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RESEARCH PAPER

Is the remobilization of S and N reserves for seed filling of winter oilseed rape modulated by sulphate restrictions occurring at different growth stages?

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Abstract

How the remobilization of S and N reserves can meet the needs of seeds of oilseed rape subject to limitation of S fertilization remains largely unclear. Thus, this survey aims to determine the incidence of sulphate restriction [low S (LS)] applied at bolting [growth stage (GS) 32], visible bud (GS 53), and start of pod filling (GS 70) on source–sink relationships for S and N, and on the dynamics of endogenous/exogenous S and N contributing to seed yield and quality. Sulphate restrictions applied at GS 32, GS 53, and GS 70 were annotated LS_{32}, LS_{53}, and LS_{70}. Long-term $^{34}SO_4^{2-}$ and $^{15}NO_3^-$ labelling was used to explore S and N partitioning at the whole-plant level. In LS_{53}, the sulphur remobilization efficiency (SRE) to seeds increased, but not enough to maintain seed quality. In LS_{32}, an early S remobilization from leaves provided S for root, stem, and pod growth, but the subsequent demand for seed development was not met adequately and the N utilization efficiency (NUtE) was reduced when compared with high S (HS). The highest SRE (65±1.2% of the remobilized S) associated with an efficient foliar S mobilization (with minimal residual S concentrations of 0.1–0.2% dry matter) was observed under LS_{70} treatment, which did not affect yield components.

Key words: Oilseed rape, sulphate restriction, ^{34}S and ^{15}N labelling, S remobilization efficiency, S utilization efficiency.

Introduction

Sulphur (S) is an important nutrient for plant growth and development. In comparison with other crops such as cereals, oilseed rape (*Brassica napus* L.) requires a relatively large amount of mineral S (Zhao *et al.*, 1997). During the last two decades, the reduced atmospheric pollution by industries has resulted in a major reduction in S emissions and, as a consequence, S deposition into the soil has strongly declined, particularly in Western Europe (McNeill *et al.*, 2005). A deficiency in S can reduce yield, and impacts on the quality of harvested products (Janzen and Bettany, 1984; McGrath and Zhao, 1996; Scherer, 2001). The technical centre for oilseed production in France (CETIOM) recommends systematic S fertilization for oilseed rape crops with ~30 kg S ha^{-1}. Therefore, more attention should be paid to S fertilization practices that need to be optimized to fulfil plant S requirements whilst minimizing cost. Similarly to nitrogen (N) uptake (Rossato *et al.*, 2001), the S requirement of oilseed rape would depend on the stage of plant development and environmental conditions. Indeed, the S requirement is not stable during the growth cycle of oilseed rape: S uptake increased from stem extension to the start of flowering, whereas little S uptake was generally (but not exclusively) observed during pod filling (McGrath and Zhao, 1996; Postma *et al.*, 1999).

Winter oilseed rape can be used to reduce N leaching during the autumn–winter period because of its high capacity to take up nitrate from the soil. N and S nutrition are tightly linked during the growth cycle (Reuveny *et al.*, 1980; Fismes *et al.*, 2000). N and S are both involved in amino acid and protein synthesis. Restriction of S supply

Abbreviations: DM, dry mass; GS, growth stage; HI, harvest index; HS, high S; LS, low S; NRE, nitrogen remobilization efficiency; NUtE, nitrogen utilization efficiency; S, sulphur; SRE, sulphur remobilization efficiency; SUtE, sulphur utilization efficiency; V-CDT, Vienna Canyon Diablo Troilite.

has been shown to depress the nitrate uptake and nitrate reductase activity in maize and spinach (Friedrich and Schrader, 1978; Prosser *et al.*, 2001), and can result in nitrate accumulation in leaves of oilseed rape (McGrath and Zhao, 1996). Fismes *et al.* (2000) reported that the S and N use efficiency of oilseed rape are synergistic at optimum rates and antagonistic at excessive levels of one of the elements. S fertilization is required to improve N use efficiency and thereby maintain a sufficient oil content and fatty acid quality of seeds (Fismes *et al.*, 2000).

During vegetative development, winter oilseed rape is at the rosette stage in winter and the leaves represent a major store of nutrients which can be remobilized thereafter to sustain growth of reproductive tissues, as shown specifically for N (Schjoerring *et al.*, 1995; Rossato *et al.* 2001; Noquet *et al.* 2004; Malagoli *et al.*, 2005*a*). For instance, nearly 75% of the N content in reproductive tissues of oilseed rape is derived from N mobilization occurring mostly in leaves and stems (Malagoli *et al.*, 2005*b*). Therefore, leaves emerging during the rosette stage would play a crucial role in seed filling and contribute to the maintenance of seed yield (Noquet *et al.*, 2004). Thus, optimizing S fertilization requires a better understanding of (i) source–sink relationships for S at the whole-plant level; and (ii) processes of S mobilization by evaluating plant S partitioning in relation to the plant growth stage and N status from stem extension to harvest.

Oilseed rape may accumulate abundant amounts of sulphate ($^{34}SO_4^{2-}$), but this anion is not mobilized efficiently from vegetative to reproductive tissues: the S Harvest Index (SHI, i.e. the S amount in seeds divided by the total S in the whole crop) is only ~20% (McGrath and Zhao, 1996), indicating that a large proportion of S is retained in the vegetative tissues. Sulphate stored in the vacuoles is the main form of S reserve in vegetative tissues (Blake-Kalff *et al.*, 1998; Scherer, 2001; Matula and Pechová, 2002). To sustain the S demand for growth of oilseed rape under S restriction occurring at the rosette stage, a strong S mobilization (mainly an $^{34}SO_4^{2-}$ mobilization), associated with an up-regulation of *BnSultr4;1* and/or *BnSultr4;2* expression (two transporters involved in efflux of sulphate from vacuoles; Kataoka *et al.*, 2004; Parmar *et al.*, 2007), was reported in leaves (Dubousset *et al.*, 2009). Smith and Lang (1988) reported that 90% of the S transported via the phloem is inorganic in soybean. Sunarpi and Anderson (1998) described S redistribution in S-deficient vegetative soybean (with an ^{35}S pulse–chase labelling method) and reported that ~25% of the mobilized S was recycled as $^{34}SO_4^{2-}$ via the root and the largest newly expanded leaf, which acts as an intermediary in the transport of S from the root to the youngest expanding leaves. S mobilization in suboptimal conditions of S fertilization was also examined in reproductive soybean (Sunarpi and Anderson, 1997; Naeve and Shibles, 2005). These authors reported that soybean leaves did not act as large reservoirs for S in conditions of suboptimal S fertilization. Nevertheless, under SO_4^{2-}-sufficient conditions, it was shown that leaves of soybean supplied the seed with 20% of its total S requirement (Naeve and Shibles, 2005). Therefore, in soybean, the amount of S mobilized from leaves at the reproductive stage appears to be reliant on the amount previously stored in roots and leaves. In oilseed rape, the source–sink relationships for S, and more particularly the contribution of leaves in the S reallocation to seeds, remains unclear. The concentration of S in leaves at early flowering was suggested to be the best index in predicting S deficiency in terms of seed yield by McGrath and Zhao (1996).

