Journal of Experimental Botany, Vol. 46, No. 282, pp. 73–81, January 1995

Journal of Experimental Botany

Partitioning and redistribution of sulphur during S-stress in *Macroptilium atropurpureum* cv. Siratro

Christopher I. Bell[1,3], David T. Clarkson[2] and W. John Cram[1]

[1] *Department of Biology, The University, Newcastle upon Tyne NE1 7RU, UK*

[2] *Department of Agricultural Sciences, University of Bristol, BBSRC Institute of Arable Crops Research, Long Ashton Research Station, Bristol BS18 9AF, UK*

Received 2 June 1994; Accepted 14 October 1994

Abstract

During the first 7 d of sulphate-deprivation stored SO_4^{2-} was redistributed and assimilated into organic forms in the tropical legume *Macroptilium atropurpureum* cv. Siratro. However, whilst the sulphate content of all tissues declined after removing the external SO_4^{2-} supply this was slowest in mature leaves. By contrast, the total S content of mature leaves declined markedly in the absence of external sulphate whilst that of both young leaves and roots increased. Furthermore, when radiolabelled SO_4^{2-} was applied to abraded surfaces of mature leaves, most of the translocated label was recovered in the root following 2 d SO_4^{2-} deprivation. By contrast, radiolabelled SO_4^{2-} applied to young leaves was mostly retained in these tissues and not translocated.

Within 3 d of removing the SO_4^{2-} supply there was a large increase in extractable APS-sulphotransferase activity in roots accompanied by a decline in nitrate reductase activity, but these effects were not seen in leaves. Five days after the removal of SO_4^{2-} there was a large increase in the content of asparagine in roots.

The results are discussed in relation to the co-ordination of NO_3^- and SO_4^{2-} uptake and assimilation and the partitioning of sulphur during S-stress.

Key words: Sulphate supply, stomatal conductance, ATP-sulphurylase, APS-sulphotransferase, nitrate reductase.

Introduction

In higher plants it has often been reported that most sulphate reduction is located in the chloroplasts and, according to Anderson (1980), reduction in non-photosynthetic tissues 'is of minor importance'. However, it is known that cell, tissue and organ cultures from a wide variety of plant parts, including roots, can be grown on media where sulphate is the sole source of sulphur (Schiff, 1983). Non-green tissues of plants such as beet discs (Ellis, 1963) and roots (Brunold and Suter, 1989) also reduce sulphate and cultured roots clearly reduce enough sulphate to provide all the S-amino acids required for protein synthesis (Hawkesford and Belcher, 1991). Thus, the contribution of roots to total SO_4^{2-} reduction is by no means negligible. In particular, there has been no examination of how the S-status of the plant affects the balance between assimilation in roots and shoots.

In a previous study on *M. atropurpureum* it was shown that SO_4^{2-} appears to be far more readily mobilized from roots to young growing tissues than from mature leaves (Clarkson *et al.*, 1983). During a 5 d period in the absence of an external SO_4^{2-} supply there was an increase in protein-S which was most marked in roots even though approximately 50% of their total S was lost during the same period. Young leaves showed a decrease in concentration in all S-fractions measured. Working on spinach plants grown for 2 weeks without external sulphate, Dietz (1989) found that the inorganic SO_4^{2-} remaining in leaves formed a distinct concentration gradient dependent on leaf age; approximately one-third of the inorganic SO_4^{2-} was located in the oldest leaves (leaf pair $1+2, \approx 0.25$ mol m^{-3}) whereas SO_4^{2-} was not detectable in the youngest leaves (leaf pair $9+10$). It appears, therefore, that sulphur is unable to move out of leaves, even in the presence of a powerful stress-produced sink. The availability of S during S-stress will, therefore, clearly affect the assimilatory capacities of root and leaf tissues.

The first two steps in assimilatory sulphate reduction are catalysed by the enzymes ATP-sulphurylase and APS-

[3] To whom correspondence should be addressed at: Horticulture Research International, Wellesbourne, Warwick CV35 9EF, UK. Fax: +44 1789 470552.

sulphotransferase, respectively, and both appear to hold key positions in regulating the rate of sulphate assimilation (Brunold, 1990). When the external sulphate supply is decreased or removed, the extractable activity of ATP-sulphurylase (Haller *et al.*, 1986) and APS-sulphotransferase (Brunold *et al.*, 1987) is frequently found to increase. There is also evidence that the absence of a readily assimilated external nitrogen source decreases the activity of both enzymes (Brunold and Suter, 1984), and APS-sulphotransferase activity can be enhanced by the addition of either readily assimilated nitrogen-containing amino acids (Suter *et al.*, 1986) or NH_4^+ (Brunold and Suter, 1984). In addition, work on tobacco cell cultures demonstrated that, when sulphate limited growth, the induction of nitrate reductase by nitrate was proportional to the initial sulphate concentration and, when nitrate limited growth, the derepression of ATP-sulphurylase was proportional to the initial nitrate concentration (Reuveny *et al.*, 1980).

The aim of the present paper is to examine this putative reciprocal regulatory coupling between the nitrate and sulphate assimilatory pathways in more detail, and attempt to relate changes in sulphur assimilation and partitioning during S-stress to tissue sulphur status and to the retranslocation of sulphur between tissues.

Materials and methods

Plant culture

M. atropurpureum was grown as described previously (Bell *et al.*, 1994), except in experiments where the redistribution of ^{35}S was followed. In such experiments 5–7-d-old seedlings were transferred to 'uptake' vessels (Bell, 1991) through which 3.5 l of culture solution was recirculated from a reservoir by means of a peristaltic pump. All 'uptake' vessels and tubing were completely sealed from external light with black adhesive tape to minimize algal growth. Culture solutions were changed every 2–3 d.

Growth rate experiments

The sulphate supply to plants was either discontinued 3 weeks after germination (−S plants) or continued throughout the experimental period (+S plants). Plants were removed at intervals, divided into roots and shoots, blotted and the fresh weight recorded.

Retranslocation and partitioning of S during sulphur-stress

Plants were grown in 'uptake' vessels with $^{35}SO_4^{2-}$-labelled culture solution from the stage at which they were young seedlings for 14 d prior to experimentation. Plants were thus assumed to be at isotopic equilibrium with respect to sulphur (the relative growth rate of *M. atropurpureum* is approximately 0.25 d^{-1} (Bell, 1991), after a 14 d period of growth in the presence of $^{35}SO_4^{2-}$, 97% of the plants would have developed during the labelling period. The remaining 3% would have had 2 weeks equilibration).

