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The role of the apoplast as an osmotic compartment in Suaeda maritima L. Dum. and Beta vulgaris L.

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Award date:
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The Role of the Apoplast as an Osmotic Compartment in *Suaeda maritima* L. Dum. and *Beta vulgaris* L.

by

Ruth Ann Lawrence

under the supervision of

Prof. A. Deri Tomos

A thesis prepared for the University of Wales Bangor for the degree of Doctor of Philosophy

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Acknowledgements

The following people have been instrumental in making my early research career a success and a delight: Prof. A. Deri Tomos, my PhD supervisor and dear friend, whose patience, enthusiasm and belief in my ability have been invaluable. Members of the Bangor Pressure Probe Group and the School of Biological Sciences Plant Group, past and present, are also thanked for their instruction, advice and encouragement - in particular Dr Paul Richardson, Dr Peter Hinde, Dr Olga Koroleva, Dr Nick Bates and Dr David Marriott. I am also deeply indebted to Dr David Wright, School of Agriculture and Forest Sciences, University of Wales Bangor, for arranging delivery of mature sugar beet taproots from Shropshire after a series of events, involving sheep and a faulty storage facility, led to a Bangor beet shortage. The technical help I received during my PhD was exceptional. Special thanks go to the multitalented Mr Eirion Owen - a very kind and generous individual who has helped me in almost every aspect of my work, Mr Andrew Davies for trusting me with the scanning electron microscope and Mrs Allison Bell with whom I spent an enjoyable couple of weeks learning how to cut and stain plant tissue sections. I would also like to thank my personal tutor Dr Anil Shirsat for guidance and support during an uncertain and tearful time. Finally, I must thank my wonderful and cherished husband Andrew, who has endured the PhD write up process without undue complaint. Financial support for this investigation was provided by a BBSRC special studentship.

Abstract

The plant cell wall is a living and dynamic compartment of the plant cell. Its many diverse functions range from cell expansion and differentiation to defence and signalling. Furthermore, there is currently a growing body of evidence which suggests that the cell wall/apoplast also plays an important role in cell water relations. The aim of this study was to highlight the importance of apoplastic solutes in plant cell water relations, particularly in turgor regulation.

The water relations parameters of two members of the family *Chenopodiaceae*, *Suaeda maritima* L. Dum. and *Beta vulgaris* L., were studied at single cell resolution using the cell pressure probe, single cell sampling and analysis techniques, and the xylem pressure probe. These species share a common peculiarity, in that certain cell types, namely the leaf epidermal cells in *Suaeda maritima* and the taproot storage parenchyma cells in *Beta vulgaris*, maintain cell turgor pressure (P_{cell}) at a level which is dramatically lower than the respective cell osmotic pressures (Π_{cell}). This phenomenon is attributed to the properties of the cell wall/apoplast. The hydrostatic component of the apoplast (P_{wall}) accounts for only a small fraction of the difference between P_{cell} and Π_{cell} in these species. In light of this the discrepancy between P_{cell} and Π_{cell} can only be due to the presence of osmotically active solutes in the adjacent apoplast (Π_{wall}).

Suaeda maritima leaf epidermal cells accumulate NaCl in response to an increase in external NaCl concentration. This accumulation of solutes leads to an increase in leaf epidermal osmotic pressure, which exactly mirrors the increase in the osmotic pressure of the external medium (Π_{ext}). Leaf epidermal turgor pressure (P_{cell}), however, is maintained at a constant level over a range of external salinities. In the short term the leaf epidermal cells are shielded from abrupt changes in Π_{ext} by the properties of the root system, and a root reflection coefficient which is close to 0. In the longer term, as NaCl accumulates in the protoplast, P_{cell} is apparently maintained by the parallel adjustment of solutes in the protoplast and apoplast.

Changes in *Suaeda maritima* leaf epidermal turgor pressure (P_{cell}), induced by modulating the solute content of the apoplast (Π_{wall}) in excised leaves, initiated a mechanism which regulated P_{cell} back to *in vivo* levels within 40 minutes. Turgor regulation was not accompanied by equivalent changes in cell osmotic pressure (Π_{cell}), suggesting that osmotic adjustment leading to turgor regulation is apoplastic rather than protoplastic in nature. This apoplastic osmotic adjustment mechanism was dependent on the permeant nature of the apoplastic solutes and on the volume of the apoplast. A comparable upward turgor regulation mechanism was observed in excised *Beta vulgaris* taproot tissue, within 40 - 80 minutes. The presence of apoplastic K^+ apparently facilitated the turgor regulation mechanism in this case. Proton efflux studies on *Beta vulgaris* taproot tissue revealed that the driving force behind this osmotic adjustment mechanism is likely to be turgor/external osmotic pressure (P_{cell}/Π_{ext}) dependent modulation of plasma membrane proton ATPase activity. It was concluded that the apoplast should be regarded as a true osmotic compartment in higher plants.

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List of abbreviations

Abbreviation	Definition
Δ	Difference/change in
EB	Erythrosin B
EDX	Energy dispersive X-ray microanalysis
ϕ	Osmotic coefficient
FC	Fusicoccin
g	Acceleration due to gravity
h	Height/depth
P	Hydrostatic pressure
P_{cell}	Cell turgor pressure
Π_{cell}	Cell osmotic pressure
PCMPS	p-Chloromercuriphenylsulphonic acid
Π_{ext}	Osmotic pressure of the external medium/osmoticum
P_{wall}	Hydrostatic component of the apoplast/cell wall
Π_{wall}	Osmotic component of the apoplast/cell wall
P_{xylem}	Xylem pressure/tension
ρ	Density of liquid
σ	Cell/membrane reflection coefficient
σ_r	Radial reflection coefficient
TS	Transverse section

To Howard M. Jones.

Chapter 1 : Introduction

1.1, The plant cell wall

One of the most striking features of plant cells is the cell wall. All plant cells (except the sperm and some egg cells) have walls (Mauseth, 1988). Plant cell walls are persistent, being preserved very well during specimen preparation and even in death. In trees, most of the wood and bark is composed of cell walls, the actual protoplast having perished. In woody plants, the walls constitute the majority of the plant body (Mauseth, 1988).

The first detailed microscopic observations of plant cell walls were made in the seventeenth century by the gentleman scientist Robert Hooke. Robert Hooke was one of the greatest experimental scientists of his time. His interests knew no bounds, ranging from physics and astronomy, to chemistry, biology and geology, to architecture and naval technology. He collaborated, or corresponded, with scientists as diverse as Christian Huygens, Antony Van Leeuwenhoek, Christopher Wren, Robert Boyle, and Isaac Newton. Among other accomplishments, he invented the universal joint, the iris diaphragm, an early prototype of the respirator, the anchor escapement and the balance spring (which facilitated the production of accurate time pieces). Hooke also served as Chief Surveyor and helped rebuild London after the Great Fire of 1666. He worked out the correct theory of combustion, assisted Robert Boyle in working out the physics of gases, worked out the physics of elastic materials, invented or improved meteorological instruments such as the barometer, anemometer and hygrometer; and the list goes on (see Newbold, 1969).

Hooke's reputation as a biologist rests largely on his book *Micrographia*, published in 1665. Hooke devised the first compound microscope (one of the finest microscopes of his time) and, with its novel illumination system, observed objects as diverse as insects, sponges, bryozoans and bird feathers. His drawings and detailed observations were truly magnificent. Perhaps Hooke's most famous microscopial observation was his study of thin slices of cork. He wrote: *"... I could exceedingly plainly perceive it to be all*

perforated and porous ... these pores or cells, ... were indeed the first microscopial pores I ever saw, and perhaps, that were ever seen, for I had not met with any writer or person, that had made any mention of them before this" (Hooke, 1665). Hooke had discovered plant cells, and indeed was the first to coin the term "cell", taken from the Latin, meaning little room. The "little rooms" Hooke described so beautifully were in fact only the ghostly remains of plant cells; the structures he viewed under his microscope were actually the plant cell walls of cells long perished.

Historically, the plant cell wall was treated as a non-living, inert secretion, that was not truly a part of the living cell. However, our current understanding portrays the wall as a dynamic, metabolically active cellular compartment, equal in status to the cytoplasm and vacuole. The plant cell wall is actively involved in defence, detoxification, signalling, cell-to-cell recognition, cell expansion, cell adhesion, cell separation, translocation, differentiation, morphogenesis (Cosgrove, 1997 and references therein), and cell water relations (this study).

1.2, Plant cell wall structure and properties

The plant cell wall is a highly complex structure, made of polysaccharides, lignin, cutin, suberin, waxes, protein, enzymes, calcium, boron, and water, that has the ability to self assemble (Carpita and Gibeaut, 1993; Cosgrove, 1997). Cell wall differentiation has enabled plants to exploit and conquer almost every microclimate on the planet. Key examples of wall differentiation include, the development of xylem and phloem transport systems; the formation of a waxy cuticle that prevents desiccation; the production of spores with cell walls impregnated with sporopollenin, a substance that prevents desiccation and is virtually completely micro-organism resistant; stems which have both rigid and flexible properties; the incorporation of lignin to provide mechanical support; and the formation of bark from specialised cork cambium (for a review on the morphology and evolution of vascular plants see Gifford and Foster, 1989).

1.2.1, Cellulose

Cellulose is the most abundant polysaccharide on the planet and is an essential component of the primary cell wall of plant cells (approximately 30 % of the primary cell wall is made up of cellulose - see Cosgrove, 1997). Cellulose is composed entirely of β-glucose monomers joined in 1-4 linkages. Parallel chains of cellulose molecules are bound together, via hydrogen bonding between hydroxyl groups on the β-glucose monomers, forming cellulose units or aggregates called microfibrils (Carpita and Gibeaut, 1993; Cosgrove, 1997). Cellulose microfibrils are between 5 and 15 nm wide and are spaced 20 - 40 nm apart (McCann *et al.*, 1990). The microfibril framework forms the main architectural component of the primary plant cell wall (Carpita and Gibeaut, 1993; Cosgrove, 1997). Cellulose has a very high tensile strength, which is equivalent to that of steel; this remarkable property of cellulose is due to its structure (Wainwright *et al.*, 1976).

1.2.2, Hemicellulose

The hemicelluloses are complex polysaccharides which are tightly bound in the primary cell wall (Cosgrove, 1997). The principal hemicellulose in the cell walls of flowering plants is xyloglucan - a branched polymer consisting of a backbone of 1-4 linked β-glucose monomers, with short side chains containing xylose, galactose, and frequently, a terminal fucose (McNeil *et al.*, 1984; Fry, 1989). Xyloglucans are arranged parallel to the cellulose microfibrils and apparently bind to and interlock the cellulose framework (Carpita and Gibeaut, 1993).

1.2.3, The pectin matrix

The fundamental cellulose-hemicellulose framework of the plant cell wall is embedded in a matrix of pectin polysaccharides (Carpita and Gibeaut, 1993). Pectins are polymers of the carbohydrate galacturonic acid and its derivatives. The chemical complexity of pectin polysaccharides varies considerably, for example, homogalacturonan is a simple linear polymer of (1-4) β-galacturonic acid, with occasional rhamnosyl residues which

distort the chain, whereas rhamnogalacturonan 1 (RG 1) has a more complex structure of repeating (1-2) α-L-rhamnosyl and (1-2) α-D-galacturonyl disaccharide subunits, with long side chains of arabinans and arabinogalactans (Carpita and Gibeaut, 1993; Cosgrove, 1997).

The esterification and de-esterification of pectins affects their physical properties. Many of the acidic residues associated with pectins are esterified with methyl, acetyl, and other unidentified groups (Kim and Carpita, 1992; McCann *et al.*, 1994). Such esterification occurs during pectin biosynthesis in the Golgi apparatus, and may be removed by esterases in the wall (Cosgrove, 1997). De-esterification creates carboxyl groups, thus increasing the charge density in the wall, which may, in turn, influence the activity of wall enzymes (Moustacas *et al.*, 1991). De-esterification promotes the assembly of helical pectin chains into a highly hydrated, gelatinous mesh by facilitating the formation of Ca^{2+} bridges, called "junction zones" (Rees, 1977), linking two anti-parallel pectin chains (Carpita and Gibeaut, 1993).

The pectin polymers are thought to perform many functions (Roberts, 1990). They apparently determine wall porosity, provide charged surfaces that modulate wall pH and ion balance (Roberts, 1990), and serve as recognition molecules that signal appropriate developmental responses to symbiotic organisms, pathogens and insects (McNeil *et al.*, 1984).

1.2.4, Structural proteins

In addition to the polysaccharides described above, structural proteins are also incorporated into the primary plant cell wall (Showalter, 1993; Cassab, 1998). These structural wall proteins are classified according to their predominant amino acid composition, for example, hydroxyproline-rich glycoproteins (HRGP), glycine-rich proteins (GRP), proline-rich proteins (PRP) and so on (Cassab, 1998). The most extensively studied group of wall proteins are the hydroxyproline-rich glycoproteins, which include a family of proteins called extensins. The extensins are important structural wall proteins which also apparently play a role in development, wound healing, and plant defence (Cassab, 1998). In dicots, extensins are expressed in

response to wounding, fungal infection, viral infection, fungal elicitors, endogenous elicitors, ethylene, red light, heat shock, gravity, tensile stress, glutathione and cell culturing (Showalter, 1993; Jose and Puigdomenech, 1993; Shirsat *et al.*, 1996 {a} and {b}).

Glycine-rich proteins are a newly discovered class of plant cell wall proteins. These are commonly localised in the vascular bundles, particularly xylem elements, and are clearly associated with cells that are going to be lignified (Showalter, 1993). In light of this, it is proposed that they play a key role in the development of vascular tissues (Cassab, 1998). The expression of these proteins is also induced by viral infection, salicylic acid, abscisic acid, drought stress, and wounding (Showalter, 1993).

Finally, proline-rich proteins also represent a relatively newly discovered class of plant cell wall proteins. They apparently participate in several aspects of development and wall differentiation, including xylem, nodule and pod differentiation, and ovary, embryo and microspore development (Cassab, 1998). In common with other wall protein groups, discussed above, proline-rich proteins are expressed in response to a number of external stimuli, such as wounding, endogenous elicitors, fungal elicitors, ethylene, light, red light and cell culturing (Showalter, 1993).

1.2.5, Cell wall enzymes

The cell wall is a metabolically active compartment of the cell (Cosgrove, 1997). Numerous enzymes have been found to be associated with the cell wall (Cassab and Varner, 1988; Fry, 1995). Four broad classes of reactions are catalysed by wall enzyme activities, hydrolysis (of glycosidic, ester, and peptide bonds), transglycosylation, transacylation (possibly involved in the synthesis of cutin), and redox reactions involving the electron acceptors O_2, H_2O_2, and NAD^+, and electron donors such as phenols, ascorbate, polyamines, and malate (see Fry, 1995).

Some enzymes act on the structure of the cell wall, for example, endoglucanases, xylosidases, pectinases, pectin methyl esterases, and xyloglucan endotransglycosylases modify the polysaccharide components of the cell wall (Fry, 1995). Peroxidases are

involved in lignification, extensin monomer cross-linking, and the repair of damaged walls (Vance *et al.*, 1980; Cassab and Varner, 1988; Bowles, 1990). While enzymes, such as the chitinases and (1-3) β-glucanases are designed to counter bacterial and fungal pathogen attack (Bowles, 1990).

1.2.6, Lignin

Lignin is a strengthening material that occurs in the cell walls of higher plants. It is one of the most abundant biopolymers on Earth (second only to cellulose), and is resistant to degradation by most micro-organisms (Kirk, 1971). Lignin is generally laid down between cellulose microfibrils, and provides mechanical support to plant tissues (Gould, 1983). The formation of lignin is considered by evolutionists to have been crucial in the adaptation of plants to a terrestrial environment, as it is an essential component of the rigid xylem vessel system, necessary to conduct water and mineral salts over long distances (see Gifford and Foster, 1989). The lignins are formed by oxidative polymerisation of p-coumaryl, coniferyl and sinapyl alcohols (Freudenberg, 1965; Alder, 1977). In addition to the strengthening function of lignin it also affords the plant some protection against attack by bacterial and fungal pathogens. Lignin synthesis is often induced at the site of a pathogen attack (Vance *et al.*, 1980).

1.2.7, Waxes, cutin and suberin

The entire shoot system of a herbaceous plant is covered by a cuticle that prevents excessive water loss from leaves, stems, flowers, fruits and seeds (Cutler *et al.*, 1980; Juniper and Jeffree, 1982). The cuticle also provides protection against some plant pathogens, and minor mechanical damage (Kolattukudy, 1987). The cuticle is largely composed of cutin and various waxes, which are attached to the pectin polysaccharides in the cell wall. Cutin is a heterogeneous polymer made up of fatty acids (Kolattukudy, 1980). The polymeric nature of cutin arises from ester bonds uniting the fatty acid hydroxyl and carboxyl groups. Small amounts of phenolic compounds are also present in cutin, and these are thought to anchor the cutin to the pectin polysaccharide component of the cell wall via ester linkages (Holloway, 1980, Kolattukudy, 1980).

Suberin forms a protective coating over underground plant tissues, such as roots. Suberin also covers cork cells formed in tree bark by the crushing action of secondary growth, and is formed by numerous cell types as scar tissue after wounding (Kolattukudy, 1980 and 1987). Suberin forms the Casparian strip of the endodermis and exodermis, and also occurs in the bundle sheath cell walls of grasses (Kolattukudy, 1980). Suberin is made up of a complex mixture of long chain fatty acids, hydroxylated fatty acids, dicarboxylic acids, long chain alcohols, and phenolic compounds of which ferulic acid is a major component (Holloway, 1983).

1.2.8, Cell wall water content

In growing plant tissues approximately two thirds of the wall mass is made up of water (Cosgrove, 1997). The bulk of this water is held within the pectin matrix, forming a dense gel (Tomos, 1988; Cosgrove, 1997). Water is a key component of the cell wall, and is apparently essential for cell wall expansion. Edelmann (1995) found that dehydration inhibits wall extension in isolated rye coleoptile cell walls. Dehydration of the wall matrix is also believed to play a direct role in growth inhibition induced by water deficits (Chazen and Neumann, 1994; Passioura, 1994). The mechanisms involved in cell expansion and cell wall extension are discussed by Cosgrove (1993) and McCann and Roberts (1994).

1.2.9, The hydrostatic and osmotic properties of the cell wall/apoplast

Neutral solutes can apparently diffuse freely throughout the aqueous phase of the cell wall (Tomos, 1988). Ions and charged molecules, on the other hand, are influenced by fixed negative charges in the polymer matrix. Pectin and other macromolecules in the cell wall have a large number of carboxyl groups (-COOH) from which hydrogen ions readily dissociate. This gives the cell wall a net negative charge (Tomos, 1988, Nobel, 1991). Cations such as Ca^{2+} are electrostatically attracted to the negatively charged components of the cell wall, leading to an exchange of H^+ for Ca^{2+} and other cations (see Richter and Dainty, 1989). This region of immobile negative charges in the wall is referred to as the Donnan phase (Tomos, 1988; Nobel, 1991). At equilibrium the Donnan phase attracts solutes of opposite charge from the aqueous phase of the wall,

thus creating an ion concentration gradient, which generates a Donnan potential between the Donnan phase and the bulk of the aqueous solution adjacent to it (Nobel, 1991).

The swarm of ions around the Donnan phase of the cell wall leads to a localised increase in osmotic pressure (Passioura, 1980; Tomos, 1988), which in turn draws water into the Donnan region. This influx of water generates a localised hydrostatic pressure, which is equivalent to the osmotic pressure generated by the Donnan phase (Passioura, 1980; Tomos, 1988). The countering action of the osmotic and hydrostatic forces of the Donnan phase means that the hydrostatic pressure (turgor pressure) inside adjacent cells is not affected by the Donnan phenomenon (Tomos, 1988).

Non-Donnan bound solutes present in the aqueous phase of the cell wall are osmotically active and influence the water relations of adjacent cells (Tomos, 1988). In the past it was assumed that cell wall/apoplastic solute concentrations were negligible (Tyree and Hammel, 1972; Sovonick *et al.*, 1974). However, in many plant species this has proven to be a false assumption. Glycophytes generally have relatively low concentrations of non-Donnan bound (free) solutes in the apoplast (Boyer and Knipling, 1965; Jachetta *et al.*, 1986; Nonami and Boyer, 1987). Halophytes and sink organs, on the other hand, accumulate high concentrations of osmotically active solutes in the wall (Meinzer and Moore, 1988; Flowers, 1985; Pomper and Breen, 1995; this study).

There is currently an increasing body of evidence, which suggests that cell wall solutes are actively maintained and controlled by the cell, and that they play an essential role in plant cell water relations, particularly turgor maintenance and regulation (Leigh and Tomos, 1983; Clipson *et al*, 1985; Tomos and Wyn Jones, 1988; Tomos, 1988; Bell and Leigh, 1996).

1.3, Primary aim

The primary aim of the work described in this study was to establish the role of apoplastic/cell wall solutes in osmotic adjustment and turgor regulation in two members of the family *Chenopodiaceae* - the halophyte *Suaeda maritima* L. Dum. and sugar beet (*Beta vulgaris* L.). These species share a common peculiarity, in that certain cell types, namely the leaf epidermal cells in *Suaeda maritima* and the taproot storage parenchyma cells in *Beta vulgaris*, maintain a cell turgor pressure (P_{cell}) which is dramatically lower than the respective cell osmotic pressures (Π_{cell}) might suggest (see Tomos, 1988). Given the observation that the hydrostatic component of the apoplast/cell wall (the transpiration tension) does not account for these differences between P_{cell} and Π_{cell} (Tomos, 1988), the only reasonable and sensible conclusion that can be drawn is that *Suaeda maritima* and *Beta vulgaris* accumulate and maintain a high concentration of solutes in the apoplast adjacent to these tissues, and that these free apoplastic solutes are instrumental in turgor regulation in these species.

The water relations of *Suaeda maritima* and *Beta vulgaris* were investigated at single cell resolution using a range of sophisticated and elegant techniques which are described fully in **Chapter 2** (see also Tomos and Leigh, 1999). **Chapters 3** through to **6** are experimental chapters, in which data are presented which highlight the remarkable properties of these species, and provide evidence which is consistent with the proposal that the apoplast can be, and is utilised as a true osmotic compartment in higher plants. In **Chapter 7**, the final discussion, turgor/osmotic dependent solute transport is discussed and the molecular basis of stimulus perception and osmotic adjustment (protoplastic and /or apoplastic), leading to turgor pressure regulation, is considered.

Chapter 2 : Techniques

2.1, Introduction

The aim of this chapter is to introduce the single-cell techniques I employed during this research programme. The experimental chapters which follow will each have a brief method section detailing the individual experiments carried out using these techniques. Full details of equipment manufacturers and chemical suppliers are given in the **Appendix**.

2.2, The cell pressure probe

2.2.1, Pressure probe set-up

A direct method for measuring the turgor pressure in higher plants at single cell resolution was described by Hüsken *et al.*, in 1978. The instrument consists of a fine, glass microcapillary attached by rubber seals (Cheshire Rubber Co.) to a perspex chamber, into a which a pressure transducer (Druck Ltd.) is set. The microcapillary and perspex chamber are filled with a non-viscous oil such as AS4 grade water saturated silicone oil (Wacker Chemicals Ltd.). A motor-driven metal rod (modified from a Hamilton syringe needle; Hamilton Co.) is used to increase or decrease the pressure within the oil filled system. In the pressure probe model generally used in Bangor, a solenoid valve (RS Components) is also in place designed to almost instantaneously return the oil in the probe to atmospheric pressure during single cell sampling (see **Section 2.3** and Malone *et al.*, 1989). A highly simplified plan of a pressure probe is illustrated in **Figure 2.1**; the plan is not drawn to scale.

Figure 2.1, A simplified plan of a cell pressure probe viewed from above

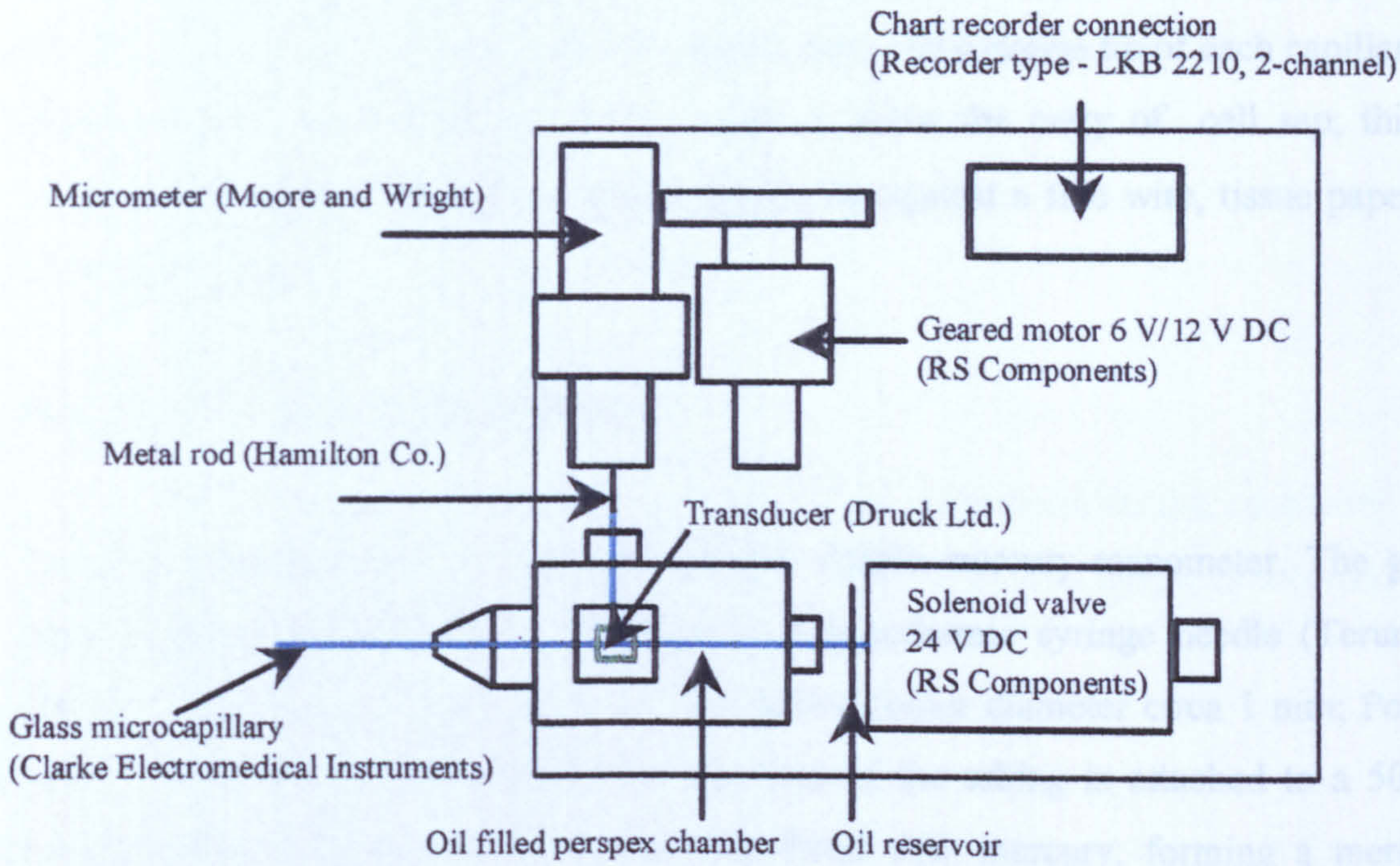
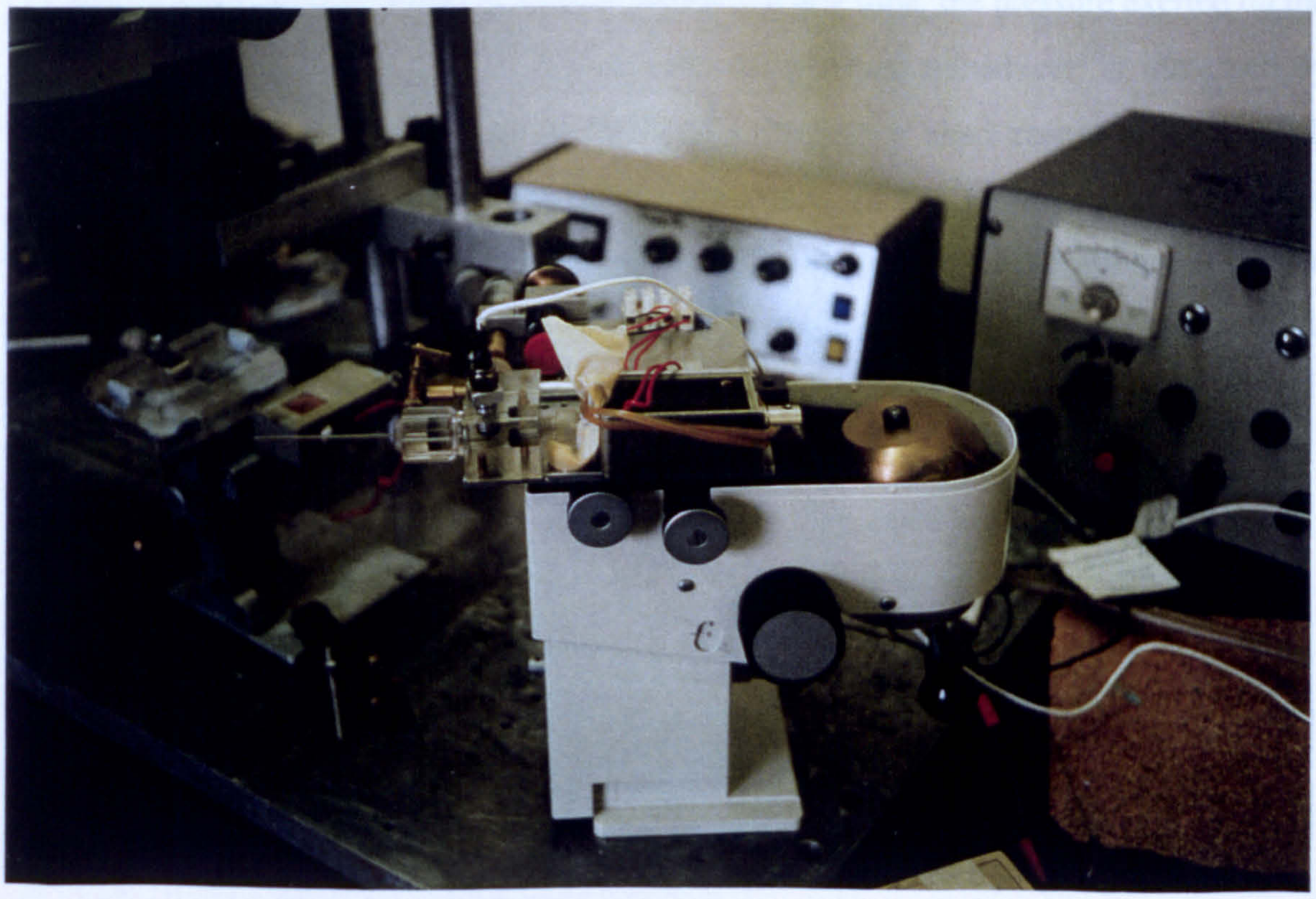


Plate 2.1, The cell pressure probe



The microcapillaries are made from 1 mm outer-diameter, 0.58 mm inner-diameter glass capillary tubing (standard wall without filament - Clark Electromedical Instruments), using a commercial capillary puller (Harvard Apparatus). Turgor pressure measurements require simple drawn out capillaries. The extreme tip of each capillary is broken slightly (to a diameter of 1 - 2 μm) to allow the entry of cell sap; this is achieved by gently brushing the microcapillary tip against a fine wire, tissue paper or even plant tissue.

2.2.2, Cell pressure probe calibration

The cell pressure probe is calibrated using a simple mercury manometer. The glass microcapillary of the probe is replaced by a hypodermic syringe needle (Terumo), which is attached to a long length of fine tubing (outer diameter circa 1 mm; Portex Ltd.) filled with mercury (BDH). The free end of the tubing is attached to a 50 ml syringe barrel (Terumo) which is partially filled with mercury, forming a mercury reservoir. The reservoir of mercury is initially positioned level with the probe's pressure transducer (at 0 m) and then raised 1 m at a time to a height of 5 m. At a height of 0 m the pressure exerted by the mercury is 0 MPa (relative to atmospheric pressure). As the level of the mercury is raised 1 m at a time, the pressure exerted on the oil in the probe chamber, and subsequently the pressure transducer, is increased by 0.1329 MPa m^{-1}. The pressure probe is connected to a chart recorder (LKB 2210, 2-channel chart recorder) which records the output of the transducer in millivolts. Pressure in MPa is plotted against number of millivolts to give a calibration plot for that particular pressure probe transducer (see **Figure 2.2**). The pressure transducer is calibrated every six months, and after any probe maintenance or repair procedure.

The relationship between pressure (MPa) and the height of mercury is shown in the following equation:

$$P = \rho g h$$

P = Hydrostatic pressure
ρ = Density of liquid (in this case mercury)
g = Acceleration due to gravity
h = Depth or height of liquid

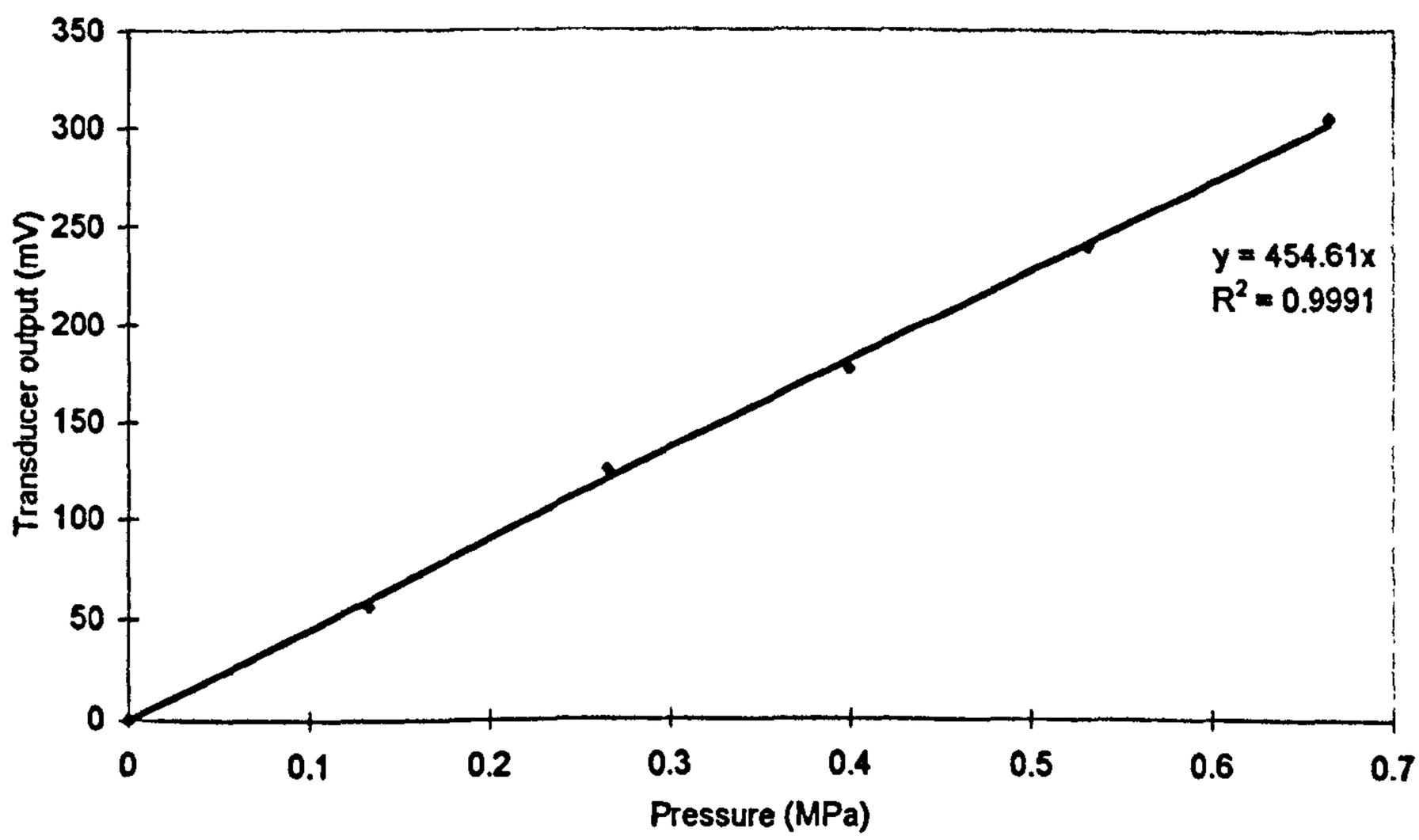
The density of mercury at 20 °C is 13550 kg m^{-3}. The acceleration due to gravity at 0 Km altitude (sea level) is 9.81 m s^{-2} (Halliday *et al.*, 1993). The height in this case is 1 m. Therefore:

$$P = \rho g h$$

$P = 13550 \text{ kg m}^{-3} \times 9.81 \text{ m s}^{-2} \times 1 \text{ m}$
$P = 132925.5 \text{ Pa}$

$P = 0.1329 \text{ MPa}$

Figure 2.2, A pressure probe transducer calibration plot



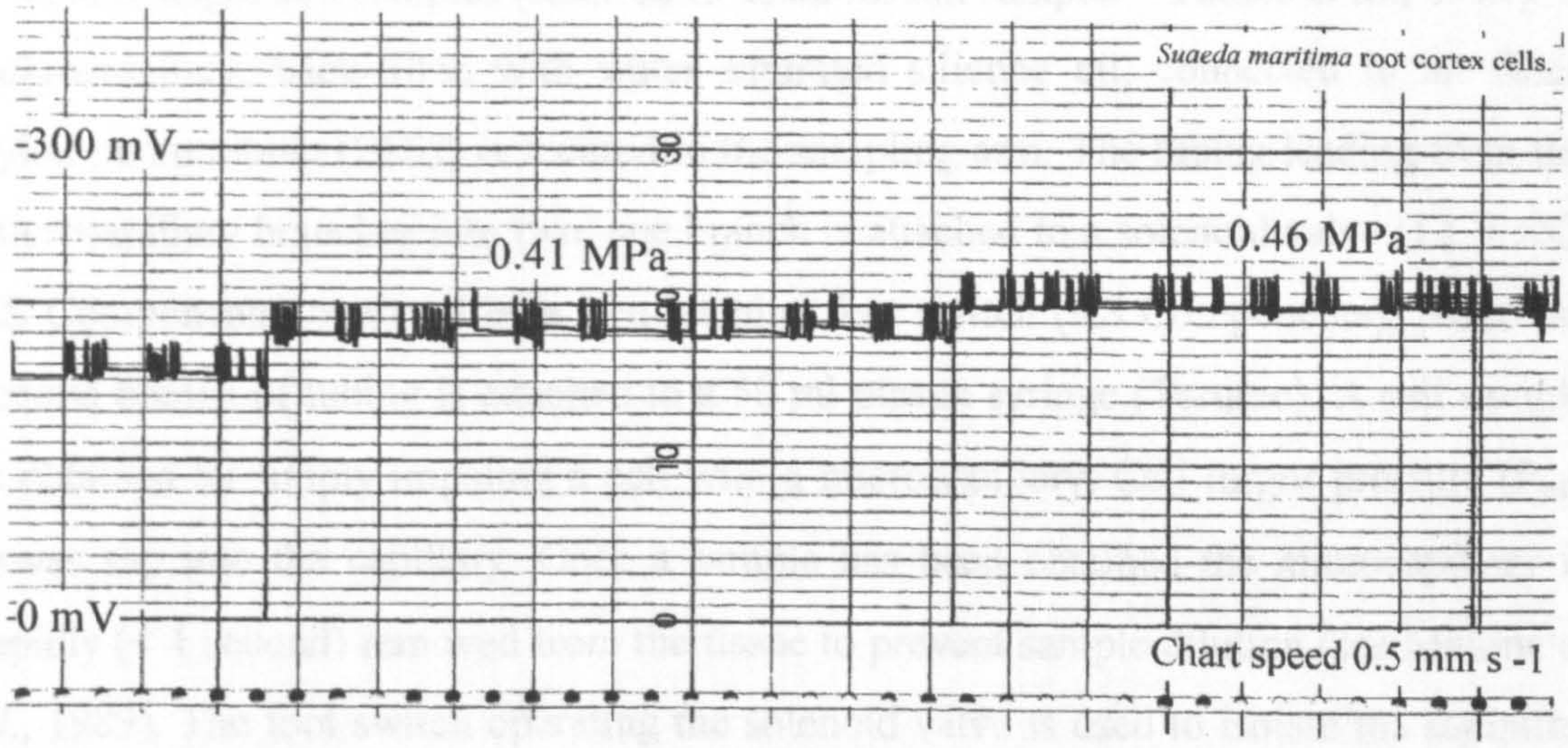
2.2.3, Turgor pressure measurement

The cell pressure probe is mounted on a micromanipulator (Leitz) allowing accurate positioning and fine adjustment of the glass microcapillary. Turgor pressure measurement involves impaling a cell with a microcapillary. Sap under pressure immediately enters the capillary forming a clearly visible sap/oil interface (referred to as the meniscus). The motor-driven metal rod is used to quickly increase the pressure within the probe chamber; ideally pushing the sap back into the cell within 1 - 2 seconds. A meniscus that cannot be freely manipulated suggests that the microcapillary tip is blocked. In this situation the microcapillary is removed and replaced, and another cell is targeted. Care is taken not to introduce any air into the system during this procedure, as the compressible nature of air can cause problems when the system is pressurised. In a cell that has been successfully impaled, the sap/oil meniscus is pushed as close as possible to the cell wall. At this point the meniscus is manipulated backward and forward by modulating the pressure in the oil filled chamber using the motor-driven metal rod. The meniscus is returned to the same position each time. Providing the cell is not leaking an output pressure trace, similar to the one illustrated in **Figure 2.3**, is obtained. The output signal recorded on the chart recorder corresponds to the pressure required to push the sap back into the cell. This is equivalent to the pressure inside the cell, i.e. the cell turgor pressure (P_{cell}). The whole procedure is viewed under a stereo microscope at maximum magnification (Wild M8, maximum magnification x 160; Leica). Depending on the application, turgor pressure measurements can be made in air (e.g. leaves) or under a liquid bathing medium (e.g. roots).

The cell pressure probe is highly sensitive to vibration. All turgor measurements are made on a vibration-resistant work bench. In Bangor the vibration-resistant benches are generally custom made using heavy concrete slabs set on breeze blocks, cushioned by cork tiles (all raw materials available from any DIY outlet). Vibration-resistant tables are available commercially from Intracel Ltd.

Figure 2.3, A typical pressure probe output trace

Each division along the horizontal axis corresponds to 1 cm.



2.3, Single cell sampling

2.3.1, Sampling with the pressure probe

The introduction of a solenoid valve to the pressure probe set-up allows the probe to be used as a single cell sampling device (Malone *et al.*, 1989; Tomos *et al.*, 1994). Once cell turgor pressure (P_{cell}) has been measured , a sample of cell sap can be obtained by opening the solenoid valve. This decreases the pressure inside the probe to atmospheric pressure, which allows cell sap, driven by the turgor pressure of the cell, to enter the glass microcapillary. The microcapillary, containing the sap sample, is withdrawn from the cell as quickly as possible to prevent sample dilution (the sampling procedure leads to a loss of cell turgor pressure, which in turn draws water into the cell by osmosis - see Malone *et al.*, 1989). The sap sample is expelled by closing the solenoid valve and increasing the pressure inside the probe using the motor-driven metal rod. Samples are expelled into water saturated paraffin oil (BDH) to prevent evaporation prior to analysis using a range of techniques.

2.3.2, Sampling with a pneumatic sampling arm

Alternatively, a simple sampling arm mounted onto the micromanipulator can be used to obtain single cell samples (referred to as an instant sampler - Tomos *et al.*, 1994). A microcapillary, back-filled with water saturated silicone oil, connected to air filled nylon pump tubing (BDH) is secured to the sampling arm. The tubing leading from the microcapillary branches into two, one branch is attached to a solenoid valve (12 V DC, RS Components) operated by a non-latching foot switch (RS Components), while the second branch of tubing is attached to a 50 ml plastic syringe (Terumo). A cell sample is obtained by simply impaling a cell with a microcapillary. Cell turgor pressure (P_{cell}) drives sap into the capillary. Once a sample has been obtained the microcapillary is rapidly (< 1 second) removed from the tissue to prevent sample dilution (see Malone *et al.*, 1989). The foot switch operating the solenoid valve is used to isolate the sampling system from the atmosphere allowing the sample to be expelled by applying pressure to the syringe barrel.

Sampling from cells under an aqueous bathing medium (e.g. root cells) may lead to cell sap dilution/contamination due to the uptake of bathing medium into the microcapillary by capillary action (Tomos *et al.*, 1994). This is detected by observing the behaviour of the sap/oil meniscus in the microcapillary. If the meniscus moves forward (away from the plant tissue) as the microcapillary tip is withdrawn from the cell, the sample is discarded. To combat this problem, the microcapillaries are silanized prior to use.

2.3.3, Microcapillary silanization

Microcapillaries are placed tip upwards in an aluminium capillary holder in a glass Pyrex beaker. The beaker is covered with aluminium foil and placed in a 200 °C oven for a minimum of 2 hours. The microcapillaries must be completely dry before silanization and are usually left in the oven overnight. The silanization procedure simply involves injecting 50 μl dimethyl-dichlorosilane (Sigma Chemicals) through the foil with a pipette and returning the microcapillaries to the oven for a further 2 hours.

The silane evaporates and coats each microcapillary, forming a hydrophobic surface. Dimethyl-dichlorosilane vapour is harmful; the silanization procedure is carried out in a fume cabinet and strict COSHH regulations are adhered to at all times. Silanized microcapillaries can be stored indefinitely.

2.4, Manipulation of single cell samples

2.4.1, Sample storage and manipulation

Single cell samples can be stored successfully for several days, or even longer, sandwiched between oil in a microcapillary at 4 °C (Tomos *et al.* 1994). However, I preferred to prepare and analyse each sample within 4 hours of sampling.

Individual single cell samples are expelled into water saturated paraffin oil to prevent evaporation. The paraffin oil is contained within an aluminium ring (custom made) which has been glued onto a microscope slide with epoxy resin adhesive. Each ring-slide is thoroughly cleaned and dried before use. The ring is washed in 10% Decon 90 (Decon Laboratories Ltd.) and hot water, and then rinsed in ethanol (BDH) and de-ionised water before being dried in a 200 °C oven for 30 minutes. The ring-slide is filled with water saturated paraffin oil and placed on an adjustable stage (Prior Scientific Instruments), under the stereo microscope. Single cell samples obtained as described in **Section 2.3.1 and 2.3.2**, are expelled into the oil and form small droplets on the glass slide. From here each droplet of cell sap can be sub-sampled and prepared for analysis.

2.4.2, Constriction microcapillaries

Sub-samples of equal volume can be taken using constriction pipettes (Tomos *et al.*, 1994). Microcapillaries are prepared using a commercial capillary tip puller as described in **Section 2.2.1**. The capillary tip is constricted using a commercial microforge (Beaudouin, Paris). The barrel of the microcapillary is carefully positioned alongside a heated wire micro-element using the microforge controls and microscope. A

constriction is formed as the microcapillary tip is drawn closer to the heated micro-element. Care is taken not to make the constriction too narrow as this will restrict the flow of sap into the capillary. Constriction pipettes of different volumes can be made by varying the diameter of the capillary barrel and/or by constricting the microcapillary further away from the tip (Lowry and Passonneau, 1972; Tomos *et al.*, 1994). The constriction pipettes used in my experiments were all approximately 10 pl in volume.

2.4.3, Sub-sample manipulation

The constriction microcapillaries are attached to a sampling arm mounted on the micromanipulator, as described in **Section 2.3.2**. Samples are drawn up into the microcapillary by closing the solenoid valve and creating a vacuum in the syringe. As the sample reaches the mid-point of the constriction, the solenoid valve is released and the sample ceases to enter the microcapillary. Sub-samples of equal volume can be drawn up using the same constriction microcapillary pipette. Silanization of the constriction microcapillaries is not essential but often makes the sample easier to manipulate within the pipette. Up to five sub-samples can be drawn into the same microcapillary, each sample sandwiched between a volume of water saturated paraffin oil. The sub-samples can then be expelled by closing the solenoid valve and pressurising the syringe.

2.5, Picolitre osmometry

2.5.1, Osmometer set-up

The osmotic pressure (Π_{cell}) of single cell samples is determined by measuring melting-point depression using a custom built picolitre osmometer (Malone *et al.*, 1989; Tomos *et al.*, 1994). A simplified diagram of a typical Bangor-built picolitre osmometer is illustrated in **Figure 2.4 {a}** and **{b}**. A pea size amount of white heat sink compound (RS Components) is placed in the middle of the recessed, copper stage.

Figure 2.4 {a}, A picolitre osmometer cooling unit (not drawn to scale)

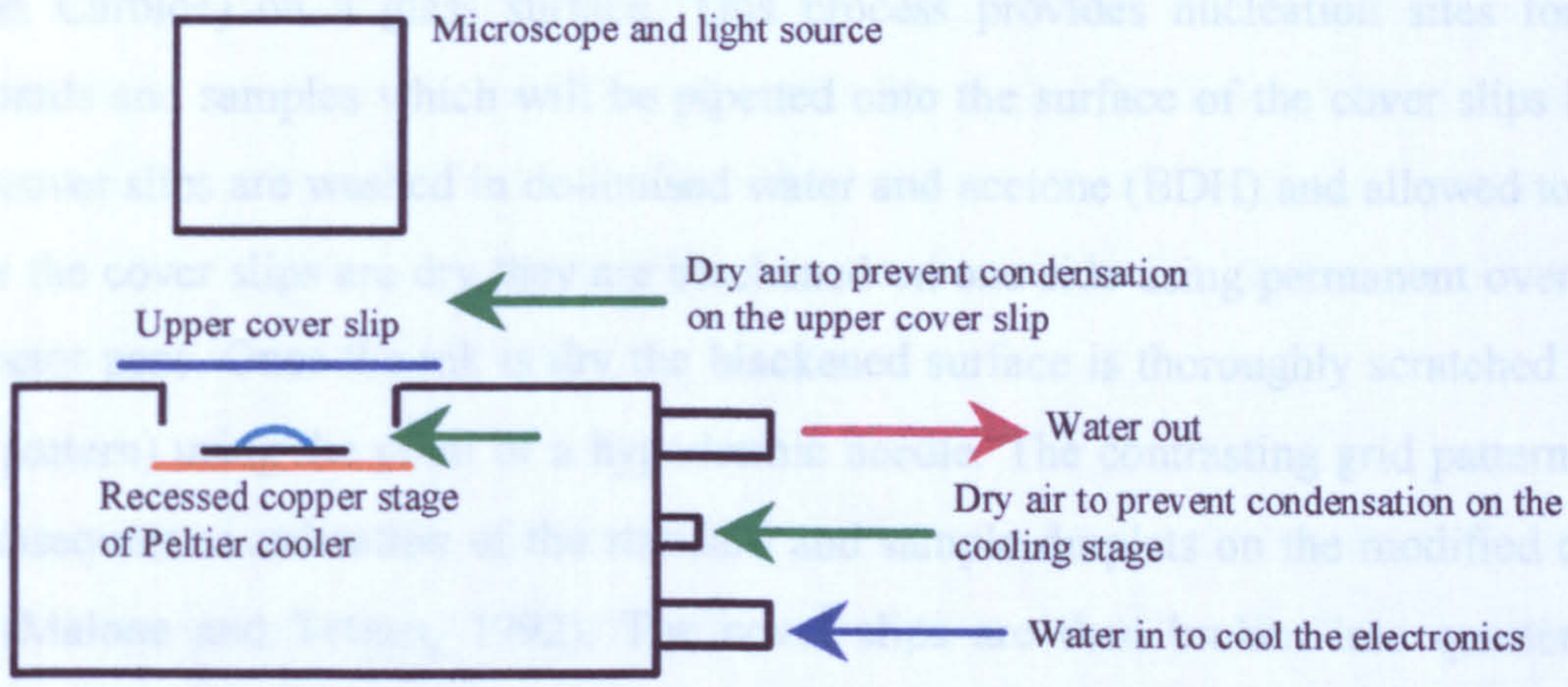
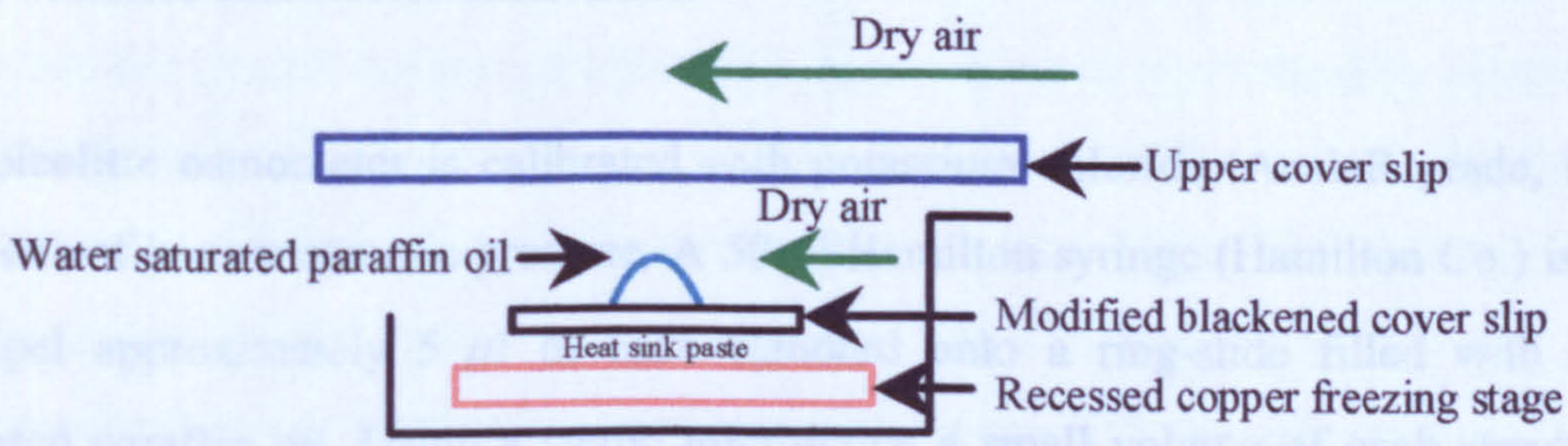


Figure 2.4 {b}, The recessed copper cooling stage (not drawn to scale)



Using forceps (curved tip N° 7; Agaraids), a modified blackened cover slip is positioned on top of the heat sink compound and gently pressed into place (**Figure 2.4 {b}**). The modified cover slips are prepared in the following way: Large cover slips (22 mm) are abraded on both sides in a slurry of abrasive alumina powder (Buehler Micropolish, Union Carbide) on a glass surface. This process provides nucleation sites for the standards and samples which will be pipetted onto the surface of the cover slips later. The cover slips are washed in de-ionised water and acetone (BDH) and allowed to dry. Once the cover slips are dry they are blackened on one side using permanent overhead projector pens. Once the ink is dry the blackened surface is thoroughly scratched (in a grid pattern) using the point of a hypodermic needle. The contrasting grid pattern aids in subsequent visualisation of the standard and sample droplets on the modified cover slip (Malone and Tomos, 1992). The cover slips are then broken into quarters by pressing down evenly with a sharp single-edged razor blade (TAAB Laboratory Equipment Ltd.). The unblackened surface is carefully cleaned with a lens tissue (Whatman International Ltd.) and ethanol before use. The cover slips are positioned blackened surface down onto the heat sink compound. Once in place, a small droplet of water-saturated paraffin oil is expelled onto the centre of the cover slip using a syringe. Standards and samples are pipetted onto the cover slip, within the droplet of oil, using the pneumatic sampling arm (**Section 2.3.2**) and appropriate microcapillary.

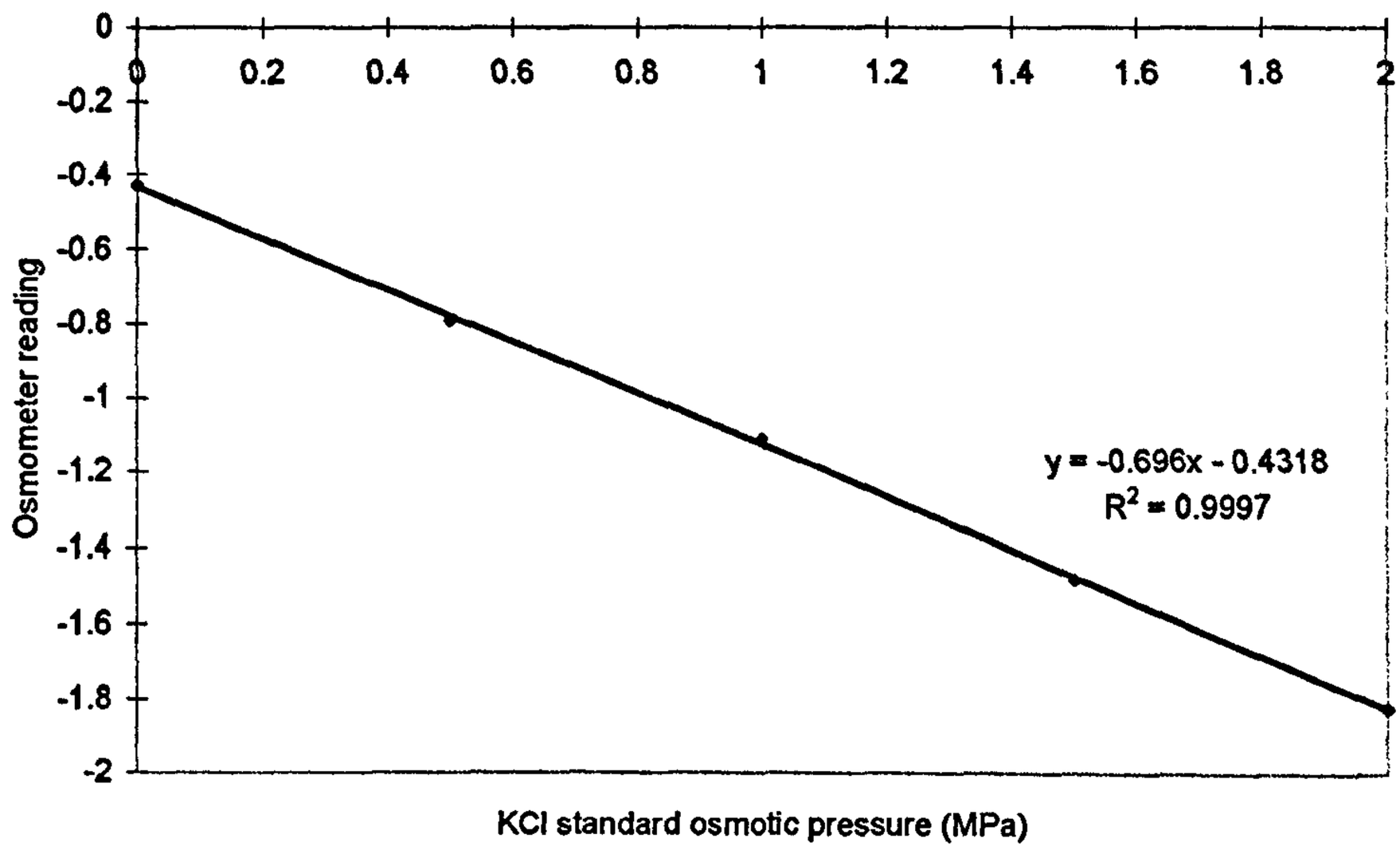
2.5.2, Picolitre osmometer calibration

The picolitre osmometer is calibrated with potassium chloride (AnalaR grade, BDH) solutions of known osmotic pressure. A 50 μl Hamilton syringe (Hamilton Co.) is used to expel approximately 5 μl of each standard onto a ring-slide filled with water saturated paraffin oil. Using a stereo microscope a small volume of each standard is picked up using the pneumatic sampling arm and a constriction microcapillary pipette (see **Section 2.3.2 and 2.4.2**). As noted earlier, up to five standards can be drawn into the pipette, each sandwiched between a volume of paraffin oil. Once the standards are safely contained within the pipette they are expelled onto the modified cover slip on the osmometer stage.

During the osmometer freezing cycle the Peltier cooler is cooled to - 40 °C to overcome the effects of super cooling and facilitate droplet freezing (Tomos *et al.*, 1994), before stabilising at around -5 °C. This freezing cycle generates a lot of heat. The osmometer is kept cool by continuously cycling water through the apparatus via tubing connected to a 15 °C water bath (Haake D3 Circulator; Fisher Scientific). A simple connection to the cold water mains supply is also adequate. Condensation is minimised by covering the recessed copper stage with a cover slip and by using a dry air supply. Air passed over silica gel crystals (BDH) is channelled beneath and/or above the upper cover slip using tubing and a three way tap system.

The osmometer hand set is used to initiate the freezing cycle. At this stage the dry air supply is passed both above and below the upper cover slip. Once the freezing cycle has begun the osmometer temperature control is turned to the coldest setting. The osmometer stage is cooled to - 40 °C, to induce freezing of the tiny droplets, rising to - 5 °C at the end of the cycle (each freezing cycle takes about 2 minutes). The frozen droplets should be clearly visible against the black and white background of the modified cover slip. The lower dry air supply is often switched off at this point as the upper air supply is generally adequate to eliminate condensation, providing that the upper cover slip is not removed. The temperature of the osmometer stage is then slowly increased until the ice crystals within the droplets begin to melt. The reading on the osmometer hand-set is recorded at the point where the ice crystals disappear. This procedure is repeated for each droplet. A calibration curve is obtained by plotting the osmometer reading against the known osmotic pressure of each standard (the standard droplets are positioned amongst the sap sample droplets). **Figure 2.5** shows a typical osmometer calibration curve. It is good practice to calibrate the osmometer at the beginning and at the end of an experiment to counter any drift that may occur.

Figure 2.5, A typical picolitre osmometer calibration plot



2.5.3, Measuring osmotic pressure in single cell samples

Samples can be placed on the osmometer stage directly from the cell, or smaller sub-samples can be taken from the ring-slide using the sampling arm and a constriction microcapillary, as described in **Section 2.4.3**. Droplets are placed on the osmometer stage while the osmometer is set at the warmest temperature setting. Whenever the upper cover slip is removed, dry air is channelled across the osmometer stage to prevent condensation forming on the surface of the oil. The freezing procedure for sample droplets is the same as described for the KCl standards (see **Section 2.5.2**). Once osmotic pressure has been measured the samples can be drawn back up into a microcapillary pipette and used for further analysis.

2.6, Energy dispersive X-ray microanalysis (EDX)

2.6.1, Protocol outline

Elements bombarded with high energy electrons emit X-rays with element specific characteristics. These can be used to quantify the amount of an element present in a sample (Morgan, 1985; Hinde, 1994; Tomos *et al.* 1994; Hinde *et al.*, 1998).

Standards and samples of fixed volume (typically 10 pl) are pipetted onto a thin 100/200-mesh copper grid (Agaraids) covered with a fine membrane of Pioloform (see Hinde, 1994; Hinde *et al.*, 1998). Each standard and sample droplet contains an equal volume of internal standard (250 mM rubidium nitrate in saturated mannitol; Sigma Chemicals and BDH respectively). The droplets are dried and analysed using a scanning electron microscope (Tomos *et al.* 1994). The resulting energy spectrum is used to calculate the concentration of elements in a sample.

2.6.2, Pipetting standards and samples onto a copper grid

Pioloform coated 200/100-mesh, folding copper grids are prepared as described by Hinde (1994). Pre-coated grids are also available commercially from Agaraids. A coated copper grid is positioned at the centre of a ring-slide, containing water saturated paraffin oil, Pioloform face up. Using curved forceps (N° 7; Agaraids), the grid is carefully lowered into the oil at a 45° angle, to prevent air being trapped beneath the membrane, and then gently positioned on the glass slide. A small volume (approximately 5 μl) of each standard (EDX standard composition is shown in **Table 2.1**), including the rubidium/mannitol internal standard, is expelled onto the ring slide, around the copper grid, using a 50 μl Hamilton syringe. The pneumatic sampling arm and a 10 pl silanized constriction pipette is used to pick up five sub-samples of each standard (see **Section 2.3.2** and **2.4.3**). The standards are then pipetted onto the Pioloform membrane. Each droplet is placed on the centre of a grid square, on the 200-mesh side of the copper grid. Using the same constriction pipette an identical volume of internal standard ($RbNO_3$/mannitol) is added to each standard droplet on the

grid. The rubidium concentration chosen is preferably equivalent to that of the major element expected in the sample. Inclusion of a rubidium internal standard allows the data to be normalised against the rubidium signal. The mannitol provides a matrix in which the elements are evenly dispersed as the sample droplet boils under the electron beam (Malone *et al.* 1991; Hinde, 1994; Tomos *et al.* 1994; Cuin, 1996, Bates, 1998; Hinde *et al.*, 1998).

Single cell samples, taken directly from the cell or from the osmometer stage, can be expelled initially onto the ring slide or onto the 100-mesh side of the copper grid. From here sub-samples are taken and pipetted onto the 200-mesh grid as described above. Care must be taken to include an equal volume of $RbNO_3$/mannitol internal standard to each sample droplet. Pipetting samples onto a grid is often a lengthy process, in order to limit evaporation from the sample and standard droplets the picolitre osmometer unit is used to cool the ring slide to approximately 4 °C. The position of each droplet pipetted on to the copper grid is noted on a grid map to aid in sample/standard identification later.

Table 2.1, EDX standard element composition and concentration

Standard	Na^+ (mM)	Cl^- (mM)	K^+ (mM)	Ca^{2+} (mM)
1	0	0	0	0
2	100	100	100	50
3	200	200	200	100
4	300	300	300	150
5	400	400	400	400

All standards were made up from the following AnalaR salts: sodium chloride, potassium nitrate and calcium nitrate (BDH).

2.6.3, Drying standard and sample droplets

The droplets are dried onto the grid in the following way: The copper grid is lifted out of the water saturated paraffin oil using curved forceps. Care is taken to avoid the 200-mesh side of the grid. The grid is dipped in a vial of water saturated hexane (Rathburn Chemicals) and gently rotated for 30 seconds to remove the paraffin oil. The grid is then transferred to a second vial of hexane and the procedure is repeated. Finally the grid is washed in a vial of water saturated isopentane (BDH) for a further 30 seconds before being allowed to dry in ambient air for a few seconds. The entire drying procedure is carried out in a fume cabinet (see Hinde, 1994; Tomos *et al.* 1994; Bates, 1998). The grids can be stored indefinitely in small plastic capsules (Agaraids) over silica gel crystals in a sealed container at room temperature.

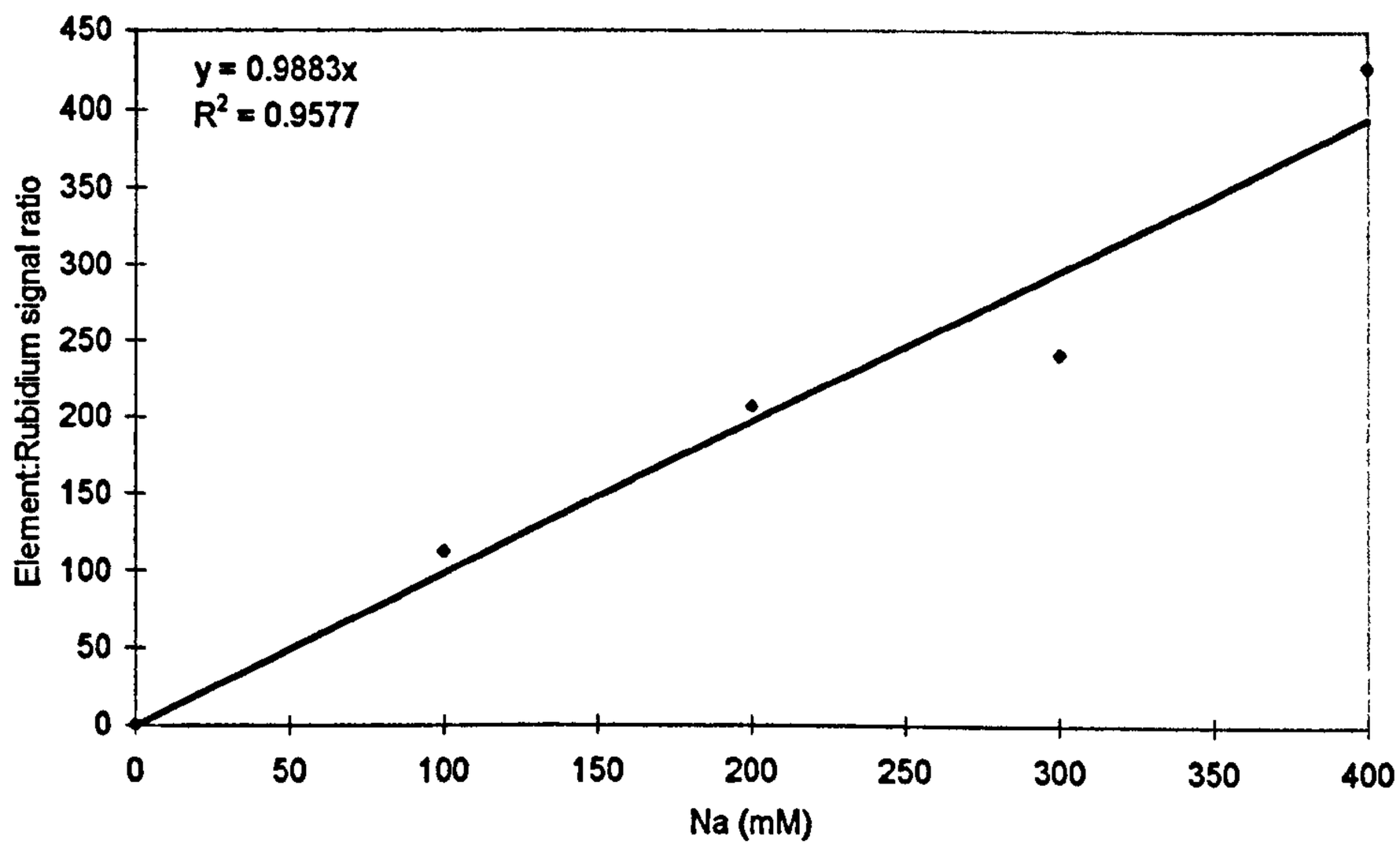
2.6.4, X-ray microanalysis

X-ray analysis is carried out using a scanning electron microscope (Hitachi S520) equipped with a Si-Li detector coupled to a Link Analytical QX2000 microanalytical system. The 100-mesh side of the grid is secured to a modified carbon block by a fine gauge sprung brass clip. The carbon block in then mounted on an aluminium stub, which in turn is positioned inside the scanning electron microscope chamber. The sample is aligned at a 45° angle to the detector and electron gun. Once a vacuum has been established in the sample chamber the dried microdroplets are individually scanned with an electron raster just large enough to cover the droplet. Analysis continues until the gross integral for rubidium has reached 4500 counts or 1000 seconds has elapsed (whichever is sooner). The accelerating voltage is set at 14 kV (Hinde, 1994; Bates, 1998; Hinde *et al.*, 1998). When K^+ and Ca^{2+} are present in the same sample the signal peaks overlap. To counter this, 10 % of the K^+ signal is subtracted from the Ca^{2+} signal (Tomos *et al.*, 1994; Hinde *et al.*, 1998).

