SLM

Soil Physics and Land Management

SALINITY STRESS

[A study of barley on hydroponics and sand]

MSc thesis by Eva van Rijsingen

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Soil Physics and Land Management

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Stressed Barley Figured Out

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PREFACE

With this thesis my intention was to integrate my background in Plant Science with the knowledge on international land and water management that I've obtained during my masters. To start with, this was already a great challenge, but that's what I imagined my master thesis would be. On top of this, I always hoped that my master thesis would contribute something useful to this world and in that, I have to admit now, I have been a bit too ambitious. However, at least to my personal development this project has been very beneficial. Sometimes I felt difficulties being very much dependent on other people, but in the end I realized that with some effort a lot can be arranged and that many people were willing to help me out. I came across many challenges, which together with many helpful people could always be tackled to sufficient extent. Furthermore, spending many enjoyable sunny days in the greenhouse, totally lit up my winter!

With this thesis I dove into two areas of research that were relatively new to me, it took some time but in the end I've learned a lot!

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ABSTRACT

Salinization of arable land is affecting food production worldwide and action is required to ensure future food availability. As improved land management and crop enhancement are promising strategies to deal with the threat of salinization, within this thesis both strategies are considered. To gain insight in plant strategies for salt tolerance and their potential for plant breeding, extensive screening is necessary. Due to high controllability and practical convenience, hydroponic systems are preferably used by researchers during screening practice. The comparison of salt tolerance on hydroponics and an inert sand medium gives insight in the applicability of hydroponic and sand screening methods for trait analysis of salt tolerance in particular. In the current research, two tolerant (G1, G2) and two more sensitive barley varieties (G3, G4) were selected to determine genetic variation for salt tolerance in response to NaCl associated salinity stress in both types of root environment. Furthermore, the aim was to keep an eye on the need of discrimination between different genotypes (tolerant/not) when incorporating ST in SWAP-WOFOST, where ST is currently included as an osmotic effect on transpiration.

In this experiment was not succeeded to design comparable stress severity in the hydroponic and sand medium. Due to the gradual increase of salt concentrations in the sand medium, plant damage was prevented. However, based on the hydroponic experiments still genetic variation for salt tolerance and contributing mechanisms could be identified. Osmotic stress effects caused reduction in potential photosynthetic area and tiller formation but also reduced stomatal conductance as well as chlorophyll content. No ion specific effects were observed as a result of the Cl^- ion, whereas, the Na^+ ion is expected to have a negative effect on leaf formation and to additionally limit stomatal conductance and chlorophyll content.

When incorporating the effects of NaCl associated stress in SWAP-WOFOST, based on the current findings, there is no need for differentiation between tolerant and sensitive varieties with regard to water loss due to transpiration. However, with regard to WUE, differentiation between tolerant and sensitive varieties is advisable.

CONTENTS

1. INTRODUCTION

1.1 The threat of salinization

Salinization of arable land is affecting food production worldwide. While the demand for food is growing, the percentage of salinized land increases. The practice of irrigation without proper drainage causes rising ground water tables and accumulation of salts in the root zone (Shani et al., 2007). Irrigated land encompasses 17% of the world's crop land and is providing 40% of our food. Due to salinization, 1-2 per cent of this area is already lost, mainly at the expense of the (semi)-arid regions (FAO, 2002) (Fariduddin et al., 2012). It was estimated that >6% of the world land area and 30% of world irrigated area is already suffering from salinity problems (Eeman et al., 2012).

In addition to bad cultivation practices, climate change enhances salinization of previously fertile soils: high temperatures cause evaporation of soil water from the soil surface, leaving behind its dissolved salt in the form of salt minerals. The increased scarcity of fresh water due to growing demand of the world population will coincide with the use of marginal (salty) water for agricultural purposes (Shani et al., 2007).

Action is required to ensure future food availability. Improved land management as well as crop enhancement should be the main strategies.

1.2 Salt Affected Soils

Three types of salinized soils can be distinguished: i) saline soils, in which it is mainly the elevated osmotic soil water potential, as a result of increased salt concentration, that causes problems with regard to root water uptake, ii) sodic soils contain relatively high amounts of monovalent anions (especially Na^+), causing structural degradation by slaking, swelling and dispersion of mineral soils (Qadir and Schubert, 2002), iii) "alkaline" soils; this type of soil has a relatively high pH-value due to the abundance of alkaline substances, bicarbonate (HCO_3^-) in particular (Beek, 2012, Bolt, 1978).

NaCl is the most abundant and soluble salt released by rock weathering. That is why salinized soils are often NaCl dominated (Türkan and Demiral, 2009). In addition, NaCl originating from the oceans is deposited by wind and rain (Munns and Tester, 2008). In coastal areas, marine deposits and salt water intrusion cause salinization mainly through capillary rise of saline ground water or its use for irrigation (De Louw et al., 2011). High concentrations of NaCl are prone to cause saline and sodic soils (saline-sodic soils). Based on the above, in this research the focus is on the most common salinization, caused by NaCl.

Nutritional disorders occurring in plants growing in this type of soil include deficiency of several nutrients, especially Ca^{2+}, N, K^+, and high levels of Na^+ and Cl^- (Qadir and Schubert, 2002). But next to this, poor physical properties of saline-sodic soils directly limit crop production.

Structural degradation

Na^+ increases the instability of soils by provoking swelling, slaking, and dispersion of soil particles. Cation Exchange Capacity (CEC) plays a crucial role in the Na^+ mediated structural soil degradation. The CEC of a soil is equal to the quantity of cations that can adsorb on the surface of soil particles (mainly clay minerals and organic matter) and is still available for exchange with the soil solution. The negative surface charge and surface area of clay minerals and organic matter (OM), at a certain pH, determine a soil's CEC. For clay aggregates adsorption of cations may occur both at their outer surface as at clay platelets within aggregates. As Na^+ ions accumulate in saline-sodic soils, the CEC may become occupied with relatively much Na^+. Because monovalent cations are less efficient in neutralizing the negative charge of solid particles than polyvalent cations, high this may lead to thick layers of adsorbed cations. A thick positively charged adsorption layer is more likely to cause electrical repulsion (swelling pressure) between clay platelets, resulting in aggregate instability. This may lead to excessive swelling and dispersion of soil particles, particularly if the overall salt concentration decreases. When air gets trapped inside soil aggregates in response to clay swelling, the outburst of this air causes disruption of aggregates in a process called slaking. Both the swelling, dispersion and slaking (platelets or micro-aggregates that detach) causes decreased pore sizes and corresponding difficulties of poor

aeration and small hydraulic conductivities. Slaking and dispersion can cause crust formation and/or hardsetting, where, due to the lack of pore space, water and air transport is restricted. Water storage capacity in these soils is poor and there are difficulties with regard to root penetration and seedling emergence. (Qadir and Schubert, 2002, Bresler et al., 1982).

1.3 Plants on salt affected soils

Due to the presence of many dissolved ions in a saline soil, the osmotic potential of its soil solution will be low, counteracting the uptake of water by the plant roots. Second, the accumulation of ions in the plant tissue can negatively affect internal processes. An indirect effect on crop growth is the detrimental effect of excess Na^+-ions on mineral soil structures. Structural soil degradation may cause plant roots to be suffocated, and hampers the uptake of nutrient containing water (Sumner, 1993). Surviving these circumstance requires energy input, energy that would probably be allocated to the formation of biomass in the absence of stress.

So far, research on salt tolerance (ST) has identified a two phased response of plants to salt stress (Munns et al., 1995). This first phase of salt stress is the osmotic stress phase; in cereals the major phenotypic effect is said to be a reduction in tiller production (Munns and Tester, 2008). During the second phase of salinity stress (1-4 weeks after stress initiation), plants start to experience difficulties due to the disturbance of ion homeostasis and specifically the high accumulation rate of Na^+ and Cl^-. The disturbance of ion homeostasis is referred to as secondary stress, whereas, the stress due to specific ions is called ionic or toxic stress (Rajendran et al., 2009) (Munns and Tester, 2008) (Nguyen Viet, 2012, Türkan and Demiral, 2009, del Martínez-Ballesta et al., 2006). The latter stress type is said to be phenotypically characterised by leaf death. If leaf death exceeds leaf growth, the photosynthetic capacity of a plant decreases (Munns and Tester, 2008).

Ionic and secondary stress effects are generally additive to the osmotic stress effects (Tavakkoli et al., 2011); (Vermue et al., 2013). However, adjustment to osmotic stress may result in a decrease of osmotic stress through time (Rajendran et al., 2009). Furthermore, genetic variation was found to be higher for ionic stress compared to osmotic stress (Munns and James, 2003) (Nguyen Viet, 2012).

Decreased osmotic soil potential

The low osmotic potential of a saline soil solution counteracts the uptake of water and dissolved nutrients by the plant roots (osmotic stress). Osmotic stress, similar to drought stress, primarily causes stomatal closure and decreased root hydraulic conductivity (del Martínez-Ballesta et al., 2006). Controlled of root water uptake, growth and photosynthesis are important to withstand osmotic stress.

Maintenance root water uptake

Root water uptake can occur through two main pathways. The apoplastic pathway involves water movement through the apoplastic space, without passing any cell membranes. Axial water flow of this pathway is blocked at the root endodermis by the casparian strips and should continue via the cell-to-cell pathway (del Martínez-Ballesta et al., 2006). The cell-to-cell pathway includes water transport via the plasma membrane, cytosol and the plasmodesmata. When water and nutrients have reached the stele, long distance transport will occur via vascular tissue whereas short distance transport will occur from cell-to-cell.

In favourable conditions, transpiration through the open stomata causes under pressure in the plant and water flow is relatively high. Since water chooses the way of least hydraulic resistance, under favourable conditions water transport is mainly apoplastic. However in saline conditions, when water flow is low, root water uptake occurs mainly via the cell-to-cell pathway. In this pathway, next to diffusion through the lipid bilayer, water uptake is well regulated by channel proteins called aquaporins.

Aquaporins belong to the major intrinsic protein family (MIP), a family of transmembrane proteins that facilitate transport of water and small neutral solutes and gases following osmotic or hydrostatic pressure gradients (del Martínez-Ballesta et al., 2006). There are two groups of aquaporins: Plasma membrane Intrinsic Proteins (PIPs) and Tonoplast Intrinsic Proteins (TIPs). Whereas PIPs are involved in transcellular transport, TIPs are expected to play a role in osmotic adjustment (since they influence water exchange between the cytoplasm

and vacuole). The change in abundance, distribution, and functionality of both PIPs and TIPs determines water flux. (del Martínez-Ballesta et al., 2006).
Root hydraulic conductivity is generally down-regulated in response to high NaCl levels and salt tolerant varieties were found capable of modulating aquaporin gating (Katsuhara et al., 2011). How the down-regulation of aquaporin abundance or functionality is initiated is not known, however, some influential factors have been discovered: ABA was found to induce closure of aquaporins (Katsuhara et al., 2011); dephosphorylation in response to decreased apoplast osmotic potential was found to cause aquaporin closure; reduced transcription of the PIP-coding gene was observed as a result of increased NaCl levels. Furthermore, hydroxyl radicals were found to be able to reversibly inactivate aquaporins; osmolytes may cause closure and bivalent cations, such as Ca^{2+} can cause short term blocking of aquaporins (del Martínez-Ballesta et al., 2006).

Growth and photosynthesis

Decreased water uptake under osmotic stress leads to decrease in cell division and elongation (Bartels and Sunkar, 2005). Furthermore, as a result of decreased CO_2 availability for photosynthesis (due to stomatal closure), O_2 is processed in the Calvin cycle in a process called photorespiration. This hampers photosynthesis and results in the formation of harmful Reactive Oxygen Species (Glenn et al.) and reduced carbohydrate production (Flexas et al., 2004). ROS play a role in cell signalling. However, excess of ROS negatively impacts the integrity of membranes, influences the activity of various enzymes, and damages functions of the photosynthetic apparatus itself (Fariduddin et al., 2012, Bartels and Sunkar, 2005, Türkan and Demiral, 2009). Photosynthesis can additionally be limited by secondary and ionic stresses (Tavakkoli et al., 2011). However, according to Munns and Tester (2008) oxidative stress in response to abiotic stress can impossibly be stronger than stress occurring in response to naturally occurring variation in light intensity. And thus, oxidative stress through dysfunctional photosynthesis should not be a major stress factor in response to salinity.

Disturbance of ion homeostasis

The altered ion availability in saline soils will result in increased uptake of Na^+ and Cl^- together with decreased uptake of other essential nutrients, especially K^+, Ca^{2+} and NO_3^- (Tavakkoli et al., 2011). The disturbance of ion homeostasis requires adaptation of cell metabolism (del Martínez-Ballesta et al., 2006). The latter is referred to as secondary stress.

Secondary stress

Plants have to deal with increased levels of Na^+ and Cl^- and have to find a way to stay functional despite the lower uptake of K^+ an Ca^{2+}, which both play a crucial role in cell functioning. In more tolerant species, at proportionally high Na^+ availability, the Na^+ concentration, as well as the Na^+/K^+ and Na^+/Ca^{2+} ratios are generally kept low. This confirms the importance of both ions (del Martínez-Ballesta et al., 2006).
The essential nutrient Ca^{2+} has structural roles in the cell wall and membranes, as counter cation for anions in the vacuole and as intracellular messenger. However, high concentration of Ca^{2+} are cytotoxic (White and Broadley, 2003). More Ca^{2+} in the external solution was found to ameliorate NaCl-associated stress (del Martínez-Ballesta et al., 2006);(Shabala, 2003);(Nedjimi and Daoud, 2009). The stimulation of aquaporins, as mentioned above, is considered as one of the possible reasons. Furthermore, a role for Ca^{2+} was found in osmotic as well as ionic stress signalling, which will be further explained below. The apoplastic pathway was found to be of major importance for Ca^{2+} uptake (White and Broadley, 2003), and Na^+ was found to inhibit apoplastic transport of Ca^{2+} (del Martínez-Ballesta et al., 2006).
The essential nutrient K^+ is important for maintenance of the membrane potential, the activity of many cytosolic enzymes and it serves as osmoticum for cell volume regulation. K^+ mediated decrease of osmotic potential in stomatal guard cells, flanking the stomatal opening, cause increased guard cell turgor and thus stomatal closure (Bartels and Sunkar, 2005, Fariduddin et al., 2012, Türkan and Demiral, 2009).
Na^+ competes with K^+ for uptake, causing an increase in the Na^+/K^+ ratio in saline conditions. Cellular K^+ efflux occurs to re-establish the membrane potential after increased uptake of Na^+. Poly Amines (Munns and

Passioura) were found to increase selective uptake of K^+ over Na^+ and to decrease K^+ shoot loss (Fariduddin et al., 2012).

Ionic stress

Ionic stress is specifically caused by excess uptake of Na^+ and Cl^-, which are both metabolically toxic if accumulated at high concentrations in the cytoplasm (Tavakkoli et al., 2011). Research on salt tolerance so far has resulted in speculations on the specific effect of Na^+ and Cl^- on salt stress in plants (Tavakkoli et al., 2011). According to Türkan et al., Na^+ causes the major toxicity problems whereas some plants are also sensitive to Cl^- (Türkan and Demiral, 2009). However, according to Munns et al., Cl^- can potentially become more toxic than Na^+ due to less sophisticated mechanisms of Cl^- transport (Munns and Tester, 2008). While traditionally research has been more focussed on the effect of Na^+, according to Tavakkoli et al., the effect of Cl^- now seems at least as important (Tavakkoli et al., 2011).

Testing the toxicity of Na^+ and Cl^- separately is difficult since the used treatments should solely differ in Na^+ or Cl^- availability, not in any other osmotic/ionic values. This is almost impossible and for this reason it is advised to make a comparison between genotype specific Na^+ and Cl^- accumulation in relation to salt tolerance (Munns and Tester, 2008). The comparison of more and less salt tolerant varieties, selected in anticipation of discriminative behaviour, provides an extra clue for interpretation of salt tolerance in response to treatment with only Na^+, only Cl^- or NaCl associated stress.

In saline conditions plants are found able to accumulate high concentrations of Cl^-, depending on salinity upto 400 mM for more tolerant species and 250mM for the more sensitive species. This implies compartmentalization of Cl^- in the vacuole. Cl^- loading into the xylem is expectedly a passive mechanism via anion channels that are down-regulated by ABA in response to salinity stress. Retrieval from the xylem is possibly an active process (Munns and Tester, 2008) Despite little research on Cl^- toxicity Tavakkloli et al. (2011) showed that Cl^- may directly damage chlorophyll and thereby inhibit Photosystem II in barley. The decreased K^+ uptake as a result of excess Na^+ is expected to be the major Na^+-toxic effect (Buschmann et al., 2000).

A quantitative trait

Some plants suffer more from salinity stress than others. This depends on the possession of a combination of characteristics that minimizes detrimental effects. Since many processes are influenced by salinity stress, there are also many characteristics that can restrain deleterious effects. Salinity tolerance is a complex trait, controlled by complex interaction of several genes, which is not unravelled at this time. Tolerance is regarded as the ability to survive the entire life cycle under the particular salt concentration and varies highly among species, even among cultivars within species (a quantitative trait)! Some plants can only tolerate concentrations up to 25 mM (chickpea) whereas others can tolerate 1000 mM NaCl (saltwater plant). (Flowers et al., 2010). The degree of tolerance depends on how well detrimental effects are kept under control under saline conditions. (Flowers and Colmer, 2008) make the distinction between sensitive and tolerant plants at tolerance to 200 mM salt. However others have drawn the line differently (Flowers et al., 1986, Breckle et al., 2004). In the corresponding classification, sensitive plants are called glycophytes, whereas tolerant plants are called halophytes. Halophytes are often native to saline soils and require higher concentrations of Na^+ and Cl^- for optimal growth (del Martínez-Ballesta et al., 2006). Halophytes are good candidates for the identification of genes for salt tolerance (Türkan and Demiral, 2009).

1.4 Mechanisms of tolerance

Salt tolerance is not the result of the expression of a single gene, it is the result of the interaction of many expressed genes (located on different loci in the genome), triggering many interacting processes that all together confer tolerance (Flowers and Flowers, 2005). In case of tolerance, the plant is able to adapt to the new unfavourable environmental conditions. But how does the plant know when to adapt, and which processes are put into action? Many researchers have put effort in revealing the secrets of salt tolerance, however up to now only segments of all involved processes are known. There are three main mechanisms of salt tolerance (Munns et al., 2000, Munns and Tester, 2008):

- Osmotic stress tolerance
- Minimization Na^+ uptake and transport towards shoot; Na^+ exclusion
- Tissue tolerance

In general there are three main steps with regard to adaptation to salinity: 1) Stress perception; 2) signal transduction; 3) action (Bartels and Sunkar, 2005). Below an overview is given of the major strategies of salt tolerance based on the current available knowledge.

Osmotic stress tolerance

Expectedly, one of the ways in which osmotic stress is sensed is when a decrease in turgor occurs. Sensors (e.g. mechanosensors, or stretch activated channels) react upon an increase in distance between the cell wall and plasma membrane by inducing the synthesis of ABA. ABA then stimulates a quick transient increase in cytosolic Ca^{2+} accumulation. As result, the transcription of the vacuolar NHX Na^+/H^+ exchanger gene is increased and thus Na^+ translocation towards the vacuole is stimulated. (Türkan and Demiral, 2009) Furthermore, the formation of Late Embryogenesis-Abundant (Lisle et al.) proteins in response to osmotic stress, was found to increase osmotic stress tolerance by amongst others the induction of ABA production (Bartels and Sunkar, 2005).

The formation of ROS during salinity stress as a result of hampered photosynthesis and other stimuli can cause destructive damage. The formation of antioxidants and ROS scavengers will protect the plant from oxidative damage. The formation of polyamines was also found to facilitate ROS scavenging (Fariduddin et al., 2012). Down regulation of photosynthesis in response to stomatal closure is another strategy to prevent oxidative damage (Chaves et al., 2009).

Minimization of Na^+ uptake and transport towards the shoot

The toxic effect of Na^+ is based on its deterimental effect on enzyme activity due to perturbation of protein structures (Bartels and Sunkar, 2005). Maintaining a low Na^+/K^+ ratio is accepted as one of the key deteriminants for salt tolerance in barley (Nguyen Viet, 2012). The latter is achieved by ensuring low rates of Na^+ transport towards the shoot, combined with a high uptake selectivity of K^+ over Na^+ (Flowers and Colmer, 2008). Limiting the entrance of Na^+ and Cl^- will limit accumulation of these toxic ions in the leaves. The uptake and translocation of Na^+ has been studied extensively (Nguyen Viet, 2012). However, still there is uncertainty about the exact mechanism behind the controlled uptake of Na^+ (to function as osmolyte in vacuole) in combination with sufficient K^+ uptake (Flowers and Colmer, 2008). Some plant families (not poaceae) have been identified to have salt glands, another option to reduce the Na^+/K^+ ratio (Flowers and Colmer, 2008).

The discovery of the salt stress induced "Salt-Overly-Sensitive" (SOS) pathway highly contributed to the understanding of how Na^+ transport is regulated in response to salinity. SOS mutants in *Arabidopsis* that were overly sensitive to NaCl led to the identification of the corresponding SOS genes and the discovery of this pathway (Bartels and Sunkar, 2005). The SOS pathway is a Ca^{2+} regulated pathway involved in Na^+ and K^+ homeostasis and thus highly influential to salt tolerance. High concentrations of Na^+ are sensed by cytosolic and membrane associated receptor molecules, which upregulate cytosolic Ca^{2+} levels. In response, the Ca^{2+}-sensor SOS3 attaches to protein kinase SOS2, and this complex activates the plasma membrane Na^+/H^+ antiporter SOS1, resulting in efflux of Na^+. The SOS pathway also stimulates the cytosolic K^+ concentration by

stimulation of the AKT1, K^+ transporter and down regulation of the HKT1 low affinity Na^+ transporter. Furthermore, SOS2 influences NHX to increase Na^+ translocation to the vacuole. (Zhu, 2003)

Tissue tolerance

The accumulation of Na^+ and Cl^- is harmful to the plant. By compartmentalization of these ions in the vacuole, they are made harmless while they still contribute to decreased osmotic cell potential. By decreasing the internal osmotic potential, the uptake of water and nutrients is facilitated. To keep the osmotic balance within the cell, inside and outside the vacuole, compatible solutes are formed and accumulated in the cytoplasm. Compatible solutes are low molecular mass compounds that do not interfere with cellular metabolism (del Martínez-Ballesta et al., 2006). Next to their function as osmolyte, (particular) compatible solutes have been found capable of ROS scavenging, protection of proteins and plasma membrane, and to function as temporary storage for C and N (Bartels and Sunkar, 2005, Nguyen Viet, 2012). The production of compatible solutes costs energy and should therefore be minimal. The utilization of Na^+ or other available ions to decrease osmolality is advantageous, but only if the plant can tolerate ion accumulation by vacuolar compartmentalization (tissue tolerance) (Flowers et al., 2010, Munns and Tester, 2008, Flowers and Colmer, 2008).

