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DIAGNOSIS OF SULPHUR DEFICIENCY IN OILSEED RAPE AND WHEAT

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DIAGNOSIS OF SULPHUR DEFICIENCY IN OILSEED RAPE AND WHEAT

by

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ABSTRACT

The reduction in sulphur dioxide emissions in Western Europe over the last decades and changes in fertilizer practices have resulted in a widespread increase in the occurrence of S deficiency in agricultural crops. However, S deficiency is dependent on soil type and prevailing climatic conditions, and does not occur uniformly across the country, hence there is a need for a reliable, practical, field-based diagnostic test to determine where it is likely to occur. In this study we examined the effect of S deficiency in oilseed rape and wheat on several diagnostic indicators, tissue variation, variations within the growth season, practicality and potential ease of implementation of techniques used.

The controlled environment experiments were performed to investigate the distribution patterns of different S pools within the plant and to examine the effects of S deficiency on growth and pool sizes in oilseed rape and wheat, with the specific objective of identifying parameters suitable as diagnostic indicators of the S-nutritional status. The results are summarised as follows:

1) In oilseed rape, the concentration of glutathione in the youngest leaves was approximately three times higher than that in the mature leaves. In wheat, before stem extension there was no significant difference between the glutathione concentrations of different leaves. In both crop species, the glutathione concentration decreased as a result of S deficiency. However, even with sufficient S supply, the glutathione concentration decreased with time.

2) In both crop species, the concentrations of sulphate decreased during S deficiency. In oilseed rape, when the external S supply was sufficient, S accumulated as sulphate, in particular in the mature leaves. During S deficiency, the sulphate concentration in the young leaves was almost completely depleted whereas in the mature leaves about 50% of total S was still present as sulphate. This suggested that the oilseed rape plants were inefficient in redistributing sulphate from the mature leaves to the young leaves when the external S supply was low and this may contribute to the high demand of oilseed rape plants for S.

3) Wheat plants were capable of redistributing S from soluble S pools in leaves to the ears at times of S shortage, but only if during the early growth stages enough S had accumulated to make redistribution possible.

4) In both crop species, protein S was maintained as long as possible at the expense of soluble S pools, so the protein S content decreased only slowly during S deficiency.

5) The effect of S deficiency on the total S concentrations was less pronounced than the effect on the sulphate concentrations. As protein S forms a large portion of total S, this was mainly due to the reason mentioned above that protein S was less affected by S deficiency.

6) In oilseed rape, glucosinolates were not a major source of S during S deficiency.

The field experiments were performed to determine whether sulphate and glutathione were suitable indicators in the field for the diagnosis of S deficiency. The following diagnostic indicators were evaluated:

1) Total S

The concentration of total S was less responsive to S deficiency than sulphate, and critical values changed during the growth season. In wheat, the critical values of total S for the prognosis of yield loss as a result of S deficiency in leaves decreased from 2.5 to 1.3 mg g^{-1} DW with time. For oilseed rape, the critical values for total S fluctuated between 4.6 and 2.8 mg g^{-1} DW during the growth season without any apparent chronological consistency. At present, some commercial laboratories have difficulties with the accurate determination of total S, so results should be treated with caution if no known plant S standards have been included in the test.

2) Sulphate

Sulphate was the most responsive S-containing compound to S deficiency, and as such potentially a good indicator. There was a large (4 to 40-fold) increase in the sulphate concentrations in leaves when crops were treated with S. However, like total S, the critical values for sulphate fluctuated during the growth season, both in oilseed rape and wheat. In wheat, the critical values of sulphate for the prognosis of yield loss as a result of S deficiency in leaves decreased from 0.5 to 0.1 mg g^{-1} DW with time. For oilseed rape, the critical values for sulphate fluctuated in young leaves between 0.6 and 1.2 mg g^{-1} DW without any apparent chronological consistency.

3) Glutathione

Glutathione was not a suitable indicator for the diagnosis of S deficiency in the field, because the concentrations decreased with time during the growth season, independently of the S treatment. As a result there was an overlap in absolute values between S-deficient and S-sufficient plants. Also glutathione increased only 1.5 to 2-fold when crops were treated with S, which is a relatively small difference. This makes glutathione impractical as a diagnostic indicator, unless the exact growth stage of the crop is known.

4) N:S Ratio

The N:S ratio in leaves was quite reliable in the diagnosis of S deficiency, with critical values of 15 for wheat and 9 for oilseed rape. A disadvantage was that two different analytical techniques needed to be used for the determination of N and S with an input of error from both. The problems with the accurate measurement of total S will also influence the accuracy of the N:S ratio. In addition, the N:S ratio reflects their relative proportion but not the actual magnitude of either. This means that it is possible to measure a low N:S ratio (suggesting sufficient S supply) when both N and S are actually deficient. Conversely, a high N:S ratio could be due to the oversupply of N even though S is sufficient. It is therefore best to use both the N:S ratio and the critical value for total S to determine whether plants are S deficient.

5) Malate:sulphate ratio

A practical and reliable indicator for S deficiency was the malate:sulphate peak area ratio, which required only one analysis using ion chromatography and was independent of the time of sampling or calibration of the samples. A malate:sulphate ratio lower than 1 indicated S sufficiency at the time of sampling, whereas a ratio higher than 1 suggested S deficiency at the time of sampling. The malate:sulphate ratio was reliable at growth stage 3.6-3.7 for oilseed rape and growth stage 22-25 for wheat, which was sufficiently early in the growth season to enable the recommendation of remedial sulphur application, if necessary.

The malate:sulphate peak area ratio looks promising as an indicator for S deficiency, but further research and development is needed to produce a practical and robust method for use by commercial laboratories. The research needs to focus on the universality of the indicator, such as the influence of other nutrients, different soil types across the UK, seasonal weather conditions and plant species. Development needs to include method improvement, such as optimisation, practicality and translation into a rapid, routine method for use by commercial laboratories.

TERMINOLOGY

anion	negatively charged ion, e.g. SO_4^{2-}, NO_3^-, Cl^-
cation	positively charged ion, e.g. Ca^{2+}, Mg^{2+}, K^+
DW	dry weight
glutathione	small peptide consisting of 3 amino acids (γ-glu-cys-gly)
GS	growth stage
malate	an organic acid produced by photosynthesis
N	nitrogen
NO_3^-	nitrate
osmoticum	solute stored in vacuoles to maintain the shape of cells
S	sulphur
SO_4^{2-}	sulphate
SO_2	sulphur dioxide
vacuole	a separate compartment in plant cells used for storage

1. Introduction

Over the last decade the occurrence of sulphur (S) deficiency in agricultural crops has increased in the United Kingdom, partly because of the reduction in SO_2 emissions and subsequent reduction in atmospheric depositions of S and partly because of changes in fertiliser practice. Due to the 1996 protocol of the Economic Commission for Europe, the SO_2 emissions are due to be reduced even further, from 2.5 million tonnes $year^{-1}$ in 2000 to 0.98 million tonnes $year^{-1}$ in 2010. This is a reduction of 80% compared to the levels of SO_2 emissions in 1980. As a result, the occurrence of S deficiency in agricultural crops is expected to increase over the next decades.

1.1 The function of S in plants

S is a constituent of the amino acids cysteine and methionine and hence of proteins. In cysteine, S is present as a thiol group (SH). The thiol groups of cysteine can provide the three-dimensional folding structure of proteins by forming S-S bridges between cysteine residues. This is important for the actual functioning of the proteins, and some of them are activated or deactivated by the formation (oxidation) or breakage (reduction) of these S-S bridges (Gilbert, 1990). The thiol group of cysteine also acts as a functional group in metabolic reactions. The most abundant water-soluble non-protein thiol is glutathione, a small peptide consisting of three amino acids: γ-glutamyl-cysteinyl-glycine. Glutathione plays an important role as an antioxidant (Alscher, 1989), is involved in the detoxification of ozone (Price et al., 1990), SO_2 (Alscher et al., 1987), and xenobiotics such as herbicides and pesticides (Lamoureux and Rusness, 1986). Glutathione also has been implicated in the adaptation of plants to environmental stresses such as drought (Burke et al., 1985) and extremes of temperature (de Kok and Oosterhuis, 1983). Methionine is an essential amino acid for humans because we can not synthesise it ourselves. The only way to acquire methionine is through our diet. In addition it is a precursor for the synthesis of glucosinolates. Glucosinolates are S-containing compounds found in the Brassicaceae and reported to be part of the plant's defence mechanism against fungi and insects (Chew, 1988). S is also a constituent of other compounds such as coenzyme A, thioredoxins, sulfolipids and glutenins. Glutenins are a group of storage proteins present in wheat grain, and contain different

amounts of cysteine residues depending on the S availability (Wrigley et al., 1980). The amount of S in these polypeptides is important for the breadmaking quality of milling wheat as shown in a recently completed HGCA project (Project No. 1221, HGCA Project report No. 197).

S is a constituent of

• cysteine: amino acid with thiol (SH) group • methionine: essential amino acid for humans • glutathione: defence against stress • glucosinolates: defence against fungi and insects • glutenins: breadmaking quality

1.2 Uptake and assimilation of S in plants

In higher plants, S is taken up by the roots as sulphate (SO_4^{2-}), and transported to the leaves. SO_4^{2-} is the most important source of S for plants, although some atmospheric S is also taken up and utilised by the aerial parts of plants. In the leaves, SO_4^{2-} is reduced to cysteine via a four-step assimilation pathway (reviewed e.g by Leustek, 1996; Schwenn, 1997; Hell, 1997). The uptake and subsequent distribution of SO_4^{2-} is closely regulated in response to demand. For example, when the S supply is sufficient, the uptake of SO_4^{2-} by the roots is down-regulated, but during S deficiency uptake increases rapidly in plants (Clarkson and Saker, 1989; Hawkesford et al., 1993). With sufficient S supply, SO_4^{2-} is stored in the vacuoles of leaf cells. This SO_4^{2-} is only released under conditions of prolonged S stress and is too slow to support new growth (Bell et al., 1995). As a result, the developing leaves are the first ones to show S-deficiency symptoms.

Sulphate uptake by the roots is the most important source of S for plants.

1.3 S-deficiency symptoms

The most common visual S-deficiency symptom is the yellowing of young leaves as a result of the inhibition of protein synthesis. In wheat, the yellowing of leaves is difficult to distinguish from nitrogen (N) deficiency. With increasing S deficiency, growth is stunted, the percentage of non-productive tillers increases and ears are smaller (Rasmussen et al., 1977). In oilseed rape, young leaves turn yellow and with increasing severity of the deficiency the leaves become thicker and of leathery texture, eventually turning bright purple. The flowers of S-deficient oilseed rape plants are much less bright yellow than those of S-sufficient plants,

and the flowers tend to drop off before pod setting. Both in oilseed rape and wheat, prolonged S deficiency can result in yield losses ranging between 10 and 50% depending on the severity and length of exposure.

A common visible symptom of S deficiency is the yellowing of young leaves.

1.4 Diagnosing S deficiency

The occurrence of S deficiency is not distributed uniformly across the country but dependent on location, soil type, and plant species grown. For example, oilseed rape plants grown on a light sandy soil in a remote area of Scotland are more likely to encounter S deficiency than wheat plants grown on a heavy clay soil downwind of a power station. Therefore there is a need for a reliable diagnosis test for S deficiency to ensure the economic and suitable use of S fertilizers and to prevent losses in yield and quality. Diagnosis can be done by soil testing, plant analysis or modelling. All these methods have their advantages and drawbacks. Soil testing can be done before drilling the seeds, but because of fluctuations in S mineralisation and S availability throughout the growth season, it is difficult to establish a reliable correlation between soil-extractable S and crop yield under field conditions (Jones, 1986). Modelling can identify high risk regions by calculating the inputs and outputs of S in an agricultural system to predict a likelihood of S deficiency, but the results are too general for use on a field scale. Plant analysis can provide a means of testing whether the S requirements of the crops are actually being met, but its success is dependent on which diagnostic indicator is used and on the determination of a reliable critical value for this indicator. It is also important that the critical value should remain relatively stable during plant development, and across different climatic conditions and soil types.

1.5 Traditional indicators of S deficiency

For many years researchers have tried to determine the best diagnostic indicator for S deficiency in different crop species (reviewed e.g. by Dijkshoorn and van Wijk, 1966; Randall and Wrigley, 1986; Zhao et al., 1999) and there is little agreement about which parameter is most reliable and what critical values should be used. Total S (Pinkerton, 1998), sulphate (Scaife and Burns, 1986), the percentage of total S as SO_4^{2-} (Spencer & Freney, 1980) and the N:S ratio (Rasmussen et al.,

1977) have all been put forward as suitable indicators for S deficiency. The critical values proposed for these indicators vary greatly as a result of differences in growth stages at which the plants were sampled, plant parts taken, analytical methods used, and whether the experiments were conducted in a controlled environment room, in a glasshouse or in the field. In addition, in field experiments the S concentrations of plants can vary between growth seasons and a S concentration classified as deficient in one year may well be classified as sufficient in the next (Rasmussen et al., 1977).

Traditional indicators of S deficiency

• Total S • Sulphate • %S as sulphate • N:S ratio

1.6 Problems and limitations of plant analysis

The main problems with plant analysis are that the methods designed by scientists are all comparative: plus and minus S treatments are compared to each other and from the differences we can conclude that the minus S treatments were S deficient. Unfortunately, farmers are not in a position to compare different treatments and therefore need absolute values to work with. Yet the absolute values of the contents of different S pools change in plants with growth stage, growth rate, plant parts tested, physiological age of plant parts tested, weather and field conditions. This problem was acknowledged as early as 1937 by W. Thomas who stated: "It is clear that no physiological significance can be attributed to the foliar diagnosis of any one fertilizer treatment (plot) considered alone. The method is comparative, just as the method of the analysis of the entire plant is comparative." This is probably the main reason why, to date, the perfect diagnostic indicator has not been found.

A second problem with using plant analysis for the diagnosis of S deficiency is that in order to determine early in the growth season whether yield losses as a result of S deficiency are likely, plants have to be S deficient at the time of sampling. No yield prognosis can be made if the plants are not deficient. Yet, when plants are small, their S requirement is usually low and S deficiency may not be a problem. The S requirement increases during periods of rapid growth and the plants are more likely to suffer from S deficiency. This can be a temporary problem from which the plants can recover without any averse effects on the yields, or it can be a

more permanent problem. By determining the S status at any single sampling time, it will not be possible to distinguish between the two types of deficiency. Only by determining the S status several times during the growth season is it possible to predict a likelihood of reduced yield or quality losses as a result of S deficiency.

1.7 Diagnosing S deficiency: alternative indicators

In this project two new potential indicators for diagnosing S deficiency were examined to try to eliminate some of the problems associated with plant analysis. Firstly, the response of glutathione to S supply was determined. Glutathione has been shown to decrease in response to S deficiency in a pot experiment with wheat (Zhao et al., 1996). The advantage of using glutathione as a diagnostic indicator would be that it may be possible to measure it with immunological methods by raising antibodies against it. This could potentially lead to a test kit for rapid diagnosis of S deficiency in the field and would make it easy to take several samples during the growth season to monitor the S status.

The second potential indicator for diagnosing S deficiency was derived directly from the results of the field experiments performed during this project and based on the ratio of malate and sulphate contents in the plants. Malate is an organic acid that accumulates in plant cells to far greater levels than other organic acids (Lance and Rustin, 1984). One of its physiological functions is to counterbalance the uptake of anions and cations in plant cells in order to maintain the anion-cation charge balance and the cytoplastic pH (Martinoia and Rentsch, 1994). When anions are taken up in excess, the cytoplasmic pH drops as a result of co-transport of anions with protons. This leads through the activation of malic enzyme to the decarboxylation of malate and the production of CO_2 and pyruvate. In contrast, when cations are taken up in excess, the cytoplasmic pH increases and a chain of reactions is set in motion resulting in an increase in the malate concentrations (Fig. 1).

Another important function of malate is that it accumulates in the vacuoles as one of the solutes important for maintaining the shape of cells (Blom-Zandstra, 1989). This type of solute is called an osmoticum. All cells are surrounded by a semi-permeable membrane which regulates the influx of water according to differences

in the solute concentrations between the vacuole and its surroundings. These solutes can be inorganic compounds such as nitrate and sulphate, but also organic compounds such as malate.

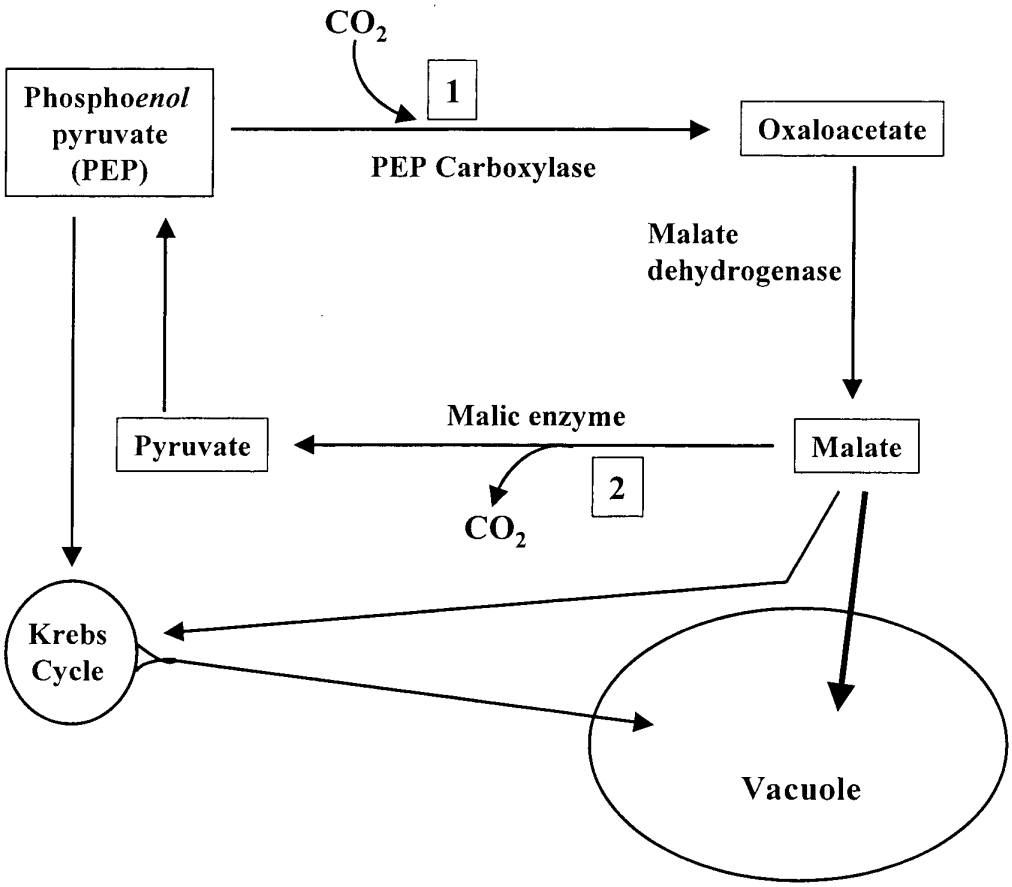

Figure 1. Schematic representation of the function of malate in maintaining the cytoplasmic pH. When cations are in excess, the enzyme PEP carboxylase (1) is increased, resulting in an increase in malate concentrations. When anions are in excess, malic enzyme (2) is increased, resulting in a decrease in malate concentrations (after Marschner, 1995).

Potential indicators of S deficiency

- **Glutathione**
- **Ratio between malate and sulphate**

1.8 Aim and objectives of the project

The aim of the project was to study the effects of sulphur deficiency on oilseed rape and wheat in order to develop reliable diagnostic methods for determining S deficiency in the field as early in the growth season as possible. More specific objectives were to answer the following questions:

1. Which S-containing compounds are the best indicators for S deficiency?
2. Do S pools change as a result of plant growth and development ?
3. Is the effect of S deficiency more pronounced in young leaves, mature leaves or stems?
4. Can we correlate the concentrations of different S pools measured early in the growth season to final yield?
5. Is it possible to develop a novel immuno-based diagnostic test?

To achieve these objectives, experiments were performed by growing wheat and oilseed rape plants a) on nutrient solutions in a controlled environment room, b) in a greenhouse at ambient temperature in pots containing soils from different areas of the UK and c) in the field at Woburn Farm, Bedfordshire.

2. Material and Methods

2.1 Glasshouse experiments with wheat

Three glasshouse experiments with wheat were performed. In the first experiment, seeds of winter wheat (*Triticum aestivum* cv. Riband) were sown in trays with moist vermiculite (medium grade), germinated and kept at 4 °C for 8 weeks. Then the seedlings were transferred to 4L pots (5 plants per pot) containing vermiculite. Every other day 600 ml of nutrient solution (see Appendix 1) was added to the pots. Five concentrations of S (10, 20, 50, 100 and 1000 μM) were added as $MgSO_4$ and Mg^{2+} was maintained at 1 mM in all treatments (4 replicates per treatment) by the addition of $MgCl_2$ when appropriate. If necessary, pots were watered with deionised water on the days when no nutrient solution was added. The pots were kept in a glasshouse at ambient temperature and light conditions. Plants were harvested 14, 24 and 38 days after transfer to the glass house.

In the second experiment, seeds of spring wheat (cv. Axona) were germinated in vermiculite and after 10 days the seedlings were transferred to 2L pots containing a continuously aerated nutrient solution as described above. After transfer to nutrient solution (see Appendix 1), seedlings were exposed to 3 different S concentrations (20, 100 and 1000 μM, respectively). The nutrient solution was initially replaced once a week but more frequent as the plants grew. At regular intervals (22, 37, 51, and 65 days after transfer to nutrient solution), 4 plants per treatment were harvested and weighed. At each harvest, the plants were separated into leaves, roots, and stems and dried at 80 °C for 24 hours. After determination of the dry weight, the plant parts were ground into a fine powder and kept at room temperature until further analysis.

In the third experiment, wheat plants (cv. Axona) were grown in a mixture of sand and perlite (1:1) in a glasshouse, and nutrient solution was added every other day containing either 20, 100 or 1000 μM of S. At the beginning of anthesis, the S supply for half of the plants within each treatment was withdrawn (=day 0), whereas the other half remained on either 20, 100 or 1000 μM S. Plants were sampled at regular intervals during ear development.

