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The distribution and mobilisation of sulphur and nitrogen in leaves of Triticum aestivum approaching senescence / by Nicholas John Bates.

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The Distribution and Mobilisation of Sulphur and Nitrogen in leaves of *Triticum aestivum* approaching senescence

by

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under supervision of

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ABSTRACT

Higher plants obtain the majority of their sulphur in the form of sulphate which is taken up directly from the soil. In industrialised areas, this uptake can be supplemented by the absorption of sulphur-containing pollutants from the air. Due to increasing environmental concerns, levels of atmospheric sulphurous pollutants have fallen dramatically in the last 30 years. This (in conjunction with increased use of sulphur-free straight and compound fertilisers), has led to the appearance of sulphur deficiency symptoms in modern-day crops. The reductions in yield associated with these sulphur deficient crops represents a threat to U.K. agriculture.

Sulphur deficiency symptoms in higher plants appear first in the lower leaves. These observations have been linked to the slow re-mobilization of sulphate from mature leaf tissue. Radio-tracer studies using ^{35}S-sulphate have indicated that mature leaves contain two populations of kinetically distinguishable cell each of which exchanges sulphate at a different rate.

Using single cell sampling and analysis techniques, the aim of the experiments described in this thesis was to investigate the behaviour of sulphur when induced to accumulate in and then re-mobilize from mature wheat leaves. This aim was addressed by inducing plants to accumulate excess sulphur (termed high S plants) which was achieved by both providing extra sulphate and restricting the supply of monovalent anions. Accumulation of sulphur in ageing leaves coincided with decreases in turgor and osmotic pressure and diminished efflux of potassium and influx of calcium.

The withdrawal of sulphate and re-instatement of nitrate supplies to high S material induced re-mobilization of epidermal and bundle sheath sulphur reserves. There was negligible re-mobilization of accumulated sulphur from mesophyll cells. It was concluded that much of the sulphur amassed by the leaf must effectively be 'immobile' and thence unavailable for re-translocation irrespective of the sulphur-status of the plant.

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To Jayne.

Companion and critic.

Chapter 1:

INTRODUCTION

1.1, SULPHUR

1.1.1, Why do higher plants require S?

Between 1850 and the mid 1900's, a flurry of interest in the growth of plants using soil-less culture systems resulted in the elucidation of all of the 'essential elements' required by green plants. In 1860, the German botanist, Knop established the importance of a source of S in such soil-less systems to ensure optimal plant growth was achieved (Benton-Jones, 1982).

Unbeknown to Knop, S represents an integral component of several biochemically important compounds including for example, the amino acids cysteine and methionine and the organic thiol glutathione (see for example, Marschner, 1995). A source of reduced S is also required for the synthesis of various secondary compounds (Marschner, 1995) and in the regulation of pollen incompatibility (Sutcliffe and Baker, 1981). Many of these so-called, secondary compounds play a significant role in the detoxification of the plant. Sulphur for example, represents a major constituent of the metallothioneins which are synthesised in response to heavy metal stress (Marschner, 1995).

Whilst the cellular metabolism of S will be discussed in detail later, the final stage in the assimilation of SO_4^{2-} (reduction pathway) is marked by the synthesis of the amino acid cysteine (see **Figure 1.1**) from its precursors O-acetylserine and sulphide in a reaction catalysed by the enzyme O-acetylserine(thiol)lyase (Gotor *et al.*, 1997). It is from this cysteine (and the methionine which may subsequently be formed) from which the synthesis of co-factors and secondary compounds may begin. In these compounds (which contain S in its reduced form), the S moiety generally forms part of a functional group involved directly in metabolism, or adopts a more structural role (Marschner, 1995). Ferredoxin and biotin (Vitamin H) for example, both contain S in a structural capacity (Marschner, 1995). Similarly, the reversible formation of disulphide (S=S) bonds between adjacent cysteine residues in polypeptide chains can influence the tertiary and quaternary structure (and hence, the function) of enzyme proteins (Marschner, 1995). In many enzymes and coenzymes (such as urease, the sulfotransferases and coenzyme A which all contain the

sulphydryl (-SH) functional group), the S moiety assumes a more active role in cellular metabolism (Marschner, 1995).

At least 70 % of total plant S is, at any one time, in the form of organic thio compounds (Thoiron *et al.*, 1981). Water-soluble thiols constitute approximately 2 % of this, the majority of this fraction being made up of the tripeptide glutathione (Marschner, 1995). Glutathione is synthesised from its constituent amino acids (glutamate, cysteine and glycine) in two enzyme-catalysed reaction steps (see Rennenberg, 1997 for a review). Many functions have been attributed to glutathione in higher plants (see Rennenberg, 1998) and one of the main roles of this tripeptide is in the defence of plants against oxidative stress caused for example, by mineral deficiencies (Cakmak and Marschner, 1992), exposure to excess O_3 (Cuin, 1996), heavy metals (such as Ag, As, Bi, Cd, Cu, Pb and Zn; Rennenberg and Brunold, 1994), cold (Brunner *et al.*, 1995) or contact with xenobiotics (Schröder *et al.*, 1990). Recent evidence has also suggested a role for glutathione in the regulation of plant S nutrition (Lappartient and Touraine, 1996; Rennenberg, 1997; Lappartient *et al.*, 1997). In this capacity, glutathione functions both as a transient storage pool for reduced S (Schütz *et al.*, 1991) and represents the form in which most reduced S is transported around the plant (see for example, Blaschke *et al.*, 1996).

Many secondary metabolites contain appreciable amounts of reduced S and some of these compounds are responsible for the characteristic taste and flavour of many plant species (Schnug, 1997). The alliins and the glucosinolates for example, are responsible for the hotness of onions (*Allium spp.*) and the bitterness of certain *Brassica* species (such as Brussels sprouts; Schnug, 1997).

Whilst the role of many secondary S compounds is not entirely clear, their involvement as defence substances to protect the plant from herbivorous predation and pathogenic attack is well documented (see for example, Greenhalgh and Mitchell, 1976). However, some secondary S metabolites (most notably the glucosinolates) also act as significant stores of reduced S which may be degraded as and when the need arises (Schnug, 1997). Degradation of glucosinolates by myrosinase for example, releases bound S thereby facilitating re-cycling of these S reserves during periods of sub-optimal S supply (Marschner, 1995). Whilst reduced S represents a major component of many biologically significant compounds, it is thought that only the sulpholipids (which are characteristic components of the inner

chloroplast and thylakoid membranes of all higher plants; Hell, 1997) utilise S in its non-reduced (sulphate ester) form (Clarkson *et al.*, 1983; Marschner, 1995).

Irrespective of whether it is utilised in its reduced or non-reduced form, the importance and relevance of S in achieving desired crop quality and obtaining optimum yields from modern agricultural crops should not be overlooked. Indeed, amongst the substances that flow into the root system, S comes fifth or sixth in quantity (Cram, 1990). In spite of this, however, the field of S biochemistry remains relatively neglected (Cram, 1990; Brunold and Rennenberg, 1997) principally because up until recently (see for example, Withers *et al.*, 1993; Withers *et al.*, 1995; Marschner, 1995; Zhao and Mc.Grath, 1997), scientists (and consequently governments) believed that S deficiency could not exist in industrialised countries (Schnug and Haneklaus, 1994).

There has been a recent surge of interest in attempts to elucidate and understand the metabolism and 'nutrient use efficiency' of S in higher plants. Much of this interest has arisen because S deficiency now represents the most frequent nutrient disorder reported across northern Europe (Schnug and Haneklaus, 1994). The many and varied requirements of S within the higher plant dictates the need for an adequate supply of S as an essential pre-requisite in maintaining the quality and yield of crop plants. A more thorough discussion of the increased incidence of S deficiency and its potential effects on European agriculture, however, will be left for later.

1.1.2, How does the plant meet these demands?

The majority of higher plants obtain their S from the soil in the form of SO_4^{2-} (see for example, Marschner, 1995; Lappartient and Touraine 1996; Massonneau *et al.*, 1997). Generally, soil SO_4^{2-} is derived from the oxidation of insoluble forms of S in the rhizosphere such as iron or zinc sulphide and gypsum (Sutcliffe and Baker, 1981). The exceptions to this, however, occur either in arid soils or in saline wetlands in which S exists predominantly as soluble sulphate (Sutcliffe and Baker, 1981) and sulphide (Rennenberg, 1984) respectively.

The S requirement for optimum plant growth varies considerably from species to species. In general, however, the optimum S content of higher plants tends to fall in the range of 0.1-1.5 % total dry weight (De Kok, 1990) although the actual amount

of S required by the plant is dependent upon its N status (Rennenberg, 1984; Cram, 1990; Clarkson *et al.*, 1989, 1993).

In a comparison of the major crop families, the requirement for S increases in the order Gramineae < Leguminosae < Cruciferae (Marschner, 1995) with members of the Brassicacea (most notably *Brassica napus* or oil seed rape) making the highest demands on soil SO_4^{2-} reserves (Schnug and Haneklaus, 1994). Whilst the S requirement of cereal crops is significantly lower (*circa* three-fold; Zhao and Mc.Grath, 1997) than that of oil seed rape, a supply of 10-30 kg ha^{-1} is still necessary for satisfactory growth and baking (or brewing) qualities (Withers *et al.*, 1995).

The uptake of SO_4^{2-} from the soil represents an energy-dependent process involving specific carrier proteins (Rennenberg, 1984; Jensén and König, 1982; Cram, 1983a; Clarkson *et al.*, 1993; Hawkesford *et al.*, 1993). Use of molecular cloning has demonstrated the existence of several different plasma membrane transporters specific for SO_4^{2-} (Clarkson, 1997). These SO_4^{2-}-specific transporters form a unique group of proteins, seemingly unrelated to any other cloned integral membrane proteins (Hawkesford and Smith, 1997) and are generally classified as either high (low K_m) or low affinity (higher K_m) (Clarkson, 1997).

In oil seed rape (Hawkesford *et al.*, 1993; Schnug and Haneklaus, 1994; Massoneau *et al.*, 1997), tomato (Hawkesford and Belcher, 1991), carrot (Cram, 1983a) and *Macroptilium* (Clarkson *et al.*, 1983), control of SO_4^{2-} uptake from the soil appears to be closely correlated with the S status of the shoot. Under normal physiological conditions (*i.e.* adequate S nutrition), the influx of SO_4^{2-} into plants via the roots is typically repressed (see for example, Clarkson *et al.*, 1989). This 'demand driven regulation' is thought to be under the control of a 'messenger molecule' which is likely to originate in the shoot and thence be transported to the roots via the phloem (Lappartient and Touraine, 1996).

Whilst the exact identity of the 'messenger' remains unknown, suitable candidates in the form of internal SO_4^{2-} (see for example, Jensén and König, 1982; Cram, 1990; Bell *et al.*, 1995a) or a product(s) of SO_4^{2-} assimilation (most likely to be glutathione; Lappartient and Touraine, 1996) have been mooted. Interestingly, Cram (1990) suggested that an inability to correctly identify the phloem messenger might be indicative of the fact that both SO_4^{2-} and reduced S-compounds act as such communicators. Irrespective of its identity, it is assumed that arrival of the shoot-root

signal at the root system evokes (at least in part) changes in the availability and/or densities of SO_4^{2-} transporters (Hawkesford *et al.*, 1993; Smith *et al.*, 1995). Whether these 'changes' in root cell membrane properties stem from modifications to the turnover rate and/or an allosteric effect on carrier activity is currently unknown (Bell *et al.*, 1995a). It is likely, however, that these modifications in the transport properties of root cell membranes arise as a consequence of modified gene expression (Hawkesford *et al.*, 1993; Massoneau *et al.*, 1997).

Some soils are unable to support plant growth either because they contain insufficient S or the S present is in an incorrect form (Rennenberg, 1984). In these instances, plants often utilise volatile S compounds (such as SO_2 and H_2S which arise as a result of the combustion of fossil fuels and from smelting processes) from the atmosphere (Rennenberg, 1984). These gaseous S compounds enter the plant mainly via open stomates (see for example Brunold, (1990)) and then subsequently dissolve in the moist apoplastic space (Herschbach *et al.*, 1995). Now in solution, these S-compounds are able to cross the plasma membrane and enter the cell (Rennenberg, 1984; Cram, 1990; Clarkson *et al.*, 1993). Once in the cytosol, the S moiety can either enter the S assimilatory pathway (Brunold, 1990) or it can be stored in the cell vacuole (presumably in the form of SO_4^{2-}) pending future use (Cram, 1990). As a result of the ability of the plant to utilise atmospheric S, the biosynthesis of S-containing primary and secondary metabolites is unaffected even when the plant is growing in a soil known to be significantly SO_4^{2-}-defficient (Rennenberg, 1984 and references therein).

However, the volatile nature of SO_2 and H_2S means that when the intake of these atmospheric S sources exceeds a certain threshold (which may vary both between and within species; De Kok, 1990), the inherent toxicity of these compounds can lead to a significant reduction in plant growth (Rennenberg, 1984 and references therein). When supplied at sub-lethal dosages, however, absorption of H_2S and SO_2 through the stomates can inhibit the uptake of soil SO_4^{2-} by the roots (Herschbach *et al.*, 1995). It would appear that the ability to absorb and subsequently utilise atmospheric S is of significant benefit to the plant, especially when the S supply to the roots is limiting (De Kok, 1990).

1.1.3, The fate of internalised sulphur

The majority of the SO_4^{2-} absorbed by root cells from the surrounding rhizosphere is transported upwards from the roots to the shoots in the xylem (see for example, Larsson *et al.*, 1991). The loading of SO_4^{2-} into the xylem is thought to represent an energy dependent process under metabolic control (Herschbach and Rennenberg, 1991). The rate of loading is determined by the amount of SO_4^{2-} taken up by the roots and although S deprivation results in increases in the rates of SO_4^{2-} uptake (see above) and xylem loading (Herschbach and Rennenberg, 1991), it is probable that the two are regulated independently (*via* different phloem-translocated signals?) by the SO_4^{2-} demand of the shoot (Kreuzwieser *et al.*, 1996).

Whilst the enzymes necessary for assimilatory SO_4^{2-} reduction can be found in both the roots and leaves of herbaceous plants (see for example, Pate, 1965), it is thought that the reduction of SO_4^{2-} and subsequent synthesis of organic S compounds is confined predominantly to the leaves (Brunold, 1990). It is possible that reduction of SO_4^{2-} in the roots is insufficient to fulfil the requirements of root tissue for reduced S (Brunold, 1990). Consequently, it is possible that the root system is re-supplied with a source of reduced S from the leaves by way of the phloem (see for example, Rennenberg *et al.*, 1979; Blaschke *et al.*, 1996; Marschner *et al.*, 1997). This apparent 'cycling' of S within the plant is considered subsequently in this chapter.

Upon arrival at the target leaf, there are, in principle, a number of different pathways which SO_4^{2-} could pursue following its unloading from the xylem. Such pathways might be fully apoplastic, partly symplastic, entirely transcellular (in which solutes (such as SO_4^{2-}) cross all cell membranes in series; Tomos and Wyn Jones, 1988) or involve vein extensions (see Leigh and Tomos (1993) for a review). Whilst the way(s) in which SO_4^{2-} travels from the xylem to the mesophyll may be unknown, the same is not true of the subsequent assimilatory steps most of which have generally been well characterised (see for example Hell, 1997; Schwenn, 1997). The assimilation of S in higher plants is summarised in **Figure 1.1**.

Despite the presence of certain key enzymes in the cytosol (such as ATP-sulphurylase and O-acetylserine(thiol)lyase; Lunn *et al.*, 1990), the assimilation of S is generally thought to be confined to mesophyll cell chloroplasts (Rennenberg, 1989; Lappartient and Touraine, 1996). Due to the inherent inertness of SO_4^{2-}, the

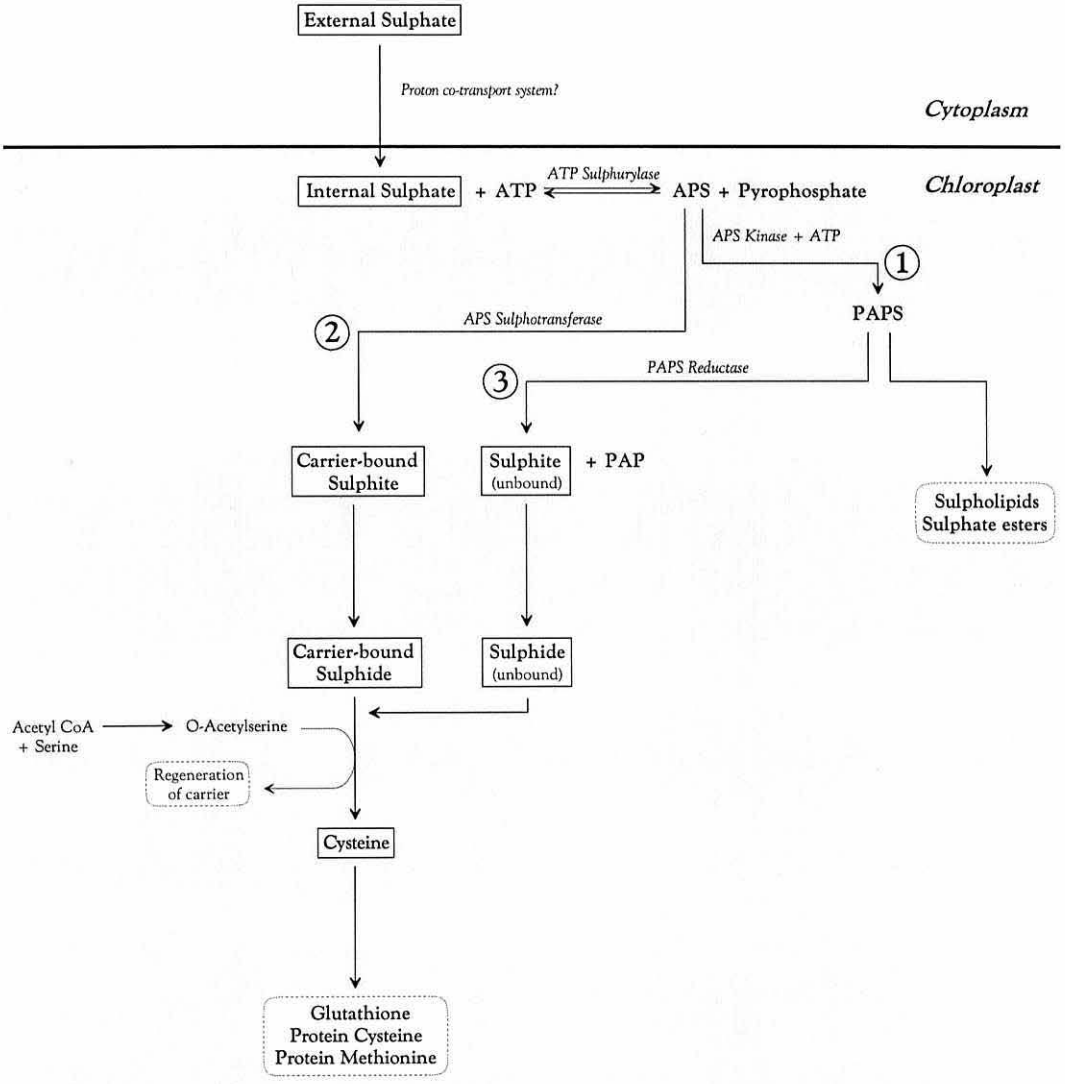
initial stage of S assimilation is characterised by the activation of the anion (Hell, 1997; Schwenn, 1997). This is achieved by the enzyme ATP sulphurylase (see **Chapter 4, Figure 4.1**) which catalyses the formation of APS (adenosine-5'-phosphosulphate, an energy-rich anhydride of sulphate and phosphate) and pyrophosphate (PP_i) from SO_4^{2-} and ATP (Hell, 1997). The activity of extractable ATP sulphurylase is affected both by environmental influences such as S and N status (Brunold, 1993; Lappartient and Touraine, 1996) and the light intensity with which plants are supplied (see for example, Ferretti *et al.*, 1995). This has led to the suggestion that centralised control of S metabolism could occur via gene regulated changes in the activity of ATP sulphurylase (Brunold, 1990).

From here on, activated SO_4^{2-} (in the form of APS) can either be reduced to sulphite (which leads to the subsequent formation of the amino acid, cysteine in the "reductive pathway") or esterified with organic compounds (for which reduction is not necessary; hence the "non-reductive pathway"). In this "non-reductive pathway", APS-kinase catalyses the conversion of APS to PAPS (3'-phosphoadenosine-5'-phosphosulphate) in a second energy-dependent reaction step (see Brunold and Rennenberg (1997) for a review). Subsequently, PAPS acts as the preferred donor in SO_4^{2-}-transfer reactions for the esterification of hydroxyl residues (Hell, 1997) which leads ultimately to the production of sulphate esters and sulpholipids (Brunold, 1990).

As the name suggests, SO_4^{2-} is progressively reduced in the "reductive pathway" thereby providing sulphide (see **Figure 1.1**) for the subsequent biosynthesis of cysteine (Hell, 1997). At the current time, however, the problematic redox-biochemistry of S compounds (see for example, Brunold and Rennenberg, 1997) means that the nature of many of the stages in this pathway remain controversial. Consequently, two possible pathways have been proposed (Hell, 1997).

In the 'APS-bound' pathway, the reduction of S begins from APS and proceeds via a bound thiosulphate (possibly glutathione?) intermediate (the formation of which is catalysed by APS-sulphotransferase) to sulphite (Hell, 1997; Schwenn, 1997). Bound sulphite is then reduced to bound sulphide by the enzyme thiosulphonate reductase (Hell, 1997).

Figure 1.1. A simplified diagram of S assimilation in higher plants. Following activation, the S moiety can be assimilated in one of three ways (labelled 1-3 in the diagram below). In pathway 1 ("non-reductive"), S is activated further to form PAPS which leads ultimately to the biosynthesis of sulpholipids and sulphate esters. In pathways 2 and 3 ("reductive"), S is progressively reduced which leads to the biosynthesis of the amino acid cysteine. In pathway 2 ('APS-bound'), reduction of the activated S moiety proceeds from APS and involves an unknown carrier compound. Pathway 3 ('PAPS-free'), proceeds from PAPS and involves no such carrier compound. Abbreviations are defined in the accompanying text.



In the 'PAPS-free' pathway, the reduction of S proceeds from PAPS in a reaction sequence similar to that found in some fungi and enterobacteria (Gutierrez-Marcos *et al.*, 1997). The reduction of PAPS by the thioredoxin-dependent PAPS-reductase releases sulphite and PAP (adenosine-5'-phosphosulphate) in a mechanism devoid of a specific carrier (Hell, 1997). Free sulphite is then reduced to free sulphide by the ferredoxin-dependent enzyme, sulphite reductase (Hell *et al.*, 1997).

A lack of molecular evidence means that the existence of these two pathways can neither be conclusively accepted nor denied and it is even possible that mixed S reduction pathways may function in plants (Hell, 1997). At the current time, unequivocal confirmation of the identity of a number of S-assimilatory enzymes (including for example, APS-sulphotransferase, APS-reductase and PAPS-reductase) is lacking (Hell, 1997). However, the recent finding that APS-kinase possesses APS-sulphotransferase activity (see Schiffmann and Schwenn, 1994) suggests that steps are being taken to address our current lack of understanding of S assimilation in higher plants.

The synthesis of cysteine (the first stable compound to contain reduced, organic S) marks the final stage of S assimilation (Hell, 1997). In a reaction catalysed by the enzyme serine acetyltransferase, serine, acetyl CoA and sulphide combine to form the intermediate O-acetylserine (OAS). This intermediary is then converted into cysteine by the enzyme O-acetylserine(thiol)lyase (OAS-TL). Cysteine biosynthesis is effectively rate-limited by the production of its intermediate, OAS (Hell, 1997). This has led to the suggestion that it is this intermediate which may represent the mediator between S and N metabolisms (Brunold, 1993). Interestingly, an up-regulation of mitochondrial OAS-TL activity has been reported in N-deficient spinach which might also tentatively suggest the existence of a further connection between S and N metabolisms at this level (Takahashi and Saito, 1996). Cysteine subsequently represents the precursor from which the synthesis of all other compounds containing reduced S is initiated (Marschner, 1995). A description of the biosynthetic pathways associated with the production of these other S compounds is considered to be outside the range of this project. Consequently, the interested reader is referred to Hell (1997) for a review of the intricacies of methionine, glutathione and secondary compound biosynthesis.

Not all of the internalised SO_4^{2-} is transported to mesophyll tissue for incorporation into organic S-compounds. Whilst some 70 % of total plant S was in the form of organic thio compounds in *Lemna minor* for example, a considerable proportion (around 25 %), was stored as free SO_4^{2-} within cell vacuoles (Thoiron *et al.*, 1981). In the mature leaves of *Macroptilium*, 20 % of the SO_4^{2-} arriving in the xylem from the root system was reduced to organic-S compounds whilst the remaining 80 % was accumulated in leaf cell vacuoles (Bell *et al.*, 1994). Ernst (1990) suggested that once accumulated within leaf cell vacuoles, SO_4^{2-} plays a role in the maintenance of cellular water relations. However, under normal physiological conditions, the vacuolar concentration of SO_4^{2-} is so low (less than 20 mM) that one would expect this 'storage pool' to contribute only minimally to cell turgor and/or osmotic pressure (Fricke *et al*, 1994b). Understandably, however, an increase in leaf SO_4^{2-} content might be expected if the delivery of S from the roots (via the xylem) exceeded the rates at which S was being assimilated and/or exported from the leaf in the phloem (Sunarpi and Anderson, 1996). However, an increase in the amounts of SO_2 or H_2S in the vicinity of leaf stomates could equally result in an elevated leaf SO_4^{2-} content (Olivares and Aguiar, 1996).

In many plant species, the S transported from the roots to the shoot in the xylem is readily re-translocated back to the roots (in the form of both SO_4^{2-} (Cooper and Clarkson, 1989; Larsson *et al.*, 1991) and glutathione; see for example, Rennenberg, 1998) in the phloem. Further evidence suggests that some of the S returned to the root system may be subsequently *re-exported* back to the shoots (presumably as SO_4^{2-}) thereby establishing a cycling process of nutrients between root and shoot (see for example, Pate *et al.*, 1979 who describes the cycling of N in the nodulated *Lupinus*). In spite of the fact that nutrient cycling appears to be a constant feature of the physiology of wheat (being detectable even when the grain is filling and ripening), the significance of the process requires further elucidation. Clarkson and co-workers (see for example, Clarkson *et al.*, 1983; Cooper and Clarkson, 1989; Larsson *et al.*, 1991) suggest, however, that the selective export of S compounds in a basipetal direction from the shoot followed by acropetal re-transportation in the xylem could prove to be an essential pre-requisite for the re-distribution of S from older source leaves to newer sink tissue within the plant.

Cooper and Clarkson (1989) estimated that a single molecule of amino-N might be expected to cycle at least once around the plant before being withdrawn from the xylem/phloem/symplast system. Although S cycles between root and shoot, the extent to which the process occurs is less than that reported for N (see Larsson *et al.*, 1991). It seems fairly safe to assume, however, that at least some of the SO_4^{2-} internalised by the plant will, at any one time, be involved in some form of cycling through the roots (see also Cooper and Clarkson, 1989).

1.1.4, What happens when plant S requirements are not met?

During the infamous London smog incident of 1952, 4000 people lost their lives in the space of a single week (Ayres, 1996 and references therein). Four years later, the first Clean Air Act was passed by the U.K. government. This new act was primarily concerned with cleaning up industrial and domestic emissions resulting from the large-scale combustion of fossil fuels (Ayres, 1996). By reducing the levels of SO_2 and particulates in the atmosphere, the government hoped that further "killer smog" incidents could be prevented (Ayres, 1996). As a consequence of this first Clean Air Act, the levels of SO_2 in the atmosphere have been falling progressively since the mid 1950's (Ayres, 1996). The total SO_2 emissions from U.K. industry for example, decreased from 6.4 million tonnes in 1970 to 2.8 million tonnes in 1994 with further reductions (below 1.0 million tonnes) predicted by the year 2010 (Zhao and Mc.Grath, 1997 and references therein). Coupled with the increasing use of the S-free straight and compound fertilisers in modern agriculture (Withers *et al.*, 1995), this decrease in the levels of atmospheric S has led to a substantial reduction in the amounts of S deposited onto arable lands (Byers *et al.*, 1987). Consequently, recent surveys of U.K. agriculture have demonstrated that the area of S deficiency in England has grown from 24 % in 1991 to 39 % in 1992 (Ceccotti and Messick, 1997 and references therein) whilst several soils in the far north of Scotland are now known to be S deficient (Chapman, 1997 and references therein). Indeed, it has been predicted that virtually all arable areas in the U.K. could be at least partially at risk from S deficiency by the year 2003 (Anon, 1995).

Since S constitutes an important structural and/or functional component of many organic compounds, it is logical that a link exists between the availability of S and the overall health of the plant (Ceccotti and Messick, 1997). In addition to

inducing the appearance of the characteristic symptoms of S deficiency however (see for example, Burke *et al.*, 1986; Green *et al.*, 1986; Dietz and Helios, 1990), a sub-optimal supply of S also results in significant reductions in the quality and yield of many modern-day crop species (Schnug, 1997). Sulphur deficiency impairs the biosynthesis of many S-containing secondary metabolites (Schnug, 1997). Since many of these compounds are involved in the protection of the plant from disease and predation (see earlier), in extreme cases, S deficiency can ultimately predispose crops to pathogenic attack (Cooper *et al.*, 1996; Schnug and Haneklaus, 1994; Schnug, 1997).

Severe S deficiency can also affect the quality of those crops grown for human or animal consumption. In the case of bread-making wheat varieties, the baking and milling quality (and thus retail value) of the crop is adversely affected by the S status of the grain (Byers *et al.*, 1987; Ceccotti and Messick, 1997). The S status of the grain in turn, is determined (in part) by the amount of S internalised by the plant. In bread making, the strength of the dough is determined by the quantity and quality of the gluten present in the wheat grain (Byers *et al.*, 1987). Specifically, it is the presence of large numbers of disulphide (S=S) bonds between adjacent cysteine residues which makes a good bread dough (Byers *et al.*, 1987; Schnug *et al.*, 1993). Indeed, attempts to bake bread from the wheat grain produced by high N:low S plants proved fruitless; the end product neither resembling bread nor proving edible (Byers *et al.*, 1987).

Since N and S are both involved in protein biosynthesis, a sub-optimal S supply can result in a reduction in the efficiency with which plants utilise NO_3^- (Ceccotti and Messick, 1997). Due to the absence of a suitable sink for N in protein metabolism, excess N accumulates within the plant as NO_3^- (Schnug, 1997) despite the down-regulation of NO_3^- uptake observed in S deficient plants (see for example, Clarkson *et al.*, 1989; Bell *et al.*, 1995b). This down-regulation of NO_3^- uptake results in an increased loss of N from agricultural soils through volatilisation and leaching into groundwater (Ceccotti and Messick, 1997). Consequently, the decrease in the NO_3^--use efficiency of S deficient crops suggests that adequate S nutrition is essential if the high N application rates (achieved via the extensive application of fertiliser to modern-day crops) are to be fully-exploited in terms of crop quality and yield (Schnug *et al.*, 1993).

Generally, symptoms of severe S deficiency in higher plants are confined to a yellowing of the younger, developing leaves whilst older and more mature material often still appears green (Schnug and Haneklaus, 1994; Bell *et al.*, 1995a). The appearance of these characteristic deficiency symptoms has been linked to the slow re-mobilization of cellular SO_4^{2-} pools from older leaf tissue (Clarkson *et al.*, 1983; Bell *et al.*, 1994, 1995a). In contrast, re-mobilization of SO_4^{2-} from young leaf tissue is considerably faster than that observed from mature tissue (Gilbert *et al.*, 1997).

Using *Macroptilium atropurpureum*, Clarkson and co-authors (1983) observed that irrespective of either shoot S-status or whether plant growth was limited by insufficient S, a relatively constant proportion of total plant S was present in the form of soluble SO_4^{2-} effectively 'trapped' within mature leaves. In the S deficient wheat of Byers *et al* (1987) for example, 28 % of total plant S was present in the form of SO_4^{2-} regardless of the fact that grain yield (at a mean of 1.48 g per plant) was substantially lower in these plants than in those supplied with adequate S (at a mean of 9.35 g per plant).

The cause(s) of the sequestration of SO_4^{2-} (even in the presence of a powerful stress-induced sink) once accumulated by mature leaves remains unknown. In previous studies, however, suggestions of slow turnover from mature leaf cells (Clarkson *et al.*, 1983; Dietz, 1989; Bell *et al.*, 1994), the lack of an active regulatory mechanism linking newly emerging sink tissues with ageing source leaves (Adiputra and Anderson, 1992, 1995; Sunarpi and Anderson, 1996) and selective phloem loading (Clarkson *et al.*, 1983; Cooper and Clarkson, 1989; Larsson *et al.*, 1991) have been made. In the absence of any reported incidence of S deficiency, this apparent inefficiency of the internal utilisation of SO_4^{2-} by higher plants (see also Cram, 1990) would presumably be of little consequence. Because of an increased incidence of S deficiency however, attempts to gain an insight into how (and why) mature leaves sequester SO_4^{2-} might prove of considerable agronomic importance in the production of more efficient crops to feed an ever-growing world population. The main aim of this investigation, therefore, was to gather data on the behaviour of SO_4^{2-} at the single cell level (the aims and aspirations of this study are discussed further in section **1.3**).

1.2, NITROGEN

1.2.1, Why is N required by higher plants?

Whilst the field of S biochemistry might have remained relatively neglected up until recently (Cram, 1990), the fundamental role of NO_3^- in plant nutrition has meant that the various pathways pertaining to the uptake and subsequent metabolism of this anion have been studied thoroughly over the last 40 years (Hoff *et al.*, 1994). As a consequence of this intense interest, N assimilation in higher plants is the subject of a large literature and in recognition of this, the treatment of N metabolism in this thesis will be less detailed than that presented earlier for S.

The roles of N within the higher plant are many and varied with N representing an integral component of many biochemically important organic compounds. Rather than directly utilising the NO_3^- absorbed from the soil however, the biosynthesis of these organic compounds necessitates prior reduction of NO_3^- to NH_4^+. The organic N compounds initially synthesised by the plant (such as amino acids, amides, peptides, amines and ureides), are of relatively low molecular weight (Marschner, 1995). Many of these low molecular weight compounds serve as intermediates in the biosynthesis of high molecular weight organic N compounds (such as proteins, nucleic acids, coenzymes, and secondary metabolites) although many have intrinsic value in their own right (Marschner, 1995). In roles analogous to those served by glutathione in S metabolism, amino acids and amides perform a valuable role in both the transient storage and long distance transport of reduced N (Marschner, 1995). Amines are components of the lipid fraction of biomembranes whilst polyamines are thought to function as secondary messengers (for example, delaying the onset of senescence; Marschner (1995) and references therein).

When N is available to the plant in amounts exceeding those required for optimum growth, the surplus is accumulated (mainly as NO_3^-) in cell vacuoles (Martinoia *et al.*, 1981; Zhen and Leigh, 1990). Cytoplasmic concentrations by contrast, remain significantly lower (Zhen *et al.*, 1991; Miller and Smith 1992; Winter *et al.*, 1993). Primarily, the vacuolar NO_3^- reserves function as storage pools thereby enabling the plant to survive short periods of N insufficiency (Zhen and Leigh, 1990; Zhen *et al.*, 1991). However, vacuolar NO_3^- also plays an important role in the maintenance of charge balance (Marschner, 1995) and turgor and osmotic pressures (Zhen *et al.*, 1991; Richardson, 1993; Schenk, 1996). The accumulation of excess NO_3^-

by plants in the field, however, indicates an imbalance between N supply (in terms of fertiliser dosage) and plant demand (Marschner, 1995). In an agronomic sense, this excess accumulation is financially uneconomical and can have detrimental effects on vegetable and forage crop quality (Marschner, 1995).

1.2.2, How does the plant fulfil its requirement for N?

Nitrogen is most frequently found in the soil in the forms of NO_2^-, NO_3^-, NH_4^+ and free ammonia which arise from the natural decay of animal and plant proteins by microbial organisms (Sutcliffe and Baker, 1981). Higher plants generally absorb their inorganic N from the soil in the form of NO_3^- or NH_4^+ or a combination of the two (Marschner, 1995; Schenk, 1996). The relative proportions of NO_3^- : NH_4^+ absorbed differ with respect to temperature, soil water pH and plant species (Schenk, 1996). Some plants however (most notably slow-growing trees in heavily polluted areas), supplement the N absorbed by the root system with atmospheric N (in the forms of NO, NO_2 or NH_3) absorbed via the stomates (Rennenberg *et al.* (1996) and references therein).

As might be expected, the N requirement for optimum plant growth varies from species to species although it generally falls in a range of between 2.0-5.0 % total dry weight (Marschner, 1995). Since N alters plant composition more than any other nutrient (Marschner, 1995), a significant deviation in the N supply either above or below that required for optimum growth can have profound environmental and economic consequence. A sub-optimal supply for example, inhibits the growth of shoots but promotes that of roots (see for example, Clement *et al.*, 1979; Robinson *et al.*, 1994) and whilst N-deficient plants will still flower and set seed, the yields which are harvested are much reduced in comparison with those made from N-sufficient plants (see for example, Mei and Thimann, 1984). Under conditions of sub-optimal N supply, the biosynthesis of amino acids and proteins takes precedence over chlorophyll production (Mei and Thimann, 1984); the classical symptoms of N deficiency (*i.e.* chlorosis and induced senescence of older leaves) consequently stem from the resultant disorganisation of chloroplasts in mature leaves (Saux *et al.*, 1987). Whilst a supra-optimal N supply might prolong leaf life, this excess of N can be equally damaging to the economics of a crop. Changes in plant morphology (specifically an increase in the shoot:root dry weight ratio) can lead to lodging in

cereal crops and impaired acquisition of nutrients in the latter stages of growth (Marschner, 1995).

The NO_3^- uptake system (in contrast with that for SO_4^{2-}; see earlier) is generally well characterised in higher plants (Clarkson, 1986) and the absorption of NO_3^- from the soil represents an energy-dependent process driven by the proton motive force through intrinsic NO_3^- transporters at the plasma membrane (see for example, Santi *et al.*, 1995; Miller and Smith, 1996; Huang *et al.*, 1996). The uptake of NO_3^- in many plants species is bi-phasic with both a high affinity transport system (HATS; K_m of between 5 and 300 μM) and low affinity transport system (LATS; K_m of $>$ 0.5 mM) having been described (see for example, Öhlén *et al.* (1995); Miller and Smith (1996); Huang *et al.* (1996) and references therein). The NO_3^- uptake system in higher plants is unusual in the sense that it is subject to induction by changes in the availability of external NO_3^- (see Kronzucker *et al.*, 1995 and references therein) Consequently, both HATS (Hole *et al.*, 1990; Kronzucker *et al.*, 1995) and LATS (Huang *et al.*, 1996) uptake systems appear to have both constitutive and NO_3^--inducible components. Generally, the HATS dominates when NO_3^- availability is low (Hoff *et al.*, 1994) whilst LATS becomes active when NO_3^- levels are higher (Hole *et al.*, 1990). It has been suggested that both HATS and LATS are subject to negative feedback regulation (Hoff *et al.*, 1994). Although the exact identity of the signal which regulates such feedback remains unknown, it is possible that either NO_3^- itself (or a product of N assimilation, such as NO_2^-, NH_3 or glutamine) could be involved (Hoff et al, 1994). A further candidate for the role of root-shoot communicator equating plant N status with control of root NO_3^- uptake appears in the form of those amino acids which cycle around the plant in the xylem and phloem (Cooper and Clarkson, 1989; Larsson *et al.*, 1991). It is tempting to speculate that a decrease in the concentrations of cycling amino acids promotes N uptake and assimilation and *vice versa* (see Cooper and Clarkson, 1989; Padgett and Leonard, 1996; Schenk, 1996).

The fact that NO_3^- assimilation is regulated principally by the N-status of the shoot comes as no real surprise. However, the fact that the metabolism of NO_3^- can also be regulated by the S-status of a plant as proposed by Reuveny and Filner (1977) is more surprising. Subsequently, there have been many reports on the effects of N and S status on the enzyme activities in their respective pathways and in N-deficient

Lemna minor L. for example, a decrease in the activities of both ATP sulphurylase and APS sulphotransferase (see **Figure 1.1**) has been observed (Brunold and Suter, 1984). In contrast, APS sulphotransferase activity is considerably enhanced in *Lemna* supplied with a source of readily-assimilated N (*i.e.* amino acids (Suter *et al.*, 1986) or NH_4^+ (Brunold and Suter, 1984)).

The suggestions of regulatory coupling between the metabolisms of N and S in higher plants could be of considerable agronomic importance in terms of nutrient use efficiency. At this stage, however, there has been no experimental demonstration of the 20 : 1 ratio of N : S uptake by the root system required to match the stoichiometry of N and S found in proteins. Therefore, whilst N and S metabolisms may be linked, it has not yet been shown that N and S *uptake* are actually linked (Cram, 1990).

1.2.3, The fate of internalised nitrate

The roots of many plant species contain appreciable nitrate reductase (NR) and nitrite reductase (NiR) activity and as such are capable of assimilating at least some of the N which they receive from the rhizosphere (Pate, 1980; Oaks, 1986 and references therein). Consequently, the relative proportions of reduced N (such as amino acids) to non-reduced N (principally NO_3^-) transported in an acropetal direction in the xylem are determined by the activities of these two enzymes in the root system (Pate, 1980 and references therein). The amount of N reduced in the roots differs between species although external factors (such as the level of NO_3^- supplied and the age of the plant concerned) can also influence the proportion of reduced to non-reduced N compounds measured in expressed xylem sap (Marschner, 1995). Generally, woody species (such as *Pyrus* or apple) transport N compounds upwards from the root almost entirely in their organic form (Pate, 1980). Similar observations have been made in plants supplied with low concentrations (*circa* 1 mM or less) of external NO_3^-, in ageing material and in those plants grown under conditions of low temperature or low light (Marschner, 1995; Crawford, 1995 and references therein).

Once unloaded from the xylem, non-reduced N is transported to the mesophyll tissue (see for example, Rufty *et al.*, 1986) although (as described previously for SO_4^{2-}), the nature of the pathway followed from vascular tissue to mesophyll cell remains unclear. Nitrate is reduced to NH_3 in a two-step reaction mechanism (see

Figure 1.2). In the first (and rate-limiting) step, NO_3^- is reduced to NO_2^- in a reaction catalysed by the cytosolic enzyme NR (Solomonson and Barber, 1990). The NO_2^- formed is then immediately reduced to NH_3 by the chloroplastic enzyme NiR (Oaks, 1986). The biosynthesis of amino acids (the first stable organic N-compounds formed by the plant) then proceeds from NH_3 by way of the glutamine synthetase (GS) glutamate synthase (GOGAT) pathway (see Lea *et al.*, 1990; Marschner 1995). **Figure 1.2** describes the assimilation of NO_3^- assimilation in higher plants in diagrammatic form.

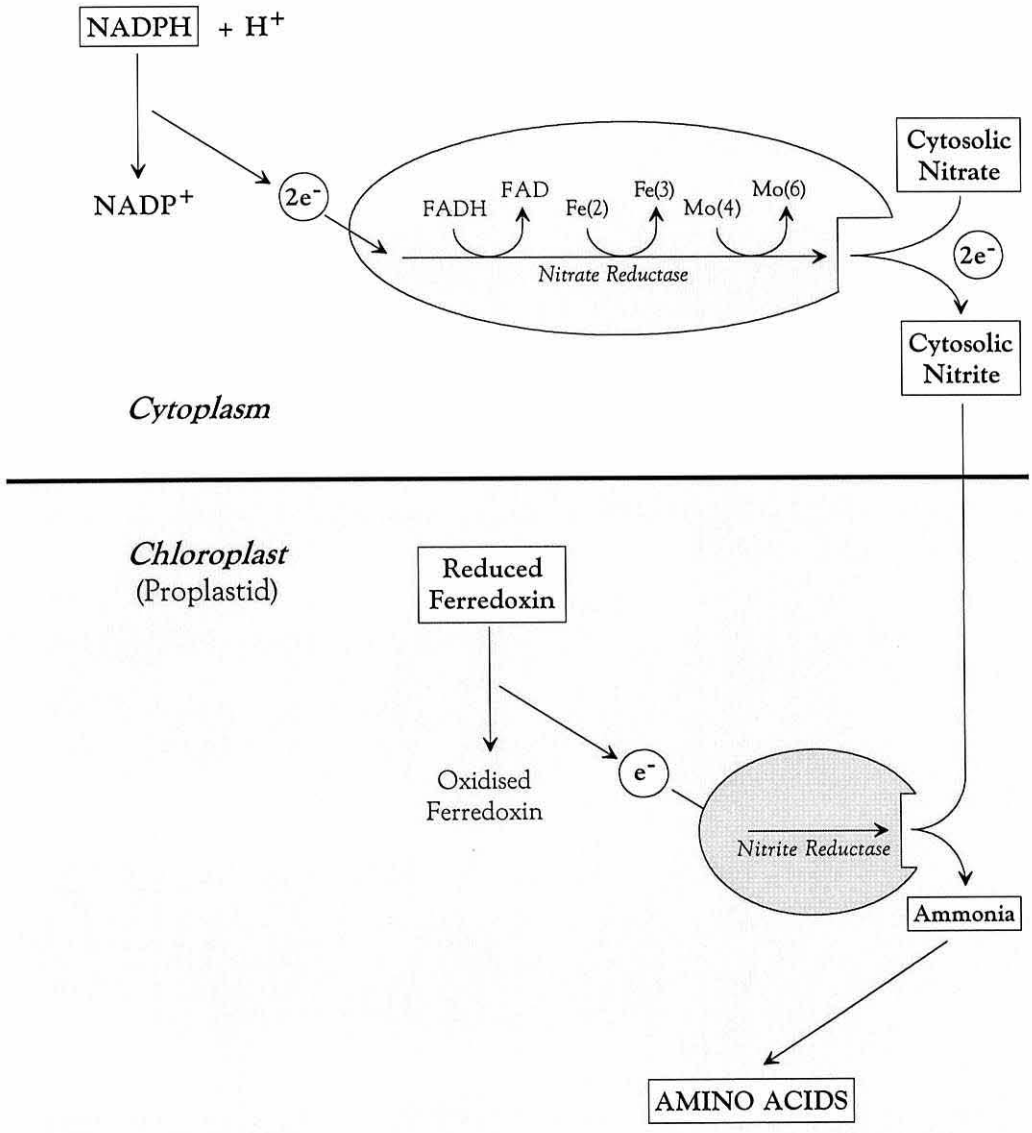
The regulation of N assimilation in higher plants is mediated through the control of NR activity which correlates closely with a number of external factors such as light intensity (Crawford, 1995 and references therein), NO_3^- availability (see for example, Solomonson and Barber, 1990), the availability of C skeletons (Campbell, 1996) and plant S status (discussed earlier). NR activity (and thus the rate of conversion of NO_3^- to NO_2^-) can be regulated in one of two ways (Campbell, 1996). When rapid modulation of activity is required (*e.g.* in response to the diurnal cycle; see Winter *et al.*, 1993; Delhon *et al.*, 1996), existing NR protein is subject to reversible phosphorylation which temporarily regulates catalytic ability (Hoff *et al.*, 1994; Huber *et al.*, 1996). More permanent up or down regulation of N metabolism is accomplished (for example, in response to changes in external NO_3^- availability; Hoff *et al.*, 1994) through genetic control which results either in the synthesis of new enzyme or the degradation of existing enzyme (Huber *et al.*, 1996; Campbell, 1996).

Not all of the N internalised by the plant is destined for immediate reduction and subsequent incorporation into large organic N compounds (such as proteins and nucleic acids). Although dependent upon a number of environmental influences such as external NO_3^- supply (see for example, **Chapters 5** and **6** this thesis) and plant growth rate (see Zhen and Leigh, 1990), a significant proportion of total plant N could at any one time be stored in root and shoot cell vacuoles primarily as NO_3^-. In hydroponic wheat supplied with 20 mM NO_3^- and grown under 'high light' conditions (*circa* 450-480 μmol m^{-2} s^{-1}) for example, accumulated NO_3^- accounted for 50-55 % of total shoot N (see Zhen and Leigh, 1990).

In addition to the N incorporated into large organic compounds and that stored as NO_3^- in cell vacuoles (see above), a significant proportion (approximately 50 % of the N flux in the xylem; Simpson *et al.*, 1982; Cooper and Clarkson, 1989) of

total plant N will, at any one time, be cycling around the plant as amino acids in the xylem and phloem (Cooper and Clarkson, 1989; Larsson *et al.*, 1991; Marschner *et al.*, 1997). The role of this cycling process in the context of N nutrition in higher plants is potentially several-fold. Some amino acids (such as lysine, threonine and homocysteine) are synthesised exclusively in the shoots (Wallsgrove *et al.*, 1983) and must, therefore, be transported in a basipetal direction in the phloem as and when required by the roots (Pate, 1980; Cooper and Clarkson, 1989). Transport of N in a basipetal direction from the shoots in the phloem could also enable part of a root system to grow through N deficient regions of a soil (Cooper and Clarkson, 1989). Conversely, transport of amino acids in an acropetal direction could provide a suitable means of re-translocating amino acids from ageing source tissue to the developing seeds, as they become increasingly important sink organs (see for example, Larsson *et al.* (1991). The importance of recovery and subsequent export of amino acids from senescing leaves to developing seeds is described in greater detail in a latter section of this chapter.

Figure 1.2. A simplified diagram of N assimilation in higher plants. The N requirements of most temperate plant species are met by absorbing NO_3^- directly from the soil. Plants are unable to utilise this NO_3^- directly in the biosynthesis of organic N compounds, however, which means that the initial stages of N assimilation are characterised by the two-step reduction of NO_3^- to NH_3. The cytosolic enzyme NR catalyses the reduction of NO_3^- to NO_2^-. The NO_2^- formed is then immediately reduced to NH_3 by the chloroplastic enzyme NiR. Amino acids are subsequently synthesised in the GS-GOGAT pathway.



1.3, LEAF SENESCENCE

1.3.1, What is leaf senescence?

Senescence represents a genetically-regulated oxidative process involving a general degradation of cellular structures and macromolecules (see for example, Mittelheuser and van Steveninck, 1971; Martinoia *et al.*, 1983). Reusable by-products of this degradation may be re-mobilized to other parts of the plant (del Rio *et al.*, 1998) and as such, the sequence of events associated with senescence constitute the very final stages of development (Smart, 1994; Gan and Amasino, 1997). The senescence of leaves is characterised by the cessation of photosynthesis, breakdown of leaf proteins, deterioration of cell membranes, loss of chlorophyll and the net export of amino acids (see the reviews by Smart (1994) and Buchanan-Wollaston (1997). The importance of this re-cycling of amino acids (in terms N-use efficiency and grain development) are covered in greater detail in the next section of this chapter.

The initiation of senescence is subject to both environmental and autonomous (or internal) factors (Smart, 1994; Buchanan-Wollaston, 1997; Gan and Amasino, 1997). Adverse environmental conditions such as nutrient stresses (particularly sub-optimal N supply; Cincerová, 1990; Barneix and Causin, 1996), drought (Feller and Fischer, 1994), excess O_3, pathogen attack and increased shading (Smart, 1994) for example, all trigger senescence. The ability of environmental factors to reduce the life span of a particular leaf has obvious adaptive values and as such, is thought to have evolved as part of a mechanism enabling the plant to complete its life cycle even when growing in adverse environments (Gan and Amasino, 1997). In the absence of environmental cues, age has a major influence on the initiation of leaf senescence, the onset of which might be triggered by a substantial drop in photosynthetic rate (Smart, 1994; Gan and Amasino, 1997).

At the current time, little is known about the molecular mechanisms behind the initiation of leaf senescence although many of the older hypotheses have been based upon the idea of an unbalanced turnover where rate of degradation exceeds rate of synthesis (Smart (1994) and references therein). Nonetheless, the control steps involved in the initiation of leaf senescence may be the same irrespective of the nature of the stimulus which triggers the process (Smart, 1994).

1.3.2, Leaf senescence and the importance of nutrient re-mobilization

The re-mobilization (and subsequent re-use) of the by-products formed from the net degradation of macromolecules in senescing leaves enables a plant to make the most economical use of its absorbed nutrients (Feller and Fischer, 1994). Those nutrients found mainly within the leaf as dissociated ions in solution (for example, K^+) require no 'metabolic processing' prior to phloem loading and are readily exported from senescing tissue (Feller and Fischer, 1994). Many other essential nutrients however (such as N and S), are bound into macromolecules which must first be broken down into smaller and more manageable compounds before they can be exported from the leaf (see for example, Waters *et al.* (1980)).

Proteins for example, constitute a large proportion of total leaf N, much of which is localised within the chloroplasts in the form of ribulose bisphosphate carboxylase (RUBISCO) (Huffaker and Peterson, 1974). This enzyme catalyses the fixation of CO_2 in the first reaction of the Calvin cycle (Weier *et al.*, 1982). As the leaf ages, the decrease in photosynthetic rate coincides with a decrease in leaf protein content as chloroplast and other leaf proteins are broken down primarily into the amino acids glutamine and asparagine (Smart, 1994). These amino acids are then exported from the leaf in the phloem to developing sinks elsewhere within the plant (Feller and Fischer, 1994). The hydrolysis of chloroplast proteins (which occurs in a sequence of reactions catalysed by a number of peptide hydrolases; Waters *et al.*, 1980) represents an essential pre-requisite for the re-distribution (and subsequent re-cycling) of organic N (Feller and Fischer, 1994). In rice for example, over 90 % of the organic N exported from ageing leaves was of chloroplast origin, the majority of which arose from the proteolysis of RUBISCO (Kamachi *et al.*, 1991). Perhaps because of its high organic N content, the degradation of chloroplasts represents one of the first casualties of leaf senescence (Matile *et al.*, 1996).

In many cereal growing areas, it is common for the level of available soil N to decrease during the grain-filling period of plant growth (Simpson *et al.*, 1983). Therefore, the N requirements of the developing grain often have to be met almost exclusively by the re-mobilization of amino acids in the phloem from vegetative organs (Pate, 1980; Barneix *et al.*, 1992). Consequently, around 90 % of the total N content of a fully developed cereal grain may have originated from vegetative tissues such as the stem and leaves (Simpson *et al.*, 1981).

1.4, PRINCIPAL OBJECTIVES OF THE INVESTIGATION

Final grain N content is determined by the ratio of nutrient export from the leaves rather than being self-regulated *per se* (Barneix *et al.*, 1992). One of the principal determinants of the baking and nutritional quality of a bread wheat is the final protein concentration of the grain (Byers *et al.*, 1987; Barneix and Guitman, 1993; Oscarson, 1996). Consequently, any factor (or combination of factors; see for example, Barneix and Guitman, 1993) which results in changes to the rate and pattern of N export from ageing leaves may have significant and far-reaching effects on crop yield and nutritional quality (Ta and Weiland, 1992). Similarly, most of the S required by the grain during development originates from vegetative tissue, and as for N, any factor(s) which impairs the re-mobilization of S from ageing tissue could potentially limit yields. In mature leaves of *Macroptilium*, Clarkson *et al.* (1983) observed that irrespective of shoot S-status or whether plant growth was limited by insufficient S, a relatively constant proportion of total plant S was present in the form of soluble SO_4^{2-}. Therefore, it would appear that a large proportion of the SO_4^{2-} stored in leaf cells is unavailable for transport irrespective of the S status of the plant. Recent radio-tracer studies using $^{35}SO_4^{2-}$ (see Bell *et al.*, 1994) have indicated that the mature leaves of *Macroptilium* contain two populations of kinetically distinguishable cell of apparently differing SO_4^{2-} content. Radio-tracer efflux from one of these cell types ('apparent mesophyll') was so slow that the turnover of SO_4^{2-} from this tissue could potentially limit the re-mobilization of S from the mature leaf (Bell *et al.*, 1994).

Using Single Cell Sampling and Analysis (SiCSA) techniques, the aim of the experiments described in this thesis was to investigate the behaviour of S when induced to accumulate in and then re-mobilize from differing cell populations within the mature leaves of wheat plants. A secondary aim of the study was to ascertain how the accumulation of S might affect water relations parameters and the behaviour of other cell solutes (with particular reference to NO_3^-). The report starts with a description of the techniques used in this investigation (see **Chapter 2**).

