Sulphur supply to peas (*Pisum sativum* L.) influences symbiotic N_2 fixation

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ABSTRACT

In a pot experiment (culture substrate: perlite with a granulometric composition 0–3 mm) with peas (*Pisum sativum* L.) we studied the influence of sulphur supply on S and N concentrations, yield formation, symbiotic N_2 fixation and available amounts of glucose and sucrose. Under S deficiency conditions S and N concentrations as well as the amounts of glucose and sucrose in shoots and nodules were significantly reduced. We assume that the reduced amounts of available photosynthate with suboptimal S supply could become limiting to energy production and as carbon skeletons for ammonia assimilation, and therefore cause a lower N_2 fixation and a reduced yield formation.

Keywords: peas; sulphur supply; carbohydrates; symbiotic N_2 fixation

In many previously sulphur sufficient areas of the world increasing S deficiency has been reported in the last years. This is because less S is being added to soils due to the use of high-analysis low-S containing fertilizers, the decreasing use of S-containing fungicides and pesticides, high yielding varieties and intensive agriculture and the reduction of sulphur dioxide immission from industrial sources (Scherer 2001, Eriksen et al. 2004).

Leguminous plant species require a large quantity of sulphur, probably because of their high protein content. Therefore S deficiency in legume crops not only affects yield formation, but also the quality and the nutritional value seeds (Sexton et al. 1998), because methionine is usually the most limiting essential amino acid in legume seeds (Friedman 1996). According to DeBoer and Duke (1982) S deficiency markedly reduced the mole percent of cysteine and methionine in the total leaf protein fraction of Lucerne (*Medicago sativa* L.) while storage proteins in legume seeds like the pea storage protein vicilin, which contains no cysteine and methione, increased (Spencer et al. 1990).

Legumes crops obtain N mainly from symbiotic N_2 fixation, which may be affected by S deprivation. In pot experiments with different legumes Scherer and Lange (1996) found a lower N accumulation and a yield reduction when S was limiting. Therefore Lange (1998) suggests that S affects leguminous plant species growth through its effect upon N_2 fixation by *Rhizobium* microorganisms. With S deficiency amino acids and other N forms, accumulating due to a lack of being synthesized into proteins, may have a feed-back repression on nitrogen fixation (Jannsen and Vitosh 1974). Furthermore S deficiency may affect N_2 fixation because of the relatively high S content of the nitrogenase (Mortenson and Thornley 1979) and of ferredoxin (Yoch 1979).

The carbon costs for N_2 fixation vary with the host species. The energy burden is approximately 6 mg C/mg N reduced. According to Pate and Herridge (1978) nodules may consume up to 50% of the photosynthates produced by legume plants. They are the ultimate source of carbon for both N_2 fixation and assimilation (Vance et al. 1998). It may be speculated that the synthesis of photosynthates is limited under S deficiency conditions, resulting in a reduced N_2 fixation. Therefore the present paper deals with investigations on the influence of the S supply on the amounts of glucose and sucrose and on the symbiotic N_2 fixation of peas (*Pisum sativum* L.).

MATERIAL AND METHODS

Experimental design. Stained seeds of peas (*Pisum sativum* L., cv. Miami) were inoculated with *Rhizobium leguminosarum biovar*: *viciae*

