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The determinants of salinity tolerance in maize (Zea mays L.)

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The Determinants of Salinity Tolerance in Maize (*Zea mays* L.)

The studies described in this thesis were carried out in the Laboratory of Plant Eco-Physiology, which is a part of the Centre for Ecological and Evolutionary Studies (CEES), Faculty of Mathematics and Natural science, University of Groningen. The candidate was financially supported by a scholarship from the Culture Affairs and Missions Sector and the Egyptian Ministry of Higher Education.

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PROEFSCHRIFT

ter verkrijging van het doctoraat in de
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op gezag van de
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door

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geboren op 3 november 1977
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Prof. dr. J. Rozema

To the soul of my beloved Mother:
Fatma Abd El-Halim.
I miss you, more than the words can tell and
more than the time can heal

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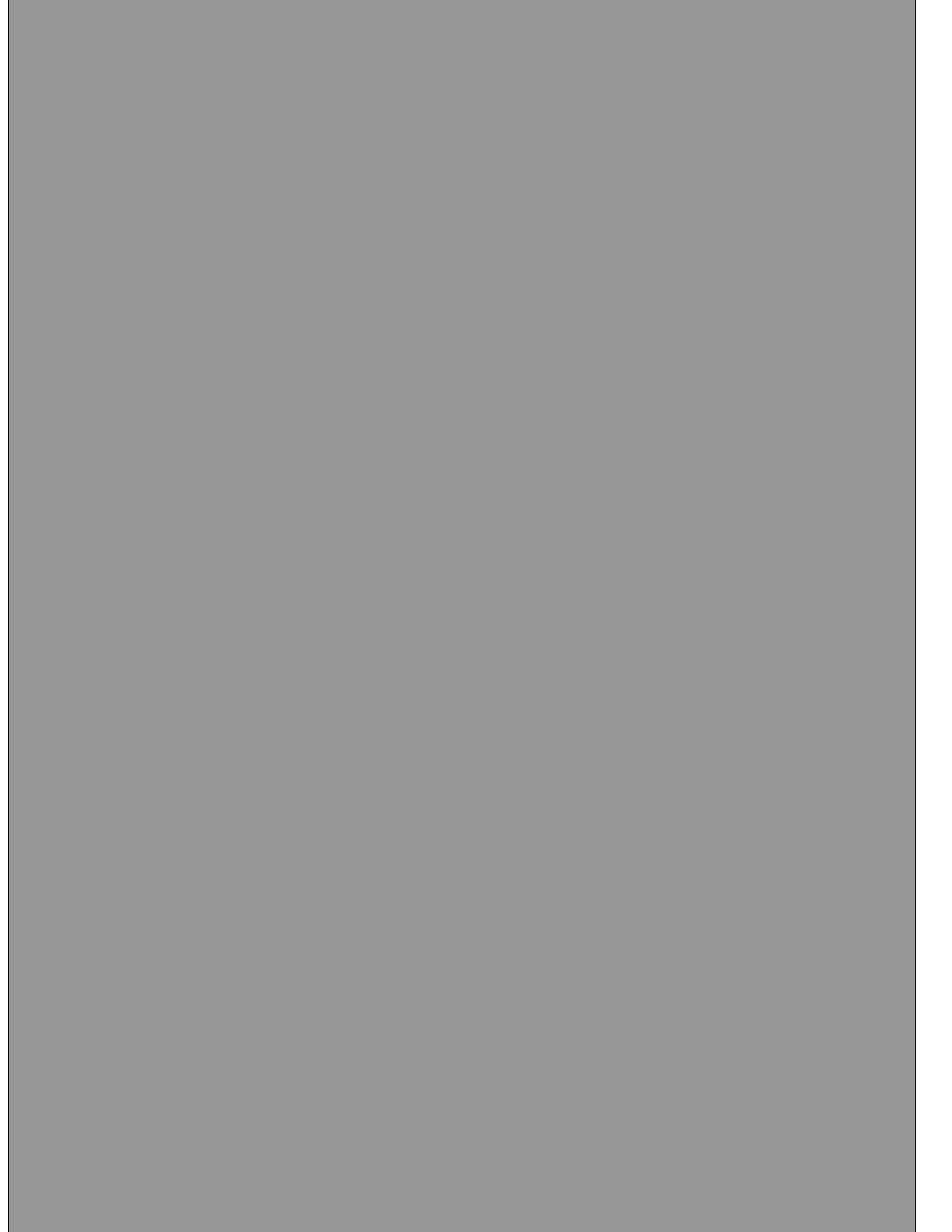
Chapter 1

General Introduction



Salinity is one of the world's oldest and most widely distributed environmental challenges. Salinity is defined as the presence of an excessive concentration of soluble salts in the soil which suppresses plant growth. A more specific definition for salinity is any irrigation water or soil solution with an electric conductivity (EC) of 400 mSm^{-1} (ca. 40 mM) or greater which reduces the growth of plant species[1]. The main salt present in a saline habitat is NaCl. However, other salts, such as Na_2SO_4, $MgSO_4$, $CaSO_4$, $MgCl_2$, KCl and $NaCO_3$, can dominate in different saline environments[2].

Most of the water on earth contains about 30 g of sodium chloride per litre. This can make earth a really salty planet. This salt has affected, and is still affecting, the land on which crops might be grown. Although the amount of salt affected land is not precisely known, its extent is sufficient to pose a threat to agriculture[3,4].

Salinity is also a threat to our food supply since most crop plants will not grow in high concentrations of salt. Only halophytes grow in concentrations of sodium chloride higher than 400 mM. Consequently, although there is currently enough food for the world population, more than 800 million people are chronically undernourished[5]. Growth of the human population by 50%, from 6.1 billion in mid-2001 to 9.3 billion by 2050 [6], means that crop production must increase if food security is to be ensured, especially for those who live on about $1 per day[6] . To reach this goal there is an urgent need for research to develop tolerant plants and soil management techniques. These approaches may keep food production at a level that meets the demand for food. We need a specific strategy to guide research in order to meet future production demands. This strategy requires close cooperation among scientists of several disciplines: plant physiology, molecular biology and crop management.

A thorough understanding of plant salt-tolerance mechanisms by plant physiologists and molecular biologists enables the identification of physiological traits and genetic markers, and thus the tools to develop or effectively breed salt tolerant cultivars of

crop plants. However, such cooperation among different scientists still needs a sustained effort to solve the puzzle of salinity tolerance.

The effects of salinity on plants can be separated into three main categories[7-9]: 1. osmotic effects, 2. ionic effects and 3. oxidative stress. The degree to which each of these components of salinity stress influences growth depends on many factors (e.g. plant species, ionic composition of the saline waters, light, humidity and stage of plant development).

Osmotic effects. Salt in the root zone decreases the water potential of the soil solution. The reaction of all plants, including halophytes, when transferred from a solution of high to one of low water potential is loss of turgor (i.e. turgor changes). This change is followed by osmotic adjustment. That is, a reduction in the internal osmotic potential of the plants[2] to compensate for the lower external osmotic potential. Some species apparently adjust fully to the new external conditions[10]. However, others apparently do not, and their growth will be permanently affected by water stress or the osmotic effect of salinity. For plants to achieve such adjustment, the biosynthesis of various osmolytes has been reported under salt stress in different plant species. These osmolytes may include amino acids, amides, imino acids, proteins, quaternary ammonium compounds and polyamines[11,12].

The detrimental effect of turgor reduction that can be caused by salt-induced water stress, on cell division and elongation and on the stomatal aperture has been well documented[12]. Turgor reduction results in stomatal closure[12,13], followed by a reduction in gas exchange (i.e. transpiration and photosynthesis). Other mechanisms besides low leaf turgor can also be affected, such as: stomatal closure, growth and photosynthesis[14,15]. Leaf expansion and stomatal closure can apparently be modified (most likely hormonally controlled) by the leaves or under the influence of signals from the roots in drought and salt-exposed plants, without any noticeable changes in leaf turgor[14].

Osmotic stress caused by salinity is not the only limiting factor at the whole plant or the cellular level. Salt toxicity does seem to have a much more pronounced effect on physiological functioning[10,14]. Therefore, in recent years a greater emphasis has been placed on ion toxicity and nutritional disturbances and less attention was paid to osmotic effects[8,14,16,17]. The reason for such a shift in focus was the realizaton that the ion relations of plants are significantly affected by salinity.

One way to distinguish the osmotic effects from the ion-specific effects are observations over time of the rate of new leaf production and the rate of increase in injury of old leaves. The effect of the osmotic stress is seen as a rapid inhibition of the rate of expansion of young leaves and reduced stomatal conductance of mature leaves. Daily measurements of the length of a growing leaf, or spot measurements of stomatal conductance with a porometer, are good indicators of growth rate and was suggested as a non destructive screening tool for salt tolerance especially in cereals[12].

Ionic effects The ion-specific phase of plant response to salinity starts when salt accumulates to toxic concentrations in the old leaves which are no longer expanding and so no longer diluting the salt transported to them, as younger growing leaves do. If the rate at which the older leaves die is greater than the rate at which new leaves are produced, the photosynthetic capacity of the plant will no longer be able to support the basal metabolic demand and growth will cease completely. More ionic stress develops over time and is due to a combination of ion accumulation in the shoot and decrease of tissue tolerance of the young leaves, which further reduces their growth rate.

Ionic effects of salinity include two primary effects on plants: direct toxicity due to excessive accumulation of toxic ions (Na^+, Cl^-) in the tissues and a nutritional imbalance caused by a reduction in some particularly essential ions (K^+, Ca^{2+})[18]. Levitt[19] summarized evidence for non-osmotic effects of salinity injury to plants, as

follows: (1) organic solutes do not injure plants at osmolalities higher than the critical concentrations for salt injury, (2) individual salts have different critical concentrations for inducing injury, (3) certain organic solutes increase the critical salt concentration for injury and (4) injurious effects of salts are antagonized by Ca^{2+}.

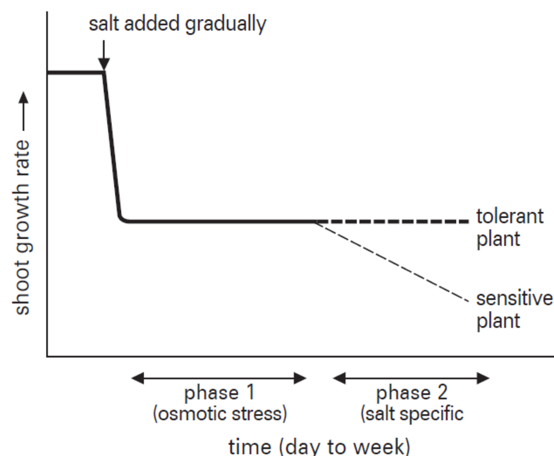


Figure 1.1. *Schematic illustration of the two-phase growth response to salinity for genotypes that differ in the rate at which salt reaches toxic levels in leafs. With annual species, the timescale is days or weeks, depending on species and salinity level. With perennial species, the timescale is months or years. During phase 1, growth of both genotypes is reduced because of the osmotic effect of the saline solution outside the roots. During phase 2, leaves in the more sensitive genotype die and reduce the photosynthetic capacity of the plant. This exerts an additional effect on growth (Adapted from[13]). If salt is added in one step, the growth rate plummets to zero or below and takes 1–24 hours to recover to the new steady rate, depending on the degree of the osmotic shock[4].*

A number of different hypotheses have been developed for the mechanisms by which ions may inhibit growth. For example, it is believed that Na^+ and Cl^- can have direct toxic effects on various metabolic processes[2,20]. Salinity can interfere with K^+ and Ca^{2+} nutrition[17] and this can cause nutrient deficiencies[21-23]. Specifically, Na^+ can disrupt membrane integrity[24,24] and inhibit the transport of these ions (K^+, Ca^{2+}) into the root[25] and up to the shoot[26,27]. High salt concentrations can induce K^+ and Ca^{2+} deficiency in different crop plants[20,28]. In several plant species, high K^+/Na^+

and Ca^{2+}/Na^{+} ratios in leaves have been correlated with salt tolerance[20,29-33]. Therefore, maintenance of high K^{+} and Ca^{2+} to Na^{+} ratios appears to be important mechanisms contributing to plant tolerance. Several studies have shown that calcium and potassium enhanced salt tolerance in various species[34-36,36]. Sodium accumulation in leaves is typified by leaf mottling and necrotic patches or by tip burn[12], or both. Salt injury to leaves often is followed by leaf shedding and twig dieback [37].

Oxidative Stress The reduced rate of photosynthesis increases the formation of reactive oxygen species (ROS), and increases the activity of enzymes that detoxify ROS[38-40]. When plants acclimate to a changed environment, they undergo adjustments in leaf morphology, chloroplast pigment composition and in the activity of biochemical processes that prevent oxidative damage to photosystems[41]. The two processes that protect from photoinhibition caused by excess light, are heat dissipation by the xanthophyll pigments and electron transfer to oxygen acceptors other than water[41].

Several studies reported that saline environments induce oxidative stress in various plant species[9,42-45]. Oxidative stress includes accumulation of reactive oxygen species such as singlet oxygen (O_2^1), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) that lead to membrane lipid peroxidation, protein oxidation, enzyme inhibition and DNA and RNA damage[41]. Accumulation of reactive oxygen species during stress results from pathways such as photorespiration, from the photosynthetic apparatus and from mitochondrial respiration. Reactive oxygen species can be viewed as cellular indicators of stress and as secondary messengers involved in the stress response signal transduction pathway[41].

Plants minimize the detrimental effects of the reactive oxygen species, by the induction of several defense systems and production of antioxidants[41,46]. The main scavenging system is superoxide dismutase, a metallo-protein that catalyzes the

conversion of superoxide radicals into hydrogen peroxide. To avoid hydrogen peroxide accumulation, a compound even more damaging than the superoxide radical, two enzymes (catalase and ascorbate peroxidase) act to detoxify this compound, yielding water and oxygen. Glutathione reductase acts by recycling oxidize glutathione using NADH as a cofactor. Many studies have reported changes in antioxidant enzyme activities in response to salinity, suggesting that the increase in these activities can be the basis for salt tolerance[42-44]. In addition to the above mentioned enzymes of the antioxidant defense mechanism, ascorbate, glutathione, α-tocopherol and cartenoids act as antioxidant and are produced in aerobic cells under stress conditions[41].

It is still doubtful that the manipulation of a single gene related to oxidative stress tolerance can enhance the tolerance to any abiotic stress[12]. Recently *Arabidopsis* mutants lacking either or both cytosolic and chloroplastic ascorbate peroxidases (H_2O_2 removal enzymes) were found to be actually more tolerant of salinity stress[47]. Several studies have found differences in levels of expression or activity of antioxidant enzymes. These differences are sometimes associated with the more tolerant genotype, and sometimes with the more sensitive genotype. Munns and Tester[12] suggested that the possible differences in antioxidant activity between genotypes are probably due to genotypic differences in controlling the stomatal opening or in other responses that affect the rate of CO_2 fixation. Alternatively, they could be due to differences in protection against photoinhibition. All these possible effects could be taken as an illustration of the plasticity and variability of reactive oxygen species regulatory pathways.

1.1 Effect of salinity on plant physiological processes and morphological structure

Salinity affects physiological functions such as seed germination, vegetative growth and gas exchange, in addition to changes in plant morphology and anatomy

1.1.1 Effect of salinity on seed germination

In both non-halophytes and halophytes, salinity reduces the total number of seeds germinating and postpones initiation of germination processes; however, within each group the responses are variable and species-specific[48-51]. Salinity influences seed germination primarily by lowering the osmotic potential of the soil solution sufficiently to retard water absorption by seeds[52-54], by toxicity to the embryo[52-54], or by alteration of protein synthesis[55]. Salinity effects on seed germination and emergence were reported to be brought about by increased accumulation of Na^+ and Cl^- and reduction of K^+. Germination and early seedling growth have been indicated to be more sensitive than later developmental stages[56]. Seeds of many halophytes accumulate less than 10% of the ionic content in shoots, indicating that they possess a mechanism which prevent excess ion accumulation in the embryo[48].

1.1.2 Effect of salinity on vegetative growth

Salinity reduces shoot growth by suppressing leaf initiation and expansion, as well as internodes growth, and by accelerating leaf abscission[57,58]. Salinity induces early leaf shedding in both angiosperms and gymnosperms. Salt stress causes a rapid and potentially lasting reduction in the rate of leaf growth[13]. A reduction of the velocity of leaf elongation results from a reduction in the number of elongating cells or a reduction in the rate of cell elongation or from both. From the biophysical point of view[59], a leaf cell of a NaCl-treated plant can expand at reduced rates because of reduced uptake rates of water or osmolytes, because of stiffened cell walls, or because of lowered turgor.

For a moderate salinity stress, an inhibition of lateral shoot development becomes apparent over weeks, and over months there are effects on reproductive development, such as early flowering or a reduced number of florets. During this time, a number of older leaves may die. However, production of younger leaves continues. All these changes in plant growth are responses to the osmotic effect

of the salt and are similar to drought responses[12]. The reduction in leaf development is mainly due to the osmotic effect of the salt, a notion that is supported by experiments using mixed salts such as concentrated Hoagland solution[60], other single salts such as KCl[61] and non-ionic solutes such as mannitol or polyethylene glycol (PEG)[61,61,62]. These different osmotica all have a similar qualitative effect as NaCl on leaf expansion.

The mechanism by which salinity inhibits plant growth depends on whether the exposure to salt is short-term (hours to days) or long-term (weeks). It is important to separate short-term and long-term effects because they can differ significantly from each other. Short-term effects usually represent an osmotic effect (water deficit) with little or no ionic effect (depending on genotype, salt composition and concentration)[13,14,17,61]. Long-term effects allow the ion toxicity symptoms to appear on the plant due to the accumulation of toxic ions as (Na^+ and Cl^-) in the cytoplasm.

1.1.3 Morphological and anatomical changes under salinity

Salinity often alters the morphology and anatomy of woody plants. Leaves of plants that grow on saline soils often are thicker and more succulent than those of trees growing on salt-free soils[63]. The epidermal cell walls and cuticles of leaves of salinized plants also are thicker. By increasing the internal surface area per unit of leaf surface, leaf succulence may increase CO_2 absorption per unit of leaf area. Increase in leaf thickness in response to salinity has been attributed to an increase in number of mesophyll cell layers or cell size, or both. In salinized *Gossypium hirsutum* plants, the leaves were thicker because the number of cell layers increased and the mesophyll cells were larger, whereas in *Citrus*, increase in the size of spongy mesophyll cells, rather than an increase in cell layers accounted for thicker leaves[64]. The large cells of leaves of salinized plants result from increased cell wall extensibility together with higher turgor pressures[14]. The xylem vessels of halophytic trees are more numerous and narrower than those in mesophytic trees[65]. Salinity often promotes suberization of the hypodermis and endodermis in roots, with formation of a well-developed casparian strip closer to the root apex than is found in non-salinized roots[65].

1.1.4 Gas exchange under salinity

Salinity reduces the rate of photosynthesis of both non-halophytes and halophytes[12,57,66]. Although both stomatal and non-stomatal factors have been implicated in the reduction of photosynthesis following treatment with saline water, most of the reduction in photosynthetic rates is the result of non-stomatal effects[13]. In the long term, total photosynthesis is reduced as a result of inhibition of leaf formation and expansion, as well as early leaf abscission[67]. However, after plants were flooded with a solution with a high concentration of salt, photosynthetic inhibition was attributed to ion toxicity, membrane disruption, and complete stomatal closure[68].

Munns and Tester[12] identified the decrease in stomatal aperture as the most dramatic and readily measurable whole plant response to salinity. They also concluded that the stomatal responses are undoubtedly induced by the osmotic effect of the salt outside the roots. Salinity affects stomatal conductance immediately, firstly and transiently owing to perturbed water relations and shortly afterward owing to the local synthesis of abscisic acid.

In addition, salinity disrupts the accumulation of several ions (Na^+, Cl^-, Ca^{2+}, and Mg^{2+}) in the leaves and this increase in leaf ionic content was considered to be the primary cause of the saline-induced reduction in photosynthetic rates[69]. In many plants, salinity lowers the efficiency of the electron transport chain and injures the light-harvesting complex [70-73].

1.2 Salinity tolerance determinants

The tolerance of a plant to salinity is usually appraised by measuring how long the plant is surviving on the saline soils, or by comparing the plant's absolute growth or yield in saline soil with that in non-saline soil[74].

Salinity affects plants at all stages of development, but it differs from one growth stage to another. It is difficult to make a comparison of the early stages of germination and emergence and those of later stages because there are different criteria that must be used to evaluate plant functioning[74]. Tolerance at emergence is based on survival whereas the tolerance after this stage is based on the decrease in yield or growth[74].

Generally, crop cultivars that tend to exclude Na^+, are more salt tolerant and do not show the damaging effects of Na^+ as much as cultivars which tend to accumulate sodium [8,75-78]. In contrast, halophytic species use Na^+ and Cl^- as cheap osmolytes, sequestering these ions in the vacuole [20]. Accumulation of Na^+ and Cl^- in the vacuole lowers its osmotic potential and the plant cell accumulates compatible solutes in the cytoplasm to balance the solute potential of the vacuole.

Specific salt-tolerance determinants are presented in the following section:

1.2.1 Effectors and signaling components

The determinants of salt stress tolerance can be divided into **effector** molecules (metabolites, proteins, or components of biochemical pathways) that lead to adaptation, and **regulatory** molecules (signal transduction pathway components) that control the amount and timing of these effector molecules.

1.2.1.1 Effector compounds

The functions of these compounds may include ion homeostasis, osmolyte biosynthesis, water uptake and transport, and long distance response coordination [9,35,79]. High salinity results in a reduction of K^+ and Ca^{2+} content and an increased level of Na^+ and Cl^- [11]. Therefore, ion homeostasis in saline environments relies on membrane transport systems that regulate ion fluxes. These transport proteins include H^+-ATPases of plasma membrane and tonoplast as well as vacuolar pyrophosphatases, Ca^{2+}-ATPases, secondary transporters, and channels[9,11]. H^+-ATPase energizes the membrane through the formation of electrochemical gradients. Both electrical and ionic gradients are used by secondary active

transporters, either to exclude Na^+ from the cytosol or to sequester Na^+ in the vacuole[11]. Cytoplasmic K^+ and Ca^{2+} regulation can also be mediated by these membrane transport systems.

Changes induced by external salinity are generally associated with the accumulation of metabolites that act as compatible solutes. These solutes do not inhibit normal metabolic reactions even at high concentrations[80]. Compatible solutes may include sugars, sugar alcohol, glycinebetaine, proline, potassium and polyamines[11]. Osmotic adjustment, protection of cellular macromolecules, storage form of nitrogen, maintaining cellular pH, detoxification of the cells, and scavenging of free radicals are proposed functions for these compounds under stress conditions [81].

High salinity causes a considerable reduction in water permeability in different plant species[81-84]. Such a reduction may be caused by changes in membrane structure and/or composition or by reducing the probability of opening water channels or by a change in their number[11] under salt stress. Water channels may have significance for water relations in stressed plants, but the available data on the control of water uptake in salt stressed roots is still insufficient for a definitive conclusions[11].

Organismal response coordination is less understood under saline conditions. Communication between root and shoot in a changing environment may be predominantly chemical, i.e., this may include ABA transport, cytokinin/auxin transport or through ethylene, and by the coordinated interplay of different growth regulators[11]. Evidence for a metabolic connection between leaf photosynthetic capacity and root Na^+ uptake is presented by Nelson *et al* [85]. These authors indicated that the salt-induced increase in the transport of inositol in the phloem from the leaves to roots is associated with increased Na^+ transport in the xylem.

1.2.1.2 Regulatory molecules

Hasegawa *et al.*[11] divided genetic determinants of salt tolerance into two classes: “encoding effectors responsible for remodeling the plant during adaptation, and regulatory genes that control the expression and activity of the effectors”. Some of these regulatory genes are MAP (mitogen-activated protein) kinases that control glycerol accumulation, AtDBF2 kinase is a cell-cycle-regulated protein kinase that modulates expression of genes involved in osmotic adaptation, and SAL1 which has a role in SO_4^{2-} reduction and phosphoinositide signaling at high salinity. More details about these regulator genes and encoding effectors are presented by Hasegawa *et al.*[11].

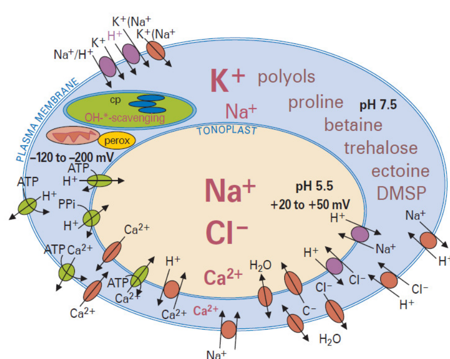


Figure 1.2. *Cellular homeostasis established after salt (NaCl) adaptation. Indicated are the osmolytes and ions compartmentalized in the cytoplasm and vacuole, transport proteins responsible for Na^+ and Cl^- homeostasis, water channels, and electrochemical potentials across the plasma membrane and tonoplast. Included are organelles (chloroplast (cp), mitochondrion (mt), and peroxisome (perox)) for which the importance of ROS-scavenging is implicated (Adapted from [86]).*

Salt tolerance of plants is a multigenic trait. Therefore, genes that regulate plant selectivity of potassium over sodium, and those controlling the expression of compatible solutes in the cytoplasm play a particularly critical role in salt tolerance and salt sensitivity[9]. The characterization of genes that contribute to salt tolerance and the underlying physiological processes could lead to the identification of specific physiological and biochemical markers for salt tolerance[86]. Such possible markers that are more broadly termed “salt tolerance determinants” can be genes, proteins, enzymes, second messengers, phytohormones, antioxidants, membrane transporters, solute compartmentation and osmolytes or compatible solutes[9].

1.3 Plasma membrane and salinity

In this section the interaction between plasma membrane and salt stress, based on studies that point to the plasma membrane as the primary site of salt effect[19,24,87], is reviewed. Several studies indicate that salinity injures cell membranes[24,88]. Salinity induced cell membrane impairment could be brought about by alteration of membrane lipid/protein structure and composition. Changes in plasma membrane proteins were reported for different plant species under saline conditions, however, these differences were not significant among genotypes differing in salt sensitivity[89-94].

Conformational alterations of the membrane structure, specifically the lipid matrix and its embedded proteins have been observed under saline conditions[89,90,92-94]. The maintenance of the membrane fluidity, regulating various membrane functions (e.g. permeability and enzyme activity), is the overriding criterion for successful membrane function under salinity stress. Several factors can affect membrane fluidity. The degree of membrane fatty acid saturation greatly regulates membrane fluidity[95]. The stability of the membrane also depends on an appropriate balance of bilayer/non-bilayer forming lipids[96,97]. NaCl induces alteration in the proportions of phosphatidylcholine and phosphatidylethanolamine, which affects membrane architecture[93,94,98].

1.4 The role of sodium transport in salt tolerance

For most of the crop plants, sensitivity to salinity is commonly (but not exclusively) due to the abundance of Na^+ in the soil. The main site of Na^+ entry in roots is uncertain, although it is more likely that as water moves across the root cortex toward the stele, the plant is working on preventing the sodium to reach to the xylem stream. Sodium exclusion has been identified as an energy consuming transport of sodium out into the apoplast or the external soil solution via plasma membrane Na^+/H^+ antiporters. Compartmentation of Na^+ into the vacuoles provides a second, efficient mechanism to prevent the accumulation of Na^+ to toxic

levels in the cytosol. Moreover, the compartmentation of Na^+ and Cl^- into the vacuole allows plants to use NaCl as an osmoticum to maintain an osmotic potential that drives water into the cells. The transport of Na^+ into the vacuoles is mediated by a Na^+/H^+ antiporter that is driven by the electrochemical gradient of protons generated by the V-type H^+- ATPase and the H^+-PPiase[99-105] . In conclusion, reducing Na^+ uptake is one important key, as well as a very efficient approach, to control Na^+ accumulation in crop plants and hence to improve their salt resistance.

At the plasma membrane level, the electrochemical gradient driving passive Na^+ entry will vary depending on the salinity level. Knowing that the electrochemical potential difference across the plasma membrane is highly negative (approximately −140 mV), even low extracellular Na^+ concentrations will establish a large Na^+ electrochemical potential gradient that will favor the passive transport of sodium from the environment into the cytosol[101]. At moderate to high salinity, the chemical driving force will become progressively larger and in salt tolerant cell types, able to withstand high levels of salinity, the ratio of Na^+ concentration across the plasma membrane might well exceed fivefold[101].

The efflux of Na^+, mediated by the plasma membrane Na^+/H^+ antiporter that couples the downhill movement of H^+ into the cell along its electrochemical gradient to the extrusion of Na^+ against its electrochemical gradient, is driven by the proton motive force generated mainly by the plasma membrane H^+-ATPase[106]. The H^+-ATPase uses the energy of ATP hydrolysis to pump H^+ out of the cell, generating the electrochemical H^+ gradient.

A cytosolically-directed electrochemical potential difference for Na^+ is also normally present across the vacuolar membrane. Working on salt stressed tissue suggest that sodium concentration is usually 2 to 8 fold higher in the vacuolar lumen than in the cytoplasm [99,107-109]. The transport of Na^+ into the vacuoles is mediated by a Na^+/H^+ antiporter, which is driven by the electrochemical gradient of protons generated by the vacuolar H^+-translocating enzymes, the H^+-ATPase and the H^+-

PPiase[110] . While salt-sensitive plants depend mainly on exclusion of Na^+ ions at the plasma membrane, salt-tolerant species accumulate large amounts of Na^+ in the vacuoles.

1.4.1 Possible candidates involved in sodium uptake

Most of the work done on the possible passage for sodium into the cytoplasm was based on the observation that Na^+ can reduce K^+ requirement[111,112] . The basic assumption was, that in the absence of external K^+, uptake of some Na^+ is better than no monovalent cation at all[113]. If Na^+ were taken up as a substitute for K^+ a decrease of the cellular pH could be avoided[114]. A low amount of Na^+ is unlikely to be toxic in the cytoplasm, and even high amounts of Na^+ are not toxic if sequestered in the vacuole[101,112]. In this section we review the transporters that possibly facilitate the transport of sodium into the cytoplasm.

1.4.1.1 NSCCs (Non-Selective cation channels)

In plants, several categories of NSCCs have been identified and subdivided according to their response to changes in membrane electrical potential[115,116] into the following major classes: (1) depolarization-activated NSCCs (DA-NSCCs), (2) hyperpolarization-activated NSCCS (HA-NSCCs), and (3) voltage-insensitive NSCCs (VI-NSCCs). Additional classification systems distinguish NSCCs by their responsiveness to certain ligands and physical stimuli and include cyclic-nucleotide-gated NSCCs (CNGCs), amino-acid-gated NSCCs (AAG-NSCCs), and reactive-oxygen-species-activated NSCCs (ROS-NSCCs).

Nonselective cation channels (NSCCs) catalyse passive fluxes of cations across the plant plasma membrane. NSCCs have a poor selectivity between monovalent cations and several are also permeable to divalent cations. Although a number of NSCC genes has been identified in plant genomes, a direct correlation between gene products and *in vivo* observed currents is still largely absent for most NSCCs[117]. In general, these NSCC channels display considerably lower selectivity for K^+ over Na^+. Electrophysiological studies suggest that Na^+

influx across the plasma membrane occurs via NSCCs in root cortical cells[118-120]. Moreover, NSCCs are considered the main pathways for Na^+ entering into root cells in high salinity conditions, based on the observed alleviation of sodium upon the blocking of the NSCCs[120].

According to Amtmann and Sanders[118] the physiological function of NSCCs might be providing sufficient background conductance to allow continuous operation of the electrogenic H^+-pump. Demidchik and Maathuis[115] summarized the physiological function of the NSCCs and suggested that NSCCs are directly involved in a number of stress responses, growth and development, uptake of nutrients and calcium signalling. NSCCs can also function in the perception of external stimuli and as signal transducers for reactive oxygen species, pathogen elicitors, cyclic nucleotides, membrane stretch, amino acids and purines.
In conclusion, NSCCs probably play a physiological role in Na^+ uptake in at least some species. However they are not the sole pathway for the transport of sodium across the plasma membrane[117,121].

1.4.1.2 HKTs (High affinity K^+ transporters)

HKTs are members of a large super family of transporters found in plants, bacteria, and fungi[122]. In a breakthrough study in 1994[123] a high-affinity transporter for potassium, originally named HKT1 (and now known as TaHKT2;1), was isolated from wheat. Its characterization as a K^+ transporter was based upon transcripts isolated from plants grown under potassium-deprivation conditions, which induce high-affinity potassium transport[124,125].

The HKT proteins can be divided into two distinct subfamilies according to their Na^+ and K^+ transport properties in heterologous expression systems: 1) **subfamily AtHKT1;1**, showing Na^+-specific transport activity and mediating Na^+ uptake in the absence of external K^+. HKTs has been identified in different plants for example: *Arabidopsis*[126,127],rice[128], wheat[128,129] and barley[128,130] 2) **subfamily TaHKT2;1** functions as a K^+-Na^+ cotransporter and its homologs have been cloned from many species of plants, including *Eucalyptus*[131,132], barley[133,134],rice[128] and wheat[123,128] .

In recent years, more and more research has been focused on the functions of HKT-type transporters in controlling retrieval of Na^+ from the xylem, reducing Na^+ accumulation in leaves and enhancing salt resistance[127,129,135,136]. Tissue specific regulation conferred through manipulation of AtHKT1;1 gene expression was shown to be critical for plant salt resistance[137]. The functions of HKT-type transporters are very complex and much more work is required to properly elucidate the functions of this very important gene family.

1.4.1.3 LCT (low-affinity cation transporters)

A further candidate for mediating Na^+ uptake is LCT1 identified from wheat[138]. LCT1 was expressed in low abundance in wheat roots and leaves and they were shown to mediate low-affinity uptake of several cations (i.e. Rb^+ Na^+ and Ca^{2+})[138]. LCT is unlikely to be the major pathway of Na^+ influx[139], because a concentration more than 1 mM for Ca^{2+} in soil (which is most likely exist) is high enough to inhibit the Na^+ transport through LCT1[140].

1.4.1.4 AKT1 (*Arabidopsis* K^+ transporters)

The first channels identified in plants were two K^+ channels from *Arabidopsis*, AKT1 (*Arabidopsis* K^+ transporter 1)[141] and KAT1 (K^+ *Arabidopsis thaliana* channel 1)[142]. It has been proposed that AKT1 could mediate a significant proportion of Na^+ uptake with an increase in external Na^+ concentrations[101,118] .