Chapter 2:

EXPERIMENTAL METHODS

2.1, INTRODUCTION

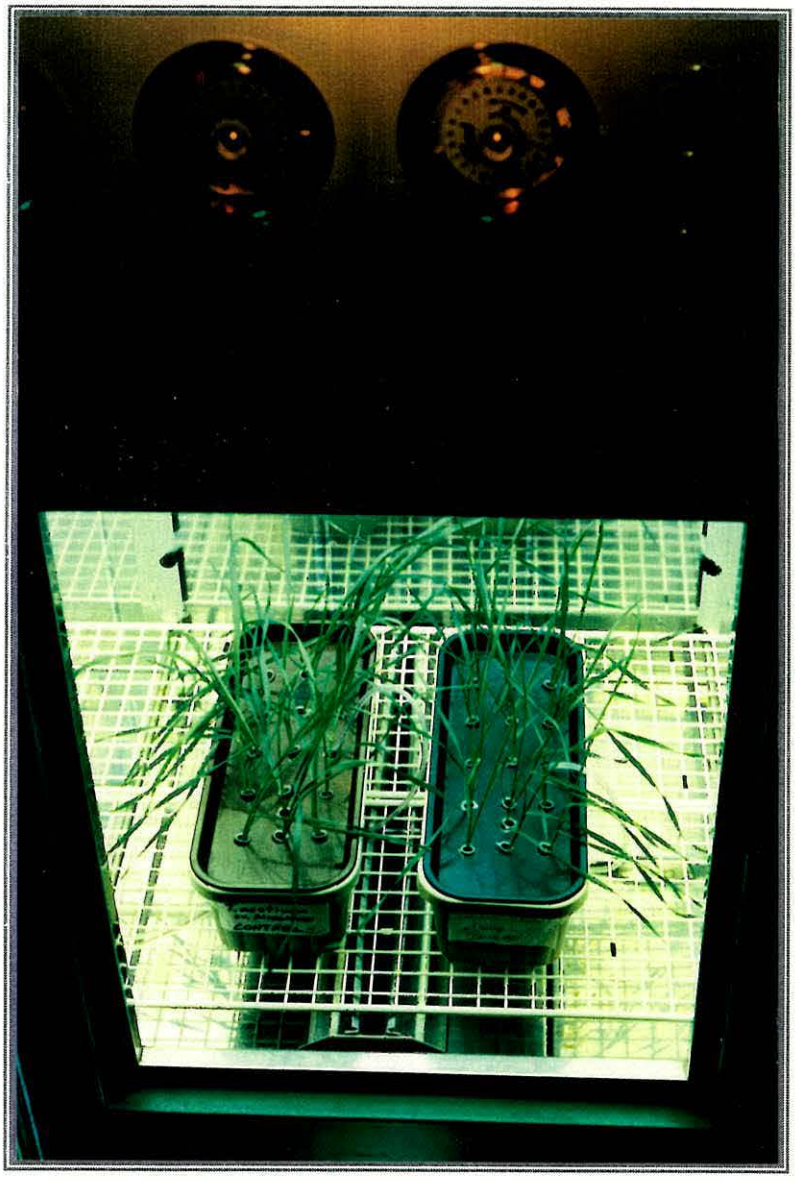
This chapter is in two sections. The first describes those techniques used to acquire data on the behaviour of the whole leaf (or a representative section of the whole leaf). The second section then describes how data was obtained on single cells *in situ* using a suite of specialist techniques. Combining these two very different approaches, it has been possible to compare the behaviour of the whole leaf with that of single cells *in situ* when plants were subjected to nitrogen or sulphur stress. The full addresses of equipment manufacturers and chemical suppliers have been excluded from the methods (to aid ease of reading) and are listed separately in **Appendix 1.**

2.2, PLANT GROWTH CONDITIONS

Seeds of *Triticum aestivum* cv. *Alexandria* were rinsed 3-4 times in water before overnight imbibition in aerated de-ionised water. Germinating seeds were transferred onto a thick polythene mesh suspended over 6 l of aerated de-ionised water so that the seeds were kept moist but not saturated. Seedlings were then grown in the dark until they reached a mean height of approximately 4 cm. At this point, they were transferred to the light and put in a Gallenkamp growth cabinet pre-set to a constant 18 °C and an 18 hour light/6 hour dark photoperiod with 55-75 % relative humidity and a light intensity of 200 μmol m^{-2} s^{-1} (at plant height) for a further 24 hours.

Seedlings were placed into 1 ml Eppendorf tubes (which were modified such that both the lid and bottom 5 mm section had been removed) and grown at a density of 15 plants per tub in a growth vessel containing 3 l of growth solution (see subsequent chapters for details of growth solutions). Plants were returned to the growth cabinet (pre-set as before) where they were left to reach the required developmental stage (see **Plate 1**). The growth medium was changed every 3 days. A thermohygrograph placed in the growth cabinet recorded any fluctuation in temperature or relative humidity (results not shown).

Plate 1. The growth system used in this project. The wheat in the left-hand side growth tub are control S plants which have been grown continuously in CON growth solution. Plants in the right-hand side tub have been subjected to a high S, low N nutrient regime (INV solution). Note the modified Eppendorf tubes in which plants are supported.



2.3, WHOLE LEAF TECHNIQUES

2.3.1, Shoot fresh and dry weights

The way in which nutrient treatments effect plant growth was measured by the determination of shoot fresh and dry weights (see Salisbury and Ross, 1992 for a review). On removing a plant from its growth tub, the supporting Eppendorf tube was removed as quickly as possible and the shoots were cut away from the plant (above the seed) at the root-shoot interface. The shoot was weighed before placing into a small paper bag to be dried in a 1000 l LEEC FCX1 drying cabinet at 65±5 °C. After a minimum period of 48 hours in the oven (and when the material gave constant weight), dry weights were recorded.

2.3.2, Preparation of bulk tissue sap

When applied to whole plant material (for example, a leaf), the process of freeze-thaw and centrifugation allows a sample of bulk tissue sap to be obtained. Providing that the plant material remains sealed during the thawing process (preventing the sample from mixing with condensation), it is possible to obtain a non-diluted sap preparation (Gorham, 1987).

A 10 cm section was removed from the middle of the third leaf of a wheat plant at the appropriate age. This was folded and placed into an Eppendorf tube which was capped before being plunged into a Dewar of liquid nitrogen at -196 °C. The use of liquid nitrogen rapidly fixes metabolic processes whilst causing gross disruption to cells (Hinde, 1994). After approximately 5 minutes, the Eppendorf tube was removed from the nitrogen and the contents allowed to thaw for a further 5 minutes. A small hole was then made in the bottom of the Eppendorf and this 'holed tube' inserted into a second (non-holed) Eppendorf tube. Centrifugation at 2800 g for 5 minutes in a Clandon MLW T52.1 bench centrifuge (VEB) was used to spin the sap out of the ruptured cells, through the hole and into the collection tube. Extracted sap was stored at -20 °C until required.

2.3.3, Tissue osmotic pressure

Determination of osmolality provides a value representing the total solute concentration of a solution. Adding a solute to a solvent affects four physically interrelated characteristics (known as colligative properties). Addition of a solute to a solvent:

1, decreases vapour pressure
2, lowers freezing point
3, increases boiling point
4, increases osmotic pressure
(when the values of the solution are referenced to the pure solvent).

Work performed by the Bangor Pressure Probe Group (for example Fricke *et al*, 1994a, b, c) has demonstrated the importance of osmotic pressure as a useful datum point against which the individual concentrations of cell sap constituents can be accounted for.

The osmometer (a Wescor 5100B), was calibrated using 290 mOsmol kg^{-1} and 1000 mOsmol kg^{-1} standards. An 8 μl sample of tissue sap (see **2.3.2**) was added to a 5 mm diameter paper disc. The disc was transferred to the sample holder of the Wescor and the osmotic pressure was determined according to the manufacturers instructions.

2.3.4, High pressure liquid chromatography

High pressure liquid chromatography (HPLC) through an ion exchange column confers many advantages for the detection of both anions and cations in large numbers of extracted cellular saps (Gorham, 1987). In the case of both anions and cations, ionic separation is determined by net charge. In the case of anion detection, the chromatography column (a low capacity styrene-divinylbenzene based HPLC column) comprises fixed positively charged sites for which anions from the sample and HCO_3^- from the eluent, compete. Once the original sample (containing cations and other unwanted components) has passed through the column, further addition of eluent will oust the bound anions from their sites on the column and allow them to be separated. Anions with a weaker overall charge tend to emerge first and have the

shortest retention times. For analyses of cations, the chromatography column comprised fixed negatively charged sites for which mono- and di-valent cations compete. Separation was, however, essentially the same as described for anions.

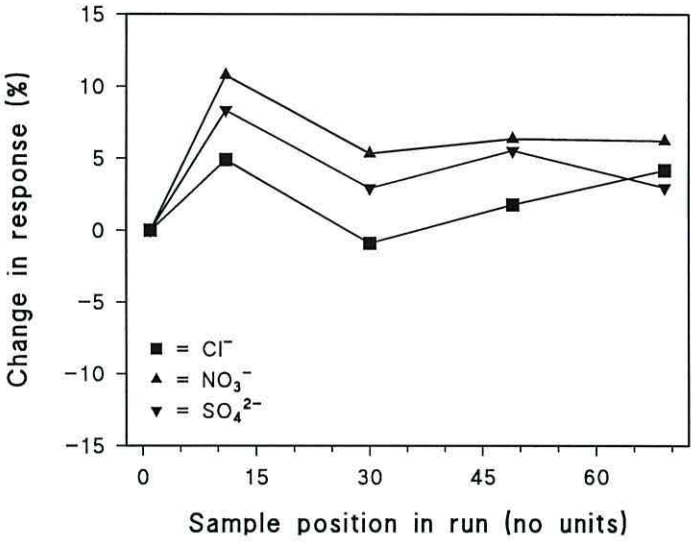
The detector used was of the two electrode type (Gorham, pers. comm.). Continual circulation of either 10 mM H_2SO_4 (during anion analyses) or 50 mM $Ba(OH)_2$ (cation analyses) in a post-column conductivity suppression system minimised background noise (Gorham, 1987).

2.3.4.1, Determination of anion concentrations in tissue sap

A tissue sap preparation (see **2.3.2**) was thawed and then spun for 5 minutes at 2800 g in the bench-top centrifuge. A 15 μl aliquot of supernatant was diluted 1:9 with 20% (v/v) isopropanol. A 100 μl volume of this diluted sap was then added to an auto-injector vial and 1.5 ml of anion eluent (4.7 mM Na_2CO_3 + 1.8 mM $NaHCO_3$ in 2.5% (v/v) propan-2-ol) was added. All eluent salts were HPLC grade from BDH. Anion quantification was performed on a Dionex 2000i ion chromatograph fitted with a PAX-500 anion column. This was coupled to a Spark Holland Marathon auto-injector in turn, linked to an Atari 1040 microcomputer. Sample loop size was 10 μl.

The area under each peak in excess of background conductivity (net integral) was converted by an integrator (Shimadzu C-R5A), into an absolute concentration based on calibration with a standard containing Cl^-, NO_3^-, SO_4^{2-} (as potassium salts, all BDH), and L-malic acid (Na_2 salt from Sigma). Anionic composition of the standard solutions was based on data from both SiCSA (Single Cell Sampling and Analyses) work and previous experiments. An example of the range of anion concentrations used in a typical calibration standard are given in the legend of **Figure 2.1**. Instrument drift during analyses of a batch of samples was checked by determination of anion concentrations in a calibration standard inserted after every 15-20 plant samples (see **Figure 2.1**).

Figure 2.1. Demonstration of drift in calibration during anion analyses using HPLC in a batch of 70 bulk tissue sap samples. Plots for Cl^-, NO_3^- and SO_4^{2-} are shown (L-malate was not measured in this particular batch). Original concentrations of Cl^-, NO_3^- and SO_4^{2} in the calibration standard were 300, 400 and 200 mM respectively. Excluding the standard measured at position 11, calibration drift during anion analyses was typically in the range of 0-5 % for all three anions during the run.



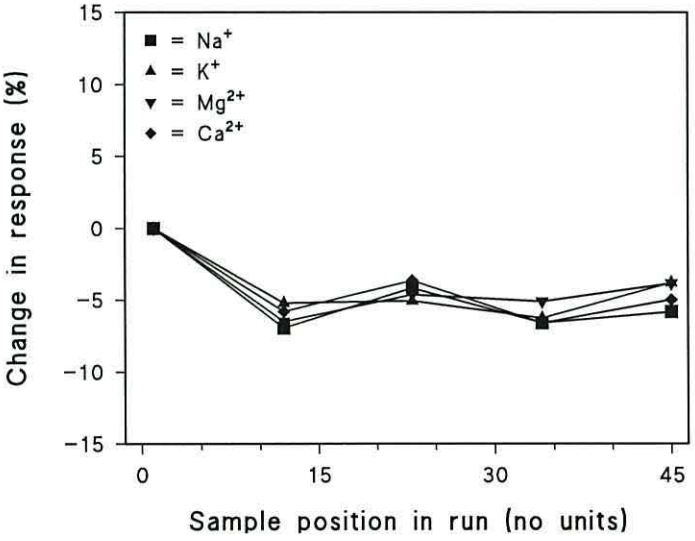
2.3.4.2, Determination of cation concentrations in tissue sap

A tissue sap preparation (see **2.3.2**) was thawed and spun for 5 minutes at 2800 g in the bench-top centrifuge. A 10 μl aliquot of supernatant was added to 1.0 ml of cation eluent (20 mM methanesulphonic acid (Fluka) in an auto-injector vial. Cation quantification was performed on a Dionex 2000i ion chromatograph fitted with a CS12 cation column (operating at 50 °C). This was coupled to a Spark Holland Marathon auto-injector in turn, linked to an Atari 1040 microcomputer. Sample loop size was 10 μl. A self-regenerating cation suppresser operating in auto-regeneration mode was also fitted. This suppresser electrolysed H_2O from the eluent and resultant OH^- could regenerate the suppresser membrane by flushing out bound mono- and di-valent anions.

The Shimadzu integrator was used to convert the area in excess of the background conductivity into an absolute concentration based on calibration with a

standard containing Na^+, K^+, Mg^{2+} and Ca^{2+} (as nitrate salts; BDH). Concentrations of cations in the top standard and instrument drift were determined as for anion method (see **Figure 2.2**).

Figure 2.2. Demonstration of drift in calibration during cation analyses using HPLC in a batch of 45 bulk tissue sap samples. Plots for Na^+, K^+, Mg^{2+} and Ca^{2+} are shown and original concentrations in the top standard were 100, 300, 100 and 200 mM respectively. Calibration drift throughout cation analyses was between 0-6.5 % for K^+, Mg^{2+} and Ca^{2+} with larger drift being observed for Na^+. However, quantification of Na^+ was of minor interest during this project.



2.3.4.3, Determination of anion concentrations in growth solution samples

A 1 ml sample of growth solution was thawed and then spun for 5 minutes at 2800 g in the bench-top centrifuge before being transferred to an auto-injector vial. As described in **2.3.4.1**, a Dionex 2000i ion chromatograph fitted with a PAX-500 column was used to quantify anion concentrations. Sample loop size was 3 μl. The area under each peak in excess of background conductivity was converted by the integrator into an absolute concentration based on calibration with a standard containing 10 mM Cl^-, 20 mM NO_3^- and 10 mM SO_4^{2-} (as K^+ salts; BDH). Instrument drift was checked during all runs.

2.3.5, Analysis of Sugars

Carbohydrate can be found in many different forms within the plant cell; as monosaccharides (for example, glucose), disaccharides (such as sucrose), oligosaccharides (for example, fructan (up to 50 individual monomers) or polysaccharides (for example cellulose (consisting of approximately 10000 glucose monomers). Extraction of carbohydrate from plant tissue exploits the solubility of the molecule as a function of its size in terms of the number of monomers within the structure.

The initial extraction step is performed in ethanol. This alcohol treatment firstly disrupts the cell membrane and secondly acts as a good solvent for mono-, di-, tri- and some tetrasaccharides. Sugars in this fraction include sucrose, hexose and low molecular weight fructans (Farrar and Farrar, 1985). The second stage of extraction is performed in water which solubilizes any oligosaccharides present (such as high molecular weight fructans).

Determination of sugar content in both ethanol and water fractions depends upon reduction of the sugar by concentrated sulphuric acid to its furfuraldehyde derivative (Ferrier and Collins, 1995). This derivative reacts with phenol to produce a coloured product which can be measured and quantified (using appropriate standards) spectrophotometrically (Dubois *et al.* 1956).

2.3.5.1, Preparation of samples

A 5 ml volume of 80 % ethanol was added to a 10 ml plastic centrifuge tube then capped with a glass marble. The tube was then transferred to a Grant BT3 heating block where the 80 % ethanol was warmed to 80°C. Approximately 0.5 g of frozen leaf tissue was added to the tube which was then incubated for 15 minutes. The heating block was switched off and the 80 % ethanol extract transferred to a fresh tube. A further 5 ml of 80 % ethanol was added to the original tube (still in the heating block and containing plant material) and a second ethanol extraction was performed at room temperature overnight.

The following day, the second ethanol wash was removed and combined with the first. The final volume of the sample was made up to 10 ml with 80 % ethanol. The tube was covered in aluminium foil to exclude light pending determination of ethanol-soluble carbohydrate. A 5 ml volume of de-ionised water was added to the

plant material remaining in the original tube which was then heated at 60 °C for 120 minutes. On cooling, the tube was sealed and stored frozen for subsequent determination of water-soluble carbohydrate.

2.3.5.2, Determination of carbohydrate with Phenol-H_2SO_4 test

For calibration; 1 ml of standard (0, 20, 40, 80 or 100 μg ml^{-1} sucrose (BDH) was added to a test tube, 1 ml of 5 % phenol solution was then added followed by 5 ml of sulphuric acid (both 'Analar', BDH). The contents of the test tube were carefully mixed. Once the reaction had cooled, the absorbance of the resulting brown solution was read at 490 nm in a Cecil CE 303 spectrophotometer equipped with a 'flow-through' cuvette system blanked on a water-phenol/sulphuric acid mix.

For determination of total carbohydrate in plant-sourced ethanol extracts, the procedure followed was as outlined for calibration except that 0.5 ml of sample plus 0.5 ml of water (instead of the 1 ml of standard) represented the initial additions to the test tube. The purpose of the 50 % dilution was to ensure that absorbance values of the plant-sourced samples were within the calibration range. For determination of total carbohydrate in plant-sourced water extracts, the procedure followed was as outlined for calibration except that 1 ml of standard was replaced with 1 ml of plant-sourced sample.

2.3.6, Determination of leaf chlorophyll

Values measured by a chlorophyll meter correspond to the relative amount of chlorophyll present in the plant leaf. The determination of leaf chlorophyll content provides a convenient parameter to record the progression of leaf senescence (Matile, 1997; Buchanan-Wollaston, 1997) or to quantify the responses of a plant to an environmental stress (Smillie and Hetherington, 1990). In the case of the Minolta meter, leaf chlorophyll content is calculated by comparing the intensities of light transmitted by a leaf following direct illumination in the red (peak λ 650 nm) and infra-red (peak λ 940 nm) regions of the light spectrum by two light-emitting diodes built into the reading head section of the meter.

Before use, the Minolta SPAD-502 chlorophyll meter was calibrated according to the manufacturers instructions. When measuring leaf chlorophyll, three replicate

measurements were taken per leaf in a 2 cm area of the mid-section. A mean value of the three readings was then calculated to represent leaf chlorophyll.

2.4, SINGLE CELL TECHNIQUES

2.4.1, Extraction of single cell vacuolar samples

Single cell samples of vacuolar sap are obtained by inserting a fine microcapillary filled with low viscosity silicone oil into a member of the required cell type. The cell turgor then forces the vacuolar contents into the microcapillary and out of the cell. Provided that the microcapillary is rapidly removed from the sampled cell and immersed under an immiscible medium (such as water-saturated liquid paraffin), then non-diluted representative samples of single cell sap can be obtained (Malone *et al*, 1989; Tomos *et al*, 1994). For epidermal cells at least, the comparative volumes of vacuole and cytoplasm mean that these samples are almost exclusively vacuolar in origin (Fricke *et al.*, 1994a). Ejection of single cell sap under oil allows determination of:

1, osmotic pressure
2, elemental composition (using X-ray microanalysis)
3, enzymatic assay for a specific solute (for example, malate or nitrate)

A single cell sap can be sub-sampled to allow a series of sequential analyses to be performed. This means that data from all three types of analyses (above) can be obtained from a single extracted cell sap (Tomos *et al*, 1994). Two types of microcapillary were routinely used in this investigation. These were:

1, drawn-out 'sampling' capillaries where exact volume was not important
2, constriction capillaries (constriction pipettes) used for measuring out identical volumes of samples, standards or reagents.

Even in the case of constriction pipettes, exact volume generally remains unknown. Samples were measured by comparison with standards using the same pipette. Where a knowledge of approximate volume is required, a droplet of water can be expelled from the pipette under paraffin oil. The diameter of the resultant

droplet can then be measured using a microscope eyepiece graticule (Tomos *et al.*, 1994) and using the mathematical formula for the volume of a sphere ($\frac{4}{3}\pi r^3$), a good approximation of pipette volume can be made.

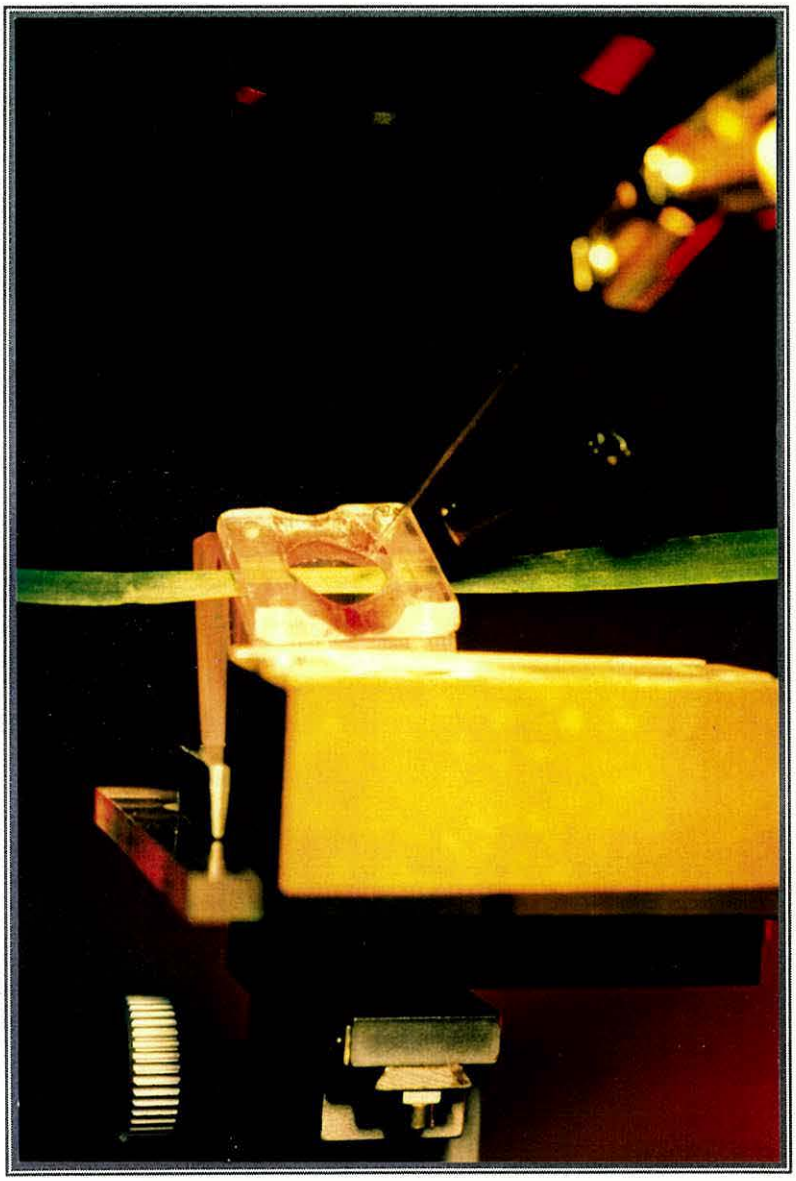
Microcapillaries were produced from 1 mm (external diameter) glass capillary tubing (Clark) using a commercial micropipette puller (Harvard). A localised source of heat and tension produced two tapered microcapillaries which were then transferred to a microforge (de Fonbrune), where, the very end of the tip was broken off by contact with a metal surface, to produce an aperture of the required diameter.

The microforge was also used to produce the constriction pipettes. A heated fine-gauge filament was aligned closely with one side of the pipette where constriction was required. Applying a current to the filament then melted the glass on the nearer side of the microcapillary leading to constriction of the lumen as the pipette bent towards the heat. Constriction pipettes of various volumes (from *circa* 10 pl through to *circa* 5 nl) can be produced in this way.

Both types of microcapillary can be made hydrophobic by silanization if required. Pipettes to be silanized were put into an aluminium block in a foil-covered Pyrex beaker which was then placed into an oven at 200 °C for a minimum period of 120 min. 50 μl of dimethyldichlorosilane (Sigma) was then carefully injected through the foil into the beaker and the tips were returned to the oven for a further 120 minutes before use. Prior to use, a microcapillary was back-filled with low-viscosity silicone oil (Grade AS4, Wacker). It was then connected to a 50 ml plastic syringe and a foot-operated solenoid valve by a series of plastic tubing. The capillary was then mounted on a micromanipulator (Leica) and moved near to the tissue for initial alignment with the naked eye. See **Plate 2**.

Use of a stereo microscope (Wild M8, Leica, maximum magnification, x160) allowed the tip of the microcapillary to be precisely aligned with the desired cell. The micromanipulator was then used to move the tip such that the targeted cell was punctured. The tip was then rapidly withdrawn from the cell and immersed under water-saturated liquid paraffin (previously centrifuged at 1300 g for 10 minutes) and the sample expelled using the syringe and solenoid valve.

Plate 2. Removal of single cell vacuolar sap samples from wheat plants. Leaves were held in a perspex sample holder and the oil-filled silanized sampling capillary was mounted on a micromanipulator via an articulated brass arm (visible here). The large brass 'box' in the foreground of the picture is the picolitre osmometer.



2.4.2, Determination of single cell osmotic pressure

The osmotic pressure of single cell vacuolar saps was measured by freezing point osmometry (Malone *et al*, 1989; Tomos *et al*, 1994). This technique exploits one of the colligative properties of solutions (see **2.3.3**). A custom-made picolitre osmometer was used to determine the freezing point of single cell vacuolar saps. The temperature of the osmometer stage was rapidly cooled to -40 °C to overcome the effects of supercooling and to induce freezing (Tomos *et al*, 1994; Richardson, 1993) before returning to *circa* -2 °C.

Using a stereo microscope, minute ice crystals in each microdroplet were seen to decrease in size as the stage temperature was slowly increased from -2 °C to *circa* +1 °C. The melting point of each droplet was then recorded as the approximate temperature displayed on the osmometer hand-set at which the last ice crystal melted and just disappeared (see **Plate 3**). It was then possible to reference this temperature against the melting points of standard solutions measured in the same way.

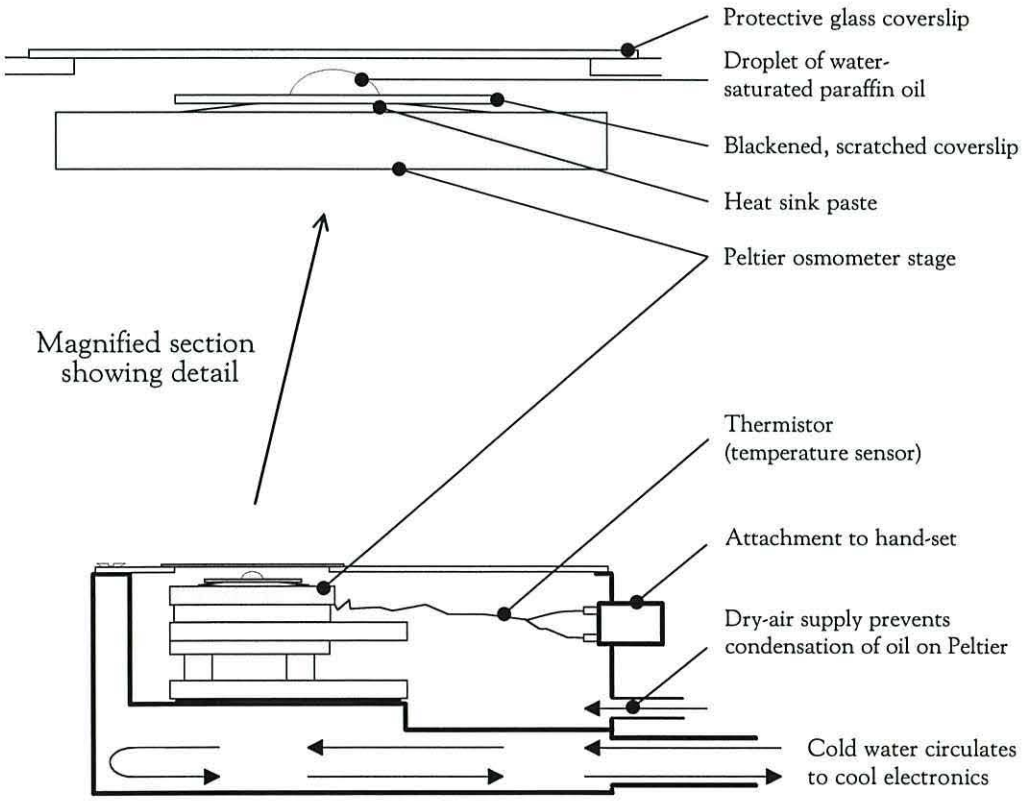
Both sides of a glass cover slip were abraded (to create nucleation sites) by rubbing on a glass plate covered in alumina slurry (Buehler Micropolish, Union Carbide). After approximately 2 minutes rubbing per side, the slurry was rinsed off first with distilled water and then with acetone. The abraded slides were put into a drying oven at a temperature of around 60 °C. When dry, one of the abraded surfaces of the coverslip was blackened with a non-water soluble marker pen. Once dry, a grid pattern was scratched into the ink with a pin to help with subsequent visualisation of microdroplets (Malone and Tomos, 1992). The blackened coverslip was then cut from 22 mm x 22 mm to approximately 10 mm x 10 mm using a razor blade.

To use, the non-scratched side of the coverslip was cleaned with ethanol-soaked lens tissue and then dried. A thin layer of heat sink paste (R.S.) was smeared onto the Peltier stage of the picolitre osmometer and the blackened coverslip (scratched side downwards) was gently lowered onto this paste. A 50 μl droplet of water-saturated liquid paraffin was then expelled from a 25 ml plastic syringe and placed centre left on the blackened coverslip. See **Figure 2.3**.

Microdroplets of KCl standards of known osmotic pressure were transferred from a microscope slide-mounted well (see Tomos *et al*, 1994 for further details) to the small area of the blackened coverslip covered with water-saturated liquid paraffin oil using a constriction pipette. The Peltier stage and blackened coverslip were protected

with a clear glass coverslip. The picolitre osmometer was calibrated by determination of osmotic pressure of the KCl standards. During the freezing process, use of a dry air supply (directed either over the Peltier stage or the top coverslip or both simultaneously) prevented formation of condensation. This dry air supply was never used, however, when determining osmotic pressures due to the high evaporation potential (and resultant increased osmotic pressure) from such small microdroplets. Upon calibration, single cell vacuolar sap samples could be routinely removed from a plant and their osmotic pressures determined from the calibration curve.

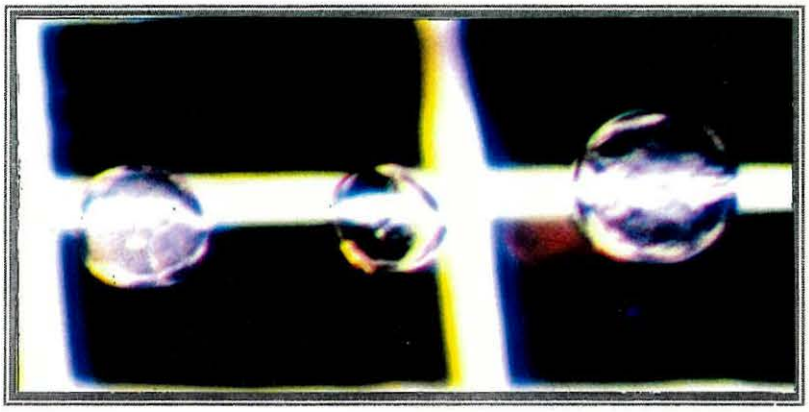
Figure 2.3. Diagrammatic representation of picolitre osmometer set-up. A full description is provided in the text.



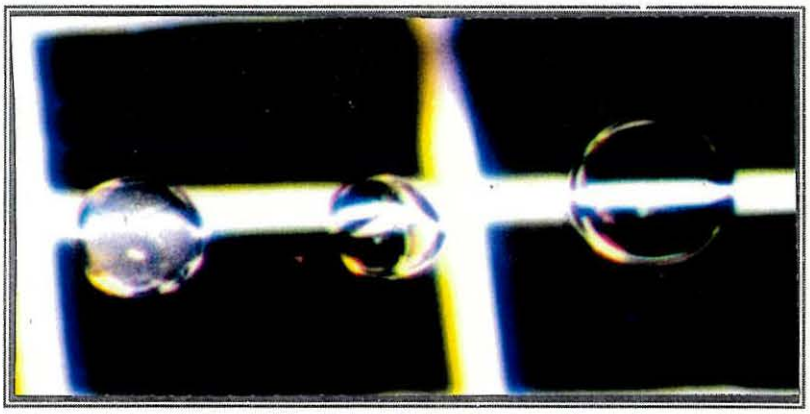
Notes: Diagram not to scale
Internal detail of osmometer has been simplified

Plate 3. Determination of osmotic pressure by picolitre osmometry. Three droplets are shown. As stage temperature is slowly increased (steps 1 to 3), the droplet furthest right thaws first (step 2). It contains the highest solute concentration of the three droplets. The droplet furthest left is the last to thaw (step 3). It contains the lowest solute concentration of the three. [Courtesy of Dr. Olga Koroleva].

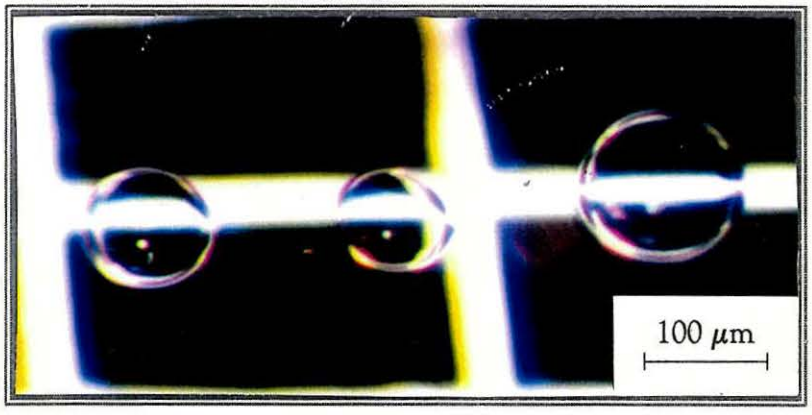
Step 1



Step 2



Step 3



2.4.3, X-ray microanalysis of dried microdroplets

Energy dispersive X-ray microanalysis (EDX) is used to quantify the elemental composition of dried single-cell vacuolar saps. The sample is bombarded with high-velocity incident electrons which cause elemental atoms (in the sample) to undergo changes in their electronic configuration. Electrons within the inner-shells of the atom are either promoted to a higher energy orbital or ejected from the atom altogether (Morgan, 1985). Loss of an electron results in an energetically-unstable, ionised atom with an inner shell electron vacancy. This vacancy is filled by displacement of an electron from a surrounding outer orbital (of the same atom) and coincides with a return to resting state. The excess energy which must be lost for electron demotion is usually emitted as an X-ray photon (Morgan, 1985). The energy quotient of the photon represents the potential energy difference between the two orbitals involved in the exchange and is a unique characteristic of the element. The electron displaced from the outer orbital in turn, creates an electron vacancy which can then be filled by an electron from a subsequent outer orbital. Ionisation of an atom can result in multiple electronic transition, each step of which yields a characteristic X-ray. Each element possesses a unique family of X-rays which is primarily dependant upon the atomic number of the atom (Morgan, 1985).

A second source of X-rays when analysing a sample is *bremsstrahlung* (or white) radiation. This is caused by interaction between an incident electron and the electrostatic cloud of a sample atom and results in inelastic scattering of the electron with subsequent X-ray emission (Morgan, 1985). *Bremsstrahlung* is present as a continually variable background 'noise' (Richardson, 1993) and does not usually represent a problem during sample analyses.

Energy spectra are derived from a plot of the number of detected impulses *versus* their energy quota. For a single element, the area enclosed by the peak (peak integral) of such a plot is proportional to its mass (Richardson, 1993). From manipulation of this data and comparison with standards containing the element of interest, a mass ratio for a single element can be obtained. The inclusion of an internal standard of a $RbNO_3$/saturated mannitol mixture (Hinde, 1994) allows normalisation of sample peaks against the Rb^+ peak integral. The incorporation of mannitol buffers both the effects of organic solutes and serves to mix the sample (as mannitol boils) under the electron beam (Malone *et al*, 1991; Cuin, 1996).

Pioloform membrane-coated 200/100 mesh folding copper grids (all from Agar Aids) were produced as described by Hinde (1994). Once preparation was complete, a membrane coated grid was immersed (pioloform surface uppermost) under water-saturated liquid paraffin in a slide-mounted well. A 5 μl aliquot of each of nine different standards (for further details, see **Table 2.1**) was then laid on the microscope slide under the water-saturated paraffin oil using a 50 μl Hamilton glass syringe.

A similar 5 μl aliquot of 250 mM $RbNO_3$/saturated mannitol mix (Sigma and BDH respectively) was then added to the microscope slide in the same way. This slide was then placed onto a 48 mm x 30 mm x 3 mm aluminium block which was covered on one side in paper on which a fine grid had been inked. Using this block as a primitive heat exchanger, subsequent micromanipulations were performed with the slide-mounted well chilled by the picolitre osmometer stage to approximately 2 °C. See **Plate 4**.

Using a silanized constriction pipette, a large droplet of the $RbNO_3$/Mannitol mix was added to the 100 mesh grid. Approximate 12 pl volumes of each of the nine standards (see **Table 2.1**) were pipetted onto the squares of the 200 mesh grid using a silanized constriction pipette. The same pipette was then used to transfer an equal volume of $RbNO_3$/Mannitol mix to each of the 12 pl droplets of standard. There were four replicates of each standard and to minimise evaporation, only one standard (of the nine) was left without its 12 pl of Rb^+/Mannitol at any time. Following removal (see **2.4.1**), single cell extracted vacuolar saps were sub-sampled four times (to establish sufficient replicates) and then placed on the 200 mesh grid as described for the standards.

Following completion of all micropipetting, the 200/100 mesh grid was removed from the water-saturated liquid paraffin and rinsed for 60 seconds in each of two separate vials of water-saturated hexane (Rathburn Chemicals) followed by a further 60 second rinse in a third vial containing isopentane (BDH). The grid was then dried gently in air before storage in a gelatine capsule over silica gel in an air-tight glass bottle at room temperature.

Just before analysis, the 100 mesh side of the 200/100 mesh grid was mounted in a modified carbon block equipped with a fine gauge sprung brass clip. The carbon block was in turn, fixed in an aluminium stub and the whole assembly was then

transferred to the scanning electron microscope (Hitachi S520 equipped with a Si-Li detector coupled to a Link Analytical QX2000 micro-analytical system (Oxford Instruments). Once a vacuum had been established in the sample chamber of the microscope, the aluminium stub (complete with carbon block and grid) was aligned at 45 ° to the X-ray detector and counting began. The dried microdroplets were scanned using an electron raster just large enough to cover the entire droplet. Analysis stopped once the gross integral for Rb^+ had reached 4500 counts or after 100 seconds (whichever was sooner). Accelerating voltage was 14 kV.

A calibration curve was produced for each element of interest. The background peak integral was automatically subtracted from the peak integral for each element giving a net integral for the element. The ratio of this net integral and the net integral for Rb^+ in the same droplet was then plotted against the concentration of the element giving a standard curve (see **Figure 2.4**). Linear regressions were fitted to the data and calibrations were only used when $r > 0.95$. The elemental concentrations of sample droplets were determined using the equation of the regression line for the standard curve after normalising them against their Rb^+ peak. When Ca^{2+} and K^+ were present in the same sample, an allowance was made for the overlap of the K^+ K_β peak with the K_α peak of Ca^{2+}. Calculation has shown that exactly 10 % of the K^+ signal must be subtracted from that of Ca^{2+} to correct for this overlap (Tomos *et al*, 1994).

Table 2.1. The elemental components (and their concentrations) of the nine different standards used in EDX. Choice of elements for inclusion in standards was made on the basis of elemental solutes known to occur naturally in plant cell vacuoles. Choice of elemental concentrations was made by reference to previous work (see for example, Fricke *et al.*, 1994a, b, c) and data obtained by whole leaf analyses. All standards were produced from Analar salts, BDH.

Standard set	Dilution (%)	[S] (mM)	[P] (mM)	[Mg] (mM)	[K] (mM)	[Ca] (mM)	[Cl] (mM)
1	100	300	300	300	0	0	0
	75	225	225	225	0	0	0
	50	150	150	150	0	0	0
	25	75	75	75	0	0	0
	0	0	0	0	0	0	0
2	100	0	0	0	400	300	400
	75	0	0	0	300	225	300
	50	0	0	0	200	150	200
	25	0	0	0	100	75	100

Figure 2.4. An example of the calibration curves obtained from standard set 1 (S, Mg^{2+} and P) and standard set 2 (K^+, Ca^{2+} and Cl^-). Datapoints represent the means of four replicate measurements ± SD. Data for Ca^{2+} have been corrected as outlined over page.

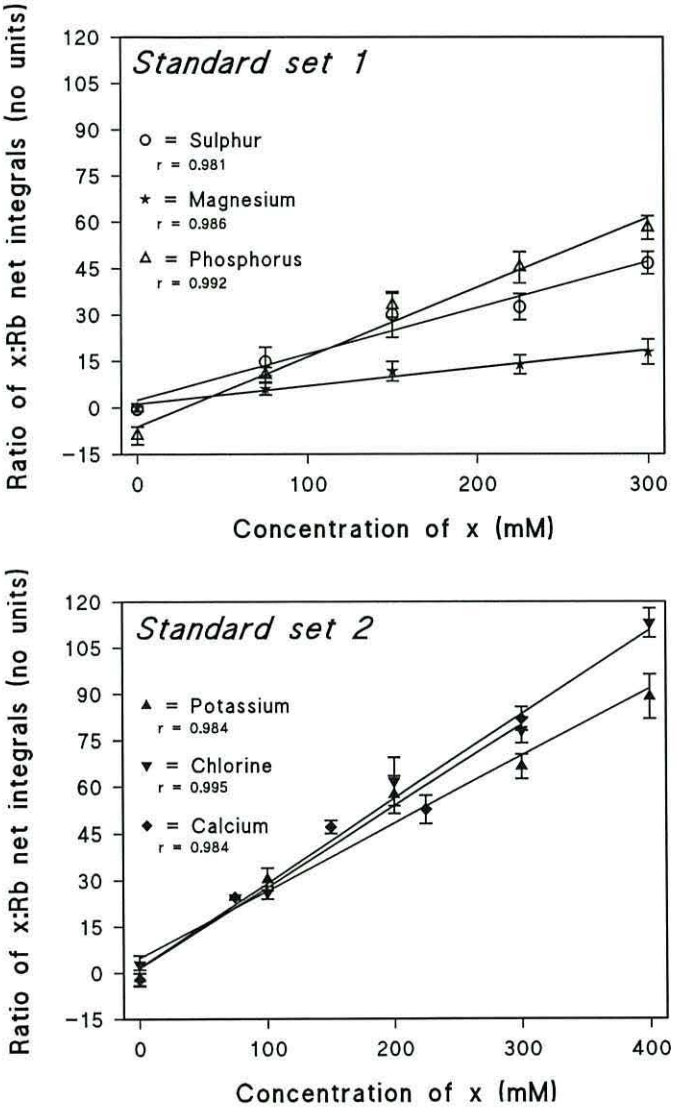


Plate 4. Adding standards to a Pioloform-coated copper grid. The grid is pictured coated-side uppermost under water-saturated liquid paraffin. The large droplets of standard are just visible around the outside of the grid and in this view the slide sits on an aluminium block, chilled by the picolitre osmometer stage.

Chapter 3:

CHOICE OF A GROWTH SOLUTION

3.1, EXPERIMENTAL OBJECTIVE

The main objective of this experiment was to identify a hydroponic growth solution which produced healthy, vigorous plants. The final choice of solution had to be amenable to easy modification of the S : N ratio with little or no interference to the concentrations of cations or other anions.

3.2, INTRODUCTION

The use of a hydroponic growth system allows nutrient availability and pH to be closely regulated and manipulated as required. Hydroponics are useful in studying nutrient deficiencies or excesses and in optimising plant growth conditions with respect to maximising yields (Hoagland, 1948). Hydroponics also allow measurement of plant growth parameters which would be very difficult (or impossible) to determine using soil culture (for example, measurements of root growth or systems where knowledge of the precise concentration of supplied nutrients is required (Epstein, 1972).

The use of hydroponic culture was particularly suited to the present investigation into the relationship between sulphur and nitrogen supply and their effects on wheat. Other methods of plant culture (for example soil or sand) would have made precise control (and monitoring) of S : N ratio difficult due to the existence of nutrient availability gradients.

Whilst the choice of a suitable growth system was relatively easy, choosing a growth solution proved far more difficult. Following satisfactory performance of barley grown on Epstein growth solution (Epstein, 1972; for example see Fricke *et al.* (1994a) or Hinde (1994)) it was decided to attempt to grow the wheat required for this project using the same solution.

This choice produced satisfactory wheat plants for preliminary analyses although it was soon evident that the presence of both NH_4^+ and NO_3^- in the growth solution meant that manipulation of S : N ratio without altering concentrations of other anions and cations would not be possible. On this basis, it was decided that the

Epstein solution should represent the 'benchmark' against which the performance of two other widely-used growth solutions would be evaluated.

The basis for the present project came from a previous study into the distribution of nitrate in wheat (Richardson, 1993) in which sulphate or chloride were used to replace nitrate in a form of Hoagland's solution (Hewitt, 1966). Choosing the same growth solution would allow a valid comparison to be made between the present and previous data with the additional advantage of the Hoagland's variant being ammonium-free.

The final growth solution to be included in this evaluation was Long Ashton (Hewitt, 1966). Long Ashton is another ammonium-free solution which has been routinely used in other work (for example, Marshall & Anderson-Taylor, 1992; Gunn *et al.*, 1996) to produce highly satisfactory plants.

3.3, METHOD

For each of the three nutrient treatments, two tubs containing 3 l of full-strength growth solution were set up (see **2.2** for further details). **Table 3.1** lists the components of full-strength Long Ashton, Epstein and Hoagland's growth solutions.

Every three days, three plants were removed from one growth tub per nutrient treatment. Shoot and root fresh weights were determined after dissection of the plant at the root-shoot interface (see **2.3.1**). Material was then separately bagged and dry weights were determined.

Growth media were replaced every three days. Using a portable pH meter (μicropHep), daily records of growth solution pH for each solution were collected (results not shown). If the pH was outside the range of 6.1-6.3, then it was corrected with 6 M HCl or 10 M NaOH as appropriate.

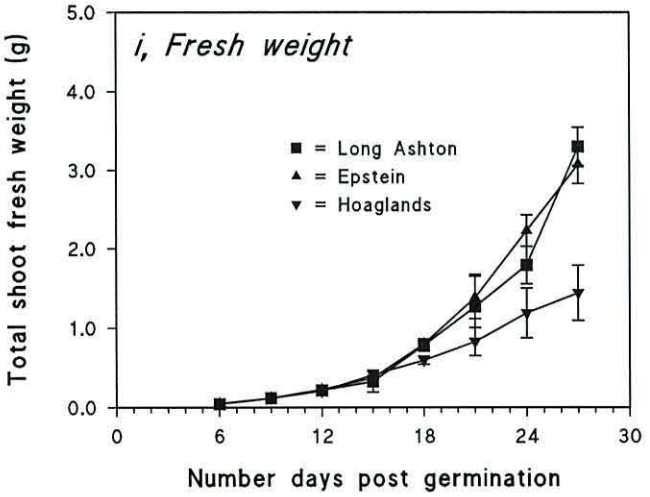
Table 3.1. Composition of the Long Ashton, Epstein and Hoagland's growth solutions as used in the comparison experiment. Concentrations are expressed in mM. For each batch of growth solution, pH was corrected with 10 M NaOH so that the final pH of the growth solution lay in the optimal range for plant growth of between pH 6.1-6.3.

Component		*Long Ashton*	*Epstein*	*Hoagland's*
Macronutrients...	Ca^{2+}	4.000	4.000	2.500
	Mg^{2+}	1.500	1.000	1.000
	NH_4^+	0.000	2.000	0.000
	K^+	4.000	6.050	3.000
	HPO_4^{2-}	1.330	2.000	0.500
	SO_4^{2-}	1.510	1.005	1.002
	NO_3^-	12.000	14.000	7.500
Micronutrients...	Si	0.047	0.000	0.094
	Fe^{2+}	0.100	0.100	0.010
	Mn^{2+}	0.010	0.002	0.012
	Cu^{2+}	0.001	5×10^{-4}	5×10^{-4}
	Zn^{2+}	0.001	0.002	0.001
	Na^+	1.378	0.000	0.189
	BO_3^-	5×10^{-5}	2.5×10^{-5}	5.8×10^{-5}
	MoO_4^{2-}	4×10^{-4}	5×10^{-4}	1.5×10^{-4}

3.4, RESULTS

Figures 3.1-3.3 demonstrate the effects of the three different growth solutions on shoot and root fresh and dry weights and on plant shoot and root growth rates.

Figure 3.1. The effects of different growth solutions on (i) fresh and (ii) dry weights of the total shoot. For each datapoint, n = 3 ± SD. At day 15, there was no significant difference in total shoot fresh weight between the three different growth solutions but by day 27, total shoot fresh weight was significantly lower (ANOVA; $p < 0.01$) in Hoagland's plants when compared with either Long Ashton or Epstein plants. These observations were also true when dry weight data were analysed statistically.



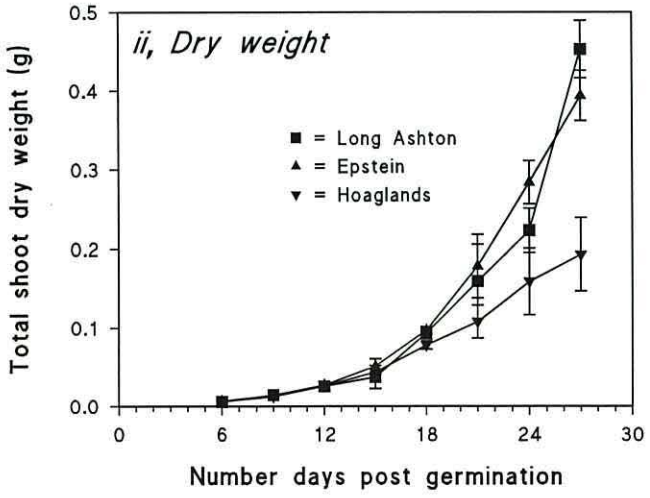
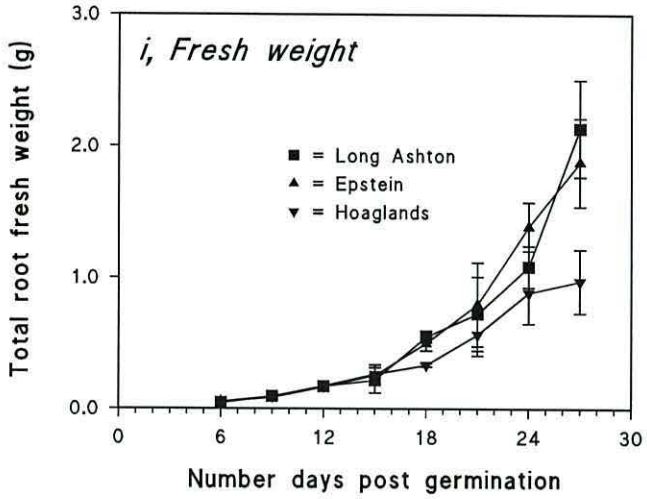


Figure 3.2. The effects of different growth solutions on (i) fresh and (ii) dry weights of the total root. For each datapoint, n = 3 ± SD. At day 15, there was no significant difference in root fresh weight between the three growth solutions but by day 27, the fresh weights of Hoagland's plants were significantly lower (ANOVA; $p < 0.05$) than those of plants grown in either Long Ashton or Epstein growth solutions. A similar observation was made for the root dry weight data.



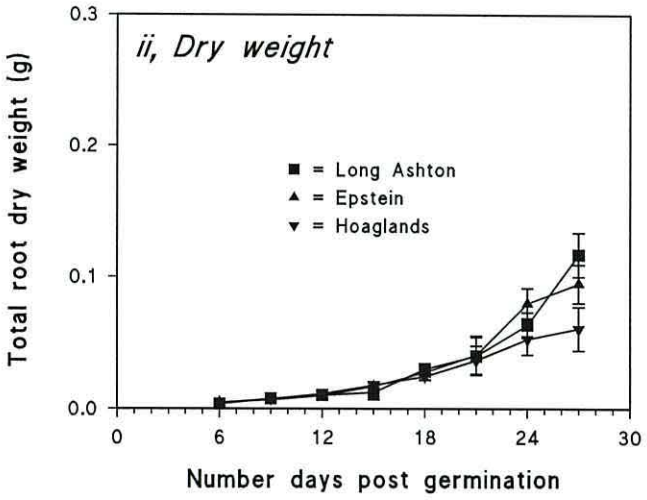


Figure 3.3. Logarithmic$_e$ plots of both (i) shoot- and (ii) root fresh weights demonstrate the effects of the three nutrient solutions on shoot and root growth rates throughout the experiment. Each datapoint represents an individual measurement and a first order regression connects datapoints. Multiple comparison tests were performed to allow the similarity of the slopes of the regression plots to be compared. Statistical comparison of shoot and root growth rates between plants grown in Epstein nutrient solution with Long Ashton plants showed that there was no significant difference in either shoot or root growth rates. However, comparison of Epstein plants with Hoagland's plants (or Long Ashton plants with Hoagland's plants) gave a highly significant difference ($p < 0.001$) for both shoot and root growth rates.

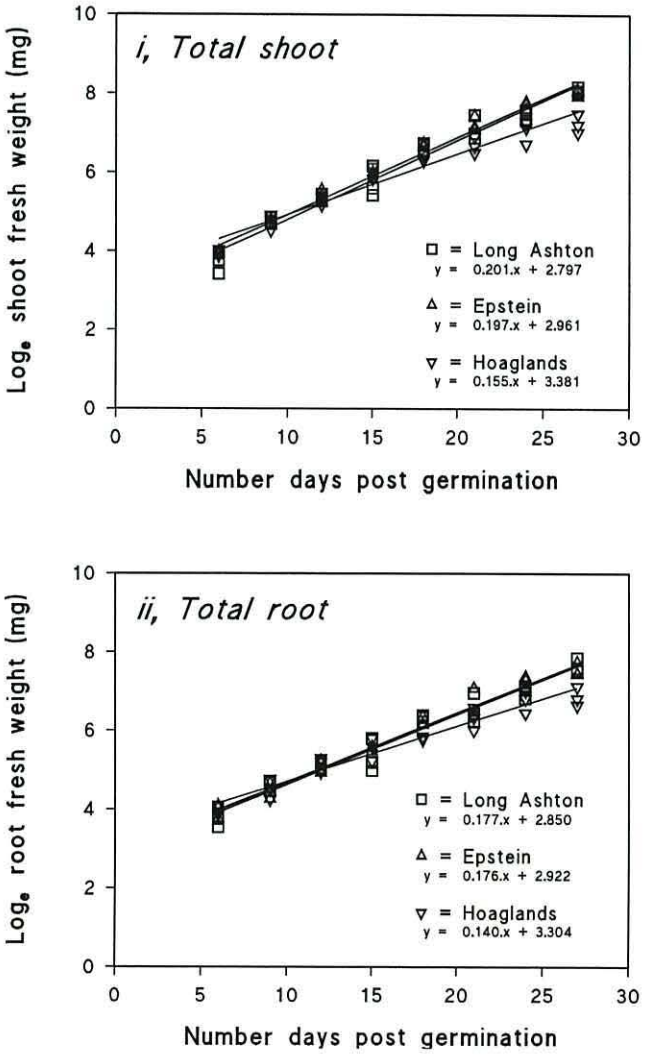
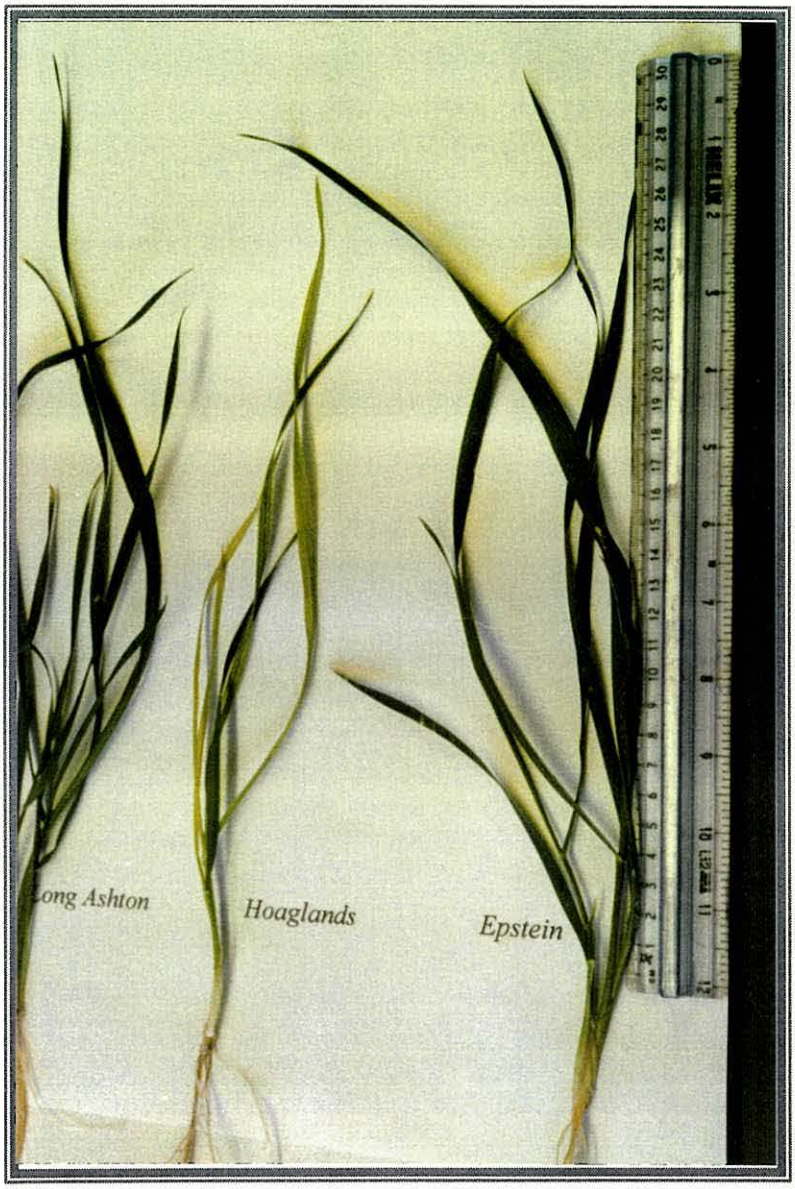


Plate 5. The effects of choice of growth solution on hydroponic growth of wheat (variety Alexandria). Plants grown on Hoagland's solution were smaller than those grown on Epstein or Long Ashton and they appeared chlorotic and unhealthy. This plate demonstrates the visible differences that existed between treatments.



