Titulo de Tesis

Mechanisms of adaptation to sulfur deficiency in higher plants

Tesis entregada a la Universidad de Chile

En cumplimiento parcial de los requisitos para optar al grado de

Doctor en Ciencias con mención en Biología Molecular, Celular y

Neurociencias.

Facultad de Ciencias

por

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Mayo, 2006

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Universidad de Chile

Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Doctorado presentada por el Candidato

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Ha sido aprobada por la comisión de Evaluación de la tesis como requisito para optar al grado de Doctor en Ciencias con mención en Biología Molecular, Celular y Neurociencias, en el examen de Defensa de Tesis rendido el día 20 de diciembre de 2005.

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Cristián Enrique Wulff Zottele nació el 21 de noviembre de 1971. En 1998 obtuvo el título de bioquímico en la Facultad de Ciencias Química y Farmacéuticas de la Universidad de Chile. Desde 1998 hasta el 2000 realizó diversas actividades de Investigación y de docencia en el área de bioquímica y biología molecular en plantas en la Facultad de Agronomía de la Universidad de Chile y en el Laboratorio de Biotecnología de la Universidad Iberoamericana de Ciencias y Tecnología (UNICYT). En el año 2001 ingresa al Programa de Doctorado en Biología Molecular y Neurociencias de la Facultad de Ciencias de la Universidad de Chile. En el año 2002 obtiene la beca del Servicio de Intercambio Académico Alemán (DAAD) para realizar el trabajo práctico de la Tesis de Doctorado en el área de Fisiología Molecular en Plantas en el Instituto Max Planck en ciudad de Potsdam en Alemania. Actualmente, él trabaja en la Sección de Química y Física del Laboratorio de Criminalística de la Policía de Investigaciones de Chile.

Agradecimientos

- Quiero agradecer y manifestar todo mi cariño a mi familia por el apoyo que me brindaron para poder realizar este trabajo. En especial a mi hija Antonia por darme la energía para continuar este trabajo.

- Agradezco al programa a los profesores del doctorado en Ciencias con mención en Biología Molecular y Neurociencias por brindarme la oportunidad por medio de la beca MECESUP de poder especializarme en el área de la fisiología molecular en plantas. En especial a los doctores Ariel Orellana, Mario Luxoro y Marco Tulio Núñez por su apoyo.

- Agradezco al servicio de intercambio académico alemán (DAAD) por brindarme la oportunidad de realizar parte de mis estudios en Alemania.

- Agradezco a los doctores Holger Hesse, Rainer Hoefgen, Lothar Willmitzer, Bertrand Gakière, Joachim Fisahn, Victoria Nokiforova y a todos los miembros del Instituto Max Planck de Fisiología Molecular en Plantas por su ayuda para poder realizar mi trabajo experimental en Alemania.

- Agradezco a Ana María Villarroel por su apoyo para terminar este trabajo.

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III. List of symbols, abbreviations and nomenclatures

APR: adenosine-5´-phosphosulfate reducatase

3PG: 3-phospho-glycerate

CC: Companion cells

CHES: Cyclohexylaminoethanesulfonic acid

CS: Cysteine synthase complex

DTT: dithiothreitol

EDTA: Ethylene diaminetetraacetate

ETR: relative electron transport rate

GABA: gamma-aminobutyric acid

GC: Gas chromatography

GC/MS: Gas chromatography coupled to mass spectrometry

GEC: gamma glutamyl cysteine

GSH: Glutathione

h: Hour

HCA: Hierarchical component analysis

HEPES: N-2-Hydroxyethylpiperazine-N¨-2-ethanesulfonic acid

HPLC: High performace liquid chromatography

IAA: indole acetic acid.

LHC II: Light harvesting complex II

MBrB: mono-bromobimane

μE : microeinsteins

Min: minutes

MS: Mass spectrometer

MSTFA: N-methyl-N-trimethyl sylil-trifluoroacetamide

MX: Methoxyaminated derivative

OPA: O-phthaldialdehyde

OAS: O-acetyl-serine

OASTL: O-acetyl-serine-thiol lyase

PC: Principal component

PCA: Principal component analysis

PFD: Photon flux density

PVPP: Polyvinylpolypyrrolidone

PSII: photosystem II

ROS: Reactive oxygen species

qP: Photochemical quenching

qN: Non-photochemical quenching

R: relative response ratio

Ra: Relative change in the metabolic composition of amino acids

RI: Retetion Index

ROS: reactive oxygen species

SAM: S-adenosyl-methionine

SAT: Serine acetyl transferase

SE: sieve-elements

SMM: S-methyl-methionine

Tris: Tris(hydroxymethyl)aminomethane

TMS: trimethylsilylated derivative

Y: Yield of Fluorescence

Resumen

Los cloroplastos de las hojas de las plantas son los mayores sitios de asimilación de azufre. La presencia de órganos fuentes (hojas maduras con capacidad de realizar fotosíntesis) y destinos (tejidos de metabolización, desarrollo y almacenamiento) en las plantas sugiere que el floema cumple una función de transporte de nutrientes que contienen azufre. Nuestra principal pregunta es referente a cómo el floema está involucrado en la respuesta de rebalance a la deficiencia de azufre en la planta y como los mecanismos de adaptación a la deficiencia de azufre que ocurren en las hojas podrían estar afectando la homeostasis del floema.

En este trabajo, técnicas de mediciones en imágenes de emisión de fluorescencia de clorofila y aproximaciones metabolómicas fueron utilizadas para estudiar las modificaciones metabólicas que se pueden observar en hojas destinos (hojas jóvenes), hojas fuentes (hojas adultas) y en exudados de floema de pepinos (*C. sativus*) crecidos en condiciones de carencia de azufre. Los resultados sugieren que la deficiencia de azufre produce fuertes cambios metabólicos como inhibición de la fotosíntesis, aumento en la fotorespiración, modificaciones en el metabolismo de serina y desbalance en la asimilación de nitrógeno en las hojas de plantas en condiciones de carencia de azufre. Sin embargo, no encontramos cambios significativos en el contenido de aminoácidos, azúcares y ácidos orgánicos en los exudados de floema cuando se comparan con las modificaciones metabólicas observadas en hojas jóvenes y adultas de plantas crecidas en carencia de azufre, indicando una regulación contra condiciones bioquímicas adversas en el floema de plantas crecidas en condiciones de carencia de azufre. También encontramos que la cantidad de ácido gama amino

butírico (GABA) aumento en exudados de floema y en paralelo a las alteraciones en la capacidad de fotosíntesis detectada en hojas jóvenes, indicando la posible función de información desde tejidos jóvenes hacia tejidos adultos a través del floema durante las primeras etapas de deficiencia de azufre. Una homeostasis del floema constante en condiciones de carencia de azufre permitiría el transporte de nutrientes y envio de comunicación entre los diferentes órganos de la planta. Estos resultados sugieren que el floema tiene una importante función en la mantención y la respuesta en toda la planta a la deficiencia de azufre, especialmente en la manutención de la homeostatis de S-adenosil metionina (SAM) en toda la planta.

Summary

Chloroplasts in leaves are the major sites of sulfur assimilation. The existence of source (mature, photosintesizing leaves) and sink (growing, metabolising, and storing tissues) organs in plants suggests a role of phloem in long distance transport with respect to sulfur nutrition. Our main question is whether phloem is involved in the rebalancing response of a plant to sulfur deprivation and whether the biochemical adaptation mechanisms that are occurring in leaves, upon sulfur starvation, could affect the phloem homeostasis. In this work, Chlorophyll fluorescence imaging and metabolomic approaches were used to study the metabolic modifications observable in sink source leaf tissues, and phloem exudates of cucumber (*C. sativus*) sulfate starved plants. Our results suggest that sulfur deprivation causes strong metabolic modifications, such as photosynthesis inhibition, increase in photorespiration, shift of metabolic pathways involved in the metabolism of serine, and nitrogen assimilation imbalance in leaf tissues. However, we found no significant modifications in the amount of amino acids, sugars and organic acids in phloem exudates in comparison to sink and source leaves of sulfur starved plants. Indicating a regulation against the adverse biochemical conditions in the phloem under sulfate deprived growth conditions. Also, we found that the amount of gamma aminobutyric acid (GABA) in phloem exudates increased in parallel to alterations in the photosynthetic performance of young leaves, which suggest a putative function in the delivery of information from sink to source organs through the phloem during the first stages of sulfur deprivation. A constant homeostasis in the phloem system maintains the function of delivery of nutrients and communication between the different organs of plants exposed to conditions of sulfate

deprivation. These results suggest that phloem has an important function in keeping homeostasis and regulating the response of the system (whole plant) to sulfur deprivation, specially in maintaining the homeostais of S-adenosyl methionine (SAM) in the whole plant.

1. Introduction

1.1. Sulfur metabolism in plants

Plants, as primary producers in natural nutrient cycles, are able to assimilate minerals into essential compounds required by the living cell. Among the various minerals essential for plant growth, the most limiting oxoanions are nitrogen, phosphorus, and sulfur (Hell et al. 2001). Thus, sulfur uptake and assimilation is a crucial determinant in plant growth and vigour, in crop yield, and even in the ability of plants to resist pests and to cope with various stresses (Hoefgen et al., 2001).

Sulfate uptake and assimilation are carried out in plants by a unique pathway different from bacteria and fungi (Leustek et al., 2000). In plants, sulfur is generally taken up as inorganic sulfate from soil by the root system, or, to a lesser extent, as volatile sulfur-containing compounds by the leaves. Proton/sulfate co-transporters localized in the plasma membrane are involved in specific functions of absorption and movement of sulfate through the plant (Yoshimoto et al., 2003; Buchner et al. 2004). Higher plants posses multiple isoforms of sulfate transporters with different affinities, capacities, and cell specific localization (Saito 2004; Buchner et al., 2004). These enable efficient uptake and internal translocation of sulfate throughout the whole plant, following the distribution to various organs through the vascular system (Leustek and Saito, 1999; Buchner et al., 2004). Inorganic sulfate is converted to the nutritionally important sulfate containing amino acids cysteine and methionine in a cascade of multiple enzymatic steps in the leaf chloroplast (Hoefgen et al., 2001; Saito 2004; Droux 2004). Important regulatory steps of integration of sulfur into organic compounds are

catalysed by adenosine-5´-phosphosulfate reductase (APR) and the cysteine synthase complex, which consist of serine acetyl transferase (SAT) and O-acetyl-thiol lyase (OASTL) (Hoefgen et al., 2001; Saito, 2004; Droux 2004). Essentially all reduced sulfur is channelled from cysteine into the biosynthesis of methionine, gluthathione, Fe/S clusters, vitamins, cofactors and proteins in order to carry out crucial structural, catalytic and regulatory functions in the plant cell, such as the involvement of sulfate in the uptake of nitrogen (Hell et al., 2001; Saito, 2004; Prossner et al, 2001) (Figure 1).

In the past, sulfur metabolism has been less thoroughly investigated than (e.g.) the metabolism of nitrogen and phosphorus. However, it has gained more attention in the last two decades, because studies at biochemical level of sulfur metabolism would provide new insights into the mechanism involved in the homeostatic regulation of amino acids in plants (Ravanel et al. 1998). Moreover, one important finding was that the availability of sulfur in the soil limits substantially the agricultural yields. The breeding of higher crop plants has increased the demand for sulfur in the past decades, although this went generally unnoticed in industrialized countries by compensatory effects of sulfur pollution. Therefore, the decreasing sulfur pollution in developed countries, since the mid-eighties legally enforced, resulted in sulfur deficiency symptoms in crops plants grown in the farms, such as chlorotic symptoms in leaves of oilseed rape (Daemmgen et al., 1998).

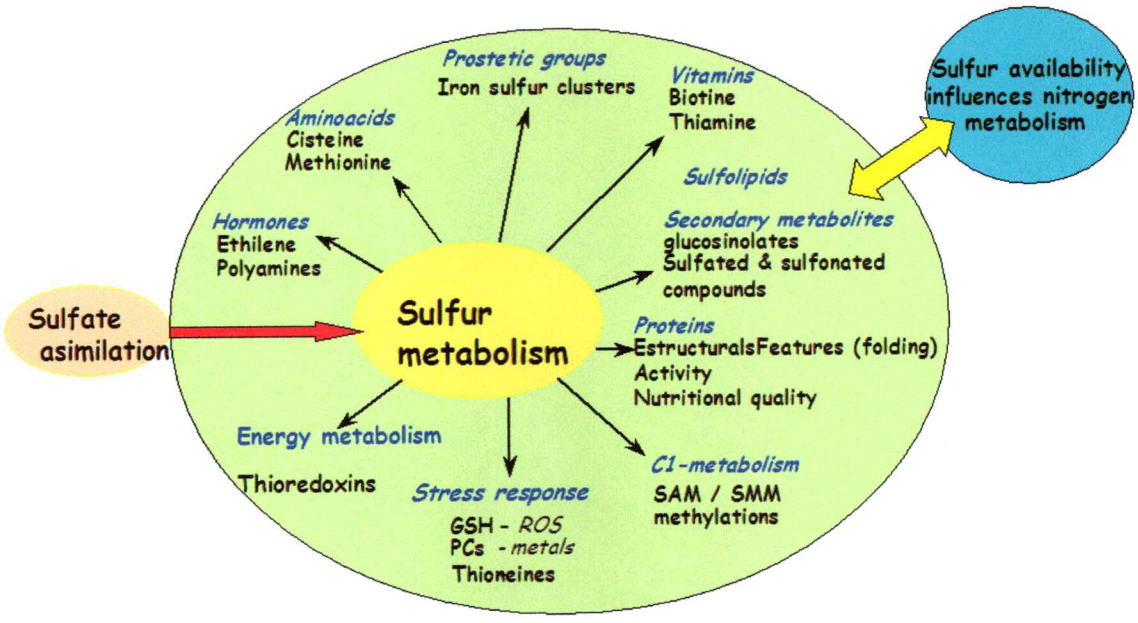

Figure 1. Involvement of sulfur in the biosynthesis of plant metabolites and physiological functions.

1.2. Sulfur deprivation

Sulfur deprivation in plants leads to symptoms of deficiency such as retarded growth, chlorosis in young leaves, bending of the leaf blade, accumulation of anthocyanines, and enhancement of the root growth, enhanced sulfate uptake capacity, and increased enzyme activities of assimilatory sulfate reduction (Bergmann, 1992; Prossner et al, 1997; Herschbach; 2002; Nikiforova et al.; 2003; Lopez-Bucio, 2003). The visual symptoms of sulfate deficiency were also shared by other nutrient stresses, such as nitrate and phosphate starvation, which already indicates that distinct stresses might in part use common signalling paths with specific response control elements (figure 2). It is notable that due to storage of sulfate in the vacuole and the pool of organic thiol compounds, such as glutathione, the appearance of symptoms is a slow

and gradual process, such as the accumulation of anthocyanines (Nikiforova et al, 2003) (figure 2). It must be therefore assumed that the several responses in different plant organs depend on the regulation steps that occur in series during the time course of sulfur deficiency. Since the major site of sulfate uptake is in the root, but the major site of reduction and biosynthetic need is the leaf, one has further to assume that the signalling mechanisms exert effects in both organ types. Thus, plants subjected to sulfur deprivation provide a good model to establish a systematic analysis of nutrient metabolism.

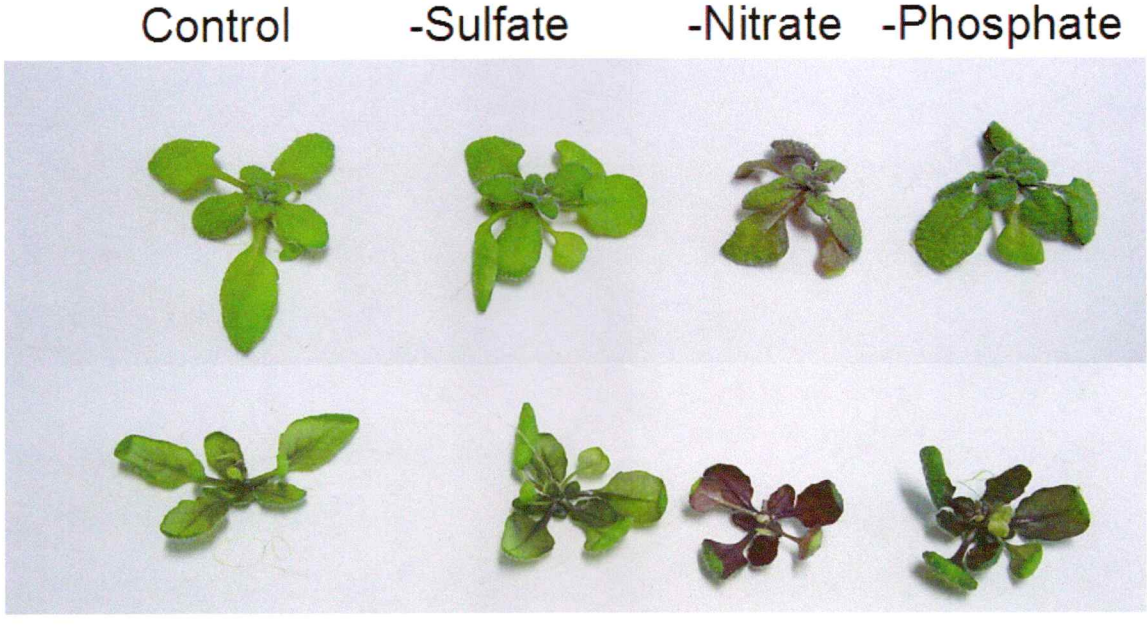

Figure 2. Visual phenotypes observed after one week in control plants and sulfur, nitrate, and phosphate starved plants grown under 400 μE controlled light conditions. Sulfate, nitrate and phosphate deprivations in *Arabidopsis* plants lead to symptoms of deficiency such as retarded growth, chlorosis in young leaves, bending of the leaf blade and accumulation of anthocyanines. The figure shows the slow accumulation of anthocyanines on the abaxial side of young leaves after one-week of sulfate deficiency. The sequence of accumulation of anthocyanines is as follows: nitrogen>phosphate>sulfate.

Various studies of sulfur metabolism in plants are reflected in publications where the molecular mechanism of gene expression in *A. thaliana* in sulfur nutrition are described

through global transcriptome analysis (Hirai et al., 2003; Maruyama et al., 2003) or combined with metabolite profiling (Nikiforova et al., 2003; Hirai et al., 2004; Nikiforova et al, 2005 a). However, the research in *A. thaliana* is limited to leaf/root analyses, omitting other important issues: the long distance transport of nutrients through the phloem and source and sink communication.

1.3. Phloem in the system response to sulfur deprivation

For living organisms with a complex hierarchical organization, such as plants, the necessity of close coordination of various elements requires systemic organization at the level of the whole organism. Additional complexity is imposed on the system through environmental variability. Due to the inability to escape unfavourable environmental conditions, plants have evolved complex mechanisms to sense and transmit external signals to the internal decision points to trigger the adaptative response program for maintenance of homeostasis. Such adaptative programs are accomplished at multiple organizational levels, e.g. gene and enzymatic activities, being finally manifested in altered metabolite concentrations (Nikiforova et al., 2005 a).

Plants adjust their capacity to carry out photosynthesis to the demand of photosynthetic products. This balance between source (mature, photosynthesising leaves) and sinks (growing, metabolising and storing tissues) is continuously adjusted in response to environmental and developmental cues (Amiard et al., 2005). Among the most important parameters for plant growth and survival is the accurate control over signal, nutrient, and assimilate transport between organs, i.e. composition and flux in plant xylem and phloem (Fiehn, 2003; Amiard et al., 2005).

The vascular system of the plant is a network of cells that interconnects all major plant organs, and it is composed of two tissues, xylem and phloem, which function to transport water and solutes, or organic compounds, respectively (Turner and Sieburth, 2002) (figure 3A and 3B). Long distance signalling in plants is thought to occur predominately through the phloem, once assumed to function solely as a conduit for nutrient dissemination. Phloem tissue contains two specialized cell types, companion cells (CC) and sieve elements (SE), which are derived from the division of the same phloem mother cell (figure 3 C). Following division, sieve elements undergo a developmental program whereby the nuclei, vacuoles and most other organelles degenerate, giving rise to cells that lack the capacity for transcription or translation but are highly specialized to deliver sugars, hormones, amino acids, proteins and RNA (Oparca and Santa Cruz, 2000; Taiz and Zeiger, 1998; van Bel et al., 2002). Sieve elements become genetically and metabolically dependent on the companion cells, because they are directly interconnected via plasmodesmata (van Bel et al., 2002). Besides, the sieve elements (SEs), are joined by perforated end walls known as sieve plates to form sieve tubes, a functional continuum of cells that is closely connected with adjoining, nucleate companion cells (CCs) and a range of associated parenchyma elements (see review Oparka and Santa Cruz, 2000).

In higher plants, sulfur is taken up by the roots as sulfate, transported via the xylem to the leaves, reduced to cysteine, and either converted to methionine or incorporated into proteins and cysteine-containing peptides such as glutathione (Leustek, 2000; Droux, 2004; Saito, 2004). The allocation of reduced sulfur between organs proceeds via the phloem system (Bourgis et al., 1999; Yoshimoto et al., 2003). The S-containing tripeptide glutathione (GSH) seems to play a major role in sulfate transport. GSH may be involved in shoot to root signalling of the sulfur status of the

plants (Lappartient et al., 1996; Lappartient et al., 1999). In addition, for several plant species, substantial amounts of reduced sulfur are transported as S-methylmethionine through the phloem from source leaves to sink tissues (Bourgis et al., 1999).

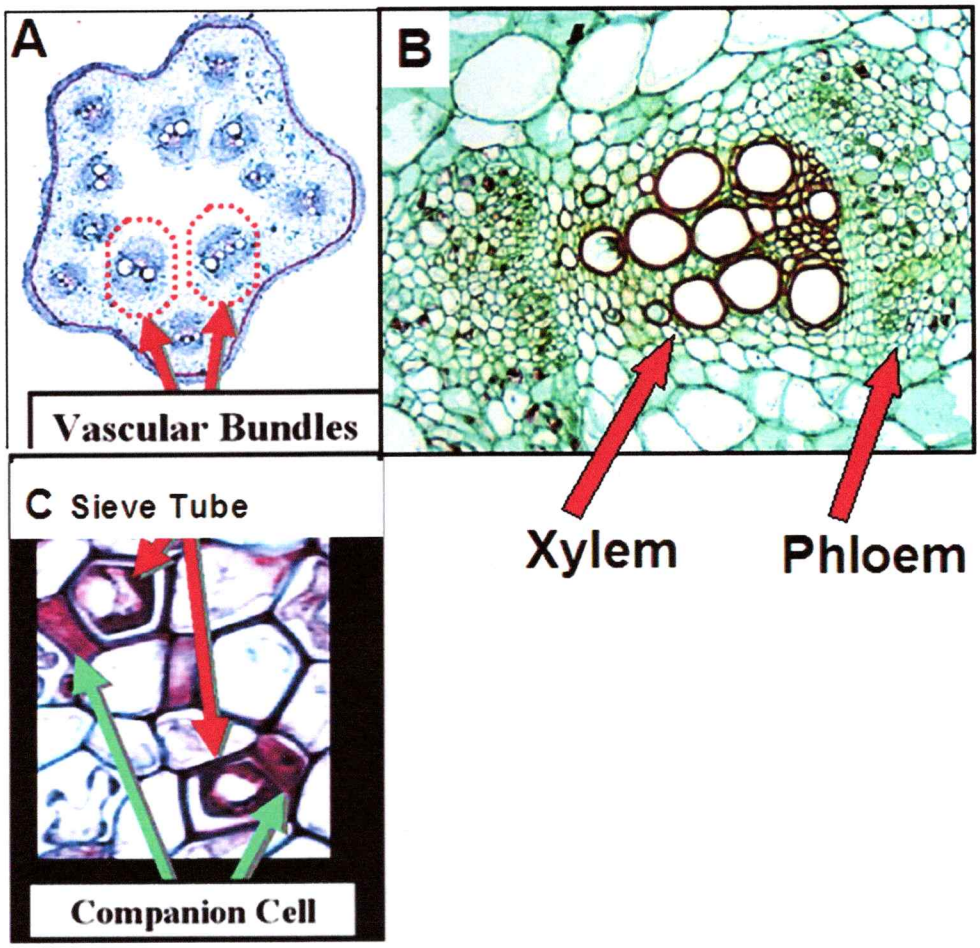

Figure 3. Transverse sections of the vascular tissue in *Cucurbitaceous* plants. Image A illustrates a cross section of Pumpkin (*Cucurbita maxima*) stems; the vascular bundles are marked with red dashed lines. Image B shows the vascular system of Cucumber (*C.sativus*). Image C illustrates the arrangement of companion cells (green arrows) and sieve elements (red arrows) in the phloem system of *C. sativus.* Original figures published in the web page http://www.botany.hawaii.edu/faculty/webb/BOT410/410Labs/LabsHTML-99/Xylem/Labxyphlo99.html.

The chloroplasts of mature leaves are the major sites of sulfur assimilation (Hell, 1997; Saito, 2004) and the phloem connects these sources to the sink tissues. As

phloem is necessary to provide organic sulfur from source leaves to sink organs, but it is not known whether the changes in the metabolic composition of source and sink tissues are reflected in the phloem metabolism of plants under sulfur deficient conditions. Considering the aforementioned facts, we decided to approach a number of questions concerning effects of holistic physiological and biochemical changes, such as those caused by sulfate deficiency, on the metabolite composition of the phloem. The task was to investigate, whether phloem is involved in maintaining the homeostasis and regulating the response of the plants to sulfur deprivation and whether the biochemical adaptation mechanisms that are occurring in leaves, upon sulfur starvation, could affect the phloem homeostasis.

1.4. Hypothesis

The present work is based on the hypothesis that the changes resulting in altered biochemical composition that occur in sink and source leaves, under sulfur deficiency, affect the phloem homeostasis. Among these changes the metabolite composition is altered.

1.5. General and specific objectives

- General objective:

The general objective of this work was to investigate the effects of sulfur deficiency in leaf tissues and phloem exudates from *C. sativus* plants at physiological and metabolic levels.

- Specific objectives

To carry out above-mentioned general objective, three specific goals were determined to enable a broad characterisation of cucumber (*C. sativus*) phloem exudates collected within a time course of sulfur starvation.

i. Characterize the physiological response of sulfur deficiency in the photosynthetic performance of young and old leaves of C. sativus plants.

ii. Study changes in the amount of low molecular weight sulfur containing metabolites of phloem exudates, sink leaves and source leaves.

iii. Evaluate differences in the phloem metabolite composition as a result of sulfur starvation.