1.4.1.5 KUP/HAK/KT (potassium transporters) group of proteins

Plants contain multiple transporters for high-affinity K^+ uptake, the AtKUP family. This family of transporters may be important for both high- and low-affinity K^+ uptake into various plant cell types[143]. K^+-uptake mediated by members of this family of transporters appears to be sensitive to low concentrations of Na^+. Both low and high-affinity K^+ uptake in *Arabidopsis* via AtKUP1 were inhibited by 5 mM or higher concentrations of NaCl[144].

1.4.1.6 CCCs (cation chloride cotransporter)

CCC proteins are secondary active transporters that mediate the movement of Cl^-, tightly coupled to that of K^+ and/or Na^+ across the plasma membrane[117]. Regarding the ions involved in the symport mechanism, CCCs are divided into three groups: K^+-Cl^- cotransporters, known as the KCC group; Na^+-Cl^- cotransporters, NCC group; and Na^+-K^+-$2Cl^-$ cotransporters, NKCC group[145] . Members of all three groups share an absolute requirement for both Cl^- and at least one cation (Na^+ and/or K^+), and that the three cotransport processes are electrically silent or electroneutral[117].

1.5 Sodium versus potassium: competition or selectivity?

In order for a plant to counteract salinity stress it should be able to maintain the K^+ level in their cytoplasm[146,147]. It is clear that net selectivity for K^+ over Na^+ differs between species and particularly between mono-and dicotyledonous halophytes[148]. Under normal conditions, with cytosolic K^+ being around 150 m M and cytosolic Na^+ in a much lower range (1-10 mM)[114], this high K^+/Na^+ ratio enables normal cell metabolism (i.e. cell osmoregulation, turgor maintenance, stomatal function, activation of enzymes, protein synthesis, oxidative metabolism and photosynthesis)[149,150]. However, under salinity stress the K^+/Na^+ ratio can fall dramatically[146]. This occurs as a result of both excessive Na^+ accumulation in the cytosol [151] and enhanced K^+ leakage from the cell[36,152], the latter resulting from NaCl-induced membrane depolarization under saline conditions[36]. For the previous reasons, it was suggested that the high K^+/Na^+ ratio is an important characteristic for salt tolerant plants, which has encouraged the idea of using it as a screening tool for salt tolerance[29].

However, some reports have pointed out that there is no relationship between salt resistance and K^+/Na^+ selectivity. It was proposed that the K^+/Na^+ selectivity of a cation channel in the plasma membrane of root cells does not differ between salt-tolerant and salt-sensitive wheat species[153]. It was found that the K^+/Na^+ ratio could not explain the variation in salt tolerance among the genotypes of bread wheat,

suggesting that Na^+ exclusion and tissue tolerance varied independently, and there was no significant relationship between Na^+ exclusion and salt tolerance in bread wheat[154]. Also, both K^+/Na^+ ratio and K^+/Na^+ selectivity were not found to be correlated with the relative salt tolerance of *Brassica* species, indicating that a high shoot K^+/Na^+ ratio or K^+/Na^+ selectivity may not be a reliable selection criterion for salinity resistance in some species[155]. In rice, bypass flow provides an additional pathway for sodium uptake, contributing to the functional and genetic independence of sodium and potassium uptake and consequently for the lesser prominence of K^+: Na^+ discrimination in rice than in wheat[156].

For glycophytes, growth in saline soils results in an over-accumulation of Na^+ in the leaf tissue causing premature leaf senescence. For several crop plants, it has been empirically observed that a significant component of salinity tolerance is the ability to exclude Na^+ from the shoot[4,13,147]. Notably, this correlation between salinity tolerance and exclusion of Na^+ from the shoot has been found in several plant species such as wheat[157] and rye[147], although exceptions have been documented in bread wheat (*Triticum aestivum*)[154] and rice[61].

1.6 Role of Na^+/H^+ antiport in sodium exclusion

In higher plants, the main mechanism for Na^+ extrusion is powered by the operation of the plasma membrane H^+-ATPase[106]. The H^+-ATPase uses the energy of ATP hydrolysis to pump H^+ out of the cell, generating an electrochemical H^+ gradient. This protonmotive force generated by the H^+-ATPase allows the operation of plasma membrane Na^+/H^+ antiporters that induces the extrusion of Na^+ against its electrochemical gradient. Na^+/H^+ antiporter activity has been reported to occur across the plasma membrane of barley[158,159], tobacco[160], red beet[161], *Atriplex nummularia*[162], tomato[163], blue-green algae[164] and the halotolerant alga *Dunaliella salina*[165]. The plasma membrane Na^+/H^+ antiporter activity increased in *Dunaliella salina*[165] and in the halophyte *Atriplex nummularia*[162] when the NaCl concentration of the external medium was increased. In *Atriplex*, the NaCl-dependent increase in Na^+/H^+ antiporter activity was correlated with an increase in plasma membrane H^+-

ATPase activity[166]. An increase in steady state transcript levels indicated an up-regulation of the *Atriplex* plasma membrane H^+-ATPase in plants treated with 400 mM NaCl [35].

Vacuolar Na^+/H^+ antiporters have been investigated as an important key to salt tolerance in plants[101]. The role of the tonoplast Na^+/H^+ antiport in salt tolerance has been indicated from several studies explaining the induction of antiport activity upon acclimation to salt[100,167-169]. On the other hand, vacuolar Na^+/H^+ antiport activity has also been detected in tonoplast vesicles isolated from glycophytic species, such as roots of barley[170], sunflower[171] and rice[172].

The absence of vacuolar Na^+/H^+ antiporter activity may be related to a general property observed in salt-sensitive plants that rely on extrusion of Na^+ ions at the plasma membrane and not in the accumulation of Na^+ in the vacuoles[100]. Nonetheless, mechanisms that regulate the activity of the vacuolar Na^+/H^+ antiporter and/or its expression under normal growth conditions and during salt stress have not yet been elucidated. The activity of the vacuolar primary H^+-pumps, that provide the driving force for the operation of the vacuolar Na^+/H^+ antiporters, is regulated by sodium. Increases in tonoplast H^+-ATPase activity in response to NaCl has been reported for barley roots[173], mung bean roots[174] and sunflower roots[171].

1.7 What about chloride?

The question is often asked: "Why focus only on Na^+, why not also consider Cl^-?" Both Cl^- and Na^+ may cause injury. The chloride toxicity is of great concern in species that accumulate high concentrations of Cl^- and not Na^+ in leaves, such as soybean, woody perennials such as avocado[8], and those species that are routinely grown on Cl^--excluding rootstocks such as grapevines and citrus[175]. For these species, Cl^- toxicity is more important than Na^+ toxicity. However, this statement does not imply that Cl^- is more metabolically toxic than Na^+. The reason for this is that these species are better at excluding Na^+ than Cl^- from the leaf

blades. For example, Na^+ does not increase in the leaf blade of grapevines until several years of exposure to saline soil, then the exclusion within the root, stem, and petiole breaks down and Na^+ starts to accumulate in the leaf blade, whereas leaf blade Cl^- concentrations increased progressively over these years[176]. Thus, Na^+ may be a more toxic solute, but because the plant is managing the Na^+ transport better than Cl^- transport, Cl^- becomes the potentially more toxic component. The evidence for this is the association between genetic differences in the rate of Cl^- accumulation in leaves and the plant's salinity tolerance. This difference may arise because Na^+ is withheld so effectively in the woody roots and stems that little reach the leaves, and K^+ becomes the major cation.

1.8 Life from different perspectives: comparison between halophytes and glycophytes.

Halophytes require electrolyte concentrations (e.g. Na^+ and Cl^-) which are higher or much higher than those found in non-saline soils for optimal growth. The range for optimal performance of halophytes can be roughly defined to be between 20 and 500 mM NaCl[2,20,148]. The metabolic machinery of halophytes is not found to be insensitive to high sodium and chloride[2,35]. Instead, plants survive and grow in saline environments by osmotic adjustment through intracellular compartmentation that partitions toxic ions away from the cytoplasm through energy-dependent transport into the vacuole[108,35,109]. Some halophytes exclude Na^+ and Cl^- through glands and bladders, which are specialized structures, enabling halophytes to survive in a very saline soil.

Osmotic adjustment of both halophytes and glycophytes is also achieved through the accumulation of organic solutes in the cytosol, and the lumen, matrix, or stroma of organelles[35]. Unlike ions, organic solutes do not affect the enzyme activities even at high concentration. A principle difference between halophytes and glycophytes is the capacity of the former to survive salt shock[35,162] and essentially have the ability to sequester toxic ions in the vacuole.

Glycophytes restrict ion movement to the shoot by attempting control of ion influx into root xylem, and sequester any salt they absorb in the root and stems thus minimizing the exposure of leaf cells to salt[8]. It seems that a major advantage that halophytes have over glycophytes is not only more responsive Na^+ partitioning, but also a more effective capacity to coordinate this partitioning with processes controlling growth, both at the cellular and whole plant level. This may explain why halophytes can use, and perhaps rely on, Na^+ and Cl^- for osmotic adjustment to supports cell expansion in growing tissues and turgor in mature organs [20,20].

In a saline environment, the ability to take up and confine Na^+ to leaves lowers the osmotic potential of aerial plant parts; this then facilitates water uptake and transport and lowers the metabolic cost for the production of osmolytes. Therefore, halophytes use this cheap strategy (i.e. ion accumulation) for osmotic adjustment. However, the osmotic benefits of storing Na^+ and Cl^- as abundant, cheap osmolytes are limited by the available vacuolar space. Therefore, continued growth, i.e. the production of new vacuoles, may be a factor limiting tolerance[148].

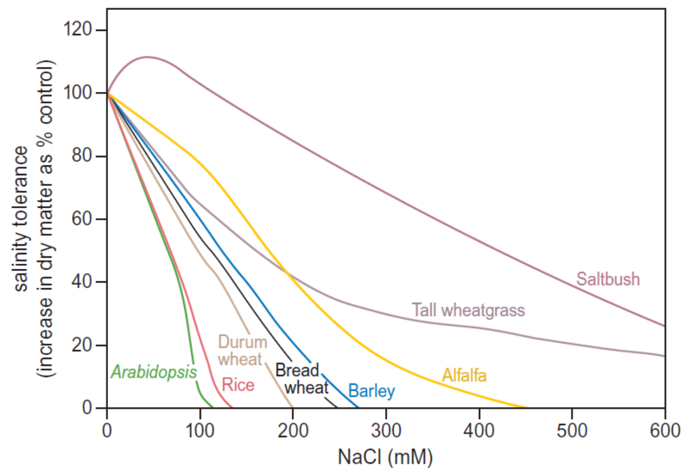


Figure 1.3. *Diversity in salt tolerance between different plant species indicated as increases in shoot dry matter relative to the control plants after growth on salt affected salt for at least three weeks (Adapted from[12]).*

1.9 Mycorrhiza and salt tolerance

Mycorrhiza is the result of a mutualistic association between a fungus and a plant. This mutualism takes place at the root level, where individual hypae extending from the mycelium of a fungus colonize the roots of a host plant, either intracellularly or extracellularly[177].

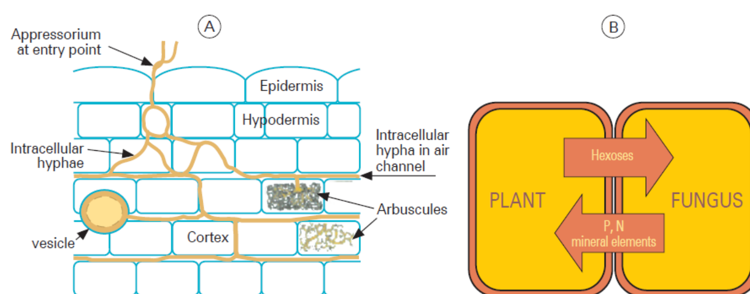


Figure 1.4. A: *Different mycorrhizal associations inside plant cells.* **B:** *Symbiotic relationship between the fungi and the plant cell*

In saline soils, Arbuscular Mycorrhiza (AM) occur naturally[178]. Although salinity might affect the formation and functioning of mycorrhizas[179,180], several studies have demonstrated that inoculation with AM fungi improves growth of plants under a variety of salinity stress conditions[181-183]. Therefore, AM fungi have been considered as bio-ameliorators of saline soils[184].

Some investigations have suggested that improvement of the plant's phosphorus (P) status is the most important mechanism of salinity stress tolerance in AM plants[181,182,185]. However, other studies have shown that mycorrhizal plants grow better than non-mycorrhizal plants under salt stress even when mycorrhizal and non-mycorrhizal plants have a similar P status[186,187], implying that the advantages of AM for plant growth and development under salt stress are not always related to P status improvement. Ruiz-Lozano *et al.* [188] and Feng *et al.* [186]concluded that the mechanisms underlying AM plant growth improvement under saline conditions, are based on physiological processes such as increased carbon dioxide exchange

rate, transpiration, stomatal conductance and water use efficiency rather than on nutrient uptake (N or P).

It was observed that arbuscular mycorrhizal plants are able to take up more water from the soil than non AM plants under water deficit conditions[189,190]. However, this capacity of AM fungi depends on the fungal species, *Glomus intraradices* is believed to be the most efficient AM fungus in enhancing plant water uptake from the soil among six fungi tested by Marulanda *et al.*,[189]. To our knowledge there is no information about the mechanism by which root hydraulic properties are influenced by AM symbiosis under salt stress.

Porcel *et al*. [191] found that, under water-deficient conditions, the AM fungus *Glomus mosseae* accelerated the decrease of a plasma membrane intrinsic proteins (*PIP)* gene expression in roots of *Glycine max* plants and diminished the expression of two *PIP* genes in roots of *Lactuca sativa* plants. On the other hand, Ouziad *et al*. [192] found that inoculation with a mixture of AM fungi decreased the expression of one *PIP* gene in the roots of tomato plants under saline conditions. Unfortunately, no measurements of root hydraulic properties were made in either study.

Ouiziad *et al.* [192] found reductions in transcript levels of a plasmalemma aquaporin gene in tomato plants subjected to salt stress. Interestingly, down-regulation of the aquaporin genes occurred at an earlier stage in mycorrhizal plants than in non-mycorrhizal plants in the study by Porcel *et al.* [192]. Despite of the focus on AM fungi in the last few decades, the effects of AM fungal colonization on the physiological characteristics of the plant growth and nutrient uptake under salt stress are still poorly understood.

1.10 The MIFE: Microelectrode ion Flux estimation

1.10.1 Principles of the MIFE ion flux measurements [193].

Newman[194] provides a very readable explanation of the theory of non-invasive MIFE ion flux measurements. In short, if an ion is taken up by a living cell or a tissue, its concentration in the unstirred layer surrounding the cell or tissue close to surface will be lower than at a position away from the surface. On the other way around, if the ion is extruded out of a living cell or tissue, there will be a pronounced concentration gradient directed away from the cell/tissue surface. Ions in solution move down a concentration gradient and also down an electrical potential gradient. Consequently, if the combined electrochemical potential gradient is measured, the net ion flux ($mol.m^{-2}.s^{-1}$) can be calculated from that gradient using the Nernst equation[194]:

$$J= c\ u\ z\ F\ g\ (dV/dx).$$

where c is the ion concentration ($mol.m^{-3}$); u is the ion mobility (ms^{-1} per Newton mol^{-1}); z is the ion valency; F is the Faraday number (96 500 C mol^{-1}); g is a factor found from the measured Nernst slope for the electrode during calibration; dV is the voltage gradient measured by the electrometer between two positions (V); and dx is the distance between two positions (Figure 1.5a). Ions crossing the tissue/cell surface are carried to or from that surface by diffusion in the unstirred layer adjacent to that surface. The MIFE technique uses a slow square-wave movement of ion-selective electrode probes between two positions, close to (position 1), and distant from (position 2) the sample surface (Figure 1.5a). Recorded at the two positions, voltage characteristics are converted into concentration parameters using the calibrated Nernst slopes of the electrodes. Net fluxes of specific ions can then be calculated from the measured voltage gradient at the surface. Different equations are used for objects of different basic geometry (e.g. having cylindrical, spherical or planar diffusion profiles)[194,195].

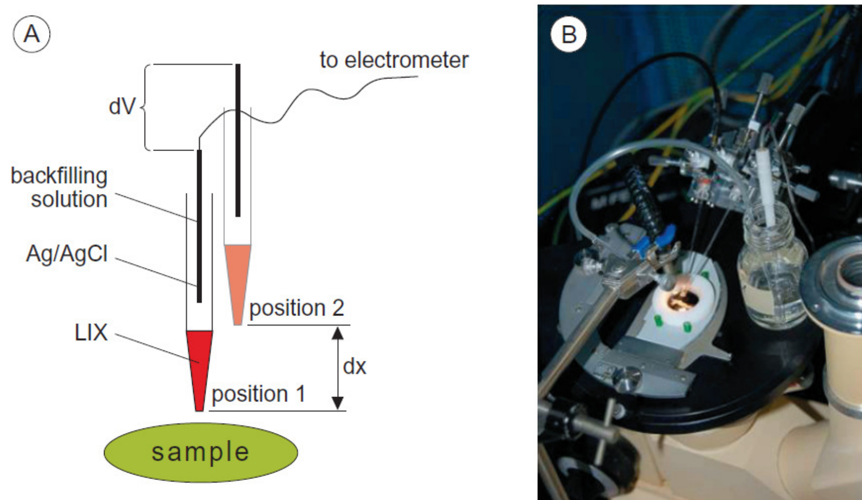


Figure 1.5. A: *Schematic representation of the vibrating microelectrode configuration, positions and movement during measuring the ion fluxes from the surface of cell/tissue.* **B:** *the setup of the measuring chamber on the stage microscope and the mounting of the electrode for the selected ion required to be measured.*

The MIFE software performs the required calculations automatically based on the geometry of the measured tissue/cell dimensions and probe distance above the cell surface and provides tabulated results of measurements as net ion fluxes (nmol^{-2} s^{-1}). The MIFE experimental setup (Figure 1.5b) is built around an inverted microscope system and the microelectrode movement is controlled by a micromanipulator connected to the MIFE computer.

1.10.2 Possible applications of the MIFE system

MIFE system was used to measure the ion fluxes from the surface of plant tissue i.e. leaf[152], stem[196] or root[29,36,197]. And recently it was also used to indicate the proton fluxes from bacterial cells[193] and from protoplasts[198]. Plants are sessile and this forces them to deal with different environmental changes in the surrounding media. Challenging plant tissue or cells by external stimuli is often accompanied by changes in ion fluxes. This makes the MIFE technique an easy way to study different stress factors i.e salt[29,152,197] and osmotic stress[199], and plant responses to

external stimuli, i.e. light[200-204] or pathogens[204-206] . In reaction to the apparent versatility of the use of the MIFE system for all kinds of plants responses, experienced in our and other laboratories, we have come to the conclusion that ”if humans responds in words, plants respond with fluxes and MIFE can read that response”.

1.11 Aim of the thesis

The aim of this thesis is to analyze the response of *Zea mays* L. cultivars, contrasting in their salt tolerance, to NaCl stress at whole plant and cellular level. Maize is a good candidate for this type of study since it is classified as a moderately salt-sensitive crop plant. Maize is also a major crop plant, globally taking the third place after wheat and rice in human daily consumption. Comparative studies are preferred as they provide insights in the tolerance mechanisms to salinity with the hope that it can also show us a way to improve the productivity of maize in saline soils. The general research question in this work is*: is there genotypic variation between the studied maize cultivars in their response to salt that can determine the level of salt tolerance?*

The second question that we addressed is: *what is the strategy of maize plants when coping with salt stress?*

1.12 The thesis outline

We start with studying the effect of salt stress on whole plant growth, development and regulation of different physiological processes upon salt stress (Chapter 2). The impact of salt stress on different physiological parameters, including photosynthesis, stomatal conductance and accumulation of solutes, was measured.

Recently[207], the MIFE technique was proposed as a fast and reliable screening tool to determine salt tolerance in barley. Changes in K^+ effluxes upon salt addition were correlated with salt tolerant in seven barley species[207]. This correlation was not confirmed for more salt sensitive cereals as wheat[199]. In Chapter 3, we use the

MIFE technique to characterize the changes in root K^+ and H^+ fluxes upon salt treatment, and study the kinetics behind the observed fluxes instantly after the addition of salt. The root of a 3 day old seedling was used as a tool to determine the profile of ion fluxes along the root and the possible effect of NaCl on it. Our main aim was to test if the NaCl-induced potassium fluxes can be used as a screening tool for salt tolerance in maize plants.

Ion fluxes measured adjacent to the root surface can be masked, as the cation exchange capacity of the cell wall can profoundly change the ion flux magnitude and dynamics. To circumvent this effect of the cell wall, we used protoplasts as a tool to study the effect of NaCl in proton transfer across the plasma membrane and the possible involvement of the p-type H^+-ATPase and the Na^+/H^+ antiport activity in salt tolerance, in chapter 4. Salt-acclimated and non-acclimated plants were used in order to highlight the changes in proton fluxes induced if the plant was more or less adjusted to salt.

Occasionally, during a flux measurement on protoplasts, probably due to slight changes in the osmotic concentration of the bath solution upon salt addition, a clear change in fluxes was observed. On the video monitor it was obvious that these strange fluxes were an indication of the release of a vacuole from the protoplast (Figure 1.6). These serendipitous recordings of tonoplast fluxes were the inspiration for the experiments reported in Chapter 5, using the MIFE system to study Na^+/H^+ antiport activity on intact maize vacuoles and compare this with fluxes on vacuoles isolated from red beet, for which Na^+/H^+ antiport acitivity is well documented[208].

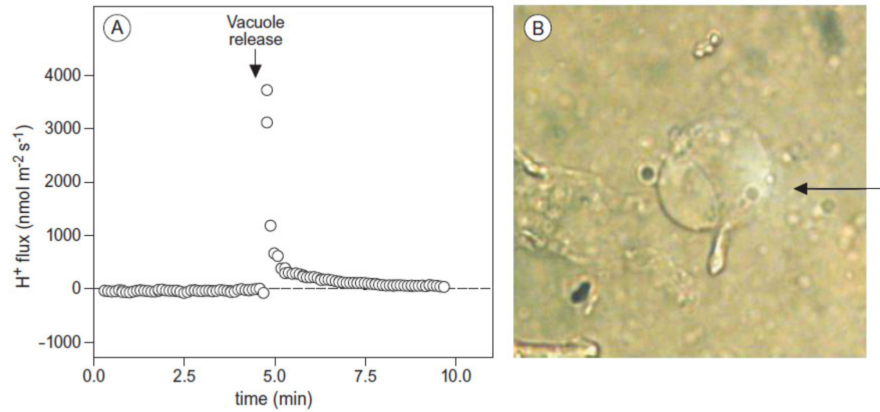


Figure 1.6. **A:** *Fluxes recorded from a protoplast isolated from the root of a 3 day old maize seedling, the arrow represent the point where the protoplasts is loosing the cytoplasmic strands and the vacuole is isolated.* **B:** *vacuoplast (vacuole with cytoplasmic residues attached to it (pointed by arrow)).*

Despite all the work on salinity over the years, the solution for salinity stress problems is still far from being achieved, but several attempts were successful in improving the crop production under salt stress[6]. In this work we test one possible bio-ameliorator for salinity stress, the arbuscular mycorrhiza (AM) fungi. In Chapter 6 the effects of arbuscular mycorrhiza fungi on plant growth and root ion fluxes under control and salt treatment are described.

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Chapter 2

The Effect of Salt on the Growth, Biochemical Parameters and Mineral Content of Maize (*Zea mays* L.)

ABSTRACT

Three cultivars of maize (Zea mays L.); Delitop, Arizona 8601 and SR15 were subjected to four salt concentrations (0, 50, 100, 150 mM NaCl) and their response to salt stress was determined by measuring shoot, root dry weight, relative growth rate and leaf area after exposure to salt for 3, 7, 10 and 14 days. In addition, the effect of exposure to salt on physiological parameters such as the leaf sap osmolality, leaf Na^+ and K^+ content and K^+/Na^+ ratio, rate of photosynthesis and stomatal conductance was measured. The results indicate the presence of genotypic variations between the maize cultivars in response to salt stress. SR15 act as a moderately salt-tolerant glycophyte which maintains plant growth, leaf area, photosynthetic rate, stomatal conductance by increasing the leaf sap osmolality, but excluding Na^+ at the root level and preventing sodium accumulation in the shoot. The other extreme, Arizona 8601, seems to survive high levels of salt by reduction of shoot growth, and increase in sodium uptake, even at short periods of salt stress, which is possibly used for osmotic adjustment. This survival strategy was most obvious at 10 and 14 days of salt exposure.

2.1 INTRODUCTION

During the last few decades the mechanism of salinity tolerance in plants, from the whole plant level to the cellular and gene level, has been the subject of numerous studies. However, parts of the mechanisms of plant salt tolerance are still mysterious[1]. Approximately 7% of the total land surface of our planet is saline[2,3]. Many arid and semi-arid regions in the world have soils and water resources that are too saline for most of the common economic crops[4]. Almost one third of the irrigated lands is suffering from secondary salinization[2] an alarming situation that is referred to as “the quiet crisis” [5,6].

Crop plants are relatively salt-sensitive and are unable to tolerate even low levels of salinity[7,8]. In this work we focus on maize which is considered the third most important cereal crop in the world, after wheat and rice, and is grown all over the world under different climatic conditions. It is considered a salt-sensitive species[9] and the most salt-sensitive of the cereals[9,10]. But cross pollination and specific breeding has caused high polymorphism through the course of natural and domestic evolution and in the enormously wide selection of maize cultivars currently present[11] salinity-tolerant varieties may exist.

We included three maize genotypes in this study: SR15 obtained from the Institute for Plant Nutrition in Giessen. This is a newly developed hybrid which was successfully established by a breeding program that depends on physiological traits, where an efficiently Na^+-excluding inbred line was crossed with inbred lines with high osmotic resistance[12]. The SR hybrids showed more salt tolerance than even the parental hybrids. Schubert *et al* [13] suggested that the SR hybrids can be an ideal tool to study physiological traits that might contribute to salt tolerance in maize.

The second maize genotype is Arizona 8601[14] a germplasm for salty soils which is the result of a natural selection program by the Arizona Agricultural Experiment

Station in Tucson, which can be grown in soils containing up to 6000-7000 ppm TSS (Total Soluble Salts) and irrigated with saline water (2000-4000 ppm TSS). Arizona 8601 performed better than Pioneer 3183 in both silage (+38%) and grain (+26%) production under the conditions in which it was selected. The physiological mechanism(s) underlying the salt tolerance of Arizona 8601 is not known.

The third genotype is Delitop, a commercial cultivar not known for its stress tolerance that has not been used in studies on salt tolerance previously. The work reported here focuses on examining the growth performance and physiological traits of three different maize hybrids exposed to various levels of salt in their growth media. The first question that we addressed is whether different mechanisms of salt tolerance are present in different maize genotypes.

According to the two phase model of growth response to salt stress proposed by Munns[15], two major physiological problems hinder the growth of plants under salt stress. In the first phase the reduction of water potential in the soil solution leads to osmotic imbalance and loss of turgor, which stunt the growth of the plant. This first phase is also called physiological drought. We studied the possible role of osmotic adjustment in protecting the plant growth under salt stress and how this develops with increasing periods of salt stress in the three genotypes.

In the second phase of salt stress, ionic toxicity due to accumulation of sodium and chloride in the cytoplasm can severely inhibit the growth of sensitive plants. For maize and other members of the Poaceae it has been shown that sodium toxicity and not chloride toxicity is the major problem in the second phase of salt stress[16,17]. Sodium exclusion was documented to be one important criterion that determines salt tolerance in some maize genotypes[18]. Sodium has its detrimental effect on cell metabolism by replacing K^+ in essential enzymatic reactions occurring in the cytoplasm. Therefore, it is not primarily the concentration of Na^+, but the K^+ to Na^+ ratio in the cytoplasm that determines toxicity[19,20]. The second aim of this study is

to evaluate the role of sodium exclusion and the potassium to sodium ratio in salt tolerance of the studied maize genotypes.

A salt-stressed plant will have an altered physiology, yellow leaf blades and leaf tips and reduced rate of CO_2 assimilation. Osmotic tolerance in cereals involves the plant's ability to tolerate the physiological drought aspect of salinity stress and to maintain leaf expansion and stomatal conductance[8]. Osmotic adjustment, or osmoregulation, is generally regarded as an important adaptation to drought or salinity. Because it helps to maintain turgor and cell volume, it is often thought that the plant which has a better osmotic adjustment, maintains growth, yield and survive better in dry or saline soils[21].

Combining two genotypes as SR15 and Arizona 8601 with a well-documented salt tolerance with a commercial cultivar (Delitop) in a study can give a better understanding of maize salt tolerance mechanisms and may have the potential on creating even a more salt-tolerant genotype, by combining or even building stable, distinguishable and heritable traits in a breeding program.

2.2 MATERIAL AND METHODS

2.2.1 Plant material

Three maize (*Zea mays* L.) cultivars were used: Delitop (provided by Syngenta Seeds, Saint Sauveur, France), Arizona 8601(a gift from Dr. Michael J. Ottman at the University of Arizona, College of Agriculture and Life Sciences, Tucson, AZ, USA) and SR15 (2005) (a gift from Prof. Sven Schubert of the Institute of Plant Nutrition, Justus-Liebig University, Giessen, Germany).

2.2.2 Plant culture

The *Zea mays* caryopses of the three cultivars were soaked in 1% sodium hypochlorite for 10 minutes and then rinsed for 15 minutes in tap water. The caryopses were germinated in a germination box on filter paper moistened with tap

water. The caryopses were then placed in a dark incubator adjusted at 23℃ for four days.

After 5 days the caryopses were transferred into 30 liter plastic containers filled with aerated full strength Hoagland solution with 20 plants per container. The containers were placed in the green house of the Biological Center in Haren, The Netherlands. Day and night temperatures were 22 and 16 ℃, respectively. The relative humidity was 35 to 45% and the photoperiod was 14 hours at a photon fluency rate of 100 ± 20 $\mu mol.m^{-2}.s^{-1}$. This experiment was conducted from the end of February till the mid of April. The Hoagland solution was renewed once a week until the formation of the fourth leaf, from then on the solution was changed twice a week.

2.2.3 Experimental design

The experimental design consisted of three different genotypes (Delitop, Arizona 8601, SR15 (2005)) with four salinity levels of 0, 50, 100 and 150 mM NaCl. NaCl was added after 6 days of growth of the seedlings on the 30 liter containers. The concentration of salt was added in increments of 25 % of the final salt concentration per day over a period of four days.

2.2.4 Plant growth

The leaf growth and expansion was quantified by measuring the width and the length of the leaf. The leaf area was calculated by multiplying length and width and dividing by two[22]. After the fresh weight of the whole plant, shoot, root, leaf, and stem were measured, the plants were dried in an oven at 80 ℃ for at least 48 h and the dry weights were determined.

The relative growth rate (RGR) was determined on a dry weight basis according to Hunt[23] as follows: $RGR = (\ln W_2 - \ln W_1)\ (t_2 - t_1)^{-1}$ ($mg.mg^{-1}.d^{-1}$). Where, W_1 is the initial total plant dry weight prior to salt treatment at time (T_1), W_2 is the final total plant dry weight after salt treatment for a period of time (T_2). The plants were

analyzed every 3, 7, 10 and 14 days on salt with n=3 per harvest being picked randomly from the 30 liter plastic container where the plants were grown.

2.2.5 Leaf sap osmolality

Osmolality was determined by squeezing 0.2 gram of the youngest fully developed leaf in a syringe. The extracted shoot sap was used to determine the osmolality with a Wescor HR-33T dew point micro voltmeter. The data is represented as mosm/g.

2.2.6 Photosynthetic rate and stomatal conductance

Photosynthetic rate was measured using the portable, open-flow gas exchange analyzer (TPS-2, PP Systems Inc., Amesbury, MA, USA). The youngest, fully developed leaf was introduced into the cuvette and the reading was taken in ambient light intensity (100 ± 20 µmole $m^{-2}s^{-1}$). The stomatal conductance of a representative area of the leaf area was measured simultaneously. The flow rate of air through the leaf chamber was set to 300 ml min^{-1}.

2.2.7 Cation analysis

The concentrations of Na^+ and K^+ were determined in ground, oven dried (80 °C) leaf samples by atomic absorption spectrometry (Varian, SpectrAA 220 FS). In the Institute of Plant Nutrition, Justus-Liebig University, Giessen, Germany).

2.2.8 Statistical analysis

Statistical treatment of the data consisted of a factorial analysis of variance using two-way ANOVA, with salt concentration and time for each genotype as independent variables. Values were considered significant if $p < 0.05$.

