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Water and Solute Relations of Salt stressed Wheat and Annual Suaeda maritima

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Water and Solute Relations of Salt stressed Wheat and Annual *Suaeda maritima*

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Dedication

To my family and to all those who taught me.

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Abstract

World agriculture is challenged by the increasing demand on diminishing resources of fresh water. Climate change and salinity are both issues, resulting in a major international research effort to breed more resistant crops. The aim of this thesis is to contribute to this effort by determining how wheat varieties respond to salt stress.

This study describes an analysis of three wheat varieties (Gamina and Bohoth 105, from Libya and of reported salt tolerance, and Hereward) representing different parts of a salt tolerance spectrum. The effect of induced salt stress on leaf growth rate and final length was investigated by analysing water and solute relations at single cell resolution, using Single Cell Sampling and Analysis (SiCSA). The parameters for the wheat were compared with those of the halophyte (salt tolerant plant) Sea Blight (*Suaeda maritima*), in order to determine which wheat variety showed the most similar response.

All four plants showed rapid cell osmotic adjustment (leaf and root) under salt stress, and leaf turgor pressure was at control levels by 48 hours after salt stress in all wheat varieties. However, varietal differences were evident in terms of cation activity, cortical cell osmotic adjustment, and root turgor regulation, with Bohoth 105 most resembling *Suaeda* in each.

An unexpected observation for wheat leaf cells was a continued increase in turgor pressure after control values had been reached at 48 h post stress, which was not accompanied by an increase in osmotic pressure. It is suggested that the epidermal apoplast played a turgor-control role, as suggested by earlier work at the Bangor laboratory. Through measuring cell volumetric modulus (ε_{cell}), it was also found that increased elasticity may be associated with increased salt tolerance, but no such association was indicated for hydraulic conductivity (*Lp*) or leaf plastochron index (PI).

In conclusion, Bohoth 105 possesses water and solute-related responses that contribute to its relative salt tolerance. This may inform future breeding of more salt tolerant wheat varieties, or indeed any crop.

Contents

Abbreviations

ANOVA Analysis of Variance

CZE Capillary zone electrophoresis

FAO Food and Agriculture Organization of the United Nation

Ha Hectare

IFAD International Fund for Agriculture Development

IPCC Intergovernmental Panel on Climate Change

CIMMYT International Maize and Wheat Improvement Center

L1 Leaf 1 (old leaf)

L2 Leaf 2 (middle leaf)

L3 Leaf 3 (young leaf)

L4 Leaf 4

L5 Leaf 5

L_p Cell membrane hydraulic conductivity

l Cell length

ΔP Changes in cell turgor

P_{cell} Cell turgor pressure

P_{wall} Apoplast hydrostatic pressure

PI Plastochron index

r Cell radius

SiCSA Cell pressure probe, associated cell sampling system and analysis

$T_{1/2}$ Relaxation half time

USDA United State Department of Agriculture

ΔV	Change in cell volume
γ	Osmotic coefficient
π_{plant}	Bulk osmotic pressure
π_{cell}	Cell osmotic pressure
π_{wall}	Apoplast osmotic pressure
Ψ_{cell}	Cell water potential
Ψ_{ext}	External water potential
$\varepsilon_{cell(i)}$	Cell instantaneous volumetric elastic modulus
$\varepsilon_{cell(s)}$	Cell stationary volumetric elastic modulus

Figures

Tables

Plates

Chapter 1: Introduction and Literature Review

1.1 Statistics

1.1.1 The Global Context

The availability of fresh water is very important for humankind. Agriculture is considered to be the sector that is the largest consumer of water. Worldwide agriculture used approximately 2.7 x 10^3 km^3 of water in 2000 (WRI, 2005), and about 276 million ha of crops are irrigated with water (FAOSTAT, 2006). According to the FAO (2002) and Pimentel *et al*. (2004), 1 m^3 of fresh water is required for producing 1 kg of wheat. For 1 kg of rice, at least 1.2 m^3 of water is needed. Water is necessary for plants to complete their life cycle. Generally, over 90 % of a plant's water requirement is lost by transpiration (Morison *et al*., 2008).

Globally, the area of land being irrigated is still increasing year on year, yet at a gradually slowing rate. From 1990 to 1994, the increase in the area of irrigated land worldwide was 4.2 million ha per year, but between 1999 and 2003 the annual increase had slowed to only 1.1 million ha (FAOSTAT, 2006). According to FAOSTAT (2006), in 2003 18 % of land worldwide was used for cropping, yet from this land, a significant 40-45% of the world's food was produced (Döll & Siebert, 2002). For this reason, the world's food supply would clearly be affected by a decline in irrigated area. Conversely lowered agricultural demand for water use would lead to less water consumed; thus maintaining water resources over a longer period of time. In North Africa, Southern Africa and the Near East, where under 2000 m^3 of quality water is available per person per year, the three most important

agriculture problems are insufficient rainfall, high evaporation rate and continuous population increase (Wallace, 2000; FAO 2003).

Fresh water demand is still increasing day-by-day due to the growth of the human population, as well as to increases in the number of other users (such as animals and plants). In many arid and semi-arid zones, the quality and quantity of the water supply is limited as the result of high competition between agriculture land extension and industry. In addition, salinisation and contamination of water resources, such as surface water and ground water, are serious problems (Khan *et al.*, 2006).

Climate predictions for the next 50 years forecast a 1^0C to 2.5^0C increase in the annual mean temperature, and the increase may even prove to be greater (IPCC, 2007). This may lead to an increase in evapotranspiration in warmer regions. In addition, precipitation patterns in temperate environments are also changing, with milder, wetter winters and warmer, drier summers being predicted for many parts of Europe (IPCC, 2007). Moreover, Danielopol *et al.* (2003) show that increasing salinisation of coastal aquifers results from rises in sea-level. All of these challenges add substantially to the strong pressures placed upon water resources, resulting in an urgent need to focus on improved water use efficiency and development of crop varieties that are adaptive to drought and salinity.

The Mediterranean climatic regions suffer from water supply problems, which restrict plant productivity. Irrigation is one of the main problems causing salinity in many parts of the Mediterranean (Turner, 2004). Soil or water salinity (or both) one pervasive threats to arid and semi-arid regions throughout the world, in that salinity substantially hampers crop growth and productivity. The FAO (2008a) reports that, taking into account both saline and sodic soils, at least 800 million ha across the globe are affected by salt. This is approximately 6 % of the world's total land area. It

is estimated that, currently, 45 million ha of agricultural land out of 230 million under irrigation (20 %), is affected by salinity, and that 32 million ha (out of 1500 million ha (2 %)) of dry land agriculture is similarly affected (FAO, 2008a). Generally, it is estimated that more than 3 ha of agricultural land around the world is lost every minute due to soil salinity (FAO, 2008a). At present, about 1.3 billion people have no access to sufficient supplies of fresh water, and 2 billion have no access to suitable sanitation. Some countries in the Middle East and North and Southern Africa have been experiencing severe water shortages and this is expected continue until at least 2025 (Rijsberman, 2006).

During a plant's life cycle, its growth rate and development can be affected by salinity at any time. The severity of the impact depends on the degree of the salinity, the damage it causes and the differences between various plants and their developmental stages. Many researchers have reported that wheat growth and production are affected by irrigation water and soil salinity. Feizi *et al.* (2007) and Maas and Poss (1989) show that, in cereal crops, the flowering and the grain filling stages are less salt sensitive but that the vegetative and early reproductive stages are less tolerant of salinity, indicating that, different growth stages respond differently to salt stress. Zeng *et al.* (2002) have also found that some plant species, such as rice, are salt tolerant at different growth stages.

Salinity affects the physiology of plants at the organ and cellular level, through both osmotic and ionic stress (Murphy *et al*., 2003). High Na^+ accumulation in the leaf apoplast leads to a decrease in P_{cell} and dehydration, and finally to death of tissue cells (Marschner, 1995). This in turn leads to changes in the activity of several enzymes, and metabolism is thus affected (Lacerda *et al*., 2003).

Salt stress enhances the accumulation of NaCl in chloroplasts of higher plants, affects growth rate, and is often associated with decrease in photosynthetic electron transport activities (Kirst, 1990). In higher plants, salt stress promotes the accumulation of Na^+ in chloroplasts. Salinity results in a reduction of K^+ and Ca^{2+} concentrations, and an increased level of Na^+ and Cl^-, which forms its ionic effects. Impaired productivity can result, and wheat and cotton yields have been found to be reduced in both saline and sodic soil (Murtaza *et al*., 2006). In general, then, salt stress can be said to affect the uptake and subsequent transport of both water and solutes in a plant, leading to changes to the plant's morphology through adaptation and/or impaired metabolism.

1.1.2. Libya

In this study, the country of Libya will be used as an example of the problems facing agriculture in an environment of water stress. This is the author's home country and will be the geographical context for the focus of the present research.

Libya is a country in the southern Mediterranean area, on the North coast of Africa, and has 1900 km of shoreline. The majority of Libyans live in Tripoli the capital and Benghazi. At least 80% of agricultural land is situated in the eastern and western coastal regions (Sadeg & Karahanoglu, 2001). Around 2.15 million ha of Libyan agricultural and permanent pasture land is near the coastal areas. This is because most of Libya is desert or semi desert (FAO, 2005). Annual crops are cultivated on about 1.82 million ha and permanent crops on about 0.34 million ha. This represents 1.2 % of the total area (FAO, 2005). There are many problems for the country's farming, such as low water quality and low soil fertility. As a result, not enough cereal can be produced to meet local demand. In 2002 a total of 253,050 metric tons was produced from 381,300 ha (FAO, 2005).

The primary cereals grown in Libya are maize, wheat and barley, but for the latter two, yields are lower than in other countries in the Mediterranean. It has been suggested from research at International Maize and Wheat Improvement Center (CIMMYT) that salinity affects between eight and ten percent of wheat crop areas in the country (Kazi & Leon, 2002). According to the FAO (2008b), in Libya the production of wheat was 27,189 metric tons in 1970; over a period of 10 years it increased to 140,500 metric tons by 1980, but then steadily decreased to 104,000 metric tons by 2007 (Fig. 1.1).

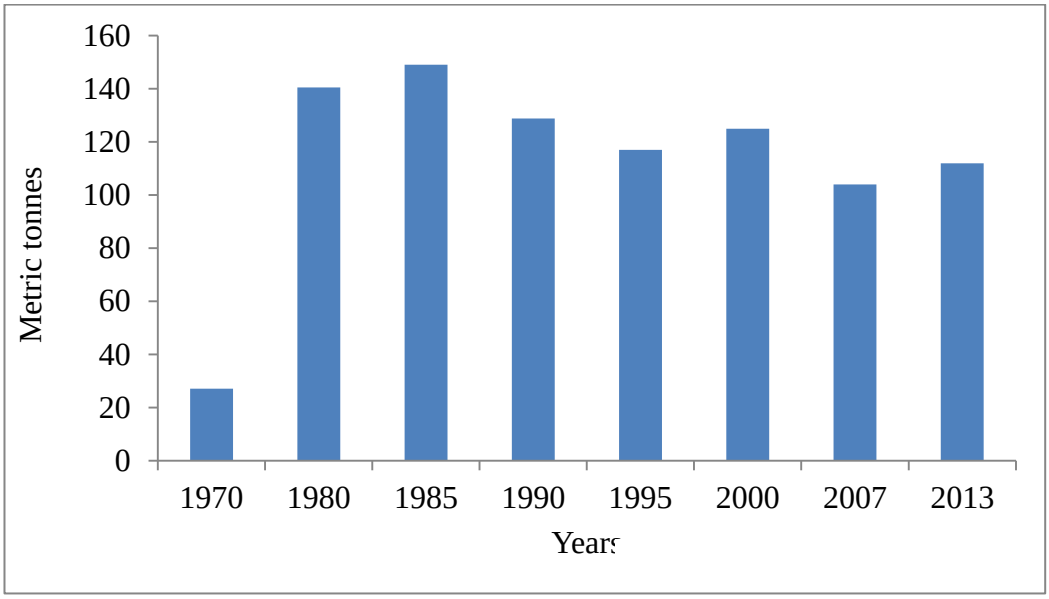

Fig. 1.1 Wheat production in Libya (FAO, 2008b)

Since the oil revolution, there has been rapid development in many activities, including agriculture, and maintaining the supply of fresh water along with managing salt water pollution have become major issues. As a result of increased agriculture, water quality has decreased because of sea water contamination. For example, in 2001 about 250 km^2 of the country suffered sea water intrusion, affecting fresh water supplies 10 km inland from Tripoli (Fig. 1.2, Sadeg & Karahanoglu, 2001). It is estimated that water demand in Libya is about 4.3×10^9 m^3 per year (FOA, 2005).

The country does not have a plan for how to meet rising future demand and so there is increased pressure on aquifers. However, many aquifers are salt contaminated so this water cannot be used. New ways in which agriculture can be advanced in such

salt-contaminated areas are currently being researched, including studies into salt-tolerant crop varieties. The present study is an attempt to contribute to the advancement of knowledge in this area, with the aim of helping to improve Libyan agriculture.

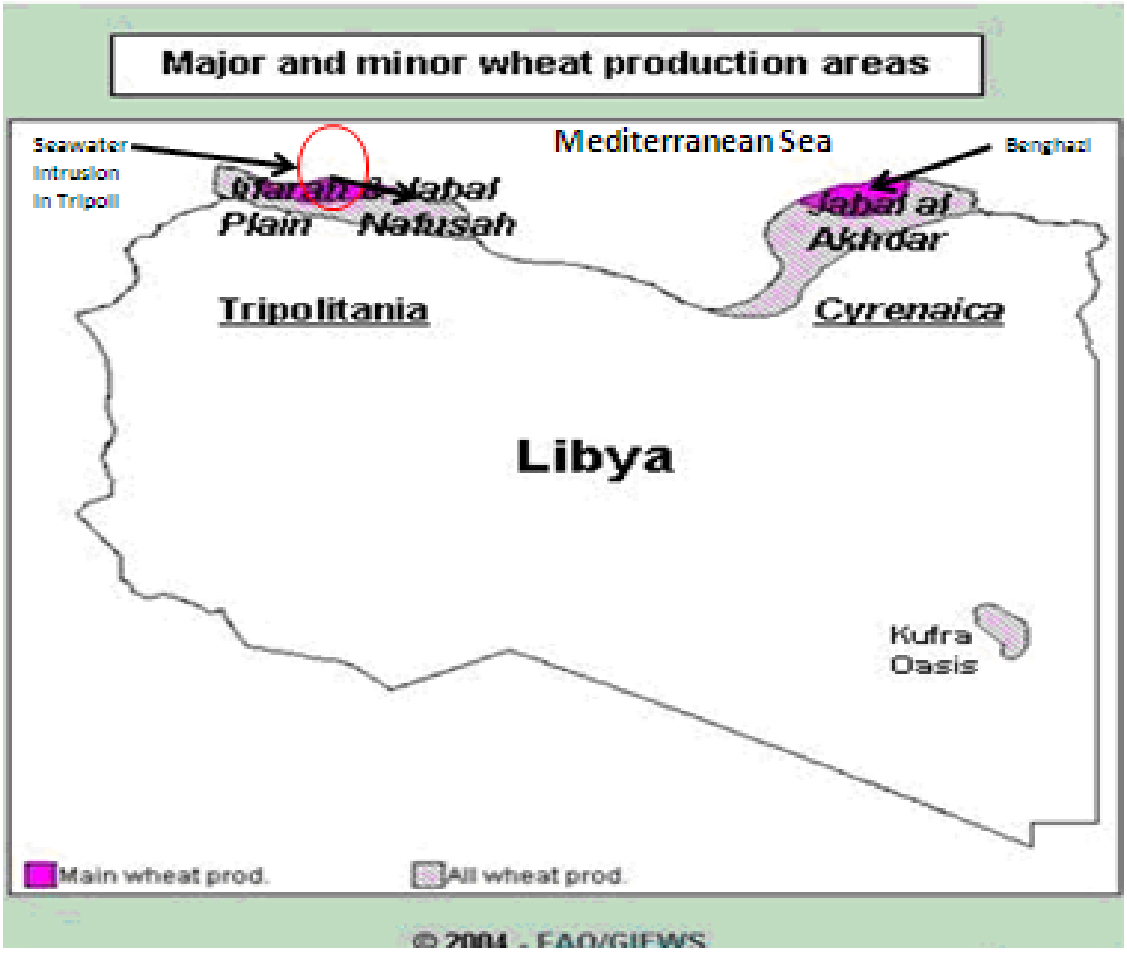

Fig. 1.2 Libyan wheat production and seawater intrusion around Tripoli (USDA, 2004). (http://www.fas.usda.gov/pecad/highlights/2004/05/libya/index.htm.)

1.2 Salt Stress

1.2.1 Why is NaCl damaging?

The majority of crop plants are considered to be glycophytes (they grow better at low NaCl levels) and are sensitive to high levels of salt (Xiong & Zhu, 2002). Cereal is

an example of this type of salt-sensitive glycophyte[1]. Wheat is a moderately salt tolerant crop species; nonetheless it is less tolerant than grain barley and sorghum (Maas, 1986) and is adversely influenced by decreased soil water potential.

Saqib *et al.* (2004) found that salt stress caused a reduction in gas exchange and photosynthesis in wheat, and affected the process of cell division and cell elongation. These changes contributed to a loss of turgor, and thus impaired the wheat's growth. In salt sensitive glycophytes such as wheat, salinity causes general a reduction in growth, both vegetative and reproductive (Zhe *et al*., 2004). However, the strategies used by plants to ensure that glycophyte plant crops survive and produce under different levels (low to moderate: Ec 4 – 10 mmhos cm^{-1})[2] of salinity is an important factor (Shannon, 1997).

Salinity affects the growth of all plants, but for grain crops, an increase in salinity of irrigation resources results in a decrease in grain yield (Cakmak *et al*., 2005; Ragab *et al*., 2008)**.**

Salt in the root medium stunts plant growth in the following ways. In the first place, water stress, or an osmotic effect, is created, and this results in a decrease in the external ψ_{ext} due to a high concentration of NaCl in the root medium of the plants and, consequently, limitation of water uptake by roots (Oliveira, 2013). This then causes a rapid decrease in growth rate, and several metabolic processes are also affected (Epstein, 1980), as are leaf growth, photosynthesis and stomatal conductance (West *et al*., 1986).

[1] Glycophyte plants exhibit various degrees of harm and decreased growth in the presence of Na^+ salt typically higher than 0.01 % of salts in the soil (Dajic, 2006).

[2] Figures related to maximum salt concentration that can be tolerated and to relative yield reduction. Plant crops can be further divided into sensitive, moderately sensitive, moderately tolerant and tolerant species (Maas, 1985).

Secondly, increasing amounts of salt entering the transpiration stream cause specific ion toxicities, the result being the harming of the cells in the transpiring leaves. This too may result in a reduction in the growth of the plant. Specific toxicity of Na^+ has been found for wheat (Kingsbury & Epstein, 1986). Sodium and chloride accumulated in leaf cells up to toxic concentrations hamper the processes of metabolism in cytoplasm and reduce the growth and development of wheat (Francois & Maas, 1993).

Thirdly, accumulation of specific ions under salt stress leads to disturbance in ionic ratios in cells. Salt stress thus induces nutritional imbalance (Hu & Schmidhalter, 2005). The Na^+ and K^+ are found to be antagonistic in uptake (Jeschke & Nassery, 1981). Salinity is therefore clearly a major issue which limits the growth and reproduction of glycophyte plants.

Under salt stress, since, a non-halophyte's leaves grow more slowly, this then increases the root:shoot ratio because root growth is less affected (Munns & Termaat, 1986). This reduced growth and increased root:shoot ratio can be seen in different varieties of salt stressed wheat, for example Hereward (Brooks, 2006), and cultivars-cv. Barkai (Botella *et al.,* 1997) and Durhum (Carillo *et al.,* 2005).

In contrast, Halophytes naturally grow well in saline environments, and absorb relatively high levels of salts (mainly Na^+ and Cl^-) from the root medium (Greenway and Munns, 1980) to achieve osmotic adjustment.[3] In the halophyte *S. maritima* (sea Blight), Flowers and Colmer (2008) have found Cl^- and Na^+ concentrations of 400 mM in leaves without any injury.

[3] An increase in π_{cell} results in a decrease in ψ_{ext} while maintaining $P_{cell.}$ When plants grown under non salt stress are suddenly exposed to a relatively high osmotic pressure in their rooting media, osmotic adjustment occurs in a short period of time (Bernstein, 1963). A higher π_{cell} can be achieved in three ways: by uptake of solutes absorbed from root media (such as Na^+, Cl^- and K^+), by synthesis of compatible organic solutes (such as sugars, amino acids) or by a loss of water (partial dehydration).

Salinity affects germination in both halophytes and glycophytes however (Pujol *et al*., 2000), although, in many halophytes, NaCl only induces seed dormancy (Debes *et al*., 2004), in glycophytes, seeds may become unviable under high salt concentrations. It has also been found that salinity can provoke seed dormancy in both types of plant (Jhamb & Sen, 1984).

The responses of halophytes and glycophytes to salt stress differ, depending on the degree of salinity. Daoud (2008) reports that under low external Ψ_{ext}, halophyte plants are able to complete their life cycle in solutions with concentrations of NaCl ranging between 100 mM and 200 mM. Studies demonstrate that halophytes grown in a saline environment accumulate levels of NaCl in the vacuole that differ in ionic compartmentation as compared to glycophytes grown in the same conditions (Shabala, 2013).

The ability of halophytes to control the influx of Na^+ into their roots is considered to be a significant mechanism for achieving lower Na^+ accumulation under salt stress in comparison with glycophyte plants (Wang *et al.*, 2006). In other words, many halophytes are Na^+ includes, whereas glycophytes are often Na^+ excluders (or K^+ includers). Halophytes transport the absorbed Na^+ to the xylem, whilst this process is limited in glycophytes, for example in *Plantago maritima* (Kuiper, 1988). In glycophytes, it is partially the accumulation of NaCl in leaf apoplast under salt stress that causes growth to be impaired, as has been reported for rice (Flowers *et al*., 1991).

It is important for excess ions to be compartmentalised within the cell because otherwise they may accumulate in the cell wall (apoplast). If this happens, the concentration in the cell wall would rise disproportionately because of its small volume (5 % of the volume of the protoplast) and relatively low water content (30 -

35 %). This concentration gradient between the apoplast and the protoplast would then lead to cell imbalance and cause water to be drawn from the protoplast by osmosis to correct the imbalance. This would cause the cell to lose P_{cell} and experience water loss or dehydration, a situation known as Oertli's hypothesis (Oertli, 1968).

The rapid accumulation of salt in the apoplast or cytoplasm that occurs when the ability of the vacuole to accommodate salt influx has been reached may result in older leaves dying (Munns, 1993). Such a process has been observed in corn when exposed to elevated Na^+ concentrations (Fortmeier & Schubert, 1995), and can be seen as a form of Na^+ toxicity in leaves (Volkmar *et al.*, 1998). Salt stress may also stimulate plasma membrane ATPase, similarly resulting in leaf apoplast Na^+ accumulation (Niu *et al.*, 1995).

Even in some halophytes, the accumulation that may occur as a result of excess Na^+ and/or Cl^- in the cell wall can lead to a loss of P_{cell} and dehydration (e.g. Clipson *et al.*, 1985). Conversely, ion toxicity may result in cases in which excessive ions are held within the protoplast but not well compartmentalised. There is evidently a balance to be struck in regulating ion transport to the shoot, particularly because excessive exclusion of ions may negatively affect osmotic adjustment.

1.2.2 Salt Tolerance of Plants

Numerous crops either possess mechanisms for excluding Na^+ from their tissue cells, or tolerate the Na^+ accumulated within their cells. Parida and Das (2005) consider that plants have developed the ability to regulate ion transport into their cells in order to protect the actively growing region and the metabolising plant cells. Salinity can result in ionic imbalance (Munns & Tester, 2008), with excess Na^+ and Cl^- ions both having a toxic effect on tissue cells (Serrano *et al.*, 1999).

To understand salt tolerance mechanisms, it is important to understand the behaviour of naturally salt-tolerant plants, i.e. those that have adaptations that allow them to survive in saline environments. For example, most halophyte plants remain relatively unaffected, in terms of growth, when exposed to 200 mM NaCl (Flowers, 2008). This suggests that there is an important potential here for learning how to raise the salt tolerance of plants. Arzani (2008) notes that in numerous crop species, salt tolerance is considered to operate at the cell level. For example, halophytes are thought to have certain cellular mechanisms for salt tolerance. During salt stress, cell wall properties are responsible for the maintaining of cell growth (Iraki *et al.*, 1989) and could be vital to salt tolerance (Seaman, 2004).

The process of growth in a plant is closely related to positive P_{cell} and water uptake. When the cell wall prepares for growth, turgor falls and so cell water potential also decreases, which in turn causes an influx of water across the plasma membrane to revive the cell's turgor. By this process, though, the cell's solutes are diluted and must be restored by more ions being taken into the cell or synthesised. Certain parameters influence the speed at which the cell volume increases, which combine to give the wall yielding property of the cell: $r = \phi (P - Y)$, where r is the growth rate, ϕ is the wall plastic extensibility (how easily cells undergo irreversible expansion), and Y is the yield threshold (critical value of minimum turgor before the wall can expand) (Lockhart, 1965).

Under salt stress, P_{cell}, cell wall extensibility, yield threshold or hydraulic conductance can be affected, so plants must either regulate their turgor (P) or adjust wall properties (ϕ and/or Y) to avoid a detrimental effect on growth. It has been noted that in the unicellular microalga *Chlorella emersonii* L, only partial turgor regulation occurs when the plant is exposed to high salt stress (Munns *et al.*, 1983).

Begg and Turner (1976) have reported that osmotic adjustment happens in crops exposed to water deficit or salinity conditions. This process increases the osmotic pressure (π_{cell}) in the protoplasts of the crop plants by the accumulation of solutes (mainly Na^+, Cl^- and K^+), and the synthesis of organic solutes (amino acids and sugars). This increases their P_{cell}, enabling them to maintain stomatal conductance and leaf growth under salt stress conditions.

Under saline conditions, plants have developed different mechanisms to adjust osmotically to the large decrease in ψ_{ext}, for example by controlling the transport of Na^+ at the plasma membrane of the root cells and xylem Na^+ loading; by Na^+ recovery from the xylem; by extrusion of Na^+ from the root; by Na^+ intracellular compartmentation (vacuoles); and by excretion of Na^+ via salt glands (Zhang *et al.*, 2010). Seaman (2004) has noted that glycophytes have low selectivity of root membrane for Na^+, Cl^- and Ca^{2+}. The only way that leaf vacuole compartmentalisation of Na^+ and Cl^- can be achieved is by their active transport into the vacuole. The tonoplast permeability to these ions is kept sufficiently low to maintain the gradients of ion concentration at an energy cost that can be continued for a significant amount of time (Seaman, 2004). Patch clamp investigations have shown that when unselective slow vacuolar ion channels open, this can result in higher plants' tonoplast conductance increasing greatly (Maathius & Prins, 1990). However, the conductance of Na^+ and Cl^- through the slow vacuolar channels must not be so great that it exceeds the ability of active transport to maintain the NaCl gradient, otherwise subsequent 'leakage' could lead to plant death (Maathius *et al.*, 1992).

Glycophytes control the movement of ions which are toxic, such as Na^+, into root cells and root xylem, in order to minimise their uptake from the soil and prevent their

transport into the shoot. The ability to do this is greater in glycophytes with higher salt tolerance, and these are also better able to maintain high K^+ levels (e.g. Flowers & Hajibagheri, 2001). Allowing K^+ influx into root cells is important as it promotes Na^+ exclusion, so the two are related. Therefore K^+ uptake helps to minimise Na^+ being taken up and distributed within the plant (Jeschke, 1972). A low Na^+:K^+ ratio can be achieved by selectivity for K^+ rather than Na^+, both in the root and in the xylem, and also by intracellular compartmentalisation of toxic ions in the cell vacuole.

Plant growth, in some cases (Luan *et al*., 2009), may match ion accumulation to maintain a balanced gradient, whilst in other species secretion is necessary, via salt glands, to remove excessive ion concentrations (Drennan & Pammenter, 1982). Since high Na^+ levels present in the cytoplasm are toxic, Na^+ has to be thus either compartmentalised in vacuoles or exported. The latter can be achieved via salt glands, as mentioned above, or bladder hairs. It can also occur through the cuticle or in the guttation fluid, or via phloem transport. Salt glands tend to be found on many aerial areas of the plant, especially the leaves, and are on, or recessed in the epidermis. Using the phloem, the plant can translocate problem ions to mature leaves, but this may be detrimental to young leaves as these are phloem sinks while they are still nascent. Alternatively, some plants have special adaptations that allow for efficient salt excretion, namely salt glands and salt hairs (bladders), whose presence is indicated by a salt crust visible on leaves and shoots. These mechanisms can effectively conduit salt out of the plant, to reduce the build-up of toxic concentrations (Popp, 1995).

However, every one of these activities has high energy costs (Zhang *et al*., 2010). ATP is needed to transport the ions through the plasma membrane and the tonoplast

(Seaman, 2004) due to an ATPase-activity-derived proton motive force. A Na^+/H^+ antiport results in the secondary active transport of Na^+ from tissue cells. For example, in plants the Na^+/H^+ antiport has been noted at both plasma membranes and the tonoplast (Fig. 1.3) (Mansour *et al.*, 2003). Apse and Blumwald (2002) found that in glycophytes and halophytes, Na^+/H^+ exchange activity is increased under low external Ψ_{ext}. After H^+ pumps have created a proton gradient, Na^+/H^+ antiporters, which are integral transport proteins, use the gradient to effect trans-membrane Na^+/H^+ exchange (Dupont, 1992 and Leigh, 1997).

Using Na^+/H^+ antiporters to transport Na^+ across the tonoplast into the vacuole from the cytoplasm is a key feature of salt tolerance. The antiports are driven by the H-electrochemical potential gradient that is mainly created by H-ATPase and V-PPase (Blumwlad, 2000) (Fig 1.3). Such antiport activity at the tonoplast has been seen in *Plantago maritima*, a salt tolerant species, but is not evident in salt sensitive *Plantago media* (Staal *et al*., 1991) or barley (Garbarino *et al*., 1988)

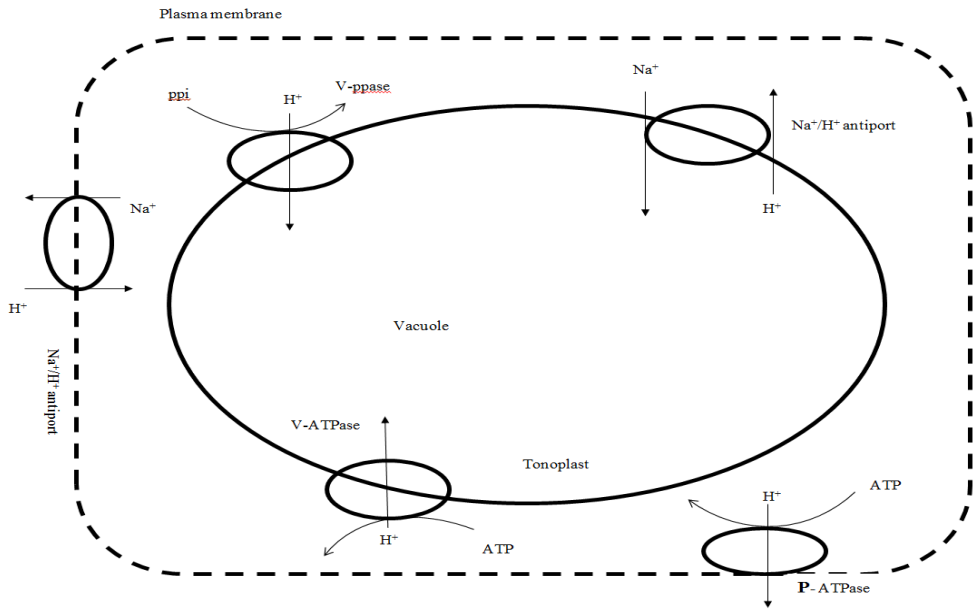

Fig. 1.3 Simple diagram representing "proton pumps and antiporters of plasma membrane and tonoplast. The primary active P-ATPase energizes the plasma membrane for secondary active transport of Na^+ by Na^+/H^+ antiport to the apoplast, and the primary active V-ATPase and V-PPase energize the tonoplast for secondary active transport of Na^+ by Na^+/H^+ into the vacuole" (Mansour *et al*., 2003).

In short, growth depends on a balance between K^+ and the highly toxic Na^+, and is promoted by high K^+ and low Na^+ (Luan *et al.*, 2009). Maintenance of a high ratio of K^+:Na^+ in the protoplast requires Na^+ extrusion and/or the compartmentalisation (mainly in the vacuole) of Na^+ (Blumwald, 2000). Using the roots to exclude salt is anther mechanism to make sure that sodium concentrations in leaves do not become toxic (Munns & Tester, 2008). Salt tolerant plants use a variety of such mechanisms.

1.3 Drought Tolerance in Plants

Water stress can be defined as decreased availability of water. This can be created either by a scarcity of water, i.e. drought, and/or by osmotic stress caused by elevated concentrations of salt. The effects of water stress include decreased photosynthesis, reduced respiration and uptake of ions, impaired metabolism, altered patterns of growth, and may even cause the death of the plant itself (Jaleel *et al.*, 2009).

Generally, plants respond to low $\psi_{ext,}$ by ensuring that tissue ψ_{cell} and water content are kept above a minimum level (similar to the unstressed level) by increasing water uptake or by the limiting of the evapotranspiration process in order that the evapotranspiration rate and water uptake remain balanced. This occurs quickly due to stomatal closure (Verslues *et al.*, 2006). Increases in the ratio of root:shoot, the tissue's water storage capacity, cuticle thickness and water conductance, together with changes in root and shoot growth, can be crucial as a longer term response (Verslues *et al.*, 2006), but for crops, it is root growth that represents the most important adaptation, in order to improve water uptake into the plant. Subjecting seedlings to low water potential (ψ_w) often results in preferential growth of roots rather than shoots, indicating that this is functioning as a salt stress response (Sharp *et al.*, 1989; Spollen *et al*, 1993). Changes in root cell wall polysaccharide

biosynthesis have been seen in wheat cultivars of varied drought tolerance upon exposure to water stress (Piro *et al*., 2003).

Moore *et al*. (2008) found that during dry spells, a number of plants under water deficit survive as drought-tolerant seeds ('orthodox' seeds) or spores, and complete their growth and development upon enough water becoming available. Another plant mechanism for avoiding water deficit (such as found in eucalypts) is to keep hydrated via extensive root systems by locating deep water sources (Moore *et al*., 2008). Moore *et al*. (2008) found that some vegetative tissues can survive without adverse effects under various stages of drought. Indeed drought resistance, to varying degrees, can be seen in the vegetative tissue of certain crop cultivars and genotypes, such as those of wheat and maize. This has been achieved through a range of adaptive mechanisms to minimise water stress damage, including water use efficiency strategies and osmotic adjustment processes (Clifford *et al.*, 1998; Iljin, 1957).

The cell wall also plays a significant part. It is a structure that causes a vulnerability to osmotic imbalance and plasmolysis, and therefore protoplasm damage. Maintaining correct P_{cell} is thus vital, as it is the mechanism that the plant cell uses in growth and division (Brett & Waldron, 1996). Turgor pressure prompts elastic (reversible) and plastic (irreversible/viscous) stretching. This in turn leads to cell growth (viscoelastic growth) (*ibid*). Osmotic adjustment results in P_{cell} maintenance and thus minimizes the physiological influence of water stress (Morgan, 1980).

Another aspect of plant adaption to environmental variation concerns the plant's stomata (Zeiger *et al*., 1987). It is transpiration through the stomata that is the driving force for uptake and transport of water from root to shoot. Open stomata are also able to take in CO_2. In times of drought, closing stomata minimises water loss, albeit at a

cost of cutting off CO_2 uptake, and allows the plant to regulate water stress. However, repeated exposure to water stress causes a change in stomatal sensitivity, making them more sensitive to indicators that prompt closure, and vice versa (Aasamaa & Sober, 2011).

Many examples exist showing how manipulation of the stomatal behaviour can contribute to cell performance under drought and related high-temperatures (Morison *et al*., 2008). Zeiger and colleagues, among others (Lu & Zeiger 1994 and Radin *et al*., 1994), found elevated stomatal conductance in high-yield Pima cotton cultivars, which had the effect of reducing leaf and canopy temperature. There is also a link between stomatal opening regulation and ion and water transport via channel proteins in the membranes of the plasma and the vacuole (Kim *et al*., 2010). The way in which the cells respond to the decrease in the ψ_{ext} during periods of water deficit dictates the amount of water loss. The reducing of P_{cell} and loss of water and π_{cell} until a new balance is set when cells are turgid is the most usual outcome.

1.4 Osmotic Adjustment

In response to salt stress, plants are able to decrease their cell ψ_{cell} and maintain positive P_{cell}, which is necessary to their continued growth (Tester & Davenport, 2003). They can also, at the same time, increase water uptake by accumulating solutes in their cells. Studying plants under water stress in the field, Acevedo *et al.* (1979) found increased π_{cell} in maize roots to maintain turgor. A similar observation was made by Michelena & Boyer (1982) in growing zones of maize leaves. Partial or complete turgor maintenance following salt stress has been studied in several species; some using indirect measurement for example in wheat (Termaat *et al*., 1985), *Acer pseudoplatanus* (Pennarun & Maillot, 1988), and barley leaves (Munns & Passioura, 1984). Other studies have used the direct pressure probe technique to

measure P_{cell} (2.5 - 3 bar), for example in *S. maritima* leaves (Clipson *et al*., 1985) and barley leaves (Thiel *et al.*, 1988).

Turgor regulation in tissue cells has been studied in marine algae by Zimmermann (1978) and Kirst (1990), in brackish or euryhaline algae by Okazaki (1996), and in *Spirogyra* cells in fresh water by Iwata (2001). Frensch & Hsiao (1994) have reported that even minor osmotic stress leads to rapid turgor regulation. Shabala & Lew (2002) report that in epidermal cells of Arabidopsis, turgor recovery is completed within 40 to 50 minutes, through the accumulation of Na^+, Cl^-, and K^+, with osmotic adjustment occurring within 2 to 10 minutes after recovery is started. Turgor regulation is achieved in marine algae by either absorbing solutes from external media or by organic solute synthesis (Kirst, 1990) and this is also the case in higher plants (Serrano, 1996). Osmotic adjustment is regarded as a significant mechanism for plants adapting to salt stress, because it is essential to the maintenance of the P_{cell} and the volume of cells. Chen and Jian-Guo (2010) have observed that Na^+, K^+, Cl^-, and Ca^{2+} contribute to osmotic adjustment. Similarly, Flowers and Colmer (2008) have found that the uptake of solutes (predominately Na^+, Cl^- and K^+) contributes to osmotic adjustment in the shoot, and leads to the uptake of water.

Orsini *et al.* (2011) report that inorganic solutes have an important role in osmotic adjustment. They suggest that K^+ contributes to osmotic adjustment by accumulating in tissue cells rather than being excreted, and thus plays an active and important role in leaf cell osmotic adjustment. Cuin and Shabala (2005) report that osmotic adjustment could be achieved by contributing osmolytes indirectly by regulating K^+ influx across the plasma membrane, and hence controlling NaCl-induced efflux of

these cations.[4] Turgor regulation enables complete turgor recovery through osmotic adjustment within 24 hours in single-cells of maize roots (measured using the pressure probe) by using organic solutes rather than K^+ and Cl^- (Pritchard *et al.*, 1996). In whole roots (grown in 200 mM mannitol), both turgor and growth recover fully (Pritchard and Tomos, 1993). Bisson and Kirst (1980) report that turgor regulation is achieved through the uptake of K^+ and Cl^- in vacuoles of *Lamprothamnium apulosum*. Under osmotic stress, turgor recovery can be completed in mung beans (*Vigna radiate*) in six hours (Itoh *et al*., 1987). Hoffmann and Bisson (1990) have found that following both hypotonic and hypertonic stress, turgor regulation is completed within two days and between five to seven day, respectively in euryhaline giant-celled algae (*Chara buckellii*).

1.4.1 Physical Osmotic Adjustment

Volumetric elastic modulus is an important physiological parameter, particularly in our understanding of the responses of plant cells and tissues to water stress and salt stress (Patakas & Noitsakis, 1997; Tomos, 2000) to maintain P_{cell} (Tomos, 1988).

It has been seen in species of wheat, olive and bean (Patakas & Noitsakis, 1997; Marshall *et al*., 1999) that increased elasticity of the cell wall may be related to the plant's ability to maintain P_{cell} or symplast volume in response to water stress. Plants may also respond to dehydration by adjusting cell wall elasticity to shrink and thus reduce turgor-loss volume, in order to maintain P_{cell} and avoid a decrease in Ψ_w (Marshall *et al*., 1999). When P_{cell} decreases, this results in the plant making physiological and biochemical adjustments to correct it. Osmotic and elastic adjustments are thus important mechanisms (Schulte, 1992) since they contribute to

[4] Ion flux and membrane potential were measured for 3-4 day old seedlings at the root elongation zone (around 3mm from tip). Noninvasive microelectrode ion flux measurements (MIFE technique) were taken of net fluxes of Na^+, K^+, H^+ and Ca^{2+}.

the maintenance of P_{cell} at lower tissue $\psi_{w.}$ In this way, plasma membranes are protected from mechanical damage.

Osmotic adjustment involves actively accumulating cell solutes, which results in an increase in π_{cell} and encourages the absorption of water. Elastic adjustment occurs when the cell wall is made more elastic so that it can contract more readily in response to dehydration (Fan *et al*., 1994). Cell wall elasticity renders cells more resistant to temporary changes in water stress, meaning that water loss will not affect P_{cell} to the same extent and turgor will be more readily maintained (Zimmermann, 1978). For plants with lower ability to effect osmotic adjustment, tissue elasticity is of particular importance (Pallardy, 2008).

The value of ε_{cell} is an important parameter, and one which plays a major role in the relative proportion of P_{cell} and π_{cell} changes in plants subject to external medium perturbations i.e. in the π_{cell} process. It is also crucial to the regulation of water balance in cells (Philip, 1958). Cell volume and shape, P_{cell}, and wall structure all affect the value of ε_{cell} (Steudle *et al*., 1977; 1982). There is considerable evidence that ε_{cell} may be the first parameter influencing P_{cell} and osmotic adjustment under plant control. In addition, Zimmermann (1978) reported that ε_{cell} plays a significant part in the regulation of P_{cell} and the processes of extension growth.

To estimate the volumetric elastic modulus (ε_{cell}), it is necessary to accurately measure instantaneous changes in cell volume ΔV and cell pressure ΔP (Zimmermann 1978;Tomos & Leigh 1999). Apparatus used in estimating the value of ε_{cell} has been improved by the development of the cell pressure probe, which allows a wider range of accuracy in the measurement of instantaneous changes in volume and pressure (Hüsken *et al*., 1978; Pritchard *et al*., 1989; Steudle, 1993; Tomos & Leigh, 1999; Tomos, 2000). This technique is not only used to measure

P_{cell} to an accuracy of approximately 0.05 bar in tissue cells, but can also be used to manipulate cell volume, allowing the calculation of ε_{cell} from the following equation (1.1) (Zimmermann & Steudle, 1978).

$$\varepsilon_{\text{cell}} = V\frac{dP}{dv} \approx V\frac{\Delta P}{\Delta v} \qquad (1.1)$$

This definition of ε_{cell} indicates the proportional change in cell volume (ΔV), related to the change in P_{cell} (ΔP), and describes the slope of volume / P_{cell} curves of the cell plant (Zimmermann & Steudle, 1978; Tomos, 1988).

This has facilitated the measurement of ε_{cell} in the single cells of a range of higher plants (Tomos *et al*., 1981; Cosgrove, 1985; Shackel, 1987; and Pritchard *et al.*, 1987) and in giant algal cells (Steudle & Zimmermann, 1971; Frey *et al*., 1988).

Zimmermann (1978) showed that P_{cell} strongly affects the ε_{cell} of the cell wall, the former being often a linear function of P_{cell} at low pressure range but constant at a high pressure range. The ε_{cell} can be seen as a function of both P_{cell} and cell volume, and in a positive relationship with both (Hüsken *et al*., 1978, Zimmermann and Steudle 1975, Steudle and Zimmermann 1974, Steudle *et al*., 1977; and Zimmermann *et al*., 1976). Zimmermann and Steudle (1978) predicted a decrease in ε_{cell} with an increase in cell size in *V. utricularis* and *M. crystallinum*, these being more spherical cells. High values of ε_{cell} show a rigid cell wall or low extensibility, meaning that large changes in pressure lead to small changes in cell volume, whilst low values of ε_{cell} indicate greater cell wall extensibility.

The instantaneous ε_{cell} (short–term elastic measurement) parameter ($\varepsilon_{cell(i)}$) is used to demonstrate rapid measurements of changes in pressure related to changes to volume (within 2 - 4 sec), while the stationary ε_{cell} (long-term elastic measurement) parameter ($\varepsilon_{cell(s)}$) is used to determine changes over a longer period (Zimmermann & Hüsken, 1980). Cosgrove and Steudle (1981) studied ε_{cell} in pea (*Pisum sativum* L.)

by using a pressure probe. The value varied from 12 - 200 bar (P_{cell} range 5 - 9 bar) for epidermal cells and from 6 - 215 bar (P_{cell} range 5 - 11 bar) for cortical cells. Hüsken *et al*. (1978) studied the water relations of tissue cells of *Capsicum annuum.* They found a volumetric elastic modulus value of between 8 - 25 bar, with ε_{cell} increasing with increasing P_{cell}, as well as a higher value in large cells.

Attention has been focused on the role of ε_{cell} under osmotic stress conditions. According to Gerdenitsch (1979), in the single celled alga *Eremosphaera viridis,* the ε_{cell} values vary between 82 - 499 bar, related to pressure/volume curves. Frey *et al*. (1988) found similar results in the same alga cell, with ε_{cell} increasing in response to osmotic stress. In higher plants, Steudle and Zimmermann (1977) reported that ε_{cell} ranged between 5 - 100 bar (P_{cell} varied from 0 to 3 - 4 bar) for *Crystallinum* bladders epidermal cells, and Steudle (1980) found that in single cells of *Kalanchoe daigremontiana,* the ε_{cell} values varied between 12 - 128 bar (P_{cell} varied 0 to 3.4 bar).

1.4.2 Biochemical Osmotic Adjustment

Biochemical osmotic adjustment is an active process in response to a change in water potential, involving transporting or metabolically generating cell solutes to recover either the original π_{cell} or the original P_{cell} (Tomos, 1988). Tomos (1988) describes the two types of active response as follows:

1) Recovery of initial π_{cell} by changing the P_{cell} to match the change in ψ_w, i.e. osmoregulation

2) Recovery of initial P_{cell} by changing the π_{cell} to match the change in the ψ_w, i.e. osmotic adjustment

1.4.2.1 Cell Wall/ Apoplast

The apoplast plays an important role in cell water relationships, and to maintain turgor and facilitates turgor regulation, cells may be able to control apoplast ions activity (Leigh & Tomos, 1983; Clipson *et al.*, 1985; Tomos & Wyn Jones, 1988; Tomos, 1988; Bell and Leigh, 1996). In beet taproot tissue, Turgor regulation is vital as high P_{cell} inhibits accumulation of sucrose (Wyse *et al*. (1986); Bell & Leigh 1996). Turgor regulation in tissue of sugar beet and red beet is attributed to apoplastic ion adjustment.

The apoplast is also critical to biochemical osmotic adjustment mechanisms (Tomos, 1988). It is more effective to use apoplast π_{cell} than protoplast π_{cell} to regulate P_{cell} (Flowers & Yeo, 1986; 1988). Na^+ and K^+ are inorganic ions involved in this adjustment (Leigh & Tomos, 1983; Bell & Leigh, 1996), which results in continuing accumulation of sucrose by maintaining turgor at a low level (Leigh & Tomos, 1983; Tomos, 1988; Bell & Leigh, 1996; Lawrence, 1999).

Dividing the flux rate into and out of any compartment by its volume will give the rate of solute concentration change for that compartment (Tomos, 1988). Since the volume of the cell wall is smaller than that of the protoplast, transport of solutes across the plasma membrane will affect π_{cell} more rapidly than trans-protoplast transport. During the former, wall-osmotic pressure creates a differential that subsequently affects P_{cell} (Tomos, 1988).

1.5 Whole Plant Response to Salt

In most plant species, cell, tissue and organ development are affected by salinity (Hu *et al*., 2005), which thus reduces, alters or restricts growth. Salt stress is associated with ion toxicity, ion deficiency and ion imbalance (Cramer, 2002; Munns & Tester,

2008), and thus continuous increase in salt in the external root medium leads to a decrease in leaf size over time (Munns *et al*., 1988). Cell division rate, expansion rate and interval of expansion are also all affected by increasing the salinity of external medium.