2. Material and methods

2.1. Chemicals

Most chemicals were purchased from Sigma-Aldrich, Merck, Carl Roth, and Macherey Nagel. Chemicals regarded as crucial for individual experimental procedure will be mentioned in the respective method description.

2.2. Plant material and growth conditions

Seeds from *Cucumis sativus* L. cv. Hoffmanns Giganta (Treppens, Berlin, Germany) were germinated in wet towel paper under controlled conditions at 22° C. After 48 hours the seedlings were transferred to boxes and grown for four weeks in hydroponic cultures using Hoagland's medium (50 μM [Fe-EDTA] SO_4, 2mM Ca $(NO_3)_2$, 0.5 mM KH_2PO_4, 0.8 mM $MgSO_4$, 10 mM KNO_3, and trace elements) under controlled conditions (12 h light, 22°C at day and night; 70% relative humidity; 700 μE m^{-2} s^{-1}). All plants were grown for 4 weeks prior to the start of each experiment at which time the solution was changed. Control plants remained in normal Hoagland's solution. $MgSO_4$ was replaced for $MgCl_2$ to impose sulfate deprivation. The only sulfate left in the sulfur deficient medium originated from $FeSO_4$, which was used to prepare Fe-EDTA resulting in a final concentration of 0.09 mM of sulfate minus S-condition. All the experiments were performed in five replicates and at least two times points (figure 4).

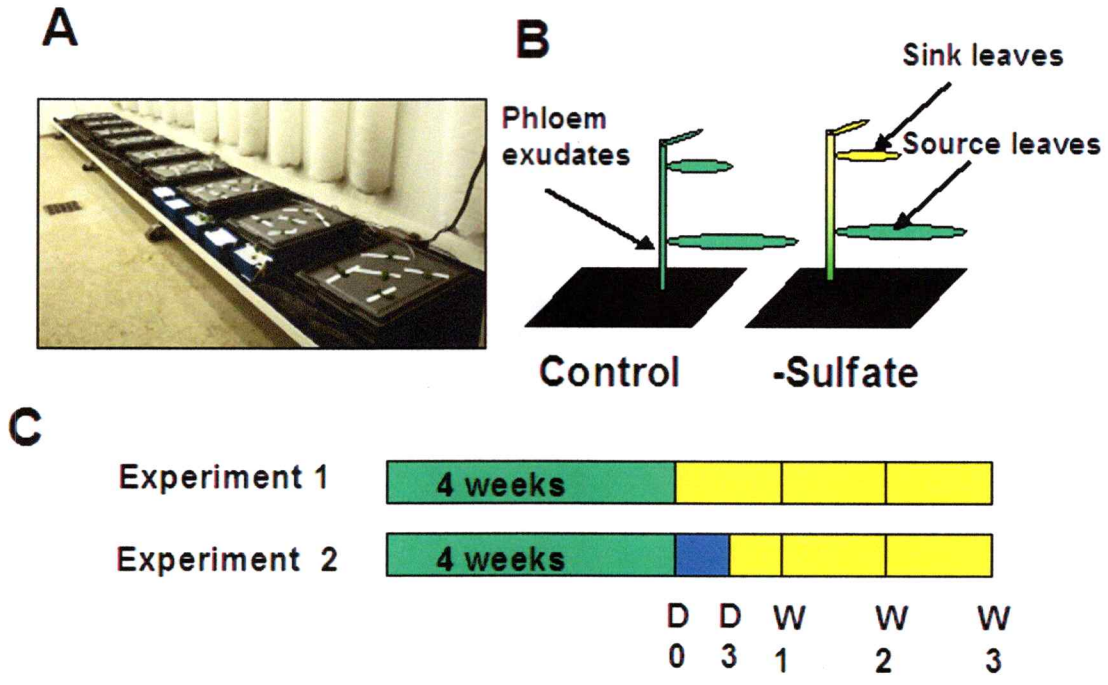

Figure 4. Experimental design used to study sulfur starvation. Cucumber (*C. sativus*) plants were grown for 4 weeks (in controlled conditions) in normal Hoagland's media (0.8 mM normal sulfate condition) (A and C). To study the effects of sulfur deficiency in phloem, the plants were grown under the same conditions as the control plants for three weeks with modified Hoagland's medium with low sulfate concentration (0.09 mM) (C). Phloem exudates and leaf tissues were collected according to the experimental design (B and C).

2.3. Chlorophyll fluorescence imaging

Images of chlorophyll fluorescence parameters of sink and source leaves of *C. sativus* plants were obtained using an IMAGING–PAM chlorophyll fluorescence imaging (Heinz Walz GmbH, Effeltrich, Germany). At the start of each measurement, a plant was dark adapted to 20 minutes to determinate F_o and F_m. Then the first level of PFD (4 μmol m^{-2} s^{-1}) was applied and a set of values was measured after 2 minutes. The same procedure was repeated for each PFD level (10, 50, 100, 225, 365, 585, and 955 μmol m^{-2} s^{-1}). Quantitative parameters analysed from chlorophyll fluorescence imaging measurements were: photochemical quenching (qP), yield of fluorescence (Y), relative electron transport rates (ETR), and non-photochemical quenching (qN) obtained at 225 and 365 photon flux density units (PFD) from the light curves.

2.4. Sampling of phloem exudates and leaves of *C. sativus*

Phloem exudates were harvested by puncturing of the stems with a clean syringe needle, and the extruding exudates were collected with a 10μl micropipette (Walz et al., 2002). Immediately, the phloem sap was diluted in an aliquot of appropriate buffers for the analysis of metabolites (See bellow), and frozen in liquid nitrogen. In parallel, young (Sink) and mature (Source) leaves were harvested with the aim to compare the biochemical status of leaves under conditions of sulfur starvation to the phloem content. All experiments were performed in five replicates and at least two time points. All tissues collected were divided for different analyses as described below (figure 4).

2.5. Quantification of total chlorophyll and total carotenoids in leaf tissues

100 mg of leaf tissues were ground to a fine powder in liquid nitrogen in a bead-mill and extracted with 80% acetone at 4°C in darkness for 1 hour. The samples were centrifuged 15 min at 15,777 g; 4°C, and the supernatant collected. 50 µl of supernatant were diluted in 950 µl of 80% acetone, and used for measurements of Chlorophyll composition following the protocol described by Lichtenthaler (1987).

2.6. Extraction and analysis of soluble thiol compounds

Individual soluble thiols were determined as the sum of their reduced and oxidized forms. 5 µl of phloem sap diluted in 200 µl of 0.1 N HCl were centrifuged (15 min at 15,777 g, 4°C). The supernatant was collected and transferred to a new eppendorf tube, and stored at –20°C until reduction and derivatization.

100 mg of fresh ground leaf material were added to 50 mg Polyvinylpolypyrrolidone (PVPP) (previously washed with 0.1 M HCl) and 500 µl of 0.1M HCl. The samples were shaken for 60 min at room temperature. After centrifugation (15 min at 15,777 g; 4°C), the supernatants were frozen at -20°C until reduction/derivatization. The levels of glutathione and cysteine were determined by high performance liquid chromatography (HPLC) based method after subsequent reduction and derivatization with monobromobimane, as described by Kreft et al. (2003).

2.7. Extraction and analysis of soluble amino acids

Soluble amino acids were determined in phloem exudates following a modified protocol from Kreft et al. (2003). 5 µl of phloem sap diluted in 200 µl of 50% ethanol were incubated for 20 minutes at 80°C. After centrifugation (10 min at 15,777 g; 4°C), the supernatant was collected and transferred to a fresh 1.5 ml microcentrifuge tube (Eppendorf ®, Hamburg, Germany), and stored at –20° C or directly subjected to HPLC (see below).

Leaf tissues (about 100 mg per plant) were ground to a fine powder in liquid nitrogen in a bead-mill and extracted three times for 20 min at 80°C: Once with 400 µl of 80% (v/v) aqueous ethanol (buffered with 2.5 mM HEPES-KOH, pH 7.5) and 10 µl of 20 µM L-norvaline (as an internal standard), once with 400 µl of 50% (v/v) aqueous ethanol (buffered as before) and once with 200 µl of 80% (v/v) aqueous ethanol. Between the extraction steps, the samples were centrifuged for 10 min at 15,777 g and the supernatants collected. The combined ethanol/water extracts were stored at -20°C or directly subjected to reverse phase HPLC using an ODS column (Hypersil C_{18}; 150 x 4.6 mm ID; 3 µm; Knauer GmbH, Berlin, Germany) connected to a Dionex HPLC system. Amino acids were measured by precolumn-derivatisation with O-phthaldialdehyde (OPA) in combination with fluorescence detection (Lindroth and Mopper, 1979). Peak areas were integrated by using Chromeleon 6.30 software (Dionex, Germany) and subjected to quantification by means of calibration curves made from standard mixtures.

2.8. Metabolite profiling using GC/MS

Phloem sap and leaf tissue extractions and derivatisations for GC/MS were performed as described by Fiehn (2003). 5 µl of phloem exudate were collected into 1.5 ml microcentrifuge tubes (Eppendorf®) containing 495 µl of distilled water and 300 µl of chloroform, and immediately frozen in liquid nitrogen until extraction and derivatisatization. The tubes were vortexed for 30 seconds to denature the proteins. After centrifugation (15,777 g for 10 minutes; 4°C), 250 µl of the upper aqueous layer were given to into fresh tubes and lyophilised to complete dryness in a speed vac concentrator.

100 mg of leaf tissues were ground to a fine powder in liquid nitrogen in a bead-mill and extracted with 300 µl of 100% methanol with 30 µl internal standards (0.2 mg/ml ribitol, 0.5 mg /ml isoascorbic acid, 1 mg/ml d4-alanine). The mixture was extracted for 15 minutes at 70°C. The extracts were mixed with 200 µl of chloroform, and shaken for 5 minutes at 37°C. Once, 400 µl H_2O was added, and vortexed. After centrifugation (14.000 rpm for 5 min, 4°C), 160µl aliquots upper aqueous layer were transferred to fresh 1.5 ml microcentrifuge tubes and dried in a speedvac concentrator.

The dried residue was redissolved and derivatised (Eppendorf ®) for 90 minutes at 30°C [in 40 µl of 20 mg/ml of methoxyamine hydrocloride (Sigma) in dry pyridine (Sigma)] followed by a 30 minutes treatment at 37°C with 70 µl N-methyl-N-trimethylsilyltrifluoro-acetamide (MSTFA, Macherey-Nagel). 10 µl of a retention time standard were added prior to trymethylsilation (Roessner et al., 2000).

Gas chromatography was performed as previously reported (Fiehn 2003, and Roesnner et al. 2000). The chromatograms and mass spectra were evaluated using the

MASSLAB 1.4v program (Finnigan, Manchester, UK). A retention time and mass spectral library for automatic peak quantification of metabolite derivatives was implemented within the MASSLAB method format. Peak finding were performed using AMDIS (http://chemdata.nist.gov/mass-spc/amdis/) (Stein, 1999).

2.9. Generation of a metabolite response matrix and statistical analysis

In accordance with Desbrosses et al. (2005) peak areas, X, were defined to represent the fragment responses (Xi, of fragment i). Fragment responses were normalized in leaf tissues by fresh weight, and/or volume in μl of phloem exudates, and response of the internal standard, ribitol (m/z=319, RI=1,733.7). This procedure corrects pipette errors and slight differences in sample amount.

The t-test was performed using the algorithm incorporated into Microsoft EXCEL (Microsoft Corporation, Seattle, WA, USA). The word significant is used in the text when the change in question has been confirmed statistically significant ($P<0.005$) whit the t-test.

Principal component analysis was performed using the software STATISTICA 6.0 (Stat Soft, Inc., Tulsa, USA). Principal component analysis (PCA) was performed after $\log_{10}$ transformation of the relative responses of each metabolite analysed, $\log_{10}$ (Ri). Missing values were either manually replaced, in the case of identified metabolites, or defined as average of the respective sample group after $\log_{10}$ transformation. If no response was retrievable for any of the samples of specific organ, $\log_{10}$ (Ri)=0 was substituted for PCA analysis.

3. Results

3.1. Physiological characterization of *C. sativus* plants subjected to sulfur starvation

For our experiments, *C. sativus* plants were grown during four weeks in hydroponic cultures in normal Hoagland's medium (Figure 4). To study the effects of sulfur deficiency in phloem, the plants were grown under the same conditions as the control plants for three weeks with modified Hoagland's medium with low sulfate concentration (0.09 mM minus sulfate condition) (see material and methods). This condition of sulfate deficiency is more similar to the sulfate deficiency conditions observed in nature than zero sulfate supply.

Plants grown in minus sulfate conditions displayed obvious phenotypic alterations such as chlorosis of sink (young) leaves, starting from the third week of sulfur deficiency (Figure 5A). From the fourth week, symptoms of chlorosis could be visualized in the source (old) leaves (figure 5B). The successive development of starvation symptoms indicates a progressive depletion of internal stores of sulfate when the external supply is reduced. The results shown in the next sections of this chapter are referred to the results obtained from the first day until the third week of sulfur starvation.

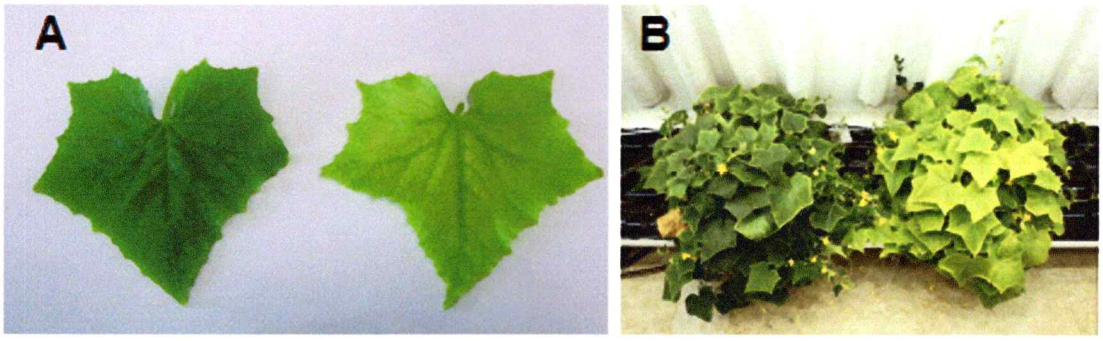

Figure 5. Visual phenotype of leaf tissues of sulfur deprived cucumber plants. Cucumber plants were grown in hydroponic cultures on Hoagland´s medium containing 0.9 mM of sulfate for control plants (left) or modified Hoagland´s with low sulfate concentration (0.09 mM) (right). (A) Sink leaves of three weeks sulfur starved plants. The leaves of sulfur-starved plants (right) show a pale green colour (chlorosis) in relation to sink leaves of control plants (left). (B) Chlorosis symptoms were detected in sink leaves and source leaves in plants after 4 weeks of sulfur deficiency (figure 5B right) compared to control plants grown in normal hydroponic cultures (figure 5b left).

3.2. Chlorophyll fluorescence measurements in sink and source leaves from *C. sativus* sulfate starved plants

As observed in the figure 5, sulfate deprivation causes visual chlorotic symptoms in leaf tissues. It is known that sulfate deprivation has an early effect on the photosynthetic apparatus and before the onset of chlorotic symptoms in leaves (Gilbert et al., 1997; Nikifova et al, 2003). We used chlorophyll fluorescence imaging to study the changes in sink and source leaves in early stages of sulfur deficiency. Chlorophyll fluorescence imaging is a non-invasive method, and it has provided the opportunity to identify photosynthetic metabolic perturbations before the onset of any visual symptoms (Maxwell and Johnson, 2000; Barbagallo et al., 2003; Oxborouhgh, 2004).

Chlorophyll fluorescence imaging was used to identify perturbations in the photosynthetic performance of the photosystem II (PSII) in sink and source leaves in the time course of sulfur depletion (figure 6 and figure 7). During the first week of

treatment, there were no visual effects of sulfur deficiency in sink and source leaves of cucumber plants (data not shown). However, a tendency for a decline of the photochemical quenching (qP), yield of fluorescence (Y) and electron transfer rate (ETR) was detected in sinks leaves beginning on the third day of sulfate starvation (figures 6A and 6C). After one week of treatment, marked changes in the photochemical quenching (qP), yield of fluorescence (Y) and electron transfer rate (ETR) were observed in sink leaves of sulfur starved plants (figures 6A and 6C). After two weeks of sulfur deficiency, we found a slight recovery of qP, Y and ETR parameters in sink leaves. Nevertheless, the increase of qP, Y and ETR chlorophyll fluorescence parameters observed in sink leaves of sulfur starved plants were significantly lower than the values detected in the same tissue of control plants (figure 6A and 6C).

No changes in the fluorescence characteristics were detected in source leaves of sulfate-starved plants during the first week. However, qP, Y and ETR declined in source leaves after two weeks of sulfur deficiency and they continued to decline throughout the experiment (figure 6B and 6D). Interestingly, the first perturbations in the Y, ETR and qP detected in source leaves were detected in parallel to the "recovery" of the same chlorophyll fluorescence parameters measured in sink leaves. Also, we found that the first changes in the chlorophyll fluorescence characteristics were localized close to the vascular tissue of sink and source leaves (figure 6A and 6B).

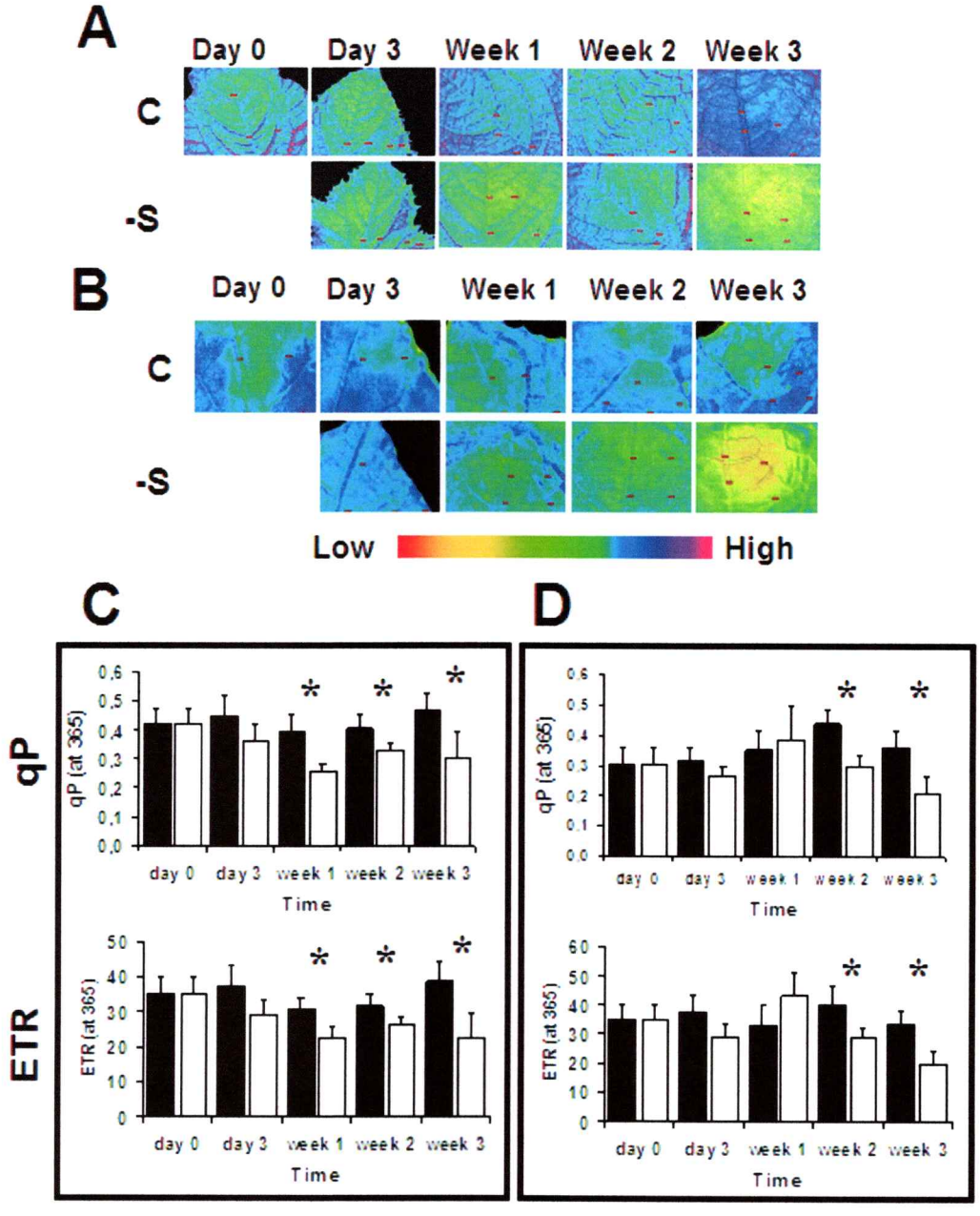

Figure 6. Effects of sulfur deficiency on plant photosynthetic performance detected with chlorophyll fluorescence imaging. Photochemical quenching (qP) images obtained at 225 PFD from sink (Figure A) and source (Figure B) leaf tissues of control (Control) and sulfur starved plants (-S) during the time course of the experiment. Quantitative parameters of photochemical quenching (qP) and relative electron transport rate (ETR) observed at 365 PFD in sink leaves (Figure C) and source leaves (Figure D) of control (black bars) and sulfur-deprived plants (white bars). Color palette shows the scale for the photochemical quenching. Each data point represents the average of 3-4 individual plants± SE. Student t-test was statistically significant ($P < 0.05$) in time points marked with asterisk (*).

Among the parameters obtained with chlorophyll fluorescence imaging, the non-photochemical quenching (qN) was measured in sink and source leaves of sulfur-starved plants during the experiment. After two weeks, the non-photochemical quenching detected in sink and source leaves was similar between control and starved plants (figure 7). Interestingly, a slight increase qN was only detected in sink leaves of sulfur-deprived plants after three weeks (figure 7A and 7B).

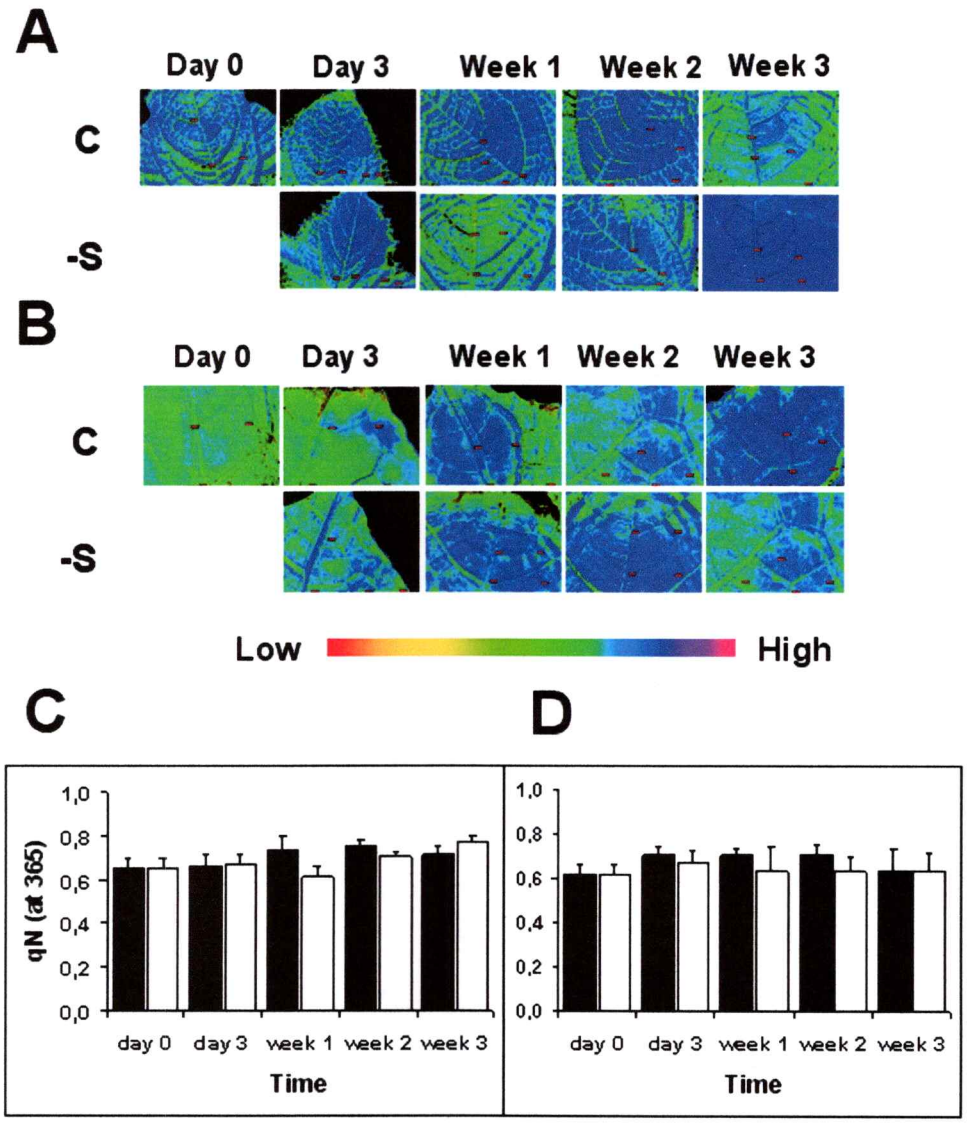

Figure 7. Non-photochemical quenching using chlorophyll fluorescence imaging in sulfur starved plants. Non-photochemical quenching (qN) images obtained at 225 PFD from sink (figure A) and source (figure B) leaf tissues from control (Control) and sulfur starved plants (-Sulfate) during the experiment. Parameters of non-photochemical quenching obtained at 365 PFD from sink leaves of control (black bars) and sulfur starved plants (white bars) (figure C). Chlorophyll fluorescence parameters in source leaves from control (black bars) and sulfur starved plants (white bars) (Figures D). Colour palette shows the scale for the non-photochemichal quenching. Each data point represents the average of 3-4 individual plants± SE. Student t-test did not provide significance (P<0.005).