3.5, DISCUSSION

This chapter has described an experiment carried out to compare the ability of Epstein, Long Ashton and Hoagland's nutrient solutions to support the hydroponic growth of wheat plants. Throughout the investigation, both Epstein and Long Ashton nutrient solutions produced comparably healthy and vigorous plants (see **Plate 5**). There were no significant differences between shoot and root fresh and dry weights and the growth rates of both shoots and roots (see **Figure 3.3**) were virtually identical.

In comparison, growth of wheat on Hoagland's nutrient solution resulted in unhealthy, chlorotic plants (see **Plate 5**) with significantly reduced shoot and root fresh and dry weights; all classical symptoms of nutrient deficiency. The differences between plants grown in Epstein/Long Ashton and Hoagland's nutrient solutions increased as material grew. Towards the end of the experiment, the shoot and root growth rates of Hoagland's plants was well below that of the other two nutrient solutions.

3.5.1, How does choice of nutrient solution affect shoot fresh and dry weights?

Between 6 and 15 days post germination, there were no significant differences either visually or in terms of total shoot fresh or dry weights due to nutrient solution. The first leaves reached full expansion at the same time (day 11 post seed germination) in all three nutrient treatments and no visible difference in either leaf structure or colour could be ascertained between treatments.

The second leaves also reached full expansion on the same day (day 16) in all three treatments. By this stage of growth, however, the first visible differences between nutrient treatments were evident. The second leaf of plants grown on Hoagland's solution showed faint signs of chlorosis when compared with Epstein or Long Ashton plants. At the next assessment on day 18, leaf 2 fresh weights of Hoagland's plants were 20 % lower (a mean of 0.149g for Hoagland's compared to a mean of 0.186 g for Epstein) and statistically significant (ANOVA; $p < 0.05$) when compared with those grown on either Epstein or Long Ashton growth solutions. These observations were mirrored by the day 18 dry weight data where a mean of 0.02 g for Hoagland's leaf 2 compared with a mean of 0.025 g for Epstein leaf 2. By this stage, growth of plants on Hoagland's solution was also significantly affecting

(ANOVA; $p < 0.05$) total shoot growth as demonstrated by both fresh and dry weights. On day 18, mean shoot fresh weight of Hoagland's plants was 0.594 g (74%) when compared with the mean weight of 0.799 g for the Epstein 'benchmark' plants. This difference increased as the plants got older. On the final assessment at day 27, differences in total shoot fresh weight between Hoagland's and Epstein/Long Ashton were still significant (ANOVA; $p < 0.001$) but the difference between the values had drastically increased such that Hoagland's mean shoot fresh weight was just 47 % (whilst dry weight was 49 %) of Epstein plants.

The symptoms demonstrated by the second leaves of Hoagland's plants were even more pronounced in the third leaf when it reached maturity on day 20 post seed germination. As was the case for the second leaves, the third leaves of all three treatments reached maturity on the same day. At assessment on day 24, the mean fresh weights of the third leaf of Hoagland's plants were just 67 % of the mean weight of Epstein third leaves (0.208 g for Hoagland's plants compared with 0.311 g for Epstein plants). As for leaf 2, statistical analyses and visual observation were fully in agreement.

3.5.2, How does choice of nutrient solution affect root fresh and dry weights?

At first glance, the root story is very similar to that of the total shoot. Between assessment days 6 and 15, there was no significant difference between either root fresh or dry weights due to choice of growth solution. However, by day 18 post germination, the fresh weight of Hoagland's plant roots were significantly different (ANOVA; $p < 0.05$) from the roots of either Epstein or Long Ashton grown plants. From day 18 onwards, however, root fresh weight data did not follow the same pattern as that for total shoot. There was a statistically significant difference between root fresh weights at day 18, which diminished at both day 21 and day 24 but returned by day 27 (ANOVA; $p < 0.01$).

There are two possible explanations for these observations. The first (influencing solely fresh weight measurement) is due to the method used for removing excess nutrient solution from the root system. This was done simply by shaking the roots prior to weighing, in hindsight, a method wide open to variability. It is unfortunate that use was never made of absorptive media such as blotting- or tissue-paper which would have given more consistent drying whilst probably

sharpening the accuracy of the data. It is likely that the significance of this source of error would increase towards the latter stages of the experiment due to corresponding increases in both the size of the root system and its capacity to retain nutrient solution.

The second major source of error was not so easy to avoid. The growth facilities used in this and subsequent investigations were seriously limiting with respect to the maximum number of plants that could be successfully grown at any one time. The size of the growth cabinet was such that only six growth tubs could be fitted inside for any one investigation. When an experiment was designed, available time, space in the growth cabinet, the number of treatments under evaluation and the need for sufficient assessment points during the experiment were all major considerations. In order to meet all four of these considerations, plants were grown at a density of 15 plants per growth tub. In the earlier stages of development, this presented no problem. A plant could easily be removed from its growth tub with only minimal disturbance to its own and its neighbours root system. However, as the plants got bigger and root systems intertwined, removal of material for analyses caused more and more disturbance to the root systems until it became impossible to remove a single plant knowing that all of its root system had been removed at the same time.

The combination of these two sources of error to different and unknown degrees means that interpretation of non-logarithmically transformed root fresh and dry weight data is difficult. Of the two analyses, dry weight data should represent the more accurate although it is still difficult to suggest whether root weights of Hoagland's plants were any different to those grown on Epstein or Long Ashton. If we believe the dry weight data, the only assessment point at which there is significant difference between Hoagland's roots and those from Epstein plants is at day 27 suggesting that Hoagland's plants did experience impaired root growth. This tentative observation is given more credence in the next section.

3.5.3, How does choice of nutrient solution affect shoot and root growth rates?

During the course of this experiment, shoot and root fresh weight data both assumed classic exponential curves (see **Figures 3.1 and 3.2**) representative of annual plants grown in a productive environment (Hunt, 1990). When this data was

logarithmically$_e$ transformed and plotted against time, it was possible to observe and compare growth rates for shoots and roots between treatments. The use of first order regressions served two purposes. Firstly, they proved that there was a linear relationship between log-transformed weight *versus* time (in all cases $r > 0.95$) and secondly they allowed comparison of growth rates but by a different method to those outlined by Hunt, 1990. In this experiment, growth rates were compared statistically using a series of multiple comparison tests (Zar, 1996). In these, the slope of the first order regression plot for either Long Ashton or Hoagland's was compared to that for the Epstein 'benchmark' growth solution.

For both total shoots and roots, there were no significant differences between growth rates of plants in Long Ashton when referenced against Epstein growth solution. However, comparison of both log shoot and log root data from plants grown in Hoagland's against those grown in Epstein indicated that there were significant differences in growth rate for both shoot and root between these two treatments.

3.5.4, Stating the case

When considering all three types of measurement performed in this investigation (total shoot, total root and growth rates), Hoagland's grown plants were consistent under-achievers when compared to plants grown on Epstein or Long Ashton nutrient solutions. Hoagland's plants had lower fresh and dry weights for both shoot and root as well as impaired growth rates again for both shoot and root; in conjunction with visual observation, such data are concomitant with a severe nutrient deficiency.

Referring to **Table 3.1**, a comparison of the three nutrient solutions reveals two components of Hoagland's which can be found in both Epstein and Long Ashton in higher concentrations. It is unlikely that the relatively minor differences between other components could have been responsible for the striking dissimilarities between Hoagland's and Epstein/Long Ashton data. For example, even though the concentration of nitrate was lowest in Hoagland's nutrient solution (7.5 mM), even this concentration is thought to represent a "luxury supply" (Richardson, 1993).

The first major difference between the three nutrient solutions is in terms of their iron (Fe^{2+}) content. In Hoagland's nutrient solution, the iron concentration is 1×10^{-5} M which represents just 10 % of the iron found in both Epstein and Long

Ashton (both 1 x 10^{-4} M). The second major difference is in phosphate (PO_4^{3-}) concentrations. Hoagland's solution contains 5 x 10^{-4} M phosphate, four-fold less (*cf.* 2 x 10^{-3} M) than is found in either Epstein or Long Ashton.

If we consider first the case for phosphate. The main deficiency symptoms are reduction in leaf expansion and leaf surface area (Fredeen *et al.*, 1989) both of which may be attributable to impaired extension of epidermal cells (Marschner, 1995). However, the protein content and chlorophyll per unit leaf area remained relatively unaffected in phosphate starved sugar beet plants (Rao & Terry, 1989). Indeed, leaf chlorophyll content may even increase under conditions of phosphorus deficiency (Rao & Terry, 1989) and it is not unknown for phosphorus deficient leaves to appear more darkly green than phosphorus-sufficient leaves (Hecht-Buchholz, 1967).

In this investigation, both the second and third leaves of plants grown on Hoagland's nutrient solution demonstrated varying degrees of chlorosis. However, no observation was made relating to impaired leaf expansion and in fact, both the second and third leaves of Hoagland's grown plants reached maturity on the same days as both Epstein and Long Ashton plants. This would not be the expected response if leaf expansion had been impaired. On the basis of this evidence, it is unlikely that the four-fold difference in phosphorus concentration between Hoagland's and Epstein/Long Ashton was responsible for the observations made in this investigation.

This then, leaves just iron content representing the only other major difference between the three growth solutions. The concentration of iron was ten fold less in Hoagland's solution when compared with either Epstein or Long Ashton solutions.

Iron deficiency has a pronounced effect on the photosynthetic machinery (primarily photosystem I). Each individual chloroplast requires around 20 iron atoms to maintain the structural and functional integrity of its thylakoid membranes. Iron is also required for the biosynthesis of chlorophyll and plays a structural role in ferredoxin (Marschner, 1995). The main symptom of iron deficiency is a measurable decrease in leaf chlorophyll content due to a reduction in chlorophyll synthesis. This is turn leads to interveinal chlorosis which is most severe in young leaves (Salisbury & Ross, 1992). A reduction in the rate of leaf growth is also possible but only in cases of very severe iron deficiency (Marschner, 1995).

From this evidence, the symptoms of chlorosis demonstrated by the second and third leaves of Hoagland's grown plants seem to have been attributable to a lack of available iron. As the plants grew, the increased biomass would result in an increase in iron requirements, in turn suggesting that the symptoms of iron deficiency would become more acute as the plants aged. Certainly, the slight chlorosis shown by leaf 2 at full expansion had become far more severe four days later when leaf 3 attained maturity. The observations (and probable prognosis) are very similar to those reported by Smith *et al.*, 1983 who found that differences in the abilities of different nutrient solutions to support growth of a plant species were normally attributable to differences in iron concentrations between the various nutrient solutions.

In addition to there being a ten-fold difference in iron concentration between Hoagland's and Epstein solutions, there may also have been a difference in the available iron that the plant could absorb. When plants are grown hydroponically using both NH_4^+ and NO_3^- as nitrogen source (as in Epstein growth solution), the pH surrounding the root zone becomes acidified due to the release of H^+ as ammonium ions are absorbed by the roots (Marschner, 1995). However, when plants are grown on nutrient solutions containing just NO_3^- as nitrogen source (for example, Hoagland's), the pH surrounding the root zone increases due to release of OH^- (Raven and Smith, 1976; Marschner, 1995). This pH increase can then reduce iron uptake by plant roots (Truog, 1948). Daily pH records collected from the three nutrient treatments confirm that prior to pH correction, there was an increase in pH in both Long Ashton and Hoagland's solutions and a decrease in pH of Epstein solution on a daily basis.

Whilst the data from this investigation is incapable of supplying a definitive explanation for the observed differences between nutrient solutions, a possible hypothesis is presented thus. Uptake of NO_3^- from Hoagland's solution resulted in an increase in solution pH. This would lead to a reduction in the availability of iron in the growth solution. This fact when coupled with the already low iron concentration of Hoagland's solution would suggest that it was a combination of these two factors that was responsible for growth of plants showing symptoms resembling iron deficiency.

3.6, CONCLUSIONS

- Growth of wheat on Hoagland's nutrient solution produced chlorotic plants with reduced shoot and root, fresh and dry weights. Both shoot and root growth rates were impaired. Iron deficiency was thought to have been responsible.

- Epstein and Long Ashton nutrient solutions produced comparable plants with no significant differences between the two in respect of any of the parameters measured.

- Long Ashton proved to be a suitable ammonium-free nutrient solution for growth of wheat plants. All future work was, therefore, based on wheat plants grown on Long Ashton solution or its derivatives modified for N and S content.

Chapter 4:

DEVELOPMENT OF AN ENZYMATIC SO_4^{2-} ASSAY

4.1, INTRODUCTION

Sulphate can be easily and routinely quantified in plant material using one of two commonly-used techniques. If data are required on the soluble S compounds present in expressed tissue sap (which would be mainly SO_4^{2-}), then HPLC anion analyses (see **2.3.4.1**) will reliably detect and quantify concentrations (Gorham, 1987). If, however, data are required on the total S content (both soluble and insoluble compounds), then acid-digestion followed by a simple, turbidimetric method involving analyses of a $BaSO_4$ precipitate (Tabatabai & Bremner, 1970) can provide a reliable means of measurement (see for example, Bell *et al*, 1994). Neither HPLC nor $BaSO_4$ precipitation would be sufficiently sensitive to determine SO_4^{2-} in extracted single cell vacuolar sap samples. The small SO_4^{2-} signal (potentially less than $2x10^{-13}$ moles) associated with such samples would effectively be swamped by background 'noise'. It is possible to quantify total S in extracted single cell vacuolar saps using X-ray microanalysis (see **2.4.3**). This technique, however, provides only a measure of S and ignores the form which it may take. It has been assumed in the past (for example Cuin, 1996) that all vacuolar S is in the form of SO_4^{2-} and work with barley mesophyll cell protoplasts has demonstrated that this is usually the case (Rennenberg, 1984; Ernst, 1997; Mornet *et al*, 1997). As yet, however, it remains to be proven that all vacuolar S is in its inorganic form in plant cells *in situ*.

The co-factors $NADP^+$ and NAD^+ act as electron acceptors in a number of enzyme-catalysed reactions within the cell. These properties are exploited in a wide range of enzymatic assays *in vitro* (Bergmeyer, 1965; Lowry and Passioneau, 1972; Boehringer, 1984) primarily because the reduced forms of these two co-factors are optically distinct from their oxidised forms in the UV-visible regions of the light spectrum (Richardson, 1993). In solution the reduced form has a higher optical density (at 340 nm) than the oxidised form. Quantification of the reduced co-factor is, therefore, possible by simple one wavelength spectrophotometric analysis (Boehringer, 1984). This approach is, however, unfeasible when wanting to quantify a specific substrate in extracted vacuolar saps from single cells. Here, the total volume and amount of absorbing co-factor is so small that single wavelength absorption

spectrophotometry would be insufficiently sensitive. Enzymatic analyses of single cell vacuolar saps, therefore, have to exploit a further property of the reduced co-factor: its ability to fluoresce at 460 nm when excited with light at its absorbing wavelength (λ_{max} = 340 nm).

The amount of co-factor reduced (or oxidised) in an enzymatic assay is stoichiometric with the quantity of substrate converted into product. Calculation of the change in fluorescence between measurements made before (*e.g.* before addition of enzyme) and after an enzyme-catalysed redox reaction provides a numerical figure representing the conversion of substrate to product. Differing concentrations of substrate result in different changes in fluorescence and microfluorometric analyses of standards of known concentrations allow standard curves to be produced.

A fluorescence microscope equipped with a photometer measures the minute changes in fluorescence occurring in these small-scale enzyme assays (see **Plate 6**). The photometer measures the light emitted by a sample at right angles to the exciting beam. This means that the general technique of fluorimetry is considerably more sensitive than that of spectrophotometry (Outlaw, 1980) since the signal is measured against a dark background rather than by detecting a small change in transmitted light (Fersht, 1985). An increase in excitation intensity results in a proportional increase in signal strength relative to background noise (Outlaw, 1980).

The use of specific microfluorometric enzymatic assays for metabolites such as nitrate, malate and glucose (Tomos *et al*, 1994) represent well-established procedures with high success rates (for example Fricke *et al*, 1994a). It was proposed that the development of a specific enzymatic assay for SO_4^{2-} could be used to ascertain whether all vacuolar S (as determined by X-ray microanalysis) was indeed in its SO_4^{2-} form. A reaction mechanism for a potential microfluorometric SO_4^{2-} assay was proposed (see **Figure 4.1**).

Plate 6. The progress of a microfluorometric enzymatic assay is monitored by detecting the change in fluorescence due to change in the oxidation state of the co-factors NAD^+ or $NADP^+$. In this picture, the reduced co-factor NADPH in these 5 nl microdroplets fluoresces at a wavelength of 460 nm (Bergmeyer, 1965) when bombarded with exciting light (λ_{max} = 340 nm) from a Hg lamp. [Courtesy of Dr. Olga Koroleva].

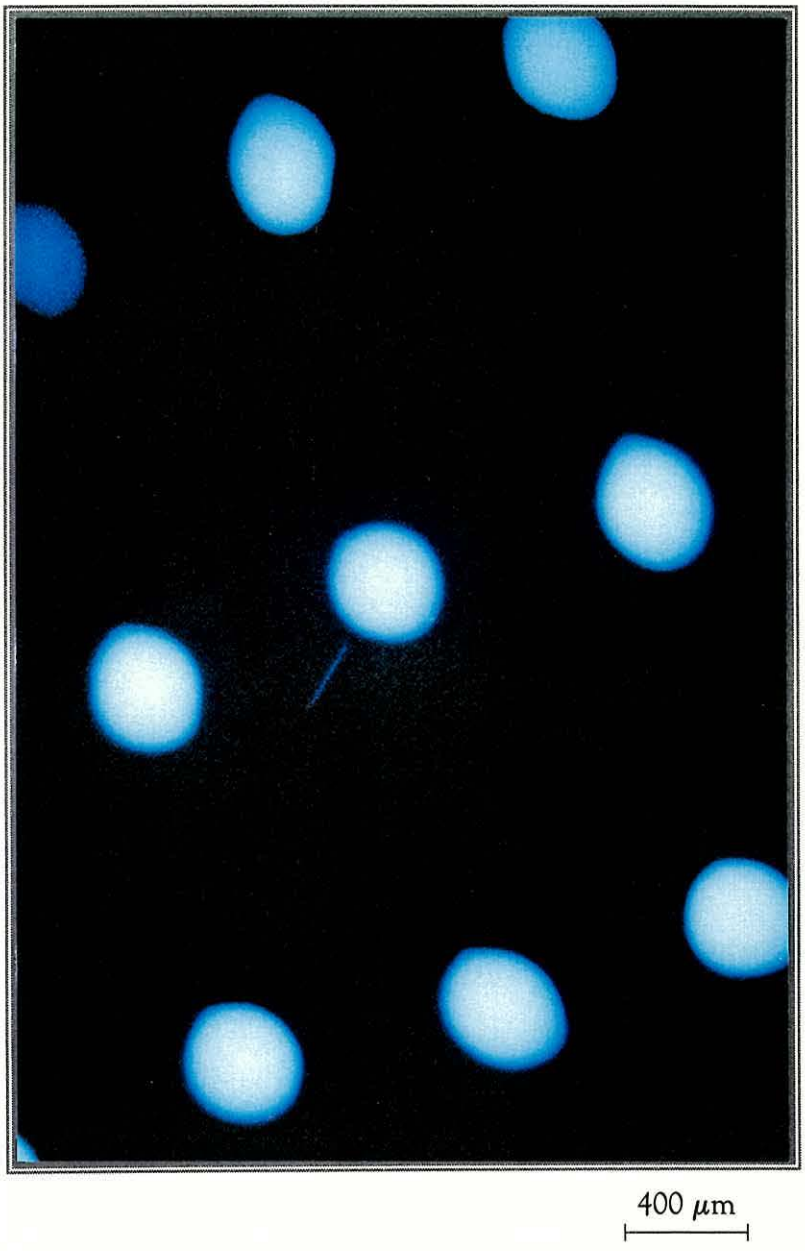
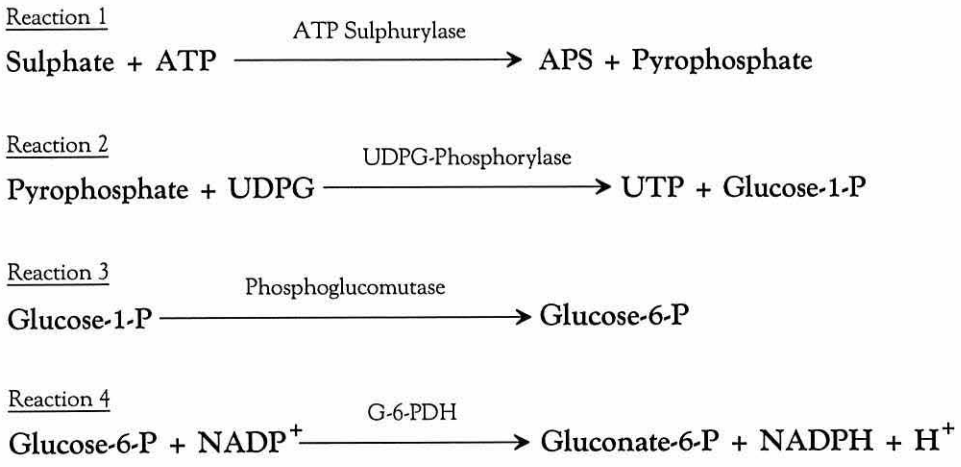


Figure 4.1. The proposed reaction mechanism for the enzymatic assay of SO_4^{2-} in extracted single cell vacuolar saps. If all reactions proceed to completion, the amount of NADPH formed as a consequence of this reaction sequence would be stoichiometric with the amount of SO_4^{2-} initially present in the system. The proposed assay was comprised of four stages. In the first (Reaction 1), commercially available ATP sulphurylase would be used to convert SO_4^{2-} and ATP into adenosine-5'-phosphosulphate (APS) and pyrophosphate (PP_i). In theory, it would then be possible to measure the PP_i formed using a published method (Boehringer, 1984). In the first step of this method (Reaction 2), PP_i and uridine-5'-diphosphoglucose (UDPG) are converted into glucose-1-phosphate (G-1-P) and uridine-5'-triphosphate (UTP) by uridine-5'-diphosphoglucose pyrophosphorylase (UDPG-ppase); G-1-P is then converted into glucose-6-phosphate (G-6-P) by phosphoglucomutase (PGluM; Reaction 3). Finally, G-6-P is converted into gluconate-6-phosphate (gluconate-6-P) by glucose-6-phosphate dehydrogenase (G-6-PDH) with subsequent reduction (and corresponding increase in fluorescence) of $NADP^+$ (Reaction 4).



4.2, EXPERIMENTAL OBJECTIVE

The main objective was to design and develop an enzymatic assay capable of quantifying SO_4^{2-} in extracted vacuolar saps. This assay would then be used to determine how much of the total vacuolar S (as measured by X-ray microanalysis) is present in this inorganic form.

The following section describes a series of experiments designed to test hypotheses and answer questions raised by the attempted (although unsuccessful) development of an enzymatic assay for SO_4^{2-}. Each individual question (or hypothesis) is presented as a separate heading whilst the experiment designed to prove or disprove it is presented beneath. Results and conclusion then follow in the normal way. The full addresses of equipment manufacturers and chemical suppliers are listed separately in **Appendix 1.**

4.3, EXPERIMENTAL METHODS

4.3.1, Can the published method for enzymatic assay of PPi work in a microtitre system?

The aim of this experiment was to produce a calibration curve for PP_i standards thus ensuring that the published method (Boehringer, 1984) for assay of PP_i worked under modified conditions. Changes were made so that the assay could be run with a final volume of 200 μl in microtitre plates. These modifications were considered necessary as it was envisaged that much of the future development work would be undertaken on the microtitre system. On the one hand, the 200 μl assay-volume was sufficiently large to allow easy manipulation of assay components whilst on the other, it was small enough to minimise the expense associated with use of larger volumes.

To each well of a microtitre plate, a 140 μl volume of assay cocktail was added. This contained the following components (purchased from Boehringer unless indicated otherwise); concentrations shown represent those in the final 200 μl assay volume; 40 mM Tris-HCl at pH 8.2, 2 mM UDPG, 6.9 mM $MgCl_2$ (BDH), 0.38 mM $NADP^+$, 0.98 U ml^{-1} G-6-PDH and 2.6 U ml^{-1} PGluM.

Before use, it was necessary to de-salt PGluM and this was performed by fast protein liquid chromatography (FPLC, Pharmacia) in which protein and salt were separated by gel filtration under high pressure. Once de-salted, the protein content of the resultant preparation was determined spectrophotometrically at 214 nm and

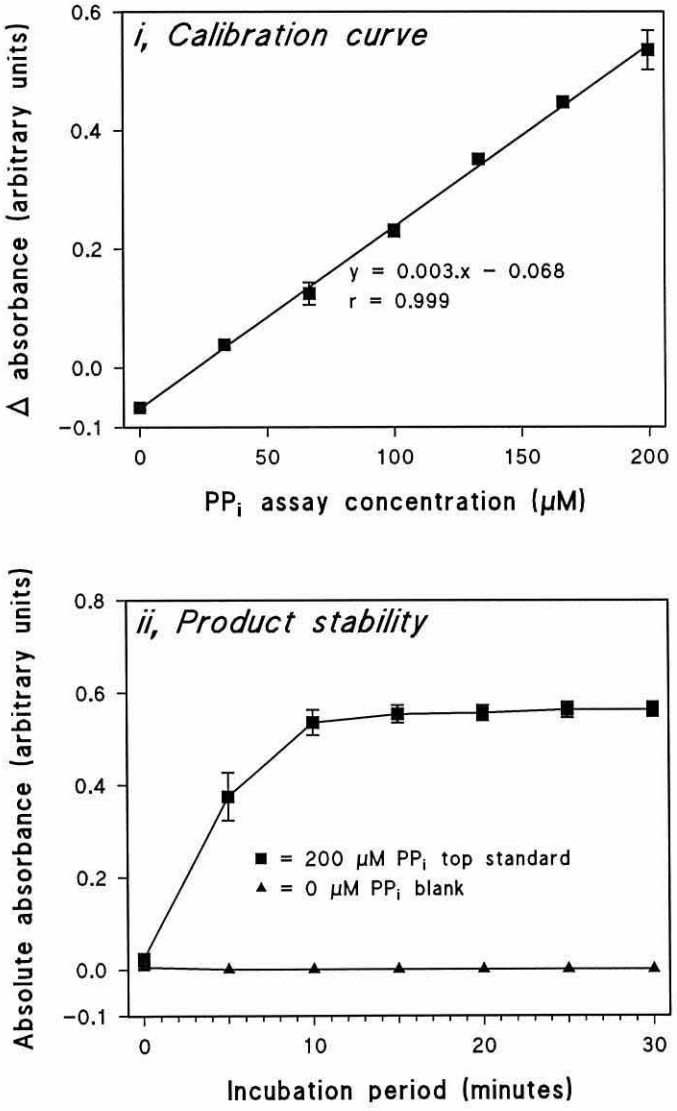
diluted to the required 22.5 U ml^{-1} with 0.05 M acetate buffer (pH 5.3). The de-salted and diluted PGluM preparation was then frozen in aliquots at -20 °C until required.

A 0, 10, 20, 30, 40, 50 or 60 μl volume of 667 μM $Na_4P_2O_7$ was added to microtitre wells containing the 140 μl assay cocktail. Final well volume was then made up to 200 μl using de-ionised water and each well was hand-stirred before the microtitre plate was covered and incubated at 23 °C for approximately 10 minutes. An initial absorbance at 340 nm was then obtained for each well using an automated microplate reader (EL340, Bio-tek) controlled by a personal computer (Viglen VPC XT120M). Reaction was started by a 2 μl addition of UDPG-ppase (3.3 U ml^{-1} final concentration in 0.5 % glycerol in a 200 μl assay volume) per well and the microtitre plate was incubated at 23 °C for a further 15 minutes before the final absorbance was measured. The results are given in **Figure 4.2**.

Conclusions

- The Boehringer PP_i assay worked at the 200 μl scale in the microtitre system.
- At 23 °C, reaction had reached 'completion' in 10 minutes; products were then stable for at least a further 20 minutes.

Figure 4.2. Testing the PP_i enzymatic assay at the 200 μl scale. Graph (i) demonstrates the calibration curve obtained from a plot of change in absorbance *versus* PP_i concentration. When absorbencies were measured every 5 minutes for a total of 30 minutes (graph (ii) it was shown that (a) reaction reached 'completion' (91 % of PP_i hydrolysed) in 10 minutes and (b) reaction products were stable for at least a further 20 minutes. For each datapoint, n = 3 ± SD.



4.3.2, Is the PP_i assay affected by adding SO_4^{2-}, ATP or APS?

The proposed coupling of the PP_i assay to the ATP sulphurylase-catalysed reaction step (see **Figure 4.1**) will lead to exposure of the existing PP_i enzymatic assay to SO_4^{2-}, ATP and APS. Any of these components could lead to partial (or even complete) inhibition of the working assay; the aim of these experiments was to investigate whether individually adding SO_4^{2-}, ATP or APS to the PP_i enzymatic assay could affect its ability to quantify PP_i substrate.

The effects of ATP

A 140 μl volume of assay cocktail (components as **4.3.1**) was added to each well of a microtitre plate. Identical 30 μl additions of both substrate (either 667 μM or 1.3 mM $Na_4P_2O_7$) and ATP were then made. Final assay concentrations of ATP were 0, 0.02, 0.2, 2 or 20 mM. Remaining method was as described in **4.3.1**. Plots of change in absorbance *versus* concentration of PP_i standard for each of the five ATP concentrations showed that only very high (20 mM) concentrations of ATP had any effect on the PP_i assay (see **Figure 4.3**).

The effects of SO_4^{2-}

In the second experiment, the effects of SO_4^{2-} inclusion on the PP_i assay were investigated. The assay was run using 100 μM PP_i (assay concentration) as substrate but with addition of suitable K_2SO_4 standards to give assay concentrations of 0, 10, 100 and 200 μM SO_4^{2-}. Resultant ratios of SO_4^{2-} : PP_i were 0 : 1, 0.1 : 1, 1 : 1 and 2 : 1 respectively. Incorporation of SO_4^{2-} had no effect on the PP_i assay (see **Figure 4.4**) and there was no difference in absorbance between wells lacking SO_4^{2-} (0 : 1 SO_4^{2-} : PP_i) and those containing the highest SO_4^{2-} concentrations (2 : 1 SO_4^{2-} : PP_i).

The effects of APS

The effects of adenosine-5'-phosphosulphate (or APS) on the PP_i assay were investigated in the final experiment in this series. The assay was run using 0, 100 or 200 μM PP_i standards in the presence of 0, 10, 25, 50, 100 or 200 μM APS. Inclusion of APS had no effect on the PP_i assay (**Figure 4.5**).

Figure 4.3. The effects of adding ATP to the PP_i assay. For each datapoint, n = 1. Data for the assay of 100 μM PP_i in the presence of 0.2 mM ATP was unfortunately lost.

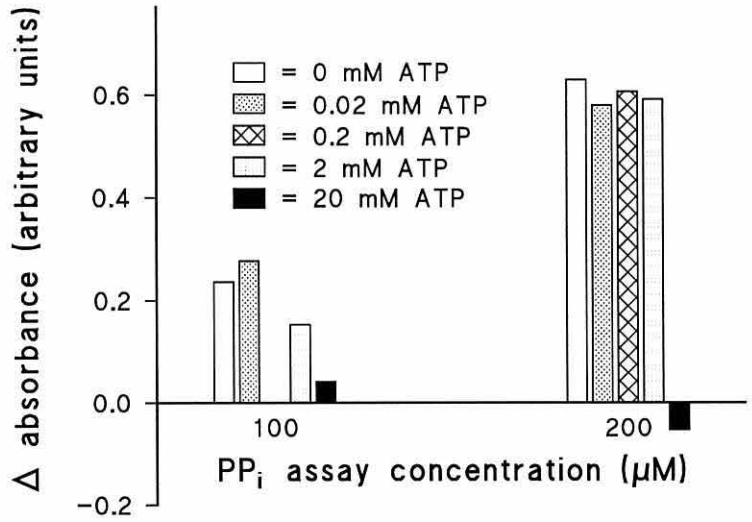


Figure 4.4. The effects of adding SO_4^{2-} to the PP_i assay. For each datapoint, n = 1.

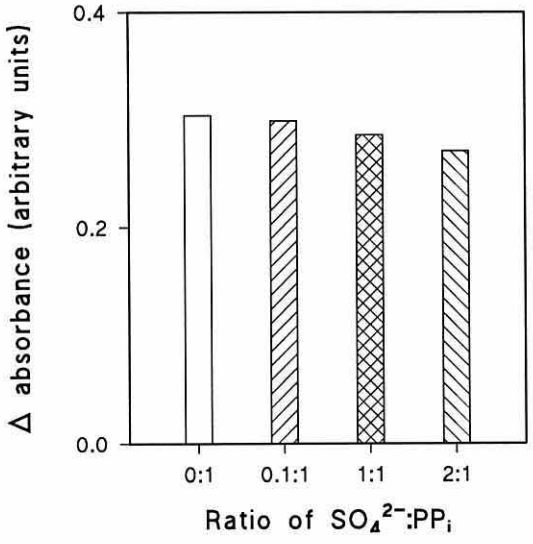
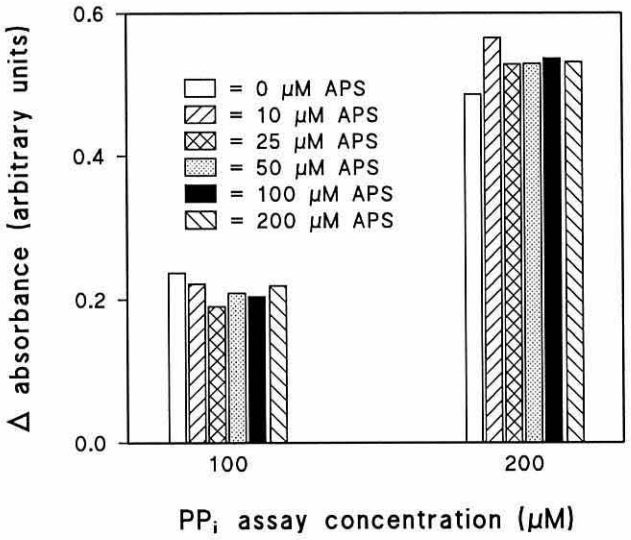


Figure 4.5. The effects of adding APS to the PP_i assay. For each datapoint, n = 1.



Conclusions

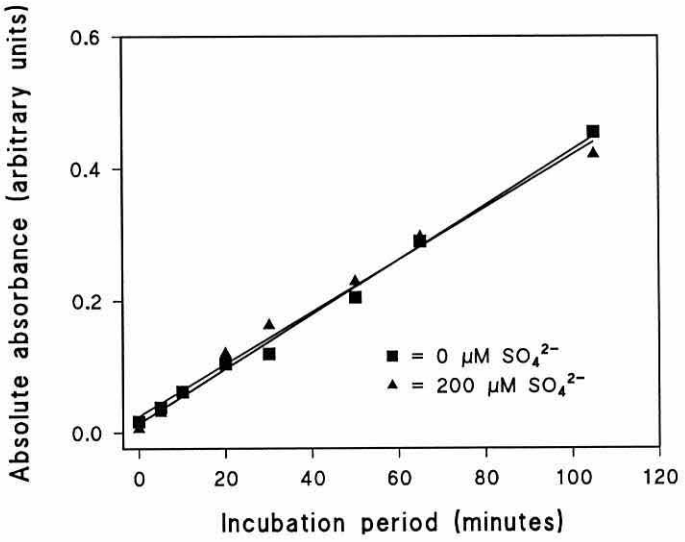
- The PP_i assay was inhibited by high concentrations (20 mM) of ATP. This did not represent a problem since 2 mM ATP is sufficient for the proposed sulphate assay.
- Neither SO_4^{2-} nor APS had any effect on the PP_i assay (SO_4^{2-} (as NH_4SO_4) can inhibit both UDPG-ppase (Boehringer pers. comm.) and G-6-PDH (Sigma pers. comm.).

4.3.3, Combining the ATP sulphurylase reaction and PP_i assay-a way to measure SO_4^{2-}?

Upon establishing that individual components of the SO_4^{2-} reaction step had no effect on the PP_i assay, it was decided to combine the two in an attempt to measure SO_4^{2-}. A 140 μl volume of new assay cocktail was added to each microtitre well. This cocktail contained 2 mM ATP (assay concentration) in addition to components described in **4.3.1**. A 60 μl volume of either 0 or 667 μM K_2SO_4 was added to each well which was stirred before incubation at 23 °C for 10 minutes. After measuring initial absorbencies, 2 μl of UDPG-ppase (assay concentration 3.3 U ml^{-1}) was added and the plate was further incubated for 15 minutes. A second reading was then made before addition of 4 μl ATP sulphurylase (prepared by adding a 100 μl volume of 0.5 % glycerol to 10 mg of ATP sulphurylase (Sigma). Absorbencies were then measured

at 5 minute intervals for a 110 minute period. The plate was returned to the incubator between readings. The results are given in **Figure 4.6**.

Figure 4.6. Combining the SO_4^{2-} reaction and PP_i assay. Initial results from the enzymatic SO_4^{2-} assay at the microtitre plate (200 μl volume) scale. Successive datapoints are joined by a first order regression and for each datapoint, n = 2.



The graph plots for both 0 and 200 μM SO_4^{2-} demonstrate a comparable increase in OD over time. This suggests that coupling the SO_4^{2-} reaction and PP_i assay has led to a substrate-independent (enzyme dependant) reaction between a component(s) of the PP_i assay and one (or more) in the SO_4^{2-} step.

Conclusions

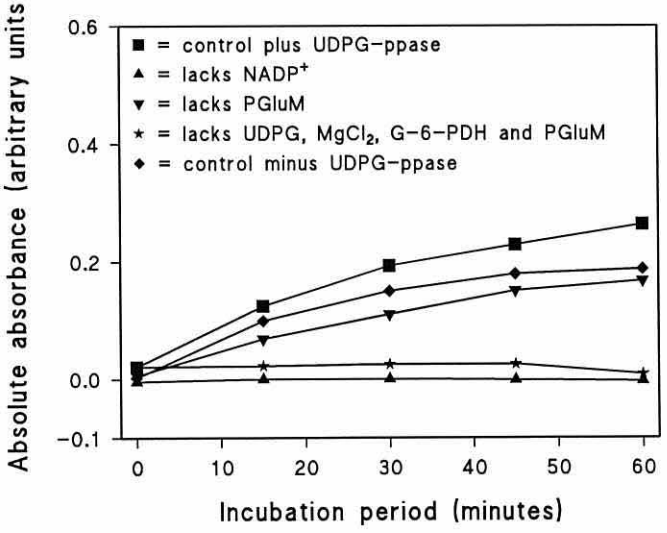
- Combining the SO_4^{2-} step and PP_i assay gave a substrate-independent reaction. Reaction rates were identical for 0 and 200 μM SO_4^{2-}.
- The rate of the substrate-independent reaction was reduced by adding less ATP sulphurylase (results not shown) which suggested that the response was enzyme dependant.

4.3.4, Substrate-independent OD increase is due to reduction of $NADP^+$?

A 140 μl volume of one of four different assay cocktails was added to a microtitre well. Assay cocktail 1 (AC 1) contained all components as described in **4.3.3**. AC 2 lacked $NADP^+$, AC 3 lacked PGluM and AC 4 was a minimal assay mix which contained just Tris buffer, $NADP^+$ and ATP. In all cases, omitted components were replaced with an equal volume of de-ionised water.

Four replicate wells were established for AC 1 whilst two replicates were set up for the remaining three assay cocktails. The only 'substrate' used in this investigation was 0 μM SO_4^{2-}. Upon measuring initial absorbencies, a 2 μl volume of UDPG-ppase was added to two of the four AC 1 wells and to all AC 2, AC 3 and AC 4 wells. Following a 15 minute incubation, absorbencies were read and 4 μl of ATP sulphurylase was added to all wells. Absorbencies were then measured at 15 minute intervals for a total of 60 minutes (**Figure 4.7**).

Figure 4.7. Effects of omission of key PP_i assay components on substrate-independent response obtained when SO_4^{2-} reaction and PP_i assay are combined. For each datapoint, n = 2.



Both omission of $NADP^+$ (AC 2) and use of minimal assay mix (AC 4) halted the substrate-independent response. This would suggest that the increase in absorbance characteristic of the response is due to $NADP^+$ reduction. However, if this reduction had occurred by a direct process, then an increase in absorbance over time in wells containing AC 4 (the minimal assay mix) would have been expected. This was not the case, suggesting that ATP sulphurylase mediates complex reduction of $NADP^+$ by interaction with other components of the PP_i assay. Further evidence of this was supplied by data from AC 3 (PGluM removed) and AC 1 minus UDPG-ppase. In both instances, absorbance values at any one time were lower than in AC 1 wells. This suggests that both enzymes may be involved in the ATP sulphurylase initiated, complex reduction of $NADP^+$.

Conclusions

- The enzyme dependant, substrate-independent reaction was due to reduction of $NADP^+$.
- The reduction process is complex; it is possible that ATP sulphurylase influences more than one of the PP_i assay stages.

4.3.5, Miniaturising the PP_i assay to run at the microfluorometric scale

Upon establishing that the PP_i assay worked at the microtitre (200 μl) scale, it was decided to miniaturise the assay to run at the microfluorometric scale (*circa* 5 nl; a 40000x reduction). This miniaturisation meant that the PP_i assay (and SO_4^{2-} reaction step) would eventually be able to quantify their specific solutes at single cell resolution.

Miniaturisation of an existing enzymatic assay can introduce a number of potential problems (Richardson, 1993) and it has been suggested that the use of small reaction volumes under oil can lead to denaturation of enzymes at the oil-droplet interface (Leese *et al.*, 1984). This leads to a subsequent loss of activity (Richardson, 1993) which has been overcome in the past, by increasing enzyme concentrations in small-scale assays for both nucleotides (by 10 times; Leese *et al.*, 1984) and nitrate (by 20 times; Zhen *et al.*, 1991). Because of this, enzyme concentrations in the microfluorometric enzymatic assay had to be greatly increased over those used in the microtitre experiments to obtain a response (results not shown).

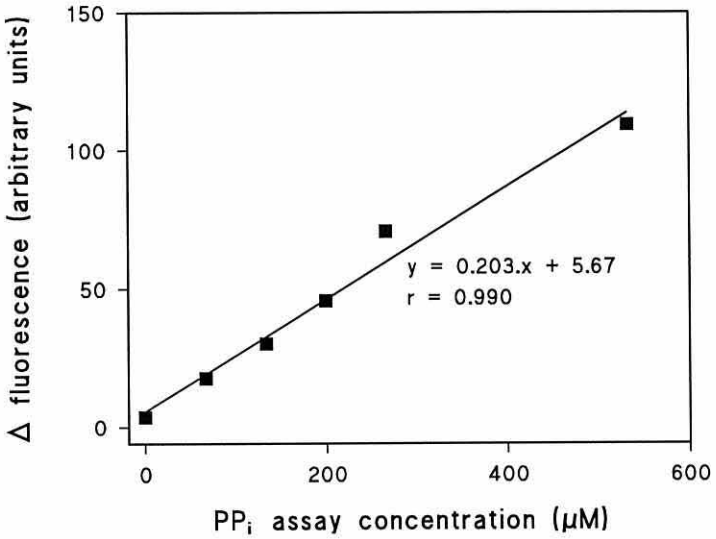
4.3.6, Final protocol for PP_i microfluorometric enzymatic assay

A 100 μl volume of assay cocktail was prepared by mixing 15 μl of 300 mM Tris-HCl (pH 8.0; found to be optimum when PP_i assay performance was compared in 300 mM Tris-HCl buffers from pH 7.4-pH 8.2 (results not shown), 15 μl 52 mM $MgCl_2$, 15 μl of 7.4 U ml^{-1} G-6-PDH, 7.5 μl of 30 mM UDPG, 9.5 μl of 143 mM $NADP^+$, 28 μl of 22.5 U ml^{-1} PGluM and 10 μl 1% w/v BSA.

Approximately 5 μl of assay cocktail, each of the following PP_i standards (0, 25, 50, 75, 100 and 200 mM $Na_2P_2O_4$) and 33.2 U ml^{-1} UDPG-ppase were transferred using a 50 μl Hamilton glass syringe to the edge of a microscope slide-mounted well (thoroughly cleaned as described by Richardson, 1993) filled with water-saturated liquid paraffin.

Under a binocular microscope with transmitted illumination (Nikon 0.8-4.0 zoom objective fitted with 20x eyepieces) the required number of assay cocktail droplets (minimum of two replicates per standard) were measured out into the centre of the well using a *circa* 4 nl non-silanized micro-constriction pipette. Spacing was such that droplets were centred at intervals of twice their diameter in order to avoid interference between neighbouring droplets when measuring fluorescence. Standards were added using a *circa* 12 pl silanized micro-constriction pipette. The slide-mounted well was transferred to the stage of an inverted microscope fluorimeter (Leitz Fluovert with MPV Compact 2 and MPV software), linked to a Vig 1 PC (Viglen). The microscope was fitted with Leitz filter block A (excitation at 270-380 nm, emission at 410-580 nm). The mercury lamp was switched on at least 30 minutes before initial readings were taken and excitation and emission windows were set such that they slightly exceeded the size of the droplet. Reference values for the oil (allowing a check to be made on the stability of the lamp) and each droplet were obtained before returning the slide-mounted well to the binocular microscope. Reaction was started by addition of a *circa* 500 pl aliquot of 33.2 U ml^{-1} UDPG-ppase added to each of the assay cocktail plus standard droplets using a non-silanized micro-constriction pipette. The mix was gently stirred using the tip of the micropipette and simultaneously, a stopwatch was started to time the progress of the reaction. Fluorescence intensity of the droplets was measured every 5 minutes until reaction was complete (**Figure 4.8**).

Figure 4.8. Calibration curve for the PP_i microfluorometric enzymatic assay obtained from a plot of change in fluorescence *versus* PP_i concentration. Reaction had reached completion after 30 minutes (results not shown). For each datapoint, n = 2.



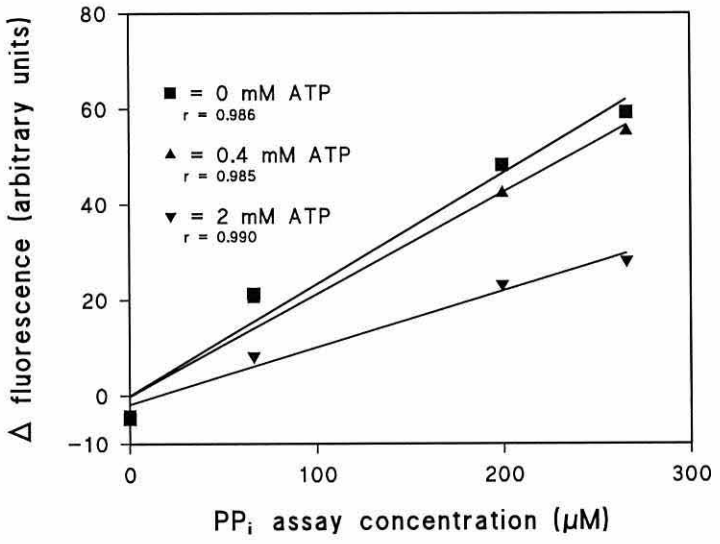
Conclusions

- Enzyme concentrations in the microfluorometric PP_i assay had to be much higher than those used in the microtitre system before a response was obtained.
- A new microfluorometric enzymatic assay (for PP_i) was established.

4.3.7, High concentrations of ATP inhibit the PP_i microfluorometric assay

Experiments described in **4.3.2** demonstrated that the PP_i assay could be inhibited in the presence of high concentrations of ATP. The objective of this experiment was to confirm these observations and determine the maximum ATP concentration which would still permit a fully functioning PP_i microfluorometric enzymatic assay. The opportunity was also taken to incorporate UDPG-ppase into the assay cocktail (rather than adding it separately as the 'initiating factor') since it was envisaged that in the proposed microfluorometric sulphate assay, the separate addition of a *circa* 500 pl volume of ATP sulphurylase would be used to start the reaction. Three new assay cocktails were designed which included UDPG-ppase (assay concentration 36.6 U ml^{-1} as previous) and ATP at concentrations of 0, 0.4 and 2 mM. Reactions were started by addition of either 0, 25, 75 or 100 mM PP_i standards (**Figure 4.9**).

Figure 4.9. The effects of ATP concentration on the PP_i microfluorometric enzymatic assay. Reaction had reached completion after 30 minutes. For each datapoint, n = 1.



Adding 2 mM ATP to the assay mix impaired performance of the PP_i microassay (see **Figure 4.9**). In contrast, addition of 2 mM ATP had little effect on the PP_i assay when run at the 200 μl microtitre scale (see **4.3.2**). Addition of 0.4 mM ATP had no appreciable effect on assay performance. Whilst this experiment was by no means exhaustive with respect to the number of different ATP concentrations tested, it would seem that 0.4 mM ATP is optimum for future development work.

Conclusions

- The optimal PP_i microfluorometric enzymatic assay ATP concentration is 0.4 mM. This represents a good compromise between a functioning PP_i microassay and a sufficient excess for the SO_4^{2-} reaction step.
- Incorporating UDPG-ppase into the assay cocktail was not detrimental to performance of the PP_i assay.

4.3.8, Combining PP_i microfluorometric assay and SO_4^{2-} reaction; is there a substrate-independent OD increase over time?

Assay cocktail was produced as outlined in **4.3.7**. Either 0 or 100 mM SO_4^{2-} was added to microdroplets, fluorescence was measured and a *circa* 500 pl volume of ATP sulphurylase (2.5 μl of 0.5 % glycerol plus 0.5 mg sulphurylase lyophilisate) was then added. There was a comparable slow increase in fluorescence over time in microdroplets containing either water or SO_4^{2-} substrate (results not shown).

Conclusion

- The substrate-independent reaction first observed in the 200 μl microtitre SO_4^{2-} assay also occurred in the miniaturised microfluorometric enzymatic assay.

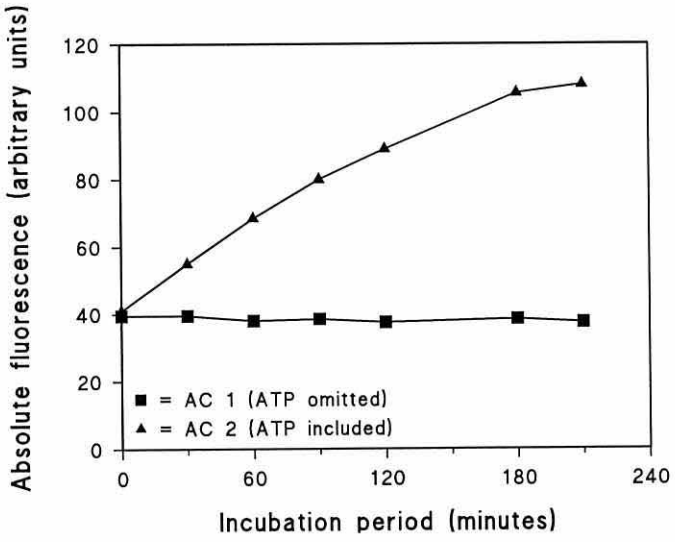
4.3.9, The substrate-independent response involves ATP

Two assay cocktails were produced. Assay cocktail 1 lacked ATP whilst assay cocktail 2 contained the optimum 0.4 mM ATP (see **4.3.7**). The only 'substrate' used in the experiment was 0 mM SO_4^{2-}. After adding ATP sulphurylase, there was no detectable change in fluorescence over time in the AC 1 microdroplets (which lacked ATP). In comparison, fluorescence increased in the AC 2 microdroplets (which contained ATP) throughout the entire 230 minute incubation period of this experiment (see **Figure 4.10**).

Conclusion

- The substrate-independent response involves ATP.

Figure 4.10. The presence of ATP in a reaction mixture appears to determine whether or not the substrate-independent response obtained from combining the PP_i microfluorometric enzymatic assay and SO_4^{2-} reaction will occur. For each datapoint, n = 2.

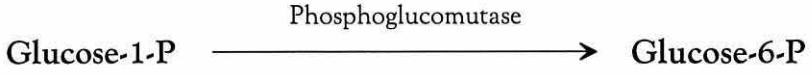


4.3.10, Which stages of the PP_i assay are affected by ATP sulphurylase?

A series of experiments were carried out to ascertain which of the three PP_i assay reaction steps (see **Figure 4.1**) was most affected by ATP sulphurylase. Three assay cocktails were prepared. In assay cocktail 1 (AC 1), UDPG, PGluM and UDPG-ppase were omitted to enable the effects of ATP sulphurylase on the third enzyme reaction of the PP_i assay (involving G-6-PDH) to be determined.

$$\text{Glucose-6-P} + \text{NADP}^+ \xrightarrow{\text{G-6-PDH}} \text{Gluconate-6-P} + \text{NADPH} + \text{H}^+$$

In assay cocktail 2 (AC 2), UDPG and UDPG-ppase were omitted so that any effect of ATP sulphurylase on PGluM could be determined. This enzyme catalyses conversion of:

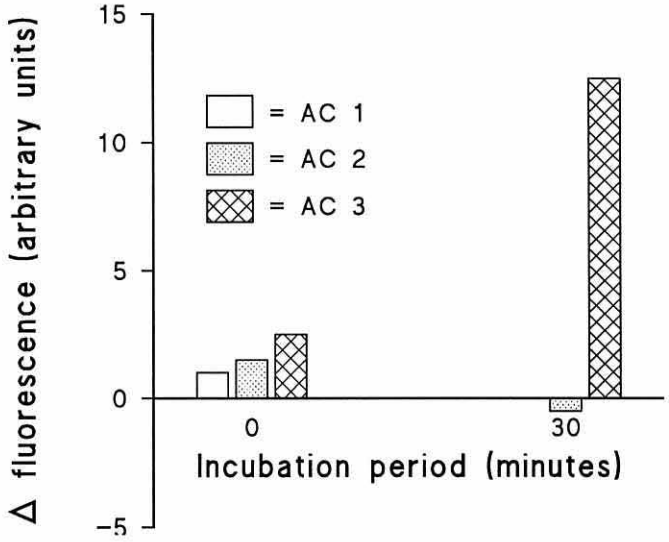


There were no omissions from assay cocktail 3 (AC 3) which contained all components as described in **4.3.6**. The inclusion of AC 3 in this investigation meant that the effects of ATP sulphurylase on the complete PP_i assay could be determined. In all cases, omitted assay components were replaced with de-ionised water. In this experiment, the addition of substrate to microdroplets was considered unnecessary.