Although mobilization of S and N from vegetative tissues is likely to be important for seed filling in oilseed rape, very little is known about the efficiency (dynamics and amounts) of S and N mobilization to the reproductive tissues. How the limitation of S fertilization impacts on the remobilization processes of S reserves and N reserves also remains largely unclear. To address these questions, the aim of this study was to determine the impact of sulphate restrictions [low S (LS) versus high S (HS)] applied at bolting (GS 32), visible bud (GS 53), and start of pod filling (GS 70) growth stages of winter oilseed rape on (i) the source–sink relationships for S and N at the whole-plant level; (ii) the remobilization of S reserves and N reserves and their contribution to developing seeds; and (iii) the seed yield and grain quality. To explore S and N reserve partitioning in oilseed rape, a greenhouse experiment was carried out for long-term steady-state labelling using stable isotopes as tracers, with $^{34}SO_4^{2-}$ and $^{15}NO_3^-$ applied at the beginning of the stem elongation stage (GS 16) for different periods (17, 30, and 44 d), before applying S restriction. In this way, the dynamics of the mobilization of S and N compounds in response to different levels of mineral S availability during the subsequent chase periods could be accurately estimated. Additionally, to determine if the foliar residual S and N concentrations were related potentially to an efficient mobilization of S and N to seeds, the S and N concentration in dead leaves in response to the different mineral S availabilities was examined in relation to their nodal positions.

Materials and methods

Experimental treatments and tissue sampling

The oilseed rape genotype chosen for this greenhouse experiment was cv. Capitol, a genotype well described in terms of N use efficiency (Malagoli *et al.*, 2004, 2005*a*, *b*; Gombert *et al.*, 2006; Etienne *et al.*, 2007; Desclos *et al.*, 2008, 2009). After surface sterilization, seeds were germinated on vermiculite in 20.0 l tanks for 24 seedlings and grown with a thermoperiod of 20 °C (day 16 h) and 15 °C (night 8 h), on 25% Hoagland nutrient solution consisting of 1.25 mM $Ca(NO_3)_2{\cdot}4H_2O$, 1.25 mM KNO_3, 0.5 mM $MgSO_4$, 0.25 mM KH_2PO_4, 0.2 mM EDTA, $2NaFe{\cdot}3H_2O$, 14 μM H_3BO_3, 5 μM $MnSO_4$, 3 μM $ZnSO_4$, 0.7 μM $(NH_4)_6Mo_7O_{24}$, 0.7 μM $CuSO_4$, 0.1 μM $CoCl_2$, renewed twice a week for 36 d. The plants were then submitted to 8 °C (day 10 h) and 4 °C (night 14 h) for 46 d for vernalization with the same nutrient solution renewed twice a week. After this period of vernalization, every plant was transferred to pots containing mixed 1/3 vermiculite and 2/3 perlite (one plant per pot) and submitted to a thermoperiod of 20 °C (day) and 15 °C (night). As indicated in Fig. 1, during different periods of growth [from GS 16 (rosette stage) to GS 32

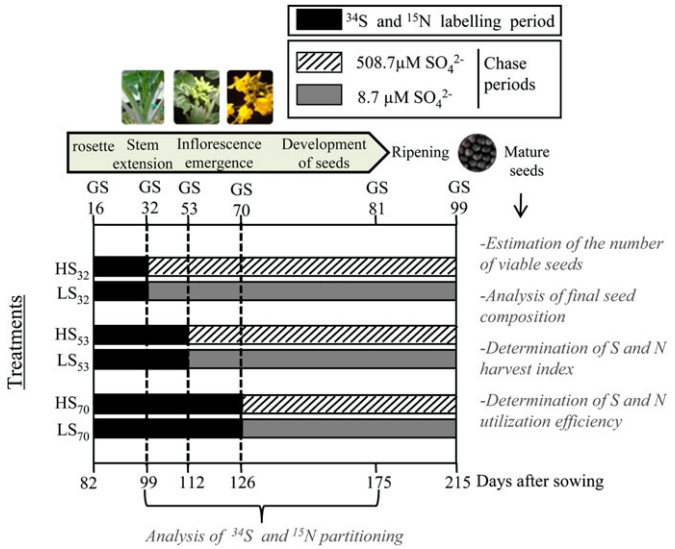

Fig. 1. Schematic diagram of the experimental design. Mineral S restriction [low S (LS)] was applied at GS 32 (bolting stage) for LS_{32}, GS 53 (visible bud stage) for LS_{53}, or GS 70 (start of pod filling) for LS_{70}, until the end of the growth cycle (GS 99). During different periods (from GS 16 to GS 32, GS 16 to GS 53, or GS 16 to GS 70), the plants were supplied with $^{34}SO_4^{2-}$ (1 atom% excess) and $^{15}NO_3^-$ (2 atom% excess) in order to obtain plants with homogeneous ^{34}S and ^{15}N labelling before applying treatments. Plants were harvested at GS 32, GS 53, GS 70, GS 81, and GS 99.

(bolting stage), GS 53 (visible bud stage), or GS 70 (start of pod filling)], plants were supplied with $^{34}SO_4^{2-}$ (1 atom% excess) and $^{15}NO_3^-$ (2 atom% excess) in order to obtain plants with homogeneous ^{34}S and ^{15}N labelling. Each day, the nutrient solution (25% Hoagland for control plants, i.e. HS treatment) was supplied automatically in an increasing volume as a function of the growth stages: 90, 120, 150, and 180 ml per plant at the start of the bolting stage, the visible bud stage, the flowering stage, and the seed maturation stage, respectively. Mineral S restriction (LS treatments) corresponding to 8.7 µM $^{34}SO_4^{2-}$ was applied at GS 32 for LS_{32}, GS 53 for LS_{53}, or GS 70 for LS_{70}, until the end of the growth cycle (GS 99).

At final harvest, the number of mature seeds per plant was accurately determined at GS 99 for four replicates per treatment. Seeds were then used for the test of viability and the determination of seed composition. At each date of harvest, the different plant parts (lateral roots, taproot, leaves, stem, floral stem, pod walls, and seeds) were weighed, freeze-dried, and then ground to a fine powder for elemental and isotope analyses. Old, mature, and young leaves were collected after determination of the relative chlorophyll concentration using the non-destructive SPAD (Soil Plant Analysis Development) chlorophyll meter (Minolta, SPAD-502 model), and measurement of the leaf area using a LI-COR 300 area meter (LI-COR, Lincoln, NE, USA). At each date of harvest, the leaves characterized by a bottom position on the plant and a chlorophyll concentration <55 SPAD units were clustered in 'old leaves'. Then, the upper leaves characterized by chlorophyll concentrations and areas >55 SPAD units and >55 cm^2 were clustered in 'mature leaves'. Finally, the younger leaves characterized by an area <55 cm^2 were clustered in 'young leaves'.

The leaf rank number was determined according to the date of leaf emergence using a labelled collar suspended on the petiole of each leaf rank after maturity. Thus, changes in the S and N concentrations in dead leaves were monitored for each nodal position, from seedling to the seed maturation stage. These leaf samples were freeze-dried, weighed for dry matter (DM) determination and then ground to a fine powder for S and N analyses.

Germination test for determination of seed viability

The viability of seeds produced by plants submitted to the different S availabilities was tested by assessment of seed germination. Mature seeds obtained for each treatment were germinated on Whatman filter paper soaked with sterile water within Petri dishes (12×12 cm). Fifty seeds per biological repetition (n=6 for HS and n=4 for each LS treatment) were sown on water for 7 days with a cycle of 8 h dark (18 °C)/16 h light (25 °C). Three technical replicates were performed for each biological repetition. The percentage of plantlets with normal development indicated the number of viable seeds for each S treatment.

Determination of oil, protein, and glucosinolate contents by NIRS

All the seed samples were scanned on a monochromator near infra red system (NIRSystem model 6500, FOSS NIRSystem Inc., Silver Spring, MD, USA) equipped with the transport module, in the reflectance mode. Intact seeds (~5 g) were placed in a standard ring cup and scanned. The results were predicted from an external calibration established for oil and total glucosinolate content (CRAW, Gembloux, Belgium). Three determinations were performed for each sample. The results were given as a percentage of oil or proteins per seed DM and in µmol of total glucosinolates per seed DM.

