A study of salinity tolerance in field pea

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Abstract

Preliminary research was aimed at identification of parental germplasm that could be used for improvement of tolerance to toxic effects of sodium chloride (NaCl) in field pea. An initial screening experiment of 780 globally-distributed Pisum L. accessions identified significant variation in response to applied NaCl, based on plant symptoms. Lines with relatively higher tolerance as compared to commercial varieties grown in Australia were most frequently identified within landraces originating from the central, eastern and southern provinces of China. The most tolerant identified accession was an unadapted landrace ‘ATC1836’ originating from Greece. Variation for salinity tolerance was validated using a sub-set of 70 accession lines. Salinity-induced toxicity symptoms were closely associated with reductions of plant growth rate, height, shoot and root dry matter and with increased concentration of Na$^+$ at the plant growing tip. The level of salinity tolerance based on these factors varied substantially and provides an important basis for genetic improvement of field pea for Australia.

Seven field pea genotypes that vary significantly for salinity tolerance showed a range of symptom development and growth responses over time under increasing levels of NaCl salinity applied using watering treatments. The genotypic responses were closely associated with Na$^+$ accumulation in leaflet tissue on the lower plant and a parallel reduction in K$^+$ concentration. Increasing salinity caused strong but variable inhibition of root and shoot dry matter accumulation and final grain yield for all genotypes. The genotype ATC1836 showed the highest relative tolerance based on measured parameters, but was comparatively slow growing. Three genotypes (03H090P-04HO2002, 03H556P-04HO2012, 99-410-2-14-2) with moderate tolerance obtained substantially more dry matter under the highest salinity treatment of (18 dsm$^{-1}$) compared to the commercial variety ‘Kaspa’. The genotype OZP0812 was also able to
maintain relatively higher growth rate at the lower salinity treatment level of 6 dsm\(^{-1}\).

The high salinity tolerance of the landrace genotype ATC1836 that is evident at early growth stages was also apparent in this study at later ontogeny, on the basis of lower biomass reduction, reduced symptom development and delayed rate of Na\(^+\) accumulation in plant tissue. In this study, sodium accumulated more rapidly and to a higher degree, and symptoms developed faster on lower growth nodes when compared to the growing tip. Plant biomass and main plant height showed lower correlation with Na\(^+\) concentration in plant tissue than plant chlorosis. The taller genotype OZP0812 produced more biomass under conditions of increasing salinity than the dwarf genotypes Kaspa and OZP0809 and the landrace genotype ATC1836. Applied salinity exerted deleterious and varying effects on seed yield components such as reduced seed and pod set and seed size. The genotype OZP0812 maintained both higher seed yield and larger seed size compared to the salinity tolerant landrace genotype ATC1836, despite accumulation of more Na\(^+\) in plant tissue. Increased salinity resulted in earlier flowering and increased Na\(^+\) concentration in seed tissue.

Segregation ratios for salinity tolerance were analysed in 3 field pea populations derived from crosses between the sensitive genotype Kaspa and the tolerant genotypes ATC1836, Parafield and Yarrum, revealing probable multigenic control. A comparatively higher proportion of tolerant progeny was observed in the ATC1836 x Kaspa population. However, a high degree of trangressive segregation for enhanced salinity tolerance was apparent in progeny from crosses of either Parafield or Yarrum with Kaspa, suggesting that parental combining abilities should also be assessed for improvement of salinity tolerance. Positive broad sense heritabilities for measures of symptom response to salinity, and repeatability of results between experiments and generations implied high potential for genetic gain from use of pot-based screening methods. Differences in assessment of salinity symptom response based on a
numerical scale as compared to percentage plant necrosis were not significant. Variation for salinity tolerance within recombinant inbred lines (RILs) progeny derived from the Kaspa x Parafield cross was documented on the basis of rate of symptom development and a salinity tolerance index.

A frequency analysis showed that the proportion of field pea germplasm with higher salinity tolerance in advanced yield testing nurseries in Australia had increased in the period 2005-2011. However, a multivariate canonical analysis based on 14 yield nurseries in 2011 indicated that the degree of salinity tolerance in advanced germplasm currently provides significantly less yield benefit than degree of boron toxicity tolerance, indicating a need for further pre-breeding efforts. Boron tolerant accessions as a group were higher yielding at 7 sites during 2011. All of these sites were in regions with highly alkaline sub-soils, and six sites had comparatively lower growing season rainfall. The high rainfall exception (Kingsford, South Australia) was affected by powdery mildew, for which resistance is positively linked with high boron tolerance. Genotypes with dual sensitivity to both boron and salinity mostly performed better at sites with higher rainfall in the growing season. For sites at which salinity tolerance was more important, the only sensitive genotypes that performed well were all early flowering (i.e. PBA Twilight). One boron sensitive genotype (119) showed specific adaptation across sites at which boron tolerance was important, and is hence suitable as a key parent for improving general adaptation of crop.

Linkage maps based on molecular genetic marker polymorphism were constructed for RIL populations of Kaspa x Parafild and Kaspa x Yarrum populations. RIL progeny and parents from these populations were screened at the seedling growth stage for growth symptom responses to salinity stress imposed by adding NaCl in the watering solution at a concentration of 18 dsm\(^{-1}\). Phenotypic variation for salinity induced symptoms was normally distributed and increased with severity over time. A salinity index was developed to quantify variation for salinity tolerance
and was used in concert with statistical correlation analysis to identify quantitative trait loci (QTLs) and flanking single nucleotide polymorphism (SNP) markers that could be useful for implementation of marker assisted selection (MAS) strategies.

This thesis has identified valuable variation in salinity tolerance in *Pisum* for field pea breeding programs. A knowledge of critical growth responses at the seedling and reproductive growth stages for salinity tolerance now provides a guide to screening of populations for useful genetic variation or marker-tagged QTLs. Preliminary investigation has identified QTLs for seedling tolerance to salinity stress for implementation of MAS for the purposes of parent building and routine screening.
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Declaration

This to certify that

i) the thesis comprises only my original work towards the PhD,

ii) due acknowledgment has been made in the text to all other material used,

iii) the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Antonio Leonforte

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Fig. 7.6. Genetic linkage map of *Pisum sativum* prepared using 120 RIL derived progeny from the Kaspa x Yarrum cross. The markers are shown on the right of the linkage groups, and map distances between markers are indicated in cM on the left. ............................... 151
Fig. 7.7. Location of two major QTLs for higher salinity tolerance based on variation between RIL progeny of the Kaspa x Parafield cross.
Thesis related publications


Chapter One

Introduction

Pea (*Pisum sativum* L.), having the chromosome constitution 2n = 14, is in the genus *Pisum* of the Viceae tribe, of the cool-season Galegoid clade in the Papilinoideae sub-family of the legume family Fabaceae (syn. Leguminosae) (Doyle and Luckow 2003). Field pea refers to crop production for dry grain, and is also referred to as dry pea. Globally the crop is widely grown and is an important pulse grain for human food and stockfeed consumption. As a cool-season legume, field peas are also used regionally for forage production and across a range of farming systems for crop rotational purposes. The crop is extensively grown as an autumn-winter sown crop across southern Australia, many Mediterranean countries, South Africa, South America and across subtropical regions within central Asia (e.g. India, Southern China) and Eastern Africa (i.e. Ethiopia). These regions have similar climates characterised by a winter-dominant rainfall pattern, but with varying growing season length. In Australia, field pea is grown over about 350,000 ha/year and is widely distributed across southern grain production regions, particularly in lower rainfall climates (i.e. growing season rainfall <230 mm) in which agronomic advantages are apparent over other pulse species such as lentil, chickpea and faba bean.

Soil salinity caused by NaCl is a major constraint to crop production across many regions of southern Australia, as it limits the availability soil water for plant growth in dry climates. High sub-soil salinity is often present naturally within the crop plant root zone, originating from natural deposition and accumulation of NaCl over time (i.e. primary salinisation), (Hingston and Gailitis 1976). However, in many instances broad scale farming practices in Australia have led to perched water tables (George *et al.* 1997, Walker *et al.* 1999) and further soil salinisation (i.e. secondary salinisation), particularly across southern Western Australia. Limited studies on small numbers of genotypes have classified pea as being moderately sensitive to soil salinity (Maas 1986, Francois and Maas 1994) when compared to cereals and canola.
(Steppuhn et al. 2001), and the threshold salinity has been estimated to be as low as 1.5 dsm\(^{-1}\) (Dua et al. 1989). No research has been undertaken to investigate the genetic variation available for improvement of salinity tolerance in pea. However, research investigating soil salinity tolerance and associated roles and genetics of physiological traits has been well reviewed in other major dryland crops such as cereals (Munns et al. 2006). This body of research is reviewed and provides a useful guide to formulating genetic approaches for improvement of salinity tolerance for pulse crops such as pea, for which an understanding of salinity tolerance has been limited.
Chapter Two
Literature Review

2.1 BREEDING OF FIELD PEA FOR AUSTRALIA

2.1.1 Crop evolution and genetic resources

Peas were domesticated as early as 7000 BC across the Fertile Crescent region of the Middle East, and later through Greece and across Europe (Ambrose 1995). Recent studies have also indicated a major source of diversity in Chinese pea germplasm (Zong et al. 2009), and suggest there may also have been strong isolated and directional selection across Eastern Asia. Domestication of peas has occurred for various uses including dry grain, as a vegetable crop and for forage, resulting in strong selection for specific plant, pod and seed traits (Davies 1976). The wild progenitor of domesticated peas was therefore probably variable, but Ben-Ze’ev and Zohary (1973) speculated that *P. sativum* subsp. *humile*, presently found in steppe habitats, is likely to represent a possible ancestral stock. The genus *Pisum* contains only one other species, the wild species *Pisum fulvum* Sibthorp & Smith, which is found in the Eastern Mediterranean regions. Pea germplasm collections globally exceed 49,000 accessions across 16 countries, including Australia which houses one of the largest. However this total is relatively small compared with the cereals (Tanksley and McCouch 1997), and probably represents much duplication. Further conservation of land races is believed to be urgently required (Ladizinsky 1998) to avoid loss of genetic diversity. Maintenance of genetic diversity in breeding programs is essential to avoid genetic bottlenecks in yield and adaptation potential. This issue may be particularly important for field pea, as strong selection has recently been applied to major genes for both plant morphology and grain traits.

2.1.2 Genomic background and research

The pea karyotype includes seven chromosomes, of which 5 are acrocentric and 2 are submetacentric. They are distinguishable by arm length,
centromere and nucleolus organiser position (Hall et al. 1997) and chromosome morphology (Fuchs et al. 1998). Pea translocation stocks were used in earlier studies to assign gene loci to chromosomes via association with translocation points (Lamm 1977, 1983, Folkeson 1990). The pea chromosomes, however, are now identifiable based on both morphology and in situ hybridization (Fuchs et al. 1998). Importantly, the pea genus includes a rich source of morphological mutants (Blixt 1972). Those affecting plant architecture (Hofer and Ellis 1998) may be useful for investigating physiological responses to abiotic stresses such as salinity. While Pisum has been used extensively in early inheritance studies (Mendel 1866), it has not been a preferred model species for genome analysis because of the relatively large and complex genome (Michaelson et al. 1991). The pea nuclear genome is composed mainly of high-copy dispersed repeated sequences which appear to be differentiated into distinct sequence classes (Murray and Thompson 1982). Diversification and expansion of this large amount of repeated sequences (the majority of which correspond to elements that are propagated through RNA intermediates, i.e. retrotransposons) may, however, provide a useful source of genetic markers for studying divergence (Ellis and Poyser 2002) particularly in relation to strong directional selection such as salinity-induced stress. In addition, use of comparative genomics using information from model legume species such as Medicago truncatula Gaertn. and Lotus japonicus L. may provide an effective means for exploitation of the useful genomic variation available in pea (Doyle et al. 1997) for plant breeding. A large number of genetic markers have been developed for pea, which have allowed the construction of moderately-dense linkage maps. These have been based on a range of molecular genetic markers including: amplified fragment length polymorphisms (AFLPs) (Vos et al. 1995), random amplification of polymorphic DNA (RAPDs) (Lacou et al. 1998), retrotransposons (Flavell et al. 1998, Pearce et al. 2000), expressed sequence tags (ESTs) (Gilpin et al. 1997) and simple sequence repeats (SSRs) (Kaur et al. 2012). Specifically, common genetic markers have permitted integration of linkage maps derived from different crosses (Gilpin et al. 1997, Lacou et al. 1998) and the development of a consensus linkage map
for pea (Weeden et al. 1998), including the many classical mutant loci (Ellis and Poyser 2002) described by Blixt (1972).

2.1.3 Focus of field pea genetic improvement in Australia

2.1.3.1 Improving agronomic potential

Field pea production across the Mediterranean-type climates of southern Australia was initially based on introduced and improved landrace plant types (i.e. Dun types) that characteristically grew vigorously over winter and were tall (i.e. long internodes). Varieties developed with this plant habit (referred to as ‘conventional-type’) exhibited a flowering pattern that was generally indeterminate and responsive to variability caused by transient climatic stress (i.e. low rainfall and frost or heat stress at podding). Adaptation improvement of conventional pea varieties for grain yield potential (i.e. Parafield, Helena and Sturt) was primarily focused on grain yield components such as seed set and size and flowering time, but yield has been generally limited by low harvest index in good seasons (Armstrong et al. 1994). The scrambling plant growth habit characteristic of these varieties has also made management for weeds, diseases and harvest difficult, and often produced poor and variable grain quality for export to human consumption markets. Globally, the most significant breakthrough for field pea was the development of modern semi-dwarf determinate types that grow erect to seed maturity and combine major genes controlling absence of leaflets (i.e. the afila trait) and reduced plant inter-node length (Redden et al. 2005). The first semi-dwarf types were developed for spring-sown environments in North America and Europe, but were not adapted for most Mediterranean-type climates, as they lacked sufficient early season biomass, flowered too early, and were generally not resistant to the more winter dominant fungal diseases such as ascochyta blight (Rubiales et al. 2009) and bacterial blight. However, they did provide a basis for improved adaptation of an improved semi-dwarf plant type for Mediterranean climate-based cropping regions.

The development of the modern semi-dwarf varieties for Australia (e.g. variety ‘Kaspa’) followed a breeding effort over more than 20 years to pyramid genes
for adaptation and plant type. This process primarily involved complex crossing and recurrent selection programs between introduced spring-type semi-leafless/semi-dwarf germplasm and Australian-adapted conventional cultivars. Specifically, the newly developed semi-dwarf germplasm was taller, more active in winter growth, and showed reproductive development that commenced in mid-spring to avoid major frost risk (Leonforte and Brouwer 1999). A critical development for Australia was the trait pyramiding of lodging resistance (Leonforte et al. 2006) with reduced pod parchment (i.e. sugar-pod trait) to reduce pod shattering at harvest. The unique combination of these traits led to the release of the first broadly-adapted semi-dwarf variety (‘Kaspa’) which has since rapidly quickly dominated production across southern Australia. The ‘Kaspa’ plant ideotype has provided a new benchmark for further improvement of adaptation of semi-dwarf germplasm for more marginal rainfall climates (i.e. < 220 mm/yr) over the last decade. The focus of recent breeding has been to optimise the phenology of the ‘Kaspa’ plant type, which is late flowering and determinate for shorter season climates, particularly in WA. This has led to recent releases such as PBA Gunyah and PBA Twilight that are early flowering, and display more stable yield than Kaspa in short season-climates (Fig. 2.1).
**Fig. 2.1.** Graph showing yield gain for new Kaspa plant type varieties ‘PBA Gunyah’ and ‘PBA Twilight’ compared to the variety ‘Kaspa’ and the conventional variety ‘Parafield’. Graphs are based on 173 experiments conducted from 2005 to 2011.

### 2.1.3.2 Improving abiotic stress tolerance

Field pea, like other pulses, is comparatively sensitive to a number of abiotic stress factors, particularly involving soil nutrition such as salinity and alkaline-induced boron toxicity, reproductive frost damage, heat stress (Dita *et al.* 2006) and specific herbicide actions. In Mediterranean-type climates, the main limitations to field pea production occur during reproductive development, as developing flowers, pods and seed are highly sensitive to abortion and damage from increasingly high and low temperature extremes and water limiting stress. As with most other crops, much lower breeding investment has been dedicated towards improvement of tolerance to abiotic constraints, compared to disease resistance. In particular, limited funding has been available for screening of tolerance to specific abiotic traits, which are highly complex and vary transiently in the field and over ontogeny. A low-cost approach of long-term and strategic selection for grain yield in high risk environments has, however, proven to be highly successful for genetically
improving grain yield reliability of the pea crop for Mediterranean climates *per se*. The component traits that contribute to higher water-limiting tolerance relate mostly to phenology and biomass production prior to flowering (Sadras *et al*. 2012). The potential opportunities to improve tolerance to or escape from specific abiotic stress factors by variable combinations of reproductive traits (i.e. flowering time and duration, units of flower, pod and seed and seed size), plant traits (i.e. leaf architecture, aerial and basal branching) and root biomass (McPhee 2005) are poorly understood. However, significant diversity exists within the species that could be explored.

Higher stress tolerance has been identified in landrace accessions for toxicity to boron (Bagheri *et al*. 1994), salinity (Leonforte *et al*. 2012) and iron deficiency (Kabir *et al*. 2012), and new seedling screening techniques to improve tolerance levels are now being routinely used in Australia (Leonforte *et al*. 2009). There is also preliminary evidence for useful variation for heat stress tolerance during flowering and podding (Petkova *et al*. 2009), and some evidence for higher tolerance to frost damage at reproductive stages on the yield response of the Australian variety ‘Sturt’ (Hawthorn 2007) and pod damage (Shafiq *et al*. 2012). Specific and general herbicide seedling stress tolerance has also been identified in controlled environment studies and validated in the field (L. McMurray, personnel communication).

### 2.1.3.4 Improving disease resistance important for Mediterranean climates

Field pea is affected by a large number of fungal and viral diseases, bacterial blight and pests, particularly for winter-sown cropping regions with a Mediterranean climate, in which a long vegetative growth phase is observed. The primary focus of field pea breeding for higher disease resistance has been to initially pyramid genes with higher disease tolerance to blackspot, particularly in earlier flowering semi-dwarf plant backgrounds (McMurray *et al*. 2010). The development of an erect-growing plant type during vegetative growth has also probably improved overall disease tolerance of the crop (Le May *et al*. 2005). Important diseases affecting Mediterranean climates can be
grouped into those which only cause damage periodically when conditions are conducive, and ascochyta blight or blackspot which causes frequent damage and yield loss.

2.2 SOIL SALINITY

Soil salinity is an excess of soluble salts in the soil that directly limits agricultural production by causing degradation of soil and restriction of plant growth. Globally, soil salinity is widely distributed across all climatic regions and is estimated to affect up to 20% of all arable land (Rhoades and Loveday 1990, Ghazssemi et al. 1995). Historically, the deleterious effects of soil salinity on crop production have long been recorded (Russel et al. 1965, Jacobsen et al. 1968) and good understanding is now available of the agricultural practices that have accelerated soil salinisation in cropping lands.

2.2.1 Classification of salt affected soils

Soil salinity is classified and measured as units of electrical conductivity (EC) of a water-saturated soil paste. The EC indicates osmotic problems and the ESP or exchangeable Na\(^+\) percentage is symptomatic of a physical dispersion problem in the soil. Units of electrical conductivity are measured in units of decisemiens per meter (ds/m) or millmhos per centimetre (mmho/cm) from filtered water of soil extracts (Rhoades 1982). A soil is considered saline if the EC is above 4 ds\(^{-1}\) (USDA 1954). Maas (1986) suggested that this threshold level is, however, dependent on a complex interaction between the relative tolerance between- and within-plant species, the variation in soil parameters and association with increased Na\(^+\), causing increased soil sodicity and subsequent plant hypoxia and nutritional imbalances; and the climatic variables that influence growth responses to increasing salinity, primarily associated with availability of water.

2.2.2 Processes leading to soil salinisation.

Soil salinity has been classified in a broad sense in relation to:
i) Origin (Ghassemi et al. 1995) as:
Primary salinity, which results from salt accumulation over long periods of time due to natural processes. In Australia, this initially derives from the deposition and accumulation of salt with rainfall and dust from prevailing ocean winds (Hingston and Gailitis 1976), and to a lesser degree from the release of salt from weathering rocks such as marine sediments. Secondary salinity, which results from human practices that alter the hydrologic balance of the soil between water applied (i.e. irrigation or rainfall) and water used by crops (i.e. transpiration). In Australia, this process is largely due to the introduction of broad-scale farming systems in which annual crops and pastures have replaced large areas of native vegetation (George et al. 1997, Walker et al. 1999). Such introduced farming systems have allowed more water to infiltrate into the soil (Dunin et al. 2001), causing saline sub-soil water tables to rise over time and accumulated concentrations of salts in the upper soil layers.

ii) In relation to soil and ground water processes (Rengassamy, 2006) as:
Groundwater associated salinity, caused by upward movement of water and salt from normally shallow water tables, as a particular feature of cropping regions of southern Western Australia. Non-groundwater-associated salinity due to natural accumulation and storage of salts in the sub-soil layers, often associated with sodicity. In dryland cropping regions (which are typically of low rainfall in Australia), salt is often not leached below the root-zone and found in high concentrations at depth leading to ‘transient salinity’. Irrigation-associated salinity, due to watering with saline water. This has been a major focus of attention in Australia along the irrigated regions within the Murray Darling Basin.

2.2.3 Sodic soils and transient salinity
Soil sodicity is caused by Na\textsuperscript{+} accumulation to a point at which sodic aggregates cause structural degradation, reduce water porosity and permeability, and increase soil strength. Together these factors can severely limit access of plant roots to water. Over 60\% of farmed soils in Australia are
considered sodic, and of these, over 80% have dense clay layers that are both highly sodic (i.e. ESP >15) and alkaline (i.e. pH > 8.5) (Rengasamy, 2002). Consequently, for many Australian cropping regions, soil sodicity, salinity and alkalinity-induced nutrient deficiencies (i.e. for Fe, Mn, Cu, Zn and P) and toxicities (i.e. boron, carbonate and aluminate) together commonly limit crop production. Individually, these factors can occur transiently by varying in degree over the soil profile spatially and temporally over the growing season.

Transient salinity is caused by accumulation of salt in the sub-soil due to restricted water movement into the sub-soil following rainfall as a result of high levels of sodicity. Transient salinity may hence often be associated with transient sub-soil water logging or a perched water table. Transient salinity is estimated to affect 67% of the cropping area in Australia (Rengasamy 2002), and much of this area does not appear to be caused by groundwater-associated salinity (Shaw et al. 1998, McArthur 1991), but rather is more likely to be a consequence of inherently high salt concentration in the sub-soil prior to land clearing for crop production (Burvill 1988). Soils subject to transient salinity generally contain concentrated salt at depth of between 4 to 16 dsm⁻¹ which, although not as high as salt concentrations in soils that are subject to groundwater-associated salinity (e.g. caused by rising water tables), are still able to restrict water uptake and plant growth (Rengasamy 2002) particularly for the cool-season grain legumes.

2.3 CROP SALINITY TOLERANCE

Salinity tolerance is a term used to describe an inherent capacity for plant growth under increasing salinity in the root zone. As high concentrations of salt are typical in Australian sub-soils (Rengasamy 2006), salinity tolerance is a major target for crop breeding programs to improve crop yield and reliability, particularly in lower-rainfall environments. Crop salinity tolerance has mostly been measured in terms of plant biomass responses which are typically immediate, due to effects on plant growth and grain yield responses on the
basis of a threshold level (Maas and Hoffman 1977). In terms of both biomass and grain yield, responsiveness of the pulse species appear to be much more sensitive to salinity when compared to cereal and oilseed crops (Francois and Maas 1994, Katerji et al. 2003) grown in similar climates. This increased sensitivity to salinity limits the adaptation of pulse crops to growth in the more arid climatic regions of Australia.

2.3.1 Effects of salinity on crop yield

Variation for crop salinity tolerance between species has been reported (Mass and Hoffman 1977, Francois and Maas 1994) and described in an agronomic context as threshold levels for yield reduction, and as regressions for yield loss with increasing salt concentrations. Based on these known parameters the relative crop yield (Y) at a given EC (ECs) can theoretically be calculated based on the threshold EC (ECT) and the linear slope (s) following regression analysis for yield loss by salinity beyond the threshold EC: Y = 100 – s(ECs – ECT) (Fig 2.2). Extensive studies have quantified the relative salt tolerance of a large number of crop species on this basis (Francois and Maas 1994, Mass and Hoffman 1977, Mass 1986), but have usually been limited to few varietal genotype and/or environments.

**Fig. 2.2.** A typical salt tolerance graph showing the threshold (EC_{t}) and slope (s) parameters.
For crop breeding purposes, use of yield response based on selection in the field to identify and select for salinity tolerance is made difficult by the transient nature of soil salinity, and the complex and often overriding effects of other abiotic and biotic stresses (Richards 1983, Daniells et al. 2001). Ultimately, sufficient selectable variation for yield response to salinity in the field is required in order for yield potential per se to be an effective strategy for improvement of salinity tolerance (Richards 1983, 1995). Examples of success in breeding include both direct selection for forage crops such as alfalfa (Johnson et al. 1992), and indirect selection for grain crops such as wheat, barley, cotton and rice (Akbar et al. 1972, Bernal et al. 1974 and Kingsbury and Epstein 1986, Rana 1986). However, in many cases critical growth responses that will improve salinity tolerance (i.e. increased survival time) within a species are often linked to inherently low yield, or identified within non-domesticated unadapted germplasm (Richards 1983).

Crop growth modelling can provide an approach to better prediction of genotypic yield response to salinity (Ferrer-Alegre and Stockle 1999), if there is a reasonable indication of critical plant growth responses to salinity and their impact on yield. In an extensive study using a lysimeter, Katerji et al. (2003) compared the yield and growth responses in response to salinity in a range of legume and other species and concluded that yield responses to salinity were best predicted by differences in leaf water potential (i.e. expressed as water stress day index) and corresponding water use efficiency, but there was no overall correlation with osmotic adjustment and leaf area (Table 2.1).
Table 2.1. Crop salinity classification according to threshold salinity, yield response and water stress day index and concurrent impact on leaf area.

<table>
<thead>
<tr>
<th>Crop</th>
<th>Classification based on soil salinity</th>
<th>Classification Based on Water Stress Day Index</th>
<th>Leaf area (impact)</th>
<th>Threshold salt sensitivity (ECₜ)</th>
<th>Slope of response (% yield reduction per ds m⁻¹)</th>
<th>Classification based on soil salinity</th>
<th>Threshold salt sensitivity (ECₜ)</th>
<th>Slope of response (% yield reduction per ds m⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broadbean</td>
<td>MT</td>
<td>S</td>
<td>Strong</td>
<td>2.8</td>
<td>14.4</td>
<td>MS</td>
<td>1.6</td>
<td>9.6</td>
</tr>
<tr>
<td>Chickpea</td>
<td>S</td>
<td>S</td>
<td>Strong</td>
<td>1.9</td>
<td>37.0</td>
<td>MS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lentil</td>
<td>S</td>
<td>S</td>
<td>Strong</td>
<td>1.7</td>
<td>62.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pea</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>MS</td>
<td>3.4</td>
<td>10.6</td>
</tr>
<tr>
<td>Sugar beet</td>
<td>T</td>
<td>T</td>
<td>Strong</td>
<td>0.0</td>
<td>0.4</td>
<td>T</td>
<td>7</td>
<td>5.9</td>
</tr>
<tr>
<td>Durum wheat</td>
<td>T</td>
<td>T</td>
<td>Strong</td>
<td>0.0</td>
<td>1.9</td>
<td>T</td>
<td>5.7</td>
<td>3.8</td>
</tr>
<tr>
<td>Potato</td>
<td>MT</td>
<td>T</td>
<td>Strong</td>
<td>0.0</td>
<td>5.6</td>
<td>MS</td>
<td>1.7</td>
<td>12</td>
</tr>
<tr>
<td>Sunflower</td>
<td>MT</td>
<td>T</td>
<td>Strong</td>
<td>0.5</td>
<td>8.7</td>
<td>MT</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maize</td>
<td>MT</td>
<td>T</td>
<td>Slight</td>
<td>1.3</td>
<td>10.5</td>
<td>MS</td>
<td>1.7</td>
<td>12</td>
</tr>
<tr>
<td>Soybean</td>
<td>MT</td>
<td>S</td>
<td>Slight</td>
<td>2.0</td>
<td>11.4</td>
<td>MT</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Tomato</td>
<td>MT</td>
<td>S</td>
<td>Slight</td>
<td>2.4</td>
<td>1.4</td>
<td>MS</td>
<td>2.5</td>
<td>9.9</td>
</tr>
</tbody>
</table>

2.4 VARIATION FOR SALINITY TOLERANCE BASED ON GROWTH RESPONSES

Salinity tolerance is a highly complex trait, due to the potentially numerous growth responses that vary in degree and timing according to genotype and environment. As a consequence of this complexity, indices for salinity tolerance have been developed based on responses, often including germination rate, conservation of relative shoot or root dry weight, shoot number, resistance to leaf damage, flower set and abortion, seed and fruit set and abortion, leaf size, canopy biomass and plant survival rates. In most studies, however, variation for salinity tolerance has been generally reported as a percent of plant biomass reduction.