Following addition of ATP sulphurylase, there was no slow increase in fluorescence in AC 1 or AC 2 microdroplets with time. The slow increase in fluorescence with time was, however, evident in AC 3 microdroplets (see **Figure 4.11**).

These observations would suggest that the substrate-independent response does not represent an interaction between ATP sulphurylase and G-6-PDH (AC 1) or ATP sulphurylase and a combination of PGluM and G-6-PDH (AC 2). Results from AC 3 indicate that the substrate-independent response could originate either from an interaction solely between ATP sulphurylase and UDPG-ppase or ATP sulphurylase and a combination of UDPG-ppase, PGluM and G-6-PDH. In a subsequent experiment, the substrate-independent response was shown to be due to interaction solely with UDPG-ppase (results not shown).

Figure 4.11. The effects of ATP sulphurylase on the individual stages of the PP_i assay. Plots for AC 2 and AC 1 demonstrate no interaction between ATP sulphurylase and PGluM or G-6-PDH respectively. The plot for AC 3 shows that interaction occurred between UDPG-ppase and ATP sulphurylase. For each datapoint, n = 2.



Conclusions

- Most of the substrate-independent response was due to an interaction between ATP sulphurylase and UDPG-ppase, leading to reduction of $NADP^+$.

4.3.11, Choice of assay buffer is responsible for non-working SO_4^{2-} assay

When the SO_4^{2-} reaction and PP_i assay were combined, the only measurable response obtained at both microtitre (200 μl) and microfluorometric (*ca.* 4 nl) scales was that of the substrate-independent reaction. The expected response from conversion of 100 mM SO_4^{2-} through 100 mM PP_i to eventual reduction of 100 mM $NADP^+$ failed to materialise. Two possible hypotheses were proposed to explain the experimental observations.

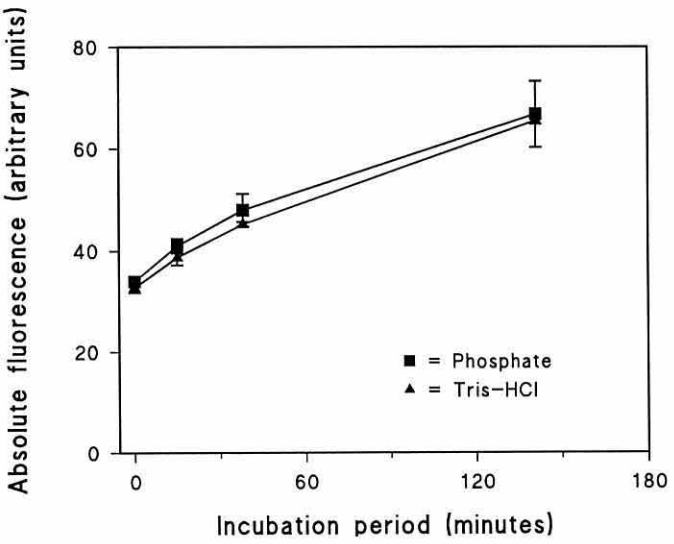
1, Reaction conditions prevent SO_4^{2-} assay from functioning correctly.

2, Unfavourable reaction kinetics prevent SO_4^{2-} assay from functioning.

Certain ATP sulphurylase preparations are known to be unstable in Tris-HCl buffer (Levi and Wolf, 1969) and an experiment was designed to test the hypothesis that incorrect choice of assay buffer was responsible for the non-working SO_4^{2-} assay. Two assay cocktails were produced. Assay cocktail 1 (AC 1) was based upon 40 mM (assay concentration) Tris-HCl (as in previous development work). AC 2 was based upon 13 mM $Na_2HPO_4^{2-}$-0.7 mM $NaH_2PO_4^-$ (BDH; as Dawson *et al.*, 1986). Phosphate buffer was chosen for this comparison because of its stabilising effects on ATP sulphurylase (Levi and Wolf, 1969).

This comparison test between Tris-HCl and phosphate buffers demonstrated that buffer choice had no effect on the SO_4^{2-} assay (see **Figure 4.12**). Again, the only measurable response was the slow increase in fluorescence over time which was identical between the two buffers.

Figure 4.12. The effects of buffer choice on the SO_4^{2-} assay. For each datapoint, n = 4 ± SD.



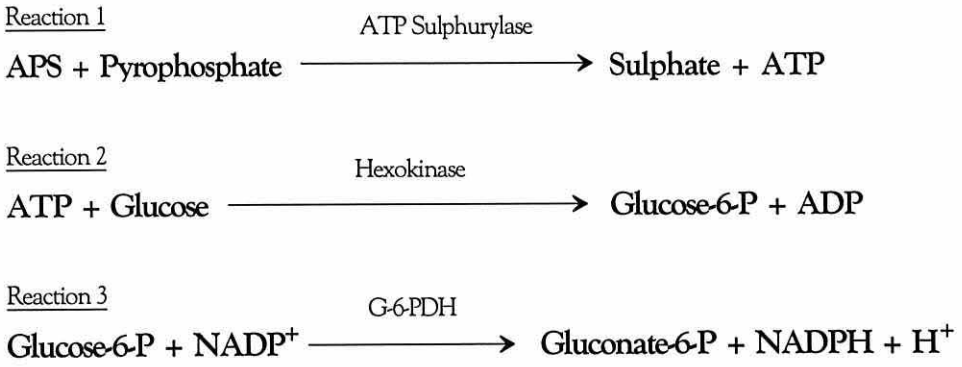
Conclusions

- Substitution of Tris-HCl with phosphate buffer had no effect on the substrate-independent reaction.

4.3.12, The ATP sulphurylase preparation has no enzymatic activity

By adapting the manufacturers quality control test procedure (*i. e.* running the assay in the opposite direction; Sigma, pers. comm.), an experiment was designed to demonstrate that the ATP sulphurylase preparation had enzymatic activity; an important characteristic which development work had so far failed to show. The assay stages are outlined in **Figure 4.13**.

Figure 4.13. The reaction pathway for the quality control test procedure for ATP sulphurylase comprises three separate reaction stages. In the first (Reaction 1), ATP sulphurylase catalyses formation of SO_4^{2-} and ATP from APS and PP_i (working in the opposite direction to that required in the SO_4^{2-} assay). ATP formed is then converted into ADP by hexokinase (HK; Reaction 2) whilst glucose is phosphorylated to form glucose-6-phosphate (G-6-P). The enzyme G-6-PDH (Reaction 3) then oxidises G-6-P to gluconate-6-P with subsequent reduction (and corresponding increase in absorbance) of $NADP^+$.

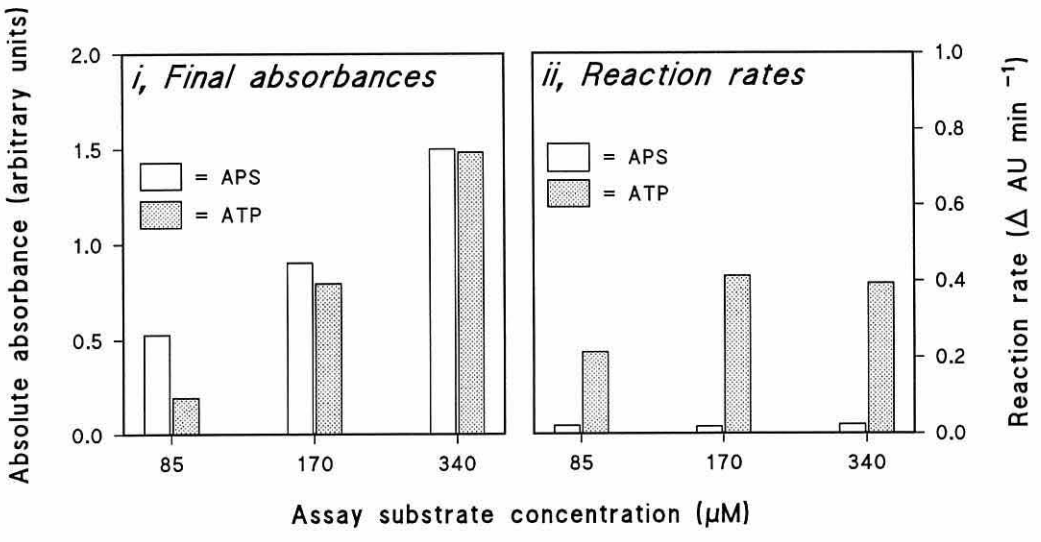


A 10 ml volume of assay cocktail was prepared by mixing 3.4 ml of 400 mM Tris-acetate (pH 8.0; Boehringer), 34 μl of 200 mM $NADP^+$ (Boehringer), 28 μl of 1 M $MgCl_2$ (BDH) and 6.5 ml de-ionised water. Before use, pH was re-measured and corrected to pH 8.0 as required. A 2.5 ml volume of this cocktail was then transferred to a quartz glass cuvette (1 cm pathlength, 3 ml volume) into which 100 μl each of 1 M glucose and a combined 40 U ml^{-1}/20 U ml^{-1} HK/G-6-PDH mix (Boehringer) were

added. Following addition of 50 μl 20 mM APS (assay substrate; assay concentration 340 μM; Sigma) and 100 μl 0.6 U ml^{-1} ATP sulphurylase, the contents of the cuvette were mixed and absorbance was monitored spectrophotometrically at 340 nm (Varian DMS 100S) until constant.

The assay was started by addition of 100 μl of 100 mM PP_i. Absorbance was measured and recorded continuously until reaction had reached completion. The experiment was repeated with lower concentrations of APS (50 μl of either 10 mM or 5 mM APS; assay concentrations 170 μM and 85 μM respectively) before APS was replaced altogether by identical concentrations of ATP (in these modified assays, reaction was started by adding 100 μl of 1 M glucose). Comparison of data from APS and ATP assays enabled a measure to be made of the efficiency with which ATP sulphurylase catalysed formation of ATP (see **Figure 4.14**).

Figure 4.14. Evaluating the performance of ATP sulphurylase using the quality control assay. Graph (i) shows the final absorbance values upon reactions reaching completion as obtained from three different assay concentrations of APS and ATP. In APS assays, NADPH formed as a result of reactions 1, 2 and 3 proceeding sequentially (see **Figure 4.13**). Assay concentrations of ATP were identical to those for APS but in ATP assays, NADPH formed as a result of reactions 2 and 3. Reaction 1 played no part in this assay. Graph (ii) shows the difference in initial reaction rates between the two assays. For each datapoint, n = 1.



With the exception of the difference in final absorbance between the 85 μM APS/ATP result (most likely to be attributable to experimental error), both APS and ATP assays gave similar final absorbance values for the three substrate concentrations (**Figure 4.14**). This means that ATP sulphurylase does have enzymatic activity and is capable of catalytic function. However, whilst the values for final absorbance are similar, it took on average 10 times longer for the APS assay to reach end-point when compared with data from the ATP assay (in which the APS to ATP conversion involving ATP sulphurylase was by-passed). It was assumed that the difference in reaction rate could have been reduced by adding further ATP sulphurylase.

Conclusions

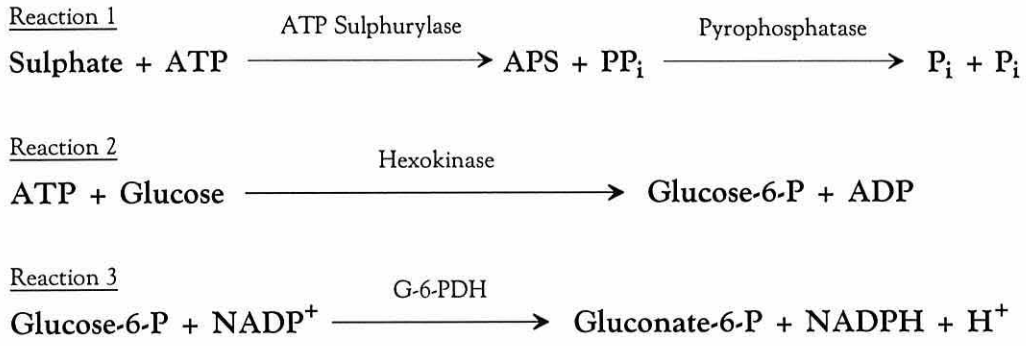
- The ATP sulphurylase preparation had catalytic ability.
- The reaction rate of the APS assay was slower than that of the ATP assay. This may have been due to differences in concentration of enzymes catalysing the first reaction step (0.02 U ml^{-1} ATP sulphurylase in APS assay *versus* 1.36 U ml^{-1} HK in ATP assay).

4.3.13, The SO_4^{2-} assay does not work due to unfavourable reaction kinetics

Once it had been shown that the ATP sulphurylase preparation possessed enzymatic activity (see **4.3.12**), the only remaining and untested explanation for the non-functioning SO_4^{2-} assay was that of unfavourable thermodynamics. There are published accounts of where ATP sulphurylase has been used to convert ATP into APS and PP_i. For example, Levi and Wolf (1969) purified ATP sulphurylase from rat liver and determined the enzymatic activity of their preparation by measuring the synthesis of labelled APS and 3'-phosphoadenosine-5'-phosphosulphate (PAPS) from ATP and $^{35}SO_4^{2-}$. The reaction was made to favour APS synthesis by inclusion of 0.1 U ml^{-1} pyrophosphatase, which hydrolysed the PP_i formed and prevented reversal of the reaction. When pyrophosphatase was omitted, enzymatic synthesis of APS was not observed (Levi and Wolf, 1969).

An alternative SO_4^{2-} assay mechanism was proposed to test the hypothesis that UDPG-ppase was not hydrolysing sufficient PP_i to force ATP sulphurylase to catalyse the forward reaction. The individual stages of the alternative SO_4^{2-} assay are described in **Figure 4.15**.

Figure 4.15. Individual reaction steps of the alternative SO_4^{2-} assay. In Reaction 1, SO_4^{2-} and ATP are converted into APS and PP_i in the presence of pyrophosphatase which immediately lyses the PP_i formed to prevent reversal of the ATP sulphurylase catalysed reaction. The reaction was then stopped by immersion in boiling water and remaining ATP was determined with a HK/G-6-PDH enzyme mix. In Reaction 2, HK catalyses formation of G-6-P and ADP from the remaining ATP and excess glucose. G-6-PDH (Reaction 3) then oxidises G-6-P to form gluconate-6-P with subsequent reduction (and corresponding increase in absorbance) of $NADP^+$.



Two separate assay systems were required. One for the ATP sulphurylase/pyrophosphatase step and the other for the assay of ATP. The ATP assay was carried out on the microtitre system. Assay cocktail contained 1.0 ml of 400 mM Tris-HCl (pH 8.0), 1.0 ml of 4 mM $NADP^+$ and 1.0 ml of 17 mM $MgCl_2$. At this stage, pH was re-measured and corrected to pH 8.0. Further 1.0 ml additions each of 242 mM glucose and a combined 9.4 U ml^{-1}/4.7 U ml^{-1} HK/G-6-PDH mix were then made. A 140 μl volume of this assay cocktail was transferred to the well of a microtitre plate and reaction was started by a 60 μl addition of 0, 0.3, 0.6, 0.9 or 1.25 mM ATP standard. A thorough evaluation of the ATP assay was necessary to establish:

1, maximum assay concentrations of ATP that could be measured (1.25 mM; results not shown)

2, the time required for the assay to reach completion (12 minutes; results not shown)

3, the effects of heating ATP at 100° C for 0.5, 1, 1.5, 2 or 10 minutes (no effect; results not shown).

Upon completion of evaluation, three further assay cocktails were prepared. Components are listed in **Table 4.1**. Three Eppendorf tubes were labelled AC 1, AC 2 and AC 3 and components (with the exception of SO_4^{2-}) were added as described in **Table 4.1**. The Eppendorf tubes were transferred to a waterbath at 30 °C and their contents were allowed to equilibrate for 5 minutes before addition of 200 μl of 1.4 mM SO_4^{2-}. Each reaction was then run for a further 30 minutes. A 100 μl aliquot of reaction mix was removed every 5 minutes and placed in boiling water for 120 seconds. The aliquot was then allowed to cool for a further 60 seconds before a 60 μl sub-sample was transferred to the well of a microtitre plate containing 140 μl ATP assay cocktail. The concentration of ATP was then determined using the ATP enzymatic assay as described previously.

There was no decrease in ATP concentration over time in either AC 1 (UDPG-ppase) or AC 2 (pyrophosphatase). This suggested that the ATP sulphurylase catalysed conversion of SO_4^{2-} plus ATP to APS and PP_i was still not occurring (results not shown). At this stage, it was decided that all development work on the SO_4^{2-} enzymatic assay should cease.

Conclusions

- The lack of response from the SO_4^{2-} enzymatic assay (even when pyrophosphatase was used to pull over the reaction equilibrium) was most likely attributable to unfavourable reaction thermodynamics.
- In previous work (Levi and Wolf, 1969), both pyrophosphatase and APS kinase were used to remove PP_i and APS respectively. From the current work, it would seem that pyrophosphatase alone is unable to pull over the ATP sulphurylase reaction equilibrium.

Table 4.1. The components of the three assay cocktails used in the comparison tests of UDPG-ppase with pyrophosphatase. Each assay cocktail contained a 200 μl volume of the following components. Final assay volume was 1.4 ml.

AC 1 (UDPG-ppase converts PP_i + UDPG to G-1-P + UTP)	**AC 2** (pyrophosphatase converts PP_i to P_i + P_i)	**AC 3** (Blank, no PP_i specific enzymes)
280 mM Tris-HCl	280 mM Tris-HCl	280 mM Tris-HCl
14 mM UDPG	De-ionised water	De-ionised water
2.8 mM ATP	2.8 mM ATP	2.8 mM ATP
49 mM $MgCl_2$	49 mM $MgCl_2$	49 mM $MgCl_2$
136.5 U ml^{-1} UDPG-ppase	136.5 U ml^{-1} pyrophosphatase	De-ionised water
ATP sulphurylase (600 μl 50 % v/v glycerol added to 0.5 mg lyophilisate)	ATP sulphurylase (600 μl 50 % v/v glycerol added to 0.5 mg lyophilisate)	ATP sulphurylase (600 μl 50 % v/v glycerol added to 0.5 mg lyophilisate)
1.4 mM SO_4^{2-}	1.4 mM SO_4^{2-}	1.4 mM SO_4^{2-}

4.4, DISCUSSION

This chapter has outlined a series of experiments carried out in an attempt to develop an enzymatic assay for SO_4^{2-}. By design, this assay was to have been sufficiently sensitive to quantify the minute amounts of SO_4^{2-} in extracted single cell vacuolar saps. The only measurable response obtained was the substrate-independent reaction; a slow increase in optical density over time, comparable in blank and SO_4^{2-}-containing assay mixes. The reason for this response is thought to have been due to some kind of interaction between ATP, ATP sulphurylase and UDPG-ppase (see **4.3.10**). In isolation, this substrate-independent response represented a problem. However, had the expected response (as resulting from conversion of mM concentrations of SO_4^{2-} to mM concentrations of NADPH) been observed, then by comparison, the contribution of this substrate-independent interaction would have been negligible.

4.4.1, Why did the enzymatic SO_4^{2-} enzymatic assay fail to work?

The standard free energy change ($\Delta G^{o'}$) for a reaction (as defined by a biochemist) is the "change in free energy of that reaction as it undergoes transformation at 25 °C, and pH 7.0. Each reactant is present at a concentration of 1.0 M and transformation occurs at 1 atmosphere" (Stryer, 1995). The $\Delta G^{o'}$ value for a reaction is related to the equilibrium constant (K_{eq}) for that reaction and the K_{eq} is derived from the ratio of forward rate constant : reverse rate constant. Whilst the presence of an enzyme can accelerate the rate of a reaction, K_{eq} remains unchanged since catalysis of the forward and reverse reactions occurs equally (Stryer, 1995).

The $\Delta G^{o'}$ for conversion of SO_4^{2-} plus ATP to APS and PP_i is 33.5 kJ mol^{-1} (Robbins and Lipmann, 1958). The reaction equilibrium lies far to the left and thermodynamically favours formation of SO_4^{2-} and ATP (Daley *et al*, 1986). This large positive $\Delta G^{o'}$ value means that only a small concentration of SO_4^{2-} and ATP will be converted into APS and PP_i. There are, however, actual examples where the reaction equilibrium has been pulled over to favour the synthesis of APS and PP_i (see for example, Robbins and Lipmann, 1958; Levi and Wolf, 1969). In both cases, two coupling enzymes were required before the forward reaction would proceed to any reasonable extent (Greenberg, 1975) and the inclusion of both pyrophosphatase and APS kinase was necessary to remove PP_i and APS respectively from the assay mix

(Levi and Wolf, 1969). Use of pyrophosphatase alone is insufficient to pull over the reaction equilibrium to favour APS and SO_4^{2-} formation (Hell, 1997).

The $\Delta G^{o'}$ for the hydrolysis of PP_i by pyrophosphatase is -29 kJ mol^{-1} (Dawson *et al*, 1986). The $\Delta G^{o'}$ for the conversion of PP_i (plus UDPG) into UTP and G-1-P by UDPG-ppase is 2.902 kJ mol^{-1} (Turnquist and Hansen, 1973). The $\Delta G^{o'}$ for the coupled reactions in which ATP sulphurylase synthesises APS and PP_i in the presence of either pyrophosphatase or UDPG-ppase can be calculated from equation (1) where,

$$\Delta G^{o'}_{\text{APS synthesis}} - \Delta G^{o'}_{\text{PPi hydrolysis}} = \Delta G^{o'}_{\text{Coupled reaction}} \qquad (1)$$

If the known $\Delta G^{o'}$ are substituted into equation (1) then:

i, Using pyrophosphatase as coupling enzyme,

$$33.5 \text{ kJ mol}^{-1} - 29 \text{ kJ mol}^{-1} = 4.5 \text{ kJ mol}^{-1}$$

ii, Using UDPG pyrophosphorylase as coupling enzyme,

$$33.5 \text{ kJ mol}^{-1} + 2.9 \text{ kJ mol}^{-1} = 36.4 \text{ kJ mol}^{-1}$$

Neither pyrophosphatase nor UDPG-ppase has a sufficiently negative $\Delta G^{o'}$ to cancel out the large positive $\Delta G^{o'}$ of the SO_4^{2-} reaction and the final $\Delta G^{o'}_{\text{Coupled reaction}}$ for both pyrophosphatase and UDPG-ppase catalysed systems remain positive. The $\Delta G^{o'}_{\text{Coupled reaction}}$ value for the UDPG-ppase system however, was most positive.

The relationship between $\Delta G^{o'}$ and K_{eq} is shown in equation (2).

$$\Delta G^{o'} = - RT\ln K_{eq} \qquad (2)$$

If equation (2) is then re-arranged to make K_{eq} the subject, then,

$$K_{eq} = e.^{-\left(\frac{\Delta G^{o'}}{RT}\right)} \qquad (3)$$

Finally, if the $\Delta G^{o'}_{\text{Coupled reaction}}$ values calculated from equation (1) are substituted into equation (3) then:

i, Using pyrophosphatase as coupling enzyme,

K_{eq} = 0.16

ii, Using UDPG pyrophosphorylase as coupling enzyme,

K_{eq} = 4.22×10^{-7}

Since K_{eq} equals the ratio of the forward rate constant : reverse rate constant, then it follows that K_{eq} is also equal to the ratio of product : substrate. If a reaction has a large K_{eq}, there will be a substantially larger amount of product present compared to substrate in a reaction mix at equilibrium. Conversely, a small K_{eq} ($K_{eq} < 1.0$) indicates the presence of larger amounts of substrate compared to product at equilibrium.

K_{eq} values for both pyrophosphatase and UDPG-ppase coupled reaction systems were less than one and at equilibrium, the concentrations of SO_4^{2-} and ATP in the assay mix would be much higher than those of P_i (pyrophosphatase) or UTP and G-1-P (UDPG-ppase). A K_{eq} of 0.16 for the pyrophosphatase-coupled system in conjunction with published accounts of using this enzyme to remove PP_i and pull over the SO_4^{2-} reaction (to favour APS synthesis) suggests that it is this system which shows the most promise as a potential future SO_4^{2-} enzymatic assay. However, the K_{eq} value for the pyrophosphatase-coupled system is so small that any decrease in ATP due to ATP sulphurylase could well lie beyond the resolution of a microfluorometric ATP enzymatic assay. Bearing in mind the K_{eq}, it is predicted that determination of even very high vacuolar concentrations of SO_4^{2-} could result in only a 16 % decrease in the ATP concentration; assumedly, such a small signal would be difficult (or even impossible) to detect against the degree of noise and error associated with the system. In contrast, the near negligible K_{eq} of the UDPG-ppase coupled reaction suggests that this enzyme would never have been able to pull over the SO_4^{2-} reaction to favour APS synthesis. The proposed reaction mechanism described in **Figure 4.1** and subsequently tested in the experiments described in this chapter would never have worked; the assay mechanism is just too thermodynamically unfavourable.

Whilst accepting the thermodynamic explanation for the failure of the UDPG-ppase coupled reaction system, one wonders why the pyrophosphatase-coupled SO_4^{2-} enzymatic assay (as described in **4.3.13**) failed to work. At least in thermodynamic terms, pulling the SO_4^{2-} reaction over to favour

APS synthesis was well within the capabilities of this enzyme and the most probable reason for the lack of measured response was the omission of APS kinase (*cf.* Levi and Wolf, 1969). The second assay was set-up only as a benchmark against which the performance of the UDPG-ppase coupled system could be compared. This meant that development work on the pyrophosphatase system was very limited and it is certainly possible that with more time investment, a detectable response should have been obtained.

Further problems may have been arisen from insufficient activity (and purity) of the ATP sulphurylase preparation. This enzyme is supplied as a lyophilisate which makes removal of minute quantities for single assays difficult. Lyophilisates are strongly hygroscopic and repeated removal of small quantities of enzyme could have had potentially damaging effects on the remaining preparation as damp lyophilisates are inherently unstable (Keesey, 1987). A related problem was knowing exactly how much ATP sulphurylase was added to the assay since enzyme activity (in units ml^{-1}) was published only for the reverse direction. In this case, 1 unit would produce 1 μmole of ATP from APS and PP_i per minute at pH 8.0 and 30 °C.

Taking into account a potentially damaged enzyme and an unknown activity, it is not inconceivable that the assay concentration (and hence assay activity) of ATP sulphurylase may have been sub-optimal. It is thought that the potential problems associated with weighing out small quantities of lyophilisate were eventually solved by reconstituting the entire preparation before freeze-drying into a number of individual Eppendorf tubes. Nevertheless, the unexpectedly slow reaction rate as demonstrated by the manufacturers quality control test procedure (see **4.3.12)** suggests that not all problems had been solved.

It is most likely that a combination of unfavourable reaction thermodynamics and sub-optimal ATP sulphurylase concentration were ultimately responsible for the non-functioning pyrophosphatase-coupled enzymatic assay. It could be argued that further time investment and use of a more highly purified ATP sulphurylase preparation could have resulted in a response from the assay. However, the very nature of the reaction thermodynamics suggest that the response (*i.e.* a decrease in ATP concentration) accompanying conversion of sub 50 mM concentrations of SO_4^{2-} from vacuolar saps could be so small as to be virtually immeasurable. Unfortunately

no amount of time investment or enzyme purification will ever change reaction thermodynamics.

4.5, CONCLUSIONS

- The development of an enzymatic assay for SO_4^{2-} was attempted but with little success. Neither UDPG-ppase nor pyrophosphatase seemed able to pull over a reaction in which SO_4^{2-} and ATP were converted into APS plus PP_i. Unfavourable reaction thermodynamics were thought to be the cause in both cases.

- Experimental objective (see **4.2**) was not met but lessons learned in enzymatic assay development proved invaluable when an enzymatic assay for NO_3^- was improved (see **6.4**).

Chapter 5:

WILL WHEAT LEAVES ACCUMULATE SULPHUR?

5.1, EXPERIMENTAL OBJECTIVES

The experiment described in this chapter was designed to address three main objectives. These were:

1, *Will S accumulate to significant concentration in the upper epidermis and whole leaf of wheat when plants are supplied with the appropriate ratio of S : N?*

2, *What effects may this epidermal accumulation have on cellular water relations and the behaviour of other vacuolar solutes?*

3, *How may the profile of solutes in the upper epidermis and whole leaf change as leaf age increases?*

5.2, INTRODUCTION

A plant accumulates sulphur when it is available in amounts exceeding those required for optimal plant growth (Singh and Singh, 1977; Olivares and Aguiar, 1996). This occurs either naturally (when plants grow in volcanic or coastal areas; Moss, 1978) or because of man, whose extensive combustion of fossil fuels leads to high atmospheric concentrations of SO_2 (Rennenberg, 1984).

The excess sulphur is stored within the cell vacuole in the form of inorganic SO_4^{2-} (De Kok, 1990). Cytoplasmic concentrations, by contrast, generally remain negligible (Rennenberg, 1984). Sulphur accumulation can also lead to a much smaller increase in organic-S compounds (Faller *et al.*, 1970) with most of this increase being attributable to raised glutathione content (Rennenberg, 1984). Accumulation of S can lead to severe and harmful changes in plant cell metabolism (Rennenberg, 1984). This would suggest that vacuolar storage of excess S may lead to gross changes in the behaviour of other vacuolar solutes with subsequent effects on water relations parameters (most notably turgor and osmotic pressures).

In this experiment, plants were grown in Long Ashton media containing either 1 : 8, 1 : 1 or 8 : 1 S : N (control S (CON), equi S (EQUI) or high S (INV) respectively) with the aim of determining whether S would accumulate within the leaf and what effects this accumulation may have on other cell solutes. The upper epidermis of graminaceous plants is composed of anatomically different cell types (see

Plate 7) and although each of these is known to differ in its solute composition (Fricke *et al.*, 1995), analyses were confined to the bulliform trough cells due to their relative ease of sampling. For this study, it was reasoned that the quantification of major anions in bulk tissue sap preparations from SiCSA-sampled plants would provide a suitable reference against which single cell work could be compared.

5.3, METHOD

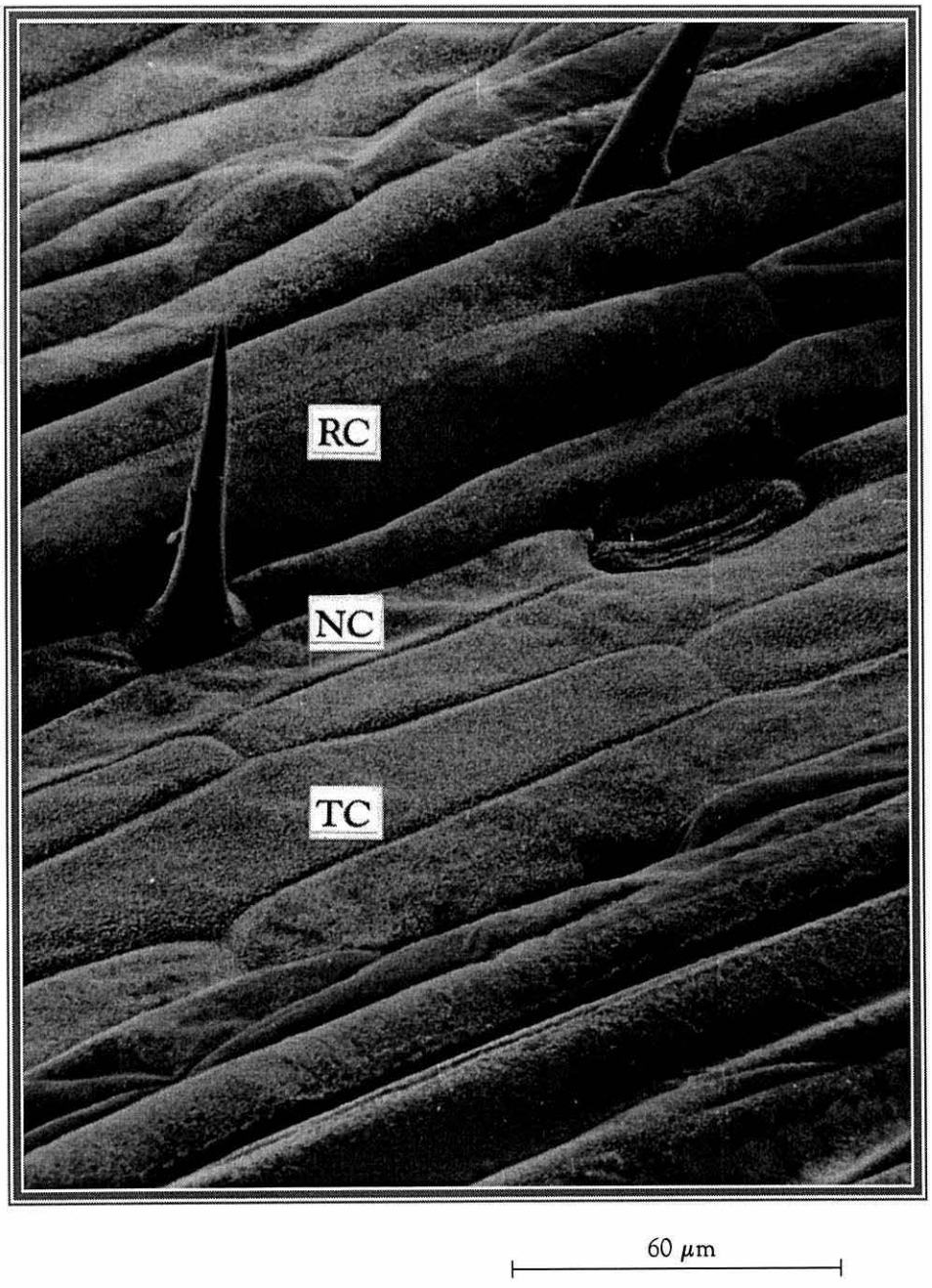
Two 3 l tubs were set up (see **2.2** for further details) for each of the three nutrient treatments. **Table 5.1** gives the composition of the control S (CON), equi S (EQUI) and high S (INV) Long Ashton growth solutions used in this experiment. Analyses began when the third leaf was approximately 2 days from full expansion and then continued every three days until the leaf had reached advanced stages of senescence (as characterised by desiccation and gross structural deformation). At each sampling point, three plants were removed from the two growth tubs per nutrient treatment and single cell vacuolar sap samples were taken from epidermal trough cells (see **2.4.1**) of the third leaf in a region 15.7 cm down from the tip (the 'statistical middle'). All of the cells sampled were situated between the 4 th and 5 th ridge on the left-hand side of the main vein. Five epidermal trough cells were sampled per plant and osmotic pressure and electrolyte concentrations were determined by picolitre osmometry (see **2.4.2**) and X-ray microanalysis (see **2.4.3**) respectively. Once single cell sampling requirements had been met, a bulk tissue sap preparation was obtained as described in **2.3.2.** This was then frozen at -20 °C pending subsequent quantification of anion concentrations by HPLC (see **2.3.4.1**).

A 1.0 ml growth medium sample was removed daily from each of the six tubs and frozen at -20 °C pending quantification of anion concentrations by HPLC (see **2.3.4.3**). In addition, daily records of growth medium pH were collected (results not shown) and the pH was corrected with 6 M HCl or 10 M NaOH (as appropriate) if outside the range of 6.1-6.3. Growth media were replaced every three days.

Table 5.1. Composition of the Long Ashton CON and modified EQUI and INV growth solutions used in this investigation. Concentrations are expressed in mM. Before use, the pH of each batch of growth solution was corrected with 10 M NaOH so that final pH lay in the optimal range for plant growth (pH 6.1-6.3).

Component		*CON* 1 : 7.95 S : N	*EQUI* 1 : 1 S : N	*INV* 7.95 : 1 S : N
Macronutrients...	Ca^{2+}	4.000	4.000	4.000
	Mg^{2+}	1.500	1.500	1.500
	K^{+}	4.000	4.000	4.000
	HPO_4^{2-}	1.330	1.330	1.330
	SO_4^{2-}	1.512	4.000	7.066
	NO_3^{-}	12.000	4.000	0.888
Micronutrients...	Si	0.047	0.047	0.047
	Fe^{2+}	0.100	0.100	0.100
	Mn^{2+}	0.010	0.010	0.010
	Cu^{2+}	0.001	0.001	0.001
	Zn^{2+}	0.001	0.001	0.001
	Na^{+}	1.378	1.378	1.378
	BO_3^{-}	5×10^{-5}	5×10^{-5}	5×10^{-5}
	MoO_4^{2-}	4×10^{-4}	4×10^{-4}	4×10^{-4}

Plate 7. A scanning electron micrograph depicting the heterogeneity of cells in the upper epidermis of the third leaf of wheat. Ridge- (RC), near-stomatal- (NC) and trough cells (TC) are shown.



5.4, RESULTS

5.4.1, Sulphur and sulphate

Sulphur accumulated in the epidermis of high S (INV) material throughout the experiment such that trough cell S concentrations were significantly higher (General Linear Model (GLM); $p < 0.001$) in INV plants than in CON or EQUI material (**Figure 5.1**).

Similar trends were seen in the whole leaf data although SO_4^{2-} concentrations in these were always lower than the epidermal concentrations measured in the same plant. There were no differences in trough cell S or leaf SO_4^{2-} concentration between CON and EQUI plants and values remained low and constant with increasing leaf age.

5.4.2, Trough cell osmotic pressure

Despite the variability in the data, epidermal OP appeared relatively constant both between nutrient treatments and throughout all stages of leaf life (see **Figure 5.2**).

5.4.3, Trough cell potassium

Epidermal trough cell K^+ concentrations decreased markedly in all three nutrient treatments as leaves aged (see **Figure 5.3**). However, the extent of this export differed between the three nutrient treatments such that this rate of loss was slowest from the trough cells of INV plants. With the exception of the initial (-2 days) assessment point, epidermal K^+ concentrations were significantly higher (GLM; $p < 0.001$) in INV plants than in CON or EQUI material.

Figure 5.1. Do the upper epidermis and whole leaf of wheat accumulate S to significant concentrations when plants are supplied with the appropriate ratio of S : N? The effects of differing external S : N ratio and increasing leaf age on (i) epidermal trough cell S and (ii) leaf sap SO_4^{2-} concentrations following growth of material in CON, EQUI or INV nutrient solutions. For each datapoint, n = 3 ± SD.

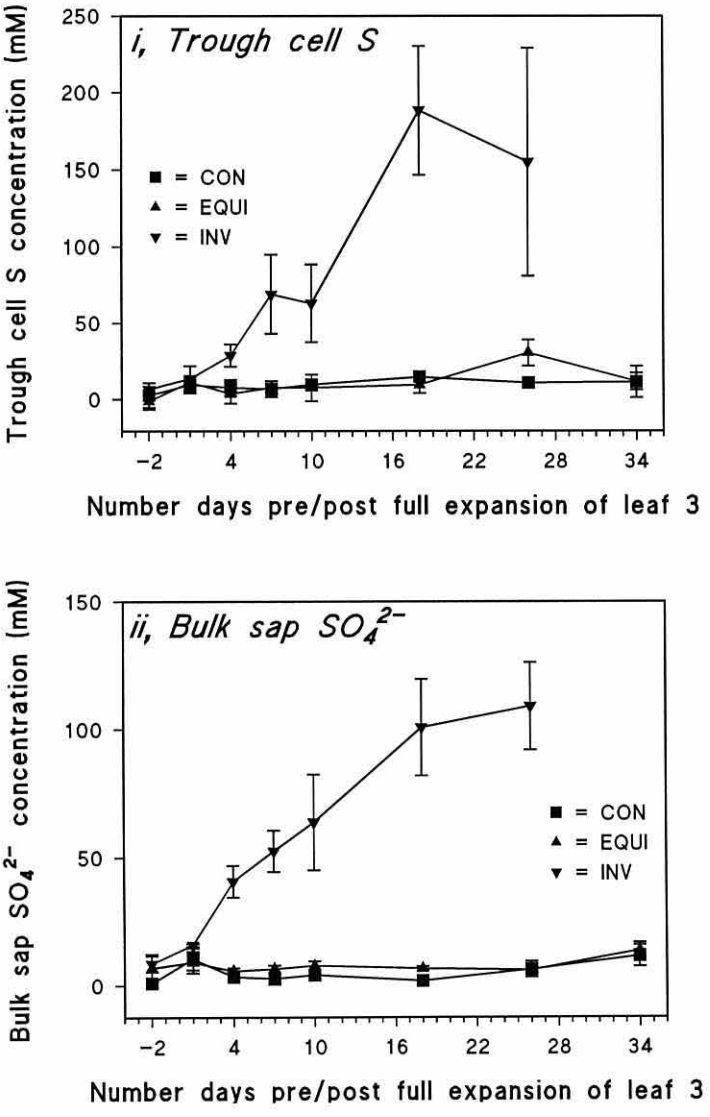


Figure 5.2. Does the accumulation of excess S in the upper epidermis affect cell water relations? The effects of differing external S : N ratio and increasing leaf age on epidermal trough cell osmotic pressures (OP) following growth of material in CON, EQUI or INV nutrient solutions. For each datapoint, n = 3 ± SD.

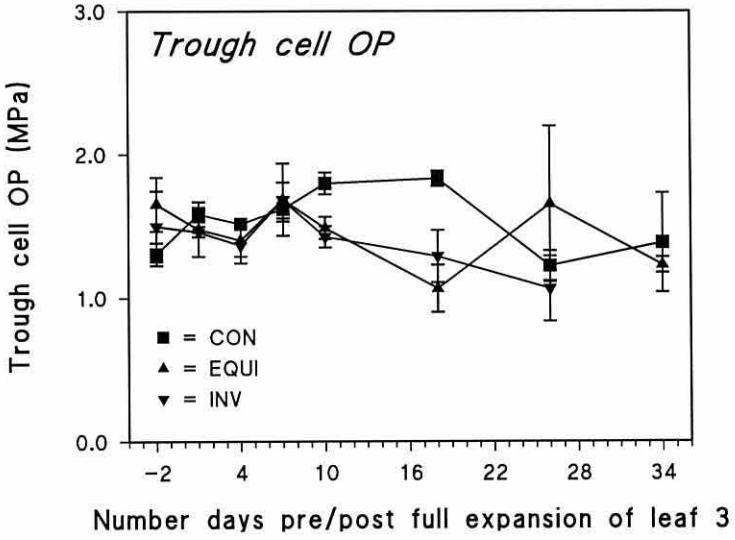
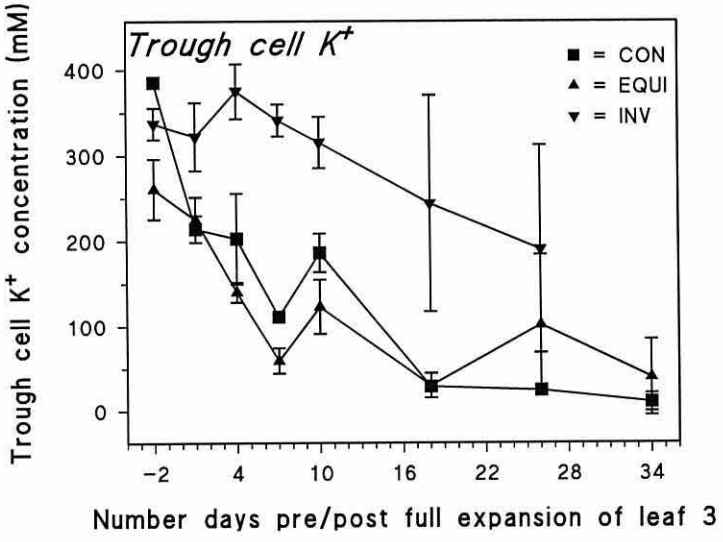


Figure 5.3. Does the accumulation of excess S in the upper epidermis affect the behaviour of other cell solutes? The effects of differing external S : N ratio and increasing leaf age on epidermal trough cell K^+ concentrations following growth of material in CON, EQUI or INV nutrient solutions. For each datapoint, n = 3 ± SD.



5.4.4, Trough cell calcium and its relationship to potassium

Epidermal trough cell Ca^{2+} concentrations increased as a function of leaf age in material from all three nutrient treatments (**Figure 5.4**). Whilst the extent of this accumulation was similar in both CON and EQUI plants, the amassment of Ca^{2+} was significantly slower (GLM; $p < 0.001$) in the epidermal trough cells of INV material.

When mean epidermal Ca^{2+} concentrations were plotted against equivalent values for K^+, a tightly-coupled 1 : 1 relationship in which Ca^{2+} replaced K^+ as leaves aged was clearly visible in CON and EQUI plants (**Figure 5.5**). In a statistical comparison of these data, there was no significant difference between the slopes of CON and EQUI treatments which suggested that the 1 : 1 relationship was maintained in both nutrient treatments. The K^+ *vs.* Ca^{2+} slope for INV plants on the other hand, differed significantly from that of CON or EQUI material at the 5 % level (MCT) which indicated that there was no coupling of K^+ and Ca^{2+} concentrations in the upper epidermis of INV plants.

5.4.5, Chlorine and chloride

In all three nutrient treatments, epidermal trough cell and bulk sap Cl^- concentrations were initially high at the start of the experiment and then decreased significantly as the leaves aged (GLM; $p < 0.001$ for both trough cells and bulk tissue sap; **Figure 5.7**). Although the highest initial Cl^- concentrations were measured in INV plants, by day 18 post full-expansion, concentrations had fallen below 25 mM in material from all three nutrient treatments.

As was the case for S (see **Figure 5.1**), bulk tissue sap Cl^- concentrations were lower than epidermal trough cell Cl^- concentrations measured in the same plant. The high Cl concentrations from days -2 to +4 are thought to represent an artefact from the addition of 6 M HCl to growth media in attempts to maintain an optimal pH for plant growth. In spite of the addition of HCl, the concentrations of Cl^- measured in daily nutrient solution samples from CON, EQUI and INV growth tubs remained low and constant throughout this experiment (see **Figure 5.6**). The implications of this are considered in greater detail in section **5.5**.

Figure 5.4. Does the accumulation of excess S in the upper epidermis affect the behaviour of other cell solutes? The effects of differing external S : N ratio and increasing leaf age on (i) epidermal trough cell Ca^{2+} concentrations and (ii) the rates of Ca^{2+} accumulation following growth of material in CON, EQUI or INV nutrient solutions. For each datapoint, n = 3 ± SD.

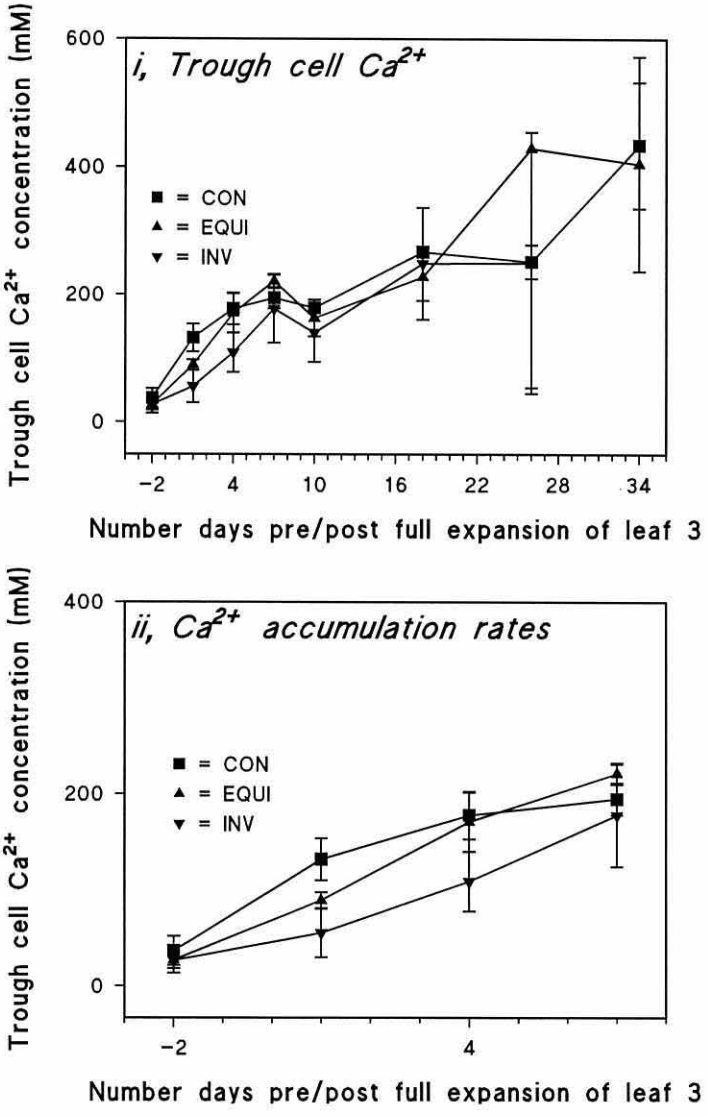


Figure 5.5. Does the accumulation of excess S in the upper epidermis affect the behaviour of other cell solutes? Plots of mean K^+ concentration (**Figure 5.3**) *versus* mean Ca^{2+} concentration (**Figure 5.4**) demonstrate the effects of CON, EQUI and INV nutrient regimes on the tightly-coupled Ca^{2+}-K^+ relationship in which Ca^{2+} replaces K^+ as leaves age. Each datapoint represents an individual measurement and a first order regression connects datapoints.

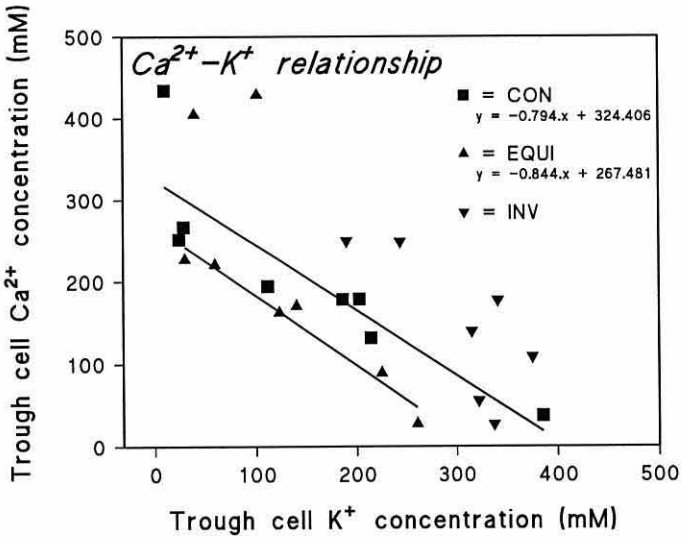


Figure 5.6. Changes in nutrient solution Cl^- concentration throughout this experiment. The availability of Cl^- to the plant was considered to be equal to the concentration measured in daily medium samples by HPLC (see **2.3.4.3**) whilst approximate uptake rates were assumed to correspond to the depletion of Cl^- from the nutrient solution. For each datapoint, n = 2.

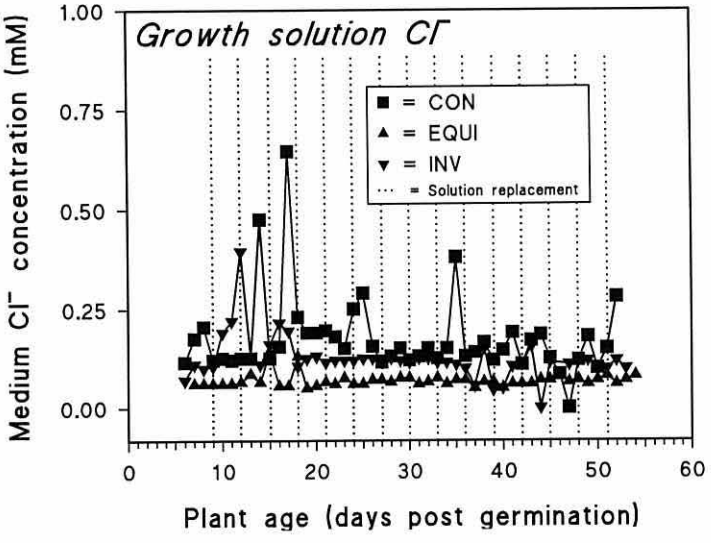
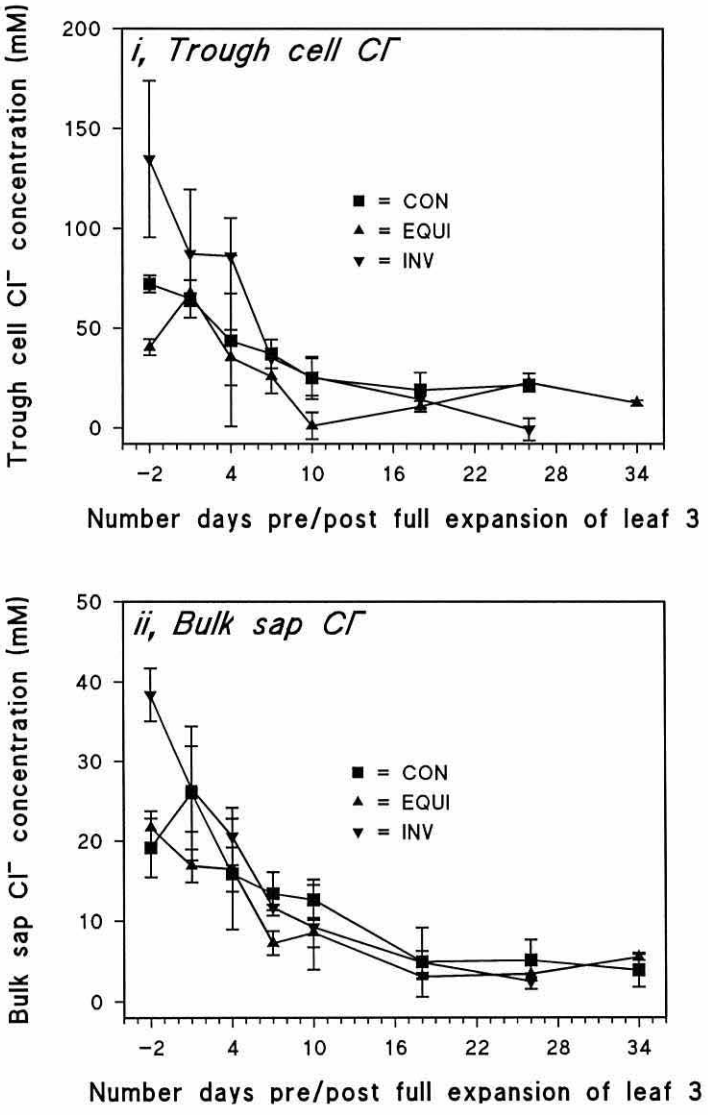


Figure 5.7. Does the accumulation of excess S in the upper epidermis affect the behaviour of other cell solutes? The effects of differing external S : N ratio and increasing leaf age on (i) epidermal trough cell Cl^- and (ii) leaf sap Cl^- concentrations following growth of material in CON, EQUI or INV nutrient solutions. For each datapoint, n = 3 ± SD.



5.4.6, Nitrate uptake and accumulation

Leaf NO_3^- concentrations were similar in both CON and EQUI material throughout this experiment (**Figure 5.8**). Concentrations increased from 2 days pre full-expansion, peaking at 4 days post full-expansion before decreasing slowly as leaves aged. In INV plants, leaf NO_3^- concentrations were significantly lower (GLM; $p < 0.001$) than those measured in CON and EQUI material and concentrations never exceeded 50 mM throughout the experiment.

Patterns of NO_3^- uptake were similar between the three nutrient treatments (**Figure 5.9**). As the plants grew, demand for NO_3^- increased as is evident from the increasingly larger depletion of solution NO_3^- in the periods between complete replacement.

Figure 5.8. Does the accumulation of excess S in the upper epidermis affect the behaviour of other cell solutes? The effects of S : N ratio and increasing leaf age on leaf sap NO_3^- concentrations following growth of material in CON, EQUI or INV nutrient solutions. For each datapoint, n = 3 ± SD.