At appropriate time intervals (both before and after the removal of external $^{35}SO_4^{2-}$) plants were harvested, divided into roots, old leaves and young leaves, blotted, weighed, and ^{35}S-sulphur fractions determined as described previously (Bell *et al.*, 1994). For convenience the sulphur-containing fractions referred to are SO_4^{2-}, 'soluble-S' (water-extractable non-sulphate-S), and 'insoluble-S' (not extractable in water). Briefly, tissue sulphate was extracted three times by freeze/thaw/grinding with liquid N_2, warming (40 min at 80 °C) in 10 mol m^{-3} $CaCl_2$ (10 ml g^{-1}), centrifugation (4 000 × *g*, 3 min) and removal of the supernatant. SO_4^{2-} concentrations were estimated turbidimetrically from the absorbance of the $BaSO_4$ precipitate at 420 nm after addition of $BaCl_2$ to the supernatant (Tabatabai and Bremner, 1970). Sulphate-sulphur was distinguished from 'soluble-S' by subtraction of the ^{35}S count after precipitation of SO_4^{2-} with $BaCl_2$ from the initial ^{35}S count. For determination of 'insoluble-S', the remaining tissue was digested in nitric/perchloric acid mixture. Samples were made up to a standard volume with water and an aliquot taken for scintillation counting.

Retranslocation of $^{35}SO_4^{2-}$ from leaves during S-stress

In order to monitor directly the flux of ^{35}S out of both mature and young leaves a 10 μl drop of carrier-free $^{35}SO_4^{2-}$ (0.2 MBq) was placed on a small area of leaf surface previously abraded with a piece of fine sandpaper. The drop of label was placed on only one of the three leaflets (leaves of *M. atropurpureum* are trifoliate) and evaporation was prevented by surrounding the drop with a plastic 'O-ring' sealed by a piece of clear plastic from above and silicon grease at the leaf surface. Two days prior to experimentation all leaves apart from the most mature and the youngest were removed in order to simplify the analysis, i.e. there could be no ambiguity in distinguishing between young and mature leaves. At appropriate time intervals roots and leaves were sampled for radioactivity. The leaflet on to which the tracer was applied was not sampled, however, as this inevitably contained $^{35}SO_4^{2-}$ which had remained on the leaf surface and had not been taken up by the plant. Results are presented on a 'percentage of total recovered' basis as the aim of the experiment was to determine differences in the mobility of ^{35}S out of both young and mature leaves either in the presence or in the absence of external SO_4^{2-}. Results are therefore purely comparative. Though the specific activity of the sulphur transported out of leaves was not known (therefore chemical flows could not be estimated), tracer flows would still be proportional to the chemical flows out of the leaf.

Amino acid analysis

Roots of *M. atropurpureum* were harvested at appropriate time intervals after the removal of external SO_4^{2-} from the culture solution. Samples (1–2 g) were freeze/thawed × 3 in liquid N_2 and amino acids extracted (10 ml g^{-1} FW) by shaking in ethanol : chloroform : water (12 : 3 : 3, by vol.). Samples were frozen overnight, defrosted, filtered (Watman No. 1), and made up to a volume of 20 ml with extraction fluid. 5 ml of chloroform was added, followed by 7.5 ml of water, and extracts were centrifuged at low speed. A 2 ml aliquot of the upper aqueous phase was evaporated to dryness at room temperature under vacuum and the residue re-disolved in 2–4 ml of 0.0025 mol m^{-3} nor-leucine. After filtration (Millipore, 0.22 μm), the amino acids were separated by HPLC (Dionex BIO LC, Amino-Pac PA1 amino acid analytical column), using a 50 μl injection, sodium buffer gradient with ninhydrin post-column derivatization (Trione reagent, Pickering Laboratories, Inc.), and visible spectroscopy. Peak areas were measured by a V.G. Multichrome Data Aquisition System (V.G. Laboratory Systems). The internal standard of 0.125×10^{-9} mol nor-leucine, included in each sample, was used to calculate any changes in response.

Enzyme assays

For determination of ATP-sulphurylase and APS-sulphotransferase (APSSTase) activity plant material was homogenized for 3×5 s in a cooled commercial homogenizer using 5 ml of extraction buffer per gram of tissue. The extraction buffer was 100 mol m^{-3} Tris/HCl (pH 7.8), containing 20 mol m^{-3} $MgCl_2$, 5 mol m^{-3} EDTA and 10 mol m^{-3} dithioerythritol (DTE). DTE was added to the extraction buffer immediately prior to use. Before assaying for enzyme activity the extract was strained through several layers of fine muslin, previously soaked in extraction buffer. For determination of nitrate reductase activity (*NRA*), crude extracts were prepared by grinding tissue in a chilled pestle and mortar using 2 ml of extraction buffer per gram tissue. The extraction buffer was 100 mol m^{-3} Na_2HPO_4/NaH_2PO_4 (pH 7.5), containing 1 mol m^{-3} EDTA, 1% BSA, 3 mol m^{-3} dithiothreitol (DTT), 10×10^{-6} mol leupeptin and 10×10^{-6} mol flavin adenine dinucleotide (FAD). The extract was squeezed through two layers af fine muslin and centrifuged at $14000 \times g$ for 15 min at 4 °C. For all enzyme assays described, extraction buffers/equipment were used at 4 °C and extracts were stored on ice until they were needed.

APSSTase was measured by the production of [^{35}S]-sulphite, assayed as acid-volatile radioactivity from [^{35}S]-APS in the presence of DTE (Brunold and Suter, 1984). ATP-sulphurylase activity was determined in the back reaction by measuring ATP formed from PPi and APS using a luciferin-luciferase system (Schmutz and Brunold, 1982). NADH-dependent nitrate reductase activity (*NRA*) was estimated by measuring the NO_2^- produced from NO_3^- as described by Fido (1985). Protein was measured according to Bradford (1976) using bovine serum albumin as a standard.

The effect of sulphur nutrition on enzymes of sulphur and nitrogen assimilation

Plants were grown as described previously until they were approximately 3-weeks-old and possessed at least two mature, fully expanded, leaves. Plants were then either maintained in complete nutrient solution or in nutrient solution −S. Tissue was harvested between 10.00 h and 12.00 h on the morning of the experiment.