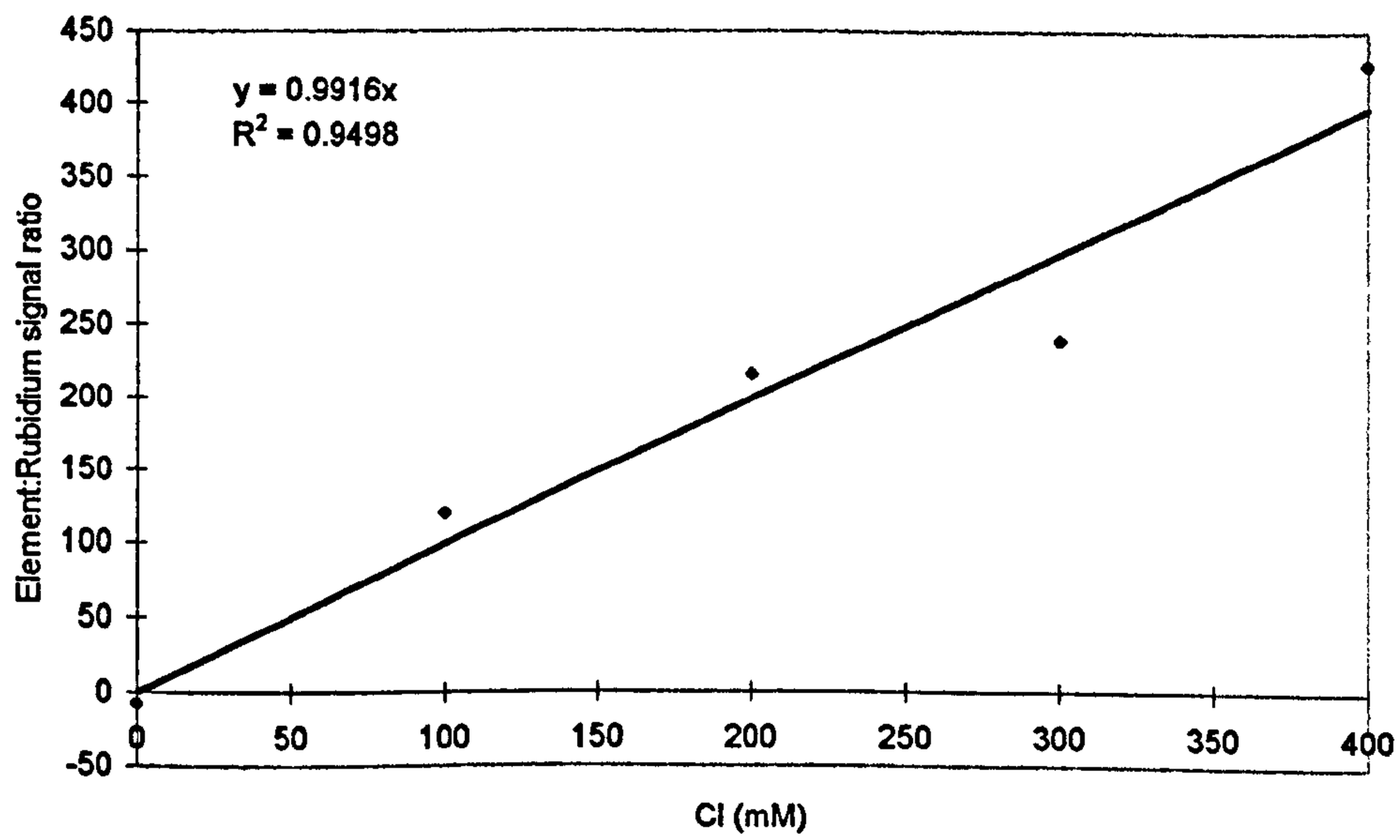
The element:rubidium signal ratio is calculated for each element. The signal ratio is then plotted against the known concentration of the element in the standard droplets to give a calibration plot (see **Figure 2.6 {a to d}**).

Figure 2.6, A typical EDX calibration plot

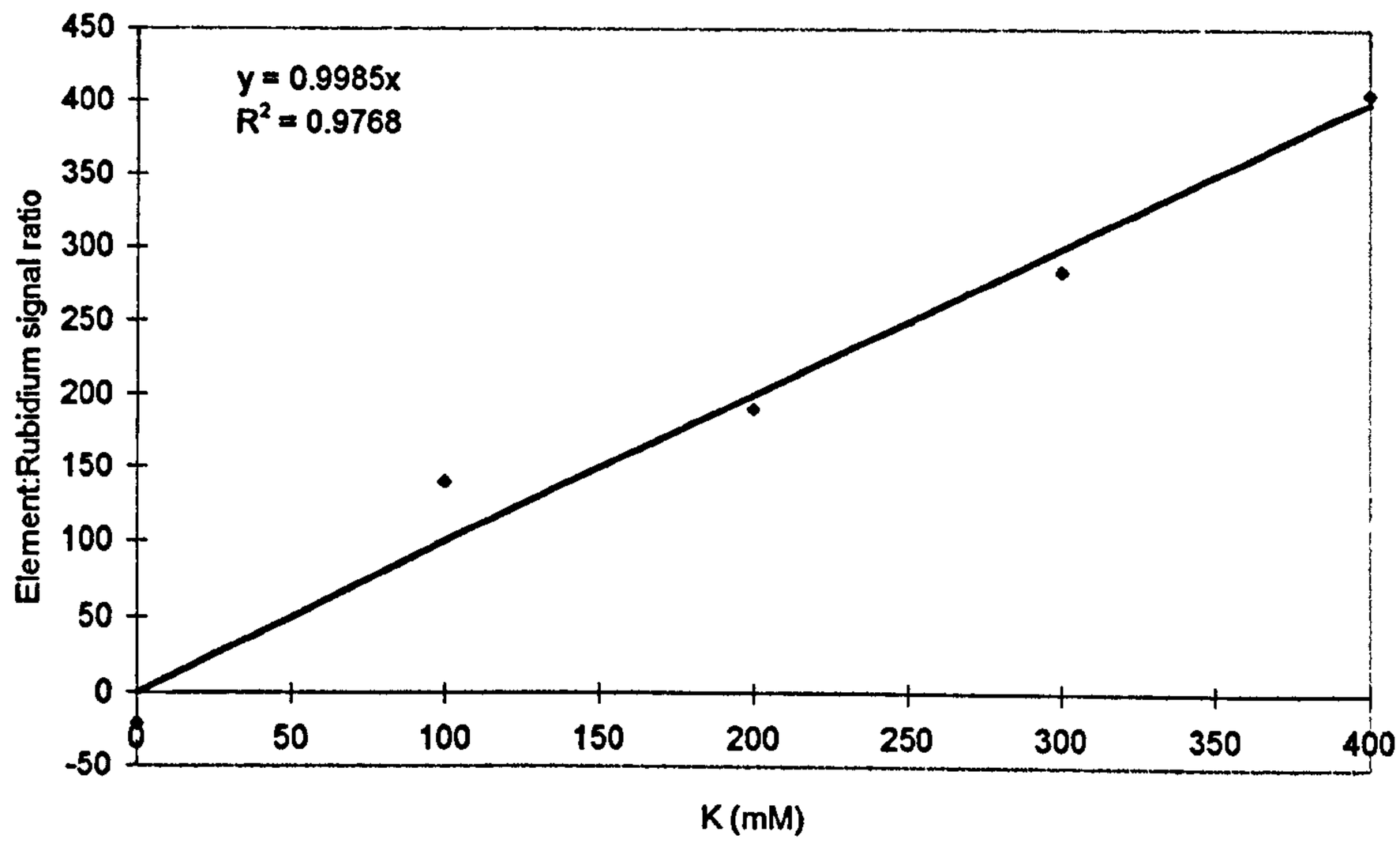
{a} Sodium



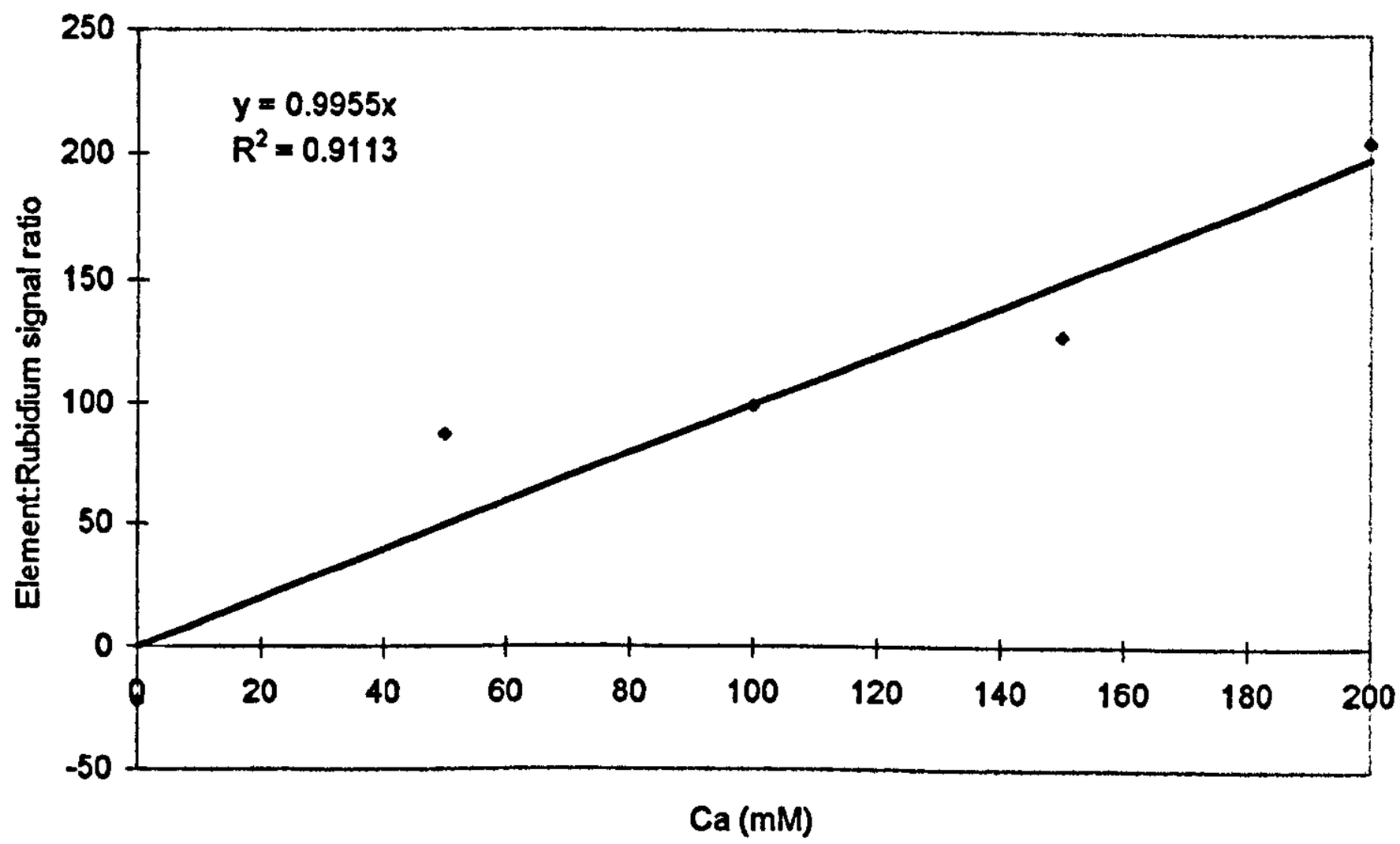
{b} Chloride



{c} Potassium



{d} Calcium



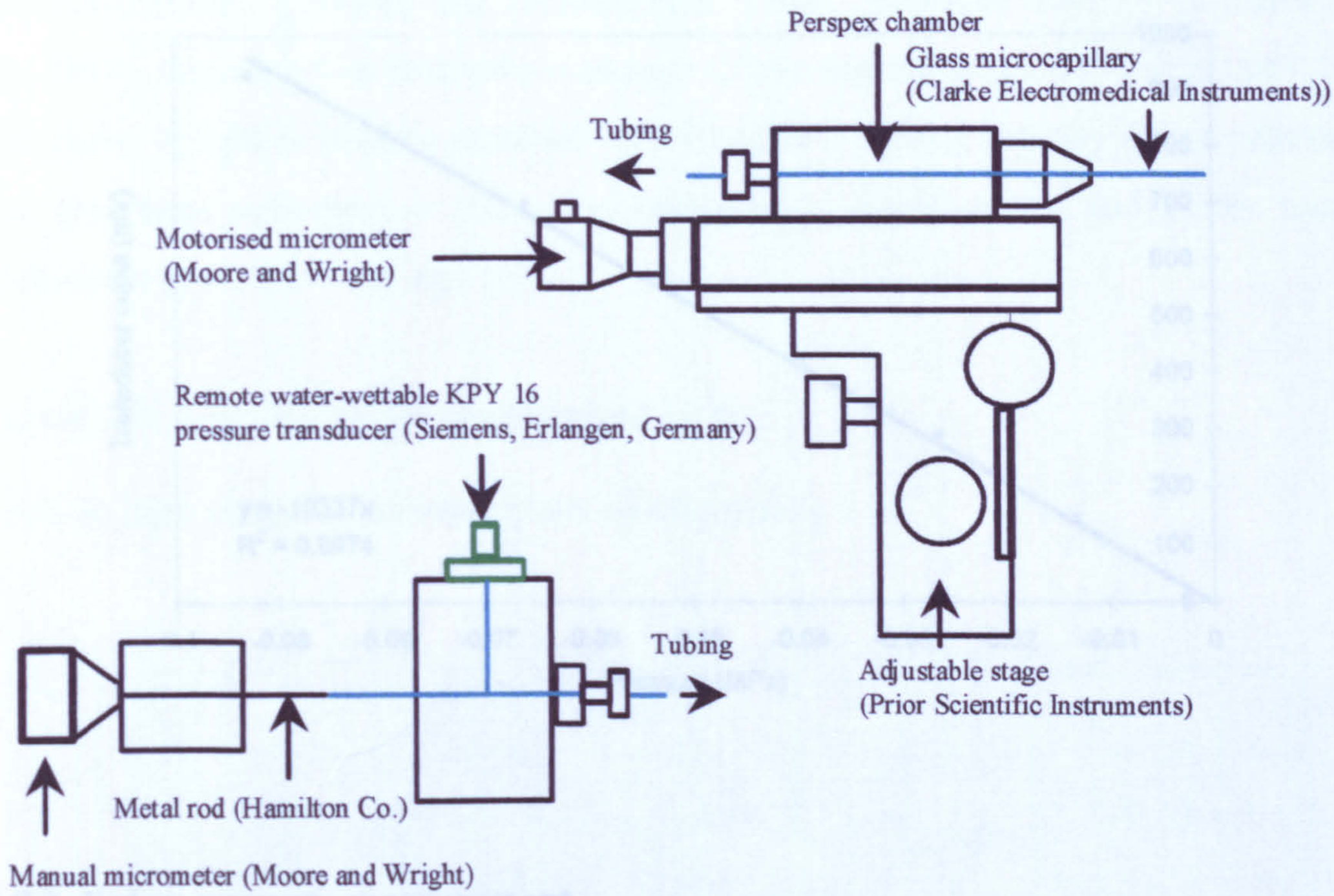
2.7, The xylem pressure probe

2.7.1, Xylem probe set-up

Balling and Zimmermann (1990) described a direct method for measuring xylem tension/pressure (P_{xylem}) in the xylem vessels of an intact plant. The xylem pressure probe has evolved from the cell pressure probe described in **Section 2.2**. The instrument consists of a fine glass microcapillary connected by rubber seals to a perspex chamber, which is attached to a remote water-wettable KPY 16 pressure transducer (Siemens, Erlangen, Germany) via fine tubing. The microcapillary and perspex chamber, through to the remote pressure transducer, are filled with degassed, de-ionised water rather than silicone oil. Water is substituted for oil in this system, as cavitation most readily occurs at the oil/sap interface during a xylem pressure measurement (Balling *et al.*, 1988; Balling and Zimmermann, 1990). The microcapillaries are made from capillary tubing, using a commercial capillary puller (as described for the cell pressure probe in **Section 2.2.1**). The extreme tip of each microcapillary is broken to a diameter of approximately 5 μm. The pressure inside the xylem probe system can be modulated using a manually-driven metal rod (Hamilton Co.), which is fed through rubber seals into the xylem probe chamber. The probe is mounted on a motorised micrometer (Moore and Wright) on a fully adjustable stage (Prior Scientific Instruments). A simplified plan of the xylem pressure probe is illustrated in **Figure 2.7** (not drawn to scale).

The xylem pressure probe is connected to a control panel which drives the motorised micrometer, which in turn allows the microcapillary to travel in and out of the plant tissue. The control panel also allows a motor cut off point to be set manually. When the xylem probe pressure transducer detects a pressure which is equivalent to or greater than that of the predetermined cut off point, the motor stops automatically. This is an essential xylem probe feature, as the probe operator cannot see the tip of the microcapillary (once it has entered the tissue) or the targeted xylem vessel. The motor cut off point is generally set between - 0.0025 MPa and - 0.005 MPa (relative to atmospheric pressure).

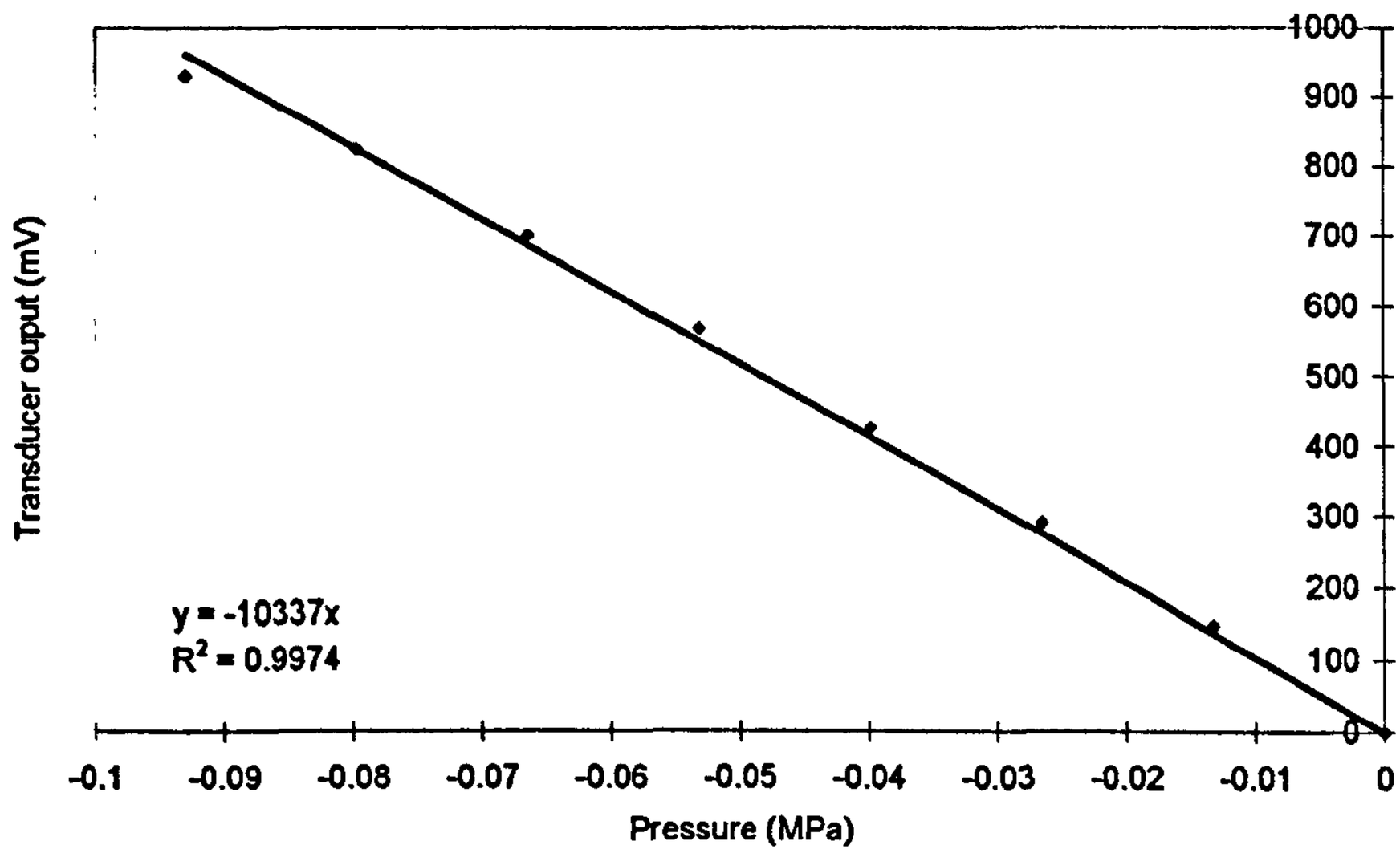
Figure 2.7, A simplified side view of a xylem pressure probe



2.7.2, Xylem pressure probe calibration

The xylem pressure probe is calibrated using a mercury manometer (as described for the cell pressure probe in **Section 2.2.2**). The xylem probe and pressure transducer are raised to a height of 1 m (from the floor) on a vibration-resistant work bench. The reservoir of mercury is initially positioned level with the pressure transducer to register 0 MPa (relative to atmospheric pressure). The mercury is then lowered, at 10 cm intervals, towards the floor. A 10 cm decrease in the level of the mercury reservoir results in a 0.01329 MPa decrease in the pressure exerted on the pressure transducer. The relationship between pressure (MPa) and height of mercury is explained in **Section 2.2.2**. The xylem probe is connected to a chart recorder which records the output of the pressure transducer in mV. A calibration curve is obtained by plotting pressure in MPa against number of millivolts **(Figure 2.8)**.

Figure 2.8, A typical xylem probe transducer calibration plot



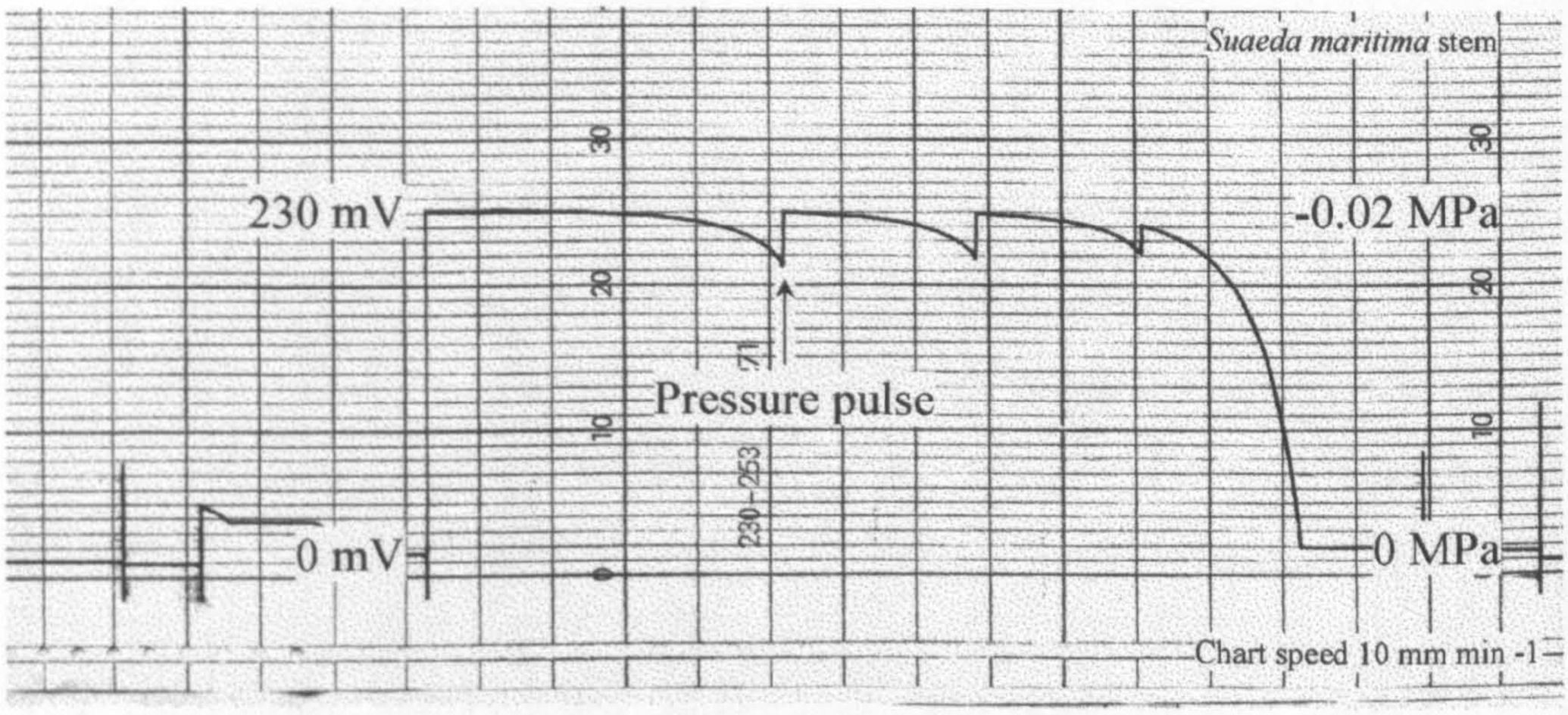
2.7.3, Xylem pressure measurement

The xylem pressure probe microcapillary is positioned adjacent to the area of stem to be probed using the adjustable stage; positioning is by eye only. The motorised micrometer advances the glass microcapillary through stem. Penetration stops immediately when the pressure transducer registers a pressure equivalent to or greater than the predetermined cut off point set on the control panel. Pressure pulses are introduced into the system using the manually driven metal rod; pressure recovery indicates that the microcapillary tip is not blocked. Providing the xylem pressure probe and plant are not disturbed in any way, xylem pressure can be measured successfully for an hour or more. A xylem probe output trace is shown in **Figure 2.9**. Unfortunately, the microcapillary tip often fails to hit a xylem vessel at all. In this situation the microcapillary is withdrawn from the tissue and a different section of stem is targeted. If the microcapillary is blocked (i.e. water cannot be freely expelled from the tip by increasing the pressure in the xylem probe chamber using the metal rod) or broken, it is replaced, taking care not to introduce any air into the probe chamber during the

procedure. Successful xylem pressure measurement requires patience and perseverance. Successful xylem pressure measurements have been obtained from the stems of *Nicotiana rustica* ((Balling and Zimmermann, 1990), *Nicotiana tabacum* (Benkert *et al.*, 1991), the mangrove *Rhizophora mangle* (Zimmermann *et al.*, 1994 {b}), and the salt marsh halophyte *Suaeda maritima* (see **Chapter 3**). More recently xylem pressure has also been measured in roots, for example, in maize, wheat and barley roots (Schneider *et al.*, 1997 {a} and {b}).

Figure 2.9, A typical xylem probe output trace

Each division along the horizontal axis corresponds to 1 cm.



2.8, Summary

Parameter	Technique	Section
Turgor pressure (P_{cell})	Cell Pressure Probe	**2.2**
Osmotic pressure (Π_{cell})	Picolitre Osmometry	**2.5**
Inorganic ions	EDX analysis	**2.6**
Xylem pressure (P_{xylem})	Xylem pressure probe	**2.7**

Chapter 3 : Salt tolerance in *Suaeda maritima* L. Dum.

3.1, Introduction

Suaeda maritima (L.) Dum. is a remarkable halophyte which has been widely used in physiological studies of the mechanism of salt tolerance in halophytes (Flowers, 1975; Flowers *et al.*, 1977; Greenway and Munns, 1983; Yeo, 1983; Clipson, 1987). *Suaeda maritima* can tolerate a wide range of NaCl concentrations, and grows optimally at 200 mM NaCl (Yeo, 1974; Flowers *et al.*, 1977; Yeo and Flowers, 1980). This species also grows successfully in the absence of NaCl (Flowers, 1971; Clipson, 1987; this chapter), however, its distribution is confined to saline areas due to the pressures of competition in the glycophytic domain (Chapman, 1947; Clapham *et al.*, 1962; Clapham *et al.*, 1987).

Using the sophisticated suite of single cell techniques introduced in **Chapter 2**, the salt tolerance mechanisms employed by *Suaeda maritima* (in steady state and fluctuating external NaCl) can be studied at single cell resolution. In this study the hydrostatic and osmotic properties of leaf epidermal cells and root cortex cells were determined using the cell pressure probe (Husken *et al.*, 1978; Tomos, 1988; **Chapter 2, Section 2.2**), picolitre osmometry (**Chapter 2, Section 2.5**) and EDX analysis (Tomos *et al.*, 1994; **Chapter 2, Section 2.6**). The hydrostatic nature of the xylem, located in the stem, was determined using the xylem pressure probe (Balling *et al.*, 1988; Balling and Zimmermann, 1990; **Chapter 2, Section 2.7**).

3.1.1, *Suaeda maritima* (L.) Dum.

Suaeda maritima belongs to the family *Chenopodiaceae*. This family is made up of about 100 genera containing at least 500 plant species (Clapham *et al.*, 1962; Clapham *et al.*, 1987). A characteristic feature of the *Chenopodiaceae* is the tolerance of its members to adverse conditions such as drought and salinity (McArthur and Sanderson, 1984); approximately half of the genera within the family include examples of halophytes (Flowers and Yeo, 1988).

The genus *Suaeda* is made up of about 40 species, consisting mainly of herbs and small shrubs. Members of this genus have a cosmopolitan distribution and are generally found in saline habitats (Clapham *et al.*, 1987).

The species *Suaeda maritima* is an annual dicot which is native to the British Isles, occurring exclusively on salt marshes and sea shores usually below the high water mark of spring tides (Chapman, 1947). This species is also common around the coasts of Europe (except the Arctic), and is found inland in saline areas of Central Europe, Russia, Eastern Asia, the East Indies and North America. Subspecies, or possibly other closely related allied species, have also been found in South America and Australia (Chapman, 1947; Clapham *et al.*, 1962 and 1987).

The Flora of the British Isles recognises three varieties of *Suaeda maritima* (L.) Dum. (Clapham *et al.*, 1962 and 1987). A brief description follows below:

- Var. *maritime* (also referred to as Var. *vulgaris* Moq. by Chapman, 1947). Plant large, branches spreading. Seed not more than 1.5 mm in diameter. Flowers August to October.

- Var. *macrocarpa* (Desv.) Moq. Plant decumbent or prostrate, rarely erect and then small. Leaves up to 10 mm, seed 2 mm in diameter. Flowers July to August.

- Var. *flexilis* (Focke.) Rouy. Usually erect, often unbranched and never branched from the base; branches are short and erect. Leaves 10 - 25 mm. Seed 1.1 - 1.4 mm in diameter. Flowers August to October.

The *Suaeda maritima* variety described in this chapter is var. *maritime*. Seeds were collected from a local salt marsh, near Brynsiencyn, on the Isle of Anglesey, North Wales. Further details regarding the site location and collection of seeds are included in the following methods section (**Section 3.2**).

3.1.2, Structure and anatomy

A simple line drawing of *Suaeda maritima* is presented in **Figure 3.1**. The cell types targeted along the axis of the plant, throughout this salt tolerance study, are illustrated in the following photomicrographs and accompanying labelled cell plans. The magnitude of magnification (indicated above each photomicrograph) refers only to the photomicrograph and not to the cell plan.

Suaeda maritima leaves emerge in pairs. Initially each leaf pair forms a discernible tier along a central axis. After 3 or 4 leaf pairs have emerged axilliary leaves and subsequently branches develop sequentially up the axis. For ease of identification each leaf pair can be allocated an appropriate number or symbol. A transverse section through a typical *Suaeda maritima* leaf approaching full expansion is illustrated in **Photomicrograph 3.1**. The leaves are cylindrical and succulent in nature. The leaf is protected by a single layer of epidermal cells which are characteristically uniform adaxially and abaxially. Pressure probe and sampling work was carried out on epidermal cells situated mainly on the adaxial surface of the leaf. This cell type was chosen due to uniformity of the tissue and ease of access.

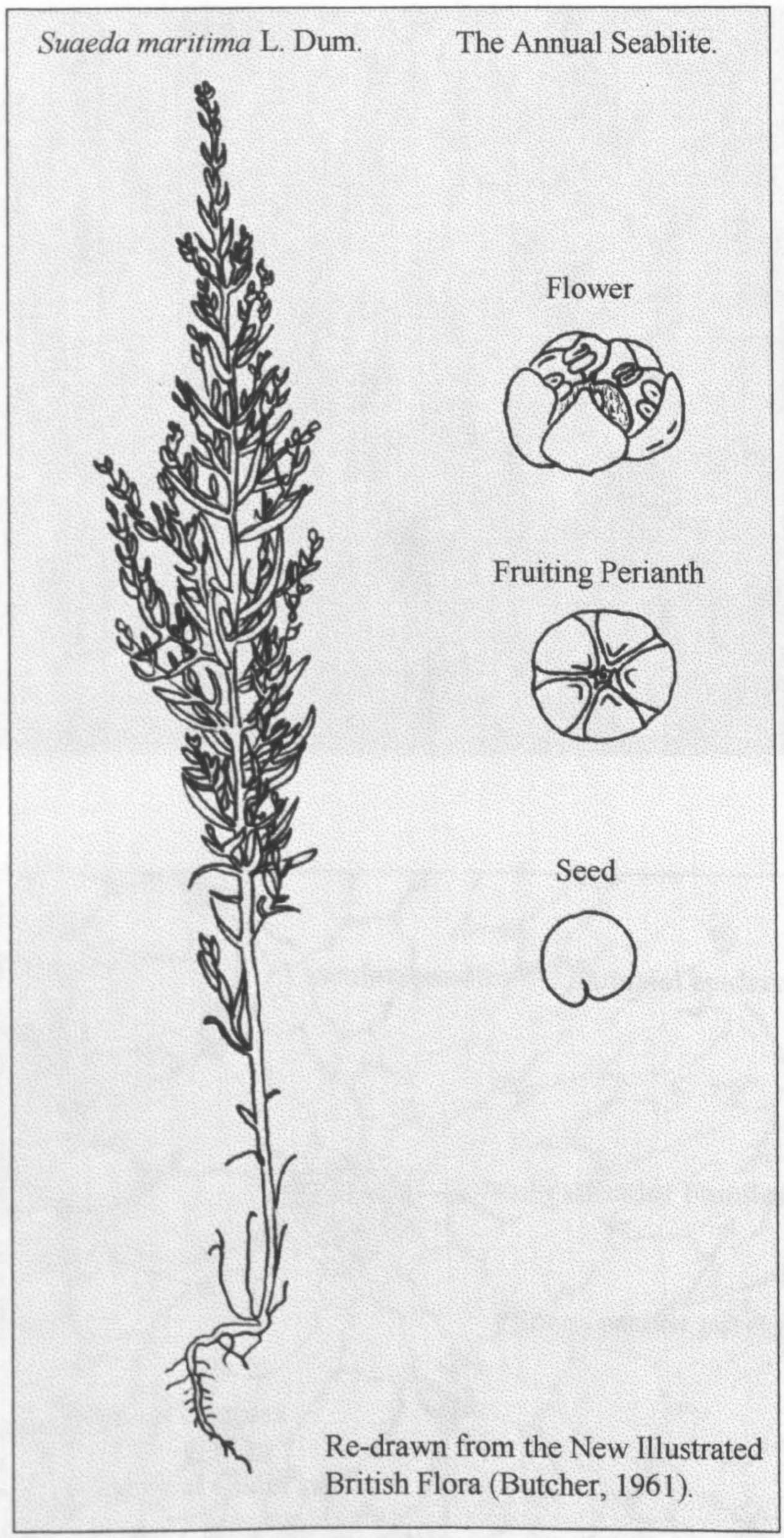
The photosynthetic parenchyma cells adjacent to the adaxial epidermis resemble elongated palisade cells, whereas the photosynthetic parenchyma adjacent to the abaxial leaf surface are rounded and spongy in nature. Towards the centre of the leaf the parenchyma cells become enlarged and irregular; these cells are referred to as water cells. The xylem and phloem tissue are arranged in vascular bundles. A large vascular bundle is positioned centrally with smaller bundles radiating away towards the extremes of the leaf.

Suaeda maritima has an extremely fine and delicate root system. A transverse section through a root, approximately 1 cm from the root tip, is illustrated in **Photomicrograph 3.2**. The outer layer of cells form the protective epidermis. Directly beneath the epidermis are three layers of large cortical cells. The root cortex is not tightly packed and has numerous air spaces. Specialised, thickened cortical cells, referred to as the endodermis encircle the vascular tissue. The vascular tissue is positioned in the centre

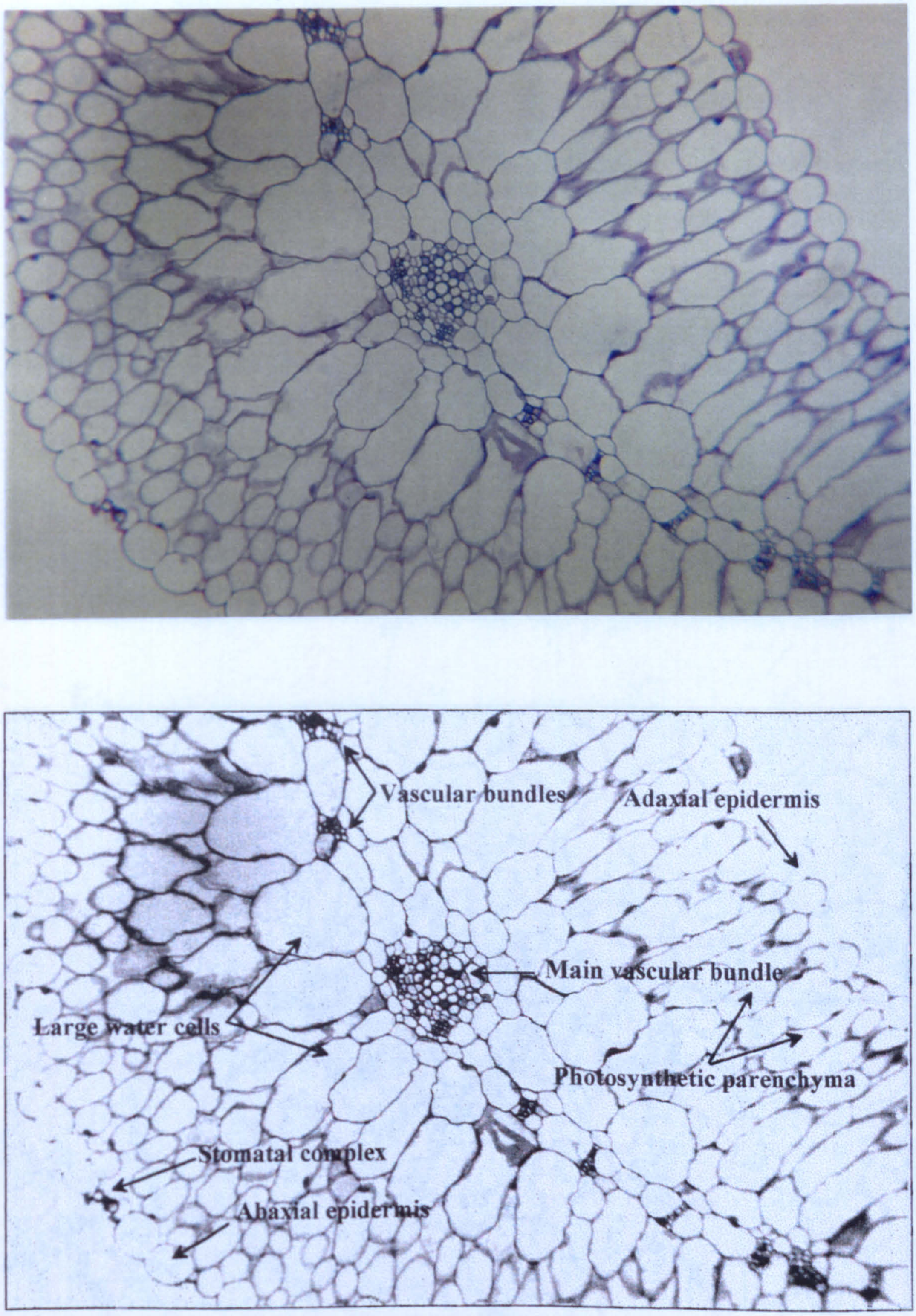
of the root, and consists of an outer pericycle and inner xylem and phloem tissue. The root tissue targeted for probing and sampling purposes was the large root cortex.

The stem (particularly below the cotyledons) is often a quite complex structure (Mauseth, 1988). Xylem pressure probe measurements were taken midway between the root and cotyledon. The structure of the stem in this region is illustrated in **Photomicrograph 3.3**. This photomicrograph beautifully illustrates how the xylem and phloem tissues are scattered almost at random throughout this tissue. The xylem vessel cells are large and numerous, which is ideal for xylem pressure probe work. The stem consists of an outer epidermis (which appears to be breaking away from the main body of tissue in this example), followed by a ring of uniform cortical cells. Below the cortex lies a more thickened tissue, referred to as sclerenchyma tissue. Embedded within the this thickened tissue are the vascular tissues. The thickened sclerenchyma and vascular tissues form a large central core within the stem.

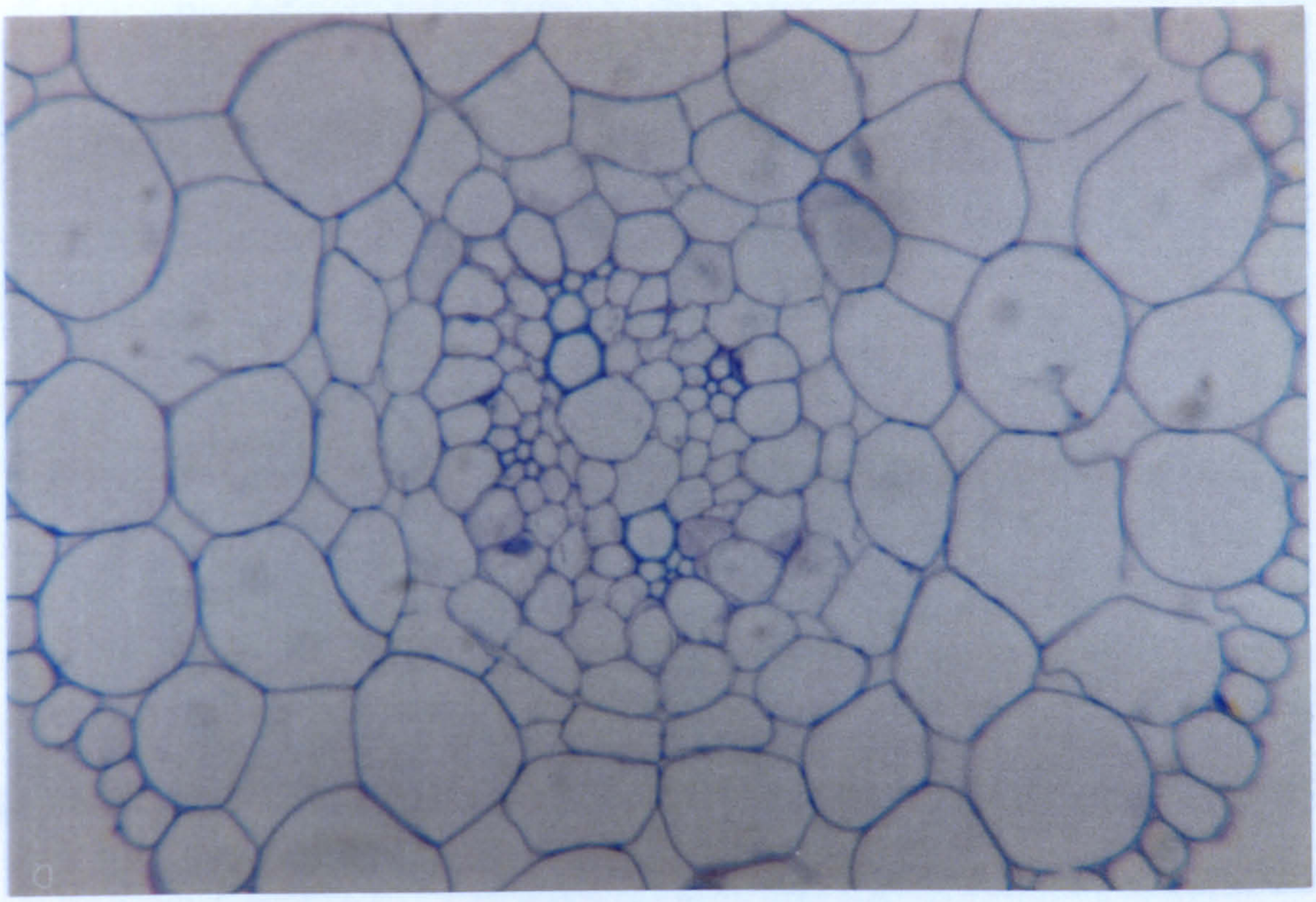
Figure 3.1, *Suaeda maritima* line drawing

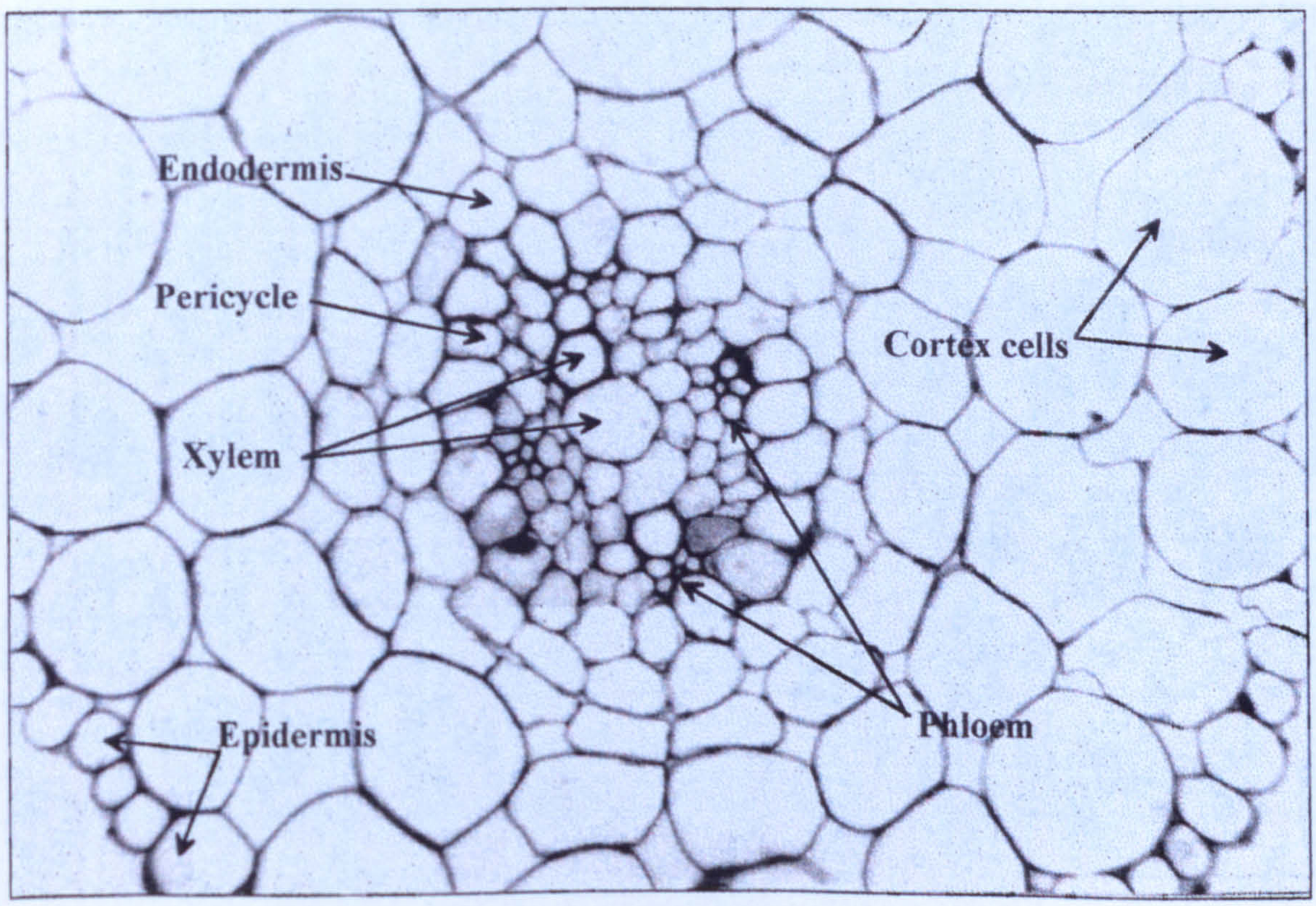


Photomicrograph 3.1, TS *Suaeda maritima* leaf (x 100)

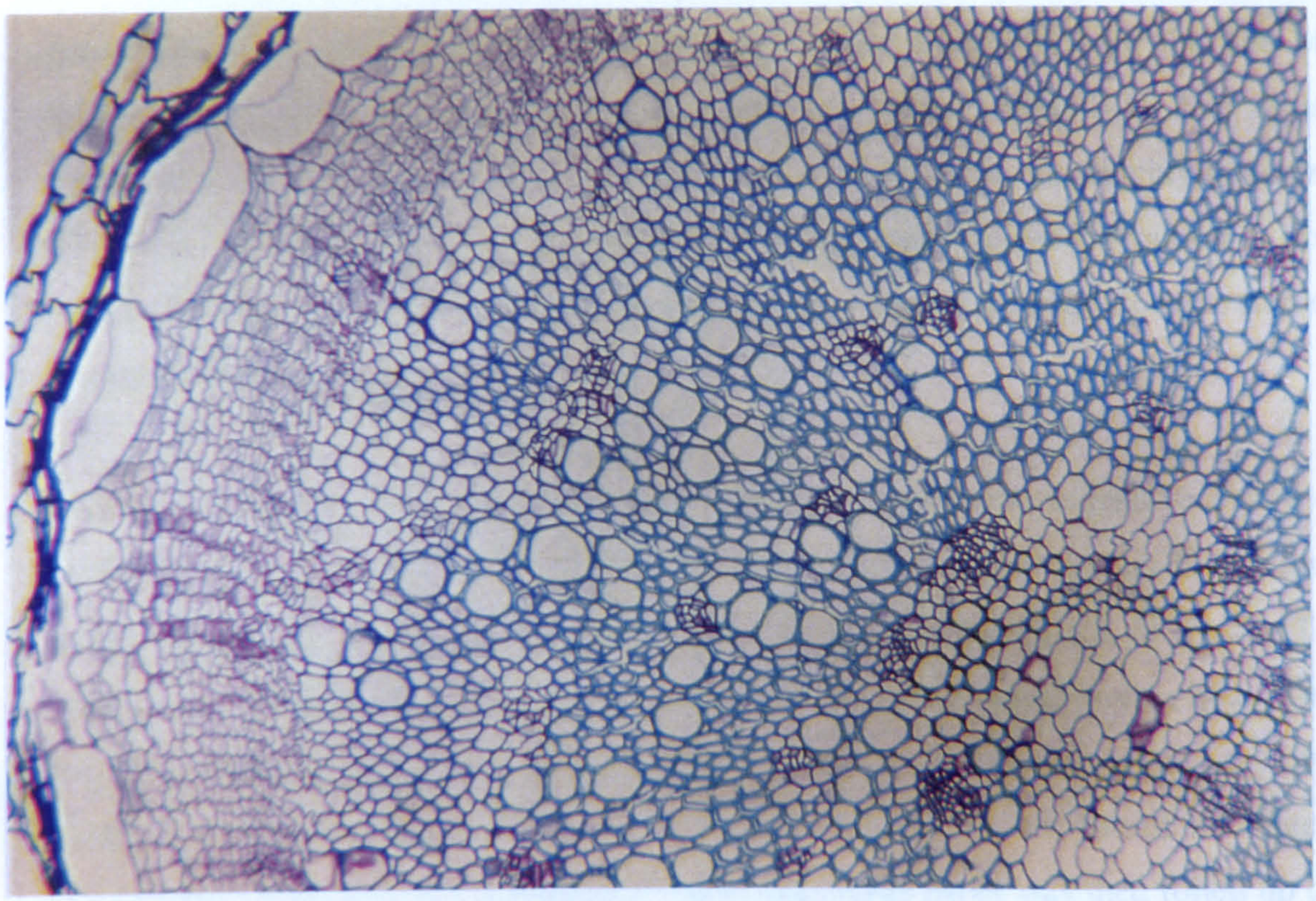


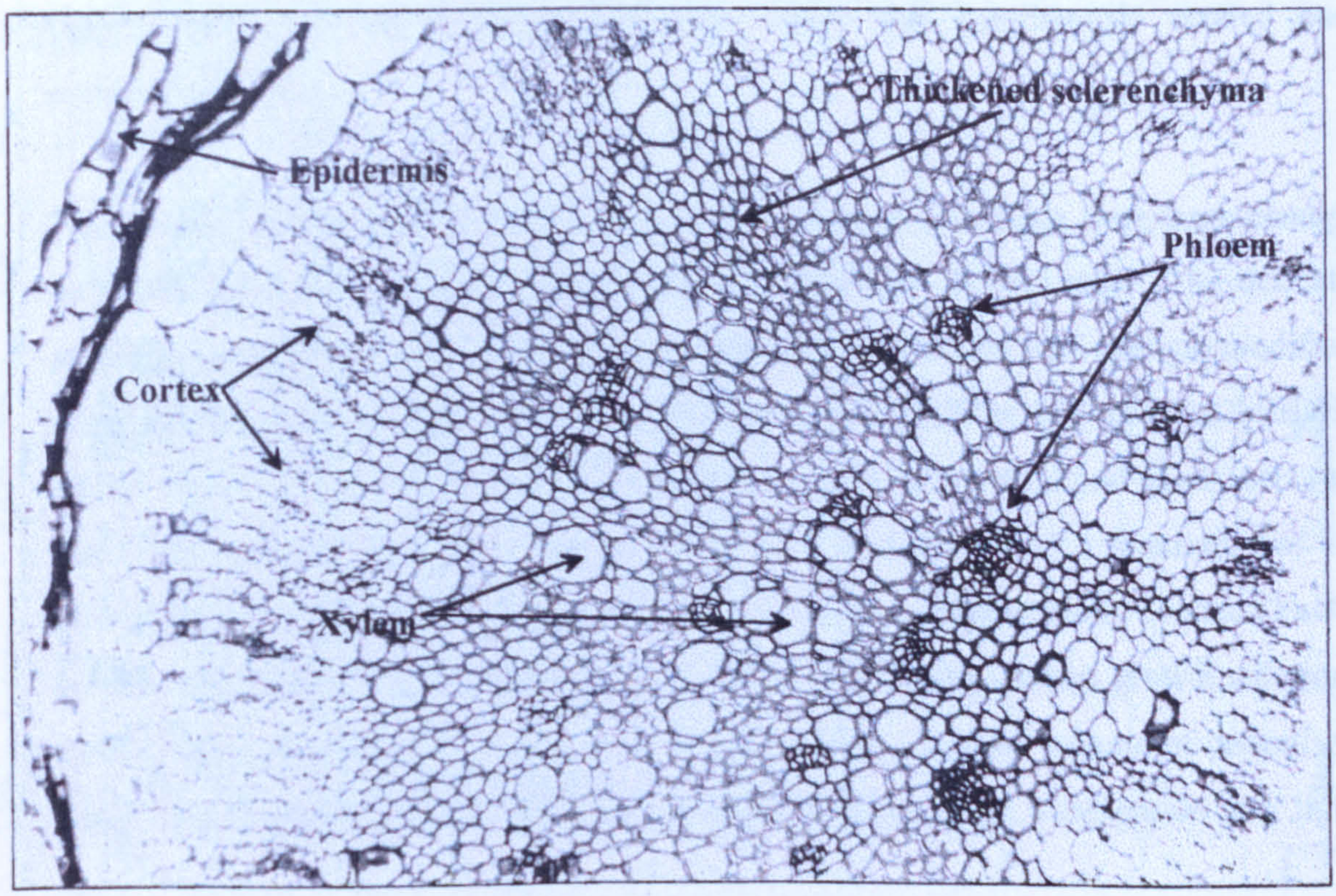
Photomicrograph 3.2, TS *Suaeda maritima* root (x 400)





Photomicrograph 3.3, TS *Suaeda maritima* stem (x 100)





3.2, Method

3.2.1, Germination and growth conditions

Suaeda maritima seeds were collected in mid October from a site on the Isle of Anglesey, grid reference SH 478650 (see also **Figure 3.2** and **Plate 3.1**). The seeds were spread out evenly on blue tissue paper and allowed to dry thoroughly at room temperature for four weeks. Following the drying procedure the seeds required a six week vernalisation period at 5 °C before germination. Any seeds not in use were stored in sealed plastic containers at 5 °C.

Prior to germination seeds were washed in de-ionised water in order to remove any mud or debris. It was unnecessary to remove the fruit coat, although many seeds had lost their coats naturally during the drying process, revealing the shiny black seed testa. To initiate germination the seeds were aerated in de-ionised water over night. The following day, using a spatula, the washed seeds were evenly spread in a thin line, two thirds of the way up a strip of blue tissue paper, which had been folded into three longitudinally and soaked in de-ionised water. The tissue paper was then rolled up to form a cylinder and placed in a 500 ml glass beaker containing a small volume (approximately 50 ml) of de-ionised water which was continuously aerated and replaced each day.

The first signs of germination were observed after 7 days. Seedlings were large enough to handle after 14 days and transferred to hydroponics at this stage. The delicate seedlings were transferred to 3 litre plastic containers containing half-strength modified Long Ashton growth solution (Hewitt, 1966) with either 0 mM or 200 mM NaCl (BDH) added. The seedlings were threaded through holes in a perspex lid and gently secured into place using custom made sponge plugs. Each container held a maximum of 16 plants. The growth medium was replaced every third day and adequately aerated (**Table 3.1** lists the components which make up a full-strength Long Ashton growth solution; the half-strength medium is simply prepared by adding an equal volume of de-ionised water to a volume of the full-strength solution). The seedlings were grown at a light

intensity of 800 μmol m^{-2} s^{-1}, under a 16 hr/8 hr light/dark regime. Temperature was maintained at 22 °C at a relative humidity of between 55 % and 60 %.

Table 3.1, Composition of full-strength Long Ashton growth solution

Macronutrient	Concentration (mM)
K^+	4
Ca^{2+}	4
Mg^{2+}	1.5
HPO_4^{2-}	1.3
SO_4^{2-}	1.5
NO_3^-	12

Micronutrient	Concentration (mM)
Si	0.05
Fe^{2+}	0.1
Mn^{2+}	0.01
Cu^{2+}	0.001
Zn^{2+}	0.001
Na^+	1.3
BO_3^-	5×10^{-5}
MoO_4^{2-}	4×10^{-4}

The growth medium was prepared using the following AnalaR grade salts supplied by BDH: potassium nitrate, calcium nitrate, sodium dihydrogenorthophosphate, magnesium sulphate, EDTA (Fe-Na salt), manganese sulphate, copper sulphate, zinc sulphate, boric acid and sodium molybdate.

3.2.2, Tissue preparation and section cutting for light microscopy

The tissue preparation and section cutting procedure used was modified from a protocol published by Feder and O'Brien in 1968. The plant material to be sectioned was fixed in 3 % glutaraldehyde (BDH) in 0.1 M phosphate buffer (BDH) in a vacuum oven at room temperature for 24 hours. The tissue was then dehydrated in a series of ethanol solutions (from 25 % to 100 % ethanol); the percentage ethanol was increased by 25 % each day. Once the dehydration procedure had been completed the plant tissue was placed in chloroform (Sigma Chemicals) for 30 minutes before being returned to 100 % ethanol.

To provide a support for the plant material during the section cutting procedure the tissue was embedded in polymerised Technovit 7100 resin (TAAB). To ensure effective infiltration of embedding medium throughout the tissue, the plant material was rotated over night in a 50/50 solution of 100 % ethanol and embedding resin. The tissue was then transferred to pure resin and left on the rotator for up to 5 days. The plant tissue was then carefully positioned in plastic moulds (TAAB) and covered in embedding resin which had been activated with a hardening agent to facilitate polymerisation and setting. The polymerisation process was complete within 3 hours. The resin blocks could then be shaped and fitted to the glass microtome ready for sectioning.

Sectioning was achieved using a glass knife microtome (Reichert-Jung, Germany). Placing a small amount of historesin glue (TAAB) on the upper edge of the resin block allowed a continuous ribbon of resin, 4 μm thick, to be cut by the microtome. The resin ribbons were immediately placed in a water bath and gently scooped onto a glass microscope slide. Once the slides were dry the sections were stained using Toluidine Blue (BDH) until the desired level of staining was achieved (60 seconds is usually adequate). The slides were then washed with de-ionised water to remove the excess stain, and dried. The sections were permanently mounted on the slide using a mounting medium and a cover slip.

3.2.3, Turgor pressure and single cell sampling and analysis

For leaf analysis, plants were selected 14 days after transfer to hydroponics (day 14), placed into individual 10 ml pots containing a small sample of the appropriate hydroponic medium, and fixed to the adjustable microscope stage adjacent to the cell pressure probe and sampling arm. The cells targeted for pressure probe and single cell sampling and analysis work were epidermal cells on leaf pair 1 and 2. Securing the plants firmly prior to probing was a problem due to the cylindrical shape and delicate nature of the leaves. This problem was overcome by threading a double length of cotton through a glass pipette to form a loop at the tip. The pipette was then fixed to the microscope stage enabling the cotton loop to be placed over a selected leaf. The loop could then be gently pulled tight and kept taught using a small weight on the free end of the cotton thread. The loop method secured a section of leaf (approximately 2 - 3 mm either side of the loop) firmly enough to pressure probe and sample successfully. The apparatus set up is illustrated in **Figure 3.3**. Measurements were made on 5 plants per treatment (approximately 3 hours into the photoperiod), and a minimum of three cells were targeted on each leaf.

The majority of the experiments were carried out on transpiring plants. However, measurements under non-transpiring conditions were achieved by coating the leaves with a thin layer of petroleum jelly, using a fine paint brush. This procedure was assumed to completely inhibit transpiration by blocking the stomatal pores. After coating the leaves the plants were returned to the growth facility for 30 minutes before any measurements were taken.

For root analysis, plants were selected at day 21. *Suaeda maritima* has a very fine root system which is too fragile to probe and sample successfully at day 14. However, by day 21 the root system is sufficiently robust to withstand the pressure probing and sampling procedure. Each plant was carefully removed from its hydroponic container, and the root system rinsed in fresh hydroponic solution for 5 minutes. A 3 cm length of root, measured from the tip towards the shoot, was secured to the base of a shallow perspex sample holder. The secured length of root was not detached from the rest of the root or shoot at any time. Once in position the entire root was covered in 10 - 15 ml of fresh

hydroponic solution. The shoot was simply allowed to rest against the side of the sample holder. The target cells were outer cortex cells situated approximately 1 cm from the root tip. Due to their small volume it was necessary to combine up to 5 individual cell samples in order to obtain an adequate volume of cell sap for analysis. Turgor pressure root analysis required 5 plants per treatment; at least 3 root cortex cells were targeted per plant. Sap samples were obtained from 15 plants per treatment.

3.2.4, Xylem pressure

Xylem pressure measurements using the xylem pressure probe (P_{xylem}) were made in the stem at day 14 and day 21. Plants were placed in a 300 ml sample holder (containing the appropriate growth medium), which was clamped firmly to a retort stand and positioned at the desired level for probing. The stem was secured to an adjustable clamp arm with cotton thread. All measurements were made three hours into the photoperiod. Data was presented in relation to atmospheric pressure rather than in absolute terms.

3.2.5, Step-up/step-down protocol

Plants subjected to a "**Step-up**" or "**Step-down**" treatment experienced an up or down osmotic shock of about 1 MPa respectively. Osmotic shock was achieved simply by swapping the hydroponic media. Plants grown initially in the 0 mM NaCl medium were given a 1 MPa step-up by being transferred to the 200 mM NaCl medium. Conversely, plants grown in the 200 mM NaCl medium were given a 1 MPa step-down by being transferred to the 0 mM NaCl hydroponic medium. Turgor pressure (P_{cell}) and osmotic pressure (P_{cell}) measurements were made in the leaves and root at the times indicated. Single cell samples for EDX analysis were taken prior to the step-up/step-down treatment and 24 hours after the treatment.

A step-up/step-down treatment during a xylem pressure probe experiment was a little more involved. A 1 MPa step-up\step-down was applied to the root system while the tip of the glass microcapillary was inside a xylem vessel in the stem. This was achieved using a modified 300 ml sample holder connected to a three way tap system and a funnel reservoir. This specialised apparatus allowed the original hydroponic medium to

be drained away and replaced by the step-up/step-down medium stored in the funnel reservoir. It was essential that the hydroponic medium was changed quickly and smoothly with minimal disturbance to the plant (any vibration could potentially dislodge the glass microcapillary and bring the experiment to an abrupt end). The whole procedure took no longer than 30 seconds.

3.2.6, Statistical analysis

The statistical analysis tool used was a paired (matched) t-Test. Microsoft Excel Version 7.0 was used to generate exact **p values** which are reported as is in the results section which follows (see **Section 3.3**). If the p value generated is equal to or less than a predetermined alpha value (which in the literature, is often 0.05 or 0.01) the Null Hypothesis put forward can be rejected. In the following experiments the Null Hypothesis to be tested states that the population mean in treatment one and treatment two are equal.

Figure 3.2, Seed collection site on the Isle of Angelsey

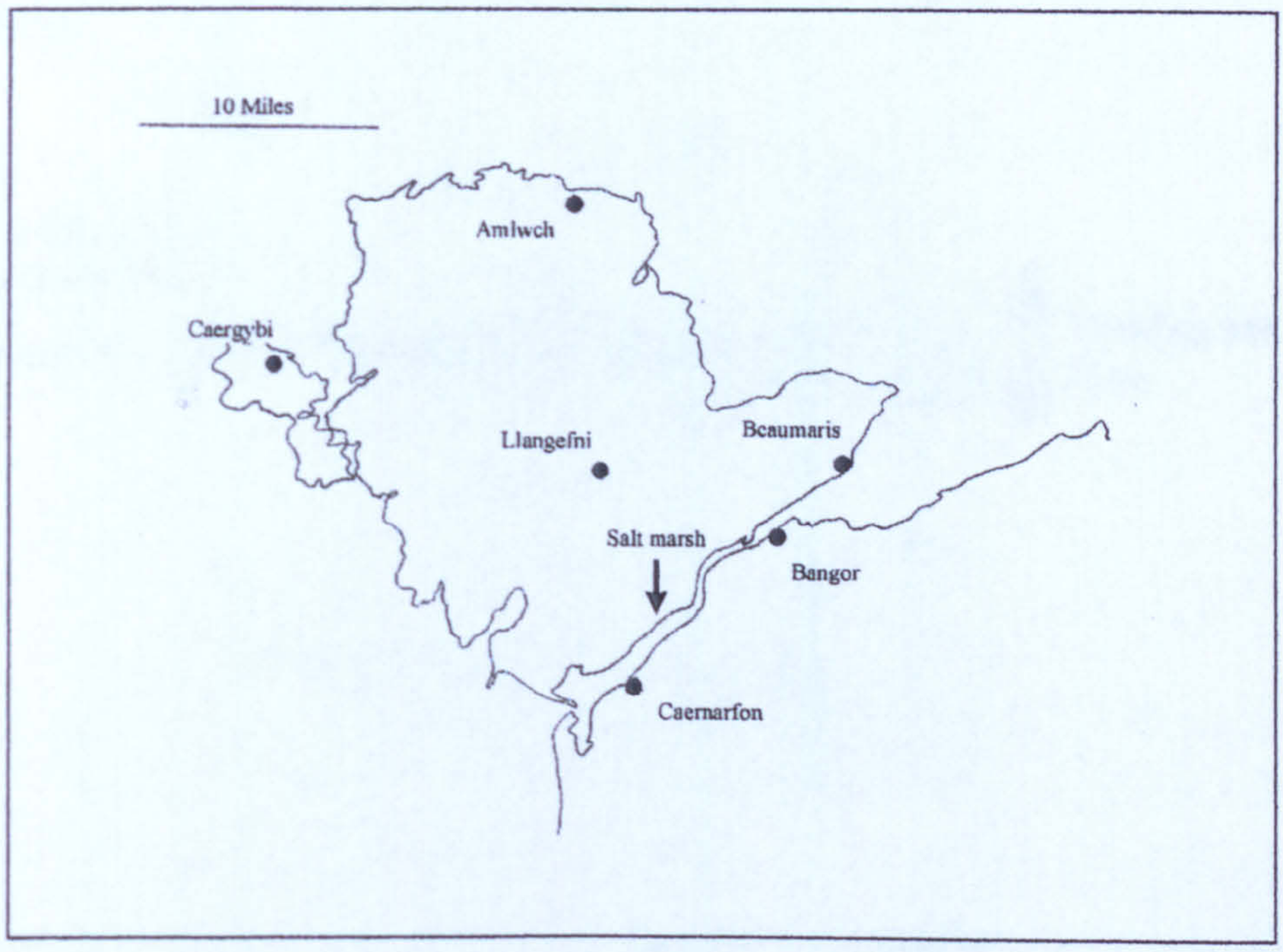
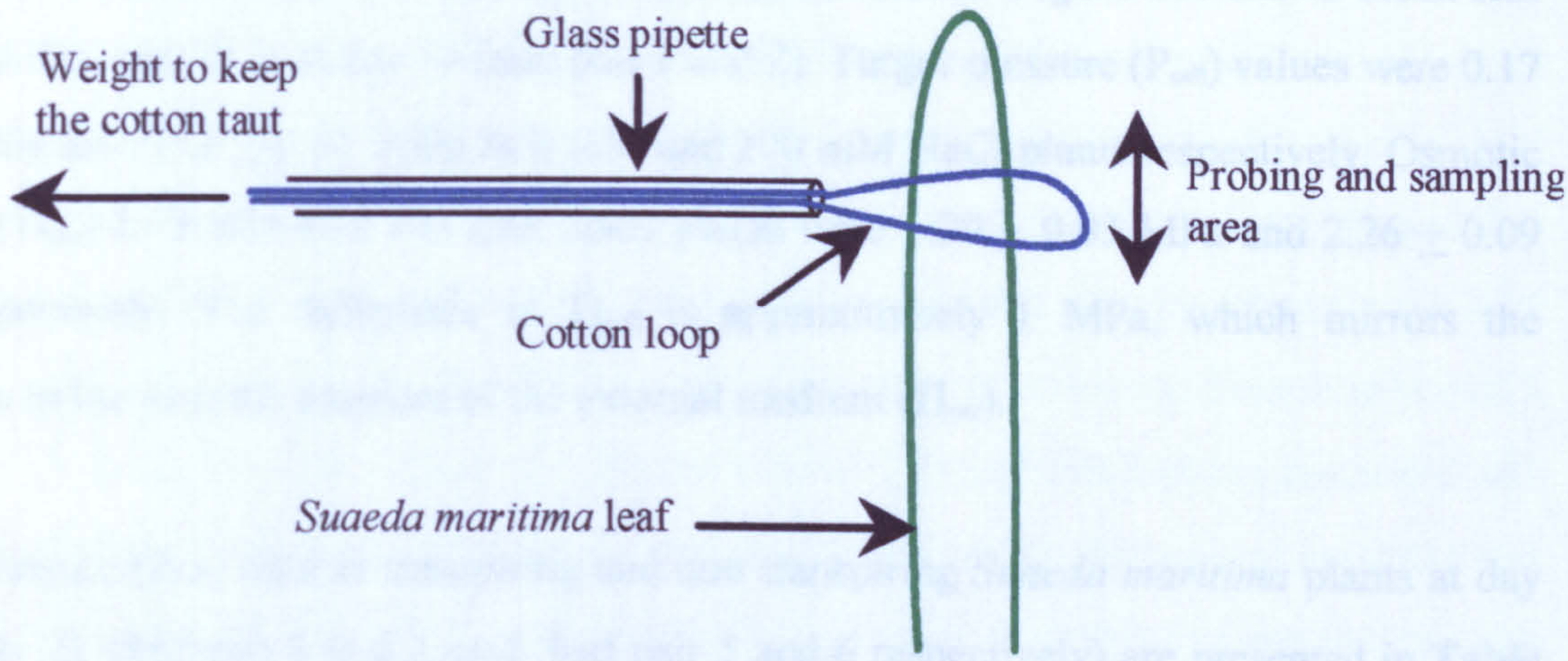


Plate 3.1, *Suaeda maritima* in its natural environment

Figure 3.3, *Suaeda maritima* leaf securing system

The glass pipette and cotton loop system used to secure a *Suaeda maritima* leaf (which is still attached to the plant) prior to turgor pressure measurements and single cell sampling.



3.3, Results

The cell pressure probe and single cell sampling and analysis techniques were used to obtain *Suaeda maritima* leaf epidermal turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) data in plants grown in 0 mM and 200 mM NaCl hydroponic media. **Figure 3.4** shows mean leaf epidermal P_{cell} and Π_{cell} at day 14 (leaf pair 1 and 2). Turgor pressure (P_{cell}) values were 0.17 $\pm$ 0.03 MPa and 0.18 $\pm$ 0.03 MPa in 0 mM and 200 mM NaCl plants respectively. Osmotic pressure (Π_{cell}) in 0 mM and 200 mM NaCl plants were 1.20 $\pm$ 0.03 MPa and 2.26 $\pm$ 0.09 MPa respectively. The difference in Π_{cell} is approximately 1 MPa, which mirrors the difference in the osmotic pressure of the external medium (Π_{ext}).