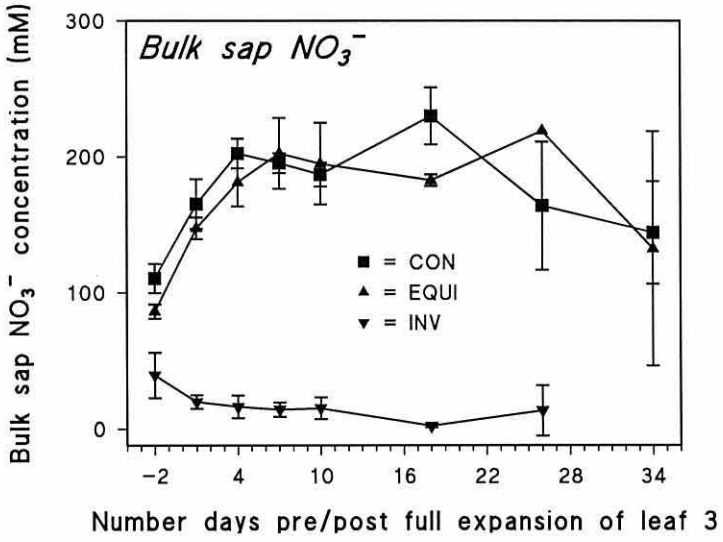
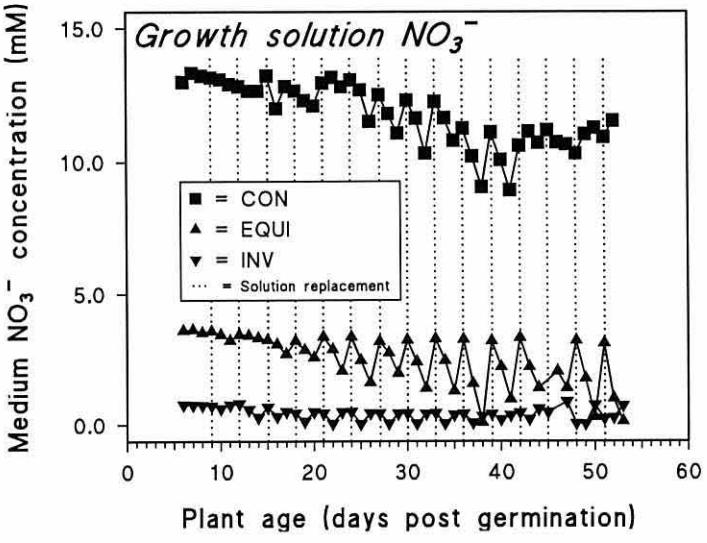


Figure 5.9. Changes in nutrient solution NO_3^- concentration throughout this experiment. The availability of NO_3^- to the plant was considered to be equal to the concentration measured in daily medium samples by HPLC (see **2.3.4.3**) whilst approximate uptake rates were assumed to correspond to the depletion of NO_3^- from the nutrient solution. For each datapoint, n = 2.



5.5, DISCUSSION

5.5.1, Do the epidermis and whole leaf function as reservoirs for excess S?

Growth of plants in the INV nutrient solution led to significant accumulation of S both in the trough cells of the upper epidermis and in representative sections of the whole leaf. Slow accumulation of S was observed throughout leaf life and at no time, was net re-mobilization detected. Even when the third leaf was in advanced stages of senescence (*circa* day 26 post full-expansion), 150 mM S was still detectable in epidermal trough cells. Similar concentrations have been reported previously in a number of CAM species which demonstrate considerable diurnal fluctuation in cell sap SO_4^{2-} concentration (Olivares and Aguiar, 1996). Generally, however, cereal SO_4^{2-} concentrations are much lower than those of CAM plants although stepwise addition of NO_3^-, Cl^- or SO_4^{2-} to a hydroponic medium is known to result in an increased vacuolar concentration of the respective ion (Rennenberg, 1984; Mornet *et al.*, 1997).

By way of comparison, there was no accumulation of S in plants grown either in CON or EQUI nutrient solutions. Epidermal and whole leaf values were generally less than 25 mM in both treatments and concentrations remained relatively constant with increasing leaf age. However, the similarity between epidermal and whole leaf SO_4^{2-} data from CON and EQUI treatments is interesting. When plants were faced with the choice of equal concentrations of both NO_3^- and SO_4^{2-} (as in the 1 : 1 S : N EQUI nutrient solution) a preference was shown for uptake of the metabolically important NO_3^- anion. This observation agrees with work by Richardson (1993) who proposed existence of an uptake affinity series in which uptake of $Cl^- > NO_3^- > SO_4^{2-}$. Our own observations, therefore, suggest that the accumulation of S by INV plants occurred through osmotic necessity as a response to a lack of monovalent anions and the inability of the epidermis to use organic ions as principal osmotica (Leigh and Tomos, 1993). Whilst this investigation has been able to show that uptake of the divalent SO_4^{2-} species results in a changed solute composition, it has not been possible to say whether these observations were specific to SO_4^{2-}.

The epidermis occupies just 21 % (by cell type) of a typical wheat leaf (Jellings and Leech, 1982). The remaining 79 % of the leaf is composed of mesophyll (38 %) and vascular tissue (41 %). Epidermal S concentrations were higher than those recorded in whole leaf sections in material from all three nutrient treatments. This would seem to suggest that SO_4^{2-} concentrations were higher in the epidermis than in

those cell types lying beneath this tissue layer. For example, the epidermal S concentrations measured in CON material were relatively constant throughout this experiment at *circa* 15 mM. If vacuolar accumulation of S had been strictly confined to the epidermal cells, then just 3 mM SO_4^{2-} would have been measurable in whole leaf sections. In fact, the mean bulk tissue sap concentration was around 5 mM suggesting that on average, those cell types lying beneath the epidermis contained three times less SO_4^{2-} than their epidermal counterparts. The difference in S concentrations between the epidermis and those cell types found deeper within the leaf was smaller (*circa* 70 % of epidermal values) in INV plants. The work of Bell *et al.* (1995c) describes a similar difference in S concentrations between epidermal (mean of 49 mM) and mesophyll cells (mean of 26 mM) when wheat plants were supplied with a comparably high ratio of S : N. The effects of S : N supply and S stress on epidermal cell and leaf cell heterogeneity are described in greater detail in **Chapters 6** and **7** respectively.

5.5.2, Does the accumulation of S affect cell water relations?

Epidermal OP were similar in material from all three nutrient treatments and values remained relatively constant throughout leaf life. Unfortunately, subsequent experiments (see **Chapters 6** and **7**) have failed to produce similar results and growth of plants in INV nutrient solution has led to striking decreases in epidermal OP (see **Figures 6.6**, **6.7** and **7.2**). It is thought that the similarity in epidermal OP between the three nutrient treatments in this investigation is attributable to the frequent addition of 6 M HCl to the growth media in an attempt to maintain an optimal pH for plant growth.

5.5.3, Does the accumulation of excess S affect the behaviour of other solutes?

Epidermal K^+ concentrations decreased exponentially in CON, EQUI and INV material as leaves aged. The extent to which this re-translocation process occurred, however, differed between the three nutrient treatments such that the decrease in epidermal K^+ concentrations over time was significantly slower (GLM; $p < 0.001$) in INV material than in CON or EQUI plants. As the third leaf entered the final stages of senescence (*circa* 18 days post full-expansion), an excess of 250 mM K^+ remained in the upper epidermis of INV plants whilst concentrations in CON and EQUI material

were less than 50 mM. It would seem, therefore, that re-translocation of K^+ from the epidermis is diminished in INV material. Due to the limitations of experimental design it is not possible to state whether this impaired export rate is due to diminished NO_3^- status (**Figure 5.8**) or to the accumulation of large amounts of SO_4^{2-} (**Figure 5.1**) in INV plants.

Calcium accumulated to significant concentration in the upper epidermis of plants from all three nutrient treatments as leaves aged. Increasing from approximately 50 mM at 2 days pre full-expansion, values had reached in excess of 200 mM by 26 days post full-expansion in CON, EQUI and INV material. Patterns of age dependant Ca^{2+} accumulation were very similar to those described by Hinde, 1994 with vacuolar sequestration thought to be related to the limited phloem mobility of the Ca^{2+} cation (see for example, Fricke *et al.* (1994a) and references therein). Once imported into the leaf via the transpiration stream, the very nature of its limited phloem mobility means that Ca^{2+} is unable to leave (Fricke *et al.*, 1995).

The replacement of K^+ by Ca^{2+} as the leaf ages would appear to be a tightly-coupled process (Hinde, 1994 and this study) when plants are grown under low light conditions (Fricke *et al.*, 1995). In this experiment, the ratio of Ca^{2+} import : K^+ export in the ageing third leaves of CON and EQUI plants was very close to 1 : 1. This ratio is markedly different from the 2 : 3 for Ca^{2+} : K^+ described by Hinde, 1994 in which three K^+ cations (and three monovalent anions) were replaced by two Ca^{2+} cations (and four monovalent anions) as material aged. This 2 : 3 ratio was calculated as precisely that required if OP was to be maintained constant throughout leaf life (Hinde, 1994). This suggests that our ratio of 1 : 1 should lead to a decrease in OP as the leaf ages. From the data presented in **Figure 5.2**, this is clearly the case as the epidermal OP of both CON and EQUI material decreases with increasing leaf age. The 1 : 1 relationship between epidermal Ca^{2+} and K^+ concentrations was identical in both CON and EQUI plants even though the first order regressions for these two treatments were not super-imposed (**Figure 5.5**).

A significantly slower (GLM; $p < 0.001$) rate of epidermal Ca^{2+} accumulation was seen in the INV plants whilst the tightly coupled 1 : 1 Ca^{2+} : K^+ relationship was absent from this material. It is possible that this loss of the 1 : 1 Ca^{2+} : K^+ relationship could have been attributable to the accumulation of excess S in the epidermis since similar observations have been made when other divalent anions (for

example, malate; Fricke *et al.*, 1995) have amassed to significant concentration in this tissue layer. This is discussed further in **Chapter 9**.

Epidermal and whole leaf Cl^- concentrations were high in CON, EQUI and INV material at the start of this experiment before decreasing considerably as leaves aged. Nevertheless, epidermal Cl^- concentrations remained on average three times higher than whole leaf values measured in the same plant. This difference further highlights the problems in using 'averaged' tissue solute concentrations when describing the behaviour of a single solute within a leaf. The problem is further exacerbated if the solute is distributed differentially within the leaf (for example, Cl^- or Ca^{2+} which are restricted to the epidermis; Leigh and Tomos, 1993; Fricke *et al.*, 1994a, b, c).

At the start of this experiment, epidermal and whole leaf Cl^- concentrations were almost twice as high in INV plants than in CON or EQUI material. These high Cl^- concentrations are thought to represent an artefact from the daily addition of 6 M HCl to the growth media to maintain an optimal pH for plant growth. On a daily basis, it was the INV nutrient solution which demonstrated the greatest increase in pH from day to day (results not shown).

The pH of a bathing solution is known to rise due to the release of OH^- when plants are grown in solutions containing NO_3^- as the sole nitrogen source (Raven and Smith, 1976; Marschner, 1995). Since the uptake of NO_3^- by root cells is considerably enhanced under conditions of N stress (see for example, Clement *et al*, 1979; Barneix and Causin, 1996 and references therein) it is possible that the INV plants absorbed proportionately more NO_3^- from their nutrient solution than did CON or EQUI plants from their respective solutions. Consequently, this enhanced uptake of NO_3^- from the high SO_4^{2-} low NO_3^- (INV) solution may have resulted in the larger daily increases in INV solution pH.

If this is true, it would follow that the INV nutrient solution received the largest additions of HCl so as to optimise pH for plant growth. Subsequent HPLC quantification of daily growth solution samples failed to demonstrate the expected high Cl^- concentrations (see **Figure 5.6**). This implies that the extra Cl^- added to INV growth tubs was absorbed by the plants to maintain internal water relations, (most notably OP) in the absence of NO_3^-. This hypothesis could explain why epidermal

and whole leaf Cl^- concentrations were highest in the INV material at the start of this experiment.

The rapid absorption of any extra Cl^- added to nutrient solution is hardly surprising since this anion is an osmotically important solute in its own right (see for example, Flowers (1988)) which can reciprocally substitute for NO_3^- to maintain plant water relations (Richardson, 1993; Pritchard *et al.*, 1996). In both CON and EQUI growth tubs, pH fluctuated less on a daily basis and as a result, proportionately less HCl was added to nutrient solutions (results not shown). Since nutrient solution Cl^- concentrations remained low and constant in all three treatments throughout leaf life suggests that (even in the presence of high concentrations of NO_3^-) extra Cl^- was rapidly absorbed by the plant. As before, this is demonstrated by the unexpectedly high epidermal and whole leaf Cl^- concentrations in both CON and EQUI material (see **Figure 5.7**).

The correction of nutrient solution pH with HCl was stopped just before the third leaf attained full expansion since it was felt that exposure of plants to excessive amounts of Cl^- could impair SO_4^{2-} accumulation. As a result, epidermal and whole leaf Cl^- concentrations decreased from 2 days pre full-expansion in plants from all three nutrient treatments. From day 18 onwards (and for the remainder of leaf life), neither epidermal nor whole leaf Cl^- concentrations changed significantly with increasing leaf age. This held true for plants from all three treatments.

Nitrate is stored almost exclusively within the cell vacuole (Pate, 1980; Martinoia *et al*, 1981; Winter *et al.*, 1993; Marschner, 1995). The extent to which this accumulation occurs is dependent upon the light intensity, length of photoperiod, temperature and the availability and form of the N supplied to the plant (Liu and Shelp, 1995). Leaf NO_3^- concentrations were similar in both CON and EQUI material throughout this experiment with values increasing from pre full-expansion to peak at around 200 mM 4 days post full-expansion. Following a long period of constant leaf NO_3^- content, values then decreased as material aged although 150 mM NO_3^- was still detectable as the leaf entered the final stages of senescence (*circa* day 26). In spite of the fact that whole leaf solute data may mask heterogeneity in the distribution of that solute within the leaf (Fricke *et al.*, 1994a, b, c; 1995), the fact that NO_3^- is distributed uniformly between epidermal, mesophyll and bundle sheath

cells (Leigh and Tomos, 1993; Fricke et al, 1994a) suggests that epidermal concentrations should have been similar to whole leaf values (Cuin, 1996).

As leaves age, (and despite a continual import of N via the xylem; Pate and Atkins, 1983; Simpson *et al.*, 1983) organic N compounds (primarily amino acids) are rapidly re-mobilized and re-translocated to areas of new growth (see **Chapter 1**). The re-mobilization of leaf NO_3^- in contrast, is much slower (Clement *et al.*, 1979; Liu and Shelp, 1995) and in the current study, whole leaf NO_3^- concentrations had decreased by only 23 % (187 mM to 144 mM) and 32 % (195 mM to 132 mM) in CON and EQUI material respectively between days 10 and 34 post full-expansion.

Whilst slow turnover of vacuolar NO_3^- may have (at least in part) been responsible for these observations, it is also possible that the concentrations of NO_3^- supplied to both CON and EQUI material were sufficiently high and constant as to fail to induce extensive re-mobilization of NO_3^- from the leaf. Liu and Shelp (1995) found that only complete withdrawal of a 15 mM NO_3^- supply led to the re-mobilization of NO_3^- from mature leaves of broccoli. From their data it was concluded that re-mobilization of shoot NO_3^- was induced only by a lowered NO_3^- availability in the nutrient solution. On this basis, the constant supply of 12 mM (CON) and 4 mM NO_3^- (EQUI) to wheat plants throughout this experiment may have been an insufficient stimulus to trigger significant re-mobilization of leaf NO_3^- reserves.

The reduction in NO_3^- in the INV (INV) nutrient solution led to a four-fold decrease in whole leaf NO_3^- concentrations when compared with values from CON or EQUI plants. Plants grown continually in the INV (INV) nutrient solution appeared stunted and chlorotic (demonstrating the classical symptoms of N deficiency; Green *et al.*, 1986) when compared with CON or EQUI material (**Plate 8**). The relationship between available NO_3^- supply and plant NO_3^- content has previously been described by Clement *et al.* (1979), Stienstra (1986), Koch *et al.* (1988) and Gojon *et al.* (1991). The lower concentrations of NO_3^- in the leaves of INV plants were rapidly re-mobilized as material aged such that by day 18 post full-expansion, there was negligible NO_3^- measurable in INV material.

HPLC analyses of nutrient solution samples indicated that patterns of NO_3^- uptake were similar in all three nutrient treatments throughout the experiment. As plants grew in size, demand for NO_3^- increased (as is evident from the decrease in

growth solution NO_3^- content in the periods between solution replacement). The reduced level of available NO_3^- in INV growth solution was rapidly taken up by the plants such that solution concentrations were generally negligible one day after replacement. The rate of NO_3^- uptake from CON solution appeared to decrease towards the end of the experiment as the plants grew older. This observation is similar to that reported by Larsson *et al.* (1991) and is most likely to be attributable to the advanced state of plant maturity (*i.e.* significant reduction in growth rate expected just prior to flowering).

The OP of a solution is directly proportional to the concentration of dissolved solute present within it. Therefore, a direct comparison of OP data with the sum of identified solutes ($\Sigma_{solutes}$) in the same sample of extracted vacuolar sap can highlight the presence of any additional, unmeasured solute. For an accurate comparison, however, data for $\Sigma_{solutes}$ must first be multiplied by an osmotic co-efficient to take into account both the deviation of a solution away from ideal behaviour and the inherent difference between osmolarity and osmolality. Throughout this investigation, the value of 0.82 (as used by Fricke *et al.*, 1994b) represented our "apparent" osmotic co-efficient although it should be remembered that the blanket application of this value (especially on INV (INV) material) may well have been inappropriate.

A selection of the data from the solute balance sheets for CON, EQUI and INV material at day 10 post full-expansion is presented in **Table 5.2.** The terms $\Sigma_{solutes}$[1], Calculated[2], Difference[3], $\Sigma_{cations}$[4], Σ_{anions}[5] and Difference[6] used in the following section of this chapter are defined in the paragraph below **Table 5.2.** In plants from all three nutrient treatments, there was a substantial difference (Difference[3]) between measured OP (Mean OP) and 'theoretical' OP (Calculated[2]) indicating that not all osmotically important components had been accounted for. Throughout this experiment, the discrepancies between $\Sigma_{solutes}$ and OP were largest in CON and EQUI material. At day 10 post full-expansion for example (see **Table 5.2**), the difference between $\Sigma_{solutes}$ and OP was 383 mOsm kg^{-1} in CON and 328 mOsm kg^{-1} in EQUI material. These values were around 100 mOsm higher than the 260 mOsm kg^{-1} difference between $\Sigma_{solutes}$ and OP recorded in the INV (INV) plants. A further discrepancy was also evident between values for $\Sigma_{cations}$ and those of Σ_{anions}. As for difference[3], the difference between $\Sigma_{cations}$ and Σ_{anions} (difference[6]) was larger in CON

and EQUI material than in INV plants. At day 10 post full-expansion (see **Table 5.2**), the difference between $\Sigma_{cations}$ and Σ_{anions} in CON, EQUI and INV material respectively was 554, 524 and 258 mEq l^{-1}.

In respect of the difference in solute shortfall between CON/EQUI and INV material, it is proposed that the identity of most of the missing solute was that of NO_3^-. In this experiment, it was not possible to quantify epidermal trough cell NO_3^- concentrations and so the actual contribution of NO_3^- to the solute balance sheets was not known. However, whole leaf NO_3^- concentrations were around ten-fold higher in CON and EQUI material than in INV plants (see **Figure 5.8**). If epidermal NO_3^- concentrations were indeed similar to whole leaf values (as suggested by Cuin, 1996 and discussed earlier), this ten-fold difference in epidermal NO_3^- between CON/EQUI and INV material would be sufficient to cancel out the significant charge and osmotic imbalance shown in **Table 5.2**.

Unfortunately, the low solubility of $CaSO_4$ (around 14 mM; Weast and Astle, 1980) complicates the relationship between OP, charge balance and quantified solutes in the INV plants. As the third leaf of INV material aged, concentrations of both Ca^{2+} and S in excess of 200 mM were recorded in the same extracted vacuolar sap (*cf.* **Figure 5.1** with **Figure 5.4**). These observations would suggest that S (presumably in the form of SO_4^{2-}; De Kok, 1990) must have formed an insoluble salt (upon contact with Ca^{2+}) in epidermal trough cell vacuoles. Moreover, crystals of this insoluble salt would have entered the *circa* 1 μm aperture of the sampling capillaries to be subsequently analysed by X-ray microanalysis although such a phenomenon was never observed.

It is the likely presence of this insoluble salt which complicates the relationship between OP and $\Sigma_{solutes}$ since the effects of this Ca^{2+}-S complex on the behaviour of other vacuolar solutes remains unclear. An additional problem concerns the identity of the 'missing' solute necessary to correct the shortfalls both between OP and $\Sigma_{solutes}$ and between $\Sigma_{cations}$ and Σ_{anions}. At day 10 post full-expansion for example (see **Table 5.2**), the absent 260 mOsm kg^{-1} (the difference between OP and $\Sigma_{solutes}$) and the 258 mEq l^{-1} (the difference between $\Sigma_{cations}$ and Σ_{anions}) was unlikely to have been due to an unmeasured inorganic solute since Cl^- (**Figure 5.7**), P (**Table 5.2**) and whole leaf NO_3^- concentrations (**Figure 5.8**) were low.

Table 5.2. A demonstration of the relationship between osmotic pressure and measured solute concentrations in individual epidermal trough cells from the third leaves of CON, EQUI and INV material at day 10. Values for osmolality and charge balance were calculated from the mean solute and OP data. Due to $CaSO_4$ being insoluble at concentrations above 14 mM (Weast and Astle, 1980), when Ca^{2+} and S were detected in the same extracted vacuolar sap in excess of 14 mM, precipitation was assumed to have occurred and any contribution of that excess to $\Sigma_{solutes}$ was discounted. In this experiment, Single cell NO_3^- concentrations were not determined and hence the contribution of NO_3^- to the solute balance sheet is not known.

	Treatment	CON	EQUI	INV
Mean Solute Concentration (mM)	[S]	*9.63*	*7.50*	*62.89*
	[Cl]	*24.94*	*0.99*	*25.52*
	[K]	*186.17*	*122.93*	*315.30*
	[Ca]	*178.52*	*162.50*	*139.74*
	[Mg]	*27.94*	*45.85*	*3.52*
	[P]	*0.00*	*0.00*	*19.43*
	$\Sigma_{solutes}$ [1]	427.20	339.78	440.62
Osmolalities (mOsmol kg^{-1})	Calculated[2]	350.30	278.62	361.31
	Mean OP	*732.82*	*606.71*	*581.50*
	Difference[3]	**382.52**	**328.09**	**220.19**
Vacuolar charge (mEq l^{-1})	$\Sigma_{cations}$ [4]	599.09	539.63	476.04
	Σ_{anions} [5]	44.20	15.99	64.38
	Difference[6]	**554.89**	**523.64**	**411.66**

$\Sigma_{solutes}$ [1] - Sum of measured solute concentrations irrespective of charge.

Calculated[2] - $\Sigma_{solutes}$ x 0.82 (osmolarity co-efficient as per Fricke *et al.*, 1994c).

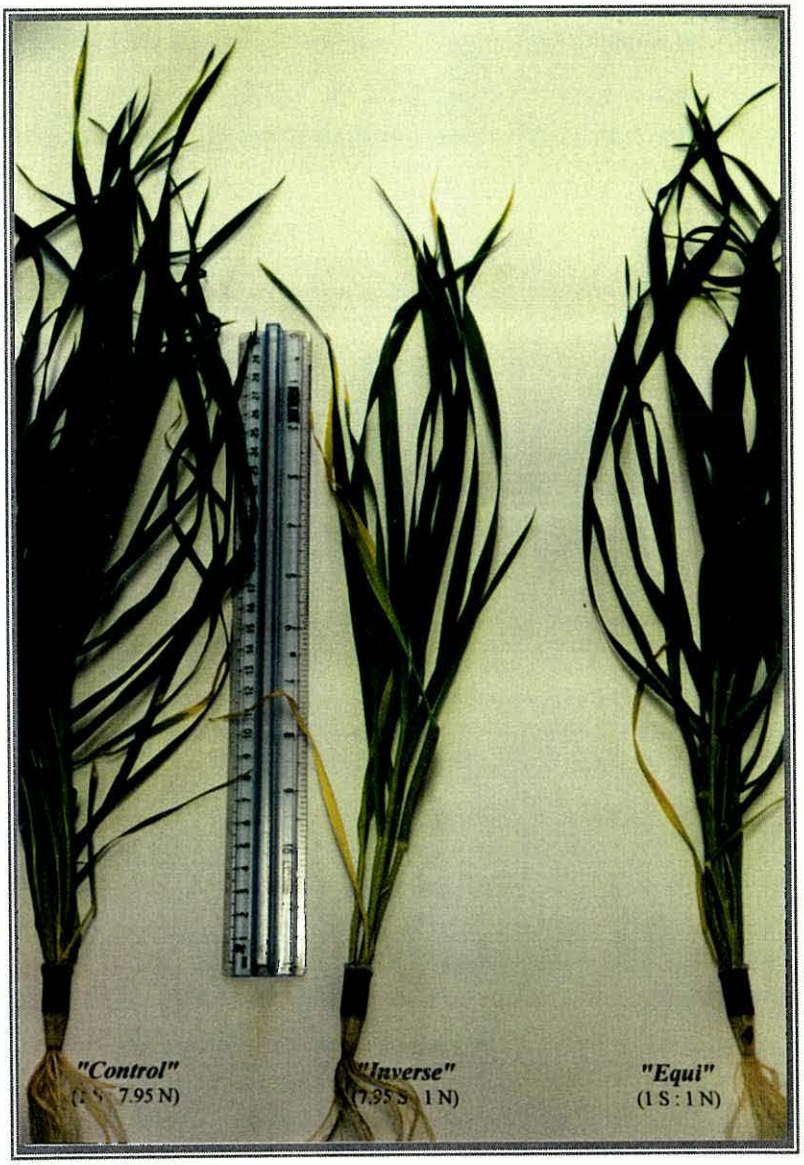
Difference[3] - Result of subtracting corrected[2] values from mean OP.

$\Sigma_{cations}$ [4] - Sum of [K], [Ca] and [Mg] respecting probable valencies.

Σ_{anions} [5] - Sum of [S], [Cl] and [P] respecting probable valencies.

Difference[6] - Product of $\Sigma_{cations}$ [4] - Σ_{anions} [5].

Plate 8. The effects of N : S ratio on the hydroponic growth of wheat (cv. Alexandria). Plants grown in the 8 : 1 S : N (INV) derivative of Long Ashton were smaller and appeared chlorotic and unhealthy in comparison with CON or EQUI material. The visible differences between nutrient treatments are thought to have been caused by the different NO_3^- concentrations in the respective growth solutions (see **Table 5.1**).



The missing solute could be organic by nature. However, it is unlikely to have been a sugar since sugar molecules are uncharged and do not tend to accumulate in the epidermis as this tissue plays no role in carbohydrate partitioning (Koroleva *et al.*, 1997). Likewise, it is equally unlikely that the identity of the missing solute was exclusively malate since plants grown under conditions of relatively low light (200 μmol m^{-2} s^{-1}) tend not to accumulate this solute to osmotically significant quantities (Fricke *et al.*, 1995).

Recent work using capillary electrophoresis (see Tomos *et al.*, 1998) has identified an organic cation which whilst found in the epidermal cells of INV plants, has not been detected in the cells of CON material. As yet this cation remains to be identified.

5.6, CONCLUSIONS

- Significant concentrations of S accumulated in both the epidermis and whole leaves of plants grown in the 8 : 1 S : N (INV) nutrient solution. Both epidermal and whole leaf concentrations increased as leaves aged and a comparison of epidermal and whole leaf values indicated that S was not distributed uniformly within the third leaf. There was no significant accumulation of S in CON or EQUI plants.

- The accumulation of S coincided with diminished K^+ export from the epidermal trough cells and concentrations in excess of 200 mM were detectable as the third leaf entered the final stages of senescence. In comparison, the epidermal K^+ concentrations of plants grown in CON or EQUI nutrient solutions were negligible at the same stage in leaf life.

- The age-dependent accumulation of Ca^{2+} by the epidermis was impaired in INV material and the tightly coupled substitution of K^+ by Ca^{2+} as the leaf aged was absent in these plants. It was assumed that much of the Ca^{2+} which accumulated in the epidermis of INV plants must have precipitated out as $CaSO_4$.

- Whole leaf NO_3^- concentrations were highest in CON and EQUI material and concentrations decreased relatively slowly as the leaves aged. A rapid rate of NO_3^- re-mobilization was, however, observed in INV material and whole leaf concentrations were negligible by the time the leaf began to senesce.

Chapter 6:

CAN EPIDERMAL CELLS RE-MOBILIZE STORED S?

6.1, EXPERIMENTAL OBJECTIVES

The experiments described in this chapter were primarily designed to answer three main questions.

1, *Are epidermal and whole leaf stores of accumulated S re-mobilized from the leaf when plant SO_4^{2-} supply is withdrawn?*

2, *Do morphologically distinct upper epidermal cells demonstrate different rates of S re-mobilization and do these correspond to the three different tracer exchange rate constants for SO_4^{2-} flux identified by Bell et al., 1994?*

3, *What is the effect of S re-mobilization on cellular water relations and the behaviour of other vacuolar solutes?*

6.2, INTRODUCTION

Symptoms of S deficiency in plants are normally confined to a yellowing of younger, developing leaves (see **Chapter 1**). However, symptoms can also be observed in older tissue if both S *and* N are limiting (Robson and Pitman, 1983). This yellowing of younger tissue is caused by a decrease in the chlorophyll content of the leaf (Burke *et al.*, 1986; Dietz, 1989) which occurs as a direct consequence of the reduced availability of the S-containing amino acids, cysteine and methionine (Archer, 1987; Marschner, 1995). In cases of extreme S deficiency, a reduction in protein synthesis throughout the whole plant can lead ultimately to a decrease in overall growth rate (Schnug and Haneklaus, 1994; Marschner, 1995).

The appearance of sulphur deficiency symptoms has been linked to the slow re-mobilization of cellular SO_4^{2-} pools from older leaf tissue (see **Chapter 1**). Whilst the exact regulatory mechanisms which control S re-mobilization are not fully understood (see **Chapter 9**), it has been shown experimentally that:

1, Extraneous SO_4^{2-} is stored in the cell vacuole (see for example, **Chapter 5**).

2, SO_4^{2-} flux is faster across the plasmalemma than the tonoplast (Cram, 1983a; Thoiron *et al.*, 1981).

3, Vacuolar SO_4^{2-} accumulation inhibits further influx of SO_4^{2-} across the tonoplast but not the plasmalemma (Cram, 1983b).

In combination, these three observations have led to the suggestion that SO_4^{2-} re-mobilization could be regulated by the tonoplast (Bell *et al.*, 1994). The SiCSA techniques used in this investigation are not readily applicable to studies of intra-cellular solute compartmentation and as such, this work is unable to directly validate the hypothesis of Bell *et al.* (1994). However, a thorough investigation of the inter-cellular compartmentation of S within the leaf is possible and such experiments are described both here and in the following chapter.

In **Chapter 5**, it was shown that S could be made to accumulate to significant quantities in both individual epidermal trough cells and representative whole leaf sections from wheat grown under a high S low N nutrient regime. On the basis of this demonstration, experiments were designed to ascertain whether epidermal and whole leaf S reserves could be re-mobilized when plant SO_4^{2-} supply was withdrawn. In a preliminary experiment, single cell analyses were confined to epidermal trough cells by virtue of their relative ease of sampling. CON and INV plants were grown and analysed as before (see **Chapter 5**) whilst the SO_4^{2-} supply to both was withdrawn at day 13 post full-expansion. It was reasoned that any noticeable decrease in vacuolar S concentration after day 13 would represent a net export of stored S-compounds from the cell. The design of the experiment enabled an investigation into the effects of S re-mobilization on the behaviour of other vacuolar solutes to be made. In combination with SiCSA data, quantification of anions in bulk leaf preparations provided further information on tissue heterogeneity.

It could be assumed that the cereal epidermis behaves as a uniform unit in terms of its solute relations. However, studies by Leigh and Storey, 1993; Hinde, 1994 and Fricke *et al.*, 1995; 1996 (amongst others) have shown that the many anatomically distinct types of epidermal cell (see **Plate 7**) differ also with respect to their vacuolar solute content. Therefore, the single cell analyses approach was extended to encompass three morphologically distinct types of epidermal cell. This enabled an investigation to be made as to whether the epidermis behaves as a uniform unit in terms of its accumulation and partitioning of S.

6.3, METHODS

6.3.1, Can trough cell S be re-mobilized?

For the preliminary experiment, two 3 l tubs were set up (see **2.2** for further details) for each of the CONtrol (1 : 8 S : N), INVerse (8 : 1 S : N) and NONe (0.0008 : 1 S : N) nutrient treatments. The composition of the CON and INV solutions used in this experiment is provided in **Table 5.1** (preceding chapter) whilst the formulation of NONe solution is presented in **Table 6.1** (this chapter).

In the case of all three nutrient treatments, plant analyses began when the third leaf was approximately 2 days from full expansion with subsequent measurements being made at 4, 10, 13, 16, 19, 22, and 34 days post full-expansion. On each occasion, three plants were removed from the two growth tubs per treatment and single cell vacuolar sap samples taken from epidermal trough cells in the middle of the third leaf in an area situated between the 4 th and 5 th ridge on the left-hand side of the main vein. Five epidermal trough cells were sampled per plant and osmotic pressure and electrolyte concentrations were determined by picolitre osmometry (see **2.4.2**) and X-ray microanalysis (see **2.4.3**) respectively. Quantification of the major anions in bulk sap preparations from single-cell-sampled plants was performed by HPLC analysis (see **2.3.4.1**). At day 13 post full-expansion, CON and INV material were transferred to the SO_4^{2-}-deficient (NO_3^--sufficient) NON solution with the aim of inducing re-mobilization of stored S by withdrawal of plant SO_4^{2-} supply. Throughout this experiment, NON material was grown continuously in the 0.0008 : 1 S : N NON nutrient solution.

A 1.0 ml growth medium sample was removed daily from each of the six tubs and frozen at -20 °C pending quantification of anion concentrations by HPLC (see **2.3.4.3**). Daily records of growth media pH were collected (results not shown) and the media renewed every three days.

6.3.2, Do different types of epidermal cell re-mobilize S at the same rate?

Three 3 l tubs were set up (see **2.2** for further details) for both CONtrol and INVerse nutrient treatments (see **Table 5.1** for the composition of CON and INV nutrient solutions). As in the first experiment, plant analyses began when the third leaf was approximately 2 days from full expansion although on this occasion, subsequent measurements were made only at 4, 10, 16 and 22 days post full-expansion. At each

of the five sampling points, three plants were removed from the three growth tubs per nutrient treatment. Single cell vacuolar sap samples were then removed from the upper epidermal trough, ridge and near-stomatal cells (see **2.4.1**) in the middle of the third leaf in an area situated between the 4 th and 5 th ridge on the left-hand side of the main vein. Three cells were sampled per plant for each of the three epidermal cell types and osmotic pressure, electrolyte and NO_3^- concentrations were determined by picolitre osmometry (see **2.4.2**), X-ray microanalysis (see **2.4.3**) and NO_3^- microfluorometric assay (see **6.4.1**) respectively. Once single cell sampling requirements had been met, a bulk tissue sap preparation was obtained as described in **2.3.2**. This was then frozen at -20 °C pending subsequent quantification of anion concentrations and OP by HPLC (see **2.3.4.1**) and vapour pressure osmometry (see **2.3.3**) respectively.

On day 13 post full-expansion, both CON and INV material were transferred to the SO_4^{2-}-deficient (NO_3^--sufficient) NON solution (see **Table 6.1**, this chapter) with the aim of inducing re-mobilization of stored S by withdrawal of SO_4^{2-} supply. From day 13 onwards, all plants were grown continually in NON solution. Growth media were sampled, analysed and replaced every three days as above (**6.3.1**).

Table 6.1. Composition of the SO_4^{2-}-deficient (NO_3^--sufficient) NON nutrient solution used in this investigation. Concentrations are expressed in mM. Before use, the pH of each batch of growth solution was corrected with 10 M NaOH so that the final pH lay in the optimal range for plant growth (pH 6.1-6.3).

Component		***NON*e** 0.0008 : 1 S : N
Macronutrients...	Ca^{2+}	4.000
	Mg^{2+}	1.500
	K^+	4.000
	HPO_4^{2-}	1.330
	SO_4^{2-}	0.012
	NO_3^-	15.000
Micronutrients...	Si	0.047
	Fe^{2+}	0.100
	Mn^{2+}	0.010
	Cu^{2+}	0.001
	Zn^{2+}	0.001
	Na^+	1.378
	BO_3^-	5×10^{-5}
	MoO_4^{2-}	4×10^{-4}

6.4, THE MICROFLUOROMETRIC ENZYMATIC NO_3^- ASSAY

Since it is not possible to quantify single cell NO_3^- concentrations with X-ray microanalysis, use of an NADPH-linked enzymatic assay is required. Nitrate reductase (NR) is the first enzyme in the N assimilation pathway of higher plants and it catalyses the two-electron reduction of NO_3^- to NO_2^- (Marschner, 1995). The electron donors for this reaction *in vivo* are the co-factors NADH and NADPH and the quantity of co-factor oxidised in the reaction is stoichiometric with the amount of NO_3^- reduced to NO_2^-. This stoichiometry (coupled with the fact that the oxidised and reduced forms of the co-factors are optically distinct (see **Chapter 4**) has led to the successful exploitation of the reaction *in vitro* as a means of quantifying NO_3^- in foodstuffs and other materials (Boehringer, 1984).

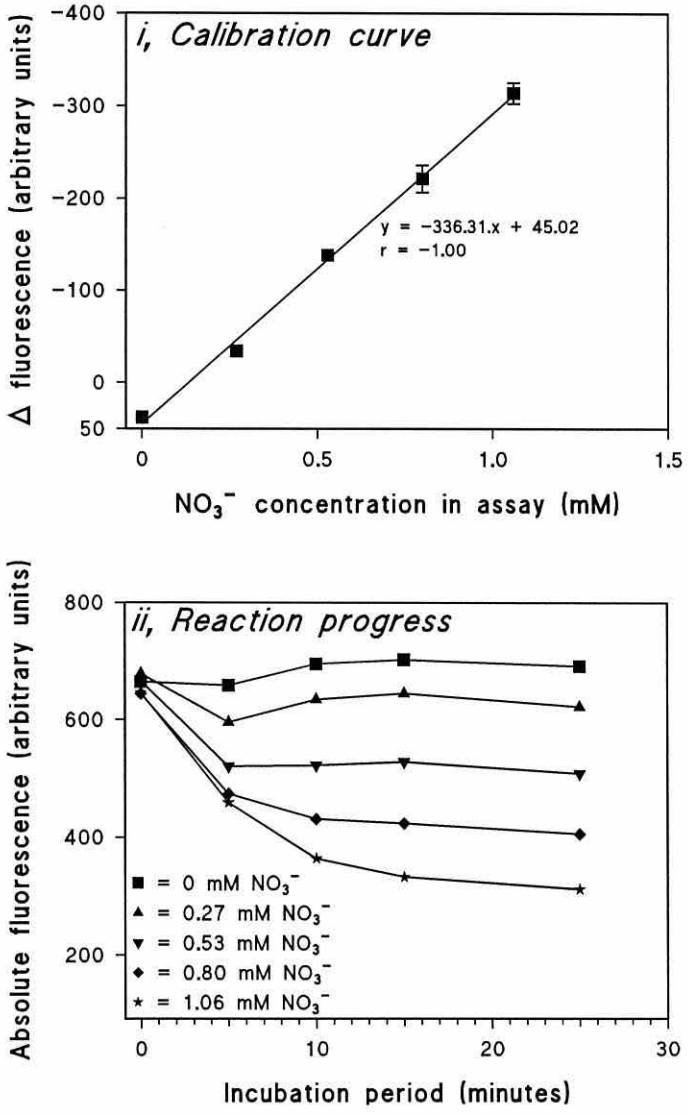
The published method for the NR-dependent NO_3^- assay (Boehringer, 1984) has been miniaturised to run at the microfluorometric scale on a number of separate occasions (see for example, Zhen *et al.*, 1991, Richardson, 1993 and Tomos *et al.*, 1994). Unfortunately, however, a problem is encountered in the transition from a 3ml final assay volume to the *circa* 5 nl required for microfluorometric enzymatic assays in that the miniaturised assay is accompanied by an enzyme-dependent, substrate-independent decrease in fluorescence (Richardson, 1993). Whilst the exact cause of this response is unknown, the inclusion of both NADPH and structurally intact NR is essential (Richardson, 1993). This suggests that the decrease in fluorescence could be due to an interaction between these two components. Following an extensive period of development (see **Appendix 2**), a new method for the microfluorometric enzymatic assay of NO_3^- was derived in which the effects of the substrate-independent reaction were either minimised or completely absent.

6.4.1, Protocol for NO_3^- microfluorometric enzymatic assay

In this revised method, the assay concentrations of imidazole buffer and FAD were identical to those originally specified by Boehringer, 1984. Final concentrations of NADPH and NR on the other hand, were much higher, resulting in an initially large fluorescence 'signal' relative to background noise and a rapid conversion of substrate to product. The optimum assay concentrations of NADPH and NR respectively were 6x (1.3 mM) and 35x (2.4 U ml^{-1}) higher than those in the original protocol (0.2 mM and 0.07 U ml^{-1}; Boehringer, 1984).

It was critical for this assay that the volumes of the three constriction pipettes was measured accurately and the method for determining volumes is described in section **2.4.1**. A 100 μl volume of assay cocktail was prepared by mixing 25 μl of 144 mM imidazole buffer (pH 7.8), 25 μl of 17 μM FAD, 25 μl of 6 mM NADPH, 10 μl of 1% w/v BSA and 15 μl de-ionised water. Approximately 5 μl of assay cocktail, each of the following NO_3^- standards (0, 100, 200, 300, 400 and 500 mM KNO_3) and 22 U ml^{-1} NR were transferred to the edge of a clean, water-saturated liquid paraffin-filled microscope slide-mounted well using a 50 μl Hamilton glass syringe. The required number of assay cocktail droplets were measured out into the centre of the well using a *circa* 4 nl non-silanized pipette and standards were subsequently added with a *circa* 12 pl silanized pipette. Microdroplet fluorescence was recorded prior to the addition of *circa* 500 pl (the 'initiating factor') of 22 U ml^{-1} NR. The fluorescence intensity of the microdroplets was assessed every 5 minutes until reaction was complete. The results are given in **Figure 6.1**. For a more thorough description of the methodology involved in running a microfluorometric enzymatic assay, see **4.3.6**.

Figure 6.1. Testing the microfluorometric NO_3^- enzymatic assay. Graph (i) shows the calibration curve obtained from a plot of change in fluorescence *versus* NO_3^- concentration. When absorbencies were measured every 5 minutes for a total of 20 minutes (graph (ii) it was shown that (a) reaction reached completion in 10 minutes and (b) the substrate-independent reaction was absent. For each datapoint, n = 2.



6.5, RESULTS

6.5.1, Sulphur and sulphate

As reported previously (see **Chapter 5**), S accumulated to significant concentrations in the upper epidermis and whole leaves of plants grown in the high S low N (INV) nutrient solution (see **Figures 6.2-6.4**). In those plants supplied with lower concentrations of SO_4^{2-} (CON and NON), epidermal and whole leaf S reserves were significantly lower (GLM; $p < 0.001$).

When the single cell analyses approach was extended to cover other types of epidermal cell (ridge and near-stomatal cells; see **6.3.2**), it became apparent that S accumulated differentially in the upper epidermis of INV plants. This differential accumulation of S, however, was absent in CON material (**Figure 6.4**). From 2 days pre to 13 days post full-expansion, the highest epidermal S concentrations in INV material were recorded in trough cells where values were significantly higher (ANOVA; $p < 0.05$) than those of either ridge or near-stomatal cells from the same plant.

In the preliminary experiment (**6.3.1**), the transferral of both CON and INV material to NON nutrient solution at day 13 resulted in a slow but significant (ANOVA; $p < 0.05$ for both treatments) decrease in epidermal trough cell S concentrations over time (**Figure 6.2**). Unfortunately, this observation was not apparent from either bulk sap SO_4^{2-} measurements (**Figure 6.3**) or from the single cell data when the experiment was repeated to assess different cell types (**6.3.2**; **Figure 6.4**). The reason for this is thought to be due to missing data rather than being attributable to variation in plant behaviour. Quite simply, it was not possible to obtain a bulk sap preparation from the heavily senesced third leaf at day 34. Similarly, differences in duration of experiment 1 (terminated when the third leaf was 34 days old) and experiment 2 (terminated when the third leaf was 22 days old) could explain the dissimilarities between the single cell datasets.

Those plants grown continuously in NON nutrient solution (see **6.3.1**) accumulated significant amounts of SO_4^{2-} (**Figure 6.2**). This observation was unexpected since NON solution contained just 12 μM SO_4^{2-} and was designed to induce S deprivation in CON and INV plants. However, a possible explanation for the accumulation of SO_4^{2-} by NON material is provided by **Figure 6.5**. Note, that as the third leaf reached full expansion, the mean SO_4^{2-} concentration in the NON tubs

increased to *circa* 1.5 mM whilst the mean NO_3^- concentration decreased simultaneously from 18 to 15 mM. These data would seem to suggest that CON nutrient solution may have been added in error to one (or both) of the NON tubs during the earlier stages of experiment 1.

6.5.2, Osmotic pressure

In both of the experiments described in this chapter, plants grown in INV nutrient solution had significantly lower single cell and bulk sap OP than CON or NON material (**Figures 6.6-6.8**). At 2 days pre full-expansion, epidermal and bulk sap OP were similar in CON, NON and INV material. However, as leaves matured INV OP decreased such that by day 4 post full-expansion, epidermal and bulk sap OP were significantly lower (GLM; $p < 0.001$ for single cell and bulk sap data from both experiments) in INV plants than in CON or NON material. This decrease in epidermal OP was not uniform between the three cell types (**Figure 6.8**) and reduction was significantly greater (GLM; $p < 0.05$) in trough and near-stomatal cells than in ridge cells. In contrast, there were no significant differences between OP values of the three epidermal cell types in CON plants throughout the entire experiment (**Figure 6.8**).

The transferral of CON material to NON nutrient solution at day 13, had (as expected) no significant effect on either epidermal or bulk sap OP (**Figures 6.6-6.8**). However, the transferral of INV material resulted in two seemingly contradictory observations from the two separate experiments of this chapter. In experiment 1, restoring NO_3^- supply to INV plants resulted in a rapid increase in epidermal OP such that values returned to CON/NON levels in less than three hours (**Figure 6.6**). In experiment 2, the transferral process had no significant effect on epidermal OP even 9 days after restoration of NO_3^- supply (**Figure 6.8**). The implications of these apparently contradictory observations will be discussed later in section **6.5**.

Figure 6.2. Can epidermal trough cell S reservoirs be re-mobilized? The effects of S deprivation on trough cell S concentrations in plants previously exposed to a S sufficient (CON) or excess S (INV) nutrient regime. Results are expressed as means ± SD for the 3 plants per sampling point in experiment 1.

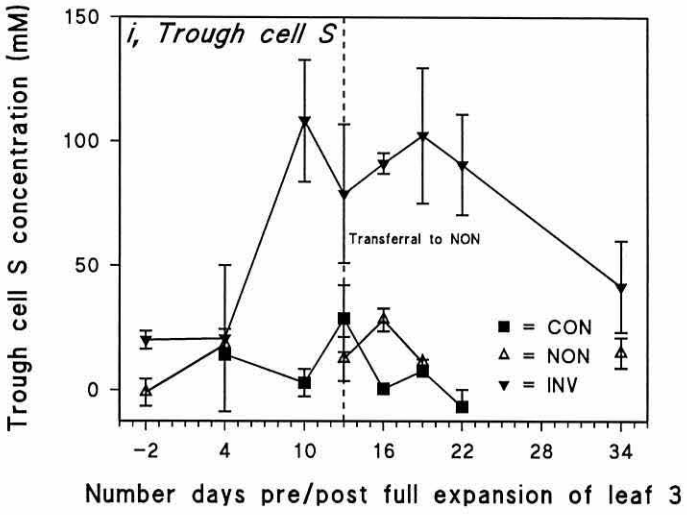


Figure 6.3. Can bulk tissue sap S reservoirs be re-mobilized? The effects of S deprivation on leaf sap SO_4^{2-} concentrations in plants previously exposed to a S sufficient (CON) or excess S (INV) nutrient regime. Results for CON and INV are expressed as means of the two experiments. Results for NON are the means ± SD for the 3 plants per sampling point in experiment 1.

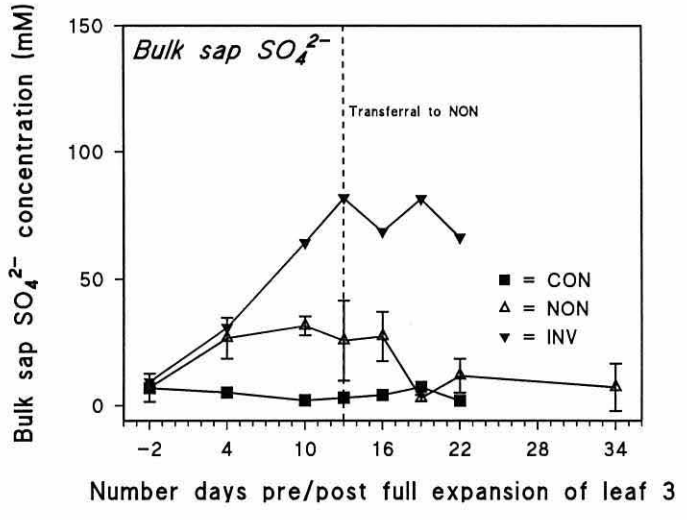


Figure 6.4. Do different types of epidermal cell re-mobilize S at the same rate? The effects of S deprivation on trough, ridge and near-stomatal cell S concentrations in plants previously exposed to a (i) S sufficient (CON) or (ii) excess S (INV) nutrient regime. Results are expressed as means ± SD for the 3 plants per sampling point in experiment 2.

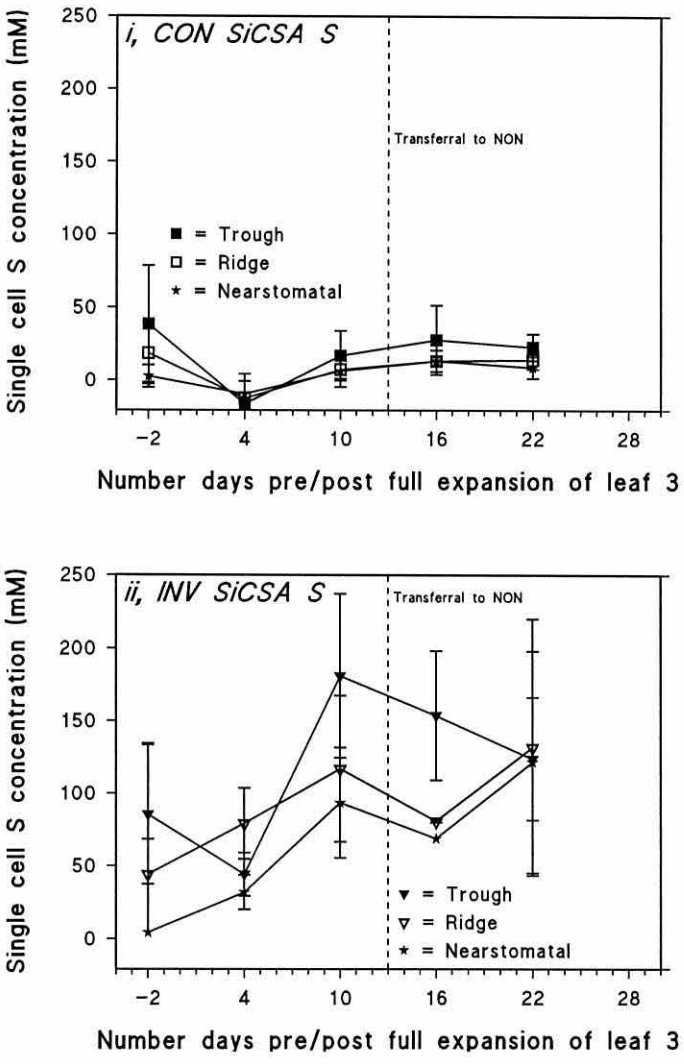
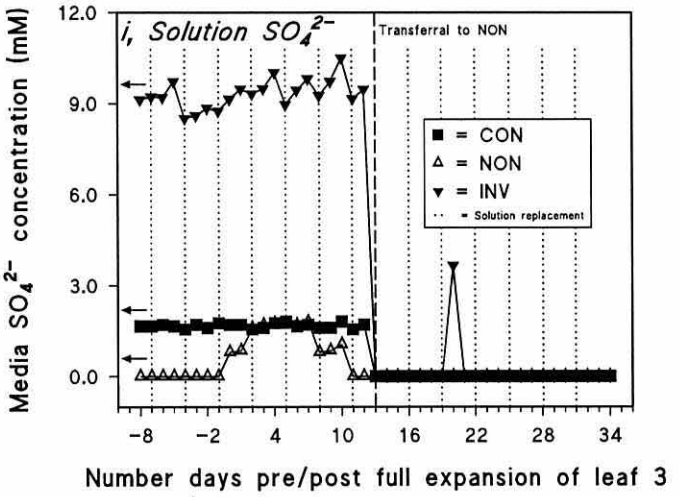


Figure 6.5. Changes in (i) SO_4^{2-} and (ii) NO_3^- supply to plants grown under the CON, NON or INV nutrient regimes of experiment 1. The availability of anions to the plant was considered to be approximately equal to the concentrations of Cl^-, NO_3^- and SO_4^{2-} measured in daily medium samples by HPLC (see **2.3.4.3**). For each datapoint, n = 2.



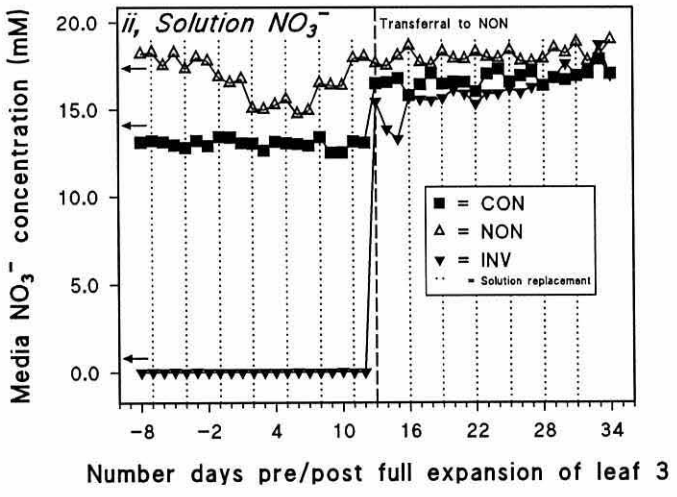


Figure 6.6. Does re-mobilization of S affect cell water relations? The effects of S deprivation on trough cell OP in plants previously exposed to a S sufficient (CON) or excess S (INV) nutrient regime. Results are expressed as means ± SD for the 3 plants per sampling point in experiment 1.

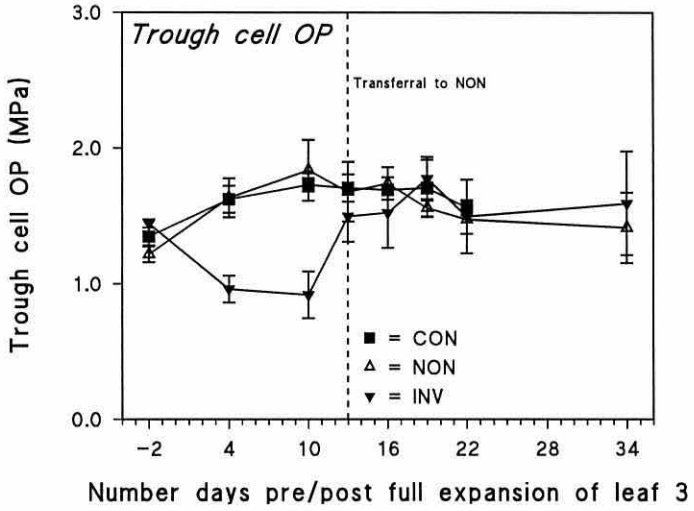


Figure 6.7. Does re-mobilization of S affect cell water relations? The effects of S deprivation on leaf sap OP of plants previously exposed to a S sufficient (CON) or excess S (INV) nutrient regime. Results are expressed as means ± SD for the 3 plants per sampling point in experiment 2.

