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**Root Pressure: A reexamination of the
infeasibility of the osmometer mechanism**

by

Linda C. Enns, B.Sc. (Hons.)

A thesis submitted to
the faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of

Master of Science

Department of Biology

Carleton University

Ottawa, Ontario

August 25, 1998

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***“There are times when I’m not certain Man is
absolutely certain what he knows
for he always finds confusion in conclusions he
concluded long ago...”***

- The King and I

The undersigned recommend to the Faculty of Graduate
Studies and Research acceptance of the thesis

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infeasibility of the osmometer mechanism”**

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the degree of Master of Science

Thesis Supervisors

Chair, Department of Biology

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Abstract

Root pressure is currently explained by the osmometer model, whereby solutes accumulate in the xylem sap to create a passive flow of water movement along an osmotic gradient from the more dilute soil solution. To test this, hydroponically-grown corn seedlings were put into a range of osmolalities of polyethylene glycol (PEG) M.W. 3500, and the elemental concentration of their xylem sap measured using EDX analysis. An external solution of 3.0 bar temporarily stopped guttation, but plants could recover their guttation after a couple of days, even though there was only about 1.5 bar worth of solutes in the xylem sap. The possibility that there might exist higher solute concentrations further down towards the base of the xylem vessels was investigated by determining the elemental composition of the xylem sap along the entire lengths of vessels of roots of guttating corn seedlings. The solute concentrations remained low all the way to the base of the LMX and EMX vessels. Higher solute levels in the living cells of the root may enable the corn seedlings to guttate in higher external osmolalities. Recovered plants in 3.0 bar PEG had about 20 mM more K in their xylem parenchyma, cortex, and endodermis. Plants which had been pre-treated with additional KNO_3 were able to guttate faster than control plants, and in higher external osmolalities of PEG. It is proposed that during root pressure, the living cells of the root are osmotically drawing in water from the soil, rather than the dead xylem vessels.

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List of Abbreviations

BeW	Be-window
c	cortex
Ci	inner cortex
CSEM	cryo-scanning electron microscopy
EDX	energy-dispersive x-ray
EMX	early metaxylem
En	endodermis
LMX	late metaxylem
LN₂	liquid nitrogen
Pe	pericycle
PEG	polyethylene glycol
SD	standard deviation
SE	standard error
Xp	xylem parenchyma
UTW	ultra-thin window

Frontispiece

Seedling of *Zea mays* L. (cv. Seneca Chief), guttating in a hydroponics solution adjusted with polyethylene glycol (PEG) M.W. 3500 to an osmolality of 3.0 bar. 1.5 X.

CHAPTER 1

General Introduction

Root pressure is the positive pressure which develops in the xylem sap of slowly or non-transpiring plants (Kramer and Boyer, 1995). It can be observed by the bleeding of solution (exudate) from the injured regions of many plants, such as when stems, roots, branches or leaves are cut across, or when a hole is bored into a maple tree in the spring (Pfeffer, 1899). It can also be visualized by the presence of guttation, a phenomenon which occurs in grasses and other herbaceous plants, whereby in the absence of transpiration, droplets of water are exuded from the margins and tips of leaves (Frontispiece) (Stocking, 1956).

The importance of root pressure is still being debated. Before 1938, most plant physiologists believed it to be inconsequential, since the maximum pressures recorded were no greater than 1.4 atmospheres, and were transitory - it was calculated that pressures of at least 13 atm would be required to push water to the top of a 350 ft tree (White, 1938). These arguments were quashed by White's dramatic experiment of 1938, showing pressures of greater than 6 atm, and estimated to be no less than 10 atm, in the cultured roots of tomato.

In spite of such experiments, some plant physiologists still maintain that root pressure plays a minor role in plant water relations (Kramer and Boyer, 1995). Evidence is accumulating, however, which implies that it may be indispensable. Root pressure is important for the repair of embolisms (Tyree and Sperry, 1989), and such embolisms have been observed to occur daily in plants such as corn, refilling even during transpiration (McCully *et al.*, 1998).

These observations imply that root pressure may be working, 24 hours a day, to capacitate water uptake and transport.

The mechanism for root pressure is highly controversial, and is the subject of this thesis. From the mid-18th to mid-19th century, root pressure was believed to be brought about by the activities of living cells of unidentified tissues in either the root or stem (Hales, 1727), but with the development of the cohesion theory of sap movement (Dixon and Joly, 1895) people began to reject the idea that living cells were necessary for the ascent of sap, and so-called “vital theories” were abandoned for more mechanical ones. The pendulum swung briefly back to possible vital theories in the mid-1900’s by dissenters such as Van Overbeek (1942), but such revolutions were quashed quickly (Eaton, 1943).

Currently, the most favoured model for root pressure, and the one which is found in most modern textbooks, is the osmometer model, which states that root pressure works by a build-up of solutes in the dead xylem vessels, which lowers the osmotic potential of the solution, or sap, below that of the external soil solution, driving the passive movement of water along an osmotic gradient (Kramer and Boyer, 1995; Kramer, 1983; Taiz and Zeiger, 1991). (The two other, less-favoured models of secretion and electroosmosis will not be discussed, but an excellent review of these and other models for root pressure can be found in Schwenke and Wagner, 1992). In mathematical terms, the osmometer model can be expressed by the equation:

$$J_v = L_p \sigma \Delta \Pi$$

Here, J_v is the total volume flow of water, L_p the hydraulic conductance, σ the reflection coefficient, and $\Delta\Pi$ the difference in osmotic potential between the xylem sap and the external solution (Kramer, 1983).

The osmometer model of root pressure has been challenged many times in the last fifty years, by observations that when detopped root systems were put into solutions encompassing a range of osmolalities, the osmolality required to stop exudation from the cut stump of the stem was much higher than that of the xylem sap (Van Overbeek, 1942; Broyer, 1951; Ginsburg and Ginzburg, 1970; Zholkevich *et al.*, 1980; Mozhaeva and Pil'shchikova, 1980).

These observations led to the proposal that there was a nonosmotic component, J_v^* to the equation for water movement by osmosis, making it:

$$J_v = L_p \sigma \Delta\Pi + J_v^*.$$

There were speculations by some that this extra component involved the injection of water from living cells into the xylem, possibly by contraction (Zholkevich *et al.*, 1980; Mozhaeva and Pil'shchikova, 1980).

There were two criticisms towards these experiments, which resulted in a failure to incorporate the new component into the water movement equation: 1) in many cases, such as in Van Overbeek's experiment, tissue-permeating solutes (ie. of small molecular weight) were used in the external solutions (Arisz *et al.*, 1951); and 2) exudate measurements from the base of the stem were not necessarily representative of what might be found in the xylem

sap further down in the root, where water was being absorbed (Arisz *et al.*, 1951; House and Findlay, 1965). A third criticism, which was never actually proposed by anyone other than this author, is that up until now, most root pressure studies have been conducted on excised root systems and not on intact plants. (An exception to this is the extensive work which has been done by Steudle using the root pressure probe (Steudle *et al.*, 1987; Steudle and Frensch, 1989; and others), but these studies do not deal with the feasibility of the osmometer model.) The three criticisms mentioned above will be explained in more detail, and summarily dealt with, in the next two chapters.

Interestingly, many modern-day studies still fail to show that solute concentrations in the exudate of isolated root systems account for all observed exudation (Zhang *et al.*, 1995), but nonetheless fail to re-examine the osmometer model as being possibly faulty.

In this thesis, the facts on which the osmometer model is based will be reexamined, and the criticisms towards Van Overbeek's and others' experiments invalidated, so as to come up with a definitive answer as to the viability of the osmometer model. Root pressure of hydroponically-grown, intact plants will be measured by exploiting the phenomenon of guttation, and impermeable solutes of large molecular weight will be used to control the osmolarity of external solutions. The composition of xylem sap will be measured *in situ*, using energy dispersive x-ray EDX analysis.

Chapter 2

Comparisons of the osmolalities of root xylem sap and of the external solution for plants generating root pressure

Introduction

One of the main reasons for the dismissal of Van Overbeek's results was his use of mannitol to control the osmolality of his external solutions. He added the mannitol dropwise to his external solution to the point where sap ceased exuding from the stump of his decapitated root system. It was believed by his critics that the mannitol was permeating his root tissue, resulting in the need for a higher mannitol concentration in his external solution (Eaton, 1943). Arisz *et al.*, 1951, condemned Van Overbeek's work because he transferred his root systems back into water before measuring the osmolality of the exudate, and any mannitol which had entered the xylem during his external solution measurements might have dissipated, resulting in a low measurement of sap osmolality.

Another objection to Van Overbeek's results was that they were not always consistent with those of others, such as House and Findlay, 1965, who used isolated maize roots to show high solute concentrations in xylem sap and to conclude that the non-osmotic component of root pressure, while present, was relatively small and unimportant.

My experiment repeated Van Overbeek's work, with two major changes to his protocol. Firstly, instead of mannitol, non-permeating polyethylene glycol (PEG) mwt 3500 was used to control the osmolality of the external solution. To precisely control the water potential of the external solution osmotically, plants were grown hydroponically - the water potential of a soil solution is

much more difficult to measure and control. While the use of a hydroponics system is the ideal way to study water relations in plants, it requires careful maintenance. Hydroponics systems must be well aerated, nutrient solutions must be prepared and changed frequently so that hydroponic solutions do not become unbalanced due to the selective uptake of ions by the plant roots, nor become toxic due to the secretion of compounds by the plant such as amino acids and carbohydrates, and the pH of the hydroponics solution must also be frequently adjusted because of plant secretions. Trace elements such as iron, in chelated form, must be adequately present in the hydroponics solution to avoid nutrient deficiencies (Klein and Klein, 1988).

Secondly, whole plants were used instead of decapitated root systems. Instead of observing and analyzing exudate from the cut end of a root system, root pressure was assessed by the presence of guttation, and the elemental composition of the root xylem sap was measured *in situ*, using energy-dispersive x-ray (EDX) analysis of bulk-frozen roots (roots frozen while still attached to the plant). EDX analysis is optimal for studying the elemental contents of cell solutions, because the freezing preparation maintains the tissue at its full water content and volume, and water soluble substances are trapped at, or close to, their positions *in vivo* (Huang et al., 1994).

Materials and Methods - General

Plant material

Seedlings of *Zea mays* L. (cv. Seneca Chief) were initially grown in well-watered Pro-mix general purpose growing medium (Premier Horticulture, Rivière-du-Loup, Qué), 6 per 12 in pot, in a growth chamber at Carleton University in 24 h of fluorescent light (CW, VHO, Phillips) at a temperature of 21° C, during the month of February. After approximately 1 week, when the first leaves had emerged from the coleoptiles, and the root systems consisted of single, approximately 20-cm-long primary roots, the plants were carefully excavated, and their roots gently rinsed with water. They were then transferred to a hydroponics system.

The hydroponics system consisted of several 1 L containers, each of which contained a dilute Hoagland's solution with enriched iron, the ideal dilution factor of which was pre-determined (Appendix 1). Per litre, the hydroponics solution contained as follows: KNO_3 , 1.3×10^{-1} g; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 1.2×10^{-1} g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6.2×10^{-1} g; KH_2PO_4 , 3.4×10^{-2} g; EDTA/ferric-sodium salt, 2.9×10^{-2} g; H_3BO_3 , 2.9×10^{-3} g; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.8×10^{-3} g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.2×10^{-4} g; MoO_3 , 8.2×10^{-5} g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 8×10^{-6} g). The solution was adjusted to a pH of approximately 4.5, using 5% HCl solution. The containers were aerated for 30 minute intervals, once an hour, twenty-four hours a day. Two plants were grown in each container,

suspended by their mesocotyls using foam plugs, so that their roots were completely immersed in solution. In all, there were 18 containers, or 36 plants. Each plant was individually labelled.

The hydroponics system was kept in the Carleton University greenhouse, under a light regime of 10 h darkness + 12 h HID metal halide illumination imposed upon 14 h of natural daylight.

The plants were grown in hydroponics for another approximately five days, after which time they had three unfolded leaves, some with the fourth leaf emerging, and a single primary root of about 40 cm. Such roots are known to have several cm of mature LMX vessels at their proximal end (see Appendix 2; also Wenzel et al, 1989). Plants which appeared small or unhealthy were discarded.