Salinity also can affect photosynthesis, synthesis of protein, and lipid metabolism and energy (Parida & Das, 2005). Munns and Sharp (1993) reported that in leaves and root, salt stress leads to reduced formation of the photosynthetic leaf area. This leads to a decrease in the influx of assimilation to the growing and meristematic zone, and also leads to decreased stomatal conductance in older leaves. Root growth however is often less affected than leaf growth, and after NaCl (or other osmotic) exposure, the rate of root elongation experiences faster recovery (Munns, 2002). It has been found that within one hour of exposure to up to 4 bar of mannitol, KCl, or NaCl, root recovery can be achieved (Frensch & Hsiao, 1994). Indeed, even exposure to concentrations of up to 150 mM of NaCl can take just a day to recover from (Munns, 2002). Unlike in leaves, full restoration of turgor is not necessary for recovery to be effected (Frensch & Hsiao, 1994).

In barley, cell division of growing tissue in leaf 3 was not affected by 175 mM NaCl added to the external medium (Munns *et al*., 1988). In contrast, in sorghum grown in 100 mM NaCl, leaf growth region and maximal growth rate were reduced (Bernstein *et al*., 1993). Delane *et al.*, (1982) and Matsuda and Riazi (1981) found that salt stress (60 – 220 mM mM NaCl) reduced growth rate within minutes in leaves of barley[5].

[5] Growth of tips of leaves of 5-6 day old hydroponic plants was measured by interval photography (Rhodes & Matsuda, 1976). Stress was induced by changing to nutrient solutions of NaCl after seedlings had been in light for 4-6 hours.

1.6 Cell Compartmentation

The ability of various species to be salt tolerant has been connected to a variety of physiological mechanisms, including exclusion and/or compartmentalising of Na^+, osmotic adjustment, potassium selectivity, and organic solute accumulation (Yeo *et al*., 1990; Barret-Lennard *et al.*, 1999). Compartmentation within the cell itself contributes to salt tolerance in a plant, and salt accumulation in aerial parts of halophytes indicates their ability to effectively sequester ions in the vacuole. This process is facilitated by the plant having both well-vacuolated cells and efficient tonoplast transport mechanisms to channel ions out of the cytoplasm, and such compartmentation in leaf and/or shoot cells is commonly found in dicotyledonous halophytic species. Other adaptive mechanisms also contribute, including water regime control and the ability to maintain metabolism in environments of low K^+ concentration (Flowers & Dalmond, 1992).

Salt inclusion and compartmentation is therefore one mechanism for tolerating salt stress, in addition to the important role played by root exclusion, and occurs at the level of the cell, the tissue and/or the organ. Root Na^+ can be transported into vacuoles or to the shoots. Sequestering inorganic ions, such as sodium, in the vacuole relieves the cytosol and allows water uptake to continue, but this must be accompanied by correcting the cytosol's osmotic balance by synthesising appropriate solutes.

1.7 Transpiration Tension

One key process that affects both water transport and cell growth is transpiration. Transpiration at the shoot creates negative pressure (water tension), and this is usually the driving force that draws water into the root xylem (Steudle, 1995). The

process of transpiration can also cause changes in cell hydraulics or membrane transport, and this may result in changes to P_{cell}. The latter is commonly expressed not as an absolute value but as a value of hydrostatic pressure relative to atmospheric pressure. Studies into the correlation between transpiration and changes in P_{cell} have been conducted, for example using sugar beet taproot (Palta *et al.*, 1987), and indicate a directly proportional relationship between the two, with P_{cell} decreasing as the transpiration rate increases.

Research is increasingly suggesting that turgor regulation and maintenance, together with other plant cell water relations, rely on the active control of cell wall solutes (Leigh and Tomos, 1983; Clipson *et al*, 1985; Tomos and Wyn Jones, 1988; Tomos, 1988; Bell and Leigh, 1996). In particular, the modulation of P_{cell} relies on π_{wall} rather than that of the protoplast (Flowers and Yeo, 1986; 1988). Since it is the ratio of the flux rate to the compartment volume that dictates the rate of change of solute concentration (Tomos, 1988), then transporting solutes across the plasma membrane will have a more rapid effect on π_{wall} due to the small relative size of the apoplast. Being able to maintain leaf P_{cell} is important as it means that transpiration can continue uninterrupted and thus that solute transport in the xylem is not inhibited (Marschner, 1995).

Using a value for transpiration tension together with values for P_{cell} and π_{cell}, the apoplast solute concentration can be calculated. There is a relationship between P_{cell}, π_{cell}, π_{wall}, and transpiration tension, i.e. the apoplast hydrostatic component (P_{wall}). Therefore, any disparity between P_{cell} and π_{cell} is the result of π_{wall} and/or P_{wall}. By means of using a pressure probe, P_{cell} in both transpiring and non-transpiring leaves can be measured to gauge the influence of P_{wall} (Hüsken *et al.*, 1978). Several

researchers have measured the transpiration tension in *S. maritima* (Clipson *et al.*, 1985; Lawrence, 1999), in wheat (Arif, 1990), and in taproot (Tomos *et al*., 1992).

1.8 Physiology and Life History of Wheat and *S. maritima*

1.8.1 Wheat- a Glycophytes

The harmful effect of salt on the growth of a non-halophyte is the result of a combination of factors and can be at the whole-plant level. An example would be a decline in growth rate. Leaves grow more slowly and there is an increase in root:shoot ratio (Blumwald *et al*., 2000). Many morphological, physiological and biochemical processes are affected by salinity, such as seed germination, plant growth, and water and nutrient uptake. However, investigations have confirmed that germination of wheat (*Triticum aestivum* L.) is retarded and lowered by low Ψ_{ext} (Hao & Dejong, 1988) and high salinity (Zhao *et al*., 2007), as well as those factors combined (Willenborg *et al*., 2004). Zhao *et al*., (2007) reported that osmotic stress resulting from salinity causes mineral deficiencies and physiological and biochemical change to crop plants.

Decreased wheat growth has also been seen under salt stress, related to turgor loss (Saqib *et al*., 2004). The latter, in turn, decreases photosynthesis and exchange of gases, and thus affects cell division and cell elongation processes.

The barley (*Hordeum vulgare* L.) crop however is more saline-tolerant than durum or bread wheat, in spite of concentrations of leaf blade Na^+ similar to those of durum wheat (Genc *et al*., 2007). This indicates larger levels of tissue tolerance to Na^+ concentration. In contrast, wheat (*Triticum aestivum* L.) has only moderate salt tolerance (Maas & Hoffman, 1977). Even so, it will produce a yield, albeit reduced, in concentrations of 100 mM NaCl, where rice (*Oryza sativa*) will not survive to

maturity. Whilst barley has the highest tolerance of the cereal crops, even this cannot cope with sustained exposure to concentrations of salt above 250 mM (equivalent to 50% seawater). There are differences between varieties, with bread wheat being more tolerant than Durum wheat (*Triticum turgidum* ssp. durum) (Maas & Hoffman, 1977).

Several authors, including Yeo *et al*., (1985) and Davenport *et al*., (2005), have found that many grasses can tolerate salt through accumulation of the ion Na^+ in the more mature leaves and the leaf sheaths, as well as by the compartmentalization of Na^+ at the whole-plant level. Moreover, Greenway and Munns (1980) reported that inducing K^+ accumulation and uptake is also crucial for salt tolerance in growing tissues. In both monocot and dicot species, Plett and Moller (2010) reported that the highest concentrations of Na^+ accumulate in the oldest leaves of shoot biomass.

Toxic concentrations of Na and Cl ions negatively affect not only cytoplasmic metabolism but also the general development of wheat. Salinity tolerance of crop plants is a function of three parameters; Na^+ exclusion; osmotic tolerance (achieved through changes to long-distance signalling, the allowing of cell expansion and the development of lateral buds, modifications to stomatal opening, and improvements to osmotic adjustment); and tissue tolerance to Na^+ concentration (Munns & Tester, 2008). Salinity tolerance in many cereal species is noted to be positively associated with Na^+ exclusion, signifying that Na^+ exclusion from the tissue cells is an important constituent of salinity tolerance (Tester & Davenport, 2003). Notable differences in Na^+ exclusion has been noted in *Triticum tauschii*, where the level of Na^+ exclusion was related to salinity tolerance (Genc *et al*., 2007). Many studies have noted positive correlations between salinity tolerance and Na^+ exclusion in bread wheat

(Poustini and Siosemardeh, 2004) and Fricke *et al*. (1994a) found K^+, Cl^- and NO_3^- in leaf epidermal cells of barley.

Osmotic and ion-specific effects result in the accumulation of Na^+ and Cl^- in plants and lead to a decrease in crop growth and yield (Munns & Tester, 2008). Munns and Tester (2008) found that cell expansion, cell division and stomatal closure are affected by the osmotic effects of salinity stress. These occur after salt dosage and it is believed they continue for the period of exposure. Due to the various mechanisms which and salinity tolerance (Colmer *et al.*, 2005), it is unsurprising that salinity stress is also a trait which is genetically complex. Some investigations have found that decrease in turgor leads to change in growth (Clipson *et al*., 1985 and Balnokin *et al*., 2005), resulting from significant levels of ions in the apoplast (Harvey *et al.*, 1981) or an adjustment in elasticity of cell wall (Touchette, 2006). Other effects probably depend on osmotic adjustment due to a lack of ability to accumulate and/or sufficient nutrient distribution; on the synthesis of sufficient organic solutes; on the ineffectual cycling of ions (Britto & Kronzucker, 2006); or on the energy demands of ion compartmentation (Yeo & Flowers, 1983).

1.8.2 *Suaeda maritima*- A Halophyte

Halophytes have developed various traits (physiological, morphological and biochemical) which allow them to tolerate high external salt concentration (Glenn *et al*., 1999). Flowers *et al*. (1986) consider that the ability to grow and reproduce when exposed to at least 200 mM NaCl defines halophytes. This condition is similar to that which may be faced in the natural environment (200 mM NaCl or more). Dubey (1999) has reported that *S. maritima* can continue growth in a saline environment of about 500 mM NaCl. However, its extracted enzymes it respond to a much lower level of NaCl (170 mM), indicating that in vivo conditions produce a lower

sensitivity to NaCl in soluble enzymes and enzymes of protein-synthesizing mechanisms than in vitro conditions do in enzymes that have been isolated.

Yeo and Flowers (1980) found optimal growth for *S. maritima* in salt concentrations of about 150 mM NaCl in saline conditions. Hajibagheri and Flowers (1989) found that when *S. maritima* was growing in 200 mM NaCl (seedling grown in culture solution, root cut from 10-20 mm behind tips of seminal root and examined by X-ray microanalysis of freeze-substituted thin section), in the cytoplasm the concentration of Na^+ was 118 mM, in the vacuole it was 432 mM and in the cell wall 95 mM. When *S. maritima* was growing in external salinity of 340 mM NaC**l** (seeds were germinated at 170 and 340 mM NaCl, and grown in culture media based on soil; fresh and dry weights of healthy seedling were analysed), in the cytoplasm the Na^+ concentration was about 150 mM. In leaf slices suspended in 600 mM NaCl, the vacuole concentration was about 600 mM (Yeo, 1974).

1.9 Objectives

The main aim of this study was to look for potential factors conferring salt tolerance in wheat, in order to further inform agricultural science in Libya. This involved several subsidiary objectives.

Firstly, the study sought to measure the effect of salt stress (created by adding NaCl to the growth medium) on three varieties of wheat, in terms of its impact on a range of growth parameters (final leaf and root length, leaf and root growth rate, and leaf plastochron index) as compared to a control. Three wheat varieties were chosen, which were expected to exhibit a range of salt tolerance. Two of the varieties, Gamina and Bohoth 105, are currently used in Libya, whilst the third, Hereward, is grown in the UK and known to be salt sensitive.

The second objective was to examine the cell water relations of the leaves and roots of the wheat varieties, in order to investigate how the cell biophysics of each variety responded to salt stress, and to assess any differences between leaf epidermal and root cortical cells. Cell water relations parameters were to include P_{cell}, π_{cell}, cation concentrations, osmotic adjustment, volumetric elastic modulus, hydraulic conductivity and bulk leaf π_{cell} and cations.

Finally, the study then sought to compare the spectrum of salt tolerance and biophysical and biochemical features of the three wheat varieties with those of a model halophyte, *S. maritima*, in order to discover which variety behaved in the most similar way to the halophyte in response to salt stress. The hypothesis was that Bohoth 105 would exhibit characteristics that were more similar to those of *S. maritima* than the other two varieties were.

Chapter 2: Materials and Methods

2.1. Materials and Growth Conditions

Three varieties of wheat were used. They were Hereward, Bohoth 105 and Gamina. The Hereward seeds were harvested in the 2007 season at Bishops Farm, Oldberrow, Henley-in-Arden, Worcs (provided by Dr K.A. Steele). The local wheat variety, Hereward, is a UK commercial variety (and presumed to be relatively salt sensitive). Variety Bohoth 105 has been classified as a salt tolerant variety, and Gamina as less salt tolerant (Libyan Agriculture Research Centre, 2010). Both are Libyan varieties, and were provided by the Agriculture Research Centre in Tripoli, Libya. The seeds were stored at 5^0C before germination. Seeds were soaked overnight in aerated tap water and then germinated on damp tissue paper in rectangular seed trays at 25^0C in the dark.

S. maritima seeds were collected in 2009 from a site on the south coast of Anglesey (Plate 2.1), and then stored at 5^0C before germination. It is an inter-tidal halophyte which has been used previously for salt tolerance research (Clipson *et al.*, 1985). For *S. maritima* germination, the seeds were spread out on moistened 90 mm tissue filter paper (Fisher brand). The wet tissue filter paper was placed in a 250 ml glass crystallizing dish (Pyrex) at 25^0C in the dark. After 5 - 7 days, the first signs of germination were observed. The wheat varieties and *S. maritima* seedlings were large enough to handle after 3 and 18 days respectively, and were transferred to 550 ml pots at this stage and grown hydroponically in aerated half-strength Long Ashton nutrient solution (Hoagland & Arnon, 1950) (Table 2.1) to allow access to the leaf and root systems.

All subsequent growth was carried out in a growth chamber (Sanyo-Gallenkamp) (Plate 2.2) under controlled conditions. Temperature was maintained at 25^0C during the light period and at 15^0C during the dark. A photoperiod of 16 hours day/8 hours' dark with 400µE.m^{-2} light at plant level and 70 % relative humidity was used.

Four or five plants were used per treatment and 3 - 12 cells in each plant were examined to measure water relation parameters (turgor pressure (P_{cell}), volumetric elastic modulus (ε_{cell}), cell hydraulic conductance (L_p)), osmolality and solute concentration, as described below.

Macronutrient	Concentration (mM)	Micronutrient	Concentration (mM)
K^+	4.0	Si (as silicate)	0.05
Ca^{2+}	4.0	Fe^{2+}	0.1
Mg^{2+}	1.5	Mn^{2+}	0.01
HPO_4^{2-}	1.3	Cu^{2+}	0.001
SO_4^{2-}	1.5	Zn^{2+}	0.001
NO_3^-	12.0	Na^+	1.3
-	-	BO_3^-	5×10^{-5}
-	-	MoO_4^{2-}	4×10^{-4}

Table 2.1 Composition of 100 % strength Long Ashton growth solution (Analar grade salts supplied by BDH). An equal volume of de-ionised water was added to a volume of the 100 % strength solution to make a 50 % strength medium.

Plate 2.1 *S. maritima* in its natural environment on the Isle of Anglesey, 2011

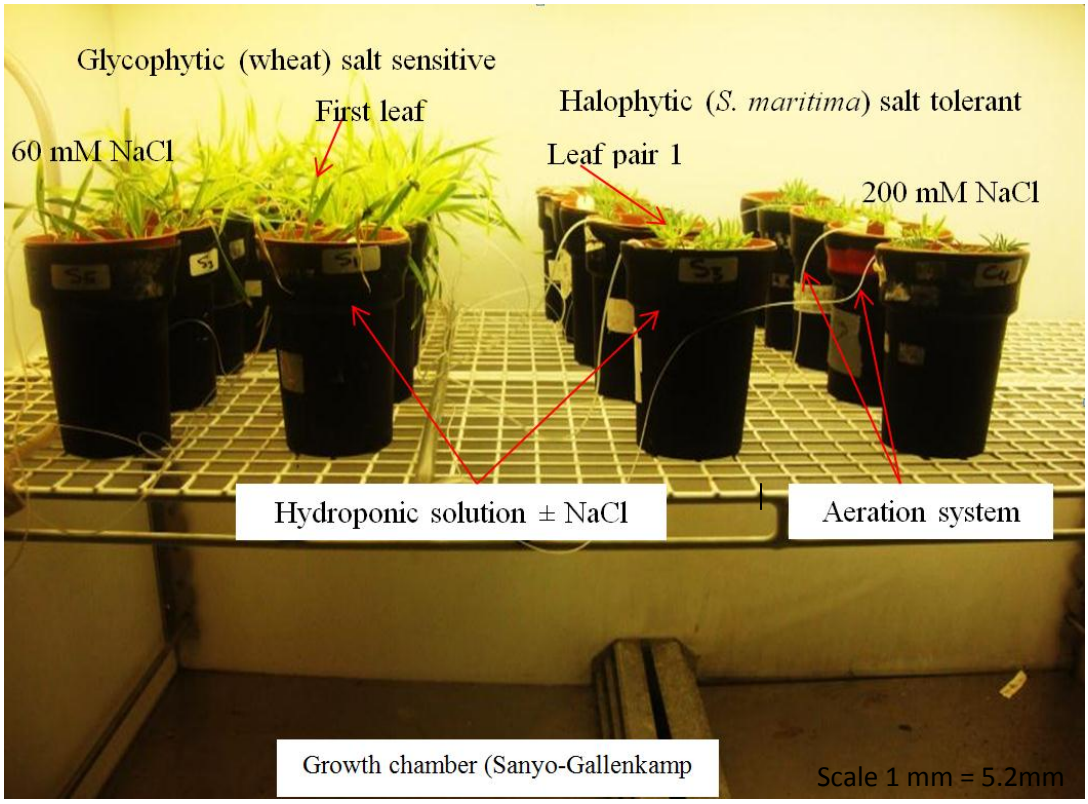

Plate 2.2 Hydroponic system used for growth of wheat varieties and *S. maritima* plants in all experiments. Plant were grown in approximately 550 ml of aerated 50 % Long Ashton solution (± salt). Conditions were 16h day (400 $\mu E.m^{-2}$)/8 h dark (25^0C /15^0C) 70 % RH. Nutrient solution was regularly replaced.

2.2. Salt Treatment

Control batches were grown in 50% Long Ashton for 22 - 23 days. During this time, salt (NaCl, BDH, grade 99.9%) was added to the test batch hydroponic solution in two stages. On day 4 after germination, 30 mM of NaCl was added to the solution, and on day 5 this was made up to a final concentration of 60 mM. The wheats were then grown on for 18-19 days.

The concentration of 60 mM was chosen as it was thought to be high enough to have an effect on the plants, but not so high as to prevent their growth altogether or cause death. According to a US Salinity Laboratory survey of salt tolerance, each of the crop and pasture species studied showed a 'bent stick' relationship between yield and salt concentration. In other words, there was a threshold salinity at which the yields started to suffer, but at concentrations lower than this, there was no effect on yield. The typical threshold of wheat was found to be around 60 - 80 mM NaCl, so 60 mM was thought to be high enough to detect an effect on the plants. (Maas & Hoffman, 1977; USDA-ARS, 2005).

Control *S. maritima* batches were grown in nutrient solution. Twenty two days after the first sign of germination for the test batch, 200 mM NaCl was added as two increments of 100 mM over two days. This concentration represents optimum conditions for *S. maritima*, which is able to grow at salinity levels which other plants cannot tolerate. A concentration of 200 mM has been used in previous studies on this species (Hajibagheri & Flowers, 1989; Maathuis *et al*., 1992; Lawrence, 1999).

2.3. Leaf and Root Growth (Wheat)

Batches of 12 plants (wheat) were used for growth analyses. From each plant, 5 leaves were measured starting from seed (zero line) every day (after emergence) by

using a ruler. The growth rate, final length and Plastochron index (the interval of time between two frequent successive events, for example leaf initiation) were determined for each leaf from these data as follows:

The leaf growth rate was measured by the linear regression of the relationship between length of leaf and time, during the initial linear growth phase (that lasted some 2 – 3 days for each leaf).

Erickson and Michelini (1957) developed a numerical index of the developmental age of plants, which they termed the "plastochron index," abbreviated to PI. This was proposed to be an indicator of plant age according to morphology, where a plastochron is the time interval between successive leaf initiations. Later, this was extended to refer not just to leaf initiation but also to any discernable point of leaf development.

Plastochron index was measured by the method as described for *Xanthium* by Maksymowych (1973), except that the cereal showed a linear rather than an exponential growth. As result, a linear rather than a log-linear equation was used. Root length was also measured daily as above. Maximum leaf and root length were determined at the point when elongation stopped.

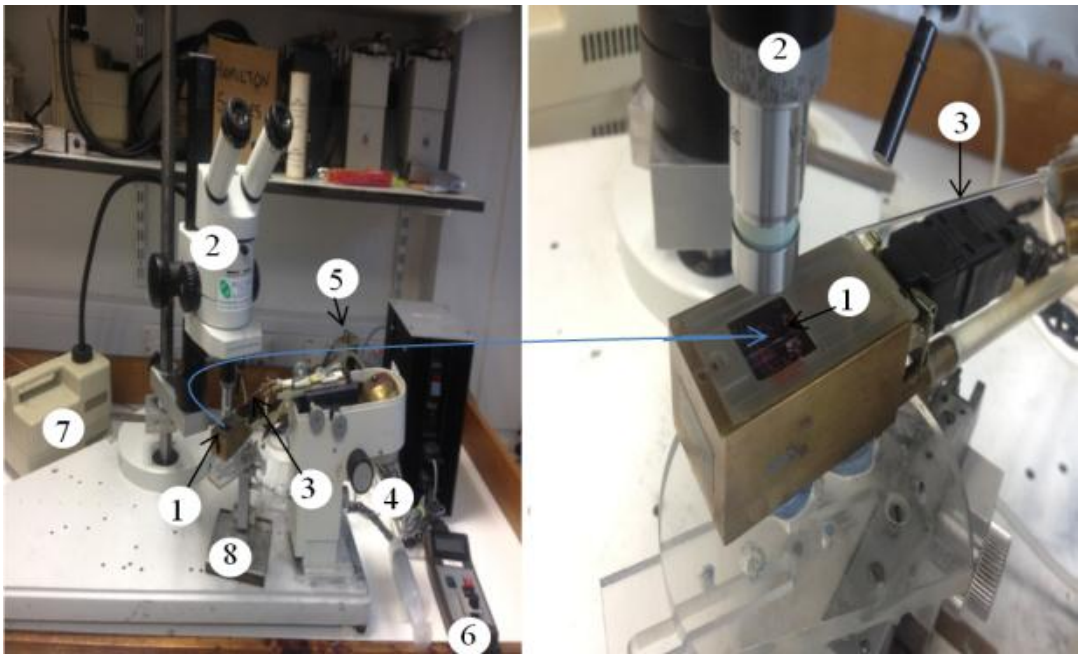

Plate 2.3 Experimental setup for sampling single cell from leaf and root. Osmotic pressure is measured by picolitre osmometer: 1: Droplet samples (10 - 60 pl) are extracted directly from plant cells *in situ*; 2: stereomicroscope 3: sampling glass microcapillary with tip 1 µm (filled with AS 4 silicon oil before sampling, capillary is manipulated into place above the copper stage, and now is ready to be used); 4: micromanipulator; 5: valve operated by foot switch; 6: osmometer hand set; 7: illuminator (fiber optics); 8: adjustment stage.

2.4. Leaf and Root Physiological Parameters

The osmolality and inorganic cations at organ level were measured from sap extracted according to Gorham *et al.* (1984). Osmolality was measured using a picoliter osmometer (Malone *et al.*, 1989; Tomos *et al.*, 1994; Fricke, 2013) (Plate 2.3) but using samples in the nl range. Inorganic cations were measured using capillary zone electrophoresis (CZE) (Bazzanella *et al.*, 1998) (Plate 2.4).

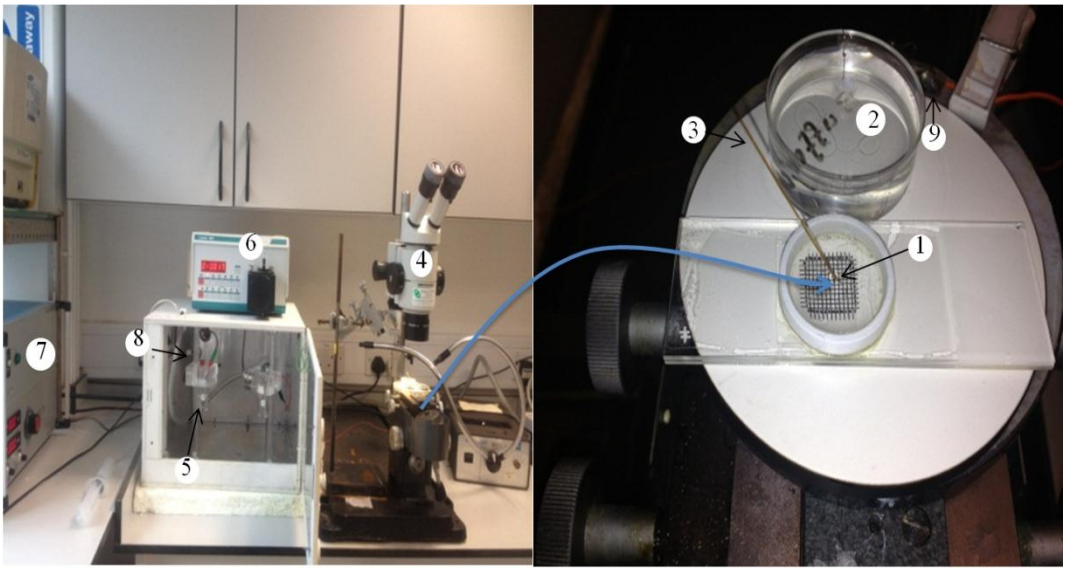

Plate 2.4 Experimental setup for injection of the prepared epidermal and cortical sample (10 - 60 pl) into the CE column; 1: droplet samples are extracted directly from plant cells *in situ*; 2: buffer solution; 3: CE column; 4: stereomicroscope; 5: outlet buffer vial; 6: spectrophotometer; 7: high voltage power supply 30 KV; 8: anode (+): 9: cathode (-).

2.5. Analytical Techniques for Single Cell Samples

2.5.1. Turgor Pressure

Single cell water relations [Turgor (P_{cell}), volumetric elastic modulus (ε_{cell}) (Fig. 2.1 and 2.2) and hydraulic conductivity (L_p)] (Fig. 2.3) of leaf epidermal and root cortical cell were measured according to Hüsken *et al.* (1978). All these parameters were measured by pressure probe (Zimmermann & Steudle, 1978; Tomos & Leigh, 1999) (Plate 2.5).

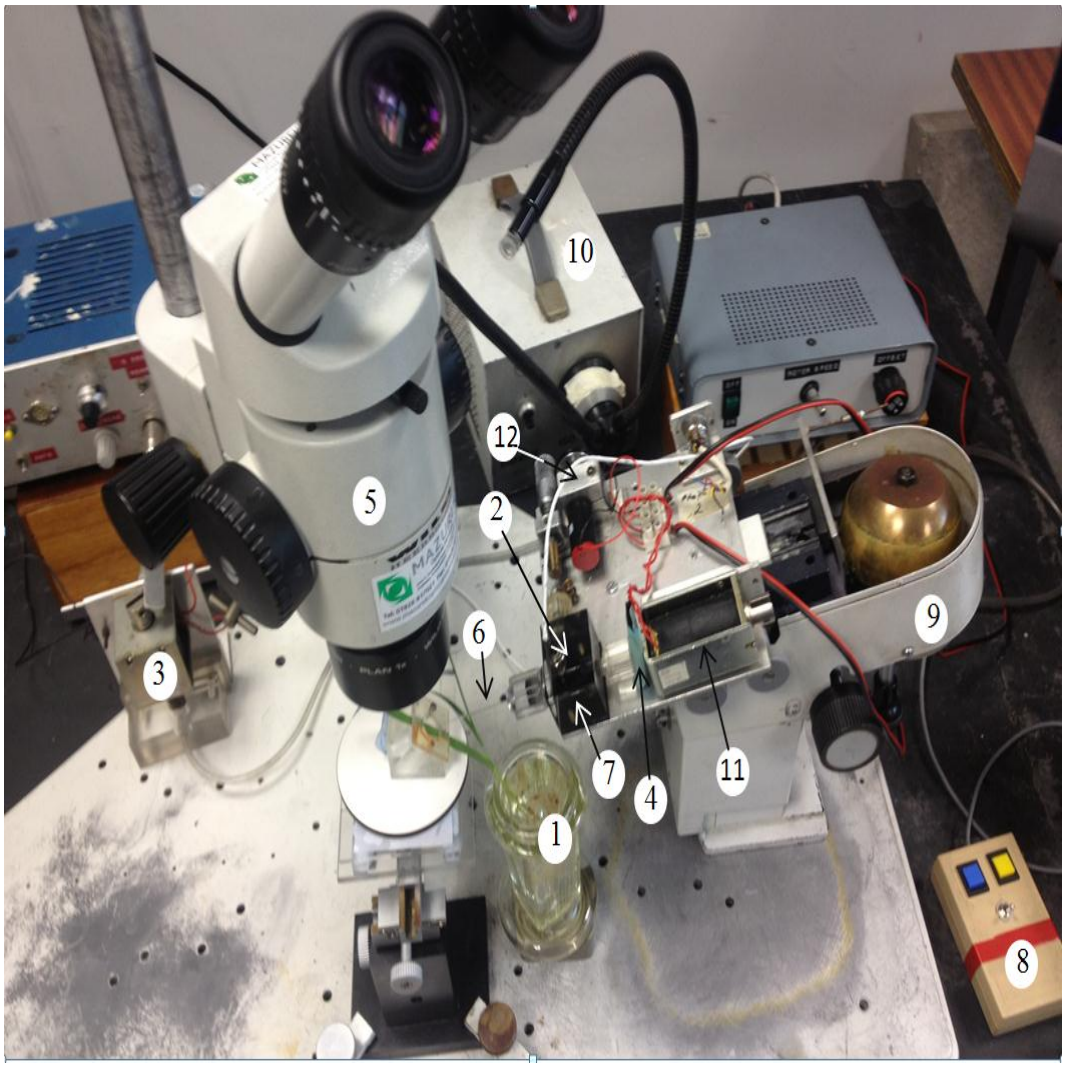

Plate 2.5 Experimental setup for measuring turgor pressure by pressure probe; 1: wheat plant in hydroponic solution; 2: pressure transducer (Druck Ltd); 3: valve operated by foot switch; 4: oil reservoir filled with silicone oil; 5: stereomicroscope; 6: glass microcapillary with tip 1 µm (filled with AS4 silicone oil before sampling) inserted in epidermal cell (Clarke Electromedical Instruments); 7: Perspex chamber; 8: hand set; 9: micromanipulator; 10: illuminator (fiber optics); 11: solenoid valve 24 V DC (RS Components); 12: geared motor 6 V/12 V DC (RS Components).

2.6 Cell Dimension Measurements

2.6.1 Root

To determine cell length and radius, a section 4 – 6 cm from the root tip was selected from 3 - 6 plants for each treatment for wheat and *S. maritima* on day 8. This region of the root was chosen for several reasons. Firstly, the cells here are outside the elongation zone, and are relatively large and so more practical for sampling with the chosen technique. Secondly, choosing a defined distance from the tip increases the

reproducibility of the results, which is important in terms of comparing measurements across varieties. Thirdly, this is a region which responds to salt, as seen in our results.

A plastic embedding technique (Feder & O'Brien, 1968) was used for preparing longitudinal and transverse sections. The root tissues were cut into small pieces (1 - 2 mm) and fixed in 3 % glutaraldehyde in 50 mM sodium cocodylate buffer under vacuum for 24 h at room temperature. Tissue samples were dehydrated in varying grades of ethanol solution (25, 50, 75 and 100 %) for 12 h each, and then subsequently transferred to ethanol and LKB (1:1 ratio) infiltration solution (LKB AB, Bromma, Sweden) on a rotator overnight. Pure LKB infiltration solution was substituted instead of bath solution and left for 4 days on the rotator. Tissue samples were placed in plastic moulds and then covered for 3 h with LKB embedding at room temperature to polymerise. The solidified resin blocks were taken out from the moulds and then placed on blocks. Tissue samples were cut to 4 µm thick using a microtome device (Supercut 2050; Reichert-Jung, Gmbh D6907, Nbloch, Germany). Samples was placed on slides and then stained with 0.05 % (w/v) toluidine blue benzoate buffer for a few minutes, mounted in distilled water. Length (l) and radius (r) were measured on a photograph taken of the slide by the microscope computer's digital camera. The surface area (A) and cell volume (V) of the cylindrical shaped cells were calculated using equation 2.1 for area and equation 2.2 for volume, as below.

$$\text{Surface area} = [2 \Pi r l) + 2[\Pi r^2] \quad (2.1)$$

$$\text{Volume} = \Pi r^2 l \quad (2.2)$$

Where, r radius (µm), l length (µm), and $\Pi = 3.14$

2.6.2 Leaf

Longitudinal sections of the plant leaves (leaf 1) were cut by hand into small pieces (1 – 2 cm). The length (*l*) and radius (r) of the epidermal cells were determined using a stereomicroscope at 400-fold magnification. Surface area and volume were calculated using equations 2.1 and 2.2, as above.

2.7 Measurement of Volumetric Elastic Modulus

The cell pressure probe, as described by Hüsken *et al*. (1978), allows the continuous measurement of P_{cell} and also the manipulation of the volume of the cell and its turgor. The P_{cell} is transferred through glass microcapillary with a tip (1 – 3 μm) filled with silicone oil (AS4; Wacker-chemie Gmbh D-81737 München Burghausen) to a pressure transducer fixed in a plexiglass block (Plate 2.5). The oil/sap meniscus was controlled electronically by a handset. Turgor pressure in leaf epidermal (except *S. maritima*) cells and cortical root cells of wheat and *S. maritima* were measured (as detailed in Chapter 3, 4, 5 and 6). P_{cell} of the plant roots was measured while the roots were bathed in external solution. P_{cell} of wheat leaves were measured in air. Instantaneous ε_{cell} was obtained by the change in volume of the cell (ΔV), which leads to changes in cell turgor (ΔP) (Figs 2.2 and 2.3). V, ΔV and ΔP represent actual cell volume. The ε_{cell} was calculated by using the equation (2.3) below (Philip, 1958; Zimmermann and Steudle, 1978).

$$\varepsilon_{cell} = V\frac{\Delta P}{\Delta V} \quad (2.3)$$

The silicone oil-cell sap meniscus was manipulated quickly. By moving the meniscus, the cell volume (V) and turgor (P_{cell}) changed and then returned to their original position. Software (Clarity Lite Chromatography, Supplied by Data Apex, Prague) was used to process the results of pressure change (ΔP) as charts on a

computer screen. The change in volume was measured from the range of movement of the silicone oil/cell sap meniscus. The cell length and radius were measured using an eye-piece graticule in the stereomicroscope. Equation 2.2 was used to calculate cell volume, with the leaf cell being assumed to be of a cylindrical shape, where = r cell radius and *l* = cell length.

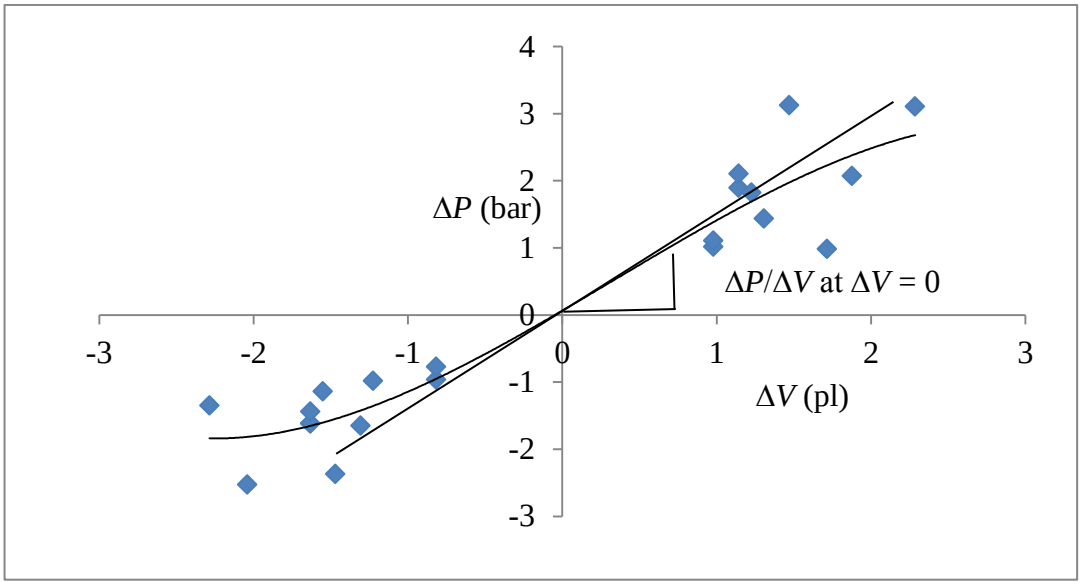

Fig. 2.1 An example of the raw data for determining volume elastic modulus (ε_{cell}). A $\Delta P/\Delta V$ curve of the leaf epidermal cell for Gamina variety as obtained by pressure probe. A pressure pulse ΔP was applied to individual cells and the resulting change in volume (ΔV) measured (The sigmoidal shape of the curve is due to water flow across the cell membrane during the experiment). The *dp/dv* used is that extrapolated to $\Delta V = 0$) see fitted line. All observations were taken from one cell.

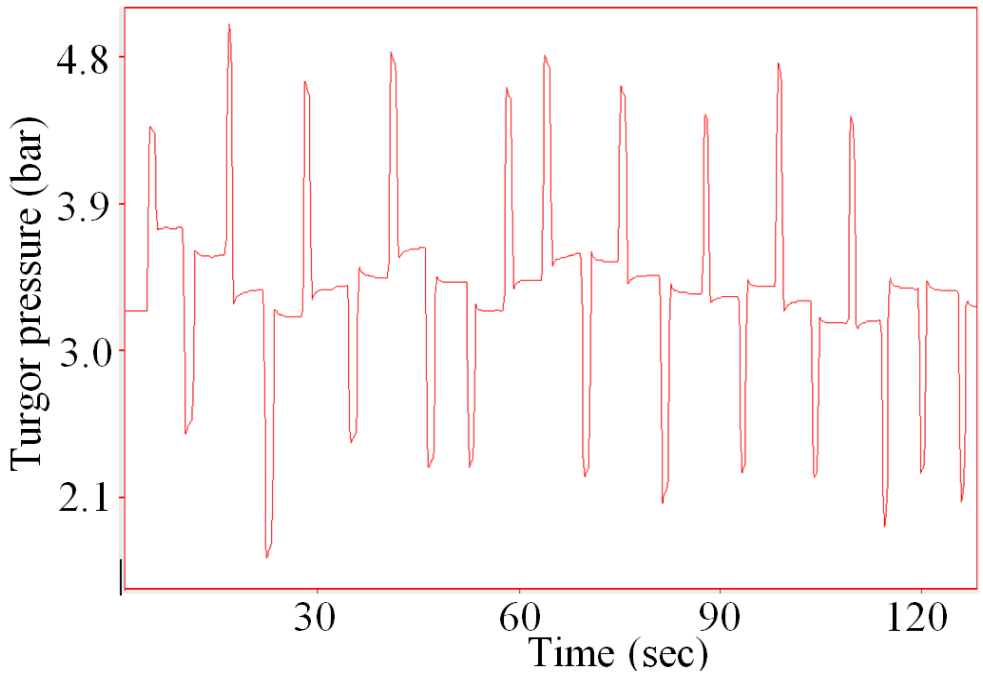

Fig. 2.2 Typical example of rapid changes in pressure to measure the volume elastic modulus (ε_{cell}). Change in pressure of the leaf epidermal cell are for Gamina variety as obtained by pressure probe. The probe mmeasure ΔP directly and calculates ΔV from the changes in meniscus position. All peaks were obtained from one cell.

2.8 Hydraulic Conductivity

Hydraulic conductivity (L_p) was measured using hydrostatic pressure relaxation (as described by exponential $P_{(t)}$ curve equation 2.4). The change in cell volume created by moving the metal rod (linked to an electric motor) to push the oil/sap meniscus to a new position leads to rapidly increased or decreased P_{cell} (Fig. 2.3). The changes in P_{cell} (ΔP) lead to outflow of water from the cell or inflow into the cell, with P_{cell} finally relaxing back to a magnitude close to the original position. Data was collected using clarity life software and then individual exponential curves were retro-fitted by eye to the pressure relaxation in order to estimate the half time ($T_{1/2}$) for the water exchange rate across the cell membrane using Equation 2.4 below. $T_{1/2}$ was calculated by the difference between $P_{cell(A)}$ and $P_{cell(E)}$ (Equation. 2.4).

$$P_{(t)} = P_{cell(E)} + (P_{cell(A)} - P_{cell(E)}).\ \exp(-k_w.t). \qquad (2.4)$$

Where $P_{cell(A)}$ is the maximum value of P_{cell}; $P_{cell(E)}$ is the end value of P_{cell}; and K_w is the rate constant of the water flow, which is the inverse of the time constant.

The π_{cell} was measured, as described in Chapters 3, 4, 5 and 6. The L_P was calculated using measurements of V, A, ε_{cell} and π_{cell}, as in Equation 2.5 below. $\Delta P/\Delta V$ and $T_{1/2}$ were obtained for the same cell, but V and A were obtained using different cells on day 8.

$$L_p = \frac{V}{A} \cdot \frac{ln2}{T_{½}} \cdot \frac{1}{(\varepsilon_{cell}+\pi_{cell})} \qquad (2.5)$$

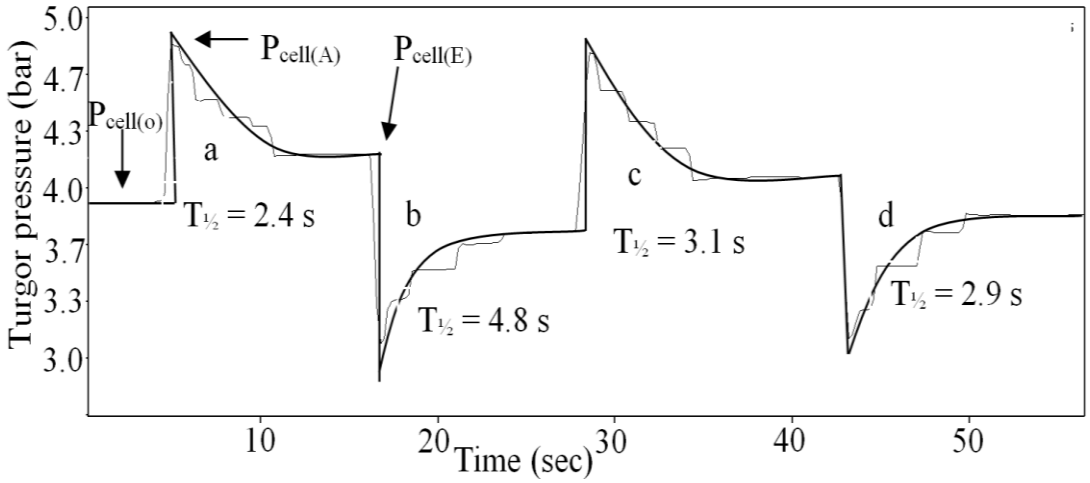

Fig. 2.3 An example of the raw data for determining *Lp* pressure–relaxation curves from single root cortical cells of Hereward wheat, as determined by the cell pressure probe. Endosmotic (a, c) and exosmotic (b, d) water flow were changing, induced by rapidly changing $P_{cell(0)}$ to an initial $P_{cell(A)}$. *P* exponentially relaxes back to $P_{cell(E)}$. Half time was measured by fitting an individual exponential curve to the pressure relaxation. The $T_{1/2}$ of water exchange was used to estimate the $L_{p.}$

2.9 Single Cell Sampling and Analysis (SiCSA)

Cell sap of single epidermal cells of leaf and cortical cells of root in intact plants *in situ* was extracted directly by using the single cell sampling and analysis performed by Tomos *et al.* (1994) and Fricke (2013) (Plate 2.6). Osmotic pressure was measured according to the picolitre osmometry method of Malone *et al.* (1989); Tomos *et al.* (1994) and Fricke (2013). Cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) were measured using the CZE system of Bazzanella *et al.* (1998) (Plate 2.4).

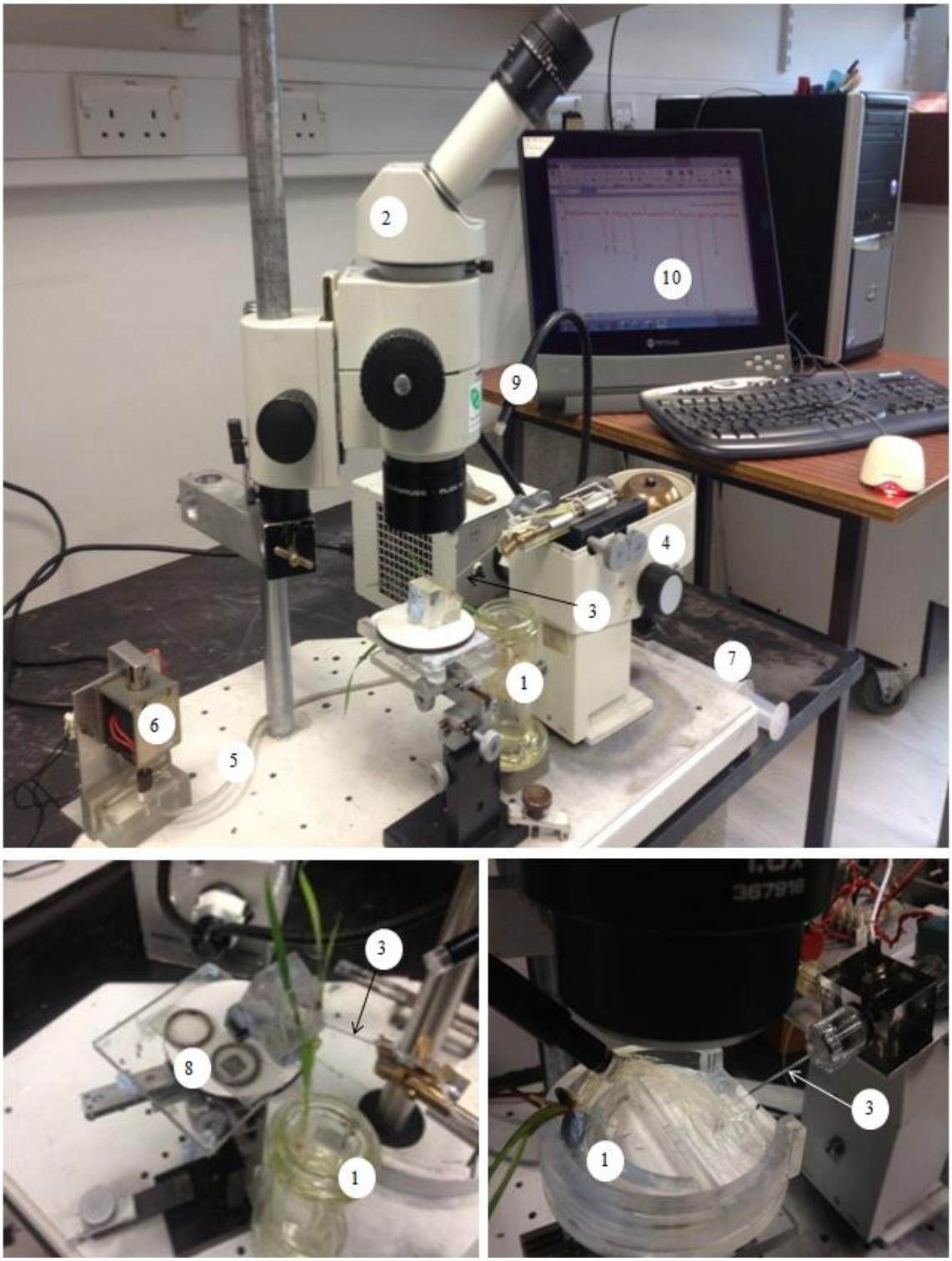

Plate 2.6 Experimental setup for sampling single cell; 1: wheat plant in hydroponic solution (sap extracted from leaf and root cells in air and under solution); 2: stereomicroscope; 3: sampling glass microcapillary with tip 1 µm (filled with AS4 silicon oil before sampling), 4: micromanipulator; 5: tubing; 6: valve operated by foot switch; 7: syringe; 8: slide with ring filed with saturated paraffin oil; 9: illuminator (fiber optics); 10: output on computer screen.