Comparison of relative salinity tolerance between crop species based on biomass reduction, as with yield response, is complicated by the degree of variation both within a given species and its interaction with other environmental factors such as availability of calcium (Lahaye and Epstein 1971, Bonilla et al. 2004). Studies comparing biomass reduction and yield response to salinity indicate that pulse species are much more sensitive relative to barley, wheat, and canola, and are likely to be severely affected when exposed to salinity levels in the field as low as 10 ds m\(^{-1}\) (Saxena et al. 1994). From separate studies both faba bean (Cordovilla et al. 1995) and pea (Hernandez et al. 2000, El-Hamdou I et al. 2003, Gomez et al. 2004), appear to be less sensitive than chickpea (Sadiki and Rabhi 2001) and lentil (Maher et al. 2003).

The impact of salt on growth responses, and ultimately yield, also varies significantly in relation to the growth stage that is exposed to toxic salt concentrations between crops. For example, sugar beet is much more sensitive during germination than at later stages (Beatty and Ehlig 1993), rice is more sensitive during the early seedling stages and at flowering (Akbar and Yubuno 1974), and grain yield is more likely to be affected than vegetative
growth (Khatun and Flowers 1995), and corn is less sensitive at the germination stage but is more sensitive at seedling growth than for ear and grain yield (Mass et al. 1983). Comparison of salinity studies conducted with pulse crops indicates that lentil (Jana 1979) and faba bean (Hamid and Talibuddin 1976, Al-Tahir and Al-Abdussalam 1997) may be more sensitive at germination than at subsequent growth stages, but the converse is likely for chickpea (Kumar 1985) and no information is currently available for peas. Reproductive growth responses to salinity can also be critical for final yield. Increasing salinity is associated with earlier flowering and grain maturity times in wheat, sorghum, oats and cotton, but appears to have little effect on barley and rye (Shannon et al. 1994) and in tomato, salinity results in delayed flowering (Pasternak et al. 1979). Reproductive responses to increasing salinity for the cool-season legumes have not yet been investigated.

Given the complexity of this trait, controlled environment experiments are essential for understanding of growth responses to salinity, as they allow tolerance to be quantified according to the salinity level (i.e. as controlled by rate of application), timing of exposure to salinity relative to growth stage (i.e. as controlled by timing of salt application), environmental factors (i.e. as controlled by soil, temperature, water, nutrition, etc) and genotype (Francois and Maas 1994). Controlled experiments also provide an accurate means of relating this tolerance to aspects of salt uptake, transport and biochemical defence mechanisms that can be directly targeted by genetic manipulation. For breeding purposes, a clear understanding is required of which specific growth responses to salinity will contribute most effectively to yield response. For more sensitive crops, like the pulse species, it may be necessary to pyramid several growth responses in order to obtain sufficient genetic gains in yield response.

2.4.1 Progressive growth responses to increasing salinity

A typical biphasic growth response pattern occurs in plants following exposure to salinity: a primary response to mostly water stress, and a secondary, gradual response to increasing salt toxicity (Munns 1993). This
general growth response pattern is supported by several salinity studies that have measured genotypic variation in growth responses of wheat, barley, triticale, maize, and rice, and the relative accumulation of Na\(^+\) in plant tissue over time (Rawson et al. 1988, Cramer et al. 1994, Munns et al. 1995, Fortmeier and Schubert 1995, Yeo et al. 1991, Rivelli et al. 2002).

2.4.1.1 Primary growth responses

2.4.1.1.1 Water stress response

The primary growth response to salinity in plants is characterised by a rapid and clear reduction in plant growth rate due to water stress, caused by decreased external water potential and water uptake (Munns 2002). Indeed growth response to water stress can be extremely fast (Passioura and Munns 2000). Support for this water stress response can be found from salinity studies that show Na\(^+\) and Cl\(^-\) concentrations following exposure of growing tissue of wheat (Hu et al. 2005) maize, (Beatriz et al. 2005), barley (Fricke 2006) and salt bush (Jeschke et al. 1986) to salinity are at levels insufficiently toxic to be associated with initial reduced growth rates. The measurable genetic variation in this initial growth phase response appears to be quite low, but may be useful for identification of superior variation for water stress tolerance (Munns 2002).

2.4.1.1.2 Ion disequilibrium response

Another primary growth response following exposure to salinity occurs due to ion disequilibrium, caused by increased Na\(^+\) ions, reducing the K\(^+\) uptake and resulting in K\(^+\) deficiency symptoms. The soil Ca\(^{2+}\) concentration will further impact on the selective uptake of Na\(^+\) and K\(^+\) ions by plants.

2.4.1.1.3 Plant hormonal responses

The unresponsiveness of leaf growth rates to water following exposure to salinity indicates that root hormonal signals may also contribute to initial growth responses (Munns 2002). Salinity-induced changes in cell division and differentiation in leaves (James et al. 2002) can reduce leaf growth but also
make leaves thicker. Abscisic acid (ABA) production increases after drought and salinity stress, and is found in xylem sap (Munns and Cramer 1996) and is therefore a likely candidate, but there are possible root signals involved (Dodd 2005). Hormone-induced responses of root cells is also evident (Rubingg et al. 2004).

2.4.2 Secondary growth responses

A secondary growth phase response to salinity occurs gradually in response to salt accumulation in plant tissue, and is characterised by plant tissue injury (i.e. leaf chlorosis and necrosis), reduced plant growth rates and early plant death. These secondary growth responses are likely to be the direct result of salt effects at the cellular level, causing:

1) Reduced cell expansion and assimilate production as a consequence of water stress;
2) Reduced photosynthate production due to effects on carbon metabolism or photophosphorylation;
3) Decreased cytosolic metabolism causing metabolic poisoning, and;
4) Increased production of reactive oxygen species (ROS) from effects of salinity on photorespiration and mitochondrial respiration. ROSs are potent oxidising agents that can lead to cell death, because of lipid peroxidation (membrane destruction), protein oxidation, enzyme inactivation and RNA/DNA damage.

Once salt concentrations reach toxic levels in cells (i.e. Na$^+$ concentrations above 100 mM) enzyme functions required for plant growth are inhibited. Indeed, no variation for enzyme inhibition has even been found between salt-tolerant halophytic species (e.g. Atriplex spongiosa or Suaeda martima) and salt-sensitive glycophytic crop species (e.g. beans and peas) (Flowers 1972, Greenway and Osmond 1972, Flowers et al. 1977).

At the cellular level, salt can only be avoided with cell compartmentalisation of toxic ions. Plant breeding should therefore be targeting those heritable whole plant traits, or physiological growth responses, that reduce or ultimately
exclude salt transport to growing tissue and/or within the cellular cytoplasm. However, in the absence of sufficient genetic variation to achieve this objective, the underlying biochemical mechanisms (e.g. plant antioxidant system) that reduce the impact of salt toxicity at the cellular level may require further exploration in order to improve salinity tolerance.

2.5 MECHANISMS FOR SALINITY TOLERANCE: WHOLE PLANT REGULATION

There are a range of plant physiological processes either in response to or independent of salinity, that result in salt exclusion from growing tissue and cells (Greenway and Munns 1980, Lauchli 1984, Jeschke 1984, Pitman 1984, Storey and Walker 1999, Munns and James, 2003). This exclusion has often been correlated with higher tolerance, in crops such as wheat (Poustini and Siosemardeh 2004) and rice (Zhu et al. 2004).

2.5.1 Regulatory salt exclusion

In the salt-tolerant halophytic species, several mechanisms act to exclude salt at the whole plant level via controlled uptake and transport within the plant and excretion via specialised structures (i.e. bladders and glands). However, excretory structures are not available in the glycophytic species, and salt exclusion is reliant on uptake and transport mechanisms. Anatomically, such regulatory exclusion can occur at the points of root uptake, transfer from root to xylem, transfer within the xylem tissue and transfer to the phloem via ion channels or transporters (Doyle et al. 1998, Amtmann and Sanders 1999) that control preferential exchange of K⁺ for Na⁺. Genetic variation for mechanisms that control uptake and transport between- and within-crop species are likely to be closely related to species evolution, breeding history and its seasonal growth cycle pattern (i.e. annual versus perennial species). For annual crops, desirable variation is likely to be available at all four anatomical points of control (Gorham et al. 1990).
2.5.2 Exclusion of salt at point of root uptake

Most land plants do not have a requirement for Na$^+$ ions for growth and do not appear to have transport systems specific to Na$^+$ uptake. However, Na$^+$ can still enter plant cells through several routes, and there is some capacity for selective uptake of Na$^+$ and Cl$^-$ ions at the root cellular level under increasing soil salinity (Husain et al. 2003). If soil solution flow is symplastic, selective uptake can occur across the epidermal and exodermis root cell membranes. Recently, in wheat (Lauchli et al. 2005) evidence was obtained for this process, from use of x-ray studies. However, passive salt uptake may also occur with flow of soil solution apoplastically across the root cortex, and in this case selective uptake needs to occur in the endodermis layer cells. A consequence of selective salt uptake is likely to be increased production of organic solutes that are required to balance the turgor pressure within roots located in soil. However, it is unknown which organic solutes are principally involved at the root level.

2.5.3 Exclusion of salt at point of transport from root to xylem cells

Plants have the capacity to regulate K$^+$/Na$^+$ concentration in the xylem sap, and in wheat this process appears to be closely correlated with tolerance (Davenport et al. 2005). Cells of the pericycle immediately within the root endodermis potentially providing a major control point (Lauchli et al. 2005, Storey et al. 2003). However, if Na$^+$ ions bypass all membranes and enter the xylem via an apoplastic rout, large amounts of Na$^+$ can be delivered into the xylem tissue (Garcia et al. 1997).

2.5.4 Exclusion of salt at point of transport within the xylem tissue

Salt transport can be selective from the xylem of upper roots to shoot tissue (Pitman 1984, Munns 2005) in cells of the stele of roots, or in the vascular bundles in stems and petioles. Consequently, Na$^+$ ions can be retained in the upper root system and in the lower portion of shoots, and hence be excluded from growing tissue.
2.5.5 Exclusion of salt at point of transport from xylem to phloem cells

There is selective transport of K⁺ / Na⁺ ions into the phloem that acts to exclude Na⁺ into the phloem, thus preventing re-translocation into growing tissue. This exclusion mechanism is a common feature of more tolerant crops (Munns et al. 1988), and there is good evidence for variation within some crop species (Munns and Rawson 1999, Wolf et al. 1991) based on concentrations of Na⁺ and Cl⁻ at growing points or within reproductive tissue following exposure to salt.

2.5.6 Non regulatory salt exclusion and avoidance.

Morphological variations can act to reduce the rate at which salt accumulates to toxic levels in photosynthetic tissue. These include factors such as a high shoot to root biomass ratio, or an absence of an apoplastic pathway in the roots (Garcia et al. 1997). Among pulse crops, peas exhibits considerably more genetic variation for plant morphology that can be manipulated to optimise shoot/root biomass to improve salinity tolerance. Inherent variation in growth patterns (i.e. dormancy, growth rate and phenology) may also reduce the exposure and impact of salinity on final yield (Pitman 1984). However, the potential to manipulate such genetic variation to improve salinity tolerance in pulse species is poorly understood.

2.6 MECHANISMS FOR SALINITY TOLERANCE: CELLULAR REGULATION

Cellular inhibition of enzyme activity initiates when salt concentrations reach about 100 mM, and there is no evidence that enzyme activity adapts to increasing salinity, but leaf tissue often continues to function in many species when salt concentrations are over twice this level (Munns et al. 1993). This observation suggests that mechanisms at the cellular level are reducing the influx of salt into the cytoplasm and causing sequestration into the vacuole of cells. Cation channel transporters can control both soil uptake of Na⁺ and intracellular transport of Na⁺ within a plant (Fig. 2.3). Cation transporter
channels refer to cell membrane proteins. Cation transporters are generally classified based on their ion-selectivity through protein pores, down an electrochemical gradient. But unselective cation channels also permit transport of Na\(^+\), K\(^+\), Ca\(^{2+}\) and NH\(_4^+\) (Demdchik et al. 2002). Na\(^+\) ions can enter plant cells through either non-selective cation channels, and/or via K\(^+\) channels or transporters, as Na\(^+\) can substitute for K\(^+\) (Amtmann and Sanders 1999, Schachtman and Liu 1999, Tyerman and Skerrett 1999, Tester and Davenport 2003). Many selective plasma membrane-located K\(^+\) channels have been identified in plant cells, capable of transporting Na\(^+\). As well as Na\(^+\), Cl\(^-\) accumulation in plant tissues is also associated with salinity tolerance in some dicot species. However, because Na\(^+\) moves more passively into the apoplast and is more difficult to exclude in plant cells, it remains the main focus of attention in salinity tolerance breeding.

**Figure 2.3.** Model indicating the general Na\(^+\) transport systems in higher plants. Regulation of Na\(^+\) uptake across the plasmalemma occurs via the uptake by selective cation transporters and channels, coupled with efflux by Na\(^+\)/H\(^+\) antiporter. The antiporter of the tonoplast sequesters Na\(^+\) in the vacuole.
2.6.1 Low affinity $K^+$ cation transporters for $Na^+$

Plant root cells have $K^+$ selective uptake channels that mostly conduct current in one direction into the cytoplasm, referred to as inward-rectifier channels. These channels are thought to be mainly responsible for $K^+$ uptake when the concentration of $K^+$ at the roots is high. These inward-rectifier channels are highly selective for $K^+$ and thus not likely to allow significant influx of $Na^+$ into plant cells (Amtmann and Sanders 1999), unless there is exposure to increasing salinity.

2.6.2 Non selective cation transporters for $Na^+$

Non-selective cation channels (Demidchik et al. 2002) can also passively transport $Na^+$ ions via an electrochemical gradient. These $Na^+$ concentration-dependent inward currents have been identified in a range of plant cells (Roberts and Tester 1997, Tyerman et al. 1997, Amtmann et al. 1997), and are influenced by the external $Ca^{2+}$ concentration. Calcium at concentrations levels typically found in soil (>0.5 mM) have been shown to significantly inhibit $Na^+$ influx through non-selective cation channels (LeHaye and Epstein 1971). This result indicates that $Ca^{2+}$ insensitive $Na^+$ uptake pathways are likely to be involved in $Na^+$ and $K^+$ uptake. Reduction of the activity of these non-selective channels may increase plant salinity tolerance, but it is unclear how these channels are regulated, and what is the nature of their genetic control in response to salt.

2.6.3 High affinity $K^+$ uptake transporters for $Na^+$

High-affinity $K^+$ carriers are active at external $K^+$ concentration in the micromolar range. The HKT (high affinity $K^+$ transporter) family in plant cells is large, and can allow the passive transport of $Na^+$ under varying conditions (Tester and Davenport 2003). This family is similar in structure to $K^+$ uptake transporters in bacteria and high-affinity $K^+$ transporters in fungal cells (Fraser et al. 1995, Deckert et al. 1998), and cDNA expression of HKT1 has been shown to occur in root cortical cells (Schachtman and Schroeder 1994). The KUP or HAK-like groups is another large family of high affinity $K^+$ transporters that may also play a role in $K^+$ acquisition and $K^+:Na^+$ selectivity of plant
cells. These plant cell transporters are like the HTK family, and also similar to K⁺ uptake transporters identified from bacteria (Schleyer and Bakker 1993) and high affinity K⁺ transporters in fungi (Banuelos et al. 1995), and many variants have been identified in higher plants. The likely role of these proteins is in the control of K⁺ homeostasis, but the degree of selectivity for K⁺ over Na⁺ in higher plants is unclear.

2.6.4 Antiporter transport pathways for Na⁺

Antiporters function as sodium and proton (usually H⁺) exchangers, and can drive efflux Na⁺ from the cytoplasm into the vacuole or across the plasma membrane and out of the cell (Barkla et al. 1995, Dupont 1991) against an electrochemical gradient. The rate of this transport is much lower than that due to ion channels, and most are non-specific to Na⁺ ions. However, the NHX family of antiporters (Na⁺/H⁺ exchangers) is selective for Na⁺. The proton pumps are needed to provide the electrical potential difference that drives Na⁺ transport from antiporters. Uptake of Na⁺ ions into the cell across the plasma membrane is driven by P-type H⁺-ATPases, that hydrolyse ATP to pump H⁺ into the cell wall (Reinhold and Guy 2002), and active ion transport into the vacuole driven by V-type H⁺-ATPases, which hydrolyse ATP to pump H⁺ into the vacuole (Binzel and Ratajczak 2002). H⁺-PPiase on the tonoplast is also important, as it hydrolyses pyrophosphate to pump H⁺ into the vacuole (Gaxiola et al. 2001, Binzel and Ratajczak 2002). Once Na⁺ ions increase in the cell they move symplastically into adjacent cells via the plasmodesmata, but can also be transported back into the cell wall or be compartmentalized in the cell vacuole via efflux transport. Efflux is controlled by the Na⁺/H⁺ antiporters on the plasma membrane (i.e. SOS1, Zhu et al. 2003). Compartmentalisation occurs through vacuolar Na⁺/H⁺ antiporters (i.e. NHX1, Blumwald et al. 2000). Cellular mechanisms that maintain low Na⁺ concentrations in cell organelles (i.e. chloroplasts and mitochondria) are not well-understood. However, a cation/proton antiporter, CHX23, which is specific to the chloroplast envelope membrane, has been identified by Song et al. (2004), and is probably essential for pH control.
2.6.5 Synthesis of organic solutes

When Na\(^+\) and Cl\(^-\) are sequestered in the vacuoles of cells, K\(^+\) and organic solutes also accumulate in the cytoplasm and organelles in order to balance the osmotic pressure. The main organic solutes that accumulate under salinity stress are proline and glycine betaine (Hasegawa et al. 2000). Organic solutes contribute to increased salinity tolerance indirectly, by not only altering osmotic balance, but also by maintenance of enzyme activity (Greenway and Munns 1980, Grumet et al. 1985, Tal et al. 1979) and repairing of oxidative damage. The plant antioxidant system includes a range of anti-oxidative enzymes or enzyme products (i.e. carotenoids, catalase, peroxidises) (Goyer and Halliwell 1976, Noctor and Foyer 1998) which can be induced by salt stress (Stevens et al. 1997).

2.7 CANDIDATE GENES FOR GENETIC MANIPULATION TO IMPROVE SALINITY TOLERANCE

There are many potential candidate genes for enhancement of salinity tolerance in higher plants using genetic manipulation. These genes can be grouped according to functionality into those that control salt uptake and transport, those that provide osmotic or protective functions (Tester and Davenport 2003), or those that control plant growth responses that can delay salt accumulation and associated effects in growing tissue (Gaynard et al. 2003, Rubio 1995, Rus et al. 2004, Zhu 2003).

2.8 PHENOTYPIC SCREENING FOR SALINITY TOLERANCE

The physiological complexity of salinity tolerance means that genotype screening in pot experiments requires adequate control of the soil water, air and temperature relations (Passioura 2006). Wang (2002) showed that coarser growing media (e.g. river sand) was better at reproducing field soil water and temperature changes than very fine media (e.g. fine silica).
Numerous pot-based experiments have been developed for different crops, but notably Munns et al. (1995) have described a pot salinity-screening approach based on use of trays and cyclical sub-irrigation (in which saline nutrient solution is gradually applied following germination) that is both quick and effective for screening of large numbers of lines for breeding purposes (Munns and James 2003), and is considered applicable to other crops.

For pulse crops, useful genetic variation for breeding efforts has been identified without too much difficulty in lentil and chickpea. In lentil, Maher et al. (2003) used a pot-based experiment to screen a diverse set of germplasm (309 accessions) from the Australian Temperate Field Crops Collection (ATFCC) for seedling resistance, based on shoot weight, leaf damage and plant height. For chickpea, Serraj et al. (2004) used pot-based screening and susceptibility as the selection criteria to identify accessions with improved tolerance within a mini-core set of chickpea lines. Sadiki and Rabih (2001) used sand benches to screen 200 Moroccan-derived chickpea accessions for salinity tolerance, based on shoot dry weight, nodulation, acetylene reduction and shoot nitrogen content and proceeded to quantify the advantages of tolerant accessions in the field both in terms of yield and nodulation advantage. There is currently no information available on salinity tolerance following screening of diverse germplasm sets of pea and faba bean. Several salinity studies, however, indicate other potential sources of salinity tolerance in lentil (Mamo et al. 1996, Ashraf and Zafar 1997 and Rai and Singh 1999), chickpea (Singh and Singh 2001, Singh et al. 2001, Soussi et al. 2003), faba bean (Gaballah and Gomaa 2004) and pea (Hernandez et al. 1995, 2000, Gomez et al. 2004) based on growth responses to salinity, but these are have been based on very restricted germplasm sets.

### 2.9 VARIATION FOR SALINITY TOLERANCE BASED ON SOIL TYPE SPECIFIC ION TOLERANCE

Relative differences in salt tolerance between crops can be closely associated with variation in soil type (Rana 1985). Significant soil
environment-by-genotype interactions for salinity tolerance are likely to exist within species, as saline soils can include a range of dissolved salts in high concentrations, and also occur across a range of soil acidity, structural and moisture content environments.

The majority of research describing variation for salinity tolerance has been based on data collected from saline water containing NaCl, and sometimes with varying amounts of calcium to avoid sodicity effects. However in some soil environments specific ion toxicity sensitivities (e.g. to boron, manganese or zinc) may also be affecting plant growth as a result of either being toxic or in limiting supply. Considerable genetic variation has been identified within crop species for tolerance to excessive accumulation, exclusion and plant tolerance to specific effects of ions associated with low and high pH. Despite reasonable tolerance of low soil pH in pea, over 80% of production in Australia is from alkaline soils that characteristically have sub-soils that are highly sodic, contain high salt and extractable boron concentrations, and are limited in availability of iron, manganese, copper, zinc and phosphorous to the plant (Naidu and Rengasamy 1993).

In pulse crops, high tolerance to boron toxicity has been identified in pea (Bagheri et al. 1994, 1996) and both lentil (Hobson et al. 2006) and faba bean are also considered sensitive (Ferreyra et al. 1997). In pea, two major genes appear to be additively contributing to high boron tolerance conferred by a mechanism of exclusion of boron uptake at the roots (Bagheri et al. 1996).

2.10 AIMS OF THIS THESIS

Field pea is considered to be the most drought tolerant pulse crop option for southern Australia. However, to expand crop production and reliability across the lower-rainfall cropping climates, improved tolerance to salinity stress was considered to be an important breeding objective. On this basis, the thesis aims to:
i) Identify useful variation for salinity tolerance in the pea species that can be used for genetic improvement. This will be based on an initial screen of plant symptom responses across a large and diverse set of *Pisum* germplasm accessions. A validation experiment will be undertaken to confirm variation of other measures of growth response to salinity (i.e. growth rate) and Na$^+$ accumulation, and to identify suitable germplasm for use as parents and for further physiological studies (Chapter 3).

ii) Identify the critical plant response and avoidance mechanisms contributing to higher salinity tolerance in field pea, as a focus for breeding and selection. This will include experiments in which genotypes of differing plant type and salinity tolerance (identified in Chapter 3) will be grown under varying salinity exposure at either seedling (Chapter 4) or adult plant (Chapter 5) stages to assess plant responses. Shoot and root biomass (i.e. growth rate, dry matter, main stem length, etc), tissue damage, Na$^+$ and K$^+$ accumulation, phenology and seed set will be analysed.

iii) Improve the understanding of genetic control for higher salinity tolerance to facilitate selection and pyramiding of important genes or traits. This will include studying progeny variation for measures of salinity tolerance (Chapter 6), assessing the relationship between grain yield response in the field and genotypic variation for salinity and boron tolerance (Chapter 6), and associating phenotypic diversity with genomic variation to identify important loci (i.e. QTLs) to facilitate selection (Chapter 7).

iv) Discuss the main outcomes of this research in the context of the potential benefits of improving salinity tolerance of the crop for Australian grain growers. Potential future directions for further research will also be discussed.
Chapter Three
Sources of high tolerance to salinity in pea
(Pisum sativum L.).

3.1 INTRODUCTION

Field pea (Pisum sativum L.) is broadly adapted and widely grown as a dryland grain crop in rotation with cereals. The annual field pea acreage over the last 20 years has fluctuated between 300,000 and 500,000 ha in Australia (ABARE, 2012). Over 70% of this production is within the lower rainfall cropping regions of southern Australia, in which rainfall during the growing season is typically in the 160-230 mm range. A high proportion of the soils in these regions have dense clay sub-soil layers that are both highly sodic (i.e. exchangeable sodium percentage (ESP) >15%) and alkaline (i.e. pH > 8.5) (Rengasamy 2002, 2006). Within such regions, soil sodicity-, salinity- and alkalinity-induced nutrient deficiencies (for micronutrients such as Fe, Mn, Cu, Zn and P) and toxicities (i.e. due to boron, carbonate and aluminate) can act together to limit crop growth and grain yield of field pea. A range of dissolved salts, can contribute to salinity stress, however NaCl is the most prevalent and important salt when considering approaches for improving salinity tolerance in Australia (Rengasamy 2002, Munns and Tester 2008). Individual sub-soil constraints occur transiently varying in spatial and temporal degrees over the soil profile. In field pea, relatively high tolerance to boron toxicity has been identified (Bagheri et al. 1994, 1996; Leonforte et al. 2009) and appears to be highly heritable (Bagheri et al. 1996; Leonforte et al. 2009). Tolerance is associated with exclusion of boron from the roots (Bagheri et al. 1996). However, no investigation of variation for tolerance to high soil NaCl has been undertaken for Pisum sativum L. Research in other major grain crops such as wheat (Munns et al. 2006) has highlighted the difficulty of using yield-based response measurements in field studies as a measure of tolerance. This is due to the complexity of interactions with abiotic and biotic stress factors, variability of NaCl in the soil profile, and differential responses according to
both growth stage and genotype. Studies of biomass reduction and grain yield changes in response to salinity under controlled environment conditions indicate that pulse species are generally much more sensitive than other major dryland crops grown in Australia, such as barley (Maas 1986, Saxena et al. 1994), wheat (Francois and Maas 1994) and canola (Steppuhn et al. 2001). Indeed, the threshold salinity level for pea has been estimated to be very low at 1.5 ds m\(^{-1}\) (Dua et al. 1989). Soils subject to transient salinity in cropping regions of Australia generally exhibit maximal NaCl concentration in B and C soil horizons between 4 to 16 ds m\(^{-1}\) (Rengasamy 2002). It is therefore highly likely that salinity levels significantly limit grain yield of pulse crops such as field pea in low rainfall environments.

From separate studies, faba bean (Cordovilla et al. 1995) and pea (Hernandez et al. 2000, El-Hamdoui et al. 2003, Gomez et al. 2004), appear to be less sensitive to NaCl than chickpea (Sadiki and Rabhi 2001) and lentil (Maher et al. 2003). Comparison of salinity tolerance studies within pulse crops indicates that lentil (Jana 1979) and faba bean (Hamid and Talibuddin 1976, Al-Tahir and Al-Abdussalam 1997) may be more sensitive at germination and as seedlings than at subsequent growth stages. However, the converse is likely to be true for chickpea (Kumar 1985), and there is no information for pea regarding variation in germination response. For cool-season grain legumes, pot-based phenotypic screening has successfully identified sources of relatively higher seedling tolerance to NaCl in both lentil (Maher et al. 2003) and chickpea (Serraj et al. 2004, Sadiki and Rabih 2001). The present study was hence aimed at identification of sources of relatively high tolerance to NaCl for genetic improvement of Australia’s field pea crop.