3.3. Content of chlorophyll and carotenoid pigments in sink and source leaves of sulfate-deprived plants

Measurements of chlorophyll fluorescence parameters have demonstrated that the photosynthetic performance of sink and source leaves of *C. sativus* plants are affected during early stages of sulfur deficiency and before the onset of visual symptoms of sulfate deficiency. The alterations on the photosynthetic performance in leaf tissues of sulfate starved plants suggest a putative sulfate dependent down-regulation of the PSII system (Oxborough, 2004), and/or inhibition of some enzymes involved in the biosynthesis of photosynthetic pigments due to the limited concentration of substrates, such as is the case for S-adenosylmethionine which is one of the substrates employed by magnesium protoporphyrin methyl transferase in the chlorophyll biosynthesis pathway (Buchanan, 2000). We studied the effects of sulfate deficiency on the total amount of chlorophylls and carotenoids extracted from sink and source leaves. The total amount of chlorophyll and carotenoids were determined using acetone extracts (Lichtenthaler, 1987)

Total amounts of chlorophyll and carotenoids remain constant in sink and source leaves of control plants during the first week of observation. However, a tendency to decline in the total amount of chlorophyll was noted from the second week in sink leaves and after 3 weeks in source leaves of plants under sulfur deprivation. After three weeks of treatment, the total amount of chlorophyll decreased significantly by 50% in sink leaves and 40% in source leaves of treated plants compared with the same tissues of control plants (figure 8A and 8C).

The total amount of carotenoids exhibited the same tendency to decline first in sink leaves after two weeks of sulfur starvation, then in source leaves after three weeks of sulfur deficiency (figure 8B and 8D).

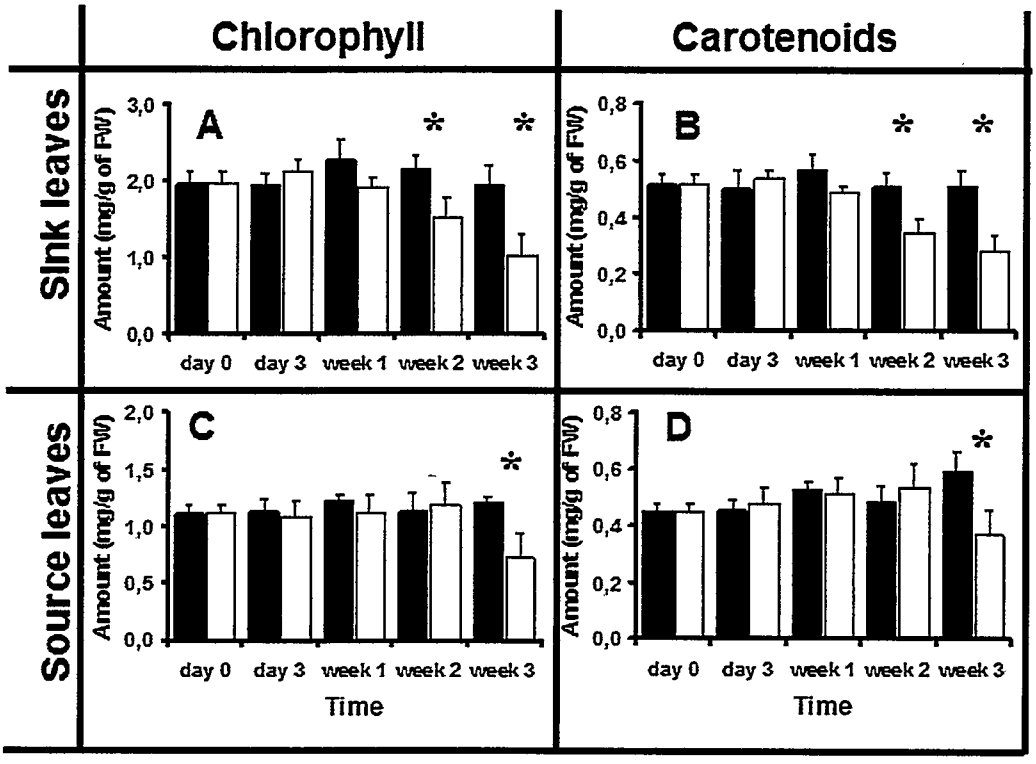

Figure 8. Quantitative analysis of the amount of photosynthetic pigments in sink and source leaves of control and sulfate deprived plants. Total amount of chlorophyll and carotene were determined using acetone extracts (described in material and methods). Total chlorophyll (figure A and C) and total carotene (figures B and D) contents of sink (figures A and B) and source leaves (figures C and D) of control (black bars) and sulfur-deprived *C. sativus* plants (white bars). Errors bars are ± SE. Student's t-test was statistically significant ($P < 0.05$) in time points marked with asterisk (*).

3.4. Relationships between parameters of chlorophyll fluorescence imaging and the amount of photosynthetic pigments in leaf tissues of sulfate deprived plants

As described in section 3.2, measurements of ETR, qP and Y have demonstrated that the photosynthetic performance of sink and source leaves of C. sativus plants is affected after one week and before the onset of visual symptoms of sulfate deficiency (figures 6 and 7). However, the total amount of chlorophyll and carotenoids exhibited the tendency to decline first in sink leaves after two weeks of sulfur starvation, and then in source leaves after three weeks of sulfur deficiency (figure 8). Further, the alterations on the photosynthetic performance before the decreasing of photosynthetic pigments in leaf tissues of sulfate starved plants suggest a putative sulfate dependent down-regulation of the electron transport from the PSII system and/or inhibition of some enzymes involved in the biosynthesis of photosynthetic pigments, such as chlorophylls, due to the limited concentration of substrates.

To study whether sulfate deficiency could affect the photosynthetic performance due to alterations in the amount of photosynthetic pigments in leaf tissues, chlorophyll fluorescence parameters, such as ETR and qN, were correlated with changes in the total amount photosynthetic pigments, like chlorophylls and carotenoids. The changes in the ETR and the total amount of chlorophyll, observed in sink and source leaves of control and sulfur starved plants, were correlated. Likewise, a correlation of the qN and total amount of carotenoids were carried out (Figure 9).

Figures 9A and 9B show the relationships between the ETR and the total amount of chlorophyll determined in sink and source leaves of control and sulfate starved plants during the experiment. No significant modifications in the relationships

between ETR and total amount of chlorophyll were observed in sink and source leaves of control plants (figures 9A and 9B). The relationship between ETR versus chlorophyll content was most strongly affected in sink leaves (figure 9A) than in source leaves (figure 9B) of sulfur-deprived plants. Interestingly, ETR values detected in sink leaf tissues declined one week before the first perturbations of the total amount of chlorophyll which was observed after two weeks of sulfur deficiency (figure 8A). In source leaves, ETR and the total amount of chlorophyll have declined after three weeks of sulfur deficiency (figure 9B).

In the case of the relationships between qN and total amount of carotenes a negative linear correlation was only found in sink leaves from the first to the third week of sulfur deficiency (figure 9C). No significant correlations were found between qN and the total amount of carotenes in source leaves of control and sulfate-deprived plants (figure 9D).

From the figures 9A it can be concluded that sulfate deficiency caused a rapid inhibition in the photosynthetic performance of the PSII system in sink leaves, and, remarkably, before the onset of alterations in the amount photosynthetic pigments. Furthermore, the increase of qN in sink leaves of sulfur-deprived plants suggests that the energy not employed in photosynthesis, due to the inhibition of the PSII system in sulfur deprived plants, is heat dissipated (figure 9C) (see review Maxwell and Johnson, 2000).

In the case of mature leaves (figure 9B), modifications in the photosynthetic parameters ETR and qP were dependent on the decrease of the amount of photosynthetic pigments, which suggest an inhibition of *de novo* biosynthesis of protein and pigment components of the PS II in old leaves from *C. sativus.*

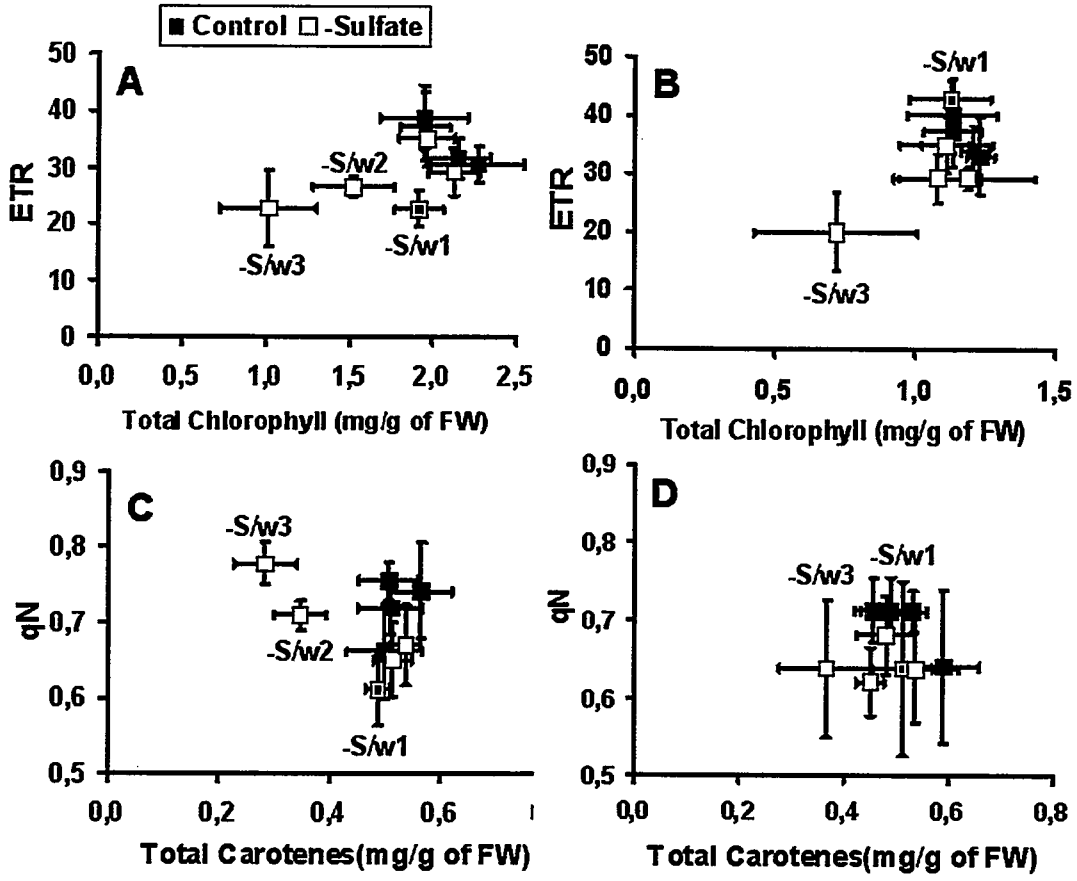

Figure 9. The relationship between photosynthetic parameters obtained with chlorophyll fluorescence imaging and the amount of photosynthetic pigments over the time course of sulfate deficiency. Relative electron transport rate (ETR) versus total amount of chlorophyll in sink (figure A) and source leaves (figure B) of control (■) and sulfate deprived plants (□). Non-photochemical quenching (qN) versus total amount of carotenes in sink leaves (figure C) and source leaves (figure D) of control (■) and sulfate deprived plants (□). Red points indicate the time point (after one week, -S/w1) in which the first symptoms of sulfate deficiency in sink leaves using chlorophyll fluorescence imaging were visualised. **–S/w2** (sulfur deficiency after 2 weeks), **-S/w3** (sulfur deficiency after 3 weeks).

3.5. Cysteine and glutathione levels are not changed in phloem at the early stages of sulfate deprivation.

Thiol content was determined as an indicator for sulfur nutrient status (as was shown earlier for glutathione in *A. thaliana* by Nikiforova et al. (2003) and *Brassica* by Blake-Kalff et al. (1998). We compared the changes of the total amounts of glutathione and cysteine in sink and source leaves between sulfur starved plants and control plants. A strong decrease in the amounts of total glutathione was observed in sink and source leaves from the first week of the time course (figure 10A and 10C). However, the amount of cysteine declined first in sink leaves after one week of sulfate deficiency, and after two weeks of sulfate deficiency in source leaves (figures 10 D and 10 F).

Interestingly, the decreased levels of total cysteine and glutathione observed during the first two weeks of sulfur starvation in sink and source leaves were not observed in phloem exudates of sulfur-starved plants (Figure 10 B and 10 E). After three weeks of sulfur deficiency, the amounts of cysteine and glutathione decreased significantly in phloem sap of plants under sulfur starvation compared with the phloem exudates of control plants (figure 10 B and 10 E). This result suggests that the sulfur assimilation homeostasis is maintained constant in phloem during long time periods of sulfur deficiency.

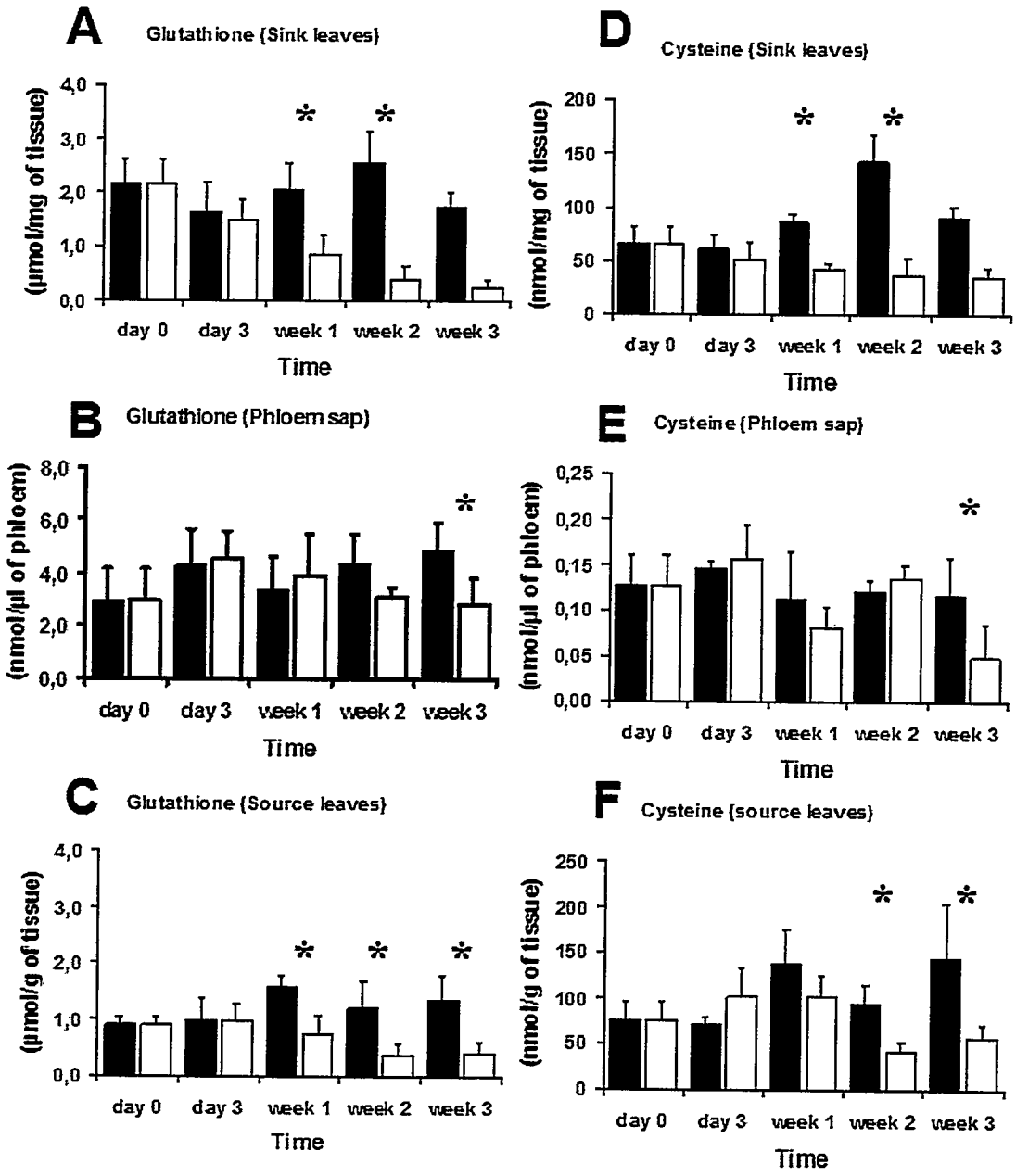

Figure 10. Modifications in the composition of thiols in phloem and leaf tissues in sulfate starved plants. Thiol compounds cysteine, total glutathione (GSH), gamma-glutamyl-cysteine and homocysteine were derivatised with monobromo-bimane (MBB), and then separated by HPLC (See materials and methods). Change in the metabolic composition of glutathione (figures A, B and C) and cysteine (figures D, E, and F) of sink (figures A and D), phloem exudates (figures B and E) and source leaves (figures C and F) of sulfur starved (white bars) in relation to control (black bars) *C. sativus* plants in the time course of the experiment. Student t-test was statistically significant ($P < 0.05$) in time points marked with asterisk (*). Measurements of thiols were repeated at least two times.

3.6. Modifications in the amino acid composition in different tissues of *C. sativus* treated with sulfur deprivation.

In addition to thiol measurements, we analysed the changes in the composition of amino acids in phloem exudates, sink and source leaf tissues in the response to sulfate deficiency by HPLC (Kreft et al., 2003). To study the amounts of soluble amino acids, we determined the composition of amino acids in phloem, sink and source leaves of sulfur starved *C. sativus* plants in relation to control plants in the time course of the experiment (Ra). Statistical significance of the differences in the composition was analysed with t-test. In all the analyses, the difference was considered significant with a probability of $P<0.05$, and $Ra<0.5$ or $Ra>2.0$. Then, each amino acid was catalogued according to the predesigned scheme of *A. thaliana* metabolism using the web page AraCyc (Zhang et al., 2005; http://www.arabidopsis.org/tools/aracyc/), the results are summarized in table I.

As shown in figure 10, cysteine and total glutathione concentrations declined at different rates in tissues of *C. sativus* during the sulfate deprivation; the effects were in the order sink leaf> source leaf>phloem exudates. For amino acids, the response to sulfur deficiency was not uniform; some amino acid levels were induced and some were not changed. In sink leaves the earliest effects were the marked accumulation of glycine, arginine, and tryptophan after one week of sulfate deprivation (Table IA). The effects of sulfate starvation on glycine, arginine, and tryptophan was less marked in mature leaves and occurred after two weeks of sulfate starvation (Table I.C.). Nevertheless, the phloem amino acid composition was more constant during the experiment, while strongest modified levels of amino acids were observed in leaf

tissues of sulfur-deprived plants (Table I.B.). Figure 11 shows examples of changes in the composition of arginine, gamma amino butyric acid (GABA), and methionine in sink leaf tissues and phloem exudates of sulfur starved and control plants.

After one week of sulfate deprivation, sink and source leaf tissues accumulated increased amounts of alanine (more than 3 fold) (Table I). Also, there were very striking proportional increases in the amounts of arginine and tryptophan over a similar time course, but only in sink leaves. In young leaves the concentrations of arginine and tryptophan were more than triple that of the control plants while there was not yet an effect on mature leaves (Table I A and figure 11). No significant effect on the amount of arginine, and tryptophan were seen in mature leaves until week 2, but by the end of the experiment (week 3) they increased more than 60 fold compared to control plants (Table I C).

The same observation was seen for glycine levels that strongly increased first in sink leaves (17 fold), and only after two weeks in source leaves (5 fold) of sulfate-deprived plants. Interestingly, the amount of glycine in sink leaves declined after two weeks of sulfate starvation (Table I.A.). After three weeks of sulfate deficiency, the same tendency of a decline in the amount of glycine was observed in source leaves (Table I.B).

The amounts of lysine and histidine in sink leaves were increased 3.6 fold and 2.8 fold after two weeks of sulfur starvation (Table I.A). An increase on the amount of lysine (2.7 fold) and histidine were also observed in source leaves, but only after three weeks of sulfur deprivation (Table I C).

It is interesting to note that the effects of sulfate deprivation on the relative amount of GABA in leaf tissues were low (less than 2.5 fold) (Table I.A, table I.C, and figure 9). However, the amount of GABA in phloem exudates of sulfate-deprived plants

was nearly triple that of control plants after one week of sulfate starvation. From the second week of sulfate deficiency, the amount of GABA declined to normal levels in phloem exudates (Table I.B. and figure 11).

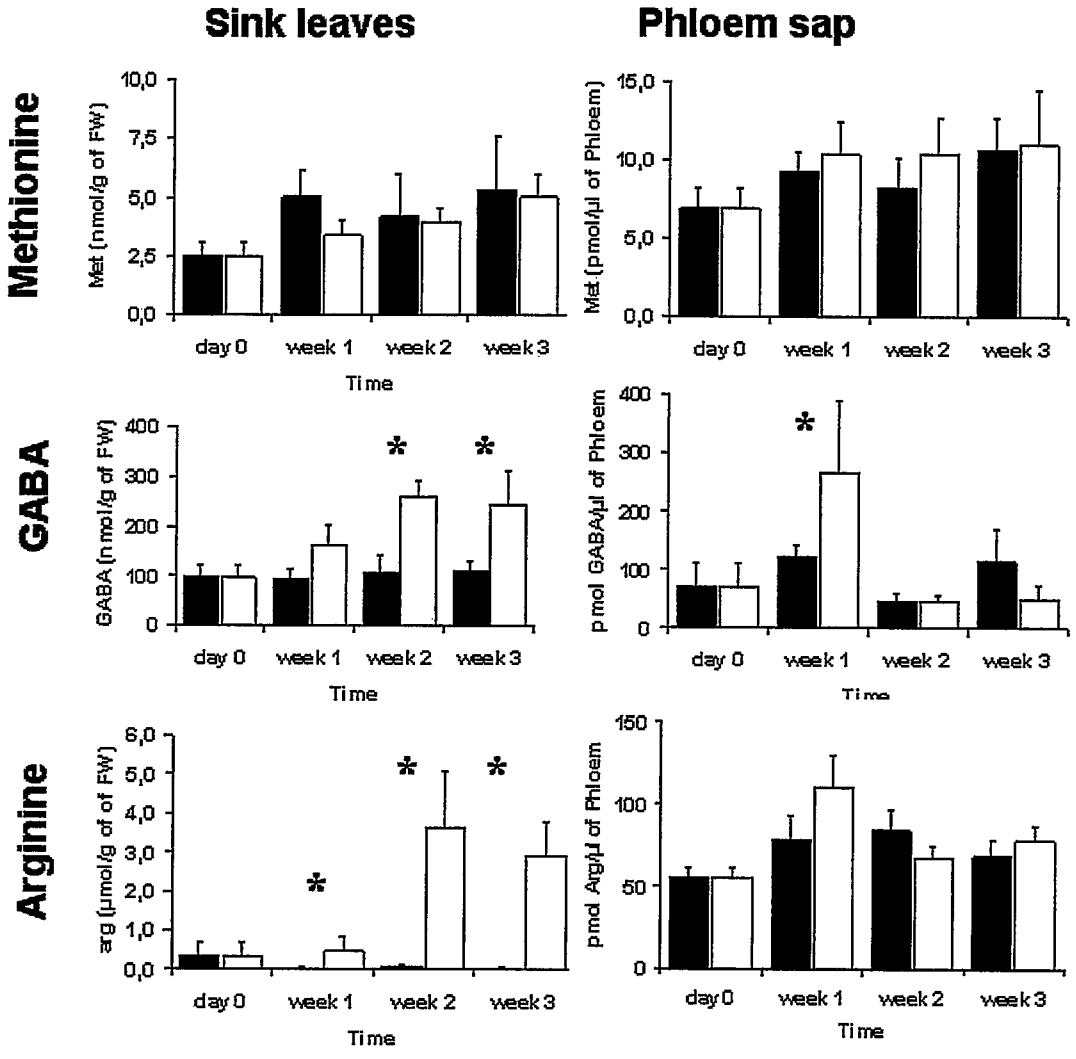

Figure 11. Analysis of the amounts of amino acids in control and sulfur starved plants. Levels of soluble amino acids methionine, gamma amminobutyric acid (GABA), and arginine in sink leaves and phloem exudates of control (black bars) and sulfur starved *C. sativus* plants (white bars). Soluble amino acids were derivatiezd with OPA (o-phtalaldehide) and separated by HPLC (See material and methods). Student t-test was statistically significant ($P < 0.05$) in time points marked with asterisk (*). Results showed in this figure were obtained in a second experimental repetition.

Sulfate deprivation had no effect on the amount of arginine, alanine, glycine, and tryptophan in phloem exudates during the experiment (Table IB and figure 11). Histidine was not detected in phloem exudates during the experiment (Table I B).

Aspartate, threonine, methionine, isoleucine, glutamite, leucine, phenylalanine, and valine did not show significant changes in phloem exudates, sink and source leaves of sulfur starved cucumber plants at all time points of the experiment (Table I).

The coelution and overlapping of unknown compounds did not allow us to identify clear changes in the serine, glutamine, asparagine, and S-methylmethionine composition in phloem sap and leaf tissues between sulfate-starved and control plants during the experiment. Furthermore, the amounts of cystathionine, tyrosine and homocysteine in samples harvested of the different tissues studied of *C. sativus* plants were less than the detection limits of this technique.

Table I. Comparison of soluble amino acid levels in sink leaves (A), phloem exudates (B), and source leaves (C) of sulfur starved and control plants by HPLC. The quotient of the mean of the amount of amino acids of sink leaves, phloem exudates, and source leaves of sulfur starved (n=5) and the mean of the amount of amino acids of sink leaves of control plants (n=5) are show in the table. Values >2 (green boxes) represent an increase in the metabolite levels in phloem exudates of sulfur starved plants in comparison to control plants; values <0.5 (red boxes) represent a decrease. The significance of the changes was evaluated by a Student t-test; bold letters indicate significant differences between the systems ($P<0.05$). (ND: metabolite not detected) (1)= Amount of cysteine detected in thiol measurements; (2)= product of serine metabolism.