2.10 Data Analyses

Data analyses were performed through IBM SPSS Statistics version 20 for Windows. Results includes the means and the standard deviations. A statistical analysis of the data was carried out on the assumption of normal distribution and homogeneity of variance. Normality was tested using the Kolmogorov-Smirnov test. Treatment effects were tested using analyses of variance and Tukey multiple comparison was performed. At single individual cell level, water relation parameters were analysed by two-way ANOVA, with repeated measurements on the various experimental time periods. The significance level was set at P=0.05.

Growth parameters for different plants were compared when different treatment (control and salt) were used over the various time periods. At whole plant level, π_{cell} and solutes were compared in the first three leaves for each of the three wheat varieties, using univariate ANOVA. The differences between the control and salt treatments for P_{cell} and π_{cell} showed an exponential relationship for all three varieties. However, when the log of the difference was plotted against time, the relationship was shown to be linear.

Chapter 3: Plant Growth Parameters of Wheat Varieties and their Responses to NaCl Stress

3.1 Introduction

Salinity conditions result in the accumulation in plants of Na^+ and Cl^-, and in water being uptaken together with solutes (K^+ and Ca^{2+}) (Hu & Schmidhalter, 1997). Salinity has detrimental effects on the plant, which can include decreased leaf elongation rate, lower maximum leaf length, and altered plastochron index (e.g. Beatriz *et al.*, 2001). Shoot growth is also impaired, resulting in a lower leaf area and shoot stunting (Läuchli & Epstein, 1990). This is because the final size of leaves depends on the rates of cell division and elongation, and these are affected by salinity. Salinity also retards the growth and development of the whole plant as well as of certain organs. Changes in the final leaf size and leaf growth rate are aspects of leaf growth that have been studied in many plants, including wheat (Auld *et al*., 1978).

From the wide range of wheat varieties available, three were chosen to represent a range of salt tolerances. The objectives of this chapter are to describe the responses of the three to the application of salt stress, grown using the hydroponic growth system available. The first five leaves and the longest seminal roots of each seedling of three wheat varieties (Hereward, Gamina and Bohoth 105) were used to measure basic growth parameters following salt stress, and to investigate the mechanism by which growth is reduced under salt stress. Growth rate, final length and leaf Plastochron Index were compared by measuring leaf (1 - 5) and root length every

day (Figs. 3.1 to 3.3; Tables 3.1 and 3.2). These measurements continued to a maximum of 21 days, in both control and 60 mM NaCl treatments. Plants were grown in hydroponic medium under controlled conditions (see Materials and Methods).

3.2 Leaf Growth

For all leaves, significant differences were seen in final leaf length between the three varieties (P = 0.00) under both control and treatment conditions (Table 3.1). The statistical different between varieties and treatment effects was also significant (P = 0.00) (Table 3.1). Salinity decreased the final leaf lengths of both Hereward and Gamina by 21 - 32 % and 10 % - 22 % respectively (Fig. 3.1 to 3.3). In contrast, this parameter in Bohoth 105 was not affected by NaCl. Concomitantly, salinity significantly inhibited the leaf growth rate of both Hereward and Gamina varieties in the range of 22 - 33 % and 8.3 - 17 % respectively. Again, the growth of Bohoth 105 was not affected by NaCl (Table 3.2).

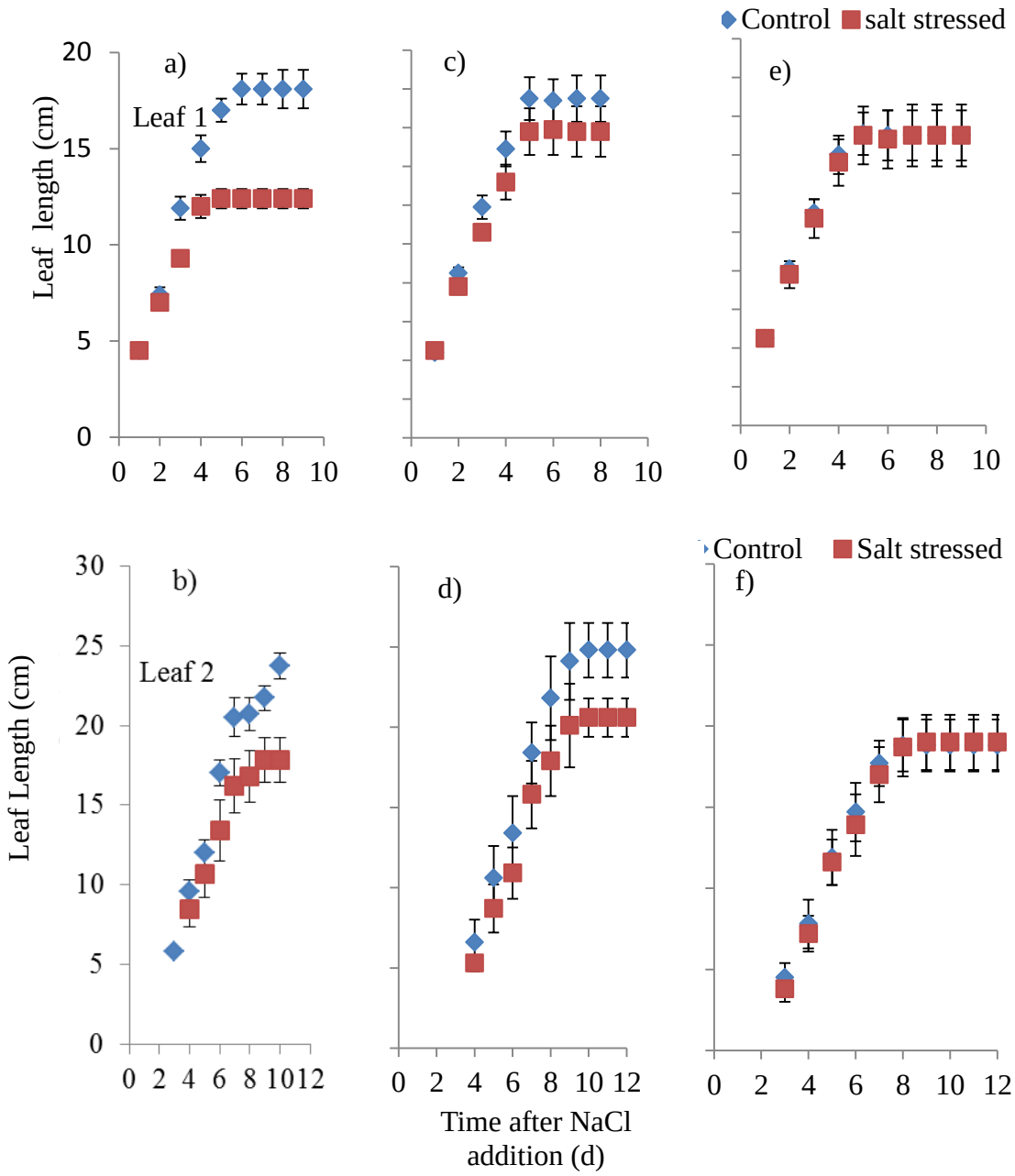

Fig. 3.1 Time course of length of leaves 1 and 2 of Hereward (a - b), Gamina (c - d) and Bohoth 105 (e - f) for 0 mM control and following application of 60 mM NaCl. Salt was added in two 30 mM doses over two days (the days are numbered at the first addition). Leaf one and two was measured using a ruler. Differences in mean final leaf length of both Hereward and Gamina varieties between treatments (control and salt) were significant, whereas differences in final leaf length of Bohoth variety between treatments (control and salt) were non-significant. Each point is the mean ± SD of 12 leaves taken from 12 individual plants. From the graph, salt stress had a strong, a moderate and no effect on final leaf length of Hereward, Gamina, and Bohoth 105 wheat varieties respectively.

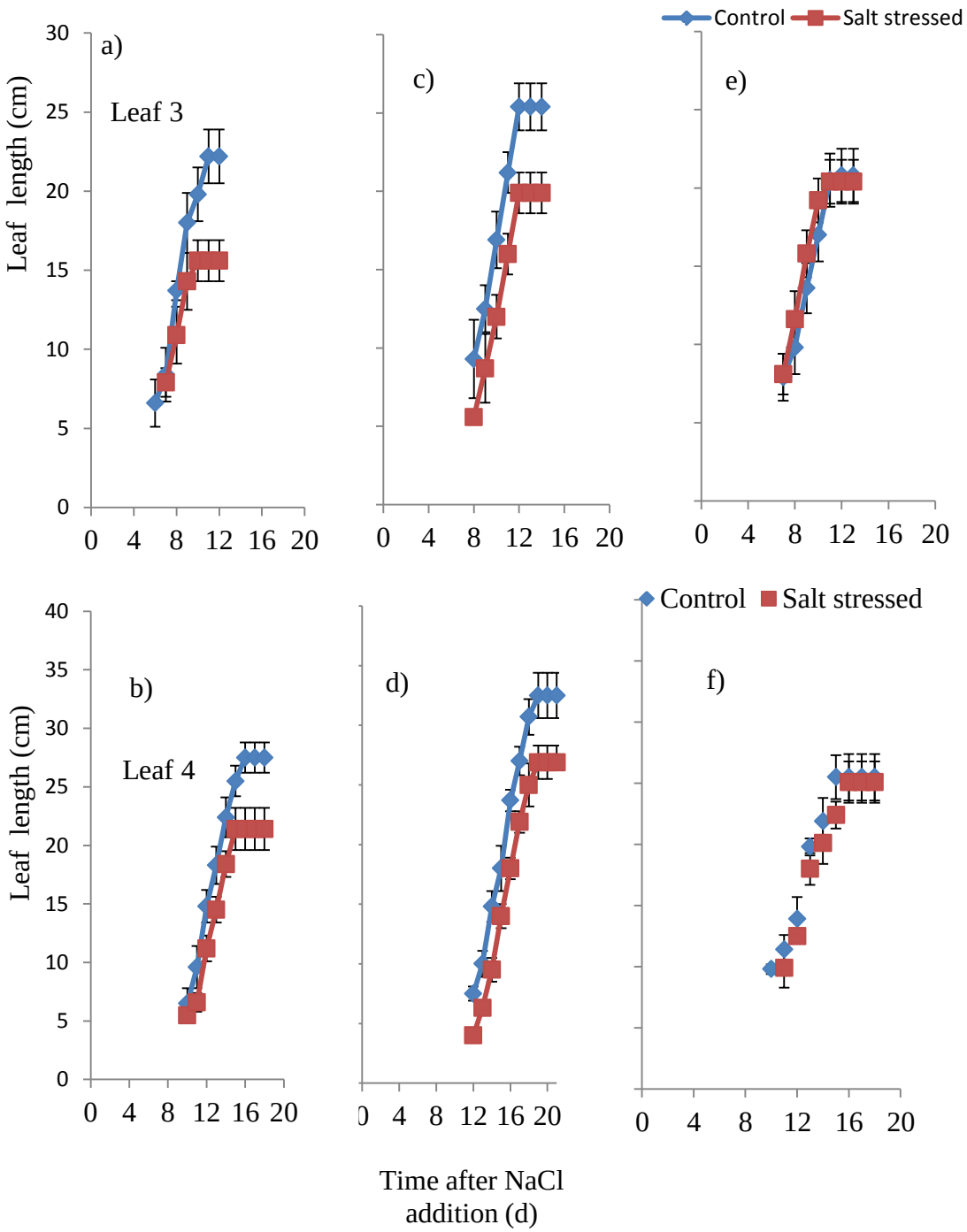

Fig. 3.2 Time course of leaf 3 and 4 length of Hereward (a - b), Gamina (c - d) and Bohoth 105 (e - f) for 0mM control and following application of 60 mM NaCl. Other details as for Fig 3.1.

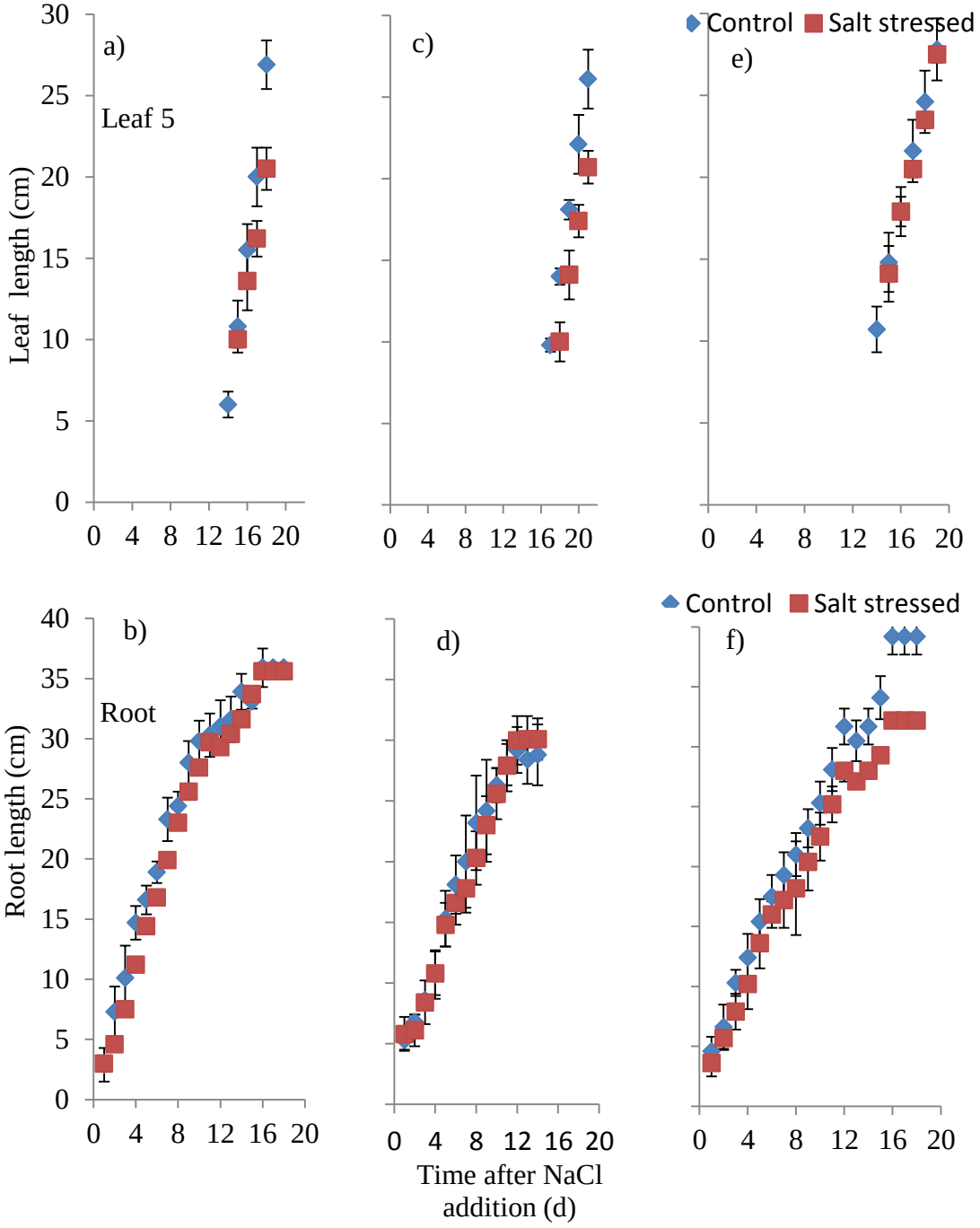

Fig. 3.3 Time course of leaf 5 length and roots of Hereward (a - b), Gamina (c - d) and Bohoth 105 (e - f) for 0 mM control and following application of 60 mM NaCl. Other details as for Fig 3.1.

Plant organs	NaCl (mM)	Final leaf length					
		Hereward		Gamina		Bohoth 105	
		(cm)	Reduction %	(cm)	Reduction %	(cm)	Reduction %
Leaf 1	0	18.1 ± 0.8^{a}	32	17.5 ± 1.2^{c}	10	15 ± 1.3^{e}	0
	60	12.4 ± 0.5^{b}		15.8 ± 1.3^{d}		15 ± 1.6^{e}	
Leaf 2	0	23.7 ± 1.8^{a}	25	24.8 ± 1.7^{c}	17	19 ± 1.6^{e}	0
	60	17.8 ± 1.4^{b}		20.6 ± 1.2^{d}		19 ± 1.7^{e}	
Leaf 3	0	22.2 ± 1.7^{a}	29	25.4 ± 1.5^{c}	22	20.8 ± 1.7^{e}	2
	60	15.6 ± 1.3^{b}		19.9 ± 1.3^{d}		20.4 ± 1.4^{e}	
Leaf 4	0	27.5 ± 1.3^{a}	21	32.5 ± 1.9^{c}	17	25.5 ± 1.9^{e}	2
	60	21.8 ± 1.8^{b}		26.9 ± 1.4^{d}		25.1 ± 1.7^{e}	
Leaf 5	0	26.9 ± 1.5^{a}	24	26.1 ± 1.8^{c}	21	27.8 ± 1.9^{e}	0.4
	60	20.5 ± 1.3^{b}		20.7 ± 1^{d}		27.5 ± 0.5^{e}	
Root	0	35.9 ± 1.6^{a}	0	29.2 ± 2^{c}	0.4	39.2 ± 1.5^{e}	10.3
	60	35.6 ± 1.7^{a}		30.1 ± 1^{c}		32.2 ± 1.8^{f}	

Table 3.1 Final length of leaves 1 to 5 and root of Hereward, Gamina and Bohoth 105 for 0 mM control and following application of 60 mM NaCl (from Figs 3.1 - 3.3). From the table, salt stress had a strong, a moderate and no effect (all roots) on final leaf length of Hereward, Gamina and Bohoth 105 wheat varieties respectively. The letters a, b, c, d, e and f are comparing between treatments. Means followed by the same letters are not significantly different at $P > 0.05$. (n = 12 in all cases).

Plant organs	NaCl (mM)	Leaf growth rate					
		Hereward		Gamina		Bohoth 105	
		cm day $^{-1}$	Reduction %	cm day $^{-1}$	Reduction %	cm day $^{-1}$	Reduction %
Leaf 1	0	3.6 ± 0.5^{a}	31	3.5 ± 0.5^{c}	17	3.1 ± 0.4^{e}	3.2
	60	2.5 ± 0.4^{b}		2.9 ± 0.4^{d}		3.0 ± 0.4^{e}	
Leaf 2	0	3.6 ± 0.4^{a}	28	3.8 ± 0.5^{c}	11	3.5 ± 0.4^{e}	0
	60	2.6 ± 0.4^{b}		3.1 ± 0.4^{d}		3.5 ± 0.4^{e}	
Leaf 3	0	4 ± 0.6^{a}	33	4.2 ± 0.6^{c}	17	3.2 ± 0.6^{e}	0
	60	2.7 ± 0.5^{b}		3.5 ± 0.5^{d}		3.7 ± 0.5^{e}	
Leaf 4	0	4.1 ± 0.6^{a}	22	3.6 ± 0.5^{c}	8.3	3.3 ± 0.6^{e}	0
	60	3.2 ± 0.5^{b}		3.3 ± 0.5^{d}		3.6 ± 0.5^{e}	
Leaf 5	0	4.7 ± 7.1^{a}	28	4.1 ± 0.7^{c}	15	3.3 ± 7.1^{e}	6.1
	60	3.4 ± 0.7^{b}		3.5 ± 0.5^{d}		3.1 ± 0.7^{e}	
Root	0	3 ± 1.6^{a}	0	2.4 ± 2.1^{c}	4.1	2.3 ± 1.7^{e}	9
	60	2.9 ± 1.7^{a}		2.3 ± 1.9^{c}		2.1 ± 1.9^{e}	

Table 3.2 Growth rate of leaves 1 to 5 and roots of Hereward, Gamina and Bohoth 105 for 0 mM control and following application of 60 mM NaCl (from Figs 3.1 - 3.6). The leaf growth rate (cm day $^{-1}$) was measured from the daily measurements of leaf length as the slope of the linear regression during the linear leaf growth phase (see Figs 3.4 – 3.6). From the table, salt stress had a strong, moderate and no effect (all roots) on the leaf growth rate of Hereward, Gamina and Bohoth 105 wheat varieties respectively. The letters a, b, c, d, e and f are comparing between treatments. Means followed by the same letters are not significantly different at $P < 0.05$ (n = 12 in all cases).

3.3 Root Growth

Salt stress did not affect final root length of either Hereward and Gamina, nor the root growth rate of any of the three varieties. However, there was a significant difference in the final root length of Bohoth 105, although not in root growth rate.

A significant difference between varieties in final root length under both control and salt treatments was observed. The statistical interaction between varieties and treatment effects was also significantly different (Table 3.1 and 3.2).

3.4 Effect of 60 mM NaCl on Leaf Plastochron Index

The relations between leaf length and time were calculated using linear regression analysis. The growth pattern of each leaf of the some plant is compared in the plots Fig. 3.4 to 3.6. These show the same data as Fig. 3.1 – 3.3, but with only the linear section of the growth phase included. In each variety, a reference length for all the examined leaves (the mean of all the final half-lengths) was calculated by Equation 3.1.

$$L_{ref.} = \frac{1}{n}\sum_{i=1}^{n}\frac{Li}{2}, \quad (3.1)$$

Where $L_{ref.}$ is reference length of leaves, n is number of leaves, L is final leaf length, i = 1, 2, 3,……n. n = 5

Using this equation (3.1), in control and salt treatments respectively, reference lengths ($L_{ref.}$) were 11.84 cm and 8.81 cm (Hereward), 12.63 cm and 10.39 cm (Gamina) and 10.81cm and 10.7 cm (Bohoth 105). The linear fit for each leaf-length time course and the reference length (Fig. 3.4 to 3.6) was used to estimate the Plastochron Index (PI). Erickson (1976) described PI as the period between the initiations of sequential pairs of leaves.

For the three varieties, no significant differences were noticed in PI between control and salt treatments (P= 0.988). The interactions between varieties and treatments were also not significant (P= 0.958). However, the PI values were highest in the control and salt treatments of Gamina, and the lowest values were in Bohoth 105.

Leaves	Plastochron Index (d)					
	Hereward		Gamina		Bohoth 105	
	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed
Leaf 1 and leaf 2	1.60	1.40	2.31	2.64	1.82	1.94
Leaf 2 and leaf 3	2.80	3.06	3.31	3.83	2.4	2.73
Leaf 3 and leaf 4	3.86	4.06	4.61	4.62	3.55	3.59
Leaf 4 and leaf 5	3.84	3.37	4.16	3.95	3.39	2.53
Mean ± SD	3.03 ± 1.1^{a}	3 ± 1.1^{a}	3.6 ± 1^{c}	3.8 ± 0.8^{c}	2.8 ± 0.8^{e}	2.7 ± 0.7^{e}

Table 3.3 Plastochron Index of leaves 1 to 5 of Hereward, Gamina and Bohoth 105 for the 0 mM control treatment and following application of 60 mM NaCl. The letters a, b, c, d, e and f are comparing between treatments in the same variety. Means followed by the same letters are not significantly different at P >0.05.

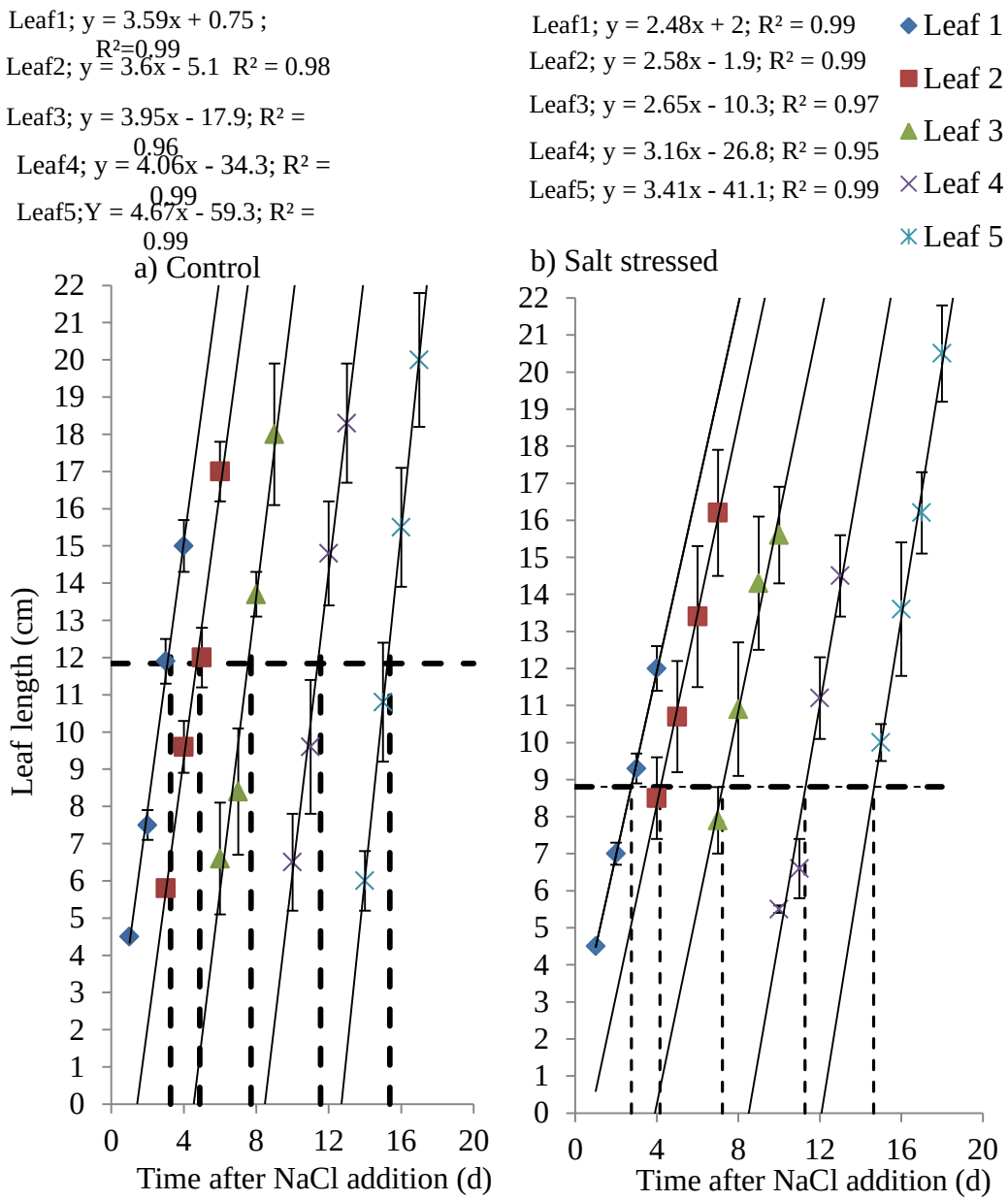

Fig. 3.4 Time course of lengths of successive leaves 1 to 5 of Hereward for 0 mM control and following application of 60 mM NaCl. Each curve applies to a single leaf. The date are those from Figs. 3.1a – b to 3.3 a - b but, only the points on the linear phase of the growth curve are included. (Note: Erickson (1976) used a logarithmic transformation for this. In current work, this was found to give a poor fit of the data and was not used). Fig. 3.1a – b, Fig. 3.2a – b and Fig.3.3a are plotted against time in Fig. 3.4. The growth rate values (slope) for leaves are given in Table 3.2. The equations for the period of linear growth for each line with R^2 values are included. Each point is the mean ± SD of 12 leaves taken from 12 individual plants. A perpendicular dashed line between two successive leaves represent the time point used to estimate the leaf Plastochron Index. The horizontal line is the $L_{ref.}$. The mean leaf Plastochron Index in both control and salt stressed plants is 3 ± 1 days (Table 3.3).

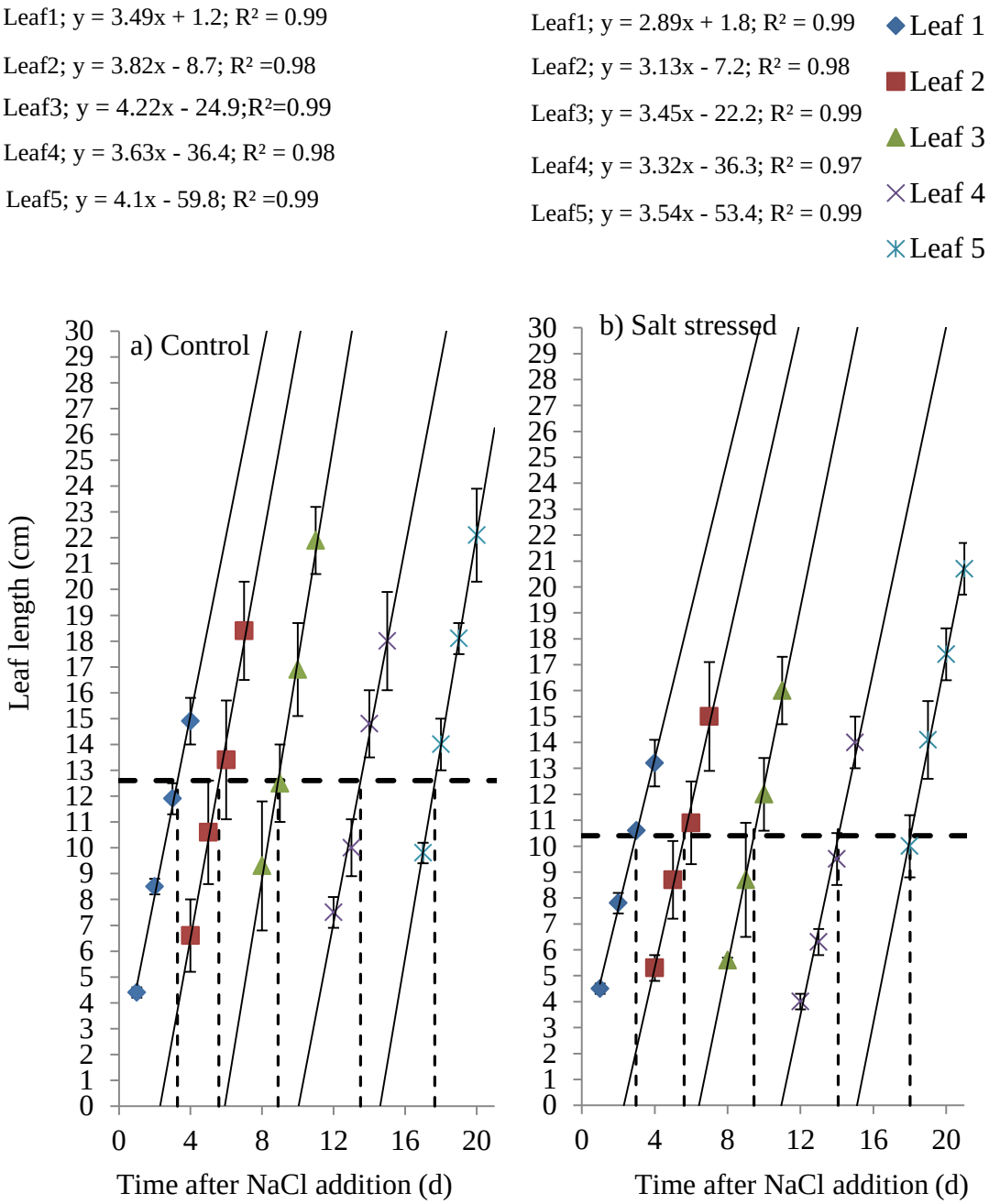

Fig. 3.5 Time course of lengths of successive leaves 1 to 5 of Gamina for 0 mM control and following application of 60 mM NaCl. Each growth curve applies to a single leaf. Leaves length in Fig. 3.1c- d, Fig. 3.2c – d and Fig. 3.3c plotted against time in Fig. 3.5. The mean leaf Plastochron Index in control was 3.6 ± 1 and in salt stressed 3.8 ± 0.8 (Table 3.3). Details as in Fig. 3.4.

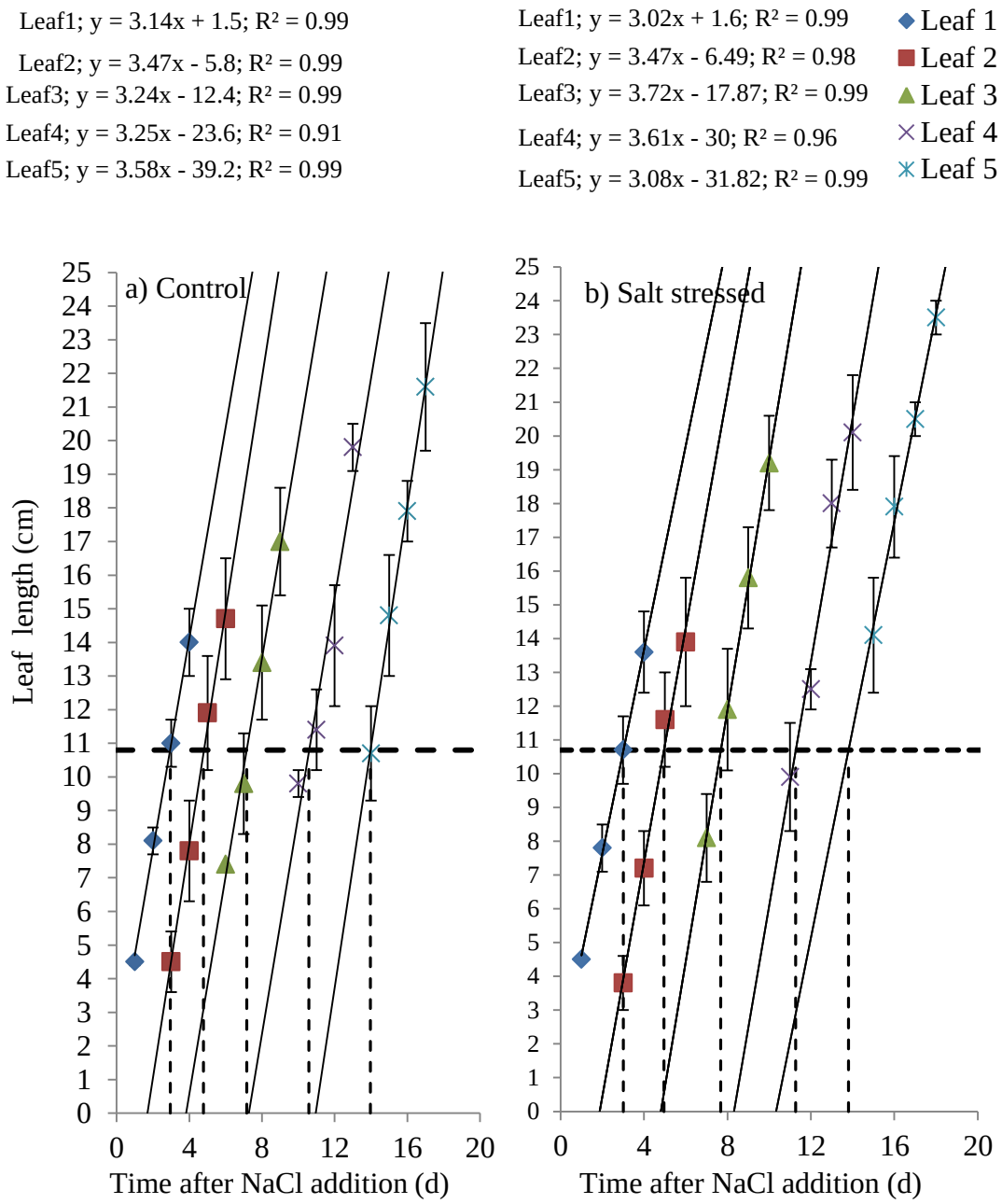

Fig. 3.6 Time course of lengths of successive leaves 1 to 5 of Bohoth 105 for control and following application of 60 mM NaCl. Each growth curve applies to a single leaf. Leaves length in Fig.3.1e – f, Fig.3.2e – f and Fig.3.3e are plotted against time in Fig. 3.6. The mean Plastochron index in control is 2.8 ± 0.8 and a salt treatment is 2.7 ± 0.7 (Table 3.3). Details as in Fig. 3.4).

3.5 Discussion

Our results show that NaCl had an adverse effect on final leaf length and growth rate of all varieties of wheat except Bohoth 105. The parameters were significantly reduced by salt stress in both Hereward and Gamina, but Hereward suffered the largest reduction (Table 3.1 and 3.2), indicating that the growth inhibitory effect of salt stress was stronger in Hereward as compared to Gamina. In contrast, the parameters of Bohoth 105 were not affected by salt stress (Table 3.1 and 3.2), suggesting that Bohoth 105 is the most salt tolerant, with Hereward the most salt sensitive and Gamina in the middle.

However, for all three varieties, neither root growth rate nor leaf Plastochron Index were affected by salt stress (Table 3.1 and 3.2). Final root length, was not affected by salt stress in Hereward or Gamina, but there was a noticeable effect in Bohoth 105.

Salt leads to growth reduction across crop species along a spectrum of responses (Brady & Weil, 1996). The cause of the reduction in final leaf length and leaf growth rate may be related to the toxic ion effect of salt and the imbalance of nutrient quantities in wheat. Chinnusamy *et al*. (2005) attribute retarded plant growth predominantly to toxic concentrations of ions rather than osmotic stress. There are, however, genetic differences in how well different species and genotypes respond and adapt to increased salinity (Wahid *et al*., 1997).

The effect of salt stress has been investigated on the growth of sorghum by Bernstein *et al.* (1993) , *Pinus pinaster* (-3 bar) by Triboulot *et al.* (1995), barley by Fricke and Peters (2002), maize by de Zevedo *et al.* (2004), and wheat by James *et al.* (2008). Sattar *et al*. (2010) found that growth reduction results from high levels of NaCl due

to either decreased plant ability to uptake water as a result of osmotic stress, or plant nutrient imbalance, or both.

The response of three wheat varieties to NaCl stress was also examined in order to evaluate the relative contribution of the different biochemical parameters. The results are reported in Chapter 4.

Chapter 4: Turgor, Osmolality and Cations in Wheat Leaves, and their Response to Salt

4.1 Introduction

Different plant varieties exhibit varying abilities to adapt to saline conditions, and there are a range of mechanisms for sustaining growth under salt stress. Crop maintenance in unfavorable conditions is of particular interest. Many studies have found great variety in salt resistance in different species and varieties of plants (Wahid *et al*., 1997; Ashraf & Foolad, 2007). Salt tolerance is the term used to refer to a plant's ability to sustain growth in saline environments, but this can be achieved through a range of different specific mechanisms (Munns & Tester, 2008), including osmotic adjustment, preferential uptake of sodium or potassium ions, the exclusion of salt, and the compartmentalising of solutes.

There can also be a difference in the way that parts of the plant respond, with a possible variation in water relation parameters between mature regions compared to growing regions of the plant. In wheat, for example, Pritchard *et al*. (1989) found lower cortex P_{cell} in mature root cells than in the growing region. Elsewhere, growing zone cells have been found to have lower ψ_w than older regions (Westgate & Boyer, 1985). This imbalance between ψ_w in differently-aged parts of the plant and between the growing zone and the vascular system could indicate a water potential gradient used for water supply.

Having established in Chapter 3 that the three varieties of wheat under investigation exhibit a demonstrable range of responses to salt stress (from Hereward, the most salt sensitive, to Bohoth 105, the least), the bulk of the work on wheat involved analysing

details of their water and solute relations. The hope was to discover differences or similarities that would indicate underlying mechanisms. In this chapter, the responses to salt stress of turgor (P_{cell}), osmolality (π_{cell}) and cations were analysed in wheat leaves. A cell pressure probe, associated with single cell sampling and analysis (SiCSA) [picoliter osmometer (freezing point depression), and capillary zone electrophoresis (CZE)], was used to measure these parameters in single epidermal cells (abaxial). The varieties Hereward, Gamina and Bohoth 105 were used. The first leaf to emerge from intact plants *in situ* was studied at 2 - 8 days after NaCl (60 mM) addition, and at 2, 5 and 8 days for the control (0 mM NaCl) treatment. Plants were grown in a hydroponic medium under controlled conditions. All methods are given in Chapter 2.

4.2 Turgor Pressure in Epidermal Cells

Figure 4.1a - c illustrates changes in P_{cell} values in response to 0 mM and 60 mM NaCl, for the period 48 hours after addition. Table 4.1 provides the values for turgor at key points in these graphs. Notably, all three varieties had similar P_{cell} values under control the condition (7.5 ± 1 bar for Hereward, 7 ± 0.03 bar for Gamina and 8.1 ± 0.8 bar for Bohoth 105). However, the effect of 60 mM NaCl on P_{cell} was significantly different at the 5 % level in cells of each variety. The amount of increase varied from 8.7 ± 1.7 bar for Hereward (Fig. 4.1a) to only 2.7 ± 0.6 bar for Gamina (Fig. 4.1b).

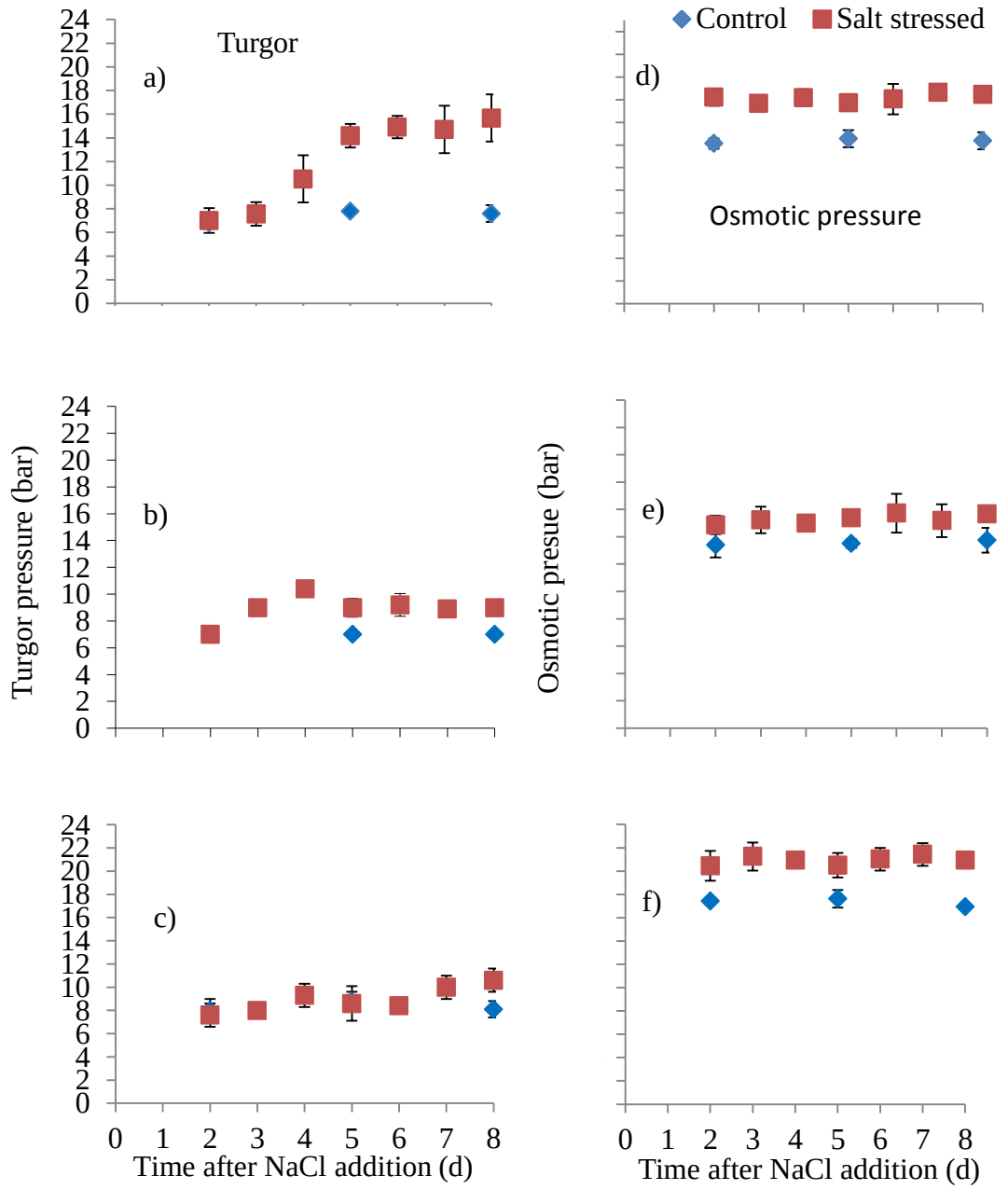

Fig.4.1 Time course of leaf epidermal (mature) cell turgor pressure (P_{cell}) and osmotic pressure (π_{cell}) of Hereward (a and d), Gamina (b and e) and Bohoth 105 (c and f) for 0 mM control and following application of 60 mM NaCl. Salt was added in two 30 mM doses over two days (the days are numbered after the first addition). Cell turgor pressure was measured using a cell pressure probe. Cell osmotic pressure was measured using single cell sampling, and picolitre osmometer (P= 0.000 two way ANOVA of day 2, 5 and 8). Each point is the mean ± SD of 3 to 12 cells taken from 3- 6 individual plants.

4.3 Osmotic Pressure in Epidermal Cells

Under the control condition, each variety maintained a constant $\pi_{cell.}$ When salt was added, π_{cell} rapidly increased in all varieties. By the end of the 48 hour period of addition, this change was complete and no further change in π_{cell} was seen. Table 4.2

and Fig. 4.1d - f show mean π_{cell} values at 2 and 8 days after the 48 hour NaCl addition (0 mM and 60 mM NaCl). For the controls, the values were 14.4 ± 0.3 bar for Hereward, 13.6 ± 0.3 bar for Gamina, and 17.5 ± 0.3 bar for Bohoth 105.

The values of π_{cell} for the three varieties were significantly different under salt stress in comparison to the control treatment. For example, π_{cell} values for 0 mM and 60 mM NaCl at day 2 in Bohoth 105 were 17.5 ± 0.3 bar and 20.5 ± 0.4 bar respectively ($P \leq 0.05$). This difference of 1.3 – 4.1 bar (Table 4.2) is of similar magnitude to the increase in π of the external root medium (60 mM NaCl ≈ 3 bar). The effects of salt stress on π_{cell} were also significantly different between Hereward, Gamina and Bohoth 105 (Table 4.2).

For Hereward, the difference in π_{cell} value between the two treatments ($\Delta\pi_{cell}$) was slightly higher (3.7 - 4.1 bar) than for the other two varieties, and for Gamina the difference was the smallest (1.3 – 2.1 bar) (Table 4.2). Hereward and Bohoth 105 cells, therefore, had accumulated more solutes than Gamina both by day 2 and by day 8. This difference between varieties ($P \leq 0.05$) may indicate that they have different capacities for the accumulation of solutes.

Variety	P_{cell} (bar)		
	0 mM (2 – 8 days)	60 mM (day 5)	60 mM (day 8)
Hereward	7.3 ± 1.3^{ax}	14 ± 1^{bx}	16 ± 2^{cx}
Gamina	7 ± 0.6^{ax}	9 ± 0.7^{by}	9.7 ± 0.6^{by}
Bohoth 105	7.6 ± 1^{ax}	8.6 ± 1.5^{by}	10.6 ± 1^{cz}

Table 4.1 Mean turgor pressure (P_{cell}) in epidermal cells (leaf 1) of Hereward, Gamina and Bohoth 105 seedling grown in 0 mM and 60 mM NaCl. Values are for 5 and 8 day for 60 mM and as average of day 2, 5 and 8 values for 0 mM. Turgor pressure was measured by a cell pressure probe. Results are given as means ± SD, n is 3 – 12 cells. Letters a, b and c are comparing the treatment where x, y, z are comparing variety level. Means followed by the same letters are not significantly different at $P>0.05$.

variety	π_{cell}			$\Delta\pi_{cell}$	
	(bar)				
	0 mM (2 – 8 days)	60 mM (day 2)	60 mM (day8)	(day 0 -2)	(day 0 - 8)
Hereward	14.4 ± 0.3^{ax}	18.1 ± 0.7^{bx}	18.5 ± 0.2^{bx}	3.7	4.1
Gamina	13.6 ± 0.3^{ay}	14.9 ± 0.1^{by}	15.7 ± 0.5^{cy}	1.3	2.1
Bohoth 105	17.5 ± 0.3^{az}	20.5 ± 0.4^{bz}	20.9 ± 0.2^{bz}	3	3.4

Table 4.2 Mean osmotic pressure (π_{cell}) for 0 mM (days 2 - 8) and 60 mM (day 2 and 8) in epidermal cells (leaf 1) of Hereward, Gamina and Bohoth 105. Osmotic pressure (π_{cell}) was measured using single cell sampling, and a picolitre osmometer. Results are given as means ± SD, n is 3 - 12 cells. (P= 0.000 ANOVA of day 2, 5 and 8). Letters a, b and c compare the treatment level and x, y and z compare variety level. Means followed by the same letters are not significantly different at (P>0.05).

