Malik *et al.* 2011). The barrier to ROL decreases the leakage of O_2 out of the root and enhances longitudinal O_2 diffusion towards the root tip in aerenchymatous roots, as well as restricting the entry of phytotoxic substances that can accumulate in reduced soils (Armstrong 1979; Jackson and Armstrong 1999; Colmer 2003). Based on these key traits, it follows that *H. marinum* can withstand the combined effects of salinity and waterlogging by maintaining better leaf ion regulation whereas wheat is more sensitive to the interactive effects of these two stresses (Malik *et al.* 2009b). *H. marinum* might therefore be used for wide hybridisations to improve the salt and waterlogging tolerance of wheat (Colmer *et al.* 2006b; Islam *et al.* 2007; Malik *et al.* 2011).

H. marinum–wheat amphiploids have been produced by AKMR Islam and some of these have been studied to assess their tolerances to salinity (Islam *et al.* 2007) and waterlogging (Malik *et al.* 2011). One of the *H. marinum*–wheat amphiploids was reported to express salt tolerance from *H. marinum* (Islam *et al.* 2007) and more recent work has shown that 14 *H. marinum*–wheat amphiploids (out of 15 tested) displayed better Na^+ ‘exclusion’ than their respective wheat parents (Munns *et al.* 2011). In terms of waterlogging tolerance, two amphiploids (out of four tested) have been found to form a ‘partial’ barrier to ROL in adventitious roots and express moderately higher root porosity (Malik *et al.* 2011). Two *H. marinum*–wheat amphiploids were reported to tolerate the combination of salt and waterlogging stresses better than the wheat parents, based on higher growth, less Na^+ and higher K^+ in leaves (Munns *et al.* 2011). However, the two amphiploids used in the work of Munns *et al.* (2011) on combined salinity and waterlogging stress shared one *H. marinum* accession (H90) as the wild parent, but had different wheat parents (one tetraploid and one hexaploid wheat), so that expression of tolerances of different *H. marinum* parents in a common wheat

background had not previously been assessed. In this study, I used four *H. marinum*–wheat amphiploids produced from four accessions of *H. marinum* and sharing a common wheat parent, to assess the extent of inherited traits associated with salinity and waterlogging tolerance from the *H. marinum* parents as expressed in Chinese Spring hexaploid wheat.

The key salt and waterlogging traits evaluated in the present study were growth responses, shoot ion concentrations (i.e. Na^+ , Cl^- and K^+), leaf tissue injury, aerenchyma formation (as root porosity) and the formation of a barrier to radial O_2 loss in roots. The hypotheses tested were that: (i) the amphiploids would inherit the salt and waterlogging tolerance traits of their *H. marinum* parent (i.e. different accessions), and (ii) the amphiploids would be more tolerant under stagnant saline conditions than their wheat parent.

5.3 MATERIALS AND METHODS

Plant material and growth conditions

Four accessions of *Hordeum marinum* (H21, H87, H109 and H155 – genome XX), and four amphiploids (genome AABBDDXX) of each of these accessions hybridised with hexaploid wheat cv. Chinese Spring (genome AABBDD) were used. Amphiploid seeds were provided by AKMR Islam (The University of Adelaide) and *H. marinum* accessions were originally provided by the Nordic Gene Bank (R. von Bothmer, Swedish Agricultural University, Alnarp). Amphiploid production by hybridisation of Chinese Spring (CS) as the male with the different *H. marinum* accessions as the female has been described by Islam *et al.* (2007).

Experimental design

The nine genotypes (described above) were subjected to two salinity treatments (0.2 or 200 mM NaCl) $\times$ two aeration treatments (aerated or stagnant) $\times$ four replicates, in a completely randomised design. The experiment was conducted in a naturally-lit phytotron (20/15°C day/night) with an average PAR at midday during the experimental period of $\sim 780 \mu\text{mol m}^{-2} \text{s}^{-1}$. The experiment was conducted between 29-May-2010 and 27-Jul-2010 at the School of Plant Biology, UWA, Perth Western Australia.

Experimental units (pots) were re-randomised weekly to reduce the effects of environmental variation within the phytotron. To ensure that the plants were all at a similar developmental stage at the start of treatments, *H. marinum* accessions were germinated three d before the wheat and amphiploids. Seeds were surface-sterilised with 0.04% NaHClO in de-ionised (DI) water for 45 s, and then rinsed thoroughly with DI water prior to being imbibed overnight in aerated 0.5 mM CaSO₄. Seeds were then transferred onto mesh floating on 10% strength aerated nutrient solution in darkness (pots covered with Al-foil) in a 20/15 °C day/night phytotron for the first four d. Seedlings were exposed to light after four d when nutrient solutions were changed to 25% strength.

Eight seedlings per pot were transplanted into 4.5 L pots containing full-strength nutrient solution on day 7. Seedlings were held in the pot lids using polystyrene foam in holes (2 cm diameter). The full-strength nutrient solution contained (mM): K⁺, 3.95; Ca²⁺, 4.0; Mg²⁺, 0.4; NH₄⁺, 0.625; Na⁺, 0.2; NO₃⁻, 4.375; SO₄²⁻, 4.4; H₂PO₄⁻, 0.2; H₄SiO₄⁻, 0.1; with micronutrients (μM): Cl⁻, 50; B, 25; Mn, 2; Zn, 2; Ni, 1; Cu, 0.5; Mo, 0.5; Fe-Sequestrene, 50. *H. marinum* can be prone to Fe-deficiency under these conditions, therefore 5 μM FeSO₄ was supplied routinely (on 7 occasions to the young plants) to prevent any risk of such deficiency, but always to all pots. The solution was also buffered with 2.5 mM 2-[N-Morpholino]ethanesulfonic acid (MES) and the pH was adjusted to 6.5 using KOH, increasing the final K⁺ concentration to ~6.0 mM.

Treatments and harvests

Treatments were imposed when plants had 2–2.5 leaves (14-d-old plants for *H. marinum* accessions and 11-d-old plants for the wheat and amphiploids). Prior to the treatments being imposed, an initial harvest of four replicate plants of each of the nine genotypes was taken to measure initial dry mass. After this, NaCl was stepped up in pots allocated to the saline treatment by 50 mM every 12 h until the final concentration of 200 mM was reached (36 h). After reaching the final NaCl concentration, pots allocated to the stagnant treatment were given a ‘hypoxic pre-treatment’ in which the solution in these pots was bubbled with N₂ for ~2 h until the dissolved O₂ concentration was less than ~0.03 mol m⁻³; these pots were then left overnight without bubbling. The following day, the nutrient solution in the stagnant pots was replaced with deoxygenated (i.e. pre-flushed with N₂ overnight) stagnant nutrient solution containing 0.1% (w/v)

agar. The agar inhibits convective movements in the nutrient solution, impeding the re-entry of O₂ and promoting the accumulation of ethylene, both important changes in the gas composition of waterlogged soils (Wiengweera *et al.* 1997). The nutrient solutions of pots allocated for aerated treatments were also renewed at the same time as those in the stagnant treatments, but these aerated solutions were without agar and continued to be bubbled with air. Solutions in all pots were renewed every 7 d and aerated pots were topped up with DI water as required.

The plants were harvested 26–29 d after the stagnant agar treatment commenced. On day 26, plants were harvested for measurements of dry mass and tissue ion analyses; on days 27–29 additional plants were used to measure radial O₂ loss and root porosity (detailed below). At each harvest, roots and the stem base were washed 3 times for 30 s in mannitol solutions iso-osmotic with the Na⁺ plus Cl[−] concentration of the nutrient solution; these wash solutions also contained 4 mM CaSO₄. The plants were then separated into four tissue classes: the youngest fully expanded leaf (YFEL), other green shoot tissues (i.e. stems, sheaths and all other green and turgid leaf blades), dead leaves including dead part of green leaves and roots.

Growth measurements

The rate of elongation of the youngest expanding leaf on the main stem was measured by recording its length over four consecutive days, starting from when the stagnant treatment was imposed. The final harvest was taken after 26 d of stagnant treatment. Tiller number, shoot and root lengths were measured before the plants were separated into the components described above. Fresh mass was recorded and dry mass of all samples was measured after being oven dried for 72 h at 65°C. The relative growth rate (RGR) was calculated from the dry mass of the whole-plant samples at the start and end of the treatment period, using the formula described by Hunt (1978):

$$\text{RGR} = (\ln DM_2 - \ln DM_1) / (t_2 - t_1)$$

Where DM_1 is the dry mass (g) at time one (initial harvest), DM_2 is the dry mass (g) at time two (final harvest), t_1 and t_2 are times one and two in d, and ln is the natural logarithm.

Measurements of radial O₂ loss

Rates of radial O₂ loss (ROL) from adventitious roots in an O₂-free medium were measured in a 20°C controlled temperature room, using plants exposed to stagnant treatments for 27–29 d. Plants were sealed in rubber lids fitted in clear Perspex chambers filled with a deoxygenated stagnant solution containing 0.1% (w/v) agar, 0.5 mM CaSO₄ and 5.0 mM KCl and, depending on the treatment, either with or without 200 mM NaCl. Intact adventitious roots that had not formed lateral roots were selected for measurements of ROL at positions 10, 20, 30, 40, 50, 60, 70 and 80 mm behind the root tip, using a root-sleeving O₂ electrode (internal diameter 2.25 mm, height 5.0 mm) fitted with guides to keep the root near the center of the electrode (Armstrong and Wright 1975; Armstrong 1994).

Three general patterns of a ‘barrier’ to ROL in roots are considered: (1) ‘strong barrier’: very low ROL from root basal zones, but high rates towards the root tip; (2) ‘partial barrier’: similar rates of ROL along root; (3) ‘weak barrier’: much higher ROL rates in basal zones than near the root tip (Colmer 2003).

Measurements of root porosity

Porosity (% gas volume per unit root volume) of adventitious roots was measured by determining root buoyancy before and after vacuum infiltration of the gas spaces in the roots with water, as described by Thomson *et al.* (1990). Porosity was measured from plants previously exposed to stagnant treatments for 27–29 d. Adventitious roots were excised with a razor blade at 100 mm behind the apex, and then cut into 50 mm segments for the measurements.

Tissue ion analyses

Concentrations of Na⁺, Cl[−] and K⁺ were determined in the different shoot tissues. Oven-dried samples were ground and extracted in 0.5 M HNO₃ by shaking for 48 h at 20–25°C. Na⁺ and K⁺ concentrations were determined in dilutions of the extracts using a flame photometer (Jenway PFP7, Essex UK). Cl[−] concentration was determined in the extracts using guanidine protocol of a chloridometer (Slamed CHLO 50 cl, Frankfurt, Germany). Blanks and a reference plant material with known ion concentrations were

taken through the same procedures. Recoveries of Na^+ , Cl^- and K^+ from the reference plant tissue taken through the same procedure as samples were 104, 101 and 95%, respectively. The data presented have not been adjusted.

Data analyses

All statistical analyses were conducted using GENSTAT 12th Edition (VSN International Ltd. Hemel Hempstead, UK). Residuals were checked for normality and no transformations were needed. Two-way ANOVA was used to identify overall significant differences between genotypes and between treatments. When significant differences were found, mean-separations were calculated using the Fisher unprotected LSD test. Unless otherwise stated, the significance level was $P \leq 0.05$.

5.4 RESULTS

Growth

The relative growth rate (RGR) was similar for all genotypes when grown under control conditions (aerated non-saline; $0.12\text{--}0.16 \text{ g g}^{-1} \text{ d}^{-1}$; Fig. 5.1A). Under non-saline conditions, hypoxia increased RGR to 114–134% of aerated controls in the *H. marinum* accessions, but had little effect on the RGR of the amphiploids and wheat (Fig. 5.1A). Salinity treatments reduced the RGR of all genotypes relative to the aerated non-saline controls. In aerated saline solutions, the RGR of *H. marinum* accessions (75–91% of controls) and amphiploids (78–89% of controls) was better maintained than for wheat (62% of control) (Fig. 5.1B). In the stagnant saline treatment, the RGRs of the *H. marinum* accessions were not significantly different to those in the aerated saline treatment. By comparison, RGR in the four amphiploids in the combined stagnant and salinity was reduced to be 58–72% of the controls and to 56% of the control in wheat (Fig. 5.1B). Treatment effects on total plant dry mass at the second harvest and the number of tillers were consistent with these general trends (see Supplementary Materials, Fig. 5.S1 and 5.S3). The proportion of dead leaf material was small (11% as a highest percentage) and with no statistical difference among genotypes at $P=0.05$, when subjected to separate stagnant or saline treatments, or when these two stresses were combined (Supplementary Materials, Fig. 5.S2).

Fig. 5.1. Whole plant relative growth rates (RGR) of *H. maritimum* accessions (H21, H87, H109 and H155), wheat (*Triticum aestivum* cv. Chinese Spring; (CS)) and their amphiploids grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. maritimum*, and 11-d-old for wheat and the amphiploids, so that plants were at a similar leaf developmental stage. Plants were treated for 26 d with aerated (control) or stagnant non-saline nutrient solution (A); or aerated or stagnant nutrient solution plus 200 mM NaCl (B). Note the different y-axes of (A) and (B). The data and errors for the saline treatment (B) are reported as % of control. Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between genotypes at $P = 0.05$, ns = not significant.

Na⁺ concentrations in the youngest fully expanded leaf (YFEL)

Na⁺ concentrations in the YFEL did not differ between genotypes in aerated non-saline controls, ranging between 240 and 298 $\mu\text{mol g}^{-1}$ DM (Fig. 5.2A). Stagnant non-saline treatment reduced Na⁺ concentrations, by 29–31% of controls for *H. marinum* accessions, 37–55% of controls for the amphiploids, and by 62% of control in wheat (Fig. 5.2A). Salinity treatments increased Na⁺ concentrations for all genotypes, and the increase was largest (relative to non-saline aerated controls) for wheat (2.4-fold), compared to only 15–68% in *H. marinum* accessions, and 39–87% in the amphiploids. Relative to the aerated saline treatment, the combined saline and stagnant treatment increased Na⁺ concentrations in all genotypes; the lowest increase was 13–45% in *H. marinum* accessions and in wheat, compared to 50–120% in the amphiploids (Fig. 5.2B).

Cl⁻ concentrations in the youngest fully expanded leaf (YFEL)

In general, Cl⁻ concentrations in the YFEL of all genotypes showed similar trends to those of Na⁺ in the various treatments (cf. Fig. 5.2A and 5.2B with Fig. 5.2C and 5.2D). Cl⁻ concentrations in the YFEL ranged from 72 to 242 $\mu\text{mol g}^{-1}$ DM for all genotypes in the non-saline aerated controls. The stagnant non-saline treatment reduced Cl⁻ concentrations in all genotypes in comparison with the aerated non-saline controls; however, in stagnant non-saline treatment all genotypes had similar Cl⁻ concentrations (Fig. 5.2C). Cl⁻ concentration increased under aerated saline conditions in all genotypes and the increase was much larger for wheat (5.4-fold), compared to an increase of only 42–89% in *H. marinum* accessions and 69–151% in the amphiploids. Relative to the aerated saline treatment, the combined salinity and stagnant treatment increased Cl⁻ concentrations by 54–241% in *H. marinum* accessions, 110–143% in the amphiploids, and 46% in wheat (Fig. 5.2D).

K⁺ concentrations in the youngest fully expanded leaf (YFEL)

All combinations of salinity and hypoxia substantially reduced K⁺ concentrations in the YFEL of all genotypes in comparison with the aerated non-saline controls (Fig. 5.2E and 5.2F). For plants in aerated non-saline controls, K⁺ concentration in the YFEL was lower in *H. marinum* accessions (980–1416 $\mu\text{mol g}^{-1}$ DM) than the amphiploids (1750–1903 $\mu\text{mol g}^{-1}$ DM) and wheat (1760 $\mu\text{mol g}^{-1}$ DM). Stagnant non-saline treatment

reduced K^+ concentrations; however, *H. maritimum* accessions had the least reduction by 15–30% of aerated control, compared to 38–53% for the amphiploids and 67% for wheat (Fig. 5.2E). Salinity treatments also reduced YFEL K^+ concentrations; in the aerated saline treatment the reductions were 1–34%, 19–39% and 42% of controls for *H. maritimum* accessions, amphiploids and wheat, respectively. In the combined stagnant plus saline treatment, concentrations of K^+ in the YFEL declined further to 32–46% of control for *H. maritimum* accessions, 41–58% of controls for the amphiploids, and 63% of control for wheat (Fig. 5.2F).

Measuring the concentration of ions (Na^+ , Cl^- and K^+) in the YFEL is a standard method used to determine the influence of salinity on plant ion relations (Munns and Termaat 1986), and is the method used here for expressing treatment effects on ion relations. However, for comparison, concentrations of Na^+ , Cl^- and K^+ were also measured in the other green tissues of the shoot, and while the concentrations of Na^+ and Cl^- were 19–41% higher with the saline and stagnant saline treatments in the bulk shoot tissues than in the YFEL, the treatment effects showed the same general trends as described above for the YFEL (see Supplementary Materials, Fig. 5.S4).

K⁺/Na⁺ ratio

Compared with aerated non-saline controls, salinity treatment reduced the K^+/Na^+ ratio in the YFEL in all genotypes; however the reduction was greatest for wheat (Table 5.1). In the aerated non-saline controls, the K^+/Na^+ ratio in the YFEL ranged between 4.0 and 6.7. By contrast, for the aerated saline treatment, the K^+/Na^+ ratio ranged between 2.1 and 3.8 for *H. maritimum* accessions and the amphiploids, but only 0.8 for wheat. The combined salinity and stagnant treatment reduced the K^+/Na^+ even further to 1.5–1.7 in the *H. maritimum* accessions, to 0.9–1.2 in the amphiploids and to 0.5 in wheat (Table 5.1).

Fig. 5.2. Concentrations of Na⁺ (A and B), Cl⁻ (C and D) and K⁺ (E and F) in the youngest fully expanded leaves of *H. marinum* accessions (H21, H87, H109 and H155), wheat (*Triticum aestivum* cv. Chinese Spring; CS) and their amphiploids, when grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. marinum*, and 11-d-old for wheat and the amphiploids. Plants were treated for 26 d with aerated or stagnant non-saline nutrient solution (A, C and E), or aerated or stagnant nutrient solution plus 200 mM NaCl (B, D and F). Note the different scales on the y-axes (E and F differ to the other parts). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between genotypes at $P = 0.05$.

Table 5.1. Ratio of K^+/Na^+ in the YFEL of *H. maritimum* accessions (H21, H87, H109 and H155), wheat (*Triticum aestivum* cv. Chinese Spring; (CS)) and their amphiploids when grown in aerated or stagnant non-saline nutrient solution, and aerated or stagnant nutrient solution plus 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. maritimum*, and 11-d-old for wheat and the amphiploids. Plants were treated for 26 d. Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot.

Species/ Accession	K^+/Na^+			
	Non-saline		Saline	
	Aerated	Stagnant	Aerated	Stagnant
H21	4.0 $\pm$ 0.6	5.1 $\pm$ 0.1	3.8 $\pm$ 0.8	1.6 $\pm$ 0.1
H87	4.8 $\pm$ 0.3	5.6 $\pm$ 0.5	2.1 $\pm$ 0.2	1.7 $\pm$ 0.1
H109	5.5 $\pm$ 0.1	5.5 $\pm$ 0.2	3.0 $\pm$ 0.6	1.6 $\pm$ 0.1
H155	5.2 $\pm$ 0.2	5.5 $\pm$ 1.1	2.2 $\pm$ 0.1	1.5 $\pm$ 0.1
Amp (H21-CS)	6.5 $\pm$ 0.1	5.9 $\pm$ 0.4	2.5 $\pm$ 0.5	1.1 $\pm$ 0.1
Amp (H87-CS)	5.9 $\pm$ 0.2	8.3 $\pm$ 1.6	3.3 $\pm$ 0.7	0.9 $\pm$ 0.1
Amp (H109-CS)	6.4 $\pm$ 0.1	7.5 $\pm$ 1.0	3.2 $\pm$ 0.6	1.2 $\pm$ 0.0
Amp (H155-CS)	6.7 $\pm$ 0.4	5.4 $\pm$ 0.1	2.9 $\pm$ 0.6	0.9 $\pm$ 0.1
Wheat (CS)	5.9 $\pm$ 0.4	5.2 $\pm$ 0.1	0.8 $\pm$ 0.2	0.5 $\pm$ 0.1
l.s.d. ^A	0.98	2.18	1.6	0.26

^AThe l.s.d. represents significant differences between genotypes at $P = 0.05$.

Adventitious root porosity

Irrespective of treatment, adventitious root porosity was greatest for the *H. marinum* accessions, intermediate for the amphiploids and lowest in wheat (Table 5.2). For the aerated non-saline controls, *H. marinum* accessions had higher root porosity (14–21%) than the amphiploids (7–15%) and wheat (6%). Exposure to stagnant solutions increased root porosity, so that the values were 21–26% in *H. marinum*, 19–21% in the amphiploids and 19% in wheat. In general, salinity alone did not cause any significant changes in root porosity relative to aerated non-saline controls and there were no significant differences amongst the genotypes. However, with the combined stagnant saline treatment, root porosity ranged from 24–38% in *H. marinum*, 16–27% in the amphiploids, and was only 16% in wheat.

