How does sulphur availability modify N acquisition of white clover (Trifolium repens L.)?

Sébastien Varin¹, Jean-Bernard Cliquet²*, Emmanuelle Personen², Jean-Christophe Avice² and Servane Lemauviel-Lavenant²

¹ Université de Caen, UMR 950, Ecophysiologie Végétale Agronomie et Nutritions NCS, INRA/Université de Caen, Esplanade de la Paix, F-14032 Caen Cedex, France
² INRA, UMR 950, Ecophysiologie Végétale Agronomie et Nutritions NCS, INRA/Université de Caen, Esplanade de la Paix, F-14032 Caen Cedex, France

Received 8 June 2009; Revised 14 August 2009; Accepted 9 September 2009

Abstract

The role of S in legume growth, N uptake, and N₂ fixation was investigated using white clover (Trifolium repens L.) as a model species. We examined whether the effect of sulphate addition on N fixation resulted from a stimulation of host plant growth, a specific effect of S on nodulation, or a specific effect of S on nodule metabolism. Clones of white clover, inoculated with Rhizobium leguminosarum, were grown for 140 d in a hydroponic system with three levels of sulphate concentration (0 mM, 0.095 mM, and 0.380 mM). Nodule morphological and biochemical traits, such as root length, nodule biomass and volume, nodule protein contents (nitrogenase and leghaemoglobin obtained by an immunological approach), and root amino acid concentrations, were used to analyse the effect of sulphate availability on N₂ fixation. The application of sulphate increased whole plant dry mass, root length, and nodule biomass, expressed on a root-length basis. N uptake proved less sensitive than N₂ fixation to the effects of S-deficiency, and decreased as a consequence of the lower root length observed in S-deficient plants. N₂ fixation was drastically reduced in S-deficient plants as a consequence of a low nodule development, but also due to low nitrogenase and leghaemoglobin production. This effect is likely to be due to down-regulation by a N-feedback mechanism, as, under severe S-deficiency, the high concentration of whole plant N and the accumulation of N-rich amino acids (such as asparagine) indicated that the assimilation of N exceeded the amount required for plant growth.

Key words: Amino acids, legumes, N₂-fixation, nitrogenase, nodules, sulphur (S) deficiency.

Introduction

Legumes are environmentally, agriculturally, and economically important in many cropping systems because of their ability to reduce atmospheric N₂ symbiotically. Because the use of nitrogen (N) fertilizer contributes substantially to environmental pollution, this biological alternative has received increasing attention in agricultural practices in the last few years. Legumes are particularly sensitive to a wide range of environmental limitations. Studies on mineral requirements of the Legume–Rhizobia symbiosis, phosphorus (P), and to a lesser extent potassium (K), have received considerable attention because N, P, and K are frequently the most limiting nutrients for plant growth in numerous ecosystems (Høgh-Jensen, 2003; Olivera et al., 2004). It is now well documented that legume growth and nitrogen (N) fixation are highly responsive to P and K supplies.

The effect of sulphur (S) supply on N₂ fixation has received less attention, as sulphate availability in soil has not been considered in the past as a limiting factor for plant growth. However, crop deficiencies of S have been reported with increasing frequency in the last decade, caused by decreasing anthropogenic S input and by the lack of input through S fertilization to compensate for exportation (Scherer, 2001). S is accumulated in plants in low concentrations compared to N, but is an essential element as...
a constituent of proteins, Cysteine-containing peptides such as glutathione, or numerous secondary metabolites. S deficiencies have been observed in Brassicaceae, Poaceae, but also in Fabaceae. Fabaceae species require a large quantity of S, probably because of their high protein concentration (Aulakh et al., 1976) and because S is crucial for N2 fixation (Scherer and Lange, 1996; Krusell et al., 2005; Scherer et al., 2008).

S-deficiency causes a decrease in N acquisition by plants as a result of a decrease in nitrate uptake (Clarkson et al., 1989; Karmoker et al., 1991) or N2 fixation (Zhao et al., 1999; Scherer et al., 2008). The low N2 fixation observed in S-deficient plants can result in lower plant growth (Scherer and Lange, 1996) or in a specific effect of S on N2 fixation (Zhao et al., 1999; Pacyna et al., 2006; Scherer et al., 2008). Low N2 fixation is due to the effect of S on nodule growth (Schulze and Drevon, 2005; Habtemichial et al., 2007) or nodule metabolism (Pacyna et al., 2006; Scherer et al., 2008). In a previous study, a specific effect of S on nodulation; (iii) a specific effect of S on N2 fixation (Scherer and Lange, 1996) or in a specific effect of S on N2 fixation (Zhao et al., 1999; Pacyna et al., 2006; Scherer et al., 2008).