Turgor pressure (P_{cell}) data in transpiring and non transpiring *Suaeda maritima* plants at day 14 and day 21 (leaf pair 1 and 2, and leaf pair 5 and 6 respectively) are presented in **Table 3.2**. At day 14 there was no statistical difference between P_{cell} in transpiring and non-transpiring plants. By day 21, however, P_{cell} in non-transpiring plants was greater than P_{cell} in transpiring plants by approximately 0.1 MPa.

Leaf epidermal (day 14, leaf pair 1 and 2) turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) measurements were taken in plants subjected to a **step-up** or **step-down** of 1 MPa in the external medium. Turgor pressure (P_{cell}) measurements were taken 10 minutes after applying the step-up/step-down treatment (given that the treatment was applied at time 0), and at 30 minute intervals thereafter for a maximum of 2 hours (**Figure 3.5**). Control measurements (prior to treatment) are indicated at time 0. Turgor pressure (P_{cell}) measurements after treatment did not deviate statistically from the observed control values (approximately 0.2 MPa). The p values generated for each treatment are included in the figure legend.

Figure 3.6 illustrates leaf epidermal P_{cell} and Π_{cell} 24 hours after the initial step-up/step-down treatment. Turgor pressure (P_{cell}) in the step-up and step-down treated plants were 0.18 $\pm$ 0.02 MPa and 0.19 $\pm$ 0.02 MPa respectively; values which are comparable to P_{cell} in the 0 mM and 200 mM NaCl control plants (0.17 $\pm$ 0.03 MPa and 0.18 $\pm$ 0.03 MPa respectively). Osmotic pressure (Π_{cell}) in step-up plants increased from 1.42 $\pm$ 0.06 MPa to 2.46 $\pm$ 0.07 MPa, an increase of 1 MPa, which is equivalent to the step-up in osmotic pressure applied to the root

system. However, Π_{cell} in step-down plants did not mirror the 1 MPa decrease in the external medium. The osmotic pressure (Π_{cell}) in step-down plants was 2.37 $\pm$ 0.1 MPa compared to 2.58 $\pm$ 0.03 MPa in the 200 mM NaCl control plants (a maximum decrease in Π_{cell} of only 0.2 MPa).

X-ray microanalysis (EDX analysis) was used to determine the mean sodium, chloride, potassium and calcium content of sap samples taken from leaf epidermal cells (day 14, leaf pair 1 and 2) in control and step-up/step-down treated *Suaeda maritima* plants. **Figure 3.7** illustrates the Na, Cl, K and Ca content of leaf epidermal vacuolar samples taken from 0 mM and 200 mM NaCl plants. The Na and Cl content in vacuolar samples from 0 mM NaCl plants were negligible. Whereas samples taken from the 200 mM NaCl plants contained 563 $\pm$ 171 mM sodium and 313 $\pm$ 91 mM chloride. The potassium concentrations in 0 mM and 200 mM NaCl plants were 152 $\pm$ 59 mM and 29 $\pm$ 26 mM respectively, and the calcium concentrations in 0 mM and 200 mM NaCl plants were and 13 $\pm$ 23 mM and -5 $\pm$ 10 mM respectively.

Changes in Na and Cl content after a 24 hour step-up/step-down treatment are illustrated in **Figure 3.8** and **Figure 3.9**. Step-up plants accumulated 397 $\pm$ 104 mM Na and 362 $\pm$ 77 mM Cl during the 24 hour treatment period. From the step-down data it could be argued that plants subjected to a 1 MPa step-down mobilised Na out of the vacuole during the 24 hour treatment. However, the decrease from 563 $\pm$ 171 mM in control plants (200 mM NaCl) to 424 $\pm$ 136 mM (step-down plants) is not statistically different at the 0.01 alpha level and is likely to be due to natural plant variation and/or sampling error. There was no evidence to suggest that chloride was mobilised out of the vacuole, chloride concentrations in step-down treated plants (286 $\pm$ 98 mM) remained comparable to the control (362 $\pm$ 77 mM). The mean and standard deviation for each element analysed in control and treated plants are presented in **Table 3.3,** along with the p values generated from the paired t-Test.

In **Table 3.4** the EDX data are presented alongside corresponding osmotic pressure (Π_{cell}) data. This allows a comparison of the Π_{cell} data with the sum of the solutes measured by X-ray microanalysis. Before a direct comparison can be made, however, the sum solute values must be multiplied by the appropriate osmotic coefficient (ϕ). The osmotic coefficient

takes into account the deviation of a solution away from ideality (see Wyn Jones and Gorham, 1983). An osmotic coefficient of 0.82 was used in this study - after Fricke *et al.*, 1994 (see also Malone *et al.*, 1991). Any discrepancy between the measured Π_{cell} and calculated Π_{cell} (calculated from the sum of solutes multiplied by the osmotic coefficient) may highlight the presence of additional, unmeasured solutes in the analysed sap samples. The largest discrepancy between measured Π_{cell} and calculated Π_{cell} values was observed in the leaf epidermal cells of 0 mM NaCl treated *Suaeda maritima* plants (see **Table 3.4**). Subtracting the calculated Π_{cell} from the measured Π_{cell} revealed that 429.3 mOsmol kg^{-1} (approximately 1.07 MPa) is unaccounted for in 0 mM NaCl plants, 309.15 mOsmol kg^{-1} (0.77 MPa) and 254.14 mOsmol kg^{-1} (0.6 MPa) is unaccounted for in step-down and step-up plants respectively and 185.76 mOsmol kg^{-1} (about 0.46 MPa) is unaccounted for in 200 mM NaCl treated plants. These data strongly suggest that other osmotically active solutes are present in the sap samples taken from *Suaeda maritima* leaf epidermal cells. Possible candidates include P_i, NO_3^- and organic solutes such as malate (Fricke *et al.*, 1994; Bates, 1998).

Corresponding turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) data were obtained from root cortex cells situated 1 cm from the root tip at day 21. Measurements were made in control and step-up\step-down treated plants. **Figure 3.10** illustrates root cortex turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in *Suaeda maritima* plants grown in 0 mM and 200 mM NaCl. Turgor pressure (P_{cell}) was comparable in both 0 mM and 200 mM NaCl plants at 0.39 ± 0.03 MPa and 0.38 ± 0.02 MPa respectively. Root cortex osmotic pressure (Π_{cell}) was 0.43 ± 0.12 MPa and 1.38 ± 0.11 MPa in 0 mM and 200 mM NaCl plants respectively. The difference in root cortex Π_{cell} is approximately 1 MPa, which corresponds with the difference in Π_{cell} observed in the leaf epidermal cells (see **Figure 3.4**), and once again mirrors the difference in the osmotic pressure of the external medium (Π_{ext}).

A 1 MPa step-up/step-down treatment dramatically influenced the turgor pressure (P_{cell}) of the root cortex cells. **Figure 3.11** illustrates root cortex P_{cell} in control plants prior to treatment (time 0) and root cortex P_{cell} in treated plants at the times indicated in the figure legend. Root cortex P_{cell} in step-up plants fell rapidly within 10 minutes of applying the treatment. Attempts to measure P_{cell} were unsuccessful indicating that P_{cell} was either extremely low or

that the cells had undergone plasmolysis. No P_{cell} recovery was observed during the experiment. In contrast, the root cortex cells in step-down treated plants experienced a huge increase in P_{cell} within the same time period; P_{cell} increased from 0.38 $\pm$ 0.02 MPa to 1.29 $\pm$ 0.03 MPa within 10 minutes. Subsequent measurements taken at 30 minute intervals indicated that P_{cell} was decreasing, suggesting that P_{cell} regulation was taking place. Seven hours (420 minutes) after applying the step-down treatment P_{cell} had reached 0.72 $\pm$ 0.03 MPa, which is a decrease of 0.6 MPa. The observed root cortex P_{cell} decrease in step-down plants was accompanied by an equivalent decrease in Π_{cell}. **Table 3.4** presents root cortex Π_{cell} data taken in control (0 mM and 200 mM) and treated (step-up/step-down) plants at the times indicated. Seven hours (420 minutes) after applying the step-down treatment Π_{cell} had decreased from 1.38 $\pm$ 0.11 MPa to 0.81 $\pm$ 0.02 MPa, a decrease of 0.6 MPa. Osmotic pressure (Π_{cell}) in step-up plants after 7 hours could not be determined due to difficulty in obtaining a sample.

Root cortex turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) data taken 24 hours after a step-up/step-down treatments are illustrated in **Figure 3.12**. Step-up and step-down treated plants regulated P_{cell} upwards and downwards respectively to a level comparable to P_{cell} in control plants (approximately 0.4 MPa), within 24 hours. Osmotic pressure (Π_{cell}) increased by 1 MPa (from 0.43 $\pm$ 0.12 MPa to 1.32 $\pm$ 0.07 MPa) in step-up plants and decreased by 1 MPa (from1.38 $\pm$ 0.11 MPa to 0.40 $\pm$ 0.05 MPa) in step-down plants over the 24 hour period. These changes in Π_{cell} exactly mirror the changes in the external medium (Π_{ext}). These data are also presented in **Table 3.5**.

Xylem pressure probe measurements were made in the stem of control and step-up/step-down treated *Suaeda maritima* plants, at day 14 and day 21. The aim was to ascertain whether applying an osmotic shock treatment to the root system would lead to the propagation of a hydrostatic signal to the shoot, via the xylem. **Figure 3.13** and **Figure 3.14** show the distribution of xylem pressure measured in *Suaeda maritima* stems at day 14 and day 21. Measurements were made in both 0 mM and 200 mM NaCl plants. The xylem pressure measurements obtained ranged from -0.005 MPa to -0.055 MPa. The level of xylem pressure most commonly observed was around -0.005 MPa in each case, although the older, larger plants (day 21) showed an increased number of greater (more negative) P_{xylem} values.

Figure 3.15 and **Figure 3.16** show typical xylem probe traces obtained from 0 mM and 200 mM NaCl plants respectively, at day 21. These images have been scanned into the text from the original chart recorder print outs. The small peaks running along the traces are pressure pulses applied manually to the system. A step-up/step-down treatment was applied to the root system approximately 5 minutes into a xylem pressure (P_{xylem}) measurement. The point at which the root medium was changed is highlighted by an arrow on each figure. No change in xylem pressure was observed after a step-up or step-down treatment. This indicates that there is either no hydrostatic signal generated or that the signal is undetectable. The xylem probe traces illustrated are representative examples of experiments carried out on at least 15 plants per treatment.

Figure 3.4, P_{cell} and Π_{cell} in *Suaeda maritima* leaf epidermal cells at day 14

Mean turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in leaf epidermal cells (leaf pair 1 and 2) at day 14. The error bars indicate standard deviation, n is 15 leaf epidermal cells (5 plants, 3 cells per plant). The statistical analysis tool used was a paired t-Test. The P_{cell} and Π_{cell} p values generated were 0.86 and 4.48 x10^{-9} respectively.

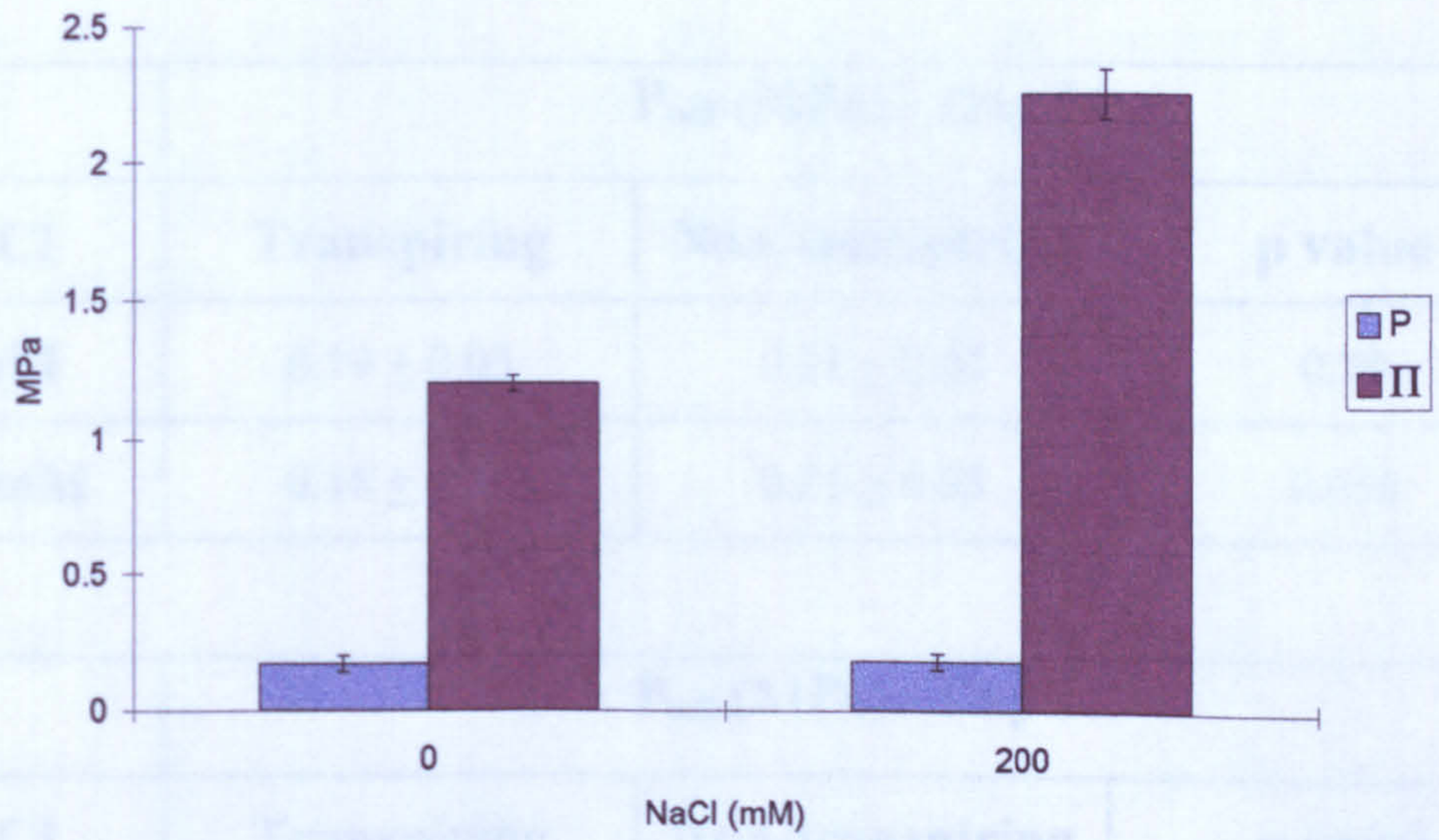


Table 3.2, Leaf epidermal P_{cell} in transpiring and non-transpiring *Suaeda maritima* plants

Mean turgor pressure (P_{cell}) in leaf epidermal cells in transpiring and non-transpiring *Suaeda maritima* plants, at day 14 (leaf pair 1 and 2) and day 21 (leaf pair 5 and 6). The ± values indicate standard deviation, n is 15 leaf epidermal cells (5 plants, 3 cells per plant). The statistical analysis tool used was a paired t-Test. The p values generated are as indicated in the table.

	P_{cell} (MPa) - Day 14		
NaCl	**Transpiring**	**Non-transpiring**	**p value**
0 mM	0.19 ± 0.03	0.21 ± 0.03	0.19
200 mM	0.18 ± 0.02	0.21 ± 0.03	0.056

	P_{cell} (MPa) - Day 21		
NaCl	**Transpiring**	**Non-transpiring**	**p value**
0 mM	0.18 ± 0.02	0.32 ± 0.02	0.0005
200 mM	0.19 ± 0.02	0.30 ± 0.02	7.34×10^{-5}

Figure 3.5, Leaf epidermal P_{cell} in *Suaeda maritima* plants subjected to a step-up/step-down treatment

Mean leaf epidermal turgor pressure (P_{cell}) in *Suaeda maritima* plants subjected to a step-up or step-down of 1 MPa in the external (hydroponic) medium at day 14 (leaf pair 1 and 2). Step-up plants were given a 1 MPa up shock from 0 mM to 200 mM NaCl, whereas step-down plants were given a 1 MPa down shock from 200 mM to 0 mM NaCl. P_{cell} measurements were taken prior to treatment (Time 0) and at the times indicated after treatment. The error bars indicate standard deviation, n is 15 cells (5 plants, 3 cells per plant). A paired t-Test was used to compare mean P_{cell} in control plants (at time 0) with mean P_{cell} at each time interval after treatment. The p values generated are as follows: Step-up plants at 10, 30, 60 , 90 and 120 minutes after treatment had p values of 1.0, 0.49, 0.91, 0.56, and 0.39 respectively. Step-down plants at 10, 30, 60, 90 and 120 minutes after treatment had p values of 0.87, 0.11, 0.43, 1.0, and 0.62 respectively.

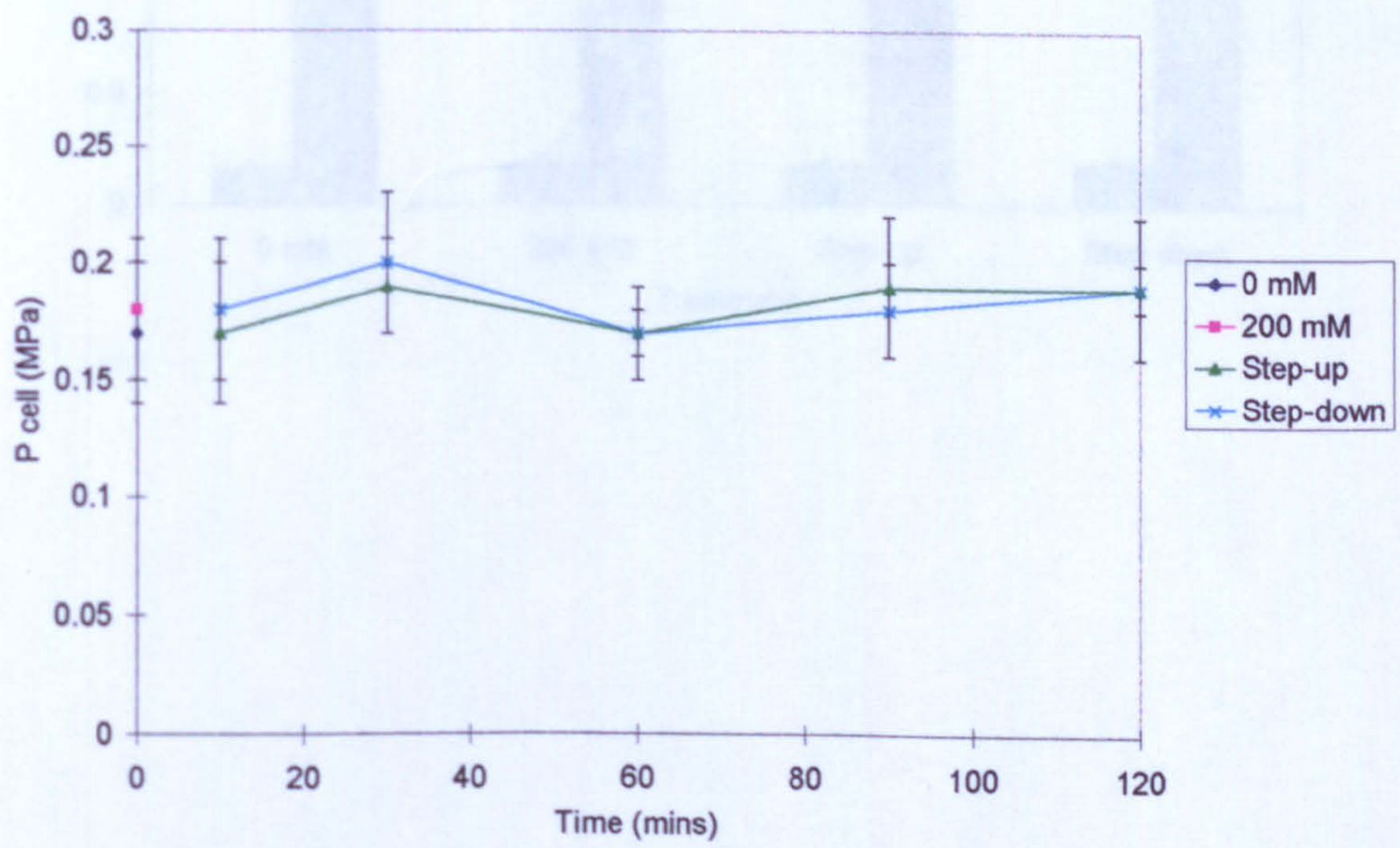


Figure 3.6, P_{cell} and Π_{cell} in *Suaeda maritima* leaf epidermal cells 24 hours after a step-up/step-down treatment

Mean turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in *Suaeda maritima* leaf epidermal cells 24 hours after a step-up/step-down treatment (day 15, leaf pair 1 and 2). Error bars indicate standard deviation, n is 15 cells (5 plants, 3 cells per plant).

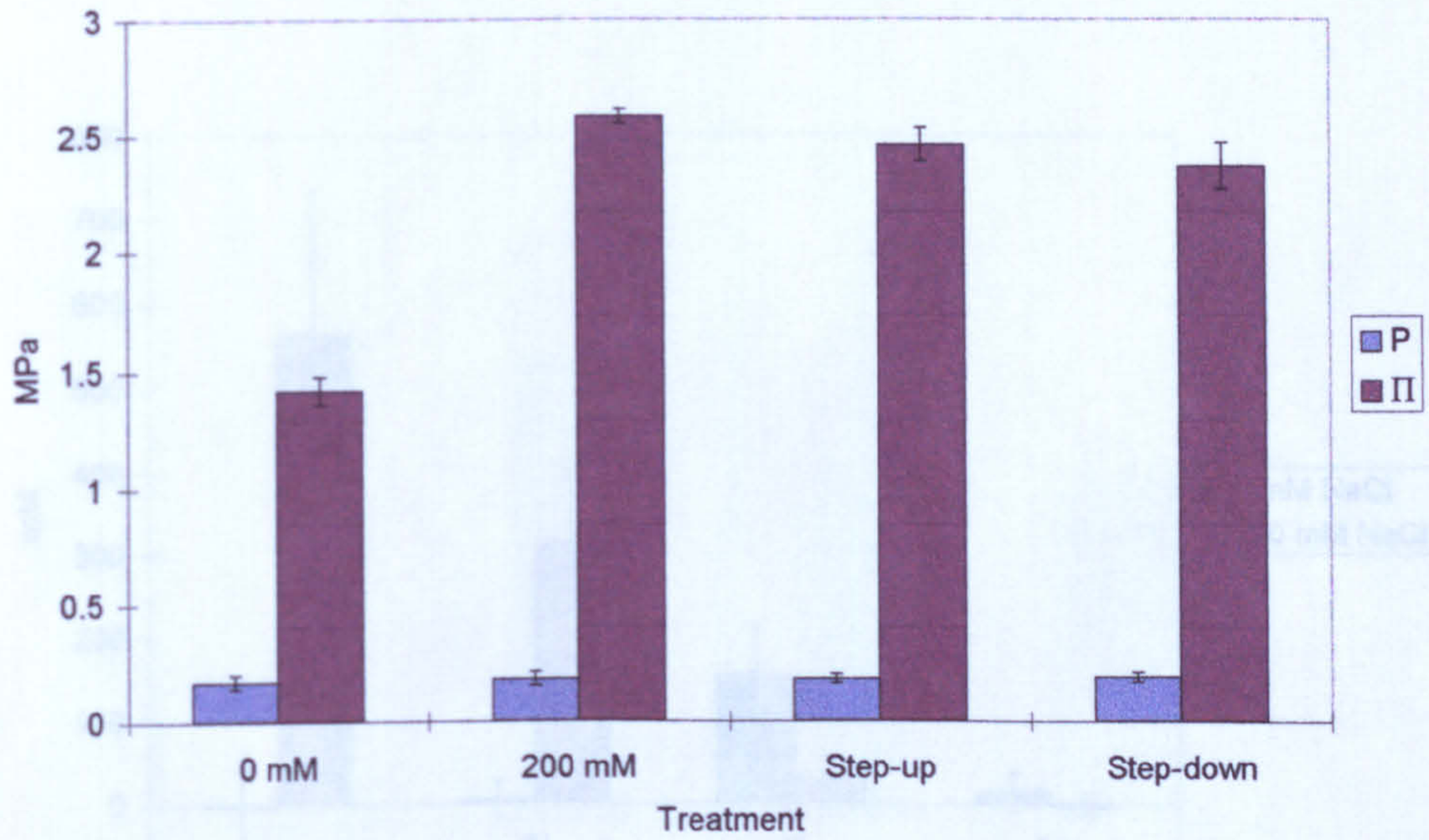


Figure 3.7, Sodium, chloride, potassium and calcium concentrations in sap samples taken from leaf epidermal cells in *Suaeda maritima* plants grown in 0 mM and 200 mM NaCl

Mean sodium, chloride, potassium and calcium concentrations in sap samples taken from leaf epidermal cells in *Suaeda maritima* plants grown in 0 mM and 200 mM NaCl at day 14 (leaf pair 1 and 2). Error bars indicate standard deviation, n is 15 cells (5 plants, 3 cells per plant).

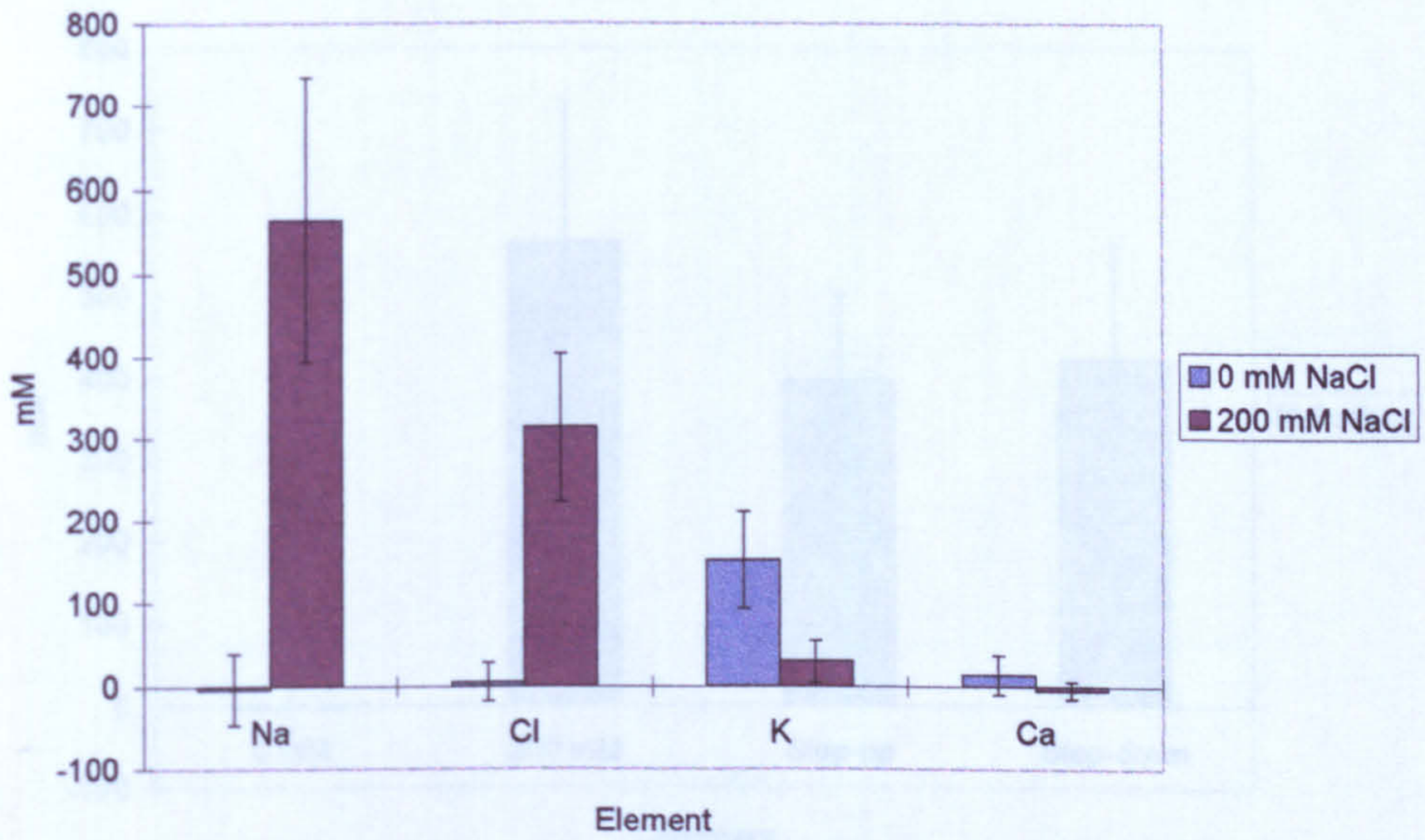


Figure 3.8, Sodium concentration in leaf epidermal sap samples taken from *Suaeda maritima* plants 24 hours after a step-up/step-down treatment

Mean sodium concentration in leaf epidermal sap samples taken from control and 24 hour step-up/step-down treated *Suaeda maritima* plants at day 14 (leaf pair 1 and 2). Error bars indicate standard deviation, n is 15 cells (5 plant, 3 cells per plant). The statistical analysis tool used was a paired t-Test. The p values generated for 0 mM/step-up and 200 mM/step-down treatments were 8.37×10^{-9} and 0.045 respectively.

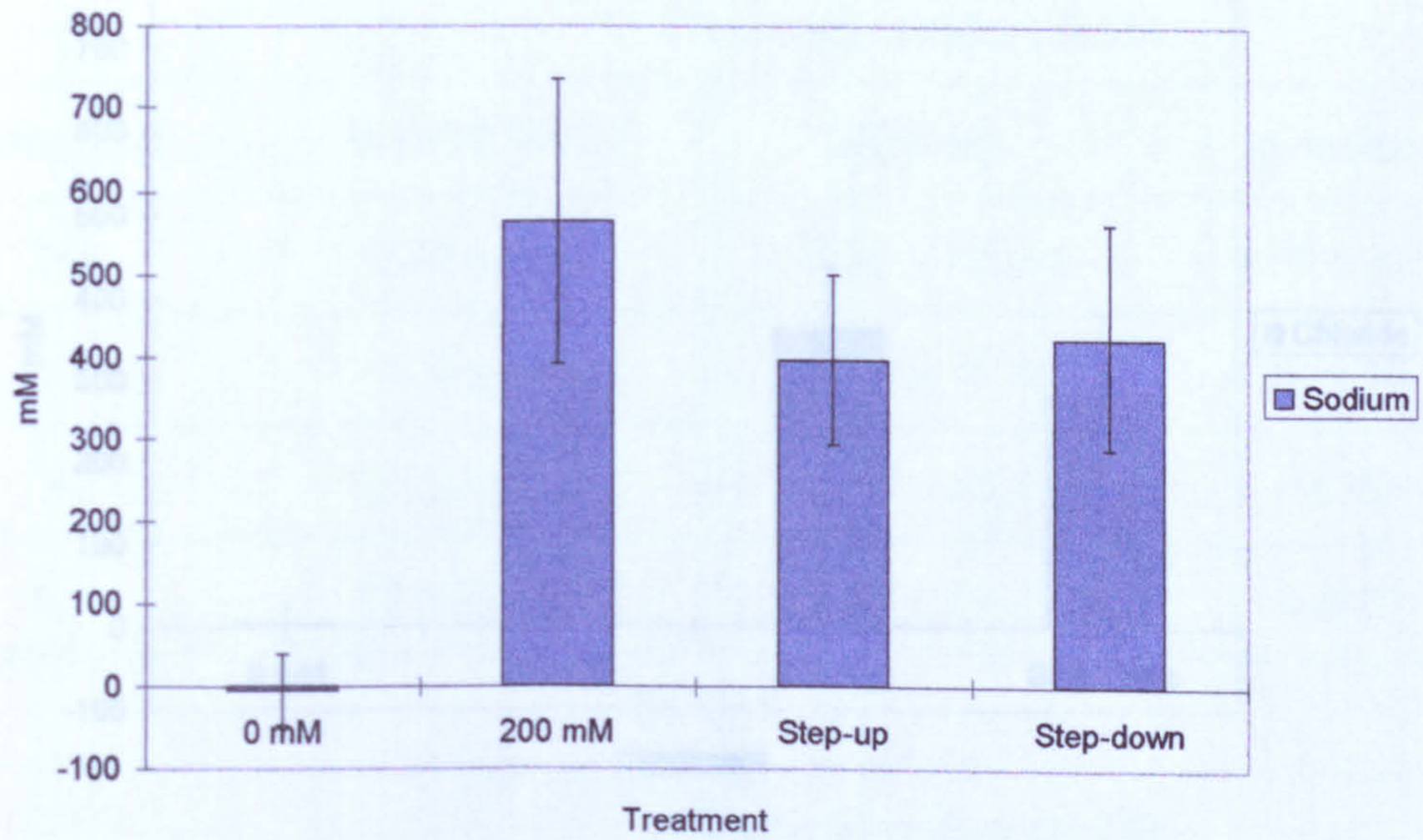


Figure 3.9, Chloride concentration in leaf epidermal sap samples taken from *Suaeda maritima* plants 24 hours after a step-up/step-down treatment

Mean chloride concentration in leaf epidermal sap samples taken from control and 24 hour step-up/step-down treated *Suaeda maritima* plants at day 14 (leaf pair 1 and 2). Error bars indicate standard deviation, n is 15 cells (5 plant, 3 cells per plant). The statistical analysis tool used was a paired t-Test. The p values generated for 0 mM/step-up and 200 mM/step-down treatments were 4.06×10^{-11} and 0.38 respectively.

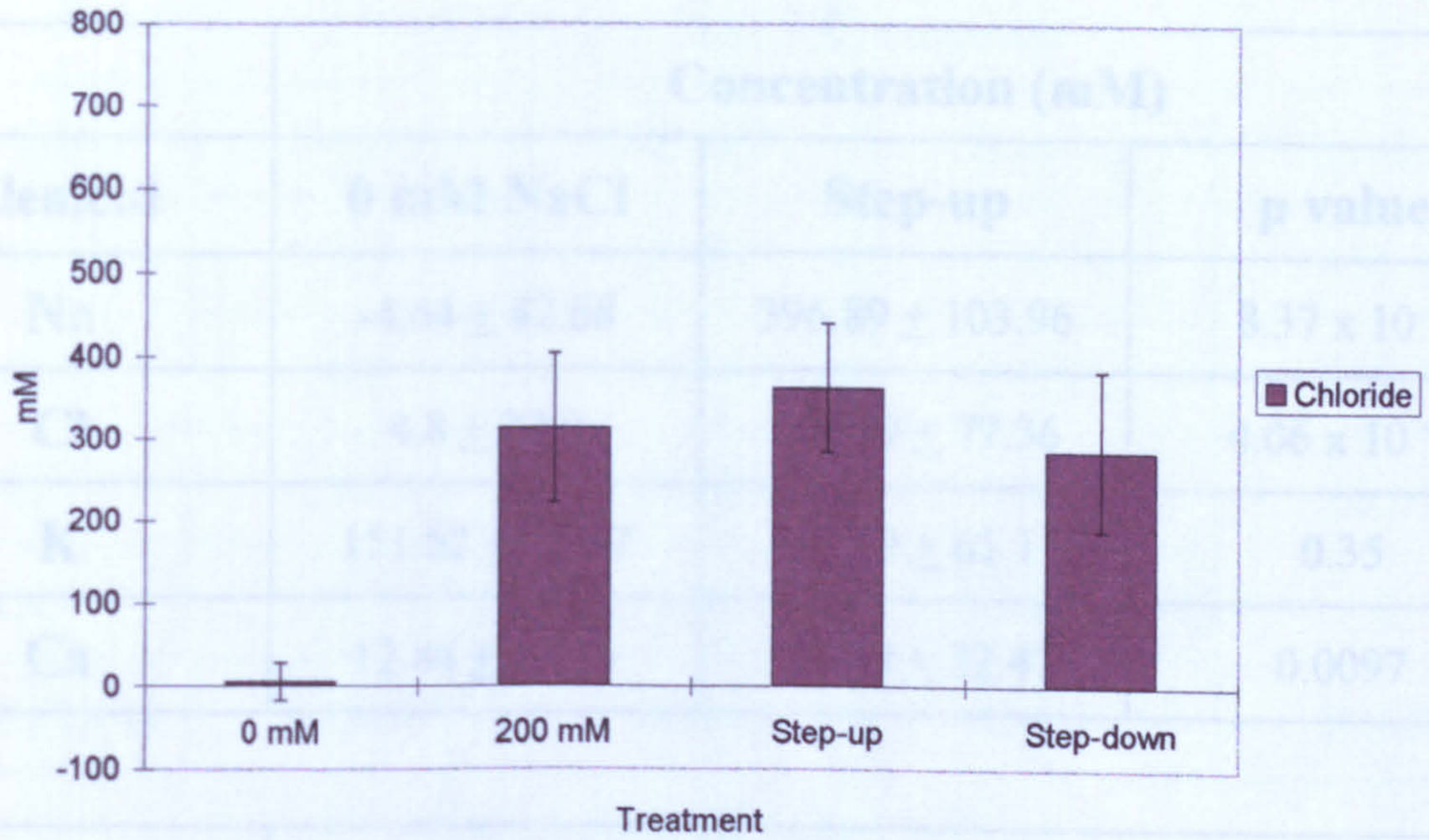


Table 3.3, EDX analysis of sodium, chloride, potassium and calcium in leaf epidermal sap samples in control and 24 hour step-up/step-down *Suaeda maritima* plants at day 14

Sodium, chloride, potassium and calcium concentrations in leaf epidermal sap samples taken from control and 24 hour step-up\step-down treated *Suaeda maritima* plants at day 14 (leaf pair 1 and 2). Data are presented as mean and standard deviation, n is 15 cells (5 plants, 3 cells per plant). The statistical analysis tool used was a paired t-Test.

	Concentration (mM)		
Element	**0 mM NaCl**	**Step-up**	**p value**
Na	-4.64 ± 42.68	396.89 ± 103.96	8.37×10^{-9}
Cl	4.8 ± 22.9	361.79 ± 77.36	4.06×10^{-11}
K	151.52 ± 58.97	131.39 ± 61.11	0.35
Ca	12.84 ± 23.35	-11.89 ± 22.47	0.0097

	Concentration (mM)		
Element	**200 mM NaCl**	**Step-down**	**p value**
Na	563.3 ± 170.56	424.04 ± 135.61	0.045
Cl	313.02 ± 90.61	285.54 ± 97.63	0.38
K	29.41 ± 25.74	69.51 ± 34.89	0.042
Ca	-5.15 ± 10.11	-25.01 ± 14.12	0.0009

Table 3.4, The relationship between osmotic pressure (Π_{cell}) and solute concentration in individual *Suaeda maritima* leaf epidermal cells

The relationship between the molarity of solutes measured by EDX X-ray microanalysis and osmolality measured by picolitre melting point depression in the same sap sample. Calculated Π_{cell} values correspond to the sum of EDX measured solutes (mM) multiplied by an osmotic coefficient of 0.82 (after Fricke *et al.*, 1994).

	NaCl Treatment			
Solute (mM)	**0 mM**	**200 mM**	**Step-up**	**Step-down**
Na	0	563.3	396.89	424.04
Cl	4.8	313.02	361.79	285.54
K	151.52	29.41	131.39	69.51
Ca	12.84	0	0	0
Sum of solutes	**169.16**	**905.73**	**890.07**	**779.09**
Osmolality mOsmol kg^{-1}				
Measured Π_{cell}	568	1,032	984	948
Calculated Π_{cell}	138.7	846.24	729.86	638.85
Difference	**429.3**	**185.76**	**254.14**	**309.15**

Figure 3.10, P_{cell} and Π_{cell} in *Suaeda maritima* root cortex cells at day 21

Mean turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in root cortex cells (1 cm from the root tip) at day 21. The error bars indicate standard deviation, P_{cell} n is 15 cells (5 plants, 3 cells per plant), Π_{cell} n = 15 plants (sap from 5 cells pooled per plant). The statistical analysis tool used was a paired t-Test. The P_{cell} and Π_{cell} p values generated were 0.51 and 9.2×10^{-9} respectively.

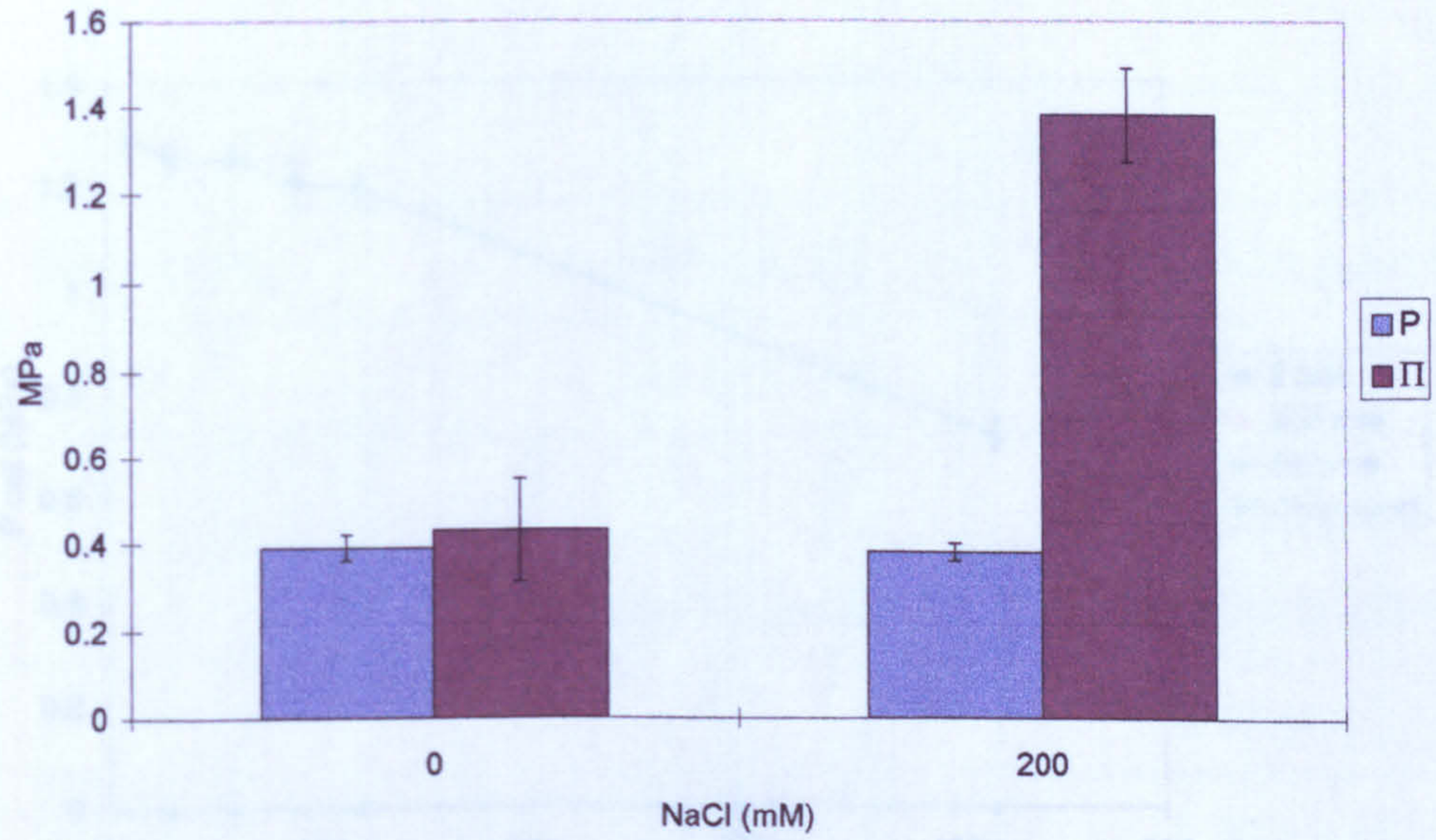


Figure 3.11, Root cortex P_{cell} in *Suaeda maritima* plants subjected to a step-up/step-down treatment

Mean root cortex turgor pressure (P_{cell}) in *Suaeda maritima* plants subjected to a step-up or step-down of 1 MPa in the external medium at day 21 (measurements were made 1 cm from the root tip). P_{cell} measurements were taken prior to treatment (Time 0) and at the times indicated after treatment. The error bars indicate standard deviation, n is 15 root cortex cell (5 plants, 3 cells per plant).

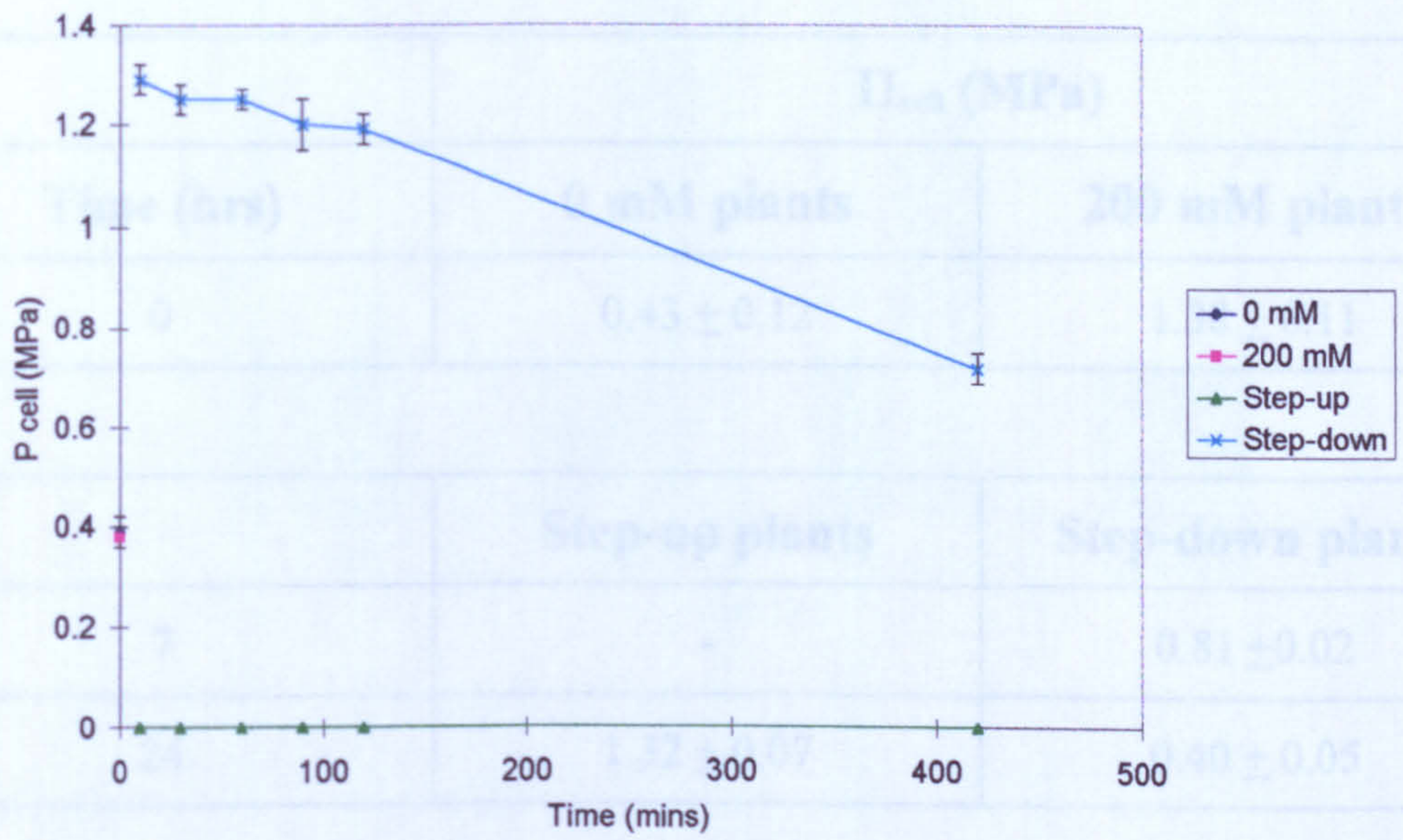


Table 3.5, Root cortex Π_{cell} in *Suaeda maritima* plants subjected to a step-up/step-down treatment

Root cortex osmotic pressure (Π_{cell}) in *Suaeda maritima* plants subjected to a step-up or step-down of 1 MPa in the external medium at day 21 (measurements were made 1 cm from the root tip). Π_{cell} measurements were taken prior to treatment (Time 0) and at the times indicated after treatment. Data are presented as mean and standard deviation, n is 15 plants (sap from 5 cells pooled per plant). A dash (-) signifies inability to obtain a cell sample due to plasmolysis.

	Π_{cell} (MPa)	
Time (hrs)	**0 mM plants**	**200 mM plants**
0	0.43 ± 0.12	1.38 ± 0.11

	Step-up plants	**Step-down plants**
7	-	0.81 ±0.02
24	1.32 ± 0.07	0.40 ± 0.05

Figure 3.12, P_{cell} and Π_{cell} in *Suaeda maritima* root cortex cells 24 hours after a step-up/step-down treatment

Mean turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in *Suaeda maritima* root cortex cells 24 hours after a step-up/step-down treatment (day 21, 1 cm from the root tip). Error bars indicate standard deviation, n is 15 plants (sap from 5 cells pooled per plant).

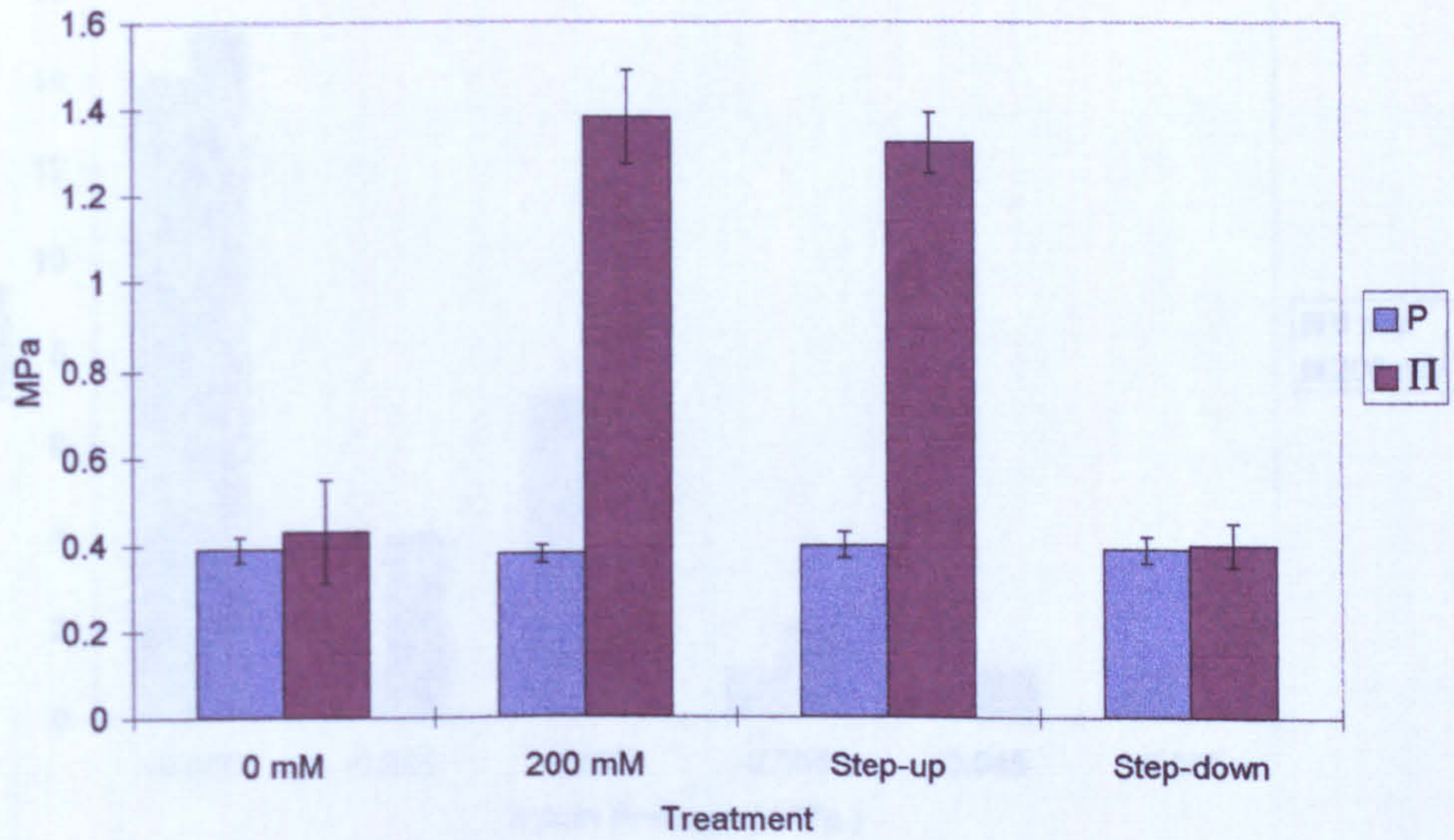


Figure 3.13, Distribution of xylem pressure (P_{xylem}) in *Suaeda maritima* stems at day 14

Distribution of xylem pressure (P_{xylem}) in the stem of *Suaeda maritima* plants grown in 0 mM and 200 mM NaCl at day 14. The data have been grouped together to form a frequency distribution chart, n is 25 plants.

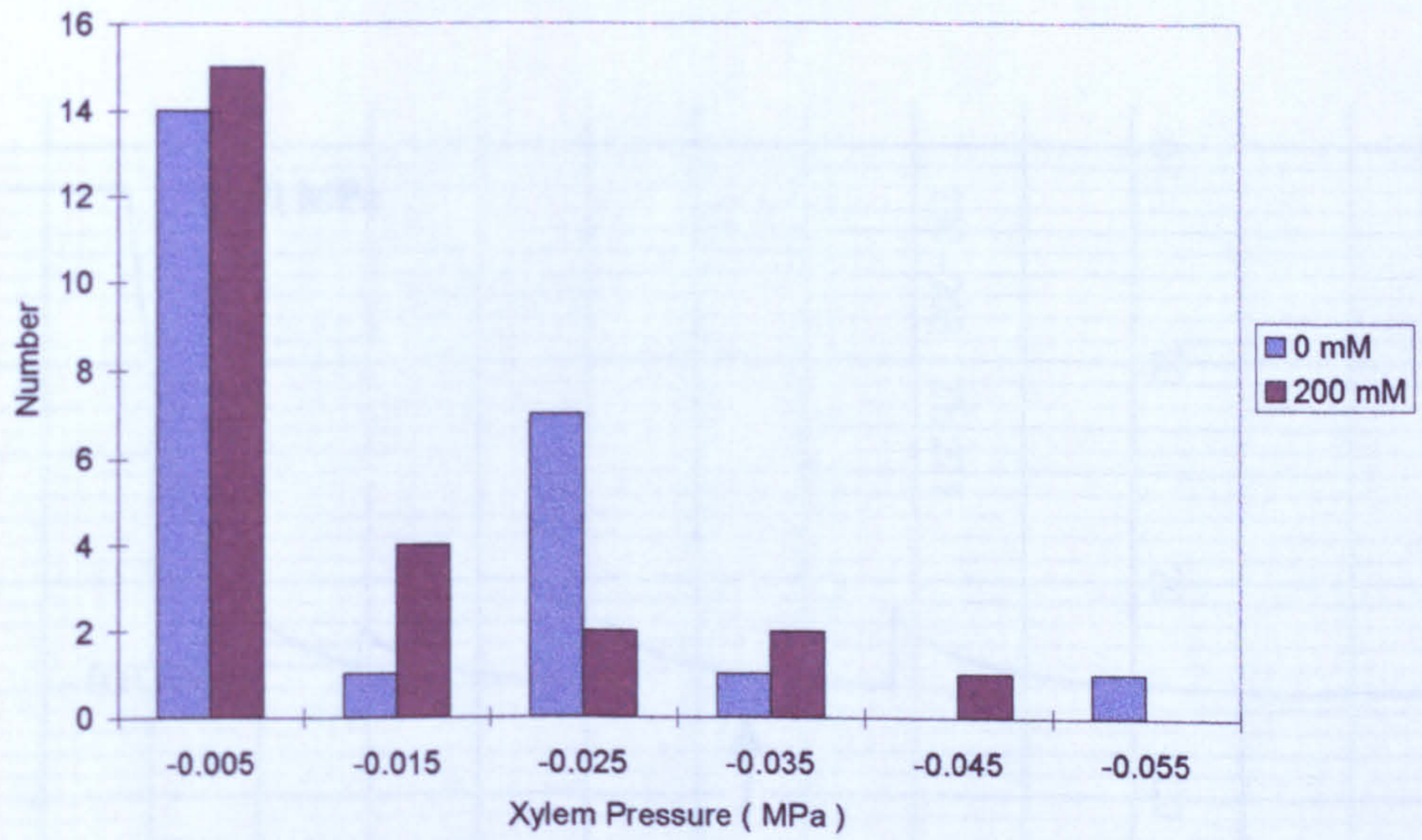


Figure 3.14, Distribution of xylem pressure (P_{xylem}) in *Suaeda maritima* stems at day 21

Distribution of xylem pressure (P_{xylem}) in the stem of *Suaeda maritima* plants grown in 0 mM and 200 mM NaCl at day 21. The data have been grouped together to form a frequency distribution chart, n = 15 plants.

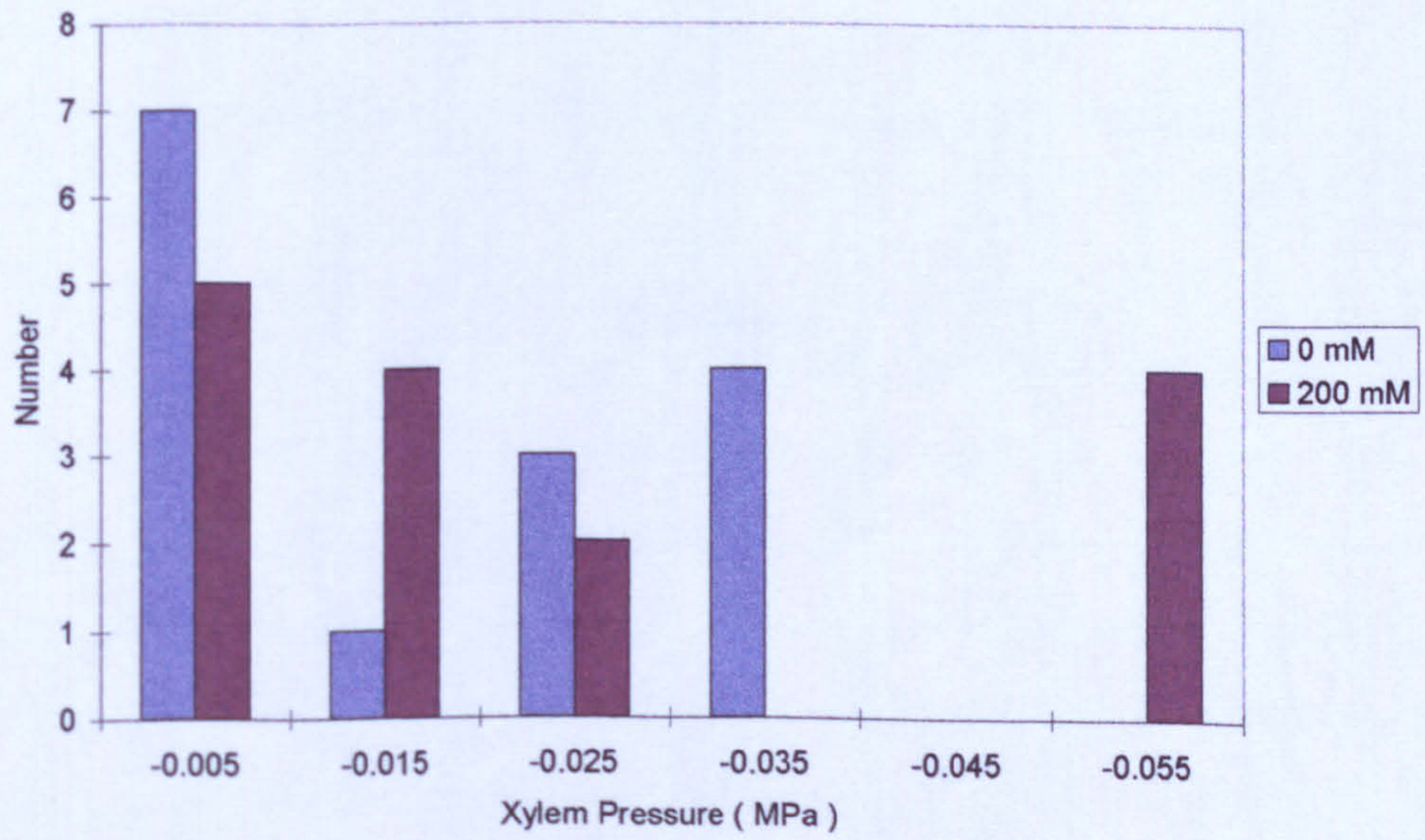


Figure 3.15, Xylem pressure (P_{xylem}) in a *Suaeda maritima* stem before and after a step-up treatment at day 21

A single xylem pressure trace obtained from the stem of a 0 mM NaCl *Suaeda maritima* plant at day 21. The arrow indicates the point at which a 1 MPa step-up treatment was applied to the root system. The peaks indicate pressure pulses applied manually to the xylem probe system. The chart recorder speed was set at 10 mm min $^{-1}$. Each division on the horizontal axis is equivalent to 1 cm.

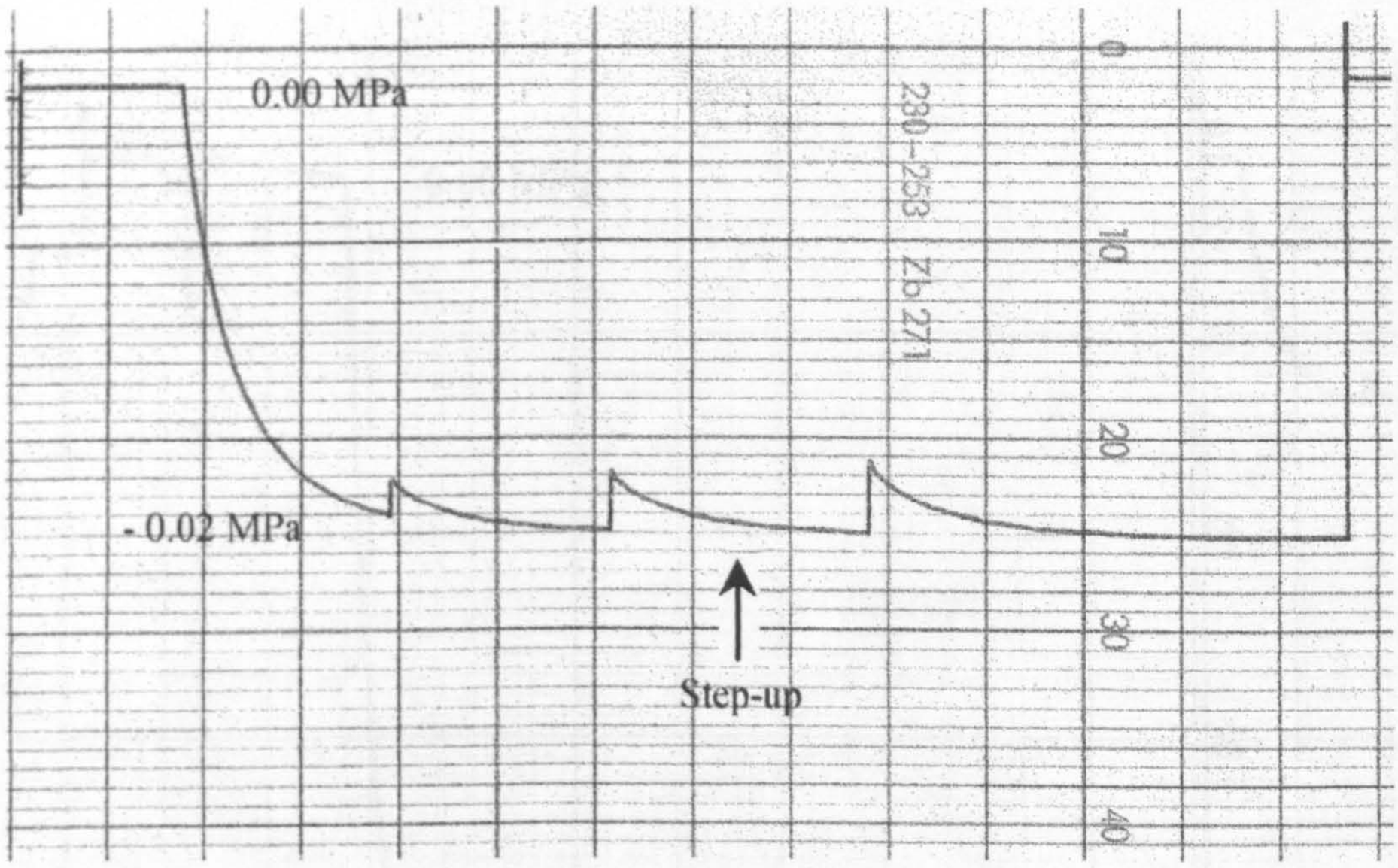
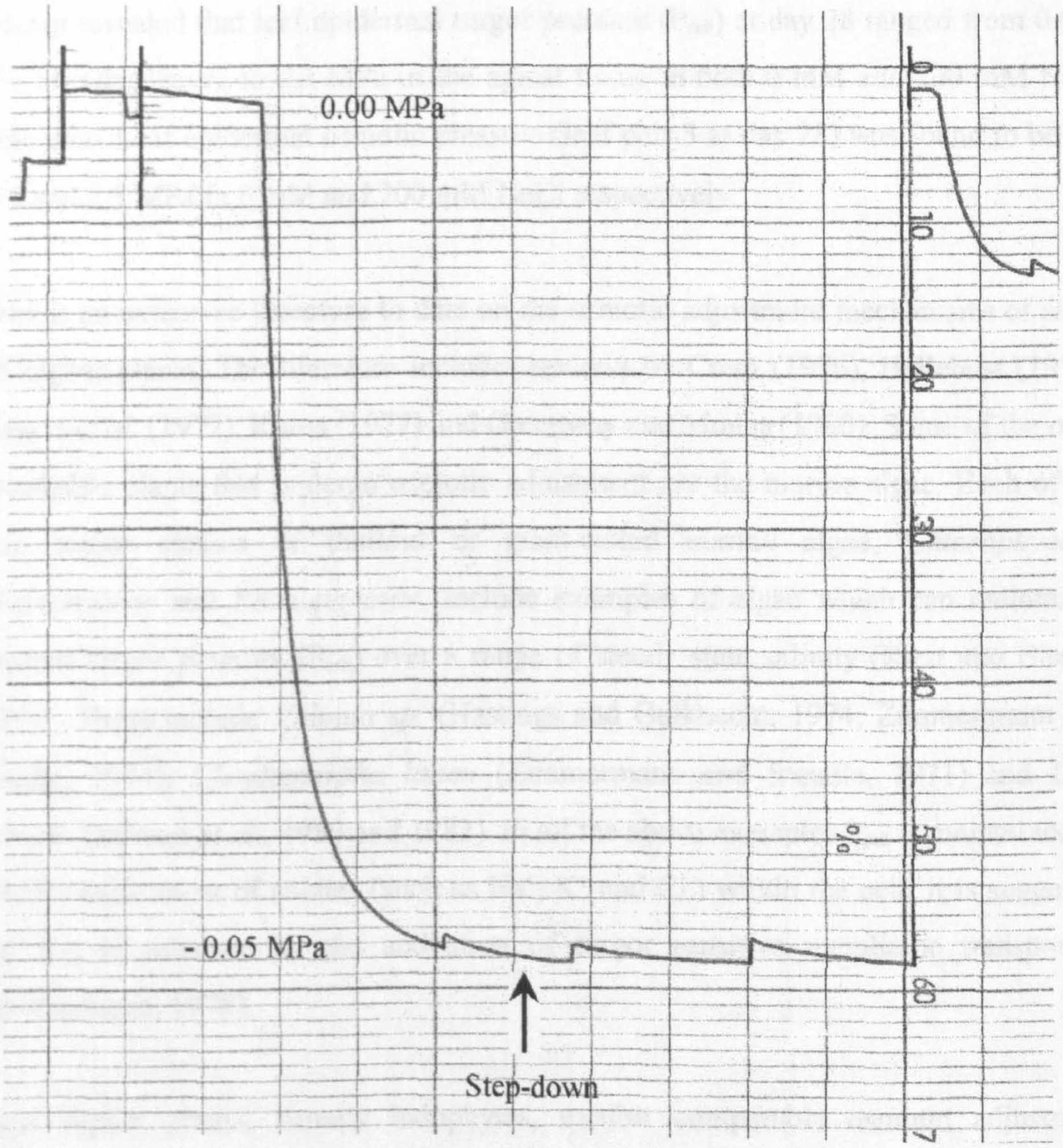


Figure 3.16, Xylem pressure (P_{xylem}) in a *Suaeda maritima* stem before and after a step-down treatment at day 21

A single xylem pressure trace obtained from the stem of a 200 mM NaCl *Suaeda maritima* plant at day 21. The arrow indicates the point at which a 1 MPa step-down treatment was applied to the root system. The peaks indicate pressure pulses applied manually to the xylem probe system. The chart recorder speed was set at 10 mm min $^{-1}$. Each division on the horizontal axis is equivalent to 1 cm.



3.4, Discussion

3.4.1, *Suaeda maritima* leaf epidermal cells

In this chapter, data is presented which demonstrates that *Suaeda maritima* plants grown in steady state 0 mM and 200 mM NaCl regimes, regulate leaf epidermal turgor pressure and undergo complete osmotic adjustment in relation to the external medium. These data are in agreement with the work carried out by Clipson *et al.* in 1985. Their findings revealed that leaf epidermal turgor pressure (P_{cell}) at day 28 ranged from 0.025 MPa in older leaves to 0.3 MPa in the apical tissue in both 0 mM and 200 mM NaCl treatments. Leaf epidermal osmotic pressure (leaf pair 3 at day 28) was found to be 1.5 MPa and 2.5 MPa in 0 mM and 200 mM NaCl respectively.

There is an extensive literature to date on the osmotic adjustment mechanisms of algae and higher plants. The literature includes reviews by Cram (1976), Hellebust (1976), Flowers *et al.* (1977), Kauss (1977) and Greenway and Munns (1980). Some of the most remarkable plants that undergo osmotic adjustment are the marine algae. Each of the three major classes of thalloid or giant-celled marine algae, *Chlorophyceae*, *Rhodophyceae* and *Phaeophyceae*, include examples of algae which can maintain a constant turgor pressure (P_{cell}) over a range of steady state salinity (Kirst and Bisson, 1979). These include *Valonia sp.* (Hastings and Gutknecht, 1974; Zimmermann and Steudle, 1974), *Chaetomorpha linum* (Zimmermann and Steudle, 1971) and *Ulva lactuca* (Dickson *et al.*, 1980 and 1982). In all the above examples P_{cell} is maintained by osmotic adjustment of solutes (such as Na^+, K^+ and Cl^-) within the cell; it is suggested that this is achieved by the activation of turgor sensitive membrane transporters (Zimmermann, 1978).

Many higher plants, namely halophytes, exhibit comparable osmotic adjustment mechanisms. An increase in external osmotic pressure (Π_{ext}) often leads to the accumulation of solutes in the shoot and root. Examples include, *Salicornia bigelovii* (Stumpf and O'Leary, 1985), *Thinopyrum bessarabicum* (Gorham *et al.*, 1985), *Suaeda maritima* (this chapter, Clipson *et al.*, 1985; Reimann, 1992), *Atriplex prostrata* (Reimann, 1992) and *Atriplex canescens* (Glenn *et al.*, 1996). *Suaeda maritima* plants

in a steady state NaCl environment have leaf epidermal osmotic pressures (Π_{cell}) which reflect the osmotic pressure of the external medium (this chapter and Clipson *et al.*, 1985). Accumulation of solutes induced by an increase in Π_{ext} enables a plant to maintain a water potential which is lower than that of the external medium, and thus facilitate the uptake of water into the plant. Halophytes exhibit a wide range of osmotic pressures which are often considerably higher than those found in glycophytes (Cram, 1976; Flowers, 1975). Salt marsh species such as *Spartina glabra*, *Juncus gerardii*, *Salicornia bigelovii* and *Atriplex hasta* have whole plant osmotic pressures of 3.5 MPa, 4.0 MPa, 4.9 MPa and 3.2 MPa respectively (Steiner, 1934). Tropical mangrove species such as *Sonnerata alba* and *Avicennia marina* have leaf osmotic pressures ranging from 3.3 MPa to 3.5 MPa and 3.5 MPa to 6.2 MPa respectively (Field, 1984 {a} and {b}).

