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The role of calcium and potassium in salinity tolerance of Brassica rapa L. cv. RCBr seed.

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The Role of Calcium and Potassium in Salinity Tolerance in *Brassica rapa* L. cv. RCBr Seed

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'The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I've found it!), but 'That's funny...'

Isaac Asimov (1920-1992)

Abstract

The possibility of manipulating calcium (Ca^{2+}) and potassium (K^{+}) levels in seeds of *Brassica rapa* by altering parent plant nutrition and investigating the potential for increased salinity tolerance during germination, given that considerable amounts of literature imply that greater amounts of available exogenous Ca^{2+} and K^{+} can ameliorate the effects of salinity on both whole plant growth and germination, was evaluated.

The investigation consisted of four growth trials. Two preliminary growth trials suggested that seed ion manipulation was possible without affecting the overall growth and vigour of the plant. After developing suitable high and low Ca^{2+} and K^{+} nutrient solutions for growth, a trial was carried out in a growth room and greenhouse, with various substrates and the seed of a certain size category was collected for subsequent ion and salinity tolerance analysis. Seed Ca^{2+} and K^{+} was significantly affected by growth substrate and nutrient solution and data showed that a significant negative regression relationship existed between seed Ca^{2+}, K^{+} and Ca^{2+} + K^{+} levels and salinity tolerance. Further experimentation using hydroponic culture attempted to remove any possible effects of substrate and also to compare size categories of seed with a view to elucidating localisation of Ca^{2+} and K^{+}. Seed Ca^{2+} was found to be significantly altered by nutrient solution in the two different sizes tested and higher Ca^{2+} nutrient solution was found to increase salinity tolerance in daughter seed. One significant negative regression correlation between salinity tolerance and seed K^{+} concentration existed in smaller seed, but disregarding seed size in a regression analysis of seed ion content and salinity tolerance, a significant negative relationship existed between seed Ca^{2+}, K^{+} and Ca^{2+}+ K^{+}. The results, especially in terms of Ca^{2+} nutrition, contradict much previous research that suggests increased salinity tolerance at germination can arise with the increased presence of Ca^{2+} and/or K^{+}. Salinity tolerance was greater in seeds of larger size across all nutritional treatments and the smaller size range exhibited increased Ca^{2+} and K^{+} per μg seed.

Ca^{2+} concentration in smaller seeds with greater surface area:volume ratios provided a clue to the potential localisation of Ca^{2+}. Cross sectional staining showed that a greater proportion of seed Ca^{2+} may reside in the coat. This was confirmed by

analysis which showed an approximate 50% split of total extractable seed Ca^{2+}, regardless of size, between coat and embryo within a seed; the majority of which, per μg, resides in the coat. Further work looked at the relative solubility of the Ca^{2+} and K^{+} in these tissues and whole seed to look at the potential bioavailability of Ca^{2+} during germination from various parts of the seed. Most water soluble Ca^{2+} exists in the embryo and most insoluble Ca^{2+} exists in the coat, but coat Ca^{2+} was found to be ionically exchangeable and therefore bioavailable. K^{+} appeared mostly water soluble in embryo and coat. In line with previous whole plant research in this species, most Ca^{2+} is readily water soluble or ionically exchangeable in form and the possible negative effects of how increasing bioavailable Ca^{2+} may reduce salinity tolerance was discussed.

Chapter 1: The Role of Calcium and Potassium in the Germination of *Brassica rapa* L. cv. RCBr Seed: General Introduction, Aims and Objectives

1.1 Salinity – An Introduction

Salinity in relation to agriculture has been defined as the occurrence of a high concentration of soluble salts in the soil or solution in which plants grow, the origins of these salts being traced back to oceanic influences (Flowers and Yeo 1986). As in seawater, the dominant ions involved in agricultural salinity are sodium (Na^+) and chloride (Cl^-), although saline conditions can be induced by a wealth of other ions including calcium (Ca^{2+}), potassium (K^+), sulphate (SO_4^{2-}), carbonate (CO_3^{2-}). Saline soils can be characterised by low activity of nutrient ions and by extreme ratios of Na^+/Ca^{2+}, Na^+/K^+, Ca^{2+}/Mg^{2+} (magnesium) and Cl^-/NO_3^- (nitrate) (Grattan and Grieve 1992, 1999b).

Saline environments are widespread with approximately 1.0 billion ha of the 14 billion ha of agricultural land available worldwide affected by excess salt and a further 6.0 billion ha classified as arid or semi arid (Christiansen 1982). Ghassemi, Jakeman, and Nik (1995), Janmohammadi, Dezfuli, and Sharifzadeh (2008) and Taalab, Okashi, and Aisha (2008) report this may have increased to between 20 and 27% of the world's irrigated lands and would account for 6% of the world's land area (Flowers and Yeo 1995, Munns and Tester 2008, Flowers, Galal, and Bromham 2010). Such environments originate predominantly from natural and man-made sources (Ashraf 1994). Salinity remains one of man's oldest problems, with historical records indicating a shift from the cultivation of wheat to more salt tolerant barley in the fertile, poorly drained and therefore increasingly saline soils of the Tigris-

Euphrates basin of ancient Mesopotamia (4000-2000 BC) (Downton 1984, Pitman and Läuchli 2004).

The natural weathering action on rocks and mineral deposits produces soils that contain concentrations of salt that affect crop production. Soils of coastal regions may receive salt as a result of sea spray or may come into direct contact with sea water within coastal marshland (Ashraf 1994).

Arid and semi arid regions often produce soils with high concentrations of salts at the surface, particularly where irrigation systems have been used for agriculture. Such soil conditions are caused by a collective distribution of salts in the soil due to high water tables and insufficient quantities of irrigation water which would otherwise leach the salts out of the soil (Ashraf 1994). The increasing area of land affected by salinity due to this phenomenon has brought into question the long term viability of irrigated agriculture (Casey 1972, Ghassemi, Jakeman, and Nik 1995, George, McFarlane, and Nulsen 1997, Ragab 2010) and with increasing global temperatures in recent years (Hansen *et al.* 2006), salt affected lands around the world are increasing (Flowers, Galal, and Bromham 2010).

There are two primary strategies to overcome the problem of soil salinity: reclamation by chemical amendments and the associated engineering involved, or by using the saline soils to grow salt tolerant plants (Ashraf and McNeilly 2004, Purty *et al*. 2008). Combating the effects of salinity by engineering is costly, with significant effort required to reclaim land and ensure that salt balances are maintained. Such procedures include recontouring land by deep ripping and land planning, installing of agricultural drains, pumping and conveyance of irrigation water and disposal of drainage water or desalinisation of water (Downton 1984). With increasing costs and economic losses

involved in such solutions, together with the competition for high quality water between agriculture and growing urban populations, it is important that different approaches are explored in terms of plant improvement (Kent and Läuchli 1985, Läuchli and Grattan 2007).

This investigation aimed to extend the data on salt stress of seed obtained by Rehman *et al*. (1996, 1998a, 1998b, 2000) in the genus *Acacia*, utilising the rapid life cycling *Brassica rapa* L. cv. RCBr (syn. *Brassica campestris* L). The work with *Acacia* suggested that the total seed Ca^{2+} and K^{+} concentrations, ratio and susceptibility of leaching of ions into water and saline solutions, could account for salt tolerance at seed germination. By using *B. rapa*, with rapid life cycle and practical seed size as a model crop plant, the effect of parent plant Ca^{2+} and K^{+} nutrition can realistically be investigated, with the resultant seed being assessed for ion content and salt tolerance. There appears to be little published work that investigates the effect of parent plant nutrition on salt tolerance at germination, yet there is a wealth of evidence that suggests externally applied Ca^{2+} ameliorates salt stress in a number of different species at the whole plant scale and during germination, along with evidence that Ca^{2+} may have a role in the initiation of plant salt stress responses. Salt stress has also been shown to have a negative effect on endogenous K^{+} levels and there is also evidence to suggest that Ca^{2+} and K^{+} may have relationships in potential pathways of the stress response.

The objectives of this research were:

- To develop a range nutritional solutions varying in Ca^{2+} and K^{+} concentrations suitable for the growth of *B. rapa* that produced plants with seed of varying

Ca^{2+} and K^+ concentration, that is likely to be attributable to the nutritional treatments administered.*

- To investigate whether the Ca^{2+} and K^+ nutrition of the parent plant affects the respective resultant seed ion concentration, by means of analysis with atomic emission spectroscopy.

- To establish whether seed Ca^{2+} and K^+ concentration influences the salt tolerance of *B. rapa* during germination, and if so, what relationships exist in terms of seed Ca^{2+} concentration, K^+ concentration, Ca^{2+}+ K^+ concentration or Ca^{2+}/K^+ ratio.

- Based upon whether seed Ca^{2+} and K^+ concentration influences the salt tolerance of seeds of *B. rapa*, to try to elucidate the potential site of any salt tolerance mechanisms and look at certain mechanisms in terms of Ca^{2+} and K^+ availability in certain tissues.

* Ideally the morphology of the plant would not be different between treatments. Nutritional effects on the mother plant that are adversely negative may affect key physiological processes such as photosynthesis, which may subsequently indirectly affect the distribution and loading of Ca^{2+} and K^+. Aspects of the parent plant morphology were tested, such as the maximum height obtained, percentage of plants which flower, time taken to flower and the number of flowers produced, as well as the physiological aspects of the seed produced that includes parameters such as seed number and weight.

Chapter 2: Literature Review

2.1 Salinity and Plants

> *Of all of the inhibitory substances that plants may encounter in their natural chemical environment, none impairs or prevents their growth on so large a scale as salt.* (LaHaye and Epstein 1969)

The plant kingdom is divided into two distinct groups in relation to their responses to saline habitats. Those species whose growth is restricted in conditions of high salinity are termed 'glycophytes', which includes the majority of global crop plants ancestors (Greenway and Munns 1980), and those plants that have developed evolutionary strategies to deal with saline habitats, tolerating high levels of salt and growing best under low-moderate saline conditions are termed 'halophytes'. The two groups are not clearly distinguished and this may arise from the fact that the growth responses of some cultivated glycophytic species overlap with that of halophytes. This should not be surprising considering that some species, *Beta vulgaris* (sugar beet) as an example, have halophytic ancestors (Greenway and Munns 1980, Daoud *et al*. 2008).

The toxicity of ions varies, and some may be more toxic to a particular species than others, but increases in soil salinity generally cause a reduction in plant growth and yield. It was originally thought that the reduced growth effects of salinity were caused by a direct influence of ions on photosynthesis; but literature suggests that the influence of salinity on photosynthesis itself is not great enough to account for such reductions in growth that are seen in plants exposed to saline conditions. According to Grattan and Grieve (1992, 1999b), Läuchli and Grattan (2007) and Munns and Tester

(2008), salinity disrupts the mineral nutrient acquisitions of glycophytes in two ways. Firstly, the ionic strength of the substrate can impose a direct effect upon the nutrient uptake and consequent translocation (osmotic effect) and secondly, the interaction of major ions in the substrate (Na^+ and Cl^-) on nutrient ion acquisition and translocation within the plant. Na^+ and Cl^- can influence nutrient absorption by competitive interaction or by affecting the ion selectivity of membranes (ionic effect). Greenway and Munns 1980, Munns and Termaat (1986) and Munns and Tester (2008) also suggest that there are two phases of response to salinity by plants. The first involves a period of water deficit via an osmotic effect, followed by a period of salt toxicity (ionic effect), which corresponds to long term exposure.

2.2 Salinity Tolerance

Salinity tolerance can be defined as the ability of a plant to withstand the effects of high concentrations of salts, mainly NaCl at the root and to grow and complete its life cycle without inducing a significant reduction in growth and metabolism (Shannon and Grieve 1999). Saline environments can affect plants in a number of ways, but two specific phases are clear. When plants encounter salinity, growth is primarily reduced by an effect of water stress. The concentration of salts at the root make it difficult for the plant to take up water osmotically, since the water potential outside the plant is lower than the water potential inside root cells. As salt is taken into the plant, salt specific reduction in growth appears, as toxic levels affect the mechanisms of metabolism. This is generally seen in older leaves, which die due to rapid rises in salt concentration in the cytoplasm when the plant can no longer sequester an intake of salt.

Natural selection has allowed halophytic species to colonise areas that would be inaccessible to glycophytes, a situation which eliminates competition for habitat. Halophytic species may adapt to their environment via a number of methods. The main evolutionary mechanisms that have developed in halophytes are salt exclusion, reabsorption and relocation, salt extrusion, salt dilution, salt compartmentation, tissue salt tolerance and Crassulacean Acid Metabolism (CAM) photosynthesis (Orcutt and Nilsen 2000).

Plants that exhibit salt exclusion are able to maintain a stable ion concentration in cell cytoplasm, by excluding ions across membranes, at the same rate at which they are introduced or at least reducing the rate of influx and increasing the efflux (He and Cramer 1993a).

Salt reabsorption and retranslocation involves a mechanism whereby ions that have passed into the transpiration stream are selectively accumulated by parenchyma cells in the xylem and retranslocated back to the roots via the phloem. A particularly good example of this mechanism is found in *Phragmites communis* (North American reed), where phloem cells in the shoot base actively reabsorb Na^+ from the xylem stream (Matsushita and Matoh 1992).

Plants which have developed techniques of salt extrusion possess physiological structures on the surface of leaves known as salt glands or salt bladders. Salt glands consist of a group of cells, containing high numbers of mitochondria and vacuoles that are covered in cuticle in order to separate them from the rest of the plant. Based on structure, two types of glands can be distinguished; two celled glands of the grasses and multicellular glands of dicotyledonous plants. These glands secrete salt and water through holes in the cuticle to the exterior environment, where wind and/or rainfall

can carry salt away from the plant (Jacoby 1999). Salt bladders or bladder hairs consist primarily of two cells, a stalk cell and a bladder cell. The stalk cell transfers salt to the bladder cell which contains a large vacuole. The bladder cells store the salt and eventually rupture and die, at the same time expelling salt to the exterior surface of the leaf.

Salt dilution relies upon the theory that salt can be diluted effectively and sufficiently within cells, so that the concentration does not become toxic to the plant and cause interference with cellular metabolism. Succulent plants have very large vacuoles, as displayed by their morphology, and this enables them to store greater concentrations of salt before the overall concentration of the vacuole becomes toxic (Jennings 1968, Hegenmeyer 1997).

Salt compartmentation away from the metabolically active areas of the cytoplasm is thought to involve the accumulation of salts in the central vacuole of cells, but such a mechanism has limitations in terms of long term adaptation, such as significant reduction in growth. However, Leigh and Storey (1991) claim that the compartmentation of nutrients between vacuole and cytoplasm is an important determinant of the response of plants to salinity. Li *et al*. (2008), Munns and Tester (2008), Chaves, Flexas, and Pinheiro (2009), Ma *et al*. (2010) and Conn and Gilliham (2010) suggest this too. Indeed, experiments have established that the ability of halophytes to withstand high concentrations of NaCl within their cells was not the result of modification of enzymes which allowed them to operate in the presence of high concentrations of NaCl, since enzymes isolated from both halophytes and glycophytes show essentially the same sensitivity to NaCl *in vitro* (Flowers, Troke, and Yeo 1977, Wyn Jones and Pollard 1983, Glenn, Brown, and Blumwald 1999,

Munns 2002, Wakeel *et al*. 2011). It was therefore suggested that the NaCl was compartmented in the vacuole where it would have no effect on the activity of salt sensitive enzymes which are located in the cytoplasm (Wyn Jones *et al*. 1977, Wyn Jones, Brady, and Spiers 1979, Zhu 2003, Conde, Chavas, and Gerós 2011).

Tissue salt tolerance comprises two requisites: compatible solute accumulation and membrane stability. Compatible solute accumulation involves the synthesis or acquisition of compounds that cause an osmotic adjustment in tissues, so that the solute potential between the vacuole and cytoplasm remains in as constant an equilibrium as possible. Examples of compatible solutes include sucrose, proline and glycine betaine. Polyols have also been implicated (Noiraud, Maurousset, and Lemoine 2001, Chen *et al*. 2007a). These organic compounds are non toxic and compatible with the cells' cytoplasmic components, not inhibiting important cellular mechanisms such as enzyme activity (Greenway and Munns 1980). These compounds may also have a protective role and although there is contradictory evidence for this in terms of whether they are merely indicative of salt tolerance, or actively involved in improving tolerance (Delauney and Verma 1993), it has been found that they contribute detoxification of reactive oxygen species, protection of membrane integrity and stabilisation of enzymes/proteins (Ashraf and Foolad, 2007). The stability of membranes in terms of their permeability and fluidity is important in maintaining metabolism during periods of exposure to salinity. Membrane composition is different in halophytes than in glycophytes and halophytic membranes contain higher quantities of membrane sterols, glycolipids, phospholipids and short chain fatty acids (Orcutt and Nilsen 2000).

Sucrose has been found to have a role in the protection of chloroplasts against injury

during desiccation and proline increases the solubility of proteins (Greenway and Munns 1980). Adams *et al*. (1992) showed accumulation of these solutes is a product of communication between different tissues and that this is required to produce the complete stress response, since the mechanism works *in vivo* but not *in vitro*. Compatible solutes may also be involved in scavenging reactive oxygen species (Chen and Murata 2002) produced within photosystems under salinity stress (primarily photosystem II) (Papageorgiou and Murata 1995, Bowler and Fluhr 2000), which may similarly account for the protection of chloroplasts by sucrose described by Greenway and Munns (1980), as sucrose may modify reactive oxygen species defence and repair mechanisms, signal transduction and cellular communication (Couée *et al*. 2006, Ramel *et al*. 2009). Murata *et al*. (1992) also reported that glycine betaine protected photosystem II complex by stabilising the association of the extrinsic proteins under salt stress. Minimising damage caused by reactive oxygen species under saline stress with K^+ has also been suggested, by reducing reactive oxygen species formation during photosynthesis and inhibiting activation of reactive oxygen generating NADPH oxidase (Cakmak 2005). Betaine and proline have also been found to have an ameliorating effect upon K^+ loss from root cells due to NaCl (Cuin and Shabala 2005), and so there may be a relationship between compatible solute production, K^+ maintenance and protection under saline stress. Attempts to engineer compatible solutes through genetic manipulation of enzyme activity have been successful, and with work by Roosens *et al*. (2002) with *Nicotiana plumbaginifolia* (tobacco) that conferred accumulation of proline, improved NaCl and mannitol tolerance, higher biomass and germination rate under osmotic conditions. Indeed, Savouré *et al*. (1995) found that exposure of *Arabidopsis thaliana* to salt and drought stresses causes an accumulation of proline and the first enzyme in the

biosynthetic pathway (Δ^1-pyrroline-5-carboxylate synthetase), encoded by gene *At-P5CS*, is strongly regulated by drought and salinity stress. However, the synthesis and distribution of these compounds generally results in lower vegetative growth at the expense of survival. Other genetic work to produce glycine betaine has conferred greater tolerance in species that would not otherwise produce it. However, compared with species that naturally produce it, the production of glycine betaine is still less and confers less salt tolerance (Rhodes and Hansen 1993, Ashraf and Foolad 2007). The benefit of such manipulation is dependent upon the increased tolerance, that may be conferred upon higher value crops that do not naturally produce glycine betaine.

CAM photosynthesis is an adaptation to arid conditions. Initially found in Crassulaceae, the photosynthetic pathway is found in over 29 families and 338 genera of flowering species (Smith and Winter 1996, Holtum *et al*. 2007) and at least 16,000 species in total, with this considered an underestimation (Dodd *et al*. 2002). The mechanisms of photosynthesis are temporally separated between day and night, with the suppression of transpiration during the day caused by stomatal closure and the fixation of CO_2 at night when transpiration rates are much lower. Genetic responses initiate the CAM pathways after approximately ten days of stress (Winter and Gademann 1991). The possible change to CAM can occur only after a juvenile stage, when the plant can produce leaves, flowers and seeds (Cushman, Michalowski, and Bohnert 1990). In the extreme halophytes, CAM is a normal developmental occurrence, even in unstressed situations, but CAM expression occurs at a much faster rate in species where plants are subjected to high salinity or drought.

Plant growth hormones have also been implicated in the mechanism of salinity tolerance, controlling reductions in growth associated with salinity. In general, an

increase in salinity is correlated with a decrease in auxin, cytokinin and gibberellin levels in tissues and an increase in abscisic acid (ABA). Jacoby (1999) discusses that ABA accelerated the adaptation of cultured tobacco cells to high salt concentrations (LaRosa *et al*. 1985) and enhanced the synthesis of 26 kD proteins, thought to be adaptive (Singh *et al*. 1987). Also, the number of days needed for the adaptation of sorghum plants in the presence of 150 mM NaCl was decreased from twenty to ten in the presence of ABA (Jacoby 1999). It has also been suggested that ABA may play a specific role in membrane stabilisation by inducing its own set of proteins as an adaptive response of plants to salinity (Maslenkova, Zanev, and Popova 1993, Zhang *et al*. 2006, Dalal *et al*. 2009). Genetic isolation of gene *btg*-26 in *Brassica juncea* (mustard) by Stroeher, Booth, and Good (1995), which is involved in adaptation to osmotic (water) stress, gives an insight into the genetic basis and potential signalling pathways in such responses. The expression of the gene was increased six-fold in plants at 81% relative water content and eleven-fold at 63%, compared with plants that were fully hydrated. The gene was also activated under high salinity, low temperature, heat shock and ABA, indicating that *btg*-26 encodes for a protein that is involved at the primary stages of general osmotic stress and specific adaptation to saline environments, even within a species, may still rely on complex pathways and a number of genes. *btg*-26 may be one of the dehydration-induced genes, of which Bartels and Sunkar (2005) state are mostly controlled by ABA. Dalal *et al*. (2009) also found that ABA induces specific late embryogenesis abundant (LEA) proteins in seeds that are key in drought and stress tolerance and are speculated to retain water molecules and prevent crystallisation of cellular components under water deficit (Park *et al*. 2005). ABA may also have a role in the production of compatible solutes, as

ABA has been found to be partly involved in regulation of expression of the aforementioned *P5CS* gene involved in proline biosynthesis (Xiong *et al*. 2001).

To be able to maintain internal turgor is perhaps one of the most important factors for a plant to maintain growth under saline conditions. This osmotic adjustment is accomplished by the uptake and distribution of ions, chiefly K^+, Na^+ Ca^{2+} and Cl^-, (Flowers and Yeo 1986, Cerda *et al.* 1995, Jacoby 1999, Chen and Jiang 2010), as well as the synthesis of organic compatible metabolites. Such ions are taken up in an attempt to provide an osmotic balance so that excessive amounts of water do not move out of the plant towards areas of lower osmotic potential, therefore maintaining cell turgidity. Past research has implied that these ions were largely restricted to the vacuoles (Flowers, Troke, and Yeo 1977, Wyn Jones *et al*. 1977, Munns, Greenway, and Kirst 1983, Stewart and Ahmad 1983), with the osmotic potential of the cytoplasm being adjusted with organic compatible solutes (Stewart and Lee 1974, Storey and Wyn Jones, 1975), although such osmotic adjustments are now known to involve a combination of the two and the vacuole has a role as both a nutrient sink and in the compartmentation of toxic ions or molecules (Conn and Gilliham 2010). Patterns of such ion accumulation in vacuoles have been used to discriminate between salt tolerant and salt sensitive species (Shannon and Grieve 1999, Munns 2002) and the specific roles of vacuoles appear to depend upon tissue, cell type and development (Martinola, Maeshima, and Neuhaus 2007).

The accumulation of these ions for the purposes of regulating turgor is limited by a plant's susceptibility to the toxicity of high salt concentrations. This cytoplasmic toxicity is ubiquitous in all eukaryotes and most bacteria, with the only exception being the ancient halophilic halobacteria, which accumulate K^+ and Cl^-, but not Na^+ to

concentrations of several moles l^{-1} (Jacoby 1999). Salt is located in the cytoplasm of these bacteria and the enzymes are adapted to high salt concentrations. However, enzymes extracted from salt adapted halophytes are severely inhibited *in vitro* at salt concentrations that would otherwise be optimal for the growth of the plants from which they are sourced (Jacoby 1999, Tester and Davenport 2003). *In vitro* isolation of enzymes from halophytes and non halophytes show similar sensitivities to NaCl and this may be suggestive that the degree of salt tolerance is dependent upon salt compartmentation and not evolutionary changes in enzymatic function (Munns and Tester 2008). Alon*g* with Flowers, Troke, and Yeo (1977), Wyn Jones and Pollard (1983) and Greenway and Munns (1980) also stated that enzymes from halophytes and non-halophytes have similar sensitivities to electrolytes and wondered if this could also apply to protein synthesis.

Aquaporins, hydrophobic proteins that facilitate osmosis by forming pores through the lipid bilayer through which water passes, rather than through diffusion (Bartels and Sunkar 2005), have also been implicated in control of water flow of cells and tissues during exposure to drought and saline stress and may be involved in the fine tuning of water availability. Four sub families have been characterised: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs) and small basic intrinsic proteins. The PIPs are further divided into two subclasses: PIP1 and PIP2 (Vandeleur *et al*. 2009). The proteins are located in the tonoplast and their structure is similar across plant species, having similar genetic sequences (Bartels and Sunkar 2005). The precise action of aquaporins remains unclear, however, with reports that dehydration and salt stress induces expression and this may cause greater water permeability and also reports that dehydration and salt stress causes the down regulation of aquaporins to allow for

cellular water conservation. López-Pérez *et al*. (2009), for example, found that although salinity increased PIP1 and PIP2 expression, there was no effect on water permeability in *Brassica oleracea* (broccoli) and down regulation of aquaporin activity has been reported (Mahdieh *et al*. 2008). Research has also suggested that aquaporins also transport molecules besides water, which include, NH_3^+, NH_4, boric acid, hydrogen peroxide, urea, glycerol, formamide, silicon, CO_2 and arsenite (Vera-Estrella and Bohnert 2011) and that aquaporins may be regulated by exogenous ABA (Ruiz-Lozano 2009) or by ABA and GA that activates the gene promoter for aquaporin expression (Siefritz *et al*. 2001).

The effect of increased salinity on photosynthesis has shown that photosynthetic membranes can become tolerant to salt stress. Maslenkova, Zanev, and Popova (1993) have stated that thykaloids isolated from plants that have been subjected to prolonged exposure to salinity, showed an increased tolerance towards high concentrations of NaCl after *in vitro* incubation. This was accounted for by an increase in resistance to salinity by the thykaloid membranes, most probably due to changes in the thykaloid membrane structure. Indeed, polypeptide composition of thykaloid membranes differ between salt sensitive and tolerant cultivars of the same species (Wang *et al*. 2009). In essence, prolonged salt treatment leads to clearly expressed adaptation to salinity stress due to reorganisation of membrane structures and a probable change in plant hormone levels such as that of ABA (Maslenkova, Gambarova, and Zeinalov 1995). This mechanism may also account for the results found by Salama *et al*. (1994), where the chloroplasts of salt tolerant *Triticum aestivum* (wheat) cultivars were seemingly not exposed to excess Na^+, whereas those of sensitive ones were.

It has been observed that salinity can have an effect upon the morphology of cells (Hameed *et al*. 2010). This may be a direct influence of salinity that negatively interferes with growth and development, or it may a mechanism whereby tolerance to salt may by increased a little more by restriction of uptake rate.

In cellular studies by Cachorro *et al*. (1995), structural changes were observed in root cells of *Phaseolus vulgaris* (common bean) under exposure to 80 mM NaCl. Roots were shorter and secondary roots appeared less developed. The shape and distribution of cells was affected, compared with control plants, with membrane vesiculation often present and increasing in size and frequency as length of exposure to NaCl increased. The vesicles appeared to originate from the plasmalemma, which was itself found to be separated from the cell wall at the cell periphery and had increased in surface area. Root cell structural changes were also found by Zidan, Azaizeh, and Neumann (1990) with *Zea mays* (maize), with reduced cell elongation and cell production.

Perhaps one of the most fundamental findings from salinity tolerance studies is that tolerance at one stage of growth is rarely related to tolerance at another (Johnson, Smith and Dobrenz 1992, Rogers *et al*. 1995, Shannon and Grieve 1999, Zeng, Shannon and Grieve 2002, Ashraf and Harris 2004, Flowers 2004, Foolad 2004, Cuartero *et al*. 2006, Ashraf *et al*. 2008). Tolerance of a given species to salinity varies across the different stages of growth. It is therefore important to examine tolerance over a number of growth stages (Rogers *et al*. 1995). Mechanisms of growth are obviously very different at various stages and it is therefore very difficult to be able to compare tolerance at one growth stage with another. Bernstein and Hayward (1958) stated that it is difficult to compare tolerance during germination with later stages because of the dissimilarity of criteria employed in the evaluation of tolerance.

Seedling salt tolerance may be evaluated by height growth, while germination salt tolerance may be measured by germination percentage, germination and growth may be affected by salinity quite differently.

There are also environmental factors that have significant interaction with salinity. Factors such as temperature, wind, humidity, light, pollution, and elevated carbon dioxide (CO_2) and ozone (O_3) levels all may affect how tolerant a certain species is at a certain stage of growth (Shannon and Grieve 1999). The ability to synthesize and/or accumulate metabolites that offer protective properties at later stages of development may enhance tolerance over earlier stages. Other thoughts on salinity tolerance include theories of the use of common defence mechanisms, that utilise common signalling systems for a range of abiotic stresses. Apart from salinity, levels of intracellular Ca^{2+} have been implicated in a number of stresses that include chilling, heat shock, anaerobic stress and drought (Trewavas and Malhò 1998), ethylene production, responses to pathogens and responses to reactive oxygen species (Bowler and Fluhr 2000, Reddy *et al*. 2011). Such cross tolerance, although useful as information that enables breeding for multiple tolerance in agricultural species and can be characterised by specific types of signal (McAinsh and Hetherington 1998, Knight and Knight 2001, White and Broadley 2003, Lecourieux, Ranjeva, and Pugin 2006, Mazars *et al*. 2011) and channels through which it passes (Bowler and Fluhr 2000, Agarwal and Zhu 2005), does complicate the study and elucidation of any cause and its effect at the whole plant and tissue level. McAinsh and Hetherington (1998) and Bartels and Sunkar (2005) do suggest that the capacity for specificity of intracellular signalling depends on the nature of the stimulus, the nature of ionic oscillations from the cells initiating the signal in terms of amplitude and frequency, cellular location of the initiated signal and the susceptibility of target effectors to the

generated incoming signalling, but "cross talking" (i.e. common components of different signal transduction pathways exist for several different stimuli) between stimuli and effect is not discounted. Plieth (2001) suggested that a form of the $[Ca^{2+}]_{cyt}$ signature could be wholly responsible for a defined physiological response was doubtful given that temperature manipulation could elicit almost any $[Ca^{2+}]_{cyt}$ perturbation and osmotic and salt stress produced similar $[Ca^{2+}]_{cyt}$ signatures (Knight, Trewavas, and Knight 1997), suggesting that other factors may be involved.

The genetic character, controlled by many genes and their interaction, and complex nature of the mechanisms of salinity tolerance (Shannon and Noble 1990, Shannon 1996, Grattan and Grieve 1999a, Ashraf and Harris 2004) have made the subject difficult to elucidate fully. Indeed, despite a wealth of published research on salinity tolerance, neither the metabolic sites at which salt stress damages plants nor the adaptative mechanisms utilised by plants to survive are well understood (Ashraf and Harris 2004), but with use of molecular biotechnology and greater understanding of genetics, there is at least a hope that fundamental mechanisms across a wide range of important crop species can be identified.

2.3 Salinity Tolerance, Calcium and Potassium

Na^+ dominated soils or solutions reduce K^+ and Ca^{2+} uptake by plants and/or affect their internal distribution (Grattan and Grieve 1992), which may suggest that the ability of plants to maintain sufficient Ca^{2+} and K^+ may have a significant effect on the degree of salt tolerance of plants (He and Cramer 1993a). Ca^{2+} and K^+ are heavily linked with the pathways of salt toxicity and mechanisms of salt tolerance and this may be due to their vital nutritional and physiological roles in plant metabolism.

Flowers, Troke, and Yeo (1977), Grattan and Grieve (1999b), Izzo, Incerti, and Bertolla (2008) and Grewal (2010) state that the uptake and translocation of Ca^{2+} and K^{+} are greatly reduced by salt stress.

2.3.1 Calcium

Ca^{2+} is essential in processes that preserve the structural and functional integrity of the plant membranes (Hanson 1984, Dudeck, Peacock, and Wildmon 1993, Ashraf 1994, Hirschi 2004, Renault 2005, Tuna *et al*. 2007, Dayod *et al*. 2010). Ca^{2+} serves to stabilise cell wall structures by binding phosphate and carboxyl groups of phospholipids at the membrane surface (Legge *et al*. 1982), regulates ion transport, controls ion exchange behaviour and cell wall enzyme activities (Demarty, Morvan, and Thellier 1984). Ca^{2+} has also been implicated in a number of functions. These include; plant hormone action, cation transport, auxin transport, abscission, senescence, ultrastructural membrane alterations, leaf movement, phototaxis, chloroplast movement and pollen tube growth. It is a regulatory component of mitosis (Vos *et al*. 2000, Tuteja 2009) and is involved in the regulation of enzymic activities through its affiliation with the protein calmodulin (Marmé 1983). Ca^{2+} also plays a multitude of roles at the membrane level. It has been proposed that the alleviating action of Ca^{2+} may be due to maintenance of integrity and function of the plasma membrane in roots and shoots and that supplemental Ca^{2+} may counteract the effects of Na^{+} (Leopold and Willing 1984, Rengel 1992, Cachorro, Ortiz, and Cerda 1994) in the sense that Na^{+} apparently competes with Ca^{2+} for uptake (Lynch, Cramer, and Läuchli 1987). Leopold and Willing (1984) go on to state that the monovalency of Na^{+} could weaken membranes by displacing the divalent bridges provided by Ca^{2+}. Epstein (1961) states that Ca^{2+} selectively modifies cation transport in plant cells by

competitive inhibition or through Ca^{2+} regulated ion transport mechanism. Lynch and Läuchli (1985) also state that NaCl inhibits Ca^{2+} transport to the shoot by inhibiting movement of Ca^{2+} into the root xylem from the external medium, but in the xylem itself, any competitive inhibition would mobilise Ca^{2+}. They concluded that NaCl may directly inhibit the release of Ca^{2+} from the symplasm to the xylem or prevent Ca^{2+} cation exchange in the apoplast, therefore blocking entry into the root. Because of its role in the maintenance of membrane integrity, in *Glycine max* (soya bean), Ca^{2+} has been shown to induce ultrastructural changes of the plasma membrane (Morre and Bracker 1976) and is involved in membrane fusion (Holz and Stratford, 1979, Portis *et al*. 1979, Ge, Tian, and Russell 2007) and associated repair mechanisms (Schapire, Valpuesta, and Botella 2009). Ca^{2+} plays a crucial role in the regulation of the salt economy of plants and specifically in the selective transport or exclusion of Na^{+} and other mineral ions by plant cell membranes (LaHaye and Epstein 1969). Rengel (1992) states that understanding the relationship between Na^{+} and Ca^{2+} may represent one of the crucial links in extending our knowledge on physiological mechanisms of salt tolerance, which has led to a wealth of literature that examines the effects of increasing and decreasing the Ca^{2+} concentration of salinized growth media on the Na^{+} and Cl^{-} concentration of root and aerial parts. Likewise, the literature also covers aspects of increasing and decreasing the concentration of NaCl, and examining the effect on concentrations of Ca^{2+} within plant organs. It is the general consensus that addition of Ca^{2+} to growth media ameliorates salt stress, and very few studies find that the relationship between Ca^{2+} and salt stress had not enabled a significant ameliorative effect (Rengel 1992).

The addition of supplemental Ca^{2+} to the growing media of plants is a recurrent research theme. Reid, Tester, and Smith (1993), working with *Chara corallina*

(stonewort), found that the toxicity of NaCl can be ameliorated by increasing the concentration of Ca^{2+} in the growth solution. Early experiments on the subject by LaHaye and Epstein (1969) found the same effect in *P. vulgaris* (common bean), and state that the experiments show the efficacy of Ca^{2+} in protecting an extremely salt sensitive species against the deleterious effects of NaCl present in the growth medium at approximately one tenth the concentration of seawater. Hawkins and Lewis (1993b), who found that additional Ca^{2+} restored the uptake of NO_3^-, proposed that Ca^{2+} may have a protective effect on the membrane, an effect on the NO_3^- transporter, or replace Ca^{2+} displaced by Na^+ (Läuchli 1990), so that Ca^{2+} binding to the membrane is maintained at near normal levels. Cabañero *et al*. (2006) also found that in the presence of NaCl, the Ca^{2+} concentration in the membrane decreased significantly with respect to the control. However, when Ca^{2+} was added no significant differences were observed with respect to the control. Cramer and Läuchli (1986) suggested that by increasing the ion activity of Ca^{2+} in saline solution, the Ca^{2+}/Na^+ competition is partially removed, therefore helping to maintain membrane integrity, ion selectivity and the integrity of ion transport systems which rely on Ca^{2+} (Hawkins and Lewis 1993a). Na^+ ions increase membrane porosity (Van Steveninck 1965, Watad *et al.* 1991) and cause membrane depolarisation (Läuchli 1990, Hua *et al*. 2008) which allows the leakage of some ions and an influx of others. Cachorro *et al*. (1995) found that addition of 80 mM NaCl to the growth medium of *P. vulgaris* (common bean) considerably increased the leakage of solutes from intact plant roots back to the solution, especially Ca^{2+} and K^+. Rengel (1992) found that the addition of Ca^{2+} to the growth media reduced Na^+ binding to cell walls and the plasma membrane, alleviated membrane leakiness and prevented declines in cell production and cell elongation, with Cachorro, Ortiz, and Cerda (1994) reporting that K^+ and Ca^{2+}

concentrations from roots to shoots were decreased by NaCl, but were restored by increasing Ca^{2+} in the growth medium. Kaya *et al*. (2002) also found that supplemental Ca^{2+} ameliorated the effects of salinity by increasing water use, and decreasing an increased membrane permeability caused by elevated NaCl concentrations in *Fragaria* × *ananassa* Duch (common strawberry). Tuna *et al*. (2007) also found that supplemental $CaSO_4$ (calcium sulphate) under salt stress of *Solanum lycopersicum* (tomato) improved yield, growth and membrane permeability, and Hua *et al*. (2008) found that addition of $CaCl_2$ (calcium chloride) reduced the extent and rate of NaCl induced membrane depolarisation. A study of adding exogenous Ca^{2+} to germinating *Arachis hypogaea* L. (peanut) under NaCl stress, increased glycine betaine concentrations in the embryonic axis above that caused by NaCl, indicating that Ca^{2+} may play an indirect role in conferring osmotic tolerance. This same work also found that the addition of $CaCl_2$ to the seedlings lowered proline concentration by increasing levels of proline oxidase and decreasing activities of γ-glutamyl kinase, the enzyme that synthesises proline (Girija, Smith, and Swamy 2002). This is supported by the work of Lutts, Kinet, and Bouharmont (1996) who found that proline did not take part in osmotic adjustment and its accumulation seemed to be a symptom of injury in *Oryza sativa* (rice).

Different species and different cultivars responded differently to supplemental Ca^{2+} when salinized (Cramer 2002) and there are instances where some species or cultivars have responded negatively to supplemental Ca^{2+} under NaCl salinity. Cramer (2002) found this to be the case with *Hibiscus cannabinus* (kenaf) and *Thinopyrum ponticum* (tall wheatgrass). Schmidt, He, and Cramer (1993) found that supplemental Ca^{2+} did not improve the salt tolerance of salt stressed *Brassica* species, but salt tolerance had been positively correlated with internal tissue Ca^{2+} concentrations in previous studies.

Although Kwon, Shaheed Siddiqui, and Harris (2009) found that supplemental Ca^{2+} did not improve growth in *Brassica rapa* 'Sani', it was shown to improve tissue K^+/Na^+ and Ca^{2+}/Na^+ and the extent to which further improvement could have occurred may be due to levels of the supplemental Ca^{2+} added to the growth substrate. Similar findings are reported by Cabot *et al.* (2009) in *P. vulgaris,* who reasoned that Ca^{2+} amelioration of responses to salinity are dependent on genetic factors.

Ca^{2+} is known to be necessary for maintenance of K^+ transport (Epstein 1961, Läuchli and Epstein 1970) and supplemental Ca^{2+} has also been found to increase the uptake and transport of K^+ (Cramer, Läuchli, and Polito 1985, Cramer *et al*. 1987, Grieve and Fujiyama 1987, Nakamura *et al*. 1990, Subbarrao *et al*. 1990, Hawkins and Lewis 1993a, Grattan and Grieve 1999a, Läuchli and Grattan 2007, Tuna *et al*. 2007, Kwon, Shaheed Siddiqui, and Harris 2009, Vaghela *et al*. 2010). It has also been suggested that supplemental Ca^{2+} maintains K^+/Na^+ exchange and selective K^+ uptake of the plasma membrane, which is one of the primary cellular sites of action of Ca^{2+} (Zhong and Läuchli 1993, Shabala *et al*. 2006, Läuchli and Grattan 2007). Together with reducing Na^+ accumulation, these effects of Ca^{2+} could be fundamental in improving the degree of salt tolerance of plants, as maintaining K^+ uptake and K^+/Na^+ can be an important mechanism of salt tolerance (Chen *et al*. 2007b).

Increasing and decreasing the concentration of NaCl supplied to a plant via the growth solution has been shown to have a marked effect on Ca^{2+} tissue concentrations. Increasing levels of NaCl provide an increase in a plant's requirement for Ca^{2+} (Gerard 1971, Kopittke, and Menzies 2005). Indeed, high concentrations of Na^+ have been shown to decrease Ca^{2+} concentrations in the tissue of many plant species (Greenway and Munns 1980, Grieve and Fujiyama 1987, He and Cramer

1992, Al-Harbi 1995, Navarro, Martinez, and Carvajal 2000, Kaya *et al*. 2002, Kopittke and Menzies 2005, Genc, Tester, and McDonald 2009). This suggests an interaction between Ca^{2+} and Na^{+}, which may occur at the plasmalemma (LaHaye and Epstein 1969). A number of hypotheses have been put forward. Suarez and Grieve (1988) suggest that in a situation of high NaCl concentration, the uptake of Ca^{2+} from the soil may decrease because of interactions with other competitive ions, and increases in ionic strength that reduce the activity of Ca^{2+}; the cation uptake process being strongly selective for Ca^{2+} against Na^{+}. As the concentration of Na^{+} in the substrate increases, the uptake system may become less discriminating and selectivity for Ca^{2+} is impaired. In essence, Ca^{2+} may be readily displaced from extracellular binding sites by competition from, and high concentrations of, other cations. This is further emphasised by Lynch and Läuchli (1985), Cramer, Läuchli, and Polito (1985), Lynch, Cramer, and Läuchli (1987) and Lynch and Läuchli (1988) who have shown that the ionic strength of saline solutions displaces Ca^{2+} from the membranes of root cells. This appears to be an accepted mechanism today (Fageria, Gheyi, and Moreira 2011). Marschner (1986) also states that Ca^{2+} is strongly competitive with Mg^{2+} for binding sites on the root plasma membrane. Working with *Leucaena leucocephala* (white leadtree), high concentrations of substrate Ca^{2+} as $CaSO_4$ usually resulted in decreased Mg^{2+} in roots, stems and leaves.

2.3.2 Potassium

K^{+} is a vital activator of many enzymes that are involved in photosynthesis and respiration, starch and protein synthesis. It also plays a role in osmotic regulation and turgor pressure via low osmotic potential in the stele of the roots that is required to drive solute transport in the xylem (Marschner 1995) and is involved in stomatal

movement (Tester 1990, Lebaudy, Véry and Sentenac 2007). K^+ is one of the most abundant cations in the plant cell, is highly mobile, exhibits long distance cycling (Szczerba, Britto, and Kronzucker 2009) and is distributed between vacuole and cytoplasm (Leigh and Storey 1991, Jordan-Meille and Pellerin 2008). In the vacuole, where it is mainly present, it has purely an osmotic role, contributing to cell turgor, especially in glycophytes. Leigh and Storey (1991) state that K^+ levels in the cytoplasm can be expected to vary in accordance with K^+ supply since K^+ salts can be replaced by Ca^{2+} and Na^+ salts, reducing sugars or amino acids but it is here that Ca^{2+} substitutions are less common, with Na^+ proven to be inhibitory to protein synthesis *in vitro*.

Halperin and Lynch (2003), using fluorescent dyes (benzofuran isophthlates), found interestingly that when roots cells of *A. thaliana* were supplied with extracellular Ca^{2+}, that the Na^+ and K^+ was confined primarily to the cytoplasm, with little K^+ signal from the vacuole within 20 min of salinisation. This could be related to findings that in the cytoplasm, K^+ has a biochemical role, with many enzymes being K^+ dependant, and a requirement for protein synthesis (Wyn Jones, Brady, and Spiers 1979, Chow, Ball, and Anderson 1990, Tyerman and Skerrett 1998).

