A comparative study of Cl^- transport across the roots of two grapevine rootstocks, K 51-40 and Paulsen, differing in salt tolerance

By

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Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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To:

my wife, Irandokht

my sons, Ali and Hesam

and

my father and mother

Abstract

Soil salinity is one of the major abiotic stresses that decreases agricultural crop production through imposition of both ionic and osmotic stresses. The accumulation of Na^+ and Cl^- in the cytosol to toxic levels inhibits metabolism. Unlike Na^+, less is known about Cl^- uptake and transport in plants. Grapevine is moderately sensitive to salinity and accumulation of toxic levels of Cl^- in leaves is the major reason for salt-induced symptoms. In this study Cl^- uptake and transport mechanism(s) were investigated in two grapevine (*Vitis* sp.) rootstock hybrids differing in salt tolerance: 1103 Paulsen (salt tolerant) and K 51-40 (salt sensitive).

Increased external salinity caused high Cl^- accumulation in shoots of the salt sensitive K 51-40 in comparison to Paulsen. Measurement of $^{15}NO_3^-$ net fluxes under high salinity showed that by increasing external Cl^- concentrations K 51-40 roots showed reduced NO_3^- accumulation. This was associated with increased accumulation of Cl^-. In comparison to Paulsen, K 51-40 showed reduced NO_3^- / Cl^- root selectivity with increased salinity, but Paulsen had lower selectivity over the whole salinity range (0-45 mM).

In order to examine if root hydraulic and permeability characterisations accounted for differences between varieties, the root pressure probe was used on excised roots. This showed that the osmotic Lp_r was significantly smaller than hydrostatic Lp_r, but no obvious difference was observed between the rootstocks. The reflection coefficient (σ) values (0.48-0.59) were the same for both rootstocks, and root anatomical studies showed no obvious difference in apoplastic barriers of the main and lateral roots. Comparing the uptake of Cl^- with an apoplastic tracer, PTS (3-hydroxy-5, 8, 10-pyrentrisulphonic acid), showed that there was no correlation between Cl^- and PTS transport. These results indicated that by-pass flow of salts to the xylem is the same for both rootstocks (10.01±3.03 % and 12.1±1.21 %) and hence pointed to differences in membrane transport to explain difference in Cl^- transport to the shoot.

$^{36}Cl^-$ fluxes across plasma membrane and tonoplast of K 51-40 and Paulsen roots showed that $^{36}Cl^-$ influx in root segments of Paulsen was greater than K 51-40 over the first 10 minutes. Unidirectional influx within 10 min loading time showed increases with increases in the external concentrations in both rootstocks but Paulsen had higher influx rate when compared to K 51-40. This appeared to be due to a greater V_{max}. There was no significant difference in K_m.

It was shown that $^{36}Cl^-$ accumulation and transport rate to the shoot of K 51-40 was higher than that of Paulsen. Compartmental analysis of $^{36}Cl^-$ efflux from intact roots confirmed that the difference in influx observed between the rootstocks was consistent with the results obtained for excised roots, although the values were not exactly the same. It was also shown that the main root of Paulsen had greater contribution to $^{36}Cl^-$ uptake than lateral roots. $^{36}Cl^-$ fluxes by lateral roots were not significantly different between the rootstocks.

Cl^- and Na^+ distribution patterns in different root cell types were determined using the X-ray microanalysis technique. It was shown that Cl^- content in the hypodermis and cortical cells was higher than the other cell types in both rootstocks, but overall Cl^- content in the root of Paulsen was higher than K 51-40. The pericycle of the main root of Paulsen accumulated more Cl^- than K 51-40. It was concluded that Cl^- loading to the xylem was different in the rootstocks and Paulsen tended to prevent the xylem Cl^- loading process. Lateral roots also displayed opposite behaviour consistent with flux analysis.

Membrane potential difference (PD) of the cortical cells showed a rapid and transient depolarization by adding 30 mM NaCl in both rootstocks that was followed by a gradual hyperpolarization. Depolarizations caused by 30 mM Choline-Cl, Na-MES and NaCl measured by the root surface potential method showed that Choline-Cl in K 51-40 and Na-MES in Paulsen caused greater depolarization than that of Na-MES in K 51-40 and Choline-Cl in Paulsen respectively. Assuming that PD measured in this method was the trans-root potential (TRP), it was concluded that the higher depolarization by Choline-Cl in K 51-40 can be due to higher Cl^- efflux rate to the xylem. Two different mechanisms were also detected for Cl^- transport: HATS which was observed in the range of 0.5-5 mM and a LATS in the range of 10-30 mM of the

external NaCl concentration. This was consistent with the concentration dependence of Cl^- influx.

In conclusion, evidence obtained from different experiments of this study indicated that in the grapevine rootstocks (Paulsen and K 51-40) Cl^- was mostly transported through the symplastic pathway. From E_{Cl} values determined for the rootstocks by the Nernst equation, a proton-driven transport system was responsible for Cl^- transport in both the HATS and LATS range of external NaCl concentrations. The rate of Cl^- transport from the root to shoot (xylem loading) was the major difference in Cl^- transport between the rootstocks in terms of salinity tolerance.

Abbreviation

ABA	Abscisic acid
AMTS	Ammonium transport system
ANOVA	Analysis of variance
cpm	Counts per minute
CW	Cell water
DW	Dry weight
E_{Cl}	Nernst potential of Cl^-
EDTA	Ethylene diamine tetra-acetic acid
FW	Fresh weight
HKT	High affinity potassium transporter
iHATS	Substrate induced high affinity transport system
cHATS	Constitutively active high affinity transport system
IBA	Indole-3- butyric acid
J_{BF}	Bypass flow of water
Lp_r	Root hydraulic conductivity
MBq	Megabecquerel
MIFE	Microelectrode ion-flux estimation
MIPs	Major intrinsic proteins
NAXT	Nitrate excretion transporter
NRT	Nitrate transporter
µCi	Microcuri
PD	Potential difference
PP	Pressure probe
PTR	Peptide transporter
PTS	8-hydroxy-1,3,6- pyrenetrisulfonic acid
S_o	Specific activity
S	Selectivity
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
SE	Standard error
S.P.Q	6-methoxy-N-(3-sulfopropyl) quinolinum

TEA	Tetraethyl ammonium chloride, K^+ channel blocker
TTX	Tetrodotoxin, Na^+ channel blocker
USL	Unstirred layer
σ	Reflection coefficient
Φ	Ion flux

Chapter 1

Introduction

1-1 Salinity stress

Salinity is a widespread and natural phenomenon (Staples & Tonniessen, 1984), which limits plant growth. On our planet, which is largely covered by salt water (about ¾ total area), the presence of salt in soils is not unusual (Flowers, 1999). Almost 1000 million ha of land (7% of all land on the earth) is affected by soil salinity (Szabolcs, 1994). In addition, of the 1.5 billion ha of land under cultivation, nearly 20% is affected by salt (Zhu, 2001, Flowers & Yeo, 1995). During the past two centuries, besides the drastic increase in human population, human demand for food has also risen (Flowers, 1999). Entry of salts into agricultural soils threatens the future of agricultural production in most parts of the world (Rus et al. 2001). In Australia, dryland salinity is an important and increasing problem in some areas, particularly in South Australia. According to the prediction of the Environment Australia-commissioned National Land and Water Resource Audit, 17 million ha of Australia's agricultural land will be confronted by salinity by 2050 (see http://audit.ea.gov.au).

It is estimated that irrigated land, in spite of being a relatively small area, provides at least 1/3 of the world's food (Munns, 2002). Irrigation waters contain varying amounts of salt (Flowers, 1999). By continuous irrigation, concentrations of salts may be increased in soils. For instance, in irrigated soils the amounts of calcium and magnesium carbonates increase. Whereas, in lands with a marine history or in areas with marine salts, which were deposited by wind, and or in areas with low rainfall, NaCl is the major accumulated salt. Furthermore, saline soils can have toxic concentrations of boric acid (Tester & Davenport, 2003) for which a boron transporter (*Bot1*) has significant effect on tolerance in barley (Sutton et al. 2007).

Soil salinity affects plant metabolic and non-metabolic processes and has negative effects on plant growth, particularly in glycophytes (Greenway & Munns, 1980). Na^+ toxicity has been studied in detail and there are many reports and articles on Na^+ transport mechanisms. However, Cl^- uptake mechanisms and compartmentation, especially in higher plants, needs to be studied in more detail with respect to salinity.

Chloride (Cl^-) is an anion and in comparison to sodium (Na^+) has a completely different transport mechanism. On the other hand, nitrate (NO_3^-) is also an anion, and plays an important role in plant nitrogen nutrition. Chloride and nitrate have nearly similar size and identical charge and may interact (e.g. competition for transport sites) (Kafkafi et al. 1982). There are a few reports on Cl^- and NO_3^- interactions, uptake kinetics and intercellular distribution in crops, and in horticultural plants.

In this study, Cl^- and NO_3^- transport and selectivity across roots was investigated in grape vines by examining the characteristics of two varieties with different Cl^- accumulation properties.

1-1-1 Definition of salinity

Salinity is an abiotic stress and it occurs when the concentration of salts such as NaCl, Na_2SO_4, Na_2CO_3 and $NaHCO_3$ increases up to a level that decreases plant growth. Among the above-mentioned salts, NaCl has the main role in causing toxicity and damage to plants, and in this review NaCl and salt stress will have the same meaning. In a large percentage of studies, Na^+ and Cl^- have been taken as the major salinisation factors in soils and plants (Gratten & Grieve, 1999).

1-1-2 Components of salt stress

Salinity (NaCl) causes several problems in plants even when present at low levels. For example even in the most tolerant varieties of crop plants (e.g barley) 50% reduction in growth rate occurs at about 100 mM NaCl. Whereas in sensitive plants (e.g beans) 60 mM NaCl has the same result in growth reduction (Greenway & Munns, 1980). Typically, the presence of salt in the soil induces three major problems (Tester & Davenport, 2003, Apse & Blumwald, 2002; Yeo, 1983; Greenway & Munns, 1980):

* Osmotic stress
* Na^+ and Cl^- toxicity
* Disruption of ion homeostasis

Soil salinity via the osmotic component decreases water uptake and it can eventually reduce plant growth. The second effect of soil salinity is due to excessive salt entry to the plant through the transpiration stream, which will damage cells in transpiring leaves and cause further growth reduction. With excessive salt loading, toxic ions will increase in the cytoplasm and disturb enzyme activity (Munns et al. 2006). Na^+ and chloride toxicity commonly affects plant growth by inhibition of metabolism.

Na^+ is absorbed and then maintained in roots and stems (especially in woody perennials such as grapevine and citrus) and chloride accumulates in the shoots (Tester & Davenport, 2003). Also accumulation of a particular cation or anion in the root or shoot must occur with charge balance in that tissue. In any case, Na^+ uptake is balanced with Cl^- uptake or K^+ efflux from the cytoplasm to outside (Tyerman and Skerrett 1999). Anion uptake is an active process and is associated with H^+ symport (Felle, 1994). It has been shown that accumulation of high Cl^- and low Na^+ concentrations in expanded leaves of many woody plants (Bernstein, 1975) for example avocado (Bingham et al. 1968) and grapevine (Bernstein et al.1969) causes chlorosis and death symptoms. The root can control internal Na^+ and Cl^- concentrations and can maintain turgor pressure at high external salinity. For example, in wheat, organic solutes (such as proline and glycinebetaine and some unknown organic solutes) together maintain turgor pressure in the absence of sufficient salt inside (e.g. K^+ content is 100 mM and decreases with salinity) (Munns et al. 2006).

In woody plants Na^+ and Cl^- distribution may be influenced by both rootstock and scion in grafted plants. Syvertsen & Yelenosky (1988) have shown the above mentioned influence in citrus plants. An effective Na^+ excluder rootstock (trifoliate orange) showed partitioning of Na^+ between leaves, stem and roots of 3%, 31% and 66% of total Na^+ accumulated in plant, respectively.

Accumulation of Na^+ and Cl^- in the plant growth medium also disrupts ion uptake by interfering with other nutrients such as K^+, Ca^{2+} and NO_3^-. Troncoso et al. (1999) showed that the K^+ content of some sensitive varieties of grapevine (*in vitro* cultured explants) decreased by increasing NaCl, while in tolerant varieties accumulation of K^+ was higher than sensitive varieties (high K^+/Na^+ selectivity). Also, Ca^{2+} content decreased in both sensitive and tolerant varieties. High salinity did not affect N and Mg contents, but P levels decreased in sensitive varieties.

Kozlowski (1997) has summarised some of the effects of salinity on plants. According to Kozlowski's description, salinity affects plant growth by:

* Destroying the cell membrane and increasing solute leakage (Leopold & Willing 1984).

* Marginal chlorosis and leaf blades burning caused by Cl^- (Harding et al.1958, Pandey & Divate, 1976) and leaf necrosis caused by Na^+ (Schaffer et al. 1994).

* Decreasing seed germination in total number and inhibition of post-germination processes (Ungar, 1982).

* Reducing vegetative growth (Kozlowski, 1997) and reproductive growth (Lumis et al.1973, Waisel, 1991, Shannon et al. 1994).

* Changing the morphology and anatomy of woody plants (Ogden, 1980).

* Inhibition of many enzymes activities *in vitro* (Greenway & Munns, 1980, Blum, 1988).

* Decreasing the rate of photosynthesis by reducing gas exchange (Downton, 1977, Walker et al. 1979, Pezeshki & Chambers, 1986).

1-1-3 Salinity tolerance

Salt tolerance is defined as the growth and reproduction capacity of a plant in the presence of salt, particularly NaCl (Jeschke, 1984). Plants take different strategies when they are confronted with environmental stresses. Some of them tolerate the stresses by resistance, while others tolerate the stresses by avoidance. Quantitative assessment of salt tolerance in plants is difficult because it varies with environmental and plant factors (Ghassemi et al. 1995). Most plants are moderately salt sensitive, especially crop plants that are unable to tolerate soil salinity for a long period (Kramer et al. 1978). Shannon et al. (1994) states that woody plants are relatively salt tolerant,

particularly during seed germination and maturity. Conversely, in post germination and young seedling stages, woody plants are very sensitive.

1-1-4 Mechanisms of salt tolerance

Plant responses to salinity and mechanisms of salt tolerance are categorized into three distinct types (Munns & Tester 2008):

1) Osmotic stress tolerance: osmotic stress reduces cell expansion and stomatal conductance. At the presence of adequate soil water, plants would be able to tolerate osmotic effects of salinity by increasing leaf area and stomatal conductance.

2) Na^+ and Cl^- exclusion: plants with Na^+ and Cl^- exclusion capacity would reduce toxic ion accumulation within leaves.

3) Tissue tolerance: some plants accumulate Na^+ and Cl^- in the vacuole to avoid toxic concentrations within the cytoplasm.

According to Zhu (2001), any changes in ion and water homeostasis cause molecular damage, growth reduction and even death. Zhu (2001) has categorized three important aspects of plant activities involved in achieving salt tolerance as follows:

1- Detoxification: Plants must decrease injuries by removing damage factor(s). For instance, they might produce stress proteins and compatible solutes to scavenge ROS (reactive oxygen species) generated by salt stress. In this case, there are many reports on producing transgenic plants with salt tolerance (e.g. Roxas et al. 1997, Allen et al. 1997, Tsugane et al. 1999). For example, expressing yeast HAL1 gene in tomato promotes salt tolerance on the basis of fruit yield. The yeast HAL1 gene is an important determinant for halotolerance (salt tolerance) in plants. It is most likely specific for Na^+ tolerance and increases K^+ accumulation by enhancing K^+/Na^+ selectivity under NaCl stress (Rus et al. 2001).

2- Homeostasis: In the new condition with the stressful medium, plants must re-establish homeostatic conditions. To do this, they need to prevent or reduce entry and accumulation of harmful ions such as Na^+ and Cl^- into the cytoplasm or organelles, with the exception of the vacuole. Therefore, it is necessary that transporters of

injurious ions across the cell membranes be identified and then blocked, or after entry, ions should be sequestrated in the vacuole (Flowers et al. 1977).

3- Growth regulation: Despite the initial reduction in plant growth, it must be started again in the presence of stress conditions. Slower growth allows plants under stress the capacity of using multiple resources (e.g building blocks and energy). One reason for decrease in plant growth is lowered photosynthesis due to stomatal closure and inhibition of CO_2 uptake. Salt stress may also prevent cell division and expansion. Some plants are very sensitive and their growth rate decreases even under mild stress. By contrast, some plants are not so responsive to stress and usually grow in a stressful environment. This may lead to the risk premature plant death. Then, precisely adjustment of this responsiveness (showing reaction to stress by reducing growth rate) would increase productivity under salt stress. For instance, the ICK1 protein is a cyclin-dependent-protein-kinase inhibitor and can reduce cell division by reducing the activity of cyclin-dependent-protein-kinase that helps to drive the cell cycle. Salt stress might inhibit cell division by causing the accumulation of abscisic acid (ABA). It has been shown that ABA can induce ICK1 in *Arabidopsis* (Zhu 2001).

1-1-5 Salt tolerance in plants that show Cl^- sensitivity

Chloride is an essential element in the nutrition of higher plants (Marschner, 1995). It is an osmotically active solute in the vacuole, and it regulates activity of certain enzymes in the cytoplasm (such as the vATPase) (Flowers et al. 1997). It also stabilizes the cell membrane potential as a counter anion (White & Broadley, 2001).
Despite this, Cl^- could have toxic effects on plants. Greenway and Munns, (1980) have classified the growth responses of plants to high Cl^- external concentrations into four categories:
1- Halophyte species for which growth is encouraged by $[Cl^-]_{ext}$ (e.g. *Suaeda maritima*).
2- Halophyte species for which growth is reduced by 200 mM $[Cl^-]_{ext}$ (e.g. *Atriplex hastata*).
3- Halophyte and non-halophyte (glycophyte) species for which growth is drastically reduced by 100 mM $[Cl^-]_{ext}$. These plants are subdivided into three groups: tolerant (e.g. barley), intermediate (e.g. tomatoes) and sensitive (e.g. beans).

4- Very salt-sensitive glycophyte species (e.g. fruit trees such as citrus).

The above mentioned classification can be attributed to changes in Cl^- concentrations by keeping Na^+ concentration at a constant level and increasing Cl^- concentration by using cations other than Na^+ (such as K^+ or Ca^{2+}). Many important cereal, vegetable and fruit crops can not tolerate excess amounts of Cl^- during cultivation. Basically, in chloride-sensitive plants 12-15 mM, and in Cl^--tolerant plants 45-150 mM of tissue Cl^- contents, are critical (toxic) concentrations (White & Broadley, 2001).

1-2 Mechanisms of entry of salt (Na^+ and Cl^-) into plant roots

It was only during the past decade that concerted experiments have concentrated on the detection of mechanisms of ion and osmotic homeostasis at the cell and molecular levels as important determinants of salt tolerance. More recently, *Arabidopsis thaliana* has been used as a plant molecular genetic model to understand both cellular and organismal mechanisms of salt tolerance (Hasegawa et al. 2000), although it has not been known as an ideal subject for physiological analysis (Xiong & Zhu 2002).

1-2-1 Transport across membrane in cells

The plasma membrane is a lipid bilayer structure that encloses the cell and in fact is impermeable to most ions. This membrane prevents leakage of ions accumulated inside the cells of a root and thereby nutrient ions contents inside the root are generally maintained higher than that of the surrounding medium. Particularly the only way for ion transport to occur across the plasma membrane is via specific proteins located in the plasma membrane (Sondergaard et al. 2004). The majority of ions are accumulated in the vacuoles (as the largest compartment inside the cell) and fewer amounts of ions may be retained in the cytoplasm and other organelles. For accumulation in the vacuole, the solutes should cross both the plasma membrane and tonoplast. Therefore, knowing the properties of these membranes and the transporters embedded within is essential for ion transport studies (Flowers & Yeo, 1992).

Ion transport across the plasma membrane can be passive or active. If the ions pass the membrane by diffusion (flow down a concentration and electrical gradient), the

movement is called passive transport. However, if the ions move from a region with lower chemical potential to higher (flow against a concentration and electrical gradient), then this process is called active transport (Nobel, 1991; Flowers & Yeo, 1992).

In normal conditions, most ions (not Na^+) are transported against a concentration gradient and/or an electrical gradient and need to be supported by energy sources (e.g ATP) (Sondergaard et al. 2004). To determine if an ion uptake through the cell membrane is active or passive, we must know the ion concentrations (chemical potential gradient) and electrical potential gradient across the membrane. All living cells show a membrane potential which is attributed to a very slightly unequal ion distribution between the inside and outside of a cell (Higinbotham, 1973). By inserting a microelectrode into a cell and measuring the voltage difference between two sides of the membrane barrier, membrane potential can be estimated. The Nernst equation (Equ.1-1) is used to calculate the membrane voltage (E_J) that would allow a particular ion (J) to be distributed in passive equilibrium across the membrane:

$$E_J = - RT / z F. \ln (C^i_j / C^o_j) \qquad \text{(Eq. 1-1)}$$

Where, R is universal gas constant, T is absolute temperature (295 °K), z is valence of the ion of interest, *F* is Faraday`s constant and C^i_j and C^o_j are internal and external concentrations of the ion (J) respectively.

According to the Nernst equation, with a negative electrochemical potential of -59 mV under standard conditions, a monovalent cation such as K^+ and a monovalent anion like Cl^- will be in electrochemical equilibrium if their activity inside the cell is 10 times higher (for K^+) and 10 times lower (for Cl^-) than in the external bathing medium. All plant cells show an electropotential difference (PD) around -100 mV (interior negative) or more across the plasma membrane. However, it may change (depolarize or hyperpolarize) by diffusion or active transport of ions (Higinbotham et al. 1964).

Using the Nernst equation it has been calculated that K^+ and Na^+ are more likely to be transported passively across the plasma membrane from the external medium into the

cell while anions such as Cl^-, NO_3^-, $H_2PO_4^-$ and SO_4^- are accumulated against their respective electrochemical gradient (Higinbotham, 1973). However, studies have shown that there are active transporters for K^+ uptake in plants (such as HKT1 and HAK1) (Schachtman & Schroeder 1994; Schachtman, 2000).

Ion channels can account for passive fluxes. They are intrinsic proteins located in the cell membranes, which allow the ions to flux through a pore when the channel is in the open state. Flux rate depends on the electrochemical gradient and for a channel it varies from 10^6 to 10^8 ions per second (Tyerman & Skerrett, 1999). In plant and animal cells ion channels are proposed to contribute in three main physiological functions: cell osmoregulation (due to high net ion fluxes in short periods), cell signalling (via electrical signals or secondary messengers such as Ca^{2+}), and membrane potential adjustment (Barbier-Brygoo et al. 2000). The main function of channels in plants is selective passive transport of organic ions (Colombo et al. 1992; Rayan et al. 1995), inorganic ions (Tester, 1990) and water (Niemietz & Tyerman, 1997).

There are various ion channels in living cell membranes and they are identified based on their ion selectivity, gating mechanism, and sequence similarity. Ion channels can be voltage-gated (e.g voltage-gated K^+ and Ca^{2+} channels) (Dreyer et al. 2005), ligand-gated (e.g Ca^{2+} channel induced by inositol 1,4,5-triphosphate (IP_3)) (Alexandere et al. 1990) , pH-gated, or mechanically gated. Ion channels are subdivided into several subtypes by considering gating criteria along with a combination of sequence similarity and ion selectivity (Blatt, 2005).

1-2-2 Na^+ transport

There is a correlation between salt tolerance and Na^+ uptake in plants. If a plant is able to maintain high K^+/Na^+ ratio in the cytoplasm at high $[NaCl]_{ext}$ it makes proper function of enzymes possible. This is the main reason for focusing many studies on the mechanism of Na^+ transport across root cell membranes (Tyerman & Skerrett, 1999). In moderate-to-high concentrations of external Na^+, Na^+ transport across the plasma membrane to the cytoplasm tends to be a passive mechanism (Cheeseman, 1982). An inside negative plasma membrane potential of about

-120 to -200 mV (Higinbotham, 1973) is the main motive force for that passive uptake. On the other hand, in low Na^+ concentrations inside a cell, Na^+ transport out of the cytoplasm or across the tonoplast will be an active mechanism. Glycophytes and halophytes maintain cytosolic Na^+ concentrations at low levels by compartmentation of Na^+ in the vacuole. Na^+ transport across the tonoplast to the vacuole is an active process. This also helps the plant cells to generate osmotic pressure (Tyerman & Skerrett, 1999).

The transport systems involved in Na^+ uptake are not yet exactly known. K^+ and Na^+ could be transported by common proteins and it has been shown that Na^+ competes with K^+ for influx to the cell (Cheesman, 1982). Physiologically, there are two transport systems for potassium and Na^+ influx: high affinity for K^+ over Na^+ and low affinity with lower K^+/Na^+ selectivity (Blumwald et al. 2000). It has been indicated that a single genetic locus (*SOS3*) regulates high and low affinity systems by encoding a single transduction intermediate (Liu & Zhu 1997). Suggested transport systems for Na^+ in plants have been categorized by Hasegawa et al. (2000). According to this classification, Hkt1 and Hak1 proteins are high affinity K^+ transporters and probably do not contribute to Na^+ uptake significantly. Amtmann & Sanders (1999) have classified ion channels involved in Na^+ transport according to their gating properties. Inward-rectifying channels (IRCs) that are mainly responsible for K^+ influx. For example in *Arabidopsis thaliana* two K^+ channels, Akt1 and Akt2 (K^+-selective inward-rectifying channels (KIRC)) have been identified that are active as low affinity systems, and have minimal contribution to Na^+ uptake (Hasegawa et al. 2000). Another possible pathway for Na^+ influx that facilitate a low affinity Na^+ uniport is encoded by the member of HKT. The HKT1 from wheat when expressed in Xenopous oocytes or yeast can catalyse a high affinity Na^+/K^+ symport and a low affinity Na^+ uniport when external Na^+ concentration is increased (Tester & Davenport 2003).
In contrast to IRCs, outward-rectifying channels (ORCs) are involved in cation (Na^+) efflux from the cell. KCO1 is a channel of ORCs group that is activated by cytosolic Ca^{2+} in the range of 150-500 nM (Amtmann & Sanders 1999). Meanwhile, Na^+/H^+ antiporters are responsible for Na^+ efflux across the plasma membrane and tonoplast (compartmentalization into vacuoles) along with H^+-ATPase which is establishing the proton gradients for Na^+ efflux (Hasegawa et al. 2000).

In addition to IRCs and ORCs, there is another group of channels in the cell membrane that are called non-selective cation channels (NSCCs). This group of channels is thought to be the major pathway for Na^+ influx (Demidchik & Tester 2002, Tyerman et al. 1997). This kind of channel has been characterized in suspension cell cultures of barley scutellum (Amtmann et al. 1997), maize root cortical cells (Robert & Tester 1997a) and wheat root cortical cells (Tyerman et al. 1997).

Cation-Cl^- cotransporter (CCC) family that has been identified for the first time in animals plays an important role in cell ionic and osmotic adjustment. Colmenero-Flores et al (2007) showed that *CCC* gene family from *Arabidopsis* (*At CCC*) encodes a cation-Cl^- transporter that catalyses co-ordinated symport of K^+, Na^+ and Cl^-. Preferential expression of CCC proteins was shown in the shoot and root xylem/symplast boundary, root tips and leaves of transgenic *Arabidopsis* plants. Under high salinity the plants lack of CCC proteins showed higher and lower amounts of Cl^- in shoots and roots compared to wild type plants with CCC proteins. It revealed that At CCC is involved in long distance Cl^- transport.

1-2-3 Cl^- Transport

Under most circumstances anion transport across the plasma membrane is an active process that is driven by co-transport with protons. Cl^- and NO_3^- are absorbed by the plant roots against an electrochemical gradient (Runge, 1983). According to many reports, the net Na^+ uptake is balanced with chloride uptake and K^+ efflux (Tyerman & Skerrett, 1999). Skerrett & Tyerman, (1994) demonstrated that at high concentrations of external Cl^- (e.g high salinity) Na^+ influx causes a depolarization in the plasma membrane. Thus, the membrane potential may decrease to a level less negative than the Cl^- equilibrium potential (which becomes more negative) and thereby the passive transport of chloride into a cell can occur via an anion channel. Under salinity and high level of external NO_3^- concentrations, Cl^- and NO_3^- may be transported into root cells through this channel. It is also sensitive to intracellular Ca^{2+} concentration (mostly active in high intracellular Ca^{2+}). Many reports show that when salt concentrations outside a cell increase, passive influx of chloride into the cell may also take place (e.g. Cram, 1973; Binzel, et al. 1988). However, in non-saline conditions with cytoplasmic Cl^- concentration of about 20 mM, Cl^- accumulation

should be an active transport process, probably via a proton symporter. Felle (1994) indicated that there is an electrogenic $Cl^-/2H^+$ symporter in the plasma membrane of root hair cells of *Sinapis alba*.

Plant cells are not able to tolerate high concentrations of Cl^- and must retain cytosolic Cl^- concentrations at low levels. According to many reports, in normal conditions cytoplasmic Cl^- concentrations are between 10-20 mM but can be higher in saline condition (Felle, 1994). It is proposed that maintaining low concentrations of internal Cl^- could be obtained either by efflux across the plasma membrane or by efflux across the tonoplast to the vacuole. It is suggested that Cl^- transport to the vacuole is possibly via a channel or a carrier. This carrier is able to couple Cl^- influx to the H^+ gradient using a +50 mV inside positive tonoplast membrane potential. Pantoja et al. (1989) suggested a Cl^-/H^+ antiport system and Martinoia et al. (1986) showed that ion channels could be involved in Cl^- efflux from the cytoplasm to the vacuole. Tyerman & Skerrett, (1999) indicated that Cl^- / H^+ antiport across the tonoplast has not been demonstrated at that time. However, they explain that an ion channel could be involved in adjusting the cytoplasmic Cl^- concentration by efflux through the plasma membrane. In addition to the Cl^- channels of the plasma membrane, tonoplast of root cells may contain an H^+/Cl^- antiporter (White & Smith 1989) that drives Cl^- accumulation in the vacuole and two hyperpolarization-activated anion channels, V_{Cl} and V_{mal}, that are involved in Cl^- fluxes in both directions across the tonoplast (White and Broadley 2001). White & Broadley (2001) have classified the above mentioned possible pathways of Cl^- transport through the cell membranes (plasma membrane and tonoplast). According to this classification, there is a $2H^+/Cl^-$ symporter in the plasma membrane of epidermal and cortical cells that drives Cl^- uptake (Felle, 1994). Also, three types of Cl^- permeable channels have been distinguished in the plasma membrane of *Vicia faba* guard cells (Cosgrove & Hedrich 1991) and *Arabidopsis* hypocotyl cells (Thomine et al. 1997):

1) Rapidly activating anion channels (R-type)
2) Slowly activating anion channels (S-type)
3) Stretch-activated anion channels

Another type of Cl^- channel which has been found in the plasma membrane of *Amaranthus tricolo*r cotyledonary tissue cells (Terry et al. 1991) is a hyperpolarization-activated anion channel.

Chloride channels found in the plasma membrane of the xylem parenchyma cells of barley roots (Kohler & Raschke 2000) are X-SLAC (slowly activating anion conductance), X-QUAC (quickly activating anino conductance) and X-IRAC (inwardly rectifying anion channel) could facilitate Cl^- efflux to the xylem (White & Broadley 2001). Gilliham & Tester (2005) identified two anion loading pathways to the xylem in protoplast of cells isolated from maize root stele: xylem-parenchyma quickly-activating anion conductance (*Zm*-X-QUAC) and xylem-parenchyma inwardly-rectifying anion conductance (*Zm*-X-IRAC). There were regulated by water stress and ABA. *Zm*-X-QUAC was the main pathway for loading most of the NO_3^- and Cl^- to the xylem of well-watered plants while in water stress condition, activity of *Zm*-X-IRAC increased and activity of *Zm*-X-QUAC decreased. ABA had an inhibitory effect on *Zm*-X-QUAC activity.

1-2-4 NO_3^- Transport

NO_3^- uptake by plants results from a balance between an active influx by H^+/NO_3^- symporter and a passive efflux. In higher plants two types of NO_3^- transporters have been characterized: NRT1 and NRT2. NRT1 is a high-affinity system while the majority of NRT2 members are low-affinity NO_3^- transporters (Tsay et al. 2007). Segonzac et al. (2007) measured in vitro E_m and NO_3^- flux using the fluorescent oxonol VI dye on isolated plasma membrane from *Arabidopsis thaliana* suspension cells. They identified nitrate excretion transporter 1 (NAXT-1) that is responsible for NO_3^- efflux across the plasma membrane and is a member of seven NAXT proteins belonging to the large NRT1/peptide transporter (NRT1/PTR) family (mostly expressed in the cortex of mature roots). They showed that the passive NO_3^- transport activity that is driven by the ATP-dependent H^+-pumping activity was inhibited by anti-NAXT antibodies. In *naxt-1* mutant plants no change in plants NO_3^- contents was observed. Acid loading and strongly acidified medium, increased unidirectional NO_3^- efflux in wild type plants. They concluded that the NAXT system is involved in

uniport of one NO_3^- per one H^+ pumped by the ATPase. It is driven by PD and is accompanied by a net negative charge transfer in the same direction.

It has been suggested that the chloride channel (CLC) protein family members in plants are anion channels that are involved in NO_3^- homestasis. De Angeli et al. (2006) showed anion transport activity in vacuolar membrane of *Arabidopsis thaliana* mesophyll cells protoplast. The NO_3^-/H^+ antiport activity of this protein (*At*CLCa) is responsible for NO_3^- accumulation in plant vacuole.

Unlike ion pumps which are highly selective for certain ions, ion channels sometimes weakly discriminate between different ions. For example H^+-coupled K^+ transporter shows selectivity more than 500:1 for K^+ over Rb^+ (Blatt et al. 1987) while some anion channels are equally permeable to NO_3^- and Cl^- (Tyerman, 1992; Skerrett & Tyerman 1994). Although ion channel protein densities in most membranes are very low, they can carry much greater current than ion pumps, thus they account largely for charge (but not H^+) balance across the cell membranes (Blatt, 2005).

In high salinity conditions, several ion channels in the plasma membranes (e.g. KIRC, KORC, non-selective cation channels and calcium channels) and tonoplast (e.g. Non-Selective cation channels in Plantago or in barley) may allow Na^+ permeation. However, in the case of Cl^- and NO_3^- there is less information. There are two major uptake systems for NO_3^-, HATS (high affinity) and LATS (low affinity), which are responsible for NO_3^- transport. These and particularly the LATS may allow chloride permeation in particular condition (Tyerman and Skerrett, 1999).

1-2-5 NO_3^- and Cl^- interactions

It has been shown that increasing nitrogen nutrition as NO_3^- reduces Cl^- toxicity in avocado (Embelton et al. 1958) and citrus (Lips, et al. 1990). Despite some reports about insensitivity of NO_3^- uptake to Cl^- levels in the external medium of the root (Cram, 1973; Glass et al. 1985), there are many other examples that confirm such an effect. For instance, in *Cucumis sativa* (Martinez & Cerda, 1989) and tomato (Kafkafi et al. 1982) the same interaction was found.

Perez-Alfocea et al. (1993) and Kafkafi et al. (1992) found another aspect of interaction between Cl^- and NO_3^- related to salt tolerance in tomato. They demonstrated that in comparison to the salt sensitive cultivars, the salt tolerant cultivars of tomato had higher NO_3^- influx rate to the roots in the presence of increasing NaCl (0-140 mM) and $CaCl_2$ (0-60 mol/m^3). Aslam et al. (1984) showed that an increase in Cl^- uptake and accumulation causes a decrease in NO_3^- concentration in barley shoot. Papadopoulos & Rendig (1983) attributed this NO_3^- reduction to substitution of Cl^- for NO_3^-. Considering the above mentioned antagonism between NO_3^- and Cl^-, Wallace & Berry (1982) concluded that reduction in plant yield is not only due to Cl^- toxicity but also can be partially caused by NO_3^- deficiency induced by the increased external Cl^- concentration. Because NO_3^- is assimilated in leaves of grapevine, NO_3^- deficiency as reduce concentration is usually observed in leaves.

In addition to the ionic antagonism, Grattan & Grieve (1994) suggested that under saline conditions, nutrient uptake by plants is inhibited by the osmotic effects of the saline solution that reduce leaf transpiration and thus the mass flow of ions to the root. A study by Abdelghadir et al. (2005) on the effect of salinity on NO_3^- uptake by rice plants was in line with Gratten & Grieve (1994). They found that NO_3^- absorption by rice shoot is strongly related to water uptake and concluded that this correlation is more effective on the inhibition of NO_3^- uptake than effects of antagonism between Cl^- and NO_3^- in saline conditions.

According to the above mentioned reports, it seems that the salinity-N relations of horticultural plants are very complex (Tyerman & Skerret, 1999) and needs to be studied in more detail, taking into account the various pathways of NO_3^- uptake across the root and interactions with water flow.

1-2-6 Different affinities of NO_3^- uptake [LATS, HATS (iHATS and cHATS)]

According to physiological studies of nitrate uptake into roots, three main nitrate uptake systems are suggested as follows (Siddiqi et al. 1991; Williams & Miller, 2001):

1- Low-affinity transport system (LATS): this uptake system, which has been reported in many plants such as barley, *Arabidopsis* and *Nicotiana tabacum*, is triggered when the external concentrations of nitrate is high (above 1 mM) and it mostly appears to be a passive transport system (Williams & Miller 2001).
2- High affinity transport system (HATS): this system is activated at low external concentrations of nitrate (between 0.1-1 mM) and is divided into two other systems:
2-1 Substrate induced (iHATS)
2-2 Constitutively active (cHATS)

In this system net uptake of nitrate is saturable and shows high affinity for nitrate (Williams & Miller 2001). Studies show that LATS can be active (ie. AMTS or NRT).

1-3 Compartmentation of Na^+ and Cl^- in plant parts

Plants that tolerate salt stress, establish cellular ion homeostasis by net intracellular Na^+ and Cl^- uptake and then compartmentalize the ions in the vacuole to protect cyto-toxic ion accumulation (Blumwald et al. 2000). It has been demonstrated that high concentrations of Na^+ in the cytoplasm can affect cellular processes by inhibition of some important Na^+-sensitive enzyme activity (Epstein & Rains, 1965). White & Broadley, (2001) have noted that there are many differences between different parts of a plant in accumulation of chloride. For example, older leaves of glycophtes accumulate more Cl^- than younger leaves.

The sequestration of toxic ions in the vacuole may also help plant cells to maintain water status under saline conditions (Rhodes & Hanson, 1993). In addition to the vacuole, other organelles like chloroplasts and mitochondria may also build up Na^+ and thereby assist the plant cells to compartmentalize Na^+ (Zhu, 2003).

It seems that Cl^- transport to and accumulation in the shoot is a main indication of Cl^- sensitivity in plants (Greenway, 1965; Downton & Millhouse 1983). There are many suggestions for the mechanism of the toxic effect of Cl^- : 1) it can be due to osmotic effects of high [Cl^-] in the cell wall (Oertli 1968); 2) it is attributed to inhibitory effects of high [Cl^-] on protein synthesis (Gibson et al. 1984) and enzyme activity (Flowers et al. 1977; Gimmler et al. 1984) in the cytosol. Therefore understanding the

subcellular sequestration and quantification of Cl^- pools will be very helpful in clarification of the mechanisms of Cl^- toxicity (Britto & Krunzuker, 2001).

1-3-1 Compartmental flux analysis

The mechanisms and compartmentation involved in chloride transport have been investigated using $^{36}Cl^-$ isotope fluxes. However, unlike Na^+, this has not detected a particular mechanism for Cl^- transport which can be directly attributed to salt tolerance (Lorenzen et al. 2004).

Knowledge about ion compartmentation and acquisition inside a cell relies intensively on radioisotope techniques. By using isotopes of the ions in question, the separate components of the net flux, influx and efflux can be measured.

NOTE: This figure is included on page 17 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1-1 Components of the fluxes into and out of each root compartment (from Pitman 1971)

Considering three compartments in series (three compartment flux model) (Fig. 1-1), root cytoplasm (c) and root vacuole (v) and the shoot or xylem (x), the ion fluxes are measurable after allowing tissues to absorb tracer, and from the subsequent loss of tracer to unlabeled solution following the uptake process. Calculation of the fluxes is based on the finding of rate constants for isotope exchange between inside and outside of a tissue. The parameters of the selected kinetic model (e.g. influx of a tracer from outside to cytoplasm or efflux from cytoplasm to outside) can be calculated by using

the rate constants (Walker & Pitman 1976). This procedure has been used for the measurement of ion fluxes and compartmentation in charophyte cells (MacRobbie & Dainty 1958; Diamond & Solomon 1959), in barley root segments (Cram & Laties 1971; Pitman, 1971) and in spruce roots (Kronzucker et al. 1995). MacRobbie, (1970a), Briggs, (1975b) and Britto & Kronzucker, (2001) have reviewed this approach in higher plants.

The ion fluxes in a plant tissue occur to and from both the cell membrane and apoplast (cell walls) of different cell layers (Shabala & Newman 2000). Using microelectrode ion-flux estimation technique (MIFE), Shabala & Newman (2000) showed that ion flux across the cell membrane was qualitatively smaller than that from the tissue surface (apoplast). They measured net fluxes of H^+ and Ca^{2+} in the mesophyll tissue and in protoplasts of the mesophyll cells of *Vicia faba*. High salinity (NaCl 90 mM) increased H^+ efflux in the protoplast and tissue, which was inhibited by vanadate (the plasma membrane H^+-ATPase inhibitor). While, NaCl had no effect on net Ca^{2+} efflux in protoplasts but a large transient Ca^{2+} efflux was observed in the tissue. The external Ca^{2+} concentrations (0.1-10 mM) had no effect on the Ca^{2+} efflux induced by NaCl. They concluded that Na^+/Ca^{2+} and H^+/Ca^{2+} ion exchange in the cell wall are responsible for Ca^{2+} fluxes in the tissue. These effects are unlikely to complicate anion fluxes because anions are not absorbed to fixed charge sites in the cell wall.