			Fresh weight (grams)				Dry weight (grams)			
3 days		[NaCl] (mM)	0	50	100	150	0	50	100	150
	shoot	SR15	2.383±0.160	2.047±0.251	2.697±0.323	1.910±0.117	0.132±0.12	0.133±0.019	0.203±0.074*	0.139±0.018
		Arizona 8601	4.260±0.242	2.937±0.490*	1.923±0.520*	2.140±0.052*	0.288±0.014	0.205±0.053*	0.141±0.042*	0.160±0.008*
		Delitop	2.357±0.653	1.817±0.420	1.740±0.216	1.740±0.235	0.153±0.042	0.126±0.018	0.144±0.028	0.143±0.021
	Root	SR15	1.463±0.055	0.943±0.253	1.627±0.049	1.090±0.045	0.067±0.005	0.039±0.007	0.074±0.010	0.054±0.004
		Arizona 8601	2.580±0.886	1.800±0.236*	1.227±0.283*	1.407±0.222*	0.138±0.022	0.116±0.028	0.089±0.028*	0.095±0.031*
		Delitop	0.883±0.306	0.990±0.321	1.113±0.106	1.063±0.066	0.038±0.014	0.037±0.015	0.057±0.008	0.053±0.003
7 days			Fresh weight (grams)				Dry weight (grams)			
		[NaCl] (mM)	0	50	100	150	0	50	100	150
	Shoot	SR15	5.007±0.987	3.950±1.187	3.730±0.078	3.250±0.580	0.336±0.065	0.285±0.119	0.354±0.059	0.253±0.057
		Arizona 8601	9.190±1.988	3.470±1.743*	4.043±0.655*	4.123±0.454*	0.643±0.143	0.238±0.146*	0.334±0.053*	0.333±0.010*
		Delitop	3.567±0.553	2.960±0.281	1.767±0.012	2.353±0.686	0.205±0.025	0.185±0.020	0.126±0.059	0.165±0.054
	Root	SR15	2.540±0.932	1.687±0.206	1.867±0.063	1.867±0.406	0.118±0.039	0.078±0.015	0.091±0.003	0.101±0.023
		Arizona 8601	6.090±1.686	3.103±1.821*	3.393±0.516*	2.897±0.748*	0.298±0.073	0.166±0.102*	0.196±0.034*	0.174±0.044*
		Delitop	1.510±0.140	1.383±0.408	1.077±0.565	0.823±0.161	0.062±0.011	0.060±0.018	0.061±0.027	0.056±0.012
10 days			Fresh weight (grams)				Dry weight (grams)			
		[NaCl] (mM)	0	50	100	150	0	50	100	150
	Shoot	SR15	5.180±0.911	3.423±1.182	4.090±0.783	4.897±1.296	0.355±0.092	0.248±0.115	0.337±0.046	0.422±0.103
		Arizona 8601	11.600±4.077	8.323±2.342	5.187±1.091*	3.420±0.684*	0.934±0.346	0.682±0.248	0.463±0.095*	0.326±0.078*
		Delitop	5.117±1.268	4.000±0.858	3.797±0.212	2.923±0.665	0.316±0.082	0.283±0.050	0.295±0.027	0.240±0.071
	Root	SR15	2.537±0.673	1.943±0.699	2.367±0.345	1.770±0.575	0.126±0.016	0.107±0.039	0.150±0.002	0.114±0.031
		Arizona 8601	6.880±1.758	5.383±2.923	5.427±1.315	2.913±0.892*	0.374±0.114	0.303±0.169	0.300±0.056	0.167±0.065*
		Delitop	1.997±0.593	2.140±0.122	2.447±0.249	1.583±0.385	0.096±0.029	0.106±0.004	0.138±0.020	0.102±0.034
14 days			Fresh weight (grams)				Dry weight (grams)			
		[NaCl] (mM)	0	50	100	150	0	50	100	150
	Shoo	SR15	8.643±2.377	6.553±1.461	6.977±0.707	5.800±1.157	0.578±0.163	0.517±0.131	0.776±0.121	0.610±0.054
		Arizona 8601	15.280±5.873	10.60±3.494	5.363±1.008*	2.670±0.297*	1.134±0.420	0.863±0.269	0.540±0.135*	0.258±0.010*
		Delitop	9.140±2.217	5.950±1.870	4.287±0.404	4.267±0.554	0.634±0.222	0.458±0.157	0.380±2.051	0.391±0.031
	Root	SR15	3.857±1.235	2.513±0.844	2.773±0.373	3.080±0.554	0.191±0.049	0.133±0.0466	0.163±0.025	0.187±0.050
		Arizona 8601	7.593±2.820	10.220±7.422	3.617±0.964	2.323±0.436*	0.450±0.124	0.560±0.406	0.239±0.049	0.133±0.030*
		Delitop	3.483±0.825	2.777±0.640	2.350±0.580	2.660±0.165	0.164±0.037	0.155±0.038	0.130±0.032	0.162±0.025

Table 2.1. *The effects of salt stress on root and shoot fresh and dry weight in three maize cultivars (SR15, Arizona 8601 and Delitop). The plants were left to grow for 3, 7, 10 and 14 days with 0, 50, 100, and 150 mM NaCl added to their nutrient solution. In the table, the shaded cells indicate a significant decrease from control and the double walled cells indicate a significant increase from control. Values is mean of n=3 ± S.D. significance is indicated if $p < 0.05$.*

2.3 RESULTS

2.3.1 Growth

Two definitions have been proposed for salt tolerance. The first definition originates from a physiologist point of view and focuses on the ability of the plant to maintain its growth under high concentrations of salt. The second definition, equally important in ecological perspective, emphasizes the period of survival on the saline conditions[24]. Plant survival in saline soil, although a useful feature for both ecologists and plant physiologists is not a useful trait for agriculturalists for whose yield reduction, which is economically unacceptable, is much more important.

In this study, four concentrations of salt were used (0, 50 100 and 150 mM NaCl), which are also referred to in the text as ‘control’, ‘moderate’, ‘high’ and ‘very high’, respectively, as they apply to a moderately salt-sensitive crop as maize. Salinity treatment had a strong impact on root and shoot growth, on both fresh and dry weight basis (Table 2.1). The impact of salinity, however, differed substantially between maize cultivars. SR15 showed a non-significant decrease in the shoot fresh weight, but even an increase in shoot dry weight with high salt concentration after 3 days of growth. SR15 was the most successful maize cultivar in maintaining its leaf area, which only was significantly decreased at a very high concentration of salt (150 mM) and not before an extended period of salt stress (14 days) (Figure 2.1).

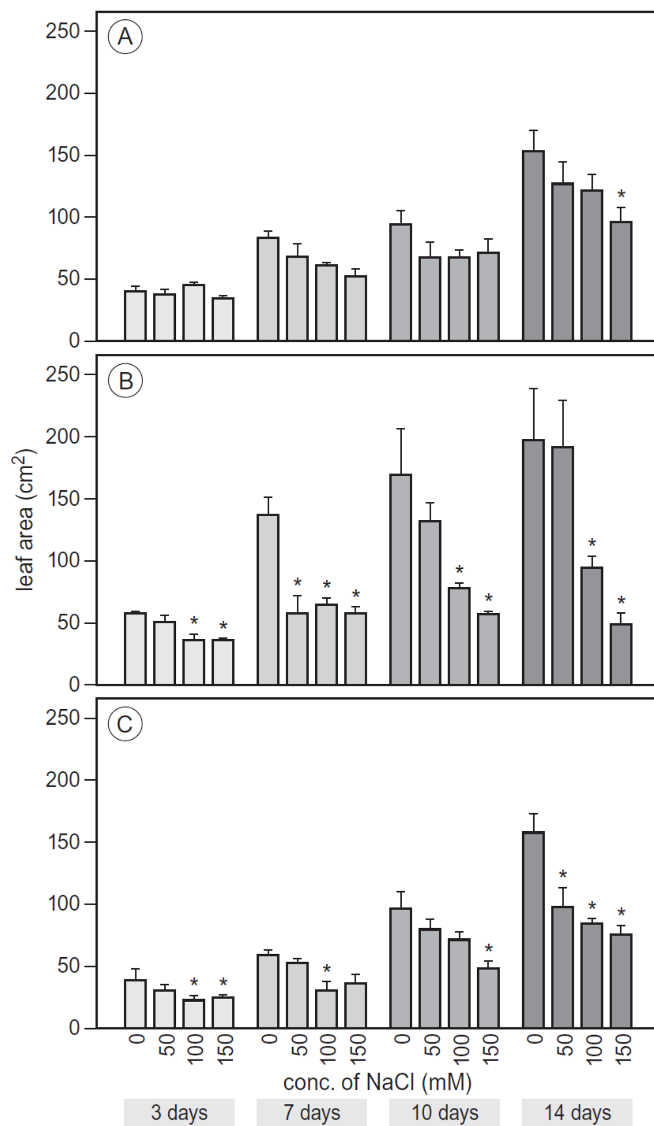

Figure 2.1. *Effect of different salt concentrations on the leaf area of* **A:** *SR15,* **B:** *Arizona 8601 and* **C:** *Delitop grown for 3, 7, 10 and 14 days on salt. Data labelled with (*) are significantly different ($P < 0.05$) between treatments at a given time point.*

Arizona 8601 showed a significant decrease in the leaf area and shoot fresh and dry weight at all the salt concentrations after 3 and 7 days of salt. However, at longer periods of exposure to salt stress the reduction in shoot growth was no longer significant at a moderate salt concentration (50 mM NaCl). The significant reduction of growth of Arizona 8601 gradually disappeared with prolonged exposure to salt (Table 2.1), which indicates an acclimation to the presence of salt in their growth media. After 10 days this characteristic of Arizona 8601 resulted in a higher biomass, compared to the other two cultivars, i.e the shoot fresh weight of Arizona 8601 after 10 days growth on 100 mM NaCl is 5.187 g compared to 4.090 and 3.797 g for SR15 and Delitop, respectively.

Delitop showed no significant decrease in shoot and root fresh weight under the different salt concentrations applied. The root dry weight matter showed the least reduction compared to the other two cultivars with only 20% decrease from the control after 14 days on high salt. The leaf area was reduced significantly with prolonged exposure to 100 and 150 mM NaCl and the effect of salt on leaf area was more significant after 14 days of growth on salt, when all salt treatments showed significant leaf area reduction.

When we want to apply the first definition of salt tolerance, taking the physiological/agricultural point of view, and equating salt tolerance with minimal growth reduction, measuring leaf growth is probably the most sensitive stress parameter[25]. Sensitivity of leaf growth has been used to identify salt tolerance among maize genotypes[22]. According to this criterion the most tolerant maize cultivar is SR15, exhibiting the least reduction in biomass and leaf area, followed by Delitop and with Arizona 8601 coming in last.

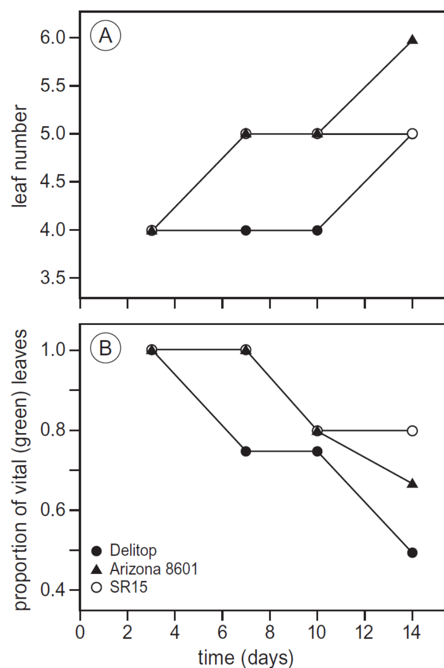

Figure 2.2. *The effect of increasing the exposure time to 150 mM NaCl in the nutrient solution on* **A:** *the formation of new leafs and* **B:** *the percentage of green to brown leafs in the three maize cultivars.*

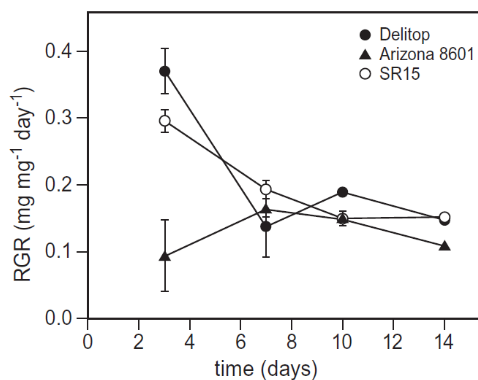

Figure 2.3. *Effect of 100 mM NaCl on RGR (relative growth rate) of three maize cultivars grown for 3, 7, 10 and 14 days on salt.*

However, even though leaf growth is inhibited most by exposure to salt in Arizona 8601, this cultivar was able to from new leaves even in 150 mM (Figure 2.2). Analyzing the RGR of the three plants after growing on 100 mM salt concentration showed that Arizona 8601 was able to maintain a stable RGR with time while in the other two cultivars the RGR decreased significantly (Figure 2.3). Maintaining the RGR under high salt concentration for a period more than 14 days indicates that Arizona 8601 employs survival mechanisms under salt stress that are different from a typical glycophyte, as for example the maize cultivar SR15.

2.3.2 Leaf sap osmolality

Generally, the concentration of osmolytes in the sap of the youngest fully developed leaf increased with salt concentration in all three cultivars. SR15 had the most pronounced increase in osmolytes even after 3 days of salt treatment, while in Arizona 8601 osmotic adjustment was only obvious at very high salt concentrations (150 mM NaCl) (Figure 2.4).

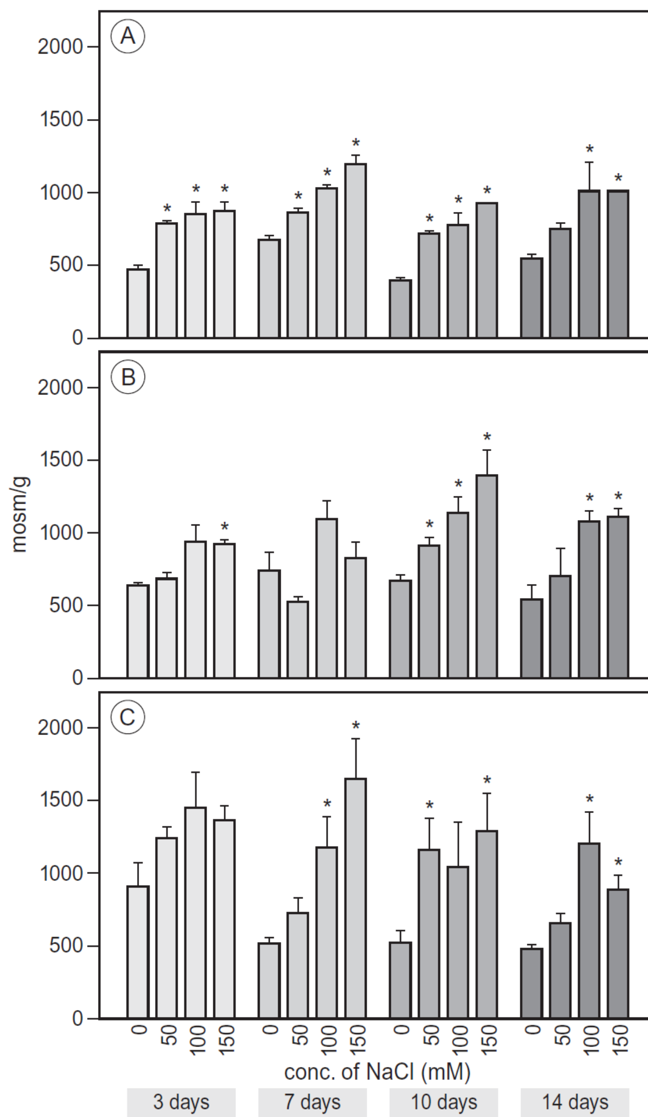

Figure 2.4. *Effect of different salt concentrations on the leaf sap concentration of* **A:** *SR15,* **B:** *Arizona 8601 and* **C:** *Delitop grown for 3, 7, 10 and 14 days on salt. Data labelled with (*) are significantly different (P < 0.05) between treatments at a given time point.*

2.3.3 Photosynthetic rate and stomatal conductance

There is a clear difference between the ways that the stomatal conductance was affected with salt stress in the three maize cultivars (Figure 2.5). In Delitop stomatal conductance was decreased significantly only after 2 days with 150 mM of NaCl and this significant decrease was also apparent in the rate of photosynthesis. Salt stress didn't affect the stomatal conductance or the assimilation rate measured on both SR15 and Arizona 8601 leaves after 2 days of growth on 150 mM NaCl.

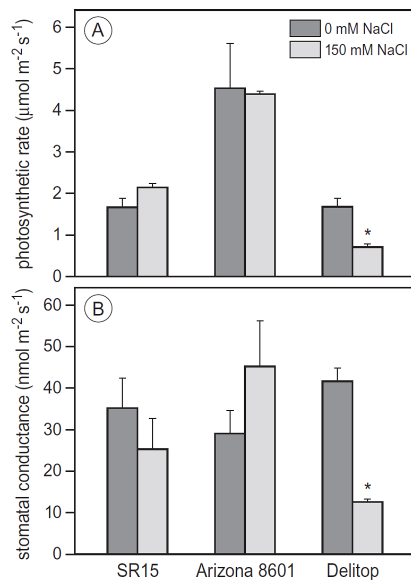

Figure 2.5. *Effect of 0 and 150 mM NaCl on* **A:** *photosynthetic rate,* **B:** *Stomatal conductance on three maize cultivars grown for 2 days on salt. Data labelled with (*) are significantly different (P < 0.05).*

2.3.4 Cation analysis

Treatment of the maize plants with NaCl resulted in a significant increase of the total leaf sodium concentration in all the three cultivars after the different periods of salt stress (Figure 2.6). During exposure to 100 mM NaCl, considered a high salt stress for maize plants, for different time periods, Arizona 8601 was better able to maintain a stable sodium concentration in the leaves than the other two cultivars (Figure 2.8a). In SR15 and Delitop extending the period of the salt stress led to an

increased in the shoot sodium concentration (Figure 2.8a). On average, Arizona 8601 showed the most pronounce increase in the sodium concentration even after 3 days of growth on salt, but this level was maintained during the following period of exposure up to 14 days (Figure 2.6). The entry of sodium during the first 3 days of salt exposure gives an indication that Arizona 8601 is using sodium ions in the leaves in order to maintain osmotic balance in the plant and be able to withstand the decrease in water potential in the growth solution.

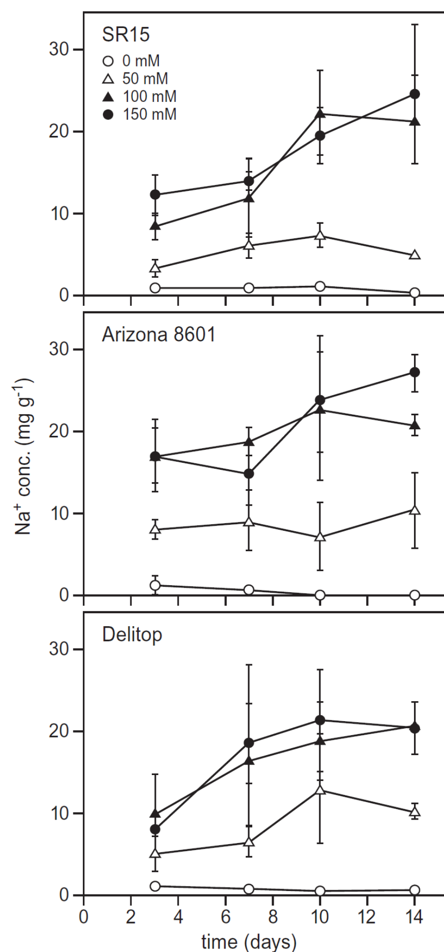

Figure 2.6. *Effect of different NaCl concentrations on the Na^+ concentration of three maize cultivars grown for 3, 7, 10 and 14 days on salt.*

In all three cultivars the salt treatment resulted in a reduction in the leaf K^+ concentration. After 14 days the potassium level decreased dramatically (≥60% from control) in both SR15 and Delitop, but in Arizona 8601 the reduction was much less (35-40% from control) (Figure 2.7). Arizona 8601 was able to maintain the leaf K^+ concentration at a fairly stable level when treated with 100 mM salt, while in SR15 and Delitop the leaf K^+ concentration gradually decreased over time (Figure 2.8 b).

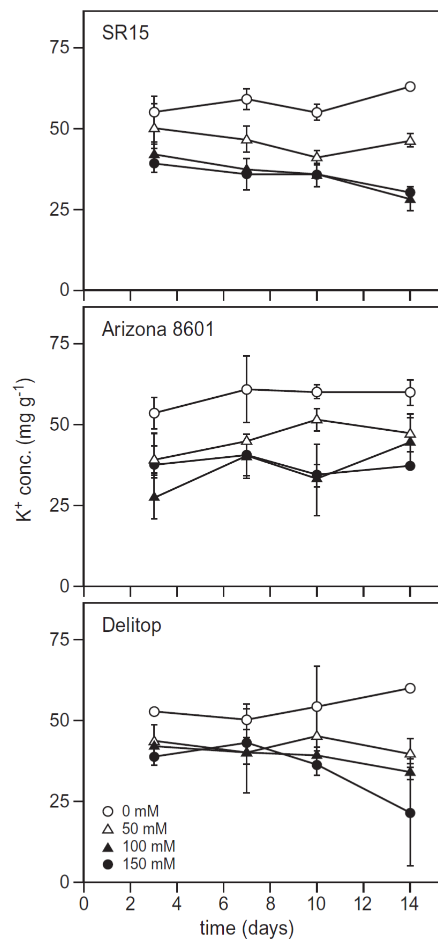

Figure 2.7. *Effect of different NaCl concentrations on the K^+ concentration of three maize cultivars grown for 3, 7, 10 and 14 days on salt.*

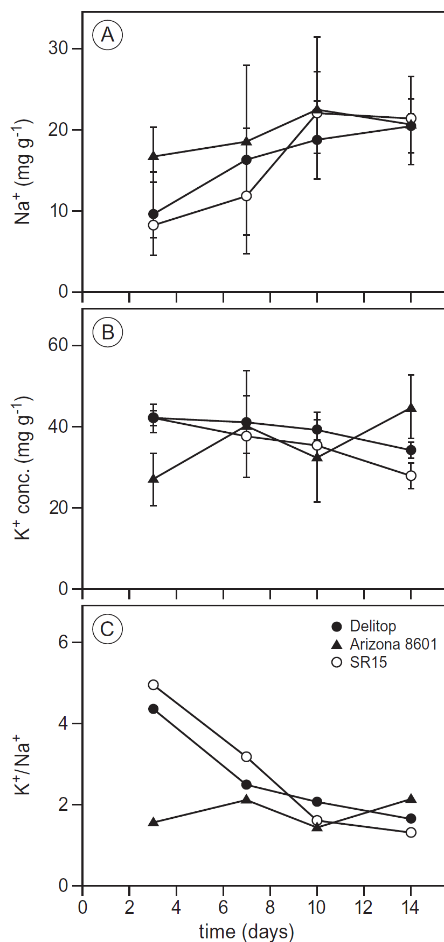

Figure 2.8. *Effect of 100 mM NaCl on* **A:** *Na^+*,**B:** *K^+ and* **C:** *K^+/Na^+ of three maize cultivars grown for 3, 7, 10 and 14 days on salt.*

The K^+/Na^+ ratio was documented to be a key parameter for salt tolerance in plants[19]. Upon exposure to 100 mM NaCl Arizona 8601 had the lowest K^+/Na^+ ratio after 3 days, however, after this initial rapid decrease the ratio was maintained during prolonged exposure up to 14 days (Figure 2.8c). In SR15 and Delitop the K^+/Na^+ was much higher on day 3, was lowered by 73% and 61%, respectively on day 14.

2.4 DISCUSSION

2.4.1 Growth

According to the two-phase model described by Munns[26] the plant can employ two fundamental sets of mechanisms to tolerate high salt concentrations in their growing medium. Mechanisms of osmotic resistance, such as osmotic adjustment can decrease the growth reduction during the first phase. After a prolonged period of salt stress ion toxicity can be the major cause for growth reduction, which can be countered by ion (sodium) exclusion either at the root surface or root xylem parenchyma[27] or by sequestration in the vacuole or salt extrusion through specialized glands.

Growth inhibition during the first phase of salt stress, as found in the present study, has been well documented in many species including maize[18,28,29,22,30]. Clear differences between the cultivars tested were observed. SR15 has the lowest reduction in shoot growth and leaf area when exposed to salt and even increases in the shoot dry weight. In a comparison between genotypes of wheat, lines with higher leaf osmotic adjustment have higher dry matter and/or grain yield under water-limited conditions[31]. In Delitop the inhibition of the shoot growth was more pronounced than that of the root growth. Delitop showed the least reduction in root biomass a trait that will benefit good root hydraulic conductivity under the water deficient conditions during the first phase of salt stress.

In Arizona 8601 the RGR is strongly decreased after addition of salt to the growth medium, but prolonged exposure to salt does not lead to a further reduction. Arizona 8601 also continued to form new leaves under high salt concentrations, which means that the mechanism of salt tolerance efficiently protected the growth initials and allows continued growth during 14 days of salt stress. The initial reduction of shoot growth could be the result of reduced turgor in shoot cells upon salt exposure, since Arizona 8601 does not rapidly increase the leaf cell sap

osmolarity. The reduction in shoot growth rate might also be interpreted as a strategy to reduce further turgor loss by a large evaporation leaf surface area and to save energy under conditions that inhibit photosynthesis. This assumption is in agreement with other reports on maize[32], wheat and barley[33]. Mladenova[32] found that the tolerant genotypes of maize had the higher growth rate reduction under salt stress, a trait that was interpreted as an energy-saving stress response. Similarly, Kuiper *et al* [33] reported that the extent of growth reduction due to salinity stress, is correlated with salt tolerance in wheat and barley.

Figure 2.9. *Adaptation of Arizona 8601 to salt stress. Picture is taken after 1 month of growth on Hoagland solution supplemented with different NaCl concentrations.*

2.4.2 Leaf sap osmolality

Munns[34] listed four criteria for osmotic adjustment in the salt stressed plants: 1) reduced osmotic potential, 2) active accumulation of solutes which lead to decrease in osmotic potential, 3) maintenance of tissue turgor and 4) maintenance of tissue growth. According to the previous criteria the SR15 cultivar is fulfilling more conditions than the other two cultivars since it is the only cultivar with a pronounced accumulation of osmolytes in the growing point and with, at the same time, the least reduction in shoot growth. As the leaf sap concentration was measured in the youngest fully developed leaf, this indicates that the increase in osmolyte concentration in SR15 after only three days of growth is mainly due to the

accumulation of compatible solutes. The role of osmotic adjustment during the first phase of salt stress, is to maintain a high turgor and growth expansion, allowing continued photosynthesis when exposed to drought or salinity[21].

Arizona 8601 possibly adopts a different osmotic adjustment mechanism to cope with the reduction of water potential caused by salt stress. After growing for three days on salt a significant increase in sodium concentration was measured in the total shoot (but obviously not in the young leaves). This resembles a mechanism employed by halophytes that accumulate Na^+, sequestered in the vacuole, to balance their osmotic potential with the decrease in soil water potential upon exposure to salt. This accumulation of sodium is a cheap alternative for the energy demanding synthesis of compatible solutes to reduce osmotic potential in glycophytes, like SR15.

2.4.3 CO_2 assimilation and stomatal conductance

Osmotic stress caused by salt often results in a rapid inhibition of the rate of expansion of young leaves and in a reduced stomatal conductance of mature leaves[8] and is mediated by perturbed water relations and the local synthesis and accumulation of ABA[35]. In Delitop a significant decrease in the stomatal conductance and assimilation rate after 2 days of growth on salt was evident, while in SR15 and in Arizona 8601 the stomatal conductance and rate of photosynthesis was not affected by growth on 150 mM NaCl for 2 days, indicative of a higher tolerance to the first, osmotic phase of salt stress in these two cultivars[8]. In Delitop, the reduced rate of photosynthesis is certainly not the sole cause of growth reduction but it might be linked to the reduction in leaf area observed[35-37].

2.4.4 Cation analysis

Ion toxicity during the second phase can be countered by sodium exclusion either at the root surface or root xylem parenchyma[27]. In addition, sequestering excess salts in the vacuole of leaf cells through intracellular compartmentation can also

delay the onset of ion toxicity. The NaCl-induced increase in Na^+ and decrease in K^+ found in the present study, was previously reported for various maize cultivars[13,27,28,38,39]. SR15 appeared to accumulate less sodium in the leaves, indicating a greater Na^+ exclusion from the shoot than the other two cultivars. The higher Na^+ exclusion efficiency of SR15 might be responsible for the lower reduction in shoot growth and photosynthetic rate. The overall K^+ and Na^+ transport characteristics in different cereals (i.e wheat)[40], were shown to be heritable. Our results confirm the report that the salt resistance of SR15 is based on the introduction of a higher Na^+ exclusion from the shoot in new SR hybrids[13].

The Na^+ concentration in the leaves of Arizona 8601 apparently did not inhibit photosynthesis, nor did it stop the initiation and formation of new leaves. This can be taken as an indication that Na^+ was either excluded to the older leaves or has been sequestered in the vacuole via the tonoplast Na^+/H^+ antiporter, a secondary transport system which is well identified in halophytes[41-44] and salt-tolerant glycophytes[45-47]. Increases in both Na^+ accumulation and Na^+ tolerance in Arizona 8601 suggests that this cultivar can indeed maintain a low cytoplasmic Na^+ concentration and at the same time accumulate Na^+ in the shoot.

The similarity in physical and chemical properties of Na^+ and K^+ allow the competitive displacement of K^+ by Na^+ from binding sites, resulting in strong impairment of a large number of K^+-dependent metabolic processes by elevated Na^+ in the cytosol. Chen el al.[48] suggested that ability of the plant to retain K^+ is more crucial for salt tolerance than their ability to prevent Na^+ uptake (i.e sodium exclusion). This hypothesis is supported by the observation that the ability of plants to tolerate salinity stress depends strongly on the status of their K^+ nutrition[19]. It is generally assumed that metabolic functions in halophytes require the same cytoplasmic K^+ concentration as in glycophytes. The few estimates of cytoplasmic concentrations of K^+ in growing cells of halophytes suggest that these are similar to those of glycophytes[49]. Arizona 8601 maintains a steady K^+ concentration in leaves

during 14 days of salt stress. In contrast, the K^+ concentrations in SR15 and Delitop decreased gradually with time when exposed to salt.

The capacity of plants to maintain a high cytosolic K^+/Na^+ ratio is likely to be one of the key determinants of plant salt tolerance[19]. In most cases salt-tolerant genotypes are capable of maintaining higher K^+/Na^+ ratios in tissues[19,48,50]. Arizona 8601 was capable to maintain a steady K^+/Na^+ ratio and maintain a balance between the Na^+ and K^+ for long periods of salt treatment. In the other two culivars the K^+/Na^+ ratio decreased with time.

The three genotypes of maize used in this study clearly differ in their response to the addition of salt. The answer to the question "Which cultivar is the most salt-tolerant?" depends on the definition used for salt tolerance. Even though Delitop is not documented as a salt-tolerant cultivar, our results show that it can tolerate moderate salt concentrations, possibly the result of the stimulation of root growth under salt stress. From the point of view of a plant breeder, concerned with the crop production and yield production, the hybrid SR15 is a good candidate. SR15 exhibits a better osmotic adjustment, less growth reduction and more sodium exclusion from the shoot. It has the characteristics of a salt-tolerant glycophyte. The situation in Arizona 8601 is completely different: the growth is stunted and the sodium concentration in the leaf increases immediately upon exposure. However, our results indicate that in Arizona 8601 the Na^+ accumulation in the leaf might in fact be a halophytic trait and helps in maintaining osmotic balance in an energy-efficient way. The high Na^+ concentration in the leaf does not seem to affect photosynthesis and leaf vitality and a high K^+/Na^+ ratio in the leaf can be maintained during prolonged NaCl exposure. As a result Arizona 8601 accumulates the highest biomass when the plants are exposed for longer periods to high salt levels.

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Characteristic Salt-Induced Changes of K^+ and H^+ Flux Profiles along Roots of Maize Cultivars with Different Salt Sensitivities

ABSTRACT

In this study, the non-invasive Microelectrode Ion Flux Estimation (MIFE) system was used to determine the net K^+ and H^+ fluxes in the unstirred layer along the roots of three different maize cultivars. The profiles of K^+ and H^+ fluxes along the roots of 3 days old seedlings were recorded under control and salt (100 and 200 mM NaCl for 1 hour) conditions. No significant effect of salt on the K^+ flux profile from the root of the salt-sensitive cultivar Delitop was observed. In the moderately salt resistant cultivar SR15 a decrease in the K^+ efflux along the root profile occurred after 1 h incubation in 200 mM NaCl. Transient changes in K^+ flux in, specifically, the salt-tolerant cultivar Arizona 8601 might indicate the higher ability of this cultivar to minimise potassium loss. Exposure to NaCl significantly increased the H^+ efflux in the elongation zone which suggests the possible involvement of the H^+-pumping ATPase in reducing the NaCl-induced membrane depolarization and its related K^+ efflux in maize roots. No correlation between the NaCl-induced K^+ effluxes and shoot K^+ concentration or growth was found for the cultivars used. It was concluded that, although clear cultivar-dependent differences in NaCl-induced K^+ fluxes in maize root are observed, they cannot be used as a screening tool for salt tolerance in maize.

3.1 INTRODUCTION

Salinity is already significant problem affecting agriculture worldwide and is predicted to become an even larger problem in the coming decades[1]. Salinity stress affects crop growth, productivity and yield[2,3]. Sodium and chloride are the two most important ions responsible for both osmotic and ion-specific damage that significantly reduces crop production[3].The osmotic effects of salinity stress can be observed immediately after salt application and are believed to continue for the duration of exposure, resulting in inhibited cell expansion and cell division, as well as stomatal closure[3-8]. Ionic stress results in premature senescence of older leaves and toxicity symptoms (chlorosis, necrosis) in mature leaves[8,9], due to the disruption of protein synthesis and the interference with enzyme activity by high Na^+ concentrations[10].

Roots are the first plant organs to face an increase in salt concentration in soil. They provide an attractive experimental system for investigating salinity effects on growth for two reasons. First, roots have definable growing regions which consist of the tip, elongation zone (dividing and extending cells) and a separate mature region, with mature elongated cells, which usually begins a few millimeters behind the tip. Second, root epidermal cells can be exposed to different salt concentrations by changing the root medium and the associated changes in ionic fluxes can be directly assayed. The Microelectrode Ion Flux Estimation (MIFE) system, a variant of the vibrating probe system, makes it possible to determine different ion fluxes (i.e. K^+, H^+, Ca^{2+} and Cl^-) under control and salt treated conditions[11]. The results were used to elucidate the mechanisms of ion transport under salt stress and their possible contributions to salt tolerance[12-17].

In the present study the focus lies mainly on potassium, a major plant nutrient, contributing up to 6 % of plant dry weight[18], participating in different physiological functions including activation of crucial enzymatic reactions, charge balance for the large excess of negative charges on proteins and nucleic acids in the cytoplasm and contribution to osmotic potential of cytoplasm and vacuole. The mechanisms

of Na^+ toxicity in the cytoplasm are largely due to competitive displacement of K^+ by Na^+ in K^+-dependent metabolic processes. Many potassium transport systems also have an affinity for sodium[19-22] and can carry Na^+ into the cytoplasm.

Traditionally, the mechanisms of Na^+ exclusion were considered much more important for salt tolerance in glycophytic plants like maize, than the mechanisms by which stressed plants retain intracellular K^+. This was based on the observation that the majority of maize species are highly sensitive to sodium accumulation and have a low tolerance for sodium in the shoot[23-25]. On the other hand, an obvious coupling between Na^+ uptake and K^+ efflux is documented for different plant species[12-16,26]. In order for a plant to counteract salinity stress it should be able to maintain the K^+ level in the cytoplasm[27]. Previously, the K^+ and Na^+ content of the whole tissue was used for the calculation of the K^+/Na^+ ratio. However, vacuolar compartmentation of sodium will lead to an increase of the cytoplasmic K^+/Na^+ ratio, compared to the calculated values based on whole tissue concentrations. Chen *et al*[12] used the MIFE technique as a non-invasive method to measure the NaCl-induced potassium efflux from seven barley genotypes, that differed in salt tolerance, and concluded that K^+ efflux measurements can be used as a reliable screening tool for salt tolerance in barely.

