PROJECT REPORT No. 272

EVALUATION OF CRITICAL PHASES OF SULPHUR SUPPLY FOR OPTIMUM YIELD AND QUALITY OF WHEAT

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EVALUATION OF CRITICAL PHASES OF SULPHUR SUPPLY FOR OPTIMUM YIELD AND QUALITY OF WHEAT

by

M J HAWKESFORD, R V PALMER, F J ZHAO AND S P McGRATH

IACR Rothamsted, Agriculture and Environment Division, Harpenden, Hertfordshire, AL5 2JQ

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CONTENTS

ABSTRACT

This project combined an agronomic study of optimum timing for S-fertilizer application with physiological and biochemical approaches to investigate sulphur metabolism in wheat. The major applied aim was to determine the optimum window for spring S-application and to provide this information as a guideline for levy payers.

In field trials undertaken in two successive years, S was applied to a winter wheat crop on a known deficient site at 4 different times throughout the spring/early summer seasons. S-application (30 kg/ha) as sulphate (gypsum) to the soil had a remedial effect of increasing yield by 30% in 2000 and by 40% in 2001. Application was effective when applied between March and May (GS 13 to 31). Application after GS 39-59 in June failed to increase grain yield.

Application of S-fertilizer also influenced grain S-concentrations, irrespective of application date (March to June, GS 13-59). Analysis of the grain from all plots receiving S-application indicated that grain S concentration was increased from 0.83 to around 1.2 or from 0.99 to 1.57 mg g^{-1} DW in 2000 and 2001, respectively. Grain nitrogen was not affected by S-fertilizer application, however N:S ratios were decreased from around 25 to below or equal to 17 by S-application. A direct consequence of the S-concentration was a modification of the protein profiles and the amino acid composition.

In glasshouse experiments in which wheat was grown on nutrient solution and continuously supplied with S at five rates, grain yield was maximal at supply rates of 0.1 mM ('adequate') to 5 mM ('substantially well-fertilized'), but decreased at rates above this. Grain sulphur ranged from 2.1 to 2.9 mg g^{-1} over a range of S-supply from adequate (0.1 mM in solution) to a substantial excess (20 mM). These high S-concentrations most likely result from the continuous supply of S throughout all growth stages which is beneficial compared to the single dose usually applied in the field. S is required for optimal vegetative development (hence a requirement for early season application) and for grain S accumulation (which may require a subsequent application). In all cases unassimilated inorganic S was present in the grain, and this greatly increased with increasing levels of supply. This represents an un-exploited resource, potentially available to improve quality parameters.

SUMMARY

This project had dual aims of answering the practical question concerning the optimum timing for sulphur application to winter wheat crops in the UK, and additionally expanding the underpinning knowledge of the basis of sulphur utilization of in wheat.

Key objectives were:

- Determine the optimum window for spring sulphur application and whether early season applied S can adequately supply S-needs for grain.
- Determine whether increased S fertilizer results in increased yield and grain quality
- Determine importance of post-anthesis S supply

Background

Sulphur (S) deficiency in crops has only recently become widespread. Previously, sufficient S to meet crop requirements was obtained from the frequent incidental additions of S to soils when N and P fertilizers, such as ammonium sulphate and single superphosphate, were applied. Industrial pollution as a result of coal combustion also contributed substantial amounts of S for plant needs by aerial deposition. Over the last two decades, however, there has been a fundamental shift in the S balance toward deficit in agricultural systems for several reasons. High analysis N and P fertilizers have gradually replaced traditional ones that contain S. In addition, yields of agricultural crops have increased markedly, and in some cases more than doubled, during the last two decades, resulting in increased removal of nutrients, including S, from soils. One of the most important reasons behind the increasing S deficiency in the UK has been the massive decrease in the inputs of S from atmospheric deposition since the early 1970s. In the UK, total emissions of sulphur dioxide decreased from 3.26 million tonnes of S in 1970 to 0.59 million tonnes in 1999. The decreasing trend in sulphur dioxide emissions, as required by international agreements, is likely to continue well into the next decade. For example , the emission target for the UK in the year 2010 is 0.31 million tonnes of S. Although wheat has a relatively low requirement for S, deficiency of S has become increasingly widespread in recent years, and UK farmers must consider appropriate management practices for sulphur fertilizers for optimum yield and quality.

Field trials

Specific aims

- To determine whether the yield benefit of S-application in a field trial is dependent on application date by comparing applications between GS 15 and 58/59.
- To determine how quality parameters such as grain S-concentration, N:S ratios and protein quality are influenced by the S-application date

Introduction

Guidelines for S-fertilizers are the application of between 15 and 25 kg/ha of S for wheat. Sulphur fertilizers are available as elemental sulphur or as the sulphate salt (e.g. ammonium sulphate and gypsum) or in liquid formulations. Elemental sulphur has advantages in providing a long-term supply of S, with a slow release of S dependent upon the rate of breakdown of particles and the oxidation of the elemental-S. This is usually applied in the autumn, however the slow release may not provide sufficient available S when demand is great. Sulphur applied as the sulphate salt is immediately available but may be susceptible to leaching following excessive rainfall.

Sulphur is required throughout the entire growth period of cereals, and particularly for optimum grain yield and quality. Previous studies have shown that sulphur increases cereal yields in many areas of the UK (HGCA project report No 115) and enhances grain quality strongly. However, late foliar applied S is not efficiently utilised (HGCA project report No 197) and it is likely from the small amount of evidence available, that only a high level of S applied to the soil, early in the season, will provide sufficient S for the grain.

Practical approaches

All field trials in the current project were conducted at Woburn Farm, Bedfordshire during the growth seasons of 1999-2000 and 2000-2001. The experimental design consisted of five treatments, including a control (no S) and four plus sulphur treatments applied in the first week of the months between March and June. Sulphur was supplied as gypsum (calcium sulphate) at a rate of 30 kg S ha^{-1} at T1 (March), T2 (April), T3 (May) or T4 (June). The growth stage at each application time was recorded. Each treatment was replicated four times in a randomised block design. Sampled plants were washed with de-mineralised water, dried, weighed and separated into fractions according to tissue type: leaf, stem or ear. Tissues were freeze-dried and re-weighed prior to being milled to a powder for analysis. Samples were analysed for ion concentrations (specifically sulphate) by ion chromatography, total S by inductively-coupled plasma atomic emission spectroscopy (ICP-AES), and total N

by a Dumas combustion method (LECO CNS Analyser). Amino acid composition of the final grain was determined and by high performance liquid chromatography (HPLC) separation of total hydrolysates.

Results

Evaluation of optimum timing for S-application during the period from March to June in a field trial at Woburn.

- In the field trial, S-applications from March to May were equally beneficial for yield compared to the zero S controls in both the 2000 and 2001 harvests. The crops were at growth stage 15 or 13/15 in 2000 and 2001 respectively for the March application and at GS 31 and 30 in 2000 and 2001, respectively at the time of the May application. Yields in 2000 were generally greater than 2001, and the probable greater carbohydrate concentration would have had a dilution effect on grain S and N pools.

- Approximate 30% or 40 % yield benefits were achieved at the first three application timings in 2000 and 2001, respectively. However in both years, the June application gave yields equivalent to the zero-S fertilizer treatment.

- Organic S in the grain also increased with the S-application, and applications from March to June were equally effective in this respect. There was an indication that the later applications were marginally the most effective resulting in a higher grain S-concentration. Inorganic S as sulphate was generally a small fraction (around 10% of the total). Late application of fertilizer (June) resulted in a small increase in sulphate (to 20%). This inorganic sulphate represents S-uptake that is not incorporated into protein and is therefore of no quality benefit. The development of varieties, or appropriate fertilizer management practices to reduce the size of this pool would be beneficial.

- S-offtake in the grain is the product of grain yield and S-concentration. Greatest offtake was observed in the plots supplied with S from March to May.

- Grain-N was not influenced by the timing of S-application. Higher grain N was observed in 2001 compared to 2000, coincident with the lower yields and illustrating the dilution effect referred to above.

- N:S ratios reflected the S-concentration, given that grain N was relatively unaffected by the timing treatment. The N:S ratio was greatest (~25) in the plots receiving no S. The N:S ratio was below 17 in all

plots receiving S. This parameter would have failed to assess S-deficiency in the crop treated with the late sulphur application.

- Samples of grain (2001 samples) from the plots receiving no S-fertilizer and S fertilizer in either May or June were hydrolysed and the total amino acid compositions were determined. Notably the concentration of the S-containing amino acids, methionine and cysteine (as cysteine and cystine), were reduced by at least 50% in the zero S-fertilizer plots. Surprisingly, several other amino acids showed decreased relative abundance in the S-deficient grain (for example valine, tyrosine, threonine, serine, proline, lysine), whereas the relative abundance of aspartic acid was increased, indicating a complex underlying perturbation of grain composition caused by the S-nutritional status. A direct consequence is a modification of the grain protein profile is illustrated in the studies presented in the next section.

- Growth rates and sulphate pools were monitored in samples collected from the field trials during 2001. Growth rates (biomass of all above ground fresh tissue) were decreased in plots prior to receiving S-application. In the case of application in June biomass never recovered. In the case of application in May, biomass recovered within about 1 month. Biomass was almost double by the beginning of July (GS 62) in plots receiving S-fertilizer in March-May compared to the zero plots and the June application plots.

- Sulphate pools fluctuated widely and were substantially influenced by fertilizer application. Following application tissue sulphate concentrations increased, particularly after April, May and June S application. Tissue sulphate concentrations following the March application only increased substantially in July and were otherwise relatively constant.

Greenhouse-based physiological experiments

Specific aims

- What is an optimal rate of continuous S-supply for greenhouse, pot grown spring wheat (Axona), and what is the effect of a range of S-supply rates on growth rates, yields and final harvest S-concentrations?
- How is S allocated between different biochemical pools of S in the various parts of a wheat plant when grown at different S-application rates?
- How important is the post-anthesis S supply to grain yields and S-concentrations in spring wheat?
- Is assimilation of sulphate into organic-S occurring in grain tissues or only in vegetative tissues from where it must be transported to the grain?

Introduction

Preliminary studies (see HGCA Project Report 217) had shown that the level of S supplied to plants throughout the vegetative growth phase substantially affected yield of the grain and S concentrations in the grain. Furthermore the removal of S-supply at the time of ear emergence resulted in almost no new S accumulating in the grain in plants previously supplied at minimal S-levels. In this project the hypothesis that with a greater S-supply, more substantial reserves could be accumulated in the plant, and that these could be utilised during grain filling was tested. The information obtained will be assessed in conjunction with field experiment data to improve the recommendations for application time and rate for UK cereal crops.

Approaches

A number of pot based experiments were conducted in the greenhouse. Plants were grown on a sand/perlite matrix and watered on alternate days with either de-ionised water or nutrient solution of defined composition, with different sulphate concentrations. Plants were sampled at regular intervals, weighed and processed for further analysis. Samples were analysed for ion concentrations (specifically sulphate) by ion chromatography, total S by ICP-AES, and total N by LECO analysis. Thiols and free amino acid concentrations were determined by HPLC.

Results

Analysis of physiological consequences of S-application rates ranging from adequate to excessive.

The effect of S-applications higher than usually recommended was investigated in a greenhouse pot experiment to determine optimum quantity of application and fate of excess application.

- Plants supplied with both 1 mM and 5 mM showed the highest growth rates (height and dry matter). Plants below and above these S-application rates grew sub-optimally due to deficiency or excess supply.

- Plants supplied with 0.1, 1 and 5 mM sulphate had the greatest grain yield. At 20 and 50 mM sulphate, yields were reduced. Tillering/number of ears was of greater significance for yield than ear weight.

- Grain protein profiles were influenced by S-application. At low S-application, the S-rich proteins were less abundant, whilst the S-poor proteins, were more abundant. This is likely to have an influence on dough qualities due to reduced cross-linking between proteins (decreased cysteine content), and result in poorer nutritional quality (low content of the essential amino acid, methionine).

- Tissue sulphate-concentrations were influenced by S supply, increasing about 2-fold when plants were supplied between 0.1 and 20 mM (a 200-fold range) sulphate in the nutrient solution. It was clear, however, that a regulatory mechanism was limiting accumulation. This regulation failed when plants were supplied with 50 mM sulphate, with a large accumulation of sulphate occurring, in parallel with the decreased yield. The tissue organic-S fraction was more constant, but also was maximal in the range of 1-20 mM external sulphate supply.

- At high S-application rates, sulphate accumulated with time and could exceed the pool of organic S at GS57. This is a considerable reserve of inorganic S, which has the potential for utilisation during grain filling.

The importance of post anthesis sulphur supply

An experiment was set up with plants grown at the two optimal S-application rates (1 and 5 mM), in which plants were continuously fed with S until anthesis, whereupon S-supply was terminated at set intervals. The effects on final grain yield, S-concentration and protein quality parameters were determined. The data obtained showed little influence of post-anthesis S supply on grain yield, however grain-S content was marginally compromised when S-supply to the roots was terminated at anthesis.

The activity of ATP sulphurylase, a key enzyme of the S assimilatory pathway was assessed. This showed a peak of activity 5-15 days post ear emergence and just following anthesis. Localisation of sulphate pools within the ear tissues indicate that assimilation of sulphate into cysteine may occur throughout the grain tissue, and therefore that delivery of sulphate is an important factor in determining grain S-concentrations. In most analyses, some sulphate was found in grain tissues, indicating that there is a potential to boost accumulation into protein if mechanisms were introduced to assimilate this.