2.2 Glasshouse experiments with oilseed rape

Three glasshouse experiments with oilseed rape were performed. In the first experiments, seeds of oilseed rape (*Brassica napus* L. cv. Apex) were sown in pots with moist vermiculite (medium grade) and germinated at a constant temperature of 20 °C, 75% humidity and a 16-h light period (280-300 μmol $m^{-2}s^{-1}$). After 7 days the seedlings were transferred to 40 L tanks (25 to 40 plants per tank) filled with a continuously aerated nutrient solution (see Appendix 1) containing either 20, 100 or 1000 μM S. The nutrient solution was replaced weekly. Depending on size, between 4 and 10 plants per treatment were harvested at regular intervals and weighed. At each harvest the plants were dissected, the equivalent leaves of each plant were pooled together in muslin cloth and immediately frozen in liquid nitrogen. Leaf 1 (L1) corresponded to the first fully exposed leaf, leaf 2 (L2) to the second etc. The frozen leaves were freeze-dried for 72 hours, and kept under vacuum at room temperature until further analysis.

In the second experiment, plants were pre-cultured for 23 days on nutrient solution (see Appendix 1) containing 1 mM SO_4^{2-} and 7 mM NO_3. After this pre-culture, the plants were transferred to nutrient solutions containing *i)* 1 mM SO_4^{2-} and 7 mM NO_3^-, *ii)* 0 mM SO_4^{2-} and 7 mM NO_3^-, *iii)* 1 mM SO_4^{2-} and 250 μM NO_3, or *iv)* 0 mM SO_4^{2-} and 250 μM NO_3^-. These nutrient solutions were replaced every three days. Three plants per treatment were harvested at days 0 (= transfer day), 2, 3, 6, 8, and 13. Each plant was dissected into the oldest leaves (L1 and L2), the middle leaves (usually L3, L4, L5, L6, L7 depending on the size of the plant) and the youngest leaves (usually L8 and L9). The three leaf fractions of each plant were frozen separately in liquid nitrogen and freeze-dried for 72 hours. The dried leaves were ground into a fine powder and stored under vacuum at room temperature until analysis.

In the third experiment, seeds of oilseed rape were sown and germinated in soils collected from 6 agricultural areas around the UK. A brief summary of the experimental conditions is presented below, but full details are described by Riley et al. (HGCA Report on Project No 1912). The plants were grown from November 1997 to June 1998 in a roofed cage in which they were exposed to ambient

temperatures and natural light, but not to rain fall. A wide range (from 3.7 to 48.9 mg kg^{-1}) of S concentrations was present in the soils. Four plants per treatment were harvested at either growth stage 1.5-1.7 (5 to 7 true leaves exposed) or at growth stage 3.3–4.3 (flower bud development). The remaining plants were grown to maturity which was approximately 240 days after sowing. Plants were sampled as described above.

2.3 Field experiments

All field trials were conducted at Woburn Farm, Bedfordshire, UK during the growth seasons of 1996-1997, 1997-1998 and 1998-1999. For agronomic details, see Appendix 2. The experiments consisted of 4 randomised block designs containing 5-6 plots (0.0036 ha $plot^{-1}$) each. Different S rates (0, 5, 10, 20, 40 or 80 kg ha^{-1}) were applied as gypsum ($Ca_2SO_4.2H_2O$) the time of sowing or reapplied in the spring. No other agrochemicals containing sulphur were used. Nitrogen was applied at a rate of 180 kg ha^{-1} in the spring. Plants were sampled at different growth stages as shown in Appendix 3. The area sampled was 0.25 m^2 each sampling time. Plants were separated into different plant parts, frozen in liquid nitrogen and freeze-dried for 72h. The dried leaves were ground into a fine powder and stored under vacuum at room temperature until further analysis.

2.4 Chemical Analysis

Total S was measured by digesting 100 mg of lyophilised plant material in a mixture of concentrated HNO_3 and 60% strength $HClO_4$ (85:15, v/v). After resuspension in 5% HCl (v/v), S was determined using inductively coupled plasma-atomic emission spectroscopy. Total N was measured using a Dumas combustion method (LECO CNS Analyser). Glutathione was measured using 2 different methods: firstly it was determined as described by Blake-Kalff et al. (1998) using the 5,5'-dithiobis(2-nitrobenzoic) acid recycling assay by Anderson (1985). Secondly, it was measured by high liquid performance chromatography (HPLC) using a Jones column (Zorbax OSD 5μm). Samples were derivatised with 50 μM monobromobimane. Sulphate, nitrate and malate concentrations were determined by extracting 50 mg of lyophilised plant material in deionised water at 90 ^{0}C for 2h, filtering the extract through filter paper (Whatman no. 42) and a 0.2 μm membrane filter, after which the concentrations were measured using ion

chromatography (Dionex DX500, with G50 gradient pump and ED40 conductivity detector). The eluent consisted of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ and was pumped isocratically over an AG9SC guard column coupled to an AS9SC separation column. Glucosinolates were extracted from 50 mg lyophilised leaf material and the concentrations of individual compounds were measured by HPLC according to the protocols of Heaney et al. (1986). Insoluble S, representing mainly protein S, was calculated by subtracting the concentrations of soluble S (e.g. sulphate, glutathione, glucosinolates) from the concentration of total S. Chlorophyll was measured using a SPAD 502 meter (Minolta). Analysis of variance was performed on all data. Least significant differences (LSD) were calculated using Genstat 5.

2.5 Antibodies

The methods for raising antibodies against glutathione are reported by Amara et al. (1994), Hjelle et al. (1994), and Pow and Crook (1993). Reduced glutathione was conjugated to the following proteins: bovine, rabbit and mouse serum albumin, keyhole limpet haemocyanin and carbonic anhydrase. The conjugates were used to immunize mice and rabbits. The reactivity of the antibodies towards glutathione was tested by adding the antibodies to ELISA plates coated with bound glutathione. After incubation, the plates were washed to remove the unbound antibodies and a secondary antibody conjugated to alkaline phosphatase was added which could be measured with a colourimetric reaction. The absorbance at 450 nm was related to the amount of antibodies bound.

3. Results and Discussion

The effects of S deficiency on wheat were quite different from the effects on oilseed rape, and therefore, in the following sections, the two crop species will initially be discussed separately. However, at the end of this chapter the results will be pulled together to highlight some general mechanisms for the diagnosis of S deficiency.

Part I: Wheat

3.1 Controlled environment experiments

3.1.1 How do S-containing compounds respond to the external S supply?

Sulphate was the most responsive compound to external S supply (Fig. 2a, Appendix 4). When plants were grown at 5 different external S concentrations, sulphate accumulated in leaves supplied with sufficient S ($\geq$ 100 μM S). At insufficient (< 100 μM S) S supply, sulphate decreased rapidly, reaching zero when exposure to S deficiency was prolonged to 38 days.

Glutathione was less responsive than sulphate (Fig. 2b). In small, 14-day old, plants the external S supply had no effect on the concentrations of glutathione. As the plants grew, the glutathione concentrations decreased in all treatments, independently of the external S supply. In addition, in 24-day and 38-day old plants, the glutathione concentrations also decreased when plants were grown on insufficient S (< 100 μM), but even with prolonged S deficiency glutathione never reached zero.

Sulphate was more responsive to the external S supply than glutathione.

3.1.2 Do S pools in different plant parts vary in their response to S deficiency?

The two major S pools in wheat are sulphate and protein S. It is essential for the survival of plants to maintain protein S as long as possible, even when the external S supply is insufficient. This is possible by depleting the non-essential pools of S and redistributing the S into proteins. Sulphate is one of the S pools which rapidly decreased when the external S supply is low, as shown in Figure 3a (Appendix 4). When plants were grown on sufficient S (1000 μM), the sulphate concentrations in leaves and stems were similar and both remained relatively stable over time. The

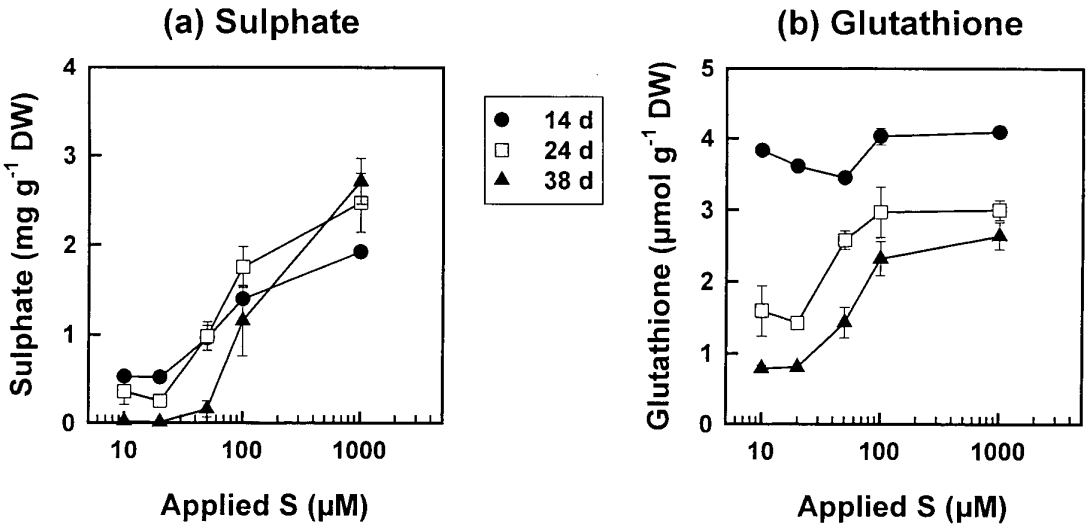

Figure 2. The effect of an increasing external S supply on the concentrations of (A) glutathione and (B) sulphate in (●) 14-day, (□) 24-day and (▲) 38-day old wheat plants.

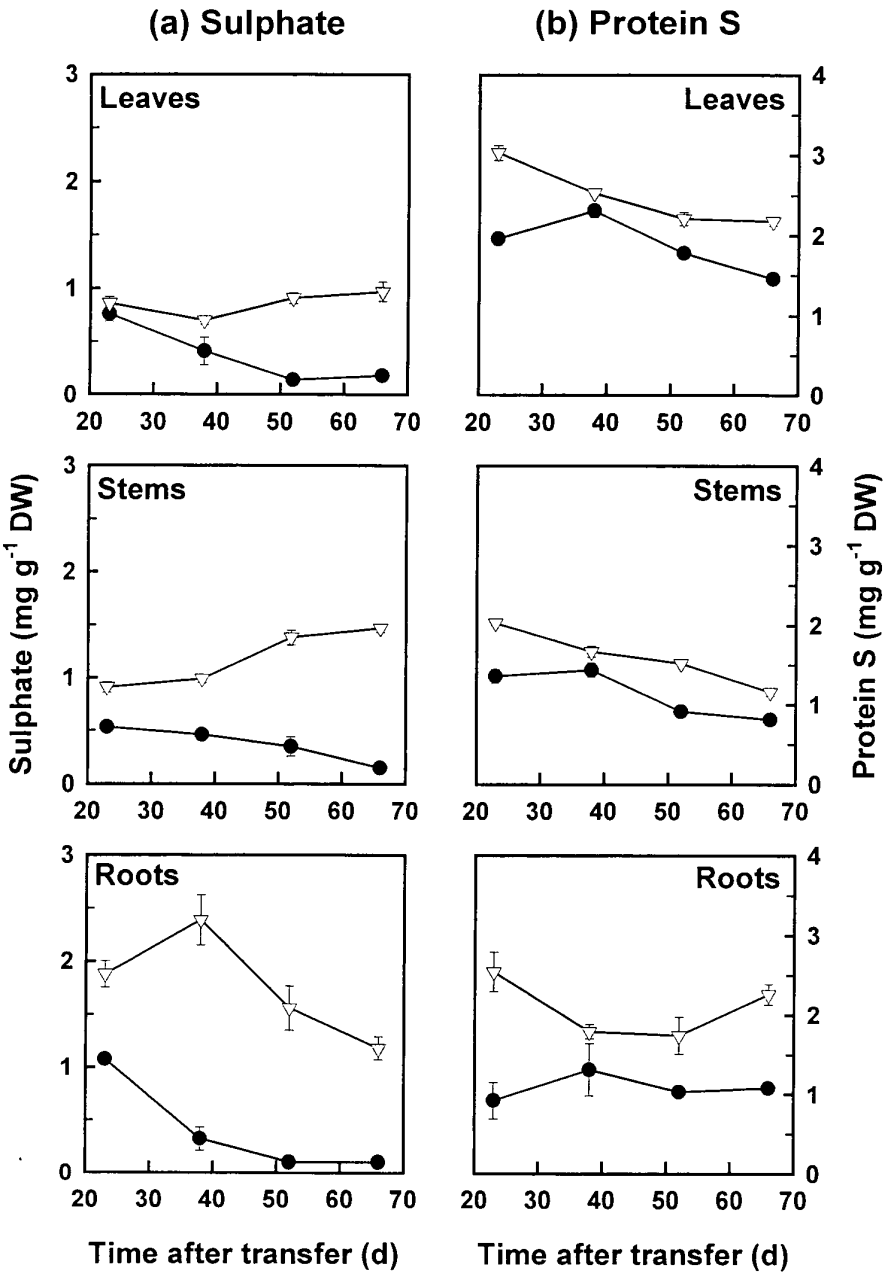

Figure 3. The concentrations of (a) sulphate and (b) protein S of wheat plants grown on nutrient solution containing either (•) 20 or (▽) 1000 μM S. Plants were between GS 20 (day 18) and GS 52 (day 65).

sulphate concentrations in roots were initially twice as high as that of leaves and stems, but decreased over time. When the external S supply was insufficient (20 μM), the sulphate concentrations decreased over time in all three plant parts, reaching a minimum in leaves and roots after 51 days.

Protein S was less affected by the external S supply (Fig. 3b). Although the concentrations of protein S in leaves and stems of S-deficient plants were only 75%, and in roots only 50%, of those found in S-sufficient plants, there was no further decrease in this proportion during the experiment. In both treatments, the concentrations of protein S decreased slightly with time, which was probably due to growth dilution. Growth dilution occurs when, during plant growth, the increase in S uptake is smaller than the increase in biomass, resulting in less S per gram dry weight.

The effect of S deficiency was similar in leaves, stems and roots: sulphate decreased, whereas protein S remained relatively stable.

3.1.3 Is S from different pools in leaves redistributed to the ears?

To study the patterns of redistribution of S from leaves to ears, four possible scenarios concerning the S supply were used: 1) the S supply was sufficient throughout experiment; 2) the S supply was deficient throughout the experiment; 3) the S supply was sufficient during early growth, but stopped at the beginning of anthesis 4) the S supply was deficient during early growth, but stopped at the beginning of anthesis. This enabled us to determine whether S pools stored early in the growth season could be redistributed from leaves to developing ears if the external S supply runs out. The full results of this experiment are presented in Appendix 5.

The weights of both flag leaf and ear in S-deficient plants (grown at 20 μM) were significantly lower than those of S-sufficient plants (grown at 1000 μM), as shown in Figure 4. However, withdrawal of S at the beginning of anthesis had little effect on the dry weights of flag leaf and ear within each treatment, suggesting that for biomass production it was more important to have an adequate S supply during the early growth stages than during the later stages.

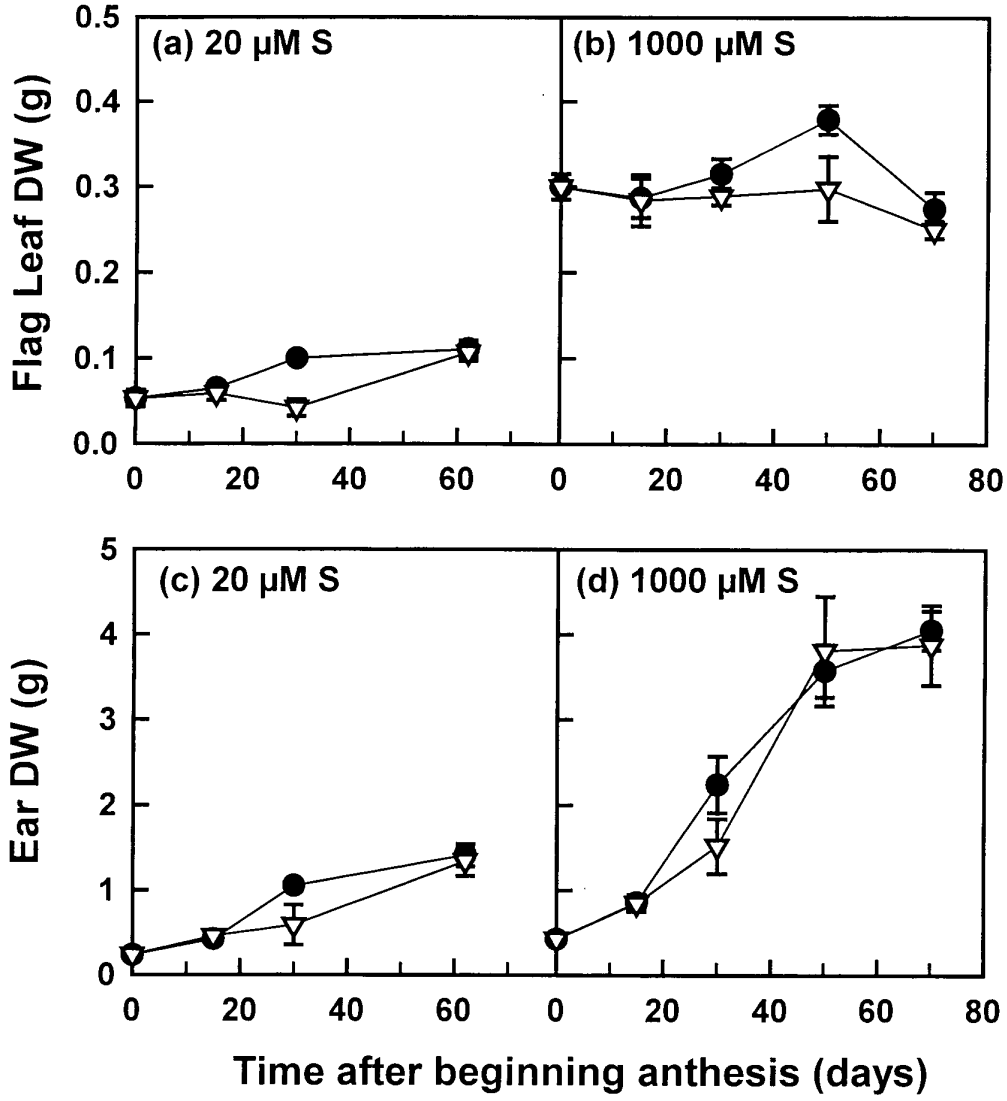

Figure 4. The accumulation of dry weight in (a,b) the flag leaves and (c,d) ears, post anthesis. Plants were grown at a S supply of (a,c) 20 or (b,d) 1000 µM up until anthesis. At anthesis, each treatment was divided, with one half continuing S supply (•) and the other half receiving no further externally supplied S (▽).

The concentrations of total S were higher in the S-sufficient plants than in the S-deficient plants, both in the flag leaf and the ear (Fig. 5). In the S-deficient plants, the withdrawal of the external S supply had little effect on the concentration of total S in the flag leaf (Fig. 5a), whereas in the ear the concentration of total S decreased by about 50% (Fig. 5c). In the S-sufficient plants, the withdrawal of the external S supply resulted in a 70% decrease in the concentration of total S in the flag leaf (Fig. 5b). Yet, in the ears of S-sufficient plants the concentration total S was only reduced by 20% when the S supply was withdrawn, suggesting that in those plants S was redistributed from other plant parts to the ears.

Like the experiment in section 3.1.2, sulphate was completely depleted in leaves of the S-deficient plants and the withdrawal of external S had no further effect (Fig. 6a). The sulphate concentrations in the ears of S-deficient plants were depleted likewise (Fig. 6c). In contrast, sulphate concentrations increased when plants were supplied continuously with sufficient S, but when the external S supply was withdrawn, sulphate concentrations decreased rapidly (Fig. 6b). In the ears of S-sufficient plants, the sulphate concentrations decreased during ear development and the withdrawal of external S had no further effect (Fig. 6d). Possibly, during ear development sulphate was assimilated into storage proteins, which may explain its decrease in S-sufficient plants.

The glutathione concentrations in leaves of S-sufficient plants were 1.5 to 2 times higher than those in S-deficient plants, but in both S treatments glutathione decreased over time (Fig. 7). In S-deficient plants, the glutathione concentrations decreased slightly faster when the external S supply was withdrawn at the beginning of anthesis (Fig. 7a), but this effect was more pronounced in the S-sufficient plants (Fig 7b). In the ears of S-deficient plants, the glutathione concentrations also decreased slightly faster when S was withdrawn than when S was supplied continuously (Fig. 7c). In the ears of S-sufficient plants, the glutathione concentrations decreased during ear development regardless of whether or not S was withdrawn (Fig 7d).