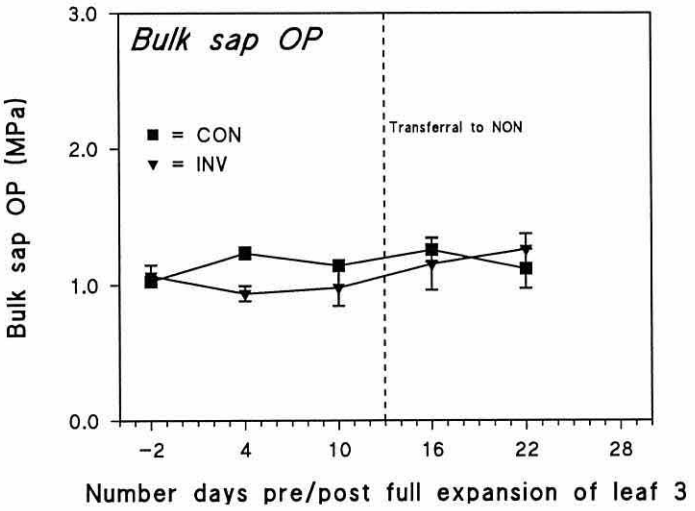
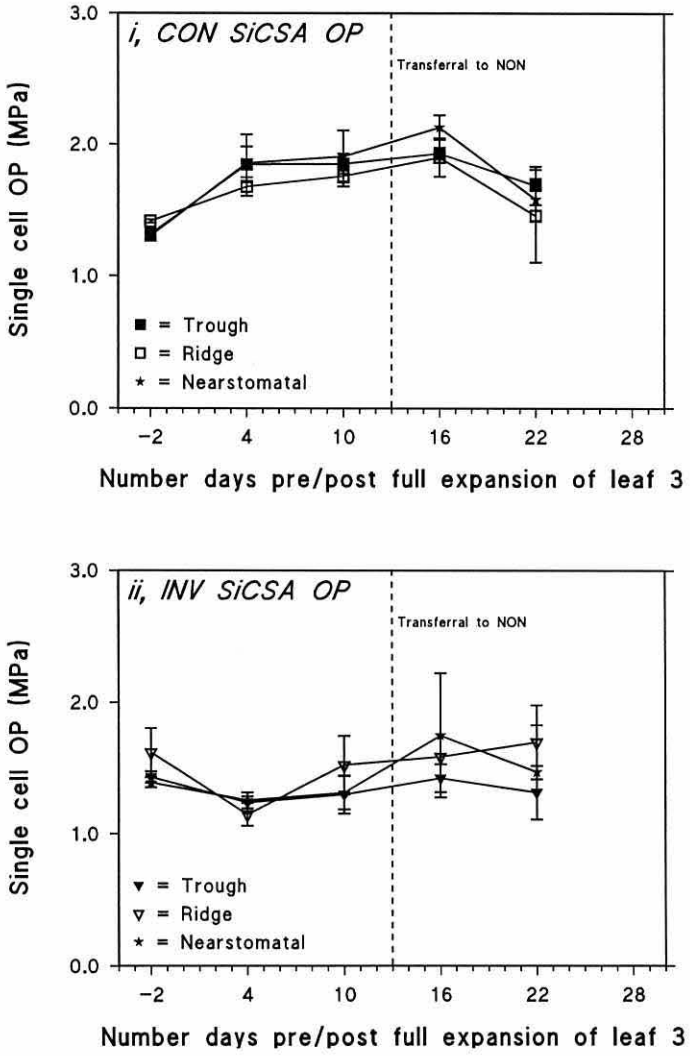


Figure 6.8. Does re-mobilization of S affect cell water relations? The effects of S deprivation on trough, ridge and near-stomatal cell OP of plants previously exposed to a (i) S sufficient (CON) or (ii) excess S (INV) nutrient regime. Results are expressed as means ± SD for the 3 plants per sampling point in experiment 2.



6.5.3, Potassium

In accordance with the results of the preceding chapter, the accumulation of excess S in the upper epidermis of INV material coincided with a significant decrease (GLM; $p < 0.001$ for experiments 1 and 2) in the amount of K^+ exported from the cell (**Figure 6.9 and 6.10**). By day 10 post full-expansion for example, the epidermal K^+ concentrations of INV material were two-fold higher than those measured in CON plants of the same age (**Figure 6.10**). In terms of epidermal heterogeneity, there were no significant differences in the export of K^+ from the three cell types in INV material. However, in CON plants, ridge cell K^+ concentrations were significantly higher (GLM; $p < 0.001$) throughout this experiment than those measured in either trough or near-stomatal cells from the same plant (**Figure 6.10**). Subsequent transferral of plants to NON solution had negligible effect on the vacuolar K^+ export rates measured in the epidermal cells from either CON or INV material. There appeared to be no difference between CON or NON treatments in terms of epidermal K^+ export.

6.5.4, Calcium

Accumulation of Ca^{2+} by epidermal cell vacuoles (as a function of leaf age) was significantly impaired (GLM; $p < 0.001$ for experiment 1; $p < 0.05$ for experiment 2) in plants grown initially in INV nutrient solution (**Figures 6.11** and **6.12**). In spite of this, the distinct distribution of Ca^{2+} between the three epidermal cell types was maintained even in the INV material. As a consequence, Ca^{2+} concentrations were highest in the trough and near-stomatal cells and significantly lower (GLM; $p < 0.05$ for both CON and INV material) in the ridge cells of both CON and INV plants. Transferral of material to NON nutrient solution had no effect on the slow rate of Ca^{2+} accumulation by INV plants. Consequently, epidermal Ca^{2+} concentrations remained generally lower in INV plants than in CON or NON material right up until the third leaf was entering senescence (*circa* day 18 post full-expansion in experiment 1). In those plants supplied with a low SO_4^{2-}, high NO_3^- nutrient regime (NON and/or CON), epidermal Ca^{2+} concentrations increased similarly as a function of leaf age. However, rate of Ca^{2+} accumulation appeared to 'tail-off' in both treatments (see **Figure 6.11**) once vacuolar concentrations had reached *circa* 300 mM.

Figure 6.9. Does re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on trough cell K^+ concentrations in plants previously exposed to a S sufficient (CON) or excess S (INV) nutrient regime. Results are expressed as means ± SD for the 3 plants per sampling point in experiment 1.

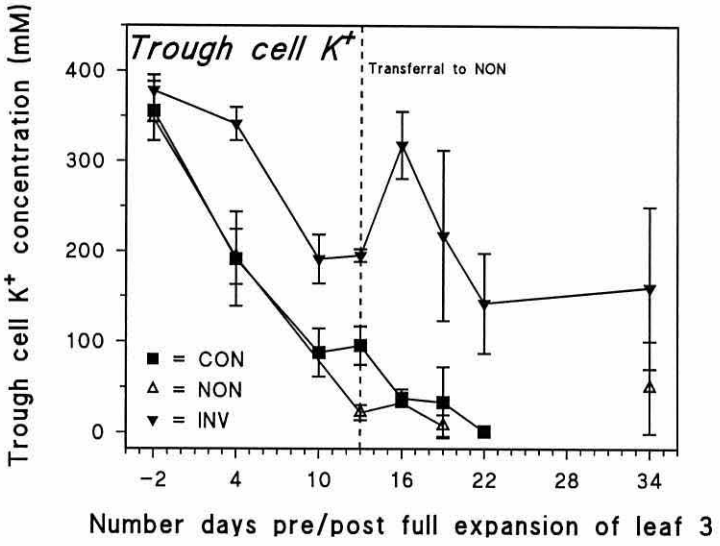


Figure 6.10. Does re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on trough, ridge and near-stomatal cell K^+ concentrations in plants previously exposed to a (i) S sufficient (CON) or (ii) excess S (INV) nutrient regime. Results are expressed as means ± SD for the 3 plants per sampling point in experiment 2.

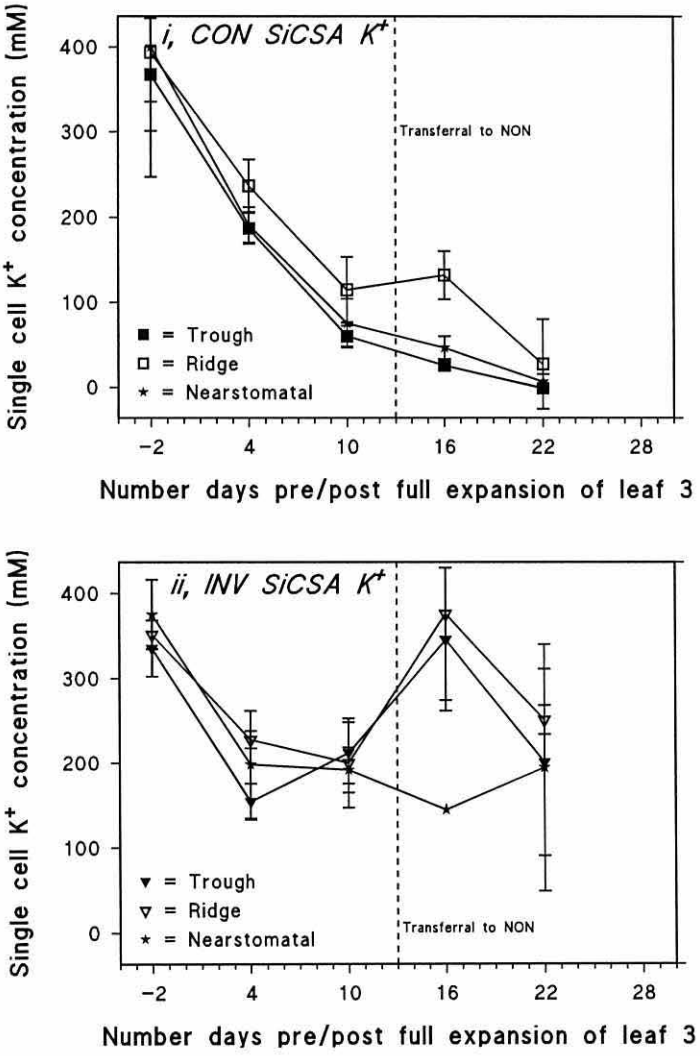


Figure 6.11. Does re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on trough cell Ca^{2+} concentrations in plants previously exposed to a S sufficient (CON) or excess S (INV) nutrient regime. Results are expressed as means ± SD for the 3 plants per sampling point in experiment 1.

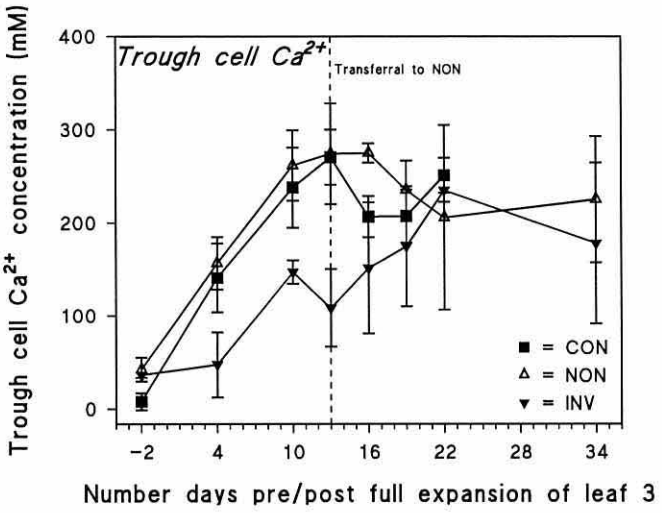
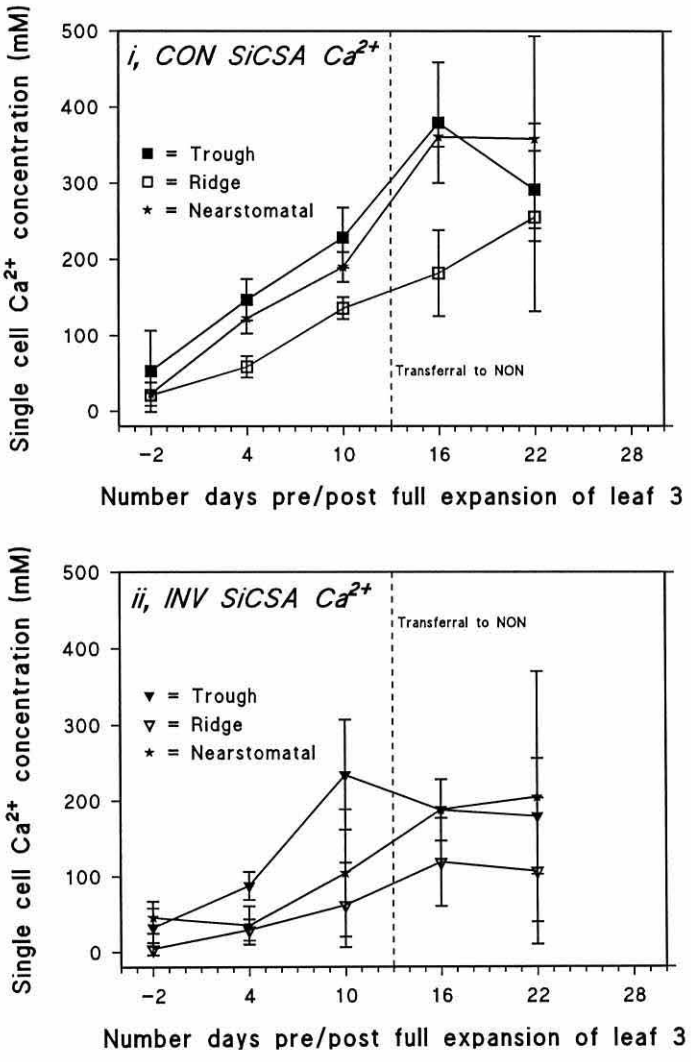


Figure 6.12. Does re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on trough, ridge and near-stomatal cell Ca^{2+} concentrations in plants previously exposed to a (i) S sufficient (CON) or (ii) excess S (INV) nutrient regime. Results are expressed as means ± SD for the 3 plants per sampling point in experiment 2.



6.5.5, Chlorine and chloride

Despite considerable fluctuation, individual epidermal cell Cl^- concentrations in CON, NON and INV material were generally low (less than 50 mM) in both experiments 1 and 2 (see **Figures 6.13** and **6.15**). In fact, values were so close to the detection limit of the EDX technique (*circa* 10 mM) that it is uncertain whether the fluctuations observed were significant. Bulk tissue sap Cl^- data fluctuated considerably less than those from single cells and mean leaf concentrations in CON, NON and INV material were consistently lower than 30 mM in both experiments (**Figure 6.14**).

6.5.6, Nitrate

Epidermal and whole leaf NO_3^- concentrations were highest in CON and NON material and significantly lower (GLM; $p < 0.001$ for bulk tissue sap and single cell data) in the INV plants (**Figures 6.16** and **6.17**). Irrespective of differences in nutrient supply however, epidermal NO_3^- concentrations in CON and INV material were remarkably conserved between the trough, ridge and near-stomatal cells (**Figure 6.17**). This may suggest that NO_3^- (rather than being distributed differentially) is in fact, accumulated more uniformly by the epidermis. There were no significant differences between the whole leaf NO_3^- concentrations of CON and NON plants (**Figure 6.16**). Under both nutrient regimes, leaf NO_3^- concentrations increased from 2 days pre full-expansion to peak at around 4 days post full-expansion. As leaves aged, NO_3^- concentration decreased and 200 mM NO_3^- was detectable as the leaf entered the final stages of senescence (*circa* day 22 post full-expansion).

As expected, the transferral of CON material to NON solution at day 13 had no effect on bulk sap NO_3^- concentrations (**Figure 6.16**). However, when NO_3^- supply was restored to INV material, there was an increase in leaf NO_3^- such that by day 22 post full-expansion, concentrations were the same in CON, NON and INV material.

Figure 6.13. Does re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on trough cell Cl^- concentrations in plants previously exposed to a S sufficient (CON) or excess S (INV) nutrient regime. Results are expressed as means ± SD for the 3 plants per sampling point in experiment 1.

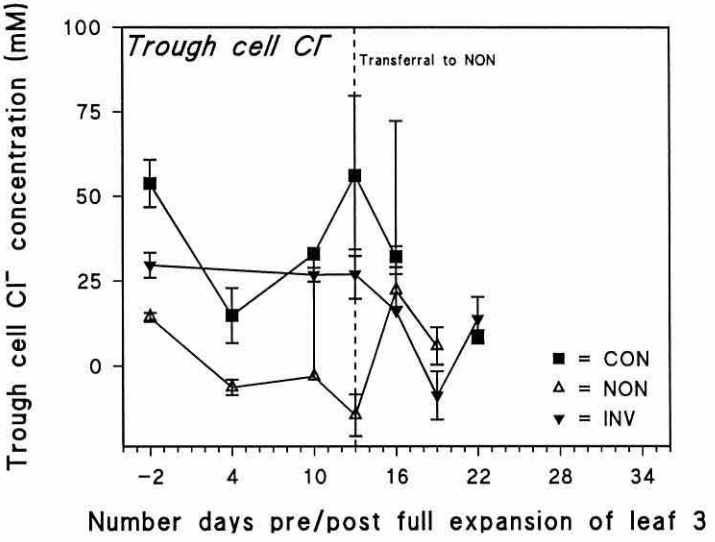


Figure 6.14. Does re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on leaf sap Cl^- concentrations in plants previously exposed to a S sufficient (CON) or excess S (INV) nutrient regime. Results for CON and INV are expressed as means of the two experiments. Results for NON are the means ± SD for the 3 plants per sampling point in experiment 1.

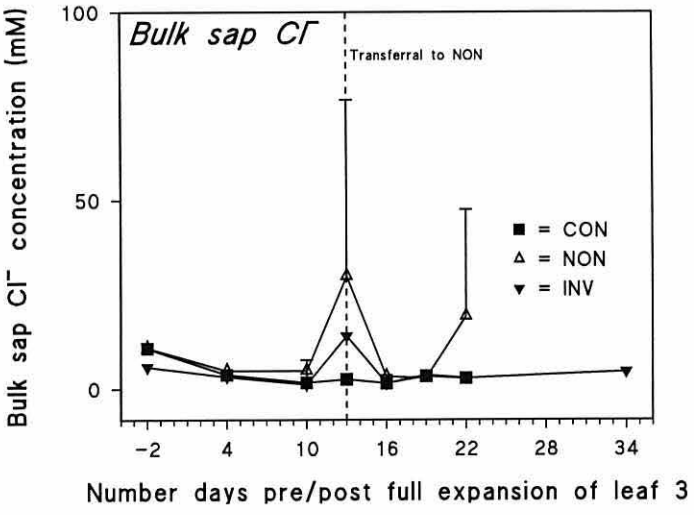
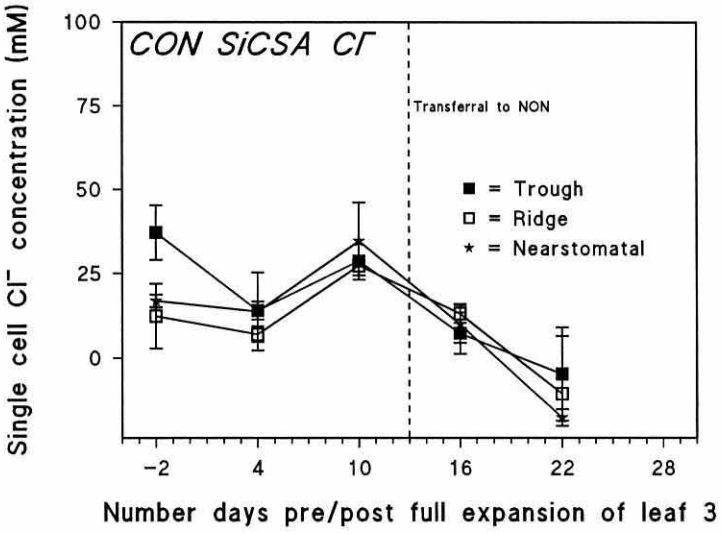


Figure 6.15. Does re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on trough, ridge and near-stomatal cell Cl^- concentrations in plants previously exposed to a (i) S sufficient (CON) or (ii) excess S (INV) nutrient regime. Results are expressed as means ± SD for the 3 plants per sampling point in experiment 2.



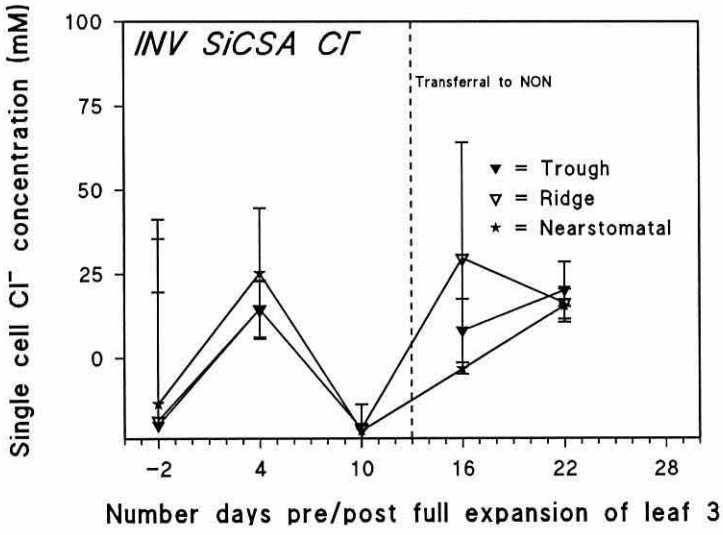


Figure 6.16. Does re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on leaf sap NO_3^- concentrations in plants previously exposed to a S sufficient (CON) or excess S (INV) nutrient regime. Results for CON and INV are expressed as means of the two experiments. Results for NON are the means ± SD for the 3 plants per sampling point in experiment 1.

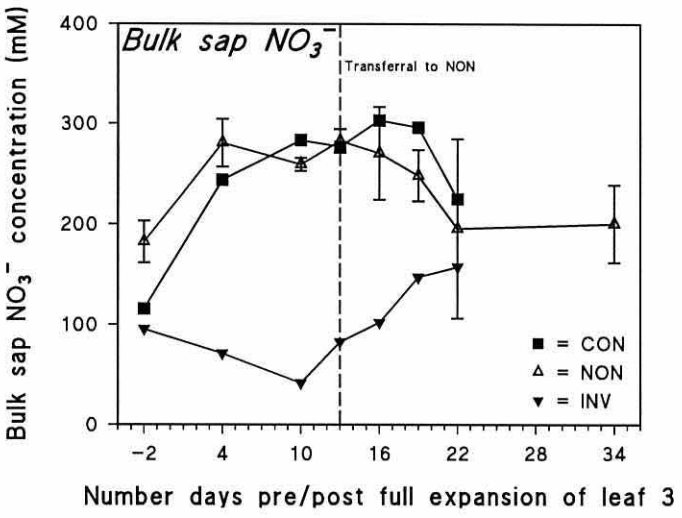
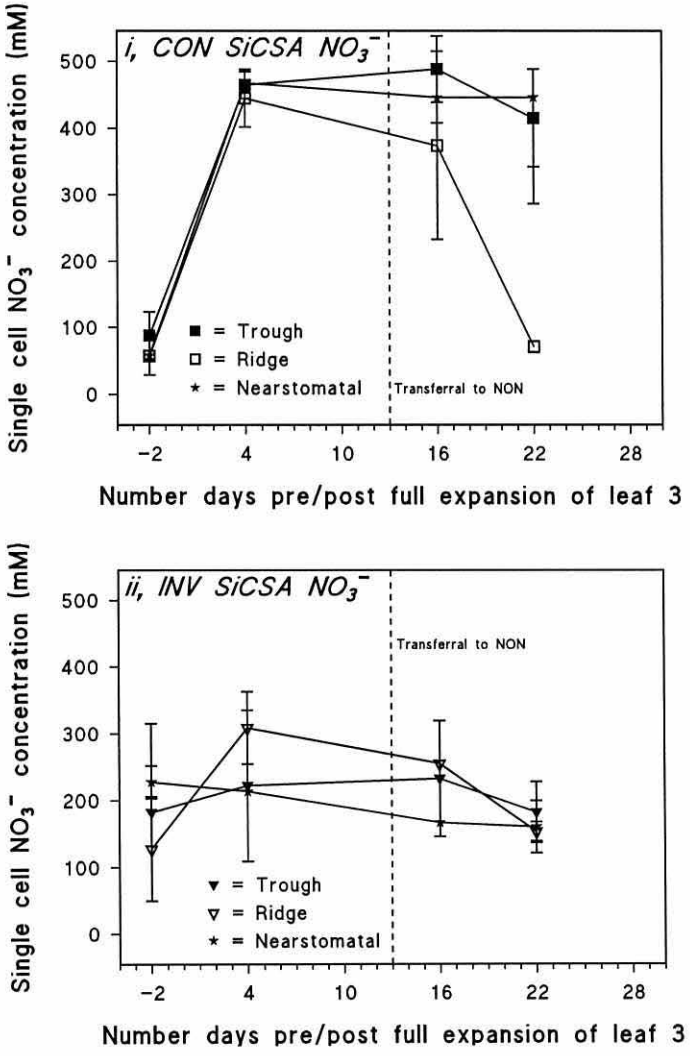


Figure 6.17. Does re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on trough, ridge and near-stomatal cell NO_3^- concentrations in plants previously exposed to a (i) S sufficient (CON) or (ii) excess S (INV) nutrient regime. Results are expressed as means ± SD for the 3 plants per sampling point in experiment 2.



6.6, DISCUSSION

6.6.1, Can epidermal trough cell and whole leaf S reservoirs be re-mobilized?

From **Figure 6.2**, it is apparent that the S which accumulates in the upper epidermis of wheat exposed to either the INV (7 mM SO_4^{2-}) or CON (1.5 mM SO_4^{2-}) nutrient regimes can be made to re-mobilize when the SO_4^{2-} supply is subsequently withdrawn. Similar observations have also been reported for tobacco (Rennenberg *et al.*, 1979), *Macroptilium atropurpureum* (Clarkson *et al.*, 1983; Bell *et al.*, 1995b), barley (Adiputra and Anderson, 1992) and wheat (Cooper and Clarkson, 1989) and there are many other accounts in which the imposition of a nutrient stress has been shown to promote the re-mobilization and redistribution of that nutrient from its source to its sink within the plant. The redistribution of N from mature leaves to developing grains for example, is enhanced when the N supply to the plants is withdrawn (for example, Pate, 1980; Simpson *et al.*, 1983; Mei and Thimman, 1984).

The re-mobilization of S from mature leaves is an inherently slow process as is evident from **Figure 6.2**. In the 21 day period from day 13 (withdrawal of SO_4^{2-} supply) to day 34 (final stages of leaf life) the mean epidermal S concentrations in INV plants decreased by just 47 % from 79 to 42 mM. Despite a lack of similar studies relating to the behaviour of S at the single cell level, accounts of whole leaf S re-mobilization in the current literature indicates that the decrease in epidermal S following the withdrawal of SO_4^{2-} supply is both slower and smaller than may have been expected. In *Macroptilium atropurpureum* for example (see Clarkson *et al.*, 1983), a 45 % decrease in mature whole leaf SO_4^{2-} concentrations was seen within a five day period following the reduction of SO_4^{2-} supply from 250 μM to 0 μM. Similarly, Bell *et al.* (1995b) recorded a 75 % decrease in mature leaf SO_4^{2-} in the same species, seven days after withdrawal of SO_4^{2-} supply. Possible explanations for the differences between the single cell and whole leaf S re-mobilization rates are provided in a subsequent discussion (see **Chapter 7**).

There was no appreciable re-mobilization of whole leaf S in CON material following withdrawal of SO_4^{2-} supply. The low concentrations measured in CON material throughout these experiments indicate that the third leaf had negligible S to export. Curiously, re-mobilization of leaf S was observed in plants supplied continually with negligible SO_4^{2-} (NON) in which the earlier accumulation of significant quantities of S had been recorded. Both of these observations were

unexpected since the NON nutrient solution was designed to induce S stress and should have contained just 12 μM SO_4^{2-}. The most plausible explanation for this unexpected accumulation of SO_4^{2-} is that the wrong growth solution was added to either one (or both) of the NON growth tubs during one (or more) of the solution changes. HPLC analyses of daily growth solution samples (see **Figure 6.5**) suggests that this may have been the case.

The contamination of NON nutrient solution meant that it was not possible to obtain SO_4^{2-} starved material in experiment 1 although interestingly, whole leaf S concentrations in NON plants were significantly higher than those measured in CON material from day 0 to day 10 post full-expansion (see **Figure 6.3**). As described for NO_3^- (see for example, Clement *et al*, 1979; Barneix and Causin, 1996 and references therein) the active uptake of SO_4^{2-} by root cells is considerably enhanced under conditions of S stress (for example, ten-fold in barley (Clarkson *et al.*, 1989), six-fold in canola (Lappartient and Touraine, 1996) and two-fold in *Brassica napus* (Hawkesford *et al.*, 1993). This enhancement of SO_4^{2-} uptake is thought to be directly controlled by the S status of the plant via a feedback mechanism (Rennenberg, 1984; Cram, 1990; Clarkson *et al.*, 1993).

It is, therefore, assumed that the capacity of the negligible-S NON plants to absorb SO_4^{2-} from a hydroponic solution would have been greatly increased if and when a SO_4^{2-} supply had become available. As such, the accidental addition of SO_4^{2-} to NON growth tubs restored the SO_4^{2-} supply which is thought to have led to an enhanced uptake of SO_4^{2-} by the plant root system. Since any S absorbed by the plant in excess of that required for optimal plant growth is accumulated (Singh and Singh, 1977) suggests that the elevated amounts of SO_4^{2-} absorbed by the previously S stressed root system would result in higher plant S concentrations. This explanation would certainly appear to be true for the NON plants in experiment 1 and similar observations have been made both for *Macroptilium atropurpureum* (Clarkson *et al.*, 1983) and barley (Adiputra and Anderson, 1995). In barley, the transferral of vegetative material from a nutrient solution containing 5 μM SO_4^{2-} (sulphur-limiting) to one of 25 μM SO_4^{2-} (sulphur-sufficient) led almost to a doubling of total leaf S within 6 days in mature, full expanded leaf tissue.

6.6.2, Do different types of epidermal cell re-mobilize S at the same rate?

The unavoidable termination of experiment 2 at day 22 meant that this study did not achieve its aim of determining whether different types of epidermal cell re-mobilize S at differing rates. Question 2 (see section **6.1**) was, therefore, unanswered. Nevertheless, it is apparent from **Figure 6.4, graph (ii)** that when supplied to excess, S is accumulated (and subsequently distributed) differentially within the upper epidermis. Prior to the termination of plant SO_4^{2-} supply, single cell S concentrations in INV plants were consistently lower in the near-stomatal cells than in the trough or ridge cells. At day 10 post full-expansion for example, the mean trough cell S concentration was 181 mM, a value 36 % and 49 % higher respectively than the mean concentrations recorded in ridge (117 mM) and near-stomatal cells (93 mM) on the same day.

There was no clear differential distribution of S within the upper epidermis of CON material which suggests that when growth solution SO_4^{2-} availability is lower, the epidermal distribution of S is more uniform. This lack of epidermal heterogeneity in low S plants is in agreement with Cuin (1996) who found that there was no difference in upper epidermal S concentrations in trough, ridge or in cells around the stomata in the mid-section of the third leaf of barley when supplied with 0.5 mM SO_4^{2-}.

As reported previously (see **Chapter 5**), whole leaf S concentrations were consistently lower than those measured in individual epidermal cells from the same plant. This was true irrespective of nutrient treatment and suggests that S may be distributed differentially within the various cell layers of the cereal leaf. A more thorough investigation into this possibility is described in the following chapter (see **Chapter 7**).

6.6.3, Does re-mobilization of S affect cell water relations?

In both of the experiments described in this chapter, the accumulation of substantial amounts of S in the leaves of INV plants coincided with a significant reduction in both whole leaf and epidermal OP. A comparison of the data from INV and CON plants at day 10 revealed that differences in OP between the two nutrient treatments lay within a range of 15 % (whole leaf and ridge cell measurements of **6.3.2**) to 50 % (epidermal trough cells in **6.3.1**).

These observations contrast with those made in a preceding chapter (see **Chapter 5**) in which epidermal OP was relatively constant in spite of the significant accumulation of S. In this previous chapter, it was suggested that these 'relatively constant' values were in fact, attributable to high plant Cl^- concentrations resulting from the daily addition of 6 M HCl to nutrient solutions. In this chapter, the Cl^- concentrations of INV plants were much lower (*cf.* **Figures 6.13-6.15** with **Figure 5.7**) whilst the accumulation of S appears to correlate with a lower OP. This suggests that the previous assumptions relating high plant Cl^- concentrations to maintenance of constant OP in INV material are correct.

The fact that epidermal OP only dropped when plant Cl^- (and NO_3^-, see **Figures 6.16** and **6.17**) concentrations were low suggests that divalent anions (such as the accumulating SO_4^{2-} in INV plants) are unable to maintain plant water relations. These observations contrast with those of an earlier study in which growth of wheat under similar conditions led to the suggestion that changes in cell solute composition occurred without any major alteration in OP (see Richardson, 1993 (also reviewed in Leigh and Tomos, 1993). In this previous investigation, it was shown that the lower epidermal cells of the second leaf of wheat were able to maintain a constant OP following exposure to a low NO_3^-, high SO_4^{2-} (0.1 mM and 4.7 mM respectively) hydroponic solution.

Whilst it is conceivable that anion availability may affect the second and third leaves of a plant differently, a more plausible explanation for the differences between this study and the investigation of Richardson (1993) relates to the frequency of measurements and the duration of the two different experiments. In the earlier study, the quantification of single cell OP was confined to a single assessment point around the time of leaf maturation. This investigation on the other hand, clearly demonstrates (see **Figures 6.2-6.17**) that differences in plant nutrition (especially with regards to SO_4^{2-} availability) have progressively more affect on epidermal and whole leaf water and solute relations as leaves age. It is certainly possible that a continuation of the study by Richardson (1993) to include more assessment points (thus encompassing a greater proportion of the leaf 'life-cycle') may have led to findings similar to those of the current investigation. Unfortunately, neither experiment 1 or 2 proved conclusively that re-mobilization of S affects cellular water relations since the imposition of SO_4^{2-} deprivation and restoration of NO_3^- supply at

day 13 resulted in two contradictory observations from the two experiments. In the first, the transferral of high S (low N) plants to a high N nutrient solution resulted in a massive increase in epidermal OP of some 60 % within three hours of the transfer. This substantial and rapid increase in epidermal OP was absent from the second experiment and even nine days after the transfer process, epidermal OP remained lower than in CON material. So which result is most plausible? The data for epidermal S, K^+, Ca^{2+}, Cl^- and whole leaf NO_3^- from experiment 1 (**Figures 6.2**, **6.9**, **6.10**, **6.13** and **6.16** respectively) fail to demonstrate the 'leap' in concentrations which may have been expected from the 0.5 MPa (204 mOsmol kg^{-1}) jump in epidermal OP between days 10 and 13. The three hour timescale for this increase in epidermal OP is also doubtful by virtue of its rapidity. On the basis of the absence of any measurable increase in solute concentration coupled with the unrealistic speed of 'recovery' (*cf.* **Figure 6.6** with **Figures 6.7** and **6.8**) it is proposed that the rapid and substantial increase in epidermal OP observed in experiment 1 was attributable to an unexplained experimental artefact.

6.6.4, Does re-mobilization of S affect the behaviour of other cell solutes?

It would appear that the behaviour of other epidermal or whole leaf solutes is affected more by the accumulated S than by any subsequent re-mobilization of this solute following withdrawal of plant SO_4^{2-} supply. In the case of K^+ for example, exposure of plants to the INV nutrient regime resulted in a significantly diminished rate of epidermal K^+ export (see **Chapter 5**) which was maintained long after the restoration of NO_3^- and withdrawal of SO_4^{2-} supply (see **Figures 6.9** and **6.11** (this chapter). This diminished export rate contrasts both with the substantial re-translocation of K^+ from mature leaves of high NO_3^-, low SO_4^{2-} (CON and/or NON) material in this investigation and that reported by other workers when plants were grown under similar conditions (for example, Wolf and Jeschke, 1987; Hinde, 1994; Fricke *et al.*, 1995).

When NO_3^- was re-supplied to the N-starved INV material of experiment 1, an increase in leaf NO_3^- concentration was observed with values doubling in the nine day period from day 13 (mean leaf NO_3^-, 83 mM) to day 22 (mean leaf NO_3^-, 157 mM). Despite this restoration of leaf NO_3^- status, the re-translocation of K from epidermal cells remained impaired. This could suggest that it is the high cell S status

(rather than the NO_3^- status) which is either directly (or indirectly) responsible for the diminished export of K^+ from the vacuole. A suitable hypothesis is proposed and discussed further in **Chapter 9**.

In CON material, K^+ was lost differentially from the upper epidermis as leaves aged such that the concentrations measured in ridge cells were significantly higher (GLM; $p < 0.001$) than those in trough or near-stomatal cells from the same plant. These observations are in part agreement with those of Fricke *et al.*, 1995 in which epidermal K^+ concentrations were higher in trough and ridge cells than in the near-stomatal cells of barley grown under similar conditions. It is possible that species-specific differences are responsible for the discrepancy concerning trough cell observations. This differential distribution of K^+ was absent from the upper epidermis of INV material. The reason for this remains unclear.

The diminished accumulation of Ca^{2+} by the upper epidermis of INV material (see **Chapter 5**) was also unaffected by the restoration of NO_3^- and withdrawal of SO_4^{2-} supply. Following maturation of the third leaf (and as described previously, see **Chapter 5**), epidermal Ca^{2+} concentrations in the INV material were consistently lower than those recorded in CON plants. Only towards the final stages of leaf life (*circa* day 22) did Ca^{2+} concentrations in the high S (INV) and control S nutrient treatments (CON and/or NON) become similar. Whilst the accumulation of significant amounts of epidermal S results in a loss of the tight K-Ca inter-relationship (see **Chapter 5**), it is still possible that the impaired influx of Ca^{2+} into epidermal cells is related to the diminished rate of K^+ export. This possibility is covered further in **Chapter 9**.

In barley, Ca^{2+} is distributed differentially within the morphologically distinct cell types of the upper epidermis (Leigh and Storey, 1993; Leigh and Tomos, 1993; Fricke *et al.*, 1995; 1996; Hinde, 1994; Cuin, 1996). This distribution is both quantitatively and qualitatively dependent on the developmental stage of the leaf (Fricke *et al.*, 1995). If also true of wheat, this could explain why previous studies (see Malone *et al.*, 1991; Richardson, 1993 in which analyses were confined to a single assessment point in young leaves) have failed to demonstrate a similar differential distribution of Ca^{2+} within the epidermis. When the single cell approach was extended to cover multiple assessment points throughout leaf life (as in this investigation), it became clear that Ca^{2+} <u>is</u> distributed differentially within the upper

epidermis of wheat irrespective of nutrient treatment (see **Figure 6.12**). The differential distribution of Ca^{2+} within the wheat epidermis is similar to that described for barley (Fricke *et al.*, 1995; 1996; Cuin, 1996). The highest epidermal Ca^{2+} concentrations were recorded in the trough and near-stomatal cells of wheat whilst in barley, the highest concentrations were recorded solely in those cells closest to the stomates (see Fricke *et al.*, 1995; 1996; Cuin, 1996).

Since none of the nutrient solutions contained Cl^-, epidermal and whole leaf concentrations were low in both experiments regardless of nutrient treatment. The low values appeared to be unaffected by the accumulation and re-mobilization of S in INV plants. In fact, plant Cl^- concentrations were much lower in this chapter than those recorded in a previous experiment (see **Chapter 5**) and it was presumed that the main cause of this difference was the decision to stop adding HCl to nutrient solutions in an attempt to regulate solution pH. In **Chapter 5**, nutrient solution pH was corrected fastidiously with the result that high epidermal and leaf Cl^- concentrations were recorded in all plants regardless of nutrient treatment. In contrast, it would appear that the omission of this 'correction step' from the experiments described in this chapter is correlated with lower plant Cl^- concentrations.

Whilst epidermal and whole leaf Cl^- concentrations were lower in experiments 1 and 2 (this chapter) than in a previous experiment (*cf.* **Figure 6.13-6.15** with **Figure 5.7**) the reverse was true for whole leaf NO_3^- concentrations with values being higher in this chapter than in the preceding one (*cf.* **Figure 6.16** and **6.17** with **Figure 5.8**). In the third leaf of CON plants at day 10 for example, mean leaf Cl^- and NO_3^- concentrations were 13 mM and 187 mM respectively in **Chapter 5** and 3 mM and 291 mM in experiment 1 (this chapter). Whilst whole leaf OP measurements are unavailable for either experiment, the mean epidermal trough cell OP of CON material at day 10 was similar in both studies (1.8 MPa and 1.7 MPa for **Chapter 5** and this chapter respectively). This maintenance of near constant epidermal OP (despite different leaf Cl^- and NO_3^- concentrations) indicates that these monovalent anions are reciprocally substitutable. Thus, if the availability of either one of these anions is restricted, the other substitutable 'partner' will accumulate to higher concentrations within the cell vacuole to maintain both turgor and osmotic pressure. Similar observations have also been made for maize roots (Pritchard *et al.*, 1996),

barley (Dietz *et al.*, 1992; Fricke *et al.*, 1994c; 1995) and wheat epidermis (Richardson, 1993 (also reviewed in Leigh and Tomos, 1993).

When both Cl^- and NO_3^- supply are limited (as in the high S, low N plants of this investigation), there is a concomitant drop in OP (see **Figures 6.6** and **6.7**) which (as discussed previously), implies that the divalent SO_4^{2-} anion is unable to maintain plant water relations. The implications of these observations are considered in a subsequent section (see **Chapter 7**).

As expected, both epidermal and whole leaf NO_3^- concentrations were higher in CON plants (NON and/or CON) than in INV material. The restoration of NO_3^- supply to INV material at day 13 resulted in a slow, progressive increase in leaf NO_3^- concentration. Irrespective of nutrient treatment, however, epidermal NO_3^- concentrations were similar in trough, ridge and near-stomatal cells implying that this solute is distributed uniformly within the upper epidermis of wheat. These observations differ from those of Fricke *et al.*, 1995 where epidermal NO_3^- concentrations were highest in trough and lower in the ridge cells of barley grown under similar conditions. In all cases, epidermal NO_3^- concentrations were higher than comparable values measured in whole leaves from the same plant. This observation is similar to that made previously for other solutes (see **5.5**).

Since the OP of a solution is directly proportional to the concentration of dissolved solute present within it, it is possible to corroborate the values obtained by the two separate techniques of picolitre osmometry (see **2.4.2**) and EDX (see **2.4.3**) by comparing OP data with that for $\Sigma_{solutes}$ in the same sample of extracted vacuolar sap. This comparison of values can also highlight the presence of any additional, unmeasured solute. A selection of the data from the solute balance sheets for CON and INV material of experiments 1 and 2 is presented in **Table 6.2**. The terms $\Sigma_{solutes}$[1], Calculated[2], Difference[3], $\Sigma_{cations}$[4], Σ_{anions}[5] and Difference[6] used in the following section of this chapter are defined in the paragraph appearing below **Table 6.2**.

In the first experiment, there was a substantial difference (Difference[3]) between measured OP (Mean OP) and 'theoretical' OP (Calculated[2]) which indicated that a significant quantity of osmotically important solute had been unaccounted for. In the second experiment, the quantification of single cell NO_3^- and the incorporation of these data into the solute balance sheets coincided with a ten-fold decrease in the OP differences (Difference[3]) when compared with equivalent values from experiment 1. In

fact values for Difference[3] in the second experiment were so small as to suggest that nearly all epidermal cell solutes had been accounted for. In spite of this, however, a further solute imbalance (Difference[6]) still remained between $\Sigma_{cations}$ and Σ_{anions} in both CON and INV material indicating that another unmeasured (probably divalent) anion was present in the epidermis.

But what could be the identity of this missing anion? Fricke *et al.*, 1994c; 1995 have shown that malate (an intermediary component of the Kreb's Respiratory Cycle) can accumulate to significant quantities (in excess of 75 mM) in the upper epidermis of barley when plants are grown under high light intensities (*circa* 400 μmol m^{-2} s^{-1}). Under these conditions, an increased metabolic demand for NO_3^- leads to the re-mobilization of vacuolar NO_3^- pools whilst malate is accumulated by the vacuole to substitute for the osmotic role of NO_3^- and maintain turgor and osmotic pressure (Fricke *et al.*, 1995).

Whilst epidermal malate concentrations were not measured in these experiments, the use of the PAX-500 HPLC anion column (see **2.3.4.1**) during quantification of bulk tissue sap preparations facilitated the determination of malate in representative sections of the whole leaf. Irrespective of nutrient treatment and leaf age, whole leaf malate concentrations remained below 30 mM (results not shown). From **Table 6.2** it can be seen that the average imbalance (Difference[6]) between $\Sigma_{cations}$ and Σ_{anions} in the CON and INV material of experiment 2 was in the order of 200 mEq l^{-1}. If the missing anion was malate, an epidermal concentration of around 100 mM would be required to cancel this charge imbalance. As found in both this chapter and the preceding one (see **Chapter 5**), the concentration of many solutes (for example Cl^- and NO_3^-) is higher in the epidermis than in the whole leaf. When coupled with the fact that malate is known to accumulate preferentially in the upper epidermis (Fricke *et al.*, 1994c), it is feasible that the 100 mM charge 'shortfall' (Difference[6]) may have been due to unmeasured epidermal malate.

Table 6.2. A demonstration of the relationship between osmotic pressure and measured solute concentrations in individual epidermal trough cells from the third leaves of CON and INV material at day 16. For each experiment, values for osmolality and charge were calculated from the mean solute and OP data. Since $CaSO_4$ is insoluble at concentrations above *circa* 14 mM (Weast and Astle, 1980), when both Ca and S were detected in the same extracted vacuolar sap in excess of 14 mM, precipitation was assumed to have occurred and any contribution of that excess to $\Sigma_{solutes}$.

	Experiment	1	2	1	2
	Treatment	CON	CON	INV	INV
Mean Solute Concentration (mM)	[S]	*0.51*	*27.66*	*91.12*	*153.63*
	[Cl]	*32.13*	*7.17*	*16.15*	*8.03*
	[K]	*37.79*	*25.47*	*318.00*	*345.62*
	[Ca]	*206.66*	*379.54*	*151.35*	*187.60*
	[Mg]	*27.53*	*22.60*	*11.45*	*28.19*
	[P]	*0.00*	*6.46*	*0.00*	*18.72*
	$[NO_3^-]$	N/A	*488.22*	N/A	*232.52*
	$\Sigma_{solutes}$ [1]	304.62	901.80	405.83	667.05
Osmolalities (mOsmol kg^{-1})	Calculated[2]	249.78	739.47	332.78	546.98
	Mean OP	*690.18*	*786.48*	*621.72*	*579.02*
	Difference[3]	**446.26**	**47.00**	**288.94**	**32.04**
Vacuolar charge (mEq l^{-1})	$\Sigma_{cations}$ [4]	506.16	774.43	461.36	469.94
	Σ_{anions} [5]	-33.15	508.31	16.15	277.99
	Difference[6]	**473.01**	**266.12**	**445.21**	**191.95**

$\Sigma_{solutes}$ [1] - Sum of measured solute concentrations irrespective of charge.

Calculated[2] - $\Sigma_{solutes}$ x 0.82 (osmolarity co-efficient as per Fricke *et al.*, 1994c).

Difference[3] - Result of subtracting corrected[2] values from mean OP.

$\Sigma_{cations}$ [4] - Sum of [K], [Ca] and [Mg] respecting probable valencies.

Σ_{anions} [5] - Sum of [S], [Cl] and [P] respecting probable valencies.

Difference[6] - Product of $\Sigma_{cations}$ [4] - Σ_{anions} [5].

6.7, CONCLUSIONS

- When the SO_4^{2-} supply to both CON and high S (INV) material was withdrawn, epidermal S concentrations decreased gradually as leaves aged. Therefore, S (presumably as SO_4^{2-}) can be re-mobilized from mature leaf tissue but the process is slow.

- Excess S was accumulated and distributed differentially within the morphologically distinct trough, ridge and near-stomatal epidermal cells of INV plants. Concentrations were highest in the trough cells and lower in ridge and near-stomatal cells. Sulphur accumulated uniformly within the epidermis of CON material.

- In both of the experiments described in this chapter, the accumulation of S by INV plants led to a sizeable drop in both epidermal and whole leaf OP. However, values returned to 'control' levels when the NO_3^- supply was restored suggesting that divalent anions are unable to maintain plant water relations.

- Restoring the NO_3^- supply to INV material had minimal effect on the diminished epidermal export of K^+ and import of Ca^{2+} first seen in **Chapter 5**. Rates remained impaired even after transferral of INV plants to the high N (NON) nutrient solution. Irrespective of nutrient treatment, the differential distribution of Ca^{2+} within the upper epidermis was maintained.

- In the CON material of both experiments 1 and 2, leaf NO_3^- concentrations were higher whilst leaf Cl^- concentrations were lower than those recorded in a previous experiment (see **Chapter 5**). This apparent correlation between leaf Cl^- and NO_3^- concentrations suggests that these monovalent anions are reciprocally substitutable.

Chapter 7:

DO DIFFERENT TISSUE LAYERS TRANSPORT S AT DIFFERING RATES?

7.1, EXPERIMENTAL OBJECTIVES

The experiment described in this chapter was designed to answer five main questions.

1, *Is S accumulated and distributed differentially within the cereal leaf?*

2, *Do the different tissue layers within the leaf re-mobilize this accumulated S at the same rate following withdrawal of plant* SO_4^{2-} *supply?*

3, *Might such potentially different rates of S re-mobilization correspond to the two tracer exchange rate constants for* SO_4^{2-} *flux from differing cell populations as identified by Bell et al., 1994?*

4, *Does the accumulation (and any subsequent re-mobilization) of S affect epidermal turgor pressure?*

5, *Does the accumulation and subsequent re-mobilization of S by bundle sheath and mesophyll cells have any effect on the water relations and behaviour of other vacuolar solutes in these cell types?*

7.2, INTRODUCTION

The recent application of micro-analytical techniques capable of solute mapping at a resolution akin to that of the individual cell (such as X-ray microanalysis of frozen-hydrated sections (see for example, Leigh and Storey, 1993), protoplast isolation and characterisation (for example, Martinoia *et al.*, 1986) and SiCSA (for example, Fricke *et al.*, 1994a; b; c; 1995)), has shown that many solutes are compartmentalised differentially within the tissue layers of the leaf. Mesophyll cells for example, contain little Ca^{2+} or Cl^- but can accumulate P (Dietz *et al.*, 1992; Leigh and Storey, 1993; Richardson, 1993; Fricke *et al.*, 1994a), sugars (Fricke *et al.*, 1994a; Koroleva *et al.*, 1997), amino acids (Dietz *et al.*, 1994; Fricke *et al.*, 1996) and malate (Fricke *et al.*, 1994a) to high concentrations. The reverse holds true for the epidermis; both Ca^{2+} (Hinde, 1994; Fricke *et al.*, 1994a) and Cl^- (Dietz *et al.*, 1992; Fricke *et al.*, 1994a; 1996) can accumulate to high concentrations but P (Richardson, 1993), sugar (Fricke *et al.*, 1994a; Koroleva *et al.*, 1997) and amino acid concentrations (Fricke *et al.*, 1994a) tend to be much lower.

Throughout previous experiments (see **Chapters 5** and **6**), epidermal S concentrations were consistently higher than the SO_4^{2-} content measured in whole leaf preparations from the same plant. As discussed previously (see **5.5**), it is this observation which would appear to support the hypothesis that S too, is distributed differentially within the cereal leaf. The primary aim of this experiment, therefore, was to ascertain whether S was accumulated and subsequently distributed differentially within the cereal leaf when plants were grown under the high S (INV) or control S (CON) nutrient regimes.

Despite its premature termination, a previous experiment showed that when supplied in excess, S accumulated (and was subsequently distributed) differentially within the morphologically-distinct cells of the upper epidermis (see **Chapter 6**). Nevertheless, the relatively small differences in epidermal S concentrations between trough, ridge and near-stomatal cells suggested that the three 'compartments' identified by Bell *et al.* (1994) (each of which exchanges SO_4^{2-} at a different rate) did not correspond to different types of epidermal cell. Since epidermal heterogeneity did not appear responsible for the three compartments of Bell *et al.* (1994), the experiment designed to investigate the effects of a high S nutrient regime on cells underlying the epidermis was modified to investigate S re-mobilization from these cell types. The questions asked were "do different tissue layers within the leaf re-mobilize S at the same rate and could such potentially different rates correspond to the three 'compartments' of Bell *et al.* (1994)?"

Control S (CON) and high S (INV) plants were grown as before with the SO_4^{2-} supply to both being withdrawn at day 13 post-full expansion (see **Chapter 6**). In this experiment though, the single cell approach was extended to encompass epidermal trough, mesophyll and bundle sheath cells. In preceding experiments (see **Chapter 6**), high epidermal S concentrations have been shown to result in a decreased OP. In this study, quantification of epidermal turgor pressures in both high S and control material would enable us to determine whether S accumulation and physiological leaf age had any effect on this water relations parameter. Quantification of cations in bulk tissue sap preparations from SiCSA-sampled material provided a reference against which single cell work could be compared.

7.3, METHOD

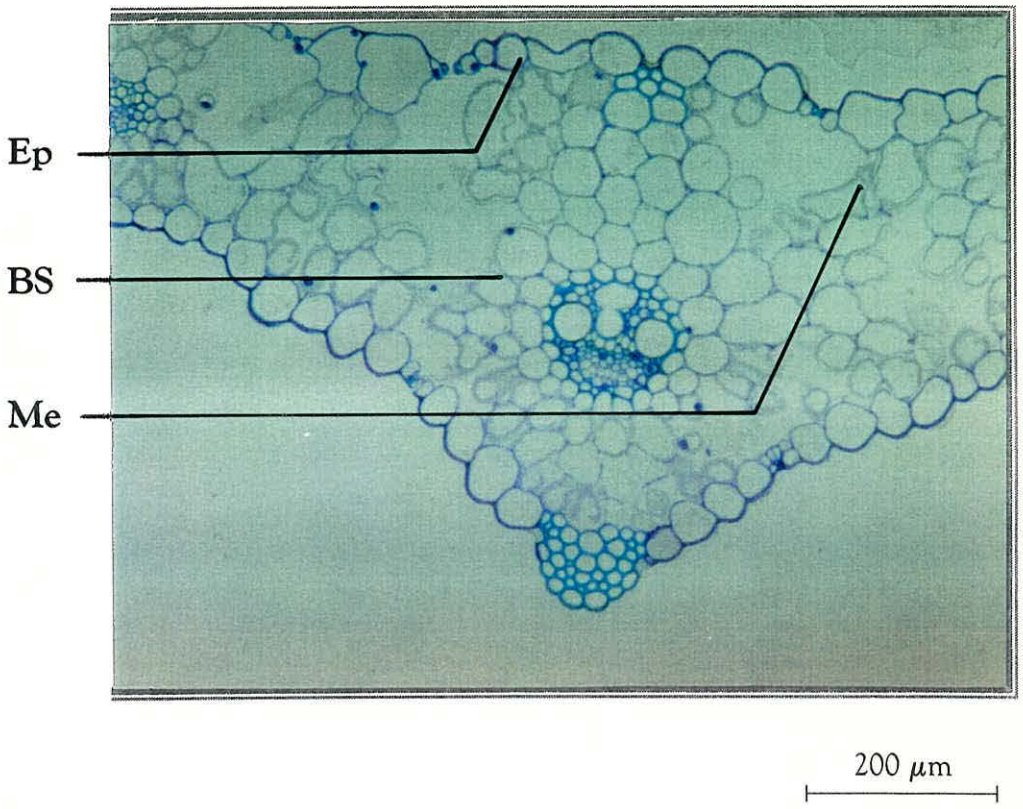
Three 3 l tubs were set up (see **2.2**) for the CONtrol (1 : 8 S : N) and INVerse (8 : 1 S : N) nutrient treatments (the composition of these nutrient solutions is given in **Chapter 5, Table 5.1**). As before, plant analyses began when the third leaf was approximately 2 days from full expansion. Subsequent analyses were then performed at 5, 12, 19 and 26 days post-full expansion. At each assessment point, six plants were removed from the three growth tubs per nutrient treatment. The first three were removed three hours into the photoperiod and for each plant, the turgor pressures of five epidermal trough cells were determined as described by Hüsken *et al.* (1978). Following completion of turgor measurements, a bulk tissue sap preparation was obtained (see **2.3.2**) which was frozen at -20 °C pending quantification of cation concentrations by HPLC (see **2.3.4.2**).

The second batch of three plants was removed nine hours into the photoperiod and it was this material from which the individual mesophyll, bundle sheath and epidermal (trough) cell vacuolar sap samples were extracted. Samples from those cell types underlying the epidermis were obtained by inserting the microcapillary (see **2.4.1**) through a suitable stomatal pore (see Fricke *et al.*, 1994a; Koroleva *et al.*, 1997). As it was not possible to directly see the extraction of these samples, a set of criteria (including position of the cell within the leaf (see **Plate 9**), volume and viscosity of the extracted sap sample and relative amount of chloroplasts in the sample) were used to distinguish between mesophyll and bundle sheath cells.

As in previous experiments (see **Chapters 5** and **6**), the extraction of single cell vacuolar sap was confined to those cells situated between the 4 th and 5 th ridge on the left-hand side of the main vein in a region 15.7 cm down from the tip (the 'statistical middle'). Three epidermal, three bundle sheath and three independent mesophyll 'pools' (each of which was formed from the summation of *circa* five individual mesophyll cell sap samples) were extracted from each plant. Following ejection from the sampling tip, these vacuolar saps were then pooled under water-saturated liquid paraffin to create an epidermal, bundle sheath and mesophyll 'sap reservoir' for each plant. Osmotic pressure and electrolyte concentrations were then determined by picolitre osmometry (see **2.4.2**) and X-ray microanalysis (see **2.4.3**) respectively following removal of the required number of sub-samples from these epidermal, bundle sheath or mesophyll cell sap reservoirs.