Material & Methods for Experiment 1 - determination of maximum osmolality of external solution and xylem sap in guttating plants

Plants were treated with solutions ranging in osmolality from 1.5 to 5 bar, made by adding polyethylene glycol mwt 3500 g/mol (PEG 3500) to the hydroponics solution. Freezing point depression and a thermocouple psychrometer (Decagon Devices; SC-10A) were both used to determine the osmolality of both the hydroponics solution and the solutions of PEG 3500. For details of these procedures and results, see Appendix 3. Solutions of PEG in hydroponics solution of 20, 50, 75, 90, and 105 g/L, were found to

have approximate osmolalities of 1.5, 2.5, 3.0, 4.0, and 5.0 bar, respectively, and the hydroponics solution was found to have an osmolality of ~1.0 bar.

Thirty-six plants were divided evenly into six sets, and each set of six plants was distributed among three containers with a common solution. Five sets of plants each had their hydroponics solution replaced by one of the five PEG/hydroponic solutions. The sixth set of plants was placed in fresh hydroponics solution only. All transfers of solution were made slowly, by adding the appropriate solution one third at a time, every six hours, over an 18-h period. Transfer to the solutions began on February 10, at 01:00 h, and was completed by 20:00 h on February 11. All solutions were then renewed, every other day, with fresh solutions of the same osmolalities, until termination of the experiment on February 15.

Guttation measurements

The rate of guttation of each of the plants was measured one night prior to, and for several nights after, the addition of the PEG/hydroponic solutions. The first guttation measurement was made on February 10, just prior to the beginning of the plants' transfer into the PEG solutions, and the next guttation measurement, on February 11, was made 4 h after the transfer was completed, as follows. Filter paper (Whatman 1), was cut into strips of approximately 2 X 0.5 cm. Each strip was put into a labelled, 1.5 mL Eppendorf microtube, and the two were weighed together.

Before measuring the rate of guttation, each plant was swabbed thoroughly with a piece of filter paper, to remove all existing guttation, and the filter paper discarded. After exactly 10 min, the entire upper surfaces and edges of all leaf laminas of each plant were then carefully swabbed with a piece of filter paper from one of the microtubes, one piece of filter paper per plant. The paper was then put back into the tube, which was closed tightly, and the plant and tube number recorded. The tube, with the filter paper swabbing inside, was then reweighed. The rate of guttation was calculated in mg/h/plant. The measurement was sometimes repeated, and a mean rate calculated.

Guttation was always measured between midnight and 01:00 h.

Preparation of the tissue for xylem sap analysis

Plants were prepared for analysis while guttating. The whole plant was removed from the hydroponics container, and the root system, still attached to the plant, was immediately plunged into liquid N₂ (LN₂) until completely frozen. Working under LN₂, the basal first 2-cm length of the primary root (containing mature LMX) was cut off and placed in a separate, labelled vial. This procedure was repeated for each of the guttating plants. Vials were held in cryostore until analysed, as follows.

From the middle of each 2-cm length, a transverse face was prepared for analysis by cutting a piece, ~ 3 mm long, under LN₂, and quickly securing it vertically in a hole in a stub using Tissue Tek

(Miles Inc. Elkhart, IA, USA). The assembly was carried under LN_2 to a cryomicrotome (CR2000, RMC Inc., USA), where the tissue piece was planed with a glass knife at -80°C to give a smooth face. The stub was then transferred under LN_2 to a cryo-transfer system (CT1500, Oxford Instruments, Eynsham, UK) and thence to a sample stage (-170°C) of a scanning electron microscope (CSEM, JEOL Ltd., Tokyo, Japan). The specimen was observed uncoated at 1 kV during etching for approximately 1-3 min at -90°C to reveal faint outlines of the cells. The etched specimens were then re-cooled and returned to the preparation chamber where they were coated with evaporated Al (50 nm), and then transferred back to the sample stage. For details of these procedures see Huang *et al.* (1994). Micrographs were recorded at 7 or 15 kV as video prints and on Kodak TMax-100 120 film.

Microanalysis

Microanalysis was done on the lumen contents of five each of the LMX and EMX vessels from the primary roots of all guttating plants. Microanalysis was done with a Link eXL, LZ-4 system (Oxford Instruments) with the Be-window (BeW) for heavy elements, and the ultra-thin window (UTW) for elements lighter than atomic number 25. The accelerating voltage was 15 kV, and the probe current was set at 1.00 nA for the BeW and 0.80 nA for the UTW. The scan raster was set at 1 μm square for x10 000 and the magnification was varied so that the raster covered most of the area of the cell or

vessel lumen to be analyzed. During analysis the stage temperature was -170°C , and the column vacuum at the gun was 3×10^{-7} Torr. Spectra were accumulated until 80 000 counts had been recorded in the Al peak, and the live time recorded for each. Counts were recorded for the different elements as net percent ratios of the Al peak, and these ratios were divided by the live time to correct for local differences in the thickness of the Al coating. Quantification of each element was by means of standard solutions frozen in 5% carbon slurry and prepared in the same way as the specimens. For details of these analytical and calibration procedures see Canny and Huang (1993) and Huang *et al.* (1994) for heavier elements, and McCully and Sealey (1996) for carbon.

Differences in the osmolalities of the xylem sap between plant sets, and between the osmolalities of the xylem sap and the external solutions, were assessed using a Student's t-test. Differences were judged to be highly significant, significant, or probably significant, based on significance levels from Moroney, 1951.

An additional control was added to evaluate further the accuracy of the microanalysis procedure, whereby solutions of KCl of known concentration were drawn by a hand vacuum pump into the vessel lumens of pieces of mature regions of maize primary roots, and their contents then analysed using the CSEM microanalysis. For details of this experiment, see Appendix 4.

Results for Experiment 1

General appearance of plants

The control plants, and the plants in the 1.5, 2.25, 3.0 and 4.0 bar PEG/hydroponic solutions continued to grow over the course of the experiment, developing an additional leaf between the time of the addition of the PEG solutions and the final guttation measurements. By the end of the experiment, they all had four leaves unfolded, and the plants which had a fourth leaf emerging before the PEG transfer now had a fifth leaf emerging. Because the plants increased in size, the measured guttation (mg/h/plant) also increased in the control plants. The plants in the PEG solutions did not appear less developed than the control plants, and also appeared healthy and green, with no signs of desiccation from the PEG.

Plants in the 5.0 bar solution appeared wilted during the day and night, and water could be seen to have retreated about 2 cm from the leaf margins. Where the water had retreated, the leaves were a lighter green in colour and appeared relatively dry; the tips of the leaves became brown and shrivelled. These plants stopped growing when put into the PEG solution, never developing beyond the three-leaf/one-leaf-emerged stage.

Guttation

The maximum osmolality in which plants could guttate was 3.0 bar (Plate 1 A; Fig. 1). Not all of the plants in the PEG solutions were able to guttate over the course of the experiment, and the number of plants which could guttate decreased as the concentration of the solution increased. While all the plants could guttate in the 1, 1.5, and 2.25 bar solutions, 40% of the plants could guttate in the 3.0 bar solution if given sufficient time to recover, and none could guttate in the 4.0 and 5.0 bar solutions, even if given several days for recovery (Table 1).

Plants which were not guttating were removed from the data set. Some plants generally had higher guttation rates than others, independent of the osmolality of the hydroponics solution. Generally, plants which had smaller or less leaves had lower guttation rates. To compensate for these inherent differences in guttation rates between plants, all data were normalized by taking the mean initial guttation rate for each plant set, before the transfer to the PEG solutions, as 100%.

Mean guttation rates are reported as mean $\pm$ standard error (SE) (n) (n represents the number of guttation measurements, not necessarily the number of plants). The mean initial guttation rate of all the plants before the transfer to the PEG solutions was 21 ± 8 (96) mg/h/plant. The effect of the PEG 3500 on the guttation rates of the corn plants varied, depending on the osmolality of the solution (Fig. 1). The control plants showed a steady guttation rate for the

first three nights, with a large increase to nearly double their initial guttation rate on the fourth night, which may have been due to the expansion of the fourth leaf. The guttation rate then remained fairly steady for the next two nights.

The guttation rates of plants put into the PEG solutions were observed to have dropped on the first night after the transfer was completed (Fig. 1). The second night after being put into the PEG, the guttation rates of the plants began to rise. This increase began between Feb 11 and 12, during which time there was no observed change in the guttation rates of the control plants (the guttation rates of the control plants increased between Feb. 12 and 13) and is interpreted as being symptomatic of the beginning of root pressure recovery in the PEG-stressed plants. Plants in the 1.5 bar and 2.25 bar solution showed a decrease to approximately 30% of their initial guttation rate, while plants in the 3.0 bar solution showed a decrease to approximately 10%. The guttation rates for all three sets of plants then increased over the next two nights, and remained steady for the remainder of the experiment.

Plants in higher concentrations of PEG generally had lower final % guttation rates - the final % guttation for plants in the control solution was 220 ± 90 (12) % of the initial guttation rate, while for the plants in the 1.5 bar and 3.0 bar solutions it was 140 ± 60 (11) % and 100 ± 35 (4) % of the initial guttation rate, respectively. The exception were the plants in the 2.25 bar solution, whose final % guttation was 180 ± 60 (6).

Xylem sap analysis

In transverse section in the CSEM, the LMX and EMX vessels had thick walls, and their lumens appeared glassy with few solute lines (Plate 1 B and C). This is a characteristic appearance of dead xylem vessels - living xylem vessels have many solute lines and often display peripheral cytoplasm.

While there were 5 - 6 plants analyzed from the each of the plant sets in the control, 1.5, and 2.25 bar solutions, translating to 25 - 30 each of LMX and EMX being measured, only two plants, representing the 40% of the plant set guttating in the 3.0 bar solution, were analyzed, translating to only 10 each of the LMX and EMX being measured.

Errors on mean elemental concentrations were calculated as standard deviations (SD). In the EMX and LMX sap from all of the plant sets, K and Ca were the predominant elements detected - C, Na and Mg were not detected, and S, P and Cl were present in very small amounts (Fig. 2 A - D). Nitrogen cannot be detected using EDX analysis because its signal is drowned by a coincident oxygen peak. Mean K concentrations of the LMX and EMX sap respectively, per plant set, ranged from 11 to 23 mM, and the range of Ca was 5 to 11 mM.

The concentrations of K and Ca were used to calculate the average osmolalities of the xylem sap. K and Ca were assumed to be present entirely as ions. It is known that while there is carbon in the sap, present below the EDX detection limits, it is predominantly

in the form of organic acids which are negatively charged and counterbalanced by K and Ca. These organic acids have been found to be the main counterbalancing anion in root xylem sap (Canny and McCully, 1988), although it is often thought that NO_3 is also very important as such (Kramer and Boyer, 1995). The approximate sap osmolality, in mosmol, can thus be calculated by $2 \times [\text{K}] \text{ (mM)} + 3 \times [\text{Ca}] \text{ (mM)}$. Calculated osmolalities were converted to the unit of bars.

Mean sap osmolalities are reported as mean $\pm$ SD (n). It was found that the mean osmolalities of the LMX sap were 1.1 ± 0.6 (25) bar, 1.3 ± 0.7 (25) bar, 1.6 ± 1.0 (25) bar and 1.7 ± 0.6 (10) bar; and the mean osmolalities of the EMX sap were 1.4 ± 0.9 (25) bar, 1.3 ± 0.9 (25) bar, 1.6 ± 1.1 (25) bar and 1.8 ± 0.8 (10) bar, for the plants in the control, 1.5 bar, 2.25 bar and 3.0 bar solutions, respectively (Fig. 3). There were probable differences in the mean osmolalities of the LMX sap between the plants in the control and the 2.25 and 3.0 bar solutions ($t=2.1, 2.6$; $P < 0.05$), and in the mean osmolality of the EMX sap between the plants in the control and 3.0 bar solutions ($t=1.2$; $P < 0.05$). More importantly, the osmolalities of the LMX and EMX sap respectively, were found to be highly significantly and significantly lower than the osmolality of the 3.0 bar PEG external solution ($t = 4.5, 3.1$; $P < 0.001, 0.01$). The osmolalities of the LMX and EMX sap were not found to be significantly lower than the other external solution osmolalities.

Material & Methods for Experiment 2 - further xylem sap analyses of plants guttating in 3 bar external solution

Trial 1

From experiment 1, it was determined that 3 bar was the maximum osmolality in which plants could guttate. Because the limited space in the hydroponics system necessitated small plant sets for experiment 1, and because fewer plants were found to guttate in the more concentrated PEG solutions, the sample size for the plants guttating in the 3 bar solution, consisting of two plants, was too small for adequate xylem sap analysis. The experiment was thus repeated, this time with only two concentrations: a control set of plants, and a larger set of plants in 3 bar PEG solution.

