PLANT ECOPHYSIOLOGY

# Sulfur in Plants

## An Ecological Perspective

Edited by
M. J. Hawkesford
and
L. J. De Kok

Springer

# SULFUR IN PLANTS

# Plant Ecophysiology

Volume 6

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**Aims & Scope:**

The Springer Series in *Plant Ecophysiology* comprises a series of volumes that deals with the impact of biotic and abiotic factors on plant functioning and physiological adaptation to the environment. The aim of the *Plant Ecophysiology* series is to review and integrate the present knowledge on the impact of the environment on plant functioning and adaptation at various levels: from the molecular, biochemical and physiological to a whole plant level. This series is of interest to scientists who like to be informed of new developments and insights in plant ecophysiology, and can be used as advanced textbooks for biology students.

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# Sulfur in Plants
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Cover photographs: The two cover photographs show an ornamental Brassica species and the slopes of Mount Etna, Sicily. Brassica species in general have a high sulfur requirement and contain high levels of organic sulfur compounds. On the slopes of Mount Etna, in addition to light and water stress, plant communities suffer occasional high levels of atmospheric sulfur from volcanic activity. Photographs by M. J. Hawkesford.

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# PREFACE

*Sulfur in Plants – an Ecological Perspective* is the 6th volume in the *Plant Ecophysiology* series. The aim is to assess the current state of knowledge in the plant sulfur field in the context of plant ecology and physiology. The volume complements previous volumes, and particularly the volume on *Nitrogen Acquisition and Assimilation in Higher Plants* by Sara Amâncio and Ineke Stulen. In recent years, substantial advances have been made in our understanding of the physiology and biochemistry of sulfur acquisition and assimilation and subsequent fate of sulfur pools *in planta*. Molecular approaches and modern genomics technologies have allowed the elucidation of the component parts of the respective pathways and systems biology is reconstructing many of the networks that are involved not only in sulfur biochemistry but also associated aspects of plant metabolism. Regular updates in this fast moving field are published as part of the International Workshop on Plant Sulfur Metabolism series. New areas have developed as the underlying importance of sulfur biochemistry to specific plant processes has become appreciated, for example in resistance to pathogens and abiotic stresses such as toxic metals, as well as in interactions with selenium, an essential component of animal health.

The majority of practical studies, which have been undertaken, have focused on agricultural species, whilst molecular studies have principally used the model species *Arabidopsis thaliana*. Outside of these very specific systems, research on plant sulfur has been quite fragmentary. Little or no systematic analysis of wild plant species in the context of their sulfur biology and ecology has been undertaken. Chapters included in this volume assess sulfur biology in a range of ecosystems including terrestrial and aquatic environments as well as summarizing the present status of sulfur in crops in the agronomic context, and includes speculation on generalised responses.

This volume highlights the central role and importance of sulfur in a wide range of responses to abiotic and biotic stresses. These innate responses are central to ecological adaptation and are important targets for breeding for practical application in agronomy and environmental protection. We hope that the approach taken here will stimulate research in a wider ecological context and facilitate the mining of new aspects of plant sulfur biology in unexplored species and ecosystems.

Malcolm J. Hawkesford
Luit J. De Kok

Chapter 1

# SULFUR AND PLANT ECOLOGY: A CENTRAL ROLE OF SULFATE TRANSPORTERS IN RESPONSES TO SULFUR AVAILABILITY

Malcolm J. Hawkesford

## INTRODUCTION

Sulfur is an essential element for plant growth, and plant requirements for sulfur are closely linked to nitrogen availability and growth rate. Adaptive mechanisms exist to optimize supply of and demand for sulfur within the plant, ranging from regulation of uptake and assimilation to modification of growth form. Availability of sulfur has a major impact on crop yield and quality influencing secondary sulfur compound content and storage protein accumulation and composition. Intensive crop production requires sulfur fertilizer inputs, especially as anthropogenic inputs have decreased in many industrial regions in recent years. Less intensive agriculture and native flora may be expected to be adequately supplied with sulfur by aerial deposition and from mineralization. However the occurrence of multiple adaptive mechanisms indicates that imbalances between supply and demand occur and potentially limit plant growth, and therefore provision of adequate sulfur is a major factor to be dealt with. Many of these adaptive mechanisms involve membrane transporters, and are the focus of this chapter. The proposition of a key role of transporters in regulating plant metabolism and function is not unique (Kunze *et al.* 2002), however here the focus is on sulfur metabolism and specifically the roles of the sulfate transporters.

A major preoccupation of sulfur research has been on the impacts of sulfur limitation. This has been both because of the need to address the sulfur requirements for crops in terms of yield and quality, but also because of the experimental usefulness of this situation. Switching from



*M.J. Hawkesford and L.J. De Kok (eds)*
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conditions of adequate to inadequate supply or comparing these treatments has highlighted physiological, biochemical, and molecular responses of individual plants and the impacts on crop yield and performance. For the most part, these represent responses to transient changes or extreme differences in availability. In many natural environments such dramatic fluctuations are less common, however fertilizer inputs or heavy rainfall will cause substantial rapid variations in availability. Crop production is often driven by nitrogen, but inadequate sulfur will influence nitrogen utilization, yield, and more subtly, quality. In agronomic situations the most common solution is to apply sulfur fertilizer, although manipulating sulfur harvest index may be an alternative and more sustainable solution:

*Sulfur harvest index (SHI) is the total sulfur in the harvested part of the plant as a fraction of the total sulfur taken up by the plant.*

Crops are mostly monocultures optimized for output by artificial and often intensive inputs. Many nutrients are removed by harvesting and the unnatural form of inputs (high doses at discrete points in time) provide a challenge for optimum plant acquisition/utilization, particularly to avoid unnatural losses, for example in runoff. In contrast, in natural communities many factors will influence productivity and species diversity including competition for individual nutrients. Species composition will be influenced by nutrient supply, particularly with regard to tolerance of extreme conditions or ability to exploit specific pools such as nutrients at depth with long roots. Seasonally, the succession of species in a given habitat will result in a fluctuating supply of nutrients, although not as dramatically variable as with fertilizer application. Critically within most natural ecosystems nutrients are recycled. Supply of sulfur must be placed in context with other overriding environmental considerations such as temperature and water availability.

Global sulfur fluxes involve terrestrial and aquatic environments and the atmosphere (Figure 1). The biota has a central role in mediating many of the physical and chemical transformations. Sulfate is the form utilized by plants and is thus central to many of the environmental fluxes apart from those involving bacteria. The availability of sulfate itself is subject to many influences and plant sulfate transporters are the key to regulating flux between the environment and the plant biota. The global sulfur cycle has been described in more detail elsewhere (Stevenson and Cole 1999).

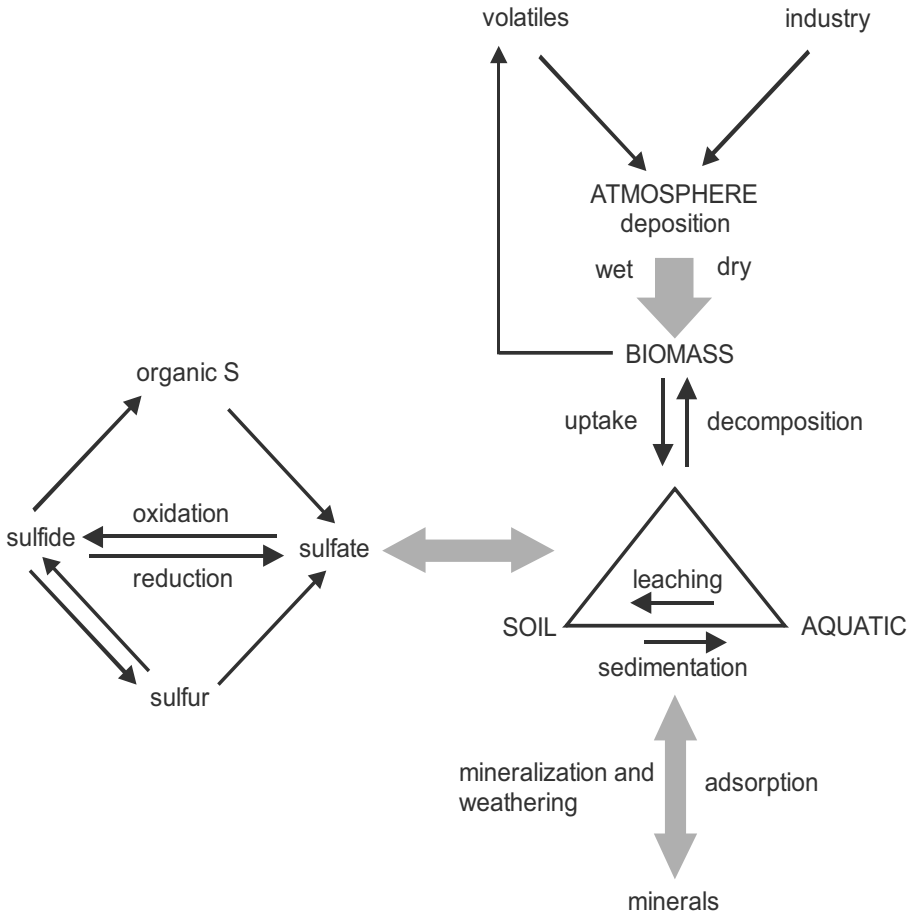


*Figure 1.* The chemical sulfur cycle and fluxes of sulfur within the environment.

## STRATEGIES TO DEAL WITH FLUCTUATIONS OF SULFUR AVAILABILITY

The largest fluctuations (10 to >500 μM) in availability in natural habitats are most likely to occur in freshwater lakes, particularly as a consequence of anthropogenic activities (Giordano *et al.* 2005; Holmer and Storkholm 2001). As a consequence freshwater algae possess adaptive strategies such as inducible transport systems (*Lemna minor* [Neuenschwander *et al.* 1991; Thoiron *et al.* 1981], *Lemna paucicostata* [Datko and Mudd 1984a,b], *Lemna gibba* [Lass and Ullrich-Eberius 1984], *Hydrodictyon reticulatum* [Rybova *et al.* 1988], *Chlorella pyrenoidosa* [Vallee and

Jeanjean 1968a,b], *Chlorella vulgaris* [Passera and Ferrari 1975], *Chlamydomonas reinhardtii* [Yildiz *et al.* 1994]). In contrast, marine environments have a very stable and ample supply of sulfur, with seawater containing around 28 mM sulfate. Organisms in this environment will have no selective pressure for sulfate scavenging but may still need to regulate and balance uptake with demand.

Sulfur availability is very variable in the terrestrial environment, ranging from very low in sandy soils to extremely abundant in gypsophilous soils or soils originating from tidal areas, where sulfides may be quite abundant (Stevenson and Cole 1999). Physiological responses of plants to environments with exceptionally high sulfur content may be divided into avoidance or tolerance (Ernst 1997). Tolerance mechanisms are generally extreme adaptations of universal homeostatic mechanisms and those relating to transport phenomenon will be considered here.

Generally soil sulfur content is related to organic matter content and chemical transformation of forms of sulfur are predominantly catalyzed by microbial action (Kertesz and Mirleau 2004) with microbial transporters playing a central role (Kertesz 2001). Sulfate is the major form accessible to plants but is also susceptible to leaching. The ephemeral nature of the sulfate pools in the soil is undoubtedly the underlying cause of the complex adaptations aimed at optimizing uptake and assimilation which have evolved in vascular land plants.

Plant strategies to respond to fluctuating sulfur availability fall into 2 major areas, namely acquisition and utilization. These concepts are familiar to crop physiologists who subdefine nutrient-use efficiency into nutrient-uptake efficiency and nutrient-utilization efficiency as independent parameters for assessing efficiency of nutrient use. Almost universally it is only nitrogen that is considered by agronomists, however the concepts are equally applicable for sulfur. These parameters may be defined as:

*Nutrient-uptake efficiency (NupE) is nutrient-uptake /nutrient-available in the soil etc.(Nup/Nav)*

*Nutrient utilization efficiency (NutE) is grain yield/N-uptake (yield/Nup) where yield only refers to harvested part of the plant, grain in the case of wheat*

*Nutrient-use efficiency (NUE) is the overall parameter and is uptake efficiency x utilization efficiency, that is:*
*NUE = Nup/Nav x yield/Nup = yield/Nav*

In the case of natural populations, the NutE parameter will be the efficiency of partitioning of resource, in this case the nutrient sulfur, into the reproductive biomass, which has a direct influence on fecundity. Whilst intrinsically related, NutE and NupE are quite distinct and each have complex traits consisting of multiple components encoded by multiple genes. Clearly individual plant species will exploit each strategy to differing degrees. Furthermore as each is a multigene trait they are many ways to achieve overall variation in efficiency. Recent breeding strategies for improving yield in domesticated plants have focussed almost exclusively on nitrogen and never on sulfur. In practice NutE (and also NHI, nitrogen harvest index, compare with SHI defined above) have been improved with the emphasis on maximum product from a given biomass. NupE has seldom been addressed, and most trials are in high input situations; a lot of scope for exploitation must exist for improving NupE in low input situations for nitrogen, sulfur, and other nutrients. Examination of diverse strategies in wild plant species, in the context of their ecology will provide targets, and even genetic material for future crop improvement in relation to sulfur (and other nutrient) use efficiency. Transmembrane transporters of ions (sulfate) are central to many of these processes and these will be considered in more detail.

## TRANSPORTERS INVOLVED IN UPTAKE AND PARTITIONING

Almost all sulfur is taken up as sulfate, via the root systems (except in aquatic and unicellular organisms). Active uptake into the cells of the epidermis, cortex, or endodermal layer is driven by a proton gradient (Hawkesford *et al.* 1993; Lass and Ullrich-Eberius 1984; Smith *et al.* 1995). Subsequently cell to cell transfer occurs symplastically via plasma-desmata or apoplastically through successive unloading and loading. Some sulfate may be stored in root cell vacuoles, involving another transmembrane transport step, some may be reduced in the roots (probably in the plastids and hence requiring a plastid uptake system), however a large proportion is loaded into the xylem for translocation to the above ground shoot tissue. The chloroplasts in the green tissues, with an abundant supply of ATP and reductant, are the major sites for reduction to sulfide and incorporation into the amino acid cysteine. Subsequently the cysteine may be directly incorporated into protein or combined into glutathione or transformed into methionine.

Transport from root to shoot involves more mass flow in the transpiration stream as there is clear evidence for selective partitioning to specific sinks, for example expanding immature leaves or seeds. Such partitioning may involve specific xylem to phloem transfer mechanisms (Anderson 2005).

Many of the transport steps are catalyzed by proteins encoded by members of a single gene family, the SulP family. Analysis of whole genomes, where available (*Arabidopsis*, rice), or systematic cloning (*Brassica*; Buchner *et al.* 2004b), wheat (Buchner *et al.* 2004a) indicates a gene family of around 12–14 genes, most, if not all of which are expressed. The 12–14 members may be divided into at least 5 subtypes on the basis of sequence similarity (Hawkesford 2003). Direct sequence homologues are usually identifiable for most of the species investigated, indicating that the gene duplication events are quite ancient; the preservation of sequence similarity indicates a selection pressure for specialized function of the isoforms. Indeed functional and expression analysis indicate specialization of subgroups and even individual isoforms, although there are exceptions. In addition some more recent gene duplication events have occurred in specific species, for example wheat (Buchner *et al.* 2004a), with little sequence divergence and no diversification of function. These represent the material for future evolutionary specialization.

The size of the gene family and the apparent specialization of function of the SulP subgroups is indicative of the complex requirements for sulfate management in terrestrial vascular plants. For example the Group 1 cluster are mostly high affinity (affinity for sulfate in the low micromolar range when expressed in yeast) types, many are transcriptionally regulated by the plant sulfur status and they are strongly, but not uniquely, expressed in the roots. Usually there are three in this group. One isoform seems to be specific for phloem cells (Yoshimoto *et al.* 2003). Generally Group 1 may be concluded to be involved in primary acquisition or in the delivery of sulfate to critical tissues. Group 2 sulfate transporters have been expressed in yeast and generally have a lower affinity for sulfate (0.1–1.2 mM range), are expressed throughout the plant, particularly in vascular tissues and show a less pronounced regulation of expression by sulfur status (Buchner *et al.* 2004b; Smith *et al.* 1995; Takahashi *et al.* 2000). Group 2 are clearly mostly involved in transfer of sulfate around the plant. Rather less is known about Group 3 sulfate transporters. The Group 3 clade is rather large and may need to be subdivided. One isoform requires the formation of a heterodimer with a Group 2 transporter for maximal activity (Kataoka *et al.* 2004a). Sulfur status does not affect expression of any of the Group 3 transporters.

There are 2 isoforms for Group 4 in *Arabidopsis*, *Brassica*, and wheat. Functional analysis suggests a role in vacuolar unloading of sulfate. Expression is increased in response to sulfur limiting conditions, a clear adaptation to facilitate utilisation of stored sulfur reserves (Kataoka *et al.* 2004b).

1–2 isoforms of the Group 5 clade have been observed, and although localizing to the tonoplast, similarly to the Group 4 sulfate transporters, no functional data has been forthcoming. Interestingly the Group 5 sulfate transporters are substantially truncated, lacking the amino and carboxyl terminal regions and show the lowest similarity with the rest of the gene family. As no candidate for vacuole loading has been identified it is tempting to speculate a role in this transport step.

Different patterns of expression in relation to tissue specificity and responses to sulfur nutritional status (see below) are seen between the isoforms and this specificity contributes to the specialization of the individual isoforms and enables a plasticity of management of sulfur in response to fluctuating supply and changing demand during the plant life cycle. Surprisingly, apparent orthologs (the equivalent gene in different species) in different species whilst usually having similar patterns of expression show contrasting expression patterns in some cases, even in closely related species such as *Arabidopsis* and *Brassica*. Quite how these anomalies would have evolved is not clear.

Most recent molecular studies have focussed on agricultural species with the notable exception of the model weed, *Arabidopsis thaliana* (Takahashi *et al.* 1996; Takahashi *et al.* 2000; Takahashi *et al.* 1997); certainly a wider examination of more wild species is required. The response to sulfur limitation of induced uptake capacity seems ubiquitous in the range of species examined at the physiological (*Macroptilium atropurpureum* (Clarkson *et al.* 1983), *Lemna gibba* (Lass and Ullrich-Eberius 1984), or molecular level (*Stylosanthes hamata* (Smith *et al.* 1995), barley (Smith *et al.* 1997), wheat (Buchner *et al.* 2004a), tomato (Howarth *et al.* 2003), maize (Hopkins *et al.* 2004), *Brassica* (Buchner *et al.* 2004b), and *Arabidopsis* (Takahashi *et al.* 1997)). One of the few sulfate transporters isolated from a wild species was a Group 3 sulfate transporter isolated from the resurrection plant, *Sporobolus stapfianus* (Ng *et al.* 1996) as part of a study on rehydration: this transporter seemed constitutively expressed (Neale *et al.* 2000).

## THE MULTIPLE RESPONSES OF METABOLISM, GENE EXPRESSION AND GROWTH FORM TO S-LIMITATION

Cellular and whole plant regulation of transport processes and flux through the assimilatory pathway attempts to balance supply with demand for growth and includes mechanisms for remobilization and redistribution of sulfur (Hawkesford and De Kok 2006). Furthermore optimization of sulfur assimilation requires coordination with carbon and nitrogen pathways and multiple processes probably contribute to this balance.

Whilst it is possible that external sulfate may be sensed and such a signal transduced to facilitate the engagement of the response pathway, it is more likely that an intracellular sensing of sulfur-status occurs. Decreases in cytosolic sulfate or a downstream metabolite such as a reduced sulfur compound or increases in the cysteine precursor, *O*-acetylserine could act as the "sensed" metabolite (reviewed in Hawkesford *et al.* 2006). Regardless of the sensed metabolite, responses to control the availability are a continual process aimed at balancing availability and demand via moderation of storage pools (Figure 2). Processes acting at the cellular level are focussed on regulating transmembrane fluxes of sulfate and or manipulating biochemical pathways. In parallel, regulation involving interorgan sulfur partitioning act at the whole plant level. Examples of such partitioning and of remobilization in response to limiting sulfur and in response to developmental cues have been documented (Anderson and Fitzgerald 2003; Anderson 2005; Blake-Kalff *et al.* 1998).

Decreased cytosolic sulfate is effectively a breakdown in the homeostasis, and the decreased availability must reflect either very high demand or exhaustion of storage reserves, for example depletion of vacuolar sulfate. Many inherent response mechanisms will have already been activated to respond to the high demand, for example increased expression of assimilatory pathway genes to maximise flux to sulfide and vacuolar efflux transporter genes to access stored vacuolar sulfate. Additional mechanisms may be engaged as the severity of the deficiency increases.

Increased capacity for sulfate uptake by roots is a classic response to sulfur limitation and is achieved by increased transcription of specific sulfate transporter genes and increased abundance of the corresponding high affinity sulfate transporter protein (Smith *et al.* 1997). This will facilitate uptake from the surrounding environment, even at low external concentrations. Expression may be particularly important in new root tips exploring underexploited areas of soil. Modification of growth form, particularly with regard to modified shoot to root ratios, occurs with

resource allocation favoring root production, a clear strategy for proliferating roots into new areas with potentially underexploited mineral reserves (Buchner *et al.* 2004b; Kutz *et al.* 2002; López-Bucio *et al.* 2003; Yang *et al.* 2003).

Control of fluxes of sulfate around the plant are critical to the coordination of responses. In the first instance remobilization from storage organs occurs, for example from older leaves to younger expanding leaves, a process which may be achieved by specific xylem to phloem transfer cells. Responses of root cells in terms of enhanced transporter expression require that they receive the "signal" of sulfur limitation. A shoot derived signal or local deficiency is required, the latter will be achieved most rapidly if flux to the shoot is partially rectified. Responses to a limiting sulfur availability in the environment will be detrimentally delayed if stored reserves from the shoot replenish root sulfur reserves to the extent that scavenging mechanisms (transporter upregulation) are repressed.

Very few comparative studies have analyzed a wide varieties of species in relation to the responses described above. It is reasonable to suspect that a unknown strategies are utilized and that there is potential for exploitation and transfer of novel solutions in to agricultural crops. Many *Brassica* species have strategies based around large sulfate storage pools, exploitation of which will require unique time-dependent patterns of expression of isoforms. For some species these pools are sulfur-containing secondary compounds, which have additional selective advantages against pathogens or herbivores.

## HOW PLANTS DEAL WITH TOO MUCH SULFUR

Great emphasis has been placed on how sulfate transporters involved in acquisition are derepressed when sulfur is limiting to a plant. The converse of this is that expression of the genes for many isoforms can be almost completely repressed when sulfur supply is in excess, however measured sulfate influxes are never completely abolished. Hence whilst cellular homeostasis in response to excess supply is predominantly achieved by moderating uptake via repression of sulfate transporter gene expression, other options are required for fine tuning internal sulfate pools. These processes become even more important in high sulfur environments (Ernst 1997). The major internal biological buffer for sulfate is the vacuole which acts as a store of sulfate and may provide an important repository to contribute to halotolerance. Excess supply of high sulfate to barley seedlings resulted in accumulation in the vacuoles of all cells but

preferentially in mesophyll rather than the epidermal cells (Kaiser *et al.* 1989). Selective control of both influx and efflux from the vacuole (by control of the respective transporters) will be required to facilitate this adaptive mechanism.

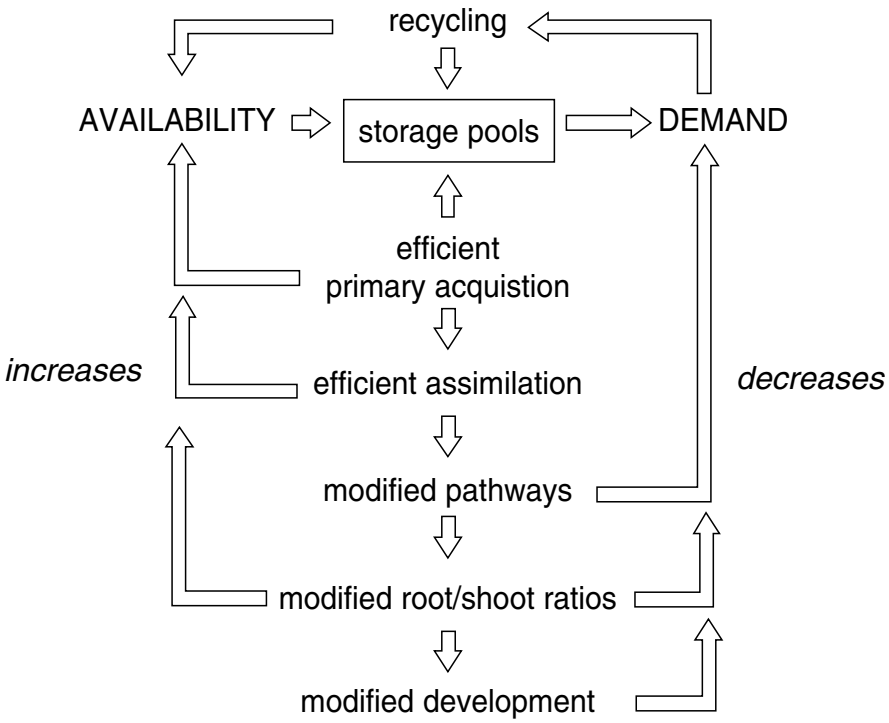


*Figure 2.* Maintaining sulfur supply to meet demand. Sulfur status (sensed via the storage pools) will depend on growth (demand) and on environmental availability.

In addition to the cotransporters of the SulP type, sulfate may be translocated across membranes via anion channels. Amongst the many characterized channel types, depolarization activated channels include R-types (rapid activated, transiently acting efflux channels, for example as found in guard cells) and S-types (slow activated and inactivated channels catalyzing prolonged anion efflux). It appears that only the R-type is permeable to sulfate (Diatloff *et al.* 2004; Frachisse *et al.* 1999; Roberts 2006). Detailed analysis indicates that this channel showed high nitrate and sulfate mediated currents, but a unique regulation by sulfate, which was able to maintain the channel in an active state and additionally was able to

affect channel density, inducing the number of active channels (Frachisse *et al.* 1999). Whilst a suggested role is in preventing toxic accumulation in the cytosol (Roberts 2006), an alternative role may be in the radial movement of sulfate from the epidermis to the xylem.

An efflux solution to respond to excess sulfur uptake is the excretion of sulfate via salt glands (Waizel 1972). This adaptive mechanism has limited long-term value as excreted sulfate will be deposited directly or washed onto the soil by rain water, only to become available for uptake once more. Unless coupled with decreased uptake, efflux mechanisms represent a poor solution to the management of sulfate.

Species diversity is generally restricted on the high sulfur-containing gypsophilous soils (Parsons 1976). Physical effects of gypsum, particularly on soil water relations may partially account for this but otherwise the particular specificity is unclear. Tolerance, for example diverting sulfur to inert internal pools, either spatially or chemically (as sulfur-secondary compounds) may be employed (see above). Excretion, dilution by development of succulence, or exclusion may all contribute and it may be that combinations of these mechanisms are particularly well developed in gypsophilous species; further investigation is required.