S, ^{34}S, N, and ^{15}N analysis

Freeze-dried samples were ground to a fine powder, weighed, and placed into tin analysis capsules. Both total S and N contents were determined with a continuous flow isotope mass spectrometer (IRMS, Isoprime, GV Instruments, Manchester, UK) linked to an analyser (EA3000, EuroVector, Milan, Italy). The IRMS analysis also provided the changes of the relative amount of ^{34}S and ^{15}N in excess in each sample derived from the tracer fed to the test plant.

The values can be calculated as:

^{34}S amount in excess=isotope abundance in sample (A%)–isotope abundance in natural standard (4.2549%)

$$\text{where } A=100\times[^{34}S/(^{34}S+^{32}S)]. \quad (1)$$

Similarly, ^{15}N amount in excess was determined as follows:

^{15}N amount in excess=isotope abundance in sample (A%)–isotope abundance in natural standard (0.3731%)

$$\text{where } A=100\times[^{15}N/(^{15}N+^{14}N)]. \quad (2)$$

$\delta^{34}S$ (‰) and $\delta^{15}N$ (‰), that were experimentally measured in each sample, are indexes generally used and defined as:

$$\delta^{34}S=(\text{Rsample}/\text{Rstandard}-1)\times 1000 \quad (3)$$

where Rsample indicates the isotopic ratio ($^{34}S/^{32}S$) in the sample, and Rstandard=0.04415206 is the internationally accepted isotope standard for S corresponding to V-CDT (Vienna Canyon Diablo Troilite).

$$\delta^{15}N=(\text{Rsample}/\text{Ratm-1})\times 1000 \quad (4)$$

where Rsample indicates the isotopic ratio ($^{15}N/^{14}N$) in the sample and Ratm indicates the isotopic ratio in the atmosphere.

Accordingly, the value of Rsample can be estimated from $\delta^{34}S$ and $\delta^{15}N$ value as follows:

$$\text{Rsample}=(\delta^{34}S\times\text{Rstandard}/1000)+\text{Rstandard} \quad (5)$$

or

$$\text{Rsample} = (\delta^{15}\text{N} \times \text{Ratm}/1000) + \text{Ratm} \quad (6)$$

Then, Equations (1) and (2) can be rewritten as:

$$A = 100 \times \text{Rsample}/(\text{Rsample} + 1)$$

From equations (1), (2), (5) and (6), ^{34}S and ^{15}N amounts in excess can be estimated from the data of δ^{34}S and δ^{15}N.

Calculation of S and N partitioning and remobilisation

Long periods of labelling allow a homogenous distribution of tracers in different organs and different biochemical fractions containing S and/or N. Normalization of the amounts of absorbed ^{34}S and ^{15}N is carried out using the average amount of each of these isotopes found throughout the whole plant for each harvest date and treatment submitted to similar periods of labelling. After normalization, the partitioning of ^{34}S and ^{15}N in plants is expressed as the percentage of total ^{34}S and ^{15}N. The method of calculation of S flows is presented below and can be transposed to the determination of N flows. The calculations of flows of remobilized S depend on the source or sink status of each organ. For the source organs, this is characterized by a loss of ^{34}S amount for a period Δt. Between the dates t_0 and $t_0+\Delta t$, the S amount remobilized (QSRsource) corresponded to:

$$\text{QSRsource} = \text{QS}t_0 \times (\text{Q}^{34}\text{S}t_0 - \text{Q}^{34}\text{S}t_0 + \Delta t)/\text{Q}^{34}\text{S}t_0$$

where $\text{Q}^{34}\text{S}t_0$=amount of ^{34}S in the source organ at t_0, $\text{Q}^{34}\text{S}t_0+\Delta t$=amount of ^{34}S in the source organ at $t_0+\Delta t$, $\text{QS}t_0$=amount of S in the source organ at t_0, and Δt=period of chase, for example between GS 70 and GS 81.

For the sink organs, this is characterized by a gain of ^{34}S amount for a period Δt. Between the dates t_0 and $t_0+\Delta t$, the S amount derived from remobilization (QSRsink) corresponded to:

$$\text{QSRsink} = (\text{Q}^{34}\text{S}t_0+\Delta t - \text{Q}^{34}\text{S}t_0) \times \Sigma\text{QSRsource}/\Sigma(\text{Q}^{34}\text{S}t_0+\Delta t - \text{Q}^{34}\text{S}t_0)$$

where $\text{Q}^{34}\text{S}t_0$=amount of ^{34}S in the sink organ at t_0, $\text{Q}^{34}\text{S}t_0+\Delta t$=amount of ^{34}S in the sink organ at $t_0+\Delta t$, ΣQSRsource=total amount of S remobilized from source organs between t_0 and $t_0+\Delta t$, and $\Sigma(\text{Q}^{34}\text{S}t_{0+\Delta t} - \text{Q}^{34}\text{S}t_0)$=total amount of ^{34}S accumulated in the sink organs between t_0 and $t_0+\Delta t$.

The inflow of S taken up (QSInflux) between two dates (i.e. for the period Δt) was calculated by subtracting the S derived from remobilization (QSRsink or source) between these two dates from the change in total S amount for this period (ΔQS):

$$\text{QSInflux} = \Delta\text{QS} - \text{QSRsink or source}$$

Statistics

The normality of the data was studied with the Ryan–Joiner test at 95%. Analysis of variance (ANOVA) and the Tukey test to compare the means were performed with MINITAB13 on Windows (Minitab Inc., State College, PA, USA). When the normality law of the data was not respected, the non-parametric test of Kruskal–Wallis was carried out and followed by Mood's median test. Statistical significance was postulated at $P < 0.05$.

Results

Seed yield and quality at GS 99

In LS_{32} conditions, the global seed DM was reduced at GS 99 by almost 45% [from 11.6±0.61 g in control (HS) to 6.30±0.66 g per plant in LS_{32}; Table 1]. In addition, the number of viable seeds decreased greatly in response to LS_{32} treatment (Table 1) and corresponded to 15.3±1.6% of the total seeds produced. Compared with control, the oil and protein content was significantly decreased by the LS_{32} treatment. In addition, a strong decrease in glucosinolate content was observed in all LS treatments and especially in LS_{32} (–69±6.7%) and LS_{53} (–82±3.1%) (Table 1). The oil content in seeds was decreased in LS_{53} conditions (Table 1). In contrast to LS_{32}, the protein content was not affected by the LS_{53} treatment as compared with the control. Interestingly, the oil and protein content in seeds was not significantly modified by the LS_{70} treatment (Table 1). LS_{70} treatment even had the benefit of lowering glucosinolate content (Table 1).

SHI, NHI, SUtE, and NUtE

The SHI (i.e. the S amount in seeds expressed as a percentage of the total S amount in plants at GS 99) corresponded to 26±1.3% of total S in control plants and was similar in LS_{32} conditions, whereas it reached 45±1.8% and 55±1.7% in LS_{53} and LS_{70} conditions, respectively (Table 1). The highest SHI was thus obtained in LS_{70} conditions and was

Table 1. *DM of total seeds and number of viable seeds, seed composition evaluated by NIRS in total seeds, S Harvest Index (SHI) and N Harvest Index (NHI) at GS 99 in plants subject to HS, LS_{32}, LS_{53}, and LS_{70} conditions*

Details of HS and LS treatments are given in Fig. 1. The values correspond to the mean ±SE (the number of viable seeds, SHI and NHI was determined with n=12 for HS, n=4 for LS_{32}, LS_{53}, and LS_{70}; the seed composition was determined with n=6 for HS, n=4 for LS_{32}, LS_{53}, LS_{70} with three technical repetitions for each analysis). Different letters indicate that mean values are significantly different ($P < 0.05$). The highest values obtained for each parameter are in bold.