An overall model may be proposed in that S-availability early in the spring determines yield, probably by affecting the yield components such as tiller formation. Assimilation of applied-S into organic S is effective throughout the growing season, including the period following anthesis, and therefore for this parameter, timing is not so critical. Excess applied S is not accumulated in plant tissue to any great extent, but will increase the availability in the soil for the duration of the growing season.

Summary of implications for levy payers

The major conclusions are:

1. At the Woburn site, S-fertilizers added to the soil, increased winter wheat yields significantly by up to 30% and 40 % in the 2000 and 2001 seasons, respectively.

2. Spring applications were effective, within a wide window between early March and early May.

3. Applications as late as June (GS 38/43 to GS 58/9), generally around anthesis, are too late to restore yield losses, although these are effective at boosting grain S-concentrations.

4. The field data are representative for the two years in the study (2000 and 2001). In the case of exceptionally dry years the window for optimum application may be narrower.

5. S is required for optimal vegetative development and hence there is a requirement for early season application. S is also required for grain S accumulation and therefore crop quality may benefit from a subsequent S-application.

TECHNICAL DETAIL

Part I: Field trials evaluating the optimum timing for sulphur-fertilizer addition

INTRODUCTION

In areas of endemic sulphur deficiency, a policy of appropriate application of S-fertilizer should be adopted. It is generally accepted that at least 15 kg ha^{-1} is required for winter wheat (HGCA Topic Sheet No. 31). An outstanding management issue concerns the optimum timing for application. **Specifically the question of whether a single early season fertilizer application to the soil provides sufficient S for the crop**, or whether a late application, which is likely to be less susceptible to leaching, can be equally beneficial.

In areas of marginal deficiency or areas in which the problem of S-deficiency is a new one, S-application is not necessarily a standard practice. In this case testing/early diagnosis is desirable followed by a strategy of remedial application of S-fertilizer. **A major issue is whether a later remedial application is beneficial to the crop.**

The Rothamsted experimental farm at Woburn, which has a light sandy loam soil and low nutrient retention characteristics, is especially useful for this type of study, and has shown strong symptoms of S-deficiency in wheat in recent years (see for example: Blake-Kalff *et al.*, 1998, 2000, 2001; Zhao *et al.*, 1996 and the HGCA Project Report No. 217)

MATERIALS AND METHODS

Field Experiments

All field trials were conducted at Woburn Farm, Bedfordshire during the growth seasons of 1999-2000 and 2000-2001. Nitrogen was applied at a rate of 180 kg ha^{-1} in the spring. The experimental design consisted of five treatments, including a control (no S) and sulphur treatments applied at different times between March and June. Sulphur was supplied as gypsum (calcium sulphate) added to the soil at a rate of 30 kg S ha^{-1} at T1 (1^{st} week of March), T2 (1^{st} week of April), T3 (1^{st} week of May) or T4 (1^{st} week of June). Each treatment was replicated four times in a randomised block design. Twenty 10 m by 4 m plots were set out in four blocks of 5 plots with each block containing one of each treatment with 2 m paths between blocks and 1 m paths between plots.

All plots were sown on the 14th September 1999 at Butt Close or the 3rd October 2000 on Lansome field at Woburn with *Triticum aestivum cv*. Hereward, at 275 seeds m^{-2}. No other S-containing agro-chemicals were used on this site during the study.

Plants were sampled at different growth stages as indicated in the relevant figures. The area sampled was 0.25 m^2 at each sampling time. The plants were washed with de-mineralised water, dried using absorbent paper, weighed for fresh weight, separated into fractions according to tissue type: leaf, stem or ear. Plant material was freeze-dried and re-weighed for dry weights prior to being milled to a fine powder prior to analysis. Samples were analysed for ion concentration (specifically sulphate), free amino acids, total S and total N.

Chemical Analysis

Total sulphur concentration of plant tissues

Total S was measured by digesting 250 mg of ground lyophilised plant 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 (ICP-AES). In house standards of wheat straw and wheat flour were analysed alongside the samples for quality assurance.

Inorganic sulphate analysis

Sulphate 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 AS9SC guard column coupled to an AS9SC separation column. Insoluble S, representing mainly protein S, was calculated by subtracting the concentrations of soluble S (i.e. sulphate) from the concentration of total S.

Total nitrogen concentration of plant tissues

Total N was measured using a Dumas combustion method. Ground lyophilised and oven dried wheat flour samples (0.2 g ± 0.001 g) were measured using a LECO CNS-2000 Elemental Combustion Analyser (LECO Corporation, Michigan, USA). A 0.2 g sample of Rothamsted in-house wheat flour standard was analysed together with each batch of samples for quality assurance.

Total amino acid analysis by HPLC (high performance liquid chromatography)

The ground wheat samples were solubilised in 0.5% HCOOH (10mg/ml). In preparation for gas-phase hydrolysis, the dissolved samples were dried under vacuum together with EDTA. 0.2 ml 6M HCl was added to the hydrolysis vessels containing a maximum of 9 samples. The tubes were successively evacuated and aerated using N_2, leaving an end-pressure of 20-40 mm Hg. The vessel was finally placed on the heating block for 22 hours at 115°C. Following hydrolysis, the free amino acids were aerated, dried under vacuum, buffered at pH 12 and finally coupled to phenylisothiocyanate. The analysis was carried out on a RP-HPLC using a Nova Pak C18 column. M. Stark and J. Schaller kindly performed analysis at the University of Bern, Switzerland.

Statistical analysis

Analysis of variance was performed on all data. Least significant differences (LSD) were calculated using Genstat (5^{th} Edition).

RESULTS

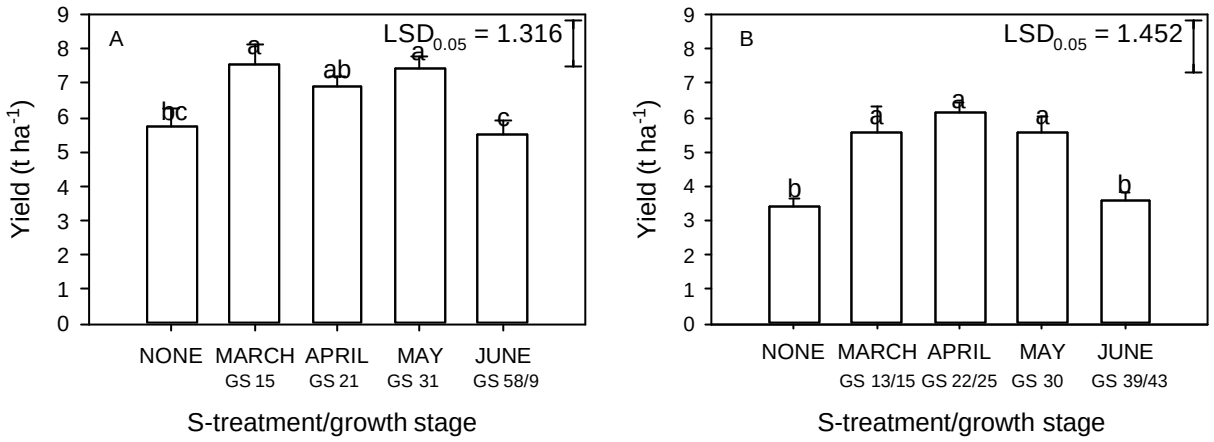


Figure 1. *Effect of timing of sulphur fertilizer application on yield. Final yield data from the experiments at Woburn in A: 2000 and B: 2001. Sulphur was applied as gypsum at 30 kg/ha at the stated times. Data are means of 4 replicate plots.*

Sulphur was applied to the winter wheat crop as gypsum (calcium sulphate), by hand, at four time points, with one treatment receiving no sulphur. Applications were on 5th March (GS 15), 6th April (GS 21), 9th May (GS 31) and 7th June (GS 58/59) in 2000 and on 15th March (GS 13/15), 9th April (GS 22/25), 2nd May (GS 30) and 1st June (GS 39/43) in 2001.

Final grain yield data are presented in Figure 1. The experimental site at Woburn has a light, sandy loam soil, and is well known as a site liable to sulphur deficiency. In both years, sulphur application in the period from March to May, (GS15 to 31) produced a significant yield response up to 30% in 2000 (Fig. 2A) and 40% in 2001 (Fig. 2B). Application in June (GS58/59) failed to elucidate this response.

S-applications are effective for obtaining a yield response in a deficient crop if applied between GS 13 and 31

Grain tissues were analyzed for their sulphur concentrations, both as inorganic, unassimilated sulphate and for total sulphur as determined by ICP-AES. Subtracting the inorganic sulphate S from the total analyses gives the fraction which is present principally as organic S. Figure 2 presents the fractions of sulphur in the sulphate-S and the calculated organic-S fraction in the grain tissues for each of the treatments in the two years of the trials. The largest fraction was always present as organic-S, and sulphate-S ranged between 3 and 20% of the total. The

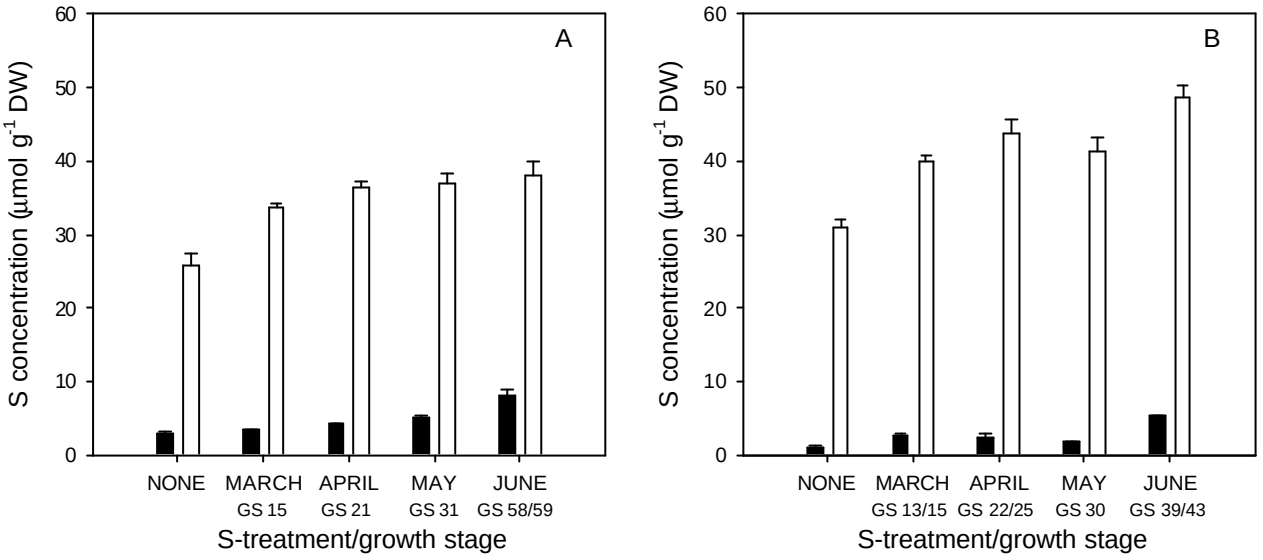


Figure 2. *Effect of timing of sulphur fertilizer application on grain sulphur pools at harvest. Data shown are from the field trials conducted at Woburn in A: 2000 and B: 2001. Solid bars: sulphate S and open bars: non-sulphate S (organic fraction). Sulphur was applied as gypsum at 30 kg/ha at the stated times in 2000 (A): March (GS15); April (GS21); May (GS31) and June (GS58/59) or in 2001 (B): March (GS15); April (GS22-25); May (GS30) and June (GS39/43). Data are the means of 4 replicate plots with error bars showing the standard error of the means.*

highest sulphate concentrations were found in the plots which received the later applications of sulphate, indicating that sulphate continued to be available to the plants to a much later date. The organic S concentration in the grain was increased by S-application by between 25 and 50% in 2001, with the largest increases occurring at the later S-applications.

Grain S concentrations were boosted by S-application in all plots, when applied between GS 15 and 58/9.

In 2001, the mean organic S concentrations in the grain were 0.97, 1.25, 1.37 and 1.5 mg/g for the zero application, March, April, May and June applications, respectively. All treatments receiving an application of S had values above the suggested critical value of 1.2 mg/g (Randall and Wrigley, 1986). An implication of this

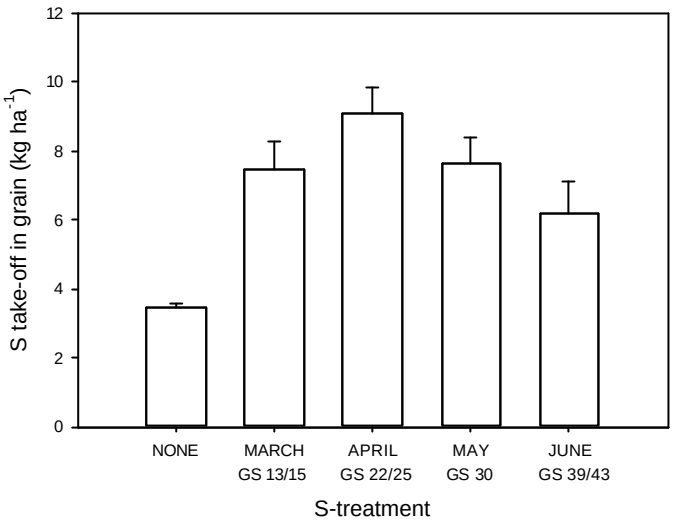


Figure 3. *Sulphur offtake in grain as influenced by timing of application of S-fertilizer at Woburn in 2001. Data are the product of the grain yields (Fig. 1B) and the total sulphur concentration of the grain (sulphate S plus non-sulphate S, Fig. 2B). Sulphur was applied as gypsum at 30 kg/ha at the stated times: March (GS15), April (GS22-25), May (GS30) and June (GS39/43). Data are the means of 4 replicate plots with error bars showing the standard error of the means.*

result is that analysis of final grain from the June-S application treatment would not indicate that the decreased yield was due to S-deficiency.