The first aim of this study is to use the MIFE technique to compare NaCl-induced K^+ fluxes in the root of three maize cultivars with different levels of salt tolerance. Second, to determine the dynamics of K^+ flux profiles along the root upon sudden salt addition in acclimated and non-acclimated roots. Third, to measure the proton flux along maize root under control and salt stress conditions, in order to understand the possible contribution of proton pumping in salt tolerance of maize.

3.2 MATERIAL AND METHODS

3.2.1 Plant material

Three maize (*Zea mays* L.) cultivars were used: Delitop (provided by Syngenta Seeds, Saint Sauveur, France), Arizona 8601(a gift from Dr. Michael J. Ottman of the College of Agriculture and Life Sciences, University of Arizona, Tucson AZ, USA) and SR15 (2005) (a gift from Prof. Sven Schubert of the Institute of Plant Nutrition, Justus-Liebig University in Giessen, Germany).

3.2.2 Plant culture

The *Zea mays* caryopses of the three cultivars were soaked in 1% sodium hypochlorite for 10 minutes and then rinsed with tap water for 15 minutes. The caryopses were germinated in a germination box on filter paper moistened with growth solution (0.1 mM $CaCl_2$ and 0.5 mM KCl). The caryopses were then placed in a dark incubator adjusted at 23°C for three days.

3.2.3 Electrodes preparation

Glass micropipettes (GC150-10; Harvard Apparatus, Kent, U.K) were used to prepare the microelectrodes. After pulling of the tip with an electrode puller (L/M-3P-A, List Medical Electronics, Darmstadt, Germany) the electrodes were silanized with tributylchlorosilane (Fluka 90974). Proton-sensitive microelectrodes were back filled with 15mM NaCl plus 40 mM KH_2PO_4, the tip of the electrode was front-filled with Hydrogen Ionophore II (Cocktail A; Fluka 95297) then calibrated in pH solution (5.1-7.8). The K^+-selective electrodes were back filled with 200 mM KCl and front filled with Potassium Ionophore I (Cocktail A; Fluka 60031) then calibrated with solutions containing 0.05, 0.5 and 5 mM KCl.

After calibration, the petri dish containing the maize seedling was then placed on an inverted microscope (Nikon TMS, Tokyo, Japan), and the microelectrode was mounted vertically in a holder connected to a three-way piezo-controlled micromanipulator (Luigs and Neumann, Ratingen, Germany) driven by a computer-controlled motor (M061-CE08; Superior Electric, Bristol, USA). The electrode was

then brought into position perpendicular to the root surface at a distance of 10 µm. During measurements, the microelectrode vibrated between two positions, close to (10 µm) and away from (50 µm) the root surface, with a frequency of 0.1 Hz. Net ion fluxes were calculated from the measured difference in electrochemical potential for ions between the two positions using diffusion equations based on cylindrical geometry[11]. Proton and potassium fluxes were measured in separate experiments and for each measurement a new seedling with a primary root length range of 2-3 cm was used.

3.2.4 Measure potassium and hydrogen flux profile along maize root

Potassium and proton flux profiles along the root were measured in control solution and after 1 h of incubation in 100 mM NaCl-containing bath solution. The primary root was supported with a glass capillary on the base of a petri dish containing 5 ml of 0.1 mM $CaCl_2$ and 0.5 mM KCl (+ 100 mM NaCl in case of salt treatment). Ion flux profile measurements were taken with 0.5 mm increments, starting from the root tip, for 2 minutes at each position. The effect of different salt concentrations (0, 100, 200 mM NaCl) on root K^+ flux profile was measured along the maize root after incubation for 1 hour in the salt containing bath solution.

3.2.5 Transient potassium flux kinetics

Net potassium fluxes were measured at a position 10 mm basipetally from the root tip for at least 10 min in control bath solution to ensure steady initial values. Then 0.1 ml of 5M NaCl stock solution was added gradually to reach to a final concentration of 100 mM NaCl in 5 ml bath solution. After NaCl addition the ion flux was measured for at least another 30 ± 5 min.

3.3 RESULTS

3.3.1 NaCl-induced changes in the potassium flux profile of maize roots

The K^+ along the roots of maize exhibited a distinct pattern: fluxes in the root tip and elongation zone were much higher than in the mature root zone. Comparison between the K^+ fluxes in control solution and after 1 h exposure to salt revealed that there was no significant difference between the K^+ flux profiles in the Delitop roots for any of the salt concentrations tested (0, 100 and 200 mM NaCl) (Figure 3.1B). In contrast, SR15 exhibited a significant decrease in the K^+ efflux upon incubation for 1 hour in 200 mM NaCl along the root profile (Figure 3.1A).

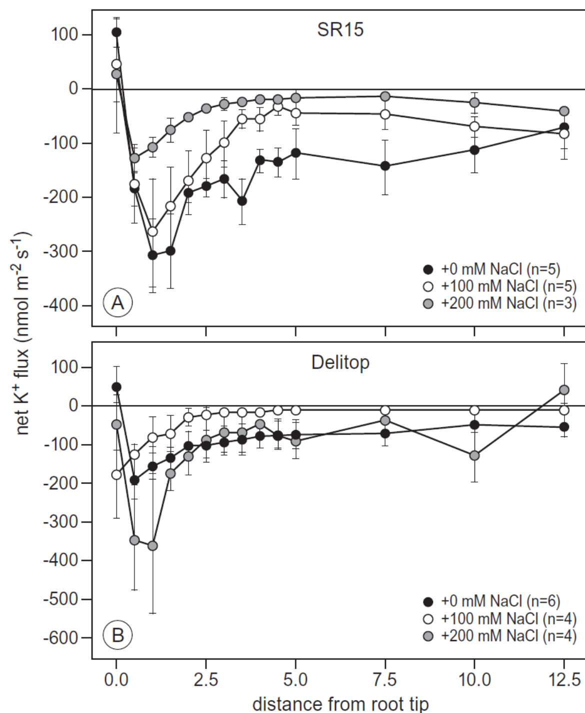

Figure 3.1. *K^+ flux profiles along the root axis of 3 d old maize seedlings,* ***A:*** *SR15;* ***B:*** *Delitop. Net K^+ fluxes were measured in control (closed symbols), after 1 h exposure to 100 mM NaCl (open symbols) and after 1 h exposure to 200 mM NaCl (grey symbols) with 0.5 mm increments, starting from the root tip. At each position, an average K^+ flux was measured for 2 min before the electrode was repositioned.*

3.3.2 Transient K^+ flux kinetics upon salt addition

In the elongation zone of the root, potassium efflux is higher than in root tip and in the mature zone. However, since the profile shows such a sharp peak in the elongation zone, any deviation in electrode positioning away from the elongation zone will lead to a significant apparent reduction in the magnitude of K^+ efflux. Therefore, to compare the fluxes of the different maize cultivars (which do show slight difference in root anatomy) the transient K^+ flux measurements were carried out in the mature zone (approximately 10 mm away from root tip), where K^+ fluxes show less variation with position along the root.

Significant differences were found between the three maize cultivars in the NaCl-induced changes in K^+ flux measured from the mature root zone in response to 100 mM NaCl addition (Figure 3.2A). Although all the cultivars showed a significant increase in K^+ efflux in response to salt addition, the magnitude of this efflux was different. Accordingly, average amount of K^+ ion lost from the mature root zone surface during the first 10 minutes after salt addition, which includes the peak of the transient NaCl-induced K^+ efflux - differed significantly between the three cultivars (Figure 3.2B). The three cultivars also exhibited differences in the steady state K^+ flux levels after the transient peak in K^+ efflux (averaged over the last 15 minutes of the measurement): in the salt resistant cultivar Arizona 8601 the K^+ flux leveled of at an influx (26.548 $nmol.m^{-2}s^{-1}$, compared to a control condition level of -11.778 $nmol.m^{-2}s^{-1}$, Figure 3.2C). In the other two, less tolerant, cultivars (SR15 and Delitop) at steady state the K^+ flux was still an efflux and not very different from the pre-salt treatment control level (Figure 3.2C).

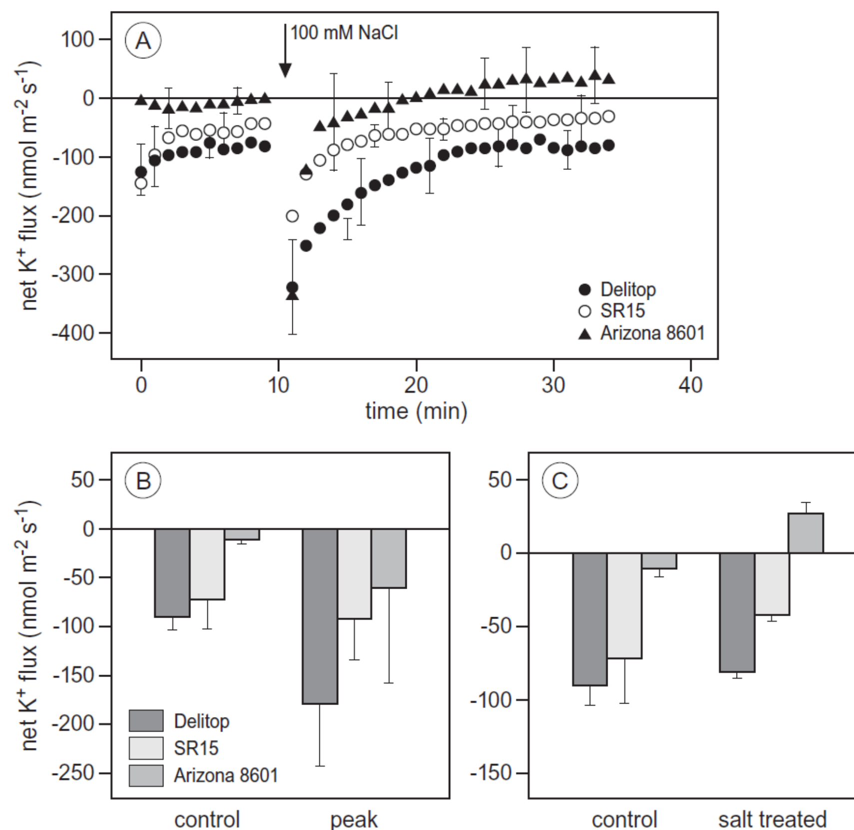

Figure 3.2. A. *Transient K^+ flux responses, measured from 3 maize cultivars after the addition of 100 mM NaCl. Fluxes were measured in mature zone, about 10 mm from root tip* **B**. *Average K^+ flux after addition of 100 mM NaCl (peak values) compared to control K^+ flux (before salt addition).* **C.** *Average K^+ flux after 10 minutes of the addition of 100 mM NaCl compared to control K^+ flux (before salt addition). Values are mean ± S.D., n=4*

3.3.3 NaCl-induced changes in the proton flux profile of maize roots

For all three cultivars a characteristic H^+-flux and pH-profile along the root was recorded (Figure 3.3). This pattern consists of a small influx at the root tip, followed by a peak of proton efflux in the elongation zone located between 2-4 mm from the root tip and the mature zone, which exhibits a gradual decrease in proton efflux

towards a steady, low proton efflux. This pH pattern was previously reported for maize root[28,29] and the region of highest proton efflux was identified to be the zone where cell elongation predominantly takes place[30].

There was no significant difference in the response in proton flux profile to the addition of 100 mM NaCl between the three cultivars. In all cultivars the addition of NaCl increased the H^+ efflux along the whole length of the root. This effect was most apparent in the acidic zone where the addition of 100 mM NaCl for 1h increased the efflux of protons by about five fold more than the proton fluxes under control conditions (Table 3.1).

In order to differentiate between the ionic and osmotic effect of salinity, an isomolar solution of mannitol (175 mM) was used instead of NaCl (100 mM) to mimic the hyperosmotic stress imposed by salinity. Exposure to a high concentration of mannitol does not invoke the same response as exposure to salt (data not shown), a clear indication that the changes in ionic fluxes induced by exposing maize roots to 100 mM NaCl are due to the ionic effect of salt stress.

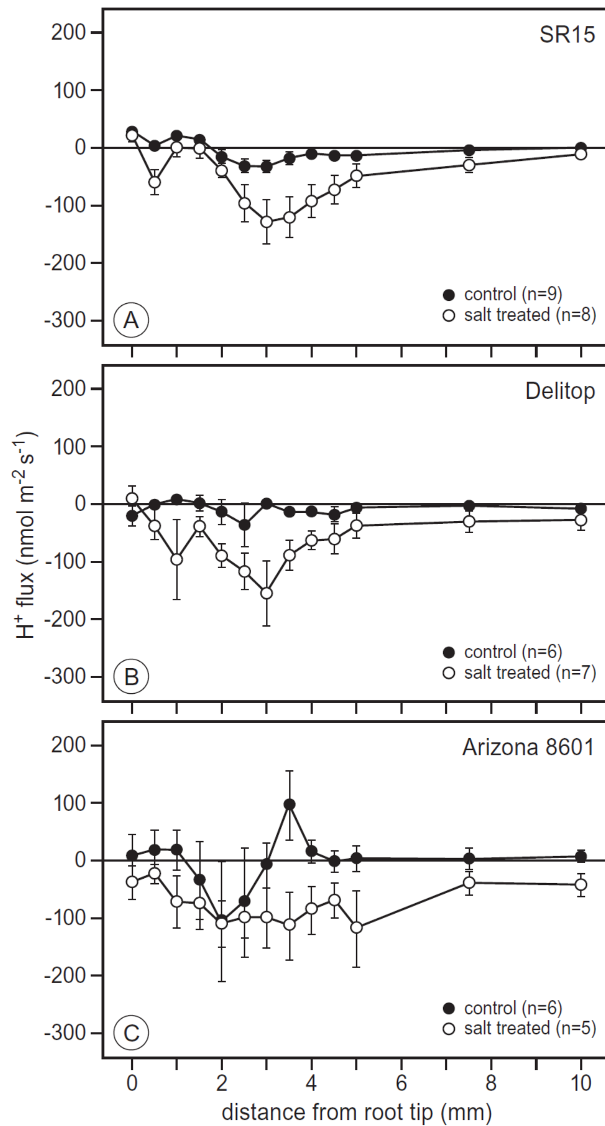

Figure 3.3. *H^+ flux profiles along the root axis of 3 d old maize seedlings,* **A:** *SR15;* **B:** *Delitop;* **C:** *Arizona 8601. Net H^+ fluxes were measured in control (closed symbols) and after 1 h exposure to 100 mM NaCl (open symbols) with 0.5 mm increments, starting from the root tip. At each position, an average H^+ flux was measured for 2 min before the electrode was repositioned.*

	Control	Salt treated
SR15	-20.680± 8.369(n=9)	-85.017±34.840(n=8)
Delitop	-14.987±39.685(n=6)	-88.614±39.685(n=7)
Arizona 8601	-17.465±64.738(n=6)	-97.049±16.582(n=5)

Table 3.1. *Average H^+ fluxes along the 3 d old maize seedlings root elongation zone. Average H^+ fluxes were calculated between distance 2 and 5 mm from the root tip in control and after 1 h exposure to 100 mM NaCl (salt treated).*

3.4 DISCUSSION

3.4.1 Potassium fluxes in different root zones

In several studies the ion uptake characteristics of the specific zones along the root have been described[12,31,32]. Potassium fluxes were found to differ between the root tip and the mature zone. Differential sensitivity of ion transporters in mature and elongation root zones in response to hormonal treatment[33], mycorrhizal colonization[34] cadmium[32], aluminum[35] and salt stress[12] has been reported. In the present study, after 1 h incubation in salt, no significant relative change in the root potassium profile was obtained. Flux levels seem to respond to the addition of NaCl identically along the root length. This indicates that potassium transporters in both the mature and meristematic zone respond with the same kinetics to salt addition. Surprisingly, when maize roots were treated with 200 mM NaCl which is considered a very high level of salt stress, SR15 showed a decrease in efflux of potassium compared to the control along the whole length of the root. An explanation for this result was provided by studying the kinetics of K^+ fluxes upon sudden addition of salt.

3.4.2 Potassium flux dynamics upon sudden addition of NaCl

A schematic representation of the NaCl-induced potassium efflux upon the addition of 100 mM NaCl and the following gradual decrease in K^+-efflux is presented in Figure 3.4. An early plant response to NaCl is the influx of Na^+ into root epidermal

cells through different plasma membrane carriers for example, AKT1, HKT1 (low and high-affinity potassium channels); NORC (Non-selective outward rectifying channels)[27]; and VIC (voltage independent channels)[36]. This Na^+ entry depolarizes the membrane and this subsequently leads to activation of K^+ efflux, possibly through K^+ outward rectifying channel (KORC) and/or non-selective cation channels (NSCC e.g., Non-selective outward rectifying channels NORC)[27].

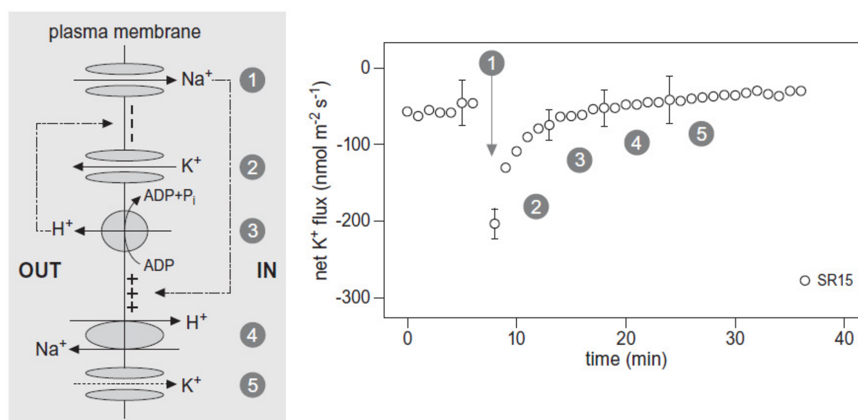

Figure 3.4. *Dynamics of K^+ vs Na^+ transport in root epidermal cell under salt stress.* **(1)** *Increase of NaCl in external medium provokes the Na^+ influx causing a depolarization of the plasma membrane.* **(2)** *Efflux of K^+ across suitable K^+- permeable channels* **(3)** *Activation of the plasma membrane H^+-ATPase by the Na^+-induced depolarization* **(4)** *Proton motive force generated by H^+-ATPase assists Na^+/H^+ antiport, further improving cytosolic Na^+/K^+ ratio and* **(5)** *facilitating the uptake of K^+.*

The observed gradual decrease of potassium efflux after the initial peak efflux was previously documented in wheat[15], which suggests that some salt-sensitive cereals are capable to re-polarize the membrane under saline conditions. Most likely the repolarization is mediated by activation of the plasma membrane H^+-ATPase (a major determinant of membrane potential)[37] and as also indicated by the increased efflux of H^+ upon NaCl exposure (see Figure 3.3). Restoration of the membrane potential allows accumulation of K^+ through passive diffusion through inward rectifying cation channels or by coupling to the `downhill' movement of H^+, possibly through a H^+-K^+ symporter[27]. H^+-ATPase activity can also fuel the sodium

exclusion through Na^+/H^+ antiport, thus increasing the K^+/Na^+ ratio in the cytoplasm.

In Arizona 8601 we observed that after a Na^+-induced transient efflux of K^+ there was a clear shift towards an influx, both compared to the other cultivars and to flux in the control solution. The mechanism behind this shift towards more influx of K^+ in the root of Arizona 8601 is not clear. Possibly, Arizona 8601 potassium channels have a higher affinity for K^+ over Na^+ than the equivalent channels in the other two cultivars, resulting in less competitive inhibition of the K^+ influx by Na^+. This trait would help to maintain a high K^+/Na^+ ratio, thus enhancing the plant performance under salt stress.

Shabala and colleges[26] have investigated the hypothesis that the Na^+ exclusion is indeed important for crop salt tolerance, but they suggested that it is an overestimated criterion, and that the ability of the plant to retain K^+ in the cytosol is a more relevant characteristic of salt-tolerant plants. A negative correlation between net K^+ efflux in 3d-old seedlings and salinity tolerance of mature barley plants was found by Chen *et al*[12-14], this correlation was not found in the present study on maize cultivars. As most of Chen *et al*'s work was done on barley which is considered the most salt-tolerant cereal crop[38] the correlation might only be valid for this type of plants. For less salt-tolerant and sensitive cereal crops, including maize, other mechanisms than the ability to retain K^+ might be more relevant. Evidence supporting our results, is the work done by the same group on wheat[15], where no significant correlation was found between salt-induced K^+ efflux 1 h after the application of 150 mM NaCl and grain production. Therefore, we conclude that salt tolerance in cereal crops is a complicated mechanism and the correlation between growth and ion fluxes in different stages of growth can not be used as a general screening tool for all cereals.

3.4.3 Salt-induced proton efflux profile

The pH along the root of maize seedlings[39] follows a pattern that is primarily determined by variations in proton pumping activity of H^+-ATPase, and possibly by

modulation of cation channel activity, in the different root zones[29] (see Figure 3.3). A similar proton flux profile was found to be instrumental to root growth and cell expansion[39]. NaCl-induced increase in proton efflux from the elongation zone was proved to be linked to the increase in the H^+-ATPase activity at the plasma membrane of plant cells[40-43]. Salt stress is known to induce plasma membrane H^+-ATPase gene expression[44]. Supporting a role of H^+ flux modulation in stress responses is the report that a gene, encoding a modulator of plasma membrane proton-pumping ATPase activity, is preferentially expressed in the maize root elongation zone under control and water-deficit condition[45]. A positive correlation was found (r^2= 0.8683, data not shown) between the NaCl-induced K^+ and H^+ from Delitop root upon sudden salt addition, this indicates that the dynamics of the K^+ transport is linked to the H^+ pumping in maize roots.

Increased H^+-ATPase activity may serve as a negative feedback mechanism, partially restoring a hyperpolarized state of the plasma membrane[46] upon the Na^+-induced depolarization (Figure 3.4). A more negative membrane potential will limit K^+ efflux through K^+ permeable channels (primarily K^+-outward rectifiers). Maintaining cytosolic K^+/Na^+ homeostasis, hence normal cell metabolism even under saline conditions, could therefore be effected by adequate up regulation of plasma membrane H^+-pumping ATPase and control over K^+ outward rectifying channel activity.

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Salt-Induced Proton Fluxes across The Plasma Membrane of Protoplasts Isolated from *Zea mays* L. Root Cortical Cells as a Tool to Screen for Salt-Tolerance Traits

ABSTRACT

The non-invasive Microelectrode Ion Flux Estimation (MIFE) technique was used to measure NaCl-induced proton fluxes in or out of root protoplasts from three maize cultivars that differ in sensitivity to salt. The maize cultivars SR15 and Arizona 8601 were previously described as salt-tolerant, whereas the commercial cultivar Delitop had never been tested for salt-tolerance before. The changes in the proton flux across the plasma membrane upon addition of 100 mM NaCl to the bath medium differed between the three maize cultivars. Also, acclimating the plants for 3 days to 100 mM NaCl in the growth medium affected the protoplast response. The dynamics of the proton fluxes across the membrane upon addition of NaCl, in the presence or absence of the plasma membrane proton pump inhibitor DCCD (N, N'-dicyclohexylcarbodiimide) and the Na^+/H^+ antiport inhibitor amiloride in the bath solution, indicate that the Na^+ extrusion in maize roots is the result of Na^+/H^+ antiport across the plasma membrane. The addition of Na^+ to the bath medium resulted in a strong efflux of H^+ in Arizona 8601, a less strong efflux of H^+ in SR15 and a weak response in Delitop. The possibility of using the Na^+-induced efflux as a proxy for H^+/Na^+ antiporter activity and thus for the ability to exclude salt from the cytoplasm is being discussed.

4.1 INTRODUCTION

Salinization is a major problem threatening the cultivated soil in more than 100 countries all over the world[1]. Accumulation of salts in the soil affects the growth of glycophytes and consequently global crop production, especially in irrigated systems where the chance of secondary salinization is more likely to happen [2].

The electrochemical gradient for Na^+ across plant membranes allows passive diffusion into the cytosol. The exact sodium transport mechanism is not yet fully understood but different plasma membrane potassium carriers can mediate the influx of sodium into the cells. For example, the low-affinity potassium channel AKT1 has a high K^+/Na^+ selectivity ratio at physiological, external K^+ and Na^+ concentrations. Nevertheless, AKT1 could mediate a significant sodium uptake when external sodium concentrations are increased[3]. HKT1 is a high-affinity K^+/Na^+ symporter which is active at external K^+ concentrations in the micromolar range. Although originally characterized as a H^+/K^+ symporter[4], it has been shown that HKT1 may also function as a Na^+/K^+ symporter[5]. Another possible pathway for the sodium transport is through Non-selective Outward Rectifying Channels (NORC), which are activated by calcium and do not discriminate between sodium and potassium. Amtmann and Sanders[6] concluded that Voltage-Independent Channels (VIC) constitute the main pathway for sodium uptake in high salt conditions.

Study of Na^+-induced ion fluxes in tissue is complicated by the possibility that changes in the ionic composition of the external medium exchanges with ions in the cell wall[7]. The induction of ion fluxes, i.e. H^+-pumping ATPase or channel activation, could by the same process lead to secondary release of ions from the cell wall[8]. Using protoplasts facilitates the study of the sodium transport through the plasma membrane, as it is not affected by the possible masking effect of the cell wall. Although the interpretations based on ion flux studies should be made cautiously, as membrane properties of protoplasts might be influenced by the protoplast isolation procedure, leading to quantitatively different results compared to the situation *in situ* [9-12]. The membrane potential of protoplasts was found to be

less hyperpolarized upon release of the protoplasts from the tissue[11,12] and the H^+-ATPase is known to be sensitive to the osmotic conditions of the external medium. Nevertheless, protoplasts have been used successfully in identifying key processes involved in salt resistance[7].

Shabala *et al*[13] used the Microelectrode Ion Flux Estimation (MIFE) system, a non invasive vibrating microelectrode technique, as a method to study cellular ion fluxes by measuring on protoplasts. The technique proved to be applicable for the study of the NaCl-induced cation fluxes in the protoplasts of bean leaf mesophyll[7]. A different non-invasive microelectrode system, Scanning Ion-selective Electrode Technique (SIET) was used to measure the NaCl-induced ion fluxes in salt-tolerant and salt-sensitive poplar species at both tissue and cellular level[14]. Both papers on bean and poplar proved that with non-invasive ion selective microelectrode techniques it is possible to record the dynamics of the ion transport across the plasma membrane under saline conditions. In this work we compare the NaCl-induced proton fluxes from the protoplasts isolated from the root cortical cells of three different maize cultivars that differ in their salt tolerance. The question that we address here: Can the MIFE technique help in identifying the salt tolerance mechanisms in isolated protoplasts from *Zea mays* root cortical cells under control (0 mM NaCl) and salt-acclimated (100 mM NaCl) conditions.

Ion transport across the plasma membrane is affected differently by exposure to saline conditions in different cultivars and species and the difference in response can be used as an indication for the level of salt tolerance[15-17]. The maize cultivars used in this study were chosen from different breeding programs for salt tolerance and are described in the introduction in Chapter 2.

Sodium exclusion in root cortical cells of maize was previously concluded to be an active process, involving Na^+/H^+ antiport activity driven by the proton motive force generated by the plasma membrane H^+-ATPase, and the main determinant of the salt tolerance in maize cultivars[16,18]. In this study, NaCl-induced proton fluxes under acclimated and non-acclimated conditions in the three maize cultivars,

Arizona 8601, SR15 and Delitop, are compared and the dynamics of these fluxes were correlated with sodium exclusion properties and salt tolerance of these cultivars.

The negative-inside plasma membrane potential, the positive-inside tonoplast potential and the low cytosolic sodium concentration allow passive sodium transport into the cytoplasm. Consequently, exclusion or compartmentation of sodium has to be an active transport process. Under saline solutions the exclusion of sodium occurs in glycophytes at the plasma membrane level[2,19]. This requires the activation of proton pumping which was proved to be essential for the Na^+/H^+ exchange and subsequently the active Na^+ extrusion to the apoplast or external environment[19-22]. In this study we test the dynamics of the proton fluxes across the membrane upon the sudden addition of NaCl. To determine the involvement of the plasma membrane H^+-ATPase and the Na^+/H^+ antiporter in maize protoplasts in the NaCl-induced proton fluxes, the inhibitors DCCD and amiloride were used.

4.2 MATERIAL AND METHODS

4.2.1 Plant material

Three maize (*Zea mays* L.) cultivars were used: Delitop (provided by Syngenta Seeds, Saint Sauveur, France), Arizona 8601(a gift from Dr. Michael J. Ottman of the College of Agriculture and Life Sciences, University of Arizona, Tucson AZ, USA) and SR15 (2005) (a gift from Prof. Sven Schubert of the Institute of Plant Nutrition, Justus-Liebig University, Giessen, Germany).

4.2.2 Plant culture

The *Zea mays* caryopses of the three cultivars were soaked in 1% sodium hypochlorite for 10 minutes and then rinsed with tap water for 15 minutes. The caryopses were germinated in a germination box on filter paper moistened with growth solution (0.1 mM $CaCl_2$ and 0.5 mM KCl). The caryopses were then placed in a dark incubator at a temperature of 23°C for three days. Typically, after this

period the primary root length was 25-30 mm. A 10 mm long section of the root was cut 10 mm away from the tip and about 5 mm from the seed. This section was used for protoplast isolation. Roots were acclimated to salt by transferring 3 days old seedlings into a second germinating chamber and grown for three days on filter paper moistened with growth solution containing100 mM NaCl.

4.2.3 Protoplasts isolation

The cut-out sections of root were finely sliced to increase the exposure area for the cell wall digestive enzymes. The enzyme media contained Cellulase RS – (Yakult Honsha, Tokyo, Japan) 1.7 % w/v, Celullysin (calbiochem, La jolla, CA) 1.7% w/v, pectolyase y-23 (Sei Shin, Tokyo, Japan) 0.026% w/v, BSA (sigma, St. Louis, MO, USA) 0.2% Gamborg (DUCHEFA Haarlem, the Netherlands) 2.325% w/v, $CaCl_2$ 2 mM, MES–KOH 10 mM, and 600 mM Mannitol, pH 5.5.

The enzyme solution was stored as 1 ml aliquots at -20 °C till the day of use. A sliced piece of root was incubated for 12-18 hours at 18-20 °C and transferred into a slightly hypo-osmotic bath solution containing 0.1 mM $CaCl_2$, 0.1 mM KCl and 500 mM mannitol, pH 5.9-6.0, to release protoplasts.

Glass cover slips were first treated with ethanol and rinsed with distilled water, dried with lens paper and then used as the bottom of the measuring chambers in which protoplasts proton flux measurements were performed. The thorough cleaning of the cover slips promoted adherence of the protoplasts to the bottom of the measuring chamber, and thus prevented movement of the protoplasts during the measurement, specifically when additions were made to the bath solution. The diameter of the protoplasts was determined before and after the measurements. Only protoplasts with a diameter in the range of 30 to 50 µm, with regular distribution of organelles and showing cytoplasmic streaming were used in the measurements (Figure 4.1).

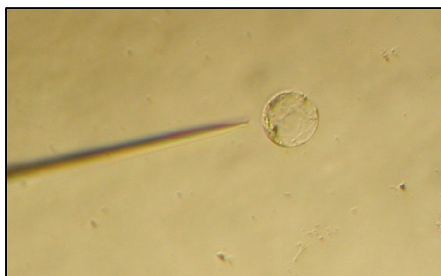


Figure 4.1. *A protoplast with regular distribution of organelles and showing cytoplasmic streaming as used in the measurements.*

4.2.4 Electrode preparation and ion flux measurements

Glass micropipettes (GC150-10; Harvard Apparatus, Kent, U.K) were used to prepare the microelectrodes. After pulling of the tip (1-2 µm) with an electrode puller (L/M- 3P-A, List medical electronic, Darnstadt, Germany) the electrodes were silanized with tributylchlorosilane (Fluka 90974). Proton-sensitive microelectrodes were back filled with 15mM NaCl plus 40 mM KH_2 PO_4 and the tip of the electrode were front-filled with Hydrogen Ionophore II (Cocktail A; Fluka 95297). The microelectrodes were calibrated in three pH buffers ranging from pH 5.1 to 7.8. The measuring chamber containing the protoplasts was placed on an inverted microscope (Nikon TMS, Tokyo, Japan), and the H^+-selective microelectrode was mounted vertically in a holder connected to a three-way piezo-controlled micromanipulator (Luigs and Neumann, Ratingen, Germany) driven by a computer-controlled motor (M061-CE08; Superior Electric, Bristol, USA). The electrode was then brought into position next to the protoplast surface at a distance of 10 µm. During measurements, the microelectrode vibrated in two positions, close to (10 µm) and away from (50 µm) the protoplast surface with a frequency of 0.1 Hz. The position of the electrode and possible protoplast movement during the measurement were observed on a video-monitor and in case of deviations from the normal positioning, the measurements were stopped and another protoplast was selected. Net ion fluxes were calculated from the measured difference in electrochemical potential for protons between the two positions using diffusion equations based on spherical geometry [23].

Steady state fluxes were measured for 2 minutes and then the electrode was moved to another protoplast; at least 4 protoplasts per chamber were measured then replaced by a new chamber with freshly isolated protoplasts. Steady state fluxes were also measured from protoplasts incubated in isomolar bath solution containing 100 mM NaCl after one hour. In case of salt acclimated seedlings the bath solution for the control measurements had 100 mM NaCl and the effect of increasing the concentration of NaCl in the bath solution to 200 mM NaCl was measured after one hour of incubation in the salt containing bath solution.

Transient proton fluxes were measured on maize root protoplasts that were exposed to sudden addition of 100 mM NaCl. Steady state proton fluxes were recorded for at least 2 minutes prior to the addition of salt. Then NaCl was added slowly from a 1M stock solution until the final concentration in the bath solution was 100 mM. The ion flux recordings were continued for at least 30 minutes.

The effect of transport inhibitors was determined by first recording 3 minutes of steady state proton fluxes and then adding amiloride (Sigma, St. Louis, MO, USA) from a 10 mM stock solution to a final concentration in the bath solution of 50 µM or N, N'-dicyclohexylcarbodiimide (DCCD, Sigma, St. Louis, MO, USA, 10 mM stock solution, 100 µM final bath solution concentration). The recording was continued in the presence of the inhibitor for at least 15 minutes before NaCl was added to a final concentration in the bath solution of 100 mM NaCl.