Results

Plant growth

Up to 5 d after the removal of external sulphate there was no significant reduction in the growth of either roots or shoots compared to plants given 0.25 mol m^{-3} SO_4^{2-} throughout (Fig. 1). However, between the fifth and seventh day of sulphate starvation shoot growth was substantially reduced, but that of roots was much less affected. During this period, and especially during longer

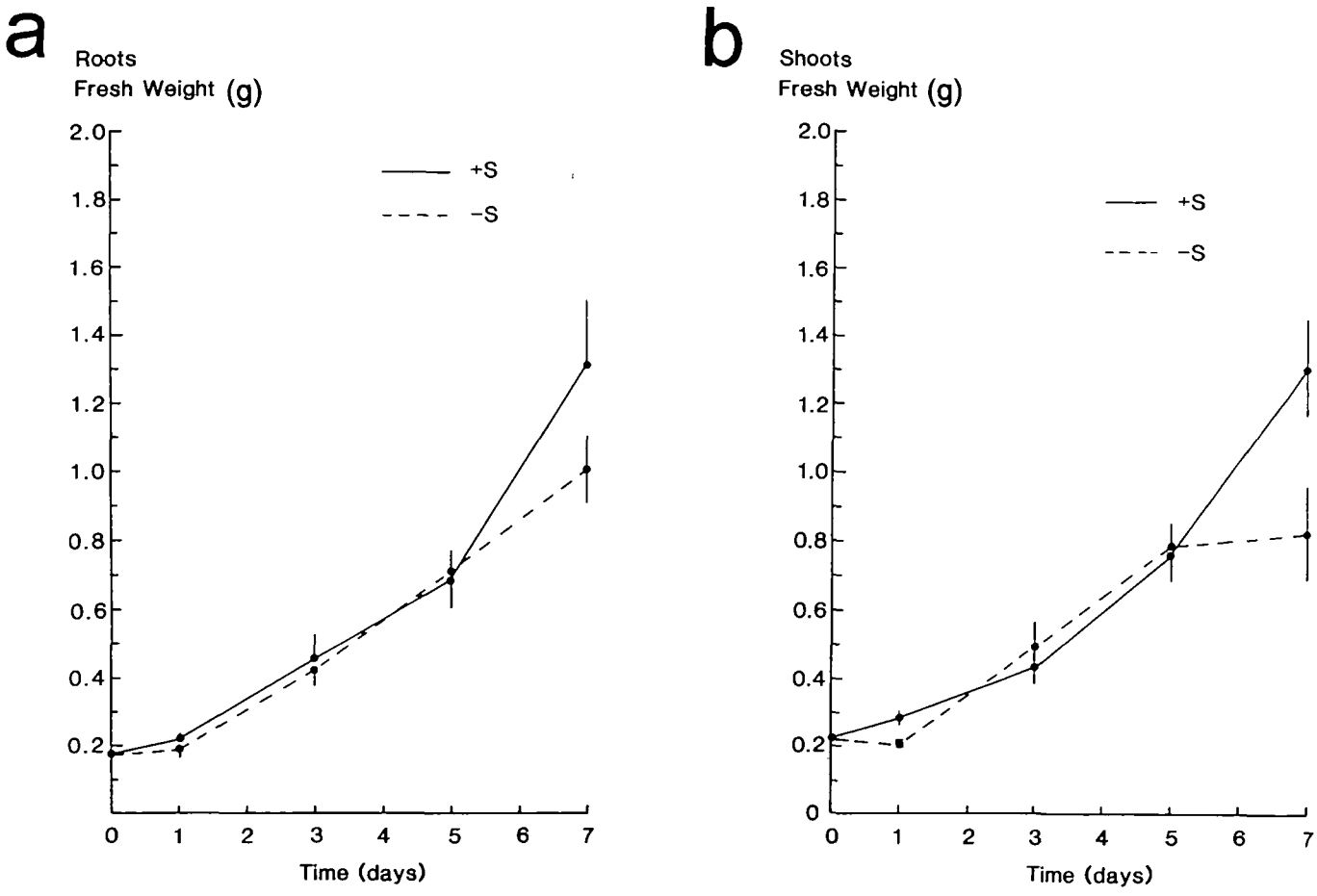

Fig. 1. Changes in fresh weight of roots (a) and shoots (b) of *M. atropurpureum* grown with (solid lines) or without (dashed lines) an external sulphate supply (0.25 mol m^{-3}). Bars represent SEM ($n = 10$).

Table 1. *Changes in the content of various S-containing fractions in tissues of plants grown in solutions lacking* SO_4^{2-}

Results are expressed in nmol equivalents based on the specific activity of the labelling solution. Values represent the means of five replicates from one representative experiment ± s.e.

S-containing fraction	Days $-SO_4^{2-}$	Tissue contents (nmoles)			
		Roots	Young leaves	Mature leaves	Stems
SO_4^{2-}	0	316±41	127±50	212±50	200±37
	3	63±36	64±20	90±23	126±50
	7	19±9	ND	51±19	4±2
'Water-soluble-S'	0	140±19	118±37	362±72	122±11
	3	380±61	177±33	614±67	166±30
	7	265±37	177±52	319±44	112±13
'Water-insoluble-S'	0	1579±28	1467±522	6045±124	1107±87
	3	3008±567	1390±297	5390±1252	847±80
	7	3188±313	1977±60	3579±471	846±26
Total S	0	2035±56	1712±342	6619±133	1429±91
	3	3451±401	1631±205	6094±802	1139±86
	7	3472±341	2154±73	3949±475	962±21

periods of sulphur starvation, newly emerging leaves appeared much paler green. Thus, the net effect of sulphate starvation was to increase the root/shoot fresh weight ratio.

Retranslocation and partitioning of S in plants deprived of SO_4^{2-}

In all tissues of *M. atropurpureum* the majority of the S is associated with water-soluble and insoluble organic compounds which are mostly protein. Inorganic SO_4^{2-} is never more than 16% of the total S. During SO_4^{2-} deprivation the internal sulphate pools were quickly used up, there being a more-or-less quantitative recovery of S in the soluble organic pool during the first 3 d following removal of external sulphate (Table 1). The total S invested in mature leaves and stems decreased markedly, there was no clear trend in the investment in young leaves, while that in roots increased. This result gives an impression of a net movement of S from the aerial parts to the roots.