Table I.A	**Sink leaves**			
Time	**Day 3**	**Week 1**	**Week 2**	**Week 3**
Compound classification	**Ra (D3)**	**Ra (W 1)**	**Ra (W 2)**	**Ra (W 3)**
1. Photorespiration				
Glycine (2)	0.78	**17.12**	**3.02**	**6.90**
2. Serine metabolism				
Cysteine (1) (2)	0.70	**0.50**	**0.26**	**0.40**
Tryptophan (2)	0.60	**3.65**	**3.67**	**8.75**
Phenylalanine	0,79	1,51	1,51	1,49
3. Aspartate pathway				
Aspartate	0.87	1.12	1.07	0.94
Lysine	1.68	0.96	**3.61**	**7.41**
Methionine	1.26	0.99	1.06	1.47
Threonine	0.68	1.61	2.10	1.85
Isoleucine	1.06	1.14	1.03	1.83
4. Glutamate metabolism				
Glutamate	0.82	0.89	1.18	0.91
Arginine	0.56	**48.34**	**35.43**	**56.08**
Histidine	0.89	1.29	**2.76**	**3.72**
GABA	0.98	1.41	1.27	1.53
5. Pyruvate metabolism				
Leucine	1.05	1.13	1.22	1.73
Valine	0.70	1.16	1.09	1.66
Alanine	1.15	**12.96**	**4.19**	**18.40**

Table I.B	Phloem exudates			
Time	**Day 3**	**Week 1**	**Week 2**	**Week 3**
Compound classification	**Ra (D3)**	**Ra (W 1)**	**Ra (W 2)**	**Ra (W 3)**
1. Serine metabolism				
Glycine (2)	0.88	0.62	1.27	1.02
Cisteine (1) (2)	1.08	0.72	1.11	**0.43**
Tryptophan (2)	0.82	1.02	1.21	1.43
Phenylalanine	0.77	0.77	1.58	1.28
2. Aspartate pathway				
Aspartate	1.06	0.97	0.63	0.78
Lysine	0.90	1.16	0.94	1.52
Methionine	0.89	1.23	1.33	1.10
Threonine	0.72	0.77	1.11	1.00
Isoleucine	0.87	1.01	0.79	1.45
3. Glutamate metabolism				
Glutamate	0.87	0.85	0.89	1.23
Arginine	1.16	1.44	0.76	1.63
Histidine	**ND**	**ND**	**ND**	**ND**
GABA	0.98	**2.85**	0.53	0.64
5. Pyruvate metabolism				
Leucine	0.67	0.99	0.63	0.77
Valine	0.62	0.80	0.83	1.30
Alanine	1.26	0.76	1.44	0.77

Table I.C.	Source leaves			
Time	**Day 3**	**Week 1**	**Week 2**	**Week 3**
Compound classification	**R (D3)**	**R (W 1)**	**R (W 2)**	**R (W 3)**
1. Photorespiration				
Glycine (2)	1.76	1.48	**5.23**	**2.97**

Table I.C. (Continuation).

Compound classification	R (D3)	R (W 1)	R (W 2)	R (W 3)
2. Serine metabolism				
Cisteine (1)(2)	1.45	0.74	**0.44**	**0.39**
Tryptophan (2)	2.27	1.49	**3.76**	**4.13**
Phenylalanine	1.37	0.76	1.14	1.56
3. Aspartate pathway				
Aspartate	0.81	1.09	0.45	1.62
Lysine	1.00	1.35	1.69	**2.73**
Methionine	0.88	1.02	0.74	1.77
Threonine	1.26	0.77	0.99	1.52
Isoleucine	1.40	1.12	1.36	1.22
4. Glutamate metabolism				
Glutamate	1.16	1.04	0.55	1.00
Arginine	0.90	2.10	**7.72**	**64.75**
Histidine	0.57	0.93	**3.03**	**4.67**
GABA	0.90	0.96	0.60	0.90
5. Pyruvate metabolism				
Leucine	1.22	1.15	1.45	0.95
Valine	1.36	0.76	1.14	1.57
Alanine	0.49	**3.77**	**2.93**	**71.86**

3.7. Metabolite profiling of phloem exudates, sink and source leaves.

In order to extend our first results of thiol and amino acid measurements, we planned to determine the changes in the metabolite composition of sink leaves, source leaves, and phloem exudates harvested from control and sulfate starved plants using GC/MS metabolite profiling (Roessner et al., 2000; Fiehn et al., 2000; Nikiforova et al., 2003). GC separates complex mixtures of metabolite derivatives into a series of compounds that enter the mass spectrometer and are subsequently ionized, fragmented and detected (Desbrosses et al., 2005). For this analysis we used the same batch of phloem exudates and leaf tissue samples used for the physiological, total amount of photosynthetic pigment, thiol, and amino acid content analyses described from sections 3.1. to 3.6. of this chapter.

GC/MS profiles of sink and source leaves of sulfate starved plants displayed significant qualitative differences in the metabolite composition compared to GC/MS profiles obtained from sink and source leaf tissues of control plants over the time of sulfur deficiency (figures 12 and 13). Nevertheless, few qualitative modifications in the metabolite composition were observed between the GC/MS profiles of phloem exudates of control and sulfate starved plants (figures 12 and 13).

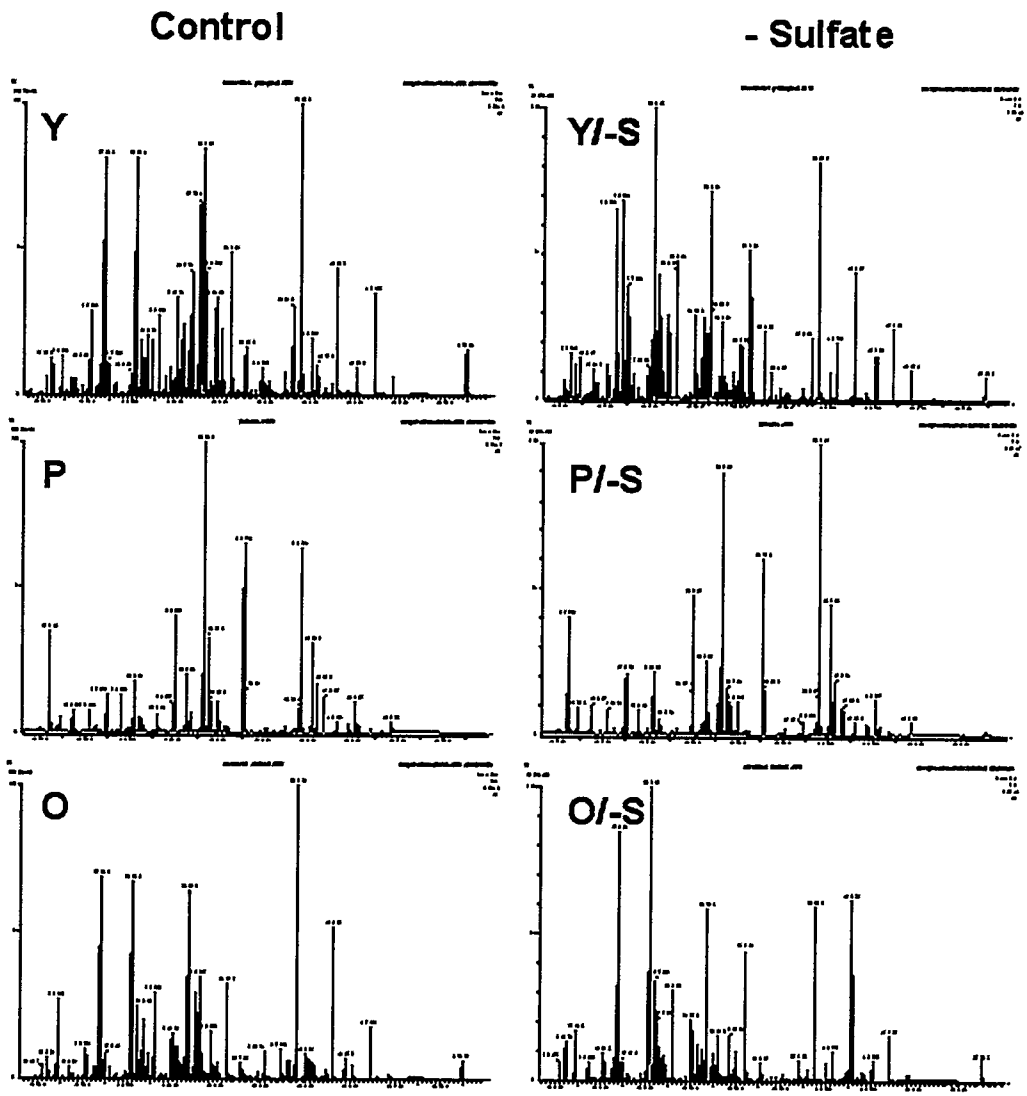

Figure 12. Metabolic profiling by GC/MS analysis of phloem exudates and leaf organs of control and sulfate deprived *C. sativus* plants. GC/MS profiles of polar extracts obtained of phloem exudates (P), sink (Y) and source leaf (O) samples of control (Control) and treated (-Sulfate) plants after one week of sulfur starvation. Extractions of polar compounds and GC/MS separation are described in material and methods.

The data obtained by this chromatographic technique were processed and analysed with the help of databases and GC/MS spectra data softwares (see material and methods) to determine the metabolic changes of phloem exudates, sink and source leaf tissues due to the effects of sulfur deficiency. Each metabolite was represented by one

to several ionic fragments of precise mass, which can serve as a tag for that metabolite. Each ionic fragment has properties that facilitate unequivocal identification of the parent metabolite, following comparison to the reference compound. The properties of ionic fragments are: (1) gas chromatographic retention, which is best characterized by a retention index (RI), (2) and specific composition of fragments, which are each characterized by mass to charge ratio (m/z) (Desbrosses et al., 2005). Then, peak areas derived from specific ion traces indicative of each analysed compound were normalized by the peak area derived from an internal standard ribitol (m/z=319, RI=1,734), present within the same chromatogram, resulting in relative concentrations for all compounds analysed (figure 13).

The changes between relative concentrations of different metabolites in phloem exudates, sink and source leaf tissues in plants grown in normal medium as a control and the same tissues of plants grown on sulfate-deficient medium were analysed by calculating the response ratio (R) of the average relative concentration of the same tissues from five plants grown on sulfate sufficient medium for each analyte. Statistical significance of the differences in relative concentrations was analysed with the t-test. In all the analyses the difference was considered significant with a probability of $P<0.05$. For all metabolites, R more than 2.0 or less than 0.5 with $P<0.05$ at least in one of the experimental points was the criteria chosen to assign statistically significant response to sulfate deprivation.

Within the chemical classes of amino acids, organic acids, sugars, and sugar alcohols detected by GC/MS, the response was not uniform; some metabolites of these classes were induced and some were reduced in sulfur starved plants. Due to the huge amount of data obtained by this technique, this study was focused to provide a catalogue of changes in the relative concentration of 75 non-redundant compounds of

known chemical structure detected by GC/MS in phloem exudates, sink and source leaves of *C. sativus* grown in sulfate deficiency. These 75 non-redundant metabolites were also analysed and characterized in a starvation response experiment to sulfate deficiency in *A. thaliana* (Nikiforova et al., 2005 a). The metabolites of unknown chemical structure are not considered further in this study (figure 13).

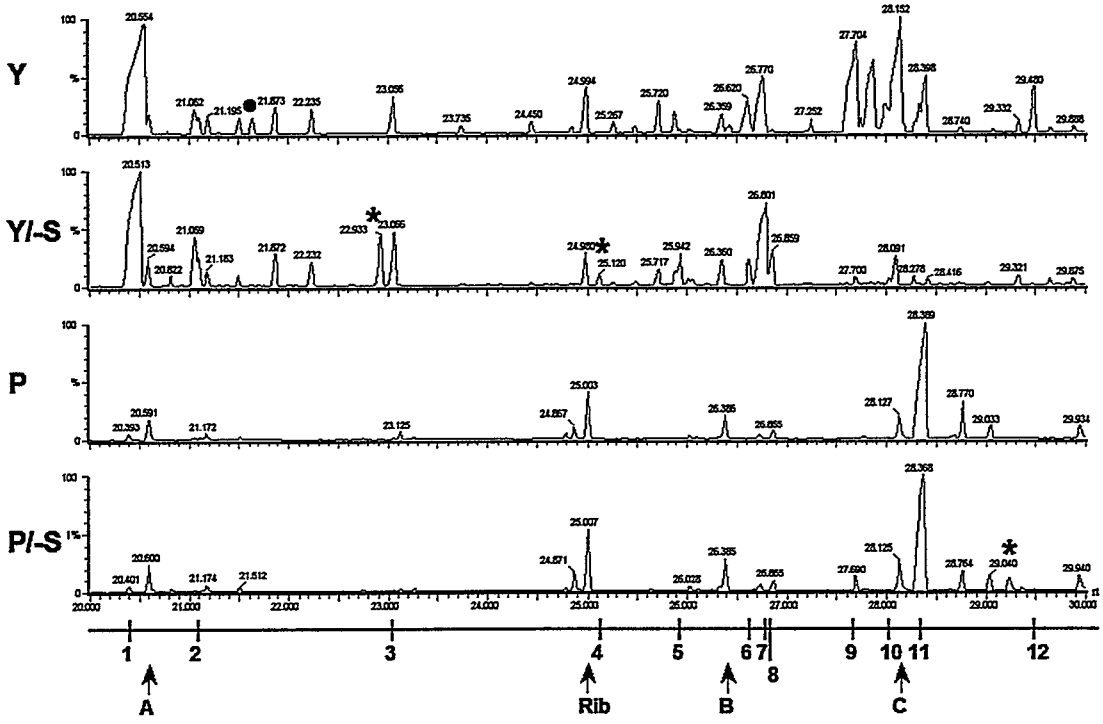

Figure 13. Demonstration of the complexity of the GC/MS metabolite profilings of samples of sink leaves and phloem exudates showed in the figure 12. Representative expansion of the GC/MS chromatograms in figure 12 of the region 20.0-30.0 min of phloem exudates (P) and sink leaves (Y) of control and sulfate deprived (-S) plants. Peak identification: **A**, pentadecane; **B**, octadecane; **C**, nonadecane; **Rib**, ribitol; **1**, malate; **2**, aspartate; **3**, glutamate; **4**, glutamine 4 TMS; **5**, glutamine 3 TMS; **6**, ornithine; **7**, citrate; **8**, arginine; **9**, fructose MX1; **10**, glucose MX1; **11**, Galactose MX1; ✱, unknown compounds detected only in samples harvested from sulfate deprived plants; ●, unknown compounds only detected in samples harvested from control plants. **A**, **B**, and **C** are internal standards used for retention index (RI) calculation.

To evaluate coordination of metabolic changes under sulfur deficiency stress, the measured metabolites were catalogued onto plant biosynthetic pathways according to the metabolic pathway schemes of AraCyc (http://www.arabidopsis.org/tools/aracyc/; Zhang et al., 2005). The results are summarized in tables II, III, and IV, and explained with more detail in different subsections of the discussion (see below).

Changes in the composition of arginine, lysine, glycine, and tryptophan in different organs of sulfur-deprived and control *C. sativus* plants determined by HPLC measurements were confirmed by GC/MS. Further, GC/MS profiles allowed to identify the modification in the amounts of serine, glutamine, asparagine, and tyrosine in phloem sap and leaf tissues, which were not detected by measurements of OPA derivatized amino acids by HPLC (Table II, III, and IV).

In the case of cysteine, the reduction in the amount of this metabolite in leaf tissues by thiol measurements by HPLC was confirmed by GC/MS (Table II and table IV). However, this technique did not allow us to identify alterations in the amount of cysteine in phloem exudates of sulfur-deprived plants. Perhaps, the amount cysteine in phloem exudates of *C. sativus* plants was less than the detection limits of this technique (Table III). A similar observation was reported in phloem exudates obtained from pumpkin plants (*C. maxima)* (Fiehn., 2003).

Table II. Comparison of metabolite levels in sink leaves of sulfate starved and control plants using GC/MS profiling. R, ratio of average relative concentration at depleted sulfur (-S) [n=5] to average relative concentration at normal sulfate (Control) [n=5]. Values R>2 and green boxes represent an increase in the metabolite levels in sink leaves of sulfur starved plants in comparison to control plants; values <0.5 and red boxes represent a decrease. The significance of the changes was evaluated by a t-test; bold letters indicate significant differences between the systems (P<0.05). (ND: metabolite not detected; M/Z: value of the specific ion used for quantifying each metabolite). (Notes: D=day, W= week, MX= methoxyaminated derivate).

Time		Day 3	Week 1	Week 2	Week 3
Compound classification	**M/Z**	**R (D3)**	**R (W 1)**	**R (W 2)**	**R (W 3)**
1. Energy					
1.1. Photosynthesis/ Glycolisis.					
Glyceric-3-P	**299**	1,84	**0,15**	1,52	0,65
Fructose-6-P MX	**315**	1,26	1,31	1,26	0,89
Glucose-6-P MX1	**387**	1,22	1,53	1,37	0,93
Glucose-6-P MX2	**387**	1,21	1,50	1,31	0,91
1.2. Photorespiration					
Serine 3	**218**	0,67	1,94	**3,66**	**0,23**
Glycine 3	**248**	1,68	**25,95**	**6,11**	0,79
Glyceric	**189**	0,73	1,53	0,88	1,56
1.3. Glycolisis.					
Sucrose	**451**	0,90	0,92	1,45	0,61
Glucose MX1	**160**	1,23	**0,27**	0,67	1,84
Fructose MX1	**307**	1,12	**0,22**	**0,38**	1,31
Fructose MX2	**307**	1,11	**0,20**	0,51	1,26
Lactic	**117**	**0,59**	1,30	1,13	1,16
1.3. TCA cycle					
Citric	**375**	1,01	1,14	1,88	1,95
Isocitric	**245**	1,13	1,78	1,97	1,61
α-keto-glutaric MX	**198**	1,83	**0,33**	1,00	**0,45**
Succinic	**247**	1,29	1,11	0,78	0,65
Fumaric	**245**	1,01	1,13	1,08	0,77
Malic	**335**	0,77	0,92	1,51	1,38

Table II (Continuation).

Compound classification	M/Z	R (D3)	R (W 1)	R (W 2)	R (W 3)
2.Amino acids					
Glutamic 3	**246**	0,85	1,21	1,23	0,97
Glutamine 4	**227**	1,25	**4,29**	**2,59**	0,90
Glutamine 3	**156**	1,24	**3,48**	**2,65**	0,55
Aspartic 3	**232**	0,76	1,58	1,15	1,41
Asparagines 4	**188**	0,66	**2,98**	**2,19**	1,29
Homoserine 3	**218**	0,91	1,70	0,74	1,12
Lysine 4	**156**	0,56	**8,56**	**13,69**	**2,38**
Cysteine 3	**220**	**0,55**	**0,58**	**0,56**	**ND**
Metionine 2	**176**	1,33	1,55	0,73	2,35
Threonine 3	**219**	0,73	1,64	**2,63**	0,53
Isoleucine 2	**158**	0,56	**2,34**	1,70	1,43
Alanine	**116**	1,52	0,45	1,00	0,33
Valine 2	**144**	0,65	1,64	1,82	1,59
Leucine 2	**158**	0,56	1,56	**2,12**	**2,02**
Shikimic acid	**372**	1,37	**0,40**	0,64	0,89
Tryptophan 3	**202**	0,47	**10,87**	**10,74**	**8,36**
Tyrosine	**218**	0,75	**2,48**	**2,77**	**5,12**
Phenylalanine 2	**192**	0,97	**2,02**	**3,13**	**2,46**
Arginine 5	**256**	**2,21**	**74,82**	**11,73**	**3,15**
3. Polyamine biosynthesis					
Ornithine 4	**142**	**2,83**	**34,80**	**10,82**	**2,83**
Putrescine 4	**174**	0,87	1,17	0,91	**2,22**
Spermidine	**144**	1,24	**86,87**	**3,21**	**3,01**
4. Sugars					
Arabinose MX	**307**	0,83	0,86	0,57	1,10
Fucose MX2	**160**	0,99	1,16	0,68	1,21
Maltose MX1	**480**	1,34	1,62	0,57	1,09
Rhamnose MX1	**160**	0,88	1,01	0,97	1,04
Melezitose	**361**	1,89	**0,43**	0,77	0,81
Trehalose	**191**	1,65	**0,31**	1,79	**0,30**
Xylose MX1	**307**	1,13	0,46	1,38	**0,45**
Galactose-6-P MX1	**299**	1,15	1,60	1,33	1,39
Mannose MX	**160**	1,12	0,51	0,51	0,48
Mannose-6-P MX1	**387**	1,41	1,72	1,41	0,89
Galactose MX1	**319**	0,83	**0,43**	0,49	0,63

Table II (Continuation).

Compound classification	M/Z	R (D3)	R (W 1)	R (W 2)	R (W 3)
5. Sugar alcohols					
Glycerol	**205**	0,99	0,70	1,46	1,29
Glycerol-1-P	**357**	0,94	0,77	**2,39**	0,19
Erythritol	**205**	1,57	0,77	**2,36**	0,75
Ribitol	**319**	1,08	1,04	1,02	0,83
Xylitol	**307**	0,96	0,89	1,07	0,92
Myo-inositol-2-P 1	**387**	0,86	1,12	1,20	1,87
6. Nutrients					
Phosphoric 3	**299**	0,96	0,67	1,30	1,11
7. Purine metabolism					
Uric 4	**456**	1,78	**11,97**	**2,23**	**2,24**
Ribose MX	**307**	1,06	0,97	0,68	**0,43**
Allantoin 4	**431**	**3,88**	**66,45**	**2,56**	**4,56**
Urea	**189**	**0,61**	0,88	1,31	1,07
8. Pyramidine metabolism					
Thymine 2	**255**	0,70	0,67	0,58	0,75
Uracil 2	**241**	1,01	**2,15**	**5,69**	**2,59**
Beta-alanine 3	**248**	0,79	1,29	**2,10**	0,69
9. Carbohydrate transport					
Galactitol	**307**	1,00	0,83	**2,00**	0,64
Raffinose	**437**	2,29	1,21	**3,08**	0,82
Myo-inosytol	**305**	1,02	1,13	1,06	0,99
10. Stress associated					
Gamma-aminobutyric 3	**174**	0,88	0,82	1,12	0,74
Proline 2	**142**	**0,61**	1,56	**2,75**	**2,22**
t-4-HO-proline	**230**	0,94	1,71	1,58	0,65

Table III. Comparison of metabolite levels in phloem exudates of sulfate starved and control plants using GC/MS profiling. R, ratio of average of relative concentration at depleted sulfate (-S) to average of relative concentration at normal sulfate (Control)[n=5]. Values >2 and green boxes represent an increase in the metabolite levels in phloem exudates of sulfur starved plants in comparison to control plants; values <0.5 and red boxes represent a decrease. The significance of the changes was evaluated by a t-test; bold letters indicate significant differences. (ND: metabolite not detected; M/Z: value of the specific ion used for quantifying each metabolite). (Notes: D=day, W= week, ND= metabolite not detected, MX= methoxyaminated derivate)

Time		**Day 3**	**Week 1**	**Week 2**	**Week 3**
Compound classification	**M/Z**	**R (D3)**	**R (W 1)**	**R (W 2)**	**R (W 3)**
1. Energy					
1.1. Glycolisis.					
Sucrose	**451**	**0,44**	**0,32**	0,97	0,86
Glucose MX1	**160**	0,96	1,17	0,92	1,46
Fructose MX1	**307**	0,95	1,01	1,86	0,69
Fructose MX2	**307**	0,95	0,84	1,78	1,28
Glucose-6-P MX2	**387**	0,70	0,72	1,40	0,99
Glucose-6-P MX1	**387**	0,74	0,70	1,11	0,90
Fructose-6-P MX	**315**	0,69	0,75	1,23	0,97
Glyceric-3-P	**299**	**ND**	**ND**	**ND**	**ND**
Lactic	**117**	0,99	0,56	**2,69**	1,13
1.3. TCA cycle					
Citric	**375**	0,73	1,39	0,96	1,16
Isocitric	**245**	1,16	1,10	1,05	0,65
α-keto-glutaric MX	**198**	0,88	0,83	0,94	1,44
Succinic	**247**	0,87	0,72	1,45	1,62
Fumaric	**245**	1,13	0,96	1,32	1,48
Malic	**335**	0,63	0,84	1,24	1,27
2. Carbohydrate transport					
Galactitol	**307**	1,00	0,62	1,02	0,87
Raffinose	**437**	1,13	0,85	0,75	2,32
Myo-inositol	**305**	1,14	1,46	0,96	2,22

Table III (Continuation).

Compound classification	M/Z	R (D3)	R (W 1)	R (W 2)	R (W 3)
3. Polyamine biosynthesis.					
Arginine 5	**157**	0,92	1,12	1,00	0,98
Ornithine 4	**142**	0,98	0,89	**4,02**	**3,63**
Putrescine 4	**174**	0,88	0,59	1,04	0,91
Spermidine	**144**	**ND**	**ND**	**ND**	**ND**
4. Amino acids					
Glutamic 3	**246**	0,97	0,73	1,45	0,63
Glutamine 3	**156**	0,84	1,51	1,17	1,04
Aspartic 3	**232**	1,05	1,26	1,09	1,18
Asparagine 4	**188**	0,77	0,54	1,11	1,39
Homoserine 3	**218**	1,07	0,94	1,49	1,06
Lysine 4	**156**	0,76	1,64	0,88	1,37
Glycine 3	**174**	0,83	0,64	0,55	1,49
Serine 3	**218**	0,79	0,53	0,96	1,55
O-acet-serine	**132**	**ND**	**ND**	**ND**	**ND**
Cysteine 3	**220**	**ND**	**ND**	**ND**	**ND**
Methionine 2	**176**	0,60	1,38	2,03	0,66
Threonine 3	**219**	0,69	0,77	1,14	1,02
Isoleucine 2	**158**	0,66	1,11	0,78	0,96
Shikimic	**372**	0,81	0,67	**2,37**	0,72
Tryptophan 2	**202**	**ND**	**ND**	**ND**	**ND**
Tyrosine	**218**	1,00	0,65	1,02	0,89
Phenylalanine 2	**192**	0,72	1,12	1,32	0,68
Valine 2	**144**	0,72	0,74	0,79	0,98
Leucine 2	**158**	0,61	0,98	0,98	0,66
5. Sugars					
Arabinose MX	**307**	**ND**	**ND**	**ND**	**ND**
Fucose MX2	**160**	**ND**	**ND**	**ND**	**ND**
Maltose MX1	**204**	0,77	0,43	1,05	1,08
Melezitose	**361**	**ND**	**ND**	**ND**	**ND**
Rhamnose MX1	**160**	0,72	0,87	1,31	1,56
Xylose MX2	**307**	1,65	1,84	1,01	1,14
Galactose-6-P MX1	**299**	0,68	0,76	1,38	1,33
Mannose MX	**160**	0,74	0,60	1,77	**0,28**
Mannose-6-P MX1	**160**	0,84	0,70	1,22	1,01
Galactose MX1	**319**	0,89	0,60	**2,18**	**0,27**

Table III (Continuation).