	DM of total seeds produced per plant (g)	No. of viable seeds produced per plant	Oil content in mature seeds (% DM)	Protein content in mature seeds (% DM)	Glucosinolate content in seeds (μmol g^{-1} DM)	SHI (% of plant S in seeds)	NHI (% of plant N in seeds)
HS	**11.6±0.61 b**	**2398±146 b**	**45±0.5 c**	**23±0.3 b**	**14±0.4 c**	26±1.3 a	**49±2.1 b**
LS_{32}	6.30±0.66 a	313±233 a	32±1.3 a	21±0.3 a	4.2±0.9 a	25 ±2.2 a	35 ±3.9 a
LS_{53}	**11.6±0.81 b**	**2325±206 b**	43±0.3 b	**22±0.2 ab**	2.5±0.4 a	45 ±1.8 b	**54 ±1.9 b**
LS_{70}	**11.7±0.49 b**	**2502±109 b**	**45±0.2 bc**	**23±0.2 b**	8.3±0.5 b	**55 ±1.7 c**	**53 ±2.7 b**

Table 2. *S utilization efficiency (mg of mature seed DM per mg of S in shoots) and N utilization efficiency (mg of mature seed DM per mg of N in shoots) at GS 99 in HS, LS_{32}, LS_{53}, and LS_{70} conditions*

Details of HS and LS treatments are given in Fig. 1. The values correspond to the mean ±SE (n=12 for HS, n=4 for LS_{32}, LS_{53}, LS_{70}). Different letters indicate that mean values are significantly different (P <0.05).

	S utilization efficiency	N utilization efficiency
HS	81±5.8 a	32±2.1 b
LS_{32}	203±26 b	18±2.7 a
LS_{53}	461±24 c	48±5.6 c
LS_{70}	379±24 c	39±3.9 bc

2-fold higher than in control, suggesting a better targeting of S mobilization to seeds in response to this treatment. The N Harvest Index (NHI, i.e. the N amount in seeds expressed as a percentage of the total N amount in plants at GS 99) was 35±3.9% in LS_{32} conditions whereas it reached 49.1±2.1% in control (Table 1). The other LS treatments did not affect the seed N amount and NHI in comparison with control.

The production of DM of mature seeds was used to calculate the S or N utilization efficiency (SUtE and NUtE, expressed as seed DM produced per unit of S or N accumulated in vegetative shoots; Table 2). The highest values of SUtE were obtained in LS_{53} and LS_{70} conditions and reached 461±24 mg and 379±24 mg of mature seed DM per mg of S in shoots, respectively (Table 2). Compared with control, the NUtE was significantly increased only in LS_{53} conditions (with 48±5.6 mg versus 32±2.1 mg of mature seed DM per mg of N in shoots in HS).

Dynamics of ^{34}S partitioning

Using double ^{34}S and ^{15}N long-term labelling it was possible to estimate for the chase period the distribution of S reserves in plants. Figure 2 illustrates the ^{34}S partitioning from GS 32 to GS 81 in response to the different sulphate availabilities. The analysis of ^{34}S partitioning as a function of growth stages allows a determination of sink–source relationships for S at the whole-plant level.

In HS_{32} conditions, all leaves (young, mature, old, and dead leaves) contained the largest proportion of the total ^{34}S from GS 32 to GS 81. As a consequence, the foliar ^{34}S remobilization efficiency (SRE_{leaf}, corresponding to the loss of ^{34}S in leaves between two growth stages, expressed in a percentage of total ^{34}S labelling) was only 26±0.8% between GS 32 and GS 81 (Fig. 2A). At GS 70, the weak proportion of ^{34}S remobilized from leaves of HS_{32} plants was transiently allocated towards the stems and roots. After GS 70, the proportion of ^{34}S in roots remained stable (15±1.8%) and high amounts of ^{34}S were lost in dead leaves at GS 81 (54±6.0%; Fig. 2A).

Compared with HS_{32}, the proportion of ^{34}S allocated to stems, floral stems, and pod walls from GS 32 (bolting stage) to GS 81 (seed colouring) was increased by LS_{32} treatment (Fig. 2A). Interestingly, in response to LS_{32} treatment, roots became a transient major sink organ (until GS 70) before becoming a source for S (from GS 70 to GS 81) (Fig. 2A). From the beginning of the chase period, ^{34}S stored in the mature and old leaves of LS_{32} plants was mobilized earlier than in HS_{32} conditions, and this ^{34}S re-allocation was to the benefit of roots and floral stem. The residual ^{34}S in dead leaves was strongly decreased at GS 81, from 54±6.0% in HS_{32} to 24±0.3% of total ^{34}S in LS_{32}. Nevertheless, a remobilization from all leaves to other plant parts did not take place between GS 70 and GS 81 in LS_{32} conditions. Indeed, the total proportion of ^{34}S in all leaves remained stable between GS 70 and GS 81 (28±0.3%; Fig. 2A).

In HS_{53}, the ^{34}S partitioning from GS 32 to GS 53 illustrates the allocation associated with the S uptake before GS 53. After GS 53 (start of the chase period; Fig. 2B), the ^{34}S partitioning illustrates the pattern of remobilization of the S previously acquired in the plant. The SRE_{leaf} from GS 53 to GS 81 (42±1.8% for HS_{53}) is higher than the SRE_{leaf} obtained between GS 32 and GS 81 (26±0.8% for HS_{32}) (Fig. 2A, B). The mobilization of ^{34}S in leaves was associated with an increasing sink status, first of the floral stems (at GS 70) and secondly of pod walls and seeds (at GS 81). Roots and stems did not act as source or sink organs for ^{34}S between GS 53 and GS 81 in HS_{53} conditions (Fig. 2B). In response to LS_{53} conditions, compared with HS_{53}, the ^{34}S was allocated particularly to stems at GS 70 (but only transiently), while the ^{34}S accumulated in leaves decreased. In contrast to the LS_{32} conditions, the LS_{53} treatment did not provoke transient redistribution of ^{34}S towards roots. In comparison with HS_{53}, the final ^{34}S partitioning in LS_{53} conditions was characterized by the highest redistribution of ^{34}S in seeds (corresponding to 46±2.0% in LS_{53} versus 27±1.7% of total ^{34}S in HS_{53} at GS 81), whereas a better remobilization of ^{34}S reserves was noticed in stems and leaves (Fig. 2B). Consequently, at GS 81, dead leaves of LS_{53} contained 20.8±0.5% of total ^{34}S versus 31.1±0.7% in HS_{53}.

A large amount of ^{34}S was allocated to stems before GS 70 in HS_{70} conditions. The decline in ^{34}S from leaves (with an SRE_{leaf} of 15±1.1% from GS 70 to GS 81) was associated with the decrease of ^{34}S in the roots and stems and this ^{34}S was re-allocated towards seeds, which reached 30±3.0% at GS 81 (HS_{70}, Fig. 2C). As compared with HS_{70}, the decrease in ^{34}S in leaves was more important in LS_{70} conditions as indicated by the value of SRE_{leaf} (–35±0.5%) observed between GS 70 and GS 81. Indeed, the dead leaves corresponded finally to 14±0.5% in LS_{70} versus 27±0.8% of total ^{34}S in HS_{70} at GS 81 (Fig. 2C). The ^{34}S in seeds finally reached 45±3.0% of total ^{34}S in LS_{70}.

Dynamics of ^{15}N partitioning

The changes in ^{15}N partitioning from GS 32 to GS 81 in response to the different S treatments are given in Fig. 3.