Root radial O₂ loss (ROL)

Under stagnant non-saline conditions all genotypes, except wheat, had a decline in ROL from near the root tip towards the root base (i.e. ROL was higher at the tip than at the base) (Fig. 5.3). Two *H. marinum* accessions (H21 and H87) formed a distinct barrier to ROL when grown in stagnant non-saline conditions, with ROL values at 50 mm from the root tip being less than ~43% of the values at 10 mm from the root tip. By contrast, the other two *H. marinum* accessions and the four amphiploids formed a partial barrier to ROL, with only modest declines, or similar ROL values at 50 and 10 mm from the root tip. By contrast, for wheat grown in the stagnant non-saline treatment, the rate of ROL was greatest in the basal zones and declined towards the root tip, indicating a weak (or absent) barrier to ROL. For wheat the ROL was 26% higher at 50 mm compared with at 10 mm behind the root tip.

The combined stagnant and saline treatment resulted in a partial barrier to ROL in all genotypes except for accession H87, which had a strong barrier to ROL, and wheat which had only a weak (or absent) barrier to ROL (Fig. 5.3). In general, rates of ROL at 10 mm behind the root tip in the stagnant plus saline treatment were relatively similar to those of stagnant non-saline treatment, in all plants except for H87-CS amphiploid. By contrast, rates of ROL at 50 mm from the root tip in the stagnant plus saline treatment were higher in accessions H21 (245%), H87 and H155 (121%), and relatively similar in the other genotypes, to those of plants in the stagnant non-saline treatment. In the stagnant saline treatment, the rates of ROL at 10 mm behind the root tip, relative to the values at 50 mm, were higher in H87 (139%) than H21 and H109 (50% and 34%),

lower in wheat (54%), H21-CS amphiploid (34%) and H109-CS amphiploid (29%) and relatively similar in the other genotypes (Fig. 5.3).

Fig. 5.3. Rates of radial O_2 loss (ROL) along adventitious roots for *Hordeum marinum* accessions (H21, H87, H109 and H155; A-D), amphiploids (E-H) and wheat (*Triticum aestivum* cv. Chinese Spring; CS; I) when in an O_2 -free root medium with or without 200 mM NaCl with shoots in air. Plants were grown for 27-29 d in stagnant non-saline nutrient solution or stagnant plus 200 mM NaCl, and measurements were conducted in deoxygenated non-saline or saline medium as appropriate. Treatments were imposed when plants were 14-d-old for *H. marinum*, and 11-d-old for wheat and the amphiploids. Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot, except for the H87-CS amphiploid (*) which had low germination so there were only two replicates in the stagnant non-saline solution and three replicates in the stagnant plus 200 mM NaCl solution.

Table 5.2. Porosity in adventitious roots of *H. maritimum* accessions (H21, H87, H109 and H155), wheat (*Triticum aestivum* cv. Chinese Spring; (CS)) and their amphiploids, when grown in aerated or stagnant non-saline nutrient solution, and aerated or stagnant nutrient solution plus 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. maritimum*, and 11-d-old for wheat and the amphiploids. Plants were treated for 27-29 d. Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot.

Species/ Accession	Adventitious root porosity (%)			
	Non-saline		Saline	
	Aerated	Stagnant	Aerated	Stagnant
H21	21 $\pm$ 5*	26 $\pm$ 5*	14 $\pm$ 2**	28 $\pm$ 3*
H87	17 $\pm$ 4**	24 $\pm$ 3	16 $\pm$ 2**	38 $\pm$ 5**
H109	12 $\pm$ 2	21 $\pm$ 5*	20 $\pm$ 2*	36 $\pm$ 8*
H155	14 $\pm$ 2*	26 $\pm$ 5	17 $\pm$ 1	24 $\pm$ 6*
Amp (H21-CS)	13 $\pm$ 3*	21 $\pm$ 3	9 $\pm$ 3*	16 $\pm$ 5*
Amp (H87-CS)	15***	20 $\pm$ 0**	21 $\pm$ 9**	27 $\pm$ 7*
Amp (H109-CS)	7 $\pm$ 1	19 $\pm$ 4	7 $\pm$ 1*	24 $\pm$ 2
Amp (H155-CS)	9 $\pm$ 1	21 $\pm$ 2	19 $\pm$ 7**	20 $\pm$ 2
Wheat (CS)	6 $\pm$ 2	19 $\pm$ 2	10 $\pm$ 3**	16 $\pm$ 4
l.s.d. ^A	9.7	ns	8.6	13.8

Some plants did not produce enough adventitious roots for a reliable measurement, therefore some have three replicates (indicated by *), two replicates (**) or one replicate (***).

^AThe l.s.d. represents significant differences between genotypes at $P = 0.05$; ns = not significant.

Relationships between some key traits (growth, leaf ion concentrations and dead leaf material) amongst the nine genotypes under aerated saline and stagnant saline conditions

Potential relationships between some key traits of salt and waterlogging tolerance were investigated using simple linear regression analyses of the data from the aerated saline and stagnant saline treatments (Fig. 5.4). Strong negative relationships between RGR and Na⁺ concentrations in the YFEL were found for plants in aerated saline solutions ($P \leq 0.05$) and in stagnant saline solutions ($P \leq 0.001$) (Fig. 5.4A). Leaf Cl⁻ concentration showed a similar negative trend against RGR as for Na⁺, with $P \leq 0.05$ in aerated saline solutions and $P \leq 0.001$ in stagnant saline solutions (Fig. 5.4B). RGR was also

negatively related to the proportion of dead leaf material in aerated saline ($P \leq 0.001$) and stagnant saline solutions ($P \leq 0.01$) (Fig. 5.4C), although the maximum proportion of shoot death of ~10% was relatively low (e.g. Munns *et al.* 1995 suggested that 20% death of the leaf tissues is required for significant impacts on growth of wheat). Nevertheless, there were also positive relationships between the proportion of dead leaf material (i.e. older leaves) and concentrations of Na^+ and Cl^- in the YFEL for plants in stagnant saline treatments ($P \leq 0.001$ for Na^+ , Fig. 5.4D; $P \leq 0.01$ for Cl^- Fig. 5.4E).

Fig. 5.4. Relationships between whole plant relative growth rates (RGR) versus Na^+ and Cl^- concentrations in the youngest fully expanded leaf (YFEL) and dead leaf material (A-C), and dead leaf material versus Na^+ and Cl^- concentrations in the YFEL (D, E) of *H. marinum* accessions (circles), amphiploids (triangles) and wheat (*Triticum aestivum* cv. Chinese Spring; CS) (diamond) when grown for 27-29 d in aerated (open symbols; dashed line) or stagnant (closed symbols; solid line) plus 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. marinum*, and 11-d-old for wheat and the amphiploids. Each value is the mean of four replicates, and each replicate is a single plant grown in a different pot; except for the H87-CS amphiploid, which had low germination so there were only three replicates for the stagnant plus 200 mM NaCl solution.

5.5 DISCUSSION

This study has evaluated tolerance to salinity and a severely hypoxic (stagnant) root-zone of four *H. maritimum*-wheat (Chinese Spring) amphiploids compared with their parents. The amphiploids were more tolerant than wheat to the combined salinity and hypoxic stress. Compared with the wheat parent, the amphiploids had higher RGR and better regulation of ion concentrations in leaves, when grown under stagnant saline conditions. The amphiploids also had higher root porosity in the stagnant saline condition and there was a noticeable barrier to ROL under stagnant solutions for three amphiploids compared with wheat. Traits associated with tolerance to salinity (lower leaf Na^+ and Cl^- concentrations and a higher ratio of K^+/Na^+) and waterlogging (root aeration) presumably contributed to the better growth of *H. maritimum*-wheat amphiploids in stagnant saline solutions, as compared with wheat.

Salt tolerance is often associated with the ability to minimise the rate of Na^+ and Cl^- transport into shoots and maintain the ratio of K^+/Na^+ in leaves (Munns 2002; Cuin *et al.* 2003; Munns and James 2003; Tester and Davenport 2003; Colmer *et al.* 2006b). The amphiploids were better able to ‘exclude’ Na^+ and Cl^- from the leaves, compared with wheat, but not to the same extent as in the *H. maritimum* accessions. For example, when grown at 200 mM NaCl, Na^+ concentration in the YFEL was 4–45% higher in the amphiploids and 1.6-fold higher in wheat than in the *H. maritimum* accessions (Fig. 5.2B). Similarly, Cl^- concentrations in the YFEL were 62–65% higher in amphiploids than in the respective *H. maritimum* parent, except for H155-CS and H155 which had similar Cl^- concentrations, whereas Cl^- was 3.7-fold higher in wheat than in the mean value for the *H. maritimum* accessions (Fig. 5.2D). The trends in leaf Na^+ and Cl^- concentrations in this study were consistent with those found by Islam *et al.* (2007), in which an amphiploid maintained lower concentrations of Na^+ and Cl^- in the YFEL, compared with wheat. Concentrations of Na^+ in leaves in this study were also consistent with results by Munns *et al.* (2011), who also found that 14 out of 15 *H. maritimum*-wheat amphiploids studied had better Na^+ ‘exclusion’ than their respective wheat parents. Together, these results support the view that salinity tolerance in wheat is associated with the ability to ‘exclude’ (i.e. restrict the rate of entry) Na^+ from leaves (Munns 2002) and in some cases also of Cl^- (Colmer *et al.* 2006b).

The K^+/Na^+ ratio in leaf tissues might also be important for salt tolerance (Maathuis and Amtmann 1999; Rubio *et al.* 1999; Cuin *et al.* 2003; Munns and Tester 2008). The amphiploids had higher leaf K^+ concentrations, which together with lower Na^+ , resulted in higher ratios of K^+/Na^+ in the leaves compared with wheat. For example, when grown at 200 mM NaCl, the ratio of K^+/Na^+ in the YFEL was reduced to 42–95% in the *H. maritimum* accessions, to 38–65% in the amphiploids and to 14% in wheat (relative to controls). Experiments have also reported a higher ratio of K^+/Na^+ in leaves of *H. maritimum* amphiploids than the respective wheat parents (Islam *et al.* 2007; Munns *et al.* 2011). These results demonstrate an improvement in K^+/Na^+ discrimination in the amphiploids, further supporting the view that salt tolerance from *H. maritimum* accessions was expressed in the *H. maritimum*-wheat amphiploids.

Growth reductions due to salinity are caused by the osmotic effect of salt outside the roots and/or toxic effects of ions when plants accumulate Na^+ and/or Cl^- in the shoot (Munns *et al.* 1995). Leaf injury from salinity is caused by accumulating ions to toxic levels, resulting in necrosis (Munns and Tester 2008), and an additional restriction on plant growth (Munns *et al.* 1995). The relationships between these key traits of salt and waterlogging tolerance were investigated using regression analyses for all genotypes grown in aerated saline or stagnant saline solutions. There were negative relationships between plant growth and concentrations of Na^+ and Cl^- in the leaves, and positive relationships between the proportion of dead leaf material and leaf concentrations of Na^+ and Cl^- (Fig. 5.4). The combination of salt and hypoxia generally increases leaf injury (necrosis) in comparison with salt alone (Barrett-Lennard 2003; Munns *et al.* 2011). Minimal dead leaf material is an important sign of plant health during salinity stress (Munns 2002) and although the proportion of shoot death was relatively small for plants in the present study, leaf injury of plants in the stagnant saline treatment was positively correlated with leaf Na^+ and Cl^- concentrations (Fig. 5.4D and 5.4E). Eventually, when leaf death removes a significant proportion of the whole shoot (e.g. 20%, Munns *et al.* 1995) this reduces photosynthetic capacity and then restricts growth and ultimately survival (Barrett-Lennard *et al.* 1999; Munns 2002). In this study, RGR was negatively correlated with the proportion of dead leaf material in aerated saline or stagnant saline treatment (Fig. 5.4C), although the maximum proportion of shoot death was relatively low (11% as a highest percentage; Fig. 5.S2). Thus, it would be of interest to assess growth responses and leaf damage of the amphiploids and wheat in

longer-term experiments, as the amount of leaf tissue death can increase with time of exposure to salinity (Munns *et al.* 1995). In addition, wheat has relatively poor root aeration when in a stagnant saline medium as it had a lower porosity and only a weak or absent barrier to ROL (data presented here; McDonald *et al.* 2001a; Malik *et al.* 2009a). This has been suggested to be a key reason for its poor ion regulation under the combination of saline and stagnant stresses (Malik *et al.* 2009a), exerting an additional restriction on growth when these two stresses occur together. By contrast, *H. marinum* accessions have superior root aeration traits, as well as superior leaf ion regulation, which make them more tolerant to combined salinity and waterlogging (Garthwaite *et al.* 2003, 2005; Malik *et al.* 2009a). The presence of the chromosomes from the *H. marinum* accessions in the amphiploids improved growth and ionic balance in the shoots, confirming the importance of ion regulation for salt tolerance and also to the combined hypoxic and salinity stresses.

H. marinum is tolerant of waterlogging (Garthwaite *et al.* 2003); this observation is consistent with its growth in marshes (von Bothmer *et al.* 1995). In the present study, whole-plant RGR of *H. marinum* was higher in stagnant (non-saline) conditions than in the aerated controls (Fig. 5.1A), although such growth stimulation was not observed in a previous study of *H. marinum* accessions (Malik *et al.* 2009a). Growth of some wetland species (Justin and Armstrong 1987) and of some accessions within a species (e.g. *Melilotus siculus*, Rogers *et al.* 2011) has previously been reported to be higher in waterlogged/stagnant conditions relative to drained/aerated controls. Waterlogging tolerance in *H. marinum* is associated with the formation of aerenchyma and a barrier to ROL in adventitious roots (Garthwaite *et al.* 2003; Malik *et al.* 2009a). In the present study two of the *H. marinum* accessions (H21 and H87) formed a strong barrier to ROL in basal root-zones, which was absent in wheat and moderate in the four amphiploids, when in stagnant non-saline nutrient solution. When in saline plus stagnant solution, accession H87 maintained a strong barrier to ROL in basal root-zones, whereas the barriers to ROL were moderate in other *H. marinum* accessions and in the four amphiploids, and absent in wheat (Fig. 5.3). Similarly, root porosity was expressed in the *H. marinum* accessions (24–38%) and the amphiploids (16–27%), which was higher than that in wheat (16%), when grown in stagnant saline solutions (Table 5.2).

High root porosity provides an internal low-resistance pathway for O₂ movement between shoot base and root tips (Armstrong 1979). Wheat had lowest root porosity and this would explain the overall lower ROL along wheat roots. By contrast, ROL from near the tip of roots of *H. maritimum* accessions was high, reflecting the higher root porosity, and the losses at the base were less than at the tip despite the expected higher internal O₂ concentrations at these positions closer to the source. Such a pattern of higher ROL at the tip than at the base is diagnostic of presence of a barrier to ROL in the basal zones (see ROL section in ‘Materials and methods’). The barrier to ROL in *H. maritimum* is likely to be suberin deposits in the walls of the hypodermis (Garthwaite *et al.* 2008). Root aeration data in stagnant non saline solution from this study that showed ROL patterns consistent with barrier development in *H. maritimum* and partial barrier functioning in some amphiploids (Table 5.2; Fig. 5.3), corresponds with results from Malik *et al.* (2011) who found that the root aeration traits of *H. maritimum* were at least partially expressed in amphiploids with wheat. In comparison with *H. maritimum*, wheat tends to form less aerenchyma in adventitious roots and has a weak or absent barrier to ROL (McDonald *et al.* 2001a; Malik *et al.* 2009a). Of note, and of potential concern for tolerance of combined salinity and waterlogging, I observed that barrier strength was apparently less in some *H. maritimum* accessions and the amphiploids when in saline plus stagnant conditions, as compared with the lower ROL from basal zones in stagnant non-saline treatment. Malik *et al.* (2009a) found strong barrier induction in roots of several *H. maritimum* accessions when in stagnant saline treatments, whereas in the present study only partial ROL barriers were evident in the stagnant saline treatment.

The interaction between salinity and waterlogging adversely impacts on most crop species due to increased rates of Na⁺ and Cl⁻ transport to the shoot resulting in increased leaf damage (Barrett-Lennard 2003). However, wetland halophytes have the ability to minimise the interaction due to high root porosity and better ion regulation (Colmer and Flowers 2008). *H. maritimum* is tolerant to salinity and waterlogging (Malik *et al.* 2009a), and this is attributed to superior root aeration traits and better ion regulation. The results of this study have shown that amphiploids of *H. maritimum*-wheat had higher porosity in adventitious roots when compared with the wheat parent, and have at least a partial barrier to ROL, when in stagnant saline conditions. Combined salinity and hypoxia has less adverse effects on the amphiploids compared with their wheat parents (Munns *et al.* 2011; data presented here), and this study demonstrates the extent of improvement in

root aeration traits and shoot ion regulation in the amphiploids, as contributed by the wild parent *H. maritimum* and expressed in the genetic background of wheat. In general, the performance of the amphiploids was intermediate relative to their *H. maritimum* and wheat parents. However, no statistical differences were recorded between the four amphiploids in most of the measurements. The *H. maritimum* accessions used as parents to make amphiploids with wheat were amongst the most tolerant (Malik *et al.* 2009a,b). To further assess the influence of *H. maritimum* accession on the variation in tolerance and trait expression in amphiploids, use of a less tolerant accession in amphiploid development would be of potential interest.

5.6 SUPPLEMENTARY MATERIALS

Fig. 5.S1. Total dry mass of *H. maritimum* accessions (H21, H87, H109 and H155), wheat (*Triticum aestivum* cv. Chinese Spring; (CS)) and their amphiploids grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. maritimum*, and 11-d-old for wheat and the amphiploids. Plants were treated for 26 d with aerated (control) or stagnant non-saline nutrient solution (A); or aerated or stagnant nutrient solution plus 200 mM NaCl (B). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between genotypes at $P = 0.05$.

Fig. 5.S2. Dead leaf material (as a percentage of shoot dry mass) in *H. maritimum* accessions (H21, H87, H109 and H155), wheat (*Triticum aestivum* cv. Chinese Spring; (CS)) and their amphiploids grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. maritimum*, and 11-d-old for wheat and the amphiploid. Plants were treated for 26 d with aerated or stagnant non-saline nutrient solution (A), or aerated or stagnant nutrient solution plus 200 mM NaCl (B). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. ns = no significant differences between genotypes at $P = 0.05$.

Fig. 5.S3. Number of tillers of *H. maritimum* accessions (H21, H87, H109 and H155), wheat (*Triticum aestivum* cv. Chinese Spring; (CS)) and their amphiploids grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. maritimum*, and 11-d-old for wheat and the amphiploids. Plants were treated for 26 d with aerated (control) or stagnant non-saline nutrient solution (A); or aerated or stagnant nutrient solution plus 200 mM NaCl (B). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between genotypes at $P = 0.05$, ns = not significant.

Fig. 5.S4. Concentrations of Na⁺ (A and B), Cl⁻ (C and D) and K⁺ (E and F) in the green tissues of the shoot other than youngest fully expanded leaves of *H. marinum* accessions (H21, H87, H109 and H155), wheat (*Triticum aestivum* cv. Chinese Spring; (CS)) and their amphiploids when grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. marinum*, and 11-d-old for wheat and the amphiploids. Plants were treated for 26 d with aerated or stagnant non-saline nutrient solution (A, C and E), or aerated or stagnant nutrient solution plus 200 mM NaCl (B, D and F). Note the different scales on the y-axes (E and F differ to the other parts). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between genotypes at $P = 0.05$.

CHAPTER 6

***Hordeum marinum* (H21)-*Triticum aestivum* amphiploid tolerates combined salinity and waterlogging: Na⁺ concentrations and levels of *HKT1;5*-like and *NHX1*-like transcripts in tissues of the amphiploid and its parents**

6.1 ABSTRACT

In a *H. marinum*-wheat amphiploid, salt tolerance has been associated with the ability to exclude Na^+ and to maintain adequate levels of K^+ in leaves (Chapter 5). *Hordeum marinum* is a wetland halophyte that has been hybridised with bread wheat to produce an amphiploid containing both genomes. The hypothesis tested in this chapter was that proteins encoded by the two candidate genes (*HKT1;5*-like and *NHX1*-like) are predicted to be involved in K^+ uptake and Na^+ sequestration into vacuoles transport, respectively, and thus might play a role in salinity tolerance in the selected *H. marinum* (H21)-bread wheat amphiploid in comparison with its parents in response to stagnant and 200 mM NaCl. In this comparative evaluation, the amphiploid tolerates combined salinity and hypoxia stresses by better maintenance of RGR in the amphiploid (68% of control) than in wheat (55% of control), but the amphiploid was more affected than *H. marinum* (75% of control). In addition, the ratio of K^+/Na^+ in leaf blade in the amphiploid was higher (0.7) than in wheat (0.3) but lower than in the *H. marinum* (2.7). The amphiploid was also capable of maintaining lower concentrations of Na^+ and Cl^- in the leaf blade than wheat (~53% lower) but was ~3.8-fold higher than in the *H. marinum*, under stagnant saline conditions. In contrast to the leaf blade, *H. marinum* accumulated higher concentrations of Na^+ and Cl^- in sheaths (4.6-fold higher in sheaths than in the leaf blade), while in the amphiploid Na^+ and Cl^- concentrations were 37% lower in the leaf blade than in sheaths. Na^+ and Cl^- concentrations were more uniform across the different leaf tissues in wheat. Therefore, *H. marinum* demonstrated a high capacity to accumulate toxic ions in older tissues, presumably in the vacuoles of the sheaths, and this was partially expressed also in the amphiploid. The level of *HKT1;5*-like transcripts across treatments in all genotypes (wheat cv. Chinese Spring, *H. marinum* (H21), their amphiploid) was higher in roots (~470-fold higher) than in leaf and sheaths. The similar abundance patterns of *HKT1;5*-like transcripts across the three genotypes, and together with the similar concentrations of K^+ in various tissues of the three genotypes suggests that K^+ homeostasis was similar in the genotypes. Under stagnant saline conditions, the level of *NHX1*-like transcripts in all tissues were higher in *H. marinum* and the amphiploid (~3.6-fold compared with those in control) than wheat. Thus, the possible role of the protein encoded by the *HKT1;5*-like transcript in transporting K^+ in favour of Na^+ mainly in roots, together with the putative role of *NHX1*-like transporter in Na^+ accumulation within leaf cell vacuoles, might have contributed to maintenance of the higher ratio of K^+/Na^+ in the leaf blade, and therefore,

contributed to the tolerance of *H. marinum* and its amphiploid to salinity, with both an aerobic or hypoxic root-zone.