### 3.2 MATERIALS AND METHODS

#### 3.2.1 Experiment 1: Identifying sources of high salinity tolerance within *Pisum L.*

Three sets of *Pisum* germplasm were sourced from the Australian Temperate Field Crops Collection (ATFCC) at Horsham, VICTDPI and included
accessions that represented the global collection based on geographic origin (418 lines), Chinese landrace accessions (355 lines) based on origin according to province and major Australian field pea varieties (7 lines). The experiment was conducted over spring (September-November in the southern hemisphere) in a semi-controlled environment (i.e. large plastic igloo) at Horsham. Six plants of each accession were sown with equidistant spacing in 13 cm diameter pots into a sand and gravel medium to a depth of 2 cm. The gravel medium was composed from a 1:1 ratio of coarse river sand and 5 mm bluestone chips. Each pot was treated daily with rainwater from sowing until emergence. From four days post-emergence, seedlings were watered with a complete nutrient solution (i.e. nitrosol, NPK ratio 12.2: 2.9: 8.5), in addition to supplementation with a calcium source (i.e. calcium nitrate). From 10 days post-emergence, NaCl dissolved in water as a concentrated stock solution was added to the watering solution. The required NaCl concentration was tested using an EC meter and was applied at an initial rate of 4 ds m⁻¹. The concentration of applied NaCl was increased by 4 ds m⁻¹ at each watering time, up to 16 ds m⁻¹, and maintained at this concentration until assessment. All watering with the nutrient and salt solution was undertaken over 3 day-intervals at a rate of 200 ml per pot applied directly to the growing medium surface. Individual plants were assessed for percentage necrosis at 9 weeks, and for a general salinity tolerance scores at 12 weeks post-sowing. The salinity tolerance score and screening method were based on a visual growth response scale (1-10), developed for lentil (Maher et al. 2003) and adapted for pea as described in Table 3.1.

The experiment was designed as a pot experiment with two replicates, in each of which pots were randomized in a grid of 80 ranges by 7 rows. A null-salt application treatment was included in order to identify any confounding effects caused by the growing climate, disease or nutritional deficiencies. The null-salt treatment consisted of pots sown in six ranges randomly located within each experimental block. A residual estimation maximum likelihood (REML) analysis of the mean salt score for each pot was performed to obtain predicted means adjusted for range and row in the experiment. Statistical analysis was conducted on the mean symptom score for each accession.
Mean symptom scores were based on at least three plants per pot. A total of 33 accessions were excluded from the analysis, as fewer than three plants per pot had germinated. To determine significant differences in mean salinity scores for accessions based on country of origin, an analysis of variance (ANOVA) was undertaken by contrasting between the national sources that had ten or more accessions represented in the screening experiment. ANOVA was also performed on the predicted mean salinity tolerance scores for 335 accessions for which site of origin was specified to 20 individual Chinese provinces.

### Table 3.1. Description of salinity symptom score.

<table>
<thead>
<tr>
<th>Salinity symptom score (1-10)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Plant healthy green, no obvious salinity symptoms.</td>
</tr>
<tr>
<td>2</td>
<td>Beginning to yellow, not very many symptoms.</td>
</tr>
<tr>
<td>3</td>
<td>Some chlorosis bottom half of plant, no necrosis, overall yellowing.</td>
</tr>
<tr>
<td>4</td>
<td>Necrosis beginning on bottom half of plant.</td>
</tr>
<tr>
<td>5</td>
<td>Chlorosis and necrosis bottom half of plant, yellowing overall (50% affected).</td>
</tr>
<tr>
<td>6</td>
<td>Chlorosis becoming more severe on upper part of plant, not necrotic on upper plant.</td>
</tr>
<tr>
<td>7</td>
<td>Chlorosis and necrosis more than half of plant.</td>
</tr>
<tr>
<td>8</td>
<td>More necrosis than 7, but still some green leaves.</td>
</tr>
<tr>
<td>9</td>
<td>Stem and very youngest leaves green, rest dead (all leaves may be dead).</td>
</tr>
<tr>
<td>9.5</td>
<td>Only top of stem (or small part stem) and very youngest leaves still green, rest dead (all leaves may be dead).</td>
</tr>
<tr>
<td>10</td>
<td>Plant dead.</td>
</tr>
</tbody>
</table>

### 3.2.2 Experiment 2: Identification and validation of lines with relatively high salinity tolerance for breeding application

For this experiment, 4 germplasm sets totalling 70 lines were used, with the following characteristics:
Set 1). Forty two accessions identified from experiment 1 (Table 9.1), based on the criteria of: low salinity symptom score (< 6), geographic diversity in origin and diversity of plant features (e.g. internode length, leaf type, and branching habit).

Set 2). Eighteen breeding lines identified from the field pea program of Pulse Breeding Australia (PBA), with relatively higher NaCl tolerance following routine screening (Leonforte et al. 2009).

Set 3) Six Australian cultivars (Kaspa, Helena, Parafieal, Excell, Yarrum and Sturt), that showed varying sensitivity in experiment 1 (Table 9.1).

Set 4). Three accessions identified from experiment 1 (Table 9.1) that showed significantly higher sensitivity on the basis of salinity tolerance score.

The sowing process and NaCl application was conducted using the same methodology as described in experiment 1. Experiment 2 was designed as a split plot consisting of 4 replicates with the main plot treatment being application of salt as NaCl at a level of 19 dsm⁻¹ or no application of salt and the sub-plot treatment being 70 germplasm accessions described above.

Once the final EC reading of the solution reached 18 dsm⁻¹ for the plus-salt treatment, a salinity symptom score (i.e. as described above) and plant height (i.e. length of the primary stem from the base of the stem to the last node) were recorded every 7th day for four weeks (i.e. 7th, 14th, 21st, 28th day) for each plant in each pot. The total plant and root matter were harvested at final assessment, dried in an oven at 70°C for 3 days, and dry matter content was recorded. Total Na⁺ concentration was measured on leaflets from the final growth node of plants at the final growth stage using inductively coupled plasma-optical emission spectrometry (ICP-OES) (Zarcinas et al. 1987) from samples digested in nitric acid/hydrogen peroxide solution. The growth rate for the main stem (i.e. cm/day) was calculated from the increase in length of the primary or main stem, and was divided by the number of growing days. Following REML analysis, the percent reduction in dry matter and growth rate or plant height was calculated from the predicted means. A principal
component analysis was undertaken using Genstat 11, and a bi-plot was developed to graphically represent the relationships between lines and measured variates.

3.3 RESULTS

3.3.1 Experiment 1: Identified sources of high salinity tolerance within *Pisum* L.

The salinity symptom scores for the null-salt treated pots were consistently measured as 1, indicating that biotic or abiotic factors were not confounding salinity symptoms as assessed in this experiment. There were major differences for symptom scores between the 747 accessions that could be compared in the plus salt treatment (Table 9.1). Over 80% of these accessions were very sensitive (i.e. symptom score was equal to, or above 7) (Table 3.2) in this screening experiment. Of the 36 accessions that had a salinity score of 4 or less, thirty originated from China. Accessions from Greece and China had the lowest symptom scores (Table 3.3, Fig. 3.2) and these were significantly lower than accessions from Afghanistan, Ethiopia, Finland, the former Soviet Union, Sweden and the United States (Table 3.4). There was no significant difference for salinity score on the basis of Chinese province of origin (Table 3.5). The regions in which higher tolerance was identified (i.e. salinity symptom score < 4) were located in neighbouring provinces in the central-eastern region (i.e. Shaanxi, Henan and Anhui) and the south central region (i.e. Yunnan, Guizhou and Guangxi) and in Qinghai province (Fig. 3.1). Variation for salinity tolerance within specific geographic locations allocated to country or to Chinese province was typically large (Fig. 3.2, 3.3). Forty two accessions were selected for further investigation in Experiment 2, on the basis of a low salinity symptom score (i.e. less than 6), (Fig. 3.4), diverse geographic origin and diverse plant morphology (e.g. internode length, leaf type, etc).
Table 3.2. Distribution of salinity symptom scores: 1 (no symptoms) to 10 (dead) for 747 accessions from the Australian Temperate Field Crops Collection screened at seedling stage for salinity tolerance.

<table>
<thead>
<tr>
<th>Range of symptom scores</th>
<th>Number of accessions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>1</td>
</tr>
<tr>
<td>2-3</td>
<td>7</td>
</tr>
<tr>
<td>3-4</td>
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<td>8-9</td>
<td>143</td>
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<tr>
<td>9-10</td>
<td>201</td>
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Table 3.3. Mean and range of salinity symptom scores of *Pisum* L. accessions grouped on the basis of global region and individual country of recorded origin in the Australian temperate field crops collection centre.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Number of accessions</th>
<th>Mean salinity tolerance score (1-10)</th>
<th>Range of salinity tolerance score (1-10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFRICA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burundi</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>53</td>
<td>8.3ab</td>
<td>5.7 – 10</td>
</tr>
<tr>
<td>Kenya</td>
<td>1</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Madagascar</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Rwanda</td>
<td>1</td>
<td>7.6</td>
<td>7.6</td>
</tr>
<tr>
<td>Tanzania</td>
<td>1</td>
<td>8.9</td>
<td>8.9</td>
</tr>
<tr>
<td>Uganda</td>
<td>1</td>
<td>9.8</td>
<td>9.8</td>
</tr>
<tr>
<td>Yemen</td>
<td>1</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Zaire</td>
<td>1</td>
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<td>7.2</td>
</tr>
<tr>
<td>Origin</td>
<td>Number of accessions</td>
<td>Mean salinity tolerance score (1-10)</td>
<td>Range of salinity tolerance score (1-10)</td>
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<td>----------------------</td>
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<td>4.9 – 10</td>
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<td>2.2 – 10</td>
</tr>
<tr>
<td>India</td>
<td>16</td>
<td>8.1</td>
<td>5.8 – 10</td>
</tr>
<tr>
<td>Mongolia</td>
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<td>8.1</td>
<td>6.3 – 10</td>
</tr>
<tr>
<td>Nepal</td>
<td>8</td>
<td>8.3</td>
<td>7.4 – 10</td>
</tr>
<tr>
<td>Pakistan</td>
<td>7</td>
<td>8.5</td>
<td>4.0 – 10.1</td>
</tr>
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<td>8.8</td>
<td>8.8</td>
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<td>4.5 – 10</td>
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<td>Armenia</td>
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<td>7.4</td>
</tr>
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<td>Belarus</td>
<td>1</td>
<td>8.7</td>
<td>8.7</td>
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<tr>
<td>Bulgaria</td>
<td>4</td>
<td>8.0</td>
<td>5.6 – 9.3</td>
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<td>Estonia</td>
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<td>5.7</td>
<td>5.7</td>
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<td>Former Soviet Union</td>
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<td>9.6ab</td>
<td>8.4 – 10</td>
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<td>7.6 – 10</td>
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<td>Kazakhstan</td>
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<td>6.7 – 10</td>
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<td>Kyrgyzstan</td>
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<td>5.3 – 10</td>
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<td>7.6 – 10</td>
</tr>
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<td>6.3 – 10</td>
</tr>
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<td>9.5 – 10</td>
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<td>8.6</td>
<td>4.5 – 10</td>
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<td>7.9 – 7.9</td>
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<td>5.7 – 9.4</td>
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<td>Greece</td>
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<td>1.1 – 10</td>
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<td>7.1 – 8.7</td>
</tr>
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<td>Libya</td>
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<td>7.9</td>
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<td>Morocco</td>
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<td>7.4</td>
<td>4.7 – 8.7</td>
</tr>
<tr>
<td>Portugal</td>
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<td>7.9</td>
<td>6.6 – 9.2</td>
</tr>
<tr>
<td>Spain</td>
<td>3</td>
<td>7.5</td>
<td>6.2 – 9.4</td>
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<tr>
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<td>7.6</td>
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<td>Origin</td>
<td>Number of accessions</td>
<td>Mean salinity tolerance score (1-10)</td>
<td>Range of salinity tolerance score (1-10)</td>
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<td>----------------------</td>
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<td>4.0 – 10</td>
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<td>6.2-10</td>
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<td>3</td>
<td>9.4</td>
<td>8.9 – 10</td>
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<tr>
<td>Israel</td>
<td>2</td>
<td>7.3</td>
<td>6.4 – 8.2</td>
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<td>9.4</td>
<td>8.7 – 10</td>
</tr>
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<td>Syria</td>
<td>4</td>
<td>7.5</td>
<td>6.2 – 8.2</td>
</tr>
<tr>
<td>NORTH AMERICA</td>
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<td>6.0-10</td>
</tr>
<tr>
<td>Canada</td>
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<td>6.5 – 9.5</td>
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<td>6.0 – 10</td>
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<td>5.7-10</td>
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<td>9.0ab</td>
<td>7.0 – 10</td>
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<td>7.6</td>
<td>4.9 – 10</td>
</tr>
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<td>Poland</td>
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<td>4.6 – 10</td>
</tr>
<tr>
<td>Sweden</td>
<td>66</td>
<td>8.4ab</td>
<td>5.3 - 10.4</td>
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<td>United Kingdom</td>
<td>5</td>
<td>6.9</td>
<td>4.9 – 9.5</td>
</tr>
<tr>
<td>Yugoslavia</td>
<td>3</td>
<td>8.5</td>
<td>6.9 - 10</td>
</tr>
<tr>
<td>Australian cultivars</td>
<td>8</td>
<td>8.6</td>
<td>7.0-10.6</td>
</tr>
</tbody>
</table>

* Means with the different letters are significantly different (F prob. < 5%) and only presented for countries represented by 10 or more accessions.
Table 3.4. P value (F pr.), for contrasts between China and other individual countries of recorded origin in the Australian temperate field crops collection centre.

<table>
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<th>Source of variation</th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
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<td>Country</td>
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<td></td>
<td></td>
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<td>18.6</td>
<td>18.6</td>
<td>6.6</td>
<td>0.01</td>
</tr>
<tr>
<td>China versus Ethiopia</td>
<td>1</td>
<td>43.6</td>
<td>43.6</td>
<td>15.4</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>China versus Finland</td>
<td>1</td>
<td>26.0</td>
<td>26.0</td>
<td>9.2</td>
<td>0.003</td>
</tr>
<tr>
<td>China versus Former Soviet Union</td>
<td>1</td>
<td>50.4</td>
<td>50.4</td>
<td>17.8</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>China versus France</td>
<td>1</td>
<td>4.2</td>
<td>4.2</td>
<td>1.5</td>
<td>0.225</td>
</tr>
<tr>
<td>China versus Greece</td>
<td>1</td>
<td>4.1</td>
<td>4.1</td>
<td>1.5</td>
<td>0.229</td>
</tr>
<tr>
<td>China versus India</td>
<td>1</td>
<td>8.4</td>
<td>8.4</td>
<td>3.0</td>
<td>0.084</td>
</tr>
<tr>
<td>China versus Sweden</td>
<td>1</td>
<td>70.3</td>
<td>70.3</td>
<td>24.9</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>China versus Turkey</td>
<td>1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.0</td>
<td>0.85</td>
</tr>
<tr>
<td>China versus United States</td>
<td>1</td>
<td>15.2</td>
<td>15.2</td>
<td>5.4</td>
<td>0.021</td>
</tr>
<tr>
<td>Residual</td>
<td>613</td>
<td>1732.7</td>
<td>2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>623</td>
<td>1925.7</td>
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Table 3.5. Mean and range of salinity symptom scores of *Pisum* L. accessions grouped on the basis of Chinese province of recorded origin in the Australian temperate field crops collection.

<table>
<thead>
<tr>
<th>Origin (Province)</th>
<th>Number of accessions</th>
<th>Mean salinity tolerance score (1-10)</th>
<th>Range for salinity tolerance score (1-10)</th>
<th>Number of accessions with salinity tolerance score &lt; 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Henan</td>
<td>9</td>
<td>5.0</td>
<td>2.2-7.5</td>
<td>3</td>
</tr>
<tr>
<td>Shaanxi</td>
<td>51</td>
<td>5.9</td>
<td>2.3-9.8</td>
<td>15</td>
</tr>
<tr>
<td>Anhui</td>
<td>11</td>
<td>6.3</td>
<td>3.5-8.5</td>
<td>1</td>
</tr>
<tr>
<td>Guangxi</td>
<td>35</td>
<td>7.2</td>
<td>3.6-9.6</td>
<td>1</td>
</tr>
<tr>
<td>Guizhou</td>
<td>14</td>
<td>7.3</td>
<td>2.8-9.9</td>
<td>2</td>
</tr>
<tr>
<td>Yunnan</td>
<td>85</td>
<td>7.9</td>
<td>2.9-10.0</td>
<td>3</td>
</tr>
<tr>
<td>Qinghai</td>
<td>39</td>
<td>8.3</td>
<td>3.1-10.0</td>
<td>1</td>
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<tr>
<td>Jiangsu</td>
<td>1</td>
<td>4.7</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Sichuan</td>
<td>16</td>
<td>7.0</td>
<td>4.4-9.7</td>
<td>0</td>
</tr>
<tr>
<td>Hubei</td>
<td>10</td>
<td>7.2</td>
<td>5.2-8.5</td>
<td>0</td>
</tr>
<tr>
<td>Guangdong</td>
<td>2</td>
<td>7.4</td>
<td>6.2-8.6</td>
<td>0</td>
</tr>
<tr>
<td>Nei Mongol</td>
<td>38</td>
<td>7.5</td>
<td>7.7-9.6</td>
<td>0</td>
</tr>
<tr>
<td>Beijing</td>
<td>2</td>
<td>7.7</td>
<td>5.6-9.8</td>
<td>0</td>
</tr>
<tr>
<td>Heilongjiang</td>
<td>1</td>
<td>7.8</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Shanghai</td>
<td>2</td>
<td>7.8</td>
<td>6.0-9.6</td>
<td>0</td>
</tr>
<tr>
<td>Gansu</td>
<td>4</td>
<td>8.1</td>
<td>6.7-9.0</td>
<td>0</td>
</tr>
<tr>
<td>Xinjiang</td>
<td>4</td>
<td>8.2</td>
<td>6.1-9.5</td>
<td>0</td>
</tr>
<tr>
<td>Xizang</td>
<td>9</td>
<td>8.5</td>
<td>6.9-10.0</td>
<td>0</td>
</tr>
<tr>
<td>Ningxia</td>
<td>2</td>
<td>8.5</td>
<td>7.8-9.2</td>
<td>0</td>
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</tbody>
</table>
Fig. 3.1. Map of China highlighting provinces in which tolerant land races (T) (i.e. mean salinity symptom score of less than 4) were identified and provinces in which only sensitive land races (S) were identified (i.e. mean salinity symptom score of 4 and above).
Fig. 3.2. Variation in salinity symptom scores within countries of origin that could be statistically compared (country represented by more than 20 accessions).
Fig. 3.3. Variation in salinity symptom scores within Chinese province of origin that could be statistically compared (province represented by more than 20 accessions).
Fig. 3.4. Salinity symptom scores for accessions that showed higher tolerance in experiment 1 and selected for validation and further investigation of whole plant growth responses in experiment 2. Australian commercial varieties are highlighted in the lighter coloured shading.
3.3.2 Experiment 2. Validation of high salinity tolerance identified in pea.

A significant reduction in growth rate and plant height was associated with the application of NaCl after 7, 14, 21 and 28 days (Table 3.5). Lines grown in the null-salt treatment did not display any salinity symptoms, and associated tolerance score values were consistently equal to 1 (Table 3.6). The plant height of the main stem increased significantly at 7, 14, 21 and 28 days for the null-salt treatment. However in the plus salt treatment there was no significant increase in mean plant height of lines after day 14 (Table 3.6). The mean plant growth rate did not significantly change over the evaluation period in the null-salt treatment but significantly decreased from day 7 to 14 to 21 with application of NaCl. There was a significant reduction in both total shoot dry matter and root dry matter at day 28 associated with the application of NaCl (Table 6). An ANOVA analysis indicated a significant interaction (e.g. F pr <0.001) between salt treatment (i.e. plus, minus) and accession line treatment for plant height, plant symptom score and plant growth rate for each consecutive recording (i.e. days 7, 14, 21, 28) and between shoot dry matter and total root dry matter at day 28.

Linear regression analysis (Table 3.7) indicates that accession growth rates for the salt treatment were closely correlated with the null-salt treatment at day 7, weakly correlated at day 14, and not correlated by day 21 and 28. The accession plant heights from the salt treatment were closely correlated with the plant heights in the null-salt treatment for each day of assessment. There was also a significant correlation between total shoot DM at day 28 in the presence or absence of applied salt.
Table 3.6. Mean plant height, salinity symptom score, growth rate at day 7, 14, 21 and 28 and final shoot and root dry matter at day 28 for plus versus minus salinity treatments.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Salt treatment</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant height (main stem: cm)</td>
<td>Salt (Nil)</td>
<td>26.2 a</td>
<td>39.7 ad</td>
<td>51.7 ae</td>
<td>60 af</td>
</tr>
<tr>
<td></td>
<td>Salt (Plus)</td>
<td>16.4 b</td>
<td>20.1 bc</td>
<td>20.4 bc</td>
<td>21.7 bc</td>
</tr>
<tr>
<td>Salinity tolerance score (1-10)</td>
<td>Salt (Nil)</td>
<td>1.0 a</td>
<td>1.0 a</td>
<td>1.0 a</td>
<td>1.0 a</td>
</tr>
<tr>
<td></td>
<td>Salt (Plus)</td>
<td>1.4 b</td>
<td>2.6 bc</td>
<td>4.9 bd</td>
<td>7.6 be</td>
</tr>
<tr>
<td>Plant Growth rate (cm/day: main stem)</td>
<td>Salt (Nil)</td>
<td>1.87 a</td>
<td>1.85 a</td>
<td>1.82 a</td>
<td>1.64 a</td>
</tr>
<tr>
<td></td>
<td>Salt (Plus)</td>
<td>1.17 b</td>
<td>0.53 bc</td>
<td>0.14 bd</td>
<td>0.18 bd</td>
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<tr>
<td>Total shoot DM (g)</td>
<td>Salt (Nil)</td>
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<td></td>
<td></td>
<td>6.63 a</td>
</tr>
<tr>
<td></td>
<td>Salt (Plus)</td>
<td></td>
<td></td>
<td></td>
<td>2.21 b</td>
</tr>
<tr>
<td>Total root DM (g)</td>
<td>Salt (Nil)</td>
<td></td>
<td></td>
<td></td>
<td>5.43 a</td>
</tr>
<tr>
<td></td>
<td>Salt (Plus)</td>
<td></td>
<td></td>
<td></td>
<td>2.51 b</td>
</tr>
</tbody>
</table>

*Means with the same letters are not significantly different (F prob. < 5%).

Table 3.7. The $R^2$ values based on linear regressions of accession growth rate, plant height, root DM and shoot DM in plus versus minus salt treatments.

<table>
<thead>
<tr>
<th>Day</th>
<th>Plant growth rate (cm/day)</th>
<th>Plant height (main stem: cm)</th>
<th>Total root DM (g)</th>
<th>Total shoot DM (g)</th>
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</thead>
<tbody>
<tr>
<td>7</td>
<td>0.84*</td>
<td>0.84*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.15*</td>
<td>0.77*</td>
<td></td>
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</tr>
<tr>
<td>21</td>
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<td>0.72*</td>
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<td></td>
</tr>
<tr>
<td>28</td>
<td>NS</td>
<td>0.70*</td>
<td>0.19*</td>
<td>0.46*</td>
</tr>
</tbody>
</table>

* F prob. < 1%

A principal component bi-plot graph of accessions by variates measured on each day of assessment in the presence of salt treatment indicates that tolerance scores were highly consistent between assessment times. High salinity symptom scores (i.e. sensitivity) were closely associated with parameters such as higher Na$^+$ and lower K$^+$ concentration in the growing tip.
tissue, lower growth rate and root and shoot dry matter at day 28. Variation in growth rates at days 7 and 21 and plant height in general did not appear to be as closely correlated with the accession salinity symptom scores (Fig. 3.5).

Based on salinity symptom scores, both unadapted and adapted breeding lines with relatively higher tolerance compared to commercial varieties were identified (Fig. 3.6). The rate at which toxicity symptoms developed over time varied significantly between lines (Fig. 3.6). In general, tested varieties showed relatively moderate to severe toxicity symptoms from day 14 when compared to the more tolerant lines identified in experiment 1 (Fig. 3.6). The most sensitive Australian variety on the basis of symptom development was Kaspa (Fig. 3.6). Accession ATC1836 showed the slowest rate of symptom development (Fig. 3.6). Some of the adapted material with lesser symptoms (i.e. low salinity symptom score) also maintained higher growth rates when compared to the unadapted lines with low symptom scores (Fig. 3.7). However all accessions showed an initial rapid reduction in growth rate from day 7, which became more gradual from day 14 to 21 (Fig. 3.7).

There was a significant association between for accession salinity symptom score between experiments (R² = 0.43; F pr. < 1%). Notable accessions with a low salinity symptom score in experiment 1 (symptoms score < 4) and slower rate of salinity symptom development in experiment 2 included ATC01836 and ATC1093 from Greece, ATC1091 from Albania and ATC04226, ATC06592, ATC06642 and ATC07157 from China.
Fig. 3.5. Principal component biplot between lines (represented by number) and the variates measured in plus salt treatment: growth rate (GRR), final root dry matter (ROOTDM), final shoot dry matter (SHOOTDM), final plant growing tip Na$^+$ concentration (NA) and K$^+$ concentration (K), plant height (HT), and salinity symptom score (SC) for days day 7, 14, 21 and 28.
**Fig. 3.6.** Salinity symptom scores from day 7 to 28 in plus salt treatment for unadapted accessions as indicated by the solid lines (−) for 1 (ATC01836), 15 (ATC01093), 23 (ATC07021), 39 (ATC04226), adapted breeding lines as indicated by the dotted lines (−−) for 44 (03H090P-04HO2002), 47 (OZP0812), 50 (99-410-2-14-2) and Australian commercial varieties as indicated by the broken lines (---) for 51 (Parafield), 53 (Helena), 60 (Yarrum), 66 (Kaspa).
Fig. 3.7. Main stem growth rate (cm/day) from day 7 to 28 in plus salt treatment for unadapted accessions as indicated by the solid lines (-) for 1 (ATC01836), 15 (ATC01093), adapted breeding lines as indicated by the dotted (···) for 47 (OZP0812), 50 (99-410-2-14-2) and Australian commercial varieties as indicated by the broken lines (--) : 51 (Parafield), 60 (Yarrum), 66 (Kaspa).
3.4 DISCUSSION

Following screening of a diverse pea germplasm set from the ATFCC, several sources of high tolerance to NaCl have been identified within the species. On the basis of country-of-origin, China provided the largest proportion of qualified accessions. Within China the central west (Henan, Anhui, Shaanxi, Guangxi), southern (Guizhou, Yunnan) and western (Qinghai) provinces showed higher proportions of tolerant accessions. These provinces are all located along three major river basins of the Yellow, Yangtze and Pearl rivers where crop irrigation has been common practice for over 2000 years and is likely associated with soil salinisation. Recent studies have highlighted the genetic distinctiveness and diversity of Chinese pea germplasm compared to the global pea germplasm collection (Zong et al. 2009), implying that pea germplasm in China has undergone strong directional selection, potentially in isolation from the Fertile Crescent region where peas are thought to have originated (Ambrose 1995). The higher frequency of salinity tolerance discovered in China in this study may be linked to divergence in natural selection between China and other global regions. However separate introgression from wild species or independent domestication also cannot be discounted. The high variability for salinity tolerance associated with province of origin in China or country is not surprising, as peas have been historically grown in diverse environments that vary significantly in terms of altitude, climate, soil type and farming systems (irrigated vs. dryland).

Pea germplasm with relatively higher salinity tolerance was morphologically diverse, and not specifically associated with non-domesticated or adapted types within the species. The most tolerant accession (ATC1836) was obtained from Greece. Unfortunately, ATC1836 displayed several negative traits that will reduce its value as a parental line. These include a high number of basal branching, relatively low early vegetative growth, very long plant internodes, thin and wiry stems, a very late flowering habit, low seed number per pod, small seed size and a dark and patterned seed coat. However the moderate salt tolerance identified in adapted plant backgrounds (i.e.
OZP0812) provides a basis for selecting recurrent parents to use in targeted breeding (Figure 3.6).

