Fluctuations of S Availability Affect Growth, S Reserves, ¹⁵N and ³⁴S Uptake in *Brassica Napus* L.

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doi:10.5618/bio.2012.v2.n1.5 || Received: 07-05-2012, Accepted: 12-07-2012, Available online: 15-07-2012

Abstract

Oilseed rape is sensitive to sulphur (S) limitation and is confronted to permanent changes of S availability, resulting from soil activity and S fertilization regimes, which may affect N and S uptake, storage and remobilization. Therefore, the present study aimed to assess the influence of initial S status and mineral S availability on i) biomass production, ii) ¹⁵N and ³⁴S uptake, iii) relative expression of genes encoding N or S transport systems and iv) ¹⁴N and ³²S remobilization. As a consequence, plants with High or Low Initial S status (HS_i or LS_i) were grown during 28 days either with High or Low S mineral availability (HS or LS).

The results showed that with a low initial S status, plants were able under ample S supply to increase N and S uptake by de-repressing gene expression of their uptake systems, and reached similar growth as control plants. With high S reserves, mostly as sulphate, plants were yet able to compensate transient S limitation, by increasing S mobilization towards the roots, and reached similar growth as control plants. Only with low S initial status and low S availability (i.e. long term S limitation), plant growth was reduced while nitrate transporter gene expression was steadily down-regulated probably as a result of amino acid accumulation in the roots.

Overall results showed that plants are able to cope with fluctuating S availability, through mobilization of S reserve when previously accumulated as sulphate, or alternatively, to increase S uptake. As a conclusion, our study supports that initial S reserves play a crucial role for oilseed rape adaptation to S limitation.

Keywords: Nitrogen, Sulphur, Uptake, ¹⁵N labeling, ³⁴S labeling, Reserve mobilization, Nitrate transporters, Sulphate transporters

1. Introduction

Sulphur (S) is an essential nutrient for crops worldwide which is mainly available as sulphate (SO₄²⁻) within the soil. Sulphate is further taken up by roots and translocated via the xylem to shoot tissues where it is reduced in cysteine (Cys) and either converted to methionine (Met) or incorporated into proteins or Cys-containing peptides such as glutathione [1, 2]. Sulphur is also important for chlorophyll synthesis (and subsequent plant photosynthetic capacities) and is incorporated in numerous secondary metabolites involved in plant defence against pathogens (SDC; Sulphur-containing Defence Compounds) like thionins, defensins, glucosinolates or crucifer phytoalexins [3]. Oilseed rape (*Brassica napus* L.), as most *brassicaceae*, presents greater S requirements than other large-crop species [4], and is therefore quite sensitive to S limitation [5]. However, the risk of soil S limitation has increased during the last three decades as a result of the decline of industrial emissions and the subsequent reduction of S deposition on agricultural lands [6]. Consequently, crops submitted to S limitation present reduced photosynthetic capacities and growth [7], which has negative consequences on agronomic performances like seed quality and yield [5, 8].

In response to a limitation of S supply, the hypothetical initial plant physiological responses involve an optimization of sulphate uptake and its subsequent utilization [9], in order to increase sulphate uptake efficiency (SUE) at the whole plant level [10]. For example, Buchner *et al.* [11] and Parmar *et al.* [12] reported that S limitation in *Brassica oleracea* L. and *Brassica napus* L. resulted

in an overall increase of the relative transcript levels of several sulphate transporters (*i.e.* Sultr). Moreover, numerous genes involved in the S assimilatory pathway, like ATP sulphurylase (ATPS), are also positively regulated in response to S limitation [13, 14, 15] in order to maximize the reduction of sulphate in S-containing amino-acids involved in protein biosynthesis.

S limitation is also accompanied by an increase of the remobilization of endogenous S reserves from vegetative tissues and their subsequent redistribution towards young growing tissues [9, 16]. In oilseed rape, Blake-Kalff et al. [17] reported that under optimal S supply, 70 to 90% of the total S in mature and oldest leaves is accumulated as sulphate, which would be remobilized during S limitation. Similarly, within rosette leaves, Noquet et al. [18] also reported that N remobilization plays a crucial role because the ablation of half of the rosette leaves present at the end of the vegetative stage results in a 30% decrease of seed yield. Lastly, Abdallah et al. [16] also reported for oilseed rape that a transient mineral S limitation (up to 28 days) occurring during the rosette stage could lead to a transient maintenance of growth related to an optimization of the recycling of endogenous foliar S compounds (particularly sulphate) from old to young leaves and roots. Despite a close interconnection between N and S metabolisms [2, 19], this was reported to be mainly achieved without any apparent modification of N uptake or N remobilization or without any acceleration of leaf senescence processes, which are particularly involved in the efficient remobilization of N reserves [16, 20]

However, and even if it has been recently reported that S limitation is accompanied by an increase of the remobilization of endogenous initial S reserves [9], the question assessing the hypothetical impact of depleted initial S reserves on growth, N and S uptake and utilization was not, to our knowledge, clearly assessed in literature. In other words, and because N and S metabolisms are closely linked at the whole plant level, with depleted initial S reserves and under S limitation, can growth, N and S uptake or mobilization patterns be reduced? With depleted initial S reserves and if supplied with optimal S, can N and S uptake be increased and will plants replenish their reserves and/or grow as control plants? These two questions also have particular relevance in the current context of sustainable agriculture and the concomitant reduction of fertilizers inputs.

As a consequence, the main aim of our study was to evaluate the influence of initial S reserves and mineral S availability on biomass production as well as on ^{15}N and ^{34}S uptake. For this purpose, a continuous ^{15}N and ^{34}S double-labeling method was therefore designed

and the expression of genes encoding relevant nitrate and sulphate transporters (*i.e.* *BnNrt* and *BnSultr*, respectively) was studied within the roots of oilseed rape. Moreover, and because a previous study revealed that there is a great reorientation of S fluxes towards the roots and that they became the main sink organ for S in case of transient S limitation [16], we specifically studied N and S partitioning and remobilization patterns within this tissue in order to determine how plants can compensate for a reduced S availability and cope with their requirements for their vegetative growth and development.