1.5 Action

Land and water management

Water management is of major importance to minimize detrimental effects of saline water and to recover already affected soils (if it is not too late). Due to water transpiration and evaporation combined with plant salt exclusion, salts are left in the soil in a reduced volume of soil water. The more saline the soil water gets, the more frequent irrigation is needed to prevent water stress (Maas and Hoffman, 1977). Drainage from time to time should prevent severe accumulation of salt, and ascertain salt leaching to the ground water. Amelioration methods have been developed for sodic soils because due to their small hydraulic conductivity adsorbed ions (in particular Na^+ in saline-sodic soils), will not leach out easily. These methods are aiming for increased aggregate stability and hydraulic conductivity by replacement of clay mineral-adsorbed Na^+ with Ca^{2+}. Examples of amelioration strategies are, leaching with salty water (containing a high concentration of especially bivalent ions such as Ca^{2+}), electro-amelioration and phytoremediation. Tillage strategies which directly improve soil structure by physical intervention, are also widely adopted, in combination with other amelioration strategies or not. (Bresler et al., 1982) (Qadir et al., 2001) Indiscrete irrigation with saline water may result in irreversible soil deterioration. Furthermore, deep percolation and drainage flows are inevitable consequences of saline-sodic soil amelioration and will translocate the harmful ions to another place. For this reason, the selection of areas where halophytes will be grown on saline water have to be selected with care. Modelling field situations can give guidelines to this respect (Vermue et al., 2013).

The Soil Water Atmosphere Plant or SWAP model, is a model used for the analysis of water management in agricultural as well as natural soils (Van Dam et al., 2008). This model is amongst others, used to optimize the Water Use Efficiency (WUE) for crop production. The crop growth simulation model World Food Studies (WOFOST), is linked to SWAP for the calculation of plant/crop transpiration and growth, on the basis of eco-physiological processes. The effect of salinity on these parameters has, however, not been incorporated in WOFOST (Boogaard et al., 2011).

Salinity clearly influences transpiration and therefore the plant's water uptake as well as the yield. In SWAP, the reduction in transpiration due to salinity is incorporated as a reduction factor on the output of WOFOST. The determination of this factor was originally described by (Maas and Hoffman, 1977) and is empirically based. In a more recent version, the osmotic pressure of the soil solution makes water less available for uptake and transpiration and again, via reduced transpiration has an impact on the primary production of plants. As may be clear from the combination of SWAP-WOFOST, the impact of salinity on plant production is exclusively a result of osmotic limitations of plant transpiration (Dam, 2000). To improve models and optimize land and water management in saline environments, it is important to get deeper knowledge on the physical as well as physiological processes that determine plant behaviour in saline conditions (Vermue et al., 2013).

Crop improvement-Barley as a model species

The use of salt tolerant varieties can be part of a solution to ensure yields in saline environments. At the same time, phytoremediation by tolerant crops could inhibit chemical/structural degradation of salty (especially sodic) soils (Qadir et al., 2001). For many of our food crops, however, no salt tolerant varieties are known (Downton, 1984). The development of salt tolerant crops contributes a lot to food security, breeding for salt tolerance however is very difficult due to the complexity of the trait (Katerji et al., 2012).

Many studies on salt tolerance are based on the response of hydroponically grown plants. The ease of application and controllability of hydroponic systems make them an attractive option. While using hydroponics, the assumption is often made that the observed plant response to salt is similar to the response that would be observed on soil. However, according to Tavakkoli et al. this turns out not to be the case for salt stress tolerance (Tavakkoli et al., 2010). In the study of Tavakkoli et al., genotypic differences were more prone to be revealed in plants grown in soil (Tavakkoli et al., 2012). However, the difference in genetic variation observed on these two media, might be shown in response to medium specific properties, instead of as a result of difference in salt tolerance.

To enable the design of efficient screening methods for the identification of genotypes with improved ST, it is important to find out which soil properties are important influential factors to salt tolerance. In previous experiments, sand media concerned a particular soil type with its own particular soil properties. These soil properties affect both the water and nutrient availability to the plant. The CEC of these soils determines nutrient and salt availability to the plant roots. For example, the Na^+/Cl^- ratio in the soil water decreases due to adsorption of Na^+ to the clay minerals. Furthermore, the soil matric potential, determined by the pore sizes, directly influences the ease of root water uptake. Additionally, due to an impeded movement and continues aeration, a nutrient gradient is prone to develop in a soil matrix in response to root water uptake. (Tavakkoli et al., 2010)

To make steps forwards in the field of crop improvement with regard to salt tolerance, it is more efficient to first focus on a few "model" species. Barley (*Hordeum vulgare* L.), a member of the Poacea family, has been used as a model species in genetics. Choosing barley is attractive in view of its short lifecycle, its few chromosomes (2n=14), being easily crossbred and having the ability to grow in diverse (harsh, salty) environments (Nguyen Viet, 2012). Furthermore, the available genetic information on barley makes it an obvious model species (Eleuch et al., 2008). Barley is grown in geographically distinct areas enabling the comparison of geographically distinct landraces[1] which have developed different properties with regard to, for example, salt tolerance. Surviving harsh environments, makes barley the fourth important cereal (food and fodder) crop worldwide. The fact that barley uses glycophytic as well as haplophytic mechanisms to cope with salinity stress, gives the opportunity to learn about both types of mechanisms (Nguyen Viet, 2012).

Research on the identification of genes responsible for salt tolerance in barley, has resulted in the localization of Quantitative Trait Loci[2] that are expected to contain important genes (Nguyen Viet, 2012).

[1] A landrace is "an ancient or primitive cultivar of a crop plant. Landraces are often genetically very heterogeneous and contain numerous alleles that contributed to the survival of the organism under natural conditions. Since intensive plant breeding can result in the loss of these alleles, landraces are a source from which plant breeders can selectively reintroduce them into highly bred cultivars." DICTIONARY OF BOTANY 2001-2002. botanydictionary.org.

[2] Quantitative Trait Loci (QTLs) are genomic areas that are expected to contain genes involved in the determination of the considered quantitative trait (Abiola et al., 2003).

1.6 Scope of this project

Although proper land management can mitigate the severity of salinity to some extent, still there is a need for salt tolerant varieties. Understanding the mechanisms behind salt tolerance is a great challenge. Analysis of large germplasm collections is required to determine potential for plant breeding and to gain insight in plant strategies for salt tolerance. Efficient screening methods for salt stress tolerance, can contribute considerably to the achievable successes. Plant growth was found to interact differently with soil and solution culture (Gregory et al., 2009, Tavakkoli et al., 2010). Furthermore, plants tend to respond to the combined effects of osmotic soil water- and soil matric potentials (Maas and Hoffman, 1977). In hydroponics the matric potential is absent, which diverges from a field situation. However, due to high controllability and practical convenience, hydroponic systems are preferably used by researchers during screening practice.
In this thesis, the focus is on mechanisms for salt tolerance used by tolerant and sensitive barley varieties during salinity stress grown on both hydroponics and sand. Different plant response to salinity stress may be expected in different root environments. The comparison of salt tolerance on hydroponics and an inert sand medium (exerting matric forces, but lacking CEC) should give insight in the applicability of hydroponic and sand screening methods for trait analysis of salt tolerance in particular. Since NaCl is the naturally most abundant and problem-causing salt, in the current thesis, salinity stress is defined as stress in a saline-sodic (NaCl-dominated) environment. As salinity stress concerns more than osmotic stress, it is appropriate to assess options on how to incorporate salinity stress in the SWAP-WOFOST model. The aim was to assess the need of discrimination between different genotypes (tolerant/not) when incorporating ST in SWAP, where ST is included as an effect on transpiration.

The corresponding objectives are:

- To determine the difference in salt tolerance and contributing mechanisms in the considered barley varieties on hydroponics and sand.
- To determine genetic variation for salt tolerance and contributing mechanisms in the considered tolerant and sensitive barley varieties under different root environments.
- To make a recommendation about the incorporation of saline-sodic stress in WOFOST.

The corresponding research questions are:

- What causes the potential difference in barley salt tolerance on hydroponics and sand?
    - Is there a difference in salt tolerance on hydroponics and sand?
    - If so, which processes/mechanisms cause this difference?

- What is the genetic variation for salt tolerance (ST) and contributing mechanisms among the considered tolerant and sensitive barley varieties, under study in the different root environments?
    - Is there a difference in ST between the considered tolerant and sensitive barley varieties?
    - Can differences in ST in response to different osmotic and/or ionic stress treatments be explained by the measured plant parameters?

- What can be recommended with regard to the incorporation of saline-sodic stress in WOFOST?
    - To what degree does salinity stress influence transpiration in tolerant and sensitive barley varieties?
    - To what degree does salinity stress influence WUE in tolerant and sensitive barley varieties?

2. MATERIALS AND METHODS

2.1 Plant material

Four genotypes were selected for this study (Table 1). Genotype 1 (G1) and genotype 3 (G3), are part of an association mapping population based on which QTLs for salt tolerance have been located (Nguyen Viet, 2012) (Wolde, 2011); G1 is regarded as salt tolerant and G3 salt sensitive. In previous experiments, genotype 2 (G2) was found to be a tolerant genotype whereas genotype 4 (G4) is regarded sensitive (Nguyen Viet, 2012). G2 and G4 have not been used for QTL mapping yet, however they show great potential. This because G2, originating from the dry and salinized parts of Ethiopia, is likely to contain many useful tolerant alleles, whereas G4 performs well under relatively favourable conditions and is not likely to contain alleles that offer tolerance under saline conditions (Nguyen Viet, 2012).

Table 1: Examined genotypes

ID	Accession name	Classification	Country of origin
G1	IG 128216	tolerant	Libya
G2	L94	tolerant	Ethiopia
G3	Volga	sensitive	France
G4	Vada	sensitive	The Netherlands

2.2 Growth conditions

Experiment 1 & 2

Experiment 1 and 2 two were conducted in parallel. In experiment 1, plants were grown on hydroponics whereas experiment 2 was executed on soil. In both experiments plants were watered with a salt and/or nutrient containing solution of which the composition can be found in Table 2. All solutions were prepared as an addition to a modified Hoagland solution (Lisle et al., 2000) as used by (Tavakkoli et al., 2010)(ANNEX I).
The Na and Cl solutions were designed to assess the specific effect of Na^+ respectively Cl^-. The Na solution does not contain Cl^- and vice versa. The PEG solution contained neither Na^+ nor Cl^-, this solution should resemble the effect of osmotic stress due to increased salt concentrations while excluding any possible toxic effects.

Table 2: Used solutions, for composition of the Hoagland solution, see Annex I.

Solution ID	Contents	Concentration salt/ ions of interest	
		Experiment 1	Experiment 2
Control	Modified Hoagland solution		
NaCl	Modified Hoagland solution + NaCl	200 mM NaCl	100 mM NaCl 200 mM NaCl
Na	Modified Hoagland solution + Na_2SO_4 (33mM) Na_2HPO_4 (33mM) $NaNO_3$ (68mM)	200 mM Na^+	--
Cl	Modified Hoagland solution + $CaCl_2$ (33 mM) KCl (66 mM) $MgCl_2$ (34mM)	200 mM Cl^-	--
PEG	Modified Hoagland solution + Polyethylene glycol (PEG) (Sigma-Aldrich, Co., Missouri, USA)(8000 g/mol)	0,1 kg/L PEG	--

Polyethylene glycol (PEG) (MW = 8000), was chosen as osmolyte since this plant uptake of this substance is regarded negligible, and is thus assumedly solely affects the osmotic potential of the solution (Lawlor, 1970). The NaCl solution was used to mimic the entire range of stresses triggered by excessive NaCl, including osmotic and ion specific toxic effects.

Seeds were germinated on silver sand. Upon appearance of the 1st leaf, the seedlings were placed on their final substrate. After one week of acclimation on the final substrate, treatment was started. Salt concentrations were gradually increased (50 mM/day). The plants were grown in a greenhouse where climate conditions are kept constant as much as possible. The average day respectively night temperatures were set at 18 and 14°C. The plants were subject to a 16 hour light period, followed by 8 eight hours of darkness. Additional lighting (100 W/m2) was used if the incoming shortwave radiation was below 200 W/m2. The environmental humidity was kept at 70%.

Experiment 1

The use a hydroponic setup allowed the experimental conditions in experiment 1 to be relatively well controlled and uniform. The used solutions, excluding the control and PEG solution, had the same molarity of dissolved salts with similar electric conductivity, as was monitored during the experimental period (ANNEX II).

The Morse equation[3] (Mansoor M. A., 2002) was used to calculate the concentration of PEG required in the PEG treatment, to create equivalent osmotic stress in all stress treatments. Calculations were based on the average osmotic stress in the other stress treatments. This corresponded with an administration of 2.9 kg PEG per litre in the PEG treatment. However, according to Shah et al., PEG in water does not behave according to the van 't Hoff's law (Shah et al., 2011) and thus Morse's equation. The relationship between [PEG] and osmotic pressure is still unclear and within literature measurements and approximations result in different osmotic potentials corresponding to a 10% PEG solution (Burlyn, 1983, Gopal and Iwama, 2007) (Table 3). Based on PEG solubility and financial restrictions was agreed on admission of 0,1 kg/l nutrient solution, resulting in a 10% PEG treatment solution. According to the Morse equation, this causes a higher osmotic potential in the PEG treatment compared to the other stress treatments, resulting in less osmotic stress for the plants (Table 3).

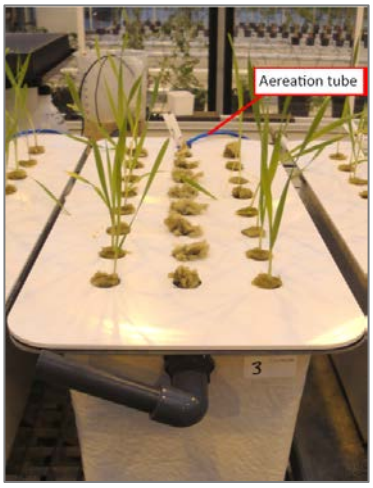

Figure 1: Hydroponic basin; cover plate filled with barley seedlings, empty holes are filled with rock wool and air is injected by the blue pipe in the back.

The pH-value of all solutions was monitored and kept constant during the entire experimental period. To decrease the pH value, sulphuric acid (out of a solution with 10 ml 41.1% H_2SO_4/L) was added, whereas Potassium hydroxide (out of a solution with 5ml KOH/L) was used to increase the pH value if necessary.

The solutions were kept in containers containing 24 litre each. On a weekly basis 25% of the solution was replaced with the corresponding fresh solution, so to limit the influence of plant water uptake on the solution composition. An electronic pump, linked to plastic pipes, was used to inject each basin with air. As soon as the first leaf appeared, 18 barley seedlings were clamped into a cover plate with 24 holes, using rockwool. The cover plates were placed on top of the basins and to prevent evaporation of the salt/nutrient solution, the remaining holes were filled with rockwool (Figure 1).

[3] Morse equation: $\pi = mRT$ with π = osmotic pressure (atm), m= molarity (Mol/l), R= the gas constant (0,08205746 L atm K^{-1} mol^{-1}) and T= temperature (K).

Table 3: Theoretical osmotic potential, calculated from the Morse equation for all treatment solutions. For the PEG treatment; i) according to the Morse equation; ii) according to Gopal and Iwama, 2007; iii) according to Burlyn, 1983.

Solution ID	Osmotic pressure (atm)		
Control	0,6		
NaCl	10,3		
Na	8,7		
Cl	8,8		
PEG	0,87[i]	0.13[ii]	1.5[iii]

To achieve statistically sound results, the experimental setup was a randomized block design. This design consisted of 9 containers (blocks) of which two replicate containers per treatment. However, due to financial restrictions, no replicate was made for the PEG treatment. To facilitate early harvests for in-between analysis of plants on the NaCl treatment, four extra containers were incorporated for that treatment (Figure 2). According to the initial plan, in each container, four plants of each genotype should have been randomly distributed. However due to lack of seedlings a redistribution had to be made: For G1 and G3 still four seedlings were planted per container, for G2 and G4 only three seedlings were planted in each container. The containers were moved around once a week to minimize the block effect. In total, 182 plants were grown on hydroponics. Figure 2 gives an overview of the experimental setup.

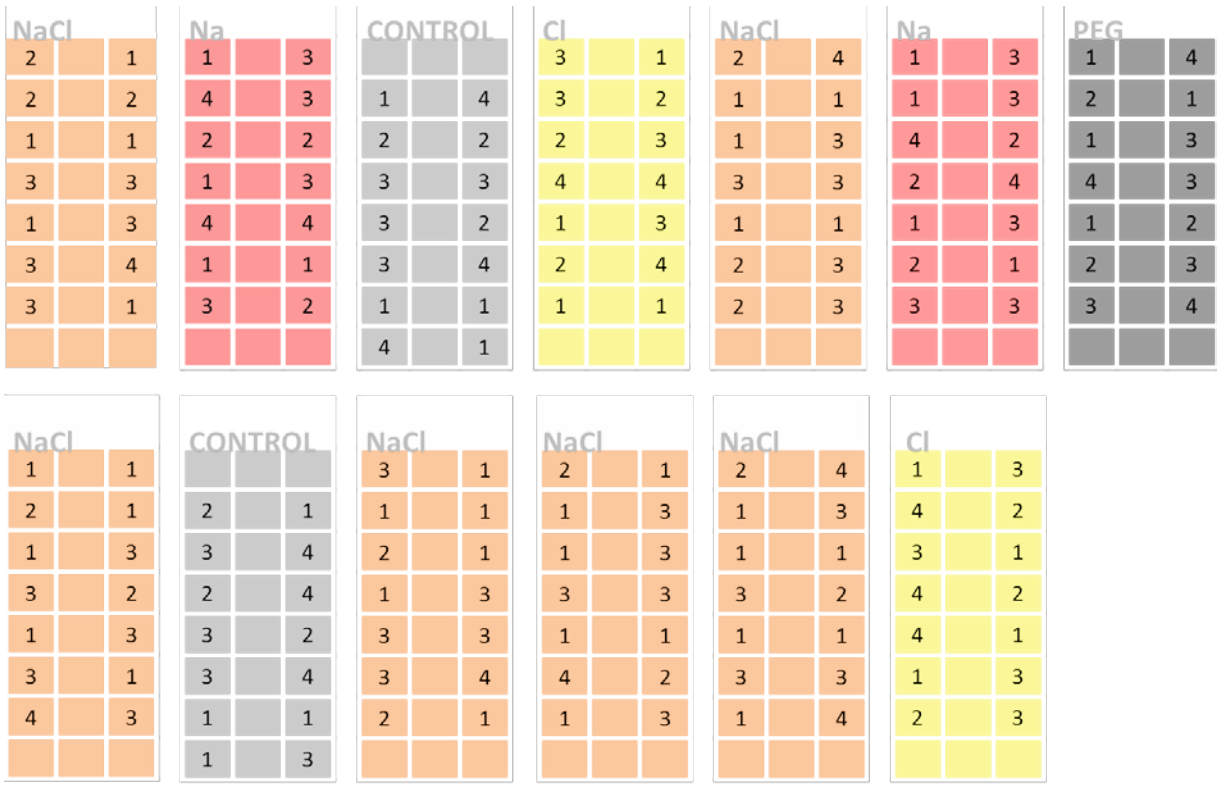

Figure 2: Setup experiment 1, five different treatment blocks; Control, NaCl, Na, Cl and PEG, in each treatment block, four units of G1 and G3 and three units of G2 and G4 are present. Genotypes are randomly distributed throughout the grid, per treatment.

Experiment 2

In previous experiments was found that pant response to salt (NaCl) is different on hydroponics and on sand (Tavakkoli et al., 2010). For this reason part of experiment 1 is repeated on soil. A closer look was taken on the plant- ion and nutrient availability in the root zone, which is influenced by the added salt- and nutrient-solution, plant behaviour and soil properties. Obtained insights contribute to the understanding of different plant behaviour on soil and hydroponics.

The NaCl treatment from experiment 1 was repeated on sand. Within this treatment, the entire range of stresses triggered by excessive NaCl was present. However, in this experiment, besides the concentration of 200 mM, also 100 mM was used to uncover the relationship between salt (NaCl) concentration and average plant response.

Mitscherlich pots (Mitscherlich, 1925), having a drainage compartment and moisture outlet at the bottom, were filled with 7 kg of silver sand (Figure 3). It was chosen to work with silver sand because this soil type is very pure, consisting of solely quartz (SiO_2). Due to the absence of OM and clay minerals, no CEC is present. This simplification of a natural soil system allows framing of the comparison of hydropnics and sand.

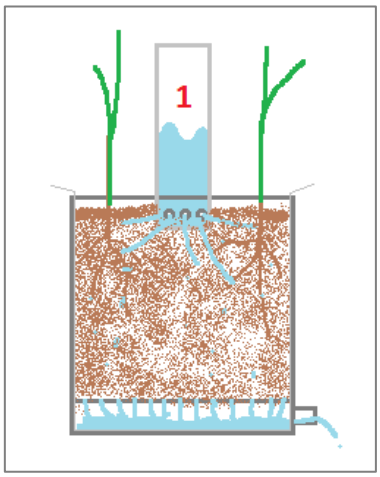

Figure 3: Schematic picture of a Mitscherlich pot with drainage compartment and watering tube (1).

