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Sulfur Deficiency Effects on Nitrate Uptake and Assimilatory Enzyme Activities in Rape Plants

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유채에 있어 황 결핍이 질산염의 흡수 및 동화관련 효소 활력에 미치는 영향

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요 약

유채 (*Brassica napus* L.)에서 황 결핍이 NO_3^- 흡수와 동화에 대한 영향을 알아보고자 SO_4^{2-} 농도를 세가지 수준 (1 mM SO_4^{2-}, 대조구; 0.1 mM SO_4^{2-}, 결핍; 0 mM SO_4^{2-}, 무공급)으로 25시간 처리한 후 NO_3^- 흡수량, 식물조직내의 nitrate reductase (NR) 및 glutamine syntheetase (GS) 활성을 분석하였다. 25시간 처리과정에서 황결핍 조건하에서의 NO_3^-의 흡수는 대조구와 큰 차이를 나타내지 않은 반면, 황 무공급구에서는 NO_3^-의 흡수는 유의적으로 감소하였다. NR 활성은 잎과 뿌리 조직에서 공히 SO_4^{2-} 농도가 감소됨에 따라 감소하였다. 특히 어린잎과 중간 잎의 황 결핍 처리구에서 각각 35% 및 황 무공급구에서 70%의 뚜렷한 감소를 보였다. 뿌리에서는 오직 황 무공급구에서 유의적인 감소 (−29%)를 보였다. SO_4^{2-} 농도에 따른 잎 조직에서 GS 활성은 잎의 성숙 정도에 따라 차이를 보였으며, 전반적으로 황 결핍과 황 무공급구에서 감소하는 경향을 보였다. 뿌리조직에서 GS 활성은 황 무공급구에서만 유의적인 감소를 보였다. 이러한 결과들은 황 공급이 제한을 받는 조건에서 질산염의 흡수가 감소되며, 식물조직체내의 질산염환원과 아미노산 합성관련 효소의 활성이 제한을 받았다는 것을 보여준다.

(Key words : Glutamine synthetase, Nitrate reductase, N uptake, Rape, Sulfate level)

I. INTRODUCTION

As an essential component of important metabolic and structural compounds, sulfur (S) has been recognized as an important nutrient for plant growth so that it is essentially required in proper amounts for most of crop plants. Sulfur is also required for the synthesis of various other compounds, such as thiols, sulpholipids and secondary sulfur compounds, which play an important role in the nutritional physiology and in the protection and adaptation of plants against stress and pests (Matsubayashi et al., 2002). It is well documented that S-deficiency decreased

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crop yields and quality, and even mild sulfur deficiency is known to influence overall plant quality (Hawkesford, 2000). Besides, sulfur application in S-deficiency soils may increase crop yield and improve the grain quality.

A closed relationship between S and N assimilatory pathways has been found in wide range of plant species. The interaction of S, nitrogen (N), and carbon (C) assimilation is clearly demonstrated by the effects that S-starvation has on overall plant metabolism. Most of proteins contain S and nitrogen (N) and it is likely that some metabolic co-ordination is necessary to ensure that the fluxes of N and S through their transport and assimilatory pathways meet the amino acid requirements for protein synthesis. There have been numerous reports of abnormal accumulations of amino compounds and amines in nutrient-deficient plants (Rabe, 1990). In addition, for the forage species, such as alfalfa (Adams and Sheard, 1966), perennial ryegrass (Millard et al., 1985), S-deficiency provoked large accumulation of the transport amino acids, glutamine and asparagines. Changes in asparagines and glutamine levels reflect the affectivity of N assimilation and C availability (Lam et al., 1996), while proline levels alter in response to exogenous stress conditions implied on the plant (Shinozaki et al., 2003; Kishor et al., 2005). S-deficiency results in reduced protein synthesis and, therefore, in the accumulation of amino acids and inhibition of photosynthesis leading to retarded growth (Gilbert et al., 1997a). Although cross influences between N and S have been described, N assimilation continues while SO_4^{2-} assimilation is impaired (Hesse at al., 2004; Kopriva and Rennenberg, 2004).