Although it has been proposed that the application of K^+ might enhance the capacity for osmotic adjustment of plants growing in saline soils (Cerda *et al*. 1995), along with overwhelming amounts of data that shows reduced uptake and translocation of K^+ by plants grown in high Na^+ substrates, according to Grattan and Grieve (1992) there is little data that shows that the addition of K^+ to saline soils improves plant growth or yield. Bar-Tal, Feigenbaum, and Sparks (1991) state that the ability of K^+ application to reduce the deleterious effects of salinity have never been conclusively

proven, since no response or negative response to K^+ nutrition of salt stressed plants (Lauter, Meiri, and Shuali 1988) has been reported. This is emphasised by Cerda *et al*. (1995), who found that K^+ did not improve the negative effect of mild saline treatment; the growth reduction in two varieties of *Zea mays* (maize) was not varied by increasing the K^+ concentration at the root of the plant. Such viewpoints may no longer be the case, as K^+ nutrition has since been implicated both positively and negatively in roles in response to salinity. Cakmak (2005) states, however, that such saline stress can be ameliorated by increased K^+ supply. Working with *O. sativa* (rice), Muhammed, Akbar, and Neue (1987) found that the addition of either Ca^{2+} or K^+ to a saline culture solution, increased the weight of the roots and shoots; it was concluded that Na^+ and Cl^- uptake may be reduced by the addition of either Ca^{2+} or K^+ to the growth medium. In *Cucumis melo* (melon), Kaya *et al*. (2007) found that supplementary K^+ ameliorated the effects of NaCl salinity on plant growth, fruit yield, dry weight, electrolyte leakage and chlorophyll content. In sugarcane, Ashraf *et al*. (2010) found that addition of K^+ significantly inhibited the uptake and transport of Na^+ from roots to shoots. Conversely, Lynch and Läuchli (1984) also found that NaCl inhibited K^+ uptake by *Hordeum vulgare* (barley) and Na^+ appeared in root xylem which was associated with an inhibition of K^+ release into the xylem. Schleiff (1978) proposed that salt resistant plants are able to take up K^+ selectively in large amounts, meaning perhaps that selectivity is the key to salt tolerance. Indeed, Watad *et al*. (1991) states that increased uptake of K^+ appears to confer tolerance rather than an increased intracellular/endogenous concentration in *Nicotiana tabacum* (tobacco). This is emphasised by Liu and Zhu (1997) and Zhu, Liu and Xiong (1998) who attributed salt sensitivity in mutant *A. thaliana* to poor capacity for uptake of K^+. Zhu (2002) and Munns and Tester (2008) who suggest relative cytoplasmic K^+/Na^+ as

being an important factor in salt tolerance. Work by Alberico and Cramer (1993) and Cachorro, Ortiz, and Cerda (1994) found that improvement in root cell membrane integrity by Ca^{2+} leads to a reduction in leakage of K^+ from the cells, essentially showing that adequate Ca^{2+} in the growth substrate influences the K^+/Na^+ selectivity (Grattan and Grieve 1999a, Tuna *et al*. 2007). Higher cytoplasmic K^+/Na^+ appears to be important in salt tolerance due to maintenance of enzymic function in the conditions of high external Na^+ (Tyerman and Skerrett 1998). This is furthered by the work of Chhipa and Lal (1995) and Poustini, Siosemardeh, and Ranjbar (2007) who found that salt tolerant varieties of *T. aestivum* (wheat) had lower Na^+/K^+ ratios, and under saline conditions maintained growth by restricting the absorption of Na^+ and favouring the absorption of K^+. Ebert *et al.* (2002) working with salt stressed *Psidium guajava* (guava) also found similar results with $Ca(NO_3)_2$ addition. $Ca(NO_3)_2$ increased leaf K^+ concentration and Na^+ transport was inhibited. Cramer, Läuchli, and Polito (1985) and Cramer *et al*. (1987) also proposed that the displacement of Ca^{2+} by high concentrations of Na^+ on the cell membrane can disrupt membrane structure which can be critical to selective K^+ uptake. Tuna *et al*. (2007) with *S. lycopersicum* (tomato) and Kaya *et al*. (2007) with *C. melo* (melon) also surmise increased leaf K^+ after the addition of $CaSO_4$ as being due to maintenance of membrane stability and permeability in roots cells and lessening K^+ cell leakage. Interestingly, it appears that increasing/decreasing externally available K^+ or Ca^{2+} may not always affect internal K^+ or Ca^{2+} under normal growth conditions (Marin *et al*. 2010) and may only be actively important under conditions of increased salinity stress. Jeschke (1984) has discussed the possible mechanisms of K^+ selectivity over Na^+ and has suggested that selectivity can occur at three membranes; the plasmalemma of the cortical root cells, the tonoplast of the root and shoot cells and the plasmalemma of the xylem and

parenchyma cells. Five mechanisms of selectivity can occur at these sites; preference for K^+ during influx (influx selectivity), K^+-Na^+ exchange at the plasmalemma (root cortex), selective Na^+ accumulation in vacuoles and Na^+-K^+ exchange across the tonoplast, selectivity during release of K^+ and Na^+ to the xylem vessels and selective readsorption of Na^+ from the xylem sap.

Investigations of the effects of NaCl on internal concentrations and distribution of K^+ have found that tissue levels of K^+ are affected by saline environments/high concentrations of Na^+ (He and Cramer 1993a). Investigating six *Brassica* species, He and Cramer found that there was a reduction in shoot K^+/Na^+ ratio, resulting from a decrease in shoot K^+ and an increase in shoot Na^+ upon exposure to saline substrate. The decline in tissue K^+ concentration was hypothesised as resulting from direct competition between K^+ and Na^+ at sites of uptake at the plasmalemma, possibly disrupting aspects of membrane structure which are critical to selective K^+ uptake, an effect of Na^+ on K^+ transport into the xylem or Na^+ induced increased efflux from the root (He and Cramer 1993b). Indeed, damage to the plasma membrane, due to an ionic toxic effect of salt, has been suggested by Leopold and Willing (1984). Following work by Bewley and Black (1982), who found that under saline conditions the re-establishment of membranes is reduced or prevented, resulting in irreversible damage and increased membrane permeability, Leopold and Willing (1984) concluded that salt toxicity produces lesions on cell membranes, generally the plasmalemma, resulting in the leakage of solutes from the cell. Leakage of K^+ from the plasmalemma as a result of Ca^{2+} displacement by Na^+ has also been suggested to account for the reduction in K^+ concentrations under salinity stress (Cramer, Läuchli, and Polito 1985, Cramer, Läuchli, and Epstein 1986, Cramer 2002, Cabot *et al*. 2009, Vaghela *et al*. 2010, Zhang, Flowers, and Wang 2010).

Apart from disruption to the plasma membrane, salinity induced changes in K^+ have also been implicated in changes to photosynthetic processes (Sudhir and Murthy 2004, Parida and Das 2005). In the halophyte *Avicennia marina* (mangrove), Ball, Chow, and Anderson (1987) concluded that NaCl salinity induced K^+ deficiency, and photosynthesis was inhibited. This was due to reduced atrazine binding sites in isolated thykaloids, causing a loss of function in photosystem II (Ball, Chow, and Anderson 1987, Lovelock and Ball 2002). The reduction of K^+ has also been shown to be linked not just to Na^+ salts of Cl^-. It also appears that the disruption of K^+ is firmly attributable to Na^+. Janzen and Chang (1987) found that barley exposed to Na_2SO_4 salinity contained one third of the concentration of K^+ in their shoots compared with non-salinised controls, further indicating that the active species affecting K^+ concentration in tissues is Na^+ regardless of its constituent compound (Paek, Chandler, and Thorpe 1988, Grattan and Grieve 1999b).

2.4 Seed Germination and Salinity

> "*In all species of vascular plants studied, salt decreases the number of seeds which germinate and the speed of germination* (Ungar 1978). *There is, however, a wide range of variability in salt tolerance between and within species* (Choudhuri 1968, Marcar 1987, Marañon, Garcia, and Troncoso 1989, Ashraf and McNeilly 2004, Noreen and Ashraf 2008, Kumar *et al*. 2009)."

Germination is one of the most critical periods for plants subjected to salinity, the salt tolerance being critical for the establishment of plants that grow in saline soil (Choudhuri 1968, Ungar 1978, Marañon, Garcia, and Troncoso 1989, Mohammed

and Sen 1990, Fowler 1991, Ungar 1995). Indeed, plants are generally most sensitive to salinity during germination (Ayers and Hayward 1948, Mayer and Poljakoff-Mayber 1989, Catalan *et al*. 1994, Ashraf and McNeilly 2004, Ashraf and Foolad 2005, Zhang *et al.* 2010) and the ability of a seed to germinate under salt stress can be an early indication that the plant genotype has potential for salt tolerance, at least at this early stage (Pearce-Pinto, Van der Moezel, and Bell 1990), but this should not be the sole indicator for whole plant tolerance, as differences in salinity tolerance are often exhibited at different growth stages (Arzani 2008, Ashraf and Akram 2009). In a saline habitat, failure to germinate is often caused by high concentrations of salt just below the soil surface, as soil solution moves upwards due to evapotranspiration leaving increasing concentrations of salt at the soil surface (Bernstein and Hayward 1958, Pasternak, Twersky, and de Malach 1979, Lamsal, Paudyal, and Saeed 1999, Nishida, Khan, and Shiozawa 2009). Water rising within 1 to 2 m, coupled with salt mobilisation and evapotransipration can cause the soil to become salt affected (Salama, Otto, and Fitzpatrick 1999). In this situation, the soil surface has a greater salt concentration than lower layers (Uhvits 1946, Dotzenko and Dean 1959, Khatib and Massengale 1966), often meaning that seed germination occurs in a more saline environment than in which the established plants grow (Esechie 1993, 1995).

Generally, saline environments result in delays in the initiation of germination, increased time to the completion of germination and lower final total germination. Seeds of halophytes can show higher resistance up to a pivotal salt concentration, and a rapid decrease in final germination thereafter (Dafni and Negbi 1978), while those of glycophytes show a constant reduction in germination with increased salinity (Ungar 1962, Macke and Ungar 1971). Seeds of halophytes also appear to be able to germinate after exposure to salinity that inhibits germination (Woodell 1985, Qu *et al*.

2008) by either inhibition of germination accompanied by no loss of viability or delay in germination that does not cause dormancy or seed death (Ungar 1978, Qu *et al*. 2008).

Soil salinity may affect the germination of seeds primarily in two ways. Firstly salt may decrease the ease with which seed may take up water osmotically, decreasing the rate of water entry through lowering the water potential, and secondly, by allowing an intake of ions in sufficient amounts to have a specific toxic effect (Uhvits 1946, Ayers and Hayward 1948, Ryan, Miyamoto, and Stroehlein 1975, Bewley and Black 1982, Norlyn and Epstein 1984, Petruzelli *et al*. 1992, Zekri 1993, Wahid, Razul, and Rao 1999, Zhang *et al*. 2010). Indeed, high concentrations of Na^+ and Cl^- within cells can prevent the metabolism associated with dividing and expanding cells (Neumann 1997, Zhang *et al*. 2010) such as changes in enzymic activity, protein metabolism, growth regulator balance and utilisation of seed reserves (Wahid, Razul, and Rao 1999). The process of germination comprises two phases: imbibition and a heterotrophic growth phase which precedes emergence. Bliss, Platt-Aloia, and Thomson (1986) have questioned whether the damage caused by salt could be different in each of the two phases. They found that during imbibition, the effect of salt was purely osmotic, until a hydration threshold was surpassed, and salt then had a combined osmotic and toxic effect. A combination of both osmotic and toxic effects has also been discussed and how different species may be more sensitive to certain toxic effects at different growth stages by Huang and Redmann (1995).

It has been noted by Igartua, Gracia, and Lasa (1994), studying *Sorghum bicolor* (grain sorghum), that salinity has a more marked effect on actual emergence than the processes of germination outlined above. Increasing salinity caused a delay in the

onset of germination, probably caused by a slower rate of water uptake due to an increase in the solution's osmotic pressure. Furthermore, delays in seedling development were observed with increasing salinity, i.e. they germinated and stopped developing. This was attributed to possible osmotic effects on imbibition and later development and possible toxic effects to emerging seedlings. The osmotic effects that lead to slow imbibition may directly cause a number of effects that could subsequently be considered toxic effects. These include changes in regulation of enzymic activities, the mobility of inorganic nutrients, nitrogen metabolism, plant growth regulators, utilisation of growth reserves and accumulation of compatible solutes and proteins (Ashraf and Foolad 2005). In some species a reduction in germination has been primarily linked to lower external osmotic potential rather than specific toxic ion effects. This was found to be the case in *Helianthus annuus* (sunflower) seeds (Zhou and Xiao 2010). The ions in the cells appeared to be used as osmolites to maintain cellular osmotic potential, lower than that of the ambient environment, allowing water entry into the cells. This was also concluded by Zhang *et al*. (2010) where the acquisition of Na^+ in a saline environment allowed *H. vulgare* (barley) seeds to absorb water more readily and germinate faster under lower osmotic conditions where they would otherwise fail to germinate. However, Wahid, Razul, and Rao (1999) state that the majority of work that they considered favoured ionic toxicity as being the greater detrimental component of saline interaction.

The biochemistry of germination under saline conditions is complex and varies between species. The ionic nature of the external environment of the germinating seed can also affect the interaction of ions both within the seed and between the seed and its germination medium.

2.4.1 Seed Germination, Salinity, Calcium and Potassium

2.4.1.1 Ionic Effectors

Two ions mentioned frequently in relation to an increase or decrease in salinity tolerance are Ca^{2+} and K^{+}. Studies from as early as 1902 have often suggested that additional Ca^{2+} can have a beneficial effect (Kearney and Cameron 1902 cited in Hu and Schmidhalter 2005, Cramer 2002, LaHaye and Epstein 1971), while increases and decreases in K^{+} can have a positive or detrimental effect. Wu, Ding, and Zhu (1996) Cakmak (2005), Kaya *et al*. (2007) and Cha-um *et al*. (2010) hint at a role for K^{+}, stating that K^{+} uptake is an integral part of a number of salt tolerance mechanisms in glycophytes. The actual mechanisms of such effects, particularly during germination, are largely unknown and the theories are unaided by differences found between species and subspecies.

Studying the germination of seeds of *T. aestivum* (wheat), Begum *et al*. (1992) found that an increase in NaCl salinity had a positive correlation with the accumulation of Na^{+} and Cl^{-}, while also decreasing the amount of K^{+} accumulated in seeds. It was found that salinity stress decreased the amount of endogenous K^{+} of the plumule and radicle, causing an adverse effect on the growth of the embryo and thus inhibiting the rate of germination. In studying twelve *H. vulgare* (barley) genotypes, Othman *et al*. (2006) found that increasing salt stress generally decreased K^{+} of the seed after 1 day of imbibition, citing the effect of competition between Na^{+} and K^{+} on the absorptive sites of the plant, reduction in K^{+} concentration that decreases the capacity for osmotic adjustment and turgor maintenance, or the negative effects on metabolic functions as protein synthesis, as contributing reasons for overall decreasing salt tolerance as salinity increases. Atak *et al*. (2006) also found that NaCl decreased the

amount of K^+ in seeds and shoots, but that K^+ increased in roots, with the hypothesis that this may be due K^+ exchange with Na^+ in the germination medium. Salt tolerant species appear to have less K^+ efflux (Al-Karaki 2001). Further hypotheses have been put forward which suggest that salinity stress may cause increases in abscisic acid (ABA) levels (Parida and Das 2005) which in turn increase K^+ efflux, borne out of research by Van Steveninck (1972) who found that ABA altered K^+/Na^+ selectivity in beet root, and Khadri, Tejera, and Lluch (2007) with *P. vulgaris*, although this may have occurred indirectly (Parida and Das 2005). Application of K^+ to the germination medium of *H. annutis* (sunflower) has also been shown to have a beneficial effect on germination under salinity (Delgado and Sánchez-Raya 1999).

Similar results have been found in roots, hypocotyls and cotelydons of *S. lycopersicum* (tomato) seedlings, with a Ca^{2+} decrease in hypocotyls and roots (Torres-Schumann *et al*. 1989), the Ca^{2+} decrease originating from Ca^{2+} displacement from the membrane by Na^+ as reported by Cramer, Läuchli, and Polito (1985) and Cramer, Läuchli, and Epstein (1986) in cotton. In *G. max* (soya bean), germination at higher tissue Na^+ concentrations has been associated with higher K^+ and Ca^{2+} concentrations in the embryo axis, suggesting that these ions may protect the seeds in the pre-germination phase against salinity (Hosseini, Powell, and Bingham 2002). It has been suggested that salinity causes some plasmalemma perturbation that stimulates Ca^{2+} release from the endoplasmic reticulum, which in turn increases Ca^{2+} efflux from the plasmalemma and reduces Ca^{2+} availability in intracellular pools; the supplementary extracellular Ca^{2+} relieving the salinity effects by increasing Ca^{2+} entry and reducing Na^+ entry (Lynch and Läuchli 1988, Zidan *et al*. 1991, Tuna *et al*. 2007). As in the case of K^+, ABA has also been linked with a mechanism of increased Ca^{2+} uptake during salinity stress that would have an ameliorative effect in

maintaining membrane integrity (Chen *et al*. 2001, Cramer 2002, Parida and Das 2005).

In terms of whole plant nutrition, salinity tends to decrease Ca^{2+} and K^{+} levels in plant tissue, with supplemental Ca^{2+} and K^{+} having an alleviating effect. It has also been reported that the addition of Ca^{2+} to the growth medium has been found to improve germination (Bliss, Platt-Aloia and Thomson 1986, Hardegree and Emmerich 1990, Ashraf and Naqvi 1991, Abd-Alla, Jones, and Abou-Hadid 1993, Cachorro, Ortiz, and Cerda 1994, Tobe, Zhang, and Omasa 2003, Bonilla, El-Hamdaoui, and Bolanos 2004, Tobe, Li, and Omasa 2004). Delgado and Sánchez-Raya (2007), using *H. annuus* (sunflower) found that Ca^{2+} additions to the saline culture medium, improved germination percentage and seedling survival, but then addition of K^{+} actually caused a decrease in survival. The addition of Ca^{2+} to the germination medium of *Gossypium hirsulum* (cotton) grown in saline solutions by Kent and Läuchli (1985), however, did not improve germination, but did prevent a reduction in root growth caused by the presence of NaCl. Indeed, root growth was greatly improved by a high Ca^{2+} supply from $CaSO_4$. In addition, it was also found that NaCl treatments caused a reduction in the K^{+} and Ca^{2+} concentrations of roots and shoots, but a high additional Ca^{2+} concentration partially offset the K^{+} reduction in the roots. The Na^{+} concentration of roots and shoots were unaffected by the elevated Ca^{2+} level in the salinised growth medium, but the cation ratios ($Na^{+}/K^{+}+Ca^{2+}$) in the salt stressed roots decreased from 5.95 to 2.25 in the presence of supplemental Ca^{2+}, possibly having a significant effect on the metabolic functions of the seedling. It is also interesting to realise that two important processes in the establishment of seedlings in a saline environment are cell elongation and the maintenance of a balanced nutrient uptake, both of which require Ca^{2+} (Kent and Läuchli 1985). Vaghela *et al*. (2009, 2010) and Patel *et al*. (2011)

concluded that the beneficial effect of additional Ca^{2+} on germination, was the counteraction of the toxic effect(s) of Na^{+}, and to maintain selectivity at the plasma membrane and the maintenance of K^{+} in tissues that may be commensurate with the repolarisation of the membrane and that Ca^{2+} may cause closure of nonselective cation channels that would restrict Na^{+} uptake. Zehra *et al*. (2012) also postulate that an increase in cytoplasmic Ca^{2+}, that could be initiated by increases in external Ca^{2+} concentration, may trigger Ca^{2+} dependent protein kinases (CDPKs), which are involved in the metabolic pathways of germination, and Ca^{2+} sensor proteins such as calmodulin that have been found in germinating *Pisum sativum* (pea) (Duval *et al*. 2002). Tobe, Zhang, and Omasa (2003) suggest that externally applied Ca^{2+} may serve to replace the salt cation-displaced Ca^{2+} on the plasma membrane, but ultimately the balance of cations available to seed at germination may be crucial to survival.

Ashraf and Naqvi (1991) report that the addition of supplemental Ca^{2+} to the saline medium had no significant effect on the germination and growth of *Brassica carinata* (Ethiopian mustard) and *Brassica campestris* (field mustard), but was beneficial for *Brassica juncea* (mustard) and *Brassica napus* (rape) at both germination and vegetative growth stages.

Salt toxicity in isolated wheat embryos has been shown to be correlated with a marked decrease in internal K^{+} concentration by Petruzelli *et al*. (1992). Table 2.1 shows the concentration of K^{+} and Na^{+} in isolated *T. aestivum* (wheat) embryos germinated in 200 mM sorbitol and 100 mM NaCl, and it is interesting to see the marked effect that NaCl has upon K^{+} concentration, compared with the control and high osmotic potential of sorbitol.

Table 2.1 - Concentration of K^+ and Na^+ in Isolated Wheat Embryos Germinated in the Presence of 100 mM NaCl or 200 mM Sorbitol for 14 h (Petruzelli *et al.* 1992).

It is interesting to read from Petruzzelli *et al*. (1992) that rapid increases in Na^+ occurred in isolated embryos, while in non-isolated embryos, lower increases in Na^+ concentration were found up to 24 h after imbibition in very high NaCl concentrations, therefore indicating that the seed coat may act as a buffer against NaCl accumulation by the embryo during germination as suggested by Poljakoff-Mayber *et al*. 1994, Esechie 1995, Wahid, Razul, and Rao 1999, Tobe, Zhang, and Omasa 1999, 2003). This appears to be particulary the case in a number of halophytic species. In the relatively salt tolerant germination of *Kosteletzkya virginica* (seashore mallow), which has high initial K^+ concentration, there was found to be no K^+ loss during germination with exposure to high external concentrations of NaCl, and internal K^+ concentration was maintained between 100 and 200 μg^{-1} seed, possibly pointing to a control mechanism of ion entry into the seed by the seed coat (Poljakoff-Mayber *et al*. 1994) also suggested by Rehman, Harris, and Bourne (1998a) with *Acacia tortilis* (umbrella thorn) and *Acacia coriacea* (wiry wattle). The toxicity of Cl^- has also been implicated in germination failure (Stone, Marx, and Dobrenz 1977, Esechie 1995, Neumann 1997). Cl^- ions are potentially toxic to embryos and their entry into the seed in high concentrations has an adverse effect on embryo viability, given that seeds and fruit have low tissue Cl^- levels and are fed via the phloem (White and Broadley 2001). Cl^- appears to be inhibitory to protein and enzyme synthesis (Wyn Jones and Pollard 1983) and high concentrations of Cl^- can be detrimental to the

integrity of the cell and affect photosynthetic processes directly through membrane damage or enzyme inhibition (Tavakkoli, Rengasamy, and McDonald 2010). Tobe, Zhang, and Omasa (2003) also found that KCl salinity induced a greater negative effect on germination than NaCl salinity, perhaps indicating that at sufficiently high levels, K^+, as well as Cl^-, is toxic to germination. It required more additional Ca^{2+} to allieviate germination inhibition, and so may more easily displace Ca^{2+} than Na^+.

Increasing Ca^{2+} and K^+ concentrations, through presowing treatments, in seeds such as *H. vulgare* (barley) (Bliss, Platt-Aloia, and Thomson 1986), *Triticum vulgare* (wheat) (Chaudhuri and Wiebe 1968, Idris and Aslam 1975), *T. aestivum* (wheat) (Iqbal and Ashraf 2007, Afzal *et al*. 2008), *Zea mays* (maize) (Ashraf and Rauf 2001), *Lolium rigidum* Gaud (wimmera rye grass) (Marcar 1986), *Tagetes* (marigold) (Afzal *et al*. 2009) and *O. sativa* (rice) (Farooq *et al*. 2006) resulted in increased germination in NaCl solutions. Iqbal and Ashraf (2007) found that seed pretreatments of $CaCl_2$, and indeed NaCl, were effective in reducing shoot Na^+ levels, and in particular, treatment of KCl significantly reduced shoot Na^+ levels in both salt sensitive and salt tolerant cultivars of wheat. Pretreatment by KNO_3 has also been implicated in amelioration of drought stress of *H. annuus* (Kaya *et al*. 2006) and *Brassica napus* (Mohammadi and Amiri 2010). However, Rehman, Harris, and Bourne (1998b) found that germination rate was not advantageously affected by the inclusion of Ca^{2+} or K^+ salts in treatment solutions in *Acacia*, but did alter the Ca^{2+} and K^+ content, loss of Ca^{2+} and K^+ and uptake of Na^+. Kamboh, Oki, and Adachi (2000) and Ashraf and Iram (2002) also found that pre-treatment of *T. aestivum* by KCl, KNO_3, $CaCl_2$ and $Ca(NO_3)_2$ did not improve germination under salinity and in *P. sativum*, Ca^{2+} or K^+ did not improve germination over distilled water pretreatment (Guerrier and Pinel 1989). An increase in osmolites, including Na^+, via seed pre-treatment may aid

germination through increased rate of water uptake of seeds of some species, as discussed by Heydecker, Higgins, and Gulliver (1973) and shown by Zhang *et al*. (2012), but this may not necessarily be the case for all species (Ashraf and Foolad 2005). Ashraf and Foolad (2005) also discuss the possible mechanisms of osmolite priming, or osmopriming, including physical aspects of creating spaces within seed tissue that facilitates water uptake, and root protrusion. The priming of seed with inorganic salts, or halopriming, may affect a number of facets of the germinating seed. The activity of enzymes has been shown to increase in seeds treated with K^+ and Ca^{2+} salts that are germinating under saline conditions, but different priming salts may also affect the uptake of nutrients differently in different parts of seed and has been shown to confer greater effectors of later growth as seedlings (Ashraf and Foolad 2005). In addition, Fenner (1992) discussed the ability of salinity applied to parental plants, which also results in a drought effect, to cause an increase in protein content of seed which then may aid salt tolerance during germination and effectively confer a mechanism of osmopriming via parental plant nutrition.

The works of Rehman *et al*. (1996, 1998a, 1998b, 2000) investigating the factors affecting the salinity tolerance of *Acacia* seeds, is primarily the basis upon which this research is founded. Using species with varying salt tolerance at germination, Rehman, Harris, and Bourne (1998a) found that greater tolerance of *A. tortilis* at germination, compared with *A. coriacea*, may be due to lower Na^+ uptake, coupled with lower leakage of both Ca^{2+} and K^+. It was also found that *A. tortilis* seeds had higher initial concentrations of Ca^{2+} and K^+ in their embryos. This work also suggests that the seed coat may be involved in the uptake of high concentrations of Na^+, consequently reducing the uptake of Na^+ by the embryo. Esechie (1995) also suggested that the seed coat may act as a buffer between the embryo and the external

NaCl solution by accumulating Cl^- and protecting the embryo from its toxic effects. In addition, Cl^- accumulation in seed coats increased with increasing NaCl accumulation.

Rehman *et al*. (1996) stated that salt tolerant *Acacia* seeds generally initially had higher Ca^{2+} and lower K^+ concentrations and lost more Ca^{2+} and less K^+ when soaked in NaCl, indicating that the salt tolerance of a seed batch could be defined, at least in part, by the Ca^{2+} and K^+ concentration of the seed, and the loss of these ions during soaking in deionised water or NaCl. The tolerance was also subject to modification by seed viability and vigour. Again, the salt tolerance of seeds was higher with high seed Ca^{2+} contents, but lower with high K^+ concentrations and in general, seeds of salt tolerant *Acacia* species had higher concentrations of Ca^{2+} and a higher Ca^{2+}/K^+ ratios than seeds of salt sensitive species. Furthermore these tolerant species showed higher Ca^{2+}/K^+ ratio of ions leached when seeds were soaked in NaCl.

The tolerant species maintained K^+ levels by minimising loss of this ion in NaCl, whilst losing Ca^{2+}, most probably present in excess. The susceptible species lost greater amounts of K^+ into NaCl solutions, possibly due to the displacement of Ca^{2+} by Na^+, allowing K^+ to leak out across the plasmalemma. Tolerant species which have an inherently higher concentration of Ca^{2+} may be able to lose Ca^{2+}, possibly replaced by Na^+, and still have sufficient levels to supply the demands of the seed and seedling while maintaining normal membrane function.

The results of the work by Rehman *et al*. (2000, 2009) have demonstrated a relationship between seed chemistry and salinity tolerance across the genus *Acacia* and permitted the development of equations that use simple and rapid analysis to enable the prediction of the likely germination of seeds under saline conditions, in which a negative relationship for K^+ and a positive relationship for Ca^{2+} were

predicted to exist for salinity tolerance (Rehman *et al*. 2009). The authors suggest that salinity tolerance could be increased by manipulation of seed K^+ and Ca^{2+} nutrition by means of correct seed priming or via mother plant nutrition, although due care should be taken to note that priming techniques may also have an osmopriming effect. The work has made a significant contribution to the understanding of salinity tolerance mechanisms in germinating seeds.

2.4.1.2 Non-ionic Effectors

As well as ions, carbohydrate molecules have been implicated in responses to salinity during germination as they play an important role in regulating the osmotic potential of cells, possibly affecting tolerance to osmotic stress (Suggett 1975, Dobrenz *et al*. 1993, Bewley 1997, Ashraf and Harris 2004, Gupta and Kaur 2005) and acting as a osmoprotectant. The link with osmotic regulation is borne from the water holding capacity of polysaccharide gels, which are networks formed by polymers (Suggett 1975). These polysaccharides have been shown to have a more complex relationship with water than that of monosaccharides (Franks 1975). Galactomannan is a polysaccharide that is found in the seed of many legume species (Stepanenko 1960) that appears to regulate carbohydrate-water interations in the seed. Other examples to be implicated in such activity are stachyose, raffinose, sucrose, glucose, fructose, arabinose, xylose (Dobrenz *et al*. 1993) and trehalose (Ashraf and Harris 2004, Zeid 2009).

At the molecular level, salinity has been shown to induce salt specific abnormalities in cells. Salt can induce inhibition of cell division, RNA, DNA and protein synthesis (Anuradha and Rao 2001, Tabur and Demir 2010). Root cells of isolated wheat embryos imbibed in NaCl have shown major structural changes in the nucleus,

(Petruzzelli *et al*. 1992). Katsuhara and Kawasaki (1996) also found such results with root mersitematic cells. Petruzzelli *et al*. (1992) found that chromatin appeared condensed, indicating the possible suppression of nucleic acid biosynthesis. Nir, Klein, and Poljakoff-Mayber (1970) using root tips of *Z. mays* (maize), Marinos and Fife (1972) using *T. aestivum* (wheat) embryos, Crevecoeur, Deltour, and Bronchart (1976) using *Z. mays* (maize) embryos and Panza *et al*. (2007) using embryos of *Euterpe edulis* (palm) found similar effects such as chromatin condensation within the nucleus of cells of early growth tissues that have been subjected to severe water stress. Salinity does not, however, appear to have any affect on nuclear size in tomato (Torres-Schumann *et al*. 1989), but can affect the size and appearance of the nucleoli. Nucleoli of cells from salt stressed plants have been found to be smaller than those in control cells, suggesting that the nucleoli of cells from salt stressed seeds are exhibiting lower transcriptional activity (Hernandez-Verdun 1986) with a lower proportion of rDNA that is actively transcribed or available for transcription. Katsuhara and Kawasaki (1996) suggest the possibility of activation of a DNA degrading endonuclease, while Tao, van Staden and Cress (2000) also found DNA degradation in *G. max* (soya bean) root meristematic cells. Also, the rates of uptake and incorporation of radioactively labelled precursors into proteins, RNA and DNA have been shown to be reduced considerably by NaCl (Petruzzelli *et al*. 1992) and RNase and DNase activity has been shown to increase in embryos of *Phaseolus aureus* (mungbean) under salinity stress (Sheoran and Garg 1978). Yupsanis *et al*. (2001) also found this to be the case under water stress, but not NaCl in *Medicago sativa* (alfalfa) seeds. However, Sheoran and Garg (1978) found that NaCl caused RNase inhibition in cotyledons and roots, but gave an increase in the embryonic axis and leaves, while DNase increased through all plant parts. This indicates that in some

species the effect on nuclear material may be organ specific. As to whether there is a specific ion or osmotic effect that causes such effects of endonucleases is still contentious and the mechanisms remain elusive (Shabala 2009) and appear to be species dependent. Recent studies have suggested that the effect may be due to osmotic stress (Tao, van Staden, and Cress 2000, Duan *et al*. 2010), but a specific ion effect has been found in some species (Katsuhara and Kawasaki 1996, Huh *et al*. 2002, Affenzeller *et al*. 2009).

Petruzelli *et al.* (1992) also reported changes in the cytoplasm, involving the plasmalemma and an increase in mitochondria. This also appears to be the case in root cells (Walker and Taiz 1988, Huang and Van Steveninck 1990, Koyro 1997 cited in Koyro 2004). Petruzzelli *et al*. (1992) interpreted the increase in the number of mitochondria as a compensation for possible impairment of mitochondrial function, since overall respiration was found to be unaffected. This theory is further enhanced by experimental material, which showed an oxygen uptake decrease, suggesting that the mitochondria had suffered damage, although appearing normal.

At its very core, salinity may affect a number of developmental processes within the seed that are primarily driven at some point by enzymatic activities, as these important biological catalysts depend upon a stable and buffered environment in which to function. Effects on metabolism of stored resources and growth of the embryo are hampered by the effect of salinity on the enzymatic activities involved in the utilisation and mobilisation of materials. Enzymes that enable the utilisation of stored lipids, such as glyoxysomal catalyse, are inhibited by salinity (Wahid, Rasul, and Rao 1999). Protein metabolism in embryos is also driven by, amongst others, enzymes such as proteases and different proteins may be produced in response to

drought or salt stress (Ramagopal 1990, Ramagopal and Carr 1991, Dell'Aquila 1992, Dell'Aquila and Spada 1993, Srivastava, Fristensky, and Kav 2004, Alam *et al*. 2011). Carbohydrates that provide reserves during embryonic expansion and emergence are regulated by enzymes such as amylases and galactosidase that breakdown complex sugars into monosaccharides and their activity is attenuated by exposure to salinity. A number of studies have shown that enzymes such as amylases (Sheoran 1980, Promila and Kumar 2000, Ashraf *et al*. 2002b, Siddiqui and Khan 2011), ribonucleases (Sheoran and Garg 1978, Gomes-Filho and Sodek 1988, Rouxel *et al*. 1989, Gomes-Filho *et al*. 2008), proteases (Sheoran and Garg 1978, Dubey and Manju Rani 1987, Ashraf *et al*. 2002a), peroxidases (Sheoran and Garg 1979, Mittal and Dubey 1991, Jbir *et al*. 2002, Aghdasi, Fazli, and Bagherieh-Najjar 2011), nitrate reductases (Billard and Boucard 1982, Köhler, Schmerder, and Shreikhave 1992, Anuradha and Rao 2003, Shahid *et al*. 2011) and carboxylases (Jbir *et al*. 2002) are affected by salt stress during germination in seeds. Sheoran and Garg (1978), Gomes Filho, and Sodek (1988), Dubey and Sharma (1990), Singh and Singh (1992), Del Zoppo *et al*. (1998) and Misra and Dwivedi (2004) have concluded that the presence of NaCl or mannitol causes limited hydrolysis of seed endosperm reserves because of the reduced water uptake and inhibits the movement of cotyledon reserves to the embryonic axis. In addition, the study of *M. sativa* by Yupsanis *et al*. (1994) revealed that the activities of protein kinase were induced and the activities of protein phosphatase (causing protein phosphorylation) were depressed by salt stress due to the ionic components of salt toxicity - since such alteration in activities were not found in iso-osmotic mannitol treated seedlings. Szczerba, Britto, and Kornzucker (2009) also suggest that protein phosphorylation may be involved in ion sensing in plants. Polyamines have also been implicated in production of ethylene forming

enzymes which may have a role in salt tolerance in seedlings (Wahid, Rasul, and Rao 1999). Organic molecules such as betaine, implicated in responses to salinity, are also produced by enzymatic activity.

Plant growth regulators are known to play important roles in germination. Gibberellins play primary roles and cytokinins are secondary agents, necessary to remove germination inhibitors in cases of stress (Khan and Downing 1968, Itai 1978, Lerner 1985). Kabar (1990) treated dicotyledonous seeds with kinetin and gibberellic acid (GA) under saline conditions and found that kinetin was much more effective at promoting total percentage germination than either GA alone or a combination of kinetin and GA. At least in dicotyledonous seeds, kinetin was shown to alleviate the osmotically induced inhibition of seed germination. Kinetin also promotes germination in salt stressed seeds by increasing the production of pregermination ethylene (Khan and Huang 1988, Shahba, Qian, and Lair 2008). It is well regarded as promoting cell division (Tabur and Demir 2010) and Wahid, Rasul, and Rao (1999) state that the effect of salinity is likely to suppress endogenous levels of growth regulators and an exogenous supply fulfils a requirement for the initiation of germination. Priming with cytokinins has also been shown to improve germination under salinity stress (Iqbal, Ashraf, and Jamil 2006). Seed dormancy, induced by salt stress, has previously been shown to be relieved by the application of GA (Khan and Ungar 1985, Khan *et al*. 1987, Kabar 1987, Gul and Khan 2008, Lee and Park 2010, Iqbal and Ashraf 2013) kinetin (Khan and Ungar 1985, Khan *et al*. 1987, Gul and Khan 2008, Iqbal, Ashraf, and Jamil 2006), a mixture of GA and kinetin (Ungar 1978, Begum *et al*. 1992, Khan, Ungar, and Gul 1998, Kaur, Gupta, and Kaur 1998), suggesting that inhibition of germination under salt stress can in part be due to variation in seed hormonal balance. External application of indole-3-acetic acid (IAA)

to seed appears to produce mixed outcomes in terms of salinity response across species. In some cases it appears to have a positive effect (Özturk *et al*. 2006) and in others it has been shown to have little or negative effects (Afzal, Basra, and Iqbal 2005, Kaur, Gupta, and Kaur 1998). Also, in a comparison of salt sensitive and salt tolerant cultivars of *G. max* (soya bean), the tolerant cultivar was shown to have higher levels of IAA than the sensitive one (Xu *et al*. 2011).

There appears to be little literature upon the nature and extent of compatible solute contribution to salinity tolerance at seed germination. Although the subject is widely covered in terms of whole plant responses to salinity, the same is not true in terms of seeds and seedlings. One compatible solute which appears to be often cited as playing a role in seed germination is betaine. Betaine is a compatible solute present in whole plants that works to alleviate the effects of excessive soil salts and is synthesised in the leaves from betaine aldehyde, catalysed by betaine aldehyde dehydrogenase (BADH). Yokoishi and Tanimoto (1994), working with seedlings of the halophyte *Suaeda japonica*, found that seedlings grown under 0.5 M NaCl stress possessed a BADH activity of five times that of the control. Consequently increases in betaine concentration and BADH activity were found during actual germination when salt stress increased, indicating that under saline conditions, betaine seemed to secure seed germination by osmotic adjustment to the environment in a similar way to that found by compatible solutes in whole plants. Working with (*A. hypogaea*) (peanut), Girija, Smith, and Swamy (2002) also found that concentrations of glycine betaine and proline increased in the embryonic axis of the seed during germination under salinity stress. Korkmaz and Şirikçi (2011) also found that pre-treatment of seeds of *Capsicum annuum* (pepper) with glycine betaine improved germination under saline conditions and modified plants producing glycine betaine have been found to have

greater salt tolerance (Holmström *et al*. 2000, Zhang *et al*. 2009). However, Holmström *et al*. (2000) questioned whether the conferred production of glycine betaine would be sufficient to generate osmotic adjustment of the cytosol and tolerance may be other effects rather than purely a role in osmoregulation. In studies by Poljakoff-Mayber *et al*. (1994) with *K. virginica*, it was found that the addition of either proline or glycine betaine to the germination medium had no significant effect upon the germination of seeds in the absence of NaCl, and was even found to inhibit germination in the presence of 100 mM NaCl. There are a number of contrasting reports on the role of proline in salt tolerance during germination and as a result, its use as a selective criterion for tolerance has been questioned (Ashraf and Harris 2004).

2.4.2 Salt Tolerance Heritability

The heritability of salt tolerance from parent plants varies among species. Often in halophytic species one would expect seeds to display increased salt tolerance due to the habitat that the parent plants often occupy. Indeed, there is a high correlation for tolerance among most halophytic species and low correlation for glycophytic species (Marinko *et al* 1992), but this is not always the case. Dafni and Negbi (1978) studied the salt tolerance heritability of *Prosopis farcta*, a species was is considered glycophytic but able to occupy saline habitats due to very deep roots systems which reach underground fresh water bodies (Bazzaz 1973), but has been included in the eHALOPH database of salt tolerant plants (Royal Botanic Gardens Kew 2008), originally compiled by Aronson (1989). Dafni and Negbi (1978) collected seeds from two Israeli populations of *P. farcta*; one from a saline area (Sedom, Dead Sea) and one from a cultivated field (Hartuv, Judean Foothills). The seeds were germinated in

increasing concentrations of NaCl, but no significant differences were found, concluding that, at least in *P. farcta*, no connection could be found between the salt tolerance of populations and the original salinity of the habitat in which the seeds were collected. Working with *Eucalyptus*, Pearce-Pinto, Van der Moezel, and Bell (1990) also found that there was no relationship between the tolerance of salinity at seed germination and the level of topsoil salinity measured near the mother trees at the time of seed collection. Indeed, of the five species located in saline soils, one failed to produce high germination at 50 mM NaCl, while of seven species which germinated well in 50 mM NaCl, three were found in soils of low salinity. Labidi *et al*. (2005) working with *A. thaliana* found that plants that had received a NaCl treatment, produced siliques with the highest levels of Na^+ and Cl^-, which produced seeds of less viability at germination, indicating that salt uptake by mother plants actually may have a detrimental effect on seed maturation and resultant seed germination.

It is therefore generally considered that salt tolerance of seed and the habitat of the parent plant is unrelated (Rozema 1975, Van der Moezel and Bell, 1987). However, some studies have shown that seed produced from plants growing on a saline soil have been more salt tolerant than seeds from plants growing on non-saline soil. Investigations by Van der Moezel and Bell (1987) showed that this was indeed the case with populations of the halophyte *Melaleuca thyoides*. In addition, Van Zandt and Mopper (2004) found that seeds from the glycophyte *Iris hexagona* (Dixie iris) plants grown under high salinity had higher total germination and germination rate than those grown under low salinity conditions when exposed to saline conditions.

There are a number of factors which need to be accounted for when investigating the salt tolerance of seeds at germination in order to prevent misinterpretations. Martinez, Valverde, and Moreno-Casasda (1992) found that under saline conditions, germination responses of tropical dune seeds differing in age (length of time in storage) differed drastically. Seeds that were stored showed a better rate of germination and final germination percentage. Similar results were found with *K. virginica* seeds, with the germination and seed coat permeability to water increasing with age. A longer storage resulted in an increased rate of complete germination. The mechanisms have not been fully investigated (Poljakoff-Mayber *et al*. 1992).

The physiological state of the seed should also be taken into consideration, as differences in seed size have been shown to have an effect in species. Marañon, Garcia, and Troncoso (1989) studying three *Melilotus* (sweet clover) species found a relationship between seed size and salt tolerance, with larger seeds showing a greater tolerance. Spring wheat plants derived from heavier seed produced larger leaves and greater shoot biomass than those derived from small seed under both saline and non-saline conditions. It follows that large seeds, that would tend to have greater seed reserves would exhibit greater tolerance and produce more vigorous seedlings (Grieve and Francois 1992), perhaps due to a dilution effect of growth that confers lower cellular toxic ion concentrations (McKimmie and Dobrenz 1991). Research with twelve halophytic *Frankenia* species showed germination rates positively correlated with seed size under saline conditions (Easton and Kleindorfer 2009), and Zhang *et al*. (2010) have suggested that this may be due to larger seeds having greater carbon reserves and therefore more growth vigour or an ability to generate a greater internal osmotic potential from compatible solutes, negating the need for Na^+ uptake or exclusion or compartmentalisation from cells, which would detract from vegetative

growth. However, Rogers *et al*. (1995) have shown that despite differences in seed weights, there were no significant differences in seedling vigour of *Trifolium repens* (white clover). Martin and Koebner (1995), studying *T. aestivum* cv. Chinese Spring), also found that seed size does not play a significant role in the variation of salinity tolerance; but variation may result from difference in the size of seed embryos and/or endosperm, and Kaya and Day (2008) found that smaller seeds of *H. annuus* (sunflower) actually germinated more rapidly than larger seeds under NaCl stress.

Soil temperature can affect evapotranspiration rates of solutions held within soil, producing an increase in salinity for consequent germination of seed. In addition, high root temperatures can lead to poor plant growth under saline conditions (Martin and Koebner 1995). Differences in germination can exist in responses to the type of salt present and the soil water content, which affects hydraulic conductivity, soil aeration, soil strength and the area of seed/soil-water contact (Hardegree and Emmerich 1990).

2.4.3 Nutritional Heritability

As stated by Fenner (1991), rather few studies have been carried out on the effect of parent plant nutrition on seed germinability. In terms of the common elements associated with growth there is almost no literature that links nutrition of the parent plant with the consequent germinability of resultant seed under saline conditions. Seeds are considered strong nutrient sinks (Patrick and Offler 2004). Providing elevated Ca^{2+} and K^{+} to seeds via parental nutrition appears highly feasible given the characteristics of calcioles is that external Ca^{2+} concentration stimulates plant Ca^{2+} accumulation (White and Broadley 2003). However, in terms of the route of delivery of the element to developing seeds, little is known. White and Broadley (2009) state

that the application of Ca^{2+} fertilizers to soil generally increases concentrations in tubers, leaves and sometimes fruit and seeds, but the application of fertilisers can increase fruit and seed Ca^{2+} concentrations markedly only when these can be supplied with Ca^{2+} via the xylem (White and Broadley 2009). Most nutrients are delivered to seeds through the phloem (Patrick and Offler 2004), but this is not the case regarding Ca^{2+} (Wolswinkel 1992) and its import pathway into the seed is uncertain (Patrick and Offler 2004). Indeed, Ca^{2+} is almost immobile in the phloem (Kinzel 1989, White and Broadley 2003) and the phloem is dominated by K^+ (Patrick and Offler 2004). Indeed, phloem mobile elements such as phosphorus (P) and K^+ are present in high concentrations as well as lesser amounts of magnesium (Mg) (Wolswinkel 1992). Fruits and seeds rely on Ca^{2+} delivery from the xylem (White and Broadley 2009, White and Broadley 2003, Moore *et al*. 2002), resulting in low Ca^{2+} concentrations. Ca^{2+} deposition is greatest per mass of tissue in the seed coat, although seed coat Ca^{2+} isn't particularly soluble and may be due to remnants of maternal loading.