4.4 Transpiration Tension in the Apoplast

The increase in P_{cell} was only 1.5 bar, but if hydrostatic tension were an important part of the water potential of the cell wall, then the P_{cell} would be expected to be much higher. This unexpected lack of correspondence between P_{cell} and π_{cell} could be due to a change in transpiration tension in the epidermal apoplast. To test this, the effect of such apoplast (cell wall) transpiration tension on P_{cell} was studied at day 8 after 60 mM NaCl addition, compared to a control of 0 mM NaCl addition (Fig. 4.2). To achieve this, the entire seedling was immersed for 30 minutes under the hydroponic medium and the turgor was measured under medium. The data in Table 4.3 show values for P_{cell} in transpiring and non-transpiring epidermal cells at day 8. After immersion, the P_{cell} was seen to have risen. It was assumed that this corresponded to the relaxation of the transpiration tension. However, all varieties showed a significant difference in P_{cell} between transpiration and non-transpiration in all treatments (Table 4.3). All varieties in both treatments appear to show a transpiration tension of approximately 1.5 bar.

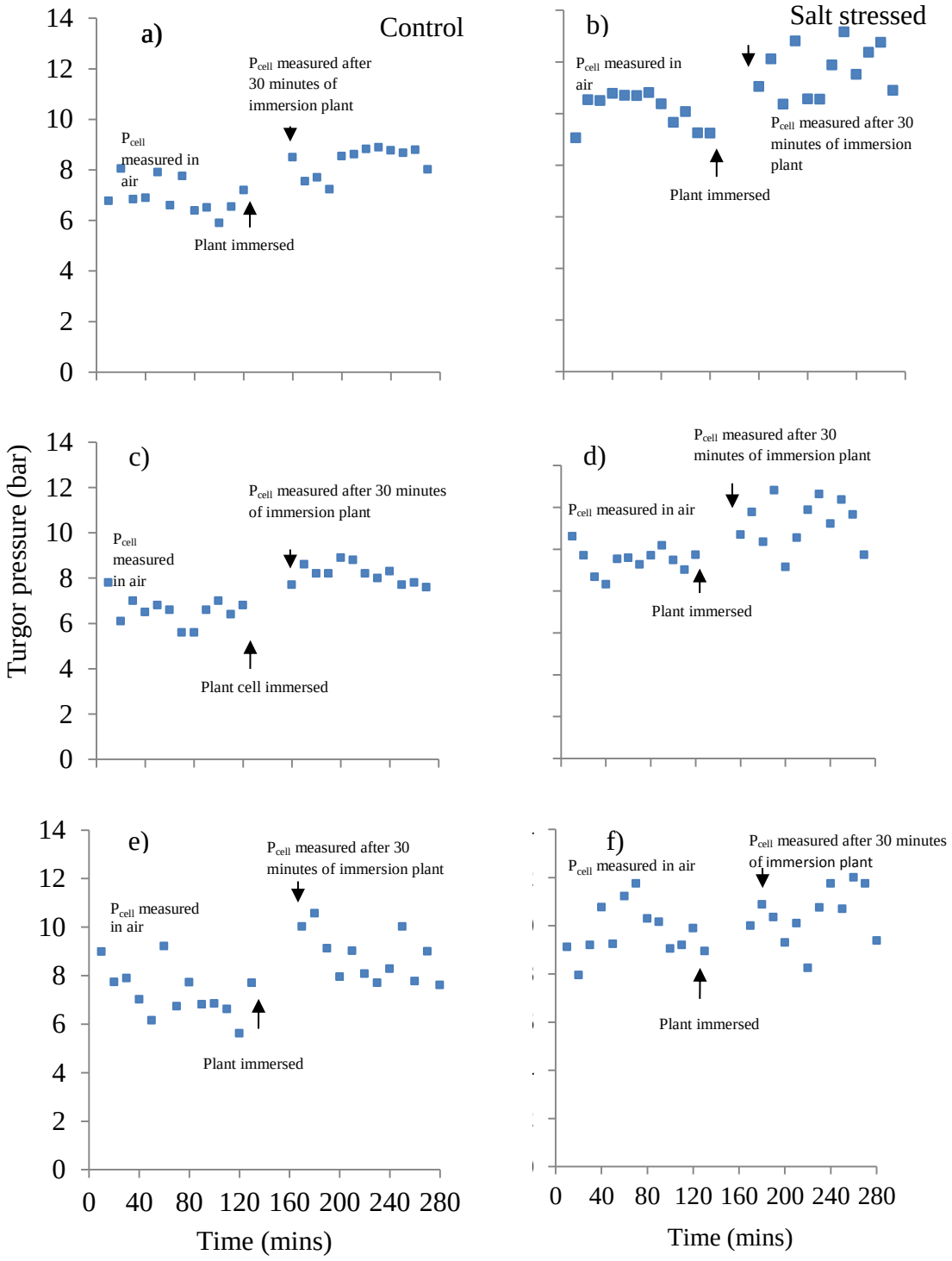

Fig.4.2 Mean turgor pressure (P_{cell}) in transpiring and non-transpiring single epidermal cells (leaf 1) of Hereward (a and b), Gamina (c and d) and Bohoth 105 (e and f) at day 8 after salt addition. Seedlings were grown in 0 mM and 60 mM NaCl. Turgor pressure was measured in air and under a liquid bathing medium (hydroponic root medium) every 10 minutes for 120 minutes for each treatment and each situation. Turgor was measured before and after immersion, using a cell pressure probe. Cells taken from 3 - 6 individual cells.

Variety	NaCl (mM)	P_{cell} (bar) at day 8		ΔP_{cell} (bar)
		Transpiring	Non-transpiring	
Hereward	0	6.9 ± 0.7^{a}	8.3 ± 0.6^{b}	1.4
	60	10.2 ± 0.7^{a}	11.7 ± 1^{b}	1.5
Gamina	0	6.6 ± 0.6^{a}	8.2 ± 0.4^{b}	1.6
	60	9.5 ± 0.6^{a}	11.2 ± 1.2^{b}	1.7
Bohoth 105	0	7.3 ± 1^{a}	8.8 ± 1^{b}	1.5
	60	10.2 ± 1^{a}	11.6 ± 0.6^{b}	1.4

Table 4.3 Mean turgor pressure (P_{cell}) in transpiring and non-transpiring epidermal cells (leaf 1) of Hereward, Gamina and Bohoth 105. Seedlings were grown in 0 mM and 60 mM NaCl. Turgor pressure was measured in air and under a liquid bathing medium (hydroponic root medium) every 10 minutes for 120 minutes for both treatments. Turgor was measured before and after immersion by using a cell pressure probe (see Fig. 4.2). The letters a and b indicate a statistical difference ($P>0.05$) between transpiring and non-transpiring conditions, for each variety and each treatment. Means followed by the same letters (not present here) are not significantly different.

4. 5 Cations in Epidermal Cells

The change in π_{cell} reported in Section 4. 2 must be due to a change in cell solutes. In order to investigate the nature of the cations contributing to osmotic adjustment, capillary zone electrophoresis (CZE) was performed on cell samples (Table 4.4).

4.5.1 Na^+ Concentration

Notably, all three varieties had a Na^+ control concentration of approximately 14 mM (Fig. 4.3). As expected from the π_{cell} data (Fig. 4.1f), Bohoth 105 was observed to have accumulated significantly ($P \leq 0.05$) higher Na^+ concentration by 2 days after salt addition. This then remained more or less at 140 mM for the remaining experimental period. Hereward had low concentrations under salt stress of around 30 mM whereas in Gamina, Na^+ concentration level increased gradually with time, reaching 68 ± 4 mM by day 8.

The comparison of Na^+ concentration was obtained from single cells and bulk leaves. The results show that similar Na^+ concentrations were found in single cells and bulk leaves of Hereward and Gamina, but in Bohoth 105 Na^+ concentration in single cells was less than in bulk leaves (Fig. 4.3 and 4.8).

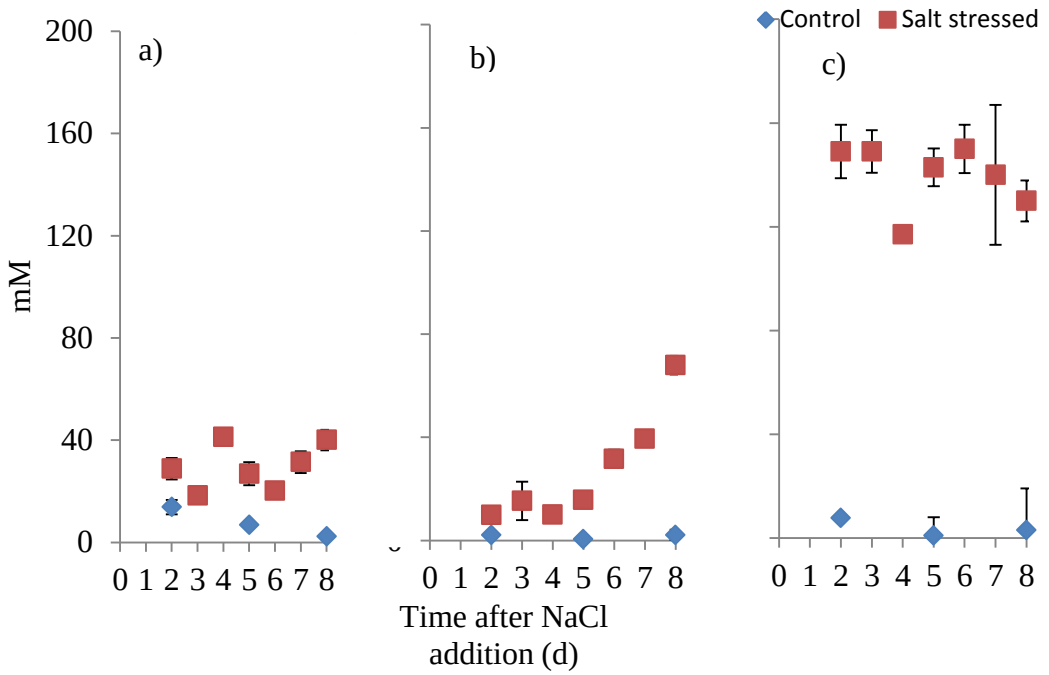

Fig.4.3 Na^+ concentration in epidermal cell sap of Hereward (a), Gamina (b) and Bohoth 105 (c) leaves. The first leaf was analysed the first day after NaCl addition. Salt was added in two 30 mM doses over two days (the days are numbered after first addition). Sodium concentration was measured using capillary zone electrophoresis (CZE). Each observation is the mean of 3 - 12 cells taken from 3 - 6 individual plants (*P*= 0.000 ANOVA of day 2, 5 and 8). Results are given as means ± SD. Bohoth 105 accumulated more Na^+ in cells. Na^+ uptake had the highest contribution to osmotic adjustment in Bohoth 105.

4.5.2 K^+ Concentration

Generally, in the control plants, K^+ concentrations were found to be the highest as compared with other ions (Fig. 4.4). The concentration was found to be around 270 mM in both Hereward and Gamina, and higher in Bohoth 105 (around 315 mM).

However, when NaCl was added to root media, whilst K^+ concentration increased in Hereward to approximately 322 mM (significant difference P=0.00, days 2, 5 and 8), in Bohoth 105 it had decreased by day 2 (significant difference P= 0.000) and then remained more or less constant (at approximately 277 mM) for the rest of the experimental period. Under salt stress, K^+ was significantly accumulated only in epidermal cells of Hereward (Fig. 4.4). Hereward inhibits Na^+ uptake to the leaf cells (i.e. through Na^+ exclusion) and maintains high selectivity of K^+ over Na^+ (Fig. 4.3 - 4.4). In Gamina, K^+ concentration immediately increased after stress, reaching 286 ± 4 mM by day 4. It then gradually decreased for the remaining experimental period. These results show that Hereward absorbed more K^+ under salt stress (60 mM NaCl) in leaf epidermal cells in comparison to the other two varieties.

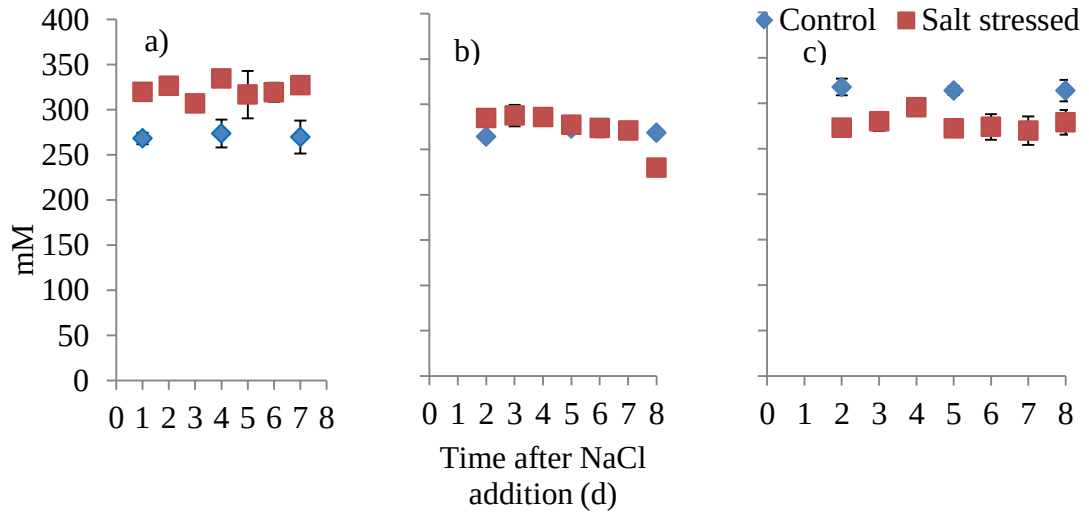

Fig. 4.4 K^+ concentration in epidermal cell sap of Hereward (a), Gamina (b) and Bohoth 105 (c) leaves. Details as in Fig. 4.3 (except for K^+). Potassium uptake had a higher contribution to osmotic adjustment in Hereward (58 mM) and had a partial contribution in the Gamina (5 mM) variety.

4.5.3 Ca^{2+} Concentration

In general, in Gamina and Bohoth 105 varieties, salt stress resulted in a significant reduction (P=0.000) in Ca^{2+} concentration level. In contrast, salt stress did not affect Ca^{2+} concentrations in epidermal cells of Hereward (P=0.770) (Fig. 4.5).

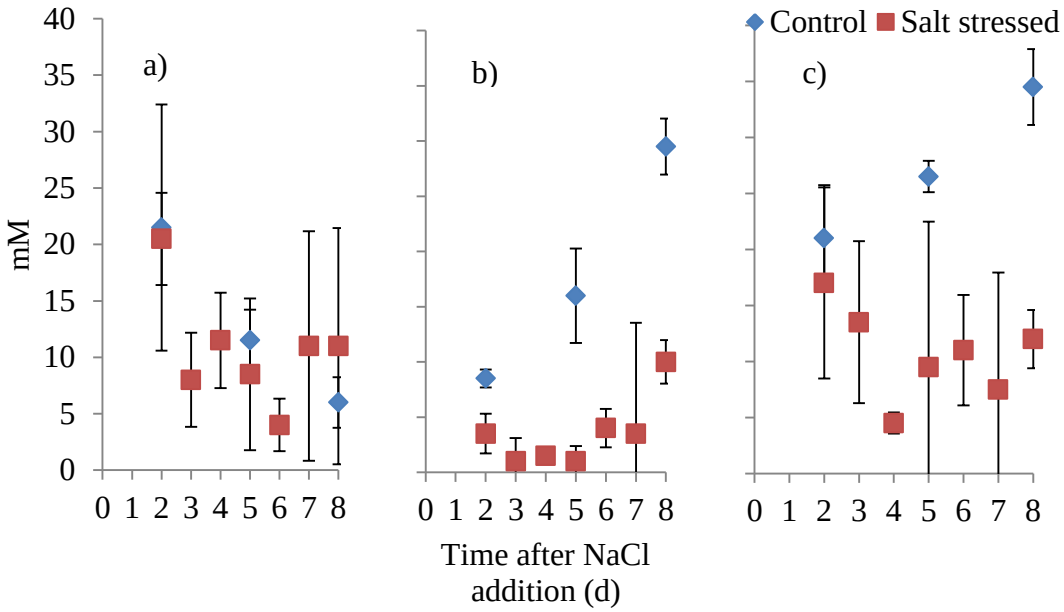

Fig. 4.5 Ca^{2+} concentration in epidermal cell sap of Hereward (a), Gamina (b) and Bohoth 105 (c) leaves. Details as in Fig. 4.3 (except for Ca^{2+}).

4.5.4 Mg^{2+} Concentration

Magnesium concentration was measured and was observed to be nearly identical (less than 6 mM) in all varieties after salt addition throughout the experimental period (Fig 4.6). Salt stress resulted in a slight reduction in Mg^{2+} concentration in Hereward (P= 0.024) and Bohoth 105 (P=0.044), but not in Gamina P=0.868). The cell level of Mg^{2+} in control conditions was less than 2 mM in Hereward, while in Gamina and Bohoth 105 it reached around 7 mM and 14 mM respectively. Under control conditions of 0 mM NaCl addition, Na^{+}, $Ca^{2+}$and Mg^{2+} were available in small amounts, whereas K^{+} was the main osmotica.

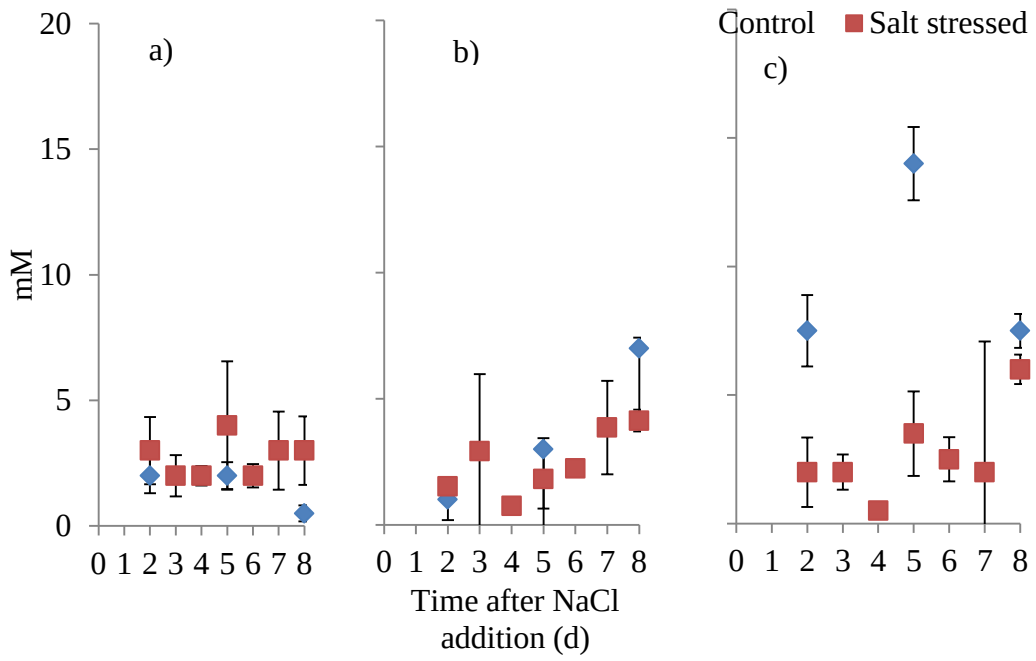

Fig. 4.6 Mg^{2+} concentration in epidermal cell sap of Hereward (a), Gamina (b) and Bohoth 105 (c) leaves. Details as in Fig. 4.3 (except for Mg^{2+}).

Variety	Time after NaCl addition (d)	Solute concentrations (mM)								∑solutes (mM)		∑charge cations (mEq)		Osmolality (mOsmol.kg-1)		π_{total}/γ		∑anions (mM)		Average charge of anions	
		Na^+		K^+		Ca^{2+}		Mg^{2+}													
		Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed
Hereward	2	14 ± 3	29 ± 4	268 ± 6	320 ± 9	22 ± 11	21 ± 4	2 ± 1	3 ± 1	306	373	330	396	565 ± 6	729 ± 10	606	782	300	410	1.1	1.0
	3		18 ± 2		326 ± 7		8 ± 4		2 ± 1		354		363		707 ± 3		759		405		0.9
	4		41 ± 2		307 ± 5		12 ± 4		2 ± 0		362		376		727 ± 10		780		418		0.9
	5	7 ± 1	27 ± 5	274 ± 15	335 ± 7	12 ± 3	9 ± 7	2 ± 1	4 ± 3	295	375	309	387	582 ± 10	709 ± 8	624	761	329	386	0.9	1.0
	6		20 ± 2		317 ± 26		4 ± 2		2 ± 0		343		348		712 ± 18		764		421		0.8
	7		31 ± 4		319 ± 10		11 ± 10		3 ± 2		364		378		746 ± 9		800		436		0.9
	8	2 ± 1	40 ± 4	270 ± 18	327 ± 3	6 ± 2	11 ± 10	0.5 ± 0	3 ± 1	279	381	285	394	575 ± 10	738 ± 7	617	792	338	411	0.8	1.0
Gamina	2	2 ± 1	10 ± 1	264 ± 2	285 ± 4	9 ± 1	4 ± 2	1 ± 0.3	1 ± 0.4	276	300	285	304	536 ± 13	595 ± 9	575	638	300	339	1.0	0.9
	3		15 ± 7		287 ± 12		1 ± 2		2 ± 3		305		307		609 ± 13		653		349		0.9
	4		10 ± .4		286 ± 4		2 ± 0.3		1 ± 0.1		298		300		600 ± 8		644		346		0.9
	5	0.5 ± 1	16 ± .5	273 ± 4	277 ± 3	16 ± 4	1 ± 1	3 ± 0.4	1 ± 1	293	295	312	297	540 ± 10	615 ± 3	579	660	287	365	1.1	0.8
	6		32 ± .3		273 ± 10		4 ± 2		1 ± .3		310		315		629 ± 19		675		365		0.9
	7		39 ± 1		271 ± 9		4 ± 10		2 ± 2		316		321		607 ± 16		651		336		1.0
	8	2 ± 2	68 ± 3.6	268 ± 3	230 ± 4	30 ± 3	10 ± 2	7.5 ± 0.4	2 ± 0.4	307	310	343	322	550 ± 13	627 ± 7	590	673	284	363	1.2	0.9
Bohoth 105	2	8 ± 2	140 ± 10	318 ± 9	263 ± 8	21 ± 5	17 ±9	7.5 ± 1	2 ± 1	355	419	383	435	695 ± 4	818 ± 17	746	878	391	459	1.0	1.0
	3		149 ± 8		280 ± 10		14 ± 7		2 ± 1		445		460		850 ± 16		912		468		1.0
	4		117 ± 3		296 ±8		4 ± 1		0.5 ± 0		418		423		837 ± 9		898		480		0.9
	5	1 ± 0	143 ±7	314 ± 5	272 ± 3	27 ± 1	10 ± 13	14 ± 1	3.5 ± 2	356	428	396	441	697 ± 3	820 ± 14	748	880	392	452	1.0	1.0
	6		150 ± 9		274 ± 14		11 ± 5		2.5 ± 1		438		451		841 ± 13		902		465		1.0
	7		140 ±27		270 ± 16		7 ± 10		2 ± 5		419		429		857 ± 13		920		500		0.9
	8	3 ± 1	130 ± 8	314 ± 12	279 ± 14	35 ± 3	12 ± 3	7.5 ± 1	6 ± 1	359	427	401	445	702 ± 2	837 ± 3	753	898	394	471	1.0	0.9

Table 4.4 Cation concentrations and osmolality of the same sap of single epidermal cells of Hereward, Gamina and Bohoth 105 leaves respectively. Epidermal cells (n = 3 - 12) were sampled from the first leaves of three to six individual plants. Cell sap was analysed every day during the salt treatment and three times during the 7 days of the control treatment. Calculations of the contribution of cations to the cell osmotic pressure (π_{cell}) were made by multiplying the sum of the concentrations by the osmotic coefficient (γ) of 0.93 of NaCl (Robinson & Stokes, 2002). The values of ≈ 1.0 for the average anion charge suggest monovalent ions (Cl^- or NO_3^-). Results are given as means ± SD.

4.6 Cations in Epidermal Cells of three Varieties

The results clearly demonstrate that the contribution of cations to osmotic adjustment under stress mainly took the form of accumulations of K^+ in Hereward and Na^+ in Gamina and Bohoth 105.

4.7 Estimate of the Anion Condition and its Valency

Table 4.4 shows the distribution of cation (Na^+, K^+, Ca^{2+}, Mg^{2+}) concentrations contributing to π_{cell} in single epidermal cells.

The relationship (and its implications) between the sum of the cations and the measured π_{cell} of samples is also shown in Table 4.4. Although anions were not measured explicitly, this relationship allows an estimate of their concentration and valency (mean ionic charge). This is based in two factors. First, the sum of positive charges must equal the sum of negative charges.

$$\sum [\text{monovalent cations}] + 2 \sum [\text{divalent cations}] = \frac{\sum \text{anions}}{\text{average charge anions}} \qquad (4.1)$$

Second, the total π_{cell} must be a function of the sum of individual solutes.

π_{total} =

$\sum$cations*γ cations + $\sum$anions*γ anions + $\sum$uncharged solutes*γ uncharged solutes. (4.2)

Where omega (γ) is the solute osmotic coefficient.

While the first factor is fairly straightforward, the second is not. This has been simplified by assuming that there are no interactions between solutes, and by using molar rather than molal concentrations of the individual solutes. A value of 0.932 has also been assumed as the same representative γ for all solutes. This is actually the value for NaCl given by Robinson and Stokes (2002).

This results in the following relationship:

$$\pi_{total} = \gamma \left(\sum [\text{cations}] + \frac{\sum[\text{monovlant catios}] + 2\sum[\text{divalent cations}]}{\text{average anions charge}} + \sum[\text{uncharged}] \right) \quad (4.3)$$

$$\text{Average anions charge} = \frac{\sum[\text{monovlant catios}] + 2 * \sum[\text{divalent cations}]}{\frac{\pi \text{total}}{\gamma} - \sum[\text{cations}] - \sum[\text{uncharged}]} \quad (4.4)$$

It can be seen that the higher the concentration of (unmeasured) neutral solutes, the higher will be the apparent average anion charge (Z). The closer the value approaches unity, the lower the expected concentration of uncharged or multi-charged anionic solutes. In Table 4.4, in order to calculate the value for Z, it was assumed that there are no significant unchanged osmotica. Previous SiCSA analyses of barley epidermis have shown that it contains negligible sugars (Koroleva *et al.*, 1997).

Variety	Leaves	Cations concentrations (mM)								∑solutes (mM)		∑charge cations (mEq)		Osmolality (mOsmol.kg^{-1})		π_{total}/γ		∑anions (mM)		Average charge of anions	
		Na^+		K^+		Ca^{2+}		Mg^{2+}													
		Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed
Hereward	Young	24 ± 3	33 ± 2	214 ± 2	320 ± 3	2 ± 1	2 ± 8	3 ± 3	6.5 ± 4	243	362	248	370	474 ± 21	711 ± 26	509	763	266	401	0.9	0.9
	Middle	17 ± 2	44 ± 10	230 ± 2	317 ± 3	6 ± 1	6 ± 7	6.5 ± 3	13.5 ± 5	259	380	271	399	498 ± 27	724 ± 14	534	777	275	397	1.0	1.0
	Old	17 ± 11	46 ± 13	250 ± 8	271 ± 13	7 ± 2	4 ± 6	7 ± 5	7 ± 5	281	328	295	339	534 ± 21	627 ± 14	573	673	292	345	1.0	1.0
Gamina	Young	5 ± 6	31 ± 11	230 ± 6	252 ± 11	2 ± 1	1.5 ± 8	3 ± .5	3 ± 3	240	289	245	295	468 ± 16	565 ± 12	502	606	262	317	0.9	0.9
	Middle	8 ± 5	46 ± 2	255 ± 6	237 ± 3	8 ± 2	3 ± 1	5.5 ± 4	5 ± 4	277	292	290	300	544 ± 11	573 ± 19	584	615	307	323	0.9	0.9
	Old	0 ± 0	82 ± 23	269 ± 4	200 ± 21	16 ± 10	5 ± 4	7 ± 5	7 ± 3	292	297	315	329	585 ± 15	600 ± 20	595	644	303	327	1.0	1.0
Bohoth	Young	21 ± 16	72 ± 4	140 ± 16	175 ± 2	2 ± 1	1 ± 0	2 ± 1	2 ± 1	165	250	169	253	319 ± 22	491 ± 6	342	527	177	277	1	0.9
	Middle	23 ± 8	132 ± 7	210 ± 12	126 ± 5	6.5 ± 3	2 ± 2	4 ± 1	2.5 ± 2	243	263	253	268	483 ± 13	518 ± 13	518	556	275	293	0.9	0.9
	Old	30 ± 16	200 ± 5	240 ± 18	104 ± 7	13 ± 4	3.5 ± 1	5 ± 3	3 ± 2	288	310	306	316	582 ± 13	621 ± 19	624	666	336	356	0.9	0.9

Table 4.5 Cation concentrations and osmolality of sap from bulk leaves of Hereward, Gamina and Bohoth 105. Wheat varieties were grown in hydroponic solution. Salt was added in two 30 mM doses over two days (the days were numbered after first addition). Samples were prepared from the young (L3), middle (L2) and old leaves (L1) of 12 individual plants. Cell sap was analysed at day 8 in control and salt treatments. Calculation of the contribution of cations to the cell osmotic pressure (π_{cell}) was calculated by multiplying the sum of the concentrations by the osmotic coefficient (γ) of 0.932 of NaCl (Robinson & Stokes, 2002). The values of ≈ 1.0 for charge suggest monovalent ions (Cl^- or NO_3^-). Results are given as means ± SD. Note: the SiCSA data in the table 4.4 refer to the old leaf (L1) in this table.

4.8 Osmotic Pressure in Bulk Plant

The picoliter osmometer was used to measure the osmotic pressure (π_{plant}) of the sap taken from the young (L3), middle (L2) and old (L1) bulk leaf of Hereward, Gamina and Bohoth 105 varieties. The data in Table 4.5 show mean π_{plant} values in L1, L2 and L3, 7 days after NaCl addition.

Under the control condition, the π_{plant} value increased with age of the leaf for each variety (Fig. 4.7). Under salt stress, a similar pattern was found in both Gamina and Bohoth 105, whereas Hereward showed the opposite trend.

Under the control condition, there was no significant difference in π_{plant} values of old leaf between Hereward and Gamina. However, a significant difference was observed in old leaf between Bohoth 105 and the other two varieties. Nevertheless, generally, the three varieties had similar π_{plant} values for old leaf within same treatment and under both conditions

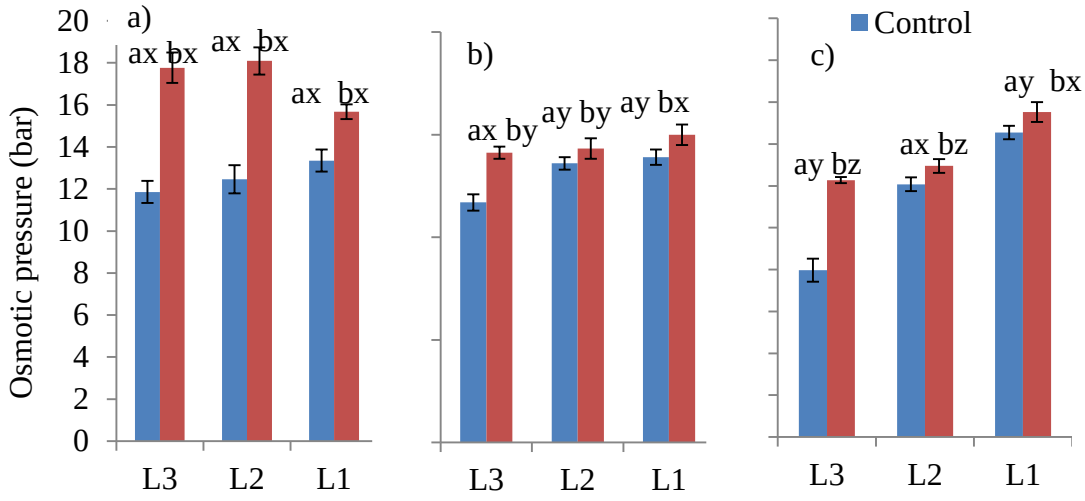

Fig.4.7 Osmotic pressure (bar) was measured using picolitre osmometry of bulk leaf extracts from Hereward (a), Gamina (b) and Bohoth 105 (c) varieties, grown in hydroponic solution. Plants were exposed to salt stress (60 mM NaCl) for 7 days. NaCl was added in two 30 mM doses over two days (the days are numbered after first addition). Young (L3), middle (L2) and old (L1) leaf sap samples were analysed at day 8 after NaCl addition. Results are given as means ± SD, n is 12 plants. with a and b comparing between 0 mM and 60mM treatment in the same leaf and variety, and x, y and z comparing between varieties for the same treatment and same age of leaf. Means followed by the same letters are not significantly different (P>0.05).

4.9 Cation Concentration in Bulk Plant Samples

Capillary zone electrophoresis was performed to examine the inorganic cation concentration of the sap samples obtained from bulk young, middle and old leaves of wheat (Hereward, Gamina and Bohoth 105) after 0 mM and 60 mM NaCl addition. The data in Table 4.5 show the distribution of inorganic cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) and their relationship to osmotic adjustment in the three varieties' bulk leaves.

4.9.1 Na^+ Concentration

The three varieties all had similar Na^+ concentration (less than 30 mM) values for young, middle and old leaf under the control condition (Fig. 4.8). In all varieties, salt stress significantly increased Na^+ accumulation for all ages of leaf (Table 4.6). As with the single cell samples, Bohoth 105 was found to accumulate the highest Na^+ concentration, and Hereward was observed to accumulate the lowest, in its youngest leaves. In general, in all varieties the lowest Na^+ concentration was found in the younger leaves and the highest Na^+ concentration was found in older leaf. Following salt stress, Na^+ concentration contributes considerably to the osmotic pressure in bulk leaf of the three varieties (Table 4.6).

	Variety		
Leaves	Hereward	Gamina	Bohoth 105
Young (L3)	27 %	84 %	71 %
Middle (L2)	61 %	83 %	83 %
Old (L1)	63 %	100 %	85 %

Table 4.6 Na^+ concentration (%) and monovalent anions of bulk osmotic pressure in young, middle and old leaf of Hereward, Gamina and Bohoth 105 following addition of 60 mM NaCl. Fig. 4.8 Na^+ concentration was measured using capillary zone electrophoresis (CZE) of bulk leaf extracts of Hereward (a), Gamina (b) and Bohoth 105 (c) varieties, grown in hydroponic solution. Plants were exposed to salt stress for 7 days (salt treatment) (Details as in Fig. 4.7).

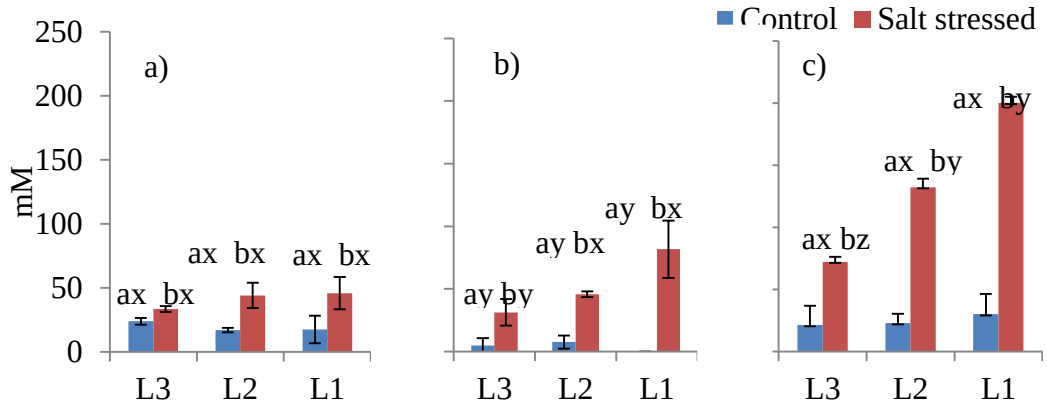

Fig.4.8 Na^+ concentration was measured using capillary zone electrophoresis (CZE) of bulk leaf extracts of Hereward (a), Gamina (b) and Bohoth 105 (c) varieties, grown in hydroponic solution. Plants were exposed to salt stress for 7 days (salt treatment). (Details as in Fig. 4.7).

4.9.2 K^+ Concentration

In the control plants, K^+ concentration was found to be highest in Gamina (whether young, middle and old leaf) (Fig. 4.9). The highest K^+ concentration was found in the oldest leaf and the lowest K^+ concentration was found in the youngest leaf of each variety. However, under salt stress, the pattern was completely different, the highest K^+ concentration was found in the youngest leaf of each variety and the lowest K^+ concentration was found in oldest leaf of Gamina and Bohoth 105. The influence of NaCl on K^+ concentration varied according to the varieties. In Hereward, for example, K^+ concentration increased significantly in all leaves whereas in Gamina and Bohoth 105 it increased only in the youngest leaves, and, in contrast, decreased in middle and oldest leaf. Furthermore, the increase in K^+ concentration contributed to an increased π_{plant} in young, middle and old leaves of Hereward by 33 %, 27 % and 8 % respectively. Potassium increased only in young leaves of Gamina and Bohoth 105, here by 9 % and 17 % respectively.

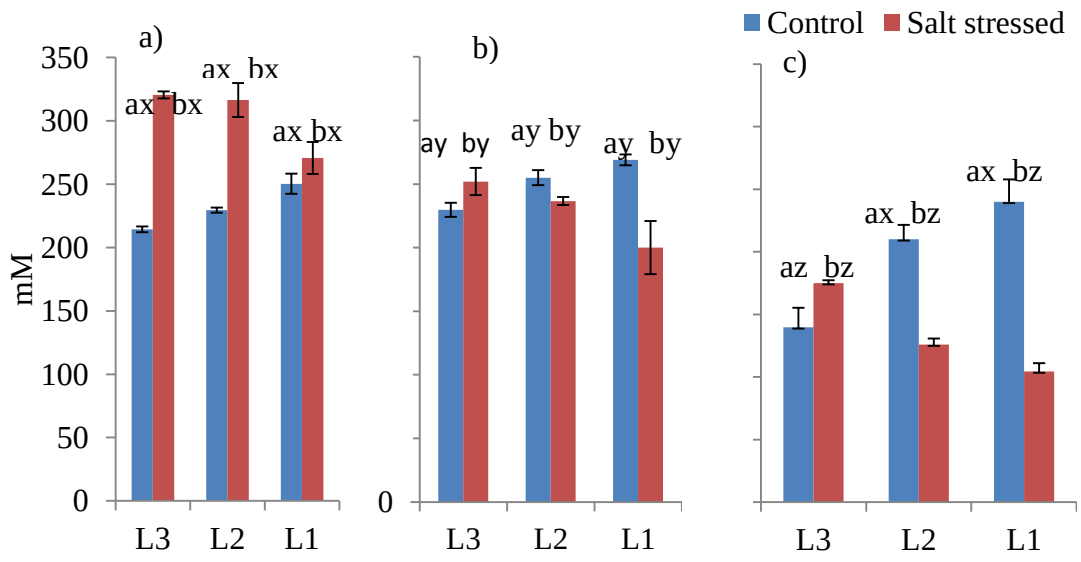

Fig.4.9 K^+ concentration was measured using capillary zone electrophoresis (CZE) of bulk leaf extracts of Hereward (a), Gamina (b) and Bohoth 105 (c) varieties, grown in hydroponic solution. Plants were exposed to salt stress for 7 days (salt treatment). (Details as in Fig. 4.7).

4.9.3 Ca^{2+} Concentration

The effect of NaCl on the Ca^{2+} concentration in all leaves is similar in each of the three varieties (Fig. 4.10). In both treatments, Ca^{2+} concentration was lowest in youngest leaf but highest in old leaf. Salt stress induces decreases (P<0.05) in Ca^{2+} concentration in all ages of leaf in Gamina and Bohoth 105, but only in old leaf in Hereward.

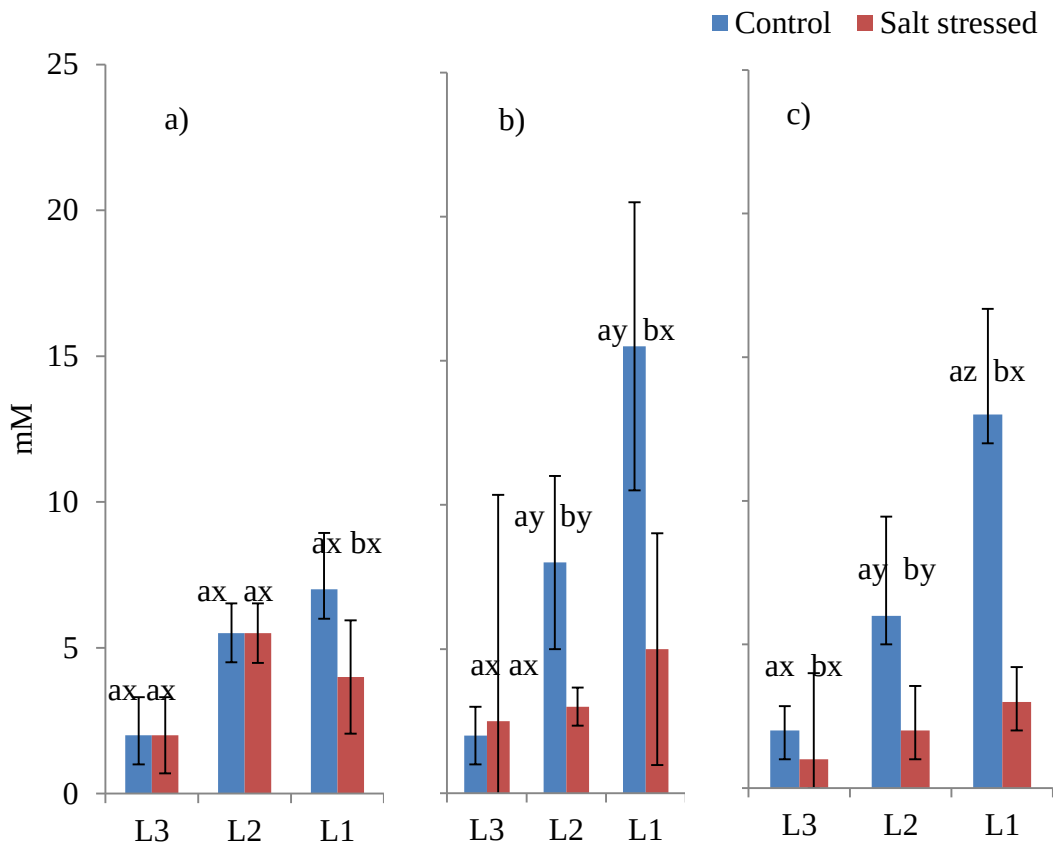

Fig. 4.10 Ca^{2+} concentration was measured using capillary zone electrophoresis (CZE) of bulk leaf extracts of Hereward (a), Gamina (b) and Bohoth 105 (c) varieties, grown in hydroponic solution. Plants were exposed to salt stress for 7 days (salt treatment). (Details as in Fig. 4.7).

4.9.4 Mg^{2+} Concentration

Mg^{2+} concentration followed a similar pattern to that of Ca^{2+}, with the lowest values being found in the youngest leaf and the highest values in the oldest leaf, for each variety and under both treatment conditions. The three varieties also had similar Mg^{2+} concentration values under the control condition (less than 7 mM) while the effect of salt stress varied between different leaf ages and varieties (up to 15 mM variation).

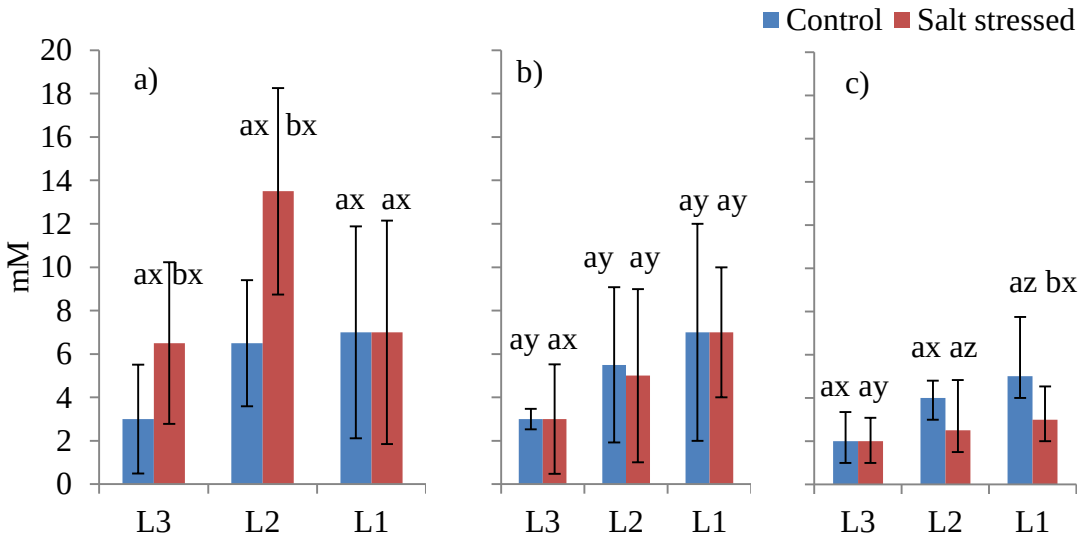

Fig. 4.11 Mg^{2+} concentration was measured using capillary zone electrophoresis (CZE) of bulk leaf extracts of Hereward (a), Gamina (b) and Bohoth 105 (c) varieties, grown in hydroponic solution. Plants were exposed to salt stress for 7 days (salt treatment). (Details as in Fig. 4.7).

4.10 Comparison of Solute Concentration and Osmotic Pressure in both Single Epidermal Cells and Bulk Leaf at Day 8.

In both cell and plant experiments, the sums of cation concentrations and the osmotic pressures in bulk old leaf (L1) for the three varieties were the same or lower than the corresponding values for single epidermal cells, with the exception of Gamina under control conditions (Table 4.7). Sodium concentration is higher in the bulk leaf than in the epidermis, while K^+ concentrations are the reverse: higher in the epidermis than in the bulk leaf. Therefore, there is discrimination between Na^+ and K^+, thereby

reducing the influx of Na^+ into the epidermis while K^+ is selectively taken up into the epidermis (Table 4.4 and 4.5).

Variety	∑Cations (bar)				Osmotic pressure (bar)			
	Control		Salt stressed		Control		Salt stressed	
	Single cell	Bulk leaf	Single cell	Bulk leaf	Single cell	Bulk leaf	Single cell	Bulk leaf
Hereward	13	13.1	17.8	15.3	14.4	13.4	18.1	15.7
Gamina	14.3	13.6	14.4	13.8	13.6	14.6	15.3	15
Bohoth 105	16.7	13.4	19.8	14.4	17.5	14.6	20.1	15.5

Table 4.7 Cation concentrations (expressed as osmotic pressure of a monovalent salt with γ of 0.932) and osmotic pressure in single epidermal cells and bulk old leaf (L1) for Hereward, Gamina and Bohoth 105 by day 8, following the addition of 60 mM NaCl.

4.11 Discussion

The objective of the experiments described in this chapter was to evaluate the effects of the addition of 60 mM NaCl on the P_{cell}, π_{cell} and cation responses of the three wheat varieties. In each variety, a noticeable increase in π_{cell} was seen by day 2, without any change in P_{cell}. Thus the cell water potential (ψ_{cell}) dropped. This drop was of a similar order of magnitude to the salt stress applied (60 mM is approximately equal to 3 bar). The ψ_{cell} of Hereward, Gamina and Bohoth 105 decreased by 4.1, ≥ 1.3, and 3 bar respectively Under the salt condition, P_{cell} values found in the epidermal cells (see Fig. 4.1a–c) were very similar to those reported for

the two Amaranth genotypes (*A. tricolor and A. cruentus*) exposed to 100 mM NaCl in a greenhouse environment by Omami (2005). In contrast, however, the data contradict aspects of the findings of Arif (1990) regarding the effect of salt concentration (25, 50, 75, 100, 125 and 150 mM) on wheat seedlings. In the latter study, direct measurements of P_{cell} did not change with time but remained constant, with osmotic pressure change in the growth zone or mature zone of the leaf. Osmotic adjustment helps the maintenance of cell turgor and volume, and is therefore seen as an important adaption of plants to saline conditions. In light of this, let us consider each variety in turn.

4.11.1 Hereward

4.11.1.1 Control

The osmotic pressure of control epidermal cells was 14.4 bar (Fig. 4.1d and Table 4.2). This π_{cell} value corresponds to reports on epidermal cell of wheat (13.9 bar) found by (Malone *et al.*, 1991), barley by (Fricke *et al.*, 1994a), (up to 12.5 bar) by (Koroleva *et al.*, 2002), and (14.5 bar) by (Cuin *et al.*, 2003). This also compares well with values for the sum of the estimated solute content. The sum of Na^+ and K^+ equates to 278 mM, with an additional 13 mM for Ca^{2+} and 2 mM for Mg^{2+} (Table 4.4, Fig. 4.3a - 4.6a). The total cation equivalence sums to 308 mEq. If this is associated with monovalent anions, such as Cl^- or NO_3^- (Fricke *et al.*, 1994a), the expected π_{cell} would be approximately 574 $mOsmol.kg^{-1}$ if the mean osmotic coefficient were 0.932 (Robinson & Stokes, 2002) for NaCl. This is equivalent to a π_{cell} of 14.6 bar.