The objective of this study was to reconcile conflicting reports on the role of S in symbiotic N fixation by investigating this effect in white clover (Trifolium repens L.), a major legume in Western Europe grasslands and a species highly sensitive to sulphate supply (Tallec et al., 2008; Varin et al., 2009a, b). The following questions were addressed. Does the stimulation of N2 fixation by S supply result from (i) a stimulation of host plant growth; (ii) a specific effect of S on nodule growth; (iii) a specific effect of S on nodule metabolism? To answer these questions, white clover plants of the same genotype have been subjected to three sulphate availability levels, and nodule morphological and biochemical parameters have been analysed in relation to the modification of plant dry mass.

Materials and methods

Plant material, clone production, and growth conditions

Clones of Trifolium repens L. cv. Huia were obtained by vegetative multiplication of the stolon of one individual chosen as an average individual from a population. Each individual consisted of a 20 mm stolon section including a node. When the primary leaf appeared, each fragment was transferred to a hydroponic system supplying a continuously aerated nutrient solution in 1.0 l black plastic bottles to maintain darkness in the rooting environment.

Plants were grown in a greenhouse with light measured at the plant level equal to 400 μmol m−2 s−1 (sodium high pressure ‘phytolaude 400 W’ and sun) providing a 16/8 h photoperiod with 25/16 °C day/night. The plants were inoculated with Rhizobium leguminosarum bv. trifolii T354 three times a week during the four weeks after planting. The nutrient solution was prepared with demineralized water, changed weekly, and contained 2 mM KNO3, 0.18 mM CaCO3, 0.4 mM KH2PO4, 0.15 mM K2HPO4, 3 mM CaCl2, 0.2 mM EDTA 2NaFe (3H2O), 14 μM H3BO3, 3 μM ZnCl2, 0.7 μM CuCl2, 0.7 μM (NH4)2MoO4, 0.1 μM CoCl2. S was added as MgSO4 and three treatments were chosen; ‘zero S’, ‘low S’ (0.095 mM SO42−), and ‘high S’ (0.380 mM SO42−). MgSO4 was partly or entirely replaced by equimolar amount of MgCl2 and KCl for the zero and low S treatments. The sulphate concentration of the zero S solution was measured by HPLC and found to be nil, showing that no other source of sulphate was available for plant growth. The nutrient solution contained 15NO3− 0.5 atom% excess to allow the measurement of NO3− absorbed from the nutrient solution and N2 fixed from the atmosphere.

Plants were harvested 70 d and 140 d after transfer into the hydroponic solution, separated into shoots, roots, and nodules and then dried at 70 °C and weighed when mass was constant. The determination of the relative chlorophyll concentration using the non-destructive SPAD (Soil Plant Analysis Development) chlorophyll meter (Minolta, SPAD-502 model, Tokyo, Japan) was also performed on leaves. Aliquots of roots and nodules were collected in an ice bath and frozen for morphological and biochemical analysis (amino acids and proteins).

Root and nodule morphological analysis

Root and nodule morphological parameters were measured using an EPSON Expression 1000XL scanner (Regent instruments Inc, Quebec, Canada). Individual root systems and nodules were spread out on a clear tray in an isotonic solution of KCl 9 g l−1 and placed on a flat-bed scanner. Root length and nodule volume were measured using the WinRHIZO software.

Isotopic analysis

All organ dry matter was reduced to a fine powder for elemental and isotopic analyses. Total N, total S, and 15N were determined from 1 mg of plant material using an elemental analyser (EA3000, EuroVector, Milan, Italy) linked to an isotope ratio mass spectrometer (Isoprime, GV instrument, Manchester, UK). The use of 15NO3− allowed the measurement of the relative contributions of N derived from the nutrient solution (N absorbed) and N derived from the atmosphere (N fixed) (Høgh-Jensen and Schjoerring, 1994):

\[
\text{N absorbed (mg) = } \left(\frac{\text{atom} \% \text{ excess of nutrient solution}}{\text{atom} \% \text{ excess of nutrient solution}}\right) \times Q_N (\text{mg})
\]

where QN is the amount of N per plant. A non-fixing plant, ryegrass, grown in the same conditions was used as a control to estimate atom% excess of nutrient solution (Høgh-Jensen and Schjoerring, 1994). The amount of N fixed was estimated as the difference between QN and N absorbed:

\[
\text{N fixed (mg) = Q}_N (\text{mg}) - \text{N absorbed (mg)}
\]

Extraction and quantification of proteins from nodules

Total proteins were extracted from nodules using the method described by Wang et al. (2003) and Desclos et al. (2009). Frozen nodule samples were ground to a fine powder with a semi-automatic crusher in the presence of liquid nitrogen and resuspended in 2 ml of cold acetone containing 10% TCA (w/v). After centrifugation at 16 000 g for 3 min at 4 °C, the supernatant was
Effect of sulphate availability on N₂ fixation | 227