6.2 INTRODUCTION

More than 900 Mha of the world's arable land is estimated to be salt affected (OECD/FAO 2012), causing a significant loss to crop production in many areas (Greenway and Munns 1980; Szabolcs 1994). Salinity impacts adversely on crops, such as wheat, by triggering both ionic and osmotic stresses (Munns 1993). Salinity is also often accompanied by waterlogging (Barrett-Lennard 2003; Barrett-Lennard and Shabala 2013), causing a very hostile environment for root respiration in most plants as soil O₂ availability is low (Huck 1970; Armstrong 1979; Webb and Armstrong 1983; Barrett-Lennard 1986). The combination of salinity and waterlogging reduces growth of many species (Barrett-Lennard and Shabala 2013), likely due to the increased rate of Na⁺ and Cl⁻ uptake to the shoot (Galloway and Davidson 1993; Barrett-Lennard *et al.* 1999; Barrett-Lennard 2003) and reduced K⁺ concentration in leaves (Akhtar *et al.* 1994; Malik *et al.* 2009a; Barrett-Lennard and Shabala 2013), relative to when grown in salinity alone. Low O₂ concentration at root-zone can influence the metabolic activities and energy production of plant cells (reviewed by Geigenberger 2003). Energy production can be reduced by 95% in O₂-deficient roots (Barrett-Lennard 2003) and the subsequent energy deficit can rapidly reduce growth and create ion imbalances (Trought and Drew 1980; Drew 1983; Barrett-Lennard 1986). These ion imbalances can cause severe tissue injury or even death of plants under conditions of combined salinity and hypoxia (Barrett-Lennard 2003). Thus, tolerance to both salinity and waterlogging is important for wheat production in salt affected areas with rising water-tables (Colmer *et al.* 2005b).

Within the Triticeae many wild species possess adaptability traits to tolerate many biotic and abiotic stresses (Jauhar and Peterson 1996). Improvement in resistance to biotic stresses in wheat has previously relied on genes sourced from wild species from within the Triticeae tribe (e.g. Friebe *et al.* 1996). Attempts have also been made to improve salt tolerance in wheat from such sources (Colmer *et al.* 2006b). Sea barleygrass (*Hordeum marinum* Huds.), a wild relative of wheat that inhabits salt marshes (von Bothmer *et al.* 1995) is more salt and waterlogging tolerant than wheat

(*Triticum aestivum* L.) (Colmer *et al.* 2005b). The tolerance of *H. maritimum* to salinity has been attributed to a high ability to 'exclude' Na^+ and Cl^- from the shoot and the better maintenance of tissue K^+ concentrations (Garthwaite *et al.* 2005). Tolerance of *H. maritimum* to waterlogging has been associated with the formation of aerenchyma and a barrier to radial O_2 loss (ROL) in the adventitious roots (McDonald *et al.* 2001a; Garthwaite *et al.* 2003; Malik *et al.* 2009a). *H. maritimum* can also withstand the combined effects of salinity and waterlogging by maintaining better ion regulation and superior root aeration, whereas wheat is relatively sensitive to the interactive effects of these two stresses (Malik *et al.* 2009b). *H. maritimum* is therefore a potential source of genes to improve the salt and waterlogging tolerance of wheat via wide hybridisations (Colmer *et al.* 2006b; Islam *et al.* 2007; Malik *et al.* 2011; Munns *et al.* 2011).

The amphiploid between *H. maritimum* (H21) and bread wheat (Chinese Spring) produced by AKMR Islam was reported to express salt tolerance (Islam *et al.* 2007) and moderate waterlogging tolerance (Malik *et al.* 2011). More recent work has shown that this amphiploid can withstand the combined effects of salinity and waterlogging by the expression of relevant traits from *H. maritimum* in the amphiploid (Chapter 5). Tolerance to combined salinity and waterlogging in *H. maritimum* and *H. maritimum*-wheat amphiploids was associated with lower concentrations of Na^+ and Cl^- , and a higher ratio of K^+/Na^+ in leaves, and the formation of aerenchyma and a barrier to radial O_2 loss (ROL) in adventitious roots (Chapter 5). Regulation of tissue ions (Na^+ , Cl^- , and K^+) is important to maintain tolerable concentrations in plant tissues (Watson *et al.* 2001). However, the mechanism(s) of regulation of leaf Na^+ concentrations in plants are complex (Tester and Davenport 2003), and are due to a combination of a number of Na^+ transport processes including Na^+ 'exclusion' from sensitive sites such as young leaves (itself involving several mechanisms), the accumulation of Na^+ in the vacuoles of cells and K^+/Na^+ discrimination by the roots (Flowers *et al.* 1977; Greenway and Munns 1980; Munns *et al.* 1995; Serrano 1996; Asch *et al.* 2000; Colmer *et al.* 2005a; Houshmand *et al.* 2005; Munns and Tester 2008). Therefore, many genes related to Na^+ transport would be likely contributing to tissue Na^+ regulation under combined salinity and waterlogging stress.

High-affinity K^+ Transporter (HKT) proteins have been reported to play a major role in controlling K^+ transport and in mediating Na^+ influx in roots (Rodriguez-Navarro and

Rubio 2006; Munns and Tester 2008). The constitutive recombinant overexpression of *TaHKT1;5* in durum wheat was shown to enhance K^+/Na^+ selectivity in loading of xylem in the roots (James *et al.* 2006). Members of the subfamily 5 of HKT1 transporters have been identified to mediate the uptake of Na^+ or K^+ from the apoplast in cereals (e.g. rice, Ren *et al.* 2005; wheat, Byrt *et al.* 2007). In addition, NHX (Na^+/H^+ eXchanger) proteins compartmentalise Na^+ into vacuoles by the exchange of Na^+ for H^+ across vacuolar membranes (Nass *et al.* 1997; Blumwald *et al.* 2000; Hasegawa *et al.* 2000; Aharon *et al.* 2003). In *Arabidopsis*, *AtNHX1* is regarded as contributing to salt tolerance by mediating the sequestration of Na^+ into the vacuole (Apse *et al.* 1999; Zhang and Blumwald 2001). Consistent with this, transcript levels of the vacuolar Na^+/H^+ antiporter *HvNHX1* in barley increase in response to salinity stress (Fukuda *et al.* 2004).

Despite the roles that orthologs of HKT1;5 and NHX1 transporters play in salt tolerance in different species, the regulation of these transporters in response to different stimuli or their expression in various tissues needs to be explored further (Maathuis 2007; Munns and Tester 2008). Little is known about the significance of HKT1;5-like and NHX1-like transporters in the salt tolerance of wheat under combined salinity and waterlogging, and for *H. marinum* information is completely lacking. The present study describes for the first time transcript-level evidence for the possible involvement of HKT1;5-like and NHX1-like transporters in salt and waterlogging tolerance of the *H. marinum* (H21)-bread wheat (Chinese Spring) amphiploid, in comparison with its parents under combined salt and hypoxia stress.

6.3 MATERIALS AND METHODS

Plant material and growth conditions

The plant genotypes used in this study were *Hordeum marinum* (H21; genome XX), hexaploid bread wheat (cv. Chinese Spring; genome AABBDD) and their amphiploid (genome AABBDDXX). Amphiploid seeds were provided by AKMR Islam (The University of Adelaide) and the *H. marinum* accession was originally provided by the Nordic Gene Bank (R. von Bothmer, Swedish Agricultural University, Alnarp, Sweden). Amphiploid production by the hybridisation of Chinese Spring (CS) as the

male with the *H. marinum* accession as the female has been described by Islam *et al.* (2007).

Experimental design

The three genotypes (described above) were subjected to two salinity treatments (0.2 or 200 mM NaCl) × two aeration treatments (aerated or stagnant) × four replicates, in a completely randomised design. The experiment was conducted in a naturally-lit phytotron (20/15°C day/night) with an average PAR at midday during the experimental period of $\sim 1160 \mu\text{mol m}^{-2} \text{s}^{-1}$. The experiment was conducted between 3-Nov-2011 and 14-Dec-2011 at the School of Plant Biology, UWA, Perth Western Australia.

To ensure that the plants were all at a similar developmental stage at the start of treatments, *H. marinum* was germinated three d before the wheat and the amphiploid. Seeds were surface-sterilised with 0.04% NaHClO in de-ionised (DI) water for 45 s, and then rinsed thoroughly with DI water before being imbibed overnight in aerated 0.5 mM CaSO₄. Seeds were then transferred onto a mesh floating on 10% strength aerated nutrient solution in darkness (pots covered with Al-foil) in a 20/15°C day/night phytotron for the first 4 d. Seedlings were exposed to light after 4 d when nutrient solutions were changed to 25% strength.

Eight seedlings were transplanted per pot into 4.5 L pots containing full-strength nutrient solution on day 7. Seedlings were held in the pot lids using polystyrene foam in holes (2 cm diameter). The full-strength nutrient solution contained (mM): K⁺, 3.95; Ca²⁺, 4.0; Mg²⁺, 0.4; NH₄⁺, 0.625; Na⁺, 0.2; NO₃⁻, 4.375; SO₄²⁻, 4.4; H₂PO₄⁻, 0.2; H₄SiO₄⁻, 0.1; with micronutrients (μM): Cl⁻, 50; B, 25; Mn, 2; Zn, 2; Ni, 1; Cu, 0.5; Mo, 0.5; Fe-EDTA, 50. *H. marinum* can be prone to Fe-deficiency under these conditions, therefore 5 μM FeSO₄ was supplied routinely (on 5 occasions to the young plants) to prevent any risk of such deficiency, but always to all pots. The solution was also buffered with 2.5 mM 2-(N-Morpholino)ethanesulfonic acid (MES) and the pH was adjusted to 6.5 using KOH, increasing the final K⁺ concentration to ~ 6.0 mM. Experimental units (pots) were re-randomised weekly to reduce any possible effects of environmental variation within the phytotron.

Treatments and harvests

Treatments were imposed when plants had 2–2.5 leaves (14-d-old plants for *H. maritimum* and 11-d-old plants for the wheat and the amphiploid). At the time the treatments were imposed, an initial harvest of four replicate plants of each of the three genotypes was taken from extra pots allocated to measure initial shoot and root dry mass (DM). NaCl was then stepped up in pots allocated to the saline treatment by 50 mM every 12 h until the final concentration of 200 mM was reached (36 h). Twelve h after reaching the final NaCl concentration, pots allocated to the stagnant treatment were given a ‘hypoxic pre-treatment’ in which the solution in these pots was bubbled with N₂ for ~2 h until the dissolved O₂ concentration was less than ~0.03 mM; these pots were then left overnight without bubbling. The following day, the nutrient solution in the stagnant pots was replaced with deoxygenated (i.e. pre-flushed with N₂ overnight) stagnant nutrient solution containing 0.1% (w/v) agar. The agar inhibits convective movements in the nutrient solution, impeding the re-entry of O₂ and promoting the accumulation of ethylene, both important changes in the gas composition of waterlogged soils (Wiengweera *et al.* 1997). The nutrient solutions of pots allocated for aerated treatments were also renewed at the same time as those in the stagnant treatments, but these aerated solutions were without agar and continued to be bubbled with air. Solutions in all pots were renewed every 7 d and aerated pots were topped up with DI water as required.

Six harvests were taken: on days 1, 2, 4, 8, 16 and 21 after stagnant agar treatment commenced. One plant from each pot was taken for the first five harvests, while three plants were allocated for the final harvest. At each harvest, roots and the stem base were washed 3 times for 30 s in mannitol solutions iso-osmotic with the Na⁺ plus Cl⁻ concentration of the nutrient solution; these wash solutions also contained 4 mM CaSO₄. At the first five harvests, plants were separated into shoot and roots then fresh mass (FM) was recorded. Samples were then immediately snap frozen in liquid N₂ and stored at -80°C for RNA extractions. For the final harvest (day 21 after stagnant agar treatment commenced), two plants were allocated for ion analyses and one plant was taken for RNA extractions. The plants of the two sets at the final harvest were separated into six tissue classes: leaf blade of leaf 5 (youngest fully expanded leaf, YFEL), leaf blade of leaf 4 (2nd YFEL), all green sheaths not suffering from symptoms of ion toxicity, seminal roots, adventitious roots and all other leaf tissues not included in the

other samples. Samples for RNA extractions were immediately snap frozen in liquid N₂ as described above. For the samples used for ion analyses, FM and DM were recorded before and after being oven-dried for 72 h at 65°C.

Growth measurements

The final harvest was taken after 21 days of stagnant agar treatment and plants were separated into the components described above. Relative growth rate (RGR) was calculated from the DM of the whole-plant samples at the start and end of the treatment period, using the formula described by Hunt (1978):

$$\text{RGR} = (\ln DM_2 - \ln DM_1) / (t_2 - t_1)$$

Where DM_1 is the dry mass (g) at time one (initial harvest), DM_2 is the dry mass (g) at time two (final harvest), t_1 and t_2 are times one and two in d, and ln is the natural logarithm.

Quantitative real-time PCR (qPCR)

Isolation of total RNA and DNase treatment

For the three genotypes, total RNA was extracted from various tissues (i.e. 2nd YFEL, sheaths and seminal roots) from all four treatments. Samples were ground to a fine powder in a high-speed homogeniser or using a mortar and pestle under liquid N₂. Total RNA from 50 to 100 mg of ground tissue was extracted according to the manufacturer (Bioline; ISOLATE Plant RNA Mini Kit), which is based on the guanidine thiocyanate protocol of Chomczynski (1993). The quality and quantity of the RNA were checked using a NanoDrop spectrophotometer ND-1000 (NanoDrop). Then genomic DNA was eliminated using RQ1 RNase-free DNase (Promega) and incubated at 37°C for 30 mins.

Reverse transcription

Quantitative reverse transcription-PCR (qRT-PCR) is the production of a single-stranded complementary DNA copy (cDNA) of the mRNA pool by reverse transcriptase (RT) enzymes. Single-strand synthesis of cDNA was performed on 1 µg of total RNA, estimated before DNase digest, by (i) denaturing RNA and oligo(dT)₁₈ primers at 70°C

for 5 min and (ii) reverse transcription by incubating the sample with M-MLV RT (Moloney Murine Leukemia Virus RT, RNase H minus point mutant, Promega) at 40°C for 10 min and 48°C for 50 min followed by a 70°C denaturation step for 15 min. Minus-RT controls were performed for each sample and primer pair in order to detect genomic DNA contaminations. The average genomic DNA contamination across samples was marginal, being $0.1\% \pm 0.03$ on average.

Optimisation of RT-PCR

For qPCR, primers for HKT1;5 (Table 6.1) were designed at the 3' end of the *H. maritimum* HKT1;5-like gene (*HmHKT1;5*, GenBank accession no. KF606928; confidential information was obtained from the author Damien Platten) and the *Triticum aestivum* HKT1;5-like gene (*TaHKT1;5*, GenBank accession no. DQ646342). Primers for a *NHX1*-like gene were designed at the 3' end of the *Hordeum vulgare* *NHX1*-like gene (*HvNHX1*, GenBank accession no. CQ969404). Using BLASTn (Altschul *et al.* 1997), a sequence alignment of *Hordeum maritimum* with *Triticum aestivum* HKT1;5 sequences shows the two sequences are 87% identical. The sequence alignment shows the forward and reverse primers located within conserved regions (Fig. 6.S1). Due to a lack of sequence information for *H. maritimum*, primers were designed based on the sequence of the *NHX1* gene of *Hordeum vulgare*. A sequence alignment of the *Hordeum vulgare* with the *Triticum aestivum* *NHX1* (GenBank accession no. CQ969406) sequence shows the two sequences were 94% identical. The sequence alignment shows the forward primer was located within conserved regions and had 100% identity with *Hordeum vulgare* and *Triticum aestivum* (Fig. 6.S2). Three reference or 'housekeeping' cDNA from wheat were used: actin (ACT-1; GenBank accession no. HX167418), elongation factor 1 alpha (EF-1; GenBank accession no. M90077) and protein phosphatase 2A subunit A3 (PP2AA3; GenBank accession no. BT009473) and all three were relatively stable in their transcript abundances across all treatments (less than ~13% different among treatments (in this experiment for all genotypes). The Cycle-threshold (Ct) $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) was used to determine the relative mRNA abundance above reference genes as internal references for normalisation.

For qPCR, primers for target (*HKT1;5*-like and *NHX1*-like sequences) and reference transcripts (*ACT-1*, *EF-1* and *PP2AA3*) were designed and highly purified salt-free

primers were generated (InvitrogenTM - USA; Table 6.1). PCR primers targeting highly conserved regions (Table 6.1) successfully amplified sequences from *H. marinum*, wheat and their amphiploid cDNA, using GoTaq DNA Polymerase (Promega). Specific melting temperatures for each primer pair are given in Table 6.1. Agarose electrophoresis was performed to visualise PCR products stained with ethidium bromide and each primer amplified a single band. Reactions for qPCR (10 µl) consisted of: 40 ng template cDNA, 0.3 µM primers and 2x Power SYBR Green master mix (Applied Biosystems[®] - USA). Standards, cDNA (4 biological replicates for each treatment x species combination) and negative controls were run in duplicate on 96-well plates. The real-time PCR amplification was conducted using a 7500 Fast Real Time PCR System (Applied Biosystems[®] - USA) following the manufacturer's standard protocol. The relative abundance of mRNAs was expressed as $40 - \Delta Ct$, with ΔCt being the difference in the threshold cycle (Ct) numbers between the target and the three reference transcripts with 40 being the maximum possible Ct value (Bari *et al.* 2006).

Table 6.1. Primer sequences used for quantitative RT-PCR of selected *HKT1;5*-like and *NHX1*-like target sequences (transcripts).

Origin	Primer names	Sequence (5' → 3')	Primer length	Tm* (C°)	Product Size (bp)
<i>Hordeum marinum</i> *	<i>HmHKT1;5F</i>	TCACCGTCGAGGTTATCAGTGCGT	24	61.9	80
	<i>HmHKT1;5R</i>	GTCGGGCGTCACTTGCCGGT	20	64.7	
<i>Triticum aestivum</i>	<i>TaHKT1;5F</i>	CCTACCACCTTACACTACATT	21	51.1	108
	<i>TaHKT1;5R</i>	TTTCCGTACGCACTGATAAC	20	56.4	
<i>Hordeum vulgare</i>	<i>HvNHX1F</i>	GGTTCAGATGAACGGTTGGT	24	61.5	144
	<i>HvNHX1R</i>	GCCGTCGGGCGTCACTTGC	19	63.2	

*These primers were designed based on a cDNA sequence of *Hordeum marinum* subsp. *marinum* (Damien Platten, unpublished results), Tm = melting temperature.

Tissue ion analyses

Concentrations of Na⁺, Cl⁻ and K⁺ were determined in the different shoot and root tissues. Oven-dried samples were ground and extracted in 0.5 M HNO₃ by shaking for 48 h at 20–25°C. Na⁺ and K⁺ concentrations were determined in dilutions of the extracts using a flame photometer (Jenway PFP7, Essex, UK). Cl⁻ concentration was determined

in the extracts using guanidine protocol of a chloridometer (Slamed CHLO 50 cl, Frankfurt, Germany). Blanks and a reference plant material with known ion concentrations were taken through the same procedures. Recoveries of Na^+ , Cl^- and K^+ from the reference plant tissue taken through the same procedure as samples were 94, 102 and 99% respectively. The data presented have not been adjusted.

Data analyses

All statistical analyses were conducted using GENSTAT 12th Edition (VSN International Ltd, Hemel Hempstead, UK). Residuals were checked for normality and no transformations were needed. Two-way ANOVA was used to identify overall significant differences between genotypes and between treatments. When significant differences were found, mean-separations were calculated using the Fisher unprotected l.s.d. test. Unless otherwise stated, the significance level was $P \leq 0.05$.