For a P_{cell} of 7.3 bar, the ψ_{cell} of control epidermal cells in transpiring plants was -7.1 bar (Fig. 4.1a–d, Tables 4.1 and 4.2, and Fig. 4.12a). This low ψ_{cell} corresponds to

reports of water potentials of maize (–8.1 to –9.6 bar), sorghum (-11 to -13.8 bar and -8 to -10 bar) (Neumann *et al.*, 1974, Jones & Turner, 1978) respectively, barley (-6 to -8 bar) (Matsuda and Riazi, 1981), salt sensitive wheat (-7.1 to -13.8 bar), salt tolerant wheat (-5.6 to -10.7 bar) (Kingsbury *et al.*, 1984), and potato (-5.3 to -8.5 bar) (Liu *et al.*, 2006). The ψ_{cell} of the control nutrient solution is approximately -0.5 bar (for further details see section on Materials and Methods). The ψ_{cell} gradient between the medium and the epidermal cell, therefore, is 6.6 bar. One explanation for this is that it is due to transpiration tension derived from stomatal evaporation.

An attempt was made to quantify the level of transpiration tension by stopping transpiration using the technique of Clipson *et al*. (1985) and Arif (1990) of immersing the entire plant in its nutrient medium. This resulted in an increase of P_{cell}, and hence ψ_{cell}, of 1.5 bar (Fig. 4.2a – b and Table 4.3). This is interpreted as indicating of 1.5 bar. Clipson *et al*. (1985) suggested that the residual ψ_{cell} might be due to osmotic solutes in the apoplast surrounding the epidermal cells being probed. In this case, wall solutes would have a value of 5.6 bar, equivalent to 224 $mOsmol.kg^{-1}$. In contrast, Arif (1990) measured a control wheat leaf epidermis ψ_{cell} of -1 bar under similar transpiration conditions. However, he did find a control ψ_{cell} of -6 bar in the growing zone of wheat seedlings, which can be seen as indicating that there are different water relation parameters in mature zone cells compared with growing zone cells.

4.11.1.2 Response to Salt

4.11.1.2.1 Phase I (0 – 2 days)

As noted above, the addition of salt solution to the root decreased the ψ_{cell} of the epidermal cells and apoplast to -11.2 bar when measured 24 hours after the second

salt addition (Fig.4.12a). This represents a decrease in ψ_{cell} of 4.1 bar, which, corresponds moderately well with the 3 bar stress applied. Despite this change in apoplast ψ_{cell}, P_{cell} at this point is unchanged from the control due to an increase of π_{cell} (decrease of ψ_{cell}) of the protoplasts of 4.1 bar. This is an example of full turgor regulation.

In Hereward, this increase in protoplast π_{cell} is due to the accumulation of 22 mEq of Na^+ and 52 mEq of K^+ (Table 4.4, Fig.4.3a and 4.4a). There is a small loss of Ca^{2+}. The measured increase of π_{cell} indicated that accompanying anions are monovalent (Cl^- or NO_3^-). It is interesting to note that this osmotic adjustment is brought about predominantly by potassium salts, despite the fact that the stress is being applied via sodium. This indicates either a preferential absorption of potassium from the medium following stress, or mobilisation (Wolf *et al.*, 1991) of an internal potassium depot during this phase of turgor regulation.

4.11.1.2.2 Phase II (2- 4 days)

The subsequent phase, from 24 to 96 hours after the second addition of NaCl, sees no further increase in π_{cell}. During this period, there is no change in the Na^+ or K^+ concentrations. However, it is accompanied by an increase in P_{cell} (Fig. 4.1a–d, 4.3a and 4.4a).

4.11.1.2.3 Phase III (after day 4)

After 96 hours after the second addition of NaCl, the P_{cell}, Na^+ and K^+ concentrations (Fig. 4.1a, 4.3a and 4.4a) stabilise and ψ_{cell} remains at about -3.4 bar (Fig. 4.12a). The immersion procedure indicates that this includes a hydrostatic tension component of -1.5 bar and a possible apoplast solute concentration of 1.9 bar or 76 $mOsmol.kg^{-1}$. (Note that under these immersed conditions ψ_{cell} of the leaf is higher than the π of the

external medium, and suggests that the water may be drawn out of the plant through the roots in these artificial non-transpiring conditions. This would result in a decrease in the pressure of the seedlings cells. Unfortunately, the immersion experiment was not run for sufficient time to determine whether this occurred.)

4.11.2 Gamina

4.11.2.1 Control

The osmotic pressure of control epidermal cells was 13.6 bar (Fig. 4.1e and Table 4.2). This π_{cell} is similar to the situation in Hereward. This again compares well with the sum of the estimated solute content, the total cation equivalence of which sums to 314 mEq (Table 4.4, Fig.4.3b - 4.6b). If this is associated with monovalent anions, such as Cl^{-1} or NO_3^-, the expected π_{cell} would be approximately 585 mOsmol.kg^{-1} if the osmotic coefficient were 0.932 (Robinson & Stokes, 2002). This is equivalent to a π_{cell} of 14.6 bar.

For a P_{cell} of 7 bar, the ψ_{cell} of control epidermal cells was approximately -6.6 bar (Fig. 4.12b). The ψ_{cell} gradient between the medium and the epidermal cell, therefore, is 6.1 bar. Again, an attempt was made to quantify the level of transpiration tension by stopping transpiration (Fig. 4.2). This again, suggested a transpiration tension of 1.5 bar and an π_{cell} in the apoplast surrounding the epidermal cells of 5.1 bar, equivalent to 204 mOsmol.kg^{-1}.

4.11.2.2 Response to Salt

4.11.2.2.1 Phase I (0 – 2 days)

Again, addition of salt solution to the root decreased the ψ_{cell} of the epidermal cell and apoplast, this time to -7.9 bar when measured 24 hours after the second salt

addition. This represents a decrease in ψ_{cell} of 1.3 bar (Fig. 4.1b–e and Fig. 4.12b). This does seem significantly less than the 3 bar applied. As in Hereward, there appears to be full turgor regulation. However, the pattern of the solute involved in this osmotic adjustment is different from that of Hereward.

In Gamina, the increase in protoplast osmotic pressure is due to the accumulation of 25 mEq of Na^+ and 5 mEq of K^+. However, there is a loss of 22 mEq of Ca^{2+} and of 5 mEq of Mg^{2+} (Table 4.4 and Fig.4.3b – 4.6b). For monovalent anions, these changes correspond to an increase in π_{cell} of 1.5 bar. This corresponds very well with the change in π_{cell} measured directly. It is interesting to note that in contrast to Hereward, this osmotic adjustment is brought about predominantly by sodium ions, which indicates that Na^+ is taken up from the external medium during this phase of turgor regulation.

4.11.2.2.2 Phase II (2- 4 days)

As with Hereward, the subsequent phase, from 24 to 96 hours after the second addition of NaCl, sees no further increase in π_{cell} and is accompanied by an increase in P_{cell}. During this period, there is no change in the concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} (Table 4.4, Fig.4.1b – e and 4.3b–4.6b).

4.11.2.2.3 Phase III (after day 4)

After 96 hours after the second addition of NaCl, the P_{cell}, Na^+ and K^+ (Fig.4.1b, 4.3b and 4.4b) concentrations stabilise and ψ_{cell} remains at about -6 bar (Fig.4.12b). As with Hereward, the immersion procedure at 168 hours indicates a hydrostatic tension component of 1.5 bar, resulting in a possible apoplast solute concentration of 4.5 bar or 180 $mOsmol.kg^{-1}$.

4.11.3 Bohoth 105

4.11.3.1 Control

For Bohoth 105, the π_{cell} of control epidermal cells was 17.5 bar (Fig.4.1f and Table 4.2). This π_{cell} is significantly higher than for Hereward and Gamina. However, it compares very well with the sum of the estimated solute content, with the sum of measured cations and estimated monovalent anions (Table 4.4 and Fig.4.3c - 4.6c), corresponding to a π_{cell} of approximately 735 mOsmol.kg^{-1} if the osmotic coefficient were 0.932 (Robinson & Stokes 2002). This is equivalent to a π_{cell} of 18.4 bar.

For a P_{cell} of 7.6 bar (Fig.4.1c and Table 4.1), the ψ_{cell} of control epidermal cells was approximately -9.9 bar (Fig.4.12c). The ψ_{cell} gradient between the medium and the epidermal cell, therefore, is 9.4 bar. Once more, as in Hereward and Gamina, there appears to be a hydrostatic tension of 1.5 bar (Fig.4.2e–f) and an apoplast osmotic pressure of 8.4 bar, equivalent to 336 mOsmol.kg^{-1}.

4.11.3.2 Response to Salt

4.11.3.2.1 Phase I (0 – 2 days)

Addition of salt solution to the root decreased the ψ_{cell} of the epidermal cell and apoplast to -12.9 bar when measured 24 hours after the second salt addition. This represents a decrease in ψ_{cell} of 3 bar (Fig.4.12c). Like Hereward, but possibly unlike Gamina, this corresponds well with the 3 bar stress applied. Again, despite this change in ψ_{cell}, P_{cell} at this point is unchanged, due to an increase in the π_{cell} of the protoplasts of 3 bar. Again, we see full turgor regulation.

Once more, the solute pattern seems to be different however. In Bohoth 105, the increase in protoplast osmotic pressure is due to the accumulation of 134 mEq of Na^+

and a loss of 39 mEq of K^+, of 33 mEq of $Ca^{2+}$and of 14 mEq Mg^{2+}(Table 4.4 and Fig 4.3c–4.6c) .

With monovalent anions, this corresponds to an increase in π_{cell} of 2.4 bar. This corresponds well with the change in π_{cell} measured directly. It is interesting to note that this osmotic adjustment is brought about predominantly by sodium ions. Accumulations of Na^+ in epidermal cells induced by an increase in π_{ext} enables a plant to maintain a ψ_{cell} which is lower than that of the external medium.

4.11.3.2.2 Phase II (2- 4 days)

As in both previous varieties, the subsequent phase, from 24 to 96 hours after the second addition of NaCl, sees no further increase in π_{cell} but is accompanied by an increase in P_{cell}. During this period, there is no change in concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} (Fig.4.1c – f and 4.3c – 4.6c).

4.11.3.2.3 Phase III (after day 4)

After 96 hours after the second addition of NaCl, the P_{cell}, Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations (Fig. 4.1c, 4.3c - 4.6c) stabilised, and ψ_{cell} (Fig. 4.12c) reached around –10.3 bar. The immersion procedure indicates, again, that this includes a hydrostatic tension component of -1.5 bar, indicating a possible apoplast solute concentration of

8.8 bar, or 352 $mOsmol.kg^{-1}$.

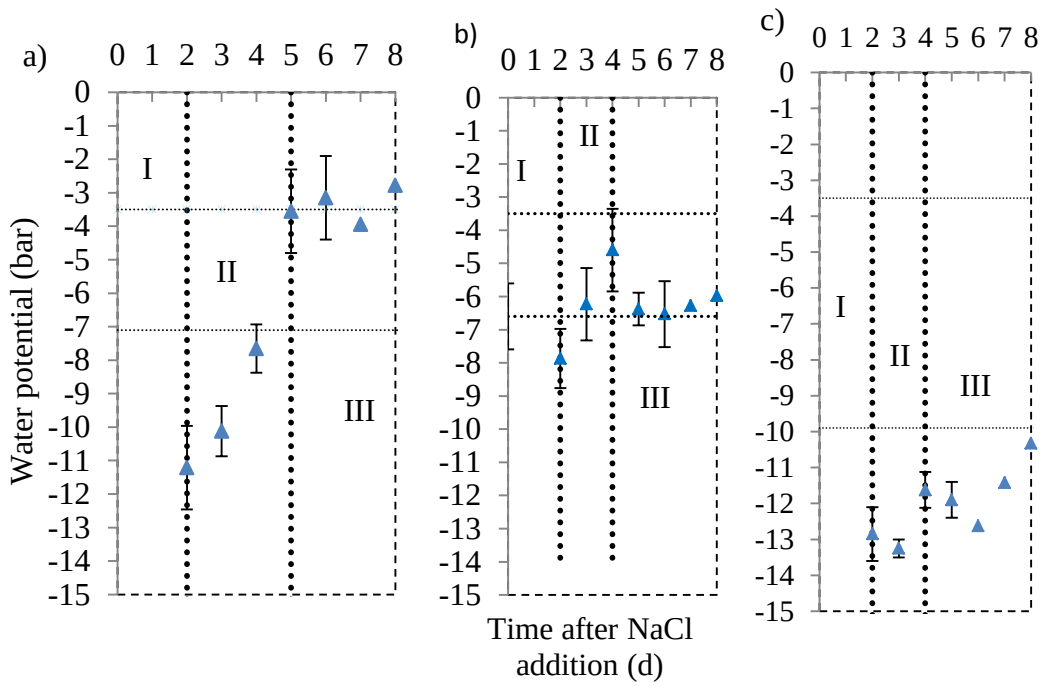

Fig. 4.12 Water potential (ψ_{cell}) change in epidermal cell of Hereward (a), Gamina (b) and Bohoth 105 (c) following addition of 60 mM NaCl. (The trajectory of the fitted curve for first 48 h is only approximate.). $\Psi_{cell} = P_{cell} - \pi_{cell}$

4.11.4 Three Varieties

The most important solutes under the control condition in the three varieties were found to be K^+ and monovalent anions (presumably Cl^- and/or NO_3^-) (Table 4.4). This is in agreement with previous studies, showing that under control conditions osmotic regulation and charge balance are achieved by inorganic solutes (mainly K^+, Cl^- and NO_3^-) in plant cells (Marschner, 1986). Potassium concentrations were found to be 270 – 315 mM in three varieties (Fig.4.4). This K^+ value corresponds well with studies of barley leaf [249 mM found by Fricke *et al*. (1996); 290 mM found by Cuin *et al*. (2003]. Under salt conditions, K^+ concentration was highest in Hereward compared with other varieties. It was found to be around 322 mM (Fig 4.4a). A similar result was reported by Fricke *et al*. (1994a, 1996) and Koroleva *et al*. (2002) for barley. In contrast, lowest Na^+ concentration was seen in Hereward by day 8 (Fig.

4.3a). The low Na^+ concentration could be related to the characteristic of Na^+ exclusion by the root system (Marschner, 1995). Highest Na^+ was in Bohoth 105 over the rest of period. It found to be between 117 – 149 mM (Fig. 4.3c). A similar result was reported by Fricke *et al*. (1996) for barley. Gamina and Bohoth 105 had lower K^+ concentration compared with Hereward (Fig. 4.4). Low K^+ concentration could be due to a competition between Na^+ and K^+ regarding their uptake.

In each variety, salt stress significantly lowered the leaf ψ_{cell} (to increasingly negative values) (Fig. 4.12). This reduction in ψ_{cell} was due to the accumulation of cations (mainly Na^+ and K^+) and monovalent anions (Cl^- and/or NO_3^-) (Table 4.4). As shown in Fig. 4.12, Gamina maintained its original ψ_{cell} values better than both Hereward and Bohoth 105 under the salt condition. The lowest ψ_{cell} was observed in Bohoth 105 and Hereward. The osmotic adjustment in epidermal cells was achieved by increased accumulation of various inorganic cations (and anions). Fricke (2004) and Munns *et al.* (2006) reported that wheat genotypes accumulate inorganic cations, mainly Na^+ and K^+. Salt stressed wheat genotypes could adjust to high salt levels by decreasing tissue ψ_π. In Hereward, this was mainly related to K^+ (Hereward has higher cell capacity to keep K^+ under salt stress) and Cl^-. In Gamina it was mainly Na^+, and in Bohoth 105, mainly Na^+, during this period. In Bohoth 105, the accumulation of Na^+ in epidermal vacuoles during the osmotic adjustment might enable the leaf epidermis to maintain high π_{cell} while concomitantly exporting K^+. Salt stress induced a progressive absorption of Na^+ from the external medium to the cells. Such patterns of accumulation of toxic ions has previous been observed in plant crops, and is referred to as salt accumulation (Turan *et al*., 2010). Atwell *et al*. (1999) reported that osmotic adjustment was largely achieved by an accumulation of Na^+ and Cl^- (such as in halophytes).

Our results with combined P_{cell} and π_{cell} (a change in ψ_{ext} leads to a change in ψ_w in epidermal cells, see Fig. 4.12) indicate that solutes were accumulated and adjusted in both the protoplast and adjacent apoplast (P_{wall}) at the same time. This suggests that wheat uses the apoplast as a dynamic osmotic controlled compartment. In this way, any changes in P_{cell}/π_{ext} trigger a chain of events that ultimately result in osmotic adjustment (protoplastic and/or apoplastic) and turgor regulation.

High Na^+ concentrations in Bohoth 105 and Gamina is caused by high Na^+ ion uptake in order to build up π_{cell} (Fig. 4.3c). Akhtar *et al*. (2005), for example, found that high Na^+ concentration in shoots could be the result of high uptake of Na^+ ions by the plant to increase osmotic pressure in the shoots. Then, the reduction of the K^+ concentration in cells of Bohoth 105 (Table 4.4 and Fig. 4.4c) could be the result of a partial substitution of K^+ by Na^+ (Rodriguez-Navarro & Rubio, 2006; Wakeel *et al.*, 2010). Fricke (2004) has shown that Na^+ increasingly replaced K^+ as the major inorganic cation counterbalancing Cl^- in barley. Turan *et al*. (2010) also reported a reduction in K^+ in epidermal cells following stress. They interpreted this as a consequence of competition between K^+ and Na^+. Thus, in agreement with Abdelmalek and Khaled (2011), salt tolerant genotypes display considerably higher shoot Na^+ concentration than salt sensitive ones. Salt tolerance is generally associated with high Na^+ accumulation in leaves of cotton plants (Leidi and Saiz, 1997; Ashraf & Ahmad, 2000). In contrast, Leidi & Saiz (1997) found high K^+ concentration in shoots of salt sensitive genotypes.

If these characteristics are reliable indications of salt tolerance, then the findings suggest that Bohoth 105 is the most salt tolerant variety of wheat, while Gamina could be moderately so, and Hereward interpreted as the most salt sensitive of the three varieties studied. However, these finding are in contrast to those of Schachtman

and Munns (1992), who argued that in wheat genotypes Na^+ exclusion was a common characteristic of salt tolerance. Ashraf (2004) reported that glycophytes are able to exclude Na^+ from shoots, while Na^+ accumulation in the shoot is an important characteristic of halophytes. Also, Gorham *et al*. (1990), in a laboratory, and Dubcovsky *et al*. (1996), in a greenhouse, each found that the maintenance of a high K^+ concentration and a low Na^+ concentration in the cytoplasm could be an important physiological mechanism response to salt stress in plants (as reported in Tester & Davenport, 2003). This mechanism may be what is occurring in Hereward.

Chen *et al*. (2009) reported that turfgrasses Diamond (*Zoysia matrella* (L.) Merr.) and Z080 (*Z. japonica* Steud.) could have decreased accumulation of Na^+ in the shoots due to salt secretion from salt glands. In contrast, variety Adalayed excluded Na^+ from shoots but also accumulated K^+. Salt tolerance has been associated with the regulation of Na^+ concentration in shoots (Taleisnik & Grunberg, 1994) and to selectivity for K^+/Na^+ ratio (Cuartero *et al*., 1992). Plants may therefore have different behaviors to prevent Na^+ from reaching to the leaves, for example by governing influx of Na^+ through the root plasmalemma (Jacoby & Hanson, 1985); by sequestering Na^+ in parenchyma cells of the root and lower stem part (Johanson and Cheeseman, 1983); or by the accumulation of Na^+ in roots after translocation from shoots via phloem (Jacoby, 1979). It could be argued that Hereward also has a salt tolerance strategy that would be different to that of Bohoth 105. Nevertheless, as seen in Chapter 3, Hereward is clearly less tolerant than Bohoth 105.

4.11.5 Bulk Leaves

Under control and salt treatments, bulk leaf π_{plant} (except salt Hereward) all ages had similar patterns in the three varieties (Fig 4.7). Generally, under both control and stressed conditions, π_{plant} increased with leaf age. In the three varieties, π_{plant}

increased following salt stress (similar results in single cell Fig 4.d - f). The differences in the sum of cations and osmotic pressure (Table 4.8) between bulk leaves and single cells are due to the former including all cell types, while the latter only relates to the epidermis. Clearly, the distribution of cations between leaves was dependent on the leaf development state. In the three varieties, bulk leaf Na^+ in a similar pattern to π_{plant} was increased with leaf age. Old leaf had progressively higher Na^+ concentration than young leaf (Fig. 4.7). This indicates that the capability of plant to control the Na^+ transport rate to the leaf was increasing with time.

A similar result was found for barley by Jeschke and Wolf (1985). They measured Na^+ transport into successive leaves under salt conditions, and noted that the accumulation rate declined in developing leaves, and that in young leaf it was low. This was also found in barley by Rawson *et al.* (1988). Again, similar findings for wheat by Wolf *et al.* (1991) and Santa-Maria and Epstein (2001) suggest that barley can tolerate salinity, and also that it has the ability to keep Na^+ in mature leaf and to limit transport of Na^+ into young leaf. In barley, the use of ion export has been demonstrated by Munns *et al*. (1986), where the phloem is used as a conduit to transport ions from shoots to roots.

Comparison of K^+ between treatments in bulk leaf of Gamina and Bohoth 105 showed that K^+ concentration increases with leaf development state under the control condition, whereas the reverse was true under salt stress (Fig. 4.9b - c). Taleisnik and Grunberg (1994) reported that K^+ is essential for the enlargement of cells, particularly in young leaf. Maathuis and Sanders (1996) and Elumalai *et al.* (2002) reported that K^+ is regards highly mobile within plant tissue and transported in both the xylem and phloem between roots and shoots. In contrast, bulk leaf K^+ of Hereward changed in the opposite direction. Under the control condition, the low K^+

concentration in youngest leaf of Hereward could indicate its relative inability to mobilise K^+ from mature to young leaves (Fig 4.9a). Though accumulation of Ca^{2+} decreased in old leaves of three varieties under salt stress (Fig. 4.10), the highest Ca^{2+} concentration was found in older leaves and the lowest in the younger. Low Ca^{2+} concentration in new leaves could be due to the limited ability of xylem to provide Ca^{2+} for growing tissues (Marschner, 1995).

4.11.6 Comparison of Single Cell and Bulk Leaf Data

When measuring the sums of cation concentrations and the osmotic pressures in bulk old leaf (L1) for the three varieties, the values were found to be the same or lower than the corresponding values for single epidermal cells. The only exception to this trend was with Gamina's value for bulk leaf osmotic pressure under control conditions, which was slightly higher than the value obtained for single epidermal cell (Table 4.7). For some varieties and treatments, the values of sum cation concentration and osmotic pressure showed very little difference between single cell and bulk leaf measurements, whereas in others, the value for bulk leaf was lower for these two parameters. This may be because bulk leaf measurements include cells and tissues such as xylem and phloem, which may affect the measurement by having more dilute solutes, thus lowering the average value.

However, when analysing particular cation concentrations, a different pattern was found. For sodium, this was more concentrated in bulk leaf than epidermal cells, for all varieties under salt stress, whereas potassium was consistently more concentrated in the epidermis than in bulk leaf. This possibly relates to Na^+/K^+ discrimination, where potassium is selectively taken up into the epidermis, while sodium is excluded (Table 4.4 and 4.5).

Chapter 5: Osmolality and Cations in Leaves of *S. maritima* in Response to NaCl Stress

5.1 Introduction

For the wheat varieties in this study, osmotic adjustment was achieved using different cations. In Gamina and Bohoth 105, the uptake and accumulation of sodium ions into the vacuole of epidermal leaf cells was used, whereas in Hereward, potassium was the main cation accumulated in epidermal protoplast, with only a small concentration of sodium uptaken.

Having seen this potential variety between the wheat varieties, this chapter describes the corresponding parameters of the model halophyte *S. maritima.* It was decided to compare the three wheats to a known halophyte in order to investigate whether there were any similarities between the profiles of the most salt tolerant wheats and the profile of a species that grows in saline conditions. It was hoped that such a comparison would help illuminate which parameters in the wheats were aiding or most associated with salt tolerance, and could therefore contribute towards future wheat breeding programmes. Single cell sampling and analysis (SiCSA) was used to measure osmolality and cation concentrations in single epidermal leaf cells of the halophyte *S. maritima* (see description of Materials and Methods in Chapter 2).

Salt-sensitive plants have their growth limited by salt in their environment, yet the growth of halophytes is, in contrast, actually promoted by salinity, even at high concentrations (Greenway & Munns, 1980). Correspondingly, π_{cell} in halophytes is, although variable, frequently noticeably higher than in glycophytes (Cram, 1976).

Salt marshes are an example of a highly saline environment, to which some species are specially adapted. The dicotyledonous halophyte *S. maritima* is one such species (Flowers *et al*., 1986). In order to cope with the low external water potential associated with such conditions, plants need to accumulate enough ions (Na^+ and Cl^-) to ensure that water potential in the leaves is lower than in the soil (Clipson, *et al.*, 1985). This accumulation needs to occur in the vacuole, in order to keep toxic ion concentrations away from the cytoplasm, which is sensitive to salt (Flowers *et al*., 1986).

S. maritima can accumulate ions in high concentrations in leaves in order to tolerate salinity (Clipson, *et al*., 1985), and in fact experiences optimum growth at levels in the region of 200 mM, although concentrations higher than this may impair growth. Indeed, non-saline growth media can be considered as nutrient deficient for halophytes (Yeo and flowers, 1980). This profile is similar to other dicotyledonous halophytes (Munns *et al*., 1983).

In terms of the measurement of parameters, whilst halophyte π_{cell} can be found in the literature (Waisel, 1972), it is hard to accurately measure water potential and P_{cell}. In Chapter 4, which reported the behavior of wheat turgor pressure, P_{cell} was described. Turgor pressure in epidermal cells increased under salt stress in all varieties, but the patterns of increase varied. However, P_{cell} for *S. maritima* proved to be too low to measure consistently (Clipson, *et al*., 1985).

To understand the water relations of halophytes, the apoplast and symplast need to be understood in terms of their osmotic and hydrostatic aspects. However, for *S. maritima*, these components are not well understood, and it is not known why its leaf P_{cell} is kept so low. Leaf epidermal P_{cell} of *S. maritima* has been found to be much

lower than expected from its π_{cell} (Clipson, *et al.*, 1985). The authors found that in an external medium of 20 bar NaCl, transpiring cells had π_{cell} of 25 bar but P_{cell} of only around 1 bar. In non-transpiration conditions, i.e. when P_{wall} is decreased, there was an increase in P_{cell} of only one more bar. This indicates that the low P_{cell} is related to the high π_{wall} (20 - 30 bar), and that the apoplast plays an important role in terms of the π_{cell} gradients. Indeed, Cram (1976) has noted that using the apoplast to control P_{cell} can be more efficient than using the protoplast.

Mature leaf of *S. maritima* has been found to have lower P_{cell} in vivo than the 1 - 7 bar found in other higher plants (when measured using a pressure probe, Tomos *et al*., 1981 and Steudle *et al*., 1983). This can be compared with other examples of halophytes, such as epidermal bladder cells of *Mesembryanthemum crystallinum*, which was found by Steudle *et al*. (1975) to have P_{cell} of 0.5 - 2.5 bar, or *Chenopodium rubrum*, found by Buchner *et al*. (1980) to have corresponding values of 1.7 - 4.1 bar. Another reason for the low P_{cell} could be that plant growth in moderate salt conditions is driven by increased solute transport, which does not increase P_{cell}. Since these points are not fully understood, it is important to investigate the differences between halophytes and glycophytes in terms of salinity resistance.

In this study, the halophyte *S. maritima* was chosen. The first leaf after the cotyledons (pair 1) to emerge from the intact plant was studied *in situ* every day from day 2 to day 8 after the addition of 200 mM NaCl. The same was done for the control treatment (0 mM NaCl addition), but at days 2, 5 and 8. It should be noted that although 0 mM NaCl was used as a control for the wheat varieties, for the halophyte, as mentioned above, this control treatment could actually be described as a type of growth stress, but was nevertheless applied in order to be consistent with the wheat

treatments. All plants tested were grown in hydroponic medium (see description of Materials and Methods in Chapter 2).

5.2 Osmotic Pressure in Epidermal Cells

For 0 mM NaCl (control) leaves, π_{cell} values were found to be 17 ± 0.5 bar (665 mOsmol.kg^{-1}), and remained more or less stable for the whole of the experimental period (see Table 5.1 and Fig.5.1). However, when 200 mM salt was added to the root media, π_{cell} after 2 days increased and then remained more or less stable for the rest of the experimental duration at 22 ± 0.4 bar (892 mOsmol.kg^{-1}). For balancing osmotic adjustment, we expected that π_{cell} would increase by about 400 mOsmol.kg^{-1}. Table 5.1 and Fig. 5.1 show that the increase in π_{cell} was found to be 230 - 279 mOsmol.kg^{-1} (around 65 %).

Time after NaCl addition (d)	Cation concentrations (mM)								∑cations (mM)		∑charge cations (mEq)		Osmolality measured (mOsmol.kg^{-1})		π_{total}/γ		∑anions (mEq)		Average charge of anions	
	Na^+		K^+		Ca^{2+}		Mg^{2+}													
	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed
2	57 ± 5	232 ± 18	256 ± 4	213± 8	1 ± 0.7	0.1 ± 0.2	24 ± 7	18 ± 5	339	463	364	481	665 ± 8	895 ± 15	714	960	375	497	1.0	1.0
3		230 ± 5		215 ± 2		0.2 ± 0.1		13 ± 19		458		471		824 ± 10		884		426		1.1
4		248 ± 9		196 ± 3		0.1 ± 0.0		17 ±3		461		478		841 ± 5		902		441		1.1
5	49 ± 5	250 ± 3	262 ± 8	195 ± 3	0.1 ± 0.1	0.1 ± 0.1	31 ± 9	17 ± 4	343	462	374	479	660 ± 6	872 ± 19	708	936	366	474	1.0	1.0
6		263 ± 3		187 ± 1		0.1 ± 0.1		17 ±2		467		484		941 ± 13		1010		543		0.9
7		302 ± 11		144 ± 9		0.0 ± 0.0		26 ± 3		472		498		921 ± 14		988		516		1.0
8	48 ± 1	354 ± 4	266 ± 8	100 ± 1	0.3 ± 0.04	0.0 ± 0.0	33 ± 11	22 ± 3	348	476	382	498	670 ± 7	949 ± 15	719	1018	371	542	1.0	0.9

Table 5.1 Cation concentrations, osmolality, and calculated charge of the sap of single epidermal cells of *S. maritima* leaves. Epidermal cells (n=3 - 12) were sampled from the first leaves after the cotyledons (pair 1) of individual plants. Cell sap was analysed every day during the salt treatment and three times during the 7 days of the control treatment. Calculations of the contribution of cations to the cell osmotic pressure (π_{cell}) were made by multiplying the sum of the concentrations by the osmotic coefficient (γ) of 0.93 of NaCl (Robinson & Stokes, 2002). The values of ≈ 1.0 for the average anion charge suggest monovalent ions (Cl^- and/or NO_3^-). Results are given as means ± SD.

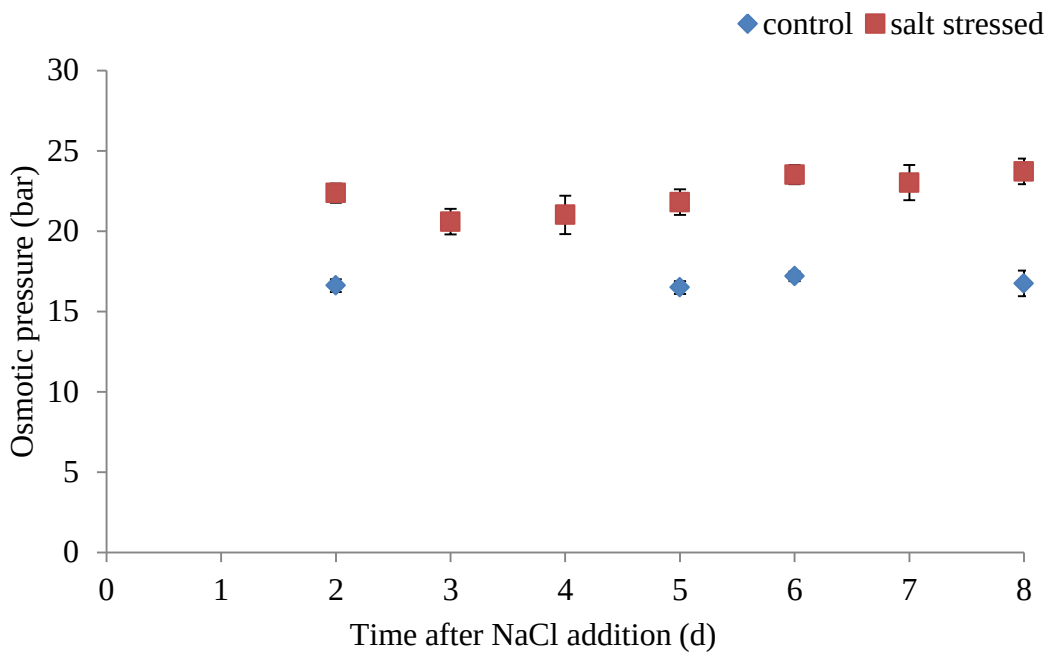

Fig. 5.1 Time course of leaf (pair 1 after the cotyledons) epidermal cell osmotic pressure (π_{cell}) of *S. maritima* for 0 mM control and following the application of 200 mM NaCl. Salt was added in two 100 mM doses over two days (the days were measured after the first addition). Osmotic pressure was measured using a picolitre osmometer. Each point is the mean ± SD of 3 - 12 cells taken from 3 - 6 individual plants.

5.3 Cation Concentrations in Epidermal Cells

To determine the identity of the inorganic cations contributing to osmotic adjustment, CZE (capillary zone electrophoresis) was performed to measure the individual cation concentrations of the sap sample obtained from epidermal cells. It showed that the solutes accumulated in the leaf epidermal cells from the plant under salt stress were largely Na^+ salts (Table 5.1).

5.3.1 Na^+ Concentration

The Na^+ concentration in the control plant cells was about 50 mM (Na^+ concentration in medium 1.3 mM see Table 2.1) throughout the experimental period (Fig. 5.2). In contrast, for the salt treatment, *S. maritima* was observed to have accumulated a considerable amount of Na^+ salt over the 2 days following NaCl addition to the medium (Fig. 5.2). After day 2, however, Na^+ concentration increased more gradually in cells. It reached 354 ± 4 mM by day 8 (Fig. 5.2). This Na^+ concentration was found to be the highest value of the whole experimental period, compared with other ions (Table 5.1). It this plays the main role in increasing π_{cell} under salt stress. By day 8, Na^+ concentration was contributing to osmotic adjustment in cells of *S. maritima* in the order of 306 mEq (100 %) (Fig. 5.1) .

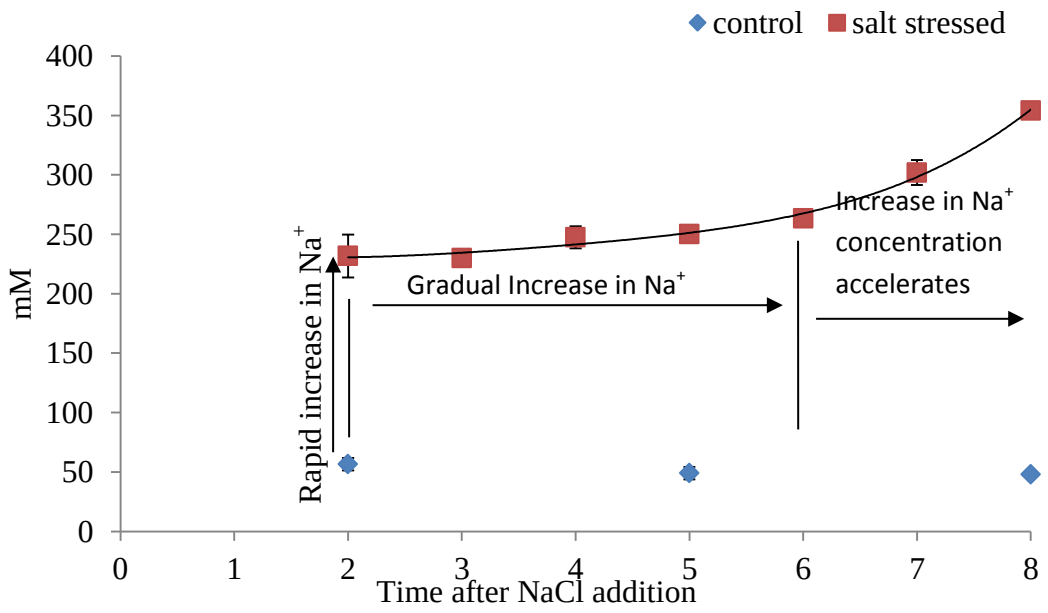

Fig. 5.2 Na^+ concentration in epidermal cell (mature) sap of *S. maritima* leaves. The first leaf (pair 1 after the cotyledons) was analysed the first day after NaCl addition. Salt was added in two 100mM doses over two days (the days were numbered after the first addition). Sodium concentration was measured using capillary zone electrophoresis (CZE). Each observation is the mean of 3 - 12 cells taken from 3 - 12 individual plants. Results are given as means ± SD. Sodium gradually increased (*P*=0.000 for day 2, 5 and 8 ANOVA) with time in response to salt stress. This was particularly rapid during the first 24 hours.

5.3.2 K^+ Concentration

Under control conditions, K^+ was the dominant cation during the whole experimental period. It was found to remain at a concentration of 261 ± 0.7 mM (Fig. 5.3). In contrast, when 200 mM NaCl was added to root media, K^+ concentration was significantly reduced (*P*= 0.000 day 2, 5 and 8 ANOVA). Potassium concentration in epidermal cells was measured as 213 ± 8 mM at day 2, and then gradually decreased for the whole experimental period. It reached 100 ± 1 mM by day 8.

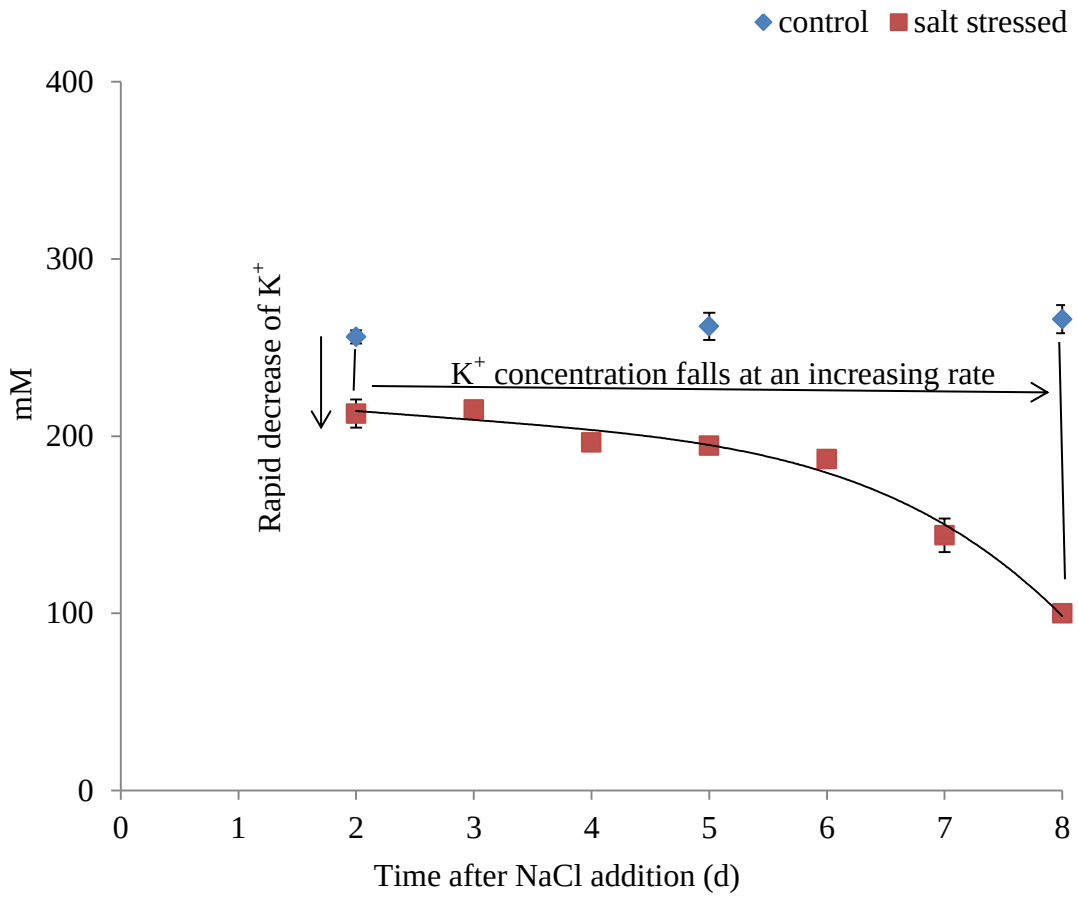

Fig. 5.3 K^+ concentration in epidermal cell sap of *S. maritima* leaves. The first leaf (pair 1 after the cotyledons) was analysed the first day after NaCl addition. Conditions as for Fig. 5.2, except for potassium. Potassium concentration decreased from the onset of stress initiation. Potassium concentration decreased significantly from day 2 - 8 (*P*= 0.000).

5.3.3 Ca^{2+} Concentration

In both treatments, Ca^{2+} was present at a low concentration (Fig. 4.5). No obvious changes were detected under salt stress during the experimental period. Epidermal cells of plant grown in both 0 mM and 200 mM NaCl had negligible (< 1 mM) amounts of Ca^{2+} in the cells.

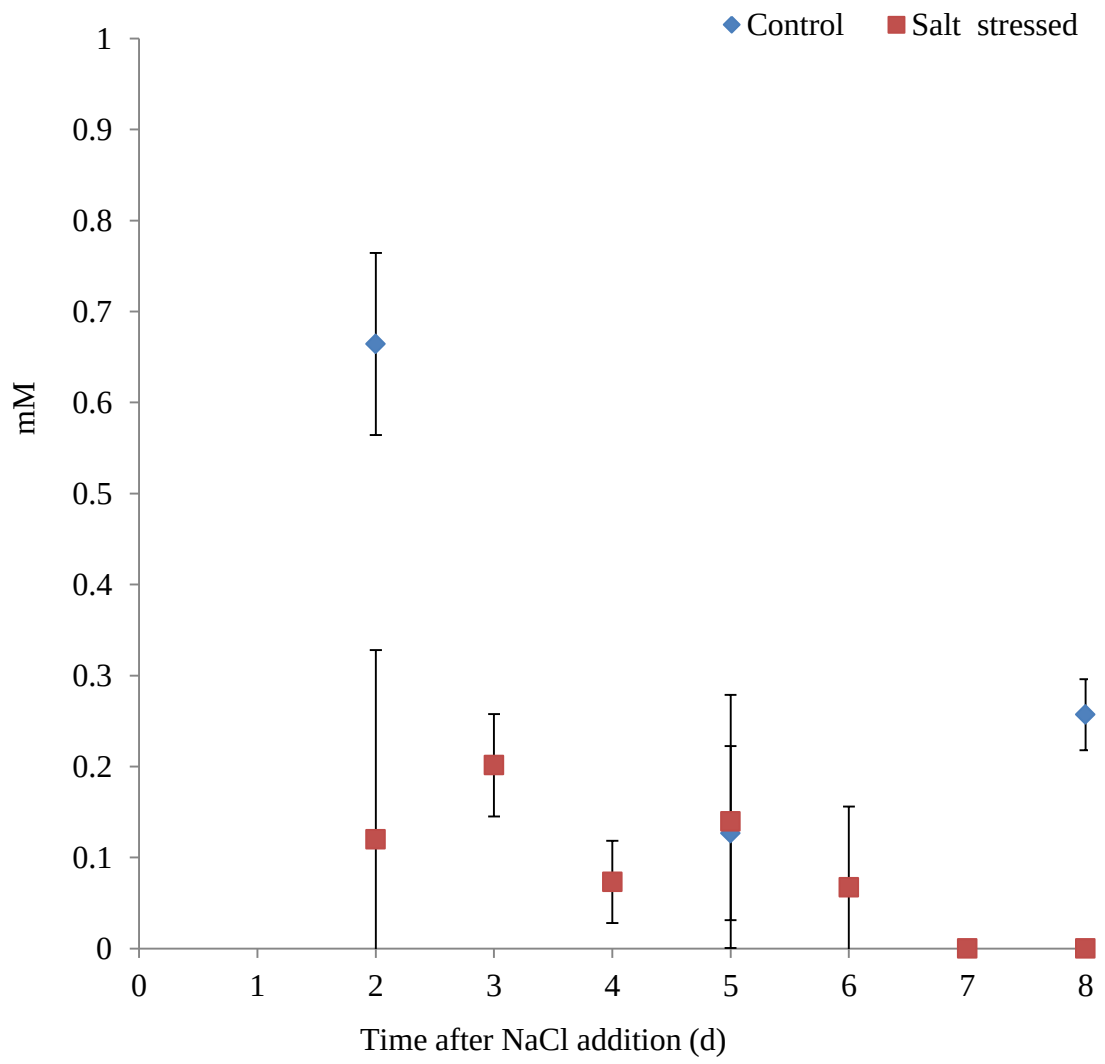

Fig. 5.4 Ca^{2+} concentration in epidermal cell sap of *S. maritima* leaves. The first leaf (pair 1 after the cotyledons) was analysed the second day after NaCl addition. Conditions as for Fig. 5.2 except for calcium.

5.3.4 Mg^{2+} Concentration

The cell level of Mg^{2+} under control conditions (0mM NaCl addition) increased from 24 ± 7 mM on day 2 to 33 ± 11 mM by day 8 (Fig. 5.5). In contrast, after salt stress, Mg^{2+} concentration remained constant. It was 18 ± 5 mM on day 2 and 22 ± 3 mM by day 8.

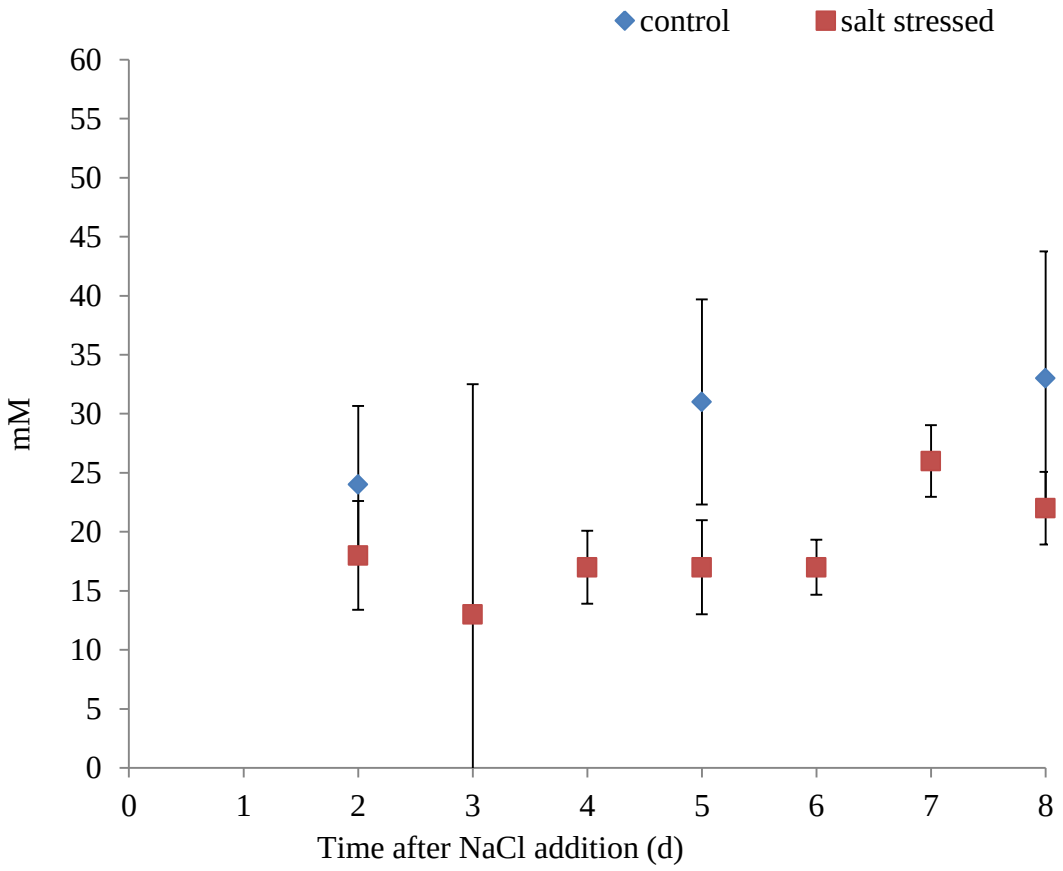

Fig. 5.5 Mg^{2+} concentration in epidermal cell sap of *S. maritima* leaves. The first leaf (pair 1 after the cotyledons) was analysed the first day after NaCl addition. Condition as for Fig. 5.2 except for magnesium. Magnesium concentration decreased significantly (*P*= 0.000).