This experiment was conducted in March. Plants were again grown to the three-leaf or three-leaf-unfolded/one-leaf-emerging stage, and 23 healthy plants selected. Seventeen plants had their hydroponic solution gradually replaced by a 3 bar PEG/hydroponic solution, as described in experiment 1, while the other six plants had their solution replaced only by fresh hydroponics solution. These transfers began at 01:00 h on March 16, and were finished by 20:00 h on March 17. All solutions were renewed every other day thereafter, until termination of the experiment on March 20.

Guttation was measured for all 23 plants, using the technique described in experiment 1, one night prior to, and for several nights after, the replacement with the PEG/hydroponics solution. The first

guttation measurements were made on March 16 just prior to the beginning of the PEG transfer and the second measurements were made on March 17, 4 h after the transfer to the PEG had been completed.

On March 20, following the final guttation measurements, plants were prepared for xylem sap analysis. Primary-root tissue was prepared as in experiment 1, for all plants demonstrating measurable guttation. Microanalysis was done as in experiment 1, on five each of the LMX and EMX vessels from five each of the control plants and of the plants in the PEG solution. Student's t-tests were again used to assess differences in osmolality as in experiment 1, as well as in differences in elemental concentrations of the xylem sap between plant sets.

Trial 2

This experiment was repeated in the month of June, to test its reproducibility. This time, only 5 plants were used for the control and 6 for the 3 bar plants.

Guttation measurements were made only once per night per plant, from June 22 - 28, and guttation rates were normalized for each individual plant as in trial 1. Microanalyses were done and Student's t-tests performed as in trial 1. Results from both trials are included.

Results for Experiment 2

Trial 1

General Appearance/Guttation

As in experiment 1, all of the plants had developed an additional leaf by the end of the experiment. The plants in both the control and the 3 bar solution appeared green and healthy, and developed at the same rate. The plants in the 3 bar solution showed no signs of wilting or desiccation.

Fourteen of the 17 plants in the 3 bar PEG solution started to guttate before the end of the experiment - a much higher percentage than in experiment 1. Because the pattern of recovery was quite different for each individual plant, the guttation rates for each plant were plotted individually, and the data were not normalized as in experiment 1.

Mean guttation rates are reported as mean $\pm$ SD (n). Initial guttation rates for all 23 plants in the experiment were highly variable, ranging from 10 to 60 mg/h, with a mean guttation rate of 38 ± 23 (54) mg/h/plant. As in experiment 1, the rate of guttation of four of the five control plants increased over time (Fig. 4 A and B). Three of the plants showed a steady increase in their rate of guttation (Fig. 4 A), while one showed a quick increase within one night, and one held a constant rate (Fig. 4 B). Except for this last plant, guttation rates approximately doubled from March 16 - 20.

As in experiment 1, the plants whose roots were immersed in the 3 bar solution showed a dramatic drop in their guttation rates, but this time guttation dropped to 0 mg/h for all plants (Fig.5 A - E). This drop was sometimes immediate (Fig 5 B and C) but sometimes occurred over two nights (Fig. 5; A,D,E). Recovery of guttation took from 1 to 2 nights, depending on the plant. Plants generally did not recover to their initial guttation rates; the mean final guttation when the plants were harvested was 59 ± 29 (28) % of the initial guttation rate.

Xylem sap analysis

Mean elemental concentrations are reported as mean $\pm$ SD (n). The elemental concentrations of the LMX and EMX sap for the plants guttating in the 3 bar solution were similar to those found in experiment 1 (fig 6 A and B). The LMX and EMX sap contained 19 ± 14 (30) mM K and 10 ± 9 (30) mM Ca, and 20 ± 13 (30) mM K and 8 ± 8 (30) mM Ca, respectively; S, P, and Cl were sometimes detected in small amounts and C and Na were not detected. These concentrations were slightly higher than those found in the control plants, which showed K concentrations of 13 ± 7 (30) mM K and 8 ± 7 (30) mM Ca in the LMX sap, and 12 ± 9 (30) mM K and 6 ± 7 (30) mM Ca in the EMX sap. The difference in the mean [K] between the control and the 3.0 bar plants for both the LMX and EMX was probably significant ($t = 2.1$, 2.7 ; $P < 0.05$), but was insignificant for the Ca concentrations ($t = 0.9$, 1.0). The concentrations of Na, C, Cl, S and P were not

significantly different between the control and the 3 bar plants.

Mean osmolalities are reported as mean $\pm$ SD (n). From the above experimental values for K and Ca, the mean osmolality of the LMX and EMX sap in the 3 bar plants was calculated to be 1.8 ± 1.0 (30) and 1.6 ± 0.9 (30) bar, respectively, compared to 1.3 ± 0.6 (30) and 1.1 ± 0.7 (30) bar, respectively, for the control plants (fig. 7). The differences between the control and the 3.0 bar plants, of the mean osmolalities of the LMX and EMX sap respectively, were probably significant ($t = 2.3, 2.4$; $P < 0.05$). The osmolalities of the LMX and EMX sap respectively, were found to be probably and highly significantly lower than that of the 3.0 bar PEG solution ($t = 2.6, 3.4$; $P < 0.05, 0.001$).

Trial 2

Guttation

This trial took two days longer than the first trial, because the plants took longer to stop guttating in the 3 bar solution, and also to recover once their guttation had stopped. By the end of the experiment, all plants had five to six leaves unfolded.

The mean initial guttation of these plants, before transfer to the PEG solution, was 48 ± 31 (20) mg/h [mean $\pm$ SD (n)]. While four out of five of the control plants showed an overall increase in their guttation by the end of the experiment, two of those plants showed

an initial drop in guttation on June 23 and 24; the fifth plant showed an overall drop in guttation over the course of the experiment (Fig. 8). Excluding this last plant, final guttations showed a broad range of approximately 160 to 300% of the initial guttation rate.

In the 3 bar PEG solution, all plants showed a complete, though not immediate loss of guttation following transfer into the PEG (Fig. 9 A - C). Four of the six plants took two days to stop guttating (Fig. 9 A and C) and two of the plants took three days (Fig. 9 B). For this trial, 100% of the plants recovered their guttation. Recovery took from two to three days following loss of guttation, and ranged from moderate recoveries of 70 to 120% (Fig. 9 A and B) to dramatic recoveries of 300 to 400% (Fig. 9 C).

LMX and EMX sap analysis

Mean elemental concentrations are reported as mean $\pm$ SD (n). The mean elemental concentrations in the LMX and EMX sap were slightly lower than those measured in the first trial (Fig. 10 A and B). The LMX and EMX vessels both had mean K concentrations of 18 ± 11 (25) mM, and mean Ca concentrations of 6 ± 5 (25) mM and 7 ± 7 (25) mM, respectively, for the 3.0 bar plants, compared to mean K concentrations of 11 ± 7 (25) mM and 15 ± 15 (25) mM respectively, and mean Ca concentrations of 6 ± 5 (25) mM and 6 ± 8 (25) mM, respectively, for the control plants. Only the difference in the mean [K] of the LMX sap between the control and the 3.0 bar

plants was of probable significance ($t = 2.6$; $P < 0.05$) As in trial 1, no Na and C were detected, and Cl, S and P were rarely detected.

Mean osmolalities are reported as mean $\pm$ SD (n). From the above experimental values for K and Ca, the mean osmolalities of the LMX and EMX sap were calculated to be 1.0 ± 0.5 (25) and 1.2 ± 1.0 (25) bar, respectively, for the control plants, and 1.4 ± 0.7 (25) and 1.4 ± 0.8 (25) bar, respectively, for the PEG-treated plants (Fig. 11). The difference between the mean osmolalities of the LMX sap of the control and the 3.0 bar plants was probably significant ($t = 2.3$; $P < 0.05$), but was not significant for the EMX sap ($t = 0.8$). Both the osmolality of the LMX sap and the EMX sap were found to be highly significantly lower than that of the 3.0 bar PEG solution ($t = 4.9, 4.3$; $P < 0.001$).

osmolarity	% plants recovered
control (1.0 bar)	100
1.5 bar	100
2.25 bar	100
3.0 bar	100
4.0 bar	0

**Table 1 (Experiment 1) -
% of plants recovered**

Plate 1.

Late metaxylem (white diamonds); early metaxylem (diamond outlines); xylem parenchyma (Xp); pericycle (Pe); endodermis (En); inner cortex (Ci).

A) Corn seedling, guttating in a 3.0 bar PEG solution. This was the highest osmolality in which this phenomenon could be observed. 1.0 X

B and C) Base of a corn primary root, transverse face. Cryo-scanning electron microscopy. At this position in the primary root, there are typically 7 - 9 LMX and approximately 15 EMX, all of which are open vessels in a four-leaf seedling. **B)** 170 X. **C)** 770 X.

Fig. 1 (Experiment 1) - guttation rates of plants in range of osmolalities of PEG 3500 ⁽¹⁾

1. Error bars represent standard error.
2. n = 12
3. n = 11
4. n = 6
5. n = 4

Fig. 2 A - D (Experiment 1) - elemental composition of xylem sap in different osmolalities of PEG 3500 ⁽¹⁾

1. Error bars represent standard deviations
2. n = 25
3. n = 20
4. n = 10

2.25 bar PEG

C

3.0 bar PEG

D

Fig. 3 (Experiment 1) - Osmolality of external solutions and xylem sap⁽¹⁾

1. Error bars represent standard deviations.
2. Osmolalities of 1, 1.5, and 2.5 bar solutions extrapolated from Fig. 25, Appendix 3. Osmolality of 3.0 bar solution taken from psychrometer measurements (Table 7, Appendix 3).
3. Sap osmolalities calculated using [K] and [Ca] from Fig. 4, A - D.

Fig. 4 A,B (Experiment 2, Trial 1) - guttation rates of individual control plants from March 16 - 20^(1,2)

1. Symbols represent guttation rates of individual plants.
2. Points with error bars represent standard deviations of 2 - 3 measurements.

Fig. 5 A - D (Experiment 2, Trial 1) - guttation rates of individual plants in 3 bar PEG solution from March 16 - 20^(1,2)

1. Symbols represent guttation rates of individual plants.
2. Points with error bars represent standard deviations of 2 - 3 measurements.

Fig. 6 (Experiment 2, Trial 1)
A - elemental composition of LMX sap
B - elemental composition of EMX sap

1. n = 30, error bars represent standard deviations.

Fig. 7 (Experiment 2, Trial 1) - Osmolality of external solution, LMX and EMX sap⁽¹⁾

1. Error bars represent standard deviations.
2. Osmolality of 3.0 bar solution measured with psychrometer (Table 7, Appendix 3).
3. Osmolality of xylem sap calculated using [K] and [Ca] from Fig. 6 A and B.

Fig. 8 (Experiment 2, Trial 2) - guttation rates of individual control plants from June 22 - 28⁽¹⁾

1. symbols represent individual control plants

Fig. 9 A - C (Experiment 2, Trial 2) - guttation rates of individual plants in 3 bar PEG solution from June 22 - 28 ⁽¹⁾

1. symbols represent individual plants

Fig. 10 (Experiment 2, Trial 2)

A - elemental composition of LMX sap

B - elemental composition of EMX sap

1. n = 25, error bars represent standard deviations.

Fig. 11 (Experiment 2, Trial 2) - Osmolality of external solution, LMX and EMX sap⁽¹⁾

1. Error bars represent standard deviations.
2. Osmolality of 3.0 bar solution taken from psychrometer measurements (Table 7, Appendix 3).
3. Osmolalities of xylem sap calculated using [K] and [Ca] from Fig. 10 A and B.

Fig. 12 A - % of plants recovered in 3 bar PEG and B - guttation rate of control plants with respect to month⁽¹⁾

1. Error bars represent standard deviations.
2. n = 5 plants
3. n = 17 plants
4. n = 6 plants
5. n = 96
6. n = 54
7. n = 20

Discussion

These experiments give definitive evidence for the absence of adequate solute concentrations in the xylem sap of guttating plants for osmotically drawing water out of an external solution of high osmotic concentration. Plants which had a calculated xylem sap osmolality of ~ 1.5 bar, could guttate in an external solution of 3 bar. Even with a large standard deviation, it is clear that the osmolality of the xylem sap is much lower than that of the external solution. This result was effectively obtained three times: once in experiment 1 and twice in experiment 2.

The results showing an absence of an osmotic driving force between external solution and xylem sap are similar to those found by Van Overbeek, 1942 and Ginzburg and Ginzburg, 1970. Van Overbeek found that a 2 bar external solution was required to stop exudation from a root system whose xylem sap had an osmolality of 1 bar, while Ginzburg and Ginzburg found that the osmotic potential of the external solution which stopped exudation from root segments was 0.5 to 2 bar lower than that of the xylem sap.