(Radicin No. 4; Jost, Germany) and germinated in sand. 10 days after germination 10 uniform seedlings at the two-leaf stage were transferred to 10 l-containers, containing Perlite (Perligran G; Deutsche Perlite GmbH, Germany; granulometric composition 0–3 mm). During a period of five weeks after transplanting until to the first harvest the following amounts of fertilisers per pot were applied: 1660 mg K (K_3PO_4, K_2SO_4 or K_2HPO_4), 654 mg P (K_3PO_4, Na_2HPO_4, NaH_2PO_4 or K_2HPO_4), 100 mg N (NH_4NO_3), 103 mg Mg ($MgCl_2$), 100 mg Fe ($FeCl_3$), 20 mg Mn ($MnCl_2$), 20 mg Zn ($ZnCl_2$), 20 mg Cu ($CuCl_2$), 5 mg B (H_3BO_3), 5 mg Mo (Na_2MoO_4), 5 mg Co [$Co(NO_3)_2$] and 2000 mg Ca ($CaCO_3$) per pot. The nutrients except Ca were applied in liquid form. Two S levels were established: 0 mg S (S_0) and 200 mg S (S_{200} = control) as SO_4^{2-}. Each treatment comprised four replications for each cropping. After the final fertilisation irrigation was continued with distilled water. The water capacity was placed at 100% and controlled gravimetrically twice a day. The plants were grown in a greenhouse and harvested 46, 53 and 60 days after sowing. The first harvest started about 6 weeks after sowing. At harvest the youngest completely developed leafs including their internodes (2 g fresh weight) were collected and immediately frozen in liquid nitrogen, followed by dividing the plants into shoots and roots. Nodules were isolated on an ice bath by forceps and kept immediately in liquid nitrogen.

N/S determination in dry matter of shoots and nodules. Shoots and nodules were oven dried at 70°C immediately after each harvest. Samples were ground to a powder and aliquots analyzed for S and N by a C/N/S-Elemental-Analyser (Euro EA).

Assay for carbohydrates. 2 g of shoot fresh matter and 1 g of nodule fresh matter, respectively, were ground in 1 ml of distilled water, transferred into micro-tubes and heated at 100°C for 10 minutes. After centrifugation (14 000 g, 15 min) glucose and sucrose were determined using test-combinations from Boehringer Mannheim (Germany)/R-Biopharm (UV method). Glucose concentration was determined before and after enzymatic hydrolysis. NADPH formed in the enzymatic reactions is stoichiometric to the amount of glucose and was measured photometrically by means of the light absorbance at 340 nm. The sucrose content was calculated from the difference of the glucose concentrations before and after enzymatic inversion. Calculation of the investigated concentrations resulted from the following general equation:

$$c = (V \times MW/\varepsilon \times d \times v \times 1000) \times \Delta A \text{ (g/l)}$$

where: V = final volume (ml), v = sample volume (ml), MW = molecular weight of the substance to be assayed (g/l), d = light path (cm) and ε = extinction coefficient of NADPH (at 340 nm = 6.3 ($L \times mmol^{-1} \times cm^{-1}$).

Statistics. Statistical analysis were performed using SPSS procedures. Significant levels were checked by the Tukey test at the 5% level.

RESULTS AND DISCUSSION

About five weeks after transplanting the seedlings shortage of sulphur was visible in treatment S_0, where plants showed yellowish leaves with chlorotic margins and a weaker growth. Reduction in chlorophyll concentrations with S deficiency has been noted (Friedrich and Schrader 1978) and chlorosis is a common symptom of S deficiency in many plant species (Stewart and Porter 1969). Confirming results of Fox et al. (1964), who observed that alfalfa yields were more than doubled with optimum S supply, the application of 200 mg S/pot resulted in a significant yield increase of the shoots (Figure 1). Under optimum growth conditions plants accumulate N and S which are proportional to that incorporated into protein.

When S is limiting protein synthesis is inhibited, resulting in lower yields. However, it should be pointed out, that the influence of S supply on the nodule formation was even more pronounced (Figure 2). In our investigation nodules were visibly smaller and fewer in treatment S_0. Already 46 days after sowing dry matter yield of nodules in treatment S_{200} was about four times as high as under S deficiency conditions, and 60 days after sowing dry matter yield of nodules was even seven times higher with optimum S supply. Already Anderson and Spencer (1950) found that in S deficient subterranean clover (*Trifolium subterraneum*) nodulation was markedly decreased. They attributed this to a decline in the requirement for N with reduced S.