2. Materials and Methods

2.1 Application of S treatments and tissue sampling. Seeds of oilseed rape (*Brassica napus* L. cv. Capitol) were sterilized by exposure to 80% ethanol for 30 sec followed by 20% sodium hydrochlorite for 20 min. After several washes in demineralized water, seeds were disposed on plastic tanks with a moist filter paper under dark conditions. Just after first leaf emergence, seedlings were transferred on demineralized water for two weeks, (18 seedlings per 12 L-plastic tank) in a greenhouse with a thermoperiod of 20°C (day) and 18°C (night). Natural light was supplemented with phytol lamps (450 $\mu\text{moles m}^{-2} \text{ s}^{-1}$ of photosynthetically-active radiation (PAR) at canopy height) for 16h. For two additional weeks, seedlings were transferred on hydroponic solution (18 seedlings per 12 L-plastic tank). The basic nutrient solution contained 0.4 mM KH_2PO_4 , 0.15 mM K_2HPO_4 , 2 mM KCL, 3 mM CaCl_2 , 0.2 mM Fe Na EDTA, 14 μM H_3BO_3 , 5 μM MnSO_4 , 3 μM ZnSO_4 , 0.7 μM CuSO_4 , 0.7 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 0.1 μM CoCl_2 . This basic nutrient solution was renewed weekly and supplemented twice a week with 1mM KNO_3 and 300 μM MgSO_4 (**Figure 1**), in order to be in optimal S conditions for growth for non-vernalized rosette plants (*i.e.* vegetative stage). After four weeks, plants were then transferred to 4 L plastic pots (one plant per pot), and were divided in two groups in order to modify the level of their initial S reserves. They were thus initially supplied either with 300 μM SO_4^{2-} (HS_i) or 15 μM SO_4^{2-} (LS_i) for 14 days. At this date (*i.e.* Day 0; Fig. 1), every set was again divided in two groups and treatments were applied during 28 days with either High S solution (300 μM SO_4^{2-} ; HS) or Low S solution (15 μM SO_4^{2-} ; LS), thus generating four treatments: $\text{HS}_i\text{-HS}$; $\text{HS}_i\text{-LS}$; $\text{LS}_i\text{-HS}$ and $\text{LS}_i\text{-LS}$. Between Day 0 and Day 28 (*i.e.* continuous labeling method), overall treatments were continuously double-labeled with ^{15}N (2.5% atom excess) and ^{34}S (2% atom excess) in order to easily follow both N and S recently taken up, as well as N and S remobilization among all different plant tissues.

Figure 1. Experimental design: After one week on demineralized water, plants were grown during two weeks on high S solution ($300 \mu\text{M SO}_4^{2-}$) and were then divided in two groups during two additional weeks. They were initially supplied either with $300 \mu\text{M SO}_4^{2-}$ (**HS_i**) or $15 \mu\text{M SO}_4^{2-}$ (**LS_i**). On Day 0, every group was again divided in two groups and treatments were applied during 28 days with either High S solution ($300 \mu\text{M SO}_4^{2-}$; **HS**) or Low S solution ($15 \mu\text{M SO}_4^{2-}$; **LS**), thus generating four treatments : **HSi-HS**; **HSi-LS**; **LSi-HS** and **LSi-LS**. In the meantime, overall treatments were continuously labelled with ^{15}N and ^{34}S and plants were finally harvested at Day 0, 14 and 28.

Four plants (*i.e.* replicates) of each of the four treatments were harvested on Day 0 and after 14 and 28 days of treatment. The whole root system, stems, leaf blades and leaf petioles were separated and weighed for determination of their fresh matter. All plant tissues were then frozen in liquid N_2 and stored at -80°C until further isotopic ratio mass spectrometry analysis, biochemical and molecular analysis. They were then finally freeze-dried and ground to fine powder for dry weight determination (**Fig. 2**).

2.2 Preparation of ^{34}S . Elemental S enriched in ^{34}S (98 % atom excess) was obtained from Trace Sciences international, France. A digestion procedure using 16.5 N HNO_3 was used to convert elemental S to sulphate according to Zhao *et al.* [21] and modified by Abdallah *et al.* [16]. Briefly, 100 mg of elemental S were weighed into a Pyrex digestion tube. Ten mL of HNO_3 were then added. Digestion was carried out in a programmable heating bloc with temperature rising up to 200°C for 2h. This operation was repeated two times. The different solutions were then recovered and K_2CO_3 was added.

The remaining solution was transferred to a 100-mL volumetric flask. Analyses of nitrate and sulphate concentrations in this stock solution were carried out using ion chromatography (Dionex DX100, CA USA, with conductivity detector). The analysis of the stock solution presented a final recovery of about 82 % of $^{34}\text{SO}_4^{2-}$ and both nitrate and sulphate concentrations were taken into account during plants N and S labeling period (*i.e.* between Day 0 and Day 28).