In HS conditions, while the proportions and dynamics of ^{15}N were similar to ^{34}S in roots (Figs 2, 3), leaves contained

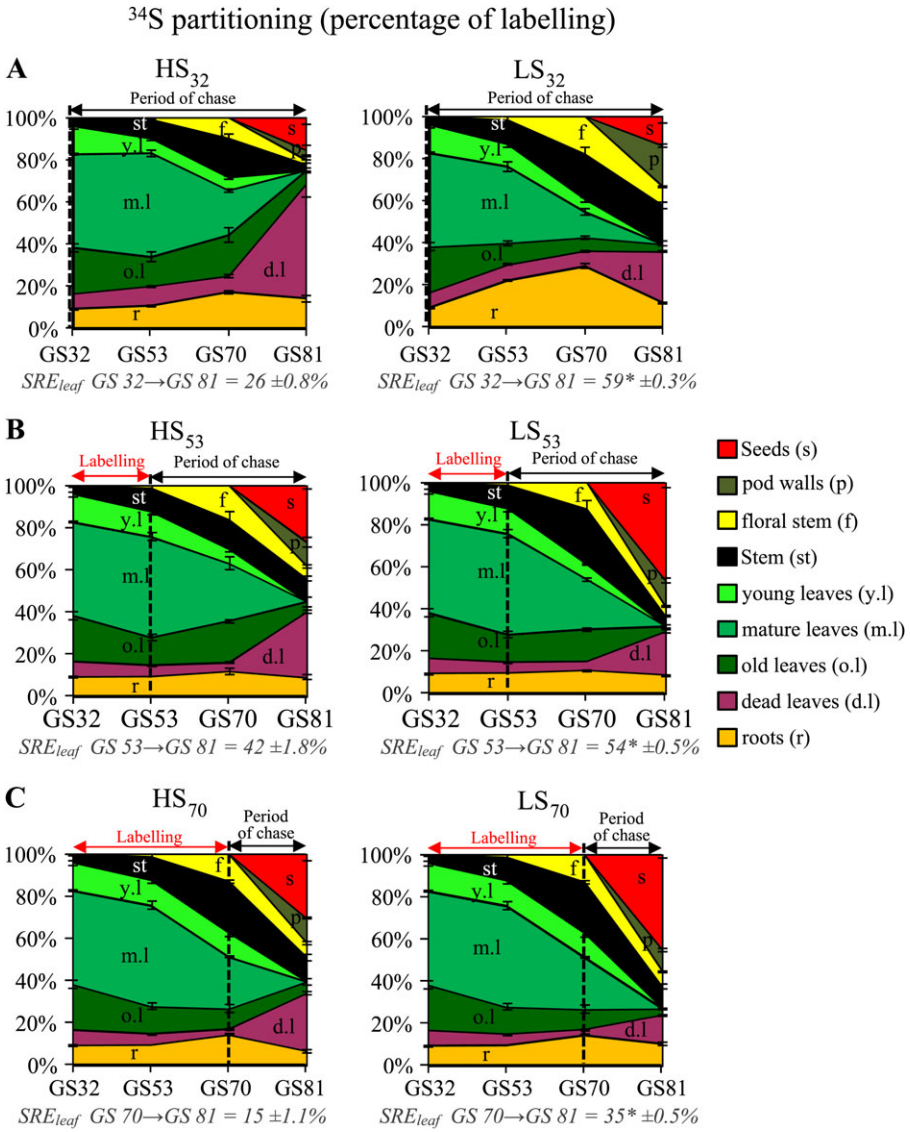

Fig. 2. ^{34}S partitioning (expressed as the percentage of total ^{34}S labelling) in the different tissues of the plants from GS 32 to GS 81 in HS_{32} and LS_{32} (A), HS_{53} and LS_{53} (B), and HS_{70} and LS_{70} conditions (C). Vertical bars indicate ±SE of the mean (n=4). The foliar S remobilization efficiency (SRE_{leaf}, corresponding to the foliar loss of ^{34}S between two growth stages, as a percentage of total ^{34}S labelling) is indicated for each chase period, and asterisks indicate that mean values in LS conditions are significantly different from control (P <0.05).

a large proportion of ^{15}N which, in contrast to ^{34}S, greatly decreased before GS 81. The foliar N remobilization efficiency (NRE_{leaf}, corresponding to the loss of ^{15}N in leaves between two growth stages, expressed as a percentage of total ^{15}N labelling) reached, on average, 71±2.9% between GS 32 and GS 81 (Fig. 3A). After GS 70, leaves were the main source of ^{15}N for seed filling and the final proportion of ^{15}N in seeds reached 47±5.2% in HS_{32} (Fig. 3A).

In response to LS_{32} treatment, the proportion of ^{15}N transiently and strongly increased in roots until GS 70 before becoming a source for ^{15}N from GS 70 to GS 81 (Fig. 3A). At GS 70, the ^{15}N in roots of plants submitted to LS_{32} conditions reached 31±3.7% of total ^{15}N versus only 18±0.8% in HS_{32}. Finally, the ^{15}N found in seeds at GS 81 in response to the LS_{32} treatment reached 34±7.3% of the total ^{15}N, and the ^{15}N in roots remained high (Fig. 3A).

In contrast to LS_{32}, the LS_{53} and LS_{70} treatments did not significantly alter the partitioning of ^{15}N in comparison with the respective controls, HS_{53} and HS_{70} (Fig. 3B, C). In LS_{53} and LS_{70} conditions, the ^{15}N reallocated to seeds corresponded to more than half of the total ^{15}N (Fig. 3B, C). Globally, all the leaves constitute the main source of ^{15}N for seed ^{15}N filling (Fig. 3B, C).

S and N flows between GS 70 and GS 81

In contrast to LS_{32} and LS_{53}, the LS_{70} treatment consisting of a restriction of sulphate supply since GS 70 (i.e. start of pod filling) did not alter seed yield and quality (Table 1). In addition, LS_{70} treatment did not affect the NUtE (Table 2) and ^{15}N partitioning (Fig. 3), and led to the most efficient seed production with high SHI (Table 1) and SUtE