Compound classification	M/Z	R (D3)	R (W 1)	R (W 2)	R (W 3)
6. Sugar alcohols					
Glycerol	**205**	1,15	1,10	0,91	0,47
Glycerol-1-P	**357**	**ND**	**ND**	**ND**	**ND**
Erythritol	**205**	1,05	0,85	0,66	1,17
Galactitol	**307**	1,00	0,62	1,02	0,87
Mannitol	**319**	**ND**	**ND**	**ND**	**ND**
Erythritol	**205**	0,70	0,85	0,66	1,17
Ribitol	**319**	0,81	1,00	1,00	1,00
Xylitol	**307**	0,69	**2,35**	**2,21**	1,13
Myo-inositol-2-P 1	**387**	**ND**	**ND**	**ND**	**ND**
7. Nutrients					
Phosphoric 3	**299**	0,96	0,99	1,03	0,93
8. Purine metabolism					
Uric 4	**456**	**ND**	**ND**	**ND**	**ND**
Ribose MX	**307**	1,04	1,96	0,87	1,34
Allantoin 4	**431**	0,65	0,80	0,97	0,94
Urea	**189**	0,69	2,77	1,77	1,35
9.Pirimidine metabolism					
Thymine 2	**255**	**ND**	**ND**	**ND**	**ND**
Uracil 2	**241**	1,28	0,87	0,79	1,24
Beta-alanine 3	**248**	1,50	0,56	2,05	0,76
10. Stress associated					
Gamma-aminobutyric 3	**174**	1,11	**2,12**	**2,98**	**0,59**
Proline 2	**142**	0,68	**0,33**	2,38	1,19
t-4-HO-proline	**230**	1,36	1,37	0,57	1,91

Table IV. Comparison of metabolite levels in source leaves of sulfate starved and control plants using GC/MS profiling. R, ratio of average of relative concentration at depleted sulfate (-S) to average of relative concentration at normal sulfate (Control)[n=5]. Values >2 and green boxes represent an increase in the metabolite levels in source leaves of sulfur starved plants in comparison to control plants; values <0.5 red boxes represent a decrease. The significance of the changes was evaluated by a t-test; bold letters indicate significant differences between the systems (P<0.05). (ND: metabolite not detected; M/Z: value of the specific ion used for quantifying each metabolite). (Notes: D=day, W= week, ND= metabolite not detected, MX= methoxyaminated derivate)

Time		**Day 3**	**Week 1**	**Week 2**	**Week 3**
Compound classification	**M/Z**	**R (D3)**	**R (W 1)**	**R (W 2)**	**R (W 3)**
1. Energy					
1.1. Photosynthesis/ Glycolisis.					
Glyceric-3-P	**299**	0,52	0,84	**0,32**	**0,15**
Fructose-6-P MX	**387**	1,46	0,74	0,65	1,33
Glucose-6-P MX1	**387**	1,77	0,54	0,82	1,30
Glucose-6-P MX2	**315**	1,31	0,70	0,73	1,39
1.2. Photorespiration					
Serine 3	**218**	1,01	1,17	0,94	0,94
Glycine 3	**248**	1,21	**6,06**	**7,63**	1,92
Glyceric	**189**	1,34	0,68	1,10	0,80
1.3. Glycolisis.					
Sucrose	**451**	1,49	**0,37**	1,16	0,81
Glucose MX1	**160**	1,11	**0,38**	1,25	**0,45**
Fructose MX1	**307**	1,53	**0,17**	1,09	**0,33**
Fructose MX2	**307**	1,47	**0,15**	1,34	**0,32**
Lactic	**117**	1,38	1,00	1,11	1,12
1.3. TCA cycle					
Citric	**375**	1,00	1,20	**0,48**	**2,85**
Isocitric	**245**	0,67	1,00	**0,33**	**4,68**
Alpha-keto-glutaric MX	**198**	1,14	**0,34**	**0,51**	0,79
Succinic	**247**	1,07	0,67	**0,39**	1,04
Fumaric	**245**	1,20	1,26	0,79	1,15
Malic	**335**	1,17	0,72	1,17	1,27

Table IV (Continuation).

Compound classification	M/Z	R (D3)	R (W 1)	R (W 2)	R (W 3)
2.amino acids					
Glutamic 3	**246**	1,00	0,87	0,94	0,97
Glutamine 3	**156**	1,92	0,98	1,89	1,15
Aspartic 3	**232**	0,87	0,93	0,92	1,71
Asparagine 4	**188**	1,08	2,39	1,69	**3,19**
Homoserine 3	**218**	1,71	1,82	0,61	**0,35**
Lysine 4	**156**	0,94	**4,15**	**11,33**	**3,26**
O-acet-serine	**174**	**ND**	**ND**	**ND**	**ND**
Cysteine 3	**220**	0,96	0,83	**0,51**	**0,56**
Methionine 2	**176**	1,64	1,90	0,88	1,01
Treonine 3	**219**	0,89	1,41	1,32	1,57
Isoleucine 2	**158**	0,81	0,94	**2,79**	1,10
Alanine	**116**	1,93	0,64	1,16	0,87
Valine 2	**144**	0,77	0,71	1,46	1,21
Leucine 2	**158**	0,87	0,99	1,68	0,80
Shikimic	**462**	0,94	0,79	**0,22**	0,51
Tryptophan 3	**202**	1,43	**24,95**	**42,26**	**6,26**
Tyrosine	**218**	0,57	**2,65**	**5,73**	**4,12**
Phenylalanine 2	**192**	0,97	1,29	**3,69**	1,81
Arginine 5	**256**	0,59	**21,01**	**6,56**	**11,34**
3. Polyamine biosynthesis.					
Ornithine 4	**142**	1,12	**13,89**	**5,97**	**12,80**
Putrescine 4	**174**	1,54	0,93	0,77	0,68
Spermidine	**144**	0,75	2,17	0,85	1,22
4. Sugars					
Arabinose MX	**307**	1,22	0,68	0,82	**0,56**
Fucose MX2	**160**	1,31	0,68	0,59	0,75
Maltose MX1	**480**	1,11	0,88	0,56	0,75
Melezitose	**361**	**0,39**	**0,38**	**0,58**	1,62
Rhamnose MX1	**160**	1,11	1,22	0,91	0,92
Trehalose	**191**	**0,15**	**0,18**	**0,35**	**0,58**
Xylose MX1	**307**	1,22	**0,33**	**2,69**	0,63
Galactose-6-P MX1	**299**	1,37	0,60	0,61	1,27
Mannose MX	**160**	1,21	1,61	0,88	**0,35**
Mannose-6-P MX1	**387**	**2,35**	1,03	0,75	1,43
Galactose MX1	**319**	1,00	0,58	1,45	0,88

Table IV (Continuation).

Compound classification	M/Z	R (D3)	R (W 1)	R (W 2)	R (W 3)
5. Sugar alcohols					
Glycerol	**205**	0,87	1,20	1,24	1,39
Glicerol-1-P	**357**	1,38	1,87	1,42	1,26
Erythritol	**205**	0,86	0,52	1,36	1,13
Mannitol	**421**	1,14	0,87	1,15	1,34
Sorbitol	**419**	1,14	0,85	1,06	1,60
Ribitol	**319**	1,16	0,97	1,01	0,92
Xylitol	**307**	1,37	0,68	0,95	1,32
Myo-inositol	**305**	1,04	0,90	1,02	1,06
Myo-inositol-2-P 1	**387**	0,85	1,23	0,61	1,59
6. Nutrients					
Phosphoric 3	**299**	1,19	0,93	0,81	1,34
7. Purine metabolism					
Uric 4	**456**	0,74	1,93	1,26	1,41
Ribose MX	**307**	0,94	0,67	0,71	0,87
Allantoin 4	**431**	1,26	**9,09**	**3,23**	1,99
Urea	**189**	0,69	0,68	0,88	0,88
8. Pyrimidine metabolism					
Thymine 2	**255**	1,38	0,63	0,95	1,14
Uracil 2	**241**	1,21	0,99	1,15	1,65
Beta-alanine 3	**248**	1,29	1,13	1,40	1,48
9. Carbohydrate transport					
Galactitol	**307**	1,18	0,88	1,50	0,95
Raffinose	**437**	0,95	0,61	1,88	0,90
Myo-inositol	**305**	1,04	0,90	1,02	1,06
melibiose MX1	**204**	0,95	1,10	**2,51**	0,69
10. Stress associated					
Gamma-aminobutyric 3	**174**	1,08	1,17	1,19	0,95
Proline 2	**142**	0,99	0,76	**2,22**	1,56
t-4-HO-proline	**230**	0,80	0,81	1,05	2,12

3.8. Metabolic phenotypes of phloem and leaf tissues of sulfur-deprived *C. sativus* plants.

In plants, the balance between source (mature, photosynthesising leaves) and sink (growing, metabolising and storing tissues) organs is continuously adjusted in response to environmental and developmental cues. This balance between source and sink organs is interceded by phloem (Fiehn, 2003; Amiard et al., 2005). Therefore, the study of metabolic interactions among different organs is a very important issue for understanding the function of phloem in the source and sinks interactions while the plants are in conditions of abiotic stress, such as sulfate deficiency.

To study the effect of sulfate deficiency on the metabolite composition, the metabolome, in phloem exudates, sink and source leaf tissues, we carried out a principal component analysis (PCA) of the data. PCA uses linear combinations of all metabolite data to generate new vectors that best explain overall variance in the data set, without prior assumptions as to how and if these data might cluster (Fiehn, 2003; Taylor et al., 2002). Each type of sample is here statistically regarded as one metabolic phenotype. Finally, if the sample classes can be clearly distinguished when projected onto any of the principal components, PCA enables identification of metabolites that distinguish the sample classes (Debrosses et al., 2005). Furthermore, PCA gives us a measure of which metabolites are the most determining in the response to sulfate deficiency.

A total of 82 known metabolites determined by measurements of thiols, soluble amino acids, and GC/MS analysis (described in sections 3.5 to 3.7) were used to generate the matrix employed for principal component analysis. This matrix describes a

total of 135 samples from phloem exudates, sink and source leaves harvested of control and sulfur starved plants during the experiment.

The first 6 principal components derived from the above data matrix encompassed 90.49% of the variance of this data set (figure 14). 85.35% of the variation in the original data is explained with the first three PCs alone (figure 15). The first component accounted for 68.89% of the variance and allowed distinction of phloem exudates from leaf organs. Sink leaves exhibited more similarity to old leaves than to phloem exudates (figure 15 A). Additionally, the first component demonstrated that sink and source samples of sulfur starved plants showed major levels of heterogeneity in the metabolic phenotype than control plants (figure 15 A and 15 B).

The second and third components encompassing 13.05% and 3.24% of the variance, respectively, demonstrated that the data set contained metabolite measurements that distinguished between leaf tissues of control plants and leaf tissues of sulfate deprived plants (figures 15 B and 15 C). The scatter plot of the second component against the third component shows that the metabolic phenotype of sink leaves displayed more variation than source leaves due to effects of sulfur deficiency (figure 15 C). These results suggest that the sink leaves are more affected by sulfur deprivation than source leaves of *C. sativus* plants.

In the case of phloem exudates, principal components 1, 2, and 3 did not allow the identification of changes in the metabolite phenotypes of phloem exudates of sulfur starved plants compared to phloem exudates harvested from control plants (figure 15). It is shown that phloem exudates of control plants and sulfate starved plants cluster together in the first three principal components analysed. These results suggest that the phloem was not significantly affected by sulfur deprivation during the time course of the experiment. No subsequent components allowed a clear discrimination between

samples types (data not shown).

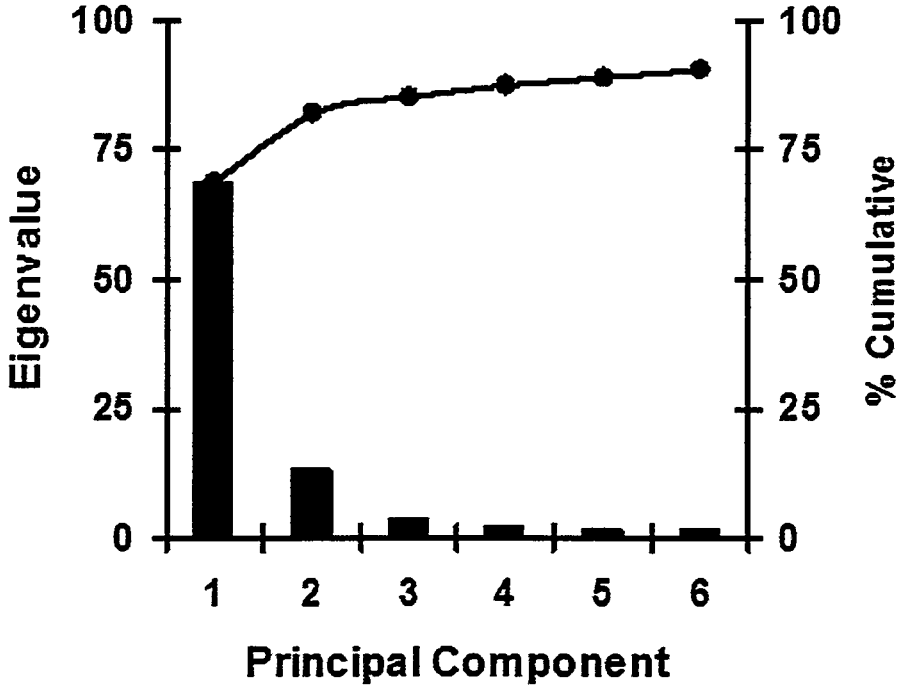

Figure 14. Variance of the data explained using principal component analysis. The figure illustrates the individual (left Y axis and black bars) and cumulative variance (right Y axis and line) that is explained by each of the first six principal components.

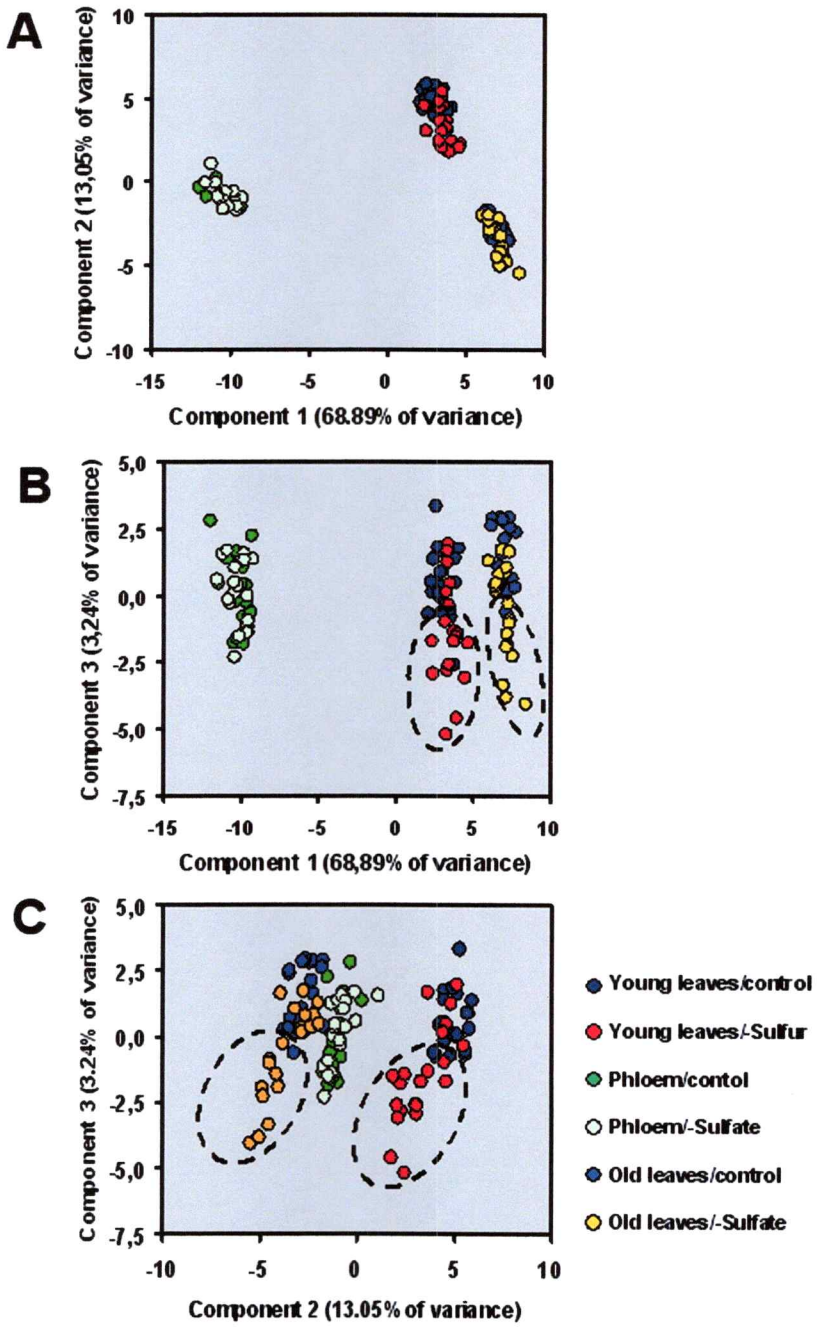

Figure 15. PCA analysis of metabolic profiles, which represents 86 non-redundant known metabolites of C. sativus organs harvested in the course of 0 day at 3 weeks of sulfur deficiency. Principal component analysis used to clustering samples of phloem exudates, sink and source leaves of control and sulfur starved C. sativus plants on different time points of the experiment. Components 1, 2 and 3 were chosen for best visualization of metabolic phenotype separation and include 85.35% of the total information content derived from metabolite variances. Clusters identified: sink (between the first to the third week of minus sulfate starvation; n=14) and source leaves (between second and third week of minus sulfate starvation; n=10) of sulfate-deprived plants (black dashed circles).

3.9. PCA reveals specific metabolites that distinguish the response to sulfate deprivation of different organs.

The contribution of each metabolite to a specific component is reflected by the loading value derived from PCA analysis. Those metabolites with highest loading values are indicated to have the strongest influence on the respective characteristics of a component (Desbrosses et al, 2005; Taylor et al., 2002; Fiehn et al. 2000). We focused on the loading values of components 1, 2, and 3. The 20 most influential metabolites of each component were analysed, and are summarised in table V. Among the metabolites involved in the systemic response to sulfur deficiency, glycerate, glutamate, homoserine, alanine, arginine, uric acid, glutathione, glycerate-3-P, and glycerol-3-P were the most influential metabolites in the first component, which described the phloem exudates and leaf organs differences, and the response to sulfur deficiency in leaf organs. The second component, which describes differences in leaf organs between sulfur deprived and control plants, was most affected by metabolites involved in sulfate metabolism [cysteine and gamma-glutamyl-cysteine (GEC)], photorespiration (glycine and glycerate), photosynthesis (glycerate-3-P), polyamine biosynthesis (spermidine), sugar transport through the phloem (raffinose), aromatic amino acid biosynthesis (tryptophan and shikimate), aspartate pathway (lysine, threonine, isoleucine, and methionine), purine and pyrimidine metabolism (beta-alanine and thymine). Interestingly, we found that tryptophan, detected by GC/MS and HPLC measurements, was one of the most influential metabolites in the component 2 (Table V).

The component 3 was influenced by other metabolites involved in purine

metabolism (histidine), nitrogen metabolism (urea), and aromatic amino acids biosynthesis (such as phenylalnine) when the plants are in conditions of sulfur deprivation. Further, the third component was also influenced with respect to sulfur response metabolites involved in the metabolic pathways of the most influential metabolites observed in component 1 and 2. Some examples are arginine detected in the component 1, and tryptophan detected in the component 2 (Table V).

Table V. The 20 most influential metabolites, which contribute to the component 1, 2, and 3 derived from the PCA analysis. Values in bold letters are metabolites involved in the response to sulfur deprivation (Nikiforova et al., 2005 a). (1)= OPA derivatized amino acids detected by HPLC, (2)= thiols compounds detected by HPLC. PC= principal component, MX= methoxyaminated derivate

PC									
1			2			3			
Metabolite		Value	Metabolite		Value	Metabolite		Value	
Glyceric		0,994	**GEC**	**(2)**	**0,318**	**Glutathione**	**(2)**	**0,403**	
Valine 2		0,986	**Shikimic**		**0,199**	**GEC**	**(2)**	**0,103**	
Glutamic 3		**0,987**	**Aspartic**	**(1)**	**0,323**	**Putrescine**		**0,095**	
Homoserine 3		**0,987**	**Threonine**	**(1)**	**0,240**	**Urea**		**0,118**	
Citric		0,991	**Isoleucine**	**(1)**	**0,397**	**Tymine**		0,254	
Fumaric		0,989	**Glyceric-3-P**		**0,371**	**Melibiose**		0,127	
Malic		0,990	**α-keto-glutaric**		**0,191**	**Arabinose MX**		0,237	
Galactose-6-P MX1		0,986	**Rhamnose MX1**		0,378	**Fucose MX2**		0,154	
Myo-inositol		**0,992**	**Glycine**	**(1)**	**-0,909**	**Maltose MX1**		0,115	
Glutathione	**(2)**	**-0,670**	**Valine**	**(1)**	-0,655	**Valine**		**-0,395**	
Alanine	**(1)**	**-0,926**	**Leucine**		-0,746	**Histidine**	**(1)**	**-0,262**	
Arginine	**(1)**	**-0,902**	**Spermidine**		**-0,792**	**Arginine**	**(1)**	**-0,274**	
Methionine		**-0,500**	**Tryptophan**	**(1)**	**-0,700**	**Phenylalanine**		**-0,259**	
Glyceric-3-P		**-0,675**	**Tryptophan 3**		**-0,739**	**Tryptophan**	**(1)**	**-0,397**	
Uric		**-0,734**	**Tymine**		**-0,789**	**Lysine**	**(1)**	**-0,451**	
Fucose MX2		-0,703	**Beta-alanine 2**		**-0,673**	**Methionine**	**(1)**	-0,489	
Glycerol-1-P		**-0,522**	**Arabinose MX**		-0,818	**Threonine**	**(1)**	**-0,607**	
Sorbitol		-0,846	**Fucose MX2**		-0,648	**Isoleucine**	**(1)**	**-0,774**	
Mannitol		-0,816	**Cysteine**	**(2)**	**0,183**	**Glycerol-1-P**		**-0,293**	

4. Discussion

4.1. Sulfur deficiency causes a rapid photosynthetic imbalance in leaf tissues

The phloem is the major long distance transport system of metabolites, and it connects distant plant tissues. Our special interest is the communication between organs involved in the sulfur containing compounds production (source leaves) and sulfur containing consumption (sink leaves) in plants exposed to sulfur deprivation. Thus, the response to sulfate deficiency of the whole system is dependent on the processes occurring in the phloem. The aim of this study was to determine at the metabolic level the response of the phloem system to sulfur deficiency in comparison to respective leaf tissues and undisturbed plant tissue controls. To study whether the metabolic alterations caused by sulfur deficiency in leaf tissues are reflected in the phloem, we used *C. sativus* plants grown in hydroponic cultures (Figure 4). After three weeks, the *C. sativus* plants displayed phenotypic alterations as expected from similar experiments of sulfur deprivation in spinach (Prossner et al., 2001), pea (Gilbert et al., 1998), rapeseed (Lencioni et al., 1997), *A. thaliana* (Nikiforova et al., 2003), and nutritional disorders observed on the field in different plant species under insufficient sulfur fertilization (Bergmann, 1992; Blake-Kalff et al., 1998) (figure 5).

However, alterations in the chlorophyll fluorescence parameters, such as Y, qP and ETR, were observed in leaf tissues before the onset of any visual effect of chlorosis (figures 6 and 7). Furthermore, the amounts of total chlorophyll and carotenoids were not decreased in parallel with the alterations observed for chlorophyll fluorescence imaging in sink leaves for sulfur deprived plants (figures 8 and 9). These results suggest a possible lack between the first alterations of the photosynthetic performance and a reduction in the

amount of photosynthetic pigments due to the decreased sulfate content in sink leaves.

The first alterations of Y, ETR and qP in sink leaves during early periods of sulfur deficiency suggest a rapid disruption or inhibition in photosynthetic activity of PSII and/or Rubisco biosynthesis in *C. sativus,* similarly to the results of a general inhibition of *de novo synthesis* of the photosynthetic apparatus (Burke et al., 1986; Hawkesford, 2000) and the decrease in the content of Rubisco before modifications in the amount of total chlorophyll in young leaves of wheat under sulfur deficiency (Gilbert et al., 1997).

The results of alterations in the photosynthetic parameters measured with chlorophyll fluorescence imaging before the first modifications in the composition of total amounts of photosynthetic pigments demonstrated that sulfur deficiency could produce a disruption on the photosynthetic performance in young leaf tissues of *C. sativus.* These results were also confirmed at the metabolic level. An increase in the amount of metabolites involved in photorespiration such as glycine and serine were found after one week of sulfur deficiency in leaf tissues (tables I, II and IV) (Somerville, 2001; Nokiforova et al., 2005 a). Similar results of an increase in the amount of glycine and serine have been described in *A. thaliana* (Nikiforova et al., 2003; Nikiforova et al., 2005 a), and sugar beet (Thomas et al., 2000) under sulfur deficiency. The levels of products of photosynthesis such as glyceric-3-phosphate and some carbohydrates (i.e. glucose, fructose) declined first in young leaf tissues after one week, and then in source leaves after two weeks of sulfur starvation (Tables II, IV and figure 13).