The study dispells the criticisms directed at Van Overbeek regarding his use of permeable solutes in his external solution. No changes in carbon above the limits of detection of the EDX microanalysis could be observed in the xylem sap, and while the limits of detection were not low enough to detect the small concentrations of organic acids in the sap, it is certain that if significant amounts of PEG were permeating the root it would be detected.

While the low concentrations of K in the xylem sap, measured to be approximately 20 mM, agree with the results of some researchers (Jeschke *et al.*, 1984; Pate and Sharkey, 1975; Ichino *et al.*, 1973), they differ from some other reports in the literature of high concentrations of K⁺ in the xylem fluid exuded by excised roots bathed in salt solutions (Pitman and Wellfare, 1978; House and Findlay, 1965). These high concentrations of K⁺, however, were found using excised roots, equilibrated in relatively high K⁺ solution (10 and 50 mM K). It is felt that measuring the xylem sap of intact plants gives more relevant and reliable measurements of sap contents. Also, recent work in our lab has shown that exudate from decapitated roots comes from both the xylem and cortex (Andrea Rowan, personal communication) and would not represent the contents of the vessels; thus, one cannot reliably say that such exudation measurements do not include the contents of living cells, nor solutes from the external solution. That the use of isolated roots does not always provide reliable evidence may explain why some researchers, such as House and Findlay, 1965, were unable to confirm Van Overbeek's work.

The observation that decreasing the osmotic potential of the external solution decreases the rate of exudation from roots is not a new one (Kramer, 1932; Tagawa, 1934), and has always been used as evidence towards the osmometer model for root pressure. That this experiment showed a continuation of guttation with a more negative potential in the external solution than in the xylem sap shows that the current osmometer model for root pressure is faulty; however,

that there is an obvious effect of external osmotic concentrations on root pressure shows that osmosis still plays a very important part in how root pressure works. This will be investigated in further chapters.

The only difference observed between the three trials was an increase in the percentage of plants which recovered their guttation in the 3 bar solution each time and in the guttation rates of the control plants (fig. 12 A and B). It is believed that this reflects an increase in osmotic tolerance which may be related to the time of year when the experiment was done. There is a possibility of a light effect, which will be discussed in chapter 3.

Chapter 3

Microanalyses along LMX and EMX vessels of guttating plants

(Based on the paper: **Enns LC, McCully ME, Canny MJ. 1998.** Solute concentrations in xylem sap along vessels of maize primary roots at high root pressure. *Journal of Experimental Botany*. In press.)

Introduction

One of the main criticisms towards Van Overbeek's and others' findings, was that concentrations of solutes in the xylem sap might be significantly higher in the roots than at the stem stump where exudate was being collected (Eaton, 1943; Klepper, 1967). These speculations arose, in part, from the observations that the stele of bean roots and stems accumulates sodium from the xylem sap (Jacoby, 1965), and that the guttation liquid from leaves of tomato plants has a higher osmotic potential than exudate from stem stumps near the soil level (Eaton, 1943; Klepper and Kaufmann, 1966). It was believed that solutes might be reabsorbed from the xylem lumen farther down in the roots of the plant than from where the xylem sap was being measured, resulting in low estimates of sap osmolality. This supposition was strengthened by the finding that K^+ uptake by the xylem parenchyma from the xylem vessels does occur by means of proton translocating ATPases (de Boer and Prins, 1985). These arguments were carried one step further, by speculations that there might exist osmotic differences within the vessel lumens of the root itself, and that salt and water absorption into the vessel lumens might be highly dependent on the position in the root (Arisz *et al*, 1951; House and Findlay, 1965). Standing osmotic gradients in solute concentration were proposed within the vessel lumens, the highest solute concentrations being at the base of the vessels (Anderson, Aikman & Meiri, 1970).

Schwenke & Wagner, 1992, extended this idea by using the test tube model of Hylmö, 1953, which assumes root pressure to be a

consequence of vessel maturation by the expansion of the intact tonoplast after cell wall rupture of the oldest living xylem element in a xylem vessel. They proposed that the continuous rupture of the plasmalemma and tonoplast of maturing vessel elements might create high solute concentrations at the base of the xylem vessel, or a standing osmotic gradient.

It has been well established that the maturation of living vessel elements in maize and other grasses takes place over many centimetres back from the apex (St. Aubin *et al.*, 1986; Sanderson *et al.*, 1988; Huang and Van Steveninck, 1988; McCully and Canny, 1988; Huang *et al.*, 1991), and that these living elements accumulate K to the highest concentration of any cells in the soybean root (McCully *et al.*, 1987). The high solute concentrations present in the immature vessel elements must be released into the vessel when the most proximal living element dies - such spasmodic injections of K⁺ into the maturing secondary xylem of sunflower stems were invoked by Canny (1995) to explain the erratic concentrations found downstream in leaf veins. The possibility, then, of such a standing gradient is quite feasible. It was therefore of interest to follow the concentration of K⁺ and other solutes in the open xylem conduits (vessels) proximal to the maturation zone.

Although the published values of concentrations of K measured *in situ* in vessels of roots were low (25 mM or less, McCully *et al.* 1987; McCully, 1994), there remains the possibility that local high concentrations of solutes might be present at some places in the vessels and have escaped notice. I therefore studied systematically

the elemental concentrations (predominantly K) *in situ* along the lengths of individual vessels from the base of the primary root of maize to the living, immature vessel elements at the distal end of each vessel. These elements, as they mature, add to the length of each vessel. For the LMX the transition between immature vessel elements and each vessel was usually around 25 cm from the root tip, and for EMX around 5 cm from the tip.

To ensure that root pressure was occurring in the plants, the roots were collected at night from guttating seedlings grown in the greenhouse. One series of measurements was made on a root grown in the field and collected at dawn, to see if there was a marked difference in xylem solutes under these conditions.

Material and Methods

Plant material

Seedlings of *Zea mays* L. (Cv. Seneca Chief) were grown in well-watered Pro-mix general purpose growing medium (Premier Horticulture, Rivière-du-Loup, Qué) in the greenhouses of Carleton University. The light regime was 11 h darkness + 13 h HID metal halide illumination imposed upon 13 h of natural daylight. After two weeks the seedlings had four unfolded leaves, a primary root 30-35 cm long, and four unbranched nodal roots about 10 cm long. Dye perfusion tests showed that at this age the open conduit of each file of LMX and EMX extended distally 5-10 cm and 25 to 30 cm respectively, from the base of the root (Appendix 2).

To ensure collection of roots that were exerting high root pressure, harvests in the greenhouse were made at midnight, of plants guttating vigorously. In all, six root systems were harvested, five from plants grown as specified, and one of the same age and size from a field plot of sandy loam soil at the Central Experimental Farm of Agriculture Canada. The field plant was harvested at 0530 h (EST) when it was already daylight and no guttation was apparent.

Preparation of tissue for analysis

At each harvest the root system was carefully separated from the soil and, still attached to the plant, was immersed in liquid N₂ until completely frozen. Working under LN₂, the primary root was cut into

1-cm lengths, starting from the base, and each length placed in a separate labelled vial. Vials were held in cryostore until analysed.

From each 1-cm length a transverse and a longitudinal planed face were prepared for analysis. Two pieces, each ~3 mm long were cut from the 1-cm length under LN_2 . One was quickly fixed vertically in a hole in a stub, the other laid horizontally in a grooved stub, each secured with Tissue Tek, and the two assemblies carried under LN_2 to a cryomicrotome, where each piece was planed as in chapter 2, experiment 1, to give a smooth face. The longitudinal piece was planed to expose LMX and EMX vessels and immature vessel elements within the stele. The two stubs with the adjoining planed root pieces were mounted together and transferred, as in chapter 2, experiment 1, to a cryo-transfer system and thence to the scanning electron microscope. Specimens were etched, coated, and photographed as in chapter 2, experiment 1.

Microanalysis

Microanalysis was done as in chapter 1, experiment 1, using the BeW and UTW. For each primary root, three kinds of information were required: 1) How far from the base toward the tip did vessels extend in a) the LMX, b) the EMX? 2) What was the concentration of chemical elements a) along each vessel, and b) in the immature elements adjoining each vessel at its distal end in both the LMX and the EMX? 3) How did the concentrations found in the xylem vessels and

immature elements compare with those in the adjacent xylem parenchyma cells? The procedure for collecting this information was as follows.

In the first basal centimetre of each root two or three of the five LMX vessels in these roots were selected to be followed down the root, and in this and the transverse faces of the distal preparations, spectra were collected from the lumens of each specific vessel. Definitive identification along the root of specific individual EMX vessels was not possible, so for each 1-cm preparation spectra were collected from five to seven (out of 10 to 14) of these vessels in each root, and means calculated from the pooled measurements. Spectra were collected from several xylem parenchyma cells at the base of each greenhouse root, and at various distances along the field root.

In each root there are two regions where the LMX and EMX vessels, respectively, end, and are succeeded by files of immature vessel elements. These transitions can be identified anatomically in the cryo-preparations (see below). Once the transition regions were identified, a few further distal samples of immature vessel elements were examined and analysed.

The LMX vessels were analysed at each cm starting from the base of the primary root. Because the EMX vessels were much longer than those of the LMX, analyses were not done for every cm. Analysis of the EMX files began near the root tip, in the living vessel elements. Working toward the base, 1-cm segments were observed

and analysed to the point where the open vessel was detected. The continuing EMX vessels were then observed and analysed at every fourth cm up to the base of the root.

By these procedures, solute concentrations were measured along the length of 15 individual LMX vessels from six roots, and the mean concentrations along the EMX vessels for five roots.

Results

Immature vessel elements had thin peripheral cytoplasm, tonoplasts and nuclei (all best seen in transverse faces, as in Plate 2 A and B; Plate 3 B and D). Along with the living cells of the root, they also usually had high electron emissivity from the many lines of solute sequestered in their vacuoles by the freezing, in contrast to the presence of only frozen liquid in the lumens of vessels which made them appeared black with few white lines or flecks of sequestered solute (low electron emissivity) (Plate 3 A and C). Vessels retained only a lignified rim where the primary end walls of the component vessel elements were removed. These rims were best seen in the longitudinal faces (Plate 2 C). Living vessel elements had intact primary end walls (Plate 2 D).

Vessels and different cell types in the transverse faces were easily recognizable and individual LMX vessels were identifiable at the different positions along each root by their position relative to

asymmetric features of the stele. An LMX vessel could thus be followed all the way down its length, and its appearance in transverse section could be related to the EDX measurements of its contents (Plate 3 A-D).

The only chemical element found in appreciable amounts in vessels was potassium. Occasionally Ca or Cl were present at their limit of detection (10 to 15 mM). The distribution of [K] in the LMX vessels of all six roots followed the pattern shown in Fig. 13 A - C: a low, even concentration (~11 mM) from the base to a point 5 to 10 cm distal, and a sudden rise in a single centimetre to a high concentration (~100 mM) where the living vessel elements started. A summary of all the measurements is given in Table 2. For the EMX, a similar pattern was found (Fig. 13 D), but the transition from vessel to living elements was nearer the tip, at 25 - 31 cm from the base. As shown in Table 3, the mean values of [K] in the vessels, and the living elements, respectively, were similar to those found in the vessels and living elements of the LMX. The one field-grown plant showed a pattern of [K] distribution no different from that of greenhouse-grown plants (Fig. 13 C). The point of transition from low [K] to high [K] always coincided with the anatomically identified transition from vessel to living elements for all the roots. Only representative graphs are included; the remaining graphs can be seen in Appendix 5.

The question of whether there is a gradient of [K] in vessels along the root was addressed by making linear regressions of [K] on

distance from the base, for all the values of [K] in vessels.

Separate regressions were made for LMX and EMX vessels because of the greater length of the latter. The values of R^2 for the two types of vessel were, LMX $R^2 = 0.02$, EMX $R^2 = 0.01$, respectively. Thus no part of the variance in concentration can be ascribed to a dependence on distance along the vessel.

In the greenhouse-grown plants, the [K] measured in xylem parenchyma cells was lower than that found in living immature vessel elements but still substantial. It ranged from 19 to 94 mM with a mean of 53 mM, compared with the overall mean of 110 mM for the immature vessel elements in Table 2. Other chemical elements were present in lesser and variable amounts, eg. S (~19 mM), P (~55 mM). Occasional cells included Ca above the level of detection, chloride was not detected. In the field-grown plant, the xylem parenchyma [K] was higher than that in the greenhouse plants. It ranged from 80 to 173 mM, with a mean of 130 ± 28 mM [mean $\pm$ SD (n)], and was not distinguishable from the concentration in the living vessel elements (Plant 6, Table 2).