## CONCLUSIONS – SUPPLY AND DEMAND AND CONSEQUENCES FOR ECOLOGICAL ADAPTATION

Sulfur supply varies greatly between ecosystems and within some ecosystems substantial temporal variations occur. Plant species differ in their ability to exploit these varied conditions. Some responses to insufficient availability appear universal, at least in the limited number of species examined. Many members of the sulfate transporter gene family, for example, show changed mRNA abundance consistent with transcriptional regulation although basal levels of transporter activity vary between species. Patterns of expression of the isoforms, their tissue specificity and their induction profiles show species specificity, again based on rather limited data sets. Correlation between transcript levels and protein abundance is not a perfect relationship and offers an additional level of species diversity. Taken together the variation available from just one gene family enables a huge plasticity of response to sulfur availability. Added to this is the plasticity of root form, the potential for engagement of alternative metabolic pathways or diversion to sulfur storage compounds, all of which facilitates the exploitation a huge range of habitats regardless of environmental sulfur status. It is likely that many of the diverse

strategies used to manage the sulfur economy of the plant have not been elucidated.

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Chapter 2

# SULFUR INTERACTIONS IN CROP ECOSYSTEMS

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## INTRODUCTION

A comprehensive examination of adaptation of crop plants to changes in sulfur (S) supply begins with an analysis of the influence of pedogenetic and climatic factors. Sulfur supply has consequences for crop productivity and nutritional quality in terms of nutritive value and health-related properties. Factors affecting S supply and the subsequent impacts on crops are discussed in this chapter.

The soil S cycle is driven by biological and physico-chemical processes, which affect both flora and fauna. For example, the knowledge of S speciation in soils is required to provide information on plant available S forms and gives indications of likely interactions between the rhizosphere and the soil matrix. An additional complexity is the high spatio-temporal variability of sulfate occurring in soils (Schnug and Haneklaus 1998), and one consequence is that the plant available sulfate concentration in soils is a poor diagnostic criterion for the S supply. The presence of allelochemicals in the soil, including S-containing compounds, not only affects plants but also other organisms such as soil microorganisms, insects and herbivores, which will have impacts on all soil processes.

Both, severe S deficiency and S toxicity may occur in plants, foodstuffs, animal feed, and the human body. Macroscopic S deficiency is a major nutritional disorder in agricultural production in Europe, whilst the detrimental impact of S pollution on crop performance is a major concern in Asia. In East Asia, where under current legislation restrictions, $SO_2$



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emissions are expected to increase 34% by 2030 (Ichikawa *et al.* 2001), excessive S deposition is an inexorably increasing problem.

Crop productivity and nutritional quality of plants are closely related to mineral nutrition. In this chapter an attempt is made to summarize the response of crop plants to different regimes of S nutrition in terms of yield and composition in order to deliver a platform for evaluating their significance for nutritive value and health. Nutrigenomics acknowledges the prominent role of nutrition for disease protection by studying interactions between bioactive compounds and the genome (Ferguson 2006). A quality parameter for foodstuffs and animal feed is, in addition to the absence of S-containing antinutritives (e.g. glucosinolates) and allergens (e.g. cysteine proteinases), an adequate cysteine to methionine ratio and a high content of health promoting metabolites (e.g. glutathione, methylsulfonylmethane). The S supply is closely related to many of these compounds. *Brassica* crops contain characteristic glucosinolates, which are antinutritives because of their goitrogenic effect. Since the introduction of double low oilseed rape cultivars in the middle of the 1980s, higher doses of extracted rapeseed meal may be fed to animals without detrimental health effects. The S supply is one of the major factors influencing the glucosinolate content in vegetative and generative tissues of oilseed rape (Schnug 1990). Whilst a high glucosinolate content is undesired in animal feed, it is one of the secondary compounds with a strong anticarcinogenic potential in humans. The intake of sulforaphan, the degradation product of glucoraphanin in broccoli, has been linked to diminished risk of prostate cancer in several epidemiological studies (Cohen *et al.* 2000; Kolonel *et al.* 2000; Giovannuci *et al.* 2003).

Thiono-S (C = S or P = S) compounds may exhibit toxic properties such as lung and liver damage, and bone marrow depression (Neal and Halpert 1982). $CS_2$ is a thiono-S compound, which is used in agriculture as a nitrification inhibitor, others are constituents of pesticides and they may enter the human body. This stresses the advantage of ecologically sound agricultural production not only for environmental protection, but also for preventing adverse health effects. A naturally occurring thiono-S compound is goitrin, which can be found after degradation of progoitrin in *Brassica species* (Fenwick and Griffiths 1981). Pigs that were fed with extracted rapeseed meal showed goitrin levels in loin muscle that were rated as being inoffensive for human consumption (Thomke *et al.* 1998).

The ratio of S per gram of protein is similar in vegetable and animal proteins, but proteins in plant products have a lower nutritional quality for humans, because the cysteine to methionine ratio is imbalanced (Massey 2003). In vegetables the cysteine to methionine ratio is lowest with a ratio varying between 1:0.5 and 1:1 (Hands 2000). Soybeans and eggs show an

intermediate ratio of 1:1.3, while meat products have distinctly higher ratios of 1:2 to 1:2.8. In most plant species, the major proportion of S (up to 70% of the total S) is present in the reduced form in cysteine and methionine residues of proteins. The S-containing amino acids cysteine and methionine play a significant role in the structure, conformation, and function of proteins and enzymes in vegetative plant tissue, but high levels of these amino acids may also be present in seed storage proteins (Tabatabai 1986).

Glutathione is an antioxidant and may play a key role in the detoxification of xenobiotics and carcinogenesis in the human body (Richie 1992). During aging a faster oxidation of the physiological S pool can be observed and thus resulting in a higher physiological demand of antioxidants for maintaining the GSH to GSSG ratio (Miquel *et al.* 2006). Friedman (1994) outlines the significance of SH-containing amino acids and peptides as a means to combat adverse effects by other food compounds, for instance aflatoxins. Asparagus is rich in glutathione with 4 mg $g^{-1}$ dry weight compared to other vegetables such as broccoli (0.7 mg $g^{-1}$), spinach (0.7 mg $g^{-1}$), or tomato (1.9 mg $g^{-1}$) (Pressman 1997). The glutathione content is closely related to the S nutritional status in such a way that an S application rate of 100 kg S $ha^{-1}$ increased the glutathione content by about 65 nmol $g^{-1}$ dry weight in leaves of oilseed rape and asparagus spears (Haneklaus *et al.* 2006).

Alliins (cysteine sulfoxides) are the characteristic S-containing secondary metabolites of *Allium* species such as onions, shallot, garlic, leek, and chives, which cause sensory characteristics and entail the pharmaceutical quality. The therapeutic effect of onions on vascular diseases such as thrombosis, arteriosclerosis, hyperlipidemia, and rheumatic arthritis of humans was attributed to the degradation of iso-alliin, which yields the lachrymatory factor (thiopropanal SO) and from this metabolite components are finally derived, which inhibit platelet aggregation (Kawakishi and Morimitsu 1994). Garlic is used against arteriosclerosis, high blood pressure, and has been shown to have antibacterial, antifungal, antiviral, and antiprotozoal activities. It also modulates the cardiovascular and immune system and has antioxidative and anticarcinogenic properties (Harris *et al.* 2001). S fertilization increased the isoalliin content in the leaves of onion up to 43-fold and doubled the alliin content in bulbs of onion and garlic (Bloem *et al.* 2001; Bloem *et al.* 2004).

After calcium (Ca) and phosphorus (P), S is the third most abundant mineral in the human body with about 0.25% (140 g S) of the total body weight (Clark 2002). While deficiency of S in the diet is rare, its toxicity has been identified as a relevant factor of concern (Komarnisky and Basu

2005). Grimble (2006) points out that high intake of L-methionine might increase the homocysteine level in plasma. Homocysteine may favor inflammatory centers, so that as a precautionary measure, increased intake of L-methionine should be avoided (Grimble 2006). High homocysteine increases the risk for cardiovascular disease, too (Borek 2006). An enhanced level of homocysteine intake is for instance possible by nutraceuticals. Methylsulfonylmethane (MSM) is a nutraceutical that alleviated symptoms of pain and physical function of humans suffering from osteoarthritis (Kim *et al.* 2006). Dietary supplements and nutraceuticals need to be critically evaluated because their regular intake may support, or even encourage, malnutrition with as yet unknown consequences for health. It is better to promote interest in and consumption of authentic foods, rich in bioactive compounds due to agro-technological measures such as S fertilization, to take advantage of the whole range of compounds in natural food and their synergetic effects.

This chapter provides an overview of various aspects of the adaptation of crop plants to changes in the S supply, in which special attention is paid to S in the rhizosphere and the effects of excessive S rates on crop performance. Previous monographs concentrated on S cycles at different scales (Haneklaus *et al.* 2003), diagnosis of the S nutritional status (Schnug and Haneklaus 1998), and various aspects of S in plant nutrition (Haneklaus *et al.* 2006). S transformation processes in the soil are closely related to management practices such as crop rotation and diversity of soil fauna. Measures, which foster plant health by combating soil-borne pathogens, as for example biofumigation, deserve a closer examination as they have the potential to substitute for pesticides by controlled amendment of S-containing allelochemicals. Data on the influence of variations in the S supply on crop productivity and quality are valuable for a better understanding of the long-term implications of anthropogenic activities causing excess or low S inputs. Even more importantly, such figures may enable an appraisal of the significance of S supply to crop plants for their nutritive value and possible health effects.

## ADAPTATION OF THE PLANT RHIZOSPHERE TO CHANGES IN THE S SUPPLY

Lorenz Hiltner (1862 – 1923) coined the term rhizosphere and underlined the significance of microbial activities in this compartment for plant nutrition and plant health. Nicholas (1965) calculated that 1 g of soil of fertile arable land contains about $10^6$–$10^9$ bacteria, $10^5$ –$10^6$ fungi, and $10^1$ –$10^3$

algae. The rhizosphere microflora, sustained by root exudates and root debris, affects plant growth in return by changing the availability of nutrients (Curl and Truelove 1986). The rhizosphere covers the space between the surface of plant roots and closely adhering soil particles and debris. Plant roots excrete among others sugars, amino acids, glycosides, organic acids, vitamins, and enzymes (Curl and Truelove 1986). The composition of the exudate varies in relation to plant species, growth stage and principal soil features (Curl and Truelove 1986). Biochemical speciation of S in soils in relation to plant species reflects interactions between root exudates and microflora.

No chemical method, as far as the extractant or extracting procedure is concerned, has found universal acceptance for analyzing plant available S in soils. Site-specific differences of soil characteristics influence plant available sulfate quantitatively, while qualitative modifications could not be verified for different S fractions (Zucker 1987). Usually plant available sulfate concentrations provide no satisfactory relationship to the plant S status or yield. The reason has to be seen in the high spatio-temporal variability of sulfate in soils.

Plant-derived allelochemicals may influence plant growth, both, positively and negatively. Glucosinolates are prominent examples of allelochemicals and their effect on soil-borne pathogens has been studied extensively as cultivation of *Brassica* species as break crops and amendment with glucosinolate-containing plant material offers the possibility to reduce the input of pesticides.

## S TRANSFORMATION PROCESSES AND S SPECIATION IN SOILS

The soil S cycle is driven by biological and physico-chemical processes, with both plants and soil biota being actively involved. The rhizosphere is a key zone with view to the mechanisms of soil S dynamics. Basic information about S speciation and transformation processes in soils is summarized below for a better understanding of soil/plant interactions and an evaluation of the chemical behavior of S species in the rhizosphere.

### *S transformation processes in soils*

Two types of processes are involved in the mineralization of S, the biological and the biochemical mineralization (McGill and Cole 1981). The biological mineralization is considered to be driven by the microbial need for organic C to provide energy, and S released as sulfate is a by-product

of the oxidation of C to $CO_2$. Microbial-mediated processes are mainly responsible for S transformations, so that the factors affecting the microbial activity, such as temperature, moisture, pH, and substrate availability will also influence the process of mineralization, immobilization, oxidation, and reduction. From a nutritional point of view, the release of plant-available sulfate is of prime interest for plant growth. This process is faster the more recently the organic matter is formed (Ghani *et al.* 1993). In comparison, biochemical mineralization relies on the release of sulfate from the sulfate-ester pool through enzymatic hydrolysis. This implies that this process is linked to the S supply. Enzymes that catalyze the degradation of sulfate esters are: aryl, alkyl, steroid, gluco-, chondro-, and myco-sulfatases (Germida *et al.* 1993). In soils, only arylsulfatase activity has been determined (Germida *et al.* 1993). The hydrolysis of ester-bonded S follows the equation (Fitzgerald 1978):

$$R\text{–}C\text{–}O\text{–}SO_3^- + H_2O \rightarrow R\text{–}C\text{–}OH + H^+ + SO_4^{2-}$$

The sulfate-ester pool seems to be important for short-term and the carbon-bonded S pool for long-term mineralization of S (McGill and Cole 1981).

The edaphon constitutes about 5% of the total organic matter in soils (Topp 1981). The $A_h$ horizon of soils typically comprises soil biota in the following ratio (dry matter $m^{-2}$ in a no-till farm soil): bacteria (50 g), fungi (100 g), amoeba (5 g), nematodes (0.2 g), arthropods (0.5 g), and worms (1–20 g) (Anthoni 2000). Farm stock amounts to 50 g $m^{-2}$ and is thus comparable to bacteria and earthworms, while fungi outweigh this number considerably (Anthoni 2000). Microbial biomass is in the range of 146 – 968 µg $g^{-1}$ soil (Roembke *et al.* 2002) with an S content of 928 – 1,355 µg S $g^{-1}$ (Saggar *et al.* 1981). Thus S in microbial biomass amounted to about 1–3% of the total organic S in agricultural soils (Saggar *et al.* 1981; Chapman 1987; Wu *et al.* 1994), but also values of up to 8.8% in vegetated soils were found (Hu *et al.* 2002). The turnover of the soil microbial biomass is fundamental for the incorporation of sulfate-S into soil organic matter. However, quantitative relationships between microbial immobilization of inorganic S, turnover of soil microbial biomass-S, and subsequent formation of organic S, as well as the extent of availability of these S fractions for plants have not been determined experimentally so far.

Mineralization of soil organic S can be influenced by farm management practices. The application of organic materials will lead to mineralization if the C:S ratio is <200:1 and immobilization at ratios of >400:1 (Eriksen *et al.* 1998). For ratios in between both processes are possible. Management

practices such as fertilization and crop rotation influence S dynamics (Tabatabai and Chae 1991) and should be steered in such way that the S supply is adapted to the S demand of the crop.

The contribution of mineralization to the S supply of plants is only small with about 1.7–3.1% of the organic S pool per year (Eriksen *et al.* 1998), because mineralization, immobilization, and possible leaching of S occur concurrently (Ghani *et al.* 1993). Thus, in soils with carbon contents between 1% and 4% C, net mineralization contributed 10–30 kg $ha^{-1}$ $year^{-1}$ S to the S balance of an agricultural soil (Bloem 1998). The studies of Eriksen *et al.* (1998) and Bloem (1998) reveal that mineralization is an important, however not cardinal S pool for plants. High-yielding crops cannot satisfy their S demand solely by mineralization and atmospheric S depositions (Schnug and Haneklaus 1998).

*S pools and transformation processes in the rhizosphere of different crops*

Crop type was shown to influence S mineralization and immobilization in soils (Freney and Spencer 1960). The rhizosphere is a key zone with a view to the mechanisms of soil nutrient dynamics. Only limited data are, however, available about interactions between soil biota and plants and how they affect different S fractions in the rhizosphere. Biological and physico-chemical processes at the soil–root interface differ considerably from those in the non-rhizosphere soil. The evaluation of the bioavailability of different S fractions in various soil–plant systems is important for a better understanding of soil/crop interactions, which may be applied in models for predicting the contribution of organic matter to the S supply of crops. Additionally agronomic and ecological impacts in relation to the site-specific S cycling in agro-ecosystems could be assessed.

*Ester-bonded S*

The distribution of S fractions in the rhizosphere and non-rhizosphere varied depending on soil type and crop species (Hu *et al.* 2002). In general, the total S content in the soil was higher in the rhizosphere than in the non-rhizosphere. Plant S uptake and mass flow of sulfate from the non-rhizosphere to the rhizosphere most likely caused this variation. Another factor is the root system: oilseed rape with a coarse root system stimulates microbial biomass and thus enhances hydrolysis of ester-bonded S (Vong *et al.* 2002). The result was a positive and significant relationship between arylsulfatase activity and sulfate uptake of oilseed rape (Vong *et al.* 2002). Arylsulfatase released by microorganisms in the rhizosphere of oilseed rape was found to be more closely related to the S demand of the crop than was the case for barley (Vong *et al.* 2002 and 2003; Dedourge *et al.* 2003).

Knauff (2000) found a distinctly higher arylsulfatase activity in the rhizosphere of *Brassica* compared to gramineous crops. Correspondingly, the amount of ester-bonded S was lower in the rhizosphere than in the non-rhizosphere (Hu and Shen 1997; Hu *et al.* 2002). Oilseed rape showed a higher enzyme activity that increased with distance from the root, whilst for winter wheat, the inverse result was found. Additionally, micro-organisms in the rhizosphere have access to energy sources such as root exudates (Yan 1993) and it is possible that living roots enhance the activity of microorganisms and enzymes.

It is presumably the exudation of glucosinolates and their degradation by myrosinase, which yields a biocidal effect when oilseed rape is grown. During senescence oilseed rape roots may secrete myrosinase at up to 20 $\mu g\ kg^{-1}$ soil (Borek *et al.* 1996). The result is a lower amount of S bound in microbial biomass or immobilized (Dedourge *et al.* 2003). Dedourge *et al.* (2003) further assumed that only a part of the microbial population takes part in S mobilization and immobilization processes, as there was a close correlation between arylsulfatase activity and S bound in microbial biomass, but none for C bound in microbial biomass. The quality of root exudates creates a host-specific environment and influences microorganism populations selectively (Angus *et al.* 1994). Vančura and Hansliková (1972) found differences in the amount of root exudates of up to 30%. Though there are no conclusive results available that changes in microbial population are related to exudation patterns, differences in the quantity and composition of exudates exist, and are apparently greater among plants that are phylogenically unrelated (Curl and Truelove 1986). Such crop-specific exudation patterns and rates further strengthen the assumption of a demand-driven adaptation to soil conditions under a limited S supply.

*Residual-S*

The amount of residual-S was higher in the rhizosphere than in the non-rhizosphere (Hu *et al.* 2003). The content of plant-available S measured in 0.1 M $CaCl_2$ and adsorbed sulfate in the rhizosphere and non-rhizosphere of oilseed rape and radish were significantly lower than those of wheat when grown on a Haplic Acrisol. The reason for these differences was presumably the significantly higher biomass production of oilseed rape and radish compared to wheat (Hu *et al.* 2002). In general, it may be expected that sulfate will accumulate in the rhizosphere when S uptake is lower than mass flow of sulfate. Vong *et al.* (2002) also determined lower sulfate concentrations of organic origin in the rhizosphere and non-rhizosphere of oilseed rape than barley. They identified the rapid S acquisition of oilseed rape as the driving force. This effect was consistent at high mineral S

conditions. The results of Hu *et al.* (2002) and Vong *et al.* (2002) suggest that crop-specific discrepancies in S uptake and crop-related differences of microbial and enzymatic activities in the root zone influence S transformation processes in soils.

Such crop-related differences in S fractions of the rhizosphere and non-rhizosphere were not only found on aerated soils, but also under waterlogged conditions (Hu *et al.* 2003). Rice utilized residual-S more intensely than oilseed rape (Hu *et al.* 2003), because its aeration tissues warrant oxidizing conditions and thus promote activity of microbes and sulfatase from the top to the roots (Han and Yoshida 1982).

Ratios of inorganic sulfate in the non-rhizosphere compared to the rhizosphere varied between 1:1.3 and 1:3.1, indicating an enrichment of sulfate in the rhizosphere (Hu *et al.* 2003). When growing oilseed rape, the ester-bonded and carbon-bonded S increased by 47% and 25% in the rhizosphere compared to the control (Hu *et al.* 2003). In contrast, the two fractions decreased by 75% and 30% in the rhizosphere of rice (Hu *et al.* 2003). These findings provide further evidence that the mineralization of organic S is related to crop type and that all fractions of organic S are on principle bioavailable.

### *Influence of S fertilization on microbial populations and on S transformation processes*

The release of organic acids by plant roots promotes growth of bacteria, and attracts bacteria and fungi towards roots (Jones 1998). Microbial arylsulfatase activity was stimulated by increasing sulfate concentrations in contrast to barley arylsulfatase activity (Ganeshamurthy and Nielsen 1990). With increasing mineral sulfate fertilization the uptake of S by barley from organic sources declined after 3 weeks (Vong *et al.* 2002). On a long-term basis, the application of compost had the strongest effect on the arylsulfatase activity when compared to manure and mineral fertilizers (Knauff *et al.* 2003). Concomitantly with an increase in organic matter, the arylsulfatase activity increased in these experiments so that a higher availability of soil organic S to plants can be expected.

The influence of elemental S applications on S-oxidizing *thiobacilli* and heterotrophic bacteria has been studied comprehensively. There exists a wide spectrum of S-oxidizing microorganisms in soils: the majority of 273 different bacteria and 70 fungi that were collected from the rhizosphere of summer oilseed rape were able to oxidize elemental S (Grayston and Germida 1991). Nevertheless, the oxidation rate of soil-applied elemental S is regularly limited because of a restricted population size. The efficacy of elemental S depends on the particle size, application rate, soil, and

climatic factors including the number as well as the activity of S-oxidizing microorganisms (Watkinson and Bolan 1998). Li *et al.* (2005) reported that repeated applications of elemental S increased the oxidation rate. Lee *et al.* (1990) found that the oxidation rate was independent of the initial number of *Thiobacillus* spp. present during incubation, while under field conditions reapplication of elemental S resulted in an increased oxidation rate because of a higher number of *Thiobacillus* spp. remaining from the first application of elemental S (Lee *et al.* 1987). Repeated applications of elemental S increased the *Thiobacillus* spp. count and population of aerobic heterotrophic S-oxidizing bacteria consistently and achieved a maximum value of $1.0 \times 10^8$ $g^{-1}$ and $5.0 \times 10^4$ $g^{-1}$ soil after the seventh and fourth application, respectively (Yang *et al.* 2006). These results suggest that soils which receive regular applications of elemental S have a higher number of S-oxidizing microbial populations and thus a substantially higher oxidation potential.

Gupta and Germida (1988) investigated the acidifying effect of repeated elemental S rates of 44 kg $ha^{-1}$ over 5 years as the reason for a decline of microbial biomass by 40%. Whilst fungi were reduced, bacteria and actinomycetes were unaffected. This resulted in a reduced number of mycophagous amoebae so that a negative impact on the control of phytopathogenic fungi cannot be excluded.

*S speciation in soils*

The spatial speciation of nutrients is relevant to soil analysis. Gassner *et al.* (2002) showed that different environmental factors resulted in the spatial speciation of P. It was possible not only to separate different pools, but also, based on the analysis of their spatial continuity, to extract different environmental parameters that resulted in the formation of these pools. For S, no correspondent investigations have been carried out so far. Usually, the speciation comprises the following S pools: total S, organically bonded S (labile and stable S fractions), and inorganic S.

Most of the S in terrestrial soils is bound in the organic fraction, which amounts usually to more than 95% of the total S content (Eriksen *et al.* 1998). Organic S in soils is a heterogeneous mixture of soil organisms, partly decomposed plant material, animal, and microbial residues. The nature of soil organic matter is highly complex and any procedure attempting to divide organically bound S into only a few biologically meaningful fractions will never match the variation of individual chemical compounds. Many different approaches were developed empirically to separate soil organic S into major fractions representing distinct forms and properties as for instance: (i) chemical extraction followed by physical-

chemical separation into humic acids, fulvic acids, and humins (Bettany *et al.* 1980); (ii) reactivity with reducing agents: carbon-bonded S (C–S) and sulfate esters (C–O–S; C–N–S and C–S–S) (Tabatabai 1982); (iii) physical separation into organo-mineral size fractions (Hinds and Lowe 1980; Anderson *et al.* 1981); and (iv) molecular weight fractionation (Scott and Anderson 1976; Keer *et al.* 1990). Details about the different procedures for fractionating different S forms are given by Eriksen *et al.* (1998).

Sulfate released from labile S fractions and microbial biomass is important for the S nutrition of crops. A soil feature, which affects the plant-available S pool is soil texture. A relative increase of the sulfate-ester pool with decreasing particle size indicates a protection of organic S from mineralization (Eriksen *et al.* 1998) and it results in a decreased availability of S to plants. This hypothesis is supported by the findings of Anderson *et al.* (1974) who showed that high molecular weight components were preferably adsorbed to clay particles and Keer *et al.* (1990) who proved that more than 75% of the total organic S was present in the form of sulfate esters with a molecular weight of >200,000 Da. These findings are in agreement with those of Bettany *et al.* (1979 and 1980), who found that a fractionation of organic matter delivered a higher percentage of S in ester form in the fulvic acid fraction that was not associated with clay minerals on arable soils than on grassland as the organic material in this fraction is usually younger and not yet bonded to clay-associated humic acids (Eriksen *et al.* 1998).

## SPATIO-TEMPORAL VARIABILITY OF S IN SOILS

Adaptations of the plant rhizosphere to changes in the S supply can be followed up by assessing the spatio-temporal variability of S species in soils. The largest scale reflects differences between soil types, the lowest scale that within a single field.

The typical range of S in agricultural soils of humid and semi-humid regions is 100–500 μg $g^{-1}$, or 0.01–0.05% S (Stevenson 1986). The total S content of soils may be as low as 20 μg $g^{-1}$ (0.002%) in highly leached and weathered soils of humid regions or as high as 35 mg $g^{-1}$ (3.5%) in marine marsh soils and up to 50 mg $g^{-1}$ (5%) in calcareous and saline soils of arid and semiarid regions (Stevenson 1986, Chapter 1). Examples for differences in S speciation for different soil types are given in Table 1. Notably the proportion of carbon-bonded S can be lower than 0.1% of the total S content (Table 1). The proportion of ester-bonded S ranged from 15% to

52% of the total S. The plant-available sulfate content varied between 1.2 µg and 40.4 µg $SO_4$-S $g^{-1}$. It has been outlined previously that inorganic sulfate content is of prime relevance for the plant S supply. S transformation processes are dynamic and the high spatio-temporal variability of sulfate reflects this (Figure 1, Schnug and Haneklaus 1998; Bloem *et al.* 2001).

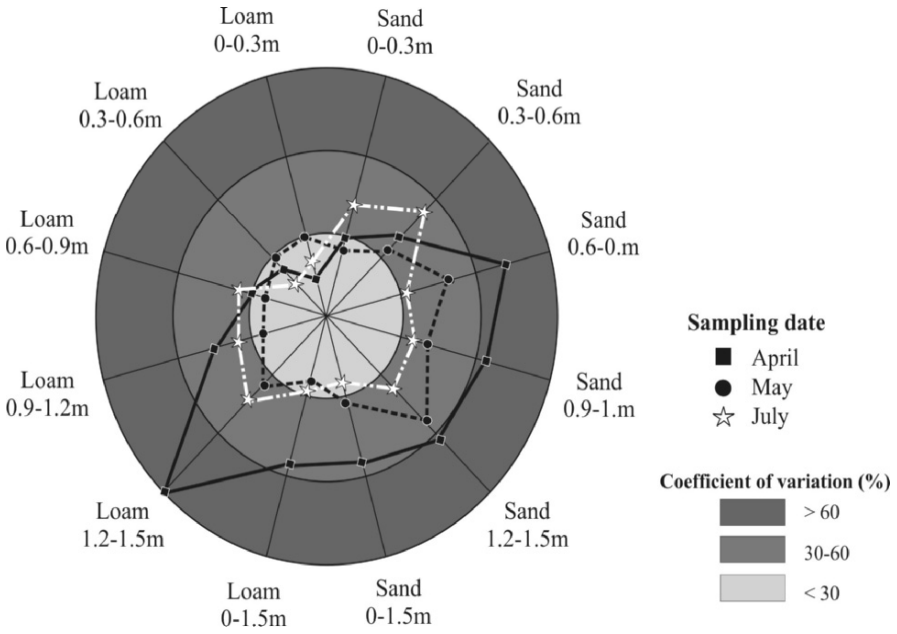


*Figure 1.* Spatio-temporal variability of the sulfate contents in different soil layers on two soil types. (Adapted from Bloem *et al.* 2001.)