As in the preceding experiments (see **Chapter 6**), control S (CON) and high S (INV) material were transferred to the SO_4^{2-}-defficient NON nutrient solution (see **Table 6.1**) at day 13 with the aim of inducing re-mobilization of stored S by withdrawal of plant SO_4^{2-} supply. All material was then grown continually in NON solution for the remainder of the experiment. Throughout this experiment, growth media were replaced every three days and at each solution change, a 1.0 ml sample was removed and frozen at -20 °C pending quantification of anion concentrations by HPLC (see **2.3.4.3**). Daily records of growth medium pH were also collected (results not shown).

Plate 9. A photomicrograph depicting tissue architecture in a transverse section from the middle of leaf three in control S (CON) material. The leaf was embedded in Historesin Plus, sectioned at a thickness of *circa* 4 μm and stained for 60 seconds with toludine blue. Epidermal (Ep), mesophyll (Me) and bundle sheath cells (BS) are shown. [Courtesy of Ms. A. Bell and Dr. Olga Koroleva].



7.4, RESULTS

7.4.1, Sulphur

Throughout this experiment, epidermal, bundle sheath and mesophyll cell S concentrations were both low and constant in CON material (**Figure 7.1**, graph (i)). From 2 days pre- to 26 days post-full expansion, single cell S concentrations were comparable in all three cell types indicating that S is accumulated more uniformly by the cereal leaf when supplied to the plant at lower concentrations.

However, S accumulated to significant concentrations in the epidermal, bundle sheath and mesophyll cells of high S (INV) plants with values in excess of 80 mM being recorded in all three cell types at day 12 post-full expansion. The extent of this accumulation, however, differed between cell types (**Figure 7.1**, graph (ii)). Prior to the withdrawal of plant SO_4^{2-} supply at day 13, single cell S concentrations were significantly higher (GLM; $p < 0.001$) in the epidermis than in the bundle sheath or mesophyll cells from the same plant (**Figure 7.1**, graph (ii)). Bundle sheath and mesophyll cell S concentrations were very similar in the period from 2 days pre to 12 days-post full expansion.

In spite of the similar accumulation of S by bundle sheath and mesophyll cells, the withdrawal of plant SO_4^{2-} supply at day 13 appeared to result in two significantly different (GLM; $p < 0.05$) rates of S re-mobilization from these two cell types. Despite some fluctuation, the S amassed by the bundle sheath cells was seemingly re-mobilized at a rate akin to that seen in the epidermis between days 13 and 26 post full-expansion (see **Figure 7.1**, graph (ii) and **Chapter 6**). In contrast, mesophyll cell S concentrations changed little in the same 13 day period following withdrawal of plant SO_4^{2-} supply. The implications of this potentially differential re-mobilization of S by the cereal leaf will be discussed fully in section **7.5**.

7.4.2, Osmotic pressure

As described previously (see **Chapter 6**), growth of plants in INV nutrient solution led to a significant reduction in epidermal trough cell OP when compared with CON material (see **Figure 7.2**, graph (ii) and **Chapter 6**). As before, the epidermal OP of CON and INV material were initially similar but as leaves aged, the epidermal OP of INV plants decreased such that values were significantly lower (GLM; $p < 0.05$) than those from CON plants by day 5 post-full expansion. Interestingly, values for bundle

sheath and mesophyll cell OP in INV plants remained higher than those measured in the epidermal trough cells throughout this experiment. In fact, bundle sheath and mesophyll cell OP were remarkably similar in CON and INV material irrespective of increasing leaf age (see **Figure 7.2**).

There were no significant differences between epidermal, bundle sheath or mesophyll cell OP in CON material and values remained relatively constant throughout leaf life. As expected, the withdrawal of SO_4^{2-} and re-instatement of plant NO_3^- supplies at day 13 had negligible effect on epidermal, bundle sheath and mesophyll cell OP in CON plants (see **Figure 7.2**, graph (i)). However, the restoration of NO_3^- supply to INV plants at day 13 resulted in substantial increases in epidermal, bundle sheath and mesophyll cell OP such that values were equivalent to control levels by day 19 post-full expansion.

7.4.3, Turgor pressure

When plants were grown in the high S (INV) nutrient solution there was a dramatic and highly significant (GLM; $p < 0.001$) decrease in epidermal trough cell turgor pressures such that by day 5, the mean of 0.23 MPa recorded in INV material was less than 25 % of the 0.96 MPa measured in CON plants (**Figure 7.3**). When NO_3^- supply was restored at day 13, the epidermal turgor pressures of INV material increased by over 130 % although values remained significantly lower (ANOVA; $p < 0.001$) than those of CON plants. From day 19 onwards however, the turgor pressures of CON and INV material decreased both progressively and similarly as leaf age increased such that by day 26 there was no significant difference between the epidermal turgor pressures of material from either nutrient treatment.

In CON plants, epidermal turgor pressures followed a distinctive profile throughout leaf life with values increasing from 2 days pre to peak at around 4 days post-full expansion of the leaf. Following several days maintenance of constant turgor pressure, values then decreased as leaves aged.

Figure 7.1. Is S accumulated and re-mobilized differentially by the different tissue layers within the cereal leaf? The effects of S deprivation on epidermal trough, bundle sheath and mesophyll cell S concentrations in plants previously exposed to (i) a S sufficient (CON) or (ii) an excess S (INV) nutrient regime. Results are expressed as the means ± SD of three plants.

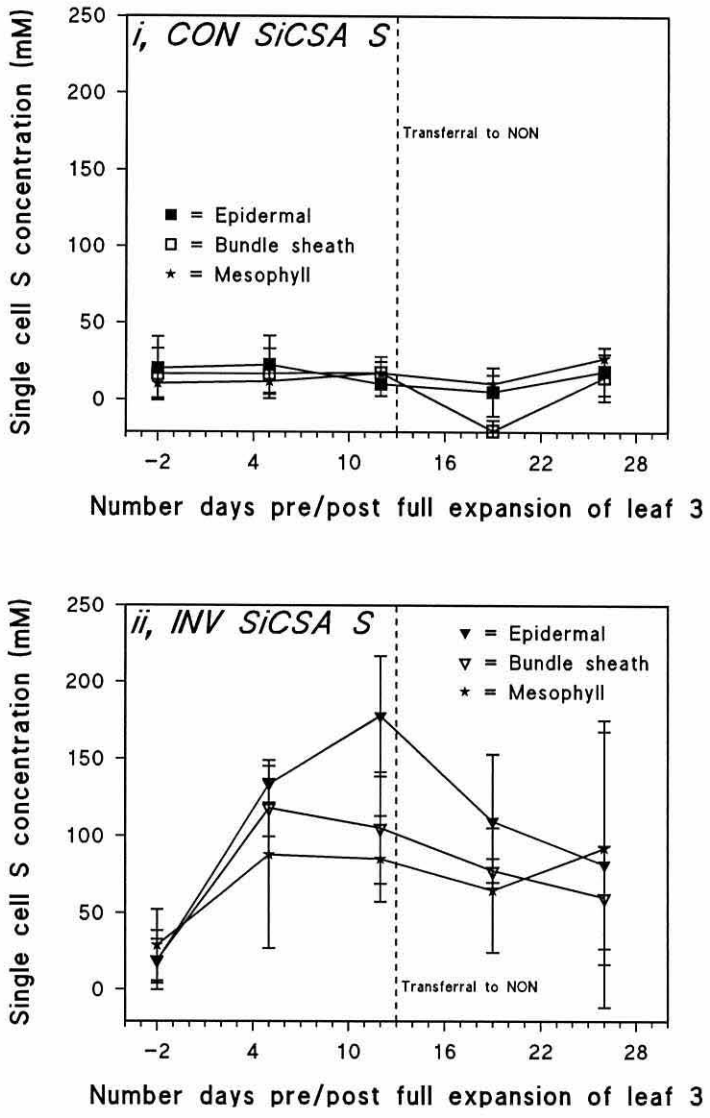


Figure 7.2. Does the accumulation and re-mobilization of S affect single cell osmotic pressure within the leaf? The effects of S deprivation on epidermal trough, bundle sheath and mesophyll cell OP in plants previously exposed to (i) a S sufficient (CON) or (ii) an excess S (INV) nutrient regime. Results are expressed as the means ± SD of three plants.

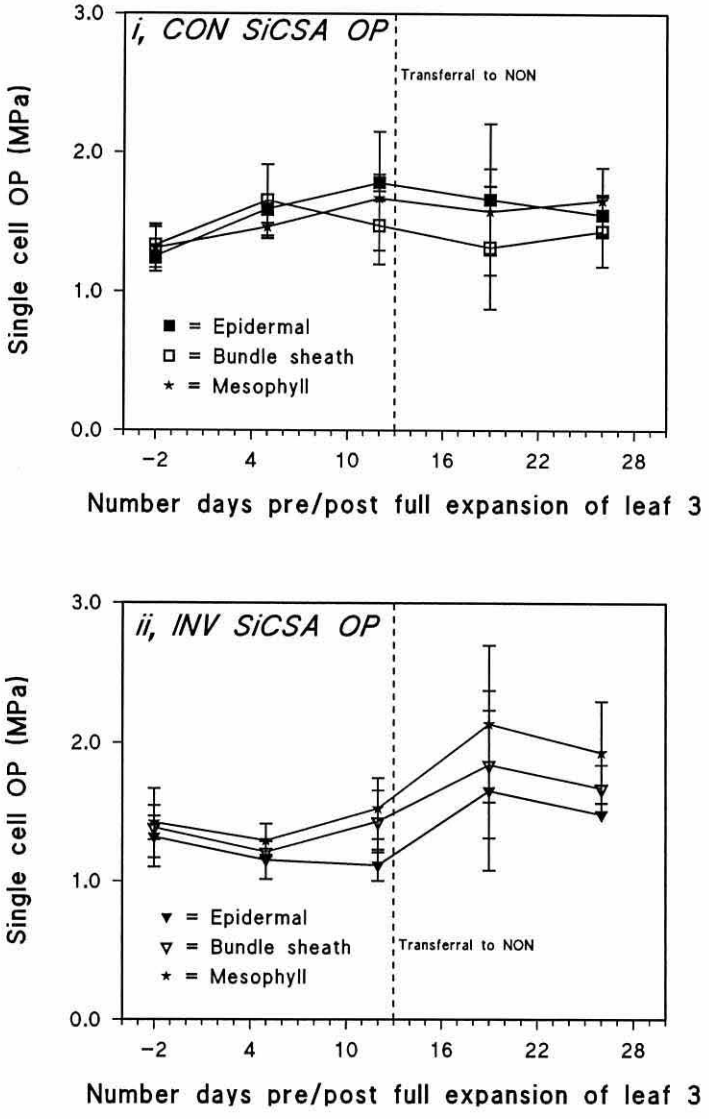
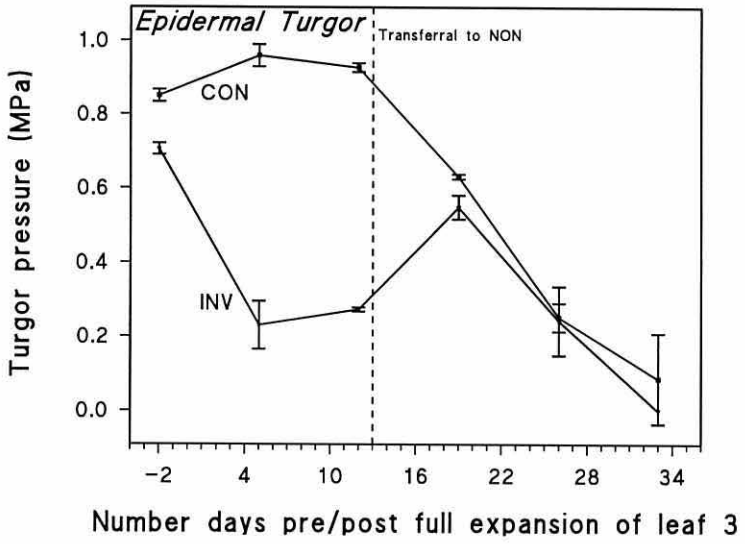


Figure 7.3. Does the accumulation and re-mobilization of S affect epidermal turgor pressure? The effects of S deprivation on epidermal trough cell turgor pressures in plants previously exposed to a S sufficient (CON) or excess S (INV) nutrient regime. Results are expressed as the means ± SD of three plants. These data are presented here courtesy of Ms. R. Lawrence.



7.4.4, Potassium

Irrespective of nutrient treatment, epidermal, bundle sheath and mesophyll cell K^+ concentrations decreased significantly (ANOVA; $p < 0.05$ for all three cell types in both CON and INV material) as leaves aged (**Figure 7.4**). In CON material, the extent of this decrease differed between the three cell types such that the drop in epidermal K^+ concentrations was significantly larger (GLM; $p < 0.001$) than that recorded in either bundle sheath or mesophyll cells (**Figure 7.4**, graph (i)).

As in previous experiments (see **Chapters 5** and **6**), accumulation of S in the third leaf of INV plants coincided with a significantly diminished (GLM; $p < 0.05$) rate of K^+ export from epidermal trough cells (**Figure 7.4**, graph (ii)). Interestingly (and despite some fluctuation), there appeared to be no significant difference in bundle sheath and mesophyll cell export rate between CON and INV material. This observation implies that these tissues were affected proportionately less than the epidermis by the accumulation of S. The withdrawal of SO_4^{2-} and re-instatement of NO_3^- supplies to both CON and INV plants at day 13 had negligible affect on epidermal K^+ concentrations (**Figure 7.4**). The cause of the substantial increase in bulk sap (**Figure 7.5**), bundle sheath and mesophyll cell (**Figure 7.4**) K^+ concentrations between days 12 and 19 remains unknown. Regardless of this 'jump' in values at day 19, however, whole leaf (**Figure 7.5**), bundle sheath and mesophyll cell (**Figure 7.4**) K^+ concentrations in INV plants were higher than those of CON material at day 26 post-full expansion.

7.4.5, Calcium

Calcium accumulated to significant concentrations in the leaves of both CON and INV material as age increased (**Figure 7.6**). Irrespective of nutrient treatment, bundle sheath and mesophyll cell Ca^{2+} concentrations remained relatively constant and were significantly lower (GLM; $p < 0.001$) than the values recorded in the epidermis as leaves aged (**Figure 7.7**). In fact, bundle sheath and mesophyll cell concentrations were remarkably conserved both within and between the two nutrient treatments (see **Figure 7.7**) implying that the distinctive distribution of Ca^{2+} within the leaf is maintained regardless of changes in nutrient availability.

Whilst Ca^{2+} accumulated in the upper epidermis of both CON and INV material, the extent of this process differed greatly between the two nutrient

treatments (**Figure 7.7**). As before (see **Chapters 5** and **6**), the accumulation of Ca^{2+} by the epidermis was significantly impaired (GLM; $p < 0.001$) at day 12 in INV material (**Figure 7.7**) and doubtless as a consequence of this, whole leaf Ca^{2+} concentrations were also significantly lower (ANOVA; $p < 0.001$) in INV than CON plants (**Figure 7.6**). As the third leaf began to senesce (*circa* day 19), the rate of Ca^{2+} accumulation appeared to level-off in plants from both nutrient treatments (see **Figures 7.6** and **7.7**).

7.4.6, Chloride

Epidermal, bundle sheath and mesophyll cell Cl^- concentrations fluctuated considerably throughout this experiment although values remained comparably low (less than 20 mM) in all three cell types from both CON and INV material **Figure 7.8**). In fact, values were often so low as to lie beyond the detection limit of the EDX technique (on which occasion, a negative value was recorded for Cl^- content).

7.4.7, Magnesium

With the notable exception of data from CON material at day 26, Mg^{2+} was distributed equally in all three cell types of both CON and INV material as leaves aged (see **Figure 7.9**). Consequently, whole leaf Mg^{2+} concentrations (**Figure 7.10**) also increased as leaves matured and as for single cell data, there were no significant differences in the extent of this accumulation between the two nutrient treatments. The withdrawal of SO_4^{2-} supply at day 13 had negligible effect on the Mg^{2+} concentrations of CON and INV plants. The observations made in this experiment imply that neither the accumulation nor the distribution of Mg^{2+} is affected by the ratio of S : N supplied to the plant.

Figure 7.4. Does the accumulation and re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on epidermal trough, bundle sheath and mesophyll cell K^+ concentrations in plants previously exposed to (i) a S sufficient (CON) or (ii) an excess S (INV) nutrient regime. Results are expressed as the means ± SD of three plants.

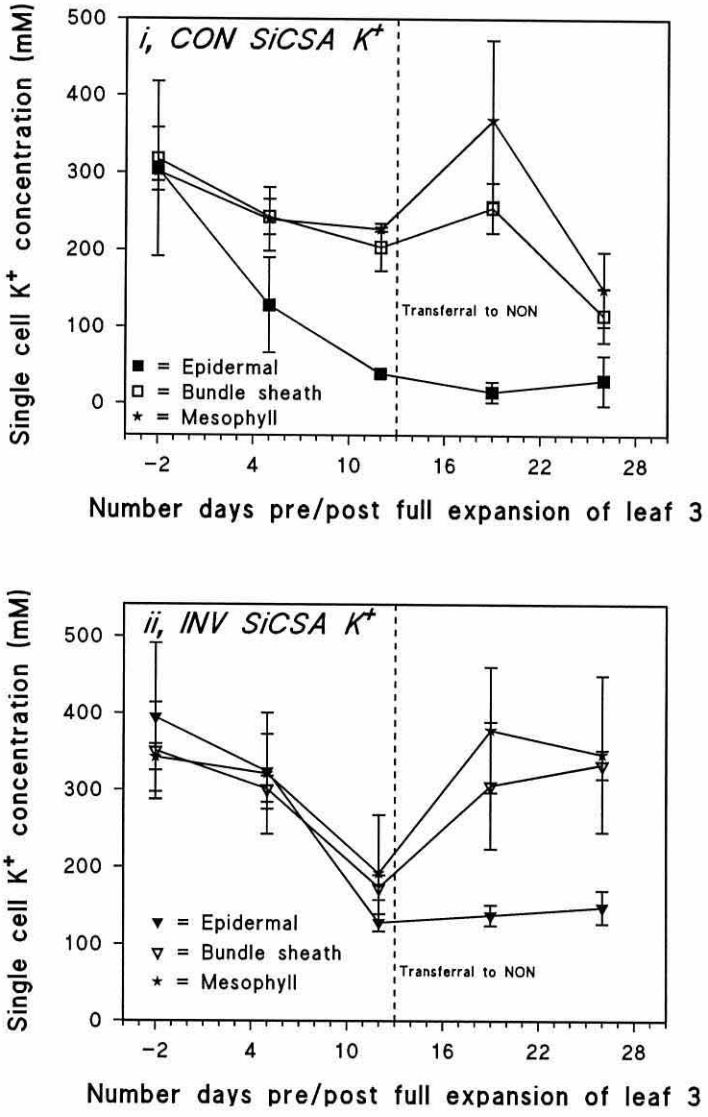


Figure 7.5. Does the accumulation and re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on leaf sap K^+ concentrations in plants previously exposed to a S sufficient (CON) or excess S (INV) nutrient regime. Results are expressed as the means ± SD of three plants.

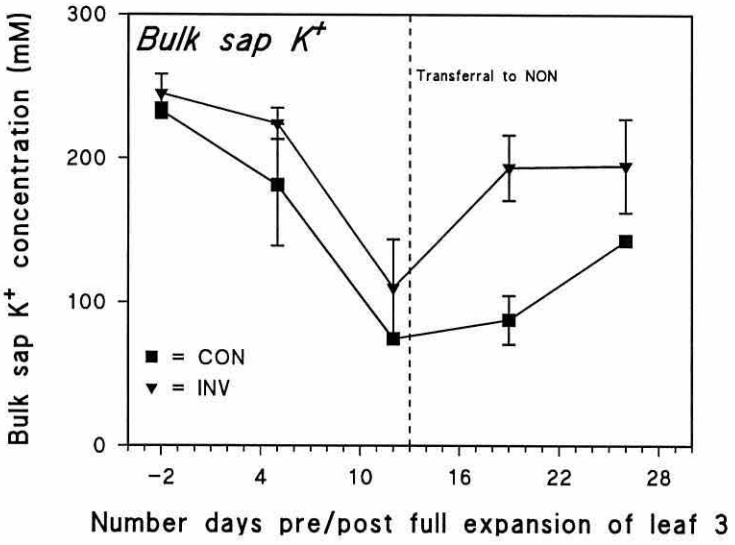


Figure 7.6. Does the accumulation and re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on leaf sap Ca^{2+} concentrations in plants previously exposed to a S sufficient (CON) or excess S (INV) nutrient regime. Results are expressed as the means ± SD of three plants.

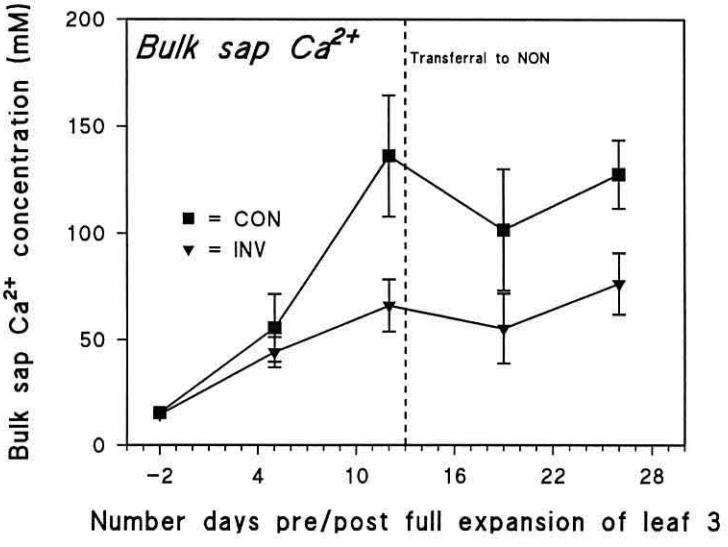


Figure 7.7. Does the accumulation and re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on epidermal trough, bundle sheath and mesophyll cell Ca^{2+} concentrations in plants previously exposed to (i) a S sufficient (CON) or (ii) an excess S (INV) nutrient regime. Results are expressed as the means ± SD of three plants.

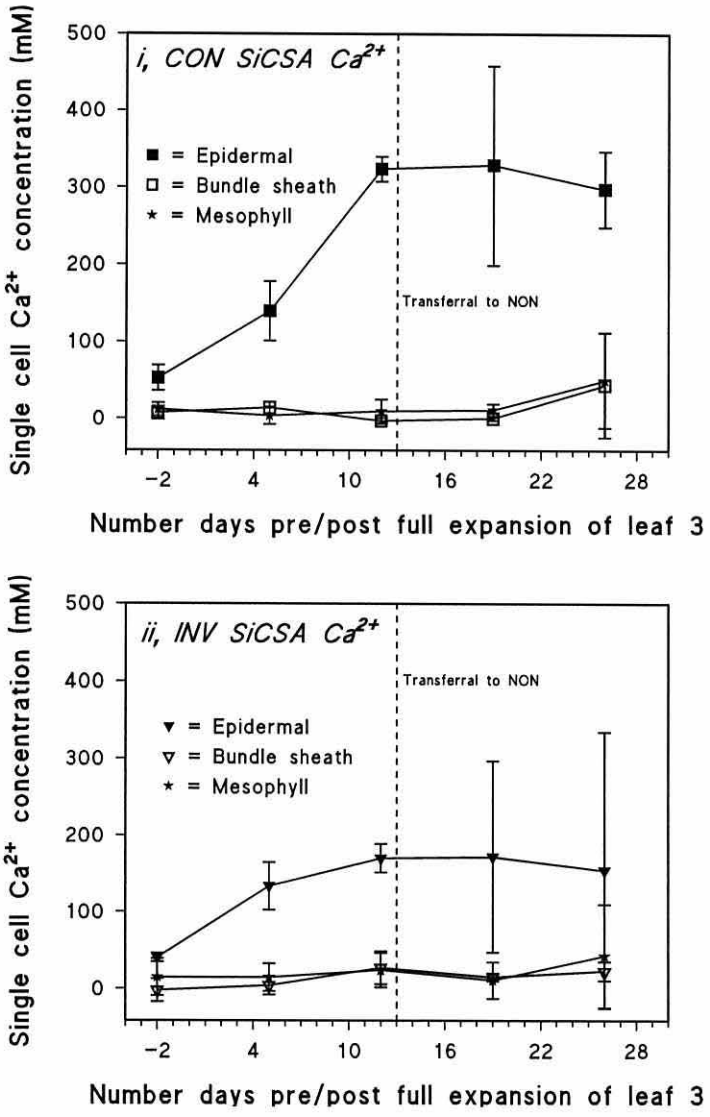


Figure 7.8. Does the accumulation and re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on epidermal trough, bundle sheath and mesophyll cell Cl^- concentrations in plants previously exposed to (i) a S sufficient (CON) or (ii) an excess S (INV) nutrient regime. Results are expressed as the means ± SD of three plants.

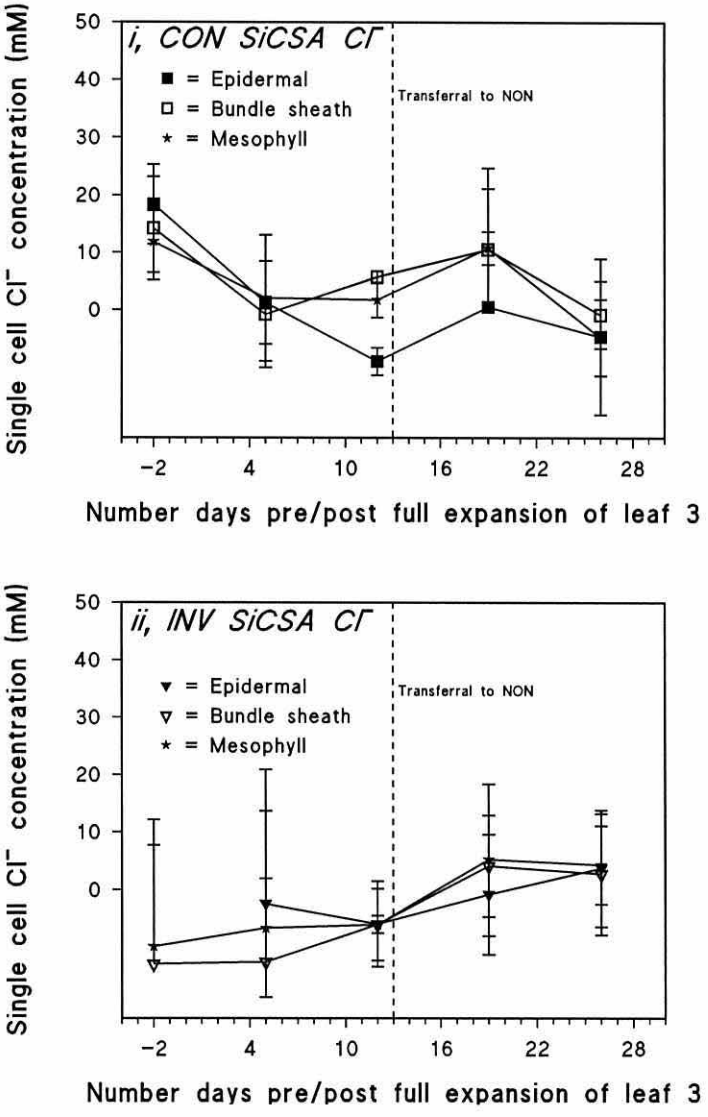


Figure 7.9. Does the accumulation and re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on epidermal trough, bundle sheath and mesophyll cell Mg^{2+} concentrations in plants previously exposed to (i) a S sufficient (CON) or (ii) an excess S (INV) nutrient regime. Results are expressed as the means ± SD of three plants.

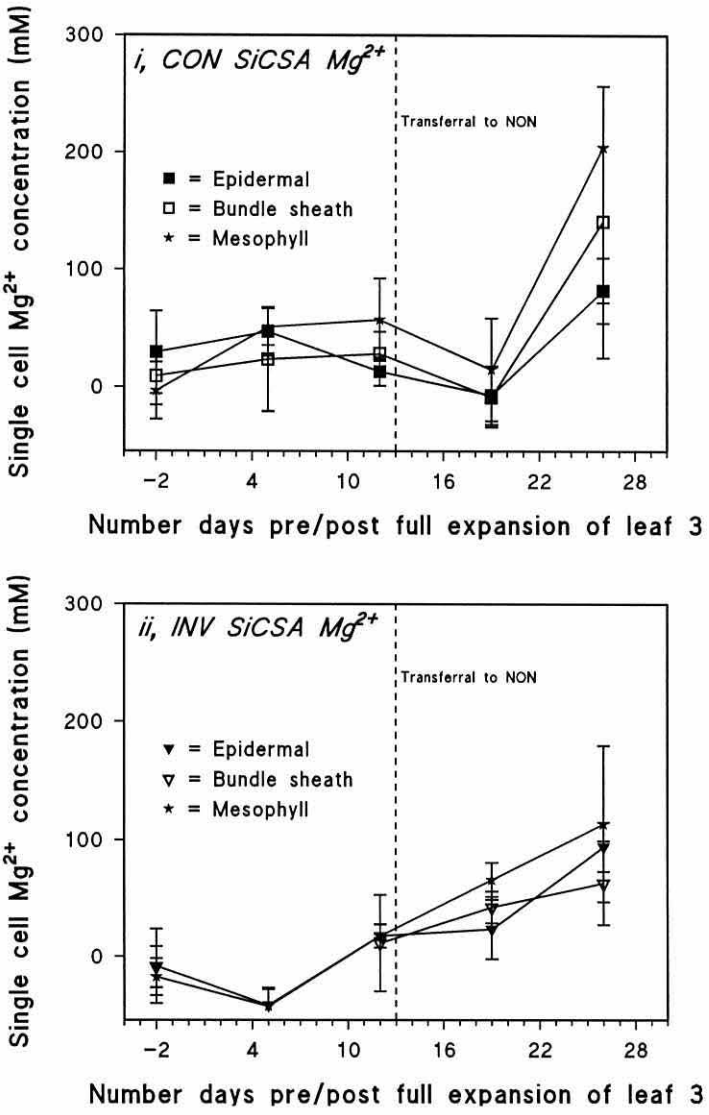
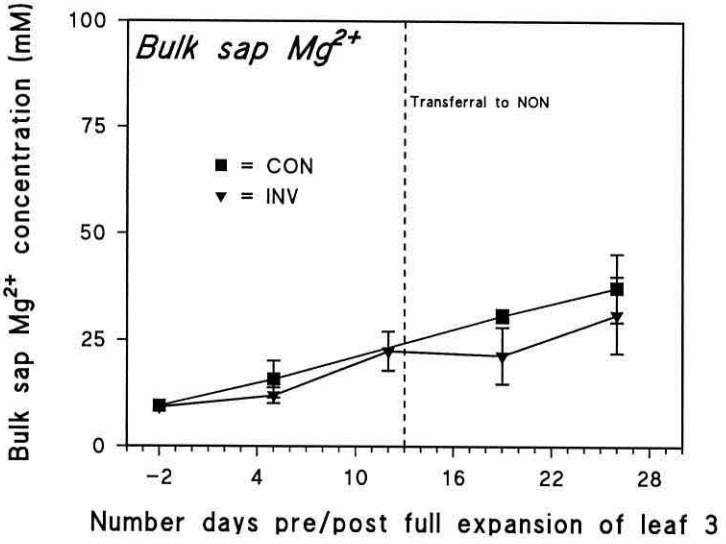


Figure 7.10. Does the accumulation and re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on leaf sap Mg^{2+} concentrations in plants previously exposed to a S sufficient (CON) or excess S (INV) nutrient regime. Results are expressed as the means ± SD of three plants.

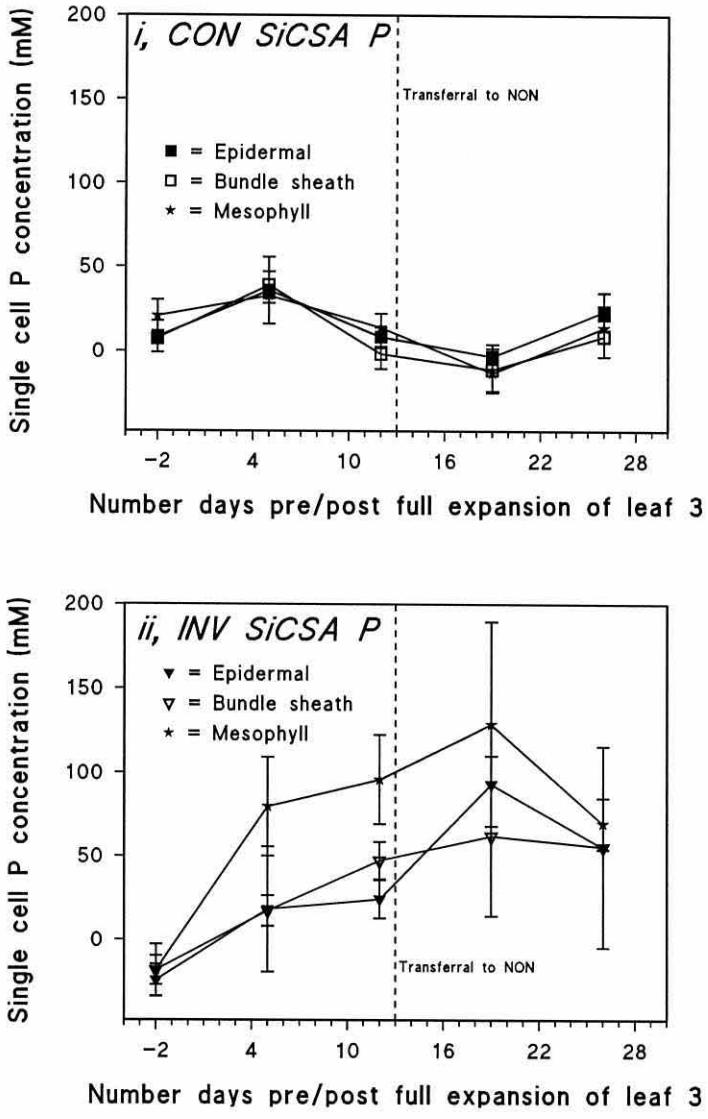


7.4.8, Phosphorus

Throughout this experiment, P (presumably as PO_4^{3-}) accumulated to significant concentrations in the epidermal, bundle sheath and mesophyll cells of INV material and values in excess of 50 mM were recorded in all three cell types towards the end of leaf life (**Figure 7.11**, graph (ii)). However, the extent of this accumulation differed between cell types such that P concentrations were significantly higher (GLM; $p < 0.01$) in mesophyll cells than in epidermal or bundle sheath cells from the same plant (**Figure 7.11**, graph (ii)). The amassment of P by INV material was unaffected by the withdrawal of plant SO_4^{2-} supply at day 13 and single cell P concentrations continued to increase until the leaf entered the very final stages of senescence (*circa* day 26 post-full expansion).

By comparison, the concentrations of P measured in the epidermal, bundle sheath and mesophyll cells of CON plants were significantly lower (GLM; $p < 0.01$ for all three cell types) than those recorded in INV material (**Figure 7.11**, graph (i)). Despite some fluctuation, P concentrations were similar in all three cell types with values remaining relatively constant throughout this experiment. The apparent correlation between high leaf S and P concentrations is discussed in greater detail in section **7.5**.

Figure 7.11. Does the accumulation and re-mobilization of S affect the behaviour of other cell solutes? The effects of S deprivation on epidermal trough, bundle sheath and mesophyll cell P concentrations in plants previously exposed to (i) a S sufficient (CON) or (ii) an excess S (INV) nutrient regime. Results are expressed as the means ± SD of three plants.



7.5, DISCUSSION

7.5.1, Does S accumulate differentially within the cereal leaf?

Irrespective of nutrient treatment and physiological leaf age, epidermal S concentrations have been consistently higher throughout this investigation than those values measured in representative sections from whole leaves (see **Chapters 5** and **6**). In a previous discussion (see **5.5**), it was proposed that this difference was attributable to SO_4^{2-} accumulating differentially within the cereal leaf with preferential compartmentation of this solute to the epidermis. Similar findings were reported by Bell *et al.* (1995c) in which an 'apparent' mean mesophyll cell SO_4^{2-} concentration (derived from the difference between epidermal and whole leaf values) of 26 mM was 47 % lower than the mean of 49 mM measured directly in (lower) epidermal cells when plants were supplied with 3.7 mM SO_4^{2-} and 0.1 mM NO_3^-.

In this experiment, bundle sheath and mesophyll cell S concentrations were measured directly by extraction of single cell saps through open stomates. When supplied to excess, it is apparent that S does accumulate differentially within the cereal leaf (see **Figure 7.1**, graph (ii)). Prior to the withdrawal of plant SO_4^{2-} supply at day 13, single cell S concentrations in INV material were considerably higher in the epidermis than in bundle sheath or mesophyll cells. At day 12 post-full expansion for example, the mean epidermal trough cell S concentration was 178 mM, a value 41 and 52 % higher than the mean concentrations of 105 and 85 mM recorded in bundle sheath and mesophyll cells respectively on the same day. Note that the difference in S concentrations between epidermal and bundle sheath/mesophyll cells are similar in this experiment and in the previous account of Bell *et al.* (1995c).

From 2 days pre- to 12 days post-full expansion, S accumulated continuously in the epidermal trough cells of INV plants yet concentrations seemed to 'tail-off' in the bundle sheath and mesophyll cells around day 4. This cessation of S accumulation by those cell types underlying the epidermis may be related to observations made concerning single cell OP (see **Figure 7.2**, graph (ii)).

As shown previously (see **Chapter 6**), growth of plants in the high S (INV) nutrient solution led to a reduction in epidermal OP when values were compared with those measured in CON material. Interestingly, however, the decrease in INV bundle sheath and mesophyll cell OP was not as great as that measured in the epidermis which would seem to imply that epidermal OP is affected more by the

restricted availability of osmotically-important inorganic electrolytes (such as Cl^- or NO_3^-) than are equivalent bundle sheath or mesophyll cell values. This is hardly surprising, however, given that the epidermal OP of plants grown under low light is generated almost exclusively by inorganic solutes (Fricke *et al.*, 1994a; Koroleva *et al.*, 1997). In contrast, the OP of those cell types underlying the epidermis is generated by a combination of both organic and inorganic solutes regardless of the intensity of illumination supplied to plants (Koroleva *et al.*, 1997).

Single cell S concentrations remained low and constant throughout leaf life in CON material and it would seem that when growth solution SO_4^{2-} availability is lower, the distribution of S within the leaf is more uniform. Dietz *et al.* (1992) came to a similar conclusion following analyses of epidermal and mesophyll cell protoplasts isolated from the primary leaves of barley. When plants were supplied with 4 mM Cl^-, 3 mM SO_4^{2-} and 17 mM NO_3^-, epidermal and mesophyll protoplast SO_4^{2-} concentrations were 0.8 and 2.4 mM respectively, leading the authors to conclude that SO_4^{2-} is distributed uniformly within the cereal leaf. An investigation was also made by Dietz and co-workers as to the effects of differing SO_4^{2-} supply on epidermal and mesophyll protoplast SO_4^{2-} concentrations. Unfortunately, however, this data is not presented in the published account meaning that a direct comparison with our own experimental values cannot be made.

By virtue of being the predominant negatively charged solute available to the growing plants, it is possible that the epidermal cells of INV material continually accumulated S in a bid to regulate their turgor and/or OP. On this basis, therefore (and in contrast to the findings of previous studies (see for example, Richardson, 1993), it is proposed that the divalent SO_4^{2-} anion is a poor osmolyte, unable to successfully substitute for either Cl^- or NO_3^- to maintain epidermal OP. Consequently, the accumulation of S by the epidermal cells of INV material fails to provide the necessary increase in OP. In a presumably cyclical process, therefore, more and more SO_4^{2-} is accumulated by epidermal cells (see **Figure 5.1**) in an attempt to regulate their cell turgor and/or OP (see also **Chapter 9**).

7.5.2, Do different leaf tissues re-mobilize S at the same rate?

The withdrawal of plant SO_4^{2-} supply from INV material at day 13 established sufficient stimulus to induce the re-mobilization of S reserves from both epidermal

and bundle sheath cells. Between days 12 and 26 post-full expansion, mean epidermal cell S concentrations decreased by some 54 % from 178 to 81 mM. In the same 14 day period, mean bundle sheath concentrations dropped from 105 to 61 mM, a decrease of some 42 %. On the basis of these observations, it would appear that both epidermal and bundle sheath cells are able to re-mobilize amassed S following imposition of a suitable S stress. Moreover, the relative rates at which this re-mobilization occurred were similar between the two cell types.

Following withdrawal of plant SO_4^{2-} supply, there was no appreciable re-mobilization of the S from INV mesophyll cell reserves. Between days 12 and 26 post-full expansion, there was no significant change in mean mesophyll cell S concentration from the 85 mM measured at day 12 to the 92 mM at day 26. As tentatively proposed by Bell *et al.* (1995c) these data would seem to support the hypothesis that epidermal and mesophyll cell vacuoles exchange SO_4^{2-} at different rates. This supposition is given further consideration in the following section.

7.5.3, Do these different rates equate to the compartments of Bell *et al.* (1994)?

In the recent investigation of Bell *et al.* (1994), $^{35}SO_4^{2-}$ radio-tracer exchange kinetics were used to estimate SO_4^{2-} fluxes in both the roots and mature leaves of *Macroptilium atropurpureum*. Radio-tracer wash-out data from root tissue was subsequently fitted by three separate exponential equations, assumed to correspond to SO_4^{2-} exchange in root cell vacuoles, cytoplasm and extra-cellular spaces. In mature leaf tissue, however, wash-out data could only be fitted by four exponentials such that SO_4^{2-} efflux obeyed published theory (Cram and Laties, 1974). In order for the wash-out data to fit these four exponential equations, it was reasoned that there had to be an equal number of discrete compartments within the tissue, with each compartment exchanging SO_4^{2-} at a different rate.

The fastest of these leaf tissue 'compartments' ($t_{1/2}$ *circa* 2 minutes) was thought to correspond to the extracellular spaces. The next fastest ($t_{1/2}$ *circa* 16 minutes) could have represented the cytoplasm whilst the two slowest exchanging compartments (the first with $t_{1/2}$ *circa* 20 hours; $k = 5 \times 10^{-4}$ min^{-1}) and the second with $t_{1/2}$ *circa* 2000 hours; $k = 5 \times 10^{-6}$ min^{-1}) were assumed to correspond to differing populations of cells.

In this experiment, the use of SiCSA techniques to study the composition of extracted vacuolar sap samples from individual epidermal, bundle sheath and mesophyll cells has shown that S is distributed differentially within the cereal leaf (see **Figure 7.1**, graph (ii)). Subsequent imposition of S stress has then indicated that these three cell types re-mobilize S differently. Do the reductions in vacuolar S concentration following withdrawal of SO_4^{2-} supply resemble the tracer exchange rate-constants identified by Bell *et al.* (1994)?

If we assume that a solute is lost from a compartment at a rate proportional to the concentration originally present in the compartment (*i.e.* first order reaction; see Cram, 1984), then the efflux of that solute fits the following equation:

$$Q_t = Q_0.e^{(-kt)} \qquad (1)$$

Where:

Q_t = concentration at time 't' (in mM)

Q_0 = initial concentration at time '0' (in mM)

k = rate constant (min^{-1})

If equation (1) is re-arranged to make the rate constant (k) the subject, then:

$$k = \frac{\ln\left(\frac{Q_0}{Q_t}\right)}{t} \qquad (2)$$

If the mean S concentrations for each of the three cell types at both day 12 (Q_0) and day 26 (Q_t) post-full expansion (see **Figure 7.1**, graph (ii)) are then substituted into equation (2):

i, For epidermal cells:

$$k = \frac{\ln\left(\frac{178\ mM}{81.5\ mM}\right)}{20160\ minutes}$$

$$k = 3.86 \times 10^{-5}\ min^{-1}$$

ii, For bundle sheath cells:

$$k = \frac{\ln\left(\frac{105\ mM}{61\ mM}\right)}{20160\ \text{minutes}}$$

$$k = 2.69 \times 10^{-5}\ \text{min}^{-1}$$

iii, For mesophyll cells:

$$k = \frac{\ln\left(\frac{85\ mM}{92\ mM}\right)}{20160\ \text{minutes}}$$

$$k_{(\text{estimate})} \leq 10^{-6}\ \text{min}^{-1}$$

Despite the variability in wheat mesophyll cell data (which precludes a precise determination of 'k'), the S efflux rate constants for epidermal and mesophyll cells in INV wheat plants are remarkably similar to the values derived by Bell *et al.* (1994) for 'apparent epidermal' ($k = 5 \times 10^{-4}\ \text{min}^{-1}$) and 'apparent mesophyll' ($k = 5 \times 10^{-6}\ \text{min}^{-1}$) populations in mature leaves of *Macroptilium*. This similarity in S efflux rate from mature leaf vacuoles of two unrelated plant species provides further evidence to support the hypothesis that epidermal and mesophyll cell populations exchange SO_4^{2-} at different rates (see Bell *et al.*, 1995c).

In INV wheat plants, derived values of SO_4^{2-} efflux (k) from both epidermal and bundle sheath tissues were similar at 3.86×10^{-5} and $2.69 \times 10^{-5}\ \text{min}^{-1}$ respectively. Since only two of the four compartments identified by Bell *et al.* (1994) were thought to correspond to different types of cell, it is proposed that the faster of these two compartments (with $t_{1/2}$ *circa* 20 hours) represented a mixture of both epidermal and bundle sheath cell SO_4^{2-} flux.

In this experiment, the rate constant for SO_4^{2-} exchange from the mesophyll cells of high S (INV) plants could potentially be as low as $10^{-6}\ \text{min}^{-1}$. Having obtained a similar value for SO_4^{2-} efflux from the 'apparent mesophyll' tissue of *Macroptilium*, Bell *et al.* (1994) suggested that the "rate of loss of SO_4^{2-} from leaf (mesophyll?) cell vacuoles could well limit redistribution of S during S-stress". Since mesophyll cells constitute a large proportion of the leaf (approximately 38 %; Jellings and Leech,

1982), it is postulated that much of the leaf SO_4^{2-} 'reserves' are effectively 'trapped' within the leaf, even as it approaches the very final stages of senescence.

7.5.4, Does the accumulation of S affect epidermal turgor pressure?

Even before the third leaf had attained full expansion, the mean epidermal turgor of INV material was (at 0.71 MPa), 17 % lower than the 0.85 MPa measured in CON plants. By day 5 post-full expansion, the difference between the two nutrient treatments had increased further such that the mean of 0.96 MPa in CON plants was over 75 % higher than the mean of 0.23 MPa in INV material. In contrast to the findings of Richardson (1993) (reviewed by Leigh and Tomos, 1993), the observations made in both this and the previous experiment (see **Chapter 6**) imply that changes in the composition of epidermal cell vacuoles do have significant and far reaching effects on both cell turgor and sap OP. A possible explanation for these differences is presented in section **6.5**.

When the NO_3^- supply was restored to INV material at day 13, there was a considerable increase in epidermal trough cell turgor pressure. Interestingly, however, this increase occurred only to the extent that epidermal turgors became similar in CON and INV material such that by day 19, values were the same in all plants regardless of nutrient treatment. This observation implies that the 'recovery' of epidermal turgor in INV material (as resulting from a sudden increase in the availability of monovalent anions) is limited by the age and condition of the cell membrane, the integrity of this presumably deteriorates with increasing leaf age.

The highest mean epidermal turgor recorded for CON material was the 0.96 MPa measured at day 5 post-full expansion. From this point onwards, values decreased substantially as the leaf aged. Nevertheless, a significant turgor pressure was measured in the epidermal cells of both CON and INV material throughout senescence with values only becoming negligible (presumably because of increased leakiness due to increasing age) towards the very end of leaf-life (*circa* day 33 post-full expansion).

Although leaves senesce asynchronously, the epidermis represents one of the last leaf tissues to perish (Feller and Fischer, 1994; Smart, 1994) as implied by the fact that a measurable turgor could be obtained from epidermal trough cells even as the leaf entered the very final stages of its life (see **Figure 7.3**). Similar observations have

been made by Feller and Fischer (1994), Smart (1994) and Matile (1997) who suggest that by maintaining membrane integrity, the leaf is able to retain its compartmentation of solutes thereby facilitating the orderly metabolism of breakdown products.

7.5.5, Does the accumulation of S affect the behaviour of other solutes in mesophyll and bundle sheath cells?

As reported previously (see **Chapter 6**) and above, the accumulation of excess S in the leaves of INV plants coincided with a significant reduction (GLM; $p < 0.05$) in epidermal OP when values were compared with CON material. When the NO_3^- supply was subsequently restored at day 13, values increased such that they became similar to those measured in CON material. Interestingly, the OP measured in the sub-surface cells of INV plants remained higher than the values obtained from the epidermal trough cells of the same plant.

In a previous discussion (see **6.6**), it was suggested that the reduced epidermal OP of INV plants growing in INV nutrient solution was attributable to the inability of divalent anions (such as the accumulating SO_4^{2-} in INV plants) to maintain plant water relations. This clearly does not represent the full story since the bundle sheath and mesophyll cells of INV plants in this experiment were able to maintain a higher (and more constant) OP in spite of the accumulation of excess S.

Organic solutes such as sugars (Fricke *et al.*, 1994a; Koroleva *et al.*, 1997) or amino acids (Fricke *et al.*, 1996) accumulate only to osmotically significant concentrations in those cells found beneath the epidermis. As a rule, concentrations of organic solutes (with the exception of malate; Fricke *et al.*, 1995) are generally so low as to be negligible in the cereal epidermis. Fricke *et al.* (1994a) showed that the concentration of sugars and free amino acids in the upper epidermis of barley was 1.6 and 0.2 mM respectively whilst mesophyll cell sugar and amino acid concentrations were 96 and 98 % higher than those measured in the epidermis at 36 and 11.2 mM respectively. The authors concluded that those cell types which are found beneath the epidermis are able to use both organic and inorganic solutes as principal osmotica. In contrast, the OP of the epidermis is generated exclusively by inorganic salts.

It is proposed, therefore, that the higher OP in the bundle sheath and mesophyll cells of high S plants growing in INV nutrient solution was due to the accumulation of organic solutes which served to counter-act the restricted availability of monovalent anions. In contrast, epidermal cells could not use sugars or free amino acids as principal osmotica and due to a lack of monovalent anions in the INV nutrient solution, epidermal OP was substantially lower.

Potassium concentrations were initially similar within the epidermal, bundle sheath and mesophyll cells of both CON and INV plants at the start of this experiment. As described previously in wheat (Hodson and Sangster, 1988), barley (Fricke *et al.*, 1994a; 1996; Leigh and Storey, 1993) and isolated barley protoplasts (Dietz *et al.*, 1992), these observations imply that K^+ is distributed uniformly within the cereal leaf. As the leaves of CON and INV material aged, the concentration of K^+ in epidermal, bundle sheath and mesophyll cells decreased. The extent of this, however, differed both between nutrient treatment and cell type. From 2 days pre to 26 days post full-expansion, the decrease in K^+ concentration in the epidermal trough cells of CON material was significantly greater (GLM; $p < 0.001$) than that measured in the bundle sheath and mesophyll cells from the same plant. By day 26 post full-expansion, the mean epidermal K^+ concentration in CON material was less than 35 mM. By way of contrast, the mean bundle sheath and mesophyll cell K^+ concentrations were 115 and 150 mM respectively. It would appear, therefore, that those cell types found beneath the epidermis retain their reserves of K^+ to a far greater extent than epidermal cells do. Since bundle sheath and mesophyll cells lack the ability to function as significant sinks for Ca^{2+} during leaf ageing (see for example, Fricke *et al.*, 1994a; Hinde, 1994), it is possible that K^+ is retained by these tissues so maintaining cellular water relations.

The re-mobilization of K^+ from epidermal trough cells was significantly diminished (GLM; $p < 0.05$) in INV plants when values were compared with those from control material. However, the extent of this re-mobilization was similar in the cell types underlying the epidermis irrespective of nutrient treatment. As expected, the loss of K^+ from the whole leaves of INV plants was less than that from CON material throughout this experiment (see **Figure 7.5**). A possible cause of the substantial 'jump' in bulk sap (**Figure 7.5**), bundle sheath and mesophyll cell (**Figure 7.4**, graph (ii)) K^+ concentrations between days 12 and 19 is that the expected influx

of NO_3^- into the third leaf following transferral of NO_3^--starved INV plants to NON nutrient solution could have been accompanied by K^+ as its counter-ion.

As reported previously (see Chapters 5 and 6), Ca^{2+} accumulated to significant concentrations in the upper epidermis of the CON and INV material in this experiment as leaves aged. Again, the extent of this amassment was significantly diminished (GLM; $p < 0.001$) in INV plants and at day 12 post full-expansion for example, the mean epidermal Ca^{2+} concentration in CON material was 323 mM, a value 47 % higher than the mean of 170 mM measured in INV plants (**Figure 7.7**, graph (ii).

Irrespective of nutrient treatment, there was no accumulation of Ca^{2+} in those cells underlying the epidermis. Throughout this experiment, bundle sheath and mesophyll cell Ca^{2+} concentrations remained low and constant (with values being close to zero) in both CON and INV plants (see **Figure 7.7**). Again using day 12 as an example, there were no significant differences between the mean Ca^{2+} concentrations of 14 and 9 mM in the bundle sheath and mesophyll respectively of CON material and the 27 (bundle sheath) and 24 mM (mesophyll) measured in INV plants once plant-plant variation had been taken into account.

Interestingly, the differential distribution of Ca^{2+} within ageing leaves was maintained regardless of the accumulation of significant amounts of S in INV plants. A similar finding was reported by Fricke *et al.* (1994a) who observed that the heterogeneous distribution of Ca^{2+} within the mature leaves of barley was maintained even after plants were supplied with 2 mM NaCl. As described by Fricke *et al.* (1994a), the Ca^{2+} concentrations of bulk sap preparations obtained from SiCSA-sampled material were approximately intermediate to epidermal and mesophyll cell values in both CON and INV plants. At day 12 post full-expansion for example, mean bulk sap Ca^{2+} concentrations were 58 % (at 136 mM) and 62 % (at 66 mM) lower than the mean epidermal concentrations of CON and INV material respectively.

Whilst the heterogeneous distribution of Ca^{2+} between epidermis and underlying mesophyll has been well documented (see for example, Hodson and Sangster, 1988; Dietz *et al.*, 1992; Leigh and Storey, 1993; Leigh and Tomos, 1993; Fricke *et al.*, 1994a, b; 1996), the reasons behind the physiological partitioning of this solute (in terms of nutrient-use efficiency or stress response; Fricke *et al.*, 1994a)

remain unclear (see review by Leigh and Tomos, 1993). Leigh and Tomos (1993) speculated that the occlusion of Ca^{2+} from mesophyll cells could be attributable to a particular sensitivity of mesophyll cell metabolism to Ca^{2+} or alternatively, an inability of the mesophyll cell tonoplast to effectively retain this solute.

A further suggestion was put forward by Fricke *et al.* (1994a) who suggested that the distinct and highly differential distribution of Ca^{2+} within mature barley leaves could be due to the need to maintain a strict spatial separation of certain solutes. Phosphate for example, can accumulate to significant concentrations in the vacuoles of mesophyll cells (Martinoia *et al.*, 1986; Leigh and Storey, 1993; Leigh and Tomos, 1993; Fricke *et al.*, 1994a) and because of this, Fricke and his co-authors suggested that strict spatial separation of Ca^{2+} and PO_4^{3-} was necessary to avoid precipitation. On countless occasions this investigation has shown that concentrations of Ca^{2+} and S well in excess of the solubility product for $CaSO_4$ (Weast and Astle, 1980) can be found in the same cellular compartment (see **Chapters 5, 6** and this chapter). These observations suggest that the rigorous segregation of those solutes which (in combination), could form insoluble salts may be less strict than implied by Fricke *et al.* (1994a). This possibility is discussed further in **Chapter 9**.

Although Cl^- is known to accumulate preferentially in epidermal cells (see for example, Hodson and Sangster, 1988; Dietz *et al.*, 1992; Leigh and Tomos, 1993; Fricke *et al.*, 1994a), the three nutrient solutions used in this experiment were not designed to stimulate Cl^- uptake and consequently, epidermal, bundle sheath and mesophyll cell Cl^- concentrations remained low in both CON and INV plants throughout leaf life. As described previously (see **6.6**), single cell Cl^- concentrations were unaffected either by the accumulation of S in INV plants or by the subsequent termination of SO_4^{2-} and re-instatement of NO_3^- supplies to these plants at day 13 post full expansion.

Similarly unaffected by the ratio of S : N supplied to plants was the accumulation and subsequent distribution of Mg^{2+} (see **Figures 7.9** and **7.10**). As leaves aged, Mg^{2+} appeared to accumulate similarly in the epidermal, bundle sheath and mesophyll cells of both CON and INV material. When supplied to excess, Mg^{2+} is known to accumulate in leaves despite its high mobility (Berchtold *et al.*, 1993). In field-grown potatoes, Berchtold and co-authors suggested that Mg^{2+} was accumulated

in order to compensate for a restricted availability of K^+. In this experiment, however, it seems unlikely that the availability of K^+ would have been limiting and therefore, the compensatory accumulation of Mg^{2+} should have been unnecessary.