6.4 RESULTS

Growth

Relative growth rate (RGR) was higher for the amphiploid and wheat (0.18 and $0.17 \text{ g g}^{-1} \text{ d}^{-1}$, respectively) than *H. maritimum* ($0.14 \text{ g g}^{-1} \text{ d}^{-1}$) in control conditions (aerated non-saline; Fig. 6.1A). Exposure of plants to the stagnant non-saline treatment increased RGR to 118% of aerated control in *H. maritimum*, but reduced the RGR to 89% and 83% of control in the amphiploid and wheat, respectively (Fig. 6.1A). The salinity treatment (200 mM NaCl) had little effect on the RGR of *H. maritimum*, but reduced the RGR to 87% of aerated control in the amphiploid and further to 72% of aerated control in wheat (Fig. 6.1B). The combined salinity and stagnant treatment reduced the RGR of all genotypes to 75–78% of their respective rates in the aerated saline treatment. The RGR of *H. maritimum* in the combined salinity and stagnant treatment was reduced to 75% of the aerated non-saline control, 68% of control in the amphiploid and only 55% of control in wheat (Fig. 6.1B). Treatment effects on shoot and root DM were consistent with these general trends described for RGR (see Supplementary Materials, Fig. 6.S3 and 6.S4).

For the P -values from the ANOVA on the data of the growth parameters described above, see Supplementary Materials Table 6.S1.

Fig. 6.1. Whole plant relative growth rates (RGR) of *H. maritimum* (H21), wheat (*Triticum aestivum* cv. Chinese Spring) and their amphiploid grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. maritimum*, and 11-d-old for the wheat and amphiploid, so that plants were at a similar leaf development stage when treatments started. Plants were treated for 21 d with aerated (control) or stagnant non-saline solution (A); aerated or stagnant plus 200 mM NaCl (B). Note the different y-axes of (A) and (B). The data and errors for the saline treatment (B) are reported as % of control. Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between genotypes at $P = 0.05$.

Na⁺ concentrations in the 2nd youngest fully expanded leaf (2nd YFEL), sheaths and seminal roots

Aerated or stagnant saline solutions substantially increased Na⁺ concentrations in all three tissues of all three genotypes in comparison with the aerated non-saline controls (Fig. 6.2). In aerated non-saline controls, for all plants and tissues, the Na⁺ concentrations were low, however, the Na⁺ concentrations across all tissues were higher in the amphiploid and wheat (~200–300 μmol g⁻¹ DM) than in *H. marinum* (~150–200 μmol g⁻¹ DM) (Fig. 6.2A). The stagnant non-saline treatment had no significant effect on Na⁺ concentrations in any of the three tissues analysed, in comparison with the tissues of the aerated non-saline controls (Fig. 6.2B). Salinity treatment increased Na⁺ concentrations in all tissues for all genotypes, and the increase was largest (relative to non-saline aerated controls) for wheat (4–7-fold), compared to the amphiploid (2–6-fold) and *H. marinum* (2–4-fold; Fig. 6.2C). Under the combined stagnant saline treatment, the Na⁺ concentration across all tissues was higher in the amphiploid (~1.5-fold higher) and in wheat (~2.5-fold higher) than in *H. marinum* (Fig. 6.2D). Relative to the aerated saline treatment, the combined stagnant saline treatment increased Na⁺ concentrations by 93% in *H. marinum* (sheaths only), 98% in the amphiploid (2nd YFEL only), and to 60% and 105% and in the 2nd YFEL and sheaths of wheat, but had little effect on seminal roots of all three genotypes, 2nd YFEL of *H. marinum* or sheaths of the amphiploid (Fig. 6.2C and 6.2D). In the saline treatments (aerated or stagnant), the Na⁺ concentration in *H. marinum* was higher in sheaths (~3.5-fold higher) and in seminal roots (~2.5-fold higher) than in the 2nd YFEL. In the amphiploid, Na⁺ concentration showed similar trends to those of *H. marinum*; higher in sheaths (~1.8-fold higher) and also higher in seminal roots (~1.3-fold higher) than in the 2nd YFEL. In wheat, Na⁺ concentration was higher in sheaths (~1.3-fold higher) than in the 2nd YFEL, but it was ~46% higher in the 2nd YFEL than in seminal roots (Fig. 6.2C and 6.2D).

Cl⁻ concentrations in the 2nd youngest fully expanded leaf blade (2nd YFEL), sheaths and seminal roots

The combinations of salinity and stagnant treatments substantially increased Cl⁻ concentrations in all three tissues of all three genotypes in comparison with the aerated non-saline controls (Fig. 6.3). In aerated non-saline controls, Cl⁻ concentrations across all tissues were higher in *H. marinum* (271 μmol g⁻¹ DM) than in the amphiploid (213 μmol g⁻¹ DM) and in the wheat (166 μmol g⁻¹ DM; Fig. 6.3A). The stagnant non-saline

treatment reduced Cl^- concentrations in comparison with the aerated non-saline controls, and the decline in Cl^- concentrations was largest for wheat (~40% of control) compared to *H. marinum* and the amphiploid (76% of control; Fig. 6.3B). Salinity treatment substantially increased Cl^- concentrations in all tissues for all genotypes, and the increase was largest (relative to non-saline aerated controls) for wheat (5–8-fold) compared to *H. marinum* (2-fold increase), with the amphiploid intermediate (3–6-fold; Fig. 6.3C). For plants in stagnant saline treatment, Cl^- concentration across all tissues was higher in the amphiploid (~1.6-fold higher) and wheat (~2.6-fold higher) than in *H. marinum* (Fig. 6.3D). Relative to the aerated saline treatment, the combined stagnant saline treatment increased Cl^- concentrations in *H. marinum* (99% in sheathes and 76% in seminal roots), the amphiploid (109% in the 2nd YFEL and 30% in seminal roots), and wheat (242% % in the 2nd YFEL, ~50% in sheathes and seminal roots), but had little effect on the 2nd YFEL of *H. marinum* and sheaths of the amphiploid (Fig. 6.3C and 6.3D).

In *H. marinum*, the average Cl^- concentration was higher in sheaths (~3.8-fold higher) and in the seminal roots (~1.5-fold higher) than in the 2nd YFEL, under saline treatments (aerated or stagnant). In the amphiploid, Cl^- concentration was higher in sheaths (~1.8-fold higher) than in the 2nd YFEL, but it was ~14% higher in the 2nd YFEL than in the seminal roots (Fig. 6.3). In wheat, Cl^- concentrations showed similar trends to those in the amphiploid; being higher in the sheaths (~1.2-fold higher) than in the 2nd YFEL, but they were ~76% higher in the 2nd YFEL than in the seminal roots (Fig. 6.3C and 6.3D).

K⁺ concentrations in the 2nd youngest fully expanded leaf blade (2nd YFEL), sheaths and seminal roots

In general, all combinations of salinity and stagnant substantially reduced K^+ concentrations in all three tissues of all three genotypes in comparison with the aerated non-saline controls (Fig. 6.4). For plants in aerated non-saline controls, K^+ concentration in all tissues was lower in *H. marinum* (~1250 $\mu\text{mol g}^{-1}$ DM) than the amphiploid and wheat (~2500 $\mu\text{mol g}^{-1}$ DM; Fig. 6.4A). The stagnant non-saline treatment had little effect on K^+ concentrations of *H. marinum*, but K^+ concentrations were reduced to ~69% of the respective aerated controls for the amphiploid and ~44% for wheat (Fig. 6.4B). Salinity treatment increased K^+ concentrations in the 2nd YFEL (146% of control) for *H. marinum*, and had little effect on sheaths, but K^+

concentrations were reduced to 48% of aerated controls in seminal roots. For the amphiploid and wheat, salinity treatment reduced K^+ concentrations to 48–65% of the aerated controls (Fig. 6.4C). Under the combined stagnant saline treatment K^+ concentration across all tissues of all three genotypes was reduced to ~32% of controls (Fig. 6.4D). Relative to the aerated saline treatment the combined stagnant saline treatment substantially reduced K^+ concentrations in all tissues for all genotypes; ranging between 39–68% of aerated saline (Fig. 6.4C and 6.4D). However, in saline treatments (aerated or stagnant), the average of K^+ concentrations in the 2nd YFEL and sheaths was higher (~56% higher) than in seminal roots for all genotypes (Fig. 6.4C and 6.4D).

K⁺/Na⁺ ratio

Compared with aerated non-saline controls, saline and stagnant saline treatments substantially reduced the K^+/Na^+ ratio in all tissues for all genotypes; however the reduction was greatest for wheat (Table 6.2). In the aerated non-saline controls, the K^+/Na^+ ratio in all tissues ranged between 5.1 and 10.5. By contrast, for the aerated saline treatment, the K^+/Na^+ ratio ranged between 0.8 and 6.2 in all tissues. The combined salinity and stagnant treatment reduced the K^+/Na^+ ratio even further to 0.2–2.7 in *H. maritimum*, to 0.4–0.7 in the amphiploid and to 0.3 in wheat (Table 6.2).

In *H. maritimum*, the K^+/Na^+ ratio was higher (40% of control) in the 2nd YFEL than in sheaths and in seminal roots (only 3–4% of control), under stagnant saline treatment. In the amphiploid, the stagnant saline treatment reduced the K^+/Na^+ ratio to only 7% of control across all tissues. In wheat, the K^+/Na^+ ratio showed similar trends to the amphiploid; but under stagnant saline treatment was only 4% of control across all tissues (Table 6.2).

For the *P*-values from the ANOVA of the tissue ion data described above, see Supplementary Materials Table 6.S2.

Fig. 6.2. Concentrations of Na^+ in the 2nd youngest fully expanded leaf blade, sheaths and seminal roots tissues of *H. marinum* (H21), wheat (*Triticum aestivum* cv. Chinese Spring) and their amphiploid grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. marinum*, and 11-d-old for wheat and the amphiploid, so that plants were at a similar leaf development stage when treatments started. Plants were treated for 21 d with aerated (control) (A); or stagnant non-saline solution (B); or aerated plus 200 mM NaCl (C) or stagnant plus 200 mM NaCl (D). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between genotypes at $P = 0.05$.

Fig. 6.3. Concentrations of Cl^- in the 2nd youngest fully expanded leaf blade, sheaths and seminal roots tissues of *H. maritimum* (H21), wheat (*Triticum aestivum* cv. Chinese Spring) and their amphiploid grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. maritimum*, and 11-d-old for wheat and the amphiploid, so that plants were at a similar leaf development stage when treatments started. Plants were treated for 21 d with aerated (control) (A); or stagnant non-saline solution (B); or aerated plus 200 mM NaCl (C) or stagnant plus 200 mM NaCl (D). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between genotypes at $P = 0.05$.

Fig. 6.4. Concentrations of K^+ in the 2nd youngest fully expanded leaf blade, sheaths and seminal roots tissues of *H. marinum* (H21), wheat (*Triticum aestivum* cv. Chinese Spring) and their amphiploid grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. marinum*, and 11-d-old for wheat and the amphiploid, so that plants were at a similar leaf development stage when treatments started. Plants were treated for 21 d with aerated (control) (A); or stagnant non-saline solution (B); or aerated plus 200 mM NaCl (C) or stagnant plus 200 mM NaCl (D). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between genotypes at $P = 0.05$; ns = not significant.

Table 6.2. Ratio of K^+/Na^+ in the 2nd youngest fully expanded leaf blade, sheaths and seminal roots of *H. maritimum* (H21; *H.m.*), wheat (*Triticum aestivum* cv. Chinese Spring) and their amphiploid (Amp) when grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. maritimum*, and 11-d-old for wheat and the amphiploid. Plants were treated for 21 d. Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot.

Tissue	Genotype	K^+/Na^+			
		Non-saline		Saline	
		Aerated	Stagnant	Aerated	Stagnant
Leaf blade	<i>H.m.</i>	6.8 $\pm$ 0.7	7.5 $\pm$ 0.6	6.2 $\pm$ 2.4	2.7 $\pm$ 0.3
	Amp	10.6 $\pm$ 2.4	9.7 $\pm$ 2.0	3.2 $\pm$ 0.7	0.7 $\pm$ 0.1
	Wheat	10.5 $\pm$ 2.1	10.7 $\pm$ 2.1	1.6 $\pm$ 0.3	0.3 $\pm$ 0.1
Sheaths	<i>H.m.</i>	9.9 $\pm$ 4.1	8.2 $\pm$ 3.1	1.9 $\pm$ 0.3	0.4 $\pm$ 0.1
	Amp	10.0 $\pm$ 1.1	8.7 $\pm$ 1.6	0.8 $\pm$ 0.2	0.6 $\pm$ 0.1
	Wheat	10.4 $\pm$ 2.1	7.3 $\pm$ 1.0	1.2 $\pm$ 0.2	0.3 $\pm$ 0.0
Seminal roots	<i>H.m.</i>	6.5 $\pm$ 2.4	5.9 $\pm$ 2.4	0.7 $\pm$ 0.1	0.2 $\pm$ 0.1
	Amp	5.1 $\pm$ 0.6	8.7 $\pm$ 2.0	0.8 $\pm$ 0.2	0.4 $\pm$ 0.0
	Wheat	5.3 $\pm$ 0.4	6.9 $\pm$ 1.4	0.8 $\pm$ 0.2	0.3 $\pm$ 0.1
l.s.d. ^A		ns	ns	ns	0.19

^AThe l.s.d. represents significant differences between genotypes at $P = 0.05$; ns = not significant.

Transcript abundance of HKT1;5-like sequences in the 2nd youngest fully expanded leaf blade (2nd YFEL), sheaths and seminal roots

The level of *HKT1;5*-like transcripts across treatments in all genotypes was higher in seminal roots (~470-fold higher) than in the 2nd YFEL and sheaths (Fig. 6.5). In aerated non-saline controls, the level of *HKT1;5*-like transcripts was higher in the 2nd YFEL of the amphiploid and wheat (~102-fold higher) than *H. maritimum*, but it was similar for sheaths and seminal roots of the three genotypes (Fig. 6.5A). There were no treatment effects on the level of *HKT1;5*-like transcripts in the various tissues, nor for any genotype, in comparison with the respective aerated non-saline controls (Fig. 6.5).

Transcript abundance of NHX1-like sequences in the 2nd youngest fully expanded leaf blade (2nd YFEL), sheaths and seminal roots

In all treatments, the level of *NHX1*-like transcripts was highest in *H. maritimum*, intermediate in the amphiploid and lower in wheat (Fig. 6.6). In aerated non-saline controls, the level of *NHX1*-like transcripts was higher in *H. maritimum* than the amphiploid (3.6-fold higher) and was 394-fold higher in *H. maritimum* than in wheat (Fig. 6.6A). Stagnant non-saline solutions increased the level of *NHX1*-like transcripts in the amphiploid (2.5-fold increase) and *H. maritimum* (1.6-fold increase), but it was similar for wheat, in comparison with the aerated non-saline controls (Fig. 6.6B). Salinity treatment had no significant effect on the level of *NHX1*-like transcripts in comparison with the aerated non-saline controls (Fig. 6.6C). Under the combined stagnant saline treatment the level of *NHX1*-like transcripts across all tissues was higher in *H. maritimum* and the amphiploid (~3.6-fold compared with those in control) than wheat (similar to control; Fig. 6.6D).

In *H. maritimum*, under stagnant saline treatment, the *NHX1*-like transcript level was higher in the 2nd YFEL and sheaths (20 and 6-fold higher, respectively) than in seminal roots, whereas, in the amphiploid and wheat the transcript abundances were similar across all tissues (Fig. 6.6 D).

For the *P*-values from the ANOVA of the transcript abundance parameters described above, see Supplementary Materials Table 6.S3.

Fig. 6.5. Relative transcript abundance of *HKT1;5*-like sequences (based on primers designed from a putative *Hordeum marinum* *HKT1;5* cDNA sequence) from qRT-PCR of tissues from *H. marinum* (H21), wheat (*Triticum aestivum* cv. Chinese Spring) and their amphiploid grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. marinum*, and 11-d-old for wheat and the amphiploid, so that plants were at a similar leaf development stage when treatments started. RNA was extracted from 2nd youngest fully expanded leaf blade, sheaths and seminal roots tissues after plants were treated for 21 d with aerated (control) (A); stagnant non-saline solution (B); aerated plus 200 mM NaCl (C) or stagnant plus 200 mM NaCl (D). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. Note that the 40- Δ Ct y-axis scale is a logarithmic one with a difference of 1 representing a 2-fold change in transcript abundance. Data were normalised using three reference genes (*ACT-1*, *EF-1* and *PP2AA3*). The l.s.d. represents significant differences between genotypes at $P = 0.05$.

Fig. 6.6. Relative transcript abundance of *NHX1*-like sequences (based on primers designed from a *Hordeum vulgare* *NHX1* cDNA sequence) from qRT-PCR of tissues from *H. marinum* (H21), wheat (*Triticum aestivum* cv. Chinese Spring) and their amphiploid grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. marinum*, and 11-d-old for wheat and the amphiploid, so that plants were at a similar leaf development stage when treatments started. RNA was extracted from the 2nd youngest fully expanded leaf blade, sheaths and seminal roots tissues after plants were treated for 21 d with aerated (control) (A); stagnant non-saline (B); aerated plus 200 mM NaCl (C) or stagnant plus 200 mM NaCl (D). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. Note that the 40- ΔC_t y-axis scale is a logarithmic one with a difference of 1 representing a 2-fold change in transcript abundance. Data were normalised using three reference genes (*ACT-1*, *EF-1* and *PP2AA3*). The l.s.d. represents significant differences between genotypes at $P = 0.05$.

6.5 DISCUSSION

The results of the present chapter describe for the first time the transcript abundances of HKT1;5-like and NHX1-like transporters in response to salt and stagnant treatments for a *H. maritimum* (H21)-bread wheat amphiploid, in comparison with its parents under stagnant saline conditions. The amphiploid was more tolerant than wheat to the combined stagnant saline conditions. The improvement in tolerance to salinity and hypoxia stress in the amphiploid was evident as higher relative growth rates (RGR), and was associated with better regulation of tissue ion concentrations and higher transcript abundance of a *NHX1*-like sequence, as compared with wheat. The *NHX* gene product is believed to function by compartmentalising Na^+ into vacuoles and thus has been shown to contribute to salt tolerance in several plant species (Pardo *et al.* 2006). Therefore higher *NHX1*-like transcript levels could be associated with salt tolerance in *H. maritimum* and this seems also to have been transferred to the amphiploid.

Regulation of Na^+ , Cl^- , and K^+ uptake and sequestration in salt-stressed plants is an important mechanism to maintain tolerable levels of these ions in plant tissues (Watson *et al.* 2001). The physiological mechanisms associated with salt resistance include: Na^+ ‘exclusion’ from the shoot (itself involving several processes), compartmentalisation of Na^+ into the vacuoles of cells, and K^+/Na^+ discrimination during uptake and transport within the plant (Colmer *et al.* 2006b; Munns and Tester 2008). When waterlogging occurs in saline soils, the lack of O_2 supply to the roots can affect ion transport processes by reducing ATP production (Greenway and Gibbs 2003). This reduction in energy production in the roots presumably increases salt uptake to the shoot system, and this has adverse impacts on the growth and survival for most crop species (Barrett-Lennard 2003). However, wetland halophytes (e.g. *H. maritimum* - Malik *et al.* 2009a; Chapter 5) can withstand the combined effects these stresses (Colmer and Flowers 2008). In the present study, the combined stagnant saline treatment had less adverse effects on *H. maritimum* than in wheat, and the effects on the amphiploid were intermediate. With the stagnant-plus 200 mM NaCl treatment, *H. maritimum* maintained a RGR of 75% of control; by contrast, the RGR of wheat was only 55% of control, while the amphiploid was intermediate (68% of control; Fig. 6.1B). The higher RGR of *H. maritimum* was associated with better ion ‘exclusion’; averaged across all tissues in *H. maritimum*, the Na^+ and Cl^- concentrations were ~35% lower than in the amphiploid and ~64% lower than in the wheat (Fig. 6.2 and Fig. 6.3).

Salt tolerant plants can restrict the entry of Na^+ and Cl^- into shoots (Munns 2005) and sequester toxic Na^+ into vacuoles so that it is kept away from sensitive enzymes in the cytoplasm (Gorham *et al.* 1985). *H. maritimum* has been identified as an ion ‘excluder’ (Garthwaite *et al.* 2005), and this was confirmed in the present study as it maintained low leaf Na^+ ($350 \mu\text{mol g}^{-1}$ DM in 2nd YFEL blade) and Cl^- ($326 \mu\text{mol g}^{-1}$ DM in 2nd YFEL blade) under stagnant saline conditions. In contrast to the leaf blade, *H. maritimum* accumulated higher concentrations of Na^+ and Cl^- in sheaths (4.6-fold higher in sheaths than in the 2nd YFEL) and seminal roots (2.1-fold higher in seminal roots than in the 2nd YFEL). Similarly, the amphiploid was better than wheat at controlling the entry of Na^+ and Cl^- , with concentrations 45% lower in the leaf blade and sheaths of the amphiploid compared to wheat under stagnant saline condition. Furthermore, concentrations of Na^+ and Cl^- in the amphiploid were ~37% lower in the 2nd YFEL than in sheaths, whereas they did not differ in wheat (Fig. 6.2D and Fig. 6.3D). This could indicate that *H. maritimum* and the amphiploid are capable of maintaining low concentrations of toxic ions in young leaves by accumulating toxic ions in older tissues, presumably in the vacuoles as the sheaths were green and not suffering from symptoms of ion toxicity. This suggests that traits from *H. maritimum* related to the control of Na^+ and Cl^- entry into the shoot are expressed in the amphiploid.