The variability of sulfate concentrations within one field can be as high as variations between different soil types in different climatic areas (Table 1, Schnug and Haneklaus 1998). This high spatio-temporal variation of plant-available sulfate concentrations under humid conditions was shown to be closely related to soil physical and hydrological parameters (Bloem 1998). Severe S deficiency in crops can occur on all soil types and is generally exacerbated by high yields, soils with a light soil texture, high permeability and low organic matter content, sites poorly connected to capillary ascending groundwater, leaching; reduced root growth and rooting intensity in acid soils, soil compaction, or low soil temperatures. In addition to the spatial variability, rapid temporal changes in soil sulfate are

a causal reason for a lack of relationship between soil analytical data and plant S status or crop yield (Schnug and Haneklaus 1998).

## S-CONTAINING ALLELOCHEMICALS

Molisch (1937) defined allelopathy as chemicals being transferred from one plant to another; these chemicals may exert positive or negative effects. Allelochemicals are secondary compounds, which affect plants, soil microorganisms, insects, and herbivores. S-containing allelochemicals are closely related to adaptations of the plant rhizosphere to changes in the S supply as they influence soil microorganisms and other plants.

Root exudates may directly affect seed germination of another plant, either by promoting the process, or inhibiting it (Curl and Truelove 1986). Bell and Koeppe (1972) showed that giant foxtail inhibited growth of maize by about 35% due to an allelopathic effect. The allelopathic effect of plants from the orders *Cruciferae*, *Resedaceae*, and *Capparidaceae* on weeds and soil-borne diseases usually focusses on the release of volatile isothiocyanates (ITCs). The degradation of glucosinolates (GSLs) by myrosinase delivers not only ITCs, but also organic cyanides, nitriles, oxazolidinethiones, and ionic ITCs all of which have allelopathic potential (Brown and Morra 1996; Mizutani 1999). Myrosinase activity was proven on fields where *Brassica* species were grown (Borek *et al.* 1996) and Yamane (1991) showed that the microorganism *Rhizopus* that can be found in the rhizosphere of *R. sylvestris* produced extracellular myrosinase. The release of about 13 µg plant$^{-1}$ day$^{-1}$ hirsutin and 9.3 µg plant$^{-1}$ day$^{-1}$ pyrocatechol by the weed yellow fieldcress (*Rorippa sylvestris*) inhibited germination of lettuce seedlings (Yamane *et al.* 1992). At lower concentrations hirsutin proved to have an inhibitory effect exclusively on noncruciferous crops (Kawabata *et al.* 1989).

ITCs may interfere with seed enzymes (Drobnica *et al.* 1977). Petersen *et al.* (2001) assumed that low concentrations induce a secondary dormancy, while high concentrations prevent germination. Basically, ITCs were shown to inhibit germination and growth of both, monocotyledonous and dicotyledonous plants (Petersen *et al.* 2001). In pot experiments, Norsworthy and Meehan (2005a, b) found the sensitivity of *Panicum texanum*, *Digitaria anguinalis, Senna otusifolia*, and *Amaranthus palmeri*, *Ipomoea lacunose*, and *Cyperus esculentus* to be related to chemical structure and concentration of ITCs. Under field conditions mustard, summer and winter oilseed rape were mixed at flowering after mowing with the soil to test their effect on seed germination (Haramoto and

Gallandt 2005). These authors could not verify any significant effect of *Brassica* crops on the delay of seed germination by weeds when compared to non-*Brassica* cover crops.

GSLs may be released by root exudates of living plants and exert their allelopathic effects. Another option is their degradation after decomposition of separated plant parts or harvest residues. Their effect on soil-borne pathogens is summarized by the term and phenomenon of biofumigation. Biofumigation might advance to a promising and ecologically sound alternative for crop protection if its efficiency can be directed.

*Biofumigation*

The efficiency of GSLs and/or ITCs against soil-borne fungal diseases, nematodes, and weeds is related to the kind of pathogen and pathotype. Additionally, GSL content and type, and quantitative release of ITCs are relevant factors (Sarwar and Kirkegaard 1998; Rosa and Rodrigues 1999; Smolinska *et al.* 2003). The toxicity of ITCs is based on their nonspecific, irreversible interactions with sulfhydryl groups, disulfide bonds, and amino groups of proteins and amino acids; thiocyanates interfere with the tertiary structure of proteins through electrostatic interaction (Brown and Morra 1996). Their, toxicity is, however lower than that of ITCs (Rosa and Rodrigues 1999). For aliphatic ITCs Sawar *et al.* (1998) found a decreasing toxicity with increasing length of side chain. The GSL content of different *Brassica* species increased in the order *B. napus* < *B. juncea* < *B. nigra* (Sarwar and Kirkegaard 1998). Propenyl-GSL was found at higher levels in *B. carinata*, *B. nigra*, and *B. juncea* and phenylethyl-GSL in *B. napus* (Kirkegaard and Sarwar 1998). Smith and Kirkegaard (2002) tested the susceptibility of 75 fungi and oomycetes, and 41 bacterial isolates against 2-phenylethyl-ITC. For fungi that showed a low susceptibility against 2-phenylethyl-ITC, the effective dose for a reduction of the mycelial growth was high and vice versa. In general, the GSL content and efficacy of *B. napus* decreased from 20.5 to 0.7 in shoots and from 31.0 µmol to 0.8 µmol $g^{-1}$ dry weight in roots from flower primordium to harvest (Kirkegard *et al.* 1996, Sarwar and Kirkegaard 1998). This dilution effect was attributed to a higher biomass production (Sarwar and Kirkegaard 1998). S fertilization was shown to significantly increase the GSL content in vegetative and generative plant materials (Schnug 1990; Haneklaus *et al.* 2006). The effect of ITCs on soil-borne fungal pathogens under laboratory conditions is summarized in Table 2.

*Table 1.* Distribution of S species in the upper layers of different soils.

| Location | Soil type | Depth (cm) | Total S | Organic S | C-bonded S | Ester-bonded S | Sulfate S[1] | Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | | (μg g$^{-1}$) | | | |
| China | Haplic Staganic Anthrosol (aerated[2]) | 0–20 | 134 | 91.4 | 13.8 | 20.1 | 28.8 | (1) |
| China | Haplic Staganic Anthrosol (waterlogged[2]) | 0–20 | 133 | 87.8 | 14.4 | 27.0 | 40.4 | (1) |
| China | Haplic Acrisol[3] | 0–20 | 182 | 144 | 18.1 | 75.3 | 28 | (2) |
| China | Hortic Anthrosol[3] | 0–20 | 130 | 106 | 0.1 | 32.4 | 24 | (2) |
| USA | 13 soils | 0–15 | 56–618 | 55–603.8 | 8.0–87.7 | 29.3–286.8 | 1.0–14.2 | (3) |
| Canada | Aridic Haploboroll | 0–15 | 312 | 310.2 | 48 | 163 | 1.8 | (4) |
| | Typic Haploboroll | 0–15 | 338 | 336.0 | 49 | 171 | 2.0 | (4) |
| | Udic Haploboroll | 0–15 | 463 | 461.8 | 52 | 220 | 1.2 | (4) |
| | Transitional | 0–15 | 225 | 223.8 | 65 | 77 | 1.2 | (4) |
| | Typic Cryoboralf | 0–15 | 144 | 141.8 | 63 | 52 | 2.2 | (4) |
| Denmark | Typic Hapludalf | 0–20 | 202 | 169 | 99 | 67 | 27.1 | (5) |
| | Typic Haplorthod | 0–20 | 122 | 146 | 81 | 49 | 1.1 | (5) |

[1]soluble sulfate; [2]soil material from nonvegetated non-rhizosphere soil; [3]soil material from fallow non-rhizosphere soil; (1) Hu *et al.* (2003), (2) Hu *et al.* (2002), (3) Tabatabai and Bremner (1972), (4) Bettany *et al.* (1973), (5) Eriksen (1996).

The fungicidal or fungitoxic effect depended on the ITC concentration in agar and headspace, respectively (Sawar *et al.* 1998). The lowest fungitoxic concentration on *Gaeumannomyces graminis* was 1.6 µmol $l^{-1}$of 2-propenyl-ITC in the headspace and 5 µmol $l^{-1}$ benzyl-ITC in agar (Sawar *et al.* 1998). With 6.2 µmol $l^{-1}$ *Bipolaris sorokiniana* proved to be least sensitive against 4-Pentenyl-ITC in the headspace and *Pythium irregulare* with 90 µmol $l^{-1}$ in the agar (Sawar *et al.* 1998). The results of these experiments showed that the toxicity of ITCs was different when incorporated into agar, or released in gaseous form. Compared to aliphatic ITCs, aromatic ITCs had a higher toxicity in agar than in gaseous form because of different vapor pressures (Sawar *et al.* 1998). Under laboratory conditions, a fungicidal/fungitoxic effect of ITCs lasted no longer than 6 days; only a continuous exposure reduced colony growth efficiently (Smolinska *et al.* 2003). In comparison, allyl-ITC had a half-life of only 20 to 60 h in soils (Borek *et al.* 1995). Another impairment of the efficacy occurs when GSL-containing plant material is used instead of pure chemicals. Only 1–8% of the potential ITC concentration was found after incorporation of plant material into soil (Brown and Morra 1996; Morra and Kirkegaard 2002). Myrosinase concentration in plant tissue was sufficient for degradation of GSLs and supplementing additional myrosinase yielded no higher fungitoxicity (Lazzeri *et al.* 2004a). Soil moisture content and a sufficient decomposition of the plant material were obviously major limiting factors for the release of ITC (Morra and Kirkegaard 2002). Another limiting factor might rely on the reaction of ITCs with inherent plant proteins and amino acids (Warton *et al.* 2001).

The efficacy of plant materials to yield a fungitoxic or fungistatic effect was related to crop type. *B. juncea and Sinapis alba* delivered better results compared to *B. napus* (Smolinska and Horbowicz 1999). Not only vegetative, but also generative plant material had a fungitoxic effect. Seed meal of mustard inhibited completely mycelial growth of *R. solani*, *G. graminis*, and *Fusarium graminearum* (Kirkegard *et al.* 1996).

As expected, the efficiency of ITCs for biofumigation declined clearly in the order *in vitro* >> pot experiment >> field experiment. Price *et al.* (2005) found an increase in the allyl-ITC concentration in relation to soil texture, soil temperature, and soil coverage, and a decrease in relation to microbial population and time after incorporation of a standardized mustard plant material. A significant decline was found after 8 h, which underlines the narrow time slot for a phytosanitary effect of ITCs. In addition, microbial degradation in soils decreased the allyl-ITC concentration. A higher allyl-ITC concentration was found on a more sandy soil, which the authors attributed to a presumably lower adsorption to the organic matter fraction (Price *et al.* 2005). With higher soil

temperature and soil coverage, a higher allyl-ITC was found, while soil water content and soil pH had no influence on the release of allyl-ITC (Price *et al.* 2005).

Under field conditions Smith *et al.* (2004) found no significant relationship between GSL content in roots of oilseed rape and phytosanitary effects, and yield of the following wheat crop in the rotation. Kirkegaard *et al.* (2000) proved that *Brassica* crops reduced the inoculum of *Gaeumannomyces graminis*. This effect coincided with root decay and a reduced content of intact GSLs at maturity (Kirkegaard *et al.* 2000), but it was also not persistent in the following wheat crop.

Under field conditions, radish showed resistance against *Meloidogyne javanica* and *Meloidogyne arenaria* that was comparable to resistant fodder sorghum, while *Brassica* crops also reduced reproduction of these nematodes (Pattison *et al.* 2006). The contribution of ITCs from *Raphanus sativus* to this resistance remains uncertain. The nematicidal effect of individual GSLs and their degradation products on *Meloidogyne incognita* and *Globodera rostochiensis* was tested *in vitro* (Buskov *et al.* 2002; Lazzeri *et al.* 2004a). ITCs differed in their nematicidal effect by factor 400; their efficacy was usually higher when exposure time was exalted (Lazerri *et al.* 2004).

Research in the field of biofumigation has shown that GSL content and pattern vary in relation to plant species, plant part, growth stage, and S supply. The potency of ITCs was found to be distinctly higher under laboratory than field conditions if at all. Soils are open systems with a much higher volume than that of sealed containers in the lab, resulting putatively in a lower ITC concentration in the headspace of pathogens. Additional obstacles under field conditions are that the incorporation of the break crop is not homogenous; the GSL content is lower in plant residues than in younger plant material and degradation of GSLs is incomplete as it requires mechanical disruption to destroy cell structures and sufficient water for a sufficiently high myrosinase activity. A solution to these problems might be a functional biofertilizer, which consists of material from different plants with highest concentrations of GSLs releasing most biocidal ITCs. Different coatings of the fertilizer will facilitate a continuous release of GSLs and ITCs.

## ADAPTATION OF PLANT GROWTH TO CHANGES IN THE S SUPPLY

S requirement differs highly between species and it varies during plant growth. The S requirement can be defined as “the minimum rate of the S

uptake and utilization, which is sufficient to obtain the maximum yield, quality and fitness", which is for crop plants equivalent to "the minimum content of S in the plant associated with maximum yield" and is regularly expressed as kg S $ha^{-1}$ in the harvest products (Haneklaus *et al.* 2006). The S demand of agricultural crops may be as low as 1 kg S $t^{-1}$ for sugar beet and as high as 17 kg S $t^{-1}$ for *Brassica* crops (Haneklaus *et al.* 2006). In physiological terms the S requirement is equivalent to the rate of S uptake, reduction and metabolism needed per gram plant biomass produced over time and can be expressed as mg S $g^{-1}$ plant $day^{-1}$ (Haneklaus *et al.* 2006). The S requirement of a crop may be predicted by scaling up the S requirement in μg S $g^{-1}$ plant $day^{-1}$ to g S $ha^{-1}$ $day^{-1}$ by estimating the crop biomass density $ha^{-1}$ (tons plant biomass $ha^{-1}$). When a plant is in the vegetative growth period, the S requirement ($S_{requirement}$) can be calculated as follows (De Kok *et al.* 2000):

$$S_{requirement} = S_{content} \cdot RGR$$

with $S_{requirement}$ (μg S $g^{-1}$ plant $day^{-1}$), S content (μg $S_{total}$ $g^{-1}$ plant biomass), and relative growth rate (RGR) of the plant (g biomass $g^{-1}$ plant $day^{-1}$). The RGR can be calculated by:

$$RGR = (\ln W_2 - \ln W_1) \cdot (t_2 - t_1)^{-1}$$

with the total plant weight in g, $W_1$ and $W_2$, at time $t_1$ and $t_2$, respectively, and the time interval (days) between two samplings $t_2$ and $t_1$.

When all other essential plant nutrients are sufficiently supplied and abiotic growth conditions are optimum, the S requirement of different crop species varies between 0.3 and 3.2 mg S $g^{-1}$ plant dry weight $day^{-1}$. Generally, the major proportion of the sulfate taken up is reduced and metabolized into organic compounds, which are essential for structural growth. However, in some plant species a large proportion of S is present as sulfate. Here, organic S content may be a better parameter for the calculation of S requirement (Haneklaus *et al.* 2006, see section below).

## YIELD STRUCTURE

*Roots*

The influence of S nutritional status on root growth is commonly neglected, though it is a major factor influencing S uptake of crops. Restricted root growth can, for instance regularly be found on headlands

due to soil compaction. Here, symptoms of S deficiency regularly appear first. Reduced root growth limits the ability of the plant to explore the soil spatially for available S and hampers its access to S resources in subsoil water (Bloem *et al.* 2000). Under humid conditions, sulfate can be leached from the root zone due to precipitation in autumn particularly on light soils, so that young plantlets do not have access to sulfate-rich capillary ascending water or groundwater. Although crops with a high S demand, such as oilseed rape, have a coarse root system which favors microbial activity and microbially-mediated degradation of ester-bonded S, this morphological modification alone might not deliver sufficient amounts of sulfate to satisfy the S demand. Whenever S supply is insufficient, this will result in the occurrence of macroscopic S deficiency symptoms, even during the very early growth stages. An increasing problem in agriculture is the enhancement of S deficiency where Tebuconazol was applied as a fungicide, as it apparently reduces not only the growth of the aboveground vegetative plant parts, but also reduces root depth and density (Bloem *et al.* 2000). Apparently this effect is also consistent in crop rotation.

Lange (1998) showed that S fertilization to leguminous crops significantly increased shoot, root, and nodule biomass of alfalfa, crimson clover, and faba bean; in the case of peas this effect was significant for shoot and nodule biomass (Figure 2). The improved root growth due to S fertilization yielded a higher number of nodules, while nodulation itself was not affected (Scherer and Lange 1996; Lange 1998).

These results strengthen the significance of a sufficient S supply in intensive farming as root growth may be inhibited and thus the risk of S deficiency enhanced. In S-deficient legumes, N that was fixed in nodules was not assimilated which caused disturbance of protein synthesis and finally resulted in the appearance of macroscopic symptoms of S deficiency (Lange 1998). At present the question cannot unequivocally be answered as to whether S deficiency affects plants and/or microsymbionts as S fertilization increased number and size of nodules, and nitrogenase activity (Singh and Raj 1988; Lange 1998).

*Yield components*

During the very early growth stages of winter cereals, severe S deficiency caused an irreversible reduction of generative yield components (Haneklaus *et al.* 1995, Figure 3). Such severe disorder could only be counterbalanced by S fertilization prior to tillering (Haneklaus *et al.* 1995). Grain yield was reduced by up to 93% if no S was applied (Haneklaus *et al.* 1995). The S nutritional status had the strongest effect on the number of kernels per ear. Cereal plants obviously retain the number of

inflorescence bearing culms at the expense of grain setting under conditions of S deficiency.

The S rate significantly influenced the number of pods per plant and seeds per pod of oilseed rape under greenhouse conditions (Schnug 1988). When the N supply was low, S fertilization had no effect on the number of pods and number of seeds per pod. When the N supply was high, S fertilization nearly doubled the number of seeds per pod. Neither variations in the N, nor in the S supply had a significant influence on the thousand grain weight (TGW). Asare and Scarisbrick (1995) could verify no significant influence of S fertilization on TGW of oilseed rape under field conditions, either. In contrast, Shukla *et al.* (2005) found a significant increase of only TGW by 9% after S fertilization under field conditions, while other changes in yield components such as the number of branches and pods per plant, seeds per pod, and seed yield were not significant so that the question arises in how far climatic conditions influenced this result.

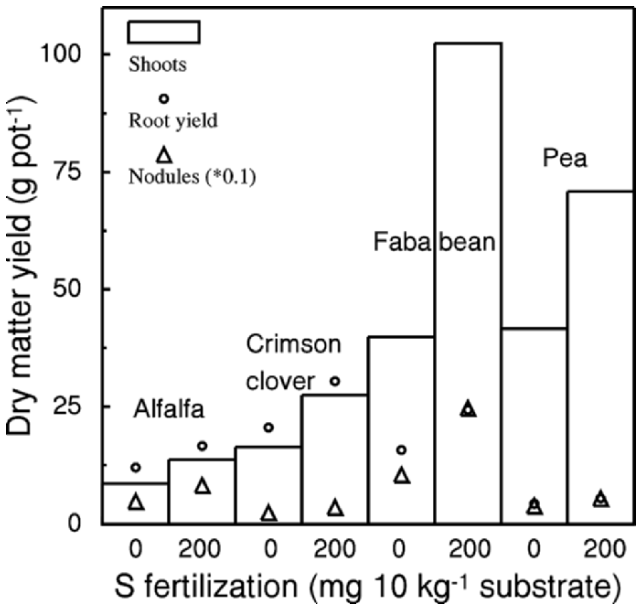


*Figure 2.* Influence of S fertilization on shoot and root biomass, and number of nodules of alfalfa, crimson clover, faba bean, and pea. (Adapted from Lange 1998.)

Investigations on the timing of S fertilization and initiation of S deficiency in oilseeds, revealed that in both cases a close and significant relationship existed between duration of S deficiency and all yield components (number of branches, number of pods per plant, number of seeds per pod, seed and straw yield) except TGW (Schnug 1988). A comparison between single and double low varieties showed that the double low cultivars had reduced components of yield structure consistently more than did the single low varieties (Schnug 1988). On average only 44% of the relative seed yield were obtained when double low plants were grown for 50% of the vegetation period under conditions of S deficiency, while the corresponding value was 57.5% for single low varieties (Schnug 1988). An assessment of the differential effect of the point of timing when S deficiency affected plant growth revealed that components of yield structure were more reduced when S deficiency occurred later during growth. From the viewpoint of plant production the area-related seed yield was reduced equally by both scenarios (Schnug 1988). Under field conditions, Nuttall and Ukrainetz (1991) recommended S fertilization to spring oilseed rape at sowing in order to avoid yield losses; otherwise with every 10 days of delay, a net yield loss of up to 7% may be incurred.

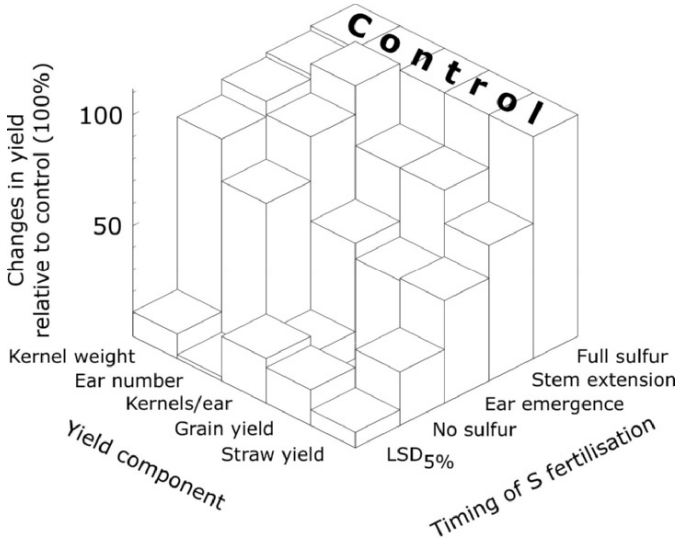


*Figure 3.* Influence of timing of S application, under conditions of severe S deficiency, on kernel weight, ear number, kernels per ear, and on grain and straw yield of wheat in comparison to a sufficiently supplied crop. (Adapted from Haneklaus *et al.* 1995.)

*Table 2*. Influence of ITCs on soil-borne fungal pathogens, bacteria, and nematodes under laboratory conditions.

| Pathogen | Effect | Ref. |
|---|---|---|
| *Aphanomyces euteiches*[a] | inhibition of mycelial growth and germination of encysted zoospores<br>reduced disease severity | (1,2) |
| *I. Bipolaris sorokiniana*[a] | retardation of mycelial growth | (3) |
| *II. Fusarium graminearum*[a] | retardation of mycelial growth (vegetative material) | |
| *III. Gaeumannomyces graminis*[a] | inhibition by mustard seed meal (25 mg) | |
| *IV. Pythium irregulare*[a] | inhibition of mycelial growth by mustard shoots (500 mg) and seed meal (5 mg) | |
| *V. Rhizoctonia solani*[a] | retardation of mycelial growth (vegetative material)<br>inhibition by mustard seed meal (25 mg) | |
| *Gaeumannomyces graminis*[a] | inhibition of mycelial growth; effect decreased with age of mycelium | (4) |
| *Fusarium oxysporum*[a] | inhibition of germination of chlamydospores | (5) |
| *Pythium irregulare*[a]<br>*Rhizoctonia solani*[a] | inhibition/retardation of mycelial growth | (6) |
| *Sclerotinia sclerotiorum*[a] | retardation of germination of sclerotia | (5) |
| *Sclerotium cepivorum*[a] | inhibition of germination of sclerotia | (5) |
| *41 isolates of bacteria*[b] | growth inhibition in relation to concentration | (7) |
| *75 isolates of fungi and oomycetes*[b] | inhibition of mycelial growth by 50% and 90% in relation to concentration | (7) |
| *Bipolaris sorokiniana*[b]<br>*Fusarium graminearum*[b]<br>*Gaeumannomyces graminis*[b]<br>*Pythium irregulare*[b]<br>*Rhizoctonia solani*[b] | fungitoxic to mycelium | (8) |
| *Fusarium oxysporum*[b] | inhibition of mycelial growth | (9) |
| *Fusarium oxysporum*[b] | suppression of conidial and chlamydospore germination | (9) |
| *Leptosphaeria maculans*[b] | inhibition of growth (except for progoitrin) | (10) |
| *Globodera rostochiensis*[b] | 100% mortality of 2nd stage juveniles | (11) |
| *Globodera rostochiensis*[b] | 100% mortality of 2nd stage juveniles | (12) |

[a]Plant material; [b]ITCs; (1) Smolinska *et al.* (1997), (2) Muehlchen *et al.* (1990), (3) Kirkegaard *et al.* (1996), (4) Angus *et al.* (1994), (5) Smolinska and Horbowicz (1999), (6) Lazzeri *et al.* (2004b), (7) Smith and Kirkegaard (2002), (8) Sarwar *et al.* (1998), (9) Smolinska *et al.* (2003), (10) Mithen and Lewis (1986), (11) Serra *et al.* (2002), (13) Pinto *et al.* (1998).