Even in a highly dilute hydroponic medium (in the absence of NaCl) *Suaeda maritima* accumulates a high level of solutes in the leaf epidermal cells (this chapter and Clipson *et al.*, 1985). This is in fact characteristic of many halophytes. Walter (1962) found that halophytes grown in a dilute hydroponic medium had osmotic pressures which were approximately double those of comparable glycophytes grown in the same growth medium. This high constitutive capacity for ion uptake may be a mechanism employed by halophytes in preparation for a sudden increase in external osmotic pressure (Π_{ext}). *Suaeda maritima* plants grown in 0 mM NaCl accumulate solutes to what appears to be a predetermined baseline level. When NaCl is included in the external medium the osmotic pressure of the leaf epidermal cells (Π_{cell}) is equal to the cell baseline plus the osmotic pressure of the external medium (this chapter, Clipson *et al.*, 1985). From the data presented in this chapter the baseline leaf epidermal cell osmotic pressure is between 1.2 - 1.4 MPa. Plants grown in a 1 MPa NaCl medium (200 mM NaCl) have a cell osmotic pressure of 2.2 - 2.4 MPa, which is equivalent to the baseline plus Π_{ext}. Full osmotic adjustment takes place despite the fact that the baseline osmotic pressure is greater than the osmotic pressure of the 200 mM NaCl medium.

The magnitude of the osmotic pressure in *Suaeda maritima* leaf epidermal cells is not reflected by the turgor pressure (this chapter and Clipson *et al.*, 1985). Clipson *et al.* (1985) demonstrated that the leaf epidermal turgor pressure in *Suaeda maritima* ranged

from 0.025 MPa in older leaves to 0.3 MPa in the apical tissue. The turgor pressure measurements described in this chapter were on leaves approaching full expansion; the leaf epidermal P_{cell} values obtained were around 0.2 MPa. Compared with other species *Suaeda maritima* has a low leaf epidermal turgor pressure; for example, the salt tolerant alga *Chara longifolia* has a turgor pressure of 0.55 MPa (Bisson *et al.*, 1995) whereas, *Tradescantia virginiana* and *Triticum aestivum* have leaf epidermal turgor pressures of 0.45 MPa (Tomos *et al.*, 1981) and 0.85 MPa (Bates, 1998) respectively.

The reason why *Suaeda maritima* regulates leaf epidermal turgor pressure to such a low level is unclear, however, how such a regulation is achieved can be explained. Assuming that the reflection coefficient (σ) of a cell is close to unity, turgor pressure (P_{cell}) can be influenced by the osmotic pressure of the cell (Π_{cell}), the osmotic pressure of the apoplast (Π_{wall}) and by the hydrostatic component of the apoplast, which is the transpiration tension (P_{wall}). This relationship can be expressed very simply in the following equation:

$$P_{cell} = (\Pi_{cell} - \Pi_{wall}) - P_{wall}$$

Assuming the reflection coefficient is 1, any difference between cell turgor pressure (P_{cell}) and cell osmotic pressure (Π_{cell}) can only be due to the osmotic (Π_{wall}) and/or hydrostatic (P_{wall}) components of the apoplast (cell wall). The influence of P_{wall} on cell turgor pressure can be measured directly by measuring turgor pressure in transpiring and non-transpiring leaves. I have shown that eliminating transpiration does not affect P_{cell} in *Suaeda maritima* plants at day 14; by day 21, however, a 0.1 MPa increase in P_{cell} is observed in non-transpiring plants. Clipson *et al.* (1985) reported similar findings; *Suaeda maritima* plants immersed in hydroponic solution for several hours had a leaf epidermal P_{cell} which was 0.1 MPa - 0.2 MPa greater than P_{cell} in freely transpiring control plants, at day 28. The increase in P_{cell} observed in older non-transpiring plants points to an increased transpiration rate which is likely to be due to an increase in leaf size and number. These data indicate that the hydrostatic component of the apoplast

exerts only a small influence on cell turgor pressure (compared to the magnitude of the difference between P_{cell} and Π_{cell}). The difference between P_{cell} and Π_{cell} is a massive 1.2 MPa and 2.2 MPa in 0 mM and 200 mM NaCl plants respectively (this chapter). Even if the hydrostatic component of the wall accounts for 0.1 MPa of this difference, the bulk of the difference is still unaccounted for. This leads to the conclusion that the difference between turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in *Suaeda maritima* leaf epidermal cells is largely due to the presence of solutes in the apoplast (Π_{wall}). X-ray microanalyses of thin sections of leaf tissue in *Suaeda maritima* plants grown in steady state 340 mM NaCl, revealed that the apoplast surrounding the mesophyll had Na^+ and Cl^- concentrations, based on apoplastic water content, of up to 550 mM and 400 mM respectively (Flowers, 1985). This is equivalent to a wall osmotic pressure of approximately 2.4 MPa. It is necessary to point out here that this method cannot distinguish between free wall solutes and Donnan bound solutes which do not exert a net osmotic effect (Tomos, 1988; Nobel, 1991).

Analysis of the solutes present in the apoplast of other species has revealed that glycophytes have a relatively low concentration of solutes in the apoplast. For example, Jachetta *et al.* (1986) analysed aliquots of sap expressed from detached sunflower leaves in a pressure chamber over intervals of 0.02 - 0.04 MPa. Three distinct fractions were successively released, a petiole/midrib fraction, a minor vein/cell wall fraction and a mixed fraction. Analysis (by vapour pressure osmometry) of the minor vein/cell wall fraction revealed that mature sunflower leaves have an apoplastic osmotic pressure (Π_{wall}) of 0.05 MPa. Nonami and Boyer (1987) applied a 0.03 - 0.04 MPa pressure to the roots of soybean and pea seedlings, using a pressure chamber. Under saturating humidity this resulted in droplets of exudate forming on the elongating region of the intact stem. This exudate was assumed to be apoplastic in nature and analysis, using a micro-osmometer (based on the thermocouple psychrometer described by Boyer and Knipling in 1965), revealed that the enlarging region of soybean and pea seedling stems have Π_{wall} values of 0.041 MPa and 0.028 MPa respectively. Meinzer and Moore (1988) collected apoplastic samples from sugarcane leaf segments (3.5 cm long, cut at different distances from the shoot apex) by centrifugation of noninfiltrated tissue. The osmotic pressure of the apoplastic sample was determined using a vapour pressure osmometer.

Apoplastic osmotic pressure (Π_{wall}) values ranged from 0.25 MPa to 0.35 MPa depending on the physiological age of the leaves; fully mature leaves having the lower apoplastic solute content.

Accumulation of solutes in the apoplast is often associated with salt stress (Oertli, 1968; O'Toole and Cruz, 1979; Flowers *et al.*, 1991). Rice cultivars exhibit leaf rolling in response to external salinity (O'Toole and Cruze, 1979; Flowers *et al.*, 1991). The leaf rolling phenomenon is believed to be induced by loss of water from the cells due to an excessive and undesirable accumulation of solutes (namely Na^+ and Cl^-) in the apoplast. Flowers *et al.* (1991) measured sodium plus potassium concentrations in the leaf apoplast of two rice cultivars (IR 2153 and Amber) grown in 50 mM NaCl for 9 days. This was achieved by X-ray microanalysis of thin transverse sections (1 - 3 mm thick) taken from the middle of the lamina of the second and third leaf. Sodium plus potassium concentrations of up to 511 mM (IR 2153) and 840 mM (Amber) were observed. This high extracellular accumulation of solutes is uncontrolled and believed to be a primary factor in salinity damage in these plants. In contrast, the high concentration of solutes present in the apoplast of *Suaeda maritima* leaf epidermal cells does not induce damage and would appear to play an important part in turgor pressure regulation (this chapter).

Steady state NaCl experiments have attracted criticism in the past from marine zoologists (for example, Stickle and Ahokas, 1974; Davenport *et al.*, 1975) because they do not reflect field conditions. In a salt marsh environment salinity levels can fluctuate wildly due to tidal inundation, evapo-transpiration, fresh water input and rainfall (Jefferies *et al.*, 1983). In this chapter, I describe an experimental regime designed to subject *Suaeda maritima* plants to a rapid step-up or step-down of 1 MPa in the hydroponic medium. *Suaeda maritima* plants grown initially in 0 mM NaCl were placed in a medium containing 200 mM NaCl (step-up), whereas plants grown initially in 200 mM NaCl were placed in a hydroponic medium which did not contain NaCl (step-down). Leaf epidermal turgor pressure (P_{cell}) measurements taken immediately after a step-up/step-down treatment revealed that P_{cell} was completely unaffected by a 1 MPa up or down osmotic shock. Measurements were taken for up to 2 hours after treatment; P_{cell} was maintained at around 0.2 MPa throughout. This is in contrast to the

situation observed in marine algae. *Ulva lactuca* undergoes short term changes in turgor pressure (P_{cell}) in response to changes in external salinity; turgor pressure is restored by long term osmotic adjustment mechanisms over a number of hours (Dickson *et al.*, 1982). The marine alga *Cladophora rupestris* also experiences changes in P_{cell} due to changes in external salinity, but in this case complete osmotic adjustment leading to turgor regulation is not achieved (Wiencke *et al.*, 1991).

The leaf epidermal cells in *Suaeda maritima* are not in direct contact with the external medium, as is the case with the marine algae, and as a result can be buffered against the immediate effects of rapid changes in salinity (the possible nature of this buffering mechanism will be discussed later). Having such a mechanism in place is likely to facilitate protoplastic osmotic adjustment. By maintaining leaf turgor pressure, transpiration can continue (loss of leaf cell turgor pressure would interrupt the transpiration stream and inhibit the transport of inorganic solutes to the shoot via the xylem - Marschner, 1995). A 1 MPa increase in external NaCl (from 0 mM to 200 mM NaCl) depresses the transpiration rate in *Suaeda maritima* by only 25 % after 6 hours. Subsequent recovery to 95 % of the control values is achieved within 24 hours (Clipson, 1987). Preservation of the transpiration stream in a fluctuating NaCl environment facilitates the transport of inorganic solutes from root to shoot, thus providing the solutes necessary for osmotic adjustment in the shoot, and preventing the build up of Na^+ and Cl^- to toxic levels in the root cortex (Clipson, 1987).

After 24 hours in the step-up/step-down treatments leaf epidermal turgor pressure (P_{cell}) remained unchanged. Changes were observed, however, in leaf epidermal osmotic pressure (Π_{cell}). *Suaeda maritima* step-up plants accumulated solutes in the leaf epidermal cells, leading to an increase in cell osmotic pressure which corresponded to the increase applied to the external medium, which was 1 MPa. Step-down plants, on the other hand, did not mobilise solutes out of the leaf epidermal cells, Π_{cell} was maintained despite the 1 MPa decrease in the external medium (Π_{ext}). In other words, *Suaeda maritima* leaf epidermal cells readily accumulate solutes, over 24 hours, in response to an increase in external NaCl. However, a decrease in external NaCl does not induce mobilisation of solutes out of the cell; this results in a massive difference

between the cell osmotic pressure (Π_{cell}) and the osmotic pressure of the external medium (Π_{ext}).

EDX analysis confirmed that the solutes accumulated in the leaf epidermal cell vacuoles of *Suaeda maritima* plants grown in steady state and step-up NaCl media were indeed largely Na^+ and Cl^-. The accumulated Na^+ and Cl^- easily accounts for the increased cell osmotic pressure (Π_{cell}) observed in these plants. Step-up plants, for example, accumulated 397 mM and 357 mM Na^+ and Cl^- respectively within 24 hours (this chapter). Taking into account the osmotic coefficient, the sum of these solutes should give rise to an increase in Π_{cell} of 1.5 MPa. Plants grown in the absence of NaCl had negligible amounts of Na^+ and Cl^- in the cell vacuoles; the main solute present was apparently potassium (this chapter).

Clipson (1987) also presented data which demonstrates that an NaCl step-up leads to an increase in shoot solute accumulation. *Suaeda maritima* plants grown in 0 mM and 200 mM NaCl media were subjected to a 1 MPa step-up 14 days after transfer to hydroponics (28 days after germination). In common with the experiments described in this chapter, the NaCl concentration was increased in a single step. Plants given a step-up from 0 mM to 200 mM NaCl increased cotyledon Π_{cell} from 1.3 MPa to 2.9 MPa, and plants given a step-up from 200 mM to 400 mM NaCl increased apical Π_{cell} from 2.0 MPa to 3.0 MPa respectively. Most leaves reached a new steady state osmotic pressure (Π_{cell}) within 24 hours. Osmotic pressure was determined by dew point hygrometry (as described in Clipson *et al.*, 1985).

Clipson (1987) took the step-up experiment a stage further and measured the rate of sodium accumulation in the shoot and the rate of sodium transport from root to shoot. Clipson's findings revealed that shoot Na^+ accumulation and Na^+ transport rates increased markedly in plants subjected to a step-up from 2 mM to 202 mM NaCl. During the first 6 hours of the step-up treatment the rate of accumulation in the shoot, and the root to shoot transport rate increased from 3 to 80 μmol Na^+ g^{-1} DWS h^{-1} and 10 to 370 μmol Na^+ (shoot) g^{-1} DWR h^{-1} respectively.

In summary, an increase in external NaCl concentration leads to complete osmotic adjustment of *Suaeda maritima* leaf epidermal cells within 24 hours (this chapter and Clipson, 1987). The solutes utilised for osmotic adjustment purposes are Na^+ and Cl^- (this chapter and Clipson, 1987). Accumulation of solutes into the cells is facilitated by an increase in root to shoot transport rate (Clipson, 1987).

Examples of other halophytes which accumulate high levels of Na^+ and Cl^- in the leaves in response to an increase in external NaCl include *Atriplex hasta* (Black, 1956), *Suaeda monoica* (Storey and Wyn Jones, 1979) and *Avicennia marina* (Downton, 1982). Each of these species accumulate Na^+ and Cl^- in the leaves to a level which exceeds the concentration in the external medium. For example, *Atriplex hasta* plants grown in media containing 100 mM and 400 mM NaCl were found to have leaf Na^+ concentrations of 658 mM and 1203 mM respectively, and Cl^- concentrations of 430 mM and 911 mM respectively (Black, 1956; Flowers, 1985).

Bacteria also accumulate high concentrations of ions within the cell, particularly potassium (Csonka, 1989; Csonka and Hanson, 1991). Some specialised halophytic bacteria possess enzymes which are only active *in vitro* in ion concentrations that are inhibitory to similar enzymes extracted from less tolerant organisms (Larsen, 1967; Peterkin and Fitt, 1971). In addition, high levels of K^+ and Mg^{2+} are required for the stability of their ribosomes (Bayley and Kushner, 1964), indicating that a high ion content is necessary for protein synthesis.

In plants, the situation is quite different. Brady *et al.* (1984) assessed the stability of ribosomes incubated in NaCl and KCl solutions at 25 °C. The ribosomes were recovered from a number of plant species, including, *Triticum aestivum*, *Hordeum vulgare*, *Capsicum annum*, *Helianthus annus*, *Pisum savitum*, *Beta vulgaris*, *Chenopodium album*, *Atriplex nummularia*, *Ulva lactuca*, *Entermorpha intestinalis*, *Cladophora* sp., and *Corallina cuvieri*. Their findings revealed that plant ribosomal systems, in both halophytic and glycophytic species, are unstable in media containing 250 mM NaCl or KCl. In *Suaeda maritima*, 100 mM Na^+ in the cytoplasm reduces ribosomal activity by 50 % (Hall and Flowers, 1973). Furthermore, enzymes isolated from halophytic plant species are inhibited by concentrations of NaCl which also inhibit comparable enzymes

in salt sensitive species (Flowers, 1972 {a} and {b}; Greenway and Osmond, 1972; Flowers *et al.*, 1976; Wyn Jones *et al.*, 1977 {a} and {b}; Wyn Jones *et al.*, 1979; Wyn Jones and Pollard, 1983). Flowers (1972 {b}) studied the effect of NaCl on enzymes from 4 halophytic species from the family *Chenopodiaceae*; *Salicornia ramosissima*, *Suaeda maritima*, *Halimione portulacoides* and the maritime sub-species of *Beta vulgaris*. Flowers found that, in general, enzymes isolated from these species were just as sensitive to NaCl as enzymes isolated from the highly salt sensitive plant *Pisum savitum* (Flowers, 1972 {a} and {b}). Therefore, in order to tolerate high levels of NaCl in the cell, halophytes such as *Suaeda maritima* must minimise the build up of Na^+ and Cl^- ions at the sites of active metabolic activity, namely the cytoplasm.

Using efflux, or compartmental analysis techniques, Yeo (1981) demonstrated that accumulated Na^+ in *Suaeda maritima* leaves is sequestered in the cell vacuoles. The concentration of Na^+ in the cytoplasm of *Suaeda maritima* leaf cells was estimated to be 165 mM (Yeo, 1981), whereas the concentration in the vacuole was between 500 mM - 600 mM (this chapter; Yeo, 1981; Yeo and Flowers, 1986). Evidence of Cl^- compartmentation has been published by Harvey *et al.*(1976). This was achieved by silver precipitation and electron microscopy, and is a qualitative rather than a quantitative method.

Due to the Na^+ electrochemical gradient, Na^+ influx across the plasma membrane and into the cytoplasm is likely to be a passive process (Niu *et al.*, 1995). The exact mechanism of Na^+ influx is unknown. The uptake of Na^+ depresses K^+ influx (Watad *et al.*, 1991; Schroeder *et al.*, 1994), suggesting that the uptake mechanisms for these ions may be similar. Efflux of Na^+ out of the cytoplasm, across the plasma membrane and tonoplast is an active process believed to be facilitated by an Na^+/H^+ antiport driven by a pH gradient generated by the plasma membrane P-type and tonoplast V-type proton ATPase respectively (Niu *et al.*, 1995). Plasma membrane Na^+/H^+ antiport activity has been stimulated by NaCl in the marine alga *Dunaliella salina* (Katz *et al.*, 1992) and the halophyte *Atriplex nummularia* (Hassidim *et al.*, 1990). Tonoplast Na^+/H^+ antiport activity increases in response to elevated NaCl concentrations in the halophyte *Mesembryanthemum crystallinum* (Barkla *et al.*, 1995), *Beta vulgaris* (Blumwald and

Poole, 1985), sunflower roots (Ballesteros *et al.*, 1997), barley roots (Garbarino and DuPont, 1988), and tobacco culture cells (Reuveni *et al.*, 1990).

Once NaCl has been sequestered in the leaf cell vacuole it is not easily remobilised out of the cell. This is observed in leaf epidermal cells of *Suaeda maritima* plants subjected to a step-down NaCl treatment (this chapter). Maathuis *et al.* (1992) suggested that ions are prevented from re-entering the cytoplasm by a very low gating frequency of non-specific slow vacuolar (SV) channels (as described by Hedrich *et al.*, 1988; Maathuis and Prins, 1990) in the tonoplast.

An osmotic balance is maintained between the vacuole and cytoplasm in *Suaeda maritima* by accumulation of K^+ and the compatible solute glycinebetaine in the cytoplasm (Wyn Jones, 1981; Gorham and Wyn Jones, 1983).

3.4.2, *Suaeda maritima* root cortex cells

In steady state 0 mM and 200 mM NaCl growth media, *Suaeda maritima* root cortex turgor pressure (P_{cell}) is maintained at a constant level (around 0.4 MPa). The root cortex osmotic pressure (Π_{cell}) in plants grown in the 0 mM NaCl medium corresponds exactly with the cell turgor pressure. Plants grown in the 1 MPa NaCl medium (200 mM NaCl plants) have a root cortex Π_{cell} which is 1 MPa greater than in plants grown in the absence of NaCl (this chapter). In other words, *Suaeda maritima* root cortex cells can regulate turgor pressure (P_{cell}) over a range of steady state NaCl concentrations and undergo complete osmotic adjustment in relation to the external medium. Under steady state conditions the root cortex cells behave in a similar fashion to the leaf epidermal cells discussed earlier (see **Section 3.4.1**), however, there are notable differences between the two cell types. The high constitutive capacity for ion uptake, in a dilute medium, in *Suaeda maritima* leaf epidermal cells, is not observed in the root cortex cells. Also, the apoplast surrounding the root cortex cells cannot be utilised as a useful osmotic compartment.

In order for a cell to exert any kind of control over the apoplast, the apoplastic compartment must be closed to the external medium. The apoplast of the root cortex cells is probably completely open to the external medium, and as a result effectively occupies the same volume as the external medium. This would make manipulation of the apoplastic compartment extremely difficult and metabolically expensive. In this case the properties of the apoplast can be considered to be the same as the properties of the external medium. Assuming a reflection coefficient of 1, turgor pressure (P_{cell}) can now be determined by simply subtracting the osmotic pressure of the external medium (Π_{ext}) from the osmotic pressure of the cell (Π_{cell}). This is illustrated in the equation below:

$$P_{cell} = \Pi_{cell} - \Pi_{ext}$$

The response of *Suaeda maritima* root cortex cells to rapid fluctuations in NaCl is very different to that observed in the leaf epidermal cells; at least in the short term. Root cortex cells subjected to a 1 MPa step-up, from 0 mM to 200 mM NaCl, experienced a rapid drop in cell turgor, and cell plasmolysis well within 10 minutes, whereas root cortex cells given a 1 MPa step-down, from 200 mM to 0 mM NaCl, underwent an equally rapid and dramatic increase in cell turgor pressure (this chapter). The root cortex cells were acting as perfect osmometers (Cram, 1976), undergoing changes in turgor pressure (P_{cell}) which mirror the changes in external osmotic pressure (Π_{ext}). Turgor pressure regulation took place by means of osmotic adjustment of solutes within the cell over a number of hours (this chapter). A decrease in root cortex turgor pressure (P_{cell}) in step-down treated plants was observed within 30 minutes, P_{cell} decreased by 0.5 MPa within 7 hours reaching 0.8 MPa; just over half way to the complete osmotic adjustment P_{cell} target of 0.4 MPa. The decrease in P_{cell} was accompanied by a corresponding decrease in osmotic pressure (Π_{cell}). Root cortex P_{cell} and Π_{cell} were both approximately 0.8 MPa 7 hours after application of the step-down treatment. No turgor regulation was observed in the step-up treated plants at this stage. This may be due to the fact that the step-up treatment induced cell plasmolysis, which due to the severe nature of plasmolysis may inhibit the membrane transport mechanisms necessary to achieve osmotic adjustment.

Within 24 hours osmotic adjustment leading to turgor regulation was complete. Root cortex turgor pressure (P_{cell}) had been restored and was comparable to control levels in both step-up and step-down treated *Suaeda maritima* plants. Root cortex osmotic pressure (Π_{cell}) in step-up plants increased by 1 MPa, whereas Π_{cell} in step-down plants decreased by 1 MPa; exactly mirroring the changes in the external medium (Π_{ext}). These data demonstrate that *Suaeda maritima* root cortex cells freely accumulate solutes in response to an increase in external NaCl and also freely mobilise solutes out of the cell in response to a decrease in external NaCl. This is a text book example of osmotic adjustment, and is comparable to the osmotic adjustment mechanism observed in the marine alga *Ulva lactuca* (Dickson *et al.*, 1980 and 1982).

Suaeda maritima leaf epidermal cells also freely accumulate solutes in response to an increase in external NaCl (this chapter), but as I discussed earlier in **Section 3.4.1**, these accumulated solutes are not remobilised out of the cell in response to a decrease in external NaCl, and are effectively trapped in the leaf epidermal vacuoles due to the properties of the tonoplast. In the root cortex the situation is somewhat different, and solutes are readily mobilised out of the cells. It is believed that remobilised solutes are preferentially transported to the shoot in order to maintain an adequate water potential gradient from root to shoot (Stelter and Jeschke, 1983; Storey *et al.*, 1983; Pitman, 1984; Yeo and Flowers, 1986). It has been calculated that more than 80% of the total sodium content of *Suaeda maritima* is found in the leaves (Pitman, 1984). Preferential transport of solutes from root to shoot has also been observed in the halophyte *Atriplex prostrata* (Reimann, 1992). In contrast, *Chenopodium album* and *Chenopodium schraderianum* (which belong to the same family as *Suaeda* and *Atriplex*; the *Chenopodiaceae*) have low Na^+ and Cl^- concentrations in the shoot and much higher concentrations in the root; this is due to a low transport rate from root to shoot, and is an example of NaCl exclusion (Reimann, 1992).

3.4.3, *Suaeda maritima* xylem pressure (P_{xylem}) in the stem

The stem xylem pressure values obtained in *Suaeda maritima* plants grown in steady state 0 mM and 200 mM NaCl ranged from -0.005 MPa to -0.055 MPa in both cases.

The majority of the values obtained were around -0.005 MPa, although P_{xylem} values of a greater magnitude were obtained more frequently in the older plants, at day 21 (all xylem pressure data is expressed relative to atmospheric pressure). By day 21 *Suaeda maritima* seedlings are considerably larger than they were at day 14. Leaf surface area has increased and up to 4 new leaf pairs have emerged (data not shown). This results in an increase in transpiration rate (as discussed in **Section 3.4.1**), which subsequently leads to an increase in xylem tension in the stem (see Benkert *et al.*, 1995; Zimmermann *et al.*, 1995). At day 21 the transpiration tension (P_{wall}) in the apoplast adjacent to the leaf epidermal cells was approximately -0.1 MPa, whereas the xylem tension (P_{xylem}) in the stem was between -0.005 MPa and -0.055 MPa (this chapter). The difference in hydrostatic tension values in the leaf apoplast and stem xylem provide evidence of a negative pressure gradient along the axis of the plant.

The large pressure gradients predicted by the original **Cohesion Theory** (Dixon and Joly, 1894 and 1895) appear to be unnecessary to achieve xylem flux. Zimmermann and co-workers have demonstrated that tension in the xylem is much lower than that required by the cohesion theory (see Zimmermann *et al.*, 1993; Zimmermann *et al.*, 1994 {a}). In light of this, Martin Canny (1995) proposed a new theory for the ascent of sap, which involves cohesion supported by tissue pressure. **The Compensating Pressure Theory** proposes that the driving force for the ascent of sap is provided by evaporation of water from the leaves, and the tensions generated in the curved menisci in the wet cell walls of the leaf. This force is transmitted by tension in the water in the tracheary elements. Thus far the new theory is in agreement with the original Cohesion Theory. However, Canny's theory goes on to suggest that this tension is kept within the cavitation threshold by compression induced by tissue pressure around the tracheary elements. This theory eliminates the need for a large axial tension gradient to sustain sap flow, however it is conceded that smaller tension gradients are necessary to overcome the resistance to water flow in the xylem and apoplast. The xylem conduits are of relatively large diameter compared to the capillaries which exist in the walls between cells. The xylem system therefore offers a pathway of low resistance to the flow of water relative to the cell walls (Nobel, 1991; Malone, 1993). Water flux through a cylinder is defined by the Hagen- Poiseuille law (see Nobel, 1991).

In order to determine whether fluctuations in external NaCl led to the propagation of a hydrostatic signal from root to shoot in *Suaeda maritima* , the xylem pressure probe was used to measure xylem pressure (P_{xylem}) during a 1 MPa step-up/step-down treatment. Remarkably (given the dramatic response observed in the root cortex cells), the step-up/step-down treatment did not induce a detectable change in xylem pressure. This can possibly be explained in terms of root reflection coefficient and shoot transpiration rate.

Solute flux across a membrane can influence the flux of water across the same membrane. The relationship and interactions between solute and water fluxes have to be considered in estimating the rate of resultant osmotic flux. This is the role of the reflection coefficient (σ). For a non-selective completely permeable membrane the reflection coefficient is 0, as there is no interaction between solute and water. If the membrane is permeable to water only, and completely impermeable to solutes (semipermeable), the reflection coefficient will be 1. Biological membranes, which are selectively-permeable, have reflection coefficient values ranging from 0 to 1, depending on the specific permeabilities for the different solutes and water (Dainty, 1963; Meidner and Sheriff, 1976; Nobel, 1991, Steudle, 1993). The radial reflection coefficient (σ_r) of a root describes the properties of the barrier between the xylem compartment and the external medium. The radial reflection coefficient can be calculated using the following equation (assuming the root elastic modulus is equal to infinity):

$$\sigma_r = \Delta P_{xylem} / \Delta \Pi_{ext}$$

σ_r = Radial reflection coefficient

ΔP_{xylem} = Change in xylem pressure

$\Delta \Pi_{ext}$ = Change in the osmotic pressure of the external medium

In the analysis of radial transport processes across roots, it has frequently been assumed that the root behaves like a perfect osmometer (Fiscus, 1977; Weatherly, 1982; Munns and Passioura, 1984). This implies that roots are completely semipermeable and have a radial reflection coefficient close to 1. There is evidence to suggest, however, that this is an over simplification. Steudle (1989, 1992 and 1993) carried out root pressure probe measurements on excised roots and found that the radial reflection coefficient was much lower (between 0.2 and 0.5). If this is indeed the case changes in xylem pressure (P_{xylem}) due to changes in the external medium would be much less than expected for a perfect osmometer. It is important to consider, however, that data obtained from excised root tissue may not tell us a great deal about the properties of that tissue in the intact plant. The net water flow through an excised root is zero, whereas the intact root is a water transport system (Schneider *et al.*, 1997 {a} and {b}). Furthermore, the stationary turgor and osmotic pressure gradients in the cortex collapse when transpiration is eliminated by root excision (Rygol and Zimmermann, 1990; Zimmermann *et al.*, 1992; Rygol *et al.*, 1993; Clarkson, 1993). This leads to unpredictable changes in the distribution of osmotically active solutes, which subsequently results in concentration-polarisation effects in the root tissue. These concentration-polarisation effects are believed to influence water relations parameters such as the radial hydraulic conductivity as well as the radial and cellular reflection coefficients (Nobel, 1983; Barry and Diamond, 1984; Dainty, 1985).

The xylem pressure probe has been used to determine the root radial reflection coefficient in a number of intact plants, under various environmental conditions. The transpiration rate is believed to play a key role in determining the radial reflection coefficient of a root system (Zimmermann *et al.*, 1994 {b}; Schneider *et al.*, 1997 {a} and {b}). A high transpiration rate often results in a radial reflection coefficient which is close to 1. Whereas a low transpiration rate leads to much lower radial reflection coefficient values. Schneider *et al.* (1997 {a}) demonstrated that freely transpiring barley plants (in dry air) subjected to a 0.46 MPa step-up/step-down regime undergo changes in xylem pressure (P_{xylem}) which correspond exactly with the changes in the external medium; in other words the root system behaves as a perfect osmometer when transpiration is maximised. For example, a 0.46 MPa step-up (from nutrient medium to

100 mM NaCl) resulted in a drop in P_{xylem} of 0.45 MPa within 10 minutes. This gives a root radial reflection coefficient of 0.98 for NaCl, as demonstrated below:

$$\sigma_r = \Delta P_{xylem} / \Delta \Pi_{ext}$$

$$\sigma_r = 0.45/0.46$$

$$\sigma_r = 0.98$$

Repeated nutrient medium/100 mM NaCl treatments revealed that the effect of NaCl on xylem pressure (P_{xylem}) was reversible. The magnitude of the drop in P_{xylem} depended on the external NaCl concentration. The change in P_{xylem} equalled that expected for a perfect osmometer.

Comparable experiments were carried out on barley plants in which transpiration had been largely eliminated (in darkness and humidified air). The response of P_{xylem} to changes in Π_{ext} was greatly reduced under these conditions. Non-transpiring plants subjected to a step-up of 0.23 MPa underwent a drop in P_{xylem} of around 0.1 MPa, resulting in a radial reflection coefficient of 0.43 for NaCl. Similar findings were also observed in wheat (Schneider *et al.*, 1997 {a}).

Xylem pressure (P_{xylem}) in the mangrove *Rhizophora mangle* does not respond to changes in NaCl concentration in the external medium. Plants transferred from tap water to a medium containing over 4.0 MPa NaCl maintained a constant P_{xylem} value (Zimmermann *et al.*, 1994 {b}). This corresponds to the *Suaeda maritima* xylem pressure data presented in this chapter. In conclusion, *Rhizophora mangle* and *Suaeda maritima* must have root radial reflection coefficients close to 0 for NaCl. This may be due to low transpiration rates in these plants. Low transpiration rates are often considered to be a characteristic feature of halophytes. The anatomy and physiology of many halophytes is geared towards reducing transpiration in order to minimise excessive and undesirable transport of solutes from root to shoot via the xylem (Flowers, 1985). Xylem pressure probe measurements have demonstrated that

Rhizophora mangle and *Suaeda maritima* do not maintain large negative xylem pressures in their xylem vessels. The xylem pressures measured in *Rhizophora mangle* and *Suaeda maritima* stems were largely + 0.05 MPa (absolute value - Zimmermann *et al.*, 1994 {b}) and - 0.005 MPa (relative to atmospheric pressure; this chapter) respectively. These low xylem tension values point to a low rate of transpiration in these plants (the relationship between transpiration rate and xylem pressure is discussed by Benkert *et al.*, 1991 and 1995). A root radial reflection coefficient which is 0 means that the effective osmotic pressure of the external medium is also 0, which may facilitate water uptake under conditions of fluctuating salinity.

Returning to the situation observed in *Suaeda maritima* root cortex cells (discussed in **Section 3.4.2**); the root cortex cells behave as perfect osmometers in response to changes in NaCl concentration in the external medium. Perfect osmometers are completely impermeable to solutes, in other words, a perfect osmometer has a permeability coefficient which is 0, and a reflection coefficient which is 1. However, the recent literature (see above) and the data presented in this chapter suggest that the root, as a whole, is not a perfect osmometer. The behaviour of a single cell or a single cell type cannot be equated with the behaviour of a whole tissue. The root cylinder is permeable to solutes, and as highlighted above, measurements on intact root systems have revealed that the radial reflection coefficient of a root is often less than 1. Transpiration appears to play an important role in determining the radial reflection coefficient (see above). However, other factors must also be taken into consideration including, root anatomy, the physiological age and development of the root, the pathways available for water and solute transport (apoplastic, symplastic and transcellular), the presence and number of water channels, and the number of such channels actively utilised at any one time. All the above parameters have been brought together by the **Composite Transport Model** (see Steudle and Peterson, 1998). This model describes the flux of water through a root, and allows for differences in water movement through individual cells as well as through various tissues (Steudle *et al.*, 1993; Steudle, 1994 {a} and {b}; Steudle and Frensch, 1996; Steudle and Peterson, 1998). The Composite Transport Model beautifully highlights the complexity and sophistication of the root system as a water transport device, and also reveals that we have much yet to learn.

The properties of the root system in *Suaeda maritima* also explains why the leaf epidermal cells do not respond to changes in NaCl concentration in the external medium (see **Section 3.4.1**). Due to the apparent low NaCl radial reflection coefficient of the root no hydrostatic signal is propagated from root to shoot via the xylem. As a result no immediate change in leaf epidermal turgor pressure (P_{cell}) would be expected.

3.5, Summary and conclusions

3.5.1, Leaf epidermal cells

- *Suaeda maritima* leaf epidermal cells maintain turgor pressure (P_{cell}) at a level which is much lower than the osmotic pressure (Π_{cell}) of the cell. Evidence suggests that this is achieved by the accumulation and adjustment of solutes in the adjacent apoplast (P_{wall}). This indicates that *Suaeda maritima* utilises the apoplast as an osmotic compartment.

- A 1 MPa increase or decrease in external NaCl has no immediate or long term effect on leaf epidermal P_{cell}.

- *Suaeda maritima* leaf epidermal cells accumulate solutes (namely NaCl) in response to an increase in external salinity. The increase in cell osmotic pressure (Π_{cell}) exactly mirrors the increase in external osmotic pressure (Π_{ext}). Complete osmotic adjustment of the protoplast takes place within 24 hours.

- Solutes are not remobilised out of the leaf epidermal cells in response to a decrease in external salinity. This is believed to be due to the properties of the tonoplast in this cell type.

3.5.2, Root cortex cells

- Root cortex cells behave as perfect osmometers in response to fluctuations in Π_{ext}.

- The root cortex cells readily accumulate solutes in response to an increase in external NaCl. The increase in root cortex Π_{cell} exactly mirrors the increase in Π_{ext}. Osmotic adjustment of the protoplast is achieved within 24 hours.

- A decrease in external NaCl leads to mobilisation of solutes out of the cortex cells. The subsequent decrease in Π_{cell} exactly mirrors the decrease in Π_{ext}. The remobilised

solutes are believed to be preferentially transported from the root to the shoot via the xylem. Once again, osmotic adjustment of the protoplast is achieved within 24 hours.

3.5.3, Xylem pressure/tension in the stem

- Xylem pressure (P_{xylem}) can be measure successfully in *Suaeda maritima* stems using the xylem pressure probe.

- A 1 MPa NaCl increase or decrease in Π_{ext} does not lead to the propagation of a detectable hydrostatic signal from root to shoot via the xylem. This is believed to be due to the properties of the root as a whole and a root radial reflection coefficient which is close to 0.

Chapter 4 : Turgor regulation by osmotic adjustment of apoplastic solutes in *Suaeda maritima* L. Dum. leaf epidermal cells

4.1, Introduction

The importance of apoplastic solutes in plant cell water relations has been highlighted by a number of authors, including: Leigh and Tomos (1983), Clipson *et al.* (1985), Tomos and Wyn Jones (1988), Tomos (1988), and Bell and Leigh (1996). Turgor regulation in beet taproot tissue is crucial, as high turgor pressure inhibits sucrose accumulation (Wyse *et al.*, 1986; Bell and Leigh, 1996). It is proposed that turgor regulation in red beet and sugar beet taproot tissue is achieved by apoplastic solute adjustment (namely apoplastic K^+ - see Leigh and Tomos, 1983; Bell and Leigh, 1996). This would allow sucrose accumulation to continue while turgor pressure was maintained at a level which facilitates membrane transport (Leigh and Tomos, 1983; Tomos, 1988; Bell and Leigh, 1996; see also **Chapter 5**).

A comparable turgor regulation mechanism is believed to take place in *Suaeda maritima* leaf epidermal cells. In order to maintain water flux into the plant, *Suaeda maritima* must accumulate NaCl in the leaves in response to an increase in external NaCl (Flowers, 1975; Clipson *et al.*, 1985; see also **Chapter 3**). In the previous chapter (**Chapter 3**), I discussed data which clearly demonstrated that solutes were present in the apoplastic space adjacent to *Suaeda maritima* leaf epidermal cells. It was also clear that under a fluctuating external NaCl regime leaf epidermal turgor pressure could only be maintained if protoplastic and apoplastic adjustment of solutes were taking place in parallel (see also Clipson *et al.*, 1985; Tomos and Wyn Jones, 1988).

This chapter describes a novel and exciting experimental protocol, which was first brought to our attention by Tomos and Wyn Jones (1988) in red beet taproot tissue. Turgor pressure in *Suaeda maritima* leaf epidermal cells is modulated by direct manipulation of the solutes present in the apoplast. Access to the apoplast is gained by slicing selected *Suaeda maritima* leaves open. The original apoplastic solutes are then washed out of the apoplast using a carefully selected osmoticum. After the washing

procedure is completed it is assumed that the only solutes left in the apoplast (at least initially) are those which were present in the osmoticum. At this stage the apoplast is either closed to the external environment using water saturated silicone oil or simply left open to the osmoticum. Turgor regulation and osmotic adjustment in the leaf epidermal cells is then monitored using the cell pressure probe and single cell sampling and analysis techniques (see **Chapter 2** and references therein). The beauty of this technique is that it allows one to access the apoplast and directly modulate the solute composition and apoplastic volume. This technique would also lend itself well to more detailed apoplastic solute flux studies using ion selective microelectrodes.

4.2, Method

4.2.1, Leaf selection and preparation

Leaves were excised from *Suaeda maritima* plants grown in 0 mM and 200 mM NaCl at day 14 (see **Chapter 3, Section 3.2.1** for germination and hydroponic culture protocols), using a sharp pair of dissection scissors. Leaf selection was limited to leaf pair 1 and 2. The leaves were preferably undamaged and roughly symmetrical. The excised leaves were placed on a suitable cutting surface (such as a glass tile), adaxial side up, and cut longitudinally from end to end with a sharp double edged razor blade.

The sliced leaves were either used immediately or after pre-treatment in an osmoticum. Pre-treatment involved aerating the longitudinal leaf slices in 100 ml of osmoticum for 15 minutes. The following osmotica were used: 300 - 1000 mOsmoles kg^{-1} NaCl and 400 - 900 mOsmoles k^{-1} melibiose (BDH). A single length of freshly cut or pre-treated leaf tissue was secured to a perspex sample holder. The tissue was immediately covered in 5 ml of water saturated silicone oil or a 5 ml aliquot of the aqueous osmoticum. Care was taken to ensure that the tissue was not exposed to the air for any length of time in order to prevent dehydration.

4.2.2, Turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) measurement

The cells targeted with the pressure probe and sampling arm (see **Chapter 2, Section 2.2 and 2.3**) were leaf epidermal cells approximately 2 -3 cells away from the cut edge of the tissue. The cells situated at the extreme edge of the tissue were avoided due to the damage and stress caused by the cutting procedure.

Turgor pressure measurements were made, using the cell pressure probe (see **Chapter 2, Section 2.2**), at 5 minute intervals for a maximum of 90 minutes. Samples for picolitre osmometry (see **Chapter 2, Section 2.5**) were taken at 10 minute intervals for a maximum of 90 minutes. The time lag between applying the water saturated silicone oil to the tissue and taking the first measurements was kept to a minimum.

4.3, Results

Leaf epidermal cells at the cut edge of *Suaeda maritima* leaves - which have been sliced in half longitudinally and placed immediately under water saturated silicone oil - had a turgor pressure (P_{cell}) which was initially too low to measure with the cell pressure probe (**Figure 4.1 and 4.2**). The act of slicing open a leaf invariably leads to cell damage. Solutes from the broken and damaged cells leach into the adjacent apoplastic space. This increase in apoplastic solute concentration leads to a decrease in turgor pressure of nearby, undamaged cells, as water is drawn out of the cells and into the apoplast. In order to determine whether the turgor pressure would recover over time, measurements were taken every 5 minutes for 90 minutes. Turgor pressure remained too low to measure successfully for approximately 15 minutes, after which time turgor pressure recovered steadily, reaching a plateau after 40 - 45 minutes. A steady turgor pressure of about 0.25 MPa was maintained for the duration of the experiment thereafter. This turgor pressure value is comparable with the leaf epidermal P_{cell} measurements obtained in corresponding leaves in intact *Suaeda maritima* plants (see **Chapter 3, Figure 3.4**). The selected leaves were taken from plants grown in 0 mM NaCl (**Figure 4.1**) and 200 mM NaCl (**Figure 4.2**) media; the turgor regulatory mechanism appeared to be identical for both (i.e. turgor regulation was achieved over the same time period in both 0 mM and 200 mM NaCl leaves).

The observed turgor regulation mechanism can be achieved either by adjustment of solutes within the protoplast or by adjustment of solutes in the apoplast. The time scale within which turgor regulation is achieved in these sliced leaves suggests that adjustment of the protoplast is unlikely; protoplastic adjustment of solutes might be expected to take a number of hours rather than minutes (Cram, 1976). Leaf epidermal osmotic pressure (Π_{cell}) measurements were taken from freshly sliced *Suaeda maritima* leaves under silicone oil in order to determine whether protoplastic solute adjustment, leading to turgor regulation, was taking place (**Figure 4.3 and 4.4**). Leaf epidermal cells on leaf slices taken from *Suaeda maritima* plants grown in 0 mM and 200 mM NaCl had initial osmotic pressure (Π_{cell}) values of approximately 1.4 MPa (**Figure 4.3**) and 2.4 MPa (**Figure 4.4**) respectively. This corresponds to the situation in the intact

plant (see **Chapter 3, Figure 3.4**). The 1 MPa difference between Π_{cell} in plants grown in 0 mM and 200 mM NaCl reflects the difference in the osmotic pressure of the external medium (Π_{ext}). The time course of Π_{cell} measurements revealed that leaf epidermal osmotic pressure was maintained at initial values throughout the experiment. This leads to the conclusion that turgor regulation is most likely achieved by adjustment of solutes in the apoplast in this case.

In order better to understand the adjustment mechanism employed by *Suaeda maritima* leaf epidermal cells it was important to gain an element of control over the initial composition of the apoplastic compartment. The leaf apoplast adjacent to the cut face of the leaf slices was effectively completely open to the external environment prior to being covered with water saturated silicone oil. This allowed manipulation of the apoplastic compartment with carefully chosen aqueous osmotica. Aerating the leaf slices for 15 minutes in an osmoticum washed out the original apoplastic solutes and replaced them with those present in the osmoticum. While the leaf slices were in the aqueous media the apoplast effectively occupied the same volume as the osmoticum, which was 100 ml in this case; this is referred to as an open system. Once the pre-treatment was complete the leaf slices were covered in water saturated silicone oil which not only prevented dehydration of the leaf tissue, but also reduced the apoplastic volume to a level which mimics the apoplastic volume *in vivo*. As the silicone oil closes off the apoplast to the external environment, this system is referred to as a closed system (Tomos and Wyn Jones, 1988).

The osmotica contained either NaCl, which was assumed could be freely transported from apoplast to cell and vice versa, or melibiose, which is impermeant in nature and cannot cross the plasma membrane (Conner and Falloon, 1993). The osmotica were either more concentrated, less concentrated, or roughly equivalent to the estimated concentration of solutes present in the apoplast in vivo. The apoplastic concentration of solutes *in vivo* was estimated from the difference between turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in the leaf epidermal cells in intact *Suaeda maritima* plants under non-transpiring conditions (see **Chapter 3, Figure 3.4** and **Table 3.2**).

Suaeda maritima leaf slices pre-treated in 600 mOsmoles kg^{-1} NaCl (0 mM NaCl plants, **Figure 4.5**) and 1000 mOsmoles kg^{-1} NaCl (200 mM NaCl plants, **Figure 4.6**) had initial leaf epidermal turgor pressures which were too low to be measured successfully. After 15 minutes turgor pressure had recovered sufficiently to be measured without undue difficulty. After 40 - 45 minutes P_{cell} reached a plateau at around 0.25 MPa, which was maintained at this level for the duration of the experiment. These data are comparable (most notably the final equilibrium P_{cell} values) to the data obtained from freshly sliced, untreated leaf tissue (**Figure 4.1 and 4.2**).

Suaeda maritima leaf slices pre-treated in less concentrated NaCl osmotica exhibited turgor regulation in the opposite direction. Leaf slices pre-treated in 300 mOsmoles kg^{-1} NaCl (0 mM NaCl plants, **Figure 4.7**) and 600 mOsmoles kg^{-1} NaCl (200 mM NaCl plants, **Figure 4.8**) had initial leaf epidermal turgor pressure values between 0.4 MPa and 0.45 MPa. Turgor pressure was regulated downwards almost immediately. Turgor pressure (P_{cell}) levelled out again at between 0.2 MPa and 0.25 MPa within 30 - 35 minutes, and was maintained at this level for the duration of the experiment.

No change in leaf epidermal turgor pressure was observed in *Suaeda maritima* leaf slices pre-treated in NaCl osmotica with an intermediate solute concentration. This suggests that the solute concentration in the osmoticum is comparable to the concentration of solutes in the leaf apoplast solution *in vivo*. Leaf slices pre-treated in 400 mOsmoles kg^{-1} NaCl (0 mM NaCl plants, **Figure 4.9**) and 800 mOsmoles kg^{-1} NaCl (200 mM NaCl plants, **Figure 4.10**) maintained turgor pressure (P_{cell}) at just over 0.2 MPa throughout the experiment. Note that this level of turgor pressure (P_{cell}) corresponds to leaf epidermal P_{cell} in the intact plant, and also corresponds to the target P_{cell} level achieved by the upward and downward turgor regulation mechanisms observed in **Figure 4.5 and 4.6**, and **Figure 4.7 and 4.8** respectively.

A control experiment was carried out to determine whether this turgor regulation phenomenon could also be observed in the open system environment. *Suaeda maritima* leaf slices were aerated in 500 mOsmoles kg^{-1} NaCl (0 mM NaCl plants, **Figure 4.11**) and 900 mOsmoles kg^{-1} NaCl (200 mM NaCl plants, **Figure 4.12**) for 15 minutes. After

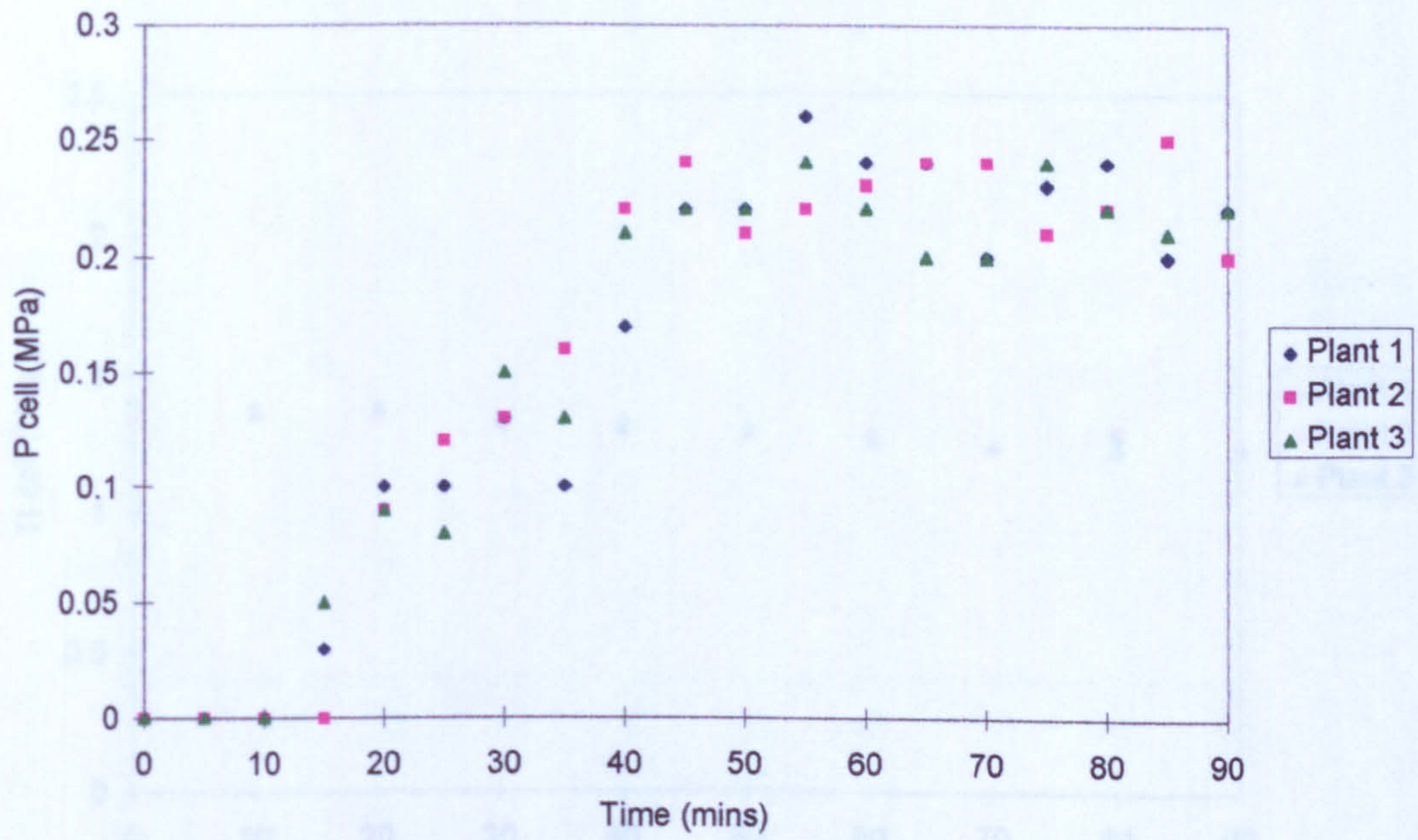
pre-treatment the leaf slices were not covered in silicone oil, but rather in a 5 ml aliquot of the bathing osmoticum. Turgor pressure (P_{cell}) measurements were taken at 5 minute intervals for 90 minutes. The osmotica induced a decrease in leaf epidermal turgor pressure. However, turgor pressure was not regulated upwards and was maintained at the initial level of between 0.075 MPa - 0.1 MPa for the duration of the experiment. This indicates that the volume of the apoplast is key to the turgor regulatory mechanism in *Suaeda maritima* leaf epidermal cells.

Returning to the closed system protocol, *Suaeda maritima* leaf slices were pre-treated in melibiose osmotica prior to being covered in water saturated silicone oil. Unlike NaCl, melibiose permeates the plasma membrane very slowly, and therefore, should not facilitate rapid turgor regulation. Leaf slices pre-treated in 500 mOsmoles kg^{-1} melibiose (0 mM NaCl plants, **Figure 4.13**) and 900 mOsmoles kg^{-1} melibiose (200 mM NaCl plants, **Figure 4.14**) had an initial leaf epidermal turgor pressure (P_{cell}) of 0.075 MPa to 0.1 MPa. This level of P_{cell} was maintained throughout the experiment; turgor pressure was not regulated upwards.

To ensure that the melibiose was not adversely affecting the leaf tissue, control osmotica were made up to a concentration which corresponded to the estimated concentration of solutes in the apoplast *in vivo*. Leaf slices pre-treated in 400 Osmoles kg^{-1} melibiose (0 mM NaCl plants, **Figure 4.15**) and 800 mOsmoles kg^{-1} melibiose (200 mM NaCl plants, **Figure 4.16**) maintained leaf epidermal turgor pressure (P_{cell}) at 0.2 MPa to 0.25 MPa, which is comparable to the to leaf epidermal P_{cell} in intact plants (see **Chapter 3, Figure 3.4**), and also corresponds to the target P_{cell} level achieved by the turgor regulation mechanisms observed in freshly cut (see **Figure 4.1 and 4.2**) and NaCl pre-treated leaf slices (see **Figure 4.5 and 4.6**, and **Figure 4.7 and 4.8**).

Figure 4.1 and Figure 4.2, Leaf epidermal turgor pressure (P_{cell}) in freshly sliced *Suaeda maritima* leaves under silicone oil (closed system)

Leaf epidermal turgor pressure (P_{cell}) in freshly cut, longitudinal slices of *Suaeda maritima* leaves under silicone oil. The leaves were excised from plants grown in 0 mM (**Figure 4.1**, upper figure) and 200 mM NaCl (**Figure 4.2**, lower figure) at day 14; measurements were made on leaf pair 1 and 2 only. Turgor pressure was measured at 5 minute intervals for 90 minutes. Each datum point represents a single turgor pressure measurement at the time indicated.



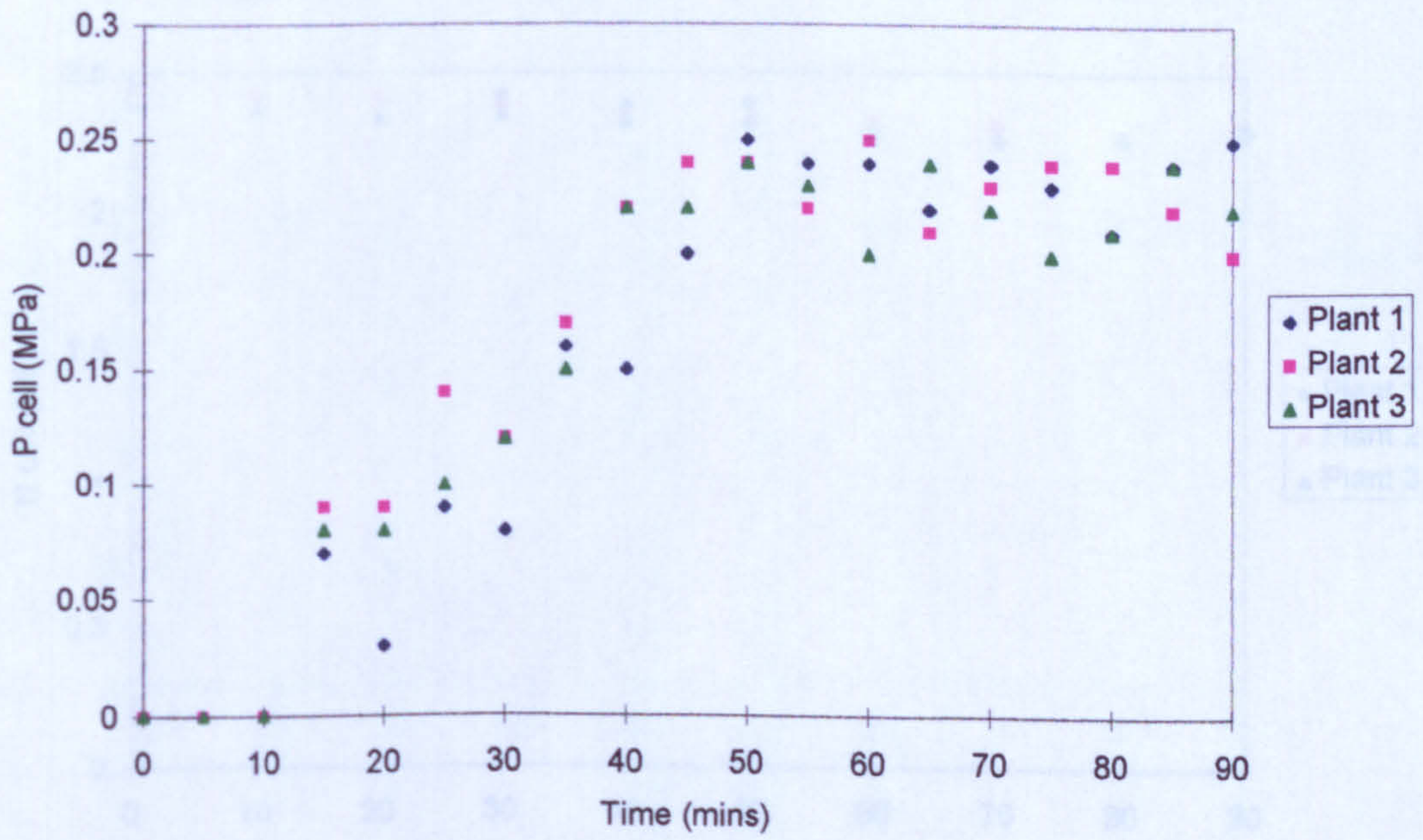
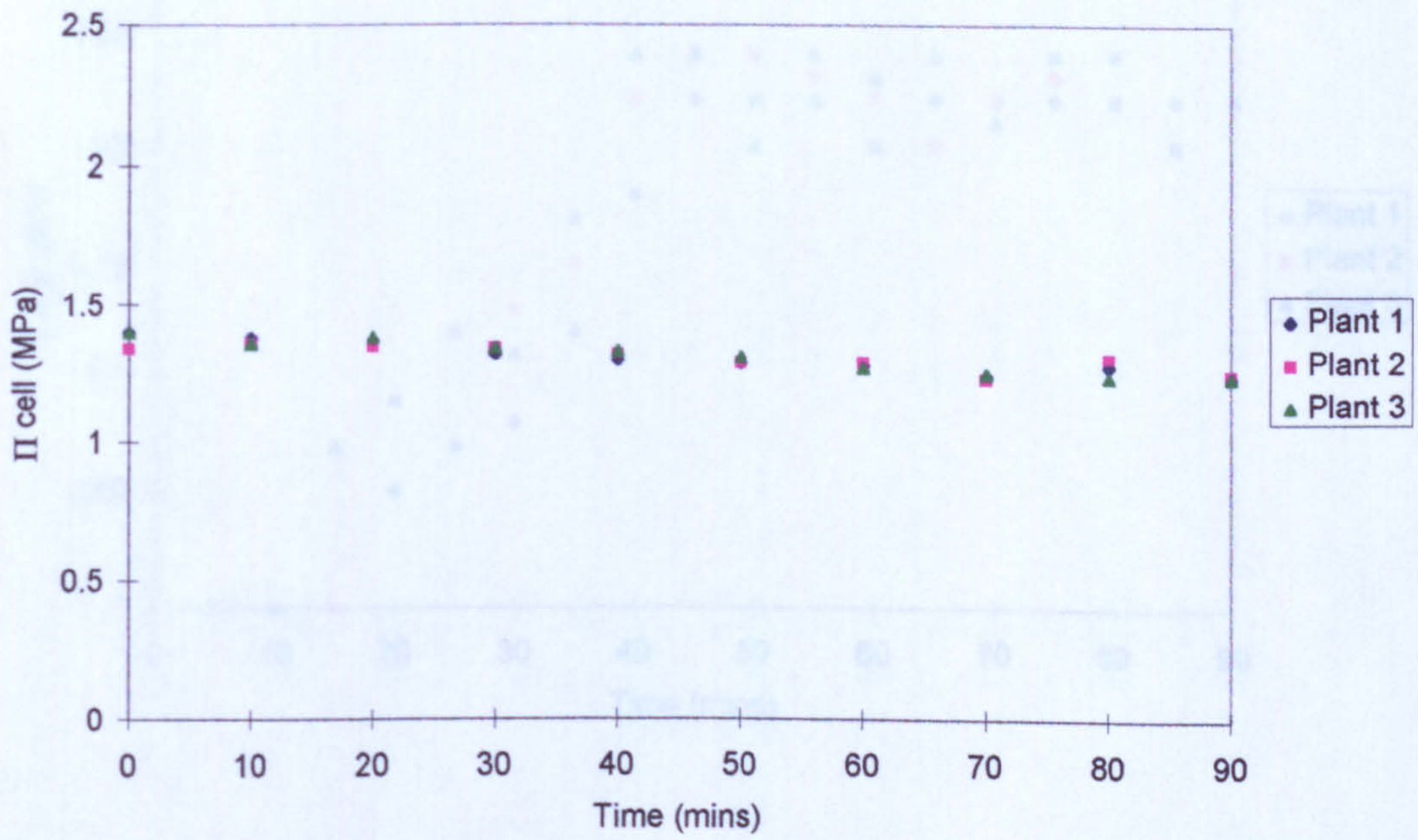


Figure 4.3 and Figure 4.4, Leaf epidermal osmotic pressure (Π_{cell}) in freshly sliced *Suaeda maritima* leaves under silicone oil (closed system)

Leaf epidermal osmotic pressure (Π_{cell}) in freshly cut, longitudinal slices of *Suaeda maritima* leaves under silicone oil. The leaves were excised from plants grown in 0 mM (**Figure 4.3**, upper figure) and 200 mM NaCl (**Figure 4.4**, lower figure) at day 14; measurements were made on leaf pair 1 and 2 only. Osmotic pressure was measured at 10 minute intervals for 90 minutes. Each datum point represents a single osmotic pressure measurement at the time indicated.



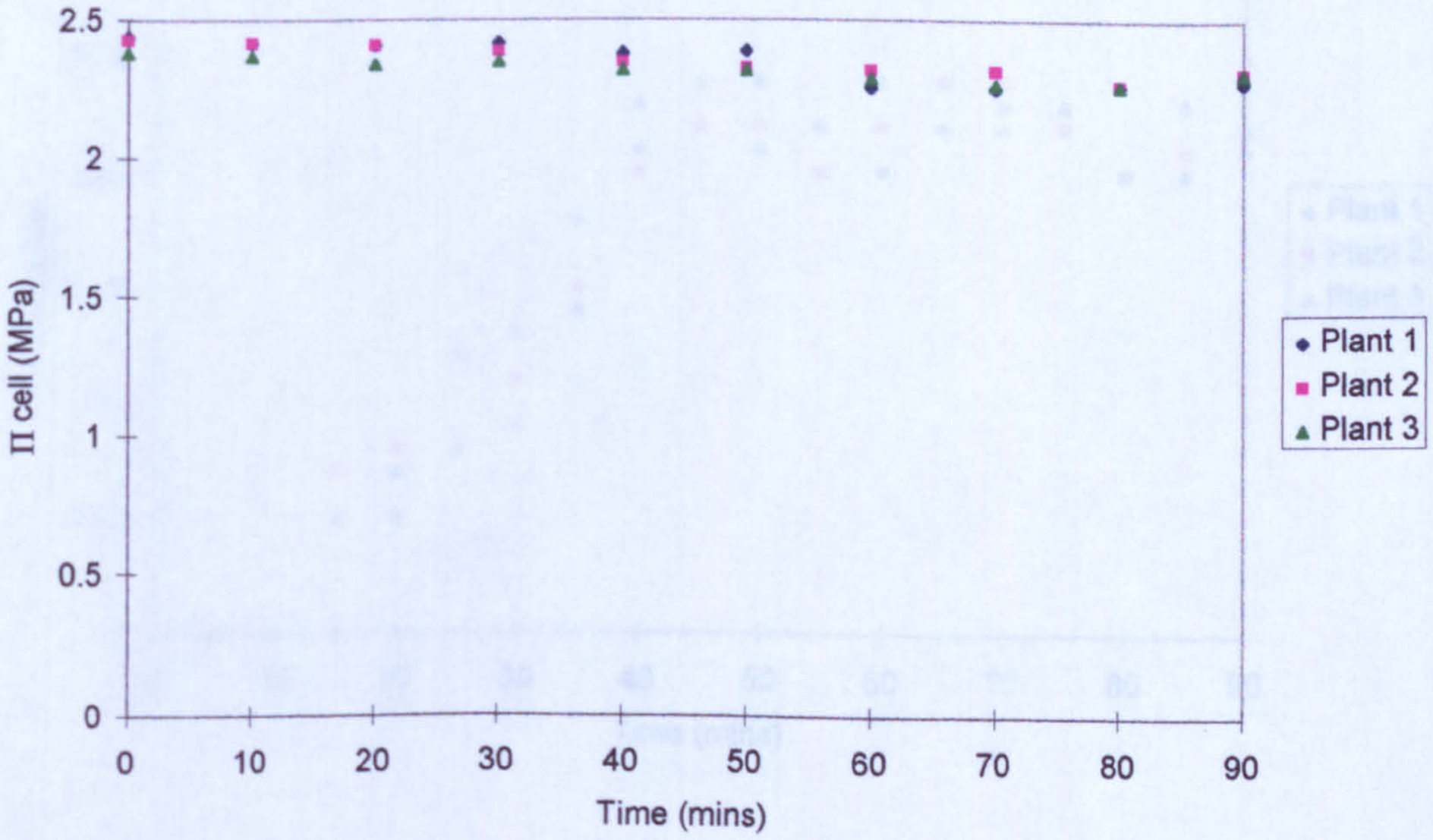
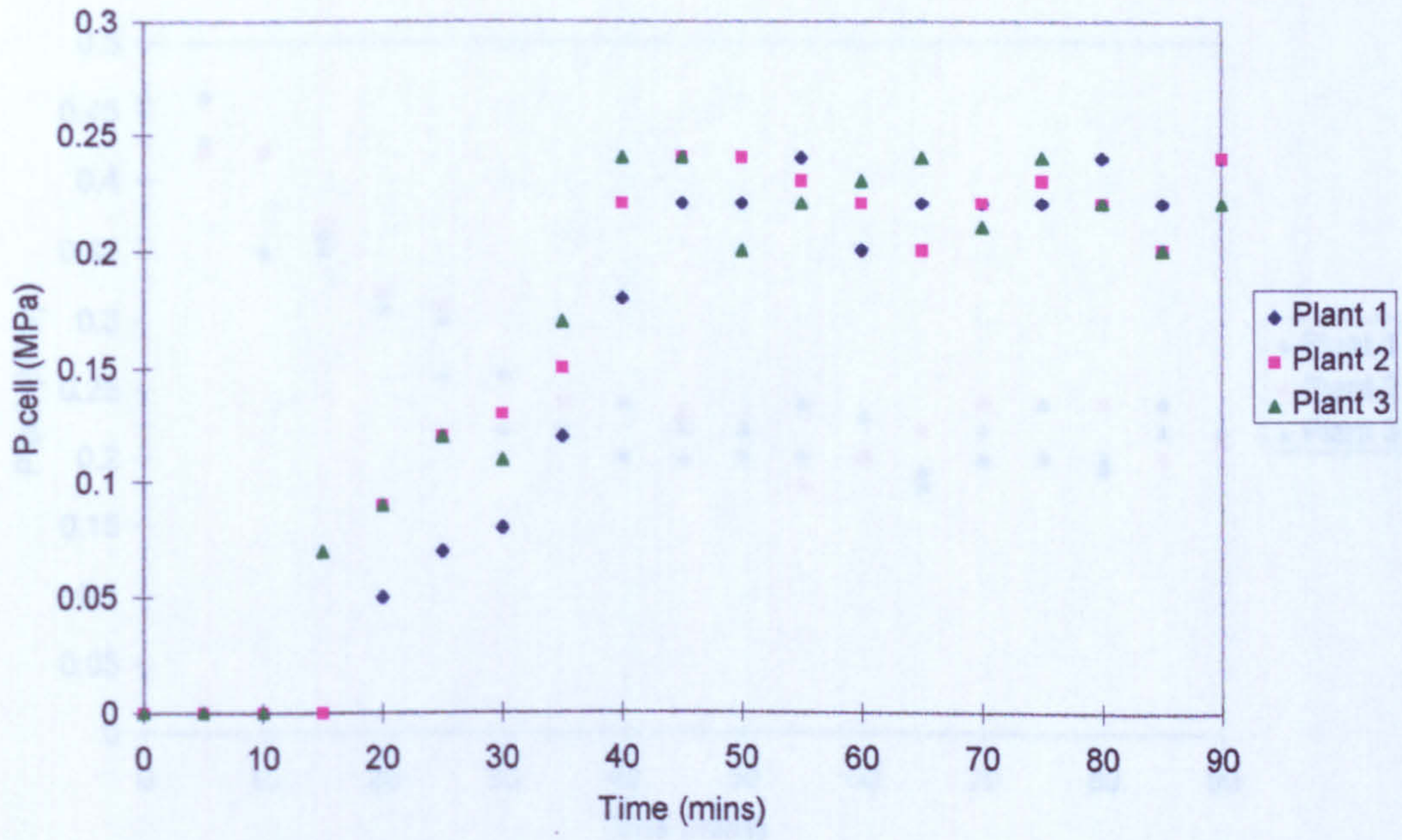


Figure 4.5 and Figure 4.6, Leaf epidermal turgor pressure (P_{cell}) in *Suaeda maritima* leaf slices pre-treated in 600 - 1000 mOsmoles kg $^{-1}$ NaCl (closed system)

Leaf epidermal turgor pressure (P_{cell}) in pre-treated *Suaeda maritima* leaf slices under silicone oil. Leaves excised from plants grown in 0 mM and 200 mM NaCl were aerated in 600 mOsmoles kg^{-1} (**Figure 4.5**, upper figure) and 1000 mOsmoles kg^{-1} NaCl (**Figure 4.6**, lower figure) respectively for 15 minutes, prior to being covered in silicone oil. Turgor pressure measurements were taken at 5 minute intervals for 90 minutes.



