



## Effects of sulphur nutrition on growth and nitrogen fixation of pea (*Pisum sativum* L.)

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### Abstract

A S-deficient soil was used in pot experiments to investigate the effects of S addition on growth and N<sub>2</sub>-fixation in pea (*Pisum sativum* L.). Addition of 100 mg S pot<sup>-1</sup> increased seed yield by more than 2-fold. Numbers of pods formed were the most sensitive yield component affected by S deficiency. Sulphur addition also increased the concentration of N in leaves and stems, and the total content of N in the shoots. The amounts of N fixed by pea were determined at four growth stages from stem elongation to maturity, using the <sup>15</sup>N dilution technique. Sulphur addition doubled the amount of N fixed at all growth stages. In contrast, leaf chlorophyll content and shoot dry weight were increased significantly by S addition only after the flowering and pod fill stage, respectively. Pea roots were found to have high concentrations of S, reaching approximately 10 mg g<sup>-1</sup> dry weight and being 2.6–4.4 times the S concentration in the shoots under S-sufficient conditions. These results suggest that roots/nodules of pea have a high demand for S, and that N<sub>2</sub>-fixation is very sensitive to S deficiency. The effects of S deficiency on pea growth were likely to be caused by the shortage of N, due to decreased N<sub>2</sub>-fixation.

### Introduction

Sulphur deficiency has become a limiting factor for crop yield and quality in many areas of Western Europe (McGrath et al., 1996; Schnug, 1991; Zhao et al., 1997). This is largely due to a massive decrease in the S inputs from atmospheric deposition over the last 2 to 3 decades. In addition, S-containing fertilisers such as single superphosphate have been superseded by fertilisers containing little or no S. During this period, the S requirements of many crops have probably increased as a result from increased yields.

Sulphur requirement and metabolism in plants are closely related to N nutrition (Hawkesford et al., 1997; Reuveny et al., 1980), whereas N metabolism is also strongly affected by the S status of plant (Duke and Reisenauer, 1986). Insufficient S supply has been shown to depress the activity of nitrate reductase in maize (Friedrich and Schrader, 1978), and to res-

ult in transient or steady-state nitrate accumulation in maize, spinach, oilseed rape and wheat (Dietz, 1989; Friedrich and Schrader, 1978; Gilbert et al., 1997; McGrath and Zhao, 1996). Sulphur deficiency also causes perturbations in specific amino acid pools. Free amides such as asparagine, and to a lesser extent, glutamine, accumulate markedly in response to S deficiency in barley and wheat (Karmoker et al., 1991; Zhao et al., 1996), whereas in alfalfa and spinach arginine is the main amino acid which accumulates under the stress of S deficiency (DeBoer and Duke, 1982; Dietz, 1989).

Legume crops obtain N mainly from symbiotic N<sub>2</sub>-fixation, although environmental conditions can exert a large influence on the contribution of symbiotic N<sub>2</sub>-fixation to N nutrition of the crop. Sulphur deficiency has been shown to decrease the concentration of N in the shoots of many legumes (Anderson and Spencer, 1950; Andrew, 1977; see also review by Robson, 1983). Whether this is due to a direct effect on symbiotic N<sub>2</sub>-fixation or an effect on the host plants is not very clear. If symbiotic N<sub>2</sub>-fixation has a greater re-

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quirement for a nutrient than the growth of host plants, a negative interaction between the additions of that nutrient and inorganic N on plant growth as defined by Robson (1983) would be expected. Anderson and Spencer (1950) found no negative interaction between S and inorganic N on the growth of clover. This led them to conclude that the restricted growth of the S-deficient clover was not due to poor N<sub>2</sub>-fixation, but to a deficiency in the host legume. In contrast, DeBoer and Duke (1982) showed that N<sub>2</sub>-fixation of alfalfa, measured as acetylene reduction, was affected early during S-deprivation and that subsequent effects of S deficiency may be due to less N<sub>2</sub>-fixation. Zaroug and Munns (1979) also observed a significant effect of S on acetylene reduction in the roots of *Lablab purpureus*, although they concluded that the S requirements for metabolism outside the nodule exceed those within the nodule, based on rather unconvincing growth data. These disagreements, together with a general lack of information, indicate the need for further study.

Sulphur deficiency in legume crops affects not only yields, but also the nutritional quality of seeds. This is because methionine is usually the most limiting essential amino acid in legume seeds (Friedman, 1996). Legume seeds also have relatively low concentrations of cysteine, which is regarded as conditionally indispensable for humans and animals. Different storage proteins in legume seeds vary considerably in their contents of the S-containing amino acids. For example, the pea storage proteins vicilin and lectin contain no cysteine and methionine, whereas legumin has 1.7% S-containing amino acids (Spencer et al., 1990). Many studies using different legumes have shown that S deficiency decreases the synthesis of S-rich storage proteins markedly, but increases the synthesis of S-poor proteins concomitantly (Blagrove et al., 1976; Gayler and Sykes, 1985; Naito et al., 1995; Spencer et al., 1990). In both field bean and pea, the 'chemical score', which is a measurement of essential amino acids compared to those in a reference protein, and the 'biological value', which was determined in N-balance trials with rats, were all increased by S fertilisation (Eppendorfer and Eggum, 1995).