The seed coat (testa) is the delivery route for nutrients into the embryo (Wolswinkel 1992) and endosperm. (Patrick and Offler 2004). In non-endospermic seeds, nutrient storage occurs in the two large cotyledons, whilst filial tissues act as compartments that store nutrients released via the seed coat and the imported nutrients themselves may act as regulatory signals for the development and role of filial cells (Patrick and Offler 2004). Seed nutrient loading may be limited by nutrient supply or by the capacity of the developing seed to utilise available nutrients and cessation of seed growth appears to be regulated by anatomical or metabolic changes, rather than from a depletion of nutrient supply (Patrick and Offler 2004). The nutritional supply to the seed can also be influenced by the position of the seed on the plant and this may be due to competition for nutrients among fruits or seeds within the same fruit (Fenner

1992). Heritability of Ca^{2+} in *G. max* (soya bean) studied by Streeter (2005), showed that Ca^{2+} supplied to plants at 3.5 times of that available in the soil had no significant effect on tissues and concentrations in seeds were essentially invariate, suggesting that Ca^{2+} concentration, especially in seeds, is difficult to manipulate. Labanauskas, Shouse, and Stolzy (1981) using *Vigna unguiculata* (cowpea) found that irrigation at different stages of growth (vegetative, flowering and pod filling) significantly affected nutrient levels in seed. While irrigation treatments did not significantly influence K^+ concentration in the seed, the concentrations of Ca^{2+} were statistically influenced by treatments, where lowest Ca^{2+} concentrations were found in water stressed plants at the pod filling stage and this was attributed to the immobility of Ca^{2+} in the phloem. The heritability of nutrition and consequent effect on germination has been studied by Labidi *et al*. (2005) with *A. thaliana,* and although using NaCl, they did conclude that there is potential for natural variability for control of ion transport to fruits and an effect on germination, albeit a negative one. However, the promotion of germination under controlled conditions has been linked to the elevation of nitrogen levels in the growth media of *S. lycospersicum* (tomato) (Varis and George 1985), *N. tabacum* (tobacco) (Thomas and Raper 1979), *Festuca arundinacea* (tall fescue) (Watson and Watson 1982) and *Chenopodium album* (white goosefoot) (Fawcett and Slife 1978), although an increased supply of parental nitrogen has been shown to have a negative effect on germination of *T. aestivum* (wheat) (Fallahi and Khajeh-Hosseini 2011). A parental supply of Ca^{2+} has, however, been shown to be important for promoting germination in *A. hypogaea* (peanut) (Coffelt and Hallock 1986). K^+ may also play a key role in some species. Harrington (1960) found that *C. annuum* (pepper) plants subjected to severe K^+ deficiency, produced seeds which tended to germinate in the fruit, indicating that K^+ may be necessary for the formation of a germination inhibitor

that was hypothesised as ABA, but this is not consistent with the work of Haeder and Beringer (1981) who found that low levels of K^+ supplied to wheat, resulted in increased levels of ABA in the immature grain, which could be expected to increase seed dormancy. Wolswinkel (1992) also categorically states that there is no clear evidence that ABA has a clear role in phloem unloading or control of seed assimilate sink strength and growth rate is not limited by endogenous concentration of ABA, stating that high occurrence of ABA in sink tissues with assimilates is simply due to its shared translocation with assimilates. Although perhaps not directly related to germinability, but sink strength, Randall, Thomson, and Schroeder (1979) working with *Pisum saltivum* (pea) showed that K^+ deficiency caused increases in seed total N, as well as physiological changes in terms of significantly decreasing seed yield and producing lower mean seed weight.

2.5 Rapid Cycling *Brassica*: Cultivation, Nutrition and Responses to Salinity

The genus *Brassica* encompasses a wide range of edible plants cultivated for over 4000 years, with the wide array of vegetables produced by different modifications to the leaf or shoot system (Shannon and Grieve 1999). *Brassica* contains about 100 species and the genus is regarded as having more important agricultural and horticultural crops than any other genus (Purty *et al.* 2008). Although the cultivated vegetables may differ widely in appearance at maturity, differences in the structure of the root, fruit and seeds are almost indistinguishable, as are their morphology as seedlings (Shannon and Grieve 1999).

B. rapa L. is a member of the *Brassicaceae*, and is also known as wild turnip or field mustard. Originally a crop native to Russia, Siberia and Scandanavian countries, it has

been grown for thousands of years as a food for both humans and animals and a range of morphologies now exist within its subspecies (Figure 2.1). These biennial, herbaceous plants are grown for their fleshy roots, large green leaves and rape seed oil. Indeed, *Brassica* oilseed species hold the third position among oilseed crops (Williams 1989, Purty *et al*. 2008); oilseed crops being in third position among crops next to cereals and legumes (Downey 1990), and are an important source of vegetable oil, fodder and industrial/medicinal products (Williams 1989, Ashraf and McNeilly 2004), including a source of biofuel as a primary use and/or as a waste product of oil.

Morphotypes of the subspecies of *Brassica rapa*

Figure 2.1 - Diagrammatic Representations of the Subspecies of *B. rapa*. (Wisconsin Fast Plants 1987).

This investigation utilises the laboratory developed rapid cycling derivative of *B. rapa* L., developed over 30 years for use in laboratory based experiments as a model crop plant by Dr Paul H. Williams at Wisconsin University, USA. (Williams and Hill 1986) from a plant found growing on the mountainsides of Nepal. A diagrammatic representation of the morphology of a typical plant is shown in Figure 2.2.

The breeding programme concentrated on selection for:

- Small plant habitat

- Minimum time from seed to flowering
- Uniformity of flower maturation
- High female fertility
- Rapid seed maturation
- Absence of seed dormancy

(Tomkins and Williams 1990)

Under optimal conditions it can pass through its life cycle, from seed to seed, in 35-40 days (Figure 2.3) and perhaps the most common use is for experiments involving Mendelian genetics, as the pollination mechanism contains a self-incompatibility complex.

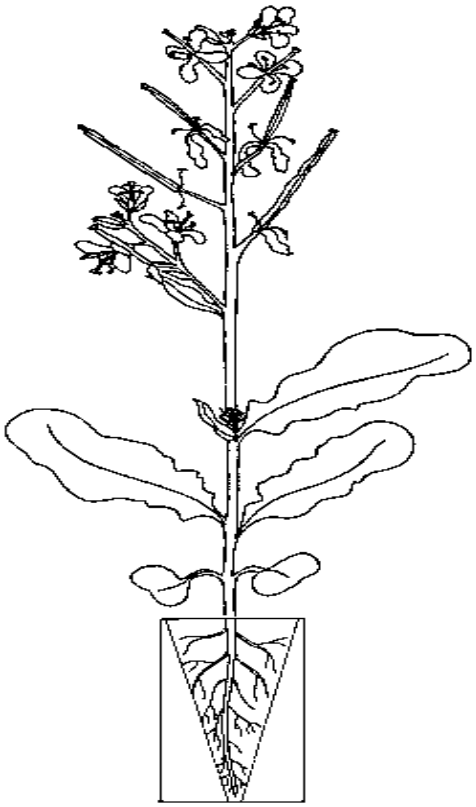

Figure 2.2 - Diagrammatic Representation of the Morphology of *B. rapa*.

0 1 2 3 4 7 9 11 13 15 18 28-35

Days

Figure 2.3 - Growth of Rapid Cycling *Brassica rapa* cv. RCBr Showing Growth Stages at Various Times from Seed to 28 -35 Days. (Adapted from Williams 1989).

Water relations and photosynthesis are disturbed by salinity stress in all brassicas and the extent varies according to the species and seed germination and early seedling growth have been reported to be relatively more sensitive towards salinity (Ashraf and McNeilly 2004, Purty *et al*. 2008). Brassicas osmotically adjust under conditions of salinity, and this is particularly true of salt tolerant species and has been found to be due to the accumulation of organic compatible solutes, such as soluble sugars, amino acids and proline (Ashraf and McNeilly 2004). Engineered production of betaine by insertion of *codA* gene that encodes for choline oxidase, which is involved in the first stage of synthesis of glycine betaine, from bacteria into *B. juncea* has showed enhanced salinity tolerance at germination (Prasad *et al*. 2000), which may indicate a role for compatible solutes during germination processes under saline conditions in *Brassica*. Protein accumulation in *B. napus* has also shown to improve salinity

tolerance under germination (Srivastava, Fristensky, and Kav 2004) and seeds of *B. napus* transformed with a LEA gene have been shown to have increased drought and salt tolerance when germinated on both MS medium and hydroponics (Park *et al*. 2005).

A number of studies by He and Cramer (1993a) involving investigation of nutritional status under conditions of salinity have been carried out on *Brassica* species. Investigating the response of six rapid cycling *Brassica* species (*B. carinata, B. campestris, B. juncea, B. napus, B. nigra and B. oleracea*) to different levels of seawater salinity, He and Cramer (1993a) found that the saline environment had significant effects on shoot and root concentrations of Na^+, K^+, Cl^-, Ca^{2+} and Mg^{2+}. They found that increases in salt concentration were negatively correlated with the Ca^{2+} concentration in shoots, as was K^+ concentration, while a positive correlation was found with Na^+. Differences in salt tolerance were found, particularly between two species; *B. carinata* (-) and *B. napus* (+). He and Cramer originally state that the difference in salinity tolerance was unlikely to be due to ion specific effect due to an indifference of ion concentrations between the two species, i.e. differences in the degree of toxicity of Na^+, Mg^{2+} and Cl^- or perhaps a capacity to maintain sufficient Ca^{2+} and K^+ in tissues. Following on from this work, He and Cramer (1993b) discovered that the K^+/Na^+ ratio in the shoots of the six species was significantly reduced by seawater salinity, due to a reduction in shoot K^+ concentration and increase in shoot Na^+ concentration. Similar results were found by Purty *et al*. (2008) with 4 day old seedlings of 11 different genotypes of *Brassica*. He and Cramer (1993b) hypothesised that the decline in tissue K^+ concentration may have resulted from direct competition between K^+ and Na^+ at sites of uptake at the plasmalemma, an effect of Na^+ on K^+ transport into the xylem, or a Na^+-increased K^+ efflux from the

root. Changes in K^+-Na^+ selectivity were found, but these were species dependent, and neither ratio or selectivity was found to be correlated with the relative salt tolerance (*B. napus, B. campestris, B. nigra, B. juncea, B. oleracea and B. carinata*) of these *Brassica* species, indicating that maintenance of a high K^+/Na^+ ratio was not a mechanism for salt tolerance during these experiments. Unpublished data was also stated to implicate ABA in the salt tolerance of *Brassica* species. However, Walker and Bernal (2005) working with *B. oleracea* and *B. carinata*, hypothesised that the greater salt tolerance of *B. carinata* was related to maintenance of shoot K^+ and restriction of Na^+ and Cl^-, and Rameeh, Rezai, and Saeidi (2004) working with eight *B. napus* genotypes concluded that salinity had no significant effect on whole plant shoot Ca^{2+} concentration, but positive correlations did exist for shoot dry weight and Ca^{2+}, K^+, Ca^{2+}/Na^+ and K^+/Na^+.

In line with the majority of experimentation investigating nutrition and salt tolerance, Schmidt, He and Cramer (1993) studied the effects of supplemental Ca^{2+} to salt treated whole plant and callus cultures of these six rapid-cycling *Brassica* species. Interestingly, they found that none of the cultures responded to the supplemental Ca^{2+}, indicating that salt tolerance was not dependent upon Ca^{2+}, but had previously found that salt tolerance was positively correlated with the internal Ca^{2+} concentration of tissue (He and Cramer 1992, 1993a). In previous germination tests by Ashraf and Naqvi (1991), it was found that the addition of supplemental Ca^{2+} to the saline medium also had no significant effect on the germination and growth of *B. carinata*, and *B. campestris*, but was beneficial for *B. juncea* and *B. napus*. Huang and Redmann (1995) also found that *B. napus* and *B. kaber* (wild mustard) responded positively with enhanced germination and seedling growth with additional Ca^{2+} under PEG and NaCl treatment.

In conclusion, the work of He and Cramer has found that the nutrition of rapid cycling *Brassica* species are significantly affected by salinity and the effects of salinity on these species at least may be primarily osmotic and not perhaps due to the toxic effects of Na^+ and Cl^-. In terms of whole plant and seed tolerance, salinity does not appear to be significantly linked to Ca^{2+} concentration, but more so K^+ in a number of *Brassica* species, although it appears that there is considerable intraspecific and interspecific variation in salinity tolerance and the mechanisms by which it may occur among *Brassica* species and their genotypes (Ashraf and McNeilly 2004, Purty *et al*. 2008).

Chapter 3: General Methods

3.1 Experimental Overview

The investigation consisted of four growth trials, two of which provided seed which was later analysed for Ca^{2+} and K^{+} concentration and assessed for salinity tolerance. Subsequent experimentation investigated the potential localisation and availability of Ca^{2+} and K^{+} to contribute to salinity responses in *B. rapa* seed during germination. The first two growth trials were preliminary in terms of testing the suitability of two differently modified Hoagland's solutions as well as sand as an initially chosen growth substrate. The third multi-substrate and fourth hydroponic growth trials utilised the preferred modification of Hoagland's solution and tested the effect of a range of different substrates and hydroponic culture. These latter trials produced enough daughter seed to enable accurate elemental analysis and salinity tolerance assays. The hydroponic trial also produced two sizes of seed which were tested in these ways as an attempt to discover if embryo to seed coat ratio had an effect on Ca^{2+} and K^{+} distribution between those tissues. During the growth trials, data were collected that reflects the vigour of growth and seed production in response to differing nutrient regimes where possible. This included, for example, information on plant height and days to flower. Further work with commercially purchased *B. rapa* looked at Ca^{2+} localisation within the seed and provided an insight into the potential chemical nature and likelihood of involvement of compounds of Ca^{2+} and K^{+} from various tissues during germination. Figure 3.1 outlines the origins and relationships of experimental procedures.

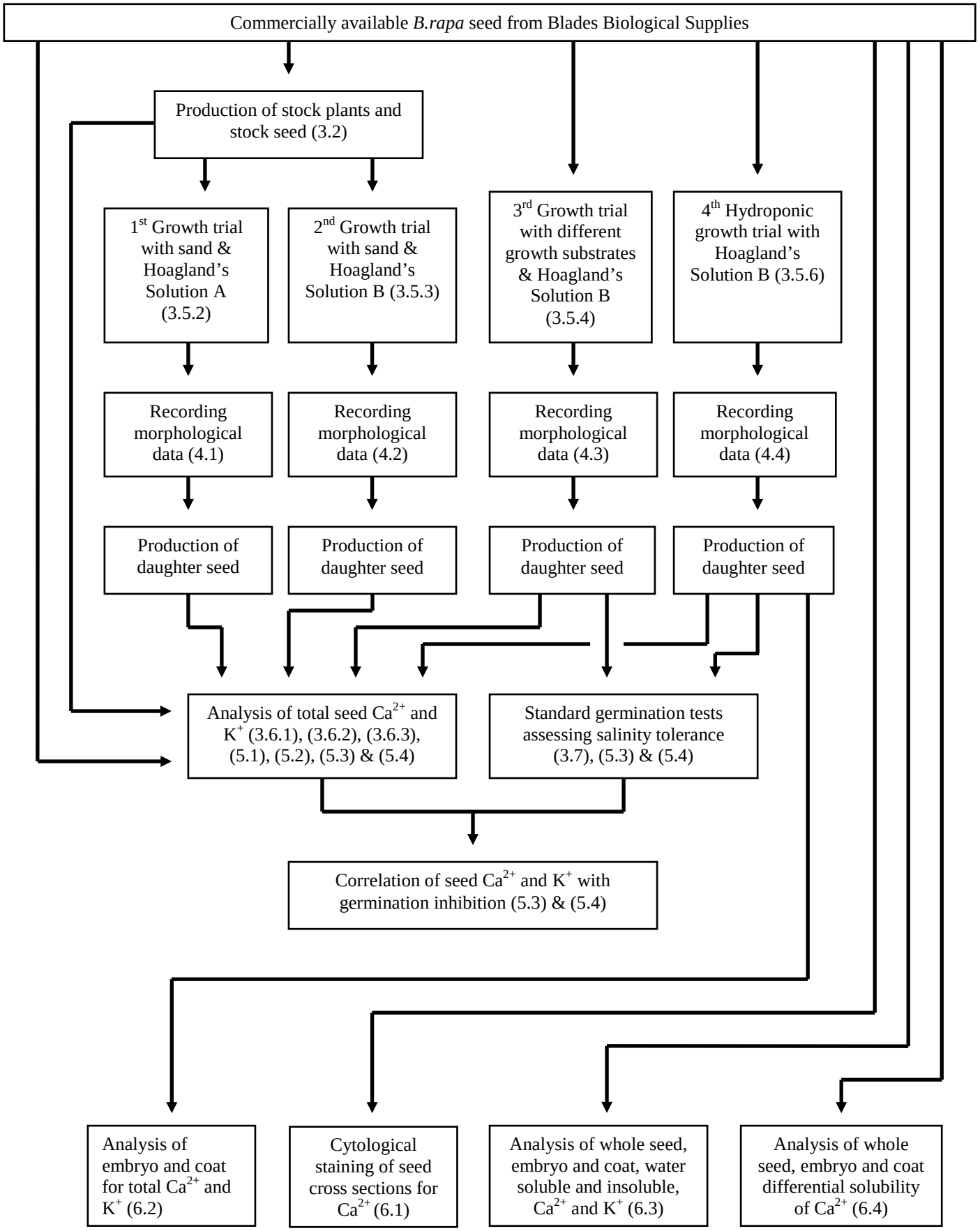

Figure 3.1 - Flow Diagram of Core Experimental Procedures (*bracketed values refer to section notation*)

3.2 Growing *Brassica rapa* Stock Plants and Producing Stock Seed

Seeds of wild type *Brassica rapa* L. were purchased commercially from Blades Biological Supplies, Cowden, Edenbridge, Kent, UK. Sixty 65 mm Ø pots, each containing 125 cm^3 of a moist general purpose sphagnum moss peat (Shamrock® Medium General Purpose Irish Sphagnum Peat, Scotts UK Professional, Ipswich, Suffolk, UK) and vermiculite (Vermiperl® Fine Grade, William Sinclair Horticulture Limited, Lincoln, UK) mixture (1:1 v/v) (Sinniah, Ellis, and John 1998) were placed in four seed trays that had been lined with capillary matting, to prevent sliding and movement (BHGS Horticultural Supplies, Evesham, Worcestershire, UK). Each pot containing the growth substrate was moistened with deionised water before the seeds were sown 5 mm deep. Sowing occurred on 13th February 2003. The seed trays were then placed under greenhouse sodium lighting supplying a 24 h daylength (Tomkins and Williams 1990), with daylight supplemented by 60 μmol $m^{-2}s^{-1}$ photosynthetically active radiant energy at bench level provided by Philips 400 W Son T sodium lamps. The minimum temperature in the greenhouse was 15°C, but maximum temperatures were not controlled. The position of the greenhouse was 52°24’ N and 1°30’ W (The National GPS Network, Ordnance Survey 2003).

Irrigation commenced 4 days after sowing with 15 ml of deionised water per pot and then alternate day irrigation of 15 ml of 0.44 g l^{-1} Phostrogen® in deionised water or 15 ml of deionised water (Sinniah, Ellis, and John 1998).

Hand pollination of flowers with a soft brush began when 50% (Table 3.1) of the plants had produced at least one flower and continued until no more flowers were produced.

Table 3.1 - Number and Percentage of Stock Plants Producing Flowers Over a 3 Day Period Prior to Pollination

Date	Number and (%)of Plants Producing at Least One Flower
03/03/03	0 (0)
04/03/03	6 (10%)
05/03/03	35 – Pollination began (58%)

After the first day of pollination, normal irrigation continued for 15 days and thereafter the volume of irrigant was reduced to 5 ml per pot in order to prevent precocious germination.

The harvesting of siliquae from the plants began when they were yellow and crinkled in appearance, being dry to the touch. Siliquae were harvested according to three distinct periods of age and development. Diagrammatic representations of each plant were drawn to identify siliquae which developed or had significantly began to develop 5 days, 10 days and 15 days after the first day of initial pollination (Plate 3.1). This was done to enable seed harvesting to be carried out on siliquae that had received the same length of exposure to seed nutrient sink, to try and eliminate any effect of late development on seed Ca^{2+} or K^{+} content. It also served to identify the likelihood and timing of silique development from flowers within the growth period and before harvesting.

During the harvesting process, siliquae were removed from the plant using sharp scissors in order to prevent the disturbance of other siliquae and then categorised into their respective age before being placed into labelled paper bags. Seed was removed from individual siliquae by hand and collected in large plastic containers in order to reduce seed loss. Debris from siliquae was separated from the seed by use of a piece of capillary matting that was in a tilted position, allowing particles to be selected by

the effect of gravity and surface resistance. The resultant seed from each physiological age category was then divided into five size categories as follows:

< 1.0 mm, 1.0 - 1.4 mm, 1.4 - 1.7 mm, 1.7-2.0 mm and > 2.0 mm Ø.

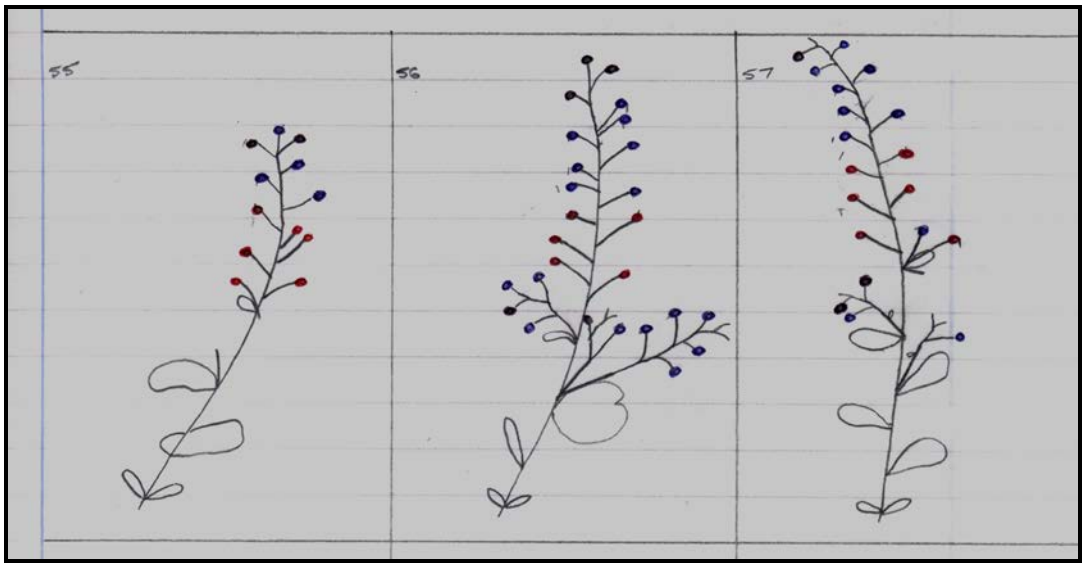

Plate 3.1 - Examples of Morphological Drawings

Separation of the seeds was achieved by passing the seeds through a selection of wire sieves with mesh sizes 1.0, 1.4, 1.7 and 2.0 mm Ø. Each set of seeds, categorised on physiological age and diameter, was assessed for their fresh weight and placed in open paper seed packets before being placed in a desiccator containing silica gel to be stored in an incubator at 15°C (Sinniah, Ellis, and John, 1998) for drying.

3.3 Nutrient Solutions for Producing Experimental Seed

3.3.1 Producing Modified Hoagland's Nutrient Solutions A and B

Based upon a recipe to produce modified Hoagland's nutrient solutions (Machlis and Torrey 1956), with full strength as control (Hoagland and Arnon 1938) the aim was to produce five solutions differing in Ca^{2+} and K^{+} nutrition. Two separate sets of five

solutions were produced which were labelled 'Solution A' and 'Solution B' and these contained different variation in the levels of Ca^{2+} and K^{+}. The five solutions that were produced included a full strength control and four solutions of higher and lower levels of Ca^{2+} and K^{+} shown in Tables 3.2 and 3.4. The completed solutions were also analysed for Ca^{2+}, K^{+} and Na^{+} and the results of this analysis can be found in Appendix 3, Tables A3.1 and A3.2.

3.3.1.1 Producing Modified Hoagland's Nutrient Solution A

The relative levels of Ca^{2+} and K^{+} in the solution are shown in Table 3.2.

Table 3.2 - Levels of Calcium and Potassium in Modified Hoagland's Nutrient Solution A, Relative to 100% Strength Control.

Control	Low Ca^{2+}	High Ca^{2+}	Low K^{+}	High K^{+}
100% strength Ca^{2+} & 100% strength K^{+}	25% strength Ca^{2+} and 100% strength K^{+}	400% strength Ca^{2+} and 100% strength K^{+}	25% strength K^{+} and 100% strength Ca^{2+}	400% strength K^{+} and 100% strength Ca^{2+}

The recipe per litre of deionised water, using 1 M stock solution constituents is described in Table 3.3.

Table 3.3 - Volumes of Constituents per Litre of Distilled Water Required to Produce Modified Hoagland's Solution A

	Volume (ml)				
Compound	Control	Low Ca^{2+}	High Ca^{2+}	Low K^{+}	High K^{+}
$Ca(NO_3)_2$	5	1.25	20	5	5
KNO_3	5	5	5	1.25	23
$MgSO_4$	2	2	2	2	2
KH_2PO_4	1	1	1	0.25	1
FeEDTA stock	1	1	1	1	1
Micronutrient stock	1	1	1	1	1
NH_4NO_3	15	18.75	-	16.5	6
$NH_4H_2PO_4$	-	-	-	0.75	-

3.3.1.2 Producing Modified Hoagland's Nutrient Solution B

The relative levels of Ca^{2+} and K^+ in the solution are shown in Table 3.4.

Table 3.4 - Levels of Calcium and Potassium in Modified Hoagland's Nutrient Solution B, Relative to 100% Strength Control

Control	Low Ca^{2+}	High Ca^{2+}	Low K^+	High K^+
100% strength Ca^{2+} & 100% strength K^+	50% strength Ca^{2+} and 100% strength K^+	200% strength Ca^{2+} and 100% strength K^+	50% strength K^+ and 100% strength Ca^{2+}	200% strength K^+ and 100% strength Ca^{2+}

The recipe per litre of deionised water, using 1 M stock solution constituents is described in Table 3.5.

Table 3.5 - Volume of Constituents per Litre of Distilled Water Required to Produce Modified Hoagland's Solution B

	Volume (ml)				
Compound	Control	Low Ca^{2+}	High Ca^{2+}	Low K^+	High K^+
$Ca(NO_3)_2$	5	2.5	10	5	5
KNO_3	5	5	5	2.5	11
$MgSO_4$	2	2	2	2	2
KH_2PO_4	1	1	1	0.5	1
FeEDTA stock	1	1	1	1	1
Micronutrient stock	1	1	1	1	1
NH_4NO_3	5	7.5	-	6	2
$NH_4H_2PO_4$	-	-	-	0.5	-

3.3.2 Notes on Constituents

3.3.2.1 FeEDTA Stock

FeEDTA is a solution of an iron complex of ethylenediamaminetetraacetic acid. FeEDTA stock was produced by dissolving 7.45 g of Na_2EDTA in 1 l of deionised water, heating until warm and subsequently adding 5.57 g of $FeSO_4.7H_2O$.

3.3.2.2 Micronutrient Stock

The micronutrients stock solution contained 2.86 g of H_2BO_3 (boric acid), 1.81 g of $MnCl_2.4H_2O$ (manganese chloride), 0.11 g of $ZnCl_2$ (zinc chloride), 0.05 g of $CuCl_2.2H_2O$ (copper chloride) and 0.025 g of $Na_2MoO_4.2H_2O$ (disodium molybdate) l^{-1} of stock solution.

3.3.2.3 pH

The pH of each solution was modified to pH 5.7-5.8 by the use of 0.1 M sodium hydroxide which was added directly to 25 l of each solution. Some precipitation was observed and it was decided that each solution should be analysed for Ca^{2+} and, K^+ as a final check and precautionary action. A 100 ml sample of each nutrient solution was filtered through a 125 mm Whatman No. 1 filter paper to remove any precipitate and analysed via an atomic emission spectrometer. The results can be found in Appendix 3, Tables A3.1 and A3.2.

3.3.2.4 Calcium, Potassium, Nitrate and Ammonium Ion Concentrations in Modified Hoagland's Solutions A and B

Due to the addition of varying amounts of $Ca(NO_3)_2$, KNO_3, NH_4NO_3 and $NH_4H_2PO_4$ in the modified Hoagland's nutrient solutions in order to alter the amounts of Ca^{2+} and K^+, but keep the nitrogen content constant, the amount of nitrogen found as NO_3^- and NH_4^+ was different for each treatment. Tables 3.6 and 3.7 outline the concentration of nitrogen as nitrate and ammonium ions as well as the concentration of Ca^{2+} and K^+ for each set of Hoagland's solutions used.

Table 3.6 - Concentration of Nutritionally Important Ions in Modified Hoagland's Solution A

	Concentration of Ions (mmoles l^{-1})			
	Ca^{2+}	K^+	NO_3^--N	NH_4^+-N
Control	5	6	30	15
Low Ca^{2+}	1.25	6	26.25	18.75
High Ca^{2+}	20	6	45	0
Low K^+	5	1.5	27.75	17.25
High K^+	5	24	39	6

Table 3.7 - Concentration of Nutritionally Important Ions in Modified Hoagland's Solution B

	Concentration of Ions (mmoles l^{-1})			
	Ca^{2+}	K^+	NO_3^--N	NH_4^+-N
Control	5	6	20	5
Low Ca^{2+}	2.5	6	17.5	7.5
High Ca^{2+}	10	6	25	0
Low K^+	5	3	18.5	6.5
High K^+	5	12	23	2

3.4 Levels of Exchangeable Calcium and Potassium in Various Growth Substrates

The levels of exchangeable Ca^{2+} and K^+ in five types of growing substrate were tested. Due to its use in growing *B. rapa* stock plants, a sample of the sphagnum peat and vermiculite mix (1:1 v/v) was tested along with a sample of the sphagnum peat and of the vermiculite alone to determine the contribution each makes to the mixture. Since the sphagnum peat and vermiculite mixture has been referred to as a fairly nutrient free medium (Tomkins and Williams 1990), it was decided that it may be suitable as a medium for growing *B. rapa* under conditions requiring low initial Ca^{2+} and K^+ concentration; this providing a second reason to test the levels of available Ca^{2+} and K^+ in the medium. A sample of horticultural sand was also tested, along with a sample of a perlite (Silvaperl® Standard Grade, William Sinclair Horticulture

Limited, Lincoln, UK) and horticultural sand mixture (2:1 v/v) to determine their suitability as a growth medium for the experiment which required a strict nutrient regime.

A sample of each medium was air dried in an oven at 80°C. Each sample was passed through a hammer mill containing a 1 mm mesh sieve (Glen Creston, London, UK). Three 5 g replicates of each sample were then transferred into separate 50 ml borosilicate glass conical flasks. Twenty millilitres of 1 M ammonium acetate was added to each flask and allowed to stand overnight. The following day, the contents of each beaker were transferred to separate funnels fitted with a 125 mm Whatman No. 1 filter paper and the filtrate was collected in a 250 ml volumetric flask. The substrate samples were continually leached with successive 25 ml volumes of 1 M ammonium acetate, with the funnel being allowed to drain between each addition, until nearly 250 ml of extract had been collected. The filtrate was made up to 250 ml and each solution was retained for the determination of Ca^{2+} and K^{+} via atomic emission spectrometry by ICP-OES. Details of the equipment used can be found in section 3.6.1. The results were then used to calculate the amount of exchangeable Ca^{2+} and K^{+} in each growth substrate. These levels were then compared with exchangeable cation ratings (m.e.100 g^{-1} or $Cmol_c\ kg^{-1}$ Ca^{++}, Mg^{++}, K^{+} and Na^{+})' (Curtis, Courtney, and Trudgill 1976) to produce Ca^{2+} and K^{+} ratings, i.e. "high", "moderate" or "low", and this was subsequently used to determine the suitability of the growth substrate for use in the growth trials requiring strict nutrient regimes. In terms of exchangeable cation ratings, sand was deemed to have very low, sand & perlite to have very low and peat and vermiculite to have very high cation exchange capacity. See Appendix 2, Tables A2.1, A2.2 and A2.3 for details of analyses.

3.5 Growing *Brassica rapa* Experimental Plants and Producing Experimental Daughter Seed

3.5.1 Growth Trial Layout

In each of the experiments, the trays were arranged in an identical Latin Square design in each location.

High Potassium A	Low Calcium A	Control A	Low Potassium A	High Calcium A
High Calcium B	High Potassium B	Low Calcium B	Control B	Low Potassium B
Low Potassium C	High Calcium C	High Potassium C	Low Calcium C	Control C
Control D	Low Potassium D	High Calcium D	High Potassium D	Low Calcium D
Low Calcium E	Control E	Low Potassium E	High Calcium E	High Potassium E

Figure 3.2 - Latin Square Design Layout for Growth Trials

3.5.2 Preliminary Growth Trial in Sand Using Modified Hoagland's Solution A in Greenhouse Conditions

The experiment was conducted under greenhouse conditions as described for the production of stock plants and seed (section 3.2). Chosen seeds ("five-day" siliquae,

Ø 1.0 - 1.4 mm) from the stock batch were removed from a desiccator that was stored in an incubator at 15°C. The seeds were suspended in a conical flask, within a spherical wire mesh over deionised water (Plate 3.2), for 16 to 17 h, within an incubator set at 20°C. This was a humidification treatment in order to prevent imbibition damage.

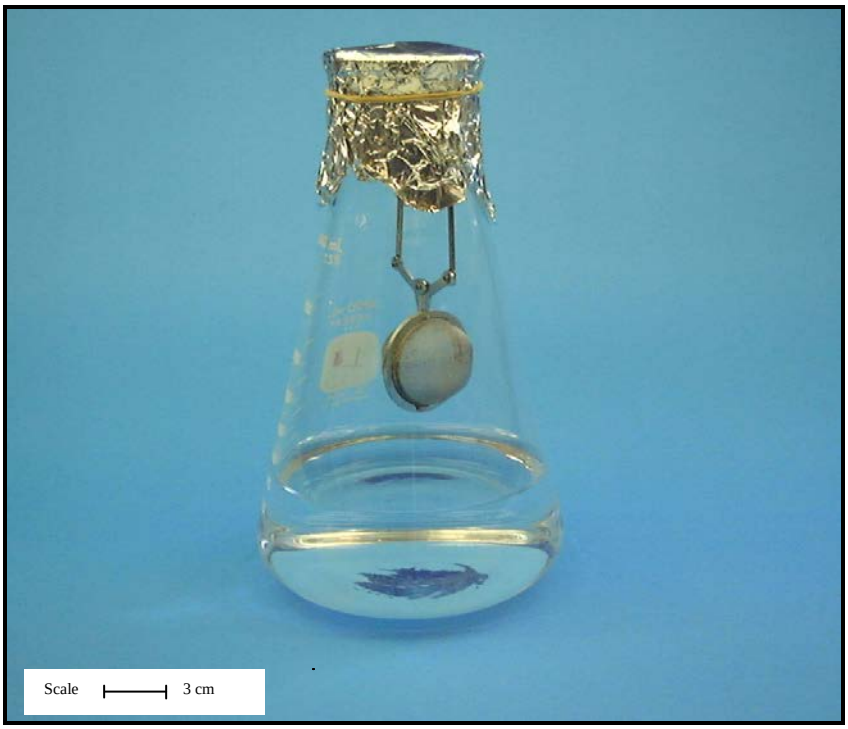

Plate 3.2 - Apparatus Used in the Humidification Treatment.

One hundred and fifty, 65 mm Ø pots were lined at their bases with 40 mm^2 pieces of capillary matting and then filled with 125 cm^3 of Gem® Horticultural Sand. Each pot had a fresh weight of sand of 170 g. The pots were placed in 75 mm Ø saucers and placed within seed trays, lined with capillary matting, with six pots and saucers per full size tray. Five trays, containing six pots, were labelled for each of the five modified Hoagland's A nutritional treatments.

The sand in each pot was irrigated to field capacity with deionised water before the humidification treated seeds were sown 5 mm deep below the surface of the sand. Sowing occurred on 15th May 2003. 150 seeds were sown (one per pot). Irrigation applied to the upper surface of sand with modified Hoagland's solution began 4 days after sowing and continued daily with each pot being supplemented with the appropriate solution so as to re-attain field capacity. Determination of attainment of field capacity was achieved by visually observing the collection of solution in the saucers placed underneath each pot, indicating that the sand was saturated with solution. Any solution collected by a saucer was discarded after allowing each pot time to drain.

3.5.3 Preliminary Growth Trial in Sand Using Modified Hoagland's Solution B in Growth Room Conditions

The experiment was conducted within a temperature controlled growth room, with a constant temperature of 25°C during periods of illumination and 21°C otherwise. Sodium lamps provided a 20 h daylength (Sinniah, Ellis, and John 1998) of 105 μmol m^{-2} s^{-1} photosynthetically active radiant energy at bench level, provided by Philips 400 W Son T sodium lamps.

Chosen seeds ("five-day" siliquae, 1.0 - 1.4 mm) from the stock batch were removed from desiccator storage and subjected to the humidification treatment described in section 3.5.2 (Plate 3.2).

Pots were prepared within seed trays as described in section 3.5.2, but 450 seeds were sown (three per pot) and were subsequently thinned out in order to accommodate for those seeds which did not germinate successfully. Further irrigation is described in

3.5.5. Sowing occurred on 16th July 2003.

3.5.4 Growth Trial in Various Growth Substrates using Modified Hoagland's Solution B in Greenhouse and Growth Room Conditions

The experiment was conducted within both greenhouse and temperature controlled growth room conditions as described in sections 3.2 and 3.5.3 respectively.

Seeds purchased from Blades Biological Supplies were subjected to the humidification treatment described in section 3.5.2 (Plate 3.2).

One hundred and twenty five, 65 mm Ø pots, lined with 40 mm^2 capillary matting, were filled with 125 cm^3 of Gem® horticultural sand. Each pot had a fresh weight of sand of 170 g. Another 125, 65 mm Ø pots, lined with capillary matting, were filled with 125 cm^3 of Silvaperl® perlite and horticultural sand mixture (2:1 v/v). Each pot contained a fresh weight of 75 g of the mixture. A further 125, 65 mm Ø pots, also lined with capillary matting, were filled with 125 cm^3 of sphagnum peat and Vermiperl® vermiculite mix (1:1 v/v), with each pot having a fresh weight of 25 g. All pots were placed in 75 mm Ø saucers and placed within seed trays, lined with capillary matting to prevent sliding and movement. Pots containing sand & perlite/sand mixture were placed in 25 seed trays, with five pots of each growth substrate per tray (i.e. 10 pots/tray in total). Pots containing sphagnum peat and vermiculite mixture were also placed in a separate set of 25 seed trays, with five pots per tray. Each set of 25 trays was then labelled for five replicates of the five modified Hoagland's B nutritional treatments. Trays containing sand and sand & perlite mixture were transferred to the growth room and those containing the sphagnum peat/vermiculite mixture were transferred to the greenhouse.

The growth substrate of each pot was irrigated as described previously in section 3.5.2. 1125 seeds were sown (three per pot) and were subsequently thinned out in order to accommodate for those seeds which did not germinate successfully. Sowing occurred on 21st October 2003.

3.5.5 Further Irrigation for Trials using Sand and Various Growth Substrates And Modified Hoagland's Solution B

For trials described in sections 3.5.3 and 3.5.4 irrigation applied to the base (capillary irrigation) of pots with modified Hoagland's solution via saucers began 5 days after sowing. Capillary irrigation continued daily with each saucer being supplemented with the appropriate solution. Irrigation applied to the top of pots was initially practised 10 days after sowing, when seedlings had developed to a stage where primary leaves were not in contact with sand and could not be covered by any sand washing over them. Irrigation from the top continued to be performed every 3 days thereafter, supplemented by capillary irrigation via saucers in order to flush salts that may have formed in the uppermost sections of the pot due to effects of surface evaporation.

Irrigation of a plant continued for 15 days after initial pollination and was then reduced to a third of the field capacity in order to prevent precocious germination.

3.5.6 Hydroponic Growth Trial using Modified Hoagland's Solution B in Growth Room Conditions

The experiment was conducted within temperature controlled growth room conditions as described in section 3.5.3.

Seeds purchased from Blades Biological Supplies were subjected to the humidification treatment described in section 3.5.2 (Plate 3.2).

Twenty five 15 l hydroponic tanks, having five replicates of each of the five modified Hoagland's B nutritional treatments were filled with 5 l of each nutrient solution. Polystyrene packaging trays, containing 30 segments and having had a 5 mm Ø hole bored to the base of each segment were used for floatation. Each hole was lightly packed with non absorbent cotton wool, with a depression created within the cotton wool to accommodate individual seeds. Gaps between the polystyrene trays and tank walls were covered by heat reflective plastic coated foil blankets that had been cut to size. This helped to prevent algae formation in the nutrient solution through light stimulation. Each tank received aeration via an air pump and network of airstones in order to prevent anoxic conditions and subsequent poor root development. Seeds were sown on 16th March 2007. Seven hundred and fifty seeds in total were sown and subsequently monitored for maximum germination, where seeds that failed to germinate were replaced within the first 3 days.

The layout of hydroponic tanks and example of polystyrene floatation trays are shown in Plates 3.3 and 3.4 respectively.

Nutrient solutions were replaced weekly and the experiment continued for 35 days. Nutrient solutions were removed after this period and the plants and siliquae were allowed to naturally dry before harvesting.

3.5.7 Pollination

Within each set of five trays for each nutrient treatment, hand pollination with a soft

brush began when the first flowers were produced and continued until no more flowers were produced within each nutrient treatment set. However, pollination only began to take place when more than one flower within each set of five trays for each nutrient treatment had fully opened, and subsequently only flowers that had fully opened were pollinated.

Plate 3.3 - Latin Square Arrangement of Hydroponically Grown *B. rapa*

Plate 3.4 - An Example of Hydroponically Grown *B. rapa* in Week 1 using Polystyrene Floatation Trays

Five days after the initial pollination of an individual plant, siliquae which had formed, or flowers which had significantly died back and showed distinct signs of developing siliquae, were noted by morphological drawings to aid future identification. For the hydroponic growth trial, no morphological drawings were made due to the number of plants grown in the trial and previous growth trials had suggested that only siliquae having at least five days maturation produced seed that would be of experimental use in terms of the size achieved.

3.5.8 Preparation of Experimental Seed

The harvesting of siliquae from experimental plants began when they were yellow and crinkled in appearance, being dry to the touch. Siliquae that were earlier identified as developed or significantly developing after five days from the date of initial pollination of a particular plant were harvested using sharp scissors, before being

placed into labelled paper bags according to their nutritional treatment. Seed was removed from individual siliquae by hand and collected in large plastic containers in order to reduce seed loss. Debris was separated from the seed and then the seed was divided into five size categories as follows: < 1.0 mm, 1.0 - 1.4 mm, 1.4 - 1.7 mm, 1.7 - 2.0 mm and over 2.0 mm Ø. For experiments using growth substrates, each set of plants within an individual tray were analysed for the total number of "five-day" seeds, the total weight of "five-day" seed produced and the size distribution of "five-day" seed produced. For the hydroponic growth trial, each set of up to 30 plants was analysed for the total number, total weight and size distribution of seed, regardless of when siliquae were produced. Harvesting in earlier trials had suggested that only siliquae having at least five days maturation produced filled siliquae and produced seed that was sufficiently large enough for experimental use.

Each set of seeds, categorised by diameter, were placed in open paper seed packets before being placed in a desiccator containing silica gel to be stored in an incubator set at 15°C (Sinniah, Ellis, and John 1998) for drying.

3.6 Seed Elemental Analysis

3.6.1 General Sample Preparation and Analysis

Samples of seed were weighed, approximately but accurately, to four decimal places for the largest available weight available given the sample size, from a uniformly mixed batch. Individual sample weights are discussed within specific methods.

Seeds were loaded into polytetrafluoroethylene (PTFE) digestion vessels and underwent microwave digestion. The PTFE digestion vessels were acid washed in 1

M sulphuric acid (H_2SO_4), as was all glassware before use. The exact nature of the equipment is discussed in individual methodologies. To each sample, concentrated nitric acid (HNO_3) and in some cases 100 vol (30% w/v) hydrogen peroxide (H_2O_2), both of trace analysis grade, were added. A control digestion was also used, containing no seed material. After cooling, the samples were decanted into separate acid washed volumetric flasks. Deionised water was then used to increase the volume of the samples to full volume with the first additions of water being made via the digestion vessel, so as to wash through any remaining sample. Analysis of Ca^{2+} and K^+ via an atomic emission spectrometer (Optima 5300DV ICP-OES, Perkin Elmer, Waltham, MA, USA) (Plate 3.5) produced triplicate readings for each sample. All standards were matrix matched, by adding the same proportion of acid as had been used to produce digested samples. Each set of triplicate ICP-OES readings was averaged to produce a replicate average. Replicate averages for each of the three viable samples were then used to produce mean overall levels (in mg l^{-1}) minus the blank readings for Ca^{2+} and K^+ per volume of sample. Calculations that followed, converted the readings to levels measured in μg g^{-1} of seed dry weight via consideration of moisture content.