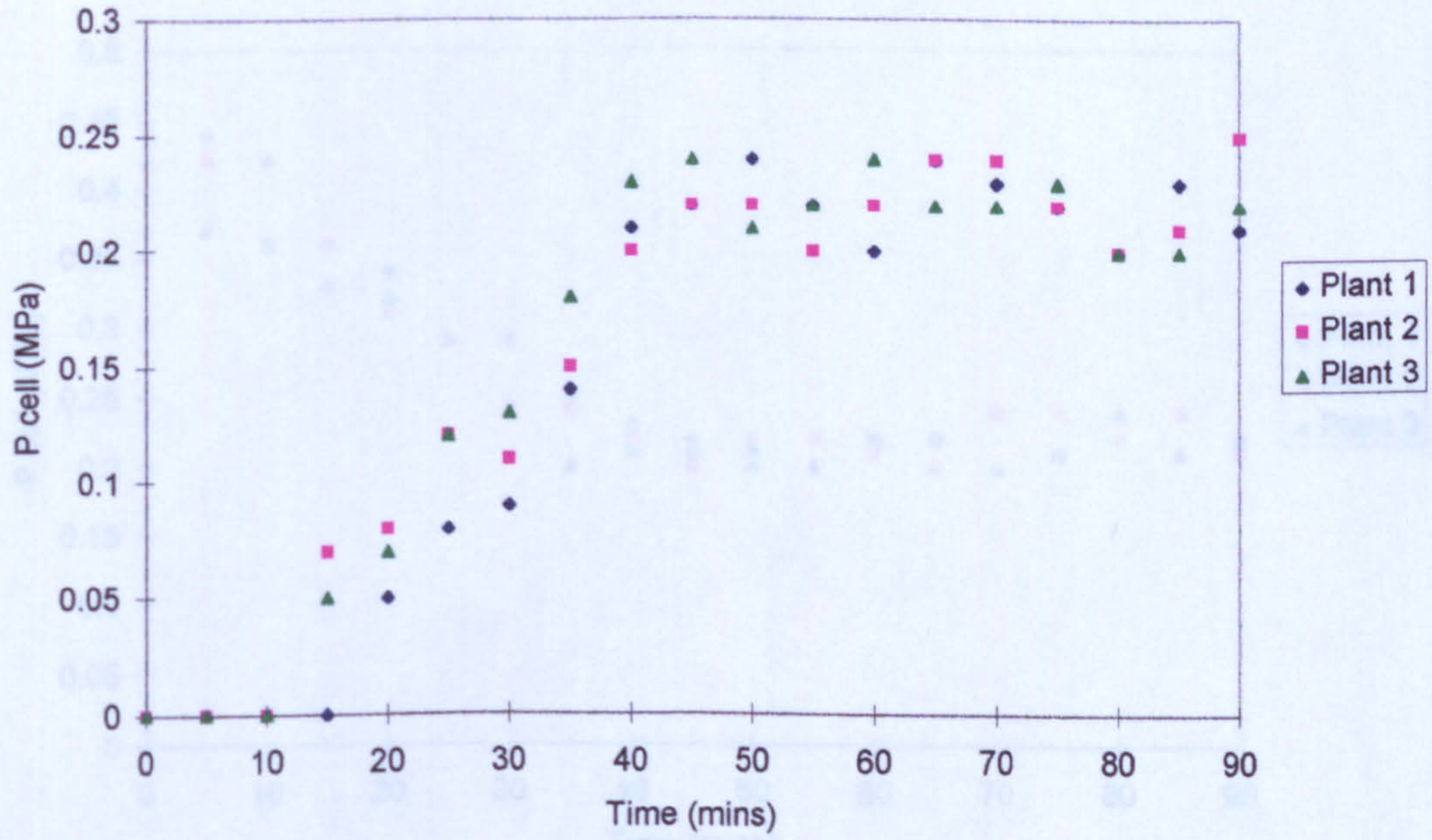
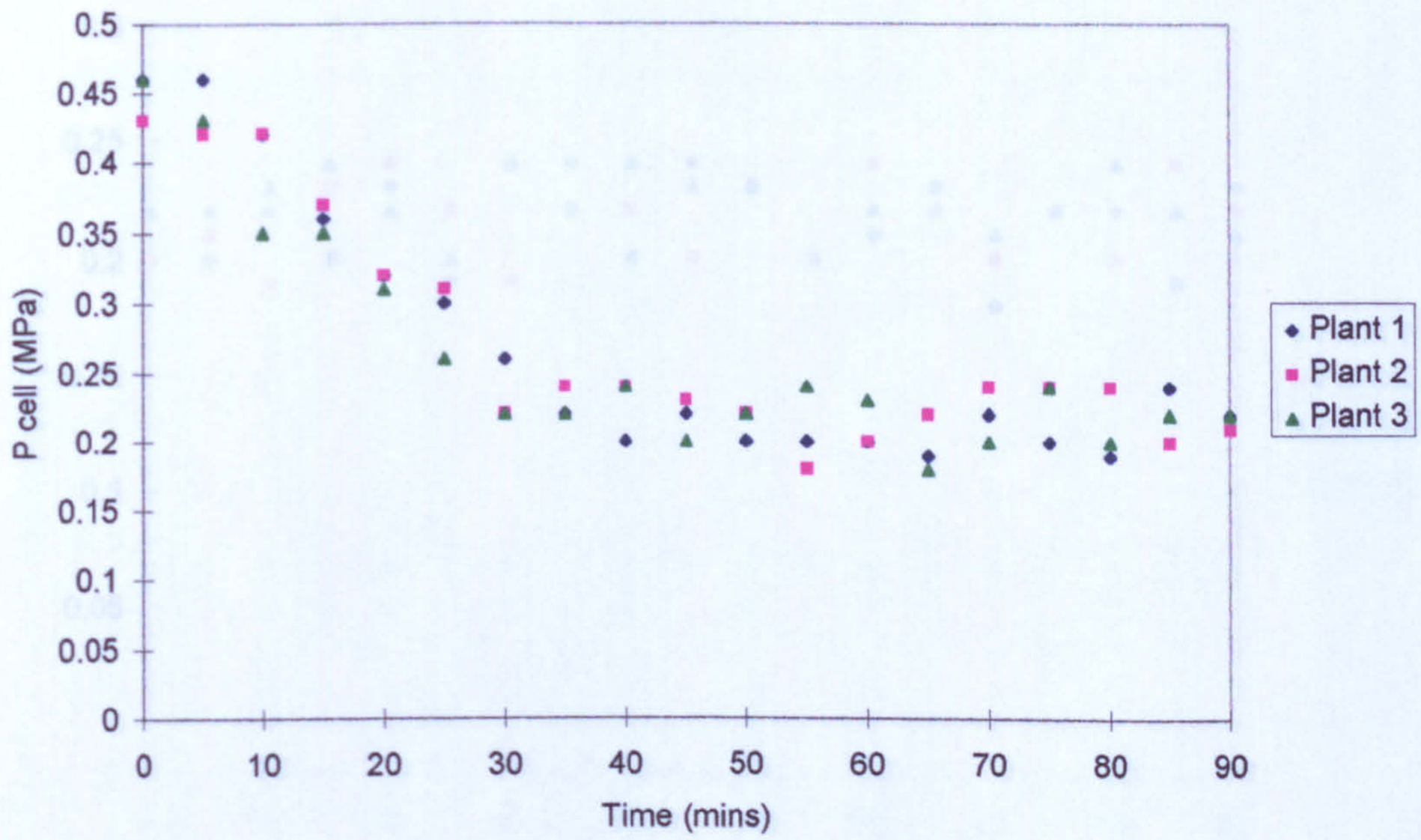


Figure 4.7 and Figure 4.8, Leaf epidermal turgor pressure (P_{cell}) in *Suaeda maritima* leaf slices pre-treated in 300 - 600 mOsmoles kg^{-1} NaCl (closed system)

Leaf epidermal turgor pressure (P_{cell}) in pre-treated *Suaeda maritima* leaf slices under silicone oil. Leaves excised from plants grown in 0 mM and 200 mM NaCl were aerated in 300 mOsmoles kg^{-1} (**Figure 4.7**, upper figure) and 600 mOsmoles kg^{-1} NaCl (**Figure 4.8**, lower figure) respectively for 15 minutes, prior to being covered in silicone oil. Turgor pressure measurements were taken every 5 minutes for a maximum of 90 minutes.



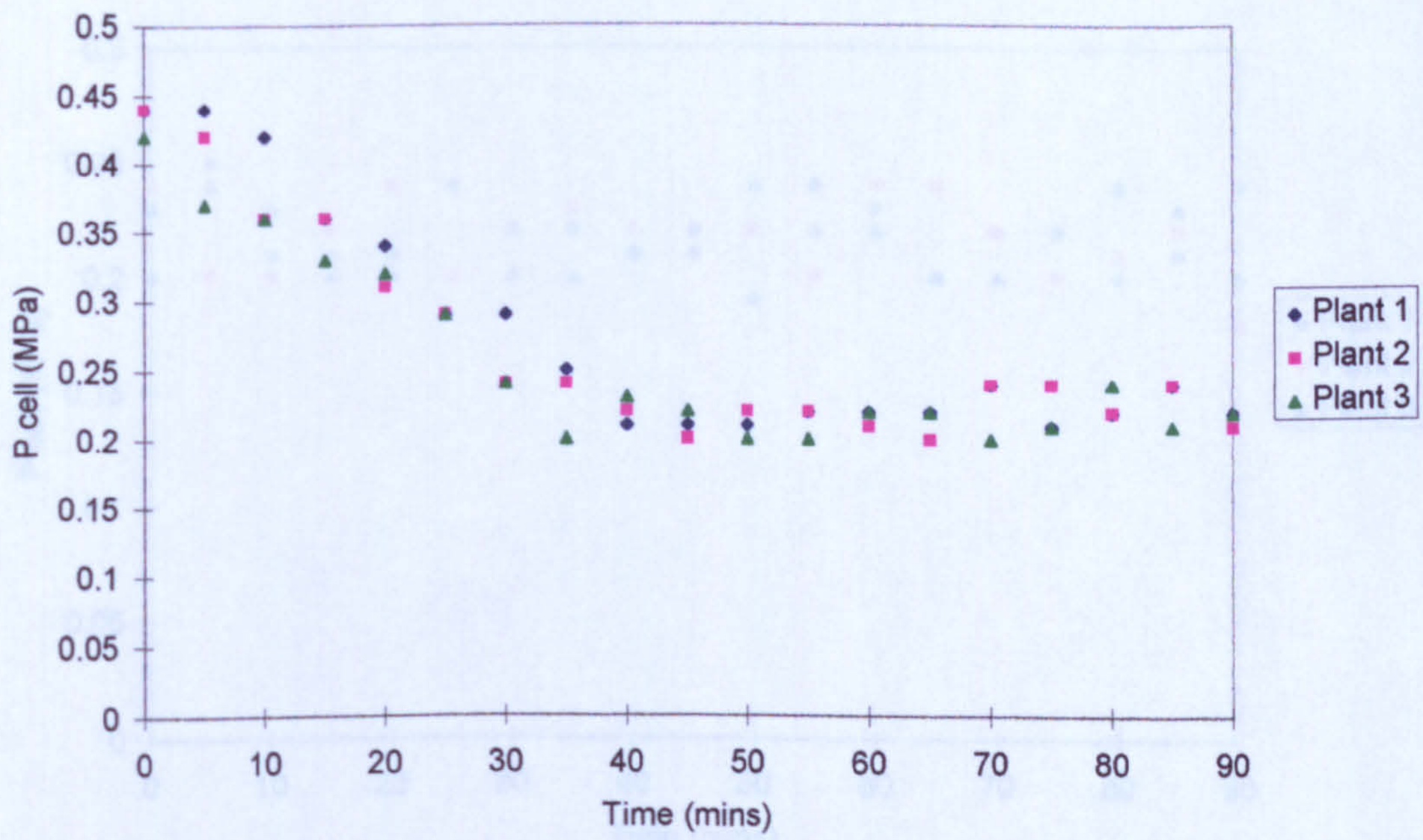
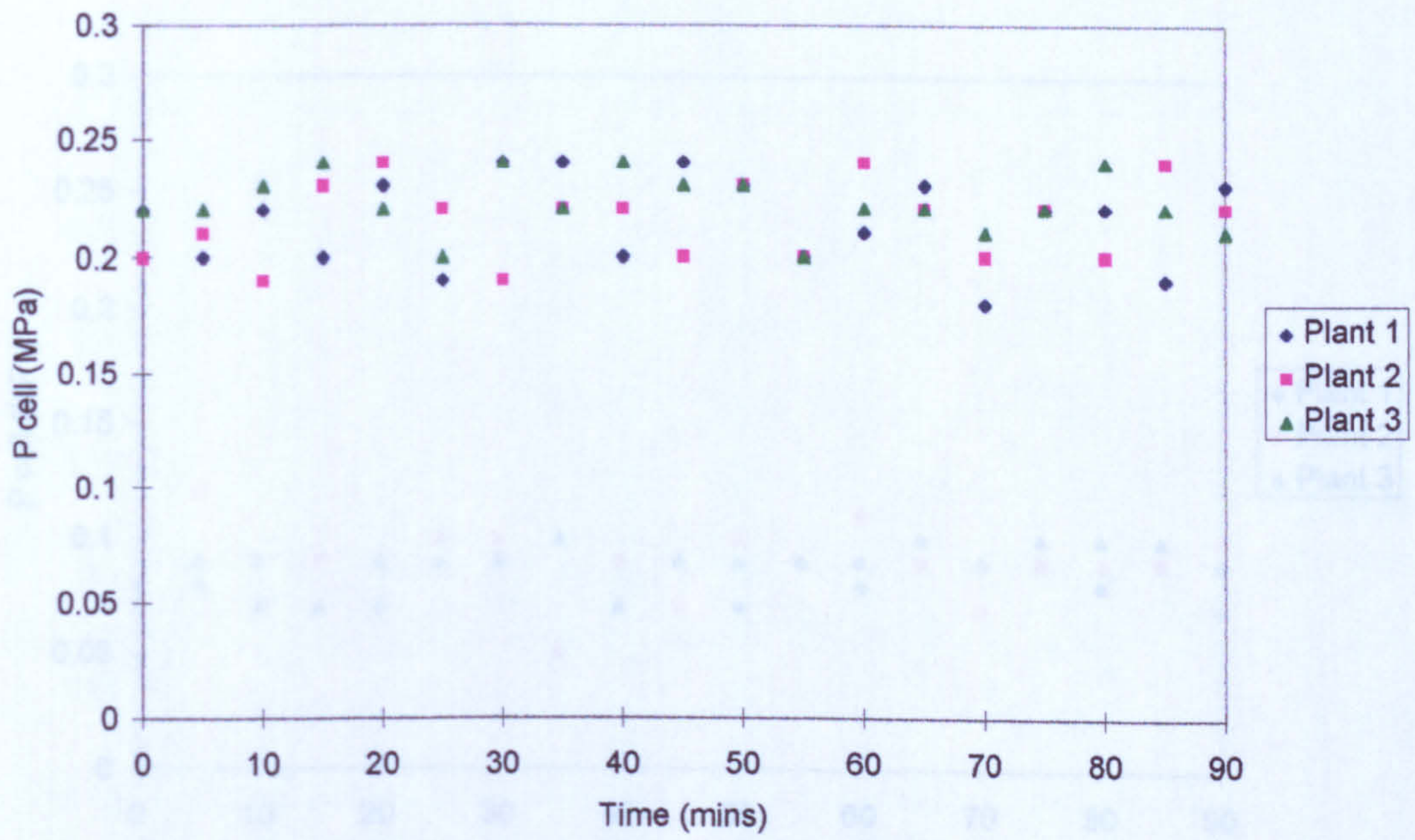


Figure 4.9 and Figure 4.10, Leaf epidermal turgor pressure (P_{cell}) in *Suaeda maritima* leaf slices pre-treated in 400 - 800 mOsmoles kg^{-1} NaCl (closed system)

Leaf epidermal turgor pressure (P_{cell}) in pre-treated *Suaeda maritima* leaf slices under silicone oil. Leaves excised from plants grown in 0 mM and 200 mM NaCl were aerated in 400 mOsmoles kg^{-1} (**Figure 4.9**, upper figure) and 800 mOsmoles kg^{-1} NaCl (**Figure 4.10**, lower figure) respectively for 15 minutes, prior to being covered in silicone oil. Turgor pressure measurements were taken every 5 minutes for 90 minutes.



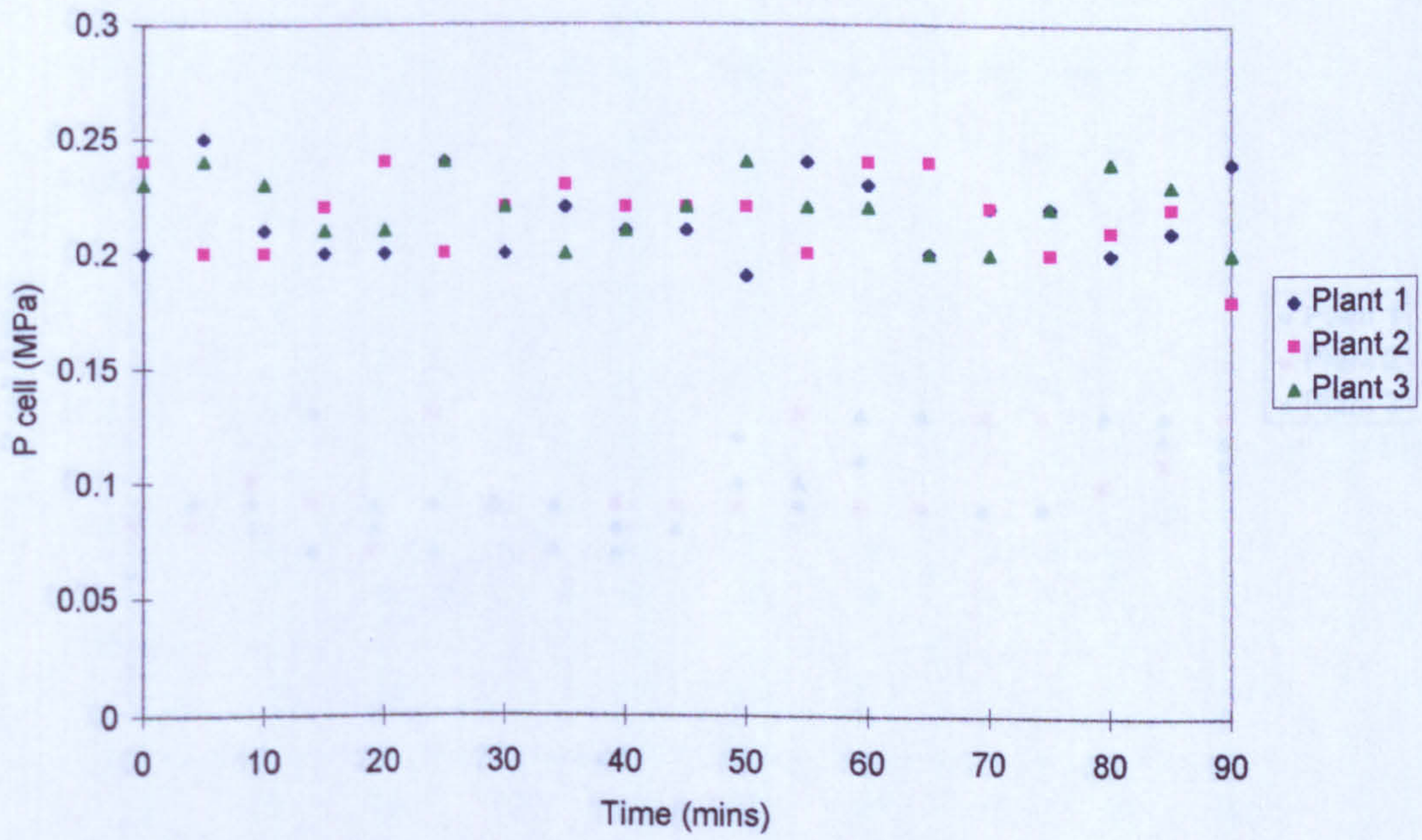
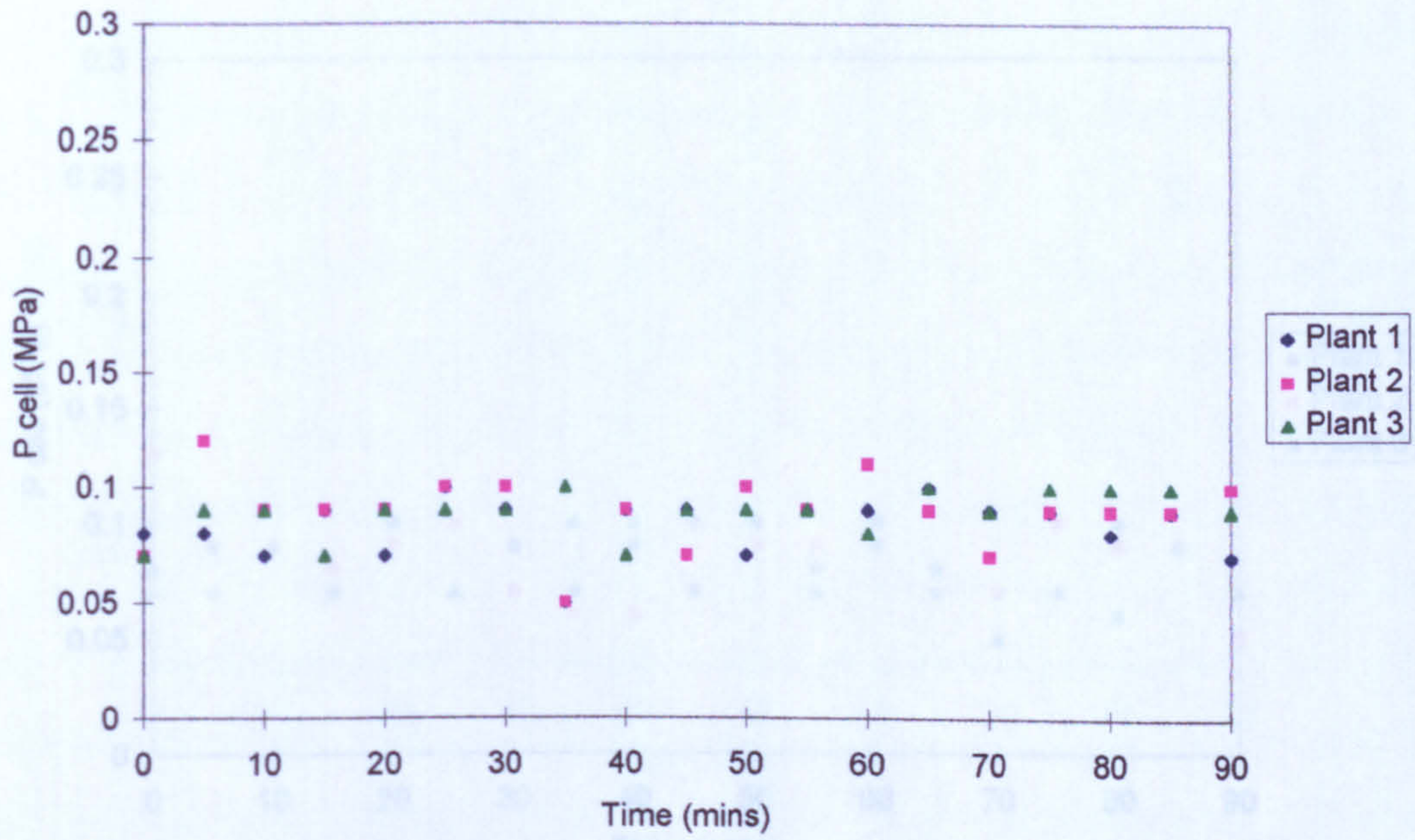


Figure 4.11 and Figure 4.12, Leaf epidermal turgor pressure (P_{cell}) in *Suaeda maritima* leaf slices bathed in 500 - 900 mOsmoles kg^{-1} NaCl (open system)

Leaf epidermal turgor pressure (P_{cell}) in *Suaeda maritima* leaf slices bathed in aqueous NaCl osmotica. Leaves excised from plants grown in 0 mM and 200 mM NaCl were bathed in 500 mOsmoles kg^{-1} (**Figure 4.11**, upper figure) and 900 mOsmoles kg^{-1} NaCl (**Figure 4.12**, lower figure) respectively. Turgor pressure measurements commenced after 15 minutes in the osmotica; P_{cell} was measured every 5 minutes for 90 minutes thereafter.



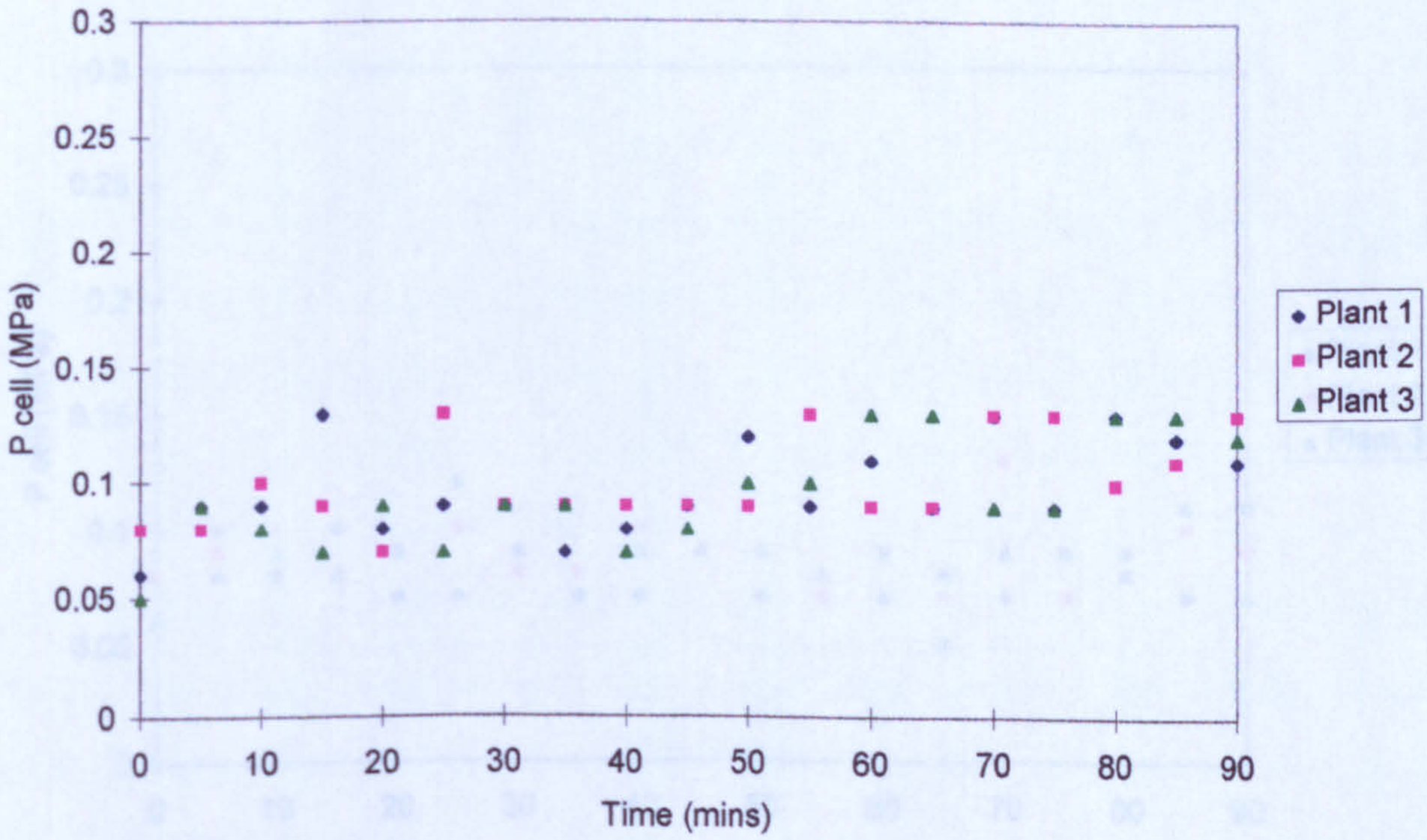
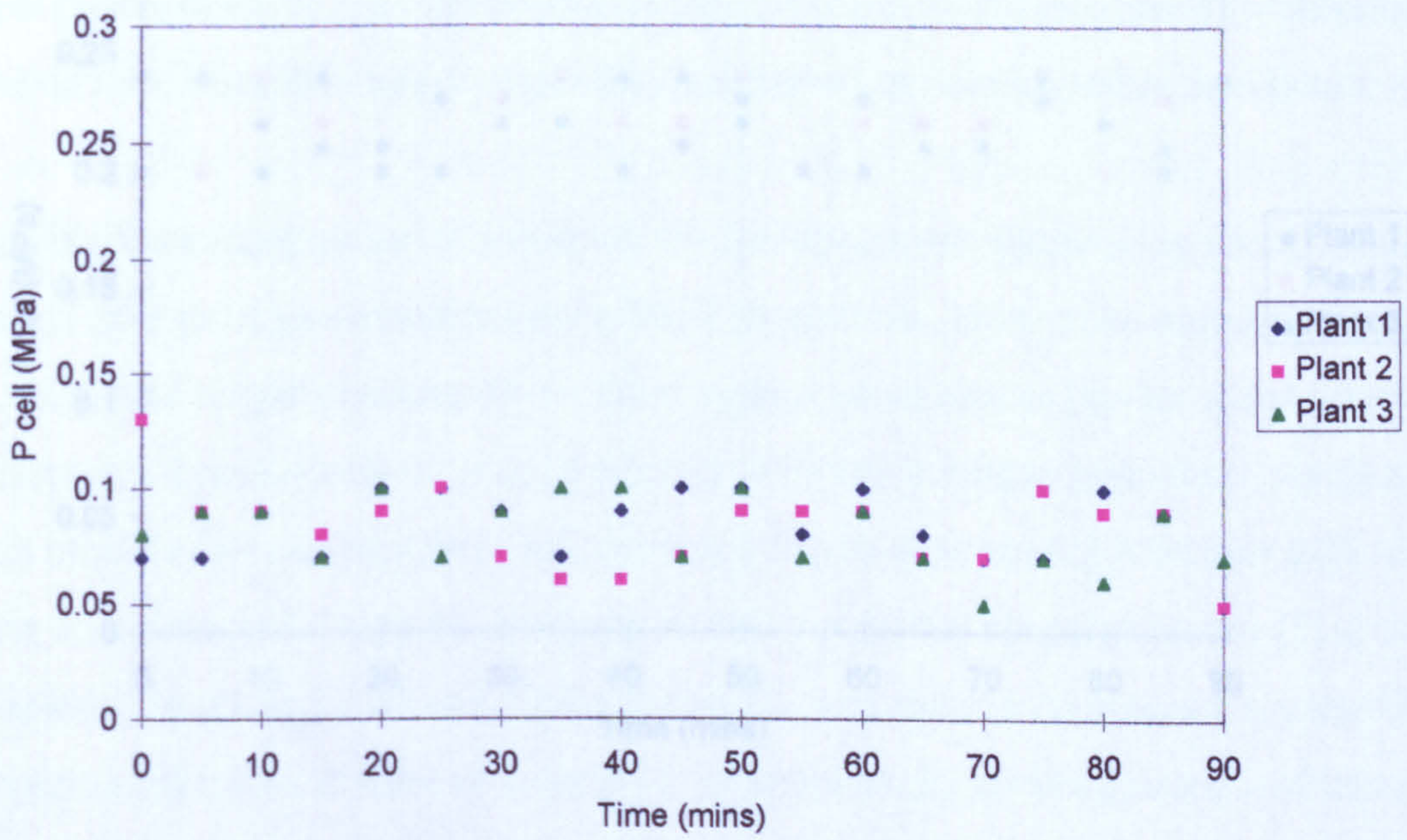


Figure 4.13 and Figure 4.14, Leaf epidermal turgor pressure (P_{cell}) in *Suaeda maritima* leaf slices pre-treated in 500 - 900 mOsmoles kg^{-1} melibiose (closed system)

Leaf epidermal turgor pressure (P_{cell}) in pre-treated *Suaeda maritima* leaf slices under silicone oil. Leaves excised from plants grown in 0 mM and 200 mM NaCl were aerated in 500 mOsmoles kg^{-1} (**Figure 4.13**, upper figure) and 900 mOsmoles kg^{-1} melibiose (**Figure 4.14**, lower figure) respectively for 15 minutes, prior to being covered in silicone oil. Turgor pressure measurements were taken every 5 minutes for 90 minutes.



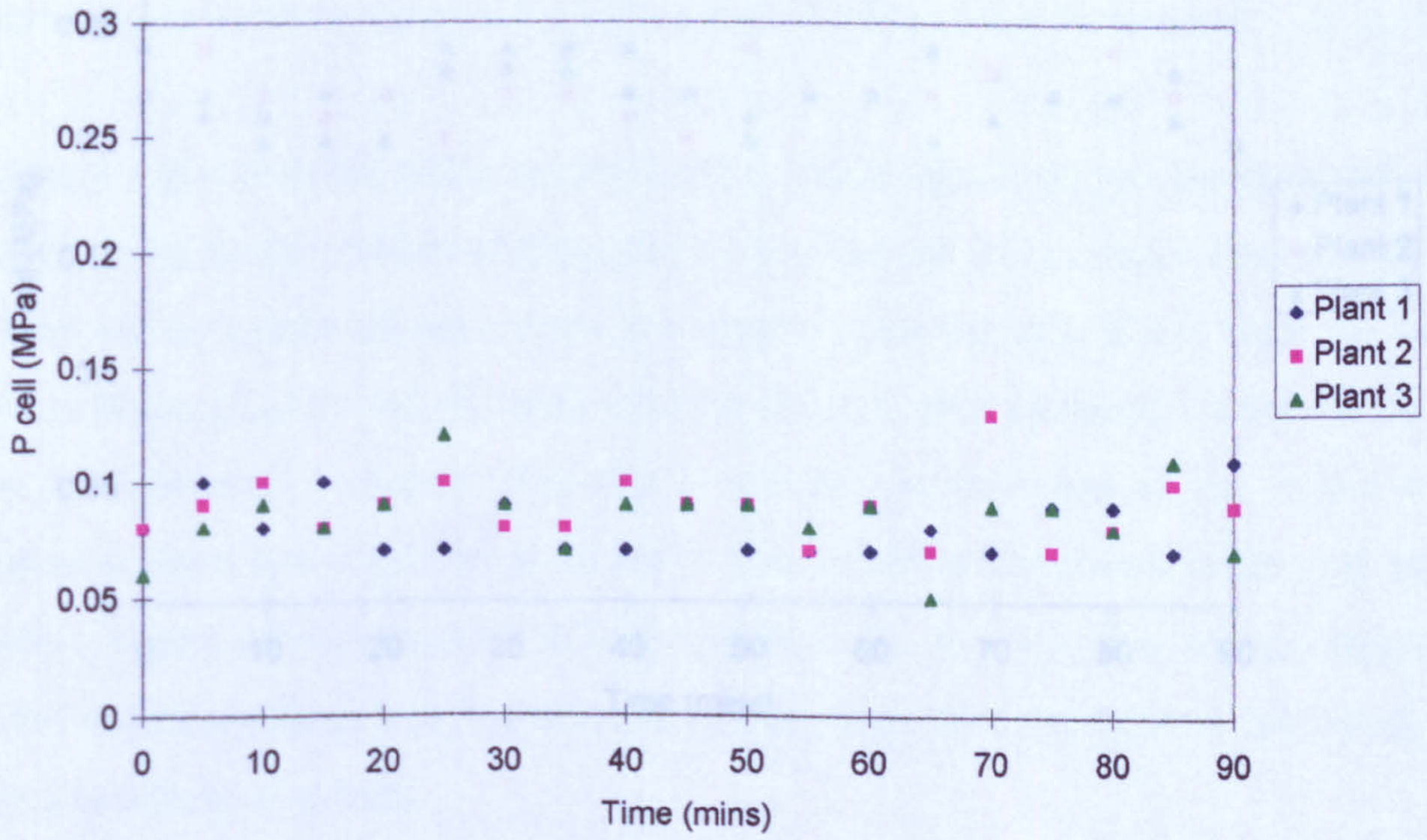
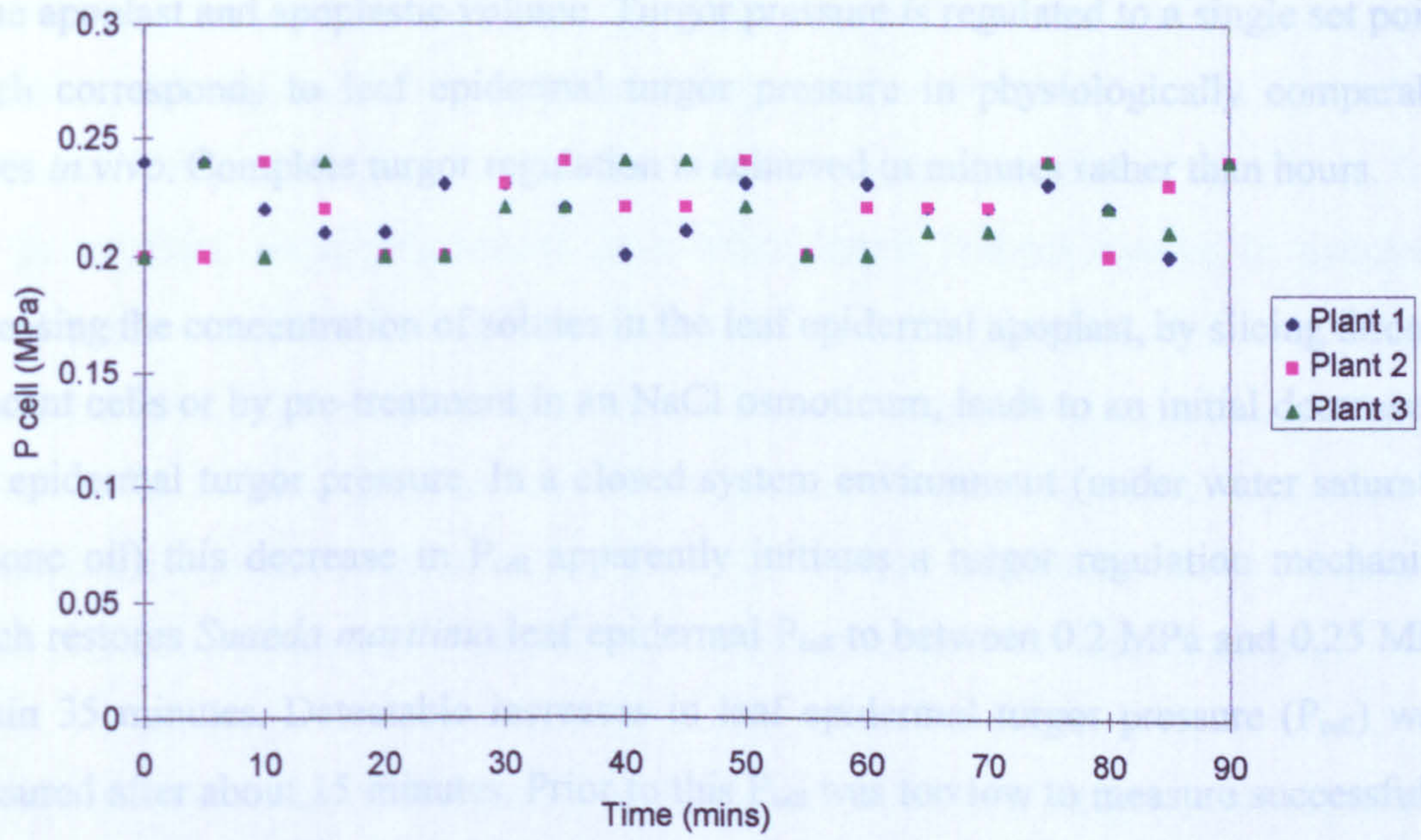
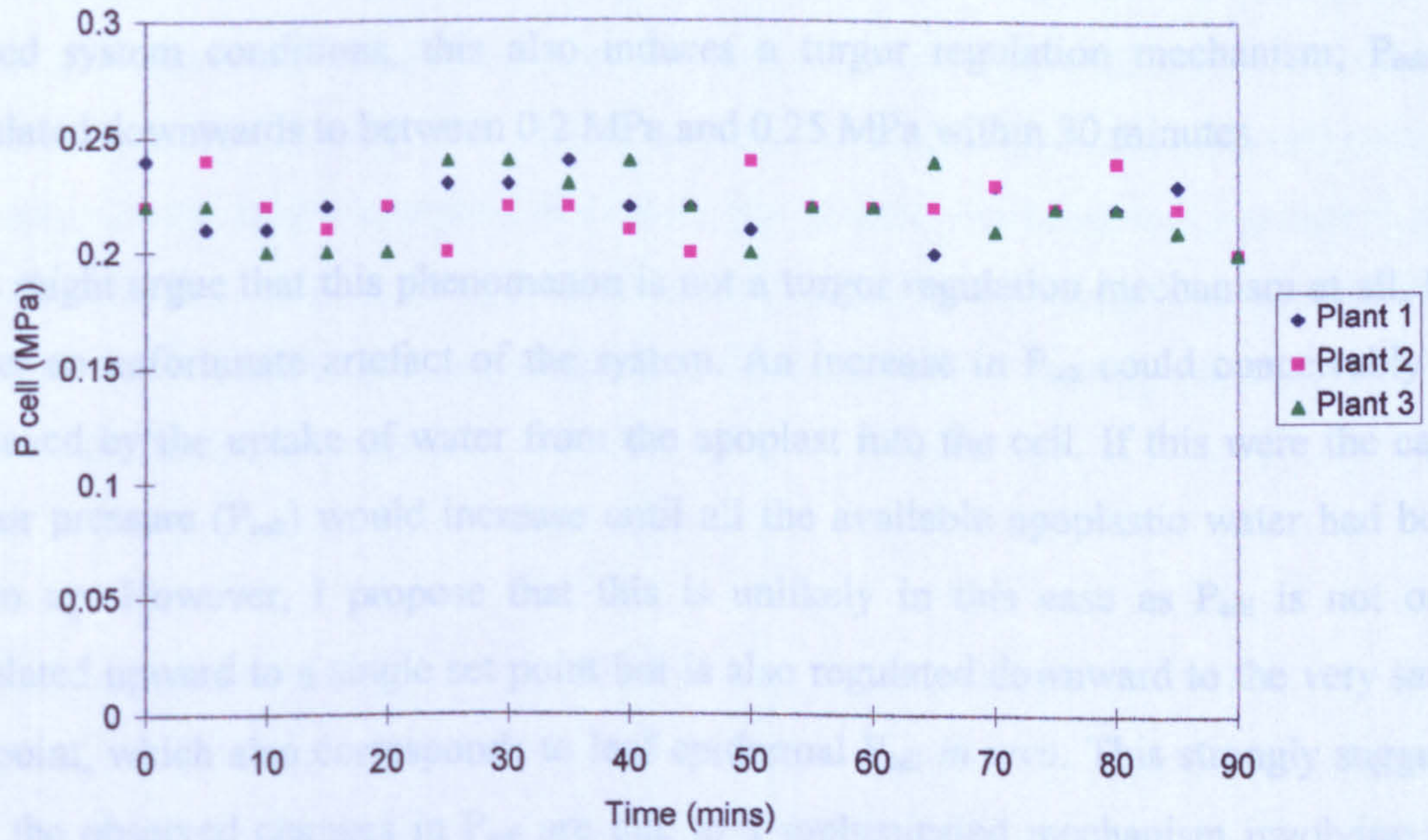


Figure 4.15 and Figure 4.16, Leaf epidermal turgor pressure (P_{cell}) in *Suaeda maritima* leaf slices pre-treated in 400 - 800 mOsmoles kg^{-1} melibiose (closed system)

Leaf epidermal turgor pressure (P_{cell}) in pre-treated *Suaeda maritima* leaf slices under silicone oil. Leaves excised from plants grown in 0 mM and 200 mM NaCl were aerated in 400 mOsmoles kg^{-1} (**Figure 4.15**, upper figure) and 800 mOsmoles kg^{-1} melibiose (**Figure 4.16**, lower figure) respectively for 15 minutes, prior to being covered in silicone oil. Turgor pressure measurements were taken every 5 minutes for 90 minutes.





4.4, Discussion

The data presented in this chapter demonstrates that *Suaeda maritima* leaf epidermal turgor pressure (P_{cell}) can be modulated by manipulating the solutes present in the adjacent apoplastic compartment. These changes in turgor pressure induce an osmotic adjustment mechanism leading to turgor regulation. The osmotic adjustment of solutes is apoplastic in nature, and strongly dependent on the permeability of the solutes present in the apoplast and apoplastic volume. Turgor pressure is regulated to a single set point, which corresponds to leaf epidermal turgor pressure in physiologically comparable leaves *in vivo*. Complete turgor regulation is achieved in minutes rather than hours.

Increasing the concentration of solutes in the leaf epidermal apoplast, by slicing through adjacent cells or by pre-treatment in an NaCl osmoticum, leads to an initial decrease in leaf epidermal turgor pressure. In a closed system environment (under water saturated silicone oil) this decrease in P_{cell} apparently initiates a turgor regulation mechanism which restores *Suaeda maritima* leaf epidermal P_{cell} to between 0.2 MPa and 0.25 MPa, within 35 minutes. Detectable increases in leaf epidermal turgor pressure (P_{cell}) were measured after about 15 minutes. Prior to this P_{cell} was too low to measure successfully. Decreasing the concentration of solutes in the apoplast, by aerating sliced leaf tissue in a dilute NaCl osmoticum, leads to an initial increase in cell turgor pressure (P_{cell}). Under closed system conditions, this also induces a turgor regulation mechanism; P_{cell} is regulated downwards to between 0.2 MPa and 0.25 MPa within 30 minutes.

One might argue that this phenomenon is not a turgor regulation mechanism at all, but rather an unfortunate artefact of the system. An increase in P_{cell} could conceivably be achieved by the uptake of water from the apoplast into the cell. If this were the case, turgor pressure (P_{cell}) would increase until all the available apoplastic water had been taken up. However, I propose that this is unlikely in this case as P_{cell} is not only regulated upward to a single set point but is also regulated downward to the very same set point, which also corresponds to leaf epidermal P_{cell} *in vivo*. This strongly suggests that the observed changes in P_{cell} are due to a sophisticated mechanism involving the active adjustment of solutes.

The short time scale in which turgor regulation is achieved in *Suaeda maritima* leaf epidermal cells in this case, suggests that the observed regulation mechanism is not due to osmotic adjustment of solutes in the protoplast; classical osmotic adjustment of the protoplast in marine algae and higher plants can often take up to 24 hours (Cram, 1976; see also **Chapter 3**). To test this hypothesis leaf epidermal cell osmotic pressure (Π_{cell}) measurements were taken during turgor regulation. The data obtained demonstrated that the concentration of solutes in the protoplast remained unchanged throughout. This leads to the conclusion that osmotic adjustment of solutes, resulting in turgor regulation, takes place in the apoplast rather than the protoplast in this case. Cram (1976 and 1980) pointed out that the flux of solutes across the plasma membrane, from cell to apoplast, invariably results in a much larger relative change in apoplastic osmotic pressure (Π_{wall}) than in that of the protoplast (Π_{cell}). This is due to very much smaller relative volume of the wall. Since turgor pressure (P_{cell}) is governed by the difference in osmotic pressure across the plasma membrane, active adjustment of solutes in the apoplast could provide a plant with a rapid and metabolically inexpensive turgor regulation mechanism that does not require protoplastic solute adjustment.

Tomos and Wyn Jones (1988) published data which pointed to an apoplastic adjustment mechanism in red beet taproot tissue. Freshly cut red beet taproot discs bathed in water saturated silicone oil had an initial turgor pressure of just below 0.2 MPa. Increases in turgor pressure were observed almost immediately, reaching a plateau after about 2 hours at between 0.2 - 0.3 MPa. There was no corresponding increase in cell osmotic pressure. These data correspond well with the *Suaeda maritima* leaf epidermal data presented in this chapter. Similar experiments carried out on sugar beet taproot discs provide data which further supports these findings. These data are discussed in the following chapter (**Chapter 5**).

4.5, Summary and conclusions

- Modulating the solute concentration in the apoplast of *Suaeda maritima* leaf epidermal cells, by slicing the leaf open longitudinally or by pre-treating leaf slices in an osmoticum, leads to changes in leaf epidermal cell turgor pressure (P_{cell}).

- Changes in P_{cell} initiate a turgor regulation mechanism which is strongly dependent on apoplastic volume and solute permeability.

- Providing the apoplast is closed to the external environment by a protective layer of water saturated silicone oil (a closed system which attempts to achieve a similar apoplastic environment and volume to that found *in vivo*), upward and downward turgor regulation is achieved within 30 - 40 minutes.

- Turgor pressure is regulated to a single set point (around 0.2 MPa) which corresponds to leaf epidermal P_{cell} in comparable intact leaves that have not been excised from the plant.

- Upward and downward turgor regulation is not accompanied by an equivalent change in cell osmotic pressure (Π_{cell}). This leads to the conclusion that osmotic adjustment leading to turgor regulation is apoplastic in nature rather than protoplastic. This provides clear evidence that apoplastic solutes are readily utilised by *Suaeda maritima* leaf epidermal cells to maintain leaf epidermal P_{cell} at a level predetermined by the plant. By utilising the apoplastic space as an osmotic compartment *Suaeda maritima* can achieve turgor regulation quickly, while minimising metabolic expenditure.

- The observed turgor regulation mechanism is an excellent example of a turgor-dependent mechanism, in which a change in P_{cell} is detected and countered by modulation of membrane transport mechanisms. The nature of the

turgor-sensing mechanism, and subsequent signal transduction pathway, which leads to membrane transport remains unclear.

Chapter 5: Turgor regulation in an open and a closed system in *Beta vulgaris* L. (sugar beet) taproot tissue

5.1, Introduction

Sugar beet *(Beta vulgaris* L.) belongs to the same family as *Suaeda maritima* - the family *Chenopodiaceae*. In common with *Suaeda maritima* leaf epidermal cells (see **Chapter 3**), sugar beet taproot parenchyma cells accumulate a high concentration of solutes in the protoplast (namely sucrose in this case), while maintaining a constant turgor pressure (Tomos *et al.*, 1992). The osmotic pressure (Π_{cell}) of a sugar beet taproot increases from 0.7 MPa to 2.0 MPa as sucrose accumulates in the parenchyma cells during maturation. Turgor pressure (P_{cell}), however, is maintained between 0.6 MPa and 0.7 MPa throughout the growing season (Tomos *et al.*, 1992).

As I pointed out in Chapter 3, assuming a reflection coefficient of 1, the turgor pressure of a cell is determined by the osmotic pressure of the cell (Π_{cell}), the osmotic pressure of the adjacent apoplast/cell wall (Π_{wall}) and by the hydrostatic component of the apoplast - the transpiration tension (P_{wall}). Therefore the difference between P_{cell} and Π_{cell} in sugar beet taproot parenchyma cells can only be attributed to properties of the apoplast. Tomos *et al.* (1992) measured turgor pressure in intact, mature taproots under transpiring (during the day) and non-transpiring (at night when the stomata are assumed to be largely closed and transpiration minimal) conditions. These data revealed that turgor pressure in non transpiring beet plants was approximately 0.1 MPa greater than in equivalent transpiring plants. This increase of 0.1 MPa accounts for only 7 % of the difference between P_{cell} and Π_{cell}. Therefore, it is reasonable to conclude that *Beta vulgaris* storage parenchyma cells utilise apoplastic solutes to maintain P_{cell}. I propose that this turgor maintenance mechanism is directly comparable to that found in *Suaeda maritima* leaf epidermal cells (**Chapter 3** and **4**).

This chapter describes experiments carried out on excised sugar beet taproot tissue. The cell pressure probe and picolitre osmometry (see **Chapter 2, Section 2.2** and **2.5**) were used to measure turgor regulation and osmotic adjustment in beet discs in an aqueous **open system** and a **closed system** (see also **Chapter 4**).

In the open system, beet discs are vigorously aerated in a range of mannitol osmotica over a number of days. This treatment is often referred to as ageing and is known to have a profound effect on the physiology and biochemistry of storage tissue; most notably an increase in protein synthesis and respiration, followed by an increase in solute uptake (for reviews see Van Steveninck, 1975; Poole, 1976). The apoplast of beet tissue in an open system is presumably completely open to the well stirred external medium (hence the term **open system** - see **Figure 5.1**), and therefore effectively occupies the same volume as the bathing osmoticum. Osmotic adjustment leading to turgor regulation is likely to be confined to the protoplast due to the artificially large volume of the apoplast.

The closed system (introduced in **Chapter 4**) attempts to mimic the apoplastic environment *in vivo* (Tomos and Wyn Jones, 1988). Sugar beet taproot discs (freshly cut or pre-treated in an osmoticum) are covered in water saturated silicone oil, which closes the apoplast and prevents tissue dehydration (hence the term **closed system** - see **Figure 5.2**). Under these conditions the apoplast occupies only a small percentage of the total tissue volume, which is comparable to the situation in the intact taproot (see Pitman, 1963; Leigh and Tomos 1983). Given the small volume of apoplast relative to protoplast, and assuming that silicone oil acts only as an inert barrier that cannot take part in the exchange of solutes or water (in either direction), the cell should be able to control and modulate the solutes in the apoplastic compartment by means of membrane transport. The closed system ensures that the protoplast and apoplast are available for osmotic adjustment purposes, and thus provides an excellent model system from which to study turgor regulation.

Figure 5.1, The open system

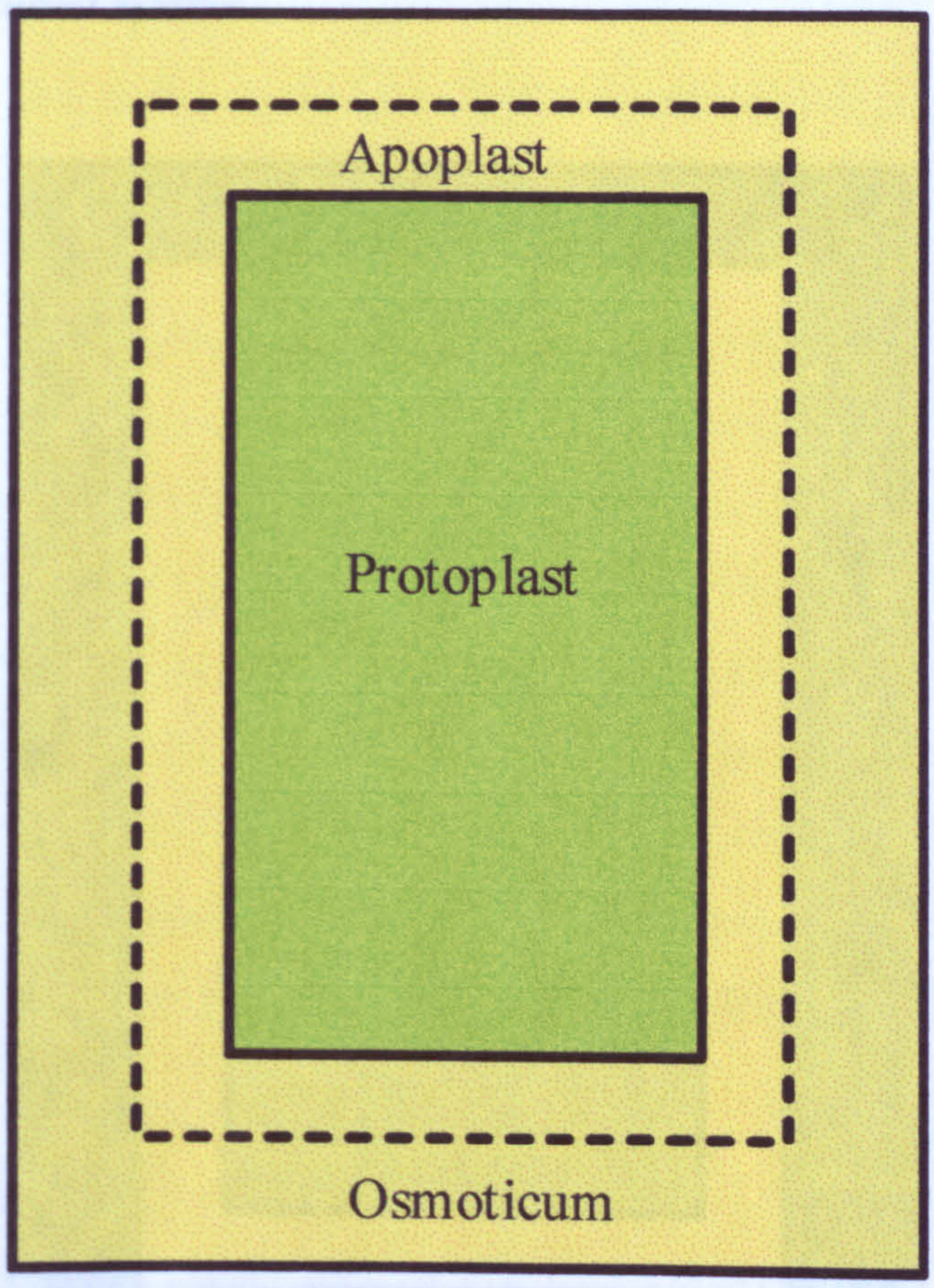
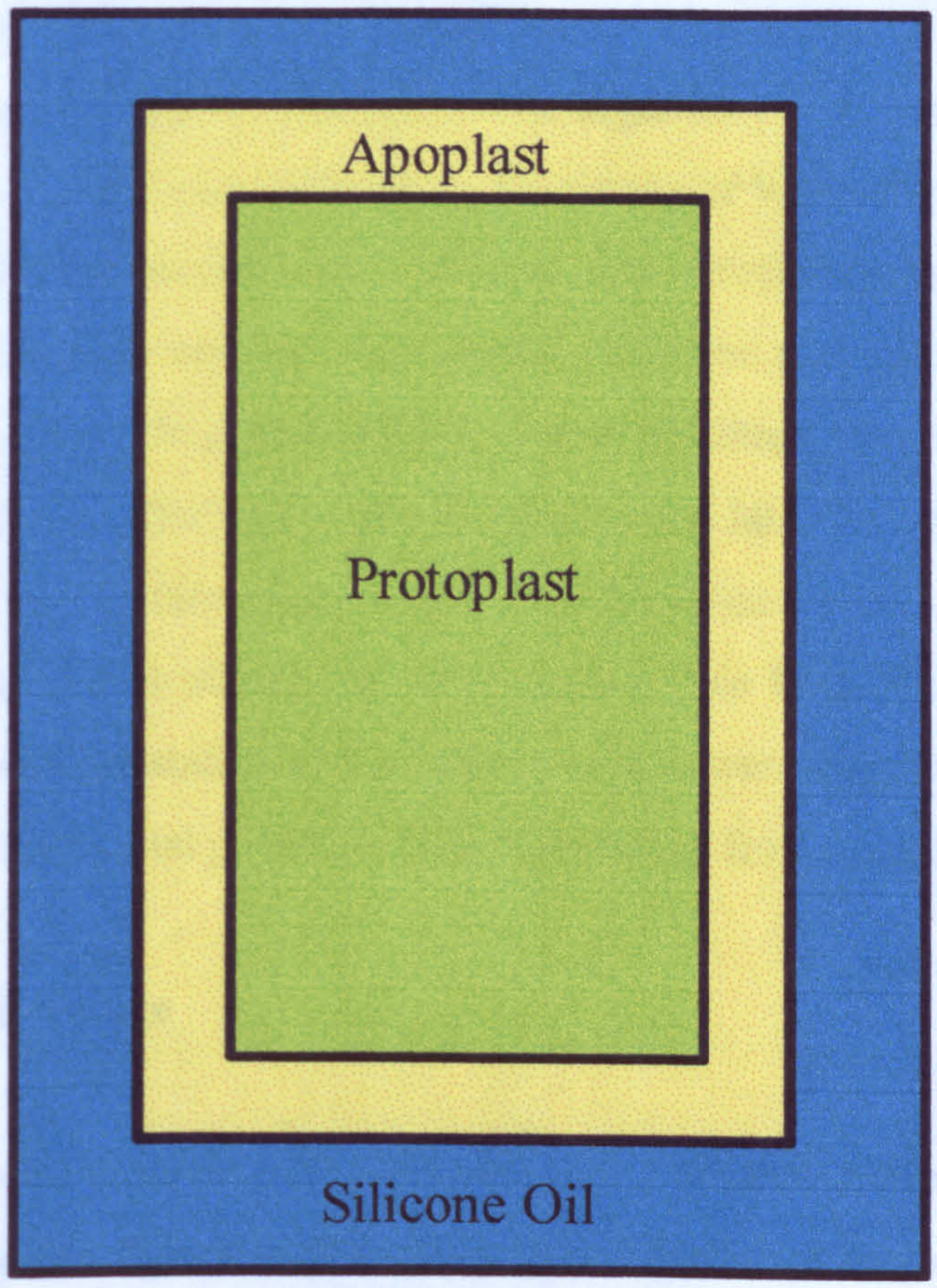


Figure 5.2, The closed system



5.2, Method

5.2.1, Germination and growth conditions

Sugar beet seeds (Gala variety) were a kind gift from Michael Coy at the English Sugar Beet Company Ltd, Lincolnshire. The seeds were in pellet form (monogerm), coated with a fungicide and insecticide treatment. The sugar beet were germinated out in the open, on the roof of the Memorial Building, University of Wales Bangor, in March. Six seeds were placed in 4 cm deep wells in small pots containing John Innes Number 1 compost. The seeds were covered with compost and the pots were watered to field capacity daily. As the seeds germinated the weaker seedlings were removed until only one seedling remained. After two weeks the plants were large enough to be transferred into larger 8 litre pots. At this stage the beet were fed twice a week using a commercial fertiliser (Phostrogen) and watered to field capacity (see **Plate 5.1)** every other day. Treatments for aphid infestation and powdery mildew were applied as required using widely available commercial products (Zeneca) obtained from a local garden centre.

5.2.2, Harvest and storage

The sugar beet were harvested after 6 months (September/October). All leaves were carefully cut away and discarded. The taproots were washed in cold water and dried thoroughly with tissue paper. After drying, the taproots were placed in plastic sacks (ten taproots per sack) between layers of dry, protective compost, and stored at 5 °C in a cold room. The sugar beet taproots were used within 8 weeks of harvest; after 8 weeks all remaining beet were discarded. During a sugar beet shortage (due to a poor harvest and cold room malfunction) Dr David Wright, School of Agriculture and Forest Sciences at the University of Wales Bangor, very kindly arranged delivery of freshly harvested beet taproots (Zulu variety) from a British Sugar beet farm in Shropshire. These taproots were cleaned and stored as described above.

5.2.3, Beet disc preparation

Suitable sugar beet taproots (intact and healthy) were selected from storage, washed and dried. The taproots were cut into three thick transverse slices using a sharp kitchen knife. The upper slice or crown was discarded. A sharp 7 mm diameter cork borer was used to cut cores of storage parenchyma tissue from the lower root slices. Each core of tissue was immediately placed in 300 mM mannitol/0.5 mM $CaSO_4$ (BDH) solution in order to prevent dehydration and loss of cell solutes. Once an adequate number of cores had been obtained a sharp double edged razor blade was used to cut 1 mm thick discs. The discs were rinsed in 300 mM mannitol/0.5 mM $CaSO_4$ before being drained and transferred to the experimental osmotica in 5 litre round bottomed flasks. Approximately 200 discs were placed in each flask. All sugar beet discs were freshly cut and used immediately (after Wyse *et al.*, 1986; Perry *et al.*, 1987).

5.2.4, The open system

Sugar beet taproot discs were aged for 7 days in 5 litre round bottomed flasks containing 0 - 600 mOsmoles kg^{-1} mannitol and 0.5 mM $CaSO_4$. Each flask was aerated via tubing connected to a compressed air supply. Care was taken to ensure that the beet discs circulated freely and did not settle at the bottom of the flasks. The bathing osmotica were changed every 12 hours to minimise the growth of bacteria.

Discs were sampled at the times indicated. Individual discs were secured to a perspex sample holder and covered in a small volume of osmoticum (5 - 10 ml). Turgor pressure (P_{cell}) was measured using the cell pressure probe (see **Chapter 2, Section 2.2**) and osmotic pressure (Π_{cell}) was measured using the picolitre osmometer (**Chapter 2, Section 2.5**). At least 5 discs were sampled from each flask, three measurements were made on each disc. The cells targeted were the large storage parenchyma cells at the periphery of the discs (after Perry *et al.*, 1987).

5.2.5, The closed System

A single core of sugar beet taproot tissue (storage parenchyma) was cut using a sharp 7 mm diameter cork borer. The core of beet tissue was sliced into 1 mm thick discs using a sharp double edged razor blade. The discs were either used immediately or after pre-treatment in an osmoticum. Pre-treatment involved aerating 10 beet discs in 100 ml of osmoticum for 15 minutes. The osmotica used were: deionised water, 300 - 600 mOsmoles kg^{-1} mannitol, 300 - 900 mOsmoles kg^{-1} sucrose with 0 - 0.5 mM KCl, 300 - 900 mOsmoles kg^{-1} sucrose/KCl (1:1 ratio), and 300 - 900 mOsmoles kg^{-1} KCl. All the osmotica contained 0.5 mM $CaSO_4$.

Only a single beet disc was required for each experiment. The selected disc was secured to a perspex sample holder and covered in 5 ml of silicone oil. Turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) measurements were made under oil every 5 - 10 minutes for a maximum of 2 hours (after Tomos and Wyn Jones, 1988).

Plate 5.1, Sugar beet plants eight weeks after germination

5.3, Results

5.3.1, Turgor regulation in an open system

The cell pressure probe and picolitre osmometry were used to measure turgor regulation and osmotic adjustment in sugar beet taproot discs aerated in 0 - 600 mM mannitol-0.5 mM $CaSO_4$ osmotica over 7 days (**Figure 5.3** and **5.4**). Sugar beet taproot discs in 0 mM mannitol-0.5 mM $CaSO_4$ had an initial (day 0) turgor pressure (P_{cell}) of 1.12 MPa, decreasing to 0.85 MPa after 48 hours (day 2). Turgor pressure was maintained at this level until the experiment terminated at day 7. The turgor pressure (P_{cell}) in beet discs in 300 mM mannitol-0.5 mM $CaSO_4$ remained constant at around 0.65 MPa, this P_{cell} value corresponds to P_{cell} in intact, mature sugar beet taproots *in vivo* (see Tomos *et al.*, 1992). Sugar beet discs aerated in 600 mM mannitol-0.5 mM $CaSO_4$ had an initial turgor pressure (P_{cell}) of 0.24 MPa which increased to a new steady value of 0.42 MPa within 48 hours (**Figure 5.3**).

Changes in turgor pressure (P_{cell}) were accompanied by corresponding changes in cell osmotic pressure (**Figure 5.4**). Beet discs in 300 mM mannitol-0.5 mM $CaSO_4$ maintained an osmotic pressure (Π_{cell}) of 1.95 MPa throughout the experiment (this corresponds to Π_{cell} in intact, mature sugar beet tissue *in vivo* - Tomos *et al.*, 1992). Discs aerated in 0 mM mannitol-0.5 mM $CaSO_4$ had an initial Π_{cell} of 1.59 MPa, decreasing to 1.24 MPa within 48 hours (day 2), while Π_{cell} in beet discs in 600 mM mannitol-0.5 mM $CaSO_4$ increased from 2.0 MPa to 2.26 MPa within the same time period (Figure 5.4). The difference between turgor pressure (ΔP_{cell}) at day 0 and day 7 and osmotic pressure ($\Delta \Pi_{cell}$) at day 0 and day 7 is shown in **Table 5.1**. There is excellent agreement between ΔP_{cell} and $\Delta \Pi_{cell}$, indicating that turgor regulation in sugar beet taproot discs, in an open system, is due to osmotic adjustment of solutes in the protoplast, and confirming that the reflection coefficient of these cells is in fact 1.

Table 5.1, ΔP_{cell} and $\Delta \Pi_{cell}$ (MPa) in sugar beet taproot discs, between day 0 and day 7, under open system conditions

Mannitol (mM)	ΔP_{cell} (MPa)	$\Delta \Pi_{cell}$ (MPa)
0	-0.33	-0.37
300	-0.06	-0.15
600	0.23	0.22

5.3.2, Turgor regulation in a closed system

Turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) of freshly cut and pre-treated sugar beet discs, water saturated silicone oil, were measured at 5 - 10 minute intervals for a maximum of 2 hours. Freshly cut, unrinsed sugar beet taproot discs in silicone oil had an initial turgor pressure (P_{cell}) of around 0.35 MPa, this is lower than P_{cell} in the intact taproot (see Tomos *et al.*, 1992) due to the broken cell solutes in the apoplast. After a lag of approximately 20 minutes, P_{cell} was regulated upwards, reaching 0.6 - 0.7 MPa after 40 minutes (**Figure 5.5**). This corresponds to P_{cell} in the intact taproot *in vivo* (Tomos *et al.*, 1992). Turgor pressure was maintained at this level until the discs became anaerobic 3 - 4 hours later (data not shown). There was no corresponding increase in cell osmotic pressure, Π_{cell} was maintained at around 2.4 MPa throughout, indicating that turgor pressure is regulated by adjustment of solutes in the apoplast rather than adjustment of solutes in the protoplast (**Figure 5.6**).

Pre-treating beet discs in an aqueous osmoticum, prior to covering the tissue in water saturated silicone oil, flushes out the original free apoplastic solutes and replaces them with the solutes present in the osmoticum. The concentrations of the osmotica used were largely determined by the osmotica used in the open system experiment (see **Figure 5.3** and **5.4**). All the osmotica were made up using 0.5 mM $CaSO_4$ stock solution.

Pre-treatment in 600 mOsmoles kg^{-1} sucrose and/or KCl (**Figure 5.7**) resulted in an initial P_{cell} of around 0.4 MPa. After a lag of between 20 - 30 minutes P_{cell} was regulated upwards, reaching 0.7 MPa (which corresponds to P_{cell} in the intact taproot) within 40 - 60 minutes. Turgor pressure (P_{cell}) was maintained at this level for the duration of the experiment. In the presence of apoplastic KCl turgor regulation is indistinguishable from that observed in the freshly cut beet tissue in **Figure 5.5** and **5.6**; the target P_{cell} was achieved within 40 minutes (after a lag of 20 minutes). However, in the absence of apoplastic KCl, beet discs under closed system conditions, took about 20 minutes longer to achieve the target P_{cell} value; beet discs pre-treated in the sucrose only osmoticum reached a P_{cell} of 0.7 MPa within 60 minutes (after a lag of 30 minutes).

Osmotic pressure (Π_{cell}) was maintained at around 2.5 MPa throughout the experiment, indicating that osmotic adjustment, leading to turgor regulation, is apoplastic rather than protoplastic in nature.

This turgor regulation mechanism in the presence of apoplastic sucrose and/or KCl is demonstrated again in **Figure 5.9**. Sugar beet discs pre-treated in 900 mOsmoles kg^{-1} sucrose and/or KCl undergo cell plasmolysis due to the magnitude of the osmotic pressure step across the plasma membrane. Turgor pressure (P_{cell}) recovered sufficiently to be measured reliably within 20 - 40 minutes, reaching 0.7 MPa within 60 - 80 minutes. Once again, beet discs pre-treated in osmotica containing KCl reached the target P_{cell} 20 minutes earlier than the beet discs pre-treated in the sucrose only osmoticum.

Pre-treatment in 300 mOsmoles kg^{-1} sucrose and/or KCl (**Figure 5.10** and **5.11**) resulted in constant turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) values of around 0.7 MPa and 2.2 MPa respectively (P_{cell} and Π_{cell} did not deviate from these values for the duration of the time course). These data largely correspond to P_{cell} and Π_{cell} *in vivo* (see Tomos *et al.*, 1992) and also correspond to P_{cell} and Π_{cell} in open system beet tissue in 300 mM mannitol-0.5 mM $CaSO_4$ (**Figure 5.3** and **5.4**).

In order to obtain closed system data which could be directly comparable to the open system data (**Figure 5.3** and **5.4**), beet discs were pre-treated in the open system mannitol osmotica, prior to being covered in silicone oil. The 0 mM mannitol-0.5 mM $CaSO_4$ pre-treated beet discs underwent a rapid increase in turgor pressure (relative to P_{cell} *in vivo*), to 1.1 MPa (**Figure 5.12**). Turgor pressure was maintained at this level for the duration of the experiment. This increase in P_{cell} did not induce a rapid downward turgor regulation mechanism in this case. Osmotic pressure (Π_{cell}) remained constant at 1.6 MPa throughout (**Figure 5.13**). These data are comparable to P_{cell} and Π_{cell} in the open system 0 mM mannitol-0.5 mM $CaSO_4$ beet discs at day 0. A 300 mM mannitol-0.5 mM $CaSO_4$ pre-treatment (**Figure 5.14** and **5.15**) resulted in constant P_{cell} and Π_{cell} values of 0.7 MPa and 2.2 MPa respectively. These values correspond to P_{cell} and Π_{cell} in the equivalent open system beet discs at day 0 to day 7, and also correspond

to P_{cell} and Π_{cell} in the intact, mature taproot *in vivo* (see Tomos *et al.*, 1992). A 600 mM mannitol-0.5 mM $CaSO_4$ pre-treatment resulted in P_{cell} and Π_{cell} vales of 0.3 MPa and 2.5 MPa respectively. Turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) were maintained at constant levels throughout the time course. Once again P_{cell} and Π_{cell} correspond to the values obtained in the equivalent open system treatment at day 0. It is assumed that turgor regulation was not achieved due to the impermeant (or slowly permeant) nature of mannitol.