Although the sulphate content of all tissues decreased, that of mature leaves decreased very much less than in the other tissues, so that after 7 d of sulphate deprivation, nearly 70% of the SO_4^{2-} in the plant was in the mature leaves.

Over the first 3 d of sulphate deprivation, roots lost SO_4^{2-} at a rate of approximately 50% d^{-1}, equivalent to a $k_v \approx 0.02\ h^{-1}$. This sulphate could be lost either by export or by local assimilation, but it is evident that there must be a net input of S into roots (Table 1). The rate constant for SO_4^{2-} loss from the mature leaves was $\approx 0.01\ h^{-1}$.

Table 2. *Percentage of* ^{35}S *recovered in various tissues after application of* $^{35}SO_4^{2-}$ *to abraded leaves in the presence or absence of an external* SO_4^{2-} *supply*

Values represent the means of two experiments made in triplicate ± s.e.

Tissue	Days ± S	$^{35}SO_4^{2-}$ applied to mature leaves		$^{35}SO_4^{2-}$ applied to young leaves	
		+S	−S	+S	−S
Roots	0	0	0	0	0
	1	52±8	43±8	18±5	0
	2	21±8	46±4	29±6	7±2
Young leaves	0	0	0	100	100
	1	27±9	24±4	70±13	91±1
	2	60±8	38±8	52±12	85±4
Mature leaves	0	100	100	0	0
	1	9±1	28±7	8±4	0
	2	6±0	8±4	16±5	0
Stems	0	0	0	0	0
	1	12±3	5±3	4±1	9±0
	2	13±1	8±2	3±1	8±2

Retranslocation of $^{35}SO_4^{2-}$ *applied to leaves during S-stress*

(i) Application of $^{35}SO_4^{2-}$ *to mature leaves*: In sulphur sufficient plants virtually no ^{35}S was recoverable in mature leaves at any time after the application of $^{35}SO_4^{2-}$ to one of the trifoliate leaflets (Table 2), i.e. the applied label was almost completely exported. One day after the addition of labelled sulphate, 52% of that detected was present in roots and almost 30% in young leaves. After 2 d, 60% of the tracer was recoverable in young leaves and just over 20% in roots.

However, after the removal of external sulphate, the greatest proportion of tracer was recoverable in roots,

both 1 d (43%) and 2 d (46%) after the application of tracer to a mature leaflet (Table 2). Two days after the removal of the sulphate supply 38% of the tracer was present in young leaves, but less than 10% was recovered in both stems and mature leaves after the same time period.

(ii) Application of $^{35}SO_4^{2-}$ to young leaves: Two days after the application of ^{35}S to leaf surfaces 52% of the tracer was retained within young leaf tissues in plants supplied with an external sulphate source (Table 2). By contrast less than 10% of the tracer recovered from that applied to mature leaves was recovered in the same tissue during the same time period (Table 2). Mature leaves, therefore, are clearly the primary 'source' leaves under S-sufficient conditions. Retention of the applied SO_4^{2-} in young leaf tissue was strongly increased after sulphate deprivation in the external medium. Here, only 15% was exported, half of which was recoverable in roots and half in stem tissues. No tracer could be detected in mature leaves following 2 d of S-starvation (Table 2).

Effect of sulphate-deprivation on the amino acid composition of roots

After the removal of external SO_4^{2-}, distinct changes were observed in the amino acid composition of roots (Table 3). There are several major points of interest. Firstly, both cysteine and methionine fell to almost unmeasurable levels within 24 h of removing the sulphate supply to roots. Neither of these amino acids could be reliably detected for the next 6 d. Secondly, the content of arginine, and, even more strikingly, asparagine, increased dramatically during the 7 d of sulphate deprivation. This was most marked between day 3 and day 7 (Table 3). Of the remaining amino acids, most showed a gradual decline in content during the 7 d −S, however, probably the most significant in this respect, were the falls in glutamine and glutamate (Table 3).

Table 3. *Changes in the contributions of individual amino acids to the total amino acid pool in roots of* M. atropurpureum *after the removal of external SO_4^{2-}*

Figures are expressed as a percentage of the total.

Amino acid	Days after the removal of external S				
	Day 0	Day 1	Day 3	Day 5	Day 7
Arg	3.09	2.78	3.50	7.92	12.5
Lys	2.35	1.58	1.66	0.85	0.42
Gln	11.1	8.10	8.23	7.35	3.23
Asn	28.9	41.5	36.2	50.6	66.2
Thr	3.98	2.94	3.87	4.53	3.29
Ala	7.47	3.64	6.34	5.78	2.08
Gly	4.49	1.68	3.75	2.90	0.91
Val	2.00	1.42	1.72	2.23	0.84
Pro	1.76	0.23	0.89	0.38	0 21
Ile	2.17	0.93	0.92	0.75	0.17
Leu	2.52	1.33	1.04	0.84	0.27
Met	1.40	0.00	0.02	0.00	0.09
His	2.43	1.83	1.59	0.92	0.86
Phe	2.06	0.41	0.76	0.69	0.24
Glu	11.2	10 4	10.6	5.86	3 34
Asp	2.63	7.21	6.71	3.72	1.99
Cys	0.79	0.16	0.03	0.01	0.00
Tyr	2.12	1.21	0.84	0.27	0.24
N-leu	7.50	13.0	11.0	4.40	3.00

Effect of sulphate-deprivation on ATP-sulphurylase, APSSTase and NR activity

From the *in vitro* assays for ATP-sulphurylase (Fig. 2) and APSSTase (Fig. 3) it is clear that roots of *M. atropurpureum* have the capacity for assimilatory sulphate reduction. Additionally, the activities of both enzymes were quite similar in roots and mature leaves, but both were more active in young leaves in plants adequately supplied with SO_4^{2-} (Figs 2, 3). During a period of declining tissue S-status the activity of ATP-sulphurylase remained at levels comparable to S-sufficient plants in leaves, but showed a slight increase in roots. By contrast, root APSSTase activity increased 7-fold within 3 d of the sulphur deprivation treatment (Fig. 3), at which point its activity was actually greater than in young leaves, and remained higher until the end of the experimental period. Though there appeared to be a transient increase in mature leaf ATP-sulphurylase after 1 d −S there were no other significant effects of sulphur nutrition on either S-assimilating enzyme. Any decreases in activity over the time-course of the experiment were, presumably, due to increasing tissue age.