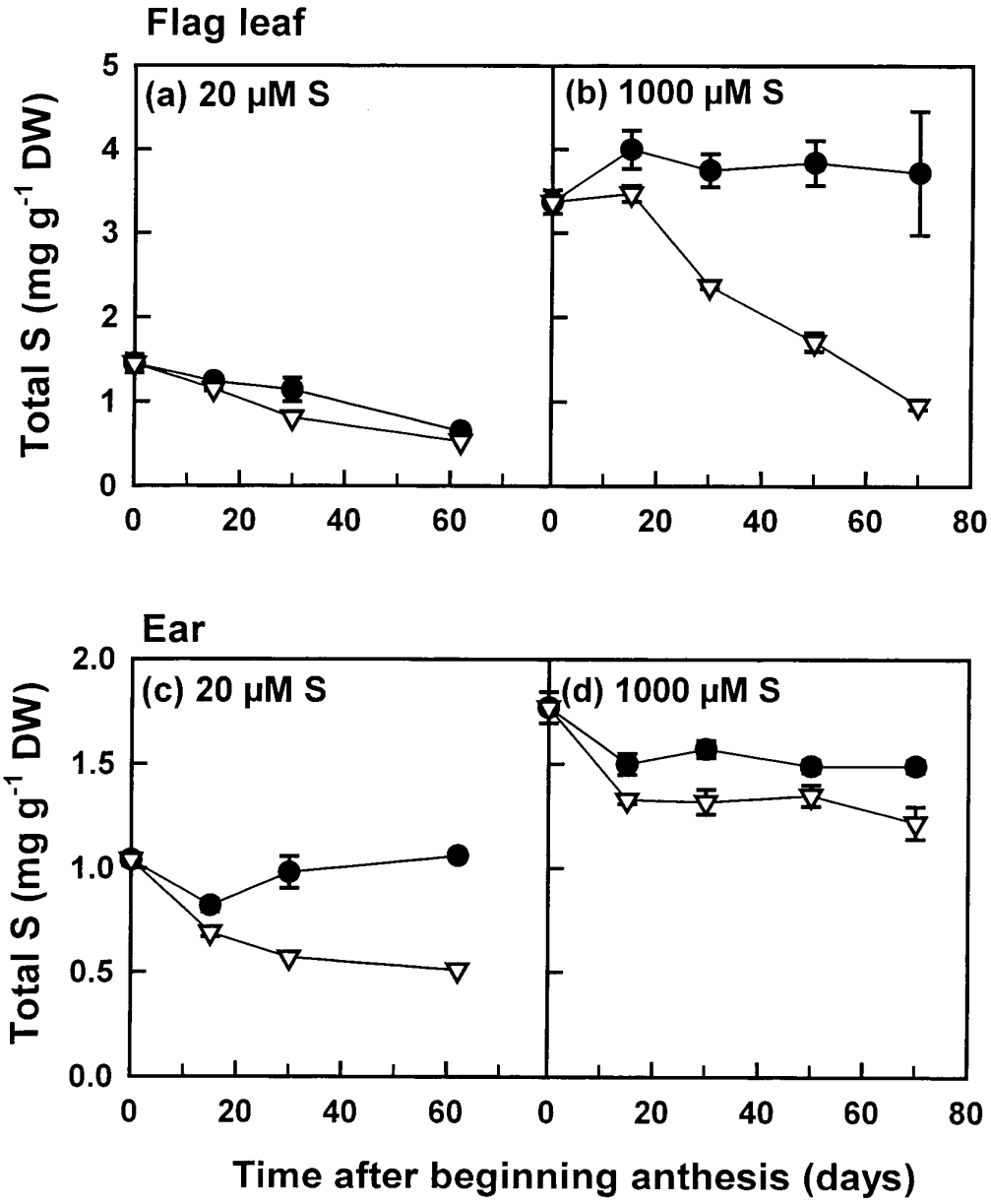

Figure 5. The concentrations of total S in (a,b) the flag leaves and (c,d) ears, post anthesis. Plants were grown at a S supply of (a,c) 20 or (b,d) 1000 µM up until anthesis. At anthesis, each treatment was divided, with one half continuing S supply (•) and the other half receiving no further externally supplied S (▽).

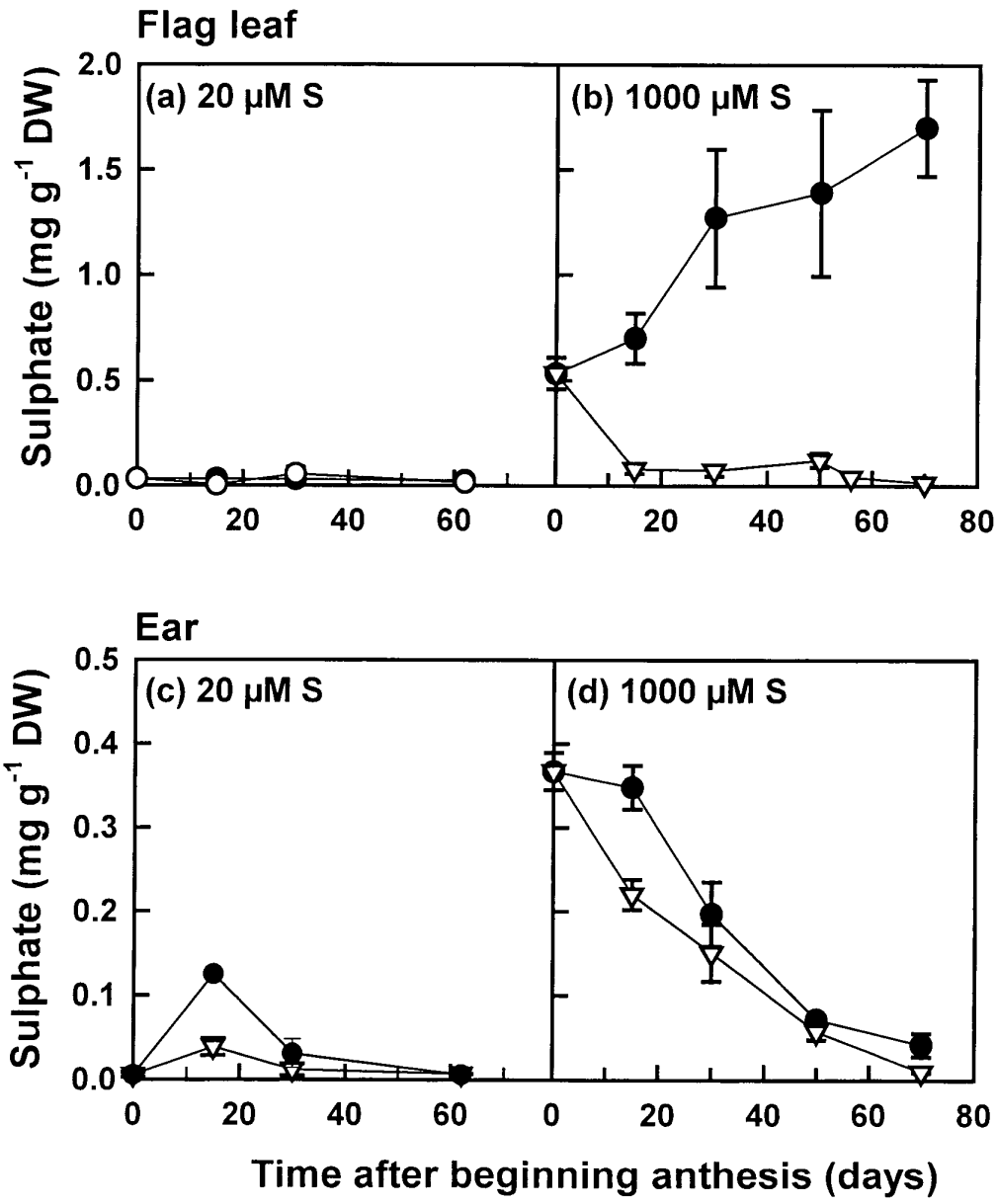

Figure 6. The concentrations of sulphate in (a,b) the flag leaves and (c,d) ears, post anthesis. Plants were grown at a S supply of (a,c) 20 or (b,d) 1000 µM up until anthesis. At anthesis, each treatment was divided, with one half continuing S supply (•) and the other half receiving no further externally supplied S (▽).

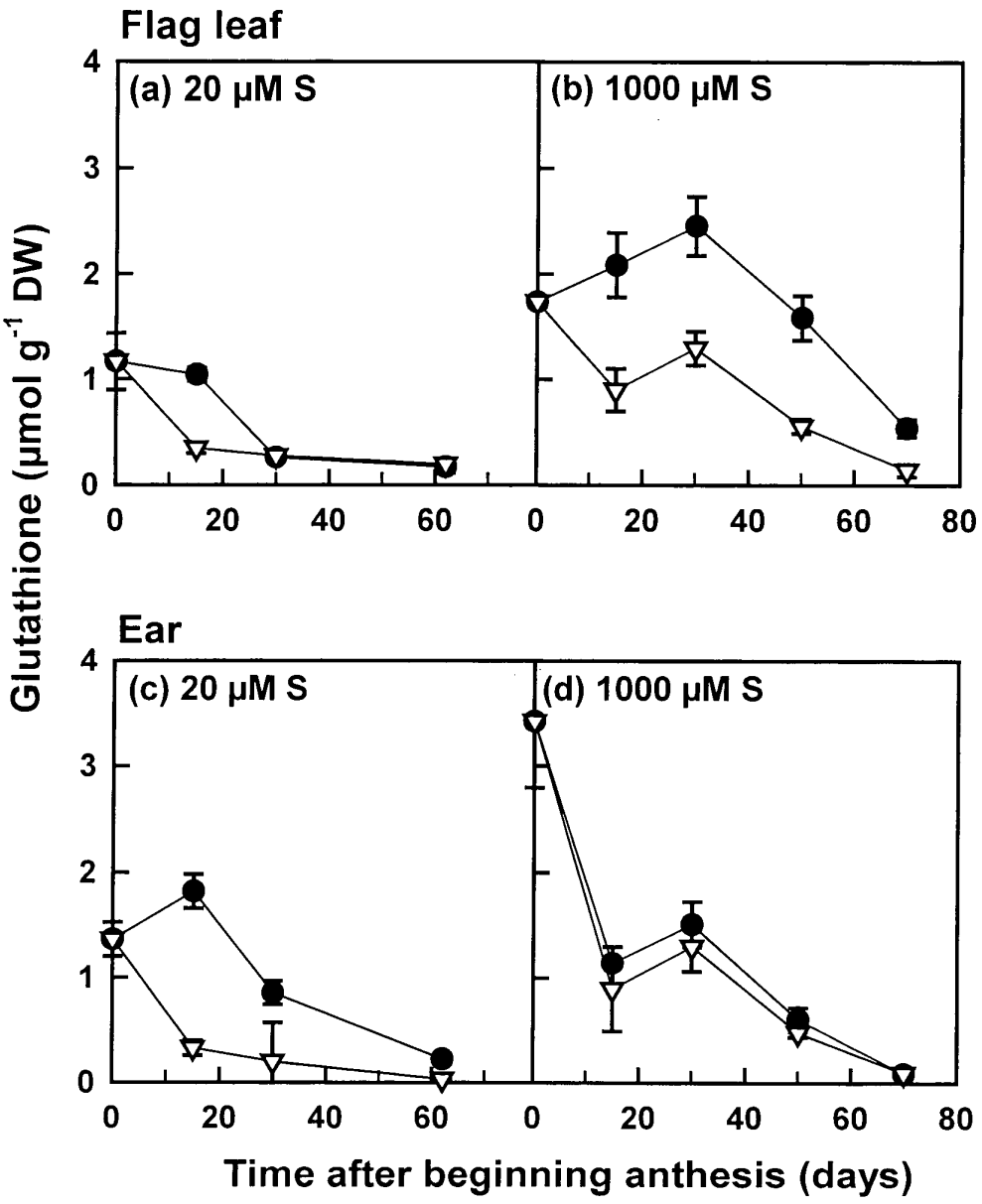

Figure 7. The concentrations of glutathione in (a,b) the flag leaves and (c,d) ears, post anthesis. Plants were grown at a S supply of (a,c) 20 or (b,d) 1000 µM up until anthesis. At anthesis, each treatment was divided, with one half continuing S supply (•) and the other half receiving no further externally supplied S (▽).

Wheat plants were capable of redistributing S from soluble S pools in leaves to the ears at times of S shortage, but only if during the early growth stages enough S had accumulated to make redistribution possible.

3.2 Field experiments with wheat

The experiments described in section 3.1 were performed to determine the effect of S deficiency under controlled growth conditions. In this section we determine whether similar responses to S deficiency occur under field conditions.

3.2.1 Are there differences in response to S between bread and biscuit making wheat varieties?

In the first experiment (1996), two wheat varieties were grown: Hereward, a bread wheat and Riband, a biscuit wheat. In May, both varieties showed S-deficiency symptoms, as shown by the chlorophyll measurements (Table 1). A lower chlorophyll measurement indicates that the leaf is yellowing, which is one of the symptoms of S deficiency. In June, S deficiency symptoms had decreased in both Hereward and Riband, but the differences were still significant.

Table 1. The effect of 0 or 20 kg ha^{-1} S on the chlorophyll content of Hereward and Riband wheat measured either in May or in June 1996.

S application (kg ha^{-1})	Chlorophyll (meter units)			
	Hereward		Riband	
	May	*June*	*May*	*June*
0	43.0	43.1	45.7	44.4
20	53.6	45.7	58.7	47.0
LSD (0.05)	*3.07*	*1.23*	*3.1*	*2.34*

The occurrence of S-deficiency symptoms was reflected in the glutathione concentrations (Fig. 8). The largest differences between S treatments were found in May in both varieties of wheat. In June, no significant differences between S treatments were observed in Hereward, whereas in Riband the 20 kg S ha^{-1} treatment contained significantly more glutathione in the mature and young leaves than the control. There was no difference in the glutathione concentration between young and mature leaves, but stems contained significantly less glutathione.

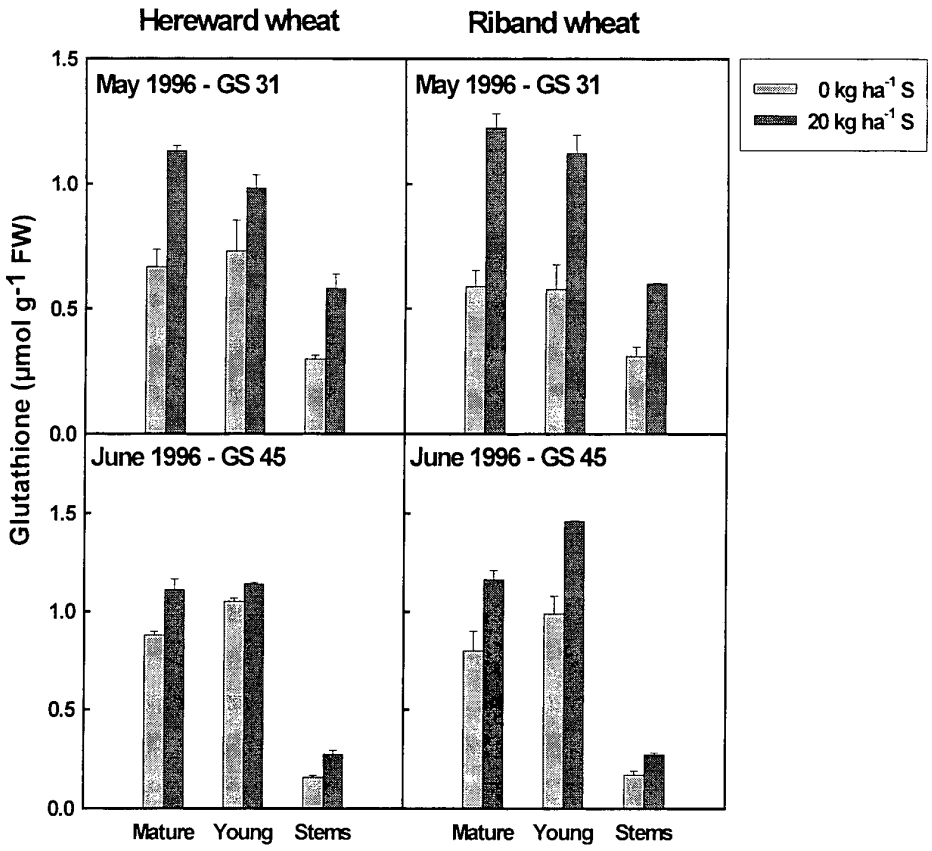

Figure 8. The effect of S applications on the concentrations of glutathione in mature leaves, young leaves and stems of field-grown Hereward and Riband wheat, sampled either in May or in June 1996.

The sulphate concentrations in both Hereward and Riband plants sampled in May were significantly higher in the 20 kg S ha^{-1} treatment compared to the control (Fig. 9). In June, this difference had disappeared in the Hereward wheat, which was consistent with the glutathione measurements. In Riband wheat, no change was observed between plants sampled in May and those sampled in June. In both varieties there was no significant difference between the sulphate concentrations in young and mature leaves, but both concentrations were higher than those in the stems.

In the field, when plants were S deficient, the concentrations of glutathione and sulphate increased in response to S, both in bread and biscuit varieties of wheat.

3.2.2 Are yield and quality affected by S deficiency?

In 1998 and 1999, the field experiments were performed with a wider range of S applications. The grain yields and concentrations of total S in the grain are shown in Table 2 for both years.

Table 2. The grain yields and concentrations of total S in grain of winter wheat harvested in 1998 (CV. Riband) and 1999 (cv. Rialto), respectively. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, indicate the levels of significant difference compared to the 0 kg S ha^{-1} treatments.

Applied S (kg ha^{-1})	Seed Yield (t ha^{-1})		Total S (mg g^{-1})	
	1998	*1999*	*1998*	*1999*
0	5.32	2.98	0.92	0.90
5	6.75	4.65*	1.01	1.00
10	6.33	4.93*	1.05	1.24***
20	5.47	5.12**	1.02	1.27***
40	6.12	6.07***	1.07	1.38***
80	6.33	5.43**	1.10	1.43***
LSD (0.05)	*1.39*	*1.51*	*0.16*	*0.15*

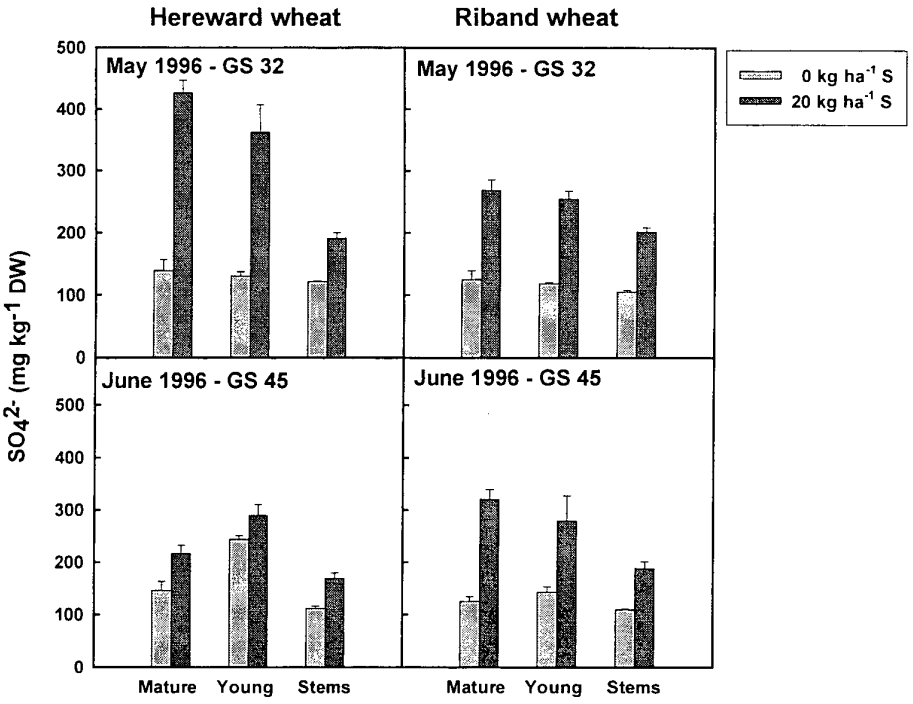

Figure 9. The effect of S applications on the concentrations of sulphate in mature leaves, young leaves and stems of field-grown Hereward and Riband wheat, sampled either in May or in June 1996.

In 1998, there was no yield response to the application of S and the concentrations of total S in the grain were not significantly increased in any of the treatments. The reason for this lack of response was that the winter of 1997-1998 was exceptionally wet, probably causing the leaching of most of the applied S. HGCA project 1912 showed that as much as 70% of S, applied as a sulphate, can leach during a winter with average rainfall, so possibly even more than 70% of S leached during the winter of 1997-1998.

To avoid problems with leaching during the winter of 1998-1999, gypsum was re-applied in the spring. During the early growth stages (25-32), S-deficiency symptoms, such as the yellowing of leaves and stunted growth, were clearly visible. In this year, yields were increased significantly in response to S (Table 2). Also, the concentrations of total S in the grain were increased significantly when more than 5 kg S ha^{-1} was applied.

In 1999, grain yield increased by 2 tonnes ha^{-1} in response to S, whereas the concentration of total S in the grain, which is important for quality, increased by about 40%.

3.2.3 How does glutathione respond to S deficiency in the field?

When the plants were S deficient, the concentrations of glutathione decreased (Fig. 10). At each sampling time, sampled at different growth stages, the concentrations of glutathione reached a maximum when S was applied at a rate of at least 20 kg ha^{-1}. However, this maximum glutathione concentration decreased with time, regardless of the S applications. As a result, there was an overlap in absolute values between S-deficient and S-sufficient plants, depending on the time of sampling. For example, a value of 2.4 μmol g^{-1} DW would be interpreted as S deficient at GS 22-25, but at all subsequent growth stages this same value would indicate that the plants were S sufficient. This makes it difficult to use the glutathione concentrations as an indicator of the S status in plants, unless the exact growth stage and time of sampling is known.

The concentrations of glutathione decreased with time regardless of applied S.

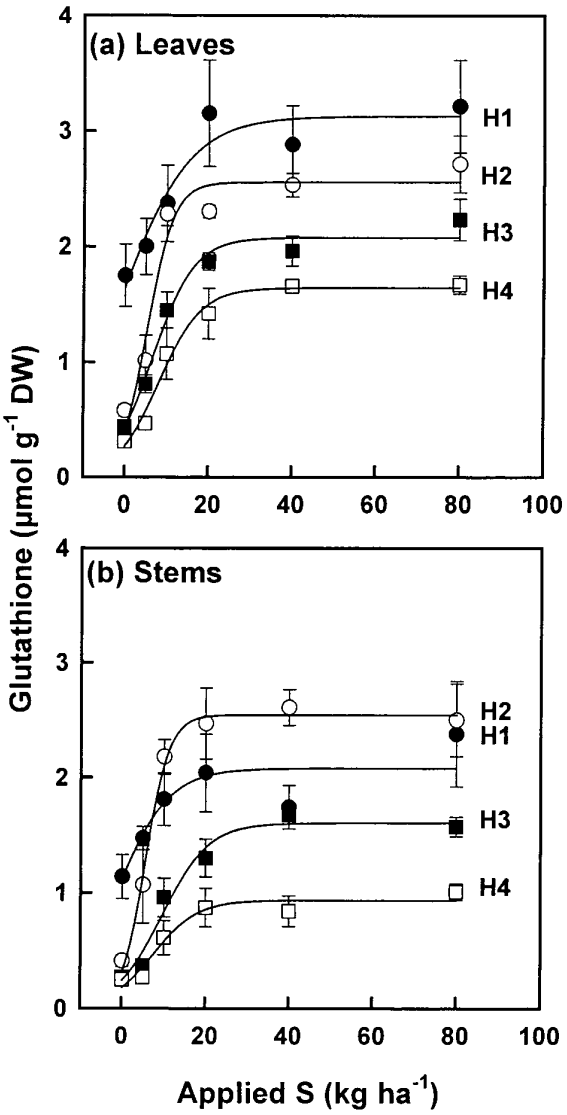

Figure 10. The glutathione concentrations in (a) leaves and (b) stems of field-grown winter wheat. Plants were sampled at growth stage stages 22-25 (H1, •), 25-30 (H2, ○) 30-31 (H3, ■), and 31-32 (H4, □).