3.6.2 Analysis of Commercially Purchased Seed

Whole seed was digested and analysed as described in section 3.6.1 with the following details. Three 0.02 g samples of the commercially purchased seed to be used for growth trials using different growth substrate and hydroponic culture (sections 3.5.4 and 3.5.6) were analysed from a uniformly mixed batch. To each sample, 3 ml of concentrated trace HNO_3 and 2 ml of H_2O_2, both of trace analysis grade, were added. A Mars 5 Microwave Accelerated Reaction System (CEM

Corporation, Matthews, NC, USA) was used for the digestion procedure. After cooling, the samples were decanted into separate acid washed 50 ml volumetric flasks and deionised water was used to increase the volume of the samples to 50 ml. The results of the analysis can be found in Appendix 1, Table A1.1

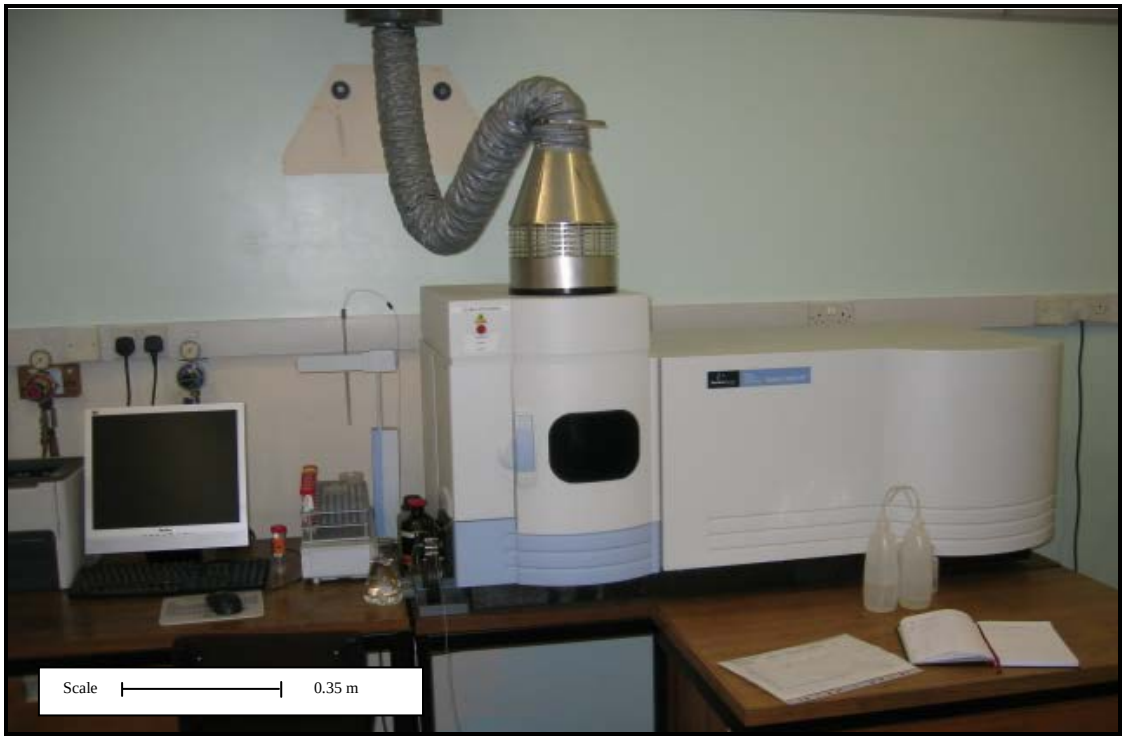

Plate 3.5 - Perkin Elmer Optima 5300DV ICP-OES

3.6.3 Analysis of Stock Plant Produced Seed

Whole seed was digested and analysed as described in section 3.6.1 with the following details. Three 0.5 g samples of the stock seed ("five-day" siliquae, size 1.0 to 1.4 mm Ø) to be used for preliminary growth trials in sand (sections 3.8.2 and 3.8.3 were analysed. To each sample, 3 ml of concentrated HNO_3 and 2 ml of H_2O_2, both of trace analysis grade, were added. A MLS 1200 Mega High Performance Laboratory Digestion Unit (Milestone, Shelton, CT, USA) was used for the digestion

procedure. After cooling, the samples were decanted into separate acid washed 50 ml volumetric flasks and deionised water was used to increase the volume of the samples to 50 ml. The results of the analysis can be found in Appendix 1, Table A1.2.

3.7 Salinity Tolerance and Standard Germination Tests

3.7.1 General Media Preparation

Two 70 mm Whatman No. 1 filter papers were placed in 9 cm Ø plastic Petri dishes. NaCl was prepared at concentrations between 0 and 400 mM with deionised water. These concentrations varied between growth trials and are discussed further within specific methods. Four millilitres of solution was added to each Petri dish.

3.7.2 Seed Germination

The seeds were placed at approximately uniform distances from one another upon the surface of the filter paper with forceps and each dish was sealed with Parafilm® before being incubated at 25°C with a 20 h photoperiod in a temperature controlled growth room. The dishes were arranged randomly and the germination rate was recorded daily over 7 days. However, only the final germination was used as a measure of salt tolerance. Germination was defined by the total emergence of the radicle.

3.7.3 Statistical Analysis

Salinity tolerance of seed germination for each replicate was estimated by fitting the data to a probit dose response model to calculate the effective NaCl concentration that

reduced germination by 50% (EC_{50}) of the unstressed control seed's germination in deionised water (Figure 3.3). This was undertaken using Statplus 2008 (AnalystSoft Inc., USA).

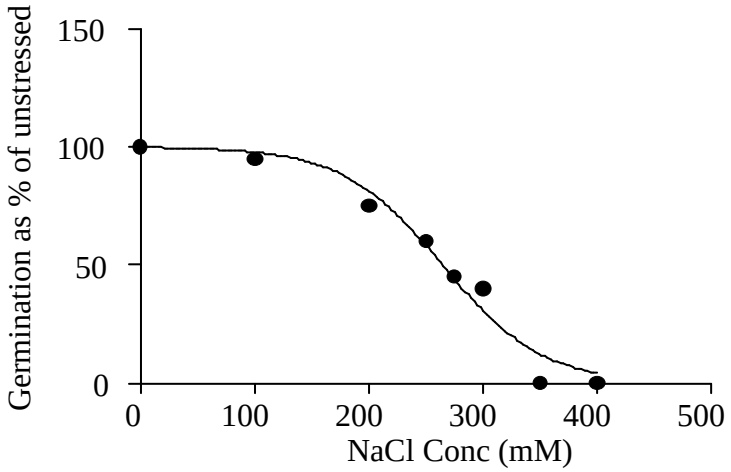

Figure 3.3 - Example of Probit Dose Response Model Used to Calculate EC_{50} (Salt Tolerance)

Regression analyses of NaCl concentration that reduced germination (EC_{50}) against seed Ca^{2+}, K^+ and Ca^{2+}+ K^+ concentration were performed for each growth substrate separately and for the whole data set, along with a calculation of Pearson's correlation significance for EC_{50} against Ca^{2+}, K^+, Ca^{2+}+K^+, Ca^{2+}/K^+ and K^+/Ca^{2+}. Regression analysis and Pearson's significance tests was performed using Minitab 13 (Minitab, UK). Seed concentrations of Ca^{2+} and K^+ were converted from μg g^{-1} to μmol g^{-1} prior to any statistical analysis or correlations that included the addition of [Ca^{2+}] and [K^+] (as per Ca^{2+}+ K^+).

Chapter 4: Nutrition, Plant Growth and Seed Physiology

4.1 Preliminary Experiment Using Sand as a Growth Substrate and Modified Hoagland's Solution A

4.1.1 Introduction

The collection of data relating to growth and vigour during the development of a suitable nutritional regime and growth substrate was undertaken to assess their suitability to produce enough seed for subsequent analysis and investigation of salt tolerance. Aspects such as seed germination percentage provide information about the suitability of the substrate during various periods of plant development. Flowering was indicative of the vigour and potential for seed production. Height obtained by a plant provided indication of nutrient availability, uptake and conversion to biomass. Seed yield data incorporating seed number, weight and 100 seed weight, gave a direct measurement of the differences due to nutrient treatments and the total seed produced over the growth trial.

4.1.2 Methods

4.1.2.1 Plant Growth

Plants were grown by the methods outlined in section 3.5.2.

4.1.2.2 Recording Data

During the period of growth, the following traits were recorded for each nutritional treatment: the percentage germination of seedlings, the percentage of plants which flowered, number of days to flowering, the cumulative numbers of flowers per plant and the height of each individual plant on a daily basis. After siliquae harvest the

number of seeds per plant, seed yield per plant (weight) and the 100 seed weight for each treatment replicate in each experiment was recorded. The seed number and total weight for each available size category was recorded and means were calculated for each treatment for those measured aspects across all size categories of seed.

4.1.2.3 Statistical Analyses

Plant developmental and seed physiology data were statistically analysed using a General Linear Model, Analysis of Variance incorporating Fisher's Protected Least Significant Difference (LSD). Data presented as a percentage (% germination, percentage flowering) underwent ARCSIN Transformation before being tested.

4.1.3 Results

The plant developmental data indicated that the control treatment appeared to have a positive effect upon percentage flowering, flowers per plant, time to flower and maximum height obtained, compared with other treatments. Table 4.1 shows that percentage flowering of control plants was higher than with any other treatment, as was the maximum height obtained. Low Ca^{2+} treated plants had lower percentage flowering and took longer to flower than any other treatment.

Table 4.1 - Plant Development Data

	% Germination	% Flowering	Mean Days to Flower	Flowers per Flowering Plant	Maximum Height (cm)
Control	83 a	71 a	20 a	27 a	20.7 a
Low Ca^{2+}	67 a	4 d	31 d	2 c	7.5 b
High Ca^{2+}	80 a	29 bc	23 bc	15 ab	7.5 b
Low K^{+}	87 a	21 c	21 ab	3 bc	5.4 b
High K^{+}	73 a	46 b	26 c	11 bc	9.6 b

Means within columns followed by different letter differ significantly at $P < 0.05$.
See Appendix 4, Tables A4.1 - A4.5 for statistical analyses.

The seed yield data provided in Table 4.2 indicated that in terms of seed yield (number of seeds plant^{-1} and mg seed plant^{-1}), the control treatment produced a higher yield compared with other treatments. The 100 seed weight values given in Table 4.2 are an indication of the density and general size of seeds produced for each nutritional treatment. Although insufficient seed was produced to accurately measure 100 seed weight for all nutritional treatments, those that were measured (Control, High Ca^{2+} and Low K^{+}) showed no differences between treatments.

Table 4.2 - Seed Yield Data

	Seed Yield (number of seeds plant^{-1})	Seed Yield (mg seed plant^{-1})	100 Seed Weight (mg)
Control	19 a	14 a	74 a
Low Ca^{2+}	0 b	0 b	#
High Ca^{2+}	4 b	3 b	99 a
Low K^{+}	0 b	0 b	#
High K^{+}	1 b	0 b	77 a

Means within columns followed by different letter differ significantly at P < 0.05. # denotes insufficient quantities of seed to undertake analyses. See Appendix 4, Tables A4.6 - A4.8 for statistical analysis.

4.1.4 Discussion

Nutritional treatment had a significant effect upon flowering, mean days to flower, flowers per plant and maximum plant height. Plants grown in low base nutrient level substrate of sand and with control treatments, produced greater levels of flowering and maximum height, indicating perhaps that Ca^{2+} and K^{+} balanced nutrition contributes to some aspects of vigour. In addition, low Ca^{2+} treatments produced plants which had very low flowering and low numbers of flowers per plant. The seed data, although limited, is also indicative of the fact that plants treated with control nutrition were more vigorous than the other treatments in terms of seed yield (no. seeds plant^{-1} and mg seeds plant^{-1}) and 100 seed weight.

4.1.5 Conclusion

The performance of the nutrient solution applied in a nutrient poor substrate, provided data that suggested that although there may appear to be differences in plant development provided by the different nutrient treatments, sufficient seed to be able to evaluate yield and perform the planned subsequent seed analysis was still required. The ability to be able to grow plants in differing nutritional regimes and to produce seed of differing nutritional quality, without substantially affecting production was of importance. The differences in Ca^{2+} and K^{+} applied between low and high treatments was considered to be too great and that plant development and subsequent seed production was being sacrificed in an attempt to alter seed nutrient loading. The development of a second modified Hoagland's solution that differed less in terms of high and low levels of Ca^{2+} and K^{+} and undertaking of a subsequent growth trial in sand would endeavour to produce greater plant vigour and seed production.

4.2 Preliminary Experiment Using Sand as a Growth Substrate and Modified Hoagland's Solution B

4.2.1 Introduction

The second modified Hoagland's solution aimed to produce plants with greater nutritional balance and vigour and subsequently more seed which could be used in the elemental and salt tolerance analysis. As with the first growth trial, using sand and modified Hoagland's Solution A, aspects of flowering and plant height were recorded to evaluate the nutrient solutions' suitability in producing viable amounts of seed. Percentage germination was less critical, given that three seeds were sown per pot and subsequently thinned to enable the best possible germination and thus plants

producing seed. Seed yield data was used as a direct indication of the production capacity of plants grown using the second modified Hoagland's solution and sand as a substrate.

4.2.2 Methods

4.2.2.1 Plant Growth

Plants were grown by the methods outlined in section 3.5.3.

4.2.2.2 Recording Data

Data was recorded as in section *4.1.2.*2

4.2.2.3 Statistical Analyses

Statistical analysis was performed as outlined in section *4.1.2.*3

4.2.3 Results

Under growth room conditions there were no differences between treatments for percentage flowering, number of flowers or height (Table 4.3). Low Ca^{2+} treated plants took longest to flower, longer than the control, low K^+ and high K^+, but not so for high Ca^{2+}**.**

Table 4.3 - Plant Development Data

	% Germination	% Flowering	Mean Days to Flower	Flowers per Flowering Plant	Maximum Height (cm)
Control	100 a	87 a	19 a	9 a	10.9 a
Low Ca^{2+}	100 a	67 a	23 b	3 a	7.1 a
High Ca^{2+}	100 a	93 a	21 ab	6 a	11.5 a
Low K^{+}	100 a	77 a	19 a	7 a	10.3 a
High K^{+}	100 a	93 a	20 a	5 a	10.4 a

Means within columns followed by different letter differ significantly at P < 0.05.
See Appendix 5, Tables A5.1 - A5.4 for statistical analyses.

There were no differences for seed yield (mg seed $plant^{-1}$), seed weight per plant and 100 seed weight between treatments (Table 4.4). Low K^{+} treatment produced plants which gave the highest seed yield (no. seeds $plant^{-1}$), higher than the control, low Ca^{2+} and high K^{+} treatments, but not so compared with high Ca^{2+}.

Table 4.4 - Seed Yield Data

	Seed Yield (number of seeds $plant^{-1}$)	Seed Yield (mg seed $plant^{-1}$)	100 Seed Weight (mg)
Control	3 b	2 a	64 a
Low Ca^{2+}	1 b	1 a	87 a
High Ca^{2+}	6 ab	3 a	49 a
Low K^{+}	8 a	4 a	50 a
High K^{+}	2 b	1 a	42 a

Means within columns followed by different letter differ significantly at P < 0.05.
See Appendix 5, Tables A5.5 - A5.7 for statistical analyses.

4.2.4 Discussion

Compared with the previous experiment that used modified Hoagland's Solution A, this growth trial used lower Ca^{2+} and K^{+} ratios among treatments compared with the control and this produced quite different results in terms of developmental and seed data. Although percentage germination was effectively engineered by experimental design, hardly any differences exist between treatments for percentage flowering,

mean days to flower, flowers per plant and maximum height. The treatments therefore appear to be more evenly matched in terms of the vigour and physiology of plants, than in the earlier trial. This is also true of seed yield and 100 seed weight (Table 4.4) with little difference between treatments.

4.2.5 Conclusion

Modified Hoagland's Solution B appeared to produce plants that were much more evenly matched in terms of development, than those produced in the preceding growth trial using modified Hoagland's Solution A. However, the number of flowers per plant and seed yield per plant was still deemed insufficient for the seed yield required to perform statistically meaningful elemental and salinity tolerance analysis. Given that plant development appeared to be much improved by the use of modified Hoagland's Solution B, but not forgetting the necessity to try to produce seed that differed in Ca^{2+} and K^{+} content, it was decided that seed yield improvement may only occur by changing the growth substrate. The dense and fine structure of sand also gave concerns to the potential of root anoxia which influenced the choice of substrates used in the subsequent growth trial.

4.3 Growth Trial Using Various Growth Substrates and Modified Hoagland's Solution B

4.3.1 Introduction

Modified Hoagland's Solution B was used with three different growth substrates; peat and vermiculite, sand and sand & perlite over two locations; a greenhouse and growth room. The problems associated with growing plants in sand and producing viable seed

yield influenced the decision to compare sand with a mixture of sand & perlite in an attempt to overcome any potential root anoxia. Peat and vermiculite mimicked the substrate used to produce stock seed, which produced successful yields. The seeds used to produce plants during germination were commercially produced and not from the stock seed batch, used during the previous two growth trials. This was to prevent and problems with seedling and plant vigour and consequent seed production that may have arisen by using second generation seed. Developmental data included those aspects previously recorded, but percentage germination was 100% as this was part of the experimental design, achieved by oversowing and subsequent thinning.

4.3.2 Methods

4.3.2.1 Plant Growth

Plants were grown by the methods outlined in section 3.5.4.

4.3.2.2 Recording Data

Data was recorded as outlined in section *4.1.2.*2 except that maximum plant height was not recorded in this growth trial, given the difficulty in accurately assessing height given the production of biomass being much greater than in previous trials and not being wholly vertical in nature, along with a need to protect siliquae.

4.3.2.3 Statistical Analyses

Statistical analysis was performed as outlined in section *4.1.2.*3

4.3.3 Results

4.3.3.1 Plant Developmental Data

Germination was not different for the type of growth substrate and site in which the plants were grown and did not differ according to the nutritional treatment supplied (Table 4.5).

Table 4.5 - Percentage Germination

	Germination (%)		
	Greenhouse Peat & Vermiculite	Growth Room Sand	Growth Room Sand & Perlite
Control	100 a	100 a	100 a
Low Ca^{2+}	100 a	100 a	100 a
High Ca^{2+}	100 a	100 a	100 a
Low K^{+}	100 a	100 a	100 a
High K^{+}	100 a	100 a	100 a

Means followed by different letter differ significantly at $P < 0.05$.

The percentage of plants which flowered was not significantly changed between site and growth substrate, and the nutritional treatments also did not produce any significant differences in flowering between plants (Table 4.6).

Table 4.6 - Percentage Flowering

	Flowering (%)		
	Greenhouse Peat & Vermiculite	Growth Room Sand	Growth Room Sand & Perlite
Control	100 a	96 a	96 a
Low Ca^{2+}	100 a	96 a	100 a
High Ca^{2+}	100 a	100 a	100 a
Low K^{+}	100 a	100 a	100 a
High K^{+}	100 a	100 a	100 a

Means followed by different letter differ significantly at $P < 0.05$.

The mean time taken in days to flower was unaffected by growth substrate, site or nutritional treatment (Table 4.7).

Table 4.7 - Days to Flowering

	Mean Days to Flowering		
	Greenhouse Peat & Vermiculite	Growth Room Sand	Growth Room Sand & Perlite
Control	20 a	19 a	20 a
Low Ca^{2+}	20 a	19 a	19 a
High Ca^{2+}	20 a	19 a	19 a
Low K^{+}	20 a	20 a	20 a
High K^{+}	20 a	20 a	20 a

Means followed by different letter differ significantly at $P < 0.05$.
See Appendix 6, Table A6.1 for statistical analysis.

The mean number of flowers per plant with sand and sand & perlite as growth substrate is shown in Table 4.8. Peat & vermiculite was not included due to the vast abundance of flowers concurrently produced on a daily basis that made it difficult to accurately elucidate the numbers produced on individual days and therefore the total amount produced over the entire growth period. In terms of plants grown in sand, there are no significant differences among the five treatments. This also true for plants grown in sand & perlite. A significant increase was found between plants grown in high K^{+} nutrition in sand and plants grown in control nutrition in sand & perlite, but otherwise it does not appear that any significant differences in numbers of flowers occurred between the two growth substrates.

Table 4.8 - Flowers per Plant

	Number of Flowers per Plant	
	Growth Room Sand	Growth Room Sand & Perlite
Control	12.6 ab	15.6 a
Low Ca^{2+}	9.0 ab	15.4 ab
High Ca^{2+}	11.6 ab	12.2 ab
Low K^{+}	9.6 ab	14.6 ab
High K^{+}	8.3 b	12.4 ab

Means followed by different letter differ significantly at $P < 0.05$.
See Appendix 6, Table A6.2 for statistical analysis.

4.3.3.2 Seed Yield Data

There was no difference in seed yield between treatments for plants grown in sand and sand & perlite in the growth room (Table 4.9). However, plants grown in a medium of peat & vermiculite in the greenhouse produced higher amounts of seed over all five treatments, than did plants grown in the other substrates. Low Ca^{2+} nutrition produced a higher number of seed than control and high K^{+}.

Table 4.9 - Number of Seeds per Plant amongst Site and Growth Substrate

	Seed Yield (number of seeds plant^{-1})		
	Greenhouse Peat & Vermiculite	Growth Room Sand	Growth Room Sand & Perlite
Control	180 b	47 d	68 d
Low Ca^{2+}	272 a	39 d	66 d
High Ca^{2+}	231 ab	68 d	64 d
Low K^{+}	200 ab	46 d	87 d
High K^{+}	194 b	53 d	52 d

Means followed by different letter differ significantly at $P < 0.05$.
See Appendix 6, Table A6.3 for statistical analysis.

As with seed yield (number of seeds plant^{-1}) (Table 4.9), plants grown in peat & vermiculite in the greenhouse produced greater amounts of seed (mg seed plant^{-1} than plants in the other two growth substrates and sites (Table 4.10). Low Ca^{2+} treatment in peat & vermiculite produced particularly high seed yield and this was significantly higher than for plants receiving control, low K^{+} or high K^{+} treatment. The weight of seeds did not vary between nutrient solutions for plants grown in sand and sand & perlite.

Table 4.10 - Seed Weight per Plant amongst Site and Growth Substrate

	Seed Yield (mg seed plant^{-1})		
	Greenhouse Peat & Vermiculite	Growth Room Sand	Growth Room Sand & Perlite
Control	238 bc	72 e	123 de
Low Ca^{2+}	342 a	46 e	126 de
High Ca^{2+}	288 ab	103 e	108 e
Low K^{+}	241 bc	52 e	142 cde
High K^{+}	222 bcd	75 e	92 e

Means followed by different letter differ significantly at P < 0.05.
See Appendix 6, Table A6.4 for statistical analysis.

Plants grown in sand & perlite had higher 100 seed weight than those plants grown in peat & vermiculite, but higher seed weight than those grown in sand, only with low Ca^{2+} and low K^{+} (Table 4.11). Results for K^{+} nutrition are also interesting. High K^{+} nutrition in the growth room substrate produced higher weights than in the greenhouse, whereas low K^{+} nutrition in the growth room substrate produced lower seed weight, compared with other treatments. In general, plants cultivated in peat & vermiculite in the greenhouse, tended to produce a greater weight and number of seeds per plant, but of lower density and size, than plants cultivated in sand & perlite in the growth room.

Table 4.11 - 100 Seed Weight amongst Site and Growth Substrate

	100 Seed Weight (mg)		
	Greenhouse Peat & Vermiculite	Growth Room Sand	Growth Room Sand & Perlite
Control	134 c	149 bc	180 ab
Low Ca^{2+}	126 d	118 f	194 a
High Ca^{2+}	126 de	158 abc	171 ab
Low K^{+}	120 e	103 h	163 abc
High K^{+}	116 g	145 bc	178 ab

Means followed by different letter differ significantly at P < 0.05.
See Appendix 6, Table A6.5 for statistical analysis.

4.3.4 Discussion

Plants that were grown in peat and vermiculite in the greenhouse produced greater amounts of flowers and produced siliquae with greater numbers of seed (Table 4.9) and seed yield in terms of mg seed plant^{-1} produced similar results (Table 4.10. These plants differed from the others in terms of both the site and growth substrate and the data highlights the differences between the growth and productivity of those plants grown in a growth medium consisting of high base nutrient levels (Tables A2.1-A2.4) and supplemented with nutrients provided by modified Hoagland's media, and those plants which were grown with almost no base levels of nutrient of the growth substrate (Tables A2.1-A2.4) and relied solely upon modified Hoagland's media for their nutrient supply. The structure of the growth substrate may have also been significant. The roots of plants grown in sand or sand & perlite may have experienced hypoxia, due to small particles and decreasing pore space and therefore reduced aeration (Lipiec and Hatano 2003), compared with the coarser structure of peat and vermiculite. Such properties of growth substrate may influence root growth; decreasing root size, retarding root penetration and rooting depth (Gliński and Lipiec 1990) and decreasing nutrient uptake (Tardieu 1988, Gliński, and Lipiec 1990, Lorenzen *et al*. 2001). Conditions of increased natural light intensity, day length and increased temperature in the greenhouse, compared with the growth room, are also very likely to have contributed to greater seed production.

Considering that low Ca^{2+} treated plants grown under greenhouse conditions with peat and vermiculite produced significantly higher numbers of seed than control and high K^+ treated plants (Table 4.9) and produced significantly higher yield (mg seed plant^{-1}) than control, low K^+ and high K^+ (Table 4.10), it may be likely that interactions

between the base levels of nutrients and supplemental modified Hoagland's media contributed to this difference. Interactions between Ca^{2+} and K^{+} may be taking place and the interaction between root chemistry and growth substrate chemistry is potentially complex. Tester (1990) noted that exogenous Ca^{2+} may inhibit the inward movement of K^{+} through the plasmalemma, whereas Ca^{2+} has also been implicated in the maintenance of adequate K^{+} transport (Epstein 1961, Läuchli, and Epstein 1970). Competition of Ca^{2+} with Mg^{2+} for uptake from nutrient solution has been observed with *L. leucocephala*. An increased concentration of $CaSO_4$ in the nutrient solution decreased Mg^{2+} in roots, stems and leaves (Grattan and Grieve 1992). Naidoo (1994) also highlighted Ca^{2+} and Mg^{2} competition for cell wall absorption sites. Competition between Ca^{2+} and other ions may explain why low Ca^{2+} solutions with peat and vermiculite produced higher seed yields than high Ca^{2+} solutions.

One hundred seed weight data suggests that those plants grown in sand & perlite generally produced seed with a greater 100 seed weight than those in the other two growth substrates, especially peat and vermiculite (Table 4.11). As a guide to the general density, the 100 seed weight data, when held alongside that described for seed yield (no. seed and mg seed $plant^{-1}$), suggest that although plants grown in peat and vermiculite produced greater amounts of seed per plant, the seed may be not as large or as dense as that produced by plants grown in sand and sand & perlite. Negative relationships between seed yield and seed size has been reported with different *Phaseolus vulgaris* genotypes (White and González 1990). These authors also reported that a positive relationship can exist between seed size and yield in certain environments, perhaps indicating that growth environment may at least partly contribute to such relationships. Although those plants grown in peat and vermiculite grew much more vigourously and produced more seed, the quality in terms of size

and density may have been sacrificed to account for increased plant biomass. Patrick and Offler (2004) concur that seed biomass gain is at the expense of other organs that compete for a finite nutrient pool and the potential size a seed may reach at maturity is positively related to cell number and inversely related to the number of seeds developing on the plant.

4.3.5 Conclusion

Growth substrate/location appears to have a significant effect on seed production, but plant development was not greatly affected in terms of flowering. The effect of growing plants in sand is that of reducing seed yield, even when mixed with perlite which was used to increase substrate pore size. Favourable plant growth occurred using peat and vermiculite as a substrate and this presented a problem in terms of accurately assessing the effect of maternally supplied Ca^{2+} and K^{+}, since peat and vermiculite was found to be very high in exchangeable Ca^{2+} and K^{+}. An abundance of nutrients in the growth substrate appears to cause increased biomass production. Additional Ca^{2+} nutrition via modified Hoagland's Solution B produced less seed yield, which may be indicative of complex interactive chemistry that may be involve Ca^{2+} as an effector of mechanisms that govern seed production. In this case, the results show that although higher levels of nutrients in growth substrate are beneficial to overall seed production, compared to nutrient poor substrates, there may be an optimum level, either side of which may start to impact upon seed production. In order to assess the sole effect of varying mother plant Ca^{2+} and K^{+} nutrition without the potential interactive complexities of a growth substrate, methods of growing plant without a substrate were considered.

4.4 Hydroponic Growth Trial using Modified Hoagland's Solution B

4.4.1 Introduction

Modified Hoagland's Solution B was used to create a hydroponic culture media in the endeavour that plants would be able to grow without any negative physical structural properties, i.e. anoxic conditions and potential background nutritional interference that using a substrate could create, along with the capability to grow many plants and to produce enough seed to carry out elemental and salt tolerance analysis and any further experiments in order to maintain experimental continuity. The seeds used to produce plants during germination were commercially produced and not from the stock seed batch that was used during the initial two growth trials. This was to prevent problems with seedling and plant vigour and consequent seed production that may have arisen by using second generation seed. Percentage germination was 100% as this was part of the experimental design, achieved by oversowing and subsequent thinning. Data acquisition comprised of seed yield data.

4.4.2 Methods

4.2.2.1 Plant Growth

Plants were grown by the methods outlined in section 3.5.6.

4.4.2.2 Recording Data

No plant developmental data was obtained due to the numbers of plants in the growth trial and the large amount of flowers that were produced. After siliquae harvest, the number of seeds per plant, seed yield per plant (weight) and the 100 seed weight for each treatment replicate in each experiment was recorded. The seed number and total

weight for each available size category was recorded and means were calculated for each treatment for those measured aspects across two size categories of seed (Ø <1.0 mm and Ø 1.0 - 1.4 mm). These size categories covered the majority of the seed that was produced.

4.4.2.3 Statistical Analyses

Statistically analysis was performed as outlined in section *4.1.2.3.*

4.4.3 Results

4.4.3.1 Seed Yield Data

Overall means for different size categories shows that seed of size Ø <1.0 mm were significantly more abundant than seed of Ø 1 - 1.4 mm (Table 4.12). There was no significant interaction between treatment and seed size. There were no differences amongst individual means. With regard to nutritional treatments, there was no difference in number of seeds produced between treatments.

Table 4.12 - Number of Seeds per Plant

	Seed Yield (number of seeds plant^{-1})		
	Ø 1.0 - 1.4 mm	Ø <1.0 mm	Mean
Control	43 a	84 a	63 *a*
Low Ca^{2+}	51 a	87 a	69 *a*
High Ca^{2+}	44 a	68 a	56 *a*
Low K^{+}	38 a	88 a	63 *a*
High K^{+}	51 a	65 a	58 *a*
Mean	45 *b*	78 *a*	

Means followed by different letter differ significantly at P < 0.05. Separate comparisons of overall means between either columns or rows are denoted by italicised letters. See Appendix 7, Table A7.1 for statistical analysis.

Overall means for different size categories shows that seed of size Ø <1.0 mm and seed of Ø 1 - 1.4 mm were not different in terms of mass of seed produced between size categories (Table 4.13). There was no significant interaction between treatment

and seed size. There were also no differences amongst individual means. With regard to nutritional treatments, there was no difference in mass of seeds produced between treatments.

Table 4.13 - Seed Weight per Plant

	Seed Yield (mg seed plant^{-1})		
	Ø 1.0 - 1.4 mm	Ø <1.0 mm	Mean
Control	43 a	46 a	44 *a*
Low Ca^{2+}	50 a	52 a	51 *a*
High Ca^{2+}	44 a	40 a	42 *a*
Low K^{+}	37 a	54 a	45 *a*
High K^{+}	54 a	38 a	46 *a*
Mean	46 *a*	46 *a*	

Means followed by different letter differ significantly at P < 0.05. Separate comparisons of overall means between either columns or rows are denoted by italicised letters. See Appendix 7, Table A7.2 for statistical analysis.

Overall means for different size categories shows that seed of size Ø 1.0 - 1.4 mm, produced a 100 seed weight value that was significantly greater than that of Ø <1.0 mm (Table 4.14). This is also reflected by the individual means across nutritional treatments. There was no significant interaction between treatment and seed size. With regard to nutritional treatments, there was no significant difference in number of seeds produced between treatments.

Table 4.14 - 100 Seed Weight

	100 Seed Weight (mg)		
	Ø 1.0 - 1.4 mm	Ø <1.0 mm	Mean
Control	100 a	54 b	77 *a*
Low Ca^{2+}	99 a	61 b	80 *a*
High Ca^{2+}	100 a	58 b	79 *a*
Low K^{+}	97 a	60 b	79 *a*
High K^{+}	105 a	59 b	82 *a*
Mean	100 *a*	58 *b*	

Means followed by different letter differ significantly at P < 0.05. Separate comparisons of overall means between either columns or rows are denoted by italicised letters. See Appendix 7, Table A7.3 for statistical analysis.

4.4.4 Discussion

Across all nutritional treatments there is consistently a significantly greater abundance of seeds produced in the siliquae of plants in the Ø < 1.0 mm category than in Ø 1.0 - 1.4 mm (Table 4.12). There is no effect of treatment on mean number of seeds per plant. This is similarly reflected in terms of mg seed produced per plant shown in Table 4.13. Nutritional treatment has no significant effect on yield across both size categories, but there was also no significant difference in the mg seed per plant between size categories, indicating that the overall mass of seed produced by both size categories did not differ between nutritional treatments or seed size, but a greater number of the smaller size category was greater in order for this to be the case.

Differences in number of seed produced per plant between the two size classes may be the result of a normal seed maturation response in a given time. Harper, Lovell, and Moore (1970) discussed the plant response to seed development during different growth stages and that under unlimited growth conditions, seed development may be initiated, but then is later not fulfilled to its full potential in subsequent growth and maturation stages. This would result in those seeds being smaller. The results may simply be indicative of the growth conditions of plants and the time in which seeds were given the opportunity to reach maturity, given that the regime imposed to reduce water uptake was different to that in previous growth trials and was carried out in order to try and avoid precocious germination of seeds within siliquae under hydroponic conditions. This is usually achieved using higher molecular weight polyethylene glycol (PEG), but it was decided that the rate at which water was reduced was difficult to determine and the onset of precocious germination would have rendered seed useless.

Positioning within the siliquae may have a role to play in determining seed mass, given the above, with competition for differences in nutrient uptake or space (Schaal 1980) or simply the distance from the nutrient source (Harper, Lovell, and Moore 1970). In some cruciferae, seeds at the stylar (distal) end of the siliquae have been reported as being larger (Salisbury 1942). Differences in the number of seeds at the two size categories, perhaps due to the water reduction technique employed, may also be due to the nature of apportionment of reproductive energy between seed size and number; a gain in seed number is counteracted by a reduction in seed size (Harper 1977) which could also be due to evolutionary tactics in terms of dispersal, predation and competition, even though seeds of a smaller size contain less endosperm, so are less likely to survive and compete (Harper 1977). Harper, Lovell, and Moore (1970) also interestingly comment on manipulation of seed number and size, citing evidence that reducing the number of seeds that are allowed to develop normally, increases mean seed weight, but obviously at a reduction in number.

Perhaps more importantly it has been shown that nutritional treatment in this case did not affect seed yield. Given the nature of the growth substrate in this particular experiment, compared with those used in the growth trial that employed various types of substrate, structure of growth substrate and the potential of differences in nutrient uptake due to the nature of the substrate is not an issue using hydroponic culture and the only effector is the level of nutrients in the culture medium, with no interactions between substrate and nutrient solutions applied. In this case, the lack of difference in seed yield between nutritional treatments provides evidence that, at least in the case of seed yield, the differences in the actual nutritional regimes applied during this investigation do not significantly affect growth patterns themselves, which is a

positive outcome given that any difference in development may affect seed nutrient loading for reasons other than the difference in nutrients available.

One hundred seed weight data (Table 4.14), in line with that of data for mg seed per plant, suggests unsurprisingly that those seeds which are of greater size (Ø 1.0 - 1.4 mm) have a significantly greater 100 seed weight than the Ø < 1.0 mm category. Mean 100 seed weight across all nutritional treatments was not significantly different and reiterates the successful lack of variation in physiological aspects of seed development that is not affected by nutrient availability in this instance.

4.4.5 Conclusion

Hydroponic culture was successful in producing seed for subsequent analysis through the number of mother plants that were grown. Seed yield (mg plant^{-1}) and 100 seed weight, was lower than that found in the growth trial using three different substrates (section *4.3.3.2*), but seed yield (no. plant^{-1}) was comparable. A hydroponic culture produced sufficient seed without incurring the effects of growth substrate on plant development and seed yield. Nutritional treatment had no effect on overall seed yield in terms of numbers or weight or 100 seed weight. This is a good indication that the nutritional treatments do not have a negative effect on plant development and seed production that would otherwise constitute a possible cause of differences in seed nutrient loading that could occur other than those which may be due to mother plant nutrition. Seed yield did differ between size categories for yield and 100 seed weight, but this has often been the case in previous studies across a number of species.

Chapter 5: Calcium and Potassium Composition and Stress Tolerance of Seeds

5.1 Preliminary Experiment Using Sand as a Growth Substrate and Modified Hoagland's Solution A

5.1.1 Introduction

Analysis of the Ca^{2+} and K^{+} composition of seed from only three nutritional treatments was possible after poor plant development and seed production (section 4.1). However, by analysing the seed produced, any obvious differences in Ca^{2+} or K^{+} concentration would indicate if mother plant nutrition could alter daughter seed nutritional loading.

5.1.2 Methods

5.1.2.1 Elemental Analysis of Experimental Seed

Owing to the small amount of experimental seed produced by this initial growth trial (sections 3.5.2 and 4.1), combined samples from each set of six replicate plants yielded samples of between 0.01 g and 0.1 g for analysis of seed. Seed from "five-day" siliquae, size Ø 1.0 - 1.4 mm was used for all analyses.

Whole seed was digested and analysed by the general method described in section 3.6.1. To each sample 3 ml of concentrated HNO_3 and 2 ml of 100 vol (30% w/v) H_2O_2, both of trace analysis grade, were added. A MLS 1200 Mega High Performance Laboratory Digestion Unit (Milestone, Shelton, CT, USA) was used for the digestion procedure. After cooling, the samples were decanted into separate, acid

washed, 50 ml, grade A volumetric flasks and deionised water was used to make the sample to volume.

5.1.3 Results

Although no statistical analysis has been performed on data for seed ion concentration (Table 5.1), the hypothesised trend is shown to be evident. High Ca^{2+} treated plants yielded the greatest seed Ca^{2+} concentration and high K^+ treated plants produced seed containing the greatest seed K^+ concentration. K^+ concentration was greater than that of Ca^{2+}.

Table 5.1 - Calcium and Potassium Concentration in Whole Seed

	Ca^{2+} ($\mu g\ g^{-1}$)	K^+ ($\mu g\ g^{-1}$)
Control	3143	14055
Low Ca^{2+}	#	#
High Ca^{2+}	4675	12138
Low K^+	#	#
High K^+	3475	16975

 # denotes insufficient quantities of seed to undertake chemical analysis.

5.1.4 Discussion

Although no statistical analysis took place, there are quite interesting trends in this preliminary data which suggest that seed ion concentration can be affected by mother plant nutrition. It is also the first indication, when looking at the K^+ concentration, that the differences between the results for control and high Ca^{2+} may suggest some form of ionic competition for uptake between Ca^{2+} and K^+, in that high Ca^{2+}, lowers seed K^+ compared with the control. The preparation of modified Hoagland's solution A differed from B (that used in later growth trials), with larger Ca^{2+} and K^+ ratios among treatments from the control and this may partly account for the poor vigour of

growth exhibited by all other treatments apart from control (section 4.1.3), possibly due to severe ionic imbalances. As a preliminary experiment, the data on the developmental aspects of growth and seed yield, aided method development in terms of the composition of modified Hoagland's solution used in subsequent experimentation and possible problems of using sand as a growth substrate especially for germination. Greenhouse temperature changes and evaporation of nutrient solution from the substrate may also have contributed to the poor vigour, growth and seed yield.

5.1.5 Conclusion

Seed production was not great enough to enable accurate analysis of seed Ca^{2+} and K^{+} concentration, but the results do suggest that altering daughter seed Ca^{2+} and K^{+} concentration is possible through changing mother plant nutrition, and greater seed production would help to accurately elucidate this. In order to achieve greater seed productivity, mother plants were produced using a set of nutrient solutions with less differential in Ca^{2+} and K^{+} levels and it was anticipated that this would improve overall seed yield, and that the seed produced would still have discernable differences in the Ca^{2+} and K^{+} loaded into seed tissue.

5.2 Preliminary Experiment Using Sand as a Growth Substrate and Modified Hoagland's Solution B

5.2.1 Introduction

The use of modified Hoagland's solution B as a nutrient solution was aimed at reducing the differential in Ca^{2+} and K^{+} levels between low and high Ca^{2+} and K^{+}

solutions and therefore achieve greater seed production in order to undertake accurate analysis and to be able to perform statistical analyses. Due to poor plant development, producing such quantities of seed was not possible and statistical analysis was not performed (section 4.2.3). However, measurement of seed Ca^{2+} and K^{+} was possible across all five nutritional treatments and would provide additional data that could be suggestive of a relationship between nutrient treatment and daughter seed Ca^{2+} and K^{+} content.

5.2.2 Methods

5.2.2.1 Elemental Analysis of Experimental Seed

Analysis of seed was performed as outlined in section *5.1.2.1* except that digestion was carried out using a Mars 5 Microwave Accelerated Reaction System (CEM Corporation, Matthews, NC, USA).

5.2.3 Results

Table 5.2 shows the data for seed ion concentration. Although no statistical analysis was undertaken due to lack of replication, high Ca^{2+} treatment produced the greatest seed Ca^{2+} concentration, considerably higher than control, low K^{+} or high K^{+} seeds. However, the Ca^{2+} concentration of low Ca^{2+} treated plants was also high. Seed K^{+} concentration was high for high K^{+} treated plants compared with control treatment. Both low Ca^{2+} and high Ca^{2+} treated plants produced seed with high seed K^{+} concentration. K^{+} concentration was greater than that of Ca^{2+}.

Table 5.2 - Calcium and Potassium Concentration in Whole Seed

	Ca^{2+} ($\mu g\ g^{-1}$)	K^{+} ($\mu g\ g^{-1}$)
Control	6548	14030
Low Ca^{2+}	14579	20183
High Ca^{2+}	17849	19894
Low K^{+}	7743	15012
High K^{+}	7160	19656

5.2.4 Discussion

Seed ion concentration shown in Table 5.2, did not undergo statistical analyses due to lack of replication, but has provided interesting data in terms of the fact that low Ca^{2+} treatment produced seed with a considerably high Ca^{2+} concentration. Low K^{+} treated plants also produced seed with a higher K^{+} concentration than control treated plants, which, although due to lack of replication may not be particularly reliable data, does not correlate with the results found in previous or subsequent growth trials for effect of Ca^{2+} or K^{+} treatment on seed ion content. The data did, however, prove that the modified Hoagland's solutions used in this experiment, and which would be used in later experimentation, was suitable and provided uniform development, flowering and seed production, albeit still low (section 4.2.3). The lack of production of seed led to the lack of possibility of replication in analysis of Ca^{2+} and K^{+} concentration, but the data did highlight the possible importance of producing enough experimental seed to undertake replication both for ion analysis and for future work on salinity tolerance. It may also be inferred that individual plant growth and development in terms of the ability to produce flowers, their number and subsequent seed, may also have an effect upon the Ca^{2+} and K^{+} concentrations found among the treatments shown in Table 5.2.

5.2.5 Conclusion

Comparing the results shown here with that of the previous preliminary growth trial's elemental analysis, it is difficult to conclude that the data may be representative of an effect of mother plant nutrition on seed Ca^{2+} and K^{+} concentration. Low nutrient treatments producing seed concentrations above that of the control cannot be a product of uniform seed nutrient loading. Again, seed production was not great enough to enable accurate analysis of seed Ca^{2+} and K^{+} content. Given the poor nature of plant development and seed production observed in sand, consideration was given to the use and comparison of different substrates and growth conditions

5.3 Growth Trial Using Various Growth Substrates and Modified Hoagland's Solution B

5.3.1 Introduction

The aim of a growth trial using three different growth substrates for the production of mother plants was to allow the investigation to consider the effect of growth substrate on seed Ca^{2+} and K^{+} loading and to try and overcome poor seed yield which had not permitted accurate analysis and statistical analysis of seed Ca^{2+} and K^{+} levels between mother plant nutritional treatments. Given successful Ca^{2+} and K^{+} analysis of seed and statistical analyses, the seed created from each type of growth substrate was then subjected to a standard germination test over a range of NaCl concentrations. Seed total percentage germination was then used to construct a probit dose response model, which calculated the effective concentration to reduce germination to 50% of the unstressed control. This salinity tolerance value (EC_{50}) was correlated with seed Ca^{2+},

K^+ and Ca^{2+}+ K^+ concentrations to investigate any relationships which may exist between those parameters for each type of growth substrate and as an overall data set.

5.3.2 Methods

5.3.2.1 Elemental Analysis of Experimental Seed

Analysis was carried as described in section *5.2.2.1* except that samples of 0.02 g were used for the analysis of each set of five replicate plants for each nutritional treatment at each site. Seed from "five-day" siliquae, size Ø 1.0 - 1.4 mm was used for all analyses. Ca^{2+} and K^+ spikes, derived from commercially available 1000 mg l^{-1} solutions, were included in the digestion procedure at concentrations close to those expected in the seed. These assessed the performance of the digestion and decanting procedures.