Probably, sulfate deficiency disrupts the regulation of some key enzymes and structural proteins involved in the reactions of photosynthesis in young leaf tissues of *C.sativus* plants. In addition, the increase of qN in sink leaves of sulfur-deprived *C. sativus* plants suggests that the energy not employed in photosynthesis is dissipated as heat (figure 9C), and may imply the possible inhibition of the PSII system in sulfur deprived plants, (see review Maxwell and Johnson, 2000). Supporting this hypothesis, sulfur

deficiency causes the down-regulation of the photosynthetic apparatus and Rubisco-encoding genes, and aggravates the problems of energy assimilation in *A. thaliana* plants (Nikiforova et al., 2003; Nikiforova et al. 2005 a). Among this group of genes a posible candidate is Lhc1B1 chlorophyll a/b binding protein (AT2G34420), because it suffers from a strong inhibition due to the effects of sulfate deficiency (Nikiforova et al., 2003). Furthermore, carrying out a search for homologous sequences using BLAST, a homology was found with a gene that codes for Lhc1B1 chlorophyll a/b binding protein (accession M16057) in *C. sativus*. Recent studies show that thiol redox mediators, such as thioredoxins, could be involved in the phosphorylation of the light harvesting complex II of pumpkin (*C. maxima*) and *A. thaliana* (Martisuo et al., 2003), and control of key enzymes involved in the Calvin cycle, such as in the case of fructose-1,6-biphosphatase (Chiadmi et al., 1999). Interestingly, these enzymes regulated by thiol containing mediators, such as glutathione and thioredoxins, are also involved in the redox signalling and regulation of several metabolic pathways localized in chloroplasts (Surpin et al., 2002; Buchanan and Balmer, 2005).

Considering the aforementioned facts, sulfate deprivation could disrupt the functions of thioredoxins and/or other thiol containing compounds involved in the control of enzymes of the light and dark reactions of photosynthesis in chloroplast of leaf plants. This question should be investigated at enzymatic level and/or proteomic analysis in leaf tissues of *C. sativus* and *A. thaliana* plants exposed to sulfur deprivation.

In source leaves the alterations in the same photosynthetic parameters appear after two weeks of sulfur deficiency (figures 6B and 6D). Similar changes in young and old leaves were observed at physiological level in wheat (Gilbert et al., 1997), and at metabolic level in spinach (Prossner et al, 2001). The alterations in chlorophyll fluorescence parameters in source leaves after long time periods of sulfur deficiency suggest that the pool of sulfate stored in vacuoles is used to maintain constant sulfur

homeostasis in mature leaves, and indicate a low remobilisation rate of sulfate from mature leaves to developmental organs, such as seed, sink leaves and roots, through the phloem. Similar results of constant source leaf homeostasis, slow exhaustation of the internal pools, and slow remobilisation of sulfate from older leaves have been described in soybean (Sunarpi and Anderson, 1996) and spinach (Prossner et al., 2001).

4.2. The composition of S-containing compounds detected in phloem exudates was not altered during early periods of sulfur deprivation

The first significant modifications in the chlorophyll fluorescence parameters were detected in parenchymatic zones next to the vascular system in sink leaves as early as one week after treatment (figures 6 and 7). Although, we found a slight recovery of ETR, qP and Y in sink leaves after two weeks of sulfur deprivation, and in parallel to the detection of the first alterations in the photosynthetic performance observed in source leaves using chlorophyll fluorescence imaging (figure 6). These results suggest that the vascular system, such as phloem, is involved in the mechanism of rebalancing the sulfur homeostasis in the whole plant system.

To study whether the phloem is involved in the rebalancing response of the system to sulfate deprivation, we planned to determine first the changes in the thiol containing compounds of sink leaves, source leaves, and phloem exudates harvested from control and sulfur starved plants. Glutathione and cysteine changes, in both the sink and source tissues, were similar to previous experiments in *A. thalina* (Nikiforova et al., 2003) and rapeseed (Lencioni et al., 1997; Blake-Kalff et al., 1998) (figure 10). Interestingly, the decreased levels of total glutathione and cysteine detected in sink and source leaves were not reflected in phloem exudates of sulfur-starved plants during the first two weeks of sulfur deprivation (figure 10 B and 10 E). Only after three weeks of sulfur deficiency, the

total amounts of glutahione and cysteine decreased in phloem exudates (figures 10 B and 10 E). Thus, it can be concluded, that sulfur deficiency in *C. sativus* may induce genes involved in the transport of sulfate and sulfate containing metabolites from leaf tissues into the phloem of *C. sativus* plants, such as sulfate transporters, with the main objective of maintaining the phloem homeostasis during sulfur deficiency. Furthermore, glutathione and cysteine produced in source organs could be transported through the phloem with the objective to keep the regulation of enzymes (i.e. ATP sulfurylase) involved in the assimilation and the uptake of sulfate in roots of *C. sativus* exposed to sulfate deprivation. Supporting this hypothesis, recent studies in *A. thaliana* showed that the levels of transcription of sulfate transporter 1;3 under sulfur deficiency condition increased. Besides, sulfate transporter 1;3 may play a role in both the remobilisation of sulfate directly towards the companion cell of the phloem under sulfate deficiency, and in the initiation of translocation of sulfur containing nutrients, such as glutathione, from source to sink in *Arabidposis* (Yoshimoto et. al., 2003; Buchner et al., 2004). The regulation of the sulfate uptake and transport in plants has been reviewed previously. Using a split root system, Lappartient and Touraine (1996) have suggested that glutathione transported in the phloem may be the signal that regulates the sulfate uptake.

Among the S-containing compounds, methionine did not show significant changes in leaf tissues and phloem exudates during the experiment (figure 11 and table I). The constant pools of methionine in leaf tissues and phloem exudates under sulfur deficiency suggest a tight control mechanism to maintain methionine homeostasis in the whole plant constant. Methionine is an essential amino acid involved in a number of biological processes in plants such as biosynthesis of proteins and secondary metabolites, i.e. S-adenosyl-methionine (SAM) and S-methyl-methionine (SMM) (Droux, 2004). SAM is used in metabolic pathways involved in the chlorophyll, ethylene, polyamines, biotin biosynthesis (Ravanel et al.1998) and organic sulfate compounds transported through the

phloem, such as S-metylmethionine (Bourgis et al., 1999).

During the experiment, dramatic changes in the amount of different metabolite products of SAM were observed in leaf tissues exposed to sulfate deficiency in relation to control plants. Such was the case for the drop of chlorophyll content in young and source leaves. Moreover, increases in the amount of polyamines (e.g. spermidine) and purine metabolism products (e.g. allantoin) were observed together with an appearance of inhibition in the photosynthetic performance in both tissues (figure 6; tables II and IV). These results suggest that the content of SAM in C. sativus leaves decreases when plants are exposed to conditions of sulfate deprivation. Furthermore, Nikiforova et al. (2005 a) recently reported that the amount of methionine did not change in sulfur deprived *A. thaliana* plants. However, they found that the SAM level decreased dramatically (less than 1%) and thus influences several metabolic pathways, which cause the inhibition of chlorophyll biosynthesis, cessation of photosynthesis, nitrogen metabolism imbalance, accumulation of polyamines (i.e. putrescine) and purine metabolism products (i.e. allantoin and uric acid)

In the case of phloem sap of plants treated with sulfate deficiency, it was not possible to detect significant changes in the content of polyamines and purine metabolism products (Table III). Furthermore, it may be concluded that the plant tries to maintain as long as possible the SAM homeostasis in phloem when it faces a condition of sulfate deficiency. Probably, the maintenance of the SAM homeostasis would be carried out through the transportation of sulfate containing compounds (e.g. glutathione and SMM) towards the phloem.

4.3. Sulfate deprivation causes an imbalance in the nitrogen assimilation of sink and source leaves of *C. sativus*

As shown in tables I, II and IV, sink and source leaf tissues of *C. sativus* plants subjected to sulfur deficiency accumulated high amounts of nitrogen rich compounds, such as amino acids, polyamines (i.e. spermidine), ureides (i.e. allantoin), and products of pyrimidine metabolism (i.e. beta-alanine and uracil). Accumulation occurs after one week in sink leaves, and after two weeks in source leaves. Major differences were found in the group of amino acids, most notably in amino acids derived from α-ketoglutaric acid such as, arginine, ornithine, and proline (tables II and IV). In addition, large increases were observed in the levels of some amino acids derived from the oxalo acetic acid, such as lysine, isoleucine, and asparagine, in leaf tissues of sulfur starved plants. Further, leaf tissues of sulfur-starved plants accumulated large amounts of aromatic amino acids, such as tryptophan, and products derived of photorespiration, such as glycine and serine.

As can be seen in table II significant accumulations of glutamine (3-4 times fold), and polyamine compounds, such as spermidine (86 fold), were detected in sink leaves in the first week of sulfate starvation. After two weeks of sulfate deficiency, the amounts of glutamine and spermidine started to decline in sink leaves. (Table II). However, the amount of glutamine and spermidine remains constant in source leaves and phloem exudates of sulfate starved plants in all time points of the experiment (Table II and III).

It is known that glutamine and asparagine are the first products of nitrogen assimilation in leaf tissues (Coruzzi, 2000). The accumulation of these amino acids involved in nitrogen assimilation in sink leaf tissues of sulfur-starved plants suggests first, a dislocation in primary assimilation of ammonia reduced from nitrate in chloroplasts, second, the re-

assimilation of photorespiratory ammonia, and third, the increase of protein degradation with the main objective to provide sulfur containing compounds in the plant system (Nikiforova et al 2005 a). Also, asparagine and glutamine could be involved in the mechanisms of sequestering the surplus of nitrogen in vacuoles of sink leaves of sulfur-deprived plants (Prossner et al., 1997), which seems to be an efficient mechanism for detoxifying sink tissues, such as young leaves, of excess of ammonia in plants exposed to sulfur deprivation (Nikiforova et al, 2005; Rabe, 1990). Similar results were observed in young leaves of spinach (Prossner et al, 2001).

In the case of spermidine, this could be produced from arginine or ornithine, and used for the stabilization of thylacoid membranes of chloroplasts with the objective to maintain constant the photosynthesis in sink leaf tissues. Spermidine is a product of arginine, ornithine, and SAM metabolism, and is an essential compound for survival and plant embryo development of *A. thaliana* (Imai et al., 2004), and may be involved in the stabilization of thylakoid membranes of chloroplast as shown for oat leaves (Besford et al., 1993).

The accumulation of N-containing compounds suggests an evident imbalance in the nitrate assimilation in different metabolic pathways and/or protein synthesis in leaf tissues from *C. sativus* deprived of sulfate. Similar results of accumulation of N-containing compounds in leaf tissues of plants have been described in spinach (Prossner et al., 1997; Prossner et al., 2001), *A. thaliana* (Nikiforova et al., 2005 a), and sugar beet (Thomas et al., 2000). Furthermore, the results show that the major changes in the amount of nitrogen rich compounds in sink leaf tissues, such as amino acids and ureides, were found early in periods of sulfate deprivation (Table II and IV), and the amounts of these compounds declined in leaf tissues after longer time periods of sulfur deprivation. These results suggest that in the case of *C. sativus* plants, sulfur deficiency causes the accumulation of N-containing compounds produced to prevent ammonia intoxication in leaf tissues

probably influences negatively the fixation of nitrogen, extending previous observations found in soybean (*Glycine max*) in response to the excess of nitrogen fertilization and drought stress (Lukaszewsky et al., 1997; King and Purcell., 2005).

4.4. Shift of metabolic pathways is a response to sulfur deficiency in leaf organs of *C. sativus.*

One of the most important points in the response to sulfur deficiency consists in the coordination of metabolic changes to maintain viability of the plant (Nikiforova et al. 2005 a). Among the several metabolic changes due to effects of sulfate deficiency, it is known that the levels of cysteine drops down and leads to the accumulation of serine, which is subsequently converted to tryptophan and favours an increased flow via down stream products including IAA as a result leaf tissues of *A.thaliana* (Nikiforova et al., 2003; Hirai and Saito, 2004). Furthermore, genes that encode for proteins involved in tryptophan and auxin biosynthesis exhibited an over expression, such as the beta subunit of tryptophan synthase and nitrilase III in *A. thaliana* indicating that the auxin stimulation is part of the sulfur starvation response (Nikiforova et 2003, Hirai and Saito, 2004).

GC/MS metabolite profilings allowed us to determine that the amount of serine did not change in leaf tissues of cucumber plants during the first stages of sulfur deficiency (Tables II and IV). However, the amount of tryptophan increased, while the amount of cysteine strongly decreased in leaf tissues of sulfur-deprived plants (Tables I, II and IV). In the case of phloem exudates of sulfur-deprived plants, the level of tryptophan did not show significant changes over the time course of the experiment (Table I and III). These results suggest that sulfur deficiency may cause the expression of genes involved in metabolic pathways that shift the utilization of the excess of serine produced during the

photorespitration to the production of tryptophan and auxins, such as indole-3-acetic acid (IAA), in leaf tissues of *C. sativus* plants treated with sulfur deficiency. Probably the auxins produced in leaf tissues are transported to roots through the phloem, and then they induce the growth of lateral roots to provide additional access to exogenous sulfur, similarly to the experimental observations of Nikiforova et al.(2003, 2005 b).

4.5. Metabolite composition in the phloem exudates was not strongly altered during sulfate deficiency compared to leaf tissues.

Few significant modifications were found in the total amounts of different metabolites analysed in phloem exudates harvested from sulfate deprived compared to control plants (Table I.B and III). These modifications were mostly found in N-containing compounds after one week of sulfate deprivation, though being modest compared to the modifications observed in sink and source leaves of the same plant (table IA, IB, II, and IV). Among the nitrogen containing compounds, the levels of ornithine, GABA and proline were the most significantly altered in phloem exudates for sulfate deprivation (Table III).

The levels of ornhitine increased in sink and source leaves after one week of sulfate starvation, and in parallel to the accumulation of arginine, lysine, glycine, and tryptophan (Table II, IV and figure 13). However, only the amount of ornithine detected in phloem exudates of sulfate-starved plants was four times higher than the amount observed in control plants after two weeks of sulfur starvation (table III and figure 13). This result suggests that the nitrogen assimilation or the regulation of transport of nitrogen containing compounds is also altered in the phloem of plants exposed to sulfate deficiency. Within this context ornithine is probably one of the principal metabolite involved in the storage of the excess of ammonia in phloem of such plants during sulfate deficiency conditions. Also,

ornithine is the substrate for the biosynthesis of proline and GABA in plants (Kishor Kavi et al., 2005).

Interestingly, the levels of GABA started to increase after one week of sulfate deprivation, while the first perturbations in the photosynthetic performance were detected after one week in sink leaves (figures 6, 11 and table I). Then, after two weeks of sulfate-starvation the levels of GABA in phloem exudates declined in parallel to the slight recovery of the photosynthetic capacity in sink leaves (figures 6, 11 and table I). In contrast, the levels of proline decreased in phloem exudates after one week of sulfur deficiency (Table III). Then, the levels of proline increased to normal levels compared to phloem exudates of control plants after two weeks of sulfate starvation. Considering these results, GABA might have functions as a compatible osmolyte in phloem or a stress-signaling molecule produced in sink organs, and then transported to source organs through the phloem. Experimental evidence supports the involvement of GABA synthesis in pH regulation, nitrogen storage, plant development and defence, compatible osmolyte and an alternative pathway for glutamate utilization (see review Shelp et al., 1999). In addition, it is known that GABA, at high concentrations, stabilizes and protects isolated thylakoids against freezing damage in the presence of salt, exceeding the cryoprotective properties of proline (Heber et al., 1971), and possesses *in vitro* hydroxyl-radical-scavenging activity, exceeding that of proline and glycine betaine at the same concentrations (16 mM) (Smirnoff et al., 1989; Shelp et al, 1999). Besides, it is established that GABA is involved in metabolic pathways that prevent the accumulation of reactive oxygen intermediates and cell death, which appear to be essential for plant defense against environmental stress (Bouché et al., 2003). Probably, GABA is synthesised with the objective of diminishing the impact of the imbalance in the nitrogen assimilation in the phloem in early periods of sulfate deprivation. The increase of GABA in the phloem may function as an osmoprotector or a hydroxy-radical-scavenging activity inductor during the first stages of

sulfate deficiency in the plant. Furthermore, this result suggests that GABA may be a stress signalling molecule synthesised due to metabolic perturbations in sink leaves. Then GABA is delivered to source organs through the phloem.

Several metabolites, such as amino acids and sugars, synthesized in source organs are delivered through the phloem to sink organs (Oparka and Santa Cruz, 2000). Within the amino acid compounds, glutamine and asparagine are the most important N-containing compounds used in the nitrogen transport from source to sink organs through the phloem (Coruzzi, 2000). As shown in table III, the internal levels of asparagine and glutamine did not show significant changes in phloem exudates of sulfur-starved compared to control plants in all the time points of the experiment, which suggests no significant modifications in the transport of N-containing compounds involved in the delivery of nitrogen from source to sink organs through the phloem, despite their increase in leaf tissues with the option of detoxifying the surplus of nitrogen (Table III). Further, the amount of carbohydrates transported through the phloem, such as raffinose, was not changed during early periods of sulfur deprivation. *C. sativus* is a symplastic loader (Zhang, 2004), and the transportation of carbohydrates through the phloem in this kind of plants mainly takes place in the form of polysaccharides like stacchyose and raffinose (Turkina et al, 1998; Zhang, 2004; Amiard et al., 2005).

Considering these results, it is possible to conclude that the delivery of amino acids and sugars from source to sink organs through the phloem is not disrupted in *C. sativus* plants exposed to sulfate deprivation. Against the effects observed in leaf tissues, phloem homeostasis is maintained.

4.6. The global scheme of metabolic regulation of sulfur nutritional stress in *C. sativus* plants.

The study of abiotic sulfur deficiency stress in plants has advanced considerably in recent years. Actually, the high interest to study sulfur metabolism is reflected in publications where the molecular mechanisms of gene expression in *A. thaliana* to sulfur nutrition are analysed through global transcriptome analysis (Hirai et al., 2003; Maruyama-Nakashita et al., 2003) or combined with metabolite profiling (Nikiforova et al., 2003; Hirai et al., 2004; Nikiforova et al., 2005 a; Nikiforova et al., 2005 b). However, the analysis in *A. thaliana* is limited to leaf/root analyses, omitting other important issues: the long distance transport of nutrients through the phloem and source to sink communication during the response to sulfate deficiency. With this study, the analysis is complemented by adding the metabolome data on the time course resulting from metabolite profiles of phloem exudates, and sink as well as source leaves of *C. sativus* plants exposed to sulfur deficiency.

Metabolite profiling of leaf tissues and phloem exudates revealed the adaptative mechanisms of the system indicating a shift to a new program to maintain the homeostasis constant as long as possible against accumulating effects of sulfate deprivation in *C. sativus* plants. By summarizing the information on the coordination between different metabolic changes, sulfate deficiency causes in leaf tissues of *C. sativus* the reduction in the amount of glutathione and cysteine, inhibition of the photosynthetic capacity, increase of photorespiration, imbalance in the nitrogen assimilation, constant homeostasis of methionine, and re-channelling of assimilated carbon to serine through and to the production of tryptophan (Table V). In addition to this, the changes in the amount of several metabolites, due to effects of sulfate deficiency, in phloem exudates and leaf

organs of *C. sativus* revealed that plants grown in the absence of external sulfate resulted in the expression of metabolic phenotypes associated to the response to sulfate deficiency; the effects were in the order young leaf and then in old leaves (figure 15). These results are in agreement with previous studies of changes of individual levels of metabolites in sink and source leaf tissues reported for various plant species in sulfur starvation experiments, such as wheat (Gilbert et al., 1997; Sunarpi and Anderson, 1996), spinach (Prossner et al., 1997; Prossner et al., 2001), oil seed rape (Lencioni et al., 1997), sugar beet (Thomas et al., 2000), rice (Resurreccion et al., 2002) and *A. thaliana* (Hirai et to, 2003; Nikiforova et al. 2003; Nikiforova et al., 2005 a; Nikiforova et al, 2005 b).

In contrast, the changes in the amount of several metabolites revealed that the metabolic phenotypes of phloem exudates obtained from sulfate-deprived plants did not show significant modifications compared to phloem exudates harvested from control plants (figure 15). These results suggest that the phloem has an important function in maintaining the balancing response of the system, such as the function of the transport of nutrients and signalling molecules (i.e. hormones) from source tissues, with the main objective to keep the metabolite provision to sink organs constant as long as possible even against the strong gradient of metabolite depletion in mature leaves. This hypothesis is supported by the observed changes in the metabolite composition and gene expression in leaf tissues from *A. thaliana* using the integrated "omics" analysis with informatic tools, which allowed the elucidation of metabolic pathways involved in the rebalancing response to sulfur deprivation (Hirai et al., 2004; Nikiforova et al., 2005 a; Nikiforova et al. 2005 b). On the other hand, these publications suggest that the rebalancing process to sulfate starvation is aimed at economizing resources for survival, and eventually for seed production (Hirai et al., 2004; Nikiforova et al 2005 a).

Furthermore, the study of changes in the metabolite composition in phloem exudates, sink and source leaves allows us to determine spatial and temporary events that happen in

C. sativus plants exposed to sulfate deficiency. It can be assumed that sulfate deficiency causes a decrease of the amount of sulfate transported from roots to young tissues through the xylem (Leustek, 2000; Buchner et al, 2004; Droux, 2004). As expected, alterations in sulfur containing compounds, such as cysteine and glutathione, occurred first in sink leaves. These changes provoked a propagation of events along the main biosynthetic pathways in sink leaves, either upstream in the accumulation of precursors, such as serine and glycine, or downstream by causing changes in the concentration of products due to limited substrate or cofactor availability, such as SAM, for further metabolic reactions (e.g. chlorophyll biosynthesis). Hence, the accumulating products lead to the activation and expression of genes involved in alternative metabolic branches (e.g. tryptophan, polyaminines and ureides biosynthetic pathways), which allows the production signal molecules involved in the response to sulfate deficiency, like hormones. An example of this is the putative cases of induction of nitrilase III (AT3G44320) genes involved in the production of auxins in sink tissues of *C.sativus* due to the excess of serine and tryptophan produced by effects of sulfate deprivation, and supported by similar observations in *A.thaliana* plants exposed to sulfate deprivation (Nikiforova et al., 2003). Afterwards, these signal molecules generated in sink leaves are delivered to roots and source leaf organs involved in the sulfur deficiency response through the phloem, and then will then trigger the response to sulfur deprivation in phloem, source organs and roots. In the putative case of auxins in *C. sativus* plants, these may be delivered to roots organs through the phloem to allow the development of enhanced roots growth which consequently allows the plant to find new sulfur sources in the soil (Nikiforva et al, 2003; Hirai and Saito, 2004).

Among the possible early responses to sulfate deprivation in the phloem, it was found that the amount of GABA (table I and figure 11) had been increased in parallel to the strong perturbations in the photosynthetic performance observed in sink leaves (figures 6A and 6C). These results suggested that GABA could be involved in the communication

between sink and source organs during the early stages of sulfate deprivation. Furthermore, it is possible that GABA has osmoprotector and/or hydroxyl-radical-scavenging functions in the phloem. In the case that GABA is putative signal molecule transported from sink organs to source leaves through the phloem, this may induce the expression of genes which code for proteins involved in the effective remobilisation of sulfate and sulfur-containing compounds, such as sulfate transporters, from source organs to the phloem, with the main objective to maintain the sulfate homeostasis in the phloem as long as possible. A constant homeostasis in the phloem system maintains the functions of delivery of sulfate, sulfur-containing nutrients, such as glutathione and SMM, sugars and amino acids between the different organs of plants, especially to sink organs. This hypothesis is supported by the identification in *A. thaliana* of a phloem-specific sulfate transporter Sultr 1;3, which it carries out the uptake of sulfate directly to the companion cell of the phloem, and probably it is involved in the translocation of sulfur containing compounds from source tissues to sink organs (Yoshimoto et al., 2003; Buchner et al, 2004).

Finally, this study on *C. sativus* grown under sulfate deficiency conditions allows to think that the transport of sulfate and sulfur containing compounds through the phloem would allow the supply of sulfate for the methionine biosynthesis, and its downstream products, such as SAM, in the whole plant system, and specially to sink organs. Then, methionine and SAM produced in sink organs are employed in different metabolic pathways with the main objective of recovery of different physiological functions essential to the plant, among which the photosynthesis (figure 6 A and 6C) and the nitrogen assimilation balance (Table II) can be included.

5. Conclusion.

Finally, this study on *C. sativus* grown under sulfate deficiency conditions allows to draw the conclusions that the metabolic perturbations indicate respective adaptation mechanisms to sulfate deficiency observed leaf tissues of *C. sativus* plants. These findings are in agreement with previous results in leaf tissues of different plant species, but extend these substantially. The metabolic responses to sulfate deficiency observed in sink and source leaf tissues did not affect the metabolite composition and thus sulfate homeostasis of phloem exudates during early periods of sulfate starvation, and suggest the plant regulates phloem metabolic composition independently of source and sink tissues keeping the metabolic composition constant against strong gradients and perturbations provoked by extended nutrient starvation, in this case continued sulfate deprivation. Besides, the results of no significant changes in the amount of sulfur-containing compounds observed in phloem exudates suggest a careful control of phloem sulfate homeostasis with the main objective to maintain vital functions of the delivery of nutrients and information between sink and source organs constant. Among theses possible functions, phloem allows the delivery of sulfur-containing compounds to sink organs for methionine biosynthesis, which is reflected in a constant methionine homeostasis in the whole plant system during long time periods of sulfate deprivation. Further, GABA is suggested as a potential signalling molecule transported through the

phloem. However, an open question still remains about whether there are alterations in the amount of SAM in the phloem and leaf tissues of *C. sativus* plants in a similar way to that detected in *A.thaliana*.

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7. Appendix

Effects of different light intensities on *Arabidopsis* treated with sulfur deficiency.

Summary

One of the most crucial functions of plant cells is the ability to respond to fluctuations in the environment. Understanding the connections between a plants initial response and the downstream events that constitute a successful adjustment response to the environment is one of the open questions of plant biology. Changes in environmental conditions, such as the quality and quantity of light, temperature or the availability of nutrients and water, modulate the function of the photosynthetic apparatus. The study of sulfate stress in plants has advanced considerably in the recent years. Nevertheless, the majority of the experiments testing the response of plants to changes in environmental conditions of sulfate were focused on a single stress treatment applied to plants under controlled conditions. However, the responses in plants to sulfur nutrition and varied light intensities during growth have not been well characterized. Chlorophyll fluorescence imaging and metabolomic approaches were used to study the metabolic modifications observable in leaf tissues of *A. thaliana* sulfate starved plants treated with low and high light intensities. Our study revealed a new pattern of response to combined sulfate and high light stresses, which shows an initial event of inhibition in the photosynthetic performance associated with alterations at metabolic levels of different metabolic pathways. Furthermore, sulfate deprivation

causes the disruption of metabolic pathways involved in the acclimatation process to high light in plants, such as the antioxidant defence system.