The S-concentrations in grain is not a reliable indicator of the S-status of wheat receiving a late S-application

With the information on the final grain yields and the sulphur concentrations in the grain it was possible to calculate the total offtake of S from the plots. This is shown in Fig. 3. Actual offtake would be much greater, as this figure does not include the straw fraction. Maximum grain offtake was in the plots with the April application at around 9 kg S per hectare. This contrasts with the plots that received no S-fertilizer, for which the grain offtake was around 3.5 kg ha^{-1}.

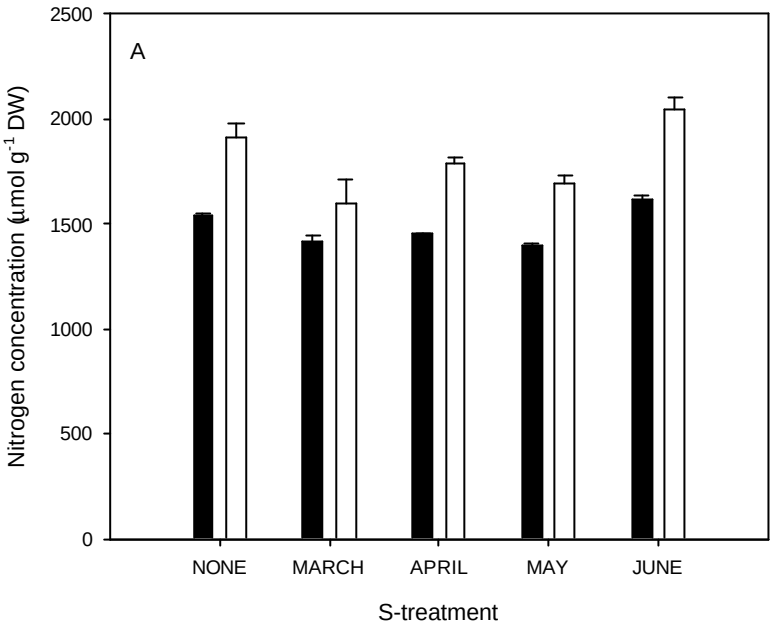


Figure 4. *Nitrogen concentrations in grain as influenced by timing of application of S-fertilizer at Woburn. Data shown are for 2000 (solid bars) and 2001 (open bars). Data are the means of 4 replicate plots with error bars showing the standard error of the means.*

The timing of S-application had no significant effect on the N-concentration of the grain (Fig. 4.). The N-concentration of the grain collected in 2001 was around 20% greater than 2000, in contrast to the yields which were reduced. This may have been due to a dilution effect by an increased carbohydrate content.

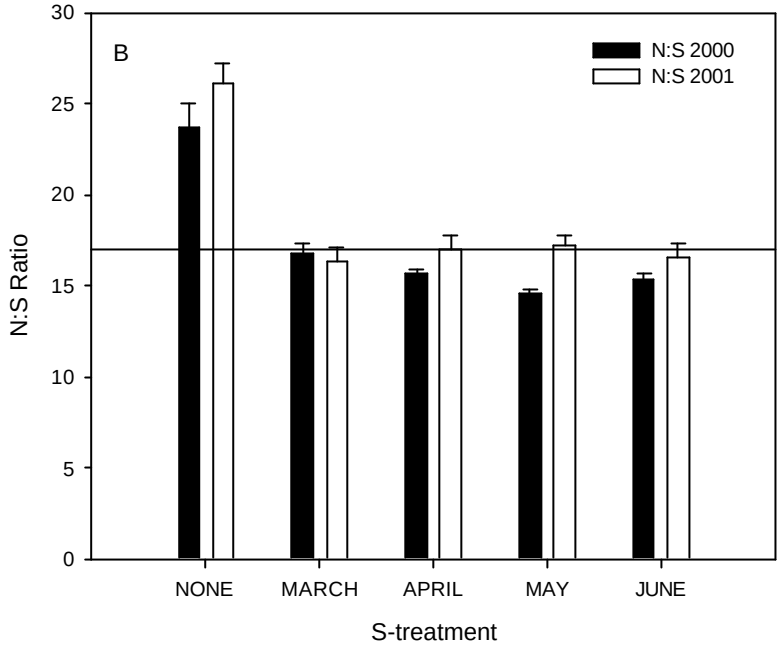


Figure 5. *Nitrogen: Sulphur ratios of grain as influenced by timing of application of S-fertilizer at Woburn. Data shown are for 2000 (solid bars) and 2001 (open bars) are the ratio of the grain total N (Fig. 4) and combined sulphate S plus non-sulphate S (calculated from Fig. 2). The horizontal bar indicates the critical value of a ratio of 17. Data are the means of 4 replicate plots with error bars showing the standard error of the means.*

The N:S ratios were obtained by combining the data for total S and total N concentrations in the grain. The N:S ratio is an indicator of grain quality and a critical value of 16-17 has been suggested (HGCA Research Review 30). As can be seen in Fig. 5, all grain samples from plots, which had received S-fertilizer, have N/S ratios that fall below this value. The control plots, which received no S-fertilizer, had grain N:S ratios of around 24-26, clearly indicative of deficiency. As with the measurements of total S, this parameter fails to diagnose that S-deficiency (as influencing yield rather than quality) was apparent in the crop harvested from the June-applied S treatment.

All grain from plots receiving S-fertilizer, irrespective of timing, had a N:S ratio below 17

Grain samples from the control (no S-application) and the plots receiving S-fertilizer in either March (early application, at GS15) or June (late application at GS39-43) from the 2000-2001 trial were hydrolyzed and subjected to total amino acid analysis. Composition of the extractable protein fraction of the grain is reported in Table 1. Compared to the total N, this represents only a fraction of the total protein. The concentration of most amino acids in the fraction extracted and analyzed were decreased in the grain from the plots receiving no

sulphate compared to the S-fertilized plots. As there was no significant difference in total N between these plots (Fig. 4), the extractable protein fraction was lower in this grain sample. As expected the decrease in the S-containing amino acids, cysteine and methionine was proportionately the greatest in the S-deficient plot.

Amino acid composition of the grain was influenced by S-nutrition

In order to determine the underlying physiology of these growth and S-concentration responses, plants in the experimental plots were assessed throughout the growing season in 2001 and samples were taken for analysis (Fig. 6). Growth data were measured as the estimated biomass (as fresh weight of all above ground tissue) between 3rd April (GS22-25) and 28th June (GS59-62) and is presented in Fig. 6A. In all cases except the June application there was a positive response to the applied S in the above ground biomass. No discernable difference was observed for the application in March and April, (T1 and T2, respectively), the biomass of the May-application plots (T3) increased within 15 d following S-application to a level comparable to the T1 and T2 treatments. No response was seen following the T4 (June application).

In summary, no significant difference at the fresh weight biomass (at GS59-62) was apparent for the treatments in which S was supplied in March, April or May, however substantially reduced biomasses were observed in the zero-S application and in the June application.

In parallel with the measurements of biomass, the sulphate concentrations in the tissues (whole plant) were measured (Fig. 6B). In all cases, following the application of S-fertilizer, a transient increase in the tissue free sulphate was observed. In the March (T1) application tissue sulphate concentrations increased from approximately 20 to 30 µmol per gram dry weight by the 3rd April. Following application at T2, T3 and T4, tissue sulphate concentrations reached 90, 95 and 140 µmol per gram dry weight, respectively, indicating effective uptake of the applied sulphur in these treatments. Tissue concentrations of sulphate in the March application treatment also rose at the final sampling point, indicating that sulphate was still available in the soil, and that there appeared to be a developmental influence on uptake. It is likely that by the 28th June (GS59/62) there was an increased demand for sulphur for grain production and that this drove the observed increased uptake.

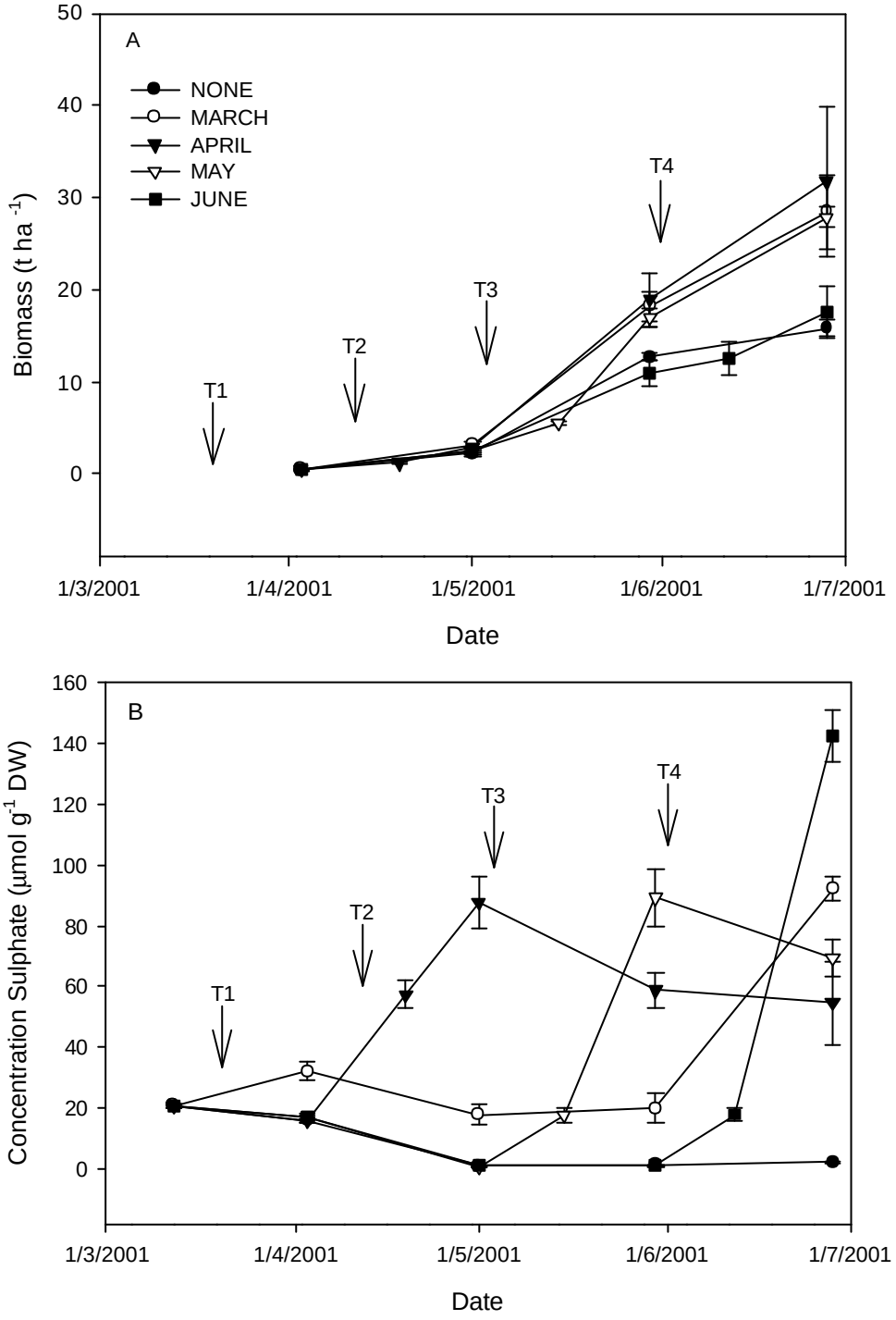


Figure 6. *Effect of S application date on mean biomass (A) and on tissue sulphate pools (B) in the 2001 field trial. Timing is relative to 1st sampling date. T1-T4 are the S-application dates. No application of S: closed circle; March (GS 15) application: open circle; April (GS22-25) application closed triangle; May (GS30) application; open triangle; June (GS39-43) application: closed square. Data are means of 4 replicate plots ±SE.*

Table 1. Total amino acid composition determined from hydrolyzed samples of grain from the plots receiving no sulphur or sulphur in May or June. Data are means ±SE for 3 replicates. C-C refers to cystine (cysteine dimer) and C- is a breakdown product of this. Cysteine may be approximated as the combination of these.