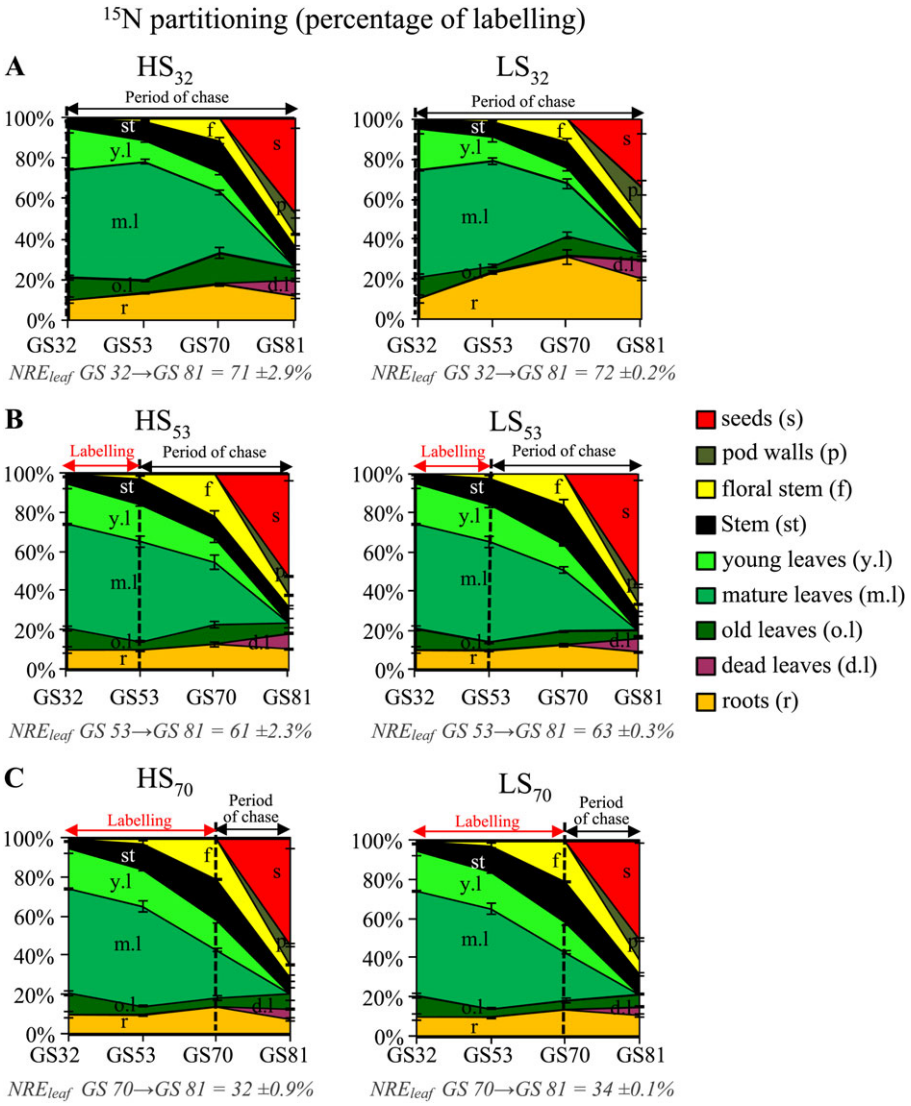

Fig. 3. ^{15}N partitioning (expressed as the percentage of total ^{15}N labelling) in the different tissues of the plants from GS 32 to GS 81 in HS_{32} and LS_{32} (A), HS_{53} and LS_{53} (B), and HS_{70} and LS_{70} conditions (C). Vertical bars indicate ±SE of the mean (n=4). The foliar N remobilization efficiency (NRE_{leaf}, corresponding to the foliar loss of ^{15}N between two growth stages, as a percentage of total ^{15}N labelling) is indicated for each chase period, and asterisks indicate that mean values are significantly different from control (P <0.05).

(Table 2). In response to this treatment, the SRE_{leaf} was more than doubled in comparison with control (35±0.5% versus 15±1.1% in HS_{70} between GS 70 and GS 81) (Fig. 2C). In these circumstances, the allocation of S and N taken up from the soil and the endogenous S and N remobilizations during the reproductive phase of oilseed rape development were examined on the basis of ^{34}S and ^{15}N enrichment (see Materials and methods for details) and illustrated for HS_{70} (Figs 4A, 5A) and LS_{70} conditions (Figs 4B, 5B).

For HS_{70} plants, little of the S taken up was allocated to the roots; the main sinks for S taken up were floral stems, pod walls, and seeds, with an equivalent allocation of S to pod walls and seeds (Fig. 4A). Leaves were the major source organ for remobilized S (60±2.2% of the total S remobilized from GS 70 to GS 81; Fig. 4A) while stems, floral stems, and roots contributed poorly to the supply of endogenous S to other tissues in control plants. The restriction of S availability (LS_{70} treatment) greatly reduced total S uptake to a level that was insignificant, whereas 67±2.2 mg of S were taken up in HS_{70} conditions (Fig. 4B). Compared with HS_{70}, LS_{70} conditions also changed the source–sink relationships for endogenous S (Fig. 4B). The LS_{70} treatment increased the SRE (i.e. the proportion of the total S amount remobilized into the plant which was recycled towards seeds) with a redistribution of 65±1.2% of S remobilized to seeds versus 44±0.9% in HS_{70} (Fig. 4A, B). Compared with HS_{70}, the highest mobilization of S for seed filling observed in LS_{70} conditions would be related to a lower loss of S by dead leaves (which was ~2-fold less in LS_{70} than in HS_{70} conditions; Fig. 4). Finally, the S amount quantified in seeds reached 41±2.3 mg in LS_{70} thanks to remobilization from vegetative plant parts. The source status of roots was significantly lower in LS_{70} than in control (7±0.1% in LS_{70}

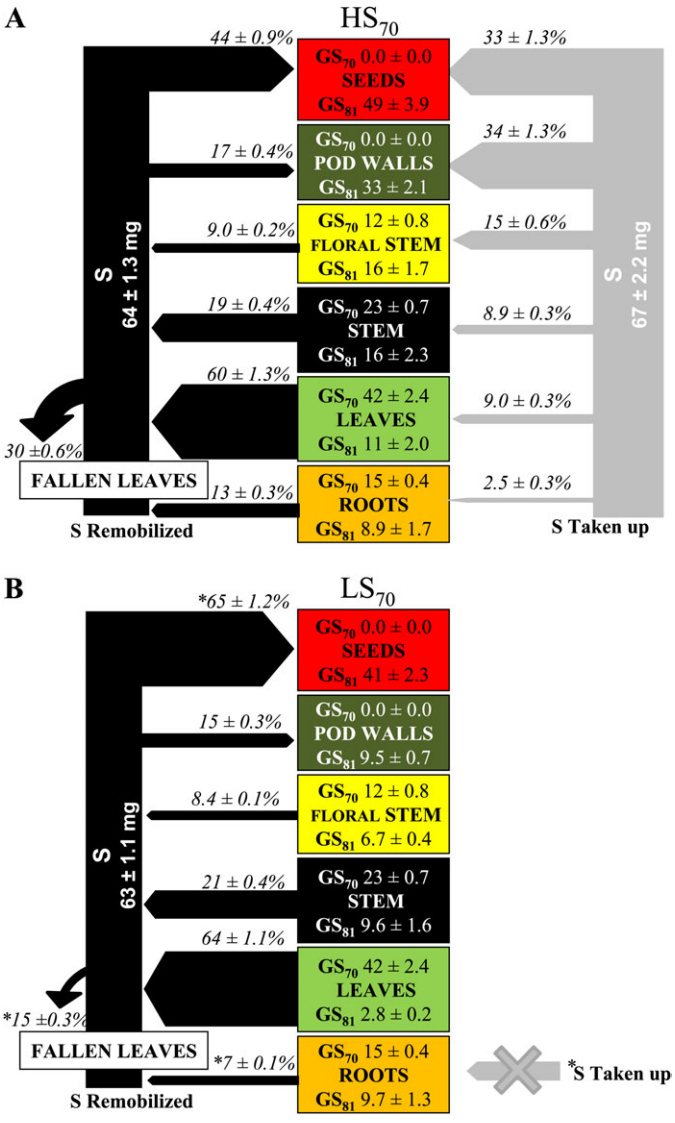

Fig. 4. Flows as a percentage of remobilized S (determined on the basis of ^{34}S enrichment; see Materials and methods for details) and S taken up (estimated from the unlabelled S), and the S amount present at GS 70 and GS 81 in seeds (in mg in seeds), pod walls, floral stems, stems, leaves, and roots of oilseed rape for control plants (HS_{70}; A) and S-deficient plants (LS_{70}; B) during the reproductive stage. Values are given as the mean ±SE (n=16 for the flows, n=4 for the S amounts in tissues). In S-deficient plants (B), the uptake of sulphate was nil during the duration of the experiment. The thickness of the arrows represents the relative importance of each flow to or from a tissue related to the S taken up or S remobilized, and asterisks indicate that mean values are significantly different from control (P <0.05).