The ability to maintain the optimal ratio of K^+/Na^+ in leaves is an important trait for salinity tolerance (Munns and Tester 2008; Barrett-Lennard and Shabala 2013). The amphiploid and *H. maritimum* had lower shoot Na^+ concentrations, resulting in higher ratios of K^+/Na^+ in the leaves compared to wheat. For example, when grown under saline conditions, the ratio of K^+/Na^+ in the 2nd YFEL was high in *H. maritimum* (2.7), intermediate in the amphiploid (3.2) and low wheat (1.6). The increase in Na^+ concentrations in sheaths and seminal roots in *H. maritimum* decreased the ratio of K^+/Na^+ to only 4% and 3% of control in sheaths and in seminal roots, respectively, but it did not differ in the amphiploid or wheat, under stagnant saline condition (Table 6.2). This result supports findings from previous studies that showed an improvement in K^+/Na^+ discrimination in the amphiploid compared to the respective wheat parents (Islam *et al.* 2007; Munns *et al.* 2011; Chapter 5), and further supports the view that salt tolerance traits were expressed in the amphiploid from *H. maritimum*.

Reducing salt-induced K^+ efflux and maintaining an optimal cytosolic K^+/Na^+ ratio can contribute to osmotic balance and therefore increase salinity tolerance (Cuin *et al.* 2008). HKT transporters involved in ion transport mechanisms, such as mediate both K^+ and Na^+ transport, are associated with salt tolerance (Rodriguez-Navarro and Rubio 2006; Munns and Tester 2008). Several genes from the *HKT* family have been studied in wheat (Benderradji *et al.* 2011). *TaHKT2;1* (*TaHKT1*) was the first *HKT* gene cloned from wheat and its transcript abundance was high in particular root and leaf regions which are important sites for mediating both K^+ and Na^+ transport (Schachtman and Schroeder 1994). However, at high external Na^+ concentrations, K^+ transport mediated by HKT1 is blocked and facilitates low-affinity Na^+ uptake, which confers salinity tolerance (Rubio *et al.* 1995). The abundance of *HKT* transcripts in wheat was associated with higher activity of functional transporters as seen in the enhanced removal of Na^+ from the xylem of the leaf sheath, reduced Na^+ entry into the leaf blade, and enhanced K^+ accumulation in leaf blades and sheaths (James *et al.* 2006). HKT1;5 is likely to function in removing Na^+ from the xylem stream (e.g. *Oryza sativa*, Ren *et al.* 2005; *Arabidopsis thaliana*, Sunarpi *et al.* 2005) which in roots might then be transported back out into the soil solution (Byrt *et al.* 2007). In bread wheat (*Triticum aestivum* L.), *HKT1;5* transcripts were more abundant in cv. Hidhab than in cv. Mahon-Demias, and this higher expression was associated with enhanced discrimination of K^+ over Na^+ (Benderradji *et al.* 2011). In the present study, levels of the *HKT1;5*-like transcripts were high in the roots of all genotypes (Fig. 6.5 and Fig. 6.S5). *HKT1;5*-like sequences were expressed in all three tissues (roots, sheath, leaf blade) of the three genotypes studied, and in salt-stressed *H. maritimum* the transcript levels were increased by 195-fold in leaf blade and ~5.5-fold in sheaths and seminal roots compared with control (Fig. 6.5). This raises the possibility that, in *H. maritimum*, *HKT1;5*-like gene products might be involved in the removal of Na^+ from the xylem stream before it reaches the leaf parenchyma cells as well as play a role in K^+ transport within the leaf blade tissue, this could be supported by the higher K^+/Na^+ ratio in leaf blade versus the lower ratio in sheaths of salt-stressed *H. maritimum*, whereas the K^+/Na^+ ratios were similar in all tissues of wheat (Table 6.2). The levels of *HKT1;5*-like transcripts across the three genotypes in this study showed similar abundance patterns (Fig. 6.5), and together with the similar concentrations of K^+ in various tissues of the three genotypes suggests that K^+ uptake occurs with comparable efficiency in all genotypes (Fig. 6.4), although such similarity in K^+ concentrations was not observed in a previous study on the YFEL of four *H. maritimum* accessions, wheat and their amphiploids (Chapter 5).

Divergence in gene function can occur by neofunctionalisation in which a gene copy acquires a new function (Force *et al.* 1999; Blanc and Wolfe 2004). For example, the function of *TaHKT2;1* in wheat is to mediate both K^+ and Na^+ co-transport (Schachtman and Schroeder 1994), while the *AtHKT1;1* (*AtHKT1*) transporter in *Arabidopsis* does not show significant K^+ transport activity (Uozumi *et al.* 2000), making gene function difficult to compare between these two species (Rus *et al.* 2006). It is likely, therefore, that *HKT1;5* orthologues in both *H. maritimum* and wheat have diverged from each other which might be reflected by different effects of each orthologue on mediating both K^+ and Na^+ transport in these two species. The function of *HKT1;5*-like transporters in *H. maritimum*, however, remains to be elucidated, and further studies are needed to obtain a greater understanding of the functional role of the product of this putative gene in *H. maritimum* response to salt stress.

In salt-stressed plants, toxic Na^+ must be sequestered away from sensitive enzymes in the cytoplasm (Gorham *et al.* 1985). Salt-tolerant plants can accumulate Na^+ in the vacuoles of older leaves, a process termed “tissue tolerance” (Yeo and Flowers 1986). The transcript abundance of the *NHX1* gene is considered one approach to assess tissue tolerance (Munns and James 2003). The *NHX* transporters (vacuolar Na^+/H^+ antiporter) compartmentalise Na^+ into vacuoles and over-expression lines have been reported to show improved salinity tolerance in *Arabidopsis*, tomato and *Brassica* (Aharon *et al.* 2003). I therefore studied the transcript abundance of a *NHX1*-like gene in *H. maritimum* and in a *H. maritimum*-bread wheat amphiploid, as I expected some contribution of expression modulation of *NHX1*-like transcripts from its halophytic parent chromosomes. Overall, the levels of *NHX1*-like transcripts were high in *H. maritimum*, intermediate in the amphiploid and lower in wheat (Fig. 6.7). Under stagnant saline conditions, the higher levels of *NHX1*-like transcripts in *H. maritimum* (1300-fold higher) and the amphiploid (370-fold higher) than wheat (Fig. 6.7D) were associated with higher RGR (75% and 68% of control in *H. maritimum* and the amphiploid, respectively, compared to 55% of control in wheat; Fig. 6.1B). It appears that *H. maritimum* has a higher capacity to accumulate high concentrations of Na^+ , presumably in the vacuoles of the sheaths in order to maintain low concentration in the leaf blade (sheaths Na^+ concentration was 4.4-fold higher than leaf blade under stagnant saline conditions; Fig. 6.2D). Such a pattern of higher levels of *NHX1*-like transcripts could indicate a higher level of tissue tolerance in *H. maritimum*. These results also clearly demonstrate increased

levels of *NHX1*-like transcripts in the amphiploid, as contributed by the wild parent *H. maritimum* and expressed in the genetic background of wheat.

This study confirmed the improvement in salt tolerance in the *H. maritimum* (H21)-bread wheat amphiploid in terms of improved plant growth, and lower tissue ion concentrations (Na^+ , Cl^-) and better maintenance of organ-level K^+/Na^+ ratios, but also identified sequences for candidate *HKT1;5*-like and *NHX1*-like transcripts as potentially having an influence on this tolerance. Tolerance to salinity in *H. maritimum* and the amphiploid seems to not only depend on Na^+ 'exclusion' from the shoot or on the ability to maintain an adequate K^+ -to- Na^+ ratio in photosynthetically active leaves, but also on a putatively higher capacity of Na^+ compartmentalisation into vacuoles as indicated by the high levels of *NHX1*-like transcripts in sheath and leaf blade tissues.

6.6 SUPPLEMENTARY MATERIALS

<i>T. aestivum</i>	957	ATGGGTTCTTTGCATGTCTCC-TC-G-AGT-GC--CACTCAACATAGCAAGCTTGAGAGGGCTTACCAACTCCTGGTTTTCCATGTGCACCCGTTCTGGCTCCAGCTCTTGTA	1070
<i>H. marinum</i>	1	ATGGGTTCTTTGCATGTCTCCGCCGAAGTACCAACTCAATATAGCAGGGTTACAGGGCTTACCAACTCTTGTTTTCCATGTGCATCCGTTCTGGCTCCAGCTCTTGTA	120
<i>T. aestivum</i>	1071	TCCATCTCCTTCTTCGGTTTGGTGATCCTCAAAGCCCTCCCCATGAAGACCAGCACGGTCCCGAGGCCCATGGATTGGACCTGATCTTCACGTCGGTCTCGGCGACCACGGTGTGAGC	1190
<i>H. marinum</i>	121	TCCATCTCCTTCTTCGGCTTCGTATGCTGAAAGCCCTCCCCATGAGGACCAGCA--T-----GCCACGGACCTAGACCTGATCTTCACGTCGGTGTGCGCGATGACGGTGTGAGC	231
<i>T. aestivum</i>	1191	ATGGTGGCCGTGGAGATGGAGTCTCTCTCAACCCCCAGCTCCTACTCCTGACCTCCTCATGCTCCTCGGCGCGAGGTGTTACAGAGCATGCTTGGCCTGCACCTTCACCTACGTC	1310
<i>H. marinum</i>	232	ATGCAGGCCGTGGAGATGGAGTCTCTCTCAACCCCCAGCTCCTACTCCTAACCTCCTCATGCTTCTCGGCGCGAGGTGTTACACAGCATCCTTGGCCTGTACTTCACCTACGTC	351
<i>T. aestivum</i>	1311	TCCAAGAAGAAAGAACACAGCACCCACGACCATGACGATGGTGACAAAGGCAAACCAGCACCATCATCTAGCCTAGAGCTCGCTGTTACCACCGCATGGATGACG-----T-C--	1421
<i>H. marinum</i>	352	TCCAAGAAAAAGAACACAGGCACCCCATGA---TGAC---GGTGCCAAAGTCAAACCAGCACCATCTAC--G-CTAGAGCTCACGGCTACCGTCTGCATGGACGACGGCACCGTACAG	462
<i>T. aestivum</i>	1422	GATCGTGTGGAGCAAGGGTTTAAGGACCAGCCCCGTTACGATCGCGCCTTCCTCACCAGGTTGCTTCTGTTTCATAGTGTGGGTATCACGTGGTGGTGACCTCGCCGGCTACTCCTTG	1541
<i>H. marinum</i>	463	GACCATATGGAGCAAGGGTTCAAGGACCAGCCCCGTTACGGCCGGCCTTCCTCACCAGGTTGCTCCTGTTTCATCGTGCTCGGCTACCACGGTGGTGACCTGGCCGGCTACTCCCTG	582
<i>T. aestivum</i>	1542	ATGCTGGTCTACCTGAGCGTGGTCTCCGGCGGAGGGCTGTGCTACCCGCAAGGGGATCAGCCTGCACACCTTCTCCGTCTTCACCGTCGTCTCGACGTTCCGCAACTGCGGCTTCGTC	1661
<i>H. marinum</i>	583	ATGCTGGTCTACCTGAGCGTCGTGTCCGGCGGAGGGTGGTGCTCGCCGCAAGGGGATCAGCATGCACACCTTCTCGATCTTCACCATCGTCTCGACGTTCCGGAAGTGGGCTTCATA	702
<i>T. aestivum</i>	1662	CCGAACAACGAAGGGATGATCGCCTTCCGGTCCTTCCCGGCCTCCTGCTCCTAGTCATGCCGCACGTCCTCCTCGGCAACACACTCTTCCCGTCTTCCCTCAGGCTGGCCATCTGGGCT	1781
<i>H. marinum</i>	703	CCGAACAACGAAGGGATGGCCTCCTTCCGGTCCTTCCCGGCCTCCTCCTCCTCGTCATGCCGCACGTCCTCCTGGGAAACACGCTCTTCCCGTCTTCCCTCAGGCTGACGATCTGGGCT	822
<i>T. aestivum</i>	1782	CTCCGAGAGTCACCAGGAGGCCGAGCTCGGTGAGCTGAGGAGCATCGGCTACGACCACCTGCTGACGAGCCGCGACACGTGGTTCTTGCTTTACCGTGGCGGCGTTCTGTGCTAGCG	1901
<i>H. marinum</i>	823	CTCCAGAGAGTCACCAAGAGGCTGAGCTCGGTGAGCTGCGGAGCATCGGCTACGACCACCTCCTGACGAGCCGGCGCACAGGTTCTTAGCGTTACCGTGGCCGCGTTCTGTGCTGGCG	942

Fig. 6.S1. Alignment of *Triticum aestivum* (GenBank accession no. DQ646342) with *Hordeum marinum* (GenBank accession no. KF606928, information was obtained from the author Damien Platten) *HKT1;5* cDNA sequences using BLASTn. Arrows above nucleotide sequences indicate forward and reverse primers used in the qRT-PCR analyses. Dashes represent gaps.

[illegible]

Fig. 6.S2. Alignment of *Hordeum vulgare* (GenBank accession no. CQ969404) with *Triticum aestivum* (GenBank accession no. CQ969406) *NHX1* cDNA sequences using BLASTn. Arrows above nucleotide sequences indicate forward and reverse primers used in qRT-PCR analyses. Dashes represent gaps.

Fig. 6.S3. Shoot dry mass of *H. marinum* (H21), wheat (*Triticum aestivum* cv. Chinese Spring) and their amphiploid grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. marinum*, and 11-d-old for wheat and the amphiploid, so that plants were at a similar leaf development stage when treatments started. Plants were treated for 21 d with aerated (control); or stagnant non-saline solution (A); or aerated or stagnant plus 200 mM NaCl (B). Each value is the mean of 4 replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between genotypes at $P = 0.05$.

Fig. 6.S4. Root dry mass of *H. maritimum* (H21), wheat (*Triticum aestivum* cv. Chinese Spring) and their amphiploid grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. maritimum*, and 11-d-old for wheat and the amphiploid, so that plants were at a similar leaf development stage when treatments started. Plants were treated for 21 d with aerated (control); or stagnant non-saline solution (A); or aerated or stagnant plus 200 mM NaCl (B). Each value is the mean of 4 replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. The l.s.d. represents significant differences between genotypes at $P = 0.05$.

Fig. 6.S5. Relative abundance of *HKT1;5*-like transcripts (based on primers designed from *Triticum aestivum* *HKT1;5* cDNA sequence) from qRT-PCR of tissues from *H. maritimum* (H21), wheat (*Triticum aestivum* cv. Chinese Spring) and their amphiploid grown in aerated or stagnant nutrient solution with or without 200 mM NaCl. Treatments were imposed when plants were 14-d-old for *H. maritimum*, and 11-d-old for wheat and the amphiploid, so that plants were at a similar leaf development stage when treatments started. RNA was extracted from the 2nd youngest fully expanded leaf blade, sheaths and seminal roots tissues after plants were treated for 21 d with aerated (control) (A); or stagnant non-saline solution (B); or aerated plus 200 mM NaCl (C) or stagnant plus 200 mM NaCl (D). Each value is the mean of four replicates $\pm$ standard error, and each replicate is a single plant grown in a different pot. Note that the 40- Δ Ct y-axis scale is a logarithmic one with a difference of 1 representing a 2-fold change in transcript abundance. Data were normalised using three reference genes (*ACT-1*, *EF-1* and *PP2AA3*). The l.s.d. represents significant differences between genotypes at $P = 0.05$. Note missing data were due to undetermined transcript abundance (i.e. no PCR products formed).

Table 6.S1. *P* values from the ANOVA of parameters fitted to the growth data set.

Parameter	S	A	Geno.	S×A	A×Geno.	S×Geno.	A×S×Geno.	Fig.
RGR	<0.001	<0.001	0.001	0.011	0.041	0.232	0.042	6.1
Non-saline	-	0.145	0.023	-	0.001	-	-	6.1A
Saline	-	<0.001	0.003	-	0.872	-	-	6.1B
Shoot dry mass	<0.001	<0.001	<0.001	0.240	0.008	<0.001	0.206	6.S3
Non-saline	-	0.011	<0.001	-	0.031	-	-	6.S3A
Saline	-	0.012	<0.001	-	0.290	-	-	6.S3B
Root dry mass	<0.001	<0.001	<0.001	0.296	<0.001	0.001	0.003	6.S4
Non-saline	-	<0.001	<0.001	-	<0.001	-	-	6.S4A
Saline	-	<0.001	<0.001	-	0.088	-	-	6.S4B

Treatments were imposed when plants were 14-d-old for *H. marinum*, and 11-d-old for wheat and the amphiploid. Plants were treated for 21 d. Four replicates each one represents a single plant grown in different pots. S = Salinity (0.2 and 200 mM NaCl); A = Aeration (Aerated and Stagnant as deoxygenated stagnant agar in nutrient solution); Geno. = Genotype (*H. marinum* (H21), wheat (*Triticum aestivum* cv. Chinese Spring) and their amphiploid).

Table 6.S2. *P* values from the ANOVA of parameters fitted to tissue ion analyses data set.

Parameter	Treat.	Geno.	Tiss.	Treat.xGeno.	Treat.xTiss.	Geno.xTiss.	Treat.xGeno.xTiss.	Fig. and Table
Na ⁺ concentration	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	Fig. 6.2
Aerated non-saline	-	<0.001	0.003	-	0.310	-	-	6.2A
Stagnant non-saline	-	0.002	0.124	-	0.055	-	-	6.2B
Aerated saline	-	<0.001	<0.001	-	0.060	-	-	6.2C
Stagnant saline	-	<0.001	<0.001	-	<0.001	-	-	6.2D
Cl ⁻ concentration	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	Fig. 6.3
Aerated non-saline	-	<0.001	<0.001	-	<0.001	-	-	6.3A
Stagnant non-saline	-	<0.001	0.052	-	0.052	-	-	6.3B
Aerated saline	-	<0.001	<0.001	-	<0.001	-	-	6.3C
Stagnant saline	-	<0.001	<0.001	-	<0.001	-	-	6.3D
K ⁺ concentration	<0.001	<0.001	<0.001	0.002	0.155	0.764	0.532	Fig. 6.4
Aerated non-saline	-	0.001	0.023	-	0.652	-	-	6.4A
Stagnant non-saline	-	0.007	0.672	-	0.384	-	-	6.4B
Aerated saline	-	0.312	0.004	-	0.464	-	-	6.4C
Stagnant saline	-	0.189	<0.001	-	0.313	-	-	6.4D
K ⁺ /Na ⁺ ratio	<0.001	0.680	0.012	0.317	0.611	0.902	0.826	Table 6.2
Aerated non-saline	-	0.472	0.394	-	0.897	-	-	
Stagnant non-saline	-	0.515	0.417	-	0.834	-	-	
Aerated saline	-	0.052	<0.001	-	0.086	-	-	
Stagnant saline	-	<0.001	<0.001	-	<0.001	-	-	

Treatments were imposed when plants were 14-d-old for *H. maritimum*, and 11-d-old for wheat and the amphiploid. Plants were treated for 21 d. Four replicates each one represents a single plant grown in different pots. S = Salinity (0.2 and 200 mM NaCl); Treat. = Treatment (Aerated and Stagnant as deoxygenated stagnant agar in nutrient solution with or without 200 mM NaCl); Geno. = Genotype (*H. maritimum* (H21), wheat (*Triticum aestivum* cv. Chinese Spring) and their amphiploid); Tiss. = Tissue (2nd youngest fully expanded leaf blade, sheaths and seminal roots).

Table 6.S3. *P* values from the ANOVA of parameters fitted to the *HKT1;5*-like and *NHX1*-like transcripts abundance data sets.

Parameter	Treat.	Geno.	Tiss.	Treat.xGeno.	Treat.xTiss.	Geno.xTiss.	Treat.xGeno.xTiss.	Fig.
<i>HKT1;5</i> -like relative transcript abundance	0.055	0.986	<0.001	0.006	<0.001	0.300	0.284	6.5
Aerated non-saline	-	<0.001	<0.001	-	0.051	-	-	6.5A
Stagnant non-saline	-	0.978	0.001	-	0.473	-	-	6.5B
Aerated saline	-	0.960	<0.001	-	0.380	-	-	6.5C
Stagnant saline	-	0.051	<0.001	-	0.388	-	-	6.5D
<i>NHX1</i> -like relative transcript abundance	0.002	<0.001	<0.001	0.258	0.027	0.065	0.055	6.6
Aerated non-saline	-	<0.001	<0.001	-	0.018	-	-	6.6A
Stagnant non-saline	-	<0.001	<0.001	-	0.552	-	-	6.6B
Aerated saline	-	<0.001	<0.001	-	0.220	-	-	6.6C
Stagnant saline	-	<0.001	0.111	-	0.125	-	-	6.6D
<i>HKT1;5</i> -like relative transcript abundance	0.082	<0.001	<0.001	0.013	<0.001	<0.001	0.091	6.S4
Aerated non-saline	-	<0.001	<0.001	-	0.669	-	-	6.S4A
Stagnant non-saline	-	<0.001	0.003	-	0.307	-	-	6.S4B
Aerated saline	-	0.024	<0.001	-	0.002	-	-	6.S4C
Stagnant saline	-	0.010	.004	-	0.044	-	-	6.S4D

Treatments were imposed when plants were 14-d-old for *H. marinum*, and 11-d-old for wheat and the amphiploid. Plants were treated for 21 d. Four replicates each one represents a single plant grown in different pots. S = Salinity (0.2 and 200 mM NaCl); Treat. = Treatment (Aerated and Stagnant as deoxygenated stagnant agar in nutrient solution with or without 200 mM NaCl); Geno. = Genotype (*H. marinum* (H21), wheat (*Triticum aestivum* cv. Chinese Spring) and their amphiploid); Tiss. = Tissue (2nd youngest fully expanded leaf blade, sheaths and seminal roots).