Because S deficiency is a relatively new development in European agriculture, research on crop S nutrition still lags far behind that on other major nutrients. Rapid progress has been made in recent years in the understanding of the S requirement for yield and quality, and diagnosis of S deficiency in major arable crops such as oilseed rape and cereals (McGrath et al.,

1996). In comparison, little attention has been paid to the S nutrition of legumes. In particular, the question regarding the relative requirements of S by symbiotic N<sub>2</sub>-fixation and by host plants has not been resolved unequivocally. In this study, we investigated the effects of S addition on growth, N<sub>2</sub>-fixation and seed yield of pea (*Pisum sativum* L.).

## Materials and methods

A S-deficient top soil (0–20 cm) was collected from Woburn Experimental Farm, Bedfordshire, UK. The soil is a loamy sand of the Cottenham series, and contained 10% clay (<2 µm), 15.1 g kg<sup>-1</sup> organic matter, 1.2 g kg<sup>-1</sup> total N, 12.6 µg g<sup>-1</sup> mineral N and 2.4 µg g<sup>-1</sup> extractable SO<sub>4</sub>-S initially. Soil pH (measured in deionised water, soil:water ratio 1:2.5) was 6.5. Moist soil was sieved through 5 mm, and mixed thoroughly. The soil had a moisture content of 13% and a water holding capacity (WHC) of 31%.

### Experiment 1

Two kg of moist soil were put into each of 48 plastic pots. Treatments consisted of six rates of S addition: 0, 5, 10, 25, 50, 100 mg S pot<sup>-1</sup>. Sulphur was applied as potassium sulphate, and the disproportionate addition of K in different treatments was counter balanced by the addition of proportionate amounts of potassium chloride to the pots. Eight replicates were included for each rate of application. Basal nutrients were also added to each pot to prevent deficiency of other nutrients. These included 70 mg P (KH<sub>2</sub>PO<sub>4</sub>), 332 mg K (KH<sub>2</sub>PO<sub>4</sub>, K<sub>2</sub>SO<sub>4</sub> or KCl), 50 mg Mg (MgCl<sub>2</sub>), 5 mg Mn (MnCl<sub>2</sub>), 5 mg Fe (FeCl<sub>3</sub>), 2 mg Zn (ZnCl<sub>2</sub>), 1 mg Cu (CuCl<sub>2</sub>), 1 mg B (H<sub>3</sub>BO<sub>3</sub>) and 0.5 mg Mo (Na<sub>2</sub>MoO<sub>4</sub>) per pot. Sulphur and other basal nutrients were dissolved in deionised water and applied to each pot in required volumes. Soils were then mixed thoroughly, and the soil moisture content was raised to 70% of WHC by adding deionised water. A saucer was placed under each pot to prevent drainage losses of nutrients. Pea seeds (cv. Eiffel) were pre-germinated on moist filter paper in the dark. Three germinated seeds were sown to each pot. The experiment was carried out in a glasshouse with the following conditions: 14 h/10 h day/night, 16 °C/12 °C day/night temperatures, and natural light supplemented with 1 kw SON-T lamps to maintain a minimum photon flux of 250 µmol m<sup>-2</sup> s<sup>-1</sup>.

At early flowering (45 days after sowing, growth stage GS 204, Knott, 1987), the chlorophyll content of the top five leaves on each plant was determined using a hand-held chlorophyll meter (Minolta SPAD 502). The SPAD meter readings have been shown to correlate closely with chlorophyll contents in different plant species (Schaper and Chacko, 1991), although the readings can vary slightly with variation in irradiance (Hoel and Solhaug, 1998). Plants in 4 replicate pots of each treatment were then harvested by cutting the shoots just above the soil surface. Plants were separated into leaves, stems and immature pods, weighed, and dried at 80 °C for 16 h before dry weights were recorded. Dried samples were ground to <0.5 mm before chemical analysis. The remaining pots were harvested at maturity (75 days after sowing, GS 303). Plants were separated into seeds, pod cases, and stems and leaves. Samples were prepared as above.

### Experiment 2

This experiment was set up to evaluate the effects of S addition on N<sub>2</sub>-fixation of pea using the <sup>15</sup>N dilution technique. A small amount (1477 mg N) of <sup>15</sup>NH<sub>4</sub><sup>15</sup>NO<sub>3</sub> (<sup>15</sup>N atom% 10.1) was dissolved in 500 mL deionised water, and added to 45 kg moist soil. The soil was brought to 50% of WHC, and homogenised using a cement mixer. The soil was then put into polyethylene bags, and transferred to a glasshouse with 16 °C/12 °C day/night temperatures. The soil was left in the dark for five weeks in order to stabilise the <sup>15</sup>N enrichment of the available soil N pool (Giller and Witty, 1987). During this period the bags were loosely tied to permit gaseous exchange, and the soil moisture content was maintained at 50% WHC. After incubation, 650 g of the soil was put into each of 64 plastic pots (9 cm); each portion of soil contained 7.5 mg of added N and 0.76 mg <sup>15</sup>N. The 64 pots were divided equally for growing pea and the reference plant Italian ryegrass (*Lolium perenne* L.). There were two S treatments, 0 and 50 mg pot<sup>-1</sup>, for both pea and ryegrass. Sixteen pots for each treatment were divided into four groups to be harvested at different growth stages. Sulphur was added as potassium sulphate and potassium chloride used to balance the K addition in the S<sub>0</sub> treatment. The amounts of basal nutrients added to each pot were half of those used in the Experiment 1. One pre-germinated pea seed (cv. Eiffel) or 0.4 g of fast growing ryegrass seeds (cv. Atalja) was sown in each pot. Deionised water was used throughout. The

experiment was conducted in the same glasshouse as in the Experiment 1.