Uptake of $^{22}Na^+$ in wheat has been investigated by Davenport et al. (2005) using a three compartment flux model. They showed that $^{22}Na^+$ is absorbed and accumulated in the cytoplasm first. In this phase, which has a short half-time of exchange (3 min), Na^+ influx is unidirectional and $^{22}Na^+$ efflux is negligible. When cytoplasmic $^{22}Na^+$ content was equilibrated with the external medium, net $^{22}Na^+$ uptake becomes linear and vacuolar influx begins. In this stage $^{22}Na^+$ efflux begins from the vacuole after $^{22}Na^+$ uptake saturation, and it causes $^{22}Na^+$ accumulation to stop (but net flux continues). To remove the cell wall $^{22}Na^+$ content, roots were washed in two successive ice-cold rinse solutions containing the same components as pre-treatment solution for a total of 3 minutes. Shoot $^{22}Na^+$ uptake begins shortly after saturation of root $^{22}Na^+$ accumulation. $^{22}Na^+$ transport to the shoot is mostly unidirectional from the root to the shoot, except after shoot $^{22}Na^+$ accumulation reaches a steady state and recirculation occurs. The first linear phase of $^{22}Na^+$ uptake was used to estimate

unidirectional influx (first 3 min) and vacuolar uptake of $^{22}Na^{+}$ was calculated from the second linear phase.

Alternatively, unidirectional ion flux estimations are possible using tracer elution methods (Walker & Pitman 1976). In this procedure the plant tissue is soaked in a labelled solution for a long period, depending on the experimental conditions (1-12 hr or even more), and then it is drained, blotted and transferred to an unlabelled solution with the same composition lacking the radioactivity, for at least the same time used for radiotracer uptake. Efflux of radioisotope from the plant tissue is expressed and plotted as reduction in the tissue radioisotope content after transferring to the nonradioactive solution.

By fitting the data with exponential components (normally two or three exponentials depending on the model), efflux rate from cytoplasm (fast component of the plot) and vacuole (slow component of the plot) as well as influx from outside to cytoplasm can be estimated. Extrapolation of the slow component of the plot to zero time will give the amount of radioactivity in the vacuole (I_v) at the start of efflux. Subtraction of vacuolar content from total tissue activity (determined by addition of the radioisotope loss of each time period) will represent efflux from cytoplasm (I_c). Then, using the following equations, the unidirectional fluxes can be estimated (Walker & Pitman 1976; Macklon, 1975):

$$\Phi_{oc} = \frac{I_v}{t_{up}} + K_c.I_c \qquad \text{(Eq. 1-2)}$$

$$\Phi_{co} = K_v.Q_v + K_c.I_c \qquad \text{(Eq. 1-3)}$$

$$\Phi_{vc} = \Phi_{oc}\frac{K_v.Q_v}{K_c.I_c} \qquad \text{(Eq. 1-4)}$$

$$\Phi_{cv} = \Phi_{oc} + \Phi_{vc} + \Phi_{co} \qquad \text{(Eq. 1-5)}$$

Where Φ_{oc} is ion influx from outside to cytoplasm, Φ_{co} is efflux from cytoplasm to outside, Φ_{vc} is efflux from the vacuole to cytoplasm, Φ_{cv} is influx from cytoplasm to the vacuole, I_v is radioisotope accumulated in the vacuole, I_c is radioisotope accumulated in cytoplasm, K_c and K_v are the rate constants of fast and slow components respectively, and Q_v and Q_c are the vacuolar and cytoplasmic contents of a particular ion.

1-3-2 X-ray microanalysis

In studies on salt compartmentation it is necessary to have a reliable way for measuring ion localization, qualitatively and quantitatively, at plant cellular and even subcellular levels (Huang & VanSteveninck 1989; Hind et al. 1998). In order to detect the mechanisms by which plants respond to unusual conditions like salinity, it is necessary to understand how ions are distributed and localized inside plant cells and tissues following uptake by roots. The interpretational and technical difficulties experienced in using specific ion electrodes and compartmental flux analysis methods have led to development of an electron microscopical procedure called X-ray microanalysis (Harvey, 1985).

X-ray micro analysis is a technique to locate different elements at cellular or even sub-cellular levels. The technique is based on energy-dispersion caused by electron incident with a particular element and measurement of emitted energy by a X-ray spectrometer. When electrons penetrate different elements they generate X-rays that have different energies. These energies are characteristic of the elements from which X-rays are emitted and are detected by an energy-dispersive X-ray spectrometer attached to a scanning (SEM) or transmission (TEM) electron microscope. Counts or amplitudes are plotted as a function of energy in order to identify elements and their abundance in the sample. This scale helps us to detect the element and estimate its concentration semi-quantitatively (Sigee, 1993).

Elements within cells observed with the microscope can be mapped using a software (Morgan et al. 1999). Fixing the elements in their natural location in the cell is an important aspect of X-ray microanalysis studies. Two different approaches can be used to fix the solutes (Yeo & Flowers, 2007): precipitation and freezing. For example: Cl^- can be precipitated in plant cells with silver which is electron dense and so directly visible with an electron microscope. Rapid freezing of plant samples is carried out by direct immersion in melting or liquid nitrogen or by using a cold metal block, a jet of propane or high pressure techniques (Hajibagheri, 1999). Frozen sample can be viewed on a cold stage located inside SEM or TEM after fracturing the sample in the microscope (freeze fracture) (Yeo & Flowers 2007). In the last 30 years, X-ray microanalysis has been developed for accurate determination of ion partitioning

in plant cells (Williams et al. 1993; Hodson & Sangster 1989). This approach includes the analysis of cell vacuoles (Leigh et al. 1986; Leigh & Storey 1993), cytoplasm (Pitman et al. 1981), cell walls (Cuenca et al. 1991) and chloroplasts (VanSteveninck et al. 1988) in bulk frozen plant tissues.

Using X-ray microanalysis, Storey et al. (2003) have investigated the effects of salinity on Cl^- accumulation in grapevine roots of a Cl^--excluding genotype (80-23) and a non-excluding genotype (80-15). In this study, hypodermis, cortex, endodermis and pericycle vacuoles were analysed for ion contents. They found that Cl^- content in the hypodermis and the endodermis is lower than pericycle and cortex. They also showed that vacuolar Cl^- contents were similar in the two genotypes except in the pericycle cells of the Cl^- excluder (80-23), which was higher than the non-excluder (80-15). They assumed that this difference was possibly due to differential regulation of Cl^- transport to the shoot between the two genotypes. They suggested that pericycle cells have an important role in Cl^- transport (particularly long distance) induced by salinity.

1-4 Pathways of Na^+ and Cl^- transport across roots to the xylem

As Flowers & Yeo, (1992) have described, solutes and water may pass across the root in three ways: apoplastic, symplastic and transmembrane pathways. The apoplastic route includes the cell wall and extracellular spaces, and the symplastic pathway is through cytoplasms and plasmodesmatal continuities. The transmembrane route includes membranes and cell walls (Fig.1-2).

The endodermis is the inner border of the root cortex with Casparian bands and up to it water and solutes are able to move through both symplastic and apoplastic pathways. The outer barrier of the root cortex to radial diffusion in the apoplast is called exodermis with Casparian bands, which acts as a barrier to water and solute radial flow (Hose et al. 2001).

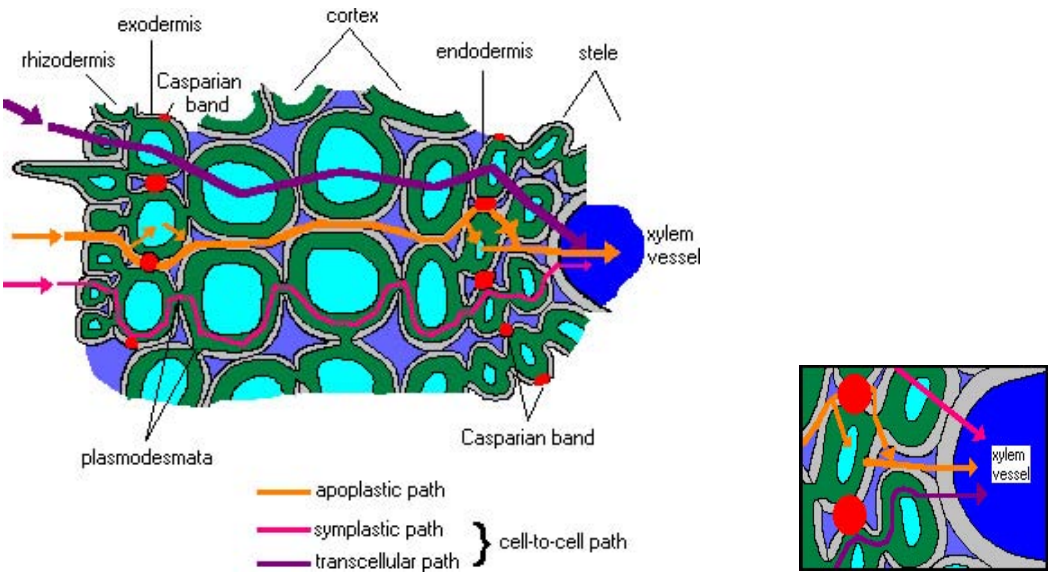

Figure 1-2 Water and solute transport pathways in plant roots (From Steudle, 2000)

During the differentiation of the endodermis, Casparian bands, which are made from substances impermeable to water, are formed and deposited in the endodermis radial cell walls. Also in this point of the cell wall, the plasma membrane is more tightly adpressed to the cell wall (Hose et al. 2001).

The Casparian band inhibits the solutes moving across the apoplastic pathway and thereby imposes membrane selectivity on solute uptake by plants. In young roots, before endodermis differentiation, and in mature roots, where the appearance of lateral roots (from primordia located in the pericycle) interrupts the endodermis, a small leakage of the solutes takes place, which is reffered to as *bypass flow* (Flowers & Yeo, 1992).

1-4-1 Apoplast pathway

Among the different possible ways for salt (NaCl) entry from epidermis to the xylem during high salinity conditions (Fig. 1-2), direct apoplastic continuity is a pathway without biological selectivity for ion transport in the regions of the root where endodermis has not been formed or has been interrupted (Yeo et al. 1987). The estimated contribution of this route (bypass flow) to ion transport through transpiration volume flow is not more than a few percent (Moon et al. 1986). Thus, the importance of this pathway becomes more dominant in high external

concentrations of salt (Pitman, 1982) and at high transpirational rates (Sanderson, 1983).

To measure bypass flow, it is required to use a compound that moves quantitatively with water flow in the apoplast. It should also not pass cell membranes and not stick to cell walls (Yeo et al. 1987). A fluorescent dye, 8-hydroxy-1,3,6-pyrenetrisulphonic acid (PTS), which includes all of the above mentioned characteristics, has been used successfully to study the apoplastic pathway of Na^+ transport under high salinity in rice roots (Yeo et al. 1987 and 1999), rice and wheat (Garcia et al. 1997), red pine root (Hanson et al. 1985), mangrove root (Moon et al. 1986), and it has been used to trace water flow (Zimmerman & Steudle 1998).

Using PTS, Yeo et al. (1987) found that rice plants with high Na^+ transport to the shoot had high shoot concentrations of PTS. They suggested that Na^+ was transported to the xylem probably via the same pathway for PTS uptake. They showed that there was a correlation between PTS uptake and Na^+ uptake amongst different varieties. They also concluded that bypass flow can increase under high salinity and it is a major contribution to Na^+ transport in rice roots under salt stress conditions.

1-4-2 Release of salt into the xylem from the symplast

Ions are transported from the soil to the shoot in two stages: from soil to root symplast and from symplast to xylem vessels (short distance ion transport) and the shoot (long distance ion transport). In general, there are opposing views on transport systems that load salts to the xylem (Zhu et al. 2007). Using the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), Pitman, (1972) showed the inhibition of Cl^- uptake and transport in barley roots. He suggested that a two-pump transport system is involved in this process: one pump for Cl^- uptake from soil to symplasm and a second pump for Cl^- transport from symplasm to the xylem. There are some other studies that are consistent with the xylem loading as an active transport process (e.g. Bowling, 1981; Clarkson, 1988).

On the other hand, there is evidence showing K^+ and anion channels in xylem parenchyma cells are the main pathways for the release of K^+ (Gaymard et al. 1998)

and anions (Gilliham & Tester 2005; Kohler and Raschke 2000) to xylem vessels. Flowers and Yeo (1992) concluded that ion channels in plant cell membranes are responsible for passive movement of ions from symplast to xylem. By using an electrophysiological method (patch clamping), Gilliham & Tester (2005) reported two Cl^- channels in protoplasts isolated from *Zea mays* root stele that may contribute to anion loading to the xylem. This evidence shows that K^+ and anion channels provide an electroneutral passive release of KCl and KNO_3 to the xylem and support the view of Dunlop & Bowling (1971) that there is an active process at the outer surface of the root that drives radial transport of ions into the root, whereas ion transport from symplast to the xylem vessels can be passive.

There are recent studies that show active transport from the xylem. Many of them have indicated a number of transporters in the xylem parenchyma cells that most likely use a H^+ gradient to drive transport. For example: Shi et al. (2002) reported two types of Na^+ transporters that are possibly involved in Na^+ reabsorption from the xylem and loading to the xylem (Hall et al. 2006). Also there is a report on an amino acid symporter that is responsible for amino acid uptake from the xylem sap (Okumoto et al. 2002), sulphate transporters that may function in reabsorbing from the xylem (Kataoka et al. 2004; Takahashi et al. 2000) and a boron transporter that is involved in boron loading to the xylem (Frommer & Von Wiren, 2002; Takano et al. 2002). Furthermore, Kohler & Raschke (1998) suggested that an electrogenic pump, presumably the H^+/ATPase is frequently active in the xylem parenchyma cells of barley roots. Also, Zhu et al. (2007) using the patch-clamp technique indicated an electrogenic pump combined with anion conductance in the xylem parenchyma cells of barley roots. According to this study, the balance between proton pump and anion channel activities influences depolarization and hyperpolarization of the membrane potential (also see Tyerman, 1992) in the parenchyma cell membrane, which in turn affects salt uptake or salt laoding to the xylem vessel.

1-5 Water relations and salinity

Water uptake capacity of a plant is reduced by salinity (Munns, 2002) therefore water and solute relations including hydraulic conductivity and reflection coefficients are affected by salinity (Azaizeh & Steudle, 1991; Tyerman et al. 1989). Water and

mineral nutrients are absorbed by growing plants from soil to the roots and transported radially across the root (short distance) and axially along the xylem (long distance). Unlike the axial pathway that includes vascular tissues, short distance transport is a non-vascular mechanism and occurs through a combination of cell membrane transport and apoplastic flow/diffusion (Johansson, 2000, and sections above). Active transport of solutes into the root and interactions between ions and water flow, play important roles in plant water relations (Steudle & Jeschke 1983).

In the apoplastic pathway of water transport in root tissue, the Casparian band, which consists of suberin and lignin, is a tight barrier to solute and water transport particularly in exodermis (Zimmerman & Steudle, 1998). Steudle, (1993) showed that in maize root the hydraulic conductivity was not significantly increased after injuring the endodermis therefore they concluded that the endodermis is not a major barrier to water transport while suberinized exodermis could be a tight barrier to water flow. However, in the presence of Casparian bands, water flow is mostly forced to pass across the plasma membrane or symplastic pathway (Johansson et al. 2000). In the pathway water molecules diffuse across the lipid bilayer or move through water channels (aquaporins). Aquaporins are membrane proteins that belong to a larger family of major intrinsic proteins (MIPs) and are responsible for most of the hydraulic conductivity (water permeability) of plant cell membranes (Tyerman et al. 1999). For example, it has been shown that water channels in *Chara* are responsible for 74% of the value of hydraulic conductivity (Schutz & Tyerman 1997).

Water transport in plants is not an active process. Flow occurs through the root tissue passively in response to water potential gradients adjusted by transpiration and ion accumulation in the xylem (Steudle, 2000). Hydraulic conductance of a root may change with root development (Peterson & Enstone, 1996) and in response to changes at external medium such as drought (Zimmerman & Steudle, 1998) and high salinity (Azaizeh & Steudle, 1991; Tyerman et al. 1989). Doing both hydrostatic and osmotic Lp_r experiments, Azaizeh and Steudle (1991) showed that salinity (NaCl, 100 mM) reduced the Lp_r 30-60% compared to control treatment (without NaCl). Adding of $CaCl_2$ (10 mM) to the medium showed ameliorative effects on Lp_r. Tyerman et al. (1989) did not find $CaCl_2$ effects on Lp_r in tobacco. Azizeh & Steudle (1991) suggested that the low growth rates in plants treated with salinity can be due to the

low Lp_r values. Similar results have been reported for paprika pepper plants (cv. Albar) by Carvajal et al. (1999). The Lp_r values obtained from different plant species show order of magnitude differences depending on the nature of the driving force (Bramley et al. 2007).

1-5-1 The pressure probe (PP) technique

Many attempts have been made for direct measurement of water relations of plant cells and measurement of cell turgor. About 36 years ago Gutknecht, (1968) inserted microcapillaries into the large cells of *Valonia* and *Nitella* and studied turgor in these cells by changing pressure manually. The pressure probe instrument has been further developed during the past decades (Zimmerman et al. 1998); Steudle & Brinckmann, 1989; Steudle, 1993). In 1983, Steudle and Jeschke used successfully the principle of the cell pressure probe technique for measuring the root pressure, and they were able to examine the water and solutes relations of excised higher plant roots (Fig. 1-3).

The active absorption of ions to the xylem produces an osmotic potential gradient that causes water uptake from soil into the root. Interaction between ions and water flow affects hydraulic relations in roots (Steudle & Jeschke 1983). Solutes are trapped inside the vessels due to the endodermis and hence water moves from surrounding cells into the root xylem in response to the osmotic potential in the xylem. Such a positive hydrostatic pressure, called root pressure, can be measured by a root pressure probe attached to an excised root (Steudle & Jeschke 1983; Tomos & Leigh 1999).

1-5-2 Using the root pressure probe to assess root permeability to ions

The root pressure probe can be used for measuring the radial flow of solutes across roots. The technique examines the response in root pressure after changes in the solute concentrations of the root medium. In the study of transport processes across roots, it has been supposed that the root behaves like an osmometer. That is, the endodermis acts as a semipermeable membrane (Steudle & Peterson 1998). This simple model may be questioned because the other compartments of a root such as the cortex are involved also in water and solute transport across the root. However, Flowers & Yeo (1992) have discussed the role of root cortex in accumulation of solutes into the

symplasm continuity. They have concluded that in many circumstances solute accumulation by the epidermis is much faster than diffusion of solutes through the cell walls of cortex cells. They have explained that the surface area of the cortex cells is not required for ion uptake because the disruption of the cortex during the forming of aerenchyma in *Zea mays* does not affect radial ion transport. Therefore according to the above mentioned discussion, it seems that treating the epidermis/cortex/endodermis as a semipermeable osmometer can most likely be acceptable.

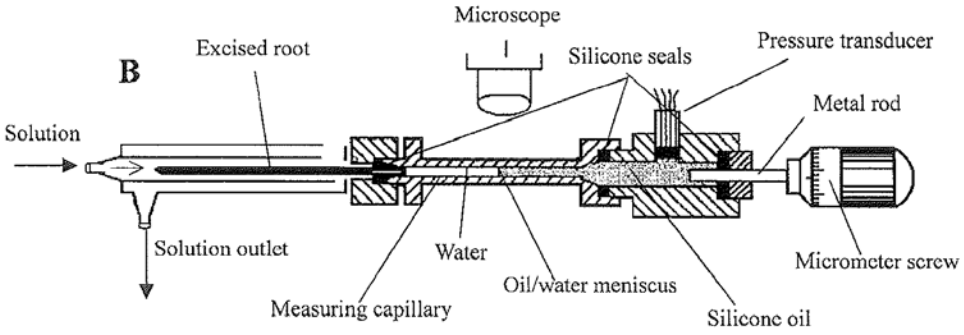

Figure 1-3 Root pressure probe technique. A root system is connected to the root pressure probe by silicone seals. The silicon seals over a length of 8-10 mm will compress the root cortex without any compression on the xylem. After proper sealing of the root in the probe, a meniscus is formed between water and silicon oil. The meniscus acts as a point of reference and is viewed by a stereo microscope. When a stable root pressure is achieved the measurements are started (from Miyamoto et al. 2001).

However, the root is not a perfect osmometer and does not act like an individual cell. The root is more likely an osmometer with a complex composite osmotic barrier built up of several layers (Steudle & Peterson 1998). A perfect osmometer is completely impermeable to solutes and the reflection coefficient is close to unity. However the reflection coefficient measured in roots of different species is between 0.2-0.8 (Steudle & Frensch 1989). This indicates that ions may pass through the root cylinder (Steudle & Peterson 1998).

The hydraulic conductivity of the root (Lp_r) measured by the root pressure probe technique shows large differences between species and from the view point of the nature of the driving force used to drive water flow. In hydrostatic experiments Lp_r is much higher than osmotic experiments. This variability of Lp_r can not be explained with a simple osmometer model (Weatherley, 1982). Passioura, (1988) has explained this variability in Lp_r as a result of valve-like action of the plasmodesmata of root cells and Fiscus, (1975) described it as a dilution effect. Variation in Lp_r can also be

attributed to changes in aquaporins activities, and by shifts in the transport pathways (apoplastic or symplastic).

Changes in water transport pathways are in turn dependent on the driving force required for water transport across a tissue (Tyerman et al. 1999). In a recent study, Bramley et al. (2007) suggested that the differences in Lp_r are probably related to the way in which the root pressure (P_r) is changed. Bramley et al. (2007) first compared hydraulic properties of the same section of a root using the pressure relaxation and pressure clamp methods. It was supposed that the above mentioned methods measure the same component of water flow in which case Lp_r values must be the same for the both methods. However, in all plant species tested in this experiment, the Lp_r values were not the same (Lp_r for pressure relaxation method was 2-3 fold greater than for the pressure clamp). It indicated that the methods did not measure the same component. Using two pressure probes connected to the two ends of an excised root Bramley et al. (2007) showed responses to induced changes in root pressure in one end recorded at the opposite end. They showed that by doing hydrostatic pressure relaxations on the root between two pressure probes, pressure values at the opposite end did not change appreciably and it indicated that xylem pressure did not rise uniformly along the length of the root. This may be due to the radial water flow resistance and capacitance particularly near the physical end of the root close to the origin of the pressure pulse (Bramley et al. 2007).

The composite transport model (Fig. 1-4) of a root that has been proposed by Steudle, (1989) has sufficiently explained some of the above mentioned variability in the reflection coefficient and the hydraulic conductivity.

As it has been mentioned in section 1-4-1, in some parts of the root (root tip and the secondary root initials) that Casparian bands have not yet developed, by-pass flow of water and solutes can occur passively. However, doing cytochemical analysis of Casparian bands, Schreiber, (1996) showed that the Casparian band is mostly built up of lignin rather than suberin. Since lignin is more hydrophilic than suberin, water leakage could also occur through the Casparian band.

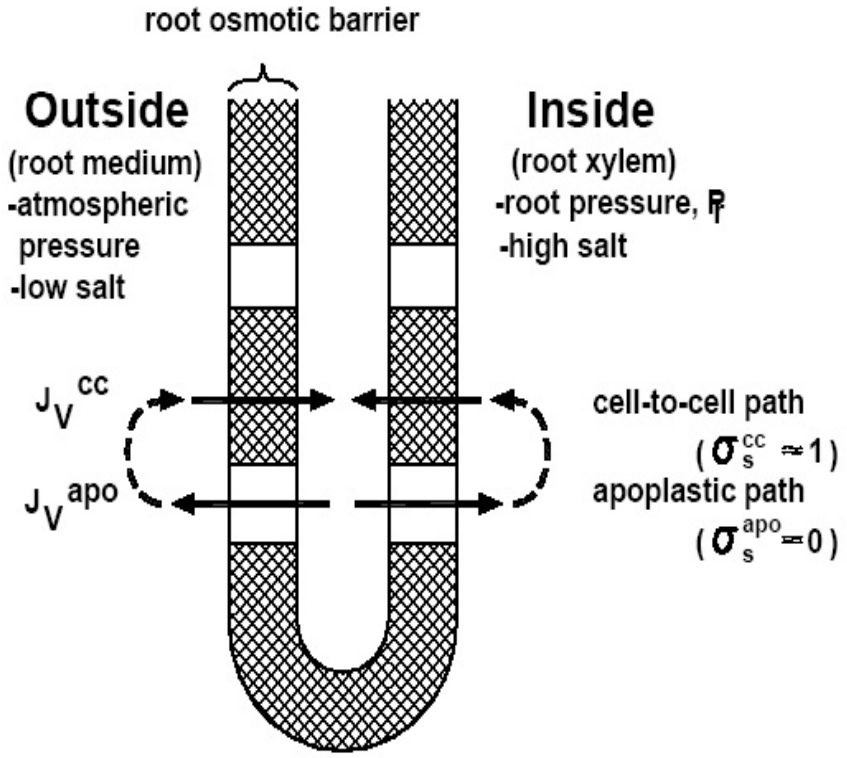

Figure 1-4 Composite transport model of the root. Along the apoplastic pathway the reflection coefficient of a cell wall is nearly zero while in cell to cell pathway both osmotic and hydraulic flow are small because of the high selectivity of cell membranes (reflection coefficient is nearly 1). This causes a circulation in water flow and low overall root reflection coefficient at low rates of transpiration (from Steudle 2000).

Steudle et al. (1993) showed that by puncturing the root with even small by-passes, this reduced the reflection coefficient. They made a small hole of 18-60 µm in diameter and 7-9 cm from the root tip by inserting a fine glass tube radially into the root. The consequence of this impalement was a small injury in the endodermis (10^{-2} to 10^{-3} % of the total surface of the endodermis). This treatment had no effect on Lp_r but decreased reflection coefficient from 0.64 to 0.41 for NaCl. According to the composite transport model, which is based on observation with the the root pressure probe reconciled with anatomical studies, the low reflection coefficients of roots can be attributed to by-pass flow of water and solutes via the apoplasmic pathway. The apoplast has high hydraulic conductance and very low reflection coefficient.

This model results in a circulation flow of water in the root and concentration gradients of solutes between the xylem and external medium of the root. Protoplastic (cell-to-cell pathway) route is semi-permeable with reflection coefficient (σs) $\approx$ 1 and in the apoplastic route there should not be selectivity between water and solute thus

$\sigma s \approx 0$. Interaction between the two pathways is proposed to cause a circulation flow of water and an overall low reflection coefficient of the root (Steudle, 2000). In some plant species like *Phaseolus coccinus* (Steudle & Brinckmann, 1989) and *Hordeum distichon* (Steudle & Jeschke, 1983) it has been shown that the cell to cell pathway dominates because of a high membrane Lp_r. Bramley et al. (2007) showed that Lp_r values in *Triticum aestivum* (wheat) obtained from both the pressure clamp and the osmotic gradient methods are the same. It shows that water flow through the apoplastic pathway may be reduced. Also in some circumstances such as the lack of hydrostatic pressure gradients or entirely blocked apoplast by Casparian band, the symplastic route is the main way for water and solute transport. In this case the role of water channels (aquaporins) in the cell membrane was suggested to be very important (Steudle & Peterson 1998).

1-5-3 Measurement of the reflection coefficient

The reflection coefficient (σ_s) is one of the solute transport parameters, and is defined as the relative permeability of a particular solute compared to that of water. It is determined from the maximum change in cell or root turgor due to a change of the osmotic pressure of a solute (Steudle & Tyerman 1983). The reflection coefficient is typically less than or equal to one (unitless). When the selectivity of a cell membrane for water is much higher than to a solute then the reflection coefficient of the solute is about 1 whereas with $\sigma_s = 0$, the solute is able to permeate as fast as water (Steudle, 2000).

Both ion permeability and reflexion coefficient for roots are measured in the same manner as the method used for isolated cells. Typically, if a permeating solute such as NaCl, is added to a cell or a root medium the turgor pressure inside the cell or root rapidly decreases due to a water flow outward from the cell or root. However after a while the turgor will increase again because of passive flow of the solute inward to the cell and consequently, the concentration of solutes on both sides (inside and outside) of the cell tends to be equilibrated. The root permeability and the reflection coefficient are analytically estimated using equations described by Steudle & Tyerman (1983).

$$\sigma s = \frac{\Delta P}{\Delta \pi} . \frac{\varepsilon + \pi i}{\varepsilon} \exp(K_s . t_{min}) \qquad \text{(Eq. 1-6)}$$

Where, σ_s is the reflection coefficient for the external solute, ΔP is the maximum change of root pressure, $\Delta\pi$ is the change of osmotic pressure of the medium, ε is the elasticity coefficient, K_s is the rate constant for the solute phase, and t_{min} is the time of pressure reduction to a minimum level. For a root the second term of the equation is used to correct the cell volume change during the osmotic process and is close to unity. The third term (exponential) of the formula is used to correct for the passive uptake or efflux of solute during the time interval required to reach the minimum.

1-6 Grapevines and salinity

Grapevines are frequently grown and irrigated in semi-arid zones where drought and salinity are common problems (Cramer et al. 2007). Salinity causes growth reduction in grapevine (Walker, 1994). Studies show that photosynthesis and stomatal conductance (Downton, 1977), ion composition in different parts of grapevine (Garcia & Charbaji 1993; Fisarakis et al. 2004) and grapevine yield (Downton, 1985; Prior et al. 1992b) are affected by salinity. Prior et al. (1992b) showed that the effect of salinity on Sultana vine i.e. yield reduction, was observed after the first season of irrigation with saline water and continued during the subsequent seasons of irrigation with saline water. Stevens et al. (1999) showed that irrigation of grapevine with saline water significantly reduced growth and yield. The berry development stage was most sensitive to soil salinity and yield was reduced by 7% (compared to control plants irrigated with non-saline water). Walker et al. (2002a) found a strong correlation between salt tolerance in Sultana grapevine and rootstocks vigor.

1-6-1 Origin of rootstocks and grapevine varieties

The genus that is important in viticulture is *Vitis* from the family *Vitaceae*. The exact number of species for the genus *Vitis* is not known but it is assumed that there are more than 40 species (Antcliff, 2000). According to archeological evidences, it is thought that the origin of wine grape, *Vitis vinifera*, is from a region between the Mediterranean basin and the Caspian sea (Olmo, 1976).

At the end of the 19th century, grape rootstocks were used in viticulture to test natural resistance against a grape pest, *Phylloxera* that originates from East North America. The modern rootstocks initially have been derived from some of the American *Vitis* species. The grape rootstocks (hybrid cultivars) are produced by crossing between American *Vitis* species (commonly *V. berlandieri, V. riparia and V. rupestris*) and sometimes between American *Vitis* and a few cultivars of *V. vinifera* (DeAndres et al. 2007). Hybrids of the species of *Vitis* were produced and used commercially for the first time in the eastern United States and then in Europe after diseases and pests from North America were introduced to Europe (Antcliff, 2000). Varieties that have originated from *Vitis vinifera* L. are the base of viticulture in Australia. These varieties have a predominant role in grape production in the world (Hardie & Cirami, 2000).

Nearly all grape vines, cultivated in Australia, are varieties of *Vitis vinifera* (Dry & Gregory, 2000). It is estimated that *V. Vinifera* has about 24,000 varieties (Viala & Vermorel,1909), although Truel et al. (1980) believe that only 5000 are authentically different varieties, and only a small number of them have commercial usage.
In Australia the total number of grapevine varieties used in production are less than 100 (Dry & Gregory, 2000).

1-6-2 Vitis rootstocks and salinity

A variety or rootstock must be screened for a specific purpose and for a suitable condition under which it is to be grown (Hardle & Cirami, 2000). Some grape rootstocks such as Ramsey, 101-14, Rupestris du Lot, 1613, Richter 99, Richter 110, and Dogridge are able to reduce Cl^- uptake and Cl^- transport to the shoot of grafted scion (Sauer, 1968).

There are many reports of the evaluation of salt tolerance in grapevine rootstocks. For example, Walker et al. (2004) have studied the effects of 7 different rootstocks on the salt tolerance in Sultana scion. In this evaluation, Sultana scion was grafted on 1103 Paulsen, Ramsey, J 17-69 rootstocks and 4 hybrids, R1, R2, R3 and R4 derived from *V. champini, V. berlandieri* and *V. vinifera*. The plants were then treated with

different concentrations of NaCl under field conditions. By measuring Na^+ and Cl^- contents in petioles, laminae and juice over the 4 years of the trial, they concluded that rootstocks with high Cl^- and Na^+ exclusion ability can increase salt tolerance in Sultana grapevines as measured by yield. According to this study, rootstock 1103 Paulsen was introduced as the best chloride excluder because of lowest Cl^- accumulation in the petiole, laminae and grape juice of the scion.

Fisarakis et al. (2001) measured ion content of lamina and roots of Sultana (*Vitis Vinifera* L.) vines, own rooted and grafted on various rootstocks. In all varieties, shoot dry weight reduced with increasing external salinity.

Table 1-1 The Effect of rootstock on Na^+ and Cl^- concentrations (% D.W.) of root and lamina of Sultana vines, 60 days after salt treatment. Different letters within columns indicate significant differences at $P < 0.05$. (from Fisarakis et al. 2001).

	Lamina		Root	
Rootstock	Na^+	Cl^-	Na^+	Cl^-
41B	0.14c	1.08 b	0.31 a	3.49 a
140Ru	0.23 b	0.78 d	0.26 a	2.79 c
1103P	0.19 bc	0.94 c	0.31 a	3.08 b
110R	0.10 c	0.82 cd	0.29 a	2.69 cd
SO_4	0.18 bc	0.77 d	0.26 a	2.48 d
Own-rooted	0.59 a	1.88 a	0.19 a	3.46 a

This study showed that Na^+ and Cl^- concentrations in vine tissues including root, stem, petiole and lamina, increased with increases of external NaCl (5, 25, 50 and 100 mM). Roots accumulated higher concentrations of Na^+ and Cl^- than other plant parts including lamina, stem, and petiole. The final Na^+ concentration of lamina was lower than that of Cl^- (Table 1-1). They concluded that in comparison to own-rooted Sultana, all other rootstocks were able to exclude Na^+ and Cl^- from shoot.

1-6-3 Na^+ and Cl^- accumulation

There are many reports on the quantity of Na^+ and Cl^- accumulation in different varieties of grapevines. Garcia & Charbaji, (1993) demonstrated that the addition of NaCl to nutrient solution causes an increase in the Na^+ content of the vegetative

organs of grapevines. Also, grapevines accumulate significant amounts of chloride, particularly in shoots (Downton, 1977, Lauchli, 1984).

Troncoso, et al. (1999), compared the responses of 11 grapevine rootstock varieties to high concentrations of NaCl. They showed that in salt tolerant varieties Na^+ and Cl^- accumulation are significant. For example they indicated that in the group of tolerant varieties Na^+ and Cl^- concentrations in plant tissue were higher than the sensitive varieties at high external [NaCl], as shown in Table 1-2.

Table 1-2 Influence of NaCl concentration of the medium on the mineral composition (%d.w.) of the plant tissue (groups of sensitive and tolerant varieties) after 45 d from the onset of *in vitro* culture (Troncoso et al. 1999)**.**

NOTE: This table is included on page 34 of the print copy of the thesis held in the University of Adelaide Library.

Walker et al. (2004) showed that mean values of Na^+ and Cl^- concentrations in petioles of Sultana on own roots were higher than those for Sultana grafted on different rootstocks except for R3 in which Na^+ concentration was higher than own-rooted Sultana vine at three salinity levels. Similar results were obtained for lamina and juice ion composition (Table 2-1). They concluded that vigor of a rootstock in combination with moderate to high Cl^- and Na^+ exclusion ability can be the best combination for salt tolerance in Sultana grapevine (Table 1-3).

Table 1-3 Four-year (1991-1994) mean values of Na^+ and Cl^- concentrations (% dry weight) in juice, petiole and lamina of Sultana on own roots or grafted to various rootstocks at three different levels of salinity (from Walker et al. 2004).

NOTE: This table is included on page 35 of the print copy of the thesis held in the University of Adelaide Library.

1-6-4 Salt tolerance in grapevines

Grapevine (*Vitis vinifera*) varieties have been classified as moderately sensitive (Maas & Hoffman, 1977) to salinity. They have used the yield response model to assess plant tolerance to salinity. According to this model, crop yield decreases almost linearly just after salinity increases beyond a threshold level of salinity.

Medium concentrations of salt (12-75 mM) may result in a growth rate reduction and budburst delay in Sultana scion grafted on different rootstocks. The time of budburst in all tested rootstocks was negatively correlated to increased Cl^- contents within canes (Downton & Crompton, 1979). Similar results have been shown by Walker et al. (2002 a) in both field grown own rooted Sultana vines and Sultana vines on a range of rootstocks. Medium salinity also decreases shoot, bunch and root number (Prior, et al.1992). After a long period of study (6 years) on own-rooted Sultana grapevine irrigated with river water or river water injected by NaCl, Prior et al. (1992) found that salinity caused a reduction in % fruitful nodes, % fruitful shoots and bunches per node and bunches per cane, and also reduction in cane number. They also found that almost 97 % of roots were located in the top 0.6 m of soil and that root density was reduced by salinity.

Excessive accumulation of chloride, particularly in leaves, is apparently the main reason for salt damage in grapevines (Downton & Millhouse, 1983; Hardie & Cirami, 2000). Bernstein et al. (1969) showed that leaf-burn symptoms were observed in older leaves of grapevine varieties treated with 25 mM NaCl. However they did not find any correlation between severity of leaf burn and leaf-chloride content. They suggested that the severity of leaf-burn symptoms in grapevine depends on the duration of accumulation of harmful levels of salt rather than actual levels of accumulation at the time of sampling. However, Ehlig, (1959) had shown the above mentioned correlation between the severity of leaf-burn injury and Cl^- contents in leaves of some grapevine varieties under hot weather conditions. They showed that marginal burn in leaves appeared when Cl^- content of the blade was about 100-150 mM on a tissue water basis. Therefore, it appears that limiting the Cl^- accumulation in leaves could be important from the view point of salt tolerance (White & Broadley, 2001). However, Walker, (1994) and Arbabzadeh & Dutt, (1987) believe that high concentrations of Cl^- may not in itself be the sole cause for saline stress in grapevine. By changing the saline medium to a non-saline condition, grapevine plants were able to recover physiologically despite the continued maintenance of high Cl^- concentrations in shoots (Walker, 1994).

There are many reports on the physiology of salinity tolerance and the responses of different grapevine varieties to high concentrations of salt from the crop improvement point of view (e.g. Troncoso et al.1999, Downton, 1977&1983, Garcia & Charbaji, 1993, Stevens et al. 1999, Storey, 2003). Troncoso et al. (1999) observed that there is an apparent relationship between root creation, number of new buds, stem growth, plant survival and salt tolerance. In other words, in salt tolerant varieties, the reduction in the above mentioned parameters were less than in sensitive rootstocks. Also, Sauer, (1968) and Bernstein et al. (1969) reported that certain grapevine rootstocks are able to decrease Cl^- accumulation within scions. Bernstein et al. (1969) investigated the Cl^- accumulation difference between 5 grapevine rootstocks. They showed that "Cardinal" variety (table grape from California, hybrid of Tokay and Ribier), accumulated Cl^- in leaves 16-fold greater than the "Salt Creek" (Ramsey) rootstock.

1-6-5 Rootstocks contrasted

In this study the grapevine rootstocks, which have been selected for comparative physiology of salt tolerance investigation, are as follows:

1- K51-40: This rootstock originated from *V. champini* x *V. riparia* . The level of scion vigour is high and root strike is good. This variety was produced for resistance to rootknot nematode at the University of California, Davis. Also, it is suggested that K51-40 is a salt sensitive variety, which is chosen for this study in order to contrast with a resistant variety (Hardie and Cirami 2000).

2- 1103 Paulsen (1103 P): This rootstock originated from *V. berlandieri* x *V.rupestris.* The scion vigor is moderate and root strike is fair. For nematode infection, this variety is moderately to highly resistant and in the case of phylloxera infection, it is highly resistant. This rootstock conveys drought resistance to scions and it is moderately salt tolerant (Hardie and Cirami 2000).

1-7 Aims of the project

High concentrations of salt (NaCl) reduce plant growth by ion toxicity, osmotic stress and nutritional disruption. Na^+ is maintained in woody roots and stems and chloride accumulates in the shoot, particularly in the leaves. There are many reports on Na^+

tolerance and transport in higher plants; however the mechanism of Cl^- tolerance and transport, especially in woody horticultural plants need to be studied in more detail. Besides the toxic effects, high concentrations of Cl^- can reduce NO_3^- uptake. Some studies have also indicated that high concentrations of NO_3^- will reduce Cl^- toxicity in certain tree crops. Hence, there are interactions between Cl^- and NO_3^-, which are very important from the view point of N-nutrition in plants.

Grapevine (*Vitis spp*) is a moderately salt sensitive woody deciduous perennial. It has been reported that the main reason for salt toxicity in grapevines is the accumulation of Cl^- at high concentrations in the shoots (Walker, 1994). Tyerman & Skerrett (1999) have pointed out that the mechanism of Cl^- influx under salinity and how its concentration in the cytoplasm is regulated, has been neglected relative to that for Na^+.