4.2.5 Viability test

The viability of the protoplasts was determined by FDA (Fluorescein Diacetate) staining[24]. A stock solution of 0.5% was prepared and 5 µl was added to the bath solution resulting in a final concentration 0.0025 %. The fluorescence was observed after 2 minutes with a Nikon (Diaphot 300, Tokyo, Japan) inverted microscope equipped with 100/W2 Osram mercury lamp, with a Filter block (MBE-34270) and a dichloric mirror (NDM505).

The protoplast that were chosen for the flux measurements, i.e. those with a diameter in the range 30-50 µm, regular distribution of organelles and showing cytoplasmic streaming, all exhibited bright fluorescence. This test was done with protoplasts under all of the experimental conditions used (100 mM NaCl, amiloride, DCCD).

4.2.6 Cell wall regeneration

The regeneration of cell wall is thought to affect the measurements of the proton fluxes from plasma membrane[13], so cell wall regeneration by isolated protoplasts was tested after two hours, which is a longer period of time than the actual duration of the recordings. The potential regeneration of the cell wall was checked for by using calcofluor White ST (0.1% w/v). Cellulose layers will fluorescence when irradiated with UV light at 366 nm and examined under Nikon (Diaphot 300, Tokyo, Japan) inverted microscope equipped with 100/W2 osram mercury lamp, with a Filter blok (MBE-34270) and a dichloric mirror (NDM505). No fluorescence could be detected on any of the protoplasts in the suspension, indicating that cell wall regeneration was at most only very limited.

4.3 RESULTS

4.3.1 Steady state fluxes

Protoplasts isolated from the root cortical cells of 3 days old seedlings of all three maize cultivars showed proton efflux under control conditions. In both Delitop and SR15 the efflux was around 100 $nmol.m^{-2}.s^{-1}$, while in Arizona 8601the efflux was much lower (25 $nmol.m^{-2}.s^{-1}$). Exposure to 100 mM NaCl for one hour significantly decreased the efflux of protons in Delitop protoplasts and induced a shift from H^+ efflux to H^+ influx in protoplasts of cultivar SR15. For Arizona 8601, the cultivar which exhibited the lowest H^+ efflux under control condition, no significant difference was observed between the control and salt-treated protoplasts (Figure 4.2A).

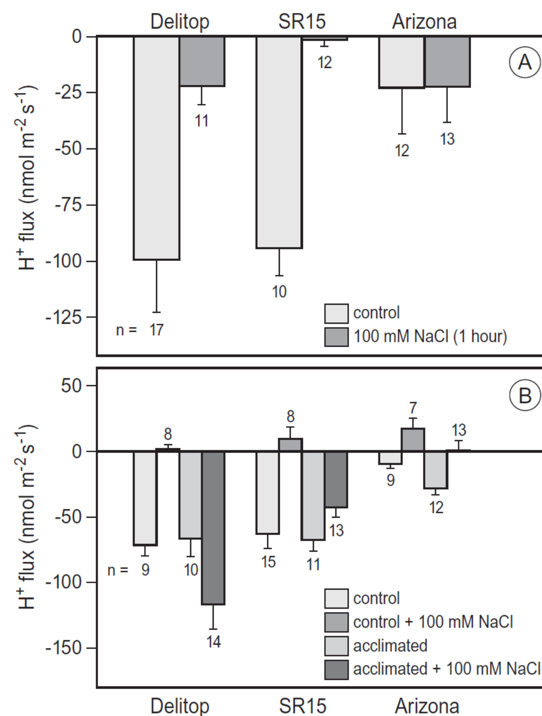


Figure 4.2. A: *Changes in the H^+ fluxes measured from protoplasts isolated from the root of non-acclimated 3 days old maize seedling root under control (0 mM NaCl) and salt (100 mM NaCl) treatment for 1 hour.* **B:** *Changes in the H^+ fluxes measured from protoplasts isolated from the root of non-acclimated 6 days old maize seedlings root under control (0 mM NaCl) and salt (100 mM NaCl) treatment for 1 hour. Acclimated seedlings were grown under control conditions for 3 days then transferred to 100 mM NaCl containing growth medium for 3 days. Bath solution for acclimated protoplast measurement contained 100 mM NaCl and extra 100 mM NaCl was added for the salt effect.*

To study the effect of acclimation, the seedlings that had been germinated for 3 days and then acclimated for 3 days on 100 mM NaCl, were compared to seedlings that had been germinated and grown for 6 days on control solution. The protoplasts from roots that were salt acclimated were released in a bath solution also containing 100 mM NaCl. The protoplasts from non-acclimated roots were kept in a bath solution with no NaCl. To measure the response of H^+ flux to the increase in NaCl concentrations, a 100 mM NaCl was added to both types of protoplasts. The H^+ flux before NaCl addition and one hour after NaCl addition

were being measured. In all three cultivars the addition of 100 mM NaCl to protoplasts from the roots of 6 days old seedlings resulted in a shift from a H^+ efflux to an H^+ influx (Figure 4.2B). These results, although the general trends are similar, are somewhat different from the results obtained with 3 days old seedlings (cf. Figure 4.2A).

In all three cultivars the acclimation to 100 mM NaCl for three days and the continued exposure to this concentration of salt in the bath solution did not lead to significant changes compared to the non-acclimated control protoplasts. However, addition of an additional 100 mM NaCl to the bath solution resulted in a strong increase in H^+ efflux in Delitop, a moderate reduction of H^+ efflux in SR15 and a very slight H^+ influx in Arizona 8601(Figure 4.2B).

4.3.2 H^+ flux dynamics after sudden salt addition

Transient H^+ flux kinetics of isolated protoplasts upon the sudden addition of 100 mM NaCl were compared between the three maize cultivars. The kinetics of H^+ fluxes exhibited a two phase response. First phase consisted of an immediate and strong, short-lived transient increase in the efflux of protons. The maximal efflux during this first phase was up to three times the value under control conditions for the more salt-tolerant cultivar (Arizona 8601) and more than nine times in the more sensitive cultivar Delitop (Figure 4.3A).

The second phase consisted of a gradual decrease in H^+ efflux towards a new steady state level. The switch from phase one to phase two varied between the three maize cultivars. Where for Arizona 8601the second phase either had already started before the recording was free of interference caused by the addition of salt or the first phase is a very slow and small increase in efflux, the recovery from the first phase for SR15 and Delitop had time constants of about 0.5 minutes. This difference in recovery from the first phase resulted in large differences in total H^+ flux (area under the curve) between the three cultivars: -1836, -4337 and -5174 $nmol.m^{-2}$ for Arizona 8601, SR15 and Delitop, respectively, Figure 4.3B). A second difference in the second phase between the cultivars is obvious when the scale of

the y-axis is expanded. In both Arizona 8601and SR15 there is a slow shift towards an influx, while in Delitop the recovery from the first phase seems to end in a new steady state immediately (Figure 4.3C).

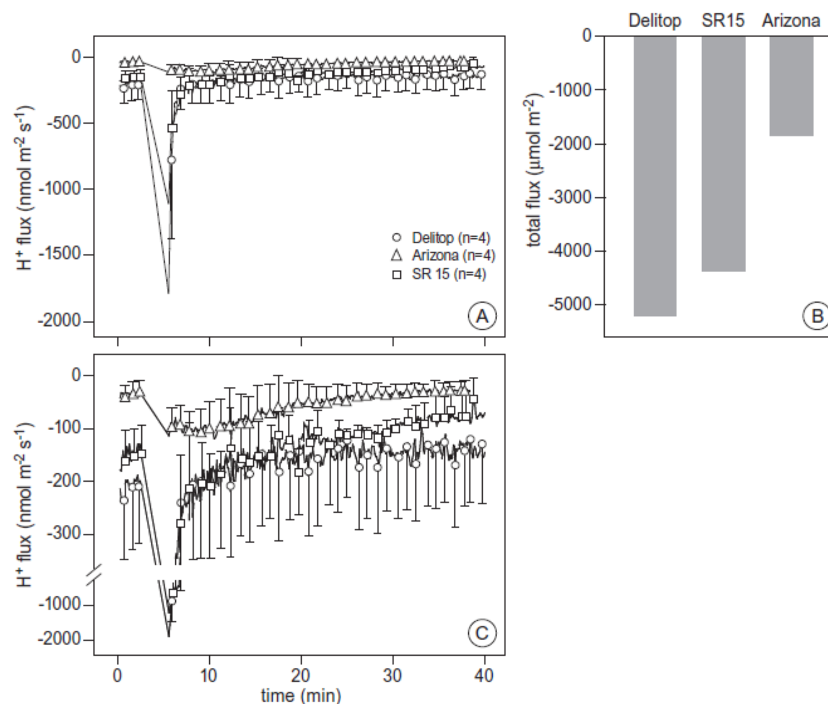


Figure 4.3. A: Effect *of the addition of 100 mM NaCl on the net H^+ fluxes from individual protoplasts isolated from non-acclimated seedlings root.* **B:** *Total H^+ fluxes (area under the curve) calculated after addition of 100 mM NaCl relative to control.* **C:** *An expanded y-axis scale to allow the comparison of the H^+ fluxes in the three maize cultivars.*

Protoplasts isolated from salt-acclimated roots also showed a two phase response to the sudden addition of 100 mM NaCl. The first phase, however, is in salt-acclimated protoplasts much faster. As a result of that the total fluxes from the protoplasts from salt-acclimated roots much smaller than from non-acclimated roots. The values for acclimated protoplasts are -311 (-83% reduction compared to non-acclimated protoplasts), -419 (-90%) and -1912 (-63%) nmol.m^{-2} for Arizona 8601, SR15 and Delitop, respectively (Figure 4.4). The reduction of total flux in

salt-acclimated protoplasts of Delitop is smaller than the reduction found for Arizona 8601 and SR15. This difference seems to be due to the fairly high efflux at steady state in Delitop, while in the other two cultivars the efflux at steady state is close to zero.

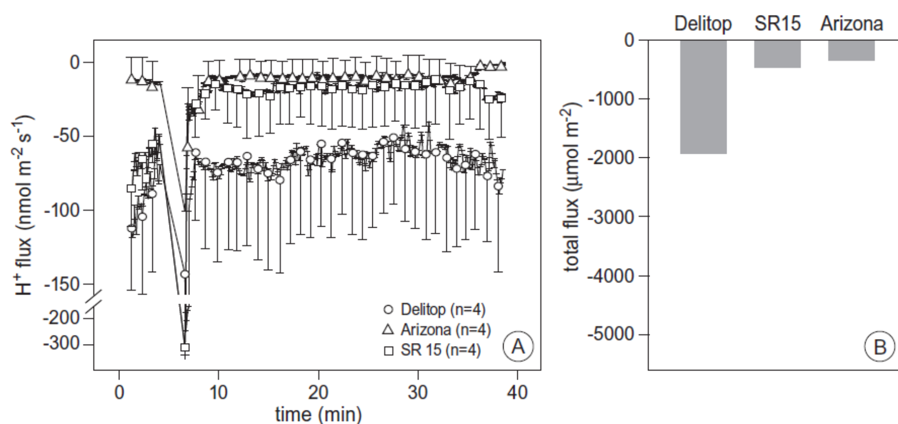
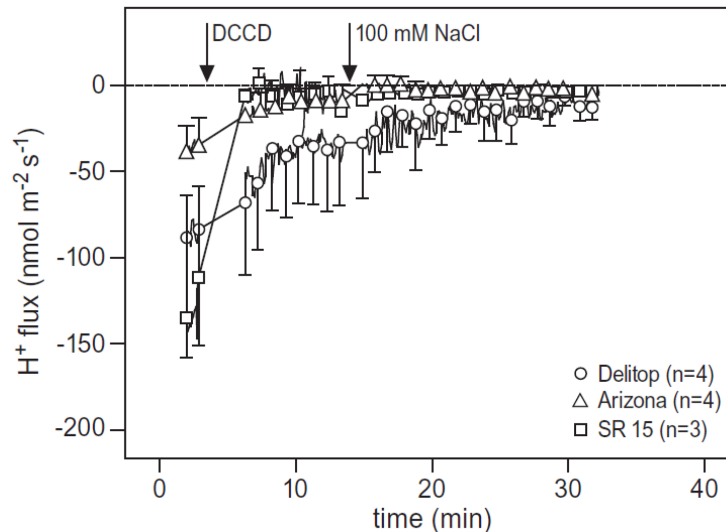


Figure 4.4. A: Effect *of the addition of 100 mM NaCl on the net H^+ fluxes from individual protoplasts isolated from acclimated seedlings root.* **B:** *Total H^+ fluxes (area under the curve) calculated after addition of 100 mM NaCl relative to control.*

4.3.3 Effect of inhibitors

DCCD (100 µM); is a toxic carboxyl reagent blocking plasma membrane H^+-ATPase activity[25] decreased the H^+ efflux from protoplasts isolated from the three maize cultivars, both under control conditions and after the sudden application of salt. The three cultivars were similar in their response to DCCD and the following addition of salt but the rate by which this response took place was different. Both in SR15 and Arizona 8601 an H^+ influx was observed, but in Delitop a H^+ efflux persisted after the addition of NaCl and DCCD (Figure 4.5).



Fig**ure 4.5.** *Effect of DCCD (100 µM) on the NaCl-induced H^+ fluxes recorded from the protoplasts isolated from non-acclimated 3 days old maize seedlings root.*

Amiloride (50 µM) acts as a competitive inhibitor of sodium transporters[26,27] including the plant plasma membrane Na^+/H^+ antiport[28]. Addition of amiloride altered the response of maize protoplasts to the sudden addition of salt and the previously explained two phase response was no longer obvious (Figure 4.6).

Delitop isolated root cortical protoplasts treated with both DCCD (100 µM) and amiloride (50 µM) in the bath media showed a decrease in proton efflux and no response to the sudden addition of 100 mM NaCl (Figure 4.7). This proves that efflux of protons observed when only DCCD was applied to the bath media is a result of a sodium transporter, possibly Na^+/H^+ antiport activity, which is inhibited by the addition of amiloride.

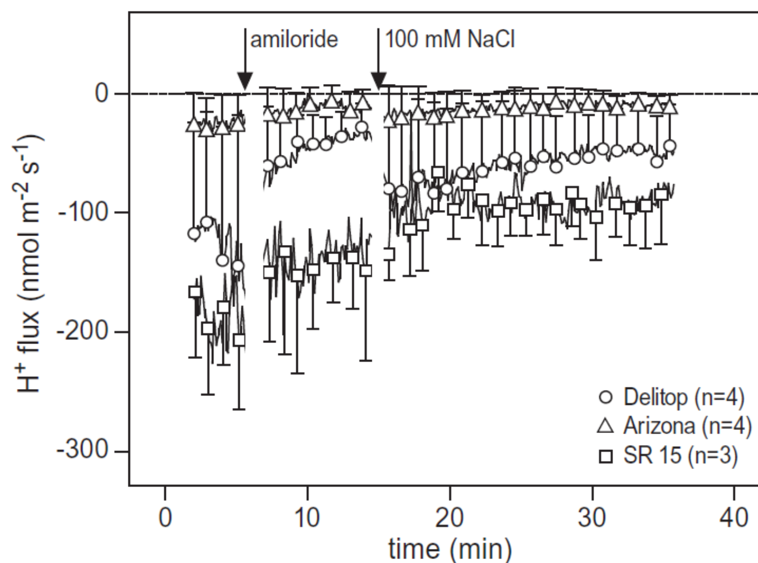


Figure 4.6. *Effect of amiloride (50 µM) on the NaCl-induced H^+ fluxes recorded from the protoplasts isolated from non-acclimated 3 days old maize seedlings root.*

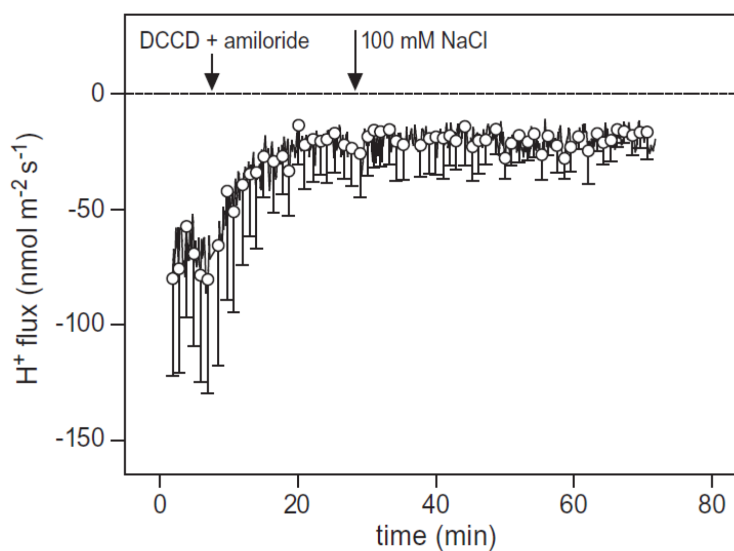


Figure 4.7. Effect *of DCCD and amiloride on the NaCl-induced H^+ fluxes recorded from the protoplasts isolated from non-acclimated 3 days old maize seedlings root (cv. Delitop, n=7).*

4.4 DISCUSSION

The aim of this work was to investigate the response of the maize root cortical protoplasts to salt stress by using the MIFE technique. Evidence was found in previous reports that the exposure to salt increased the apoplast pH in maize which may be due to the alteration of ion distribution and plasma membrane H^+-ATPase activity in the salt sensitive cultivars[29]. This makes working on protoplasts a valuable tool to study the different plasma membrane transporters and the NaCl induced fluxes in the maize plasma membrane without the masking effect of the cell wall or the NaCl-induced changes in the apoplast pH.

4.4.1 NaCl- induced proton fluxes from maize plasma membrane surface

The steady state measurements under control and NaCl treatment showed that the proton fluxes switched from efflux under control condition to more influx especially in case of Arizona 8601 and SR15. This change from efflux of protons into influx indicates the presence of a mechanism which is induced by the presence of the NaCl in the bath solution and is responsible for the reversal of the fluxes. This mechanism was tested with the sudden addition of salt and was proved to involve a primary transport system (H^+ pump) and secondary transport system (Na^+/H^+ antiport) which responded to the sudden addition of salt with different kinetics. The extent of this response could be an indication of the level and persistence of the Na^+-induced depolarization and thus of the permeability for Na^+ through the cation transporters in the plasma membrane. In this context it is interesting to note that non-selective cation channels exposed for a fairly short period to Na^+ have a higher Na^+ conductivity. The persisting increase in H^+ efflux upon the sudden addition to NaCl in salt-acclimated Delitop could be the result of a higher Na^+ permeability. An uncontrolled Na^+ conductance and high external Na^+ levels could lead to failure to recover from the salt-induced depolarization and a long term activation of the H^+-ATPase.

4.4.2 The dynamics of H^+ fluxes upon salt addition

Binzel *et al.*[30] measured the cytosolic sodium concentration, under normal physiological conditions, to be in the range of 1 to 10 mM. However, plants under stress conditions may have a higher Na^+ concentration. Cytosolic concentration of sodium in salt stressed barley roots were found to be between 10 and 30 mM[31]. Under even weakly saline conditions, the negative-inside membrane potential in root of approximately -140 mV[32] will provide a strong driving force that facilitates the passive entry of sodium into the cell. Sodium can be transported into the cell through various channels[6, 19] and transporters[33]. A major determinant of salt tolerance of a plant is the capability to limit sodium transport activity across the plasma membrane[2].

Electrogenic transport of Na^+ across the membrane (i.e. through ion channels) will lead to a depolarisation of the membrane. This depolarisation could result in an activation of the plasma membrane H^+-ATPase. Exposure to NaCl was shown to increase the H^+-ATPase activity at the plasma membrane of plant cells[7,34-36]. This might provide a first explanation for the instantaneous increase in H^+ efflux upon the sudden addition of 100 mM NaCl observed on isolated protoplasts of all three cultivars. The increased H^+-ATPase activity will repolarise the membrane, restoring the proton motive force for transport processes that are coupled to an inward transport of protons.

The fact that the Na^+-induced H^+ efflux is abolished by the addition of DCCD seems to confirm a role of the H^+-ATPase in the fast H^+ efflux observed in the first phase. On the other hand amiloride also inhibit the first phase of H^+ efflux since amiloride is known to block other Na^+ transport pathways[37]. Application of amiloride could have prevented NaCl-induced membrane depolarization and activation of the H^+-ATPase, and thus the fast H^+ efflux response through its effect on H^+-ATPase activity since it can compete with ATP for ATP-binding sites[38]. Amiloride was proven to have a wide spectrum of effects on membrane transport, due to the fact

that amiloride and many of amiloride analogues may accumulate in different cell compartments and collapse the pH gradients across the plasma membrane.

4.4.3 Na^+/H^+ antiport activity and its role in sodium exclusion in maize

The efflux of protons observed with the sudden addition of salt could provide the driving force required for the operation of Na^+/H^+ antiport. Na^+/H^+ antiport couples the downhill movement of protons along its electrochemical gradient with the extrusion of Na^+ against its electrochemical gradient. Entry of protons through the antiporter could explain the gradual decrease in protons efflux during the second phase response. This mechanism would prevent the accumulation of the toxic sodium inside the cytoplasm and thereby determine, together with the permeability for Na^+ of transporters mediating cation influx, the level of sodium exclusion in the root of the three maize cultivars studied.

Na^+/H^+ antiporter activity has been reported to occur across the plasma membrane of barley[39], tomato[40], and wheat[41] where the NaCl-dependent increase in the H^+-ATPase activity was correlated to the Na^+/H^+ antiport activity. Shabala and Newman[7], indicated the presence of Na^+/H^+ antiport in the plasma membrane of bean mesophyll and the importance of the H^+ pump for activation. Sun *et al.* [14] found a difference in the NaCl-induced proton fluxes between the salt tolerant *Populus euphratica* and the salt sensitive *Populus popularis* and concluded that the Na^+ extrusion in salt stressed *P.euphratica* is the result of an active Na^+/H^+ antiport across the plasma membrane where the Na^+/H^+ antiport system in *P. popularis* was insufficient to exclude Na^+ at both the tissue or cellular levels.

Our data show that protoplasts of root cortical cells of the three maize cultivars in this study differ in the slow shift from efflux to more influx, a trait tentatively associated with the presence of a plasma membrane Na^+/H^+ antiporter. The more tolerant cultivars SR15 and Arizona 8601 should according to this hypothesis have a higher Na^+/H^+ antiport activity. This conclusion is based on the following observations: 1- the two phase response to salt stress was not observed after the

ATP- dependant H^+ pump was inhibited by DCCD which is responsible for the efflux of protons from the plasma membrane surface and apparently forms a significant role in the response of the plasma membrane of the three studied maize cultivars to salt stress. 2- The application of amiloride to the bath solution restricted the influx of protons and confirmed the role of the Na^+/H^+ antiport in the second phase response. 3- When both amiloride and DCCD were added to the bath media no distinctive two phase response was observed due to the inhibition of the proton pump and the Na^+/H^+ antiport.

These results indicate that for different maize cultivars the degree of tolerance to exposure to salt could be the result of permeability of the cation transporters to Na^+, in combination with the ability to extrude Na^+ through plasma membrane Na^+/H^+ antiporters driven by a H^+-ATPase generated proton motive force. The results indicate that maize cultivars show distinct differences in these traits.

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Chapter 5

Comparison of The Na^+/H^+ Antiport Activity in *Beta vulgaris* and *Zea mays* L. Intact Vacuoles Using Ion Selective Microelectrodes

ABSTRACT

The activity of the vacuolar Na^+/H^+ antiport was measured for the first time by the use of the Microelectrode Ion Flux Estimation (MIFE) system, a variant of the vibrating microelectrode technique. Changes induced by the addition of NaCl to the bath medium in both pH and the proton fluxes could be measured on individual vacuoles. In vacuoles isolated from red beet storage tissue, the addition of MgATP to the bath solution induced an influx of proton across tonoplast and an increase in the pH in the unstirred layer. The subsequent addition of NaCl (50 mM) caused a proton efflux and acidification of the bath solution. As the efflux of protons only was observed when NaCl or $NaNO_3$ was added and not when KNO_3 or choline chloride was used the effect seems to be sodium-specific. The Na-induced H^+ efflux was inhibited by amiloride, a Na^+/H^+ antiport inhibitor. Two salt tolerant maize cultivars, SR15 and Arizona 8601, were tested for the presence of Na-induced H^+ efflux activity. Evidence was found for the presence of Na^+/H^+ antiport in tonoplast of the root cortical cells of cultivar Arizona 8601 as the addition of 50 mM NaCl resulted in a H^+ efflux comparable to the fluxes observed in red beet vacuoles. Na^+/H^+ antiport activity was not detected in vacuoles isolated from root cells of cultivar SR15.

5.1 INTRODUCTION

Plant cells maintain a K^+/Na^+ ratio in their cytosol with relatively high K^+ (100–200 mM), and low Na^+ (1–10 mM) concentrations[1]. Thus, the efficient exclusion of Na^+ from the cytoplasm by transporting it into the vacuole is one of the main mechanisms for the adaptation of plants to salt stress. Accumulation of Na^+ in the vacuole is typically carried out by a secondary transporter known as Na^+/H^+ antiporter, which is driven by the proton-motive force generated by the H^+-ATPase and H^+-pyrophosphatase in the tonoplast[2,3].

Vacuolar Na^+/H^+ antiporters have been identified as a key feature in salt tolerance in plants[4]. In earlier studies the role of the tonoplast Na^+/H^+ antiport in salt tolerance has been inferred from the induction of antiport activity upon acclimation to salt[5-9]. Vacuolar Na^+/H^+ antiport activity has, however, also been detected in tonoplast vesicles isolated from salt-tolerant glycophytic species, such as red beet[10] and barley[11], and some less salt-tolerant species, such as sunflower[12], tomato[13] and rice[14].

In *Arabidopsis thaliana* the vacuolar Na^+/H^+ antiporter AtNHX1, which is a homologue of the yeast Na^+/H^+ exchanger NHX1, was cloned and functionally expressed in *S. cerevisiae*[15]. Plants over-expressing the gene gained salt resistance to 200 mM NaCl, a concentration that damaged wild-type plants[16]. Since then, several NHX homologues have been characterized in plants, i.e. red beet[17], rice[14], cotton[18], wheat[19], grape berry[20] and maize[21]. This molecular and genetic approach has been suggested to be a robust tool for engineering salt-tolerance in plants[16,22]. However the validity of this approach on highly salt-sensitive plant species, including maize, still requires further study.

A vacuolar Na^+/H^+ antiporter acitivity was directly measured by Blumwald and Poole[10] first in 1985 in purified tonoplast vesicles from red beet storage tissue by measuring the quenching of acridine orange fluorescence in response to the salt addition. Since then, this method has been widely used to estimate the Na^+/H^+

antiport activity in different plant species[5,9-12,23-25]. However, application of the acridine orange fluorescence quenching method requires a time consuming preparation of purified tonoplast vesicles from several grams of plant material. This limits its application only to tissue of which large quantities of homogeneous tissue is available, like sugar beet storage tissue, or when tissue- or cell type-specificity of the antiport activity is not relevant. Since these measurements are performed on tonoplast vesicles the preparation will contain vesicles of both the inside-out and inside-in orientation in an unknown proportion, limiting the resolution even in comparative studies. We propose that by use of the vibrating microelectrode technique some of these disadvantages of the acridine orange technique can be avoided. The technique depends on the isolation of protoplasts and the subsequent osmotic release of the vacuole. This can be a fairly quick procedure and allows measurements on individual vacuoles of specific cell types and with a known, outside-out, membrane orientation.

In this work we introduce the use of the vibrating microelectrode (MIFE: Microelectrode Ion Flux Estimation) as a technique that has the potential to complement salt tolerance studies at higher integration levels (tissue or organ), but also on the plasma membrane and tonoplast level. For the first time, using the storage tissue of the salt-tolerant glycophyte *Beta vulgaris* with a well documented vacuolar Na^+/H^+ antiport activity[10] the vibrating microelectrode is used to study Na^+/H^+ antiport activity. The technique is further used to compare the importance of Na^+/H^+ antiport activity in vacuolar Na^+ sequestration in two moderately salt-tolerant maize cultivars.

5.2 Materials and methods

5.2.1 Plant material

Two maize (*Zea mays* L.) cultivars were used: Arizona 8601(a gift from Dr. Michael J. Ottman of the College of Agriculture and Life Sciences, University of Arizona, Tucson AZ, USA) and SR15 (a gift from Prof. Sven Schubert of the Institute of Plant Nutrition Justus-Liebig University, Giessen, Germany). Red beet roots were purchased from local grocery on the day of the measurements.

5.2.2 Plant culture

The *Zea mays* caryopses of the two cultivars were soaked in 1% sodium hypochlorite for 10 minutes and then rinsed with tap water for 15 minutes. The caryopses were germinated in a germination box on a filter paper moistened with growth solution (0.1 mM $CaCl_2$ and 0.5 mM KCl). The caryopses were then placed in a dark incubator adjusted at 23℃ for three days. Typically, after this period the primary root length was 25-30 mm. A 10 mm long section of the root was cut 10 mm away from the tip and about 5 mm from the seed. This section was used for vacuole isolation. Red beet roots were used for the control study where the outer layer was peeled off and then a slice of 1 cm^2 area was used for the isolation of vacuoles.

5.2.3 Vacuole isolation

Root material was finely sliced to increase the exposure of tissue to the cell wall digesting enzymes. The enzyme media contained Cellulase RS – (Yakult Honsha, Tokyo, Japan) 1.7 % w/v, Celullysin (calbiochem, La jolla, CA) 1.7% w/v, pectolyase y-23 (Sei Shin, Tokyo, Japan) 0.026% w/v, BSA (sigma, St. Louis, MO, USA) 0.2% Gamborg (Duchefa, Haarlem, the Netherlands) 2.325% w/v, $CaCl_2$ 2 mM, MES–KOH 10 mM, and 600 mM Mannitol at pH 5.5. The enzyme solution was stored in 1 ml aliquots at -20 ℃ till the day of use.

Glass cover slips were first treated with ethanol, and rinsed with distilled water, dried with lens paper and then used as the bottom of the measuring chambers in which vacuolar proton flux measurements were performed. The thorough cleaning of the cover slips promoted adherence of the vacuoles to the bottom of the measuring chamber, and thus prevented movement of the vacuoles during the measurement, specifically when additions were made to the bath solution.

Vacuoles were isolated according to the osmotic shock method described by Maathuis and Sanders[26]. A sliced root segment was incubated in the enzyme solution for 12-18 hours at 18-20 °C and transferred into a slightly hypo-osmotic bath solution containing 0.1 mM $CaCl_2$, 0.1 mM KCl and 4 mM Na-ATP (pH adjusted to 7.8 with NaOH) and 400 mM mannitol in case of red beet and 250 mM mannitol in case of maize cultivars. Vacuoles were visually inspected under the microscope and only those without residues of plasma membrane attached to its tonoplast were used for measurements. The diameter of the vacuoles ranged from 30-50 µm and was always measured before and after the H^+ flux recordings. In case of doubt FDA (Fluorescein Diacetate) staining was applied to test for contamination of the tonoplast by plasma membrane fragments and to verify the visual selection of clean, uncontaminated vacuoles.

5.2.4 Electrode preparation and ion flux measurements

Glass micropipettes (GC150-10; Harvard Apparatus, Kent, U.K) were used to prepare the microelectrodes. After pulling of the tip (1-2 µm) with an electrode puller (L/M- 3P-A, List medical electronic, Darnstadt, Germany) the electrodes were salinized with tributylchlorosilane (Fluka 90974). Proton-sensitive microelectrodes were back-filled with 15 mM NaCl plus 40 mM KH_2PO_4 and the tip of the electrode was front-filled with Hydrogen Ionophore II (Cocktail A; Fluka 95297). Microelectrodes were calibrated in three pH buffers ranging from pH 5.1 to pH 7.8. The measuring chamber containing the vacuoles was placed on an inverted microscope (Nikon TMS, Tokyo, Japan), and the H^+ selective microelectrode was mounted vertically in a holder connected to a three-way piezo-controlled

micromanipulator (Luigs and Neumann, Ratingen, Germany) driven by a computer-controlled motor (M061-CE08; Superior Electric, Bristol, USA). The electrode was then brought into position close to the vacuole surface at a distance of 10 µm. During measurements, the microelectrode vibrated between two positions, close to (10 µm) and away from (50 µm) the vacuole surface with a frequency of 0.1 Hz. The position of the electrode and possible vacuole movement during the measurement were observed on a video-monitor and only measurements without any deviations from the original position were included in the analysis. For every measurement a new preparation of vacuoles was prepared. Net ion fluxes were calculated from the measured difference in electrochemical potential for protons between the two positions using diffusion equations based on spherical geometry.

Steady state proton fluxes were measured for at least 5 minutes before 1mM $MgCl_2$ was added to the bath solution to activate the tonoplast proton-pumping ATPase. The proton flux measurement continued for another 5 minutes before the addition of 50 mM of salt (NaCl, $NaNO_3$, KNO_3 or choline chloride) to the bath media and the resulting changes in proton flux and pH were recorded. The contribution of Na^+/H^+ antiporter activity to the proton fluxes was determined by adding 100 µM amiloride (Sigma, St. Louis, MO, USA) to the bath solution prior to the addition of NaCl.

5.2.5 Statistical analysis

The effect of salt addition on the proton fluxes and the pH were analyzed by plotting the data with Graphpad Prism (Graphpad Sofware Inc., San Diego, CA, USA). A one phase exponential decay curve was fitted to the data points to determine time constant and span of the change in pH and H^+ flux induced by the addition of Na^+.

5.3 RESULTS

The addition of the $MgCl_2$ to activate the tonoplast H^+-pumping ATPase resulted in either a clear increase in H^+ influx with a typical time constant of around 70 seconds, or in hardly any change in H^+ flux at all (Figure 5.1). In tonoplast where $MgCl_2$ failed to induce a H^+ influx also exhibited a very small NaCl-induced efflux of H^+, whereas in the tonoplasts that did show activation of the H^+-pumping ATPase, NaCl induced strong reversal of the H^+ influx. Staining of the vacuoles with fluorescein di-acetate revealed that non-$MgCl_2$ responsive vacuoles were likely to still be surrounded by a plasma membrane and thus should be regarded as so-called vacuoplast. Supporting evidence for this assumption is provided by the similarity in response to addition of salts between these vacuoplasts and protoplasts as presented in Chapter 4. In the analysis only measurements from true vacuoles with obvious acidification after $MgCl_2$ addition are used.