3.2.4 How does sulphate respond to S deficiency in the field?

Like the experiments under controlled conditions, in the field sulphate was depleted to almost zero when plants were S deficient (Fig. 11a, b). The highest sulphate concentrations were found at sampling time H2 (GS 25-30). This might have been due to the re-application S as gypsum just two weeks before this sampling time, providing a sudden boost in available S for the plants. Therefore the results from this sampling time were probably not representative but they do illustrate how sulphate concentrations can fluctuate rapidly in response to environmental conditions.

In addition, we measured the concentrations of malate in leaves and stems (Fig. 11c, d). The malate concentrations were of the same magnitude as the sulphate concentrations. There was no clear correlation between the malate concentrations and the S applied. Even so, because the concentrations of malate and sulphate were of the same magnitude, the ratio could be used as an indicator of S status in plants, as will be explained further in section 3.4.6.

The concentrations of sulphate decreased towards zero with increasing sulphur deficiency.

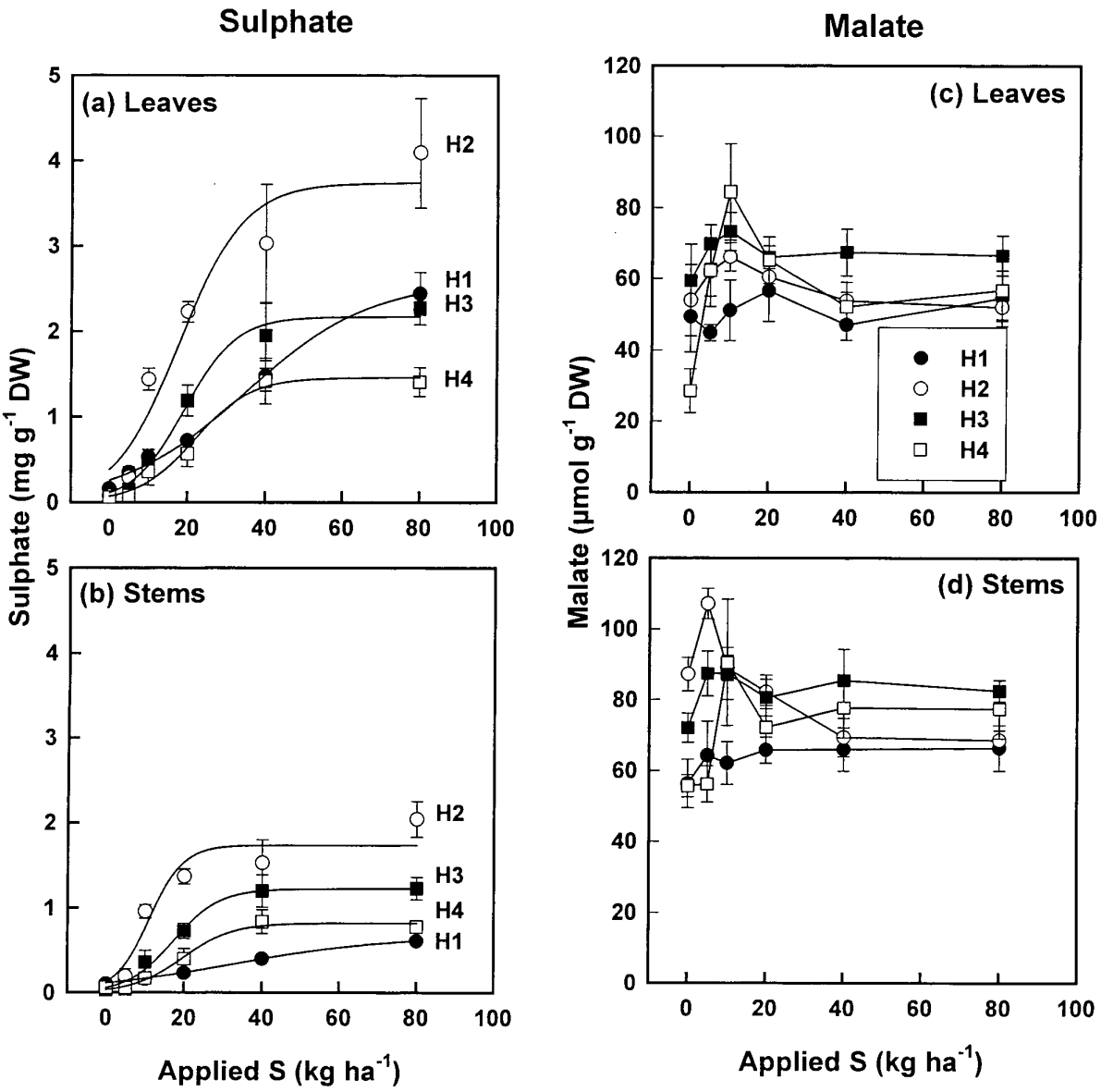

Figure 11. The concentrations of sulphate (a, b) and malate (c, d) in leaves (a, c) and stems (b, d) of field-grown winter wheat plants.

Part II: Oilseed rape

3.3 Controlled environment experiments

3.3.1 How is S distributed between different leaves?

In this experiment, we determined whether there were differences in the concentrations of glutathione, sulphate and total S between individual leaves and how these S pools responded to S deficiency. The knowledge gained is important for recommending which leaves to sample for the diagnosis of S deficiency.

In S-sufficient plants (grown on 1000 μM S), three trends were observed: 1) The concentration of glutathione in the youngest leaves was always higher than that in the oldest leaves (Fig. 12a); 2) The concentration of sulphate was higher in the oldest leaves than in the youngest leaves (Fig. 12b); 3) The concentration of total S was fairly evenly distributed among leaves (Fig. 12c).

In plants grown on 20 μM S, when the plants were only 10 days old the same trends were observed as described above. The reason for this was that at this age the S requirements were low and the plants were not deficient. As the plants grew and became S deficient, glutathione decreased to a minimum concentration in all leaves, whereas sulphate decreased rapidly to zero in the youngest leaves. Yet, although sulphate was completely depleted in the youngest leaves after 20 days growth on low S, approximately 6 mg g^{-1} DW of sulphate still remained in the oldest leaves. This suggested that once sulphate had accumulated in the oldest leaves, it was not easily redistributed unless the plants were severely S deficient, which was at 41 days. The concentrations of total S decreased much slower than sulphate, probably because it was essential for the survival of the plants to maintain protein S as long as possible, as explained for wheat in section 3.1.2.

When S was sufficient:

- **glutathione concentrations were higher in young leaves than in old leaves.**
- **sulphate concentrations were lower in young leaves than in old leaves.**

When S was deficient:

- **glutathione and sulphate concentrations decreased more rapid in young leaves than in old leaves.**

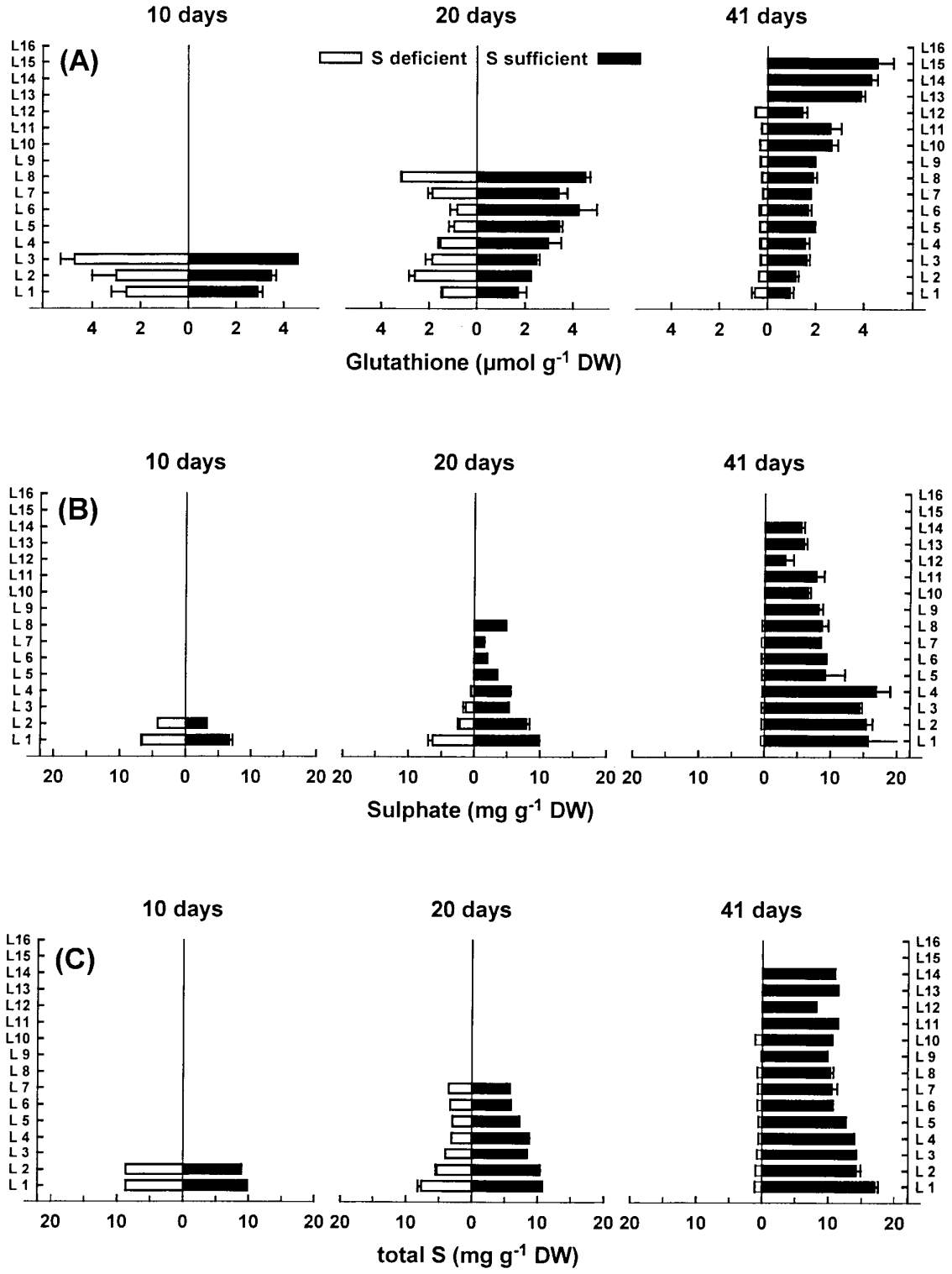

Figure 12. Distribution patterns of the concentrations of (A) glutathione, (B) sulphate and (C) total S within individual leaves of oilseed rape grown either on 20 µM S (□, S deficient) or 1000 µM S (■, S sufficient). L1 is the oldest leaves and the top leaf the youngest.

3.3.2 How is S distributed across different metabolic pools?

In oilseed rape plants, there are four major S pools: proteins, sulphate, glutathione and glucosinolates. Glucosinolates are S-containing compounds predominantly found in the Brassicaceae and reported to be part of the plant's defence mechanism against fungi and insects (Chew, 1988). It has been suggested that oilseed rape plants have a high demand for S because of the production of glucosinolates and that these glucosinolates can be a source of S when the external supply is insufficient (Schnug and Haneklaus 1993). Yet, when we determined the sizes of the different S pools as a percentage of total S, glucosinolates were only a minor pool compared to, for example, sulphate or protein S (Table 3).

Table 3. The relative sizes of S pools as a percentage of total S in oilseed rape plants continuously grown on sufficient S (+ S) and for plants grown on sufficient S for 3 weeks after which the S supply was withdrawn for 13 days (- S).

	Oldest leaves		**Youngest leaves**	
	+ S	*- S*	*+ S*	*- S*
Glutathione	0.2	0.6	0.8	0.5
Glucosinolates	0.1	0.3	8.2	2.2
SO_4^{2-}	91.2	49.2	42.4	0.0
Protein S	8.6	49.9	48.6	97.3
Total S	100	100	100	100

In the oldest leaves, almost all S was present as sulphate when S was sufficient. In these leaves, the percentages of glucosinolates and glutathione were less than 1% of total S and the remainder was present in proteins. When the external S supply was withdrawn, although the concentration of total S was reduced by 60%, half of the remainder of total S was still present as sulphate.

In the youngest leaves, the percentage of S in glucosinolates was 8% of the total, whereas the remainder of S was mainly divided between sulphate and proteins. When the external S supply was withdrawn, the concentration of total S decreased by 80%, the remainder of which was predominantly present in proteins, apart from 2% which was present as glucosinolates. It seems therefore unlikely that

glucosinolates play a vital role as a S source in the event of S deficiency as was suggested by Schnug and Haneklaus (1993).

- **When S was sufficient, sulphate was the largest pool of soluble S, especially in mature leaves.**
- **Glucosinolates contained less than 10% of the total amount of S in young leaves, and less than 1% in mature leaves.**

3.3.3 How do different S pools respond to S deficiency?

The effect of withdrawal of S supply is shown for oldest and youngest oilseed rape leaves (Fig. 13). For clarity, only a selection of the results are presented here, and the full results are given in Appendix 6. In the oldest leaves, the decrease in total S was predominantly due to a decrease in the sulphate concentrations (Fig. 13a). The sulphate concentrations in those leaves were not affected until day 3, after which it decreased rapidly. Yet, even after 13 days there was still 2.7 mg g^{-1} DW of sulphate-S present in the oldest leaves, enough to sustain protein S (Fig. 13c), glutathione (Fig. 13e) and glucosinolates (Fig. 13g). None of these S pools decreased in the oldest leaves during the 13 days of S withdrawal.

In the youngest leaves, all S pools decreased when the external S supply was withdrawn. The concentrations of sulphate (Fig. 13b) and glutathione (Fig. 13f) decreased immediately from the day of transfer. The concentrations of glucosinolates decreased only slowly from day 3 onwards (Fig. 13h). Protein S also decreased from day 3 onwards, and after 13 days only 50% of the initial concentration remained (Fig. 13d).

In the youngest leaves, soluble S pools were remobilised to maintain protein S.

3.3.4 Can yield losses as a result of S deficiency be predicted by measuring S pools early in the growth season?

Oilseed rape plants were grown in pots with soils collected from 6 agricultural areas around the UK, as described by Riley et al. (2000). The plants were grown in a roofed cage, so that they experienced the same temperature changes during growth as if they would have been growing in a field, but all other inputs, such as water and S fertilizers, were controlled.

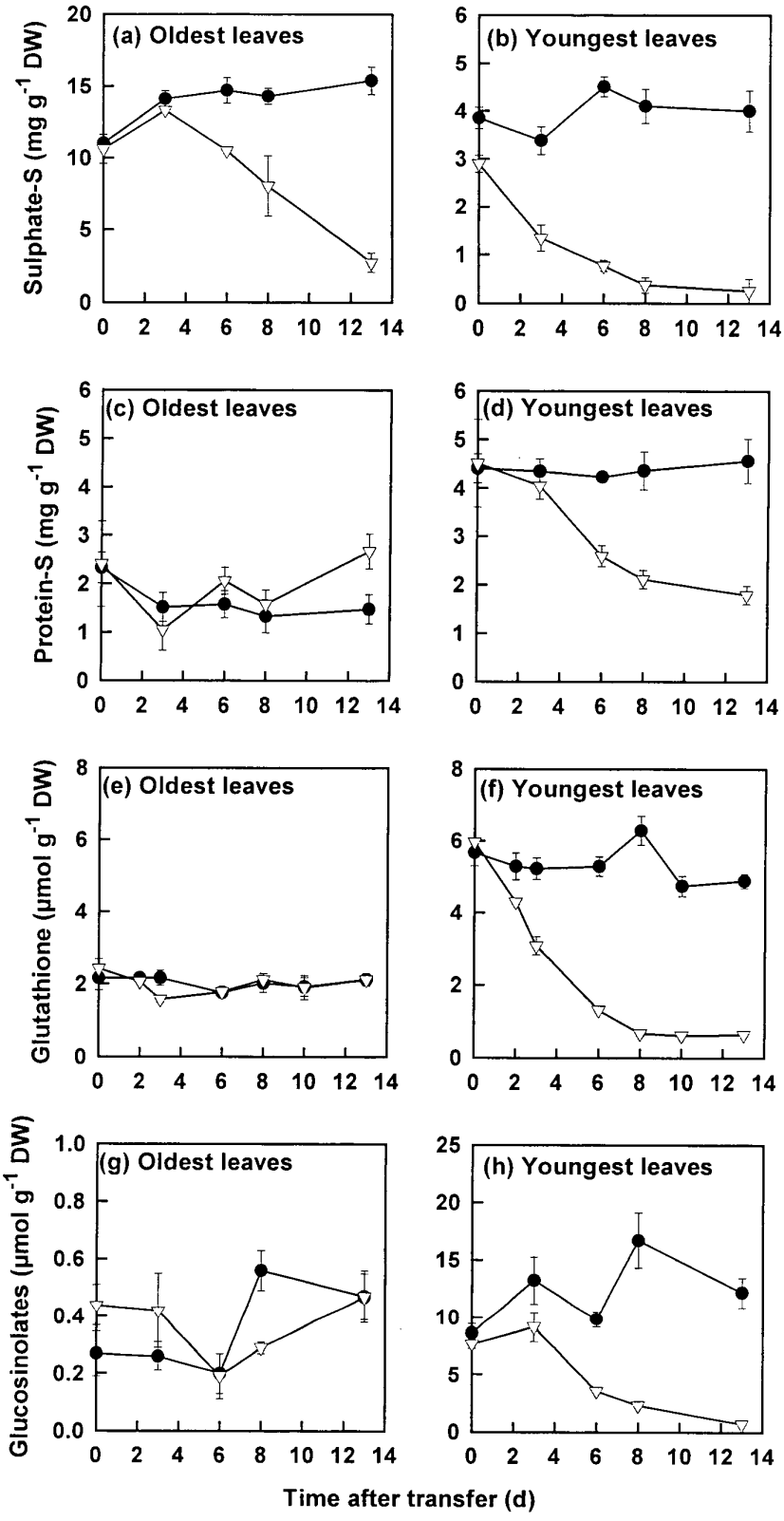

Figure 13. The concentrations of (a) total S, (b) sulphate, (c) sulphate and (D) Protein S in the oldest, middle and youngest leaves of oilseed rape plants grown in the presence (•) or absence (▽) of external S supply. Plants were grown for 3 weeks on 1 mM S before transfer to the two different treatments at day 0.

Plants samples were collected at either GS 1.5-1.7 or GS 3.3-4.3, and separated in young and mature leaves. The concentrations of SO_4^{2-} and glutathione measured at these growth stages were related to the seed yield in order to determine a critical value.

The sulphate concentrations decreased with time between GS 1.5-1.7 and GS 3.3-4.3 (Fig. 14). This was probably because the S supply in pots was limited and decreased rapidly as the plants grew. At GS 1.5-1.7, the critical values for SO_4^{2-} in mature and young leaves correlating with a 90% maximum seed yield were 2.6 and 2.5 mg g^{-1} DW, respectively (Fig. 14a). At GS 3.3-4.3, the critical values for SO_4^{2-} decreased to 0.3 and 0.2 mg g^{-1} DW for mature and young leaves, respectively (Fig 14b). However, some sulphate concentrations correlated to a seed yield near zero were similar to sulphate concentrations correlated to seed yields of 8-12 g pot^{-1}. As a result, these sulphate concentrations could either indicate S deficiency or sufficiency.

The glutathione concentrations in mature and young leaves also decreased with time between GS 1.5-1.7 and 3.3-4.3 (Fig. 15). It was not possible to derive a reliable critical value for the glutathione concentrations in mature and young leaves at GS 1.5-1.7 because the data were too scattered (Fig. 15a). At GS 3.3-4.3, a critical value of 0.7 μmol g^{-1} DW was derived for the glutathione concentrations in young leaves (Fig. 15b). In the mature leaves, the concentrations were too scattered to determine a reliable critical value.

From the experiments in section 3.3.1 it was shown that, at sufficient external S supply, the concentrations of glutathione in the youngest leaves were always higher than those in the mature leaves. Because S deficiency affected the youngest leaves first, before the mature leaves, the glutathione concentrations in the young leaves decreased before the glutathione concentrations in the mature leaves were affected. Hence the ratio between the glutathione concentrations of young over mature leaves (glutathione ratio Y/M) decreased as well. Therefore, in this experiment, the use of the glutathione ratio (Y/M) as diagnostic indicator of the current S status was determined (Fig. 16).

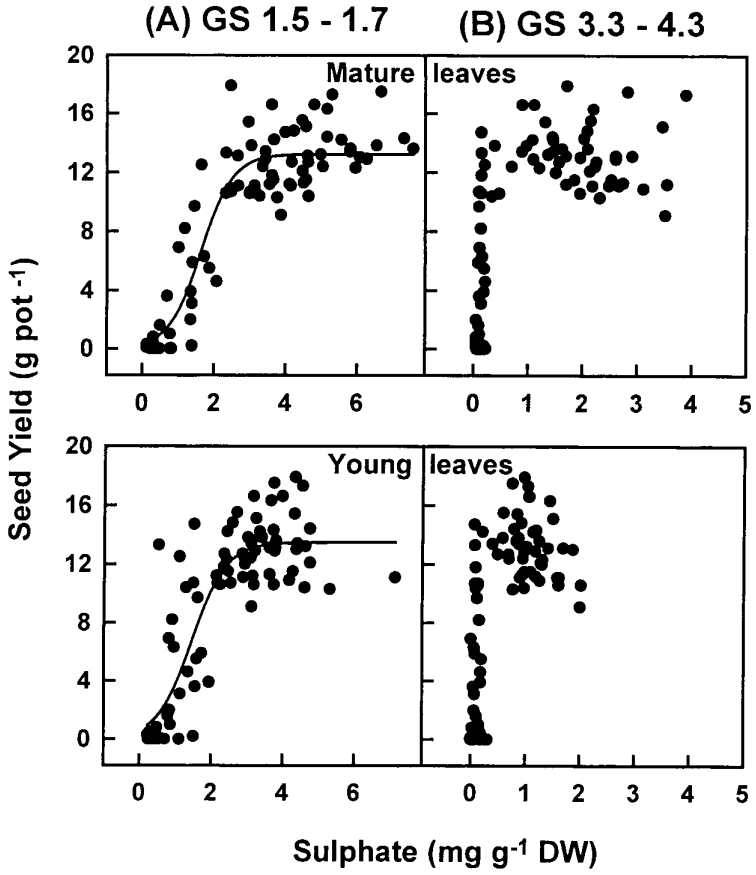

Figure 14. The relationship between the SO_4^{2-} concentrations in mature and young leaves of oilseed rape sampled at (A) GS 1.5-1.7 or (B) GS 3.3-4.3 and the final seed yields. Plants were grown at ambient temperature in pots containing soils from across the UK.