*Table 3.* Influence of excessive S supply on yield components and selected plant characteristics.

| Crop | Highest S rate | | Effect of highest S rate | Ref. |
|---|---|---|---|---|
| Bean | 600 | mg pot$^{-1}$ | reduced biomass (–58%), amino acid (–59%) and protein content (–50%) in leaves | (1) |
| Broccoli | 180 | mg kg$^{-1}$ | reduction of (market) yield and total biomass | (2) |
| Cabbage | 360 | mg kg$^{-1}$ | reduction of (market) yield and total biomass | (2) |
| Cabbage | 90 | kg ha$^{-1}$ | reduction of (market) yield | (3) |
| Cabbage | 100 | kg ha$^{-1}$ | reduction of (market) yield | (4) |
| Grass | 300 | kg ha$^{-1}$ | increased shoot and root biomass | (5) |
| Kidney beans | 6,000 | kg ha$^{-1}$ | no effect on yield components and protein content of seeds (S application in previous year) | (6) |
| Maize | 120 | kg ha$^{-1}$ | reduction of yield components with S >60 kg ha$^{-1}$ | (7) |
| Onion | 115 | mg kg$^{-1}$ | no influence on yield | (2) |
| Pea | 400 | mg kg$^{-1}$ | reduced seed yield in 1 out of 3 genotypes (reduced seed number and seed weight) | (8) |
| Pea | 75 | kg ha$^{-1}$ | reduced vegetative biomass, seed yield, seed protein, no of effective nodules, leghaemoglobin content | (9) |
| Potato | 150 | kg ha$^{-1}$ | reduced tuber yield | (10) |
| Soybean | 90 | kg ha$^{-1}$ | highest grain yield (regular fertilization over 6 years) | (11) |
| Soybean | 60 | kg ha$^{-1}$ | S fertilization increased number of nodules/plant, active nodules, d.w. of nodules, and chlorophyll content | (12) |
| Soybean | 240 | kg ha$^{-1}$ | reduced biomass and seed yield (strength of effect N-related) | (13) |
| Tomato | 222 | mg l$^{-1}$ | Ca imbalance in plants; no significant influence on fruit yield and quality | (14) |
| Tomato | 666 | mg pot$^{-1}$ | reduced photosynthetic capacity and protein N content; no effect on biomass | (15) |
| Wheat | 224 | kg ha$^{-1}$ | decrease of forage and grain yield in relation to year and location (regular application over 7 years) | (16) |
| Cultivation on post-mining land/amelioration of salinity and alkalinity | | | | |
| Alfalfa | 4,730 | mg kg$^{-1}$ | high plant available sulfate-S in soils did not yield over-proportional S uptake (0.23–0.48% S); accumulation of non-protein-N compounds with higher S level | (17) |
| Alfalfa | 2,800 | mg kg$^{-1}$ | gypsum amendment enhanced salt tolerance and thus maintained yield | (18) |
| Tomato | 1,700 | mg l$^{-1}$ | decreased fruit weight and size | (19) |
| Tomato | 900 | kg ha$^{-1}$ | increased yield and fruit weight; less unripe fruit | (20) |
| Pawpaw | 3,500 | kg ha$^{-1}$ | up to 73% higher lateral branch extension and 100% higher dry matter production | (21) |

(1) Ruiz *et al.* (2005), (2) Blankenburg (2002), (3) Rhoads and Olson (2001), (4) McKeown and Bakker (2003), (5) Olson and Jacobsen (1999), (6) Hojjati (1976), (7) Khan *et al.* (2006), (8) Randall *et al.* (1979), (9) Singh and Raj (1988), (10) Singh *et al.* (2001), (11) Saha *et al.* (2001), (12) Ganeshamurthy and Reddy (2000), (13) Abbès *et al.* (1992), (14) Lopez *et al.* (2002), (15) Xu *et al.* (1996), (16) Girma *et al.* (2005), (17) Pucek and Pys (1999), (18) Vaughan *et al.* (2002), (19) Cerda *et al.* (1984), (20) Di Candilo *et al.* (1993), (21) Picchioni *et al.* (2004).

In sunflower, S deficiency delayed floret initiation and anthesis, but not maturity under controlled growth conditions (Hocking *et al.* 1987). Additionally, the number of seeds per plant and TGW were reduced. The authors concluded that a sufficient S supply before floret initiation is important for initiating a maximum number of florets and thus potential seeds.

## BIOMASS DEVELOPMENT

There are small differences in patterns of uptake of different macronutrients during the vegetation period. S uptake runs more or less parallel to biomass development and is proportional to seed yield. Oilseed rape for instance may take up about one-third of its total S demand before winter resting. Usually, under conditions of S deficiency, S fertilization significantly increases vegetative and generative plant biomass production. Lack of response is often related to experimental conditions such as site and climatic conditions (Kowalenko 2000).

Growing leguminous crops such as soybean, which have been previously multiplied on S-deficient soils, increases the susceptibility of young plantlets against an insufficient S supply, as the proportion of S-containing storage proteins is reduced (Hitsuda *et al.* 2005). S deficiency in the vegetative stage reduced biomass (Randall and Wrigley 1986) and a lower plant dry matter of sunflower was closely related to the N supply in such a way that no impact was found at a low N input, however, severe losses were recorded when the N supply was high (Hocking *et al.* 1987).

The influence of S deficiency on vegetative and generative yield has been studied in detail for agricultural crops and is comprehensively summarized for instance by Pedersen *et al.* (1998) and Aulakh (2003).

### *Plant growth under excessive S availability*

While numerous studies have investigated the influence of S fertilization on crop productivity under limiting conditions, the impact of excessive S input in temperate regions has only been dealt with sporadically. An exception is the influence of atmospheric S pollution on plant growth. In comparison, extremely high S rates are applied, for instance, in desert agriculture for the amelioration of salinity and alkalinity, and in the course of cultivating post-mining land (Table 3).

S is commonly considered as being highly biocompliant such that excess S neither diminishes productivity, nor impairs quality of the plant products. There are, however, indications that overrated S fertilization may

reduce crop yield and that this effect is related to crop type (Table 3). A major handicap of a proper attribution of effects to an excessive S rate (Table 3) is the lack of information about other growth limiting factors, antagonistic effects with other essential plant nutrients, and the S nutritional status itself.

Even more important than detrimental effects of an excess S supply on crop parameters is a possibly detrimental effect on animal health. Prominent examples of adverse effects of high S intake on ruminants are polioencephalomalacia, a neurological disorder and haemolytic anaemia (Stoewsand 1995; Gould *et al.* 2002). The risk of polioencephalomalacia exists when grass which contains more than 0.38% S is eaten by the animals (Gould *et al.* 2002).

Excess S may cause a premature leaf fall (Motavalli *et al.* 2006). Even a uniform application rate of 134 kg $ha^{-1}$ S causes site-specifical yield increases and depressions as was shown for forage grass (Kowalenko 2000). These results fit to the observations of Donald and Chapman (1998) who found indications of S toxicity at rates of 200 kg $ha^{-1}$ S to grass and clover. Forage yield at stem extension was reduced by about 5% at 224 kg $ha^{-1}$ S, while the corresponding value for grain yield was even as high as 11% (Girma *et al.* 2005). Khan *et al.* (2006) found that 120 kg $ha^{-1}$ S reduced dry-matter yield of maize significantly compared to a sufficiently supplied crop, such that the yield level equaled that of the S deficient control plots. Excessive S produced the lowest grain yield, and also TGW (Khan *et al.* 2006). This growth-depressive effect was observed at total S concentrations of about 6–9 mg $g^{-1}$ S dry weight at silking stage.

Other reports from McKeown and Bakker (2003) and Sanderson (2003) delivered contradictory results. Cabbage yield decreased when S rates exceeded 55 kg $ha^{-1}$ S; this effect was not significant for the harvest products of broccoli though biomass production was reduced 8–10 times (McKeown and Bakker 2003). In contrast, S rates of up to 670 kg $ha^{-1}$ S proved to be compliant for broccoli (Sanderson 2003). In both experiments the S source was gypsum so that a Ca effect might be excluded. Using a different S source it might be possible that excessive S rates induce Ca deficiency as was shown for tomatoes in hydroponics, which revealed blossom end, rot symptoms (Lopez *et al.* 2002). In further experiments, S fertilizer rates of 45–90 kg $ha^{-1}$ S reduced cabbage yield with the head size being affected in particular (Rhoads and Olson 2001); in the pot experiments of Blankenburg (2002) a change of the S supply from sufficient to excess resulted in a reduction of head and floret yield of cabbage and broccoli by 16.5% and 18.4%; the corresponding increase of the total S content was from 7.9 to 9.6 mg $g^{-1}$ S and 8.8 to 10.9 mg $g^{-1}$ S, respectively.

Disproportionate S rates significantly reduced shoot biomass of beans in a pot experiment with the S concentration in the leaf tissue more than doubled with values of 1.25% S under optimum supply increasing to 2.71% S dry weight under excessive S supply (Ruiz *et al.* 2005).

The effects of extreme S applications when used in desert agriculture are also not consistent (Table 3). For pawpaw, Picchioni *et al.* (2004) found that 15 t $ha^{-1}$ gypsum significantly improved growth parameters; the total S concentration in roots (1.9 mg $g^{-1}$ S) and trunks (0.7 mg $g^{-1}$ S) was not significantly increased because of a dilution effect through increased plant growth. In comparison, stems of tomato plants were thinner, leaves darker green and smaller when grown under excessive S and symptoms became more pronunced with plant age and affected the aboveground biomass more than root growth (Cerda *et al.* 1984, Table 3). Fruit yield, both fruit weight and size, was reduced by up to 52%, whilst the number of fruits was not affected. In comparison, severe S deficiency reduced fruit yield by 58% (Cerda *et al.* 1984). Relative increases in organic S concentrations in different plant parts, for instance from 0.2 under conditions of S deficiency to 0.33% S under excess S in leaves at flowering were determined, but which were distinctly lower than the corresponding values found for sulfate (0.1% and 1.79% $SO_4$-S, respectively).

*Critical nutrient values and ranges*

For the evaluation of S nutritional status and prognosis of crop yield, different S species such as organic S, sulfate, total S, and the N:S ratio of various plant parts are determined, usually during the vegetation period and results are interpreted by employing diverse statistical approaches. It is the large variation in experimental conditions and mathematical procedures which make it more or less impossible to compare results from different experiments (Haneklaus *et al.* 2006). Thus the main objective, the reliable deduction of critical values is confronted with major limitations. Important threshold markers for the S supply are: the symptomatological value, which reflects the S concentration below which deficiency symptoms become visible; the critical nutrient value, which stands for the S concentration above which the plant is sufficiently supplied with S for achieving the maximum potential yield or yield reduced by 5%, 10%, and 20%; and the toxicological value, which indicates the S concentration above which toxicity symptoms can be observed. A comprehensive overview of crop-specific deficiency and sufficiency ranges of S supply has been compiled by Haneklaus *et al.* (2006), and the major outcome can be summarized as follows: severe to moderate S deficiency is indicated generally by sulfate concentrations of <0.15 mg $g^{-1}$ sulfate-S and total S

concentrations of <1.7 mg $g^{-1}$ S; for *Poaceae* and non-*Brassica* vegetables total S concentrations may be lower with 0.9 mg $g^{-1}$ S or higher with 2.9 mg $g^{-1}$ S, respectively. An adequate S supply is reflected by total S concentrations of 1.7–4 mg $g^{-1}$ S; *Brassica* crops show a higher optimum range with values of 4.8 (oil crops) to 7.5 (vegetables) mg $g^{-1}$ S. For N:S ratio and sulfate concentrations, values of 16–20 and 150–1,600 mg $kg^{-1}$ sulfate-S, respectively reflect a sufficient S supply. In the literature, S concentrations, which impair crop performance are rare for S. An excessive S supply can be expected if plants contain more than 2.8 mg $g^{-1}$ sulfate-S; for fodder crops total S concentrations of only 3.2 mg $g^{-1}$ S may be already excessive, while the corresponding value for non-*Brassica* vegetables would be 10 mg $g^{-1}$ S (Haneklaus *et al.* 2006). In general, it can be expected that yield depressions occur at lower S concentrations in plants when green matter is harvested, such as forage grasses and cabbage (see Table 3).

The boundary line approach is a robust tool to evaluate without bias the relationship between individual growth factors and yield and to determine optimum values and ranges of the soil and plant nutrient status of a crop (for a detailed description of *Bolides*, the upper boundary line development system see Haneklaus *et al.* 2006). The boundary line approach has been applied to determine threshold values for S deficiency, sufficiency, and excess in oilseed rape, cereals, and sugar beet (Table 4). The interpretation of cereal and oilseed rape values is based on more than 5,000 data pairs from greenhouse and field experiments as well as field surveys which have been compiled since 1973 and 1980, respectively. Details for sugar beet are given by Haneklaus *et al.* (1998).

Comparing these threshold values with median values from literature (Haneklaus *et al.* 2006), it is striking that total S concentrations which can be found when macroscopic symptoms are visible are in good agreement. The same applies for threshold concentrations indicating a sufficient S supply of cereals and sugar beet, although for oilseed rape significantly higher values were determined. The reason is most likely that the yield of oilseed rape crops was distinctly lower in many studies; only for the 75% percentile of literature data was there a sufficient S supply indicated by a S concentration of 6.7 mg $g^{-1}$ S (Haneklaus *et al.* 2006). For the first time upper critical S concentrations in cereals and oilseed rape, which result in yield depressions of 10% have been calculated by a robust statistical procedure. For sugar beet upper critical S concentrations were determined before by *Bolides* (Haneklaus *et al.* 1998).

*Table 4.* Threshold values for total S concentrations (mg $g^{-1}$ S, d.w.) in younger leaves of oilseed rape and sugar beet, and whole aboveground biomass of cereals at start of stem extension and canopy closing.

| | Deficiency | Sufficiency | | Excess |
|---|---|---|---|---|
| Crop | Symptomatological threshold | Lower critical value (–5% yield) | Maximum yield[1] | Upper critical value (– 10% yield) |
| Cereals | <1.2 | 3.2 | 4.0 | >7.5 |
| Rape | <2.8[2] and <3.5[3] | 5.5 | 6.5 | >14.0 |
| Sugar beet | <1.7 | 3.0 | 3.5 | >4.5 |

[1]seed (oilseed rape), grain (cereals), root and sugar (sugar beet) yield; [2]single low and [3]double low varieties

At present the physiological background of sulfate toxicity is unknown but some speculations about regulatory mechanisms may be formulated. A first hint of possible metabolic dysfunctions comes from the fact that excessive S supply to tomatoes induces Ca deficiency which becomes visible as blossom end rot (Cerda *et al.* 1984). May *et al.* (1998) assumed interactions between Ca and redox based signaling processes. The reactivity of the enzyme serine acetyltransferase, which catalyzes the first reaction in the biosynthesis of cysteine from serine was regulated by Ca-dependent protein kinase phosphorylation in soybean (Liu *et al.* 2006). Kim and Kim (2002) showed that sulfhydryl containing metabolites controlled the increase of cellular $Ca^{2+}$ under conditions of S amino acid deprivation in rat heptoma cells, which is a further reference to a redox-state regulation of Ca. Additionally, pool sizes of ascorbic acid and GSH, and functional and regulatory interactions between them might be involved in growth inhibition under excessive S stress; a similar mode of action was proposed for boron deficient plants (Lukaszewski and Blevins 1996). Thus it might be possible that under excessive S stress crosstalk between Ca and S metabolic pathways hampers S homeostasis and thus unfolds its toxic effects. The identification of genes that govern the plant ionome might elucidate the mechanisms controlling S accumulation.

## DRY MATTER COMPOSITION

The dry matter composition of plant products is an important quality parameter of foodstuffs and animal feed. The S nutritional status of crops has a significant influence on the nutritive value and sensory features of

plant products. S-containing flavor compounds are, for example, cysteine in fruits (Shankaranarayana *et al.* 1973), asparagusic acid, 3-mercaptoisobutyric acid, 3-methylthioisobutyric acid, diisobutyric acid disulfide, and 3-*S*-acetylthio-methacrylic acid in asparagus (Tressel *et al.* 1977), and glucosinolates and alliins in mustard, radish, onion, and garlic (Bloem *et al.* 2004). The influence of S fertilization on secondary S-containing compounds has been comprehensively summarized by Haneklaus *et al.* (2006).

*Cysteine and methionine*

Vegetable proteins have been recognized as being of lower nutritional value than animal proteins. The reason is the imbalanced cysteine to methionine ratio rather than the lower S content per gram of protein (Massey 2003, see above). The amino acids cysteine and methionine are the major end products of sulfate assimilation in plants and bind up to 90% of the total S (Giovanelli *et al.* 1980). A significant relationship between S supply and S-containing amino acids exists only under extreme S deficiency where macroscopic symptoms are visible (Haneklaus *et al.* 2006). Under conditions of S deficiency, firstly a decrease of S-containing amino acids in proteins is found (Schnug 1997). As the amino acid composition is genetically determined this effect is, however limited, and thereafter the total protein content will be reduced (Schnug 1997). The transition point to a reduced protein content matches the appearance of severe S deficiency symptoms (Schnug 1997). An insufficient S supply in the vegetative stage reduced biomass, the amino acid composition was only slightly influenced, however significant changes were observed in generative parts (Randall and Wrigley 1986). The authors attributed this to the fact that leaf proteins are mainly functional, while seed proteins are mainly for storage.

Eppendorfer and Eggum (1992) found the biological value of proteins in potatoes reduced from 94 to 55 by S deficiency at high N supply and from 65 to 40 and 70 to 61 in kale and field beans, respectively. Whilst the essential amino acid concentrations declined due to S deficiency, the content of amino acids of low nutritional value, such as arginine, asparagines, and glutamic acid, increased (Eppendorfer and Eggum 1992). The final influence of the S nutritional status is closely related to the N supply and they should therefore be assessed together. At low N supply, S deficiency increased the starch content in vegetative parts of kale and ryegrass, and seeds of oilseed rape, while this effect was not found at high N input. At high N levels, S deficiency reduced the methionine content in vegetative tissue of kale and ryegrass more severely than the cysteine

content, whereas in seeds of oilseed rape and field bean the cystine content was more strongly reduced (Eppendorfer and Eggum 1992).

The composition of seeds reflects an adaptation of plants to the S supply. Species with a low TGW, such as oilseed rape, typically rely on oil and fat as energy sources for the embryo. The total protein content of their seeds is uniform and more or less independent of the S supply. An increase of cysteine and methionine in total protein from about 0.8% to 1.1%, and 0.75% to 1.4%, respectively with increasing S supply from moderate deficiency towards sufficient supply (Mortensen *et al.* 1992), did not cause any significant changes in total S bound in the protein fraction. This was confirmed using the X-RF method for the indirect determination of GSLs by determining the total S content (Schnug and Haneklaus 1990). Adaptation of the metabolic sink to the S supply is maintained solely by the number of seeds produced (Schnug and Haneklaus 1994).

The endosperm of cereals which has a distinctly higher TGW, consists mainly of carbohydrates as the main energy reserve. S deficiency impairs the baking quality of wheat before crop productivity is reduced and a lack of protein or S could partly be compensated by increased concentrations of either compound (Haneklaus *et al.* 2006). The supply before anthesis is critical for wheat grain yield and quality as results of Haneklaus and Schnug (1992), Haneklaus *et al.* (1992 and 1995), and Anderson and Fitzgerald (2001) reveal. So, the S content of plants deprived of S from start of anthesis equaled that of plants fully supplied with S throughout the vegetation period, whereby sulfate was derived presumably from uptake by roots and GSH translocation from flag leaves (Anderson and Fitzgerald 2001).

In legumes, which have a high TGW, the cotyledons have a major storage function, whilst the proportions of embryo and endosperm are minor. Krishnan *et al.* (2005) found that soybean cultivars with high protein content had a low content of S-containing amino acids and vice versa. Under conditions of S deficiency these plants reduce the amount of the S-rich fractions. In pea seeds, legumin-type globulin proteins contained a higher proportion of S-containing amino acids than vicilin-type globulins (Randall *et al.* 1979). Extreme S deficiency yielded a decrease in the legumin content, whilst both increases and reductions were found when S was excessively applied to different genotypes (Randall *et al.* 1979). Excess S was accumulated as sulfate and the nonprotein amino acid *S*-methylcysteine in lupin and peas (Randall and Wrigley 1986). Sexton *et al.* (2002) showed that pods and seeds seemed to be the major sites of S reduction and that it was the S supply during reproductive growth which influenced protein-S in soybean seeds. In accordance with these results, Sunarpi and Anderson (1997) determined that 87% of the S in seeds was

taken up by roots during seed filling, with the balance coming from redistribution. A sufficient S supply before floret initiation proved to be nevertheless important for inserting maximum number of florets in sunflower (Hocking *et al.* 1987). Schroeder (1984) suggested that a sufficient S supply during seed filling might contribute to a significant improvement of the nutritive value of peas.

TGW, protein, and fat content of oilseed rape seeds were only affected by the S supply under conditions of extreme S deficiency (Schnug 1988), otherwise no significant influence could be verified under field conditions (Schnug 1988; Asare and Scarisbrick 1995). In contrast, Eppendorfer and Eggum (1992) and Shukla *et al.* (2005) found a significant increase in TGW by S fertilization. S deficient sunflower plants produced seeds with a lower TGW, while the oil content was not influenced (Hocking *et al.* 1987).

Crosstalk between S and N metabolic pathways will not only influence yield structure, biomass development, and dry matter composition, but also N-use efficiency of agricultural crops. Under conditions of S deficiency, nitrate and non-S-containing amino acids accumulate which may reduce the nitrate reductase activity (Srivastava 1980; Schnug 1997). Randall and Wrigley (1986) determined an increase from <5% to 30% of nonprotein N in seeds under conditions of severe S deficiency. S fertilization promotes nitrate reduction and thus reduces the nitrate content in vegetative plant tissues. Disproportionate N fertilization enforces the negative impact of an insufficient S supply on plant quality and it is inevitably linked to avoidable N losses to the environment. On average, per kilogram of insufficient S required to satisfy the demand of the crop, 15 kg of N are prone to be lost to the environment (Schnug 1997). The solution of this problem cannot be an excess S dose as adverse effects on crop productivity and quality are possible, and in any case, this is not compliant with a sustainable use of resources. A holistic appraisal of S interactions in crop ecosystems from field to fork should therefore always be a part of the farm management system.

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Chapter 3

# SULFUR IN FOREST ECOSYSTEMS

Michael Tausz

## INTRODUCTION

The relationship of forest ecosystems and sulfur is historically, and in the public perception, dominated by the impact of sulfurous air pollutants, which are still leading to environmental disasters such as forest decline and tree dieback in many parts of the world. Unfortunately, this seems to have masked the basic fact that sulfur is an essential nutrient element for all plants including forest trees, and that sulfur compounds play crucial roles in the defence of trees against environmental stress factors.

The main distinguishing feature of forest ecosystems is the dominance of the tree life-form, and whilst the biochemistry of sulfur metabolism in tree cells is not fundamentally different from plant cells in general (principles laid out elsewhere in this volume), modifications of whole-plant metabolism related to the typical biology of trees, for example long life spans, long internal transport distances, and large volumes of woody tissues, are significant. This chapter, therefore aims to characterize those ecophysiological aspects of sulfur metabolism that set trees and forests apart from agricultural and other ecosystems dominated by short-lived herbaceous plants.

## SULFUR NUTRITION AND METABOLISM OF TREES

Sulfur is an essential macroelement for all organisms including forest trees. Plants normally take up sulfur from the soil and subsequently reduce it (if taken up in a higher oxidation state than -II, e.g. as sulfate), incorporate it into the essential amino acid cysteine, and from there into all other organic



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sulfur compounds, and distribute it into all organs (Figure 1). Reduction, incorporation, and distribution of S do not necessarily happen in this sequence, because the extent to which the different reactions operate depends on the tissue and organ, seasonal variation, environmental conditions, and the growth form. In particular the distribution and cycling of S in trees is different from herbaceous plants, because trees have to redistribute their resources depending on the seasonal cycles, have access to large volumes of potential storage tissues in their stems and the transport distances are considerably longer than in herbaceous plants.

*Sulfur content and sulfur compounds in trees*

The sulfur-containing amino acids cysteine and methionine are essential protein constituents comprising a large proportion of the total sulfur content in plants, a fact that is reflected by the relatively uniform N/S ratio in tissues (Hogan and Rennenberg 1998). Additional organic S compounds present in abundance in most plants are the nonprotein tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH) and its precursor γ-glutamyl-cysteine, sulfolipids, thionins, thioazoles, and others, as well as a large number of secondary compounds specific to certain species and/or induced only under particular conditions. Inorganic sulfur is mainly present as sulfate, because sulfite and sulfide are metabolized at high rates and their tissue concentrations are usually kept very low.

Total S contents and the ratio of inorganic to organic S in tree tissues may vary according to the species, the tissue type, environmental conditions, developmental stage, seasonal fluctuations, and supply with S and other nutrients. In needles of spruce (*Picea abies*) seedlings, for example, the proportion of inorganic S in total S varied between 10% and 20% (between control trees and trees subjected to additional atmospheric S sources, Tausz *et al.* 2003). As for most nutrients, deciduous foliage has a higher S content on a dry weight basis, which simply reflects the fact that it contains less sclerenchymatic elements and cell wall material (Table 1). Total S contents in foliar tissues are used in diagnosis of nutritional deficiencies and as an indication for an overoptimal S supply due to airborne S input. For example, values above 1.5 mg S $g^{-1}$ needle dry weight indicate sulfurous air pollution impact on *Pinus* (Huttunen *et al.* 1985).

*Sulfur uptake in trees*

As for most plants, the normal sulfur source for trees is sulfate taken up from the soil via fine roots. Sulfate uptake into the roots and loading into the xylem proceeds via specific, energy dependent transporters, which are

well characterized on the molecular level for herbaceous plants, where at least 14 different forms exist in *Arabidopsis* for example (Chapter 1, Hawkesford 2003). Analysis of the poplar genome indicates a similar large gene family. Functional analysis of sulfate uptake kinetics into the roots identified at least two distinct root uptake systems in trees (*Populus*, *Fagus*, *Quercus*), a high affinity and a low affinity system. Given the low sulfur concentrations in forest soil water, only the high affinity systems (apparent $K_m$ between 5 μM and 15 μM sulfate) may be of ecophysiological significance (Herschbach and Rennenberg 2001). Feedback regulation of root sulfate uptake by phloem translocated glutathione (as shown for herbaceous plants) was not corroborated for trees (*Populus*). Instead, the sulfate/glutathione ratio in the phloem was suggested as a potential regulator (Herschbach *et al.* 2000). Cross regulation by N availability, probably by *O*-acetylserine, the substrate of sulfur incorporation into amino acids, seems also important (Herschbach and Rennenberg 2001).

*Table 1.* Some literature examples for typical total sulfur contents (μmol $g^{-1}$ dry weight) in organs of selected tree species (evergreen conifer, deciduous broadleaf, sclerophyllous evergreen broadleaf). Averages (minima–maxima).

| | *Picea abies* | *Fagus sylvatica* | *Eucalyptus spp.* |
|---|---|---|---|
| Foliage | 32 (17–43)[1] | 52 (37–70)[1] | 28[4]; 47[5] |
| | | | 56 (19–81)[6] |
| | 50[3] | 43-56[2] | 81 (53–103)[7] |
| Stem | | 37 (32–42)[2] | 7[4]; 8[5] |
| Roots | 78[3] | 45 (40–49)[2] | |

[1]Bauer *et al.* 1997; mature trees (>100 years) at 5 (*Fagus*) to 7 (*Picea*) forest stands across Europe. [2]Peuke and Rennenberg 2004; seedlings from 11 provenances under greenhouse conditions. The roots refer to mixed samples of the total root systems. [3]Tausz *et al.* 2003; seedlings under growth chamber conditions. The root values refer only to fine roots (<2 mm diameter). [4]Judd *et al.* 1996; average of a range of *Eucalyptus* species in mature (>40 years) forest stands; "stem" values refer to total branch concentrations. [5]Judd *et al.* 1996; average of a range of *Eucalyptus* species in young (10 years) plantations. [6]Judd *et al.* 1996; plantation grown young *Eucalyptus grandis* [7]Judd *et al.* 1996; (mostly glasshouse grown) *Eucalyptus grandis* seedlings.

In a forest ecosystem, the role of mycorrhiza, the symbiosis between tree roots and fungi, on tree sulfur nutrition needs to be taken into account. Mycorrhiza can improve the nutritional state of plants and virtually all forest trees are partners in mycorrhiza. However, mycorrhiza associations do not improve sulfur uptake in trees (shown for *Populus, Quercus*, and *Picea*), although mycorrhized trees can become more resistant to short-term

sulfur starvation (Herschbach and Rennenberg 2001). Even though mycorrhiza may not increase uptake rates, the pathway of sulfate into the plant will be different from non-mycorrhized plants. At least in ectomycorrhiza, the predominant mycorrhiza-form of many forest trees, roots have little plant tissue available outside of a dense fungal mantle, which is near-impenetrable for sulfate. This means that uptake must proceed through the fungal hyphae, and potentially different regulatory mechanisms may apply (Taylor and Peterson 2005).