The main objective of this study was to compare the Cl^- fluxes through the plant roots and interactions with NO_3^- uptake in two grapevine varieties, K 51-40 as a salt sensitive and 1103 Paulsen as a salt tolerant variety.
In this project, the following subjects were investigated:
1- The nature of the interaction between Cl^- and NO_3^- entry to roots
2- The effect of the increased external salinity on Cl^- accumulation inside the plant
3- The differences between salt tolerant and salt sensitive varieties of grapevine in accumulation and compartmentation of Cl^-
4- The main pathway and mechanism for Cl^- entry across the cell membranes in grapevine

Chapter 2

NO_3^- / Cl^- selectivity in grapevine roots

2-1 Introduction

Soil salinity unfavourably affects absorption and assimilation of water and mineral nutrients by plant roots. Among the mineral nutrients required for plants, inorganic nitrogen is very important and nitrogen metabolism is linked to yield potential in crop plants (Leidi et al. 1992). Nitrogen deficiency is a limiting factor of plant growth, and the interaction between nitrogen fertilizers and salinity has been studied to find approaches for decreasing or inhibiting effects of high concentrations of salt on crop production (Flores et al. 2004). There are many reports that show that under salt (NaCl) stress conditions, uptake of nitrogenous inorganic ions is severely reduced (Pessarakli & Tucker 1985). For example:

- Decreases of NO_3^- and NH_4^+ contents in red kidney beans (Frota & Tucker, 1987)
- Inhibition of N and P absorption in rice and wheat (Palfi, 1965; Mahajan & Sonar, 1980)

There are variable results from the literature on Cl^- and NO_3^- interactions. In some reports, it has been shown that NO_3^- uptake is insensitive to Cl^- levels in the root medium. Cram, (1973) and Smith, (1973) showed that net NO_3^- absorption by barley is not inhibited by Cl^- in the root medium. Similar result was obtained for $^{13}NO_3^-$ absorption by barley roots in the presence of Cl^- (Glass et al. 1985). On the other hand, there are a number of studies that show an antagonism of Cl^- on NO_3^- absorption (Cerezo et al.1999).

Glass & Siddiqi (1985) examined the interaction between NO_3^- and Cl^- and showed that by increasing external NO_3^-, accumulation of Cl^- in barley plants decreased via reduction of Cl^- influx (Fig. 2-1). Also, Bar et al. (1997) showed that adding NO_3^- to the irrigation water decreased Cl^- accumulation in avocado and citrus plants. In

tomato, it has been shown that by increasing NO_3^- concentration in the external medium, Cl^- content in the plant is decreased (Kafkafi et al. 1982). In addition, net NO_3^- uptake can be reduced by increasing Cl^- concentration in the external medium

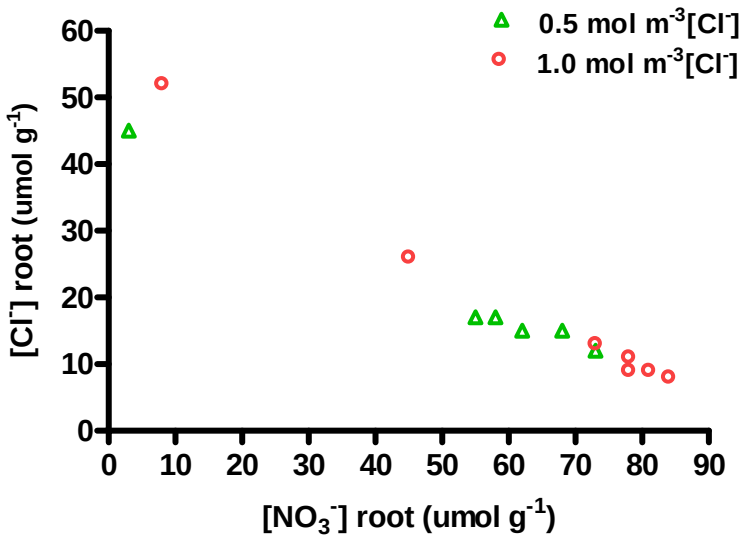

Figure 2- 1 Root $[Cl^-]$ as a function of root $[NO_3^-]$ in barley (Glass & Siddiqi 1985).

(Peuke & Jeschke 1999). They showed that HATS in barley root is inhibited by NaCl with a K_i of about 60 mol m^{-3} NaCl. In another experiment, Abdelgadir et al. (2005) have examined the relationship between NO_3^- and Cl^- uptake in two rice cultivars, Koshihikari and Pokkali, under NaCl and Na_2SO_4 treatment. They concluded that there is an antagonism between NO_3^- and Cl^-, with a significant negative correlation between NO_3^- and Cl^- contents in shoot and root of the two cultivars.

High salinity has probably different effects on the NO_3^- LATS and HATS transport systems. It has been shown that high external salinity inhibited net NO_3^- uptake by HATS whereas high NaCl concentrations did not inhibit net nitrate absorption at high concentration (LATS) in two species of citrus, Troyer citrang and Cleopatra mandarin (Cerezo et al. 1997). They also confirmed the above mentioned results in another cultivar, Carrizo citrange, by examining the effect of salt pre-treatment on $^{15}NO_3^-$ absorption (Cerezo et al. 1999). They suggested that Cl^- accumulation in tissues is the main reason for the inhibition of $^{15}NO_3^-$ uptake rate by *Citrus* plants. This finding was also consistent with the results provided by Cram, (1973). According to Cram, (1973),

NO_3^- and Cl^- transport system(s) activity in barley and carrot roots is a function of the total accumulation of these two ions inside the vacuole.

The main aims of the experiments reported in this chapter are as follows:

- To examine the selectivity between NO_3^- and Cl^- uptake in two grapevine varieties (K 51-40 and 1103 Paulsen) that differ in salt tolerance.
- To determine Cl^- contents accumulated in different parts of the tested varieties.
- To measure ^{15}N uptake into the plants in the presence of different NaCl concentration.
- To test a finding by Cram, (1973) and Cerezo et al. (1997) that NO_3^- uptake is sensitive to Cl^- status of the tissue in grapevine.

2-2 Materials and Methods

2-2-1 Plant material

Grapevine cuttings of two varieties, K 51-40 and 1103 Paulsen, were collected from the Coombe vineyard at Waite Campus, University of Adelaide. K 51-40 is salt sensitive (Nicholas, 1997) and originates from *Vitis champini x Vitis riparia*. 1103 paulsen is a salt tolerant variety (Walker et al. 2004) and originates from *Vitis berlandieri x Vitis rupestris*. The basal parts of stem cuttings were immediately soaked in IBA (0.1 % w/v in 50% ethanol) for 5-10 s. They were fixed in a growing medium (a sheet of the engineered foam root cubes) and put in a growth chamber (humid heat bed) to initiate roots. After 3-4 weeks, rooted cuttings were transferred to plastic pots with sand.

2-2-2 Experimental design

For the first 3-4 weeks the plants in pots were watered with ¼ strength Hoagland solution containing 0.25 mM $NH_4H_2PO_4$, 1.5 mM KNO_3, 1 mM Ca $(NO_3)_2$, 0.5 mM $MgSO_4$, and micronutrients 576.25 µM H_3BO_3, 114.25 µM $MnCl_2$, 9.5 µM $ZnSO_4$, 4 µM $CuSO_4$, 3 µM H_2MoO_4 and 88 µM iron tartrate, to produce a single stem with 3-4 leaves. In order to increase N demands of the plants and also to measure net fluxes of ^{15}N later, plants were irrigated with N-depleted ¼ Hoagland solution (0.25 mM

KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 1.5 mM KCl and micronutrients as mentioned above) for another 3 weeks. Salinity (NaCl) was imposed as 15, 30 and 45 mM in ¼ strength Hoagland solution lacking N, for 7 days. The above mentioned range of salinity was selected according to the previous studies carried out on grapevine (i.e. Walker et al. 2004). After pre-treatment with NaCl, the plants were exposed to 4 mM $K^{15}NO_3$ in Hoagland solution lacking N compounds plus specified NaCl concentrations for 3 days to measure net fluxes of nitrate. During the ^{15}N treatment, all pots were irrigated with 300 mL of the solution two times per day to field capacity (pots drained).

2-2-3 Determination of NO_3, Cl and ^{15}N

At the end of the experiment (1 or 2 days after ^{15}N treatment period) plants were harvested and plant parts including leaves, stem, cane and roots were weighed separately and dried at 70^oC for 48 hours. Dried plants were well ground to increase sample homogeneity by a grinder (Labtech Essa LM1-P).

100 mg of ground plant materials were put in plastic vials containing 10 mL distilled water. The vials were boiled in a hot water bath for 20 min. After cooling the vials, their contents were transferred to 10 mL plastic tubes to centrifuge at 5000xg for 15 min. Then supernatants were poured in new 10 mL plastic tubes and made up to 10 mL with distilled water. The extracts were stored in a fridge until doing NO_3^- and Cl^- measurements.

Chloride concentration in water extracts was determined by silver ion titration using a chloride analyzer (Corning 926). The instrument was adjusted by standard solution (200 mg Cl/L). Then 0.5 ml of the above mentioned extracts was injected into the titration solution containing combined acid buffer. Cl^- concentration was read in mg L^{-1} and converted to mg Cl^- content per gram dry weight of the tissue.

Total nitrate concentration was measured using a spectrophotometer according to the modified method of Cataldo, (1975). Extracts (0.5 mL) were pipetted into 10 mL plastic tubes containing 0.3 mL 5% salicylic acid in concentrated H_2SO_4. After 10 minutes at room temperature, 7.2 mL NaOH (2M) was gently added to tubes. NaOH helps to make a solution with pH above 12 which is suitable for NO_3^- and acid interaction. Absorbance at 410 nm of each sample was measured by a

spectrophotometer (BIO-RAD SmartSpec 3000). Standards containing 5, 10, 15, 20, 25 and 30 µg NO_3^--N in a 0.5 mL aliquot were analyzed with each set of samples. Blank solution was made of 0.5 mL H_2O, 0.3 ml 5% salicylic acid in concentrated H_2SO_4 and 7.2 mL NaOH (2N).

^{15}N contents of the tissues was determined using a mass spectrometer (Geo 20-20, Europa Scientific). 4-4.5 mg of well powdered plant parts was weighed and put in a small tin capsule (4x6 mm). The capsules were folded several times and put inside the sinks on a tray. References including 25 capsules of EDTA (0.9-1.5 mg) per 100 samples, 15 capsules of ammonium sulphate (20 uL of 0.05897 g in 2.5 mL distilled water) per 100 samples and 15 capsules of glycine (20 uL of 0.06699 g in 2.5 mL distilled water) per 100 samples were analyzed with each set of samples. ^{15}N contents of the plant tissues were measured as atom % :

$$\text{atom \%} = 1/(1+(^{15}N/^{14}N)) \times 100 \qquad \text{(Eq. 2-1)}$$

and then were calculated as µg g^{-1} (dry wt). All data were analysed statistically and plotted with GraphPad Prism-4 program.

2-2-4 Statistics

In this chapter and throughout the thesis all means are given with the Standard Error of the means (SEM). Error bars on graphs are SEM. When required significance tests were performed. These were either t-test (for equality of variance in each test), one-way analysis of variance with post tests for differences between means, or two-way analysis of variance with post tests. Different letters were used to indicate significant difference between the compared means within the columns. Significance level was $P < 0.05$ for all tests.

2-3 Results

Interaction between Cl^- and NO_3^- uptake under salinity (NaCl) stress was studied in the two grapevine varieties grown in sand pots and watered with ¼ strength Hoagland solution. Concentrations of Cl^- in both varieties increased in roots after 7 days treatment with different concentrations of NaCl (Fig. 2-2).

In comparison to the salt tolerant Paulsen, the salt sensitive K 51-40 accumulated more Cl^- in the shoot (Fig. 2-2 A) while Cl^- accumulated in roots of both varieties was similar although it significantly increased with increased NaCl concentrations in Paulsen (Fig. 2-2 B).

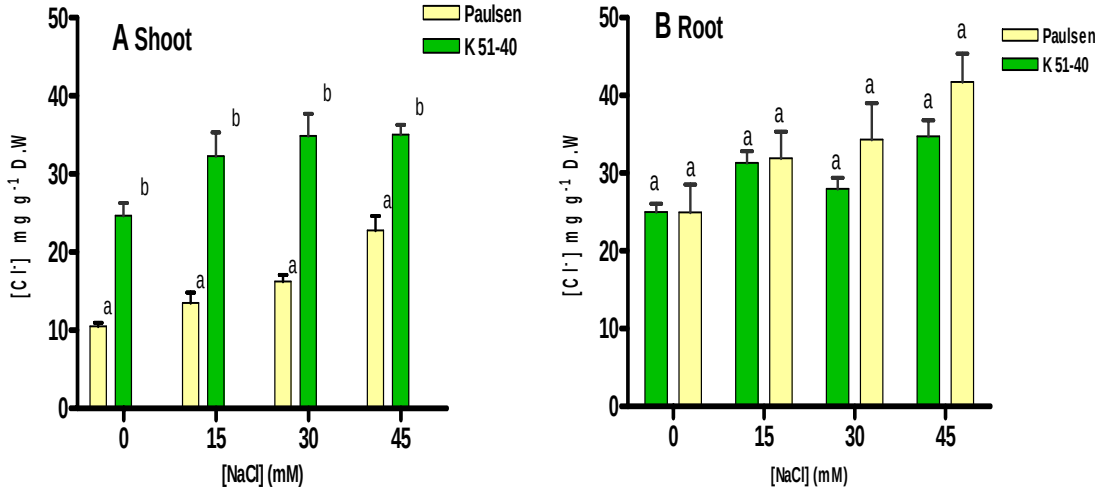

Figure 2- 2 Chloride concentration in shoot (A) and root (B) of K 51-40 and Paulsen treated with different concentrations of NaCl for 7 days. Bars are SE of the means (n=4) (two way ANOVA, $P < 0.05$). Different letter indicates significant difference between the varieties in each treatment (t-test).

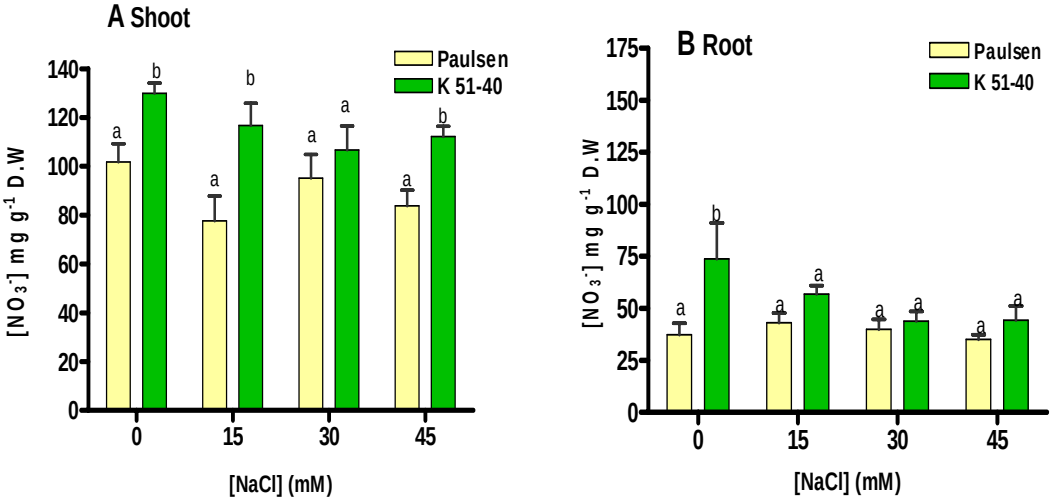

Figure 2- 3 Total NO_3^- concentration in shoot (A) and root (B) of K 51-40 and Paulsen treated with different concentrations of NaCl for 7 days. Bars are SE of the means (n=4) (two way ANOVA, $P < 0.05$). Different letter indicates significant difference between the varieties in each treatment (t-test).

Total NO_3^- concentration of the shoot in the salt sensitive K 51-40 was higher than salt tolerant Paulsen, but with increasing NaCl concentrations no significant difference between different salt treatments was observed (Fig. 2-3 A). However,

NO^-_3 concentrations in roots were reduced in K 51-40 while it had no significant effect on Paulsen (Fig. 2-3 B).

Comparing Cl^- and NO_3^- concentrations in shoot and root of each variety, it can be observed that in K 51-40 (salt sensitive) the NO_3^- concentration was reduced with increased Cl^- concentrations in roots (Fig. 2-4B). Also shoot NO_3^- content of K 51-40 was lower than shoot Cl^- concentration (Fig. 2-4 A).

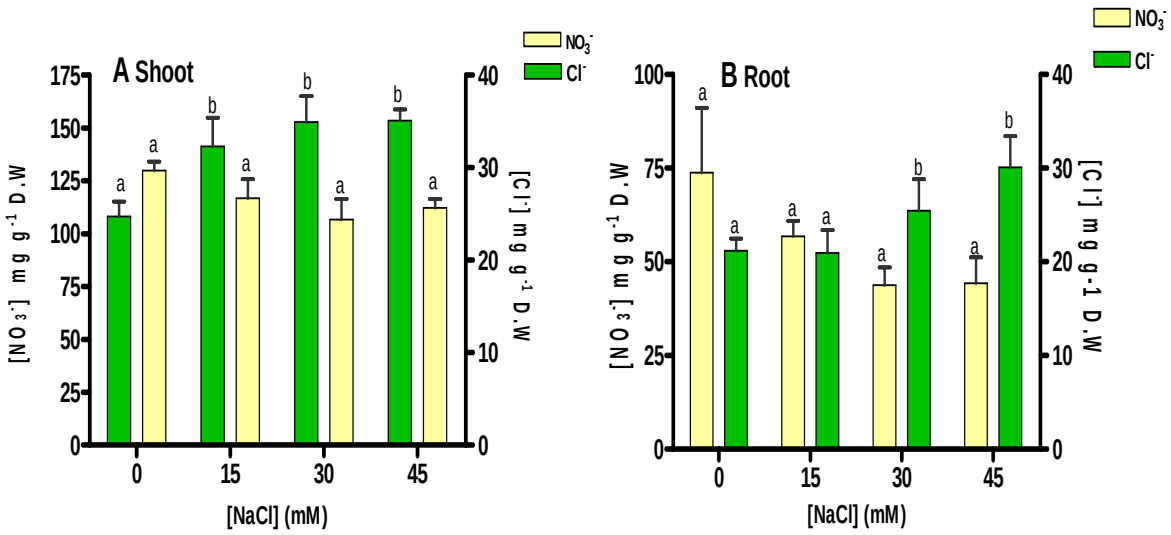

Figure 2- 4 NO_3^- and Cl^- concentrations in shoot (A) and root (B) of K 51-40. Bars are SE of the means (n=4) (two way ANOVA, $P < 0.05$). Different letter indicates significant difference between NO_3^- and Cl^- values in each treatment (t-test).

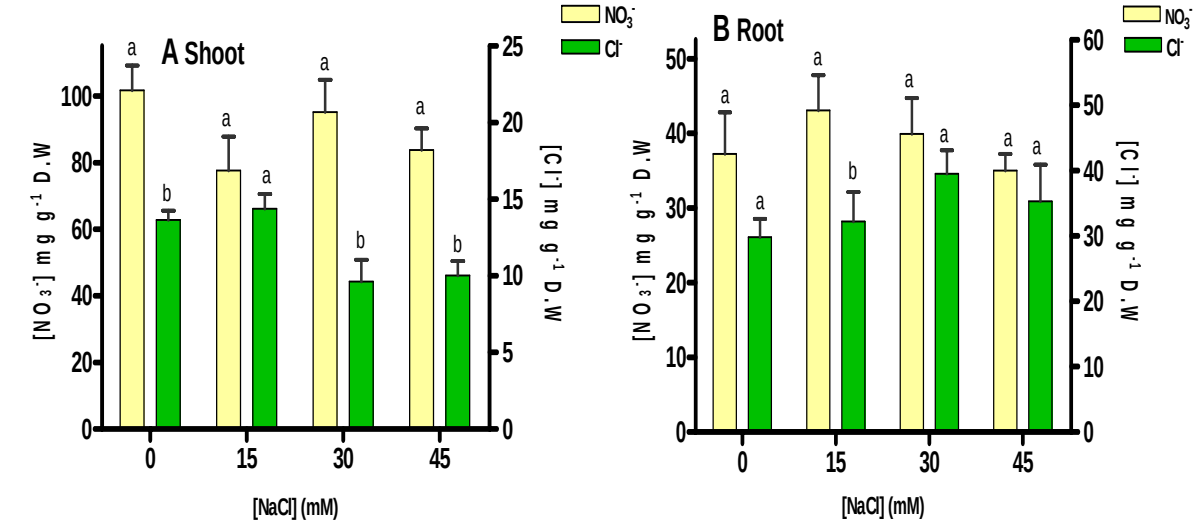

Figure 2- 5 NO_3^- and Cl^- concentrations in shoot (A) and root (B) of Paulsen. Bars are SE of the means (n=4) (two way ANOVA, $P<0.05$). Different letter indicates significant difference between NO_3^- and Cl^- values in each treatment (t-test).

In Paulsen (salt tolerant), shoot NO_3^- content was higher than shoot Cl^- concentration particularly in high NaCl concentrations (30 and 45 mM) (Fig. 2-5 A). However, high

concentrations of Cl^- had no significant effect on NO_3^- accumulation in Paulsen at high salinity (Fig. 2-5 B).

$$NO_3/Cl\ Selectivity = \frac{tissue\ (root)\ NO_3/Cl}{external\ NO_3/Cl} \qquad (Eq.\ 2\text{-}2)$$

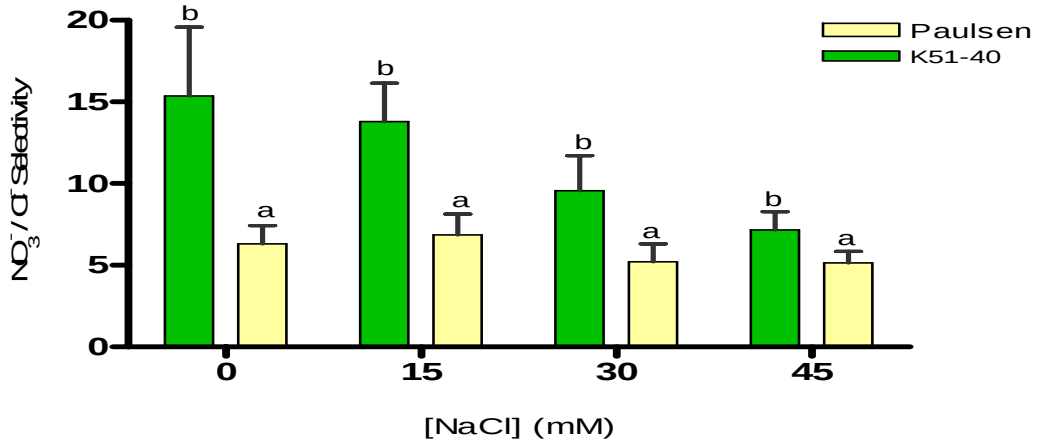

Figure 2- 6 NO_3^-/Cl^- selectivity in root of K 51-40 and Paulsen. Bars are SE of the means (n=4) (two way ANOVA, $P< 0.05$). Different letter indicates significant difference between the varieties in each treatment (t-test).

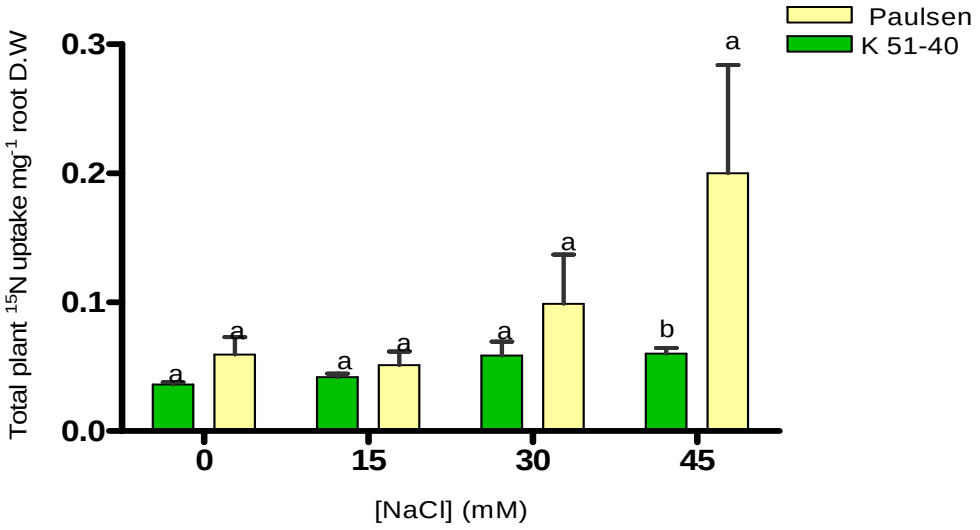

Figure 2- 7 The total plant uptake of $^{15}NO_3^-$ in K 51-40 and Paulsen per root dry weight. Bars are SE of the means (n=4) (two way ANOVA, $P< 0.05$). Different letter indicates significant difference between the varieties in each treatment (t-test).

A NO_3^- / Cl^- selectivity (SNO_3^-/Cl^-) calculated from tissue concentrations relative to medium concentrations (Eq. 2-2) showed significant differences between the two varieties; K 51-40 showed reduced root selectivity with increased salinity but had initially a higher *S*NO3/Cl (Fig. 2-6).

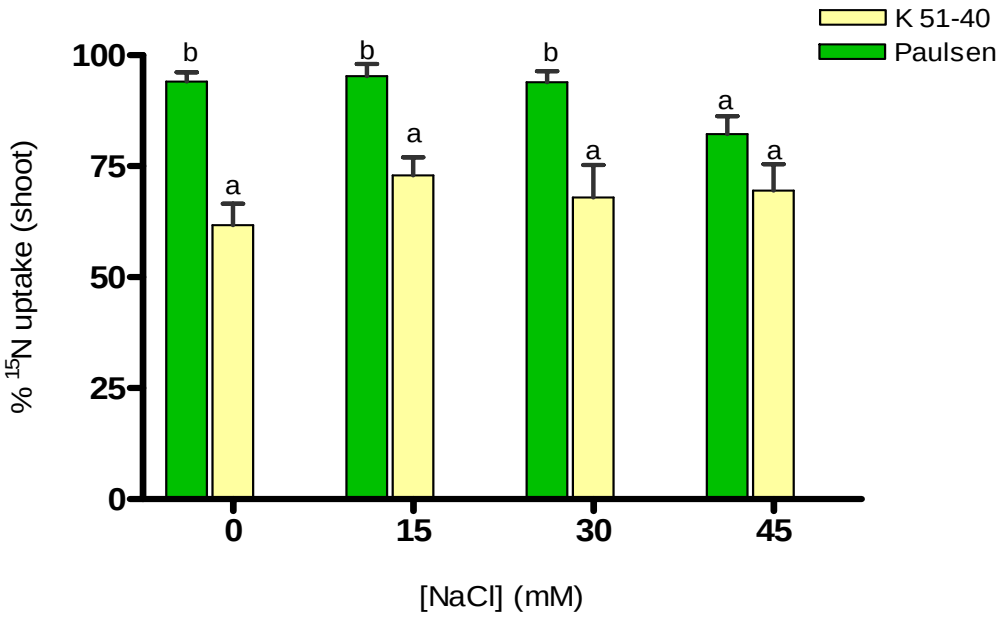

Figure 2- 8 Total fraction of ^{15}N uptake transported to the shoot of K 51-40 and Paulsen calculated from the ratio of shoot ^{15}N to whole plant ^{15}N content (two way ANOVA, $P< 0.05$). Different letter indicates significant difference between the varieties in each treatmt (t-test).

In both rootstocks the rate of NO_3^- uptake (total accumulated in shoot + root per root dry weight) was similar at low concentrations of NaCl but more than doubled at high salinities in Paulsen (salt tolerant) (Fig. 2-7).The fraction of total uptake of ^{15}N transported to the shoot was greater in Paulsen than for K 51-40 (Fig. 2-8).

2-4 Discussion

Paulsen (salt tolerant), in comparison to K 51-40 (salt sensitive) is able to maintain lower Cl^- accumulation in the shoot (Fig. 2-2 A). This is in line with a finding reported by Walker et al. (2004). They showed that Paulsen is the best Cl^- excluder rootstock because it had lowest Cl^- concentrations in petiole, lamina and grape juice

of field grown vines. The higher root/shoot Cl^- concentrations ratio indicates the Cl^- retaining ability of the Paulsen roots, which can be a determinant of Cl^- tolerance (Miklos et al. 2000). High Cl^- accumulation in the shoot of K 51-40 is the most likely reason for salt sensitivity in this variety.

Despite some reports that show insensitivity of net NO_3^- uptake to Cl^- levels in the root medium (Cram, 1973; Smith, 1973; Glass et al. 1985), there are many other studies that indicate antagonism and interaction between NO_3^- and Cl^- transport (Cerezo et al. 1999; Glass & Siddiqi 1985; Bar et al. 1997; Kafkafi et al. 1982; Abdelgadir et al. 2005). Results presented in this chapter showed that probably high external Cl^- concentration reduces NO_3^- accumulation in shoot and root of K 51-40 (salt sensitive), but it had no significant effect on Paulsen (salt tolerant). NO_3^- accumulation decreased with high concentration of Cl^- in the root of K 51-40 (Fig. 2-4 A).

In grapevine (various cultivars), it has been shown that by increasing NaCl concentrations in the external medium the NO_3-N concentrations in all parts of the tested grapevine cultivars were decreased after 60 days (Fisarakis et al. 2004). Miklos et al. (2000) treated grapevine cultivars with high NO_3^- and high Cl^- concentrations and showed interaction between NO_3^- and Cl^- and also the inhibitory effects of high NO_3^- and Cl^- on the transport of other ions. They showed 30-60% reduction of NO_3^- uptake due to high Cl^- concentrations in the medium. Inversely, treatment of the plants with 50 mM NO_3^- decreased Cl^- contents in the root of various grapevine cultivars. Thus, the results of the experiments in this thesis are consistent with these studies.
Furthermore, NO_3^- and Cl^- interference could occur via various transport mechanisms. Tyerman & Findlay (1989) found that NO_3^- and Cl^- permeat the same anion channels in tonoplast membrane, and that NO_3^- permeability is approximately twice that of Cl^-. The interaction between anions can be attributed to the competition of anions for transport across the tonoplast and/or at the location of anion release to the xylem (Jaenicke et al. 1996).

Overall, Paulsen is a Cl^- excluder rootstock that accumulates low Cl^- in the shoot. As salinity increased in the external medium the NO_3^-/Cl^- selectivity was maintained in Paulsen by increase in NO_3^- uptake by roots and a greater proportion being transferred to the shoots. Since root concentrations of Cl^- and NO_3^- were nearly similar in both

varieties, it is likely that the xylem-loading transport for Cl^- and NO_3^- differs between the two varieties in terms of regulation and/or selectivity.

Chapter 3

Water and solute relations of grapevine root under salinity

3-1 Introduction

Plants under salt stress suffer from osmotic effects and ion toxicity. Decreasing water potential in the rhizosphere can reduce water and nutrient uptake from the soil (Azaizeh et al. 1992). The root hydraulic properties differ between species and with growth (environmental) conditions (Steudle & Peterson 1998). During salt stress water relations, including hydraulic conductivity and turgor pressure, as well as solute relations, including reflection and permeability coefficients, are changed significantly. Azaizeh & Steudle, (1991) showed that the Lp_r (root hydraulic conductivity) of maize root decreased by salinization (100 mM NaCl) of the growth medium. The Lp_r variability can also be related to the nature of the driving force (hydrostatic or osmotic). There are large differences between osmotic and hydrostatic water flow across roots. According to Steudle et al. (1993) in maize root, the hydraulic conductivity (2.7×10^{-7} m s^{-1} MPa^{-1}) determined by hydrostatic pressure gradients as the driving force, was larger than that measured from osmotic gradients (2.2×10^{-8} m s^{-1} MPa^{-1}). The formation of an exodermis with Casparian band also may decrease the root capacity to take up water (Steudle & Peterson, 1998).

Bramley et al. (2007) used a new technique to study the transmission of pressure over the length of a root tissue that was assumed to be the reason for the variability of Lp_r. They observed much less difference between hydrostatic and osmotic Lp_r when hydrostatic Lp_r of maize root was measured under steady state conditions (pressure clamp instead of pressure relaxation method).

Steudle et al. (1993) showed that injuring the endodermis by a capillary needle did not increase the hydraulic conductivity of maize root connected to the pressure probe.

They concluded that the endodermis is not a major barrier to water flow. However, the exodermis with Casparian bands and suberin lamellae when present, may decrease hydraulic conductivity of some roots such as the basal zone of onion root (Melchior & Steudle, 1993).

Using a two pressure probe technique, Bramley et al. (2007) showed that by applying a pressure pulse at the basal end of an excised root that the pressure was dissipated along the root length due to radial water flow. There was also a time delay before the maximum pressure change occurred at the apical end of the root. It was concluded that the pressure pulse for a pressure relaxation experiment was not induced uniformly over all the root length. It can be a reasonable explanation for the observation by Steudle et al. (1993) in which puncturing the endodermis did not increase hydrostatic Lp_r.

According to the composite transport model (see section 1-5-2), low reflection coefficient (less than unity) for solute transport by the root, which for cell membranes is expected to be practically unity, can be due to water flow through apoplastic by-passes (Steudle & Peterson, 1998). In woody species Lp_r is smaller than herbs (Rudinger et al. 1994). They showed that Lp_r of spruce root obtained from hydrostatic gradients (6.4×10^{-8} m s^{-1} MPa^{-1}) is much larger than that obtained from osmotic gradients (0.017×10^{-8} m s^{-1} MPa^{-1}). This large difference between results of the two types of experiments (hydrostatic and osmotic) was consistent with results of herbaceous species (results from Steudle et al. 1993). However, Lp_r in spruce was lower than maize root.

The reflection coefficient for some osmotica (K_2SO_4 or Na_2SO_4) in spruce root (0.14-0.28) (Rudinger et al. 1994) was smaller than that obtained from maize for NaCl (0.37-0.92) (Steudle et al. 1993). It was concluded that in woody plants, root reflection coefficient is highly affected by apoplastic by-pass flows (Steudle & Peterson, 1998).

Water uptake by roots is regulated by physical and physiological processes. The root hydraulic conductivity can be regulated either by switching between the pathways for water transport, which is a physical phenomenon and is done by apoplastic barriers, or

by water channel activity which is under metabolic control (Miyamoto et al. 2001). There is a relation between hydraulic properties of roots and their anatomy (Steudle & Peterson, 1998). It has been assumed that Casparian bands (a deposite of suberin and/or lignin) in the endodermis and exodermis are hydrophobic and are major barriers to water uptake. However, some studies (e.g Steudle, 1993) showed that injuring the endodermis did not affect the hydraulic conductivity. On the other hand, North & Nobel (1991) indicated that the development of suberin lamella in the endodermis decreases water flow across the root. These variable results about the role of the endodermis and exodermis in water uptake show that the transport properties in a root may change through root development stages (Steudle & Peterson. 1998). In other words, the hydraulic conductivity of a root can be varied through different stages of development of endo- and exodermis. For example, suberised Casparian bands of exodermis are developed during later stages are correlated with decreased hydraulic conductance in the root (Steudle, 2000).

In this chapter the hydraulic conductivity (L_{pr}) and the reflection coefficient (σ_s) for NaCl and $NaNO_3$ were measured in K 51-40 and 1103 Paulsen by using the root pressure probe technique for the purpose of testing if there were differences between the two rootstocks from the view point of bypass flow of salts to the xylem. Root anatomical studies also were carried out to find differences in apoplastic barriers or root branching for the rootstocks.

3-2 Materials and methods

3-2-1 Plant material

Cuttings of K 51-40 and 1103 Paulsen were provided as described in section 2-2-1. Rooted cuttings were transferred to an aerated culture solution in plastic containers, containing ¼ strength Hoagland solution (composition as mentioned in section 2-2-2). After 1-2 weeks, the main roots of the plants before lateral roots were used in pressure probe experiments and root anatomical studies.

3-2-2 Measurement of root hydraulic conductivity (L_{pr}) and reflection coefficient (σ_s)

Water and solute transport between the external medium and the xylem were measured using a root pressure probe. Unbranched root segments (50-100 mm) from apical end were cut under the culture solution. The cut end of a segment was connected to the pressure probe using a silicone seal (about 10 mm long) provided from silicone material (Optosil-Xantopren, Heraeus Kulzer) (Fig. 1-3, Chap.1). In order to prevent solution leakage, the seal around the root was tightened gently by a screw around silicon seal until the root pressure started to increase. The capillary between probe and root was filled with distilled water and silicone oil and it was checked that no air bubbles were left in the system. A meniscus, formed between water and oil, was monitored using a microscope. Steady state root pressure (0.3-0.7 bars) was obtained after 10-20 min. After each experiment the root at the seal was cut and the fast decrease in pressure showed the proper functioning of the seal. 4-7 replicates were used in each experiment depends on the experiment conditions. The solutions used in all the experiments were circulated by a peristaltic pump along the root segments connected to the pressure probe (Fig. 1-3, Chap. 1).

In hydrostatic experiments, radial water flow was induced across the root by moving the meniscus either forward (exosmotic water flow caused by an increase in the xylem pressure) or backward (endosmotic water flow caused by a decrease in the pressure of the xylem) using a movable metal rod in the pressure probe. Doing hydrostatic pressure relaxation, water rate constant (K_{wr}) or relaxation time ($T_{1/2}$) was obtained and from which hydraulic conductivity (L_{pr}) of a given root was calculated using the following equations (Steudle & Jeschke, 1983):

$$K_{wr} = \ln (2) / T_{1/2} \qquad \text{(Eq. 3-1)}$$

$$L_{pr} = K_{wr} / A_r . \beta \qquad \text{(Eq. 3-2)}$$

Where, A_r is the geometric root surface area and β is the elastic modulus. A_r was calculated from the root length and diameter:

$$A_r = 2\pi.r.l \qquad \text{(Eq. 3-3)}$$

Where, π= 3.14, (r) is the root radius determined using an ocular micrometer calibrated against a slide micrometer gauge, and (l) is the root length.
The root elastic modulus (β) was estimated by moving the metal rod by a certain amount, in the root pressure probe instantaneously determining the change in volume and recording the changes in the pressure.

In osmotic experiments with different permeating solutes (NaCl and $NaNO_3$), root pressure relaxations are usually biphasic (Steudle & Jeschke, 1983). The first rapid phase (water phase) is attributed to the change in osmotic pressure of the external medium. The second (slower) phase (solute phase) is attributed to the passive transport of the solutes into or out of the root. It in turn causes water uptake resulting from changes in the osmotic gradient across the root. Osmotic Lp_r was estimated from the first phase of relaxation curves using Eq. 3-2.
The reflection coefficient (σ_r) was calculated using the following relationships (Azaizeh and Steudle, 1991):

$$\sigma s = \frac{\Delta P}{\Delta \pi} . \frac{\varepsilon + \pi i}{\varepsilon} \exp(K_s . t_{\min}) \qquad \text{(Eq. 3-4)} \qquad K_s = 1 / \tau_s \qquad \text{(Eq. 3-5)}$$

Where, σs is the reflection coefficient of the root, ΔP is the maximum change of root pressure P_{ro}-P_{min}, $\Delta\pi$ is the change of osmotic pressure in the external medium of permeating solute (in this experiment NaCl or $NaNO_3$), ε is the elastic modulus of the xylem, π_i is the osmotic pressure in the xylem, K_s is the rate constant for the solute phase, τ_s is the time constant for the external solute flow, and t_{min} is the time of pressure reduction to a minimum level. It is assumed that the factor $\varepsilon+\pi_i / \varepsilon$ is equal to unity because ε is much bigger than π_i of the xylem.

3-2-3 Anatomy

Root cross sections were made at 20-50 mm behind the root tip of K 51-40 and Paulsen. The plants were grown in hydroponics as mentioned in section 3-2-1. Hand-cut root sections were stained using the berberine-aniline blue fluorescent staining

procedure (Brundrett et al. 1988). The sections were put in mesh-bottomed holders and then transferred into staining plates. They were stained with 0.1% (w/v) berberine hemisulphate in distilled water for 1 h. The sections in the holders were rinsed with distilled water several times. The holders were transferred into 0.5% (w/v) aniline blue in distilled water for half an hour and then rinsed as above. The sections were placed in 0.1% $FeCl_3$ in 50% (v/v) glycerol for 2-3 min and then were put on slides and mounted in the same solution. A fluorescent microscope (Zeiss, Axiophot Pol, Photomicroscope) was used to observe the mounted sections.

3-3 Results

3-3-1 Root hydraulic conductivity and reflection coefficient

Hydraulic root pressure relaxations were measured in K 51-40 and Paulsen roots (Fig. 3-1). From the half-time ($T_{1/2}$) of water exchange obtained in this experiment, hydraulic conductivity (Lp_r) of the roots was calculated using Eq. 1-3. Hydrostatic Lp_r for K 51-40 and Paulsen was 5.7 x 10^{-7} (m $s^{-1}MPa^{-1}$) and 6.1 x10^{-7} (m $s^{-1}MPa^{-1}$) respectively. There was no significant difference between the Lp_r of the rootstocks (t-test) (Table 1-3 and Fig. 3-2).