Root *NRA* was found to be much greater than leaf *NRA* regardless of sulphur nutrition (Fig. 4). In addition, there appeared to be a substantial decrease in root *NRA* within 1 d of removing the external SO_4^{2-} supply (Fig. 4). This effect was still apparent after 7 d −S. As with some of the assays for ATP-sulphurylase and APSSTase activity, a decrease in *NRA* (root) was commonly observed during the course of the experimental period −S. This may reflect the attempt to reduce the variability found in *NRA* (younger roots tended to have higher *NRA*) by using mostly mature root tissue and avoiding newer growing regions. The decrease in *NRA* with time may be a consequence of using 'ageing' roots, taken from the same part of the root system in all plants assayed.

Discussion

During the first 7 d of sulphate deprivation both roots and leaves either mobilized or assimilated most of their SO_4^{2-}, though this was most rapid in roots. At the same time there was a dramatic decrease in the quantity of 'insoluble-S' in mature leaves, a large increase in roots, and a small but significant increase in young leaves (Table 1). However, although sulphur is redistributed

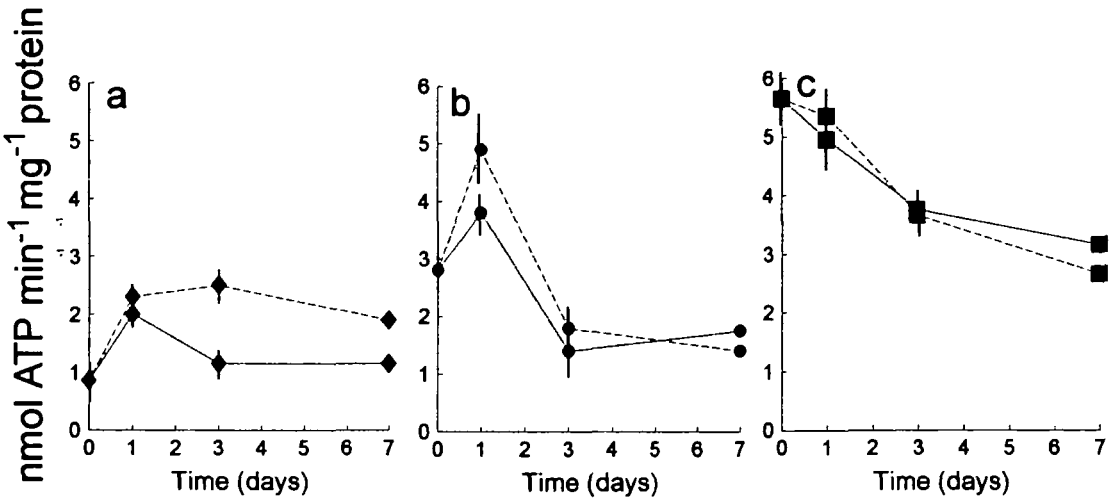

Fig. 2. Effect of removing the external sulphate supply on ATP-sulphurylase activity in roots (a), mature leaves (b) and young leaves (c) of *M. atropurpureum*. Solid lines correspond to control treatments given SO_4^{2-} at 0.25 mol m^{-3} and dashed lines to plants where the sulphate supply was removed at day 0.

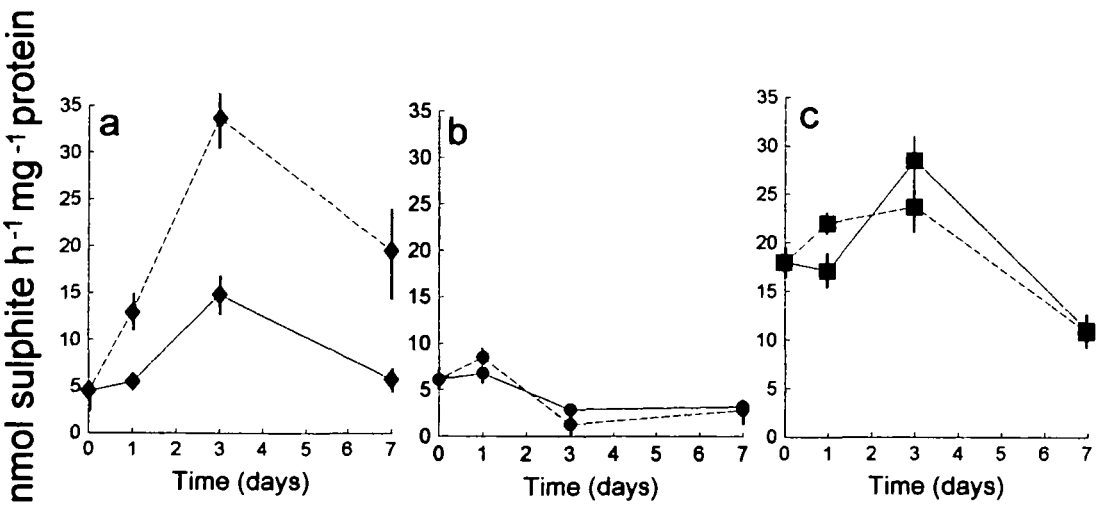

Fig. 3. Effect of removing the external sulphate supply on APS-sulphotransferase activity in roots (a), mature leaves (b) and young leaves (c) of *M. atropurpureum*. Solid lines correspond to control treatments given SO_4^{2-} at 0.25 mol m^{-3} and dashed lines to plants where the sulphate supply was removed at day 0.

between inorganic and organic forms and between mature leaves and roots it reaches the young, developing, foliage in only very limited quantities, 1:3 compared to roots (Table 1).