5.3.2.2 Standard Germination Test and Analysis

A standard germination test was performed by the methods outlined in section 3.7, with 75 Petri dishes that were labelled with seven NaCl concentrations to be tested: 0, 100, 200, 250, 300, 350 and 400 mM. The seed produced from each set of the five replicate sets of five plants for each nutrient treatment from the three sites/growth substrate, was analysed independently (25 sets of plants x 3 substrates x 7 concentrations, 525 in total). Each Petri dish contained either 10 or 20 seeds dependent upon the number of seeds available, tested with each of the seven NaCl concentrations.

5.3.2.3 Statistical Analyses, Correlation and Regression

Seed elemental analysis data underwent a General Linear Model Analysis of Variance incorporating Tukey's Honestly Significant Difference Test (Clewer and Scarisbrick 2001) incorporating pairwise comparison. This was undertaken using Minitab 13.

Correlation plots and regression analysis was undertaken using Microsoft Excel 2007. Along with regression analysis, analysis of covariance (Clewer and Scarisbrick 2001) was used to study the regression lines of Ca^{2+}, K^{+} and Ca^{2+}+ K^{+} concentrations against salinity tolerance. Analysis of covariance was performed using Minitab 13.

5.3.3 Results

5.3.3.1 Seed Elemental Analysis

Plants grown in sand produced seed containing higher overall levels of Ca^{2+} than those grown in sand & perlite and peat & vermiculite. Sand & perlite produced seed containing overall higher levels than those grown in peat & vermiculite (Table 5.3) Spike recovery ranged between was 107 and 115% for Ca^{2+}. There was no significant interaction between nutrient solution and substrate/site.

Plants grown with high Ca^{2+} nutrition produced seed with the highest overall mean concentration of Ca^{2+}, higher than low Ca^{2+} and high K^{+} treatments. Low Ca^{2+} treatment of plants produced seed containing lower concentration of Ca^{2+} than plants grown in control, high Ca^{2+} and low K^{+} solutions.

Table 5.3 - Calcium Concentration of Whole Seed

	Ca^{2+} (μg g^{-1})			
	Peat & Vermiculite	Sand	Sand & Perlite	Mean
Control	3615 de	5391 abc	4902 bcd	4636 *ab*
Low Ca^{2+}	3063 e	4273 bcde	4136 bcde	3824 *c*
High Ca^{2+}	4289 bcde	6906 a	4920 bcd	5371 *a*
Low K^+	4072 cde	5798 ab	4946 bcd	4939 *a*
High K^+	3483 de	4672 bcde	4407 bcde	4187 *bc*
Mean	3705 *c*	5408 *a*	4662 *b*	

Means followed by different letter differ significantly at P < 0.05. Means followed by different letter differ significantly at P < 0.05. Separate comparisons of overall means between either columns or rows are denoted by italicised letters. See Appendix 8, Table A8.1 for statistical analysis.

Substrates of peat & vermiculite and sand produced seed with higher concentrations of K^+ than sand & perlite. Plants grown in sand and peat & vermiculite produced seed that overall did not differ in K^+ (Table 5.4). There was no overall effect of treatment. Within each different type of substrate there was also no significant effect of treatment. Spike recovery ranged between 100 and 103% for K^+. There was no significant interaction between nutrient solution and substrate/site.

Table 5.4 - Potassium Concentration of Whole Seed

	K^+ (μg g^{-1})			
	Peat & Vermiculite	Sand	Sand & Perlite	Mean
Control	12472 abc	12567 abc	10038 c	11693 *a*
Low Ca^{2+}	12297 abc	13432 ab	11246 abc	12325 *a*
High Ca^{2+}	12989 abc	11754 abc	10327 c	11690 *a*
Low K^+	11736 abc	12512 abc	10750 bc	11666 *a*
High K^+	14315 a	12703 abc	11397 abc	12805 *a*
Mean	12762 *a*	12594 *a*	10751 *b*	

Means followed by different letter differ significantly at P < 0.05. Means followed by different letter differ significantly at P < 0.05. Separate comparisons of overall means between either columns or rows are denoted by italicised letters. See Appendix 8, Table A8.2 for statistical analysis.

Plants grown in peat & vermiculite produced seed with higher K^+/Ca^{2+} ratios overall, when compared with seed produced by plants growing in sand and in sand & perlite (Table 5.5). Nutritional treatment was also found to have a significant effect on

overall K^+/Ca^{2+} ratio, with low Ca^{2+} treatment and high K^+ treatment giving greater overall mean seed K^+/Ca^{2+} ratio, compared with high Ca^{2+} and low K^+ treatment. There was no significant interaction between nutrient solution and substrate/site.

Table 5.5 - Ratio of Potassium/Calcium in Whole Seed

	K^+/Ca^{2+}			
	Peat & Vermiculite	Sand	Sand & Perlite	Mean
Control	3.496 ab	2.397 bcd	2.063 cd	2.652 *b*
Low Ca^{2+}	4.058 a	3.173 abc	2.738 bcd	3.323 *a*
High Ca^{2+}	3.054 abc	1.786 d	2.106 cd	2.315 *b*
Low K^+	2.194 abcd	2.194 cd	2.177 cd	2.429 *b*
High K^+	4.162 a	2.766 abcd	2.641 bcd	3.190 *a*
Mean	3.537 *a*	2.463 *b*	2.345 *b*	

Means followed by different letter differ significantly at P < 0.05. Means followed by different letter differ significantly at P < 0.05. Separate comparisons of overall means between either columns or rows are denoted by italicised letters. See Appendix 8, Table A8.3 for statistical analysis.

5.3.3.2 Salinity Tolerance

The germination of unstressed seeds on deionised water varied between 80 and 100% between replicates. There was no significant effect of substrate or nutrient solution on germination and no interaction between these factors. In some cases, the total germination of seed of the unstressed control was less than the germination of seeds at 100 mM NaCl, which is normal considering that the growth of most plants is stimulated at low Na^+ concentrations (Cramer 2002). This consequently led to values above 100%, when germination was expressed as a percentage of the unstressed control. For individual replicates, EC_{50} NaCl tolerance ranged from 162 mM to 296 mM. Table 5.6 shows the mean concentration of NaCl which reduced germination to 50% of the unstressed control for seeds originating from plant grown in the three growth substrates, each with five different nutrient solutions. Two-way analysis of variance of data suggested no significant differences in EC_{50} between substrate or nutrient solutions. Using mean values of salinity tolerance proved insensitive because

of the considerable variation between replicates. However, by correlating the salinity tolerance data with the appropriate seed ion parameters, a more sensitive test of seed NaCl tolerance was introduced.

Table 5.6 - Salinity Tolerance of Seeds Expressed as Mean Concentration of NaCl Required to Reduce Germination to 50% of the Unstressed Control (EC_{50})

	EC_{50} (mM)		
Nutrient Solution	Peat & Vermiculite	Sand	Sand & Perlite
Control	266	245	239
Low Ca^{2+}	246	219	244
High Ca^{2+}	238	198	261
Low K^{+}	265	220	250
High K^{+}	203	264	233

See Appendix 9, Table A9.1 for statistical analysis.

5.3.3.3 Seed Ion Concentration Correlation with Salinity Tolerance

Pearson's correlation coefficients found between Ca^{2+}, K^{+} and Ca^{2+}+ K^{+} and EC_{50} were significant (Table 5.7). The highlighted cells indicate significant correlation at the probability shown by the table of critical values. Values for K^{+} and Ca^{2+}+ K^{+} correlation are particularly high ($P < 0.001$) and Ca^{2+} correlation is significant at $P = 0.05$. Correlations between Ca^{2+}/K^{+} or K^{+}/Ca^{2+} ratios and salinity tolerance were not significant.

Table 5.7 - Pearson's Correlation Coefficients for Correlation Between Seed Ion Concentration and EC_{50}

Ca^{2+}	K^{+}	Ca^{2+}+ K^{+}	Ca^{2+}/K^{+}	K^{+}/Ca^{2+}	*n*
-0.284*	-0.402***	-0.536***	-0.055	-0.004	70

Critical Values

P	*d. f.* 68
0.001	0.385***
0.01	0.306**
0.05	0.235*

*Cells highlighted by * denote probability as shown by critical values and degrees of freedom.*

The regression of salinity tolerance, expressed as the EC_{50}, against seed Ca^{2+} concentration, combining data for all three substrates is shown in Figure 5.1. The overall linear trend is significant (P = 0.0143, Table 5.8) and negative, indicating that as seed Ca^{2+} concentration increases, salinity tolerance decreases.

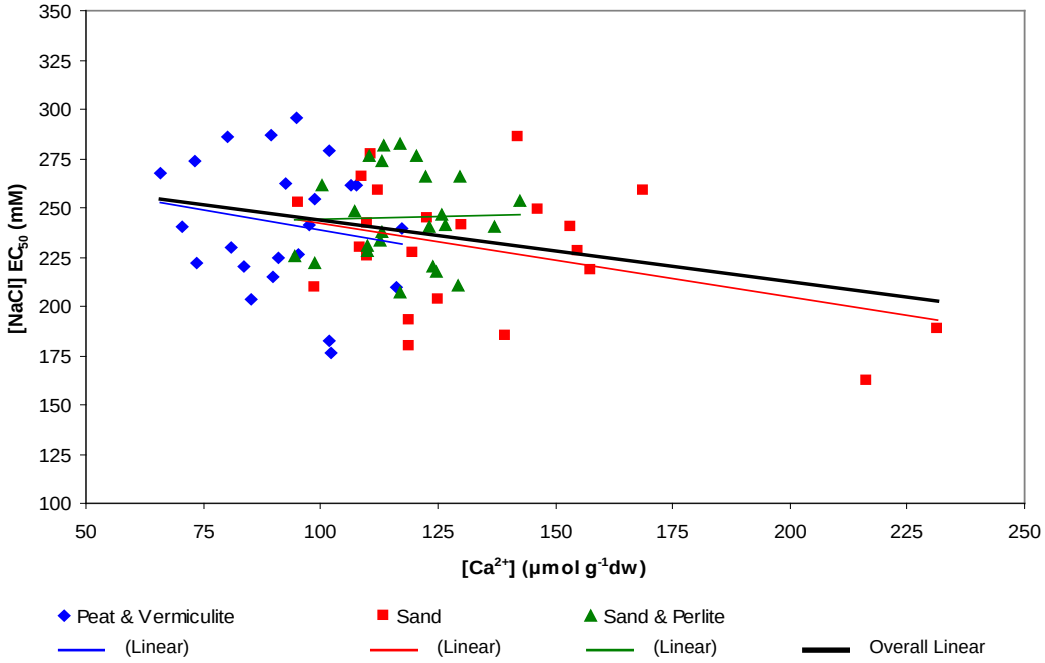

Figure 5.1 - Regression of [NaCl] Required for 50% Germination Inhibition with Seed [Ca^{2+}] for Three Types of Growth Substrate, Showing an Overall Linear Trend.

Table 5.8 - Regression Analysis of [NaCl] for 50% Germination Inhibition Correlation with Seed [Ca^{2+}].

Overall R^2	0.085
P Value	0.014
Overall Trend	y = -0.3119x + 274.59
Peat & Vermiculite	y = -0.4223x + 280.67
Sand	y = -0.3742x + 279.36
Sand & Perlite	y = 0.0503x + 239.35

See Appendix 9, Table A9.2 for statistical analysis.

Seeds produced from parent plants growing in sand seem to have a larger distribution of points for Ca^{2+} against salinity tolerance than the other two types of growth substrate (Figure 5.1). The overall R^2 value of 0.0851 (Table 5.8) is very low and

indicates a large scatter of data around the trend line, therefore making the line unsuitable for the accurate prediction of salinity tolerance at germination from seed Ca^{2+} concentration. concentration.

The regression for seed K^+ concentration is similar to that for seed Ca^{2+} (Figure 5.2). The overall linear trend is significant and negative (Table 5.9), indicating that as seed K^+ concentration increases, salinity tolerance decreases.

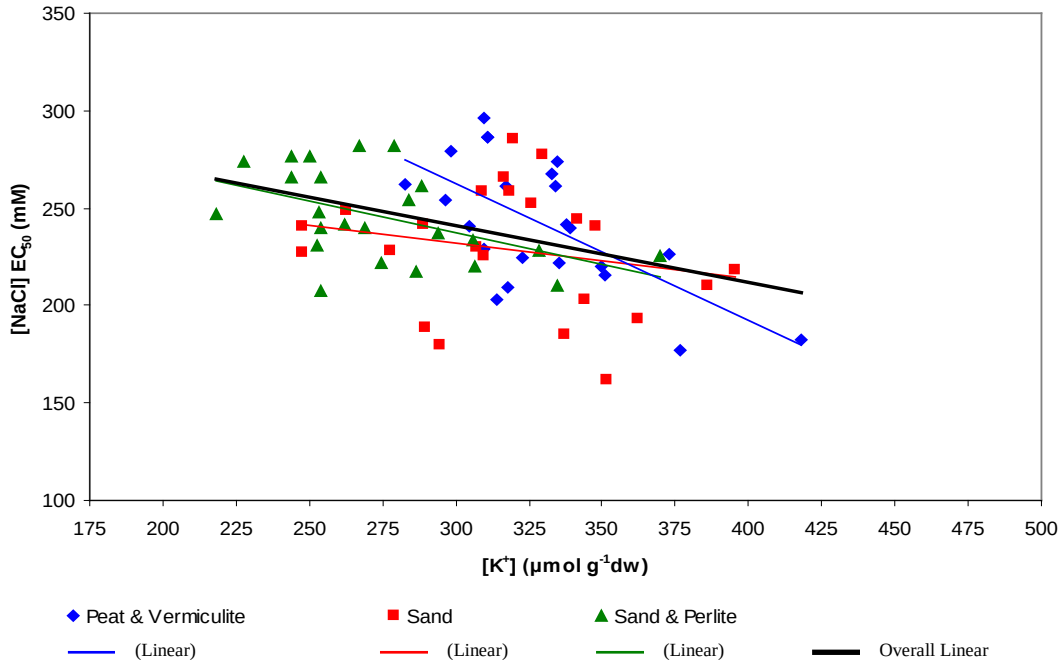

Figure 5.2 - Regression of [NaCl] Required for 50% Germination Inhibition with Seed [K^+] for Three Types of Growth Substrate, Showing an Overall Linear Trend.

Table 5.9 - Regression Analysis of [NaCl] for 50% Germination Inhibition Correlation with Seed [K^+].

R^2	0.162
P Value	<0.001
Overall Trend	y = -0.2896x + 327.68
Peat & Vermiculite	y = -0.6980x + 471.84
Sand	y = -0.1799x + 286.12
Sand & Perlite	y = -0.3230x + 334.12

See Appendix 9, Table A9.3 for statistical analysis.

As with the correlation of EC_{50} with seed Ca^{2+} concentration (Figure 5.1), seed produced from plants growing in sand, appear to have a wider distribution that the other two growth substrates. The R^2 value of 0.1616 (Table 5.9) indicates a large range of overall distribution and although higher than in the case of seed Ca^{2+} vs EC_{50} correlation, is still low, and would also not be suitable for the accurate prediction of seed salinity tolerance from seed K^+ concentration.

The regression for seed Ca^{2+}+ K^+ concentration vs. EC_{50} is shown in Figure 5.3. The overall trend is highly significant ($P < 0.001$) and negative (Table 5.10), indicating that as combined seed Ca^{2+} and K^+ concentration increases, salinity tolerance decreases.

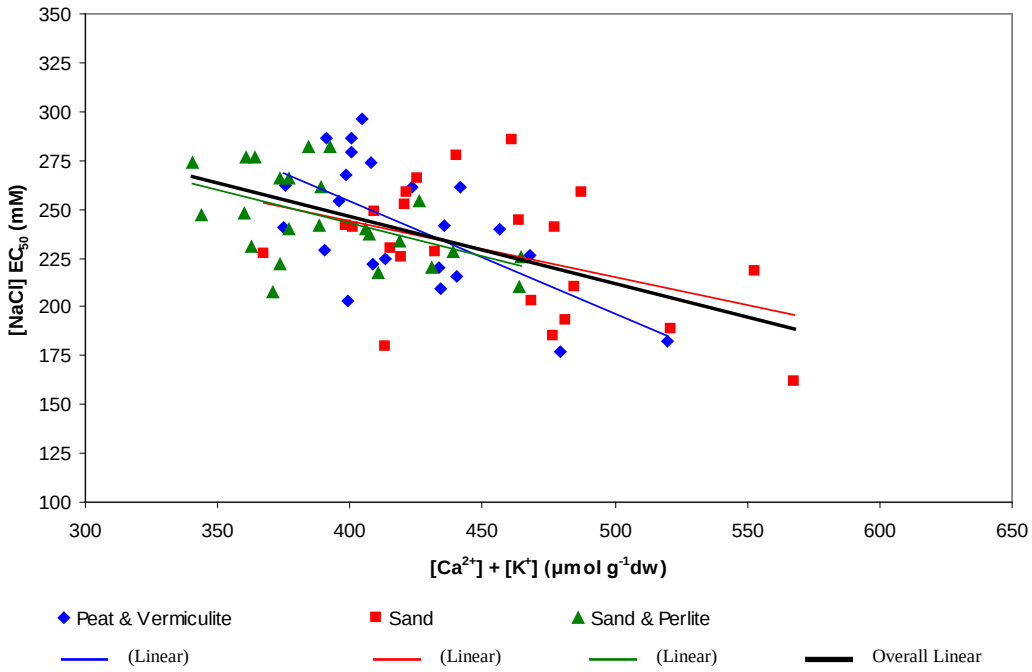

Figure 5.3 - Regression of [NaCl] Required for 50% Germination Inhibition with Seed [Ca^{2+}] + [K^+] for Three Types of Growth Substrates, Showing an Overall Linear Trend.

Table 5.10 - Regression Analysis of [NaCl] for 50% Germination Inhibition Correlation with Seed $[Ca^{2+}] + [K^{+}]$.

R^2	0.287
P Value	<0.001
Overall Trend	y = -0.3456x + 384.54
Peat & Vermiculite	y = -0.5810x + 486.72
Sand	y = -0.2884x + 359.47
Sand & Perlite	y = -0.3385x + 378.12

See Appendix 9, Table A9.4 for statistical analysis.

The R^2 value of 0.2871 (Table 5.10) is higher than the regression of Ca^{2+} or K^{+} concentration alone against salinity tolerance, but the trend line would not be suitable for the accurate prediction of salinity tolerance from seed Ca^{2+}+ K^{+} concentration.

Analysis of covariance was used to study the regression lines of seed Ca^{2+}, K^{+} and Ca^{2+}+ K^{+} concentrations against salinity tolerance. This analysis (Clewer and Scarisbrick 2001) fits three models for each of the three ion parameters: a single line for all data for that parameter, separate parallel lines for each substrate for that parameter and independent lines for each substrate for that parameter. Comparison of these models allows a calculation of the probability that the slope and intercept differs significantly among the three substrates for each ion parameter. Table 5.11 shows that for Ca^{2+}, K^{+} and Ca^{2+}+ K^{+} concentrations, there was no significant difference at P = 0.05 among the slopes of the three substrates, and no significant difference among the intercepts either. Therefore, there was no significant benefit in plotting separate regression lines for each substrate/site, rather than a single regression line for all data for each ion parameter as shown in Figures 5.1, 5.2 and 5.3.

Table 5.11 - Comparison of Individual Regression Lines of Correlation of [NaCl] EC_{50} (mM) with Seed [Ca^{2+}], [K^{+}] and [Ca^{2+}] + [K^{+}] for Three Types of Growth Substrate.

	Ca^{2+}	K^{+}	Ca^{2+}+K^{+}
P Value (Slope)	0.607	0.322	0.456
P Value (Common Intercept)	0.328	0.147	0.758

All values above P = 0.05, no significant difference. See Appendix 10, Tables A10.1- A10.3 for statistical analysis.

5.3.4 Discussion

The seed analysis data provides an insight into the effect that nutritional treatment and growth substrate may have on ion concentration of resultant seed and how this may affect salt tolerance of the seed at germination. Seed Ca^{2+} concentration was significantly affected by growth substrate and nutritional treatment (Table 5.3). Seed produced from plants grown in sand had a significantly higher mean overall Ca^{2+} concentration over five treatments than seed from the other two growth substrates, and sand & perlite produced seed with a higher mean Ca^{2+} concentration than did peat and vermiculite. Nutritional treatment also affected seed ion concentration, with high Ca^{2+} and low K^{+} treatment producing seed with a significantly greater overall mean concentration of Ca^{2+} over all three substrates, than low Ca^{2+} and high K^{+} treatments. The Ca^{2+} concentration of seeds in this investigation thus appears to be governed by a combination of factors. Plants grown in peat and vermiculite, which produced seed of significantly lower Ca^{2+} concentration, had greater productivity and produced a greater seed yield (Tables 4.9 & 4.10); perhaps indicating that the uptake and distribution rate of Ca^{2+} to developing seeds is finite and increased seed productivity may effectively dilute seed ion concentration (Jarrell and Beverly 1981). Soil or intracellular chemistry may also be a factor. Low Ca^{2+} and high K^{+} treated plants produced seed with lower mean levels of Ca^{2+} compared with high Ca^{2+} and low K^{+}

treated plants. In the case of varying Ca^{2+} nutrition, this would be an expected outcome, but the resultant low Ca^{2+} concentration with high K^+ nutrition may indicate a form of competition between Ca^{2+} and K^+ uptake either at a growth media level, during plant ion uptake or distribution to developing siliquae.

Seed K^+ concentration was significantly affected by growth substrate but not nutritional treatment (Table 5.4). Seed from plants grown in peat & vermiculite and sand had significantly higher mean K^+ concentration than those grown in sand & perlite, the mechanisms of which may in part account for the low Ca^{2+} concentration of seed grown in peat & vermiculite (Table 5.3). The initial nutritional status of the growth substrate, the physical properties of the substrate and/or environmental conditions in the greenhouse may affect the balance of competition for ionic selectivity at the root level. No significant differences in seed K^+ concentration were found between nutritional treatments. The plentiful supply of K^+ for plants grown in peat & vermiculite may have been sufficient to overcome the demand sustained by increased biomass and seed productivity. This approach is favoured by the results of the K^+/Ca^{2+} ratios, which were significantly greater for seed produced from plants grown in peat & vermiculite (Table 5.5). Mean K^+/Ca^{2+} ratio values between nutritional treatments indicate significantly higher ratios for low Ca^{2+} and high K^+ than for other treatments, over the three substrates, highlighting potential competition between Ca^{2+} and K^+ and indicating possible congruency of the possible mechanisms discussed earlier. In this instance, low Ca^{2+} and high K^+ treatments are shown to produce seeds that have a proportionally greater content of K^+ over Ca^{2+}, which at least suggests that nutritional treatment has an influence over Ca^{2+} and K^+ seed uptake.

Increases in seed Ca^{2+}, K^+ or Ca^{2+}+ K^+ concentrations, significantly decreased salinity tolerance, implying that as total seed Ca^{2+}+ K^+ increases, there is a decrease in the NaCl concentration required to reduce germination (Table 5.7)

The correlation between seed Ca^{2+}+ K^+ and EC_{50} was, when compared to correlations between Ca^{2+} and K^+ individually, the most significantly negative, produced a linear regression which strongly followed the data and, although low, produced an R^2 value that was greater than any other correlation. In terms of seed ion concentration and EC_{50}, increasing seed Ca^{2+} concentration reduces salt tolerance as does increasing K^+ concentration. The combined effect of increasing both Ca^{2+} and K^+ concentration appears to further augment the reduction of salt tolerance at germination.

These results do not seem to correspond to the current wealth of data generated by studies of whole plant or seed exposure to salinity. In the present study, it has been shown that an increase in seed Ca^{2+} concentration decreases salinity tolerance, in contrast to the consensus that addition of Ca^{2+}, albeit to growth substrates, ameliorates salt stress in almost all studies conducted (Rengel 1992). With supplemental additions of Ca^{2+} during germination, either in the growth medium, or as part of a presowing treatment, Ashraf and Naqvi (1991) found increased germination in *B. napus* and *B. juncea* L. in saline culture. Bonilla, El-Hamdaoui, and Bolanos (2004) found that the addition of extra Ca^{2+} to the germination medium of *P. sativum* (pea) seeds prevented a reduction in development at 75 mM NaCl and Tobe, Li, and Omasa (2004) have reported that the addition of $CaCl_2$ at low concentrations favoured the germination of *Haloxylon ammodendron* (saxaul) in NaCl. Using *T. vulgare* both Chaudhuri and Wiebe (1968) and Idris and Aslam (1975) also found that increasing Ca^{2+} and K^+ levels through presowing treatments, resulted in increased seed germination in NaCl

solutions. However, there is evidence that in some instances, such results are not the case. Kamboh, Oki, and Adachi (2000) found that in two varieties of *T. aestivum* (wheat) differing in salt tolerance, pre-treatment of seeds with KCl, KNO_3, $CaCl_2$ and $Ca(NO_3)_2$ did not lead to a significantly higher final germination in 200 mM NaCl compared with those that had been treated with distilled water. A similar lack of effect of exogenous Ca^{2+} or K^+ on salinity tolerance was found by Rehman, Harris, and Bourne (1998b) with *Acacia* species.

Seed Ca^{2+} and K^+ concentrations were clearly affected by growing conditions of the parent plant, with an increase in substrate available Ca^{2+} and decrease in K^+ resulting in increased seed Ca^{2+} concentration. This was accompanied by a proportionally greater increase in seed K^+ than seed Ca^{2+} by high K^+ and low Ca^{2+} nutritional treatment, suggesting that there may be an antagonistic uptake of these nutrients into seed. This is interesting given the differing uptake pathways of Ca^{2+} into seed and its relative immobility in the phloem.

5.3.5 Conclusion

Growth substrate affected seed K^+ and growth substrate and nutritional treatment affected seed Ca^{2+} concentration. Ca^{2+}/K^+ ratios of seed between nutritional treatments was indicative of a relationship between treatments that provide higher K^+ over Ca^{2+} compared to control (i.e. low Ca^{2+}, high K^+) and seed Ca^{2+} and K^+ concentration. Correlation of seed Ca^{2+} and K^+ concentration with EC_{50} produced significant negative correlations and the Ca^{2+} and K^+ concentration of seeds affected their salinity tolerance, with increases in seed Ca^{2+}, K^+, Ca^{2+}+ K^+ concentrations decreasing salinity tolerance. The results in terms of Ca^{2+} nutrition especially, appear

to challenge a wealth of literature that suggests a beneficial effect of increasing Ca^{2+} availability on salinity tolerance. In terms of K^+ nutrition, increases in NaCl salinity have been shown to reduce plant and seed K^+ concentration and an increase in K^+ might partially offset this effect and lead to increased salinity tolerance, but the results shown here indicate that increased seed K^+ concentrations may actually contribute to a decline in salinity tolerance. The nature of growth substrates used and the high base nutrient level of peat and vermiculite, before the addition of modified Hoagland's B solution, make assessing the effect of varying mother plant Ca^{2+} and K^+ nutrition on the Ca^{2+} and K^+ concentration of daughter seed and subsequent salinity tolerance, with a defined nutritional treatment, difficult. Given the potential interactive complexities of growth substrates, highlighted by plant development and seed yield data during this growth trial, methods of growing mother plants without a substrate were considered as a method of being able to better define the effect of nutritional treatment of mother plants on the nutrient loading of daughter seed.

5.4 Hydroponic Growth Trial using Modified Hoagland's Solution B

5.4.1 Introduction

Hydroponic culture using modified Hoagland's solution B for the production of mother plants allowed the investigation to consider the effect of nutritional treatment on seed Ca^{2+} and K^+ loading without the potential background nutritional interference that a solid substrate creates and without environmental differences due to growth taking place in different locations. Many more plants than in previous trials were grown which increased seed production even though the vigour of plants was not matched to that found by using peat and vermiculite as a substrate (section *4.3.3.*2,

Tables 4.9 and 4.10). Successful production of seed allowed accurate Ca^{2+} and K^{+} analysis of two seed sizes and statistical analysis was carried out to determine if nutritional treatment or size had an effect on seed Ca^{2+} and K^{+}. The analysis of two seed sizes (Ø <1.0 and Ø 1.0 - 1.4 mm) was undertaken in an attempt to show that seed surface area to volume ratio may have an influence on seed nutrient loading and that this may be suggestive of a difference in nutrient loading of the coat and embryo. Both size categories of seed were then subjected to a standard germination test over similar NaCl concentrations to those in the previous growth trial. It was considered that differences in salinity tolerance between seed sizes are well documented and any differences that may have arisen, may not be due solely to any difference in Ca^{2+} and K^{+} distribution in seed. Seed total percentage germination was used to construct a probit dose response model, which calculated the (EC_{50}). This salinity tolerance value was correlated with seed Ca^{2+}, K^{+} and Ca^{2+}+ K^{+} concentrations to investigate any relationships that may exist between those parameters for each seed size.

5.4.2 Methods

5.4.2.1 Elemental Analysis of Experimental Seed and Localisation of Calcium and Potassium

In order to try and elucidate the location of Ca^{2+} and K^{+} between embryo and coat, not only was the usual Ø 1.0 - 1.4 mm seed chosen to be analysed, but also the smaller size category of Ø <1.0 mm, with the premise that the smaller seeds collectively contain a larger surface area and hence greater amount of seed coat per gram of seed and greater coat to embryo ratio. This was carried out in order to i) provide more resolute data and ii) investigate the possible effect of seed coat to embryo ratio upon Ca^{2+} and K^{+} levels.

Analysis was carried out as outlined in section *5.2.2.1* using duplicate 0.02 g samples of the seed from each treatment and size category of Ø < 1.0 mm and Ø 1.0 - 1.4 mm, except that to each sample 4 ml of concentrated trace analysis grade HNO_3 was added, since digestion was deemed just as efficient without the use of H_2O_2 as used in previous procedures. Ca^{2+} and K^+ spikes were included in the digestion procedure as described in section *5.3.2.1*.

5.4.2.2 Statistical Analyses

Seed elemental analysis data underwent a General Linear Model Analysis of Variance as outlined in section *5.3.2.2*.

5.4.2.3 Standard Germination Test and Analysis

A standard germination test was performed by the methods outlined in section 3.7, with 50 Petri dishes that were labelled with eight NaCl concentrations to be tested: 0, 100, 200, 250, 275, 300, 350 and 400 mM. The seed produced from each set of the five replicate sets of five plants for each nutrient treatment with two seed sizes was analysed independently (25 sets of plants x 2 seed sizes x 8 concentrations, 400 in total). Each Petri dish contained 20 seeds tested with each of the eight NaCl concentrations.

Plotting of regression of EC_{50} against seed [Ca^{2+}], [K^+] and [Ca^{2+}+ K^+] (Figures 5.5 - 5.7) was performed on identical axes as those used for the multi-substrate growth trial (section 5.3 and Figures 5.2 - 5.4). This was to allow comparison of the range results of EC_{50} and seed ion concentrations obtained via hydroponic mother plant growth and the three different types of substrate used in the multi-substrate growth trial.

5.4.3 Results

5.4.3.1 Seed Elemental Analysis

There is a significant effect of seed size on Ca^{2+} concentration. Smaller seeds had a higher Ca^{2+} concentration and a low Ca^{2+} nutritional treatment is also shown to cause a significantly lower Ca^{2+} concentration over the two seed sizes, compared with all other nutritional treatments (Table 5.12). There was no significant interaction between treatment and seed size. Spike recovery ranged from 91 to 102% for Ca^{2+}.

Table 5.12 - Calcium Concentration of Whole Seed

	Ca^{2+} (μg g^{-1})		
	Ø 1.0 - 1.4 mm	Ø <1.0 mm	Mean
Control	4824 c	6076 a	5450 *a*
Low Ca^{2+}	4298 c	4969 bc	4634 *b*
High Ca^{2+}	4877 c	6096 a	5487 *a*
Low K^{+}	4776 c	5614 ab	5195 *a*
High K^{+}	4561 c	5649 ab	5105 *a*
Mean	4667 *b*	5681 *a*	

Means followed by different letter differ significantly at P < 0.05. Separate comparisons of overall means between either columns or rows are denoted by italicised letters. See Appendix 11, Table A11.1 for statistical analysis.

These results are similar to those found in the multi-substrate growth trial, where high Ca^{2+} nutrition produced seed of significantly higher Ca^{2+} concentration than low Ca^{2+} nutrition did (section *5.3.3.1*, Table 5.3). Seed of Ø <1.0 mm grown with a control and high Ca^{2+} treatment, produced seeds with significantly higher Ca^{2+} concentration than low Ca^{2+} grown seed. Within the size category of Ø 1.0 - 1.4 mm, there was no significant difference between treatments. The higher Ca^{2+} per g of seed in smaller than in larger seeds, may infer a higher Ca^{2+} concentration in the coat, rather than in other parts of the seed, as smaller seeds will tend to have a higher surface area per gram than larger seeds and hence a higher proportion of coat to embryo.

There is a significant effect of size on K^+ concentration per gram of seed. The smaller size category is shown to have a significantly higher concentration of K^+ per gram. Nutritional treatment is not shown to have a significant overall effect, which was also shown to be the case in the multi-substrate growth trial (Table 5.4). There was no significant interaction between treatment and seed size. Spike recovery ranged from 96 to 104% for K^+.

Table 5.13 - Potassium Concentration of Whole Seed

	K^+ (μg g^{-1})		
	Ø 1.0 - 1.4 mm	Ø <1.0 mm	Mean
Control	14913 c	16798 ab	15856 *a*
Low Ca^{2+}	15456 bc	17145 a	16300 *a*
High Ca^{2+}	14833 c	16567 ab	15700 *a*
Low K^+	15596 abc	16525 ab	16061 *a*
High K^+	14802 c	16352 abc	15577 *a*
Mean	15120 *b*	16677 *a*	

Means followed by different letter differ significantly at P < 0.05. Separate comparisons of overall means between either columns or rows are denoted by italicised letters. See Appendix 11, Table A11.2 for statistical analysis.

Seeds of Ø 1 - 1.4 mm were found to have a higher K^+/Ca^{2+} ratio than seeds of Ø <1.0 mm. Nutritional treatment also had a significant effect with low Ca^{2+} treated plants having a significantly higher ratio than any of the other nutritional treatments (Table 5.14). There was no significant interaction between treatment and size. The results are similar to those found in the multi-substrate growth trial (Table 5.5), where low Ca^{2+} nutrition had a significantly higher ratio than control, high Ca^{2+} low K^+ treatments. The results also indicate that seeds of greater size may contain proportionally more K^+ compared to Ca^{2+} than smaller seeds, probably due to the relative larger embryo volume, as opposed to the seed coat.

Table 5.14 - Ratio of Potassium/Calcium in Whole Seed

	K^+/Ca^{2+}		
	Ø 1 – 1.4 mm	Ø <1.0 mm	Mean
Control	3.130 abc	2.764 c	2.947 *b*
Low Ca^{2+}	3.597 a	3.470 ab	3.534 *a*
High Ca^{2+}	3.061 abc	2.723 c	3.061 *b*
Low K^+	3.278 abc	2.946 bc	3.112 *b*
High K^+	3.252 abc	2.905 c	3.079 *b*
Mean	3.264 *a*	2.961 *b*	

Means followed by different letter differ significantly at P < 0.05. Separate comparisons of overall means between either columns or rows are denoted by italicised letters. See Appendix 11, Table A11.3 for statistical analysis.

5.4.3.2 Salinity Tolerance

The total germination of unstressed seeds in deionised water varied between 85 and 100% between replicates. Salinity tolerance of two sizes of seed was expressed as the concentration of NaCl that reduced germination to 50% of the unstressed control. As in the growth trial using various growth substrates, in some cases the germination of the unstressed control was less than the germination shown at 100 mM NaCl. This led to values above 100% when germinaton was expressed as a percentage of the unstressed control. For individual replicates, EC_{50} ranged from 123 mM to 268 mM for seeds of Ø <1.0 mm and from 202 mM to 339 mM for seeds of Ø 1.0 - 1.4 mm. Table 5.15 shows the mean concentration of NaCl which reduced germination to 50% of the unstressed control. Two way analysis of variance of the data suggested significant differences for EC_{50} between treatment and size. There was no significant interaction between treatment and size. Seeds of Ø 1.0 - 1.4 mm showed a significantly greater salt tolerance than seed of Ø <1 mm and, in terms of treatment, high Ca^{2+} treated mother plants produced seed that had significantly higher salt tolerance than seeds grown with control and low Ca^{2+} nutrition. Although high Ca^{2+} treatment produced seed with a higher salinity tolerance than control and low Ca^{2+} treatments, the seed produced did not significantly differ in Ca^{2+} concentration

compared with control treatment (Table 5.12). Over both size categories, salinity tolerance of seeds produced under high Ca^{2+} treatment was higher than under low Ca^{2+} treatment. It should be noted that this is quite insensitive given the limited number of replicates (5) and the considerable variation in salinity tolerance within treatments and size categories. The significantly higher EC_{50} of high Ca^{2+} treatment over control and low Ca^{2+} may be product of a statistical anomaly within this variable data. It is important to note that the ANOVA compared the effect of mother plant nutritional treatment on seed salinity tolerance rather than the effect of seed ion concentration, and that seed ion concentration varied greatly within each nutritional treatment. Therefore, correlating salinity tolerance data with the appropriate ion parameters gave a more sensitive test of NaCl tolerance.

Table 5.15 - Salinity Tolerance of Seeds Expressed as Mean Concentration of NaCl Required to Reduce Germination to 50% of the Unstressed Control (EC_{50}).

	EC_{50} (mM)		
	Ø 1.0 - 1.4 mm	Ø <1.0 mm	Mean
Control	262 abc	176 d	219 *b*
Low Ca^{2+}	245 abc	182 d	214 *b*
High Ca^{2+}	294 a	239 abcd	267 *a*
Low K^{+}	270 ab	196 cd	233 *ab*
High K^{+}	264 ab	218 bcd	241 *ab*
Mean	267 *a*	202 *b*	

Means followed by different letter differ significantly at P < 0.05. Separate comparisons of overall means between either columns or rows are denoted by italicised letters. See Appendix 12, Table A12.1 for statistical analysis.

5.4.3.3 Seed Ion Concentration Correlation with Salinity Tolerance

Pearson's correlation coefficients found between seed ion concentration and germination/salinity tolerance for seeds in the two size categories indicate significant correlation at the probability shown by the table of critical values and *n* (Table 5.16). No values for the size category Ø 1.0 - 1.4 mm were significantly correlated with any

of the ion parameters shown. However, for the seeds of Ø <1.0 mm significant correlations exist between K^+, Ca^{2+}/K^+ and K^+/Ca^{2+} at $P = 0.05$. When considering the data from both size categories jointly, there is a significant negative correlation between EC_{50} and Ca^{2+} at $P = 0.01$ and seed K^+ and $Ca^{2+}+ K^+$ at $P = 0.001$. These significant correlations are similar to those found in the multi-substrate growth trial (Table 5.7).

Table 5.16 - Pearson's Correlation Coefficients for Correlation Between Seed Ion Concentration and EC_{50}

Size	Ca^{2+}	K^+	$Ca^{2+}+ K^+$	Ca^{2+}/K^+	K^+/Ca^{2+}	*n*
Ø 1.0 -1.4 mm	0.118	-0.333	-0.256	0.258	0.251	25
Ø <1 mm	0.291	-0.497*	-0.299	0.444*	-0.432*	25
Overall	-0.415**	-0.702***	-0.690***	-0.055	0.054	50

Critical Values

P	*d. f.* 23	*d. f.* 48
0.001	0.618***	0.451***
0.01	0.505**	0.361**
0.05	0.396*	0.279*

*Cells highlighted by * denote probability as shown by critical values and degrees of freedom.*

The regression of salinity tolerance (EC_{50}) against seed Ca^{2+} concentration for two seed sizes (Ø 1.0 - 1.4 mm and Ø <1.0 mm) is shown in Figure 5.4. The linear trends for individual seed sizes were not significant at $P = 0.05$. However, the overall linear trend for both seed sizes was (Table 5.17). This indicates that, discounting seed size as a factor, as seed Ca^{2+} concentration increases, salinity tolerance decreases. The data points from low and high Ca^{2+} treatments are also highlighted with reference to the higher salinity tolerance found between the treatments over both size categories via analysis of variance (Table 5.15). Between high and low Ca^{2+} treatments, the data points are spatially separated and when averaged may account for the significant

difference found in salinity tolerance, but variation in salinity tolerance between low and high Ca^{2+} treatments is high (Table 5.15) and no significant differences existed within size categories. This may suggest that a higher mean salinity tolerance of high Ca^{2+}, compared with low Ca^{2+}, may be a statistical anomaly and not truly representative of the distribution of data points within these two treatments.

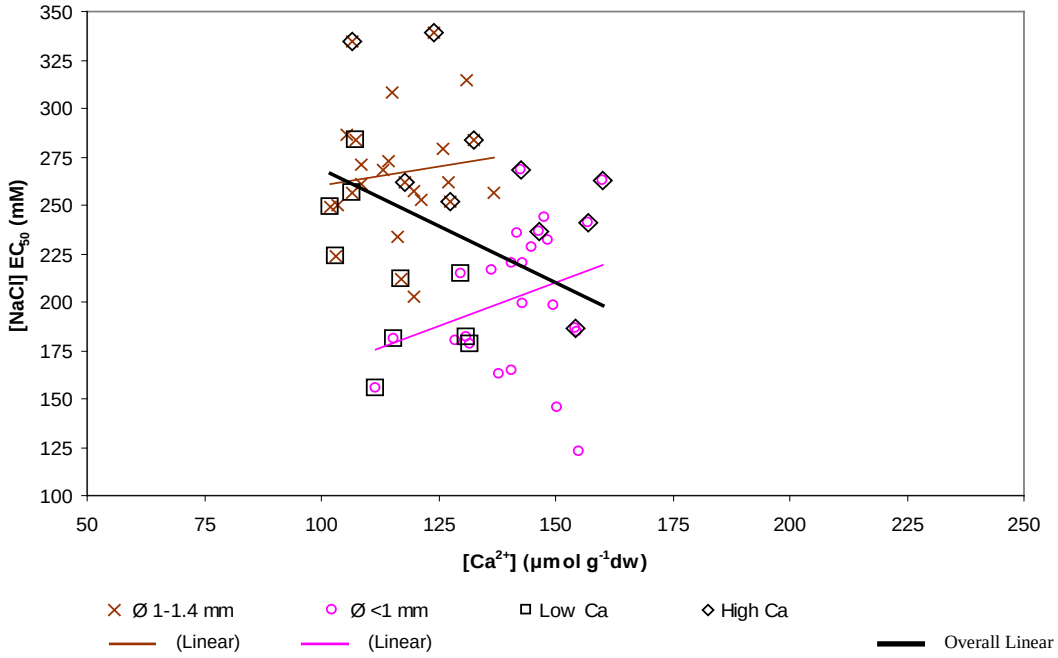

Figure 5.4 - Regression of [NaCl] EC_{50} with Seed $[Ca^{2+}]$ for Two Seed Diameters Produced from Plants Grown Hydroponically.

Table 5.17 - Regression Analysis of [NaCl] for EC_{50} Correlation with Seed $[Ca^{2+}]$.

	Ø 1.0 - 1.4 mm	Ø <1.0 mm	Overall
R^2	0.014	0.085	0.1724
P Value	0.573	0.158	0.0027
Line	y = -0.3919x + 221.37	y = -0.8984x + 74.949	y =-1.1779x + 386.68

See Appendix 12, Tables A12.2 - A 12.4 for statistical analyses.

Figure 5.5 shows a regression salinity tolerance (EC_{50}) against seed K^+ concentration for the two seed sizes. Although the trend for Ø 1.0 - 1.4 mm is not significant , for Ø <1.0 mm, it is. Considering the overall linear trend for both seed sizes the correlation is significantly negative (Table 5.18). This indicates, for Ø <1.0 mm and when

discounting seed size as a factor, that as seed K^+ concentration increases, salinity tolerance decreases.

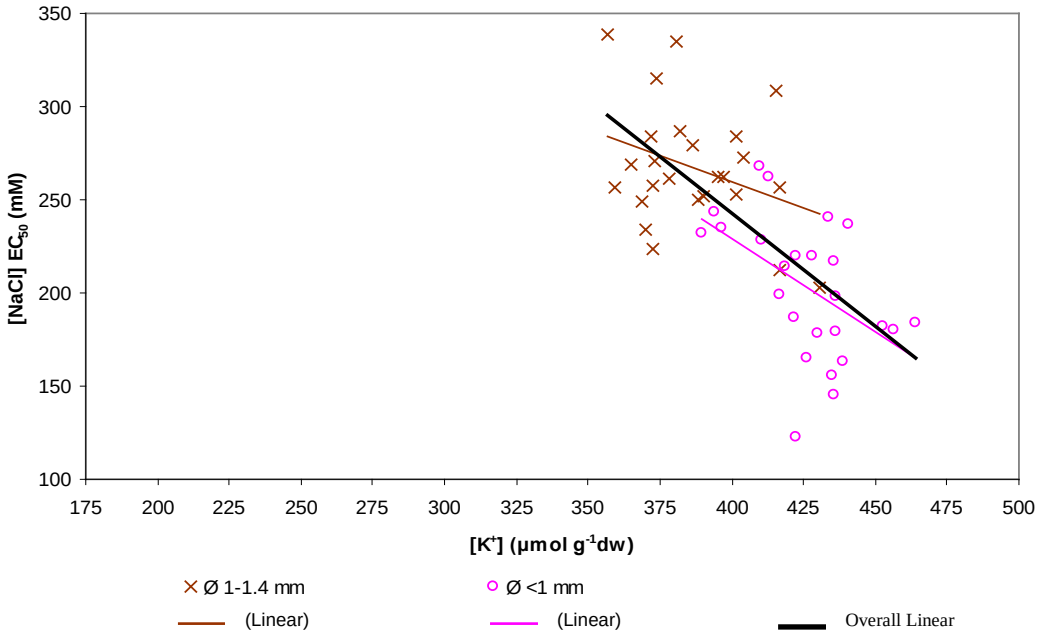

Figure 5.5 - Regression of [NaCl] EC_{50} with Seed $[K^+]$ for Two Seed Diameters Produced from Plants Grown Hydroponically.