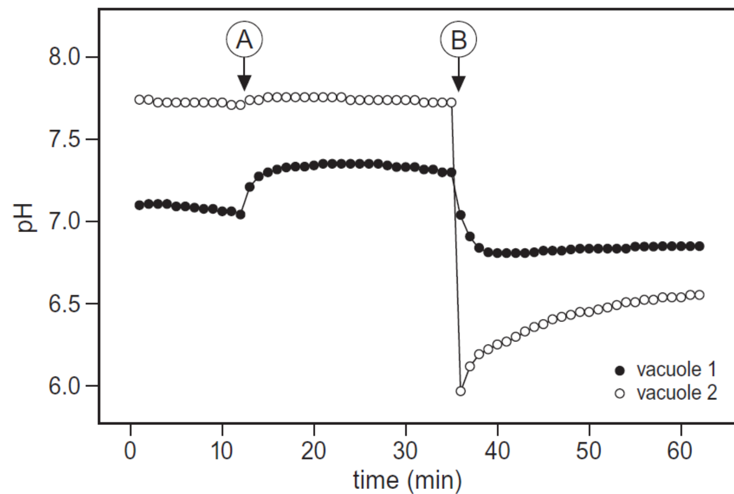

Figure 5.1. *Changes in pH induced by the addition of A: 1mM $MgCl_2$ and B: 50 mM NaCl.*

A telltale sign of cation/proton exchange in tonoplast vesicles is an inward movement of cations in exchange for protons moving outward. In red beet vacuoles, released by osmotic shock in a Na-ATP containing bath solution, consistently a proton efflux was observed prior to the addition of $MgCl_2$ and

activation of V-ATPase. This activation of the proton pump resulted in a H^+ influx and alkalinazation of unstirred layer around the vacuole. Addition of 50 mM NaCl reversed this process and the net efflux of protons was induced again. In Figure 5.2 this sequence is illustrated as **A:** Activation of the V-ATPase and increase in H^+ influx; **B:** Addition of a high extravacuolar concentration of Na^+ drives the Na^+/H^+ antiport resulting in increased H^+ efflux.

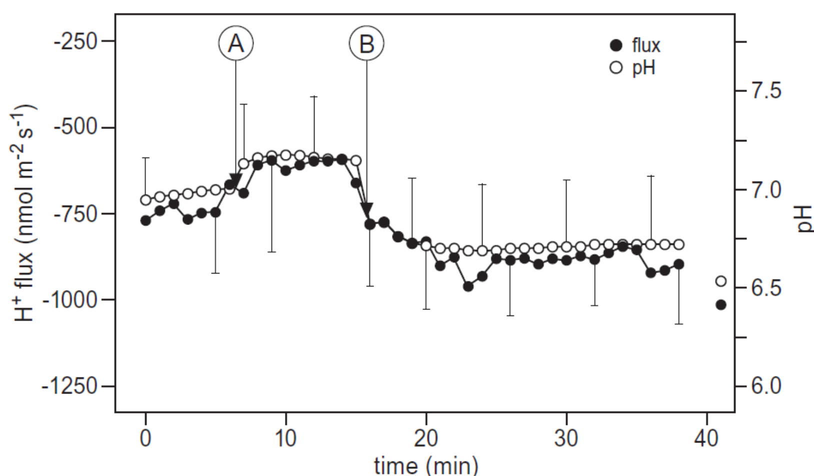

Figure 5.2. *Changes in pH and proton fluxes from beet root vacuoles (n=3) by the addition of* **A:** *1 mM* $MgCl_2$ *and* **B:** *50 mM NaCl.*

Amiloride, an inhibitor of Na^+/H^+ exchange in many eukaryotes, has been reported to competitively inhibit the Na^+/H^+ exchange in red beet tonoplast vesicles[10]. The addition of amiloride to the bath solution decreased the NaCl induced proton efflux from vacuoles, thus indicating that the cation/proton exchange was carried out by the Na^+/H^+ antiport upon the addition of NaCl (Figure 5.3).

Addition of 50 mM NaCl results in a gradual increase in H^+ efflux and decrease in pH (change in H^+ flux of 372 $nmol.m^{-2}s^{-1}$ and change in pH of 0.4, Figures 5.3A and 5.3B, Table 5.1). Similar results were obtained with the addition of 50 mM $NaNO_3$ (800 $nmol.m^{-2}s^{-1}$ and 0.5 respectively). Significantly smaller changes in H^+ flux and pH were measured with 50 mM KNO_3 (Figure 5.3). While after addition of 50 mM

choline chloride an increased H^+ influx was observed. Possibly, a chloride/H^+ symporter is responsible for the additional flux of H^+ into the vacuole upon increasing the extra-vacuolar Cl^- concentration. This result indicates that the increased proton efflux, upon addition of NaCl or $NaNO_3$, is sodium-specific and likely due to an active secondary transport system functioning as a Na^+/H^+ antiport.

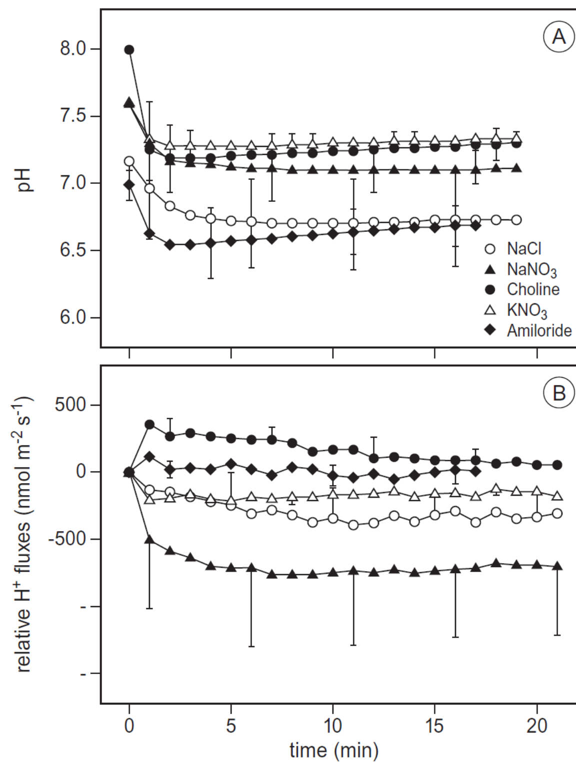

Figure 5.3. *Effect of different salt (50 mM) addition on* **A:** *the pH and* **B:** *relative proton fluxes from red beet vacuoles (n≥3). Y=0 represents the values before the addition of the salt*

Salt	Span pH	Span flux
NaCl	0.357±0.06	372.26±277.17
$NaNO_3$	0.492±0.14	800.5±588.21
Choline chloride	n.a.	n.a.
KNO_3	n.a	n.a.
Amiloride	n.a	n.a.

Table 5.1. *Comparison between the changes in pH and relative proton fluxes in different red beet vacuoles (n≥3) in response to salt, values represent the span of curves ± S.D. Data analysed by one* phase exponential decay analysis of curves.

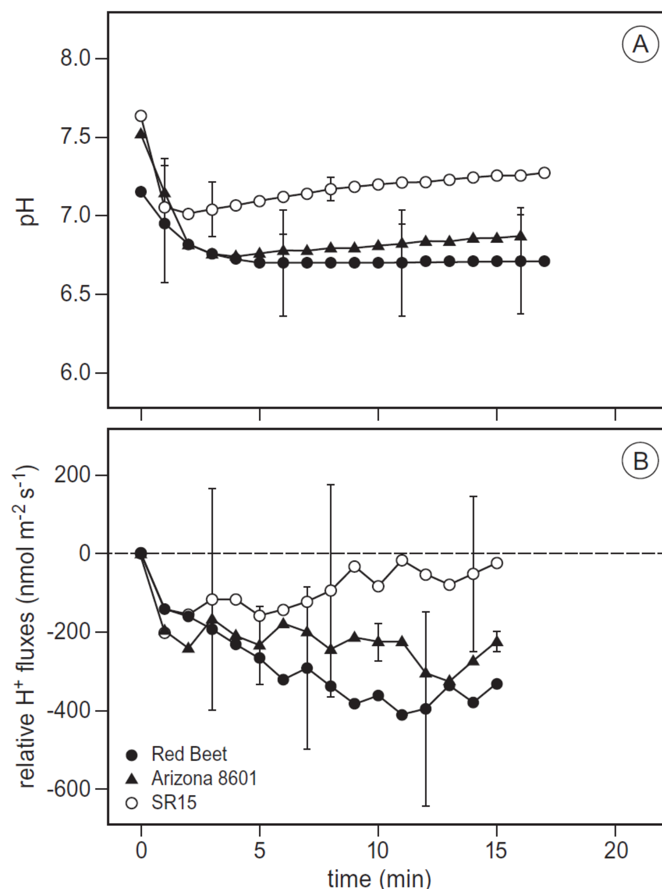

Figure 5.4. *Effect of NaCl (50 mM) addition on* **A:** *the pH and* ***B*****:** *relative proton fluxes from maize and red beet vacuoles (n≥ 3). Y=0 represents the values before the addition of the salt*

NaCl	Span pH	Span flux
Red beet	0.357±0.06	371.660±280.98
Arizona 8601	0.981±0.23	301.500±136.61
SR15	n.a.	n.a

Table 5.2. *Comparison between the changes of maize and beet root vacuoles pH and relative proton fluxes in response to NaCl (50 mM). Values represent the span of curves ± S.D. (n≥3). Data analysed by one* phase exponential decay analysis of curves.

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The maize cultivars in this study showed different levels of response upon addition of NaCl. The response of cultivar Arizona 8601 was comparable to the response found for red beet vacuoles (301 and 372 $nmol.m^{-2}s^{-1}$, respectively. Figure 5.4B,

Table 5.2), while in cultivar SR15 the response was much reduced. Similar to red beet vacuoles, 50 mM $NaNO_3$ induced a significant decrease in pH and an increased efflux of protons in cultivar Arizona 8601, while 50 mM choline chloride did not invoke these changes (Figures 5.6A and 5.6B). In cultivar SR15 these effects were not observed (Figures 5.5A and 5.5B), which indicates the presence of an active Na^+/H^+ antiporter activity in Arizona 8601, which enables this cultivar to sequester sodium in the vacuole when growing under saline conditions. In SR15 no indications for antiporter activity could be detected.

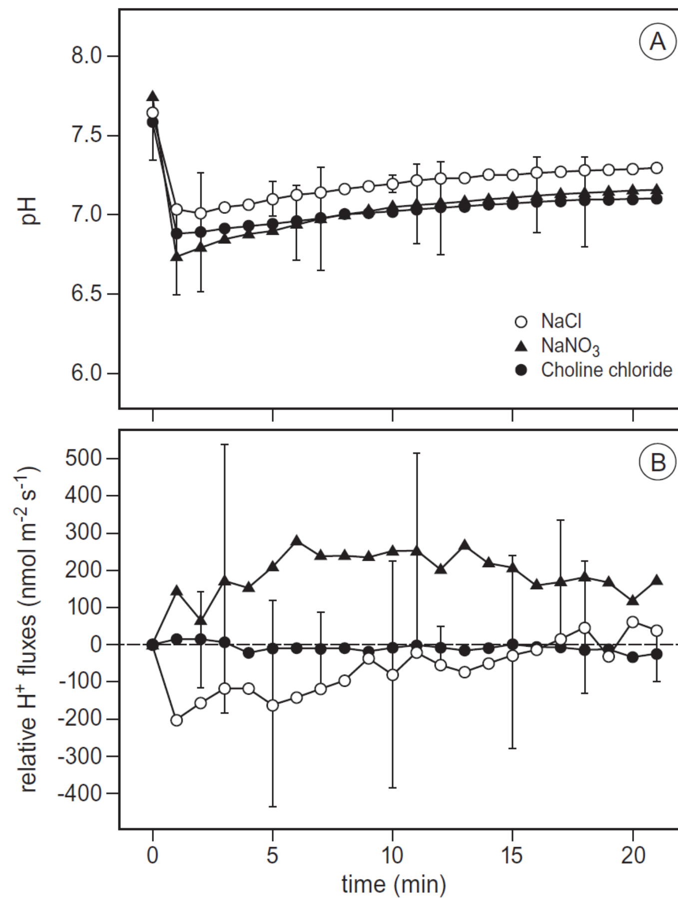

Figure 5.5. *Effect of different salt (50 mM) addition on* **A:** *the pH and* **B:** *relative proton fluxes from SR15 vacuoles (n≥3). Y=0 represents the values before the addition of the salt.*

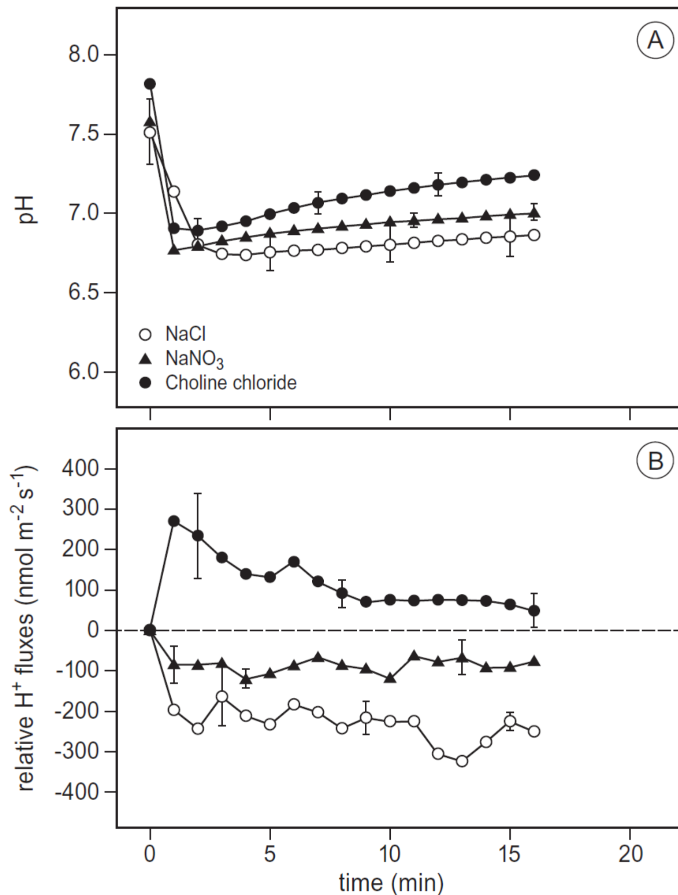

Figure 5.6. *Effect of different salt (50 mM) addition on* **A:** *the pH and* **B:** *relative proton fluxes from Arizona 8601vacuoles (n≥3). Y=0 represents the values before the addition of the salt.*

5.4 DISCUSSION

Our results show that the MIFE system is sensitive enough to study ion fluxes, likely carried by antiporters across the tonoplast of root cells. To our knowledge this is the first time that the vibrating microelectrode technique is applied for the measurement of ion fluxes from individual vacuoles.

Plant vacuoles have been a subject to patch clamp studies which has resulted in the characterization of many ion channels, particularly those conducting cations. However, the patch clamp technique cannot be applied to transporters that, like the Na^+/H^+ antiporter, exchange a cation for a cation and therefore carry no net current. The sensitivity and time resolution of the ion-sensitive microelectrode vibrating probe technique does not have this drawback and can be a useful, additional tool to study membrane transport processes.

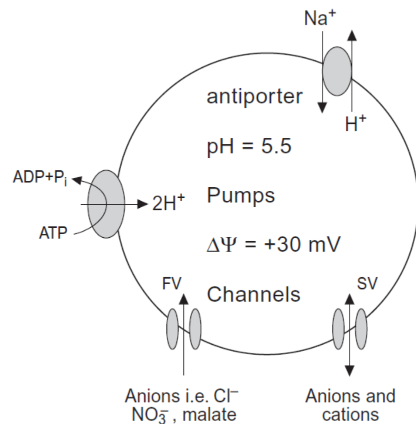

Figure 5.7. *The possible tonoplast transporters (proton pumps, antiporters) and channels involved in the changes in pH and H^+ flux activity due to the addition of salts (NaCl, $NaNO_3$, choline-chloride and KNO_3).*

The possible transporters (proton pumps, antiporters and channels) involved in the changes in pH and flux activity due to the addition of salts (NaCl, $NaNO_3$, choline chloride and KNO_3) which are represented in Figure 5.7.

- The proton pump creates the proton motive force across the tonoplast which drives all the secondary transporters. The proton pumps in the tonoplast are V-ATPase and PPase (fuelled by PPi). The presence of ATP in the bath solution and the addition of $MgCl_2$ only activate the V-ATPase. The H^+ gradient and the electrical potential difference (together from the proton motive force) generates the pump and is used to activate the secondary transport systems.
- Secondary transporters i.e. Na^+/H^+ antiport. When the extra-vacuolar Na^+ concentration is increased, the inward-directed Na^+ gradient can drive the antiporter and force an efflux of H^+ through the antiporter. This increased

efflux can become obvious as a decrease of a H^+ influx or as an increased net efflux.

- Channels conduct passive fluxes of ions, transported down their electrochemical gradient. The selectivity properties of ions are important for selective uptake or excretion of ions and therefore play a role in salt tolerance[27-29]. The electrical potential difference generated by the H^+-pumping V-ATPase can facilitate the passive transport of anions, specially the chloride. Fast vacuolar anion channel probably constitutes the principle transport pathway into the vacuole for chloride[30].

The regulation of the V-ATPase, the tonoplast Na^+/H^+-antiporter and vacuolar ion channels is a crucial process in salt tolerance. Accumulation of toxic ions in the vacuole and prevention of re-entering in the cytoplasm, can improve plant performance under salt stress. Sodium/proton antiporters are not featuring in all plant cells[13] and species. Some plant species, both tolerant and non-tolerant to sodium do not have Na^+/H^+ antiporter activity in either the plasma membrane or tonoplast (or both). In the absence of Na^+/H^+ antiporters moderately salt-tolerant species exhibit other mechanisms to protect the cytoplasm from accumulation of sodium to toxic levels. Apparently, other mechanisms than vacuolar accumulation must be present in SR15 to give it the salt-insensitive characteristic that is attributed to this cultivar. In contrast, Arizona 8601, a moderately salt-tolerant cultivar, is considered one of a very few maize cultivars in which the vacuolar Na^+/H^+ antiport was detected[31,32]. With the MIFE technique it has now been demonstrated that the antiporter is indeed physiologically active.

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Effect of Arbuscular Mycorrhiza on Plant Growth and Root H^+ Flux Profile of Maize (*Zea mays* L.) under Salt Stress

ABSTRACT

*The effect of arbuscular mycorrhiza (*Glomus intraradices*) colonization on the growth and root H^+ flux profile of maize under salt stress is examined in a greenhouse experiment. Roots of the maize cultivar Delitop were colonized by* G. intraradices *(spores and growing hyphae) and after one month of growing on sand culture 60% colonization of the root was obtained. The effect of 0 and 100 mM NaCl concentration on the growth and development of mycorrhized and non-mycorrhized maize plants was studied by determining dry mass, fresh mass, leaf area, chlorophyll concentration and leaf sap osmolarity after 7 and 14 days. The H^+ flux profiles along roots of mycorrhized and non-mycorrhized plants were examined under control conditions and after 1 h incubation in 100 mM NaCl containing bath solution. Mycorrhization succeeded in maintaining plant growth under salt stress, an effect that was even more obvious in the newly developed leaves that completed their growth during the period of salt stress. The leaf area of the newly developed leaves was significantly less inhibited in mycorrhized plants, especially after 7 days of salt stress. Mycorrhization increased the osmolarity of cells sap of the maize plants by 100% only after 7 days under salt stress, which indicated a better osmotic adjustment at the growing points. The H^+ flux profiles along the root were different between mycorrhized and non-mycorrhized roots, with higher levels of efflux in the mycorrhized plants, suggesting induction or activation of plasmamembrane H^+-ATPase by mycorrhizal colonization. Exposure of the roots to salt did not affect the H^+ flux profile of maize roots. It is concluded that mycorrhization assists maize plants in maintaining their growth rate under salt stress by osmotic adjustment of leaf tissue, which will help to maintain leaf cell turgor and expansion.*

6.1 INTRODUCTION

Arbuscular mycorrhiza (AM) is so ancient and widespread, Paleobotanical and molecular sequence data suggest that the first land plants formed associations with Glomalean fungi from the Glomeromycota about 460 million years ago[1]. This is estimated to be some 300–400 million years before the appearance of root nodule symbioses with nitrogen-fixing bacteria. Arbuscular mycorrhiza (AM) symbioses can be formed with a very wide range of plant species, as many as 250 000. Only 150–200 species of AM fungi have so far been distinguished on the basis of morphology, but DNA-based studies suggest that the true diversity of these symbionts may be very much higher[2].

Roots are the site of carbohydrate and mineral nutrient exchange between AM fungi and host cells. The symbiosis is characterized by highly branched fungal structures called arbuscules, which grow intracellularly without penetrating the host plasmalemma. When an AM hypha penetrates a root cell, a newly synthesized plant cell membrane extends from the plasma membrane to surround it, so that the fungus is topologically in the cellular apoplast. A narrow interfacial matrix where most of the nutrient exchange in the symbiosis takes place separates the interface between the arbuscules and plasma membrane. Glucose, primarily, is transferred from the plant to the fungus and, conversely, water and minerals from the fungus to the host[3,4] (Figure 6.1).

Arbuscular mycorrhiza (AM) occurs naturally in saline soils[5]. Although salinity might affect the formation and function of mycorrhizas[6,7], several studies have demonstrated that inoculation with AM fungi improves growth of plants under a variety of salinity conditions[8-11]. Therefore, application of AM fungi has been considered as a method for bio-amelioration of the effect of salinity[12]. But the mechanism by which the AM improves the plant performance under salt stress is not fully understood. Some physiological changes occur in mycorrhized plant under salt stress, which includes: regulation of the water and nutrient uptake in the root and increase of the osmotic adjustment of the root cells. On the shoot level,

mycorrhiza helps in inducing a higher photosynthetic rate[13-15], K^+/Na^+ ratio[15]. More control over the stomatal conductance was found necessary for mycorrhized plants to perform better under salt stress [14].

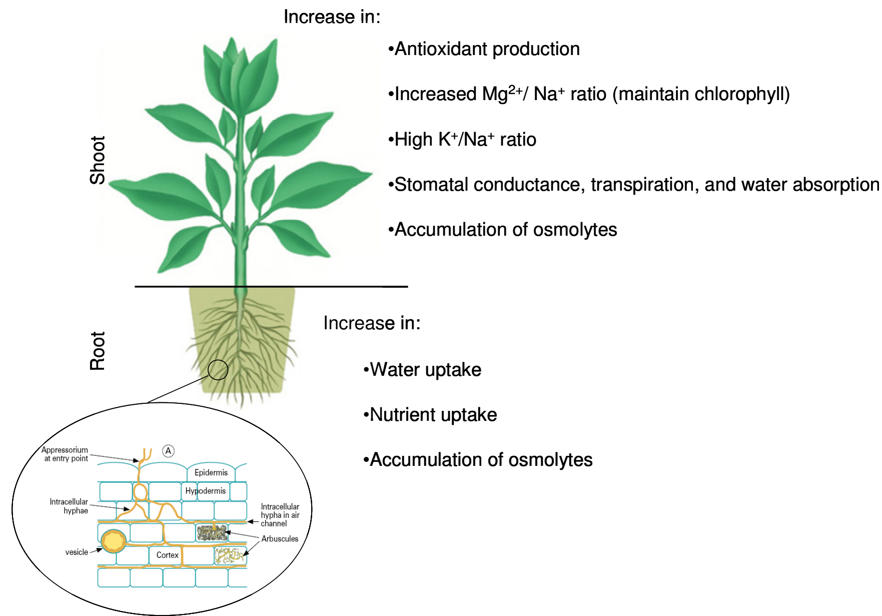

Figure 6.1. *Summary of the possible mechanisms that the arbuscular mycorrhiza endures to enhance the plant performance under salt stress.*

In the previous chapters we have shown that the maize hybrid Delitop is a relatively salt-sensitive maize cultivar and its exposure to 100 mM NaCl in hydroponic nutrient solutions resulted in reduction of shoot growth, leaf area and less osmotic adjustment in the growing leaves, if compared to the other more tolerant maize cultivars studied (Arizona 8601 and SR15). The purpose of this study is to investigate the effect of AM colonization on plant growth, leaf expansion, chlorophyll concentration and osmotic adjustment on the salt sensitive maize

cultivar (Delitop). The question that we try to answer here is: can AM colonization enhance Delitop performance under salt stress?

Very little is known about the mechanisms by which different molecules and ions are transferred between the plant and the AM fungus at the symbiotic interface. Membrane transport proteins responsible for the transfer of ions (i.e. P and carbon compound) are not yet identified. However, increasing information about the plasma membrane H^+-ATPase is being obtained by the use of molecular techniques, in order to understand its role in the active transport of ions in the plant and/or the fungal plasma membrane. Recently, the vibrating microelectrode technique was used to identify the hypothesis that extracellular H^+ fluxes could constitute a "pH signaling" signature that would promote the electrical phenomena related to the host recognition, appressorium formation and root colonization[16]. Ramos et al[16] have discussed the possible involvement of the fungal and the plant P-type H^+-ATPase in all these processes. In this study, we use the Microelectrode Ion Flux estimation (MIFE) system to identify the changes in the proton flux profile along the roots of a mycorrhized and non-mycorrhized plants of the maize cultivar Delitop and to measure the possible changes of root proton fluxes under salt stress.

6.2 MATERIALS AND METHODS

6.2.1 Plant material and culture

Maize seeds (*Zea mays* L. cv. Delitop, provided from Syngenta seeds, Saint Sauveur, France) were soaked in 1% sodium hypochlorite for 10 minutes and then rinsed with tap water for 15 minutes. The experiment was conducted from the last week in July till the mid of September in the green house of the department of Ecophysiology of Plants in Haren, The Netherlands. Day and night temperatures were 25 and 16 °C, respectively. The relative humidity was 35 to 45% and the photoperiod was 14 hours at a photon fluency rate of 100 ± 20 µmole $m^{-2}s^{-1}$.

Maize seeds (*Zea mays* L. cv. Delitop) were sown in plastic pots containing 1 Kg of autoclaved sand. It was observed from several trial experiments that Sand substrate was the most suitable substrate that can be used for our type of study since it is easily washed away from the root, which minimize the loss of ions and root material with intensive washing of soil particles.

Three seeds were sown in each pot and seedlings were thinned to one per pot after germination. The plants were supplied with 50 ml nutrient solution twice a week. The composition of the nutrient solution was as follows: 1 mM Mg $SO_4.7H_2O$, 2.5 mM Ca $(NO_3)_2.4H_2O$, 0.115 mM KH_2PO_4, 2.5 mM KNO_3, 23.2 µM H_3BO_3, 4.6 µM $MnCl_2.4H_2O$, 0.48 µM $ZnSO_4.7H_2O$, 0.16 µM $CuSO_4.5H_2O$, 0.26 µM $Na_2MoO_4.2H_2O$ and 45 µM Fe^{3+}EDTA. The nutrient solution pH was adjusted to 6.5 ± 0.3.

6.2.2 AM fungal inoculum

Glomus intraradices spore solution (Mycorise ASP, Premier Tech. Biotechnologies, Quebec, Canada) was cultured for three months on *Daucus carota* hairy roots *in vitro* cultures[17] (Figure 6.2), and was kept in dark at 25 C°. This *In Vitro* culture plates were cut into small squares (1 cm^2) and each square was used as a mycorrhizal inoculum per 1 liter pot. An additional 0.5 ml spore solution was added in each pot to ensure and speed the colonization process. Regeneration of mycorrhizal hyphae with plant roots and using them as an innoculum seems to be the fastest way to get colonization, and the use of axenic spores are highly preferable in order to avoid unfavorable fungal contamination in the root.

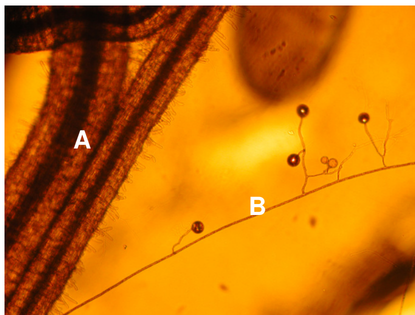

Figure 6.2. Daucus carota *hairy roots grown on* in vitro *culture in association with arbuscular mycorrhiza (*Glomus intraradices*). The picture illustrates how the fungal hyphae looked like before used as an innoculum in the autoclaved sand substrate were the maize plants were left to grow.* **A:** *Daucus carota hairy roots,* **B:** *spores and growing hyphae of (*Glomus intraradices*).*

For the non-mycorrhizal control pots a control *in vitro* culture plates were used (i.e. only *Daucus carota* hairy roots grown on the phytagel media without *Glomus* spores) and an autoclaved spore solution was added in each pot. After a month the roots were positively infected with mycorrhiza and then the mycorrhized and non-mycorrhized plants were subjected to two concentrations of salt (0 and 100 mM NaCl) added to the nutrient solution.

6.2.3 Clearing and staining of mycorrhizal roots

Trypan blue in lactoglycerol is used to stain maize roots that have been cleared by heating in KOH[18,19].A representative sub-sample (0.2 g) from the root system was cut in 1 cm pieces and placed in glass tube containing 10% KOH. The tubes were incubated at room temperature overnight and then at 80°C for 3 hours in a water bath. Roots were rinsed with water, acidified with 1% HCl solution and stained overnight at room temperature in 0.1% Trypan blue lactoglycerol solution.

6.2.4 Root colonization

The slide method proposed by Giovanetti & Mosse[20] was used. Root segments, each approximately 1 cm long, were selected at random from a stained sample and mounted on microscope slides in groups of ten. Presence or absence of infection was recorded in each of the ten pieces and the result again expressed as a percentage. The check was repeated three times. Each replicate comprised an additional sample of 10 root pieces.

6.2.5 Plant growth

The fresh weight of the whole plant was determined, then the root, leaf, and stem were separated and left to dry in an oven at 80 °C for at least 48 h for the determination of the dry weight. The plants were analyzed after 7 and 14 days on salt with n=4 per harvest. The leaf area was determined by multiplying the length and width of the measured maize leaf and divides it by two[21].

6.2.6 Leaf sap concentration

Osmolality was determined by freezing, sawing and then squeezing 0.2 g of the fifth leaf (fully developed under salt stress) in a syringe and the extracted shoot sap was used to determine the osmolality by dew point micro voltmeter (Wescor HR-33T, USA).

6.2.7 Pigment analysis

Two leaf discs were cut from the fifth leaf, the leaf discs was grind with liquid nitrogen then 2 ml of 80% acetone was added and stored overnight at 4 °C in the dark. Next day the samples were centrifuged in a swing-out Sorvall bench centrifuge at 2000 rpm for 10 minutes. One mL of the supernatant is taken in a cuvette and the absorbance is measured at 663.2, 646.8 and 470 nm on Uvikon 940 spectrophotometer against 80% acetone as blank. The chlorophyll a, b concentration was calculated according to Lichtenthaler[22] as µg pigment/cm^2 with the following equations:

Chl a: $12.25*A_{663.2} - 2.79*A_{646.8}$

Chl b: $21.5*A_{646.8} - 5.1*A_{663.2}$

Where A_i is the optical density at the wavelength i.

6.2.8 Electrodes preparation

Glass micropipettes (GC150-10; Harvard Apparatus, Kent, U.K) were used to prepare the microelectrodes. After pulling of the tip with an electrode puller (L/M-3P-A, List Medical Electronic, Darmstadt, Germany) the electrodes were silanized with tributylchlorosilane (Fluka 90974). Proton-sensitive microelectrodes were back filled with 15 mM NaCl plus 40 mM KH_2PO_4, the tip of the electrode was front-filled with Hydrogen Ionophore II (Cocktail A, Fluka 95297) then calibrated in solutions ranging from pH 5.1-7.8.

After calibration, the petri dish containing a piece of maize root, cut at a distance 2cm away from the root tip was placed in front of an inverted microscope (Nikon

TMS, Tokyo, Japan), and the H^+ microelectrode was mounted vertically in a holder connected to a three-way piezo-controlled micromanipulator (Luigs and Neumann, Ratingen, Germany) driven by a computer-controlled motor (M061-CE08; Superior Electric, Bristol, USA). The electrode was then brought into position perpendicular to the root surface at a distance of 10 µm. During measurements, the microelectrode vibrated in two positions, close to (10 µm) and away from (50 µm) the root surface with a frequency of 0.1 Hz. Net ion fluxes were calculated from the measured difference in electrochemical potential for ions between the two positions using diffusion equations based on cylindrical geometry[23].

6.2.9 Measure hydrogen flux profile along maize root axis

After checking the colonization by staining the roots of a representative sample of maize plants, a maize plant was picked randomly and the sand around the root was washed carefully in distilled water then the whole plant was kept in solution containing 0.1 mM $CaCl_2$ and 0.5 mM KCl for at least 2 hours. A small piece of root was cut 2 cm away from the root tip and supported with a glass capillary on the base of a petri dish containing 5 ml of 0.1 mM $CaCl_2$ and 0.5 mM KCl (+ 100 mM NaCl in case of salt treatment). After 15 minutes in the bath solution, root H^+ flux profile measurements started from the root tip and were carried out with 0.5 mm increments, with net ion fluxes measured for 2 minutes at each position. Proton flux profiles along the root axis were measured in control and after 1 h of incubation in 100 mM NaCl-containing bath solution. After completion of the measurement the root segment was checked for AM fungus colonization and only the profiles from colonized roots were included in the 'mycorrhizal root profile' results.

6.2.10 Statistical analysis

All growth data were subjected to factorial analysis of variance using two-way ANOVA, in order to determine how the plant growth parameter is affected by two factors, mycorrhization and salt concentration. Values were considered significant if $p < 0.05$.

6.3 RESULTS

6.3.1 Mycorrhizal colonization

None of the maize plants from the non-inoculated treatments were colonized by *G. intraradices*. Plants inoculated with *G. intraradices* had root colonization of 60%, and the extent of mycorrhizal colonization was not significantly affected by salt (Figure 6.3).