From results presented in Table 2 it appears that S is able to move out of both mature and young leaves during S-stress, though, it must be emphasized that this sulphur is not vacuolar in origin (as virtually all of the 'stored' SO_4^{2-} would be). Previous workers have also reported translocation of externally applied $^{35}SO_4^{2-}$ mainly as SO_4^{2-}, but also 7–8% as reduced sulphur (mostly as glutathione) out of mature leaves of tobacco (Rennenberg *et al.*, 1979) and *Ricinus* (Bonas *et al.*, 1982). However, in *Ricinus*, only 1–2% of the absorbed radiosulphur was exported within 2–3 h. (Bonas *et al.*, 1982). From these results it appears that, on average, less than 0.01% of the absorbed $^{35}SO_4^{2-}$ may be exported from leaves, in organic form, each hour. Recently, however, Schneider *et al.* (1994) reported that up to 77% of the externally applied ^{35}S-labelled glutathione which was taken up by a 1-year-old spruce needle was exported in the first 3 h, but the proportion of ^{35}S exported and its destination was influenced strongly by season (Schupp *et al.*, 1992). A more likely explanation, perhaps, of why S is not retranslocated from mature to young leaves, is that roots become the predominant sink during S-deprivation. Indeed, in *M. atropurpureum*, after the removal of external S, there is a 'shift of destination' for the sulphur translocated out of mature leaves towards the roots, at the expense of that translocated to the young leaves (Table 2). During the

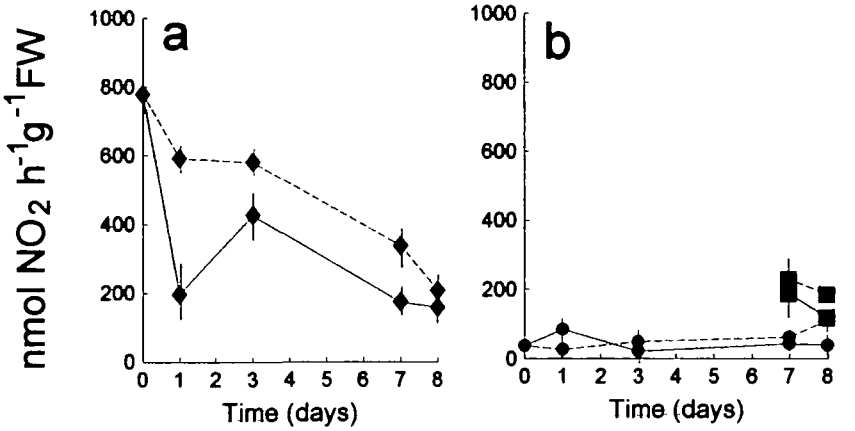

Fig. 4. Effect of removing the external sulphate supply on nitrate reductase activity in roots (a), and mature (●) and young leaves (■) (b) of *M. atropurpureum*. Solid lines correspond to control treatments given SO_4^{2-} at 0.25 mol m^{-3} and dashed lines to plants where the sulphate supply was removed at day 0.

first 24 h after the application of $^{35}SO_4^{2-}$ to mature leaves the same proportion of ^{35}S was transported to roots in the presence (and in the absence) of an external SO_4^{2-} supply, but, during the following 24 h, the proportion of ^{35}S in the roots fell. This can tentatively be viewed as recycling of S between roots and shoots. However, in the absence of external SO_4^{2-}, roots appeared to retain sulphur translocated from mature leaves (less is recycled). Indeed, exchange of reduced sulphur (glutathione) between phloem and xylem has been reported in spruce trees (Schneider *et al.*, 1994). These results, discussed above, are consistent with relative enlargement of the root system compared to the shoot during prolonged periods of S-stress (Fig. 1, Clarkson *et al.*, 1983).

Accompanying all of the symptoms of sulphur deficiency described thus far there was also a massive increase in the asparagine content and a large increase in the arginine content of roots (Table 3). These effects became most apparent after the first 3 d of sulphur deprivation and before root growth became restricted (Fig. 1). These accumulations in S-deprived roots resemble the situation reported for barley (Karmoker *et al.*, 1991). In barley, it was shown that the rate of protein synthesis had not been perturbed by SO_4^{2-}-deprivation at a time when asparagine concentrations had increased 4–5-fold. The specific increases in the amide and arginine concentration can not be because protein synthesis has stalled. The present analysis does not indicate the source of the accumulating amino compounds. It is possible that more amino-N is delivered to the roots than can be used (due to lack of S), and asparagine is an 'economic' storage compound (Rabe, 1990). Raven (1987) has calculated that in marine algae, although asparagine costs 11 times the energy needed to store the same amount of N as NO_3^-, in terms of water relations, the N storable in a given volume of vacuole as asparagine is 3.2 times that storable as KNO_3. A restriction in export to the xylem would also be part of the explanation of accumulation if the amino compounds were of shoot origin. Experiments with wheat and barley plants indicate that 30% of the amino-N delivered to the roots in the phloem is cycled by phloem/xylem interchange, without mixing with the N in the root. Only transport amino acids cycle in this way (Cooper and Clarkson, 1989). Thus, a small adjustment in the rate of cycling would cause the build-up observed in the present work.

How does the retranslocation of sulphur and the redistribution of SO_4^{2-} from inorganic to organic forms relate to the processes of assimilatory SO_4^{2-} reduction?

Under many conditions roots do not seem to contribute appreciably to the needs of plants for reduced sulphur (Brunold, 1990). The specific activities of both ATP-sulphurylase (Adams and Rinne, 1969) and APSSTase (Schmidt, 1976) in roots have been commonly found to be 10% or less of that in shoots. However, working on enzyme localization in pea seedling roots, Brunold and Suter (1989) reported that the total root activities of ATP-sulphurylase and APSSTase were 50% and 30%, respectively, of those in the shoot. This indicates that the root has the potential for sulphate reductive assimilation at least equal to providing for its own needs. From results presented in Figs 2 and 3 it also appears that the highest specific activity of ATP-sulphurylase and APSSTase, respectively, in *M. atropurpureum* is in young leaves under conditions where sulphate is adequately supplied. The activity of both enzymes in roots was comparable to that in mature leaves, but, much less than that in young leaves. Thus, the activity of both ATP-sulphurylase and APSSTase in roots could approach 50% of the total plant activity (Figs 2,3), given that mature leaves account for most of the foliar tissue, and that the root/shoot fresh weight ratio is close to 1 (Fig. 1). It is evident, therefore, that roots of *M. atropurpureum* are not necessarily dependent on the shoots for reduced sulphur compounds.