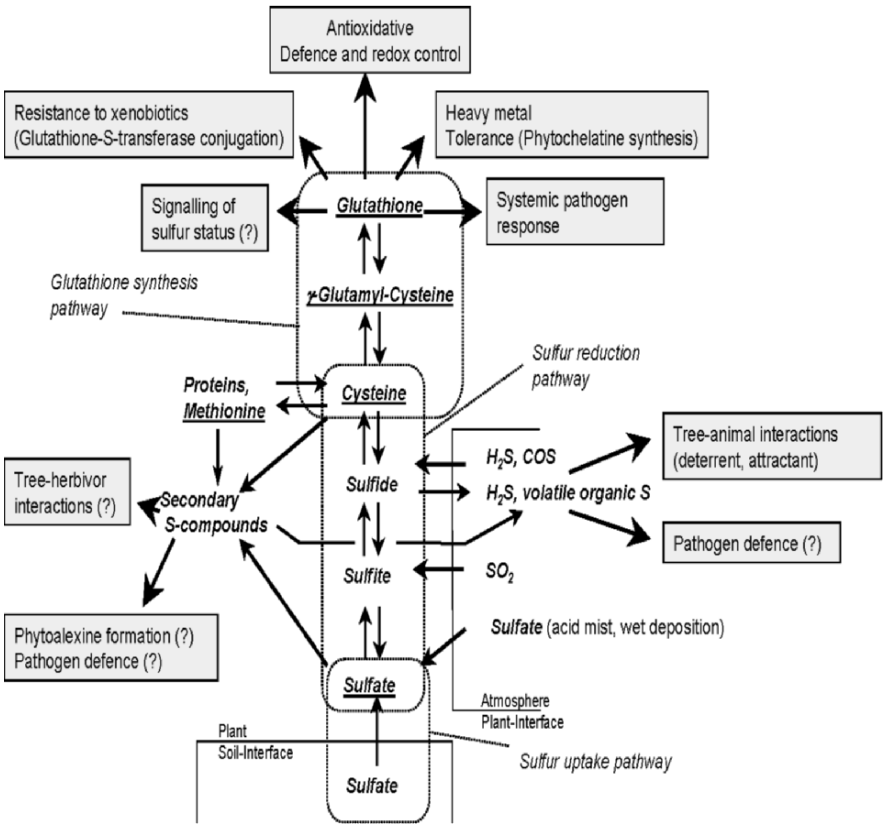


*Figure 1.* Some relationships between tree sulfur metabolism and tree–environment interactions. Ecophysiological functions of sulfur compounds are marked by *grey boxes*, main long-distance transport forms are *underlined*, (?) indicates that this role has not been clearly established in forest trees.

*Whole tree regulation of sulfur metabolism*

A number of in-depth studies (reviewed by Rennenberg and Herschbach 1995; Herschbach and Rennenberg 2001) compared sulfur nutrition of the deciduous broad leaf species, beech (*Fagus sylvatica*), to the evergreen conifer, spruce (*Picea abies*), and found appreciable differences related to the different life cycles of these trees (Rennenberg and Herschbach 1995). The evergreen *Picea abies* takes up sulfate, transports it into the canopy, where it is reduced mainly in older needles. Young needles or buds have only low activities of the enzymes of sulfur reduction and receive reduced sulfur, imported as glutathione, from older needles. Glutathione is exported at high rates from older needles during the night, and translocated in xylem and phloem towards the younger needles, where it may support day and night protein synthesis. Under normal conditions, spruce trees do not seem to transport reduced sulfur (at least not in form of the thiols glutathione, cysteine, or γ-glutamylcysteine) downwards from the canopy towards trunk and roots. Reduced sulfur requirements of the organs below the canopy may be met by root sulfur reduction, as reduced sulfur compounds are found in appreciable amounts in the xylem sap of the trunk (Kostner *et al.* 1998). However, under exceptional conditions, for example with high S uptake from the atmosphere directly into the foliage, spruce trees seem capable of transporting organic sulfur compounds (most probably glutathione) from the needles into the roots (Tausz *et al.* 2003).

In deciduous beech, in contrast, sulfur nutrition of the developing leaf tissues at bud break is supported by both reduced organic sulfur in form of thiols (mainly cysteine and some glutathione) and sulfate supplied in the xylem. Cysteine seems to originate mainly from storage proteins in the trunk, which accumulate during the vegetation period through import of glutathione and sulfate (rather than cysteine) from leaves into the trunk.

## SULFUR TOXICITY ON TREES AND FOREST ECOSYSTEMS

Forests have been subject to excess atmospheric sulfur, mainly derived from sulfur dioxide originating from the burning of fossil fuels, for the major part of the 20th century, and in central Europe from as early as the 1870s (Schulze 1989). Exposure to high levels of atmospheric sulfur dioxide leads to the “classical smoke damage to forests” as has commonly been observed in Central Europe with the beginning of industrialization (Kandler and Innes 1995). Such damage and dieback can be directly

attributed to acute toxic effects of $SO_2$ on trees (Pfanz and Beyschlag 1993), with conifers being highly susceptible. Emission control technologies decreased atmospheric $SO_2$ concentrations in many regions of the world (e.g. from 75 nl $l^{-1}$ in the late 1960s to less than 11 nl $l^{-1}$ in the late 1980s in the Ruhr area in Germany, Kandler and Innes 1995), which decreased the incidence of acute $SO_2$ effects on forests. However, $SO_2$-related problems may still persist in some regions of Europe (*cf.* Augustin *et al.* 2005), and are possibly on the rise in developing countries, where they are poorly studied and documented.

In addition to direct toxic effects of the gas, atmospheric $SO_2$ is oxidized to sulfate, which is then deposited into forest ecosystems via precipitation. Atmospheric sulfate deposition can also have direct effects on trees, but even more significant effects on the forest ecosystem level (e.g. nutrient cycling), and hence contribute to a general decline in forest ecosystem health. It is worth noting that due to generally high atmospheric sulfur deposition, forest ecosystems deficient in sulfur have formerly only been described from remote areas in the northwestern United States, in Australia, and in East Africa (Johnson and Mitchell 1998). On the other hand, due to high leaching loss rates of sulfur from soil parent materials, atmospheric sulfur seems to be the major sulfur source for all forest ecosystems, even those in low sulfur input areas (Johnson and Mitchell 1998).

*Direct exchange of sulfur compounds between tree foliage and atmosphere*

In addition to sulfur dioxide ($SO_2$), a number of other sulfurous gases such as hydrogen sulfide ($H_2S$), carbonyl sulfide (COS), carbon disulfide ($CS_2$), dimethyl sulfide ($CH_3SCH_3$, DMS), and methyl mercaptan ($CH_3SH$) are present as trace gases in the atmosphere (Chapters 4 and 5). While their concentrations are low (in the range of pl $l^{-1}$) in remote rural areas, they can be substantially higher in the vicinity of industrial (both primary and secondary) and volcanic activities.

All sulfurous gases are taken up by trees mainly via stomata, but the mechanisms limiting their uptake rates are different for oxidized ($SO_2$) and reduced ($H_2S$) gases. Due to the fast decomposition of $SO_2$ in the aqueous phase of mesophyll cell walls (forming sulfuric acid), internal concentrations are close to zero, hence the concentration gradient driving its uptake is only dependent on the outside concentration, and uptake rates increase linearly with increasing concentration (De Kok and Tausz 2001, Chapter 5). Uptake rates of $H_2S$, on the other hand, show saturation at high outside concentrations, which suggests a limitation by internal metabolic processes. *O*-acetylserine(thiol) lyase, the enzyme responsible for incorporating sulfide

into cysteine, seems to be the rate limiting step (De Kok and Tausz 2001, Chapter 5).

If sulfide accumulates in leaves, it may be (re)emitted as $H_2S$ following the equilibrium between dissolved sulfide and $H_2S$ at the liquid–gas interface (Chapter 5). While this is not directly measurable under $H_2S$ exposure (but possibly contributes to the saturation of uptake rates), $H_2S$ reemission from tree foliage has been demonstrated after $SO_2$ exposure or from excess sulfur in the soil, and even in absence of excess sulfur. $H_2S$ release has been regarded as a means of rapidly adjusting the sulfur assimilation rates to changing needs, which might be of particular importance in trees (Hogan and Rennenberg 1998).

Metabolic processes may also limit the potential uptake rates of volatile organic sulfur compounds (Geng and Mu 2006; Kesselmeier *et al.* 1993; Xu *et al.* 2002, Chapter 5). Under field conditions with ambient atmospheric concentrations of these gases, forests and trees are sources for DMS and methylmercaptane, but can be both sources and sinks for COS and $CS_2$, depending on the species (Xu *et al.* 2002) and on physiological factors, such as assimilation rate and stomatal aperture (Geng and Mu 2006; Xu *et al.* 2002, Chapter 5).

Although stomata are considered relatively impenetrable to aqueous ion uptake; trees exposed to sulfate-containing acid mist exhibited higher foliar sulfate concentrations. It is assumed that uptake is possible through the incomplete cuticles in young leaves. Interestingly, such foliar absorbed sulfate accumulates in the apoplast, whereas excess sulfate from soil uptake and other processes is usually located in vacuoles (Sheppard 1994).

*Metabolism and toxicity of atmospheric sulfur in trees*

Both $SO_2$ and $H_2S$ have long been known as phytotoxic gases (De Kok 1990; De Kok *et al.* 1998, Chapter 5). Effective concentrations, which may cause chronic injury, can be as low as 10 nl $l^{-1}$ for $SO_2$ and 30 nl $l^{-1}$ for $H_2S$ (Posthumus 1998, Chapter 5). Acute injury to sensitive plants has been observed at concentrations as low as 30 nl $l^{-1}$ for $SO_2$, but only at much higher concentrations of 300 nl $l^{-1}$ for $H_2S$ (Posthumus 1998). Due to its prevalence in forest decline issues, $SO_2$ effects on trees have been intensively studied and many countries have derived air quality standards to protecting forest trees. Much less is known about the effects of $H_2S$ on trees, and hardly any data exist on the effects of other sulfurous gases on trees.

Trees incorporate sulfur from atmospheric sources into their normal sulfur metabolism and hence can use sulfurous gases as sulfur sources (Figure 1). Under elevated $SO_2$, trees accumulate high levels of sulfate in leaves, which is widely used as a diagnostic tool similarly to total sulfur

content. Labeling experiments with spruce showed that the major part of sulfate accumulation comes directly from the $SO_2$ (Tausz *et al.* 2003), which implies the oxidation of sulfite (formed through the solution of $SO_2$ in water) to sulfate, a step not yet fully clarified. Superoxide-mediated free radical mechanisms seem to contribute in some cell compartments (Miszalski and Ziegler 1992), but a specific sulfite oxidase, which was recently characterized in herbaceous plants, may also play a significant role in trees (Hänsch *et al.* 2006). Furthermore, sulfite can also be channelled into the sulfur-reduction pathway leading to increases in reduced sulfur such as glutathione (albeit quantitatively at a much lower level than sulfate accumulation) and organic sulfur. $H_2S$, on the other hand, can be incorporated in organic compounds without prior reduction, leading to marked increases in glutathione content. However, part of the sulfur still shows up as increased sulfate, which has to be produced by oxidations (Tausz *et al.* 2003). Both $H_2S$ and $SO_2$ are used to synthetize organic sulfur compounds thus decreasing the utilization of soil sulfate. It seems, however, that contrary to herbaceous plants, trees do not respond with a strong decrease of root sulfate uptake, possibly indicating a relatively poor canopy–root signaling in trees (Herschbach 2003; Tausz *et al.* 2003).

It seems surprising that after many decades of concern and research on $SO_2$ effects on trees, aspects of the toxicity mechanisms are still unclear. Acute injury, which encompasses a number of morphological, cytological, and physiological effects (Hogan and Rennenberg 1998) may be caused by severe acidification brought about by the formation of sulfuric acid upon contact of $SO_2$ and water, by toxic levels of sulfite in the cells or by the superoxide-mediated free radical chain oxidation of sulfite to sulfate (De Kok 1990). Furthermore, acidic reactions on the leaf surface may lead to direct cation leaching from the foliage and disturb the nutrient element balances (Hogan and Rennenberg 1998). Some hypotheses have been put forward to explain chronic $SO_2$ injury: firstly, acidification of cell compartments can severely impact on their function (Pfanz *et al.* 1987), and secondly, more general interactions of $SO_2$, sulfite, or resulting products with a number of cellular components may lead to deregulations of cell metabolism and a reduced fitness (De Kok 1990). In this respect, the disturbance of glutathione metabolism has been put forward as a crucial factor, because glutathione is a central regulator of cell metabolism, stress responses, and gene expression (De Kok and Tausz 2001, see below).

A specific effect of sulfate-containing acid mist on tree frost hardiness has been described and it is hypothesized that apoplastic sulfate accumulation, which specifically occurs upon direct sulfate uptake into the foliage,

leads to plasma membrane dysfunctions and so exacerbates susceptibility to frost (Sheppard 1994).

*Ecosystem effects of atmospheric sulfur on forests*

Sulfur input – as gaseous $SO_2$ or sulfate in mist or precipitation, can have acidifying effects not only on tree tissues, but on the whole forest ecosystems including the soils, which is thought to have contributed to various forest damage and decline events (Guderian 1977). Soil acidification can mobilize nutritional cations (Ca, Mg, K), which can be leached from the ecosystem and lead to symptoms of mineral deficiencies. Apart from disturbances of the nutritional cycles, a number of further negative effects of soil acidification on ecosystem health, such as the disturbance of mycorrhiza communities, have been described (Guderian 1977).

Hence, critical levels for sulfate inputs into forest ecosystems have been defined to guarantee the long-term steady state conditions of ecosystems (de Vries 1993). A large-scale survey of European forests showed clear correlations between exceeding of these critical loads and soil pH values, indicating that the problems relating to forest ecosystem effects are not over in Europe (Augustin *et al.* 2005).

## SULFUR COMPOUNDS IN TREE DEFENCE AGAINST ENVIRONMENTAL STRESS

Sulfur-containing compounds are essential in the defence reactions of trees to many biotic and abiotic stress factors. A number of low-molecular-weight sulfur metabolites are involved in plant defence and have collectively been named sulfur-containing defence compounds (SDCs; Rausch and Wachter 2005, Figure 1). Sulfur-containing defence compounds include multifaceted primary metabolites such as glutathione and its derivatives (e.g. phytochelatins), potential roles for elemental sulfur and $H_2S$, but also specific roles for nonubiquitous secondary SDCs.

*Glutathione and the cellular redox balance*

In addition to its role as a long-distance transport form of reduced sulfur, glutathione plays multiple roles in tree–environment interactions and defence. It functions as an antioxidant and as a redox buffer to protect tissues from reactive oxygen species (ROS) produced under abiotic and biotic stress (Tausz 2001). In this role it has been suggested as a general redox sensor and signaling agent in plant cells (Meyer and Hell 2005). As a substrate in the glutathione *S*-transferase conjugation reaction it

detoxifies xenobiotics and toxic metabolic products by channelling them into the vacuole (Schröder 2001). Moreover, it is the substrate for phytochelatin synthesis, which serves to complex and detoxify heavy metals in plants (Rauser 2001).

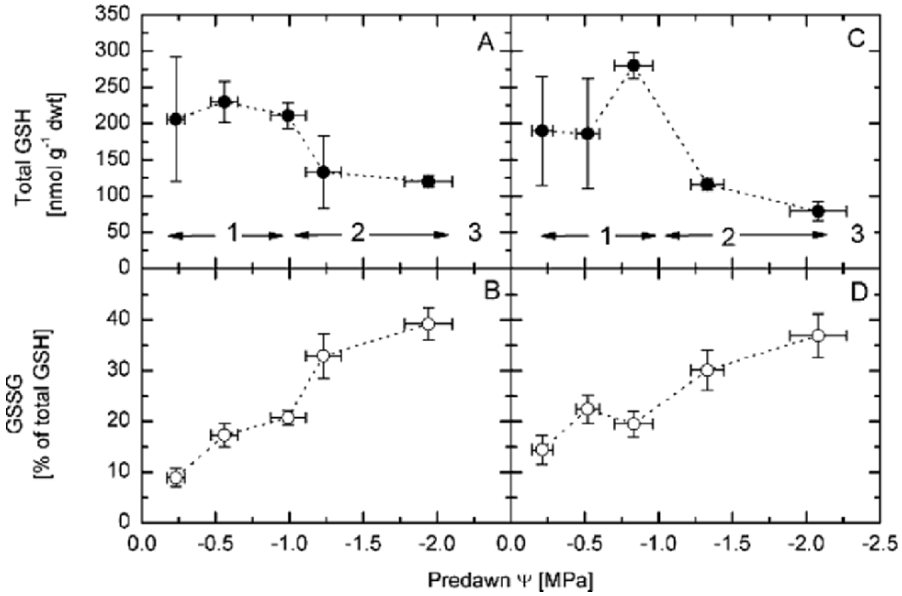


*Figure 2.* Responses of the foliar glutathione system of two apple (*Malus domesticus*) cultivars to progressing drought. GSH glutathione; GSSG oxidized glutathione, dwt leaf dry weight. **A**, **B** cultivar Jonagold, **C**, **D** cultivar Elstar. 1 initial acclimatory stress response; 2 severe stress and degradation; 3 tissue death. (Redrawn from data in Šircelj *et al.* 2005.)

Trees under stress seem to generally require and synthesize higher concentrations of glutathione, underlining the central role of this compound in plant cells under stress (Tausz 2001). However the results seem to be highly inconsistent. A recent study on apple trees showed that the response of the glutathione system to progressing drought stress is dynamic and polyphasic (Šircelj *et al.* 2005), which may explain some of the discrepancies reported in the literature. There is a tendency towards increased glutathione levels at the early stage of the stress response, which can be interpreted as an acclimation effect to increase resistance. With increasing stress levels, glutathione concentrations decrease with the degradation of the system just before cell and tissue death occurs (Figure

2). The glutathione redox state responds quickly to the onset of stress, which is thought to trigger an array of defensive responses (Mullineaux and Rausch 2005). However, further and probably less controlled oxidation of the glutathione pool occurs in relation to destructive processes. Sampling at different points of this stress response without taking into account the dynamic nature can give inconsistent results (Tausz *et al.* 2004).

It seems surprising that the role of glutathione in stress responses and resistance of trees is still not fully understood, given that transgenic trees with manipulated glutathione metabolism have been available for more than a decade (Herschbach and Kopriva 2002). A number of transgenic approaches succeeded in producing poplar trees with elevated glutathione levels, and some of those trees were apparently more resistant to xenobiotics (Gullner *et al.* 2001) and more efficient in removing heavy metals from contaminated soils (Bittsanszky *et al.* 2005; Koprivova *et al.* 2002). The role of glutathione in detoxifying xenobiotics or heavy metals is straightforward, as it is the sole conjugating agent for xenobiotics (Schröder 2001) or the exclusive substrate (apart from a starter molecule) for phytochelatin synthesis (Rauser 2001). Hence, higher glutathione concentrations may easily translate to higher detoxification capacity. In contrast, higher glutathione status did not improve tree resistance to other types of stress, e.g. oxidative stress (Herschbach and Kopriva 2002). It has to be taken into account that the role of glutathione in response to oxidative stress is a multifaceted one, and glutathione is only one part of a complex network of antioxidants, enzymes, and redox balances (Tausz 2001). It is not surprising that manipulation of only one element cannot increase the efficiency of the whole system.

*Sulfur-containing substances in tree defence against pathogens*

Although elemental sulfur is considered man's oldest pesticide, the discovery that it is a component of plant defence reactions is very recent (Cooper 2004). Up to now it has been found in a number of species from different families, among them the tree species *Theobroma cacao* (Sterculiaceae). In *T. cacao*, elemental sulfur possibly in the form of $S_8$ rings, appeared in the xylem after infection with the pathogen *Verticillium dahliae* (Resende 1996). Sulfur is a potent fungicide and local concentrations were considered effective. The pathway of elemental sulfur formation is uncharacterized, although first results link it to increased levels of glutathione and sulfate, and a high sulfur supply seems to be a prerequisite for this to occur (Cooper 2004). There is currently no further

information available as to whether elemental sulfur is of significance in other tree species or forest ecosystems.

Secondary sulfur compounds, such as glucosinolates, alliins, or derivatives induced upon attack (phytoalexins) are being intensively investigated with respect to protective effects against predators and parasites in crops (Bloem *et al.* 2005). A number of sulfur-containing secondary metabolites can be found in tree or shrub species. Examples include glucosinolates in the horticulturally important *Carica papaya* (Caricaceae; Rodman *et al.* 1998), or *Moringa* species (Moringaceae; Bennett *et al.* 2003), or sulfur-containing amides and sulfur-containing flavanols in *Glycosmis* species (Rutaceae; Grayer and Harborne 1994; Wang *et al.* 2005). No information on the potential roles of sulfur containing secondary metabolites in forest tree–pathogen interactions is currently available.

The emission of volatile S compounds, particularly $H_2S$ (see above), but also $CS_2$ and COS have been discussed as potential factors in pathogen resistance (Bloem *et al.* 2005). It has been shown that the activity of cysteine-desulfhydrase, an enzyme potentially responsible for the release of $H_2S$, is elevated upon infestation *Brassica napus* with a pathogen (Bloem *et al.* 2005), but the potential role of $H_2S$ itself remains obscure even in crops (Rausch and Wachter 2005). To date no studies have been done to link volatile sulfur emissions from trees directly to pathogen attack and resistance.

Pathogen response also involves an additional role for glutathione, which forms part of the systemic response of plants to pathogen attack. Given its mobility in xylem and phloem, glutathione may well act as a signaling substance spreading the message to the whole plant, and may trigger an array of specific responses (Gullner and Kömives 2001). Considering the long distances within a tree, such a role would be even more important than in herbaceous plants.

*Sulfur-containing substances in tree–animal interactions*

The sulfur amino acids cysteine and methionine are essential for most herbivores and hence a potential determinant of the quality of the feed. Interestingly, highly reduced sulfur contents in acorns translated directly to high cysteine status of feeding mites (Grill *et al.* 2003). It has been hypothesized that reduced sulfur compounds in the bark are a determinant for the breeding success of bark beetles, but a study on spruce (*Picea abies*) and *Ips typographus* failed to establish a clear connection (Mattanovich *et al.* 2001).

Specific roles of volatile sulfur compounds emitted by trees have also been described in the relationship between forest trees and animals. A study in tropical rainforests in French Guyana (Berkov *et al.* 2000) suggests that the "foul odour" mainly produced by emissions of *S*-methylmethionine in certain tree species of the Brazil nut family (*Couratari stellata* and *Gustavia hexapetala*, Lecythidaceae) deters wood-boring beetles specialized on that tree family (Cerambycidae). Volatile sulfur compounds (mainly sulfur methyl esters, and organic sulfides) contribute to the strong scent of tree flowers designed to attract pollinating bats, a pollination strategy widely distributed in tropical rainforests (Pettersson *et al.* 2004).

*Sulfur-related defence and sulfur nutrition*

The central role of sulfur-containing compounds in plant stress responses implies increased sulfur requirements of plants under stress, or, from the opposite viewpoint, would suggest that plants with high sulfur status (e.g. attained by sulfur fertilizer application) have improved resistance. This latter concept has been verified as "sulfur-induced resistance" (SIR) for a range of crop–pathogen interactions (Bloem *et al.* 2005). However, it is unclear whether such results can be generalized to tree species and extended to the resistance to abiotic stress factors.

On the other hand, it appears that sulfur deficiency renders plants more susceptible to stress, because for example glutathione levels can drop to 25% of the controls in sulfur starved *Arabidopsis* plants (Kandlbinder *et al.* 2004). This was not corroborated for trees, because short-term sulfur starvation did not decrease glutathione levels in poplar (Kopriva *et al.* 2004).

## CONCLUSIONS

The change in pollution towards significant reductions of sulfur deposition and the challenges of global changes on forest ecosystems will lead to a significant shift in the forest research interests related to sulfur. In the late 1980s, crops began to exhibit sulfur deficiency symptoms in regions where they were unknown before, and research in crop physiology refocussed on the beneficial roles of sulfur (Bloem *et al.* 2005). Analogous effects in forests have not been clearly identified yet (but note that sulfur deficiency (!) was discussed as a potential cause in "novel forest decline" events during the 1980s; *cf.* Kandler and Innes 1995). Given the much longer response times in trees and forests we can anticipate such a scenario in the

longer term (Johnson and Mitchell 1998). In this respect, clearly most of our knowledge on sulfur-containing defence compounds and sulfur-induced resistance refers to herbaceous plants. Compared to this, forest tree physiology is way behind. We can assume that the roles of sulfur in resistance and adaptation of forest trees to stress and environmental change will get more attention in the future.

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Chapter 4

# SULFUR IN THE MARINE ENVIRONMENT

Jacqueline Stefels

## INTRODUCTION

In open ocean waters, sulfate concentrations are approximately 29 mM and can thus be regarded as a nutrient in excess. Maybe it is this excess in sulfate that has instigated the development of a biochemical pathway in micro and macro algae leading to the production of dimethylsulfoniopropionate (DMSP), a compound almost exclusively found in the marine environment. DMSP can be regarded possibly as the most important organic sulfur compound in seawater due to its prominent role at many different levels: going from the molecular, through the ecosystem and up to the global level.

It was as early as 1935 that DMSP and its enzymatic cleavage product, dimethyl sulfide (DMS), were found to be produced by marine macroalgae (Cantoni and Anderson 1956; Challenger 1951; Haas 1935). However DMS came to prominence in the 1980s, when DMS was hypothesized to play an important role in climate regulation (Bates *et al*. 1987; Charlson *et al*. 1987). From that time on, research interest sharply increased. Apart from its role in algal physiology, DMSP, or one of its cleavage products DMS or acrylate, was found to affect grazing activity, by acting as a repellent for microzooplankton (Strom *et al*. 2003), thereby affecting the structure of the microbial food web. Recently, DMSP has been recognized to be the most important source of reduced sulfur for marine bacteria. It may potentially cover 50–100% of total bacterial sulfur demand (Kiene *et al*. 2000), which is unprecedented for a single compound.

Due to its biological origin, production, and conversion of DMS and DMSP are strongly linked to the growth season. As a result there are large variations in the DMS flux to the atmosphere in time and space (Kettle *et al*. 1999). After its release from algae, a complex network of production



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and consumption pathways of both DMSP and DMS involves most of the microbial food web. Physical and chemical ecosystem parameters all affect this network, potentially resulting in dramatic shifts in the DMS flux to the atmosphere (Stefels *et al*. 2007). Although our knowledge on the qualitative aspects of the marine sulfur cycle has improved considerably during the last two decades (Bentley and Chasteen 2004), it is still difficult to quantify the effects of controlling factors on the various pathways. During the last decade, many excellent reviews have been written on several aspects of the marine sulfur cycle (Kiene *et al.* 2000, Stefels 2000; see Stefels *et al*. 2007 for an overview). The emerging picture is that this cycle is not only of interest for global climate, but that DMS and DMSP are compounds which are central to the microbial food web in their own right.

## THE MARINE SULFUR CYCLE AND GLOBAL CLIMATE

DMS accounts for 50–60% of the total natural reduced sulfur flux to the atmosphere, including emissions from volcanoes and from vegetation (Andreae 1990; Bates *et al*. 1992; Spiro *et al*. 1992). By providing 95% of the flux to the atmosphere, the oceans are the main source for DMS, with estimates of its emission ranging between 15 and 33 Tg S $year^{-1}$ (Kettle and Andreae 2000; Watts 2000). Once in the atmosphere, a cascade of oxidation processes occurs, leading to the production of sulfur dioxide ($SO_2$), dimethylsulfoxide (DMSO), and methane sulfonic acid (MSA). Subsequently, sulfate particles are formed, which act as condensation nuclei for water vapor. These nuclei affect the radiative properties of the atmosphere and clouds, with implications for climate. Higher numbers of condensation nuclei will deflect more incoming solar radiation back into space and thereby reduce the temperature on earth. The hypothesis that this process may modulate the greenhouse effect of increased anthropogenic $CO_2$ input to the atmosphere as put forward by Charlson *et al*. (1987) is now the subject of many modeling efforts (Bopp *et al*. 2003). The results of such modeling exercises show that both increased and decreased DMS fluxes can be found, depending on the hydrography and biology of a particular ocean area. Thus, depending on the direction of the change in DMS flux, the subsequent climate changes induced by sulfur products could either alleviate or amplify the greenhouse effect (Bopp *et al*. 2004).