Both pea and ryegrass were harvested at 28, 38, 56 and 73 days after sowing, corresponding to the pea growth stages of mid-stem elongation (GS 107), first open flower (GS 203), pod fill (GS 207) and maturity (GS 303), respectively. Plant shoots were cut just above the soil surface and washed with deionised water. The roots were separated from the soil, and washed first with tap water and then with deionised water. Plant samples were dried and ground as before. In the first 3 harvests, leaf chlorophyll content of pea was measured using the chlorophyll meter before the plants were harvested. Chlorophyll content was not determined in the last harvest because leaves had senesced.

### Chemical and data analysis

The concentration of total S in plant samples was determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES; Fisons ARL Accuris) following a digestion with HNO<sub>3</sub>/HClO<sub>4</sub> (Zhao et al., 1994). Total N concentration was determined using a combustion method (LECO CNS Analyzer). Total N and S concentrations were expressed on a dry weight basis. Atom% of <sup>15</sup>N in the plant materials from Experiment 2 was determined using a mass spectrometer (Europa Scientific Integra-CN).

Weighted mean <sup>15</sup>N atom% of plants was calculated for each pot, taking into account the N content and the <sup>15</sup>N atom% of different plant parts. Nitrogen fixation in pea was calculated using the formula of Fried and Broeshart (1975):

$$\begin{aligned} \% \text{ N of plants derived from N}_2\text{-fixation} = \\ [1 - (^{15}\text{N atom\% in pea} / ^{15}\text{N atom\% in ryegrass})] \\ \times 100 \end{aligned}$$

The amount of N fixed in pea was calculated as follows:

$$\begin{aligned} \text{N fixed (mg pot}^{-1}\text{)} \\ = \% \text{ N fixed} \times \text{Total N content in pea (mg pot}^{-1}\text{)} \end{aligned}$$

Analysis of variance was performed on all data sets. Least significant difference (LSD) was used to compare treatment means.

## Results

### Experiment 1

Differences in plant height and leaf colour were visible between S treatments at early flowering. The chlorophyll content of the top 5 leaves increased significantly from 36 SPAD units in the  $S_0$  treatment to 47 SPAD units in the  $S_{100}$  treatment (Figure 1a). At early flowering, dry weight of shoots per pot increased by 72% with the addition of 100 mg S  $\text{pot}^{-1}$ , compared to the  $S_0$  treatment (Figure 1b). The effect of S was most noticeable from the addition of the first 25 mg S  $\text{pot}^{-1}$ . The effect of S addition on pea growth was even greater by the crop maturity stage. Compared to the  $S_0$  treatment, addition of 100 mg S  $\text{pot}^{-1}$  increased the dry weight of shoots by 145%, and of seeds by 203% (Figure 1c). Seed yield components are shown in Table 1. This indicates that the main effect of S was on the pod number, with S-sufficient plants producing twice as many pods as plants grown in the  $S_0$  treatment. Whereas the number of seeds per pod was also increased slightly by the S addition, single seed weight was hardly affected. Because the effect of S on total seed weight was greater than that on the total weight of plants, the harvest index was increased significantly by the S addition (Table 1).

The concentrations of S in different plant parts at early flowering and maturity are shown in Figure 2a and 2b, respectively. At early flowering, leaves contained much more S than stems, whereas at maturity seeds had the highest concentrations of S. All plant parts analysed at the two growth stages responded to S addition significantly. For example, leaf S concentration at early flowering increased from 0.9 mg  $\text{g}^{-1}$  in the  $S_0$  treatment to 2.0 mg  $\text{g}^{-1}$  in the  $S_{50}$  and  $S_{100}$  treatments. Similarly, the concentration of S in the seeds at maturity increased from 0.9 mg  $\text{g}^{-1}$  to 1.8 mg  $\text{g}^{-1}$ .

Sulphur addition also increased the concentrations of N in leaves and stems at early flowering and at maturity significantly (Figure 3a and 3b). The effect was most noticeable on leaf N concentration at early flowering, which was increased from 22 mg  $\text{g}^{-1}$  at  $S_0$  to 33 mg  $\text{g}^{-1}$  at  $S_{100}$ . At maturity, the concentrations of N in seeds and pod cases were not significantly influenced by the S treatments.

Sulphur uptake increased significantly with the increasing rate of S addition at both harvests (Figure 4a). At maturity, maximum S uptake in the shoots was only a quarter of the S added in the  $S_{100}$  treatment. There

was little increase in the S uptake from early flowering to maturity in the  $S_0$  treatment, indicating that the S supply from the soil was almost exhausted by early flowering. Sulphur addition also increased the total content of N in the shoots significantly at both harvests (Figure 4b), with the effect being most noticeable from the addition of S up to 25 mg  $\text{pot}^{-1}$ .

### Experiment 2

There were little differences in either shoot dry weight or leaf chlorophyll content between the  $S_0$  and  $S_{50}$  treatments at the first harvest (mid-stem elongation, Figure 5). But at the second (early flowering) and third harvest (pod fill), leaf chlorophyll content was significantly ( $p < 0.01$ ) higher in the  $S_{50}$  treatment than in the  $S_0$  treatment. The difference between the two treatments in shoot dry weight was still relatively small and not significant at the second harvest, and became significant ( $p < 0.05$ ) only at the last two harvests (Figure 5).