Concentration ($\mu mol\ g^{-1} DW$)	**Sulphate supplied**		
	Zero	**May**	**June**
Alanine	13.1 ± 1.75	21.8 ± 4.74	20.1 ± 5.25
Arginine	9.36 ± 1.40	15.5 ± 4.96	12.6 ± 2.90
Aspartic acid	28.3 ± 3.90	20.6 ± 5.28	20.5 ± 5.16
C-	0.27 ± 0.03	0.78 ± 0.15	0.80 ± 0.17
C-C	0.61 ± 0.07	1.50 ± 0.24	1.47 ± 0.23
Glutamic acid	81.5 ± 11.9	101 ± 16.7	99.0 ± 14.8
Glycine	17.5 ± 2.18	30.3 ± 6.48	27.7 ± 8.25
Histidine	4.69 ± 0.60	8.33 ± 1.77	7.87 ± 2.04
Isoleucine	7.86 ± 0.97	13.0 ± 2.10	13.2 ± 2.98
Leucine	15.3 ± 1.86	26.2 ± 4.48	25.4 ± 5.36
Lysine	5.46 ± 0.67	9.10 ± 2.53	8.54 ± 2.59
Methionine	1.88 ± 0.21	4.44 ± 0.84	3.15 ± 0.28
NH_3	21.0 ± 0.35	21.5 ± 0.98	21.8 ± 1.23
Phenylalanine	10.1 ± 1.37	14.4 ± 2.37	14.4 ± 2.53
Proline	33.6 ± 4.66	46.8 ± 5.41	46.3 ± 6.56
Serine	13.8 ± 1.73	22.5 ± 3.64	21.4 ± 4.62
Threonine	7.93 ± 0.94	13.2 ± 2.19	12.5 ± 3.06
Tyrosine	2.85 ± 0.62	5.67 ± 1.37	4.39 ± 0.75
Valine	10.1 ± 1.19	18.7 ± 3.64	18.0 ± 4.65

Part II: Physiological analysis of sulphur-pools in wheat

INTRODUCTION

A series of trials was performed to evaluate the partitioning of S between inorganic and organic (principally protein) fractions in wheat under a variety of conditions of S-supply.

- One pot trial evaluated the effectiveness of a range of S-supply levels (0.1, 1, 5, 20 or 50 mM sulphate in nutrient solution) on growth and S-pools. The total S, amino acid, glutathione and sulphate concentrations were determined for wheat plants grown in sand/perlite culture and supplied with different concentrations of sulphate throughout the growth period. The range of S concentrations chosen focused on hypothetical adequate and excess rates of S-supply. The N:S ratio of the grain at all the different S supplied concentrations was determined and compared to the protein composition of the grain.

- A second experimental approach examined the impact of the removal of S-supply to the roots during the post anthesis period. The total S and sulphate concentrations in wheat were determined in plants grown in sand/perlite culture and supplied with sulphate via nutrient solution until set intervals during anthesis. Two nutrient S concentrations were used during this study chosen to be at the extremes of the optimum levels of S supply as determined in the first part of this chapter. The relationships between the protein S and sulphate pools in leaf and ear tissue, the N:S ratio of the grains and the protein composition of the final grains of the plants as influenced by the removal of the post anthesis S-supply were examined. The aim of this study was to assess the importance for gain production of a root S supply in the post anthesis period.

- Biochemical analysis of developing grain was performed in hydroponically-fertilized, pot-grown wheat. The activity of the sulphate assimilatory pathway in the grain was measured to evaluate the capacity of the grain to utilise available inorganic S and convert this into the protein fraction. The activity of the assimilatory pathway was assessed by measuring ATP sulphurylase activity, the first enzyme of the pathway.

All of these factors when examined collectively, will be used discuss utilisation of the S supplied with regard to crop yield and quality, and particularly the potential for increasing the reserves of S by increasing rates of supply during vegetative growth. The potential for manipulating S-use efficiency in crop plants, either by fertilizer management practices, or by the development of specific variety traits has been discussed (Hawkesford, 2000; Hawkesford and Wray 2000). Many previous studies have documented the effects of S-deficiency at low S-supply (Adiputra & Anderson, 1992; Adiputra & Anderson, 1995; Blake-Kalff *et al*., 1998; Fitzgerald *et al*., 1999; Zhao *et al*., 1996; Zhao *et al*., 1997).

MATERIALS AND METHODS

Growth of plants on sand/perlite

Triticum aestivum cv. Axona seed, a spring wheat variety, was used for all pot trials Seeds were sown in 20 cm pots containing 50/50 (v/v) mix of coarse horticultural sand (2EW, Petersfield products) and Silverperl™ graded horticultural perlite (William Sinclair Horticultural Ltd. Lincoln). Plants were raised under glass-house conditions at 12-16ºC with a photo-period of 16 hours provided by 1000 W high pressure sodium lamps. Plants were watered with 1 l of de-mineralised water on Tuesday, Thursday, Saturday and Sunday, or with nutrient solution on Monday, Wednesday and Friday, unless otherwise stated. Nutrient solution contained: 7 mM KNO_3, 1 mM KH_2PO_4, 1.7 mM $Mg(NO_3)_2$, 0.01 mM NaCl, 0.6 mM EDTA FeNa, 5 mM $Ca(NO_3)_2$, 2 mM $CaCl_2$, 1.9? µM Zn acetate, 49.0 µM H_3BO_3, 2.2 µM $Cu(NO_3)_2$, 1.6 µM ammonium molybdate, 19.7 µM $Mn(NO_3)_2$ and sulphate as $MgSO_4$ as required. All pots were free draining.

Determination of glutathione and cysteine by HPLC

0.1 g aliquots of plant material were added to 1.5 ml of 0.1 M HCl containing 0.1 g of acid washed polyvinylpoly pyrrolidone. After 1 h the samples were centrifuged at 10 000 g for 5 min, and 0.5 ml of the supernatant was filtered (0.22 µm). For derivation: 100 µl of 0.25 M 2-[cyclohexylamino]-ethanesulphonic acid (CHES) buffer at pH 9.4-9.8 was added to 100µl aliquots of extract to bring the samples to approximately pH 8. 70 µl of 10 mM dithiothreitol was added and incubated at room temperature for 1 hr. Subsequently, 10 µl of 25 mM mono-bromobimane was added and the sample was incubated at room temperature for 15 min in the dark. The reaction was terminated with 220 µl of 100 mM methanosulphonic acid. The samples were centrifuged at 10 000 g at 4°C for 20 min and then analysed by HPLC (Kontron™) on a Zorbax ODS 5µ column (Jones Chromatography, Mid Glamorgan, UK) with a gradient of 10-90% (v/v) methanol in 0.25% acetic acid (pH 4.9). Derivatives were detected fluorimetrically (excitation 380 nm, emission 480 nm).

Free amino acid analysis by HPLC

0.1 g fresh, liquid nitrogen ground, plant tissue was extracted in 2 ml of 70 % (v/v) ethanol. The sample was centrifuged at 10 000 g for 5 min and 1 ml of the supernatant was dried under vacuum. This was re-dissolved in 450 µl of 0.05 M ammonium acetate at pH 6.8 and 50 µl of α-amino butyric acid was added as an internal standard to the samples and filtered through 0.22 µm PTFE filters into 2 ml clear glass vials for analysis on a DIONEX™ HPLC. HPLC analysis of the amino acids on a Spherisorb 3 µm ODS2 analytical column used a gradient system, comprising two solvents: 0.05 M ammonium acetate, pH 6.8 and 0.1 M ammonium acetate with 50% (v/v) acetonitrile, pH 6.8.

Protein estimation

A dye-binding assay was used to determine the protein concentration of samples as laid out in the manufacturer instructions (Biorad, Hemel Hempsted). Bovine serum albumin was used as a standard and absorbance was determined at 595 nm.

Separation of protein from wheat grain by electrophoresis

Grain storage proteins were analysed according to the method of Shewry *et al* (1995). Lyophilised grain (single grain) was mixed with 1 ml of protein extraction buffer, consisting of 0.0625 M Tris-HCl, pH 6.8, 2 % (w/v) SDS, 1.5 % (w/v) dithiothreitol, 10 % (v/v) glycerol, 0.002 % (w/v) bromophenol blue. The samples were incubated at room temperature for 2 hr prior to heating at 95 °C for 2 min. The samples were cooled and centrifuged at room temperature for 5 minutes at 5 000 g. 20 µl of each sample was loaded onto a SDS-polyacrylamide gel for gel electrophoresis. A Tris-Borate gel electrophoresis system was used to separate high molecular weight subunits of wheat glutenin. Separating gel buffer consists of 15 % (w/v) Tris, 3.8 % (w/v) boric acid and 1 % (w/v) SDS with a pH of 8.9. Stacking gel buffer consists of 1.0 M Tris-HCl, pH 6.8, 10 % (w/v) SDS and 10 % (w/v) ammonium persulphate. Sample buffer consists of 6.55 ml stacking buffer (pH 6.8), 3.3 % SDS, 10 ml (v/v) glycerol and 1.54 % (w/v) dithiothreitol (100 mM final concentration). Running buffer consists of separating buffer diluted 10-fold. Acrylamide (40 % w/v), NN'-methylenebisacrylamide (2 % w/v) and N,N,N',N'-Tetramethylethylenediamine solutions were purchased from Sigma. Gels were stained with Coomassie Blue R250 to visualise proteins.

ATP sulphurylase enzyme assays

Approximately 0.2 g of fresh tissue was chopped and ground in a pestle and mortar with 3 ml of ice-cold extraction buffer before being transferred to vials and centrifuged in a bench top centrifuge (Sigma-Aldrich Co, Poole, Dorset, UK) for 10 minutes at 10000 g. The supernatant was removed and assayed for ATP sulphurylase activity. The reaction catalysed by ATP sulphurylase is:

$$SO_4^{2-} + ATP \leftrightarrow APS^{2-} + PPi$$

It is convenient to assay the enzyme by measuring the reverse reaction by adding excess pyrophosphate (PPi) and measuring the ATP formed. The assay is coupled to the luciferin/luciferase bioluminescence assay to measure ATP. An adenosine 5' – triphosphate (ATP) bioluminescent assay kit (FL-AA, Sigma-Aldrich, UK,) was used and the luminescence measured on a LKB Model 1250 Luminometer (Wallac Oy, Finland).

RESULTS

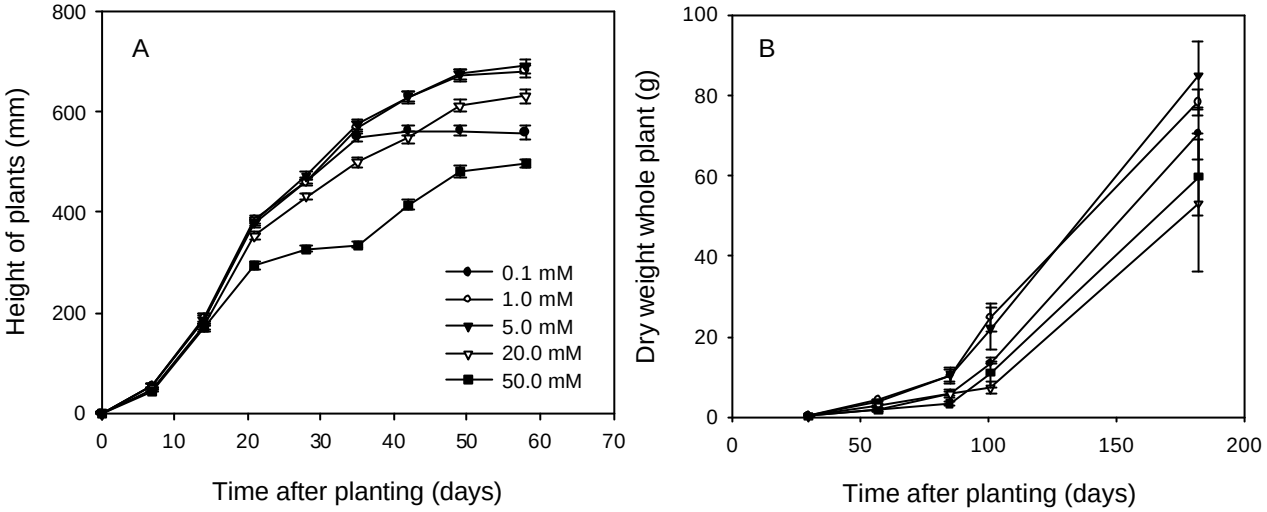


Figure 7A. *Growth response determined by leaf/plant measurements of wheat plants grown on different S concentrations. Measurements were taken over the first eight weeks of growth. Closed circles, 0.1 mM sulphate; open circles, 1 mM sulphate; closed triangles, 5 mM sulphate; open triangles, 20 mM sulphate and closed squares, 50 mM sulphate. Data are means of 4 replicates ±SE.*

Figure 7B. *Dry weight of whole plant with different S-supplies. All plants were grown on a sand/perlite mix 50% (v/v) and supplied with S containing nutrient solution at different concentrations. The data shown represents whole plant tissue. Closed circles, 0.1 mM sulphate supplied; open circles, 1.0 mM sulphate supplied; closed triangles, 5.0 mM sulphate supplied; open triangle, 20.0 mM sulphate supplied and closed squares, 50.0 mM sulphate supplied. Data are means of 4 replicates ±SE.*

The effect of a range of levels of sulphate supply on growth and S-pools in wheat was examined in a pot trial. Five concentrations of sulphate were chosen, ranging from 0.1 mM, previously ascertained to be adequate for growth (Blake-Kalff *et al.*, 2000) to a maximum of 50 mM sulphate, judged to be well in excess of requirements. Sulphate was supplied in nutrient solution, and plants were watered on alternate days with de-ionised water to avoid accumulations of nutrients in the sand/perlite support matrix. Growth parameters are shown as plant heights (Fig. 7A) or as dry weights (Fig. 7B). Maximum growth was observed in plants supplied with 1 and 5 mM sulphate. Lower growth rates in the plants supplied with 0.1 mM sulphate indicated deficiency. The higher concentrations (20 and 50 mM) of supplied-S were also not optimal.