Exposure to salt treatment significantly reduced plant height and growth rate over time, and resulted in rapid onset of plant toxicity symptoms and early plant death. Importantly, however, this study identified in the pea germplasm a wide diversity of responses in terms of the rate of symptom development and growth responses that can be exploited in breeding higher tolerance. Relative salt tolerance based on tissue-specific symptoms was validated and highly consistent across experiments. The growth response to salinity in pea appears to be similar to the biphasic response observed with other crops, which is characterised by an initial sudden reduction in growth, mostly likely due to the exposure to a solution of low osmotic potential and then followed by a gradual response to increasing salt toxicity (Rawson et al. 1988, Cramer et al. 1994, Munns et al. 1995, Fortmeier and Schubert 1995, Yeo et al. 1991, Rivelli et al. 2002). Plant height as controlled by internode length, does not appear to be directly associated with NaCl sensitivity in pea. This is an important finding, as international breeding efforts are focused on the development of semi-dwarf types with a high harvest index. Selection for higher plant biomass may, however, be useful in order to dilute and delay Na⁺ toxicity effects (Almodares et al. 2011). For peas, scope exists to increase plant biomass as a salt stress avoidance mechanism, even within semi-dwarf plant backgrounds via the selection for, increased internode length and leaflet number, larger plant structures (i.e. tendrils, stipules) and greater basal and aerial branches (e.g. ATC1836). In Australia early flowering time is generally a breeding priority for improving reliability of yield of field pea, as production is mostly within short season environments (Sadras et al. 2012). Despite this the rate and timing of maximum biomass accumulation can still be significantly increased during reproductive development (Mahli et al. 2007) in early flowering germplasm.

The growth symptoms used as a basis for symptom assessment were not confounded by the growing conditions, and developed rapidly in response to application of salt. As expected germplasm with higher salinity symptoms also
displayed the greatest reduction in root and shoot dry matter. The Na\(^+\) and K\(^+\) concentrations at the growing tips were closely correlated with symptom scores, indicating that plant symptoms are highly predictive of Na\(^+\)-induced plant tissue toxicity. As growing tips of more tolerant pea lines had lower Na\(^+\) and inversely higher K\(^+\) concentration, Na\(^+\) exclusion (Demidchik et al. 2002) may be involved as a mechanism. Knowledge of how Na\(^+\) transport may be regulated in pea and at what point (e.g. roots (Lauchli et al. 2005), xylem (Davenport et al. 2005, Pitman 1984, Munns 2005) or phloem tissue (Munns and Rawson 1999, Wolf et al. 1991)) requires further investigation to facilitate selection. Consecutive salinity score assessments did not vary significantly and little advantage was gained in assessing symptoms after 14 days of salt exposure. However, the optimum timing for assessment is likely to vary with the concentration of exogenous salt and the climate for growth (e.g. temperature and applied water), as these factors have an interactive effect on osmotic regulation and transpiration rate (Blum 2005). For pea the low cost and rapid semi-hydroponic screening methodology described in this study appears very effective to identify new sources of NaCl tolerance in the species. Interestingly the major field pea variety grown (Kaspa) in Australia appears to be quite sensitive to salinity even when compared to other commercial varieties. This may partially explain the unreliability of this variety in some short season climates (Sadras et al. 2012) and the sometimes unexpected poor growth and early senescence observed in field testing in Western Australia (I. Pritchard, personal communication) where salinity is likely to be more severe. On this basis any incremental gain in salinity tolerance could have a major impact on crop reliability in Australia.

The positive variation for salinity tolerance in pea appears substantial and available across diverse plant backgrounds and origins. Significant genetic improvement based on direct phenotypic selection alone is therefore highly likely to be possible. Consequently targeted backcross and recurrent selection breeding is being undertaken using variation identified in this study. Further research to validate how this tolerance varies across ontogeny, understand genetic control and identify major genes or DNA molecular markers in field pea are now planned.
Chapter Four
Growth responses of field pea (*Pisum sativum* L.) to varying concentration of applied NaCl salinity from seedling growth stage.

4.1 INTRODUCTION

Transient soil salinity is a major constraint for crop growth and grain yield across southern Australia (Rengasamy 2002), particularly in lower rainfall regions (i.e. when annual rainfall is less than 400 mm) in which limited water supply compounds salinity stress (Läuchli and Grattan 2007). In Australia, improving salinity tolerance of field pea has increasingly become more important onwards from the mid 1990s as production (Siddique and Sykes 1997) has continued to shift towards regions with lower and more variable rainfall climates. This shift in production is likely to continue, and is attributable to both the relative agronomic advantage of field pea in lower rainfall regions (Farhoodi *et al.* 2004) and grain price disadvantage in higher rainfall regions when compared to other grain legumes such as lentil. Genetic improvement in Australia has led to the rapid development of better-adapted, shorter-season varieties (PBA Twilight, PBA Oura) intended for low rainfall climates (Sadras *et al.* 2012). However, significant improvements to both soil boron and salinity toxicity tolerance are required to improve yield reliability in comparison with cereals and to encourage crop adoption across the lower rainfall Mallee regions (Leonforte *et al.* 2009).

Significant phenotypic variation for NaCl-induced salinity tolerance has been identified in pea (Leonforte *et al.* 2013), based on early plant growth responses. Sodium-induced salinity effects on plant growth are complex in nature, as they cause multiple and varying responses in terms of whole plant physiology and cellular function (Läuchli and Grattan 2007). Initially, salinity
causes sudden plant growth reduction due to osmotic adjustment (Chen and Jiang 2010). Minimal genotypic variation for this trait has been observed in salinity tolerance studies of crop species. The secondary growth responses are generally more gradual effects, resulting from increasing Na⁺ concentration in plant tissue (Jacoby 1999). Variation in tolerance appears to be mostly determined by an ability over time to prevent or delay Na⁺ accumulation in leaves and thereby delay associated toxicity (Munns and Tester 2008). Most crops appear to display relatively more salt tolerance at germination compared to emergence, and vegetative growth and root and shoot growth is generally inhibited by increasing salinity (Läuchli and Grattan 2007). Abiotic stress causing strong inhibition of early plant growth (Neumann 1997) will in most cases severely limit reproductive development and thus potential for grain yield, particularly in more sensitive crops such as pulses. However, exposure to transient salinity during reproductive development due to root growth into more saline sub-soil layers (Nutall et al. 2009) may in some regions cause larger total productivity losses than seedling-induced salinity stress. In wheat, salt stress seems to accelerate reproductive growth and inhibits spike development (Maas and Grieve 1990), and thus grain yield potential. In rice, which is relatively more salt sensitive, lower yield is primarily associated with reduction in tiller number and sterility of spikelets (Zeng and Shannon 2000). A comparable understanding is hence needed for field pea to better focus genetic improvement.

This study was aimed at obtaining a more detailed understanding of plant responses and Na⁺ accumulation for 7 pea genotypes that differ significantly in salinity tolerance (Leonforte et al. 2012) following exposure to varying concentration levels applied to roots from the early seedling growth stage.

4.2 MATERIALS AND METHODS

Seven genotypes were sown in a replicated pot experiment to assess growth responses to varying concentration of salt (NaCl) from the seedling growth stage (3-4 nodes). Six genotypes were selected on the basis of higher salinity tolerance (Leonforte et al. 2013), including three breeding lines (03H090P-
04HO2002, 03H556P-04HO2012, 99-410-2-14-2) from the Pulse Breeding Australia field pea program, and three unadapted accession lines (ATC01836, ATC04226 and ATC07021) from the Australian Temperate Field Crops Collection (ATFCC). The variety Kaspa was also included in the experiment as a salinity sensitive control genotype (Leonforte et al. 2010).

The experiment was conducted over spring (September-November in the southern hemisphere) in a semi-controlled environment (i.e. large plastic igloo) at Horsham, Victoria. Six plants of each accession were sown with equidistant spacing in 13 cm diameter pots into a sand and gravel medium to a depth of 2 cm. The gravel medium was composed from a 1:1 ratio of coarse river sand and 5 mm bluestone chips. Each pot was treated daily with rainwater from sowing until emergence. From 4 days post-emergence, seedlings were watered with a complete nutrient solution (i.e. nitrosol, NPK ratio 12.2: 2.9: 8.5), in addition to supplementation with a calcium source (i.e. calcium nitrate). The average day-night temperatures were maintained at 23 / 16°C. The required NaCl concentration was tested using an EC meter and was applied at an initial rate of 3 dsm⁻¹ from day 9 post-emergence. The concentration of applied NaCl was increased by 3 dsm⁻¹ at each watering time to avoid sudden osmotic adjustment, up to a final rate of either 0 (null treatment), 6, 12 or 18 dsm⁻¹, and maintained at this concentration until assessment of the salinity treatments. All watering with the nutrient and salt solution was undertaken over 3 day-intervals at a rate of 200 ml per pot applied directly to the growing medium surface. The null-salt application control (no added NaCl) was included in order to quantify effects of salinity exposure. Plants were trellised onto plastic lattice to avoid splashing of salt solution during watering, and to avoid shading effects and plant damage during assessment.

Individual plants in each pot were assessed for symptom development using salinity symptom score of 1 (no symptoms) to 10 (severe symptoms) as described by Leonforte et al. (2013), and plant growth of the primary or main stem as measured in centimetres from the first node to the last node. This assessment was undertaken from 21 days post-emergence, and then on
every 7th day until day 70 for symptom score, and until day 56 for main stem length. Pod set, seed number set per pod and total plant and root dry matter were recorded at day 70 post-emergence.

Leaflets were harvested from the fourth primary node from one of the 6 plants in each pot and pooled across reps at each assessment time for salinity symptom score. Leaflet Na+ and K+ concentration were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES) (Zarcinas et al. 1987) following sample digestion in nitric acid/hydrogen peroxide solution.

The pot experiment was designed as a split plot with 13 replications. This included 4 whole-plot salinity treatment levels for varying concentration of applied NaCl in watering solution (0, 6, 12 and 18 dsm⁻¹) and 7 sub-plot genotype treatment levels described above. An ANOVA analysis was undertaken of all variables measured using Genstat 12. Analysed treatment data is presented in either tabular or graphic form for interpretation.

4.3 RESULTS

4.3.1 Salinity symptom score

There was a significant and successive increase in salinity symptom score associated with root exposure to higher NaCl (Table 4.1, Fig. 4.2), but mostly from day 35 post-emergence. Beyond day 63 post-emergence, natural senescence associated with the onset of reproductive development appeared to confound assessment of salinity symptom score. Exposure to higher salinity caused significantly higher toxicity symptoms (as reflected by salinity symptom score) irrespective of genotype. However the rate of increase in symptom development varied significantly between the seven genotypes. The 12 dsm⁻¹ salinity treatment appeared to be most discriminatory for identification of variation for symptom score. The genotype ATC1836 and the variety ‘Kaspa’ showed the slowest and fastest rates of symptom development, respectively.
4.3.2 Changes in growth rate

The seven genotypes varied significantly in relation to main stem growth in the null-salinity treatment. However, all genotypes showed a significant reduction in main stem plant growth associated with exposure to increasing salinity (Table 4.2). The degree of plant growth reduction varied significantly between genotypes, across salinity treatments and over time (Fig. 4.4.1, 4.4.2, 4.4.3). Only a minor effect on main stem growth was observed at the 6 dsm⁻¹ salinity treatment compared to the null treatment (Table 4.2) for all 7 genotypes. At 42 days post-emergence the genotype Kaspa ceased growth in both the 12 and 18 dsm⁻¹ salinity treatments, while the equivalent effect for genotype ATC4226 was in response to the 18dsm⁻¹ treatment. The remaining 5 genotypes maintained increased main stem growth for all salinity treatments through until day 56 post-emergence (Table 4.2).

4.3.3 Final root and plant dry matter

Significant differences in genotype were detected for plant and root dry matter production in the null-salinity treatment at days 70 post emergence (Table 4.3). All seven genotypes exhibited significantly lower plant dry matter associated with increasing salinity treatments. Relative to the null treatment, genotype Kaspa showed the largest reduction in plant dry matter associated with increasing salinity (Table 4.4). There was a significant reduction in root dry matter associated with higher salinity, but no significant effect of genotype by salinity. The most salinity tolerant genotype ATC1836 had significantly higher root dry matter, and the most sensitive genotypes, ATC4226 and Kaspa, had the lowest plant dry matter at day 70 post-emergence.

4.3.4 Seed and pod set components

By day 70 post-emergence, all of the seven genotypes showed significant variation for number of pod and seeds set per plant (Table 4.5). The genotype ATC1836 was very late flowering, resulting in few seeds and pods being set by day 70 post-emergence in the null salinity treatment, and consequently the effect of salinity could not be determined. The genotype Kaspa failed to set any seed under any of the salinity treatment regimes. The remaining 5
genotypes set both seed and pods at the 6 and 12 dsm\(^{-1}\) levels. However, at the 18 dsm\(^{-1}\) salinity level treatment, seed set was very low.

**4.3.5 Concentration of Na\(^+\) in plant tissue**

The concentration of Na\(^+\) in leaflet tissue increased significantly in response to higher salinity, and successively over time for all genotypes. The concentration of Na\(^+\) that had accumulated in plant tissue varied significantly between genotypes, but was closely associated with measures of symptom development and reduced plant growth. Specifically, the more tolerant genotype ATC1836 showed the slowest and lowest rate of Na\(^+\) accumulation, while the sensitive genotype Kaspa displayed the highest and most rapid accumulation of Na\(^+\) in leaflet tissue (Fig. 4.1). The degree and rate of K\(^+\) concentration accumulation in plant tissue varied significantly between genotypes. All showed a negative association with increasing Na\(^+\) concentration in plant tissue. The ratio for K\(^+\) to Na\(^+\) decreased significantly in all salinity treatments but the ratio was much higher for ATC1836 in the 6 and 12 dsm\(^{-1}\) salinity treatments when compared to the other genotypes.
Table 4.1. Salinity symptoms (salinity symptom score 1 (low) to 10 (high)) for 7 genotypes for 4 salinity watering treatments varying in applied concentration of NaCl: 0 (null), 6, 12, and 18 dsm⁻¹ over 8 assessment times.

<table>
<thead>
<tr>
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<th>Genotype</th>
<th>Salinity x Genotype x Day</th>
<th>Salinity x Genotype</th>
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</tr>
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<td>Day</td>
<td>Day</td>
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Least significant differences of means (F prob. <5%)

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<td>0.33</td>
<td>0.76</td>
</tr>
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<td>Except when comparing means with the same level(s) of</td>
<td></td>
<td></td>
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<tr>
<td>Salinity</td>
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<td>0.23</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>Salinity x Genotype</td>
<td>0.61</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salinity x Day</td>
<td>0.74</td>
<td></td>
<td></td>
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</tbody>
</table>
Table 4.2. Main stem growth (length in cm’s) for 7 genotypes for 4 salinity watering treatments varying in applied concentration of NaCl: 0 (null), 6, 12, and 18 dsm⁻¹ over 6 assessment times.

| Salinity (NaCl dsm⁻¹) | Genotype            | Salinity x Genotype x Day | Salinity x Genotype | Salinity
|-----------------------|----------------------|---------------------------|---------------------|-----------
<p>|                       |                      | Day 21  28  35  42  49  56 | Mean                | Mean      |
| 0 dsm⁻¹ (null)        | 03H090P-04HO2002     | 113  230  366  621  858  1083 | 545                |
|                       | 03H556P-04HO2012     | 112  214  333  533  818  1063 | 513                |
|                       | 99-410-2-14-2        | 101  219  333  582  821  1054 | 519                |
|                       | ATC01836             | 90   147  183  272  425  566  | 280                |
|                       | ATC04226             | 66   129  174  320  502  729  | 320                |
|                       | ATC07021             | 76   153  198  371  572  800  | 362                |
|                       | Kaspa                | 63   129  192  338  455  596  | 295                |
| 6 dsm⁻¹               | 03H090P-04HO2002     | 118  223  335  548  723  913  | 256                |
|                       | 03H556P-04HO2012     | 116  209  303  522  718  910  | 222                |
|                       | 99-410-2-14-2        | 108  200  295  479  755  929  | 260                |
|                       | ATC01836             | 89   142  168  240  302  417  | 135                |
|                       | ATC04226             | 68   125  162  286  454  604  | 95                 |
|                       | ATC07021             | 74   137  184  326  479  613  | 149                |
|                       | Kaspa                | 69   132  190  292  389  475  | 85                 |</p>
<table>
<thead>
<tr>
<th>Salinity (NaCl dsm⁻¹)</th>
<th>Genotype</th>
<th>Salinity x Genotype x Day</th>
<th>Salinity x Genotype</th>
<th>Salinity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day</td>
<td>Day</td>
<td>Day</td>
</tr>
<tr>
<td>12 dsm⁻¹</td>
<td>03H090P-04HO2002</td>
<td>118</td>
<td>202</td>
<td>265</td>
</tr>
<tr>
<td></td>
<td>03H556P-04HO2012</td>
<td>113</td>
<td>193</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>99-410-2-14-2</td>
<td>108</td>
<td>198</td>
<td>253</td>
</tr>
<tr>
<td></td>
<td>ATC01836</td>
<td>89</td>
<td>131</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>ATC04226</td>
<td>71</td>
<td>117</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>ATC07021</td>
<td>72</td>
<td>122</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>Kaspa</td>
<td>68</td>
<td>113</td>
<td>130</td>
</tr>
<tr>
<td>18 dsm⁻¹</td>
<td>03H090P-04HO2002</td>
<td>118</td>
<td>178</td>
<td>211</td>
</tr>
<tr>
<td></td>
<td>03H556P-04HO2012</td>
<td>114</td>
<td>181</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>99-410-2-14-2</td>
<td>112</td>
<td>180</td>
<td>208</td>
</tr>
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<td></td>
<td>ATC01836</td>
<td>87</td>
<td>124</td>
<td>126</td>
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<td></td>
<td>ATC04226</td>
<td>67</td>
<td>104</td>
<td>92</td>
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<td>ATC07021</td>
<td>71</td>
<td>112</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>Kaspa</td>
<td>61</td>
<td>85</td>
<td>78</td>
</tr>
<tr>
<td>Salinity (NaCl dsm(^{-1}))</td>
<td>Genotype</td>
<td>Salinity x Genotype x Day</td>
<td>Salinity x Genotype</td>
<td>Salinity</td>
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<tr>
<td></td>
<td></td>
<td>Day</td>
<td>Day</td>
<td>Day</td>
</tr>
<tr>
<td>Salinity x Day</td>
<td></td>
<td>21</td>
<td>28</td>
<td>35</td>
</tr>
<tr>
<td>0 dsm(^{-1}) (null)</td>
<td></td>
<td>89</td>
<td>174</td>
<td>254</td>
</tr>
<tr>
<td>12 dsm(^{-1})</td>
<td></td>
<td>91</td>
<td>154</td>
<td>186</td>
</tr>
<tr>
<td>18 dsm(^{-1})</td>
<td></td>
<td>90</td>
<td>138</td>
<td>148</td>
</tr>
<tr>
<td>6 dsm(^{-1})</td>
<td></td>
<td>92</td>
<td>167</td>
<td>234</td>
</tr>
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</table>

Least significant differences of means (F prob. <5%)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Salinity</th>
<th>Salinity x Genotype</th>
<th>Salinity x Day</th>
<th>Salinity x Genotype x Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13</td>
<td>27</td>
<td>19</td>
<td>45</td>
</tr>
</tbody>
</table>

Except when comparing means with the same level(s) of

| Salinity | 25       | 15     | 44 |
| Salinity x Genotype | 38       |  | |
| Salinity x Day | 44       |  | |
Table 4.3 Genotype plant and root dry matter (grams) at day 70 post emergence for 4 salinity watering treatments varying in applied concentration of NaCl: 0 (null), 6, 12 and 18 dsm⁻¹.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NaCl dsm⁻¹</th>
<th>Plant DM</th>
<th>Root DM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 dsm⁻¹</td>
<td>6 dsm⁻¹</td>
<td>12 dsm⁻¹</td>
</tr>
<tr>
<td>03H090P-04HO2002</td>
<td>16.06</td>
<td>13.80</td>
<td>5.74</td>
</tr>
<tr>
<td>03H556P-04HO2012</td>
<td>17.16</td>
<td>13.17</td>
<td>4.47</td>
</tr>
<tr>
<td>99-410-2-14-2</td>
<td>18.47</td>
<td>12.13</td>
<td>2.40</td>
</tr>
<tr>
<td>ATC01836</td>
<td>12.79</td>
<td>9.76</td>
<td>4.65</td>
</tr>
<tr>
<td>ATC04226</td>
<td>14.20</td>
<td>11.27</td>
<td>3.21</td>
</tr>
<tr>
<td>ATC07021</td>
<td>14.87</td>
<td>12.13</td>
<td>4.90</td>
</tr>
<tr>
<td>Kaspa</td>
<td>13.98</td>
<td>9.41</td>
<td>1.41</td>
</tr>
</tbody>
</table>

**Least significant differences of means (F prob. <5%)**

<table>
<thead>
<tr>
<th>Plant dry matter</th>
<th>Root dry matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Salinity x Genotype</td>
</tr>
<tr>
<td></td>
<td>0.86 0.69 1.52</td>
</tr>
</tbody>
</table>

Except when comparing means with the same level(s) of

| Salinity | 1.37 | ns |
Table 4.4. Percentage of plant dry matter reduction for seven genotypes at day 70 post emergence in the 3 plus salinity watering treatments varying in applied concentration of NaCl: 6, 12 and 18 dsm$^{-1}$.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NaCl dsm$^{-1}$</th>
<th>Plant DM (% reduction / plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6 dsm$^{-1}$</td>
</tr>
<tr>
<td>ATC01836</td>
<td>24</td>
<td>64</td>
</tr>
<tr>
<td>03H090P-04HO2002</td>
<td>19</td>
<td>67</td>
</tr>
<tr>
<td>03H556P-04HO2012</td>
<td>14</td>
<td>64</td>
</tr>
<tr>
<td>99-410-2-14-2</td>
<td>23</td>
<td>74</td>
</tr>
<tr>
<td>ATC04226</td>
<td>34</td>
<td>87</td>
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<tr>
<td>ATC07021</td>
<td>21</td>
<td>77</td>
</tr>
<tr>
<td>Kaspa</td>
<td>33</td>
<td>90</td>
</tr>
</tbody>
</table>
Table 4.5. Pod and seed number of 7 genotypes at day 70 post emergence for 4 salinity watering treatments varying in applied concentration of NaCl: 0 (null), 6, 12 and 18 dsm⁻¹.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NaCl dsm⁻¹</th>
<th>Seed number / plant</th>
<th>Pod number / plant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 dsm⁻¹</td>
<td>6 dsm⁻¹</td>
<td>12 dsm⁻¹</td>
</tr>
<tr>
<td>03H090P-04HO2002</td>
<td>33.2</td>
<td>2.6</td>
<td>0.9</td>
</tr>
<tr>
<td>03H556P-04HO2012</td>
<td>41.2</td>
<td>5.1</td>
<td>0.7</td>
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<td>99-410-2-14-2</td>
<td>48.7</td>
<td>18.8</td>
<td>0.8</td>
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<td>ATC01836</td>
<td>2.5</td>
<td>0.0</td>
<td>0.0</td>
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<td>ATC04226</td>
<td>61.8</td>
<td>35.8</td>
<td>8.2</td>
</tr>
<tr>
<td>ATC07021</td>
<td>21.8</td>
<td>14.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Kaspa</td>
<td>20.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Mean</td>
<td>32.7</td>
<td>11.0</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Least significant differences of means (F prob. <5%)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Seed number</th>
<th>Pod number</th>
</tr>
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<tbody>
<tr>
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<td>Salinity x Genotype</td>
<td>Salinity x Genotype</td>
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<td>Salinity Genotype</td>
<td>Salinity Genotype</td>
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<tr>
<td>4.9</td>
<td>4.0</td>
<td>8.9</td>
</tr>
<tr>
<td>1.0</td>
<td>0.90</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Except when comparing means with the same level(s) of Salinity

| Salinity | 8.0 | 1.8 |
Fig. 4.1. Concentration of Na\(^+\) from leaflet tissues at the fourth node of the primary stem for seven genotypes for 4 salinity watering treatments varying in applied concentration of NaCl: 0 (null), 6, 12 and 18 dsm\(^{-1}\) at days 21, 28, 35, 42, 49, 56 and 70 post emergence. Error bar indicates l.s.d. f prob <5%.
Fig. 4.2. Concentration of $K^+$ from leaflet tissues at the fourth node of the primary stem for seven genotypes for 4 salinity watering treatments varying in applied concentration of NaCl: 0 (null), 6, 12 and 18 dsm$^{-1}$ at days 21, 28, 35, 42, 49, 56 and 70 post emergence. Error bar indicates l.s.d. f prob <5%.
Fig. 4.3. Ratio of $K^+$ to $Na^+$ (weight basis) from leaflet tissues at the fourth node of the primary stem for seven genotypes for 4 salinity watering treatments varying in applied concentration of NaCl: 0 (null), 6, 12 and 18 dsm$^{-1}$ at days 21, 28, 35, 42, 49, 56 and 70 post emergence. Error bar indicates l.s.d. f prob <5%.

Ratio of concentration of $K^+$ to $Na^+$ in plant tissue

Salinity treatment: 0=Null, 6,12,18 dsm$^{-1}$, genotype, and day: 21, 28, 35, 42, 49, 56, 70 from left to right within salinity x genotype treatment groups.
Fig. 4.4.1. Line graph showing \([\text{Na}]\), symptom score and plant growth of main stem for three contrasting genotypes (03H556P-04HO2012, ATC01836 and Kaspa) varying in salinity tolerance for plus salinity watering treatments of \(\text{NaCl}\) at 6 dsm\(^{-1}\), from 21 to 70 days post emergence.

**Salinity treatment: 6 dsm\(^{-1}\)**
Fig. 4.4.2. Line graph showing [Na], symptom score and plant growth of main stem for three contrasting genotypes (03H556P-04HO2012, ATC01836 and Kaspa) varying in salinity tolerance for plus salinity watering treatments of NaCl at 12 dsm⁻¹, from 21 to 70 days post emergence.
**Fig. 4.4.3.** Line graph showing [Na], symptom score and plant growth of main stem for three contrasting genotypes (03H556P-04HO2012, ATC01836 and Kaspa) varying in salinity tolerance for plus salinity watering treatments of NaCl at 18 dsm⁻¹, from 21 to 70 days post emergence.
4.4 DISCUSSION

The broad differences in salinity tolerance documented by Leonforte et al. (2013) were validated in this study. In particular, the higher sensitivity of the widely grown Australian variety ‘Kaspa’ and the greater tolerance of the landrace accession ATC1836, which originates from Greece, were confirmed.

The genotypes that were investigated were all significantly and incrementally affected by exposure to higher NaCl-induced salinity over time. In particular, significant genotype differences were identified for rate of symptom development, plant growth and Na⁺ accumulation in plant tissue in response to varying salinity concentration. These genotypic responses for field pea corroborate results for variation found in other crops that suggest a strong relationship between rate (Munns et al. 2002) and biphasic manner (Munns 2005) of symptom development and growth responses, with increasing accumulation of Na⁺ ions in plant tissue (Lauchli and Grattan 2007).

Although at the lowest salinity treatment level (6 dsm⁻¹) no genotypes reached a plateau for growth rate or symptom score, the results indicate that a crop of the major Australian field pea variety ‘Kaspa’ that is exposed to even low levels of salinity from seedling stage can suffer significant reductions in growth and grain yield potential. At this lowest salinity treatment level, all lines except the genotype ATC1836 set pods in this study. The absence in pod set of ATC1836 was, however, probably due a later flowering habit rather than an effect of salinity stress per se. The number of pods that were set was severely reduced for the remaining 6 genotypes. This effect could be due to Na⁺-induced growth inhibition and effects on phenology (i.e. flowering time and duration) and deleterious effects on specific components of grain yield (i.e. seed size and seed set). Growth and reproductive responses at later stages of ontogeny require better understanding for field pea, particularly because increases of transient salinity (Rengasamy 2006) are expected as crop production continues to shift towards drier climates in which root systems will receive greater exposure to salinity (Nuttall et al. 2009). Salinity research showing effects on yield components in other grain crops, including legumes
such as mungbean (Shakil 2009) and chickpea (Vadeza 2012), provide good models for investigating this variation in field pea.