Figure 5.3 and 5.4, Turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in aged sugar beet taproot discs (open system)

Changes in turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in sugar beet discs aerated in 0 - 600 mM mannitol-0.5 mM $CaSO_4$ for 7 days. Data are presented as mean and standard deviation, n is 15 (5 beet discs , 3 cells per disc).

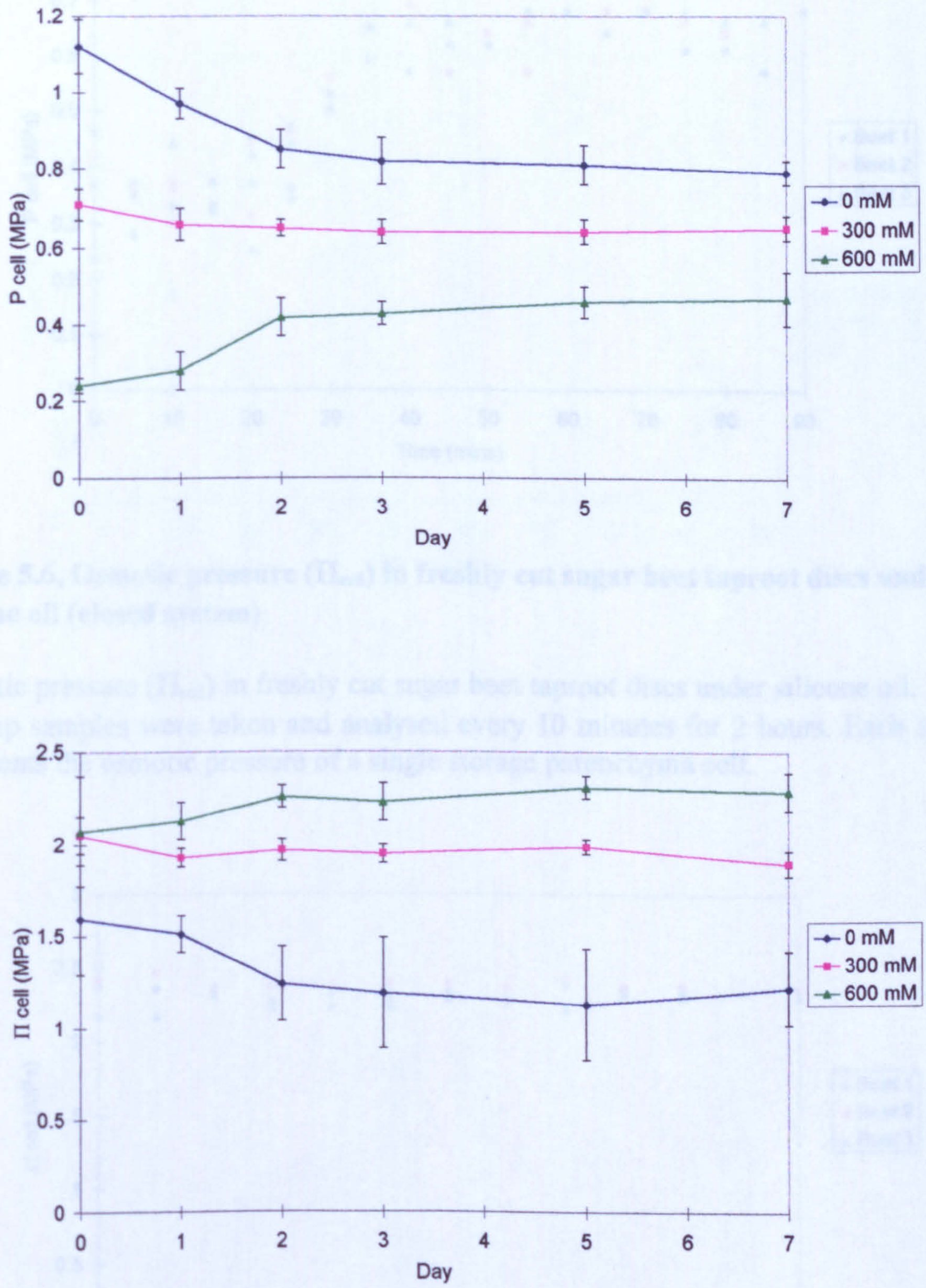


Figure 5.5, Turgor pressure (P_{cell}) in freshly cut sugar beet taproot discs under silicone oil (closed system)

Turgor pressure (P_{cell}) in freshly cut sugar beet taproot discs under silicone oil. Turgor pressure was measured at 5 minute intervals for 90 minutes. Each symbol represents a single turgor pressure measurement from a single storage parenchyma cell.

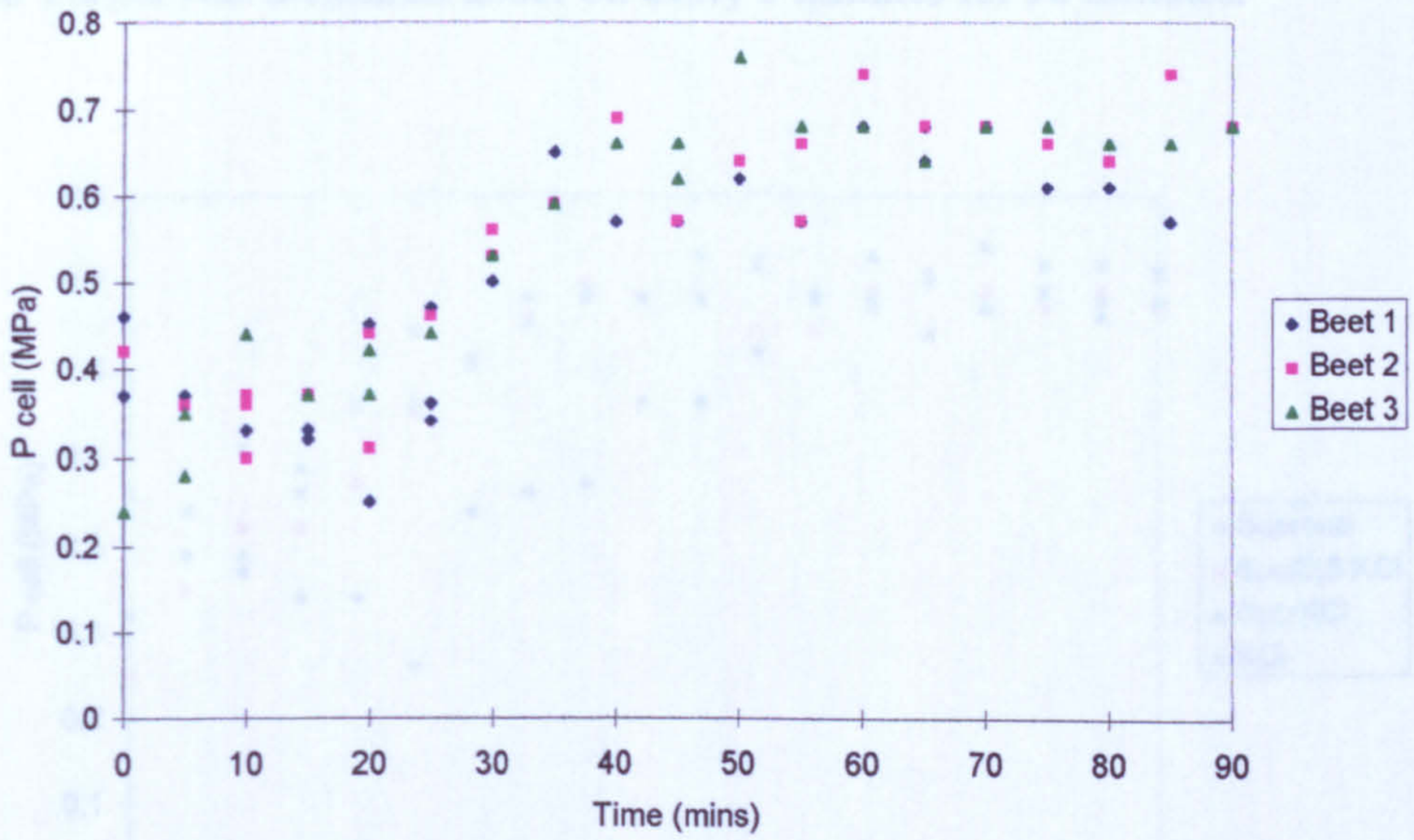


Figure 5.6, Osmotic pressure (Π_{cell}) in freshly cut sugar beet taproot discs under silicone oil (closed system)

Osmotic pressure (Π_{cell}) in freshly cut sugar beet taproot discs under silicone oil. Single cell sap samples were taken and analysed every 10 minutes for 2 hours. Each symbol represents the osmotic pressure of a single storage parenchyma cell.

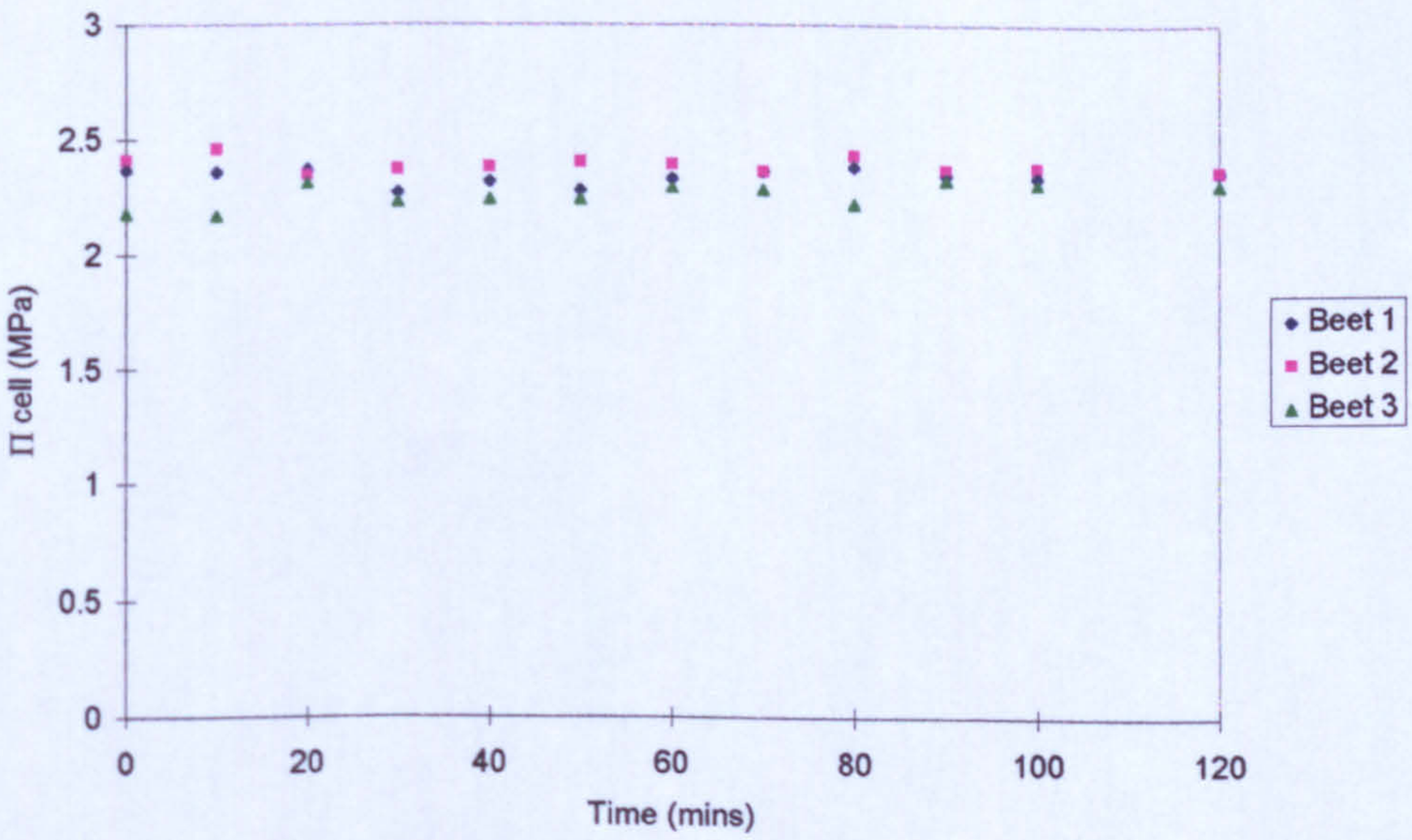


Figure 5.7, Turgor pressure (P_{cell}) in sugar beet taproot discs pre-treated in 600 mOsmoles kg^{-1} sucrose and/or KCl (closed system)

Turgor pressure (P_{cell}) in sugar beet discs aerated in either 600 mOsmoles kg^{-1} sucrose, 600 mOsmoles kg^{-1} sucrose with 0.5 mM KCl, 600 mOsmoles kg^{-1} sucrose/KCl (300 mOsmoles kg^{-1} of each), or 600 mOsmoles kg^{-1} KCl for 15 minutes prior to covering in silicone oil. All the pre-treatment osmotica were made up using 0.5 mM $CaSO_4$ solution. Turgor was measured under oil every 5 minutes for 90 minutes.

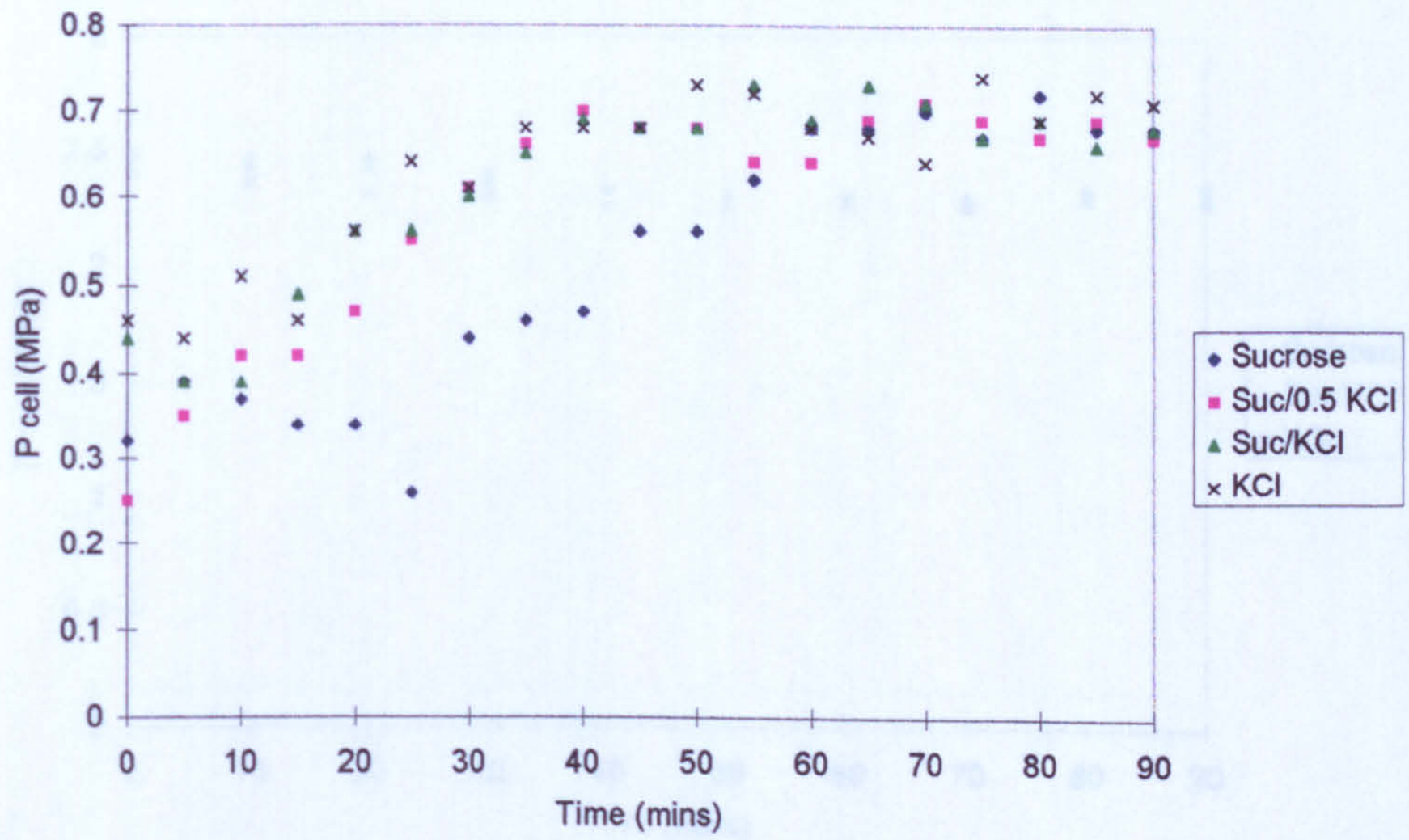


Figure 5.8, Osmotic pressure (Π_{cell}) in sugar beet taproot discs pre-treated in 600 mOsmoles kg^{-1} sucrose and/or KCl (closed system)

Osmotic pressure (Π_{cell}) in sugar beet discs aerated in either 600 mOsmoles kg^{-1} sucrose, 600 mOsmoles kg^{-1} sucrose/KCl (300 mOsmoles kg^{-1} of each), or 600 mOsmoles kg^{-1} KCl for 15 minutes prior to covering in silicone oil. All the pre-treatment osmotica were made up using 0.5 mM $CaSO_4$ solution. Osmotic pressure was measured under oil every 10 minutes for 90 minutes.

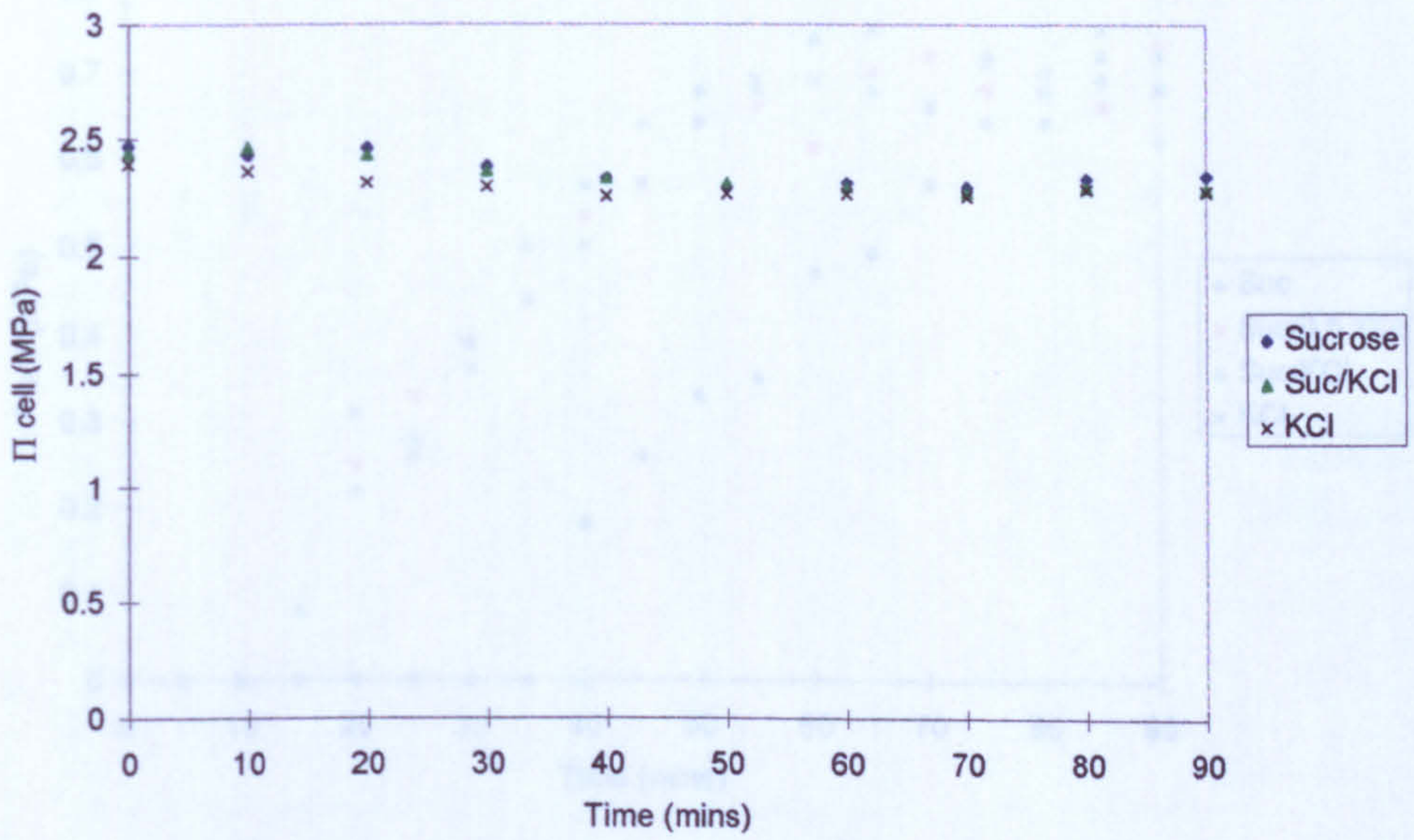


Figure 5.9, Turgor pressure (P_{cell}) in sugar beet taproot discs pre-treated in 900 mOsmoles kg^{-1} sucrose and/or KCl (closed system)

Turgor pressure (P_{cell}) in sugar beet discs aerated in either 900 mOsmoles kg^{-1} sucrose, 900 mOsmoles kg^{-1} sucrose with 0.5 mM KCl, 900 mOsmoles kg^{-1} sucrose/KCl (450 mOsmoles kg^{-1} of each), or 900 mOsmoles kg^{-1} KCl for 15 minutes prior to covering in silicone oil. All the pre-treatment osmotica were made up using 0.5 mM $CaSO_4$ solution. Turgor was measured under oil every 5 minutes for 90 minutes.

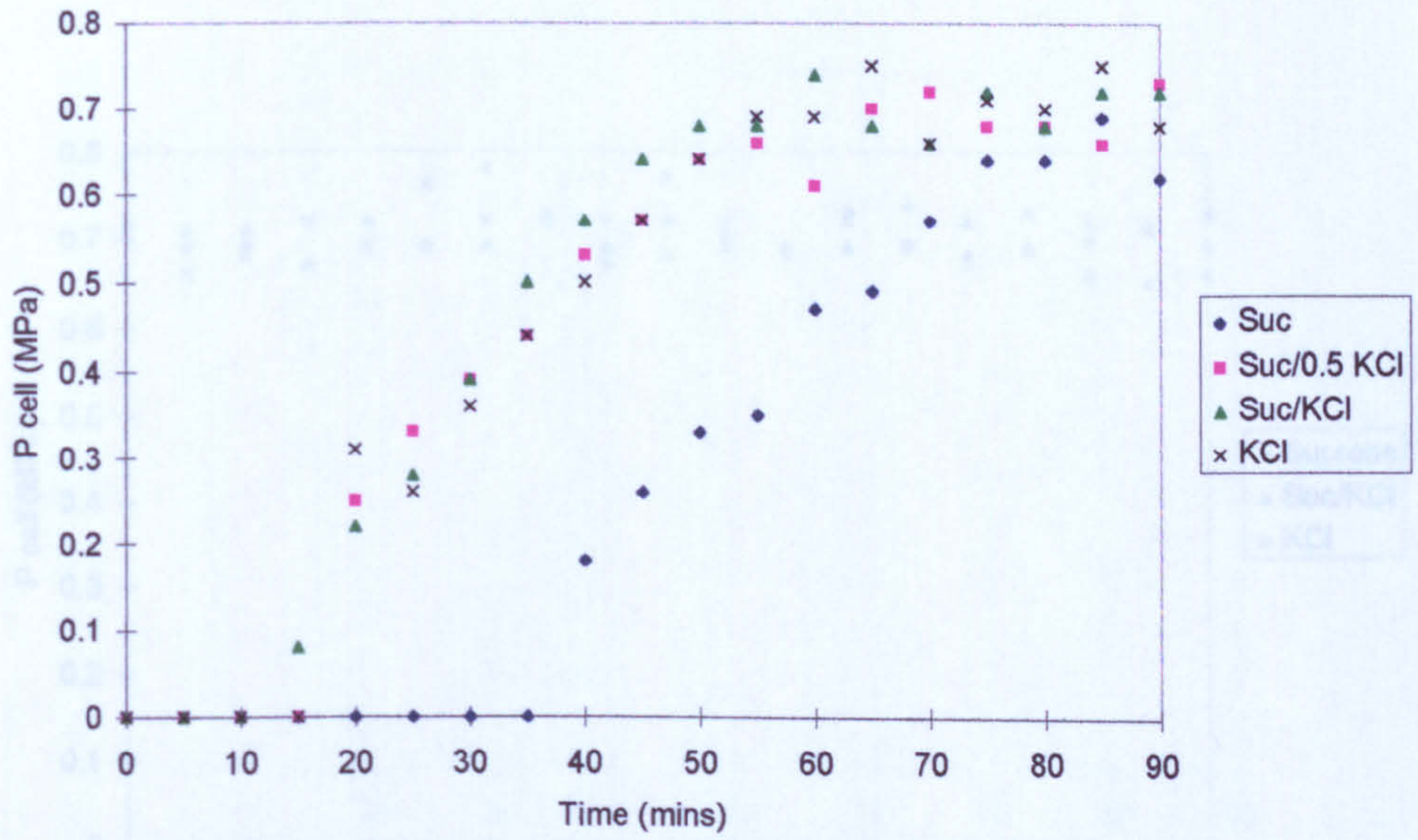
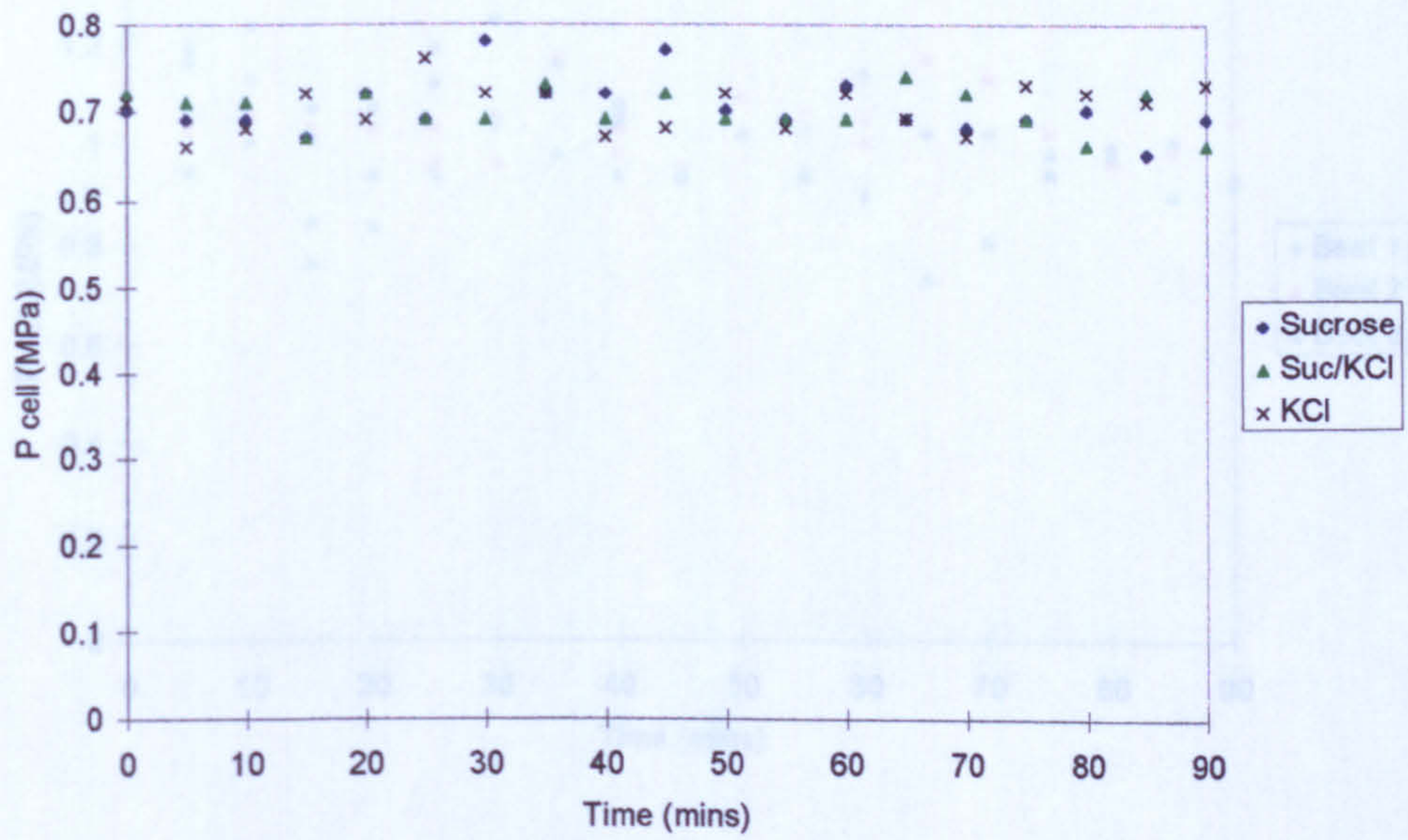


Figure 5.10 and 5.11, Turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in sugar beet taproot discs pre-treated in 300 mOsmoles kg $^{-1}$ sucrose and/or KCl (closed system)

Turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in sugar beet discs aerated in either 300 mOsmoles kg^{-1} sucrose, 300 mOsmoles kg^{-1} sucrose/KCl (150 mOsmoles kg^{-1} of each), or 300 mOsmoles kg^{-1} KCl for 15 minutes prior to covering in silicone oil. All the pre-treatment osmotica were made up using 0.5 mM $CaSO_4$ solution. Turgor pressure and osmotic pressure were measured under oil every 5 and 10 minutes respectively, for 90 minutes.



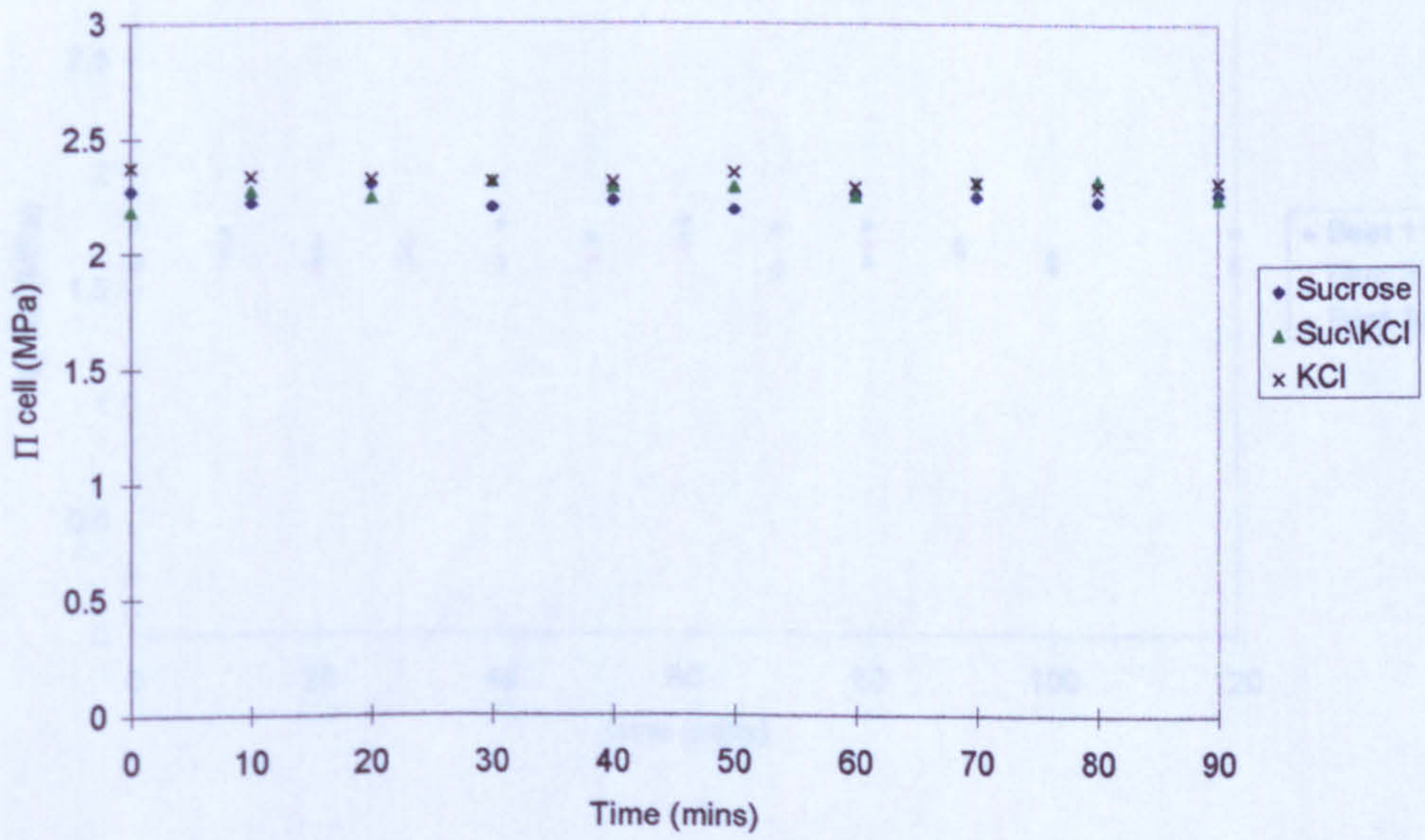
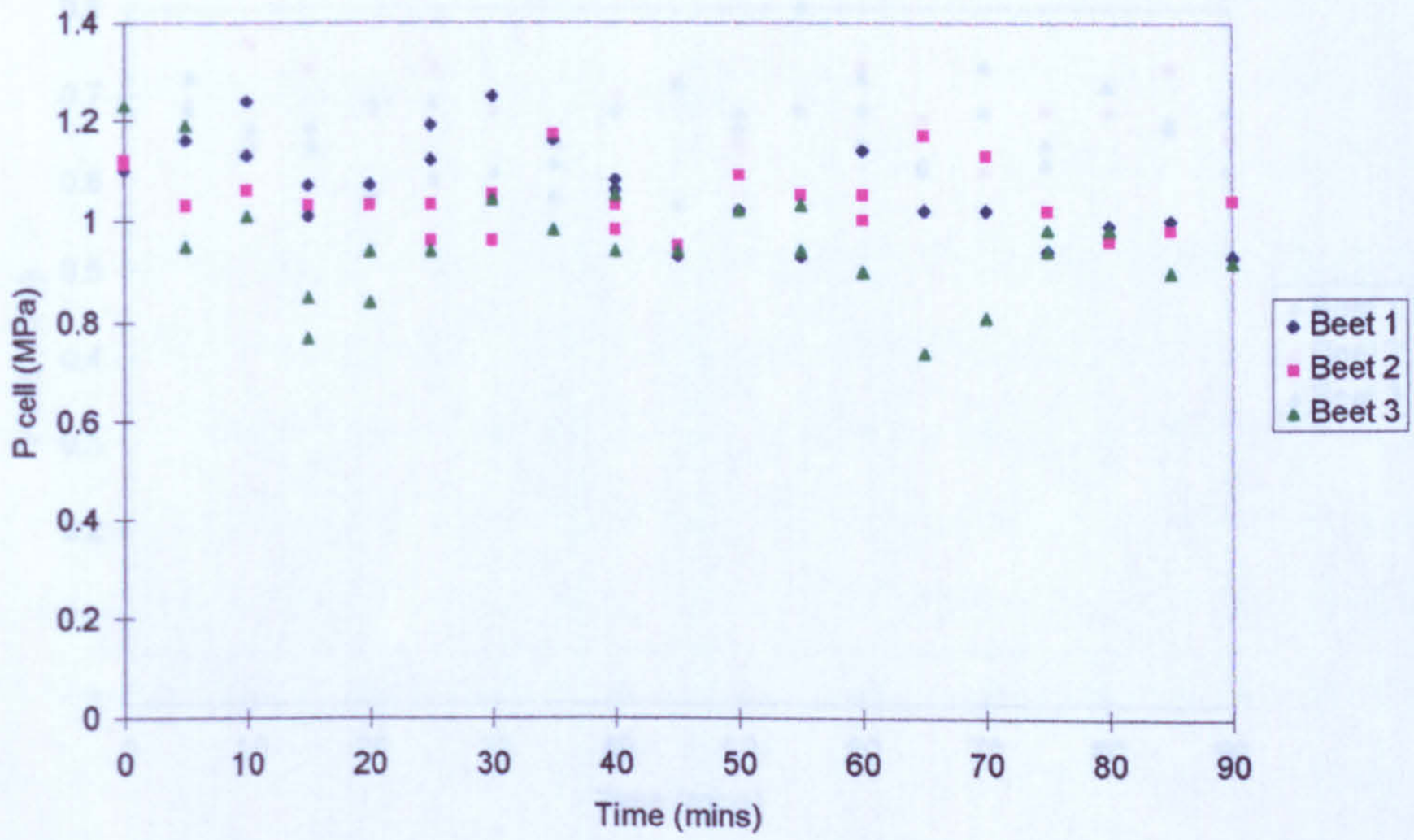


Figure 5.12 and 5.13, Turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in sugar beet taproot discs pre-treated in 0 mM mannitol-0.5 mM $CaSO_4$ (closed system)

Turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in sugar beet taproot discs pre-treated in 0 mM mannitol-0.5 mM $CaSO_4$ for 15 minutes prior to covering in silicone oil. Turgor pressure and osmotic pressure were measured at 5 and 10 minute intervals respectively, for a maximum of 2 hours.



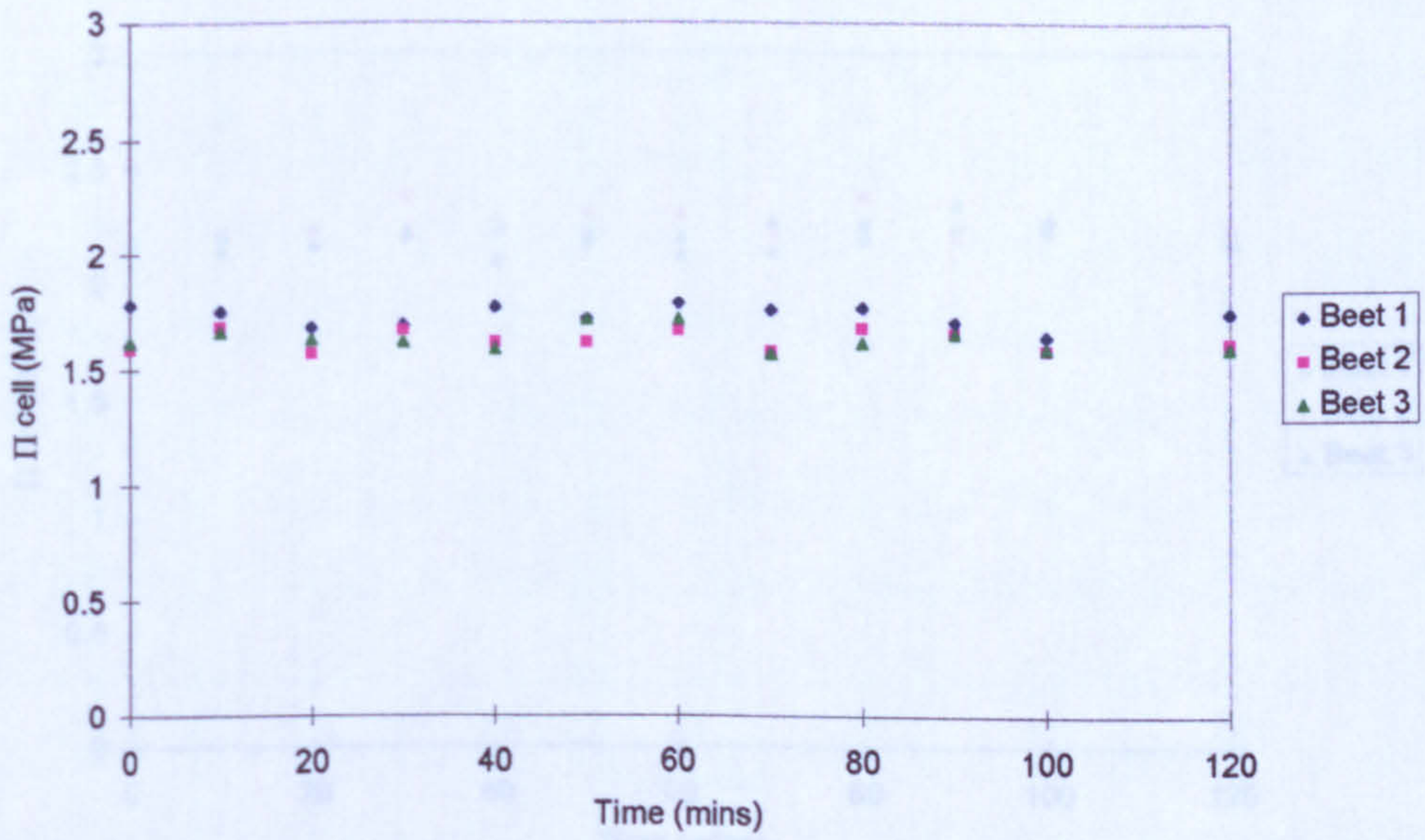
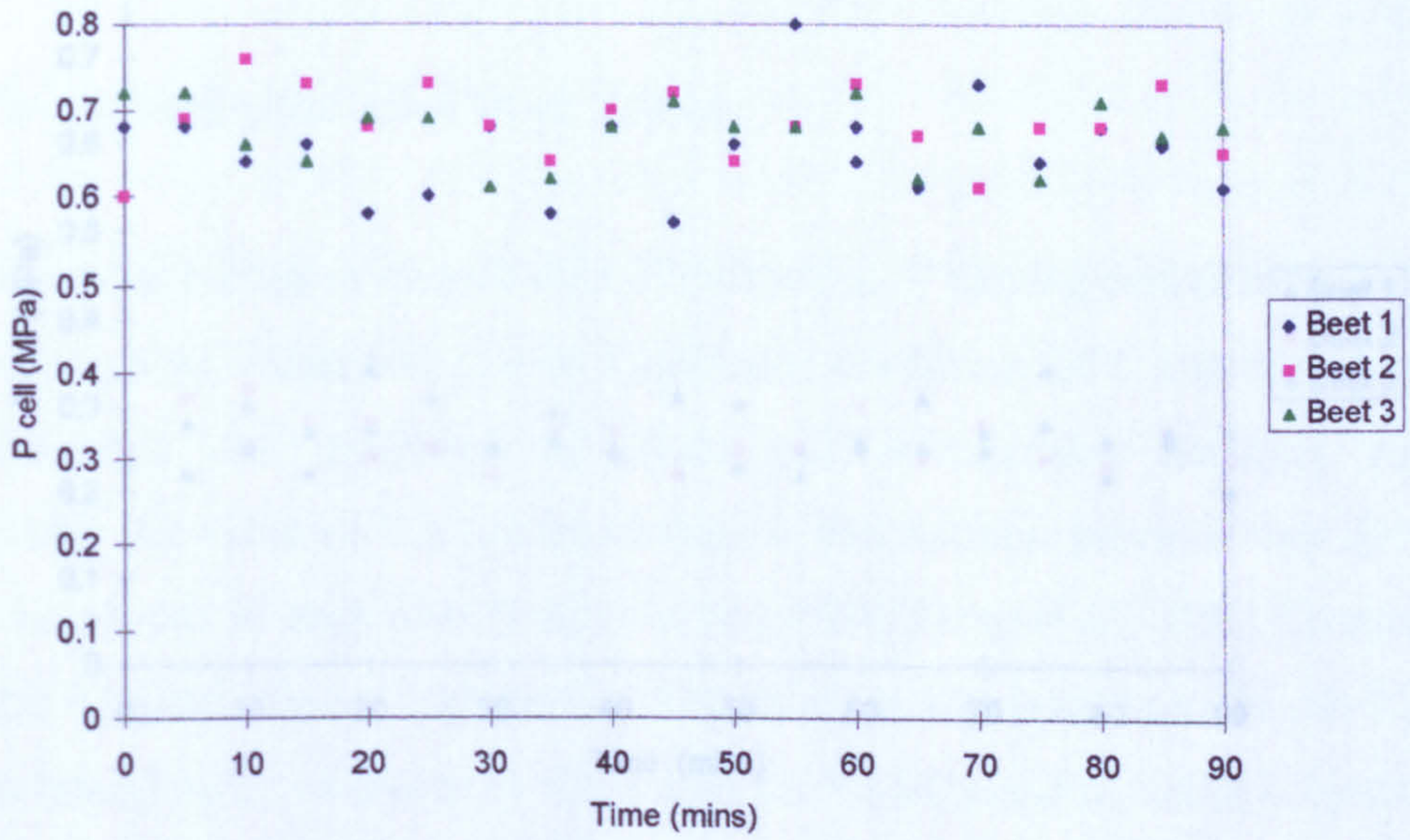


Figure 5.14 and 5.15, Turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in sugar beet taproot discs pre-treated in 300 mM mannitol-0.5 mM $CaSO_4$ (closed system)

Turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in sugar beet taproot discs pre-treated in 300 mM mannitol-0.5 mM $CaSO_4$ for 15 minutes prior to covering in silicone oil. Turgor pressure and osmotic pressure were measured at 5 and 10 minute intervals respectively, for a maximum of 2 hours.



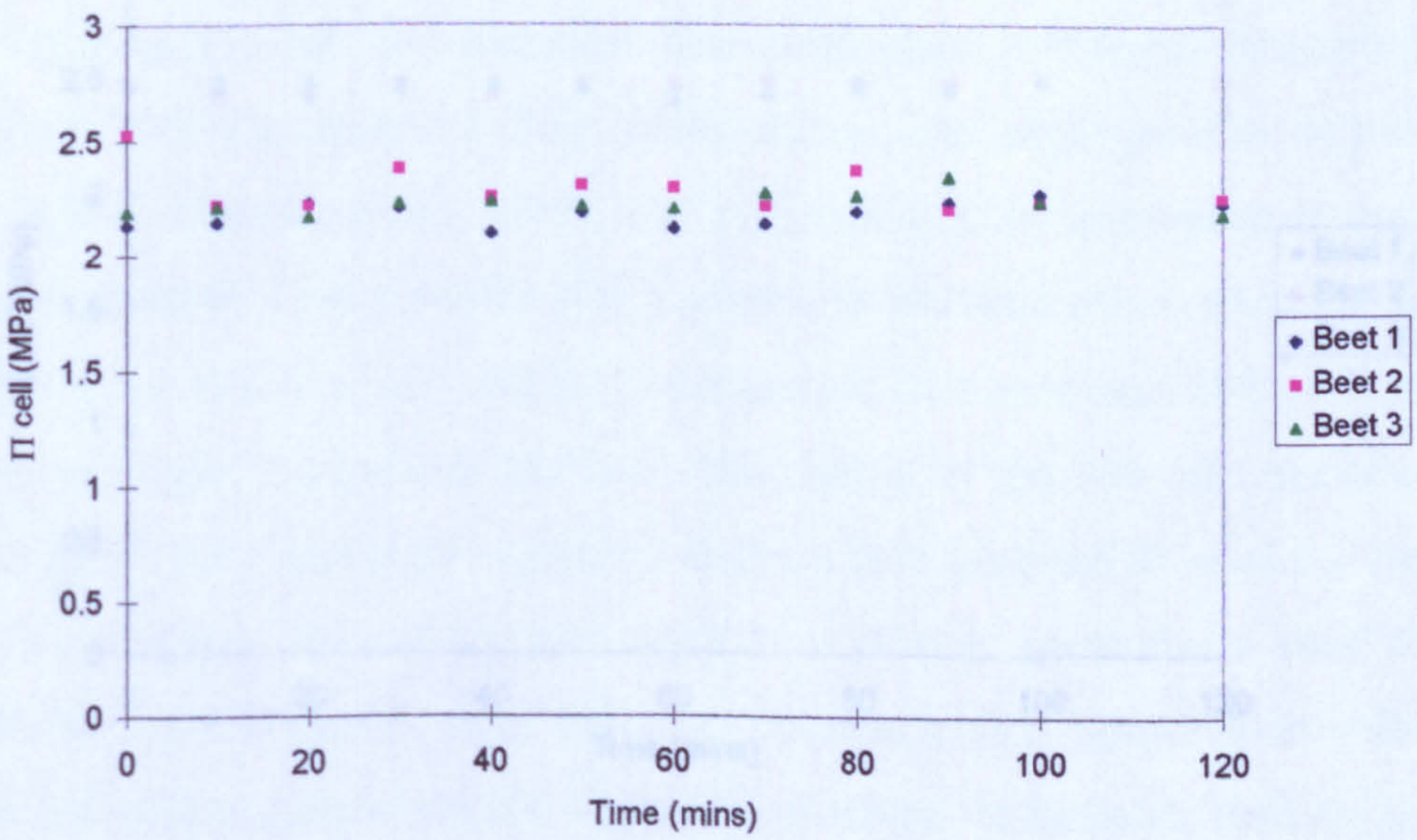
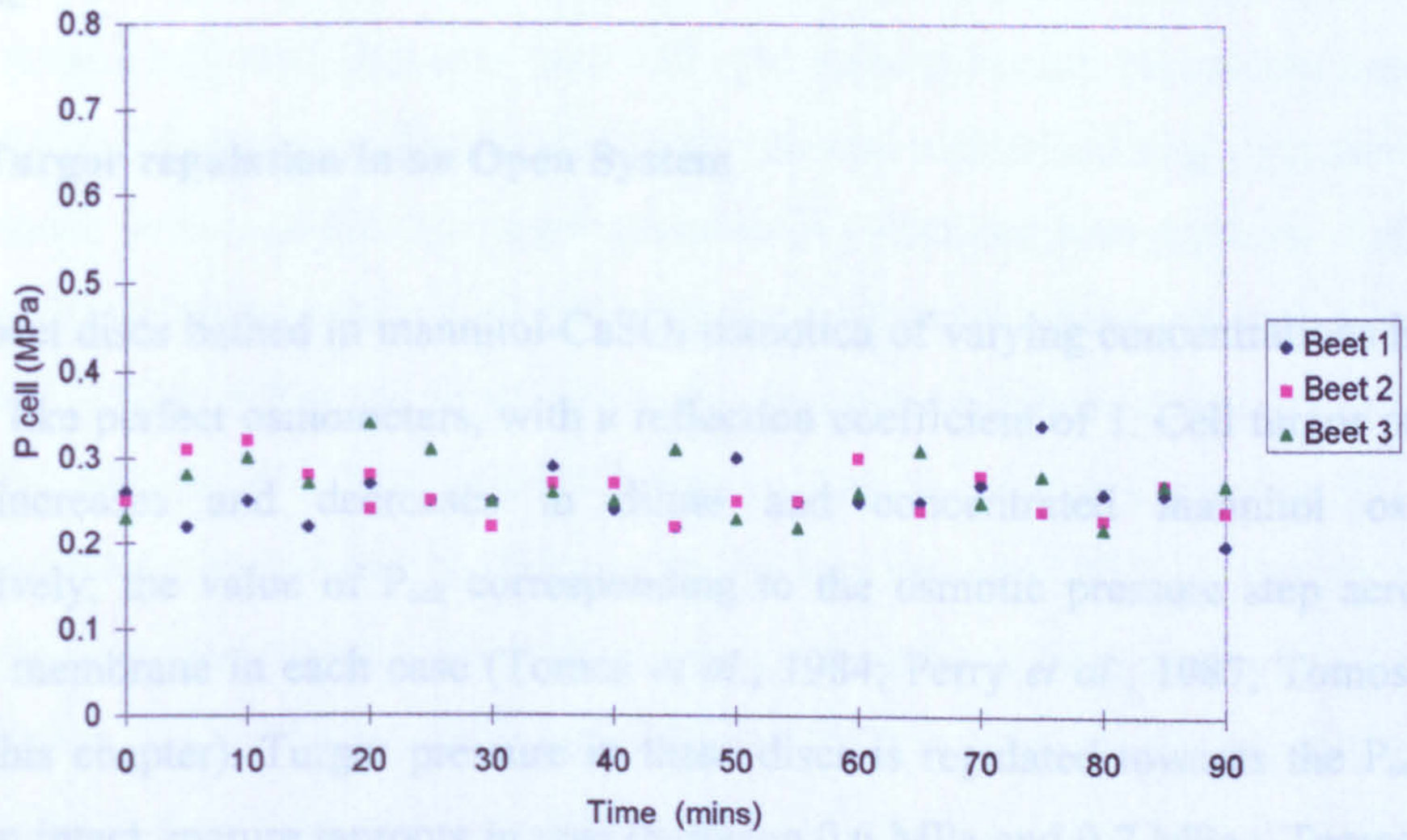
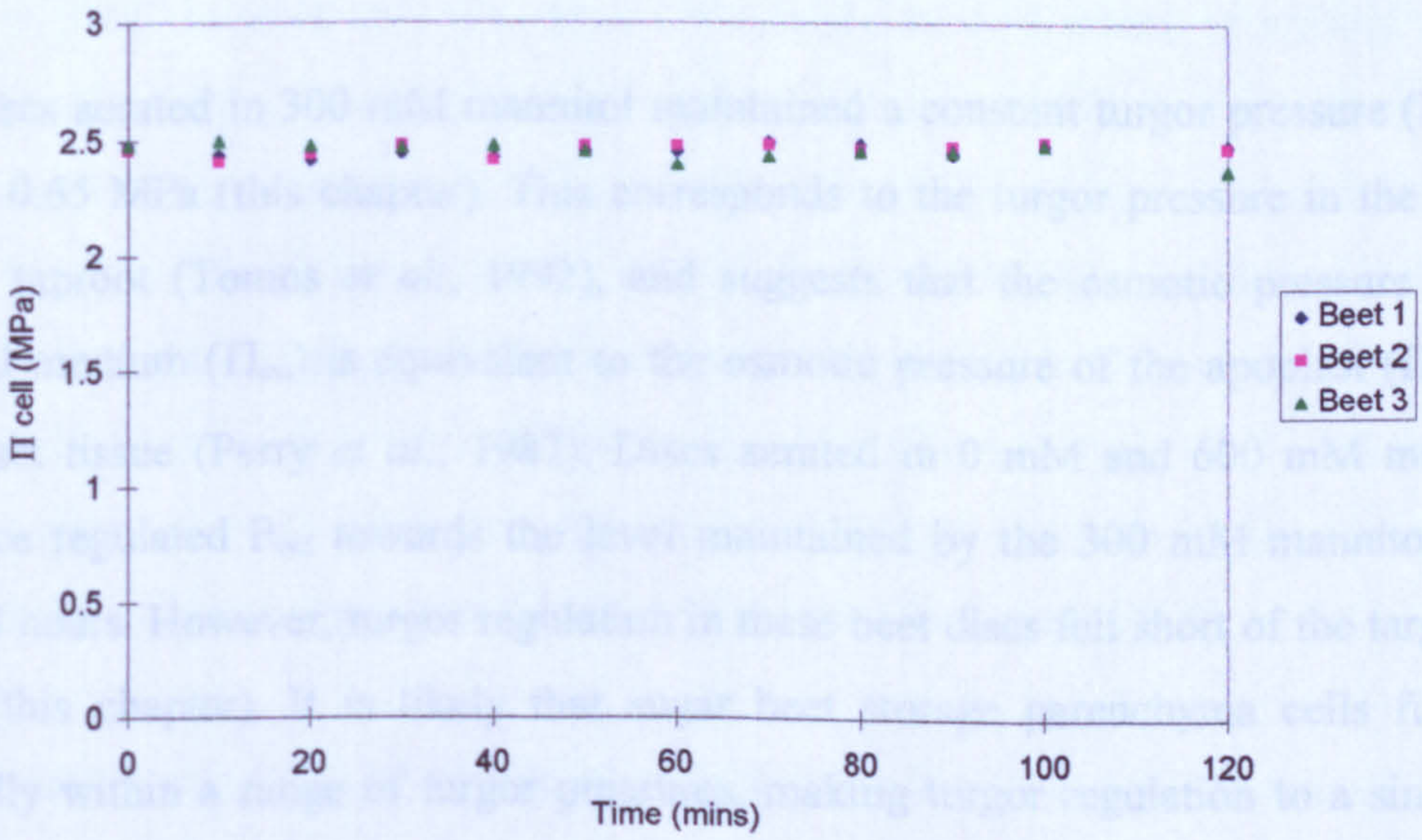


Figure 5.16 and 5.17, Turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in sugar beet taproot discs pre-treated in 600 mM mannitol-0.5 mM $CaSO_4$ (closed system)

Turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) in sugar beet taproot discs pre-treated in 600 mM mannitol-0.5 mM $CaSO_4$ for 15 minutes prior to covering in silicone oil. Turgor pressure and osmotic pressure were measured at 5 and 10 minute intervals respectively, for a maximum of 2 hours.





5.4, Discussion

The data presented in this chapter demonstrates that sugar beet taproot discs in an open system regulate cell turgor pressure by adjustment of solutes in the protoplast over 48 hours. Whereas beet discs in a closed system environment apparently regulate cell turgor pressure (in one direction) by rapid and subtle adjustment of solutes in the apoplast.

5.4.1, Turgor regulation in an Open System

Sugar beet discs bathed in mannitol-$CaSO_4$ osmotica of varying concentrations initially behave like perfect osmometers, with a reflection coefficient of 1. Cell turgor pressure (P_{cell}) increases and decreases in dilute and concentrated mannitol osmotica respectively; the value of P_{cell} corresponding to the osmotic pressure step across the plasma membrane in each case (Tomos *et al.*, 1984; Perry *et al.*, 1987; Tomos *et al.*, 1992; this chapter). Turgor pressure in these discs is regulated towards the P_{cell} level found in intact, mature taproots *in vivo* (between 0.6 MPa and 0.7 MPa - Tomos *et al.*, 1992), within 48 hours (**Figure 5.3** and **5.4**). After 48 hours P_{cell} is maintained at a constant level for up to 7 days (this chapter).

Beet discs aerated in 300 mM mannitol maintained a constant turgor pressure (P_{cell}) of around 0.65 MPa (this chapter). This corresponds to the turgor pressure in the intact, mature taproot (Tomos *et al.*, 1992), and suggests that the osmotic pressure of the external medium (Π_{ext}) is equivalent to the osmotic pressure of the apoplast (Π_{wall}) in the intact tissue (Perry *et al.*, 1987). Discs aerated in 0 mM and 600 mM mannitol osmotica regulated P_{cell} towards the level maintained by the 300 mM mannitol discs over 48 hours. However, turgor regulation in these beet discs fell short of the target P_{cell} value (this chapter). It is likely that sugar beet storage parenchyma cells function optimally within a range of turgor pressures, making turgor regulation to a single set point unnecessary and metabolically expensive. It is also important to consider that beet discs in an open system are in a completely artificial and alien environment which affects the physiology and biochemistry of the cell (Van Steveninck, 1975).

Changes in turgor pressure (P_{cell}) were accompanied by corresponding changes in cell osmotic pressure (Π_{cell}), demonstrating that P_{cell} regulation is achieved by adjustment of solutes in the protoplast (this chapter). Adjustment of apoplastic solutes would be impossible in an open system as the apoplastic volume is effectively equivalent to the volume of osmoticum surrounding the discs (which is 5 litres in this case).

Perry *et al.*, in 1987, described a similar adjustment mechanism in red beet taproot tissue aerated in 0 mM, 200 mM, and 350 mM mannitol (each osmoticum containing 0.5 mM $CaSO_4$). Red beet discs in 200 mM mannitol maintained turgor pressure at 0.5 MPa, which is comparable to turgor pressure in intact red beet taproots (Leigh and Tomos, 1983). Red beet discs in 0 mM mannitol underwent a decrease in P_{cell} from 0.88 MPa to 0.7 MPa within 48 hours, while P_{cell} in discs in 350 mM mannitol increased from 0.12 MPa to 0.31 MPa within 48 hours. Changes in turgor pressure (P_{cell}) were accompanied by corresponding changes in osmotic pressure (Π_{cell}). The decrease in Π_{cell} in the 0 mM mannitol red beet discs was found to be due to solute efflux out of the cell. The increase in Π_{cell} in red beet discs aerated in 350 mM mannitol was found to be due to sucrose hydrolysis, subsequently leading to an increase in the total sugar content of the cell.

Aerating plant tissue in an open system is referred to as washing or ageing. Ageing induces profound physiological and biochemical changes within the cell, most notably rapid increases in protein synthesis and respiration, followed by an increase in solute uptake (for reviews see Van Steveninck, 1975; Poole, 1976). In sugar beet and red beet taproot tissue, the ageing phenomenon induces vacuolar acid-invertase activity (Bacon *et al.*, 1965; Leigh *et al.*, 1979; Milling *et al.*, 1993; Marvier *et al.*, 1997). Induction of acid-invertase activity coincides with a decrease in sucrose concentration and accumulation of reducing sugars (Leigh *et al.*, 1979, Perry *et al.*, 1987).

Acid-invertase activity is undetectable in freshly cut beet tissue. Induction of activity occurs after 24 hours, and is maximal after 72 hours (Milling *et al.*, 1993; Marvier *et al.*, 1997). Marvier *et al.* (1997) detected a maximum acid-invertase activity of 0.12 μmol.sucrose hydrolysed min^{-1} mg^{-1} in red beet taproot discs after 72 hours of ageing.

Initially the rate of sucrose decline was dependent on the concentration of the external medium (Π_{ext}). Perry *et al.* (1987) found that, during the first 24 hours, the decrease in sucrose concentration was greatest in discs aerated in 0 mM mannitol and slowest in discs aerated in 350 mM mannitol. However, after 24 hours sucrose concentration decreased at a constant rate of 3.5 mM h^{-1} in all treatments. Sucrose had disappeared from discs in 0 mM mannitol after 46 hours and from all other treatments by 118 hours. This decrease in sucrose concentration was accompanied by an accumulation of reducing sugars (glucose and fructose). Accumulation of glucose and fructose began after a lag of 24 hours and increased steadily at a rate of 4.4 mM h^{-1} before reaching a plateau after 50 hours. The final concentration of reducing sugars was dependent on the concentration of the external medium. After 118 hours (approximately 5 days) of ageing, red beet discs in 0 mM, 200 mM and 350 mM mannitol had 85 mM, 126 mM, and 167 mM reducing sugars respectively.

In the open system described above turgor regulation can only be achieved by osmotic adjustment of the protoplast. Uptake of mannitol from the external medium occurs only very slowly due to its largely impermeant nature (Cram, 1984; Perry *et al.*, 1987). Perry *et al.* (1987) demonstrated that the solutes necessary for turgor regulation, in open system beet discs, originate from the protoplast itself; hydrolysis of sucrose induced by the ageing phenomenon provides hexoses that can be used to regulate turgor pressure. The final concentration of reducing sugars (hexoses) is dependent on the concentration of the external medium (Π_{ext}), which in this case is also the concentration of the apoplast (Π_{wall}). Modification of cell osmotic pressure (Π_{cell}) by accumulation (or loss) of reducing sugars directly influences the turgor pressure (P_{cell}) of the cell (Perry *et al.*, 1987).

5.4.2, Turgor regulation in a closed system

In a closed system environment sugar beet taproot discs, freshly cut or pre-treated in a concentrated sucrose and/or KCl osmoticum, undergo upward turgor regulation to a single set point (between 0.6 and 0.7 MPa) within 40 - 80 minutes (this chapter). This turgor pressure (P_{cell}) level corresponds to P_{cell} maintained by the 300 mM mannitol open

system beet discs (this chapter), and also corresponds to P_{cell} in intact, mature taproots in vivo (Tomos *et al.*, 1992). The time taken to achieve turgor regulation is dependent on the concentration of the pre-treatment osmoticum and the presence or absence of KCl.

Freshly cut, unrinsed beet discs have an initial P_{cell} value which is lower than P_{cell} *in vivo* (Tomos *et al.*, 1992). Beet disc preparation inevitably damages the tissue; cells rupture and release their contents into the apoplast leading to a decrease in P_{cell}. Pre-treating beet discs in 600 mOsmoles kg^{-1} sucrose and/or KCl has a comparable effect on P_{cell} (this chapter). Turgor regulation in freshly cut and 600 mOsmoles kg^{-1} sucrose and/or KCl pre-treated discs largely follows the same pattern. Following a lag of approximately 20 minutes, P_{cell} was regulated upwards, reaching 0.6 MPa - 0.7 MPa within 40 minutes. The exception being beet discs pre-treated in an osmoticum which was completely devoid of KCl. In the absence of apoplastic KCl turgor regulation was achieved within 60 minutes, after a lag of up to 30 minutes. Due to the larger osmotic pressure step/difference across the plasma membrane in beet discs pre-treated in 900 mOsmoles kg^{-1} sucrose and/or KCl, turgor regulation took 60 minutes in discs with apoplastic KCl and 80 minutes in discs without apoplastic KCl, after a lag of 20 and 40 minutes respectively. Providing the overall osmolarity of the sucrose and/or KCl pre-treatment was the same - micromolar (0.5 mM/500μM) and millimolar (300 - 900 mOsmoles kg^{-1}) apoplastic KCl resulted in an indistinguishable turgor regulation pattern (this chapter). Changes in turgor pressure (P_{cell}) were not accompanied by equivalent changes in cell osmotic pressure (Π_{cell}), indicating that osmotic adjustment leading to turgor regulation is apoplastic rather than protoplastic (this chapter).

The observed lag period, prior to the initiation of turgor regulation in closed system beet discs, may simply be an undesirable effect of beet disc preparation. The act of obtaining a core of beet tissue, followed by slicing the discs may produce a wounding response which affects the membrane transport processes necessary to achieve osmotic adjustment. For example, the plant plasma membrane proton ATPase (proton pump) acts as a primary transporter by pumping protons out of the cell, thereby creating pH and electrical potential differences across the plasma membrane. Transport of many solutes into and out of the cell involves secondary transporters whose ability to function

is directly dependent on the proton motive force created by the proton ATPase (for reviews see Serrano, 1989 and 1990; Michelet and Boutry, 1995). Inhibition of plasma membrane proton ATPase activity has been observed after mechanical shock to plant tissues (Hanson and Trewavas, 1982); mechanical shock includes cutting and slicing. The pH and solute content of the pre-treatment osmotica must also be considered as potential membrane transport inhibitors. The pre-treatment procedure almost certainly abolishes the acid-exterior pH gradient generated by the proton pump. The osmotica used all had a pH which was close to neutrality (data not shown), whereas the apoplast *in vivo* is generally acidic (Tetlow and Farrar, 1993; Husted and Schjoerring, 1995; Felle, 1998). The presence or absence of particular apoplastic solutes may also inhibit membrane transport. Apoplastic potassium (K^+) stimulates plasma membrane ATPase activity (Briskin, 1990; Briskin and Hanson, 1992). Evidence pointing to an inhibition of membrane transport due to the absence of apoplastic K^+ is presented in this chapter. As I highlighted above, beet discs pre-treated in an osmoticum which is devoid of KCl, achieved complete turgor regulation (to the target P_{cell} level of 0.6 MPa - 0.7 MPa) within 60 - 80 minutes (depending on the concentration of the sucrose pre-treatment osmoticum); 20 minutes longer than turgor regulation in the presence of apoplastic KCl (this chapter).

Potassium is a major nutrient in higher plants, where it plays a crucial role in turgor maintenance, charge balance and protein synthesis (Leigh and Wyn Jones, 1984). Addition of K^+, at micromolar concentrations, to K^+ starved barley and maize roots elicits membrane depolarisation and net H^+ efflux (Behl and Raschke, 1987; Kochian *et al.*, 1989). This net increase in H^+ efflux is due to stimulation of the plasma membrane proton ATPase (Briskin, 1990; Briskin and Hansen, 1992; Briskin and Gawienowski, 1996). From early work on this enzyme, it was suggested that stimulation of activity by K^+ might reflect the direct transport of this cation by the proton ATPase (Leonard, 1982 and references therein). Subsequent investigations, however, have revealed that this is not the case. Stimulation of the enzyme activity of a primary transporter by a cation is not always indicative of a direct role in the transport of that cation. For example, the ATPase activity of the animal sarcoplasmic reticulum Ca^{2+}-ATPase is stimulated by monovalent cations, including K^+, this clearly does not reflect direct K^+ transport by this enzyme (see Tonomura, 1986). However, K^+ stimulation of the sarcoplasmic reticulum

Ca^{2+}-ATPase is also accompanied by an increase in phosphoenzyme turnover in the enzyme reaction mechanism, suggesting that K^+ acts as an effector or modulator of ATPase activity (Tonomura, 1986 and references therein). Briskin and Gawienowski (1996) propose that this may also point to the mode of proton ATPase stimulation, by K^+, in higher plants.

The proton ATPase may not facilitate K^+ uptake into the cell directly, however, the proton motive force generated by the proton ATPase is essential in driving the secondary transport of K^+ into the cell (Briskin and Gawienowski, 1996; Maathuis and Sanders, 1996 and 1997). Two pathways for K^+ uptake across the plasma membrane have been identified - the high affinity K^+ pathway (Maathuis and Sanders, 1996 and 1997; Maathuis *et al.*, 1997) and the low affinity K^+ pathway (Maathuis and Sanders, 1996 and 1997). High affinity K^+ uptake from micromolar external K^+ levels is coupled to H^+ transport in a 1 : 1 H^+/K^+ symport. Low affinity K^+ uptake, induced at relatively high external K^+ levels in the millimolar range, is achieved by passive K^+ transport (down an electrochemical K^+ gradient) via a plasma membrane inwardly rectifying K^+ channel (K^+ IRC channel). A H^+/K^+ symporter and an IRC channel are illustrated in **Chapter 6, Figure 6.3**.

From the sucrose and/or KCl upward turgor regulation data presented in this chapter, it would appear that both sucrose and KCl are utilised for osmotic adjustment purposes in sugar beet taproot tissue. Wyse *et al.* (1986) presented data which demonstrated that a decrease in turgor pressure (P_{cell}) in sugar beet taproot discs, induced by an increase in external mannitol concentration, leads to an increase in sucrose uptake into the cell, driven by turgor dependent modulation of plasma membrane proton ATPase activity (the turgor dependent nature of the proton ATPase is demonstrated and discussed in **Chapter 6**). The turgor dependent regulation of sucrose transport in beet tissue is described in greater detail in the concluding discussion (**Chapter 7**).

Turning to the role of apoplastic K^+ in turgor regulation. Turgor pressure (P_{cell}) in red beet taproot tissue (also *Beta vulgaris* L.) is maintained at 0.5 MPa throughout the growing season (Perry *et al.*, 1987), whereas the osmotic pressure (Π_{cell}) reaches

approximately 1.0 MPa as the taproot reaches maturity (Perry *et al.*, 1987). Efflux analysis of solutes present in the cell wall of red beet taproot tissue has revealed that free apoplastic K^+ is present at concentrations sufficient to account for the difference between P_{cell} and Π_{cell} in this tissue (Leigh and Tomos, 1983). Furthermore, Bell and Leigh (1996) showed that turgor dependent K^+ transport in sugar beet taproot tissue modulates the K^+ concentration of the apoplast, thus facilitating turgor regulation. For example, an increase in turgor pressure, induced by decreasing the external mannitol concentration, leads to the initial loss of sucrose and K^+ out of the cell. Sucrose is selectively retrieved from the apoplast, whereas the K^+ is not. This leads to an accumulation of K^+ in the adjacent apoplast. This in turn, increases the osmotic pressure of the apoplast (Π_{wall}), resulting in a decrease in turgor pressure (P_{cell}) and turgor regulation (Bell and Leigh, 1996). The work carried out by Bell and Leigh (1996) is described further in **Chapter 7**. All the available evidence suggests that beet taproot tissue, *in vivo*, utilises K^+, rather than sucrose, for apoplastic osmotic adjustment purposes (Leigh and Tomos, 1983; Tomos *et al.*, 1992; Bell and Leigh, 1996).