Currently, anthropogenic $SO_2$ production exceeds natural $SO_2$ production by a factor of 2 (Chapter 5), but the impact of the former on

aerosol production is largely confined to industrialized areas of the Northern Hemisphere. The oceans, on the other hand, cover approximately 70% of the earth's surface and much of this area is remote from man-made atmospheric contaminants. Consequently, the exchange of marine DMS is of high regional importance and may affect climate globally. For example, the Southern Ocean appears to be an important source area for DMS, with implications for climate over the total Southern Hemisphere (Gondwe *et al*. 2003).

## BIOCHEMICAL PATHWAYS OF DMSP PRODUCTION

Algae that produce high amounts of DMSP appear to be confined to a few classes of marine micro- and macroalgae (Blunden *et al*. 1992; Keller *et al*. 1989; Reed 1983), although in almost all classes some species can be found that produce it in small amounts. Observations of DMSP production in higher plants are rare, with the exception of a few species that experience regular salinity fluctuations, such as *Spartina* species, some sugarcanes and the coastal strand plant *Wollastonia biflora* (Chapter 5). On a global scale, phytoplanktonic producers are most important, especially species of the classes Dinophyceae (dinoflagellates) and Haptophyceae (including the coccolithophorids and *Phaeocystis* sp.; Keller *et al*. 1989).

The production and regulation of DMSP in marine algae is still enigmatic. Typical values for intracellular concentrations of DMSP are 50 –400 mM. In those cases, DMSP-sulfur can comprise 50% to almost 100% of the total cellular organic sulfur (Keller *et al*. 1999; Matrai and Keller 1994). Much of our knowledge about processes involved in the assimilation of sulfate up to the incorporation of sulfur into DMSP has been derived from experiments with higher plants (Figure 1).

Methionine is derived from cysteine. Although the major pathway for methionine metabolism is the utilization of its methyl group in transmethylation reactions via *S*-adenosylmethionine (AdoMet), this is not a true sink for methionine (Giovanelli 1987). The incorporation into protein and – if applicable – the production of DMSP are therefore the only sinks for methionine. Currently, there is strong evidence that the biochemical pathway from methionine to DMSP has evolved independently at least three times through different intermediates (Figure 2). The best-studied DMSP-containing plant is *Wollastonia biflora* (Compositae), a common Indo-Pacific strand plant. In *W. biflora*, *S*-methylation is the first step in the sequence, which results in the production of *S*-methylmethionine (SMM) and subsequently DMSP- aldehyde (Hanson

and Gage 1996; Hanson *et al*. 1994; James *et al*. 1995, Figure 2A). Most higher plants, including non-DMSP containing plants, have the enzymes

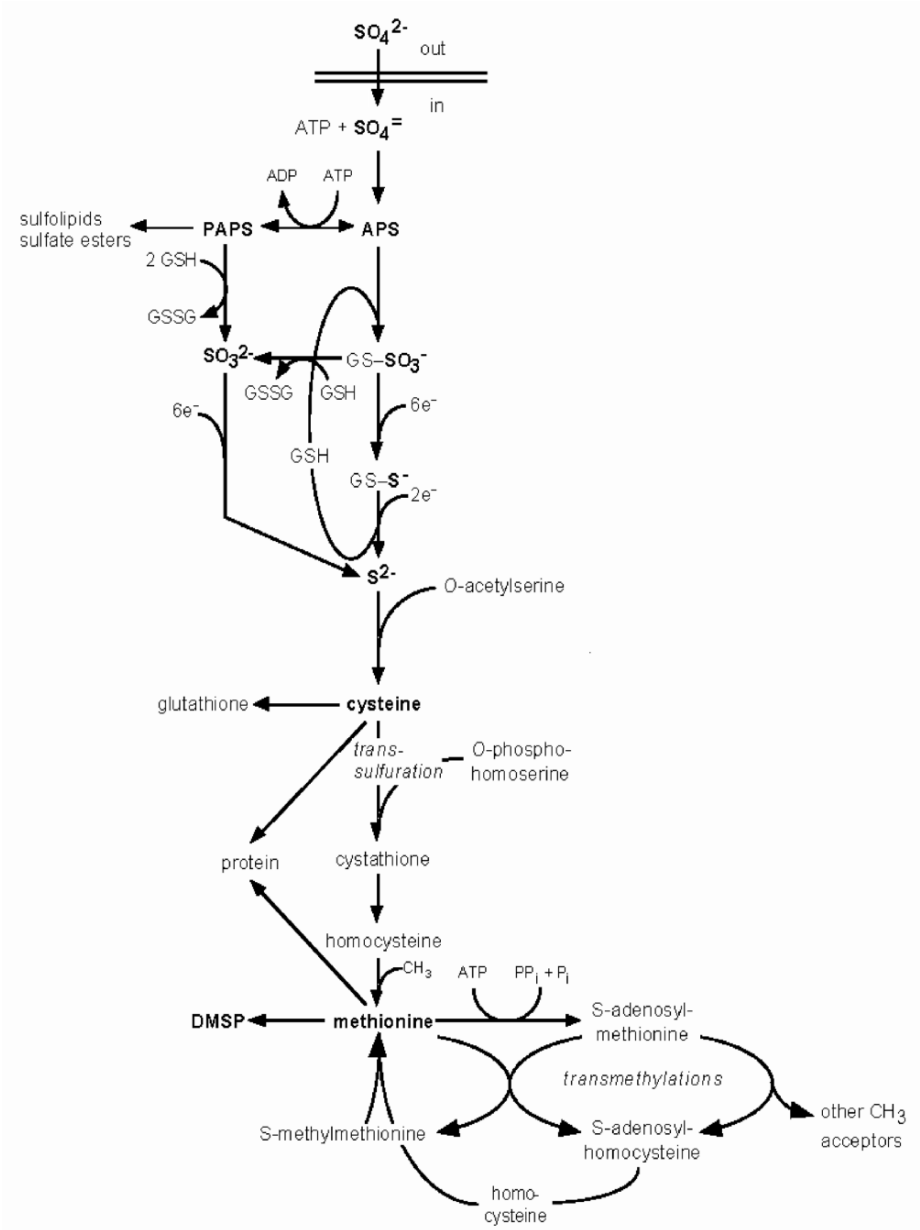


*Figure 1*. Sulfate assimilation and synthesis of DMSP. (Adapted from Stefels 2000.)

to mediate the methylation of methionine and the oxidation of DMSP-aldehyde, but it is the conversion of SMM to DMSP-aldehyde that is specific for DMSP synthesis. In *W. biflora*, the methylation reaction occurs in the cytosol. SMM is transported to the chloroplast, where the conversion into DMSP-aldehyde and DMSP takes place. The oxidation reaction has strong similarities with betaine aldehyde dehydrogenase (Trossat *et al*. 1996).

A second pathway has been identified in *Spartina alterniflora* (Gramineae; Kocsis and Hanson 2000; Kocsis *et al*. 1998). In this sea grass, DMSP-amine was identified as an intermediate between SMM and DMSP-aldehyde (Figure 2B). SMM is decarboxylated by a pyridoxal 5′-phosphate-dependent decarboxylase, which yields equimolar amounts of $CO_2$ and DMSP-amine (Kocsis and Hanson 2000). The conversion of DMSP-amine to DMSP aldehyde is catalyzed by DMSP amine oxidase, which requires $O_2$ for activity (Kocsis and Hanson 2000). The specific production of DMSP-amine in grasses, suggests that the DMSP-specific pathway from SMM to DMSP-aldehyde had evolved independently in the Compositae and Gramineae (Kocsis *et al*. 1998).

A third and entirely different pathway was identified in the green macroalga *Enteromorpha intestinalis* (Gage *et al*. 1997; Summers *et al*. 1998, Figure 2C). The first step is a transamination of methionine to form 4-methylthio-2-oxobutyrate (MTOB), which is followed by a NADPH-linked reduction to 4-methylthio-2-hydroxybutyrate (MTHB). Then an AdoMet-dependent methylation occurs, which yields 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB), followed by an oxidative decarboxylation to DMSP. The first two steps appear reversible; they are widespread among a variety of higher and lower plants, though much higher activities are found in DMSP containing algae. The conversion of MTHB to DMSHB seems to be specific for DMSP synthesis. DMSHB was also found in three planktonic species: *Emiliania huxleyi* (a prymnesiophyte), *Melosira nummuloides* (a diatom), and *Tetraselmis* sp. (a prasinophyte) (Gage *et al*. 1997). All three were able to convert supplied DMSHB to DMSP and it was therefore suggested that they have the same pathway as *E. intestinalis*.

## DMSP AS A COMPATIBLE SOLUTE

DMSP is a multifunctional compound and there is no doubt that it has a role as a compatible solute in cell metabolism (Kirst 1996; Stefels 2000). Many unicellular algae are wall-less cells or have a cell wall with a low elastic modulus (coefficient of elasticity), which implies that they are not

able to build up high turgor pressures inside the cell. Since open ocean salinity is around 34 PSU (practical salinity units), which is equivalent to approximately 1,000 mosmol $kg^{-1}$, cells need to produce high concentrations of osmotically active compounds in order to maintain their intracellular water potential at a comparable level. High concentrations of ions in the cytoplasm, however, would jeopardize enzymatic reactions. Therefore, cells need to produce organic solutes, which are noninhibitory to metabolism. These so-called compatible solutes are low molecular weight organic compounds and accumulate in the cytoplasm of cells at low water potential, whereas high ion concentrations are mainly found in vacuoles.

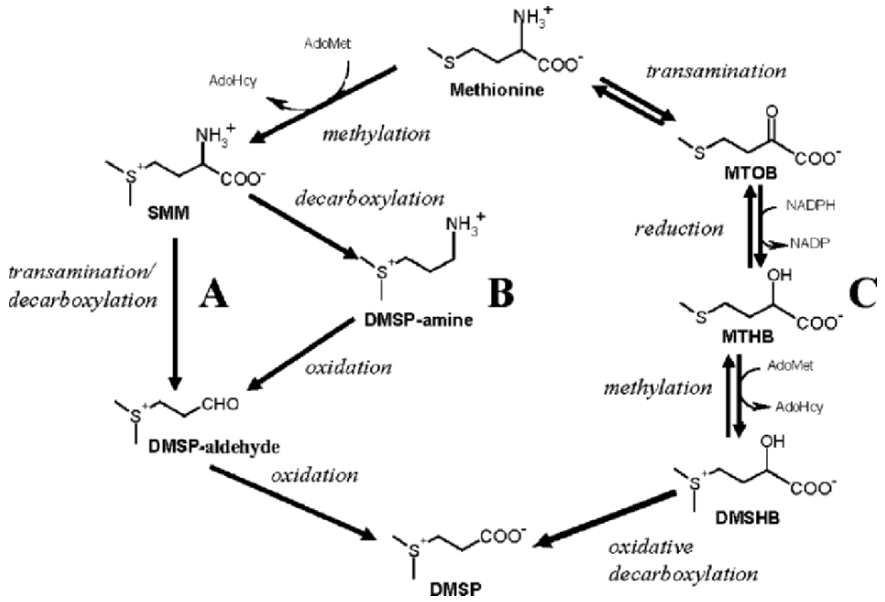


*Figure 2*. Pathways of DMSP biosynthesis in plants and marine algae. **A**, Compositae; **B**, Gramineae; **C**, marine algae. (Adapted from Stefels 2000.) AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; DMSHB, 4-dimethylsulfonio-2-hydroxybutyrate: MTHB, 4-methylthio-2-hydroxybutyrate; MTOB, methylthio-2-oxobutyrate; SMM, *S*-methylmethionine.

Compatible solutes like sugars, polyols, and heterosides, are directly produced from photosynthesis, whereas amino acids, betaines, and DMSP are produced after glycolysis of carbohydrates. Not all algae use the same set of solutes. Their composition appears to be taxonomically defined, but

also is affected by the physiological condition of the cell, since the energy cost and the requirement for carbon and nitrogen vary between compounds. Changes in condition may therefore result in changes in the relative concentrations of these solutes. Given the structural similarity between DMSP and glycine betaine, it has long been hypothesized that DMSP could replace glycine betaine under nitrogen limitation. A direct coupling between these two compounds has, however, not been observed (Keller *et al*. 1999), although there are some indications of increased DMSP concentrations under N limitation. Especially under low temperatures, the property of DMSP to stabilize enzymatic reactions improves, hence the conclusion that DMSP is an effective cryoprotectant (Nishiguchi and Somero 1992). This is confirmed by the observation that high concentrations of DMSP are found in ice algae (Kirst *et al*. 1991).

The physiological function of DMSP, playing a role in maintaining cell water potential, seems very straightforward, but the puzzling aspect about this compound is that the regulation of its internal concentration is still unresolved. On a timescale of hours to days, salinity effects the intracellular DMSP concentration, but not on a timescale of minutes to 1 hour, as would be expected of a compound that is actively involved in osmoregulation. We therefore cannot assign DMSP as an osmolyte in the strict sense of being responsible for osmotic balance, although it greatly contributes to the cell's osmotic potential due to the high intracellular concentration. Kirst (1996) suggested therefore that DMSP only may act as a buffer during the initial period after hyperosmotic shock, when immediate cell volume changes result in concomitant changes of intracellular solute concentrations; an effect which takes place without active production or degradation of the solute.

## WHAT CONTROLS THE PRODUCTION OF DMSP?

The lack of response in DMSP-production rates upon salinity shifts has inspired many researchers to look for other factors that may control production. DMSP production is coupled to cell growth, but also continues at a low rate during growth-limited conditions, no matter what the limiting factor is. For instance, in experiments with *Phaeocystis globosa*, a prolific DMSP producer of temperate coastal areas, in which the cultures experienced a salinity range from 25 to 50 PSU and growth was limited by either nitrate or phosphate, the total DMS plus DMSP production was correlated with salinity and continued in the stationary phase of growth, although at a lower pace. Although distinct differences could be observed

between the N- and P-limited cultures with respect to cell size and cell lysis during the stationary phase of growth, the DMSP content of cells was comparable. It appeared that the total DMSP production, including DMSP that has been released from the cell or converted into DMS, was directly coupled to the growth rate of the cells, irrespective of the condition of the cultures (Figure 3). The fact that the regression coefficient deviates from "1" reflects the observation that under unlimited (high) growth rates, cells tend to divide faster than they grow in terms of carbon, which results in a cell-size reduction during exponential growth. The positive Y-intercept indicates that at limited (low) cell growth, DMSP production continues under all conditions, even when cell numbers decline (negative growth).

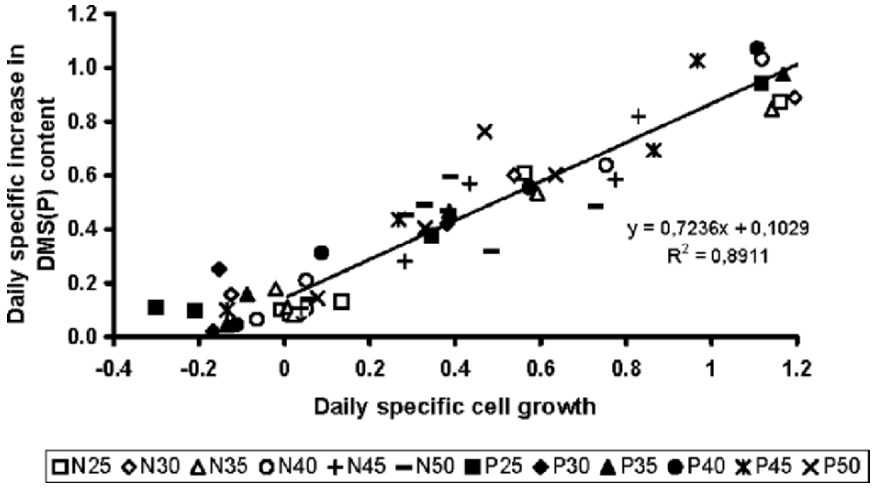


*Figure 3.* Daily specific cell growth of *Phaeocystis globosa* versus the increase of the total DMS and DMSP pool at various levels of salinity (25–50 PSU) and nitrate (N) or phosphate (P) limitation. Temperature (11C) and light (70 µE) were kept constant during cultivation.

In an attempt to explain these phenomena, it was hypothesized that the production of DMSP might serve as an overflow mechanism for excess reduced sulfur under conditions of unbalanced growth, when carbon and nitrogen flows are out of tune (Stefels 2000). This hypothesis was based on a well-known mechanism in higher plants, in which a reciprocal regulatory coupling exists between the pathways of assimilatory sulfate and nitrate reduction (Figure 4, Brunold 1993; Giovanelli 1990). This mechanism

ensures the appropriate proportions of sulfur-containing and other amino acids for protein synthesis, and is associated with a strong negative feedback coupling of *de novo* synthesis of methionine, which needs to be maintained at a concentration of around 10 μM. In higher plants this mechanism may result in inhibited sulfate reduction under nitrogen limitation, and vice versa. This is a sensible mechanism for environments where both N and S can become growth-limiting nutrients. The marine environment is, however, rich in sulfate and the continued production of DMSP under various growth-limiting conditions suggests that algae have a different mechanism to regulate their methionine equilibrium. During stress conditions, high protein turnover rates are observed, which allows the cell to reutilize amino acids and to adapt enzyme systems to the new situation. The continued production and possible loss of DMSP, could serve as a sink for excess carbon and at the same time regenerate intracellular nitrogen from recycled methionine, which can then be used for synthesis of other amino acids (Figure 4). Although such a mechanism seems wasteful, the benefits are the continuation of the metabolic machinery. The continued production of DMSP keeps cysteine and methionine concentrations at a low level, thereby preventing possible feedback mechanisms from coming into action. This allows continued sulfate assimilation even under nitrogen-limited conditions. In addition, an increased DMSP concentration may reduce the requirement for nitrogen-containing compatible solutes. One may compare this potential mechanism with the commonly observed exudation of carbohydrates by cells at high light and low nutrient concentrations. If indeed DMSP production is connected to an overflow metabolism, this requires that DMSP is mainly located in the cytosol and that the intracellular equilibrium concentration is regulated by its degradation or loss from the cell rather than by its production. Although this hypothesis can explain many of the observed changes in DMSP content presented in the literature (Stefels 2000), direct evidence is difficult to obtain.

Sunda *et al*. (2002) presented another hypothesis for the physiological function of DMSP and how its internal concentration may be regulated. These authors suggested that DMSP and its breakdown products DMS, acrylate, dimethylsulfoxide (DMSO), and possibly methane sulfinic acid (MSNA) and MSA together form a cascade of radical scavengers that may serve as an efficient antioxidant system. This mechanism would need to be regulated in part by the enzymatic cleavage of DMSP, through which DMS and acrylate is formed. An active mechanism of this kind would suggest that the production of DMSP and its enzymatic cleavage is likely to be located in the chloroplast, where most reactive oxygen species (ROS) are produced. There are indeed indications for a chloroplastic location of

DMSP production in plants that exhibit the first production pathway (Figure 2A, Trossat *et al*. 1996), but there is no conclusive evidence for this in marine algae. A complicating factor is that with the common techniques for DMS(P)-analysis, it is as yet impossible to measure the fluxes through this cascade of compounds.

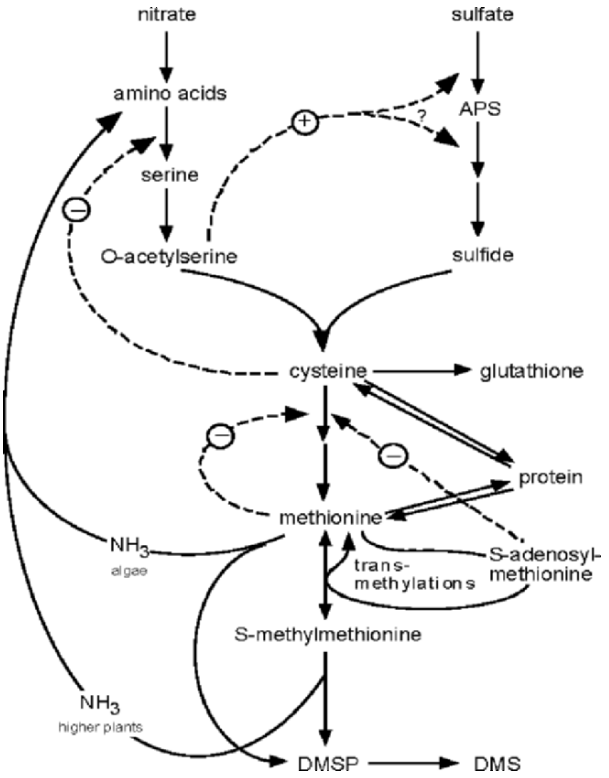


*Figure 4.* Regulatory coupling between the assimilatory nitrate and sulfate reduction pathways. *Solid lines* represent reaction pathways. *Dotted arrows* indicate negative (-) or positive (+) regulatory effects. (After Stefels 2000.)

Sunda and coworkers proposed the antioxidant hypothesis on the basis of elevated concentrations of intracellular DMSP under stress conditions, where cell growth had ceased. In the process of radical scavenging, DMSP will be converted into one of its breakdown products. Therefore, a loss of DMSP is expected, unless the stress reaction results in increased *de novo* synthesis (upregulation) of DMSP. Only in those cases, a subsequent

overshoot production may lead to increased intracellular concentrations of DMSP and/or one of the downstream products. In several subsequent publications, it appeared that the reaction of increased DMSP concentrations under stress conditions appears to be confined to species with low DMSP concentrations, such as the diatom *Thalassiosira pseudonana* (Bucciarelli and Sunda 2003; reviewed by Stefels *et al*. 2007). DMSP concentrations in this species are an order of magnitude lower than in, for example, *Phaeocystis globosa*. Possibly, the high concentrations in the latter species can buffer any consumption effect due to the scavenging process, thereby masking an effect in the concentration, whereas a comparable absolute consumption of DMSP in the diatom would be clearly visible. Alternatively, it may be that the production pathway of DMSP in different algal groups has developed independently and that the functionality of this compound also differs between groups.

Since unbalanced growth and the production of ROS often co-occur under high irradiance or nutrient-limited conditions, it is difficult to test the two hypotheses individually without detailed investigation of the physiological condition of the cells and of the fluxes through relevant biochemical pathways. In this context, a method for the measurement of *de novo* synthesis of DMSP is clearly warranted. The two hypotheses do not necessarily need to be mutually exclusive, since a function in oxidative stress management does not exclude additional functions in cell metabolism. So far, the prime function of DMSP in algal physiology still seems to be the one of being a compatible solute, especially under cold conditions.

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Chapter 5

# ATMOSPHERIC SULFUR

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## INTRODUCTION

Atmospheric sulfur gases originate from both natural and anthropogenic sources (Bates *et al.* 1992; Dämmgen *et al*. 1998; Watts 2000; Stern 2005). There is natural emission of $SO_2$ and $H_2S$ from volcanic and geothermic activity, however, the predominant proportion of the natural sulfur emissions are formed biologically and emitted as $H_2S$ or organic sulfur gases *viz.* DMS (dimethyl sulfide), COS (carbonyl sulfide), and $CS_2$ (carbon disulfide), which are predominantly formed over oceans, wetlands, salt marshes, and estuaries by algae and bacteria (Watts 2000). The concentration of sulfur gases in the atmospheric is the final balance of the emission, transport, and the lifetime of the gases in the atmosphere. Lifetimes may vary from less than a day (e.g. DMS) to more than a year (COS; Schröder 1993; Dämmgen *et al*. 1998; Kesselmeier 2005). The total natural sulfur emission was estimated at 34 Tg S per year, predominantly as $H_2S$ and DMS at 7.7 and 24.5 Tg S per year, respectively (Watts 2000). Part of the volatile sulfur in the atmosphere may originate from vegetation, since the plant shoot may emit trace amounts of specific sulfur gases *viz.* $H_2S$, COS, and DMS, though their actual contribution to global sulfur emissions is quite uncertain due to the lack of data (Watts 2000; Kesselmeier 2005). Emissions of $SO_2$, $H_2S$ and organic sulfur gases are of current special interest in global change research, as important sources for stratospheric sulfate aerosols which are highly significant in stratospheric ozone chemistry. Hydroxyl and nitrogen oxide radicals oxidize $SO_2$, $H_2S$, and the organic sulfur gases in the atmosphere and their oxidation products are important in cloud formations as precursors of cloud condensation



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nuclei (Andreae and Crutzen 1997; Dämmgen *et al*. 1998; Alfonso and Raga 2002; Kesselmeier 2005; Sanderson *et al.* 2006).

Anthropogenic sulfur is mainly emitted as $SO_2$, from coal, oil, industrial processes, and biomass burning and in 2000 its global emission was estimated at 68 (Smith *et al.* 2001) and 55.2 Tg S per year (Stern 2005), which is higher than the natural sulfur emission. $SO_2$ may react in the atmosphere with water and atmospheric oxygen to form sulfuric acid and forms together with nitrogen oxides the basis for acid rain (Badr and Probert 1994; Sanderson *et al.* 2006). Since 1990, as the consequence of legislatory control on sulfur gas emissions for the combustion of fossil fuels, sulfur emissions have dramatically been decreased in Western Europe and the USA in order to diminish the negative effects of acid rain deposition. Global sulfur emissions have decreased (Smith *et al.* 2001; Stern 2005), at an estimated average rate of 2.7% per year since 1990 (Stern 2005). However, the major sources of emissions has been shifted towards East and South Asia, where several countries have experienced an unprecedented period of industrial development and economic growth accompanied with strongly increased energy demand (Dämmgen *et al*. 1998; Kesselmeier 2005; Stern 2005).

It has become evident that dry and wet deposits from atmospheric $SO_2$ pollution may substantially contribute to sulfur nutrition of agroecosystems (De Kok 1990; Schnug and Evans 1992; Dämmgen *et al*. 1998; Haneklaus *et al.* 2003). Plants may benefit from atmospheric sulfur deposited on soil via wet deposition (sulfur gases removed from the atmosphere by precipitation, e.g. rain, snow, fog) or via dry deposition (foliar absorption of the gas). Modern fertilizers are low in sulfur and the ongoing decrease in atmospheric sulfur deposition appears to be one of the primary causes of sulfur deficiency of crop plants. For instance in Western Europe, total atmospheric sulfur deposition has decreased from 70 kg $ha^{-1}$ $year^{-1}$ in 1970s to less than 10 kg $ha^{-1}$ $year^{-1}$ presently (McGrath *et al.* 2002), which is far from optimal for most crop plants and additional sulfur fertilization is necessary to avoid economic losses (Schnug and Evans 1992; Ceccotti and Messick 1997; Zhao *et al.* 1999).