2.3 Determination of total N and S content. An aliquot of each freeze-dried plant organ (roots, stems, leaf blade and leaf petioles) was placed into tin capsules for isotopic analysis. The total N (^{14}N , ^{15}N) and S contents (^{32}S , ^{34}S) in plant samples (**Fig. 3**) were determined with a continuous flow isotope mass spectrometer (Isoprime, GV Instrument, Manchester, United Kingdom) linked to a C/N/S analyzer (EA3000, Euro Vector, Milan, Italy):

Total N (N_{tot}) content in a tissue “i” at a given time “t” was calculated as:

$$\text{N}_{\text{tot } i,t} = \% \text{N}_{i,t} \times \text{DW}_{i,t} / 100$$

Natural ^{15}N abundance ($0.3663 \% \pm 0.0004$) of atmospheric N_2 was used as a reference for ^{15}N analysis. Nitrogen derived from current N uptake (N_{upt}) in a given organ was calculated as follows:

$$\text{N}_{\text{upt}} = \text{N}_{\text{tot}} \times (\text{E}_{\text{N}} (\%) / \text{E}_{\text{N Sol}} (\%)),$$

where $\text{E}_{\text{N}} (\%)$ is the atom % ^{15}N excess in a given organ and $\text{E}_{\text{N Sol}}$ is atom % ^{15}N excess (2.5%) in the nutrient solution

As for N, total S (Stot) content in a tissue “i” at a given time “t” was calculated as:

$$\text{Stot } i,t = \% \text{S}_{i,t} \times \text{DW}_{i,t} / 100.$$

For ^{34}S enrichment, the natural isotope abundance of 4.255% was withdrawn to the sample ^{34}S abundance, then sulphur derived from current S uptake (S_{upt}) in a given organ was calculated as follows:

$$\text{S}_{\text{upt}} = \text{Stot} \times (\text{E}_{\text{S}} (\%) / \text{E}_{\text{S Sol}} (\%)),$$

where ES (%) is the atom % ^{34}S excess in a given organ and ES_{Sol} is atom % ^{34}S excess (2%) in the nutrient solution.

2.4 Determination of S and N partitioning and mobilization. On Day 28, N and S partitioning derived from uptake of labeled sources in whole leaf-blades and roots was expressed as a percentage of whole plant (roots+stems+leaf blade+leaf petioles) total N or S, respectively (**Table I**).

Thereafter, the remobilized N content (Nmob) from or towards each tissue between two dates (*i.e.* Day 28 - Day 0) was calculated by subtracting the N derived from uptake between these two dates (Nupt) to the change in total N content ($\text{N}_{\text{tot Day 28}} - \text{N}_{\text{tot Day 0}}$) according to the following equation:

$$\text{Nmob}_{(\text{Day 28}-\text{Day 0})} = (\text{N}_{\text{tot Day 28}} - \text{N}_{\text{tot Day 0}}) - \text{Nupt}.$$

Therefore, positive values of mobilized N represent N that is mobilized towards this tissue (*i.e.* imported), while negative values correspond to a net mobilization of N from this tissue (*i.e.* exported; **Table I**).

As for N, the mobilized S content (Smob) from or towards each tissue between two dates (*i.e.* Day 28 - Day 0) was calculated by subtracting the S derived from uptake between these two dates (Supt) to the change in total S content ($\text{S}_{\text{tot Day 28}} - \text{S}_{\text{tot Day 0}}$) according to the following equation:

$$\text{Smob}_{(\text{Day 28}-\text{Day 0})} = (\text{S}_{\text{tot Day 28}} - \text{S}_{\text{tot Day 0}}) - \text{Supt}.$$

Therefore, positive values of mobilized S represent S that is mobilized towards this tissue (*i.e.* imported), while negative values correspond to a net mobilization of S from this tissue (*i.e.* exported; **Table I**).

2.5 Determination of NO_3^- and SO_4^{2-} contents. Nitrate and sulphate contents (**Fig. 5**) were measured by extracting 30 mg of freeze-dried plant material in 1.5 mL of 50% ethanol solution at 40°C for 1h. After centrifugation (20 min, 10 000 g) the supernatant (called S1) was recovered and 1.5 mL of 50% ethanol were added to the pellet. After a new incubation (1h, 40 °C) and centrifugation (20 min, 10 000 g), the remaining supernatant was taken up and added to the previous supernatant (S1). All these operations (*i.e.* incubation and centrifugation) were repeated twice but now with 1.5 mL ultra-pure water and incubation at 95 °C. All supernatants were finally pooled then air-dried for 16 h without heating. The dry residues containing both nitrate and sulphate were solubilized in 1 mL of ultra-pure water. Thereafter, nitrate and sulphate contents in the extracts were determined by using ion chromatography (Dionex DX100, with conductivity detector; **Fig. 5**). The eluent solution consisted of 1.8 mM Na_2CO_3 and 1.7 mM Na_2HCO_3 and was pumped isocratically through a AS17 guard column.

2.6 RNA extraction. Total RNA was extracted from 200 mg of roots fresh matter. Frozen samples were

grounded to a fine powder with a pestle in a mortar containing liquid nitrogen. The resulting powder was suspended in 750 μL extraction buffer (0.1 M Tris, 0.1 M LiCl, 0.01 M EDTA, 1% SDS (w/v), pH 8) and 750 μL of hot phenol (80°C, pH 4). This mixture was vortexed for 30 sec. and after addition of 750 μL of chloroform / isoamylalcohol (24:1), the homogenate was centrifuged at 15 000 g (5 min, 4°C). The supernatant was transferred into 4 M LiCl solution (w/v) and incubated overnight at 4°C. After centrifugation (15 000 g, 30 min, 4°C), the pellet was suspended in 250 μL of sterile water. Fifty μL of 3 M sodium acetate (pH 5.6) and 1 mL of 96% ethanol were added to precipitate the total RNA for 1 h at -80°C. After centrifugation (15 000 g, 20 min, 4°C), the pellet was washed with 1 mL of 70% ethanol, then centrifuged at 15 000 g for 5 min at 4°C. The resulting pellet was dried for 5 min at room temperature and re-suspended in sterile water containing 0.1% SDS and 20 mM EDTA. Quantification of total RNA was performed by a spectrophotometer at 260 nm (BioPhotometer, Eppendorf, Le Pecq, France) before Reverse Transcription (RT) and quantitative PCR (Q-PCR) analysis.

2.7 Relative expression of BnNrt.1;1, BnNrt.2;1 and BnSultr.1 and BnSultr.4 genes using Q-PCR. Q-PCR amplification and relative expression of *BnNrt.1.1* and *BnNrt.2.1* genes which are supposed to encode root high and low affinity nitrate transporters, respectively, were performed as previously described by Leblanc *et al.* [22].