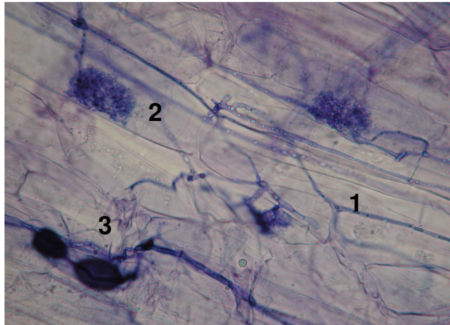

Figure 6.3. *Maize root (Delitop cv.) cortical cells infected with arbuscular mycorrhiza (*Glomus intraradices*). The picture indicates the presence of three distinct structures of mycorrhizal colonization which is: 1- the hyphae, 2- the arbuscules and 3- the vesicles*.

6.3.2 Plant growth

In plants of which the roots were colonized by *G. intraradices* salt treatment had a reduced effect on plant growth, especially in the short period of salt stress (7 days) where the total plant dry mass didn't show any significant decrease relative to the control (Figure 6.4). Salt-stressed mycorrhized plants maintained their leaf, stem and root dry weight after 7 days on salt, however, when the period of exposure to salt was extended to 14 days, a significant decrease in root dry mass was measured, similar to reduction in root dry mass of the non-mycorrhized plants (Figure 6.5). The total leaf area of the mycorrhized plants was maintained under salt stress, while a significant reduction in total leaf area was observed between the control mycorrhized and the salt stressed non-mycorrhized maize plants (Figure 6.6).

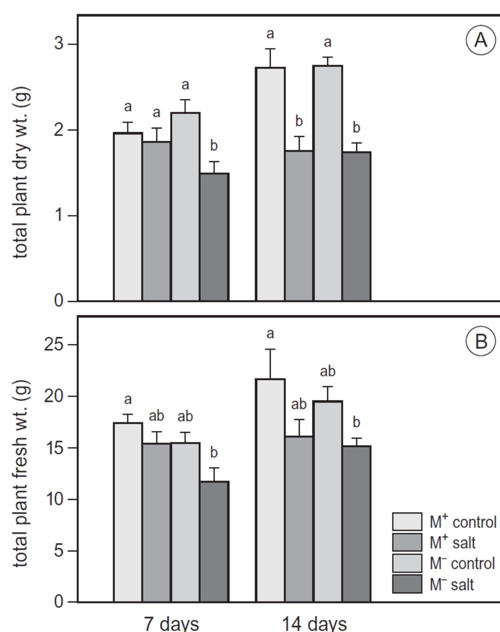

Figure 6.4. *Effect of* Glomus intraradices *on* **A:** *plant dry weight,* **B:** *plant fresh weight of Delitop at 0 (control) and 100 mM NaCl (salt).* M^+ *and* M^- *represent with and without inoculation of G. intraradices. Data labelled with different letters are significantly different (P < 0.05) between treatments at a given time point (7 or 14 days).*

Generally, most of the effect of salt exposure on growth is masked by the small effect of reduced accumulation of new plant mass, relative to the total plant mass assimilated in the period (one month) before the addition of salt. For this reason we also measured the leaf area of the youngest two leaves (number five and six), which can be considered as the actively growing zone of the plant. The effect of mycorrhiza on maintaining plant growth and leaf expansion under salt stress was more obvious in the growing region of the maize plants (Figure 6.7). The newly developed leaves of non-mycorrhized plants were sensitive to salt and a reduction of 53% and 31% compared to control plants was observed after 7 and 14 days of growth on salt. The salt treatment did not significantly affect the growth and expansion of the youngest leaves of mycorrhized plants.

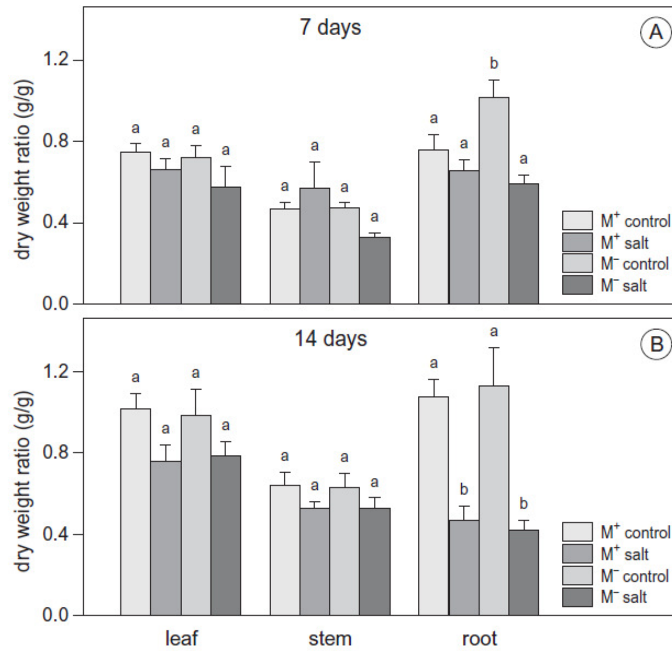

Figure 6.5. *Effect of* Glomus intraradices *on the dry weight of different maize tissue after* **A:** *7 days,* **B:** *14 days, at 0 (control) and 100 mM NaCl (salt). M^+ and M^- represent with and without inoculation of* G. intraradices*. Data labelled with different letters are significantly different (P < 0.05) between treatments at a given time point (7 or 14 days).*

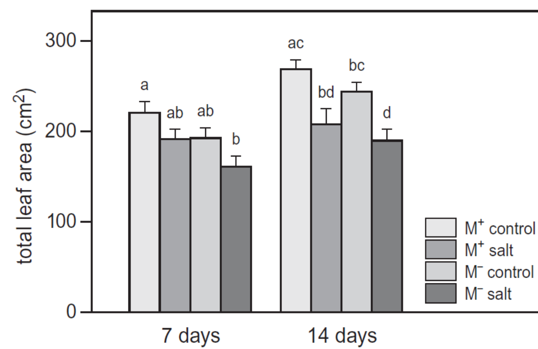

Figure 6.6. Effect of *Glomus intraradices* on the total leaf area of Delitop cv. at 0 (control) and 100 mM NaCl (salt). M^+ and M^- represent with and without inoculation of *G. intraradices*. Data labelled with different letters are significantly different ($P < 0.05$) between treatments at a given time point (7 or 14 days).

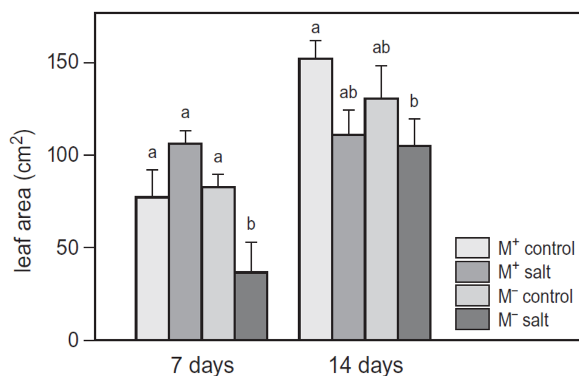

Figure 6.7. *Effect of* Glomus intraradices *on the leaf area of the youngest leafs (leaf no. 5 and 6) developed during the period of salt stress at 0 (control) and 100 mM NaCl (salt).* M^+ *and* M^- *represent with and without inoculation of* G. intraradices*. Data labelled with different letters are significantly different (P < 0.05) between treatments at a given time point (7 or 14 days).*

6.3.3 Chlorophyll

No significant effect was observed on the amount of chlorophyll a+b concentration in the fifth leaf of maize grown under control or saline conditions in mycorrhized or non-mycorrhized maize plants (Figure 6.8).

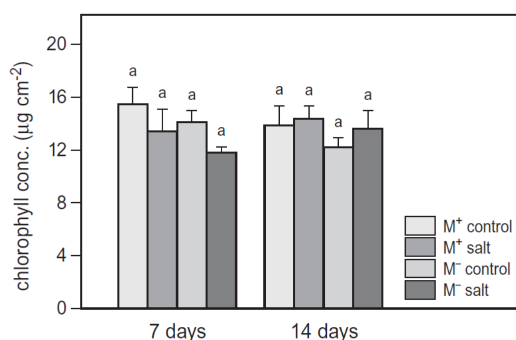

Figure 6.8. *Effect of* Glomus intraradices *on the chlorophyll a+b content of maize (Delitop cv.) leafs, at 0 (control) and 100 mM NaCl (salt).* M^+ *and* M^- *represent with and without inoculation of* G. intraradices*. Data labelled with different letters are significantly different (P < 0.05) between treatments at a given time point (7 or 14 days)*

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6.3.4 Osmolality

Salt treatment significantly increased the osmolality of the cell sap of the fifth leaf of mycorrhized plants by 100% (after 7 days) and 22% (after 14 days). In non-mycorrhized plants the effect on leaf sap concentration was less obvious, only after 14 days of salt treatment an increase in the accumulation of solutes in the leaves was observed (Figure 6.9).

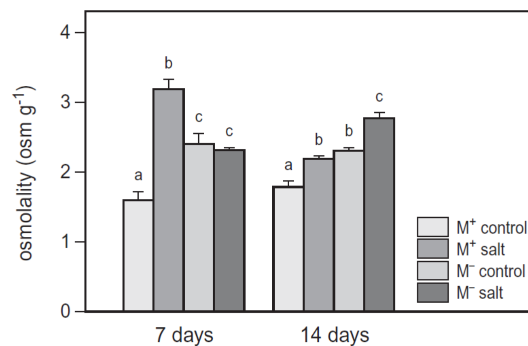

Figure 6.9. *Effect of* Glomus intraradices *on leaf sap concentration of maize (Delitop cv.), at 0 (control) and 100 mM NaCl (salt). M$^+$ and M$^-$ represent with and without inoculation of* G. intraradices. *Data labelled with different letters are significantly different ($P < 0.05$) between treatments at a given time point (7 or 14 days).*

6.3.5 Root H^+ flux profile

A characteristic proton flux profile along the root, similar to the Delitop cultivar (see chapter 3), was observed: a small influx at the root tip, a maximal efflux in the elongation zone and a decrease in proton efflux towards the mature root zone. As the elongation zone is not at a fixed, absolute distance behind the root apex, but at a variable distance depending on the root elongation rate, we plotted the fluxes of each root relative to the elongation zone (identified as the area of maximum efflux). Mycorrhized roots exhibited a higher proton efflux in the mature zone than the non-mycorrhized roots (Figure 6.10). Upon salt addition no significant changes in the root H^+ flux profile was detected in the mycorrhized and non-mycorrhized roots (Figure 6.11).

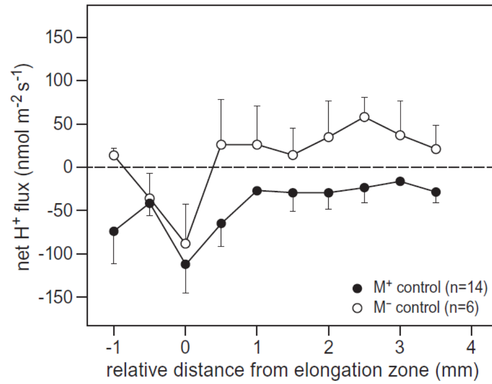

Figure 6.10. *Effect of* Glomus intraradices *on root H^+ flux profile of maize (Delitop cv.). M^+ and M^- represent with and without inoculation of* G. intraradices*. x_i determines the position of the electrode on the root. x_0 indicates the zone of elongation on each measured root (the area of maximum efflux). When x_i equals a negative value this indicates the direction to the root tip, and x_i is positive then the electrode is moved*

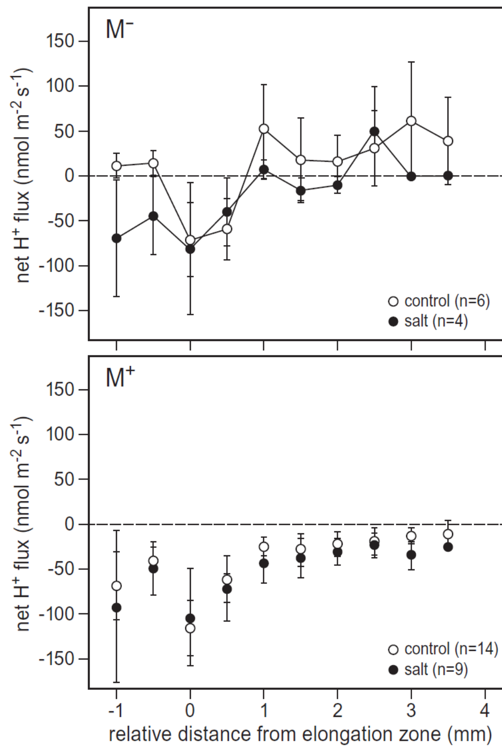

Figure 6.11. *Effect of* Glomus intraradices *on root H^+ flux profile of maize (Delitop cv.) at 0 (control) and 100 mM NaCl (salt). M^+ and M^- represent with and without inoculation of* G. intraradices*. X_i determines the position of the electrode on the root. X_0 indicates the zone of elongation on each measured root (the area of maximum efflux). When X_i equals a negative value this indicates the direction to the root tip, and X_i is positive then the electrode is moved to the direction of the mature zone.*

6.4 DISCUSSION

In this study, we show that plant dry weight and leaf area of mycorrhizal maize plants are less affected by NaCl stress conditions, compared with non-mycorrhizal plants. This indicates that root colonization by *G. intraradices* can alleviate the deleterious effects of NaCl stress. Also in other studies AM-inoculated plants grew better than non-inoculated plants under salt stress[8-12,15,24-26]. Enhanced growth of AM-inoculated plants has been partly attributed to enhanced nutrient acquisition, especially better P nutrition[27,28]. This is primarily regulated by the supply of nutrients to the root system[25] and increased transport (absorption and/or translocation) by mycorrhiza[9,28]. Unfortunately no elemental analysis was done in this study to confirm this hypothesis.

In maize, salt stress may reduce the assimilate supply to the growing shoot by partially inhibiting photosynthesis[29] or by reducing the rate of transport of assimilates to the growing points. This reduction of assimilate supply could also be responsible for shoot growth inhibition during the first phase of salt stress. In addition, the supply of assimilates influences the plant's capacity to maintain turgor through osmotic adjustment[30]. In AM colonized plants an increase in osmolality of the cell sap of the youngest leaf by 100% after 7 days on salt was observed. This higher accumulation of solutes in the growing tip appears to be a mechanism induced by AM fungi to increase tolerance to salt-induced osmotic stress. This enhanced osmotic adjustment could be the key mechanism for maintenance of leaf growth and expansion under mycorrhized condition[31]. Considering the level of increase in osmolality this cannot be attributed to an improved uptake of phosphate. The increase in osmolality can only be attributed to either organic compatible solutes or to major ions, possibly potassium.

Earlier studies have indicated that AM colonization had a beneficial effect on salt-induced chlorophyll loss[25]. By enhancing the uptake of Mg^{2+}, mycorrhized plants could support a higher chlorophyll concentration[32]. However, in our study no

significant effect on the chlorophyll content was observed between the mycorrhized and non-mycorrhized maize cultivars in control or salt treated condition. Similar results were reported for other plants under salt stress[33-35]. Apparently, under the conditions applied salt primarily affects growth in maize, while the supply of Mg^{2+} is sufficient and does not require an AM-induced stimulation.

Evidence that the fungal growth and host signal perception are mediated by ion fluxes across the plasma membrane have recently become available and in most cases the participation of H^+ fluxes seem to be consistently involved[16]. During plant and fungal growth, cells pump out protons by two different mechanisms: (1) the activity of the plasma membrane H^+- ATPases[36], and (2) plasma membrane redox reactions[37,38]. The exact proportions in which both mechanisms contribute is generally unclear, but in most plant cells the plasma membrane H^+-ATPase seems to be the dominant process. The first analysis of the H^+-ATPase distribution in AM root consisted of cytochemical staining for ATP. ATPase activity was detected along the plasma membrane surrounding the arbuscules in the infected roots of *Allium cepa*[39]. No ATP-dependent enzyme activity was found in the sites where no active mycorrhyzal colonization occurred (i.e. old arbascules or non-colonized cells). Based on this early observation and the results obtained in our study we propose that the increased amount of efflux measured on the mycorrhizal maize roots were mainly due to the active proton pumping induced by the H^+-ATPase. Different studies on barley[40], tomato[41], tobacoo[42] and *Medicago tranculata*[43] concluded that the AM fungal colonization modulates the expression of the H^+-ATPase gene. Further studies are necessary to ascertain whether mycorrhizal-specific H^+-ATPase induction and activation is a general phenomenon of the symbiotic interaction of different plant species. In maize the increased proton efflux is possibly instrumental in the increased osmotic adjustment under salt stress, allowing continued growth by increased uptake capability of osmotically active, non-toxic, ions like K^+. In future studies, this hypothesis will be further tested.

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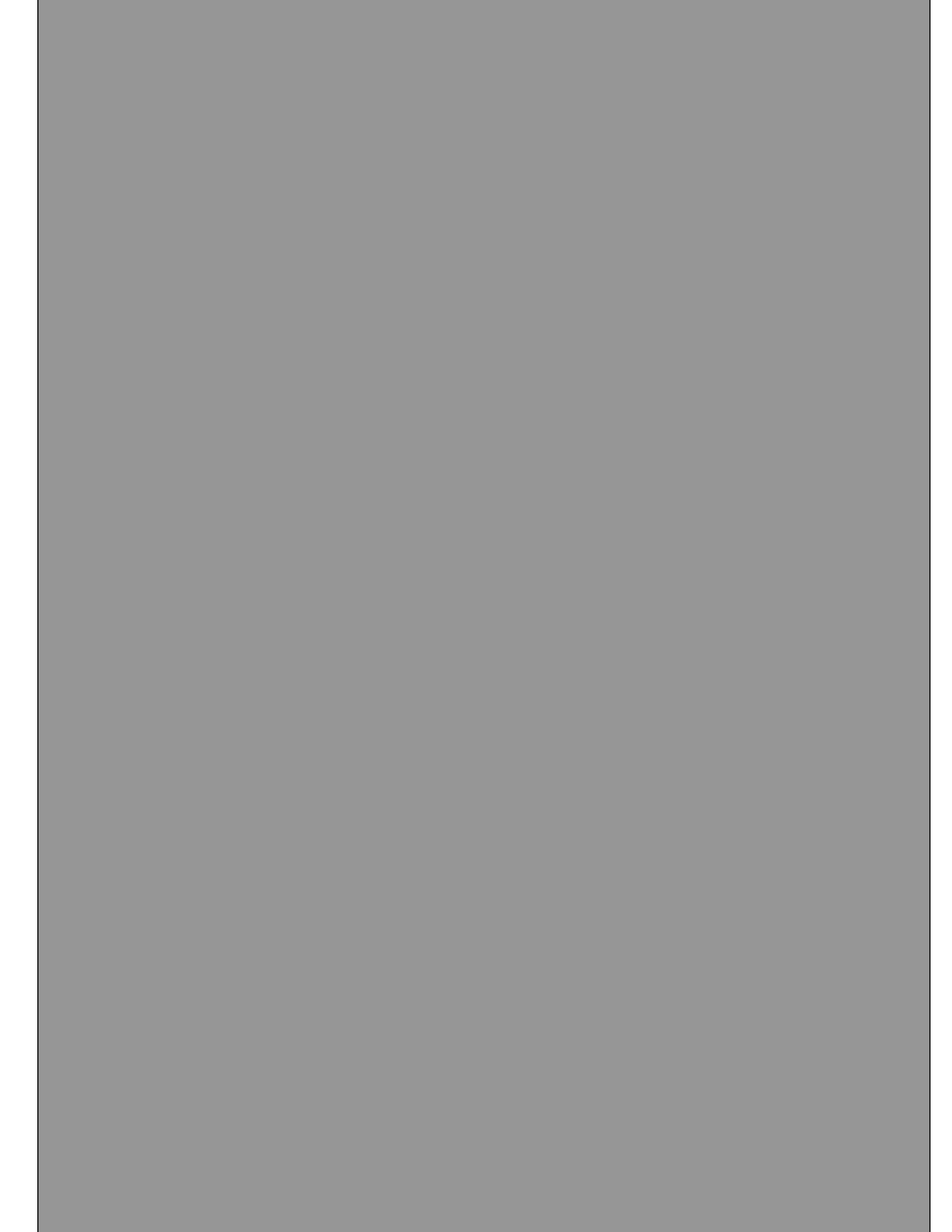
Chapter 7
Summary and Synthesis



We started the work on this thesis by asking the question: *what are possible strategies or mechanisms of salt tolerance in maize?* Now, at the end of the thesis it has become obvious that maize plants can use multiple mechanisms to cope with the problems caused by salinity which include: osmotic stress, ion toxicity, and ion imbalance. We conclude that these mechanisms include: regulation of plant growth, reduction in leaf area, maintaining a high K^+/Na^+ ratio, extrusion of sodium across the plasma membrane of root cortical cells and storage of excess sodium in the vacuoles. It is likely that a number of specific molecular mechanisms including several regulating steps are responsible for these results. In Table 7.1, we try to summarize the performance of the three studied maize cultivars under salt stress; the aim of this comparison is to highlight the possible mechanisms involved in the salt tolerance in each maize cultivar.

SR15 is considered a remarkable result of the most recent plant breeding programs in maize[1], where two major lines for salt tolerance were combined to form the newly developed SR15 hybrid. These two lines are selected for better sodium exclusion and better osmotic adjustment, respectively. SR15 acted as a moderately salt tolerant cultivar, with a typical, but efficient, 'glycophytic' strategy against the increase in salt concentration of the soil solution. This strategy includes moderate reduction in plant growth and leaf area, increasing the concentration of the compatible solutes in the growing leaves, and exclusion of NaCl from the shoot at low to moderate levels of soil salinity. These observations indicate that SR15 has indeed inherited the osmotic adjustment from one parent line and the ability to exclude more sodium from entering the root from the other, and forms a hybrid that is superior to both his parents.

Arizona 8601 applies strategy under salt stress aimed at survival. The stunted growth of the shoot and the reduction of leaf area both minimize the transpiration surface. This limits the need for water uptake and thus the accumulation of sodium in the shoot. It might not be the best economically attractive maize cultivar, but the results documented in this work and in that of Day[2] show that Arizona 8601,

compared to that of SR15 and Delitop in our study or Pioneer 3183 in Day's, yielded more plant biomass under saline conditions. Delitop, a cultivar that was not specifically bred for salt resistance, did tolerate exposure to salt for a period up to 14 days better than expected from a typical glycophyte. Possibly, longer exposure to salt of Delitop will result in signs of toxicity and growth reduction.

Table 7.1. *Comparison between the responses of the three maize cultivars in this study to salt stress.*

Salt tolerance criteria tested	**Arizona 8601**	**SR15**	**Delitop**
Regulation of plant growth	Stunt growth as survival strategy	Maintain the whole plant growth relative to control	A relative reduction in shoot growth but maintenance of root mass
Maintenance of Leaf area	Significantly decreased	Non significant decrease unless with very high concentration of salt (150 mM)	Significant decrease with high salt concentration
Photosynthetic rate and stomatal conductance	Maintained under salt stress	Maintained under salt stress	Reduced
Na^+ exclusion from the shoot	More Na^+ conc. in leaf	Least conc. of Na^+ in leaf	Less conc. of Na^+ in leaf
K^+ content	More decrease	Least decrease	Less decrease
K^+/Na^+	More decrease	Least decrease	Less decrease
Less K^+ leakage from root	More control on K^+ leakage, induce K^+ influx with prolonged treatment	An induced K^+ influx with the increase in salt concentrations to 200 mM NaCl	Less control on the K^+ leakage than the other two cultivars
plasma membrane Na^+/H^+ anitport activity	least efflux of protons with application of salt	Less efflux of protons with application of salt	More efflux of protons with application of salt
Vacuolar compartmentation	Na^+/H^+ antiport activity is used for vacuolar loading on sodium	No Na^+/H^+ antiport activity	Not tested

7.1 The role of sodium exclusion and tissue tolerance in determining salt tolerance in maize

The mechanism of Na^+ exclusion enables the plant to avoid or postpone the problem of ion toxicity, but unless it is compensated for by the uptake of K^+ or the synthesis of organic, compatible solutes for osmotic adjustment[3] the exclusion of Na^+ will exacerbate the osmotic imbalance between the saline soil solution and the cell. The synthesis of organic solutes represents an additional cost in terms of energy. In a more philosophical description Munns and Tester[3] stated that "the plant must steer a course through ion toxicity on the one hand, and turgor loss on the other, in analogy to the Scylla versus Charybdis dilemma faced by Ulysses."

Using Na^+-induced K^+ efflux as an indication for Na^+ exclusion properties and utilizing it as a screen for salt tolerance, has been a subject of debate. The K^+ efflux appears to correlate with salt tolerance for some plant species, but this correlation doesn't exist in others [4-6].

A negative relationships between salinity tolerance and leaf Na^+ concentration was found in the studied maize cultivars after 3, 7 and 10 days of growth on 100 mM salt containing nutrient solution (Figure 7.1). The higher intercept on the x-axis indicates an increased tolerance to the low osmotic value of the soil solution by elevating the internal concentrations of Na^+. This correlation was also reported for different cereal crops, like rice[7], durum wheat[8] and bread wheat[9], while it was less obvious in barley[10].

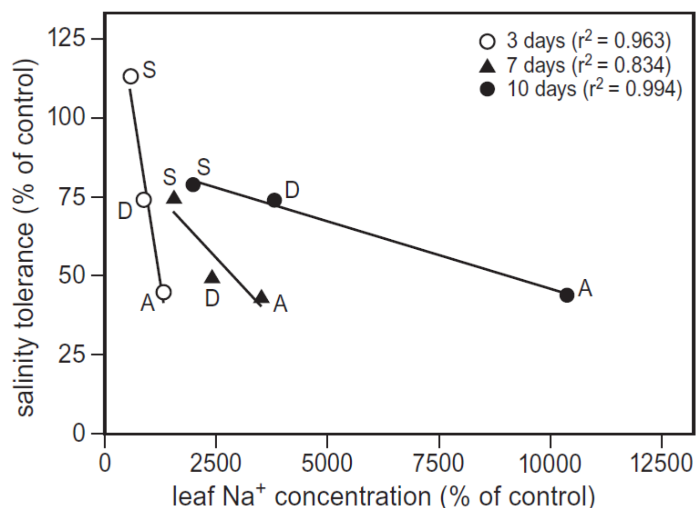


Figure 7.1. *Relationships measured between salinity tolerance (shoot fresh weight in 100 mM NaCl as a % of shoot fresh weight in 0 mM NaCl conditions) and leaf Na^+ concentration (as % from control in different maize cultivars). Negative correlations were obtained after 3, 7, 10 days of growth on salt. A= Arizona 8601, S= SR15 and D= Delitop.*

Cheeseman[11] stated back in 1988 that 'it is unclear how many different types of transporters must actually be involved in Na^+ transport'. About two and a half decades of intensive and novel research later, plant physiologists are still puzzled by Na^+ transport[12]. In some ways, the complexity of sodium transport in plants appears to exceed that of most other ions. Na^+ transport includes influx, efflux, sequestration, long-distance transport and recirculation. Possibly, this complexity is due to the low value of Na^+ as a nutrient, resulting in it being shuffled around from cell to cell and tissue to tissue, just to avoid reaching toxic levels. Plants seem to cope with saline environments by utilizing very different strategies[13], and breeders probably also should not be tempted to aim for a single 'cure all'. In this study we focused on two major sodium transport pathways in maize leading to low cytoplasmic Na^+ levels: exclusion (Chapters 2 and 4) and vacuolar loading (Chapter 5).

The possibility that maize was able to compartmentalize sodium into the vacuole was doubtful, since only few reviews indicated the presence of a vacuolar Na^+/H^+ antiporter in maize[14,15]. The finding that in cultivar Arizona 8601 vacuolar Na^+/H^+ antiport activity could be measured is novel. This trait of Arizona 8601 allows accumulation of Na^+ in the vacuole to increase the osmotic potential of the cell and decrease the toxicity of sodium in the cytoplasm. Osmotic adjustment to a low external water potential by accumulating Na^+ in the vacuole can be considered the "cheapest" way to do the trick.

7.2 K^+ concentration in the stressed leaves

Contrary to some studies in which the absolute K^+ content of a plant correlated positively with the salt tolerance[16, 17], we did not find such a correlation between the growth rate under salt stress and leaf K^+ concentration in the three maize hybrids (Figure 7.2). However, we did find a positive correlation between salt tolerance and K^+/Na^+ ratio in the shoot after 3, 7 and 10 days on 100 mM NaCl (Figure 7.3). This is in agreement with the suggestion of Maathuis and Amtmann[17] that it is not the absolute amount of K^+ concentration in the cytoplasm that determines salt tolerance but the K^+/Na^+ ratio.

The optimal cytosolic K^+/Na^+ ratio can be maintained by either restricting Na^+ accumulation in plant tissues or preventing potassium loss from the cell. Due to the lack of correlation between the salt tolerance and the amount of K^+ efflux from the root of a 3 day old maize seedling observed in chapter 3, we are tempted to conclude that the control of the K^+/Na^+ ratio in the leaf is established by restricting the rise in sodium level in the shoot rather than in preventing the loss of potassium from the tissue.

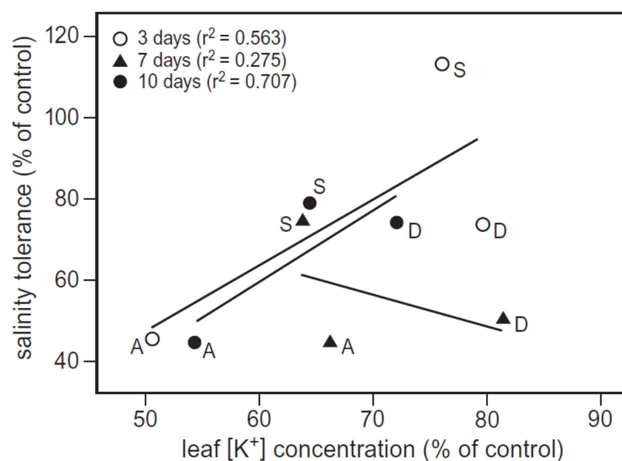


Figure 7.2. *Relationships measured between salinity tolerance (shoot fresh weight in 100 mM NaCl as a % of shoot fresh weight in 0 mM NaCl conditions) and leaf K^+ concentration (as % from control in different maize cultivars). No correlations were obtained after 3, 7, 10 days of growth on salt. A= Arizona 8601, S= SR15 and D= Delitop.*

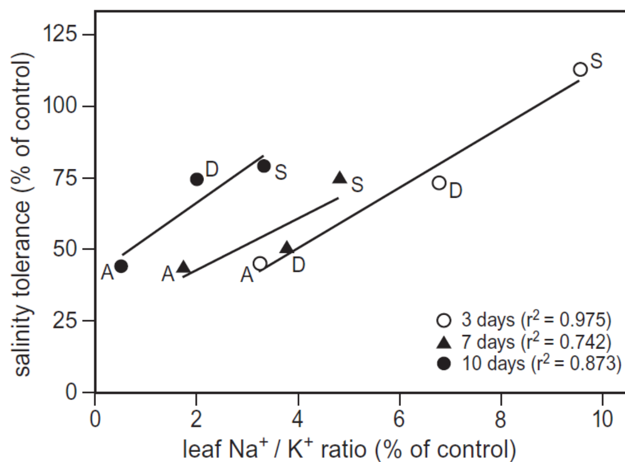


Figure 7.3. Relationships measured between salinity tolerance (shoot fresh weight in 100 mM NaCl as a % of shoot fresh weight in 0 mM NaCl conditions) and leaf K^+/Na^+ ratio (as % from control in different maize cultivars). Positive correlations were obtained after 3, 7, 10 days of growth on salt. A= Arizona 8601, S= SR15 and D= Delitop.

7.3 K^+ and H^+ fluxes in protoplasts as indicators of the K^+ transport kinetics under salt stress.

As proposed by Chen *et al*[18] K^+ efflux is probably mediated by outward rectifying K^+ selective channels. The activity of these channels is determined by the degree of the salt-induced membrane depolarization. This depolarization is also likely to stimulate H^+-ATPase activity, which can be measured as an increased DCCD-sensitive H^+ efflux. Plotting the NaCl-induced K^+ efflux upon sudden salt addition (described in chapter 3) and the DCCD sensitive H^+ fluxes measured on protoplasts after salt addition (described in chapter 4), a positive correlation was found between the K^+ efflux from the root surface and the H^+ efflux measured from protoplasts isolated from the same root zone of 3 days old maize seedlings upon salt addition (Figure 7.4). All the three maize cultivars had a correlation value >0.8.

This correlation indicates that the dynamics of K^+ and H^+ transport is similar in each of the tested cultivars. These results are in agreement with the observation by Chen *et al*[18] and the explanation given by Shabala and Cuin[19] on the H^+ efflux, possibly mediated by the plasma membrane H^+-ATPase, and the K^+ efflux upon sudden exposure to Na^+ in the external solution. Depending on severity and the time of exposure to salt stress, one of these two components dominates, resulting in either increased K^+ uptake (at mild salinity levels and/or more salt tolerant plants), or in a massive K^+ leak (at more severe salt concentrations and/or salt sensitive plants) from the cell or tissue.

As summarized in Table 7.1, SR15 and Arizona 8601 have more salt-tolerance criteria than Delitop. The performance of Delitop under salt stress could be improved by Arbuscular mycorrhiza (AM) fungal colonization (Chapter 6). This positive effect of AM colonization was more obvious in the newly developed leaves that completed their growth during the period of salt stress. Growing Delitop on salt containing growth media resulted in a decrease in leaf area after 3 days of salt stress and in inhibition of the formation of new leaves after prolonged periods of

salt stress (Chapter 2). The inhibition by salt of leaf area was much reduced in mycorrhized plants, especially in the newly developing leaves and after short term salt stress. Mycorrhization increased the osmolarity of cells sap of Delitop leaves by 100%, indicative of better osmotic adjustment in the growing points. Using the MIFE technique, we were able to measure the H^+ flux profiles along the roots of mycorrhized and non-mycorrhized plants. Different H^+ flux pattern between mycorrhized and non-mycorrhized roots were observed, with a higher efflux in the mycorrhized plants, suggesting induction or activation of plasma membrane H^+-ATPase by mycorrhizal colonization. Exposure of the roots to salt did not affect the H^+ flux profile of maize roots.