Sugar beet disc pre-treatment in a highly dilute osmoticum, such as 0 mM mannitol-0.5 mM $CaSO_4$, leads to a large increase in turgor pressure (P_{cell}), due to water influx. Under closed system conditions this increase in P_{cell} does not initiate a downward turgor regulation mechanism; turgor pressure is maintained between 1.0 MPa and 1.2 MPa for the duration of the 90 minute time course (this chapter). However, I am inclined to be suspicious of these data. Wyse *et al.* (1986) and Bell and Leigh (1996) have clearly demonstrated that an increase in beet taproot turgor pressure (P_{cell}) induces solute efflux. In theory, solute efflux, under closed system conditions, should lead to the accumulation of solutes in the apoplast and a decrease in P_{cell}. It could be argued that failure to achieve downward turgor regulation, in closed system beet tissue (this chapter), casts some doubt on the apoplastic solute adjustment hypothesis. However, the upward turgor regulation data (this chapter) is far too compelling to ignore. As I highlighted in **Chapter 4**, an apparent upward turgor regulation mechanism in closed system plant tissue, may be due to water uptake from the apoplast. However, this scenario cannot account for the fact that turgor pressure (P_{cell}) is regulated upward to a single set point, which corresponds to P_{cell} in intact taproots *in vivo*. Furthermore, a

turgor pressure increase facilitated by apoplastic water uptake cannot account for the observed optimisation of turgor regulation in the presence of KCl.

I propose that the magnitude of the osmotic pressure step across the plasma membrane, leading to a rapid and large increase in cell turgor pressure, may inhibit membrane transport. Wyse *et al.* (1986) demonstrated that proton efflux, due to plasma membrane proton ATPase activity, is sharply reduced at high turgor pressures (see also **Chapter 6**). Inhibition of this major primary transporter is likely to inhibit the subsequent secondary transport of solutes necessary to achieve osmotic adjustment. In hindsight less extreme pre-treatment osmotica (100 - 200 mOsmoles kg^{-1} sucrose and/or KCl) should also have been used to determine whether downward regulation of P_{cell} is achieved in beet tissue under closed system conditions.

5.5, Summary and conclusions

- Turgor pressure (P_{cell}) in open system sugar beet taproot discs, aerated in mannitol osmotica of varying concentration, is initially determined by the osmotic pressure step across the plasma membrane.

- Over 48 hours P_{cell} is regulated upward and downward (depending on the concentration of the mannitol osmoticum) towards the P_{cell} level found in intact (physiologically comparable) taproots *in vivo* (0.6 - 0.7 MPa - Tomos *et al.*, 1992).

- Changes in P_{cell} are accompanied by corresponding changes in cell osmotic pressure (Π_{cell}), indicating that osmotic adjustment, leading to turgor regulation, is protoplastic in nature in this case.

- Under closed system conditions an increase in apoplastic solute concentration, brought about by the rupture of adjacent cells or by pre-treatment in an osmoticum, leads to an initial decrease in P_{cell}.

- This decrease in P_{cell} initiates a turgor regulation mechanism which is strongly dependent on the permeant properties of the apoplastic solutes.

- In freshly cut beet discs and beet discs pre-treated in sucrose and/or KCl osmotica, upward turgor regulation is achieved within 40 - 80 minutes. Turgor (P_{cell}) is regulated to a single set point (about 0.65 MPa) which corresponds to P_{cell} in intact (physiologically comparable) taproots *in vivo*.

- Turgor regulation in the presence of apoplastic KCl is achieved 20 minutes sooner than turgor regulation in the absence of apoplastic KCl, suggesting that apoplastic KCl facilitates membrane transport.

- Turgor regulation in a closed system is not accompanied by corresponding changes in Π_{cell}. This indicates that osmotic adjustment of solutes, necessary to achieve turgor regulation, is apoplastic rather than protoplastic in nature.

- An increase in P_{cell}, induced by beet disc pre-treatment in a highly dilute osmoticum does not initiate downward turgor regulation. This may be due to membrane transport inhibition induced by the magnitude of the osmotic pressure step across the plasma membrane.

- The upward turgor regulation mechanism in closed system sugar beet discs is comparable to the closed system turgor regulation mechanism observed in *Suaeda maritima* leaf epidermal cells (**Chapter 4**).

Chapter 6 : The turgor dependent nature of the plasma membrane proton ATPase in *Beta vulgaris* L. (sugar beet) taproot tissue

6.1, Introduction

The osmotic adjustment mechanisms described in **Chapter 3** through to **Chapter 5** are largely achieved by the stimulation and modulation of membrane transport processes. In plants, solute transport across the plasma membrane is driven by the electrochemical and pH gradients generated by the plasma membrane proton ATPase (Slayman, 1974; Poole, 1978; Spanswick, 1981; Sze, 1985; Serrano, 1989 and 1990; Michelet and Boutry, 1995). For, example, the secondary transporters which facilitate the transport of cations, anions (Michelet and Boutry, 1995), amino acids (Reinhold and Kaplan, 1984), sugars (Giaquinta, 1983), and growth regulators (Goldsmith, 1977) are (with few exceptions) dependent on plasma membrane proton ATPase activity (a primary transporter). **Figure 6.1, 6.2** and **6.3** illustrate the secondary transport of Na^+, sucrose and K^+ respectively.

6.1.1, The plasma membrane proton ATPase

Proton ATPases are the main electrogenic pumps in higher plants (Sze, 1985). The structure and mechanism of these enzymes are similar to that of other ATPases including the Na^+/K^+ ATPase (sodium pump) of animal cells, the gastric H^+/K^+ ATPase, and the Ca^{2+} ATPase of the sarcoplasmic reticulum (Serrano, 1989). These enzymes are collectively referred to as P-type ATPases because they form a phosphorylated intermediate during their reaction cycle (Serrano, 1989 and 1990).

The plant plasma membrane ATPase, a protein with a molecular mass of about 100 kD, is composed of a singe polypeptide that is predicted to be anchored in the plasma membrane by 10 membrane spanning regions (Wach *et al.*, 1992). It transports one proton per molecule of ATP hydrolysed, has a pH optimum of 6.6, and is inhibited by vanadate, dicyclohexylcarbodiimide, diethylstylbestrol, and erythrosin B (Michelet and

Boutry, 1995). Described as the "Master Enzyme" by Serrano in 1989, the plasma membrane proton ATPase plays a central role in plant physiology, controlling many important cellular functions, including: energisation of secondary solute transport, regulation of turgor pressure, cell wall extension, and intracellular pH regulation (Michelet and Boutry, 1995).

6.1.2, Modulation of plasma membrane proton ATPase activity

Plant plasma membrane proton ATPase activity can be modulated by a diverse range of stimuli. Light stimulates proton ATPase activity in guard cells, this drives K^+ influx, which in turn leads to an increase in guard cell turgor pressure and opening of the stomatal aperture (Assmann *et al.*, 1985; Shimazaki *et al.*, 1986; Serrano *et al.*, 1988). Plant growth regulators also interact with the proton ATPase. Auxin activates the enzyme in the growing regions of shoots, promoting cell elongation (Brummell and Hall, 1987; Rayle and Cleland, 1992). Abscisic acid, however, inhibits plasma membrane proton ATPase activity in a number of plant tissues, for example, proton ATPase inhibition by abscisic acid induces stomatal closure, prevents seed germination, and inhibits xylem loading in roots (Serrano, 1989 and references therein). Fusicoccin is a phytotoxin produced by the fungus *Fusicoccum amygdali* (Ballio *et al.*, 1964). In many ways fusicoccin mimics the plant growth regulator auxin in that it stimulates plasma membrane proton ATPase activity and hence proton efflux (Lanfermeijer and Prins, 1994; Jahn *et al.*, 1996; De Michelis *et al.*, 1996; De Boer, 1997; this chapter). Due to its growth regulator like mode of action, fusicoccin has been widely studied by plant physiologists (Marré, 1979). More recently, however, interest in fusicoccin has shifted to its receptor site on the plasma membrane and the role of this receptor in signal transduction (Aducci *et al.*, 1995; Ferl, 1996; Jahn *et al.*, 1997).

The plasma membrane proton ATPase modulators with the most relevance to the work described in this study (Chapter 3 through to 5) are NaCl and turgor pressure. In halophytes, NaCl stimulates proton ATPase activity (Ayala *et al.*, 1996; Wu and Seliskar, 1998). Ayala *et al.* (1996) found that *Salicornia bigelovii* plants grown in 200 mM NaCl had a 60 % higher plasma membrane proton ATPase activity than equivalent plants grown in the absence of NaCl. Wu and Seliskar (1998) reported similar findings

in the C_4 plant *Spartina patens*. Callus cultures of *Spartina patens* grown on 340 mM NaCl had a plasma membrane proton ATPase activity which was 250 % greater than in calli cultured in the absence of NaCl. Furthermore, studies on *Atriplex nummularia* have revealed that optimum plasma membrane proton ATPase activity is only achieved in the presence of NaCl in this species (Braun *et al.*, 1986).

Turning to the affect of turgor pressure (P_{cell}) on plasma membrane proton ATPase activity. A decrease in cell turgor pressure, induced by an increase in the concentration of the external medium, stimulates proton ATPase activity leading to an increase in proton efflux and a greater transmembrane potential difference. This turgor induced phenomenon has been observed in a number of plant tissues, including: *Arabidopsis thaliana* cell cultures (Curti *et al.*, 1993; Teodoro *et al.*, 1998), broad bean mesocarp cells (Ze-Sheng and Delrot, 1987), pea internode segments (Marré *et al.*, 1973; Cleland, 1975), oat coleoptiles (Cleland, 1975), *Senecio mikanioides* leaf segments (Reinhold *et al.*, 1984), carrot cell cultures (Reuveni *et al.*, 1987) and sugar and red beet taproot tissue (Kinraide and Wyse, 1986; Wyse *et al.*, 1986; Tomos, 1989; this chapter). It has been suggested that this mechanism plays an essential role in turgor regulation (Giaquinta, 1983; Reinhold *et al.*, 1984; Ze-Sheng and Delrot, 1987). In this chapter, the turgor dependent nature of proton efflux in sugar beet taproot tissue is investigated using a delightfully simple method. The turgor pressure (P_{cell}) of sugar beet taproot discs is modulated using mannitol osmotica of varying concentration. Proton efflux is determined by titration with potassium hydroxide (KOH), while the molecular basis of the proton efflux mechanism is determined using inhibitors and activators of membrane transport.

Figure 6.1, Sodium efflux

Sodium efflux, via a H^+/Na^+ antiport system, driven by plasma membrane proton ATPase activity (see Rausch *et al.*, 1996).

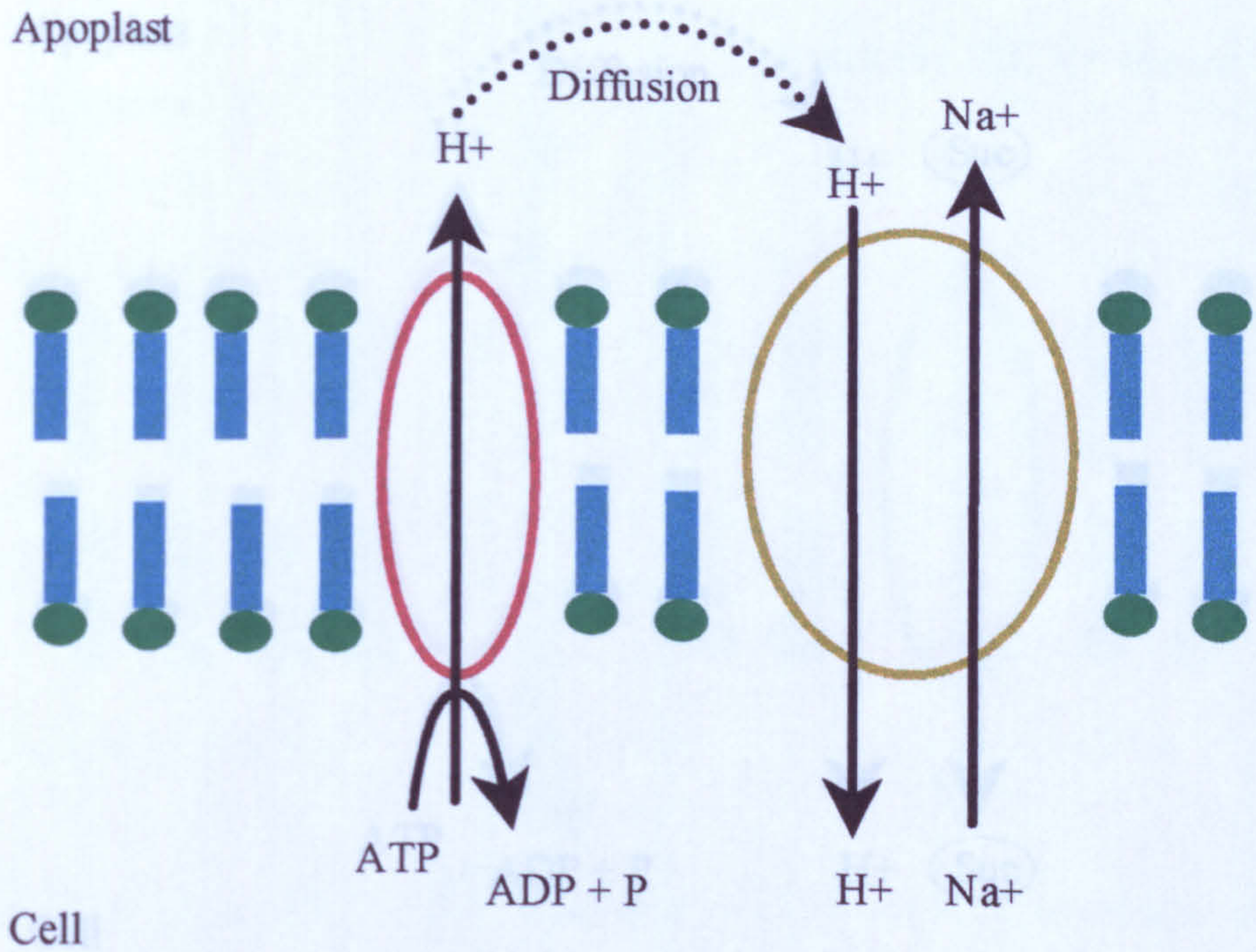


Figure 6.2, Sucrose influx

Sucrose uptake into the cell via a H^+/ sucrose symport system, driven by plasma membrane proton ATPase activity (see Giaquinta, 1983).

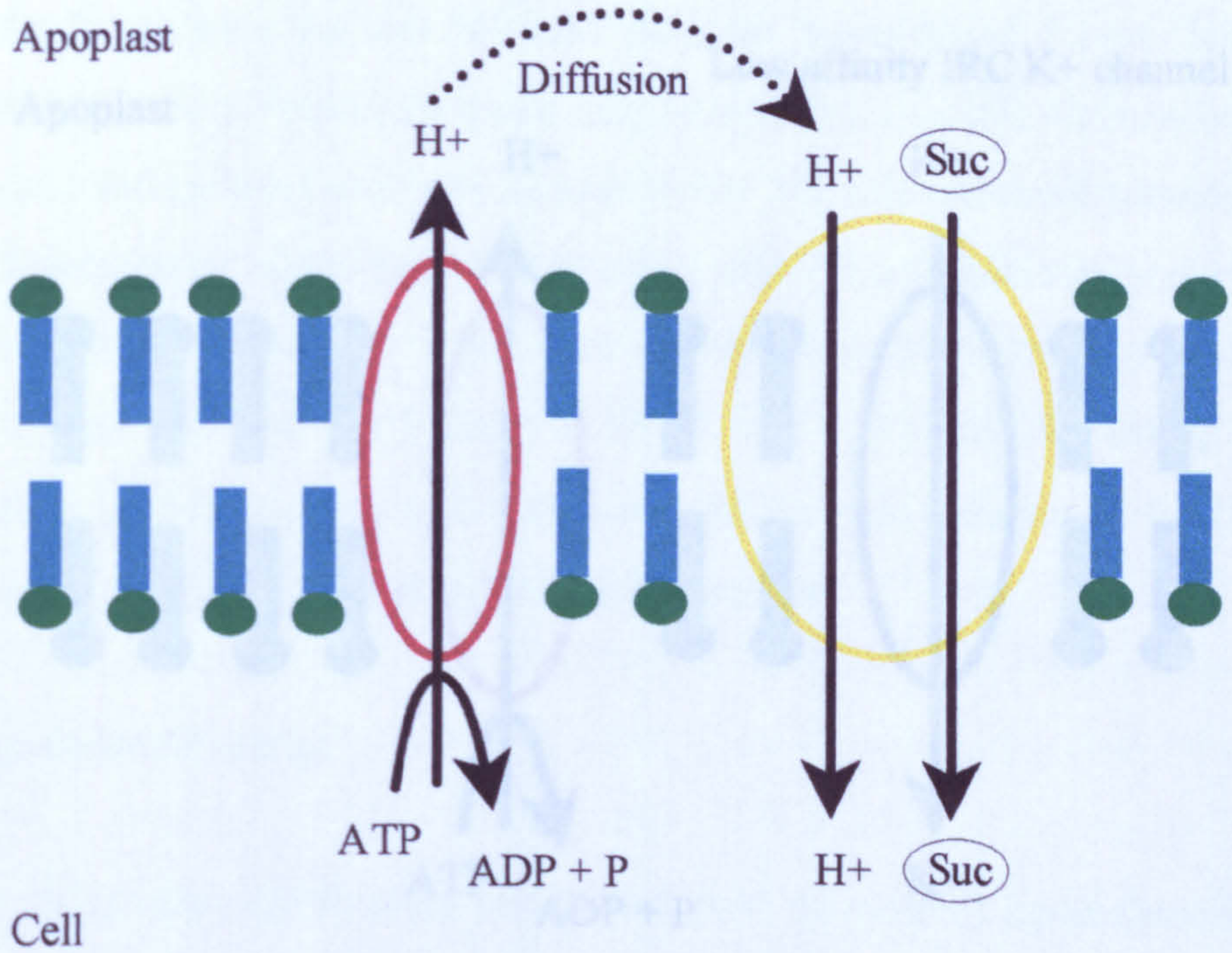
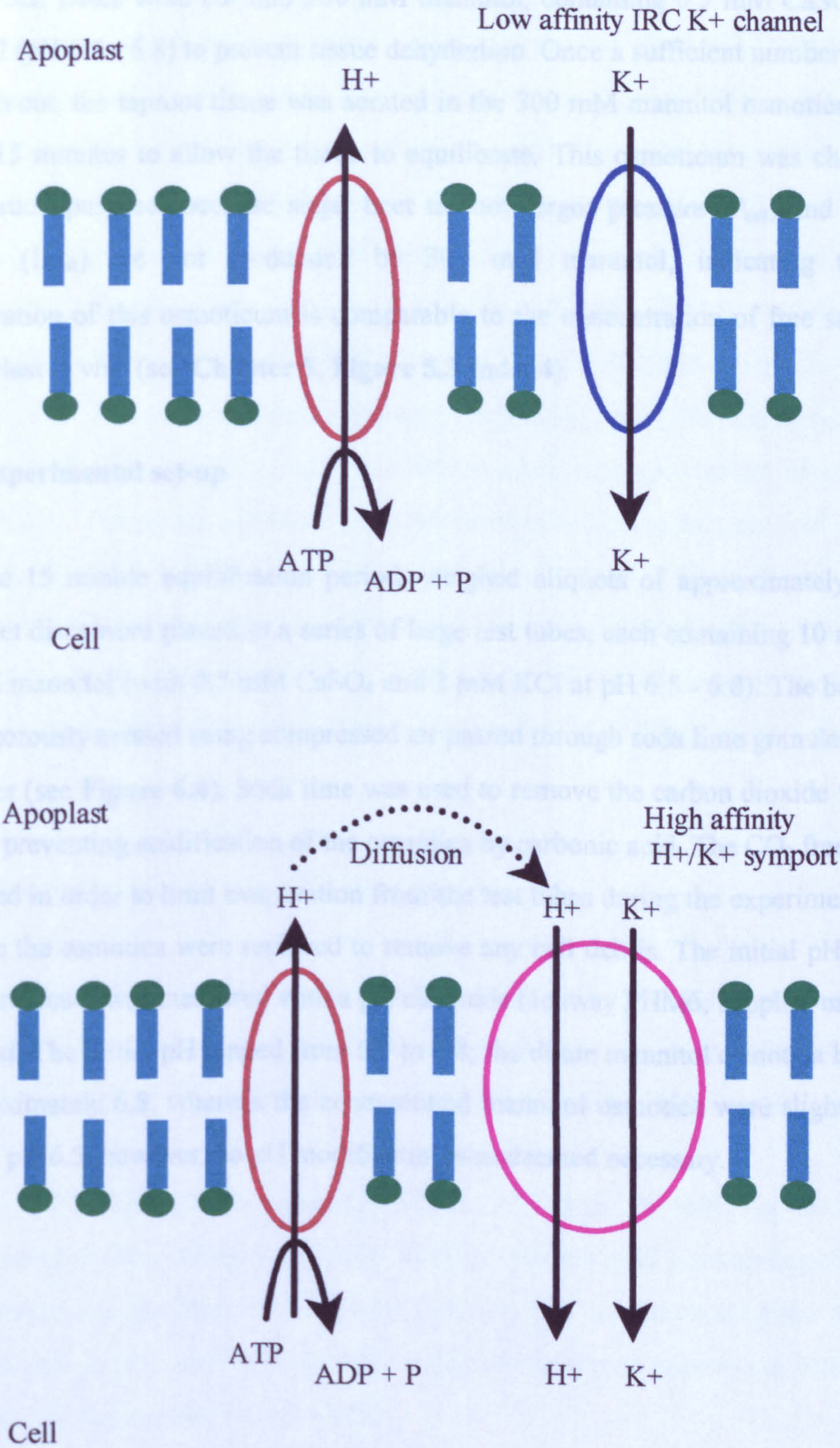


Figure 6.3, Low affinity and high affinity K^+ influx

Low affinity K^+ uptake, by diffusion down an electrochemical gradient, via an inwardly rectifying (IRC) K^+ channel, and high affinity K^+ uptake achieved by a 1 : 1 symport with H^+ (see Maathuis and Sanders 1996 and 1997).



6.2, Method

6.2.1, Sugar beet disc preparation

Sugar beet taproot discs were prepared from mature taproots as described in **Chapter 5, Section 5.2**. Discs were cut into 300 mM mannitol, containing 0.5 mM $CaSO_4$ and 2 mM KCl (pH 6.5 - 6.8) to prevent tissue dehydration. Once a sufficient number of discs had been cut, the taproot tissue was aerated in the 300 mM mannitol osmoticum for a further 15 minutes to allow the tissue to equilibrate. This osmoticum was chosen for equilibration purposes because sugar beet taproot turgor pressure (P_{cell}) and osmotic pressure (Π_{cell}) are not modulated by 300 mM mannitol, indicating that the concentration of this osmoticum is comparable to the concentration of free solutes in the apoplast *in vivo* (see **Chapter 5, Figure 5.3** and **5.4**).

6.2.2, Experimental set-up

After the 15 minute equilibration period, weighed aliquots of approximately 2 g of sugar beet discs were placed in a series of large test tubes, each containing 10 ml of 0 - 700 mM mannitol (with 0.5 mM $CaSO_4$ and 2 mM KCl at pH 6.5 - 6.8). The beet discs were vigorously aerated using compressed air passed through soda lime granules (BDH) and water (see **Figure 6.4**). Soda lime was used to remove the carbon dioxide from the air, thus preventing acidification of the osmotica by carbonic acid. The CO_2 free air was moistened in order to limit evaporation from the test tubes during the experiment. After 1 minute the osmotica were replaced to remove any cell debris. The initial pH of each fresh osmoticum was measured with a pH electrode (Jenway PHM6; supplier unknown) and noted. The initial pH ranged from 6.5 to 6.8; the dilute mannitol osmotica had a pH of approximately 6.8, whereas the concentrated mannitol osmotica were slightly more acidic at pH 6.5, however, no pH modification was deemed necessary.

6.2.3, Proton efflux determination

Proton efflux was determined by titration with potassium hydroxide (analytical grade; BDH). A pipette (Gilson; BDH) was used to expel 20 μl of 10 mM KOH into each test tube. Using a stopwatch and the pH electrode, the time taken for the pH to return to a predetermined level (pH 6.6) was noted. At pH 6.6 a further 20 μl of 10 mM KOH was added, and the procedure repeated - this continued for 4 hours. **Figure 6.5** shows a chart recorder trace of change in osmoticum pH with time. The time taken for each KOH addition (of known volume and concentration) to be neutralised by the proton efflux mechanism was then used to calculate the rate of proton efflux in nmol min^{-1} / 2 g tissue. The calculation was carried out as follows: 10 mM KOH is equivalent to 10 mmol l^{-1}. Therefore in 20 μl or 0.00002 l there is 10 x 0.00002 mmol/0.0002 mmol/200 nmol of KOH. This value divided by the time (in minutes) taken for the added KOH to be neutralised gives the rate of proton efflux in nmol min^{-1} per weight of sugar beet taproot tissue. The proton efflux data are presented as the mean and standard deviation of individual KOH additions in a representative experiment. Each experiment was repeated at least 4 times (data not shown). The n number varies from treatment to treatment due to the differential nature of the proton efflux mechanism (the n number stated in each figure legend corresponds to the number of KOH titrations carried out in 4 hours).

6.2.4, Membrane transport inhibitors and activators

Inhibitors and activators of membrane transport were used to determine the nature of the proton efflux mechanism. **Table 6.1** gives details of the membrane transport modulators used. Additional information is presented in bullet form below:

- **Anoxia** - sugar beet discs were starved of oxygen by bubbling the mannitol osmotica with nitrogen gas rather than air. The nitrogen gas was passed through soda lime granules and water in the same way as the compressed air in the control experiment. The recovery phase of the anoxia experiment was induced by simply restoring the air supply.

- **Potassium cyanide** - a 1 mM stock solution of potassium cyanide was carefully prepared under a fume hood. Due to the harmful nature of cyanide, the exact volume required was calculated to avoid any unnecessary excess. Potassium cyanide is strongly alkaline in aqueous solution. The mannitol osmotica made up with 1 mM KCN had an initial pH of between 10.5 and 10.8. The pH of the osmotica was adjusted with concentrated HCl (BDH) to between pH 6.5 - 6.8 prior to adding the sugar beet tissue. This procedure was carried out under supervision as acidifying KCN solution promotes the evolution of volatile HCN. Proton efflux determination, in the presence of cyanide, was carried out under a fume hood. Strict COSHH regulations were adhered to at all times.

- **p-Chloromercuriphenylsulphonic acid (PCMPS)** - a stock solution of 1 mM PCMPS was prepared and used to make up the mannitol-$CaSO_4$-KCl osmotica as required.

- **Erythrosin B (EB)** - erythrosin B is relatively insoluble in water, but readily dissolves in an alkali. A 100 μM stock solution of EB was made up by diluting aliquots of potassium hydroxide, containing EB, in de-ionised water. The pH of the EB stock solution was modified to pH 6.5 - 6.8 as required, using HCl.

- **Verapamil** - verapamil is insoluble in water. A 100 μM stock solution was prepared by dissolving the verapamil in 1 ml of absolute ethanol before making up the solution with de-ionised water. An identical volume of ethanol was also added to the control osmotica.

- **Nifedipine** - nifedipine is also insoluble in water and is only sparingly soluble in ethanol. A 100 μM stock solution was prepared by dissolving the nifedipine in 1 ml of acetone (BDH) before making up the solution with de-ionised water. An identical volume of acetone was also added to the control osmotica.

- **Fusicoccin (FC)** - a 10 μM stock solution was prepared in de-ionised water. The stock FC solution was then used to make up the 0 - 700 mM mannitol osmotica (with 0.5 $CaSO_4$ and 2 mM KCl).

Table 6.1, The membrane transport modulators used to determine the nature of the proton efflux mechanism in sugar beet taproot discs

Treatment	Concentration	Mode of action	Grade/Supplier
Nitrogen gas	N/A	Respiratory chain inhibitor	BOC
KCN	1 mM	Respiratory chain inhibitor	Analytical grade / BDH
PCMPS	1 mM	Proton ATPase inhibitor	Analytical grade / Sigma
EB	100 μM	Proton ATPase inhibitor	Analytical grade / Sigma
Verapamil	100 μM	Calcium channel blocker	Analytical grade / Sigma
Nifedipine	100 μM	Calcium channel blocker	Analytical grade / Sigma
FC	10 μM	Proton ATPase activator	Analytical grade / Sigma

Figure 6.4, Experimental set-up

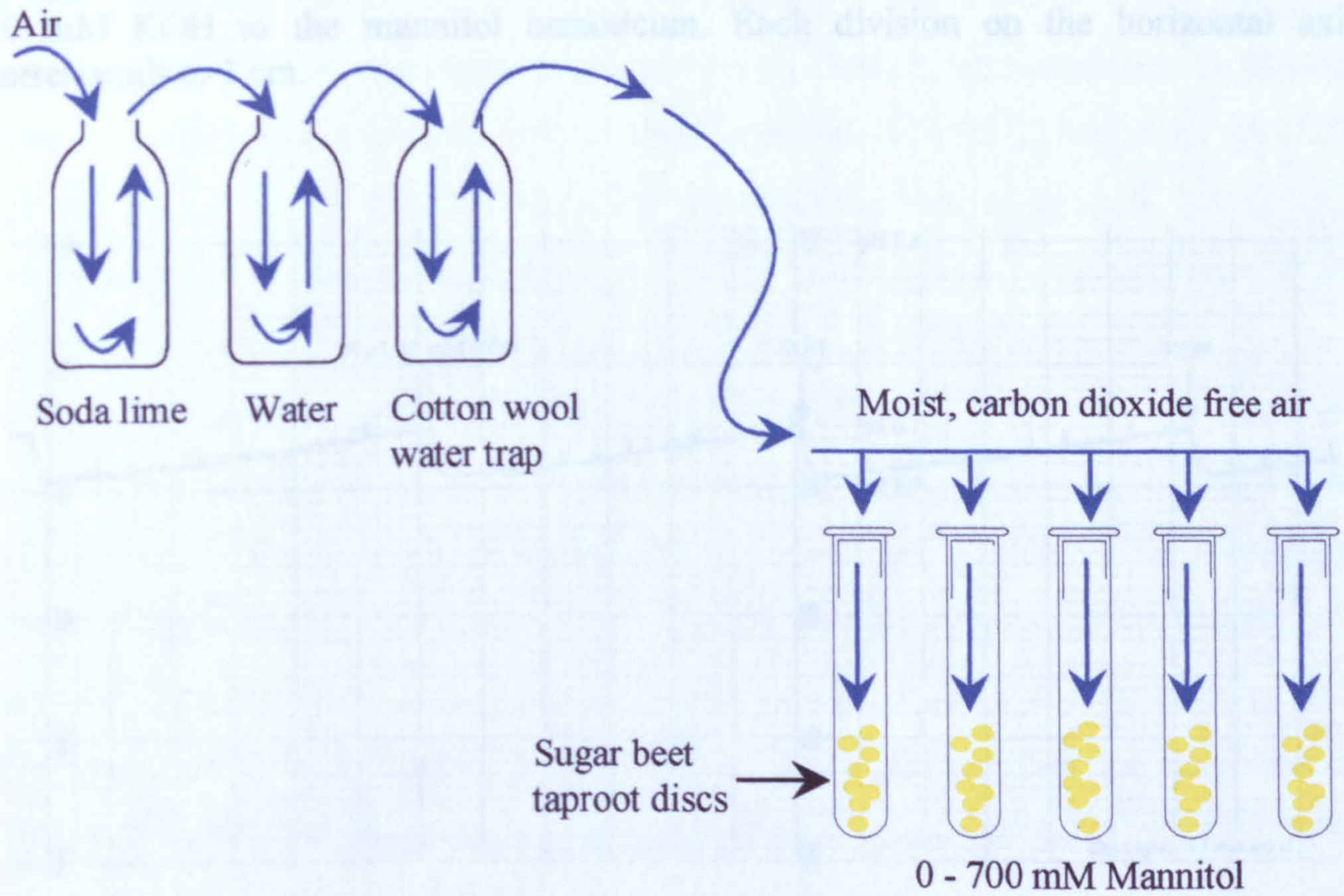
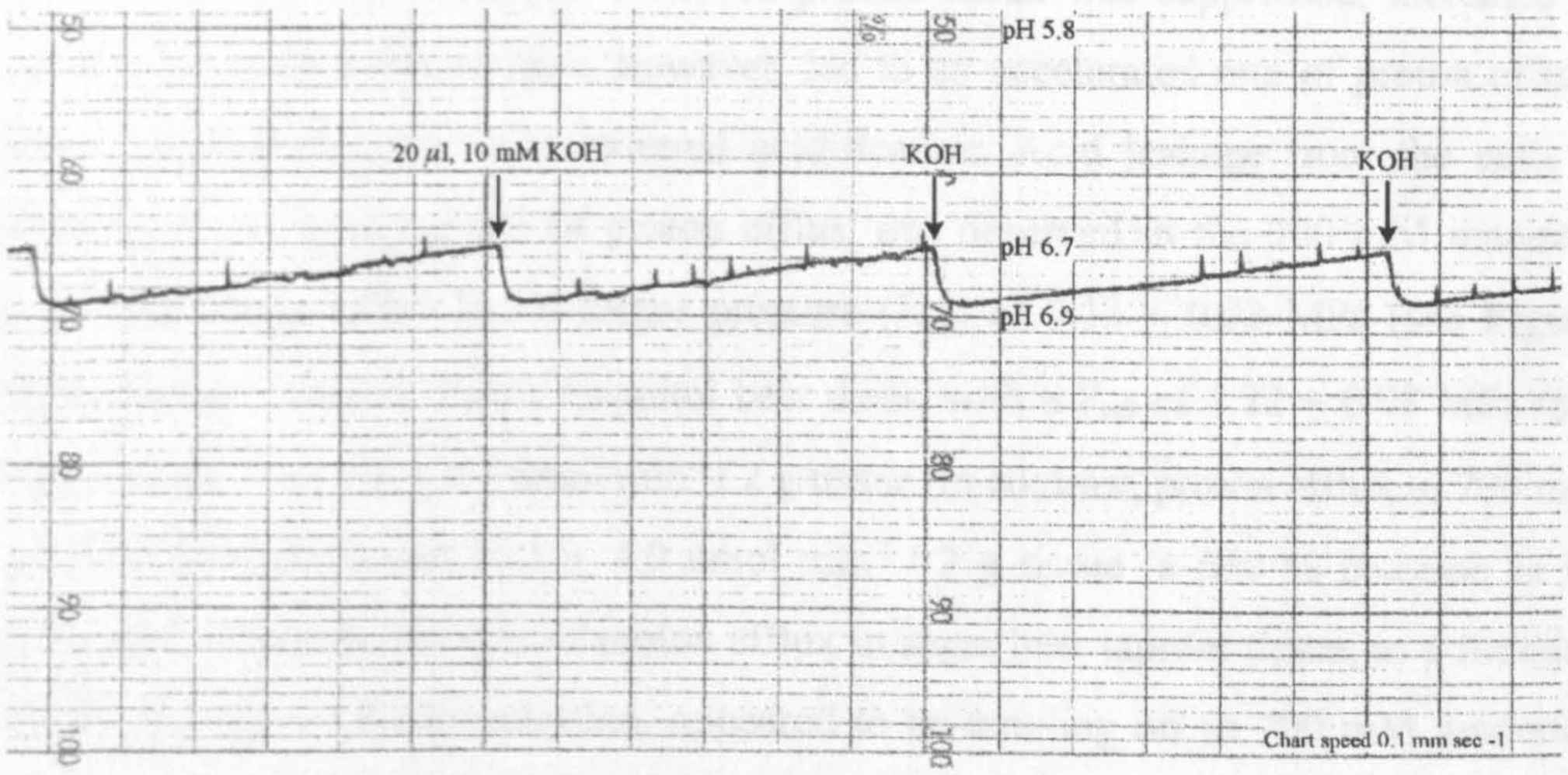


Figure 6.5, Acidification of an external osmoticum by sugar beet taproot tissue

Acidification of 10 ml of 700 mM mannitol (containing 0.5 mm $CaSO_4$ and 2 mM KCl) by approximately 2 g of sugar beet taproot tissue. Arrows indicate the addition of 20 μl, 10 mM KOH to the mannitol osmoticum. Each division on the horizontal axis corresponds to 1 cm.



6.3, Results

Turgor dependent proton efflux in sugar beet taproot tissue is demonstrated in **Figure 6.6**. Proton efflux was determined by titration with KOH, in approximately 2 g of sugar beet taproot discs, aerated in 0 - 700 mM mannitol with 0.5 mM $CaSO_4$ and 2 mM KCl, for 4 hours. In dilute mannitol osmotica proton efflux was suppressed, increases in external mannitol concentration, however, led to an accelerated rate of proton efflux, and a subsequent increase in external acidification. Acid leakage from the cells is unlikely, as the greatest rate of proton efflux was observed in the 700 mM mannitol sugar beet discs, which had a turgor pressure (P_{cell}) of 0.13 $\pm$ 0.03 MPa (see **Figure 6.14**). Proton efflux in 0 mM mannitol beet discs, with a P_{cell} of 1.22 $\pm$ 0.08 MPa (see **Figure 6.14**), was 3.5 $\pm$ 0.2 nmol min^{-1} / 2 g tissue. In contrast, proton efflux in 700 mM mannitol beet discs was 26.1 $\pm$ 4.0 nmol min^{-1} / 2 g tissue (a 650 % increase in H^+ extrusion). However, the rate of proton efflux in sugar beet taproot tissue, as a function of external mannitol concentration, appeared to be tapering off in 700 mM mannitol, suggesting that proton efflux was approaching its optimal level at this point. These data demonstrate that sugar beet storage parenchyma cells respond to a turgor/osmotic related stimulus by modulating a membrane transport mechanism, leading to changes in proton efflux.

Inhibitors and activators of membrane transport were used to determine the nature of this proton efflux mechanism. Anoxia and KCN are crude membrane transport inhibitors in that they inhibit aerobic respiration and subsequently inhibit ATP production. Inhibition of proton efflux, in sugar beet taproot tissue, by anoxia and KCN would be consistent with this mechanism being an active process requiring oxygen and ATP. **Figure 6.7** shows that anoxia suppressed proton efflux across the range of mannitol treatments. Proton efflux in 700 mM mannitol beet discs starved of oxygen was 6.7 $\pm$ 1.5 nmol min^{-1} / 2 g tissue, whereas in oxygen sufficient 700 mM mannitol discs proton efflux was 25.8 $\pm$ 4.1 nmol min^{-1} / 2 g tissue. The turgor dependent pattern of proton efflux was not completely abolished by the anoxia treatment. This may be due to the fact that the test tubes, in which the experiments were carried out, were completely open to ambient air. Inhibition by anoxia was found to be rapidly and

completely reversible. Proton efflux was restored to control values once the air supply was restored (**Figure 6.7**). Potassium cyanide (KCN) completely abolished proton efflux in all mannitol treatments (**Figure 6.8**).

Membrane transport inhibitors, more specific in nature, such as PCMPS and erythrosin B also inhibited proton efflux in sugar beet taproot tissue (**Figure 6.9 and 6.10**). PCMPS is a widely used sulfhydryl group inhibitor, which markedly inhibits sucrose uptake into excised sugar beet leaf and taproot tissue by inactivating the plasma membrane proton ATPase (Giaquinta, 1976 and 1983; Reinhold *et al.*, 1984; Wyse *et al.*, 1986). **Figure 6.9** illustrates that proton efflux was largely inhibited by 1 mM PCMPS across the range of mannitol treatments. Proton efflux was not completely abolished in the 300 mM, 500 mM and 700 mM mannitol osmotica, but only reached 1.0 ± 0.02 nmol min^{-1} / 2 g tissue, 1.3 ± 0.05 nmol min^{-1} / 2 g tissue, and 1.4 ± 0.05 nmol min^{-1} / 2 g tissue respectively. Erythrosin B (EB) is an effective and highly specific plasma membrane proton ATPase inhibitor (Cocucci and Marré, 1986; Beffagna and Romani, 1988; Curti *et al.*, 1993; Walker *et al.*, 1995). As little as 100 mM EB completely abolished proton efflux in sugar beet taproot discs, in all treatments (**Figure 6.10**).

To summarise, sugar beet taproot storage parenchyma cells respond to a decrease in cell turgor pressure (P_{cell}) or an increase in external osmotic pressure (Π_{ext}) by increasing plasma membrane proton ATPase activity. However, the mechanism of stimulus detection, leading to the modulation of the proton ATPase remains unclear.

The role of calcium as a possible secondary messenger in the sequence of events leading to plasma membrane proton ATPase modulation was investigated using widely studied calcium channel blockers (for a review on calcium regulation and signalling in plants see Bush, 1995). There are currently two major groups of chemically distinctive calcium channel blockers, the phenylalkylamines, such as verapamil, methoxyverapamil, desmethoxyverapamil, diltiazem and bepridil, and 1,4-dihydropyridines, such as nifedipine and nitrendipine (Nayler and Horowitz, 1983; Miller and Freedman, 1984; Graziana *et al.*, 1988). Two calcium channel blockers (an

example from each group) were chosen to determine the effect of calcium flux inhibition on proton efflux in sugar beet taproot tissue. **Figure 6.11** and **6.12** illustrate that verapamil and nifedipine had no effect on proton efflux. The proton efflux pattern in calcium channel blocker treated beet discs was indistinguishable from that in control sugar beet discs. However, no positive control was carried out to determine whether the calcium channel blockers were effective. Therefore, I cautiously conclude that Ca^{2+} is not involved in the turgor/osmotic sensing mechanism, leading to plasma membrane proton ATPase modulation in this case.

Fusicoccin (FC) is a phytotoxin which strongly stimulates proton efflux in plant tissue (Rayle, 1973; Senn and Goldsmith, 1988; Lüthen *et al.*, 1990; this chapter, Figure 6.13). This increase in H^+ extrusion in the presence of fusicoccin is due to plasma membrane proton ATPase stimulation (Johansson *et al.*, 1993; Rasi-Caldogno *et al.*, 1993; Lanfermeijer and Prins, 1994; Jahn *et al.*, 1996). This stimulation of proton efflux by fusicoccin is demonstrated in **Figure 6.13**. The presence of 10 μM FC increased proton efflux, in sugar beet taproot discs, to around 40 nmol min^{-1} / 2 g tissue throughout the range of mannitol treatments. The differential response to turgor pressure (P_{cell}) or external osmotic pressure (Π_{ext}) was apparently abolished. These data suggest that the site of turgor/osmotic responsiveness, in sugar beet storage parenchyma cells, is the same as or "up stream" from that of fusicoccin sensitivity.

Figure 6.14 demonstrates the modulation of sugar beet taproot cell turgor pressure by external mannitol osmotica of varying concentration. Turgor pressure in sugar beet discs aerated in 0 mM, 100 mM, 300 mM, 500 mM and 700 mM mannitol (each containing 0.5 mM $CaSO_4$ and 2 mM KCl), for 4 hours, were 1.22 $\pm$ 0.08 MPa, 0.97 $\pm$ 0.08 MPa, 0.65 $\pm$ 0.1 MPa, 0.39 $\pm$ 0.05 MPa and 0.13 $\pm$ 0.03 MPa respectively. Turgor pressure was measured using the cell pressure probe (see **Chapter 2, Section 2.2**). The data are presented as mean and standard deviation, n is 15 (5 beet discs, 3 cells per disc).

Figure 6.6, Turgor/osmotic dependent proton efflux in sugar beet taproot discs

Mean proton efflux in approximately 2 g of sugar beet taproot tissue aerated in 10 ml of 0 - 700 mM mannitol, with 0.5 mM $CaSO_4$ and 2 mM KCl, over 4 hours. Data are presented as mean and standard deviation of individual KOH additions, n is 5, 15, 25 and 30 in the 0 - 100 mM, 300 mM, 500 mM and 700 mM mannitol osmotica respectively.

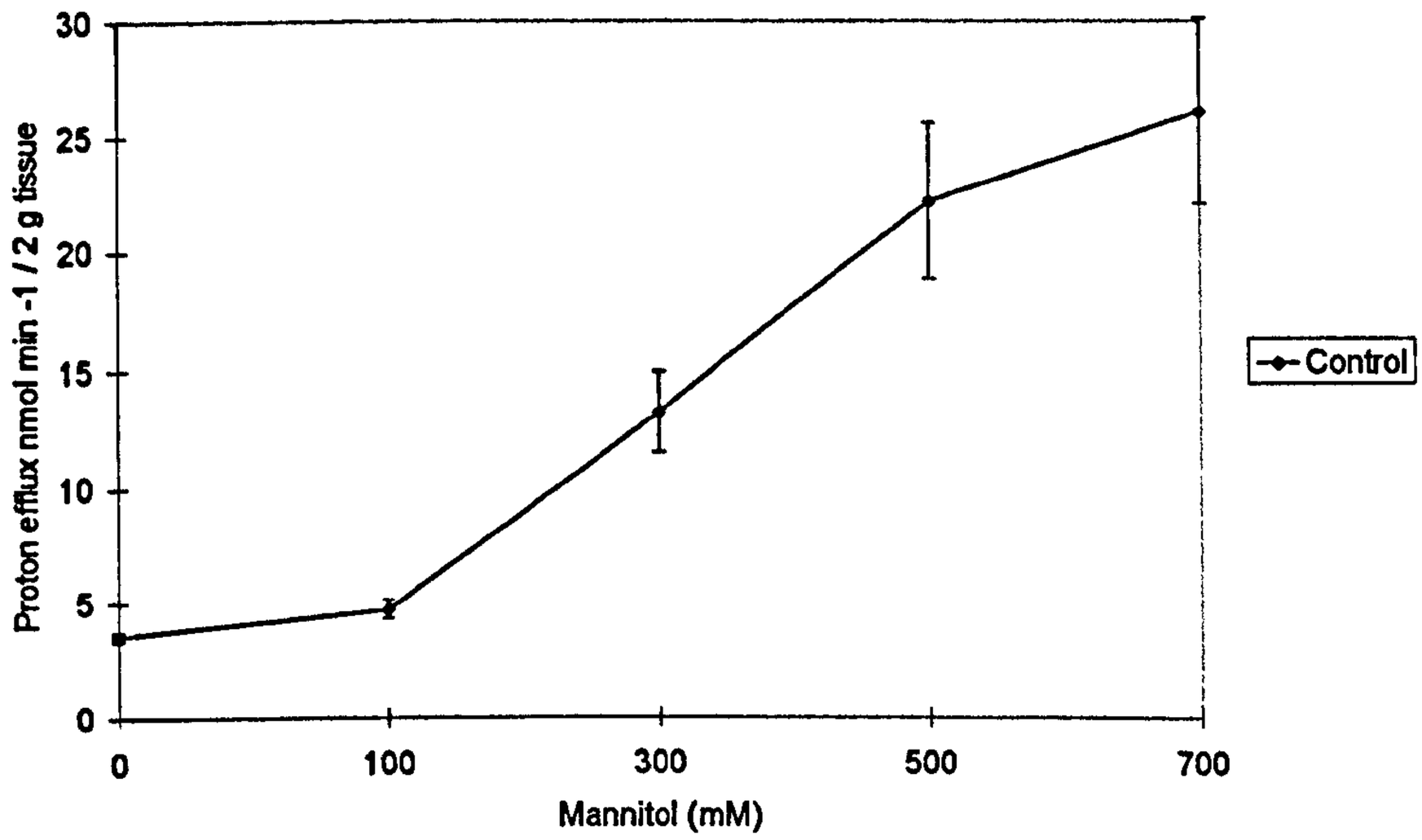


Figure 6.7, Reversible inhibition of proton efflux by anoxia

Mean proton efflux in sugar beet taproot tissue in 10 ml of 0 - 700 mM mannitol, containing 0.5 mM $CaSO_4$ and 2 mM KCl, with sufficient or in the absence of oxygen, over 4 - 8 hours. Data are presented as mean and standard deviation of individual KOH additions, n is 10, 30, 50 and 60 in control 0 - 100 mM, 300 mM, 500 mM and 700 mM mannitol osmotica respectively (over 8 hours). In the anoxia treated discs n is 2 and 5 in 0 -100 mM and 300 - 700 mM mannitol osmotica respectively (over the first 4 hours). In the recovery beet discs n is 1, 5, 15, 25, and 30 in 0 mM, 100 mM, 300 mM, 500 mM and 700 mM mannitol osmotica respectively (over the last 4 hours).

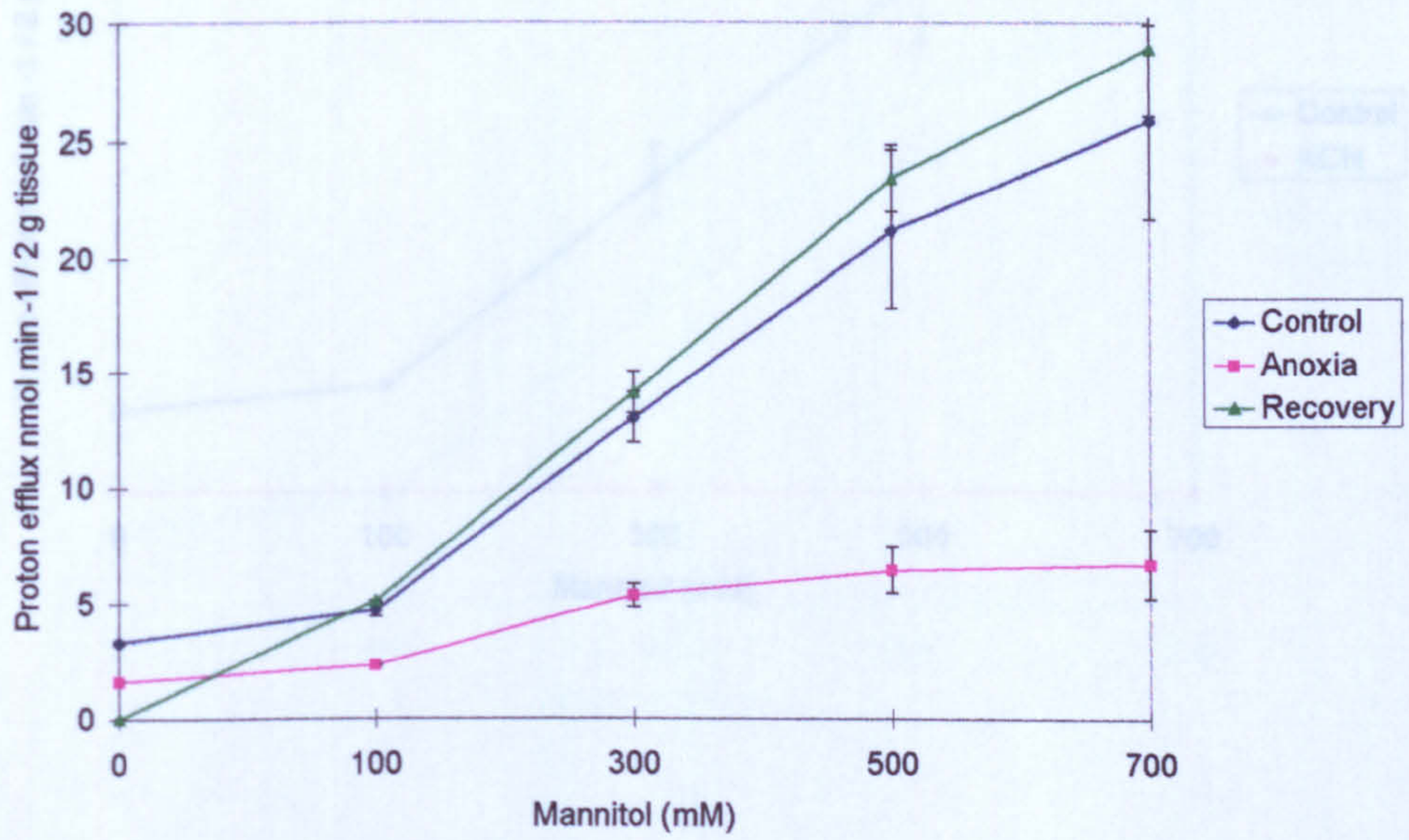


Figure 6.8, Complete inhibition of proton efflux by potassium cyanide

Mean proton efflux in sugar beet taproot tissue aerated in 10 ml of 0 - 700 mM mannitol, containing 0.5 mM $CaSO_4$ and 2 mM KCl, in control and 1 mM KCN treated sugar beet discs, over 4 hours. Data are presented as mean and standard deviation of individual KOH additions, n is 5, 15, 25 and 30 in control 0 - 100 mM, 300 mM, 500 mM and 700 mM mannitol osmotica respectively. In KCN treated discs n is 1.

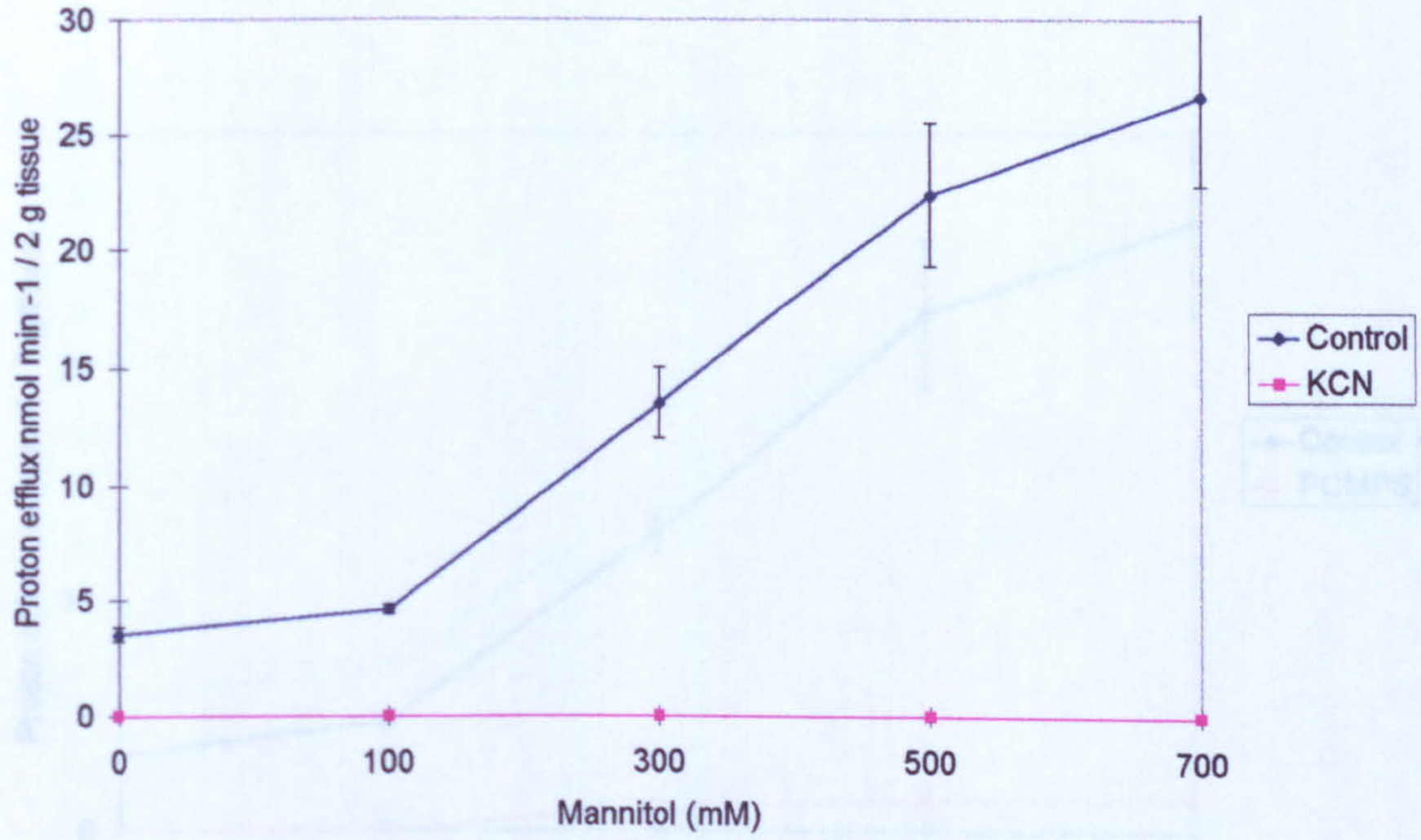


Figure 6.9, Inhibition of proton efflux by PCMPS

Mean proton efflux in sugar beet taproot tissue aerated in 10 ml of 0 - 700 mM mannitol, containing 0.5 mM $CaSO_4$ and 2 mM KCl, in control and 1 mM PCMPS treated sugar beet discs, over 4 hours. Data are presented as mean and standard deviation of individual KOH additions, n is 5, 15, 25 and 30 in control 0 - 100 mM, 300 mM, 500 mM and 700 mM mannitol osmotica respectively. In PCMPS treated discs n is 1 and 5 in 0 - 100 mM and 300 - 700 mM mannitol osmotica respectively.

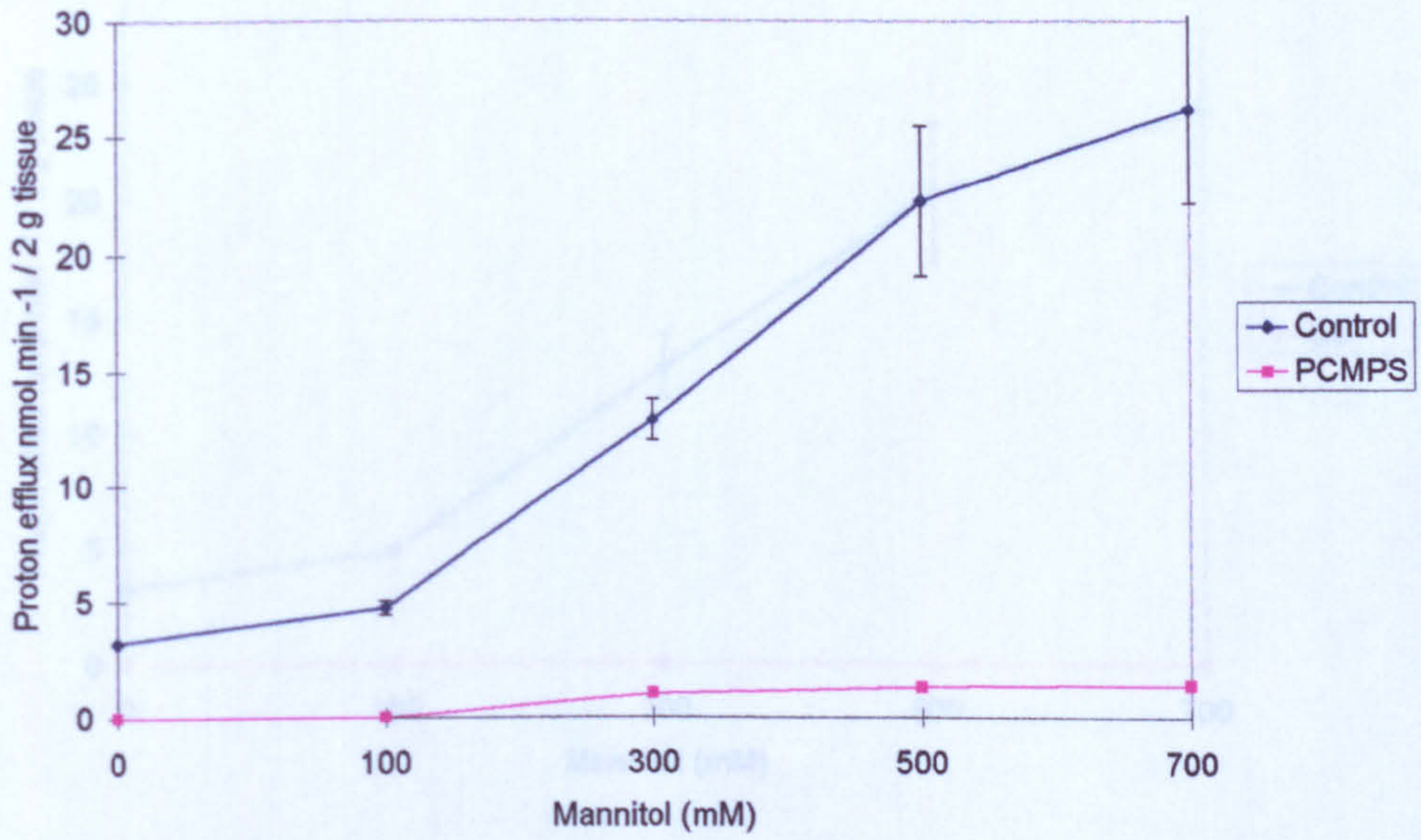


Figure 6.10, Complete inhibition of proton efflux by erythrosin B (EB)

Mean proton efflux in sugar beet taproot tissue aerated in 10 ml of 0 - 700 mM mannitol, containing 0.5 mM $CaSO_4$ and 2 mM KCl, in control and 100 μM EB treated sugar beet discs, over 4 hours. Data are presented as mean and standard deviation of individual KOH additions, n is 5, 15, 25 and 30 in control 0 - 100 mM, 300 mM, 500 mM and 700 mM mannitol osmotica respectively. In EB treated discs n is 1.

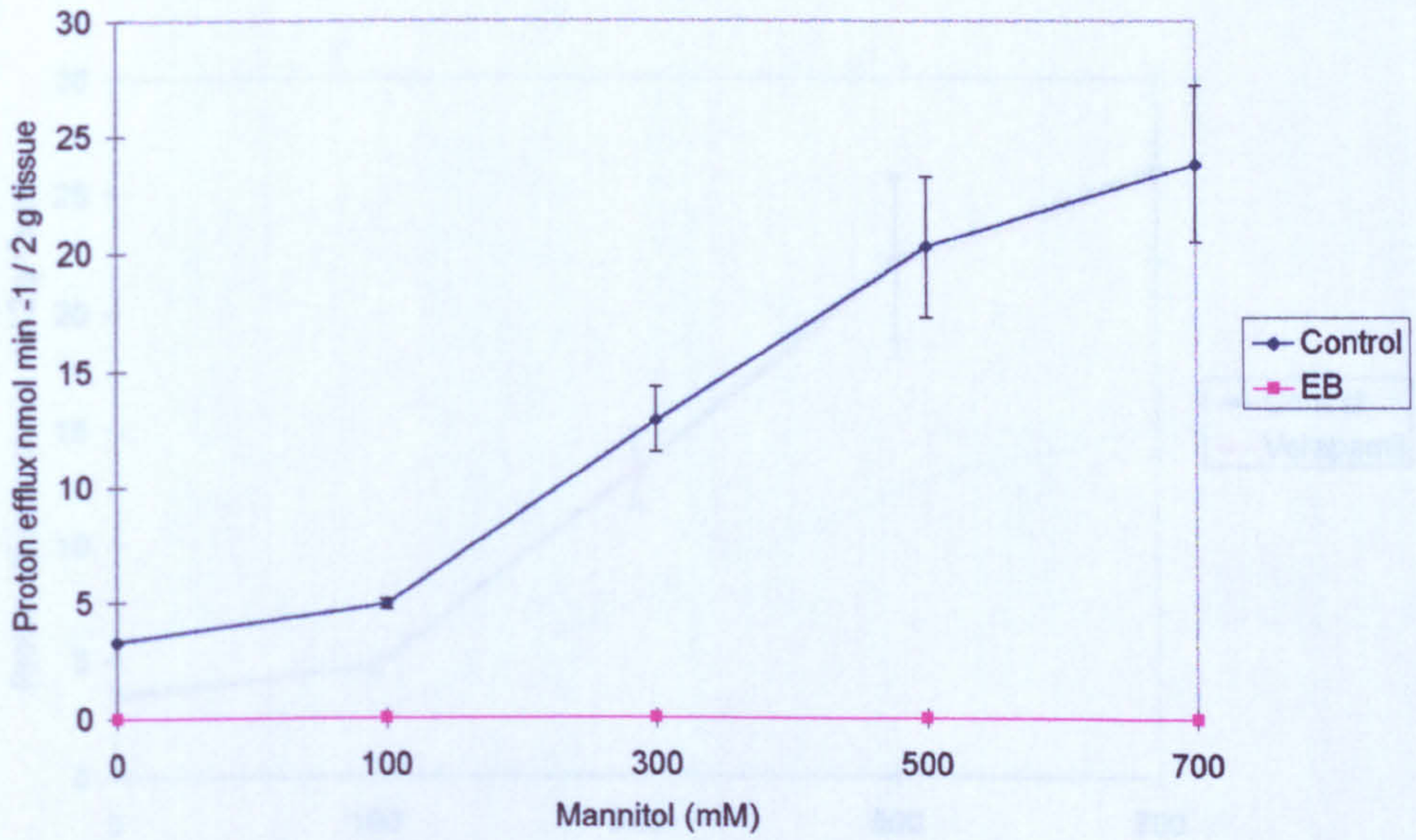
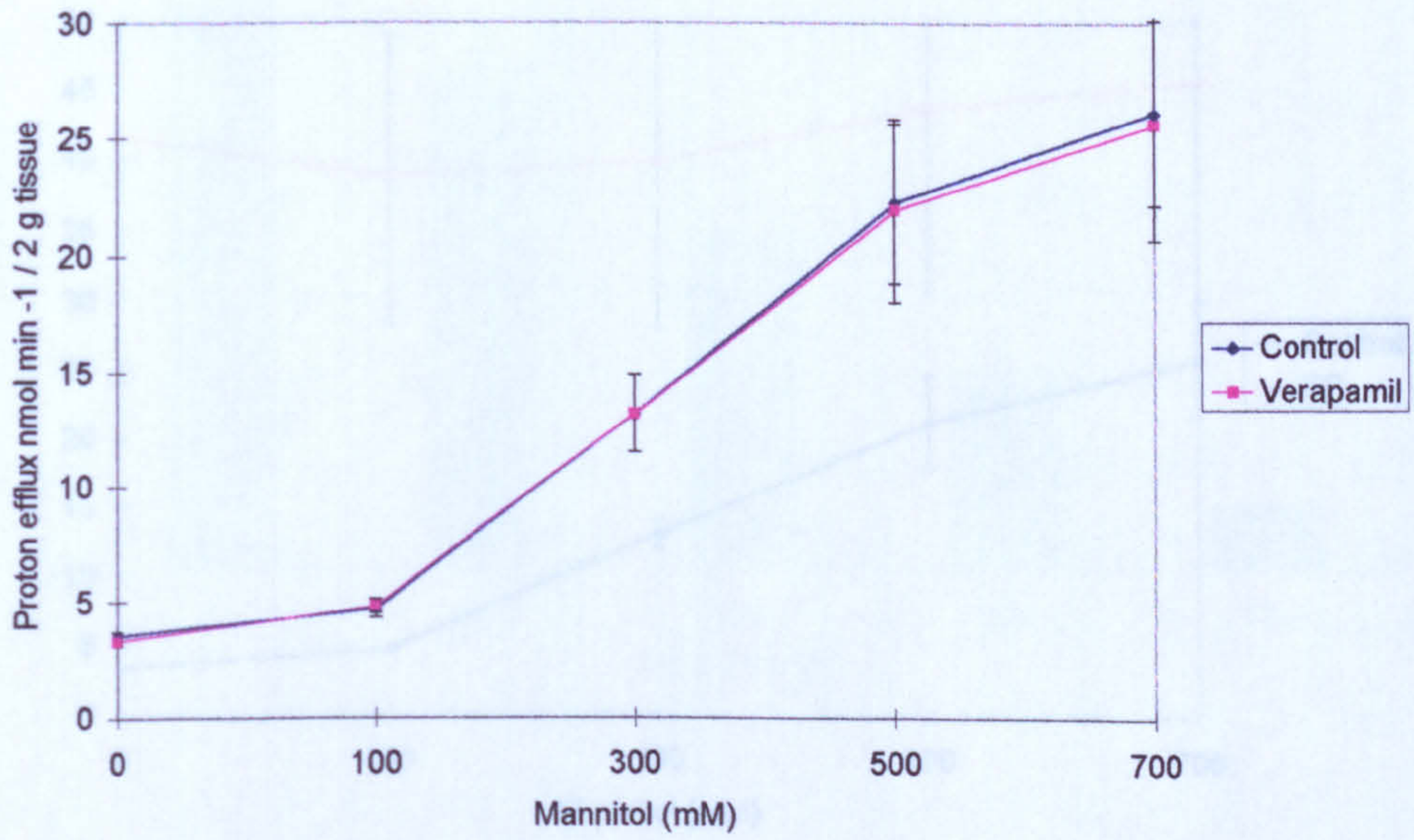


Figure 6.11 and 6.12, Proton efflux in the presence of verapamil and nifedipine

Mean proton efflux in sugar beet taproot tissue aerated in 10 ml of 0 - 700 mM mannitol, containing 0.5 mM $CaSO_4$ and 2 mM KCl, in control and 100 μM verapamil (**Figure 6.11**, upper figure) and nifedipine (**Figure 6.12**, lower figure) treated sugar beet discs, over 4 hours. Data are presented as mean and standard deviation of individual KOH additions, n is 5, 15, 25 and 30 in control and treated 0 - 100 mM, 300 mM, 500 mM and 700 mM mannitol osmotica respectively.



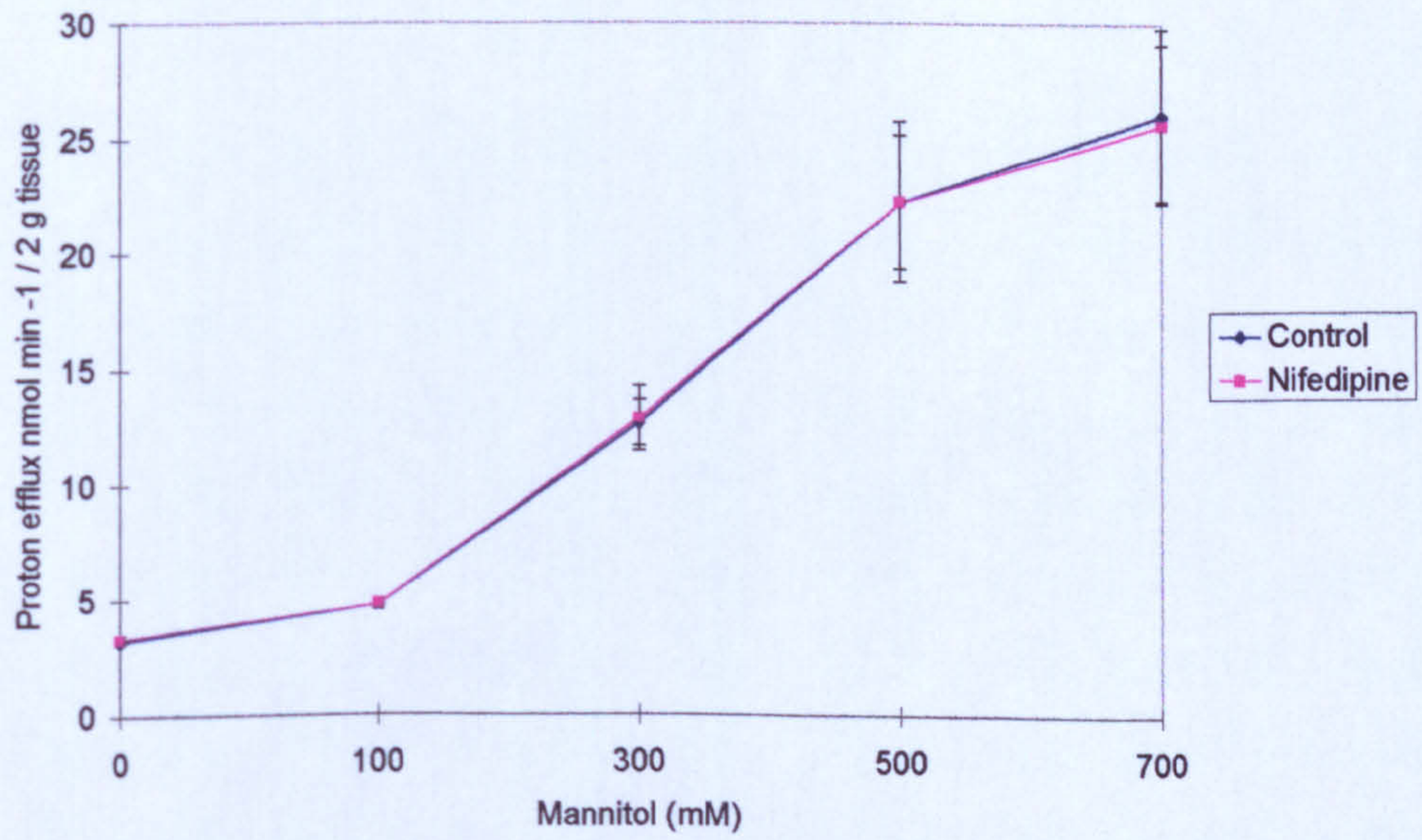


Figure 6.13 , Proton efflux stimulation in the presence of fusicoccin (FC)

Mean proton efflux in sugar beet taproot tissue aerated in 10 ml of 0 - 700 mM mannitol, containing 0.5 mM $CaSO_4$ and 2 mM KCl, in control and 10 μM FC treated sugar beet discs, over 4 hours. Data are presented as mean and standard deviation of individual KOH additions, n is 5, 15, 25 and 30 in control 0 - 100 mM, 300 mM, 500 mM and 700 mM mannitol osmotica respectively. In FC treated discs n is 40 throughout.