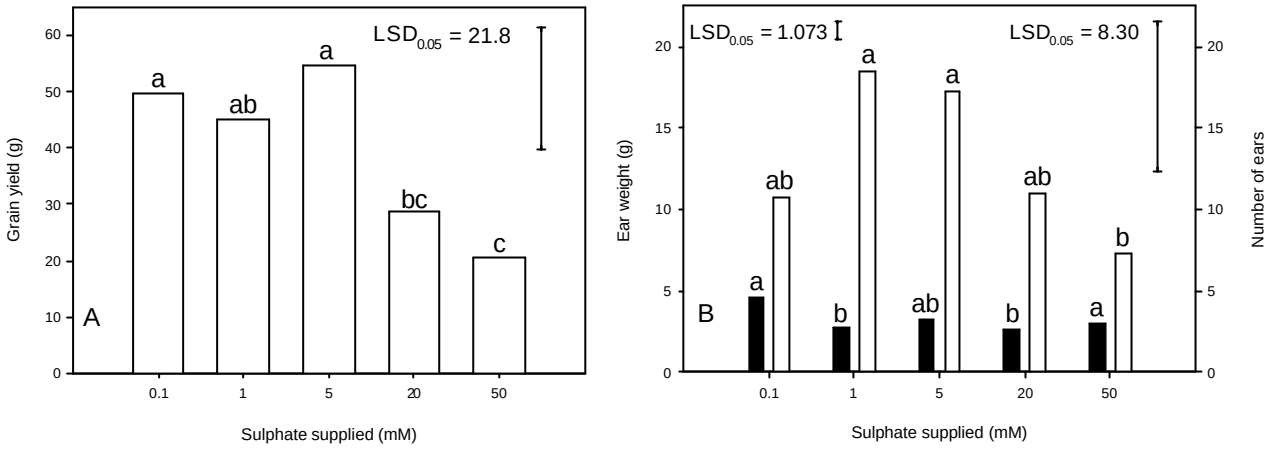


Figure 8A. *Final grain yield per plant for wheatgrown on different S concentrations. LSD $_{0.05}$ = 21.8.*

Figure 8B. *Ear weight and number of ears per plant for wheat plants grown on different S concentrations. Black bars, ear weight and white bars, number of ears. Ear weight $LSD_{0.05}$ = 1.073. Number of Ears $LSD_{0.05}$ = 8.30.*

Final grain yield was influenced by sulphur supply. With nutrient solution contain sulphate at between 0.1 and 5 mM sulphate, there was no significant difference in grain yields (Fig. 8A), although there was a shift in the yield components. At 1 and 5 mM supplied sulphate, ear weights were decreased compared to 0.1 mM sulphate, however the number of ears per plant was substantially increased, resulting in an overall compensation. At higher concentrations of supplied S (20 and 50 mM sulphate), grain yield was decreased, mainly as a result of a decreased number of ears per plant (Fig. 8B).

Fig. 9 shows the distribution of S between sulphate and non sulphate-S pools during the vegetative growth phases (up to anthesis/GS 60) for each of the five S-treatments. Data are presented as the S per plant (Fig. 10A-E) and as a tissue concentration based on dry weight (Fig. 10F-J).

With S supplied in solution at concentrations between 0.1 and 20 mM, the total non-sulphate sulphur increased to a maximum (approximately two-fold increase) at 5 mM supplied-S. There was a small decease at 20 mM and 50 mM supplied-S. These trends reflected the plant tissue weights, however when the non-sulphate-S concentrations were plotted as concentrations (Fig. 9 F-J) the same trends were observed. This contrasted with

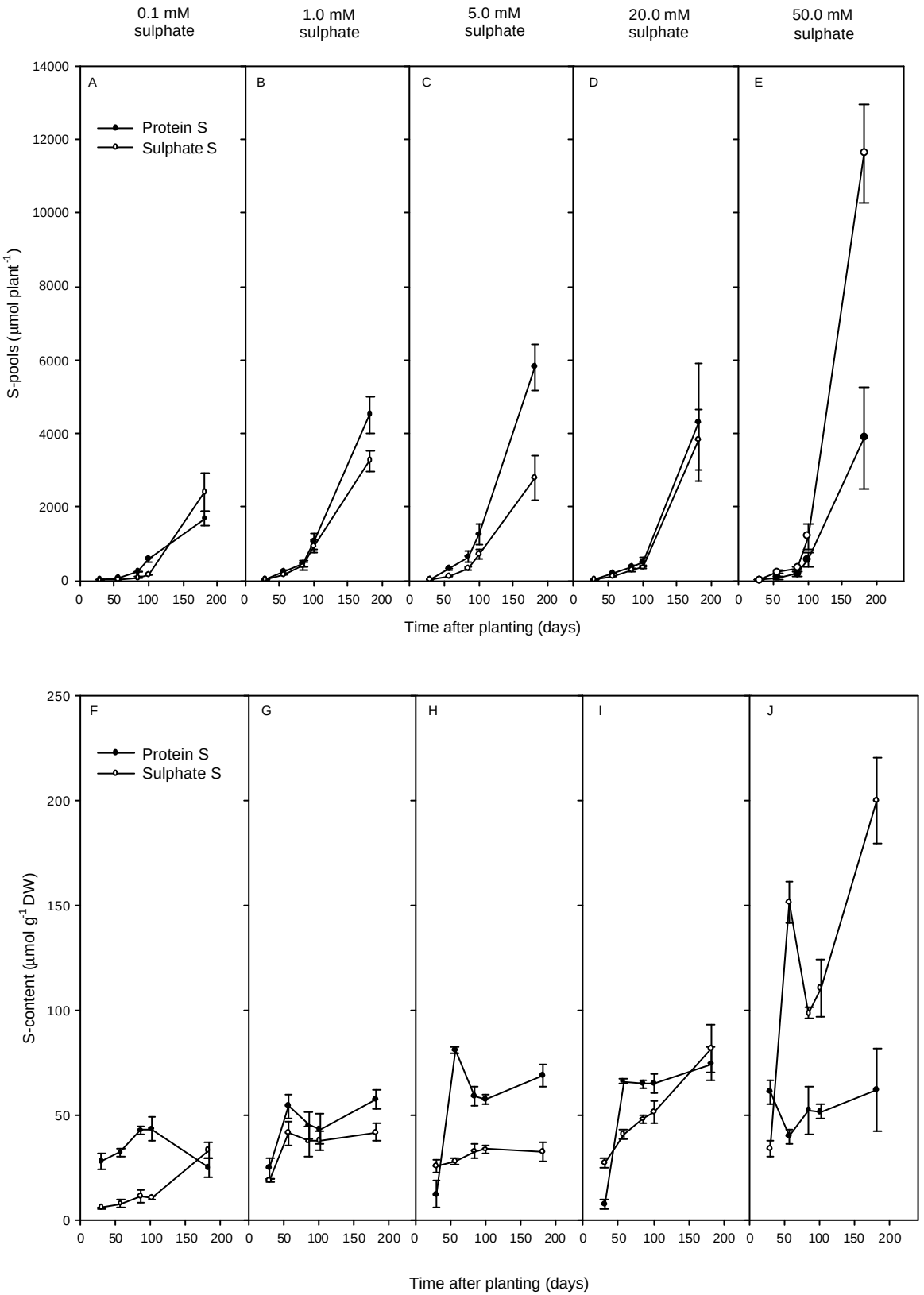


Figure 9. *Sulphate and non-sulphate S concentrations in wheat plants grown on different S concentrations. Closed circles, non -sulphate sulphur and open circles, sulphate-sulphur. Data are expressed on a per plant basis (A-E) or as a concentration (F-J). Plants were grown on 0.1 (A&F), 1 (B&G), 5 (C&H), 20 (D&I) or 50 (E&J) mM sulphate-containing nutrient solution.*

the data for the inorganic sulphate pools. The sulphate concentrations in the plants grown with a 0.1 mM supply were very low, were approximately similar for the plants grown at 1 and 5 mM sulphate, slightly increased at 20 mM supplied-S and increased substantially at 50 mM supplied-S. These same trends were again observed when the data were presented as tissue concentrations. The remarkably constant tissue organic-S content and relatively small rise in sulphate content in plants supplied at between 0.1 and 20 mM sulphate (a 200-fold range of externally supplied sulphate) clearly shows the effectiveness of the regulatory processes which control and limit S-uptake and incorporation. The large ratio of inorganic to organic S at the highest supplied rate of S indicated that regulatory processes were insufficient to maintain homeostasis.

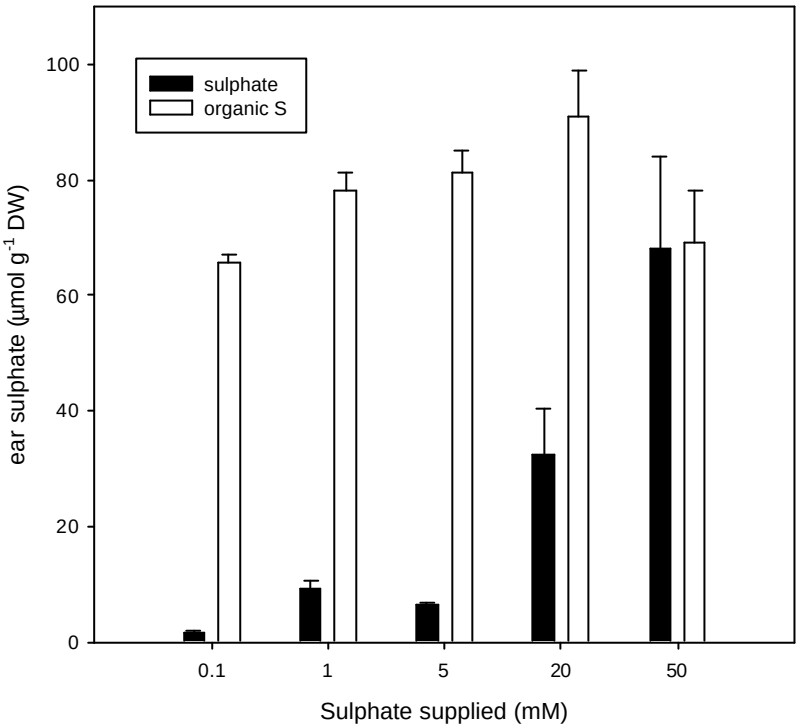


Figure 10. *Distribution of sulphur between inorganic and organic fractions in ears of wheat at harvest as influenced by S-supply. Data presented are µmol sulphate and organic S per g dry weight of ear tissue at final harvest of wheat plants. Plants received 1L of nutrient solution containing 0.1mM, 1.0mM, 5.0mM, 20.0mM or 50.0mM sulphate, 3 times a week. Treatment was continued until plant maturity. Plants were grown 5 per pot under controlled conditions on a 50% v/v sand/perlite mixture. Data are the means of 4 replicates with error bars representing the SE of the means.*

The distribution of sulphur between the inorganic sulphate pools and the non-sulphate (organic) fraction in the grain derived from the plants grown on the five different sulphate concentrations is shown in Fig. 10. The organic S concentration increased (from 64 to 90 µmol g^{-1} DW) as the sulphate concentration of the nutrient

solution increased from 0.1 to 20 mM sulphate, however the organic S-concentration decreased to 70 µmol g^{-1} DW in the plants supplied with 50 mM sulphate. Free sulphate pools in these tissues showed a marked increase in sulphate concentrations with increasing external supply, with the greatest increases occurring at 20 and 50 mM external sulphate. When plants were supplied with 50 mM external sulphate the amounts of sulphate in the two pools were approximately equal. The mechanisms regulating the sulphate content in the grain do not seem to be as efficient at these high external concentrations. Although this S (sulphate) contributes to the measured total grain-S, it is of no nutritional value and will not contribute to the quality of the grain.