7.1. Introduction

Light is essential for photosynthesis and it is crucial for the survival of plants, though it can also cause oxidative stress. Exposure of a plant to light exceeding what is utilized in photosynthesis leads to inactivation of photosynthetic functions and the production of reactive species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radicals ($^{\cdot}OH$), and singlet oxygen ($^1O_2$ $\cdot$). The ROS produced by exposure to excessive light originates from three sites in the photosynthetic light apparatus, the light-harvesting complex associated with PSII reaction center, the PSII center, and the PS I acceptor site (Niyogi, 1999). The effects of these ROS species can be the oxidation of lipids, proteins, and enzymes necessary for proper functioning of the chloroplast and the cell as a whole (Foyer et al., 1994). Besides excess of light, a range of abiotic environmental conditions such as ozone (O_3), salt, toxic metals, and temperature can induce increased production of ROS by limiting the availability of a plant to utilize light energy through photosynthesis (Conklin and Last, 1995; Richards et al., 1998, Shinozaki and Yamagushi-Shinozaki, 2000).

Antioxidant defence systems are often constitutive multifactorial elements of the plant cell metabolism that are up regulated under the impact of biotic and abiotic stressors. Among the elements of the antioxidant defence system, glutathione, a sulfur containing compound, and ascorbate are well known antioxidants, and they are involved in the cellular defence against oxidative stress produced in the photosynthetic reactions in the chloroplasts (Grene, 2002; Tausz et al., 2004). Furthermore, it is known that the limitation of sulfate in the environment causes a decreasing in the amount of total glutathione in plants, and then the induction of genes involved the antioxidant

defence system in plants (Nikiforova et al., 2003; Yokota and Saito, 2004; Nikiforova et al., 2005 a).

The study of sulfate stress in plants has advanced considerably in the recent years. Nevertheless, the majority of the experiments testing the response of plants to changes in environmental conditions were focused on a single stress treatment applied to plants under controlled conditions (Hirai et al., 2003; Nikiforova et al., 2003; Nikiforova et al, 2005a; Nikiforova 2005b). Within their natural habitat, the plants are subjected to a combination of abiotic conditions that include stresses such as different light intensities and availability of nutrients, such as sulfur limitation. A number of studies examined the effect of a combination of drought and heat stress on the growth and productivity of maize, different grasses and Arabidopsis (Rizhsky et al., 2004). However, the responses in plants to sulfur nutrition and varied light intensities during growth have not been well characterized. Only, it is described that the combined stresses of sulfur deficiency and high light intensities causes an increasing in the total amount of nitrogen, and decreasing in the total amount of Rubisco and Chlorophyll in leaf tissues of rice (*Oryza sativa*) plants, but not in rice plants under sulfate deficiency and low light intensities (Resurrección et al., 2002).

The objective of this study was to investigate at physiological and metabolic the effects of sulfate deficiency in leaf tissues of *Arabidopsis* plants treated with different light intensities. Our study revealed a new pattern of response, which shows an initial event of inhibition in the photosynthetic performance, and is associated with alterations at metabolic level of different metabolic pathways (i.e. maltose associated to starch degradation) during the first two days of sulfur deficiency and high light. Followed by recovery of the photosynthetic capacity after three days of treatment. This was linked to alterations in the amounts of putrescine and sugars transported trough the phloem. In

addition, redox parameters such as glutathione, ascorbate, and dehydroascorbate were altered in plants treated with sulfate starvation and high light.

7.2. Experimental design

Arabidopsis thaliana (Col-0, g-1) were grown in hydroponic cultures using Hoagland's medium (50 μM [Fe EDTA] SO_4, 1.5 mM $Ca(NO_3)_2$, 0.5 mM KH_2PO_4, 0.75 mM $MgSO_4$, 1.25 mM KNO_3, and trace elements) in the greenhose (temperature 20-25°C). All the plants were grown for 3 weeks prior to the start of each experiment at which time the solution was changed. Control plants remained in normal Hoagland's media solution. For starvation, the complete medium was changed to medium in which [Fe-EDTA] NO_3 and chloride salts substituted for sulfate, respectively. The population of control and starved plants were divided in two subsets for further growth in the greenhouse (temperature 20-25°C) and under high light conditions. Experiments with plants under changing PFD (photon flux density) during growth were carried out in a growth chamber fixed to 20°C, 60-70% relative humidity, 16 hours of light at 700-1,000 $\mu mol^{-2} s^{-1}$).

Images of Chlorophyll fluorescence of *Arabidopsis* young leaves of control and sulfur starved plants under the different light regimes were obtained using an IMAGING–PAM chlorophyll fluoremeter (Heinz Walz GmbH, Effeltrich, Germany). At the start of each measurement, a plant was dark adapted for 20 minutes for determination of F_o and F_m. Then the first level of PFD (4 $\mu mol\ m^{-2} s^{-1}$) was applied and a set of values was measured. The same procedure was repeated for each PFD level (10, 50, 100, 225, 365, 585, and 955 $\mu mol\ m^{-2} s^{-1}$). All experiments were performed in triplicates and repeated at least two times.

In parallel to the chlorophyll fluorescence measurements, young leaves of a total of 30 plants were harvested with the aim to compare the biochemical status of leaves

under conditions of sulfur deficiency. Once, the leaf tissues were frozen in liquid nitrogen, all tissues collected were ground in a mortar, divided and used for GC/MS metabolite profiling, chlorophyll and thiol content analyses, previously described in material and methods of the thesis.

To study the effect of different light intensities on the metabolite composition, the metabolome of plants treated with sulfate starvation, PCA analysis of the data was carried out.

7.3. Results and discussion

In plants, ROS are produced by exposure of chloroplasts to excessive light conditions, and they are metabolised by the antioxidant systems of the plant cell. The exposure of green tissues to potentially damaging light intensities involves redox sensing molecular events throughout the plant, originating at the plastoquinone (PQ) pool in the thylakoid membrane. Major defense genes which expression is affected by the redox state of the PQ pool include both cytosolic and chloroplast ascorbate peroxidases (APX) (Karpinska *et al.,* 2000). Furthermore, glutathione, a sulfate-containing compound, is one of the most important molecules involved in the redox control in the cell of the plants (Tausz et al., 2004). Thus, the response to high light conditions is dependent of the concentration of components, such as glutathione and ascorbate, and the expression of genes involved in the antioxidant defence systems of the plant (Surpin et al., 2002).

In this study we performed an analysis of the photosynthetic performance using chlorophyll fluorescence imaging in plants treated with sulfate deficiency and different light intensities (figures 1, 2, 3 and 4). In parallel, we carried out an analysis of changes of the metabolic composition of *Arabidopsis* leaf tissues treated with a combination of sulfur deficiency and high light, using GC/MS profiling, chlorophyll, and thiol measurements (figures 5, 6 and 7). No significant changes in the photosynthetic capacity and metabolic composition were found in sulfate-deprived plants treated with low light intensities during the experiment. However, chlorophyll fluorescence imaging and GC/MS metabolite profiling revealed a new pattern of metabolic response showing an initial event of inhibition in the photosynthetic performance associated with

alterations at the metabolic level of different pathways during the first two days of sulfate deficiency and high light (figures 1, 3 and 7).

Among the first metabolic changes, an increase in the amount of maltose was observed during the first day of sulfate deprivation and high light, which suggests a possible inhibition in the photosynthetic capacity (Figure 8D). It is known that transitory starch and their degradation products (e.g. maltose), act as energy reserves, providing the plant with carbohydrate during the night when the sugars cannot be generated by photosynthesis (Smith et al., 2005; Weise et al., 2005). Furthermore, these results suggest the expression of genes required for starch degradation, such as β-amylase, and sucrose biosynthesis in *Arabidopsis* plants exposed to sulfate deficiency and high light. Similar results were observed in *Arabidopsis* plants.subjected to a combination of drought stress and heat stress (Rizhsky et al, 2005). Besides, as a second step of the response a recovery in the photosynthetic capacity after three days of treatment was observed. This was associated to an increase in the amount of putrescine (Figures 8E and 8F), several sugars, such as raffinose (figure 6 and 8C), and inhibition of the chlorophyll production (figure 5A). Putrescine might accumulate due to the limited amounts of SAM, both being necessary precursors of polyamine biosynthesis as has been shown for long time periods of sulfate deprivation in *Arabidopsis* leaf tissues (Nikiforova et al., 2005). Subsequently ,the excess of putrescine generated during sulfate deficiency in plants under high light conditions might be used for the stabilization of thylacoid membranes of chloroplasts with the objective to restablish and maintain constant the photosynthesis (figure 1 and figure 8F), similarly to the observations of increase in the amount of polyamines in leaf tissues of *C. sativus* plants exposed to sulfate deprivation (Discussed in section 4.3 of the Ph. D. manuscript). Moreover, SAM is one of the substrates employed by magnesium protoporphyrin methyl transferase in

the chlorophyll biosynthesis pathway (Buchanan, 2000). These results allow to conclude that high light conditions stimulated the response to sulfate deficiency in leaf tissues of *Arabidopsis* plants, in which a possible key metabolite could be SAM, extending previous observations found in leaf tissues of *A. thaliana* (Nikiforova et al., 2005) and *C. sativus* (Discussed in Ph. D. manuscript) in response to the deficiency of sulfate fertilization.

To study the effects of sulfate deficiency and different light intensities on the metabolite composition, the metabolome, in Arabidopsis leaf tissues, principal component analysis (PCA) of the data was carried out. (figure 9) (Fiehn et al., 2000; Desbrosses et al. 2005). A total of 92 known metabolites determined by measurements of thiols, photosynthetic pigments and GC/MS analysis were used to generate the matrix employed for principal component analysis. This matrix describes a total of 60 samples from young leaves harvested from control and sulfate starved plants grown under different light intensities. The first 6 principal components derived from the above data matrix encompassed 86.70% of the variance from this data set (figure 9 A). 71.24% of the variation in the original data is explained with the first two PCs alone. The first component accounted for 55.39% of the variance and allowed distinction between plants grown in the greenhouse and plants transferred to high light conditions. Also, the first component demonstrated that leaf tissues of plants after two days of treatment with sulfur deficiency and transferred to high light conditions cluster together in an independent group (figure 9 B). The second component encompassing 15.85% of the variance, demonstrated that the data set contained metabolite measurements that distinguished between leaf tissues of control plants and leaf tissues of sulfate deprived plants transferred to high light conditions starting from the third day of treatment (figure 8 B). Furthermore, the scatter plot of the first component against the second component

shows that the metabolic phenotype of plants transferred to high light conditions displayed more variation than plants grown in low light conditions. These results suggest that the data set contained metabolite measurements that distinguished between leaf tissues of sulfur deprived plants grown in the greenhouse and transferred to high light conditions.

Among the metabolite measurements that distinguished between plants grown in conditions of sulfur deficiency and different light intensities, glutathione and ascorbate were found in the 20 most influential metabolites in the componenet 1 and 3 (Table I). In addition, metabolites involved in the antioxidant stress response (e.g. glutathione, ascorbate, and dehydroascorbate) and their degradation products , such as threonic acid, were strongly altered in sulfur starved plants under high light conditions compared to sulfate deficient plants grown under low light intensities, especially after two days of sulfate deficiency and high light treatments (figure 10). These results suggest that ascorbate and glutathione are involved in regulating the acclimatation responses of plants treated with limited sulfate nutrient supply and high light intensities.

As expected, the amount of glutathione decreases after two days of combined stress of sulfate deficiency and high light conditions (figure 10A). Interestingly, an increase in the amount of ascorbate was observed after three days of sulfate starvation and high light conditions (figure 10B). Additionally, the amount of threonic acid remained constant from the second day of sulfate deficiency in plants treated with sulfate deficiency and high light intensities (figure 10 D),. This result, associated with the changes observed in the amount of glutathione and ascorbate, suggests the expresion of genes involved in the up-regulation and resource-saving of ascorbate, which then allows the provision of low molecular antioxidants employed to maintain several functions, such as the photosynthesis and direct quenching of ROS species, in

plants exposed to combined low sulfate supply and high light conditions. These results are supported by similar observations of changes in the amount of glutathione and ascorbate in *Arabidopsis* plants grown under low light intensities and treated with sulfate deficiency (Kandlbinder et al., 2004).

Ascorbic acid and glutathione have each been shown to act as antioxidants in the detoxification of ROS. These compounds have central and interrelated roles, acting both non-enzymatically and as substrates in enzyme-catalyzed detoxification reactions located within the aqueous phase of the peroxysome, chloroplast, cytosol and the mitochondrion (See review Grene, 2002). Anti-ROS mechanisms were one of the most important points in the response to sulfur deficiency and high light conditions in *Arabidopsis* leaf tissues. Therefore, the response to high light and sulfate deficiency conditions is dependent on the concentration of those components, such as glutathione and ascorbate, and might be by the regulation of genes involved in the antioxidant defence systems, specially the expresion of genes involved in the biosynthesis and degradation of ascorbate metabolism. This possible metabolic shift allows the provision of ascorbate as long as possible in leaf tissues of plants exposed to combined stresses of sulfate deficiency and high light intensities.

7.4. Conclusion

The responses of plants to reduction of sulfur nutrition and varied light intensities during growth have not been well characterized. Analysis of plants under combined sulfate and high light stresses provides one of the first studies, in which the response to sulfate deficiency under different light intensities is characterized by physiological and high-throughput methods. High light intensities have a strong effect on the activation of the adaptation mechanisms to sulfate deficiency in leaf organs of *Arabidopsis* plants. The metabolic perturbations in the photosynthetic capacity, starch degradation, sugars transported thorugh the phloem, such as raffinose, and polyamine biosynthesis, indicate respective adaptation mechanisms to sulfate deficiency and high light intensities observed in leaf tissues of *A. thaliana* plants. These findings are in agreement with previous results in leaf tissues of rice plants, but extend these substantially about the effects of the light intensity at metabolic level. Furthermore, sulfate deprivation causes the disruption of metabolic pathways involved in the acclimatation process to high light in plants, such as the case of the antioxidant defence system.

7.5. Figures and tables of results

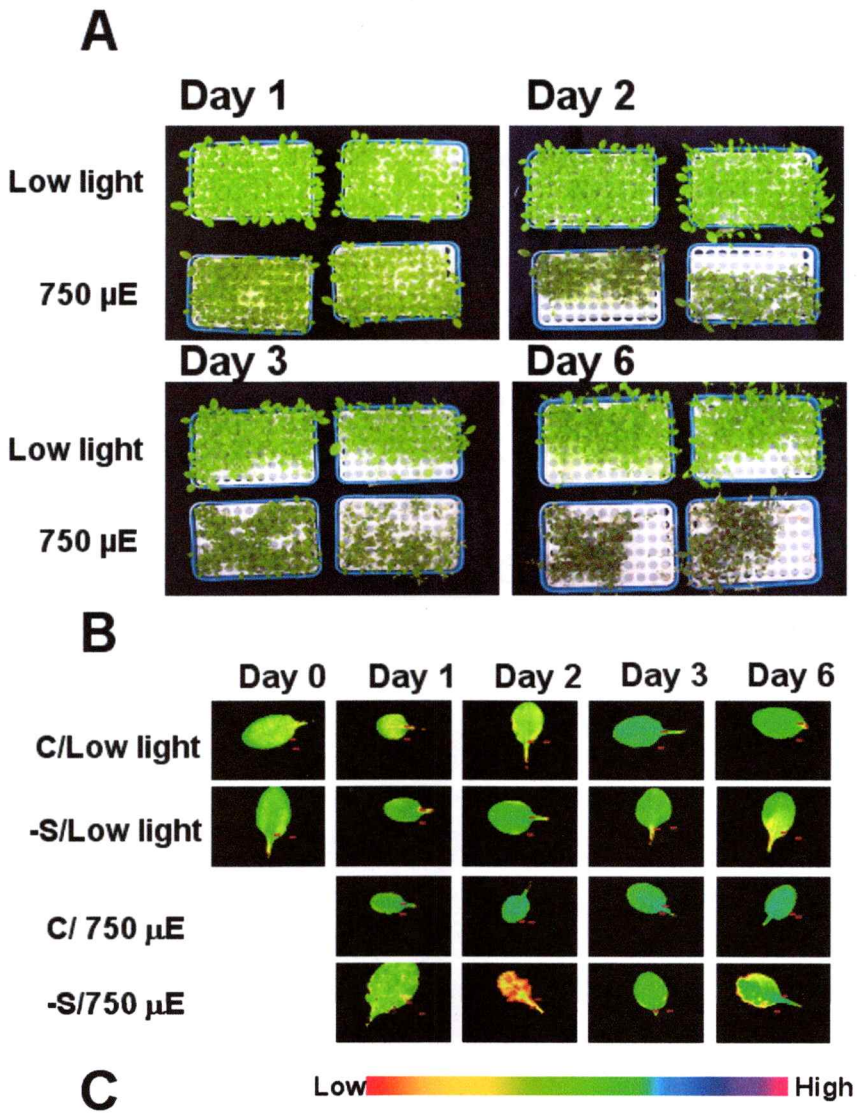

Figure 1. Detection of the effects of sulfur deficiency in different light intensities on the photosynthetic performance using chlorophyll fluorescence imaging. Visual phenotypes observed in control and sulfur starved *A. thaliana* plants growth in the green house (Low light) and trasnfered to 750 µE after 1, 2, 3, and 6 days of treatment (figure 1A). Figure B, images of the yield (Y) of fluorescence detected 365 PFD on *A. thaliana* leaves of control and sulfur starved plants in different light regimes during the experiment. Yield of fluorescence have been mapped to the color palette shown below C.

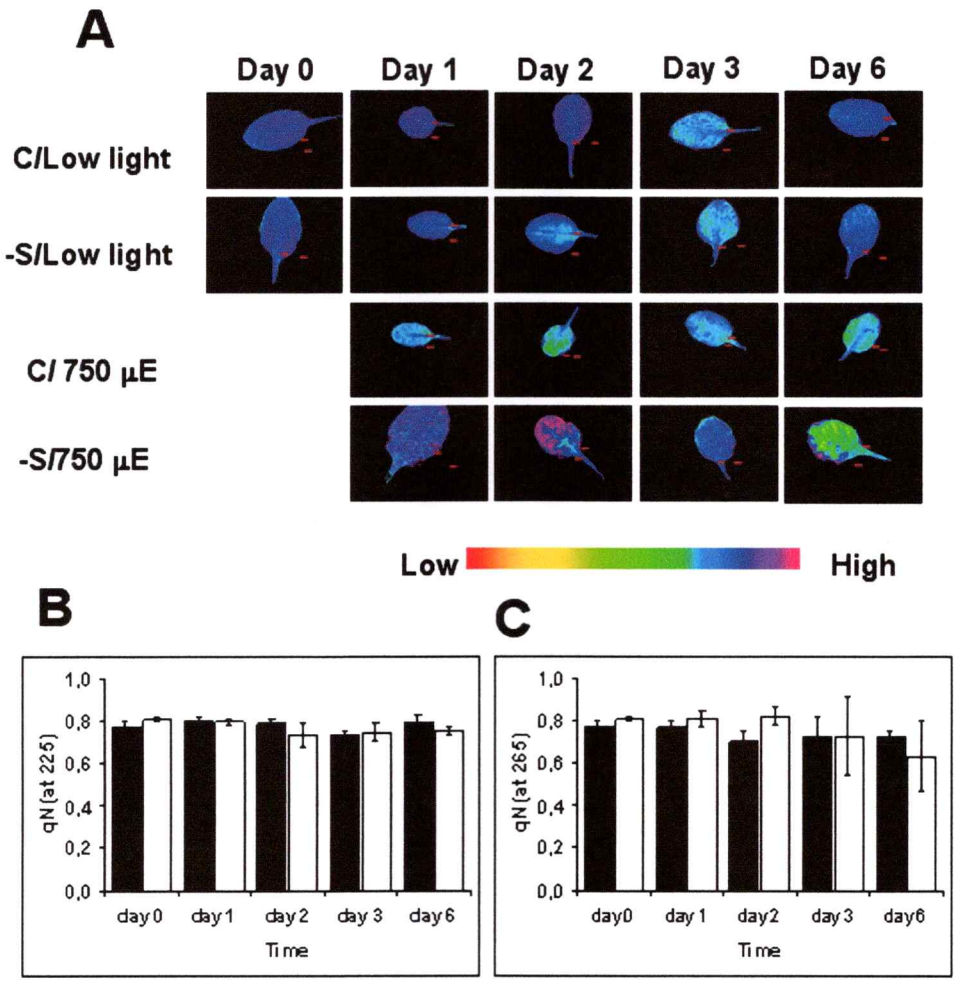

Figure 2. Detection of the effects of sulfur deficiency and different light intensities on photosynthetic performance using chlorophyll fluorescence imaging. Figure A, images of the non-photochemical quenching (qN) detected 365 PFD on *A. thaliana* leaves of control and sulfur starved plants in different light regimes during the experiment. qN of fluorescence have been mapped to the color palette shown below figure A. Changes in the fluorescence parameter qN (figures 3A and 3C) obtained of control (black bars) and sulfur-starved (white bars) *A thaliana* plants grown in the greenhouse (figure B) and/or transferred to 750 µE (figure C) over the time course of the experiment. qN was obtained during stepwise increase of 225 PFD from the same set of plants as results shown in the figure 1. Each data point represents the average of 3-5 individual plants ± SE.

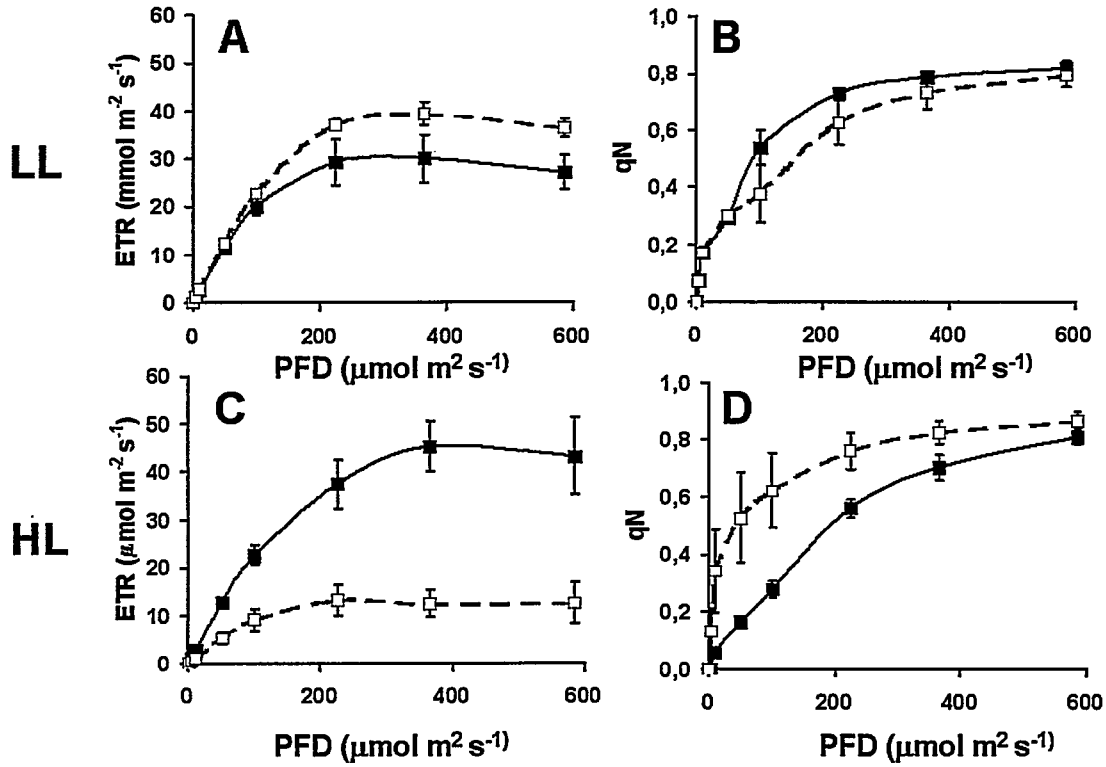

Figure 3. Chlorophyll fluorescence parameters of *A. thaliana* under sulfur starvation and different light conditions after 2 days. Relative electron transport rates in the electron transport chain (ETR) (figures 2A and C) and non-photochemical quenching (qN) coefficients (figure B and D) were obtained during stepwise increase of PFD (See material and methods). Plants were grown at constant light in the greenhouse (figures A and B) or transferred at 750 µE (figures C and D). Data were obtained from the same set of plants as results shown in the figure 1. Each data point represents the average of 3-5 individual plants ± SE.