Table 5.18 - Regression Analysis of [NaCl] for EC_{50} Correlation with Seed $[K^+]$.

	Ø 1.0 - 1.4 mm	Ø <1.0 mm	Overall
R^2	0.111	0.247	0.4974
P Value	0.104	0.012	<0.001
Line	y = -0.5579x + 482.67	y = -0.1.007x + 631.93	y = -1.2212x + 731.20

See Appendix 12, Tables A12.5 -A12.7 for statistical analyses

Figure 5.6 shows the regression of salinity tolerance (EC_{50}) against seed Ca^{2+}+ K^+ concentration for the two seed sizes. The negative trends for Ø 1.0 - 1.4 mm and Ø <1.0 mm are not significant; however the overall linear trend for both seed sizes was (Table 5.19). This indicates that, discounting seed size as a factor, as seed Ca^{2+}+ K^+ concentration increases, salinity tolerance decreases. (Table 5.19).

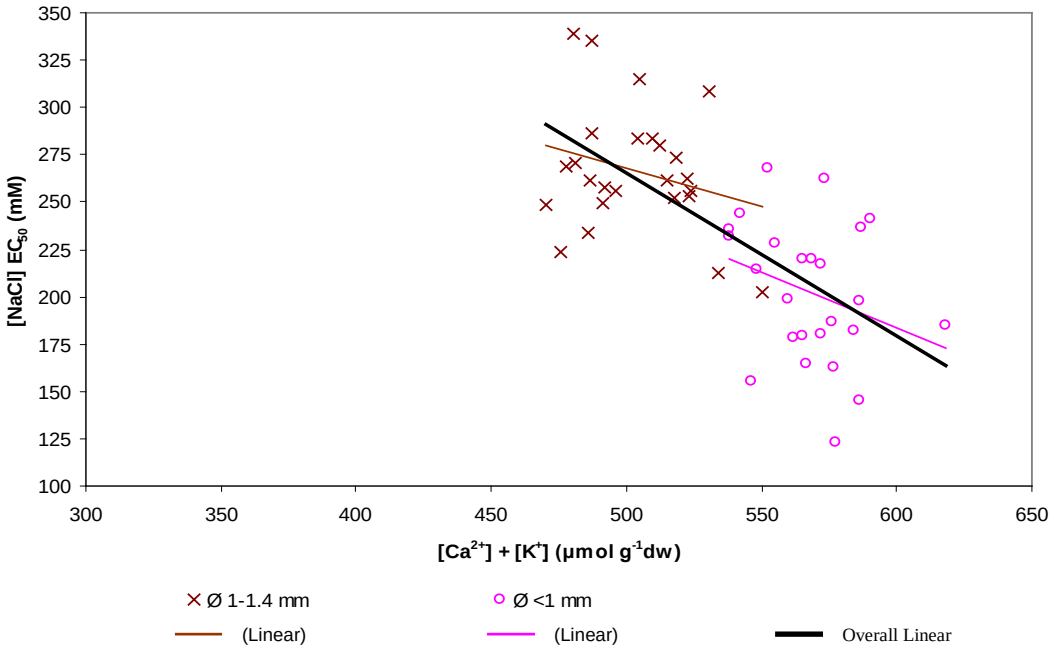

Figure 5.6 - Regression of [NaCl] EC_{50} with Seed $[Ca^{2+}] + [K^{+}]$ for Two Seed Diameters Produced from Plants Grown Hydroponically.

Table 5.19 - Regression Analysis of [NaCl] for EC_{50} Correlation with Seed $[Ca^{2+}] + [K^{+}]$.

	Ø 1.0 - 1.4 mm	Ø <1.0 mm	Overall
R^2	0.066	0.089	0.4755
P Value	0.216	0.147	<0.001
Line	y = -0.4047x + 470.58	y = -0.5917x + 538.56	y = -0.8589x + 694.76

See Appendix 12, Tables A12.8 and A12.10 for statistical analyses.

Analysis of covariance was used to study the regression lines of seed Ca^{2+}, K^{+} and $Ca^{2+} + K^{+}$ for different seed sizes as was undertaken in section *5.3.3.2*. Table 5.20 shows that for Ca^{2+}, K^{+} and $Ca^{2+} + K^{+}$ concentrations, there was no significant difference at $P = 0.05$ among the slopes of the two seed sizes although there was a significant difference in intercept. Therefore a single regression line for both seed sizes was plotted, as there was no benefit in plotting separate regression lines for each seed size for each ion parameter as shown in Figures 5.4, 5.5 and 5.6.

Table 5.20 - Comparison of Individual Regression Lines of Correlation of [NaCl] EC_{50} (mM) with Seed $[Ca^{2+}]$, $[K^+]$ and $[Ca^{2+}] + [K^+]$ for Two Size Categories of Seed.

	Ca^{2+}	K^+	$Ca^{2+}+K^+$
P Value (Slope)	0.587	0.367	0.712
P Value (Common Intercept)	<0.001	0.014	0.087

Values above P = 0.05 indicate no significant difference. See Appendix 13, Tables A13.1-A13.3 for statistical analysis.

5.4.4 Discussion

Hydroponic culture of mother plants appeared to create fewer effects on plant growth and yield than those produced in the multi-substrate growth trial, but also interesting elemental data between different seed sizes. The seed elemental analysis provided information regarding the potential effect of nutritional treatment and seed size on seed Ca^{2+} and K^+ concentration, along with how this may affect salinity tolerance during germination. Seed Ca^{2+} concentration was significantly affected by seed size and nutritional treatment (Table 5.12). Seeds of Ø 1.0 - 1.4 mm had significantly lower Ca^{2+} concentration than seed of Ø < 1.0 mm over all five nutritional treatments, with high Ca^{2+} treatment having a significantly higher Ca^{2+} concentration than low Ca^{2+} in the Ø < 1.0 mm category. Seeds of Ø 1.0 -1.4 mm had no significant differences in Ca^{2+} amongst the five treatments. Seed produced from plants growing in low Ca^{2+} treatment had significantly lower Ca^{2+} concentration when taking into account both seed sizes compared to all other nutritional treatments, which is similar to the results obtained in the multi-substrate growth trial (Table 5.3).

The effect of seed size on Ca^{2+} concentration may be attributable to aspects of seed morphology between the two size categories. Differences in seed size may confer two factors which contribute to the result obtained by a total seed analysis. Firstly, smaller

seeds have a smaller embryo than larger seeds and, secondly, the relative ratio of coat to embryo increases with decreasing size. An increase in Ca^{2+} concentration per g of smaller seeds would appear to contrast with the first factor if a relatively large proportion of Ca^{2+} is a constituent of the embryo. Since the coat/embryo volume ratio increases with decreasing size, it could be concluded that Ca^{2+} may be a greater constituent of the coat. Such an association would, however, depend on the relative distribution differences between the two types of tissue. For example, an increase of analyte in μg per g seed with decreasing size, could initially point to an increase in seed surface area, from which the analyte concentration may be seen to increase in the outer embryo/seed coat, even if there was an even distribution of concentration per g within the seed between the actual coat and embryo tissue. However, if the analyte is almost solely distributed in the embryo, any decrease in size and effective increase in surface area, would have a lesser effect on overall concentration and could therefore theoretically increase the overall concentration of the analyte in terms of μg per g seed.

Seed K^+ concentration was also affected by size. Seeds of Ø 1.0 - 1.4 mm produced seed of lower K^+ concentration than Ø < 1.0 mm over all five nutritional treatments. Amongst the five treatments, there was no significant difference in seed K^+ concentration within size categories or the overall mean of treatments. As with seed Ca^{2+} concentration, the differences in seed K^+ concentration between seed sizes may be attributable to a difference in relative distribution in seed K^+ concentration amongst the coat and the embryo. Like seed Ca^{2+} concentration, since the coat/embryo volume ratio, in terms of μg per g seed, increases with decreasing size, it could be concluded that seed K^+ concentration may have a relationship with this factor, but

again it may be dependent on the distribution of the element between the two types of tissue.

A relative decrease in K^+ as shown by K^+/Ca^{2+} ratios in smaller Ø < 1.0 mm seeds compared with Ø 1.0 - 1.4 mm, may suggest that seeds of Ø 1.0 - 1.4 mm contain proportionally more K^+ per Ca^{2+} than seeds of a smaller size (Table 5.14), where conversely smaller seeds per gram, tended to otherwise have greater Ca^{2+} and K^+ compared with larger ones (Table 5.12 and 5.13). This may also be suggestive that Ca^{2+} may appear in tissue closer to the seed surface as this is a factor in considering an increase in surface area to volume ratio as discussed. The same is true of K^+, but importantly the results of K^+/Ca^{2+} ratio suggest that in larger seed, K^+ increased proportionally more than Ca^{2+} relative to a smaller size. This may also suggest a volume increase over surface area as size increases, perhaps indicating that K^+ may be located in the embryonic tissue to a greater extent than Ca^{2+}, or Ca^{2+} being located in the testa to a greater extent than K^+.

A wealth of factors may, however, contribute to a decrease in salinity tolerance in seeds, especially of a smaller size, where a general decrease in germination compared to larger seeds is attributed to a smaller embryo and less reserves (see sections 1.4.2 and 7.1), but analysis of the relative distribution of elements between embryo and testa would help to resolve the proposal that they differ between tissues.

In terms of an overall effect of nutrition on Ca^{2+} concentration of seed, the results of the hydroponic trial also show similar trends shown by the multi-substrate trial for seed Ca^{2+} concentration over three different types of growth substrate. In the multi-substrate growth trial, it was shown that high Ca^{2+} and low K^+ treatments produced seed of significantly higher Ca^{2+} concentration than low Ca^{2+} treated seeds and the

same was true in the hydroponic trial, further suggesting that a difference in seed Ca^{2+} concentration can be achieved by varying nutritional treatment of the mother plant. However, in the multi-substrate trial, the results were partially attributed to differences in growth and dilution of resources due to an accumulation of biomass that increases more rapidly than nutrient accumulation (Jarrell and Beverly 1981) among plants that grew particularly well, whereas this was not the case in the hydroponic trial. Also, soil and possible interactive root chemistry was also presented as a possible factor, but in the hydroponic trial, properties of the growth substrate are less of a factor than when dealing with substrates that contain appreciable amounts of exchangeable ions in addition to that which is provided in the nutritional treatments. The results obtained through use of hydroponic culture may be a better reflection of the effect of altering parent plant nutrition, but over all four substrates (peat & vermiculite, sand, sand & perlite and hydroponic culture), there is consistently a significant increase in seed Ca^{2+} with increasing Ca^{2+} nutrition when comparing low Ca^{2+} and high Ca^{2+} treated mother plants.

In terms of the lack of effect of nutritional treatment on overall seed K^+ concentration, the hydroponic culture method in this experiment produced results similar to those shown in the multi-substrate trial (Table 5.4). In this case, although differences in seed Ca^{2+} between nutritional treatments could be partially attributed to various aspects of the growth substrate, this should also be the case for K^+. The fact that nutritional treatment has no effect on seed K^+ concentration in both varied substrate and hydroponic culture, may point to the fact that the uptake and distribution dynamics of K^+ may be different from those of Ca^{2+}. It should also be taken into account that K^+, compared with Ca^{2+}, may be taken up more readily and therefore there may be less limiting factors regarding its distribution throughout a plant.

Mean values of K^+/Ca^{2+} between nutritional treatments (Table 5.14) indicated that low Ca^{2+} treatment had a significantly higher K^+/Ca^{2+} ratio than other treatments. This indicates that, over both size categories of Ø < 1.0 mm and Ø 1 - 1.4 mm, seeds produced with low Ca^{2+} in the growing medium have lower Ca^{2+} uptake (Table 5.12), or higher K^+ uptake, which may not necessarily be the case as indicated by Table 5.13. These results are similar to those shown in the multi-substrate growth trial (Table 5.5), wherein seed from the low Ca^{2+} treatment had a significantly higher K^+/Ca^{2+} ratio than seeds from high Ca^{2+} and control treatments.

Seeds of Ø 1.0 - 1.4 mm had a significantly higher salt tolerance than seeds of Ø <1.0 mm (Table 5.15), which is not unexpected given that there is literature that discusses an increase/decrease in reproductive capacity in larger and smaller seed, respectively, regardless of any additional abiotic stresses imposed. Harper (1977) suggests that this size dependent reproductive capacity is a function of intraspecific competition and self thinning, with the death of a significantly larger number of seedlings derived from small seeds than of seedlings from large seeds and this is determined by the seed resources, However, the long term nature of the environment and competitiveness may have a significant effect in determining seedling survival (Gross 1984). Harper (1977) also states that a reduction in embryonic tissue decreases seedling survivorship. This is perhaps amplified by the addition of saline stress. Grieve and Francois (1992) also found, using two *T. aestivum* cultivars grown under a mixture of NaCl and $CaCl_2$, that large seeds consistently gave more yield than small seeds. This is further emphasised by Khan and Ungar (1984) using halophytic *Atriplex triangularis*, Bayuelo-Jiménez, Craig, and Lynch (2001) using *Phaseolus* species and Easton and Kleindorfer (2009) with *Frankenia* species. There is, however, evidence

that an increase in seed size can have a negative effect on germination (Cideciyan and Malloch 1982).

Nutritional treatment was shown to have a significant effect on overall salt tolerance, where high Ca^{2+} treatment produced seed that had a significantly overall higher salt tolerance than control and low Ca^{2+} treatments when combining both size categories (Table 5.15). These results appear contradictory to those found during the multi-substrate trial, where no significant difference in salt tolerance was found amongst the different nutritional treatments, and also contradictory within the hydroponic growth trial in terms of Pearson's correlation (Table 5.16) and regression analysis (Figure 5.4, Table 5.17), which all suggest significant negative relationships between seed Ca^{2+} concentration and salinity tolerance. Given that the multi-substrate and hydroponic trial Ca^{2+} seed analysis is suggestive of increased mother plant Ca^{2+} nutrition increasing seed Ca^{2+} concentration in relation to low and high Ca^{2+} treatments (Tables 5.3 and 5.12) and that correlation of seed Ca^{2+} concentration and salinity tolerance shows a negative relationship (Figures 5.1 and 5.4), the idea that high Ca^{2+} treatment produces more salt tolerant seed than low Ca^{2+} treatment appears incongruous. Highlighting of the low and high Ca^{2+} data points in Figure 5.4 illustrates the spread of data for EC_{50} for both nutritional treatments and given the lack of significant difference between treatments within the seed size categories, the significant difference in mean values for these treatments may not be truly representative of the distribution of replicate data for each treatment. However, it should not be discounted that there may be an unknown factor that means that although a seed receives high Ca^{2+} nutrition, it is not high Ca^{2+} nutritional levels that are imparted on the seed itself that are able to primarily govern salinity tolerance, even though high Ca^{2+} nutrition produces higher seed Ca^{2+} than low Ca^{2+} nutrition (Tables 5.3 and 5.12).

Pearson's correlation coefficients between seed ion parameters for both seed sizes and EC_{50} (Table 5.16) indicate that there was no significant relationship for seeds of Ø 1.0 - 1.4 mm for Ca^{2+}, K^+, Ca^{2+}+ K^+, Ca^{2+}/K^+, K^+/Ca^{2+} and EC_{50}. However, for seeds of Ø < 1.0 mm, increases in seed K^+ concentration decreased salinity tolerance at P = 0.05, along with a significant negative relationship with K^+/Ca^{2+} and positive relationship between Ca^{2+}/K. When considering the correlation regardless of seed size, significant negative relationships existed between salinity tolerance and seed Ca^{2+}, K^+ and Ca^{2+}+ K^+ (Table 5.16). These results are similar to those of the multi-substrate growth trial (Table 5.7), where significantly negative relationships occurred between seed Ca^{2+}, K^+ and Ca^{2+}+ K^+ concentration for seeds of Ø 1.0 - 1.4 mm across the different growth substrates. Regression analysis of the overall data sets in the hydroponic growth trial also showed significant negative relationships with seed Ca^{2+} (Figure 5.4 and Table 5.17), K^+ (Figure 5.5 and Table 5.18) and Ca^{2+}+ K^+ (Figure 5.6 and Table 5.19) concentration. These results are also similar to those relationships found in the multi-substrate growth trial for salinity tolerance with seed Ca^{2+} (Figure 5.1 and Table 5.8), K^+ (Figure 5.2 and Table 5.9) and Ca^{2+}+ K^+ concentrations (Figure 5.3 and Table 5.10), which were all negative and significant. This may suggest that the similarities in both salt tolerance and correlations with ion parameters between the two growth trials, may arise due to factors other than growth substrate. The hydroponic growth trial should be considered lesser affected by factors that may have arisen by physical and chemical attributes of the substrate and its affect on plant growth, but it is clear from the correlations of salinity tolerance and ion parameters of both growth trials, that an increase in seed Ca^{2+}, K^+ and Ca^{2+}+ K^+ may have a role in a reduction in salinity tolerance.

Both growth trials appear to confirm that a significant increase in seed Ca^{2+} concentration arises from high Ca^{2+} treatments, compared with low Ca^{2+} treatments supplied to the mother plant (Tables 5.3 and 5.12), and that there is a significant negative relationship between subsequent seed Ca^{2+} concentration and salt tolerance. Conversely, seed K^+ concentration does appear to have a negative relationship with salt tolerance in both multi-substrate and hydroponic trials, but mother plant nutritional treatment appears not to affect subsequent daughter seed K^+ concentration (Tables 5.4 and 5.13).

5.4.5 Conclusion

Disregarding seed size as a factor, higher seed Ca^{2+} and K^+ concentrations were shown to cause a decrease in salinity tolerance and this is interesting considering that, in terms of nutrition supplied to the mother plant, high Ca^{2+} nutrition appeared to provide greater salinity tolerance than low Ca^{2+} nutrition (Table 5.15). Seed size affected seed K^+ concentrationand size and nutritional treatment affected seed Ca^{2+} concentration and was suggestive that Ca^{2+} may be distributed differently than K^+ between seed embryo and coat. However, conversely it may be that the nutrient status of the parent plant may have affected the seed size as this may be determined at the time of flower bud initiation when nutrients are translocated from the vegetative tissues to seeds (Fenner 1992). The effect of mother plant Ca^{2+} and K^+ nutrition on seed Ca^{2+} concentration was similar to that shown by the results of the multi-substrate trial, with a potential relationship between both mother plant supplied Ca^{2+} and K^+, and Ca^{2+} concentration of seed across both size categories. High Ca^{2+} and low K^+ treatments produced seed of higher Ca^{2+} concentration than low Ca^{2+} treatment. Ca^{2+}/K^+ ratios also showed that seeds from a low Ca^{2+} treatment had higher K^+/Ca^{2+}

ratios, therefore seeds produced with a low Ca^{2+} treatment had a relatively lower Ca^{2+} uptake over K^+ compared with other treatments.

Seed size had a significant effect upon salt tolerance and overall high Ca^{2+} nutritional treatment of mother plants produced seed with higher salt tolerance than control and low Ca^{2+} treatments, but there was no significant correlation between daughter seed Ca^{2+} and Ca^{2+}+ K^+ concentration and salinity tolerance between size categories. However, seed K^+ and K^+/Ca^{2+} ratios did have a significant and negative relationship in smaller seed and a positive relationship for Ca^{2+}/K^+ ratio, which is suggestive that as seed K^+ concentration increases proportionally over seed Ca^{2+} concentration, salinity tolerance decreases and as seed Ca^{2+} concentration increases over K^+ concentration, salinity tolerance increases. Given that K^+/Ca^{2+} ratios are greater for seeds of greater diameter, yet they exhibit greater salt tolerance, such interpretation is unsafe. The localisation of Ca^{2+} and K^+ within the seed and how these change relative to seed size together with the extent to which they may have a role in salinity tolerance may help in understanding if any definitive relationships exist between Ca^{2+}, K^+ and salinity tolerance.

5.5 Summary

The multi-substrate growth trial, along with the hydroponic growth trial using modified Hoagland's B, yielded data that was suitable for statistical analyses. A diagrammatic representation summarising the outcomes of the multi-substrate and hydroponic trials is shown in Figure 5.7. Bracketed values refer to the following statements. The multi-substrate growth trial indicated via analysis of variance that an increase in Ca^{2+} mother plant nutrition (i.e. high Ca^{2+} vs. low Ca^{2+}), produced seed of

higher Ca^{2+} concentration[1]. Through Pearson's correlation and regression analysis, the trial indicated that increases in seed Ca^{2+}, K^{+} and $Ca^{2+} + K^{+}$ concentration results in a reduction in salinity tolerance (EC_{50}) at germination[2-7].

The hydroponic trial provided statistically significant data. Analysis of variance here also indicated that over both seed sizes, an increase in Ca^{2+} mother plant nutrition produced seed of higher Ca^{2+} concentration[8] and that seed Ca^{2+} was affected by seed size with smaller seeds (Ø < 1.0 mm) having significantly higher seed Ca^{2+} per gram of seed than larger ones (Ø 1.0 - 1.4 mm)[9]. Seed K^{+} per gram was also significantly greater in smaller seeds[10]. Disregarding any differences due to seed size, salinity tolerance also increased with increased Ca^{2+} mother plant nutrition[11], but the scatter of data and lack of replicates may make this an anomalous result as the statistical analysis is insensitive to actual seed ion concentration and salinity tolerance. Salinity tolerance was found to be greater for larger seeds when comparing seed size[12]. Pearson's and regression analysis both showed that an increase in seed Ca^{2+}, K^{+} and $Ca^{2+}+ K^{+}$, decreased salinity tolerance overall[13-18], but when comparing seed size, higher K^{+} in smaller seed led to a decrease in salinity tolerance[19-20].

Growth Trial			
Multi-substrate (P&V, S, S&P)	Hydroponic		
	Overall	Ø < 1.0 mm	Ø 1.0 - 1.4 mm
	Anova		
↑ Ca^{2+} Nutritional Treatment = ↑ Seed Ca^{2} (1)	↑ Ca^{2+} Nutritional Treatment = ↑ Seed Ca^{2+} (8)	↑ Seed Ca^{2+} vs. Ø 1.0 - 1.4 (9) ↑ Seed K^{+} vs. Ø 1.0 - 1.4 (10)	
	↑ Ca^{2+} Nutritional Treatment = ↑ EC_{50} (11)		↑ EC_{50} vs. Ø < 1.0 (12)
	Pearson		
↑ Seed Ca^{2+} = ↓ EC_{50} (2)	↑ Seed Ca^{2+} = ↓ EC_{50} (13)		
↑ Seed K^{+} = ↓ EC_{50} (3)	↑ Seed K^{+} = ↓ EC_{50} (14)	↑ Seed K^{+} = ↓ EC_{50} (19)	
↑ Seed Ca^{2+}+ K^{+} = ↓ EC_{50} (4)	↑ Seed Ca^{2+}+ K^{+} = ↓ EC_{50} (15)		
	Regression		
↑ Seed Ca^{2+} = ↓ EC_{50} (5)	↑ Seed Ca^{2+} = ↓ EC_{50} (16)		
↑ Seed K^{+} = ↓ EC_{50} (6)	↑ Seed K^{+} = ↓ EC_{50} (17)	↑ Seed K^{+} = ↓ EC_{50} (20)	
↑ Seed Ca^{2+}+ K^{+} = ↓ EC_{50} (7)	↑ Seed Ca^{2+}+ K^{+} = ↓ EC_{50} (18)		

Notes: ↑ denotes increase, ↓ denotes decrease.

Figure 5.7 - Diagrammatic Representation of Experimental Outcomes of Multi-substrate and Hydroponic Growth Trials

Chapter 6: Calcium and Potassium Localisation: Seed Coat and Embryo Qualitative and Quantitative Assessment

6.1 Calcium Localisation - Seed Staining

6.1.1 Introduction

The potential for a difference of distribution of Ca^{2+} and K^{+} between seed coat and embryo is an interesting concept that may highlight specific areas that are reactive to salt tolerance. A quick and simple way to look for differences in tissue is through staining techniques. Given that previous results may suggest Ca^{2+} increasing proportionally over K^{+} in smaller whole seed, implicating that Ca^{2+} may be more abundant towards the seed outer surfaces, seeds were dissected, mounted and stained with glyoxal bis-(2-hydroxyanil) (GBHA), which has been used for the cytological examination of plant tissue since the late 1950s, to determine if any visual differences between coat and tissue were obvious.

6.1.2 Methods

6.1.2.1 Producing Seed and Stain

Samples of non-experimental commercially purchased seeds were carefully dissected in half with a double edged razor blade that had been washed in distilled water and dried. A cross sectional view of seed Ca^{2+} localisation in a single field of view was required, and localisation of intracellular Ca^{2+} was not of particular importance. Preliminary investigations indicated that the use of a microtome was difficult and the best results were obtained by disturbing the seed structure as little as possible. Seeds were stained with GBHA according to a method used by Crean and Haisman (1964)

for the examination of the cytological distribution of Ca^{2+} in *P. sativum* (peas). GBHA is a highly selective Ca^{2+} indicator developed by Goldstein and Stark-Mayer (1958) that stains red in the presence of Ca^{2+} (Crean and Haisman 1964, Val *et al*. 2008). GBHA however, is not able to be localise Ca^{2+} present as insoluble salts (Kashiwa and Atkinson, 1962). GBHA was prepared as a saturated solution in 95% ethanol. This was applied to the sample as a single drop, followed by one drop of a solution of 5% KOH (potassium hydroxide). After 3 minutes the sample was washed by flooding the section with 95% ethanol and mounted using DPX, making sure the cut surface was as level as possible in order to obtain as even view as possible of the seed structure/staining across the field of view. Samples were examined under a dissection microscope with an incidental light source.

6.1.3 Results

Plate 6.1 shows that there is a noticeable dark red staining of the seed coat and outermost cells of the embryo, with staining highlighting the outline of the cotyledons, endosperm and hypocotyl.

6.1.4 Discussion and Conclusion

GBHA staining indicated that there was likely to be a difference between the relative amounts of Ca^{2+} between the coat and the embryo. A quantitative assessment of the concentrations of Ca^{2+} between the coat and embryo was required to confirm this was indeed the case.

Plate 6.1 - GBHA (Red) staining on the seed cross sections showing distinct areas of calcium localisation, particularly highlighted by the dark staining of the seed coat (1), between the cotyledons (2) and endosperm (3) and around the hypocotyl (4).

6.2 Coat and Embryo Analysis

6.2.1 Introduction

As outlined by the results of the analysis of different seed sizes during the hydroponic growth trial, the potential for a differential distribution of Ca^{2+} and K^{+} between seed embryo and coat was highlighted by differences in K^{+}/Ca^{2+} ratios. Localisation of Ca^{2+} or K^{+} between these two different tissues could narrow the search for potential mechanisms that are linked with saline stress tolerance or intolerance.

6.2.2 Methods

6.2.2.1 Obtaining Tissue

In order to investigate the concentrations of Ca^{2+} and K^{+} in different seed tissue, it was necessary to separate the coat from the embryo. This strategy to initiate germination with limited soluble nutrient loss was achieved by suspension of the seeds via a semi-spherical wire mesh containing the seeds within a distilled water atomising chamber. Previous trials involved either positioning the seeds in direct contact with relatively large volumes of water, or suspension over water which failed to initiate any separation of seed coat from embryo. This method appeared to initiate germination using the minimum amount of water possible, thus any Ca^{2+} or K^{+} that may be soluble and therefore lost, was theoretically kept to a minimum. Seeds of Ø 1.0 - 1.4 mm from each parental nutritional treatment (Control A to High K^{+} E) produced during the hydroponic growth trial were suspended in the wire mesh in the humid atmosphere overnight in order to begin germination. The atomising chamber consisted of a plastic tank with glass rails to suspend the mesh containing the seed, an atomiser connected to an external air pump, and cling film covering the tank to create

a humid environment (Plate 6.2). Water contained within the tank acted as sump to which the atomiser was connected. Seeds were suspended in the wire mesh in the humid atmosphere overnight in order to begin germination.

Plate 6.2 - Humidification Tank

After the initiation of coat and embryo separation, embryos were teased out of coats using fine forceps and placed in clean micro beakers, before being dried in an oven at 80°C to dry weight overnight. The coats and embryos were then placed in a dessicator until analysis.

6.2.2.2 Tissue Elemental Analysis

Samples of coat and embryo were digested and analysed by the general method described in section 3.6.1. Samples of 0.02 g of the coat and between 0.05 and 0.07 g of embryo from each nutritional treatment were used. To each sample 4 ml of

concentrated trace analysis grade HNO_3 was added. A Mars 5 Microwave Accelerated Reaction System (CEM Corporation, Matthews, NC, USA) was used for the digestion procedure. Ca^{2+} and K^+ spikes were included in the digestion procedure as described in section *5.3.2.1*. After cooling, samples were decanted into 20 ml Grade A volumetric flasks and made to volume with deionised water. Concentrations of Ca^{2+} and K^+ (µg g^{-1} d.w.) of each type of tissue were calculated.

6.2.3 Results

6.2.3.1 Coat and Embryo Analysis

Table 6.1 shows the mean concentration of Ca^{2+} and K^+ in the coat and embryo tissue for each nutritional treatment. Spike digestions produced recoveries of 101 to 105% for both Ca^{2+} and K^+.

Seed coats were found to contain higher concentrations of Ca^{2+} than K^+ and embryos contained higher concentrations of K^+ than Ca^{2+}. Ca^{2+} concentration was greater in the coat than in the embryo and K^+ was greater in the embryo than the coat. Mother plant nutritional treatment had a significant effect on seed coat Ca^{2+} and K^+ concentration. Considering firstly Ca^{2+} concentration for seed coat, there was no significant difference in concentration between control and low Ca^{2+}, high Ca^{2+}, low K^+ and high K^+ treatments. Low Ca^{2+} treatment, however, produced lower Ca^{2+} concentration than high Ca^{2+} and high K^+ treatments, suggesting that Ca^{2+} nutrition may have had an effect upon the amount of Ca^{2+} delivered to the seed coat and that high K^+ treatment may have a positive effect on Ca^{2+} delivery to the coat. High K^+ nutrition may allow for greater uptake of Ca^{2+} by the seed coat. Considering K^+ concentration for seed coat, there was again no significant difference between control, high Ca^{2+}, low K^+ and

high K^+ treatments, but low Ca^{2+} did have a significantly higher K^+ concentration than all other nutritional treatments, which is also interesting as this could suggest that Ca^{2+} and K^+ uptake by the seed coat is connected and that lower Ca^{2+} uptake may allow for greater uptake of K^+. This is important considering the results of the previous growth trials, whereby K^+ nutrition appeared to have no effect on overall seed Ca^{2+} or K^+concentration, but Ca^{2+} nutrition has been implicated in changes to overall seed Ca^{2+}. It may be presented that although high K^+ nutrition appears to have little effect on overall seed Ca^{2+} concentration, it may have a significant effect on seed coat Ca^{2+}, but differences in this mass which represents a small proportion of seed would not contribute significantly to the overall seed Ca^{2+}concentration. Ca^{2+} nutrition, however, appears to have a significant effect on seed coat Ca^{2+} concentration and it may be considered that the differences in overall seed Ca^{2+} concentration that are attributed by low and high Ca^{2+} nutrition, may arise because the differences in seed coat are large enough, and larger than those that may arise through varying K^+ nutrition, to have an effect on overall seed Ca^{2+} concentration. Ca^{2+} and K^+ concentration in the embryo tissue showed no significant difference between nutritional treatments.

Table 6.1 - Calcium and Potassium Concentration in Seed Coat and Embryo

	μg g^{-1}			
	Coat		Embryo	
	Ca^{2+}	K^+	Ca^{2+}	K^+
Control	11014 ab	4790 b	3800 a	13611 a
Low Ca^{2+}	9980 b	6784 a	3459 a	13827 a
High Ca^{2+}	11759 a	4485 b	3387 a	14895 a
Low K^+	10679 ab	4580 b	3635 a	14353 a
High K^+	11468 a	4783 b	3479 a	14056 a
Mean	10980 *aA*	5084 *bB*	3552 *aB*	14148 *bA*

Means within columns followed by different letter differ significantly at P < 0.05. Means between columns are marked by italicised letters. Lower case italics show differences of means between tissues and upper case italics show differences of means between analytes. See Appendix 14, Tables A14.1-A14.5 for statistical analysis.

6.2.4 Discussion

White and Broadley (2009) state that mineral elements are not homogenously distributed in seed and that the concentrations of many mineral elements are the highest in the husk or aleurone layer. This is the case for Ca^{2+} and K^{+} in terms of the seed coat and embryo analysed here. Although the results do not take into account masses of the tissues relative to the whole seed, the results per gram of tissue do show clear differences in the distribution of two elements between seed tissue. However, it should be safe to surmise that total seed K^{+} is greater than Ca^{2+}, due to the high amounts of K^{+} in the embryo and the larger proportion of embryo which constitutes the seed. This would be expected given the macronutrient status of K^{+}. Ca^{2+} should conversely be considered as being much lower in the overall seed, but the much higher amounts found in the coat may be the first site of contact with a saline environment. Moraghan and Grafton (2002) analysing *P. vulgaris* (common bean) and looking at the relative distribution found that for Ca^{2+}, 80% of the total seed Ca^{2+} was located in the coat, but K^{+} was distributed much more evenly. The former was attributed to formation of CaC_2O_4 (calcium oxalate). Ilarslan *et al*. (1997) measuring oxalate levels in whole seeds of *G. max* (soyabean) found that up to nearly a quarter of the seed was oxalate, three quarters of which was insoluble CaC_2O_4 and associated its presence with Ca^{2+} storage and seed storage protein synthesis. Streeter (2005) also investigating soyabean, found that although seed Ca^{2+} remained invariate during seed pod development, oxalate concentrations actually declined. Given the low amounts of oxalate found by a number of investigations at the whole plant level in species of the *Brassicaceae* compared with other plants, (Savage *et al*. 2000, Judprasong *et al*. 2006, Lucarini *et al*. 1999, Kamchan *et al*. 2004, Santamaria *et al*. 1999), seed oxalate as a compound for Ca^{2+}, would also perhaps be expected to be low in this case.

Mother plant nutritional treatments also appear to have a significant effect upon the distribution of Ca^{2+} and K^{+} between coat and embryo and most importantly within the coat. High Ca^{2+} treatment increased coat Ca^{2+} concentration and low Ca^{2+} decreased coat Ca^{2+} concentration which may indicate that in terms of whole seed Ca^{2+} concentration variability in response to mother plant nutritional treatment, seed coat may actually possess differences in Ca^{2+} uptake, which is then reflected in the whole seed. Ca^{2+} nutritional treatment affects not only the coat Ca^{2+} concentration, but also coat K^{+} concentration. Low Ca^{2+} mother plant nutrition appears to decrease coat Ca^{2+}concentration, which would be expected, but at the same time significantly increases coat K^{+} concentration. This may be indicative of a how Ca^{2+} nutrition may affect the nutritional loading of the coat in terms of K^{+}, but this may also be the case for other elements. The embryo however, appears to be much more stable and less prone to the effects of any differences in mother plant Ca^{2+} and K^{+} nutrition.

6.2.5 Conclusion

Seed coats contained more Ca^{2+} than K^{+} and embryos contained more K^{+} than Ca^{2+} per gram of tissue. Mother plant nutritional treatment from the hydroponic growth trial appeared to affect the nutritional loading of the coat. For Ca^{2+}, there was a significantly greater amount of Ca^{2+} in coats from seeds produced by high Ca^{2+} nutrition, compared with low Ca^{2+} nutrition. For K^{+}, low Ca^{2+} nutritional treatment gave higher seed coat K^{+} concentration than other treatments. Seed embryos contained around four times more K^{+} than Ca^{2+} per gram of tissue, but there was no effect of mother plant nutritional treatment on daughter seed embryo Ca^{2+} or K^{+} concentrations.

These results confirm the ideas that resulted from K^+/Ca^{2+} ratios found in different seed sizes and that high Ca^{2+} mother plant nutritional treatment results in lower K^+/Ca^{2+} ratios compared with low Ca^{2+} mother plant treatment. They also confirm the results shown by staining with GBHA in that Ca^{2+} must be present in high concentrations in the seed coat, given the relative mass of the embryo compared to coat.

6.3 Soluble and Insoluble Calcium and Potassium - Preliminary Extractions

6.3.1 Introduction

Total extractions of tissues provide pertinent information regarding seed tissue nutrient loading with different mother plant nutrient treatments. However, when considering a biological mechanism that may rely upon particular elements, it is important to consider the viability of that element to take part in any pathway. In biological systems it would safe to assume that only compounds which are soluble *in vivo* would be available to exert any effect, whereas those which combine with other chemical species that render them insoluble *in vivo* become biologically inert. One such example would be CaC_2O_4. CaC_2O_4 is an insoluble Ca^{2+} salt that is prevalent in a range of plant species. Its insolubility means that in terms of ion exchange capacity, potential for cell signalling and its role in salt tolerance, CaC_2O_4 may not have any active function. In order to elucidate the proportions of Ca^{2+} and indeed K^+ which are soluble and insoluble in *B. rapa* seeds and to investigate the potential of elucidating Ca^{2+} and K^+ viability in response to mechanisms, a simple sequential extraction was performed on samples of whole seed, seed coat and embryo with water.

6.3.2 Methods

6.3.2.1 Extraction Procedure

An initial trial extraction to find whether the experimental procedures were feasible was carried out. Throughout the procedure, all glassware and digestion vessels were acid washed in 1 M H_2SO_4. Samples of 0.2 g of non-experimental commercially purchased whole seeds, 0.02 g coat and 0.1 g embryo measured at dry weight, after undergoing separation by humidification treatment outlined in the previous assay (section *6.2.2.1*), were placed into a micro homogeniser vial (Plate 6.3) and 4 ml of distilled water was added. The sample was homogenised for 30 min and the blades and spindle were washed of sample into the vial before it was sealed with parafilm and placed in a 40 khz ultrasonic waterbath for 20 min at a temperature of 80°C (Savage *et al*. 2000, Judprasong *et al*. 2006). In order to harvest any CaC_2O_4 crystals, homogenate was then filtered through a 0.22 μm (Tiselius 1980, Dawson, Grover and Ryall 1998, Chauvet and Ryall 2005) pore mixed cellulose ester (MCE) filter membrane (Millipore Corporation, USA) that had previously been washed in deionised water, folded into a funnel whilst still wet and dried in a dessicator. Samples were decanted via the filter membrane to 20 ml Grade A volumetric flasks, via continuous washing of the digestion vessel and made up to volume with deionised water. This was considered to be the water soluble fraction of the extraction procedure. Whilst wet, the filter membranes were compressed into a more tubular form, so as to allow easy loading of the membrane filters to a PTFE digestion vessel.

Plate 6.3 - Microhomogeniser, Vial, Blades and Spindle

6.3.2.2 Tissue Elemental Analysis

After being redried in a dessicator, the filter membranes containing the water insoluble fractions were digested in 4 ml of concentrated trace analysis grade HNO_3. (Birnbaum *et al*. 2008) via a Mars 5 Microwave Accelerated Reaction System, CEM Corporation, USA. The resultant digests were decanted into 20 ml Grade A volumetric flasks and made up to volume with deionised water. Analysis of both fractions was carried out using the procedure outlined for whole seed in 3.6.1. Three replicates were used, but in some cases either the soluble or insoluble fraction were compromised by the experimental procedure, in which case the remaining replicates were used. Soluble and insoluble Ca^{2+} and K^{+} concentrations were calculated in µg g^{-1} d.w. of seed or tissue and the soluble and insoluble fractions were expressed as a percentage of the total seed or tissue Ca^{2+} or K^{+}. This total was considered as the sum of soluble and insoluble fractions for each analyte for seed or tissue.

6.3.3 Results

Table 6.2 shows the relative solubility of Ca^{2+} and K^{+} in the whole seed and then between embryo and coat. The solubility of Ca^{2+} over the whole seed is split comparatively evenly, with 46.56% soluble and 53.44% insoluble. However, when the embryo and coat are analysed separately, the solubility of Ca^{2+} in each component differs to a much greater extent. Within the embryo, Ca^{2+} is markedly more soluble with 73.96% being soluble, compared with 26.04% being insoluble. Within the coat, the opposite was found, with the greater proportion (87.68%) being found to be insoluble and a lesser 12.32% being soluble. K^{+} over the whole seed was mainly soluble (91.96%). The majority of K^{+} in the embryo and the coat was also found to be soluble (94.42% and 81.97% respectively).

Table 6.2 - Relative Percentage Soluble and Insoluble Calcium and Potassium Within Whole Seed and Tissues

	Ca^{2+}		K^{+}	
	Soluble	Insoluble	Soluble	Insoluble
Whole Seed	46.56	53.44	91.96	8.04
Embryo	73.96	26.04	94.42	5.58
Coat	12.32	87.68	81.97	18.03

6.3.4 Discussion

Although the seed coat of *B. rapa* has been shown to contain more Ca^{2+} per gram of tissue than the embryo which occupies the majority of the mass of a seed, the Ca^{2+} present seems to be largely insoluble in water. K^{+} appears to be mostly soluble in the embryo and in the coat. The preceding experiment (6.2) showed that the nutrient loading of tissues is affected by mother plant nutrition, but the nature of that element's incorporation into the seed is unknown. Although seed may contain greater

amounts of a specific element, there is no guarantee that it will be soluble or exchangeable, and therefore considered useful in response to environmental stimuli. Ca^{2+} may be uptaken and sequestered as an immobile and chemically reactive void compound under conditions *in vivo*. CaC_2O_4 is a good example in many oxalate producing species, but the compound is seldom recorded in *Brassica* species (Savage *et al*. 2000, Judprasong *et al*. 2006, Lucarini *et al*. 1999, Santamaria *et al*. 1999, Kamchan *et al*. 2004). Other forms of Ca^{2+} deposition may be present such as those which are ionically exchangeable and which would be important during germination in NaCl and compounds other than CaC_2O_4 which would still be insoluble *in vivo* such as calcium phosphate, ($Ca_3(PO_4)_2$), calcium sulphate ($CaSO_4$), and calcium pectinate (Kostytschew and Berg 1929, Kinzel 1989).

6.3.5 Conclusion

Most K^+ within the seed is soluble, but this is not the case for Ca^{2+}, with differing solubilities found between different types of tissue. The embryo contains the highest amount of soluble Ca^{2+} and the coat has the lowest. This may have an importance for the mobilisation of reserves and which tissues are likely to be primarily involved in responses to salinity and osmotic stress.

6.4 Further Solubility Extractions - Calcium

6.4.1 Introduction

Through the literature reviewed here, Ca^{2+} has widely been implicated in tolerance to salinity at the whole plant level and in some cases, at the tissue level. Given the relative abundance of literature and that most of the K^+ present in seed is soluble

(Table 6.2), the following investigation concentrates primarily on Ca^{2+} solubility and thus aimed to find how readily available the element would be *in vivo* and the extent to which different compounds of the element exist in the tissue tested, by using different extractants. The extractions endeavoured to provide information of the amount of metabolically active forms of Ca^{2+} which are soluble and from what cellular constituents they may originate from. Given that any excess Ca^{2+} provided by the mother plant is likely to be metabolically active and soluble in *B. rapa* and because oxalate compounds tend to be rare, this may provide an insight into which organelles are the most likely sink and could raise hypotheses as to why an increase in seed Ca^{2+} appears to decrease EC_{50} as found in the multi-substrate growth trial. Two size categories of seed were used in order to assess any differences in seed tissue Ca^{2+} between different extractants.

Relative distribution of Ca^{2+} between the coat and embryo of the two seed size categories was calculated to ascertain if seed size affected distribution and to examine the difference in Ca^{2+} between the two tissues on a single seed basis. In previous extractions, although the coat was found to have a greater concentration of Ca^{2+}, the distribution of Ca^{2+} between the tissues relative to the whole seed has been unknown.

6.4.2 Methods

6.4.2.1 Tissue Extraction and Analysis

Three different types of *B. rapa* seed tissue were used. Non-experimentally produced commercially purchased whole seed, coat and embryo were investigated over two size categories of Ø 1.0 - 1.4 mm and Ø 1.4 - 1.7 mm diameters. These diameters were used as the majority of the purchased seed fell within the size range Ø 1.0 - 1.7 mm.

Five replicates of each tissue were used for each solvent at each seed size category. Each type of tissue was ground to a homogenous powder before approximately, but accurately, 0.03 g dry weight of whole seed, 0.04 g of embryo and 0.01g of coat were weighed into 15 ml plastic centrifuge tubes. Using an accurately dispensed volume of 10 ml, solvents of 80°C Water (H_2O), 1 M salt (NaCl), 1 M acetic acid (CH_3COOH) and 1 M hydrochloric acid (HCl) were dispensed into the tubes containing the samples and the tubes were placed on a mechanical shaker for 1 h and then allowed to settle before being analysed directly via atomic emission spectroscopy using the equipment described in section 3.6.1.