The S concentrations of the shoots, which were significantly influenced by the S supply, decreased in both treatments with each successive harvest (Table 1). They ranged between 0.064 and 0.032% in the dry matter in treatment S_0 and between 0.171 and 0.140% in treatment S_{200}. This study further showed surprisingly high S concentrations in the nodules. Even in the treatment S_0 nodules

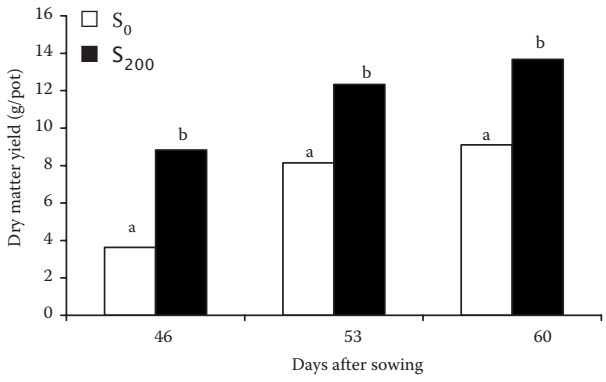

Figure 1. Influence of S supply on dry matter (dm) yield of shoots; different superscripts denote significant statistical difference at the 5% level (ANOVA)

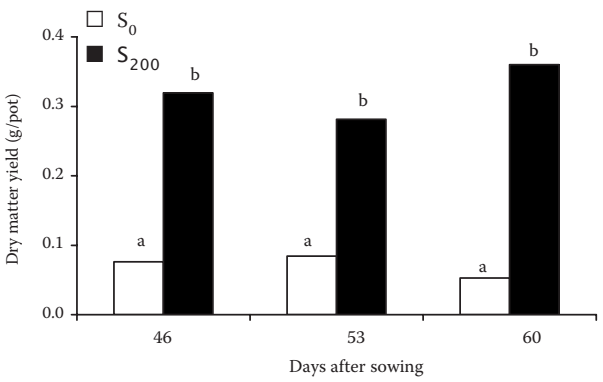

Figure 2. Influence of S supply on dry matter (dm) yield of nodules; different superscripts denote significant statistical difference at the 5% level (ANOVA)

still had higher S concentrations than shoots of the control. These high S concentrations in nodules probably reflect the high S requirement for the functions of the nodules. Ferredoxin as well as nitrogenase, which contain Fe-S-clusters and play a vital role in N_2 fixation, are rich in S (Duke and Reisenauer 1986).

N concentrations of the shoots of peas were influenced by the S supply (Table 1). They were significantly higher with optimum S supply and ranged between 2.89 and 3.10% in the time-course in the control and between 1.85 and 2.21% in treatment S_0. Independent of the S supply the N concentrations of the nodules were higher as compared with those the shoots. However, S deficiency resulted in lower N concentrations of nodules. The decrease in N concentration under S deficiency implies a parallel decrease in N_2 fixation, which may be indicative of a S limitation on protein synthesis.

N_2 fixation of peas was calculated, assuming that all of the N applied to the plants as mineral fertilizer (100 mg/pot) in our experiment was taken up completely. During the time-course it ranged between 20.1 and 113.9 mg N/pot in treatment S_0 and between 235.1 and 365.0 mg N/pot in treatment S_{200}. The results suggest that symbiotic N_2 fixation was substantially impaired by insufficient S supply. Taking the differences between the treatments S_0 and S_{200} in total N accumulation at the final harvest as a contribution of symbiotic N_2 fixation, additional 250 mg N/pot were fixed as a result of an optimum S supply. As put forward by Parsons et al. (1993) a nitrogen feedback mechanism could be the main trigger for regulating both nodulation and N_2 fixation, both being reduced under S deficiency conditions.