To prevent the rusty inside of the pot from influencing the experiment, prior to filing, the pots were coated with a plastic bag that was perforated at the bottom side. Soil moisture content is kept constant, at 60% of the flow limit, during the entire experiment. While filling the pots, 180 cl of nutrient solution was thoroughly mixed with each kilo of silver sand. In this way the correct moisture content was obtained. Directly after filling the pots, the total weight was noted on the pot. By adding nutrient solution on a daily basis, the total weight of each pot and thus the soil moisture content, was kept stable. Water availability should not be a limiting factor. An estimation of the biomass was made using plants grown on a spare hydroponic container. Watering is done via an, at the down side wall perforated, plastic tube in the middle of the pot (Figure 3). The tube facilitates soil water uptake with minimal deterioration of the soil structure. Due to the lack of CEC, aggregate formation in silver sand is very unstable. Upon wetting, dispersion is likely to occur and the air in between the sand particles will be forced out. Due to low water holding capacity of this soil, most water will leach out. When water is leaching, it are the adhesive forces between water and soil particles and cohesion among water particles that cause closure of pore spaces, resulting in a cement like soil structure (hardsetting). This soil structure should be prevented because lack of aeration and water transport impede plant survival. By preventing the detaching force of water falling down on the soil surface at conventional watering, the tube prevents the dispersion of aggregates at the surface which would result in crust formation impeding the infiltration of water and air. (Morgan, 2005) The presence of holes in the sides of the tube allow horizontal transport of water into the non-disturbed soil.

To achieve statistically sound results, the experimental setup consisted of a completely randomized design. For each of the treatments 16 pots were available, 4 for each genotype. Per pot three plants of one genotype were planted. The pots were moved around the available space on a weekly basis (Figure 4).

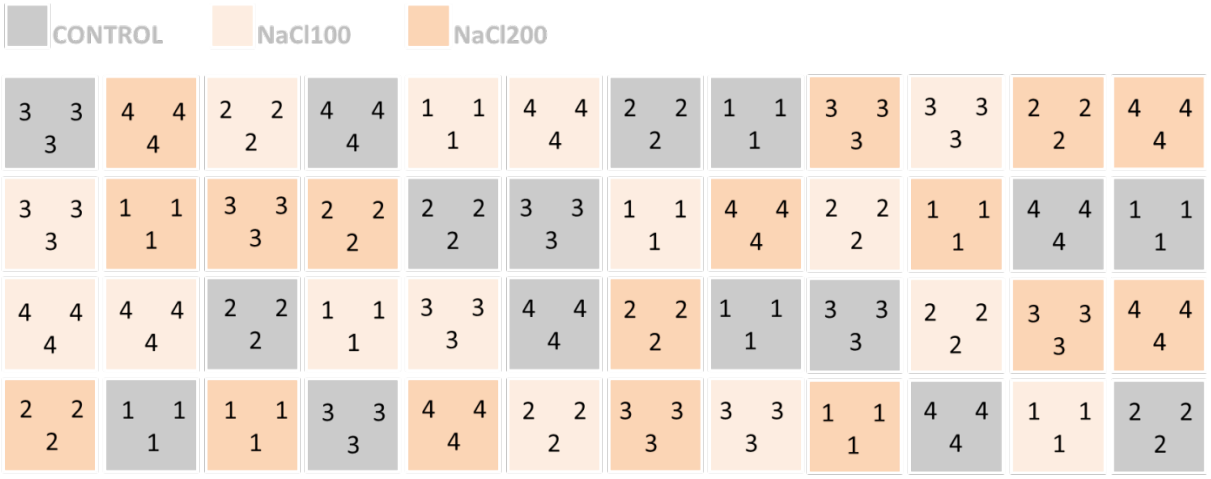

Figure 4: Setup experiment 2, 16 pots for each of the treatments, Control (grey); NaCl100(light orange); NaCl200 (deep orange) are randomly distributed. Per pot three units of one genotype were planted. Within each treatment four replicates per genotype

2.3 Measurements

Data collection for experiment 1 and 2 was largely similar. During the experimentation period, data collection took place on a weekly basis. All plants were harvested three weeks after the final salt concentration was reached for all treatments.

Experiment 1 & 2

Data collection throughout the experiment

Once a week, starting one week after the final salt concentrations were reached for all treatments, the following measurements/observations were done:

- *Transpiration/stomatal conductance (mmol/m^2/s)*
  A Decagon SC-1 Leaf Porometer (Decagon Devices, Inc., Pullman, Hopkins Ct, Washington) was used to measure stomatal conductance in the youngest fully expanded leaf. The sensor head was placed in the mid-area of the leaf, with the diffusion block pointing downwards, measuring stomatal conductance of stomata on the downside of the leaf (Willmer and Fricker, 1996). All measurements took place between 10 am and 2 pm. For experiment 2, stomatal conductance was measured for two plants per pot.

- *Chlorophyll content leaves (mg/g dry weight)*
  A SPAD-502 chlorophyll meter (Minolta Camera Co., Osaka, Japan) was used to measure the chlorophyll content of the two youngest fully expanded leaves. Two measurements were done per leaf, one close to the stem and one close to the leaf tip. For experiment 2, chlorophyll content was measured for all three plants per pot.

- *Leaf survival/death (nr.)*
- *Tiller formation (nr.)*
- *Leaf elongation*
  The leaf elongation of the youngest leaf was measured until it was fully expanded. Where after was continued with the present newest leaf.

Data collection after final harvest

For the following measurements shoot and root were considered separately.

- *Fresh Weight (g)*

Fresh weight of the plant as a whole was measured, where after the fresh weight of only the shoot was determined. Root fresh weight was calculated afterwards. Due to this quick processing, moisture loss was minimized and a more representative estimation of the fresh weight was obtained.

- *Dry Weight (g)*
  Roots and shoots were dried at 70°C for 16 hours, after which the dry weight was determined.

- *Ion accumulation (mmol/g dry weight)*
  Prior to determination of ion accumulation, shoot and root samples were grinded with a hammer mill using a 1 mm sieve. During this step, for experiment 1 identical genotypes were pooled per container. For experiment 2, all plants per pot, which always had the identical genotype, were pooled.
  Ion accumulation was measured using the Ion Chromatography (IC) system 850 Professional (Metrohm, Herisau, Switzerland) according to the corresponding protocol (ANNEX III). Circa 25 mg of grinded sample material was ashed for 5 hours at 575°C and mixed with 1ml of 3M formic acid (15 min, 99°C). Solvability was poor, so after adding 1 ml of MilliQ again was shaken for 30 minutes at 99°C. After this step ashes in most samples were solved entirely, samples with unsolved ashes were noted. 100 µl of this solution was put in 9.9 ml MilliQ and analysed for Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, SO_4^{2-}, and PO_4^{3-} content.

Experiment 1

For the plants growing on the NaCl solution in experiment 1, early harvests were done at one and two weeks after the final salt concentration is reached. Three plants of each genotype were harvested at a time. The plants were divided in shoot and root. They were analysed upon the following characteristics, in the same manner as described above:

- *Dry Weight (g)*
- *Water content (g)*
- *Ion accumulation (mg/g dry weight)*

At the same time, one and two weeks after final salt concentration was reached, and at final harvest time, three plants per genotype were harvested for the following analyses:

- *Transcription analysis of genes related to salt stress resistance.*
  The youngest fully expanded leaf together with non fully expanded leaves, were cut form the plants and directly frozen using liquid nitrogen. The plant material was stored at minus 80°C, ready for RNA extraction.

- *Leaf osmotic potential (osmol/kg)*
  The second and third youngest fully expanded leaves were pooled and analysed for the osmolality of their leaf sap. A Gonotec Osmomat 030 freezing point osmometer was used according to the corresponding protocol. For the isolation of leaf sap, a disc of glass micro-fibre filter paper was placed in the barrel of a 2 ml syringe, to cover the outlet hole. The leaf was put in the barrel and the plunger was reinserted. After sealing the tip with parafilm the syringe was frozen in liquid nitrogen. Hereafter the frozen leaf was thawed to ambient temperature, the plunger and parafilm were removed. The barrel of the syringe was placed in a 15 ml centrifuge tube with its tip in a 1.5 ml Eppendorf tube. After centrifugation at 2500 g for 10 min at 4 °C, the osmolality of the collected sample was measured.

During the final week of experimentation solution samples were taken directly from all containers in experiment 1. Availability (mmol/l) of Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, SO_4^{2-}, and PO_4^{3-} in the media was determined using the using Ion Chromatography (IC) (system 850 Professional, Metrohm, Herisau, Switzerland). Prior to analysis, all samples were diluted 50 times with MilliQ.

Experiment 2

In experiment 2, soil water samples were taken from the root zone by means of Rhizons® (Rhizosphere research Products, Wageningen, The Netherlands) according to the manufacturer's protocol. Since salt and nutrient availability are influenced by root exudates (Dakora and Phillips, 2002, Bertin et al., 2003), samples ideally should have been taken from the rhizosphere. In the pots, containing three plants each, two rhizons are inserted (Figure 5). One is inserted diagonally through the root zone of one plant and another is inserted diagonally through the area in between two root zones. This was done to test difference in ion availability at different distance from the rhizosphere. A different availability at both locations may indicate that the plants influence ion availability in their own surrounding.

According to the initial plan, samples should have been taken twice a week. However due to difficulties with delivery of the rhizons and their application soil water samples were taken only once, during the last week of the experiment. All samples were diluted 50 times with MilliQ before analysis of their ion content. Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, SO_4^{2-}, and PO_4^{3-} content (mmol/l) was quantified using Ion Chromatography (IC) (system 850 Professional, Metrohm, Herisau, Switzerland).

Figure 5: Mitscherlich pot containing three barley seedlings. Two Rhizons (arrow) (marked red) are inserted in the pot, the soil water is collected in the connected syringes.

2.4 Data Analysis

All obtained data were inspected and the relevance of treatment, genotype and genotype-by-treatment interactions were assessed by analysis of variance using GenStat15. GenStat15 is a renowned statistical package used worldwide, particularly in plant breeding. It is used for genetic analysis and to examine different treatment effect on crops, but next to this, a range of other statistical analysis is available.

Data analysis is based on the entire dataset for the corresponding variate. However, if comparison was made of relative performance (as in relative to the control situation) under the different stress treatments, the control treatment was taken out of the dataset.

The data obtained from experiment 1 formed an unbalanced dataset. This because more replicate blocks from the NaCl treatment were present, containing different number of units per replicate, compared to the other treatments (due to in between harvests). Furthermore, for the PEG treatment no replicate was present. The unbalanced ANOVA option (ANNEX IV) from GenStat was used to analyse these data. Multiple comparison is not possible for unbalanced data, however all Least Significant Differences (LSDs) at a significance level of 5% could be calculated.

Data obtained from experiment 1 were analysed using ANOVA for complete randomized designs in GenStat15. Fishers Protected LSD was used to clarify significant differences between means.

In general experiment 1 and 2 are regarded separately. However if comparison between both treatments was made, unbalanced ANOVA was used. In comparison between means, always $P<0.05$ was used unless mentioned differently.

3. RESULTS

3.1 Ion availability

To determine the availability of ions, (soil) water samples were analyzed for ion concentration. In experiment 2, a comparison was made of the ion availability in the root zone and in the non-root zone within one pot. Only for PO_4^{3-} a significantly higher concentration was found in the direct root zone (on average 2.05 mM in the direct root zone compared to 1.47 mM in the non-direct root zone). For all other analysed ions no significant difference in concentration was found ($P<0.05$). For this reason, both samples taken from one pot are treated as replicates in all further determinations.

Ion availability of Na^+ and Cl^- in both 200 mM NaCl treatments (NaCl (hydroponics) and NaCl200 (sand)) does not significantly differ (Figure 6). However, it should be noted that ion concentrations of Cl^+ and Na^+ in the control situation were higher in sand (experiment 2) than in hydroponics. The difference was about 7 mM, which is not significant according to the unbalanced ANOVA.

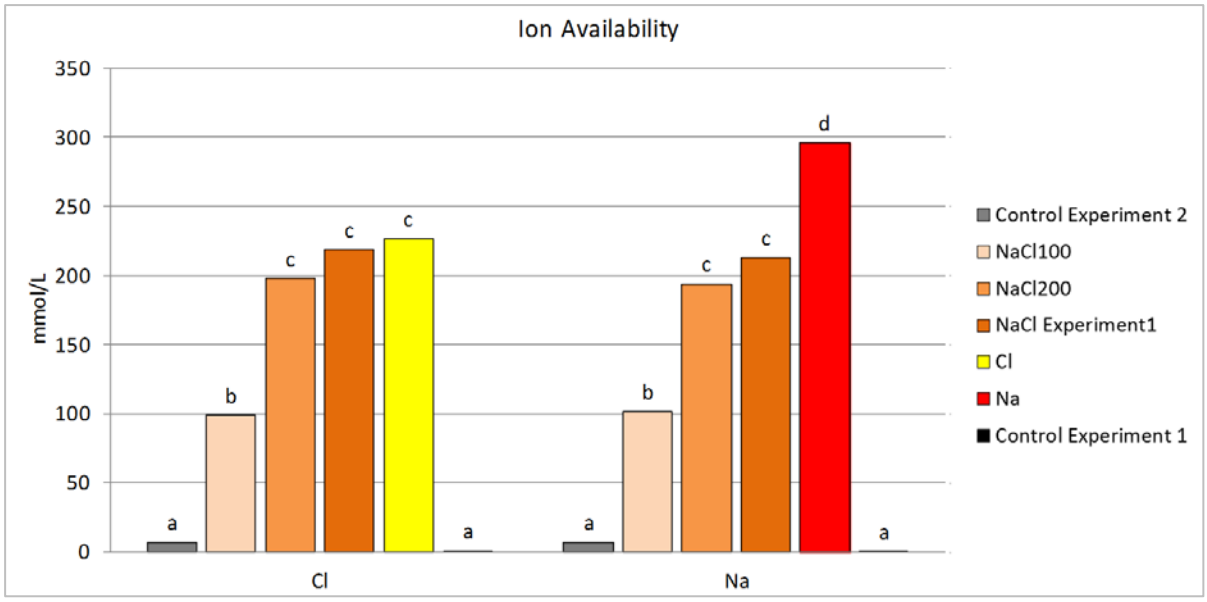

Figure 6: Sodium and Chloride availability in all Na and/or Cl containing treatments. The treatments, Control; NaCl100 and NaCl200 are part of experiment 2 whereas the other four treatment are part of experiment 1. Means that are significantly different do not have a letter in common.

In the NaCl treatments on sand, elevated levels of Ca^{2+}, Mg^{2+} and K^+ were found compared to the hydroponic NaCl treatment. With the predicted mean of 5.17 mM K^+ in the hydroponic NaCl treatment; 7.75 mM in the NaCl100 treatment and 9.23 mM for the NaCl 200 treatment, K^+ is the only ion which was present at significantly higher levels in the soil- compared to the hydroponics experiment. For Ca, Mg and K^+, significantly higher concentrations were measured for the NaCl 200 treatment compared to the NaCl 100 treatment. The differences were very small, from 0.4 to 1.5 mM

The purpose of the Na and Cl treatments was to create equal Na^+ and Cl^- concentrations as in all NaCl treatments. The Na^+ concentration in the Na treatment was found to be significantly higher than in the NaCl treatments. The Cl^- concentration in the Cl treatment did not significantly differ from concentrations found in the NaCl treatments (Figure 6). As was expected, the counter ions used for the admission of Na^+ and Cl^- in these treatments were present in high concentrations compared to the other treatments. For the PEG treatment no values deviating from control circumstances were found.

Root environment monitoring

Environmental factors such as the pH value of the soil solution influence the solubility of salts. Different salts are soluble to a different extend at certain pH values. And thus, plants should be grown in an environment where all necessary salts are soluble to sufficient extend. During the whole experimental period the pH of all hydroponic treatments was monitored and ranged between pH 5 and 7(ANNEX V). Within this pH range, ion availability off all essential ions should be assured (Cornell, 2010). The pH value of the solutions that were used to water the pots in experiment 2, was measured and ranged between 4 and 5.

Furthermore, for experiment 1, the Electrical Conductivity (EC) as a measure for the amount of solved ions was monitored during the experiment. The EC values measured for all salt treatments in hydroponics are comparable, pointing towards similar ion concentrations. However, between 4 and 7 Dec (two weeks after final salt concentrations were reached), the EC value of the Na and Cl treatment decreased approximately 5 mS/cm, where after the EC value stabilized again (ANNEX II).

3.2 Salt tolerance

Salt tolerance (ST) is defined as the production of shoot dry matter in the stress treatment, relative to shoot dry weight matter in the control situation. The shoot (stem and leaves) represents photosynthetic capacity and therefore indirectly gives information about the capacity of transpiration.

Reduction in ST was found as a result of all treatments. However, stress seems to be experienced differently on hydroponics compared to sand. Comparing the NaCl treatment from experiment 1 and the NaCl200 treatment from experiment 2, a vastly different average ST was found (Figure 8)(Table 4). ST was much higher in experiment 2 (68 % ST vs. 21 % in experiment 1). Furthermore, experiment 2 suggests that salt tolerance does not proportionally decrease with increasing salt concentration (Figure 8)(Table 4).

The effect of Na^+ separately, in the "Na" treatment, on ST, appeared (insignificant according to the LSD ($p<0.05$)) more severe than that of NaCl. The effect of high levels of Cl^- in the Cl treatment, was found significantly less severe (Figure 7). The effect of the PEG treatment represents the effect of increased osmotic potential, of the available solution, on the plant. Less stress (reduction in shoot growth) was experienced in the PEG treatment compared to the Na and NaCl treatments. (Figure 8).

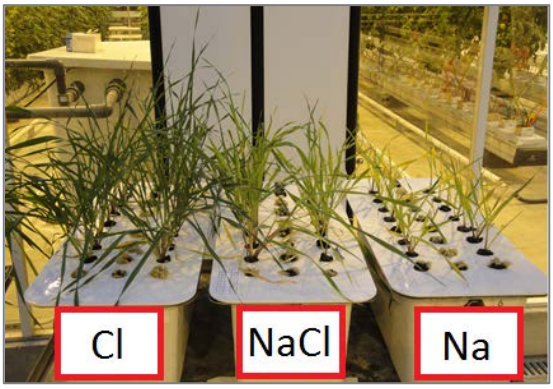

Figure 7: Phenotypical differences between the Cl, Na and NaCl treatments.

Table 4: Average salt tolerance per treatment. Predicted means for experiment 1 as a result of the unbalanced ANOVA(left), and the means for experiment 2 (right) compared in an ANOVA for complete randomized designs and a Fishers protected LSD test. Significantly different means are followed by different letters.

Experiment 1		Experiment 2	
Treatment	**Predicted mean (%)**	**Treatment**	**Mean (%)**
Cl	49.42a	NaCl200	68.11d
Na	12.58b	NaCl100	84.65e
NaCl	21.1bc		
PEG	32.80ac		

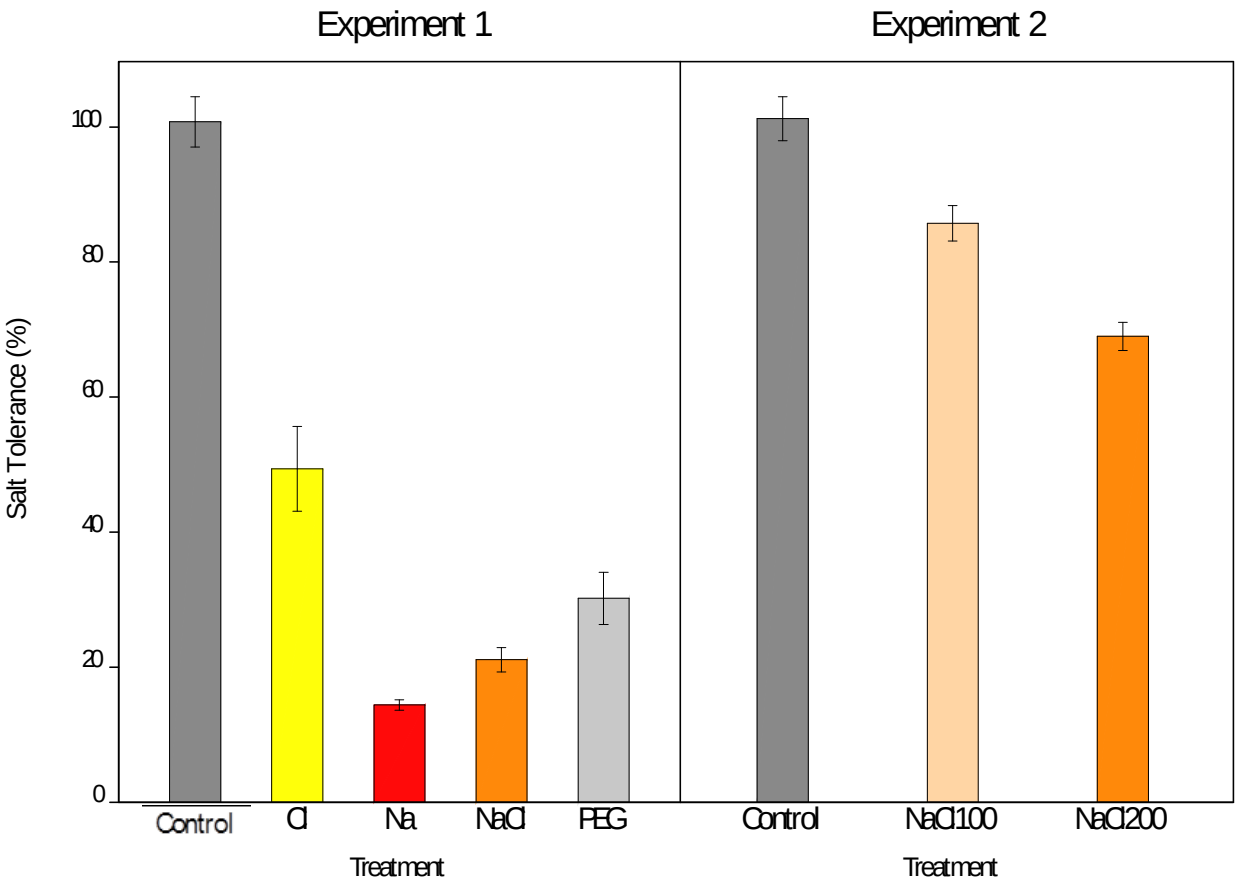

Figure 8: Average salt tolerance per treatment in experiment 1 (left) and experiment 2 (right). Error bars depict standard deviation.