Table 3- 1 Hydraulic conductivity (Lp_r) and the reflection coefficient (σ_s) of K 51-40 and Paulsen calculated using root surface area (Ar), the elasticity coefficient (β), the half-time of water ($T^w_{1/2}$) and solute ($T^s_{1/2}$) exchange, the rate constant of water (K_w) and solute (K_s) transport parameters.(n=15) (t-test was used to compare the mean values of Lp_r and σ_s , $P< 0.05$). Different letters indicste significant difference between osmotic and hydrostatic Lp_r.

	Ar	β	$T^w_{1/2}$	K_w	$T^s_{1/2}$	K_s	Lp_r		σ_s	
	(mm^{-2})	(MPa / µl)	(s)		(s)		Hydrostatic	Osmotic		
							m s^{-1} MPa^{-1}	m s^{-1} MPa^{-1}	NaCl	$NaNO_3$
K 51-40	183.93	0.27	32.97	0.026	123	0.008	[a] 5.4 x10^{-7}	[b] 2.16 x10^{-7}	[a] 0.51	[a] 0.48
	± 7.03	± 0.019	± 4.8	± 0.003	± 15.33	± 0.001	± 0.053	± 0.028	± 0.047	± 0.052
Paulsen	171.42	0.31	41.47	0.032	237	0.007	[a] 6.1 x10^{-7}	[b] 1.95 x10^{-7}	[a] 0.59	[a] 0.51
	± 11.1	± 0.028	± 6.85	± 0.01	± 69.66	± 0.0009	± 0.8	± 0.23	± 0.048	± 0.047

In osmotic experiments conducted on the roots of both rootstocks, NaCl and $NaNO_3$ were applied as permeating salts (Fig. 3-3 and Fig. 3-4). The half time ($T_{1/2}$) of the

water phase in relaxation curves was used to calculate the hydraulic conductivity (Lp_r) of the roots.The osmotic Lp_r for K 51-40 and Paulsen was 2.6 $x10^{-7}$ (ms^{-1} MPa^{-1}) and 1.95 $x10^{-7}$ (ms^{-1} MPa^{-1}) respectively. It showed that the Lp_r values were almost similar for the both rootstocks (Table 3-1 and Fig.3-2 A).

However, the Lp_r values of the both rootstocks obtained from osmotic and hydraulic experiments were remarkably different as compared in Fig. 3-2 A and Table 3-1.

The reflection coefficient (σ_s) of the applied solutes (NaCl and $NaNO_3$) on the roots was calculated from the ΔP and half-time of solute phase obtained from the biphasic root pressure relaxation curves using Eq. 3-4 and 3-5.

As shown in Table 3-1 and Fig. 2-3 B, the reflection coefficient (σ_s) of NaCl (30 mM) for K 51-40 and Paulsen roots was 0.51 and 0.59 respectively and of $NaNO_3$ (30 mM) was 0.48 and 0.51 for K 51-40 and Paulsen respectively which were not significantly different (t test, $p < 0.05$).

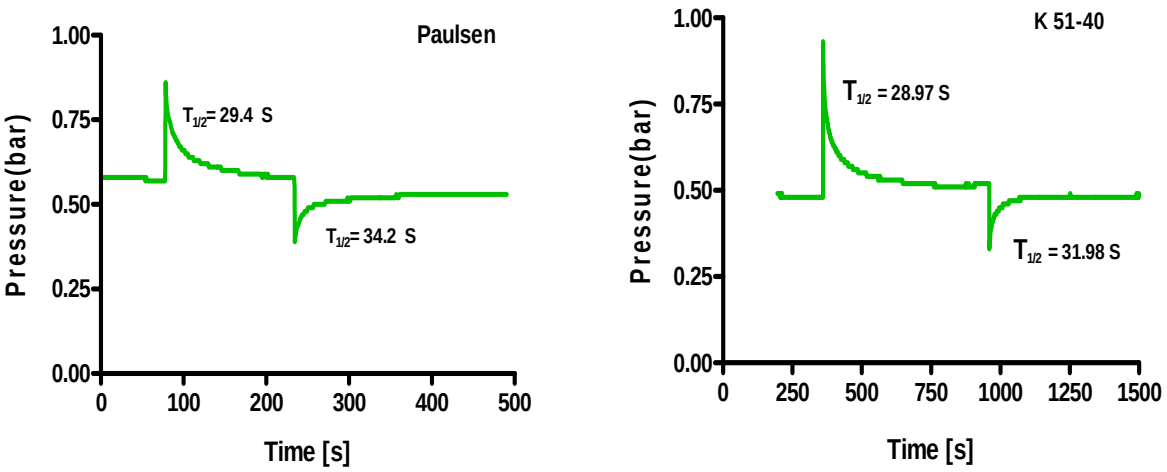

Figure 3- 1 Typical graphs of hydrostatic root pressure relaxation experiments on excised roots of Paulsen and K 51-40 using the root pressure probe equipment. The $T_{1/2}$ of water exchange was used to estimate the hydraulic conductivity (Lp_r).

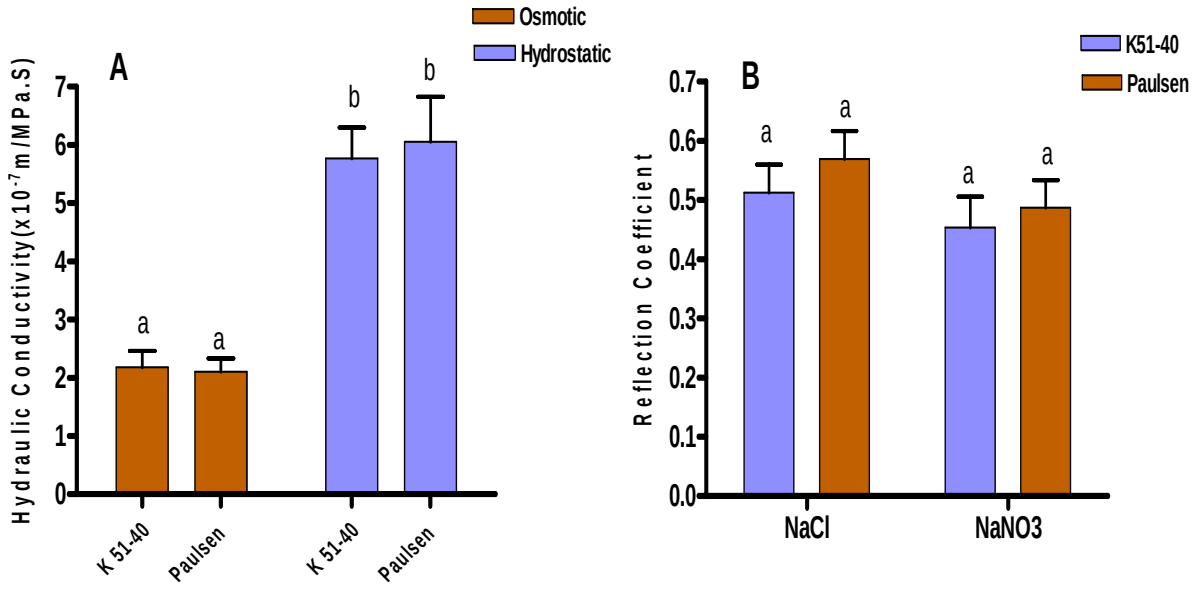

Figure 3- 2. The osmotic and hydrostatic hydraulic conductivity (Lp_r) (A) and the reflection coefficient (σ_s) (B) of K 51-40 and Paulsen roots. Bars are SE of the means (n=15) (t-test, $P< 0.05$). Different letters indicate significant difference between osmotic and hydrostatic Lp_r.

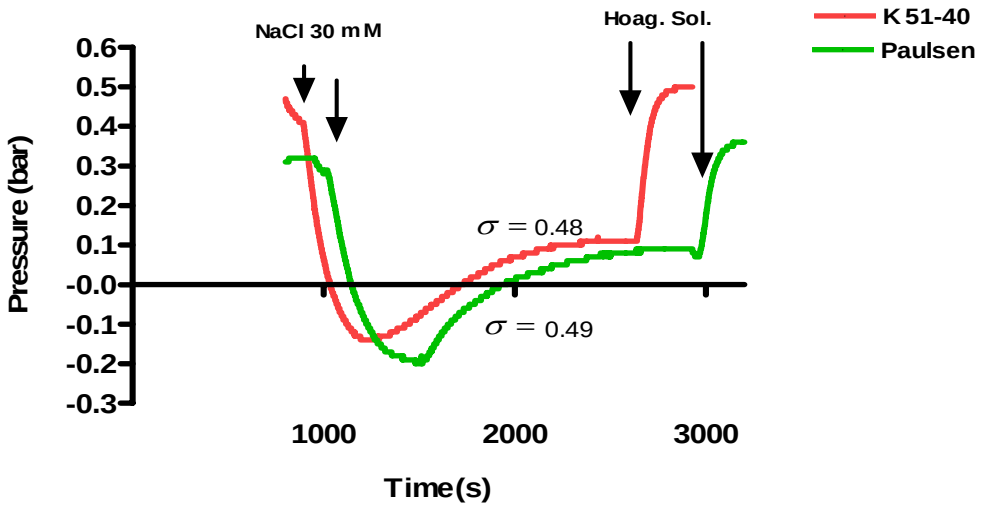

Figure 3- 3 A comparative graph of osmotic root pressure relaxation experiment on K 51-40 and Paulsen roots. NaCl (30 mm) was applied as the osmoticum on the roots.

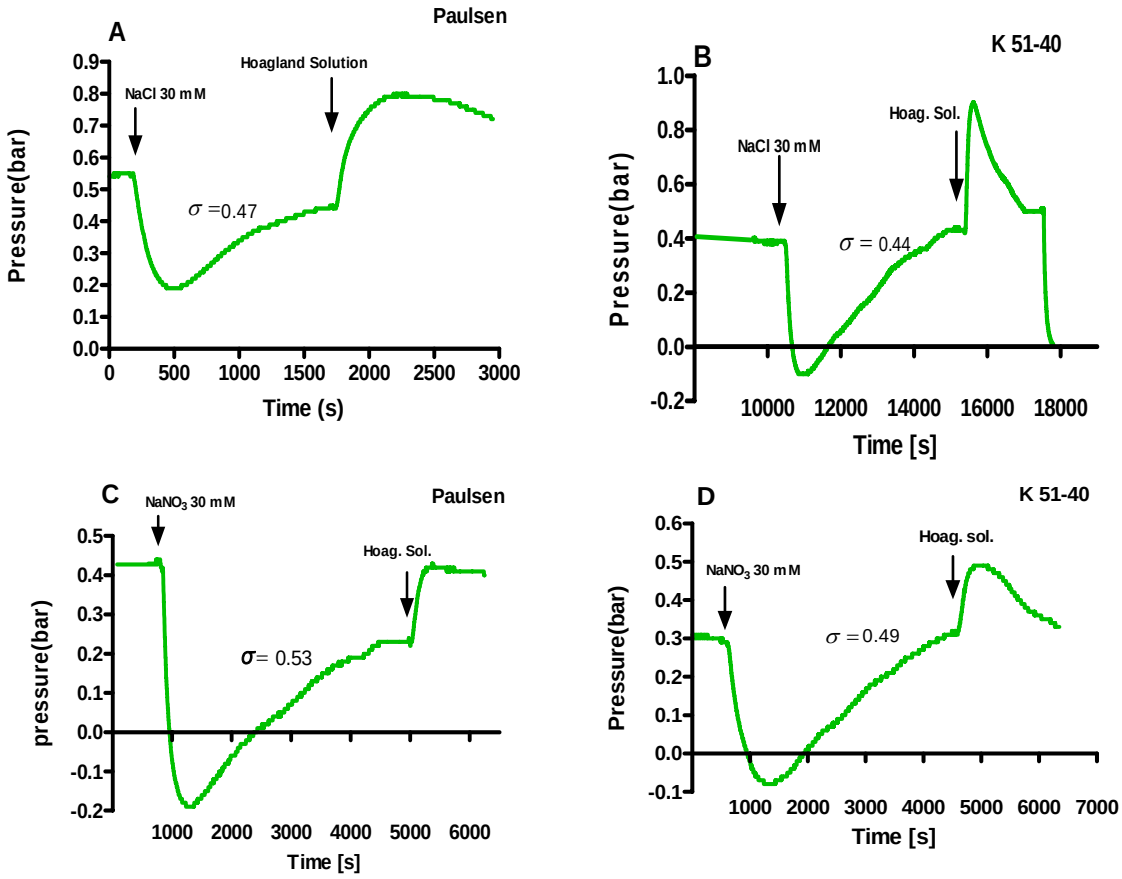

Figure 3- 4 Typical graphs of osmotic root pressure relaxation experiments on excised roots of Paulsen (A and C) and K 51-40 (B and D) using the root pressure probe equipment. NaCl (30 mM) (A and B) and $NaNO_3$ (30 mM) (C and D) were applied as osmotic solutes to the excised roots. The half-time of water exchange ($T_{1/2}$ of the water phase) was used to calculate the osmotic Lp_r. The time constant for the external solute flow (τ_s of the solute phase) was used to estimate the reflection coefficient (σ_s).

The results showed that the reflection coefficients of NaCl and $NaNO_3$ for the roots of the both rootstocks were not significantly different (Table 3-1 and Fig.3-2 B). The reflection coefficients of the solutes applied on the tested rootstocks have been exemplified as some typical graphs in Fig. 3-3 and 3-4.

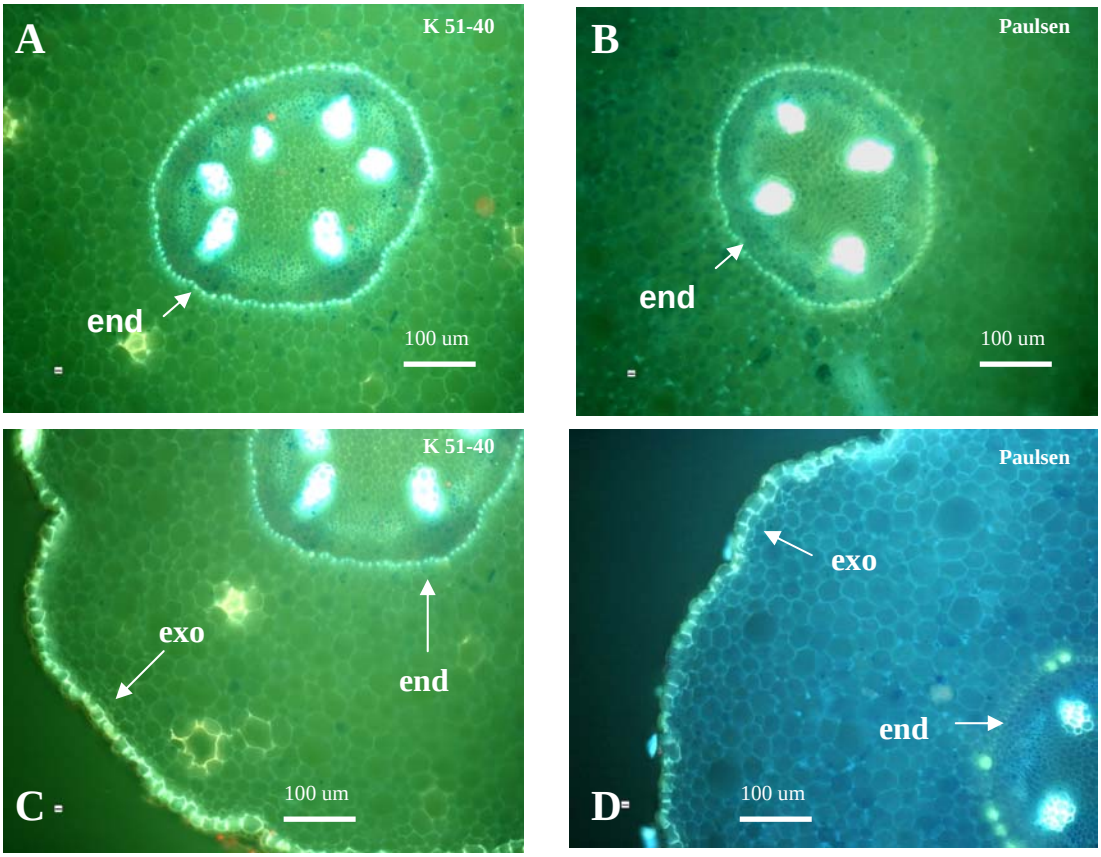

Figure 3- 5 Photomicrographs of K 51-40 (A and C) and Paulsen (B and D) main roots. Aand C, cross section of a K 51-40 root 20-50 mm behind the root tip. B and D, cross section of a Paulsen root 20-50 mm behind the root tip. Casparian bands in endodermis (end) and exodermis (exo) were stained by berberine hemisulfate (0.1% w/v) and counterstained with aniline blue (0.5% w/v).

3-3-2 Root anatomy

Photographs, taken from cross sections of main roots at the distance of 20-50 mm behind the root tip, showed that there were no obvious differences in endodermis and exodermis between the two rootstocks (Fig. 3-5). At the distance of 20-50 mm, endodermis and exodermis with Casparian bands in radial walls were developed around vascular tissue and under the epidermis (hypodermis) respectively. The role of these layers in water transport will be discussed in relation to the root Lp_r and solute σ_s next.

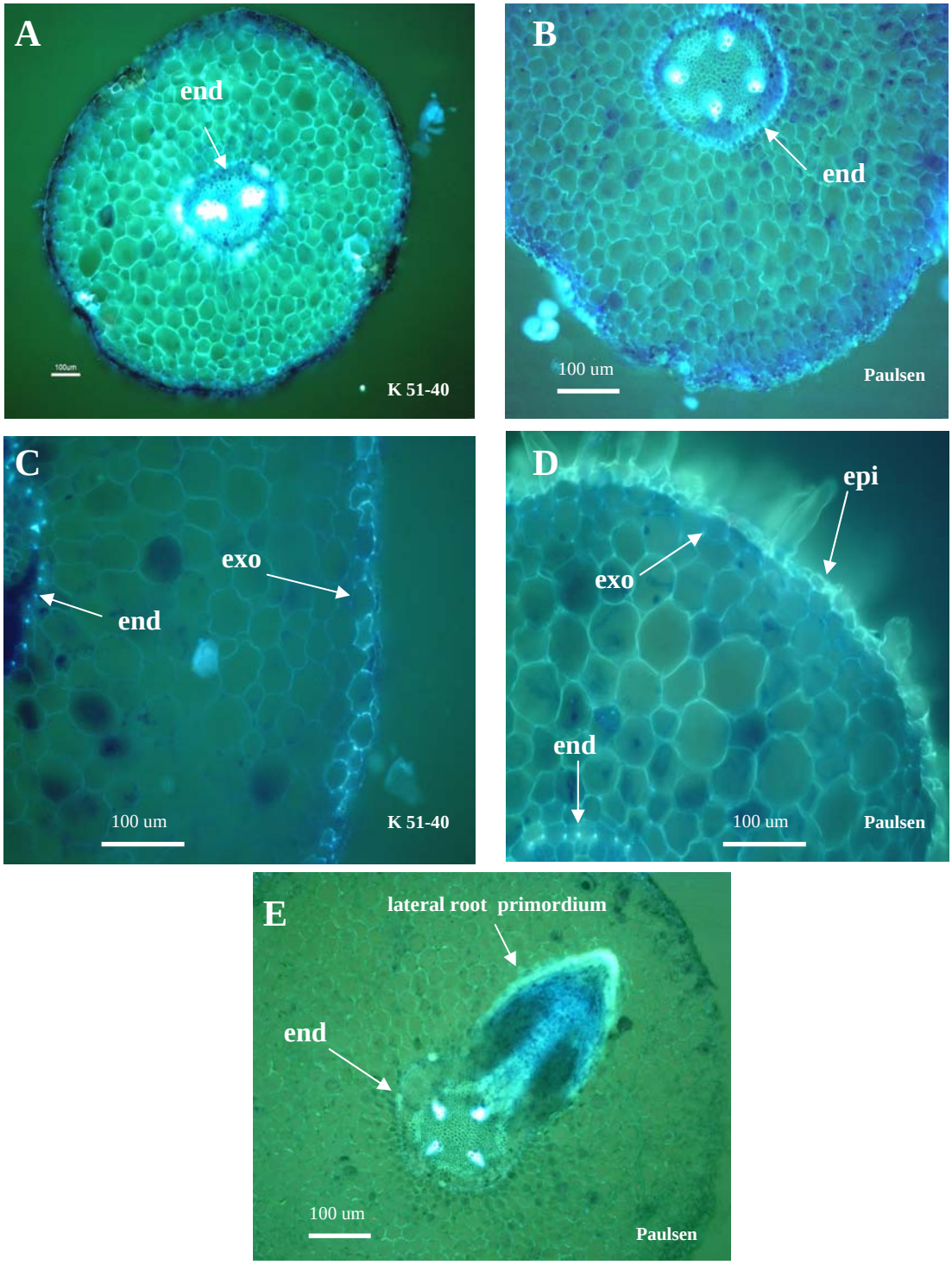

Figure 3- 6 Photomicrographs of K 51-40 (A and C) and Paulsen (B and D) lateral roots. The cross sections of the roots were taken at a distance of 5-20 mm from the root tip. Casparian bands in radial wall of endodermis (end) and immature exodermis (exo) were stained by berberine hemisulfate (0.1% w/v) and counterstained with aniline blue (0.5% w/v). Epidermis (epi) is observed in photograph D. (E) Initiation of a lateral root in a main root of Paulsen and disturbance of endodermis.

The lateral root cross sections were taken in a similar procedure with the main roots sections at a distance of 5-20 mm from the root tip. Casparian bands of endodermis are distinguished in radial walls as bright spots (Fig. 3-6).

Unlike endodermis, it seems that Casparian bands of exodermis in both rootstocks were in the first stages of development (immature) and were not as detectable as the Casparian bands in the main roots (Fig. 3-6 C and D in comparison with Fig. 3-5 C and D). Discontinuity in the endodermis Casparian band which is caused by initiation of a lateral root (Fig. 3-6 E), was commonly observed in the main roots. This will be examined later in respect of an apoplastic bypass for water and solute transport.

3-4 Discussion

3-4-1 Lp_r and σ_s

Using the root pressure probe technique, hydrostatic and osmotic Lp_r and σ_s of two grapevine rootstocks, K 51-40 and Paulsen, were measured. The results showed that Lp_r of the two rootstocks was similar when the driving force of water flow was hydrostatic or osmotic. The nature of the driving force affected the Lp_r values in both rootstocks similarly. The hydrostatic Lp_r was significantly greater than the osmotic Lp_r for the rootstocks (Fig. 3-2 A). It has been found that there are up to three orders of magnitude differences between osmotic and hydrostatic water flow (Steudle, 2000). This variable Lp_r of roots has been already reported in many herbaceous and woody plants as summarized by Steudle & Peterson (1998). It has been suggested that a dilution of the xylem contents due to water uptake and consequently a decrease of osmotic driving force (Fiscus, 1975) and an opening of valve like plasmodesmata in the presence of hydrostatic pressure gradients (Passioura, 1988) can be the reasons for the above mentioned variability in Lp_r. In addition, the composite transport model has also been proposed to explain both the variability of root hydraulic properties and the difference between hydrostatic and osmotic Lp_r (Steudle et al. 1993). This model has also been discussed in section 1-5-2 of chapter-1 in detail.

Recently, a new model (pressure propagation along a root) has been developed by Bramley et al. (2007). They carried out a series of pressure clamps by increasing

5 KPa in root pressure at each clamp for 60-120 s. Lp_r was determined as the slope of linear regression between water flow rate and applied pressure. They suggested that variability in the values of hydrostatic and osmotic Lp_r can be minimized by using the pressure clamp method. Because the pressure clamp method measures Lp_r under steady-state conditions compared with transition conditions observed in the pressure relaxation method, it was argued that there will be a uniform pressure change along the root xylem. The Lp_r values obtained for grapevine rootstocks in this experiment showed 2-3 times difference between hydrostatic and osmotic Lp_r. This magnitude of difference could be due to the above mentioned discussion by Bramley et al. (2007).

On the other hand, Knipfer et al. (2007) has argued against the application of pressure clamp technique to measure hydraulic Lp_r. According to this explanation, the pressure clamp forces larger amounts of water across the root in a direction opposite to the transpiration stream and hence causes a concentration polarization or unstirred layer (USL) effect due to considerable accumulation of solutes in front of the endodermis. This in turn, will reduce water flow and show an underestimation of Lp_r. They concluded that measuring initial phase of water flow through the first step of pressure relaxation in root pressure probe experiments could be used to determine the real Lp_r. Currently there is no clear consensus on how to measure hydrostatic Lp_r and osmotic Lp_r may be also underestimated by unstirred layers.

The reflection coefficient (σ_s) values reported in this chapter for K 51-40 (0.51 and 0.48 using NaCl and $NaNO_3$ respectively) and Paulsen (0.59 and 0.51 using NaCl and $NaNO_3$ respectively) were not remarkably different and were less than unity, thus it could be consistent with the composite model. Furthermore, low reflection coefficient indicates apoplastic by-passes in the absence of Casparian bands for example in endodermis disrupted by lateral root initiation and before differentiation of the endodermis in young roots or water movement through Casparian bands (Steudle & Peterson, 1998). The nearly similar reflection coefficients (σ_s) for Cl^- and NO_3^- salts used in this measurement showed that by-pass flow of salts to the xylem is the same for both rootstocks and anions.

3-4-2 Root anatomy

The endodermis of all vascular plants and the exodermis of many angiosperms possess a Casparian band that is developed in the radial and tangential wall of their cells (Karahara et al. 2004). The Casparian band is made of suberin and /or lignin that is deposited in the cell wall (Zeier & Schreiber, 1998).

Water permeability in a root is mostly related to changes in suberisation of roots that increases with age and stressful environments (Steudle, 2000). Since suberisation in woody roots is greater than herbaceous plants, the Lp_r of trees is often lower than that of herbs (Steudle & Frensch, 1996). Miyamoto et al. (2001) found identical values of Lp_r for two rice varieties (IR64 and Azucena) and suggested that it can be due to the same root anatomy.

Photomicrographs taken from freehand cross-sections showed that the root anatomy did not obviously differ for K 51-40 and Paulsen (Fig. 3-5 and 3-6). No obvious differences were observed in apoplastic barriers (endo- and exodermis) of the main and lateral roots between the two rootstocks. Thus the similar Lp_r and the reflection coefficient (σ_s) of the rootstocks could be related to the similar root anatomy.

In conclusion, the results obtained from measurements with the root pressure probe showed that although there was a difference in hydrostatic and osmotic Lp_r for each rootstock, the Lp_r (hydrostatic or osmotic) of both rootstocks was similar. The roots also had a similar σ_s for Cl^- and NO_3^- salts indicating that perhaps bypass flow of salts to the xylem is similar for both rootstocks. According to the anatomical study, there was no noticeable difference in apoplastic barriers between the two rootstocks. This could be the reason for the same Lp_r for the rootstocks. These results taken together with the results from NO_3^- and Cl^- interaction experiments (Chapter 2), point to differences in membrane transport properties between the rootstocks. However, there is still a question about the role of water channels in root hydraulic properties. Water transport at the cellular level is highly affected by water channel activity (Steudle, 2000). Hence, it is suggested that more studies be carried out using the cell pressure probe technique to measure the hydraulic conductivity at the root cell level to compare with the overall hydraulic properties of the root.

Chapter 4

Apoplastic Flux (Fluorescent Dye Approach)

4-1 Introduction

Radial water and solute flow across a root includes a combination of three different pathways: apoplastic, symplastic and trans-cellular (Steudle, 2000). Experimentally, separation between the symplastic and vacuolar pathways is not currently possible, although the contribution of the cell to cell pathway (symplastic + vacuolar) to water uptake can be measured by using the cell pressure probe (Steudle, 1993). The apoplastic pathway includes water and solute movement around the protoplasts via cell walls, intercellular spaces and the lumen of xylem vessels (Steudle & Peterson, 1998). In the root, Casparian bands that consist of deposits of suberin or lignin in the radial and tangential walls of the endodermis, obstruct apoplastic transport of water and solutes (Ranathunge et al. 2004).

Comparison between the hydrostatic Lp_r and that of root cells using the pressure probe, shows that bypass flow of water and solutes occurs via the apoplastic route lacking Casparian bands or even partially across the endodermis (Radin & Matthews, 1989; Birner & Steudle, 1993). Furthermore, measurement of solute uptake into roots has revealed apoplastic bypass flow using an apoplastic tracer PTS (3-hydroxy-5,8,10- pyrenetrisulphonic acid) (Flowers & Yeo 1995).

PTS is a water soluble, membrane impermeant fluorescent dye that has been used to study apoplastic continuity in regions of the root where endodermis either has not formed or has been interrupted due to lateral root initiation (Yeo et al. 1987). It should be mentioned that PTS entry into vacuoles may change with age of the root, the concentration of PTS, and time of incubation in PTS (Cholewa & Peterson 2001).

The importance of the apoplastic bypass flow in ion transport is mainly obvious at high external concentrations of solutes (Pitman, 1982) and during high transpirational rates (Sanderson, 1983). Using PTS, Yeo et al. (1987) and Garcia et al. (1997) showed the importance of the transpirational bypass flow for Na^+ uptake by rice roots. Moon et al. (1986) used PTS to show the contribution of the apoplastic route to Na^+ and Cl^- uptake by the mangrove (*Avicennia marina*) and concluded that bypass flow is of minimal importance in this species even at high external concentrations.

In this chapter the contribution of the transpirational bypass flow to Cl^- uptake under high external NaCl concentrations was assessed in two grapevine rootstocks, K 51-40 and Paulsen. The fluorescent dye was applied to the root systems in the presence of high external concentrations of NaCl.

4-2 Materials and Methods

4-2-1 Plant material

The cuttings of K 51-40 and 1103 Paulsen were provided as described in section 2-2-1. Rooted cuttings were then transferred to an aerated culture solution in plastic containers, containing ¼ strength Hoagland solution (composition as described in section 2-2-2). After 1-2 weeks plants with 3-4 leaves were transferred to black plastic pots containing different solutions depending on experimental design.

4-2-2 Experimental design

Three different treatments with 4 replications were considered in this experiment:
1) control plants grown in ¼ strength Hoagland solution without NaCl and PTS; 2) plants that were grown in ¼ strength Hoagland solution with NaCl (30 mM) without PTS; 3) plants that were grown in ¼ strength Hoagland solution with NaCl (30 mM) + PTS (0.0125 % w/v). All pots were aerated and carefully sealed using plastic bags to prevent evaporation from the lids as shown in Fig. 4-1. Plants were grown in the above mentioned media for 4 days in glasshouse conditions. The pots were weighed before and after the four days incubation of the plants in the solutions to estimate total transpiration.

4-2-3 Determination of Cl and PTS

Chloride contents in different parts of the plants were extracted and measured as mentioned in section 2-2-3, Chapter 2.

PTS concentrations in root, stem, cane and leaves were measured using a fluorescence spectrometer (excitation wavelength: 405 nm; emission wavelength: 510 nm). PTS extraction was carried out using the chloride extraction procedure mentioned in section 2-2-3, Chapter 2.

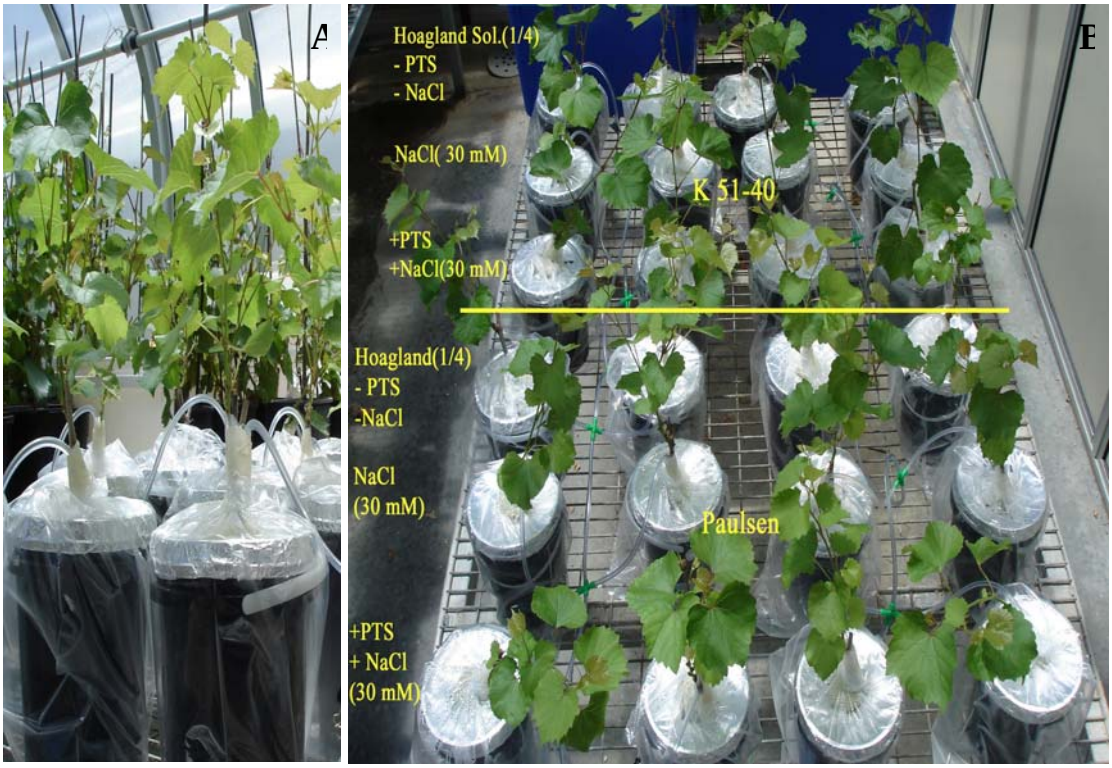

Figure 4- 1 Well aerated hydroponic culture of K 51-40 and Paulsen in black plastic pots sealed with plastic bags to prevent evaporation from the lids (A) Experimental design (B) conducted for each rootstock as three different treatments: control (without NaCl and PTS), NaCl (30 mM) (without PTS) and NaCl (30 mM) + PTS (0.0125 % w /v), (n=4).

4-3 Results

4-3-1 Chloride

Similar to the results obtained for Cl^- in Chapter 2, shoot Cl^- content of K 51- 40 was higher than that of the root in all treatments (Fig. 4-2 B). Unlike K 51-40, shoot Cl^- contents of Paulsen showed no significant difference with root in NaCl treatments (Fig. 4-2 A). As expected, Cl^- concentrations in shoot and root of Paulsen and K 51-

40 in the treatments containing NaCl were higher than that of the control plants (Fig. 4-2 A and B).

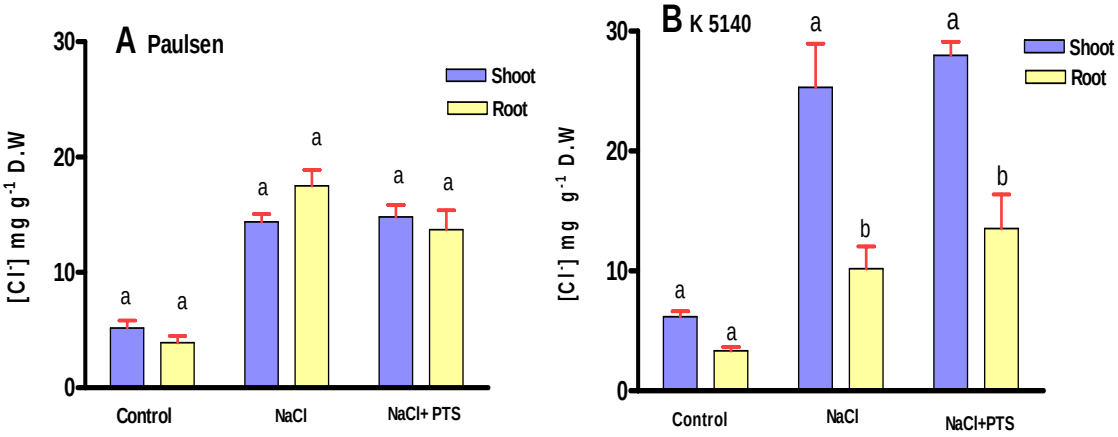

Figure 4- 2 Shoot and root chloride contents of Paulsen (A) and K 51-40 (B). Plants were grown under different treatments: Control, NaCl (30 mM) and NaCl (30 mM) + PTS (0.0125 % w / v). Bars are SE of the means (n=4) (two way ANOVA, P< 0.05). Different letters indicate significant difference between shoot and root in each treatment (t-test).

Comparing K 51-40 and Paulsen, it was shown previously that shoot Cl^- concentration of K 51-40 was higher than that of Paulsen. However, K 51-40 and Paulsen root Cl^- content was similar in the third treatment (NaCl+PTS) and was high for Paulsen in the second treatment (NaCl) (Fig. 4-2 A and B).

4-3-2 PTS

Determination of the fluorescent dye (PTS) contents showed that there was no significant difference between root and shoot PTS contents of K 51-40 and Paulsen. Both rootstocks also had no difference in accumulation of PTS in their root and shoot (Fig.4-3 A)

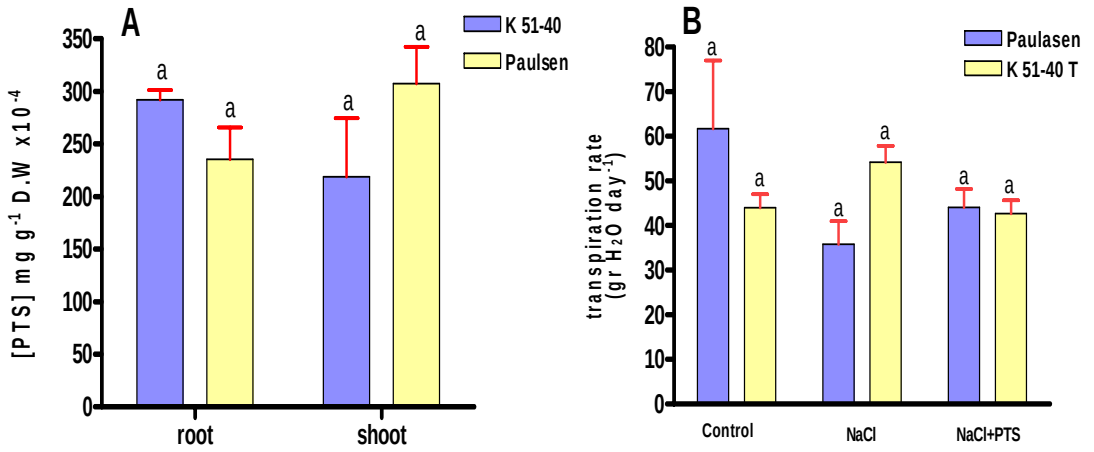

Figure 4- 3 The apoplastic tracer (PTS) concentrations in shoot and root (A) and transpiration rate (B) of K 51-40 and Paulsen. Plants were grown in hydroponic culture contain NaCl (30 mM) and PTS (0.0125 % w / v). Bars are SE of the means (n=4) (two way ANOVA, P<0.05). Different letters indicate significant difference between the varieties in each treatment (t-test).

Total transpiration rate measured under the glasshouse conditions demonstrated that both rootstocks had similar rates of transpiration particularly in the third treatment (NaCl+PTS).

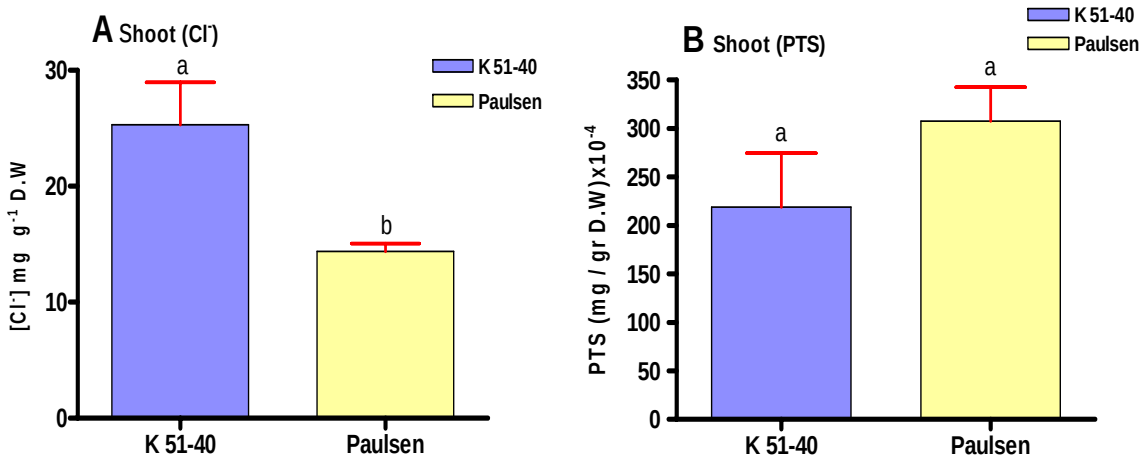

Figure 4- 4 Chloride (A) and PTS (B) accumulation in the shoot of K 51-40 and Paulsen. Plants were grown in hydroponic culture contain NaCl (30 mM) and PTS (0.0125 % w / v). Bars are SE of the means (n=4) (t-test, P < 0.05). Different letters indicate significant difference between the varieties.

Shoot and root PTS and Cl^- concentrations were compared in both rootstocks. The results showed that there was no positive correlation between Cl^- and PTS transport to the shoot. Cl^- accumulation in the shoot of K 51-40 was higher than that of Paulsen

(Fig. 4-4 A) whereas PTS content in the shoot of K 51-40 tended to be lower than that of Paulsen (Fig.4-4 B).