Lower N concentrations as well as a reduced N_2 fixation under suboptimum S conditions were also established in other legumes (Shock et al. 1984, Collins et al. 1986), which may be partly due to limiting the synthesis of the enzymatic machinery for reducing inorganic N (DeBoer and Duke 1982). According to Sexton et al. (1997) S deficiency at first influences protein synthesis and later on photosynthesis. Work of Wheeler (1971) has established marked diurnal variation in N_2 fixation, indicating that this process is quite sensitive to supply of photosynthetic assimilates

Table 1. Influence of S supply on S and N concentrations of shoots and nodules of peas; different superscripts denote significant statistical difference at the 5% level (ANOVA)

	Treatment	S (%)			N (%)		
		days after sowing					
		46	53	60	46	53	60
Shoots	S_0	0.064^a	0.034^a	0.032^a	2.21^a	1.85^a	1.90^a
	S_{200}	0.171^b	0.148^b	0.140^b	3.10^b	2.95^b	2.89^b
Nodules	S_0	0.25^a	0.21^a	0.23^a	5.34^a	5.41^a	3.92^a
	S_{200}	0.59^b	0.45^b	0.37^b	6.54^b	5.93^b	5.03^b

Table 2. Influence of S supply on symbiotic N_2 fixation of peas; different superscripts denote significant statistical difference at the 5% level (ANOVA)

Treatment	Days after sowing		
	46	53	60
	(mg N/pot)		
S_0	20.1[a]	97.9[a]	113.9[a]
$S_{20}0$	235.1[b]	327.1[b]	365.0[b]

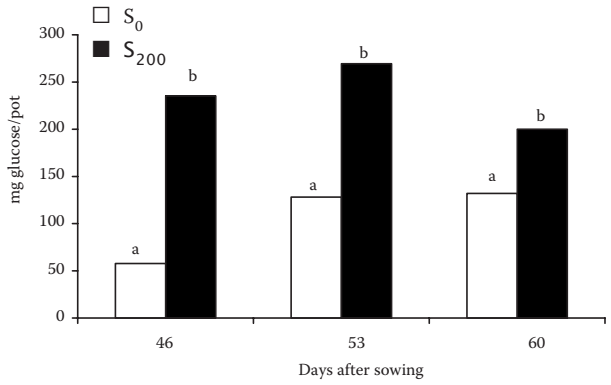

Figure 3. Available amounts of glucose in shoots; different superscripts denote significant statistical difference at the 5% level (ANOVA)

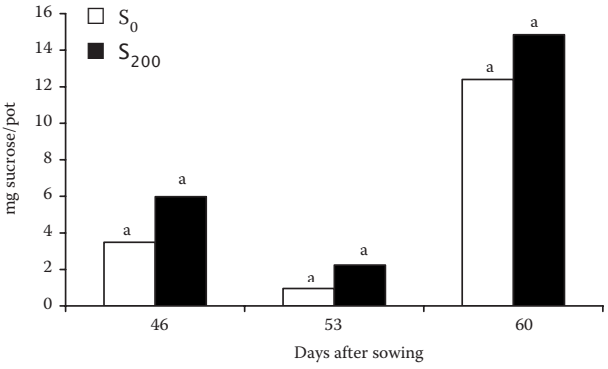

Figure 4. Available amounts of sucrose in shoots; different superscripts denote significant statistical difference at the 5% level (ANOVA)

The ultimate source of energy for symbiotic N_2 fixation in the nodule are carbohydrates and the most important functions of the carbon metabolism are the provision of energy for the host cell and the bacteroids and to provide carbon skeletons for the transport of fixed nitrogen. To show the totally available amounts of carbohydrates, we present the total amounts of glucose and sucrose, respectively, on per pot base. During the time-course of the experiment the amounts of glucose in the shoots of peas were influenced by the S supply (Figure 3). With suboptimum S supply they were significantly lower and ranged between 58 and 132 mg glucose/pot, while in the control they ranged between 200 and 235 mg glucose/pot. In the control the amount of glucose was highest 53 days after sowing and declined at pod setting. We assume that this decline is the result of a sink competition between the developing seeds and the nodules. Besides glucose the amounts of sucrose and results of Lawn and Brun (1974) indicate that the decline in nodule activity is associated with the development of the pods of soybeans as a competing assimilate sink, since the decline in nodule activity coincided with the time when pod growth rate first exceeded total crop growth rate. For this reason it may be assumed that photosynthate translocation to roots and nodules may also become a limiting factor in N_2 fixation of S starved legumes.