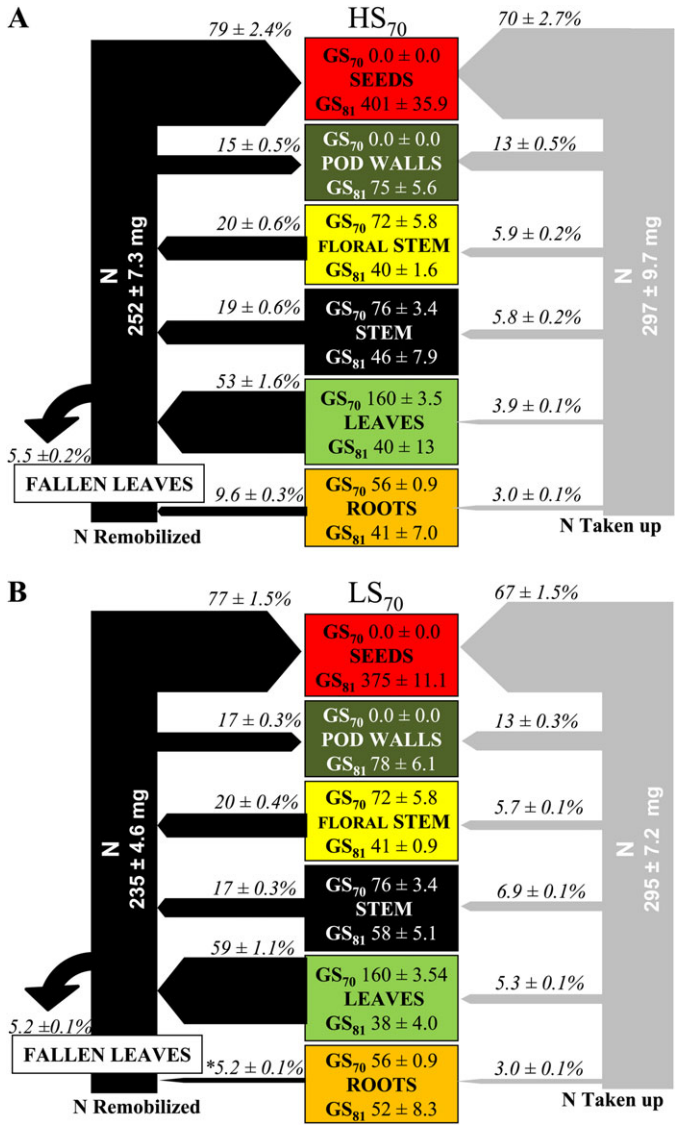

Fig. 5. Flows as a percentage of remobilized N (determined on the basis of ^{15}N enrichment; see Materials and methods for details) and N taken up (estimated from the unlabelled N), and the N amount present at GS 70 and GS 81 in seeds (in mg in seeds), pod walls, floral stems, stems, leaves, and roots of oilseed rape for control plants (HS_{70}; A) and S-deficient plants (LS_{70}; B) during the reproductive stage. Values are given as the mean ±SE (n = 16 for the flows, n=4 for the S amounts in tissues). The thickness of the arrows represents the relative importance of each flow to or from a tissue related to the N taken up or N remobilized, and asterisks indicate that mean values are significantly different from control (P <0.05).

versus 13±0.3% of total endogenous S recycled in HS_{70} plants, Fig. 4A, B).

The restriction of S availability applied at GS 70 did not significantly reduce the total N uptake between GS 70 and GS 81 (with an average of 297±10 mg of N taken up) and did not drastically change the N partitioning within the different plant tissues (Fig. 5A, B). The N remobilization efficiency (NRE) to seeds in LS_{70} conditions reached 77% and was not significantly different from the control (Fig. 5A, B). Finally, about half of the total N in seeds at GS 81 was derived from mobilization in both treatments. It appeared that leaves represented the major source organ for N, to the main benefit of the seeds, and to a lesser extent to the pod walls. The residual N lost by dead leaves (14±2.1 mg of N) was unchanged by the treatment restricting S availability. Compared with control, the N remobilization from roots

towards reproductive tissues was reduced in LS_{70} (5.2±0.1% in LS_{70} versus 9.6±0.3% of total endogenous N recycled in HS_{70} plants; Fig. 4A, B). Whatever the treatment, and as observed for S, roots therefore contributed poorly to the supply of endogenous N to other plant tissues.

Residual S and N concentrations in leaves

Since the residual DM of each leaf rank was not affected by LS treatments (data not shown), the S and N concentration in dead leaves in response to the treatments was examined in relation to their nodal positions (Fig. 6A, B). The average of residual S in leaf ranks below nodal position #13 was 0.67±0.03% of DM while the residual S concentration was >0.8% of DM in upper leaf ranks (ranging from 0.88±0.05% of DM in leaf rank #14 to 1.78±0.22% of DM in leaf rank #16). These upper leaves corresponded to the smallest leaves (with a leaf area <6 cm^2, data not shown) that appeared at the visible bud stage. As expected, in response to mineral S restriction treatments, the residual S concentration in leaves was significantly reduced. The S concentration in dead leaves of LS_{32} plants was significantly affected from node #5 while this decrease happened in leaves above node #7 for LS_{53} and above node #9 for LS_{70} plants (Fig. 6A). Minimal values of residual S concentration in dead leaves (comprised between 0.1% and 0.2% of DM) were observed in response to the three LS treatments, particularly in leaves above leaf #11 (emerged at GS 32).

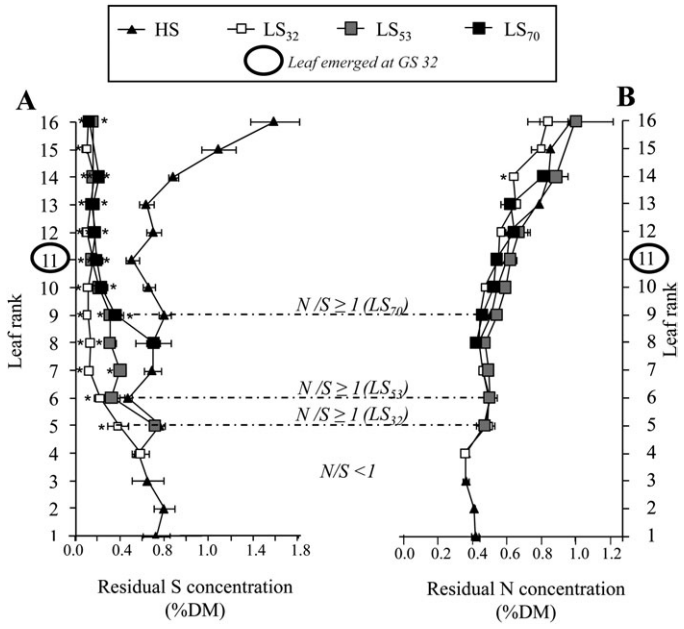

Fig. 6. Residual S (A) and N (B) concentrations of dead leaves in oilseed rape grown under a high level of sulphate (HS) or under a low level of sulphate (LS_{32}, LS_{53}, and LS_{70}). Horizontal bars indicate ±SEM (n=8) when larger than the symbol. Asterisks indicate that mean values are significantly different from control (P <0.05). The intersection points between S and N concentrations were illustrated for each treatment by dotted lines corresponding to the transition between N/S <1 and N/S>1.

These minimal foliar S concentrations were observed earlier for the LS_{32} treatment (from node #7).

In comparison with HS, and with the exception of leaf rank #14 (with a concentration of N significantly reduced in response to the LS_{32} treatment; Fig. 6B), the residual N concentration in leaves was not affected by sulphate restriction treatments. Residual N gradually increased from basal to upper leaves and was globally below 1% of DM (Fig. 6B). While the residual S concentration in the control was higher than the residual N concentration in leaves emerged before the 11th rank (emerged at GS 32), it is interesting that cross-talk between S and N concentrations (corresponding to an N/S ratio of 1) was observed in lower leaf ranks in response to LS treatments (Fig. 6). The residual N concentration was higher than the S concentration for leaf ranks ⩾#5 for LS_{32}, for leaf ranks ⩾#6 for LS_{53}, and for leaf ranks ⩾#9 for LS_{70} (Fig. 6).