Table 2. *Free amino acid concentrations in wheat tissue grown at different S supply rates sampled at GS 23 (30 days after planting). Data are means of 3 replicates ±SE.*

Concentration	**Sulphate supplied**				
(nmol g^{-1} FW)	**0.1 mM**	**1.0 mM**	**5.0 mM**	**20.0 mM**	**50.0 mM**
Alanine	2377 ± 604	1999 ± 505	1950 ± 467	1289 ± 573	743 ± 310
Arginine	182 ± 46.26	413 ± 298	466 ± 362	720 ± 339	194 ± 79
Asparagine	4448 ± 2187	4430 ± 2528	3273 ± 1304	3181 ± 1120	11921 ± 7160
Aspartic acid	3374 ± 816	3688 ± 984	3620 ± 935	2983 ± 839	1228 ± 613
Cysteine	11.7 ± 2.85	8.95 ± 3.18	6.43 ± 3.17	9.03 ± 4.27	18.3 ± 12.64
Glutamic acid	9313 ± 2020	9027 ± 2330	9063 ± 2136	8164 ± 2210	4072 ± 2052
Glutathione	210 ± 13.3	158 ± 26.9	153 ± 47.3	197 ± 36.4	168 ± 77.1
Glycine	3192 ± 577	2198 ± 422	2485 ± 657	2662 ± 641	2551 ± 1456
Histidine	181 ± 26.9	220 ± 36.9	400 ± 125	1736 ± 786	586 ± 496
Isoleucine	154 ± 50.5	142. ± 49.4	141 ± 44.9	192 ± 67.3	271 ± 192
Leucine	130 ± 48.3	138 ± 53.7	132 ± 50.8	124 ± 41.4	209 ± 149
Lysine	62.3 ± 26.5	70.9 ± 27.2	85.0 ± 23.6	63.7 ± 27.7	87.0 ± 20.4
Methionine	622 ± 181	685 ± 219	689 ± 244	393 ± 145	589 ± 333
Phenylalanine	672 ± 549	1310 ± 650	183 ± 89.5	675 ± 544	690 ± 543
Proline	374 ± 151	345 ± 175	387 ± 181	326 ± 30.5	731 ± 404
Serine	4397 ± 2631	6014 ± 1984	5980 ± 1671	5366 ± 1599	6146 ± 3811
Threonine	6920 ± 3283	6614 ± 3088	6553 ± 3134	5421 ± 2393	9752 ± 7684
Tryptophan	98.1 ± 17.7	98.4 ± 39	117 ± 30.7	102 ± 22.3	85.2 ± 23.8
Valine	531 ± 196	448 ± 152	406 ± 128	406 ± 181	567 ± 295

Table 2 shows concentrations of free amino acids in 30 day old wheat plants (GS 23) grown at different S levels. The concentration of proline in the tissues was influenced by S supply; the levels of proline remained fairly constant in the 0.1 mM, 1.0 mM, 5.0 mM and 20 mM S-grown plants, however when the 50.0 mM plants were analysed the levels of proline increased substantially. Accumulation of proline in plant tissues is an indication of salt stress. It is likely that the decreased biomass and grain yield in the 50 mM treatment (Figure 8) was due to salt stress. A similar pattern was observed with asparagine: the concentration remained constant in all plants except the 50.0 mM S-grown plants where the level of asparagine increased dramatically. The concentration of the S containing amino acids, methionine and cysteine, and the thiol, glutathione, remained at a constant concentration regardless of S treatment.

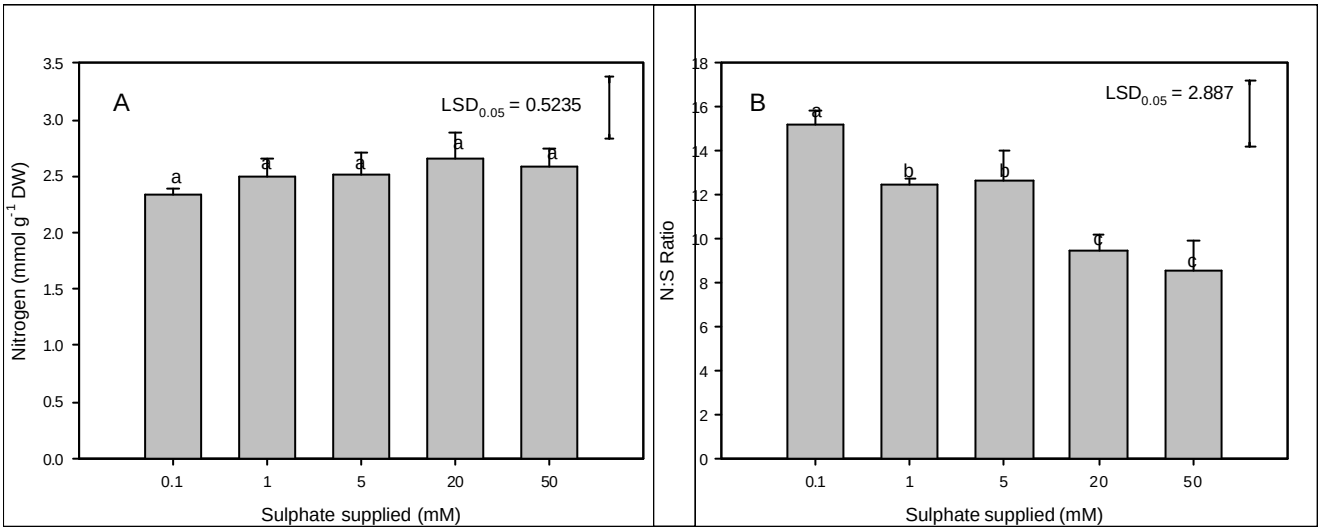


Figure 11. *Effect of S-supply on (A) the N-concentrations and (B) N:S ratio of mature wheat grain from plants grown on different S concentrations until maturity. For the calculation of N:S ratios the total S (inorganic + organic) fraction was used. L.S.D.$_{(0.05)}$ = 2.887.*

N-concentrations were not significantly affected by the S-supply (Fig. 11A). With increasing S-supply the N/S ratio decreased (Fig. 11B), a trend accounted for principally by the accumulation of sulphate in the grain (Fig. 10). Even at the lowest rate of S-supply, the N:S ratio was below 16, and therefore these plants would not be classified as deficient.

The consequence of the imbalance of N and S on the protein fraction of the grain is shown in Fig. 12. Grain consists of multiple proteins, and some plasticity exists which determines the relative abundance of S-poor compared to S-rich proteins. These are visualized by analysis with SDS-PAGE. Effects of S-nutrition on grain

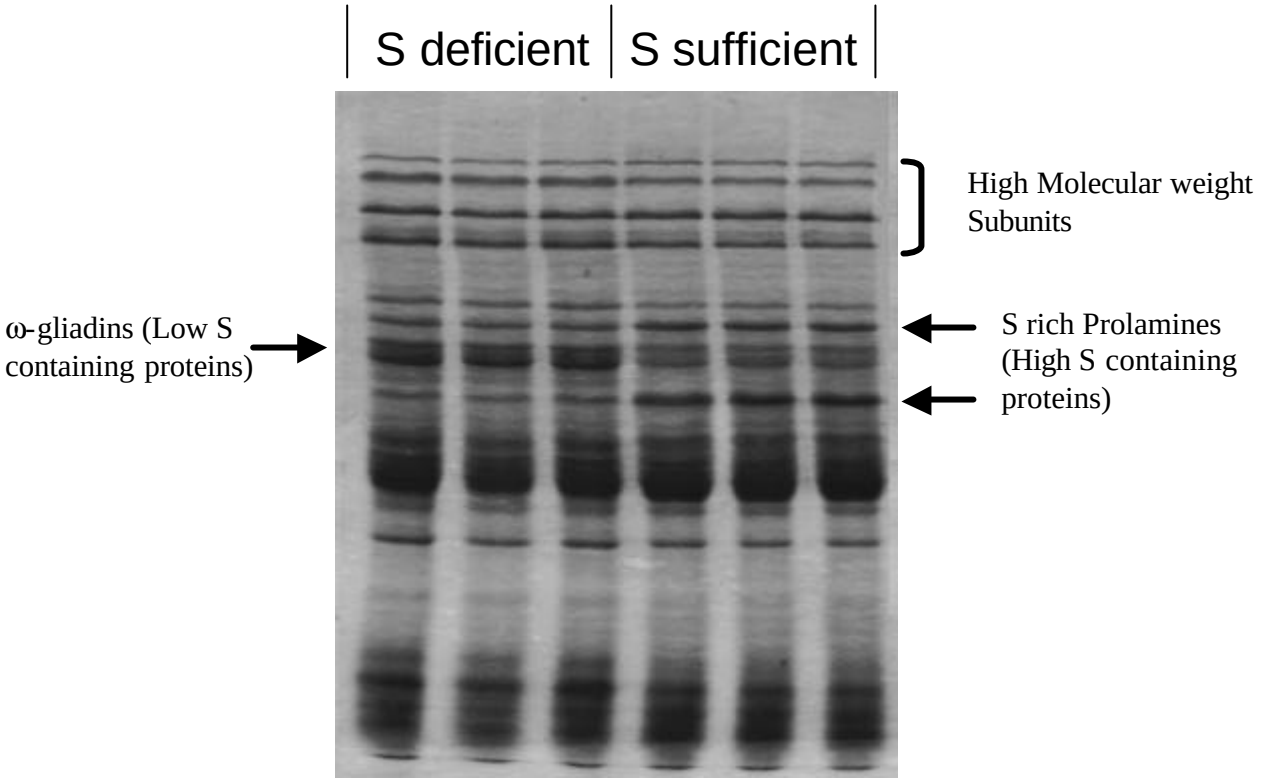
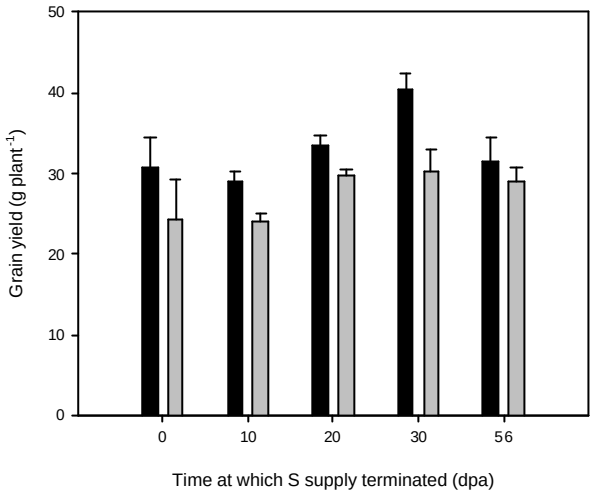


Figure 12. *SDS-PAGE analysis of protein composition of grain endosperm. Comparison of protein extracted from S-deficient and S-sufficient grain, harvested from plants supplied with 10 or 1000 µM S, respectively.3 replicate samples for each treatment are shown.*

protein composition are summarized in Zhao *et al.*, 1999 and in the HGCA Project Report No. 197. It is these changes in protein composition which influence dough elasticity and breadmaking quality.

In an effort to investigate the importance of post-anthesis sulphur supply, an experiment was carried out in which plants were grown up to anthesis on two rates of S supply (1 and 5 mM, chosen as upper and lower extremes of optimum supply) and were then subsequently deprived of S at defined periods after anthesis (Figs. 13 and 14). Grain yields (Fig. 13) were not substantially affected by the length of the post anthesis S-supply at either S-concentration. The small difference between the 1 and 5 mM treatments was probably due to factors other than nutritional supply. Grain S-pools were examined in the plants supplied with 1 mM sulphate (Fig. 14). The grain organic S-fraction (Fig. 14, black bars) was slightly decreased if sulphate supply to the roots was terminated at

anthesis, rather than continued beyond anthesis. An analysis of the sulphate concentrations in the grain (Fig. 14,



white bars) indicated surprisingly that sulphate concentrations were greater when supply to the roots was

Figure 13. *Grain yields data for wheat plants grown at 1 mM and 5 mM sulphate with S supply removed at different times after anthesis. Sulphate supply removed at either 0, 10, 20, 30 or 56 days post anthesis, after which all plants were left to scenesce. 1 mM Black bars; 5 mM grey bars. Data are the means of 4 replicates and error bars indicate standard error of the means.*

terminated at anthesis rather than continuously supplied. This may reflect different processes of sulphate redistribution from tissue reserves occurring in plants deprived of S compared to continuously supplied with S.

The capacity of the ear tissue to assimilate sulphate was investigated by measuring activity of the first step of the biosynthetic pathway. Figure 15 shows the activity of the enzyme, ATP sulphurylase in ear tissue for the period following anthesis. When expressed on a per milligram protein basis there was a clear rapid increase in activity of this enzyme following anthesis, which then slowly decreased over a period of about 20 d. This indicates a substantial capacity of ear to assimilate sulphate into an organic fraction, a process which would supplement any delivery of reduced S from leaf tissues via the phloem. The maximal activity (Fig. 15A) corresponds to the peak period of protein accumulation in the seed (Fig. 15B), as would be expected as the provision of reduced S as cysteine would be an essential component of all protein synthesis, and especially of S-rich protein synthesis. Whilst protein concentration did not increase after 5 dpa, cell expansion and ear growth would, require continued protein synthesis to maintain the concentration. During this period there is a gradual decrease in ATP sulphurylase activity, perhaps indicating a decreased importance of synthesis of S-rich proteins. The protein accumulation may mask the actual trend of enzyme activity (as this is expressed on a per protein basis) and

therefore the enzyme activity data are also expressed as per g of ear fresh tissue (Fig. 15C). Here again a large post anthesis peak of activity was seen.