Concentrations of total S in the shoots were maintained at around 2.3–2.8 mg  $\text{g}^{-1}$  dry weight in the  $S_{50}$  treatment throughout the 4 harvests (Table 2). This compared to a decrease from 1.0 mg  $\text{g}^{-1}$  to 0.4 mg  $\text{g}^{-1}$  in the  $S_0$  treatment. Sulphur addition produced a large increase in the total S concentration of roots (Table 2). The concentration of S in the roots decreased slightly from 10.8 mg  $\text{g}^{-1}$  in the first harvest to 7.4 mg  $\text{g}^{-1}$  dry weight in the fourth harvest in the  $S_{50}$  treatment, whereas in the  $S_0$  treatment, root S concentration varied between 1.1 and 1.6 mg  $\text{g}^{-1}$  dry weight. The concentration of N was slightly higher in the roots than in the shoots (Table 2). Similar to the results from Experiment 1, addition of S generally increased the concentrations of N in both shoots and roots.

The amount of N fixed in pea was more than doubled by the addition of S throughout the four harvests (Figure 6a), with the effect being significant ( $p < 0.05$ ) at all harvests. The percentage of the N in pea plants that was derived from  $\text{N}_2$ -fixation increased considerably from the first to the final harvest. This percentage was also increased by the S addition, with the effect being significant in the first three harvests (Figure 6b).

## Discussion

Experiment 1 was carried out to establish the effects of a range of S additions to a S-deficient soil on the

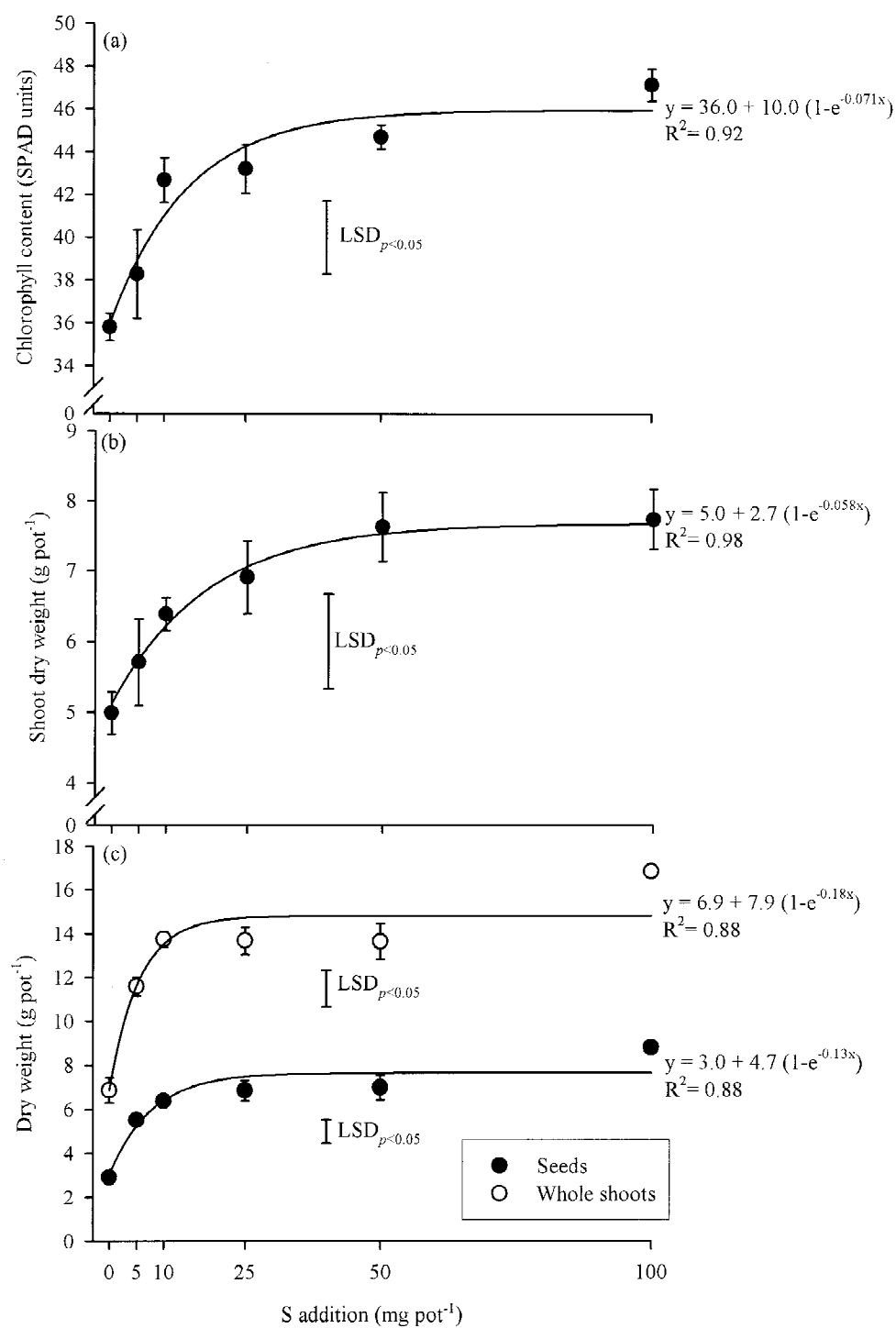


Figure 1. Effects of S addition on: (a) the chlorophyll content of pea leaves at early flowering; (b) shoot dry weight at early flowering; and (c) dry weights of shoots and seeds at maturity in Experiment 1. Lines represent best-fit exponential curves. Vertical bars associated with symbols are  $\pm$ SEs.