Protein spots of interest were manually excised from the gel, washed several times with water, and dried for a few minutes. Trypsin digestion was performed overnight with a dedicated automated system (MultiPROBE II, PerkinElmer). The gel fragments were subsequently incubated twice for 15 min in a H₂O/CH₃CN solution to allow extraction of peptides from the gel pieces. Peptide extracts were then dried and dissolved in starting buffer for chromatographic elution, consisting of 3% CH₃CN and 0.1% HCOOH in water. Peptides were enriched and separated using lab-on-a-chip technology (Agilent, Massy, France) and fragmented using an on-line XCT mass spectrometer (Agilent). The fragmentation data were interpreted using the DataAnalysis program (version 3.4, Bruker Daltonic, Billerica, USA). For protein identification, tandem mass spectrometry peak lists were extracted and compared with the protein database using the MASCOT Daemon (version 2.1.3; Matrix Science, London, UK) search engine. The searches were performed with no fixed modification and with variable modifications for the oxidation of methionines, and with a maximum of one missed cleavage. Tandem mass spectrometry spectra were searched with a mass tolerance of 1.6 Da for precursor ions and 0.8 MS/MS fragments. The LC MS/MS data were converted into DTA-format files which were further searched for proteins with MASCOT Daemon. Peptides matching an individual ion score >51 were considered. Proteins with two or more unique peptides matching the protein sequence were automatically considered as a positive identification. Measured peptides were searched in the NCBI–protein sequence databases, Viridiplantae (green plants) and Bacteria (Eubacteria).

**Amino acid extraction and analysis**

Frozen plant material was freeze-dried and amino acids were extracted from 10±5% mg of milled plant material in 400 ml of methanol containing 200 μM 3-aminobutyric acid as an internal standard and samples were agitated at 1500 rpm for 15 min. Subsequently, 200 ml of chloroform was added and samples were agitated again for 5 min. Finally, 400 ml of ultra-pure water was added and samples were then vigorously vortexed and centrifuged at 13 000 g for 5 min. The upper phase was transferred to a microtube and dried under vacuum over-night. Dry residues were resuspended in an appropriate volume of ultra-pure water and 10 μl of the resulting extract was sampled for amino acid derivatization according to the AceQ Tag Ultra Derivitization Kit protocol (Waters Corp., Milford, USA). Amino acids were analysed using an Acquity™ UPLC system (Waters Corp., Milford, USA) by injecting 1 μl of the derivatization mix onto an Acquity™ UPLC BEH C18 1.7 μm 2.1×100 mm column heated at 55 °C. Amino acids were eluted at 0.7 ml min⁻¹ flow with a mix of 10-fold diluted AceQ Tag Ultra Eluent (A) and acetonitrile (B) according to the following gradient: initial, 99.9% A; 0.54 min, 99.9% A; 6.50 min, 90.9% A; 7.85 min, 78.8% A, curve 6; 8.90 min, 40.4% A, curve 6; 9.50 min, 40.4% A, curve 6; 9.60 min, 99.9% A, curve 6; 10.10 min, 99.9% A. Derivatized amino acids were detected at 260 nm using a photo-diode array detector.

**Statistical analysis**

Five replicates per treatment were used. Because some data did not fit the parametric test conditions, the choice was made to analyse the effect of S treatments by the Kruskall–Wallis non parametric test (Sokal and Rohlf, 2003), and then by the Signed-Ranks test.

**Results**

**Growth parameters**

An increase in sulphate application markedly increased the total biomass of plants grown for 140 d (Fig. 1; $H=12.50,$
Values are presented as means (\(\pm \) SE) (n=5). Different letters indicate significant differences between S treatments (\(P < 0.01\)).

### Sulphur and nitrogen acquisition

A higher N concentration was observed in nodules, and was independent of S treatments in this organ (Table 1). Sulphate application increased N concentration in roots but decreased N concentration in leaves and stolons. A higher S concentration was also observed in nodules (Table 1), and in this organ, S concentration was increased by sulphate application. S concentration was lower and independent of S treatments in all other organs. As a result of the stimulation of plant biomass by sulphate application, the amount of nitrate absorbed from the nutrient solution and the amount of N derived from nitrogen fixation were both significantly higher under the high S treatment (Table 2). However, S supply appeared to have no significant effect on nitrate uptake when the results are expressed per unit of root length (Table 2). By contrast, S supply appeared to have a specific effect on \(\text{N}_2\) fixation, as a higher amount of N fixed per metre of roots was observed for the high S treatment. Moreover, the percentage of N derived from fixation increased from 38\(\pm\)2\% for plants grown in the absence of S to 59\(\pm\)2\% of total N for the high S treatment, confirming that \(\text{N}_2\) fixation is more dependent on S nutrition than N uptake.

### Nodulation parameters

In order to clarify the ways that a deficiency in S diminishes atmospheric \(\text{N}_2\) fixation, nodule morphological and biochemical traits were further investigated. A very strong effect of S nutrition on nodulation was observed. As Fig. 2A shows, a very small number of nodules were observed when plants were grown in the absence of S, and this number increased dramatically with S treatment (\(H=12.50, P < 0.01\)). S supply had a similar effect on nodule dry mass per plant (\(H=11.18, P < 0.05\)) (data not shown), and nodule volume per plant (\(H=12.02, P < 0.05\)) (Fig. 2B), with these two parameters being closely correlated (Pearson correlation: \(r=0.893, P < 0.001\)). S appeared to have a specific effect on nodulation as nodule dry mass increased strongly when expressed per unit of root length (\(H=12.02, P < 0.01\); Fig. 2C). Plants grown in the absence of S exhibited a very low nodule mass per root length and a higher nodule mass per root length was observed for white clover plants grown under the high S treatment. The nodule morphological parameters (nodule DM/root length; total nodule volume per plant) were correlated significantly with the percentage of N derived from fixation (Pearson correlation: \(r=0.714, P < 0.01\) and \(r=0.873, P < 0.05\), respectively). In addition, the nodules of plants grown in the absence of S appeared pale in contrast with the pink nodules observed in the presence of S.