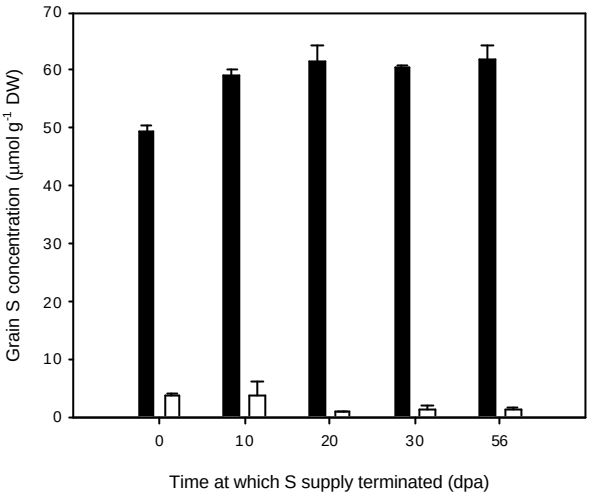


Figure 14. *Sulphur and sulphate concentrations in grains harvested from wheat plants grown at 1mM sulphate with S supply removed at different times after anthesis. Sulphate supply removed at either 0, 10, 20, 30 or 56 days post anthesis, after which all plants were left to scenesce. Black bars: organic S fraction, white bars: sulphate S fraction. Means of 4 replicates with error bars indicating standard errors of the means.*

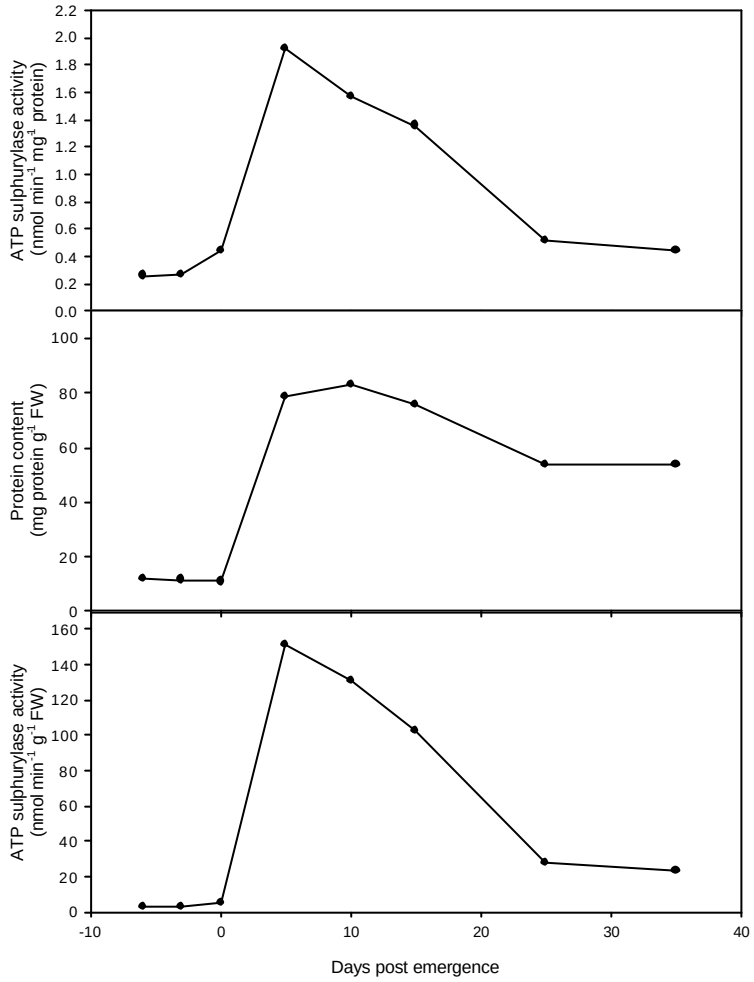


Figure 15. *Activity of S-assimilatory pathway in developing grain. Whole spikelets including grain were homogenized and assayed for ATP sulphurylase activity (A and C) and protein concentrations (B). Replicate assays on the same extract were averaged. The experiment was performed 3 times and a representative result is presented.*

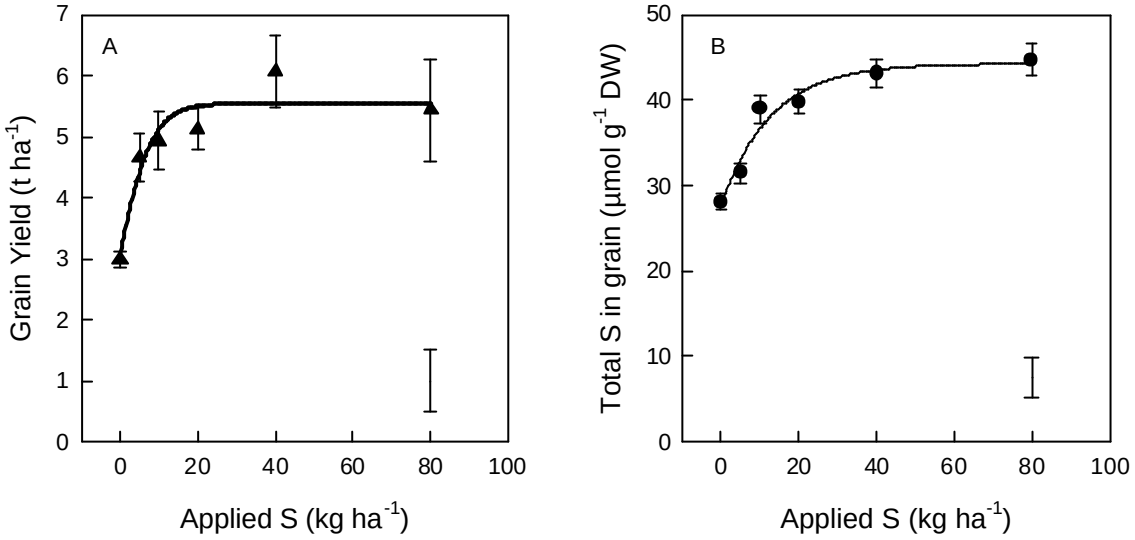


Figure 16. *The effect of increasing S applications on the (A) grain yield and (B) grain sulphur concentrations in winter wheat in 1998. Data are the means and ±SE of four separate plant samples. LSD ($p<0.05$) are shown by vertical bars. Figure adapted and redrawn from Blake-Kalff et al., 2000.*

DISCUSSION

We have previously investigated the effect of increasing S-application on grain yield and grain S-concentrations in wheat (Blake-Kalff *et al.*, 2000). Data from this publication is reproduced in Figure 16. Little increase in grain yield was observed above applications of 20 kg ha^{-1}. Grain S reached a plateau at around 45 µmol g^{-1} dry weight (1.44 mg g^{1} S), well above the suggested 1.2 mg g^{1} S critical value (Randall and Wrigley, 1986). In the experiments reported here, the field experiments produced grain with values ranging from 0.93 (inorganic + organic S fractions combined) in the zero S application plots in 2000 (Fig. 2A) to 1.73 (June S-application, 2001) (Fig. 2B). These values may be compared to those obtained in the pot experiments (Figs. 11 and 14) where grain S (organic fraction alone) ranged from 2.1 to 2.9 mg g^{-1} S depending upon S-supply (Fig. 11) or 1.6 to 1.9 mg g^{-1} S depending upon post-anthesis supply (Fig. 14). The range of grain S-concentrations reported in Zhao *et al*. (1995) (and reviewed in Zhao *et al.*, 1999) ranged from 1-2 mg g^{-1} S in a survey of grain samples collected between 1981 and 1993 from around the UK. The pot experiments indicated that there is a potential to increase

grain S, however studies such as those described in this report and in elsewhere (Blake-Kalff *et al*., 2000) indicate that this is not easy to realise in the field. A critical difference in the pot experiments is that S is continuously supplied, rather than the typical single doses applied to field crops.

The data presented in this report clearly demonstrates that early season (March-May) application of S-fertilizer was able to restore yield in an S-deficient wheat crop. Late application (June) was ineffectual at restoring yield. It is likely that optimum S-nutrition at approximately GS 15-30 is required for maximum vegetative growth, which will have a direct bearing on yield potential. Application of S-fertilizer at any time in the period studied (March-June) increased grain S-concentrations above generally accepted critical values.

It is possible that successive applications of S may be beneficial. The early season application to boost vegetative development and ensure yield potential, and an additional later application to boost quality parameters (grain S-concentrations). This later application may be particularly important when foliar N dressings are applied to avoid N/S imbalances. Such multiple S-application strategies need to be investigated.

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APPENDICES

Appendix A: Timings Field Experiment 2000

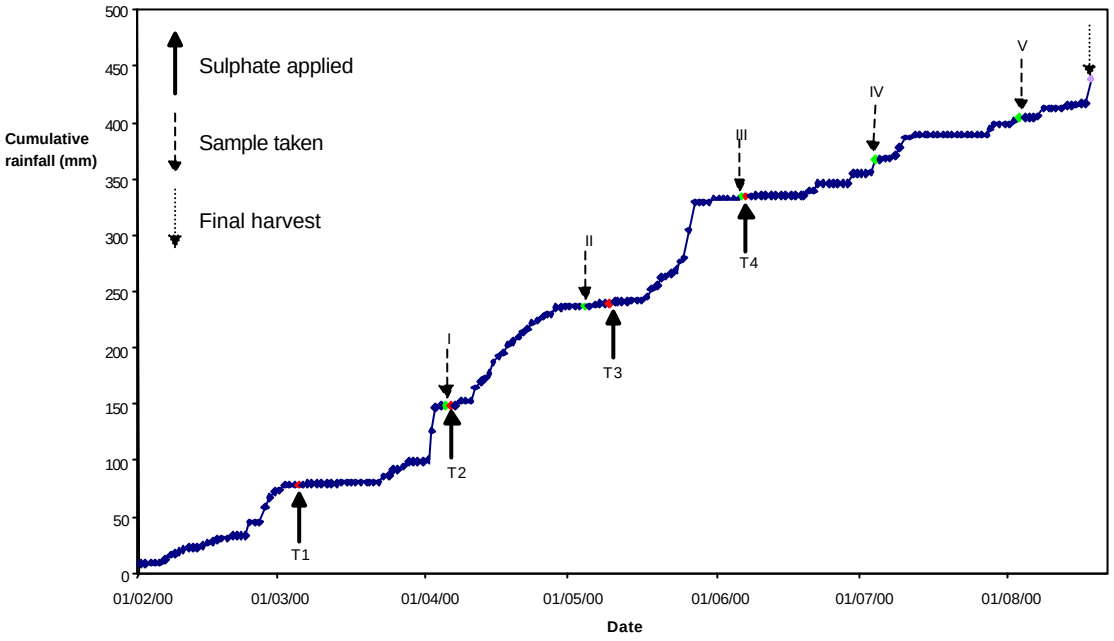
Sampling date	S treatment date + (kg ha^{-1})	Plant tissue	Yield (t ha^{-1})	Biomass (t ha^{-1})	Total S (µmol g^{-1} DW)	Sulphate S (µmol g^{-1} DW)	Protein S (µmol g^{-1} DW)	Nitrogen (mmol g^{-1} DW)	N:S Ratio
05.04.00	0	Leaf	-	2.50	94.13	21.56	72.57	-	-
05.04.00	Mar (30)	Leaf	-	2.82	112.59	37.13	75.46	-	-
05.04.00	Apr (30)	Leaf	-	2.33	97.49	20.30	77.19	-	-
05.04.00	0	Leaf	-	-	98.36	20.00	78.36	-	-
05.04.00	0	Leaf	-	-	90.71	19.67	71.04	-	-
19.04.00	Apr (30)	Leaf	-	4.62	-	-	-	-	-
04.05.00	0	Leaf	-	9.67	41.85	5.16	36.69	-	-
04.05.00	Mar (30)	Leaf	-	12.09	58.92	14.32	44.60	-	-
04.05.00	Apr (30)	Leaf	-	10.31	78.83	33.14	45.69	-	-
04.05.00	May (30)	Leaf	-	10.08	47.78	5.80	41.98	-	-
04.05.00	0	Leaf	-	-	43.43	6.09	37.34	-	-
19.05.00	May (30)	Leaf	-	18.31	-	-	-	-	-
06.06.01	0	Leaf	-	25.04	24.03	5.21	18.82	-	-
06.06.01	Mar (30)	Leaf	-	33.62	33.03	7.88	25.15	-	-
06.06.01	Apr (30)	Leaf	-	28.05	43.02	14.40	28.62	-	-
06.06.01	May (30)	Leaf	-	27.75	65.14	32.47	32.67	-	-
06.06.01	Jun (30)	Leaf	-	26.54	21.96	4.55	17.41	-	-
21.06.00	Jun 30	Leaf	-	27.93	-	-	-	-	-
28.06.00	0	Leaf	-	28.67	24.07	4.85	19.22	-	-
28.06.00	Mar (30)	Leaf	-	34.94	30.79	6.40	24.39	-	-
28.06.00	Apr (30)	Leaf	-	33.12	39.47	8.99	30.48	-	-
28.06.00	May (30)	Leaf	-	32.42	51.86	21.60	30.26	-	-
28.06.00	Jun (30)	Leaf	-	24.77	26.95	10.77	16.18	-	-
18.08.01	0	grain	5.76	-	28.64	2.75	25.89	1538.39	23.72
18.08.01	Mar (30)	grain	7.56	-	37.01	3.45	33.56	1416.61	16.77
18.08.01	Apr (30)	grain	6.89	-	40.60	4.15	36.44	1451.25	15.65
18.08.01	May (30)	grain	7.41	-	42.13	5.13	37.00	1400.00	14.56
18.08.01	Jun (30)	grain	5.49	-	46.14	8.01	38.13	1617.14	15.36