Indeed Brunold and Suter (1989) have demonstrated that excised roots and roots of intact 5-d-old pea plants assimilated comparable amounts of radioactivity from $^{35}SO_4^{2-}$ into the amino acid and protein fractions during a 1 h period, indicating that these plants were also not solely dependent on the shoots for reduced sulphur compounds. Root APSSTase activity in *M. atropurpureum* increases greatly once the external SO_4^{2-} supply is removed (Fig. 3). Though there may also be a small increase in root ATP-sulphurylase activity during S-deprivation (Fig. 2), there were no other marked effects on ATP-sulphurylase or on APSSTase activity either in young or mature leaves (Figs 2, 3). Generally, the activities of both ATP-sulphurylase (Adams and Rinne, 1969; Passera and Ghisi, 1982; Archer, 1987; Schmutz and Brunold, 1984; Brunold *et al.*, 1981) and APSSTase (Schmutz and Brunold, 1984; Brunold *et al.*, 1987) have been found to increase in leaves during sulphur deprivation, though this effect is more pronounced in cell cultures (Reuveny and Filner, 1977; Bergmann *et al.*, 1980; Zink, 1984; Haller *et al.*, 1986). Additionally, in *M. atropurpureum*, the increase in APSSTase root activity observed after the removal of external SO_4^{2-} (Fig. 3) was accompanied by a decrease in nitrate reductase activity (Fig. 4).

These data bear on the question of coupling of the nitrate and sulphate assimilatory pathways first suggested from work with tobacco cell cultures by Reuveny and Filner (1977). Subsequently, there have been many reports on the effects of N and S status on enzyme activities in their respective assimilatory pathways (Brunold and Suter, 1984). Although a more thorough investigation of this aspect is called for, there does appear to be a certain amount of regulatory coupling in the nitrate and sulphate assimilatory pathways in *M. atropurpureum*. It might be noted that *NRA* did not appear to be more severely repressed after 3–5 d of sulphate deprivation, when the levels of asparagine were found to increase very markedly (Table 3). Thus, there is no evidence to support the idea that this amide can repress the expression of *NRA*. Glutamine, whose concentration was seen to increase with sulphate deprivation in barley roots (Karmoker *et al.*, 1991) did not increase in *M. atropurpureum*.

Working with *Lemna minor*, Brunold *et al.* (1987) reported close correlations between APSSTase activity and the content of asparagine, glutamine, non-protein thiols, and SO_4^{2-}. Specifically, when the tissue sulphate and thiol concentrations fell, the extractable APSSTase activity rose along with the tissue asparagine and glutamine contents. These findings prompted the authors to postulate a negative control mechanism induced by SO_4^{2-} and thiols, and a positive mechanism induced by amides. Suter *et al.* (1986) also reported 50–110% increases in APSSTase activity within 6 h of feeding 2 mM asparagine, arginine and glutamine to *Lemna* plants. It has also been suggested that the reduced uptake of NO_3^- observed in S-stressed barley plants might be due to feedback inhibition on transport at the plasmalemma due to an accumulation of 'transport amino acids' in the roots (Clarkson *et al.*, 1989).

In conclusion, therefore, it appears that removal of the external SO_4^{2-} leads to a decrease in the tissue SO_4^{2-} content of the roots followed by an increase in APSSTase activity (and possibly ATP-sulphurylase) and a build-up of transport amides in roots. The subsequent effect of this build-up of amino-N compounds appears to be 2-fold. Firstly, it may stimulate further APSSTase activity, and secondly, it inhibits further uptake of nitrate into the roots. Further to this, Neyra and Hagemann (1975), found that the rate of induction of nitrate reductase in maize roots was regulated by the rate of nitrate flow into the roots, i.e. the pattern of induction of nitrate reductase was coincident with the pattern of nitrate uptake, as a function of time and increasing nitrate concentrations. The inhibition of nitrate uptake during S-stress appears to affect nitrate reductase activity directly in plant roots. The build-up of transport amides thus provides a common mechanism by which the regulatory coupling of nitrate and sulphate assimilatory pathways could be achieved. As mentioned previously, however, the build-up of asparagine in roots of *M. atropurpureum* appears to occur after the repression of *NRA* activity, though these measurements were from different experiments. The correlation between the build-up of asparagine and the increase in APSSTase activity in roots is, however, better — both peak on day 3 of S-deprivation.

The coupling of nitrogen assimilation (into *O*-acetylserine) with sulphur assimilation into cysteine may also require the presence of an, as yet unidentified, nitrogen-containing compound (Giovanelli, 1990). From data presented here, asparagine is a prime candidate for this role, at least in *M. atropurpureum*.

Acknowledgements

The gift of ^{35}S-APS from Professor C. Brunold is gratefully acknowledged. Thanks are also due to Lez Saker for help with the amino acid analysis. CIB acknowledges the financial support of the SERC.

References

Adams CA, Rinne RW. 1969. Influence of age and sulphur metabolism on ATP sulphurylase activity in the soybean and survey of selected species. *Plant Physiology* **44,** 1241–5.

Anderson JW. 1980. Assimilation of inorganic sulphate into cysteine. In: Miflin BJ, ed. *Biochemistry of plants.* New York: Academic Press, 203–25.

Archer MJ. 1987. Effect of differential sulphur nutrition on the

specific activity of ATP sulphurylase in grains from five wheat cultivars. *Australian Journal of Plant Physiology* **14,** 239–44.

Bell CI. 1991. The efficiency of sulphur use and its regulation of transport in *Macroptilium atropurpureum* cv. Siratro. PhD thesis, University of Newcastle upon Tyne.

Bell CI, Cram WJ, Clarkson DT. 1994. Compartmental analysis of $^{35}SO_4^{2-}$ exchange kinetics in roots and leaves of a tropical legume *Macroptilium atropurpureum* cv. Siratro. *Journal of Experimental Botany* **45,** 879–86.

Bergmann L, Schwenn JD, Urlaub H. 1980. Adenosine triphosphate sulphurylase and *O*-acetyl serine sulphydrylase in photoheterotrophically and heterotrophically cultured tobacco plants. *Zeitschrift für Naturforschung* **35c,** 952–7.

Bonas U, Schmitz K, Rennenberg H, Bergmann L. 1982. Phloem transport of sulphur in *Ricinus*. *Planta* **155,** 82–8.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein–dye binding. *Analytical Biochemistry* **72,** 248–54.

Brunold C. 1990. Reduction of sulphate to sulphide. In: Rennenberg H, Brunold C, DeKok LJ, Stulen I, eds. *Sulfur nutrition and sulfur assimilation in higher plants*. The Hague: SPB Academic Publishing, 13–31.