Q-PCR amplification and relative expression of plasmalemma (*i.e.* *BnSultr.1.1* and *1.2*) and tonoplasmic (*i.e.* *BnSultr.4.1* and *4.2*) sulphate transporters were performed as previously described by Dubousset *et al.* [20] and Abdallah *et al.* [16].

EF1- α gene (Accession no: DQ312264) was used as an internal control gene. For each sample, the subsequent Q-PCR reactions were performed in triplicate and the relative expression of the different nitrate or sulphate transporters in each sample were compared to the control sample (corresponding to control plants (+S) at Day 28) and were determined with the delta-delta Ct method using the following equation [23]:

$$\text{Relative expression} = 2^{-[\Delta\text{Ct sample} - \Delta\text{Ct control at Day 28}]},$$

with $\Delta\text{Ct} = \text{Ct}_{\text{BnNrt1.1 or 2.1 or BnSultr.1.1; 1.2; 4.1; 4.2}} - \text{Ct}_{\text{EF1-}\alpha}$

where Ct refers to the threshold cycle determined for each gene in the exponential phase of PCR amplification. Using this analytic method, relative expression of the different nitrate or sulphate transporter genes in the control sample at Day 0 was equal to one [23], and the relative expressions of other treatments were then compared to the control at Day 0, on this basis (**Fig. 6**).

2.8 Statistical analysis. Results are presented as mean values $\pm$ s.e. for four replicates ($n = 4$). The effects of initial S status and mineral S limitation were determined by ANOVA method, and according to a comparison of the means (Tukey t-Test), with MINITAB13 on windows (Minitab Inc, State College, PA, USA). When the normality law of data was not respected, the non-parametric test of Kruskal - Wallis was used. Statistical significance was postulated for $P < 0.05$ and letters indicate that mean values are significantly different at a given date between treatments.

3. Results

3.1 Whole Plant analysis:

3.1.1 Plant biomass analysis. Figure 2 presents the influence of initial S status and sulphur availability on whole plant, whole leaf-blade and root biomasses for High initial S - High S (HS_i -HS), High initial S - Low S (HS_i -LS), Low initial S - High S (LS_i -HS) and Low initial S - Low S (LS_i -LS) plants during the 28 days of the experiment of oilseed rape at vegetative stage. On Day 0, overall biomasses of LS_i plants were significantly decreased when compared to HS_i plants. During the experiment, whole plant, whole leaf blade and roots biomasses progressively increased for overall treatments, but LS_i -HS whole plant biomass was much more increased and finally reached values close to that of control plants (*i.e.* HS_i -HS) on Day 28 with 32 ± 1.6 g DW. plant⁻¹ in average. By contrast, and even if overall biomasses of LS_i -LS plants were slightly increased during the experiment, these biomasses were however significantly reduced by about 4 fold in average on Day 28, when compared to control plants. At this date, roots represented about 25% of whole plant biomass for overall treatments, and were even the largest for LS_i -HS plants.

3.1.2 Whole plant N and ¹⁵N content. The influence of initial S status and sulphur availability on total N and ¹⁵N contents was presented in figures 3A and B, respectively. On Day 0, total N content was significantly lower for LS_i plants, when compared to HS_i plants (figure 3A). During the experiment, but to a lower extent for LS_i -LS plants, total N content was greatly increased for overall treatments and reached about 375 mg. plant⁻¹ in average on Day 28. This increase was however greater for LS_i -HS plants than for control plants. By contrast, total N content of LS_i -LS plants was only slightly increased during the experiment and was thus reduced of one third, when compared to control plants on Day 28.

Figure 2. Changes of (A) whole plant, (B) whole leaf blades and (C) roots total biomass of plants initially grown either with High S (HS_i ; solid line) or Low S (LS_i ; dotted line) and submitted during 28 days to High S (HS; closed circles) or Low S (LS; open circles) treatments. Vertical bars indicate $\pm$ s.e. of the mean for $n = 4$ when larger than the symbol. Different letters indicate that mean values are significantly different at a given date between treatments ($P < 0.05$).

For overall treatments, the whole plant cumulated ¹⁵N content (*i.e.* derived from uptake) was greatly increased during the first 14 days of experiment (Figure 3B). Thereafter, the cumulated ¹⁵N content solely progressed at the same rate for LS_i -HS plants while it was significantly reduced for all of the other treatments (HS_i -HS, HS_i -LS and LS_i -LS) in the meantime. Nevertheless, on Day 28, the whole plant cumulated ¹⁵N content was greater for HS plants than for LS plants, irrespective of their initial S status.

3.1.3 Whole plant S and ³⁴S contents. Figures 3C and 3D present the influence of initial S status and sulphur availability on whole plant total S and ³⁴S contents, respectively. On Day 0, total S content was approximately 50 mg S. plant⁻¹ for HS_i plants, against solely about 5 mg S. plant⁻¹ for LS_i plants. Thereafter, total S content was increased as a function of mineral S availability. Grown on low S, total S content was very slightly increased (Figure 3C). This was due to a negligible ³⁴S uptake of less than 3 mg S. plant⁻¹ in average, as shown in Figure 3D. Conversely, when plants were grown on high mineral S, their total S content was greatly increased, according to a massive

cumulated S uptake throughout the experiment (Figure 3D). However, this increase was markedly more important for LSi-HS plants where S content (Figure 3C) was increased by approximately 20 times against solely less than two times for HS_i-HS plants at the end of the experiment. As a consequence, S deriving from uptake represented about 90 and 60% of total S content for LSi-HS and HS_i-HS plants on Day 28, respectively, against less than 5% in average for LS plants.