These solvents have in previous literature allowed categorisation of Ca^{2+} compounds into distinct solubility categories (Kostytschew and Berg 1929, Kinzel 1989). An 80°C H_2O soluble fraction contains all the compounds that would primarily come from the vacuoles, cytoplasm, organelles and partly from the cell wall that would dissolve *in vivo*. A NaCl soluble fraction contains those considered water soluble and those that are exchangeable or that are electrostatically absorbed onto cell walls or polyanions in vacuoles. A CH_3COOH soluble fraction contains water soluble, NaCl soluble and Ca^{2+} compounds in the form of calcium phosphate ($Ca_3(PO_4)_2$) and calcium pectinate. A final HCl soluble fraction contains water soluble, NaCl soluble, CH_3COOH soluble and Ca^{2+} bound to oxalate. The amount of Ca^{2+} and K^+ released by a particular extractant was calculated by the cumulative subtraction of lesser performing extractants, i.e. the amount of Ca^{2+} considered only soluble by HCl would be equivalent to the total Ca^{2+} extracted by HCl minus the Ca^{2+} extracted by water, NaCl and CH_3COOH.

6.4.2.2 Calculation of Relative Distribution of Calcium Between Seed Coat and Embryo

The relative distribution of Ca^{2+} between the coat and embryo of the two seed size categories was calculated as µg g dry weight per seed by factoring in the relative proportion of mass of embryo or coat relative to a whole seed within each size category. This was achieved by measuring the dry weight of 100 seeds and finding the average weight of 1 seed for each size category and multiplying by the relative proportion of coat and embryo calculated from the splitting of tissues from a batch of seed. Seeds of Ø 1.0 - 1.4 mm had 82.47% and 17.53% of their mass made up by embryo and coat respectively and seeds of Ø 1.4 - 1.7 mm had 84.41% and 15.59% of their mass made up by embryo and coat respectively.

The addition of the Ca^{2+} extracted by each extractant for coat and embryo for each size category was compared with the µg g Ca^{2+} measured per whole seed and checked for likeness. The difference in the sum of embryo and coat Ca^{2+} compared with total seed Ca^{2+} was +5.37% for seeds of Ø 1.0 - 1.4 mm and -0.02% for Ø 1.4 - 1.7 mm. The Ca^{2+} for seed, coat and embryo for each replicate extraction was expressed as a percentage of the average maximum cumulative extraction of Ca^{2+} achieved for each extractant for seed, coat and embryo respectively (Tables 6.3 and 6.4).

6.4.3 Results

6.4.3.1 Ø 1.0 - 1.4 mm

The extraction of Ca^{2+} from whole seed, along with coat and embryo tissue was initially expressed as µg of Ca^{2+} per seed and in the case of the coat and embryo tissue, is expressed in terms of the proportionate mass of the coat or embryo which

would constitute the mass of a whole seed as dry weight as calculated in section *6.4.2.2*.

The extraction of seed and tissue of Ø 1.0 - 1.4 mm is shown in Figure 6.1. Water extractable Ca^{2+} is greatest in the seed, followed by NaCl, HCl and much lower by CH_3COOH. In the embryo, much of the Ca^{2+} is extractable by water, with decreasing amounts removed by NaCl, HCl and CH_3COOH. In the coat, however, there appears to be very little water extractable Ca^{2+}, with the majority extractable by NaCl. Following the trend of whole seed and embryo, much less appears to be extractable by HCl and CH_3COOH.

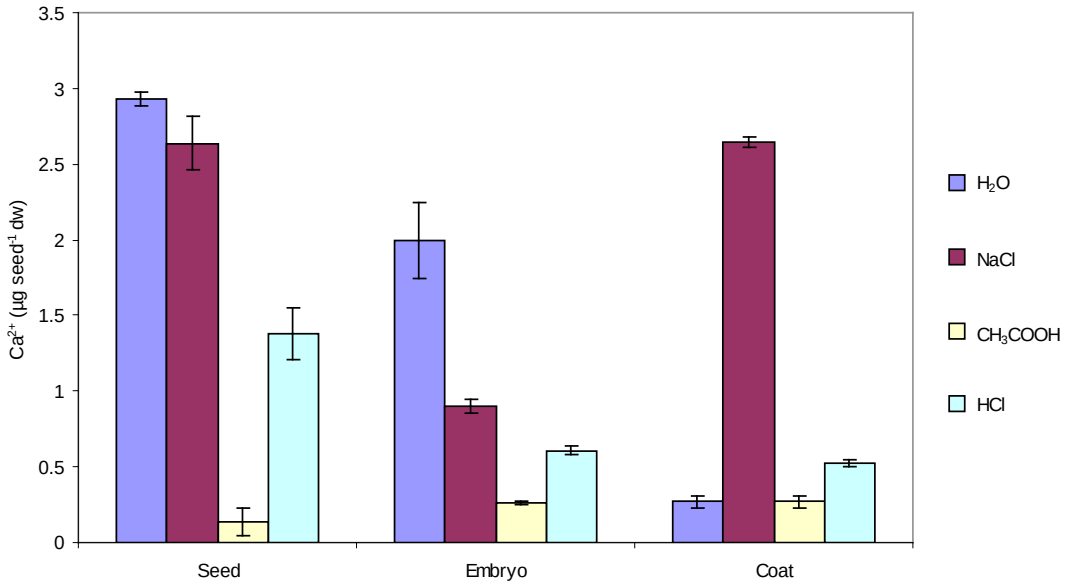

Figure 6.1 - Calcium Extracted From Seed and Tissues of Ø 1.0 - 1.4 mm and Expressed as µg Ca^{2+} per Whole Seed

6.4.3.2 Ø 1.4 - 1.7 mm

The extraction of Ca^{2+} from seed/tissue of seed of Ø 1.4 - 1.7 mm is shown in Figure 6.2. Similar results were found in this size category as were found for Ø 1.0 - 1.4 mm. In whole seed, water extractable Ca^{2+} predominates followed by NaCl, HCl and

CH_3COOH, although much more appears from this particular fraction in larger seeds. In terms of embryonic tissue, like that in seeds of Ø 1.0 - 1.4 mm, much of the Ca^{2+} is extractable by water, with lower amounts of HCl, but interestingly higher amounts of Ca^{2+} extracted by CH_3COOH. In seed coat tissue, the results reflect those found in smaller seeds with lesser Ca^{2+} extractable by water, compared to NaCl which extracts the most. The HCl and CH_3COOH fractions produced ever decreasing amounts of Ca^{2+} respectively.

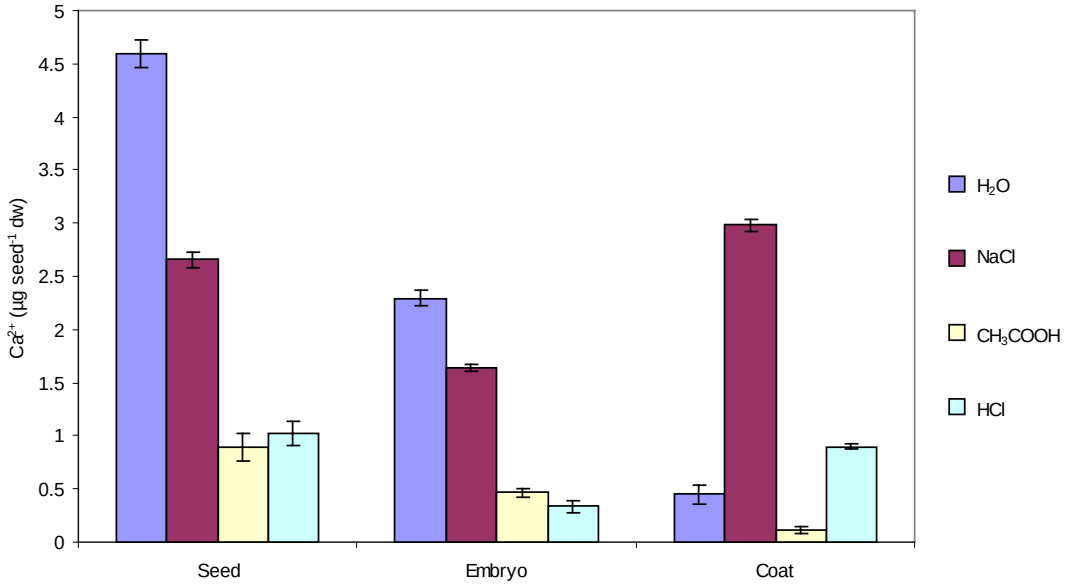

Figure 6.2 - Calcium Extracted From Seed and Tissue of Ø 1.4 - 1.7 mm and Expressed as µg Ca^{2+} per Whole Seed

The results presented as average percentage removal for each type of extractant (the Ca^{2+} removed by the extractant expressed as a percentage of the highest Ca^{2+} removed by the best performing extractant) for each seed size (Tables 6.3 and 6.4). In terms of different seed size, there may be a trend between removal by extractant and seed tissue type. Water extractable Ca^{2+} in embryonic tissue in both seed sizes appears to be significantly greater than by any other extractant (53.10 and 48.53%). Similarly in seed coat, NaCl appears to extract a significantly greater amount of Ca^{2+} over both

seed sizes (71.34 and 67.20%), compared with any other extractant of that tissue. Ideally, more than five replicates for each type of tissue for each seed size would be analysed, but the assay is restrictive, especially regarding coat tissue in terms of the production of tissue needed to provide accuracy for analyses. The results do, however, show, at least in this case, that around 45-55% of Ca^{2+} in the embryo is water extractable and therefore may have biological significance and 65-75% of Ca^{2+} in the seed coat is ionically exchangeable and also may have a role to play during germination *in vivo*.

Table 6.3 - Mean Percentage Removal of Calcium for each Extractant for Seed of Ø 1.0 - 1.4 mm

	Ca^{2+} (%)		
Extractant	Seed	Embryo	Coat
H_2O	41.36	53.10	7.27
NaCl	37.24	23.85	71.34
HCl	19.47	16.20	14.09
CH_3COOH	1.93	6.85	7.30

Table 6.4 - Mean Percentage Removal of Calcium for each Extractant for Seed of Ø 1.4 - 1.7 mm

	Ca^{2+} (%)		
Extractant	Seed	Embryo	Coat
H_2O	50.16	48.53	10.08
NaCl	28.96	34.60	67.20
HCl	11.14	7.06	20.26
CH_3COOH	9.74	9.81	2.46

An interesting point derived from this data is that the CH_3COOH extraction, prior to undergoing subtraction of Ca^{2+} removed by other extractants as described in section *6.4.2.1*, removed the most overall Ca^{2+} compared with all other extractants. In both seed size fractions, CH_3COOH extracted more overall Ca^{2+} and HCl appeared to provide an intermediate extraction, rather than extracting all possible Ca^{2+} compounds as predicted by Kinzel (1989). The differences between CH_3COOH and HCl shown

in Figures 6.1 and 6.2, is derived from the difference in performance between each extractant (i.e. the difference in Ca^{2+} relieved from tissue by CH_3COOH and HCl).

The distribution of Ca^{2+} as calculated using the extraction techniques used to determine mean percentage removal of Ca^{2+} by extractants is shown in Table 6.5. With previous experiments, a greater concentration of Ca^{2+} was found in the seed coat than embryo per g of tissue (section *6.2.3.1*), but whether this changes with seed size was unknown. Given that the coat occupies a relatively small mass of the total seed, the percentage of extractable Ca^{2+} found in the embryo and in the coat is approximately 50% for each and differs very little between seed sizes, the percentage of extractable Ca^{2+} in the coat is slightly higher in smaller seeds and also lower in terms of embryo, which may suggest that surface to volume ratios do increase in smaller seeds and this may increase Ca^{2+} concentration in seed coat, but considering the lack of replicate data, lack of range of sizes and the opportunity to undertake statistical analyses, it would appear that the distribution is proportionally indifferent (Table 6.5) and such small differences could not solely account for the differences in salt tolerance found between different seed sizes. Indeed, it appears that in terms of salt tolerance, seed size and the hypothesised differences in Ca^{2+} distribution, may not itself have a role in tolerance itself. This is substantiated by the salinity tolerance and seed Ca^{2+} regressions in both the multi-substrate and hydroponic trial (Figures 5.2, and 5.5), which showed that regardless of seed size, as seed Ca^{2+} increased, salinity tolerance decreases. Thus, it may be that salt tolerance at germination may be more importantly linked to seed reserves, which is a characteristic of larger seeds, rather than specific ions or ratios between them at a specific site.

Table 6.5 - Relative Distribution of Calcium Between Seeds of Different Sizes

	Ca^{2+} Relative to Whole Seed (%)	
Size	Coat	Embryo
Ø 1.0 - 1.4 mm	49.61	50.39
Ø 1.4 - 1.7 mm	48.41	51.59

6.4.4 Discussion

Schmid (1968) analysed the leaves of the *Brassicaceae* species, *Arabis turrita* (tower rockcress), by this method and found that in terms of Ca^{2+}, the majority was water soluble, with decreasing amounts in subsequent fractions. Although translocated levels of Ca^{2+} may differ between plant organs, the generic principal of the ability to sequester Ca^{2+} as a certain molecule of a given solubility may apply throughout plant parts, including seed embryo and coat. Kinzel (1982) and Kinzel and Lechner (1992) also concur that notably the *Brassicaceae* do not mineralise appreciable quantities of CaC_2O_4, but instead accumulate significant quantities of water soluble Ca^{2+} complexes in their vacuoles, such as $CaSO_4$ in sulphate storing *Brassicaceae* (Kinzel 1989). However, no reliable and accurate methods are present which are able to reveal which fraction of the water soluble Ca^{2+} is available as free Ca^{2+} *in vivo*. Work with ion sensitive electrodes, although prone to cross sensitivities and interferences of companion ions, only found a small portion of water soluble Ca^{2+} to be in this form, thus suggesting that just because a form of Ca^{2+} is water soluble, it is not necessarily dissociated and does not necessarily mean that it is useful *in vivo* (Buresch 1989 cited in Kinzel 1989).

6.4.5 Conclusion

In interpreting the results shown here, we can surmise that the majority of Ca^{2+} in *B. rapa* seed is either water soluble or ionically exchangeable. More water soluble fractions are present in the embryo and greater amounts of ionically exchangeable Ca^{2+} are found in the seed coat. Seed size appears to have little effect on the approximate 50% distribution of total Ca^{2+} between tissues in whole seed. The water and NaCl soluble fractions found in seed are representative of the proportions found in other *Brassicaceae* tissues, but interpretation as being potentially biological active and available Ca^{2+} should be taken under consideration since free Ca^{2+} is difficult to elucidate. It would, however, be safe to assume that soluble/exchangeable Ca^{2+} would contain the potential to contain free Ca^{2+}, which may be the key to providing salt tolerance in tissue.

Chapter 7: General Discussion

7.1 Summary of Results

The multi-substrate and hydroponic growth trials, using a variety of different growth substrates, provided little evidence to suggest that the different nutritional treatments supplied had any adverse effect on rate and completion of developmental growth such as flowering. In terms of seed yield, however, the peat & vermiculite substrate in the multi-substrate growth, trial showed that yield may be increased by the availability of nutrients due to the chemical composition of the substrate or the physical nature of the substrate. The hydroponic growth trial highlighted that greater numbers of smaller seed are produced (Table 4.12), which may be due to loss of seed maturity under unlimited growth conditions as discussed by Harper, Lovell, and Moore (1970) or to the nature of apportionment of reproductive energy between seed size and number as discussed by Harper (1977), but this was not linked to differences in mother plant nutrition. The chosen concentrations of Ca^{2+} and K^{+} had little effect on actual developmental growth which may have always raised issues regarding the validity of ion content data from seeds produced via differing nutritional treatments. This appears not to be the case amongst the data linked to salinity tolerance, although the scope for differentiation of concentration of Ca^{2+} and K^{+} between nutritional treatments does potentially remain, should further experimentation warrant trying to obtain greater differences in mother plant fed seed Ca^{2+} and K^{+} concentrations.

Concentrations of Ca^{2+} and K^{+} in seed were significantly affected by nutritional treatment in both major growth trials (multi-substrate and hydroponic). Low Ca^{2+} treatment produced seed with lower Ca^{2+}concentration and K^{+} treatments appeared to have no effect on the K^{+} concentration of seeds, perhaps indicating that any change in

K^+/Ca^{2+} ratio amongst seeds differing in nutritional background must originate from the levels of Ca^{2+} found within them. Considering low Ca^{2+} treatment, K^+/Ca^{2+} ratios for seeds produced in both the multi-substrate and hydroponic growth trials were higher than all other nutritional treatments with the exception of high K^+ treatment in the multi-substrate trial (Tables 5.5 and 5.14), which is not beyond feasibility considering an increase in seed K^+ concentration would increase K^+/Ca^{2+} ratio, suggesting that seed Ca^{2+} concentration may be more prone to manipulation than perhaps seed K^+ concentration.

Contrasting results were obtained between the multi-substrate and hydroponic growth trials, regarding the effect of nutritional treatment on salinity tolerance of seeds. EC_{50} did not differ between nutrient solutions for the multi-substrate trial and this may be due to the effect of growth substrate (Table 5.6). However, salinity tolerance was affected by nutritional treatment for the hydroponic trial when considering the mean EC_{50} over both seed diameters (Table 5.15). High Ca^{2+} treatment appeared to confer a higher EC_{50} salinity tolerance than control and low Ca^{2+} treatments across both size categories of seed used, but within a size category no significant differences were found between treatments. This may be indicative of an effect of seed size on the outcome of mother plant nutrient regime, but appears to be counter intuitive to the results of the regression analysis and Pearson's correlation of the overall data sets of the multi-substrate and hydroponic trial; both of which indicate that EC_{50} is significantly reduced as seed Ca^{2+} concentration increases. There may be a factor that means that although a seed receives high Ca^{2+} nutrition, it is not high Ca^{2+} nutritional levels that are imparted on the seed itself that primarily governs salinity tolerance, even though high Ca^{2+} nutrition produces higher seed Ca^{2+} concentration than low Ca^{2+} nutrition (Tables 5.3 and 5.12). The differences found between multi-substrate

and hydroponic growth trials, for the effect of mother plant nutrition on seed EC_{50} (Tables 5.6 and 5.15), may serve to highlight a possible effect of substrate structure and chemistry on other effectors that may contribute to the degree of salinity tolerance of daughter seed.

The Pearson's correlation of salinity tolerance showed that for the multi-substrate growth trial, increases in seed Ca^{2+}, K^+ and Ca^{2+}+ K^+ concentration had significant negative relationships with salt tolerance (Table 5.7) and for the hydroponic growth trial, increases in seed K^+ concentration and K^+/Ca^{2+} in seeds of Ø < 1.0 mm, and seed Ca^{2+}, K^+ and Ca^{2+}+ K^+ concentration over both seed sizes caused a significantly negative correlation with EC_{50} (Table 5.16). There was no effect of any ionic parameter on salinity tolerance for seeds of Ø 1.0 - 1.4 mm

Seed size may almost certainly give rise to other facets that affect salt tolerance. Perhaps one of the key results is that if seed K^+ concentration increases, salinity tolerance decreases and this may or may not be a force in the increase in K^+/Ca^{2+} ratio increases which were also seen to cause a negative relationship with salinity tolerance in smaller seed (Table 5.16). Given the wealth of literature which is supportive of increases in external Ca^{2+} that provide an increase in salt tolerance, the data of the multi-substrate and hydroponic growth trials is not supportive. The results of both experiments, however, do suggest other factors may affect salt tolerance as well as integral seed Ca^{2+} and K^+, which make the true response of increasing or decreasing these nutrients difficult to elucidate. There may also be factors whereby the usefulness of Ca^{2+} and K^+ in terms of storage, *in vivo* availability, and what fraction of the total Ca^{2+} and K^+ contents found in a seed would be useful in counteracting the effects of

salinity, that could conceal the effects of additional Ca^{2+} and K^{+} and their impact on salinity tolerance.

7.2 Calcium, Cell Signalling and Potential for Cytotoxicity

Possible influences on Ca^{2+} and K^{+} uptake, movement and effects of translocation in relation to salinity tolerance in various plant tissues and whole plant systems are of interest in light of the results obtained and the following discussion is aimed at highlighting the potential complexity of mechanisms involved in plant responses to saline conditions.

Within the plant cell, Ca^{2+} may exist as insoluble CaC_2O_4 deposits, as in "oxalate plants", as calcifuges or "potassium plants" with little Ca^{2+}, either mineralised or water soluble, having a high K^{+}/Ca^{2+} ratio, or as free Ca^{2+} found in plants termed calciotrophs of which *Brassicaceae* is one. Instead of forming CaC_2O_4 crystals in response to excess Ca^{2+}, increases in water soluble Ca^{2+} compounds are found within vacuoles (Kinzel 1982), as Ca^{2+} is utilised as a counter cation for both inorganic and organic anions. This appears to be in line with the results obtained in section 6.5 which looked at the relative solubility of Ca^{2+} in whole seed, coat and embryo. Most of the Ca^{2+} extracted in the *B. rapa* embryo tested was either water soluble or ionically exchangeable (Tables 6.3 and 6.4). Ca^{2+} deposited outside the cytoplasm and organelles is also found in cytoplasmic membranes and the cell walls where it provides a building block for cell walls and a basic latticework of pectin. Considerable amounts are also found as $CaCO_3$ (calcium carbonate) and several *Brassicaceae* are known to contain $CaSO_4$ and $CaCO_3$ (Kinzel 1989).

In the cytoplasm, Ca^{2+} has been shown to be involved in cell message signalling in a number of pathways for different environmental stimuli. Ca^{2+} appears to have roles in signalling changes in cell metabolism in response to temperature, osmotic salinity stress and the regulation of other elements through cation channels and changing conformation of protein and enzymes and hence their activity, which may make such cell signalling processes a candidate for the negative response to salinity under increasing Ca^{2+} concentration at a cellular level during seed germination which was demonstrated by the results of analysis of correlation of seed $[Ca^{2+}]$ and $[Ca^{2+}] + [K^{+}]$ and EC_{50} from both the multi-substrate and hydroponic growth trials. Regulation by other elements may account for the results of seed K^{+}/Ca^{2+} with mother plant nutrition, which was shown to be significantly higher for low Ca^{2+} nutritional treatments in the multi-substrate and hydroponic growth trials.

A number of studies have investigated the role of Ca^{2+} at the cellular level under environmental stress during plant developmental stages. Intracellular Ca^{2+} is found primarily within vacuoles, endoplasmic reticulum (Dewald *et al*. 2001, Pauly *et al*. 2001) and also mitochondria, chloroplasts and the nucleus (Knight 2000). Since Ca^{2+} is immobile in the phloem, it is delivered to the plant via Ca^{2+} permeable channels in plasma membranes and the xylem and transverses the root via the symplast (via cytoplasm) or apoplastic (between cells) pathway. A wealth of literature discusses the fluctuations in concentration and role of Ca^{2+} in the cytoplasm in response to various external stimuli (e.g. Hedrich, Busch, and Raschke 1990, Ward and Schroeder 1994, Ward, Pei, and Schroeder 1995, Knight, Trewavas and Knight 1997, Cessna, Chandra, and Low 1998, Blumwald 2000, Kiegle *et al*. 2000, Knight 2000, Dewald *et al*. 2001, Pauly *et al*. 2001, Moore *et al*. 2002, White and Broadley 2003).

Given the results of the multi-substrate growth trial that found no direct effect of mother plant nutrition on salinity tolerance, but negative relationships with seed Ca^{2+}, K^{+} and Ca^{2+}+K^{+}, there are a number of potential avenues, some of which may be interlinked, by which an increase in embryonic, and therefore most probably, intracellular Ca^{2+}, could cause a negative impact upon cell growth and the potential to germinate. They are outlined as follows:

1. Growth of a mother plant in excessive Ca^{2+} may have a direct negative effect upon the ability of resultant seed to germinate, which is not necessarily controlled by the seed itself.
2. Excess Ca^{2+} is taken up and distributed in higher quantities to seed via the xylem, where it has direct cytotoxic effect. (i.e. the combining of Ca^{2+} with other molecules exerts a toxic effect). Ca^{2+} at concentrations higher than those normally found in organelles or cytoplasm may combine with negatively charged molecules and may exert a toxic effect (Reddy *et al*. 2011) and this may either be through the formation of a toxic compound or by reducing the mechanism of which the negatively charged molecule was involved.
3. Excess Ca^{2+} is taken up and stored in intracellular stores within the cells of a developing seed. Signal transduction in response to drought and salt stress at germination, causes a toxic influx of Ca^{2+} into the cytosol.
4. Excess Ca^{2+} is taken up and affects the activity/absorption/release of other mineral ions important in the germination process, especially perhaps K^{+} or organic molecules.
5. Excess Ca^{2+} is uptaken, but because it is stored intracellularly, permeation of Na^{+} via voltage independent cation channels (VICs) is not inhibited as it would have been by elevated extracellular Ca^{2+} (Blumwald 2000).

6. An increase in Ca^{2+}, through its affinity as a permeable counter cation, gives rise to increased Cl^- uptake in low Cl^- phloem fed tissues such as seeds (Xu *et al*. 2000), due to a high affinity of Cl^- uptake in plants deprived of Cl^- (Lee 1982), which then directly exerts a toxic effect on a developing embryo.

A high Ca^{2+} concentration of the cytosol ($[Ca^{2+}]_{cyt}$), which is above low micromolar levels is toxic to cells (Knight 2000, Broadley *et al*. 2003, White and Broadley 2003), with a prolonged increase in $[Ca^{2+}]_{cyt}$ being lethal. Sustained high $[Ca^{2+}]_{cyt}$ is implicated in apoptosis during normal development and hypersensitive responses to pathogens (Levine *et al*. 1996 in White and Broadley 2003). Excessive Ca^{2+} in the rhizosphere may cause plants to suffer Ca^{2+} toxicity, which may reduce plant growth rate and more importantly in the context of this study, prevent the germination of seed (White and Broadley 2003). The cytotoxicity of Ca^{2+} has been directly implicated in causing cell damage through cold-stress induced high Ca^{2+} levels (Minorsky 1985), although the direct effect may be still possible, the view is less popular (Knight 2000). It has also been suggested that this pathological effect of toxic ion cell entry has evolved into a basis of a signalling system (Knight 2000). Sanders, Brownlee, and Harper (1999) also propose that the evolution of a maintenance of $[Ca^{2+}]_{cyt}$, is borne out of the fact that Ca^{2+} combines with PO_4^{2-} to form Ca_2PO_4 which is not particularly soluble, and that cells would need a lower $[Ca^{2+}]_{cyt}$ than $[Ca^{2+}]$ of seawater. This therefore required a mechanism to remove Ca^{2+} from the cytosol, which has subsequently evolved into a signalling system. The same authors also noticed that Ca^{2+} can co-ordinate six to eight uncharged oxygen atoms (Katz *et al*. 1996) which may have by chance made the evolution of proteins that change conformation upon binding Ca^{2+} possible, thus initiating a signal (Garza, Vega, and Soto 2006). Since cells are sensitive to any long terms or significant elevations in

$[Ca^{2+}]_{cyt}$, any increases must be small or transient (White and Broadley 2003). Bulk cell Ca^{2+} is quite different to that of changes in Ca^{2+} involved in signalling processes (Ottow *et al* 2005), but fundamentally any pathway by which there is an increase in $[Ca^{2+}]_{cyt}$ may potentially be toxic, whether from a toxic influx during stress signalling from intracellular stores, or via less ideal conditions in the cytosol prior to stress taking place.

Maintenance of low submicromolar $[Ca^{2+}]_{cyt}$ is achieved normally by Ca^{2+}ATPases and H^+/Ca^{2+} antiporters which remove Ca^{2+} to the apoplastic pathway or to intracellular storage such as the vacuole or endoplasmic reticulum. These enzymes are also responsible for restoring $[Ca^{2+}]_{cyt}$ following any increases in the cytosol and removing any other divalent cations from the cytosol (White and Broadley 2003).

A variety of stresses can initiate intracellular signalling that involves Ca^{2+}. Chilling, freezing, osmotic stress, salinity, oxidative stress, anoxia, heat and mechanical stress are examples of the wide range of environmental stimuli that appears to have an effect on $[Ca^{2+}]_{cyt}$ (Knight 2000), that acts as a signal to initiate metabolic processes that enable a plant to resist stress and is essential for producing a physiological response. Szczerba, Britto, and Kronzucker (2009) discuss Ca^{2+} signalling as fundamentally central to ion sensing in plants, where an initial elicitor causes secondary reaction that may involve other molecules such as calmodulin, the activation/deactivation of other ion transporters or processes that lead to modification of gene transcription. The response generally involves an increase in $[Ca^{2+}]_{cyt.}$, caused by an influx of Ca^{2+} into the cytosol from the the apoplastic pathway, across the plasma membrane or from primary intracellular stores such as the vacuole and endoplasmic reticulum. The influx into the cytosol is regulated by Ca^{2+} permeable ion channels and the type, location

and abundance will influence the spatial notation of the signal and targeting of different cellular responses, since the local increase dissipates quickly after the channel has closed (White and Broadley 2003). Changes in $[Ca^{2+}]_{cyt}$ are often biphasic waves and it has been suggested that initial influxes of Ca^{2+} into the cytoplasm might produce secondary messengers such as inositol triphosphate (IP_3) that activate other Ca^{2+} channels (Trewavas 1999) that are dependent upon IP_3. Knight, Trewavas and Knight (1997), using inhibitors of inositol suggested that under mannitol induced osmotic stress, the elevation in $[Ca^{2+}]_{cyt}$ was due to Ca^{2+} release from the vacuole via IP_3 dependent channels. This is has also been suggested by Alexandre, Lassalles, and Kado (1990) and Allen, Muir, and Sanders (1995). Moore *et al*. (2002) also concur that Ca^{2+} release from intracellular stores greatly contributes to an increase in $[Ca^{2+}]_{cyt}$ when root endodermal cells are subjected to salt stress and that the $[Ca^{2+}]_{cyt}$ changes follow a biphasic pattern where $[Ca^{2+}]_{cyt}$ increases immediately following exposure and then has a second prolonged exposure, where in part the increase in $[Ca^{2+}]_{cyt}$ is due to a release of Ca^{2+} from IP_3 dependent vacuolar stores. Working with *A. thaliana* root cells, Moore *et al*. (2002) also found that salt stress caused elevations in suberised endodermal cells, concluding that at least in this instance, Ca^{2+} influx would be unlikely from the apoplastic pathway and Ca^{2+} does not influx across the plasma membrane strongly suggesting that the increase in $[Ca^{2+}]_{cyt}$ due to salt stress must originate from intracellular stores. The authors also reason this theory by pointing out that Ca^{2+} channels that are found in the plasma membrane of the root cells are non selective between cations and permeable to Na^+ and since root cells must endeavour to resist Na^+ influx, the contribution of Ca^{2+} via the plasma membrane may be limited. Blumwald (2000) also suggests that this is the case and the similarity of the hydrated ionic radii of Na^+ and K^+ makes it difficult to

distinguish between them (Schachtman and Liu 1999, Blumwald 2000, Kaya *et al*. 2007, Zhang, Flowers, and Wang 2010), which may account for some of the results found in this study in terms of the antagonistic role of seed K^+ levels in decreasing salinity tolerance highlighted by the multi-substrate growth trial. However, uptake of externally supplied Ca^{2+} over Na^+, that has been shown by much literature to alleviate salt stress at the whole plant level, does suggest that Ca^{2+} is selected preferentially over Na^+ to some degree. Roberts and Tester (1997a) using *Z. mays* (maize) root cortical protoplasts, found that increasing extracellular Ca^{2+} inhibited channel activity carrying Na^+ and decreased up to 50% of cell Na^+ dependent current under Na^+ stress, indicating that there are both Ca^{2+} sensitive and Ca^{2+} insensitive components to Na^+ influx into a cell. There is also evidence that $[Ca^{2+}]_{cyt}$ is implicated in affecting the abundance and functionality of aquaporins under saline stress. Martinez-Ballesta *et al*. (2008) report that an increase in $[Ca^{2+}]_{cyt}$ associated with stress perception may cause the aquaporins to close, lowering water permeability in root protoplasts and plasma membrane vesicles in *C. annum* (pepper) and that extra Ca^{2+} could lead to an upregulation of aquaporin activity that could help maintain optimum apoplastic concentration of Ca^{2+} required for transport of water through the plasma membrane, giving rise to suggestion that regulation between the activities could lead to fine adjustment of aquaporin activity. The authors explain that this would account for the reduction in $[Ca^{2+}]_{cyt}$ by NaCl exposure and how externally applied Ca^{2+} would counteract this effect, which is such a predominant finding across literature.

Drought and high salt is known to also induce expression of other Ca^{2+} dependent biochemical factors including Ca^{2+} dependent protein kinases (CDPK) (Wimmers, Ewing and Bennett 1992), salt stress induces expression of Ca^{2+}ATPase (Perez-Prat *et al*. 1992) and osmotic shock induces Ca^{2+} binding proteins (Ko and Lee 1995) such as

calmodulin which are activated by an influx of Ca^{2+} into the cytosol (Kinzel 1989), although it should be remembered that some stresses may overlap with salt stress causing both a direct toxic effect of Na^{+} and Cl^{-} as well as an osmotic stress. Previously mentioned biosynthesis of proline by (Δ^{1}-pyrroline-5-carboxylate synthetase), encoded by gene *At-P5CS* (Savouré *et al*. 1995) which is induced by drought and salinity stress has been shown, in part at least, to be regulated by intracellular Ca^{2+} via signalling pathways (Knight, Trewavas, and Knight 1997). The authors also suggest that an influx of external Ca^{2+} is important, but that internal Ca^{2+} is required for full gene expression as well as other signalling factors. This may be a predominant factor in the differences in results found during these studies in terms of a collaborative effect of decreasing salinity tolerance with $[Ca^{2+}]$ and $[K^{+}]$, and those prevalent in literature which have found a primarily positive salinity tolerance response to externally supplied Ca^{2+}. The stress response may not always be primarily directly regulated by Ca^{2+} itself, which then produces a secondary elicitor, but in some cases can involve an intermediate response to initiate change in $[Ca^{2+}]_{cyt.}$. ABA levels, for example, greatly implicated in stress response (section *1.2 Salinity Tolerance*), causes the increase in $[Ca^{2+}]_{cyt.}$ which triggers stomatal closure in response to drought stress (Ward and Schroeder 1994), albeit through non selective Ca^{2+} channels, indicating a close coupling of ABA receptors with the ion channels (Ward, Pei, and Schroeder 1995). Further work has indicated a role of IP_3 in the signalling chain, with an increase in ABA, causing an increase in IP_3 (MacRobbie 1992), which as discussed, may well have a role in Ca^{2+} release from intracellular stores.

7.3 Salinity and Intracellular Movements of Potassium and their Potential for Governance by Calcium

7.3.1 Plasma Membrane Potassium Channels

There is some involvement of K^+ channels in the mediation of responses to drought stress in plants (Knight, Trewavas, and Knight 1997) and tolerance to salt may be regulated by the uptake of K^+ (Watad *et al*. 1991), rather than a change in or initial endogenous levels. Watad *et al*. (1991) also states that uptake of K^+ in *N*. *tabacum v. gossii* (tobacco) by plasma membrane H^+-ATPases with enhanced capacity for K^+ transport may appear to confer tolerance, perhaps by not affecting intracellular levels directly, but by providing an ability to be able to regulate intracellular levels. Between salt adapted and non adapted cells, Watad *et al*. (1991) found no differences in intracellular K^+. Increases in seed K^+ concentration were shown to confer lesser salt tolerance (EC_{50}) in both the multi-substrate trial and seed of Ø <1.0 mm in the hydroponic trial (sections *5.3.3.3* and *5.4.3.3 Seed Ion Concentration Correlation with Salinity Tolerance*), which if the above may be true, may infer that the relationships found between cell K^+ and salinity tolerance is purely circumstantial and that the levels of indigenous K^+ in a seed may not necessarily have a bearing upon the measured EC_{50}. It may be that a seed's affinity for K^+ uptake and regulation during salt stress is what determines its salinity tolerance. Therefore, a measurement of K^+ concentration in presalinised seeds may not necessarily be a good indicator of salinity tolerance *in vivo*.

Since halophytic enzymes are not adapted to high salt levels and display the same sensitivity as enzymes from glycophytes, plants must respond by being able to maintain low Na^+ concentrations and a high cytosolic K^+/Na^+ ratio. Strategies include

Na^+ extrusion or compartmentalisation mainly within the vacuole (Blumwald 2000). An example of Na^+ exclusion/extrusion is a K^+ transporter, HKT1, which has been found to be Na^+ coupled. At toxic millimolar concentrations of Na^+, HKT1, has a low affinity uptake for Na^+, which blocks the uptake of K^+ (Gassman, Rubio, and Schroeder 1996, Roberts and Tester 1997a) and Szczerba, Britto, and Kronzucker (2009) suggest that these transporters may be much more important in Na^+ uptake by plants. K^+ outward rectifying channels (KORCs), which could mediate the efflux of K^+ and influx of Na^+, have been shown to have high selectivity of K^+ over Na^+ in barley roots (Wegner and Raschke 1994), but also a lower K^+/Na^+ in *Arabidopsis* root cells (Maathius and Sanders 1995). KORCs have also been shown to be permeable to Ca^{2+} (Roberts and Tester 1997b), which could suggest that the opening of KORCs could result in an increase in cytosolic Ca^{2+} which could trigger activation of other ion channels within the cell. These channels also show a large dependence on $[Ca^{2+}]_{cyt.}$ (Ketchum *et al*. 1995), so may effectively be a Ca^{2+} inducible, Ca^{2+} release mechanism. The initiation of channel opening due to Ca^{2+} itself has been questioned and it appears that this is not the case and Ca^{2+} is electrically silent to these channels (Gilliham *et al*. 2006). Blatt and Armstrong (1993) have also shown that K^+ efflux can be mediated by KORCs in the plasma membrane and KORC currents can be regulated by ABA which may be linked to an intracellular Ca^{2+} release. Similar voltage gated channels termed NORCs (non selective outward rectifying conductance), that may act concurrently with KORCs, do not discriminate between cations and are activated by increased $[Ca^{2+}]_{cyt.}$ (de Boer and Wegner 1997). These could play a role in the efflux of Na^+ via indirect Ca^{2+} signalling.

K^+ inward rectifying channels (KIRCs) may also have a relationship with intracellular Ca^{2+}, with the channel's inhibition mediated by IP_3 (Blatt, Thiel, and Trentham 1990)

and an ABA induced intracellular Ca^{2+} release (Lemtiri-Chlieh and MacRobbie 1994). A mechanism involving a KIRC named AKT1 has been elucidated and is shown to rely upon cytosolic Ca^{2+}. AKT1 interacts with with a protein kinase (CIPK23) that activates the channel by phosphorylation and is targeted by calcineurin B-like Ca^{2+} sensors (CBL1 and CBL9) that are originally activated by Ca^{2+}. Unfortunately the mechanism of activation of the Ca^{2+} signal to low K^+ is unknown (Li *et al*. 2006, Lee *et al*. 2007 and Xu *et al*. 2006). Such channel mechanisms may have a role to play in a response to salinity and although related in this case to extracellular Ca^{2+}, is indicated by the previously cited statements of Epstein (1961) and Läuchli and Epstein (1970), that Ca^{2+} is known to be necessary for maintenance of K^+ transport and supplemental Ca^{2+} has also been found to increase the uptake and transport of K^+ (Cramer, Läuchli, and Polito 1985, Cramer *et al*. 1987, Grieve and Fujiyama 1987, Nakamura *et al*. 1990, Subbarrao *et al*. 1990, Hawkins and Lewis, 1993a). Zhong and Läuchli (1993) also suggested that supplemental Ca^{2+} maintains K^+/Na^+ exchange and selective K^+ uptake of the plasma membrane, which is one of the primary cellular sites of action of Ca^{2+}. Although, there is no conclusive evidence to suggest that extracellular Ca^{2+} may have a direct bearing on those mechanisms which have been found to be governed solely by intracellular Ca^{2+}, it is interesting that the observations of relationships between Ca^{2+} and K^+ seen at the external level may apply intracellularly through the mechanisms of cation channels and intrinsic levels of Ca^{2+} and K^+.

7.3.2 Vacuolar K^+ Channels

Cell vacuole K^+ may also have a role in maintaining turgor and osmotic pressure (Maathius *et al*. 1997), which a lack of, is quite symptomatic of non woody plant

species struggling to cope with saline environments. K^+ release from the vacuole into the cytoplasm has been proposed to be controlled by K^+ channels and a number are thought to exist which also have a dependence on $[Ca^{2+}]_{cyt}$. The first type is slow activated vacuolar (SV) channels, which transport K^+ into the vacuole with other monovalent cations along with Mg^{2+} and Ca^{2+}. Indeed, these have also been proposed to play a role in Ca^{2+} mediated Ca^{2+} release, in this case from the vacuole (Ward and Schroeder 1994, Ward, Pei, and Schroeder 1995) since a rise in $[Ca^{2+}]_{cyt}$. has been proposed to open and promote SV channel activity (Maathius *et al*. 1997, Hedrich and Neher 1987), possibly through ABA induction or IP_3 induced Ca^{2+} release (Ward, Pei, and Schroeder 1995).

The second type of channel is vacuolar K^+ (VK) channels. These are highly selective for K^+ and are normally active at physiologically normal $[Ca^{2+}]_{cyt.}$ (Ward and Schroeder 1994, Ward, Pei, and Schroeder 1995). H^+-ATPases drive the vacuolar K^+ release, but depend on the presence of cytosolic Ca^{2+}. It has also been proposed that the Ca^{2+} dependent K^+ flux through VK channels from the vacuole to the cytoplasm may activate SV channels (Ward and Schroeder 1995).

The third type of channel is fast vacuolar (FV) channels which is not particularly selective among cations, but opens at low $[Ca^{2+}]_{cyt}$, is inactivated at by high $[Ca^{2+}]_{cyt.}$ (Hedrich and Neher 1987) and transports cations from the vacuole to the cytoplasm. It has also been hypothesised that these channels, while essentially dependent on low $[Ca^{2+}]_{cyt.}$, could allow for Ca^{2+} independent K^+ release (Allen and Sanders 1996). Both SV and FV channels have been seen to take prominence during low and high $[Ca^{2+}]_{cyt.}$ respectively in sugarbeet storage tissue vacuoles, pointing to the fact that the channels exist independently or are profoundly different in form, rather than a

conversion that is dependent upon $[Ca^{2+}]_{cyt.}$ (Hedrich and Neher 1987). The authors also found Ca^{2+} dependence of vacuolar channels in photosynthetic and phototrophic tissue and hypothesise that this mechanism may therefore be a general mechanism for the regulation of ion transport in plant vacuoles.

Hedrich, Busch, and Raschke (1990) also support the idea that K^+ channels may be governed, at least in part, by $[Ca^{2+}]_{cyt.}$. K^+ release through channels of the plasma membrane in guard cells was shown to be elicited by a rise in $[Ca^{2+}]_{cyt.}$, together with the presence of nucleotides, which can also be implicated in the control of other ion channels. K^+ channels may also be regulated by Ca^{2+} interaction with guanine nucleotide-binding proteins (Kelly, Esser, and Schroeder 1995).

7.3.3 Identification of Mechanisms by Genetic Mutation

Work with salt hypersensitive mutants with *Arabidopsis* has also identified a genetic basis of salt tolerance in glycophytes that is linked to both Ca^{2+} and K^+. *SOS* (salt overly sensitive) genes, which include as examples here, *SOS1* (Wu, Ding, and Zhu 1996) and *SOS3* (Liu and Zhu 1997, Ishitani *et al*. 2000). Loci mapped to chromosomes II and V respectively, share phenotypic traits regarding growth under salt and low K^+. Both *SOS* loci appear to confer greater sensitivity to Na^+ and an inability to grow on media containing low levels of K^+. Wu, Ding, and Zhu (1996) showed that very low levels of K^+ (<1 mM) caused lack of growth and that the *SOS1* mutants do not have high affinity K^+ uptake and that the plants become deficient in K^+ when treated with NaCl, thus indicating that the *SOS1* gene encodes for a high K^+ affinity uptake system, possibly a K^+ transporter, that is required, at least in part, for an increase in salt tolerance. Shi *et al* (2003) found that the overexpression of the

gene, which was found to encode a Na^+/H^+ plasma membrane antiporter that improved salt tolerance of seeds during germination, and of calli, which accumulated less Na^+. High affinity K^+ uptake itself may also be inhibited by Na^+, by causing a reduction in abundance or activity of transporters (Szczerba, Britto, and Kronzucker 2009).

The *SOS3* mutant has been identified as having a relationship with extracellular Ca^{2+} levels. Liu and Zhu (1997) found that an increase in extracellular Ca^{2+} relieved the poor growth of *SOS3* on low K^+ or 50 mM NaCl, but this was not the case with the aforementioned *SOS1* mutants. *SOS3* mutants were found to accumulate Na^+ to a greater extent than the wild-type and less K^+ than the wild-type but extracellular Ca^{2+} increased selectivity for K^+ over Na^+ by more than twice as much. Ishitani *et al*. (2000) demonstrated *SOS3* to be a unique Ca^{2+} binding protein and that the mutation reduced the capacity of *SOS3* to bind Ca^{2+} and interact with an affliated serine/threonine protein kinase (*SOS2*) (Bartels and Sunkar 2005), indicating that Ca^{2+} binding is required for *SOS3*'s role in salt tolerance mechanisms and more importantly it may have a role to play in regulating uptake of K^+. Indeed, Bartels and Sunkar (2005) state that molecular interaction and complementation analysis indicates that *SOS3* is required for activation of *SOS2* that regulates *SOS1* transcription. This may occur at the plasma membrane in order to interact with extracellular Ca^{2+} levels, since Na^+ displaces plasma membrane Ca^{2+}, leading to an inability to maintain intracellular K^+ levels (Rains 1972, Watad *et al*. 1991). An overexpressed Ca^{2+} binding mechanism may therefore result indirectly in an ability to better regulate intracellular K^+.