On the other hand, rape plants (*Brassica napus*) including oil seed and forage type are known as one of high S-demanding plants. The productivity and seed quality have often limited under S-deficient condition. However, little information for the capacity of NO_3^- uptake, assimilation, internal distribution translocation in response to the S levels has been available. This experiment was designed to determine the effect of sulfur deficiency on NO_3^- uptake and its assimilation. N absorption and the activity of key enzymes related to N assimilation in leaves with different age and root tissues in response to the exogenous SO_4^{2-} levels (control, 1.0 mM SO_4^{2-}; S-deficient, 0.1 mM SO_4^{2-}; S-deprivation, 0 mM SO_4^{2-}) were measured.

Ⅱ. MATERIALS AND METHODS

1. Plant culture

Seeds of rape (*Brassica napus* L. cv. Mosa) were germinated in the bed soil which contained 14~19% peatmot, 8~12% perlite, 67~73% cocopeat and 5~9% zeolite at 25℃ in the dark. The seedlings at the growth stage of three leaves were transferred on hydroponic culture pots (Kim et al., 1991). The seedling were grown in a greenhouse with day/night mean temperature 27/20℃. Natural light was supplemented by metal halide lamps and sodium lamps that generated approximately 200 μmol $m^{-2}s^{-1}$. Nutrition solution was continuously aerated and renewed every 5 days. Eight-week-old plants with good uniformity were selected for the treatments.

The control plants were exposed to complete nutrient solution (1.0 mM SO_4^{2-}) as shown at Table 1. For S-deficient (0.1 mM SO_4^{2-}) and S-deprivation (0 mM SO_4^{2-}) treatments, SO_4^{2-} concentration in the nutrient solution was decreased to 0.1 mM or withdrawn SO_4^{2-}

Table 1. Composition of nutrient solution used for the hydroponic culture

Macro element	Concentration (mM)	Micro element	Concentration (μM)
$CaCl_2 \cdot 2H_2O$	1.5	H_3BO_3	14
NH_4NO_3	1.0	$MnSO_4 \cdot 4H_2O$	5.0
K_2SO_4	0.5	$ZnSO_4 \cdot 7H_2O$	3.0
$MgSO_4$	0.5	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.7
KH_2PO_4	0.5	$CuSO_4 \cdot 5H_2O$	0.7
Fe · Na · EDTA	0.2	$CoCl_2 \cdot 6H_2O$	0.1

source from the control solution with controlling cation/anion balance. Three plants per treatment were harvested at 25 hour after treatment. Each plant was separated into old leaves, middle leaves, young leaves, and root. Samples were immediately frozen in liquid N_2 and stored at −80℃ in deep-freezer for further analysis.

2. Determination of nitrate uptake

Nitrate uptake was estimated by depletion method that determined the reduced concentration of specific ion in the complete nutrient solution. At the solution sampling time, the volume of nutrition solution was corrected to 400 ml with distilled water and 2 ml of well mixed solution was taken for analysis. NO_3^- concentration in sample solution was determined by ion chromatography (Dionex, DX-120, USA) equipped with an IonPac AS14A column and AG4A-SC guard column, using an isocratic $Na_2CO_3/NaHCO_3$ eluent (1.8 mM/1.7 mM), flow rate 2.3 ml min^{-1}.