Table 1. Effects of S on yield components and harvest index of pea (Experiment 1)

S addition (mg pot <sup>-1</sup> )	Pod number	Seed number per pod	Single seed weight (g)	Harvest index
0	4.5	3.0	0.22	0.42
5	7.3	3.4	0.23	0.48
10	8.5	3.2	0.24	0.47
25	9.0	3.3	0.23	0.50
50	8.3	3.7	0.23	0.51
100	9.8	3.7	0.25	0.52
LSD <sub>p&lt;0.05</sub>	1.6	0.57	NS	0.027

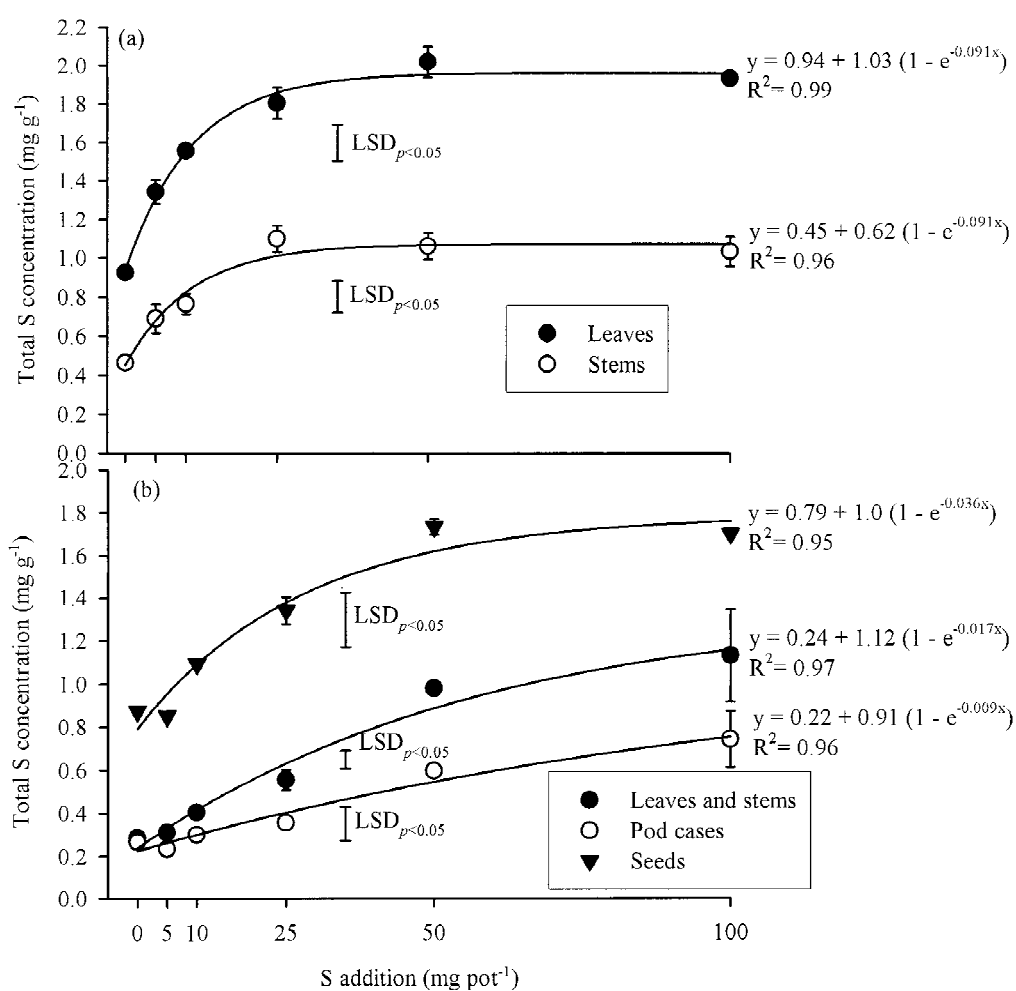


Figure 2. Effects of S addition on the concentrations of S in different tissues of pea at early flowering (a) and at maturity (b) in Experiment 1. Lines represent best-fit exponential curves. Vertical bars associated with symbols are  $\pm$ SEs.