### Nodule proteins

Nodule protein concentration increased with S supply (Table 3). Using 2-DE and immunological analysis, 10 spots were immunodetected using anti-FeMoCo nitrogenase antibodies (Fig. 3A, B). Identification by LC MS/MS demonstrated that five of these proteins are nitrogenase
Fe-Mo β-chain (Spots 3 to 7) and one (Spot 10) is nitrogenase Fe protein (Fig. 3C).

Both nitrogenase and leghaemoglobin were detectable in nodules of white clover grown in the absence and in the presence of sulphate in the nutrient solution (Fig. 4). Image analysis of SDS-PAGE electrophoresis of nodule soluble proteins (Fig. 4A) and a comparison with data from immunoblots (Fig. 4B) indicated that the amount of both nitrogenase Fe-Mo protein and leghaemoglobin were dependent on S supply (Table 3). Nodules of plants grown in the absence of S contained significantly less nitrogenase Fe-Mo protein and less leghaemoglobin than plants grown with the high S treatment. The lowest nitrogenase Fe protein content was observed for the 0.095 mM S treatment, the zero S treatment being intermediary between treatments and not significantly different from other treatments.

**Nodulated root amino acids**

A strong accumulation of amino acids was observed in nodulated roots of white clover grown in the absence of S (Table 4), with the amino acid concentration in roots being 4-times higher in the zero S treatment than in the high S treatment. A similar effect of S availability on amino acids was observed in shoots (data not shown). Asparagine was the major amino acid found in white clover roots, and the proportion of asparagine increased from 71±7% of total free amino acids for the high S concentration nutrient solution, to 85±2% for the zero S roots. A strong accumulation of other N-rich amino acids, arginine and histidine, was also observed for zero S roots, while the concentration of other amino acids like glutamate and aspartate was independent of S availability. The concentration of cysteine was too low in all treatments to be measured accurately, and the other sulphur amino acid, methionine, constituted less than 0.1% of total amino acids in all treatments. As expected, this proportion of methio-

| SO$_2^-$ (mM) | N absorbed (g plant$^{-1}$) | N fixed (g m$^{-1}$ roots) |
|---------------|-----------------------------|---------------------------|
| 0            | 0.23 a                      | 0.14 a                    |
| 0.095        | 0.35 b                      | 0.29 b                    |
| 0.380        | 0.51 c                      | 0.74 c                    |
| H            | <0.01                       | <0.01                     |
| P            | ns                          | <0.01                     |

*Fig. 2. Nodulation of white clover plants grown for 140 d at three sulphate concentrations in the nutrient solution (0, 0.095, and 0.380 mM). (A) Number of nodules per plant, (B) total volume of nodules per plant, and (C) dry mass (DM) of nodules per unit of root length. Values are presented as means ±SE (n=5). Different letters on bars indicate significant differences between S treatments (P <0.05).*

Discussion

**Plant growth and N acquisition**

Plants are able to use both pedospheric S (sulphate) and atmospheric S as sources of S to support growth. The observation that white clover can grow hydroponically without S added to the nutrient solution suggests that this legume is able to use atmospheric sulphur as a significant source of S because the amount of S contained in each plant at the beginning of the experiment (67 μg) was not sufficient to support plant growth for 140 d. Atmospheric S can
Table 3. Protein concentrations (expressed as mg g\(^{-1}\) fresh mass) of nodules of white clover plants grown for 140 d at three sulphate concentrations in the nutrient solution.

White clover plants were grown for 140 d at three sulphate concentrations of the nutrient solution (0, 0.095, and 0.380 mM). Total soluble proteins were determined by the Bradford method (1976). Nitrogenase and leghaemoglobin were quantified from image analysis of SDS-PAGE gels shown in Fig. 4. Values are presented as means (\(n=5\)). Different letters indicate significant differences between S treatments (\(P<0.05\)). \(H\) and \(P\): Kruskall–Wallis statistics (ns: non significant).