3. Analysis of nitrate reductase

0.2 g fresh tissues were rapidly ground with liquid nitrogen in Pre-cooled mortars, and then extracted by 1.0 ml phosphate buffer (pH 7.5, containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethanesulphonyl fluoride (PMSF)) at 1:5 of sample buffer ratio. The samples with extract buffer were rotated about 1 hour at 4℃ and then centrifuged at 13000 rpm for 10 min at the same temperature. The supernatant was transferred to analyze the nitrate reductase activity. The assay for NR activity contained in a total volume of 1 ml: enzyme extract, 200 μl; 0.1 M KNO_3, 100 μl; 0.1 M phosphate buffer (pH 7.5), 500 μl. The reaction was initiated by the addition of 200 μl nicotinamide adenine dinucleotide (NADH) and incubated for 15 minutes at 30℃. Finally, The reaction was terminated by rapid addition of 0.5 ml of sulfanilamide reagent followed by 0.5 ml of the N (1-naphthyl) ethylenediamine dihydrochloride reagent. The color is allowed to develop for 30 minutes prior to reading at 540 nm. One unit of nitrate reductase activity was defined as the amount of enzyme that liberated 1 μmol of NO_2^- produced per minute g^{-1} FW.

4. Analysis of glutamine Synthetase

200 mg fresh tissues were ground with liquid nitrogen and then immersed into the extract buffer (pH7.5), which contained 50 mM KH_2PO_4, 100 mM KCl, 5 mM EDTA and 1 mM PMSF. After 1 hour's rotation at 4℃, centrifugation was carried out at 13000 rpm for 10 min at same temperature. The supernatant was used to

determine the glutamine synthetase activity. The standard reaction mixture contained 50 mM L-glutamate, 10 mM ATP, 30 mM $MgSO_4$, 20 mM NH_2OH and 100 mM Tris-HCl (pH 8.0). 50μl extract was added to start the reaction giving a total assay volume of 1 ml. After incubation at 27℃ for 15 minutes, the reaction was terminated by adding 2 ml 2.5% (w/v) $FeCl_3$ and 5% (w/v) trichloroacetic acid in 1.5 M HCl. The absorbance of the supernatant was read at 540 nm and then the concentration of r-Gluh (r-glutamylhydroxamate) can be determined. The GS activity was expressed as μmol r-Gluh produced g^{-1} fresh weight min^{-1}.

Ⅲ. RESULTS

1. NO_3^- uptake by root

NO_3^- uptake measured for 25 h after applying three levels of sulfate are presented at Fig. 1. NO_3^- was constantly absorbed by the root under three treatments throughout the sampling time. Compared with the control (1 mM SO_4^{2-}) plants, S-deficient treatment stimulated NO_3^- uptake, while, S-deprivation slightly repressed it for the first 3 h. In viewing overall experimental period, S-deprivation resulted in a reduction of NO_3^- uptake for the early 8 h after treatment. At the final measurement (25 h), NO_3^- uptake in the control and S-deficient plants was similar level, representing 26.7 μmol/g FW in average, while decreased to 21.31 μmol/g FW in S-deprived plants.

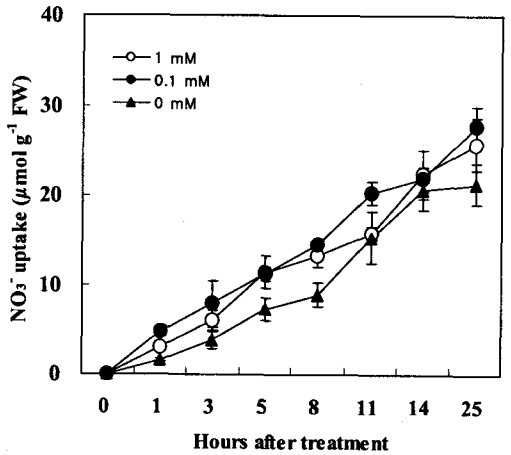

Fig. 1. Changes of NO_3^- uptake measured at the complete S supply (control, 1.0mM SO_4^{2-}), S deficiency (0.1 mM SO_4^{2-}) and S deprivation (0 mM SO_4^{2-}) during 25 h after treatment. The values are means ± SE of three replicates.