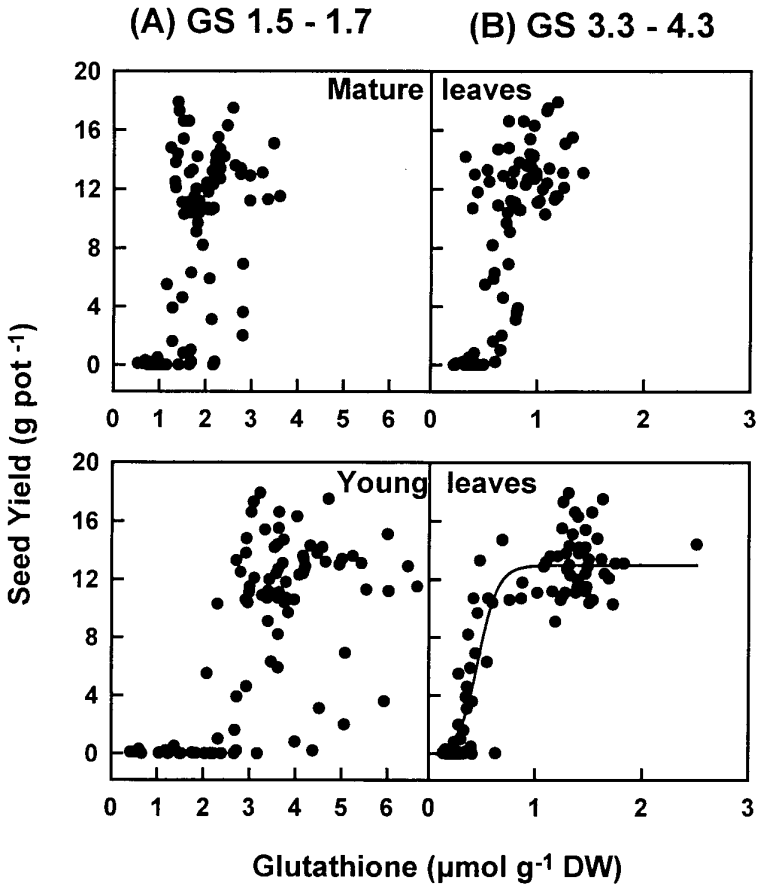

Figure 15. The relationship between the glutathione concentrations in mature and young leaves of oilseed rape sampled at (A) GS 1.5-1.7 or (B) GS 3.3-4.3 and the final seed yields. Plants were grown at ambient temperature in pots containing soils from across the UK.

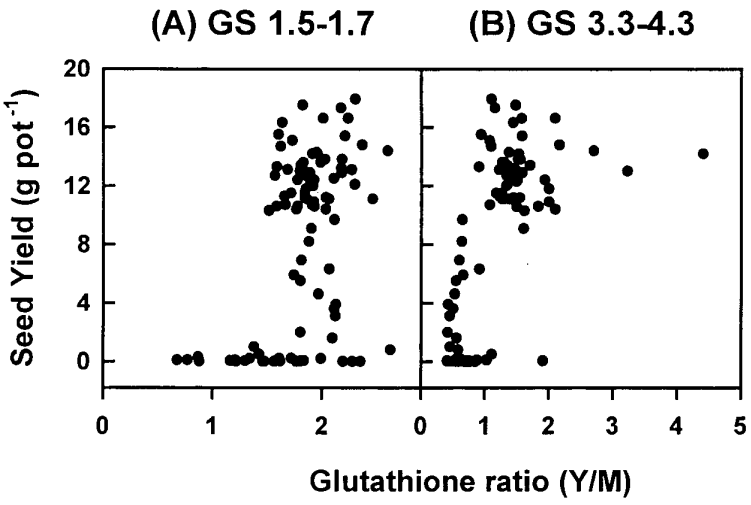

Figure 16. The relation between the ratio of glutathione concentrations in young over mature leaves of oilseed rape plants sampled at (A) GS 1.5-1.7 or (B) GS 3.3-4.3 and the final seed yields.

At GS 1.5-1.7, there were no S-sufficient plants with a glutathione ratio (Y/M) less than 1.5, meaning that in S-sufficient plants the young leaves contained at least 1.5 times more glutathione concentration than the mature leaves. Conversely, all plants with a glutathione ratio less than 1.5 produced no seeds due to severe S deficiency. Unfortunately, some of the plants producing yields below the 90% maximum seed yield, including plants producing no seeds at all, had a glutathione ratio larger than 1.5, meaning that a glutathione ratio larger than 1.5 would not give a conclusive answer regarding the S status of the plants.

At GS 3.3-4.3, the glutathione ratio (Y/M) gave a more conclusive answer regarding the S status of the plants and the likelihood of yield loss as a result of S deficiency: in general, when the glutathione ratio (Y/M) was equal or larger than 1 no yield losses as a result of S deficiency occurred, whereas with a glutathione ratio less than 1, a yield loss as the result of S deficiency was likely.

Critical values for oilseed rape grown in pots with soil:

	GS 1.5-1.7	GS 3.3-4.3
sulphate (mg g^{-1} DW)	2.5-2.6	0.2-0.3
glutathione (µmol g^{-1} DW)	not applicable	0.7
glutathione ratio (Y/M)	1.5	1

3.4 Field experiments with oilseed rape

3.4.1 Winter oilseed rape

Winter oilseed rape was grown in 1996-1997 and produced a yield of about 4.5 t ha^{-1}, but no significant differences in yield between the various S applications were observed (data not shown). The sulphate and glutathione concentrations were measured throughout the growth season. For clarity, only the 0 and 80 kg S ha^{-1} treatments are shown (Fig. 17), and the full results are presented in Appendix 7.

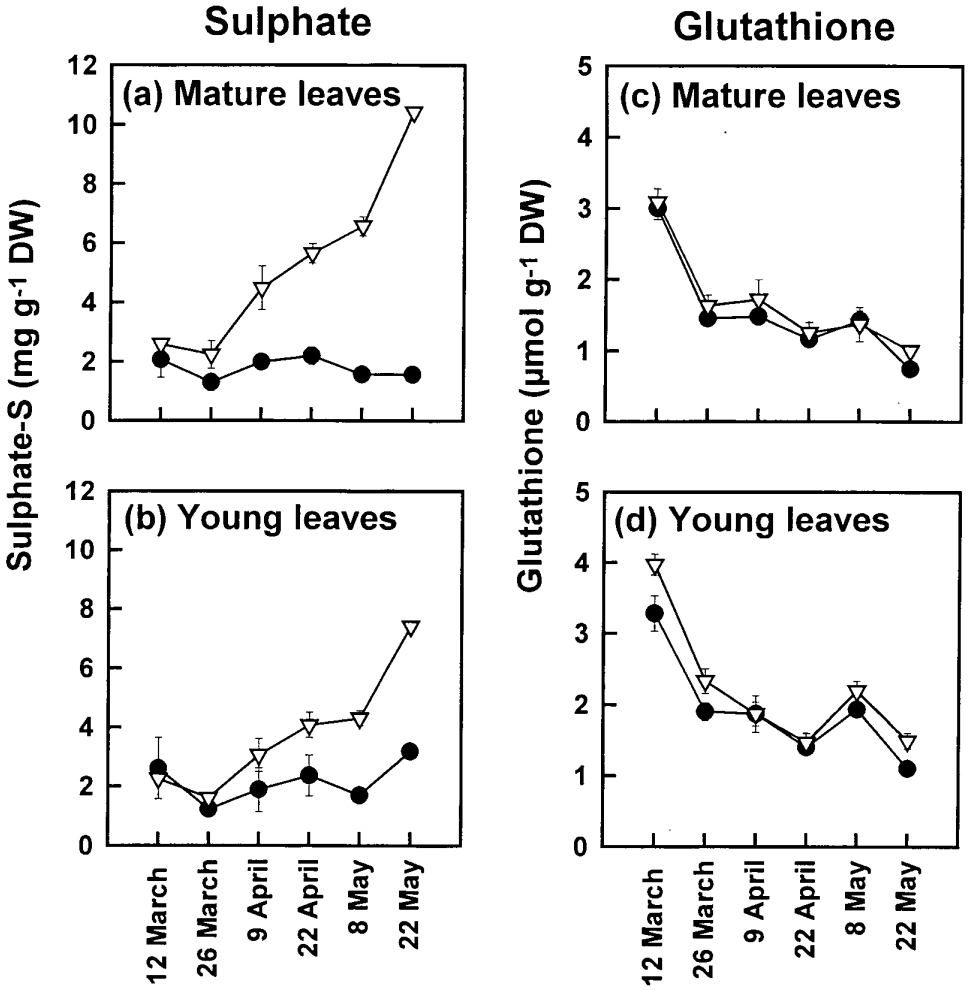

Figure 17. The concentrations of (a, b) sulphate and (c, d) glutathione in mature (a, c) and young (b, d) leaves of field-grown winter oilseed rape sampled in 1997. The S applications were either 0 kg S ha^{-1} (•) or 80 kg S ha^{-1} (▽).

The SO_4^{2-} concentrations increased with time in the 80 kg S ha^{-1} treatment, whereas no accumulation was observed in the 0 kg S ha^{-1} treatment (Fig. 17a). The relatively constant SO_4^{2-} concentrations (± 2 mg g^{-1} DW) in the 0 kg S ha^{-1} treatment during the course of the experiment suggested that the plants were not S deficient in the 1997 growth season. This was probably due partly to the type of soil which, especially in the subsoil, contained marginal S concentrations and partly to the lack of rainfall in March and April 1997, which caused the plants to root deeper.

There was no significant effect of the various S treatments on the concentrations of glutathione (Fig. 17b). The glutathione concentrations in young leaves were higher than those in the mature leaves, which was in agreement with results from the controlled environment experiments (section 3.3.1). During the growth season, glutathione decreased, probably as a result of growth dilution as explained for wheat in section 3.1.2.

Because of the lack of S deficiency in the winter oilseed rape, subsequent trials were grown on another type of soil which contained lower S concentrations in the subsoil. Unfortunately, in 1997-1998, the germination of winter oilseed rape was very poor, so the trials were changed to spring oilseed rape.

3.4.2 Is the yield of spring oilseed rape affected by S deficiency?

Spring oilseed rape was grown for three consecutive years. The yields and the concentrations of total S in the seeds are shown in Table 4. Seed yields were generally low compared to yields of commercially grown spring oilseed rape, and a significant yield increase in response to S was only observed in 1999. In 1998, there was a yield increase of about 0.3 t ha^{-1} in response to S, but this was not significant because the variation in yield between the four treatment blocks was larger than the yield differences caused by the various S treatments. The total S concentrations in seeds increased significantly in all three seasons with increasing S applications.

Table 4. The seed yield (t ha^{-1}) and concentration of total S in seeds (mg g^{-1}) for spring oilseed rape grown in 1997, 1998 and 1999, respectively. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, indicate the levels of significant difference compared to the 0 kg S ha^{-1} treatments.

Applied S (kg ha^{-1})	Seed Yield (t ha^{-1})			Total S (mg g^{-1})		
	1997	*1998*	*1999*	*1997*	*1998*	*1999*
0	2.05	1.20	0.47	3.51	2.89	2.73
5	----	1.34	0.57	----	3.13^{*}	2.57
10	2.09	1.46	0.81^{**}	3.91^{*}	3.29^{**}	3.32^{*}
20	1.96	1.67	0.90^{***}	4.20^{**}	3.66^{***}	3.33^{*}
40	2.07	1.50	0.96^{***}	4.45^{***}	3.86^{***}	4.01^{***}
80	----	1.54	0.82^{***}	----	4.29^{***}	3.94^{***}
LSD (0.05)	*0.21*	*0.54*	*0.21*	*0.39*	*0.23*	*0.62*

In 1999, the yield of spring oilseed rape increased by about 0.4 t ha^{-1} in response to S, whereas the concentration of total S in the seeds increased by 44%.

3.4.3 Does glutathione respond to S deficiency in the field?

The effect of different S applications on the concentrations of glutathione and sulphate in mature and young leaves of spring oilseed rape were determined for the three growth seasons. For clarity, only the 0 and 40 kg S ha^{-1} treatments are shown, but the full results are presented in Appendix 8. The glutathione concentrations in mature and young leaves are shown in Figure 18. This graph illustrates the variation in response to S treatments within different growth seasons. In mature leaves, the glutathione concentrations remained relatively constant in 1997 (Fig. 18a) and 1999 (Fig. 18c), whereas in the young leaves the concentrations decreased with time (Fig. 18d, f). In those years, the glutathione concentrations in leaves treated with 40 kg S ha^{-1} were approximately 1.5 to 2-fold higher than those in the 0 kg S ha^{-1} treatment but this difference decreased with time, especially in the young leaves. In contrast, in 1998 the glutathione concentrations increased with time both in mature (Fig. 18 b) and young leaves (Fig. 18e) and there was no significant difference between the two treatments.

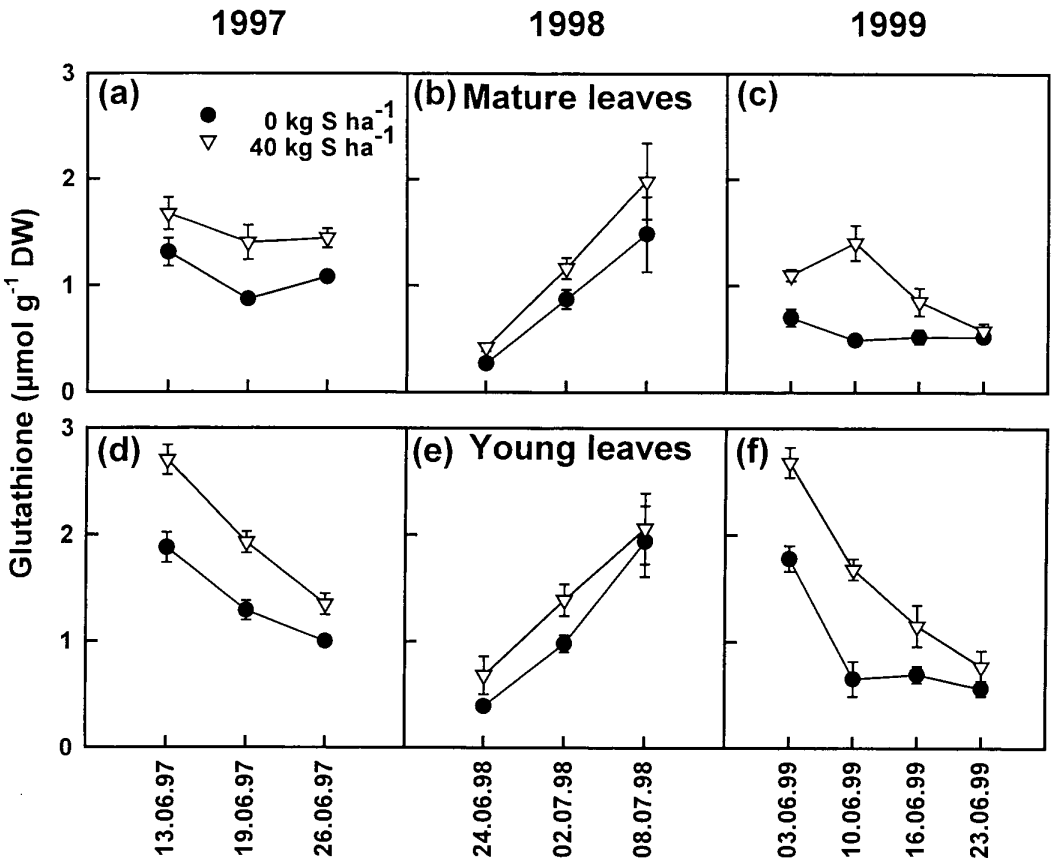

Figure 18. The concentrations of glutathione in mature (a, b, c) and young (d, e, f) leaves of field-grown spring oilseed rape sampled during 1997 (a, d), 1998 (b, e) and 1999 (c, f). The S applications were either 0 kg S ha^{-1} (•) or 40 kg S ha^{-1} (▽).

The increase in the glutathione concentrations in 1998 suggested that in this year the plants recovered from S deficiency during the growth season, which was confirmed by the disappearance of visual S-deficiency symptoms at the last sampling time.

The glutathione ratio between mature and young leaves changed with and within each growth season (Appendix 8) and no consistent results were produced between S-deficient and S-sufficient treatments. This suggested that in the field the glutathione Y/M ratio was not a suitable diagnostic indicator.

The difference in glutathione concentrations between plus and minus S treatments was small and changed during the growth season and between years.

3.4.4 Does sulphate respond to S deficiency in the field?

The sulphate concentrations were more responsive to applied S than the glutathione concentrations (Fig. 19). In all three years, the sulphate concentrations were less than 1.5 mg g^{-1} when no S was applied, both in mature and young leaves. When 40 kg S ha^{-1} was supplied, between 4 and 12 mg g^{-1} DW of sulphate accumulated in mature leaves depending on the year and sampling time, whereas in the young leaves sulphate remained around 4 mg g^{-1} DW in 1997 (Fig. 19d) and 1999 (Fig. 19f) but increased to 8 mg g^{-1} DW in 1998 during the growth season (Fig. 19e). This confirms that in 1998 the external S supply improved during the growth season, perhaps because the growing roots reached a subsoil layer with slightly higher S concentrations.

The large difference in sulphate concentrations between plus and minus S treatments suggested that sulphate was a more suitable indicator than glutathione for determining the S status of plants in the field. Yet, as with glutathione, the problem with sulphate was that the absolute values changed during the growth season and between different years. We therefore looked for a way of improving the reliability and practicality of determining the S status in oilseed rape plants whilst using the changes in sulphate concentrations as a basis.

The changes in sulphate concentrations with different S applications were larger than the changes in glutathione concentrations.

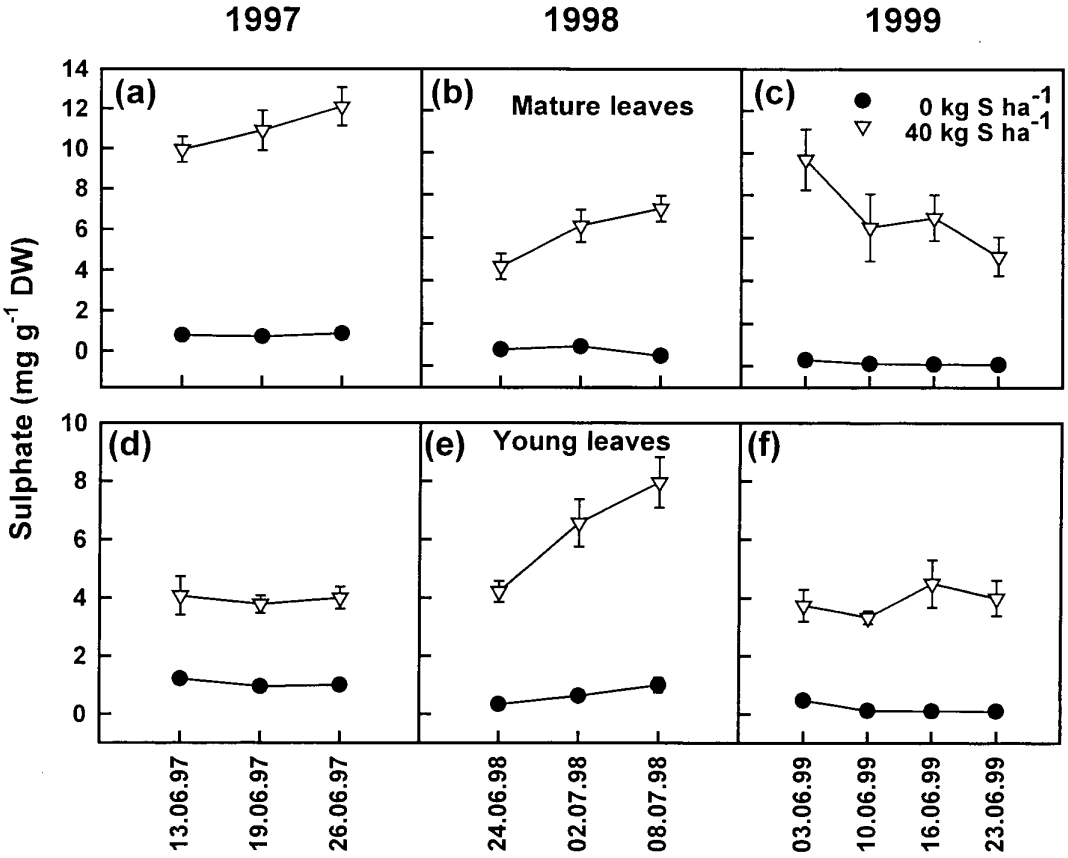

Figure 19. The concentrations of sulphate in mature (a, b, c) and young (d, e, f) leaves of field-grown spring oilseed rape sampled during 1997 (a, d), 1998 (b, e) and 1999 (c, f). The S applications were either 0 kg S ha^{-1} (•) or 40 kg S ha^{-1} (▽).

3.4.5 Can we improve the reliability of sulphate as a diagnostic indicator?

The use of absolute values as a diagnostic indicator has two major drawbacks. Firstly, the determination has to be accurate, which is dependent on precise sample preparation, extraction methods and the accurate calibration of equipment used. All these steps are vulnerable to the introduction of small errors which may influence the final outcome. Secondly, there is a lot of variation between plants depending on the position of the plant in the field, the time of sampling and the prevailing climatic conditions. These drawbacks can be avoided if there is another indicator which also changes in relation to the S status of plants, so that a ratio can be calculated. In general, ratios are more constant than absolute values and less likely to fluctuate with time.

We examined whether a correlation could be found between sulphate and other compounds in relation to the S status of the plants. Malate and nitrate were useful candidates because all three compounds could be determined in a single analysis on an ion chromatograph. The concentrations of sulphate, malate and nitrate in young leaves of plants treated with increasing S applications are shown in Figure 20. As shown before, the sulphate concentrations increased with increasing external S, and with time more sulphate accumulated (Fig. 20a). In this graph the sulphate concentrations are expressed in μmol g^{-1} DW for comparison with the malate and nitrate concentrations but can be converted to mg g^{-1} DW by multiplying by 0.032.