Root ⁽³⁾	LMX file	[K] (mM) ⁽¹⁾		Length (cm) ⁽²⁾
		Vessel	Immature elements	
1	1	6 ± 0 (4)	109 ± 12 (2)	4
	2	8 ± 5 (4)	95 ± 3 (2)	4
2	1	17 ± 8 (6)	94 ± 27 (3)	5
	2	13 ± 5 (6)	61 ± 15 (3)	5
	3	9 ± 4 (6)	94 ± 12 (2)	6
3	1	8 ± 3 (7)	124	6
	2	10 ± 3 (6)	86 ± 16 (2)	5
	3	14 ± 8 (7)	118	6
	4	14 ± 8 (7)	123	6
4	1	18 ± 8 (10)	243	9
	2	15 ± 8 (9)	75	9
	3	18 ± 6 (10)	112	9
5	1	13 ± 9 (6)	134 ± 62 (2)	5
	2	8 ± 3 (5)	107 ± 68 (3)	4
	3	7 ± 3 (7)	132	6
6	1	6 ± 0.5 (4)	113 ± 17 (3)	5
	2	10 ± 7 (4)	143 ± 23 (3)	5
Mean		11±9 (108)	110 ± 45 (32)	

Table 2 - Potassium concentrations in the vessels and the oldest immature vessel elements in individual files of late metaxylem (LMX) in primary roots of maize. [mean ± SD (N)]

1) Mean of determinations in all 1-cm lengths of the vessel in each LMX file.

2) Length of LMX vessel.

3) Roots 1-5, greenhouse grown, root 6 field-grown.

Root	[K] (mM) ⁽¹⁾		Length (cm) ⁽²⁾
	Vessels	Immature elements	
1	13 ± 8 (8)	123 ± 7 (2)	30
2	12 ± 2 (7)	165 ± 13 (2)	26
3	20 ± 6 (8)	137 ± 13 (2)	27
4	15 ± 6 (8)	101	27
5	13 ± 7 (7)	116 ± 21 (4)	24
Mean	15 ± 3 (38)	128 ± 24 (11)	

Table 3 - Potassium concentrations in vessels and immature elements in early metaxylem (EMX) files of primary roots of maize [mean ± SD (N)]

1) Mean of determinations in all 1-cm lengths of EMX vessels.

2) Length of EMX vessels.

Plate 2.

All micrographs are of cryo-planed faces of bulk-frozen maize primary roots viewed by cryo-scanning electron microscopy. Faces in (A) and (B) are transverse, (C) and (D) longitudinal. Late metaxylem (white diamonds); early metaxylem (diamond outlines).

A) Root 2 (Table 3). Early metaxylem (EMX) file, 27 cm from the root base. The EMX element has a thin shell of peripheral cytoplasm and an intact tonoplast (arrowhead), indicating that it is still alive. The mean [K] of the EMX elements at this position in this root was 177 mM. The mean analyses along the selected EMX files in this root are shown in Fig. 13 D. 2200 X.

B) Adjacent LMX files, both elements are alive. The tonoplast is apparent in both the right and left elements (arrowheads), and the nucleus (N) in the left. 950 X.

C) Portions of two mature LMX elements forming part of a vessel. The primary transverse wall has gone but the lignified rim of secondary wall remains (arrow). 650 X.

D) Intact primary crosswall (arrow) between two living LMX elements. 770 X.

Plate 3.

All micrographs are of cryo-planed, transverse faces of bulk-frozen maize primary roots viewed by cryo-scanning electron microscopy.

A) Root 1, LMX file 2 (Table 2). Four cm from the root base. The LMX element is mature, forming a portion of the open conduit (vessel).

[K] at this position in the vessel was 6 mM. The analyses along this LMX file are shown in Fig. 13 A. 1440 X.

B) Same LMX file as in (A) but 5 cm from root base. Here the LMX element is still alive, as indicated by the peripheral cytoplasm and the intact tonoplast (arrowhead). 2600 X.

C) Root 3, LMX file 4 (Table 2). Six cm from root base. The element is dead and forms part of the vessel. [K] was 18 mM. The analyses along this file are shown in Fig. 13 B. 1300 X.

D) Same LMX file as in (C), but at 7 cm from the base. The element is alive, the tonoplast is indicated by an arrowhead. [K] was 123 mM. 1050 X.

Figure 13. Typical data for [K] measured *in situ* in individual vessel lumens. Distances are from the base of the root.

(A) [K] at each cm along the full length of an LMX vessel in root 1 file 2 (table 2). The vessel extended 4 cm distally from the base of the root. [K] in the immature vessel elements in the more distal 2 cm of the same xylem file is shown.

(B) As in Fig. 13(A) but for an LMX vessel in root 3 file 4 (Table 2). This vessel extended 6 cm distally from the base of the root. [K] in the immature vessel element in the next more distal cm is shown.

(C) Data for [K] in the lumen of the vessel in LMX files 1 and 2 of root 6 (Table 2). For this particular root, additional measurements of [K] were taken over 3 cm along the file of immature vessel elements beyond the transition point 5 cm distal to the root base.

(D). Typical data for [K] in the lumens of EMX vessels in root 2, (Table 3) measured *in situ* along their length (to 26 cm from the base of the root), and in the immature vessel elements in the next distal cm of the same xylem files. Each point is the mean of measurements in 5 vessels, or immature vessel elements. Error bars represent standard deviations.

Discussion

The cryo-analytical technique used in this study has allowed me to follow, for the first time, solute concentrations along the full length of vessels in the roots of intact plants at full root pressure. I did not find the hypothesized high concentration of solutes or gradients of concentrations anywhere along the vessels of either the EMX or the LMX. The solutes accumulated by the living vessel elements, which must be released when these cells die and become parts of vessels, did not accumulate in the distal ends of the vessels. They must either be diluted and dispersed by incoming water, or taken up quickly by the surrounding parenchyma.

For reasons mentioned in chapter 1, other elements may have been present at lower concentrations, but would be balanced by the cation K^+ . Calcium was not present in measurable amounts in the xylem sap as in chapter 1, presumably because these plants were not grown hydroponically and their soil solution would have a much different composition. It can therefore be concluded that measurements of K^+ in the vessels of these plants are representative of the total sap osmolality.

With this experiment, the last of the criticisms against Van Overbeek's and others' work has been dispelled. It is felt that this is the final evidence required to definitively discredit the osmometer model for root pressure.

Chapter 4

**Microanalyses on living root cells of plants guttating in
3 bar PEG**

Introduction

It has been observed that increasing the osmolality of the external solution decreases the rate of guttation of the plant (Chapter 2). This is not a new observation (Arisz *et al.*, 1951); it has, however, always been used as evidence for the osmometer model, because it seems to support the idea that root pressure is osmotically generated. Because definitive evidence has now been provided showing the absence of an osmotic driving force between the xylem sap and soil solution in guttating plants (Chapter 2), a new osmotic model needs to be produced.

If root pressure works by an osmotic mechanism, and the xylem sap does not have an adequate osmotic potential to account for water being drawn from high osmotic solutions, it seems an obvious hypothesis that the living cells are providing the osmotic power for the movement of water, as proposed by Pfeffer, 1899; Zholkevich *et al.*, 1980; Mozhaeva and Pil'shchikova, 1980; and others.

If such a hypothesis were correct, one would expect to see decreases in the osmotic potentials of living cells in the roots of plants which had recovered their guttation in high osmotic external solutions. This decrease in osmotic potential would be reflected in solute changes which might be detectable using EDX analysis. The experiment described in this chapter used EDX analysis to analyze the elemental contents of the living cells of the roots harvested from the plants in chapter 1, to see if there

were any such changes in solute levels.

Another technique for looking at changes in a cell's osmotic potential is plasmolysis, the separation of the protoplasm from the cell wall caused by water loss from the cell sap to a solution of lower osmotic potential (Oparka, 1994). Plasmolysis has been used to determine the solute concentrations of individual cells (Geiger *et al.*, 1973; Evert, Eschrich and Heyser, 1977; Fisher and Evert, 1982; Russin and Evert, 1985). I also employed this technique to look at solute changes in PEG-stressed root cells.

Material and Methods

EDX analysis of cell contents

Only a small piece of the harvested primary root tissue from the control plants and the plants guttating in 3 bar PEG solution was used for xylem sap analysis from the two trials done of chapter 2, experiment 2; the remaining tissue was put back into cryostorage. This tissue was taken out of storage to be used for the analysis of the living cell contents from the primary roots of both of these sets of plants. One set of analyses was done for the plant material from each of the two trials, as follows.

From the stored primary-root material, transverse faces were prepared for the scanning electron microscope as described in chapter 2, experiment 1. No changes were made to the protocol for transfer, etching, and coating. Micrographs were again recorded at 7 or 15 kV on Kodak Tmax 100 120 roll film.

Microanalyses were again done with a Link eXL, LZ-4 system (Oxford Instruments) as described in chapter 2, experiment 1. The Be- and UT-window were used for the root tissue from experiment 2, trial 1, and the Be-window only for the root tissue from experiment 2, trial 2.

For each piece of root, the elemental contents of five cells from 4 different living cell types were analyzed: xylem parenchyma (X.P.), pericycle (Pe), endodermis (En), and inner cortex (Ci). For

living cells, the analyses were of vacuolar contents, unlike the xylem sap analyses of chapter 2 which were done on the contents of vessel lumens. Measurements were made on five each of the control plants and of the plants which had recovered their guttation in the 3 bar PEG solution, for each of the two trials performed in chapter 2, experiment 2.

Mean elemental concentrations were calculated for each of the cell types, for both the control and the PEG-stressed plants, for each of the two trials, and expressed as mean $\pm$ SD (n). Student's t-tests were used to assess the differences in the elemental concentrations between the control and the PEG-stressed plants, for each of the cell types.

Plasmolytic measurement of vacuole osmotic potential

Plant material

Twelve plants were grown as described in chapter 2, experiment 1, by transferring seedlings of *Zea mays* L. (cv. Seneca Chief) from soil to a hydroponics system. The seedlings were grown in hydroponics from May 21 - June 5, until they had six leaves unfolded, at which time six of the plants were transferred slowly, using the gradual replacement described in chapter 1, into a solution of 90 g / L PEG. Such a solution has an osmolality of 4 bar (appendix 1). Six of the plants had their solutions replaced by fresh hydroponics solution only.

After complete transfer to the PEG solution, both the PEG solutions and the hydroponic solutions of the control plants were renewed every other day for one week, at which time, on June 12, the primary roots were harvested for plasmolysis.

Sectioning/staining

Roots were harvested during the day. One sample each was prepared for the control plants and for the plants in the 4 bar solution, as follows. The basal approximately four-centimetre region of the primary roots of each plant was cut off and immediately put into water. Root pieces from the control plants were mixed together into one sample, and the same was done for the 4 bar plants. Each sample thus consisted of six four-centimetre pieces of primary root.

From each four-centimetre piece of root, approximately 20 longitudinal sections were hand-cut into watchglasses containing potassium phosphate buffer, 0.025 M, pH 7.8. For each of the control and the 4 bar samples, the sections from the six root pieces were mixed together, amounting to over one hundred longitudinal sections for each sample. The sections from each sample were kept in separate watchglasses. Both watchglasses were covered to prevent evaporation of buffer, and the sections were left in buffer for 45 minutes.

The vacuoles of unstained cells are extremely difficult to see in a microscope, even using phase-contrast optics. Many kinds of

stains kill cells, however, which would make plasmolytic measurements impossible. Vital stains, such as neutral red, are accumulated by the vacuoles of living cells, and are often used to test cells' vitality (Stadelmann and Kinzel, 1972). In order to make the vacuoles of the cells more visible, the sections were transferred to 0.1 % neutral red (Chroma-Gesellschaft, Stuttgart-Untertürkheim, 1B469) dissolved in phosphate buffer, 0.025M, pH 7.8. After 15 minutes in neutral red, sections were put back into phosphate buffer for a few seconds rinsing.

Plasmolysis

Solutions of mannitol (BDH Inc., B29148) in potassium phosphate buffer, 0.025 M, pH 7.8 were prepared, ranging in osmolality from 5 to 9 bar in 0.5 bar increments, using published osmolalities (Weast, 1983). Two sets of solutions were made, one for each sample of root tissue. Solutions were put into separate glass containers and covered to prevent evaporation.