7.4 Conclusions and Suggestions for Further Work

7.4.1 Physiological Aspects

Discounting some of the results obtained for the developmental data in the preliminary growth trial using modified Hoagland's Solution A due to a lack of data, the other growth trials showed very few differences in the developmental traits tested amongst the different treatments, suggesting that plant growth and reproductive cycle in this instance was not affected by the nutritional treatment. This suggests the application of nutritional treatment did not adversely affect the growth and development of mother plants. Germination was unaffected by treatment also. In terms of seed number, nutritional treatment and substrate was shown to have an effect in the pot grown trials but this was not the case in trials using hydroponic conditions. The hydroponic growth trial did, however, highlight the fact that overall seed yield (number and weight) appears to be unaffected by nutritional treatment, implemented only by solutions of different nutritional treatment not influenced by substrate chemistry or structure, and that *B. rapa* tends to produce a greater number of smaller seed per plant over all treatments (Table 4.12), but the total seed mass produced between the two size categories examined here was not significantly different (Table 4.13). Taking into account the relative lack of physiological effect in varying ion content, there is potential scope to alter the nutritional treatment and hence response of nutritional loading to seed sinks may exist. Such an approach would hope to accentuate any differences that nutritional treatment may infer to salt tolerance also. and is perhaps an avenue to be explored further as discussed in section 7.4.3.

7.4.2 Ionic Relations and Salinity Tolerance

Altering seed ion concentration by manipulating levels available in the growth substrate was possible. Discounting the data gained from preliminary growth trials in sand, due to lack of replication, mother plant nutritional treatment had a significant effect on the Ca^{2+} concentration of seed from plants grown in sand in the multi-substrate growth trial where high Ca^{2+} and low K^{+} mother plant treatments gave the highest seed Ca^{2+} (Table 5.3) and over the three substrates used, there was a significant difference in the Ca^{2+} in seed between low Ca^{2+} and high Ca^{2+} treatments. In the hydroponic trial, variation in seed ion concentration appeared more pronounced in smaller Ø <1.0 mm seeds. In these seeds, high Ca^{2+} treatment produced significantly greater Ca^{2+} concentration than low Ca^{2+} fed mother plants. Considering data over both Ø 1.0 - 1.4 mm and Ø <1.0 mm seed, this was still the case. Seed K^{+} concentration appeared not to be affected to the same degree. Within each type of growth substrate in the multi-substrate growth trial, there was no difference in seed K^{+} concentration between treatments and consequently no effect over the three types of substrate used. This was similarly mirrored by the data found by the hydroponic growth trial. Within each size category there was no effect of treatment on seed K^{+} concentration and consequently, again, no overall effect over both size categories. K^{+}/Ca^{2+} ratios were also significantly greater in low Ca^{2+} treatments over high Ca^{2+} treatments in both the multi-substrate and hydroponic growth trial. Ca^{2+} was clearly more manipulatable in daughter seed than was K^{+}. The degree to which different seed Ca^{2+} concentrations could be induced by mother plant nutritional regime was significant between high Ca^{2+} and low Ca^{2+}, and high Ca^{2+} and high K^{+} treatments. Differences in growth substrates used in the multi-substrate growth trial and lack of substrate in the hydroponic growth trial had a bearing on the amount of both Ca^{2+} and

K^+ deposited in the seed. Ion availability and potential to increase biomass through uptake of other nutrients may have had an effect when using various growth substrates, whereas the data from using hydroponic culture may be more dependable as this experimental variable was removed.

The salinity tolerances of manipulated seed appeared to differ between the multi-substrate and hydroponic growth trial in terms of the nutritional treatment applied. The multi-substrate trial showed no statistically significant effect of differing nutritional treatments in terms of affecting daughter seed EC_{50}, but since increases in the measured amounts within seed of Ca^{2+}, K^+ and Ca^{2+}+ K^+ produced negative relationships with EC_{50}, it may be important to recognise that growing mother plants in the substrates chosen may not influence EC_{50} directly at least in relation to seed Ca^{2+} and K^+. However, factors such as other substrate chemistry and substrate structure, giving rise to effects of other ions and their uptake, along with factors which could affect delivery to developing seeds, may be of consequence in terms of the role of Ca^{2+} and K^+ are perceived to have in a response to salinity. The hydroponic growth trial, considering both seed size categories together, gave rise to a high Ca^{2+} treatment that produced seed with a significantly higher EC_{50} than low Ca^{2+} and control treatments. Smaller seed had a significantly lower salinity tolerance, which is an expected outcome, which may infer that embryonic reserves may be a factor in salinity tolerance at germination. When seed ion concentration is correlated with EC_{50} for the multi-substrate trial, and hydroponic trial when correlating EC_{50} regardless of seed size, there was a significant negative relationship with Ca^{2+}, K^+ and Ca^{2+}+ K^+. This infers that for both trials, as the levels of the two ions increases, salinity tolerance decreases. However, in all cases the R^2 value was poor, indicating a

relatively large scatter of data around any trendline and making any prediction of salinity tolerance from ion concentration untenable.

Further work to try and elucidate the localisation of ions within seed, the nature of the Ca^{2+} and K^{+} content and hypothesising potential mechanisms of ion movement under saline stress yielded interesting data. Staining seed cross sections with GBHA identified the coat as a major sink of Ca^{2+} and separation of coat and embryo for quantitative analysis of seed produced under the different nutritional regimes, showed that seeds produced under high Ca^{2+} treatment had a significantly greater amount of Ca^{2+} in their coat compared with low Ca^{2+} treatment. Analysis for K^{+} interestingly showed that coat K^{+} in seed produced via low Ca^{2+} treatment, was significantly higher than seed coats tested from any other treatment. Analysis of embryos produced no differences between treatments for either Ca^{2+} or K^{+}, but the relative amounts of Ca^{2+} and K^{+} per gram of seed highlighted that Ca^{2+} appears to be more concentrated in the coat per gram of tissue as opposed to the embryo. The reverse was true of K^{+}, which was shown to be prevalent in the embryonic tissue rather than the coat.

The solubility of the mineral elements in question was measured in an attempt to understand the degree of mobility of seed reserves that exist in differing tissue types, given their disproportionate distribution over the whole seed. In terms of Ca^{2+}, 74% of what exists in the embryo and 12% of what exists in the coat was soluble in water at 80°C. The solubility of K^{+} was much greater with 94% soluble in the embryo and 82% soluble in the coat. From this data, the embryonic tissues appear to have a role in any potential response to salinity and osmotic stress, more so than coat in terms of Ca^{2+}. Further solubility extractions with NaCl, HCl and CH_3COOH indicated that Ca^{2+} in embryonic tissue is primarily soluble by H_2O and then NaCl, while in coats,

most is extractable by NaCl, followed by H_2O. This indicated that although Ca^{2+}, which has heavy deposits in the seed coat, is insoluble by H_2O, it is extracted by NaCl which according to Kinzel (1989) is indicative of Ca^{2+} that is mostly exchangeable and is electrostatically absorbed onto cell walls or are part of polyanions in vacuoles, so therefore may actually have a part to play in the mobilisation of reserves *in vivo*.

The potential mechanisms of salinity tolerance and the wealth of literature that discusses the preceding cellular signalling does implicate a pivotal role for Ca^{2+} and/or K^+. Indeed, an increase in either cation or a relationship between the two, since K^+ channels have been implicated in changing $[Ca^{2+}]_{cyt}$, which may be site specific within the cell, may induce an effect upon the primary mechanisms of cellular signalling. However, the magnitude of possible interaction of Ca^{2+} and K^+ with other cellular species is vast, with the scaling of signal initiation affecting the degree of signal initiation, and may be different between different cell types making common mechanisms difficult to distinguish. Other influences such as nutritional loading of mineral ions other than Ca^{2+} and K^+ to the seed during development may constitute an effect, as perhaps would the accumulation, or lack of, certain organic molecules. A true Ca^{2+} or K^+ initiated response may also be accompanied by other signalling pathways which could amplify or counter one another and affect the final combined response outcome in terms of salinity tolerance. The pathway may also be subject to the coincidental control of certain responses via $[Ca^{2+}]_{cyt}$ perturbations not eliciting only response specific signalling (Trewavas 1999). As also suggested by Trewavas (1999), a network of $[Ca^{2+}]_{cyt}$ signalling pathways may be subject to a number of facets which may affect the specific intended physiological outcome. For example, the spatial separation of pathway cellular components relies upon the location of those components in the successive transport of signals. Additional stresses may modify the

ability of signal transduction to take place because the secondary stress factor has affected the spatial separation or abundance of components that are involved the pathway. In a similar manner, a $[Ca^{2+}]_{cyt}$ response to a particular stress may be prevented or be non effective due to a lag period whereby a previous stress has elicited a previous different $[Ca^{2+}]_{cyt}$ signal. The results presented are not conclusive but do highlight a difference in seed response to intracellularly stored seed Ca^{2+} as opposed to extracellularly applied Ca^{2+} during germination and that mother plant growth conditions may have a strong effect on mineral transduction to seed which may consequently affect salinity tolerance either directly or via salinity induced response pathways. Definitive mechanisms may not exist solely; they may interact and may be significantly different between difference species and subspecies and therefore it may make hypothesising a definitive mechanism in response to salinity complex and difficult, given the relatively small amount of information which is to be gained about this single subspecies.

7.4.3 Future Work

An increase in the range of Ca^{2+} and K^{+} given to mother plants would potentially lead to a greater difference between ion levels in seed produced by the nutritional treatments. If it would be possible to do this, without affecting the growth rate and physiological nature of the mother plant, it may allow for the production of seeds that have greater differences in Ca^{2+} and K^{+} that would enhance any potential response to salinity and the mechanisms involved. Given the growth physiology data from the multi-substrate and hydroponic trials, this would feasible, but would be carried out on a trial and error basis.

The measurement of extractable or possibly free Ca^{2+} and K^{+} from seed tissue and correlating these with EC_{50} for salinity tolerance may help to elucidate the effects of *in vivo* available ions rather than total elemental content. This would be of particular use since high Ca^{2+} treatment in the hydroponic growth trial appears to increase salt tolerance and low Ca^{2+} treatments across the multi-substrate and hydroponic growth trials show a higher K^{+}/Ca^{2+} ratio. The question of whether high Ca^{2+} treatment produces a proportionally greater increase in non-available Ca^{2+}, rather than free available ions that confer a higher salt tolerance as shown in the hydroponic growth trial, perhaps through hydroponic culture, would be an interesting idea regarding as to why an increase in total Ca^{2+} appears to confer significantly negative correlation with salt tolerance. Testing a range of seed sizes for both HNO_3 extractable and H_2O, NaCl, HCl and CH_3COOH extractable Ca^{2+} relative to whole seed in numerous replicate numbers may be able to confirm whether the data, which showed that Ca^{2+} distribution between coat and embryo between seeds of Ø 1.0 mm - 1.4 mm and Ø 1.4 - 1.7 mm differed very little (Table 6.5), could be significant. This may at least go on to investigate whether as seed diameter decreases and volume to surface area increases, solubility of Ca^{2+} of seed coat, compared to embryo, differs between seed size for various extractants and therefore if it may have some contributory role in salt tolerance.

The isolation of different types of embryonic cells and investigating movements of ions and opening of specific channels in differing levels of NaCl by techniques such as bioluminescent indicators (Knight, Trewavas, and Knight 1997, Cessna, Chandra, and Low 1998, Takahashi *et al*. 1999, Kiegle *et al*. 2000, Pauly *et al*. 2001, Moore *et al*. 2002) and patch clamp technique (Hedrich and Neher, 1987, Hedrich, Busch, and Raschke 1990, Ward and Schroeder 1994, Roberts and Tester 1997a, 1997b) would

perhaps provide a greater depth of information regarding the mechanisms of ion movement and cellular ion channel response of embryonic and seed coat tissue to salinity, since research in this area appears not to exist, yet the techniques involved appear to be well practiced. Microarray studies have also identified several hundred genes that are involved in response to drought or salt stress in plants (Bartels and Sunkar 2005), and analysis of the individual genes and their transcription may also be able to elucidate pathways in those species which have been heavily sequenced. Indeed, the recent acquisition of the *B. rapa* genome sequence (Wang *et al*. 2011) can only further the analysis of the genetic basis of salinity tolerance in *Brassicaceae*.

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Appendices

1. Analysis of Ion Content of Stock Seed and Commercially Purchased Seed

Table A1.1 - Calcium and Potassium Concentration of Commercially Purchased Seed

Element	Concentration ($\mu g\ g^{-1}$)
Ca^{2+}	3272
K^{+}	9392

Table A1.2 - Calcium and Potassium Concentration of Stock Seed

Element	Concentration ($\mu g\ g^{-1}$)
Ca^{2+}	964
K^{+}	11033

2. Levels of Exchangeable Calcium and Potassium in Various Growth Substrates

Five types of growth substrate were assessed for their levels of exchangeable Ca^{2+} and K^{+} in order to determine their suitability as relatively nutrient free substrate for the production of experimental seed.

Table A2.1 - Exchangeable Calcium Analysis in Five Types of Growth Substrate

Key: P = Peat
V = Vermiculite
VP = Vermiculite/Peat (1:1)
S = Horticultural Sand
PrS = Perlite/Horticultural Sand (2:1)

Sample	Replicate Average ($mg\ l^{-1}$)	Mean of Sample ($mg\ l^{-1}$)	Exchangeable Ca^{2+} ($Cmol_c\ kg^{-1}$)
P_1	32.81		
P_2	16.19		
P_3	17.15	22.05	5.50
V_1	54.34		
V_2	53.77		
V_3	54.04	54.07	13.48
VP_1	133.30		
VP_2	125.03		
VP_3	126.93	128.42	32.02
S_1	3.07		
S_2	3.34		
S_3	4.81	3.74	0.93
PrS_1	2.05		
PrS_2	2.26		
PrS_3	3.42	2.58	0.64

Table A2.2 - Exchangeable Potassium Analysis in Five Types of Growth Substrate

Sample	Replicate Average ($mg\ l^{-1}$)	Mean of Sample ($mg\ l^{-1}$)	Exchangeable K^{+} ($Cmol_c\ kg^{-1}$)
P_1	1.21		
P_2	0.68		
P_3	0.87	0.92	0.12
V_1	18.86		
V_2	18.52		
V_3	18.77	18.72	2.39
VP_1	13.26		
VP_2	13.22		
VP_3	13.39	13.29	1.70
S_1	0.44		
S_2	0.45		
S_3	0.14	0.34	0.04
PrS_1	1.31		
PrS_2	1.17		
PrS_3	1.25	1.24	0.16

The values of exchangeable Ca^{2+} and K^{+} for peat were deemed at this stage unreliable. Extraction with ammonium acetate proved very difficult with samples of dry peat; the sample floating without absorbing the ammonium acetate solution. A wetting agent may have proved beneficial. However, taking into consideration the exchangeable cation values found for samples of vermiculite and the vermiculite and peat mixture (Table A2.2 and A2.3), peat alone was identified as possessing levels of Ca^{2+} and K^{+} that were too high to be considered suitable as relatively nutrient free substrate for the production of experimental seed.

Table A2.3 - Exchangeable Cation Ratings (m.e.100 g^{-1} or $Cmol_c\ kg^{-1}$) Ca^{++}, K^{+}. In *Soils in the British Isles Chapter 1- Soil Genesis in Britain*. (Curtis, Courtney, and Trudgill, 1976). 55.

Rating	Ca^{2+}	K^{+}
Very High	20	1.2
High	10-20	0.8-1.2
Moderate	5-10	0.5-0.8
Low	2-5	0.3-0.5
Very Low	2	0.3

Table A2.4 - A Comparison of Exchangeable Cation Ratings (m.e/100 g or $Cmol_c\ kg^{-1}$) Ca^{++}, K^{+} for five types of growth substrate. In: *Soils in the British Isles Chapter 1- Soil Genesis in Britain*. (Curtis, Courtney, and Trudgill, 1976). 55.

	Ca^{2+}	Comment	K^{+}	Comment
Range	2 - 20		0.3 – 1.2	
Peat	5.50	Low-Moderate	0.12	Very Low
Vermiculite	13.48	High	2.39	Very High
Vermiculite/Peat (1:1)	32.02	Very High	1.70	Very High
Horticultural Sand	0.93	Very Low	0.04	Very Low
Perlite/Horticultural Sand (2:1)	0.64	Very Low	0.159	Very Low

Disregarding the ratings produced for peat, the ratings of exchangeable Ca^{2+} and K^{+} for vermiculite and the vermiculite/peat mixture were found to be high and very high

respectively (Table A2.4). Horticultural sand, having very low base levels of exchangeable Ca^{2+} and K^{+}, was chosen as a preferred medium in which to carry out the growth experiments when applying nutrient solutions containing known concentrations of Ca^{2+} and K^{+}.

3. Producing Modified Hoagland's Nutrient Solutions A and B

The full strength modified Hoagland's solutions A and B were tested to be certain of their exact content in terms of Ca^{2+} and K^{+}. Na^{+} levels were also tested due to its addition as NaOH, which was used to raise the pH to around 5.7/5.8. The levels of Ca^{2+} and K^{+} in relation to the control solution shown in Tables A3.1 and A3.2 correspond well to the desired levels that were required, proving that the solutions were prepared correctly and as accurately as possible. The levels of Na^{+} are fairly constant throughout the range of solutions and show very low levels.

Table A3.1 - Calcium, Potassium and Sodium Concentration (mg l^{-1}) in Modified Hoagland's Solution A.

Sample	Ca^{2+}	K^{+}	Na^{+}
Control	222	281	12
Low Ca^{2+}	52	275	9
High Ca^{2+}	944	276	13
Low K^{+}	227	66	11
High K^{+}	212	1033	10

Table A3.2 - Calcium, Potassium and Sodium Concentration (mg l^{-1}) in Modified Hoagland's Solution B

Sample	Ca^{2+}	K^{+}	Na^{+}
Control	222	253	7
Low Ca^{2+}	96	202	5
High Ca^{2+}	411	250	8
Low K^{+}	202	92	5
High K^{+}	225	490	4

4. Preliminary Growth Trial Using Sand and Modified Hoagland's Solution A Plant Developmental Statistical Data

Table A4.1 - Germination

Analysis of Variance for Asin Ger, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	1190.2	1190.2	297.5	1.24	0.347
Row_2	4	195.4	195.4	48.8	0.2	0.932
Column_2	4	3453.8	3453.8	863.4	3.59	0.038
Error	12	2885.7	2885.7	240.5		
Total	24	7725.0				

Table A4.2 - % Flowering

Analysis of Variance for Asin Fl%, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	10161.3	10161.3	2540.3	28.42	<0.001
Row	4	966.3	966.3	241.6	2.70	0.081
Column	4	1206.8	1206.8	301.7	3.38	0.045
Error	12	1072.5	1072.5	89.4		
Total	24	13406.8				

Table A4.3 - Mean Days to Flower

Analysis of Variance for Days to, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	365.55	356.43	89.11	6.14	0.001
Row	4	102.13	137.47	34.37	2.37	0.068
Column	4	83.33	83.33	20.83	1.43	0.239
Error	43	624.35	624.35	14.52		
Total	55	1175.36				

Table A4.4 - Flowers per Flowering Plant

Analysis of Variance for Days to, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	4475.8	4492.9	1123.2	10.63	<0.001
Row	4	104.0	104.7	26.2	0.25	0.909
Column	4	244.5	244.5	61.1	0.58	0.680
Error	43	4541.6	4541.6	105.6		
Total	55	9366.0				

Table A4.5 - Maximum Height (cm)

Analysis of Variance for Greatest, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	3703.64	3875.89	968.97	11.22	<0.001
Row_1	4	543.86	499.30	124.83	1.45	0.224
Column_1	4	415.86	415.86	103.97	1.20	0.314
Error	104	8982.76	8982.76	86.37		
Total	116	13646.13				

Table A4.6 - Seed Yield (number of seeds per plant)

Analysis of Variance for No seeds, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	1353.01	1353.01	338.25	9.05	0.001
Row_3	4	266.17	266.17	66.54	1.78	0.198
Column_3	4	185.58	185.58	46.40	1.24	0.345
Error	12	448.61	448.61	37.38		
Total	24	2253.37				

Table A4.7 - Seed Yield (mg seed per plant)

Analysis of Variance for Wt seeds, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	716.19	716.19	179.05	8.85	0.001
Row_3	4	154.34	154.34	38.59	1.91	0.174
Column_3	4	90.08	90.08	22.52	1.11	0.395
Error	12	242.90	242.90	20.24		
Total	24	1203.50				

Table A4.8 - 100 Seed Weight (mg)

Analysis of Variance for 100 seed, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	2	1649.95	1993.5	996.75	25.47	0.139
Row_4	4	4904.18	5247.42	1311.85	33.52	0.129
Column_4	4	3218.53	3218.53	804.63	20.56	0.164
Error	1	39.14	39.14	39.14		
Total	11	9811.80				

5. Preliminary Growth Trial Using Sand and Modified Hoagland's Solution B Plant Developmental Statistical Data

Table A5.1 - % Flowering

Analysis of Variance for Arcsin F, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	2354.3	2354.3	588.6	2.73	0.080
Row	4	865.2	865.2	216.3	1.00	0.444
Column	4	1650.7	1650.7	412.7	1.91	0.173
Error	12	2589.3	2589.3	215.8		
Total	24	7459.5				

Table A5.2 - Mean Days to Flower

Analysis of Variance for Days to, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	158.82	151.87	37.97	2.68	0.035
Row_1	4	27.43	30.20	7.55	0.53	0.712
Column_1	4	46.22	46.22	11.55	0.82	0.518
Error	112	1587.65	1587.65	14.18		
Total	124	1820.11				

Table A5.3 - Flowers per Flowering Plant

Analysis of Variance for Total F, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	337.3	395.40	98.85	3.60	0.008
Row	4	360.1	359.33	89.83	3.28	0.014
Column	4	64.39	64.39	16.10	0.59	0.673
Error	111	3044.62	3044.62	27.43		
Total	123	3806.42				

Table A5.4 - Maximum Height (cm)

Analysis of Variance for Greatest, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	356.3	356.30	89.08	6.08	<0.001
Row_1	4	53.15	53.15	13.29	0.91	0.462
Column_1	4	142.11	142.11	35.53	2.43	0.051
Error	137	2006.30	2006.3	14.64		
Total	149	2557.87				

Table A5.5 - Seed Yield (number of seeds per plant)

Analysis of Variance for No seeds, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	162.32	162.32	40.58	4.82	0.015
Row_2	4	67.77	67.77	16.94	2.01	0.157
Column_2	4	216.17	216.17	54.04	6.42	0.005
Error	12	101.02	101.02	8.42		
Total	24	547.28				

Table A5.6 - Seed Yield (mg seed per plant)

Analysis of Variance for Wt/plant, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	31.03	31.03	7.76	2.88	0.069
Row_2	4	13.08	13.08	3.27	1.21	0.355
Column_2	4	48.32	48.32	12.08	4.49	0.019
Error	12	32.29	32.29	2.69		
Total	24	124.71				

Table A5.7 - 100 Seed Weight (mg)

Analysis of Variance for 100 Seed, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	4134.5	2078.8	519.7	1.20	0.389
Row_3	4	1210.9	1117.1	279.3	0.65	0.647
Column_3	4	772.8	772.8	193.2	0.45	0.772
Error	7	3027.1	3027.1	432.4		
Total	9	9145.3				

6. Growth Trial Using Various Growth Substrates and Modified Hoagland's Solution B Plant Developmental Statistical Data

Table A6.1 - Days to Flowering

Analysis of Variance for Days to, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Site	2	34.98	35.12	17.56	2.57	0.078
Treatment	4	11.27	11.27	2.82	0.41	0.799
Site*Treatment	8	41.28	41.28	5.16	0.76	0.641
Error	357	2434.95	2434.95	6.82		
Total	371	2522.47				

Table A6.2 - Number of Flowers per Plant

Analysis of Variance for Total F, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Site_4	1	902.50	902.50	902.50	13.77	<0.001
Treatment	4	365.56	365.56	91.39	1.39	0.237
Site_4*Treatment	4	230.92	230.92	57.73	0.88	0.476
Error	240	15731.12	15731.12	65.55		
Total	249	17230.10				

Table A6.3 - Seed Yield (number of seeds per plant)

Analysis of Variance for No. Tota, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Site	2	10262794	10262794	51393197	192.32	<0.001
Treatment	4	226934	226934	56733	2.13	0.089
Site*Treatment	8	583776	583776	72972	2.73	0.012
Error	60	1600890	1600890	26682		
Total	74	12674395				

Table A6.4 - Seed Yield (mg seed per plant)

Analysis of Variance for Wt. Tota, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Site	2	13.11	13.11	6.56	127.32	<0.001
Treatment	4	0.44	0.44	0.11	2.16	0.085
Site*Treatment	8	1.20	1.20	0.15	2.91	0.008
Error	60	3.09	3.09	0.05		
Total	74	17.85				

Table A6.5 - 100 Seed Weight (mg)

Analysis of Variance for 100 seed, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Site	2	38751.4	38751.4	19375.7	53.46	<0.001
Treatment	4	5959.5	5959.5	1489.9	4.11	0.005
Site*Treatment	8	8137.6	8137.6	1017.2	2.81	0.010
Error	60	21745.7	21745.7	362.4		
Total	74	74564.2				

7. Hydroponic Growth Trial Using Modified Hoagland's Solution B Plant Developmental Statistical Data

Table A7.1 - Seed Yield (Number of Seeds per Plant)

Analysis of Variance for No. Tota, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Size	1	13504.4	13504.4	13504.4	21.00	<0.001
Treatment	4	1047.5	1047.5	269.9	0.41	0.802
Size*Treatment	4	1996.2	1996.2	499.0	0.78	0.547
Error	40	25721.0	25721.0	643.0		
Total	49	42269.0				

Table A7.2 - Seed Yield (mg Seeds per Plant)

Analysis of Variance for Wt. Tota, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Size	1	258	258	258	0.00	0.976
Treatment	4	758615	758615	189654	0.69	0.602
Size*Treatment	4	1084898	1084898	271224	0.99	0.424
Error	40	10962054	10962054	274051		
Total	49	12805825				

Table A7.3 - Seed Yield (100 Seed Weight)

Analysis of Variance for 100 seed, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Size	1	0.0218656	0.0218656	0.0218656	1107.30	<0.001
Treatment	4	0.0001127	0.0001127	0.0000282	1.43	0.243
Size*Treatment	4	0.0001856	0.0001856	0.0000464	2.35	0.071
Error	40	0.0007899	0.0007899	0.0000197		
Total	49	0.0229538				

8. Growth Trial Using Various Growth Substrates and Modified Hoagland's Solution B Seed Analysis Statistical Data

Table A8.1 - Seed Calcium Analysis

Analysis of Variance for Ca^{2+} ($\mu g.g^{-1}$) using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Site	2	32721216	34215006	17107503	29.95	<0.001
Treatment	4	23717123	22995461	5748865	10.07	0.000
Site*Treatment	8	7299561	7299561	912445	1.60	0.145
Error	60	34267547	34267547	571126		
Total	74	98005448				

Table A8.2 - Seed Potassium Analysis

Analysis of Variance for K^{+} ($\mu g.g^{-1}$) using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Site	2	61843984	63183324	31591662	16.81	<0.001
Treatment	4	15788437	17321432	4330358	2.30	0.069
Site*Treatment	8	14299919	14299919	1787490	0.95	0.482
Error	60	112741084	112741084	1879018		
Total	74	204673423				

Table A8.3 - Potassium/Calcium Ratios

Analysis of Variance for K^{+}/Ca^{2+} using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Site	2	18.8986	19.9177	9.9588	38.04	<0.001
Treatment	4	13.1356	13.1490	3.2872	12.56	0.000
Site*Treatment	8	1.6722	1.6722	0.2090	0.80	0.606
Error	60	15.7082	15.7082	0.2618		
Total	74	49.4146				

9. Growth Trial Using Various Growth Substrates and Modified Hoagland's Solution B Salinity Tolerance and Seed Ion Concentration Relationships Statistical Data

Table A9.1 - Salinity Tolerance Analysis of Variance

Analysis of Variance for EC_{50} using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Site	2	3444.1	3598.2	1799.1	3.05	0.055
Treatment	4	2792.6	3359.8	839.9	1.42	0.238
Site*Treatment	8	24147.1	24147.1	3018.4	5.12	<0.001
Error	55	32422.5	32422.5	589.5		
Total	69	62806.4				

Table A9.2 - Correlation Between Ca^{2+} Concentration and EC_{50} Salinity Tolerance

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.2917212
R Square	0.0851013
Adjusted R Square	0.0716469
Standard Error	29.069258
Observations	70

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	5344.903227	5344.9	6.3252	0.014272893
Residual	68	57461.47924	845.02		
Total	69	62806.38247			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	274.58543	14.65643935	18.735	1E-28	245.3389576	303.831896	245.338958	303.8318956
X Variable 1	-0.311897	0.124015134	-2.515	0.0143	-0.559364962	-0.0644283	-0.55936496	-0.06442827

Table A9.3 - Correlation Between K^+ Concentration and EC_{50} Salinity Tolerance

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.4020011
R Square	0.1616048
Adjusted R Square	0.1492755
Standard Error	27.827348
Observations	70

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	10149.81598	10150	13.107	0.000561473
Residual	68	52656.5665	774.36		
Total	69	62806.38247			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	327.68007	24.78077697	13.223	2E-20	278.230803	377.129343	278.230803	377.1293429
X Variable 1	-0.289604	0.079992195	-3.62	0.0006	-0.449226156	-0.1299823	-0.44922616	-0.12998228

Table A9.4 - Correlation Between Ca^{2+}+ K^+ Concentration and EC_{50} Salinity Tolerance

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.5358382
R Square	0.2871226
Adjusted R Square	0.2766391
Standard Error	25.659894
Observations	70

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	18033.12997	18033	27.388	1.75105E-06
Residual	68	44773.2525	658.43		
Total	69	62806.38247			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	384.53638	28.02062616	13.723	3E-21	328.6220912	440.450668	328.622091	440.4506678
X Variable 1	-0.345568	0.06603182	-5.233	2E-06	-0.47733279	-0.2138039	-0.47733279	-0.2138039

10. Comparison of Individual Regression Lines of Correlation of EC_{50} with Seed $[Ca^{2+}]$, $[K^{+}]$ and $[Ca^{2+}]+ [K^{+}]$ for Three Types of Growth Substrate.

Table A10.1 - Ca^{2+}

Intercept
Analysis of Variance for EC50, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Ca	1	4304.0	2803.1	2803.1	3.27	0.075
T_1	2	1943.2	1943.2	971.6	1.13	0.328
Error	66	56559.2	56559.2	857.0		
Total	69	62806.4				

S = 29.2738 R-Sq = 9.95% R-Sq(adj) = 5.85%

Term	Coef	SE Coef	T	P
Constant	271.62	18.55	14.65	0.000
Ca	-0.2870	0.1587	-1.81	0.075

Slope
Analysis of Variance for EC50, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Ca	1	4304.0	213.8	213.8	0.25	0.622
T_1	2	1943.2	659.9	329.9	0.38	0.686
T_1*Ca	2	875.4	875.4	437.7	0.50	0.607
Error	64	55683.8	55683.8	870.1		
Total	69	62806.4				

S = 29.4968 R-Sq = 11.34% R-Sq(adj) = 4.41%

Term	Coef	SE Coef	T	P
Constant	254.30	26.11	9.74	0.000
Ca	-0.1165	0.2350	-0.50	0.622
Ca*T_1				
1	0.0909	0.3418	0.27	0.791
2	-0.2577	0.2575	-1.00	0.321

Table A10.2 - K^+

Intercept
Analysis of Variance for EC50, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
K	1	9853.7	9399.2	9399.2	12.42	0.001
T_1	2	2989.7	2989.7	1494.8	1.97	0.147
Error	66	49963.1	49963.1	757.0		
Total	69	62806.4				

S = 27.5139 R-Sq = 20.45% R-Sq(adj) = 16.83%

Term	Coef	SE Coef	T	P
Constant	338.94	28.64	11.83	0.000
K	-0.32742	0.09292	-3.52	0.001

Slope
Analysis of Variance for EC50, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Ca	1	9853.7	10197.9	10197.9	13.53	0.000
T_1	2	2989.7	2235.1	1117.5	1.48	0.235
T_1*K	2	1739.9	1739.9	870.0	1.15	0.322
Error	64	48223.1	48223.1	753.5		
Total	69	62806.4				

S = 27.4497 R-Sq = 23.22% R-Sq(adj) = 17.22%

Term	Coef	SE Coef	T	P
Constant	344.56	28.86	11.94	0.000
Ca	-0.34322	0.09329	-3.68	0.000
K*T_1				
1	-0.1835	0.1363	-1.35	0.183
2	0.1633	0.1273	1.28	0.204

Table A10.3 - Ca^{2+} + K^{+}

Intercept
Analysis of Variance for EC50, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Ca+K	1	16908.4	13847.7	13847.7	20.08	0.000
T_1	2	383.5	383.5	191.7	0.28	0.758
Error	66	45514.5	45514.5	689.6		
Total	69	62806.4				

S = 26.2605 R-Sq = 27.53% R-Sq(adj) = 24.24%

Term	Coef	SE Coef	T	P
Constant	390.21	33.96	11.49	0.000
K	-0.35993	0.08032	-4.48	0.000

Slope
Analysis of Variance for EC50, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Ca	1	16908.4	14291.6	14291.6	20.59	0.000
T_1	2	383.5	1122.1	561.1	0.81	0.450
T_1*Ca+K	2	1102.5	1102.5	551.3	0.79	0.456
Error	64	44412.0	44412.0	693.9		
Total	69	62806.4				

S = 26.3427 R-Sq = 29.29% R-Sq(adj) = 23.76%

Term	Coef	SE Coef	T	P
Constant	401.65	35.72	11.24	0.000
Ca	-0.38889	0.08569	-4.54	0.000
Ca+K*T_1				
1	-0.1508	0.1288	-1.17	0.246
2	0.1005	0.1075	0.93	0.354

11. Hydroponic Growth Trial Using Modified Hoagland's Solution B Seed Analysis Statistical Data

Table A11.1 - Seed Calcium Analysis

Analysis of Variance for Ca^{2+} ($\mu g.g^{-1}$) using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Size	1	12846962	12846962	12846962	124.52	<0.001
Treatment	4	4712699	4712699	1178175	11.42	<0.001
Size*Treatment	4	631914	631914	157979	1.53	0.212
Error	40	4126838	4126838	103171		
Total	49	22318413				

Table A11.3 - Seed Potassium Analysis

Analysis of Variance for K^+ ($\mu g.g^{-1}$) using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Size	1	30319082	30319082	30319082	55.16	<0.001
Treatment	4	3325926	3325926	831482	1.51	0.217
Size*Treatment	4	1375853	1375853	343963	0.63	0.647
Error	40	21987093	21987093	549677		
Total	49	57007953				

Table A11.4 - Potassium/Calcium Ratios

Analysis of Variance for K^+/Ca^{2+} using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Size	1	1.14219	1.14219	1.14219	16.39	<0.001
Treatment	4	2.54549	2.54549	0.63637	9.13	<0.001
Size*Treatment	4	0.09689	0.09689	0.02422	0.35	0.844
Error	40	2.78671	2.78671	0.06967		
Total	49	6.57128				

12. Hydroponic Growth Trial and Modified Hoagland's Solution B Salinity Tolerance and Seed Ion Concentration Relationships Statistical Data

Table A12.1 - Salinity Tolerance Analysis of Variance

Analysis of Variance for EC_{50} using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Site	1	52219.1	52219.1	52219.1	52.62	<0.001
Treatment	4	17506.7	17506.7	4376.7	4.41	0.005
Site*Treatment	4	2397.9	2397.9	599.5	0.60	0.662
Error	40	39696.9	39696.9	992.4		
Total	49	111820.7				

Table A12.2 - Correlation Between Ca^{2+} Concentration and EC_{50} for 1.0 - 1.4 mm Seed

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.1183452
R Square	0.0140056
Adjusted R Square	-0.0288637
Standard Error	33.683477
Observations	25

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	370.670993	370.671	0.32670423	0.573150254
Residual	23	26095.26288	1134.577		
Total	24	26465.93387			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	221.36514	79.99980335	2.767071	0.01096768	55.87293949	386.85734	55.8729395	386.857342
X Variable 1	0.391271	0.68454236	0.57158	0.57315025	-1.024812711	1.8073548	-1.0248127	1.8073548

Table A12.3 - Correlation Between Ca^{2+} Concentration and EC_{50} for <1.0mm Seed

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.290958
R Square	0.0846566
Adjusted R Square	0.044859
Standard Error	36.314122
Observations	25

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2805.146091	2805.146	2.12718073	0.158228501
Residual	23	30330.45528	1318.715		
Total	24	33135.60138			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	74.949159	87.6154303	0.855433	0.40113511	-106.2971662	256.19549	-106.29717	256.195485
X Variable 1	0.8984085	0.615987164	1.458486	0.1582285	-0.375858016	2.172675	-0.375858	2.17267504

Table A12.4 - Correlation Between Ca^{2+} Concentration and EC_{50} for Combined Seed Sizes

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.41522189
R Square	0.17240921
Adjusted R Square	0.15516774
Standard Error	43.9084672
Observations	50

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	19278.92	19278.92	9.999679	0.002713539
Residual	48	92541.77	1927.953		
Total	49	111820.7			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	386.67998	48.4881	7.974739	2.4E-10	289.1881156	484.1718	289.1881	484.1718
X Variable 1	-1.17791972	0.372497	-3.16223	0.002714	-1.92687492	-0.42896	-1.92687	-0.42896

Table A12.5 - Correlation Between K^+ Concentration and EC_{50} for 1.0 - 1.4 mm Seed

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.3325462
R Square	0.110587
Adjusted R Square	0.0719169
Standard Error	31.991266
Observations	25

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2926.788417	2926.788	2.85975265	0.104329708
Residual	23	23539.14546	1023.441		
Total	24	26465.93387			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	482.67412	127.7385883	3.778608	0.00097327	218.4267141	746.92152	218.426714	746.921517
X Variable 1	-0.5578884	0.329900589	-1.69108	0.10432971	-1.240339754	0.124563	-1.2403398	0.12456297

Table A12.6 - Correlation Between K^+ Concentration and EC_{50} [NaCl] for <1.0 mm Seed

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.4966115
R Square	0.246623
Adjusted R Square	0.2138675
Standard Error	32.945013
Observations	25

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	8172.002385	8172.002	7.52920502	0.011563015
Residual	23	24963.59899	1085.374		
Total	24	33135.60138			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	631.92846	156.7138975	4.03237	0.00051908	307.7410612	956.11585	307.741061	956.115851
X Variable 1	-1.0072273	0.367073397	-2.74394	0.01156302	-1.766576433	-0.2478781	-1.7665764	-0.2478781

Table A12.7 - Correlation Between K^+ Concentration and EC_{50} for Combined Seed Sizes

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.7052423
R Square	0.4973667
Adjusted R Square	0.48689517
Standard Error	34.2189031
Observations	50

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	55615.88	55615.88	47.49705	1.07736E-08
Residual	48	56204.8	1170.933		
Total	49	111820.7			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	731.196564	72.2166	10.12505	1.68E-13	585.9953608	876.3978	585.9954	876.3978
X Variable 1	-1.22120781	0.177197	-6.89181	1.08E-08	-1.57748614	-0.86493	-1.57749	-0.86493

Table A12.8 - Correlation Between Ca^{2+}+ K^+ Concentration and EC_{50} for 1.0 - 1.4 mm Seed

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.2564014
R Square	0.0657417
Adjusted R Square	0.0251218
Standard Error	32.787868
Observations	25

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1739.914955	1739.915	1.61845884	0.216019161
Residual	23	24726.01892	1075.044		
Total	24	26465.93387			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	470.58171	160.2150631	2.937188	0.00740226	139.151607	802.01182	139.151607	802.011822
X Variable 1	-0.4047407	0.318145674	-1.272187	0.21601916	-1.062875142	0.2533938	-1.0628751	0.25339379

Table A12.9 - Correlation Between Ca^{2+}+ K^{+} Concentration and EC_{50} for < 1.0 mm Seed

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.2985372
R Square	0.0891244
Adjusted R Square	0.0495212
Standard Error	36.225387
Observations	25

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2953.191941	2953.192	2.2504305	0.147180415
Residual	23	30182.40943	1312.279		
Total	24	33135.60138			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	538.55737	224.2703772	2.401375	0.0248108	74.61874662	1002.496	74.6187466	1002.49599
X Variable 1	-0.5917017	0.394430079	-1.500143	0.14718041	-1.407642495	0.2242391	-1.4076425	0.22423906

Table A12.10 - Correlation Between Ca^{2+} + K^{+} Concentration and EC_{50} for Combined Seed Sizes

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.68958794
R Square	0.47553153
Adjusted R Square	0.4646051
Standard Error	34.9542627
Observations	50

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	53174.26	53174.26	43.52123	3.0522E-08
Residual	48	58646.42	1221.8		
Total	49	111820.7			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	694.760953	69.92551	9.935729	3.13E-13	554.1662913	835.3556	554.1663	835.3556
X Variable 1	-0.85891527	0.130197	-6.59706	3.05E-08	-1.12069313	-0.59714	-1.12069	-0.59714

13. Comparison of Individual Regression Lines of Correlation of EC_{50} with Seed $[Ca^{2+}]$, $[K^{+}]$ and $[Ca^{2+}]+ [K^{+}]$ for Hydroponic Growth Trial

Table A13.1 - Ca^{2+}

Intercept
Analysis of Variance for EC50, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Ca	1	19279	2809	2809	2.32	0.134
Size	1	35749	35749	35749	29.58	0.000
Error	47	56793	56793	1208		
Total	49	111821				

S = 34.7614 R-Sq = 49.21% R-Sq(adj) = 47.05%

Term	Coef	SE Coef	T	P
Constant	145.51	58.65	2.48	0.017
Ca	0.6902	0.4527	1.52	0.134

Slope
Analysis of Variance for EC50, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Ca	1	19279	2374	2374	1.93	0.171
Size	1	35749	1870	1870	1.52	0.223
Size*Ca	1	367	367	367	0.30	0.587
Error	46	56426	56246	1227		
Total	49	111821				

S = 35.0235 R-Sq = 49.54% R-Sq(adj) = 46.25%

Term	Coef	SE Coef	T	P
Constant	148.16	59.29	2.50	0.016
Ca	0.6448	0.4636	1.36	0.171
Ca*Size				
1	-0.2536	0.4636	-0.55	0.587

Table A13.2 - K^+

Intercept
Analysis of Variance for EC50, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
K	1	55616	10223	10223	9.73	0.003
Size	1	6826	6826	6826	6.50	0.014
Error	47	49379	49379	1051		
Total	49	111821				

S = 32.4131 R-Sq = 55.84% R-Sq(adj) = 53.96%

Term	Coef	SE Coef	T	P
Constant	545.77	99.86	5.47	0.000
K	-0.7652	0.2453	-3.12	0.003

Slope
Analysis of Variance for EC50, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
K	1	55616	10628	10628	10.08	0.003
Size	1	6826	578	578	0.55	0.463
Size*K	1	876	876	876	0.83	0.367
Error	46	48503	48503	1054		
Total	49	111821				

S = 32.4716 R-Sq = 56.62% R-Sq(adj) = 53.80%

Term	Coef	SE Coef	T	P
Constant	557.3	100.8	5.53	0.000
K	-0.7826	0.2465	-3.17	0.003
K*Size				
1	0.2247	0.2465	0.91	0.367

Table A13.3 - $Ca^{2+} + K^{+}$

Intercept
Analysis of Variance for EC50, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Ca+K	1	53174	4529	4529	3.86	0.055
Size	1	3574	3574	3574	3.05	0.087
Error	47	55073	55073	1172		
Total	49	111821				

S = 34.2310 R-Sq = 50.75% R-Sq(adj) = 48.65%

Term	Coef	SE Coef	T	P
Constant	495.8	132.9	3.73	0.001
Ca+K	-0.4875	0.2480	-1.97	0.055

Slope
Analysis of Variance for EC50, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Ca+K	1	53174	4668	4668	3.91	0.054
Size	1	3574	74	74	0.06	0.804
Size*Ca+K	1	164	164	164	0.14	0.712
Error	46	54908	54908	1194		
Total	49	111821				

S = 34.5494 R-Sq = 50.90% R-Sq(adj) = 47.69%

Term	Coef	SE Coef	T	P
Constant	504.6	136.2	3.70	0.001
Ca+K	-0.4982	0.2519	-1.98	0.054
Ca+K*Size				
1	0.0935	0.2519	0.37	0.712

14. Seed Coat and Embryo Analysis

Table A14.1 - Coat Calcium Analysis

Analysis of Variance for Ca^{2+} ($\mu g.g^{-1}$) using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	9677413	9677413	2419353	4.61	0.008
Error	20	10492390	10492390	524619		
Total	24	20169803				

Table A14.2- Coat Potassium Analysis

Analysis of Variance for Ca^{2+} ($\mu g.g^{-1}$) using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	18394024	18394024	4598506	7.96	0.001
Error	20	11559448	11559448	577972		
Total	24	29953472				

Table A14.3 - Embryo Calcium Analysis

Analysis of Variance for Ca^{2+} ($\mu g.g^{-1}$) using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	549161	549161	137290	1.70	0.191
Error	20	1619789	1619789	80989		
Total	24	2168950				

Table A14.4 - Embryo Potassium Analysis

Analysis of Variance for K^{+} ($\mu g.g^{-1}$) using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	4999660	4999660	1249915	2.24	0.100
Error	20	11135149	11135149	556757		
Total	24	16134808				

Table A14.5 - Analysis of Variance for Tissue and Analyte

Source	SS	df	MS	F	P-value	F crit
Analyte	1.38E+08	1	1.38E+08	193.7758	9.22E-25	3.940163
Tissue	16721430	1	16721430	23.4594	4.89E-06	3.940163
Interaction	1.7E+09	1	1.7E+09	2384.975	1.33E-69	3.940163
Within	68427033	96	712781.6			
Total	1.92E+09	99				