While the onset of reproductive development and associated natural senescence (Lim et al. 2007) was a probable confounding effect on salinity symptom score, the differences between genotypes remained significant. In particular, the sensitive genotype Kaspa displayed a much more sudden and rapid symptom response to increasing salinity for the 12 and 18 dsm⁻¹ salinity treatments. In contrast, the most tolerant genotype displayed a much slower rate of Na⁺ accumulation and a much more gradual rate of salinity symptom development. The remaining 6 genotypes that were screened were intermediate and varied in their response to varying salinity treatments. The three breeding lines were all much taller growing per se, but 03-556P-04HO2012 showed lower dry matter loss in the 6 dsm⁻¹ salinity treatment and as such, potentially a higher salinity tolerance phenotype in terms of growth response. Further research is needed to understand this difference, and to determine whether and which non-Na-specific effects of salinity, such as osmotic stress, may be contribute to this tolerance (Rahnam et al. 2010). The genetic potential to pyramid the two characters of higher early plant growth rate and vegetative biomass prior to flowering under salinity stress is also likely to be important for development of more reliable forage field pea varieties for Australia. Although the genotype ATC1836 appears to be highly tolerant, much slower plant growth rate is apparent compared to the other 6 genotypes. Therefore, tolerance could be a partial consequence of this factor rather than an ability to exclude or tolerate higher Na⁺ accumulation. This is an important consideration, as inherently low early plant vigour or biomass growth will probably be detrimental to field pea production in short-season or water-deficit prone environments of Australia (Sadras et al. 2012).

There was a significant reduction in final root dry matter from increasing salinity exposure, but no genotypic effect. The reduction in root biomass could be due to a combination of direct effects of Na⁺ on growth inhibition (Tester and Davenport 2003), or indirect effects of Na⁺-reduced plant shoot biomass (Läuchli and Grattan 2007). Variation for higher root biomass and architecture
in pea (McPhee 2005) can improve tolerance to root diseases (Kraft and Boge 2001) and damage by herbicides (Ali-Kahn and Snoad 1977), so the combination of higher salinity tolerance and root biomass that is apparent in ATC1836 may be valuable to breeders.

All genotypes showed decreased K$^+$ content in leaf tissues in response to increasing NaCl$^+$ salinity in the root media, which is consistent with numerous studies for a range of other crop species (Grattan and Grieve 1999). The ratio of K$^+$ to Na$^+$ in plant tissue was higher in the more tolerant genotype ATC1836, and probably associated with low Na$^+$ uptake and thus K$^+$ exchange (Fox and Guerinot 1998). Potassium is important for cell expansion, osmotic regulation and cellular and whole–plant homeostasis (Schachtman et al. 1997) and there is a high stomatal K$^+$ requirement for photosynthesis (Chow et al. 1990). Sodium-induced K$^+$ deficiency has been implicated in growth and yield response in a number of field crops such as barley (Britto et al. 2010), and selection for a higher K$^+$/Na$^+$ ratio (Maathuis and Amtmann 1999) may hence be a useful strategy for improving salinity tolerance.

Seedling tolerance to salinity appears critical for field pea in order to reduce significant damage or even plant death (Leonforte et al. 2012). This study indicates the potential to select for salinity responses associated with timing and rate of symptom development, growth rate, Na$^+$ and K$^+$/Na$^+$ plant tissue concentration, plant height, root biomass and final grain yield. Depending on genetic correlations and heritability values, these salinity responses could be targeted by either independent or co-dependent breeding strategies (Shannon 1985) to improve tolerance of field pea. Clearly, screening and selection based on seedling plant symptom responses is an effective, reliable and low-cost approach to rapidly pyramiding genes for salinity tolerance. However because grain yield appears to be so strongly inhibited by salinity, a long-term and targeted selection strategy based on yield response in high risk environments is also likely to be useful for breeding higher salinity tolerance *per se*. 
Chapter Five
Growth responses of four *Pisum sativum* L. genotypes to increasing NaCl salinity applied just prior to the commencement of flowering.

5.1 INTRODUCTION

Subsoil factors including high transient salinity and boron are recognised as major limitations to crop productivity and reliability across southern Australia (Cartwright *et al.* 1984, Ralph 1991, Nuttall *et al.* 2003, Rengasamy 2006). There are limited physical solutions for improvement sub-soils constraints in dry land cropping (Adcock *et al.* 2007), and so breeding for higher tolerance based on genetic improvement is critical. Field pea is relatively more sensitive to salinity than other crops that are commonly grown in Australia, such as barley, wheat and canola (Maas and Hoffman 1977, James *et al.* 2006). Field pea production in Australia is shifting towards more arid climates with growing season rainfall less than 200 mm, in which saline sensitivity is expected to be greater (Bray, 2000). Therefore, genetic improvement is becoming an increasing priority.

A complicating factor associated with estimation of salinity tolerance is that variation can occur across ontogeny (Lunin *et al.* 1963) from germination to the seedling stage, and through to seed set (Shannon *et al.* 1994). For many crops, such as barley (Mano and Takeda 1997) and wheat (Kingsbury and Epstein 1984, Almansouri *et al.* 2001), salinity tolerance at germination and emergence has not been a useful measure of tolerance at later growth stages. However, several crops including pulse species such as field pea (Leonforte *et al.* 2013), lentil (Jana 1979) and faba bean (Hamid and Talibuddin 1976, Al-Tahir and Al-Abdussalam 1997) exhibit very high sensitivity at early growth stages (Blum 1984) that can severely limit crop
establishment. While salt toxicity causing seedling death can occur in localised lower-lying paddock locations with water logging, this scenario is not common for field pea in Australia, as the crop is predominately grown on sandy loam soils. The more significant and widespread affects of NaCl salt are less apparent, and probably caused by transient salinity (Rengasamy 2010), in which roots are exposed to high subsoil salinity at depth (Nuttal et al. 2009).

Salinity-induced crop growth symptoms in field pea are commonly seen across southern Australia, and are typically associated with lower growth node leaf necrosis on the margins and leaf chlorosis, stunted plant growth and reduced grain yields. However, the complicated patterns of expression for these symptoms in field nurseries due to interactions with other abiotic stresses as well as seasonal diseases makes direct selection difficult. High tolerance has been identified in landraces through use of pot studies, and significant variation also exists within Australian adapted germplasm (Leonforte et al. 2012). As with other crops, a range of traits have been used to quantify this tolerance, including Na\(^+\) accumulation in plant tissue, plant tissue injury and growth measures associated with shoot biomass (Leonforte et al. 2012). Specifically, rate of Na\(^+\) accumulation in the leaves at the seedling stage appears to be an important attribute of tolerant field pea genotypes (Leonforte et al. 2012). This is consistent with findings from other crops such as rice (Yeo and Flowers 1986) and wheat (Schachtman et al. 1991). Salinity-induced growth responses at the seedling stage observed in field pea (Leonforte, unpublished) appear similar to those from studies of other crops, indicating a strong correlation between rate and mode of plant symptom development (i.e. biphasic) (Munns et al. 2002, Munns 2005), with increasing accumulation of Na\(^+\) ions in plant tissue (Lauchli and Grattan 2007).

Salinity-derived effects on reproductive development may reduce the capacity of a crop to adapt to environmental conditions, and therefore need to be considered during breeding. For example, increasing salinity is associated with earlier flowering and grain maturity time in wheat, sorghum, oats and
cotton, but appears to have little effect in barley and rye (Shannon et al. 1994), while in tomato, flowering is delayed (Pasternak et al. 1979). In addition, deleterious effects of salinity on growth at later ontogenetic stages often include reduced plant biomass and higher sterility and abortion, as reported in rice (Zeng and Shannon 2000) and wheat (Maas and Grieve 1990). The present study was undertaken to assess the effect of increasing salinity when applied at later ontogeny (i.e. just prior to flowering) on growth responses of four field pea varieties that vary for salinity tolerance at the early growth stage.

5.2 MATERIALS AND METHODS

Four genotypes were sown in a replicated pot experiment to assess growth responses to varying concentrations of NaCl salt applied via watering treatments from 6 weeks post-emergence. The genotypes were selected on the basis of salinity tolerance at the seedling stage, as documented by Leonforte et al. (2012) and included the tolerant genotype ATC1836, the moderately tolerant genotype OZP0812 (previous synonym name 03H556P-04HO2012), the moderately susceptible genotype OZ0809 and the susceptible genotype Kaspa (Leonforte et al. 2010).

The experiment was conducted in a temperature controlled glasshouse at Horsham, Vic, maintained at average day-night temperatures of 23 / 16°C in 2011. Six plants of each accession were sown with equidistant spacing in 25 cm diameter pots into a sand and gravel medium to a depth of 2 cm. The gravel medium was composed from a 1:1 ratio of coarse river sand and 5 mm bluestone chips. Each pot was treated daily with rainwater from sowing until emergence. From 4 days post-emergence, seedlings were watered with a complete nutrient solution (i.e. nitrosol, NPK ratio 12.2: 2.9: 8.5), in addition to supplementation with a calcium source (i.e. calcium nitrate). The required NaCl concentration was tested using an electrical conductivity (EC) meter and was applied at an initial rate of 3 dsm⁻¹ from 6 weeks or 42 days post-emergence. The concentration of applied NaCl was increased by 3 dsm⁻¹ at each watering time to avoid sudden plant osmotic adjustment, up to final
treatment rates of either 6, 12 or 18 dsm⁻¹, and maintained at this concentration until assessment was complete. All watering with the nutrient and salt solution was undertaken over 3 day-intervals at a rate of 350 ml per pot applied directly to the growing medium surface. A null-salt application treatment (no added NaCl) was included in order to identify effects of salinity exposure. Plants were trellised onto plastic lattice to avoid splashing of salt solution during watering and to avoid shading effects and plant damage during assessment.

From 7 days post-application of salinity treatment, measurements were made of percentage of plant tissue chlorosis and main stem length (cm). The symptom assessment for chlorosis was undertaken separately for the whole plant and for leaf tissue at the 5th growing node. Main stem length was measured from the first growth node to the last growth node. Whole plants were harvested and dry matter (g / plant) was determined following oven-drying for 2 reps, every 7th day until maturity, from 7 days post-application of salinity treatments. Commencement of flowering was recorded as number of days post-germination, when all plants in each pot had one fully open flower. Number and weight of seeds per plant, number of seeds per pod and seed size were determined at maturity for each treatment.

Leaf stipule tissue was harvested from the 5th and 10th growth nodes and the growing tip of each primary stem, from one of the 6 plants in each pot every 7 days post-application of salinity treatments. Plant tissue and seed for each treatment were pooled across replications, and Na⁺ concentration was determined using inductively coupled plasma-optical emission spectrometry (ICP-OES) (Zarcinas et al. 1987) following sample digestion in nitric acid/hydrogen peroxide solution.

The pot experiment was designed as a randomised complete block experiment (number of replications: 16) with 4 salinity treatment levels by 4 genotype treatment levels described above. An ANOVA analysis was undertaken of all variables measured using Genstat 13 software. Analysed treatment data is presented in either tabular or graphic form for interpretation.
5.3 RESULTS

5.3.1 Plant tissue chlorosis

The genotype ATC1836 showed comparatively slower development of chlorosis with increasing salinity at both the whole plant level and at the fifth growing node (Fig. 5.1, 5.2). For all genotypes, chlorosis at the 5th growth node was higher and developed more rapidly in relation to the whole plant (Fig. 5.1, 5.2). There was a strong linear association ($R^2 = 0.92$, t pr. <0.001) and correlation (Table 1) between chlorosis at the 5th growth node and the whole plant.

5.3.2 Plant growth responses

All genotypes showed significant reductions in plant growth associated with lower main stem growth and lower dry matter accumulation with increasing salinity (Fig. 5.3). The semi-dwarf genotypes Kaspa and OZP0809 appear to have the most significant reduction in dry matter accumulation, particularly at the higher salinity treatment (18 dsm$^{-1}$) (Fig. 5.3, 5.4). However proportionally, the four genotypes appear to have similar growth reduction over time associated with increasing salinity. The taller genotype OZP0812 was able to produce significantly more dry matter and grow significantly taller when compared to the other 3 genotypes in response to application of salinity (Fig. 5.3, 5.4). There was a strong linear association ($R^2 = 0.66$ t pr. <0.001) and correlation (Table 5.1) between dry matter and main stem length.

5.3.3 Plant flowering time, seed and pod responses

All genotypes had a significant reduction in the number of seeds set per pod associated with higher salinity (Fig. 5.5). However the adapted (i.e. domesticated lines OZP0812, OZP0809 and Kaspa) genotypes showed a more gradual reduction when compared to the genotype ATC1836. The genotype OZP0812 had the least reduction, with no significant difference in seed set between the null, 6 and 12 dsm$^{-1}$ salinity treatments. All genotypes showed significant reductions in seed set per plant at the 18 dsm$^{-1}$ salinity
treatment. ATC1836 produced a very large number of seeds per plant, and showed the largest reduction in seed set resulting from the null treatment. However, at the highest salinity treatment ATC1836 still exhibited significantly higher seed numbers per plant.

All genotypes had a significant reduction in total plant seed weight (g) yield associated with increasing salinity (Fig. 5.6). However, the genotype OZP0812 had significantly higher plant seed yield across all plus-salinity treatments.

All genotypes had a significant reduction in seed size with increasing salinity (Fig. 5.7). However, OZP0812 maintained relatively larger seed size in the plus-salinity treatments. The genotype OZP0809 showed no difference for seed size between the 6 and 12 dsm⁻¹ salinity treatments. The non-adapted genotype ATC1836 produced very small seeds in comparison to the adapted genotypes, and there was no significant difference between the plus-salinity treatments for seed size.

There was no significant reduction in pod set per plant between the null and 6 dsm⁻¹ salinity treatments for any genotypes (Fig. 5.9). Genotypes OZP0809 and ATC1836 showed significant reductions in pod set between the 6 and 12 dsm⁻¹ salinity treatments. All genotypes showed significant reductions in pod set between either the 6 or 12 dsm⁻¹ and the 18 dsm⁻¹ salinity treatments.

The pod weight per plant varied significantly in the null salinity treatment across genotypes (Fig. 5.9). All genotypes showed significant reductions in pod weight per plant at the highest salinity treatment (18 dsm⁻¹), and varying responses at lower salinity treatments. The genotype ATC1836 pod weight per plant was significantly greater in the 6 dsm⁻¹ salinity treatment when compared to the null treatment.

There were no significant differences in the number of seeds set per pod between the null and 6 dsm⁻¹ salinity treatments for Kaspa and OZP0809, and between the null, 6 and 12 dsm⁻¹ salinity treatments for OZP0812 (Fig. 5.10).
Kaspa and OZP0809 produced higher number of seeds per pod compared to OZP0812. ATC1836 produced very small pods in comparison to the adapted genotypes, and there was no significant difference between the plus-salinity treatments for this genotype.

There were significant differences in the number of days to flowering between genotypes in the null-salinity treatment. All four genotypes showed a propensity to flower earlier in response to increasing salinity (Fig. 5.11). Relative genotype differences in flowering time remained consistent regardless of salinity treatment.

5.4.4 Na$^+$ concentration in plant and seed tissue

All four genotypes generally showed the highest concentration of Na$^+$ in plant tissue harvested from the 5$^{th}$ growth node, and the lowest Na$^+$ concentration in the growing tips (Fig. 5.13, 5.14, 5.15, 5.16). However, this relationship varied between genotypes, particularly at the highest salinity treatment (18 dsm$^{-1}$). All genotypes showed increasing concentration of Na$^+$ in plant tissue over time for all plus-salinity treatments. However, the rate of Na$^+$ accumulation varied between genotypes and between the plant nodes and growing tip. The genotype ATC1836 showed the slowest rate of accumulation of Na$^+$ in plant tissue. Specifically for the 6 and 12 dsm$^{-1}$ salinity treatments, Na$^+$ concentration at the growing tip of ATC1836 was the same as that recorded in the null-salinity treatment. A delay in Na$^+$ accumulation in the growing tip was observed compared to the growing nodes for the 6 and 12 dsm$^{-1}$ treatments for all genotypes, with the exception of OZP0812 at the 12 dsm$^{-1}$ salinity treatment. The genotype ATC1836 also showed a significant delay in Na$^+$ accumulation in the growing tip at the 18 dsm$^{-1}$ salinity treatment. Accumulation of Na$^+$ at the growing tip for the other three lines at the 18 dsm$^{-1}$ level was much more rapid, particularly for genotypes Kaspa and OZP0812. The concentration of Na$^+$ at either growth node or the growing tip was more strongly correlated with tissue chlorosis than measures of plant biomass (Table 5.1).
Sodium concentration in seed tissue for all 4 genotypes was much higher than that of the null-salinity treatment, which varied between 8 to 50 mg/Kg (Fig. 5.12). Higher seed Na\(^+\) concentration was associated with higher salinity. The genotype ATC1836 had the lowest seed Na\(^+\) concentration in seed tissue at the 18 dsm\(^{-1}\) salinity treatment.
Fig. 5.1. Percentage chlorosis at the 5th growth node for four genotypes watered with varying NaCl salinity treatments: 0(null), 6, 12, 18 dsm⁻¹. Error bar refers to l.s.d. at fprob <5%.
Fig. 5.2. Percentage chlorosis of the whole plant for four genotypes watered with varying NaCl salinity treatments: 0(null), 6, 12, 18 dsm⁻¹. Error bar refers to l.s.d. at fprob <5%.
Fig. 5.3. Plant growth as measured by the main stem length (cm) for four genotypes watered with varying NaCl salinity treatments: 0(null), 6, 12, 18 dsm$^{-1}$. Error bar refers to l.s.d. at fprob <5%.
Fig. 5.4. Plant growth as measured by dry matter (g) for four genotypes watered with varying NaCl salinity treatments: 0(null), 6, 12, 18 dsm⁻¹. Error bar refers to l.s.d. at fprob <5%.
**Fig. 5.5.** Final plant seed number for four genotypes watered with varying NaCl salinity treatments: 0(null), 6, 12, 18 dsm$^{-1}$. Error bar refers to l.s.d. at \( \text{tprob} < 5\% \).
Fig. 5.6. Final plant seed yield (g) for four genotypes watered with varying NaCl salinity treatments: 0(null), 6, 12, 18 dsm\(^{-1}\). Error bar refers to l.s.d. at fprob <5%.

![Plant seed yield (g)](image)

Fig. 5.7. Seed size (i.e. 100 seed weight in grams) for four genotypes watered with varying NaCl salinity treatments: 0(null), 6, 12, 18 dsm\(^{-1}\). Error bar refers to l.s.d. at fprob <5%.

![Seed size](image)
Fig. 5.8. Final plant pod number for four genotypes watered with varying NaCl salinity treatments: 0(null), 6, 12, 18 dsm⁻¹. Error bar refers to l.s.d. at fprob <5%.

![Pod number per plant](image)

Fig. 5.9. Final plant pod weight (g) for four genotypes watered with varying NaCl salinity treatments: 0(null), 6, 12, 18 dsm⁻¹. Error bar refers to l.s.d. at fprob <5%.

![Pod weight per plant](image)
**Fig. 5.10.** Number of seeds per pod for four genotypes watered with varying NaCl salinity treatments: 0(null), 6, 12, 18 dsm$^{-1}$. Error bar refers to l.s.d. at fprob <5%.

![Number of seeds per pod](image)

**Fig. 5.11.** ‘Days to flowering’ (post emergence) for four genotypes watered with varying NaCl salinity treatments: 0(null), 6, 12, 18 dsm$^{-1}$. Error bar refers to l.s.d. at fprob <5%.

![Days to flowering](image)
Fig. 5.12. Seed tissue Na\(^+\) concentration (mg/Kg) for four genotypes watered with varying NaCl salinity treatments: 0(null), 6, 12, 18 dsm\(^{-1}\). Error bar refers to standard deviation from mean.
**Fig. 5.13.** Plant tissue Na$^+$ concentration (mg/kg) taken at the 5th and 10th growth nodes and the growing tip for four genotypes from 7 days post application of NaCl salinity treatments: 0(null), 6, 12,18 dsm$^{-1}$ for ATC1836.
Fig. 5.14. Plant tissue Na\(^+\) concentration (mg/kg) taken at the 5\(^{th}\) and 10\(^{th}\) growth nodes and the growing tip for four genotypes from 7 days post application of NaCl salinity treatments: 0(null), 6, 12,18 dsm\(^{-1}\) for Kaspa.
**Fig. 5.15.** Plant tissue Na\(^+\) concentration (mg/kg) taken at the 5\(^{th}\) and 10\(^{th}\) growth nodes and the growing tip for four genotypes from 7 days post application of NaCl salinity treatments: 0(null), 6, 12, 18 dsm\(^{-1}\) for OZP0809.
**Fig. 5.16.** Plant tissue Na$^+$ concentration (mg/kg) taken at the 5$^{th}$ and 10$^{th}$ growth nodes and the growing tip for four genotypes from 7 days post application of NaCl salinity treatments: 0(null), 6, 12,18 dsm$^{-1}$ for OZP0812.
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5.4 DISCUSSION

Higher NaCl salinity was associated with earlier development of plant tissue chlorosis in this study. This occurred more rapidly in the lower plant when compared to the whole plant, and corresponded to higher accumulation of Na\(^+\) in lower leaf tissue compared to tissue at higher growth nodes. Accelerated tissue damage and loss of older leaves is expected due to greater cumulative transpiration and consequent Na\(^+\) ion accumulation under increasing salinity (Shannon et al. 1994). Genotypic variation for rate of Na\(^+\) accumulation and induced tissue damage is closely associated with availability of photosynthate for growth and seed set in crops (Munns et al. 2006). The more tolerant genotype ATC1836 (Leonforte et al. 2013) maintained higher tolerance with exposure to salinity at later ontogeny in this study, based on Na\(^+\) accumulation and chlorosis. For the three adapted genotypes (i.e. Kaspa, OZP809 and OZP0812) there were only minor differences in the rate of chlorosis development at the whole plant level. More rapid chlorosis development in the earlier flowering genotype OZP812 at the lower growth node may be partially due to prior translocation of sugars associated with early reproductive development (Roche et al. 1988). The accumulation of dry matter, and to some extent main stem growth, showed much lower levels of correlation with Na\(^+\) concentration in plant tissue than tissue injury based on chlorosis. This result could reflect genetic differences in internode length and branching habit rather than variation in response to salinity, and highlights the potential use of plant morphology traits to reduce sensitivity of the crop. In particular, the results suggest that a semi-dwarf type genotype like Kaspa or OZP0809 could undergo serious stunting if exposed to salinity just prior to flowering. This is crucial, as biomass accumulation prior to flowering appears to be critical for grain yield, particularly in climates with shorter growing seasons (Sadras et al. 2012). However, in Australia excess biomass produced with tall growing field pea germplasm can often result in reduced yield potential due to low harvest index and poor crop light interception, and poor grain quality and high yield loss due to harvest difficulties (Armstrong et al. 1994).
As with other crops such as wheat, reproductive development in terms of flowering time appears to be accelerated by salinity stress in field pea. This effect may have important implications for increasing sensitivity to frost risk (Shafiq et al. 2012) or ascochyta blight disease (Timmerman et al. 2004, Boros and Marcinkowska 2010), which are major constraints to field pea production in Australia. Strong salinity-induced inhibition was observed for all measured seed yield components, including seeds per pod, plant yield, seed size and pod number. The genotype ATC1836 produces a comparatively larger number of small pods per plant and very small seeds, yield loss in response to increasing salinity being mostly attributable to loss of pod number. The genotype OZP0812 produced the highest seed yield per plant with increasing salinity exposure, and appeared to remain more fertile in terms of seed and pod number set. OZP0812 also maintained larger seed size. Differences in pod weight between the genotype Kaspa and other genotypes are probably attributable to the reduced pod parchment layer in the pod wall that is characteristic of Kaspa (McGee and Baggett 1992), rather than pod yield. The genotype ATC1836 produced the same number of pods for the 6 dsm⁻¹ and the 0 (null) salinity treatment, but the overall pod weight per plant was greater for the 0 (null) treatment. A possible explanation for this effect may be an increase in partitioning of assimilate (Jeuffroy and Warembourg 1991) to the pod wall, due to increased seed abortion at the 6 dsm⁻¹ salinity treatment.

Seed tissue Na⁺ concentration increased dramatically with increased salinity, which can have important implications for seed quality and seed production in more saline environments, as seen in lentil (Ghassemi-Golezani et al. 2012). This possibility requires further investigation for field pea. The present study shows that there is likely to be significant variation for effects of salinity stress on seed size. This is an important consideration, as seed size affects the end-use marketability of grain for human consumption (Redden 2005) and seed quality for sowing in field pea (Singh et al. 2009).
Tolerance to salinity following salt exposure at a juvenile plant stage appears to be retained when it occurs at later ontogeny, in terms of biomass reduction, tissue symptoms and timing and rate of Na\(^+\) accumulation. However, this study suggests that the final effects on seed yield reduction will also be strongly influenced by salinity affects and interactions with phenology and fertility (i.e. seed and pod abortion). Specific plant morphology traits such as internode length, branching, pod structure and seed size may provide some degree of avoidance, or delay effects to increasing salinity and should also be investigated further for consideration in variety development. In particular, this study shows that genetic studies and breeding need to target pyramiding not only of genes for salinity tolerance, but traits that optimise grain yield responses under salinity stress. The genotype OZP0812, for example, has been used in breeding to develop a new dual-type variety (i.e. for forage or grain production) for low rainfall regions (PBA Coogee) that combines high tolerance to both high boron and salinity with powdery mildew resistance.
Chapter Six
Breeding field pea with higher salinity and boron tolerance for Australia.

6.1 INTRODUCTION

Field pea in Australia is sensitive to a number of abiotic stress factors, particularly water limiting stress associated with low and variable rainfall, and sensitivity to both high soil salinity and alkaline-induced boron toxicity (Rengasamy 2006). The crop is most vulnerable to yield loss when vegetative growth is stunted prior to flowering (Sadras et al. 2012), or from stress-induced abortion and damage of flowers, pods and seed (Roche et al. 1998). A low cost approach to genetic improvement of abiotic stress tolerance in Australia has involved a strategy of screening for yield in higher risk environments (Arus et al. 2008). This approach has been critical for delivery of more broadly adapted 'Kaspa'-type germplasm for Australia, which is characterised by a semi-dwarf, semi-leafless, erect growing, pod shatter-resistant plant type and production of spherical grain with a uniformly tan-coloured seed coat (Siddique et al. 2013). Specifically, selection for earlier-flowering Kaspa-type germplasm has led to release of recent varieties, such as PBA Gunyah and PBA Twilight, that exhibit higher stability of yield in lower rainfall regions.

Water use efficiency is the main limitation to crop production in Australia (Richards et al. 1993), and the major genetic factors contributing to higher drought tolerance for field pea appear to relate to phenology and biomass accumulation prior to flowering (Sadras et al. 2012). However, sensitivity to soil boron toxicity and salinity are likely to limit yield potential of the crop in Australia in low rainfall climates (Cartwright et al. 1984, Ralph 1991, Nuttall et al. 2003).
**Tolerance to boron**
Sources of boron tolerance were identified over 20 years ago in field pea (Bagheri *et al.* 1994), but have only recently been incorporated into newly released adapted varieties (Leonforte *et al.* 2009). Genetic control of boron tolerance is highly heritable, possibly involving only two major genes (Bagheri *et al.* 1994). Incorporation of high boron tolerance has been accelerated in the Australian breeding program since 2000 through use of glasshouse-based seedling screening experiments (Leonforte *et al.* 2009). Previous research conducted with field pea (Paull *et al.* 1992) has shown that boron tolerant accessions accumulate less boron in plant tissue when grown in soils with high boron concentration. This could be associated with mechanisms that reduce or exclude uptake at the roots as with tolerant barley (Hayes and Reid 2004) or reduce translocation from roots to the shoot as found with tolerant *Brassica rapa* (Kaur *et al.* 2006). The benefit of boron tolerance has been difficult to validate on the basis of yield performance and for wheat in Australia, but it could be as high as 11% (Moody *et al.* 1991). An understanding of yield and reliability of yield benefits that higher boron tolerance provides to field pea across regional Australia is needed to better target variety development.