As a rule, P_i accumulates preferentially in mesophyll cells (Martinoia *et al.*, 1986; Dietz *et al.*, 1992; Richardson, 1993; Fricke *et al.*, 1994a; 1996) where concentrations in excess of 100 mM can be detected (Dietz *et al.*, 1992; Fricke *et al.*, 1996). Epidermal concentrations on the other hand, are generally much lower (less than 10 mM; Dietz *et al.*, 1992; Fricke *et al.*, 1994a; 1996) which implies that these cells do not form significant sinks for P_i. In this experiment, epidermal, bundle sheath and mesophyll cell P concentrations were similar in all three cell types from CON material with values remaining low and constant throughout leaf life. In fact, values were often so low as to lie close to the 10 mM 'detection limit' of the EDX technique. However, as leaves aged, P accumulated in all three cell types from INV material, with concentrations being significantly higher (GLM; $p < 0.01$) in the mesophyll cells than in epidermal or bundle sheath cells from the same plant. The withdrawal of SO_4^{2-} and re-instatement of NO_3^- supplies at day 13 post full-expansion had negligible effect on the accumulation of P by epidermal, bundle sheath and mesophyll cells; values remained significantly higher (GLM; $p < 0.01$) than those measured in CON plants for the remainder of the experiment.

The EDX technique measures elemental composition (see **2.4.3**) and is thus unable to distinguish between the various chemical or physical states in which an element can occur (Richardson, 1993). It is safe to assume that many inorganic solutes (*i.e.* K or Cl), are present as their dissociated ions in solution (*i.e.* K^+ and Cl^-). However, the increase in single cell P concentrations in INV plants could theoretically be attributable to any number of P-containing compounds including sugar phosphates, nucleotides or P_i (Koyro and Stelzer, 1988). Addressing a similar problem, Fricke *et al.* (1994a) suggested that the majority of the P in epidermal samples would be in its inorganic form since P_i represents the dominant form of this solute in the cell vacuole (Lee and Ratcliffe, 1983; Roby *et al.*, 1987). In samples from bundle sheath and mesophyll cells, however, unavoidable cytoplasmic contamination (a problem discussed subsequently in **Chapter 9**) implies that P_i may not necessarily represent the major form of P found in the extracted saps from these sub-surface cells. Regardless of the form taken by P in the various leaf tissues, the question remains as

to why the epidermal, bundle sheath and mesophyll cells of INV plants accumulated P to significant concentration when values in CON material remained low and constant?

Using epidermal and mesophyll cell protoplasts isolated from barley, Dietz *et al.* (1992) demonstrated that the differential compartmentation of P within the cereal leaf was maintained even in plants supplied with 17 mM NO_3^-, 4 mM Cl^-, 3 mM SO_4^{2-} and supplemented further by an extra 100 mM SO_4^{2-}. In wheat supplied with substantially lower concentrations of anions in the nutrient solution (0.1 mM NO_3^-, 4.7 mM SO_4^{2-} and 0.1 mM Cl^-; see Richardson (1993) or 0.9 mM NO_3^-, 7.0 mM SO_4^{2-} and no Cl^-; the INV material in this experiment) significant concentrations of P are found in the upper epidermis.

Whilst it is conceivable that barley and wheat may differ with respect to the stimulus required to induce accumulation of P in epidermal cells, it is proposed that the strict compartmentation of P in the cereal leaf can be over-ridden when necessary in an attempt to regulate turgor and/or osmotic pressure. Therefore, the accumulation of P in the epidermis (rather than being attributable to high plant S status) is thought to occur as a consequence of the restricted availability of monovalent anions.

7.6, CONCLUSIONS

- When supplied to excess, S accumulates differentially within the cereal leaf. Concentrations were highest in the epidermis and significantly lower in bundle sheath and mesophyll cells. In CON material, concentrations were similar in all three cell types implying that this solute accumulates uniformly in the leaves of plants supplied with lower concentrations of SO_4^{2-}.

- Following withdrawal of plant SO_4^{2-} supply, significant re-mobilization of amassed S was observed in both the epidermal and bundle sheath cells of INV plants. The extent of this re-mobilization was similar between the two cell types. There appeared to be little or no appreciable re-mobilization of amassed S from mesophyll cells.

- Bell *et al.* (1994) identified four discrete compartments in mature leaves each having a different SO_4^{2-} efflux rate. The two slowest compartments were thought to correspond to the epidermis (faster) and mesophyll (slower). In this experiment, use of SiCSA techniques has made it possible to quantify the extent of S re-mobilization from the

epidermal and mesophyll cells of INV plants following withdrawal of SO_4^{2-} supply. In a subsequent calculation of S efflux rates, the values derived in this experiment were similar to those of Bell *et al.* (1994).

- Accumulation of excess S in the leaves of INV plants coincided with a significant decrease in epidermal turgor and osmotic pressure. The divalent SO_4^{2-} anion would appear to be a poor osmolyte, unable to fully substitute for either Cl^- or NO_3^- to maintain epidermal turgor and/or OP. In CON plants, turgor decreased progressively as leaves aged but even in the final stages of senescence, a significant turgor was detectable.

- Amassment of excess S in INV plants had negligible effect on (i) K^+ export from and (ii) the accumulation of Ca^{2+} by bundle sheath and mesophyll cells. However, P accumulated to significant concentrations in all three cell types as leaves aged, in contrast with control material.

Chapter 8:

DOES ACCUMULATION OF S AFFECT GROWTH OR LEAF CHLOROPHYLL AND CARBOHYDRATE CONTENT?

8.1, EXPERIMENTAL OBJECTIVES

The experiment described in this chapter was designed to address two main objectives. These were:

1, *Does the relative growth rate of those plants supplied with high SO_4^{2-} and low NO_3^- concentrations differ significantly from control material?*

2, *Does the accumulation of S in the leaves of high S plants have any significant effect on the relative chlorophyll and carbohydrate content of the third leaf?*

8.2, INTRODUCTION

It was decided that the determination of leaf sugar and chlorophyll contents and measurement of fresh and dry weights would enable an investigation to be undertaken into the effects of S accumulation on the photosynthetic capability, chlorophyll content and growth rate of high S plants.

In this experiment, control S (CON) and high S (INV) plants were grown as before whilst the SO_4^{2-} supply to both was withdrawn at day 13 post-full expansion (see **Chapter 6**). The experiment is described in detail in the following section.

8.3, METHOD

Three 3 l tubs were set up (see **2.2**) for the CONtrol (1 : 8 S : N) and INVerse (8 : 1 S : N) nutrient treatments (the composition of these nutrient solutions is given in **Chapter 5, Table 5.1**). As before, plant analyses began when the third leaf was approximately 2 days from full expansion. Subsequent analyses were then performed at 5, 12, 19 and 26 days post-full expansion. At each assessment point, six plants were removed from the three growth tubs per nutrient treatment. The first three were removed three hours into the photoperiod and leaf chlorophyll content was determined as described previously (see **2.3.6**).

The second batch of three plants was removed *precisely* nine hours into the photoperiod and a *circa* 2-3 cm transverse section was removed from the middle of each third leaf. This was weighed before being sealed in an Eppendorf tube and

plunged into a dewar of liquid nitrogen. After approximately 20 minutes, the Eppendorf tube (containing the frozen leaf section) was removed from the liquid nitrogen and stored at -20 °C pending sugar analysis (see section **2.3.5**). Fresh and dry weights of the remaining shoot were then determined as described previously (see **2.3.1**).

As in the preceding experiments (see **Chapter 6**), CON and INV material were transferred to the SO_4^{2-}-defficient NON nutrient solution (see **Table 6.1**) at day 13. All material was then grown continually in NON solution for the remainder of the experiment. Throughout this experiment, growth media were replaced every three days and at each solution change, a 1.0 ml sample was removed and frozen at -20 °C pending quantification of anion concentrations by HPLC (see **2.3.4.1**). Daily records of growth medium pH were also collected (results not shown).

8.4, RESULTS

8.4.1, Leaf chlorophyll

The relative chlorophyll content in the third leaf of those plants supplied with excess SO_4^{2-} and insufficient NO_3^- decreased with increasing leaf age until day 12 post-full expansion where values were significantly lower (ANOVA; $p < 0.001$) than those recorded in CON material (see **Figure 8.1**). When NO_3^- supply was restored at day 13, there was no significant change in the leaf chlorophyll content of these INV plants and values remained constant for the rest of the experiment as leaves aged and began to senesce.

In contrast with the observations made in INV plants, the chlorophyll content of CON material decreased more rapidly with increasing leaf age after day 12 such that by day 33 post full-expansion, values were significantly lower (ANOVA; $p < 0.001$) than those recorded in INV plants (see **Figure 8.1**). These observations indicate that accumulation of excess S by the leaf may interfere with the breakdown of chlorophyll as leaves senesce and this is given further consideration in section **8.5**.

8.4.2, Plant growth

With the exception of those data from the initial assessment point (day 18 post-germination), the fresh and dry weights of INV material were significantly lower (GLM; $p < 0.001$ for the remainder of the experiment) than those recorded in CON plants (**Figure 8.2**, *cf.* graph (i) with graph (ii)). The magnitude of this difference between fresh and dry weight data of the two nutrient treatments increased as plants grew and appeared unaffected by the withdrawal of SO_4^{2-} and re-instatement of NO_3^- supply at day 13. By the end of the experiment (*circa* day 46), the fresh and dry weights of INV material were just over half those recorded for CON plants.

A logarithmic plot of shoot fresh weight data demonstrates the effects of the control and high S nutrient regimes on shoot growth rates (**Figure 8.3**). Subsequent statistical analyses of these data (see **Chapter 3** for description of analysis) demonstrated that the growth rate of INV plants was significantly lower (MCT; $p < 0.001$) than that measured in CON material. The re-instatement of NO_3^- supply at day 13 appeared to have no effect on the impaired growth rate of INV material and the full implications of this and the other plant growth observations are discussed fully in the following section (see **8.5**).

Figure 8.1. Is leaf chlorophyll content affected by changes in the ratio of SO_4^{2-} : NO_3^- supplied to the roots? The effects of the S sufficient (CON) and excess S (INV) nutrient regime on the relative chlorophyll content of the third leaf. Results are expressed as the means ± SD of three plants.

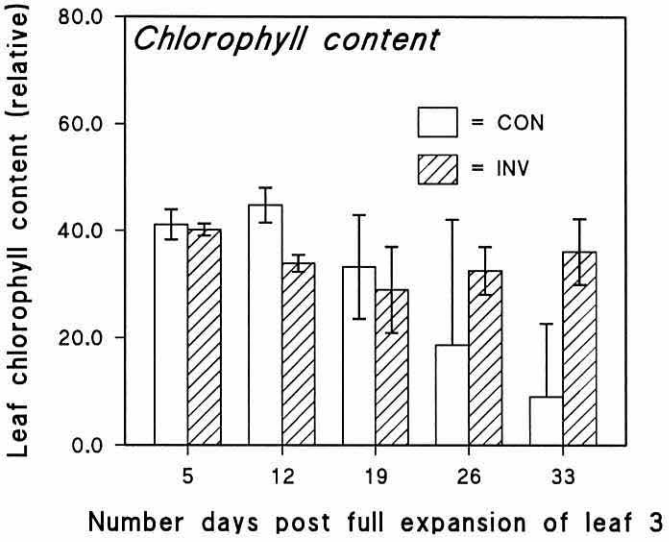


Figure 8.2. Is plant growth rate affected by changes in the ratio of SO_4^{2-} : NO_3^- supplied to the roots? The effects of the S sufficient (CON) and excess S (INV) nutrient regime on the (i) fresh and (ii) dry weights of the total shoot. Results are expressed as the means ± SD of three plants.

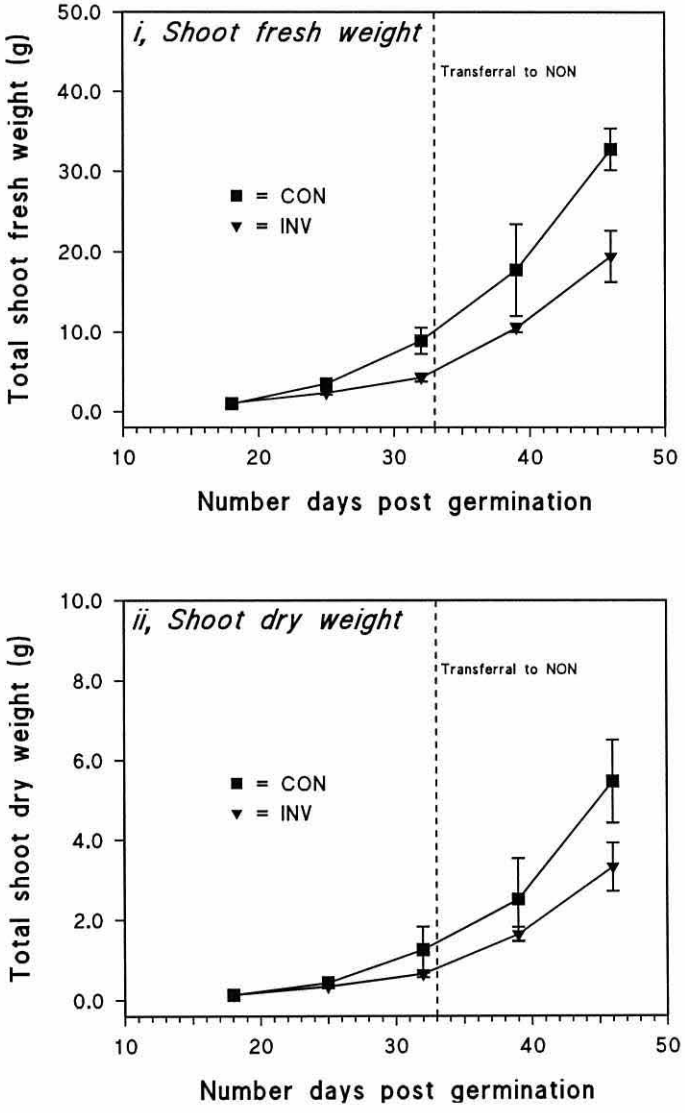


Figure 8.3. Is plant growth rate affected by changes in the ratio of SO_4^{2-} : NO_3^- supplied to the roots? Logarithmic plots of shoot fresh weight demonstrate the effects of the S sufficient (CON) and excess S (INV) nutrient regime on shoot growth rates throughout the experiment. Each datapoint represents an individual measurement and a first order regression connects datapoints.

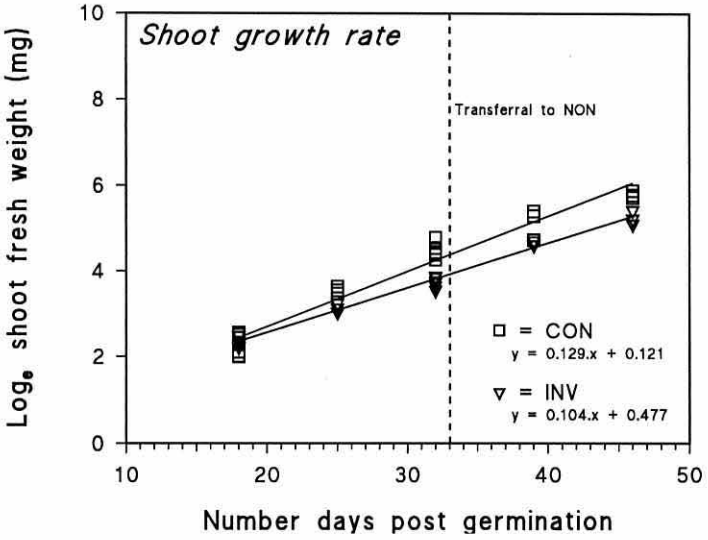
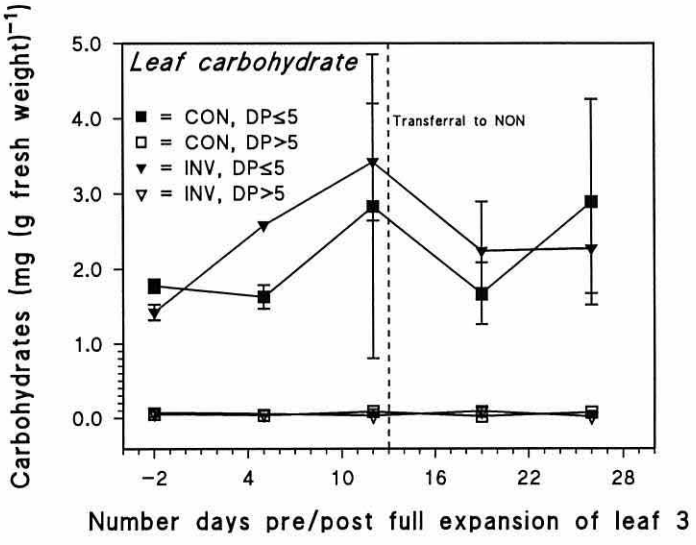


Figure 8.4. Is the soluble carbohydrate content of the leaf affected by changes in the ratio of SO_4^{2-} : NO_3^- supplied to the roots? The effects of the S sufficient (CON) and excess S (INV) nutrient regime on both the ethanol soluble (degree of polymerisation (DP) < 5) and ethanol insoluble (water soluble, DP > 5) carbohydrate content of the third leaf. Results are expressed as the means ± SD of three plants.



8.4.3, Soluble leaf carbohydrate content

Despite some fluctuation, the ethanol soluble (DP < 5) carbohydrate content of whole leaves remained relatively constant throughout this experiment with similar values being recorded in both CON and INV material regardless of the withdrawal of SO_4^{2-} supply at day 13 (see **Figure 8.4**). The ethanol insoluble (water soluble, DP > 5) carbohydrate content of whole leaves was considerably lower (*circa* 50 fold) than the ethanol soluble content although it too remained constant throughout this experiment, being unaffected by nutrient treatment or increasing leaf age. In combination, these observations suggest that neither changes in N : S ratio nor increasing leaf maturity have any effect on the accumulation of sugars (such as sucrose or hexose) with DP < 5 or those with DP > 5 (such as high molecular weight fructans, see **2.3.5**).

8.5, DISCUSSION

8.5.1, Does the accumulation of S affect plant growth rate?

Extreme N deficiency can result in an inhibition of both root and shoot growth (Smart, 1994), although development of the root system is affected less than that of the shoot (Scheible *et al.*, 1997). It is thought that by maintaining root development at the expense of shoot growth, the plant improves its chances of nutrient acquisition when N is limiting (see for example, Bloom *et al.*, (1985)). No measure was made of root fresh and dry weights in this experiment (for the same reasons as discussed in section **3.5**). However, it is presumed that differences in shoot fresh and dry weights and plant growth rate (see **Figures 8.2** and **8.3**) between CON and INV material were attributable to insufficient NO_3^- in INV nutrient solution.

The senescence of leaves represents a highly-regulated sequence of biochemical and physiological events which constitutes the very end of the development process (see **1.3**). Senescence is frequently induced in those plants denied an adequate supply of essential nutrients (Usuda, 1995). In N deficient plants for example, chloroplasts are smaller, diminish more quickly after attaining full size (Kutík *et al.*, 1993) and contain less chlorophyll (Martinoia *et al.*, 1983; Mei and Thimman, 1984) than those from N sufficient plants. It would appear that protein synthesis takes precedent over chlorophyll production in those plants deprived of N (Mei and Thimman, 1984).

8.5.2, Does the accumulation of S affect leaf chlorophyll content?

By day 12 post-full expansion, the leaf chlorophyll content of the N deficient INV plants was significantly lower (ANOVA; $p < 0.001$) than that of CON material supplied with sufficient NO_3^-. Interestingly, however, following restoration of NO_3^- supply at day 13, the relative leaf chlorophyll content of INV plants remained relatively constant even as the leaf entered the very final stages of senescence (*circa* day 33). In contrast, the chlorophyll content of CON leaves decreased progressively as material aged such that by day 33, the mean value was 75 % lower than that of INV plants.

The loss of greenness from the leaf (which characterises the final stages of senescence) is attributable to the breakdown of chlorophyll. This complex process begins with the opening of the porphyrin macrocycle and effectively concludes with the dumping of unwanted catabolites in the central vacuoles of mesophyll cells

(Matile *et al.*, 1996 and references therein). On the basis of the observations made in this experiment, it would appear that the breakdown of chlorophyll in the mature leaves of INV plants is impaired by the accumulation of excess S.

8.5.3, Does the accumulation of S affect the soluble carbohydrate content of the leaf?

Throughout this experiment, the ethanol and water soluble carbohydrate content of the third leaf remained relatively constant irrespective of nutrient treatment or increasing leaf age. In both CON and INV material, the amount of ethanol soluble sugars (with DP $<$ 5) consistently exceeded the amount of ethanol insoluble/water soluble carbohydrate (with DP $>$ 5) measured in the same leaf. Therefore, it appears that the ratio of N : S supplied to the plant has negligible affect on the accumulation of sugars within mature leaves. Interestingly, Reddy *et al.* (1996) demonstrated the existence of an inverse relationship between the N and sucrose contents of the young leaves of pima cotton plants. Clearly though, the measurements made in this experiment failed to indicate the existence of a similar trend.

Leaf accumulation of carbohydrate is influenced both by the rate of synthesis of the sugars and by the subsequent demand made on these by the growing plant. It is possible that carbohydrate synthesis would have been impaired in the N deficient INV plants as both photosynthetic rate (Dietz and Helios, 1990; Reddy *et al.*, 1996) and leaf area (Bänziger *et al.*, 1994) are known to be reduced in nutrient deficient plants. Logically, the significantly reduced growth rate shown by INV plants (see **Figure 8.3**) may have been expected to lead to a decreased demand for the products of photosynthesis. Therefore, the reduced rate of carbohydrate synthesis could effectively have been cancelled out by the decreased demand for photosynthetic products; the carbohydrate content of both CON and INV material would, therefore, appear similar.

8.6, CONCLUSIONS

- (i) Shoot fresh weight, (ii) shoot dry weight and (iii) plant growth rate were significantly reduced in INV plants when compared with CON material. The re-instatement of NO_3^- supply at day 13 had negligible affect on these values. In contrast, the soluble carbohydrate content of the leaf was unaffected by the ratio of S : N supplied to plants.

- The leaf chlorophyll contents of CON and INV plants were initially similar at the start of the experiment. As leaves reached 12 days of age, however, the accumulation of S in INV plants appeared to coincide with a substantial drop in leaf chlorophyll content such that values were significantly lower than those measured in CON material of equivalent age. In contrast to CON plants, when NO_3^- was restored to INV material, the leaf chlorophyll content remained constant for the remainder of the experiment. Values were significantly higher than those measured in CON plants where the leaf chlorophyll content decreased progressively as leaves aged. It was proposed that the catabolism of chlorophyll was impaired in INV plants.

Chapter 9:
FINAL DISCUSSION AND CONCLUDING REMARKS

9.1, FINAL DISCUSSION

The experiments described in chapters 5, 6, 7 and 8 were designed to investigate the behaviour of S when induced to accumulate in and subsequently re-mobilize from the mature third leaves of wheat plants. Although far from numerous, there are some other examples in the published literature of similar studies having been carried out in spinach (see for example, Dietz, 1989), barley (Adiputra and Anderson, 1992; 1995), *Macroptilium atropurpureum* (Bell *et al.*, 1994) and soybean (Sunarpi and Anderson, 1996). However, the extensive use of SiCSA techniques in this investigation has, for the first time, made it possible to obtain quantitative data on the ways in which the solute composition of anatomically and spatially distinct leaf cells are affected by the ratio of S : N supplied to growing plants. On this basis, therefore, the experiments described in this thesis represent the first of their kind.

When supplied to excess, S (presumably as SO_4^{2-}) accumulates in leaves to significant concentrations (in excess of 100 mM; see **Figure 5.1**). Not surprisingly, therefore, epidermal, bundle sheath and mesophyll cell S concentrations were significantly higher than those recorded in CON plants (see **Figure 7.1**). This observation raises the first in a series of questions.

9.1.1, Why does S accumulate in the leaves of high S plants?

In **Chapter 1**, emphasis was placed on the increasing problems of S deficiency in U.K. agriculture. The experiments described in this thesis, however, appear in some way irrelevant to these concerns as some plants were induced to accumulate excess S by supplying them with a modified INV nutrient solution. Why then, were plants loaded with S in order to investigate the problems associated with S deficiency?

Throughout this investigation, epidermal S concentrations were consistently lower than 30 mM in CON plants (see **Figures 5.1, 6.2, 6.4** and **7.1**). It is possible to map solutes at a very fine spatial resolution using SiCSA techniques (see for example, Hinde, 1994; Fricke *et al.*, 1994a, b, c; 1995; Cuin, 1996; Pritchard *et al.*, 1996) and generally, the resolution of this type of analysis is in the order of 10 mM (Fricke *et al.*, 1994a). Whilst on some occasions, it has been possible to detect (and quantify) the

re-mobilization of S amassed by the epidermis of these 'low S' plants (see **Figure 6.2**), closer inspection of these data reveal how easy it would be to attribute this decrease in epidermal S concentrations to experimental variation, rather than to the true efflux of this solute out of cell vacuoles.

By inducing plants to accumulate S, it was possible to establish a large initial S signal in the vacuolar saps extracted from what were subsequently termed 'high S' (or INV) plants. When plant SO_4^{2-} supply was then withdrawn, the small reduction in the size of the S signal was relatively fluctuation-free. In fact, it became difficult to attribute the reduction in signal size to anything other than the re-mobilization of S from the vacuole. Although the SO_4^{2-} status of the plants used in this investigation was considerably higher than one might expect to find in the field (*cf.* Marschner, 1995), it is argued that loading plants with excess S represents an important pre-requisite in studying the efficiency with which field-grown crops use SO_4^{2-}.

When plants were supplied with a 1 : 1 ratio of S : N (see **Chapter 5**, EQUI nutrient treatment), there was no significant accumulation of S within the leaves (see **Figure 5.1**). This lack of accumulation in equi S material implies that when plants are supplied with equal concentrations of NO_3^- and SO_4^{2-}, uptake of NO_3^- is preferential to the uptake of SO_4^{2-}. This implies, therefore, that the amassment of S in the leaves of INV plants occurred, perhaps, as a joint response to an insufficient supply of monovalent anions (electrical requirement?) and the inability of epidermal cells to use organic ions as principal osmotica (Leigh and Tomos, 1993).

It is concluded, therefore, that S will only accumulate to excess in those plants grown in hydroponic solutions containing high concentrations of SO_4^{2-}. This elevation in nutrient solution SO_4^{2-} content alone, however, is insufficient to induce the accumulation of S and the supply of monovalent anions (Cl^- and NO_3^-) must be simultaneously restricted.

The accumulation of excess S in the leaves of INV plants has had significant and far reaching effects on many of the parameters measured in this investigation. The catabolism of chlorophyll for example, appeared to be impaired in INV plants (see **Chapter 8**). Turgor and osmotic pressures too, were significantly affected by the accumulation of excess S. Some of these observations have been easy to interpret and full discussions appear in the relevant chapters. The decrease in epidermal turgor and osmotic pressures of INV plants for example, has been attributed to the inability of

divalent anions (such as the accumulating SO_4^{2-} in INV plants) to maintain plant water relations. This observation was subsequently discussed in detail in section **6.5**. Interpretation of many of the other experimental observations has proved considerably more challenging. These observations will now be considered in detail in this section of the thesis.

9.1.2, Why is the export of K^+ and accumulation of Ca^{2+} diminished in high S vacuoles?

Potassium often represents the dominant vacuolar cation in the epidermal cells of young leaves (Dietz *et al.*, 1992; Hinde, 1994; Fricke *et al.*, 1994a, b, c). As these leaves subsequently age, considerable amounts of this K^+ are lost (see **Figures 5.3, 6.9-6.10** and **7.5-7.6**, this study; Greenway and Pitman, 1965; Bogemans *et al.*, 1990; Berchtold *et al.*, 1993). However, it would appear that the initiation of this process differs according to the light intensity under which plants are grown (compare for example, Greenway and Pitman (1965) with Bogemans *et al.* (1990) or Hinde (1994) with Fricke *et al.* (1995). It has been suggested that the net export of leaf K^+ prior to the onset of senescence indicates the efficiency with which this solute is regulated within the plant (Bogemans *et al.*, 1990).

Leaf Ca^{2+} concentrations increase significantly as leaves age (see **Figure 7.7**) and in unstressed plants grown in relatively low light, this accumulation of Ca^{2+} osmotically offsets the loss of K^+ such that the vacuolar turnover of these two solutes are linked in a reciprocal relationship (see section **5.5** and Hinde, 1994). Throughout this investigation the induced accumulation of excess S in the leaves of INV material coincided with diminished rates of both K^+ export and Ca^{2+} import (see **Figures 5.3-5.4, 6.9-6.12** and **7.5-7.8**). This may have led to the loss of the 1 : 1 Ca^{2+} : K^+ relationship as seen in the epidermis of CON plants (see **Figure 5.5**). The cause of the diminished K^+ export and Ca^{2+} import remains unknown although due to the reciprocal relationship existing between these two solutes in the leaves of CON plants, it is possible that the cause of the diminution in Ca^{2+} and K^+ turnover rates could be closely related. Theoretically, the diminution could be attributable to a significant change in the level(s) of another inorganic cell solute(s) stored within the vacuole. With the exception of Ca^{2+} and K^+, the concentrations of amassed NO_3^- and

SO_4^{2-} varied most between CON and INV plants (by virtue of the differing ratios of S : N supplied to plants).

Initially, it was thought that the reduced NO_3^- status of INV vacuoles might have been responsible for the diminished export of K^+ and import of Ca^{2+} as leaves aged (see **Chapter 5**). In subsequent experiments, however (see **Chapters 6** and **7**) the increase in NO_3^- status of INV vacuoles (following withdrawal of SO_4^{2-} and re-instatement of NO_3^- supplies) had no significant effect on either epidermal Ca^{2+} or K^+ concentrations. In fact, neither Ca^{2+} nor K^+ turnover rates were affected by the change in the S : N ratio supplied to plants (see **Figures 6.9-6.12** and **7.5-7.8**). On the basis of these observations, it is proposed that the diminution of Ca^{2+} import and K^+ export are attributable to the elevated SO_4^{2-} status of the epidermal vacuoles from INV plants. A similar loss of the Ca^{2+} : K^+ inter-relationship has been reported in the upper epidermis of barley induced to accumulate malate (Fricke *et al.*, 1995).

If we accept that the increase in vacuolar SO_4^{2-} status was responsible for the diminution of Ca^{2+} import and K^+ export, two hypotheses can be proposed in order to explain why the accumulation of excess S in the leaves of INV plants could have led to a substantial retention of epidermal K^+. In the first hypothesis, we propose that epidermal K^+ reserves were maintained in order to compensate for the restricted availability of monovalent anions supplied to the plant in the high S (INV) nutrient solution (see **Figure 9.1**). By retaining epidermal K^+ reserves, the plant may have been effectively trying to minimise the impact of the substantial decrease in epidermal turgor and/or osmotic pressure caused by the sub-optimal supply of monovalent electrolytes. In turn, the retention of K^+ would have presumably led to a reduction in the amount of Ca^{2+} required to maintain charge neutrality within the vacuole. Therefore, retention of K^+ (as a direct response to a decrease in turgor and/or OP) might have regulated the influx of Ca^{2+} in the epidermal vacuoles of the INV plants in this investigation. In effect, this conjecture contradicts that proposed by Hinde (1994) who theorised that Ca^{2+} influx (as a function of leaf age) controlled the loss of K^+ from epidermal vacuoles in barley. Whilst species-specific differences may be responsible for this apparent contradiction, it is more likely that these two contrasting theories have arisen as a result of the substantially different S and N status of the INV wheat in this investigation and the barley used by Hinde (1994).

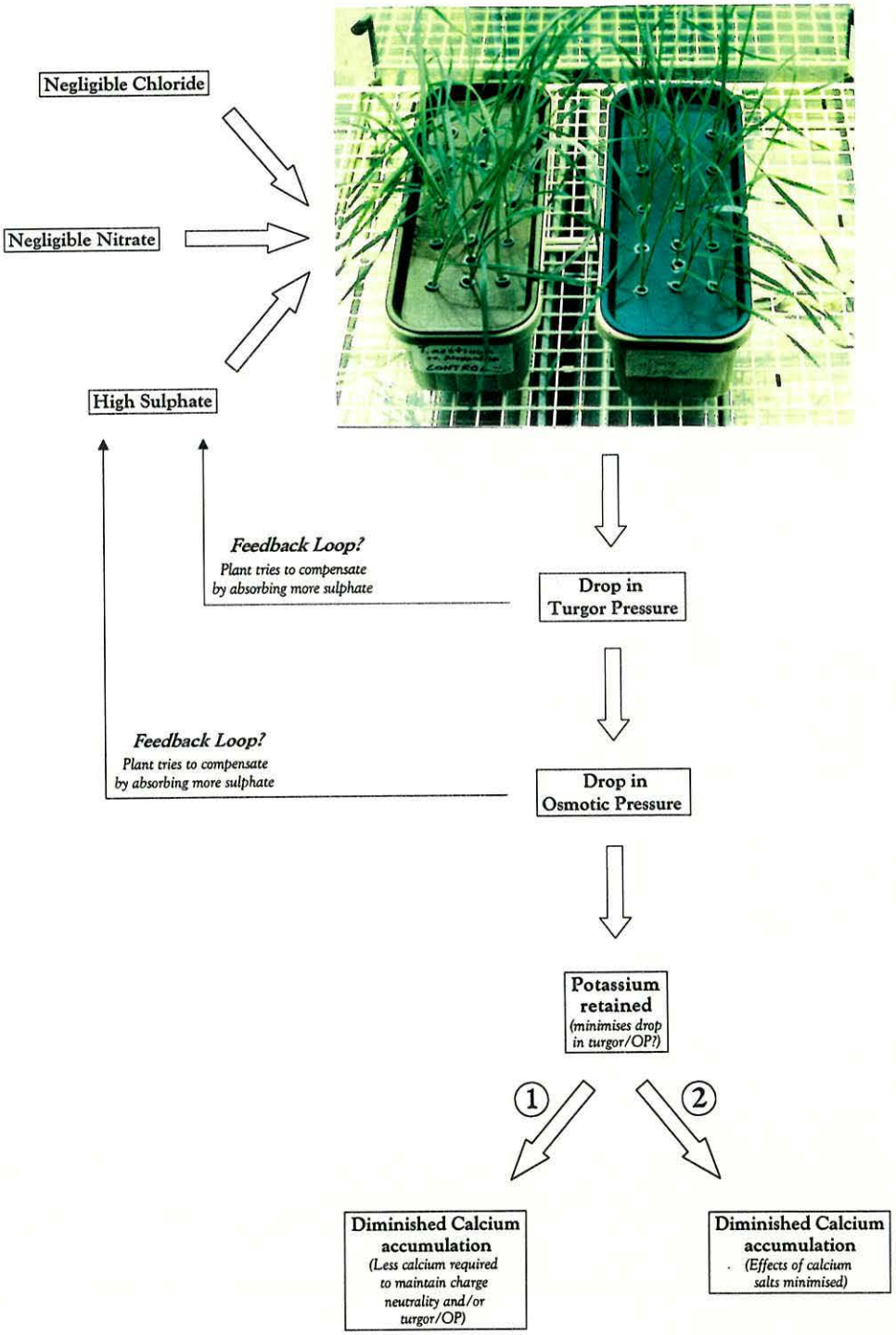
In the second hypothesis (see **Figure 9.1**), we propose that the retention of K^+ was necessary to avoid the excess precipitation of Ca^{2+} salts in epidermal cell vacuoles. The solubility of $CaSO_4$ is very low (*circa* 14 mM; Weast and Astle, 1980) and it is possible that a mechanism could exist to limit the export of K^+ from the vacuole thereby restricting the accumulation of Ca^{2+} as a function of leaf age (Hinde, 1994; Fricke *et al.*, 1994a, 1995). By limiting the epidermal amassment of Ca^{2+}, the impact of the precipitation expected from 100 mM Ca^{2+} and 100 mM S measured in the same cell vacuole could be minimised. Presumably, however, since Ca^{2+} represents an essential requirement for cellular metabolism, epidermal accumulation was not entirely prevented, even in INV plants (see **Figures 5.4, 6.11-6.12, 7.7-7.8**).

The situation is made even more complex by the accumulation of significant amounts of P_i in the epidermal cells of INV plants (associated discussion appears in section **7.5**). As for $CaSO_4$, the solubility of calcium phosphate salts are low (Weast and Astle, 1980) which implies that the INV epidermis contained considerable quantities of two insoluble calcium compounds. By amassing these salts, it would appear that the upper epidermis of INV plants functioned as little more than a 'dumping ground' for unwanted and insoluble compounds.

In spite of the accumulation of insoluble salts in the upper epidermis and the severely restricted supply of monovalent electrolytes, the strict compartmentation of Ca^{2+} within the leaves of INV plants was rigorously maintained (see **Chapter 7**). As suggested by Leigh and Tomos (1993), these observations would seem to indicate that the exclusion of Ca^{2+} from sub-surface cells is of paramount importance to the plant, maybe indicating a "particular sensitivity of mesophyll processes to these ions."

In conclusion, therefore, the amassment of excess S in the leaves of INV plants had significant (but as yet, not fully understood) effects on K^+ re-translocation and Ca^{2+} accumulation. It would be interesting to know whether these observations are specific to the amassment of SO_4^{2-} or whether they are also seen when other divalent, inorganic electrolytes accumulate to significant concentrations.

Figure 9.1. A summary of the proposed inter-relationship(s) which may exist between high vacuolar S concentrations and diminished K^+ export and Ca^{2+} import in the epidermal trough cells of INV plants. Two hypotheses have been proposed to explain these observations. In hypothesis (1), less Ca^{2+} is required to maintain charge balance and water relations but Ca^{2+} influx might also be diminished if the cell was attempting to minimise the effects of the precipitation of two insoluble calcium salts in its vacuole (hypothesis (2)).



9.1.3, Why is S accumulated differentially within the leaves of high S plants?

When supplied to excess, S (presumably as SO_4^{2-}) appears to accumulate differentially within the cereal leaf with epidermal concentrations being significantly higher than those of the sub-surface cells (see **Figure 7.1**). As for many other inorganic solutes (see for example, **Chapters 6** and **7**, this study; Hodson and Sangster, 1988; Dietz *et al.*, 1992; Leigh and Storey, 1993; Fricke *et al.*, 1994a) the reason for this differential distribution of S within the leaf remains unclear. In spite of this, however, there can be little doubt that intercellular gradients in vacuolar solute concentrations play a major role in leaf physiology (Fricke *et al.*, 1996).

It has been suggested that a possible sensitivity of mesophyll metabolism to Ca^{2+} (see above) and Cl^- (Dietz *et al.*, 1992; Leigh and Storey, 1993) is responsible for the exclusion of these ions from sub-surface tissues. In the case of Cl^-, preferential sequestration within the epidermis may represent a stress-tolerance mechanism whereby exclusion of Cl^- from sub-surface cells would enable leaf metabolism to continue even during conditions of severe salt stress (Fricke *et al.*, 1996). In the case of S, however, the fact that significant concentrations were measured in sub-surface cells prior to the withdrawal of SO_4^{2-} supply (see **Figure 7.1**) suggests that bundle sheath and mesophyll metabolism is not particularly sensitive to the accumulation of this solute.

Using the cytoplasmic marker enzyme malate dehydrogenase (MDH), Fricke *et al.* (1994a) found that single cell sap samples extracted from epidermal cells were almost exclusively vacuolar in origin. In contrast, those taken from bundle sheath or mesophyll cells often contained a mixture of both vacuolar and cytoplasmic components. In this investigation, no measure was made of MDH activity in the samples taken from bundle sheath and mesophyll cells although the frequent appearance of chloroplasts in these sub-surface samples (results not shown) implies that these extracts were at least partly contaminated with cytoplasm.

The unavoidable cytoplasmic contamination of single cell sap samples taken from sub-surface cells (as identified by Fricke *et al.* (1994a) and in this study by the presence of chloroplasts in bundle sheath and mesophyll cell extracts) could potentially lead to a dilution of the solute contents of the vacuole since cytoplasmic concentrations of NO_3^-, Cl^- and SO_4^{2-} are substantially lower than those of the corresponding vacuole (Mornet *et al.*, 1997). Therefore, the concentration of a solute

(such as S) in single cell sap samples from sub-surface cells could be lower than the concentration of the same solute in the epidermis by virtue of the higher proportion of cytoplasmic contamination rather than by differences in the extent of the accumulation of the solute between tissue types *per se*.

Unfortunately, it is not possible to ascertain the extent of the contribution made by cytoplasmic contamination to the differences in cellular S concentrations seen in epidermal and sub-surface tissues. In barley leaves for example, the central vacuole constitutes 99 % of epidermal cell volume whilst the same structure occupies just 61 % of mesophyll cell volume (Winter *et al.*, 1993). Consequently, if the vacuole were to constitute similar proportions in wheat epidermal and mesophyll cells, a 40 % difference between the concentration of a solute in these two cell types might be expected (assuming of course, that the entire contents of the cell were extracted during removal of a sample). Interestingly, the mean mesophyll cell S concentration in the INV plants of this investigation (see **Chapter 7**) was 48 % lower than the mean epidermal concentration and we wonder how much of this 'apparent' heterogeneity between tissue types was attributable to cytoplasmic contamination of sub-surface samples.

In some cases, the concentration of a solute (for example Ca^{2+}) differs so much between epidermal and mesophyll tissues to suggest the involvement of a factor other than cytoplasmic contamination in establishing the heterogeneous distribution of that solute within the leaf. If we re-consider as an example, the single cell Ca^{2+} data from the 12 day old CON material of **Chapter 7**, the mean Ca^{2+} concentrations were 323, 14 and 9 mM for epidermal, bundle sheath and mesophyll cell populations respectively.

The difference between the epidermal and sub-surface Ca^{2+} concentrations is substantial (in excess of 300 mM) and it seems improbable that a difference of this magnitude was caused exclusively by the cytoplasmic contamination of samples from sub-surface cells. Besides, the heterogeneous distribution of Ca^{2+} between epidermal and mesophyll tissues has been demonstrated by techniques other than SiCSA, including X-ray microanalysis of frozen-hydrated sections (Hodson and Sangster, 1988; Leigh and Storey, 1993) and protoplast isolation and characterisation (Dietz *et al.*, 1992). In spite of the relatively rudimentary knowledge concerning the movement of solutes within whole leaves (Leigh and Tomos, 1993), it is possible to propose two

mechanisms by which the heterogeneous distribution of a solute (such as Ca^{2+}) may be established.

Firstly, one may expect the composition of bundle sheath, mesophyll and epidermal cells to differ if these three cell types were supplied with differing proportions of solute depending upon their location within the leaf. Thus, certain cells may accumulate particular solutes because these are the only solutes with which they are supplied (Leigh and Tomos, 1993). Selective retention of solutes (such as P_i by the mesophyll cells for example) could ultimately lead to a reduced availability of P_i in the local apoplast surrounding epidermal cells. Alternatively, transportation of those solutes destined for the mesophyll may follow a different pathway to those designated as exclusively epidermal (Tomos and Wyn Jones, 1988; Canny, 1993; Leigh and Tomos, 1993; Fricke *et al.*, 1994c). Again, if this were the case, one might expect that the solute composition of epidermal and sub-surface cells would differ substantially.

Solutes are transported across the plasma membrane and into the cell by a variety of specific transporters and ion channels (Allen and Sanders, 1997). Recent application of molecular (and other innovative) techniques has resulted in the identification of specific tonoplast carriers for K^+, Ca^{2+}, NO_3^- (Bassham and Raikhel, 1996), malate and SO_4^{2-} (Mornet *et al.*, 1997). In recognition of the differing ultrastructure (see for example, Weier *et al.* (1982) and subsequent function of epidermal and mesophyll cells, one may expect to find differences in the expression, type and maybe density of the tonoplast carrier proteins were it possible to directly compare the tonoplast membranes from these two cell types (Fricke *et al.*, 1995). Consequently, if all solutes were supplied equally to all cells, it is possible that the differences in the suites of membrane transporter proteins expressed on the tonoplast of epidermal and mesophyll cells could further result in differing cell solute compositions.

A plausible conclusion for this section could be thus: the non-uniform accumulation (and subsequent distribution) of S in the leaves of INV plants may have arisen (i) as a result of differences in the levels of expression of SO_4^{2-} transporters on epidermal and mesophyll cell tonoplasts or (ii) unavoidable cytoplasmic contamination of single cell sap samples extracted from sub-surface cells.

9.1.4, Why is S re-mobilized differentially from the leaves of high S plants?

This investigation has shown that when the SO_4^{2-} supply to plants induced to accumulate S is suddenly withdrawn, differential re-mobilization of amassed S reserves can be seen. Furthermore, it seems unlikely that cytoplasmic contamination could negate the value of these data (see **Figure 7.1**) since the turnover of SO_4^{2-} in this compartment is considerably faster than that in the vacuole (for a more thorough discussion, see section **7.5**). Consequently, the S stored in the cytoplasmic pool is depleted faster than that in the vacuolar pool under conditions of SO_4^{2-} starvation (Cram 1983a, b; Bell *et al.*, 1994; Mornet *et al.*, 1997).

Re-mobilization of amassed S from epidermal and bundle sheath cells is relatively rapid (see section **7.5.3**). In contrast, the S accumulated by mesophyll cells effectively appears 'immobile' once sequestered (presumably) into the central vacuole. Despite the use of separate, unrelated techniques on differing species of plant grown under different growth conditions, estimates of rates of S efflux (see **7.5.3**) from the epidermal and mesophyll tissue of mature wheat leaves are remarkably similar to the values derived by Bell *et al.* (1994). Therefore, differing populations of leaf cell exchange SO_4^{2-} at different rates although the question remains, however, as to how this can occur.

Solutes can be transported across the tonoplast by a variety of specific transporters and ion channels involving either active or passive mechanisms (Pantoja *et al.*, 1989; Allen and Sanders, 1997). At the present time, the largest body of information pertains to the identification and characterisation of cation transporters on the tonoplasts of epidermal guard cells by virtue of the rapidity and size of the current associated with the cationic fluxes in these cells (see for example Allen *et al.*, 1998). Far less is known about the behaviour of anion channels and at the time of writing, only tonoplast-resident channels specific for vacuolar influx of malate and Cl^- have been identified (Allen and Sanders, 1997). In spite of this lack of information, it is concluded that differences in SO_4^{2-} flux between epidermal and mesophyll cells might be attributable to different levels of expression, leading to differences in the densities and maybe even specificities of SO_4^{2-} channels in the tonoplasts of these two cell types.

9.2, CONCLUDING REMARKS

This investigation has been successful in achieving its primary aims (see **1.4**) of identifying the two cellular compartments described by Bell *et al.* (1994). Whilst the reason(s) for the difference might remain unclear, the use of SiCSA techniques has shown that epidermal and mesophyll tissues re-mobilize amassed S to differing extents (see **Chapter 7**). The negligible re-mobilization of mesophyll S (see **Figure 7.1**) implies that turnover of amassed S from this tissue could, potentially, be too slow to make a significant contribution to the S economy of the plant (see **7.5.3**). Since the mesophyll cells may constitute approximately 38 % of total leaf area (Jellings and Leech, 1982), it must be concluded that a significant proportion of the S amassed by the leaf is effectively 'immobile' and thence unavailable for re-translocation irrespective of the S-status of the plant.

Throughout this investigation, growth of material in the modified INV Long Ashton nutrient solution has had significant and far-reaching effects on both the water relations and behaviour of other solutes within the ageing third leaf. Both K^+ export and Ca^{2+} import were significantly diminished in the leaves of INV plants (see **Figures 6.9-6.12, 7.5-7.8**) and it was proposed that these observations were attributable to the substantial decreases in epidermal turgor and osmotic pressure (see **Figures 6.6-6.8, 7.2-7.3**). In turn, this decrease in turgor and OP was assumed to have been caused by the inability of divalent anions to maintain plant water relations (see sections **6.6.3** and **7.5.4** for discussion of epidermal OP and turgor pressure respectively).

From a personal point of view, SiCSA techniques have fulfilled their usefulness in acquiring the data necessary to describe the behaviour of SO_4^{2-} when induced to accumulate in and then re-mobilise from the differing cell types within the cereal leaf. With the exception of further replication of the experiments described in this thesis, the logical continuation of this study requires the application of molecular and electrophysiological techniques.

9.3, SUGGESTIONS FOR FUTURE WORK

In spite of the recent advances made in cloning plasma membrane SO_4^{2-} transporters from higher plants (see for example, Hawkesford and Belcher, 1991; Hawkesford *et al.*, 1993; Hawkesford and Smith, 1997), little or no progress has been made on characterising the transporter(s) responsible for either the influx or efflux of SO_4^{2-} into (or out of) the cell vacuole.

Faced with our current lack of understanding of the uptake and behaviour of SO_4^{2-} in the vacuoles of higher plant cells, the practical sequent necessary to address the aims and hypotheses (with respect to how SO_4^{2-} is re-mobilized differentially from differing populations of cells) generated both by this investigation and that of Bell *et al.* (1994) could prove difficult if not impossible to address at the present time. It is clear, however, that future investigation into the re-mobilization of cellular S should be concerned with those events occurring principally at the tonoplast.

Electrophysiological (patch-clamp) measurements on epidermal and mesophyll cell vacuoles facilitates the characterisation of ion channels expressed at the level of the tonoplast (Allen and Sanders, 1997). This type of approach has proved very successful in the characterisation of a number of specific vacuolar ion channels for Ca^{2+}, K^+ and Cl^- (Allen and Sanders, 1997). Unfortunately, SO_4^{2-} fluxes across the tonoplast are both slow and small (**Chapter 7**, this study; Martinoia *et al.*, 1986; Mornet *et al.*, 1997). Consequently, the current associated with such fluxes (upon which patch-clamp measurements rely) could either be very small or completely undetectable using existing electrophysiological techniques (Maathuis, *pers. comm.*).

A molecular approach, therefore, appears the more feasible step forward for this study. The direct visualisation of cellular location and determination of the density of tonoplast SO_4^{2-} ion channels by, for example, tagging with green fluorescent protein (see Marshall *et al.*, 1995; Hughes *et al.*, 1998; Müller-Röber *et al.*, 1998; Makhina and Nichols, 1998) or use of micro-immunolocalization (Leech and Marrison, 1996) awaits the cloning of the gene responsible for these channels. Only when this gene(s) has been identified, will the cause of the heterogeneous distribution and subsequent re-mobilization of SO_4^{2-} within ageing leaf tissue become clearer.

Appendix 1:
LISTINGS OF EQUIPMENT MANUFACTURERS AND CHEMICAL SUPPLIERS

Refered to in text as:	Full address:
Agaraids	Agaraids, Stansted, Essex, U.K.
BDH	BDH Ltd., Poole, Dorset, U.K.
Boehringer	Boehringer, Lewes, East Sussex, U.K.
Bio-tek	Bio-tek Instruments Inc., Luton, U.K.
Cecil	Cecil Instruments Ltd., Cambridge, U.K.
Clark	Clark Electromedical Instruments, Reading, U.K.
Dionex	Dionex (U.K.) Ltd., Camberley, Surrey, U.K.
Fluka	Fluka, Gillingham, Dorset, U.K.
de Fonbrune	de Fonbrune, Alcatel, Annecy, France.
Gallenkamp	Gallenkamp, Loughborough, Leicestershire, U.K.
Grant	Grant Instruments, Cambridge, U.K.
Hamilton	Hamilton Co., Reno, Nevada, U.S.A.
Harvard	Harvard Apparatus, Sheerness, Kent, U.K.
Jung	Jung, Germany.
LEEC	LEEC Ltd., Nottingham, U.K.
Leica	Leica Ltd., Milton Keynes, U.K.
Leitz	Ernst Leitz Wetzlar GmbH, D-6330 Wetzlar, Germany.
µicropHep	µicropHep, Hanna Instruments, BDH Ltd., Poole, U.K.
Minolta	Minolta (U.K.) Ltd., Milton Keynes, U.K.
Pharmacia	Pharmacia (U.K.) Ltd., St. Albans, U.K.
Rathburn Chemicals	Rathburn Chemicals Ltd., Walkerburn, Scotland U.K.
Reichert	Reichert Jung, Germany.
R. S.	R. S. Components, Corby, Northamptonshire, U.K.
Oxford Instruments	Oxford Instruments, High Wycome, U.K.
Shimadzu	Shimadzu Corp., Kyoto, Japan.
Sigma	Sigma, Poole, Dorset, U.K.
Spark Holland	Spark Holland B.V., The Netherlands.
Varian	Varian Techtron Pty. Ltd., Victoria, New Zealand.
VEB	MLW Zentrifugeubau, Engelsdorf, Germany.
Viglen	Viglen, London, U.K.
Wacker	Wacker Chemicals Ltd., Walton on Thames, U.K.
Wescor	Wescor Inc., Logan, Utah, U.S.A.

Appendix 2:

SUMMARISED DEVELOPMENT OF NO_3^- ASSAY

Hypothesis/Aim	Results	Conclusions
Does the microfluorometric NO_3^- assay of Tomos et al. (1994) work?	Assay failed to work.	Assay requires further development.
Does NO_3^- assay work when scaled-up to run on microtitre system?	A good calibration obtained but slow enzyme independent oxidation of NADPH observed.	Enzyme-independent oxidation of NADPH merits further study.
Are NADPH or FAD photo-labile?	Change in OD with time of both full assay and minimal mix (containing NADPH, buffer and BSA only) was measured. No change in the OD of either mix was recorded irrespective of whether mixes were continuously exposed to light or placed in the dark. Experiment was repeated with measurement at 450 nm (FAD λ_{max}). OD decreased over time regardless of the presence or absence of light.	FAD breaks down with time. Process is not light-dependent.
Does the light independent breakdown of FAD increase when incorporated into microdroplets exposed to fluorescent light?	Two sets of microdroplets (containing NADPH and FAD) were laid down under oil. Set 1 was bombarded with fluorescent light every three minutes, set 2, every 15 minutes for a total of 60 minutes. Set 1 showed the largest decrease in fluorescence.	The breakdown of FAD (leading to a subsequent decrease in fluorescence) is more pronounced in microdroplets.
Is the 0.44 U ml^{-1} assay concentration of NR in the microfluorometric assay of Tomos et al. (1994) too low to catalyse reaction?	Following a number of unsuccessful assays, the concentration of NR was increased ten fold (see Leese and Barton, 1984) to 4.4 U ml^{-1}. Response was obtained.	0.44 U ml^{-1} NR is too low to catalyse reaction.
Does use of frozen aliquots of FAD influence the NO_3^- microassay?	Following more unsuccessful assays (all of which used frozen FAD), a fresh FAD preparation was made up and used in an assay. A good calibration curve was obtained.	FAD loses efficacy when frozen.
BSA is used as a protein protectant. Does this use of BSA affect the NO_3^- assay?	The response of the microtitre NO_3^- assay to a series of graded standards was the same regardless of the presence or absence of BSA.	Inclusion of BSA has no effect on the NO_3^- assay.

Continued overpage

Hypothesis/Aim	Results	Conclusions
The original NO_3^- assay (Boehringer, 1984) was re-miniaturised with a 10x increased concentration of NR (Leese and Barton, 1984). Does this new microfluorometric assay work?	The concentration of NADPH in the Boehringer NO_3^- assay was too low to be detected by the phototube of the fluorescence microscope.	Further modifications are required to the NO_3^- protocol of Boehringer (1984).
What assay concentration of NR is necessary to optimise the performance of the assay whilst minimising the substrate independent response.	Assay reached completion in less than five minutes whilst substrate-independent response was negligible when 500 pL of 22 U ml^{-1} NR was added to microdroplets.	Optimal concentration of NR in microfluorometric assay is 2.4 U ml^{-1}.
Does paraffin oil denature NR at the oil-enzyme interface?	3 µl droplets of NR were stored under water saturated paraffin oil for 0, 30, 60, 120 and 240 minutes before being removed and used to start a microtitre NO_3^- assay. The incubation of NR under oil led to a decrease in activity although this followed no clear trend.	The enzymatic activity of NR is impaired by incubation under oil.
Can the performance of the microfluorometric NO_3^- assay be improved further by changing the buffer concentration in the assay mix?	The NO_3^- assay of Boehringer (1984) uses 32 mM imidazole as the assay buffer. A previous microassay (see Richardson, 1993) was based on 100 mM imidazole. In this experiment, the quantification of KNO_3 standards was unaffected by buffer strength. Reaction rates were identical regardless of buffer concentration.	The concentration of imidazole in the NO_3^- microassay has no affect on assay performance.
Is the assay specific for NO_3^-?	A comparison of two calibration curves (one from 'normal' KNO_3 standards, the other from sap 'spiked' standards) demonstrated that assay response was specific for NO_3^-.	The response of the NO_3^- assay is specific for its substrate.

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