3.1.4 Whole plant N and S partitioning and remobilization. Table I presents the influence of initial S status and mineral S availability on ¹⁵N and ³⁴S partitioning (derived from uptake) and ¹⁴N and ³²S (*i.e.* deriving from initial endogenous N and S present on day 0) remobilization within whole leaf-blades and roots on Day 28, as well as total N and S amounts and total remobilized N and S at the whole plant level. Other tissues such as leaf petioles and stems are not presently given. Irrespective of the treatment, ¹⁵N and ³⁴S taken up were mainly partitioned within the leaf-blades, and secondly within the roots (both tissues thus represented 65 to 85% of whole plant total N or S). However, and except for N in LSi-LS plants, both ¹⁵N and ³⁴S partitioning within the leaf-blades was

severely reduced for LS plants, when compared to HS plants. This reduction was always accompanied by a parallel increase of the partitioning to the roots. For example, S partitioning in HS_i-LS roots reached 26.5%, against solely 15.6% within the roots of control plants.

Except for ¹⁴N in LSi-HS, leaf blades also always acted as source tissues for ¹⁴N and ³²S (*i.e.* endogenous initial N and S). Moreover, irrespective of their initial S status, there was an average 10 fold increase of the ¹⁴N remobilization within the leaf-blades in LS plants, when compared to HS plants. Except for ¹⁴N of LSi-LS plants, this greater remobilization was mainly at the benefit of the roots, which always acted as a sink tissue for ¹⁴N or ³²S. Moreover, 43 and even 100% of the total remobilized ¹⁴N at the whole plant level were recovered within the roots of LSi-LS and HS_i-LS plants, against less than 8% for control plants. For ³⁴S, and irrespective of the treatment, almost all of the total remobilized S at the whole plant level was coming from leaf blades, and the main part of it was then recovered within the roots on Day 28. The S remobilization to the roots was then increased by previous (LS_i) or following N (HS_i-LS) deficiency.

Figure 3. Changes of (A) whole plant N and (B) cumulated ¹⁵N (*i.e.* deriving from uptake) and (C) whole plant S and (D) cumulated ³⁴S (*i.e.* deriving from uptake) of plants initially grown either with high S (HS_i; solid line) or low S (LS_i; dotted line) and submitted during 28 days to High S (HS; closed circles) or Low S (LS; open circles) treatments. Vertical bars indicate ± s.e. of the mean for n = 4 when larger than the symbol. Different letters indicate that mean values are significantly different at a given date between treatments (P<0.05).

Table 1. Day 28 partitioning of total N (N_{tot}) or S (S_{tot}) in whole leaf-blades and roots as % of whole plant (roots, stems, leaf blade and leaf petioles) total N or S, and total remobilized N (¹⁴N, present on Day 0) and S (³²S, present on Day 0) within whole leaf-blades and roots of oilseed rape plants, initially grown either with high S (HS_i) or low S (LS_i) and submitted during 28 days to High S (HS; closed circles) or Low S (LS; open circles) treatments. Values are given as the mean ± s.e. (n = 4). Different letters indicate that mean values are significantly different between treatments (P<0.05).

Day 28		HS _i -HS	HS _i -LS	LS _i -HS	LS _i -LS
Whole leaf-blades	Partitioning N _{tot}	59.5 ± 2.6 ^c	38.0 ± 1.5 ^a	54.1 ± 2.6 ^b	50.0 ± 3.2 ^b
	(%) S _{tot}	58.2 ± 1.6 ^b	38.3 ± 2.5 ^a	61.0 ± 2.9 ^b	37.1 ± 4.9 ^a
	Remobilization ¹⁴ N	-1.75 ± 0 ^c	-20.8 ± 0.2 ^a	1.7 ± 0 ^d	-14.9 ± 0.1 ^b
	(mg. plant ⁻¹) ³² S	-11.9 ± 0.2 ^b	-14.6 ± 2.2 ^a	-9.2 ± 0.3 ^b	-3.9 ± 0.3 ^c
Roots	Partitioning N _{tot}	24.3 ± 1.8 ^{ab}	32.3 ± 5.6 ^b	31.9 ± 2.9 ^b	19.6 ± 1.8 ^a
	(%) S _{tot}	15.6 ± 1.6 ^a	26.5 ± 2.7 ^b	25.2 ± 1.0 ^b	30.2 ± 1.6 ^{bc}
	Remobilization ¹⁴ N	14.6 ± 0.1 ^c	5.4 ± 0 ^b	21.9 ± 0.2 ^d	-19.2 ± 0.3 ^a
	(mg. plant ⁻¹) ³² S	3.2 ± 0.7 ^a	7.3 ± 1.0 ^b	10.5 ± 0.3 ^c	2.1 ± 0.8 ^a
Whole plant	(mg. plant ⁻¹) N _{tot}	411.2 ± 18.1 ^c	357.6 ± 28.8 ^c	443.2 ± 38.9 ^b	274.1 ± 29.9 ^a
	S _{tot}	116.8 ± 10.3 ^c	53.1 ± 3.2 ^c	93.6 ± 0.7 ^b	8.8 ± 2.3 ^a
	Remobilization ¹⁴ N	-21.2 ± 0.7 ^a	-20.8 ± 0.2 ^a	-23.7 ± 0 ^b	-34.2 ± 2.0 ^c
	(mg. plant ⁻¹) ³² S	-11.9 ± 0.2 ^b	-14.6 ± 2.2 ^c	-13.4 ± 3.0 ^b	-3.9 ± 0.3 ^a

3.2 Roots analysis

3.2.1 Total N, total S and cumulated ¹⁵N and ³⁴S contents. Figure 4 presents the effect of initial S status and S availability on total N, total S and cumulated ¹⁵N and ³⁴S contents within the roots of oilseed rape plants during the 28 days of experiment. At the beginning of the experiment, total N content was about 25 mg N. roots⁻¹ in average for all treatments (Figure 4A). This content was then progressively increased for overall treatments but there was a faster and greater increase for HS plants than for LS plants during the first 14 days of experiment. Until this date, this increase remained greater for LS_i-HS plants than for other treatments. These overall results were confirmed by the dynamics of ¹⁵N content (*i.e.* deriving from recent N uptake), where it was significantly higher for plants grown on high S than on low S (Figure 4B). Moreover, more than 90% of total N found in the roots was finally deriving from recent N uptake for HS plants, against less than 60% for LS plants.