**Tolerance to NaCl salinity**
Sources of high tolerance to, or escape mechanisms from salinity have only recently been identified (Leonforte *et al.* 2013). Selection for higher salinity tolerance using pot-based seedling screening methods (Leonforte *et al.* 2009) was used in the breeding program from 2002, but mostly utilised variation available in adapted germplasm. Inheritance of salinity tolerance in pea is likely to be quantitatively controlled, given the complexity of this trait (Flowers 2004), and requires further investigation. Progeny testing of germplasm derived from parents varying for a specific trait of interest is a useful approach for investigation of genetic control and complexity of inheritance (Sprague 1967). In the present study, $F_2$ progeny from 3 pair-wise crosses between divergent parents were screened for salinity tolerance. A recombinant inbred line (RIL) population was also screened. Assessment of the shift in
germplasm trait frequencies can identify the rate of genetic gain via selection (Condon et al. 2009). In the present study, the shift towards higher tolerance for salinity tolerance of advanced germplasm in the Pulse Breeding Australia (PBA) field pea breeding program is described.

**Complexity of interaction between boron and salinity tolerance for grain yield**

There is likely to be significant potential for increased crop yield based on pyramiding of genes for higher boron and salinity stress tolerance with those for other desirable genetic variation, in order to drive regional adaptation in Australia (Siddique et al. 2013). However, direct field-based selection for tolerance to sub-soil constraints such as high boron and salinity is difficult, as highly complex interactions often occur simultaneously with sub-soil induced stress (Shannon et al. 1994). Salinity and boron stress can display transient spatial and temporal variation (Rengasamy 2006) and genotypic responses may also be non-uniform across growth stages (Lunin et al. 1963). Adding to this complexity, reproductive development, flower, pod and seed set and seed size, plant traits relating to growth and architecture (Armstrong et al. 1994) and root biomass (McPhee 2005) may also exhibit plasticity in response to stress.

Providing sufficient genetic variation for abiotic stress tolerance is present in advanced stages of yield testing, multivariate statistical analysis can be a useful tool to clarify genotype x environment (G x E) interactions for yield response, to quantify genetic gain and to identify key parental germplasm for crossing (Jaradat 2006). In the present study multivariate analysis was used to enhance understanding of relationships between boron and salinity genotype tolerance based on results from multi-location (14 sites) yield testing in 2011 across southern Australia.
6.2 MATERIALS AND METHODS

6.2.1 Progeny testing for salinity tolerance

The salinity sensitive cultivar Kaspa (Leonforte et al. 2013) was used in reciprocal pair-wise crosses with three lines that vary in sensitivity to soil salinity: ATC1836 (tolerant), Parafiel (moderately tolerant) and Yarrum (moderately sensitive to moderately tolerant) (Leonforte et al. 2013). F₂ progeny from the three crosses were sown in three large randomised block experiments to evaluate variation for salinity tolerance (2010 experiment). RILs derived from the F₂ generation at the F₈ (eighth filial) generation for Kaspa x Parafiel were sown in a randomised block experiment to evaluate variation for salinity tolerance (2012 experiment).

The experiments were conducted over the spring (2010) or autumn (2012) seasons in a semi-controlled environment (i.e. large plastic igloo) at Horsham, Victoria. Six seeds of each derived line were sown with equidistant spacing in 13 cm diameter pots into a sand and gravel medium to a depth of 2 cm. The gravel medium was composed from a 1:1 ratio of coarse river sand and 5 mm bluestone chips. Each pot was treated daily with rainwater from sowing until emergence. From 6 days post-emergence, seedlings were watered with a complete nutrient solution (i.e. nitrosol, NPK ratio 12.2: 2.9: 8.5), in addition to supplementation with a calcium source (i.e. calcium nitrate). The average day-night temperatures were maintained at 16-23°C. The required NaCl concentration was tested using an EC meter and was applied at an initial rate of 3 dsm⁻¹ from day 9 post emergence. The concentration of applied NaCl was increased by 3 dsm⁻¹ at each watering time to avoid abrupt osmotic shock, up to a final rate of 18 dsm⁻¹, and maintained at this concentration until assessment (salinity treatments). All watering with the nutrient and salt solution was undertaken over 3 day-intervals at a rate of 200 ml per pot applied directly to the growing medium surface. A null-salt application treatment (no added NaCl) was included for control lines (parents) and randomized in the experiment in order to verify that effects of salinity exposure are not caused by other stress factors. Tall growing plants were
trellised onto plastic lattice to avoid splashing of salt solution during watering, and shading effects and plant damage during assessment.

For the F\textsubscript{2} population screening experiments in 2010, individual plants in each pot were assessed for symptom development quantified on a salinity symptom score from 1 (no symptoms) to 10 (severe symptoms) (Leonforte \textit{et al.} 2013) and plant percentage necrosis. Assessment for percentage plant necrosis was undertaken at 28 and 35 days post-emergence (designated percentage necrosis 1 and 2). Assessment for symptom score was undertaken at 28, 35 and 42 days post emergence (designated symptom score 1, 2 and 3). Genotype pot averages for percentage necrosis and plant symptom score were calculated from individual plant assessments, and used to estimate genotype average values using REML spatial row-column analysis. Principal component analysis was undertaken with genotype mean values in order to find orthogonal linear combinations of variates (i.e. symptom score and percentage necrosis) using the correlation matrix method described in Genstat 13. Frequency distribution histograms were developed from F\textsubscript{2} genotypes for symptoms scores and percentage necrosis for each population. Broad sense heritabilities for symptom score and percentage necrosis were calculated on the basis of variance components (i.e. genotype vs phenotype). Genetic correlations between symptoms score and percentage necrosis assessments were determined for each population.

Segregation for the leaf \textit{afila} trait controlled by a single major gene (af = no leaflets, Af af or Af = leaflets present) was determined for F\textsubscript{2} progeny of both Kaspa x Parafield and Kaspa x ATC1836.

For the Kaspa x Parafield F\textsubscript{2}-derived RIL population experiment in 2012, individual plants in each pot were assessed for symptom development as described above at 28, 35, 42, 49, 63, 70, 77, 85 days post-emergence designated RIL symptom score 1 to 8). Genotype pot averages for plant symptom score were calculated from individual plant assessments and used to estimate genotype average values for symptom score using REML spatial
row-column analysis. A graph of symptom score by assessment time was constructed for both progeny lines that vary for salinity tolerance and parent genotypes. A salinity tolerance rating was determined using a salinity index value, calculated by summation of weighted symptom scores for each genotype according to assessment time. The weighting of specific symptom scores was based on degree of variance. Salinity screening experiments for \( F_2 \) and RIL progeny were used to undertake a canonical variates analysis using symptom score at varying assessment times as vectors (Genstat 13). Salinity tolerance rating from the RIL screening experiment was used as a factor to specify grouping of genotypes for this analysis.

6.2.2 Estimating breeding progress for NaCl salinity tolerance in Australia

Advanced breeding lines in the PBA field pea breeding program have been screened annually for salinity tolerance each spring from 2005 to 2011. Salinity screening was undertaken using the pot method as described above, using a final NaCl concentration of 18 dsm\(^{-1}\). The degree of seedling salinity tolerance within semi-dwarf and semi-leafless plant type germplasm in 2011 was determined following 14 days of salinity treatment. Normal distribution curves fitted for symptom scores of advanced breeding lines from 2005 to 2011 were interpreted to indicate genetic gain in breeding for tolerance. Average symptom scores for key parental germplasm and cultivars were indicated on this graph as a reference points for discussion.

6.2.3 Comparative G x E analysis for grain yield of advanced genotypes varying in salinity and boron tolerance

Data from screening of seedlings for salinity and boron toxicity tolerance in 2011, for 132 advanced generation lines (including commercial cultivars), was used to undertake a canonical multivariate analysis based on yield response vectors for 14 field nurseries conducted across southern Australian in 2011. Boron sensitivity screening was based on a glasshouse seedling screening methodology described by Leonforte \( et \, al. \) (2009) in which seed of genotypes
were sown as rows of 5 plants in non draining plastic tubs (25cm x 25cm) (4 rows per tub) that had been filled with loam soil spiked with boric acid (Boron concentration: 10 ppm). These screening experiments were set out as randomised complete block designs. Low boron concentration boxes and known tolerant and sensitive genotypes were used as control factor. Toxicity symptoms were used as basis for rating tolerance. The basis for canonical analysis was using variation for tolerance to high boron toxicity and salinity as factors to specify grouping of genotypes. The 14 field nurseries were designed as replicated alpha lattice randomised designs in Agrobase generation II. All experiments contained the same genotype entry lists, and seed was all sourced from breeding nurseries located at Horsham, Victoria, in 2010. The nurseries were experimentally managed to optimise grain yield according to best practice by regional farmers for sowing rate and time, herbicide and pesticide application and harvest time. Yield data was analysed using REML spatial row-column analysis in Genstat12 to estimate genotype site specific yields. Selection of the 14 experiments for canonical analysis was on the basis of low experimental error for grain yield (i.e. coefficient of variation < 12%). Growing season rainfall was estimated for each site based on rainfall (mm) from time of sowing to estimated grain maturity. A 10% proportion of total summer – autumn rainfall prior to sowing was included in the growing season rainfall estimate for each site. Genotype-specific variation for internode length, leaf type, flowering time and disease resistance to powdery mildew, downy mildew bacterial blight, PSbMV virus and BLRV virus, as described by the Pulse Breeding Australia Field pea breeding program, were used to identify any statistical correlations with genotypic variation for boron or salinity tolerance.

6.3 RESULTS

6.3.1 Progeny testing results for salinity tolerance

F₂ progeny lines derived from crosses between the sensitive cultivar Kaspa and genotypes with higher salinity tolerance, such as ATC1836, Parafield and revealed a broad distribution of values for symptom score and percentage
necrosis. Based on principal component biplots (Fig. 6.1, 6.2, 6.3) the three populations showed a similar relationship between measured variates and variance distribution of genotypes, such that percentage necrosis or symptom score were highly correlated with each other according to timing of assessment. Variation for symptom score assessed at day 42 was more informative in terms of relative distribution of genotypes. For all three populations, higher sensitivity of lines was closely associated with increased value of PC-1 scores (graphed as X values), which accounted for most of the variation. The PC-2 (graphed as Y values) appeared to account for variation associated with timing of assessment. The parental genotypes showed comparatively similar variation for tolerance on biplot charts and means when graphically displayed (Fig. 6.6 and 6.7) across the three experiments. Specifically, the results confirm the higher relative sensitivity of the parent genotype Kaspa when compared to Parafied, Yarrum and ATC1836. F₂ progeny lines in Kaspa x Parafied and Kaspa x Yarrum populations with significantly higher tolerance to either the Yarrum or Parafied parental genotypes were identified based on plant symptoms (Fig 6.1, 6.2, 6.3 and Table 6.1). Variation associated with timing of assessment indicated that variation for percentage necrosis and symptoms were normally distributed (Fig. 6.4.1, 6.4.2, 6.4.3, 6.5.1, 6.5.2, 6.5.3). The first assessments for symptom score and percentage necrosis were, however, more skewed towards lower values. Proportionally, the F₂ progeny population derived from Kaspa x ATC1836 demonstrated the slowest average increase in salinity symptoms, and included a higher proportion of genotypes with high tolerance. Broad sense heritability (h²B.S) values for measured variates were generally above 0.5. However, percentage necrosis at day 35 exhibited a comparatively low h²B.S value. Genetic correlations between percentage necrosis and symptom score across times of assessment were high for all three populations (Table 6.2). Segregation for presence or absence of the *afila* trait (characterised by absence of leaflets on tendrils) showed a 3:1 ratio, as expected, in both the Kaspa x Parafied and Kaspa x ATC1836 F₂ progeny.
Kaspa x Parafield RILS varied significantly for tolerance based on symptom score (Fig. 6.9). Highly tolerant and sensitive lines identified as both $F_2$ genotypes and RILs were relatively consistent in terms of phenotype (Fig. 6.9). RIL progeny genotypes rated as salinity tolerant expressed a much slower rate of symptom development as compared to sensitive progeny (Fig. 6.8).

6.3.2 Estimated breeding progress for NaCl salinity tolerance in Australia

The frequency distribution of breeding lines was shifted towards higher salinity tolerance, as based on symptom score, during the period from 2005 to 2011 (Fig. 6.11). In 2005, the mean symptom score of advanced lines in PBA’s field pea breeding program following 14 days of salt water application at a concentration of 18 dsm$^{-1}$ was 8, similar to that of variety Kaspa. By 2011, this value had shifted to a mean symptom score of around 5.5, located between the estimated mean symptoms score values for cultivars PBA Wharton and Yarrum. Significant visual differences for salinity induced toxicity symptoms / tolerance for advanced semi-dwarf and semi-leafless type germplasm in the PBA breeding program was demonstrated graphically (Fig. 6.10) following 14 days of screening treatment with a saltwater solution of 18 dsm$^{-1}$ in 2011.

6.3.3 Comparative G x E analysis for grain yield of advanced genotypes varying in salinity and boron tolerance

Advanced lines with higher, or even varying, tolerance to high boron concentrations exhibited comparatively higher yield performance as a group across 7 sites in 2011 (i.e. Hopetoun, Victoria (VIC), Beulah VIC, Minnipa, South Australia (SA), Kingsford SA, Snowtown SA, and to a lesser extent Balaklava SA). Advanced lines rated as sensitive to both boron and salinity in general yielded comparatively better at higher rainfall locations (Fig 6.13) with the exception of Kingsford, SA (Fig 6.12). A weaker relationship between higher salinity tolerance and site yield performance was apparent. Higher
salinity tolerance was associated with better yield performance at three sites (Dallwallinu, Western Australia (WA), Scadden, WA and Yenda, New South Wales (NSW). However, boron- and/or salt-sensitive genotypes performed comparatively well at the Dallwallinu site. At the Scadden and Yenda sites, very few boron and/or salt-sensitive lines were among the highest yielding genotypes, and these were earlier maturing types (i.e. PBA Twilight). Lines exhibiting both boron and salinity tolerance were mostly clustered within the same group as boron tolerant lines. As a group, they appeared to have performed better at Snowtown SA, but only comprised 5 lines.

Based on the location of genotypes in the canonical biplot (Fig. 6.12, Table 6.4) the only traits that are likely to have significantly confounded or contributed additively to performance of boron and/or salinity tolerant genotypes appear to be resistance to powdery mildew and flowering time. For the 55 genotypes that could be compared, powdery mildew resistance and boron tolerance appear to be positively associated (Table 6.4). However powdery mildew resistance was only present at a significant level at Kingsford, SA. The boron and salt sensitive genotypes 119 and PBA Pearl showed much higher adaptation in those locations for which boron tolerance appeared to be more important, and are hence likely to be valuable parents.

Growing season rainfall was a significant limitation to mean site-specific grain yield (Fig. 6.13). Six of 7 sites at which boron tolerance appeared to be important were generally lower yielding and displayed lower rainfall during the growing season. The exception was the Kingsford site. No clear relationship was apparent between the 3 sites at which salinity tolerance appeared to be important in 2011. Dallwallinu and Scadden are both located in WA, and have shallow sandy loam soil types, but are contrasted in terms of growing season rainfall and final grain yield.
Fig. 6.1. Principal component biplot showing phenotypic variation of $F_2$ progeny genotypes derived from Kaspa x ATC1836, for successive measures of salinity plant symptoms (i.e. symptoms score 1, 2, 3 (1-10) and % necrosis (1, 2) in pot screening using a NaCl watering concentration of 18 dsm$^{-1}$. 

PCP Biplot (94%)

PC-1 (81.2%)
Fig. 6.2. Principal component biplot showing phenotypic variation of $F_2$ progeny genotypes derived from Kaspa x Parafield, for successive measures of salinity plant symptoms (i.e. symptoms score 1, 2, 3 (1-10) and % necrosis 1, 2) in pot screening using a NaCl watering concentration of 18 dsm$^{-1}$. 
Fig. 6.3. Principal component biplot showing phenotypic variation of $F_2$ progeny genotypes derived from Kaspa x Yarrum, for successive measures of salinity plant symptoms (i.e. symptoms score 1, 2, 3 (1-10) and % necrosis 1, 2) in pot screening using a NaCl watering concentration of 18 dsm$^{-1}$. 
Fig. 6.4.1. Progeny genotype frequencies for successive measures of % plant necrosis (Necrosis 1, 2) of F₂ progeny genotypes derived from the cross Kaspa x ATC1836 in pot screening using a NaCl watering concentration of 18 ds m⁻¹.

![Graph showing percentage necrosis vs frequency of progeny for Kaspa x ATC1836.](image-url)
Fig. 6.4.2. Progeny genotype frequencies for successive measures of % plant necrosis (Necrosis 1, 2) of F$_2$ progeny genotypes derived from the cross Kaspa x Parafield in pot screening using a NaCl watering concentration of 18 dsm$^{-1}$. 

![Kaspa x Parafield Graph](image-url)
Fig. 6.4.3. Progeny genotype frequencies for successive measures of % plant necrosis (Necrosis 1, 2) of F$_2$ progeny genotypes derived from the cross Kaspa x Yarrum in pot screening using a NaCl watering concentration of 18 dsm$^{-1}$. 

![Graph showing the percentage of necrosis and the frequency of progeny genotypes for Kaspa x Yarrum.](image-url)
Fig. 6.5.1 Progeny genotype frequencies for successive measures of plant symptom score (1-10) (Score 1,2,3) of $F_2$ progeny genotypes derived from the cross Kaspa x ATC1836 in pot screening using a NaCl watering concentration of 18 dsm$^{-1}$.
Fig. 6.5.2. Progeny genotype frequencies for successive measures of plant symptom score (1-10) (Score 1,2,3) of F$_2$ progeny genotypes derived from the cross Kaspa x Parafield in pot screening using a NaCl watering concentration of 18 dsm$^{-1}$. 

Kaspa x Parafield

<table>
<thead>
<tr>
<th>Plant symptom score (1-10)</th>
<th>Frequency of progeny (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>Score 1</td>
</tr>
<tr>
<td>2-3</td>
<td>Score 2</td>
</tr>
<tr>
<td>3-4</td>
<td>Score 3</td>
</tr>
<tr>
<td>4-5</td>
<td></td>
</tr>
<tr>
<td>5-6</td>
<td></td>
</tr>
<tr>
<td>6-7</td>
<td></td>
</tr>
<tr>
<td>7-8</td>
<td></td>
</tr>
<tr>
<td>8-9</td>
<td></td>
</tr>
<tr>
<td>9-10</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 6.5.3. Progeny genotype frequencies for successive measures of plant symptom score (1-10) (Score 1,2,3) of $F_2$ progeny genotypes derived from the cross Kaspa x Yarrum in pot screening using a NaCl watering concentration of 18 dsm$^{-1}$. 
Fig. 6.6. Percentage necrosis for parent genotypes (Kaspa, Parafield, Yarrum, ATC1836) at 14 (Necrosis 1) and 21 (Necrosis 2) days post application of salinity treatment (18 dsm$^{-1}$) for three progeny experiments (Exp 1: Kaspa x ATC1836, Exp 2: Kaspa x Parafield, Exp 3: Kaspa x Yarrum). Error bars refer to l.s.d. (frob <0.05).
Fig. 6.7. Plant symptom score (1-10) for parent genotypes (Kaspa, Parafield, Yarrum, ATC1836) at 14 (Score 1), 21 (Score 2) and 28 (Score 3) days post application of salinity treatment (18 dsm\textsuperscript{-1}) for three progeny experiments (Exp 1: Kaspa x ATC1836, Exp 2: Kaspa x Parafield, Exp 3: Kaspa x Yarrum). Error bars refer to l.s.d. (frob <0.05).
Table 6.1. Mean, range and broad sense heritability’s ($h_{B.S}^2$) values of successive salinity symptom measures (% plant necrosis (% Necrosis 1, 2) and symptom score (Symptom score 1, 2, 3)) for parents and F2 progeny genotypes (Kaspa x ATC1836, Kaspa x Parafield and Kaspa x Yarrum crosses) in salinity screening (18 dsm$^{-1}$).

<table>
<thead>
<tr>
<th>Variates</th>
<th>h$_{B.S}^2$</th>
<th>Tolerant Parent</th>
<th>Sensitive Parent</th>
<th>Progeny No. of lines</th>
<th>Progeny Range</th>
<th>Progeny Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Necrosis 1</td>
<td>0.60</td>
<td>5</td>
<td>56</td>
<td>106</td>
<td>0 - 73</td>
<td>22.1</td>
</tr>
<tr>
<td>% Necrosis 2</td>
<td>0.24</td>
<td>11</td>
<td>69</td>
<td></td>
<td>0 - 86</td>
<td>32.2</td>
</tr>
<tr>
<td>Symptom score 1</td>
<td>0.44</td>
<td>2.0</td>
<td>6.5</td>
<td></td>
<td>1.1 - 8.1</td>
<td>4.0</td>
</tr>
<tr>
<td>Symptom score 2</td>
<td>0.69</td>
<td>3.1</td>
<td>7.3</td>
<td></td>
<td>1.5 - 4.9</td>
<td>4.9</td>
</tr>
<tr>
<td>Symptom score 3</td>
<td>0.57</td>
<td>4.7</td>
<td>8.9</td>
<td></td>
<td>1.7 - 9.4</td>
<td>6.7</td>
</tr>
<tr>
<td>% Necrosis 1</td>
<td>0.75</td>
<td>10</td>
<td>71</td>
<td>449</td>
<td>0 - 83</td>
<td>19.6</td>
</tr>
<tr>
<td>% Necrosis 2</td>
<td>0.54</td>
<td>33</td>
<td>73</td>
<td></td>
<td>0 - 87</td>
<td>34.9</td>
</tr>
<tr>
<td>Symptom score 1</td>
<td>0.88</td>
<td>3.2</td>
<td>7.3</td>
<td></td>
<td>1.0 - 8.3</td>
<td>3.9</td>
</tr>
<tr>
<td>Symptom score 2</td>
<td>0.47</td>
<td>4.7</td>
<td>7.5</td>
<td></td>
<td>1.0 - 8.7</td>
<td>5.1</td>
</tr>
<tr>
<td>Symptom score 3</td>
<td>0.52</td>
<td>6</td>
<td>8.8</td>
<td></td>
<td>1.7 - 8.7</td>
<td>6.5</td>
</tr>
<tr>
<td>% Necrosis 1</td>
<td>0.99</td>
<td>14</td>
<td>73</td>
<td>329</td>
<td>0 - 88</td>
<td>12.8</td>
</tr>
<tr>
<td>% Necrosis 2</td>
<td>0.53</td>
<td>34</td>
<td>83</td>
<td></td>
<td>0 - 93</td>
<td>27.3</td>
</tr>
<tr>
<td>Symptom score 1</td>
<td>0.94</td>
<td>3.8</td>
<td>7.8</td>
<td></td>
<td>1.2 - 8.7</td>
<td>3.6</td>
</tr>
<tr>
<td>Symptom score 2</td>
<td>0.47</td>
<td>5.3</td>
<td>8.4</td>
<td></td>
<td>1.7 - 9.3</td>
<td>4.7</td>
</tr>
<tr>
<td>Symptom score 3</td>
<td>0.84</td>
<td>7.2</td>
<td>9.6</td>
<td></td>
<td>2.5 - 10</td>
<td>6.4</td>
</tr>
</tbody>
</table>
Table 6.2. Genetic correlations between measures of successive salinity symptom measures (% plant necrosis (% Necrosis 1, 2) and symptom score (Symptom score 1, 2, 3)) within 3 F$_2$ progeny salinity screening (18 dsm$^{-1}$) experiments (Kaspa x ATC1836, Kaspa x Parafield and Kaspa x Yarrum).

<table>
<thead>
<tr>
<th>Population</th>
<th>Variate</th>
<th>% Necrosis 1</th>
<th>% Necrosis 2</th>
<th>Symptom score 1</th>
<th>Symptom score 2</th>
<th>Symptom score 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaspa x ATC1836</td>
<td>% Necrosis 1</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>% Necrosis 2</td>
<td>0.67</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Symptom score 1</td>
<td>0.92</td>
<td>0.69</td>
<td>1.00</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Symptom score 2</td>
<td>0.66</td>
<td>0.97</td>
<td>0.72</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Symptom score 3</td>
<td>0.74</td>
<td>0.82</td>
<td>0.85</td>
<td>0.87</td>
<td>1.00</td>
</tr>
<tr>
<td>Kaspa x Parafield</td>
<td>% Necrosis 1</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>% Necrosis 2</td>
<td>0.59</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Symptom score 1</td>
<td>0.95</td>
<td>0.65</td>
<td>1.00</td>
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<tr>
<td></td>
<td>Symptom score 2</td>
<td>0.60</td>
<td>0.96</td>
<td>0.67</td>
<td>1.00</td>
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</tr>
<tr>
<td></td>
<td>Symptom score 3</td>
<td>0.68</td>
<td>0.73</td>
<td>0.81</td>
<td>0.79</td>
<td>1.00</td>
</tr>
<tr>
<td>Kaspa x Yarrum</td>
<td>% Necrosis 1</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>% Necrosis 2</td>
<td>0.67</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Symptom score 1</td>
<td>0.92</td>
<td>0.69</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Symptom score 2</td>
<td>0.66</td>
<td>0.97</td>
<td>0.72</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Symptom score 3</td>
<td>0.74</td>
<td>0.82</td>
<td>0.85</td>
<td>0.87</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Table 6.3. Segregation for afila trait (af recessive gene = leaflets absent, Af dominant gene = leaflets present) in the F₂ progeny populations derived from Kaspa x Parafield and Kaspa x ATC1836. Chi squared test (P<5%) confirms 3:1 segregation for expected single gene affect.

<table>
<thead>
<tr>
<th>Population</th>
<th>Trait variation</th>
<th>Gene segregation</th>
<th>Observed</th>
<th>Expected</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaspa x Parafield</td>
<td>Non afila : Afila</td>
<td>(Af  Af/af) : (af)</td>
<td>2128 : 720</td>
<td>3 : 1</td>
<td>0.12*</td>
</tr>
<tr>
<td>Kaspa x ATC1836</td>
<td>Non afila : Afila</td>
<td>(Af  Af/af) : (af)</td>
<td>319 : 116</td>
<td>3 : 1</td>
<td>0.60*</td>
</tr>
</tbody>
</table>

* P < 5%: not statistically significant deviations from expected ratio (1:3).
Fig. 6.8. Line graph showing specific differential responses of more sensitive and tolerant Kaspa x Parafield RIL progenies and parents (Kaspa, Parafield) in terms of symptom score (1-10) measured over 8 weeks in pot screening (18 dsm\(^{-1}\)). Qualitative ratings for tolerance were based on a calculated salinity tolerance index value. Average l.s.d (fprob <5%) indicated by vertical error bar.
**Fig. 6.9.** A canonical variates analysis biplot showing genotype variation for successive measures of salinity symptom score for F$_2$ (F2 score 1, 2, 3) and derived RIL progeny (RIL Score 1, 2, 3, 4) lines of the Kaspa x Parafield population in pot screening (18 ds$m^{-1}$). Qualitative ratings for salinity were used as factors to specify grouping of genotypes and are based on a salinity index value.
Fig. 6.10. Visual variation in NaCl salt induced plant symptom damage (symptoms score (1-10) and ratings T = tolerant, MT = moderate tolerance, MS = moderate sensitivity, S = sensitive) for advanced afila and semi-dwarf germplasm in the Pulse Breeding Australia field pea breeding program in pot screening following 14 days of treatment (watering with salt solution (18 dsm\(^{-1}\)) commencing at early seedling stage.
**Fig. 6.11.** Shift in normalised frequency distributions of advanced breeding lines from 2005 to 2011 for salinity symptom scores (1-10) assessed at seedling stage in pot screening (18 dsm$^{-1}$). Mean symptom score for conventional and semi-dwarf cultivars and key germplasm are indicated as reference points.
Fig. 6.12. A canonical variates analysis biplot based on yield vectors for 14 field nurseries conducted across southern Australian in 2011 using tolerance to boron and NaCl induced salinity as factors to specify grouping of genotypes.