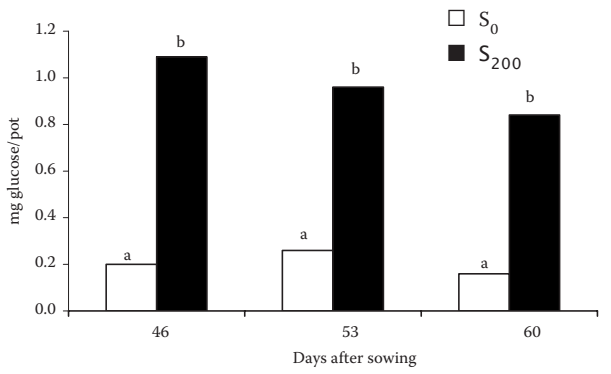

Figure 5. Available amounts of glucose in nodules; different superscripts denote significant statistical difference at the 5% level (ANOVA)

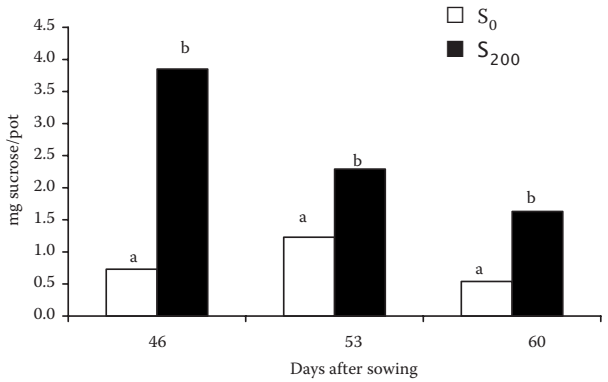

Figure 6. Available amounts of sucrose in nodules; different superscripts denote significant statistical difference at the 5% level (ANOVA)

in the shoots were influenced by the S supply. However, the differences between S_0 and S_{200} were not significant. Independent of the S supply the amounts of sucrose decreased after the first harvest and drastically increased after the second harvest (Figure 4). However, this observation cannot be explained.

The high demand of available carbohydrates for N_2 fixation under optimum conditions is reflected in the amounts of glucose (Figure 5) and sucrose (Figure 6) in the nodules, provided by the shoots. After the transport of carbohydrates into the nodules, they are metabolized to form organic acids, to cover the energy demand of nitrogenase (Vance et al. 1998). As compared to treatment S_0 in the control treatment the amounts of glucose and sucrose were up to five times higher. Both declined during the time-course, indicating a stronger sink of the developing pots later in the growing period. Under S deficiency conditions the amounts of glucose and fructose stayed on the same low level, indicating that vegetative and reproductive growth of the plant has preference. These observations suggest that available photosynthate could become limiting to energy production and as carbon skeletons for ammonia assimilation, resulting in a lower N_2 fixation and a reduced yield formation.

REFERENCES

Anderson A.J., Spencer D. (1950): Sulphur in nitrogen metabolism of legumes and non-legumes. Aust. J. Sci. Res. B, *3*: 431–449.

Collins M., Lang D.J., Kelling K.A. (1986): Effects of phosphorus, potassium and sulphur on alfalfa nitrogen fixation under field conditions. Agron. J., *78*: 959–963.

DeBoer D.L., Duke S.H. (1982): Effects of sulphur nutrition on nitrogen and carbon metabolism in lucerne (*Medicago sativa* L.). Physiol. Plant., *54*: 343–350.