## ATMOSPHERIC SULFUR AND PLANT FUNCTIONING

In rural areas the atmosphere generally contains only trace levels of atmospheric sulfur gases (nl $l^{-1}$ levels). Natural high atmospheric sulfur gas may occur locally in areas with volcanic and geothermic activity *viz.* volcanoes, fumaroles, sulfur springs, and geothermal wells. Here atmospheric

$SO_2$ or $H_2S$ concentrations may exceed the minimal active concentration of these sulfur gases for plants and may become locally a severe threat for the natural vegetation (Ernst 1993, 1997; Posthumus 1998; Yang *et al*. 2002, 2005). Potentially phytotoxic levels of these sulfur gases may occur in industrialized areas, caused by the refining of oil and utilization of fossil fuels. It is evident that in developing countries not only natural but also agricultural vegetation is at risk from elevated $SO_2$ levels, since the latter is often grown close to emission sources. For instance, in China 22.4% of cities had in 2002 a higher than 0.024 μl $l^{-1}$ annual average $SO_2$ level, and peak levels were much higher, for example in 1996 the daily average $SO_2$ levels in some cities exceeded 0.36 μl $l^{-1}$ (Yang *et al.* 2002, 2005, 2006a). These concentrations exceed the minimal effective concentration of $SO_2$ for susceptible plants (Posthumus 1998, Table 1). For comparison, in Europe an annual mean concentration of 0.008 μl $l^{-1}$ (20 μg $m^{-3}$) has been set for $SO_2$ as air quality standard for ecosystems (http://europa.eu.int/comm/environment/air/). In the USA there are both short- and long-term National Ambient Air Quality Standards NAAQS for $SO_2$ (http://www.epa.gov/air/airtrends/sulfur2.html). The short-term (24 h) standard of 0.14 μl $l^{-1}$ (365 μg $m^{-3}$) is not to be exceeded more than once per year and the long-term standard specifies an annual arithmetic mean, which may not exceed 0.030 μl $l^{-1}$ (80 μg $m^{-3}$). There are no clear air pollution standards for other sulfur gases. However, in the vicinity of surface water pollution by paper mills and farina factories, and in areas with intensive bioindustry, locally elevated levels of $H_2S$ and organic sulfur gases may be found, exceeding the odor threshold (>0.02 μl $l^{-1}$), which may affect plant functioning (De Kok *et al.* 2002; Durenkamp and De Kok 2004).

In addition to the negative consequences of soil acidification as the consequence of wet deposition, dry deposition of sulfur gases (their foliar uptake) may affect plant functioning. The physical/biochemical background of toxicity of $SO_2$ can be ascribed to the negative consequences of acidification of tissue/cells upon the dissociation of the foliarly absorbed $SO_2$ and/or the direct reaction of the formed sulfite with cellular constituents and metabolites. Likewise, the toxicity of $H_2S$ also may be ascribed to a reaction of sulfide with cellular components, for instance metallo-enzymes appear to be particularly susceptible to sulfide, in a reaction similar to that of cyanide (De Kok 1990; De Kok *et al.* 1998, 2002). The susceptibility of plants toward sulfurous air pollutants varies between species (Table 1) and the developmental stage of the plant. For susceptible species, the minimal effective concentrations of $SO_2$ and $H_2S$ are as low as 0.01–0.03 μl $l^{-1}$, respectively, and exposure to higher

concentrations may negatively affect growth and fitness of plants (Posthumus 1998; De Kok *et al.* 2000). In contrast, in some species exposure to low concentrations of $H_2S$ (0.03–0.1 μl $l^{-1}$) resulted in a slightly enhanced biomass production at sulfur-sufficient soil conditions (De Kok 1990; Durenkamp and De Kok 2005). Evidently the impact of these sulfur gases on plants is ambiguous, since they may act as toxin or plant nutrient upon foliar deposition. It is unclear to what extent metabolism of the foliarly absorbed sulfur contributes to its detoxification, since there is often no clear-cut transition in the level/rate of metabolism of the absorbed sulfur gases and their toxicity and their impact on plant functioning may depend on the soil sulfur status (De Kok 1990; Yang *et al.* 2006b).

There is little information on the impact of the organic sulfur gases on plants, since the impact of chronic low levels on plant functioning has barely been studied. It has been observed that in contrast to the observations with $SO_2$ and $H_2S$, extremely high levels (1.8–3.6 μl $l^{-1}$) of the most common organic sulfur gases did not induce acute plant injury (Taylor and Selvidge 1984).

*Table 1.* Susceptibility of crop species and maximum allowable $SO_2$ concentration for crop protection in China (derived from the "Environmental Standards" in "State Environmental Protection Administration of China" (www.sepa.gov.cn/).

| Susceptibility to $SO_2$ | Average concentration during growing season | Average daily concentration | Peak concentration |
|---|---|---|---|
| High: alfalfa, apple, barley, buckwheat, cabbage, clover, cucumber, grape, lettuce, pear, potato, pumpkin, ryegrass, sesame, soybean, spinach, sugar beet, wheat | 0.02 μl $l^{-1}$ | 0.06 μl $l^{-1}$ | 0.19 μl $l^{-1}$ |
| Medium: apricot, carrot, cherry, corn, cotton, eggplant, oat, orange, peach, plum, rice, sorghum, tobacco, tomato | 0.03 μl $l^{-1}$ | 0.10 μl $l^{-1}$ | 0.27 μl $l^{-1}$ |
| Low: broccoli, horse bean, rape, strawberry, sunflower, taro | 0.05 μl $l^{-1}$ | 0.12 μl $l^{-1}$ | 0.31 μl $l^{-1}$ |

## EXCHANGE OF GASES BETWEEN PLANTS AND ATMOSPHERE

The gas exchange between the atmosphere and the plant shoot or canopy can be described according to Fick's law for diffusion (De Kok *et al.* 1991; Baldochi 1993; De Kok and Tausz 2001):

$$J = \Delta c.g$$

Where J represents the rate of the gas exchange (nmol $cm^{-2}$ $s^{-1}$), $\Delta c$ the gas concentration gradient between the atmosphere and the plant shoot or canopies (nmol $cm^{-3}$) and g the overall diffusive conductance of the plant shoot or canopy towards the gas (cm $s^{-1}$).

*Table 2.* Chemical and physical properties of the major atmospheric sulfur gases. (The Henry's law constants of reduced sulfur gases are derived from De Bruyn *et al*. 1995.)

| | Molecular mass (g $mol^{-1}$) | Boiling point (ºC) | Vapor pressure (bar, 20ºC) | Henry's law constant (mol $l^{-1}$, 25ºC) |
|---|---|---|---|---|
| Carbon disulfide | 76.1 | 46 | 0.4 | 0.054 |
| Carbonyl sulfide | 60.1 | –50 | 12.5 | 0.022 |
| Dimethyl sulfide | 62.1 | 37 | 0.6 | 0.474 |
| Hydrogen sulfide | 34.1 | –60 | 18.2 | 0.086 |
| Methyl mercaptan | 48.1 | 6 | 1.7 | 0.201 |
| Sulfur dioxide | 64.1 | –10 | 3.4 | 1.23 |

For foliar deposition (*viz.* absorption) or emission of a sulfur gas, its exchange between the atmosphere and the plant occurs mainly via the stomates, since the cuticle is hardly permeable for gases and is in general a negligible factor in total gas exchange (Lendzian 1984). In addition to the stomatal conductance (degree of stomatal opening), the exchange strongly depends on the mesophyll conductance toward the gas, which is determined by the physical and biochemical characteristics of the gas, *viz.* solubility, dissociation, reactivity, and by the rate of synthesis or metabolism of the gas in the mesophyll. According to Henry's gas law, the amount of a gas dissolved in the mesophyll water phase would be directly proportional to the *in situ* partial pressure of that gas in equilibrium with the mesophyll water phase (at a constant temperature). However, the solubility of the different sulfur gases in water vary as is illustrated by their

Henry's law constants (Table 2) and is temperature dependent (solubility increases with a decrease in temperature). Furthermore, the chemical/ physical properties of a specific gas *viz.* its reaction with and dissociation in water, its vapor pressure (increases with temperature), and also rates of metabolism or synthesis in the mesophyll, may all affect the exchange of gases between the shoot and the atmosphere. It is evident that depending on their ambient atmospheric levels, the plant shoot may be both source and sink of atmospheric sulfur gas species (Baldocchi 1993; Schröder 1993).

## FOLIAR DEPOSITION OF SULFUR GASES

At elevated atmospheric levels of $SO_2$, $H_2S$, and COS plant foliage may form a potential sink for these sulfur gases (dry deposition), however, the pattern and kinetics of uptake by the shoot differs between gases. The uptake of $SO_2$ by the shoot is determined by the chemical/physical properties of the gas, whereas that of $H_2S$ and COS is largely determined by their rate of metabolism in the plant.

At ambient levels of $SO_2$, there is generally a linear relation between its uptake by the shoot and the atmospheric concentration (De Kok 1990; De Kok and Tausz 2001). The overall shoot conductance towards the gas appears often to be close to the stomatal conductance, which means that stomatal opening is the limiting factor for the uptake of $SO_2$ by the shoot. Indeed, the mesophyll conductance to $SO_2$ is relatively high, since the gas is highly soluble in the water of the mesophyll apoplast and symplast; in equilibrium there is about 40 times more $SO_2$ dissolved than is present in the atmosphere. This is not solely based on its high Henry's law constant, but also on the fact that this gas is reacting with water and is dissociated, whereby $H^+$ ions are liberated:

$$SO_2 + H_2O \rightarrow HSO_3^- + H^+ \quad (pK_a = 1.8)$$
$$HSO_3^- \rightarrow SO_3^{2-} + H^+ \quad (pK_a = 6.9)$$

The (bi)sulfite formed may be either enzymatically or nonenzymatically oxidized to sulfate or reduced in the chloroplast and assimilated into organic sulfur compounds (De Kok 1990; De Kok and Tausz 2001, Figure 1). In case the buffering capacity is not sufficient, the liberated $H^+$ ions upon hydration of $SO_2$ and/or the sulfate formed after its oxidation may be the basis of a possible acidification of the mesophyll cells, which is likely to be part of the physiological basis for the toxicity of $SO_2$.

The uptake of $H_2S$ by shoots shows saturation kinetics with respect to the atmospheric concentration and strongly decreases with temperature (De Kok *et al.* 1998; De Kok and Tausz 2001; De Kok *et al.* 2002). The uptake at high atmospheric $H_2S$ levels and low temperatures appears to be limited by mesophyll rather than stomatal conductance. The mesophyll conductance towards $H_2S$ is determined by its rate of metabolism rather than the chemical/physical properties of this gas and it varies between species. Evidently at the apoplastic pH of the mesophyll cells, which varies between 5 and 6.4, the absorbed $H_2S$ is largely undissociated:

$$H_2S \rightarrow HS^- + H^+ \quad (pK_a = 7.0)$$

In this form it will easily pass through the membrane and is subsequently metabolized with high affinity into cysteine and subsequently into other sulfur metabolites. $H_2S$ uptake appears to result from the activity of *O*-acetylserine(thiol) lyase, the affinity of the enzyme for sulfide and the *in situ* availability of *O*-acetylserine and it coincides with the sulfur requirements of a species for growth (De Kok 1990; De Kok *et al.* 1998; De Kok and Tausz 2001; De Kok *et al.* 2002, Figure 1).

Shoots may form a sink for atmospheric COS, which is taken up via the stomates and in the mesophyll cells it may be hydrolyzed to yield $H_2S$ and $CO_2$ by a carbonic anhydrase (Protoschill-Krebs *et al.* 1996; Sandoval-Soto *et al.* 2005; Geng and Mu 2006, Figure 1), an enzyme which in at least C3 plants, is predominantly present in the chloroplast and might function in diffusion of $CO_2$ and $HCO_3^-$ across the chloroplast (Badger and Price 1994).

## FOLIAR EMISSION OF SULFUR GASES

Plants may emit substantial levels of sulfur gases mainly as $H_2S$ via their shoots into the atmosphere, especially when they are previously exposed to high levels of atmospheric sulfur, or when the sulfate uptake by the roots is bypassed and sulfur either in the oxidized or reduced form is directly supplied to foliar tissue (De Kok 1990; Schröder 1993). It was presumed that emission of reduced sulfur compounds occurred as a regulatory step in the homeostasis of the sulfur pools in plants (Rennenberg 1984; Schröder 1993; Haneklaus *et al.* 2003). $H_2S$ may be formed prior to or after the synthesis of cysteine in the latter case by cysteine desulfhydrase (Schröder 1993; Haneklaus *et al.* 2003; Riemenschneider *et al.* 2005a,b, Figure 1). However, to what extent $H_2S$ emission has physiological significance

under natural conditions appears to be unclear (Ernst 1990). It is evident that plants grown under normal sulfur conditions may emit minute levels of $H_2S$ (Schröder 1993; Haneklaus *et al.* 2003), though the rate is a negligible proportion of the total sulfur flux in plants (Stulen and De Kok 1993).

Plants are also reported to emit COS (Schröder 1993; Sandoval-Soto *et al.* 2005; Geng and Mu 2006), however, the compensation point for COS of different species appears to vary from 0.09 to 0.8 nl $l^{-1}$, which is close to the natural ambient COS levels (approximately 0.5 nl $l^{-1}$, Kesselmeier and Merk 1993; Geng and Mu 2006). Plants also may emit DMS (Schröder 1993, Sandoval-Soto *et al.* 2005; Geng and Mu 2006), whose emission is highly significant in some plant species from marine ecosystems, e.g. Spartina. In these species the source of DMS is dimethylsulfonio-propionate, which may accumulate in leaves upon excessive sulfur supply and may enzymatically be degraded and yield DMS (Ernst 1990; Bentley and Chasteen 2004, Chapter 4). Specific species (e.g. *Allium* and *Brassica*) may emit a variety of other organic sulfur gases including DMS, which are likely degradation products of sulfurous amino acids and secondary sulfur compounds (Lanzotti 2006). It has been proposed that sulfur gas emission might be involved as a factor in sulfur induced resistance of plants against pests and diseases (Haneklaus *et al.* 2003).

## METABOLISM OF SULFUR GASES

In addition to wet-deposited atmospheric sulfur, which may be taken up as sulfate by the root, plants are also able to utilize foliarly taken up sulfur gases as sulfur source for growth (De Kok 1990; De Kok *et al.* 1998, 2002; De Kok and Tausz 2001, Figure 1). The absorbed $SO_2$ and $H_2S$ may directly enter the sulfur assimilatory pathway and be metabolized into organic sulfur compounds and contribute to plant sulfur nutrition (De Kok 1990; De Kok *et al.* 1998, 2002; Stulen *et al.* 1998; De Kok and Tausz 2001; Yang 2006a,b, Figure 1, Table 3). For instance, it has been established, that a continuous exposure of curly kale (*Brassica olereacea*) to ≥0.06 µl $l^{-1}$ $H_2S$ appeared to be sufficient to cover the sulfur requirement for growth, in the absence of sulfate in the root environment (De Kok *et al.* 1998, 2002; Buchner *et al.* 2004). However, the ability of Chinese cabbage (*Brassica pekinensis*) to utilize $SO_2$ as sulfur source strongly depends on the sulfur status and/or developmental stage of the plant, and prolonged sulfate-deprived plants benefited only little from $SO_2$ exposure (Yang *et al.* 2006b). It was unclear to what extent the latter

effects can be explained by an interfering toxicity of $SO_2$ in the absence of sulfate supply.

Both $SO_2$ and $H_2S$ exposure may substantially enhance the size and alter the composition of the thiol pool of the shoot (De Kok 1990; De Kok and Stulen 1993; De Kok *et al.* 1998, 2002; De Kok and Tausz 2001). Exposure generally results in a rapid enhancement of the water-soluble nonprotein thiols, up to 5-fold depending of the atmospheric levels, though a maximum is reached within hours. In addition to glutathione, usually the most abundant thiol compound present in plant tissue, strongly enhanced levels of cysteine (up to 30-fold) and, in the dark high levels of γ-glutamyl-cysteine (up to 20-fold) may occur in the shoot (De Kok 1990; De Kok *et al.* 1998, 2002). The physiological background of the altered composition of the thiol pool in the shoot upon exposure to atmospheric sulfur gases is still largely unclear. Sulfate taken up by the root is reduced and metabolized into cysteine in the chloroplast, whereas the foliarly taken up sulfur gases might in part be metabolized outside of the chloroplast beyond strict regulatory feedback control. The accumulation of γ-glutamyl-cysteine in the dark, upon exposure to sulfur gases, could be attributed to a subcellular shortage of glycine for glutathione synthesis, since its accumulation was prevented by the additional supply of glycine to the leaf tissue, yielding in glutathione accumulation (Buwalda *et al.* 1990). An altered thiol size and composition has no direct impact on plant growth and functioning (De Kok 1990; De Kok *et al.* 1998, 2002).

Shoots of $SO_2$-exposed plants may contain an enhanced total sulfur content, in some species even at relatively low atmospheric levels, which is in general due to an enhanced sulfate content. Evidently, upon oxidation of the foliarly absorbed $SO_2$, the formed sulfate is transferred into the vacuole, wherein it is accumulated and remains accessible for metabolism (De Kok 1990; De Kok and Tausz 2001). Similarly, for some plant species, $H_2S$ exposure also may result in an enhanced sulfate content of the shoot, though to a lesser extent than with equal concentrations of $SO_2$ (De Kok 1990). Some plant species have the potential to synthesize secondary sulfur compounds *viz.* γ-glutamyl peptides and alliins in *Allium* species (e.g. onion, garlic, leek). These compounds are synthetized from cysteine, via γ-glutamylcysteine or glutathione and their levels or that of their precursors and/or degradation products in the shoot may, in addition to sulfate, be strongly enhanced upon $H_2S$ exposure (Durenkamp and De Kok 2002, 2003, 2004).

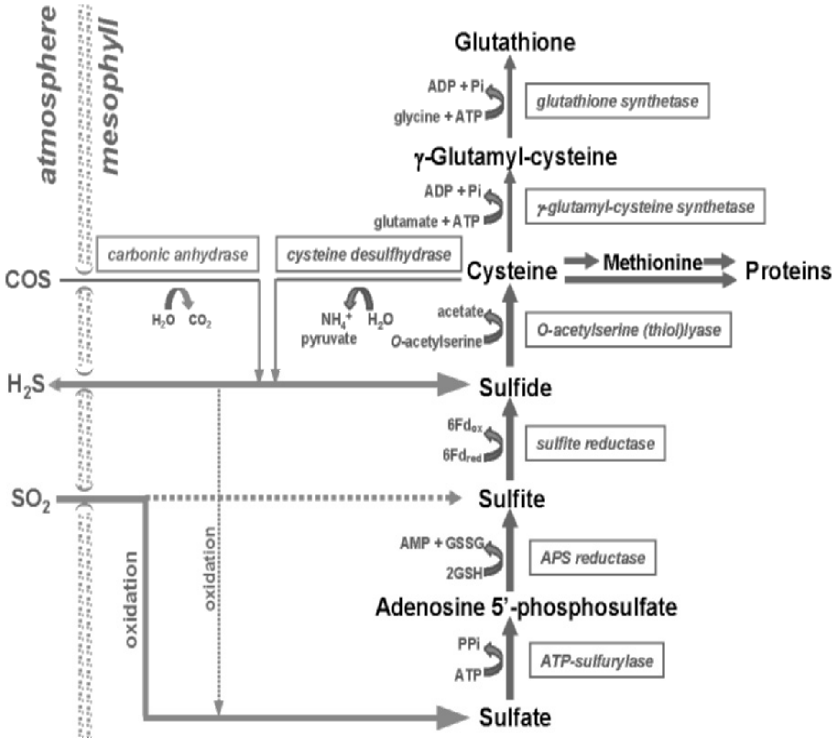


*Figure 1.* Deposition and emission of COS, $H_2S$ and $SO_2$, and their interaction with plant sulfur metabolism. APS reductase, adenosine 5'-phosphosulfate reductase; $Fd_{red}$, $Fd_{ox}$, reduced and oxidized ferredoxin; GSH, GSSG, reduced and oxidized glutathione.

At the whole plant level, the uptake of sulfate by the root and its transport and assimilation in the shoot is coordinated by and balanced with the actual sulfur requirement for growth (Hawkesford and De Kok 2006). Exposure of plants to $SO_2$ and $H_2S$ may affect the uptake of sulfate by the root, and its transfer to and its reduction in the shoot (De Kok 1990; Herschbach *et al.* 1995a,b, 2000; Westerman *et al.* 2000a,b, 2001a,b). For instance, upon $H_2S$ exposure of *Brassica oleracea* at ≥0.2 μl $l^{-1}$, this species stitched from utilizing sulfate taken up by the root to sulfide taken up by the shoot as the sulfur source for structural growth, which resulted in a partial decrease in the uptake of sulfate by the root (Westerman *et al.* 2000a,b, 2001a) and in a decrease in the APS reductase activity in the shoot (Westerman *et al.* 2001b). It is as yet unsolved to what extent the impact of $H_2S$ exposure proceeds via an allosteric inhibition and/or a repression of the genes involved in expression of the sulfate transporters

and APS reductase activity and to what extent sulfate itself (sulfate uptake by the root) and cysteine or glutathione (APS reductase activity) were involved in the signal transduction pathway.

Sulfate deprivation of the root generally induces multiple responses enabling an enhanced sulfate uptake efficiency on a whole plant basis. For instance, sulfate deprivation generally results in a rapidly induced mass expression of the sulfate transporters mRNAs accompanied with an enhanced sulfate uptake capacity by the roots, whereas more prolonged sulfate deprivation results in an altered shoot to root biomass partitioning in favor of that of the root (Stuiver *et al.* 1997; Buchner *et al.* 2004; Yang *et al.* 2006a,b, Chapter 1). Despite the fact that plants are able to transfer from sulfate taken up by the root to absorbed $SO_2$ and $H_2S$ as sole sulfur source for growth, the enhanced sulfate uptake capacity, a mass expression of the various sulfate transporters in the root and the altered shoot to root partitioning in favor of that of the root upon sulfate deprivation were not rapidly alleviated upon exposure (Buchner *et al.* 2004; Durenkamp and De Kok 2005; Yang *et al.* 2006a,b,). Apparently in the absence of sulfate in the root environment there was a poor shoot to root signaling for the regulation of sulfate uptake and shoot to root partitioning.

*Table 3.* Possible contribution of foliar uptake of $SO_2$ to the sulfur requirement of a plant at various relative growth rates (RGR). The sulfur contribution was estimated from the theoretical rate of $SO_2$ uptake by the leaves (see above, nmol $cm^{-2}$ $s^{-1}$), the plant leaf area ($cm^{-2}$ $g^{-1}$ plant fresh weight), the shoot to root ratio and the organic sulfur content (nmol $g^{-1}$ plant fresh weight). Values are for spinach (*Spinacia oleracea*) and derived from Stulen *et al.* (1998).

| RGR ($g\ g^{-1}day^{-1}$) | Sulfur contribution from foliar $SO_2$ uptake (as % of requirement) | | |
|---|---|---|---|
| | 0.03 $\mu l\ l^{-1}$ $SO_2$ | 0.1 $\mu l\ l^{-1}$ $SO_2$ | 0.3 $\mu l\ l^{-1}$ $SO_2$ |
| 0.05 | 40 | 100 | 100 |
| 0.10 | 20 | 61 | 100 |
| 0.15 | 14 | 41 | 100 |
| 0.20 | 10 | 31 | 92 |

## CONCLUSIONS

Throughout the world natural and agroecosystems are subjected to enhanced atmospheric sulfur levels as the consequence of volcanic activity and anthropogenic sulfur emissions. Wet and dry deposition of atmospheric

sulfur may locally and substantially contribute to plant sulfur nutrition. Foliarly absorbed sulfur gases may be directly metabolized by the sulfur assimilatory pathway. Sulfur gases are also potentially phytotoxic and there is no clear-cut transition in the level/rate of metabolism of foliarly absorbed sulfur gases and their phytotoxicity. The paradoxical effects of sulfur gases on plant functioning complicate the establishment of cause–effect relationships of these air pollutants and their acceptable atmospheric concentrations. Plants are also able to emit minute amounts of $H_2S$ and a variety of organic sulfur gases, however, the nature and their significance to the global sulfur budget needs to be further evaluated.

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Chapter 6

# SULFUR IN PLANTS AS PART OF A METABOLIC NETWORK

Rainer Hoefgen and Holger Hesse

## INTRODUCTION

Sulfur was at the basis of early life on earth as an energy source and as a reactant in biochemical processes. Remnants of this life under reducing atmospheric conditions are seen in some genera of archeae and lithotrophic bacteria where $H_2S$ serves as an energy source in the oxidizing sulfur pathway, much as water in photosynthesis, or as electron acceptor within the sulfate reducing pathway (Österberg 1997; Nisbet and Sleep 2001). Plants have retained parts of the basic principles of these pathways. Sulfur is next to N, P, and K, one of the central metabolites directly or indirectly involved in numerous plant biosynthetic and physiological processes (Nikiforova *et al.* 2004). Plant growth is dependent on and affected by environmental, biotic, and abiotic factors inducing biochemical and physiological adaptation processes: insufficient availability of sulfate affects other metabolic pathways in a pleiotropic manner demonstrating that sulfur is an integral part of plant metabolism and impairs plant productivity and vitality. Genomics approaches now provide tools to dissect, describe, and contribute to our understanding of sulfate metabolism in these processes with the aim of achieving a systems biology description (Hesse and Hoefgen 2006); the response of *Arabidopsis thaliana* to sulfur starvation is a suitable model for such a systems level analysis of plant nutrient physiology.

For land plants sulfur is an indispensable inorganic nutrient usually taken up as sulfate. Uptake and assimilation processes resemble those known for phosphate and nitrate (Kopriva and Rennenberg 2004; Hesse *et al.* 2004b). Sulfate uptake and transport is mediated by sulfate transporters



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in the root and in the whole plant (Maruyama-Nakashita *et al.* 2004; Buchner *et al.* 2004; Kataoka *et al.* 2004; Hawkesford 2003; Hawkesford *et al.* 2003; Saito 2000, Chapter 1). Excess sulfate is either channeled to sulfolipids (Benning 1998; Harwood and Okanenko 2003; Frentzen 2004) or reduced to sulfide and incorporated into cysteine while the remainder is stored in the vacuole. Cysteine is an integral part of proteins determining structure and function, for example being involved in redox reactions. Further, cysteine is converted to the nutritionally important amino acid methionine, as well as to a wide range of sulfur-containing metabolites, predominant among them are glutathione (GSH) and *S*-adenosylmethionine (SAM) (Hesse *et al.* 2004a; Hesse and Hoefgen 2003; Matthews 1999; Hell and Rennenberg 1998; Hell 1997; Azevedo *et al.* 1997; Anderson 1990). The control of cysteine and methionine biosynthesis has been the target of numerous studies at the biochemical and molecular biology level (Riemenschneider *et al.* 2005b; Hesse *et al.* 2004a; Wirtz *et al.* 2004; Hell *et al.* 2002; Nikiforova *et al.* 2002; Galili and Höfgen 2002; Berkowitz *et al.* 2002; Höfgen *et al.* 2001). These proteinogenic amino acids are essential for humans and livestock such as monogastric animals and birds (Hawkesford *et al.* 2006). Further, enzyme activities depend on Fe/S clusters as prosthetic groups and vitamin cofactors such as biotin and thiamine. Numerous derived compounds such as GSH, phytochelatins(PCs), thioredoxins, sulfated and sulfonated compounds, Co-enzyme A, SAM, and *S*-methylmethionine (SMM) have essential functions in plant metabolism. In relation to thiol-based activation of metabolites, the thiol group of Coenzyme A (CoA) for example, is involved in numerous cellular processes where activation of molecules is necessary to allow further reactions. Examples include the serine activation to *O*-acetylserine to form cysteine, or pyruvate activation catalyzed by pyruvate dehydrogenase converting pyruvate to the versatile metabolic precursor acetyl-CoA, which feeds into the tricorboxylic acid (TCA) cycle and indirectly through these anaplerotic reactions into numerous compounds such as amino acids, pyrimidines, alkaloids, porphyrins, fatty acid and terpene biosynthesis, or protective agents such as cyanogenic glucosinolates. Fatty acid biosynthesis would be impossible without binding of the growing fatty acid chain to a thiol group of the acyl carrier protein and the repetitive delivery of acetyl-CoA. Fragrances and tastes are often determined through sulfur-containing compounds or their breakdown products.