Group A: MT-Tolerant to high boron
Group A: Segregating for high boron tolerance
Group D: MT-Tolerant to high boron and salinity
Group C: MT-Tolerant to high salinity and segregating for high boron tolerance
Group C: MT-Tolerant to high salinity
Group B: Sensitive to high boron and salinity

Site vector id: 1 = Hopetoun, Vic, 2 = Ardlethan, NSW, 3 = Beulah, Vic, 4 = Minnipa, SA, 5 = Kingsford, SA, 6 = Snowtown, SA, 7 = Yenda, NSW, 8 = Scaddlen, WA, 9 = Dallwallinu, WA, 10 = Balaklava, 11 = Horsham, 12 = Wagga, 13 = Merredin, 14 = Williamulka.

Genotype id: Co = PBA Coogee, Wa = PBA Wharton, Gu = PBA Gunyah, Pa = Parafield, Pe = PBA Percy, St = Sturt, Ya = Yarrum, Ka = Kaspa, Ou = PBA Oura, Pl = PBA Pearl, Tw = PBA Twilight.
Fig. 6.13. Linear regression of site growing season rainfall versus, final grain yield (t/ha) in 2011.
Table 6.4. Frequency of genotypes varying in tolerance to high boron and resistance to powdery mildew in advanced testing in 2011. Based on genotypes for which there is sufficient screening data for comparison only (55). Correlation coefficient between Boron rating and powdery mildew resistance adjusted for ties: 0.743. Spearman rank coefficient significant, t probability < 1%.

<table>
<thead>
<tr>
<th>Boron tolerance rating (value and term)</th>
<th>Powdery mildew rating (value and term)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3: Resistant</td>
</tr>
<tr>
<td>5: Tolerant</td>
<td>100%</td>
</tr>
<tr>
<td>4: Moderately tolerant</td>
<td>70%</td>
</tr>
<tr>
<td>3: Segregating</td>
<td>57%</td>
</tr>
<tr>
<td>2: Moderately sensitive</td>
<td>12%</td>
</tr>
<tr>
<td>1: Sensitive</td>
<td></td>
</tr>
</tbody>
</table>
6.4 DISCUSSION

Phenotypic variation for salinity symptom responses was normally distributed in the progeny from each cross, implying that higher tolerance is likely to be quantitatively inherited (i.e. controlled by multiple genes). This result is expected given currently known mechanisms of Na\(^+\) induced plant toxicity and growth inhibition (Munns 2002). Crosses with the more tolerant Greek landrace parent ATC1836 produced a higher proportion of tolerant progeny, suggesting the presence of a higher proportion of positive alleles at contributory gene loci for tolerance, or more alleles with pronounced dominance effects.

Progeny showing significantly higher tolerance than the parents were identified from the Kaspa x Yarrum and Kaspa x Parafied crosses, indicating the presence of transgressive segregation. This phenomenon may be due to recombination between loci with alleles of additive effect, epistatic effects between alleles, or over-dominance caused by heterozygosity at specific loci (Rieseberg et al. 1999). Consistency of performance between Kaspa x Parafied F\(_2\) progeny and derived RILs, suggests that over-dominance mechanisms are not likely to provide an explanation.

The results from progeny screening demonstrate the need to assess plant salinity symptom variation over time, as both the timing and rate of symptom development indicate variation in degree of tolerance (Leonforte et al. 2013). Specifically, progeny testing RILs confirmed that genotypes with higher tolerance have a significantly slower rate of symptom development, probably associated with slower Na\(^+\) accumulation in leaf tissue (Leonforte unpublished). Assessment of salinity symptoms on the basis of a numerical score (Leonforte et al. 2010, 2013) as compared to assessment based on estimated percentage of plant leaf necrosis in this study produced no obvious difference in rating for salinity tolerance. As a consequence, genotypic screening using a more rapid approach based on measurement of percentage plant necrosis should be sufficient for breeding and selection purposes. Genetic variance or broad sense heritability for measures of salinity
symptom were of reasonable magnitude. Also variation for tolerance between progeny and parental genotypes displayed high repeatability between pot experiments, indicating good potential for genetic improvement using the screening method as described.

Salinity indexes are frequently used to better classify plant accessions in support to selection for higher salinity tolerance (Bchini et al. 2011), as genotype responses can be complex, may vary with ontogeny and interact with other environmental factors (Shannon 1985). In this study, a salinity index based on weighted symptom scores was used for more accurate identification of RIL progeny genotypes with higher parental value for use in targeted crossing programs. Specifically, the documented phenotypic variation for salinity tolerance between Kaspa x Parafield RIL progeny lines can be used to statistically identify associations with regions of genomic variation, based on use of molecular genetic markers such as simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs) (Kaur et al. 2012), and therefore identify controlling major genes or quantitative trait loci (QTLs). Marker assisted selection may then facilitate selection for specific salinity tolerance genes. However, tight chromosomal linkages with deleterious alleles at other closely adjacent genes are likely to occur frequently when using non-domesticated germplasm in breeding applications (Zamir 2001). Also, for complex traits such as salinity tolerance, the extent of the chromosomal region containing the identified QTL may often be too large (Asins 2002) to be useful for selection. In such instances, assuming that only single loci have been detected in the target region, further cycles of finer-structure genetic mapping, based on higher densities of markers and larger numbers of progeny, are required to refine the interval and mitigate the effects of potential co-selection of deleterious gene loci.

Pot-based screening used for routine selection resulted in a significant shift in the frequency of advanced breeding lines that were classified with higher tolerance in the PBA breeding program. This has been important in order to
reduce the number of sensitive genotypes evaluated for yield in advanced nurseries, particularly as advanced testing is costly because of the need for increased experimental replication and multi-location testing (Allen et al. 1978). Various pre-breeding strategies are being used in Australia to improve salinity tolerance of the pea crop. These also mainly rely on the pot-based screening techniques described in this study, and include recurrent selection aimed at incremental co-assembly of tolerance genes from different germplasm sources, and backcross breeding aimed specifically at introgression of tolerance into earlier flowering germplasm with the ‘Kaspa’-like ideotype. Mass selection for tolerance based on biomass production in low rainfall environments has also been used to develop boron and salt tolerant forage field pea cultivars (i.e. PBA Coogee).

Substantial differences in salinity tolerance were identified by seedling screening between soon-to-be-released semi-dwarf accessions (i.e. PBA Wharton), and more sensitive lines that are currently marketed (i.e. PBA Gunyah or Kaspa (Fig. 6.10). However, such differences may only translate to seasonal yield advantages, as rainfall is highly variable and salinity is transient in nature in many low rainfall climates (Rengasamy 2006). Concurrent genetic gains for higher boron toxicity tolerance, are also likely to provide an additional advantages in adaptation in low rainfall regions. In 2011, higher boron and salinity tolerance were sufficiently frequent in advanced stage yield testing to conduct a linked multivariate analysis for grain yield between genotype and environment based on degree of tolerance. In this study, genotypes with higher boron tolerance were closely associated with higher genotype yield at 7 of 14 locations of evaluation. These were mostly sites located in ‘Mallee’-type regions of SA and VIC, but also included Kingsford in SA and Ardlethan in southern NSW. All 7 sites were in regions in which sub-soils are typically highly alkaline. With the exception of Kingsford, the 7 sites were on average lower yielding, and exhibited lower growing season rainfall when compared to sites at which sensitive genotypes performed comparatively well. Even genotypes varying for boron tolerance on average performed relatively better than boron-sensitive genotypes at these 7
locations. Notably, a sensitive genotype (119) showed higher specific adaptation across these sites, and may therefore provide additional genes for improving adaptation. This genotype is unique in that it produces non-*aflila* and semi-dwarf plants similar to the Czechoslovakian-bred variety Bohatyr. Boron tolerance is controlled by one, or probably two, major genes (Bagheri *et al.* 1994) and appears to be in coupling linkage with alleles for resistance to powdery mildew in Australian-bred germplasm. This close linkage may explain why boron-tolerant lines performed relatively well at Kingsford, as this was the only site among 7 at which damaging incidence of powdery mildew was observed. This inferred genetic linkage is also likely to explain why genotypes with resistance to powdery mildew are regularly identified as unexpectedly higher yielding in low rainfall seasons and at locations in SA at which powdery mildew disease is not causing damage (Larn McMurray, personal communication).

From the multivariate analysis, a much weaker relationship was apparent between higher salinity tolerance and site yield performance than that identified for boron tolerance. Genotypes with higher salinity tolerance as a group performed relatively better only at 3 of the 14 sites (Dallwallinu WA, Scadden, WA and Yenda, NSW). Of these, the Dallwallinu site displayed the weakest association, but also experienced comparatively higher rainfall during the growing season. Dallwallinu is therefore likely to have been affected to a lesser extent by salinity induced water stress or reduced osmotic potential (Nuttal *et al.* 2009). In contrast, the only salinity sensitive genotypes that yielded well at the Scadden and Yenda sites showed relatively earlier maturity (i.e. PBA Twilight). Genotypes with both higher boron and salinity tolerance were mainly clustered within the same group as boron-tolerant genotypes, suggesting that the latter trait was the predominant factor contributing to yield, in this instance. This finding is not surprising, as high boron tolerance is more common in advanced germplasm compared to salinity tolerance (Leonforte *et al.* 2009). Hence, the degree of salinity tolerance available in the field pea breeding program is unlikely to deliver the same value in terms of yield response, and an ongoing pre-breeding effort to
enhance the prevalence of this trait is therefore required. It may not be possible to properly assess the additive value of incremental combinations between salinity and boron tolerance until a higher proportion of such genotypes are present in advanced testing, or until the high tolerance from unadapted types such as ATC1836 has been transferred in adapted backgrounds with high yield.

Regression analysis of mean site yield in 2011 against rainfall during the growing season confirmed, as expected, that water availability is the major limitation to grain yield in field pea. Tolerance to abiotic factors that reduce water use efficiency, such as high boron and salinity, will hence be critical to improving crop adaptation in Australia. The present study has indicated significant genetic potential to improve salinity tolerance in the crop, and highlights the opportunities for gain of yield in lower rainfall climates by improvement of tolerance to both boron- or salinity-induced stress.
Chapter Seven
Construction of linkage maps and identifying QTL’s associated with higher salinity tolerance in field pea.

7.2 INTRODUCTION

Field pea (*Pisum sativum* L.) is widely cultivated as an important pulse crop on a global basis for human nutrition and stock-feed consumption. This species is also used for forage production (Kocer and Albayrak 2012), in rotations with cereals for provision of soil nitrogen (Omokanye *et al.* 2011), and to provide disease breaks.

Development of sustainable high-yielding varieties which persist under biotic and abiotic stresses is a prerequisite for meeting the growing world population. Molecular breeding strategies have been adopted for improvement programs in several crops, including legumes such as soybean and common bean (Chamarthi *et al.* 2011), and are suitable for application to field pea. Most breeding gains for field pea grain yield in Australia have been achieved by optimisation of crop architecture (i.e. reduced internode length), harvest index and phenology traits with growing season length and rainfall (Redden *et al.* 2005). Breeding practice has also primarily focused on pyramiding of genes for resistance to locally important fungal diseases such as ascochytta blight, powdery and downy mildew, and viruses such as pea seed-borne mosaic virus (PSbMV) and bean leaf roll virus (BLRV). However, comparatively little effort has been directed towards the improvement of tolerances to physiologically complex and putatively multigenic traits such as salinity (Kumar *et al.* 2011).

Genetic improvement for complex traits will be facilitated by new genomic tools for identification and selection of superior gene variants. Historically, progress for pulse crops has been limited by insufficient resources for such a
purpose (Varshney et al. 2009), and so adoption of marker assisted selection (MAS) has been slow (Kumar et al. 2011). However, advances in DNA sequencing have recently delivered large-scale transcriptome sequence data sets for field pea (Franssen et al. 2011, Kaur et al. 2012). This data can be exploited for design of DNA-based genetic markers such as SSRs and SNPs, supporting linkage mapping, analysis of genetic diversity and trait-dissection by QTL detection, (Jing et al. 2007, Fondevilla et al. 2011), as well as tagging for MAS (Collard et al. 2005).

For pea, a large number of genetic linkage maps have been developed using various types of markers such as restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), randomly amplified polymorphic DNAs (RAPDs) and SSRs (Timmermann-Vaughan et al. 1996, Mc-Callum et al. 1997, Weeden et al. 1998, Pilet-Nayel et al. 2002, Prioul et al. 2004, Loridon et al. 2005, Fondevilla et al. 2011). Of these, RFLPs are co-dominant in nature, but require large quantities of DNA and are expensive to screen. The PCR based markers, AFLP and RAPD are dominant systems and therefore not practical for MAS. The SSR markers are mostly co-dominant, highly polymorphic and have becoming increasingly popular for pedigree analysis in crop breeding and genetics research. Genotyping of large populations using this technique is however probably still too costly for application to breeding programs for minor crops such as pulses (Kumar et al. 2011). The SNP markers in contrast can be more effectively used for genetic and association mapping, to study genetic diversity or for genome-wide selection. A number of methods have been developed for genotyping of SNP markers. Medium- to high-throughput array-based SNP genotyping systems are now available, depending on the number of samples and markers to be analysed, such as GoldenGate and Infinium from Illumina Inc., SNPStream from Beckman Coulter, and GeneChip from Affymetrix (Deulvot et al. 2010).

In order to understand complex biological processes in plants, comparative genetic analysis with model species has been used extensively. In concert with extensive genomic resources that are available for a number of species
of the legume sub-family Papilionoideae (e.g. M. truncatula (http://www.medicago.org webcite), L. Japonicas (Sato et al. 2008), chickpea (Varshney et al. 2013, soybean Schmutz et al. 2012) and pigeon pea (Varshney et al. 2013), such analysis provides opportunities for translational genomics to assist breeding of other, less well-studied crop legumes, such as field pea.

Salinity tolerance in field pea has become increasingly important in Australia as crop production has shifted geographically towards environments characterised by shorter seasons, greater water limitation and marginal soils with higher transient soil salinity (Nutall et al. 2009). High salinity and sodicity effects are chiefly due to levels of the Na$^+$ cation, and in Australia are commonly associated with highly alkaline (pH > 8.5) soils (Rengasamy 2002, 2006). Research with other major dry-land crops such as wheat (Munns et al. 2006) have demonstrated the difficulty of using yield-based response measurements from field studies as a measure of salinity tolerance, due to complexity of interactions with other stress factors, Na$^+$ variability in the soil profile, and differential responses according to both growth stage and genotype. However, low-cost and reliable pot-based glasshouse screening methodologies have been developed for a range of crops, including pea (Leonforte et al. 2013), that can be used to identify useful variation at the seedling stage for breeding purposes. Considerable potential for genetic improvement appears to be available, on the basis of screening experiments (Leonforte et al. 2010, 2013). Identification and marker-tagging of genomic regions containing QTLs for aspects of salinity stress tolerance would highly facilitate the targeted introgression of this trait into otherwise unadapted germplasm.

The objectives of this study were to better understand the genetic basis of salinity tolerance, identify genomic regions controlling salinity stress tolerance in field pea and molecular genetic markers linked to tolerance QTLs for MAS.
7.3 MATERIALS AND METHODS

7.3.1 Population development

Crosses were made between genotypes of Kaspa and Parafield and Kaspa and Yarrum at Horsham VIC DPI in 2007 and progeny of the F$_2$ generation were produced. Single seed descent seed increase was undertaken from F$_2$ progeny derived genotypes for 6 generations in glasshouses at Horsham DPI, from 2008 to 2011.

7.3.2 Phenotypic screening

Recombinant inbred line (RIL) populations derived from Kaspa (sensitive to salinity) x Parafield (moderately tolerant to salinity) and Kaspa x Yarrum (moderately sensitive to moderately tolerant to salinity) (Leonforte et al. 2013), were screened for response to NaCl salt-induced stress applied at the seedling stage.

The experiments were conducted over autumn (Kaspa x Parafield) and winter (Kaspa x Yarrum) of (2012) in a semi-controlled environment (i.e. large plastic igloo) at DEPI-Horsham, Victoria in both years. Screening was undertaken by sowing six plants of each RIL-derived line at equidistant spacing in 13 cm diameter pots into a sand and gravel medium to a depth of 2 cm in two replications. This provided 12 plants as replications for each RIL line for phenotypic analysis. The gravel medium was composed from a 1:1 ratio of coarse river sand and 5 mm bluestone chips. Each pot was treated daily with rainwater from sowing until emergence. From 6 days post-emergence, seedlings were watered with a complete nutrient solution (i.e. nitrosol, NPK ratio 12.2: 2.9: 8.5), in addition to supplementation with a calcium source (i.e. calcium nitrate). The required NaCl concentration was tested using an EC meter and was applied at an initial rate of 3 dsm$^{-1}$ from day 9 post emergence. The concentration of applied NaCl was increased by 3 dsm$^{-1}$ at each watering time to avoid abrupt osmotic adjustment, up to a final rate of 18 dsm$^{-1}$, and maintained at this concentration until assessment. All watering with the nutrient and salt solution was undertaken over 3 day-intervals at a rate of 200 ml per pot applied directly to the growing medium surface. A null-
salt application treatment (no added NaCl) was included for control lines (parents) and randomised in the experiment to validate effects of salinity exposure were not caused by other stress factors. Tall growing plants were trellised onto plastic lattice to avoid splashing of salt solution during watering and to avoid shading effects and plant damage during assessment. Individual plants in each pot were assessed for symptom development (symptom score) as described by Leonforte et al. (2013) from 28 days post emergence and there after every 7th day until plant death. Final plant biomass cuts were also taken and seed set was recorded for each genotype. Genotype pot averages for plant symptom score were calculated from individual plant assessments and used to estimate genotype average values for symptom score using REML spatial row-column analysis. A salt index was used to quantify genotypic salinity tolerance values and describe tolerance levels according to sensitivity. The salt index was calculated by adding weighted symptom score values (weighted on the basis of phenotypic variance) measured over the duration of the experiment and final plant biomass.

7.3.3 Constructions of linkage maps and QTL analysis

Plant tissue DNA from RIL progenies were extracted and used for genomic analysis. Genomic analysis was undertaken at AgriBio, DEPI-Bundoora.

1) DNA extraction: Frozen leaf tissue from each progeny genotype was ground for extraction using a Mixer Mill 300 (Retsch®, Haan, Germany), and genomic DNA was obtained using the DNeasy® 96 Plant Kit (QIAGEN, Hilden, Germany). DNA was resuspended in 1 x TE buffer to a concentration of 50 ng/µl and stored at -20°C.

2) SSR genotyping: Genomic and EST-derived SSRs (Kaur et al. 2012) were screened on mapping parents for polymorphism detection. Previously described publicly available SSR primer pair sequences attributed to field pea genetic maps were also obtained for anchoring purposes. Primer synthesis was carried out following the method described in Schuelke (2000). PCR products were analysed using an ABI3730xl capillary electrophoresis
platform. SSR products were combined with the ABI GeneScan LIZ500 size standard and allele sizes were scored using GeneMapper® 3.7 software package.

3) SNP genotyping: A preliminary list of SNPs was selected for GoldenGate™ primer design using as criterion of the absence of any other SNP within the 20bp segment flanking the targeted SNP analysed. A designability rank score (0 to 1) was calculated for each SNP. In total 768 SNPs with designability scores between 0.6 and 1.0 were finally selected for genotyping. SNP genotyping was performed using Illumina GoldenGate™ assays. The PCR products were hybridised to the bead chips via the address sequence for detection on an Illumina iSCAN Reader. The automatic allele calling was done using the Illumina Genome Studio software v2011.1 with a GeneCall threshold of 0.20.

4) Genetic linkage mapping: Marker data was tested for Mendelian segregation ratios using Pearson’s $\chi^2$ tests for the expected 1:1 ratio of individual markers. Markers with a $\chi^2$ score >10 were not included in further analysis. The genetic linkage map was generated using Map Manager Software version QTXb19 (Manly et al. 2001). Map distances were calculated using the Kosambi mapping function (Kosambi 1944) at a threshold of $p = 0.0001$. Linkage groups (LGs) were assigned on the basis of common marker loci (Loridon et al. 2005) from publicly available P. sativum linkage maps, and by comparison with M. truncatula chromosomes. Linkage groups were drawn using Mapchart software v 2.2 (Voorrips 2001, 2002).

5) QTL analysis: Genotype means from each experiment were used to generate frequency distribution histograms. Narrow sense heritabilities ($h^2$) were calculated for the trait by considering the spatial trends in the experiment using best linear unbiased predictions (BLUPS) analysis. QTL detection was conducted using simple interval mapping (SIM) and composite interval mapping (CIM) in Windows QTL Cartographer version 2.5 (Wang et al. 2003). One thousand permutations were used to determine LOD significance levels (0.05). The co-factors were specified as five marker loci
identified by stepwise regression that explained the most variation for a given trait. Regression ($R^2$) values taken at the peak LOD score of a QTL were used to indicate the percentage of the phenotypic variation explained by the QTL. The type of gene action (additive/dominant) was estimated using QTL Cartographer.

7.4 RESULTS

7.4.1 Phenotypic screening

Plant symptom response data from salinity screening Kaspa x Parafield and Kaspa x Yarrum RIL populations at seedling stage indicates that variation for tolerance was normally distributed and therefore likely to be quantitatively controlled (Fig. 7.1, 7.2, 7.3, 7.4). Two different phenotypic measurements, including salt index and mean symptom score (average of symptom scores obtained at up to 35 days) were used to detect salt tolerance QTLs (Figure 7.5), with LOD scores of 3.2 (salt index) and 2.5 (symptom score) as minimum significance levels. The estimated narrow sense heritability ($h^2$) for salt index was 0.55 in the Kaspa x Parafield population, indicating that about 45% of the variation was due to non-genetic factors.

7.4.2 Constructions of linkage maps and QTL analysis

Linkage maps for Kaspa x Parafield and Kaspa x Yarrum RIL populations were constructed using SSR and SNP markers (Fig. 7.5, 7.6). Associated QTLs linked to seedling salinity tolerance were identified and are graphically presented (Fig. 7.7, 7.8). The percentage of accounted phenotypic variation and flanking markers associated with QTLs are indicated on these graphs. For the Kaspa x Parafield population two QTLs were identified on Ps III and Ps VII, explaining 12% and 19% of phenotypic variance ($V_p$) for salt index score, and 12% and 17% for the symptom score, respectively (Table 7.1). QTL analysis was also performed using symptom scores obtained at different time points (day 7, 14, 21, 35), which identified the same QTL locations and accounted for similar proportions of $V_p$ (data not shown). The phenotypic data for symptom scores obtained at day 42, 49, 56 deviated from normality, and
was consequently not used for QTL analysis based on mean symptom score. For the Kaspa x Yarrum population the phenotypic variance (Vp) explained by interval mapping (IM) statistical approach was low at 15% (Fig 7.8).
Fig. 7.1. Frequency distribution for symptom score (1 to 10) for RIL progeny derived from the Kaspa x Parafield cross at 7, 14, 21, 35, 42, 49 and 56 days post application of NaCl in watering solution at 18 dsm$^{-1}$. 
Fig 7.2. Frequency distribution for salinity index value and qualitative rating (T (tolerant), MT-T (moderately tolerant to tolerant), MS-S (Moderately sensitive to sensitive), S (sensitive), HS (high sensitivity) for RIL progeny derived from the Kaspa x Parafield cross following salinity treatment of 18 dsm⁻¹.
**Fig. 7.3.** Frequency distribution for symptom score (1 to 10) for RIL progeny derived from the Kaspa x Yarrum cross at 7, 14, 21, 35, 42, 49, 56, 63, 70 and 77 days post application of NaCl in watering solution at 18 dsm⁻¹.
Fig 7.4. Frequency distribution for salinity index value and qualitative rating (T (tolerant), MT (moderately tolerant), MS (moderately sensitive), S (sensitive) for RIL progeny derived from the Kaspa x Yarrum cross following salinity treatment of 18 dsm$^{-1}$. 

![Frequency distribution for salinity index value and qualitative rating](image)
Fig. 7.5. Genetic linkage map of *Pisum sativum* prepared using 120 RIL derived progeny from the Kaspa x Parafield cross. The markers are shown on the right of the linkage groups, and map distances between markers are indicated in cM on the left.
Fig. 7.6. Genetic linkage map of *Pisum sativum* prepared using 120 RIL derived progeny from the Kaspa x Yarrum cross. The markers are shown on the right of the linkage groups, and map distances between markers are indicated in cM on the left.
Fig. 7.7. Location of two major QTLs for higher salinity tolerance based on variation between RIL progeny of the Kaspa x Parafield cross.
Table 7.1. Identification of QTLs for salt tolerance in Kaspa x Parafield mapping populations using CIM.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Flanking markers</th>
<th>Position</th>
<th>LOD threshold</th>
<th>Max LOD score</th>
<th>% Phenotypic variance</th>
</tr>
</thead>
<tbody>
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<td>Salt index_QTL 1</td>
<td>SNP_100000318</td>
<td>218 - 222</td>
<td>3.2</td>
<td>4.7</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>SNP_100000130</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salt index_QTL 2</td>
<td>SNP_100000313</td>
<td>179 - 190</td>
<td>3.2</td>
<td>3.75</td>
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<td></td>
<td>SNP_100000190</td>
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</tr>
<tr>
<td>Final salt rating_QTL 1</td>
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<td>218 - 222</td>
<td>2.5</td>
<td>5.9</td>
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<td>SNP_100000353</td>
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Fig. 7.8. Graph showing QTL analysis for salinity tolerance using Kaspa x Yarrum population. The phenotypic variance (Vp) explained by interval mapping (IM) statistical approach was low at 15%.
7.5 DISCUSSION

Several field pea linkage maps have been previously developed with successive adoption of new molecular marker technologies (Fondevilla et al. 2011, Timmerman-Vaughan et al. 1996, Loridon et al. 2005). The linkage maps constructed in the present study exhibits a regular marker distribution, but a significantly longer cumulative genetic map (1916 cM) than would be expected on the basis of typical chiasma frequency (1–2 per bivalent) at meiotic prophase. Such expansions of the pea genetic linkage map were also previously reported (1700 cM (Ellis et al. 1992); 2202.7 cM (Moreno, 2009). Several factors may be responsible, including the genetic constitution of different mapping populations, mapping strategies, number and type of mapped loci, the choice of mapping software and ratio between number of markers and population size (Liu et al, 2008, Shirawawa et al, 2013).

Plant response to salt tolerance is influenced by various physiological mechanisms, which are likely to be controlled by multiple genes across different environments (Foolad 2004). The present study suggested a quantitative basis for seedling-induced salinity tolerance derived from adapted and high-yielding parental field pea genotypes. Two QTL loci were identified, each accounting for moderate proportions of the phenotypic variation in the Kaspa x Parafield population. In the Kaspa x Yarrum population one QTL was identified but only accounted for a minor component of phenotypic variation. This difference could be explained by lower genetic contributions to variation and/or differences between screening environments (autumn vs winter). It was noticeable that salinity-induced symptoms developed much slower in the Kaspa x Yarrum progeny (screened over winter) when compared to Kaspa x Parafield progeny (screened over autumn), possibly due to differences in temperature and humidity (Subbarao and Johansen 1999). Equivalent studies of different physiological traits associated with salt tolerance in M. truncatula and C. arietinum identified up to 20 putative genomic regions in the linkage map (Arraouadi et al. 2012, Samineni 2010), substantially larger than in this study. In previous studies (Hernandez et al. 1995) salt stress was shown to induce a range of responses in salt tolerant pea plants, leading to enhanced
production of activated oxygen species and antioxidants. As for previous studies of a range of crop species, genetic markers linked to QTLs for a given target trait could be implemented for a MAS program. However, in practice this has rarely happened for genetic improvement of salinity, as the trait is highly physiologically complex (Ashraf and Foolad 2012). In such circumstances, breeders will need to select for varying and multiple genomic regions or response mechanisms found in different germplasm, different screening environments and within different ontogenic stages.