| SO\(_{2}^{2-}\) (mM) | Total soluble proteins (mg g\(^{-1}\) FM) | Nitrogenase Fe Mo \(\beta\) chain (mg g\(^{-1}\) FM) | Nitrogenase Fe protein (mg g\(^{-1}\) FM) | Leghaemoglobin (mg g\(^{-1}\) FM) |
|----------------------|------------------------------------------|-------------------------------------------------|------------------------------------------|---------------------------------|
| 0                    | 1.97 a                                   | 0.16 a                                          | 0.13 ab                                  | 0.06 a                          |
| 0.095                | 3.18 ab                                  | 0.49 b                                          | 0.06 a                                   | 0.16 ab                         |
| 0.380                | 5.51 b                                   | 0.32 b                                          | 0.22 b                                   | 0.33 b                          |
| \(H\)                | 8.61                                     | 10.35                                           | 9.74                                     | 10.35                           |
| \(P\)                | <0.05                                    | <0.01                                           | <0.01                                    | <0.01                           |

Fig. 3. Proteomic maps of nodule proteins from white clover plants grown for 140 d at three sulphate concentrations in the nutrient solution (0, 0.095, and 0.380 mM). (A) Nodule proteins were separated by 2-D SDS-PAGE (12% acrylamide) and stained with silver nitrate. (B) Nodule proteins were revealed after immunoblot with anti-NiFH. Immunodetected spots are numbered from the highest to the lowest molecular mass. (C) Identification of nodule proteins immunodetected by the anti-FeMoCo-nitrogenase antibodies. Experimental and theoretical pI and molecular mass are indicated as Exp. and theor. pI/Mr. The assigned protein of best match is given with the organism in which it was identified and its GenBank protein accession number: PM: number of LC-MS/MS matched peptides, SCb (%): percentage of sequence coverage of the protein.
replace soil sulphate as the sulphur source for other plant species, especially when the sulphur supply to the roots is low (Cowling et al., 1973; De Kok et al., 2007). The main sources of atmospheric S are H$_2$S reduced in cysteine via O-acetylserine lyase and SO$_2$ oxidized to sulphate via sulphite oxidase (Van Der Kooij et al., 1997; Hänisch and Mendel, 2005; Durenkamp et al., 2007). Our study suggests that clover can use these sources and a more detailed experiment is needed to quantify the ability of legume plants such as clover to use H$_2$SO$_4$ or SO$_2$ as S sources.

Work by Zhao et al. (1999) has shown that the effect of S-deficiency on pea growth was likely to be caused by the shortage of N, due to decreased N$_2$ fixation. Accordingly, the effect of S-deficiency on clover growth was associated here with a strong reduction of N$_2$ fixation. However, the higher N concentration in shoot tissues observed for the zero S treatment indicates that shoot growth was not limited by N assimilation. The low amount of N acquired from the nutrient solution and from the atmosphere shows that both pathways of N assimilation, soluble N uptake and N$_2$ fixation, were reduced in S-deficient clover. A decrease in nitrate uptake in S-starved plants has previously been observed in barley (Clarkson et al., 1989; Karmoker et al., 1991) and spinach (Prosser et al., 2001). In clover, the reduction of nitrate uptake is mainly explained by the strong reduction in root length in the zero S and low S treatments rather than a specific effect of S availability on soluble N uptake, as shown by the similar amount of nitrate taken up per unit of root length that was observed in all treatments.

S-deficiency had a stronger effect on the process of atmospheric N$_2$ fixation than on soluble N uptake as shown by the low amount of N fixed per metre of roots for the zero S and low S treatments. The high requirement of S for the process of N fixation, also evidenced by the high nodule S concentration, is in accordance with previous work (Pacyna et al., 2006; Zhao et al., 1999).

**Nodule morphology and metabolism**

Nodulation was evaluated here as the number, volume, and dry mass of nodules. The strong reduction of nodulation observed with the zero and low S treatments resulted mainly in a much lower dry mass of nodules per unit of root length, and not only in a lower root production as observed for other legumes (Gilbert and Robson, 1984; Scherer and Lange, 1996). This result confirms that traits associated with N$_2$ fixation are more responsive to S availability than host plant growth. This specific effect of S on nodulation can be explained by the role of S in stimulating the formation of nodules in the early events of symbiosis.
establishment (Schwedock and Long, 1992; Crockard et al., 2002). The effect of S-deficiency appears similar to those of P deficiency in preventing noduleation in white clover (Almeida et al., 2000). This strong reduction of noduleation could result from an N feedback mechanism, as a strong accumulation of amino acids was observed in the zero and low S treatments. It has been shown that such an accumulation can reduce nodule growth (Parsons et al., 1993).

The close relationship observed between nodule volume per plant and the percentage of N derived from fixation suggests that the low N2 fixation observed can be primarily explained by the low noduleation. However, our biochemical and immunological approach shows that nodule metabolism was also markedly dependent on sulphate availability. A close relationship was found between S supply and protein content in the nodules, and both the proteins measured, nitrogenase and leghaemoglobin, were dependent on S supply. It has been proposed that low N2 fixation observed in S-deficient legumes is due to a low ATP supply and to an inhibition of nitrogenase by high levels of free O2 (Pacyna et al., 2006; Scherer et al., 2008). ATP was not measured in the present study, but the low N2 fixation could also be due to a high level of O2 in clover nodules. Indeed, it was observed that leghaemoglobin content in clover roots is highly dependent on sulphate availability, confirming a recent result by Scherer et al. (2008) working with pea and alfalfa. As the role of leghaemoglobin in legume nodules is to maintain a low free O2 concentration within the nodule (Gordon et al., 2001), the decrease in its content may have resulted in a higher O2 concentration.