CHAPTER 7

GENERAL DISCUSSION

To improve plant production in saline areas subject to waterlogging, there must be a greater understanding of the mechanisms, and underlying genes, associated with tolerance of crops to salinity, waterlogging and to the combined stresses. The general aim of this thesis was to evaluate tolerance to combined salinity and waterlogging in amphiploids produced by the hybridisation of *H. marinum* with wheat in comparison with their parents, and to identify possible physiological mechanisms of tolerance to the combined salinity and waterlogging stress. In the case of the regulation of tissue Na^+ concentrations, the expression of some underlying candidate genes (*HKT1;5* and *NHX1*) with products involved in regulation of Na^+ transport (and for *HKT1;5*, also K^+) were also assessed. The main traits assessed in my experiments were those that would contribute to better ion regulation, decreased leaf injury and improved root aeration (reviewed in Chapter 2). These evaluations will assist in understanding the interactions between these two important stress factors on plants; of importance for much of the world's saline land.

Mechanisms of salinity and waterlogging tolerance in H. marinum

The physiological evaluations of *H. marinum* accessions in this study (Chapters 3, 4 and 5) were in general agreement with previous studies; *H. marinum* can tolerate high salinity (450 mM NaCl; Garthwaite *et al.* 2005) and the combination of salinity and waterlogging (stagnant plus 200 mM NaCl; Malik *et al.* 2009*a,b*). The salinity tolerance of *H. marinum* was due to the regulation of ions (Na^+ , K^+ and Cl^-) in the shoot (cf. Garthwaite *et al.* 2005), while waterlogging tolerance was associated with improved root aeration (cf. McDonald *et al.* 2001*a*; Garthwaite *et al.* 2003). In the present study, two of the *H. marinum* accessions (H109 and WA9) were found to be more tolerant to the combination of salinity and stagnant treatments than six other accessions evaluated. Salt tolerant *H. marinum* accessions had: (i) highly effective Na^+ and Cl^- 'exclusion' to keep these ions at relatively low concentrations in leaves (Chapter 3), (ii) better maintenance of shoot K^+ concentrations and therefore a higher ratio of K^+/Na^+ in the shoot (Chapter 3), and (iii) a greater localisation of Na^+ and Cl^- in older leaves in one accession (H109) before leaf death (Chapter 4). Under stagnant saline conditions, accession H109 avoided ion toxicity in photosynthetically active leaves by restricting entry of Na^+ and Cl^- to the shoot, minimising the concentration of these ions in the young leaves, but accumulating toxic ions in older tissues. This greater capacity to tolerate salt accumulated in the leaf tissues presumably contributed to tolerance to the

combination of salinity and waterlogging (Chapter 4). In response to low O₂; in comparison with wheat, *H. maritimum* accessions had larger percentages of root porosity. In addition, measurements of high radial O₂ loss (ROL) near the tips of adventitious roots and low rates of ROL closer to the shoot base suggested that *H. maritimum* accessions induced a barrier to ROL (Chapter 5). The lower ROL in the root basal zones of *H. maritimum* occurred despite the fact that internal O₂ concentrations would have been higher closer to the shoot base than at the root tip (cf. Colmer 2003). These better root aeration and leaf ion regulation characteristics confirm that tolerant *H. maritimum* accessions have the potential to improve the salt and waterlogging tolerance of wheat via wide hybridisations.

Interest in *H. maritimum* has grown because of the potential for its use as a source of genes for salinity and waterlogging tolerance in wheat. The *H. maritimum*–wheat (Chinese Spring) amphiploids produced by AKMR Islam showed greater salt and waterlogging tolerances than wheat, based on traits inherited from *H. maritimum* parents (Chapters 5 and 6). One of the *H. maritimum*–wheat amphiploids was previously reported to express salt tolerance from *H. maritimum* (Islam *et al.* 2007), and more recent work has shown that 14 *H. maritimum*–wheat amphiploids (out of 15 tested) displayed better Na⁺ ‘exclusion’ than their respective wheat parents (Munns *et al.* 2011). In terms of waterlogging tolerance, two amphiploids (out of four tested) had been found to form a ‘partial’ barrier to ROL in adventitious roots and to be of higher root porosity (Malik *et al.* 2011). In terms of tolerance to the combination of salt and root-zone O₂ deficiency, two *H. maritimum*–wheat amphiploids were reported to tolerate the combination of stresses better than the wheat parents, with higher growth, less Na⁺ and higher K⁺ in leaves (Munns *et al.* 2011). However, the two amphiploids used in the work of Munns *et al.* (2011) shared one *H. maritimum* accession (H90) as the wild parent, but had different wheat parents. Lacking in the studies described above is an evaluation of tolerance to combined salinity and waterlogging of amphiploids developed from a range of *H. maritimum* accessions, but with one common wheat parent, to evaluate whether variation in salt and waterlogging tolerance in amphiploids is proportional to the variation in tolerances of the *H. maritimum* accessions used as parents. This thesis addressed this issue by studying four *H. maritimum*–wheat amphiploids produced from four accessions of *H. maritimum* and sharing a common wheat parent, which enabled me to assess the extent of expression of traits associated with salinity and waterlogging

tolerance from the wild parents in the wheat generic background. One structural problem with the present study is that there is little incentive for a cytogeneticist to prepare amphiploids using poorly adapted (e.g. H546) *H. maritimum* accessions, especially as the program of AKMR Islam was funded to be potential pre-breeding work. Therefore, all the accessions used as parents to make amphiploids with wheat were amongst the most tolerant (Malik *et al.* 2009b) and, the four amphiploids were therefore relatively similar for most of the measurements (Chapter 5). Use of less tolerant *H. maritimum* accessions to produce amphiploids would certainly be of interest in assessing the influence of *H. maritimum* accession on the variation in tolerance and trait expression in amphiploids.

Some of the existing amphiploids (e.g. H87–CS Amphiploid) do not seem to have been developed using the most salt and waterlogging tolerant wild parents. For example, amongst the *H. maritimum* accessions (Chapter 3), the accession with one of the highest growth rate under saline/waterlogged conditions was WA9. Unfortunately, an amphiploid has never been developed using this accession, possibly due to a number of reasons such as: Australian availability post quarantine, incomplete phenotype data at the time of parent selection, matching of flowering times when various sets of accessions were grown, and numerous possible technical difficulties. On the other hand, the *H. maritimum* accession with the lowest Na⁺ concentration in the shoots under saline/waterlogged conditions was H155, and an amphiploid has been developed with this accession and is an important genotype to focus on in future work. For comparative purposes it would also be useful to have amphiploids created using *H. maritimum* accession H546 which had poor ion regulation or low rates of growth under stagnant saline conditions. The *H. maritimum* accession with the highest concentration of Na⁺ in the shoots under saline/waterlogged conditions was H826. It would have been interesting to see if the lack of ion regulatory ability in this accession would also be conveyed to an amphiploid.

In general, in the present work the combination of salinity and hypoxia has less severe effects on the *H. maritimum*–wheat amphiploids than on their wheat parent. In response to 200 mM NaCl, *H. maritimum*–wheat amphiploids had lower leaf Na⁺ and Cl⁻ concentrations (~57% lower) and a 3.75-fold higher ratio of K⁺/Na⁺ than the wheat parent (Chapter 5). While in stagnant growth conditions, the *H. maritimum*–wheat

amphiploids had improved root aeration traits (i.e. formation of aerenchyma and a partial barrier to ROL) compared with the wheat parent. In stagnant saline solutions adventitious root porosities in the amphiploids were up to 27%, which was higher than in wheat (16%). A moderate barrier to ROL formed in basal root-zones of the amphiploids under stagnant conditions, whereas this was absent in wheat. This improvement in growth, root aeration traits and shoot ionic balance in the amphiploids was presumably because of the influence of genes contributed by the wild parent *H. maritimum* chromosomes added to the wheat genome. These findings also confirm the importance of ion regulation for tolerance to salinity and combined stagnant and saline stresses (Chapter 5).

H. maritimum has the ability to restrict entry of Na^+ to leaves and maintain leaf K^+ concentrations (Garthwaite *et al.* 2005; Islam *et al.* 2007; Malik *et al.* 2009a); this trait was also expressed in the *H. maritimum*–wheat amphiploids (Islam *et al.* 2007; Munns *et al.* 2011; this study). Therefore, this study also focused on the expression of genes encoding transporters related to Na^+ - K^+ regulation and associated with salt tolerance, namely HKT1;5 and NHX1 (Chapter 6).

HKT transporters have previously been found to contribute to the uptake of Na^+ from the soil solution when K^+ limits the growth of a plant, and to reduce Na^+ accumulation in leaves by both removing Na^+ from the xylem sap and loading Na^+ into the phloem sap (reviewed extensively in Rodriguez-Navarro and Rubio 2006; Munns and Tester 2008). The *TaHKT1;5* transporter has been identified to mediate the uptake of Na^+ or K^+ from the apoplast (Byrt *et al.* 2007), and therefore, HKT1;5 transporter might cause K^+ to be transported unfavourably (cf. Cotsaftis *et al.* 2012). In this study, *HKT1;5*-like sequences were highly expressed in the roots (~900-fold higher than in the leaf and sheath) of all genotypes under salt treatment (Chapter 6). Transcript levels of *HKT1;5*-like sequences in salt-stressed *H. maritimum* increased by up to 195-fold over control, coinciding with the higher leaf K^+/Na^+ ratio of salt-stressed *H. maritimum* compared to wheat. This suggests that in *H. maritimum*, HKT1;5 might be involved in removal of Na^+ from the xylem of the leaf sheath as well as transport of K^+ to leaf blades, despite the fact that the role of *TaHKT1;5* in Na^+ and K^+ homeostasis during salinity stress have not been completely defined yet (Hauser and Horie 2010). However, the constitutive overexpression of *HKT* genes was shown to enhance the removal of Na^+ from the xylem of the leaf sheath, and was shown to enhance K^+ accumulation in leaf blades and

sheaths of durum wheat (James *et al.* 2006). In this study, the similar concentrations of K^+ together with similar abundance patterns of *HKT1;5*-like transcripts across various tissues of *H. maritimum*, wheat and their amphiploid suggests that K^+ uptake occurs with comparable efficiency in these genotypes (Chapter 6), although, *H. maritimum* accessions and the amphiploids showed greater ability to maintain higher K^+ concentrations younger tissue (the YFEL) compared with wheat (Chapter 5).

Halophytes have the ability to compartmentalise Na^+ intracellularly into vacuoles (Munns and James 2003); yet, to my knowledge, this has not been assessed among *H. maritimum* accessions, despite the finding that these accessions show considerable variations in leaf Na^+ concentration (Malik *et al.* 2009a,b; this thesis). *NHX1* gene products are believed to compartmentalise Na^+ into vacuoles (Nass *et al.* 1997; Blumwald *et al.* 2000; Hasegawa *et al.* 2000; Aharon *et al.* 2003); thus, the expression of this gene could provide a useful screening tool when assessing intracellular compartmentation of Na^+ (Munns and James 2003). The combined hypoxic and salinity stresses reported to reduce the transcript level of *NHX1* (Xue *et al.* 2004; Pardo *et al.* 2006; Teakle *et al.* 2010). The compartmentalisation of Na^+ into vacuoles depend on trans-membrane H^+ gradients which are impaired by O_2 deficiency (Xue *et al.* 2004; Pardo *et al.* 2006). Furthermore, differences in compartmentation of Na^+ into vacuoles could explain differences in salinity tolerance between closely related species (Munns and Tester 2008). Therefore, I measured the transcript abundance of *NHX1*-like transcripts to determine the potential role of this gene in contributing to salt tolerance of a *H. maritimum* (H21)-Chinese Spring amphiploid and the *H. maritimum* parent under salt and hypoxic stresses. The high levels of *NHX1*-like transcripts in various tissues were higher in *H. maritimum* (1300-fold) and the amphiploid (370-fold) than wheat. These differences in *NHX1*-like tissue expression were associated with higher Na^+ concentrations in the sheath of *H. maritimum* (4.6-fold higher) and the amphiploid (38% higher) than leaf, whereas in wheat Na^+ concentrations were similar in leaf and sheaths, under salt and hypoxic stresses. This finding suggests that *H. maritimum* and the amphiploid might have higher capacities than wheat to sequester Na^+ in the vacuoles of the sheath.

The greater transcript levels of *NHX1*-like in the amphiploid can be attributed to the influence of the wild parent *H. maritimum*, which had higher levels of *NHX*-like transcript in all treatments (Chapter 6). Higher levels of *NHX1*-like transcript could indicate an

improvement in the compartmentalisation of Na^+ into the vacuoles in the amphiploid, as contributed by the wild parent *H. maritimum* and expressed in the genetic background of wheat. To my knowledge, this is the first study to measure changes in the transcript levels of ion transporters on wheat, *H. maritimum* and their amphiploid, in response to the interaction between salinity and waterlogging. The significant finding that the higher transcript levels of *NHX1*-like genes were associated with tolerance to stagnant saline treatments, and therefore this suggests that the root aeration could be linked to ion transport processes. However, further work is needed to verify the function of the *HKT1;5* and *NHX1* genes for ion regulation, and their role in salinity tolerance of *H. maritimum* accessions as well as their amphiploids.

Implications of the thesis for using wild relative (H. maritimum) to improve wheat

The results from this thesis demonstrate the successful attempts to transfer traits associated with salt and waterlogging tolerance from *H. maritimum* to wheat via the production of an amphiploid (Chapters 5 and 6). These results are in accordance with earlier results showing that *H. maritimum* accessions had significant variation in salt and waterlogging tolerance (Malik *et al.* 2009a,b; Chapters 3) and also that *H. maritimum* has some of the highest tolerance to these stresses within the Triticeae (Colmer *et al.* 2006b). The tolerance in *H. maritimum* accessions (Chapters 3 and 4) could enable the incorporation of desired traits to further improve crops against salt and waterlogging stresses. Studying mechanisms of tolerance within closely related species provides a unique opportunity to identify traits associated with tolerance to salinity and waterlogging. The traits identified by experiments in this thesis which are important targets for breeding wheat with improved tolerance to combined salinity and waterlogging include root aerenchyma formation, a barrier to ROL in roots, and tissue Na^+ and K^+ regulation. The *H. maritimum*-Chinese Spring amphiploids provide novel genetic stocks in wheat and showed improvement in tolerance to salinity and waterlogging, due to traits transferred from the *H. maritimum* parent. The amphiploids can withstand the combined effects of salinity and waterlogging by maintaining better leaf ion regulation and root aeration, compared with the wheat parent.

It is unclear whether the *H. maritimum*-wheat amphiploids will ever be used in agriculture, and no salt-tolerant hybrids have ever been commercially released (Colmer *et al.* 2006b). This may be because of the low productivity and poor seed production of the amphiploids (summarised in Islam and Shepherd 1992). Although restoration of the

amphiploid fertility might be achieved by backcrossing the amphiploid with wheat cultivars (Islam and Colmer 2008), the amphiploids would not be expected to produce grain of commercial bread wheat quality, but the resulting crop could be a crop variety suitable for grazing, hay and/or a feed grain (Colmer *et al.* 2006b).

Limitations and future studies

Prior to the experiments completed as part of this thesis, there has been only one study on the effects of combined salinity and waterlogging tolerance on *H. marinum*–wheat amphiploids (Munns *et al.* 2011). This thesis is the first study to evaluate the interactive effects of salinity and waterlogging on *H. marinum*–wheat amphiploids with a common wheat parent (Chinese Spring), allowing differences in the amphiploids to be unequivocally attributed to their *H. marinum* parent (Chapter 5). However, this thesis has identified several significant correlations that will need to be further evaluated by future research. The key relationships arising from this work that still require detailed assessment include:

1- Comparative efficiency assessment of the intracellular compartmentation of Na^+ within *H. marinum* accessions. *H. marinum* has been identified as the most salt tolerant species in the genus *Hordeum*, including the domesticated species barley (Garthwaite *et al.* 2005), which has been classified as a species with ‘tissue tolerance’ to Na^+ due to the relatively high levels of leaf Na^+ that barley can tolerate (Munns and James 2003). The data presented in this thesis (Chapter 6) suggests higher levels of tissue tolerance in *H. marinum* and the amphiploid compared with wheat, as evidenced by higher transcript levels of *NHX1*-like sequence, and high levels of sheath Na^+ . Furthermore, *H. marinum* accessions showed significant variation in response to salt (Malik *et al.* 2009a,b). Therefore, measurements of the intracellular compartmentation of Na^+ could help to determine the variability in tissue tolerance of *H. marinum* accessions, for example by scanning electron X-ray microanalysis studies. It would then be interesting to make comparisons with *NHX1* gene expression also, as well as protein abundance and Na^+/H^+ exchange activity/capacity at the tonoplast.

2- Distribution of Na^+ and Cl^- concentrations in different tissues in *H. marinum*–wheat amphiploids. The ability to accumulate Na^+ and Cl^- in older leaves to avoid ion toxicity in the photosynthetically active younger leaves is a key trait for salt tolerance in cereal crops (Colmer *et al.* 2005b), which was identified in *H. marinum* (Chapter 4). A

limitation of the results presented in this thesis was a lack of an assessment of ion distribution in the different-aged leaves of amphiploids which could reveal useful information about ion accumulation mechanisms, and therefore salt tolerance inherited from wild parents.

3- Further research on the function of *TaHKT1;5* and *HmHKT1;5* orthologues in plants and their role in controlling ion regulation are required to assess the mechanisms by which the products of these genes contribute to the salt tolerance of *H. maritimum* and wheat. One limitation of the results presented in this thesis was that the function of these genes was not evaluated and the understanding of the overall control of Na⁺ transport is limited. Further work on *HKT1;5* and other *HKT* family members using a cell specific analysis of gene expression and concentration of Na⁺ and K⁺, is needed to confirm the roles of these genes and their products under salt stress.

Conclusions

The results from this thesis have extended our understanding of the complicated interactive effects of salinity and waterlogging on plants. The present work is the first to conduct a comparative evaluation of the changes in the transcript abundance of two ion transporters in a *H. maritimum*-wheat amphiploid in comparison with its parents, in response to combined salinity and waterlogging stresses. The main research finding of this thesis was that the amphiploids displayed greater salt and waterlogging tolerances than wheat, which was most likely to have come from the genetic contribution of the *H. maritimum* chromosomes. Traits associated with salt and waterlogging tolerance in *H. maritimum* accessions were identified (Chapter 3), and the diversity was discussed in relation to the degree of capacity for ion regulation and reduced leaf death (Chapter 4). The improvement in salt tolerance in the *H. maritimum*-wheat amphiploid in terms of plant growth and tissue ion concentrations were confirmed (Chapter 5), and also transcript abundances and responses to various salt and stagnant treatments were assessed for two candidate genes (*HKT* and *NHX* genes) involved in salinity tolerance (Chapter 6). The presence of the chromosomes from *H. maritimum* improved growth and ionic balance in the amphiploid, as compared with the wheat parent, confirming the importance of ion regulation for salt tolerance and also to the combined stagnant and salinity stresses.

LITERATURE CITED

- Aceves-N E, Stolzy LH, Mehuys GR (1975) Combined effects of low oxygen and salinity on germination of a semi-dwarf Mexican wheat 1. *Agronomy Journal* **67**, 530–532.
- Aharon R, Shahak Y, Wininger S, Bendov R, Kapulnik Y, Galili G (2003) Overexpression of a plasma membrane aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions but not under drought or salt stress. *Plant Cell* **15**, 439–447.
- Akhtar J, Gorham J, Qureshi RH (1994) Combined effect of salinity and hypoxia in wheat (*Triticum aestivum* L.) and wheat-*Thinopyrum* amphiploids. *Plant and Soil* **166**, 47–54.
- Amtmann A, Sanders D (1998) Mechanisms of Na⁺ uptake by plant cells. *Advances in Botanical Research* **29**, 75–112.
- Apse MP, Aharon GS, Snedden WA, Blumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar Na⁺/H⁺ antiport in *Arabidopsis*. *Science* **285**, 1256–1258.
- Armstrong W (1975) Waterlogged soils. In: Etherington, J.R. (Ed.) *Environment and Plant Ecology* Chichester, United Kingdom: Wiley, pp. 181–218.
- Armstrong W (1979) Aeration in higher plants. *Advances in Botanical Research* **7**, 225–332.
- Armstrong W (1994) Polarographic oxygen electrodes and their use in plant aeration studies. *Proceedings of the Royal Society of Edinburgh* **102**, 511–527.
- Armstrong W, Beckett PM (1987) Internal aeration and development of stela anoxia in submerged roots. *New Phytologist* **105**, 221–245.
- Armstrong W, Cousins D, Armstrong J, Turner DW, Beckett PM (2000) Oxygen distribution in wetland plant roots and permeability barriers to gas-exchange with the rhizosphere: a microelectrode and modelling study with *Phragmites australis*. *Annals of Botany* **86**, 687–703.
- Armstrong W, Wright EJ (1975) Radial oxygen loss from roots: the theoretical basis for the manipulation of flux data obtained by the cylindrical platinum electrode technique. *Physiologia Plantarum* **35**, 21–26.
- Asch F, Dingkuhn M, Dörffling K, Miezan K (2000) Leaf K⁺/Na⁺ ratio predicts salinity induced yield loss in irrigated rice. *Euphytica* **113**, 109–118.
- Ashraf M (2003) Relationships between leaf gas exchange characteristics and growth of differently adapted populations of Blue panicgrass (*Panicum antidotale* Retz.) under salinity or waterlogging. *Plant Science* **165**, 69–75.
- Bari R, Datt Pant B, Stitt M, Scheible WR (2006) PHO₂, MicroRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiology* **141**, 988–999.
- Bramley H, Turner NC, Turner DW, Tyerman SD (2010) The contrasting influence of short-term hypoxia on the hydraulic properties of cells and roots of wheat and lupin. *Functional Plant Biology* **37**, 183–193.