5.4 Estimate of the Anion Concentration and its Valency

The relationship (and its implications) between the sum of the cations and the measured π_{cell} of samples is shown in Table 5.1 (see Section 4.7). Cations are associated with anions, and Table 5.1 shows that to balance π_{cell} and net charge, this relationship corresponds to 375 mEq, 366 mEq and 371 mEq (day 2, 5 and 8) anions of mean charge

1.0 under the control condition. While under the salt condition, this corresponds to 797 mEq, 474 mEq and 542 mEq (for example day 2, 5 and 8) anions of mean charge 1.0. The charge balance was close to 1.0 (Table 5.1), suggesting monovalent anions, for instance Cl^- or NO_3^-. In Table 5.1, in order to calculate the value for Z, it was assumed that these was no significant unchanged osmotica. (As in Chapter 4, the concentration of uncharged solutes was assumed to be negligible).

5.5 Inter-Relationship of Cations Na^+ and K^+

When 200 mM NaCl was added to root media, Na^+ increased within 48 hours (from 57 to 232 mM). Its increase then slowed to only gradual rise (from 232 to 353 mM) with time. Potassium began to decrease (From 253 to 213 mM), at an increasing rate rapid with time (from 213 to 100 mM) (Fig. 5.2 and 5.3). A 1:1 linear relation was found between Na^+ and K^+ concentration (Fig. 5.6). In summary, sodium concentration increased from 57 mM to 232 mM while K^+ decreased from 256 mM to 213 mM. These changes at day 8 are due to an accumulation of 306 mEq of Na^+ and a loss of 166 mEq of K^+(Fig. 5.2 and 5.3). During this period, osmotic adjustment was mainly achieved by the accumulation of Na^+ and the loss of K^+. After 2 - 3 days following NaCl addition, the linear exchange of Na^+ for K^+ started. Concomitantly, π_{cell} remained unchanged with time (Fig. 5.2 and 5.3).

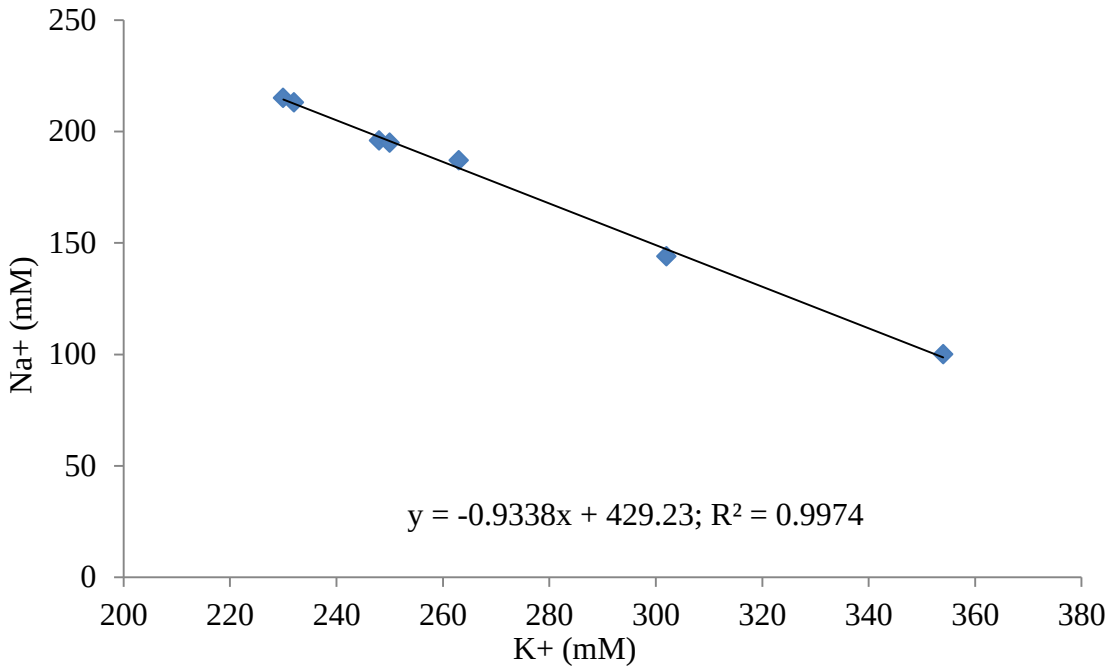

Fig. 5.6 Relationship between Na^+ and K^+ concentration in epidermal cell sap of *S. maritima* leaves. Sodium concentration increased gradually from the onset of stress initiation and during the rest of the experimental period. In contrast, K^+ concentration decreased gradually with time.

5.6 Discussion

The objective of this study was to compare the response of wheat varieties of varying salt tolerance to the impact of 200 mM NaCl with the responses in epidermal cells of the halophyte *S. maritima*. Unfortunately, *S. maritima* has a very low P_{cell} (Clipson *et al.*, 1985, Lawrence, 1999), which was hard to measure, so data from the literature were used to complete the comparison. Problems in measuring P_{cell} were due to the older cells having very low P_{cell} and also to the fact that the tip of the capillary of the pressure probe is frequently blocked or broken by the leaf cuticle being thickened and waxy (Hajibagheri *et al*., 1983).

On addition of NaCl, leaf epidermal π_{cell} increased in *S. maritima*. Since $P_{cell} << \pi_{cell}$, water potential (ψ_{cell}) approximates π_{cell}. This approximation is used in the following discussion.

As with the three varieties of wheat, the epidermal ψ_{cell} dropped on salt stress. In this case it fell by approximately 5.8 bar, which is less than the order of magnitude of the salt stress applied, since 200 mM is approximately equal to 10 bar (Clipson *et al.*, 1985; Lawrence, 1999). The π_{cell} of *S. maritima* control epidermal cells was 16.6 bar (Table 5.1 and Fig. 5.1), which is similar to that of Bohoth 105 (about 17.4 bar) and to previous reports on epidermal cells of *S. maritima* (15 bar, see Clipson *et al.*, 1985 and Lawrence, 1999).

The π_{cell} of control epidermal cells compares well with the sum of the estimated cation content, where 51 mM of Na^+, 268 mM of K^+, 0.5 mM of Ca^{2+}, and 30 mM of Mg^{2+} were found (equivalent to 342 mM) (Table 5.1, Fig. 5.2-5.5). The total cation equivalence sums to 373 mEq, and for electrical balance, this must be accompanied by 373 mEq of anions. If we assume that neutral solute concentrations are negligible (Malone *et al*., 1991), then the sum of anions and cations must correspond to the measured π_{cell} (multiplied by a suitable osmotic coefficient). In this case, π_{cell} = 665 $mOsmol.kg^{-1}$, meaning that 714 mM of this 343 mM are cations, leaving 371 mEq of anions. The charges are these 373 mM to balance the cations. The average ionic charge is 373/371 = 1.0. This corresponds well to the presence of Cl^- or NO_3^-.

If we assume the $P_{cell} << \pi_{cell}$, ψ_{cell} corresponds to π_{cell}. The ψ_{cell} of control epidermal cells was -16.6 bar (Fig. 5.7), which is much lower (>1.5 times more negative) than in epidermal cells of wheat (see Section 4.3). Using the same control nutrient solution as for the wheat, the ψ_{cell} gradient between medium and epidermal cell is 16.1 bar, which is about double that found with wheat. Transpiration tension was only 1.5 bar (Clipson *et al.*, 1985, Lawrence, 1999), and the residual ψ_{cell} could be related to osmotic solutes in

the apoplast surrounding the epidermal cells (Clipson *et al*., 1985). The residual value is 15.1 bar, which is equal to 604 mOsmol.kg^{-1}. Clipson *et al.* (1985) and Lawrence (1999) measured a control leaf epidermis ψ_{cell} in the same plant of approximately -14.7 bar.

The addition of 200 NaCl to the root medium decreased the ψ_{cell} of the epidermal apoplast to about -22 bar when measured 24 - 196 hours after the second salt addition (Fig. 5.7). A similar finding has been reported in the literature (Clipson *et al.*, 1985). This change in apoplast ψ_{cell} is maintained for the rest of the experimental period (Fig. 5.7). This increase in protoplast osmotic pressure is caused by an accumulation of 217 mEq Na^+, and a loss of 82 mEq K^+ and 22 mEq of Mg^{2+} (Table 5.1, Fig. 5.2, 5.3, 5.5 and 5.6). For monovalent anions, this corresponds to an increase of 5.6 bar, which is identical to the change in π_{cell} as measured directly. It is interesting to note that this osmotic adjustment is brought about by sodium uptake but is also a linked function of K^+ loss (cf. Bohoth 105 and Gamina in Chapter 4). This indicates preferential absorption of Na^+ from the medium following stress during turgor regulation.

Solutes were accumulated and adjustment occurred in both protoplast and the adjacent apoplast at the same time. Similar results were found in the three wheat varieties (see Chapter 4). Fig. 5.6 indicates a rapid flooding of the leaf epidermis with 160 mM Na^+ salt (presumably Cl^-) during the first 24 – 48 h. This was achieved with little change in epidermal K^+. After 48 h however, starting slowly but later accelerating, K^+ was lost in a 1:1 ratio with Na^+ uptake. By day 8, 60 % of the initial K^+ was lost. (the trajectory of the fitted curve for first 48 h.) (Fig. 5.7). A comparison of this figure with Fig 4.12 illustrates the difference between wheat and *S. maritima*, but shows that of the wheat varieties, Bohoth 105 is the most similar.

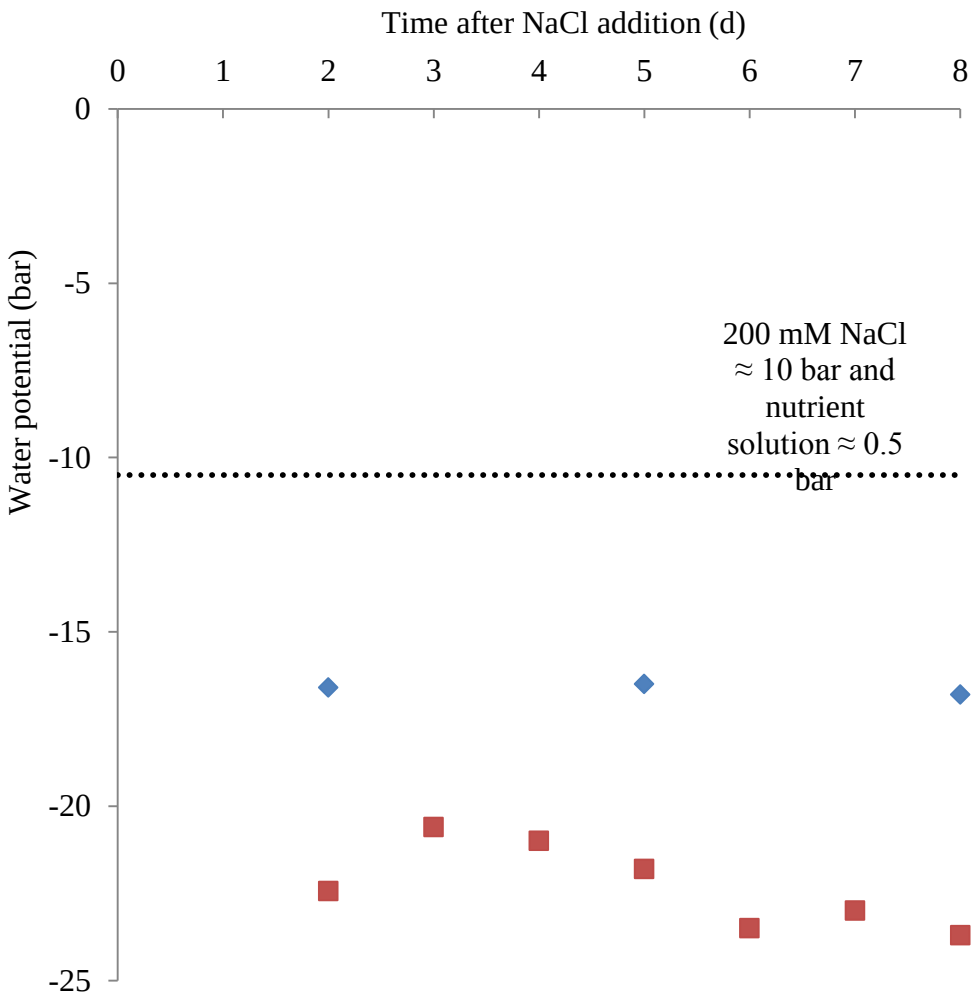

Fig. 5.7 Water potential in epidermal cell of *S. maritima* following addition of 200 mM NaCl. $\Psi_{cell} = P_{cell} - \pi_{cell.}$

Chapter 6: Turgor, Osmolality and Cations in Roots of the Wheat Varieties and their Response to NaCl Stress

6.1 Introduction

Root growth in plants is essentially in competition with leaf growth in terms of nutrition (i.e. photosynthetic products, water, and nutrients). However, the growth of each organ takes place in a coordinated way, with this dynamic balance varying according to changes in environmental conditions. The relative sizes of roots and leaves can thus respond to external conditions in order to optimize the whole plant's resource use (Wilson, 1988).

While saline conditions generally impede growth in plants due to nutritional disorders created by salt toxicity (Niu *et al*., 1995), roots have been found to be more salt-tolerant than leaves (Munns & Tester, 2008), despite being the first parts of the plant to be affected. The precise reasons for this are as yet unclear, but could be related to a reduction in leaf growth relative to root growth in order to minimize water needs (*ibid*.). Indeed it has been seen for both wheat and barley that root pressurisation induces changes to leaf water potential, which in turn alters leaf elongation rate (Passioura & Munns, 2000). Salinity-induced water stress results in reduced water potential and thus low cell turgor in root cells. Since the roots are then less able to uptake water, plant growth is slowed (Greenway & Munns, 1980).

Under conditions of high salinity, however, ion imbalance or toxicity can require more specific response mechanisms. Osmotic adjustment is one, involving the regulation of solute levels in tissues (Greenway & Munns, 1980), commonly Na^+. Sodium ions are actively transported across the tonoplast and accumulated in the vacuole in order to protect the cytoplasm (Yokoi *et al*., 2002). Such salt sequestration lowers the leaf osmotic potential and thus increases turgor potential, allowing leaf growth and stomatal conductance to continue (Beeg & Turner, 1976). In short, plants respond to salt stress in a variety of ways, both with specific response mechanisms, but also with physiological changes to the relative growth of different parts of the plant, i.e. roots and leaves.

In the present study, having completed the analysis of the leaves, the equivalent analysis was then conducted in the root cells, starting with wheat. The cell pressure probe and its associated single cell sampling and analysis (SiCSA) were used to measure P_{cell}, osmolality and cation concentrations in single cortical cells of three wheat varieties [Hereward, Gamina and Bohoth 105 (see Materials and Methods)] following salt stress.

6.2 Turgor Pressure in Cortical Cells

Cells four to six cm away (see Chapter 2.6.1 for explanation) from the root tip of intact plants were studied *in situ* every day from day 2 to day 8, 14 and 21 after the addition of 60 mM NaCl, and at 2, 5, 8, 14 and 21 days for the control treatment (0 mM NaCl addition). All three varieties had similar P_{cell} values under control conditions (5.4 ± 0.8 bar for Hereward, 5.2 ± 0.5 for Gamina and 4.6 ± 0.5 for Bohoth 105; n = 3 - 12). In each variety, after 2 days following salt addition, P_{cell} decreased rapidly. After that, there was an exponential increase with time until a new stationary state was reached (Fig. 6.1a - c).

When salt was added to root media, it is believed (from previous experience) that the P_{cell} dropped immediately by about 2 - 3 bar (according to Vant't Hoff's law). P_{cell} regulation was subsequently achieved, by cell osmotic adjustment (Fig. 6.1d – f) to preserve a constant difference in the π_{cell} between cortical cells and the external medium. The regulation (recovery) rate varied between different varieties. However, complete P_{cell} recovery was achieved by day 8 with a $T_{1/2}$ of 6.3 days in Hereward, 7.9 days in Gamina and 1.7 days in Bohoth 105. It was lowest in Bohoth 105 and highest in Gamina (Table 6.2).

In control plant roots, the magnitude (approximately 6 bar) of the π_{cell} is reflected by the P_{cell} (Fig. 6.1a - f). A significant difference in P_{cell} ($T_{1/2}$) at day 2 - 8 between the control and salt treatments was observed for all varieties. The three wheat varieties studied presented the biphasic response (fast P_{cell} decrease and slower P_{cell} recovery) after 60 mM NaCl addition. Ultimately, the P_{cell} increase reflected the solute uptake rate or/and synthesis in single cortical cells. In the first phase of the biphasic response, P_{cell} could followed the loss of water of the cortical cells to the external medium. In second phase, it was likely to be due to an increase in π_{cell} of single cells as result of transport, synthesis of solutes, or both. In addition, this result suggests that Bohoth 105 shows more rapid recovery of P_{cell} (within 1.7 days) than either Hereward (6.7 days) or Gamina (7.9 days) during the course of the 7 day experimental period.

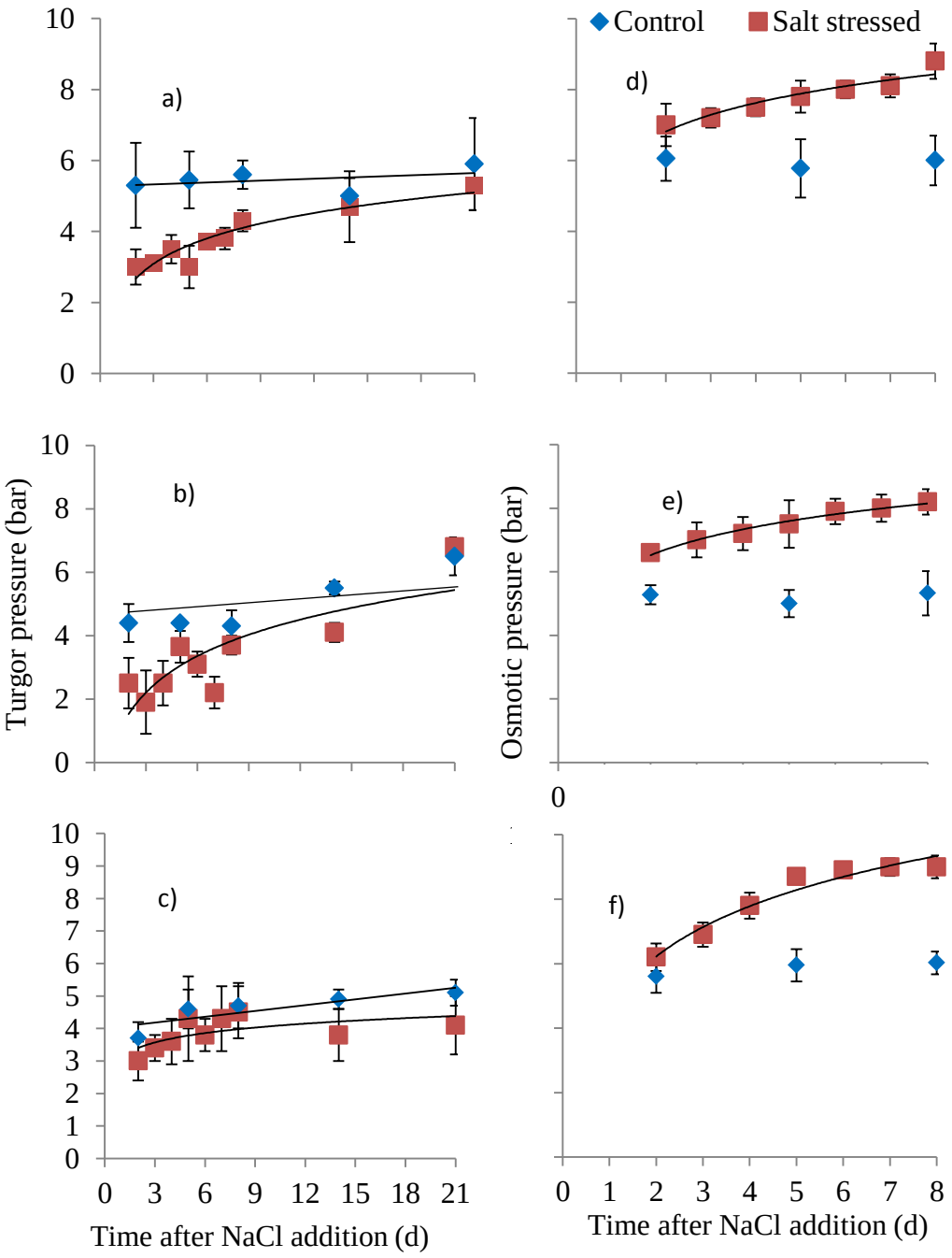

Fig. 6.1 Time course of root cortical cell turgor pressure (P_{cell}) and osmotic pressure (π_{cell}) of Hereward (a and d), Gamina (b and e) and Bohoth 105 (c and f) for control and following application of 60 mM NaCl. Salt was added in two 30 mM doses over two days (the days are numbered after the first addition). Cortical cells were prepared 4 - 6 cm from the root tip of individual plants. Cell turgor pressure was measured using a cell pressure probe. Cell osmotic pressure was measured using a single cell sampling, and picolitre osmometer. Difference in means for P_{cell} and π_{cell} between treatments were always highly significant (P = 0.000). Each point is the mean ± SD of 3 - 12 cells taken from 3 - 6 individual plants.

Variety	Time after NaCl addition (d)	Cations concentrations (mM)								∑solutes (mM)		∑charge cations (mEq)		Osmolality (mOsmol.kg-1)		π_{total}/γ		∑anions (mM)		Average charge of anions	
		Na^+		K^+		Ca^{2+}		Mg^{2+}													
		Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed
Hereward	2	9 ± 3	46 ± 7	113 ± 4	97 ± 4	5 ± 4	2 ± 2	5 ± 1	6 ± 0.4	132	151	142	159	242 ± 25	280 ± 24	260	300	128	149	1.112	1.064
	3		90 ± 12		56 ± 5		4 ± 1		5 ± 0.6		155		164		288 ± 11		309		154		1.065
	4		107 ± 10		48 ± 3		2.5 ± 2		5 ± 0.3		163		170		300 ± 10		322		159		1.067
	5	17 ± 2	126 ± 7	108 ± 5	30 ± 8	10 ± 11	7 ± 4	9 ± 1	4 ± 0.4	144	167	163	178	247 ± 33	312 ± 18	265	335	121	168	1.347	1.061
	6		124 ± 13		36 ± 10		5 ± 1		5 ± 0.7		170		180		320 ± 10		343		173		1.038
	7		130 ± 11		32 ± 5		1.5 ± .4		5 ± 0.3		169		175		324 ± 13		348		179		0.977
	8	17 ± 3	149 ± 10	104 ± 2	27 ± 8	9 ± 12	14 ± 0.4	9 ± 2	8 ± 0.4	139	198	157	220	240 ± 28	352 ± 20	258	378	119	180	1.325	1.224
Gamina	2	3 ± 3	49 ± 7	103 ± 7	85 ± 13	2 ± 3	13 ± 8	5 ± 2	6 ± 1	113	152	120	171	211 ± 12	264 ± 4	226	283	113	131	1.065	1.303
	3		79 ± 9		63 ± 9		24 ± 9		7 ± 2		172		203		280 ± 22		300		128		1.585
	4		70 ± 20		74 ± 12		5 ± 4		6 ± 3		156		167		288 ± 21		309		153		1.089
	5	4 ± 3	96 ± 33	104 ± 9	56 ± 17	2 ± 3	11 ± 5	5 ± 2	7 ± 5	115	170	122	188	200 ± 17	300 ± 30	215	322	100	151	1.216	1.244
	6		123 ± 18		30 ± 6		25 ± 21		11 ± 17		189		226		316 ± 16		339		150		1.506
	7		130 ± 10		32 ± 2		23 ± 8		5 ± 1		190		218		320 ± 17		343		154		1.421
	8	5 ± 3	133 ± 6	115 ± 11	32 ± 2	3 ± 3	17 ± 0.2	6 ± 2	1 ± 1	129	183	138	201	213 ± 28	328 ± 16	229	352	99	169	1.395	1.185
Bohoth 105	2	8 ± 2.2	51 ± 7	109 ± 11	72 ± 15	3 ± 4	5 ± 4	9 ± 3	4 ± 1	129	132	141	142	224 ± 20	248 ± 17	240	266	112	134	1.265	1.057
	3		61 ± 16		76 ± 18		3 ± 2		10 ± 2		150		163		276 ± 15		296		146		1.114
	4		94 ± 4		63 ± 6		3 ± 1		7 ± 0.3		168		179		312 ± 16		335		167		1.072
	5	3 ± .2	94 ± 8	125 ± 9	81 ± 6	2 ± 0.3	6 ± 3	7 ± 0.3	11 ± 1	136	193	144	211	238 ± 20	348 ± 9	255	373	119	180	1.207	1.172
	6		106 ± 5		73 ± 4		4 ± 1		11 ± 1		194		208		356 ± 7		382		188		1.109
	7		109 ± 5		72 ± 4		3 ± 1		10 ± 0.8		194		207		360 ± 11		386		192		1.075
	8	6 ± 0.3	112 ± 8	118 ± 8	71 ± 6	3 ± 0.5	2 ± 1	6 ± 0.5	9 ± 2	131	194	138	205	241 ± 14	360 ± 14	259	386	128	192	1.077	1.066

Table 6. 1 Cation concentrations and osmolality of the sap of single cortical cells of Hereward, Gamina and Bohoth 105 root. Cortical cells (n = 3 - 12) were selected from 4 - 6 cm away from the root tip of 3 - 6 individual plants. Cell sap was analysed every day during the salt treatment and three times during the 7 days of the control treatment. The relationship is between the osmolality of cations obtained by picoliter osmometer and molarity obtained by capillary zone electrophoresis (CZE). Calculations of the contribution of cations to the cell osmotic pressure (π_{cell}) were made by multiplying the sum of the concentrations by the osmotic coefficient (γ) of NaCl 0.932 (Robinson & Stokes, 2002).

6.3 Osmotic Pressure in Cortical Cells

Single cell sampling and a picoliter osmometer were used to measure the π_{cell} of the sap taken from similar single root cortical cells of the three wheat varieties. Figs. 6.1d - f demonstrates mean π_{cell} in root cortical cells in response to 0 mM and 60 mM NaCl in root medium. Notably, all three varieties had similar π_{cell} values under the control condition (5.9 ± 0.7 bar for Hereward, 5.2 ± 0.5 for Gamina and 5.9 ± 0.5 for Bohoth 105; n = 3 - 12) (Fig. 6.1 d - f).

When salt was added to the root media, π_{cell} increased continuously with time starting from 7 ± 0.6 bar, 6.6 ± 0.1 bar and 6.2 ± 0.4 bar at day 2 (for Hereward, Gamina and Bohoth 105), and then reaching 8.8 ± 0.5, 8.2 ± 0.4 and 9 ± 0.4 by the end of the experimental period. The difference in π_{cell} between the control and salt treatment at day 8 is approximately 3 bar, which reflect the π_{cell} of the external root medium (60 mM NaCl). In salt treated plants, by day 8, $T_{1/2}$ of π_{cell} was found to be 4.6 days in Hereward, 4.3 days in Gamina and 2.1 days in Bohoth 105. A statistically significant difference in $T_{1/2}$ between varieties was observed only in Bohoth 105 (Table 6.3).

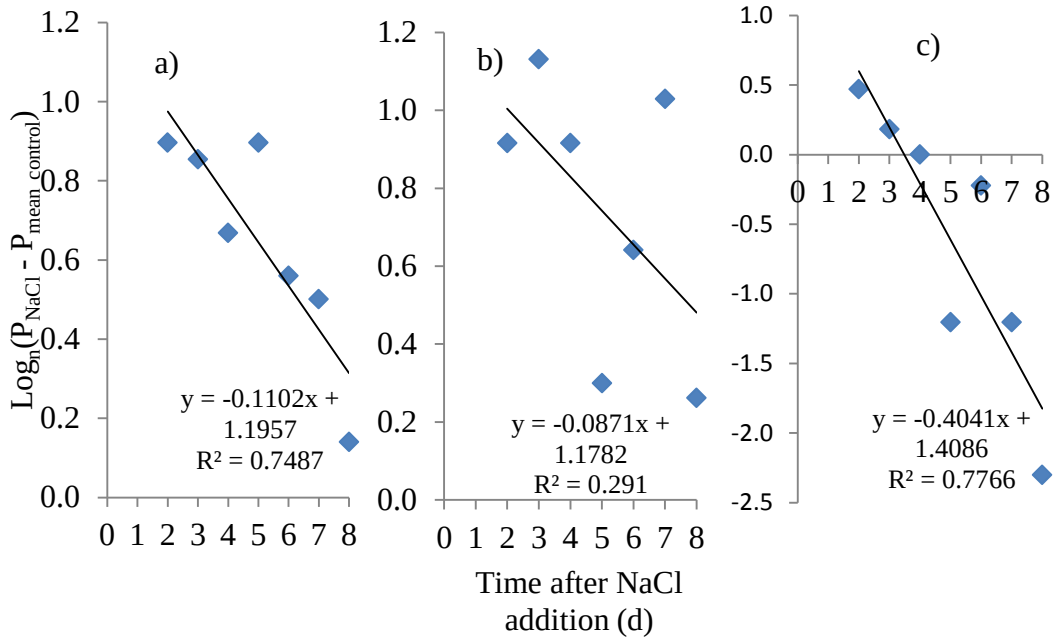

Fig. 6.2 Log plot of the time course of root cortical cell turgor pressure (P_{cell}) during turgor recovery following salt stress of Hereward (a), Gamina (b) and Bohoth 105 (c). See Fig. 6.1 for details of cells. The difference between control and salt turgor pressure in Fig.6.1a – c is plotted logarithmically log(P_{NaCl} − $P_{mean\ control}$) against time in Fig. 6.2. Half time ($T_{1/2}$) calculated from log(2)/slope. The equations for the curves for each variety together with R^2 values are included.

	Hereward	Gamina	Bohoth 105
$T_{1/2}$ (days)	6.3[a]	7.9[b]	1.7[c]
R^2	0.75	0.29	0.78

Table 6.2 Half time ($T_{1/2}$) of root cortical cell turgor pressure (P_{cell}) on day 2 to 8 of Hereward, Gamina and Bohoth 105 for the control treatment and following application of 60 mM NaCl. The difference between the control and salt turgor pressure in Fig.6.1a – c is plotted logarithmically (log(π_{NaCl} − $\pi_{mean\ control}$) against time in Fig. 6.2. Half time ($T_{1/2}$) calculated from log(2)/slope, the equations for the period of liner curve for each variety with R^2 values in Fig. 6.2. Letters, a, b and c comparing between varieties. Means followed by the same letters are not significantly different at $P > 0.05$.

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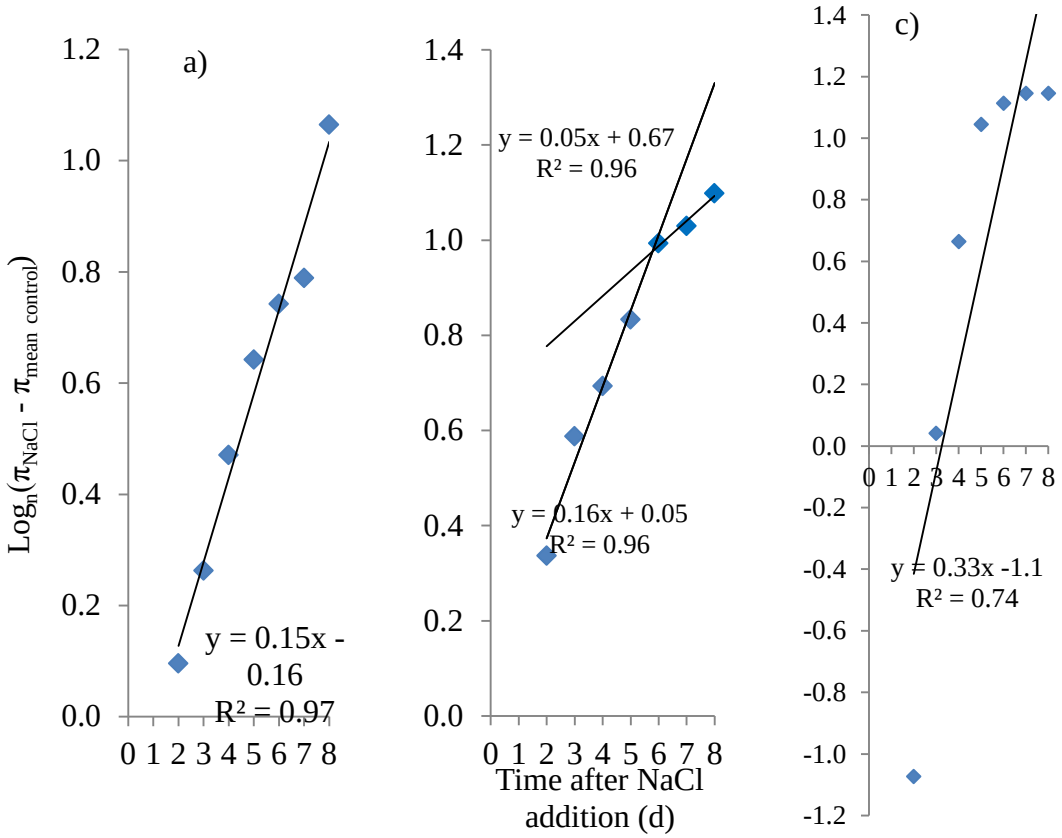

Fig. 6.3 Log plot of the time course of root cortical cell osmotic pressure (π_{cell}) of Hereward (a), Gamina (b) and Bohoth 105 (c) at day 2 to 8 control treatment and following application of 60 mM NaCl. Each curve applies to osmotic pressure. Log($\pi_{NaCl} - \pi_{mean\ control}$) in Fig.6.1d – f is plotted against time in Fig. 6.3. Half time ($T_{1/2}$) estimated from log(2)/slope; the equations for the curves for each variety together with R^2 values is included.

2 to 8 (days)

	Hereward	Gamina	Bohoth 105
$T_{1/2}$ (days)	4.6[a]	4.3[a]	2.1[b]
R^2	0.97	0.96	0.74

Table 6.3 Half time ($T_{1/2}$) of root cortical cell osmotic pressure (π_{cell}) of Hereward, Gamina and Bohoth 105 at day 2 to 8 for the control treatment and following application of 60 mM NaCl. The difference between control and salt osmotic pressure in Fig.6.1d – f is plotted logarithmically against time in Fig. 6.3. Half time ($T_{1/2}$) estimated from log(2)/slope, with the equations for the curves for each variety together with R^2 values in Fig 6.3. Letters, a and b compare between varieties. Means followed by the same letters are not significantly different at P>0.05.

6.4 Osmotic Adjustment

Data in Fig. 6.1d - f show osmotic adjustment had begun by 2 days after NaCl addition by the accumulation of solutes in the cells. However, complete osmotic adjustment was only achieved by day 8, with a $T_{1/2}$ of 4.6 days in Hereward, 4.3 days in Gamina, and 2.1 days in Bohoth (Fig. 6.1d – f and Table 6.3). Cell osmotic pressure (π_{cell}) increased as a consequence of net solute accumulation. That in turn led to water uptake into the cell, which maintains P_{cell}. The data presented is considered to be supporting evidence that turgor recovery in the three varieties was due to equivalent changes in π_{cell} by building up osmotically-active solutes in the protoplast via osmotic adjustment.

6.5 Cation Concentrations in Cortical Cells

To determine the inorganic cations contributing to osmotic adjustment, CZE was performed on sap samples obtained from single cortical cells of wheat root in 0 mM and 60 mM NaCl (Fig. 6.4 – 6.7). Table 6.1 demonstrates the distribution of cation (Na^+, K^+, Ca^{2+}, Mg^{2+}) concentrations in the three examined varieties of wheat. CZE confirmed that the solutes accumulated in the root cortical cells of all varieties under salt stress were largely Na^+ salts. This accumulation resulted in the observed increase in π_{cell}.

6.5.1 Na^+ Concentration

All three varieties had Na^+ concentrations in control roots of less than 17 mM (Fig 6.4). The NaCl addition increased root Na^+ concentrations in Hereward, Gamina and Bohoth 105 at day 8 by 149 ± 1.3 mM, 133 ± 14 mM and 122 ± 8 mM respectively. Na^+ concentration significantly increased with time (P = 0.000, ANOVA of day 2, 5 and 8) after 2 days following salt addition in the three varieties (Fig. 6.4). In all three varieties of salt-treated plants, Na^+ concentration and π_{cell} followed the same patterns. In salt-treated plants, Na^+ concentration was found to be the highest throughout the whole experimental period, as compared with other ions. In addition, data in Table 6. 1 demonstrate that Na^+ concentration contributed to the π_{cell} in cortical cells by 5.6 bar for Hereward, 5.8 bar for Gamina and 6 bar mM for Bohoth 105 at day 8.

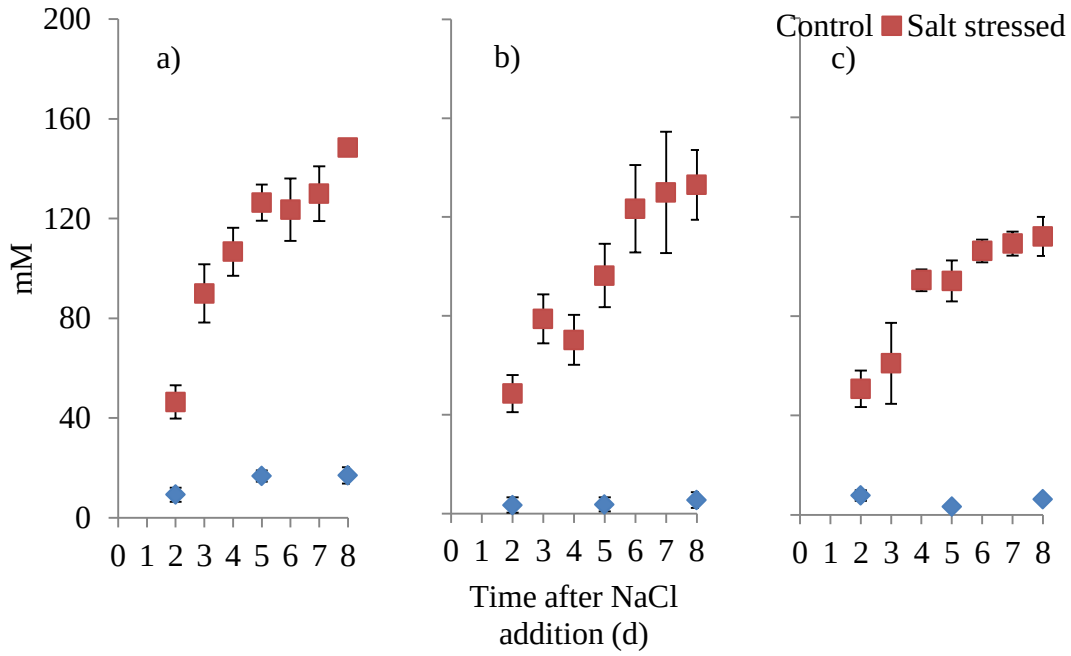

Fig. 6.4 Na^+ concentration in root cortical cell sap of Hereward (a), Gamina (b) and Bohoth 105 (c). Root cortical cells 4 - 6 cm from the root tip of individual plants was analysed on the first day after NaCl addition. Salt was added in two 30 mM doses over two days (the days were numbered after the first addition). Sodium concentration was measured using CZE. Each observation is the mean of 3 - 12 cells taken from 3 – 6 individual plants. Difference in means Na^+ concentration between treatments were always highly significant (P = 0.000 ANOVA of day 2, 5 and 8). Results are given as means ± SD. The three varieties accumulated Na^+ in their cells.

6.5.2 K^+ Concentration

In control plants, K^+ concentration was found to be dominant cations (Fig. 6.5), in the range of 100 - 130 mM. However, when NaCl was added to root media, the K^+ concentration values had decreased by 2 days after NaCl addition (highly significant difference P = 0.000 between treatments). By the end of the experimental period, K^+ in Hereward and Gamina varieties had reached 27 ± 8.3 mM and 32 ± 3.1 mM respectively. In these two varieties of salt treated plants, K^+ concentration followed the same patterns. However, in Bohoth 105, K^+ concentration values had decreased by 2 days after NaCl addition (highly significant difference P = 0.000 between treatments), but then remained more or less (approximately 73 mM) constant for the rest of the experimental period.

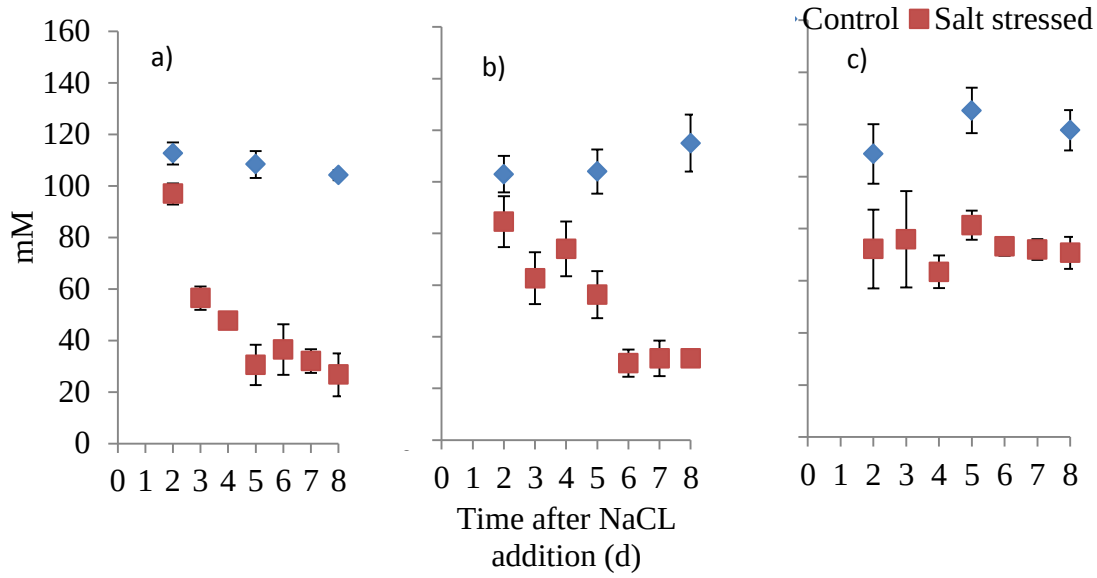

Fig. 6.5 K^+ concentration in root cortical cell sap of Hereward (a), Gamina (b) and Bohoth 105 (c). Details as for Fig. 6.4, except for K^+. Difference in means K^+ concentration between treatments were always highly significant (P=0.000). Results are given as means ± SD. Potassium concentration decreased significantly (P= 0.000) in the three wheat varieties.

6.5.3 Ca^{2+} Concentration

In Gamina, salt stress caused a significant increase in Ca^{2+} concentration (P = 0.007), which it was found to be average 20 mM for the whole experimental period (Fig.

6.6). In contrast, salt stress did not appear to affect Ca^{2+} concentrations in cortical cells of Hereward and Bohoth 105 varieties (Fig. 6.6).

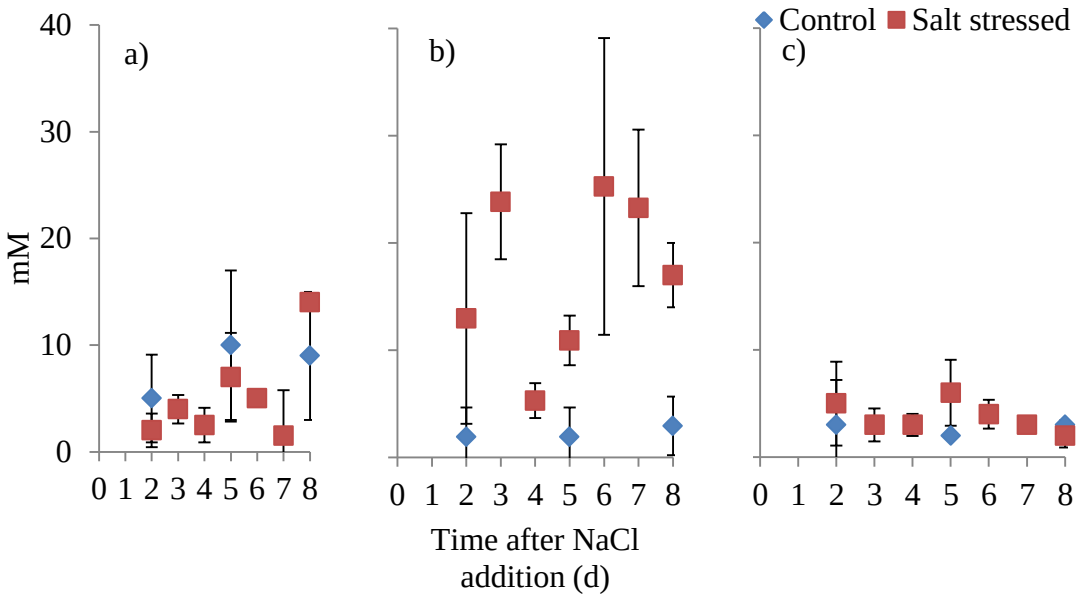

Fig. 6.6 Ca^{2+} concentration in root cortical cell sap of Hereward (a), Gamina (b) and Bohoth 105 (c). Details as for Fig. 6.4, except for Ca^{2+}.

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6.5.4 Mg^{2+} Concentration

In Hereward variety, salt stress caused a decrease in Mg^{2+} concentration between day 3 and 7 (P = 0.027) (Fig. 6.7). It was found to be as average of 5 mM for the whole experimental period. However, Mg^{2+} concentration values in Bohoth 105 variety statistically increased (P = 0.034), and had an average of 9 mM. In contrast, no statistically significant differences were found in Mg^{2+} concentration for Gamina.

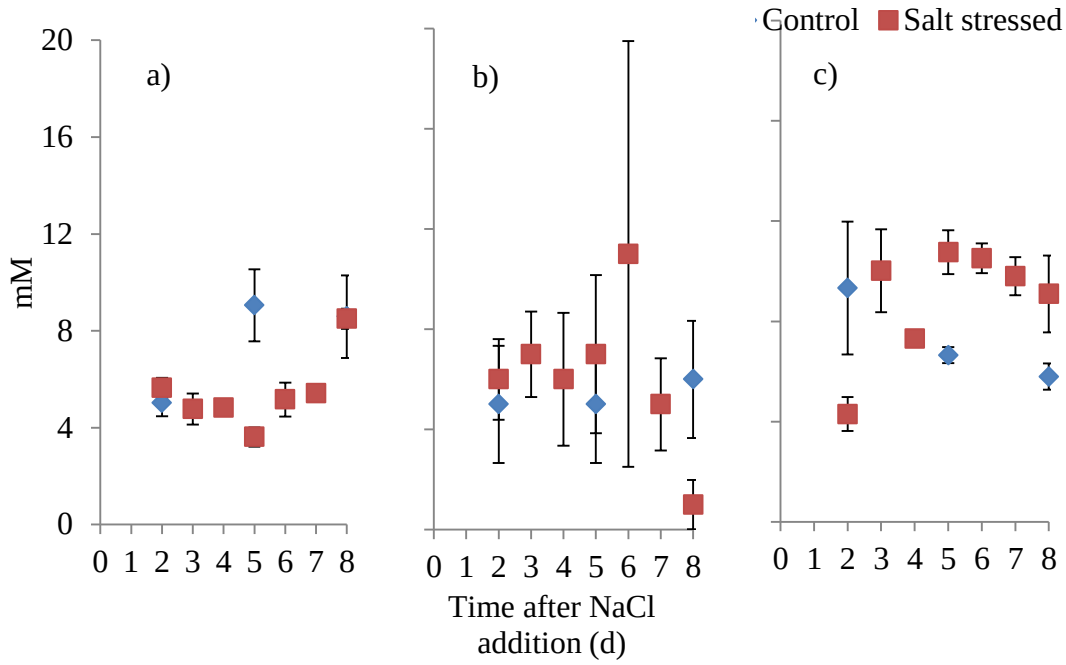

Fig. 6.7 Mg^{2+} concentration in root cortical cell sap of Hereward (a), Gamina (b) and Bohoth 105 (c). Details as for Fig. 6.4, except for Mg^{2+}.