Salt tolerance per genotype

based on average ST per genotype (over all treatments), genotype three was least tolerant (Table 5). In general genotype one and two were found to be more salt tolerant, but their performance did not always differ significantly from less performing genotypes. ST per analysed genotype in the NaCl treatment from experiment 1, showed that G1 and G2 were significantly more tolerant than G3 and G4 ($P<0.05$). This is not confirmed in experiment 2 where G4 outperforms G1, however, the difference is small and insignificant (Table 5).

To further unravel the underlying causes of genotype performance in the NaCl treatments, the performance of each genotype under the other stress treatments are of interest. In this case ST refers to tolerance to the particular stress factor administered in the corresponding treatment (figure 9).

Table 5: Average Salt Tolerance per genotype. Predicted means for experiment 1 as a result of the unbalanced ANOVA ($P<0.05$) (left), and the means for experiment 2 (right). Means that are significantly different do not have a letter in common. To enable comparison between the genotypic effect in the NaCl treatments from both experiments, the obtained data for the NaCl treatment on hydroponics is mentioned separately from the other stress treatments in experiment 1 (third column).

	Experiment 1		Experiment 2
	All stress treatments	NaCl treatment	All stress treatments
Genotype	Predicted mean	Predicted mean	Mean
1	31.27a	26.75a	73.55ab
2	35.49a	30.28a	82.27b
3	18.19ab	11.42b	70.18a
4	24.67a	15.75b	79.53b

Differences in ST among genotypes per treatment seemed more obvious in experiment 1 (table 5); more phenotypic variation among genotypes was found in experiment 1. This was confirmed by ANOVA ($P<0.05$), giving an F-value of <0.001 for genotypic variation in the NaCl treatment of experiment 1. Whereas the F-value for genotypic variation in experiment 2 is 0.04 (ANNEX VI).

Genotype 1 was shown to perform significantly better in the Cl treatment compared to the PEG treatment. For all other genotypes, similar ST is found in both treatments. So in general, Cl^-'s expected combined osmotic and toxic effect was found to affect ST to the same extent as the osmotic effect of the PEG treatment. For G1 however, this is not the case. G1, was shown to deal better with the stress effect caused by the Cl treatment. Furthermore, G1 and G4 were found to be less affected by the addition of extra NaCl (comparing NaCl100 and NaCl200) in experiment 2 (Figure 9).

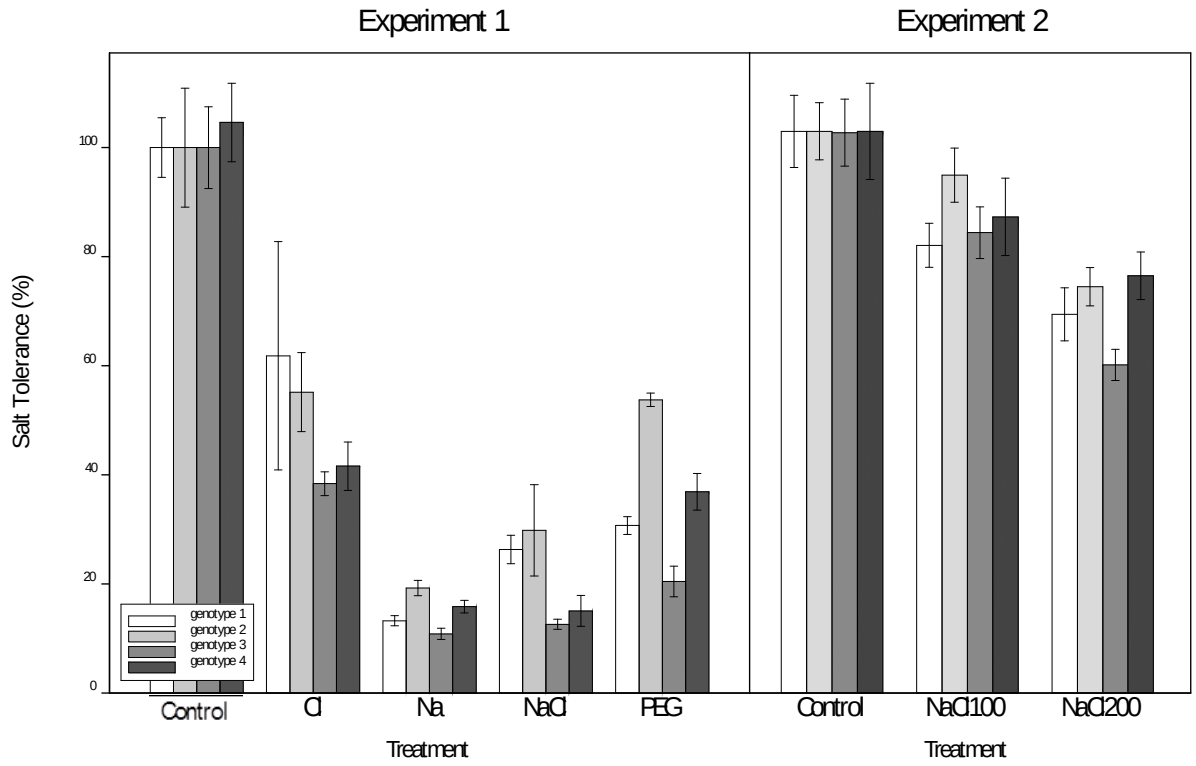

Figure 9: Average salt tolerance per genotype for each treatment. Error bars depict standard deviation.

3.3 Phenotypic characterisation

Potential photosynthetic area

For plants subject to stress, leaf death increased throughout time. In the control treatment no leaves died. The amount of live and dead leaves gives insight in the reduction of the potential photosynthetic area (A). Here the photosynthetic area is assumed to be strongly correlated to the amount of functional leaves. The reduction in potential photosynthetic area is described as follows:

$$\text{A reduction} = \frac{\text{(leaf death) Treatment X}}{\text{(leaf survival + leaf death) Treatment X}}$$

Where the denominator describes the number of leaves that have died in the treatment of interest (X), and the nominator describes the total amount of leaves that were formed in that particular treatment.

In experiment 2, no significant reduction of potential photosynthetic area was measured for any of the treatments. However G3 did show significantly higher leaf death in the stress treatments (m=1.83) compared to the other genotypes. In experiment 1, G3 showed the highest average (over all stress treatments) decrease in potential photosynthetic area. G4 followed by G3 and G2 showed the highest leaf death (Table 6). Compared to the other treatments, significantly more leaves died in the PEG, NaCl and Cl treatment. But A reduction was shown to be strongest in the Na-, NaCl- and PEG treatment, where leaf formation apparently compensated leaf damage to a lesser extend (Figure 10).

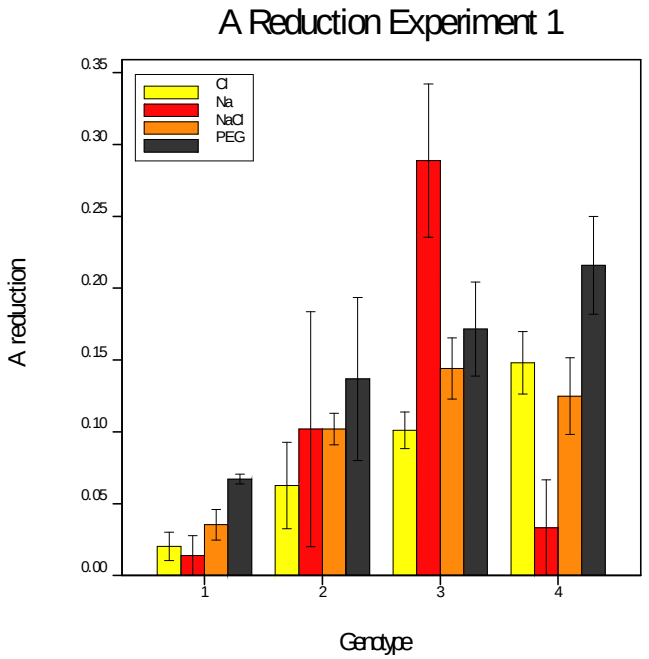

Figure 10: Average reduction of photosynthetic area for each genotype, under all stress treatments from experiment 1. Error bars depict standard deviation.

Table 6: Average leaf death and reduction of potential photosynthetic area per genotype in the stress treatments from experiment 1.

Experiment 1	Leaf death	A reduction
Genotype	Predicted mean	Predicted mean
1	0.30a	0.02a
2	0.64b	0.07b
3	0.83b	0.14c
4	0.92bc	0.08b

Tiller formation

Tiller formation was decreased by all stress treatments. However, the effect on hydroponics was much stronger than on sand.

There was no significant difference between the hydroponic treatment effects. In experiment 1, a significant difference in average tiller formation over all stress treatments was found between G3 and G1, and G4 and G1 (Table 7). Furthermore, tiller formation in G1 and G2 decreased least compared to the control situation (relative tiller formation) (Table 7). This indicates that with respect to tiller formation G1 and G2 were less severely affected by the stress treatments than G3 and G4.

Tiller formation is more strongly inhibited throughout time in the NaCl200 treatment compared to the milder NaCl100 treatment in experiment 2 (Figure 11). This was especially obvious for G3. However, there is no significant difference between average tiller formation in both treatments at any particular point in time. Furthermore, no significant differences in tiller formation were found among genotypes in experiment 2.

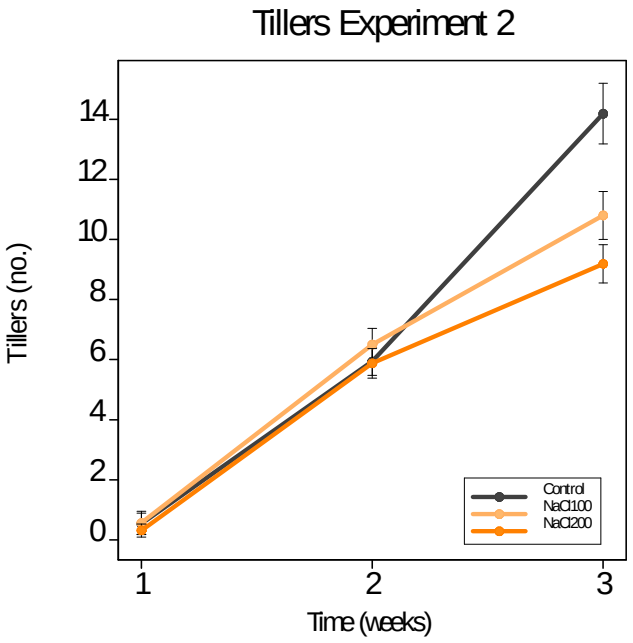

Figure 11: Average tiller number throughout time, per treatment for experiment 2. Measurements were performed at respectively 1, 2, and 3 weeks after final salt concentrations were reached. Error bars depict standard deviation.

Table 7: Absolute and relative average tiller formation in the stress treatments of experiment 1, for all genotypes of analysis.

	Experiment 1	
Genotype	**Tiller formation**	**Rel. tiller formation**
1	5.24a	0.51a
2	3.9b	0.56a
3	2.4bc	0.36b
4	2.7bc	0.38b

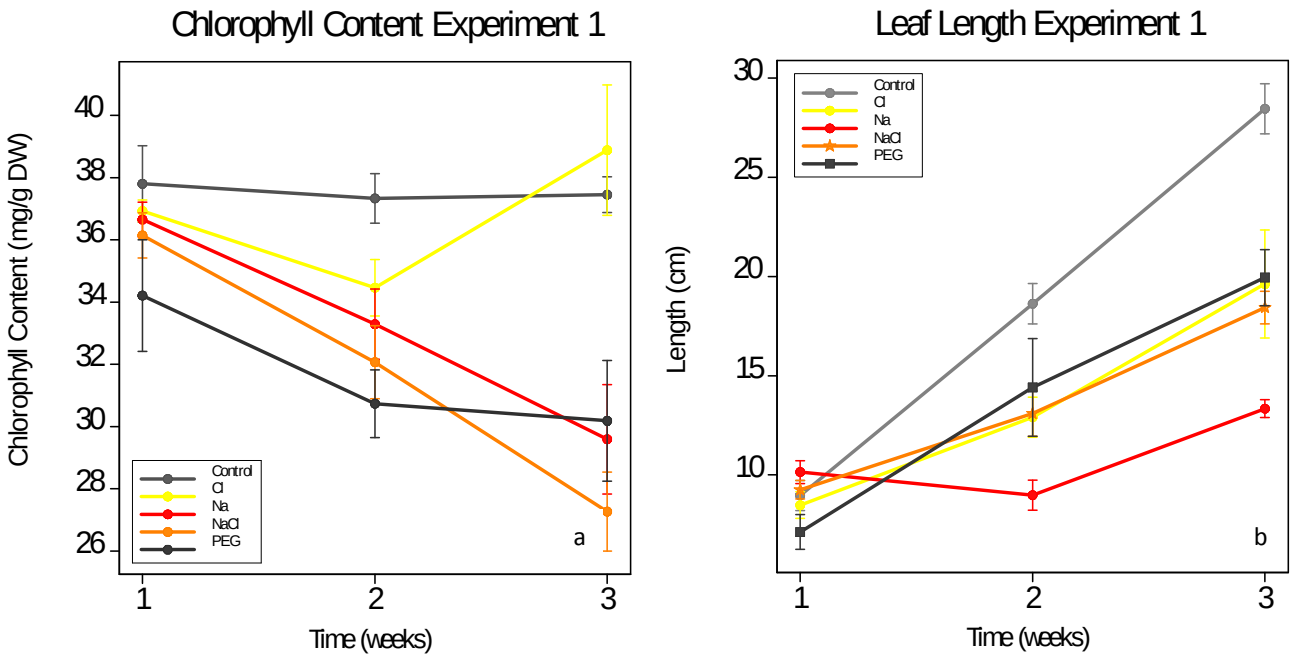

Figure 12: a) Average chlorophyll content of the youngest fully expanded leaf throughout time for all treatments in experiment 1 (left). b) Average leaf length of the youngest fully expanded leaf throughout time (right). Error bars depict standard deviation.

Chlorophyll content

In experiment 1, significant decreases in chlorophyll content were measured for all but the Control and Cl treatments. Average chlorophyll content reduced throughout time in the Na and NaCl treatments. In the Cl treatment, higher average values were measured at the third time point for all genotypes (Figure 12a). However only for G2 this increase was significant. With respect to genotype specific behaviour per treatment, the following findings are notable: G4's chlorophyll content hardly changed in the Na treatment, whereas the NaCl treatment had a stronger impact on chlorophyll content in G4 compared to the other genotypes. In general, looking at chlorophyll content over all stress treatments, G2 and G4 were found to maintain a relatively high and constant chlorophyll level throughout the experiment (Figure 13).

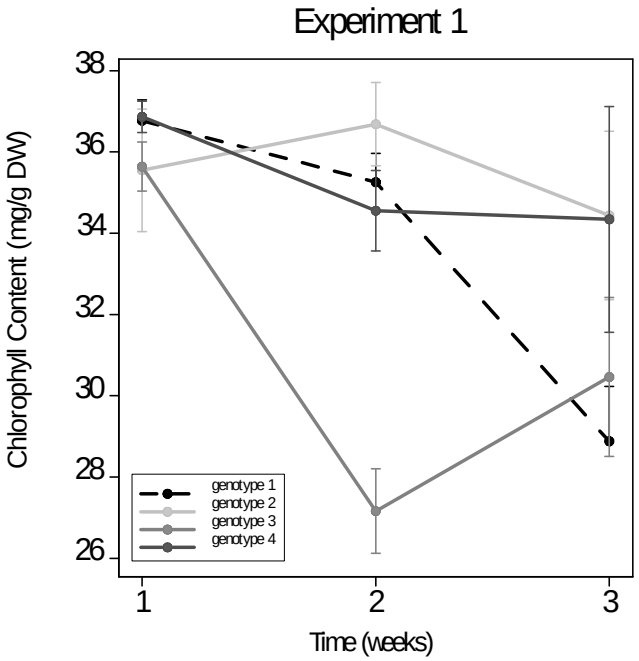

Figure 13: Average chlorophyll content per genotype in the stress treatments of experiment 1. Error bars depict standard deviation.

A low chlorophyll content does not per see corresponds to decreased chlorophyll production. Possibly, chlorophyll production did not increase to sufficient extend to prevent leaf elongation from causing a decrease in chlorophyll concentration per unit of leaf area. In the Cl, NaCl and PEG treatments, leaf length at time of chlorophyll measurement was similar (Figure 12b). In the Na treatment, which had the smallest average leaf length, the concentration of chlorophyll per unit of leaf area was low. But because leaf elongation in the latter treatment was low compared to the elongation in the NaCl treatment, total chlorophyll content in leafs from both treatment is probably more similar

In experiment 2 no changes in average chlorophyll content were detected (ANNEX VII). Salinity stress does not seem to affect chlorophyll content in any of the genotypes. The available data on leaf length do not point to any significant differences in leaf length between the treatments at any time.

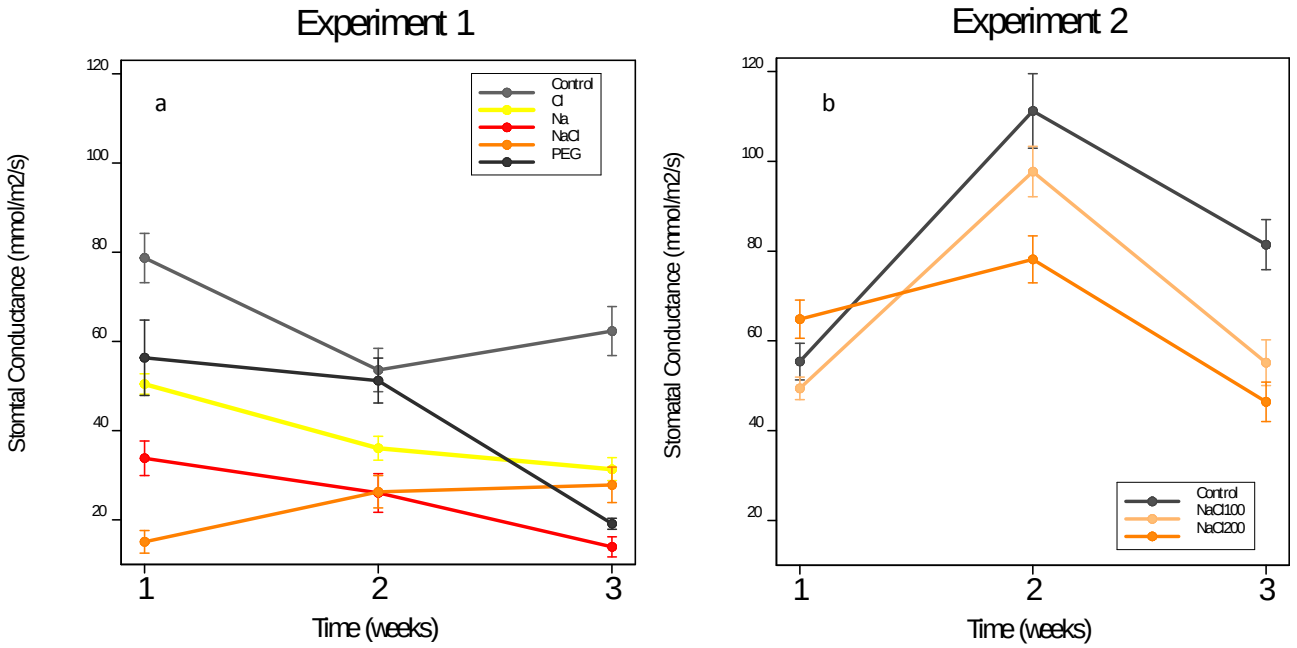

Figure 14: Absolute stomatal conductance in mmol/m2/s for all genotypes per treatment, for experiment 1 (a) and experiment 2 (b). Error bars depict standard deviation.

Stomatal conductance

Stomatal conductance (g_s) was decreased by all stress treatments.

In experiment 1, the NaCl treatment, initially had a significantly stronger effect on stomatal conductance than the Na and Cl treatment separately. At time point one and three, stomatal conductance was significantly lower in the Na treatment than in the Cl treatment. Based on ANOVA for unbalanced designs, stomatal conductance of plants in the NaCl treatment was found to be stable throughout time. For all other treatments stomatal conductance had significantly decreased in time (Figure 14a).

The effect of salinity stress on stomatal conductance was found to be less severe in experiment 2 compared to experiment 1. Furthermore, whereas stomatal conductance increased up to time point two for the other treatments in experiment 2, for the NaCl200 treatment no significant increase was measured. At time point three, stomatal conductance in both stress treatments of experiment 2 was equal (Figure 14b).

In experiment 1, variations in stomatal conductance among genotypes have been observed. G2 had the highest stomatal conductance compared to the other genotypes in both the Cl and Na treatment and maintained relatively high stomatal conductance in the NaCl treatment. G4 was able to maintain relatively high levels of stomatal conductance in the NaCl and PEG treatments. For both G1 and G4, no decrease in stomatal conductance was observed as a result of the PEG treatment. (Figure 15a)

In experiment 2, again G2 maintained relatively high levels of stomatal conductance. In the NaCl200 treatment however, G1, G2 and G3 transpired similarly whereas G4 had significantly lower stomatal conductance. In G1 stomatal conductance was stable over both NaCl treatments from experiment 2. For G2 and G4 a gradual decrease as a result of increased NaCl concentration was detected. For G3 no clear pattern could be observed to this regard (Figure 15b).