- Barrett-Lennard EG (1986) Effects of waterlogging on the growth and NaCl uptake by vascular plants under saline conditions. *Reclamation and Revegetation Research* **5**, 245–261.
- Barrett-Lennard EG (2003) The interaction between waterlogging and salinity in higher plants: causes, consequences and implications. *Plant and Soil* **253**, 35–54.
- Barrett-Lennard E, Leighton P, Buwalda F, Gibbs J, Armstrong W, Thomson C, Greenway H (1988) Effects of growing wheat in hypoxic nutrient solutions and of subsequent transfer to aerated solutions. i. Growth and carbohydrate status of shoots and roots. *Australian Journal of Plant Physiology* **15**, 585–598.
- Barrett-Lennard EG, Malcolm CV, Bathgate A (2003) Saltland Pastures in Australia. A practical guide, land, water and wool sustainable grazing on saline lands.
- Barrett-Lennard EG, Shabala SN (2013) The waterlogging/salinity interaction in higher plants revisited-focusing on the hypoxia-induced disturbance to K⁺ homeostasis. *Functional Plant Biology* **40**, 872–882.
- Barrett-Lennard EG, van Ratingen P, Mathie MH (1999) The developing pattern of damage in wheat (*Triticum aestivum* L.) due to the combined stresses of salinity and hypoxia: experiments under controlled conditions suggest a methodology for plant selection. *Australian Journal of Agricultural Research* **50**, 129–136.
- Benderradji L, Brini F, Amar SB, Kellou K, Azaza J, Masmoudi K, Bouzerzour H, Hanin M (2011) Sodium transport in the seedlings of two bread wheat (*Triticum aestivum* L.) genotypes showing contrasting salt stress tolerance. *Australian Journal of Crop Science* **53**, 233–241.
- Bennett SJ, Barrett-Lennard EG, Colmer TD (2009) Salinity and waterlogging as constraints to saltland pasture production: A review. *Agriculture, Ecosystems & Environment* **129**, 349–360.
- Bernstein L, Hayward HE (1958) Physiology of salt tolerance. *Annual Review of Plant Physiology* **9**, 25–46.
- Blanc G, Wolfe KH (2004) Functional divergence of duplicated genes formed by polyploidy during *Arabidopsis* evolution. *The Plant Cell Online* **16**, 1679–1691.
- Blumwald E, Aharon GS, Apse MP (2000) Sodium transport in plant cells. *Biochimica et Biophysica Acta* **1**, 1–2.
- Bradford KJ, Hsiao TC (1982) Stomatal behaviour and water relations of waterlogged tomato plants. *Plant Physiology* **70**, 488–492.
- Buwalda F, de Kok LJ, Stulen I, Kuiper PJC (1988) Cysteine, γ -glutamyl-cysteine and glutathione contents of spinach leaves as affected by darkness and application of excess sulfur. *Physiologia Plantarum* **74**, 663–668.
- Byrt CS, Platten JD, Spielmeier W, James RA, Lagudah ES, Dennis ES, Tester M, Munns R (2007) HKT1;5-like cation transporters linked to Na⁺ exclusion loci in wheat, *Nax2* and *Knal*. *Plant Physiology* **143**, 1918–1928.

- Cakirlar H, Bowling DJF (1981) The effect of salinity on the membrane potential of sunflower roots. *Journal of Experimental Botany* **32**, 479–485.
- Chen Z, Cuin TA, Zhou M, Twomey A, Naidu BP, Shabala S (2007a) Compatible solute accumulation and stress-mitigating effects in barley genotypes contrasting in their salt tolerance. *Journal of Experimental Botany* **58**, 4245–4255.
- Chen Z, Newman I, Zhou M, Mendham N, Zhang G, Shabala S (2005) Screening plants for salt tolerance by measuring K^+ flux: a case study for barley. *Plant, Cell & Environment* **28**, 1230–1246.
- Chen Z, Pottosin II, Cuin TA, Fuglsang AT, Tester M, Jha D, Zepeda-Jazo I, Zhou M, Palmgren MG, Newman IA, Shabala S (2007b) Root plasma membrane transporters controlling K^+/Na^+ homeostasis in salt-stressed barley. *Plant Physiology* **145**, 1714–1725.
- Chomczynski P (1993) A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* **15**, 532–534.
- Colmer TD (2003) Long-distance transport of gases in plants: a perspective on internal aeration and radial oxygen loss from roots. *Plant, Cell & Environment* **26**, 17–36.
- Colmer TD, Islam AKMR (2002) Development of a cereal tolerant to salinity and waterlogging 8th national conference and workshop on the productive use and rehabilitation of saline lands (PURSL), Fremantle, Western Australia. 16-20 September 2002. Promaco Conventions, pp. 241–247.
- Colmer TD, Cox MCH, Voisenek LACJ (2006a) Root aeration in rice (*Oryza sativa*): evaluation of oxygen, carbon dioxide, and ethylene as possible regulators of root acclimatizations. *New Phytologist* **170**, 767–778.
- Colmer TD, Epstein E, Dvořák J (1995) Differential solute regulation in leaf blades of various ages in salt-sensitive wheat and a salt-tolerant wheat $\times$ *Lophopyrum elongatum* (Host) A. Love amphiploid. *Plant Physiology* **108**, 1715–1724.
- Colmer TD, Flowers TJ (2008) Flooding tolerance in halophytes. *New Phytologist* **179**, 964–974.
- Colmer TD, Flowers TJ, Munns R (2006b) Use of wild relatives to improve salt tolerance in wheat. *Journal of Experimental Botany* **57**, 1059–1078.
- Colmer TD, Garthwaite AJ, Islam AKMR, Islam S, Malik AI, von Bothmer R (2005a) ‘Salinity and waterlogging tolerance in wild *Hordeum* species: Physiological basis and prospects for use in cereal improvement.’ pp. 8-9. (The 15th International Plant Nutrition Colloquium: China)
- Colmer TD, Greenway H (2011) Ion transport in seminal and adventitious roots of cereals during O_2 deficiency. *Journal of Experimental Botany* **62**, 39–57.
- Colmer TD, Munns R, Flowers TJ (2005b) Improving salt tolerance of wheat and barley: future prospects. *Australian Journal of Experimental Agriculture* **45**, 1425–1443.

- Cotsaftis O, Plett D, Shirley N, Tester M, Hrmova M (2012) A two-staged model of Na⁺ exclusion in rice explained by 3D modeling of HKT transporters and alternative splicing. *PLoS One* **77**, e39865.
- Cramer WA, Tae GS, Furbacher PN, Böttger M (1993) The enigmatic cytochrome b-559 of oxygenic photosynthesis. *Physiologia Plantarum* **88**, 705–711.
- Cuin TA, Betts SA, Chalmandrier R, Shabala S (2008) A root's ability to retain K⁺ correlates with salt tolerance in wheat. *Journal of Experimental Botany* **59**, 2697–2706.
- Cuin TA, Miller AJ, Laurie SA, Leigh RA (2003) Potassium activities in cell compartments of salt-grown barley leaves. *Journal of Experimental Botany* **54**, 657–661.
- Cuin TA, Shabala S (2005) Exogenously supplied compatible solutes rapidly ameliorate NaCl-induced potassium efflux from barley roots. *Plant and Cell Physiology* **46**, 1924–1933.
- Cuin TA, Tian Y, Betts SA, Chalmandrier R, Shabala S (2009) Ionic relations and osmotic adjustment in durum and bread wheat under saline conditions. *Functional Plant Biology* **36**, 1110–1119.
- Davenport RJ, Tester M (2000) A weakly voltage-dependent, nonselective cation channel mediates toxic sodium influx in wheat. *Plant Physiology* **12**, 823–834.
- Demidchik V, Davenport RJ, Tester M (2002) Nonselective cation channels in plants. *Annual Review of Plant Physiology* **53**, 67–107.
- Demidchik V, Tester M (2002) Sodium fluxes through nonselective cation channels in the plasma membrane of protoplasts from *Arabidopsis* roots. *Plant Physiology* **128**, 379–387.
- Dionisio-Sese ML, Tobita S (2000) Effects of salinity on sodium content and photosynthetic responses of rice seedlings differing in salt tolerance. *Journal of Plant Physiology* **157**, 54–58.
- Drew MC (1983) Plant injury and adaptation to oxygen deficiency in the root environment: A review. *Plant and Soil* **75**, 179–199.
- Drew MC, He CJ, Morgan PW (2000) Programmed cell death and aerenchyma formation in roots. *Trends in Plant Science* **5**, 123–127.
- Drew MC, Jackson MB, Giffard S (1979) Ethylene-promoted adventitious rooting and development of cortical air spaces (aerenchyma) in roots may be adaptive responses to flooding in *Zea mays* L. *Planta* **147**, 83–88.
- Drew MC, Läuchli A (1985) Oxygen-dependent exclusion of sodium ions from shoots by roots of *Zea mays* (cv Pioneer 3906) in relation to salinity damage. *Plant Physiology* **79**, 171–176.
- Dunne JA, Williams RJ, Martinez ND (2002) Network structure and biodiversity loss in food webs: robustness increases with connectance. *Ecology Letters* **5**, 558–567.

- Evans DE (2004) Aerenchyma formation. *New Phytologist* **161**, 35–49.
- Flowers T, Yeo A (1995) Breeding for salinity resistance in crop plants: where next? *Australian Journal of Plant Physiology* **22**, 875–884.
- Flowers TJ, Dalmond D (1992) Protein synthesis in halophytes: The influence of potassium, sodium and magnesium in vitro. *Plant and Soil* **146**, 153–161.
- Flowers TJ, Troke PF, Yeo AR (1977) Mechanism of salt tolerance in halophytes. *Annual Review of Plant Physiology* **28**, 89–121.
- Flowers TJ, Hajibagheri MA, Clipson NJW (1986) Halophytes. *The Quarterly Review of Biology* **61**, 313–337.
- Folzer H, Dat JF, Capelli N, Rieffel D, Badot PM (2006) Response of sessile oak seedlings (*Quercus petraea*) to flooding: an integrated study. *Tree Physiology* **26**, 759–766.
- Force A, Lynch M, Pickett FB, Amores A, Yan YL, Postlethwait J (1999) Preservation of duplicate genes by complementary, degenerative mutations. *Genetics* **151**, 1531–1545.
- Friebe B, Jiang J, Raupp WJ, McIntosh RA, Gill BS (1996) Characterization of wheat-alien translocations conferring resistance to diseases and pests: current status. *Euphytica* **91**, 59–87.
- Fukuda A, Nakamura A, Tagiri A, Tanaka H, Miyao A, Hirochika H, Tanaka Y (2004) Function, intracellular localization and the importance in salt tolerance of a vacuolar Na⁺/H⁺ antiporter from rice. *Plant and Cell Physiology* **45**, 146–159.
- Galloway R, Davidson NJ (1993) The response of *Atriplex amnicola* to the interactive effects of salinity and hypoxia. *Journal of Experimental Botany* **44**, 653–663.
- Garciadeblás B, Senn ME, Banuelos MA, Rodriguez-Navarro A (2003) Sodium transport and HKT transporters: the rice model. *Plant Journal* **34**, 788–801.
- Garthwaite AJ, Armstrong W, Colmer TD (2008) Assessment of O₂ diffusivity across the barrier to radial O₂ loss in adventitious roots of *Hordeum marinum*. *New Phytologist* **179**, 405–416.
- Garthwaite AJ, von Bothmer R, Colmer TD (2003) Diversity in root aeration traits associated with waterlogging tolerance in the genus *Hordeum*. *Functional Plant Biology* **30**, 875–889.
- Garthwaite AJ, von Bothmer R, Colmer TD (2005) Salt tolerance in wild *Hordeum* species is associated with restricted entry of Na⁺ and Cl[−] into the shoots. *Journal of Experimental Botany* **56**, 2365–2378.
- Geigenberger P (2003) Response of plant metabolism to too little oxygen. *Current Opinion in Plant Biology* **6**, 247–256.
- Genc Y, McDonald GK, Tester M (2007) Reassessment of tissue Na⁺ concentration as a criterion for salinity tolerance in bread wheat. *Plant, Cell & Environment* **30**, 1486–1498.

- Ghassemi F, Jakeman AJ, Nix HA (1995) 'Salinisation of land and water resources: human causes, extent, management and case studies.' University of New South Wales Press, Sydney. pp. 526.
- Gorham J (1993) Genetics and physiology of enhanced K/Na discrimination. In 'The Fourth International Symposium on Genetic Aspects of Plant Mineral Nutrition'. Canberra, Australia. (Ed. PJ Randall) pp. 151–158. (Kluwer Academic Publishers)
- Gorham J, Bristol A, Young EM, Jones RGW, Kashour G (1990) Salt tolerance in the Triticeae: K/Na discrimination in barley. *Journal of Experimental Botany* **41**, 1095–1101.
- Gorham J, Bristol A, Young EM, Wyn Jones RG (1991) The presence of the enhanced K/Na discrimination trait in diploid *Triticum* species. *Theoretical and Applied Genetics* **82**, 729–736.
- Gorham J, Forster BP, Wyn Jones RG, Miller TE, Law CN (1986) Salt tolerance in the Triticeae: solute accumulation and distribution in an amphidiploid derived from *Triticum aestivum* cv. Chinese Spring and *Thinopyrum bessarabicum*. *Journal of Experimental Botany* **37**, 1435–1449.
- Gorham J, McDonnell E, Budrewicz E, Jones RGW (1985) Salt tolerance in the Triticeae: growth and solute accumulation in leaves of *Thinopyrum bessarabicum*. *Journal of Experimental Botany* **36**, 1021–1031.
- Grable AR (1966) Soil aeration and plant growth. *Advances in Agronomy* **18**, 57–106.
- Greenway H (1968) Growth stimulation by high chloride concentrations in halophytes. *Israel Journal of Botany* **17**, 169–177.
- Greenway H, Armstrong W, Colmer TD (2006) Conditions leading to high CO₂ (>5 kPa) in waterlogged-flooded soils and possible effects on root growth and metabolism. *Annals of Botany* **98**, 9–32.
- Greenway H, Gibbs J (2003) Review: Mechanisms of anoxia tolerance in plants. II. Energy requirements for maintenance and energy distribution to essential processes. *Functional Plant Biology* **30**, 999–1036.
- Greenway H, Munns R (1980) Mechanisms of salt tolerance in nonhalophytes. *Annual Review of Plant Physiology* **31**, 149–190.
- Hasegawa P, Bressan R, Zhu J, Bohnert H (2000) Plant cellular and molecular responses to high salinity. *Annual Review of Plant Physiology* **51**, 463–499.
- Hauser F, Horie T (2010) A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K⁺/Na⁺ ratio in leaves during salinity stress. *Plant, Cell & Environment* **33**, 552–565.
- Horie T, Yoshida K, Nakayama H, Yamada K, Oiki S, Shinmyo A (2001) Two types of HKT transporters with different properties of Na⁺ and K⁺ transport in *Oryza sativa*. *Plant Journal* **27**, 129–138.

- Houshmand S, Arzani A, Maibody SAM, Feizi M (2005) Evaluation of salt-tolerant genotypes of durum wheat derived from in vitro and field experiments. *Field Crops Research* **91**, 345–354.
- Huang B, Johnson JW, Box JE, Nesmith DS (1997) Root characteristics and hormone activity of wheat in response to hypoxia and ethylene. *Crop Science* **37**, 812–818.
- Huang B, NeSmith DS, Bridges DC, Johnson JW (1995) Responses of squash to salinity, waterlogging, and subsequent drainage: II. Root and shoot growth. *Journal of Plant Nutrition* **18**, 141–152.
- Huang CX, van Steveninck RFM (1989) Longitudinal and transverse profiles of K⁺ and Cl⁻ concentration in 'low-' and 'high-salt' barley roots. *New Phytologist* **112**, 475–480.
- Huang S, Spielmeyer W, Lagudah ES, Munns R (2008) Comparative mapping of HKT genes in wheat, barley, and rice, key determinants of Na⁺ transport, and salt tolerance. *Journal of Experimental Botany* **59**, 927–937.
- Huck MG (1970) Variation in taproot elongation rate as influenced by composition of the soil air. *Agronomy Journal* **62**, 815–818.
- Hunt R (1978) 'Plant growth analysis.' (Edward Arnold Limited: London).
- Islam AKMR, Colmer TD (2008) Attempts to transfer salt- and waterlogging tolerances from Sea barleygrass (*Hordeum marinum* Huds.) to wheat. In 'The 11th International Wheat Genetics Symposium proceedings.' (Eds R Appels, R Eastwood, E Lagudah, P Langridge, M Mackay Lynne). (Sydney University Press: Sydney)
- Islam AKMR, Shepherd KW (1992) Production of wheat-barley recombinant chromosomes through induced homoeologous pairing. *Theoretical and Applied Genetics* **83**, 489–494.
- Islam S, Malik AI, Islam AKMR, Colmer TD (2007) Salt tolerance in a *Hordeum marinum*-*Triticum aestivum* amphiploid, and its parents. *Journal of Experimental Botany* **58**, 1219–1229.
- Jackson MB, Armstrong W (1999) Formation of aerenchyma and the processes of plant ventilation in relation to soil flooding and submergence. *Plant Biology* **1**, 274–287.
- Jackson MB, Drew MC (1984) Effects of flooding on growth and metabolism of herbaceous plants. In 'Flooding and Plant Growth'. (Ed. TT Kozlowski) pp. 47–128. (Academic Press: New York)
- Jackson MB, Hall KC (1987) Early stomatal closure in waterlogged pea plants is mediated by abscisic acid in the absence of foliar water deficits. *Plant, Cell & Environment* **10**, 121–130.

- James RA, Blake C, Byrt CS, Munns R (2011) Major genes for Na⁺ exclusion, *Nax1* and *Nax2* (wheat *HKT1;4* and *HKT1;5*), decrease Na⁺ accumulation in bread wheat leaves under saline and waterlogged conditions. *Journal of Experimental Botany* **62**, 2939–2947.
- James RA, Davenport RJ, Munns R (2006) Physiological characterization of two genes for Na⁺ exclusion in durum wheat, *Nax1* and *Nax2*. *Plant Physiology* **142**, 1537–1547.
- James RA, Rivelli AR, Munns R, Caemmerer SV (2002) Factors affecting CO₂ assimilation, leaf injury and growth in salt-stressed durum wheat. *Functional Plant Biology* **29**, 1393–1403.
- James RA, von Caemmerer S, Condon AG, Zwart AB, Munns R (2008) Genetic variation in tolerance to the osmotic stress component of salinity stress in durum wheat. *Functional Plant Biology* **35**, 111–123.
- Jauhar PP, Peterson TS (1996) *Thinopyrum* and *Lophopyrum* as sources of genes for wheat improvement. *Cereal Research Communications* **24**, 15–21.
- Justin SHFW, Armstrong W (1987) The anatomical characteristics of roots and plant response to soil flooding. *New Phytologist* **106**, 465–495.
- Justin SHFW, Armstrong W (1991) Evidence for the involvement of ethene in aerenchyma formation in adventitious roots of rice (*Oryza sativa* L.). *New Phytologist* **118**, 49–62.
- Kao WY, Tsai TT, Shih CN (2003) Photosynthetic gas exchange and chlorophyll a fluorescence of three wild soybean species in response to NaCl treatments. *Photosynthetica* **41**, 415–419.
- Kravchik M, Bernstein N (2013) Effects of salinity on the transcriptome of growing maize leaf cells point at cell-age specificity in the involvement of the antioxidative response in cell growth restriction. *BMC Genomics* **14**, 1471–2164.
- Kriedemann PE, Sands R (1984) Salt resistance and adaptation to root-zone hypoxia in sunflower. *Australian Journal of Plant Physiology* **11**, 287–301.
- Lawlor DW, Tezara W (2009) Causes of decreased photosynthetic rate and metabolic capacity in water-deficient leaf cells: a critical evaluation of mechanisms and integration of processes. *Annals of Botany* **103**, 561–579.
- Lazof D, Bernstein N (1998) The NaCl-induced inhibition of shoot growth: the case for disturbed nutrition with special consideration of calcium nutrition. *Advances in Botanical Research* **29**, 113–189.
- Leigh RA (2001) Potassium homeostasis and membrane transport. *Journal of Plant Nutrition and Soil Science* **164**, 193–198.
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{- $\Delta\Delta C_T$} Method. *Methods* **25**, 402–408.