Brunold C, Suter M. 1984. Regulation of sulphate assimilation by nitrogen nutrition in the duckweek *Lemna minor* L. *Plant Physiology* **76,** 579–83.

Brunold C, Suter M. 1989. Localization of enzymes of assimilatory sulphate reduction in pea roots. *Planta* **179,** 228–34.

Brunold C, Suter M, Lavanchy P. 1987. Effect of high and low sulphate concentrations on adenosine 5′-phosphosulphate sulphotransferase activity from *Lemna minor*. *Physiologia Plantarum* **70,** 168–74.

Brunold C, Zryd J-P, Lavanchy P. 1981. Regulation of enzymes of assimilatory sulphate reduction in aerated cell suspension cultures of *Nicotiana sylvestris*. *Plant Science Letters* **21,** 167–74.

Clarkson DT, Hawkesford M, Saker LR. 1989. The sulphate transporter in roots: physiological behaviour and attempted identification. In: Dainty J, DeMichelis MI, Marrè E, Rasi-Caldogno F, eds. *Plant membrane transport: the current position*. Amsterdam/New York: Elsevier, 401–2.

Clarkson DT, Smith FW, Van den Berg PJ. 1983. Regulation of sulphate transport in a tropical legume *Macroptilium atropurpureum* cv. Siratro. *Journal of Experimental Botany* **34,** 1463–83.

Cooper HD, Clarkson DT. 1989. Cycling of amino nitrogen and other nutrients between shoots and roots in cereals; a possible mechanism integrating shoot and root in the regulation of nutrient uptake. *Journal of Experimental Botany* **40,** 753–62.

Dietz K-J. 1989. Leaf and chloroplast development in relation to nutrient availability. *Journal of Plant Physiology* **134,** 544–50.

Ellis RJ. 1963. Cysteine biosynthesis in beet discs. *Phytochemistry* **2,** 129–36.

Fido RJ. 1985. Spinach nitrate reductase: *In vitro* reconstitution, purification and production of monoclonal antibodies. PhD thesis, University of Bristol.

Giovanelli J. 1990. Regulatory aspects of cysteine and methionine biosynthesis. In: Rennenberg H, Brunold C, DeKok LJ, Stulen I, eds. *Sulfur nutrition and sulfur assimilation in higher plants*. The Hague: SPB Academic Publishing, 33–48.

Haller H, Suter M, Brunold C. 1986. Regulation of ATP sulphurylase by the sulphur and nitrogen source in heterotrophic cell suspension cultures of Paul's scarlet rose. *Journal of Plant Physiology* **125,** 275–83.

Hawkesford MJ, Belcher AR. 1991. Differential protein synthesis in response to sulphate and phosphate deprivation: identification of possible components of plasma membrane transport systems in cultured roots. *Planta* **185,** 323–9.

Karmoker JL, Clarkson DT, Saker LR, Rooney JM, Purves JV. 1991. Sulphate deprivation depresses the transport of nitrogen to the xylem and the hydraulic conductivity of barley (*Hordeum vulgare* L.) roots. *Planta* **185,** 269–78.

Neyra CA, Hagemann RH. 1975. Nitrate uptake and induction of nitrate reductase in excised corn roots. *Plant Physiology* **56,** 692–5.

Passera C, Ghisi R. 1982. ATP-sulphurylase and *O*-acetyl-L-serine sulphydrylase in isolated mesophyll protoplasts and bundle sheath strands of S-deprived maize leaves. *Journal of Experimental Botany* **33,** 432–8.

Rabe E. 1990. Stress physiology: the functional significance of the accumulation of nitrogen-rich compounds. *Journal of Horticultural Science* **65,** 231–43.

Raven JA. 1987. The role of vacuoles. *New Phytologist* **106,** 357–422.

Reuveny Z, Filner P. 1977. Regulation of ATP-sulphurylase in cultured tobacco cells: effects of sulphur and nitrogen sources on formation and decay of the enzyme. *Journal of Biological Chemistry* **252,** 1858–64.

Reuveny Z, Dougall DK, Trinity PM. 1980. Regulatory coupling of nitrate and sulphate assimilation pathways in cultured tobacco cells. *Proceedings of the National Academy of Science USA* **77,** 6670–2.

Rennenberg H, Schmitz K, Bergmann L. 1979. Long-distance transport of sulphur in *Nicotiana tabacum*. *Planta* **147,** 57–62.

Schiff JA. 1983. Reduction and other metabolic reactions of sulphate. In: Pirson A, Zimmerman MH, eds. *Encyclopedia of plant physiology*, Vol. 15A. Berlin/Heidelberg/New York: Springer-Verlag, 401–21.

Schmidt A. 1976. The adenosine-5′-phosphosulphate sulphotransferase from spinach (*Spinacea oleracea* L.). Stabilization, partial purification and properties. *Planta* **130,** 257–63.

Schmutz D, Brunold C. 1982. Rapid and simple measurements of ATP sulphurylase activity in crude plant extracts using an ATP meter for bioluminescence determination. *Analytical Biochemistry* **121,** 151–5.

Schmutz D, Brunold C. 1984. Intercellular localization of assimilatory sulphate reduction in leaves of *Zea mays* and *Triticum aestivum*. *Plant Physiology* **74,** 866–70.

Schneider A, Schatten T, Rennenberg H. 1994. Exchange between phloem and xylem during long-distance transport of glutathione in spruce trees (*Picea abies* [Karst.] L.). *Journal of Experimental Botany* **45,** 457–62.

Schupp R, Schatten T, Willenbrink J, Rennenberg H. 1992. Long-distance transport of reduced sulphur in spruce (*Picea abies* L.). *Journal of Experimental Botany* **43,** 1243–50.

Suter M, Lavanchy P, Von Arb C, Brunold C. 1986. Regulation of sulphate assimilation by amino acids in *Lemna minor* L. *Plant Science Letters* **44,** 125–32.

Tabatabai MA, Bremner JM. 1970. A simple turbidimetric method of determining total sulphur in plant materials. *Agronomy Journal* **62,** 805–6.

Zink MW. 1984. Regulation of ATP-sulphurylase by various nitrogen and sulphur sources in cultured *Ipomea* sp. *Canadian Journal of Botany* **62,** 2107–13.