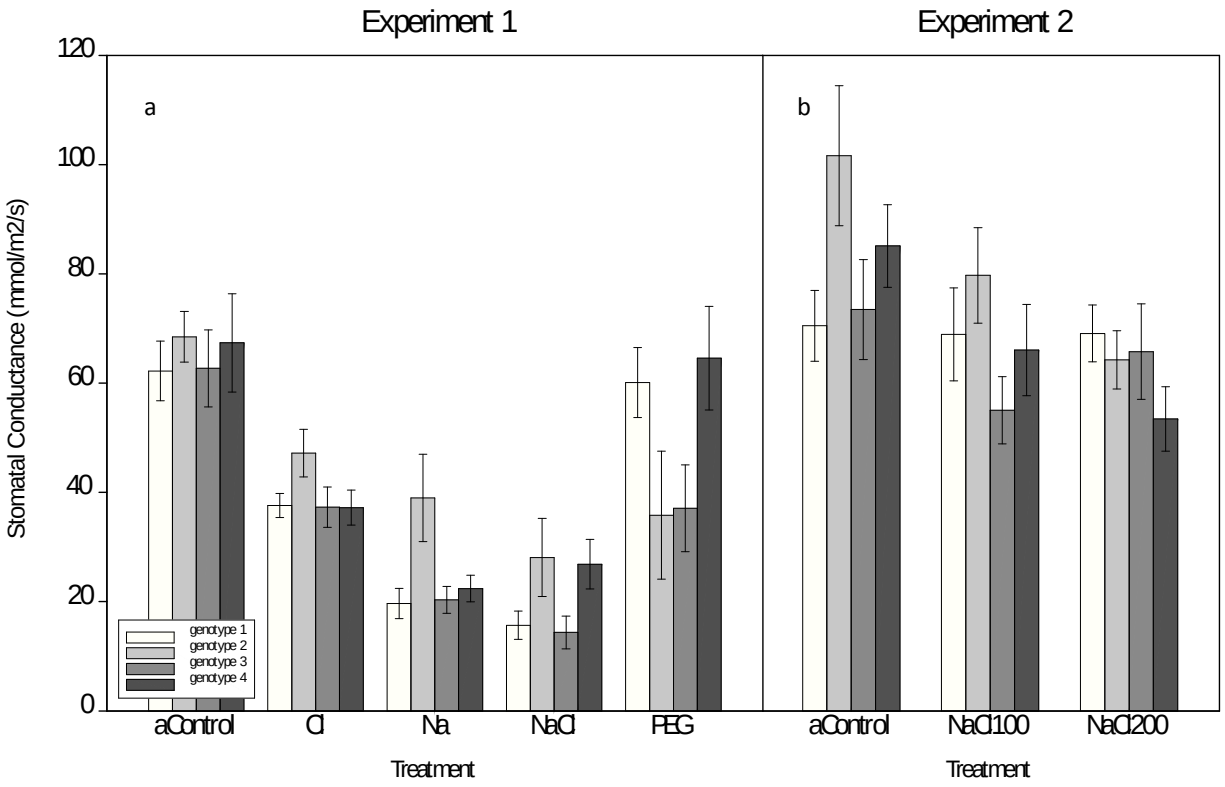

Figure 15: Average stomatal conductance per genotype for all treatments from experiment 1(a) and experiment 2(b). Error bars depict standard deviation.

Leaf osmotic potential

The osmotic potential of the second and third youngest leaf was measured for G1, G2 and G3 grown on the NaCl-hydroponic treatment at one, two and three weeks after final salt concentrations were reached. Measurements are based on three replicates per treatment. For G2 no measurement was done at the second time point. To be able to make a comparison between the obtained leaf osmotic potentials and the corresponding potentials found in the control situation, at each time point, measurement should have been done on plants from the control treatment. This was not done due to lack of available plants. Only at the last time point measurements were done on G1 and G2 from the control treatment.

Based on the measurements it is likely that leaf osmotic potential in G3 increased throughout time. Whereas the osmotic potential in G1 and G2 increased, it was kept more stable after an expected initial increase. Due to the lack of a control/reference measurements for G3 nothing can be said about the leaf osmotic potential under non NaCl-stress conditions (Figure 16).

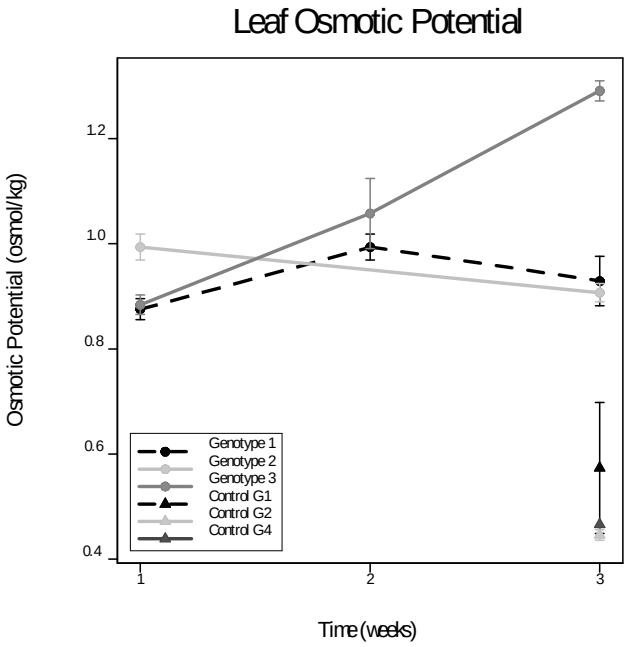

Figure 16: Leaf osmotic potential in the second youngest leaf of plants grown on the NaCl hydroponic (dots) and control hydroponic treatment (triangles) at 1, 2 respectively 3 weeks after final salt concentration was reached. Error bars depict standard deviation.

3.4 Ion Content

Hydroponics versus Sand

For all ions but Ca^{2+}, significantly less root-content was measured in experiment 2 (sand) compared to experiment 1 (hydroponics) (Figure 17). For shoot ion content, no outstanding differences were found between experiment 1 and 2. However, significantly more Cl^- and significantly less Mg^{2+} and SO_4^{2-} accumulated in shoots of plants grown in the hydroponic control situation (Figure 17).
Furthermore, in experiment 2, a higher standard deviation is found for especially K^+ and Na^+ content.

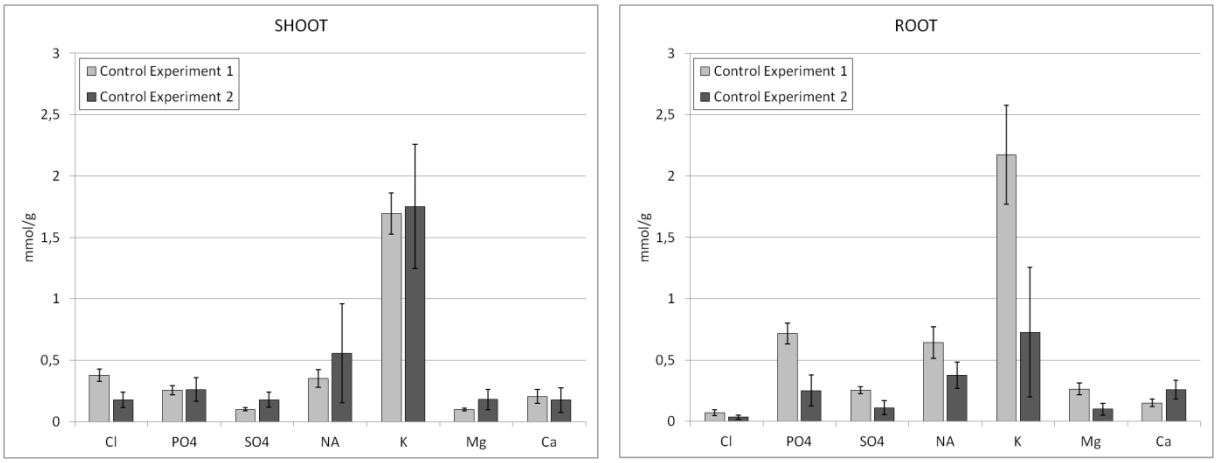

Figure 17: Average ion accumulation in control situation experiment 1 versus experiment 2 in mmol per gram dry weight. Error bars depict standard deviation.

Treatment effect

Differences in ion accumulation between treatments might tell us something about the processes involved in salt tolerance. In the following sections, the average ion accumulation per treatment is presented.

PEG treatment

Shoot content shows significant decrease for all measured ions compared to the control situation (Figure 18). The reduction in K^+ content is most outstanding. Whereas in the root, Cl^-, PO_4^{2-}, Mg^{2+} and Ca^{2+} were still accumulated to the same or even a bigger extent as in the control situation, translocation of the ions to the shoot was not sufficiently efficient to prevent significantly lower ion content in the shoots (compared to the control situation). Furthermore, the high root Ca^{2+} content is remarkable.

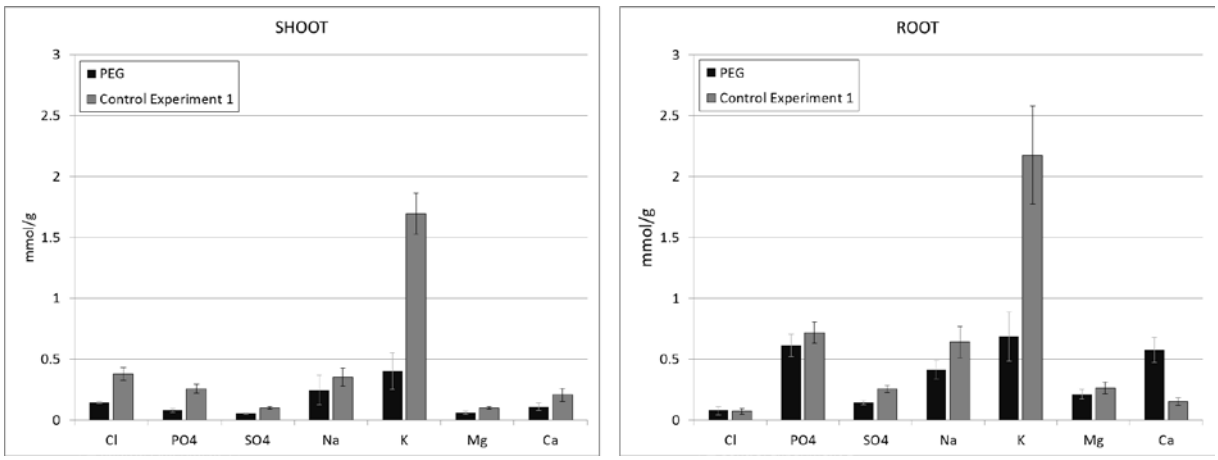

Figure 18: Average ion accumulation for all genotypes in the PEG treatment. Error bars depict standard deviation.

Na and Cl treatments

Chloride

Concentrations of the Cl^- counter ions, Mg^{2+} and Ca^{2+} are high in Cl-stressed plants compared to the control situation. This can be explained by the higher availability of these ions in the "Cl" treatment. K^+ was also used as a Cl-counter ion but was significantly underrepresented in the shoot. Based on these findings, the increased Cl^- accumulation in root and shoot together with a slight decrease in shoot K^+ content, may be stress factors in this treatment (Figure 19).

Sodium

A significant decrease in K^+ root content was found for plants that were exposed to the Na treatment. Root Ca^{2+}- accumulation had increased significantly compared to the control situation, which cannot be explained by Ca^{2+} availability in the medium. Furthermore, a significant increase in Na^+ accumulation was measured in root as well as in shoot. Ion accumulation in shoots was even more affected than root accumulation. Transport from root to shoot of Cl^-, Mg^{2+} and Ca^{2+} seems to have been at the expense of Na^+-counter ion transport (Figure 19). In short, decreased K^+ root accumulation and decreased shoot accumulation of K^+, Mg^2 and Ca^{2+}, together with increased Na^+ (and SO_4^{2-}) shoot and root accumulation, may be stress factors in the Na treatment.

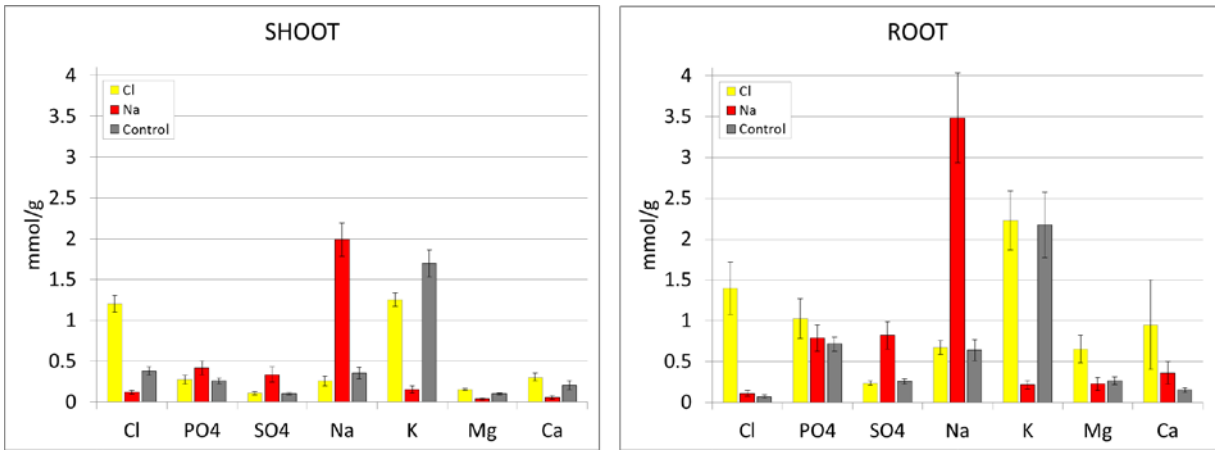

Figure 19: Average ion accumulation for all genotypes in the Na and Cl treatments. Error bars depict standard deviation.

NaCl treatments

In roots, deviations from the control situation have been observed for K^+ and Ca^{2+} content (Figure 20). Significantly lower amount of K^+ has accumulated, and content appears inversely proportional to the NaCl concentration that was found available in the treatments (Figure 20). An increase in Ca^{2+} root-content was observed for the NaCl-hydroponic treatment, similar to what has been observed in the PEG and Cl treatments (and to a lesser extend in the Na treatment). Furthermore, in shoot, K^+, Mg^{2+} and Ca^{2+} content decreased substantially compared to the control situation. In both root and in shoot, increased Na^+ and Cl^- content was measured. Outstanding is that Cl^--root content for all NaCl treatments had increased more than Na^+-root content ($P< 0.05$) (Figure 20). Furthermore, there was no significant difference between ion accumulation in general in treatment NaCl100 and treatment NaCl200. Next to this, Na^+-and Cl^--root accumulation in hydroponically grown plants (experiment 1) was found to increase more profoundly in comparison with the control situation, than in plants grown on sand (experiment 2).

Based on the findings above, can be stated that decreased accumulation of K^+, Mg^{2+}, Ca^{2+} and increased accumulation of Cl^- and Na^+, result from treatment with (excess) NaCl.

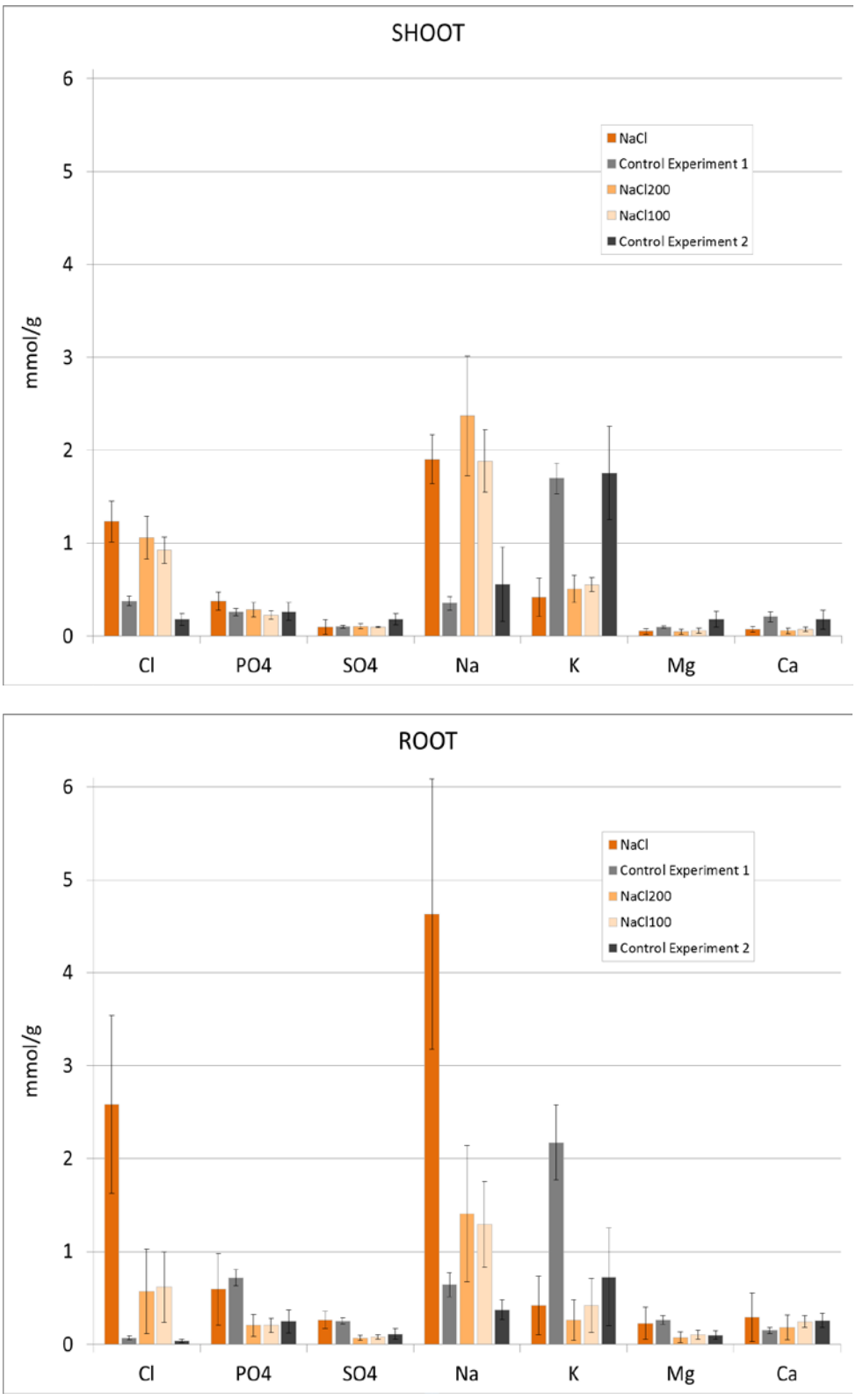

Figure 20: Average ion accumulation for all genotypes in the NaCl treatments. Error bars depict standard deviation.

Genotype effect

High standard deviations on the bars for average ion accumulation per treatment might point towards differences in ion accumulation among genotypes. Ion accumulation of PO_4^{2-} and SO_4^{2-} was not considered in this analysis because these ions were shown to be present to a similar extend in all treatments, will unlikely reveal any genotypic variation. Furthermore, previously leaf growth was shown to be more sensitive to salinity stress than root growth (Munns and Termaat, 1986). In accordance to this, the previous sub-paragraph shows that shoot ion content is generally more strongly affected by stress treatment than root ion content. Next to this, the consistent difference in root-ion content between experiment 1 and 2 does not facilitate comparison between both experiments. For these reasons was decided to focus on genotypic behaviour with respect to Na^+, Cl^-, K^+, Mg^{2+} and Ca^{2+} shoot content.

PEG treatment

Genotype 2 was found to accumulated more Na^+ and less K^+ compared to the other genotypes in the PEG treatment, thus under osmotic stress. Furthermore, no differences are observed for ion accumulation between genotypes. Lack of replicates make observations in the PEG treatment less powerful (n=2).

Cl treatment

All genotypes show similar ion accumulation in the Cl treatment. As was expected, all ions, except for Cl^-, are present in similar quantities to the control situation. G1 has the highest absolute Cl^- accumulation, followed by G4 and G3 had the lowest. Cl^- accumulation increased to the same extend for all genotypes. (n=4)

Na treatment

For G3 in the Na treatment Na^+ accumulation was high compared to accumulation in the other genotypes. For G2 and G4 less Na^+ accumulation was measured compared to the other genotypes. However, according to the unbalanced ANOVA there are no significant differences in ion accumulation between different genotypes. (n=4)

NaCl treatment

In all genotypes, similar concentrations of Na^+ and Cl^- are found as a result of the NaCl stress treatments. Analysis was based on average ion accumulation in the NaCl, NaCl100 and NaCl200 treatments per genotype. The severity of stress response, as in increased ion accumulation, does differ between genotypes. Na^+ accumulation increased stronger in G1 and G3 than it did in the other two genotypes. Cl^- accumulation in G1 and G2 increased more with respect to the control situation than in G3 and G4. (n= 12-23)

Altogether this gives more insight in shoot ion content of the different genotypes. In general, total ion content per gram dry weight is similar in all genotypes in response to the same treatments and thus, the total ion content per genotype over all treatments does not significantly differ. Nevertheless, with regard to Na^+ and Cl^- content, the highest average accumulation was found for G1 and the lowest for G2 (Table 8). All differences between genotypes described in this sub-paragraph are statistically insignificant. This, however, does not mean that these findings cannot be regarded informative.
In short, the following findings are done. G1 seemed to react relatively strong on changes in Cl^- and Na^+ ion availability by increasing corresponding ion accumulation. In G1, Cl^- accumulated to relatively high amounts if present in abundance. In G3 Na^+ accumulation increased relatively strong a response to increased Na^+ availability. Furthermore, as a result of the Na treatment, accumulation of Na^+ was highest in G3.

	Ion Accumulation	
Genotype	**Cl^- (mmol/g)**	**Na^+ (mmol/g)**
1	1.0773a	2.037a
2	0.7918a	1.578a
3	0.9956a	1.853a
4	0.9072a	1.852a

Table 8: Average Cl^- and Na^+ accumulation over all treatments per genotype.

4. DISCUSSION

4.1 Materials and methods

Growth conditions

Determination of ion availability in experiment 1 was based on samples taken from the hydroponic basins. It should be noted that in the NaCl treatment the amount of plants growing on one basin was different among replicates. This might have influenced the ion concentrations in the nutrient solution among replicates. Furthermore, most likely transpiration has caused the total volume of available nutrient solution to decrease throughout time. This might have caused an increase in ion concentrations throughout time. Since ion availability is only determined in the final week of the experiment, obtained values might give an overestimation of ion availability during the rest of the experiment.

The ion availability in the soil water samples from experiment 2, taken in the final week, can neither be regarded representative for the ion availability throughout the entire experiment. This is because the silver sand medium only allowed the addition of little amount of salt/nutrient solution per day, especially in the beginning of the experiment when transpiration was low, resulting in a slow build-up of soil salinity. In the hydroponics treatment salt concentrations we increased with 50mM per day, resulting in a quick switch-over to the intended stress level. In the end plants grown on sand are subject to a more gradual increase of salinity and, in total, experience less stress.