6.5 Estimate of the Anion Concentration and its Valency

The relationship between the sum of the cations measured and π_{cell} is shown in Table 6.1. As in previous chapters, anions were not measured. However some attempt can be made to describe them. The total cations are associated with anions. Table 6.1 shows that to balance both π_{cell} and net charge, this relationship corresponds to around 110 mEq of all the varieties anions of mean charge 1.06 - 1.39 under control condition (see explanation of calculation in Section 4.7). Under the salt condition, this corresponds to average of 161 mEq, 148 mEq and 171 mEq anions of mean

charge 0.977 - 1.585 for Hereward, Gamina and Bohoth 105 respectively. In the three varieties, the charge balance up to 1.6 (Table 6.1). The high value of Z indicates either the presence of uncharged solutes and/or multivalent anions such as malate (as well as monovalent). In Table 6.1, in order to calculate the value for Z, it was assumed that there were no significant unchanged osmotica. Previous SiCSA analyses of barley epidermis have shown that it contains negligible sugars (Koroleva *et al.*, 1997).

6.7 Inter-Relationship of Cations (Na^+ and K^+) and Osmotic Pressure.

Fig. 6.8 shows the relationship between Na^+ and K^+ concentration in root cortical cells of wheat. When NaCl was added to root media, Na^+ concentration increased with time in all varieties (Fig. 6.2). Potassium concentration showed the opposite pattern under salt stress. However, in Bohoth 105, K^+ concentration initially decreased after salt stress and then became more or less constant for the whole experimental period (Fig. 6.3). From Fig. 6.8, the relationship between Na^+ and K^+ concentration can be seen to be linear in Hereward and Gamina, but not in Bohoth.

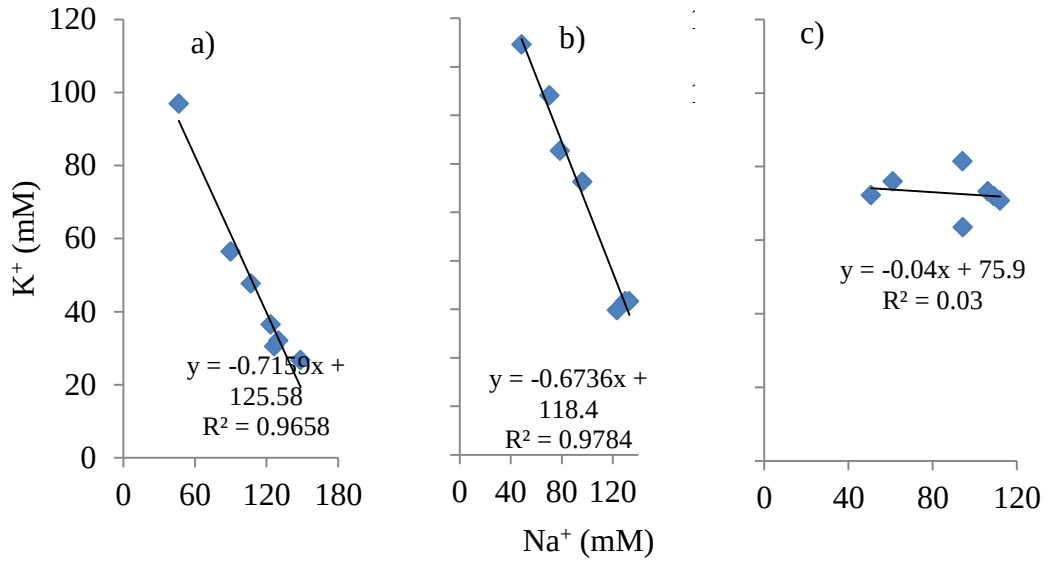

Fig. 6.8 Relationship between Na^+ and K^+ concentration in root cortical cell sap in Hereward (a), Gamina (b) and Bohoth 105 (c). (Data from Fig. 6.4 and 6.5).

6.8 Discussion

In the three varieties, the P_{cell} of the root cortical cells dropped rapidly within 48 hours by ≤ 3 bar, and turgor recovery occurred after 48 - 72 hours (Fig. 6.1a - c). In a similar pattern to P_{cell}, π_{cell} also rapidly increased following salt stress and then increased gradually with time before remaining constant at about 3 bar by 144 - 169 hours which reflect the change in the external medium (about 3 bar NaCl in medium). Clearly, P_{cell} recovery varied between the different varieties. Fig. 6.1a - c shows that half time ($T_{1/2}$) took at least 6.3 days for Hereward, 7.9 days for Gamina and 1.7 days for Bohoth 105 for the P_{cell} to reach the stable adjusted level (Table 6.2). However, also a lower osmotic pressure $T_{1/2}$ (2.1 days) was found in Bohoth 105 than in the others (Table 6.3). Cortical cells of Bohoth 105 had faster osmotic adjustment and rapid π_{cell} than change the other varieties (Table 6.2 and 6.3).

The total contribution of ion uptake in the cortical cell osmotic adjustment is according to van t'Hoff law (see Shabala *et al.*, 2000), about 3 bar and hence changes in π_{cell} caused by salt stress would be achieved by the uptake of Na^+ (Table 6.1 and

Fig. 6.2). This means that osmotic adjustment depended on the uptake of Na^+ and the loss (mainly) of K^+. In the three varieties, the π_{cell} of control cortical cells was about 5.7 bar (Table 6.1 and Fig.4.1d - f). This π_{cell} agrees with a report on cortical cells of barley (7.1 bar) by Pritchard (1996), and compares well with the overall estimated solute content. The overall cations equal about 131 mM and sum to 140 mEq. If this was associated with monovalent anions, for instance as Cl^- or NO_3^- (Chapters 4 and 5), we expected π_{cell} would be about 6.5 bar (approximately 260 mOmol.kg^{-1}).

For a P_{cell} value of about 5 bar in the three varieties (Fig 6.1), the ψ_{cell} of control cortical cells was about -1 bar, shows much higher values than that of epidermal leaf cells (Chapter 4). This ψ_{cell} is equivalent to reports of that of cortical cells of maize (-2 bar) by Pritchard *et al.* (1996). And of *Pinus pinaster* (-3 bar) by Triboulot *et al.* (1995).

In all three varieties, addition of salt to the root medium dropped the ψ_{cell} of the root cortical protoplast by about 4 - 4.7 bar during the whole experimental period (Fig 6.9). These changes could be due to π_{cell} changes and, hence, it reflected the 3 bar stress applied. This change represents an increase of π_{cell} (decline of ψ_{cell}) of the cortical protoplast.

The contribution of ions to the cortical protoplast osmotic pressure was found to be 132 mEq of Na^+, 10 mEq of $Ca^{2+}$and a loss of 79 mEq of other cations (K^+ and Mg^{2+}) for Hereward; 128 mEq of Na^+, 28 mEq of Ca^{2+} and a loss of 93 mEq of other cations for Gamina; and 106 mEq of Na^+ and a loss of 55 mEq of other cations for Bohoth 105 by day 8 (Table 6.1 and Fig. 6.2 - 6.5). In all varieties, this corresponds to an increase of 3.5 bar of monovalent anions.

Potassium concentration in Hereward and Gamina was decreased significantly by salt stress with time compared with control plants. A similar finding was found by Tavakkoli *et al*. (2010) in barley. While K^+ concentration was increased within 24 h and remained unchanged with time. The conclusion is that Na^+ salt was found to be predominant and that similar amounts were found in cortical cells of three varieties (Fig. 6.4). In Bohoth 105 cells, turgor recovery was found to be faster than in Hereward and Gamina. However, osmotic recovery ($T_{1/2}$ = 1.7 days) was found to be faster in Bohoth 105 as compared with other varieties (Table 6.2), suggesting that Bohoth 105 is the more salt tolerant plant.

The condition of the mature root cells in the samples in this study was affected by the fact that they were grown hydroponically. The mature cells were being used for transport, and not redundant, meaning that they needed to be kept alive by the plant. For this, they had to be able to effect osmotic adjustment in response to osmotic stress, and maintain P_{cell}. While root endodermal cells actively exclude sodium, to prevent its translocation, mature root cells use Na^+ for osmotic adjustment. Different parts of the root therefore play different roles. The findings in this study were specifically for mature cortical cells, which were chosen for pragmatic reasons.

Having considered root turgor, osmolality and cations for the three wheat varieties in the above, the next chapter will consider the same parameters for the halophyte *S. maritima.*

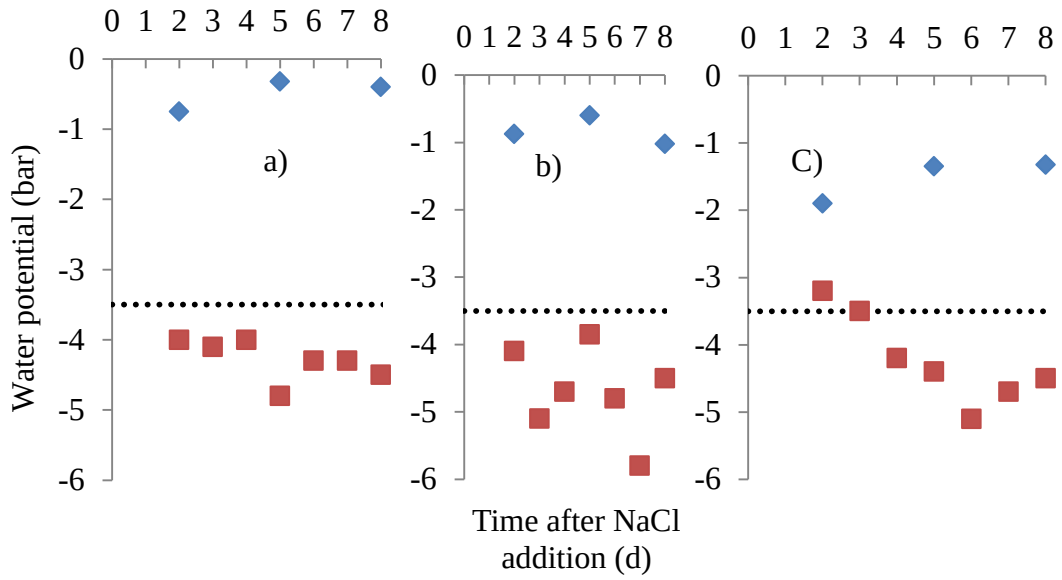

Fig. 6.9 Water potential in root cortical cell of Hereward (a), Gamina (b)and Bohoth 105 (c) following addition of 60 mM NaCl. $\Psi_{cell} = P_{cell} - \pi_{cell.}$

Chapter 7: Turgor, Osmolality and Cations in Roots of *S. maritima*

7.1 Introduction

As mentioned earlier in the introduction, this study uses the known halophyte *S. maritima* as a comparison to the wheat varieties in order to investigate the extent to which their parameters are similar. This chapter therefore turns to the water and solute relations of *S. maritima* roots.

Cells four to six cm from the root tip of intact plants were studied with regards P_{cell}, π_{cell} and cations *in situ* every day from days 2 to 8 (salt π_{cell}), plus days 14 and 21 (salt P_{cell}) after the addition of 200 mM NaCl. They were also studied at day 2, 5 and 8 (control π_{cell}), plus at days 14 and 21 for the control treatment P_{cell} (0 mM NaCl).

7.2 Turgor Pressure in Root Cortical Cells

After application of NaCl, P_{cell} of root cortical cells changed exponentially increased with time until a new (nearly) stationary state was reached (Fig. 7.1a). Cells had P_{cell} values under the control condition of 6.4 ± 1.1 bar (n = 3 – 12). A significant difference (P = 0.000) in the P_{cell} of the cells was found between 0 mM and 200 mM NaCl treatments.

When salt was added to the root medium, P_{cell} decreased by 5.3 bar and then increased gradually until it reached 6.7 bar by day 21 (Fig. 7.1a). Turgor pressure recovery had started by 48 h from stress initiation. Cortical P_{cell} was restored over some 8 days, with a $T_{1/2}$ of 3.2 days (Fig7.1a). This result shows the biphasic response (fast P_{cell} decrease and slower P_{cell} recovery) after 200 mM NaCl addition.

The turgor pressure increase reflected the solute uptake rate and/or synthesis in the root cortical cells. The first phase of the biphasic P_{cell} relaxation is due to the loss of water of the cortical cells to the external medium, whilst the second phase is due to an increase in π_{cell} of the cells as result of transport, of synthesis of solutes, or of both.

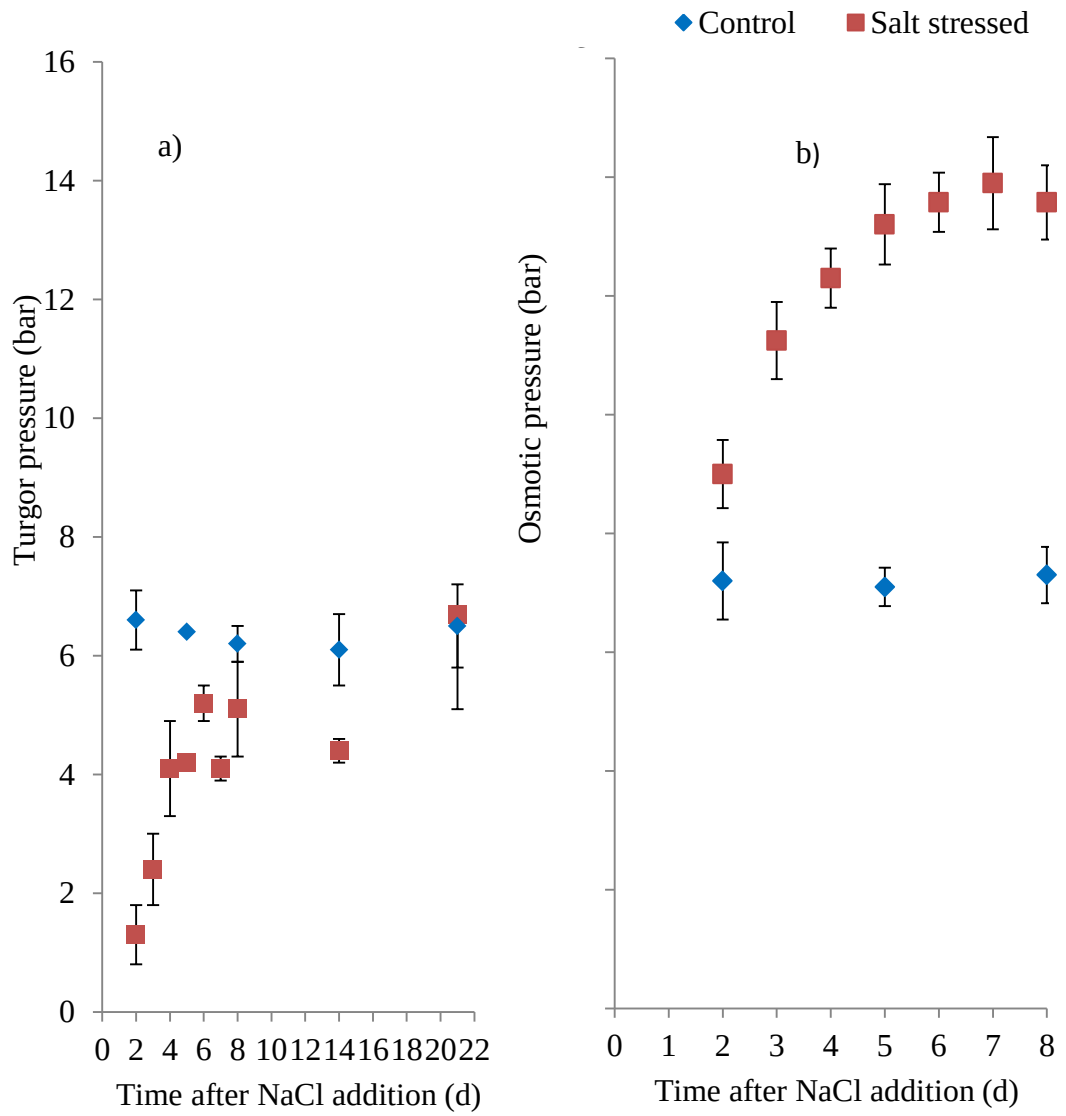

Fig. 7.1 Time course of root cortical cell turgor pressure (P_{cell}) and osmotic pressure (π_{cell}) of *S. maritima* for 0 mM control and following application of 200 mM NaCl. Salt was added in two 100 mM doses over two days (the days are numbered first addition). Root cortical cells were prepared 4 - 6 cm from the root tip, for 3 - 12 cells taken from 3 - 6 individual plants. Cell turgor pressure was measured using a cell pressure probe. Cell osmotic pressure was measured using single cell sampling, and a picolitre osmometer. Difference in means P_{cell} and π_{cell} between treatments were always highly significant (P= 0.000 ANOVA of day 2, 5 and 8). Each point is the mean ± SD.

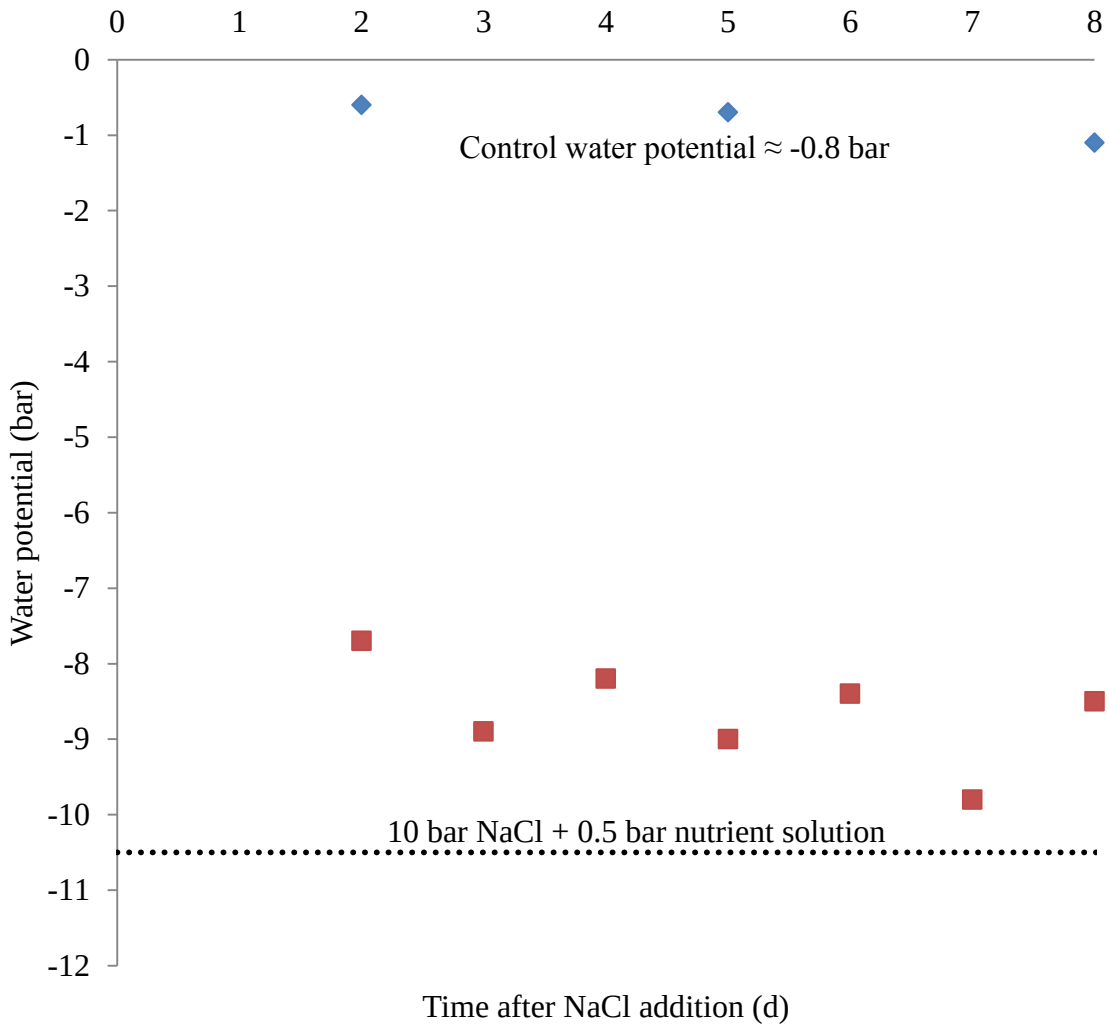

Fig. 7.2 Water potential in root cortical cell of *S. maritima* following addition of 200 mM NaCl. $\Psi_{cell} = P_{cell} - \pi_{cell.}$

Time after NaCl addition (d)	Cations concentrations (mM)								∑cations (mM)		∑charge cations (mEq)		Osmolality (mOsmol.kg^{-1})		π_{total} /γ		∑anions (mM)		Average charge of anions	
	Na^+		K^+		Ca^{2+}		Mg^{2+}													
	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed	Control	Salt stressed
2	30 ± 5	125 ± 18	69 ± 4	60 ± 8	21 ± 0.7	1 ± 0.2	10 ± 7	2 ± 5	130	188	161	191	292 ± 26	360 ± 23	313	386	183	198	0.9	1
3		161 ± 5		53 ± 2		2 ± 0.1		3 ± 19		219		224		450 ± 26		483		264		0.8
4		166 ± 9		50 ± 3		3 ± 0.0		3 ± 3		222		228		490 ± 20		526		304		0.8
5	25 ± 5	155 ± 3	86 ± 8	52 ± 3	13 ± 0.1	4 ± 0.1	9 ± 9	3 ± 4	133	214	155	221	284 ± 13	528 ± 27	305	567	172	353	0.9	0.6
6		187 ± 3		50 ± 1		4 ± 0.1		4 ± 2		245		253		543 ± 20		583		338		0.7
7		220 ± 11		39 ± 9		5 ± 0.0		6 ± 3		270		281		558 ± 31		599		329		0.9
8	15 ± 1	240 ± 4	99 ± 8	18 ± 1	9 ± 0.04	1 ± 0.0	6 ± 11	6 ± 3	129	265	144	272	292 ± 19	543 ± 25	313	583	184	318	0.8	0.9

Table 7. 1 Cation concentrations and osmolality of the sap of single cortical cell of *S. maritima* root. Cortical cells (n = 3 - 12) were selected 4 - 6 cm from the root tip individual plants. Cell sap was analysed every day during the salt treatment and three times during the 7 days of the control treatment. The relationship between the osmolality of cations was obtained by picoliter osmometer and molarity was obtained by capillary zone electrophoresis (CZE). Calculations of the contribution of cations to the cell osmotic pressure (π_{cell}) were made by multiplying the sum of the concentrations by the osmotic coefficient (γ) of NaCl 0.932 (Robinson & Stokes, 2002). The values of ≈ 1.0 for the average anion charge suggest monovalent ions (Cl^- or/or NO_3^-). Results are given as means ± SD.

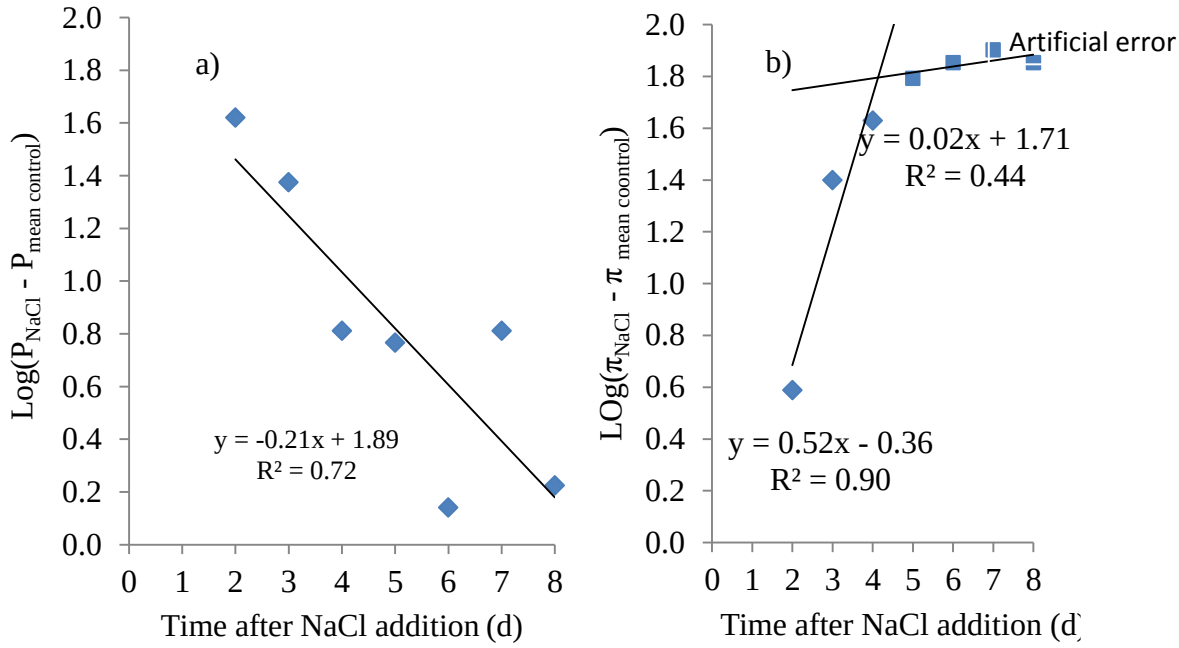

Fig. 7.3 Log plot of the time course of root cortical cell turgor pressure (a) and osmotic pressure (b) during turgor recovery following salt stress of *S. maritima*. See Fig. 7.1 for details of cells. The difference between control and salt turgor pressure in Fig.7.1a plotted logarithmically ($\log(P_{NaCl} - P_{mean\ Control}$) against time in Fig. 7.3. Half time ($T_{1/2}$) calculated from log(2)/slope. The equations for the curves for each variety together with R^2 values are included.

7.3 Osmotic Pressure in Cortical Cells

Cells had similar π_{cell} values of 7.1 ± 0.5 bar (n = 3 - 12) under control conditions during the whole experimental period. When salt was added to the root medium, π_{cell} increased gradually over the subsequent days and reached 13.6 bar for the rest of the experimental period (Fig. 7.1b). Significant differences between treatments were observed at the 5 % level (Fig. 7.1b). The difference in π_{cell} between control and salt treatment is around 6.3 bar by day 8, which reflects the increased π_{cell} of the external root medium in the 200 mM NaCl treatment. These data (Fig. 7.1) show that the magnitude (approximately 7.1 bar) of the π_{cell} in control root cells is reflected by the P_{cell}.

7.4 Osmotic Adjustment

Data in Fig. 7.1b shows osmotic adjustment had started by day 2 after NaCl addition, by accumulation of solutes in the cells. Root π_{cell} increased as a consequence of net solute accumulation. It is possible that this, in turn, led to water uptake into the cell, thus maintaining P_{cell}. In root cortical cells, turgor recovery was followed by equivalent changes in π_{cell} by building up solutes in the protoplast by osmotic adjustment.

7.5 Cation Concentrations in Single Cortical Cells

To determine the inorganic cations contributing to osmotic adjustment, CZE was performed to examine the mean of cation concentration of the sap sample obtained from the single cortical cells. Table 7.1 demonstrates the distribution of cation (Na^+, K^+, Ca^{2+}, Mg^{2+}) concentrations contributing to osmotic adjustment. The results show that the cations corresponded to the osmotic adjustment in the cells. The solutes accumulated in the root cortical cells of *S. maritima* under salt stress were largely Na^+ salts.

7.5.1 Na^+ Concentration

Cells had Na^+ concentration in the control (0 mM NaCl) of less than 30 mM (Fig. 7.4). Root cortical cells accumulated significantly higher Na^+ concentration ($P \leq 0.05$) by 2 days after salt addition. The concentration increased gradually during the whole experimental period until it reached 240 ± 15 mM (Table 7.1 and Fig. 7.4). Potassium and Na^+ concentrations were found to have the highest values in both the control and salt treatments during the whole experimental period as compared with other ions (Fig.7.4 and 7.5). Under salt stress, Na^+ concentration contributed to the π_{cell} of cortical cells of plant roots by 225 mEq at day 8.

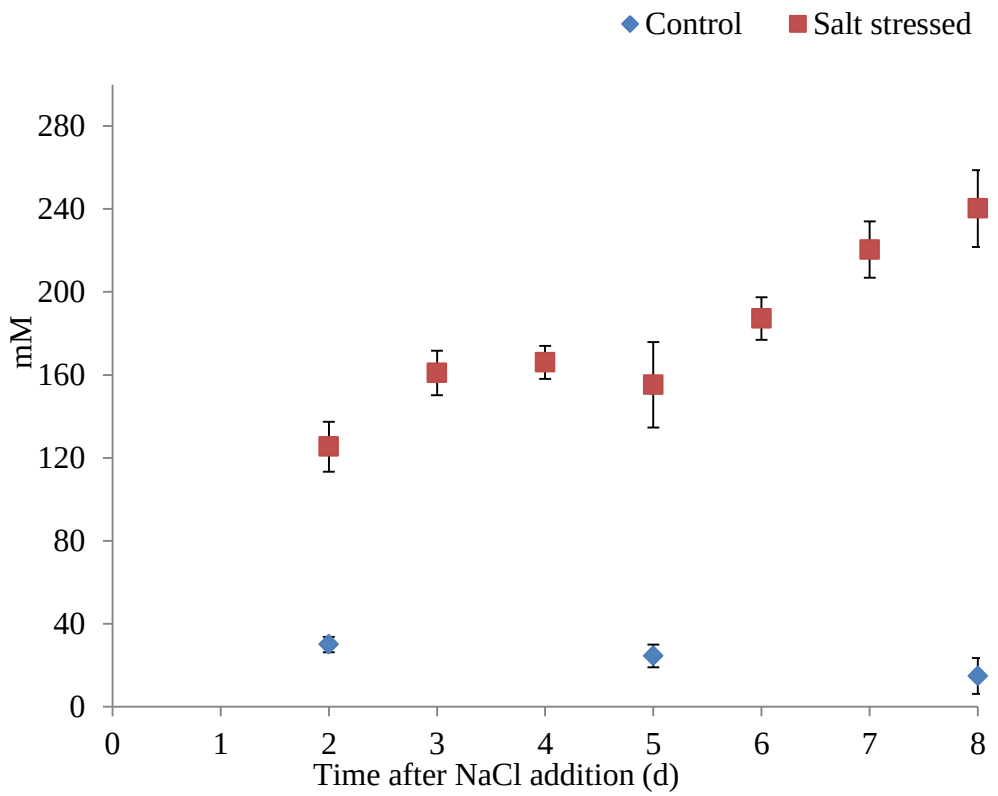

Fig. 7.4 Na^+ concentration in cortical cell sap of *S. maritima* roots. Cortical cells from 4 - 6 cm away from the root tip of individual plants were analysed at the first day after NaCl addition. Salt was added in two 100 mM doses over two days (the days are numbered after first addition). Na^+ concentration was measured using capillary zone electrophoresis (CZE). Each observation is the mean of 3 - 12 cells taken from 3 - 6 individual plants. Difference in means Na^+ concentration between treatments were always highly significant (P = 0.000 at 5 % level).Results are given as means ± SD. From the graph, plants accumulated more Na^+ in root cells under stress. Sodium uptake played a major contribution to osmotic adjustment.

7.5.2 K^+ Concentration

Generally, in 0 mM NaCl plants, K^+ concentration increased with time and was found to have the highest values for the rest of the experimental period as compared with other ions (Table 7.1). Potassium concentrations ranged from 69 ± 3 mM at day 2 to 99 ± 10 mM at day 8. When 200 mM NaCl was added to root media, K^+ concentration decreased from 60 ± 3 mM at day 2 to 18 ± 3 mM at day 8 (Fig. 7.5). Potassium was found in the reverse pattern to Na^+.

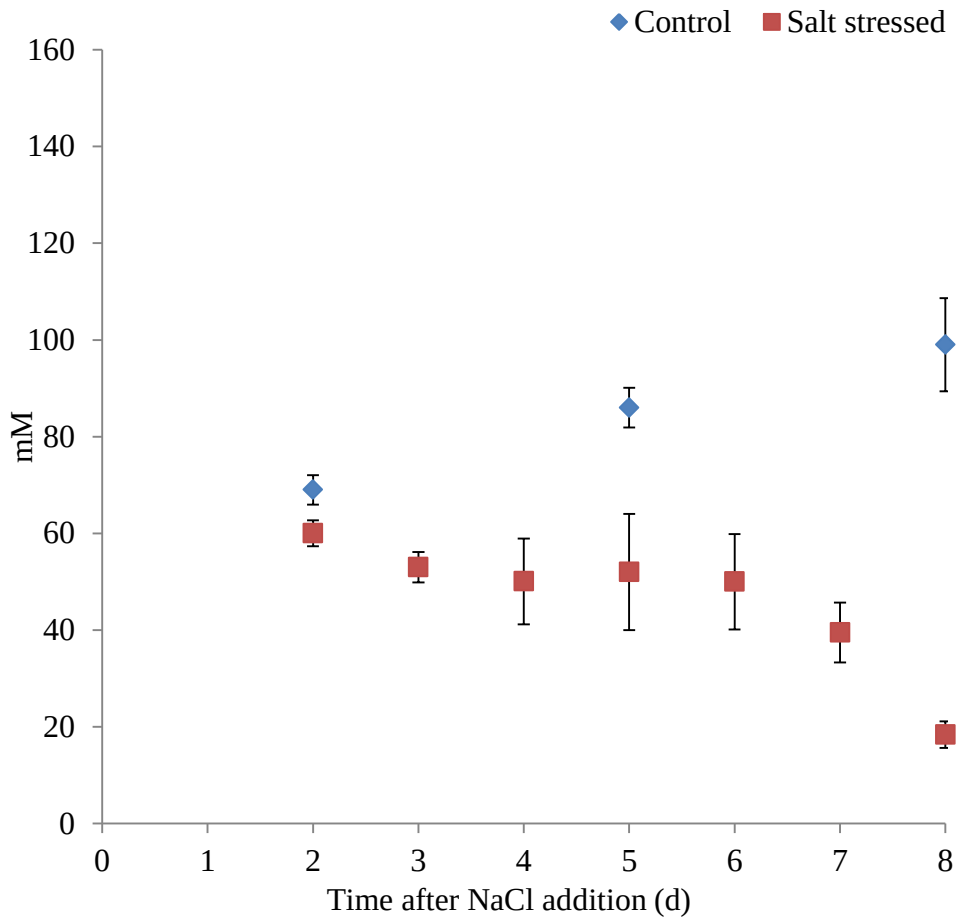

Fig. 7.5. K^+ concentration in cortical cell sap of *S. maritima* roots. Details as in Fig. 7.3

7.5.3 Ca^{2+} Concentration

Under the control condition the Ca^{2+} concentration was less than 21 ± 1.6 mM, and decreased with time (Fig. 7.6). Salt stress caused a significant reduction in Ca^{2+} concentration, but with a small subsequent recovery.

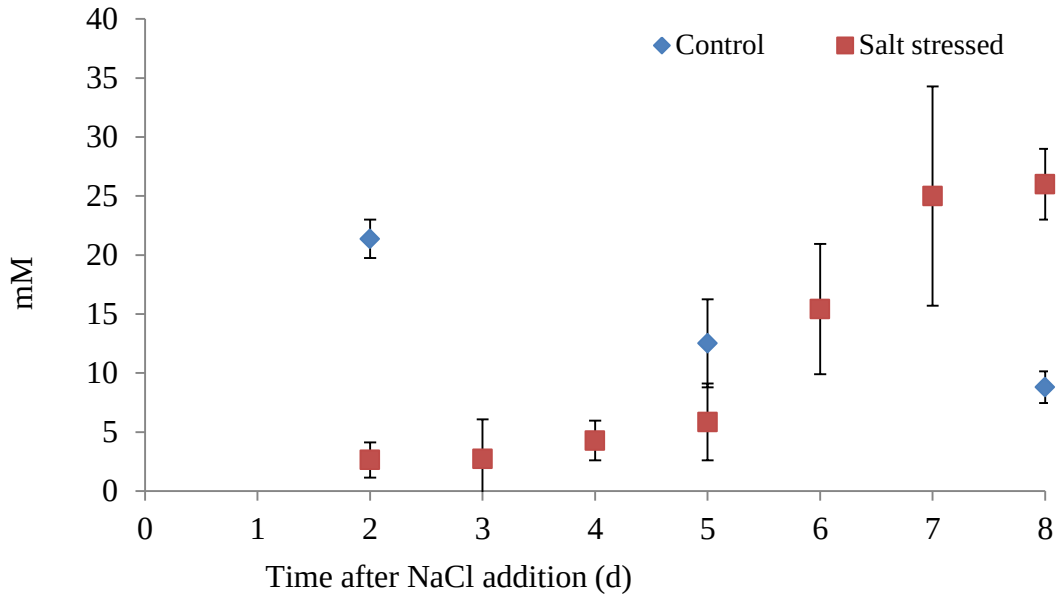

Fig. 7.6 Ca^{2+} concentration in cortical cell sap of *S. maritima* roots. Details as in Fig. 7.3.

7.5.4 Mg^{2+} Concentration

The cell level of Mg^{2+} in control conditions (0 mM NaCl addition) was less than 10 mM (Fig. 7.7). Salt stress caused a reduction in Mg^{2+} concentration for the rest of the experimental period, although with a small recovery. Magnesium control and salt treatment plants had similar patterns to Ca^{2+}.

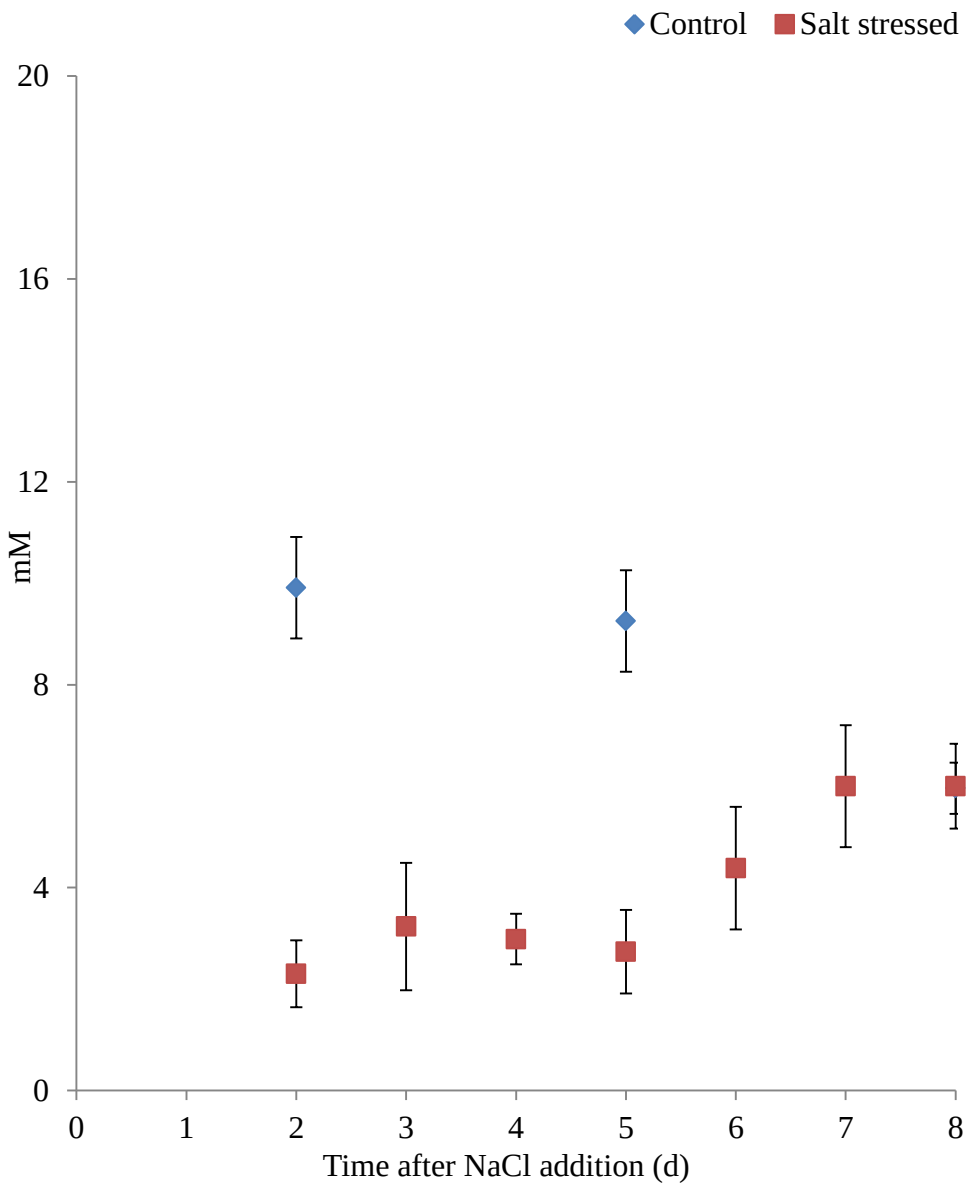

Fig. 7.7 Mg^{2+} concentration in cortical cell sap of *S. maritima* roots. Details as in Fig. 7.3.

7.6 Estimate of the Anion Concentration and its Valency

The relationship (and its implications) between the sum of the cations and the measured π_{cell} of samples is shown in Table 7.1. The calculated charges for the anions indicate monovalent ions, presumably chloride (see Section 4.7) if no uncharged solutes are present. In Table 7.1, in order to calculate the value for Z, it was assumed that there were no significant unchanged osmotica. Previous SiCSA analyses of barley epidermis have shown that it contains negligible sugars (Koroleva *et al.*, 1997).

7.7 Inter-Relationship of Cations (Na^+ and K^+) and Osmotic Pressure.

When 200 mM NaCl was added to root media, cell Na^+ concentration increased with time (Fig. 7.4). Potassium however, showed the opposite pattern (Fig. 7.5). Fig. 7.8 shows that a 3:1 linear relationship is found between the two ions.

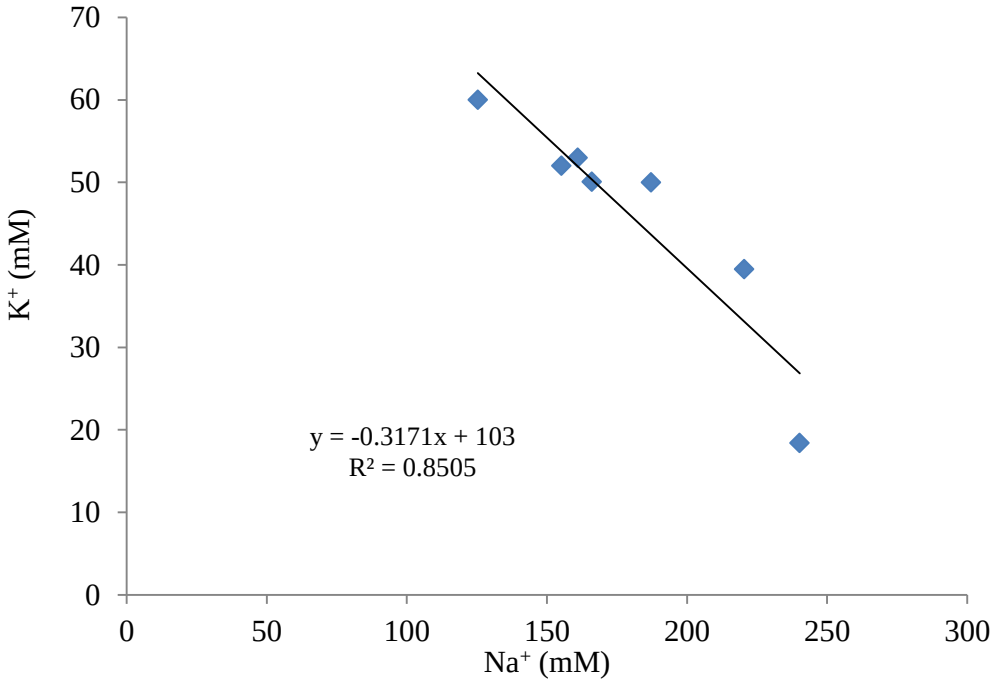

Fig. 7.8 Relationship between Na^+ and K^+ concentration in root cortical cell sap in *S. maritima*.

7.8 Discussion

The aim of this work was to compare the response of wheat varieties of varying salt tolerance with the impact of 200 mM NaCl on the π_{cell} and cation responses in root cortical cells of the halophyte *S. maritima*. The osmotic pressure of cortical cells increased with time in *S. maritima*. At the same time, P_{cell} decreased rapidly by about 5.3 bar, this drop was of half the order of the 200 mM (≈10 bar) NaCl applied. The change in π_{cell} was followed the change in P_{cell}. Turgor pressure recovery started to increase within 48 hours, and the half time ($T_{1/2}$) took at least 3.2 days for the turgor to reach that stable adjusted level, while $T_{1/2}$ in π_{cell} was found after 1.3 days (Fig. 7.3).

Out of the three wheat varieties, the most similar results to this were obtained for Bohoth 105 (Chapter 6), which had a half time for P_{cell} of 1.7 days and for π_{cell} of 2.1 days, suggesting that Bohoth 105 and *S. maritima* had the most similar behaviour response to salt stress.

The osmotic pressure for control root cortical cells of *S. maritima* was 7.1 bar, the P_{cell} was 6.4 bar, ψ_{cell} of was -0.8 bar (Fig.7.2). These findings were similar to those obtained for cortical cells of wheat (Chapter 6). Total cations were equal to 129 mM by day 8. This value is equivalent to a sum of 144 mEq. If this is associated with anions (monovalent), for instance as Cl^- or NO_3^- (Chapter 4, 5 and 6) (Fricke *et al*., 1994a and b), we anticipate π_{cell} could be about 268 mOsml.kg^{-1} (6.7 bar).

When salt was added to the root medium, ψ_{cell} of cortical apoplast decreased by -7.7 bar (decrease in ψ_{cell}) after the second addition of salt, and then remained stable around -8.8 bar over the remaining period of the experiment (Fig. 7.2). These are

similar results to those observed for leaf epidermal cells of Gamina (Chapter 4), and double that of root cortical wheat cells. This change could be due to the change in π_{cell}, which is equivalent to the change in the external medium of the root (200 mM NaCl ≈ 10 bar).

The increase in π_{cell} is due to the accumulation of 225 mEq of Na^+ and the loss of 97 mEq of both K^+ and Ca^{2+} (Table 7.1). For monovalent anions, this is equivalent to an increase of 6.4 bar (Table 7.1). The total contribution of ions accumulated during the osmotic adjustments of the cells was 6.4 bar (256 mOmol.Kg^{-1}). This change in π_{cell} due to salt stress would be achieved by the accumulated Na^+ salt (Table 7.1 and Fig. 7.3). Sodium accumulated in cortical *S. maritima* was twice as high as in cortical cells of wheat. This result is in agreement with previous studies (Mori *et al*., 2010). It has been found that Na^+ is the main inorganic cation in tissues of *S. salsa* contributing to the maintenance of osmotic pressure under salt stress. However, in *S. maritima* osmotic adjustment was accomplished by both Na^+ uptake and loss of K^+ and Ca^{2+} (Table 7.1 and Fig. 7.3 – 7.5).

In the above chapters, the parameters of turgor pressure, osmotic pressure and cations have been considered, for both leaves and roots. The next chapter will turn to the biomechanical parameter of cell wall elasticity and to the hydraulic conductivity of the cell membrane.

Chapter 8: Volumetric Elastic Modulus, Half Time and Hydraulic Conductivity in Wheat and *S. maritima*

8.1 Introduction

Turgor and osmotic pressure are not the only water relations parameters that could contribute to salt tolerance. In this final chapter, we look to several other such parameters (Zimmermann & Steudle 1978) for evidence of their potential contribution to salt tolerance in wheat and *S. maritima*.

Volumetric elastic modulus (ε_{cell}) is a biomechanical parameter that contributes to the plant's response to changing water potential in the external environment by affecting the balance of turgor regulation and osmotic adjustment processes. Indeed, it is considered that when under stress conditions, changing the ε_{cell} is the first response of plant cells in the series of adjustments made to minimize the effects of osmotic stress (Tomos, 1988). By increasing the cell wall elasticity with the onset of stress, the decrease in cell volume maintains turgor pressure (Hsiao & Jing, 1987). An alternative strategy is to decrease elasticity in order to lower leaf water potential and thus decrease water loss (Kramer and Boyer, 1995; Navarro *et al*., 2007). Whilst it is clear that cell wall elasticity is an important mechanism, the ways in which it is used in different species is not completely understood (De Diego *et al*., 2013).

Having more rigid cell walls may indeed have benefits, despite their inability to maintain turgor. One is that they may help successful rehydration when stress is removed, in terms of preserving the integrity of the physiology of a plant, which has

adjusted osmotically by accumulating solutes. Another is that they may be better than elastic walls at maintaining low Ψ_{cell} and thus increasing water potential gradient (Bowman & Roberts, 1985).

A second important parameter to consider is hydraulic conductivity. Under saline conditions, both the ionic balance and water relations are affected, but the latter is a more immediate response than the former. Hydraulic conductivity (L_p) controls the process of changing the cell water potential. Indeed, physically, cereals can be seen as hydraulic conductors that profit from the natural water potential gradient between soil and atmosphere to upwardly conduct both water and solute nutrients (Knipfer and Fricke, 2010).