For each of the two root samples, 10 longitudinal sections were put into each of the 10 mannitol solutions, and left, covered, for 30 minutes. The sections from each of the solutions were then mounted on glass slides, in their corresponding solutions, under glass coverslips. Sections were observed using an Olympus Vanox AHB-LB microscope with Nomarski optics. Each section was individually assessed for plasmolysis by studying the cortical cells only - the section was judged to be plasmolysed if any plasmolysis

could be seen in over 50% of the intact cortical cells present.

Sections were photographed using an Olympus C-35A0-2 or C-35A0-4 camera, on Kodak TMax 100 film.

Results

EDX analysis

General appearance of cells

Under the scanning electron microscope, the ultrastructure of all cell types seemed quite well preserved by the freezing procedure. Nuclei, peripheral cytoplasm, and tonoplasts could frequently be observed in the xylem parenchyma, endodermis, pericycle and cortex (Plate 4 A - D).

There were many solute lines present in all cell types, contrasting with the glossy appearance of the empty LMX and EMX vessels (Plate 4 D).

Microanalyses

The results for the LMX and EMX sap analyses from chapter 1 are included for comparison. The two trials generally gave similar results for elemental contents of all cell types (Fig. 14 - 19). Errors are reported as standard deviations.

The predominant element present in all cell types was potassium (Fig. 14 A and B). Both trials showed increases of ~ 20 mM K in the endodermis, inner cortical cells and xylem parenchyma of the plants in the 3 bar solutions - much higher than the observed increases in the K concentrations of the LMX and EMX sap in chapter 2, experiment 2. The standard deviations, however, for the mean K concentrations in these cells for both the control plants and the 3 bar plants were very high (~ $\pm$ 20 mM) and sometimes exceeded the observed increases. The differences in the mean [K] between the control and the 3.0 bar plants yielded significance levels as follows: for the xylem parenchyma, trial 1, insignificant ($t = 1.7$), and trial 2, probably significant ($t = 1.9$; $P < 0.05$); for the endodermal cells, trial 1, insignificant ($t = 1.8$), and trial 2, highly significant ($t = 3.7$; $P < 0.001$); and for the cortical cells, trial 1, significant ($t = 3.1$; $P < 0.01$) and trial 2, highly significant ($t = 5.4$; $P < 0.001$).

Ca was present in measurable concentrations in all cell types, predominantly in the cortical cells (Fig 15 A and B). Trial 2 showed a 20 mM decrease in the mean Ca concentration of the cortical cells of the 3 bar plants, but this decrease was found to be insignificant ($t = 1.8$), and no decrease was observed in trial 1. There were no large changes in Ca observed in the other cell types.

All cell types also contained measurable concentrations of S, and although small decreases of ~ 5 -10 mM in the mean S concentration of all cell types of 3 bar plants from trial 1 were observed, these changes were not apparent from measurements done on the plants from trial 2 (Fig.16 A and B).

Some P was detected in the xylem parenchyma and pericycle cells, but was rarely detected in the endodermis and inner cortex (fig. 17 A and B). Changes in concentration between the control and the 3 bar plants were very slight, and probably insignificant.

Cl and C were rarely detected in any of the cell types (Fig. 18 A and B; Fig. 19).

Plasmolysis

General appearance of plants

By the time the roots were harvested, both the control and the 4 bar plants had developed about eight unfolded leaves. Their root systems were quite developed, with a long primary root and first and second tier nodal roots, and short (< 5 cm) third tier nodal roots. The lengths of the roots were not recorded. The plants in the 4 bar solution did not appear smaller or less developed than the control plants.

Over the course of the experiment, the plants in the 4 bar solution did not guttate, although the control plants guttated prodigally.

Appearance of cells

The longitudinal sections had many intact cells, which appeared dark red due to the neutral red in their vacuoles (Plate 5 A-E). Cells which had been ruptured or killed when the sections were cut did not take up neutral red and were colourless. All cell types could be observed in the longitudinal sections, and the only cells which did not take up neutral red were the endodermal cells (Plate 5 A). Using Nomarski optics, cytoplasmic streaming could be easily observed in the cortical cells; this phenomenon was more difficult to observe in the other cell types.

Plasmolysis was easiest to observe in the cortical cells, because of their large size. Plasmolysis was observed as a recession of the neutral-red filled vacuole from the cell wall (Plate 5 B and C), and was often accompanied by a loss of cytoplasmic streaming in the cells.

Plasmolysis was much more difficult to observe in the other cell types. It was impossible to see in the endodermis, because these cells did not take up any neutral red, presumably because the thick lignified and suberized cell walls excluded the dye (Wenzel and McCully, 1991). The less lignified walls of some cells, such as the xylem parenchyma and pericycle, while not excluding the dye, tended to stain with neutral red, obscuring the view of plasmolysis. Many of the cell types in the stele, such as the xylem parenchyma and pericycle, were much smaller in diameter, and there tended to be more than one layer of these cells in longitudinal section, making it

difficult to resolve individual cells. Plasmolysis of the xylem parenchyma was especially hard to observe, as these cells were quite long and often ruptured during sectioning. In spite of these obstacles, it was occasionally possible to observe whether or not a xylem parenchyma cell was plasmolysed (Plate 5 D and E).

It was due to the ease with which plasmolysis could be observed in the cortical cells, that these cells were chosen to assess plasmolysis in the sections.

Plasmolysis

The results of the experiment were surprisingly binary (Table 4). For both the control and the 4 bar plants, 100% of the sections demonstrated over 50% plasmolysis of the cortical cells at a given osmolarity of mannitol.

The cortical cells of the control plants were unplasmolysed at 6 bar (Plate 6 D) , and plasmolysed at 6.5 bar (Plate 6 E). The cortical cells of the 4 bar plants were unplasmolysed at 7.5 bar (Plate 6 A) and plasmolysed at 8.0 bar (Plate 6 B and C). Thus, the osmotic potential of the control cortical cells was approximately -6.2 bar, and that of the plants adjusted to a -4 bar solution was approximately -7.8 bar, making the decrease in the vacuolar osmotic potential less than half that of the decrease in the outside solution.

		Osmolality of mannitol (bar)								
		5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
% sections plasmolysed	control	0	0	100	100	100	100	100	100	100
	4 bar PEG	0	0	0	0	0	0	100	100	100

Table 4 - % root sections plasmolysed in range of osmolalities of mannitol for control and PEG-treated plants

Plate 4.

All micrographs are of cryo-planed, transverse faces at the base of bulk-frozen maize primary roots, viewed by cryo-scanning electron microscopy. LMX (white diamonds); EMX (diamond outlines); xylem parenchyma (Xp); pericycle (Pe); endodermis (En); inner cortex (Ci); nuclei (N).

A, B and C) Pericycle, endodermis, and inner cortical cells. The ultrastructure of these cells has been well preserved by the freezing process, and peripheral cytoplasm, tonoplasts (arrowheads) and nuclei (N) can be observed. Prominent lines of sequestered solute are also apparent in the vacuoles of these cells, which contrast with the glossy, black appearance of the dead LMX and EMX vessels (seen in (A) and (D)). **A)** 1330 X. **B)** 830 X. **C)** 2200 X.

D) LMX vessel and corresponding xylem parenchyma, which also show well-preserved ultrastructure. 2050 X.

Plate 5.

All micrographs are of longitudinal sections taken at the base of maize primary roots, treated with neutral red, and viewed with Nomarski optics. Cortical cells (c); xylem parenchyma (Xp); endodermis (En). Arrowheads show where the plasma membranes of plasmolysed cells have pulled away from the cell walls.

A) Unplasmolysed, turgid cortical cells. The plasma membranes are pushed right up against the cell walls by the vacuoles, which occupy the majority of the cell volume and which are full of neutral red dye. The endodermal cells do not take up any neutral red, although the lignin in their walls stains a bright red. 280 X.

B and C) Plasmolysed cortical cells. The plasma membranes of the cells have pulled away from the cell walls (white arrowheads) because of water loss from the vacuoles and a corresponding loss of cell turgidity. **B)** 280 X. **C)** 700 X.

D) Unplasmolysed, turgid xylem parenchyma cell. 600 X.

E) Plasmolysed xylem parenchyma cell. 600 X.

Plate 6.

All micrographs are of longitudinal sections of cortical cells (c) taken at the base of maize primary roots, treated with neutral red, and viewed with Nomarski optics. Black arrows show where the plasma membranes of plasmolysed cells have pulled away from the cell walls.

A, B and C) Cortical cells from the roots of corn seedlings which have been in a 4.0 bar PEG solution for one week. These sections have been treated with a range of osmolalities of mannitol. **A)** 7.5 bar mannitol. The cells are still turgid. 300 X. **B and C)** 8.0 bar mannitol. The cells are plasmolysed. 600 X.

D and E) Cortical cells from the roots of control seedlings, which have also been treated with mannitol. **D)** 6.0 bar mannitol. Most cells are turgid, although the beginnings of plasmolysis may be seen in some cells - such cells are not considered to be plasmolysed, as the separation of the plasma membrane from the cell wall is very slight. 600 X. **E)** 6.5 bar mannitol. The cells are plasmolysed. 600 X.

Fig. 14 A and B - $[K]$ in LMX, EMX and cell sap of control and 3 bar plants. A) trial 1; B) trial 2.
X.P.=xylem parenchyma; Pe=pericycle;
En=endodermis; Ci=inner cortex

1. $n = 25$, error bars represent standard deviations.

Fig. 15 A and B - [Ca] in LMX, EMX and cell sap of control and 3 bar plants. A) trial 1; B) trial 2.

1. n = 25, error bars represent standard deviations.

(A)

(B)

Fig. 16 A and B - [S] in LMX, EMX and cell sap of control and 3 bar plants. A) trial 1; B) trial 2.

1. n = 25, error bars represent standard deviations.

Fig. 17 A and B - [P] in LMX,EMX and cell sap of control and 3 bar plants. A) trial 1; B) trial 2.

1. n = 25, error bars represent standard deviations.

Fig. 18 A and B - [Cl] in LMX, EMX and cell sap of control and 3 bar plants. A) trial 1; B) trial 2.

1. n = 25, error bars represent standard deviations.

Fig. 19 - [C] in LMX, EMX and cell sap of control plants and 3 bar plants from trial 1.

1. n = 25, error bars represent standard deviations.

Discussion

Microanalysis of the 3 bar plants showed an approximate increase in K of 20 mM for the xylem parenchyma, cortex, and endodermis corresponding to about 1 bar of osmotic pressure. While the standard deviations were very large, and exceeded the actual osmotic differences observed between the control and experimental plants, both trials done of the experiment showed similar increases, and a Student's t-test on these results usually showed significant differences. The increases of approximately 5 mM K in the xylem sap, while probably significant, are small in comparison to the increases shown by the living cells, and do not translate to a very large change in osmotic potential (~ 1/4 of a bar).

Plasmolysis of the corn roots which were immersed in a 4 bar solution of PEG, showed an osmotic adjustment of approximately 2 bar in the cortical cells. From the equation:

$$\psi_{\text{solution}} = \psi_o^{\text{vacuole}} + \text{TP}$$

where ψ_{solution} is the total water potential of the cell, ψ_o^{vacuole} is the osmotic potential of the cell vacuole, and TP is the turgor pressure inside the cell, for 1/4 strength Hoaglands solution,

$$-1 = -6.2 + \text{TP}$$

$$\therefore \text{TP} = 5.2 \text{ bar}$$

and for -4 bar PEG,

$$-4 = -7.8 + \text{TP}$$

$$\therefore \text{TP} = 3.8 \text{ bar}$$

The observed adjustment of vacuolar ψ_o is thus sufficient to retain

considerable tissue turgor, but does not represent a full recovery of the PEG-stressed cells. This is expected, as the PEG-stressed plants never recovered the ability to guttate.

This type of osmotic analysis proved much more suitable than the EDX microanalysis, because several cells could be observed at once to calculate an approximate osmotic potential for all of the cortical cells. The EDX microanalysis technique only allows individual measurements of cells, and each measurement is time-consuming and costly; hence, only a small population of cells can be examined. This results in the very high standard deviations shown in Fig. 14 - 19. The disadvantage to the plasmolysis technique is that it only allowed for cortical cell measurements, as the other cells were too small in diameter to observe plasmolysis clearly. The osmotic adjustment measured with the plasmolysis was greater than that measured using EDX analysis for the cortical cells of the 3 bar plants in experiment 1 (an increase of 20 mM K translates to a change of ~ 1 bar), but the 3 bar plants were exposed to a lower osmolality of PEG, for a shorter period of time. The plants of experiment 2 never did recover their guttation, and it would be desirable to repeat the plasmolysis experiment again for plants in a 3 bar solution.