Upon analysis of ion availability, the IC was used once per sample to analyse the content of all considered ions. It would have been more precise to measure each sample twice: Once to detect ions present at smaller concentrations (based on a 50x diluted sample), and once to detect Na^+ and Cl^- which could be present in much higher concentrations (based on a 3000x diluted sample). This would decrease the dispersion of obtained data per measurement, allowing more accurate calibration based-conversion to concentrations.

Furthermore, the fact that the pH and EC were not monitored and kept constant in experiment 2, gives some uncertainty with regard to comparison of both experiments. However, since the ion availability did not show large deviations among both experiments, we can assume that the EC as well as the pH in the soil water were similar to the EC and pH in the hydroponic solution.

Furthermore, in experiment 1, the PEG treatment was used to mimic osmotic stress. PEG is often used for this purpose (Anithakumari et al., 2011), but the precise effect of PEG is unknown. Most likely, slight uptake of this substance causes unknown side effects. However, according to Carpita et al., PEG with a molecular weight above 6000 g/mol, cannot penetrate cell wall pores (CARPITA et al., 1979). Adverse effect of PEG may be the blockage of root surfaces, hampering water and nutrient uptake, and limiting O_2 movement through the soil solution (Gopal and Iwama, 2007, Verslues et al., 1998, Lawlor, 1970). Furthermore, according to Shah et al., PEG does not behave according to the Morse equation and should be seen as a matricum instead of as an osmoticum (Shah et al., 2001). If this is the case, than the PEG treatment from experiment 1 would more resemble the effect of a solid medium where matric forces play a role. Comparison of results from the PEG treatment and the NaCl200 treatment (with questionable stress intensity) do not confirm this statement.

Measurements

Due to high sensitivity of the trait, variation in stomatal conductance is generally high. Since measurements were done on different time points, coinciding with different weather conditions (light intensity, temperature, humidity), it is very unlikely that all observed variations in stomatal conductance through time can be allocated to plant physiological changes based on genetic variation.

Chlorophyll measurements in the two youngest fully expanded leaves gave insight in chlorophyll formation in new leaves in response to the different considered environments. If measurements on chlorophyll content (as well as on stomatal conductance) would have been based on one particular aging leaf, the degree of senescence in response to different environments could have been measured, which may be more valuable information (Munns and Passioura, 1984).

Measurements were performed on leaf length to determine leaf elongation through time. However, due to lack of marking, it is uncertain whether the same leaf was measured through time. For this reason the obtained data could not be used to represent leaf elongation.
Due to the incomplete execution of measurements on osmotic leaf potential (incomplete analysis of all genotypes and lack of replicates (through time)), no conclusions can be drawn from the obtained results. However, the results can serve to hypothesize on genetic variation with regard internal osmotic changes and to support other experimental findings.
The determination of root and shoot ion content is based on measurement of dissolved ions in processed samples. Incomplete solution of root and shoot tissue results in unrepresentative estimation of total root and shoot ion content. Furthermore, despite the fact that roots were well cleaned after harvest, some sand ended up in the roots samples. This sand did contribute to the total weight from which ion content was measured, but does not release any ions. This may have resulted in an overestimation of ion root content. Next to this, should be kept in mind that measurements for ion content do not give a complete overview of all essential nutrients, for example the primary macro nutrient N is not detected.
Finally, during the final harvest, some roots of hydroponically grown plants were found highly entangled in each other. To facilitate easy harvest it would be better to separate the roots on a regular basis throughout the experimental period.

Data analysis

Statistically seen, the experimental setup has not been in the ideal shape. The lack of seeds made it impossible to plant the intended amount of plants (units). For this reason, experiment 1 had to be analysed as an unbalanced design. This impeded statistical analysis based on the available tests in GenStat15, and required manual calculations. Ensuring balanced data, would have saved a lot of work. Next to this, more plants should have been incorporated to facilitate in between harvest of plants from the control treatment, and ideally more replicates from the NaCl treatment, within experiment 1.
Furthermore, a significant block effect was often found within experiment 1. This points out, unintended, high variation in environmental conditions between replicates. It has been useful to work with blocks, because this allows correction for unintended deviations caused by environmental differences. Within experiment 1, three blocks(basins) were placed together on one movable table, not allowing the movement of these blocks independently from each other. To diminish the block effect, It would have been better to enable independent movement of all blocks throughout the available space on a more frequent basis.

4.2 Results

Hydroponics versus Sand

Ion Availability

K^+ availability was found to be significantly higher in the sand medium. In the NaCl200 treatment from experiment 2, on average 6.23 mM K^+ was found available compared to 5.17 mM K^+ in the NaCl treatment from experiment 1. With respect to availability of Na^+, Cl^- and the other detected ions (Ca^{2+} Mg^{2+}, PO_4^{3-}, SO_4^{2-}), both treatments were comparable.
Experiment 1 and 2 were performed with the same nutrient solution, but possibly because the sand medium could not be washed well (leached), more K^+ was present in this medium. The composition of the hydroponic solution is expected to remain similar to the initial composition due to replacement of 25% with the original salt/nutrient solution on a weekly basis and homogenization by aeration. The higher chance of ion accumulation together with a possible lower K^+ uptake in the sand medium may have caused the increased K^+ availability in experiment 2. Anyway, the lower K^+ content experiment 1 is sufficient to prevent K^+ deficiency (Hoagland and Arnon, 1950).
The significantly higher concentration of PO_4^{3-}, found in the direct root zone (on average 2.05 mM) compared to the non-direct root zone (on average 1.47 mM) in experiment 2, may be caused by increased accumulation in the root zone through suction of the roots and limited uptake of PO_4^{3-} due to excess availability.

Salt Tolerance

Average salt tolerance was found significantly higher in plants grown on sand (68% in the NaCl200 treatment) compared to hydroponically grown plants (21% in the NaCl treatment). Ion availability at time of measurement was similar for both treatments. Possibly a difference in concentration development between both treatments caused a difference in total amount of stress experienced throughout the experiment. Thus, despite of the similar ion availability at the end of the experiment, ion availability may have been lower in earlier phases of experiment 2. For this reason it can be postulated that the observed differences in ST are caused by the gradual increase of salt concentrations in the sand medium that prevents plant damage and allows adaptation to the more salty root environment in experiment 2. Monitoring the EC throughout time, would have given an overview of concentration development.

Ion content

Despite the difference in ST, shoot ion contents in the NaCl treatments of experiment 1 and 2 were similar. Based on the equal ion availability in both experiments this is not surprising. In general, average root ion content for all measured ions (except for Ca^{2+}), was higher in hydroponically grown plants than in plants grown on sand. This implies a difference in root-shoot transport efficiency and possibly in underlying mechanisms, in both media. However, another explanation is the direct nutrient availability at the entire root surface in hydroponics. In sand, roots are party covered with soil particles decreasing the area for water and nutrient uptake; increasing resistance to inflow (Verslues et al., 1998). As the root surface is not blocked with sand particles, in hydroponics, the apoplast is likely to be fully infiltrated with the nutrient solution. Ions present in the apoplast did not (yet) pass any cell membrane and will thus not pass the endodermis, but still this may cause detection of high ion contents. Only if entrance to the stele, via the symplastic pathway, is accomplished, ions can be transported by the vascular tissue towards the shoot. Briefly rinsing the hydroponically grown roots with tap water might have decreased the deviation in root ion content between experiment 1 and 2. To check the latter hypothesis, calculations were done on the volume of ion/nutrient solution that upon adhesion to the root would cause the observed differences in ion content (based on nutrient concentrations as can be found in ANNEX I). Calculations reveal that the observed differences in ion content cannot be caused by a consistent volume of ion/nutrient solution. As adhesion of a more or less similar volume, dependent on root size, in each hydroponic treatment is expected, rejection of the hypothesis seems fair. However, still it is possible that ion accumulation in the apoplastic space may occur to concentrations higher than in the original ion/nutrient solution. This accumulation may be ion dependent, resulting in different degree of accumulation for different ions, not fully dependent on their concentration in the original nutrient solution.

The high standard deviation found for ion content (of especially K^+ and Na^+) in experiment 2 might indicate that genetic variation is more prone to be revealed (with regard to these ions) on sand.

Genetic variation and contributing mechanisms

Treatment effects

Ion availability

In plants grown in the Na treatment a significantly higher concentration of Na^+ was found than in plants that have been subject to the NaCl treatment. Only at similar Na^+ concentration, the stress effect observed in the Na treatment would represent the Na^+-specific stress effect, combined with corresponding osmotic stress, as occurring in the NaCl treatment. The too high Na^+ concentration is probably caused by a mistake during salt addition. Furthermore, high concentration of Cl^-- and Na^+-counter ions in the Cl- and Na treatments are likely to affect plant growth. Ca^{2+}, one of the Cl^-- counter ions was shown to directly ameliorate the effects of salinity stress (del Martínez-Ballesta et al., 2006, Shabala, 2003, Nedjimi and Daoud, 2009) whereas both K^+ and Mg^{2+} are also essential nutrients that have to compete with Na^+ for uptake. Next to this, increased levels of HPO_4^{2-}, SO_4^{3-} and especially NO_3^-, as found in the Na treatment, are beneficial to plant growth. So, the treatment effects of the Cl- and Na treatment are likely alleviated by the effect of Cl^- and Na^+ counter ions, respectively.

The fact that the ion concentration of Ca^{2+}, Mg^{2+} and K^{+} in the soil solution of the NaCl200 treatment was found to be significantly higher than in the NaCl100 treatment, may be explained by the impeding effect of Na^{+} on the uptake of the other nutrients (del Martínez-Ballesta et al., 2006, Fariduddin et al., 2012).

Salt tolerance

Average salt tolerance was found to be highest in the Cl treatment, lower in the NaCl treatment and lowest in the Na treatment. Based on these results, the Cl^{-} ion may interact with the stress effect of Na^{+} to make it less severe. It should be kept in mind that the concentration of available Na^{+} in the Na treatment was shown to be about 100 mM higher than in the NaCl treatment, this will be (partly) responsible for the additional stress effect in the Na treatment compared to the NaCl treatment. The fact that salt tolerance was higher in the PEG treatment compared to the NaCl and Na treatments, may be attributable to Na^{+} toxicity. Furthermore, whereas, the osmotic pressure of the nutrient solution in the Cl treatment is expected to be higher than in the PEG treatment (Table 3), ST was found to be higher in the Cl treatment compared to the PEG treatment. It may be the Cl^{-}-counter ions, with Ca^{2+} in particular, that counteract possible negative effects of Cl^{-} associated ionic and osmotic stress (Shabala et al., 2003) (del Martínez-Ballesta et al., 2006, Fariduddin et al., 2012), but also the previously described adverse effects of PEG might play a role here (Gopal and Iwama, 2007, Verslues et al., 1998, Lawlor, 1970).

Experiment 2 suggests that salt tolerance does not decrease proportionally with increasing salt concentration as was also found by Wolde (2011) (Figure 8) (Table 4). However, the final salt concentration was probably reached sooner in the NaCl100 treatment than in the NaCl200 treatment (Materials & Methods §2.2). This causes the total amount of stress experienced in both treatments of experiment 2 to be more similar than would be expected based on their definition.

Figure 21: Roots from hydroponically grown barley varieties, grown in the PEG treatment a) and in the control treatment b).

Phenotypic characterisation of treatment effects

To gain more insight in the effect of the different treatments. average phenotypic characterization was considered. The following findings should be noted:

- The fact that reduction of potential photosynthetic area in the PEG treatment was high suggests that osmotic stress is an important determinant for leaf death and formation. The fact that despite the low number of dead leaves in the Na treatment, reduction of the potential photosynthetic area in this treatment is high, suggests a negative effect of Na^{+} on leaf formation. In experiment 2, no significant reduction of potential photosynthetic area was measured for any of the treatments.
- The effect of all treatments on tiller formation in experiment 1 was similar. This may indicate that reduction in tiller formation is caused by osmotic stress. Which corresponds to statements of Rahnama et al. (Rahnama et al., 2010). Furthermore, according to Cabeza et al., reduction in tiller formation is one of the first signs of sensitivity to water stress (Cabeza et al., 1993). Experiment 2 is in line with this finding; although statistically insignificant, tiller formation was more strongly inhibited through time in the NaCl200 treatment compared to the milder NaCl100 treatment (Figure 11). The latter effect may also be partly due to increased ionic stress in the NaCl200 treatment, but as ionic stress of 100 mM is also substantial, the major difference in effect of both “severe” stress treatments

will be due to osmotic stress (Husain et al., 2003). In hydroponics, tiller formation decreased much stronger in response to salinity stress than on sand.

- All stress treatments from experiment 1, except for the Cl treatment, which had the highest Cl^- shoot content, caused a decrease in chlorophyll content. These findings are not in agreement with findings of Tavakkoli et al. (2011) who found that Cl^- shoot content negatively impacts leaf chlorophyll content. In the Na and NaCl treatments, chlorophyll content kept decreasing through time. This may indicate an additional decrease in chlorophyll content as a result of Na^+ content as was also found by Husain et al. (Husain et al., 2003). In experiment 2, stress severity may not have crossed a certain threshold for reduction in chlorophyll content because no changes in chlorophyll content were observed.
- All stress treatments resulted in decreased stomatal conductance (g_s). The high sensitivity of the trait as well as the measurement for g_s resulted in deviations that make interpretation of the obtained data difficult. At time point one, few measurements were done on g_s because many leaves were too narrow to allow valid measurement. By excluding the data from time point one, the more representative data are left for analysis: Na^+ strongly decreased g_s, as was found by Tavakolli et al (2011), whereas the effect of Cl^- was less. The degree to which the NaCl treatment limited g_s was in between the separate Na^+ and Cl^- effects. Between time point two and three, stomatal conductance in the PEG treatment decreased severely, more than would be expected as a result of osmotic stress when looking at the effect of the Cl treatment which should cause higher osmotic pressure. This may point towards blockage of the transpiration pathway due to PEG probably at the vein endings, causing reduced water uptake and reduced stomatal conductance (Figure 14a) (Lawlor, 1970, Reid et al., 1978). The average root dry weight in the PEG treatment was 0.68 gram compared to 0.62 gram in the control treatment, whereas the roots from the PEG treatment appeared small (Figure 21). This could point towards the accumulation of high molecular weight PEG molecules at the root surface.

  In experiment 2, the effect of NaCl on stomatal conductance was only detected after the second time point. An early decrease in transpiration, as was observed in experiment 1, might have been prevented in the sand medium because the salinity of the soil solution increased more gradually and was thus not right away present to the full extent. In the NaCl200 treatment, ion concentrations increased more rapidly than in the NaCl100 treatment, causing a faster decrease in stomatal conductance. It might have been the slower build-up of salt concentrations that causes a generally higher stomatal conductance at time point three in experiment 2 compared to experiment 1. In experiment 2 plants may have been able to gradually adapt/acclimatize to a more salty root environment, and thus maintain higher levels of stomatal conductance (Jones and Rawson, 1979).

  The fact that, at time point three, stomatal conductance in the NaCl100 and NaCl200 treatment was similar despite the difference in NaCl concentration at this time point (100 mM, 200 mM respectively), was unexpected. The high standard deviation among measurements indicates that this observation is less reliable. Possibly, if the experiment would have continued for some time, g_S of both treatments would stabilize at different values.

Summarizing: Osmotic stress effects expectedly caused reduction in potential photosynthetic area and tiller formation but also reduced stomatal conductance as well as chlorophyll content. No ion specific effects were observed as a result of the Cl^- ion, whereas, the Na^+ ion is expected to have a negative effect on leaf formation and to additionally limit stomatal conductance and chlorophyll content.

In experiment 2, generally little or no impact of the stress treatments on the measured parameters was detected. Observations on retarded reduction in g_s in experiment 2 compared to experiment 1 suggest that the salinity of the soil solution increased more gradually and was not right away present to the full extent. This implies that plants from experiment 2 may have had a more extensive opportunity to acclimatize to salinity stress and possibly suffered from a less severe salinity stress in comparison to plants from experiment 1 (as was also suggested by the high ST in experiment 2).

Effect of treatment on ion content

K^+ content is affected in all stress treatments. Root and shoot K^+ content decreased the least (insignificantly in root) in the Cl treatment followed by the NaCl and most in the Na treatment. The lower K^+ content in plants resulting from the Na treatment may be due to the higher Na^+ availability in the Na treatment (about 100 mM more) compared to the NaCl treatment: Na^+ may be involved in K^+ exclusion, probably due to competition for the same uptake system as well as maintenance of ion homeostasis (Schachtman and Liu, 1999).

Furthermore, in all hydroponic stress treatments, increased Ca^{2+} root content, together with lower shoot Ca^{2+} content was observed in comparison to the control situation. The increased root Ca^{2+} content may be the result of Ca^{2+} mediated stress signalling in root tissue. The absence of increase in Ca^{2+} root content in plants from the stress treatments in experiment 2, again suggest that little stress may be experienced here. Next to this. the lack of difference in plant ion content in the NaCl100 and NaCl200 treatment may indicate that stress in both treatments is similar.

In the PEG treatment, general reduction in ion content, and of K^+ in particular, was observed. This might indicate that K^+ uptake is especially sensitive to osmotic stress. Furthermore, a high Ca^{2+} root content was found compared to the control situation.

In the Cl treatment increased content of Cl^- and its counter ions, with the exception of K^+, was found. Root K^+ content did not increase proportionally with the extra K^+ addition. And compared to the control situation a lower K^+ shoot content was found. This might be an effect of osmotic stress as observed in the PEG treatment. The fact that the assumedly sole osmotic effect of the PEG treatment had a much stronger effect on K^+ content that the Cl treatment, which causes a higher osmotic pressure (Table 3), is remarkable. An explanation may be that blockage of the root surface with the PEG molecules impeded ion uptake. If the latter is the case, than it would be more advisable to use the Cl treatment to test osmotic stress effects since, in the current experiment, no additional adverse effects of Cl^- uptake have been found so far. However, an unobserved effect of the Cl treatment may for example be a decrease in NO_3^- uptake (De Wit, 1964).

In the Na treatment, decreased K^+ uptake together with increased root Ca^{2+} content was measured. Transport from root to shoot of Mg^{2+}, Ca^{2+} and Cl^- seems to have been at the expense of Na^+-counter ion transport. Due to high availability of Na^+ and its counter ions their uptake was increased. Possibly it was the tendency to maintain electrical neutrality that stimulated transport of anions towards the shoot in case of high Na^+ transport. The efficiency of K^+ translocation seems to be maintained to a bigger extend, pointing towards the importance of the Na^+/K^+ ratio for cellular functioning (Epstein, 1998, Garg et al., 2002). Still, according to ANOVA, transport from root to shoot for all ions is similarly efficient as in the control treatment and for all ions the relative decrease from root to shoot concentration is alike.

In the NaCl treatments as well as in the Na treatment, decreased K^+ uptake and reduced root-shoot transport of Mg^{2+} and Ca^{2+}, likely due to increased anion transport, were observed. Next to this, a general increase in Na^+ and Cl^- content was measured. Furthermore, for the NaCl hydroponic treatment again an increased root Ca^{2+} content was measured in comparison to the control situation.

In the control situation, where little Cl^- and Na^+ were available, Cl^- uptake was limited since high concentrations were not needed by the plant. Whereas Na^+ availability was comparable, Na^+ uptake was higher. The fact that as a result of the NaCl treatment, Cl root content was found to increase more profoundly than Na^+ root content, may be explained by the passive diffusion of excess Cl^- into the roots (Munns and James, 2003). In shoot, both Na^+ and Cl^- concentrations are similar, which means that translocation of Cl^- towards the shoot is less efficient than that of Na^+. Chloride cation co-transporters may be involved in translocation of Cl^- from endodermis to xylem (Mian et al., 2011) and may thus in be a limiting factor for Cl^- transport.

Genetic variation

Salt tolerance

As was expected (Nguyen Viet, 2012, Wolde, 2011), based on experiment 1, ST in G1 and G2 was significantly higher than in G3 and G4 (Table 5). Genotype 1, was shown to deal better with the stress effect caused by the Cl treatment than by the PEG treatment. It might be the adverse effects of PEG that cause this difference.

Furthermore, G1 and G4 were found less responsive to increased NaCl administration in experiment 2 (comparing the NaCl100 and NaCl200 treatments) (Figure 9).
Since ST is generally high in experiment 2, it should be questioned how stressful its stress treatments have actually been. If the plants have experienced little salt stress, genotypic variation with regard to ST will not show. This might explain why a different order of ST was found for the genotypes in experiment 2, where G4 was found more tolerant than G1, compared to experiment 1.
Genetic variation for ST was higher in the NaCl treatment of experiment 1 than in the NaCl treatments of experiment 2. Since the genetic variation in both experiments does not seem to be caused by a comparable amount of stress, nothing can be stated on whether genotypic differences were more prone to be revealed on hydroponics or on sand.
Previously the considered genotypes were tested on salt tolerance (Nguyen Viet, 2012, Wolde, 2011). A hydroponic study, including G2 and G4, by Nguyen Viet (2012) gave different results for ST at 200mM NaCl: 65% for G2 and 30% for G4, against 30% respectively 16% in the present study. This indicates that the hydroponic treatment in the current study has been more stressful.
A study on salt tolerance including G1 and G3 on silver sand performed by Wolde (2011), gave different result for ST at 200mM NaCl: G1's ST was 64% against 78% in the present study, and G3's ST was 38% against 60% in the present study. These differences may be explained by the difference in soil management in both experiments. In the current study, soil structure was protected by keeping the right soil moisture content and application of water via the watering tubes. In the experiment by Wolde (2011), pots filled with silver sand were surface irrigated on a daily basis. Possibly, this resulted in compaction of the soil structure and corresponding lack of aeration an hydraulic conductivity. This in turn influences plants behaviour in saline conditions; lack of oxygen is known to impede cellular metabolism (Drew, 1997) and will, in all probability, reduce ST. This might indicate that the lower ST for G3 in the experiment of Wolde (2011) was not solely caused by sensitivity to salinity stress but by the combined effect of salinity stress and root suffocation. Next to this, the regime of soil management in the current experiment allowed the addition of little amount of water every day, especially in the beginning of the experiment where transpiration was low. This caused a slow increase of salt levels in the soil solution, resulting in the slow build-up of soil salinity. In the experiment of Wolde (2011) soil salinization was probably a much faster process, resulting in a longer period of intense stress for the plants and thus a lower ST after an experimental period of three weeks.