Roots S content was 6 fold lesser for LS_i plants than for HS_i plants on day 0. Thereafter, and except for LS_i-LS plants where it was kept rather constant over time, root S content was greatly increased for overall treatments during the experiment (Figure 4C). However, it was much more increased for LS_i-HS plants than for control plants and roots S content therefore reached values not significantly different from control plants.

As expected, roots ³⁴S content showed a positive and constant increase for plants grown with high mineral S, but there was no significant differences between HS_i or LS_i plants (Figure 4D). In the meantime, it remained to a negligible level for plants grown with low mineral S availability. Therefore, roots ³⁴S content was 13 fold greater for HS plants than for LS plants on day 28. Overall data from figures 4C and D shows that the faster increase of S content in roots was achieved by internal S remobilization rather than by an increased S uptake.

Figure 4. Changes of (A) roots total N and (B) cumulated ^{15}N (i.e. deriving from uptake) and (C) roots total S and (D) cumulated ^{34}S (i.e. deriving from uptake) of oilseed rape plants, initially grown either with high S (HS_i ; solid line) or low S (LS_i ; dotted line) and submitted during 28 days to High S (HS; closed circles) or Low S (LS; open circles) treatments. Vertical bars indicate $\pm$ s.e. ($n = 4$) when larger than the symbol. Different letters indicate that mean values are significantly different at a given date between treatments ($P < 0.05$).

3.2.2 Nitrate, sulphate and amino acids content.

The influence of initial S status and S availability on the kinetics of both nitrate-N, sulphate-S and amino acids contents within the roots of oilseed rape are presented on **Figure 5A, B and C**, respectively. On Day 0, root nitrate-N content was almost 4 times greater for LS_i than for HS_i plants and represented less than 4% of total N (according to **Figure 4A**). Thereafter, and irrespective of the initial S status, roots nitrate-N content was fastly and severely reduced when plants were grown on high S. However, it was increased by about 50% between Day 0 and Day 28 for LS_iLS plants.

Conversely, and when compared to LS_i plants where it was close to negligible values, roots sulphate-S content was greater for HS_i plants on Day 0 (**Fig. 5B**). On this date, sulphate-S content even represented 44% of roots total S for HS_i plants, against solely 12% for LS_i plants. During the experiment and irrespective of the S availability, roots sulphate-S content was reduced for HS_i plants but this decrease was greater for $\text{HS}_i\text{-LS}$ plants. In the meantime, roots sulphate-S content was greatly increased for $\text{LS}_i\text{-HS}$ plants, whereas it remained stable and close to negligible values for $\text{LS}_i\text{-LS}$ plants. As a consequence, and despite very different initial values, roots sulphate-S content was similar for plants grown on HS on Day 28 with $1.7 \text{ mg S-SO}_4^{2-} \cdot \text{g}^{-1} \text{ DW}$ on average. Overall, when all treatments are considered together, nitrate was accumulated in the roots at value higher than $0.3 \text{ mg N-NO}_3^- \cdot \text{g}^{-1} \text{ DW}$,

only when sulphate concentrations were below $0.2 \text{ mg S-SO}_4^{2-} \cdot \text{g}^{-1} \text{ DW}$.

On Day 0, amino acids content was almost two fold greater for LS_i plants than for HS_i plants. Thereafter, and except for $\text{LS}_i\text{-LS}$ plants where it remained constant, amino acids content was decreased during the experiment, and more particularly for $\text{LS}_i\text{-HS}$ plants. On Day 28, despite greater initial values, it was therefore not significantly different from control plants. On this date, root amino acids content was greater for LS than for HS plants, irrespective of their initial S status.

3.2.3 Relative expression of nitrate and sulphate transporters.

The influence of initial S status and mineral S availability on the kinetics of both nitrate (*BnNrt.1;1* and *BnNrt.2.1*) and sulphate plasmalema root transporters (*BnSultr.1;1* and *BnSultr.1;2*) as well as on sulphate tonoplastic root transporters (*BnSultr.4;1* and *BnSultr.4;2*) are reported on **Figure 6**. During the first 14 days of treatment, and except for control plants (i.e. $\text{HS}_i\text{-HS}$) where it remained significantly unchanged, there was a progressive decline in the expression pattern of *BnNrt.1;1* for overall treatments, when compared to values measured on Day 0 (**Figure 6A**). This decline was however more important for $\text{LS}_i\text{-HS}$ plants where there was a 4 fold decrease, against less than one third in average for others. Thereafter, the relative expression of the root plasmalema nitrate transporter *BnNrt.1;1* was nearly constant for overall treatments.

Figure 5. Changes of (A) nitrate, (B) sulphate and (C) amino acids contents in roots of oilseed rape plants initially grown either with high S (HS_i) or low S (LS_i) and submitted during 28 days to High S (HS; closed circles) or Low S (LS; open circles) treatments. Vertical bars indicate ± s.e. (n = 4) when larger than the symbol. Different letters indicate that mean values are significantly different at a given date between treatments (P<0.05).

On Day 0, the relative expression of the root plasmalemma nitrate transporter *BnNrt.2;1* was decreased in LS_i plants. Then, except for LS_i-LS plants where it was nearly constant over time, there was an increase of the relative expression of *BnNrt.2;1* for overall treatments (Figure 6B). This increase was however far more important for LS_i-HS plants when compared to other treatments. When compared on Day 0, the relative expression of *BnNrt.2;1* was thus increased of more than 10 times after 14 days, against solely 2 to 3 times for HS_i-HS or HS_i-LS plants.