The results from the EDX analysis, coupled with the plasmolysis experiment, show that osmotic changes of PEG-stressed root cells in corn do occur, and that these changes do coincide with a recovery of guttation in such plants. Such osmotic changes in the face of water stress are well documented in the literature as

“osmotic adjustment”, an accumulation of solutes in higher plants in response to water deficits which has been shown to maintain full or partial turgor in leaves, hypocotyls, roots, and reproductive organs (Turner and Jones, 1980). While osmotic adjustment is known, in the face of osmotic stress, to be beneficial in the maintenance of cell elongation, stomatal opening, photosynthesis, and survival of dehydration, (Turner and Jones, 1980), it has never before been associated with the maintenance of root pressure. (For an excellent review of osmotic adjustment, refer to Morgan, 1984.)

Osmotic adjustment has been shown in many plants such as rice, sorghum, sunflower, maize and wheat, under water stress, focussing mainly on the leaves (Cutler *et al.*, 1980; Simmelsgaard, 1976; Sionit *et al.*, 1980; Turner *et al.*, 1978; Sanchez-Diaz and Kramer, 1978); however some work has been done showing substantial osmotic adjustment in roots (Iljin, 1957; Greacen and Oh, 1972). Because most work on osmotic adjustment has been done on leaves, studies have focussed on internally generated solutes such as sugars and amino acids (Acevedo *et al.*, 1979; Ackerson, 1981; Jones *et al.*, 1980; Munns and Weir, 1981) and to some extent organic acids (malate, citrate), nitrate, and chloride ions (Cutler and Rains, 1978; Cutler *et al.*, 1977). It is believed, however, that changes in potassium may also contribute substantially to osmotic adjustment (Ford and Wilson, 1981; Gutknecht, 1968; Zimmerman and Steudle, 1974). The results of the EDX analyses imply that potassium is the significant contributor to the changes observed using plasmolysis, in the vacuolar osmotic potential of the root cortical cells of the

PEG-stressed plants. The UTW analyses revealed no detectable changes in carbon in the different cell types of the root, implying no role for carbon-based solutes in osmotic adjustment in these cells.

It was proposed many years ago by critics of the osmometer model, that the living cells of the root might be providing the osmotic driving force for the movement of water into the root (Zholkevich *et al.*, 1980; Mozhaeva and Pil'shchikova, 1980).

If this is indeed true, it might be necessary for cells to lower their osmotic potential in external solutions of low osmolality, in order to maintain root pressure.

The observed osmotic adjustments in the living cells of the root may account for the recovered guttation observed in chapter 2 by plants in 3 bar PEG solutions. By accumulating K^+ and subsequently lowering their osmotic potentials, the living cells may have played a primary role in the plants' recovery of their ability to draw water out of the solution and to guttate.

Osmotic adjustment may also provide an explanation for the phenomenon described in chapter 2, whereby a higher percentage of PEG-tolerant plants was found with each successive experiment. As each experiment was performed, the summer months approached and the days lengthened; light intensity also increased. It has been shown that light intensity affects osmotic adjustment (Acevedo *et al.*, 1979; Hsiao *et al.*, 1976; Ackerson, 1981). While this type of research has focussed on increased photosynthesis and sucrose levels in leaves, ion uptake is also an energy-dependant process and could be affected by higher light levels. It is possible that the

plants grown in later months tended to have slightly higher solute concentrations in their cells, enabling more of them to guttate when water-stressed.

Chapter 5

Guttation rates of plants pre-treated with KNO_3

Introduction

From chapter 4, it was found that plants which had recovered their guttation in 3.0 bar PEG solution had higher solute levels in their cells than control plants which were not exposed to PEG. It was hypothesized that the observed increases in the solute levels of the cells of these PEG-stressed plants were what was causing their recovery, and that higher solute levels might enable plants to guttate in external solutions of higher osmotic concentration.

In order to test this hypothesis, corn seedlings were pre-treated with extra KNO_3 in their hydroponics solution, and their ability to guttate in different osmolalities of PEG was then assessed and compared with that of the plants from the previous chapters. EDX analysis was used to confirm that the plants had indeed taken up the extra potassium.

Material and Methods

Plant Material/ Microanalysis

A batch of 18 plants was set up in a hydroponics system as described in chapter 2, experiment 1. On April 13, when the plants were at the four-leaf-unfolded stage of development, half of the plants were transferred to a hydroponics solution containing an additional 50 mM KNO_3 . The other half of the plants were transferred

to the regular, dilute hydroponics solution used in chapter 2, experiment 1 and 2. All plants were grown in their respective solutions for five days, renewing the solutions every other day, at which time they had six leaves unfolded and a seventh emerging.

At this time, the five largest, healthiest looking plants were selected from each of the control and of the high potassium sets of plants, and tissue was prepared from the primary roots for microanalysis, as described in chapter 2, experiment 1.

For each of the ten plants, microanalysis was again done on five each of the following six cell types described in experiment 2 (LMX, EMX, xylem parenchyma, pericycle, endodermis, and inner cortex). Average K concentrations were calculated for each of the cell types for the control and for the high-potassium plants.

Guttation in PEG 3500

A batch of 30 plants in hydroponics was set up as above, but this time, on May 4, all plants were transferred to the high potassium hydroponics solution. Plants were grown as above, for four days, until they had five leaves unfolded. They were divided into 5 sets of six, and each set of plants was put into one of the following PEG/hydroponics solutions: 2.25, 3.0, 3.5, 4.0 and 4.5 bar. These solutions were made up as in experiment 1, using the information shown in Appendix 3, and the gradual transfer procedure to these new solutions was also identical.

The guttation rates of all guttating plants were measured,

using the procedure described in chapter 2, experiment 1, on the first night after the transfer to the PEG solutions was completed. The guttation rates were averaged for the plants in each PEG solution, and compared to the mean guttation rates on the first night after PEG transfer of the 2.25 bar plants from chapter 2, experiment 1, the 3.0 bar plants from chapter 2, experiment 2, and the 4.0 bar plants from the plasmolysis experiment of chapter 4.

Results

Microanalyses

The roots of plants grown in the high-potassium hydroponics solution had much more potassium in their living cells, and slightly more potassium in their LMX and EMX vessels, than the control plants (Fig. 20). The concentrations of K in the high-potassium plants were ~ 100 mM higher for the xylem parenchyma and pericycle, ~ 80 mM higher in the endodermis and cortex, and ~15 mM higher in the LMX and EMX, when compared with the control plants. K concentrations in the cells of the control plants were similar to those found in the control plants of earlier experiments (chapter 4). Standard deviations for both sets of plants were quite high, for reasons discussed in chapter 4. The differences in the K concentrations of all of the cell types between the control and the high-potassium plants were found to be highly significant ($P < 0.001$), with the

exception of the EMX vessels, for which the difference was significant at the 1% level. The t values for the differences in [K] between the control and high-potassium plants are as follows: for the LMX, $t = 3.8$; for the EMX, $t = 2.8$; for the xylem parenchyma, $t = 4.0$; for the pericycle, $t = 5.8$; for the endodermis, $t = 4.9$; and for the inner cortex, $t = 4.7$.

Guttation

When the guttation measurements on the KNO_3 -treated plants were done, the plants had five leaves unfolded. This was one leaf larger than the 2.25 and 3.0 bar plants to which the plants were being compared, and three leaves smaller than the 4.0 bar plants. Mean guttation rates are presented as mean $\pm$ SD (n). The high-potassium plants exhibited much stronger guttation than the lower-potassium plants from other experiments - the differences were quite dramatic (Fig. 21). On the first night after transfer to the PEG solutions, the high-potassium plants in the 2.5 and 3.0 bar PEG solutions were guttating at 64 ± 14 (4) and 24 ± 11 (5) mg/h respectively, compared with 34 ± 16 (6) and 12 ± 10 (6) mg/h respectively for the low-potassium plants. The difference between the guttation rates of the 2.5 bar plants was probably significant ($t = 2.7$; $P < 0.05$) but not significant at a 5% level for the 3.0 bar plants ($t = 1.7$). The high-potassium plants could also guttate in a 4 bar solution of PEG, at a rate of 16 ± 11 (5) mg/h, while the twelve low-potassium plants from chapter 4 which were used for plasmolysis,

never managed to guttate at all in the 4.0 bar PEG, even after being left in the solution for several days to potentially recover. This difference was found to be highly significant ($t = 4.7$; $P < 0.001$).

Fig. 20 - [K] in control and KNO₃-treated plants

1. n = 25, error bars represent standard deviations.

Fig. 21 - rate of guttation of control and KNO₃-treated plants for different osmolalities of PEG ⁽¹⁾

1. Error bars represent standard deviations.
2. for 2.5 bar, n = 6
3. for 3.0 bar, n = 6
4. for 4.0 bar, n = 12
5. for 2.5 bar, n = 4
6. for 3.0 bar, n = 5
7. for 4.0 bar, n = 5

Discussion

The results of this experiment are quite dramatic, and show clearly that plants which have been pre-treated with KNO_3 can exude much more strongly when osmotically stressed. The main flaw in this experiment is that the guttation rates of the high-potassium plants in the 2.25 bar and 3.0 bar solutions were compared to those of plants which were grown earlier in the year, in February and March respectively. Chapter 2 showed that plants became increasingly osmotically tolerant as the winter progressed to spring, and it can be argued that the high-potassium plants, grown in May, had an advantage over plants grown earlier in the year. It is also known from chapter 2 that larger plants guttate more, and the high-potassium plants in the 2.25 and 3.0 bar solutions were one leaf larger than the plants to which they were being compared. It is believed, however, that the dramatic differences between the guttation rates of the high and low-potassium plants far transcend any differences which might be attributed to time of year, or the size of the plant. Most importantly, the low-potassium plants in the 4.0 bar solution, from chapter 4, experiment 2, were grown in the month of June - if there were any seasonal advantages then these plants would have had the stronger advantage over the high-potassium plants. These plants were also given a full week to recover in the 4.0 bar PEG solution - unlike the high-potassium plants which were measured immediately - and were grown to a much larger size (8 leaves unfolded compared to 5 leaves for the high-potassium plants), and still the high-potassium plants could

guttate where the low-potassium plants could not. These results are thus quite definitive.

Increasing the root pressure of plants by pre-treating them with osmotic solutions is not a new accomplishment. Similar findings were reported by Arisz *et al.*, 1951, who found that adapting roots to mannitol made them exude more strongly when put back into Hoagland's solution. He interpreted his results to mean that the xylem vessels were decreasing the osmotic potential of their sap in mannitol, and used this as further evidence to invalidate Van Overbeek's xylem sap measurements, which had been made after his plants were transferred back into water.

Nitrate is another osmolyte which has been recognized by many to increase root pressure (Minshall, 1964, 1968; Ashcroft *et al.*, 1972; Ezeta and Jackson, 1975) and has been hypothesized to act by either increasing the osmotic gradient between the external solution and xylem sap, or by increasing the radial hydraulic conductivity of roots (Ezeta and Jackson, 1975). Barthes *et al.*, 1996, found that detopped seedlings exuded more when treated with KCl and KNO₃. When they found no correlation between changes in exudation rate and sap osmolality, they assumed that their findings were the result of increases in hydraulic conductivity, and did not consider nor measure cell sap osmotic changes. It is thought that increased nitrate enhances water transfer between cells, and possibly between cells and apoplasm by somehow controlling the opening and closing of water channels (Hoarau *et al.*, 1996). The possibility that the nitrate may be influencing the osmotic potential of the living

cells of the root, and may thus be affecting root pressure, has not been considered.

Chapter 6

General Conclusions

The observations drawn from this thesis can be summarized as follows:

- 1) Plants whose roots are put into non-permeating PEG solutions of high osmolality can still generate root pressure, even though their xylem sap does not contain adequate solute levels to drive the osmotic movement of water into the root.
- 2) The mature xylem vessels do not contain high solute levels anywhere along their length; therefore, there is not an osmotically driven uptake of water farther down towards the base of the vessels.
- 3) Root pressure must still have an osmotic component, since a plant's rate of guttation decreases as the osmolality of the external solution increases.
- 4) Plants in solutions of high osmolality recover their ability to generate root pressure, and this recovery coincides with an increase in the osmotic power (or a decrease in the osmotic potential) of the living cells of the root.
- 5) Plants which have higher solute levels in their living cells can guttate faster, and can guttate in external solutions of higher osmolality, than plants with lower solute levels.