Phenotypic characterisation
To gain more insight in the mechanisms behind ST phenotypic characterization took place. The following findings should be noted, average performance of genotypes is not mentioned:

- G1, followed by G2 showed least leaf death and A reduction in the stress treatments compared to the other genotypes. G3 was shown especially sensitive to excess Na^+ (A reduction).
- Tiller formation of G1 and G2 was less affected by the stress treatments than that of G3 and G4. G3 was found especially sensitive to the increase in [NaCl] (comparing the NaCl100 and NaCl200 treatments).
- G4's chlorophyll content reduced significantly more in the NaCl than in the Na treatment. This suggest a role of Cl^- in reduction of chlorophyll content as found by Tavakkoli et al. (2011). However, the Cl treatment did not negatively affect chlorophyll content of G4, nor any other genotype. Chlorophyll formation in new leaves of G2 and G4, over all treatments, was found to maintain relatively high and constant throughout the experiment. For both G1 and G4, no decrease in stomatal conductance was observed as a result of the PEG treatment. (Figure 15a).
- With regard to stomatal conductance, G2 outperformed the other genotypes in both the Cl and Na treatment and had a shared first place with G4 in the NaCl treatment.
  Due to little effect of the stress treatments in experiment 2, genetic variation with regard to $_{GS}$ is less revealed. But, in experiment 2, again G2 was found to maintain a relatively high level of stomatal conductance.

Based on interpretation of phenotypic characterisation of the genotypes and the phenotypic characterisation of the treatment effects the following expectation can be formulated. G1, has well developed mechanisms to deal with osmotic stress. G2 has well developed mechanisms to deal with osmotic stress as well as with high Na^+ availability. The mechanisms to withstand osmotic stress as well as Na^+ associated ionic stress are little developed in G3 compared to the other genotypes. In G4 photosynthesis is maintained but the total photosynthetic area is smaller; biomass production is reduced which results in low ST (with the current definition of ST).

Ion content per genotype

Based on the result of ion content per genotype, presumptions with regard to the mechanisms underlying the expectations above can be formulated. All differences between genotypes described in this sub-paragraph are statistically insignificant. This, however, does not mean that these findings cannot be regarded informative.

G1 & G2- tolerant

G1 had the highest Cl^- content in the Cl treatment and Na^+ en Cl^- content increased relatively strong in the NaCl treatment. In the latter treatment the Na^+/K^+ ratio of G1 was high (Figure 22), still G1 was most salt tolerant compared to the other genotypes. Most probably, Na^+ and Cl^- uptake and compartmentalization, allows osmotic adjustment and reestablishment of the Na^+/K^+ ratio in the cytoplasm, so to maintain cell functioning, resulting in salt tolerance.

G2 seems to be able to deal with relatively high Na^+/K^+ ratios. Already under control conditions, as well as in the PEG treatment the Na^+/K^+ ratio is high compared to the other genotypes. This while ST for G2 is also high in the PEG treatment. Probably, the mechanisms of Na^+ uptake and compartmentalization, allowing osmotic adjustment and reestablishment of the Na^+/K^+ ratio in the cytoplasm is very well developed in G2, better than in G1. The relatively high Cl^- content as a result of the NaCl treatments also points out the use of Cl^- uptake for osmotic adjustment, necessarily followed by compartmentalization. Furthermore, in the Na and NaCl treatment, G2 contained relatively little Na^+ and much K^+, suggesting a strategy of selective uptake of K^+ over Na^+.

G3 & G4- sensitive

G3 contained high concentrations of Na^+ in the Na treatment and Na^+ content increased relatively strong as a result of the NaCl treatment. This suggest low capacity with regard to Na^+ exclusion.

G3- and G4's Cl^- content increased less compared to the other two genotypes in response to the NaCl treatments. However, this did not result in a higher ST. Ion content of G3 and G4 reveals that high concentrations of Na^+ seem to coincide with lower ST. Increased Cl^- content does not seem to decrease ST as was observed from the Cl treatment.

Relatively low Na^+ content was measured in the Na treatment for G4, still its ST did not increase compared to G1 and G2. This implies high sensitivity of G4 to Na^+ associated stress effects. The latter together with fact that average ion content in all genotypes is similar (Table 8) and average ST in the NaCl stress treatments is lowest in G3 and G4 (Table 5), suggest that the process of internal compartmentalization of Na^+ and Cl^-, in G3 and G4, is not as well developed as in G1 and G2, leading to damaging effects of relatively low Na^+ (and Cl^-) concentrations. As internal compartmentalization turns out not the way for G3 and G4, the glycophytic strategy of reduced Na^+ uptake, selective uptake of other ions over Na^+, might be used by these genotypes to increase ST. However, the current experiment does touch upon this hypothesis.

5. CONCLUSIONS

5.1 Hydroponics versus Sand

Within this experiment was not succeeded to design comparable stress severity in the hydroponic and sand medium. This impedes comparison of ST in the different barley varieties on the different media. Average salt tolerance was found significantly higher in plants grown on sand (68% in the NaCl200 treatment) compared to hydroponically grown plants (21% in the NaCl treatment). The observed difference in ST is most probably caused by the gradual increase of salt concentrations in the sand medium that prevents plant damage and allows adaptation to the more salty root environment in experiment 2. Results obtained from experiment 2 suggest that plants have experienced little stress and thus, genetic variation with regard to ST was not revealed in this experiment.

Furthermore, the direct nutrient availability at the entire root surface in hydroponics and associated higher average root ion content in hydroponics compared to the sand medium, may have contributed to the difference in ST between both treatments. Next to this, the lack of rinseability in the sand medium allows less control over the plant available nutrient solution. In case of excess, ions accumulation may occur with the corresponding positive (in case of K^+ accumulation) or negative consequences.

5.2 Genetic variation and contributing mechanisms

G1 (IG 128216) and G2 (L94) turned out more tolerant to saline-sodic environments (NaCl-associated stress) than G3 (Volga) and G4 (Vada), where G2 showed the highest salt tolerance and G3 was least salt tolerant. Osmotic stress effects caused reduction in potential photosynthetic area and tiller formation but also reduced stomatal conductance as well as chlorophyll content. No ion specific stress effects were observed as a result of the Cl^- ion, whereas, the Na^+ ion is expected to have a negative effect on leaf formation and to additionally limit stomatal conductance and chlorophyll content.

G1 was found very well able to deal with osmotic stress. Most probably, Na^+ and Cl^- uptake and compartmentalization, allowing osmotic adjustment and reestablishment of the Na^+/K^+ ratio in the cytoplasm, provide maintenance of cellular functioning and salt tolerance.

G2 was identified with well developed mechanisms to deal with osmotic stress as well as with high Na^+ availability. G2 was found able to deal with relatively high Na^+/K^+ ratios. The mechanisms of Na^+ as well as Cl^- uptake and compartmentalization, allowing osmotic adjustment and reestablishment of the Na^+/K^+ ratio in the cytoplasm, are developed even better than in G1. Furthermore, selective uptake of K^+ over Na^+ expectedly increases ST of G2.

The mechanisms to withstand osmotic stress as well as Na^+ associated ionic stress are little developed in G3 compared to the other genotypes. In both G3 and G4, mechanisms for internal compartmentalization of Na^+ and Cl^- seem less well developed, resulting in sensitivity to Na^+. In G4 photosynthesis seems relatively well maintained but biomass production is reduced, which, with the current definition of ST, results in low ST.

5.3 Incorporation of saline-sodic stress in SWAP-WOFOST

With regard to land and water management in agriculture, it is of interest how water can be allocated most efficiently. Optimization of crop production together with minimization of water allocation and soil deterioration is the goal. In saline-sodic root environments, as was observed in the current experiment, transpiration decreases significantly. 200 mM NaCl associated stress caused a decrease in stomatal conductance of at least 50%. This highly impacts soil water uptake. Genetic variation in stomatal conductance within this experiment does not reveal a clear distinction between more tolerant and sensitive barley varieties. When incorporating the effects of saline-sodic stress in SWAP-WOFOST, based on the current findings, there is no need for differentiation between tolerant and sensitive varieties with regard to water loss due to transpiration.

The intention is to maintain crop production, and thus, the influence of a saline-sodic root environment on the plants WUE is of interest. As all plants in the NaCl treatment had the same amount of water and nutrients

available, their production of dry weight under these conditions is indicative of WUE. Dependent on the crop, above ground or below ground dry weight production may be of major interest. In case of barley, the focus is on the production of above ground biomass. ST, representing the change in WUE, was found different in more tolerant compared to sensitive genotypes. Barley is a tolerant species and here is shown that even within this species ST can vary to a great extent, meaning that differences among species might be even bigger. So, with regard to WUE, when incorporating the effects of saline-sodic stress in SWAP-WOFOST, based on the current findings, differentiation between tolerant and sensitive varieties is advisable.
Due to the unexpected lack of stress in experiment 2, it was not possible to hypothesise on the relation between NaCl concentration and water use efficiency, an interesting relationship to be incorporated in water use models.

6. RECOMMENDATIONS

To allow comparison of salinity stress on hydroponics and silver sand, it is important that the transition to stress follows the same course in both media. The addition of proportionally high salt concentration, that will be diluted with the known volume of present soil water, will speed up the process of salinization in the silver sand medium without deterioration of the soil structure. Another option is to slow down concentration development in the, highly controllable, hydroponic solution. The use of rhizons to monitor salinity of the soil solution on a regular basis, as was the intention in this experiment, allows monitoring of the actual stress severity in the sand medium. This transparency might lead to intervention.
Furthermore, to validate the assumption of strong correlation between above ground biomass production before seed development and seed formation in the end, experiments should be carried out for all genotypes.
To substantiate findings on genetic variation with regard to salt tolerance and underlying mechanisms, an expression study on expectedly involved genes in all four genotypes would be useful. Genes involved in osmotic stress resistance, for instance the gene coding for a tonoplast intrinsic protein, involved in water exchange between the cytoplasm and vacuole, is expected to be most up regulated in G1 and G2. A gene expression study on genes involved in Na^+ compartmentalization such as the gene coding for SOS2, involved in Na^+ translocation towards the vacuole should reveal high expression in G2. The expression of the genes, involved in selective uptake of K^+ over Na^+ should be highest in G2 whereas the expression of this gene in G4 and G3 should be high in comparison to less tolerant varieties or species.
Furthermore, to find the relation between salt stress and plant transpiration, transpiration should not be quantified using a porometer. By weighing, water loss from a closed basin on which plants are grown, transpiration can be monitored much more precisely.
In this research attention was merely paid to salinity stress as in stress caused by NaCl. However, high water allocation on saline soils to facilitate leaching out of salts and to decrease osmotic stress, may cause water ponding. In real life, salinity stress may thus coexist with stress due to root O_2 deficiency (Qadir et al., 2001). By the use of soil water content/crop-simulation models such as SWAP-WOFOST, water lodging and associated tress should be prevented, but still it might be worthwhile to focus research on the development of varieties with a combined tolerance to salinity stress and O_2 deficiency.
Furthermore, salinity stress is not necessarily present the entire year round. Weather conditions, seasons, are highly influential to the degree of salinity especially in coastal areas. It would be worthwhile to test the impact of the duration of stress periods as well as the frequency on ST/ WUE. Possibly short periods of stress can be overcome by the plant without intervening in land/water management.

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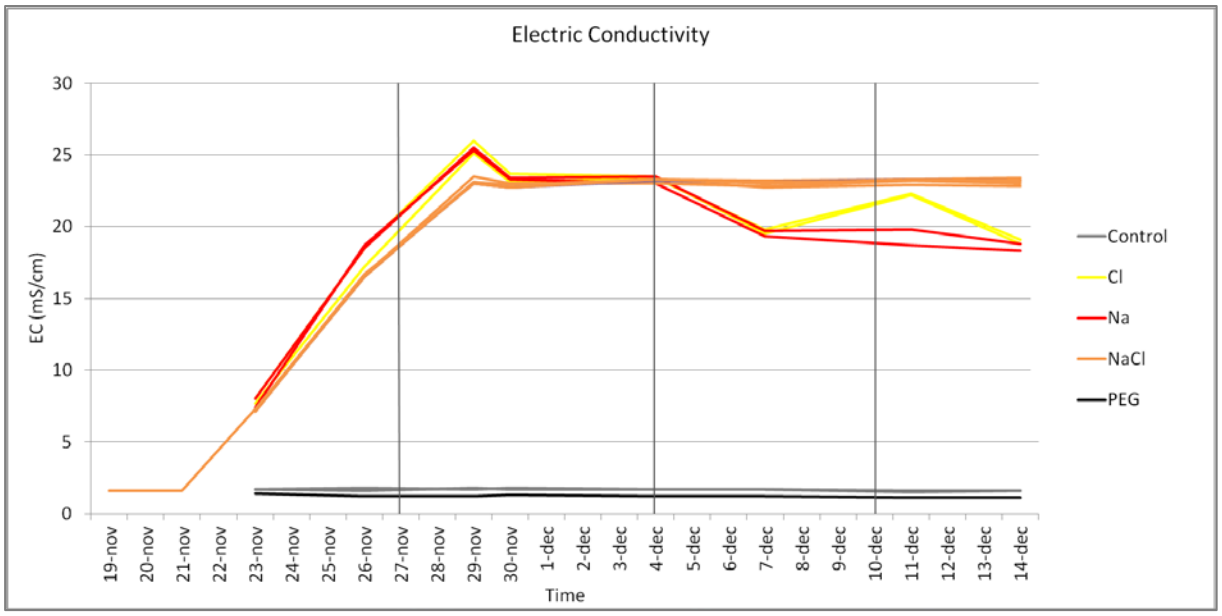
ANNEX I

Modified Hoagland solution (Lisle et al.2000) (Tavakkoli et al., 2011)(in mM):

-	ammonium nitrate:	NH4NO3	0.2	mM
-	potassium oxide:	K2O	5.5	mM
-	calcium nitrate:	Ca(NO3)2	2	mM
-	magnesium nitrate:	Mg(NO3)2	1	mM
-	Magnesium sulfate	MgSO3	1	mM
-	Nitric acid	HNO3	2	mM
-	Phosphoric acid:	H3PO4	1	mM
-	Iron (III) (from Argifeed Fe-extra 3%*)	Fe-DTPA/EDDHSA	0.025	mM
-	boric acid:	Mg2BO3	0.0437	mM
-	manganese (II) chloride:	MnSO4	0.0118	mM
-	zinc sulfate:	ZnSO4	0.00175	mM
-	copper (II) sulfate:	CuSO4	0.000125	mM
-	sodium molybdate:	Na2Mo4	0.00052	mM

** Agrifeed Fe-extra 3% contains a mixture of DTPA and 10% EDDHSA iron chelate.*

ANNEX II



Electric Conductivity of hydroponic treatment solutions throughout the experimental period. Black vertical bars at 27 Nov, 4 Dec and 10 Dec represent dates where phenotypic characterization took place; respectively one, two , three weeks after final salt concentrations were reached.

ANNEX III

Manual Ion chromatography (Metrohm)

Solvent preparation

NOTE: use always clean and dry glassware, calibrated pipettes and dispensers.

3M formic acid solution

Formic acid:

Molecular weight = 46.03 g / mol

Density: 1.22 kg/l

Purity: 99%

* 3 mol formic acid = 46.03 * 3 = 138.09 g → 3M formic acid = 138.09 g formic acid/liter milliQ.

* To change the weight into a volume: 138.09/1.22 = 113.19 *(100/99) = 114.3 ml/l to give a 3M formic acid solution

* To prepare 250ml of 3M formic acid you need 114.3*(250/1000) = 28.6 ml of formic acid / 250ml milliQ.

Anion buffer preparation

- Stock solution: add 2120mg of Na_2CO_3 and 1680 mg of $NaHCO_3$ into a 1000ml measuring glass and add 900ml milliQ and 100ml acetone (20mM Na_2CO_3, 20mM $NaHCO_3$, 10% acetone). This solution can be used for 1 month.
- The buffer concentration needed to analyze the anions is 10x diluted: 100ml stock solution + 900ml milliQ. This gives a concentration of 2mM Na2CO3, 2mM $NaHCO_3$, 1% acetone. This solution can be used for 2 weeks.

Cation buffer preparation

- Stock solution: add 2080µl of 65% HNO_3 /l into a 1000ml measuring glass containing 900ml milliQ and add 100ml acetone (30mM HNO_3, 10% acetone). This solution can be used for 1 month.
- The buffer concentration needed to analyze the cations is 10x diluted: 100ml stock solution + 900ml milliQ. This gives a concentration of 3mM HNO_3, 1% acetone. This solution can be used for 2 weeks.

Regeneration solvent 100mM H_2SO_4 (cation system)

- Add 5.56 ml 96% H_2SO_4 /l into a 1000ml measuring glass containing already some milliQ water, add 10 ml acetone and refill to 1000ml volume with milliQ.
- Put the solvent into the empty, clean and dry flask belonging to the apparatus.

Sample preparation

NOTE: first prepare and analyze a few samples to optimize the sample amount and time in the ashing oven.

- Weigh 25-50 mg of dry and grinded sample in a screw cap tube.
- Put the tubes in the ashing oven for a minimum of 5h at a maximum temperature of 575ºC.

NOTE: for the next steps it is important to calibrate the pipettes and dispensers before use.

- After cooling down the ashed samples add 1 ml of 3M formic acid and shake the capped tubes at 99ºC for 15min and allow to cool down.

- Look at the dissolvability of the sample:

→ if the ash is dissolved add 9 ml of milliQ water (10x dilution) and mix the solution properly. Put 100µl from this sample into 9.9ml milliQ (the sample is now 1000x diluted) and mix properly.

→ if the solvability is poor add 9ml of milliQ and put it in the shaker for 30min at 80ºC. When the ash is dissolved put 100µl from this sample into 9.9ml milliQ water (the sample is now 1000x diluted) and mix properly. When the ash is not dissolved ask Annemarie what to do in this case.

Calibration standard preparation

NOTE: 1ppm = 1mg/l = 1µg/ml

Anions: Chloride (Cl^-), Sulfate (SO_4^{2-}), Phosphate (PO_4^{2-})

It is also possible to analyze fluoride, bromide and nitrate.

Cations: Sodium (Na^+), Potassium (K^+), Magnesium (Mg^{2+}), Calcium (Ca^{2+}) It is also possible to analyze lithium.

The software recognizes the following standards:

Standard 1 → 0.5 ppm cations	Standard 6 → 0.5 ppm anions
Standard 2 → 1 ppm cations	Standard 7 → 1 ppm anions
Standard 3 → 2 ppm cations	Standard 8 → 2 ppm anions
Standard 4 → 4 ppm cations	Standard 9 → 4 ppm anions

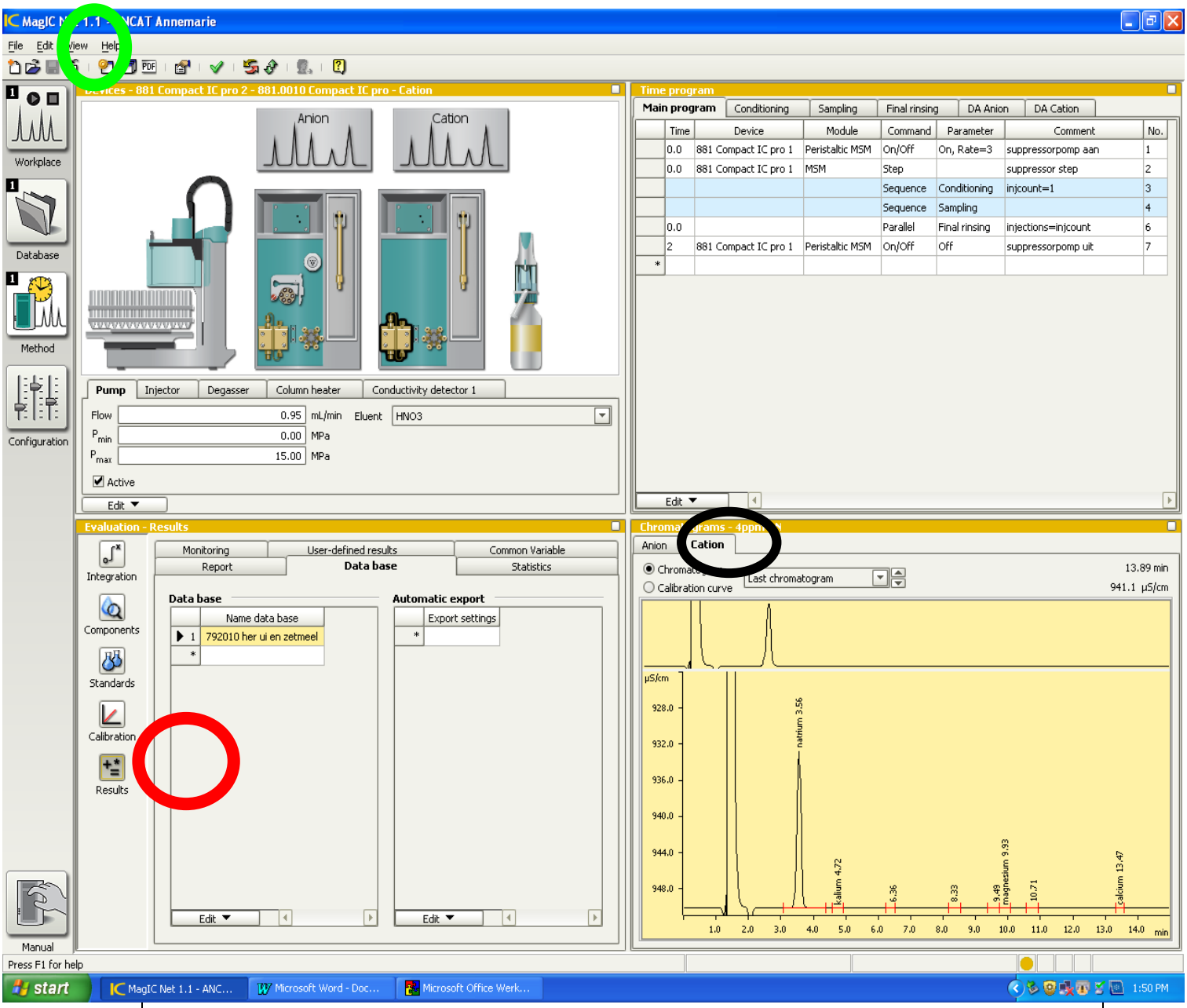
- Prepare the standards needed to quantify the ions of your interest.
- The stock solutions available are 10mg/ kg = 10ppm. To make standard 1: dilute 0.5ml *cation* stock solution with 9.5ml milliQ. For standard 2: dilute 1ml *cation* stock solution with 9ml milliQ, standard 8: 2ml *anion* stock solution with 8ml milliQ…etc. mix properly.
- Prepare also a blank (1ml 3M formic acid and 9 ml milliQ → 100 µl in 9.9ml milliQ).