In contrast to sulphate, the malate concentrations decreased with increasing external S (Fig. 20b). The highest malate concentrations were found when S was deficient. The decrease in malate concentrations was in the same concentration range as the increase in sulphate concentrations. For example, at GS 4.6-4.8 (H4), the sulphate concentrations increased by 375 μmol g^{-1} DW (12.8 mg g^{-1} DW) from 0 to 80 kg S ha^{-1}, whereas the malate concentration decreased by 320 μmol g^{-1} DW. This suggested that there was an inverse relation between malate and sulphate: when the sulphate concentrations increased, the malate concentrations decreased and vice versa. A possible explanation is that in oilseed rape stored sulphate in the vacuoles acts as an osmoticum (see section 1.7) which may explain why oilseed rape plants have a high demand for S.

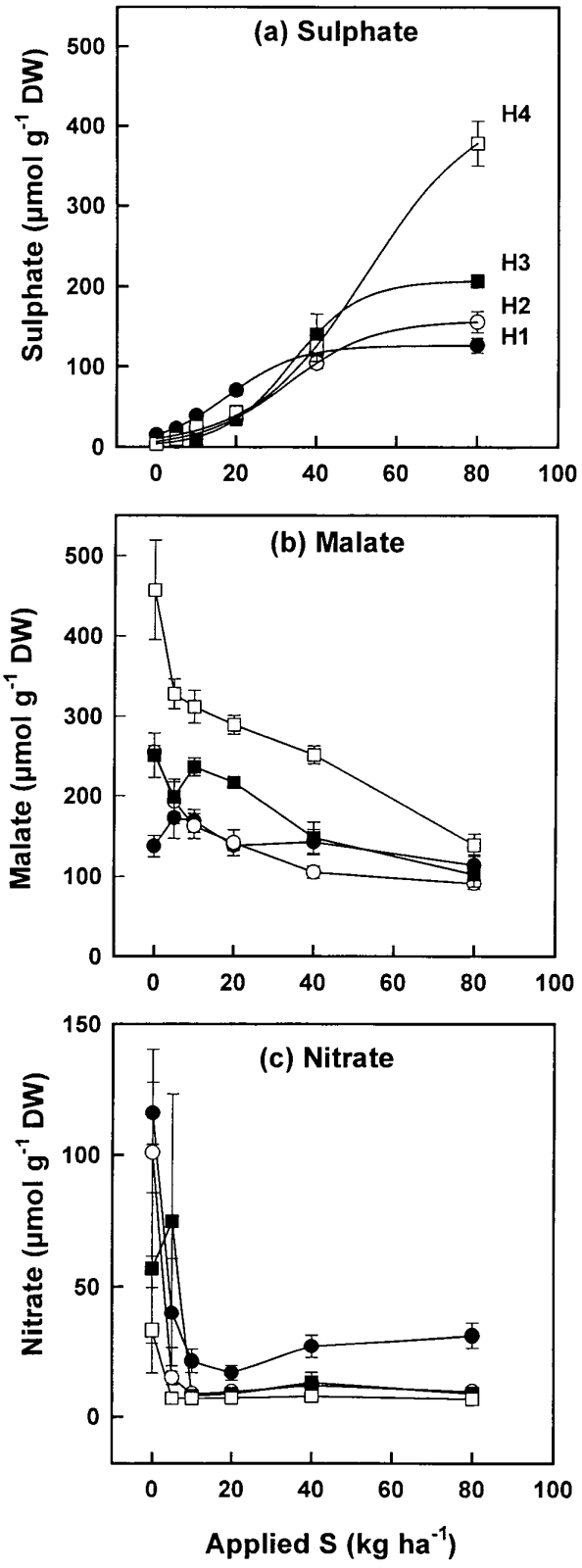

Figure 20. The concentrations of (a) sulphate, (b) malate and (c) nitrate in young leaves of field-grown spring oilseed rape. The sulphate concentrations expressed in µmol g^{-1} DW can be converted to mg g^{-1} DW by multiplying by 0.032.

When the SO_4^{2-} concentrations decrease as a result of insufficient external S supply, the stored SO_4^{2-} is replaced by malate, as has been suggested in lettuce for nitrate (Blom-Zandstra et al., 1990). Alternatively, the inverse relation between SO_4^{2-} and malate could be due to the maintenance of the cytoplasmic pH (Fig. 1), as was shown for field-grown *Vicia faba* L. (Kropff, 1991).

Although some nitrate accumulated in the S-deficient plants (Fig. 20c), this accumulation was not consistent and there was no direct relation either between the nitrate and sulphate concentrations, or nitrate and malate concentrations.

When the sulphate concentrations increased, the malate concentrations decreased and vice versa.

3.4.6 Can we use the ratio between malate and sulphate as a more reliable and practical diagnostic indicator than sulphate alone?

Malate and sulphate could be measured within a single analysis using ion chromatography, which reduced the introduction of errors with the sample preparation and extraction. When S was deficient, the malate peak was higher than the sulphate peak (Fig. 21a). When S was sufficient, the malate peak was lower than the sulphate peak (Fig. 21b). The ratio was determined by dividing the peak areas. The advantage of using the peak area ratio was that no calibration was needed, thus reducing the chances of errors.

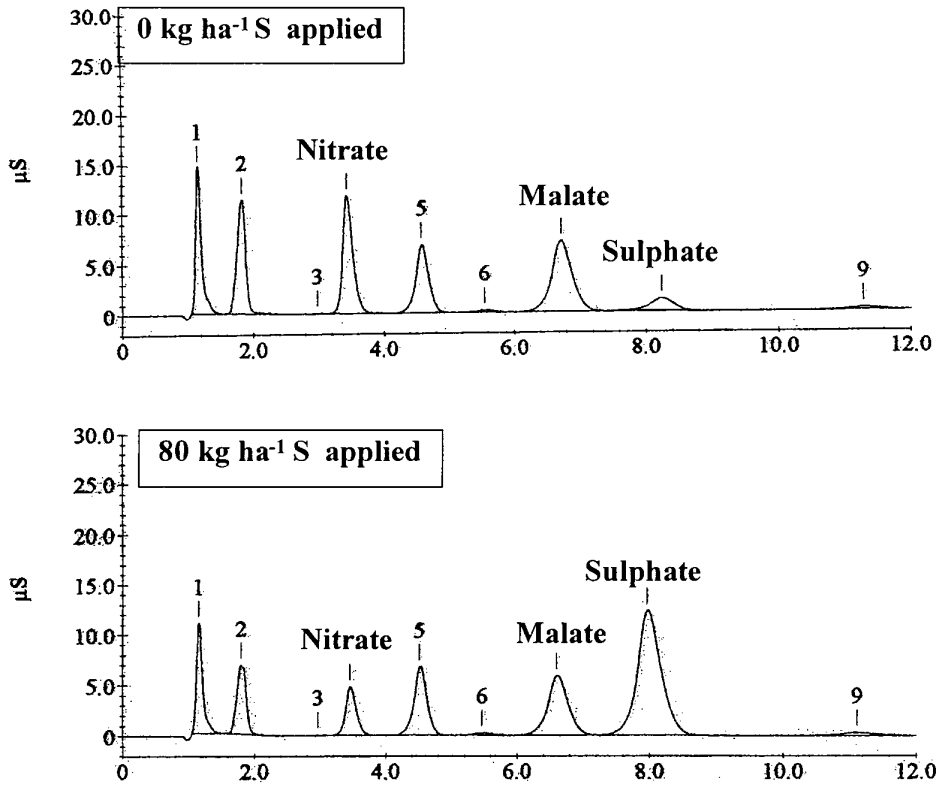

Figure 21. Example of typical ion chromatograms of oilseed rape plants grown in the field treated with 0 or 80 kg S ha^{-1}.

Part III: Diagnosis of S deficiency

In Part I and II we determined which compounds might be reliable for diagnosing S deficiency in wheat and oilseed rape. In this part we determined the suitability of all these parameters by correlating the concentrations or ratios measured at the time of sampling to the final seed yield. S deficiency was defined as occurring when the yield was less than 90% of the maximum. It should be emphasised that this was a strict criterion for S deficiency because of the indefinite relationship between yield and plant nutrient composition measured at a single growth stage: deviations in the S status at the time of measurement may only reflect the start of a nutrient imbalance from which the plant may or may not recover. In our field experiments, significant yield losses occurred only when the plants were S deficient for prolonged periods during the growth season. For example, S deficiency symptoms were observed at the stem extension stage in winter wheat in 1996 and spring oilseed rape in 1998. Yet, both crops were recovered from S deficiency at the later stages and yield losses were not significant. Conversely, sometimes yield losses occurred for reasons other than S deficiency, for example because of pests or an unfavourable position in the field. It was therefore assumed that yield losses in plants treated with high S applications (in wheat $\geq$ 10 kg S ha^{-1}, in oilseed rape $\geq$ 20 kg S ha^{-1}) were unlikely to be due to S deficiency, but due to other reasons.

3.5 Critical values

The critical values for diagnosing S deficiency were determined for total S, sulphate, glutathione, N:S ratio and malate:sulphate peak area ratio. There was no advantage in determining the percentage S as sulphate, because changes in total S were predominantly determined by changes in sulphate as was shown for wheat in section 3.1.2 and for oilseed rape in section 3.3.3. In fact, Scaife and Burns (1986) pointed out that not only is there no advantage in using the %S as SO_4^{2-}, but it also involves twice as much analytical work.

The critical values for wheat are shown in Table 5. The critical values for total S and SO_4^{2-} at GS 25-30 were exceptionally high, probably due to the fact that gypsum was reapplied only two weeks before this sampling time, resulting in a large and sudden influx of SO_4^{2-}. The critical values for this sampling time should therefore be treated with caution. The critical value of total S at GS 31-32 is lower

than has previously been reported (McGrath et al. 1999), probably because the plants were experiencing severe S deficiency at this growth stage. The critical values for oilseed rape are shown in Table 6.

Table 5. Summary of critical values for the prognosis of a reduced yield as the result of S deficiency in winter wheat using the 1999 data. Critical values were calculated for all leaves.

	Growth stage			
Indicator	20-25	25-30	30-31	31-32
Total S (mg g^{-1})	2.5	4.5	1.9	1.3
Sulphate (mg g^{-1})	0.5	1.3	0.2	0.1
N:S ratio (in mg g^{-1})	15.4	13.4	14.9	17.5
Glutathione (μmol g^{-1})	2.9	2.1	1.6	1.2
Malate:Sulphate ratio	0.8	0.6	1.0	1.1

Table 6. Summary of critical values for the prognosis of a reduced yield due to S deficiency in spring oilseed rape using the 1999 data. Critical values were calculated for the young leaves.

	Growth stage			
Indicator	3.5-3.6	3.9-4.2	4.2-4.6	4.6-4.8
Total S (mg g^{-1})	4.6	2.8	3.7	3.1
Sulphate (mg g^{-1})	1.2	0.6	1.1	0.8
N:S ratio (in mg g^{-1})	10.0	9.6	7.8	7.2
Glutathione (μmol g^{-1})	2.6	1.3	1.0	0.9
Malate:Sulphate ratio	1.2	1.0	0.8	1.1

The critical values presented in Tables 5 and 6 were valid for the relevant plant species, growth stage, type of tissue, soil type and climate conditions at the time of sampling. However, it was difficult to extrapolate these results to general conditions. The main problem was that the concentrations of total S, sulphate and glutathione changed over time with different growth stages and growth rates. For

example, the glutathione concentrations decreased with time, independently of the S applications, and as a result an overlap occurred in absolute values between S-deficient and S-sufficient plants. So a value of 1.6 μmol g^{-1} DW in young leaves of oilseed rape would indicate S deficiency when measured at GS 3.6-3.7, but a week later, at GS 3.9-4.2, this same value would indicate S sufficiency. In wheat, a value of 2.4 μmol g^{-1} DW would be interpreted as S deficient at GS 22-25, but at all subsequent growth stages as S sufficient. This makes glutathione unpractical as a diagnostic indicator, unless the exact growth stage of the crop is known.

It has been suggested that critical values should never be considered as absolute, but rather as a representation of a possible range which can serve as a guide (Melsted et al., 1969). Yet, successful diagnosis depends strongly on the choice of critical value. If the critical value is too high, many S sufficient plots will receive S fertiliser unnecessarily which defeats the objective of trying to diagnose S deficiency because money is wasted. If the critical value is too low, S deficiency will not be diagnosed which also results in economic loss. An additional problem is that the technique for the measurement of total S has not been standardised among laboratories, and a recent HGCA project (Crossland et al. 1998) testing the performance of commercial soil and plant testing laboratories showed that results provided by these laboratories for the total S concentration of identical plant material varied by approximately 30%. This means that the results from wheat leaves sampled at GS 20-25 and containing a total S concentration of 3 mg g^{-1} DW would have ranged from 2.1 to 3.9 mg g^{-1} DW depending on which laboratory it was sent to. With the critical value at GS 20-25 determined at 2.5 mg g^{-1} DW, some laboratories would have diagnosed this sample as S deficient and recommended S fertilisation, whereas other laboratories would have diagnosed the same sample as S sufficient.

The critical values of total S, sulphate and glutathione changed with time.

3.6 Ratios

As suggested in section 3.4.5, in general, ratios are more constant than absolute values and less likely to fluctuate with time.

3.6.1 N:S Ratio

The N:S ratio has been frequently used as an indicator of S deficiency and is based on the interactions between nitrogen and sulphur metabolism. For wheat, the average critical N:S ratio in leaves during the season was 15, which was in agreement with previous results (Spencer and Freney, 1980; Hue et al. 1984). This critical value enabled the correct diagnosis of all S-deficient plots (Fig. 22a). If, early in the growth season (GS 22-25), the N:S ratio was higher than 15, the likelihood of yield losses as a result of S deficiency was 60%. This takes into account that plants may recover when S deficiency is diagnosed early in the season.

For oilseed rape, the critical N:S ratio decreased during the growth season (Table 6). The critical ratios were in the same range as those reported previously (Hocking et al. 1996, Zhao et al. 1997). An average ratio of 9 enabled the correct diagnosis of all S-deficient plots (Fig. 22b). If, early in the growth season (GS 3.5-3.6), the N:S ratio was higher than 9, the likelihood of yield losses as a result of S deficiency was 69%.

Although the N:S ratio is quite reliable in diagnosing S deficiency, there are two problems associated with its use. Firstly, the N:S ratio requires two analytical determinations, hence twice as much work and an input of error from both methods (Jones, 1986). The problems with accurate determination of total S has already been described in section 3.5, and will also have an effect on the use of the N:S ratio (Crosland et al., 1998). Secondly, the N:S ratio reflects their relative proportion but not the actual magnitude of either. This means that it is possible to measure a low N:S ratio (suggesting sufficient S supply) when both N and S are actually deficient. Conversely, a high N:S ratio could be due to the oversupply of N even though S is sufficient (Sumner, 1978). It is therefore best to use both the N:S ratio and the critical value for total S to determine whether plants are S deficient.

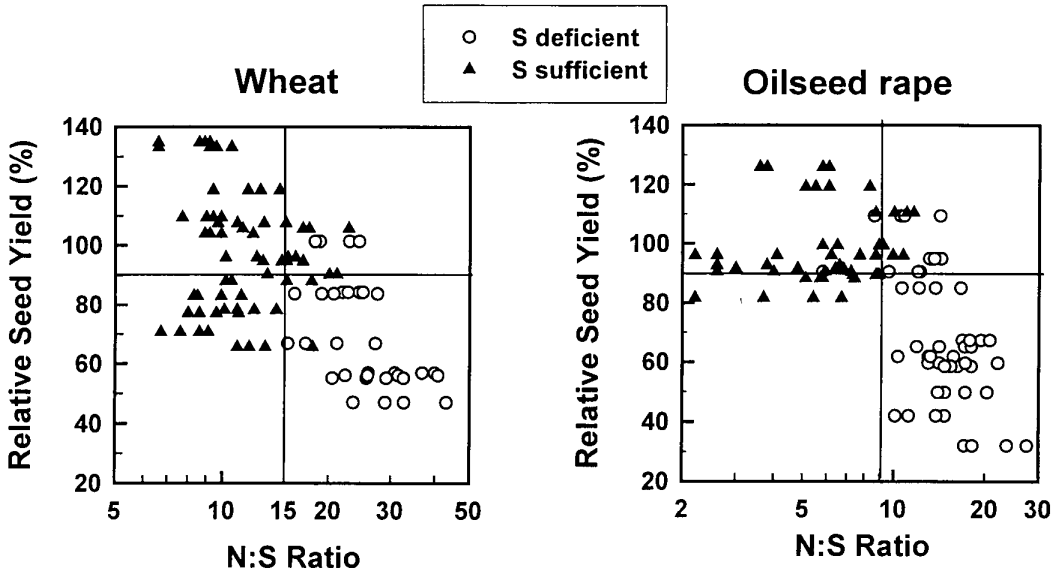

Figure 22. The relationship between the N:S ratio in wheat plants sampled during a 4-week period between GS 22-32 or oilseed rape plants sampled between GS 3.6-4.8, and the relative seed yield. (▲) = S sufficient (for wheat, treatments ≥ 10 kg S ha^{-1}, for oilseed rape, treatments ≥ 20 kg S ha^{-1}), (○) = S deficient sufficient (for wheat, treatments < 10 kg S ha^{-1}, for oilseed rape, treatments < 20 kg S ha^{-1}). The horizontal line depicts the 90% maximum seed yield, the vertical line the average critical value (for wheat, 15; for oilseed rape, 9).

For example, a N:S ratio > 15 and a total S concentration of 1 mg g^{-1} DW should be diagnosed as S deficient, whereas a N:S ratio >15 and a total S concentration of 3 mg g^{-1} DW should not be diagnosed as deficient.

3.6.2 Malate:sulphate peak area ratio

The inverse relationship between malate and sulphate in oilseed rape plants provided a practical and reliable method to determine the S status in plants. The use of peak area ratios rather than absolute values avoided problems with accurate calibration as reported for the measurements of total S (Crosland et al., 1998). Absolute values of malate and sulphate increased over time but the ratios remained similar at all sampling times.

As with all the other diagnostic indicators, the malate:sulphate ratio could only indicate the S status of the oilseed rape plants at the time of sampling (Fig. 23b): if the ratio was ≤ 1, the S status of the plant at the time of measurement was sufficient and if the ratio was > 1, the S status at the time of measurement was deficient. Similar assumptions could be made for wheat plants (Fig. 23a), even though there was no clear relationship between the sulphate and malate concentrations in leaves. In fact, using the malate:sulphate ratio in wheat plants we could diagnose that the reduced yield in one of the plots receiving 10 kg S ha^{-1}, therefore assumed to be S sufficient, was probably due to S deficiency after all, since the malate:sulphate ratio at 3 out of 4 sampling times was ≥ 1.

For wheat, if early in the growth season (GS 20-25), the malate:sulphate peak area ratio was higher than 1, the likelihood of yield losses as a result of S deficiency was 67%, whereas for oilseed rape this likelihood was 69%. The malate:sulphate ratio was a more sensitive indicator of the S status than the N:S ratio, because sulphate changed more rapidly than total S, as shown in section 3.1.2 for wheat and 3.3.3 for oilseed rape.

The malate:sulphate ratio is a more sensitive indicator of the S status in crops than the N:S ratio.

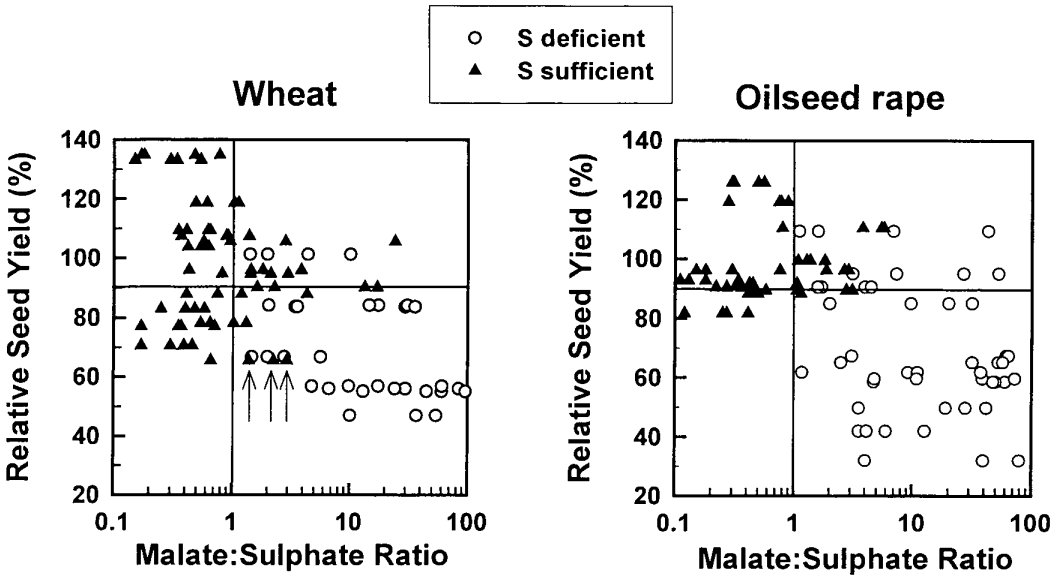

Figure 23. The relationship between the malate:sulphate peak area sampled during a 4-week period between GS 22-32 or oilseed rape plants sampled between GS 3.6-4.8, and the relative seed yield. (▲) = S sufficient (for wheat, treatments ≥ 10 kg S ha^{-1}, for oilseed rape, treatments ≥ 20 kg S ha^{-1}), (○) = S deficient sufficient (for wheat, treatments < 10 kg S ha^{-1}, for oilseed rape, treatments < 20 kg S ha^{-1}). The horizontal line depicts the 90% maximum seed yield, the vertical line the average critical value. The arrows point at wheat samples which were treated with 10 kg S ha^{-1} and therefore assumed to be S sufficient, but which probably were S deficient after all, as diagnosed with the malate:sulphate ratio.

3.7 Development of an immuno-assay for glutathione

Before the start of this project, results from a pot experiment with wheat had shown that glutathione decreased in response to S deficiency (Zhao et al., 1996). Therefore, it was included in this project to raise antibodies against glutathione, which could potentially lead to a test kit for rapid diagnosis of S deficiency in the field. However, the results in this project showed that although glutathione decreased in response to S deficiency, the concentrations also decreased with time. Also it turned out to be more difficult to raise antibodies against glutathione than previously expected. The results and problems with raising antibodies against glutathione are explained in this section.