S starvation generally leads to the accumulation of non-S-containing amino acids and O-acetylserine, the precursor for the synthesis of the first organic S compound, cysteine, and a decrease in numerous S compounds, primarily cysteine, methionine, and S-adenosyl methionine (SAM), (Prosser et al., 2001; Nikiforova et al., 2003; Saito, 2004; Hoefgen and Nikiforova, 2008). In clover leaves, a reduction in chlorophyll concentration was observed, as mentioned for other species grown with insufficient S supply (Lunde et al., 2008). This chlorosis could be explained by the decrease in the SAM that served as a methyl-group donor in different branches of plant metabolism and especially in chlorophyll synthesis (Hoefgen and Nikiforova, 2008). In nodules, this disruption of N metabolism was evidenced by the decrease in protein content and the strong accumulation of non-S-containing amino acids. The low level of the nitrogenase Fe–Mo protein is in line with reports showing that iron–sulphur proteins and haem biosynthesis can be repressed when sulphate availability decreases (Nikiforova et al., 2003; Touraine et al., 2004; Hausman et al., 2008). Both components of the nitrogenase complex, the Fe protein and the Fe–Mo protein, contain high proportions of S (Krusell et al., 2005; Curatti et al., 2006). However, the differentiated behaviour of both nitrogenase components with respect to S availability makes the relationship between S availability and nitrogenase difficult to interpret. It is noteworthy that nodulated clovers grown in the absence of S in the nutrient solution were able to form both components of the nitrogenase complex, and the strikingly high total S concentrations in nodules for all S treatments confirms that nodules are a strong sink for S in legumes. A full metabolic analysis could help to clarify the interactions between S and N metabolism in nodules.

Even though noduleation and Fe–Mo nitrogenase content were very low under S-deficiency, 38% of the total N assimilated by these plants originated from N2 fixation. Nodule dry weight was reduced by 99% in zero S clover compared with high S clover and the amount of N fixed per plant was only decreased by 81%. This indicates that S-deficiency inhibited noduleation to a greater extent than N2 fixation. Specific nitrogenase activity was not measured directly in the present study, and it is difficult to estimate this activity expressed on a quantity, volume or mass of nodules basis as neither the kinetics of fixation nor those of nodule development are known. Nevertheless, this comparison suggests that S-deficiency could increase the specific nitrogenase activity expressed g−1 of nodule. A similar increase in specific nitrogenase activity has been found under other conditions that hindered the development of nodules such as under P deficiency (Almeida et al., 2000) or high mineral N supply (Zanetti et al., 1998). In the case of P deficiency, it has been proposed that this high specific nitrogenase activity could be the result of an increase in O2 permeability, and further work is needed to investigate the effect of S-deficiency on O2 permeability in the nodules.

Concluding remarks

From our experimental data, it is proposed that the reduced symbiotic N2 fixation observed in S-deficient white clover is an adjustment to the lower demand for N due to a lower plant growth. The high N concentration observed in leaves and stolons of S-deficient clover, compared with other treatments, shows that shoot growth was not limited by N assimilation under S-deficiency and thus supports this hypothesis. The lowered N2 fixation may have resulted from an N-feedback mechanism down-regulating nodule development and nitrogenase and leghaemoglobin production, as indicated by the increased asparagine concentration observed. Such regulatory behaviour has already been observed for other nutrient stresses in white clover and other legumes (Hartwig, 1998; Almeida et al., 2000; Vadez et al., 2000; Høgh-Jensen et al., 2002).

Acknowledgements

We are grateful to Anne-Françoise Ameline, Marie-Paule Bataillé, Michelle Coustenoble, Josiane Pichon, Dominique Ballois, Patrick Beaucclair, Hugues Renault, and Raphaël Séguira for technical assistance. We wish to thank Jessica Bryant and Laurence Cantrill for critical comments and advice on the manuscript.
References

Almeida JPF, Hartwig UA, Frehner M, Nöbsberger J, Lüscher A. 2000. Evidence that P deficiency induces N feedback regulation of symbiotic N₂ fixation in white clover (Trifolium repens L.). Journal of Experimental Botany 51, 1289–1297.

Aulakh MS, Dev G, Arora BR. 1976. Effect of sulphur fertilization on the nitrogen-sulphur relationships in alfalfa (Medicago sativa L. Pers.). Plant and Soil 45, 75–80.

Blum H, Beier H, Gross HJ. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamid gel. Electrophoresis 8, 93–99.

Bolaniños L, Martin M, El Hamdaoui A, Rivilla R, Bonilla I. 2006. Nitrogenase inhibition in nodules from pea plants grown under salt stress occurs at the physiological level and can be alleviated by B and Ca. Plant and Soil 280, 135–142.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Clarkson DT, Saker LR, Purves JV. 1989. Depression of nitrate and ammonium transport in barley plants with diminished sulphate status. Evidence of co-regulation of nitrogen and sulphate intake. Journal of Experimental Botany 40, 953–963.