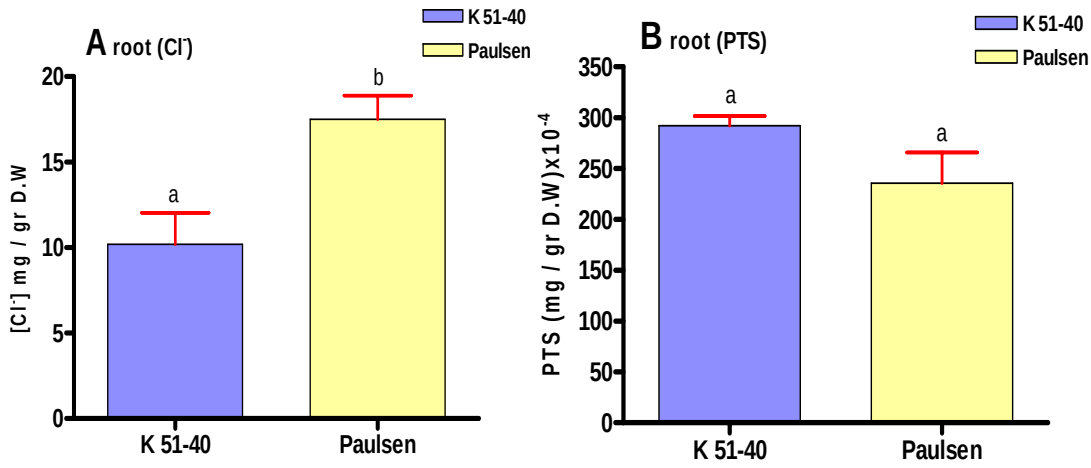

Figure 4- 5 Chloride (A) and PTS (B) accumulation in the root of K 51-40 and Paulsen. Plants were grown in hydroponic culture contain NaCl (30 mM) and PTS (0.0125 % w / v). Bars are SE of the means (n=4) (t-test, $P < 0.05$). Different letters indicate significant difference between the varieties.

Similar to the shoot, no positive correlation was observed between Cl^- and PTS accumulation in the root. The results demonstrated that Cl^- content in the root of K 51-40 was lower than that of Paulsen (Fig. 4-5 A) while PTS concentration had no significant difference between the two rootstocks (Fig. 4-5 B).

In order to estimate the fraction of Cl^- and PTS transport to the shoot via an apoplastic pathway, the transpirational bypass flow as a percentage of the total water flow over the experimental period and Cl^- concentration in the xylem were calculated using the procedure described by Yeo et al. (1987) and Garcia et al. (1997). Briefly, the percentage contribution of bypass flow to water transport was estimated by dividing the fluorescent (PTS) contents in the plant shoot by the volume of water transpired. It gave the apparent concentration of PTS in the transpiration stream or $PTS_{(xyl)}$. Dividing $PTS_{(xyl)}$ by the external concentration of $PTS_{(ext)}$ gives the leakage of PTS to the xylem as a percentage. Then, an empirical correction factor was applied (7.75; obtained for rice by Yeo et al. 1987) to give bypass flow of water expressed as a percentage (Table 4-1). PTS is a larger molecule (diameter of 1.1 nm) in comparison to water molecule (diameter of 0.39 nm). PTS penetrates through the cell wall

microfibrillar spaces slower than water molecules. So, the correction factor is applied to minimize underestimation of bypass flow of water.

Table 4- 1 PTS transport by K 51-40 and Paulsen. The shoot PTS contents [$PTS_{(Sh)}$] for the rootstocks were divided by the volume of water transpired (J_t) by the plants to give the apparent concentration of PTS in the xylem [$PTS_{(xyl)}$]. The leakage of PTS [$PTS_{(xyl)}$ / $PTS_{(ext)}$] was given by dividing $PTS_{(xyl)}$ by the external PTS concentration [$PTS_{(ext)}$]. An empirical correction factor (7.57; see discussion for details) was applied to the PTS leakage values to estimate the water bypass flow (J_{BP}) as a percentage of total flow (n=4) (t-test, $P < 0.05$). No significant difference observed between the varieties.

Rootstock	J_t (ml)	$PTS_{(Sh)}$ (mg/g. DW)	$PTS_{(xyl)}$ (mg/ml)	$PTS_{(ext)}$ (mg/ml)	$PTS_{(xyl)}/PTS_{(ext)}$ (%)	J_{BF} (%)
K 51-40	[a] 170.75 ± 12.14	[a] 0.28 ± 0.06	[a] 0.0017 ± 0.0005	0.125	[a] 1.34 ± 0.4	[a] 10.1 ± 3.03
Paulsen	[a] 176.5 ± 16.45	[a] 0.34 ± 0.004	[a] 0.002 ± 0.0002	0.125	[a] 1.6 ± 0.16	[a] 12.1 ± 1.21

Furthermore, the apparent concentration of Cl^- in the xylem [$Cl_{(xyl)}$] was calculated by dividing Cl^- concentration of the shoot by the volume transpired. The result then divided by the external concentration of Cl^- [$Cl_{(ext)}$] to give the apparent net concentration of Cl^- in the xylem which is expressed as a percentage (Table 4-2).

The results shown in Table 4-1 and Table 4-2 revealed that the values of the PTS leakage and the percentage of water bypass flow were not significantly different between the rootstocks (Table 4-1). In contrast, the apparent net concentration of Cl^- in the xylem was two times bigger in K 51-40 than that of Paulsen (Table 4-2).

Table 4- 2 Cl^- transport by K 51-40 and Paulsen. The shoot Cl^- concentrations [$Cl_{(Sh)}$] for the rootstocks were divided by the volume of water transpired (J_t) by the plants to give the apparent concentration of Cl^- in the xylem [$Cl_{(xyl)}$]. The apparent net concentration of Cl^- in the xylem [$Cl_{(xyl)}$ / $Cl_{(ext)}$] was given dividing [$Cl_{(xyl)}$] by the external Cl^- concentration [$Cl_{(ext)}$]. It is expressed as a percentage of total Cl^- transport (n=4) (t-test, $P < 0.05$).

Rootstock	J_t (ml)	$Cl_{(Sh)}$ (mM)	$Cl_{(xyl)}$ (mM/ml)	$Cl_{(ext)}$ (mM)	$Cl_{(xyl)}$ / $Cl_{(ext)}$ (%)
K 51-40	[a]170.75 ± 12.14	[a] 7.88 ± 0.32	[a] 0.047 ± 0.002	30	[a] 0.16 ± 0.007
Paulsen	[a]176.5 ± 16.45	[b] 4.18 ± 0.24	[b] 0.025 ± 0.003	30	[b] 0.08 ± 0.01

4-4 Discussion

There have been some studies in which the contribution of transpirational bypass flow to water and solute uptake has been assessed (Moon et al. 1986; Yeo et al. 1987; Kamaludin & Zwiazek 2001; Garcia et al. 1997; Hanson et al. 1985). There are variable results reported about the relative extent of apoplastic bypass flow of water and ions. Hanson et al. (1985) and Moon et al. (1986) demonstrated that the apoplastic pathway in *Pinus resinosa* roots contributes less than 1% of total uptake. In contrast, using La^{3+} uptake, Lawton et al. (1981) showed that the apoplastic route in *Avicennia marina* was significant. However, they did not present any estimation in terms of symplastic or total ion uptake. Gracia et al. (1997) estimated the importance of the transpirational bypass flow in rice and wheat. They concluded that the transpirational bypass flow in rice is 10 times greater than that of wheat and found a high correlation between Na^+ uptake and the bypass flow magnitude in rice but not in wheat.

The results reported in this chapter showed that this variability does not exist between K 51-40 and Paulsen. PTS contents of the shoot and the root in both rootstocks were similar (Fig. 4-3 A) and the results obtained for the leakage of PTS [$PTS_{(xyl)}$ / $PTS_{(ext)}$] and the bypass flow of water (J_{BF}) shown in Table 4-1, were also not significantly different in the two rootstocks. The leakage of PTS and J_{Bf} for K 51-40 were 1.34 % and 10.1 % and for Paulsen were 1.6 % and 12.1 % respectively. It can be compared to the values obtained for rice by Garcia et al. (1997) which were 0.722 % and 5.74 % for the leakage of PTS and J_{BF} respectively. The large difference between the leakage of PTS and the bypass flow of water (J_{BF}) in grapevine (at least in K 51-40 and Paulsen) can possibly be due to smaller spaces distributed between cell wall microfibrills whose diameters are less than the diameter of PTS molecule (1.1 nm; Moon et al. 1986). Thus water molecules with diameter of 0.39, Na^+ with 0.6 and Cl^- with 0.5 nm (Lauchli, 1979) will freely move through the spaces. This was also the reason that Yeo et al. (1987) used the empirical correction factor (7.57; see results) in calculations to correct underestimation of bypass flow of water.

PTS is a highly fluorescent dye that can move through unmodified cell walls, but it is blocked by walls with Casparian bands (Vesk et al. 2000). Similar values of PTS transport obtained for K 51-40 and Paulsen (Fig. 4-3 A; Table 4-1) was also

consistent with the findings in Chapter 3. Anatomical studies reported in Chapter 3 showed that there were no obvious differences in apoplastic barriers (endodermis and exodermis) between the two rootstocks.

Although there is evidence that shows a strong correlation between PTS transport and ion transport, for example for Na^+ transport in rice (J_{BF} =5.47 % and the leakage of Na^+ = 5.65 %) (Garcia et al. 1997; Yeo et al. 1987; Gong et al. 2006), no correlation was observed in the grapevine rootstocks (K 51-40 and Paulsen) between PTS and Cl^- transport to the shoot (J_{BF} = 10.1 % and 12.1 % and the apparent net concentration of Cl^- = 0.16 % and 0.08 % for K 51-40 and Paulsen respectively) (Fig. 4-4 and Fig. 4-5). The results shown in Table 4-2 indicated that the apparent net concentration of Cl^- in the xylem of K 51-40 is two times greater than that of Paulsen. In fact the higher apparent net concentration of Cl^- in the xylem [$Cl^-_{(xyl)}$] for K 51-40 (Tables 4-1 and 4-2) can be attributed to higher Cl^- loading into the xylem in K 51-40 when compared to Paulsen. It was consistent with the findings reported in Chapters 2 and 4 that showed that in comparison to Paulsen more Cl^- was accumulated in the shoot of K 51-40 (Fig. 1-2 A Chapter 1; Fig. 4-1 B Chapter 4).

In conclusion, the results showed that there was an apoplastic bypass flow for PTS and it was similar for both rootstocks. There was not a correlation between PTS and Cl^- transport. Although, there is a bypass flow for Cl^- transport, it does not account for differences observed between the rootstocks.

Chapter 5

$^{36}Cl^-$ compartmentation and flux characteristics in grapevine

5-1 Introduction

Unlike Na^+, the mechanisms of Cl^- transport to the root and its uptake to the shoot and / or efflux under salt stress are unclear (White & Broadley 2001). Some anion channels that are permeable to Cl^- have been characterized in the root cell plasma membrane of different plant species using the patch-clamp technique. For example, a depolarization-activated outward rectifying anion channel (anion influx) has been detected in wheat (Skerrett & Tyerman 1994), maize (Pineros & Kochian, 2001) and white lupin (Zhang et al. 2004a). A voltage-independent inward rectifying anion channel (X-IRAC) has been found in barley root by Kohler & Raschke (2000). Gilliham & Tester (2005) showed that an hyperpolarization-activated inward rectifying anion channel (anion efflux) in the xylem parenchyma of maize root, was equally permeable to Cl^- and NO_3^-.

Little is known about the molecular structure of plant Cl^- channels. Lurin et al. (1996) isolated ClC-Nt1 from tobacco as a new member of the ClC gene family that is expressed in a broad range of tissue types. It was expressed in Xenopus oocytes and proposed as a voltage-dependent Cl^- channel in plant membranes. Hechenbereger et al. (1996) cloned four genes (AtClC-a, AtClC-b, AtClC-c and AtClC-d) in to the ClC family of Cl^- channels from *Arabidopsis thaliana* cell memmbranes. AtCLC-b is expressed in root while AtCLC-a and c are expressed in leaf. AtCLC-d is mainly expressed in the fruit.

Despite a variety of hypotheses, anion transport parameters such as pH, membrane potential and concentration gradients of the anion are used by almost all investigators

to explain anion uptake by plants (Babourina et al. 1998a). At high external Cl^- concentrations under saline conditions and depending on membrane potential, passive transport of Cl^- could take place through an outward rectifying anion channel permeable to NO_3^- and Cl^- (Skerrett & Tyerman 1994). In contrast, Cl^- transport in low concentrations and with hyperpolarized membrane potential is an active process that is accomplished by a ΔpH-driven Cl^- / nH^+ symport (Felle, 1994; Yamashita et al. 1997; Babourina et al. 1998a).

Estimation of ion fluxes can be useful in understanding regulation of the ion transport into the plant root. It has been done in different plants by using various experimental procedures. For example, Lorenzen et al. (2004) demonstrated passive Cl^- influx under salt stress using the recombinant fluorescent probe (anion indicator) CLOMELEON. They showed that Mg^{2+} and Ca^{2+} inhibited Cl^- influx to *Arabidopsis thaliana* root while La^{3+} completely blocked it. This was consistent with the findings of Skerrett & Tyerman (1994).

Much information about Cl^- transport mechanisms has been obtained by using a well known approach, the radioisotope $^{36}Cl^-$ as tracer. In this procedure, the unidirectional Cl^- fluxes can be measured after absorbing tracer ($^{36}Cl^-$) by tissue. Using $^{36}Cl^-$, Cram & Laties (1971) studied Cl^- influx and efflux in barley root segments and showed saturation kinetics for Cl^- uptake to the vacuole at external Cl^- concentrations higher than 10 mM, while the plasmalemma influx increased linearly. Pitman, (1971) and (1972) suggested a two-pump model for Cl^- transport from the external medium to the xylem.

Using $^{42}K^+$, $^{22}Na^+$ and $^{36}Cl^-$, Macklon, (1975) suggested that all three ions were actively transported from outside into the cortical cells of *Allium cepa*, but only Na^+ was actively accumulated in the vacuole.

Britto et al. (2004) provided compartmental analysis of Cl^- transport in intact barley root system for the first time by using $^{36}Cl^-$. They showed that by increasing the external concentrations of Cl^- to salinity levels, cytoplasmic content of Cl^- increased. Also, Cl^- flux to the xylem showed saturation kinetics probably indicating an active transport system for xylem loading.

"Ion fluxes" can refer to different components that are defined as follows (White & Broadley 2001):

Influx is defined as the movement of an ion into a cell. In the case of Cl^-, influx is determined by measuring $^{36}Cl^-$ accumulation in plant tissues after a short period of time.

Efflux is defined as the transport of an ion from inside a cell to the outside (external medium or xylem) or from the vacuole to the cytoplasm and is measured by loading the radiotracer (e.g. $^{36}Cl^-$) into the plant tissues in a given time and estimation of radioisotope loss from the tissues in non-radiolabled solutions over different time periods (White & Broadley 2001). In most studies *net ion uptake* is measured to describe the ion uptake kinetics, but net tracer uptake to the vacuole is more complicated as is net tracer efflux from vacuole since it depends on fluxes across two membranes in parallel. Cl^- uptake process is the result of various transport systems. Under non-saline condition Cl^- influx across the plasma membrane is active via nH^+ / Cl^- symport (Felle, 1994). Cl^- efflux across the plasma membrane and fluxes across tonoplast are probably passive through Cl^- channels. It is also loaded into the xylem down an electrochemical gradient. When the external concentration of Cl^- is high (i.e. due to high salinity) a passive transport system may dominant influx (White & Broadley 2001).

In this study, the mechanisms of Cl^- import, retranslocation and/or extrusion in grapevine root and shoot under saline conditions were studied. $^{36}Cl^-$ influx and efflux were measured respectively in grapevine root segments and whole plants.

5-2 Materials and methods

5-2-1 Plant material

The cuttings of K 51-40 and 1103 Paulsen were provided as described in section 2-2-1, Chapter 2. In addition to stem cuttings provided as explained in section 2-2-1, leaf cuttings were also made from grapevines as described by Schachtman & Thomas (2003). The leaves with lignified petioles were selected, excised and immersed in

0.1% indol butyric acid (IBA) in a 50% ethanol solution for 30-60 s. They were then placed in a tray containing a moist medium of 50% vermiculite and 50% perlite. The lamina was located above the surface of the tray. The tray was put on a heat bed and covered with a clear dome to maintain high humidity around the shoot. The cuttings were watered with ¼ strength Hoagland solution. After 2 weeks callus and root initiations began to appear (Fig. 5-1 B).

Rooted cuttings (stem and leaf) were then transferred to an aerated culture solution in plastic containers, containing ¼ strength Hoagland solution (composition as mentioned in section 2-2-2). Plants were pretreated with NaCl (20 and 30 mM depending on the experiment`s conditions) for 5-7 days before used in flux experiments.

5-2-2 Measurement of $^{36}Cl^-$ fluxes

5-2-2-1 Experiment 1 (Initial influx): For measuring net uptake of $^{36}Cl^-$ in K 51-40 and Paulsen, root segments (8-12 cm and including lateral roots and the tip) of plants pretreated for 5-7 days in 30 mM NaCl + ¼ Hoagland solution, were soaked in aerated loading solutions labelled with $^{36}Cl^-$ (9.25 MBq, 250 µCi, Amersham Biosciences) for different periods (2-120 min with 4 root segments of each rootstock for each time point). After the loading period, the root segments were transferred to an aerated ice-cold non-labelled solution to wash the root surface and the apoplastic $^{36}Cl^-$ contents for 5 min. In the next step, the root segments were taken out, blotted and weighed and put in 5 ml plastic scintillation vials containing 2 ml SDS (10% w/v) and boiled for 20-30 min in a water bath. After cooling the vials, 3 ml scintillation liquid was added to the vials. Each vial was counted by a liquid scintillation counter (TRI-CARB 2100TR, PACKARD). Specific activity of $^{36}Cl^-$ in the external solution (S_o) which is defined as counts per minute (cpm) per µM of Cl^- in the external medium, was the same for all experiments. Influx was calculated as tissue content (counts per gram fresh weight of tissue / counts per µmole of chloride in the external solution) divided by the loading time and plotted with Graphpad Prism.

5-2-2-2 Experiment 2 (short period influx): This experiment was conducted to estimate $^{36}Cl^-$ influx in the first 20 min (short period) of loading time. All steps were

similar to experiment 1 as described above except the rinsing process. In this experiment, after loading the root segments in the labelled solution they were transferred to aerated ice-cold non-labelled solutions in series for 1 and 5 min. After 1 min washing, the root segments were well blotted and then put in the second washing solution for 5 min.

5-2-2-3 Experiment 3 (Concentration Kinetics of $^{36}Cl^-$ influx): Similar to the previous experiment, root segments were pretreated for 5-7 days with NaCl (30 mM) and then were placed for 10 min in the required concentrations of NaCl (1, 3, 5, 10, 15, 20, 30 mM) labelled with $^{36}Cl^-$ and similar specific activity determined exactly for each concentration. After the rinsing period (1 and 5 min in an ice-cold non-labelled solutions) the root segments of each concentration was lifted up, blotted, weighed (FW), placed in the vials and counted. $^{36}Cl^-$ content was calculated as in the first experiment.

5-2-2-4. Experiment 4 ($^{36}Cl^-$ uptake by main and lateral roots): In this experiment, $^{36}Cl^-$ uptake by main and lateral roots were separately estimated. Plants were pretreated in NaCl (5 days in 30 mM and the last 24 h in 20 mM). According to the results obtained in experiment 3, influx rate was nearly the same for 20 and 30 mM external concentration of NaCl therefore to prevent excessive build up of salt in the plants over a reasonable acclimation period, the 20 mM treatment was selected.The root segments were placed in the loading solution for 10 min and were then washed, blotted, weighed and counted the same as in previous experiments. $^{36}Cl^-$ content was calculated and plotted as in experiment 1.

5-2-2-5 Experiment 5 (uptake of $^{36}Cl^-$ to the shoot): Rooted leaves were used to measure $^{36}Cl^-$ uptake to leaves instead of whole plants. The plants were pretreated with NaCl as described for Experiment 4 and were loaded in a solution labelled with $^{36}Cl^-$ for various times (1, 3, 6 h). After the loading periods, root, lamina and petiole were separated, weighed, placed in the vials and counted by liquid scintillation counting. Roots were washed for 1 and 5 min in aerated ice-cold non-labelled solutions in series before putting them in the vials. $^{36}Cl^-$ content was measured and plotted the same way as mentioned in previous experiments.

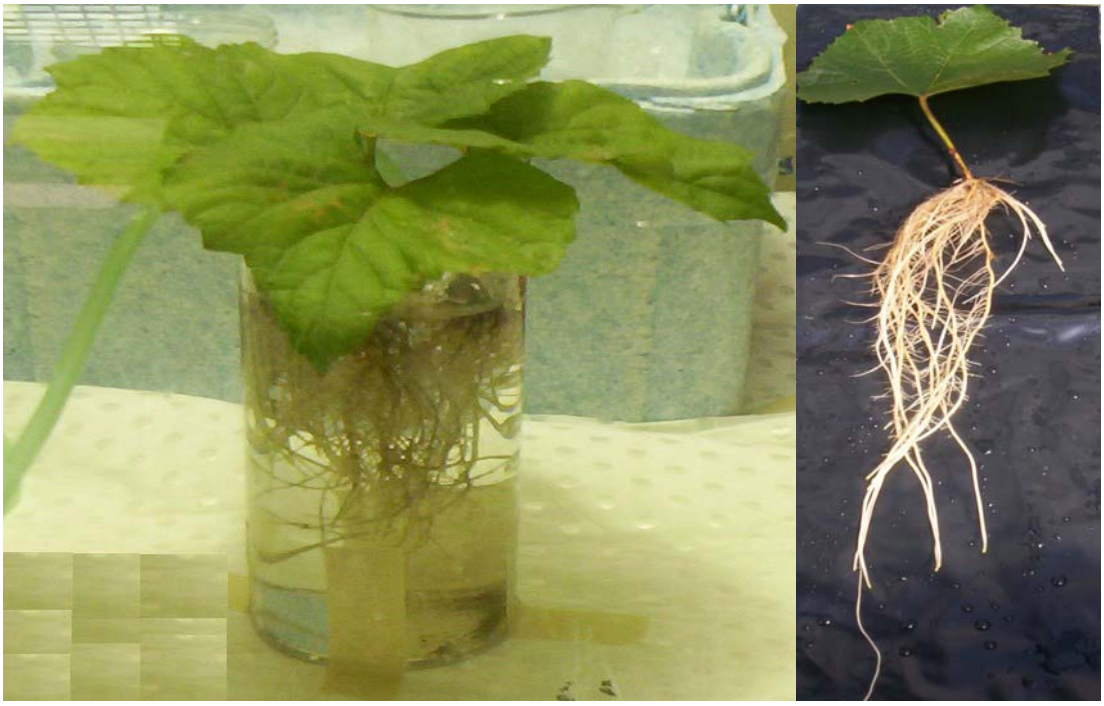

Figure 5- 1 The loading of rooted leaves in a labelled solution (A) and a rooted leaf (B)

5-2-2-6 Experiment 6 (efflux of $^{36}Cl^-$): *$^{36}Cl^-$ efflux from whole root systems*: To measure $^{36}Cl^-$ efflux from the whole root, 6 intact plants (pretreated leaf or stem cuttings same as in experiment 4) were loaded with $^{36}Cl^-$ for 12 h (Fig. 5-1 A) under light and then transferred to aerated successive washing solutions for varying time periods (1-720 min) after 5 min wash in a non-labelled solution. 12 h was quite enough time for ion loading and transport into the plant parts. Within 12 h, cytosolic compartment as well as the vacuole are labeled by $^{36}Cl^-$ and there will be enough time for the isotope to be transported to the shoot (Davenport et al. 2005; Cram & Laties 1971; Britto et al. 2004). The radioactivity in the rinse solutions and that remaining in the root and shoot (if applicable) tissue were counted. Data were plotted as $^{36}Cl^-$ remaining inside the root tissue and ion fluxes into / out of each root compartment (Fig. 5-2) were calculated using compartmental analysis (Walker & Pitman 1976).

$^{36}Cl^-$ efflux from lateral roots: After loading the pretreated intact plants with $^{36}Cl^-$ for 12 h, lateral roots of each plant were separated from the main root and floated in aerated successive washing solutions for varying time periods (1-720 min) after 5 min wash in a non-labelled solution.

NOTE: This table is included on page 79 of the print copy of the thesis held in the University of Adelaide Library.

Figure 5- 2 Components of the fluxes into and out of each root compartment. J is the flux in the directions indicated by the arrows and the subscripts; solution(*O*), cytoplasm (*C*), vacuole (*V*), xylem vessels (*X*). J_n is net flux into the root and J_{nx} is net flux to the vessels. J to the vessels through free spaces (after Pitman 1971).

Because of their small size and in order to handle them easily and quickly, the lateral roots of each plant were put inside a mesh-bottomed holder. The radioactivity counting and data calculation and plotting procedure was the same as for whole roots.

5-2-2-7 Analysis of efflux experiments:

This experiment was carried out for whole roots (intact plants) and excised lateral roots separately. At the end of each efflux experiment the amount of $^{36}Cl^-$ remaining in the root was counted and added to the values obtained for each successive rinse solution. Data were analysed assuming a two compartment model for efflux to the external medium (cytoplasm and vacuole) and were analysed fitting to the sum of two exponentials (Walker & Pitman 1976). The real number of phases was 3 but we assumed that free space component could be neglected because of 5 min wash in a non-labeled solution. There will also be a component to the xylem (Pitman, 1972). The final slower part of the efflux curve is defined as vacuolar efflux (the slowest efflux component) and by extrapolation to zero time, the amount of ^{36}Cl- in the vacuole at the beginning of the efflux can be determined. The efflux from the cytoplasm (the faster-exchanging component) is the first part of the curve and theoretically is defined as subtraction of the vacuolar part from the total tissue contents. It was assumed that the free space component of efflux was negligible because of the 5 min wash in a non-labelled solution before the main

elution process (Macklon, 1975). Parameters obtained from two exponential fitting analysis of the curves were used to calculate $^{36}Cl^-$ fluxes. The amount of radioactivity in the vacuole (the intercept of the slowest efflux component or I_v) and that of cytoplasm (the intercept of the faster efflux component or I_c), the rate constant of the cytoplasmic efflux (K_c) and the rate constant of the vacuolar compartment (K_v) were used to calculate $^{36}Cl^-$ influx from outside to cytoplasm (Φ_{oc}), influx from cytoplasm to the vacuole (Φ_{cv}) , net influx (Φ_{net}), and efflux from cytoplasm to outside (Φ_{co}), and to the xylem vessel (Φ_{cx}) and from the vacuole to cytoplasm (Φ_{vc}) using the equations given in the Appendix of this chapter.

5-3 Results

5-3-1 Experiment 1:

$^{36}Cl^-$ accumulation in the root of Paulsen was higher than that of K 51-40 over the long period of loading (2 h) (Fig. 5-3 A) and influx rate in the two rootstocks decreased remarkably after the first 10 min (Fig. 5-3 B). The cytoplasmic unidirectional $^{36}Cl^-$ influx estimation over the first 10 min loading period showed that in Paulsen $^{36}Cl^-$ uptake (0.12 ± 0.013 µmol g^{-1} F.W. min^{-1} SEM) was more than 2 times greater than that of K 51-40 (0.05 ± 0.005 µmol g^{-1} F.W min^{-1} SEM) (Table 5-1).

5-3-2 Experiment 2:

This experiment was carried out to estimate $^{36}Cl^-$ unidirectional influx to the cytoplasm in a short period of time (20 min). Similar to experiment 1 (long period loading), the results showed that $^{36}Cl^-$ accumulation in Paulsen was higher than K 51-40 over 20 min of loading time (Fig. 5-4 A) and influx rate was decreased after the first 10 min (Fig. 5-4 B). This can be attributed to $^{36}Cl^-$ efflux from the cytoplasm. Influx rate estimation showed that the cytoplasmic unidirectional $^{36}Cl^-$ influx rate in Paulsen (0.059 ± 0.013 µmol g^{-1} F.W min^{-1} SEM) was high when compared with K 51-40 (0.03 ± 0.007 µmol g^{-1} F.W min^{-1} SEM) (Table 5-1).

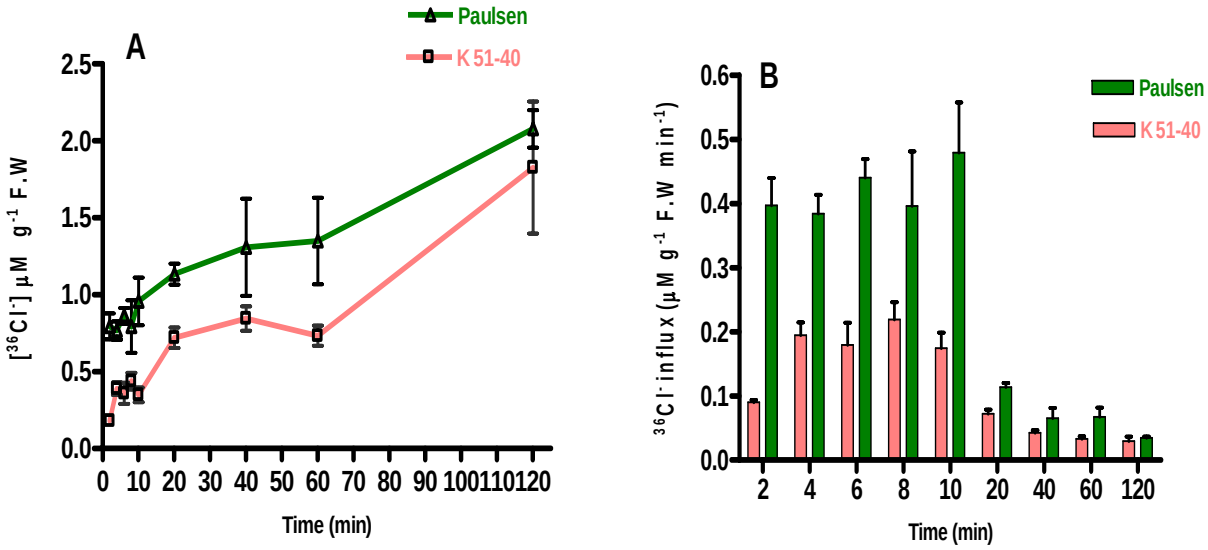

Figure 5- 3 (A) Root $^{36}Cl^-$ net uptake as $^{36}Cl^-$ accumulation in K 51-40 (□) and Paulsen (Δ) root segments. (B) Root $^{36}Cl^-$ influx rate. Whole plants were pretreated with NaCl (30 mM) and root segments (including lateral roots) were cut 8-12 cm from root tip. They were loaded by the labelled solution for 2 h and washed for 5 min. Bars are SE of the means (n = 4).

5-3-3 Experiment 3:

The results obtained from experiments 1 and 2 indicated that the cytoplasmic Cl^- pool becomes fully labelled within 10 min of radioisotope load. Over the first few minutes Cl^- efflux is negligible and the uptake rate indicates unidirectional influx.

Table 5- 1 $^{36}Cl^-$ transport to the cytoplasm estimated as $^{36}Cl^-$ influx rate (slope of linear regressions) to the root segments of K 51-40 and Paulsen over the first 10 min of radioisotope loading period in experiments 1 and 2.

	Cytoplasmic net influx rate (µmol g^{-1} F.W min^{-1})	
Experiment	K 51-40	Paulsen
1	0.05 ± 0.005	0.12 ± 0.013
2	0.03 ± 0.007	0.059 ± 0.013

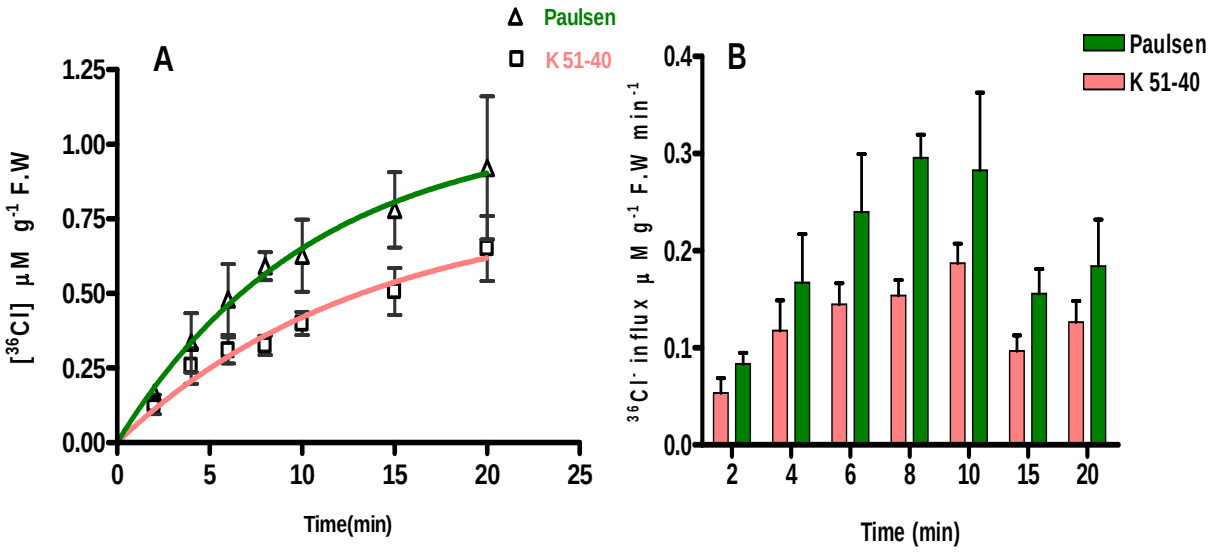

Figure 5- 4 (A) Root $^{36}Cl^-$ influx as $^{36}Cl^-$ accumulation in K 51-40 (□) and Paulsen (Δ) root segments. Data were fitted using one phase exponential association. (B) Root $^{36}Cl^-$ influx rate. Whole plants were pretreated with NaCl (30 mM) and root segments (including lateral roots) were cut 8-12 cm from root tip. They were loaded by the labelled solution for 20 min and washed for 1 and 5 min. Bars are SE of the means (n = 4).

After fully labelling of the cytoplasmic pool, Cl^- is effluxed. To study the effect of external Cl^- concentrations on $^{36}Cl^-$ influx, unidirectional cytoplasmic $^{36}Cl^-$ influx was estimated over 8 min loading time and in a range of external Cl^- concentrations (1, 3, 5, 10, 15, 20, and 30 mM) for plants pretreated in 30 mM NaCl.

The values of influx rate showed that by increasing external Cl^- concentration, influx rate increased in the both rootstocks; however this increase in Paulsen was higher than K 51-40 (Fig. 5-5). V_{max} and K_m values for K 51-40 were 0.059 ± 0.026 (µm g^{-1} F.W. min^{-1}) and 24.27 ± 18.51 (mM) and for Paulsen were 0.127 ± 0.042 (µm g^{-1} F.W. min^{-1}) and 28.68 ± 15.76 (mM) respectively. The large error in K_m is probably because the flux still had not saturated at 30 mM external Cl^-.

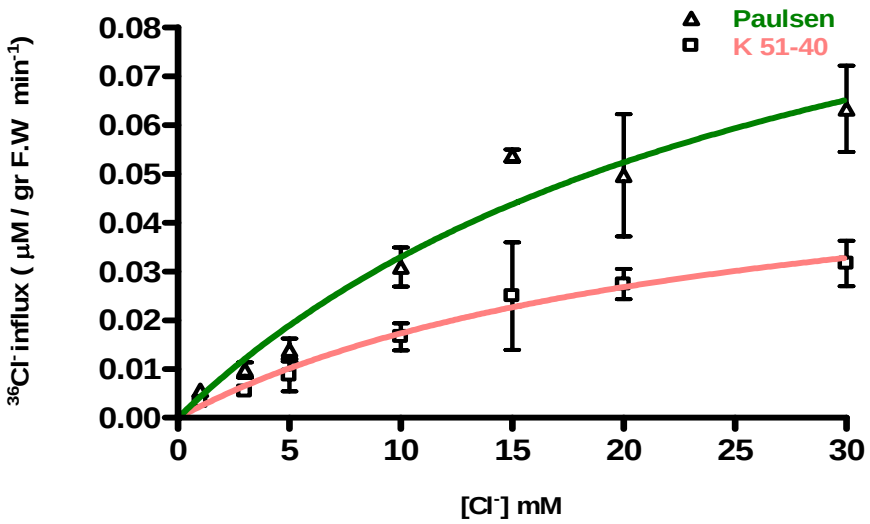

Figure 5- 5 Unidirectional cytoplasmic $^{36}Cl^-$ influx in K 51-40 (□) and Paulsen (Δ) estimated as $^{36}Cl^-$ influx rate over 8 min loading time in the presence of different external Cl^- concentrations. Whole plants were pretreated with NaCl (30 mM) and root segments (including lateral roots) were cut 8-12 cm from the root tip. They were loaded by the labelled solution for 10 min and washed for 1 and 5 min. Data were analysed fitting to Michaelis-Menten. Bars are SE of the means (n = 4). Comparison of fits indicated significant difference between the curves. V_{max} was significantly different between the varieties at 30 mM [Cl^-] but no difference observed for K_m.

5-3-4 Experiment 4:

The previous experiments showed differences between the two rootstocks from the view point of $^{36}Cl^-$ accumulation and influx rate. One of the possibilities for describing this difference could be different physiological capability of lateral roots in their contribution to Cl^- uptake. To test this hypothesis, the cytoplasmic $^{36}Cl^-$ accumulation was measured in the main and lateral roots separately. The results showed that the lateral roots $^{36}Cl^-$ influx is nearly similar in the two rootstocks; however the main root of Paulsen accumulated $^{36}Cl^-$ about three times faster than for K 51-40. $^{36}Cl^-$ accumulation in lateral root was higher than main root in K 51-40, whereas main root of Paulsen significantly accumulated more $^{36}Cl^-$ than lateral roots (Fig. 5-6) during a 10 min load.

5-3-5 Experiment 5:

The rootstocks showed a large difference in $^{36}Cl^-$ uptake to the shoot. As shown in (Fig. 5-7 A), accumulation of $^{36}Cl^-$ to the shoot of K 51-40 and Paulsen increased over

time, however $^{36}Cl^-$ content and influx rate (slope of linear regression) in K 51-40 (0.83 ± 0.33 µmol g^{-1}F.W h^{-1}) was higher in comparison to Paulsen (0.52 ± 0.14 µmol g^{-1}F.W h^{-1}).

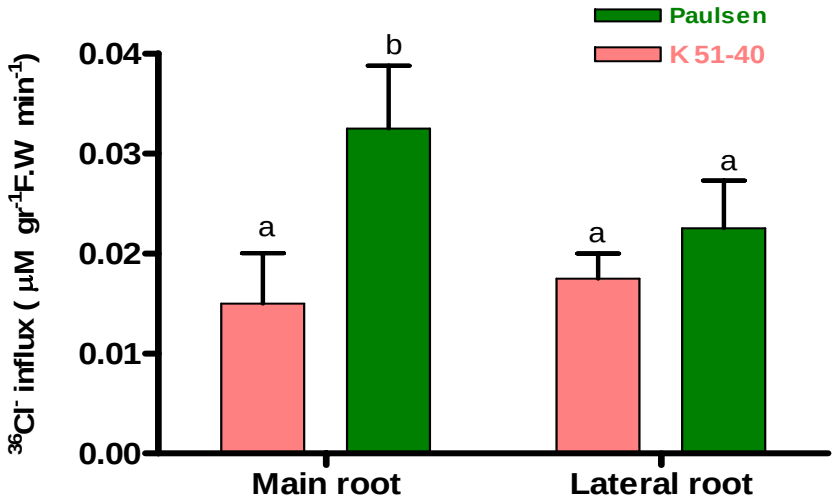

Figure 5- 6 Main and lateral root $^{36}Cl^-$ influx in K 51-40 and Paulsen. Plants were pretreated by NaCl (30 mm for 5 days and 20 mM for 24 h) and root segments (main and lateral roots separately) were loaded by $^{36}Cl^-$ for 10 min. Bars are SE of the means (n = 4) (t-test, $P < 0.05$). Different letters indicate significant difference between the varieties.

The same trend was observed in the values of $^{36}Cl^-$ uptake per root g F.W estimated in the rootstocks (Fig. 5-7 B). Similar results were obtained for petiole and lamina separately (data not shown). In K 51-40, $^{36}Cl^-$ transport to petiole and lamina was higher than that of Paulsen.

5-3-6 Experiment 6:

All the results of the efflux experiments carried out on whole and lateral roots of K 51-40 and Paulsen have been summarized in Table 5-2 and as a diagram (Fig. 5-10). The efflux curves for whole roots of K 51-40 and Paulsen (Fig. 5-8) showed that the initial rate of loss of the tracer (R) (Appendix, Eq.8) in Paulsen (0.99 µmol g^{-1} FW min^{-1}) was about three times higher than that of K 51-40 (0.34 µmol g^{-1} FW min^{-1}). In Paulsen, $^{36}Cl^-$ influx from the external medium to the cytoplasm (Φ_{oc}) (1.04 µmol g^{-1} FW min^{-1}) was much higher than K 51-40 (0.368 µmol g^{-1} FW min^{-1}). Similar results were observed for $^{36}Cl^-$ efflux from cytoplasm to the external medium (Φ_{co}).