It is likely that the genetic variation for seedling symptom responses identified in this study is a consequence of lower and less rapid Na\textsuperscript{+} uptake (Leonforte et al. 2013). Numerous crop-based studies have identified QTLs for reduced Na\textsuperscript{+} uptake under salinity stress (Ashraf and Foolad 2012). However, the value of such QTLs has often not been validated, as lower Na\textsuperscript{+} accumulation or exclusion only contributes a partial component of the total salinity tolerance response. The QTLs for lower Na\textsuperscript{+} uptake identified at a given growth stage may not be effective over other stages of ontogeny (Ashraf and O'Leary 1994, Chartzoulakis and Klapaki 2000, Qasim and Ashraf 2006) critical for grain yield. The QTLs may also have been identified from non-domesticated germplasm, or through use of atypical growing conditions, and hence may not be directly applicable.

The QTLs identified in the present study are associated with seedling growth-stage salinity tolerance. Similarly, QTLs for seedling growth tolerance have been identified in numerous grain crops, including rice (Alam et al. 2011), barley (Zhou et al. 2012), soybean (Hamwieh et al. 2011) and wheat (Ma et al. 2007). Mechanisms related to other growth-response QTLs occurring at germination (as for tomato (Foolad et al. 1997, 1998), rice (Cheng et al. 2008), barley (Mano and Takeda 1997) and wheat (Ma et al. 2007)) or during reproductive development (as for rice (Manneh et al. 2007), barley (Xue et al. 2010) and tomato (Villalta et al. 2007)) are likely to be significant for field pea and warrant investigation. The significant variation in degree and timing of salinity-induced growth responses within- between-crop species highlights complexity of the trait that requires understanding. Selection for specific
Salinity-induced growth response traits or QTLs may hence need to be undertaken separately for different growth stages as part of a targeted breeding strategy to pyramid useful genes.

A number of studies have associated oxidative defence systems with salinity tolerance. However, regulation of some oxidative defences may be a secondary response to salinity stress (Ashraf 2009). The production of organic osmolytes during plant stresses such as salinity or drought has been shown to improve osmoregulation and protect enzyme function in the cytosol (Ashraf and Foolad 2007), as validated in recent transgenic studies (Hussain et al. 2011) including tobacco (Ziaf et al. 2011) and potato (Rahnama et al. 2011). Little research to date has been performed to identify QTLs controlling production of different osmoprotectants, apart from studies of proline accumulation (Siahsar and Narouei 2010). The effectiveness of different osmolytes for improvement of salinity tolerance may also vary between species (Ashraf and Foolad 2012), which may provide another avenue for identification of useful genes for tolerance to salinity in pea.

The complexity of salinity tolerance in terms of developmental regulation and genotype-by-environment effects on plant responses indicates that plant breeders may need to utilise different QTLs representing different components of tolerance for successful MAS implementation. It therefore may be necessary to quantify the adaptive nature (Collins et al. 2008) of different QTLs according to varying salinity stress, and to allocate genomic values akin to index-trait based selection. Advances in genome sequencing and genotyping capacity, especially genotyping-by-sequencing (GBS), offer the potential for genome-wide marker analysis (Elshire et al. 2011) and the capacity to identify all loci contributing to a trait such as saline stress tolerance, irrespective of effect magnitude. Such data may be used to develop breeding value estimates based on all trait-linked markers, in order to identify key parental lines for targeted introgression programs.
Field pea was introduced by the first European settlers, and has a long history of production in Australia. However, major crop production only initiated in the mid-1980s. Until the mid 1990s, most of the field pea crop was grown on the more fertile soils and within the higher rainfall districts (i.e. >380 mm average annual rainfall) of southern VIC, south-eastern NSW, lower mid-north cropping regions of SA and southern cropping regions of WA. Expansion of the crop into lower-rainfall (i.e. < 380 mm average annual rainfall) ‘Mallee-type’ cropping regions has increased significantly, in part due to improved variety options, but additionally for economic reasons (i.e. the monetary value of field pea grain). Crop yield reliability in low rainfall regions has been significantly improved with the development of ‘Kaspa-type’ germplasm, which has delivered higher harvest index, pod shatter resistance and more erect growth. New, shorter season ‘Kaspa-type’ varieties (i.e. PBA Gunyah, PBA Twilight), with pyramided disease resistance (PBA Oura, PBA Wharton) are also now becoming available. However, high sub-soil boron, salinity and sodicity are major constraints for dryland grain crop production across much of southern Australia (Carwright et al. 1984, Riley 1987, Nuttall et al. 2003), and have not been adequately addressed for field pea using genetic improvement. The high levels of salt sensitivity observed for pea when compared to cereals (Maas 1986, Saxena et al. 1994, Francois and Maas 1994) and canola (Steppuhn et al. 2001) provides a strong indication that increased tolerance will significantly improve crop potential in lower rainfall regions.

This Ph.D. program was undertaken to improve field pea adaptation for lower rainfall regions through identification of salinity tolerance (NaCl) in field pea germplasm, by investigating important growth responses conferring higher tolerance, and undertaking preliminary research to understand genetic control. A screening method was developed for field pea based on studies
undertaken by Maher et al. (2003), and was used to screen for higher salinity tolerance. Higher tolerance was validated in subsequent pot experiments in which germplasm was exposed to salinity at varying concentrations, at the seedling stage, and from the onset of flowering. Growth response mechanisms that are useful for breeding of higher tolerance were identified for this trait. RIL populations were developed on the basis of initial screening results (Chapter 3) and used to investigate the complexity of trait control at the genetic level (based on QTL detection) through the use of genetic linkages maps populated with SSR and SNP markers specific for pea (Kaur et al. 2012). The contribution of higher salinity and boron tolerance in advanced breeding germplasm was investigated by comparison of yield responses across 14 breeding nurseries grown in southern Australia in 2011. This study has highlighted the need to genetically improve salinity tolerance in field pea.

8.1 SOURCES OF HIGH TOLERANCE TO SALINITY IDENTIFIED IN PEA

An initial screening experiment using 780 globally-distributed Pisum L. accessions identified significant variation in response to applied NaCl, based on plant symptoms (Leonforte et al. 2013). Lines with relatively higher tolerance, as compared to commercial varieties grown in Australia, were most frequently identified within landraces originating from the central, eastern and southern provinces of China. The higher frequency of salinity tolerance discovered in China in this study may be linked to divergence in natural selection between China and other global regions (Zong et al. 2009). The most tolerant identified accession was an unadapted landrace (ATC1836) that originated from Greece.

Variation for salinity tolerance was validated using a sub-set of 70 accession lines. Salinity-induced toxicity symptoms were closely associated with reductions in plant growth rate, height, shoot and root dry matter and with increased concentration of Na\(^+\) at the plant growing tip. Plant height as controlled by internode length did not appear to be directly associated with
NaCl sensitivity in pea. This was an important finding, as international breeding efforts are focused on the development of semi-dwarf types with a high harvest index. Selection for higher plant biomass may, however, be useful in order to mitigate and delay Na\(^+\) toxicity effects (Almodares et al. 2011). The level of salinity tolerance based on these factors varied substantially, and provides an important basis for genetic improvement of this trait in field pea, for Australia.

8.2 SALINITY INDUCED GROWTH RESPONSES AT SEEDLING STAGE

A total of seven field pea genotypes that exhibit varying effects for salinity tolerance showed a range of symptom development and growth responses over time under increasing NaCl salinity. The genotype responses were closely associated with Na\(^+\) accumulation in plant leaflet tissue on the lower plant, and a parallel reduction in K\(^+\) concentration. Increasing salinity caused strong, but varying, inhibition of root and shoot dry matter and final grain yield for all genotypes. These results corroborate observations from other crops that reveal close associations between rate (Munns et al. 2002) and biphasic manner (Munns 2005) of symptom development and growth responses, with increasing accumulation of Na\(^+\) ions in plant tissue (Lauchli and Grattan 2007).

The ATC1836 genotype showed the highest relative tolerance based on measured parameters, but was comparatively slow-growing. This is a concern, as inherently low early plant vigour or biomass growth will probably be detrimental to field pea production in shorter seasons or in water-deficit environments in Australia (Sadras et al. 2012). Three genotypes (03H090P-04HO2002, OZP0812, 99-410-2-14-2) with moderate tolerance produced substantially more dry matter under the highest salinity treatment of 18 dsm\(^{-1}\) when compared to the commercial variety ‘Kaspa’. The OZP0812 genotype was also able to maintain relatively higher growth rate at the lower salinity treatment of 6 dsm\(^{-1}\). Further research is required to understand this
difference and to determine whether non-Na-specific effects of salinity such as osmosis (Rahnam et al. 2010) may be contributing to this tolerance, and if so, which.

There was a significant reduction in final root dry matter from increasing salinity exposure, but no significant genotypic effect. The reduction in root biomass could be due to a combination of direct effects of Na\(^+\) on growth inhibition (Tester and Davenport 2003), or indirect effects of Na\(^+\)-reduced plant shoot biomass (Läuchli and Grattan 2007). Variation for higher root biomass and architecture in pea (McPhee 2005) can improve tolerance to root diseases (Kraft and Boge 2001) and herbicide damage (Ali-Kahn and Snoad 1977), so the combination of higher salinity tolerance and root biomass that is apparent in ATC1836 may be useful.

All genotypes showed decreased K\(^+\) content in leaf tissues with increasing NaCl salinity in the root media, which is consistent with numerous studies for a range of crop species (Grattan and Grieve 1999). The ratio of K\(^+\) to Na\(^+\) in plant tissue was higher for the more tolerant genotype (ATC1836) and likely to be associated with low Na\(^+\) uptake and thus K\(^+\) exchange (Fox and Guerinot 1998). Potassium is important for cell expansion, osmotic regulation and cellular and whole–plant homeostasis (Schachtman et al. 1997), and there is a high stomatal K\(^+\) requirement for photosynthesis (Chow et al. 1990). Sodium-induced K\(^+\) deficiency has been implicated in growth and yield response for a number of field crops (Britto et al. 2010), and selection for a higher Na\(^+\)/K\(^+\) ratio (Maathuis and Amtmann 1999) may be a useful strategy for improving salinity tolerance.

8.3 SALINITY INDUCED GROWTH RESPONSES AT ONSET OF FLOWERING

High salinity tolerance of the landrace genotype ATC1836 at early growth stages was evident at later ontogeny, on the basis of lower biomass reduction and symptom development and delayed rate of Na\(^+\) accumulation in plant
tissue. In this study, Na\textsuperscript{+} accumulated more rapidly and to a higher level, and symptoms developed faster on lower growth nodes when compared to the growing tip. Accelerated tissue damage and loss of older leaves is expected, due to greater cumulative transpiration and consequent Na\textsuperscript{+} ion accumulation under increasing salinity (Shannon et al. 1994). Plant biomass and main plant height showed lower correlations with Na\textsuperscript{+} plant tissue concentration than plant chlorosis. The results in this study, however, indicate that comparison of development of chlorosis symptoms may become confounded when assessed for genotypes of varying phenology, due to associated timing of translocation of sugars for seed development (Roche et al. 1988). The taller genotype (OZP0812) produced more biomass with increasing salinity when compared to the dwarf genotypes Kaspa and OZP0809 and the landrace genotype ATC1836. This could reflect genetic differences in internode length and branching habit rather than variation in response to salinity, and highlights the potential use of plant morphology traits to reduce sensitivity of the crop.

As with many other crops (such as wheat), reproductive development in terms of flowering time appears to be accelerated with salinity in field pea. This may have important implications for increasing sensitivity to frost risk (Shafiq et al. 2012) or ascochyta blight disease (Timmerman et al. 2004, Boros and Marcinkowska 2010), which are major constraints to field pea production in Australia.

There was strong salinity-induced inhibition of all measured seed yield components, which included seeds per pod, plant yield, seed size and pod number. The genotype ATC1836 produced a comparatively larger number of small pods per plant, and the very small seeds. Yield loss in response to increasing salinity for this line appeared to be mostly attributable to loss of pod number. The OZP0812 genotype produced the highest seed yield per plant with increasing salinity exposure, and appeared to remain more fertile in terms of seed and pod number set, as well as maintaining larger seed size. Differences in pod weight between Kaspa and other genotypes are probably attributable to a reduced pod parchment layer in the pod wall (McGee and
Baggett 1992), rather than pod yield. The ATC1836 genotype produced the same number of pods in the 6 dsm$^{-1}$ and null salinity treatment, but the overall pod weight per plant was greater. A possible explanation for this may be an increase in assimilate partitioning (Jeuffroy and Warembourg 1991) towards the pod wall, due to increased seed abortion at the 6 dsm$^{-1}$ salinity treatment.

Seed tissue Na$^+$ concentration increased dramatically with increased salinity, and this may have important implications for seed quality and seed production from more saline environments, as with lentil (Ghassemi-Golezani et al. 2012). This requires further investigation for field pea.

This study also revealed significant variation for effects of salinity stress on seed size. This is an important consideration, as seed size effects the end-use marketability of grain for human consumption (Redden 2005), as well as seed quality for sowing (Singh et al. 2009).

8.4 UNDERSTANDING GENETIC CONTROL FOR HIGHER SALINITY TOLERANCE

Progeny segregation for salinity tolerance across 3 populations derived from either ATC1836, Parafield or Yarrum, when crossed to the sensitive genotype Kaspa, indicate probable multigenic control. A comparatively higher proportion of progeny tolerance was observed from the ATC1836 x Kaspa population. However, a high degree of transgressive segregation for higher salinity tolerance was apparent in progeny from crosses between Parafield or Yarrum x Kaspa, suggesting that parental combining abilities should also be assessed for improvement of salinity tolerance. Positive broad sense heritabilities for measures of symptom response to salinity, and repeatability of results between experiments and generations, imply that high potential for genetic gain can be achieved using pot-based screening methods as described. Differences in assessment of salinity symptom response based on a scaled (1-10) symptom score when compared to percentage plant necrosis were not significant. Variation for salinity tolerance within RIL progeny derived
from the Kaspa x Parafield and Kaspa x Yarrum crosses were documented on the basis of rate of symptom development and a salinity tolerance index. Linkage maps based on molecular genetic marker polymorphism were constructed for RIL populations of Kaspa x Parafield and Kaspa x Yarrum populations. The salinity index values were used to identify QTLs associated with variation for tolerance, and flanking closely linked SNP markers. As from numerous studies over a range of crop species, such identified QTLs could be exploited in a MAS program. However, in practice this has rarely happened for improvement of the salinity tolerance trait, which is physiologically (Ashraf and Foolad 2012) and genetically complex, implying the requirement to identify multiple genomic regions each of which is likely to contribute relatively small proportions of genetic variance. For complex traits like salinity tolerance, it is likely that breeders will need to select for varying numbers of genetic markers relating to differing response mechanisms found in different germplasm, under different screening environments and within different ontogenic stages.

8.5 POTENTIAL IMPACT OF PYRAMIDING HIGHER BORON AND SALINITY TOLERANCE

A frequency analysis showed that the proportion of field pea germplasm in advanced yield testing nurseries in Australia with higher salinity tolerance had increased from 2005 to 2011. However, a multivariate canonical analysis based on 14 yield nurseries in 2011 indicated that the degree of salinity tolerance in advanced germplasm currently provides significantly less yield benefit when compared to the degree of boron toxicity tolerance. This highlights the need for further pre-breeding effort. Boron-tolerant germplasm as a group was higher yielding across 7 sites in 2011. All of these sites were in regions with highly alkaline sub-soils, and six sites experienced comparatively lower rainfall in the growing season. The exception (Kingsford, SA) was affected by powdery mildew, for which resistance is positively linked with high boron tolerance. Genotypes with dual sensitivity to both boron and salinity generally performed better at sites with higher growing season rainfall.
For sites where salinity tolerance was more important, the only sensitive genotypes that performed well were all early flowering (e.g. PBA Twilight). A single boron sensitive genotype (119) showed specific adaptation across sites at which boron tolerance was important, and is recommended as a key parent for improving general adaptation of the crop.

8.6 RECOMMENDATIONS FOR GENETIC IMPROVEMENT

The degree of salinity tolerance that was identified in *Pisum* is substantially greater than that present in commercial field pea crop varieties (Leonforte et al. 2012). However, selection for variation present in adapted germplasm appears to be sufficient to provide some moderate incremental gains in adaptation, especially if combined with higher boron tolerance when already available in elite germplasm (Leonforte et al. 2009). As the sources of germplasm with the highest tolerance are non-domesticated accessions that possess numerous deleterious traits, pre-breeding strategies will be required to first develop parental lines in order to exploit this variation more effectively in conventional breeding. Given the complex multigenic nature of salinity tolerance, as substantiated in this thesis, it would be prudent for pre-breeding efforts to focus on a recurrent selection strategies using parents that contribute different or varying responses to salinity stress. Based on findings presented in this thesis, important components of salinity tolerance for field pea are likely to include selection for lower Na\textsuperscript+ uptake, reduced plant toxicity affects (at seedling stage), higher plant growth potential particularly prior to flowering, beneficial changes in flowering response, lower abortion rates of flowers, ovules and pods and lower seed size loss.

The plant growth responses observed for pea germplasm in this study are typical of the biphasic salinity model described by Munns (2005). Despite this developmental complexity, routine pot screening conducted at an early seedling stage appears to provide a quick, repeatable, low-cost selection tool for advancement of germplasm in breeding programs. The identification of QTLs may further facilitate selection, but will probably be more useful at the
pre-breeding phase, in which germplasm is still highly morphologically diverse and may hence exhibit unpredictable responses to salinity screening.

8.7 POTENTIAL FOR FURTHER RESEARCH

While extensive screening was undertaken in this study to identify sources of higher tolerance, more intense investigation of germplasm originating from Greece is warranted to identify parental germplasm that is closer to domesticated types for pre-breeding. A total of 95 landrace accessions originating from Greece have been identified in the Australian Temperate Field Crops collection for such a study.

Relevant RILs and unselected and mass selected bulk populations have been developed during the course of this thesis as a basis for investigating different selection strategies for improving salinity tolerance in field pea. The RIL populations, in particular, provide an opportunity to screen for the presence of specific salinity tolerance QTLs associated with varying environments and from different genetic backgrounds. For example, assessment of RILs across field locations varying in sub-soil salinity over time may identify useful QTLs associated with yield response, and better quantify the value of higher tolerance to final grain yield. In addition, experiments in controlled environments could be undertaken to identify specific QTLs for different growth responses associated with varying salinity stress across different ontogenic periods. RIL populations could also be useful for validation of the contribution of oxidative defence systems or organic osmolytes to increased tolerance in pea. In the longer-term, a combination of genotyping systems based on high density genome-wide distributed SNPs and customised germplasm collections subjected to detailed phenotypic assessment may be applied to genome-wide association studies (GWAS), allowing identification of multiple QTLs of low magnitude from a broad range of genetic backgrounds.
The pyramiding of the higher salinity tolerance trait into already adapted boron tolerant elite pea germplasm will probably improve the adaptation of the crop in lower rainfall regions, as high soil B and salinity commonly co-exist in Australian cropping regions (Rengasamy 2006). Phenotypic screening of germplasm under combined high B and salinity stress may identify additional genes for improving crop adaptation.
References


homologous to the Kup system of *Escherichia coli* has a high concentrative capacity. *The European Molecular Biology Organization Journal* **14**, 3021–3027.


Schleyer M, Bakker EP (1993) Nucleotide sequence and 3′-end deletion studies indicate that the K⁺-uptake protein Kup from *Escherichia coli* is composed of a hydrophobic core linked to a large and partially essential hydrophilic C-terminus. *Journal of Bacteriology* **175**, 6925–6931.


Table 9.1. Mean salinity symptom scores based on a 1 (no symptoms) to 10 (dead) scale for 780 accessions screened during the vegetative period following application of NaCl. Australian commercial varieties are highlighted in bold.

<table>
<thead>
<tr>
<th>Mean salinity tolerance score</th>
<th>Accession: Australian Temperate Field Crops Collection ID number or variety name.</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>ATC07082, ATC07047, ATC01091, ATC06952, ATC07156, ATC07057, ATC06642</td>
</tr>
<tr>
<td>&gt;3.0 – 4.0</td>
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</tr>
<tr>
<td>&gt;4.0 – 5.0</td>
<td>ATC00872, ATC07141, ATC07074, ATC07053, ATC05658, ATC06914, ATC07021, ATC04265, ATC07204, ATC03362, ATC04059, ATC07200, ATC07115, ATC07164, ATC07060, ATC07043, ATC02703, ATC02702, ATC02707, ATC07140, ATC02549, ATC01479, ATC07067</td>
</tr>
<tr>
<td>&gt;5.0 – 6.0</td>
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<td>&gt;6.0 – 7.0</td>
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<tr>
<td>Mean salinity tolerance score</td>
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| ATC07133, ATC05762, ATC07103, ATC07218, ATC06634, ATC07133, ATC05762, ATC07103, ATC07218, ATC06634, ATC07133, ATC05762, ATC07103, ATC07218, ATC06634 | **Parafield**, ATC00762>
| ATC07168, ATC01502, ATC06893, ATC07202, ATC07185, ATC01036, ATC00931, ATC07160, ATC07273, ATC06636, ATC01088, ATC07040, ATC02444, ATC03489, ATC07032, ATC00742, ATC01042, **Bundi**, ATC03755, ATC03844, ATC06951, ATC00541, ATC07113, ATC06646, ATC06611, ATC05752, ATC05744, ATC07089, ATC01630, ATC07112, ATC03355, ATC07128, ATC03416, ATC00995, ATC03472, ATC07023, ATC02308, ATC00952, ATC02564, ATC03462, **Excell**, ATC01391, ATC07039, ATC01455, ATC01564, ATC02354, ATC02653, ATC04998, ATC02369, ATC01491, ATC07107, ATC06989, ATC04207, ATC07292, ATC07298, ATC07111, ATC05089, ATC07137, ATC07153, ATC03801, ATC03445, ATC07309, ATC00925, ATC07187, ATC07215, ATC04322, ATC02504, ATC02799, ATC07124, ATC07207, ATC07055, ATC02709, ATC02933, ATC07166, ATC07125, ATC06624, ATC07070, ATC07162, ATC04359, ATC01614, ATC06905, ATC06958, ATC02894, ATC01749, ATC01034, ATC02427, ATC01058, ATC01294, ATC02422, ATC07300, ATC00524, ATC00548, ATC04038, ATC04120, ATC06979, ATC02567, ATC04465, ATC07026, ATC00919, ATC07081, ATC02393, ATC04383, ATC06982, ATC07092, ATC02372, ATC05721, ATC02383, ATC02926, ATC00385, ATC06655, ATC02438, ATC01521, ATC07314, ATC06899, ATC07174, ATC04937, ATC03816, ATC01452, ATC06936, ATC01481, ATC01748, ATC00730, ATC00953, ATC00948, ATC05139, ATC06901, ATC07076, ATC05399, ATC05133, ATC06902, ATC05780, ATC07058, ATC02412, ATC01576, ATC05153, ATC02388, ATC00898, ATC07020, ATC01498, ATC01772, ATC0757, ATC01467, ATC02710, ATC03101, ATC00731, ATC01825, ATC07285, ATC02381, ATC01742, ATC05138, ATC01541, | >7.0 – 8.0

> 8.0 – 9.0
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| >9.0 - 10                   | ATC01405, ATC06649, ATC01550, ATC02918, ATC01026, ATC06897, ATC01387, ATC02544, ATC07196, ATC00930, ATC02385, ATC07313, ATC00921, ATC00962, **Yarrum**, ATC04107, ATC01651, ATC07093, ATC04229, ATC04181, ATC00940, ATC03980, ATC07199, ATC07286, ATC06997, ATC00903, ATC00945, ATC00949, ATC07280, ATC07097, ATC07171, ATC02389, ATC00967, ATC07109, ATC06966, ATC00721, ATC07306 ATC02364, ATC00922, ATC03454, ATC01223, **Sturt**, ATC01760, ATC02361, ATC01472, ATC02411, ATC06921, ATC05110, ATC03799, ATC07308, ATC00533, ATC01520, ATC06970, ATC06615, ATC00936, ATC07152, ATC03450, ATC01643, ATC06640, ATC06657, ATC04258, ATC00992, ATC07146, ATC07008, ATC00779, ATC06623, ATC04106, ATC07175, ATC01194, ATC02353, ATC07281, ATC06983, ATC01559, ATC0109, ATC03808, ATC07291, ATC06631, ATC03992, ATC00680, ATC07059, ATC06909, ATC07096, ATC00899, ATC06962, ATC01562, ATC03989, ATC05132, ATC02184, ATC04906, ATC01487, ATC03466, ATC02380, ATC01039, ATC02435, ATC00959, ATC00089, ATC01318, ATC02371, ATC07078, ATC00525, ATC06896, ATC07158, ATC02539, ATC03962, ATC06617, ATC07005, ATC05700, ATC06969, ATC06610, ATC07004, ATC02386, ATC07279, ATC06908, ATC03991, ATC03994, ATC06939, ATC00978, ATC00508, ATC02515, ATC03198, ATC06906, ATC01057, ATC03979, ATC00956, ATC02313, ATC00723, ATC02433, ATC07116, ATC01321, ATC00703, ATC00980, ATC00514, ATC02562, ATC06994, ATC06954, ATC04040, ATC01350, ATC00537, ATC02540, ATC02538, ATC02524, ATC01217, ATC01052, ATC01553, ATC06625, ATC03456, ATC00538, ATC01454, ATC00759, ATC06910, ATC02362, ATC00574, ATC06990, ATC06973, ATC07151, ATC04471, ATC01295, ATC05140, ATC02558, ATC01469, ATC02359, ATC04621, ATC06894, ATC00247, ATC04070, ATC01028, ATC06626, ATC04472, ATC01246, ATC01086, ATC02539, ATC02390, ATC00760, ATC01283, ATC00916, ATC01732, ATC07303, ATC04233, ATC00950, ATC02384, ATC03805, ATC07296, ATC02363, ATC00926, ATC03096, ATC03754, ATC00049, ATC07012, ATC04235, ATC01030, ATC04489, ATC02568, ATC02395, ATC06622, ATC00535, ATC02375, **Moonlight**, ATC06974, ATC04257, ATC00176, ATC02392
Table 9.2. Trait variation for Australian field pea cultivars and advanced the breeding line 119 also highlighted on the canonical biplot (Fig. 6.12).

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<th>Genotype</th>
<th>Boron tolerance</th>
<th>Salinity tolerance</th>
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<th>Leaf type</th>
<th>Flowering time</th>
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<th>Downy mildew</th>
<th>Bacterial blight</th>
<th>PSbMV</th>
<th>BLRV</th>
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<td>Ta</td>
<td>C</td>
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<td>R</td>
<td>S</td>
<td>MS</td>
<td>S</td>
<td>S</td>
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<td>MT-T</td>
<td>SD</td>
<td>SL</td>
<td>Early to Mid</td>
<td>R</td>
<td>MR</td>
<td>S</td>
<td>R</td>
<td>MR-R</td>
</tr>
<tr>
<td>PBA Gunyah</td>
<td>S</td>
<td>MT</td>
<td>SD</td>
<td>SL</td>
<td>Early</td>
<td>S</td>
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<td>MS</td>
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<td>S</td>
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<td>MR-R</td>
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<td>S</td>
<td>MR</td>
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<tr>
<td>PBA Oura</td>
<td>S</td>
<td>S</td>
<td>SD</td>
<td>SL</td>
<td>Early</td>
<td>S</td>
<td>MR</td>
<td>MR</td>
<td>S</td>
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</tr>
<tr>
<td>PBA Pearl</td>
<td>S</td>
<td>S</td>
<td>SD</td>
<td>SL</td>
<td>Early to Mid</td>
<td>S</td>
<td>MR</td>
<td>MS</td>
<td>S</td>
<td>MR</td>
</tr>
<tr>
<td>PBA Twilight</td>
<td>S</td>
<td>S</td>
<td>SD</td>
<td>SL</td>
<td>Early</td>
<td>S</td>
<td>MR</td>
<td>S</td>
<td>S</td>
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</tr>
<tr>
<td>119</td>
<td>S</td>
<td>S</td>
<td>SD</td>
<td>C</td>
<td>Mid</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

S = Sensitive or susceptibility, MS= Moderate sensitivity or susceptibility, MT = Moderate tolerance, T = Tolerant, MR = Moderate resistance. Ta = Long internodes or tall plant type, SD = Shorter internodes or semi-dwarf, C = Leaflets present on tendril, SL = Leaflets absent on tendril. Downy mildew rating: Based on average response across pathovar’s. BLRV= Bean leaf roll virus, PSbMV = Pea seed borne mosaic virus.
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