2. Nitrate reductase activity

The nitrate reductase activities (NRA) in the different age of leaf and root tissues after 25 h of treatment are presented at Fig. 2. As the Sulfur supply level decreased, NRA was repressed significantly in all leaf tissues. In the

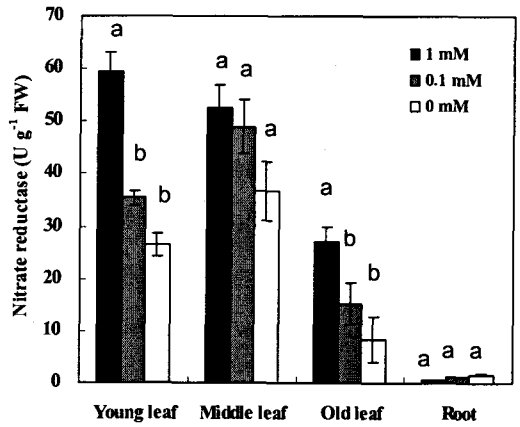

Fig. 2. Nitrate reductase activity in different leaf tissues and root after 25 h of treatment (control, 1.0 mM SO_4^{2-}; S-deficiency 0.1 mM SO_4^{2-} and S-deprivation 0 mM SO_4^{2-}). The values are means ± SE of three replicates. Bars labeled with the same letters are not significantly different ($P > 0.05$) according to Duncan's multiple range test.

young leaves, compared with that in the control, NRA significantly decreased by 35.9% and 72.7 % respectively in S-deficient and S-deprivation treatment. In the middle leaves, S-deficient and S-deprivation treatment repressed NR by 39% and 89% respectively. However, in old leaves, non significant change was appeared between S-deficient and S-deprived plants. A gradual decrease in NRA was found, representing a significant difference only between control and S-deprivation treatment.

3. Glutamine Synthetase (GS) activity

The glutamine synthetases (GS) activities in the different age of leaf and root tissues after 25 h of treatment are presented at Fig. 2. In young leaves, GS was depressed significantly only in S-deprived plants (−29%). In the old leaves, compared with the control, GS activity significantly depressed by 42% and 62%, respectively, in S-deficient and S-deprived plants. Nevertheless, the response of GS activity in the middle leaves was less constant compared to other leaf tissues. In root which showed the lowest activity, a significant decrease (−30%) was observed only active between S-deficient and S-deprivation treatment.

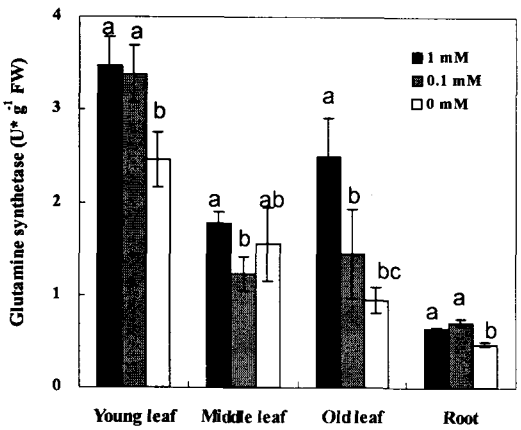

Fig. 3. Glutamine synthetase activity in different leaf tissues and root after 25 hours of treatment (control, 1.0 mM SO_4^{2-}; S-deficiency, 0.1 mM SO_4^{2-} and S-deprivation, 0 mM SO_4^{2-}). The values are means ± SE of three replicates. Bars labeled with the same letters are not significantly different ($P>0.05$) according to Duncan's multiple range test.

Ⅳ. DISCUSSION

NO_3^- uptake was not significant between control and S-deficient treated plants for most of measuring times, while significantly depressed especially for the early 8 h in S-deprived plants (Fig. 1). Similar results had been found that the uptake of NO_3^- distinctly declined in respond to sulfur deprivation, especially at higher external NO_3^- concentration (Prosser et al., 2001). The positive effect of S uptake and N uptake was observed in cereals and oil seed rape (McGrath and Zhao, 1996; Zhao et al., 1997). The response of leaf chlorophyll content and leaf N concentration to S nutrition was similar, with the two measurements in the leaves of wheat (Gilbert et al., 1997b). This suggests that the decreased chlorophyll content in a low S availability is likely to be caused by a shortage of N, as result of decreased NO_3^- uptake. The results suggested that higher NO_3^- uptake requires a proper SO_4^{2-} level taken up from the soil medium.