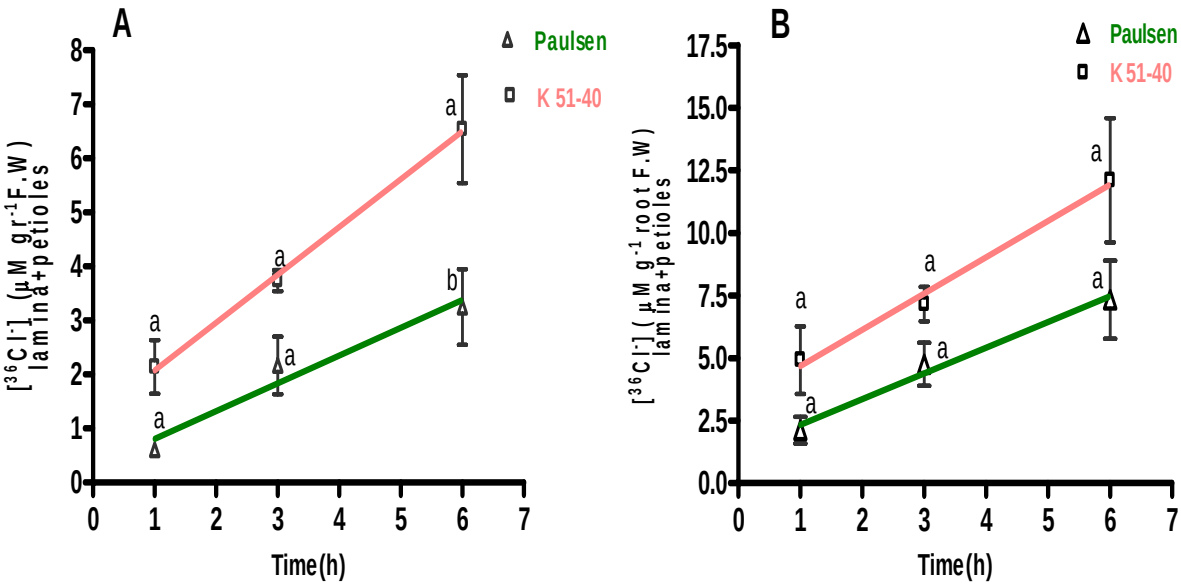

Figure 5- 7 Shoot (petiole+lamina) $^{36}Cl^-$ uptake estimated as shoot $^{36}Cl^-$ content (A) and shoot $^{36}Cl^-$ uptake per root FW (B) in K 51-40 (□) and Paulsen (Δ). Plants (rooted leaves) were pretreated by NaCl (30 mM for 5 days and 20 mM for 24 h) and loaded with radiolabelled solution for various times (1, 3, and 6 h). Data (A) were analysed using linear regression. Bars are SE of the means (n = 4) (two way ANOVA, $P < 0.05$). Different letters indicate significant difference between the varieties within the columns.

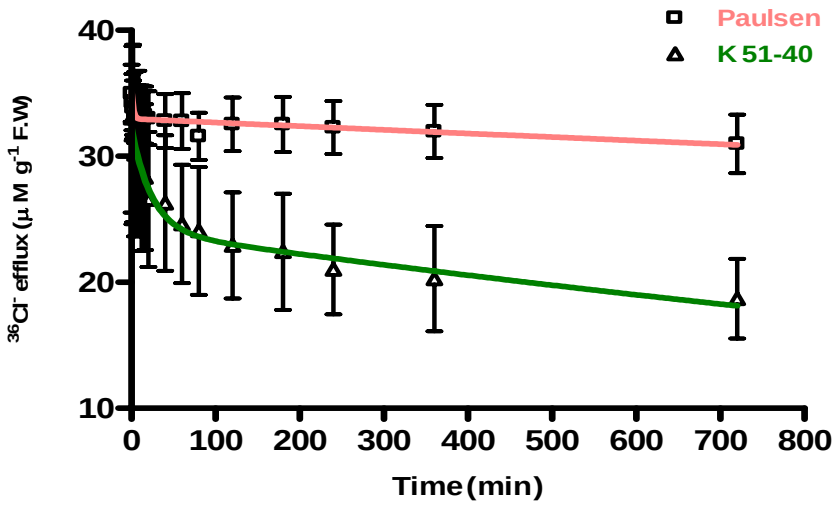

Figure 5- 8 Efflux of $^{36}Cl^-$ from intact whole roots of K 51-40 (Δ) and Paulsen (□) plotted as decrease in tissue $^{36}Cl^-$ content expressed as µM/gr F.W. after transfer to non-labelled solution. Plants were pretreated by NaCl (30 mM for 5 days and 20 mM for 24 h) and loaded with $^{36}Cl^-$ for 12 h. Plants then were transferred to aerated successive washing solutions for 1-720 min. Data were fitted with a double exponential equation to calculate fluxes. Rate constant for Bars are SE of the mean (n = 6). Comparison of fits indicated significant difference between the curves.

The results also showed that the tracer influx from the cytoplasm to the vacuole (Φ_{cv}) in Paulsen was higher than K 51-40 but unlike influx rate, the efflux rate from the vacuole to the cytoplasm (Φ_{vc}) in K 51-40 was higher than Paulsen. It was also calculated that the $^{36}Cl^-$ efflux from cytoplasm to the xylem in K 51-40 (0.014 µmol g$^-$

^{1} FW min^{-1}) was about two times greater than that of Paulsen (0.0087 µmol g^{-1} FW min^{-1}).

In comparison to whole roots, the parameters estimated for the lateral roots did not show significant differences between the two rootstocks, although the values for the lateral roots of K 51-40 were slightly higher than those of Paulsen (Table 5-2) (Fig. 5-9).

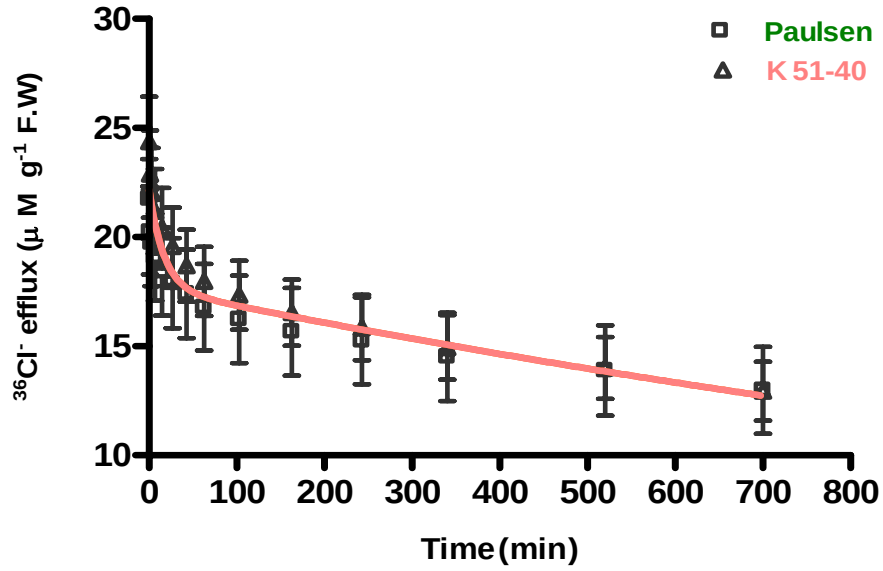

Figure 5- 9 Efflux of $^{36}Cl^-$ from lateral root segments of K 51-40 (Δ) and Paulsen ( ) plotted as decrease in tissue $^{36}Cl^-$ content expressed as µM after transfer to non labelled solution. Plants were pretreated by NaCl (30 mM for 5 days and 20 mM for 24 h) and loaded with $^{36}Cl^-$ for 12 h. The root segments were then transferred to aerated successive washing solutions for 1-720 min. Data were fitted with a double exponential equation to calculate fluxes. Bars are SE of the mean (n = 6). No significant difference observed between the varieties.

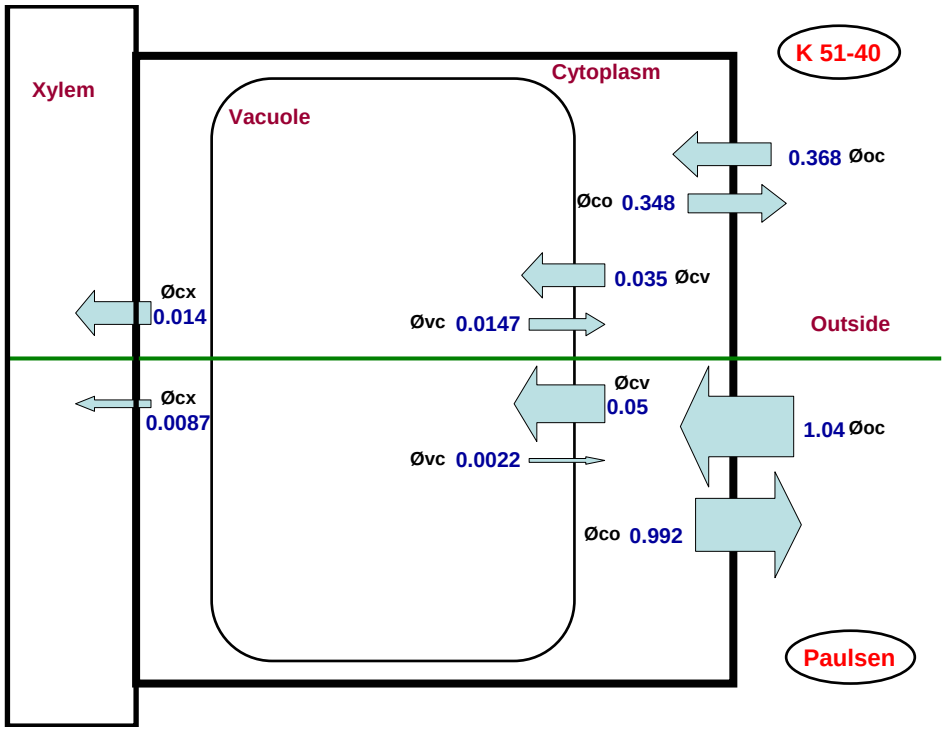

Figure 5- 10 Schematic demonstration of the whole root fluxes values estimated as µM g^{-1} FW min^{-1} from the influx and efflux experiments (compartmental analysis) summarized in Table 5-2. The growth condition as described for experiment 6.

Table 5- 2 Compartmental analysis of $^{36}Cl^-$ fluxes (experiment 6) including influx from outside to cytoplasm (Φ_{oc}), cytoplasm to vacuole (Φ_{cv}) and from cytoplasm to outside (Φ_{co}), vacuole to cytoplasm (Φ_{vc}), net influx (Φ_{in}), net flux to cytoplasm (Φ_{net}), efflux from cytoplasm to the xylem (Φ_{cx}) and the rate of loss of tracer (R) in whole and lateral roots estimated as µM g^{-1} FW min^{-1} using parameters which were obtained from fitting the efflux data to two exponential equation (Table 5-3, Appendix). The plants were pretreated by 30 and 20 mM NaCl for 5 days and 24 h respectively. The plant roots (K 51-40 and Paulsen) were loaded in a $^{36}Cl^-$- labelled solution for 12 h and then washed in successive non-labelled solutions for various times (1-720 min) (n = 6).The radioactivity in the tissue and those of the washing solutions were counted. Errors are SEM. Data were plotted as $^{36}Cl^-$ remaining in the tissue and fluxes were calculated using equations given in the Appendix.

	Φ_{oc} (Eq. 5-1) ($\mu M\ g^{-1}$ F.W. min^{-1})	Φ_{co} (Eq. 5-2) ($\mu M\ g^{-1}$ F.W. min^{-1})	Φ_{vc} (Eq. 5-3) ($\mu M\ g^{-1}$ F.W. min^{-1})	Φ_{cv} (Eq. 5-4) ($\mu M\ g^{-1}$ F.W. min^{-1})	R (Eq. 5-9) ($\mu M\ g^{-1}$ F.W. min^{-1})	Φ_{in} (Eq. 5-10) ($\mu M\ g^{-1}$ F.W. min^{-1})	Φ_{net} (Eq. 5-11) ($\mu M\ g^{-1}$ F.W. min^{-1})	†Φ_{cx} ($\mu M\ g^{-1}$ F.W. min^{-1})
Whole root								
K 51-40	0.368 ± 0.005	0.348 ± 0.007	0.0147 ± 0.002	0.035 ± 0.001	0.34 ± 0.02	0.034 ± 0.005	0.039 ± 0.004	0.014 ± 0.002
Paulsen	1.04 ± 0.087	0.992 ± 0.083	0.0022 ± 0.0002	0.05 ± 0.002	0.99 ± 0.02	0.05 ± 0.008	0.06 ± 0.01	0.0087 ± 0.001
Lateral root								
K 51-40	0.228 ± 0.003	0.215 ± 0.004	0.016 ± 0.0025	0.029 ± 0.002	0.21 ± 0.04	0.027 ± 0.005	-	-
Paulsen	0.198 ± 0.005	0.182 ± 0.004	0.01 ± 0.0016	0.026 ± 0.004	0.18 ± 0.02	0.025 ± 0.004	-	-

† is the slope of the linear regression of $^{36}Cl^-$ uptake to the shoot (experiment 5)

5-4 Discussion

The main aim of this chapter was to compare component Cl^- fluxes in roots of the two grapevine rootstocks, K 51-40 and Paulsen. In comparison to a cell, the study of tracer fluxes in a root are more complex because there is a net flux to the xylem and, depending on pre-treatment, also to the cortex. Thus the amount of tracer transported to the shoot and that which remains in the root should be estimated (Pitman, 1972). Cram (1973) showed that the initial Cl^- influx to the cortical cells of maize root isolated from stele was independent of the stele function. Also, Cl^- influx across plasma membrane increased linearly by increasing the external concentration and was an active process up to 20 mM (Cram, 1973). At high external concentrations, greater influx was observed to the cytoplasm than to the vacuole. Both plasmalemma influx and influx of $^{36}Cl^-$ to the vacuole were decreased by pre-treatment of barley roots with 25 mM KCl and 5 mM $CaCl_2$ for 22 h (Cram & Laties 1971).

In this investigation $^{36}Cl^-$ fluxes across different membranes (plasma membrane and tonoplast) were estimated from influx and efflux experiments on roots of whole plants and root segments of K 51-40 and Paulsen. The results obtained from influx experiments showed that the rootstocks differed in Cl^- influx (Fig. 5-3 A and 5-3 A; Table 5-1). Paulsen had almost 2 times more Cl^- net influx than K 51-40. It was consistent with my previous finding in Chapter 2 in which it was shown that Cl^- content in root of Paulsen was higher than that of K 51-40. Unidirectional influx values obtained in experiment 6 (Φ_{in} values from Table 5-2) were also consistent with the values in Table 5-1 particularly for experiment 2.

Unidirectional influx values of experiment 1 (Table 5-1) showed a similar ratio between rootstocks with Φ_{in} values of table 5-2, although they were different to experiment 1. It is noteworthy to explain here that unlike experiments 1 to 4, experiment 6 of this chapter was carried out on whole plants under light, thus it is possible that differences observed between the values obtained from experiment 6 with those of the other experiments can be due to differences in experimental materials and environmental conditions (*i.e.* root segments vs intact root and transpiration). Meanwhile, fluxes were calculated for the first minute of experiment 6 whereas, net influx values of experiment 1 were slopes of linear regression analysis over 10 minutes.

Davenport et al. (2005) showed that $^{22}Na^{+}$ accumulation in roots of two durum wheat genotypes (salt tolerant *line149* and salt sensitive *Tamaroi*) was fast over the first 5 min. They also suggested that in the first 3 min of uptake, $^{22}Na^{+}$ accumulated in the root cytoplasm first with negligible efflux. $^{22}Na^{+}$ efflux to the external solution and to the shoot was started after 3 min (after saturation of the cytoplasmic pool). Similarly in the two rootstocks investigated here when the cytoplasmic $^{36}Cl^{-}$ pool became labelled over 10 min, $^{36}Cl^{-}$ influx to vacuole or efflux from the cytoplasm to outside and / or efflux to the shoot was started. Reduction in the rate of $^{36}Cl^{-}$ accumulation after 10 min (Fig. 5-3 B) can be due to the beginning of $^{36}Cl^{-}$ efflux to the external medium or to the shoot and/or to the vacuole.

In experiment 3 of this chapter, unidirectional influx kinetics showed that Paulsen had high influx rate over 10 min and in the presence of various external Cl^{-} concentrations (Fig. 5-5). Cram, (1973) and Cram & Laties (1971) showed that influx across plasmalemma increased by increasing the external concentrations of Cl^{-} in isolated maize root cortex and suggested that in the low external Cl^{-} the plasmalemma influx can be the rate-limiting step in the influx of Cl^{-} to the vacuole. Furthermore, at high external concentrations of Cl^{-}, influx to the cytoplasm continued to increase but the Cl^{-} influx to the vacuole remained constant. In the two rootstocks examined here, increases in Cl^{-} influx rate were due to increased external concentrations of Cl^{-} over 10 min. V_{max} value for Paulsen (0.127 ± 0.042 µM g^{-1} F.W. min^{-1}) was greater than K 51-40 (0.059 ± 0.026 µM g^{-1} F.W. min^{-1}) but K_m difference between the rootstocks was not significant. This showed that unidirectional influx of Cl^{-} in Paulsen was significantly higher than K 51-40 because of higher V_{max} in Paulsen.

There is evidence that show the importance of lateral roots and root hairs in nutrient uptake by plants. Volder et al. (2005) found that nitrate uptake and respiration rate decreased quickly after lateral roots of grape aged. Ma et al. (2001) investigated the role of root hairs and lateral roots in the Si uptake by two mutants of rice. They showed that silicon uptake by RH2 mutant (defective in the formation of root hairs) was similar to wild type but RM109 mutant (defective in the formation of lateral roots) had much less Si uptake than wild type. In experiment 4 of this chapter, $^{36}Cl^{-}$ uptake by main and lateral roots of K 51-40 and Paulsen was studied. The results

showed that $^{36}Cl^-$ fluxes by lateral roots of the rootstocks were not significantly different, which was consistent with the results of experiment 6 (Table 5-2). In comparison to K 51-40, main roots of Paulsen had greater contributions to $^{36}Cl^-$ uptake. As it can be seen in Fig. 5-4 and Table 5-2, the influx ratio of main to lateral roots was similar in both experiments (4 and 6), although the values were different. More investigations need to be done to clearly indicate the effect of lateral roots in Cl^- uptake by grapevine under different conditions (such as intact plant and natural soil condition).

The rootstocks showed a significant difference in $^{36}Cl^-$ accumulation in the shoot. The salt sensitive K 51-40 accumulated higher levels of $^{36}Cl^-$ in shoot (petiole +lamina) than the salt tolerant Paulsen. The rate of $^{36}Cl^-$ transport (slope of linear regression) was also higher in K 51-40 than Paulsen. This result is consistent with the result obtained in Chapter 2. As it has been shown in Fig. 2-2 A, Chapter 2, shoot Cl^- content of K 51-40 was significantly higher than that of Paulsen.

Davenport et al. (2005) discussed a similar difference in two genotypes of durum wheat; salt tolerant line149 and salt sensitive Tamaroi. They suggested that because of the same influx rate to root and similar vacuolar accumulation of $^{22}Na^+$ in roots of the genotypes, the higher transport rate of $^{22}Na^+$ to shoot in the salt sensitive genotype (Tamaroi) can be attributed to higher efflux rate of $^{22}Na^+$ from the parenchyma cells to the mature xylem vessels. The same possibility may be suggested for $^{36}Cl^-$ in the salt sensitive K 51-40. This was supported by the results of compartmental analysis of $^{36}Cl^-$ efflux experiments. As it has been shown in Table 5-2, efflux rate from the cytoplasm to the xylem (Φ_{cx}) in K 51-40 was nearly two times greater than Paulsen. This difference can be probably due to a difference in the number of transporters or channels that load Cl^- into the xylem of main roots. Also, higher cytoplasmic Cl^- concentration in K 51-40 (Table 5-3, Appendix) could be another reason for higher Cl^- efflux to the xylem. The lower estimated cytoplasmic Cl^- concentration in Paulsen can be due to higher vacuolar storage or higher efflux to the external medium. Furthermore, the results summarized in Table 5-2 and Fig. 5-10 showed the same differences between the rootstocks observed in various influx experiments of this chapter. However, Fig. 5-10 is a reductionist diagram for only one cell and influences

of different cell types and existence of cell layers in a whole root must be considered in measurement of the flux values.

Britto et al. (2004) studied Cl^- fluxes and compartmentation in barley under high external Cl^- concentartion (100 mM). They found that Cl^- influx into the cytoplasm, flux from cytoplasm to vacuole, Cl^- transport from root to shoot and efflux from the cytoplasm to the external medium increased with increases in the external Cl^- concentration. Particularly efflux values increased to greatest levels compared to the other fluxes as it was 90 % of influx at the highest level of external Cl^- (100mM). In experiment 6 of this chapter $^{36}Cl^-$ efflux from the cytoplasm to the external medium in Paulsen was three times greater than that of K 51-40 and it was nearly 95 % of influx in both rootstocks (Table 5-2). This apparently futile cycling of Cl^- between outside and the cytoplasm might be further investigated.

In conclusion, $^{36}Cl^-$ flux experiments showed that the cytoplasmic $^{36}Cl^-$ influx was higher in Paulsen than K 51-40 and similar results were observed for the cytoplasmic $^{36}Cl^-$ efflux. The $^{36}Cl^-$ influx from outside to cytoplasm increased with increases in external Cl^- concentrations with a saturating effect at high external Cl^-. Lateral roots of K 51-40 were likely more effective than main root in Cl^- uptake; however it needs to be discussed after more experiments. In comparison to Paulsen, $^{36}Cl^-$ transport to shoot and uptake rate in K 51-40 was significantly higher. Compartmental analysis of $^{36}Cl^-$ fluxes showed that the cytoplasmic $^{36}Cl^-$ efflux rate to the medium in Paulsen was greater than K 51-40 as the same results was already shown for influx rate. It also showed higher efflux from the vacuole to the cytoplasm in K 51-40. Furthermore, high efflux rate from the cytoplasm to the xylem (Φ_{cx}) in K 51-40 was assumed to be the reason for high $^{36}Cl^-$ transport to the shoot. These differences between the rootstocks can be due to a difference in the number of transporters or channels in the cell membranes and their response to the external and cytoplasmic concentrations of Cl^- as shown in experiment 3 of this chapter. Higher V_{max} for $^{36}Cl^-$ influx in Paulsen in comparison to K 51-40 may be attributed to high density of transporters and channels in the plasma membrane. Electrophysiological experiments along with positional cloning (Davenport et al. 2005) could indicate Cl^- transport mechanism(s) across different cell membranes in the two rootstocks.

5-5 Appendix

Fitting data collected from efflux experiment to double exponential equation, two important parameters of efflux kinetics were determined; the efflux rate constants of cytoplasm (first phase) and vacuole (second phase) (K_c and K_v) and cytoplasmic and vacuolar tracer contents (I_c and I_v) (Table 5-3).

Table 5- 3 The values of parameters of efflux kinetics (experiment 6) for whole and lateral roots of K 51-40 and Paulsen obtained from fitting data to double exponential equation. I_c and I_v represent cytoplasmic and vacuolar tracer contents and K_c and K_v represent the efflux rate constants of cytoplasm (fast component) and vacuolar (slow component) respectively (n = 6).

Experiment 6	I_c (µmol g^{-1} F.W.)	K_c (min^{-1})	I_v (µmol g-1 F.W.)	K_v (min^{-1})
Whole root				
K 51-40	8.16 ± 0.61	0.041 ± 0.008	24.06 ± 0.55	3.93e-04 ± 6.96e-05
Paulsen	2.05 ± 0.40	0.482 ± 0.218	32.98 ± 0.15	9.01e-05 ± 1.87e-05
Lateral root				
K 51-40	5.26 ± 1.72	0.038 ± 0.032	18.59 ± 1.48	4.23e-04 ± 2.14e-04
Paulsen	3.94 ± 1.91	0.044 ± 0.057	17.22 ± 1.55	3.33e-04 ± 2.43e-04

$^{36}Cl^-$ fluxes (Φ) were calculated using the following equations (Mackleon, 1975):

$$\Phi_{oc} = \frac{I_v}{t_{up}} + K_c.I_c \qquad \text{(Eq. 5-1)}$$

$$\Phi_{co} = K_v.Q_v + K_c.I_c \qquad \text{(Eq. 5-2)}$$

$$\Phi_{vc} = \Phi_{oc}\frac{K_v.Q_v}{K_c.I_c} \qquad \text{(Eq. 5-3)}$$

$$\Phi_{cv} = \Phi_{oc} + \Phi_{vc} + \Phi_{co} \qquad \text{(Eq. 5-4)}$$

Where I_c and K_c are the intercept and rate constant for the faster-exchanging component and I_v and K_v are the intercept and rate constant for the slow component. Q_v is the vacuolar contents of Cl^- which were determined chemically in the tissue. Total Cl^- quantity in the tissue, $Q_T = (Q_v + Q_c)$, was assumed to be identical to Q_v because of small size of the cytoplasm. Qc was calculated from the following equations:

$$Q_c = Q_T - Q_v \qquad \text{(Eq. 5-5)}$$

or

$$Q_c = (\Phi_{co} + \Phi_{cv}) / K_c \qquad \text{(Eq. 5-6)}$$

Assuming some parameters, it is possible to calculate the cytoplasmic Cl^- concentration in terms of mM/L. If a plant cell water content (CW) is 0.8 ml g^{-1} F.W. and the fraction of cytoplasm as cell water (Cyt) is 0.1 ml g^{-1} F.W. (Dracup et al. 1989), then:

$$[Cl^-] = I_c / (CW)\,(Cyt) \qquad \text{(Eq. 5-7)}$$

From Table 5-3 cytoplasmic $^{36}Cl^-$ content (I_c) is 8.16 µmol g^{-1} F.W and 2.05 µmol g^{-1} F.W for K 51-40 and Paulsen respectively. Using Eq. 5-7 cytoplasmic Cl^- concentration will be 102 mM/L for K 51-40 and 25 mM/L for Paulsen.

The tissue tracer content (Q^* ; µmol g^{-1} FW) and rate of loss of tracer (R) in any time of elution (t) were given by following equations (Macrobbie, 1981):

$$Q^* = I_c.e^{-Kct} + I_v.e^{-Kvt} \qquad \text{(Eq. 5-8)}$$

$$R = I_c.K_c.e^{-Kct} + I_v.K_v.e^{-Kvt} \qquad \text{(Eq. 5-9)}$$

The rate of tracer uptake (Φ_{in}) or net influx which is a difference between tracer influx and efflux was estimated as follows (Walker & Pitman 1976):

$$\Phi_{in} = \Phi_{cv} \,.\, \Phi_{oc} \,.\, S_o / (\Phi_{cv} + \Phi_{co}) \qquad \text{(Eq. 5-10)}$$

Where S_o is specific activity of the external labelling medium.

Net flux of tracer to cytoplasm (Φ_{net}) was given by (Siddiqi et al. 1991):

$$(Q^*_T / S_o) \;/\; \text{Root FW}$$

$$\Phi_{net} = \frac{Q^*_T}{t_{up}} \qquad \text{(Eq. 5-11)}$$

Where Q^*_T is total $^{36}Cl^-$ contents of root plus shoot.

Flux to the xylem (Φ_x) is estimated by:

$$\Phi_x = \frac{(Q^*_{sh} / S_o) / \text{Root FW}}{t_{up}} \qquad \text{(Eq. 5-12)}$$

Where Q^*_{sh} is $^{36}Cl^-$ contents of shoot.

To calculate tracer efflux from the cytoplasm to the xylem (Φ_{cx}), the slope of the linear regression of $^{36}Cl^-$ uptake to the shoot (Fig. 5-5 A) was used to indicate the rate of the tracer release to the xylem (Davenport et al. 2005).

Chapter 6

Cl^-, Na^+ and K^+ distribution in grapevine root pretreated with NaCl (X-ray microanalysis)

6-1 Introduction

Cl^- as an essential micronutrient must be transported to the shoot (Su et al. 2000). However, under saline conditions where Na^+ and Cl^- are presented at toxic levels, plant roots play a critical role in preventing high concentrations of Na^+ and Cl^- accumulating in the shoot (Storey et al. 2003). Genetic differences between plant species and varieties in the restriction Na^+ and Cl^- uptake from the soil, or reduction of ion transport to the xylem could be an influential factor in diminishing the accumulation of those ions in leaves (Munns, 2002). There is considerable variation between grapevine varieties in Cl^- transport to the shoot. Comparing various species of grapevine, Walker et al. (2004) showed that Cl^- accumulation was reduced in petioles and leaves of Sultana when grafted on Ramsey or 1103 Paulsen rootstocks in comparison to own-rooted vines. Husain et al. (2004) showed genetic differences between durum wheat genotypes in sequestration of Na^+ and Cl^- in roots and shoots. They found that in comparison to other genotypes, *Line 149*, *Line 151 and Janz* were able to retain more Na^+ and Cl^- in roots.

Many studies have attempted to determine Na^+ and Cl^- distribution patterns in different parts of plants under salt stress using energy dispersive X-ray microanalysis. It is a reliable technique for measuring ion contents within cells and subcellular levels such as the cytoplasm and vacuole (Huang & VanSteveninck 1989). K^+, Na^+ and Cl^- distributions in roots of barley were investigated by Pitman et al. (1981) by means of the X-ray microanalysis method. They showed that the K^+/Na^+ ratio in root cortex of plants grown in solution containing NaCl (5 mM) was higher than that of the stele. Vacuolar content of Cl^- was greater than that of the cytoplasm in cortex cells but there was no result for vacuolar Cl^- content in the stele to compare with the cortex.

Hajibagheri et al. (1987) studied K^+, Na^+ and Cl^- distribution within root cells of salt sensitive and salt tolerant maize varieties grown under high external NaCl concentrations (100-200 mol m^{-3}). The cytoplasmic Na^+ and Cl^- contents and Na^+ / K^+ ratio of the root cells of the salt sensitive variety was greater than that of a salt tolerant variety (Protador and Across 8024). The ratio of Na^+ / K^+ in the root cells decreased from epidermis inward to the stele. The X-ray microanalysis technique was also used by Hodson & Sangster (1989) to show subcellular localization of some nutrient elements in the roots of wheat.

There are also several studies on ion concentration profiles in upper plant parts using X-ray microanalysis technique. For example; Na^+ and Cl^- concentrations in the epidermal and mesophyll cells of the leaf lamina and sheath of barley (Huang & Van Steveninck 1989), K^+, Cl^- and Ca^{+2} concentrations in the vacuole of barley leaf mesophyll, epidermis and bundle sheath cells (Williams et al. 1993), investigation of Ca^{+2} and K^+ distribution patterns in different cell types of citrus leaves (Storey & Leigh 2004), localization of 'bound Na^{+}' in extracellular matrix of rice shoot (Anil et al. 2005) and study of K^+, Na^+ and Cl^- cellular and subcellular partitioning in the leaves of salt stressed barley and durum wheat (James et al. 2006). These studies have highlighted the importance of different cell types in sequestration of ions.

Using x-ray microanalysis, Storey et al. (2003) demonstrated the intracellular distribution of Ca^{+2} in different regions of the primary root of *Vitis vinifera* (var. Biancone). They showed Ca-accumulating cells (idioblasts) in the outer region of the meristematic zone of *Vitis* roots in the shape of a discontinuous cone of cells and in the older regions of the root located at the margin of the cortex. They suggested that these specialist cells act as a Ca^{+2} reservoir for cell wall synthesis in the elongation zone.

In grapevine, it has also been shown that Cl^- transport to the shoot is controlled by the root (Downton, 1977; Walker et al. 2004) through either Cl^- exclusion or restriction of uptake and sequestration in root vacuoles (Lauchli, 1984). In order to understand how grapevine roots control Cl^- accumulation in the shoot, Storey et al. (2003) conducted a comparative study on K^+, Na^+ and Cl^- distribution pattern in different cell types of salinized grapevine roots. They found that Cl^- content in the vacuole of pericycle cells

of Cl^- excluder genotype (80-23) was greater than the non-excluder genotype (80-15) and suggested that pericycle cells have an important role in regulation of Cl^- transport to the shoot under salt stress probably to keep cytoplasmic Cl^- concentration low.

In this chapter a comparative investigation was carried out to demonstrate K^+, Na^+ and Cl^- distribution in different root cell types (epidermis, hypodermis, cortex, endodermis and pericycle) of salt sensitive (K 51-40) and salt tolerant (Paulsen) grapevine rootstocks pretreated with NaCl.

6-2 Materials and methods

6-2-1 Plant material

The cuttings of K 51-40 and 1103 Paulsen were provided as described in section 2-2-1. Rooted cuttings were transferred to an aerated culture solution in plastic containers, containing ¼ strength Hoagland solution (composition as mentioned in section 2-2-2). Plants with 2-3 fully expanded leaves were pretreated with NaCl (30 mM) in ¼ strength Hoagland solution for 5-7 days. They were then transferred in small plastic containers containing the pre-treatment solution, from glasshouse to a preparation room to make root samples for X-ray microanalysis.

6-2-2 X-ray microanalysis

Root segments (4-5 mm) were excised 20-30 mm from the root tip of K 51-40 and 1103 Paulsen and the pair of segments placed vertically in a Brass stub supported by a drop of deionized water. The samples were rapidly frozen in liquid nitrogen slush. The frozen sections were then transferred under vacuum to a cryo-microtome system (Cressington CFE50) at -180 $^{\circ}$C and planed flat using the cryo-microtome blade. The samples were then transferred under vacuum to a CT1500-HF cryo transfer stage (Oxford instruments) warmed to -92 $^{\circ}$C to etch (remove the frost and expose cellular detail) for 1.5 min.

The samples were re-cooled to below -110 °C and coated with a thin layer of platinum using splutter-coating for 2 min. Argon was loaded into the cryo transfer stage chamber to replace residual oxygen during the splutter coating.

The samples were then inserted into the stage of the microscope (Philips, XL.30) to perform x-ray microanalysis with a scanning electron microscope (EDAX-energy-disperssive- X-ray detector - EDAX.international) (Fig. 6-1 A). Images were taken at 12 kV, spot size 4 (4 μm^2), 1000 (cps), 9000 x magnification and 10 mm distance from the sample surface for data (spectra) collection time of 60 live sec. The spectra for K^+, Na^+ and Cl^- (Fig. 6-1 B) obtained from each cell of hypodermis, outer cortex, inner cortex, endodermis and pericycle (Fig. 6-2) were analysed using eDXi-software (EDAX). Cryo-SEM X-ray microanalysis of Cl^-, Na^+ and K^+ distribution was conducted on the cells from hypodermis (Hy), outer cortex (OC), inner cortex (IN), endodermis (En) and pericycle (Pe) of 6 roots and 5 cells of each part of the root. All data were expressed in P-B/B (peak-background/ background) ratios, to give a semi-quantitive measurement of ion (K^+, Na^+ and Cl^-) concentration in the tissues. Average P-B/B would not reflect chemical analysis of the whole root, because the P-B/B averages do not account for different relative volumes of cell types.

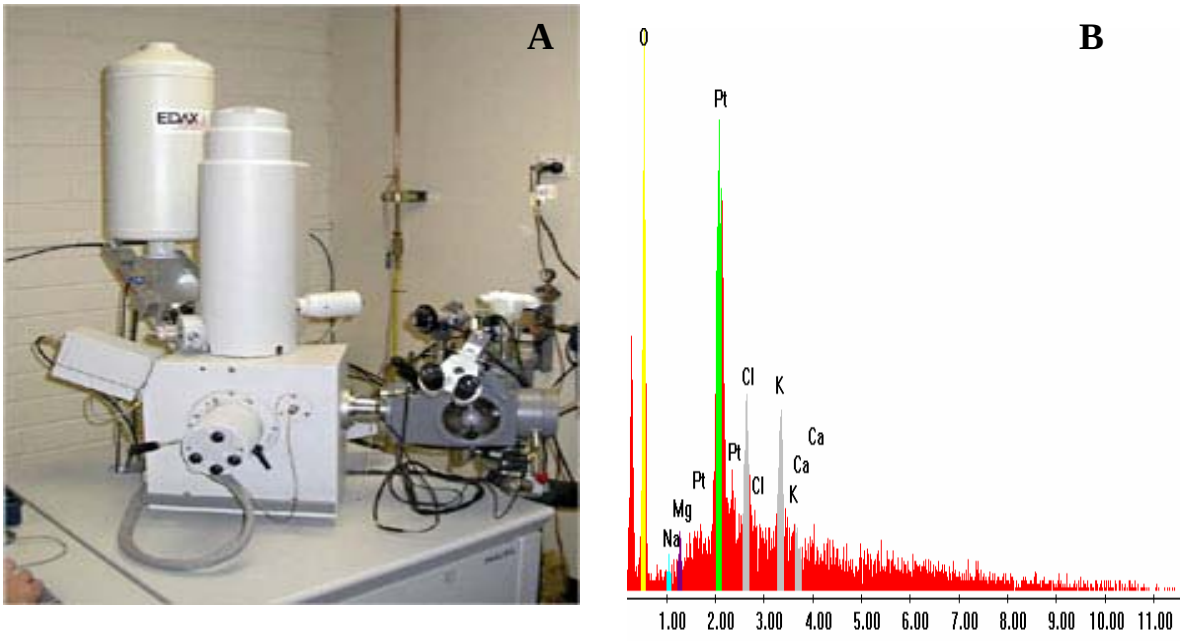

Figure 6- 1 X-ray microanalysis instrument set (A) and a diagram of x-ray microanalysis spectra of K51-40 root hypodermis cell (B). Vertical scales are in counts and horizontal scales are in Kiloelectron volts (keV).

6-3 Results

Transverse fracture faces of the cells of K 51-40 and Paulsen main and lateral roots are shown in Fig. 6-2. All X-ray microanalysis of Cl^-, Na^+ and K^+ distribution patterns were performed on the cells from hypodermis, outer cortex, inner cortex, endodermis and pericycle. Root samples were made over a range of between 20-30 mm from the root tip. As it can be seen in Fig. 6-2 particularly in picture B, all cells contained a large vacuole which occupies the majority of the cell volume. Thus, the ion contents obtained by X-ray microanalysis in this experiment represent the vacuolar concentrations.

Analysis of the distribution pattern of Cl^- in main root showed that Paulsen and K 51-40 retained more Cl^- content in hypodermis, outer and inner cortex than endodermis and pericycle; however, Cl^- content in all cells across the root and also total Cl^- contents in the main root of Paulsen was greater than those of K 51-40 (Fig. 6-3 A and D). K^+ contents across the main root (except in the hypodermis) and total K^+ content of K 51-40 was higher than that of Paulsen (Fig. 6-3 B and D). The rootstocks showed an increase for Na^+ content across the main root (from hypodermis to pericycle) but Na^+ contents in all parts of the main root (except in hypodermis) and total root Na^+ content of Paulsen was higher than that of K 51-40 (Fig. 6-3 C and D).

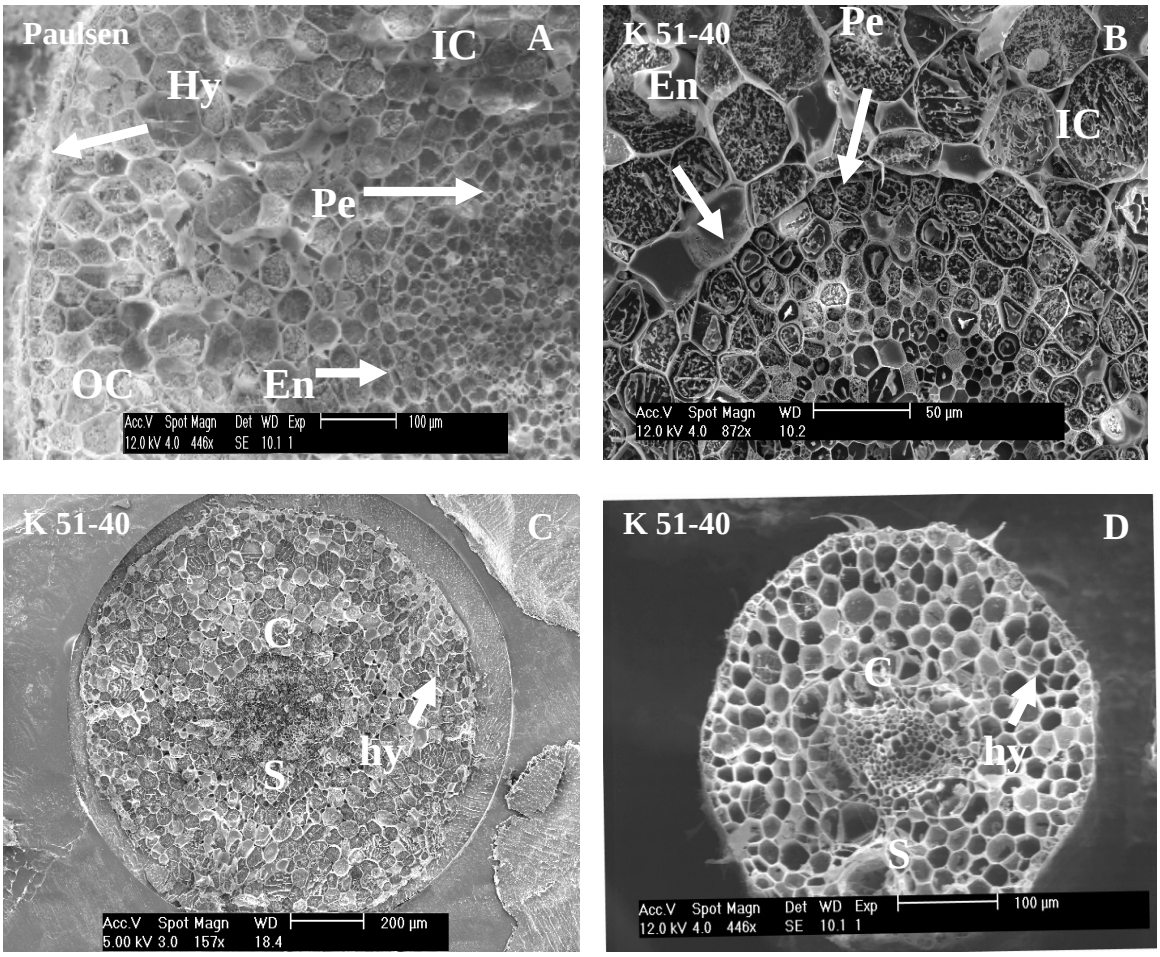

Figure 6- 2 Scanning electron micrographs of cryo-planed main (A,B, and C) and lateral (D) roots of K 51-40 and Paulsen. (C) and (D) Overview of K 51-40 main and lateral roots respectively showing stele (s), cortex (c) and hypodermis (hy). Plants were pretreated with NaCl (30 mM) for 5-7 days.All sections were made in the range of 20-30 mm from the root tip.