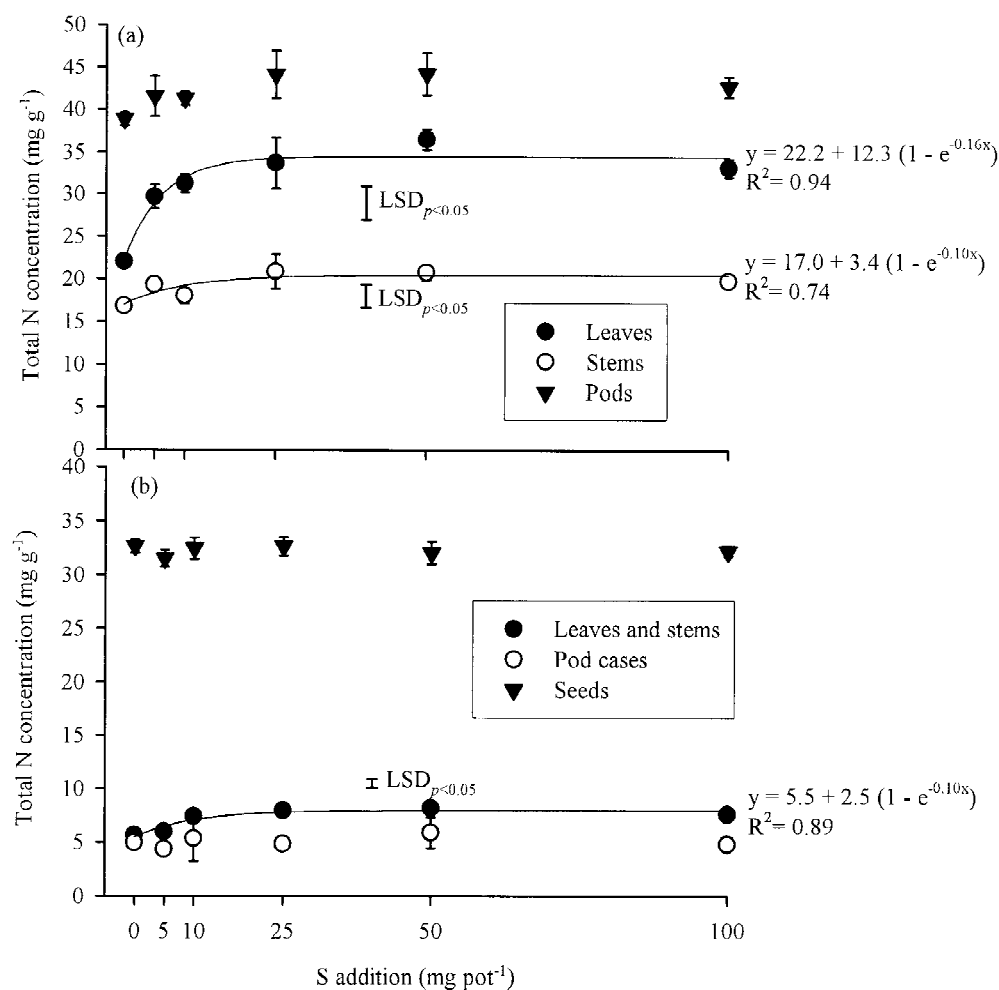


Figure 3. Effects of S addition on the concentrations of N in different tissues of pea at early flowering (a) and at maturity (b) in Experiment 1. Lines represent best-fit exponential curves. Vertical bars associated with symbols are  $\pm$ SEs.

growth and yield of pea. This showed that the seed yield of pea was increased markedly by the additions of S up to an optimal level of 50 mg pot<sup>-1</sup>. Furthermore, S additions also increased shoot N concentration significantly. Experiment 2 was then set up to determine the effects of S at the optimal rate of addition (50 mg pot<sup>-1</sup>) on the time-course of N<sub>2</sub>-fixation under identical conditions to those used in Experiment 1.

Experiment 2 showed that N<sub>2</sub>-fixation in pea was highly sensitive to S deficiency. The effect of S supply on N<sub>2</sub>-fixation was large and occurred well before the effects on leaf chlorophyll content or other dry matter production became apparent. The positive effect of S on the concentration of N in pea shoots was not observed in non-legume crops such as cereals and oilseed rape (McGrath and Zhao, 1996; Zhao et al.,

1996). The response patterns of leaf chlorophyll content and leaf N concentration to S were very similar, with the two measurements correlating linearly in Experiment 1 ( $R^2=0.65$ ,  $n=24$ ). This suggests that the decreased chlorophyll content in S-deficient pea leaves was likely to be caused by a shortage of N, as a result of decreased N<sub>2</sub>-fixation. Chlorosis in S-deficient pea plants occurred in all leaves, a feature that is different from non-legume plants in which yellowing usually occurs in the young leaves.

The results from this study support that of DeBoer and Duke on alfalfa (1982). These indicate that S deficiency has a direct effect on N<sub>2</sub>-fixation, and do not support the hypothesis that S deficiency affects photosynthesis first, resulting in a shortage of C and energy sources for N<sub>2</sub>-fixation (Zaroug and Munns, 1979).