Glutathione is a small molecule containing only three amino acids, and to increase its antigenity it was conjugated to three different carrier proteins and antibodies were raised. These antibodies were tested for cross reactivity, because if antibodies raised against, for example, a bovine serum albumin-glutathione conjugate recognise the carboanhydrase-glutathione conjugate this would suggest that the antibodies recognise the glutathione part of the conjugates, since that is the only part they have in common.

Several polyclonal antibodies in mice and rabbits were selected and the reactivity towards glutathione determined. The reactivity was tested in two different ways: firstly, the antibodies were added to ELISA plates coated with glutathione and the increase in absorption was used as a measure of the reactivity towards glutathione (Fig. 24a). Although the reactivity increased with increasing glutathione concentrations, large changes in the concentrations resulted in relatively small changes in the signal.

Secondly, the reactivity was determined by adding free glutathione with the antibodies (Fig. 24b). In theory these standard solutions could be replaced by plant tissue extracts for the determination of their glutathione concentration. Although the free glutathione inhibited the absorption, suggesting competition between free and bound glutathione for the antibodies, again the changes in absorption were small and the assay was not sensitive enough to detect small changes in glutathione concentration.

Although at present the immuno-assay for measuring glutathione is not sensitive enough for plant tissue extracts, the results show that it is possible to raise antibodies against glutathione and potentially develop an immuno-assay. The antibodies used in these assays were polyclonal, but some monclonal lines against glutathione have been obtained. The reactivity towards monoclonal lines still need to be tested.

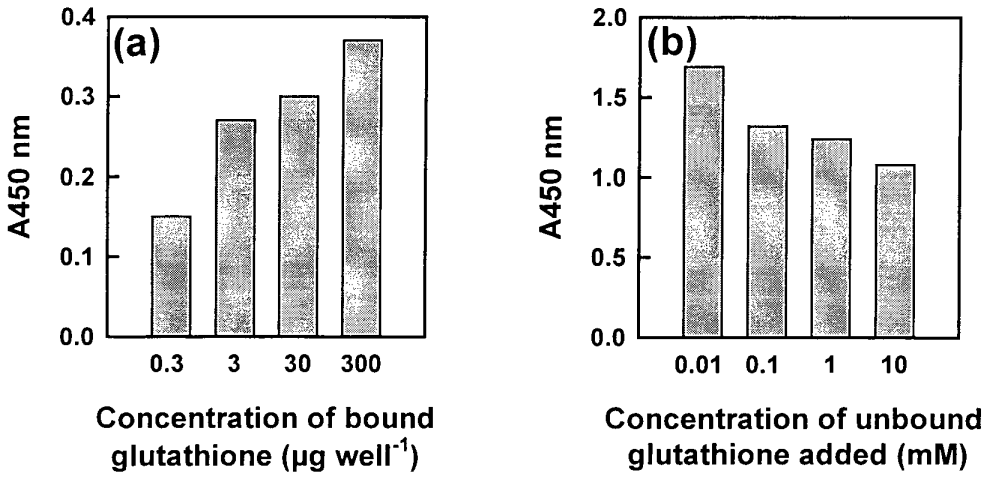

Figure 24. (a) The reactivity of polyclonal antibodies to bound glutathione and (b) competition by the addition of free glutathione to block antibody binding to bound glutathione.

4. Summary and Conclusions

4.1 Controlled environment experiments

The controlled environment experiments were performed to investigate the distribution patterns of different S pools within the plant and to examine the effects of S deficiency on growth and pool sizes in oilseed rape and wheat, with the specific objective of identifying parameters suitable as diagnostic indicators of the S-nutritional status. The results are summarised as follows:

1) In oilseed rape, the concentration of glutathione in the youngest leaves was approximately three times higher than that in the mature leaves. In wheat, before stem extension there was no significant difference between the glutathione concentrations of different leaves. In both crop species, the glutathione concentration decreased as a result of S deficiency. However, even with sufficient S supply, the glutathione concentration decreased with time.

2) In both crop species, the SO_4^{2-} concentrations decreased during S deficiency. In oilseed rape, when the external S supply was sufficient, S accumulated as sulphate, in particular in the mature leaves. During S deficiency, the sulphate concentration in the young leaves was almost completely depleted whereas in the mature leaves about 50% of total S was still present as sulphate. This suggested that the oilseed rape plants were inefficient in redistributing sulphate from the mature leaves to the young leaves when the external S supply was low and this may contribute to the high demand of oilseed rape plants for S.

3) Wheat plants were capable of redistributing S from soluble S pools in leaves to the ears at times of S shortage, but only if during the early growth stages enough S had accumulated to make redistribution possible.

4) In both crop species, protein S was maintained as long as possible at the expense of soluble S pools, so the protein S content decreased only slowly during S deficiency.

5) The effect of S deficiency on the total S concentrations was less pronounced than the effect on the sulphate concentrations. As protein S forms a large portion of total S, this was mainly due to the reason mentioned above that protein S was less affected by S deficiency.

6) In oilseed rape, glucosinolates were not a major source of S during S deficiency.

4.2 Field experiments

The field experiments were performed to determine whether sulphate and glutathione were suitable indicators in the field for the diagnosis of S deficiency. The following diagnostic indicators were evaluated:

4.2.1 Total S

The concentration of total S was less responsive to S deficiency than sulphate, and critical values changed during the growth season. In wheat, the critical values of total S for the prognosis of yield loss as a result of S deficiency in leaves decreased from 2.5 to 1.3 mg g^{-1} DW with time. For oilseed rape, the critical values for total S fluctuated between 4.6 and 2.8 mg g^{-1} DW during the growth season without any apparent chronological consistency. When total S is used as a diagnostic indicator, it is important to know the growth stages of the plants sampled, so that the appropriate critical value is used. At present, some commercial laboratories have difficulties with the accurate determination of total S, so results should be treated with caution if no known S standards have been included in the test.

4.2.2 Sulphate

Sulphate was the most responsive S-containing compound to S deficiency, and as such potentially a good indicator. There was a large (4 to 40-fold) increase in the sulphate concentrations in leaves when crops were treated with S. However, like total S, the critical values of sulphate fluctuated during the growth season, both in oilseed rape and wheat. In wheat, the critical values of sulphate for the prognosis of yield loss as a result of S deficiency in leaves decreased from 0.5 to 0.1 mg g^{-1} DW with time. For oilseed rape, the critical values for sulphate fluctuated in young

leaves between 0.6 and 1.2 mg g^{-1} DW without any apparent chronological consistency.

4.2.3 Glutathione

Glutathione was not a suitable indicator for the diagnosis of S deficiency in the field, because the concentrations decreased with time during the growth season, independently of the S treatment. As a result there was an overlap in absolute values between S-deficient and S-sufficient plants. Also glutathione increased only 1.5 to 2-fold when crops were treated with S, which is a relatively small difference. This makes glutathione impractical as a diagnostic indicator, unless the exact growth stage of the crop is known.

4.2.4 N:S Ratio

The N:S ratio in leaves was quite reliable in the diagnosis of S deficiency, with critical values of 15 for wheat and 9 for oilseed rape. A disadvantage was that two different analytical techniques needed to be used for the determination of N and S with an input of error from both. The problems with the accurate measurement of total S will also influence the accuracy of the N:S ratio. In addition, the N:S ratio reflects their relative proportion but not the actual magnitude of either. This means that it is possible to measure a low N:S ratio (suggesting sufficient S supply) when both N and S are actually deficient. Conversely, a high N:S ratio could be due to the oversupply of N even though S is sufficient. It is therefore best to use both the N:S ratio and the critical value for total S to determine whether plants are S deficient.

4.2.5 Malate:sulphate ratio

A practical and reliable indicator for S deficiency was the malate:sulphate peak area ratio, which required only one analysis using ion chromatography and was independent of the time of sampling or calibration of the samples. A malate:sulphate ratio lower than 1 indicated S sufficiency at the time of sampling, whereas a ratio higher than 1 suggested S deficiency at the time of sampling. The malate:sulphate ratio was reliable at growth stage 3.6-3.7 for oilseed rape and growth stage 22-25 for wheat, which was sufficiently early in the growth season to enable the recommendation of remedial sulphur application, if necessary.

4.3 Future work

The malate:sulphate peak area ratio looks promising as an indicator for S deficiency, but further development is needed to produce a practical and robust method for use by commercial laboratories. We need to answer the following questions:

1. How do we optimise the sample preparation for maximum simplicity and reliability?
2. Can the method be applied universally or are there differences between soil types?
3. Are there interactions with other nutrients, especially nitrogen?
4. Can we translate the experimental method into a rapid, routine method suitable for use by commercial laboratories?

5. Acknowledgements

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6. References

Alscher RG, Bower J, and Zipfel W (1987) The basis for different sensitivities of photosynthesis to SO_2 in two cultivars of pea. *J. Exp. Bot.* **38**, 99-108

Alscher RG (1989) Biosynthesis and antioxidant function of glutathione in plants. *Physiol. Plant.* **77**, 457-464

Amara A, Coussemacq M. and Geffard M (1994) Antibodies to reduced glutathione. *Brain Res.* **659**, 237-242

Anderson ME (1985) Tissue glutathione. *In* RA Greenwald, ed, Handbook of Methods for Oxygen Radical Research, CRC Press, Boca Raton, FL, pp 317-323

Bell CI, Clarkson DT, and Cram WJ (1995) Sulfate supply and its regulation of transport in roots of a tropical legume *Macroptilium atropurpureum* cv. Siratro. *J. Exp. Bot.* **46**, 65-71

Blake-Kalff MMA, Harrison KR, Hawkesford MJ, Zhao FJ, McGrath SP (1998) Distribution of sulfur within oilseed rape leaves in response to sulfur deficiency during vegetative growth. *Plant Physiol.* **118**, 1337-1344

Blom-Zandstra M (1989) Nitrate accumulation in vegetables and its relationship to quality. *Ann. Appl. Biol.* **115**, 553-561

Blom-Zandstra M, Koot HTM, van Hattum J, Borstlap AC (1990) Interactions of influxes of malate and nitrate into isolated vacuoles from lettuce leaves. *Planta* **183**, 10-16

Chew FS (1988) Biological effects of glucosinolates. *In* Biologically Active Natural Products: Potential Use in Agriculture, HG Cutler, ed, American Chemical Society, Washington, DC, pp 155-181

Clarkson DT, and Saker LR (1989) Sulfate influx in wheat and barley roots becomes more sensitive to specific protein-binding reagents when plants are sulfate-deficient. *Planta* **178:** 249-257

Crosland AR, Zhao FJ and McGrath SP (1998) Evaluation of the performance of commercial soil and plant testing laboratories for analysis of sulphur and nitrogen. Home-Grown Cereals Authority, Project Report OS26

Dijkshoorn W and van Wijk A L (1966) The sulphur requirement of plants as evidenced by the sulphur-nitrogen ratio in the organic matter: A review of published data. *Plant Soil* **26**, 129-157

Gilbert HF (1990) Molecular and cellular aspects of thiol-disulfide exchange. *Adv. Enz. Rel. Areas Mol. Biol.* **63**, 69-172

Hawkesford MJ, Davidian J-C and Grignon C (1993) Sulfate/proton cotransport in plasma-membrane vesicles isolated from roots of *Brassica napus* L.: increased transport in membranes isolated from sulfur-starved plants. *Planta* **190**, 297-304

Heaney RK, Spinks RK, Hanley AB, and Fenwick GR (1986) Analysis of glucosinolates in rapeseed. Technical Bulletin, AFRC Food Research Institute, Norwich. 28 pp.

Hell R (1997) Molecular physiology of plant sulfur metabolism. *Planta* **202,** 138-148

Hjelle OP, Chaudhry FA and Ottersen OP (1994) Antisera to glutathione: characterization and immunocytochemical application to the rat cerebellum. *Eur. J. Neurosci.* **6**, 793-804

Hocking PJ, Pinkerton A, Good A (1996) Recovery of field-grown canola from sulfur deficiency. *Aus. J. Exp. Agric.* **1**, 79-85

Hue NV, Adams F and Evans CE (1984) Plant-available sulfur as measured by soil-solution sulfate and phosphate-extractable sulfate in a ultisol. *Agron. J.* **76**, 726-730

Jones MB 1986 Sulfur availability indexes. In: *Sulfur in agriculture*, Vol 27. Ed. MA Tabatabai. pp 549-566. American Society of Agronomy, Madison, Wisconsin, USA.

de Kok L, and Oosterhuis FA (1983) Effects of frost-hardening and salinity on glutathione and sulfhydryl levels and on glutathione reductase activity in spinach leaves. *Physiol. Plant.* **58**, 47-51

Kropff MJ (1991) Long-term effects of SO_2 on plants, SO_2 metabolism and regulation of intracellular pH. *Plant Soil* **131**, 235-245

Lamoureux GL, and Rusness DG (1986) Xenobiotic conjugation in higher plants. In: *Xenobiotic Conjugation Chemistry*, (Paulson, G.D., Cadwell, J., Hutson, D.H., Menn, J.J. eds.), American Chemical Symposium Series, N0. 299, American Chemical Society, Washington D.C., pp 62-105

Leustek T. (1996) Molecular genetics of sulfate assimilation in plants. *Physiol. Plant.* **97**, 411-419

Marschner H (1995) Cation-anion relationships. In: *Mineral nutrition of higher plants,* 2nd edition, Academic Press, London, pp 45-50

McGrath SP, Zhao FJ, Withers PJA, Evans EJ, Monaghan JM, Salmon SE and Shewry PR (1999) yield and breadmaking quality responses of winter wheat to sulphur fertilizer. HGCA Report 197.

Melsted SW, Motto HL and Peck TR (1969) critical plant nutrient composition values useful in interpreting plant analysis data. *Agron. J.* **61**, 17-20

Pinkerton A (1998) Critical sulfur concentrations in oilseed rape (*Brassica napus*) in relation to nitrogen supply and to plant age. *Aus. J. Exp. Agric.* **38**, 511-522

Pow DV and Crook DK (1993) Extremely high titre polyclonal antisera against small neurotransmitter molecules: rapid production, characterisation and use in light- and electron-microscopic immunocytochemistry. *J. Neurosci. Meth.* **48**, 51-63

Randall PJ, Spencer K and Freney JR (1986) Effects of sulphur supply on the yield, composition, and quality of grain from cereals, oilseeds and legumes. In: *Advances in Cereal Science and Technology*, Vol 8. Ed. Y Pomeranz. pp 171-206. American Association of Cereal Chemists, St. Paul, MN.

Rasmussen PE, Ramig RE, Ekin LG and Rohde CR (1977) Tissue analyses guidelines for diagnosing sulfur deficiency in white wheat. *Plant Soil* **46**, 153-163

Riley NG, Zhao FJ and McGrath SP (2000) Availability of different froms of sulphur fertilizers to wheat and oilseed rape. *Plant Soil* (in press)

Scaife A and Burns IG (1986) The sulphate-S/total S ratio in plants as an index of their sulphur status. *Plant Soil* **91**, 61-71

Schnug E and Haneklaus, S (1993) Physiological backgrounds of different sulfur utilisation in *Brassica napus* varieties. *Asp. Appl. Biol.* **34,** 235-242

Schwenn JD (1997) Assimilatory reduction of inorganic sulphate. In: *Sulphur metabolsim in higher plants*, W.J. Cram, L.J. de Kok, I. Stulen, C. Brunold, H. Rennenberg, eds., Backhuys Publishers, Leiden, pp 39-58

Smith IK and Lang AL (1988) Translocation of sulfate in soybean (*Glycine max* L. Merr). *Plant Physiol.* **86,** 798-802

Spencer K and Freney JR (1980) Assessing the sulfur status of field-grown wheat by plant analysis. *Agron. J.* **72**, 469-472

Sumner ME (1978) Interpretation of nutrient ratios in plant tissue. *Commun. Soil Plant Anal.* **9**, 335-345

Sylvester-Bradley R and Makepeace RJ (1984) A code for stages of development in oilseed rape (*Brassica napus* L.). *Aspects of Applied Biology* **6**, 399-419.

Thomas W (1937) Foliar diagnosis. *Plant Physiol.* **12**, 571

Wrigley CW, Du Cros DL, Archer MJ, Downie PG and Roxburgh CM (1980) The sulfur content of wheat endosperm proteins and its relevance to grain quality. *Aus. J. Plant Physiol.* **7**, 755-766

Zhao FJ, Hawkesford MJ, and McGrath SP (1999) Sulphur assimilation and the effects on yield and quality of wheat. *J. Cereal Sci.* **30**, 1-17

Zhao FJ, Hawkesford MJ, Warrilow AGS, McGrath SP and Clarkson DT (1996) Responses of two wheat varieties to sulphur addition and diagnosis of sulphur deficiency. *Plant Soil* **181**, 317-327

Zadok JC, Chang TT and Konzak CF (1974) A decimal code for the growth stages of cereals. *Weed Res.* **14**, 415-421

APPENDICES 1 –8

Appendix 1: Nutrient solution

The nutrient solution used in the experiments contained 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnCl_2$, 2 μM $ZnCl_2$, 0.5 μM $CuCl_2$, 0.5 μM $(NH_4)_6Mo_7O_{24}$ and 20 μM NaFeEDTA. The pH of the solution was adjusted to 5.5 with KOH. $MgSO_4$ was added as indicated in the experiments and Mg^{2+} was maintained at 1 mM in all treatments by the addition of $MgCl_2$ when appropriate.

Appendix 2: Agronomic details of field experiments

Oilseed rape

Harvest year	Site	Soil Series	Sow date	Seed rate (seeds/ m^2)	Variety	Previous Crop	Harvest date
1997	Far Field II	Lowland	02.09.96	225	Apex	w.wheat	05.08.97
1997	Stackyard I	Cottenham	18.04.97	250	Starlight	w.wheat	02.09.97
1998	Stackyard AII	Cottenham	29.04.98	225	Rebel	Mixed	02.09.98
1999	Stackyard AII	Cottenham	31.03.99	180	Starlight	s. oilseed	23.08.99

Wheat

Harvest year	Site	Soil Series	Sow date	Seed rate (seed/m^2)	Variety	Previous Crop	Harvest date
1996	Stackyard AI	Cottenham	05.10.95	375	Hereward, Riband	lupins	19.08.96
1997	Butt Close	Cottenham	03.10.96	350	Hereward	grass	14.08.97
1998	Butt Close	Cottenham	14/10/97	385	Riband	s.beans	13.08.98
1999	Butt Close	Cottenham	25/09/98	380	Rialto	w.wheat	20.08.99

Appendix 3a: The growth stages at which oilseed rape plants were sampled. Growth stages were determined using the code of Sylvester-Bradley and Makepeace (1984). Y: young leaves, M: mature leaves.

Experiment	Sampling date	Growth stage	Description	Plant parts sampled
1997	12.03.97	1.10-1.13	10 to 13 fully expanded leaves	Y & M
	26.03.97	3.3	flower buds visible	Y & M
	09.04.97	3.6-3.7	1st flower stalks extending to 1st flower buds yellow	Y & M
	22.04.97	4.6-4.8	60 to 80% of buds on raceme flowering	Y & M
	08.05.97	5.6-5.8	60 to 80% of pods more than 2 cm long	Y & M
	22.05.97	6.1	seeds present	Y & M
	08.07.97	6.8	most seeds black and hard	seeds
1997	13.06.97	3.3	flower buds visible	Y & M
	19.06.97	3.6-3.7	1st flower stalks extending to 1st flower buds yellow	Y & M
	26.06.97	4.6-4.9	60 to 90% of buds on raceme flowering	Y & M
	06.08.97	6.8	most seeds black and hard	seeds
1998	24.06.98	3.6-3.7	1st flower stalks extending to 1st flower buds yellow	Y & M
	02.07.98	4.4-4.9	40 to 100% of buds on raceme flowering	Y & M
	08.07.98	5.1-5.6	10 to 60% of pods more than 2 cm long	Y & M
	12.08.98	6.8	most seeds black and hard	seeds
1999	03.06.99	3.5-3.6	flower buds raised above the leaves to 1st flower stalks extending	Y & M
	10.06.99	3.9-4.1	more than half of flower buds yellow to 1st flowers opened	Y & M
	16.06.99	4.2-4.6	20 to 60% of buds on raceme flowering	Y & M
	23.06.99	4.6-4.8	60 to 80% of buds on raceme flowering	Y & M
	12.08.99	6.8	most seeds black and hard	seeds

Appendix 3b: The growth stages at which the wheat plants were sampled. Growth stages were determined according to Zadok et al. (1974).