Discussion

Optimization of SRE is required to maintain NUtE, seed yield, and grain quality in response to S restriction

This double ^{34}S and ^{15}N labelling experiment (Fig. 1), undertaken in control conditions, was designed to follow the course of remobilization of endogenous S and N in oilseed rape with particular attention to leaves that correspond to the main source organs for S and N (Figs 2, 3). Except for LS_{70}, the SHI values obtained were noticeably lower than those observed for the NHI (Table 1), indicating that S is remobilized to seeds less efficiently than N (Sexton *et al.*, 1998). The results obtained for yield and quality of seeds reveal that the mineral S availability between GS 32 and GS 70 would be a determinant for seed filling processes and seed quality. In response to the LS_{32} treatment, the NHI and NUtE were reduced and the seed composition was affected (Table 1). Fismes *et al.* (2000) have shown using field-grown oilseed rape that S deficiency can reduce NUtE and protein level in seeds. The present results indicate that an S fertilization regime with the ability to satisfy the growth needs of oilseed rape until GS 53 is required to maintain a sufficient NUtE and protein level in seeds. In response to the LS treatment consisting of a restriction of sulphate supply from GS 70 (LS_{70}), oilseed rape was able to optimize its SUtE (Table 2) in order to produce high quality seeds (Table 1). The LS_{70} treatment led to the highest SRE to seeds, with a redistribution of 65±1.2% of remobilized S towards seeds, in contrast to the 4 ±0.9% observed in HS_{70} (Fig. 4A, B).

The enhanced remobilization of endogenous S towards the seeds observed in response to the LS_{53} or LS_{70} treatments was not associated with noticeable modifications of the source–sink relationships for N (Figs 3B, C, 5). This shows that the interaction of the two nutrients is strongly affected by development. Thus, the altered seed yield and quality in response to the LS_{32} treatment would be partially attributed to significant modifications in N dynamics. After

GS 53, it appears that oilseed rape can optimize the mobilization of endogenous S to seeds in response to S restriction, independently of the N distribution.

The efficiency of seed S and N filling is related to S and N remobilization from vegetative aerial organs rather than from root reserves established before GS 70

About half of the N content in reproductive tissues of oilseed rape was derived from N mobilization (Fig. 5) occurring mostly in leaves and stems. The roots did not significantly contribute to endogenous S remobilization. The lack of S remobilization from roots suggests sequestration of sulphate and/or the presence of a high proportion of organic S reserves that were difficult to mobilize. In HS plants at GS 81, 35±2.4% of the total S in roots was in the sulphate form (data not shown).

Hoefgen and Nikiforova (2008) suggested enhanced lateral root formation thanks to activation of auxin-inducible genes as a possible adaptation to prospect for available S in soil in the case of sulphate deficiency. In response to the drastic restriction of mineral S which started from a younger stage (LS_{32} treatment), there was a transient and strong increase of S and N demands in roots (increase of sink status until GS 70) (Figs 2A, 3A). Compared with control, this temporary sink status of roots in response to severe S restriction (LS_{32}) in oilseed rape was also associated with an accumulation of N amount (but not of S) and a higher DM production in roots (data not shown).

These results underline the importance of S mineral availability before flowering and emphasize the level of S reserves in vegetative aerial tissues. It appears that oilseed rape will more efficiently mobilize previously acquired S and N towards the seeds if S is supplied in adequate amount to support growth of the plant up to the beginning of seed formation. After GS 70, S taken up later (in HS_{70}) was more likely to be disproportionally allocated to the pods (and seeds) (Fig. 4), and was not necessary for the maintenance of seed yield and quality (Table 1).

The importance of leaves for N storage and mobilization to seeds has been well established (Noquet *et al.*, 2004; Malagoli *et al.*, 2005*a*) and was verified in the present experiment (Figs 3, 5, 6). In contrast, the contribution of leaves to S storage and subsequent S distribution to sustain seed formation and filling remains unclear in oilseed rape (Hawkesford and De Kok, 2006). While Sunarpi and Anderson (1997) reported that soybean leaves contribute little to seed S filling, the present work underlined that leaves of oilseed rape would be crucial for their role as a major source organ for S in response to S restriction (Figs 2, 3). More specifically, if S limitation occurred at GS 70, leaves may improve their SRE in order to cover the demand for S for seed growth (Fig. 4). Interestingly, despite an enhanced remobilization of foliar S reserves (Fig. 6), the lifespan of the leaves emerging during the whole of the growth cycle was unaltered by the LS treatments (data not shown). Besides, the total N amount in dead leaves was not significantly different between HS and LS treatments (Figs 5, 6B), suggesting that LS conditions improved the S mobilization in leaves independently of N [higher SRE_{leaf} (Fig. 2) versus unaltered NRE_{leaf} (Fig. 3)]. To sustain the S demand for growth under S limitation, a strong SO_4^{2-} mobilization in leaves was already reported at the rosette stage without any acceleration of leaf senescence (Dubousset *et al.*, 2009).

The mobility of S stored in leaves of oilseed rape depends on mineral S supply

While the leaves have been shown to be the primary donors of N for mobilization to seeds (Noquet *et al.*, 2004; Malagoli *et al.*, 2005*a*), their importance as a major source organ for S has been demonstrated as well. In LS_{70} conditions, leaves supplied the seed with up to 75±3.7% of the mobilized S during reproductive development. The present study showed that leaves of control plants had high S concentrations (0.67±0.03% of DM for nodes 1–13) when they abscised, indicating that a significant proportion of leaf S was not mobilized before abscission (as verified in Fig. 2). In the absence of deficiency of sulphate, the high proportion of residual S of dead leaves characterized in controlled conditions (Fig. 6) is in accordance with the potential sequestration of S in leaves (in sulphate form) suggested by previous studies (Blake-Kalff *et al.*, 1998; Hawkesford, 2000; Matula and Pechovà, 2002). Under restricted sulphate availability, the residual concentration of S in dead leaves seemed clearly to reflect the balance between supply and demand of S for growth and seed filling. The present experiment suggests that the conjunction of a residual S concentration of 0.1–0.2% of DM with a value of the N/S ratio $\geqslant 1$ in dead leaves (corresponding to leaves emerged before the bolting stage) could be used as indicators of S deficiency leading to alteration in seed quality (Fig. 6). Nevertheless, the N/S ratio in leaves depends on S and N availability, which leads to difficulties in using this ratio as an accurate diagnosis of the plant S status (Blake-Kalff *et al.*, 2002).

Analysis of the effects of sulphate limitations applied at different growth stages on S and N partitioning reveals disruption between S and N distribution patterns in oilseed rape in response to this nutrient deficiency. By using stable isotopes (^{34}S, ^{15}N) as a tracer system (Monaghan *et al.*, 1999), the determination of $^{34}S/^{15}N$ partitioning and S/N flows allowed characterization of the contribution of each organ to seed S/N filling. The data obtained in the present work confirm that S is relatively immobile in plants in control (HS) conditions, as the proportion of S redistributed from leaf tissue was considerably smaller than that of N. Under recommended levels of S fertilization, the loss of S through leaf fall from *Brassica napus* L. cv. Capitol in field conditions can reach 22±0.7 kg S ha^{-1} (LD, unpublished results). This also indicates that S is not recycled during leaf senescence if oilseed rape is grown under optimal S nutrition. In response to LS_{70} treatments, the highest S remobilization (SRE) to seeds was associated with a high foliar mobilization of S (leaves supplied the seed with

~75±3.7% of the SRE between GS 70 and GS 81). The minimal values of S concentration, comprised between 0.1% and 0.2% of DM in dead leaves, would indicate an enhanced remobilization in response to mineral S restriction. Results observed in response to LS_{70} treatment indicate the possibility of increasing the SRE (without altering seed quality) by limiting the practice of fertilization. Considering that the presence of glucosinolates in seeds restricts the use of meal in animal feed (Zhao *et al.*, 1994), a limited fertilization with sulphate after GS 70 did not alter the seed quality for meal. To adapt S inputs, the residual S concentration of leaves that emerged before GS 32 may serve as an indicator of a sufficient S reserve status for reproductive growth if it is >0.5% of DM. These results should be taken into account for the development of field diagnosis tests to determine whether plants are deficient in mineral S.

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