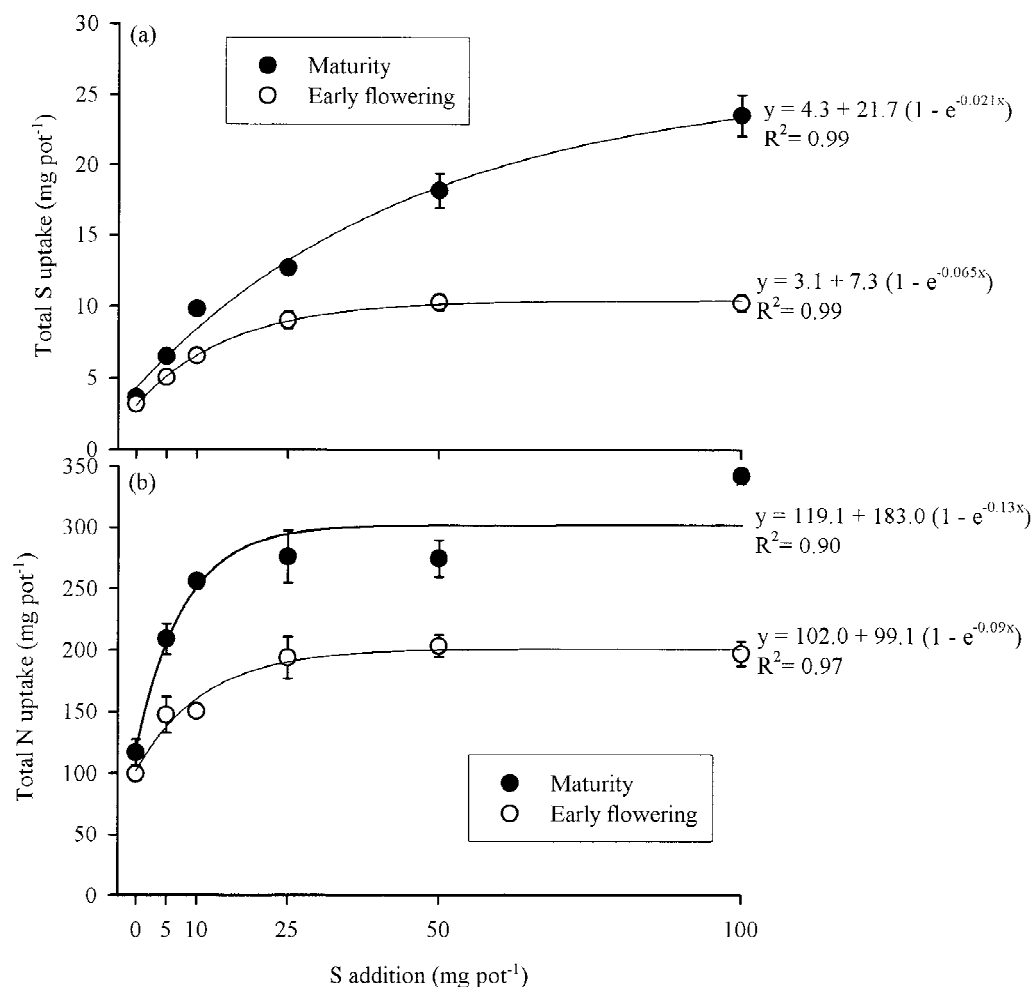


Figure 4. Effects of S addition on the uptake of S (a) and the total content of N in the shoots (b) at early flowering and at maturity in Experiment 1. Lines represent best-fit exponential curves. Vertical bars associated with symbols are  $\pm$ SEs.

Table 2. Effects of S on the concentrations of S and N in shoots and roots of pea (Experiment 2)

Days after sowing  (Harvest)	S concentration (mg g <sup>-1</sup> )				N concentration (mg g <sup>-1</sup> )			
	Shoots		Roots		Shoots		Roots	
	S <sub>0</sub>	S <sub>50</sub>	S <sub>0</sub>	S <sub>50</sub>	S <sub>0</sub>	S <sub>50</sub>	S <sub>0</sub>	S <sub>50</sub>
28 (1)	0.97	2.60***	1.44	10.81***	24.2	26.1	25.1	30.4
38 (2)	0.67	2.34***	1.58	10.40***	20.7	26.5*	25.8	28.1
56 (3)	0.56	2.67***	1.09	9.63***	17.9	22.6*	19.8	28.0***
73 (4)	0.37	2.83***	1.28	7.38***	16.4	20.7*	19.5	22.1

The level of significance from ANOVA: \*  $p < 0.05$ , \*\*\*  $p < 0.001$ .



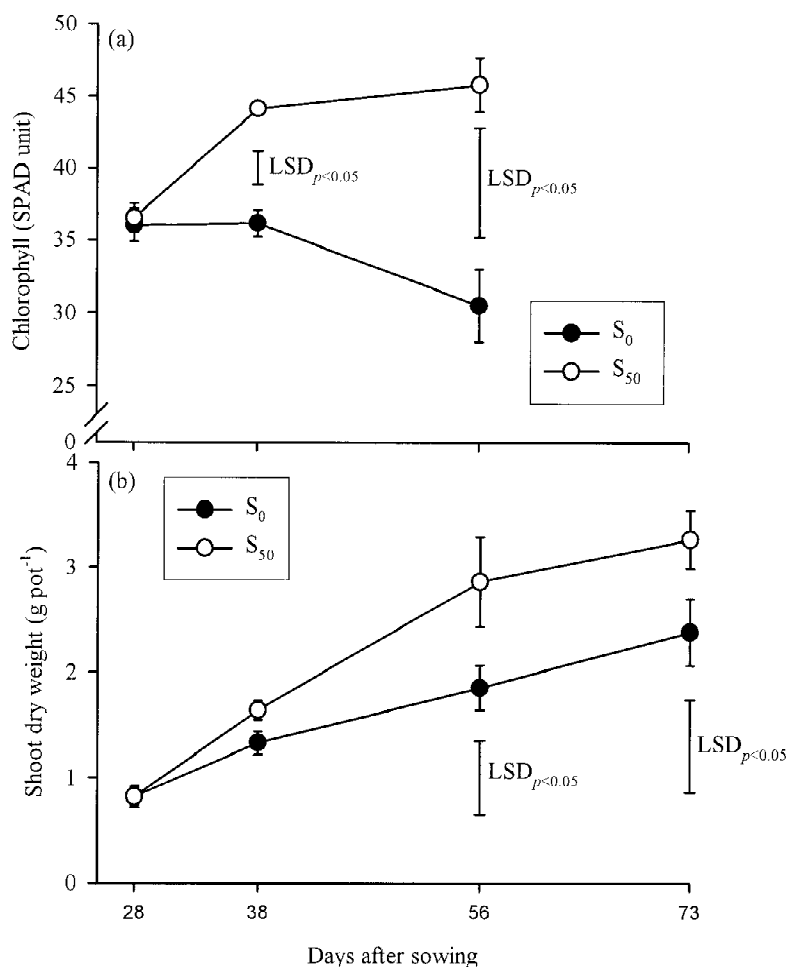


Figure 5. Effects of S on leaf chlorophyll content (a) and shoot dry weight of pea (b) at 4 growth stages in Experiment 2. Vertical bars associated with symbols are  $\pm$ SEs.