Ion chromatography Metrohm

Starting up the system

NOTE: Before starting the system make sure every buffer, rinsing fluid etc. is completely filled and the waste bottles are empty (sample holder + big can beside apparatus)

- Start the pumps by pressing the switch at the back of the pumps nearby the electric cord.
- Put on the computer
- Start Magic Net 1.1
- Computer and apparatus are connected when the parameters of the system are shown.
- If you changed the buffer flasks you need to get rid of the air bubbles in the system by purging the system (ask Annemarie to show how to do this).
- Go to DATABASE → file → database manager → edit → new → *'name your new database (give a unique name)'* (delete any other databases) → OK → OK → close
- Go to METHOD → file → open → ANCAT Annemarie→ open → *see print screen below:*
- Click on results (red marked), go to database (black marked)→ edit → delete → new → select your previous named database and go to file (green marked) → save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Microsoft Office Werk...
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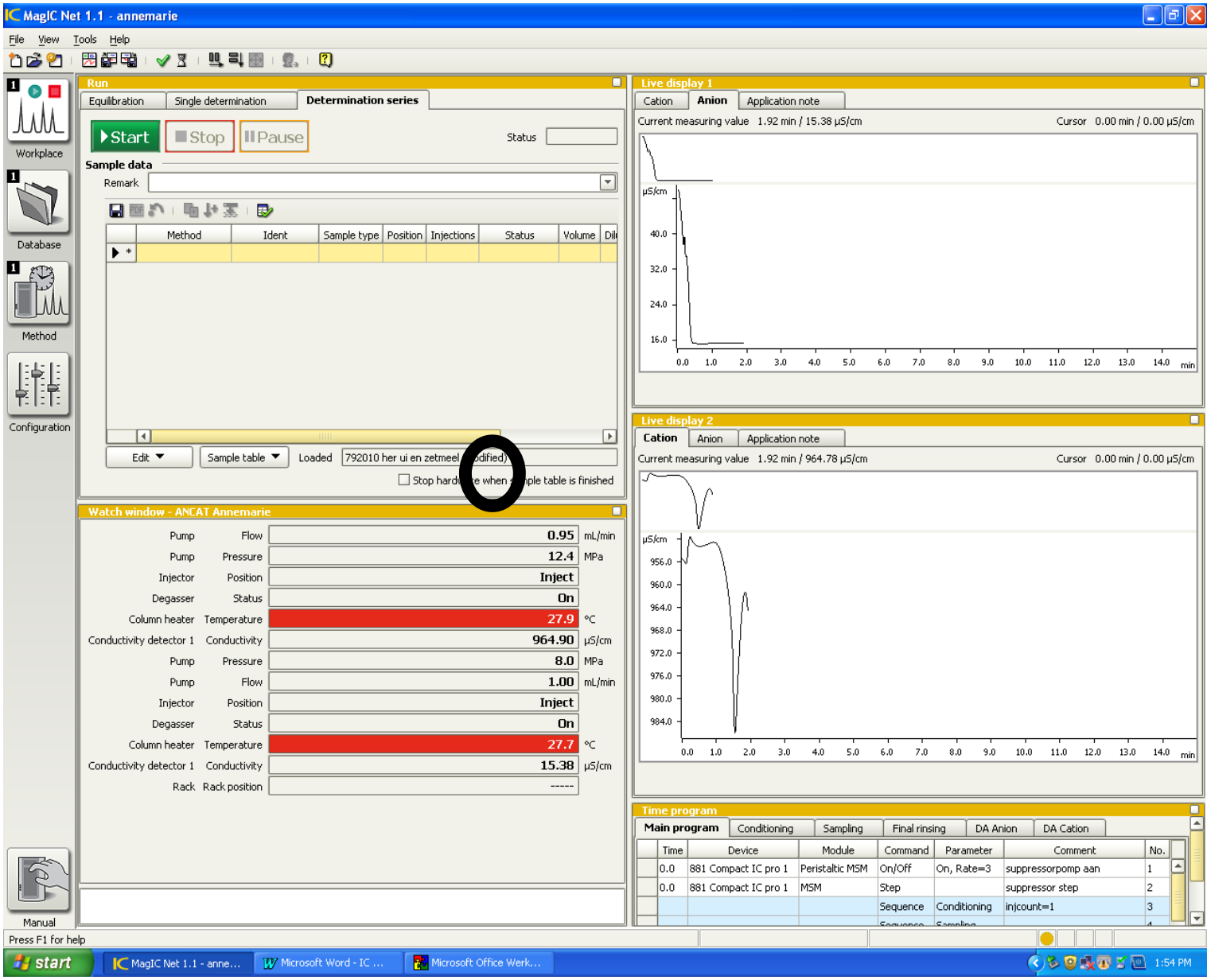
- Go to WORKPLACE → *see print screen below:*



- Click on determination series → sample table → new
- Dubbel click with the left mouse button on the empty line → a small window appears.
- Fill in:

Method: select the correct method (ANCAT Annemarie)
Ident: "*name of the sample*"
Sample type: Sample. Do not select the sample type "blank" but give a blank the type SAMPLE!
Position: 1,2,3..etc. Make sure you give the right position of the sample in the sample holder.
Injections: 1
Volume: 20µl
Dilution: 1
Sample amount: 1
Info 1: leave empty

→ press apply

If you want to move to the next sample press the buttons (below in small window) to move to the next/previous line.

In general: Start the serie with a run of the blank, the first run of a serie is always a bad one. After the blank the system is stabilized and the system can start the analyses of a standard containing your ions of interest to check if all the peaks are detected and recognized correctly. If this is not the case it is best that you adjust the retention times before all the samples are analyzed.

When you finish the serie it is very important to activate the function: Stop hardware when sample table is finished (black marked in print screen above). After this you can go to sample table → Save as *"give a name"*

NOTE: each injection consumes 2ml of sample so if you have 10ml solution in the tube you can inject 4x (needle is not going to the lowest point of the tube).

Before starting the serie let the system equilibrate. This can be done by pressing the green START button on the tab 'equilibrate' (so NOT in the tab 'determination series'). The system is ready when the baselines are flat. Press the red STOP button.

To start the serie go to determination serie en press the green START button.

MOST IMPORTANT: **Check in time if there is enough solvent and empty in time the waste bottles or else the system will be extremely damaged!**

Results

To check the results you go to Database → file → open→ select the correct database

When the serie has ended check the results in the database: most important is that every peak is recognized. If not follow these instructions:

- Select the sample(s) you need to adjust or

- Click on Reprocess (green marked in print screen below)
- Go to local disk (C) → Magic results → your file → copy to external hard disk

1: If there has been a shift in retention time for a peak which is causing the software to not recognizing the compound →

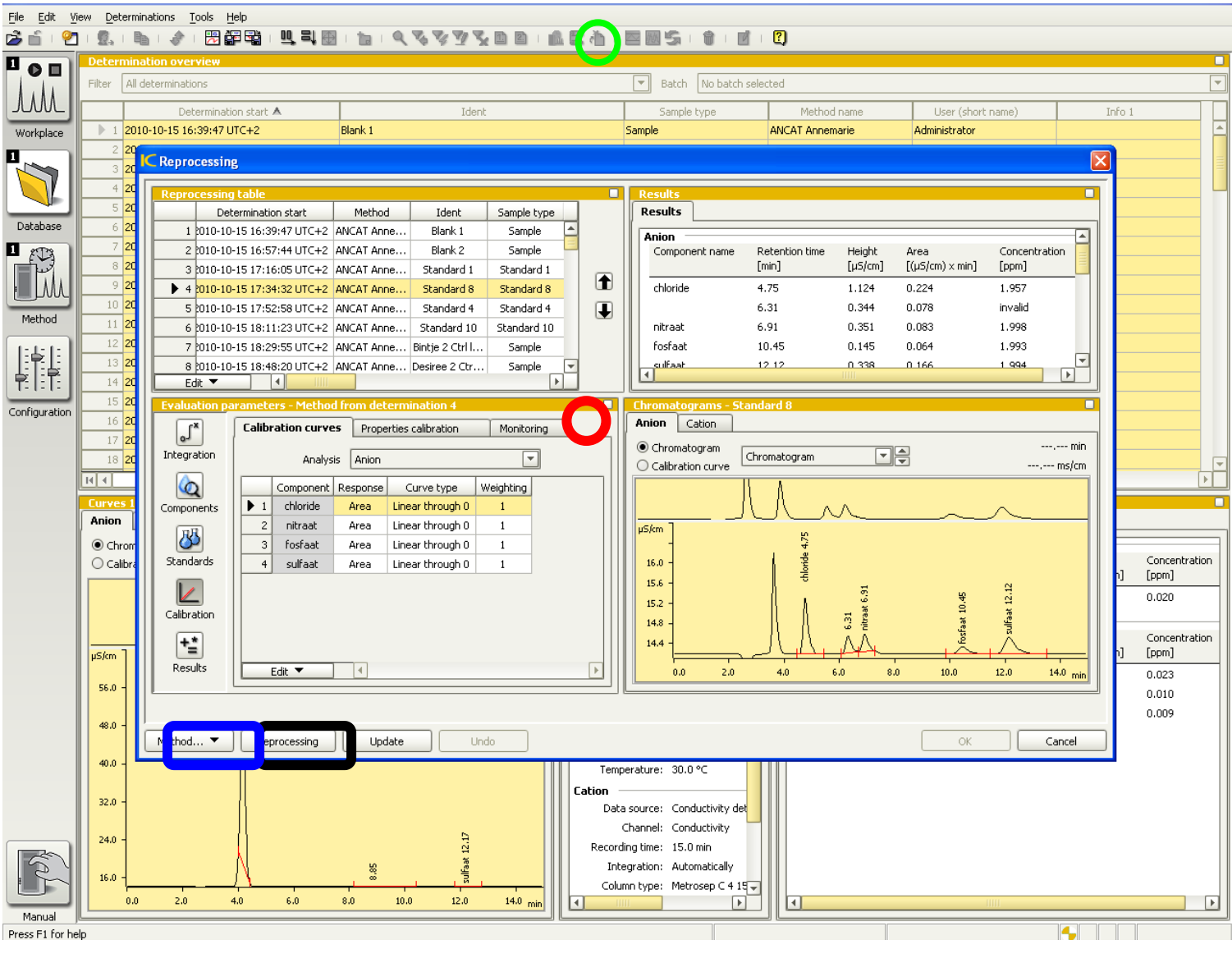
- Click on Components in the window Evaluation Parameters (bottom left). If you need to adjust a cation select this in the line Analysis or else use anions. Activate the line containing your ion of interest.
- Go to the window Chromatograms (bottom right). You can enlarge these windows by using the square in the upper right corner.
- Adjust the blue line to the maximum height of the peak using the right mouse button.
- Click on Update Retention Time in the Evaluation Parameters window.
- Click on Update (black in print screen)
- Click on Reprocess (bleu in print screen) and select the option: From selected determination.

The reprocessing will take some time. When it is finished check if the adjustments you made are implemented in all the data measured in this serie.

- If everything is oke → click OK

Export results

- Tools → Export templates → retention time + peak area
- Choose Properties
- Choose Target directory (1st time make a new directory)
- Choose File type *CSV, Comma Separated



- Check under options → Field separator: **,** Record Separator: **CR/LF**
- File name: give a name
- OK → OK → Close
- Go to Edit →select all
- Go to Determinations → Export → all selected data records
- Export template → conc. + peak area

Calculations

1ppm = 1mg/litre = 1µg/ml

So if the concentration is 1 ppm = 1ug/ml --> 1*10 (ml) = 10ug/10ml , this came out of 100µl which came out of 10 ml: 10*100 = 1000µg/10ml.

1000µg came out of the *x* mg (ashed) sample. So 1000µg / *x* mg sample = concentration in µg/mg.

A Reprocessing window appears.

ANNEX IV

Statistical background unbalanced ANOVA
(Citation from GenStat help function)

"The ANOVA directive analyses balanced designs. These include most of the commonly occurring experimental designs such as randomized bocks, Latin squares, split plots and other orthogonal designs, as well as designs with balanced confounding, like balanced lattices and balanced incomplete blocks. Many partially balanced designs can also be handled, so a very wide range of designs can be analysed. The necessary condition of *first-order balance* is explained algorithmically by Wilkinson (1970) and Payne & Wilkinson (1976), and mathematically by James & Wilkinson (1971) and Payne & Tobias (1992). However, ANOVA can itself detect whether or not a design can be analysed, so if you are not sure whether or not a particular design is analysable, you can run it through ANOVA and see what happens! (If it is unbalanced, you can use the AUNBALANCED procedure for designs with a single error term, or the REML[1] directive for those with several.)

Unbalanced ANOVA
The unballanced ANOVA menu allows you to fit completely general models to unbalanced data. The analysis of variance is carried out using the regression facilities in GenStat. The method takes account of any blocking structure, however, it cannot produce stratified analyses, and is able to estimate treatments and covariates only in the "bottom stratum". So, for example, the full analysis can be produced for a randomized block design, where the treatments are all estimated on the plots within blocks, but it cannot produce the whole-plot analysis in a split plot design.

Unbalanced analysis of variance can also be found on the General Analysis of Variance menu by selecting the Unbalanced Treatment Structure design.

Y-Variate
This should be set to the variate containing the data values.

Treatment structure
The treatment terms to be fitted are specified by entering a model formula.

Blocking (Nuisance Terms)
Blocking, or nuisance, terms to be removed before fitting treatments.

Factorial limit on treatment terms
Controls the factorial limit to be used for treatment terms to be fitted.

Available Data
This lists data structures appropriate to the current input field. It lists either factors for use in specifying the treatment structure, or variates for specifying the data and additional covariates. The contents will change as you move from one field to the next. Double-click on a name to copy it to the current input field; alternatively, you can type the name directly into the input field.

Operators
This provides a quick way of entering operators in the treatment model formula. Double-click on the required symbol to copy it to the current input field. You can also type in operators directly. See model formula for a description of each.

Covariates
Select the check box if you want to perform analysis of covariance. This produces an additional field in which you can list the covariates for the analysis. If there is more than one, their names must be separated by spaces or commas."

References

James, A.T. & Wilkinson, G.N. (1971). Factorisation of the residual operator and canonical decomposition of non-orthogonal factors in analysis of variance. *Biometrika*, **58**, 279-294.

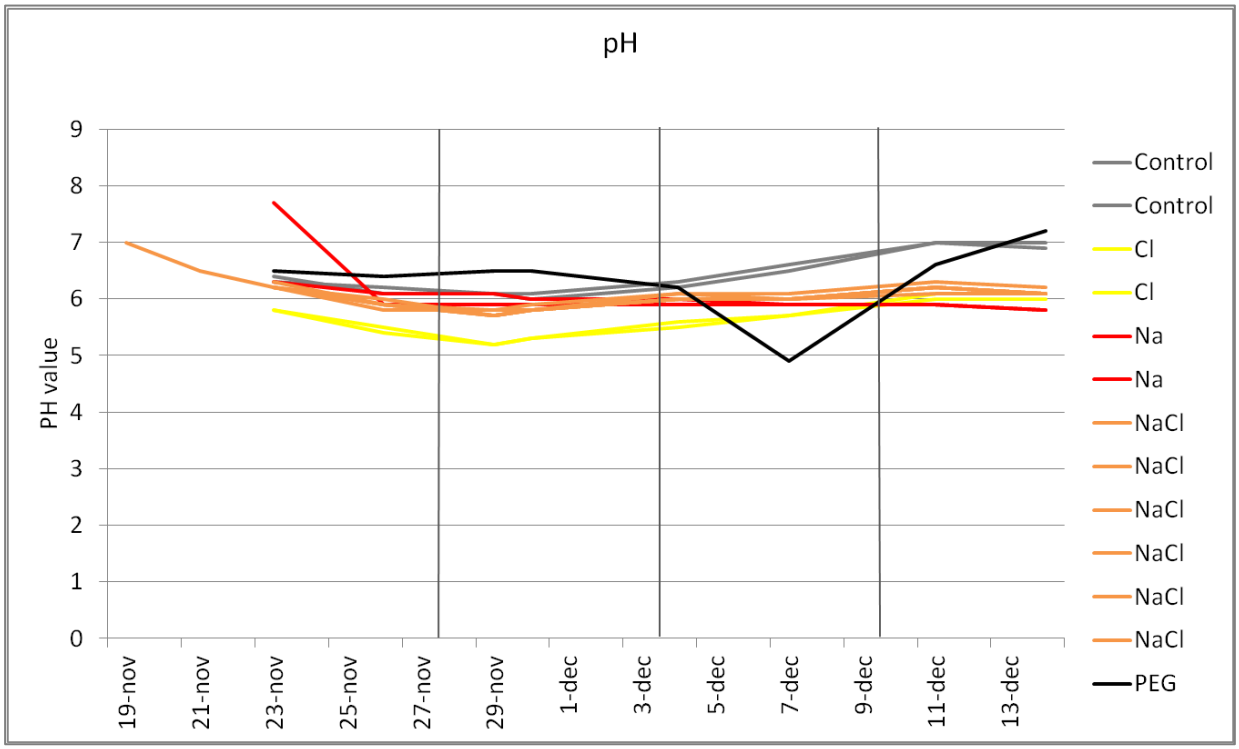
Payne, R.W. & Wilkinson, G.N. (1977). A general algorithm for analysis of variance. *Applied Statistics*, **26**, 251-260.

Payne, R.W. & Tobias, R.D. (1992). General balance, combination of information and the analysis of covariance. *Scandinavian Journal of Statistics*, **19**, 3-23.

Wilkinson, G.N. (1970). A general recursive algorithm for analysis of variance. *Biometrika*, **57**, 19-46.

[1]The method of residual maximum likelihood (REML) was introduced by Patterson & Thompson (1971).

ANNEX V



pH values of hydroponic treatment solutions throughout the experimental period. Black vertical bars at 27 Nov, 4 Dec and 10 Dec represent dates where phenotypic characterization took place; respectively one, two , three weeks after final salt concentrations were reached.

ANNEX VI

Experiment 1: Analysis of an unbalanced design using GenStat regression

Variate: Salt Tolerance					
Change	d.f.	s.s.	m.s.	v.r.	F pr.
replicate	5	20848.5	4169.7	12.53	<.001
genotype	3	6641.5	2213.8	6.65	<.001
treatment	4	108954.1	27238.5	81.87	<.001
genotype.treatment	12	3190.3	265.9	0.80	0.651
Residual	128	42586.2	332.7		
Total	152	182220.6	1198.8		

Experiment 2: Analysis of variance

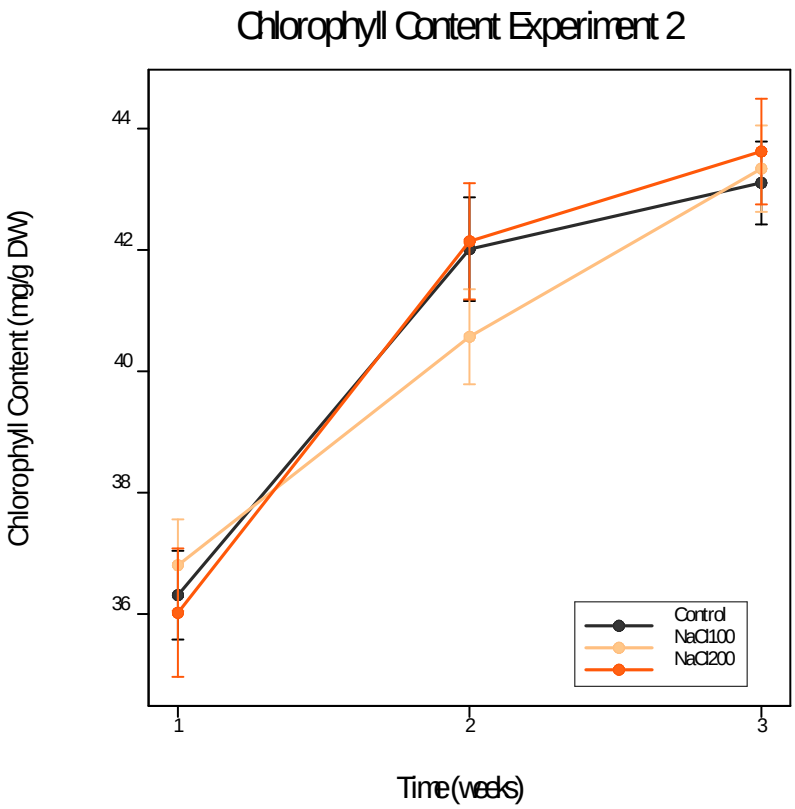
Variate: Salt Tolerance					
Change	d.f.	s.s.	m.s.	v.r.	F pr.
replicate	3	641.7	213.9	0.85	0.471
genotype	3	2184.3	728.1	2.89	0.040
treatment	1	6566.3	6566.3	26.03	<.001
genotype.treatment	3	689.1	229.7	0.91	0.439
Residual	85	21442.2	252.3		
Total	95	31523.6	331.8		

Experiment 2: Chlorophyll content, summary table

	Nobservd	Mean	s.d.
treatment			
Control	48	40.48	4.229
NaCl100	48	40.24	3.995
NaCl200	48	40.60	5.037

Experiment 2: Chlorophyll content, analysis of variance

Variate: Chlorophyll Content					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
replicate stratum	15	320.19	21.35	1.09	
replicate.*Units* stratum					
treatment	2	3.1	1.58	0.08	0.922
Residual	126	2462.91	19.55		
Total	143	2786.27			



(genotype, treatment, time)- Graph