Comparing K^+ and Na^+ contents across the main root (from hypodermis to pericycle) of the both rootstocks, it was shown that the K^+ content decreased while Na^+ increased in Paulsen (Fig. 6-3 B and C). However there was no significant difference between K^+ and Na^+ contents across the main root of K 51-40, except in hypodermis (Fig. 6-3 B and C). The ratio of total K^+ / Na^+ in K 51-40 main root (1.68) was higher than that of Paulsen (0.99).

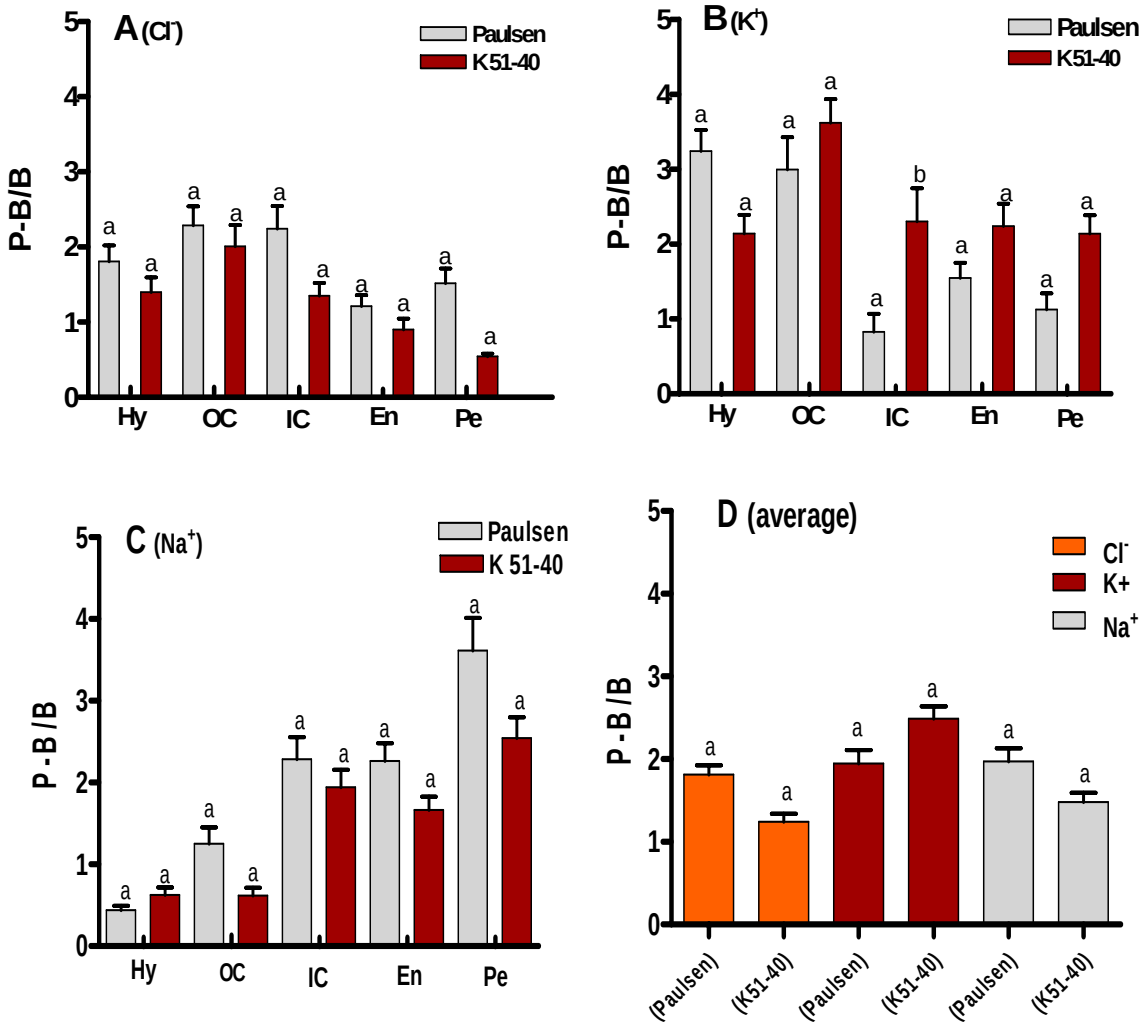

Figure 6- 3 Ion content (P-B/B) of K 51-40 and Paulsen main root cells. Plants were pretreated with NaCl (30 mM) for 5-7 days and Cl^- (A), K^+ (B) and Na^+ (C) were determined in hypodermis (Hy), outer cortex (OC), inner cortex (IC), endodermis (En) and pericycle (Pe) using X-ray microanalysis technique. Average Cl^- ,Na^+ and K^+ contents (Hy + OC + IC + En + Pe) in the main root of the two rootstocks is shown in (D). 5 cells of each part of the root were analysed for the ion distribution. Bars are SE of the means (n=6) (two way ANOVA, $P < 0.05$). Different letters indicate significant difference between the varieties within the columns.

The second analysis of ion distribution pattern was performed on the cells of K 51-40 and Paulsen lateral roots. The results obtained from the lateral roots of K 51-40 showed that the highest level of Cl^- was observed in the outer cortex and then reduced in the inner cortex, endodermis and pericycle. Whereas, there was no obvious difference between the cells across the lateral root of Paulsen (except a small reduction in pericycle Cl^- content) (Fig. 6-4 A). Total Cl^- concentration in the cells across the lateral root of K 51-40 was significantly (about 3-fold) higher than Paulsen (Fig. 6-4 B and C). In both rootstocks, K^+ concentrations had no significant changes not only across the cells of the roots but also between the rootstocks, except

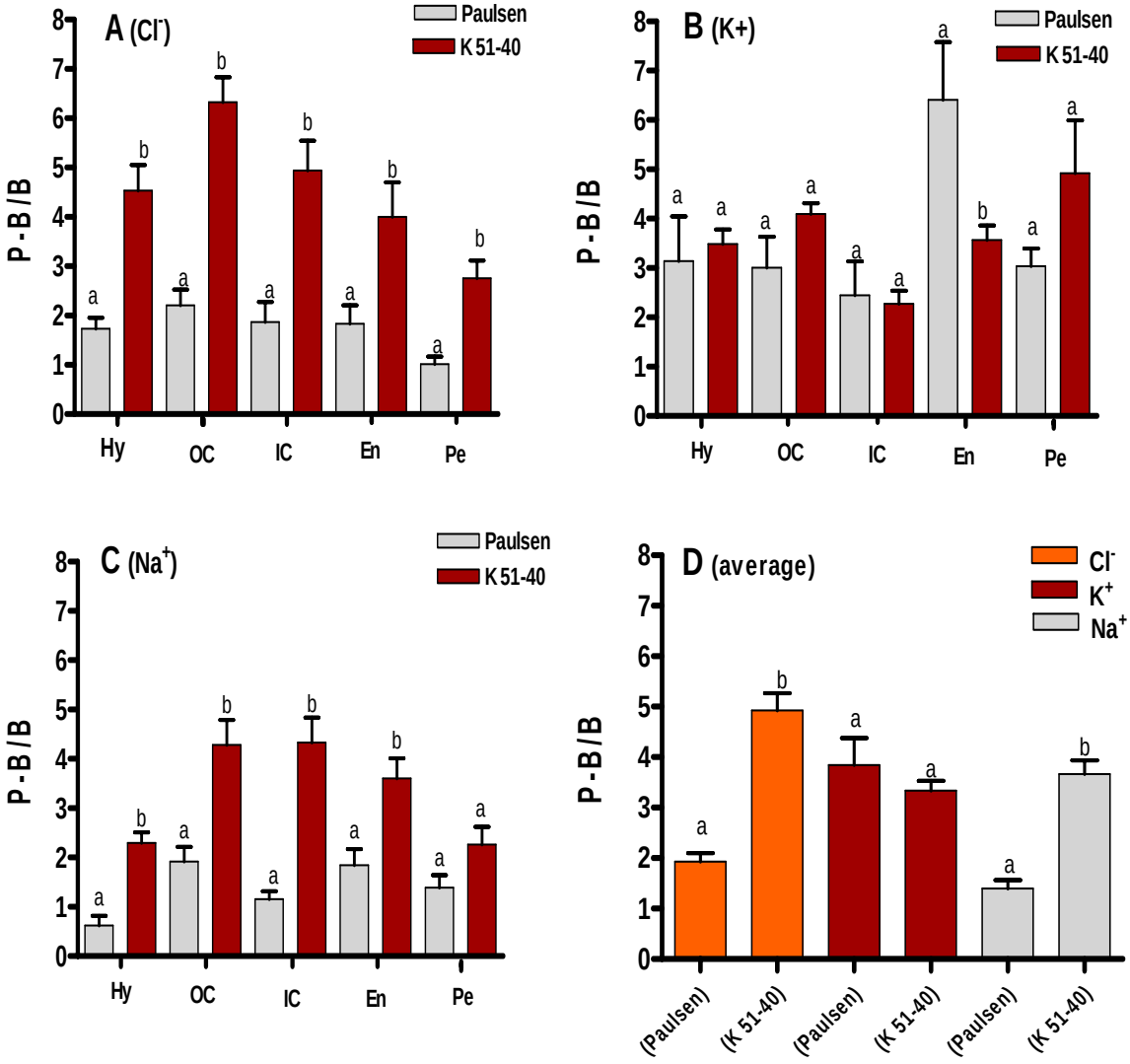

Figure 6- 4 Ion content (P-B/B) of K 51-40 and Paulsen lateral root cells. Plants were pretreated with NaCl (30 mM) for 5-7 days and Cl^- (A), K^+ (B) and Na^+ (C) were determined in hypodermis (Hy), outer cortex (OC), inner cortex (IC), endodermis (En) and pericycle (Pe) using X-ray microanalysis technique. Average Cl^-, Na^+ and K^+ contents (Hy + OC + IC + En + Pe) in the lateral root of the two rootstocks is shown in (F). 5 cells of each part of the root were analysed for the ion distribution. Bars are SE of the means (n=6) (two way NOVA, $P < 0.05$). Different letters indicate significant difference between the varieties within the columns.

in endodermis and pericycle in which higher K^+ was observed in endodermis of Paulsen than K 51-40 and higher K^+ in pericycle of K 51-40 in comparison to Paulsen (Fig. 6-4 B). There was no obvious difference between the rootstocks from the view point of total K^+ content in the cells of lateral roots (Fig. 6-4 D).

Similar to Cl^-, the highest levels of Na^+ were observed in the cells of outer and inner cortex of lateral root of K 51-40 while there was no significant difference between the cells of lateral root of Paulsen except in hypodermis that was lower than the other cell

types. However, in comparison to Paulsen, Na^+ content in all cell types of K 51-40 was remarkably high (Fig. 6-4 C). Total Na^+ content in the cells of K 51-40 was about 2-fold greater than that of Paulsen (Fig. 6-4 D). Comparative analysis of K^+ and Na^+ contents in the cells of Paulsen and K 51-40 showed that nearly all the cell types of lateral root of Paulsen accumulated more K^+ than Na^+ (Fig. 6-4 B and C) but in K 51-40, higher K^+ content was observed only in pericycle (Fig. 6-4 B). The ratio of total K^+ / Na^+ in Paulsen (2.75) was significantly higher than K 51-40 (0.91).

6-4 Discussion

X-ray microanalysis was used to characterize cellular (mainly vacuolar) Cl^-, K^+ and Na^+ distribution patterns across salinized main and lateral roots of two grapevine rootstocks, K 51-40 and Paulsen. The results obtained for Cl^- accumulation in main root showed that the hypodermis and cortical cells (inner and outer) of both rootstocks accumulated more Cl^- than the other cell types. Investigating vacuolar Cl^- content in subapical regions of rice root, Zheng & Yan (1996) indicated that in comparison to the other cell types, the cortical cells accumulated higher levels of Cl^-. Kelday & Bowling (1980) determined Cl^- concentrations in the cells across the roots of *Commelina communis* grown in a solution with a high Cl^- concentration, by extracting vacuolar sap using a microsampling technique. They showed that Cl^- contents in the cortical cells were greater than those of epidermis or endodermis.

Higher total Cl^- concentration of the main root (Cl^- content of all cell types across the main root) of Paulsen in comparison to K 51-40 was consistent with my previous findings that showed that Paulsen is able to accumulate more Cl^- in the root than K 51-40 (Chapter 2 and 4). It also indicated that in Paulsen, Cl^- transport to the shoot is reduced by accumulating Cl^- in the vacuole of root cortical cells. Under high salinity, vacuolar Cl^- channels (Tyerman, 1992; Wissing & Smith 2000) are probably involved in Cl^- storage in the vacuoles.

The results also showed that Cl^- and Na^+ concentrations in pericycle of the main root of Paulsen are greater than for K 51-40. This difference can be due to lower Cl^- and Na^+ loading to the xylem under salt stress in Paulsen by the stelar parenchyma cells in

comparison to K 51-40. It is also consistent with my finding in Chapter 1 and 5 that showed high Cl^- transport to shoot by K 51-40.

In the hypodermis and outer cortex of the rootstocks, K^+ content was high compared to other cell types. While in both rootstocks, Na^+ concentration increased from hypodermis to pericycle. This is consistent with a finding by Storey et al. (2003) in which the vacuolar Na^+ content increased across salinized roots of grapevine genotypes (80-15 and 80-23), whereas K^+ content had no obvious difference. A similar result has been reported for lupin by Van Steveninck et al. (1980). However, the results that have been reported by Pitman et al. (1981) for barley are not consistent with the results obtained for grapevine. They showed high Na^+ contents in the outer layers of barley cortical cells when compared to K^+ concentration. In grapevine this difference could be due to low activity of H^+ / Na^+ antiporter in tonoplast of the outer cortex cells (Storey et al. 2003). In comparison to Paulsen, the high K^+ /Na^+ ratio in the main root of K 51-40 indicated that the assumed H^+ / Na^+ antiport system in tonoplast of the root cells (most likely in the cortical cells) was less active or in K 51-40 Na^+ can be mostly transported to the xylem (shoot). Downton, (1977) showed that the lamina Na^+ content of Sultana grapevine increased with high external salinity.

The results obtained for lateral roots of the rootstocks were almost opposite from those estimated for the main root. Unlike main root, K 51-40 lateral roots accumulated higher Na^+ and Cl^- contents than Paulsen and the K^+ / Na^+ ratio of Paulsen lateral roots was about three times greater than K 51-40. Robinson, (1994) showed a higher lateral root growth when high nutrient concentrations were supplied for grapevine root systems in soil. These new and young roots had a high capacity to uptake N. Similarly, under high salinity, the lateral roots of K 51-40 were probably developed with a high capacity to uptake and accumulate Na^+ and Cl^- in the vacuoles. Bloom et al. (2002) suggested that the very youngest maize roots accumulated NO_3^- (without assimilating it) in the vacuoles of the cells in the elongation zone and used it as an osmoticum to drive root cell expansion. Similar function could be suggested for the lateral roots of K 51-40 that is, Na^+ and Cl^- were probably accumulated by new young roots to generate turgor required for cell expansion. However, in order to find a definite interpretation for the above mentioned results, the pressure probe experiment

and some morphological investigations on different stages of the lateral roots lifespan are required.

In conclusion, the results collected from X-ray microanalysis showed that the main root of Paulsen accumulated more Cl^- and Na^+ than K 51-40 particularly in the cortical and pericycle cells. This may be indicative of low Cl^- transport to the shoot in Paulsen. In contrast the lateral roots of K 51-40 had a high capacity to uptake and retain Cl^- and Na^+ in the vacuoles and likely use them as osmotica for root cell expansion. It is suggested that measuring the ratio of lateral / main roots may reveal new differences between the rootstocks and will be helpful to explain the differences in ion uptake capacity between rootstocks.

Chapter 7

Membrane potentials of grapevine root cortical cells and root surface-potential under high salinity

7-1 Introduction

The electrical potential difference between two aqueous electrolyte solutions separated by a membrane, mainly results from imbalanced charge transport of various ions across the membrane (Dainty, 1962). Ion transport across the plasma membrane is regulated by the ion concentrations on both sides of the membrane and membrane potential itself (Clarkson, 1993). The imbalanced ion distribution is caused either by a Donnan potential or ion pumps. The Donnan potential is a result of indiffusible ions located inside a cell. Ion pumps are located in the cell membrane and in plants, the activity of the primary H^+- ATPase within the plasma membrane generates most of the electropotential gradient (Dainty, 1962). It has been shown that all living plant cells display an electropotential difference (PD) of about -100 mV (internal negative) across cell membranes. The Nernst equation (Chapter 1, Eq.1-1) describes how the membrane potential is balanced by a concentration gradient for a particular ion, and it can be predicted if an ion moves passively across the cell membrane (Higinbotham, 1973).

It is possible for K^+ and Na^+ as monovalent cations to be be passively transported into the cell through ion channels if the cell membrane potential (E_m) is more negative than the K^+ and Na^+ equilibrium potentials (E_k and E_{Na}) (Clarkson, 1993). On the other hand at low external concentration, Cl^- as an anion is transported against its electrochemical gradient probably by nH^+/Cl^- symport with n >1 (Felle, 1994; Sanders, 1984). Ion channels may be responsible for passive Cl^- transport under high external salinity but allow passive efflux from the cytoplasm to outside under normal conditions. Unlike cations, in this condition, the Cl^- equilibrium potential (E_{Cl}) is

negative of the cell membrane potential. When ions pass through ion channels, the membrane PD between inside and outside of the cell will change as ions carry electrical charge. Consequently all active transport systems as well as voltage-sensitive ion channels will be affected by the above mentioned change in PD (Skerret & Tyerman 1994).

Measuring the PD and an ion concentration at the two sides of the membrane, it is possible to determine whether there is passive or active transport of the ion in question. Maathuis & Sanders (1993) measured the membrane electrical potential and K^+ concentrations of cytosol and external medium in root cells of *Arabidopsis thaliana*. They showed that K^+ transport into the root epidermal and cortical cells must be active at external K^+ concentrations less than 1 mM although the PD was -160 mV. They concluded that K^+ channels were not involved in K^+ transport at micromolar external K^+ concentration.

In most physiological conditions, the electrochemical gradient for Cl^- drives it out of the cell. In order to be imported, Cl^- must overcome the above mentioned electrochemical barrier using a considerable amount of metabolic energy (Felle, 1994). Substitution of 1 mM $CaSO_4$ by 1 mM $CaCl_2$, Ulrich & Novacky (1990) showed that Cl^- uptake into *Limnobium stoloniferum* root cells depolarized the membrane potential about 60 mV, and cytosolic pH decreased about 0.25 pH units. They also showed that increasing the external K^+ decreased Cl^- uptake and induced an extracellular acidification. At these lower Cl^- concentrations they suggested a $2H^+/Cl^-$ symport mechanism for Cl^- uptake by *Limnobium*. However, under high salt conditions, Cl^- permeable channels (such as NO_3^- permeable channels) may allow Cl^- influx when cytoplasmic Cl^- concentration is initially low and the membrane is depolarized. In this condition, Na^+ influx through non-selective cation channels, deactivating the K^+ channel (outward rectifying) and reduction in the proton pump activity may depolarize the membrane (Steudle & Tyerman 1994).

In a study by Gilliham & Tester (2005), anion loading pathways into the xylem and the regulation of the pathways by stress and ABA were investigated in the root stele cell protoplasts of maize. In comparison to xylem-parenchyma inwardly-rectifying anion conductance (*Zm*-X-IRAC), xylem- parenchyma quickly-activating anion

conductance (*Zm*-X-QUAC) was the common pathway for loading most of the NO_3^- and Cl^- ions into the xylem of well-watered plants. *Zm*-X-IRAC activity increased in water stressed plants while the activity of Zm-X-QUAC decreased. ABA applied to protoplasts of well-watered plants inhibited Zm-X-QUAC activity.

7-1-1 The root surface potential.

If an intact plant root system is divided into two parts longitudinally and each part is put in two electrically isolated media, the PD measured between the two rooting media might be equal to the difference in the two trans-root potentials. In this condition any treatments in one side will generate a gradient in surface potential through the xylem stream. Extending this idea, surface potential of a short length of a root can be measured in two different points on the surface of the root, which is related to the trans-membrane potential. Due, (1993) suggested a model in which the root is considered as a three-conductor electrical cable including cortical cell wall, symplasm and xylem vessels. Using this method, rapid changes in the surface potential between two pools of solution, about 10 mm apart, can be measured. This measurement will show the difference in the average plasma membrane PD. of the cortical cells in the two pools.

Practically, the PD across the cortical cell membranes which is called the trans-membrane potential, is measured by inserting a glass microelectrode into the tissue. Due, (1993) has discussed some advantages of the surface potential measurement where plant tissue is not wounded, and probably does not include the potential difference across the tonoplast. It also measures PD of many cells in the tissue instead of a single cell. The root surface potential measurement can be used as a complementary way to support cell microelectrode measurements.

In this chapter the effect of high external salinity (30 mM NaCl) on the root cortical cell PD of Paulsen (salt tolerant) and K 51-40 (salt sensitive) was studied in salt pre-treated and nonpretreated root segments. Because Choline and MES are large molecules that would not be expected to pass through the plasma membrane, Choline-Cl and Na-MES (2- (N- morpholino) ethane sulfonic acid) were used to demonstrate just the effects of Cl^- and Na^+ separately on the membrane potential. As far as I am aware there is no evidence for a substantial MES permeability from the literature. However, recently

there was identified a trasnporter in *Arabidopisis* that could facilitate Choline transport (Lelandais-Briere et al. 2007). It is not clear how much trasnport would occur in a short period. This would probably not be very substantial. The root surface-potential method was also used to measure depolaraization currents caused by ion transport in the above mentioned conditions.

7-2 Materials and methods

7-2-1 Plant material

The cuttings of K 51-40 and 1103 Paulsen were provided as described in section 2-2-1. Rooted cuttings were transferred to an aerated culture solution in plastic containers, containing ¼ strength Hoagland solution (composition as mentioned in section 2-2-2). Plants with 2-3 fully expanded leaves were pretreated with NaCl (30 mM) in ¼ strength Hoagland solution for 5-7 days or were grown in Hoagland solution and were used as nonpretreated plants.

7-2-2 The cortical cell membrane potential

The apical 30-50 mm of the excised main root of the rootstocks was placed in a Perspex chamber. The constantly flowing solution (gravity feed) was maintained on the root surface using vacuum suction at one end of the chamber. Various solutions were used depending on the experimental conditions without disturbing the preparation. The chamber with the root was held and kept under microscopic observation (Nikon, SMZ 645).

Micropipettes were pulled from filament-containing borosilicate glass capillaries (Clark, GC150F-10) with a vertical puller (NARASHIGE, PP-83, Japan). The measuring electrode was filled with 3 M KCl and inserted into a microelectrode holder which was located on the micromanipulator. Different filling solutions (1 M KCl and 200 mM K^+-acetate) were tested according to Blatt et al. (1987) but measurements were unsuccessful. The reference electrode was filled with 3 M KCl in 2% agar. It was placed within the flowing solution in the chamber down stream of the root. The measuring microelectrode was inserted horizontally and gently into the root cortical cells using a high precision micromanipulator (NARASHIGE, MX-2, Japan).

Membrane potentials were determined measuring the potential difference between the root cortical cells and bathing medium. An amplifier (Neuroprobe, Model 1600) connected to a calibrated oscilloscope (Tektronix, 5111A) was used to measure electrical potential changes. Data were collected by USB Data Acquisition modules (DT 9816) and recorded by DataAcq software and plotted using Excel.

Composition of solutions bathing root segments during impalement was different depending on experimental design. They consisted of ¼ strength Hoagland solution with or without 30 mM NaCl and of ¼ strength Hoagland solution containing 0.1 mM $GdCl_3$ or 0.1 mM $GdCl_3$+30mM NaCl. All solutions were supplied from reservoirs via plastic tubes.

7-2-3 Measurement of the root surface potential

The surface potential of the root segments was measured using the method developed by Walker, (1994). A root segment was placed in a Perspex chamber containing two small pools of solution 5 mm in diameter and 10 mm apart and the compartment between the pools was insulated with high vacuum grease. Two electrodes were filled with 3 M KCl in 2% agar and placed in the pools. Solutions consisted of ¼ strength Hoagland solution with 0.5, 1, 3, 5, 10, 20, and 30 mM NaCl and ¼ strength Hoagland solution containing 0.5-30 mM Choline-Cl or 0.5-30 mM Na-MES were maintained (1 ml per 40 sec) on the root surface in one pool (the root tip side) for various time periods and depleted from the same pool using a vacuum pump. The other pool, where the proximal end of the root segment was located, was filled with ¼ strength Hoagland solution. Data were collected and plotted in a similar way as that for the impalement experiment.

7-3 Results

7-3-1 The cortical cell membrane potential

Membrane potentials in the range between -85 to -120 mV were observed when the root segments of K 51-40 and Paulsen were impaled in ¼ strength Hoagland solution. An average membrane potential difference (PD) of -112 ± 2.27 mV (n = 70) was

observed for the cortical cells of the NaCl pretreated (-108 ± 3.42 mV, n=16) and non-pretreated roots (-116 ± 2.1 mV, n= 54) at the beginning of impalement. Membrane PD was not different for the cortical cells of both rootstocks after pretreating with 30 mM NaCl and using the same perfusion solution (Fig. 7-1 A and B).

In non-pretreated plants, introducing 30 mM NaCl to the root segments caused a relatively rapid and transient depolarization in both rootstocks. This initial depolarization was followed by slow hyperpolarizations to nearly steady values that were more negative than initial values (Fig. 7-2 A and B).

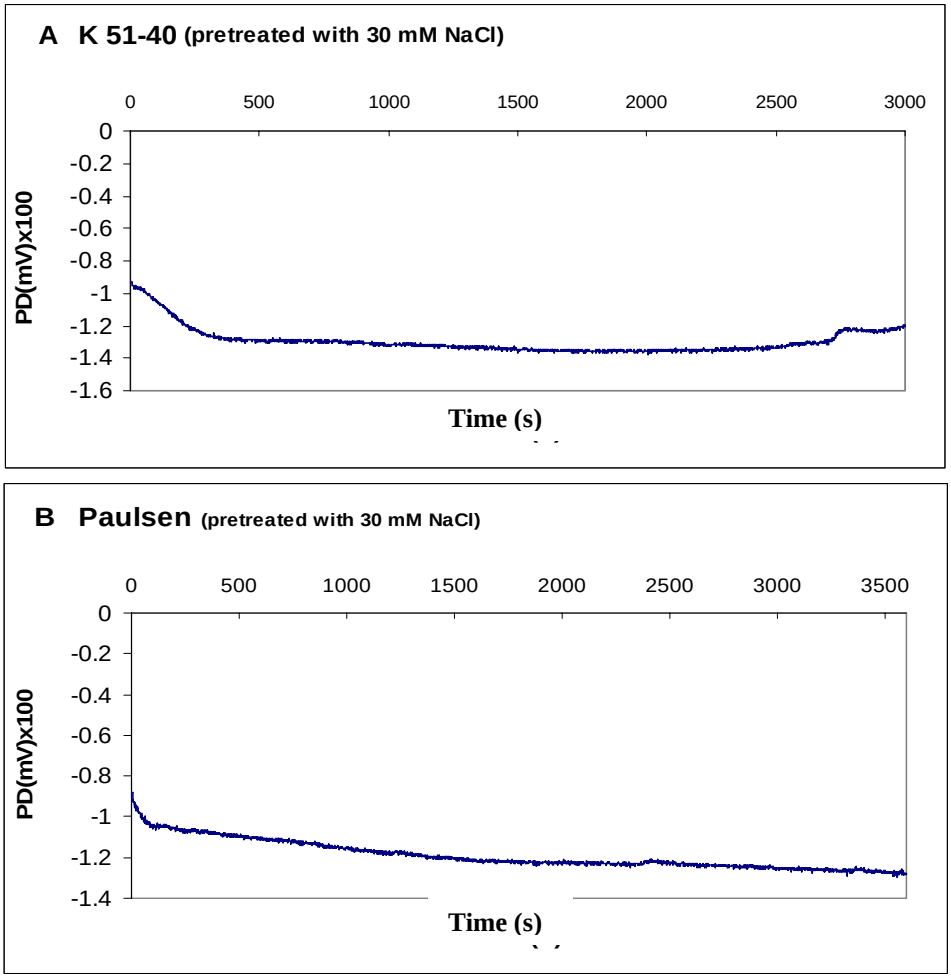

Figure 7- 1 Representative graphs of the effect of 30 mM NaCl pre-treatment of K 51-4 0 (A) and Paulsen (B) roots on the cortical cell membrane potential difference (PD) (0 time = impalement). Plants were pretreated with NaCl (30 mM) for 5-7 days and the root segments were impaled under perfusion of the same solution used for pre-treatment (n = 12).

Table 7- 1 Effect of 30 mM NaCl on membrane potential of the cortical cells of non-pretreated K 51-40 and Paulsen root. The growth condition, root impalement and solution combination as described in legend of the Figure 7-2. Hyperpolarization and depolarization times represent the time between minimum and maximum PDs, and the time between the start point of depolarization and minimum PD respectively. The average initial PD was -110 mV. Data are mean values ± SE (n = 12). No significant differences observed between varieties within the columns (t test, P <0.05).

NaCl (30 mM)	Maximum PD (mV)	Minimum PD (mV)	Depolarization Time (s)	Hyperpolarization Time (s)	Depolarization (mV)
K 51-40	-121± 13.22	-97.25 ± 6.24	134.3 ± 17.21	744.7 ± 109.2	13.75± 1.41
Paulsen	-126.2 ± 4.59	-95.1± 6.26	158.4 ± 17.58	618.3 ± 131.8	16.33 ± 2.27

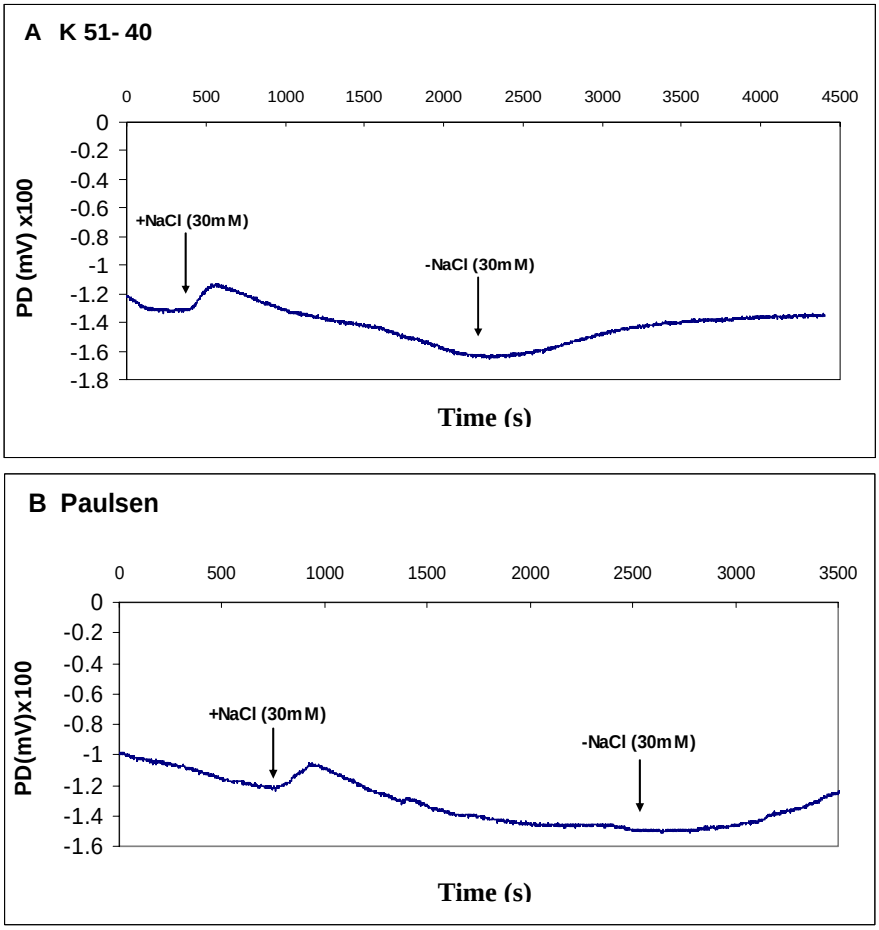

Figure 7- 2 Effect of 30 mM NaCl on membrane potential of the cortical cells of K 51-40 (A) and Paulsen (B). Plants were grown in ¼ strength Hoagland solution and impalement was made 2-5 min after transferring the root segments into the chamber. Arrows show times at which changes were induced between NaCl-free and 30 mM NaCl-containing solutions (n = 12). Depolarization values caused by 30 mM NaCl were not significantly different in both rootstocks although it was slightly greater in Paulsen (Table 7-1). Maximum and minimum PD also were not significantly different in the rootstocks but depolarization time in Paulsen was greater than K 51-40. However, hyperpolarization time in Paulsen was less than K 51-40 (Table 7-1).

Adding 0.1 mM $GdCl_3$ had slight depolarization effect on the cortical cell membrane potential in comparison to depolarizations caused by 0.1 mM $GdCl_3$ and 30 mM NaCl in combination (Fig. 7-3 A and B; Table 7-2).

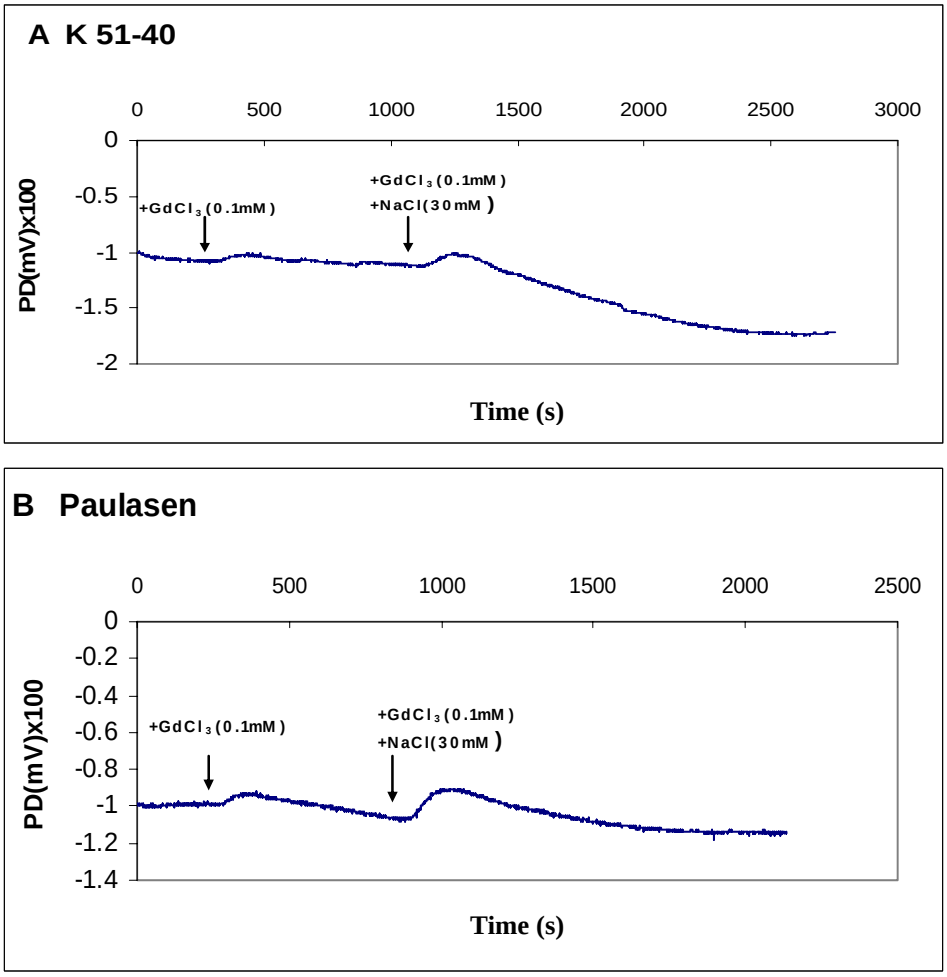

Figure 7- 3 Representative graphs of the effect of cation channel blocker ($GdCl_3$) on the response of the K 51-40 (A) and Paulsen (B) cortical cell membrane potential to 30 mM NaCl. Plants were grown in ¼ strength Hoagland solution and impalement was made on the root segments 2-5 min after transferring into the chamber. Arrows indicate times at which 0.1 mM $GdCl_3$ was added to the perfusion solution with or without 30 mM NaCl (n = 10).

Table 7- 2 Effect of 30 mM NaCl + 0.1 $GdCl_3$ on membrane potential of the cortical cells of non-pretreated K 51-40 and Paulsen root. The growth condition, root impalement and solution combination as described in legend of the Figure 7-3. Hyperpolarization and depolarization times as described in Table 7-1. The magnitude of the depolarization (mV) is a difference between the steady state and minimum PDs. Data are mean values ± SE (n = 10). No significant differences observed between varieties within the columns (t test, P <0.05).

$GdCl_3$ (0.1 mM) ± NaCl (30 mM)	Maximum PD (mV)	Minimum PD (mV)	Depolarization Time (s)	Hyperpolarization Time (s)	Depolarization (mV)	
					$GdCl_3$	$GdCl_3$+ NaCl
K 51-40	-167.5 ± 7.1	-137.2 ± 9.2	200 ± 36.56	541 ± 138.5	a 1.98 ± 0 .56	b 9.21± 1.8
Paulsen	-132 ± 4.34	-106 ± 3.95	112.1 ± 22.46	606 ± 99.5	a 2.21 ± 0.38	b 9.36 ± 2.1

7-3-2 The root surface potential

The results obtained from root surface-potential experiments showed that there is a similar trend in surface-potential changes induced by different solutions in both rootstocks; that is, by increasing of Choline-Cl, Na-MES and NaCl concentrations in the range of 0.5 – 30 mM, depolarization increased in both rootstocks (Fig. 7-4 A-F and 7-5 A and B). In the range of 0.5 – 5 mM of the above mentioned solutions, fast and relatively large hyperpolarizations were observed after small depolarizations in all treatments. In other words, the higher the external concentrations, the lower were the hyperpolarizations. There were larger depolarizations in the range between 5-30 mM of the solutions without any hyperpolarization (Fig. 7-4 and 7-5).

Comparison between Choline-Cl and Na-MES effects on the magnitude of the depolarization in K 51-40 and Paulsen root surface-potential showed that depolarization in root surface-potential of K 51-40 made by Choline-Cl in lower concentrations was greater than that of Paulsen whereas, Na-MES showed greater effect on Paulsen (Fig. 7-5 A-F). Fitting to the Michaelis-Menten equation, in the concentration range between 0.5-5 mM, showed saturation kinetics of the depolarization in both rootstocks (Fig. 7-5 C and E) whereas, no indication of saturation was observed in the concentration range between 10-30 mM (Fig. 7-5 D and F). For K 51-40 in the range of low concentrations (0.5-5 mM), V_{max} (12.73 ±

1.34 mV) and K_m (0.56 ± 0.23 mM) for Choline-Cl were higher than that of Na-MES (V_{max} = 5.27 ± 1.1 mV and K_m = 0.29 ± 0.32 mM) (Table 7-3). In Paulsen and in the same range of concentration, V_{max} (6.91 ± 0.25 mV) for Choline-Cl was lower than calculated for Na-MES while K_m (0.12 ± 0.04 mM) for Choline-Cl was nearly similar to K_m (0.10 ± 0.04 mM) for Na-MES. V_{max} for NaCl in K 51-40 were the same as Paulsen (17.24 ± 1.44 mV and 17.89 ± 1.85 mV respectively), however K_m value for NaCl in Paulsen was two times greater than that of K 51-40 (0.25 ± 0.15 mM and 0.11 ± 0.1 mM respectively). This shows that K 51-40 is more sensitive to NaCl than for Paulsen. In the range of higher concentrations (10-30 mM) of the treatments no saturation was observed in both rootstocks (Fig. 7-5 D and F).

Using NaCl did not show considerable difference between the rootstocks from the view point of magnitude of depolarization on surface-potential particularly in lower concentrations (0.5-5 mM) (Fig.7-5 A-F and Table 7-3).