Appendix B: Timings field Experiment 2001

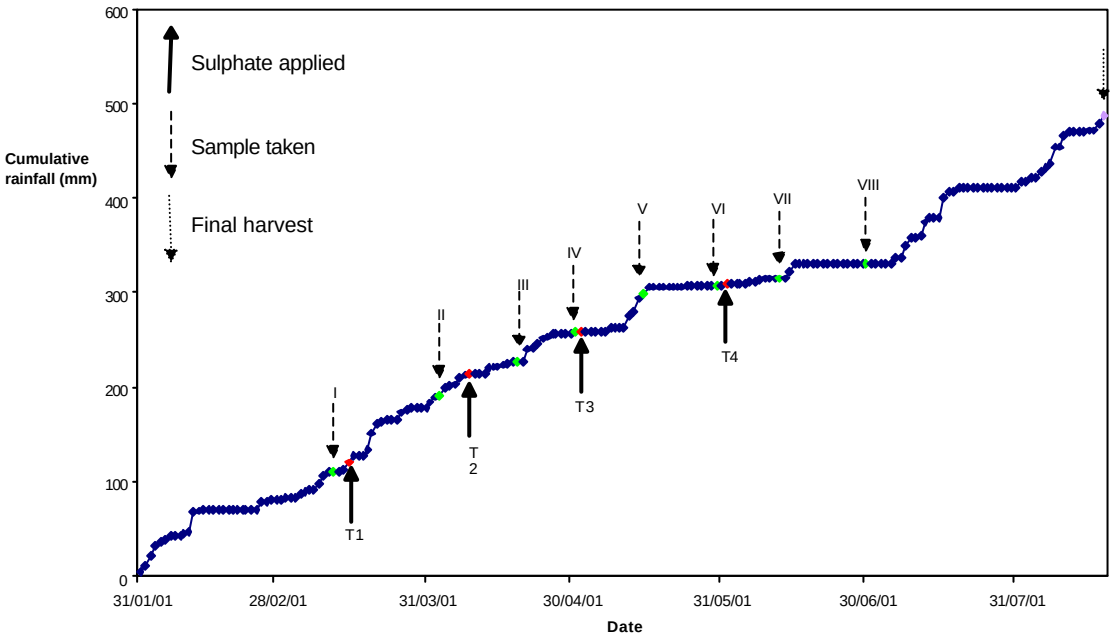
Sampling date	S treatment (kg ha^{-1})	Plant tissue	Yield (t ha^{-1})	Biomass (t ha^{-1})	Total S (µmol g^{-1} DW)	Sulphate S (µmol g^{-1} DW)	Protein S (µmol g^{-1} DW)	Nitrogen (µmol g^{-1} DW)	N:S Ratio	S take off (kg ha^{-1})
12.03.01	Pre treatment	Whole	-	-	108.6	20.9	87.7	3228	13.0	-
03.04.01	0	Leaf	-	0.45	121.5	15.81	105.71	-	-	-
03/04/01	Mar (30)	Leaf	-	0.50	136.8	32.23	104.54	-	-	-
03/04/01	Apr (30)	Leaf	-	0.46	121.8	16.00	105.78	-	-	-
03/04/01	0	Leaf	-	0.47	121.6	17.34	104.29	-	-	-
03/04/01	0	Leaf	-	0.47	126.4	17.05	109.33	-	-	-
19.04.01	Apr (30)	Leaf	-	1.21	162.9	57.37	105.53	-	-	-
01.05.01	0	Leaf	-	2.26	75.4	0.97	74.41	-	-	-
01.05.01	Mar (30)	Leaf	-	3.20	119.9	17.86	102.02	-	-	-
01.05.01	Apr (30)	Leaf	-	2.89	205.6	87.84	117.76	-	-	-
01.05.01	May (30)	Leaf	-	2.55	69.4	0.77	68.60	-	-	-
01.05.01	0	Leaf	-	2.59	69.5	1.06	68.45	-	-	-
15.05.01	May (30)	Leaf	-	5.57	95.1	17.62	77.51	-	-	-
30.05.01	0	Leaf	-	12.72	47.1	1.24	45.83	-	-	-
30.05.01	Mar (30	Leaf	-	18.19	92.4	19.94	72.41	-	-	-
30.05.01	Apr (30	Leaf	-	18.92	146.5	58.75	87.74	-	-	-
30.05.01	May (30	Leaf	-	16.91	176.5	89.22	87.24	-	-	-
30.05.01	Jun (30	Leaf	-	10.93	43.6	1.00	42.62	-	-	-
12.06.01	Jun (30	Leaf	-	12.50	63.0	17.99	44.99	-	-	-
28.06.00	0	Leaf	-	15.83	37.6	2.26	35.32	-	-	-
28.06.00	Mar (30)	Leaf	-	28.42	184.9	92.14	92.75	-	-	-
28.06.00	Apr (30)	Leaf	-	31.73	142.6	54.56	88.01	-	-	-
28.06.00	May (30)	Leaf	-	27.88	172.8	69.23	103.56	-	-	-
28.06.00	Jun (30)	Leaf	-	17.61	232.0	142.40	89.61	-	-	-
18.08.01	0	grain	3.42	-	32.05	0.98	31.07	1911.61	26.16	3.48
18.08.01	Mar (30)	grain	5.57	-	42.38	2.55	39.82	1593.93	16.40	7.47
18.08.01	Apr (30)	grain	6.15	-	46.22	2.44	43.79	1790.18	17.04	9.12
18.08.01	May (30)	grain	5.57	-	43.02	1.81	41.21	1689.64	17.24	7.66
18.08.01	Jun (30)	grain	3.57	-	53.96	5.24	48.72	2041.43	16.59	6.17

Appendix C: Cumulative rainfall data, S-application and sampling dates.

Cumulative Rainfall 2000



Cumulative Ra infall 2001



Appendix D: Height of plants, pot experiment on variable rates of S-supply

Time (days)	S treatment (mM)	Plant height (mm)
0	0.1	0
0	1	0
0	5	0
0	20	0
0	50	0
7	0.1	55.93
7	1	55.23
7	5	54.96
7	20	48.96
7	50	44.56
14	0.1	185.73
14	1	189.55
14	5	186
14	20	173.61
14	50	169.37
21	0.1	379.64
21	1	386.12
21	5	382
21	20	354
21	50	295.48
28	0.1	462.44
28	1	460.32
28	5	473.76
28	20	430.84
28	50	327.2
35	0.1	551.3
35	1	568.75
35	5	575.35
35	20	500.45
35	50	335.75
42	0.1	562.35
42	1	629.75
42	5	630.55
42	20	550.05
42	50	414.35
49	0.1	562.2
49	1	673.9
49	5	675.25
49	20	611.7
49	50	481.25
56	0.1	558.53
56	1	681.8
56	5	690.93
56	20	631.2
56	50	496.53

Appendix E: Tissue S pools and grain final data, pot experiment on variable rates of S-supply.

Time (days)	S treatment (mM)	Dry Weight (g)	Total S (µmol plant)	Sulphate (µmol plant)	Protein S (µmol plant)	Total S (µmol g^{-1} DW)	Sulphate (µmol g^{-1} DW)	Protein S (µmol g^{-1} DW)
30	0.1	0.42	14.8	2.41	12.4	33.8	5.69	28.1
30	1	0.42	19.2	8.01	11.2	43.5	18.8	24.6
30	5	0.39	16.1	9.50	6.57	40.0	24.2	15.8
30	20	0.32	11.2	8.71	2.46	34.7	28.0	6.73
30	50	0.21	19.5	7.03	12.5	94.8	33.8	61.0
57	0.1	1.97	74.2	13.2	61.0	40.0	7.83	32.2
57	1	4.12	394	162	231	95.7	41.4	54.2
57	5	3.97	433	112	321	109	27.9	81.0
57	20	2.99	319	121	198	107	40.9	66.2
57	50	1.64	305	234	70.5	192	152	39.9
85	0.1	5.64	305	66.9	239	54.2	11.5	42.7
85	1	10.26	839	395	444	82.6	37.5	45.1
85	5	10.56	976	332	644	91.8	32.9	58.9
85	20	5.68	634	270	364	113	48.0	64.9
85	50	3.32	504	331	173	151	98.7	52.5
101	0.1	13.54	725	145	580	54.1	10.6	43.5
101	1	24.69	1975	927	1048	81.0	37.7	43.3
101	5	21.94	1966	707	1259	91.3	33.7	57.6
101	20	7.26	832	346	486	117	51.5	65.1
101	50	11.08	1766	1200	566	163	110.8	51.7
182	0.1	70.74	4092	2404	1688	57.9	32.9	25.0
182	1	78.33	7765	3253	4512	99.0	41.8	57.2
182	5	85.05	8592	2785	5807	101	32.2	68.7
182	20	53.30	8137	3836	4301	156	81.7	74.5
182	50	59.81	15508	11628	3880	262	200	62.1

Time (days)	S treatment (mM)	Dry Weight (g)	Grain Yield (g)	No ears	Ear weight (g)	Grain N:S Ratio	Grain Nitrogen
182	0.1	70.74	49.71	10.75	4.61	15.25	2.34
182	1	78.33	45.2	18.5	2.7	12.51	2.50
182	5	85.05	54.69	17.25	3.22	12.65	2.51
182	20	53.3	28.53	11	2.63	9.47	2.65
182	50	59.81	20.53	7.25	2.98	8.58	2.58

Appendix F: Post anthesis S-supply data

Time after anthesis (days)	S treatment (mM)	Grain yields (g)	Grain Sulphur (µmol g^{-1} DW)	Grain Sulphate (µmol g^{-1} DW)	Grain Protein S (µmol g^{-1} DW)
0	1	30.8	49.4	7.38	42.0
10	1	29.1	59.0	10.0	49.0
20	1	33.4	61.6	12.0	49.7
30	1	40.4	60.6	13.0	47.6
56	1	31.4	61.9	11.8	50.1
0	5	24.4	52.6	8.26	44.3
10	5	23.9	61.1	10.8	50.3
20	5	29.6	65.3	13.2	52.0
30	5	30.2	64.9	14.7	50.2
56	5	29.0	65.3	14.4	50.9

Appendix G: ATP sulphurylase activity measurements in spikelets relative to anthesis (day 0).

GS	Day	ATP sulphurylase activity (nmol min^{-1} g^{-1} FW)	ATP sulphurylase activity (nmol min^{-1} mg^{-1} protein)	Protein content (mg protein g^{-1} FW)
51	-6	3.12	0.261	12.26
55	-3	3.08	0.265	11.63
60	0	4.92	0.447	11.00
65	5	151	1.918	78.72
69	10	131	1.576	83.03
73	15	103	1.359	75.53
83	25	27.8	0.516	53.95
85-87	35	23.8	0.441	53.91

Appendix H: The growth stages at which the wheat plants were sampled. Growth stages were determined according to Tottman (1987).

Experiment	Sampling date	Growth stage	Description	Plant parts sampled
Timings field trial 2000	05.04.00	21	Main shoot and 1 tiller	Whole plant taken
	04.05.00	31	First node detectable	Whole plant taken
	06.06.00	58-59	Emergence of inflorescence completed	Whole plant taken
	04.07.00	76-77	Late milk	Whole plant taken
	18.08.00	92	Caryopsis hard	Grain
Timings field trial 2000 Timings	12.03.01	13-15	3-5 leaves unfolded	Whole plant taken
	03.04.01	22-25	Main shoot and 2-5 tillers	Whole plant taken
	19.04.01	24-25	Main shoot and 4-5 tillers	Whole plant taken
	01.05.01	30	Ear at 1 cm (pseudostem srect)	Whole plant taken
	15.05.01	32	Second node detectable	Whole plant taken
	30.05.01	39-43	Flag leaf ligule just visible – Boots just visibly swollen	Whole plant taken
	12.06.01	51-55	First spikelet of inflorescence just visible – ½ of inflorescence emerged	Whole plant taken
	28.06.01	59-62	Emergence of inflorescence completed – Beginning of anthesis	Whole plant taken
	18.08.01	92	Caryopsis hard	Grain
S-rate pot trial	11.11.98	21	Main shoot and 1 tiller	Whole plant
	08.12.98	31	First node detectable	Leaf and Stem
	05.01.99	39	Flag leaf ligule just visible	Leaf and Stem
	21.01.99	60-65	Beginning to half-way anthesis	Leaf, Stem and Ear
	13.04.99	92	Caryopsis hard	Leaf, Stem and Ear
Post anthesis S-supply 1 mM plants	05.05.99	60	Beginning of anthesis	Leaf, Stem and Ear
	15.05.99	69	Anthesis complete	Leaf, Stem and Ear
	25.05.99	75	Medium milk	Leaf, Stem and Ear
	04.06.99	85	Soft dough	Leaf, Stem and Ear
	30.06.99	92	Caryopsis hard	Leaf, Stem and Ear
Post anthesis S-supply 5 mM plants	10.05.99	60	Beginning of anthesis	Leaf, Stem and Ear
	20.05.99	69	Anthesis complete	Leaf, Stem and Ear
	30.05.99	77	Medium milk	Leaf, Stem and Ear
	09.06.99	85	Soft dough	Leaf, Stem and Ear
	06.07.99	92	Caryopsis hard	Leaf, Stem and Ear
ATP sulphurylase experiment	Various at GS intervals	51	First spikelet of inflorescence just visible	Grain
		55	½ of inflorescence emerged	Grain
		60	Beginning of anthesis	Grain
		65	Anthesis ½ way	Grain
		69	Anthesis complete	Grain
		73	Early milk	Grain
		83	Early dough	Grain
		85-87	Soft dough – hard dough	Grain