The conclusions which are drawn from this thesis are: 1) the

osmometer model of root pressure is faulty; and 2) the living cells of the root must be responsible for drawing water out of the external solution.

This thesis does not provide a new model for root pressure, but rather serves to prove that root pressure is much more complicated than the osmometer model would have us believe. In order to come up with a better model, we must first accept that the current model is infeasible; second, we must acquiesce to the rudimentary but important idea that the living cells of the root are doing more than just providing a semi-permeable membrane between the soil solution and the xylem sap - they are taking up water from the soil osmotically and then passing it on to the xylem vessels in a way which produces a positive pressure inside those vessels. This idea does not dispel the theory that the root acts as an osmometer, but rather changes the location of the inside of the osmometer from the xylem vessel lumens to the vacuoles of the living cells.

This thesis fails to provide a new model for it does not explain how the plant finally generates pressure inside the root. The root may take in the water osmotically, but what keeps the pressure inside the root from dissipating? In order for pressure to build, and be sustained inside the root, there must be two valves within the system: one which removes water from the system at the top, and one which prevents a backflow of water, and hence a dissipation of pressure, at the bottom. The valve at the top is obviously guttation, for water is forcibly exuded from the plant at the leaf margins, and cannot flow back into the system. It has been speculated that the

valve at the bottom may be the endodermis (Canny, 1998).

Canny's model proposes that pressure builds up in the stele of the root and forces water from the xylem parenchyma cells into the xylem vessels; this idea corresponds well with observations made by McCully *et al.*, 1998, who used cryo-SEM to show water being squeezed from the xylem parenchyma into embolised vessels through pits (thin, unlined portions of the common wall) during transpiration. The mechanism by which water passes from these living cells into the mature xylem is still unknown, but it is in the plasma membranes of these xylem parenchyma, as well as in the endodermis, where water channels, called "aquaporins", are being predominantly located (Maurel, 1997; Chrispeels and Maurel, 1994). While work on aquaporins in plants is still nascent and largely controversial, the belief is spreading that these channels may be responsible for the semi-permeable properties of cell membranes. Is the water from the xylem parenchyma virtually being squeezed into the vessels via these channels? It has been shown that red beet vacuoles have a stretch-activated channel that is sensitive to osmotic pressure (Alexandre and Lassalles, 1992) - might the pressure build-up in the stele, proposed by Canny, 1998, be a way of regulating these water channels?

This author believes that it is time to come up with a new model for how plants generate root pressure which incorporates such modern observations. The osmometer model has been shown, definitively, to be faulty. The mechanism for root pressure is far from being the simple process that the current dogma would have it

be, but this should not be surprising - plants are complicated organisms.

APPENDICES 1-5

Appendix 1 - determination of ideal Hoagland's concentration

Based on the observation that corn seedlings did not guttate very strongly in full-strength Hoagland's, and assuming that this was because of a deleterious osmotic effect, the concentration in which plants would guttate at a maximum rate was determined.

Full-strength Hoagland's solution was diluted to 1/2, 1/4, 1/10, 1/20, 1/50 and 1/100 strength. Corn seedlings at the one-leaf stage of development were transferred to a hydroponics system; six plants were put in each of the six solutions. The seedlings were grown to approximately the three-leaf-unfolded/one-leaf-emerged stage, at which time the guttation rates for each of the plants was measured. For details of these procedures, see chapter 2, experiment 1. For each set of six plants in each solution, the mean guttation rate was calculated.

Results are shown in Fig. 22. It was found that the maximum guttation rate, of 34 ± 12 (10) mg/h [mean $\pm$ SE (n)], occurred in the 1/4-strength Hoagland's solution. At 1/10 and 1/20-strength, the guttation rate started to drop, and at 1/50 and 1/100-strength, the plants did not guttate at all, and showed stunted growth and signs of iron deficiency.

Fig. 22 - guttation rate with respect to concentration of Hoagland's solution^(1,2)

1. error bars represent standard error
2. n = 10

Appendix 2 - determination of approximate length of mature, open vessels of 4-leaf-emerged corn seedlings

While it was already known that there are several cm of open LMX at the base of the primary roots of 4-leaf seedlings (Wenzel, 1989), it was necessary to confirm this for the hydroponically-grown plants used in these experiments, for two reasons: it was important to confirm that the contents of open vessels were being analysed in chapter 2, and knowing the approximate length of the mature, open vessels was helpful when looking for the ends of the vessels in chapter 3.

Five primary roots of 4-leaf plants, grown hydroponically as in experiment 1, were excised and individually examined, as follows. Working underwater so as not to introduce embolisms into the root, and starting from the base of the root, 2 cm pieces were excised - each piece was examined before the next piece was cut off. Still working underwater, the branch roots were cut off of the root piece using a pair of dissection scissors, and ~ 1 cm of cortex was gently scraped off of one end of the root piece using a razor blade.

A plastic tip for an Eppendorf pipette was snugly fitted into one end of a length of 1/8" i.d. vinyl capillary tubing, and the end was trimmed, to reduce the diameter of the opening of the tube. The scraped end of the root piece was pushed firmly into the conical tip to form a water-tight seal, and the junction sealed with Terostat (Teroson GmbH, Hedelberg Bund, Germany). The other end of the capillary tubing was fitted to a hand-vacuum pump (OTC, 7559),

which was used to pull a solution of 1% aqueous basic fuchsin through the root piece (Diagram 1). At no time was the root piece exposed to air.

Diagram 1 - vacuum pump apparatus

Transverse sections from the middle of the root piece were hand-cut and examined with an Olympus Vanox AHB-LB microscope with

bright-field optics. Sections were photographed using an Olympus C-35AO-2 or C-35AO-4 camera, on Agfa 100 film.

Results

Where the vessels were mature and open, the basic fuchsin was pulled through the root piece and stained the vessels walls a bright-red colour (Plate 7 A and B). It was found that the LMX and EMX vessels were open for 5 - 10 cm, and 25 - 30 cm respectively, at the base of the primary root.

Plate 7.

Both micrographs show transverse sections taken from segments of a corn primary root through which 1 % aqueous basic fuchsin was pulled using a hand vacuum pump, and viewed with bright-field optics. LMX (white diamonds); EMX (diamond outlines).

A) Base of the root. All of the LMX and EMX are open vessels, shown by the staining of the walls where the dye was pulled through. 130 X.

B) Eight cm from the base of the root. All of the EMX are still open, but five of the eight LMX are living xylem elements through which no dye has passed. 130 X.

Appendix 3 - osmolality of PEG 3500

A home-made freezing point depression apparatus and a commercial thermocouple psychrometer (Decagon Devices, SC10A) were used to measure the osmolality of a range of PEG concentrations and of Hoagland's solution.

Freezing point depression

The freezing apparatus was set up as in diagram 2.

Diagram 2 - freezing point depression apparatus

Approximately 10 mL of the solution to be measured was put into a test tube, which was suspended with a plastic stopper into a glass receptacle. The test tube was also stoppered, and a mixing wire and thermocouple were suspended through holes in the stopper, into the solution. Care was taken to ensure that the thermocouple did not touch any side of the test tube. The thermocouple was attached to a digital temperature readout, which gave temperatures to the nearest 0.1 °C.

The glass receptacle, containing the test tube, was lowered into a freezing bath containing antifreeze, and the temperature of the bath was lowered slowly (~ 0.5 °C/minute). The solution was stirred, every few minutes, with the stirring wire.

The temperature of the solutions always dropped several tenths of a degree Celsius below 0 °C, at which time rapid agitation with the stirring wire caused rapid freezing into a ice/solution slush. As the solution froze, the temperature immediately jumped to the eutectic temperature, still below 0 °C, and remained steady. This temperature was recorded as the freezing point.

Known solutions of sucrose were first tested, creating a calibration curve (Fig. 23) of osmolalities which closely matched those published in Weast, 1984.

Solutions of PEG 3500 in dH₂O were then tested, and the freezing points were used to extrapolate their osmolalities from the calibration curve (Fig. 25).

The osmolality of full-strength Hoagland's solution was

measured. A solution of 140 g PEG per L Hoagland's instead of dH_2O was then prepared and measured, to see if the final osmolality was the sum of the two osmolalities of 140 g PEG/ L dH_2O and Hoagland's. Once it was determined that the osmolalities were indeed cumulative (Table 5), the osmolality of 1/4 strength Hoagland's was measured and the osmolalities of all of the PEG concentrations in 1/4 strength Hoagland's were calculated (Table 6).

A solution of 75 g PEG per L of 1/4 strength Hoagland's was also prepared and measured (Table 7). Once again, the measured osmolality was very close to the calculated osmolality in Table 4.

Thermocouple psychrometer

A calibration curve was first constructed by measuring several known concentrations of KCl (Fig. 24). Solutions of PEG in dH_2O were then prepared and measured, and their osmolalities were extrapolated from the calibration curve (Fig. 25). The resultant curve corresponded closely to the curve calculated from the freezing point depression, up to an osmolality of about 3.5 bar. The curves started to diverge significantly beyond this point.

A solution of 75 g PEG per L/Hoagland's was prepared and measured, and the resultant osmolality was close to that measured using freezing point depression (Table 7).

Osmolality (bar)		
PEG (140 g/L)	Hoagland's solution (full strength)	PEG + Hoagland's solution
5.0 ± 0.7 (5)	1.5 ± 0.5 (5)	6.9 ± 0.4 (5)

Table 5 - freezing point depression showing cumulative osmotic effect of adding PEG and Hoagland's solution [mean ± SD (n)]

[PEG] (g/L)	Osmolality (bar)		
	PEG	Hoagland's (1/4 strength)	Calculated total osmolality
0	0 ± 0 (5)	0.9 ± 3 (5)	0.9 ± 0.3
75	2.2 ± 0.4 (5)	0.9 ± 3 (5)	3.1 ± 0.5
105	3.5 ± 0.3 (5)	0.9 ± 3 (5)	4.4 ± 0.4
140	5.0 ± 0.7 (5)	0.9 ± 3 (5)	5.9 ± 0.8

Table 6 - calculated osmolalities for PEG in 1/4 strength Hoagland's solution [mean ± SD (n)]

Osmolality (bar)	
Freezing point depression	Psychrometer
3.8 ± 0.6 (2)	3.0 ± 0.1 (5)

Table 7 - measured osmolalities of 75 g/L PEG/ L of 1/4 strength Hoagland's solution [mean ± SD (n)]

Fig. 23 - calibration for freezing point depression apparatus using sucrose, experimental and published values.

1. $n = 5$, error bars represent standard deviations.

Fig. 24 - calibration curve for psychrometer using KCl⁽¹⁾
($r^2 = 0.999$)

1. for each point, $n = 1$

Fig 25 - Osmolality of PEG 3500 determined using freezing point depression and psychrometer

1. $n = 5$, error bars represent standard deviations.

Appendix 4 - Evaluation of accuracy of EDX microanalysis

Using the technique described in Appendix 2, solutions of 40 mM KCl were drawn by a hand vacuum pump into the vessel lumens of two-centimetre pieces taken from mature regions of maize primary roots. (These root pieces were debranched, and the cortex removed from one end, as described in Appendix 2.) Several millilitres of solution, representing many times the volume of the LMX and EMX vessels, were drawn through the pieces of root. Once the vacuum was released, the pieces were immediately frozen by plunging into liquid nitrogen, and portions taken from the middle of the root piece were attached to stubs, planed, etched, coated and analysed exactly the same way as the specimens in chapter 2, experiment 1.

This control, originally used with fractured, uncoated specimens (McCully et al., 1987), showed that the microanalysis of the test solutions within the vessel lumens agreed closely with their known concentration. Mean elemental concentrations are reported as mean $\pm$ SD (n). The 40 mM KCl solution gave mean [K] and [Cl] of 42 ± 9 mM and 42 ± 10 mM respectively, n =13.

Appendix 5 - Additional data for [K] measured *in situ* in lumens of LMX and EMX vessels, from chapter 3.

The figures in chapter 3 (Fig. 13 A - D) are representative graphs of a much larger body of data. The remaining data are presented in Fig. 26 A - I and Fig. 27 A - D.

Fig 26 A - I - Additional data for $[K]$ measured *in situ* in individual LMX vessel lumens (chapter 3). Distances are from the base of the root.

Fig 27 A - D - Additional data for mean [K], measured *in situ* in EMX vessel lumens (chapter 3). Distances are from base of root. Error bars represent standard deviations.

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