Colding DW, Jones LHP, Lockyer DR. 1973. Increased yield through correction of sulphur deficiency in ryegrass exposed to sulphur dioxide. Nature 243, 479–480.

Crockard MA, Bjourson AJ, Dazzo FB, Cooper JE. 2002. A white clover nodulin gene, dd23b, encoding a cysteine cluster protein, is expressed in roots during the very early stages of interaction with Rhizobium leguminosarum biovar trifolii and after treatment with chitooligosaccharide Nod factors. Journal of Plant Research 115, 439–447.

Curatti L, Ludden PW, Rubio LM. 2006. Nif B-dependent in vitro synthesis of the iron-molybdenum cofactor of nitrogenase. Proceedings of the National Academy of Sciences, USA 104, 5207–5301.

De Kok LJ, Durenkamp M, Yang L, Stulen I. 2007. Atmospheric sulfur. In: Hawkesford MJ, De Kok LJ, eds. Sulfur in plants: an ecological perspective. Dordrecht: Springer-Verlag, 91–106.

Desclos M, Etienne P, Coquet L, Cosette P, Bonnefoy J, Segura R, Reze S, Ourry A, Avice J-C. 2009. A combined ¹⁵N tracing/proteomics study in Brassica napus reveals the chronology of proteomics events associated to N remobilization during leaf senescence induced by nitrate limitation or starvation. Proteomics 9, 3580–3608.

Durenkamp M, De Kok LJ, Kopriva S. 2007. Adenosine 5’-phosphosulfate reductase is regulated differently in Allium cepa L. and Brassica oleracea L. upon exposure to H₂S. Journal of Experimental Botany 58, 1571–1579.

Gilbert MA, Robson AD. 1984. The effect of sulphur supply on the root characteristics of subterranean clover and annual ryegrass. Plant and Soil 77, 377–380.

Gordon AJ, Lea PJ, Rosenberg C, Trinchant JC. 2001. Nodule formation and function. In: Lea PJ, Morot-Gaudry JF, eds. Plant nitrogen. Berlin, Heidelberg, New York: Springer-Verlag, 101–146.

Goulaš E, Le Dily F, Teissedre L, Corbel G, Robin C, Ourry A. 2001. Vegetative storage proteins in white clover (Trifolium repens L.): quantitative and qualitative features. Annals of Botany 88, 789–795.

Habtemichial KH, Singh BR, Aune JB. 2007. Wheat response to N₂ fixed by faba bean (Vicia faba L.) as affected by sulfur fertilization and rhizobial inoculation in semi-arid southern Ethiopia. Journal of Plant Nutrition and Soil Sciences 170, 412–418.

Hartwig UA. 1998. The regulation of symbiotic N₂ fixation: a conceptual model of N feedback from the ecosystem to the gene expression level. Perspectives in Plant Ecology, Evolution and Systematics 1, 92–120.

Hausmann A, Samans B, Lill R, Mühlenhoff U. 2008. Cellular and mitochondrial remodeling upon defects in iron–sulfur protein biogenesis. Journal of Biological Chemistry 283, 8318–8330.

Hänsch R, Mendel RR. 2005. Sulfite oxidation in plant peroxisomes. Photosynthesis Research 86, 337–343.

Hoejsgaard MA, Nikiforova VJ. 2008. Metabolomics integrated with transcriptomics: assessing systems response to sulfur-deficiency stress. Physiologia Plantarum 132, 190–198.

Høgh-Jensen HH. 2003. The effect of potassium deficiency on growth and N₂-fixation in Trifolium repens. Physiologia Plantarum 119, 440–449.

Høgh-Jensen H, Schjoerring JK. 1994. Measurement of biological dinitrogen fixation in grassland: comparison of the enriched ¹⁵N dilution and the natural ¹⁵N abundance method at different nitrogen application rates and defoliation frequencies. Plant and Soil 166, 153–163.

Høgh-Jensen H, Schjoerring JK, Soussana JF. 2002. The influence of phosphorus deficiency on growth and nitrogen fixation of white clover plants. Annals of Botany 90, 754–753.

Karmoker JL, Clarkson DT, Saker LR, Rooney JM, Purves JV. 1991. Sulphate deprivation depresses the transport of nitrogen to the xylem and osmotic osmotic hydraulic conductance of barley (Hordeum vulgare L.) roots. Planta 185, 269–278.

Krusell L, Krause K, Ott T, et al. 2005. The sulfate transporter SST1 is crucial for symbiotic nitrogen fixation in Lotus japonicus root nodules. The Plant Cell 17, 1625–1636.

Laemmli UK. 1970. Cleavage of structural proteins during the heat denaturation of Escherichia coli. Nature 227, 680–685.

Lunde C, Zygadlo A, Simonsen HT, Nielsen PL, Blennow A, Haldrup A. 2008. Sulfur starvation in rice: the effect of photosynthesis, carbohydrate metabolism, and oxidative stress protective pathways. Physiologia Plantarum 134, 508–521.