Lastly, when compared to the relative expression observed for HS_i plants on Day 0, there was a significant increase for overall sulphate transporters (Figures 6C, D, E and F). This increase was even close to 6000 times for *BnSultr.1;1*, which was the most induced sulphate transporter amongst all on Day 0. Thereafter, and except for *BnSultr.4;1* where it was nearly constant over time, there was a progressive decrease of the relative expression of overall sulphate

transporters for LS_i-LS plants. Conversely, there was a significant and progressive induction of the relative expression of overall transporters for HS_i-LS plants. For example, *BnSultr.1;1* relative expression increased from nearly none to 2000 times between Day 0 and the end of the experiment (Figure 6C). Lastly, the relative expression of overall plasmalemma and tonoplastic sulphate root transporters was greatly and fastly reduced for LS_i plants grown on high S. As a consequence, and except for *BnSultr.4;1* where there was only a 50% decrease, the relative expression of overall root sulphate transporters was severely decreased to negligible values after only 14 days of experiment.

4. Discussion

It is generally admitted that S limitation has negative consequences on seed yield and quality [5, 8, 24] in winter oilseed rape (*Brassica napus* L.) and induces a broad range of physiological and molecular adaptations [12, 25, 26, 27], like an optimization of both sulphate uptake and utilization [9], as well as an increase of S reserves remobilization from vegetative tissues [16, 20]. Soil S concentration fluctuation, which is largely affected by S fertilizers may generate situations where plants are confronted to variable S availability, that may be favourable or not to S uptake, storage and mobilization. Because final seed yield and quality are also largely dependent on events occurring during the vegetative growth of the plant in oilseed rape [28, 29], the present experiment was designed in order to obtain plants (at vegetative stage) with high (HS_i) or low (LS_i) initial S reserve status followed by a period of extended S starvation (LS) or S availability (HS). Biomass production, ¹⁵N and ³⁴S uptake and expression of genes encoding relevant root nitrate and sulphate transporters, as well as N and S partitioning and remobilization were assessed

4.1 S limitation modifies plant initial S status.

When plants were initially (*i.e* two weeks before Day 0) grown with low sulphate availability, this resulted in a significant decrease of whole plant biomass (Fig. 2) and whole plant total N and S contents (Fig. 3), as previously reported in oilseed rape [4, 9, 17, 29] or wheat (*Triticum aestivum* L.) [30] and maize (*Zea mays* L.) [31]. As a consequence, overall results clearly show that when oilseed rape plants were initially S limited during two weeks, this resulted in a large modification of the initial plant S status, and more precisely of the initial level of S reserves (mainly as sulphate, which represents almost 85% of whole plant total S for control plants). In the meantime, both total S and sulphate-S contents were also severely decreased within the roots on Day 0 (sulphate accounting for about 10% of root total S, against about 50% for plants grown on High S; Fig. 5). In these LS_i plants, all sulphate transporter genes

Figure 6. Relative expression of root nitrate transporters (A) *BnNRT1.1*, (B) *BnNRT1.2* and root sulphate transporters (C) *BnSultr1.1*, (D) *BnSultr1.2* (E) *BnSultr4.1* and (F) *BnSultr4.2* in oilseed rape plants, initially grown either with high S (HS_i) or low S (LS_i) and submitted during 28 days to High S (HS ; closed circles) or Low S (LS ; open circles) treatments. Vertical bars indicate $\pm$ s.e. ($n=4$) when larger than the symbol. Values are given as the mean $\pm$ s.e. ($n=3$) of Q-PCR analysis and are expressed relatively to the Day 28 value observed for control plants (*i.e.* $HS_i - HS$). Different letters indicate that mean values are significantly different at a given date between treatments ($P<0.05$).

were up regulated (**Fig. 6**), while the root nitrate high affinity transporter was down-regulated, probably as a result of an increase of root amino acid contents (**Fig. 5C**) as Clarkson *et al.* [32] or Nikiforova *et al.* [33] suggested it for *Brassicaceae*.

4.2 A low initial S status increased N and S uptake under ample supply. With depleted initial S reserves (*i.e.* LS_i plants), whole plant, whole leaf-blades and roots biomass production (**Fig. 2**), as well as plant total N and S (**Fig. 3A** and **B**) were significantly increased during the experiment when plants were grown on High S. This was mainly achieved according to the large increase of ^{15}N and particularly ^{34}S uptake (**Fig. 3C** and **D**), which was concomitant with higher gene expression levels of roots plasmalemma sulphate transporters (**Fig. 6C** and **D**). This indicates a possible de-repression mechanism, probably mediated by O-acetyl-serine (OAS) as previously reported by Koralewska *et al.* [34] and

Hirai *et al.* [35]. Conversely, genes encoding roots nitrate transporters were either unaffected (low affinity, **Fig. 6A**) or repressed (high affinity, **Fig. 6B**) in plants with depleted initial S reserves. This repression could be triggered by a higher root amino acid content, as Abdallah *et al.* [36] already reported it for oilseed rape. Several studies also demonstrated that amino acids can repress the expression of high affinity nitrate transporters [37, 38, 39]. Accordingly, *Nrt2.1* expression as well as NO_3^- uptake (**Fig. 3B**) were then de-repressed under S ample supply, with a concomitant decrease of amino acid content in roots (**Fig. 5C**).

Consequently, with low initial S status but grown on High S, oilseed rape has an increased S uptake capacity (**Fig. 3D**), which allows maximum growth and development as well as a rather complete replenishment of S reserves (mainly as sulphate, **Fig. 5B**) within the different plant tissues. Moreover, most

of the sulphate taken up by the roots was distributed within the shoot (**Table I**), where it represented more than 80% of total S in young leaf-blades (data not shown). Therefore, sulphate could be further used as a transient store of S for the future needs of the plant for example during reproductive development during which S is required for seed filling [20, 36]. For example, Dubousset *et al.* [28] reported in oilseed rape that a limitation of S supply during late vegetative growth (*i.e.* just before bolting) resulted in a significant decrease of seed yield as well as seed protein content. While Sunarpi and Anderson [40] reported that unlike in soybean (*Glycine max* L. Merr.) where leaves contribute little to seed filling, our present work, taken together with our previous studies [36] and that of Dubousset *et al.* [28] underline that leaves of oilseed rape would be crucial for their role as a major source organ for S in response to S limitation. Moreover, and unlike in wheat [41], this mobilization of S stores within the leaves is achieved at the rosette (vegetative) stage without any acceleration of leaf senescence, which has a large positive impact on nitrogen use efficiency (NUE) or sulphur use efficiency (SUE) [28, 36].