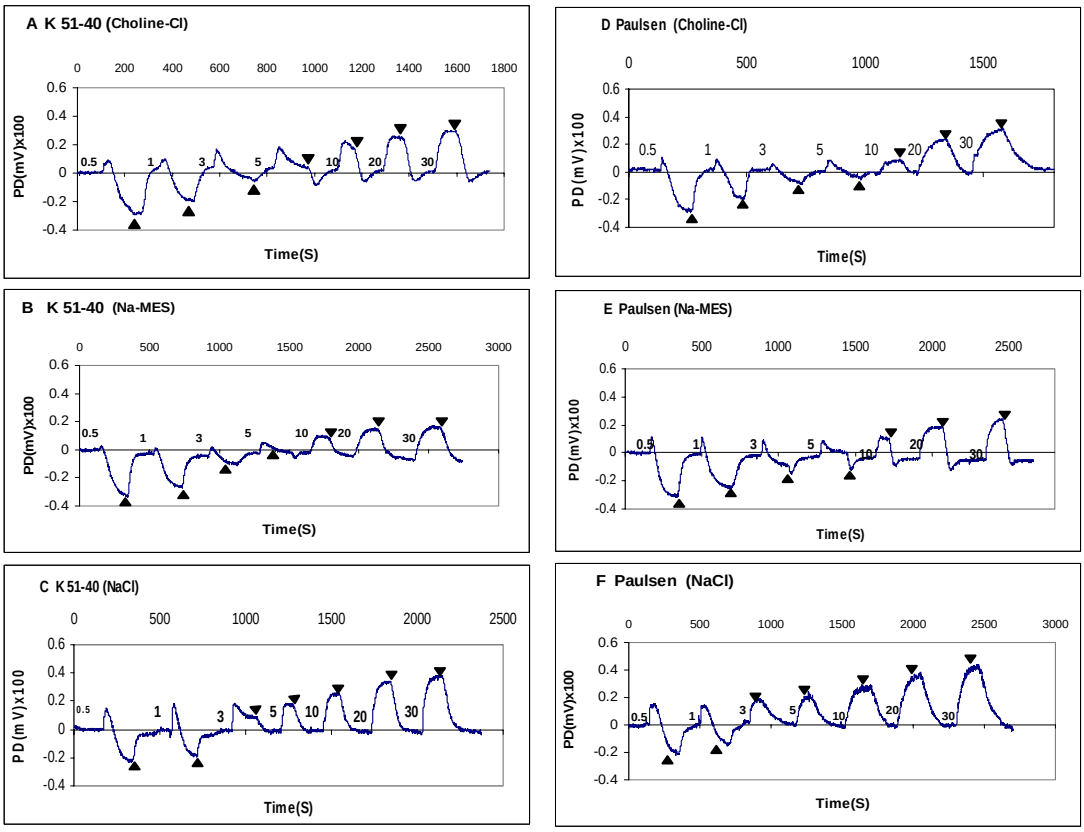

Figure 7- 4 Representative graphs indicating the effect of Choline-Cl (A and D), Na-MES (B and E) and NaCl (C and F) on the root surface-potential of K 51-40 (A, B, C) and Paulsen (D, E, F). Plants were grown in ¼ strength Hoagland solution and the surface-potential of the root segments were measured under perfusion (1 ml per 40 sec) of different concentrations (0.5-30 mM) of Chloine-Cl, Na-MES and NaCl solutions. The numbers also show adding points of the solutions to the perfusion stream. Arrows indicate times at which the above mentioned solutions were removed from the root surface and ¼ strength Hoagland solution was substituted (n = 12).

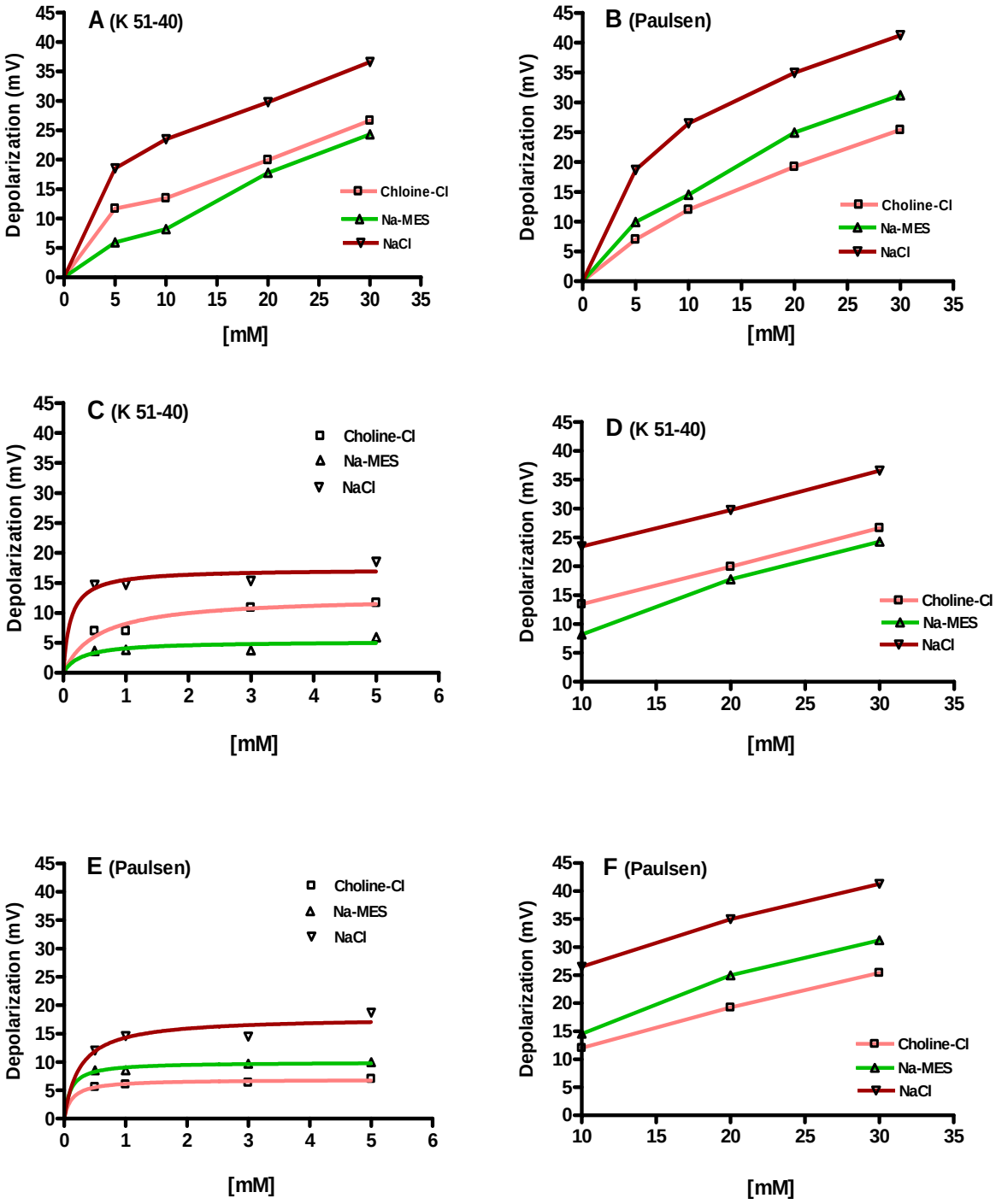

Figure 7- 5 Effect of various concentrations (0.5-30 mM) of Choline-Cl, Na-MES and NaCl on depolarization values observed in K 51-40 (A) and Paulsen (B) root surface-potential. Depolarizations depending on Choline-Cl, Na-MES and NaCl concentrations have been shown separately for the ranges of 0.5-5 and 10-30 mM of the treatments for K 51-40 (C and D) and Paulsen (E and F) fitting to Michaelis-Menten equation. The growth condition, root surface-potential measurement and solution combination as described in legend of Figure 7-4 (n = 12).

Table 7- 3 V_{max} and K_m values obtained from fitting the depolarizations magnitudes caused by low concentrations of the treatments plotted in Fig. 7-5 (C and E) to Michaeilis-Menten equation (n=12). Asterisks indicate significant differences between varieties within the rows of each parameter (t test, P<0.05).

	V_{max} (mV)		**K_m** (mM)	
	K 51-40	Paulsen	K 51-40	Paulsen
Choline-Cl	* 12.73 ± 1.34	* 6.91 ± 0.25	* 0.56 ± 0.23	* 0.12 ± 0.04
Na-MES	* 5.27 ± 1.1	* 9.96 ± 0.38	* 0.29 ± 0.32	* 0.10 ± 0.04
NaCl	17.29 ± 1.44	17.89 ± 1.85	* 0.11 ± 0.1	* 0.25 ± 0.15

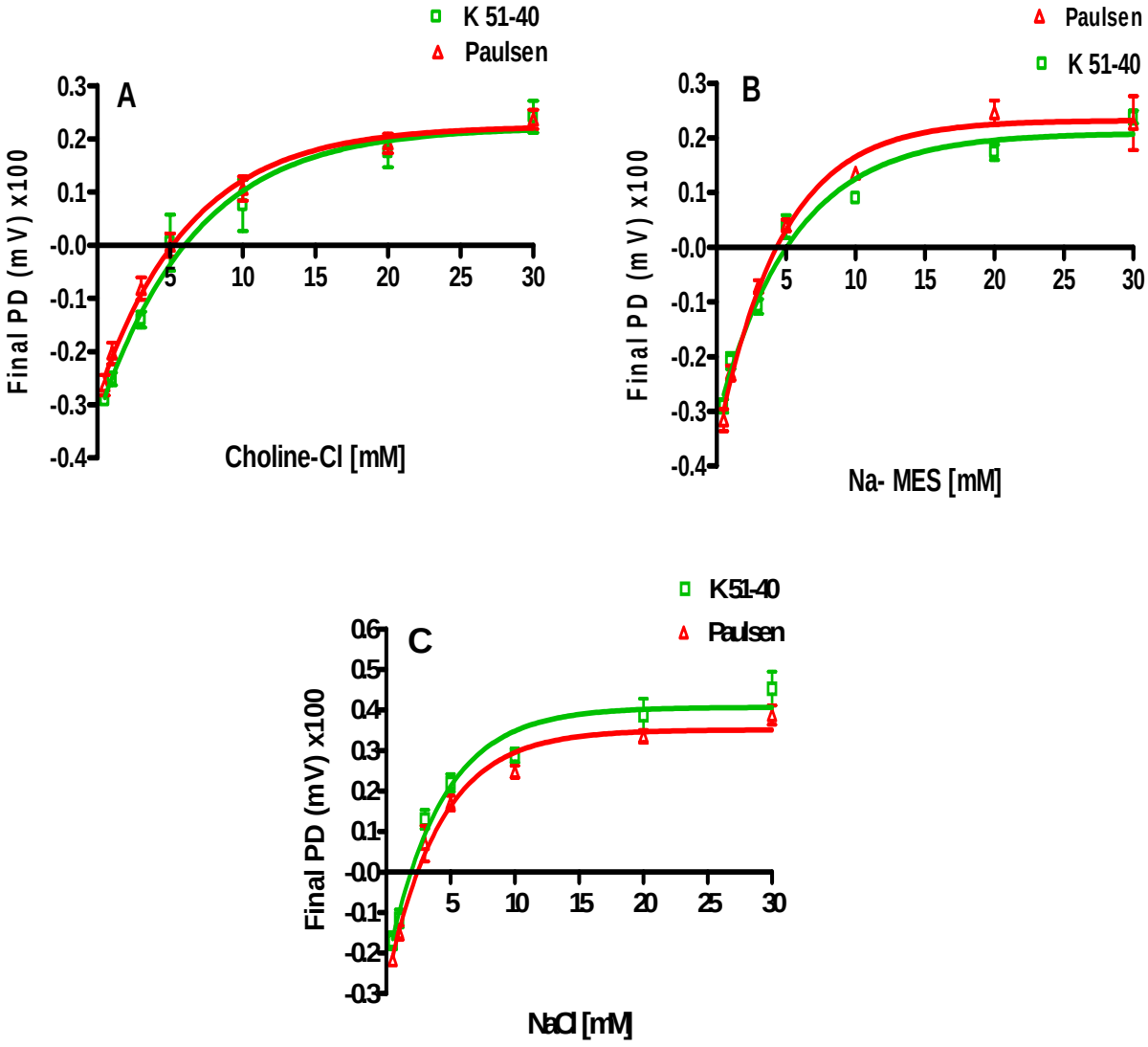

Figure 7- 6 Final surface-potential change including depolarization and hyperpolarization made by various concentrations of Choline-Cl (A), Na-MES (B) and NaCl (C) in K 51-40 and Paulsen roots. The growth condition, root surface-potential measurement and solution combination as described in legend of the Figure 7-4. Bars are SE of the means (n=12).

The final steady state surface-potential difference caused by Choline-Cl, Na-MES and NaCl, was similar in both rootstocks (Fig. 7-6 A, B and C). It can be seen that the magnitude of depolarizations made by NaCl (5-30 mM) was greater than that of Cl^- and Na^+ at the same range of concentrations (Fig. 7-4, 7-5 and 7-6).

7-4 Discussion

Membrane potential measurement of the root cortical cells displayed the same pattern in K 51-40 (salt sensitive) and Paulsen (salt tolerant) after 5-7 days pre-treatment with 30 mM NaCl. Studying electrophysiological characteristics of *Plantago maritima* (salt tolerant) and *Plantago media* (salt sensitve), Maathuis & Prins (1990) did not find any difference in the membrane potential (-115 to -120) between the species when measured in growth medium (1/4 strength Hoagland solution) with or without NaCl (25 mM). Similarly, in non-pretreated K 51-40 and Paulsen roots, average free-running membrane potential was -110 mV, which was the same as membrane potential difference in the pretreated rootstocks. Similar E_m has been reported for the cortical cells of many higher plants, *i.e* -105 mV for *Arabidopsis thaliana* and -119 mV for *Thellungiella halophila* (Volkov & Amtmann, 2006); 102 to -109 mV for *Avena sativa* and -119 to -123 mV for *Pisum sativum* (Etherton, 1963).

Volkov & Amtmann (2006) measured root cell membrane potential difference in *Arabidopsis thaliana* (salt sensitive) and *Thellungiella halophila* (a salt tolerant relative of *Arabidopsis*) before and after salt (100 mM NaCl) introduction to the root. They showed that membrane potential in the two species was not different when measured upon introduction of salt-free perfusion solution to the roots. After adding 100 mM NaCl, membrane depolarization occurred in both species, however it was smaller in *T. halophila* (37 ± 17 mV) than that of *A.thaliana* (63 ± 27 mV).

Rapid and transient depolarizations of cortex cell PD were observed in K 51-40 and Paulsen after adding 30 mM NaCl. It was followed by a gradual hyperpolarization which was identical in both rootstocks. The magnitude of depolarizations, in both rootstocks was nearly similar although it was slightly greater for Paulsen (16.33 ± 2.27 mV) than K 51-40 (13.75 ± 1.41 mV). McClure et al. (1990) reported similar electrical

responses of maize roots to 0.1 mM NO_3^-. Maize root cell membrane potential decreased 1 to 12 mV after introduction of 0.1 mM NO_3^- to nitrate-grown plants. They also showed the same response by maize root to Cl^- (0.4 mM). This response was attributed to $2H^+$ /A^- co-transport mechanism and was independent from Cl^- pre-treatment because maize roots that were grown in $CaSO_4$ or $CaCl_2$ showed the same pattern. They attributed the subsequent hyperpolarization to plasma membrane proton pump activity that was secondary to the operation of the co-transport probably due to cytoplasm acidification (Glass et al. 1992; Felle, 1994).

It has been shown that Gd^{3+} is able to block Ca^{2+} uptake in *Arabidopsis* root epidermal cells (Demidchik et al. 2002a). Gd^{3+} is a non-specific blocker of voltage-independent non-selective cation channels (Demidchik & Tester 2002). Tyerman et al. (1997) suggested that under salt stress non-selective cation channels are major pathways for Na^+ entry into plant roots, thus Na^+ uptake as well as Ca^{2+} uptake can be inhibited by Gd^{3+} (Demidchik & Tester 2002). In comparison to depolarizations caused by 30 mM NaCl in K 51-40 and Paulsen, introduction of 0.1 mM $GdCl_3$ to the perfusion solution containing 30 mM NaCl decreased depolarization by about 4.5 mV in K 51-40 and 7 mV in Paulsen. If this can be interpreted as Gd^{3+} inhibited Na^+ uptake by blocking non-selective cation channels, then it suggest that Paulsen may be more sensitive to Na^+ than K 51-40 i.e. perhaps due to more NSC channels in Paulsen.

It was also shown in this study that Na-MES contribution to depolarization in Paulsen was greater than K 51-40 (Fig. 7-5 B and E).These observations indicated that Gd^{3+} decreased initial depolarization made by Na^+ entry into the cortical cell cytoplasm by perhaps blocking Na^+ permeable channels. Application of other types of channel blockers such as La^{3+}, TEA (tetraethylammonium chloride), DPC (diphenylamino-2-carboxylic acid) is recommended.

Using the Nernst equation (Chapter 1, Eq. 1-1), it is possible to determine if Cl^- transport should be active into the cortical cells. Taking into account:

- the average membrane potential of the cortical cells of both varieties (-110 mV) assuming measured PD is across plasma membrane,

- the external Cl^- concentration (30 mM) and the cytoplasmic Cl^- concentrations (25 and 102 mM) calculated in Chapter 5 (Appendix) for Paulsen and K 51-40 respectively.

Then E_{cl} will be - 4.7 mV and + 31.2 mV for Paulsen and K 51-40 respectively. These calculations show that in order for Cl^- to be passively transported across the cell membrane, the PD would have to be well depolarized for both rootstocks. Hence, in this experiment Cl^- was actively transported against an electrical and concentration gradients across the plasma membrane. It is interesting to note that the gradient for Cl^- was considerably greater for K 51-40 than for Paulsen.

The root surface potential

To describe the results obtained from the root surface potential measurement in the root segments of K 51-40 and Paulsen, two possible explanations will be discussed below. According to Due, (1993), the symplasm of the epidermal and cortical cells of a root is electrically continuous along the whole root through plasmodesmata and it is separated from cell wall by the plasma membrane of the epidermal and cortical cells. When Choline-Cl, Na-MES and NaCl were added to the medium of K 51-40 and Paulsen with the concentration range between 0.5 to 30 mM, rapid depolarizations occurred depending on the external concentrations (Fig. 7-4). The depolarizations can be attributed to a rapid response by the cells located in the other pool of the root chamber (see materials and methods) through the above mentioned electrical continuity particularly in the epidermal cells. However, in the range of lower concentrations (0.5-5 mM), because of active transport of the applied ions into the epidermal cells, the hyperpolarizations were expected due to proton pump activity secondary to the $2H^+$ / A^- co-transport. In the higher range of concentrations (5-30 mM), rapid depolarizations were caused by the same mechanism described for the lower range of concentrations, but because of the lack of the active mechanism and moreover because of dominating ion channels activity in the epidermal and probably in outer cortical cells, no indication of hyperpolarization was observed in this range of concentrations.

The trans-root potential (TRP) is a driving force for the ion transport from cortex to the shoot and it is defined as the potential difference between the external solution and xylem sap (Graham & Bowling, 1977). Like the membrane potential, TRP includes a

passive and an active component. Measuring TRP, House & Findlay (1966) showed that increasing the external KCl concentration increased (getting more negative) the osmotic potential of the maize root xylem sap in less than 5 min due to increased KCl diffusion. Dunlop & Bowling (1971) suggested that after increasing the external KCl concentration there is a rapid depolarization in the epidermal cells, which are directly connected to the stellar exudate via the symplasm. Diffusion into the cortical cell walls is slow but will subsequently depolarize the membrane potential of the cortical cells. They assumed that the major component of TRP is in the epidermal cell membrane and the inner edge of the symplastic component in the root is located adjacent to the xylem vessels, thus the transport characteristics of the xylem parenchyma cell membranes may be dependent on the composition of the external solution via epidermal, cortical and endodermal cells symplasmic continuity.

In Paulsen, depolarizations caused by Na^+ were higher than that of Cl^- and saturation kinetics was observed for both ions (Fig. 7-5 B and E). It is possible that at lower concentrations Cl^- and Na^+ were actively transported to the xylem (higher pump activity for Na^+ efflux from the xylem parenchyma to the xylem) but in the higher range of concentrations passive transport through ion channels was dominant.
The results obtained for Cl^- and Na^+ with assumed non-permeating cation and anion and NaCl in this chapter were consistent with previous findings for NO_3^- and K^+ (Glass et al. 1992; Walker, 1994; Hayes et al. 2001). Glass et al. (1992) studied the effect of various external concentrations of NO_3^- on potential difference of the barley root cortical cell. They showed two NO_3^- uptake mechanisms, HATS (high affinity transport system) and LATS (low affinity transport system) by measuring depolarization values in the cell membrane. Below 1 mM NO_3^-, depolarization showed saturation kinetics with 60 µM K_m value, while no indication of saturation was observed in the concentrations more than 1 mM even at 10 to 20 mM. Measuring root surface-potential, Walker, (1994) showed similar saturation kinetics for K^+ uptake by the roots of *Elodea* and Hayes et al. (2001) showed that depolarizations due to K^+ uptake increased by increasing the external K^+ concentrations in the range of 3 to 100 µM in wheat root. They suggested that the depolarization is a measure of high affinity transport of K^+ in the above mentioned range of concentration.

In conclusion, membrane potential characteristics of K 51-40 and Paulsen were studied. The results showed that in grapevine, at least in K 51-40 and Paulsen, pre-treatment with 30 mm NaCl made no difference to membrane potentials of cortical cells between the two rootstocks when measured in growing medium. Adding 30 mM NaCl into the external solution caused a rapid and transient membrane potential depolarization that was followed by a gradual hyperpolarization. This hyperpolarization probably resulted in the subsequent activity of proton pumps with no considerable difference in both rootstocks. Depolarizations caused by 30 mM NaCl decreased 4.5 mV in K 51-40 and 7 mV in Paulsen in the presence of $GdCl_3$, a non-selective cation channel blocker.

Root surface-potential measurement in K 51-40 and Paulsen showed depolarizations made by Choline-Cl, Na-MES and NaCl in the range of 0.5 to 30 mM external concentrations. Choline-Cl in K 51-40 and Na-MES in Paulsen had larger effects on depolarization in TRPs. Two possible transport mechanisms were detected in the rootstocks: HATS which was detectable in lower range (0.5-5 mM) and LATS, which was observed in higher range (10-30 mM) of concentrations. It is also recommended that using other types of channel blockers and uncouplers will be useful to test the above mentioned possibilities.

Chapter 8

General Discussion and Future Perspectives

8-1 Discussion

Accumulation of Cl^- in grapevine leaves in toxic concentrations, leads to marginal chlorosis and necrosis (Walker, 1994). Certain grapevine rootstocks have Cl^- restriction and exclusion capacity that can reduce Cl^- uptake and transport to the shoot (Walker et al. 2004). Little is known about Cl^- uptake and compartmentation mechanisms in plants (Britto et al. 2004). In this study Cl^- transport mechanisms were investigated in two grapevine rootstocks with different ability of Cl^- exclusion, using K 51-40 as a salt sensitive and Paulsen as a salt tolerant variety.

There are abundant reports on influences of salinity on nutrient uptake and sequestration (Fisarakis et al. 2004; Britto et al. 2004; Abdelghadir et al. 2005). Wallace & Berry (1981) showed that yield reduction of wheat was due to NO_3^- deficiency induced by Cl^- toxicity. Increasing NO_3^- levels in soil had an ameliorative effect on Cl^- toxicity on avocado and citrus (Bar et al. 1997). I have studied Cl^- and NO_3^- selectivity in two grapevine rootstocks differing in salt tolerance. In K 51-40, salt sensitive rootstock, NO_3^- accumulation in root and shoot decreased by increasing the external Cl^- concentrations but no obvious effect of Cl^- on NO_3^- accumulation was observed in Paulsen, the salt tolerant rootstock. NO_3^- / Cl^- selectivity was decreased by increasing the external NaCl in K 51-40 while in Paulsen it showed no significant difference between salt treatments. Cl^- concentration in the shoot of Paulsen was less than that of K 51-40 whereas greater proportion of NO_3^- was transported to the shoot of Paulsen (Chapter 2). This was consistent with Walker et al. (2004) who showed low Cl^- concentration in petiole, lamina and juice of sultana on Paulsen roots in comparison to sultana on own roots. NO_3^- concentrations in roots of both rootstocks were nearly similar thus it was concluded that xylem-loading transport is probably different in the rootstocks in terms of regulation and/or selectivity.

Water and solute flow across a root occurs via a combination of three pathways: apoplastic, symplastic and trans-cellular (Steudle, 2000). The apoplastic pathway including cell wall, intercellular spaces and lumen of xylem vessels, is blocked by Casparian bands of the endodermis and exodermis (Ranathunge et al. 2004). However, in the absence of Casparian bands (interruption of endodermis due to development of lateral roots primordia and non-developed endodermis) bypass flow of water and solutes may occur (Yeo et al. 1987). From other studies, where closely related species or cultivars have been compared, it is possible that transport to the shoot was linked to by-pass flow through the apoplast (Yeo et al. 1999; Anil et al. 2005; Yeo & Flowers 1982). In three experiments (Chapter 3 and 4) of this study it was attempted to provide some evidence to show possible differences between the rootstocks concerning the bypass flow pathway in water and solute transport.

Using the pressure probe technique, it was shown that hydrostatic and osmotic hydraulic conductivity (L_{p_r}) and reflection coefficient (σ_s) of K 51-40 and Paulsen were similar. Both varieties showed differences when the nature of the driving force on water changed. For both rootstocks, the hydrostatic L_{p_r} was considerably greater than osmotic L_{p_r} (Chapter 3). This is consistent with previous findings where there are 2-3 orders of magnitude differences between osmotic and hydrostatic water flow (Steudle, 2000). This variability can be due to dilution of the xylem contents resulting from water uptake (Fiscus, 1975), or involvement of plasmodesmata that could act like pressure sensitive valves (Passioura, 1988).

Interaction between the protoplastic and apoplastic pathways (the composite transport model) with the reflection coefficient close to unity and close to zero respectively, may cause a low overall reflection coefficient. This model was proposed by Steudle et al. (1993) to explain the variability of root hydraulic properties. Recently, Bramley et al. (2007) suggested that applying a series of pressure clamps to the xylem by the root pressure probe, gives a reliable estimate for L_{p_r} and decreases the above mentioned difference observed for hydrostatic and osmotic L_{p_r}. The reflection coefficient (σ_s) values obtained for K 51-40 and Paulsen using NaCl and $NaNO_3$ showed less than unity values (0.48-0.59) with no differences between the rootstocks. It was consistent with

the composite model and indicated that by-pass flow of salts to the xylem is the same for both rootstocks.

Casparian bands are made of suberin and lignin depositions in radial and tangential walls of endodermis and exodermis. They are known as apoplastic barriers that prevent water and solutes flow through cell walls (Steudle & Frensch 1996). There are variable results in the literature about the role of endodermis and exodermis in water uptake. Steudle, (1993) showed that puncturing of endodermis had no effect on the hydraulic conductivity. Whereas, North & Nobel (1991) showed that water flow decreased as suberin lamella developed in the endodermis. Considering these variable results about endodermis and exodermis functions in water permeability, Steudle & Peterson (1998) suggested that root transport properties could change over development stages.

Root anatomical studies of K 51-40 and Paulsen (Chapter 3) showed that there were no obvious differences in apoplastic barriers of the main and lateral roots. The similar root hydraulic conductivity of the rootstocks was related to similar root anatomy.

In addition to water, measurement of solute uptake using an apoplastic tracer, PTS (3-hydroxy-5, 8, 10-pyrentrisulphonic acid) showed that an apoplastic by-pass pathway is involved in ion transport at high external concentrations (Pitman, 1982). The importance of the transpirational by-pass flow in Na^+ uptake across rice root has been studied by Garcia et al. (1997). There was a high correlation between Na^+ uptake and the transpirational by-pass flow in rice. The contribution of the transpirational by-pass flow to Cl^- uptake by roots of K 51-40 and Paulsen was tested using PTS. At high external NaCl concentrations (30 mM), nearly similar values of PTS transport to the xylem were estimated for K 51-40 and Paulsen. However, the leakage of Cl^- to the xylem of K 51-40 was about two times greater than that of Paulsen. It was consistent with $^{36}Cl^-$ fluxes to the xylem obtained in Chapter 5 (Table 5-2). Also no correlation was found between Cl^- and PTS transport. These results were consistent with the results including similar apoplastic by-pass flow of both rootstocks and high Cl^- accumulation in shoot of K 51-40 obtained from previous experiments. Taken together these results agree with the findings of the previous experiments, it can be concluded that there are differences in membrane transport properties. Hence, more studies at the root cell level in comparison to overall hydraulic properties of the root are suggested.

Study of $^{36}Cl^-$ fluxes into intact and excised roots is a simple and reliable method to investigate Cl^- dynamics at the cellular level (White and Broadley 2001). $^{36}Cl^-$ fluxes across plasma membrane and tonoplst of K 51-40 and Paulsen root cells was studied conducting influx and efflux experiments (Chapter 5). The results showed that over the first 10 minutes, $^{36}Cl^-$ influx rate into the root of Paulsen was greater than K 51-40. A similar result was found from $^{36}Cl^-$ efflux. Unidirectional influx kinetics over 10 min showed that by increasing external concentrations of Cl^-, the cytoplasmic $^{36}Cl^-$ influx rate increased in both rootstocks but it was higher for Paulsen. It seems that Paulsen shows high energy inefficient cycling. Cram, (1973) and Cram & Laties (1971) showed that by increasing external Cl^-concentrations, Cl^- influx across the plasmalemma of isolated root cortical cells of maize increased. Paulsen had a higher V_{max} than K 51-40 but K_m was not significantly different.

The importance of lateral roots of grapevine in nitrate uptake has been studied by Volder et al. (2005). They showed that by aging the lateral roots, NO_3^- uptake decreased. In Paulsen the main root had greater contribution to $^{36}Cl^-$ uptake than lateral roots (Chapter 5). It has been shown that $^{36}Cl^-$ transported by the roots of intact plants are mostly translocated to the shoot (Pitman, 1971). I showed that $^{36}Cl^-$ accumulation and transport rate to the shoot of K 51-40 was greater than that measured for Paulsen. It was consistent with the results of Chapter 2 which showed high Cl^- contents in the shoot of K 51-40 in comparison to Paulsen. Davenport et al. (2005) showed high $^{22}Na^+$ contents in the shoot of salt sensitive wheat genotype (Tamaroi) in comparison to a salt tolerant genotype (line149). Because influx rate to the root in two genotypes was similar, it was suggested that efflux rate of $^{22}Na^+$ from the xylem parenchyma cells to the xylem vessels of salt sensitive genotype (Tamaroi) must be higher than the salt tolerant genotype (line 149). I estimated that efflux rate from the xylem parenchyma cells to the xylem vessels (Φ_{cx}) in K 51-40 (salt sensitive) was 2 times greater than for Paulsen (salt tolerant).

Compartmental analysis of $^{36}Cl^-$ efflux showed that the difference in influx rate (Φ_{oc}) observed between the rootstocks was consistent with measures of rapid $^{36}Cl^-$ uptake (Exp. 1 and 2 in Ch. 5). Compartmental analysis also showed that the ratio of main to lateral root influx rate was the same as the result obtained for $^{36}Cl^-$ uptake (Exp. 4 in Ch. 5).

It was also shown that the estimated cytoplasmic Cl^- concentration in K 51-40 was higher than for Paulsen. Higher $^{36}Cl^-$ efflux (95 % of influx) in the rootstocks was consistent with the finding by Britto et al. (2004). They showed that Cl^- efflux reached to 90 % of influx with increased $[Cl^-]_{ext}$ in barley roots. The results obtained in experiment 6 of Chapter 5 showed that Paulsen could keep the cytoplasmic Cl^- concentration low possibly by greater efflux to the vacuole or outside. Overall, the results of the flux experiments indicated different properties of cell membranes in the root of K 51-40 and Paulsen concerning transporters or channels.

According to the results collected from the above mentioned experiments, it can be concluded that the root of the tested rootstocks controled Cl^- accumulation in the shoot. In order to know how this control is exerted by the root, X-ray microanalysis technique was used to determine Cl^-, Na^+ and K^+ distribution patterns in different cell types of the main and lateral roots of K 51-40 and Paulsen (Chapter 6). The hypodermis and cortical cells of both rootstocks accumulated high content of Cl^- than other cell types. It was consistent with findings by Zheng & Yan, (1996) in rice root and Kelday & Bowling (1980) in *Commelina communis* root. In both plants, vacuoles of the cortical cells accumulated higher level of Cl^- than other cell types. Overall Cl^- content in the root of Paulsen was higher than K 51-40. It was consistent with the results obtained in Chapter 2 and 4. These results taken together with the higher level of Cl^- and Na^+ estimated in pericycle of the main root of Paulsen in Chapter 6, indicated that Cl^- and Na^+ loading to the xylem was different in the rootstocks Paulsen tended to prevent the ion loading process by vacuolar sequestration and efflux across the plasma membrane in the cortex to keep the cytoplasmic Cl^- concentration low. It has been reported that by increasing nutrient concentration in soil, development of new and young lateral roots with high N uptake capacity increased in grapevine (Robinson, 1994). Unlike the main root, lateral roots of K 51-40 accumulated more Cl^- and Na^+ than Paulsen. In both rootstocks, Cl^- and Na^+ accumulated by lateral roots can be probably used as osmotica in root cell expansion.

Passive or active transport of an ion can be determined by measuring membrane potential difference and from estimation of the ion concentrations at the two sides of the membrane. In most circumstances, Cl^- is driven out of a cell due to electrochemical gradient, thus Cl^- import requires overcoming the electrochemical barrier (Felle, 1994). The effect of high NaCl concentration (30 mM) on the potential difference of the

cortical cells and on the root-surface potential was studied in K 51-40 and Paulsen root segments using microelectrodes (Chapter 7). The average membrane potential of the cortical cells of the rootstocks was -110 mV, which is similar to that reported for many higher plants. A rapid and transient depolarization was observed by adding 30 mM NaCl to perfusion solution in both rootstocks. It was slightly greater in Paulsen (16.33 ± 2.27 mV) than K 51-40 (13.75 ± 1.41 mV). This depolarization was followed by a gradual hyperpolarization.

McClure et al. (1990) showed a similar depolarization (1-12 mV) in the cortical cells of maize root by addition of 0.1 mM Ca $(NO_3^-)_2$. Such a response was attributed to $2H^+ / A^-$ co-transport and later proton pump activity in plasma membrane resulted in the co-transport process (McClure et al. 1990). Adding $GdCl_3$ (0.1 mM) decreased depolarization induced by 30 mM NaCl (4.5 and 7 mV for K 51-40 and Paulsen respectively). Gd_3^+ is a non-selective cation channel blocker. Non-selective cation channels may be a major pathway for Na^+ transport into plant roots under salinity (Tyerman et al. 1997). It can be concluded that Gd^{3+} decreased Na^+ transport by blocking non-selective cation channels and then decreased depolarization in both rootstocks. Furthermore, Paulsen seemed to be more sensitive to Na^+ than K 51-40 based on the Gd^{3+} effect and the surface potential but K 51-40 seemed more sensitive to Cl^-.

The root surface potential method was used to measure depolarization caused by Choline-Cl (30 mM), Na-MES (30 mM) and NaCl (30 mM) (Chapter 7). Of the salts, Choline-Cl in K 51-40 and Na-MES in Paulsen caused large depolarization. It was shown that $^{36}Cl^-$ efflux from the xylem parenchyma cells to the xylem vessels was higher in K 51-40 than Paulsen (Chapter 5). It was concluded that the higher depolarization by Choline-Cl in K 51-40 can be likely due to higher Cl^- efflux rate to the xylem. Measuring the trans-root potential, two transport mechanisms were detected for Cl^-: HATS was observed in the range of 0.5 to 5 mM and LATS was detected in the range of 10-30 mM of the external solution.

Assuming the cytoplasmic Cl^- concentrations, 102 mM for K 51-40 and 25 mM for Paulsen (calculated in Chapter 5, Appendix), E_{Cl} of various external Cl^- concentrations applied in the surface potential experiment were calculated using the Nernst equation

(Chapter 1, Eq. 1-1) (Fig. 8-1; Table 8-1). The measured cortical cell membrane potential was -110 mV (Chapter 7). Figure 8-1 shows that Cl^- transport in the roots of both rootstocks was active in the range of 1-30 mM external Cl^- concentration. In both rootstocks there was an electrochemical gradient that decreased with increases in external Cl^-. This gradient was greater in K 51-40 than Paulsen. It means that in the range of 1-30 mM or more of external concentrations, Cl^- must overcome the above mentioned electrochemical gradients to be transported in both rootstocks. Hence, Cl^- transport will be an active process in the range of 1-30 mM of Cl^- concentration.

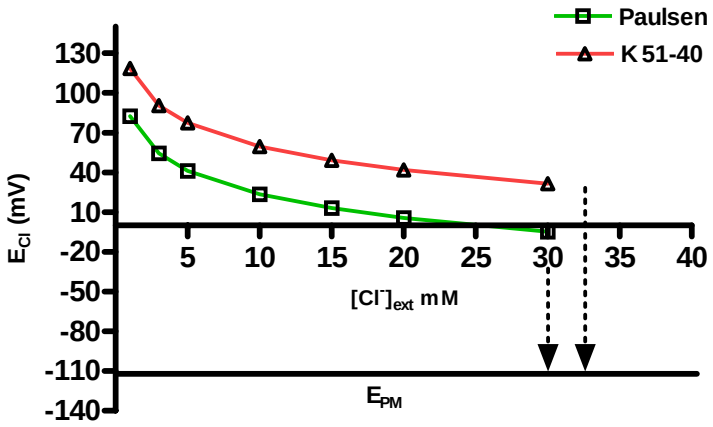

Figure 8- 1 E_{Cl} values calculated from various external Cl^- concentrations. Electrochemical gradients of Cl^- transport have been shown by dotted lines between the measured and calculated potential difference values.

It is assumed that $[Cl^-]_{cyt}$ was constant and the same as that measured by $^{36}Cl^-$ efflux in 30 mM external $[Cl^-]$. This is unlikely; $[Cl^-]_{cyt}$ is more likely lower in the lower $[Cl^-]_{ext}$.

8-2 Future perspectives

The results obtained in this study showed obvious differences between the rootstocks in Cl^- transport not only from outside to root (radial transport) but also from root to shoot (axial transport). It was shown that such a difference can be attributed to different cell membrane properties rather than the involvement of bypass flow pathway or apoplastic barriers. The root pressure probe experiment showed similar bypass flow of salt to the xylem for both rootstocks. However, the cell pressure probe

technique can be used to show possible difference between the rootstocks from the view point of solute transport and selectivity through the plasma membrane.

It is possible to study Cl^- transport directly in isolated membrane vesicles. Using isotopes and measuring membrane potential difference across the isolated membranes have been examined in many studies (Martinoia et al. 1986; Pope & Leigh 1987; Bennett & Spanswick 1983). Probing Cl^- transport with isolated membrane vesicles using a fluorescence technique can also give direct measurement of Cl^- fluxes through transporters. S.P.Q (6-methoxy-N-(3-sulfopropyl) quinolinum) is a high water solute and membrane permeant fluorescent Cl^- indicator dye. This technique is based on the quenching of S.P.Q by Cl^-. S.P.Q is loaded into the vesicles and Cl^- transport can be followed via S.P.Q fluorescence quenching at the presence of Cl^- using a spectrofluorometer.

The cell membrane potential and the root surface potential were measured in this study. Concentration kinetics experiment showed two transport mechanisms for Cl^- transport. High affinity transport system (HATS) was active in the range of 0.5-5 mM and low affinity system was responsible for Cl^- transport in the range of 10-30 mM. In order to obtain more details and evidences about ion transport mechanism (s) in grapevine roots under salinity, it is recommended to use different channel blockers such as TTX, TEA, La^{3+}, Niflumic acid, Anthracene-9-carboxylic acid (A9C) and Ethacrynic acid.

The patch clamp is a technique by which ion channels activities across the cell membranes can be analysed. It is also applied for measurement of current through ion pumps (Findlay et al. 1994). High salinity can change ion channels activity due to sensitivity of opening and closing of some channels to salinity. Ion channels are able to alter membrane potential difference. Voltage clamp, using ion channel blockers, vesicle technique and measuring ion channel selectivity are some of the many methods which are currently used in electrophysiological assay of ion channels (Tyerman & Skerrett, 1999). The results obtained in this study indicated that higher Cl^- efflux from the cytoplasm of cortical cells to external medium in Paulsen and higher Cl^- efflux from the cytoplasm of the xylem parenchyma cells to the xylem vessels in K 51-40 could possibly be through Cl^- channels. Using patch clamp

technique on isolated cortical and stellar protoplasts, it is possible to investigate Cl^- efflux current across the above mentioned cell membranes. This current may depend on the number of channels and on the opening rate. Also, it was shown in this study that the cytoplasmic Cl^- concentration differs between the rootstocks, so the concentration dependence of Cl^- efflux can be comparatively studied by whole cell patch clamp in the rootstocks.

Different salt tolerance capacities between grapevine varieties probably can be a reason for involvement of particular gene or genes in this process. Sykes, (1987) suggested that Cl^- accumulation in the petioles and leaves of grapevine is controlled by a single dominant gene in grapevine. Abel, (1969) proposed *Ncl* and *ncl* gene symbols for the dominant chloride excluder and the recessive chloride includer, respectively. However, up to now, there is no direct evidence in isolating any anion (Cl^-) channel gene in grapevine. Further work and the final aim of this study can be the comparison of the two rootstocks by identification of the probable genes coding for Cl^- transporters. Considering that grapevine genome has been sequenced recently, microarray technique can be used to compare the salt sensitive and salt tolerant rootstocks in terms of expression profiling of stress-inducible genes.

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