Mechin V, Consoli L, Le Guilloux M, Damerval C. 2003. An efficient solubilization buffer for plant proteins focused in immobilized pH gradients. Proteomics 3, 1299–1302.

Nikiforova VJ, Freitag J, Kempa S, Adamik S, Hesse H, Hoejsgaard MA. 2003. Transcriptome analysis of sulfur depletion in Arabidopsis thaliana: interlacing of biosynthesis pathways provides response specificity. The Plant Journal 33, 633–650.

Noquet C, Avice JC, Rossato L, Beauchlair P, Henry MP, Ourry A. 2004. Effects of altered source–sink relationships on N allocation and vegetative storage protein accumulation in Brassica napus L. Plant Science 166, 1007–1018.
Olivera M, Tejera N, Iribarne, Ocana A, Lluch C. 2004. Growth, nitrogen fixation and ammonium assimilation in common bean (Phaseolus vulgaris): effect of phosphorus. Physiologia Plantarum 121, 498–505.

Pacyna S, Schulz M, Scherer HW. 2006. Influence of sulphur supply on glucose and ATP concentrations of inoculated broad beans (Vicia faba minor L.). Biology and Fertility of Soils 42, 324–329.

Parsons R, Stanforth A, Raven JA, Sprent J. 1993. Nodule growth and activity may be regulated by feedback mechanism involving phloem nitrogen. Plant, Cell and Environment 16, 125–136.

Prosser IM, Purves JV, Saker LR, Clarkson DT. 2001. Rapid disruption of nitrogen metabolism and nitrate transport in spinach plants deprived of sulphate. Journal of Experimental Botany 52, 113–121.

Saito K. 2004. Sulfur assimilatory metabolism. The long and smelling road. Plant Physiology 136, 2443–2450.

Scherer HW. 2001. Sulphur in crop production: invited paper. European Journal of Agronomy 14, 81–111.

Scherer HW, Lange A. 1996. N2 fixation and growth of legumes as affected by sulphur fertilization. Biology and Fertility of Soils 23, 449–453.

Scherer HW, Pacyna S, Spto KR, Schulz M. 2008. Low levels of ferredoxin, ATP, and leghemoglobin contribute to limited N2 fixation of peas (Pisum sativum L.) and alfalfa (Medicago sativa L.) under S deficiency conditions. Biology and Fertility of Soils 44, 909–916.

Schultze J, Drevon JJ. 2005. P-deficiency increases the O2 uptake per N2 in alfalfa. Journal of Experimental Botany 56, 1779–1784.

Schwedock JS, Long SR. 1992. Rhizobium meliloti genes involved in sulfate activation: the two copies of nodPQ and a new locus, saa. Genetics 132, 899–909.

Sokal RR, Rohlf FJ. 2003. Biometry, 3rd edn. New York: WH Freeman and Company.

Touraine B, Boutin JP, Marion-Poll AM, Briat JF, Pettier G, Lobréaux S. 2004. Nfu:2 a scaffold protein required for (4Fe-4S) and ferredoxin iron-sulphur cluster assembly in Arabidopsis chloroplasts. The Plant Journal 40, 101–111.

Tallec T, Diquelou S, Lemauniel S, Cliquet JB, Lesuffleur F, Ourry A. 2008. Nitrogen:sulfur ratio alters competition between Trifolium repens and Lolium perenne under cutting: production and competitive abilities. European Journal of Agronomy 29, 94–101.

Vadez V, Sinclair TR, Serraj R. 2000. Asparagine and ureide accumulation in nodules and shoots as feedback inhibitors of N2 fixation in soybean. Physiologia Plantarum 110, 215–223.

Van Der Kooij TAW, De Kok LJ, Haneklaus S, Schnug E. 1997. Uptake and metabolism of sulphur dioxide by Arabidopsis thaliana. New Phytologist 135, 101–107.

Varin S, Lemauniel-Lavenant S, Cliquet JB, Diquelou S, Michaelson-Yeates TPT. 2009a. Functional plasticity of Trifolium repens L. in response to sulphur and nitrogen availability. Plant and Soil 317, 189–200.

Varin S, Leveel B, Lemauniel-Lavenant S, Cliquot JB. 2009b. Does the white clover response to sulphur availability correspond to phenotypic or ontogenetic plasticity? Acta Oecologica 35, 452–457.

Wang W, Scali M, Vignani R, Spadafora A, Sensi E, Mazzuca S, Cresto M. 2003. Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds. Electrophoresis 24, 2369–2375.

Zanetti S, Hartwig UA, Nosberger J. 1998. Elevated atmospheric CO2 does not affect per se the preference for symbiotic nitrogen as opposed to mineral nitrogen of Trifolium repens L. Plant, Cell and Environment 21, 623–630.

Zhao FJ, Wood AP, McGrath SP. 1999. Effects of sulphur nutrition on growth and nitrogen fixation of pea (Pisum sativum L.). Plant and Soil 212, 209–219.