Our conclusion appears to contradict that of Anderson and Spencer (1950) and Robson (1983), who suggested that host plants have a greater requirement for S than the process of symbiotic N<sub>2</sub>-fixation. However, it is important to recognise the clear differences in the experimental approach. Firstly, this study and that of DeBoer and Duke (1982) measured N<sub>2</sub>-fixation directly, whereas Anderson and Spencer (1950) relied on the data of plant growth and N concentrations under different S and N treatments. Secondly, we examined the time-course of the effects of S deficiency, whereas Anderson and Spencer (1950) observed the effects of treatments at a single time point when plants had already developed severe deficiency symptoms. When S deficiency symptoms have developed, it is not surprising that photosynthesis and N metabolism

in leaves are disrupted (Friedrich and Schrader, 1978; Sexton et al., 1997).

In this study, nodules were visibly fewer and smaller in the S-deficient plants, although the number and weight of nodules were not determined. It is not clear which of the following phases, growth of the rhizobia, infection and nodule development, or nodule function, is affected more by S deficiency. Few studies have reported the concentration of S in roots. This study showed that the concentration of S in the roots of S-sufficient pea was surprisingly high, being 2.6–4.4 fold of that in the shoots. Even in the S<sub>0</sub> treatment, pea roots still had higher concentrations of total S than shoots. It is unusual for roots to have a much higher S concentration than shoots, because the latter usually contain more proteins than the former. The high S concentrations found in the pea roots probably

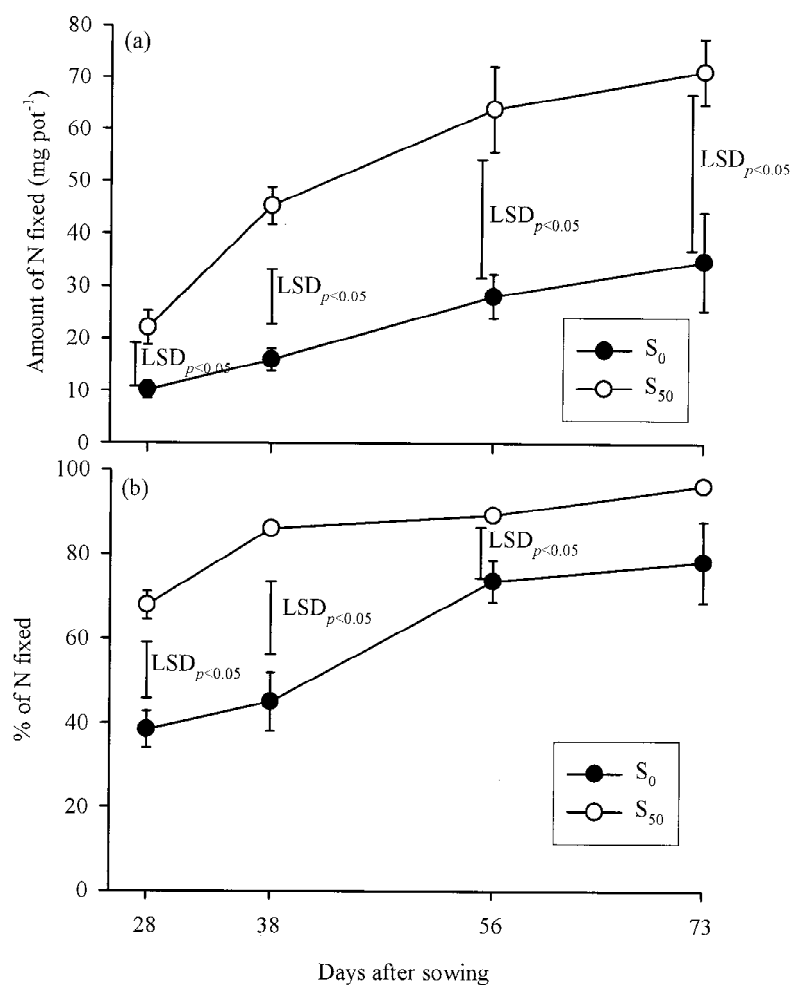


Figure 6. Effects of S on N<sub>2</sub>-fixation in pea in Experiment 2: (a) amount of N fixed and (b) percentage of N in pea that was derived from N<sub>2</sub>-fixation. Vertical bars associated with symbols are  $\pm$ SEs.

reflect a high requirement of S for the functions of nodules. Nitrogenase and ferredoxin, which play vital roles in N<sub>2</sub>-fixation, are rich in S, both containing Fe-S clusters (Duke and Reisenauer, 1986). Further studies are needed to quantify different S pools in the roots and nodules of legume plants, and to evaluate the effects of S on nodule production and functioning.

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