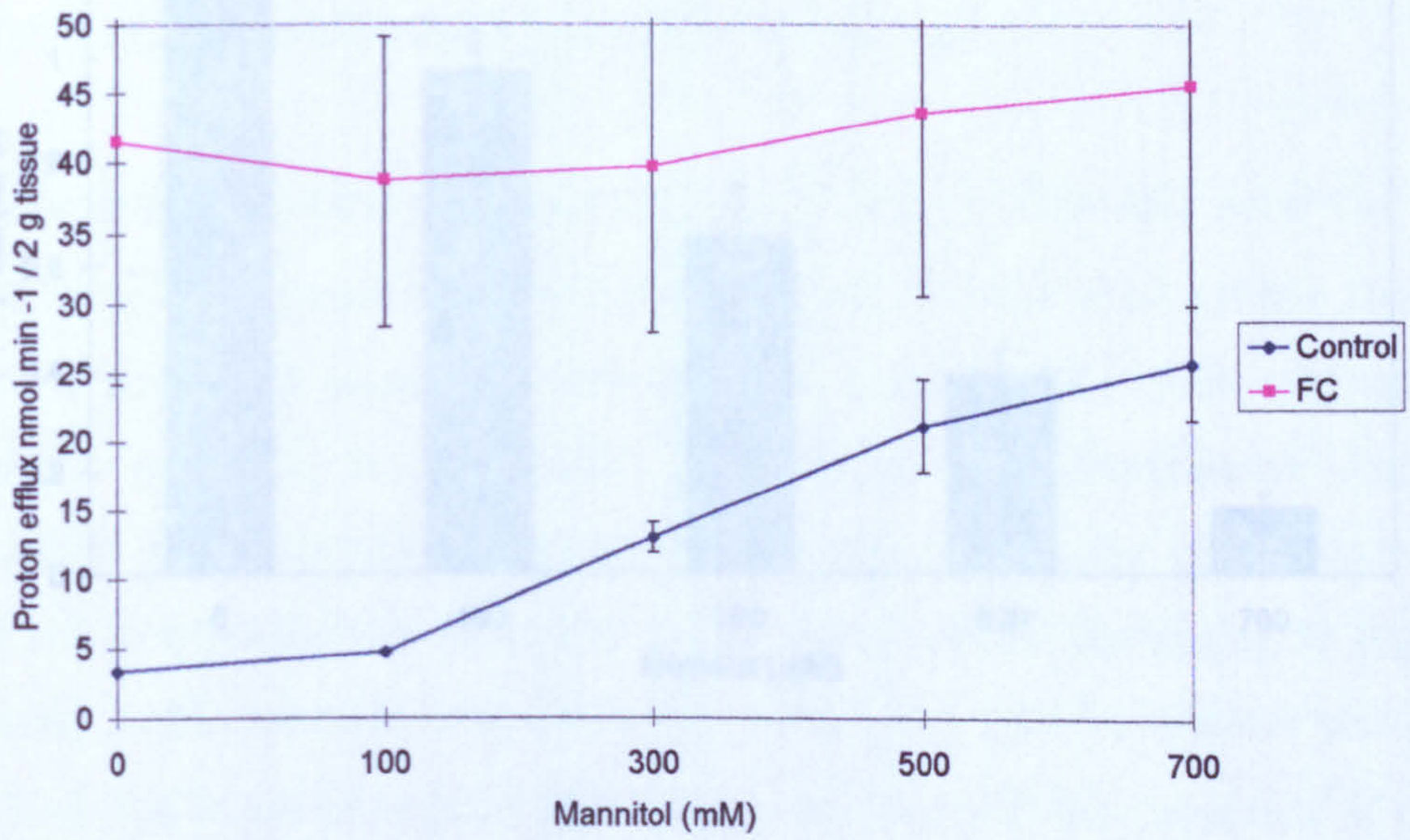
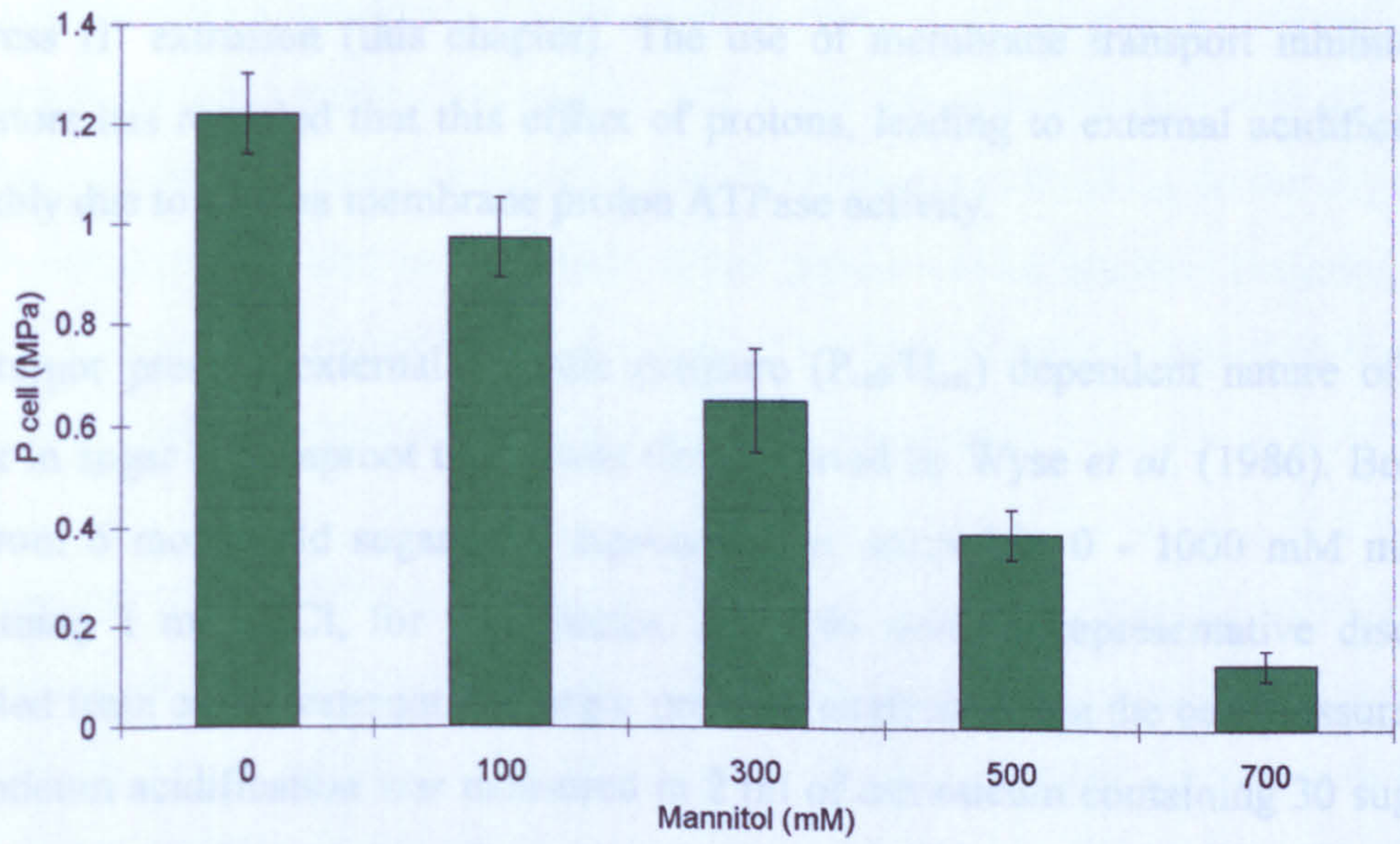


Figure 6.14, Turgor pressure (P_{cell}) modulation by external mannitol

Turgor pressure (P_{cell}) in sugar beet taproot discs aerated in 0 - 700 mM mannitol, containing 0.5 mM $CaSO_4$ and 2 mM KCl, for 4 hours. Data are presented as mean and standard deviation, n is 15 (5 beet discs, 3 cells per disc).



6.4, Discussion

Changing the turgor pressure of sugar beet taproot discs, using mannitol osmotica of varying concentration, modulates the rate of proton efflux from the tissue. A decrease in cell turgor pressure (P_{cell}) stimulates proton efflux, whereas an increase in P_{cell} appears to suppress H^+ extrusion (this chapter). The use of membrane transport inhibitors and activators has revealed that this efflux of protons, leading to external acidification, is probably due to plasma membrane proton ATPase activity.

The turgor pressure/external osmotic pressure (P_{cell}/Π_{ext}) dependent nature of proton efflux in sugar beet taproot tissue was first reported by Wyse *et al.* (1986). Beet discs cut from 6 month old sugar beet taproots were aerated in 0 - 1000 mM mannitol, containing 1 mM KCl, for 90 minutes. After 90 minutes representative discs were sampled from each treatment for turgor pressure analysis, using the cell pressure probe. Osmoticum acidification was measured in 2 ml of osmoticum containing 30 sugar beet discs, using a pH electrode. The change in pH (Δ pH) over 90 minutes was plotted against beet disc turgor pressure. Their findings revealed that the magnitude of external acidification was dependent on cell turgor pressure (P_{cell}). Acidification increased as P_{cell} decreased, reaching an optimum level at 0.3 MPa (Δ pH 0.7 - 1.0), before trailing off towards the lower extremes of cell turgor pressure.

The data presented by Wyse *et al.* (1986) are largely in agreement with the data presented in this chapter. However, the Wyse *et al.* (1986) data points to an acidification optimum at a turgor pressure (P_{cell}) of 0.3 MPa, whereas the data in this chapter suggests that acidification continues to decrease, with decreasing turgor pressure, beyond 0.3 MPa. The greatest rate of proton efflux was measured in sugar beet discs aerated in 700 mM mannitol, with a P_{cell} value of approximately 0.1 MPa. However, proton efflux did appear to be levelling out at this point, suggesting that a further decrease in P_{cell} may suppress any further increase in H^+ extrusion. This discrepancy between the Wyse *et al.* (1986) data and the data presented here, may be attributed to the pH of the external osmotica. The initial pH, and the magnitude of pH change over time due to proton efflux, is likely to affect membrane transport processes.

For example, the plasma membrane proton ATPase has a pH optimum of about 6.6 (Michelet and Boutry, 1995), any deviation from this pH value suppresses proton ATPase activity. Lanfermeijer and Prins (1994) presented data which showed the pH dependent nature of proton ATPase activity. At pH 5.5, 6.5, and 7.5 proton ATPase activity, in *Avena sativa* (oat) plasma membrane vesicles, was 2.0, 3.5, and 1.0 μmol min^{-1} mg^{-1} protein respectively. In this chapter, the pH of the external osmotica were maintained within a narrow range (6.6 - 6.9) by frequent titration with KOH, thus ensuring that the only variable parameter in the system was P_{cell}/Π_{ext}. In the Wyse *et al.* (1986) investigation, and in the majority of proton efflux studies discussed below external acidification was allowed to continue unchecked over a period of time, thus introducing a second variable parameter (pH) into the system.

Kinraide and Wyse (1986) looked at the P_{cell}/Π_{ext} dependent nature of proton efflux in sugar beet taproot tissue by measuring the membrane electrical potential difference (E_m) of tissue bathed in mannitol osmotica of varying concentration. Tapering wedges of tissue, approximately 6 mm in cross section, were excised from mature sugar beet taproots. The sugar beet tissue was bathed in a medium containing 1 mM NaCl, 1 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 0.25 mM $MgSO_4$ and 0 - 800 mM mannitol, at pH 5.0. Measurement of E_m was carried out using microelectrodes, as described by Kinraide and Etherton (1980). The data obtained revealed that membrane electrical polarity increased as the external mannitol concentration increased. At 0 mM, 400 mm and 800 mM mannitol concentrations, membrane E_m was - 80 $\pm$ 2 mV, - 129 $\pm$ 4 mV and - 136 $\pm$ 17 mV respectively. These data reflect the more vigorous H^+ extrusion from sugar beet taproot tissue at the lower end of the turgor pressure (P_{cell}) range.

Turgor/external osmotic pressure (P_{cell}/Π_{ext}) induced proton efflux has been reported in a number of other plant tissues. Reinhold *et al.* (1984) measured external acidification in mannitol osmotica containing *Senecio mikanioides* leaf segments. *Senecio mikanioides* leaves, approaching full expansion, were selected and the lower epidermis removed. The leaves were cut into 4 mm^2 sections and equilibrated for 90 minutes in 0.1 mM $CaSO_4$. After the equilibration period, 0.5 g aliquots of tissue were placed into 10 ml volumes of 0 - 300 mM mannitol, containing 0.2 mM $CaSO_4$ and 10 mm KCl. The pH

of the osmotica was measured initially and after 3 hours, using a pH electrode. During this time the pH of the 0 mM and 300 mM mannitol osmotica decreased by 0.2 and 0.7 pH units respectively, indicating that the *Senecio mikanioides* leaf segments in the 300 mM mannitol osmoticum had an accelerated rate of H^+ extrusion.

Teodoro *et al.* (1998) measured P_{cell}/Π_{ext} dependent proton efflux in *Arabidopsis thaliana* cells (0.1 g ml^{-1}) suspended in 0.5 mM BTP-MES buffer, pH 6.25, containing 0.5 mM $CaSO_4$, 2 mM KCl and 0 - 400 mM mannitol. Cells were harvested from the culture medium and equilibrated in 0.5 mM BTP-MES buffer, pH 5.0, for 18 hours prior to mannitol treatment. Proton efflux (μmol g^{-1} fw h^{-1}) was measured by back titration with NaOH, and plotted against concentration of mannitol (mM). Their findings revealed that *Arabidopsis thaliana* cells also exhibit P_{cell}/Π_{ext} dependent proton efflux. Proton efflux in the 0 mM mannitol osmoticum was below 5 μmol g^{-1} fw h^{-1}, however, as the concentration of the external mannitol osmotica increased, proton efflux also increased, peaking at 15 μmol g^{-1} fw h^{-1} in the 300 mM mannitol osmoticum, before decreasing to 14 μmol g^{-1} fw h^{-1} in the 400 mM mannitol osmoticum. This pattern of proton efflux in *Arabidopsis thaliana* cells is comparable to the pattern of external acidification observed by Wyse *et al.* (1986), in sugar beet taproot tissue (see above).

Turgor/external osmotic pressure (P_{cell}/Π_{ext}) dependent proton efflux is also induced by other osmotica such as polyethylene glycol (Curti *et al.*, 1993) and sorbitol (Reuveni *et al.*, 1987). Reuveni *et al.* (1987) found that hyper-polarisation of carrot cells and acidification of the external medium occurred following exposure of the cells to increasing external osmotic pressures, induced by sorbitol. Approximately 6 x 10^5 cells were incubated in 15 ml of 0 - 800 mM mannitol, with 0.2 mM $CaSO_4$, with shaking. Change in osmoticum pH was determined in cell free aliquots of the media at 60 minute intervals for 120 minutes. Membrane hyper-polarisation was determined by measuring the accumulation of tetraphenylphosphonium over the same time period. Acidification and membrane polarisation increased with increasing sorbitol concentration. Maximal hyper-polarisation and acidification (Δ pH 0.5) occurred at 200 mM sorbitol. At higher

sorbitol concentrations a decrease in acidification and membrane polarisation was observed.

The proton efflux data presented in this chapter, together with the proton efflux and external acidification data taken from the literature, demonstrate that many plant tissues respond to a turgor/osmotic related stimulus by modulating the rate of proton efflux from the cells. Active proton efflux from plant tissue is generally associated with plasma membrane proton ATPase activity (Serrano, 1989 and 1990; Michelet and Boutry, 1995). In this study, the role of the plasma membrane proton ATPase in P_{cell}/Π_{ext} dependent proton efflux in sugar beet taproot tissue was investigated using membrane transport inhibitors and activators.

In order to establish that P_{cell}/Π_{ext} dependent proton efflux in sugar beet taproot tissue is an ATP requiring process, beet discs were subjected to treatments designed to prevent ATP production, by inhibiting the respiratory chain. Briefly, ATP is generated by a process which couples electron-transfer with oxidative phosphorylation (Mitchell, 1979). Multiprotein electron-transfer complexes in the mitochondrial inner membrane reoxidise the NADH and $FADH_2$ generated by the citric acid cycle (Baldwin and Krebs, 1981) and fatty acid oxidation (Wakil *et al.*, 1983) in the adjacent mitochondrial matrix. These complexes pass electrons stepwise to molecular oxygen at the end of the chain. This flow of electrons is coupled indirectly, but tightly, to the synthesis of ATP; as electrons move through some of the electron-transfer complexes, protons are taken up from the solution from the solution in the mitochondrial matrix and released at the outer surface of the inner membrane, setting up a gradient of pH and electric potential across the membrane. Protons flow back into the mitochondrial matrix through an ATP-synthase enzyme, which uses the free energy liberated by the proton to drive ATP synthesis (see Hinkle and McCarty, 1978; Mitchell, 1979; Dickerson, 1980; Boyer, 1989; Chan and Li, 1990).

The citric acid cycle through to the electron-transfer chain and oxidative phosphorylation are all oxygen requiring processes. In the absence of oxygen (anoxia) aerobic respiration leading to ATP synthesis is inhibited. Potassium cyanide (KCN)

inhibits a specific site on the electron-transfer chain, the final electron-transfer complex - cytochrome oxidase (Nicholls, 1982). Each cytochrome is made up of a porphyrin ring, with a central iron atom (Dickerson, 1972). Negative ions such as CN^- and N_3^- interact with the ferric form of iron at the centre of the porphyrin ring, thus preventing the final stage of electron-transfer to molecular oxygen (see Nicholls, 1982; Nicholls and Ferguson, 1992). The inhibition of P_{cell}/Π_{ext} dependent proton efflux in sugar beet taproot discs subjected to anoxia and KCN is consistent with this mechanism being an oxygen and ATP requiring process (this chapter).

In order to determine the molecular basis of P_{cell}/Π_{ext} dependent proton efflux, specialised membrane transport inhibitors were selected, which target specific sites on the plasma membrane. P-Chloromercuriphenylsulphonic acid (PCMPS) is a non-penetrating chemical modifier, which targets sulfhydryl groups (-SH) on the plasma membrane (Giaquinta, 1976). It has been extensively employed to investigate surface-located carrier proteins involved in the transport of amino acids, sugars and ions in a variety of plant tissues, including sugar beet leaf discs (Giaquinta, 1976), *Vicia faba* leaves (Delrot *et al.*, 1980) and *Vicia faba* and *Pisum sativum* seed coats (Wolswinkel and Ammerlaan, 1983; De Jong *et al.*, 1996).

Many plant ATPases cannot function if their -SH group integrity has been compromised (Poole, 1978). Therefore, modulation of -SH groups on the plasma membrane proton ATPase is likely to suppress proton ATPase activity and inhibit the secondary transport of solutes, such as sucrose. Giaquinta (1976) demonstrated that PCMPS markedly inhibits sucrose uptake into sugar beet leaf tissues. Sucrose accumulation was determined by ^{14}C analysis in sugar beet leaf discs incubated in 20 mM K-phosphate buffer (pH 7.0), containing varying concentrations of PCMPS. Sucrose accumulation in leaf discs, treated with 2 mM PCMPS for 15 minutes, decreased by 60 %. This inhibition of sucrose accumulation was not accompanied by a decrease in photosynthesis or respiration indicating that the site of PCMPS inhibition was confined to the plasma membrane. Giaquinta (1977) proposed that sucrose accumulation in sugar beet leaf tissue was facilitated by H^+/sucrose symport, driven by the plasma membrane proton ATPase (see also Giaquinta, 1983), and later, presented data demonstrating that

PCMPS also inhibits plasma membrane proton ATPase mediated proton efflux (Giaquinta, 1979). Approximately 2 g fresh weight of 0.5 cm^2 sugar beet leaf discs were incubated in 100 ml of 0.2 mM KH_2PO_4, 0.2 mM $CaCl_2$, containing 0 - 0.5 mM PCMPS, at pH 6.0. After 15 - 20 minutes medium acidification was measured with a pH electrode and proton efflux determined by back titration with NaOH. Proton efflux in control treated sugar beet leaf tissue (in the absence of PCMPS) was 1.46 μmol g^{-1} fw h^{-1}, whereas in the PCMPS treated tissue proton efflux was completely abolished. These data are in agreement with the data presented in this chapter. Proton efflux in sugar beet taproot tissue was largely abolished by 1 mM PCMPS throughout the range of mannitol treatments (this chapter).

Sulfhydryl groups are abundant on the plasma membrane (Rothstein, 1970). It is therefore likely that an -SH group modifier such as PCMPS interacts with the plasma membrane at numerous sites. Erythrosin B (EB - an iodinated derivative of fluorescein), on the other hand, is a specific membrane transport inhibitor, which targets the plasma membrane proton ATPase (Cocucci, 1986; Cocucci and Marré, 1986; Gimmler, 1988). In this study 100 μM EB completely abolished proton efflux in sugar beet taproot tissue, indicating that turgor/external osmotic pressure (P_{cell}/Π_{ext}) dependent proton efflux in this tissue is driven by the plasma membrane proton ATPase. Similar findings have been observed in radish seedlings (Cocucci and Marré, 1986), *Ricinus* cotyledons, cucumber hypocotyls (Ball *et al.*, 1987), *Elodea densa* leaves (Beffagna and Romani, 1988), and *Arabidopsis thaliana* cells (Curti *et al.*, 1993).

Cocucci and Marré (1986) demonstrated that a relatively low concentration of EB (100 - 300 μM) inhibits both basal and fusicoccin (FC) induced proton efflux, and depolarises the membrane potential, in 24 hour old radish seedlings. Proton efflux experiments were carried out on 1 g fresh weight of radish seedlings (approximately 50 seedlings) in 10 ml of 1 mM MES-K, 3 mM K_2SO_4, 0.5 mM $CaSO_4$, pH 6.2, at 27 °C, with shaking, in the dark. Change in pH (Δ pH) was determined with a pH electrode after 60 minutes. In control (without FC) and 100 μM EB treated radish seedlings Δ pH was - 0.23 $\pm$ 0.01 and 0.03 $\pm$ 0.01 respectively. In 10 μM FC and 10 μM FC plus 100 μM EB treated radish seedlings Δ pH was -1.20 $\pm$ 0.04 and -0.75 $\pm$ 0.02 respectively.

Transmembrane electric potential measurements were carried out on apical root segments (2 cm in length). Selected seedlings were aerated in 1 mM MES-K, 0.3 mM $CaSO_4$, and 1 mM KCl prior to membrane potential determination in the same medium, at 22 °C. In control (without FC) and 10 μM FC treated radish seedlings membrane potential was -120 mV and -150 mV respectively. Addition of 300 μM EB to the FC treated seedlings depolarised the membrane, bringing the membrane potential value back to - 120 mV.

Beffagna and Romani (1988) reported the EB inhibition of K^+ and FC induced proton efflux in *Elodea densa* leaves. Mature *Elodea densa* leaves were harvested and incubated for 2 hours in 0.5 mm $CaSO_4$ solution at 20 °C, under agitation. After the incubation period the leaves (0.2 g fresh weight 10 ml^{-1}) were transferred to a solution containing 0.5 mM $CaSO_4$, 0.3 mM MES, 5 μM DCMU (a photosynthetic inhibitor), 2.5 mM K_2SO_4, 100 μM FC, and 0 - 300 μM EB. External acidification was measured with a pH electrode and proton efflux was determined by back titration with NaOH after 90 minutes. Their findings revealed that 100 μM - 300 μM EB completely inhibited external acidification and demonstrated that 30 μM EB completely abolished titratable H^+ extrusion.

Finally, Curti *et al.* (1993) demonstrated the EB inhibition of P_{cell}/Π_{ext} induced proton efflux in *Arabidopsis thaliana* cells. Freshly harvested cells (0.1 g ml^{-1}) were agitated in 0.5 mM BTP-MES buffer, pH 6.25, containing 0.5 mM $CaSO_4$ and 0 - 150 mM mannitol. External acidification was determined with a pH electrode in cell free, 120 μl aliquots of osmoticum. The change in pH (Δ pH) in control 0 mM and 150 mM mannitol treated cells, over 180 minutes, was -0.17 and -0.59 pH units respectively. Addition of 100 μM EB completely suppressed H^+ extrusion, resulting in a Δ pH of 0.50 and 0.43 in 0 mM and 150 mM mannitol respectively.

To summarise briefly, the respiratory chain inhibitors, anoxia and KCN demonstrated that P_{cell}/Π_{ext} dependent proton efflux in sugar beet taproot tissue is an oxygen and ATP requiring process. Inhibition of proton efflux by PCMPS and erythrosin B (EB) established that this H^+ extrusion mechanism is due to plasma membrane proton

ATPase activity (this chapter). Therefore, in conclusion, sugar beet storage parenchyma cells apparently respond to a turgor/osmotic related stimulus by modulating the activity of a major primary transporter - the plant plasma membrane proton ATPase. However, the mode of stimulus detection and signal propagation, leading to proton ATPase modulation has not been established.

Changes in cytosolic calcium concentrations have been implicated in the response of plants to a number of stimuli (Bush, 1995), including plant growth regulators (Felle, 1988; Gilroy *et al.*, 1990; Schroeder and Hagiwara, 1990; Hahm and Saunders, 1991; Gilroy and Jones, 1992), light (Chae *et al.*, 1990; Russ *et al.*, 1991), temperature (Knight *et al.*, 1991; Braam, 1992), touch (Knight *et al.*, 1991), salinity and drought (Knight *et al.*, 1997). It has been suggested that Ca^{2+} acts as an intracellular messenger, conveying information about the nature of a particular stimulus or stress impinging on a cell to target proteins that guide the cellular response (Bush, 1993 and 1995).

Levels of cytosolic calcium are established by Ca^{2+} uptake and extrusion mechanisms, in which both influx and efflux transporters are active. The balance between the Ca^{2+} transporters determines the Ca^{2+} concentration in the cytosol (Bush, 1993 and 1995). Observations of stimulus induced changes in cytosolic Ca^{2+} indicate that there is both short-term and long-term regulation of this uptake and extrusion mechanism. Short-term regulation leads to abrupt and usually transient changes in response to stimuli that suddenly and preferentially alter the activity of an influx or an efflux transporter. Long-term regulation on the other hand, leads to the establishment of resting or steady state levels of cytosolic Ca^{2+} and the restoration of the uptake/extrusion balance (Bush, 1993 and 1995).

The change in cytosolic Ca^{2+} induced by most stimuli is transient and involves short-term regulation (Bush, 1993 and 1995). Preventing Ca^{2+} influx into the cytoplasm, by blocking calcium channels should, in theory, prevent the propagation of a stimulus induced calcium signal. The possible role of Ca^{2+} in the turgor/osmotic response, leading to plasma membrane proton ATPase modulation was investigated using the calcium channel blockers verapamil and nifedipine.

Verapamil belongs to the phenyalkylamine family of molecules. A moderate to high density of phenylalkylamine receptor sites have been reported in maize (Harvey *et al.*, 1989), carrot (Graziana *et al.*, 1988) and courgette membranes (Andrejauskas *et al.*, 1985). Also partial purification of a verapamil binding protein from maize coleoptiles has been achieved by Harvey *et al.* (1989). Furthermore, it has been demonstrated that verapamil suppresses cytokinin and exogenous Ca^{2+} stimulated bud formation in the moss *Funaria* (Saunders and Hepler, 1982 and 1983) and inhibits Ca^{2+} influx into carrot microsomes (Graziana *et al.*, 1988) and *Amaranthus tricolor* protoplasts (Rengel and Elliott, 1992; Terry *et al.*, 1992).

Nifedipine belongs to the 1-4, dihydropyridines group of calcium channel blockers. Low levels of 1-4, dihydropiridine receptor proteins have been reported in pea shoot membranes (Hetherington and Trewavas, 1984). However, no 1-4, dihydropiridine receptor sites have been detected in carrot (Graziana *et al.*, 1988) or courgette (Andrejauskas *et al.*, 1985), which is consistent with the absence of effect of this type of calcium channel blocker on Ca^{2+} influx in these examples (Andrejauskas *et al.*, 1985; Graziana *et al.*, 1988). Despite these findings, Terry *et al.* (1992) demonstrated that nifedipine and related molecules affected plasma membrane cation outward rectifiers in *Amaranthus tricolor* protoplasts, causing a 50 % block of time-dependent current, at concentrations between 50 - 100 μM. The phenylalkylamines were found to be consistently more potent, however, and capable of blocking all time-dependent outward currents at concentrations as low as 15 μM.

Turgor/external osmotic pressure (P_{cell}/Π_{ext}) dependent proton efflux was unaffected by 100 μM verapamil and nifedipine. The pattern of proton efflux, throughout the range of mannitol osmotica, was indistinguishable from that of the control (this chapter). Assuming the calcium channel blockers used were effective, these data suggest that Ca^{2+} is not involved in the signal-transduction pathway, leading to plasma membrane proton ATPase modulation in this case. Knight *et al.* (1997), however, found that mannitol and NaCl treatments led to transient increases in cytoplasmic free calcium concentration (of up to 1.5 μM) in *Arabidopsis thaliana* seedlings *in vivo*. These

cytoplasmic calcium elevations were inhibited by pretreatment with the calcium channel blocker lanthanum.

The fungal toxin fusicoccin (FC) promotes H^+ extrusion in plant tissues, examples include, sugar beet taproot tissue (this chapter), *Arabidopsis thaliana* cells (Curti *et al.*, 1993; De Michelis *et al.*, 1996), radish seedlings (Cocucci and Marré, 1986; De Michelis *et al.*, 1996), maize coleoptiles (Jahn *et al.*, 1996) and *Avena* coleoptiles (Rayle, 1973; Senn and Goldsmith, 1988). It has been demonstrated that this FC induced stimulation of proton efflux is due to an increase in plasma membrane proton ATPase activity (Rasi-Caldogno and Pugliarello, 1985; Rasi-Caldogno *et al.*, 1986; Aducci *et al.*, 1988; Marra *et al.*, 1992; De Michelis *et al*, 1996; Jahn *et al.*, 1996).

Jahn *et al.* (1996) measured FC induced external acidification and plasma membrane proton ATPase activity in *Zea mays* coleoptiles. Fifty coleoptiles were split and pre-incubated, with agitation, in 8 ml of 0.5 mM BTP-MES (pH 5.5), 0.1 mM KCl, 0.1 mM $CaCl_2$ and 0 - 5 μM fusicoccin. External acidification was measured using a pH electrode, over 60 minutes. After pre-incubation, plasma membranes were isolated from control and FC treated *Zea mays* coleoptile segments (see Larsson *et al.*, 1994). Proton ATPase activity was determined by assay, in which ATP hydrolysis was enzymatically coupled to the oxidation of NADH. The rate of ATP hydrolysis was measured as the absorbance decrease at 340 nm. Fusicoccin (5 μM) caused a dramatic decrease in medium pH, from 5.55 to 4.60 within 60 minutes. ATP hydrolysis increased by 100 %, from 0.5 ADP mg^{-1} protein min^{-1} in control plasma membrane preparations to 0.1 ADP mg^{-1} protein min^{-1} in FC plasma membrane preparations.

De Michelis *et al.* (1996) measured plasma membrane proton ATPase activity in control and fusicoccin (FC) treated *Arabidopsis thaliana* and *Raphanus sativus* (radish) seedlings. The seedlings were pre-treated in 0 - 5 μM FC for up to 3 hours, prior to plasma membrane fraction isolation, using a method described by Olivari *et al.* (1993) and Rasi-Caldogno *et al.* (1995). Plasma membrane proton ATPase activity was determined by measuring the release of P_i from ATP according to the method of Ames (1966), at pH 7.5. Proton ATPase activity in *Arabidopsis thaliana* plasma membranes,

pre-treated *in vivo* with 0 μM and 5 μM FC, was 82 nmol min^{-1} mg^{-1} protein and 330 nmol min^{-1} mg^{-1} protein respectively. Radish plasma membrane fractions had a control (0 μM FC) and FC treated proton ATPase activity of 53 nmol min^{-1} mg^{-1} protein and 142 nmol min^{-1} mg^{-1} protein respectively. Pre-treatment, *in vivo*, with 5 μM fusicoccin resulted in a 300 % and 170 % increase in proton ATPase activity in plasma membrane fractions isolated from *Arabidopsis thaliana* and radish seedlings respectively.

Fusicoccin (FC) does not interact with the plasma membrane proton ATPase directly, but binds to a specific FC receptor site on the plasma membrane, which in turn interacts with the proton ATPase by displacing the enzymes C-terminus (Meyer *et al.*, 1989; De Michelis *et al.*, 1996; Jahn *et al.*, 1997). The C-terminus region of the ATPase is an auto-inhibitory domain; displacement of this domain activates the proton ATPase (Aducci *et al.*, 1995; Jahn *et al.*, 1997). The fusicoccin receptor site belongs to a family of proteins called 14-3-3 proteins (Marra *et al.*, 1994). The 14-3-3 family is a highly conserved class of hydrophilic proteins with multiple, often regulatory, functions in a wide range of organisms (for a review see Aitken, 1996).

In this study, 10 μM fusicoccin (FC) maximised proton efflux in sugar beet taproot discs, throughout the range of mannitol concentrations, apparently abolishing the P_{cell}/Π_{ext} dependent properties of the system. This suggests that the site of turgor/osmotic responsiveness on the plasma membrane is either the same as or "up stream" from that of fusicoccin sensitivity. The fusicoccin receptor protein (14-3-3 protein) may hold the key to turgor/osmotic related stimulus detection in sugar beet taproots and other plant tissues. The 14-3-3 family of proteins and their mode of action are discussed further in the following chapter, **Chapter 7**.

6.5, Summary and conclusions

- Changes in cell turgor pressure (P_{cell}), induced by mannitol osmotica of varying concentration, modulate the rate of proton efflux from sugar beet taproot tissue. An increase in P_{cell} inhibits proton efflux, whereas a decrease in P_{cell} stimulates proton efflux.

- Inhibition of this turgor/external osmotic pressure dependent (P_{cell}/Π_{ext}) proton efflux mechanism by respiratory chain inhibitors (anoxia and KCN), confirms that this process requires oxygen and ATP.

- The plasma membrane proton ATPase inhibitors PCMPS and erythrosin B abolish proton efflux, throughout the range of mannitol osmotica, demonstrating that proton efflux is due to plasma membrane proton ATPase activity.

- From these data it is clear that sugar beet storage parenchyma cells respond to a turgor/osmotic related stimulus by modulating the activity of an important primary transporter - the plasma membrane proton ATPase.

- The possible involvement of calcium as a secondary messenger in the chain of events leading from stimulus detection to proton ATPase modulation is tentatively ruled out. The calcium channel blockers verapamil and nifedipine have no effect on proton efflux in this system.

- Fusicoccin maximises proton efflux and apparently abolishes the P_{cell}/Π_{ext} dependent nature of the mechanism. This suggests that the site of turgor/osmotic responsiveness on the plasma membrane is either the same as or "up stream" from that of fusicoccin stimulation.

Chapter 7 : Final discussion and concluding remarks

7.1, Final discussion

The work carried out on *Suaeda maritima* L. Dum. and *Beta vulgaris* L. in this study demonstrates that changes in cell turgor pressure/external osmotic pressure ($\Delta P_{cell}/\Delta \Pi_{ext}$) initiate a sequence of events leading to osmotic adjustment (protoplastic and/or apoplastic) and turgor regulation (see **Chapter 3, 4** and **5**). Cells are apparently responding to a hydrostatic or osmotic related stimulus by modulating membrane transport processes. In **Chapter 6**, evidence is presented which suggests that osmotic adjustment is achieved by turgor/external osmotic pressure (P_{cell}/Π_{ext}) dependent modulation of plasma membrane proton ATPase activity. It is assumed that this, in turn, drives the secondary transport of solutes necessary to achieve osmotic adjustment. The P_{cell}/Π_{ext} dependent nature of solute transport has been widely documented in the literature, in a range of organisms - including bacteria, algae and higher plants.

7.1.1, The turgor/external osmotic pressure dependent transport of solutes in bacteria, algae and higher plants

In a fluctuating external environment bacteria behave as perfect osmometers, with a reflection coefficient which is close to 1 (Csonka, 1989; Csonka and Hansen 1991). An increase in external osmotic pressure leads to water efflux and a decrease in turgor pressure, whereas a decrease in the concentration of the external medium leads to water influx and an increase in cell turgor pressure. Changes in cell turgor pressure induce a turgor dependent response which leads to osmotic adjustment of solutes within the cell and subsequent restoration of cell turgor pressure (for reviews see Csonka, 1989; Csonka and Hansen 1991).

Escherichia coli, for example, maintains turgor pressure (P_{cell}) at 0.3 MPa, over a range of external osmotic pressures (Π_{ext}). This is primarily achieved by turgor dependent adjustment of K^+ (Laimins *et al.*, 1981). Potassium ions are the most prevalent cations

in the cytoplasm of bacteria, and consequently play an important role in osmotic adjustment and turgor regulation (Csonka, 1989). The internal K^+ concentration in *Escherichia coli* is determined by the osmotic pressure of the external medium (Π_{ext}), and ranges from 100 mM in dilute media to 600 mM in media with an osmolarity of 1200 mOsmoles kg^{-1} (Epstein and Schultz, 1965). Small increases in Π_{ext} are countered by accumulation of K^+ (McLaggan *et al.*, 1994), however, larger increases in Π_{ext} also require the accumulation of glutamate (Csonka, 1989; Csonka and Hansen, 1991; McLaggan *et al.*, 1994).

The turgor dependent nature of membrane transport processes has been most comprehensively studied in the marine algae (Gutknecht, 1968; Zimmermann and Steudle, 1974; Cram, 1976; Kirst and Bisson, 1979). The marine algae regulate cell turgor pressure (P_{cell}), in response to changes in the external medium, by modulating membrane transport processes, which leads to osmotic adjustment of the protoplast (Cram, 1976; Gutknecht *et al.*, 1978; Zimmermann, 1978). The main ions involved in osmotic adjustment are K^+, Na^+ and Cl^- (Kirst and Bisson, 1979).

In *Valonia*, for example, turgor regulation is achieved by modulation of an inwardly directed K^+ pump (Steudle and Zimmermann, 1974; Hastings and Gutknecht, 1974). The turgor dependent nature of this transport mechanism was first demonstrated by Gutknecht (1968) in *Valonia ventricosa* cells. Using a pressure perfusion system, the turgor pressure (P_{cell}) of the cell was increased from 0 MPa to 0.1 MPa. This 0.1 MPa increase in P_{cell} gave rise to a 70 % decrease in K^+ influx (K^+ efflux also increased slightly). Zimmermann and Steudle (1974) modulated P_{cell} in *Valonia* by increasing or decreasing the osmotic pressure of the external medium (Π_{ext}). This work demonstrated that K^+ influx continues to decrease steadily up to a pressure level of 0.2 MPa in cells of *Valonia utricularis*. Above this P_{cell} level K^+ influx became increasingly independent of pressure. Potassium efflux, however, showed a remarkable and progressive increase over the entire pressure range; up to 0.5 - 0.6 MPa, which is the point at which larger cells usually burst (Zimmermann and Steudle, 1974; Zimmermann, 1977 and 1978). Conversely, decreasing turgor pressure (P_{cell}) in *Valonia macrophysa* from 0.15 MPa (the P_{cell} level maintained over a range of external salinities *in vivo*) to 0.05 MPa leads

to a 300 % increase in K^+ influx (the decrease in P_{cell} was achieved by pressure perfusion in this case - Hastings and Gutknecht, 1974).

Chloride (Cl^-) transport also exhibits turgor dependence in some species of marine algae. Work carried out by Bisson and Gutknecht (1975) and Graves and Gutknecht (1976) on *Codium decorticatum* and *Halicystis parvula* respectively, suggests that turgor dependent Cl^- transport plays a key role in turgor regulation in these species; in both cases Cl^- influx is stimulated by a low P_{cell} and inhibited by a high P_{cell}.

There are several lines of evidence that turgor pressure (P_{cell}) can affect ion transport in higher plants. Meidner and Edwards (1975) clearly demonstrated that turgor dependent fluxes are encountered when stomata open and close. Luttge *et al.*, (1975) suggested that the diurnal oscillations of malate levels in the crassulacean acid metabolism of *Bryophylium daigremontianum* leaf cells may be subject to direct turgor pressure control. It is also believed that a similar phenomenon plays a key role in the circadian rhythm of K^+ fluxes in the pulvinule cells of *Trifolium repens* (Scott and Gulline, 1975).

More recently, work carried out on sugar beet taproot tissue has demonstrated the turgor dependent nature of sucrose and K^+ transport in this tissue (Wyse *et al.*, 1986; Bell and Leigh, 1996). Wyse *et al.* (1986) showed that cell turgor pressure (P_{cell}) is an important sucrose uptake regulator in sugar beet taproot tissue. Changes in P_{cell}, induced by mannitol osmotica, resulted in the modulation of ^{14}C sucrose uptake. Sucrose uptake was measured in beet discs bathed in 20 mM ^{14}C sucrose in 25 mM MES/BTP pH 6.5 buffer, containing 0 mM or 400 mM Mannitol, over 60 minutes. Beet discs bathed in the control medium, which was devoid of mannitol, had a sucrose uptake rate of $0.50 \pm 0.05\ \mu mol\ h^{-1}\ g^{-1}$, whereas the mannitol treated discs had a sucrose uptake rate of $1.16 \pm 0.21\ \mu mol\ h^{-1}\ g^{-1}$ (an increase of 132 %). Proton efflux, attributed to plasma membrane proton ATPase activity, was also found to be turgor dependent. A decrease in P_{cell} stimulated proton ATPase activity (with an optimum at 300 mM mannitol external concentration). Wyse *et al.* (1986) proposed that the secondary transport of sucrose is reduced at high P_{cell} as a result of inhibition of the plasma membrane proton ATPase.

The turgor/external osmotic pressure (P_{cell}/Π_{ext}) dependent nature of the plant plasma membrane proton ATPase was demonstrated in **Chapter 6**.

Other authors have also highlighted the turgor dependent nature of sucrose transport in beet tissue. Ehwald *et al.* (1980) reported that the leakage of sucrose from slices of sugar beet was strongly reduced in solutions of high osmotic pressure (0.8 MPa and above), and Perry *et al.* (1987), working on red beet, found that solutes were leaked in proportion to their intracellular concentration following an increase in P_{cell}. However, Bell and Leigh (1996) took the investigation of turgor dependent membrane transport in beet taproot tissue a stage further. They investigated the effects of turgor pressure on transport at specific membranes, i.e. the plasma membrane and tonoplast. The solutes of interest in this study where sucrose and K^+. Sucrose and K^+ fluxes were determined by ^{14}C and ^{86}Rb analysis respectively. Rubidium is an analogue of K^+, and there is generally little discrimination between them in terms of solute flux, for example in barley tissues (Epstein, 1966; Pitman *et al.*, 1974) and rye (White *et al.*, 1991). Bell and Leigh (1996) demonstrated that P_{cell}/Π_{ext} dependent sucrose and K^+ transport across the tonoplast largely followed the same pattern. Sugar beet taproot discs were incubated in BTP/MES buffer (pH 6.5), containing 50 mM sucrose, 5 mM KCl, 1 mM $CaSO_4$ and 0 mM/600 mM mannitol. A decrease in turgor pressure (P_{cell}), induced by an increase in external mannitol concentration, resulted in an increase in the rate of both sucrose and potassium uptake into the vacuole. Sucrose and K^+ influx was initially determined under steady state 0 mM/600 mM mannitol conditions for 60 - 100 minutes. After a maximum of 100 minutes, the beet discs were subjected to a 1.5 MPa step-up/step-down treatment, in which 0 mm mannitol beet discs were transferred to a medium containing 600 mM mannitol and vice versa. Sucrose and K^+ influx was measured at intervals for a further 4 hours. The decrease in P_{cell}, induced by the step-up treatment, lead to an increase in the rate of both sucrose and K^+ uptake into the vacuole of 100 % and 70 % respectively (sucrose uptake increased from 1 to 2 μmol h^{-1} g^{-1}, and K^+ uptake increased from 0.75 to 1.25 μmol h^{-1} g^{-1}) within 4 hours.

Conversely, an increase in P_{cell}, induced by the step-down treatment, largely inhibited sucrose and K^+ uptake into the vacuole and accelerated solute efflux out of the tissue.

For example, under 600 mM mannitol steady state conditions, the flux of K^+ from the cytoplasm outward, into the apoplast/external osmoticum was 2.33 μmol g^{-1} h^{-1}, a step-down into 0 mM mannitol resulted in a massive 340 % increase in K^+ efflux to 8 μmol g^{-1} h^{-1}.

At the plasma membrane, however, changes in P_{cell}/Π_{ext} elicited different sucrose and K^+ transport responses. An increase in P_{cell}, induced by the 1.5 MPa step-down treatment decreased the rate of K^+ uptake, but increased the rate of sucrose uptake over time (a decrease in P_{cell} was found to induce the opposite effect). Potassium transport across the plasma membrane, into the cytoplasm, in discs bathed in 0 mM and 600 mM mannitol osmotica was 3 μmol h^{-1} g^{-1} and 4 μmol h^{-1} g^{-1} respectively after 4 hours. Whereas sucrose uptake from the apoplast was 12 μmol h^{-1} g^{-1} and 5 μmol h^{-1} g^{-1} in 0 mM and 600 mM treated sugar beet discs respectively, after 5 hours. Therefore, although both sucrose and K^+ may be lost from cells following an increase in turgor pressure (P_{cell}), it appears that sucrose is selectively retrieved from the apoplast, but K^+ is not. Given this scenario, K^+ will tend to accumulate in the apoplast during adjustment to high P_{cell} in sugar beet storage taproots. The loss of osmotically active ions from the protoplast reduces P_{cell} by decreasing the internal osmotic pressure (Π_{cell}) of the cell, but more importantly the accumulation of solutes in the apoplast increases apoplastic osmotic pressure (Π_{wall}) and further facilitates P_{cell} reduction. These data add weight to the findings discussed in **Chapter 3, 4,** and **5,** and further highlight the potential importance of the apoplast as an osmotic compartment.

Moving away from beet taproot tissue, another interesting example of turgor dependent membrane transport is described by Teodoro *et al.* (1998) in *Arabidopsis thaliana* cells. The authors demonstrated that hyper-osmotic stress (200 mM mannitol) rapidly reduces Cl^- efflux by up to 70 % (from 0.48 $\pm$ 0.05 μmol g^{-1} FW in control cells in 100 mM mannitol, to 0.14 $\pm$ 0.01 μmol g^{-1} FW in 200 mM mannitol treated cells, after 10-30 minutes). Inhibition of Cl^- efflux under these conditions was found to be independent of a mannitol induced stimulation of Cl^- influx, and was rapidly reversible after removal of the hyper-osmotic shock treatment. The inhibition of Cl^- efflux was accompanied by plasma membrane proton ATPase stimulation (H^+ efflux). Both inhibition of Cl^- efflux

and H^+ efflux were dependent on the concentration of mannitol in the external medium. These data point to a correlation between the inhibition of Cl^- efflux and proton extrusion. However, a direct relationship between the two is ruled out by Teodoro *et al.* (1998). The authors argue that if hyper-osmotic stress primarily reduces Cl^- efflux (leading to hyper-polarisation of the plasma membrane), proton ATPase activity should not be stimulated (see Bellando *et al.*, 1995). Conversely, if hyper-osmotic shock primarily stimulates proton ATPase activity (which would once again lead to hyper-polarisation of the membrane) Cl^- efflux should not be inhibited. Teodoro and co-workers (1998) also demonstrated that direct stimulation of the proton ATPase by the fungal toxin fusicoccin (FC) did not affect Cl^- efflux. This further strengthens the view that the inhibition of Cl^- efflux can not be a direct consequence of proton ATPase activity.

The hypothesis put forward by the Teodoro *et al.* (1998) to explain these findings, suggests that the first target of hyper-osmotic stress, in other words the turgor/osmotic sensor, may in fact be the Cl^- channel itself. Inactivation of the Cl^- channel would induce a decrease in Cl^- efflux and, consequently, lead to transmembrane electric potential (E_m) hyper-polarisation. This change in E_m would then allow larger influxes of solutes such as K^+ and Cl^- (symported by two protons) or a lower K^+ efflux. Both the increase in intracellular K^+ concentration (tending to reduce E_m hyper-polarisation) and the decrease in cytoplasmic pH due to the entry of protons accompanying the Cl^- anion would activate the plasma membrane proton ATPase (see Beffagna and Romani, 1991). Returning briefly to **Chapter 5**, the above hypothesis may explain the observed facilitation of turgor regulation in sugar beet taproot discs, under closed system conditions, in the presence of KCl (see **Figure 5.7** and **5.9**).

The Teodoro *et al.* (1998) paper suggests that an ion channel (in this case a Cl^- channel) may play a role in actually sensing turgor pressure and/or osmotic pressure changes. The turgor sensor reacts to a stimulus by directly or indirectly activating the plasma membrane proton ATPase which subsequently drives the secondary transport of solutes necessary to achieve osmotic adjustment and turgor regulation. It is to turgor-sensing mechanisms that this discussion now turns.

The evidence that turgor pressure can directly influence membrane transport (examples of which are discussed above) has led to an extensive discussion of the possible mechanisms involved in turgor-sensing (Zimmermann, 1977 and 1978; Bisson and Gutknecht, 1980; Tomos, 1988). The aim is to understand the individual steps taken in the transformation of a hydrostatic/osmotic signal into changes in membrane transport. The main consensus of opinion in the scientific community is that the basic steps of the turgor-sensing mechanism are mechanical and electrochemical in nature. This led to the formulation of the electromechanical turgor-sensing model (Zimmermann *et al.*, 1974; Coster and Zimmermann, 1975; Coster *et al.*, 1976; Zimmermann *et al.*, 1977; Zimmermann, 1977 and 1978). The fundamental concept behind this model is that mechanical forces can act on the mechanics of membranes in two ways: first by direct compression of the membrane, and secondly by membrane stretching induced by a pressure gradient across the membrane. In walled cells, stretching of the cell membrane in response to pressure gradients is controlled by the elastic properties of the cell wall to which the plasma membrane is coupled. Pressure gradient induced stretching may also change membrane thickness. The electromechanical model postulates that the pressure-induced changes in the geometric dimensions of the membrane, or certain membrane areas which may be preferentially involved in turgor sensing, are transformed into electrochemical changes within the membrane, which in turn lead to membrane transport.

Plasma membrane pressure-induced changes are beautifully illustrated in a paper by Peng and Jaffe (1976). They prepared freeze-fractures of a *Pelvetia* embryo plasma membrane over a range of cell turgor pressures. In a fully turgid cell, imprints of cell wall microfibrils were numerous and clearly visible. At intermediate turgor pressures microfibril imprints were still discernible, but much less prominent and less numerous. A fully plasmolysed cell showed no microfibril imprints whatsoever. These observations demonstrate that changes in turgor pressure induce physical changes in the plasma membrane/cell wall complex.

From the literature I propose that there are three possible candidates that could fulfil the role of a plasma membrane bound turgor-sensor in bacteria, algae and higher plants.

These are primary transporters such as the P-type ATPases, mechanosensitive ion channels, and molecular switches such as the 14-3-3 proteins.

7.1.2, Primary transporters as turgor-sensors

To regulate internal osmolarity, *Escherichia coli* accumulate K^+ by two distinct transport mechanisms. Under most growth conditions K^+ is taken up by the constitutive TrkA system, which has a high rate of transport and a low affinity for K^+ (Rhoads *et al.*, 1976). At very low external K^+ concentrations, K^+ is taken up by the high-affinity Kdp system, which has a K_m of 2 μM (Rhoads *et al.*, 1976). The Kdp system is ATP driven (Rhoads and Epstein, 1977) and requires the expression of four closely linked genes, Kdp A to D (Epstein and Davies, 1970; Rhoads *et al.*, 1978). Three of these genes, KdpA, KdpB and KdpC , form an operon that codes for three inner membrane proteins (Laimins *et al.*, 1978). The fourth gene, KdpD, codes for a positive regulator and is located on the promoter-distal end of the KdpABC operon (Rhoads *et al.*, 1978).

High external K^+ represses the Kdp operon, whereas very low external K^+ leads to Kdp operon expression (Laimins *et al.*, 1981). However, external K^+ concentration is not believed to be the stimulus that leads to Kdp expression. The specific signal which leads to Kdp expression appears to be low turgor pressure (P_{cell}), induced by an increase in the concentration of the external medium (Laimins *et al.*, 1981; Walderhaug *et al.*, 1992). At a given internal concentration of K^+, Kdp expression can be turned on or off by varying the medium K^+ concentration, whereas at a given external K^+ concentration, Kdp expression is dependent on the osmotic pressure of the external medium (Laimins *et al.*, 1981). The magnitude of Kdp expression is also determined by the magnitude of the osmotic shift in the external medium (Laimins *et al.*, 1981).

Control of expression by turgor pressure also explains the role of external K^+ concentration in regulation of Kdp expression. Kdp expression occurs at an external K^+ concentration which is low enough to adversely affect cell growth. When K^+ uptake is inadequate (due to low K^+ concentration in the external medium), the intracellular volume increases that accompany growth lead to a reduction in internal osmotic pressure. This reduction in internal osmotic pressure (Π_{cell}) is accompanied by a

corresponding decrease in turgor pressure (P_{cell}) which in turn leads to Kdp expression (Laimins *et al.*, 1981; Walderhaug *et al.*, 1992).

The Kdp transport system in *Escherichia coli* is a K^+ ATPase, a member of the E_1 - E_2 class of transport ATPases (Skou, 1975; Inesi, 1985). These particular transport ATPases are also often referred to as P-type ATPases (Pederson and Carafoli, 1987) to distinguish them from ATPases that do not form a phosphorylated intermediate (Epstein *et al.*, 1990).

Another well documented turgor dependent P-type ATPase is the plant plasma membrane proton ATPase (see **Chapter 6**). As a key primary transporter it is believed to play a central role in osmotic adjustment (Reinhold *et al.*, 1984; Serrano, 1989 and 1990; Michelet and Boutry, 1995). The turgor/external osmotic pressure (P_{cell}/Π_{ext}) dependent nature of the plasma membrane proton ATPase is demonstrated and discussed in **Chapter 6**. To briefly recap, a decrease in turgor pressure (P_{cell}), induced by an increase in the concentration of the external medium (Π_{ext}), leads to plasma membrane proton ATPase stimulation, whereas an increase in P_{cell} inhibits proton ATPase activity.

Reinhold *et al.* (1984) proposed that the operation of the plasma membrane proton ATPase is directly affected by the hydrostatic pressure gradient across the plasma membrane/cell wall complex, possibly due to deformation of the plasma membrane by the cell wall. An increase in proton ATPase activity, directly induced by physical changes in the membrane, would generate the pH and electrochemical gradient (or proton-motive force) necessary to drive the secondary transport of solutes across the plasma membrane and thus achieve osmotic adjustment (see Serrano, 1989 and 1990; Michelet and Boutry, 1995). The benefit of a single detector/effector was also pointed out by Reinhold *et al.* (1984). The dual functioning of a system, both as detector of changes in cell turgor pressure (P_{cell}) and effector of changes in cell osmotic pressure (Π_{cell}) and/or wall osmotic pressure (Π_{wall}), would obviate the postulated need for information transfer between detector and effector.

7.1.3, Mechanosensitive ion channels as turgor-sensors

Mechanosensitive channels have been found in a variety of animal cell types, as well as microbial and plant cells (for a review see Morris, 1990). In walled cells, such as *Escherichia coli* (Martinac *et al.*, 1987; Delcour *et al.*, 1989), yeast (Gustin *et al.*, 1988), and cultured tobacco cells (Falke *et al.*, 1988). However, the study of stretch activated or mechanosensitive ion channels has mainly concentrated on stomatal guard cells. Changes in guard cell volume and pressure can be large (3 : 1 volume change and more than 1 MPa pressure change) and are largely controlled by the release and uptake of ions, principally K^+, malate and Cl^-, through co-ordination of ion channels and ion pumps (Raschke, 1979; Outlaw, 1983; Zeiger, 1983; Macrobbie, 1988; Hedrich and Schroeder, 1989; Mansfield *et al.*, 1990). Guard cells deal with a variety of internal and external stimuli (light, CO_2, plant water status, and growth regulator concentration) and adjust the stomatal aperture accordingly. This would suggest that they possess a sophisticated and finely tuned mechanism for regulating volume and turgor pressure (Raschke, 1970; Farquhar *et al.*, 1978).

The role of mechanosensitive channels in turgor pressure regulation was investigated by Cosgrove and Hedrich (1991). Using the patch clamp technique in *Vicia faba* leaves they examined guard cells for mechanosensitive channels which might respond to plasma membrane deformations that accompany changes in cell turgor pressure. Such channels could include both stretch-activated and stretch-inactivated channels. The work carried out by Cosgrove and Hedrich (1991) identified three stretch-activated channels (one of each for Cl^-, K^+ and Ca^{2+}). They were distinguishable from their non stretch-activated counterparts with respect to their conductance, selectivity and kinetics, as well as in their sensitivity to membrane tension. Compared to other cells (see Morris, 1990), guard cells have an unprecedented variety of mechanosensitive channels; which suggests that guard cells possess multiple, highly specialised mechanisms for sensing and controlling turgor pressure.

In the plasma membrane of other walled cells, stretch-activated channels have been characterised with lower ion specificity, larger amplitudes, and/or larger mean opening times than the guard cell stretch-activated channels. These properties indicate that the

channels in these other cells act directly as pressure valves against excessive pressure (Morris, 1990; Cosgrove and Hedrich, 1991). For example: cultured tobacco cells have a stretch-activated anion channel with a single channel conductance 5 - 10 times larger than the equivalent stretch-activated channel found in guard cells, an open time 100 times longer, and less selectivity for Cl^- over K^+ (Falke *et al.*, 1988; Cosgrove and Hedrich, 1991). The yeast stretch-activated channel has a conductance (40 pS) similar to values found for some guard cell stretch-activated channels, but has poor ion selectivity and longer open times (Gustin *et al.*, 1988). More extreme is the stretch-activated channel reported for *Escherichia coli*, with a conductance of 1000 pS, negligible ion selectivity, and long open times (Martinac *et al.*, 1987; Delcour *et al.*, 1989). In animal cells, stretch-activated channels are similar in many ways to those found in guard cells; having a lower conductance, shorter open times and greater ion selectivity (reviewed in Morris, 1990).

7.1.4, 14-3-3 type proteins as turgor-sensors

The 14-3-3 proteins initially were described as a part of an extensive characterisation of the acidic, soluble proteins within the mammalian brain (Moore and Perez, 1967). The study involved the purification of brain proteins of unknown function; names were assigned based on banding patterns on electrophoresis gels. The interesting observations at the time were that many of the proteins (including the 14-3-3 proteins) were abundant in the brain (up to 1 % of the total soluble protein in the case of 14-3-3 proteins), were found exclusively in the brain, and were immunologically conserved among animals. This led to the conclusion that 14-3-3 proteins were uniquely important to brain and neuronal function (Boston *et al.*, 1982 {a} and {b}; Erikson and Moore, 1980; Ichimura *et al.*, 1991).

By the mid 1980s the 14-3-3 proteins were understood to be a heterogeneous family of proteins with subunit masses of 30 kDa and with various isoelectric points (Yamauchi *et al.*, 1981; Ichimura *et al.*, 1988). It was also known that they were found as dimers of 50 - 60 kDa in their native form and that their amino termini were blocked (Ichimura *et al.*, 1988). The first clue to 14-3-3 protein function came in 1987. Ichimura and co-workers (1987) observed that 14-3-3 proteins were similar in amino acid

composition to a protein known to activate tyrosine and tryptophan hydroxylases. Later experiments confirmed that the biochemical characteristics of 14-3-3 proteins were indistinguishable from those of purified activators of tyrosine and tryptophan hydroxylases (Ferl, 1996). Tyrosine and tryptophan hydroxylases are the first enzymes in the metabolic pathways that lead to serotonin and dopamine production; important neurotransmitters. This provided the scientific community with an important clue to 14-3-3 protein function - signal transduction (Ichimura *et al.*, 1987; Ferl, 1996).

More recently, 14-3-3 proteins have been discovered in many biological systems. They are not brain specific, or indeed animal specific. They are highly conserved and found in a broad range of tissues. Different isoforms have different functions, and no eukaryotic cell type has been identified which does not contain members of the 14-3-3 protein family (Robinson *et al.*, 1994; Ferl, 1996). It would be impossible to assign a single function to the 14-3-3 proteins, however, it is clear that they play an important and crucial role in signal transduction and regulation (reviewed by Ferl, 1996).

A variety of external stimuli are known to influence 14-3-3 protein expression in higher plants. *Arabidopsis thaliana* has cold-inducible 14-3-3 proteins, expressed in stems, leaves and flowers at 4 °C (Jarillo *et al.*, 1994). Hypoxia induces a 300 % increase in the 14-3-3 protein, GF14-12, in *Zea mays* (De Vetten and Ferl, 1995). A tobacco homolog 14-3-3 protein is down regulated during acclimation to high NaCl (Chen *et al.*, 1994), and a rice isoform is regulated by both salt and low temperature (Kidou *et al.*, 1993). A barley 14-3-3 homolog is induced after infection with the powdery mildew fungus *Erysiphe graminis*, apparently linking pathogen attack to signal transduction (Brandt *et al.*, 1992). Shinozaki and Yamaguchi-Shinozaki (1997) include 14-3-3 type proteins in a list of other important regulatory proteins, expressed in response to water stress.

The 14-3-3 proteins regulate the plant enzyme nitrate reductase (Sehnke and Ferl, 1996). Nitrate reductase catalyses a key step in nitrate assimilation - the reduction of absorbed nitrate to nitrite (Campbell, 1996). The expression of nitrate reductase is inducible and depends on both a light signal and the presence of the substrate nitrate. The enzyme is also subject to post-transcriptional regulation by phosphorylation in response to stimuli such as light levels, CO_2 concentration, and nitrate availability

(Campbell, 1996). Nitrate reductase can be inactivated in a matter of hours in response to darkness. This inactivation occurs by a two step process: phosphorylation of the enzyme, followed by binding to an inhibitory protein (Spill and Kaiser, 1994; Douglas *et al.*, 1995; Su *et al.*, 1996; Bachmann *et al.*, 1996). Binding of the nitrate reductase inhibitory protein (NIP) only takes place in the presence of adequate Mg^{2+} (Sehnke and Ferl, 1996). Once binding has occurred it is believed that the NIP specifically interferes with electron flow between the two metal-dependent cofactor domains of nitrate reductase (Bachmann *et al.*, 1996), presumably by altering its conformational state. Nitrate reductase is reactivated upon dephosphorylation and release of the NIP. The NIP protein is a member of the 14-3-3 family of proteins (Bachmann *et al.*, 1996; Moorhead *et al.*, 1996).

Another known association of 14-3-3 proteins in plants is with the fungal toxin, fusicoccin (Korthout and De Boer, 1994). Fusicoccin causes wilt in almost all higher plants, because it induces stomatal opening and thus water loss. This phytohormone-like effect, and other associated physiological effects of fusicoccin, have been attributed to the activation of the plant plasma membrane proton ATPase (Johansson *et al.*, 1993; De Michelis *et al.*, 1996; see also **Chapter 6**). Moorhead *et al.* (1996) showed that fusicoccin can disrupt binding of 14-3-3 proteins to phosphorylated nitrate reductase, suggesting there is competition for binding domains on the 14-3-3 protein. These findings, and the report that the plasma membrane proton ATPase is regulated by phosphorylation (Xing *et al.*, 1996), suggests a possible mechanism of proton ATPase activation by fusicoccin. It is suggested that fusicoccin competes for, and removes, inhibitory 14-3-3 proteins from the plasma membrane proton ATPase molecules, thus initiating activation.

Taken together, the nitrate reductase and proton ATPase studies point to a common mechanism for enzyme regulation by 14-3-3 proteins. The target enzyme would be regulated by phosphorylation and binding of a 14-3-3 protein; this process would be contingent upon the cellular levels of 14-3-3 protein, the kinase and phosphatase that act on the target enzyme, and Mg^{2+} availability. This complex contingency, along with the large number of possible 14-3-3 isoform combinations (10 identified to date in

Arabidopsis thaliana - Sehnke and Ferl, 1996), allows for multiple regulatory controls on target enzyme activity (Sehnke and Ferl, 1996).

In addition to the regulatory functions of 14-3-3 proteins in plants, it is becoming increasingly apparent that 14-3-3 proteins also have a sensory function. Moorhead *et al.* (1996) speculated that the NIP 14-3-3 protein (discussed above) directly senses changes in nitrate reductase activity. Furthermore, the plasma membrane bound, fusicoccin sensitive 14-3-3 protein (which directly interacts with fusicoccin molecules), was designated a true receptor/sensory type protein by Aducci *et al.* in 1995. In **Chapter 6**, it was suggested that this fusicoccin-sensitive 14-3-3 protein may also be involved in turgor pressure and/or osmotic pressure perception at the plasma membrane. Sadly, there is no molecular evidence to back up this hypothesis as yet (see Ingram and Bartels, 1996).

Given the highly conserved nature and abundance of 14-3-3 proteins in plant tissue (Ferl, 1996), it is reasonable to conclude that 14-3-3 proteins are essential molecular switches which respond to a number of stimuli, by initiating a regulatory response. The extent and range of these stimuli has yet to be determined. However, in the search for a hydrostatic/osmotic sensor on the plasma membrane, I believe the membrane bound 14-3-3 proteins are a sensible place to start.

7.2, Concluding remarks and future work

The primary aim of this study was to establish the importance of the apoplast as an osmotic compartment in two members of the family *Chenopodiaceae* - *Suaeda maritima* L. Dum. and *Beta vulgaris* L. I believe that my endeavours to achieve this objective have been successful. A water relations study, at single cell resolution, revealed that osmotically active solutes accumulate, and are maintained at relatively high concentrations, in the apoplast/cell wall of these species. These apoplastic solutes are instrumental in maintaining cell turgor pressure (P_{cell}) at a level which is much lower than the cell osmotic pressure (Π_{cell}). Furthermore the accumulated apoplastic solutes are apparently under the full control of the cell, and are rapidly adjusted in response to changes in cell turgor pressure (ΔP_{cell}), induced by modulation of the concentration of the external medium ($\Delta \Pi_{ext}$).

Suaeda maritima leaf epidermal cells accumulate NaCl in response to an increase in external NaCl. This protoplastic osmotic adjustment mechanism, necessary to maintain water flux into the plant, is accompanied by a simultaneous adjustment of apoplastic solutes, enabling the leaf epidermal cells to maintain a constant turgor pressure (P_{cell}) throughout (**Chapter 3** and **4**). Increases in *Beta vulgaris* (sugar beet) storage parenchyma cell osmotic pressure (Π_{cell}), due to sucrose accumulation, is also apparently countered by the accumulation and adjustment of solutes in the apoplast, allowing P_{cell} to be maintained at a constant level (**Chapter 5**).

The elegant single cell techniques (see **Chapter 2**) utilised during this study allowed the direct measurement of cell turgor pressure (P_{cell}), cell osmotic pressure (Π_{cell}), and cell solute content, from which indirect inferences were made about the apoplastic milieu. However, the tantalising challenge of directly measuring apoplastic solute fluxes during osmotic adjustment still remains. The apoplastic spaces of a plant are manifold and their ionic environments are difficult to gain access to. In spite of this, ion-selective microelectrodes have been applied successfully to measure apoplastic free ion concentration in leaves and roots. Bowling (1987) measured the apoplastic activities of K^+ and Cl^- in the leaf epidermis of *Commelina communis* in relation to stomatal activity.

Rhythmic and light-dependent K^+ and Cl^- fluxes have been measured in the pulvini of *Samanea saman* (Lee and Satter, 1989) and *Phaseolus vulgaris* (Starrach and Mayer, 1989). In roots, Henriksen *et al.* (1992) measured net fluxes of NH_4^+ and NO_3^- in the unstirred layer immediately external to the root surface in barley seedlings, and Felle (1998) measured apoplastic pH, K^+ and Ca^{2+}, under varying conditions, in the root cortex of *Zea mays*.

The use of ion-selective microelectrodes in conjunction with the closed system method described in **Chapter 4** and **5** could further facilitate the direct measurement of solute fluxes in the apoplastic compartment. Applying the closed system to excised tissue allows the researcher to access the apoplast of plant tissues which would otherwise be out of reach. The closed system maintains an apoplastic environment, in terms of volume, which is comparable to the apoplast *in vivo*, and also allows an element of control over the initial free solute content of the apoplast, which is simply not possible in intact tissues. Given the opportunity to continue working on the apoplastic compartment of higher plants, I would first seek to exploit the closed system as a possible tool to enhance and complement the fascinating work already carried out using ion-selective microelectrodes.

Turning from the measurement of protoplastic/apoplastic osmotic adjustment to the molecular basis of these regulatory mechanisms - protoplastic and apoplastic osmotic adjustment is largely achieved by the modulation and manipulation of membrane transport processes. The data presented in **Chapter 6** suggests that the secondary transport of solutes necessary to achieve osmotic adjustment (in either the protoplastic or apoplastic compartment) is driven by the turgor/external osmotic pressure (P_{cell}/Π_{ext}) dependent nature of the plasma membrane proton ATPase. However, how the cell perceives changes in P_{cell}/Π_{ext} remains a mystery that only the molecular biologists can realistically tackle. In **Chapter 6**, I proposed that a plasma membrane bound, fusicoccin sensitive, 14-3-3 type protein may play a role in P_{cell}/Π_{ext} sensing. In order to establish whether this is the case or not, the development of genetically modified plants, which are devoid of this particular protein would certainly be useful. The current

understanding of the molecular basis of water stress tolerance in plants is reviewed by Ingram and Bartels (1996).

Appendix

In text	Address
Agaraids	Agaraids, Stansted, Essex. UK.
BDH	BDH Ltd., Poole, Dorset, UK.
Beaudouin, Paris	Beaudouin, 1-3 Rue Rataud, Paris.
Cheshire Rubber Co.	Cheshire Rubber Company, Unit 4-5, Alexander House, CCE Centre, Windmill Lane, Manchester, UK.
Clarke Electromedical Instruments	Clarke Electromedical Instruments, Reading, UK.
Decon Laboratories Ltd.	Decon Laboratories Ltd., Conway Street Hove, East Sussex, UK.
Druck Ltd.	Druck Ltd., Fir Tree Lane, Groby, Leicester, UK.
Fisher Scientific	Fisher Scientific UK., Bishop Meadow Road, Loughborough, Leicestershire, UK.
Hamilton Co.	Hamilton Company, Reno, Nevada, USA.
Harvard Apparatus	Harvard Apparatus, Sheerness, Kent, UK.
Intracel Ltd.	Intracel Ltd., Unit 4, Station Road, Shepreth, Royston, Herts., UK.
Leica	Leica Ltd., Milton Keynes, UK.
Leitz	Ernst Leitz Wetzlar GmbH, D-6330 Wetzlar, Germany.
Moore and Wright	Moore and Wright Micrometer Head Tools Ltd., Sheffield, UK.
Portex Ltd.	Portex Ltd., Hythe, Kent, UK.
Prior Scientific Instruments	Prior Scientific Instruments Ltd., Unit 4, Wilberham Road, Fulbourn, Cambridge, UK.

Rathburn Chemicals	Rathburn Chemicals Ltd.,Walkerburn, Scotland, UK.
RS Components	RS Components Ltd., Corby, Northamptonshire, UK.
Sigma Chemicals	Sigma-Aldrich Chemicals, Fancy Road, Poole, Dorset, UK.
TAAB Laboratory Equipment	TAAB Laboratory Equipment Ltd., 3 Minerva House, Calleva Industrial Park, Aldermaston, Berkshire, UK.
Terumo	Terumo Europe, N.V. 3001 Leuven, Belgium.
Wacker Chemicals Ltd.	Wacker Chemicals Ltd., Walton on Thames, UK.
Whatman	Whatman International Ltd., Maidstone, Kent, UK.

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