Water flow through all parts of a plant is limited by hydraulic resistance. An analogy can be drawn with Ohm's Law (Frensch, 1997). However, it is the root hydraulic conductivity that is a particularly important parameter that controls flow rate (Steudle, 2000), and root water movement can be seen as an osmotic pressure. Root membranes are therefore a key part of this process (Knipfer and Fricke, 2010), since root structures are determinants of their physical characteristics and must be understood in this context (Steudle, 2000; Steudle and Peterson, 1998). A membrane's hydraulic conductivity and osmotic water permeability are proportionally related, and its ability to transport water depends on these gradients (Steudle and Henzler, 1995).

In order to identify any trend that might be related to the ability of the plant to withstand salt stress, the response to salt stress of single cell volumetric elastic modulus (ε_{cell}), half time ($T_{1/2}$) and hydraulic conductivity (L_p) were analysed in wheat and *S. maritima* leaves and roots. The cell pressure probe (see Chapter 2) was

applied to the same cell types exposed to the same treatment as described in Chapters 3 - 7.

Variety	Treatment (mM NaCl)	A (mm^2)	V (pl)	$\Delta P/\Delta V$ (bar/pl)	$T_{1/2}$ (s)	Π (bar)	ε_{cell} (bar)	Lp x 10^6 ($cm.s^{-1}bar^{-1}$)
Hereward	0	0.0134	108	1.8 ± 0.1	2.4 ± 0.3[a]	14.4 ± 0.3	177 ± 20[a]	1.02 ± 0.02[a]
	60	0.0133	107	1.7 ± 0.3	2.8 ± 0.6[a]	18.5 ± 0.2	198 ± 19[b]	1.04 ± 0.24[a]
Gamina	0	0.0137	111	1.6 ± 0.2	2.8 ± 0.4[a]	13.6 ± 0.3	181 ± 23[c]	1.16 ± .015[a]
	60	0.0136	110	1.5 ± 0.3	3.3 ± 0.9[a]	15.7 ± 0.5	167 ± 28[c]	1.11 ± 0.03[a]
Bohoth 105	0	0.0136	109	1.4 ± 0.2	2.8 ± 0.5[a]	17.5 ± 0.3	182 ± 31[e]	1.13 ± 0.04[a]
	60	0.0131	103	1.7 ± 0.3	3 ± 0.4[a]	20.9 ± 0.2	156 ± 35[f]	1.21 ± 0.02[a]

Table 8.1 Cell water relations parameters of leaf epidermal cells of control and salt treated Hereward, Gamina and Bohoth 105 (60 mM NaCl). Change of turgor pressure per change in volume ($\Delta P/\Delta V$), half time for water exchange ($T_{½}$), osmotic pressure (π_{cell}), and volumetric elastic modulus [ε_{cell} values have been calculated using several cell volume taken from living cells (see Chapter 2)] and Lp values of the cell membrane. Results are given as means ± SD. Letters a, b, c, d, e and f are comparing between control and salt treatments. Means followed by the same latters are not significantly different at P>0.05.

8.2 Volumetric Elastic Modulus of Wheat Leaf Epidermal Cells

Examples of $\Delta P/\Delta V$ curves for each of the wheat varieties are shown in Fig. 2.2. Mean cell volumes determined from longitudinal sections were used (Chapter 2). Salt stress significantly increased the ε_{cell} in Hereward (Table 8.1) and significantly decreased the ε_{cell} in Bohoth 105, while there was not a difference between the control and salt treatment in Gamina (Table 8.1).

8.3 Half-Time of Water Exchange and Hydraulic Conductivity of Wheat Leaf Epidermal Cells

Pressure-relaxation curves for exosmotic and endosmotic water flows from epidermal cells revealed rapid equilibrium. The pressure-relaxation curves were

fitted by eye (Fig. 8.3 – 8.5). It can be seen from the figures that the nature of the raw data makes a precise estimate of $T_{1/2}$ very difficult. The value of L_p derived can only be approximate. All three varieties showed no significant changes in $T_{1/2}$ water exchange in response to salt stress throughout the experimental period (Table 8.1). The ε_{cell} and the $T_{1/2}$ value, together with average value for cell volume (V), and surface area (A) were used to calculate Lp (see Chapter 2 for detail). The cells chosen for P_{cell} and elasticity measurements were cylindrically shaped to improve the estimates of V and A. The cell volumes were similar between treatments and varieties (Table 8.1).

8.4 Volumetric Elastic Modulus in Root Cortical Cells of Wheat and *S. maritima*.

Examples of $\Delta P/\Delta V$ curves for each of the wheat varieties and for *S. maritima* are shown in Figs. 8.1 and 8.2. Mean cell volumes were determined from root longitudinal and transverse sections (Chapter 2). Salt stress significantly decreased the ε_{cell} in all plants (Table 8.2). The ε_{cell} decreased in root cortical cell by 26 %, 43 %, 33 % and 54 % in Hereward, Gamina, Bohoth 105 and *S. maritima* respectively (Table 8.2). In both Hereward and Gamina, ε_{cell} decreased after salt addition by day 2 and then started to recover its value reaching 192 bar and 158 bar by day 8 respectively. In contrast, in Bohoth 105 and *S. maritima*, ε_{cell} was decreased after salt addition by day 2 and then remained unchanged (Table 8.2).

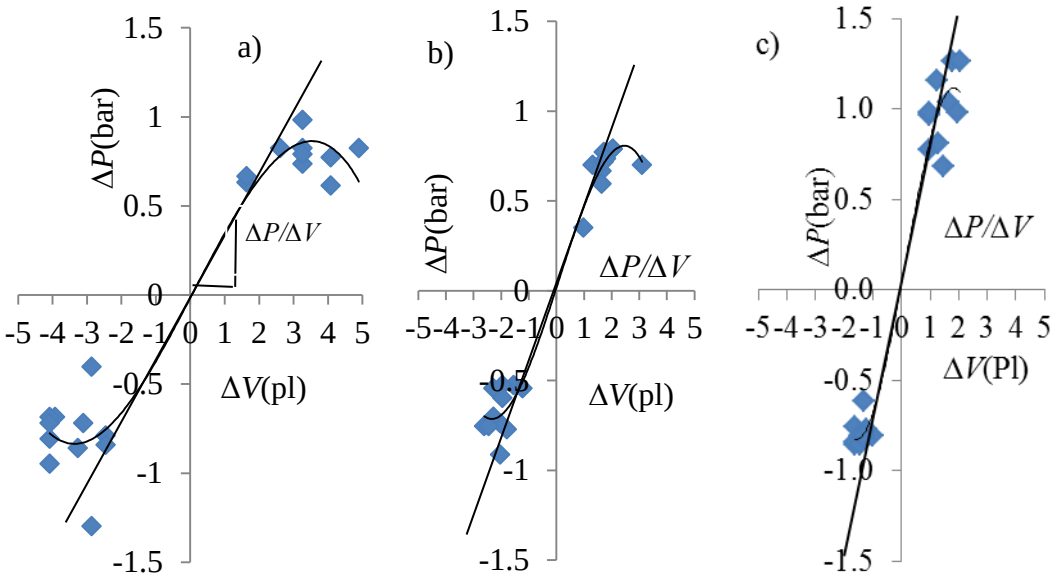

Fig. 8.1 $\Delta P/\Delta V$ curves for root cortical cells of Hereward (a), Gamina (b) and Bohoth 105 (c) varieties as obtained by pressure probe (cell wall elasticity measurements). A pressure pulse ΔP is applied to individual cells and the resulting change in volume (ΔV) is measured (The sigmoidal shape of the curve is due to water flow across the cell membrane during the experiment). The *dp/dv* used is that extrapolated to $\Delta V = 0$).

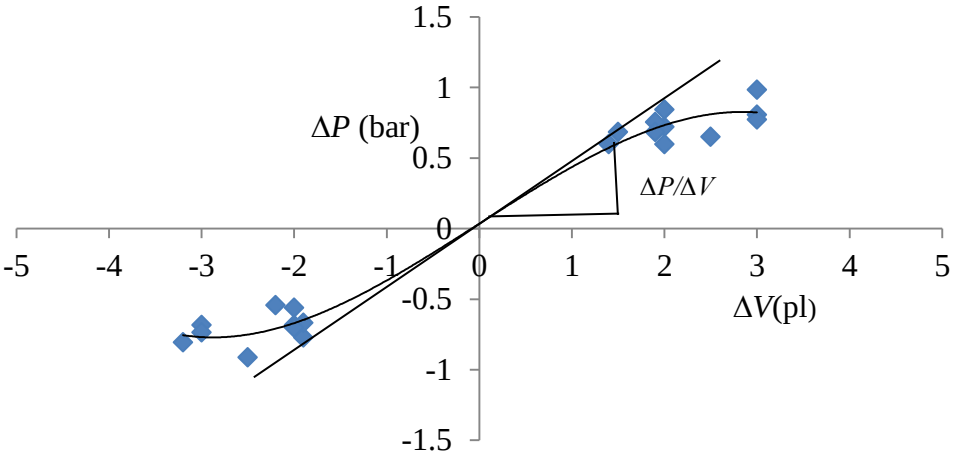

Fig. 8.2 $\Delta P/\Delta V$ curve for *S. maritima* root cortical cells. Details as in Fig. 8.1.

8.5 Hydraulic Conductivity in Root Cortical Cells of Wheat and *S. maritima.*

Pressure-relaxation curves for exosmotic and endosmotic water flows from root cortical cells revealed rapid equilibrium (Table 8.2). The cortical cell volume for

both the control and the salt treatment was broadly similar for the three varieties. In *S. maritima*, a higher cell volume was found for the salt condition as compared to the control. Cell volume in Bohoth 105 most closely resembled this, whilst Hereward and Gamina had similar values to each other. No significant change in water exchange (L_p) was seen in any of the varieties.

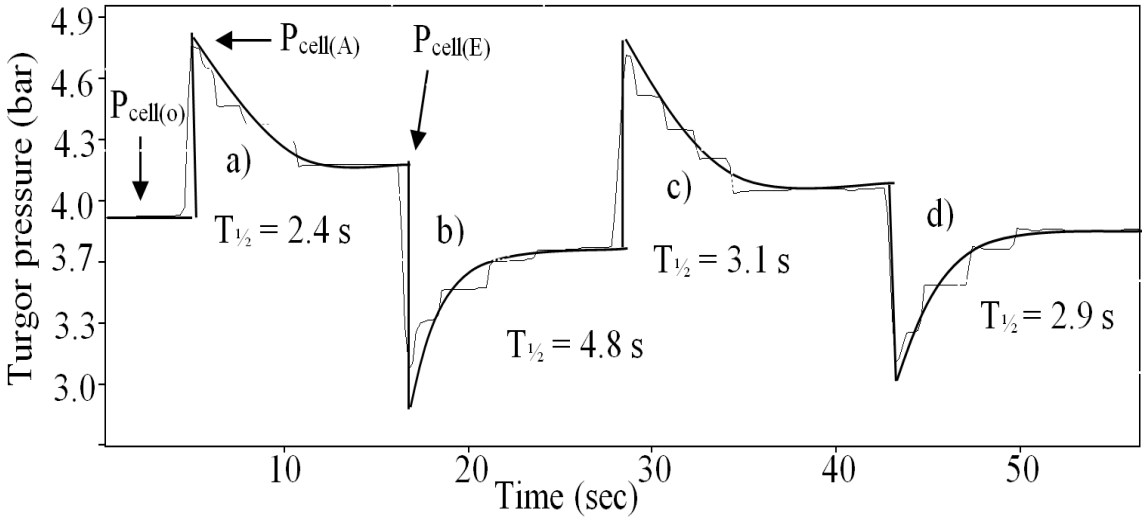

Fig. 8.3. Pressure – relaxation curves from root cortical cells of Hereward wheat determined by the cell pressure probe. Details as in Fig. 8.2.

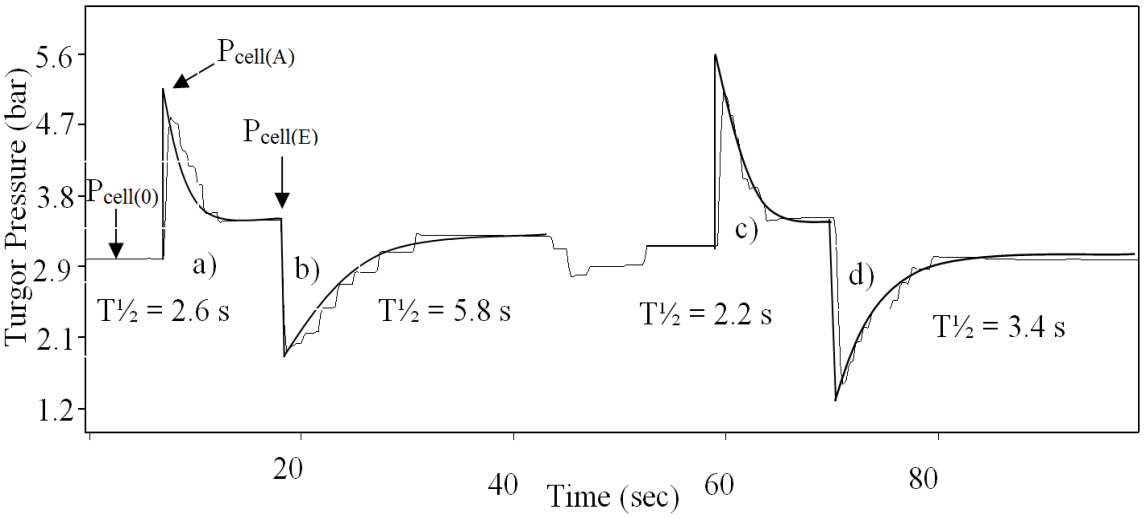

Fig. 8.4 Pressure – relaxation curves from root cortical cells of Gamina wheat determined by the cell pressure probe (Chapter 2). Details as in Fig. 8.2.

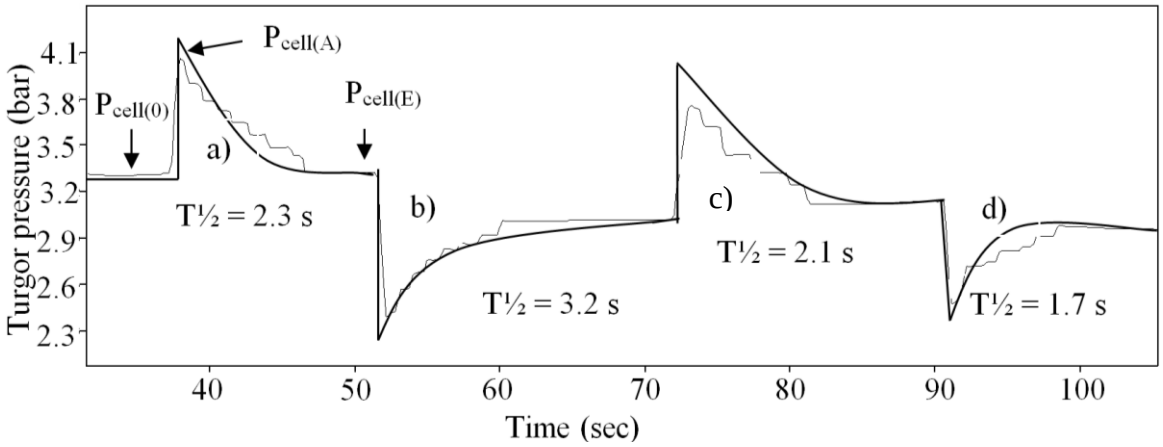

Fig. 8.5 Pressure – relaxation curves from root cortical cells of Bohoth 105, as determined by the cell pressure probe Details as in Fig. 8.2

Variety	Treatment	Time (d)	A (mm2)	V (pl)	Δp/Δv (bar/pl)	$T_{1/2}$ (s)	π_{cell} (bar)	ε_{cell} (bar)	Average ε_{cell} (bar)	*Lp* x 10^6 (cm s^{-1} bar^{-1})	Average *Lp* x 10^6 (cm s^{-1} bar^{-1})
Hereward	0	2 to 8	0.03	270	0.5 ± 0.4	2.3 0.5	6	135	135 ± 62[a]	1.7	1.7[a]
	60	2	0.04	275	0.36 ± 0.1	2.2 0.6	7	99	95 ± 35[b]	2.3	2.0[a]
		3			0.29 ± 0.1	-	7.2	80		-	
		4			0.4 ± 0.1	2.9 0.7	7.5	110		1.6	
		5			0.26 ± 0.1	2.6 0.9	7.8	71		2.7	
		6			0.4 ± 0.1	2.8 0.9	8	110		1.6	
		7			0.3 ± 0.1	2.6 0.9	6.8	82		2.3	
		8			0.4 ± 0.2	2.5 1.1	8.1	110		1.8	
Gamina	0	2 to 8	0.03	279	0.68 ± 0.2	2.7 2.7	5.3	190	190 ± 54[c]	1.1	1.1[b]
	60	2	0.03	263	0.3 ± 0.2	2.3 0.2	6.6	79	109 ± 33[d]	2.8	1.9[b]
		3			0.3 ± 0.1	2.9 0.1	7	79		2.2	
		4			0.4 ± 0.01	2.6 0.6	7.2	105		1.9	
		5			0.6 ± 0.3	2.8 0.03	7.5	158		1.2	
		6			0.6 ± 0.2	2.5 0.9	7.9	158		1.3	
		7			0.3 ± 0.01	2.5 0.7	8	79		2.5	
		8			0.4 ± 0.1	3	8.2	105		1.6	
Bohoth 105	0	2 to 8	0.01	229	0.65 ± 0.3	2.3 0.4	6	149	149 ± 50[e]	1.6	1.6[a]
	60	2	0.01	218	0.5 ± 0.1	2.1 0.4	6.2	109	100 ± 20[f]	2.4	1.9[a]
		3			0.3 ± 0.1	2.1 0.3	6.9	65		3.8	
		4			0.4 ± 0.1	2.1 0.1	7.8	87		2.9	
		5			0.5 ± 0.1	2.4 0.3	8.7	109		2.1	
		6			0.5 ± 0.1	2.7 0.3	8.9	109		1.8	
		7			0.4 ± 0.2	2.4 0.6	9.0	87		2.5	
		8			0.6 ± 0.8	2.7 0.3	9.0	131		1.5	
S. maritima	0	2 to 8	0.01	242	1.1 ± 0.4	5.2 0.8	7.3	267	267 ± 79[g]	3.5	0.35[a]
	200	2	0.01	275	0.4 ± 0.1	4.1 1.2	9	110	122 ± 14[h]	0.53	0.76[a]
		3			0.4 ± 0.1	2.6 0.7	11.3	110		0.83	
		4			0.4 ± 0.2	2.8 0.3	12.3	110		0.76	
		5			0.5 ± 0.2	2.6 0.5	13.2	137		0.67	
		6			0.5 ± 0.3	1.8 0.3	13.6	137		0.96	
		7			0.5 ± 0.1	-	13.9	137		-	
		8			0.4 ± 0.1	2.8 0.1	13.6	110		0.76	

Table 8.2 Cell water relations parameters of root cortical cells of control and salt treated Hereward, Gamina, Bohoth 105 (60 mM NaCl) and *S. maritima* (200 mM NaCl). Change of turgor pressure per change in volume ($\Delta P/\Delta V$), half time for water exchange ($T_{½}$), osmotic pressure (π_{cell}), volumetric elastic modulus. *V* and *A* determination was from fixed and sectional material. Results are given as means ± SD. Letters a, b, c, d, e, f, g and h compare between control and salt treatments. Means followed by the same letters are not significantly different at $P>0.05$.

8.6 Discussion

In terms of our results for volumetric elastic modulus, the values for all varieties of wheat under control conditions were comparable to those found in epidermal cells of *Tradescantia virginiana* (Zimmermann *et al.*, 1980) and in *Avicennia germinans* (L) (Suarez *et al.*, 1998). In the salt condition, for Hereward, ε_{cell} was 198 bar, as compared to 177 bar in the control (Table 8.1). Elsewhere, it has been found that elastic modulus increases in salt stress conditions for shoot bulk in *Alternantherap hiloxeroides* (Mart.) Griseb, due to the higher concentrations of solutes (Bolanos & Longstreth, 1984), and in three species from mangrove ecosystems (Rada *et al.*, 1989).

Meanwhile, Bohoth 105 leaf had increased cell wall elasticity in the salt condition (lower ε_{cell}) (Table 8.1). This can be interpreted as a salt-adaptive mechanism. Similar changes have been seen in *Gutierrezia sarothrae* (Wan *et al.*, 1998) and in *Avicennia germinans* (Suarez *et al.*, 1998). In Gamina, however, salt stress did not result in a change in cell wall elasticity.

A decrease in ε_{cell} together with an increase in solute concentration can help plants cope with hypersalinity, as seen in *H. vulgare* seedlings (Perez-Lopez *et al.*, 2010), by aiding turgor maintenance and water uptake. Additionally, the risk of damage to cells is minimized (Ruiz-Sanchez *et al.*, 1997).

For *S. maritima,* the values of ε_{cell} for cortical root cells under salt stress were around half of those obtained in the control plants (control, 267 ± 79 bar; NaCl-treated, 122 ± 14 bar). These findings indicate a more rigid cell wall for *S. maritima,* which then tended to become more elastic under salt stress. In comparison with the three wheat varieties, under control conditions cortical root cells in *S. maritima* had a

significantly higher ε_{cell} than the wheat controls (Table 8.2), again suggesting more rigid cells. A similar decrease in ε_{cell} under salt stress has been observed in field beans (Elston *et al*., 1976) and two genotypes of wheat (Rasico *et al*., 1988). For all plants, the values for ε_{cell} in control root cortical cells were comparable to values that have been found in control barley cortical cells (Steudle & Jeschke, 1983).

In terms of hydraulic conductivity, for all plants the *Lp* values of leaf epidermal cells and root cortical cells showed no significant difference between the salt and control treatments (Table 8.1 and 8.2).

Chapter 9: Final Discussion

9.1 Context

In this study, we investigated in two plants (three varieties of wheat and *S. maritima*) differing in their tolerance to salt, the water relations of single cells and the biophysical parameters controlling plant growth under salt stress. We also looked at the cation accumulation that may contribute to salt tolerance and at which parameters and cations contribute to the osmotic adjustment that occurs under salt stress. The main finding was that one of the varieties of wheat – Bohoth 105 – behaved under salt stress in a way that was noticeably more similar to the halophyte (*S. maritima*) than the other two varieties.

Data presented in the previous chapters of this study show that all plants grown in low external water potential can regulate leaf epidermal and root cortical cell turgor and complete their osmotic adjustment to adapt to change in the external medium. It is well known that several plants accumulate solutes in leaves and roots in response to a decrease in external water potential by using osmotic adjustment mechanisms. Examples of these are *Thinopyrum bessarabicum* (Gorham *et al*., 1985), *S. maritima* (Clipson *et al.*, 1985 and Reimann, 1992), *Atriplex canescens* (Glenn *et al*., 1996) and *Chenopodium quinoa* Wild (Orsini *et al.*, 2011). All of these plants used Na^+, Cl^- and K^+ to achieve osmotic adjustment and maintain P_{cell}.

The data in the previous chapters (Chapters 4, 5, 6 and 7) indicate that osmotic adjustment in epidermal and cortical cells is an important component of the salt tolerance mechanisms of both wheat and *S. maritima*. This conclusion is based on several observations. For example, for in the three wheat varieties under salt stress,

turgor regulation was achieved by solute uptake in plant cells. As has elsewhere been suggested (Wyn Jones & Pritchard, 1989; Bohnert *et al.,* 1995), changes in solute uptake in response to low external water potential provide rapid (within a few minutes) osmotic adjustment and maintain turgor. The response in the cortical root of the three wheat varieties studied after 60 mM NaCl addition was biphase (a rapid decrease in P_{cell}, followed by slower P_{cell} recovery), which was as expected, i.e. an initial passive response followed by active osmotic adjustment.

In the present study, turgor pressure was measured starting from 48h after the first salt addition, i.e. 24h following the second salt addition. No measurement was taken before this point, so it is not possible to report on the speed of the response to the initial onset of salt stress. However, many other studies have tracked turgor pressure response immediately following salt addition to the external medium. Arif (1990), for example, used the same techniques and species as this study, and found wheat leaf turgor pressure dropped within a space of 15-20 minutes after the application of NaCl. Also, the magnitude of the decrease was found to be roughly proportional to the concentration of salt applied.

Comparably rapid responses in turgor pressure have been found in barley under salt stress (Thiel *et al*., 1988) and in soybean under water stress (Nonami & Boyer, 1989). For roots, Itoh *et al*. (1987), using the same technique, found a drop in turgor pressure in mung bean root within between 10 and 20 minutes, depending on the concentration of salt in the external medium.

In this study, turgor regulation in leaf epidermal cells of wheat (varieties Hereward, Gamina and Bohoth 105) and *S. maritima* was achieved within 24 h after the second salt addition. The regulation was completed by the adjustment of solutes in both the

epidermal protoplast and epidermal apoplast. Similar turgor recovery has been found by Arif (1990) under similar conditions and using the same technique in mature leaf region of wheat, where recovery was complete after 24 and 48 - 72 h after addition of salt (low and high concentrations respectively). He considered that turgor recovery occurs due to osmotic adjustment, which is in turn caused by solute accumulation in tissue cells (Flowers *et al.*, 1991). Several researchers have studied the role of solutes in the apoplast in the water relations of plant cells (Leigh & Tomos, 1983; Clipson *et al.*, 1985; Tomos, 1988; Tomos & Wyn Jones, 1988; & Bell and Leigh, 1996).

Nonami and Boyer (1989) found similar results in the mature region of soybean stems, using the same methods. Turgor recovery occurred after 45 h when plants were exposed to 2.85 bar NaCl. Dracup and Greenway (1988) similarly show that Na^+ and/or Cl^- content contributes to turgor regulation in tobacco cells. Plants were exposed to 82 mM or 150 mM of NaCl in the medium culture, and ion accumulation occurred faster in plants under salt stress in relation to the control plants. The authors suggest a solute accumulation rate is a system for regulating turgor.

9.2 Transpiration Tension

The pressure probe (Hüsken *et al.*, 1978) is considered the most powerful technique so far for measuring many parameters in plant cells, including transpiration tension in the apoplast, and is a tool that is particularly well exploited within Bangor University laboratories. Changes in cell osmotic pressure (π_{cell}), apoplast osmotic pressure (π_{wall}), and also the apoplast hydrostatic component (P_{wall}) (transpiration tension) can cause changes in P_{cell}, all of which can be measured directly or indirectly with the pressure probe.

Hydrostatic pressure is one component of water potential in a plant (Dainty, 1963, Nobel, 1991), and transpiration tension plays a central role in the governance of cell water potential. It can be seen that differences between P_{cell} and π_{cell} are not correlated to the P_{wall} (Tomos, 1988). Our results, in Section 4.4, show that for both the control and the salt conditions, this parameter was -1.5 bar. Clipson *et al.* (1985) researched *S. maritima* leaves at day 28, using a similar technique to that employed in this study, to measure transpiration tension. The plants were immersed for several hours in hydroponic solution, and it was found that the transpiration tension was 1 to 2 bar. Arif (1990) similarly measured transpiration tension in leaf of wheat plant in the growing and mature region. When immersed in hydroponic root medium for 1 h, an increase in P_{cell} of 1 bar was found.

These studies suggest that, proportionally, P_{wall} does not have a major impact on the P_{cell}, decreasing the P_{cell} by about 20 %. This agrees with previous results, for example Nonami and Boyer (1987) used isopiestic psychometry to measure apoplast water potential in the elongating zone of stems in intact plant of soybean, and found it was 1.5 to 3 bar. They claimed that growth induced water potential gradient, not transpiration tension, was responsible for this.

9.3 The Effect of Turgor on Growth Parameters

The results in Section 4.2 indicate that P_{cell} in epidermal cells increased gradually over the 2 – 3 days subsequent to salt stress in all three varieties of wheat (Fig. 4.1a – c). In contrast, final leaf length and growth rate in both Hereward and Gamina significantly decreased over the rest of the experiment period under salt stress. No significant effect was found for the same parameter in Bohoth 105 (Table 3.1 and 3.2).

Due to the difference between the mature leaf cells measured and the unmeasured growing cells of the plant, no clear direct relationship between turgor and growth can be inferred. The relationship could perhaps be related to other water relation parameters (such as volumetric elastic modulus and/or hydraulic conductivity) or growth could have been decreased due to salts as a consequence of the osmotic effect and imbalance of ions (Grattan *et al.*, 1999; Muranaka *et al.*, 2002) and the accumulation of large Na^+ and Cl^- ions due to the primary toxicity of specific ions (Chinnusamy *et al.*, 2005).

Several authors have found no correlation between turgor and decrease in growth. For instance, Arif and Tomos (1993) reported this for wheat exposed to a range of salt stress, as did Fricke *et al.* (1997) for barley under N-limitation. Each of them measured P_{cell} in single cells. Termaat *et al.* (1985) used a pressure bomb on the entire leaf and suggested that in plants under stress, turgor does not constrain growth. However, it should be noted that the pressure bomb technique does not allow differentiation between cell types. i.e. between the mature zone and the growing zone. Matsuda and Riazi (1981) and Michelena and Boyer (1982) found similar results, i.e. that turgor does not change, in barley and maize respectively.

Other researchers have also observed a reduction in growth related to cell wall properties. For instance, Thomas *et al.* (1989) reported that in leaves of *Lolium temulentum* at low temperature, turgor does not change, and growth reduction is caused by cell wall behaviour. Several studies have been reported that show that the reduction in growth can be attributed to changes in cell wall properties, such as cell extensibility and yield threshold (Cramer & Bowman, 1991; for reviews see Barlow, 1986; Hsiao & Xu, 2000). Cosgrove and Sovonick-Dunford (1989) investigated pea stem elongation as affected by gibberellins. They suggest that the reduction in

growth is the consequence of a reduction in cell wall extensibility and an increase in the yield threshold.

9.4 Solutes Used for Osmotic Adjustment

The results of this study show that control epidermal cells of all plants had a higher K^+ concentration compared with other ions (Table 4.1 and 5.1, Fig. 4.4 and 5.3). Similar results were found in leaf cells for sorghum by De Lacerda *et al.* (2003). Schachtman and Munns (1992) also point out that wheat genotypes excluded Na^+. This is considered a common characteristic of salt tolerance. Salt tolerant plants accumulated larger Na^+ concentrations than sensitive genotypes. In general, osmotic adjustment of salt tolerant genotypes is achieved by maintaining a distribution of Na^+ and Cl^- between the vacuole and cytoplasm, with a higher concentration of these ions in the former.

The concentrations of K^+ found here in epidermal cells of the three wheat varieties under both control and salt conditions were in the same range as those found in epidermal cells of barley grown under salt stress (0 and 100 mM NaCl) by Fricke *et al.*, 2006. Specifically, in Hereward, salt stress results in an increase in π_{cell} in epidermal cells, with 57 mEq of K^+ and 38 mEq of Na^+ contributing to osmotic adjustment, alongside a loss of other ions. Therefore, the main solute used by Hereward for osmotic adjustment is K^+. This is in agreement with results for maize leaves (Premachandra *et al.*, 1992; Erdei & Taleisnik, 1993). Erdei and Taleisnik (1993) indicated that higher accumulation of K^+ and lower accumulation of Na^+ in leaves of sorghum under salt stress could be related to a characteristic associated with superior performance of the plant. In general, solute content changes are important for osmotic adjustment. For example, Fricke *et al*. (2004) found that in growing leaf 3 of barley, there were changes in osmotic pressure in epidermal cells

and in entire leaf after 20 h exposure to stress (100 mM NaCl), with osmotic pressure increasing by around 2 bar in leaf mature zone. The increase of osmotic pressure in the growing zone is the result of osmotic adjustment caused by an increase in solute content. It is also interesting that potassium seems to be often taken up, where available, in greater quantities than necessary, a phenomenon known as 'luxury consumption' (Leigh & Wyn Jones, 1984).

For the plants in this study, osmotic pressure increased in epidermal cells of Gamina, Bohoth 105 and *S. maritima* by the accumulation of 66 mEq, 127 mEq and 306 mEq respectively of Na^+ by day 8, which contributed to osmotic adjustment. This increase in Na^+ concentration is similar to previous results for wheat leaves (Gorham *et al.*, 1985), barley leaves (Delane *et al.*, 1982), rice (Yeo & Flowers, 1986), *S. maritima* (Yeo, 1981, Gorham & Jones, 1983), perennial Triticeae leaves (Gorham *et al.*, 1985 , Gorham *et al.*, 1986), in barley (Perez-Lopez *et al.*, 2010) and in Quinoa (*chenopodium quinoa* Wild.) leaves (Eisa *et al.*, 2012). Although this Na^+ accumulation evidently contributes to the maintenance of turgor, it is still important for leaf cells to maintain a level of K^+, since this is essential for plant metabolism (Marschner, 1995).

In the present study, under control conditions, epidermal and cortical cell Ca^{2+} concentrations were found to be low in all plants. Davenport *et al.* (1997), Kinraide (1999), and Reid and Smith (2000) reported that plants need only a low concentration of around 2 to 3 mM Ca^{2+} for optimum growth and for control of tissue Na^+ uptake in cases of low external water potential. Similar results to this study for all ions have been previously found by Boughalleb *et al.* (2012). They studied the effect of salt stress on a xero-halophyte plant (*Nitraria retusa* L.) and found that osmotic pressure and Na^+ and Cl^- concentrations noticeably increased, but that K^+

and Ca^{2+} decreased, whilst Mg^{2+} was only slightly affected. This indicates that the plant had a high capacity for osmotic adjustment. Under the salt condition, Ca^{2+} epidermal cell concentration decreased or was not affected in wheat and *S. maritima.* Ca^{2+} concentration in root cortical cells increased only in Gamina and *S. maritima* (end of experimental period). For many plant species, high levels of external Na^{+} result in lower K^{+} and Ca^{2+} tissue concentrations (Hu & Schmidhalter 1997). There are several possible explanations for this reduction in K^{+} concentration in plant tissue, such as the competition between Na^{+} and K^{+} at sites of root uptake, the subsequent impact of Na^{+} on the transport of K^{+} into the xylem (Lynch & Lauchli 1984), or inhibited uptake mechanisms (Suhayda *et al*., 1990).

Control epidermal and cortical cell Mg^{2+} concentrations were found to be small in all plants (Table 4.4, 5.1, 6.1 and 7.1, and Fig. 4.6, 5.5, 6.7 and 7.5). Hinde (1994) found similar results, with Mg^{2+} concentration in leaves of barley ranging from 5 - 10 mM. In all plants under the salt condition, Mg^{2+} decreased or was not affected, with the exception of root cortical cells in Bohoth 105.

Munns *et al.* (2006) stated that osmotic adjustment under salt stress occurs mostly by an increased uptake by cells of both Na^{+} and Cl^{-}. Such large ion accumulation in leaves leads to recovery of elongation growth following stress, as reported in barley by Fricke and Peters (2002), Fricke (2004), Fricke *et al.* (2006), and Matsuda and Riazi (1981), and in maize under water stress by Premachandra *et al.* (1992).

In terms of osmotic adjustment, all wheat varieties in this study showed similar behaviour, completing adjustment in epidermal cells during 48 h after salt addition. Over this initial 2 day period, P_{cell} remained unchanged, but after that it increased. In contrast, π_{cell} increased 48 h after salt addition, but then stabilized and remained unchanged over the whole of the rest of the period. An interpretation of this is that

over the first 48 hours, solutes are accumulated both in epidermal protoplasts and in the cell wall (apoplast) in similar proportions. Subsequently, the solutes appear to be removed from the apoplast and the cell turgor increases.

Several studies have similarly shown osmotic adjustment achieved by solute accumulation in the apoplast in leaves of *S. maritima* (Clipson *et al.*, 1985; Lawrence, 1999), wheat leaves (Arif, 1990) and sugar beet (Lawrence, 1999). This suggests that epidermal cells of Gamina, Bohoth 105 and *S. maritima* compartmented Na^+ in vacuoles and in the apoplast to protect the toxicity of the cytoplasm. Läuchli and Grattan (2007) documented that, under salt stress in growing leaves, osmotic adjustment was achieved by the accumulation of Na^+, since if the Na^+ is firstly compartmentalised in leaf cell vacuoles, the cytoplasm cannot be affected. Since most of a cell's volume is occupied by the vacuole, it is a suitable compartment for the role of solute storage (including of toxic substances) (Canut *et al.*, 1993; Andreev, 2001) and for keeping nutrients available to the cytosol (Conn & Gilliham, 2010). High Na^+ accumulation while keeping a balance of ions is believed to be an important tolerance mechanism in durum wheat and barley (Genc *et al.*, 2007).

Halophytes successfully achieve osmotic adjustment to salt stress by accumulating a large amount of ions, for instance Na^+ and Cl^-, in tissue cells. As a consequence, they are able to take up water under high salt stress conditions. Similarly, high uptake of Na^+ in leaves and roots of *S. maritima* could be one of the key mechanisms by which the cells decrease their ψ_{cell}. Our findings show that *S. maritima* was capable of uptaking water by keeping a high osmotic pressure in its cells due to the uptake of inorganic solutes, mainly Na^+ (Table 5.1, 7.1 and Fig. 5.2 and 7.2). For Hereward, although both K^+ and Na^+ were used for adjustment, K^+ was the main ion used, accumulated in the protoplast under salt stress.

Under the control condition, K^+ salt was predominantly found in epidermal and cortical cells in all plants. However, under the salt condition K^+ was consistently found in an inverse pattern to Na^+, with Na^+ increasing and K^+ decreasing. Similar findings have been noted by Husain *et al.* (2004) and Läuchli *et al.* (2008).

The data showed a different response in plant root cells (Chapters 6 and 7). In roots, turgor rapidly decreased within 48 h after salt addition to the external medium, then recovered over 72 h (Fig.6.1a – c and 7.1a). Turgor recovery was observed in all plants. Root cortical P_{cell} recovery was completed by day 8, with a $T_{1/2}$ of 6.3 days in Hereward, 7.9 days in Gamina, 1.7 days in Bohoth 105, and 3.2 days in *S. maritima* (Chapter 6 and 7). Osmotic adjustment was observed to be faster in Bohoth 105 and *S. maritima*. This indicates that these plants are able to use osmotic adjustment mechanisms to maintain turgor and continued growth under low external water potential.

Bisson *et al.* (1995) noticed that in the salt tolerant alga of *Chara longifolia,* turgor was fully regulated within 72 h. Since this study is the first ever to comprehensively investigate the roots of *S. maritima*, no previous data is available to which to compare our results for this plant. Hoffmann and Bisson (1990), however, have found that following hypotonic stress and hypertonic stress, turgor regulation is completed within two days and between five to seven days, respectively, in euryhaline giant-celled algae (*Chara buckellii*).

In all plants, root cortical cells had accumulated Na^+ salt in response to external lower water potential by day 8 (Table 6.1, 7.1 and Fig. 6.2 and 7.2). Lauchli *et al.* (2008) studied the response of root cortex of durum wheat to 50 mM NaCl by using scanning electron microscopy, and found similar results for Na^+ and K^+ to our results for wheat (Table 6.1). Sodium concentrations of leaf epidermal cells were 71 % and

48 % lower than in root cortical cells of Hereward and Gamina by day 8 respectively. Similar results have been found for wheat genotypes by Abdelmalek and Khaled (2011). In contrast, Bohoth 105 had higher Na^+ concentration in leaf epidermal cell than root cortical cells, similar to *S. maritima*. Sodium concentration in epidermal cells was 17 % and 26 % higher than in cortical cells for Bohoth 105 and *S. maritima* respectively by day 8.

In glycophytes, the maintenance of Na^+ within roots is considered as a Na^+ tolerance mechanism that allow them to increase their turgor and continue to grow against an increasingly low external water potential (Munns & Tester, 2008). However, Flowers and Hajibagheri (2001) and Làuchli *et al*. (2008) found that sensitive varieties of barley and wheat accumulate more Na^+ in their roots compared with other varieties (tolerant varieties). It is clear then that for plant roots, Na^+ is a critical part of the response to salt stress.

Comparing the response of single epidermal cells to that of the whole plant, in both experimental conditions, salt and control, values for osmotic pressure and cation concentration in single cells was always identical to or in excess of corresponding values for bulk leaf in the three varieties (Table 4.7). In bulk measurements of all wheat varieties, Na^+ in younger leaves increased statistically following salt stress, as compared to the non-salt treatment. It was observed in all wheat varieties that Na^+ concentration increased in young leaves less than in old leaves, and so proportionally more Na^+ accumulated in the old leaves. Strikingly, in Bohoth 105, the bulk leaf accumulation of Na^+ was much higher than in the other two varieties, for all leaves. This again shows that this variety behaves differently under salt stress.

In contrast, K^+ was inversely accumulated (Table 4.6 and Fig. 4.8 and 4.9). These findings are similar to the findings of Khelil *et al.* (2007), who suggested that for tomato (*Solanum lycopersicum* cv. Volgogradskij), the decreased Na^+ concentration uptake was associated with improved maintenance of K^+ concentration in young leaves compared to older ones. Plants have a mechanism to compartmentalise Na^+ in basal old leaves, which is thought to protect the young leaves from the effects of ions (Khelil *et al.*, 2007) and ultimately the plants sacrifice the old leaves for the young (Cheeseman, 1988). In cotton plants, Khan *et al*. (2004) similarly found that lower Na^+ concentration accumulates in younger leaves compared with older leaves. In contrast, however, *S. maritima* has been found to give opposite results.

Having measured the cations (Na^+, K^+, Ca^{2+} and Mg^{2+}), it was possible to estimate the anions. We found a net charge of 1.0, and interpreted that the anions are Cl^- and/or NO^-_3. However, this involved the assumption of no uncharged solutes. This may be justifiable for cereal leaves (Koroleva *et al.*, 1997), but may not be so for *S. maritima* as no equivalent data is available.

In terms of growth, final leaf length and growth rate were reduced by salt stress (Table 3.1 and 3.2). This decrease in leaf length and growth was in spite of a clear increase in turgor (in the later stages, following an initial period of no change) and an initial increase in osmotic pressure (after 48 h) (Fig 4.1a, b, d and e) in epidermal cells of Hereward and Gamina, indicating no positive relation between these parameters. The increase in turgor occurs with the accumulation of Na^+ and K^+ in Hereward and Na^+ in Gamina.

This observation is difficult to account for, but raises the possibility that it is the decreased growth that in fact causes the increased turgor, related to the surplus of

imported solutes. Similar results found by Pritchard *et al.* (2004) showed that in maize root exposed to 5 mM D-galactose in the external medium, growth decreased in both the growing zone and mature zone, while both turgor and water potential initially increased, but later did not change. The authors suggested that this was related to certain behavior, such as feedback regulation of ion influx into the cell, or the rise of osmotic level, by outflow, or one of either respiration or polymerisation.

9.5 Summary and Conclusion

In the plants studied, epidermal cells in the leaves and cortical cells in the root both adjusted to increased external osmotic pressure through increases in the osmotic pressure of cells. Under the salt condition, root turgor recovery was noted in all plants, with P_{cell} recovery being completed by day 8. This was achieved by increasing solute loads. Under all conditions and for all plants, inorganic solutes (K^+ and/or Na^+) in leaves and roots contributed significantly to the osmotic pressure of the epidermal and cortical cells studied. Under the control condition, K^+ was the main ion found in leaf epidermal and root cortical cells in all plants and varieties. However, under salt stress, osmotic adjustment was achieved mainly by the uptake and accumulation of sodium ions in the vacuole in each of Gamina, Bohoth 105 and the halophyte *S. maritima.*

Interestingly, the concentration of sodium accumulated in Bohoth 105 was noticeably closer to that of *S. maritima* than was Gamina's. In contrast, Hereward used a different strategy, by primarily accumulating even more potassium in the protoplast to achieve osmotic adjustment, with sodium being uptaken in only a smaller concentration. These results indicate that epidermal leaf cells were able to use both the apoplast (compartment) as an osmoticum and the protoplast to build up osmotic

pressure. In contrast, root cortical cells of all plants accumulated inorganic cations (Na^+ rather than K^+) in the protoplast only.

The varieties each behaved slightly differently. For example, root cortical cells of Hereward preferentially accumulated more Na^+ in order to protect the leaves and to encourage leaf K^+ uptake, since this is essential to maintain P_{cell} and continued growth. However, a similarity between Bohoth 105 and *S. maritima* was consistently noted. For instance, recovery was observed to be faster in both Bohoth 105 and *S. maritima*, suggesting that these plants are able to effectively employ osmotic adjustment mechanisms to maintain turgor and continued growth under low external water potential conditions. Another difference between Bohoth 105 and *S. maritima,* on the one hand and the other two wheat varieties on the other, relates to the relative distribution of sodium between the leaves and the roots. In Hereward and Gamina, sodium concentrations of leaf epidermal cells were lower than those in root cortical cells. In contrast, Bohoth 105 and *S. maritima* both had higher Na^+ concentrations in leaf epidermal cells compared with root cortical cells, i.e. the inverse of the other two wheat varieties. Finally, the response of the Hereward wheat variety to salt stress reduced the final length and growth rate, whereas the Gamina variety had a more moderate response to the salt condition. In contrast, the response of the Bohoth 105 variety in terms of final length and growth rate was to remain unaffected, being the same as the control.

To conclude, the focus of this study was to compare the physiological characteristics of wheat and of a halophyte (*S. maritima*), with the aim of identifying novel features that could be usefully bred into future wheat crops, using either conventional or molecular genetics methods. The hypothesis was that Bohoth 105 would be the variety most similar to the halophyte, and Hereward the least, with Gamina

somewhere in the middle. Taken together, the above results indicate that Bohoth 105 is indeed the wheat variety that is most similar to the model – *S. maritima* – and is therefore the most salt tolerant of the three, while Gamina has moderate tolerance and Hereward is salt sensitive. This means that the initial hypothesis of this study is supported by the data collected.

It would appear then that Bohoth 105 would be a useful variety to study further in relation to developing stress-resistant wheat varieties for agriculture. By focusing on the parameters related to the plant's water relations, and using conventional and/or molecular genetics techniques, it may be possible to improve the response of wheat to salt stress and thus enable a greater geographical range for this crop worldwide. Such research could usefully contribute to the aim of minimizing global food shortage.

9.6 Future Work

Although some ancestral varieties of crops have some salt tolerant features, a past focus within conventional breeding and selection on yield improvement has meant that modern cultivars have lost the ability to tolerate stresses. Current renewed interest within conventional breeding in encouraging salt tolerance has led to some successes, such as the salt resistant bread wheat (*Triticum aestivum* L.) cultivars S24 and KRL1-4 (Ashraf & Akram, 2009). However, conventional methods have been limited by the low natural gene pool diversity, the reproductive barriers when using wild relatives, and also the risk of introducing undesirable genes. In addition, this method does not directly contribute to an understanding of the underlying mechanisms and pathways associated with salt tolerance (*ibid.*).

As a result, the search for a genetic connection between physiological traits and salt tolerance has taken precedence. Although the multigene aspect of the salt tolerance trait makes genetic research complex, geneticists have been focusing on identifying genes which encode certain specific features involved in salt resistance, such as ion transport proteins (*ibid.*). Our research showed that the wheat variety most similar to the halophyte – Bohoth 105 – accumulated more Na^+ in the vacuole than the other wheat varieties, indicating that this is an important feature of salt tolerance. This suggests that genetic modification targeted at altering Na+ transport could be worth investigating. Indeed, Xue *et al.* (2004) studied this possibility by over-expressing the gene for vacuolar Na^+/H^+ antiporters (AtNHX1), sourced from *A. thaliana* L., in wheat (*Triticum aestivum* L.). They found that this resulted in better germination, improved biomass production, improved yield, higher K^+ accumulation, and lower leaf Na+. This study was unusual in two ways; firstly, it took account of yield as well as growth, and secondly it used both greenhouse and field conditions.

Studies such as this represent a way forward in efforts to improve agriculture in a time of water stress. It would be interesting to continue in this vein, testing genes for other aspects, such as Na^+ exclusion (even though Genc *et al.* (2007) found no clear relationship between this and salt tolerance). Other avenues include looking at more phases of wheat's life cycle, to see whether the cultivars show beneficial effects through to maturity; conducting studies using salts other than NaCl (since this is not always the only salt found in soils); or investigating the performance of salt resistant cultivars on normal soils (Ashraf & Akram, 2009).

In general, more studies of this type need to be conducted for food crop plants, not just cash crops, such as tobacco, and there needs to also be a continued focus on yield in addition to just salt tolerance. Also, field testing must become more

commonplace, as field conditions cannot be fully replicated in the lab or in greenhouses. At the same time, our understanding of plant stress response needs to carry on being developed, in order to better inform genetics programs.

Looking to the future, it is likely that continued developments in genetics techniques will aid studies on multigenic traits, such as salt tolerance, rather than being restricted to focusing on single genes. This will make it easier to feed knowledge of salt tolerance mechanisms into genetics research.

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