The present study also revealed that plant total N content (**Figure 3A**), cumulated N taken up (**Figure 3B**) and *BnNrt2.1* de-repression were increased for plants which had depleted initial S reserves (**Figure 3A and B**) but further grown with ample S supply. It is usually reported that S availability may influence N use efficiency (NUE) of oilseed rape and *vice-versa* [29, 42], indicating that mineral S and N availabilities closely interact on S and N management by the plant [43]. Hesse *et al.*, [44] also reported that sulphur interacts with nitrogen in such a way that the lack of one reduces the uptake and assimilation of the other. Under these conditions, the increased S uptake and assimilation would have certainly led to an increased *de novo* synthesis of S-containing amino-acids and, as a consequence, of an increased proteic synthesis which thus led to decrease the root amino acids contents. This decrease of root amino acid concentration, according to their associated regulatory control on N uptake transporters, could explain the highest N uptake, as Gojon *et al.* reported it for *Arabidopsis thaliana* [45].

4.3 Low S availability can be compensated for by high initial S status. With High initial S status, the limitation of S supply led to a strong limitation of whole plant S uptake (**Fig. 3D**) and to a concomitant induction of overall roots plasmalemma sulphate transporters (**Fig. 5**), but without significant S uptake (**Fig. 3D**). For example, *BnSultr.4;1* relative expression was greatly increased from about 2000 times between day 0 and the end of the experiment (**Figure 5C**). Several authors already reported such de-repression of sulphate transporters genes in plants grown under S limited conditions [11, 12, 14].

However, in our experimental conditions, plant growth remained surprisingly unchanged (**Fig. 1A**). This was mainly achieved by a large remobilization of S reserves and by the subsequent depletion of sulphate pools, for example from the roots (**Fig. 5B**). Moreover, this was also due to a greater remobilization of initial S reserves (*i.e.* ^{32}S) from shoot to roots tissues, which acted as the strongest sink for S (**Table I**). Thus, almost all of the ^{32}S entering the roots was coming from leaf blades for LSi plants, against less than 50% of values reported for HSi plants.

As a consequence, our results clearly show for the first time that winter oilseed rape, with high initial S reserves, can easily compensate for a transient S limitation, mainly through a large remobilization of S reserves and a redistribution of S fluxes towards growing tissues like young leaves (data not shown) or roots. Surprisingly, this also allows optimal growth like control plants. This particular behaviour of oilseed rape is different from wheat or maize where it was reported that despite a sufficient S availability during early development (*i.e.* vegetative growth), a S limitation can seriously impact final seed yield and quality [30]. This is however related to the weaker requirements for S for these two species, when compared to oilseed rape, as Abrol and Ahmad reported it [46].

4.4 Low initial S status affects N metabolism when plants are S limited. With low initial S reserves and grown on low S, and despite an overall induction of S uptake transporters, there was few S uptake and overall S reserves, mainly sulphate, were already depleted in all plants tissues. As a consequence, whole plant total remobilized ^{32}S was two fold reduced and leaf blade remobilization towards the roots was also significantly reduced, when compared to control plants (**Table I**). These overall processes therefore severely affected whole plant biomass production, as several authors reported it (for review, see Anjum *et al.* [47]). However, irrespective of their initial S status, there was an average 10 times greater increase of the ^{14}N remobilization within the leaf blades of S limited plants, when compared to HS plants (**Table I**). This effect of a large S limitation throughout the vegetative growth cycle was already observed for most large crop species like soybean, wheat or maize where an average 30% was observed in response to a severe S limitation [30, 40]. Our study also showed that the relative expression of *BnNrt.2;1*, a gene supposed to encode root plasmalemma high affinity nitrate transporter, is under permanent repression (**Figure 6B**) probably as a result of a root amino-acid concentration that was maintained to its highest value (**Figure 5C**). It could be easily suggested, even if individual amino-acids were not analyzed, that it was the result of an accumulation of non S-containing

amino acids like glutamine or asparagine, related to a reduced protein synthesis.

It was also found that root nitrate-N content was almost 4 times greater for LS_i than for HS_i plants (**Figure 5A**). Thereafter, and irrespective of the initial S status, root nitrate-N content was fastly and severely reduced when plants were grown on High S. Conversely, it was increased by about 50% during the experiment for LS_i-LS plants. In *Brassicaceae*, it was demonstrated that a sub-optimal S nutrition results in large accumulations of nitrate [4, 48]. Nitrate and sulphate concentrations in roots followed opposite changes, being increased and decreased by low S supply, respectively (**Figure 5**). To our knowledge, this has never been described previously but it could be the results of a compensatory process due to their involvement in the osmotic potential of root cells as shown for example in *Lolium perenne* L. between nitrate and chloride [49]. Alternatively, this nitrate accumulation could be the result of a lower nitrate reductase activity, as already suggested by Reuveny et al., [19], who reported that a deficiency of sulphur represses nitrate reductase activity even in the presence of an adequate supply of nitrate. Overall results therefore have particular importance for oilseed rape crop management systems and underline that, unlike for wheat or maize, much attention should be paid during early (vegetative) growth.

Acknowledgements

This work was supported by a PhD grant to Abdallah M. from the Egyptian government. Authors are grateful to Marie Paule Bataillé, Raphael Segura, Anne Sophie Desfeux, and Sandrine Rezé for their skillfull help with isotopic mass spectrometry, plant culture and molecular analysis.

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