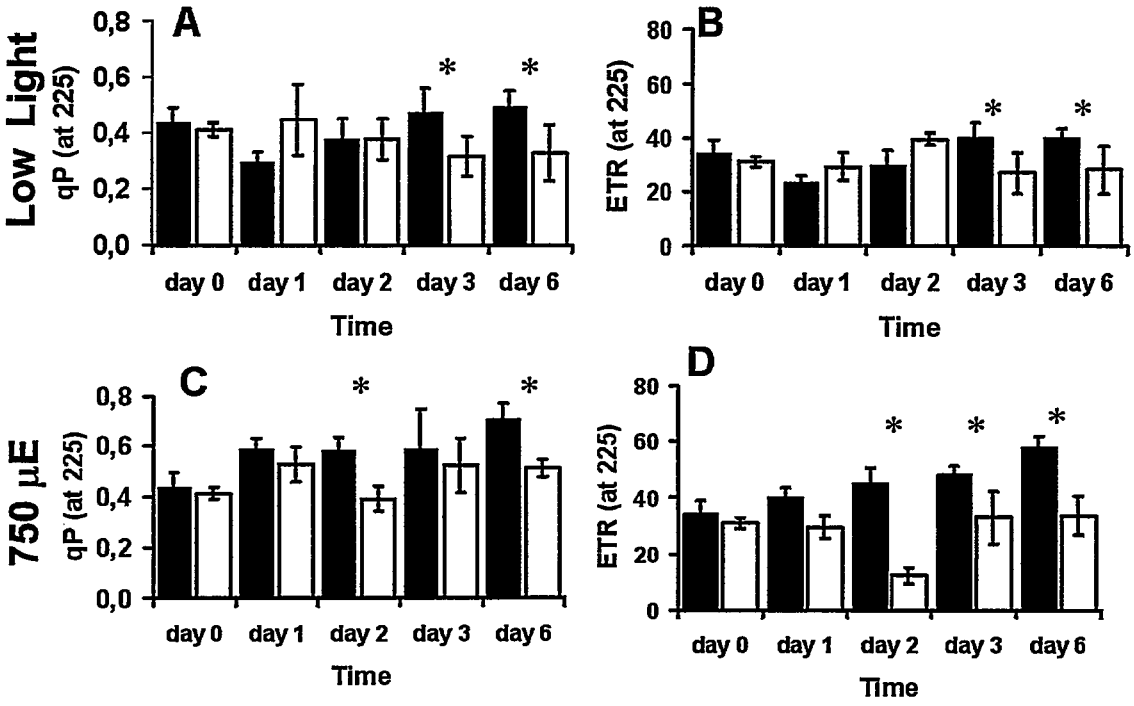

Figure 4. The effects of sulfate deficiency and different light intensities on chlorophyll fluorescence parameters measured. Changes in the fluorescence parameters qP (figures 3A and 3C) and ETR (figures 3B and 3D) obtained of control (black bars) and sulfur-starved (white bars) *A thaliana* plants grown in the greenhouse (figure 3A and 3B) and/or transferred to 750 µE (figure 3C and 3D) over the time course of the experiment. qP and ETR were obtained during stepwise increase of 225 PFD from the same set of plants as results shown in the figure 1. Each data point represents the average of 3-5 individual plants ± SE.

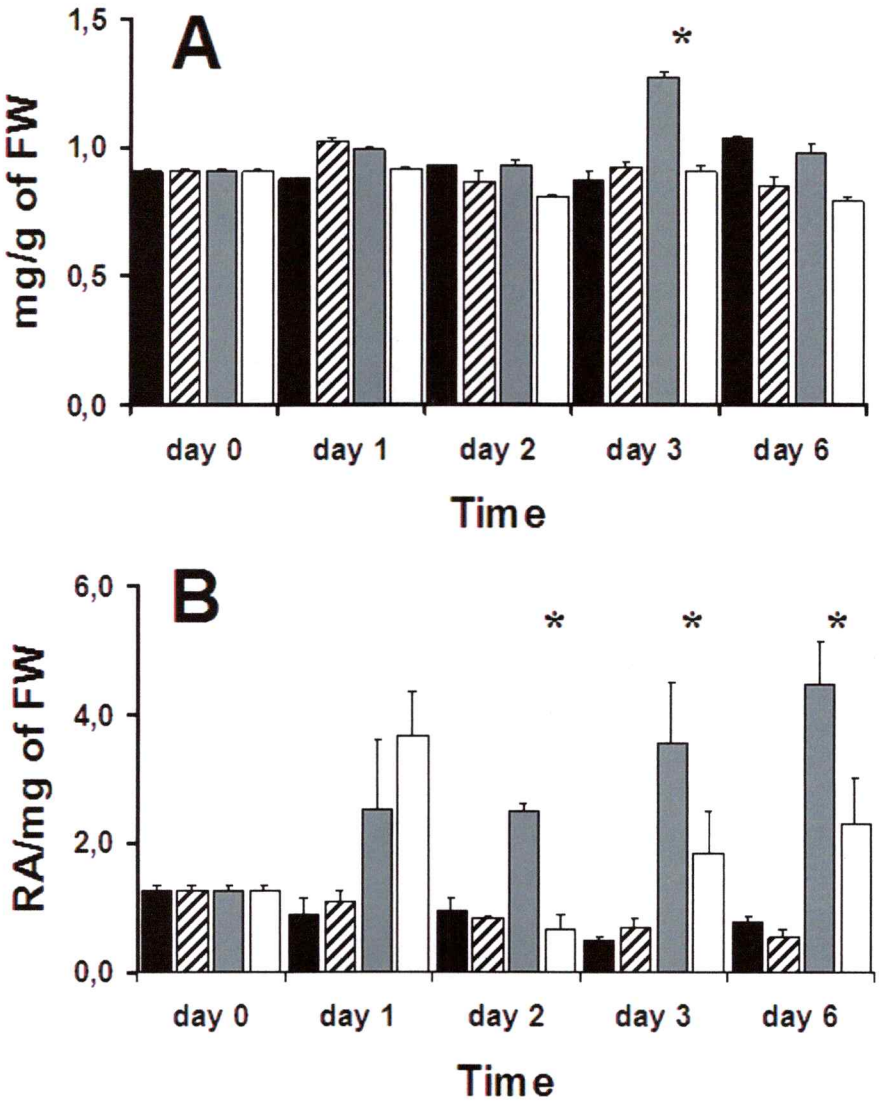

Figure 5. Quantitative analysis of the amount of photosynthetic pigments and cysteine in leaf tissues of control and sulfate deprived plants in different light intensities. Levels of the total amounts of chlorophyll (figure 4A) and cysteine (figure 4B) in control plants (black bars) and plants subjected to sulfur deficiency (dashed bars), under high light (Gray bars), or a combination of sulfate deficiency and high light (white bars). Values ± SE characterise the average of at least three independent samples. (RA= relative amount of cysteine). Student t-test was statistically significant ($P < 0.05$) in time points marked with asterisk (*).

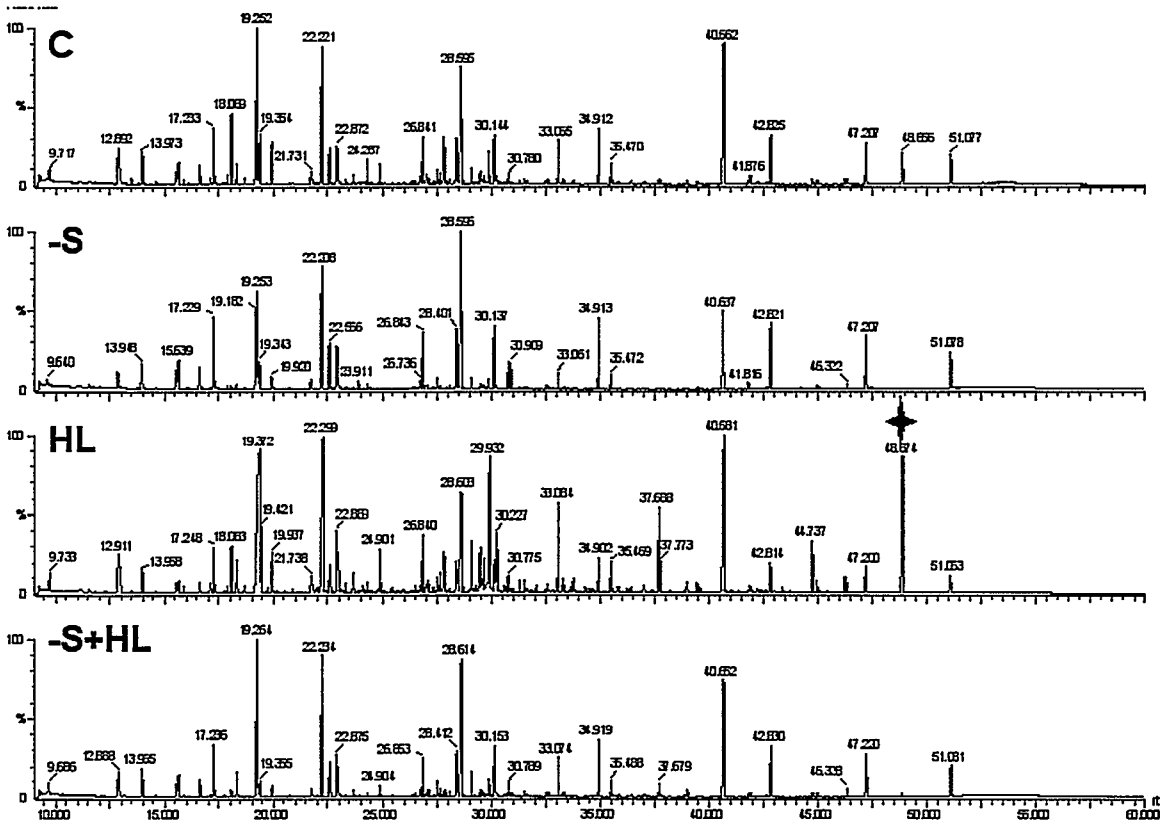

Figure 6. Metabolic profiling by GC/MS analysis of leaf tissues of control and sulfate deprived *Arabidopsis* plants treated with different light intensities. GC/MS profiles of polar extracts obtained from control plants and plants subjected to sulfur deficiency (-S), high light (HL), or a combination of sulfur deficiency and high light (S+HL). All the samples were obtained after 2 days of sulfur deficiency and different light intensities. Polar compound extractions and GC/MS separations are described in material and methods. Raffinosse is marked by symbol.

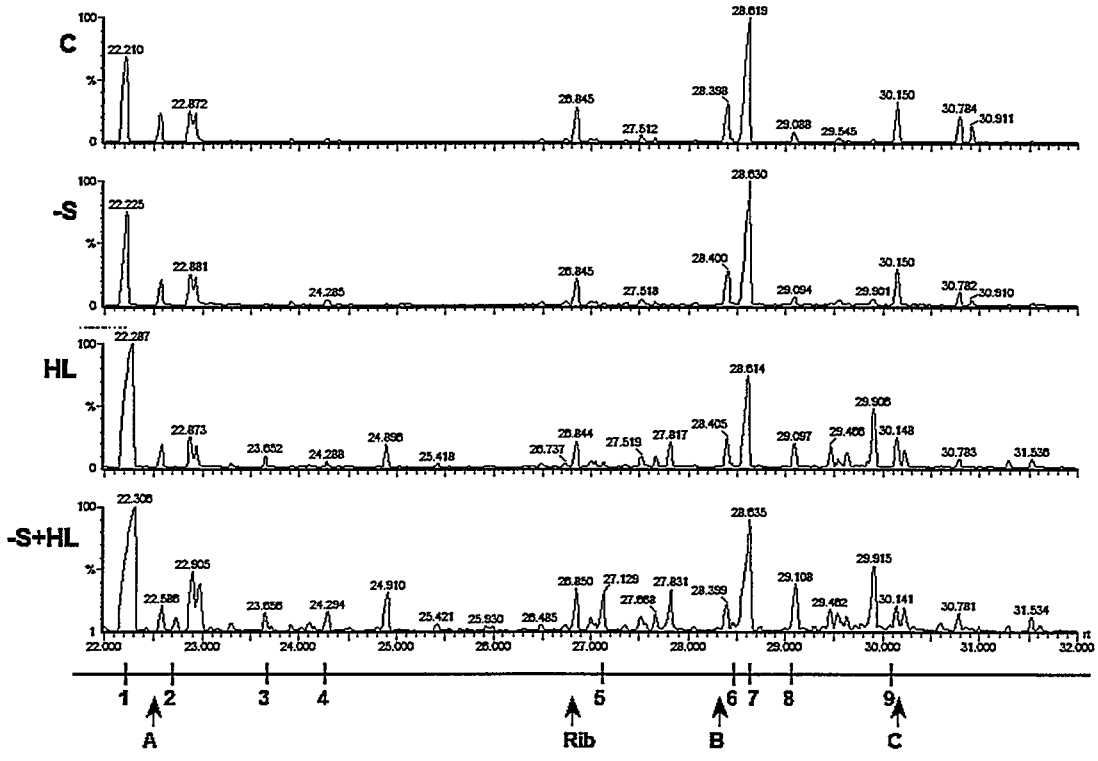

Figure 7. Demonstration of the complexity of the GC/MS metabolite profilings of samples of control and sulfate deprived plants after 3 days of treatment with different light intesities. Representative expansion of the GC/MS chromatograms of the region 22.0-32.0 min of control plants and plants subjected to sulfur deficiency (-S), high light (HL), or a combination of sulfur deficiency and high light (S+HL). Peak identification: **A**, pentadecane; **B**, octadecane; **C**, nonadecane; **Rib**, ribitol; **1**, malic acid; **2**, putrescine 3TMS; **3**, threonic acid; **4**, glutamine 4 TMS; **5**, Putrescine 4 TMS; **6**, Shikimic acid; **7**, citric acid; **8**, dehydroascorbic acid; **9**, Tyrosine. Unknown compounds deteted in the chromatograms are not showed. **A**, **B**, and **C** are internal standards used for retention index (RI) calculation.

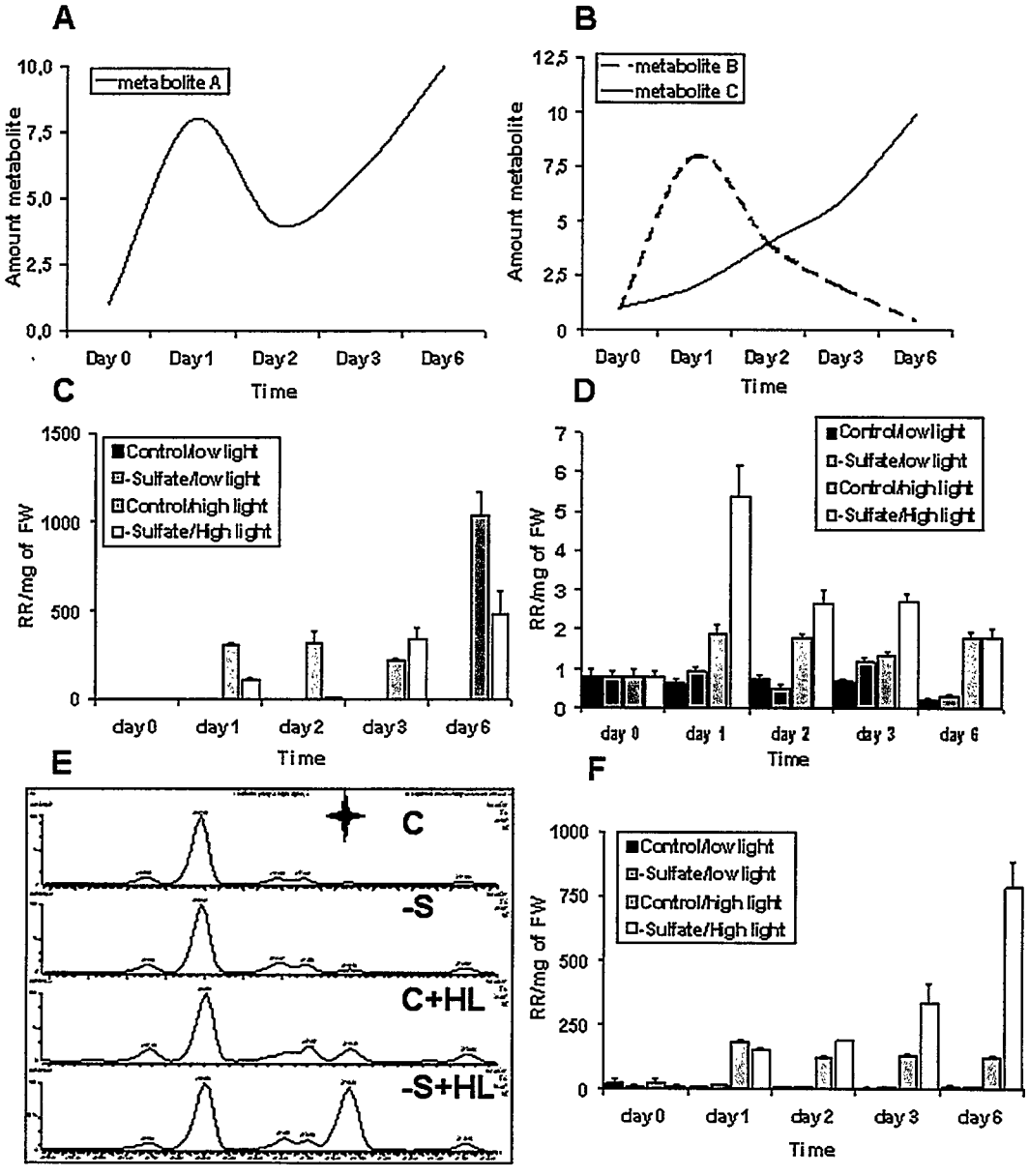

Figure 8. Metabolic patterns expected over the time course of the experiment. Figures A and B show metabolic patterns expected in Arabidopsis plants treated with sulfate deficiency and high light intensities. Levels of raffinose (figure C), maltose (figure D), and putrescine (figures E and F) obtained by GC/MS metabolite profilings of control plants (black bars) or plants subjected to sulfur deficiency (dark gray bars), high light (light gray bars) or combined sulfur deficiency and high light (white bars). Figure E, demonstration of different modifications in the amount of purtrescine (marked with start) after three days of different treatments by a representative expansion of the chromatograms for the region 26.5-27.5 minutes. Values ± SE characterise the average of at least three independent samples. (RR= relative response of metabolite).

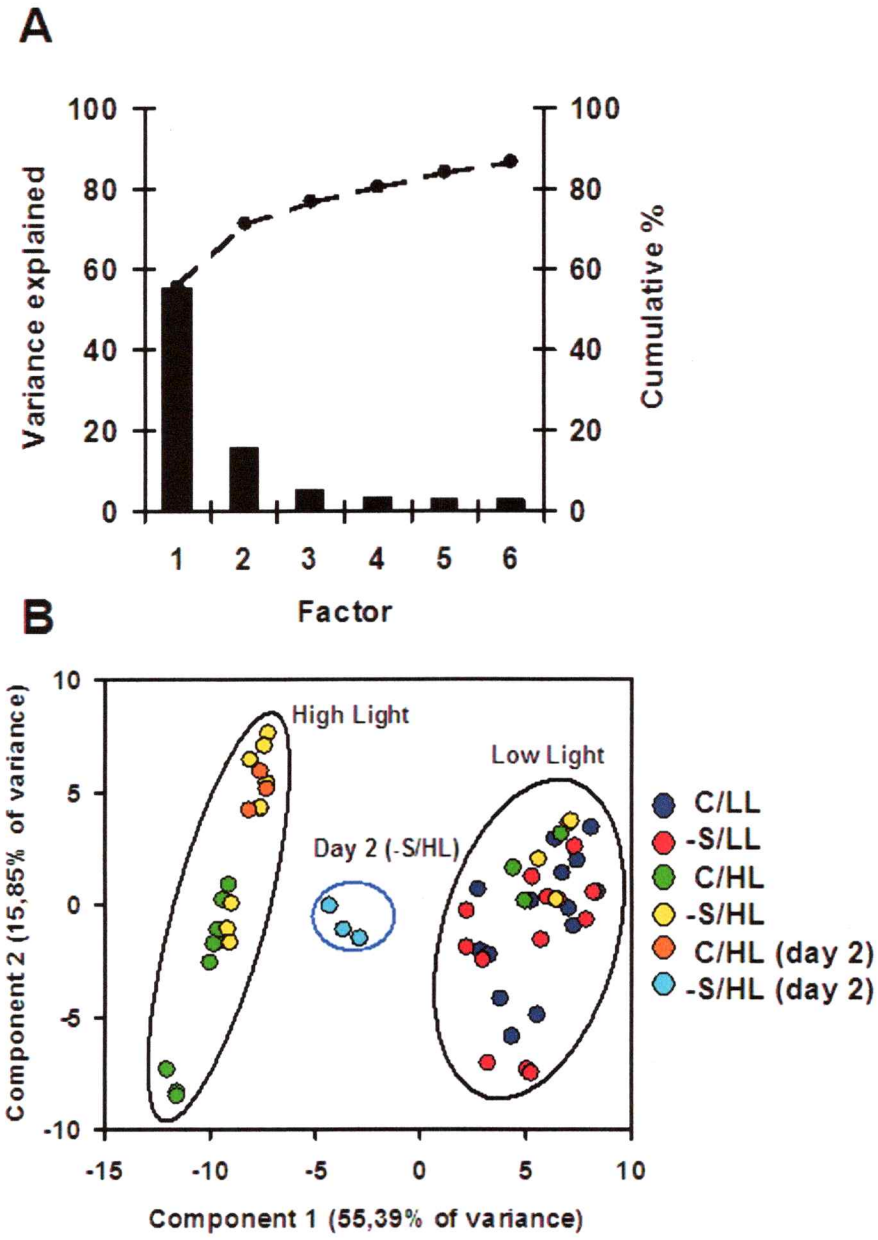

Figure 9. Metabolic phenotype clustering of control and sulfur starved plants in different light intensities. Figure A shows the variance of the data (left Y axis and black bars), and percentage of cumulative variance (right Y axis and line) explained by the first seven principal components. Figure B, clusters (circles) found after principal componet analysis (PCA) of total metabolite data of samples harvested during the experiment of sulfur deficiency and different light intensities. Each point represents a linear combination of 92 known metabolites from an individual sample. Vectors 1 and 2 were chosen for best visualization of phenotype separation and include 69% of the total information content derived from metabolite variance.

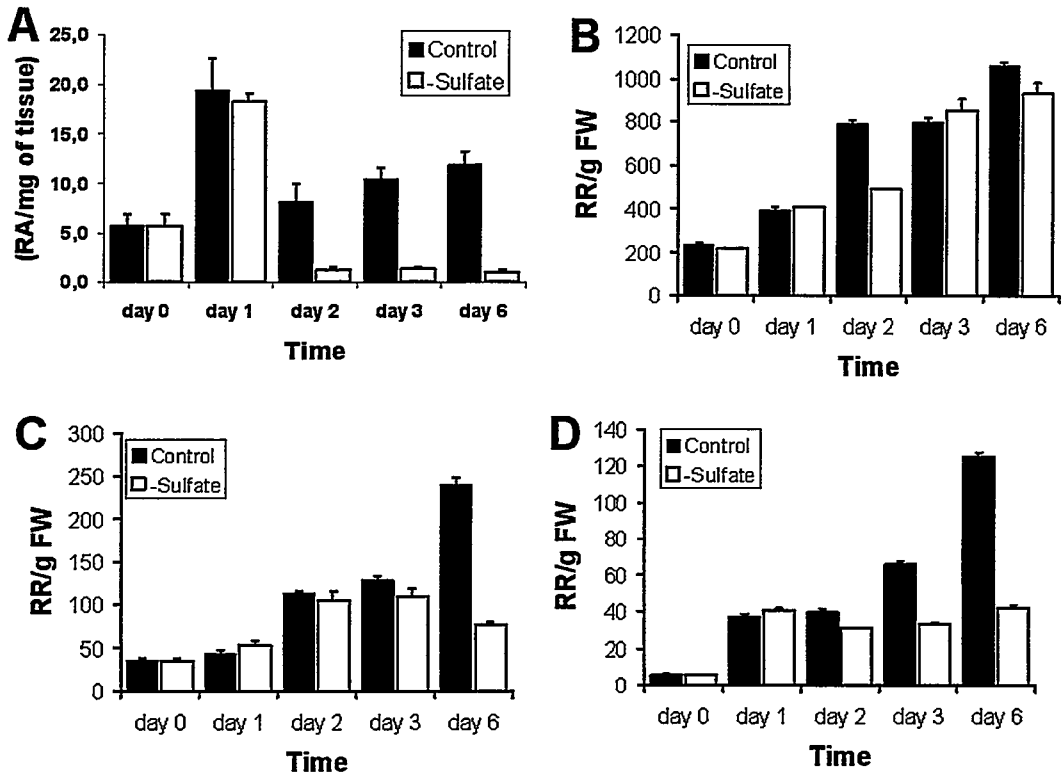

Figure 10. Levels of low molecular weight antioxidants and their metabolic products in *A. thalina* leaves under sulfur deficiency and high light. Levels of total glutathione (A), ascorbate (B), dehydroascorbate (C), and threonic acid (D) in control (black bars) and sulfur starved (white bars) *A. thaliana* plants transferred to 750 µE. Values ± SE characterise the average of at least three independent measurements. (RA= relative amount of total amount of glutathione; RR= relative response).

Table I. The 20 most influential metabolites, which contribute to the component 1, 2, and 3 derived from the PCA analysis. Values in bold letters are metabolites involved in the response to sulfur deprivation (Nikiforova et al., 2005 a) and results thesis. (1)= thiols compounds detected by HPLC. PC= principal component, MX= methoxyaminated derivate

Metabolite	Factor 1	Metabolite	Factor 2	Metabolite	Factor 3
Gluthathione (1)	**0,41730**	**Glutamine 4**	0,38284	**L-ascorbic**	**0,250403**
Quinic	**0,69663**	**arginine 5**	**0,30541**	GABA	0,486449
Benzoic	0,24937	**ornithine** 4	**0,35000**	**tryptophan 3**	**0,264064**
aspartic 3	**0,04825**	homoserine 3	0,32921	**o-acet-serine**	**0,434444**
methionine 2	**0,20921**	**o-acet-serine**	**0,47264**	glyceric-3-P	**0,261098**
Lactic	0,36609	**uric4**	**0,50560**	malic	0,300760
Citric	**0,21476**	**allantoin 5**	**0,30563**	**allantoin 5**	**0,346805**
phosphoric 3	0,41646	**maltose MX1**	**0,30529**	**allantoin 4**	**0,236444**
Aconitic	0,21444	glycerol-1-P	**0,36184**	erythritol	0,306194
Palmitic	**0,11466**	sorbitol-6-P	**0,41086**	gluconic	0,259989
galactose MX1	**-0,93640**	aspartic 3	**-0,83644**	**Gluthathione (1)**	-0,494260
valine2	**-0,93606**	lactic	-0,74912	man-6-P MX	-0,376808
glutamine 3	**-0,94650**	citric	-0,92668	**Chl_a**	-0,378272
glucose MX2	**-0,93442**	thymine	**-0,78363**	t-caffeic	-0,387111
α-kt-glutaric MX	**-0,96143**	uracil2	**-0,87785**	glycine	-0,424721
Raffinose	**-0,95473**	phosphoric 3	-0,81298	**glutamine 4**	-0,359772
β-alanine 3	**-0,97728**	glycerol	-0,89900	**homoserine 3**	-0,541078
Arabinose	-0,98124	glutaric	-0,72540	**methionine 2**	-0,539471
Trehalose	**-0,94984**	palmitic	**-0,91473**	**threonine**	-0,403336
Threonic	**-0,97950**	stearic	**-0,90297**	Myo-inositol-2-P1	-0,639195

7.6. Bibiography

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