- Maas EV (1986) Salt tolerance of plants. *Applied Agricultural Research* **1**, 12–26.
- Maas EV, Hoffman G J (1977) Crop salt tolerance – current assessment. *Journal of the Irrigation and Drainage Division* **103**, 115–134.
- Maathuis FJM, Amtmann A (1999) K⁺ nutrition and Na⁺ toxicity: The basis of cellular K/Na ratios. *Annals of Botany* **84**, 123–133.
- Maathuis FM (2007) Monovalent cation transporters; establishing a link between bioinformatics and physiology. *Plant and Soil* **301**, 1–15.
- Malcolm CV (1980) Production from salt-affected soils. Report of the senior research officer on a visit to India and Pakistan. Soil Research and Survey Section. Western Australian Department of Agriculture.
- Malik AI, English JP, Colmer TD (2009a) Tolerance of *Hordeum marinum* accessions to O₂ deficiency, salinity and these stresses combined. *Annals of Botany* **103**, 237–248.
- Malik AI, English JP, Shepherd KA, Islam AKMR, Colmer TD (2009b) Tolerance of combined salinity and O₂ deficiency in *Hordeum marinum* accessions from the grain-belt of Western. In International Plant Nutrition Colloquium XVI, 1277 Davis USA.
- Malik AI, Islam AKMR, Colmer TD (2011) Transfer of the barrier to radial oxygen loss in roots of *Hordeum marinum* to wheat (*Triticum aestivum*): evaluation of four *H. marinum*-wheat amphiploids. *New Phytologist* **190**, 499–508.
- Marcar N (1993) Waterlogging modifies growth, water use and ion concentrations in seedlings of salt-treated *Eucalyptus Camaldulensis*, *E. tereticornis*, *E. robusta* and *E. globulus*. *Functional Plant Biology* **20**, 1–13.
- Mäser P, Gierth M, Schroeder J (2002) Molecular mechanisms of potassium and sodium uptake in plants. *Plant and Soil* **247**, 43–54.
- McDonald MP, Galwey NW, Colmer TD (2001a) Waterlogging tolerance in the tribe Triticeae: the adventitious roots of *Critesion marinum* have a relatively high porosity and a barrier to radial oxygen loss. *Plant, Cell & Environment* **24**, 585–596.
- McDonald MP, Galwey NW, Ellneskog-Staam P, Colmer TD (2001b) Evaluation of *Lophopyrum elongatum* as a source of genetic diversity to increase the waterlogging tolerance of hexaploid wheat (*Triticum aestivum*). *New Phytologist* **151**, 369–380.
- Møller IS, Tester M (2007) Salinity tolerance of *Arabidopsis*: a good model for cereals? *Trends in Plant Science* **12**, 534–540.
- Munns R (1993) Physiological processes limiting plant growth in saline soils: some dogmas and hypotheses. *Plant, Cell & Environment* **16**, 15–24.
- Munns R (2002) Comparative physiology of salt and water stress. *Plant, Cell & Environment* **25**, 239–250.

- Munns R (2005) Genes and salt tolerance: bringing them together. *New Phytologist* **167**, 645–663.
- Munns R, Greenway H, Kirst GO (1983) Halotolerant eukaryotes. In: Lange OL, Nobel PS, Osmond CB, Zeigler H, eds. Physiological Plant Ecology. III. Responses to Literature Cited 143 the Chemical and Biological Environment. *Encyclopedia of Plant Physiology, New Series*. Berlin: Springer-Verlag, 59-135.
- Munns R, James RA (2003) Screening methods for salinity tolerance: a case study with tetraploid wheat. *Plant and Soil* **253**, 201–218.
- Munns R, James R, Islam A, Colmer T (2011) *Hordeum marinum*-wheat amphiploids maintain higher leaf K^+Na^+ and suffer less leaf injury than wheat parents in saline conditions. *Plant and Soil* **348**, 365–377.
- Munns R, James R, Lauchli A (2006) Approaches to increasing the salt tolerance of wheat and other cereals. *Journal of Experimental Botany* **57**, 1025–1043.
- Munns R, James RA, Xu B, Athman A, Conn SJ, Jordans C, Byrt CS, Hare RA, Tyerman SD, Tester M, Plett D, Gilliam M (2012) Wheat grain yield on saline soils is improved by an ancestral Na^+ transporter gene. *Nature Biotechnology* **30**, 360–364.
- Munns R, Rebetzke GJ, Husain S, James RA, Hare RA (2003) Genetic control of sodium exclusion in durum wheat. *Australian Journal of Agricultural Research* **54**, 627–635.
- Munns R, Schachtman DP, Condon AG (1995) The significance of a two-phase growth response to salinity in wheat and barley. *Australian Journal of Agricultural Research* **22**, 561–569.
- Munns R, Tester M (2008) Mechanisms of salinity tolerance. *Annual Review of Plant Physiology* **59**, 651–681.
- Musgrave ME (1994) Waterlogging effects on yield and photosynthesis in eight winter wheat cultivars. *Crop Science* **34**, 1314–1318.
- Nass R, Cunningham KW, Rao R (1997) Intracellular sequestration of sodium by a novel Na^+/H^+ exchanger in yeast is enhanced by mutations in the plasma membrane H^+ -ATPase: insights into mechanisms of sodium tolerance. *Journal of Biological Chemistry* **272**, 26145–26152.
- Netondo GW, Onyango JC, Beck E (2004) Sorghum and Salinity: II. Gas exchange and chlorophyll fluorescence of sorghum under salt stress. *Crop Science* **44**, 806–811.
- NLWRA (2001) Australian dryland salinity assessment 2000: Extent, impacts, processes, monitoring and management options. Commonwealth of Australia, Turner, ACT.
- OECD/Food and agriculture organization of the united nations (2012) OECD-FAO agricultural outlook 2012, OECD Publishing. [10.1787/agr_outlook-2012-en](https://doi.org/10.1787/agr_outlook-2012-en)

- Ohnishi M, Fukada-Tanaka S, Hoshino A, Takada J, Inagaki Y, Iida S (2005) Characterization of a novel Na⁺/H⁺ antiporter gene InNHX2 and comparison of InNHX2 with InNHX1, which is responsible for blue flower coloration by increasing the vacuolar pH in the Japanese morning glory. *Plant Cell Physiology* **46**, 259–267.
- Olgun M, Kumlay AM, Adiguzel MC, Caglar A (2008) Effects of waterlogging in wheat (*T. aestivum* L.). *Acta Agriculturae Scandinavica, (Section B). Plant and Soil Sciences* **58**, 193–198.
- Omielan JA, Epstein E, Dvořák J (1991) Salt tolerance and ionic relations of wheat as affected by individual chromosomes of salt-tolerant *Lophopyrum elongatum*. *Genome* **34**, 961–974.
- Pang JY NI, Mendham N, Zhou M, Shabala S (2006) Microelectrode ion and O₂ fluxes measurements reveal differential sensitivity of barley root tissues to hypoxia. *Plant, Cell & Environment* **29**, 1107–1121.
- Pannell DJ, Ewing MA (2006) Managing secondary dryland salinity: Options and challenges. *Agricultural Water Management* **80**, 41–56.
- Pardo JM, Cubero B, Leidi EO, Quintero FJ (2006) Alkali cation exchangers: roles in cellular homeostasis and stress tolerance. *Journal of Experimental Botany* **57**, 1181–1199.
- Perera LKRR, Mansfield TA, Malloch AJC (1994) Stomatal responses to sodium ions in *Aster tripolium*: a new hypothesis to explain salinity regulation in above-ground tissues. *Plant, Cell & Environment* **17**, 335–340.
- Pérez-López U, Robredo A, Lacuesta M, Mena-Petite A, Munoz-Rueda A (2012) Elevated CO₂ reduces stomatal and metabolic limitations on photosynthesis caused by salinity in *Hordeum vulgare*. *Photosynth Research Journal* **111**, 269–283.
- Platten JD, Cotsaftis O, Berthomieu P, Bohnert H, Davenport RJ, Fairbairn DJ, Horie T, Leigh RA, Lin HX, Luan S, Maser P, Pantoja O, Rodriguez-Navarro A, Schachtman DP, Schroeder JI, Sentenac H, Uozumi N, Very AA, Zhu JK, Dennis ES, Tester M (2006) Nomenclature for HKT transporters, key determinants of plant salinity tolerance. *Trends in Plant Science* **11**, 372–374.
- Plett DC, Møller IS (2010) Na⁺ transport in glycophytic plants: what we know and would like to know. *Plant, Cell & Environment* **33**, 612–626.
- Ponnamperuma FN (1984) Effects of flooding on soils. In 'Flooding and Plant Growth'. (Ed. T Kozlowski) pp. 9–45. (Academic Press: New York)
- Qureshi RH, Barrett-Lennard EG (1998) Saline Agriculture for Irrigated Land in Pakistan: a Handbook. Monograph No. 50, Australian Centre for International Agricultural Research, Canberra. 142 pp.
- Rahnama A, Poustini K, Munns R, James RA (2010) Stomatal conductance as a screen for osmotic stress tolerance in durum wheat growing in saline soil. *Functional Plant Biology* **37**, 255–263.

- Raven JA (1985) Tansley Review No. 2. Regulation of pH and generation of osmolarity in vascular plants: A cost-benefit analysis in relation to efficiency of use of energy, nitrogen and water. *New Phytologist* **101**, 25–77.
- Redondo-Gómez S, Mateos-Naranjo E, Davy AJ, Fernández-Muñoz F, Castellanos EM, Luque T, Figueroa ME (2007) Growth and photosynthetic responses to salinity of the salt-marsh shrub *Atriplex portulacoides*. *Annals of Botany* **100**, 555–563.
- Ren ZH, Gao JP, Li LG, Cai XL, Huang W, Chao DY, Zhu MZ, Wang ZY, Luan S, Lin HX (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nature Genetics* **37**, 1141–1146.
- Rengasamy P (2006) World salinization with emphasis on Australia. *Journal of Experimental Botany* **57**, 1017–1023.
- Rengasamy P, Chittleborough D, Helyar K (2003) Root-zone constraints and plant-based solutions for dryland salinity. *Plant and Soil* **257**, 249–260.
- Richards RA (1995) Improving crop production on salt-affected soils: by breeding or management? *Experimental Agriculture* **31**, 395–408.
- Rodríguez-Navarro A, Rubio F (2006) High-affinity potassium and sodium transport systems in plants. *Journal of Experimental Botany* **57**, 1149–1160.
- Rogers ME, Colmer TD, Nichols PGH, Hughes SJ, Frost K, Cornwall D, Chandra S, Miller SM, Craig AD (2011) Salinity and waterlogging tolerance amongst accessions of messina (*Melilotus siculus*). *Crop and Pasture Science* **62**, 225–235.
- Rogers ME, West DW (1993) The effects of root-zone salinity and hypoxia on shoot and root growth in *Trifolium* species. *Annals of Botany* **72**, 503–509.
- Romero-Aranda R, Soria T, Cuartero J (2001) Tomato plant-water uptake and plant-water relationships under saline growth conditions. *Plant Science* **160**, 265–272.
- Romick TL, Fleming HP, McFeeters RF (1996) Aerobic and anaerobic metabolism of *Listeria monocytogenes* in defined glucose medium. *Applied and Environmental Microbiology* **62**, 304–307.
- Rubio F, Gassmann W, Schroeder JI (1995) Sodium-driven potassium uptake by the plant potassium transporter HKT1 and mutations conferring salt tolerance. *Science* **270**, 1660–1663.
- Rubio F, Schwarz M, Gassmann W, Schroeder JI (1999) Genetic selection of mutations in the high affinity K⁺ transporter HKT1 that define functions of a loop site for reduced Na⁺ permeability and increased Na⁺ tolerance. *Journal of Biological Chemistry* **274**, 6839–6847.
- Rus A, Yokoi S, Sharkhuu A, Reddy M, Lee BH, Matsumoto TK, Koiwa H, Zhu JK, Bressan RA, Hasegawa PM (2001) *AtHKT1* is a salt tolerance determinant that controls Na⁺ entry into plant roots. *Proceedings of the National Academy of Sciences USA* **98**, 14150–14155.

- Saqib M, Akhtar J, Qureshi RH, Nasim M (2013) Selection and characterization of wheat genotypes for saline soils prone to water-logging. *Journal of Plant Nutrition and Soil Science* **176**, 131–137.
- Schachtman DP, Munns R, Whitecross MI (1991) Variation in sodium exclusion and salt tolerance in *Triticum tauschii*. *Crop Science* **31**, 992–997.
- Schachtman DP, Schroeder JI (1994) Structure and transport mechanism of a high-affinity potassium uptake transporter from higher plants. *Nature* **370**, 655–658.
- Serrano R (1996) Salt tolerance in plants and microorganisms: toxicity targets and defense responses. *International Review of Cytology* **165**, 1–52.
- Setter TL, Waters I (2003) Review of prospects for germplasm improvement for waterlogging tolerance in wheat, barley and oats. *Plant and Soil* **253**, 1–33.
- Shabala S (2000) Ionic and osmotic components of salt stress specifically modulate net ion fluxes from bean leaf mesophyll. *Plant, Cell & Environment* **23**, 825–837.
- Shabala S, Cuin TA (2008) Potassium transport and plant salt tolerance. *Physiologia Plantarum* **133**, 651–669.
- Shabala S, Demidchik V, Shabala L, Cuin TA, Smith SJ, Miller AJ, Davies JM, Newman IA (2006) Extracellular Ca^{2+} ameliorates NaCl -induced K^{+} loss from *Arabidopsis* root and leaf cells by controlling plasma membrane K^{+} -permeable channels. *Plant Physiology* **141**, 1653–1665.
- Shabala S, Shabala L, Volkenburgh EV (2003) Effect of calcium on root development and root ion fluxes in salinised barley seedlings. *Functional Plant Biology* **30**, 507–514.
- Shiono KTH, Colmer TD, Nakazono M (2008) Role of ethylene in acclimations to promote oxygen transport in roots of plants in waterlogged soils. *Plant Science* **175**, 52–58.
- Shoresh M, Spivak M, Bernstein N (2011) Involvement of calcium-mediated effects on ROS metabolism in the regulation of growth improvement under salinity. *Free Radical Biology & Medicine* **51**, 1221–1234.
- Smedema L, Shiati K (2002) Irrigation and Salinity: a perspective review of the salinity hazards of irrigation development in the arid zone. *Irrigation and Drainage Systems* **16**, 161–174.
- Smethurst CF, Rix K, Garnett T, Auricht G, Bayart A, Lane P, Wilson SJ, Shabala S (2008) Multiple traits associated with salt tolerance in lucerne: revealing the underlying cellular mechanisms. *Functional Plant Biology* **35**, 640–650.
- Sunarpi, Horie T, Motoda J, Kubo M, Yang H, Yoda K, Horie R, Chan WY, Leung HY, Hattori K, Konomi M, Osumi M, Yamagami M, Schroeder JI, Uozumi N (2005) Enhanced salt tolerance mediated by *AtHKT1* transporter-induced Na unloading from xylem vessels to xylem parenchyma cells. *Plant Journal* **44**, 928–938.
- Szabolcs I (1994) Soils and salinization. In: Handbook of plant and crop stress (Ed. M.Pessarakali) 3–11.

- Taeb M, Koebner RM, Forster BP (1993) Genetic variation for waterlogging tolerance in the Triticeae and the chromosomal location of genes conferring waterlogging tolerance in *Thinopyrum elongatum*. *Genome* **36**, 825–830.
- Teakle N, Bazihizina N, Colmer T, Shabala S, Barrett-Lennard E, Rodrigo Moreno A, Läuchli A (2013) Differential tolerance to combined salinity and O₂ deficiency in the halophytic grasses *Puccinellia ciliata* and *Thinopyrum ponticum*: the importance of K⁺ retention and membrane potential maintenance in roots. *Environmental and Experimental Botany* **87**, 69–78.
- Teakle N, Flowers T, Real D, Colmer T (2007) *Lotus tenuis* tolerates the interactive effects of salinity and waterlogging by 'excluding' Na⁺ and Cl[−] from the xylem. *Journal of Experimental Botany* **58**, 2169–2180.
- Teakle NL, Amtmann A, Real D, Colmer TD (2010) *Lotus tenuis* tolerates combined salinity and waterlogging: maintaining O₂ transport to roots and expression of an *NHX1*-like gene contribute to regulation of Na⁺ transport. *Physiologia Plantarum* **139**, 358–374.
- Tester M, Davenport R (2003) Na⁺ tolerance and Na⁺ transport in higher plants. *Annals of Botany* **91**, 503–527.
- Tezara WMVJ (1999) Water stress inhibits plant photosynthesis by decreasing coupling factor and ATP. *Nature* **401**, 914–917.
- Thomson CJ, Armstrong W, Waters I, Greenway H (1990) Aerenchyma formation and associated oxygen movement in seminal and nodal roots of wheat. *Plant, Cell & Environment* **13**, 395–403.
- Thomson CJ, Colmer TD, Watkin ELJ, Greenway H (1992) Tolerance of wheat (*Triticum aestivum* cvs Gamanya and Kite) and triticale (*Triticosecale* cv. Muir) to waterlogging. *New Phytologist* **120**, 335–344.
- Thomson WW, Oross JW (1988) Salt glands. In: Baker, DA HJ, Eds. Solute transport in plant cells and tissues., Harlow E, UK: Longman, 498–537.
- Trought MCT, Drew MC (1980) The development of waterlogging damage in wheat seedlings (*Triticum aestivum* L.). *Plant and Soil* **56**, 187–199.
- Uozumi N, Kim EJ, Rubio F, Yamaguchi T, Muto S, Tsuboi A, Bakker EP, Nakamura T, Schroeder JI (2000) The *Arabidopsis* HKT1 gene homolog mediates inward Na⁺ currents in *Xenopus laevis* oocytes and Na⁺ uptake in *Saccharomyces cerevisiae*. *Plant Physiology* **122**, 1249–1259.
- Van Der Moezel PG, Watson LE, Bell DT (1989) Gas exchange responses of two *Eucalyptus* species to salinity and waterlogging. *Tree Physiology* **5**, 251–257.
- von Bothmer R, Jacobsen N, Baden C, Jørgensen RB, Linde-Laursen I (1995) An ecogeographical study of the genus *Hordeum*. 2nd Edition. (International Plant Genetic Resources Institute: Rome)
- Watson R, Pritchard J, Malone M (2001) Direct measurement of sodium and potassium in the transpiration stream of salt-excluding and non-excluding varieties of wheat. *Journal of Experimental Botany* **52**, 1873–1881.

- Webb T, Armstrong W (1983) The effects of anoxia and carbohydrates on the growth and viability of rice, pea and pumpkin roots. *Journal of Experimental Botany* **34**, 579–603.
- White PJ, Broadley MR (2001) Chloride in soils and its uptake and movement within the plant: A Review. *Annals of Botany* **88**, 967–988.
- Wiengweera A, Greenway H (2004) Performance of seminal and nodal roots of wheat in stagnant solution: K^+ and P uptake and effects of increasing O_2 partial pressures around the shoot on nodal root elongation. *Journal of Experimental Botany* **55**, 2121–2129.
- Wiengweera A, Greenway H, Thomson CJ (1997) The use of agar nutrient solution to simulate lack of convection in waterlogged soils. *Annals of Botany* **80**, 115–123.
- Wolf O, Munns R, Tonnet ML, Jeschke WD (1991) The role of the stem in the partitioning of Na^+ and K^+ in salt-treated barley. *Journal of Experimental Botany* **42**, 697–704.
- Xue ZY, Zhi DY, Xue GP, Zhang H, Zhao YX, Xia GM (2004) Enhanced salt tolerance of transgenic wheat (*Triticum aestivum* L.) expressing a vacuolar Na^+/H^+ antiporter gene with improved grain yields in saline soils in the field and a reduced level of leaf Na^+ . *Plant Science* **167**, 849–859.
- Yao SX, Chen SS, Xu DS, Lan HY (2010) Effects of different salinity stress on K, Na content and relevant genes expression in leaves of *Chenopodium album* in Xinjiang. *Guang Pu Xue Yu Guang Pu Fen Xi* **30**, 2281–2284.
- Yeo A (1998) Molecular biology of salt tolerance in the context of whole-plant physiology. *Journal of Experimental Botany* **49**, 915–929.
- Yeo AR, Flowers TJ (1983) Varietal differences in the toxicity of sodium ions in rice leaves. *Physiologia Plantarum* **59**, 189–195.
- Yeo AR, Flowers TJ (1986) Ion transport in *Suaeda maritima*: its relation to growth and implications for the pathway of radial transport of ions across the root. *Journal of Experimental Botany* **37**, 143–159.
- Zhang HX, Blumwald E (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nature Biotechnology* **19**, 765–768.
- Zhang HX, Hodson JN, Williams JP, Blumwald E (2001) Engineering salt-tolerant *Brassica* plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. *Proceedings of the National Academy of Sciences USA* **98**, 12832–12836.
- Zhang X, Jiang D, Zheng C, Dai T, Cao W (2011) Post-anthesis salt and combination of salt and waterlogging affect distributions of sugars, amino acids, Na^+ and K^+ in wheat. *Journal of Agronomy and Crop Science* **197**, 31–39.
- Zhu JK (2000) Genetic analysis of plant salt tolerance using *Arabidopsis*. *Plant Physiology* **124**, 941–948.
- Zhu JK (2001) Plant salt tolerance. *Trends in Plant Science* **6**, 66–71.

- Zhu JK (2002) Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology* **53**, 247–273.