In this study, nitrate reductase activity (NRA) tended to decreased when S-availability decreased, especially in young and middle leaves, and the absolute activity was much higher in young and middle leaves than in old leaves regardless of applied S level (Fig. 2). This clearly showed that the response of NRA to S nutrition reflects a

sink-source relationship for their metabolites among different ages of leaf. These results suggest that NO_3^- may be assimilated much quickly in the developing tissues as like young and middle leaves, while more slowly in mature leaves. Such responses to S availability occurred to SO_4^{2-} assimilation in spinach leaves (Prosser et al., 2001), which showed that the SO_4^{2-} assimilated very soon in young leaves while much slowly (e.g. as much as 30% of the stored SO_4^{2-} for 6 days after S-deprivation) in old matured leaves. These results clearly indicate that younger leaves are so susceptible to short-term effects of S-limited nutrition, especially S-deprivation in this study. In addition, in roots, the difference in NRA was less clear between neighboring levels of S external supply (Fig. 2). It could be suggested that root plays roles in the storage or transport of NO_3^- rather than an active assimilatory site. In fact, the increase in S content of root tissues in response to external S level was found in the associated work (data not shown). The response of glutamine synthetase (GS) to the external S level was much less distinct compared with that of NR. In young leaves, a relatively higher GS activity was found for three all treatments. This indicates that young leaves would be the primary site of amino acid synthesis, which needs to meet protein synthesis and plant growth. In addition, it is noteworthy that the highest rate of decrease in GS activity was found in S-deprived old leaves. These results could be suggested that low levels of S nutrition promoted the loss of N from mature leaves and the gain of N in developing leaves, vice versa for S content against N nutrition (Sunarpi and Anderson, 1997). Thus, the source of N assimilation and amino acid synthesis might be derived from mainly the newly absorbed N, while from stored N in old mature leaves. Similarly, when a developing leaves was labeled by applying pulse of ^{35}S-SO_4^{2-} to the nutrient solution, the fate of the label was also strongly affected by the level of N nutrition (Sunarpi and Anderson, 1996). In viewing overall result, S-limited conditions, S-deficient and S-deprived treatment, resulted in a reduction of GS activity. Glutamine acid, which is the primary product of the GS-GOGAT pathway of nitrogen metabolism, therefore, leading to decrease of glutamine synthetase.

From a practical viewpoint, this work emphasizes how important it is maintain an adequate S-supply to rapidly growing leaf crop, such as forage rape plant. These results combined with our analysis suggest that the lowed NR and GS activities by limited S nutrition may result in accumulation of nitrate in leaf tissue. Thus the early identification of S-deficiency has implications for the quality of crops. Its early diagnosis is most important since S-deficiency can be ameliorated by the addition of fertilizer S.

V. ABSTRACT

Rape plants, especially forage type, are known as one of high S-demanding plants. Their productivity and quality have often limited under S-deficient condition. To investigate the effect of S-deficiency on N uptake and its assimilation, NO_3^- absorption, nitrate reducatse (NR) and glutamine synthetase (GS) activity in leaf and root tissues as affected by different S-supplied level was determined. NO_3^- uptake was not significant between control and S-deficient treated plants, while significantly depressed in S-deprived plants for the early 8 h. NR activity

decreased as S-availability decreased, especially in young and middle leaves, representing more than 35% of decrease in S-deficient and 70% in S-deprived plants when compared with control. In roots, a significant decrease (−29%) in NR was observed only in S-deprived plants. Relatively higher GS activity was found in young leaves for three all treatments. As a whole leaf tissue, S-limited conditions resulted in a reduction of GS activity. In root which showed the lowest activity, a significant decrease (−30%) was observed only in S-deprived plants.

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