Experiment	Sampling date	Growth stage	Description	Plant parts sampled
1996	May 96	32	2 cm stem extension	flag leaf, leaves, stems
	June 96	45	boots swollen	flag leaf, leaves, stems
1997	21.05.97	30-32	up to 2 cm stem extension	flag leaf, leaves, stems
1998	22.04.98	25-29	Main shoot and 5 to 9 tillers	leaves
	06.05.98	30-31	1 cm stem extension to first node detectable	leaves
	02.06.98	52-57	¼ to ¾ of inflorescence emerged	leaves
	18.06.98	60-65	Beginning to half-way anthesis	leaves
	06.08.98	92	Caryopsis hard	grain
1999	15.04.99	22-25	Main shoot and 2 to 5 tillers	leaves, stems
	29.04.99	25-30	Main stem and at least 5 tillers to 1 cm stem extension	leaves, stems
	06.05.99	30-31	One cm stem extension to first node detectable	leaves, stems
	13.05.99	31-32	first and second node detectable	leaves, stems
	03.08.99	92	Caryopsis hard	grain

Appendix 4: Controlled environment experiments with wheat

L=leaf S=Stem R=Root

Time (days)	S treatment (µM)	Plant Tissue	Total S (mg/g DW)	Sulphate (mg/g DW)	Protein S (mg/g DW)	Glutathione (µmol/g DW)
14	10	L		0.53		3.83
14	20	L		0.52		3.61
14	50	L		0.96		3.45
14	100	L		1.39		4.03
14	1000	L		1.92		4.09
24	10	L		0.36		1.59
24	20	L		0.25		1.42
24	50	L		0.98		2.58
24	100	L		1.75		2.97
24	1000	L		2.47		3.09
38	10	L		0.02		0.79
38	20	L		0.01		0.81
38	50	L		0.16		1.43
38	100	L		1.15		2.32
38	1000	L		2.71		2.64
23	20	L	2.72	0.76	1.96	
23	20	S	1.89	0.53	1.36	
23	20	R	2.00	1.08	0.92	
23	1000	L	3.88	0.86	3.03	
23	1000	S	2.94	0.91	2.03	
23	1000	R	4.42	1.88	2.54	
38	20	L	2.71	0.41	2.31	
38	20	S	1.91	0.46	1.44	
38	20	R	1.22	0.32	1.31	
38	1000	L	3.23	0.70	2.53	
38	1000	S	2.66	0.99	1.67	
38	1000	R	4.18	2.39	1.79	
52	20	L	1.92	0.14	1.78	
52	20	S	1.22	0.35	0.91	
52	20	R	1.13	0.10	1.03	
52	1000	L	3.12	0.70	2.21	
52	1000	S	2.91	1.38	1.52	
52	1000	R	3.30	1.56	1.74	
66	20	L	1.64	0.18	1.46	
66	20	S	0.95	0.15	0.81	
66	20	R	1.18	0.10	1.08	
66	1000	L	3.15	0.97	2.18	
66	1000	S	2.64	1.47	1.16	
66	1000	R	3.44	1.18	2.26	

Appendix 5: Pot experiment with wheat

C= continuous WaA= withdrawn at anthesis

Time (days)	S treatment (µM)	S supply	Plant Tissue	FW (g)	Total S (mg/g DW)	Sulphate (mg/g DW)	Protein S (mg/g DW)	Glutathione (µmol/g DW)
0	20	C	Leaf	0.05	1.45	0.032	1.42	1.17
0	20	WaA	Leaf	0.05	1.45	0.032	1.42	1.17
0	20	C	Ear	0.23	1.04	0.006	1.03	1.37
0	20	WaA	Ear	0.23	1.04	0.006	1.03	1.37
0	100	C	Leaf	0.16	2.43	0.026	2.40	1.31
0	100	WaA	Leaf	0.16	2.43	0.026	2.40	1.31
0	100	C	Ear	0.55	1.24	0.150	1.09	2.64
0	100	WaA	Ear	0.55	1.24	0.150	1.09	2.64
0	1000	C	Leaf	0.30	3.36	0.534	2.83	1.74
0	1000	WaA	Leaf	0.30	3.36	0.534	2.83	1.74
0	1000	C	Ear	0.42	1.77	0.366	1.40	3.42
0	1000	WaA	Ear	0.42	1.77	0.366	1.40	3.42
15	20	C	Leaf	0.07	1.24	0.038	1.20	1.04
15	20	WaA	Leaf	0.06	1.15	0.031	1.12	0.35
15	20	C	Ear	0.42	0.82	0.124	0.70	1.82
15	20	WaA	Ear	0.42	0.69	0.039	0.65	0.33
15	100	C	Leaf	0.17	2.26	0.018	2.24	1.46
15	100	WaA	Leaf	0.20	1.81	0.021	1.79	0.79
15	100	C	Ear	1.22	1.2	0.124	1.08	1.00
15	100	WaA	Ear	1.34	0.99	0.039	0.95	0.32
15	1000	C	Leaf	0.29	4	0.703	3.30	2.08
15	1000	WaA	Leaf	0.28	3.47	0.790	2.68	0.90
15	1000	C	Ear	0.86	1.5	0.347	1.15	1.14
15	1000	WaA	Ear	0.85	1.33	0.216	1.11	0.89
30	20	C	Leaf	0.10	1.14	0.054	1.09	0.26
30	20	WaA	Leaf	0.04	0.81	0.029	0.78	0.28
30	20	C	Ear	1.05	0.98	0.007	0.97	0.86
30	20	WaA	Ear	0.59	0.57	0.012	0.56	0.20
30	100	C	Leaf	0.30	1.6	0.020	1.58	0.37
30	100	WaA	Leaf	0.33	1.2	0.028	1.17	0.13
30	100	C	Ear	2.89	1.06	0.016	1.04	0.97
30	100	WaA	Ear	2.46	0.69	0.014	0.68	0.12
30	1000	C	Leaf	0.32	3.74	1.272	2.47	2.45
30	1000	WaA	Leaf	0.29	2.36	0.720	1.64	1.30
30	1000	C	Ear	2.24	1.57	0.197	1.37	1.51
30	1000	WaA	Ear	1.52	1.32	0.151	1.17	1.30
50	1000	C	Leaf	0.38	3.84	1.389	2.45	1.59
50	1000	WaA	Leaf	0.30	1.71	0.117	1.59	0.56
50	1000	C	Ear	3.57	1.49	0.073	1.42	0.61
50	1000	WaA	Ear	3.81	1.35	0.058	1.29	0.48
56	100	C	Leaf	0.24	1.34	0.057	1.28	1.03
56	100	WaA	Leaf	0.22	0.57	0.039	0.53	0.18
56	100	C	Ear	4.22	1.19	0.082	1.11	0.33
56	100	WaA	Ear	3.67	0.67	0.052	0.62	0.11
62	20	C	Leaf	0.11	0.65	0.015	0.64	0.17
62	20	WaA	Leaf	0.11	0.54	0.023	0.52	0.20
62	20	C	Ear	1.41	1.06	0.006	1.05	0.23
62	20	WaA	Ear	1.34	0.51	0.007	0.50	0.03
70	1000	C	Leaf	0.27	3.72	1.704	2.02	0.54
70	1000	WaA	Leaf	0.25	0.96	0.041	0.92	0.14
70	1000	C	Ear	4.05	1.49	0.042	1.45	0.09
70	1000	WaA	Ear	3.88	1.22	0.010	1.21	0.09

Appendix 6: Controlled environment experiments with oilseed rape

Blake-Kalff et al. 1998 Plant Physiol. 118, 1337-1344. O = old M = Middle Y = young leaves

Time (days)	N supply (mM)	S supply (µM)	Leaf Type	Glutathione (µmol/g DW)	Glucosinolate (µmol/g DW)	Total S (mg/g DW)	Sulphate (mg/g DW)	Protein S (mg/g DW)	Chloro-phyll (units)
0	7	1	O	1.08	0.27	13.35	11.02	2.33	34.5
0	7	1	M	2.03	1.40	11.24	7.75	3.49	42.0
0	7	1	Y	2.83	8.64	7.87	3.85	4.03	41.3
0	7	0	O	1.22	0.44	13.02	10.62	2.41	36.0
0	7	0	M	1.84	1.07	11.30	7.64	3.66	43.8
0	7	0	Y	2.96	7.77	8.94	2.90	6.04	39.6
0	0.25	1	O	1.17	0.66	14.73	12.28	2.46	37.7
0	0.25	1	M	1.82	1.04	11.11	7.71	3.41	45.1
0	0.25	1	Y	2.58	7.69	8.02	3.46	4.55	43.3
0	0.25	0	O	1.09	0.37	14.17	11.81	2.36	34.5
0	0.25	0	M	1.69	1.00	11.45	7.75	3.71	45.9
0	0.25	0	Y	2.70	8.41	8.06	3.57	4.49	44.7
3	7	1	O	1.08	0.26	15.62	14.10	1.52	35.8
3	7	1	M	1.63	1.42	11.82	9.01	2.81	45.7
3	7	1	Y	2.61	13.30	8.66	3.38	5.28	43.3
3	7	0	O	0.79	0.42	14.32	13.27	1.05	35.2
3	7	0	M	1.38	1.21	7.91	4.84	3.07	44.7
3	7	0	Y	1.54	9.12	6.04	1.34	4.69	43.4
3	0.25	1	O	1.08	0.27	15.57	13.54	2.03	39.1
3	0.25	1	M	1.91	1.70	12.34	9.28	3.06	48.0
3	0.25	1	Y	2.57	12.53	9.68	4.33	5.36	45.3
3	0.25	0	O	0.87	0.12	15.06	13.69	1.37	34.6
3	0.25	0	M	1.57	1.41	9.11	5.39	3.72	45.8
3	0.25	0	Y	1.54	7.27	5.38	1.15	4.23	47.4
6	7	1	O	0.88	0.20	16.29	14.71	1.58	34.4
6	7	1	M	1.67	1.51	12.97	8.84	4.13	44.8
6	7	1	Y	2.64	9.90	9.35	4.51	4.84	44.8
6	7	0	O	0.89	0.19	12.57	10.50	2.07	34.5
6	7	0	M	0.98	0.66	5.06	2.29	2.77	43.3
6	7	0	Y	0.66	3.56	3.61	0.77	2.83	41.0
6	0.25	1	O	0.97	0.52	17.23	14.73	2.50	35.0
6	0.25	1	M	1.97	2.12	12.19	8.74	3.44	45.2
6	0.25	1	Y	2.64	17.00	9.03	4.16	4.87	47.6
6	0.25	0	O	1.13	0.67	9.95	7.94	2.01	35.4
6	0.25	0	M	1.25	1.26	5.16	2.33	2.83	47.9
6	0.25	0	Y	0.91	6.69	3.65	0.74	2.92	47.9
8	7	1	O	1.01	0.56	15.68	14.35	1.33	31.8
8	7	1	M	1.83	2.24	13.41	10.50	2.91	43.1
8	7	1	Y	3.14	16.70	9.64	4.11	5.53	42.8
8	7	0	O	1.06	0.29	9.63	8.05	1.58	37.4
8	7	0	M	0.52	0.33	3.21	1.14	2.07	40.0
8	7	0	Y	0.32	2.32	2.80	0.54	2.27	34.6
8	0.25	1	O	1.17	0.74	16.81	15.89	0.93	34.0
8	0.25	1	M	1.93	4.44	10.73	7.27	3.46	49.4
8	0.25	1	Y	2.82	22.80	8.27	2.96	5.30	46.0
8	0.25	0	O	1.00	0.48	10.76	9.30	1.46	32.7
8	0.25	0	M	0.82	1.74	4.19	1.54	2.65	46.7
8	0.25	0	Y	0.66	7.12	3.54	0.50	3.04	47.4
13	7	1	O	1.06	0.47	16.93	15.44	1.48	30.4
13	7	1	M	1.89	1.85	13.39	10.20	3.19	45.9
13	7	1	Y	2.44	12.10	9.42	4.00	5.42	45.1
13	7	0	O	1.06	0.47	5.42	2.73	2.69	33.7
13	7	0	M	0.35	0.42	1.85	0.39	1.46	37.4
13	7	0	Y	0.28	0.69	1.85	0.51	1.34	27.1
13	0.25	1	O	1.08	1.40	12.35	9.85	2.50	31.5
13	0.25	1	M	2.11	6.98	10.44	6.31	4.13	49.5
13	0.25	1	Y	2.62	25.90	10.45	4.51	5.94	52.2
13	0.25	0	O	0.98	0.99	5.35	3.44	1.92	32.7
13	0.25	0	M	0.43	1.00	1.94	0.29	1.66	45.6
13	0.25	0	Y	0.34	3.25	2.34	0.23	2.10	41.9

Appendix 7: Field experiment with winter oilseed rape

M = mature Y= young leaves

Sampling date	S treatment (kg/ha)	Plant Tissue	Total S (mg/g DW)	Sulphate (mg/g DW)	Protein S (mg/g DW)	Glutathione (µmol/g DW)
12.03.97	0	M	4.99	2.07	2.92	3.00
12.03.97	0	Y	6.11	2.61	3.50	3.28
12.03.97	5	M	4.97	2.16	2.81	2.88
12.03.97	5	Y	6.23	2.49	3.74	3.29
12.03.97	10	M	5.41	2.04	3.37	2.89
12.03.97	10	Y	6.17	2.02	4.15	3.46
12.03.97	20	M	5.42	2.18	3.24	3.15
12.03.97	20	Y	6.66	2.18	4.48	3.78
12.03.97	40	M	5.54	2.46	3.08	3.36
12.03.97	40	Y	6.39	2.21	4.18	3.15
12.03.97	80	M	6.32	2.60	3.72	3.09
12.03.97	80	Y	7.05	2.26	4.79	3.97
26.03.97	0	M	4.35	1.29	3.06	1.45
26.03.97	0	Y	4.29	1.23	3.06	1.90
26.03.97	5	M	4.71	1.80	2.91	1.35
26.03.97	5	Y	4.27	1.16	3.11	1.53
26.03.97	10	M	4.50	1.48	3.02	1.44
26.03.97	10	Y	4.73	1.28	3.45	1.94
26.03.97	20	M	4.82	1.68	3.14	1.62
26.03.97	20	Y	4.53	1.19	3.34	1.60
26.03.97	40	M	5.06	2.22	2.84	1.34
26.03.97	40	Y	4.66	1.34	3.32	1.75
26.03.97	80	M	5.30	2.24	3.06	1.63
26.03.97	80	Y	5.04	1.61	3.43	2.33
09.04.97	0	M	3.51	1.99	1.52	1.48
09.04.97	0	Y	3.65	1.89	1.76	1.87
09.04.97	5	M	3.41	2.06	1.35	1.10
09.04.97	5	Y	3.36	2.09	1.27	1.52
09.04.97	10	M	3.79	2.03	1.76	1.46
09.04.97	10	Y	3.94	1.90	2.04	1.75
09.04.97	20	M	4.12	2.29	1.83	1.61
09.04.97	20	Y	4.08	2.40	1.68	1.87
09.04.97	40	M	4.18	3.14	1.04	1.69
09.04.97	40	Y	4.13	2.79	1.34	1.65
09.04.97	80	M	5.40	4.48	0.92	1.72
09.04.97	80	Y	4.58	3.06	1.52	1.87
22.04.97	0	M	3.35	2.20	1.15	1.16
22.04.97	0	Y	3.59	2.37	1.22	1.40
22.04.97	5	M	3.72	2.45	1.27	1.08
22.04.97	5	Y	3.85	2.52	1.33	1.29
22.04.97	10	M	3.56	2.64	0.92	1.02
22.04.97	10	Y	4.16	3.18	0.98	1.37
22.04.97	20	M	5.30	3.76	1.54	1.28
22.04.97	20	Y	4.65	4.03	0.62	1.50
22.04.97	40	M	5.15	3.91	1.24	1.12
22.04.97	40	Y	4.78	3.78	1.00	1.21
22.04.97	80	M	6.80	5.65	1.15	1.25
22.04.97	80	Y	5.35	4.07	1.28	1.47
08.05.97	0	M		1.57		1.42
08.05.97	0	Y		1.70		1.93
08.05.97	80	M		6.56		1.37
08.05.97	80	Y		4.31		2.19
22.05.97	0	M		1.56		0.74
22.05.97	0	Y		3.19		1.10
22.05.97	80	M		7.41		1.00
22.05.97	80	Y		10.41		1.49

Appendix 8: Field experiments with spring oilseed rape

M = mature Y = young leaves

Sampling date	S treatment (kg/ha)	Plant Tissue	Total S (mg/g DW)	Sulphate (mg/g DW)	Protein S (mg/g DW)	Glutathione (µmol/g DW)	Glutathione Ratio (Y/M)
13.06.97	0	M		0.77		1.31	1.43
19.06.97	0	M		0.71		0.87	1.48
26.06.97	0	M		0.86		1.08	0.93
13.06.97	0	Y		1.22		1.88	
19.06.97	0	Y		0.95		1.29	
26.06.97	0	Y		1.00		1.00	
13.06.97	10	M		3.16		1.48	1.34
19.06.97	10	M		3.78		1.20	1.26
26.06.97	10	M		4.87		1.24	0.94
13.06.97	10	Y		3.26		1.99	
19.06.97	10	Y		1.78		1.51	
26.06.97	10	Y		1.85		1.16	
13.06.97	20	M		6.39		1.74	1.35
19.06.97	20	M		8.49		1.20	1.45
26.06.97	20	M		7.31		1.40	0.93
13.06.97	20	Y		4.28		2.35	
19.06.97	20	Y		3.38		1.74	
26.06.97	20	Y		2.44		1.30	
13.06.97	40	M		9.95		1.67	1.62
19.06.97	40	M		10.90		1.40	1.38
26.06.97	40	M		12.10		1.44	0.94
13.06.97	40	Y		4.07		2.70	
19.06.97	40	Y		3.78		1.93	
26.06.97	40	Y		4.00		1.35	

Appendix 8 (Continued)

M = mature Y = young leaves

Sampling date	S treatment (kg/ha)	Plant Tissue	Total S (mg/g DW)	Sulphate (mg/g DW)	Protein S (mg/g DW)	Glutathione (µmol/g DW)	Glutathione Ratio (Y/M)
24.06.98	0	M	2.32	0.77	1.55	0.27	1.44
02.07.98	0	M	2.62	0.92	1.70	0.87	1.13
08.07.98	0	M	2.69	0.48	2.21	1.48	1.31
24.06.98	0	Y	2.71	0.34	2.37	0.39	
02.07.98	0	Y	2.88	0.63	2.25	0.98	
08.07.98	0	Y	3.30	1.00	2.30	1.94	
24.06.98	5	M	2.95	0.54	2.41	0.36	1.36
02.07.98	5	M	3.30	1.09	2.21	0.95	1.28
08.07.98	5	M	2.88	0.97	1.91	1.65	1.27
24.06.98	5	Y	3.02	0.43	2.60	0.49	
02.07.98	5	Y	3.78	1.10	2.68	1.22	
08.07.98	5	Y	3.81	1.34	2.46	2.10	
24.06.98	10	M	3.23	1.01	2.22	0.40	1.05
02.07.98	10	M	4.61	1.92	2.68	1.45	1.31
08.07.98	10	M	3.78	1.51	2.26	1.96	
24.06.98	10	Y	3.58	0.91	2.68	0.55	
02.07.98	10	Y	5.22	2.31	2.91	1.52	
08.07.98	10	Y	4.96	2.26	2.70	2.56	
24.06.98	20	M	4.99	2.47	2.52	0.34	1.59
02.07.98	20	M	6.05	3.47	2.58	1.30	1.24
08.07.98	20	M	5.92	3.39	2.53	1.95	1.40
24.06.98	20	Y	4.13	1.53	2.60	0.54	
02.07.98	20	Y	6.66	6.55	0.10	1.61	
08.07.98	20	Y	7.33	4.96	2.37	2.73	
24.06.98	40	M	7.20	4.67	2.53	0.42	1.62
02.07.98	40	M	9.63	6.56	3.07	1.16	1.20
08.07.98	40	M	10.18	7.37	2.81	1.98	1.04
24.06.98	40	Y	6.34	4.22	2.12	0.68	
02.07.98	40	Y	9.38	6.56	2.82	1.39	
08.07.98	40	Y	10.50	7.95	2.55	2.06	
24.06.98	80	M	13.28	12.35	0.93	0.51	1.75
02.07.98	80	M	13.50	10.14	3.36	1.60	1.10
08.07.98	80	M	17.18	13.38	3.81	2.63	1.05
24.06.98	80	Y	9.22	8.61	0.61	0.89	
02.07.98	80	Y	12.06	9.44	2.62	1.76	
08.07.98	80	Y	13.28	10.75	2.53	2.76	

Appendix 8 (Continued)

M = mature Y = young leaves

Sampling date	S treatment (kg/ha)	Plant Tissue	Total S (mg/g DW)	Sulphate (mg/g DW)	Protein S (mg/g DW)	Glutathione (µmol/g DW)	Glutathione Ratio (Y/M)
03.06.99	0	M	2.21	0.29	1.92	0.70	2.54
10.06.99	0	M	1.82	0.11	1.71	0.49	1.33
16.06.99	0	M	1.98	0.09	1.89	0.52	1.33
23.06.99	0	M	1.76	0.07	1.69	0.52	1.08
03.06.99	0	Y	3.26	0.48	2.78	1.78	
10.06.99	0	Y	2.05	0.13	1.91	0.65	
16.06.99	0	Y	2.14	0.11	2.03	0.69	
23.06.99	0	Y	1.86	0.11	1.75	0.56	
03.06.99	5	M	2.30	0.34	1.96	0.85	2.51
10.06.99	5	M	1.98	0.12	1.87	0.66	1.29
16.06.99	5	M	2.05	0.09	1.96	0.49	1.14
23.06.99	5	M	1.79	0.13	1.66	0.45	1.51
03.06.99	5	Y	3.94	0.72	3.21	2.13	
10.06.99	5	Y	2.05	0.21	1.84	0.85	
16.06.99	5	Y	2.14	0.21	1.93	0.56	
23.06.99	5	Y	2.30	0.32	1.99	0.68	
03.06.99	10	M	2.62	0.40	2.22	0.93	2.80
10.06.99	10	M	2.56	0.33	2.23	0.83	1.20
16.06.99	10	M	2.18	0.11	2.06	0.52	1.42
23.06.99	10	M	2.18	0.38	1.79	0.44	1.34
03.06.99	10	Y	4.77	1.24	3.53	2.60	
10.06.99	10	Y	2.78	0.74	2.05	1.00	
16.06.99	10	Y	2.53	0.31	2.21	0.74	
23.06.99	10	Y	2.69	0.80	1.89	0.59	
03.06.99	20	M	3.97	1.25	2.72	1.11	2.41
10.06.99	20	M	3.10	0.74	2.37	0.93	1.33
16.06.99	20	M	3.65	1.12	2.53	0.74	1.34
23.06.99	20	M	2.98	0.93	2.05	0.62	1.15
03.06.99	20	Y	6.18	2.27	3.90	2.67	
10.06.99	20	Y	3.36	1.12	2.24	1.24	
16.06.99	20	Y	3.74	1.15	2.59	0.99	
23.06.99	20	Y	3.62	1.38	2.24	0.71	
03.06.99	40	M	10.91	9.70	1.22	1.10	2.44
10.06.99	40	M	9.41	6.50	2.91	1.40	1.20
16.06.99	40	M	9.38	6.94	2.43	0.85	1.35
23.06.99	40	M	7.26	5.15	2.11	0.58	1.33
03.06.99	40	Y	7.74	3.74	4.00	2.68	
10.06.99	40	Y	5.73	3.33	2.40	1.68	
16.06.99	40	Y	7.07	4.48	2.59	1.15	
23.06.99	40	Y	6.40	4.00	2.40	0.77	
03.06.99	80	M	11.33	9.47	1.86	1.39	2.02
10.06.99	80	M	13.41	12.70	0.70	1.30	1.19
16.06.99	80	M	13.38	10.27	3.10	0.92	1.21
23.06.99	80	M	13.47	12.61	0.86	0.76	1.28
03.06.99	80	Y	8.77	4.03	4.74	2.81	
10.06.99	80	Y	6.94	4.99	1.95	1.55	
16.06.99	80	Y	9.22	6.62	2.59	1.11	
23.06.99	80	Y	13.34	12.10	1.25	0.97	