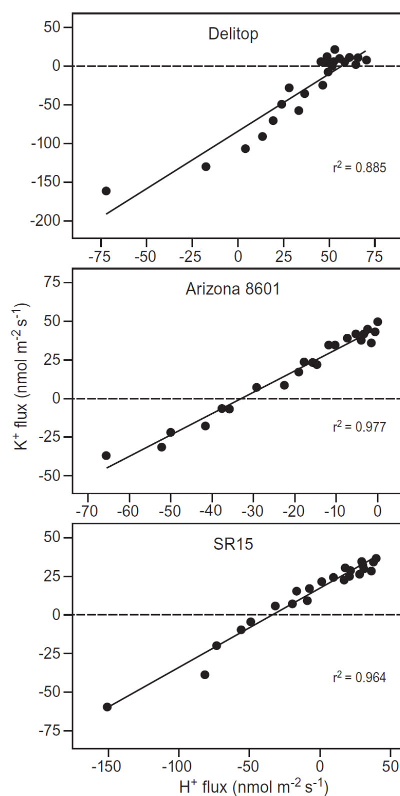


Figure 7.4. *Relationships between the NaCl-induced K^+ fluxes measured on the root surface at the mature zone (10 mm away from the root tip) and NaCl-induced H^+ fluxes measured from the surface of protoplasts isolated from the mature zone of the root cortical cells. Positive correlation was obtained between H^+ and K^+ flux in the three maize cultivars.*

7.4 Ideas for future work

From the work presented in this thesis it has become clear that the application of the MIFE technique helped in identifying the different cation fluxes associated with salt stress, at tissue, cellular and for the first time, at vacuolar level. This confirms the conclusion of Chen *et al*[16] that the MIFE technique is an easy and reliable screening tool for salt tolerance.

Measuring ion fluxes from protoplasts is considered a unique tool to measure different plasma membrane transport mechanisms without the interference created by the cell wall. In this work we have studied the effect of NaCl on the induction of H^+ fluxes, which have given us an insight in the role of the H^+-ATPase activity in restoring the maize membrane potential. For the study of Na^+/H^+ antiport activity, either in the plasma membrane or in the tonoplast, the application of Na^+ selective microelectrode will make it possible to couple the observed H^+ fluxes to Na^+ counter fluxes. Possible differences in compartmentation of ions and in ion transport and translocation between different plant cell types in shoot and root could be studied by the isolation of the protoplasts from different plant tissues.

The vacuolar membrane's positive membrane potential allows the passive diffusion of anions. The study of vacuolar chloride channels and other anion porters with the MIFE technique will be of considerable interest when trying to elucidate the contribution of Cl^- to salt stress. In principle, using the vibrating microelectrodes to study ion channels and transporters in an individual vacuole, i.e. using *Arabidopsis* knock-out mutants, would be a valuable complement to patch clamp studies on specific channels.

The use of the MIFE technique in microbiological studies and specially plant microbe interaction has been discussed in several papers[20, 21], however still more focus is required on the role of plant-micro-organism interaction in nutrient uptake and hormonal signalling. In our study, we demonstrated that H^+ fluxes are changed

by the association with fungal hyphae, a result that might indicate that a molecular and/or ionic dialogue between AM fungi and host plant is taking place. With the MIFE we might be able to eavesdrop on this conversation.

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S. Root plasma membrane transporters controlling K^+/Na^+ homeostasis in salt-stressed barley. *Plant Physiology* **145**, 1714-1725 (2007).

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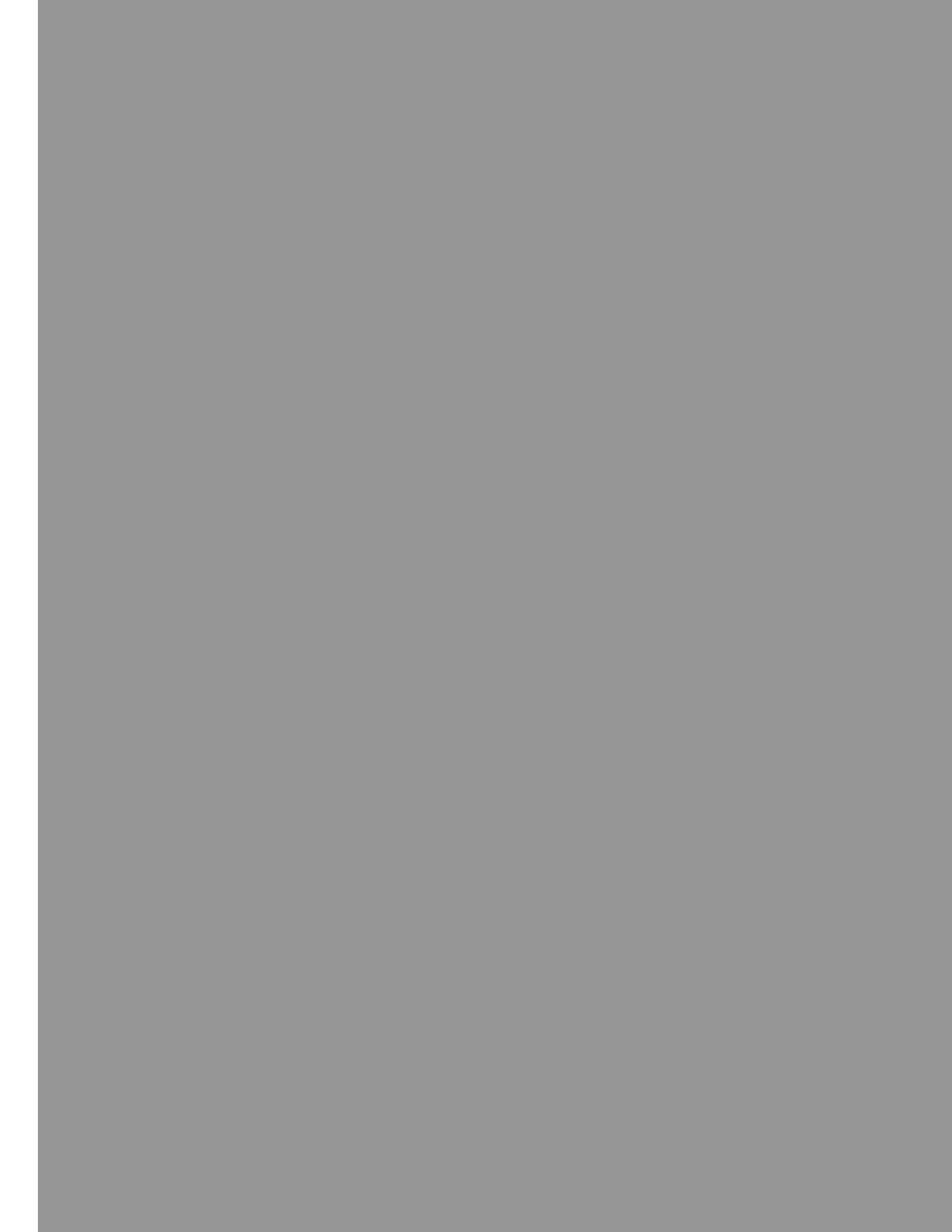
Samenvatting en Synthese

Dutch Summary and Synthesis

Translated by: Marten Staal and Theo Elzenga



Het werk voor dit proefschrift begon met het stellen van de volgende vraag: Wat zijn de mogelijke strategieën of mechanismen voor zouttolerantie in maïs? Nu, aan het eind van dit proefschrift, is het duidelijk geworden dat maïs meerdere mogelijkheden heeft om met problemen veroorzaakt door verzilting van de bodem, zoals osmotische stress, natrium toxiciteit en een verkeerde verhouding van K^+en Na^+ ionen om te gaan. De opties zijn: aanpassing van de groeisnelheid, vermindering van bladoppervlakte, handhaving van een hoge K^+/Na^+ ratio, uitscheiden van Na^+ over het plasmamembraan van de cortex cellen van de wortel en het opslaan van een overmaat aan Na^+ ionen in de vacuole. Het is aannemelijk dat specifieke moleculaire mechanismes, waaronder een aantal regulerende stappen, verantwoordelijk zijn voor deze resultaten. Tabel 7.1 geeft weer hoe de drie bestudeerde maïs cultivars zich gedragen bij zoutstress.

De cultivar SR15 wordt beschouwd als een opmerkelijk resultaat van een recent plantenveredelingprogramma voor maïs[1], waarin twee belangrijke zouttolerante lijnen werden gecombineerd tot de SR hybrides. De twee ouder lijnen zijn geselecteerd op, respectievelijk, het vermogen om Na^+ buiten de cel te houden en het vermogen om zich beter osmotisch aan te passen aan verzilting in de bodem. SR15 gedraagt zich als een gemiddeld zouttolerante plant met een strategie die typisch is voor glycofieten (zoutmijdende), maar een die wel heel efficiënt wordt toegepast. Deze strategie bestaat uit verminderding van de plantengroei en bladoppervlakte, toename van organische oplosbare stoffen in groeiende bladeren, en een mechanisme om zout buiten de spruit te houden bij lage tot middelmatige bodemverzilting. Deze resultaten zijn in overeenstemming met de genetische achtergrond van SR15. Het is een hybride die beter bestand is tegen zout dan zijn beide ouders, waarvan deze zowel het vermogen om zout buiten te sluiten van de ene ouder, als een efficiënte osmotische aanpassing van de ander overgeërfd is.

De cultivar Arizona 8601 heeft een overlevingsstrategie onder zoutstress. De verminderde groei van de spruit en de vermindering van het totale bladoppervlak zorgen voor een verkleining van het verdampingsoppervlak. Dit beperkt de

behoefte om water op te nemen en daarmee ook de opeenhoping van Na^+ in de spruit. Economisch gezien lijkt deze cultivar niet bijzonder aantrekkelijk, maar zowel de resultaten beschreven in dit proefschrift als de resultaten van Day[2] laten zien dat Arizona 8601 een hogere biomassa productie heeft onder zoute omstandigheden dan SR15, Delitop (dit proefschrift) en Pioneer 3183[2].

Table 7.1. *Comparison between the responses of the three maize cultivars in this study to salt stress.*

Salt tolerance criteria tested	Arizona 8601	SR15	Delitop
Regulation of plant growth	Stunt growth as survival strategy	Maintain the whole plant growth relative to control	A relative reduction in shoot growth but maintenance of root mass
Maintenance of Leaf area	Significantly decreased	Non significant decrease unless with very high concentration of salt (150 mM)	Significant decrease with high salt concentration
Photosynthetic rate and stomatal conductance	Maintained under salt stress	Maintained under salt stress	Reduced
Na^+ exclusion from the shoot	More Na^+ conc. in leaf	Least conc. of Na^+ in leaf	Less conc. of Na^+ in leaf
K^+ content	More decrease	Least decrease	Less decrease
K^+/Na^+	More decrease	Least decrease	Less decrease
Less K^+ leakage from root	More control on K^+ leakage, induce K^+ influx with prolonged treatment	An induced K^+ influx with the increase in salt concentrations to 200 mM NaCl	Less control on the K^+ leakage than the other two cultivars
plasma membrane Na^+/H^+ anitport activity	least efflux of protons with application of salt	Less efflux of protons with application of salt	More efflux of protons with application of salt
Vacuolar compartmentation	Na^+/H^+ antiport activity is used for vacuolar loading on sodium	No Na^+/H^+ antiport activity	Not tested

Delitop, een cultivar die niet specifiek voor zouttolerantie is veredeld, handhaafde zich beter na te zijn blootgesteld aan zout gedurende 14 dagen, dan zou mogen worden verwacht van een typische glycofiet. Langere blootstelling aan zout zal vermoedelijk echter wel leiden tot vergiftigingsverschijnselen en een verminderde groei.

De rol van het buiten de cel houden van Na^+ en weefseltolerantie in het bepalen van zouttolerantie bij maïs

Het buiten de cel houden van Na^+ voorkomt, in ieder geval tijdelijk, dat de plant last heeft van natrium toxiciteit. Een vereiste is dan wel dat de plant extra K^+ opneemt of organische oplosbare stoffen aanmaakt om osmotisch in balans te blijven met de zoute omgeving. De aanmaak van extra organische oplosbare stoffen kosten extra energie. Munns en Tester[3] beschreven het op de volgende filosofische wijze: “de plant moet zijn weg zien te vinden tussen ion toxiciteit aan de ene en het verlies van turgor aan de andere kant, analoog aan het Scylla versus Charybdis dilemma waar Odysseus mee werd geconfronteerd”.

Een negatieve relatie tussen zouttolerantie en de Na^+ concentratie in het blad werd gevonden in de gebruikte maïs cultivars na 3, 7 en 10 dagen groei op een voedingsoplossing met daarin 100 mM NaCl (Figuur 7.1). Hoe verder naar rechts het snijpunt met de x-as wordt gevonden, hoe hoger de tolerantie van de plant tegen een lage osmotische waarde van de bodemoplossing d.m.v. een verhoging van de interne Na^+ concentratie. Deze correlatie is ook beschreven voor verschillende andere graangewassen, zoals rijst[4], durum tarwe[5] en broodtarwe[6], terwijl voor gerst deze correlatie in mindere mate op ging[7].

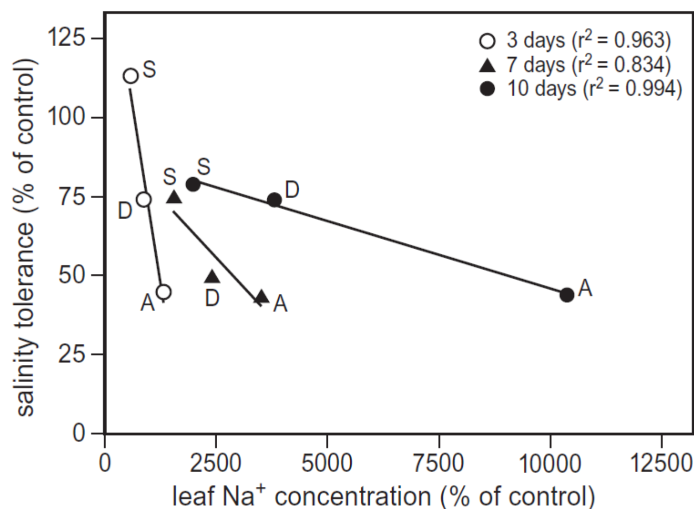


Figure 7.1. *Relationships measured between salinity tolerance (shoot fresh weight in 100 mM NaCl as a % of shoot fresh weight in 0 mM NaCl conditions) and leaf Na^+ concentration (as % from control in different maize cultivars). Negative correlations were obtained after 3, 7, 10 days of growth on salt. A= Arizona 8601, S= SR15 and D= Delitop.*

In 1988 beweerde Cheeseman[8] dat het onduidelijk is hoeveel verschillende typen transport moleculen er nu eigenlijk betrokken zijn bij het transport van Na^+. Ongeveer 25 jaren van intensief en innovatief onderzoek later, zijn plantenfysiologen nog steeds bezig het raadsel van het Na^+ transport te ontrafelen[9]. Op de één of andere manier lijkt het Na^+ transport complexer dan het transport van andere ionen. Netto Na^+ transport bestaat uit influx, efflux, het opslaan, lange afstand transport en (re)circulatie. Mogelijk is de complexiteit van het transport te wijten aan het feit dat Na^+ een lage voedingswaarde heeft, waardoor het heen en weer wordt getransporteerd tussen cellen en weefsels om toxische concentraties te vermijden. Planten kunnen zich handhaven in een zout milieu door verschillende strategieën te gebruiken[10] en plantenveredelaars zouden zich daarom niet moeten focussen op één enkele eigenschap. In dit onderzoek hebben we ons beperkt tot 2 belangrijke Na^+ transport wegen, die tot lagere Na^+ concentraties in het cytoplasma leiden te weten: het buiten de cel houden (hoofdstuk 2 en 4) en opslag in de vacuole (hoofdstuk 5).

De mogelijkheid dat maïs in staat is om Na^+ in de vacuole op te slaan was twijfelachtig, omdat slechts een enkele keer de aanwezigheid van een Na^+/H^+ antiporter, het transporteiwit dat verantwoordelijk is voor het transport van Na^+ in de vacuole, op de tonoplast is beschreven[11,12]. Dat in dit proefschrift in cultivar Arizona 8601 de activiteit van de Na^+/H^+ antiporter in geïsoleerde vacuoles kon worden gemeten is een noviteit. De vacuolaire Na^+/H^+ antiporter in Arizona 8601 is hoogstwaarschijnlijk verantwoordelijk voor opeenhoping van Na^+ in de vacuole, waardoor de osmotische potentiaal van de cel toeneemt en de toxiciteit van Na^+ in het cytoplasma afneemt. Deze osmotische aanpassing van de plant aan een lagere waterpotentiaal d.m.v. opslag van Na^+ in de vacuole wordt beschouwd als de "meest voordelige" manier van aanpassen.

K^+ concentratie in aan bladeren die zijn blootgesteld aan zoutstress

In tegenstelling tot studies waarbij het absolute K^+ gehalte van een plant positief is gecorreleerd met de zouttolerantie[13,14], werd een dergelijke correlatie tussen de groeisnelheid onder zoutstress en de K^+ concentratie in de bladeren van de drie bestudeerde maïs cultivars in de huidige studie niet gevonden (figuur 7.2). Wel werd een positieve correlatie tussen zouttolerantie en de K^+/Na^+ ratio in de spruit na een behandeling gedurende 3, 7 en 10 dagen op 100 mM NaCl gevonden (figuur 7.3). Dit is in overeenstemming met de suggestie van Maathuis en Amtmann[14] dat het niet de absolute K^+ concentratie is, die de zouttolerantie bepaalt maar de K^+/Na^+ ratio.

De optimale K^+/Na^+ ratio kan worden bereikt door de ophoping van Na^+ in de plant te voorkomen of door het verlies van K^+ uit de cel te voorkomen. Het gebruik van Na^+-geïnduceerde K^+ efflux als een indicatie voor het buiten de cel houden van Na^+ en de mogelijkheid om dit te gebruiken als een screeningsmethode voor zouttolerantie, is in de wetenschappelijke literatuur een onderwerp van discussie. De correlatie tussen K^+ efflux en zouttolerantie gaat op voor een aantal plantensoorten, maar voor anderen niet[15-17].Omdat we geen correlatie hebben

gevonden tussen de zouttolerantie en de K^+ efflux van drie dagen oude maïs wortels (hoofdstuk 3), concluderen we dat de K^+/Na^+ ratio in de plant bepaald wordt door het voorkomen van Na^+ ophoping in de spruit en niet zozeer door het beperken van het verlies van K^+.

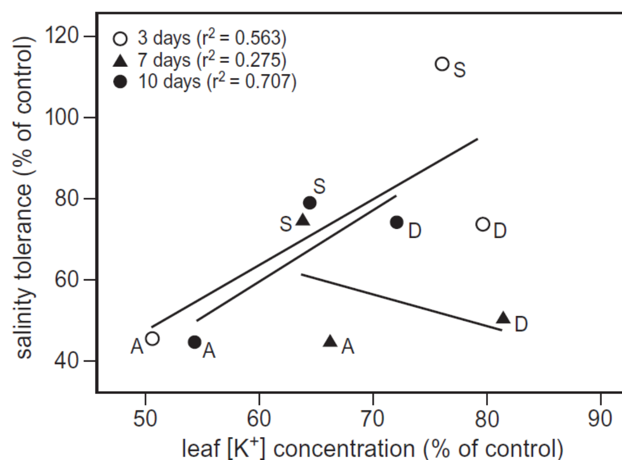


Figure 7.2. *Relationships measured between salinity tolerance (shoot fresh weight in 100 mM NaCl as a % of shoot fresh weight in 0 mM NaCl conditions) and leaf K^+ concentration (as % from control in different maize cultivars). No correlations were obtained after 3, 7, 10 days of growth on salt. A= Arizona 8601, S= SR15 and D= Delitop.*

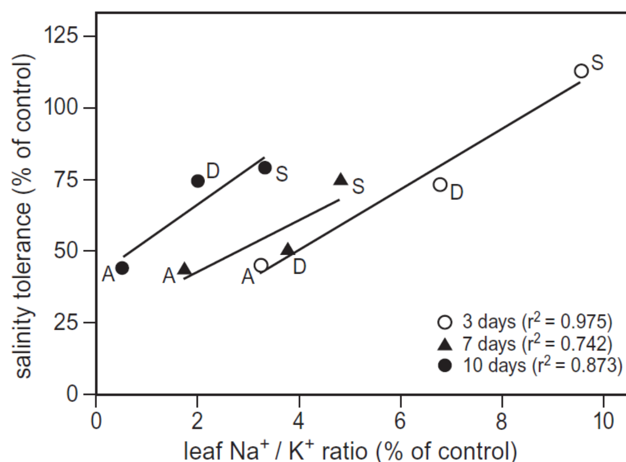


Figure 7.3. Relationships measured between salinity tolerance (shoot fresh weight in 100 mM NaCl as a % of shoot fresh weight in 0 mM NaCl conditions) and leaf K^+/Na^+ ratio (as % from control in different maize cultivars). Positive correlations were obtained after 3, 7, 10 days of growth on salt. A= Arizona 8601, S= SR15 and D= Delitop.

K^+ en H^+ fluxen in protoplasten als een maat voor K^+ transport onder zoutstress

De K^+ efflux wordt hoogstwaarschijnlijk gefaciliteerd door zogenaamde 'outward-rectifying, K^+-selectieve kanalen[18]. Deze kanalen worden actief door depolarisatie van de membraanpotentiaal ten gevolge van de opname van Na^+. De H^+-ATPase activiteit, gemeten als een toename van de DCCD gevoelige H^+ efflux, wordt ook gestimuleerd door deze depolarisatie. Als de NaCl-geïnduceerde K^+ efflux (hoofdstuk 3) wordt uitgezet tegen de DCCD gevoelige H^+ efflux, gemeten aan protoplasten van 3 dagen oude maïs wortels (hoofdstuk 4) dan is er een positieve correlatie tussen beide fluxen. Alle drie de gebruikte maïs cultivars hebben een correlatie coëfficiënt >0.8. De gevonden correlatie duidt erop dat de dynamiek voor zowel het K^+ als het H^+ transport hetzelfde is in de drie bestudeerde cultivars. Deze resultaten komen overeen met de observaties van Chen *et al*[18] en de verklaring die door Shabala en Cuin[19] wordt gegeven voor de H^+ efflux door het plasmamembraan gebonden H^+-ATPase en de K^+ efflux na blootstelling aan Na^+ in het externe medium. Afhankelijk van de intensiteit en de duur van blootstelling aan zoutstress zal één van deze twee componenten domineren, wat resulteert in een toename van de K^+ opname (bij lichte zoutstress en/of bij zouttolerantere planten) of in aanzienlijke K^+ lekkage van de cel of weefsel (in het geval van hogere zout concentraties en/of zout gevoelige planten).

Zoals samengevat in tabel 7.1 voldoen SR15 en Arizona 8601 meer aan de zouttolerantie criteria dan Delitop. Het functioneren van Delitop onder zoutstress zou verbeterd kunnen worden door vorming van arbusculaire mycorrhiza, een symbiose van een plant met een schimmel, (hoofdstuk 6). Het positieve effect van arbusculaire mycorrhiza was duidelijk te zien aan de jonge bladeren die onder zoutstress zijn gevormd en uitgegroeid. Het groeien van Delitop op een voedingsoplossing met zout resulteerde in een afname van de bladoppervlakte na 3 dagen van zoutstress en de vorming van nieuwe bladeren was geremd bij langdurige zoutstress (hoofdstuk 2). De zout-geïnduceerde vermindering van het

bladoppervlak was niet zo sterk in planten met mycorhiza's. In Delitop resulteert de aanwezigheid van mycorrhiza's in een verhoging van de osmolariteit van het celsap van bladeren met 100%. Dat zou kunnen duiden op een betere osmotische aanpassing in de groeizones. Met de MIFE techniek zijn de H^+ flux profielen langs wortels van gekoloniseerde en niet gekoloniseerde planten gemeten. De wortels met mycorrhiza vertoonden een hogere H^+ efflux . Dat zou kunnen wijzen op inductie of activatie van het plasmamembraan gebonden H^+-ATPase door kolonisatie van mycorrhiza-vormende schimmels. De aanwezigheid van zout had geen effect op de H^+ flux profiel van maïs wortels, waarbij het niet uitmaakte of de wortels geïnfecteerd waren of niet.

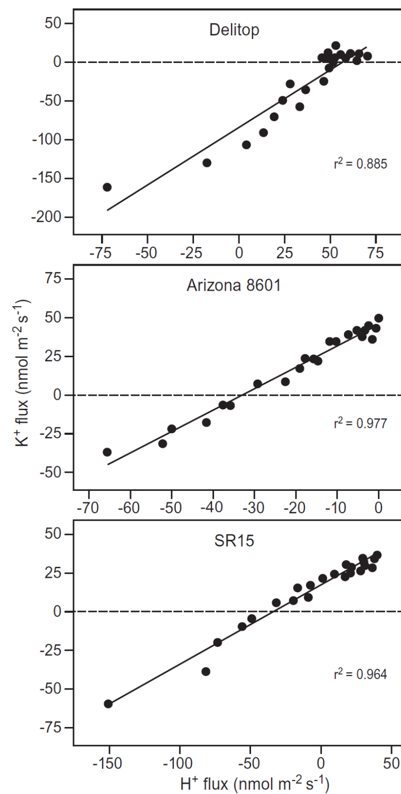


Figure 7.4. *Relationships between the NaCl-induced K^+ fluxes measured on the root surface at the mature zone (10 mm away from the root tip) and NaCl-induced H^+ fluxes measured from the surface of protoplasts isolated from the mature zone of the root cortical cells. Positive correlation was obtained between H^+- and K^+ flux in the three maize cultivars.*

Ideeën voor toekomstig werk

Uit het werk beschreven in dit proefschrift komt duidelijk naar voren dat de MIFE techniek kan worden toegepast om de diverse kationen fluxen, die een rol spelen bij zoutstress op zowel weefsel-, cel- en (nu voor het eerst beschreven) op vacuoleniveau te onderscheiden. We onderschrijven daarom de conclusie die werd getrokken door Chen *et al*[16] dat de MIFE techniek een gemakkelijke en betrouwbare screening methode is voor zouttolerantie.

Het meten van ionen fluxen aan protoplasten wordt beschouwd als een unieke methode om verschillende plasmamembraan-gebonden transportprocessen te meten zonder de storende aanwezigheid van de celwand. In dit proefschrift is het effect van NaCl op de inductie van H^+ fluxen beschreven. En dit gaf inzicht in de rol die de H^+-ATPase activiteit heeft in het herstellen van de membraanpotentiaal van maïs cellen. Om Na^+/H^+ antiport activiteit in het plasmamembraan en/of tonoplast te bestuderen, is het meten van Na^+ fluxen met een Na^+-selectieve elektrode een manier om deze Na^+ flux te koppelen aan de gemeten H^+ flux. Mogelijke verschillen in de verdeling van ionen over verschillende compartimenten en in ionentransport tussen verschillende celtypes uit spruit en wortel, zouden kunnen worden bestudeerd m.b.v. protoplasten, geïsoleerd uit de verschillende plantenweefsels.

De positieve membraanpotentiaal van de vacuole is een drijvende kracht voor de passieve opname van anionen. Het bestuderen van chloride kanalen en andere anionen transport eiwitten met de MIFE techniek zou een goede manier zijn om de rol van Cl^- in zoutstress te bepalen. Het zou een waardevolle aanvulling kunnen zijn op patch clamp studies om flux metingen met ion-specifieke micro-elektrodes te verrichten aan vacuoles van knock-out mutanten van anionen transporters in *Arabidopsis*.

De MIFE techniek is al eerder toegepast in microbiologische studies, in het bijzonder naar de interactie tussen plant en micro-organisme[20,21]. Meer onderzoek

is echter nodig om de interactie tussen plant en micro-organisme in nutriënten opname en hormoon signalering te bestuderen. In dit proefschrift hebben we laten zien dat de H^+ flux, gemeten aan wortels, verandert door kolonisatie van de wortel met een schimmel. Dit zou kunnen duiden op een moleculaire en/of door ionen gedragen communicatie tussen arbusculaire micorrhiza schimmel en gastheer plant. De MIFE techniek stelt ons mogelijk in staat "mee te luisteren met dit gesprek".

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Curriculum Vitae

Fatma Zaki Mostafa Ali was born in November 3, 1977 in Cairo, Egypt. In 1995 she finished high school and joined the science faculty. She obtained a bachelor degree in botany from the Botany Department, Faculty of science, Ain Shams University in Cairo, Egypt in 1999. She worked since 2000 till 2005 as demonstrator in the Botany Department, Faculty of Science Ain Shams University. In 2005 she obtained a master in plant physiology, and was promoted to assistant lecturer in the same department.

She was granted a four years paid scholarship from the Egyptian Ministry of Higher Education, Cultural and Missions Sector to obtain her doctoral degree from the Netherlands. In January 2007 she started her PhD program in the department of plant Eco-physiology, University of Groningen, the Netherlands, under the supervision of Prof. Dr. J. Theo. M. Elzenga. During this time she participated in several conferences and courses listed below.

Courses and International Meetings

- Dynamic presentation course (University of Groningen)
- SPSS course (University of Groningen).
- Publishing using word course (University of Groningen).
- Reference manager course (University of Groningen).
- Five days of training on the in vitro mycorrhiza culture. (Université Paul Sabatier, endo- mycorrhizal symbiosis and cell signaling group, under supervision of Prof. Guillaume Bécard).
- Attended the 13 th school of pure and applied biophysics. On the subject “Electrogenic transport in plant cells and organelles”. The school has been

held in Venice at the Instituto Veneto di Scienze Lettere ed Arti, January 26-30, 2009.

- EPS 2010, Lunteren.
- International group meeting: Plant Eco-physiology Groningen (NL) & Frankfurt (D), 11th February 2010 in Frankfurt/ Germany.
- Writing a grant proposal course (Rubicon). “University of Groningen”.

الملخص العربى

بدأنا العمل فى هذه الرسالة عن طريق طرح السؤال التالى: ما هى الاستراتيجيات الممكنة لمقاومة نبات الذرة للملوحة. وفى النهاية أصبح واضحاً أن نبات الذرة يستخدم آليات متعددة للتعامل مع المشاكل التى تسببها الملوحة وهذه الآليات يمكن أن تتلخص فيما يلى: تنظيم نمو النبات، الحد من مساحة الورقة، والحفاظ على نسبة عالية من أيونات البوتاسيوم مقابل الصوديوم، التخلص من أيونات الصوديوم الزائدة فى الفجوات الخلوية. و لقد تم تحديد كيفية التحكم فى هذه الآليات على المستوى الجزيئى.

لقد تم استخدام ثلاثة أصناف من الذرة مختلفة فى درجة تحملها الملوحة وهذه: إسى أر 15، أريزونا 8601، وداليتوب. و قد تبين من البحث أن إسى أر 15 هو صنف نموذجى لدراسة آليات مقاومة الملوحة فى الذرة و تشمل استرتيجية هذا النبات على (1) الحفاظ على نمو النبات ومساحة الورقة (2) زيادة تركيز المواد المذابة خاصة فى الأوراق حديثة النمو (3)استبعاد الصوديوم من الوصول إلى الجزء الخضرى من النبات.

أما صنف أريزونا 8601- الذى قد لا يكون اقتصاديا مربح ولكنه بمقارنة انتاجيته مع الأصناف الأخرى تحت تركيز عالى من الملوحة- فقد لوحظ إنه ينمو بصورة أكبر فى حالة الإجهاد الملحى بالمقارنة بالأصناف الأخرى، و كأنه يطابق النباتات المحبة للملح. أما صنف داليتوب يعتبر الصنف الوسطى بين الصنفيين الوارد ذكرهما.

تم عمل دراسة شاملة لدور(آلية) استبعاد الصوديوم فى مقاومة الإجهاد الملحى على ثلاثة مستويات: مستوى الأنسجة، مستوى الغشاء البلازمى ، و مستوى الفجوة الخلوية. و لقد تم اثبات علاقة عسكية بين مقاومة الذرة للملوحة وتركيز الصوديوم فى الأوراق. و كذلك لم يتم التوصل إلى أى علاقة بين تركيز البوتاسيوم فى الأوراق و مقاومة النبات للإجهاد الملحى. و لكنه بحساب نسبة الصوديوم إلى البوتاسيوم تأكد أن تأثير النسبة بين الصوديوم للبوتاسيوم له دور أهم فى مقاومة الذرة للملوحة من القيمة المطلقة لهذين العنصرين.

فى هذه الرسالة تم تحديث أسلوب متابعة تدفق الأيونات باستخدام الميكروالكترود (الأقطاب الدقيقة)، و قد تم تطوير هذه التقنية حتى تتناسب مع قياس معدل تدفق الأيونات عبر الغشاء البلازمى على مستوى الخلية الواحدة. وقد تم التوصل إلى وجود علاقة طردية بين مستوى تدفق البوتاسيوم و الهيدروجين و الاجهاد الملحى مما يشير إلى نشاط المضخات الهيدروجينية الموجودة على الغشاء البلازمى، ومن ثم فقد تكون هى المسئولة عن استبدال البوتاسيوم بالصوديوم داخل الخلية مما له من نواحى ضارة للعمليات الحيوية داخل الخلية.

و فى سابقة هى الأولى من نوعها، تم استخدام تقنية الميكرو الكترود لقياس تدفق الأيونات عبر الغشاء البلازمى الفجوى ومنه تم تأكيد وجود ألية التبادل الأيونى بين الصوديوم والهيدروجين فى جذور صنف (أريزونا 8601) و الذى يجعله يحمل صفة مقاومة ملوحة التربة بصورة عالية.

و للعمل على تحسين أداء نبات الذرة تحت ظروف الإجهاد الملحى فقد تم استخدام الميكروريزا- و هو نوع من التكوينات التكافلية داخل الجذور- ولقد تم توضيح الأثر الإيجابى لهذه العلاقة التكافلية على نمو نبات الذرة (صنف الداليتوب) الحساس للتركيزات العالية من الملح فى التربة عن طريق رفع الضغط الأسموزى فى الأوراق ،مما يدل على تكيف النبات بصورة أفضل. كما لوحظ أيضا زيادة ضخ الهيدروجين من الخلايا المصابة بالفطر، مما يدل على نشاط و كفاءة الغشاء البلازمى تحت تأثير هذه العلاقة التكافلية.

و من هذه الرسالة نستنتج أن استخدام تقنية الميكروالكترود قد ساعد على مقارنة الأصناف المختلفة من الذرة و يحدد الطرق المختلفة لمقاومتها للإجهاد الملحى.

مقاومة نبات الذرة للإجهادالملحى

رسالة مقدمة للحصول على درجة الدكتوراة فى العلوم

(فسيولوجيا النبات)

من

فاطمة زكى مصطفى على

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قسم النبات

(1999)

تحت إشراف

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