Duke S.H., Reisenauer H.M. (1986): Roles and requirements of sulfur in plant nutrition. In: Tabatabai M.A. (ed.): Sulfur in agriculture. Agron. Monogr. No. 27, Am. Soc. Agron., Madison, Wisconsin, USA: 123–168.

Eriksen J., Thorup-Kristensen K., Askegaard M. (2004): Plant availability of catch crop sulphur following spring incorporation. J. Plant Nutr. Soil Sci., *167*: 609–615.

Fox R.L., Olson R.A., Rhoades H.F. (1964): Evaluating the sulfur status of soils by plants and soil tests. Soil Sci. Soc. Amer. Proc., *28*: 243–246.

Friedman M. (1996): Nutritional value of proteins from different sources: a review. J. Agr. Food Chem., *44*: 2–29.

Friedrich J.W., Schrader L.E. (1978): Sulfur deprivation and nitrogen metabolism in maize seedlings. Plant Physiol., *61*: 900–907.

Janssen K.A., Vitosh M.L. (1974): Effect of lime, sulphur, and molybdenum on N_2 fixation and yield of dark red kidney beans. Agron. J., *56*: 736–740.

Lange A. (1998): Influence of sulphur supply on N_2-fixation of legumes. [Ph.D. Thesis.] Univ. Bonn, Germany.

Lawn R.J., Brun W.A. (1974): Symbiotic nitrogen fixation in soybeans. I. Effect of photosynthetic source-sink manipulations. Crop Sci., *14*: 11–16.

Mortensen L.E., Thornley R.N.F. (1979): Structure and function of nitrogenase. Ann. Rev. Biochem., *48*: 387–418.

Parsons R., Stanforth A., Raven A.J., Sprent J.I. (1993): Nodule growth and activity may be regulated by a feedback mechanisms involving phloem nitrogen. Plant Cell Environ., *16*: 125–136.

Pate J.L., Herridge D.F. (1978): Partitioning and utilization of net photosynthate in nodulated annual legumes. J. Exp. Bot., *29*: 401–412.

Scherer H.W. (2001): Sulphur in crop production – invited paper. Eur. J. Agron., *14*: 81–111.

Scherer H.W., Lange A. (1996): N_2 fixation and growth of legumes as affected by sulphur fertilization. Biol. Fertil. Soils, *23*: 449–453.

Sexton P.J., Batchelor W.D., Shibles R. (1997): Sulfur availalability, rubisco content, and photosynthetic rate of soybean. Crop Sci., *37*: 1801–1806.

Sexton P.J., Paek N.C., Shibles R. (1998): Soybean sulphur and nitrogen balance under varying levels of available sulphur. Crop Sci., *38*: 975–982.

Shock C.C., Williams W.A., Jones M.B., Center D.M., Phillips D.A. (1984): Nitrogen fixation by subclover associations fertilized with sulphur. Plant Soil, *81*: 323–332.

Spencer D., Rerie W.G., Randall P.J., Higgins T.J.V. (1990): The regulation of pea seed storage protein genes by sulphur stress. Aust. J. Plant Physiol., *17*: 355–363.

Stewart B.A., Porter L.K. (1969): Nitrogen sulfur relationships in wheat, corn, and beans. Agron. J., *61*: 276–271.

Vance C.P., Miller S.S., Driscoll B.T., Robinson D.L., Trepp G., Gantt J.S., Samas D.A. (1998): Nodule carbon metabolism: Organic acids for N_2 fixation. In: Elmerich E. et al. (eds.): Biological Nitrogen Fixation for the 21st Century. Kluwer Acad. Press Publ., the Netherlands: 443–448.

Wheeler C.T. (1971): The causation of the diurnal changes in nitrogen fixation in the nodules of *Alnus glutosina*. New Phytol., *70*: 487–495.

Yoch D.C. (1979): Electron-transport systems coupled to nitrogenase. In: Hardy R.W.F. et al. (eds.): A Treatise on Dinitrogen Fixation. John Wiley and Sons, New York: 605–652.

Received on June 3, 2005

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