In order to dissect this complex integrated system, genomics approaches provide tools for analysis. Ideally, high-throughput analytical technologies provide systematic unbiased data sets of multiple parallel or sequential samples. Data analysis should enable a systems level interpretation

of the different functional components of plant cells, organs, and entire plants by predicting their properties through quantitative simulation models. To reach systems level knowledge, mathematical and computational methods for modeling and simulating complex biological systems have to be employed. The ideal result would be detailed, accurate and quantitative predictions of the behavior of biological systems, including predictions of the effects of systems modifications, i.e. simulations. These predictions can be tested to refine the model and allow ingenious optimization of plant processes through plant breeding, either by classical or transgenic means. At the analytical level, systems biology relies on the comprehensive profiling of large numbers of elements. These approaches are commonly referred to as transcriptomics (Holtorf *et al.* 2002; Oliver *et al.* 1998), proteomics (Blackstock and Weir 1999; Thiellement *et al.* 1999; van Wijk 2001), and metabolomics (Fiehn *et al.* 2000; Trethewey *et al.* 1999; Trethewey 2001, 2004). The use of these "omics" technologies to gain comprehensive data sets has increased rapidly during recent years, especially with respect to studying mechanisms underlying plant growth and plant responses to perturbations. The new high-throughput tools of genomics have provided the potential to systematically analyze perturbed biological systems and monitor the responses. The challenge of systems-based approaches now lies in extracting information from the multivariate experiments and in building models that incorporate all of the data. With the development of computational-based statistical methods, it is now possible to extract the maximum amount of information from experiments involving genome-scale data. In systems biology, bioinformatic tools are not only required to analyze the genomic data but, most importantly, to determine the experimental parameters needed for model building. Testing the derived models *in vivo* with mutants completes the circle. Thus, by combining new tools in genomic biology and bioinformatics, systems biology paves the way to comprehend complex biological systems.

The knowledge provided through these molecular analyzes and interpretations will have a bearing on plant breeding strategies and agricultural practices and, hence, environmental and ecological issues (Figure 1). In agricultural practice even moderate sulfate limitation leads to effects on yield and plant performance such as stress and pathogen resistance, or more generally, insufficient sulfate availability impairs the ability of plants to cope with additional stresses. Severe insufficiencies lead to acute growth and yield depressions often coupled to inappropriate fertilizer use regimes which may negatively affect the environment (Haneklaus *et al.* 2003; Hesse and Hoefgen 2001; Blake-Kalff 2000). Despite this importance for plant biochemistry, plant sulfur metabolism has been much less thoroughly investigated than that of carbon, nitrogen,

or phosphorus metabolism in plants. It has, however, gained much more attention in the past three decades after the unexpected observation of sulfur limitation affecting agricultural production due to reduced air pollution by $SO_2$, mainly derived from fossil fuels, i.e. of biogenic origin again being converted to sulfuric acid when dissolved in water (Chapter 4).

## PLANT SULFUR NUTRITION

On a wider level, nutrient availability in general has an impact on diversity and productivity of vegetation and, hence, fauna in ecological systems (Scherber *et al.* 2006; Kahmen *et al.* 2005, 2006; Palmborg *et al.* 2005; Perner *et al.* 2005). For example, iron is generally limiting in oceans and thus import of iron from rivers or Sahara storms trigger algal blooms, experimentally validated through "seed" experiments dumping tons of iron into aquatic systems allowing primary producers to build up enormously harnessing these resources (Jickells *et al.* 2005; Morel and Price 2003; Siegenthaler and Sarmiento 1993). As well in terrestrial systems nutrient management and understanding nutrient fluxes is an integral part of ecosystem development (Figure 1). There are intentions to put this on a basis that allows calculation by ecological programs such as the SAVANNA model for wildlife conservation areas (Coughenour 1992, Christensen *et al.* 2003; Augustine 2004, 2003). For example, the coexistence of Maasai population and wildlife in the Serengeti produces accumulation of nutrients in Maasai settlements, called Bomas, where livestock drops faeces and, when abandoned in regular shifts of the half-nomad lifestyle, this allows the development of bush islands which would normally not occur in the open savannah steppe. These islands foster plant and animal life locally and in the savannah through provision of a more complex ecosystem – based on an improved supply of plant available nutrients. The influence of N and P has been investigated, though it can well be speculated that also S plays a major role, as sulfur import by rain is extremely scarce in the semidesert environment far from the sea.

Quantitatively, plant need for sulfate is about 10% that of nitrogen for optimal plant growth. Probably, this is one of the reasons for under-estimating the importance of sulfate in agriculture as this is already in the area of contaminations in classical nitrogen phosphor potassium (NPK) fertilizers or farm manure (Blake-Kalff 2000). Fertilization and resource management of other plant nutrients, N, P, and K were long established – the importance of the macronutrient S has long been underestimated as being in abundance through air pollution. Natural sources of sulfate are

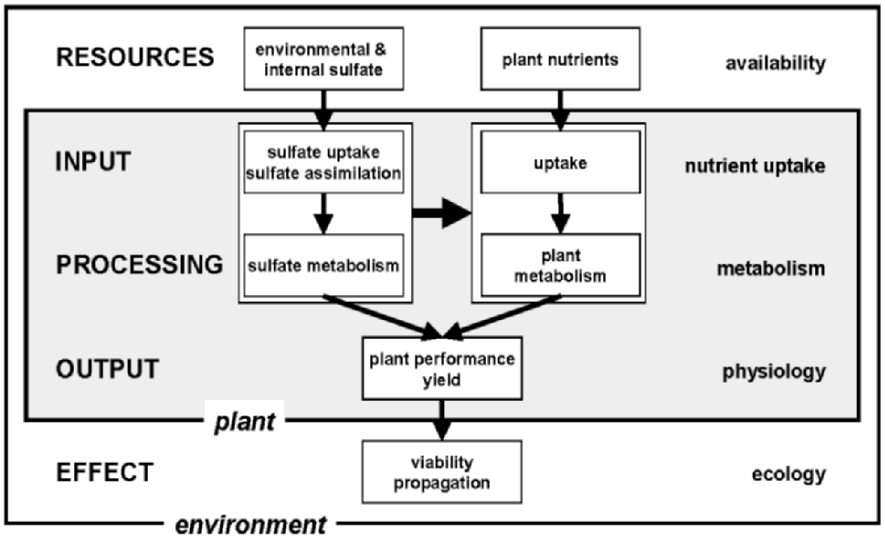


*Figure 1.* Plants are complex systems within a complex multifactorial environment and have evolved the ability to respond to various resources and input situations to achieve competitiveness and to propagate in a complex ecosystem comprised of abiotic and biological factors. Sulfate metabolism as an integral part of plant metabolism is influenced by and influences itself plant metabolism. As a macronutrient sulfate availability has a significant impact on the physiological responses of the plant to environmental factors and is a crucial integral determinant of plant vigor, yield, viability and propagation.

bacterial degradation of deteriorating plant material, gaseous compounds from volcanoes ($SO_2$) or marine algae (DMS) being deposited by rainfalls as sulfite or sulfate, resulting in an ocean-cloud-land-ocean sulfur cycle. Gaseous air pollutions from fossil fuels throughout the industrial revolution attained wide interest as causing sulfuric rains and water body acidification (Curtis *et al.* 2005). The resulting massive ecological problems stirred a public debate forcing political decisions culminating in clean air acts effectively reducing total $SO_2$ output from industries. Only then, the agronomical importance of sulfate provision to the crop plant was realized as decreasing S inputs to the fields resulted in agronomical problems of reduced yields and plant health. This agronomical interest together with the progress in plant biochemistry, molecular biology, and physiology boosted our knowledge on details of sulfur metabolism in

plants, though the basic principles had been worked out earlier, often in bacterial systems (Bryan 1980, 1990).

Plants are able to adapt to and cope with a variety of soil-borne nutrient conditions. Under natural conditions characteristic plant associations develop on certain stands as the most adapted plants compete more efficiently leading to typical associations of seaside vegetation, nutrient rich or nutrient poor meadows, vineyards, etc. Under agricultural conditions crop species artificially dominate the ecosystems and though often not being competitive under "natural" conditions the agricultural practice as well as the use of herbicides allow them to establish. Further, high-yielding elite crop varieties are bred to fit to certain agricultural practices, e.g. short straw wheat's would normally be overgrown by tall grasses or they need high fertilizer dosages for optimal growth. Changes in agricultural practices such as organic farming, no tilling, use of marginal lands, reduction of fertilizer and pesticide inputs either due to economic or environmental concerns and altered consumer behavior ask for a better understanding of plant physiology, biochemistry and molecular biology to eventually provide breeders with opportunities to breed new, and better varieties.

Above all, the major pressure on agricultural practices and policies will be the need to feed a growing world population which is expected to reach 10 billion in 2050 asking for an increase of 100% of agricultural primary production, i.e. another "green revolution" (Cakmak 2002). For example wheat yield in a long-term experiment could be increased from 3 ton $ha^{-1}$ on average before 1920 to 10 ton $ha^{-1}$ nowadays (Miflin 2000). The challenge is even bigger as increasingly arable land is lost due to urbanization, desertification, salinization, or global climate changes. On the other hand, the need for high-quality protein increases with more countries advancing from developing to threshold or industrialized countries, thus asking for higher yielding crop cultivars and more plant-based-high protein feed for increased livestock production (FAO 2002). As the impact of cropping techniques and agrochemicals cannot be assumed to further increase substantially, future potentials to increase yield and quality of crops have to be mainly expected from plant breeding and green biotechnology. The luxury attitude of the consumers in some of the developed countries, e.g. with respect to nonacceptance of GMO-based plant products, just ignores immanent world wide tendencies and camouflages problems necessary to be solved pragmatically. Not meeting this challenge will lead to malnutrition, especially negatively affecting children under 5 years leading to retarded physical and mental development (Tabe and Higgins 1998; Pinstrup-Andersen 2002). Understanding sulfate metabolism and its integration into plant metabolism will contribute to improve

plant agricultural performance, yield, plant vigor, and product quality at various aspects as nutritional quality or low fungal toxin contaminations.

## SULFUR ASSIMILATION AND REDUCTION

The primary source of sulfur for plants is sulfate, though plants are also able to take up gaseous sulfur compounds and either incorporate them ($H_2S$, $SO_2$) immediately into cysteine or to retrieve it through catabolic processes (sulfite oxidases; Hänsch *et al.* 2006; Hänsch and Mendel 2005; Durenkamp *et al.* 2005; Riemenschneider *et al.* 2005a; Yang *et al.* 2006; Durenkamp and De Kok 2005). In general, after uptake and activation of the almost inert sulfate molecule, plants reduce sulfate to sulfide and synthesize the thiol amino acid cysteine as the first common intermediate of all downstream reactions. An important branch is the use of activated sulfate without further reduction to synthesize sulfonates such as sulfolipids or sulfate metabolites (Figure 2).

Sulfate in soils moves with the capillary water. Interestingly, a substantial amount of sulfate in rich organic soils is bound in the organic material fraction rather than the mineral constituents and is released through the deteriorating activity of bacteria (Kertesz and Mirleau 2004). Whether plants are able to actively stimulate beneficial bacterial associations by providing, e.g. carbohydrates is still unknown. While bacteria, fungi, and seawater organisms use ATP-binding cassette (ABC) sulfate permeases, plants have developed highly specific sulfate transporters which can grossly be grouped into high ($K_m$ 1 – 10 μM) and low ($K_m$ 0.1 – 1 μM) affinity proton/cotransporters (Anderson 1990; Hawkesford 2000; Saito 2000; Grossman and Takahashi 2001, Chapter 1). The uptake and transport of sulfate within the plant probably takes the same combined apoplastic/ symplastic route as nitrate and phosphate. Sulfate is either reduced and incorporated into organic molecules, central among them the thiol amino acid cysteine, or it can be deposited to substantial amounts in vacuoles then being much less mobile than other ions. Here then, it might be retrieved under insufficient supply situations from the soil or during grain filling.

Prior to reduction the sulfate ion is activated (by binding to ATP) by ATP sulfurylase to form adenosine 5′-phosphosulfate (APS; Figure 2). ATP sulfurylase (ATP-S) isoforms in plants are located either in plastids or in the cytosol. The APS serves as a substrate for two branches: sulfate reduction or phosphorylation by APS kinase to yield 3′-phosphoadenosine

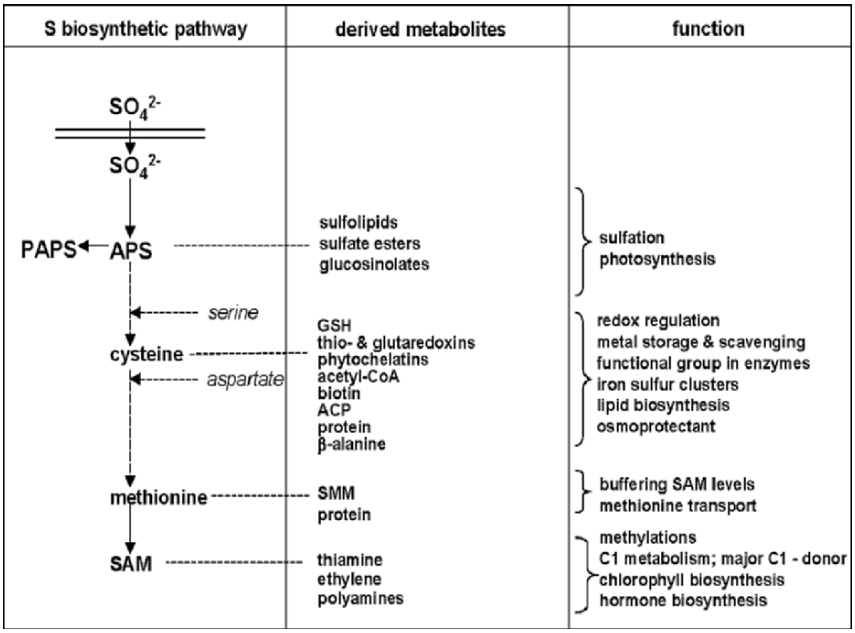


*Figure 2.* The core of the sulfur biosynthetic pathway comprises the uptake of sulfate through the plant membrane, the activation of the inert molecule to form APS and either its further activation to PAPS serving as precursor for sulfate transfer reactions or its reduction to sulfide and incorporation into serine to form cysteine, the central precursor of all plant metabolites containing reduced sulfur moieties. Furthermore the central pathway culminates in synthesis of methionine and the eventual pathway end product, S-adenosylmethionine (SAM). Numerous compounds are derived from this primary pathway and, hence, numerous functions can be assigned to the various metabolites within plant metabolism.

5′-phosphosulfate (PAPS). PAPS is the substrate of sulfotransferases, which catalyze the sulfatation of a range of metabolites including plastidial sulfolipids, flavanols, choline, betaines, and glucosides. In the reductive pathway, APS bound sulfate is reduced to sulfite by the plastid-localized APS reductase (APR) probably with GSH as a reductant, as a domain of the enzyme resembles a GSH-dependent reductase. APR is highly regulated at the molecular and biochemical level through numerous stimuli such as sulfate availability, hormones, nitrogen and others, obviously integrating various signal inputs of various parallel primary anabolic pathways. Sulfite is reduced to sulfide in a ferredoxin-dependent reaction by plastidial sulfite reductase (SIR). *O*-acetyl serine-(thiol) lyase (OAS-TL) eventually transfers sulfide to an activated serine, *O*-acetyl serine (OAS), resulting in

cysteine (2-amino-3-mercapto-propionic acid) formation. Cysteine is the common precursor of all following metabolic steps employing reduced sulfur. Serine acetyltransferase (SAT) catalyzes the formation of the activated OAS and is located in the cytosol, chloroplasts, and mitochondria and feedback inhibited by micromolar concentrations of cysteine. Upon sulfur starvation the mRNA levels of plastid SAT increase. SAT is associated with OAS-TL in the cysteine synthase complex to synthesize OAS. The complex, though, is inefficient in synthesising cysteine and rather free OAS-TL appears to be responsible for cysteine synthesis.

The sulfate reduction pathway is present in different cellular compartments, the cytosol, plastids, and mitochondria, though partially incomplete in the cytosol and mitochondria. The reason for this is not unequivocally proven but might have to do with necessary detoxification mechanisms of released sulfide resulting from catabolic processes. A complex regulatory pattern, first at the biochemical, and second at the molecular level, results in a strict regulation of sulfate uptake and assimilation adapting biosynthesis to sink demands. For example, heavy metal treatment increases GSH and phytochelatin biosynthesis (Cobbett 2000a,b; Cobbett and Goldsbrough 2002)

## METHIONINE BIOSYNTHESIS

Methionine is synthesized in three steps in the chloroplast catalyzed by cystathionine γ-synthase (CGS), cystathionine β-lyase (CBL), and methionine synthase (MS; Ravanel *et al.* 2004; Hesse and Hoefgen 2003; Ravanel *et al.* 1998). Free methionine only occurs in marginal amounts in plants. About 20% of the methionine is incorporated into proteins while 80% is converted to SAM, thus essentially constituting the end product of the methionine biosynthetic pathway. CGS catalyzes the formation of the thioether cystathionine from the substrates cysteine and *O*-phosphohomoserine (OPHS). The common branchpoint at OPHS of the methionine and threonine biosynthetic pathway in plants requires an effective regulation. There is no evidence suggesting the occurrence of feedback inhibition of CGS activity by either methionine or SAM. However, in *Lemna* and *Arabidopsis* the stability of the CgS mRNA, respectively, seems to be regulated by methionine/SAM levels as shown through feeding studies with methionine in *Lemna paucicostata* (Thompson *et al.* 1982; Giovanelli *et al.* 1985a,b) and the analysis of an *A. thaliana* mutant, *mto*1, in which a mutation of the CgS gene increases the stability of the mRNA in the presence of increased levels of methionine (Inaba *et al.*

1994; Chiba *et al.* 1999). Potato CGS RNA stability, however, appeared not to be regulated by methionine (Zeh *et al.* 2001; Kreft *et al.* 2003). However, threonine synthase (TS) activity is positively regulated by *S*-adenosyl-methionine (SAM), a direct product of methionine and to be imported from the cytosol (Ravanel *et al.* 2004), thus favoring carbon flow into threonine biosynthesis when sufficient SAM is available. Under these conditions the $K_m$-values of TS for OPHS have been shown to be 250- to 500-fold lower as compared to the competing enzyme, CgS (Madison and Thompson 1976; Curien *et al.* 1996; 1998, Laber *et al.* 1999; Giovanelli *et al.* 1985b). Regulation of TS occurs at the level of enzyme activity rather than at the level of gene expression (Casazza *et al.* 2000). This suggests an autoregulation of methionine synthesis by modulating metabolite flux via the TS/CgS branch point, but plant specific differences have to be considered for applied exploitation. Likewise, spatial and developmental differences in TS and CGS expression have to be taken into account (Casazza *et al.* 2000; Bartlem *et al.* 2000; Inaba *et al.* 1994).

CBL is essential but not rate limiting for methionine biosynthesis. A Met mutant was isolated from protoplast cultures of *Nicotiana plumbaginifolia* that was severely stunted in growth and development (Negrutiu *et al.* 1985). Complementation of the plant mutant with a plastidially targeted bacterial CGS restored the wild-type phenotype (Frankard *et al.* 2002). This finding is supported by the observation that transgenic potato plants expressing antisense CbL RNA showed a comparable phenotype resulting in increased levels of the upstream metabolites cysteine, cystathionine, and homoserine, while threonine remained constant and methionine was only slightly reduced (Maimann *et al.* 2000). Overexpression of CBL had no effect on metabolite composition (Maimann *et al.* 2001).

The last step of methionine synthesis is localized in the plastids and in the cytosol and is catalyzed by cobalamine-independent methionine MS, which methylate homocysteine to form methionine, using polyglutamated N5-methyltetrahydrofolate as a methyl group-donor and SAM as a cosubstrate as shown in *Catharanthus roseus* and *A. thaliana* (Ravanel *et al.* 2004). The function of this enzyme is on the one hand the *de novo* synthesis of methionine in the chloroplast and on the other hand the regeneration of SAM from *S*-adenosylhomocysteine after methylation reactions or after ethylene biosynthesis. Sucrose or photoassimilates seem to regulate MS gene expression in *C. roseus* and *Solanum tuberosum* (Eckermann *et al.* 2000; Zeh *et al.* 2001). The whole system seems to be highly and independently regulated at the transcriptional and posttranscriptional level

and to be indispensible as neither overexpression nor down regulation by antisense could be achieved in potato.

## METABOLISM OF CYSTEINE AND METHIONINE

Cysteine is converted to GSH, both metabolites apparently being in equilibrium (Figure 2). GSH is a tripeptide synthesized through the sequential activity of γ-glutamyl-cysteine synthase and GSH synthase. Further, phytochelatin synthases polymerize multiple blocks of this basic unit to synthesize PCs which probably act in heavy metal scavenging and transport, possibly as a storage form and play a role in tolerance to some heavy metals such as cadmium. PCs are synthesized by a γ-glutamyl cysteine dipeptidyl transpeptidase transferring the glutamyl cysteinyl moiety of GSH on to another GSH or on to a growing PC (Zenk 1996; Chen *et al.* 1997; Cobbet *et al.* 1998, 2000a,b; Vernoux *et al.* 2000). Usually a degree of polymerization of 5 is reached. The terminating amino acid can either be glycine, glutamate, serine, or β-alanine. GSH is a less reactive storage form of cysteine and a transport form of reduced sulfur. It is involved in stress tolerance responses by directly scavenging active oxygen species (AOS) or indirectly by reducing oxidized ascorbate in the ascorbate–gluathione cycle while itself being oxidized to a GSH dimer linked via an S–S bridge (GSSG; Noctor 2006; Leustek *et al.* 2000; Schürmann and Jacquot 2000; Jacquot *et al.* 2002; Noctor *et al.* 2002). The ratio of GSH to GSSG provides information on the redox status of the cell and, thus, stress levels. GSSG is reduced back to GSH by glutathione reductase and NAD(P)H. Further, cysteine serves as precursor for *S*-containing metabolites such as co-enzyme A, the vitamine B1, thiamine, methionine and its derivatives SAM and SMM, and many secondary compounds such as *S*-methylcysteine, *S*-alkylcysteine, glucosinolates, and phytoalexins (Schmidt and Jäger 1992; Ravanel *et al.* 1998; Matthews 1999; Hesse and Hoefgen 2003).

Methionine undergoes two major fates: first, incorporation into proteins or, second, conversion to SAM (Figure 2). SAM is a methyl donor used in DNA and RNA modification and in synthesis of abundant plant structural components, including lignin precursors, choline and its derivatives, chlorophylls, and pectin (methyl esters of polygalacturonic acid). The carbon skeleton of the methionine moiety of SAM is also used as a precursor for the plant hormone ethylene, the vitamins biotin (vitamin H) and thiamine (vitamin B1), and for polyamines. Radiotracer experiments indicate that more than 80% of the label from $^{14}$C-methyl labeled

methionine was incorporated into lipids, pectins, chlorophyll, and nucleic acids, whereas less than was found in 20% in protein (Giovanelli *et al*. 1980; Giovanelli 1990). Thus, apparently the majority of methionine is converted into SAM for transmethylation reactions in plants. As control of fruit ripening is of substantial commercial interest the pathway to ethylene synthesis has received some attention in terms of biotech applications (White 2002). Furthermore, methionine is converted to SMM which appears to be a phloem localized transport form of reduced S in plants, as is GSH (Bourgis *et al.* 1999; Ranocha *et al.* 2001). The SMM to GSH ratios vary among different plant species. SMM is synthesized from methionine and SAM by SAM:methionine *S*-methyltransferase releasing *S*-adenosylhomocysteine (MMT). SMM can be reconverted to methionine by homocysteine *S*-methyltransferase (HMT) methylating homocysteine and yielding two molecules of methionine. Essentially this appears to be a shortcut of the SAM methylation cycle and it is speculated currently that its main function is the downregulation of SAM levels in plants. However, as SMM is also transported in the phloem it might well contribute to reduced sulfur supply to sinks. In plants such as wheat, substantial amounts of reduced sulfur are transported as SMM from source leaves to sink tissues.

## GENOMICS OF PLANT SULFATE METABOLISM – SULFUR AS PART OF METABOLIC NETWORKS

In order to resolve plant sulfate metabolism at a systems level, high-throughput genomics technologies such as transcript, metabolite, and protein profiling are applied (Figure 3). To deduce the systems response, controls with sufficient sulfate supply are compared to plants or tissues grown under varied sulfate availability conditions or mutants in genes putatively affecting sulfur metabolism in plants. The first aim is the collation of a parts list, essentially a list of responding elements (transcripts, metabolites, proteins, or other determinants), which is followed by attempts to model the system and thus predict metabolic and physiological responses.

*Transcriptome analysis of* Arabidopsis thaliana *in response to sulfur deprivation*

Genomics tools, such as DNA microarrays (DNA chips), have enabled the simultaneous measurement of multiple gene expression changes in response to an experimental treatment (i.e. system perturbation) or to developmental changes (endogenous programmes). In *A. thaliana*, more than a dozen studies have been reported using chip technology to describe the transcriptome of exogenously perturbed systems or ontogenetic programs. Examples include analysis of the circadian rhythm (Harmer *et al.* 2000); hormone action (Goda *et al.* 2002; Müssig *et al.* 2002; Rashotte *et al.* 2003), stress response (Kreps *et al.* 2002; Seki *et al.* 2002; Hammond *et al.* 2003; Oono *et al.* 2003), cell cycle (Menges *et al.* 2002), developmental programs (Menges *et al.* 2002; Tepperman *et al.* 2001; Che *et al.* 2002; Honys and Twell 2003; Köhler *et al.* 2003), responses to pathogens (Puthoff *et al.* 2003; Tao *et al.* 2003), plants with altered metabolism (Laule *et al.* 2003), and plants under different nutrient regimes such as nitrogen (Wang *et al.* 2000, 2001; Colebatch *et al.* 2002), phosphate (Wang *et al.* 2002), iron (Thimm *et al.* 2001; Negishi *et al.* 2002; Wang *et al.* 2003), potassium (Wang *et al.* 2002; Maathuis *et al.* 2003), and sulfate alterations (Hirai *et al.* 2003; Maruyama-Nakashita *et al.* 2003; Nikiforova *et al.* 2003).

Transcriptome profiling of *Arabidopsis* subjected to changing sulfur availability has been used to provide a systems-level description with the aim of identifying novel *Arabidopsis* genes that respond to minus sulfur treatment (Figure 4). Consistent with previous reports, genes encoding proteins that are directly involved in sulfate transport, reduction, and assimilation are induced. Sulfate starvation results in the depletion of endogenous vacuolar stores and of derived organic compounds due to impaired biosynthesis of the respective metabolites. In sulfate-deprived plant tissues, sulfate levels, total elemental sulfur levels and the levels of the main organic molecules carrying reduced sulfate, i.e. cysteine, GSH, and proteins are reduced (Nikiforova *et al.* 2003, 2004, 2005a). The system aims at retaining metabolic homeostasis or constant fluxes through finely tuned biosynthetic (Riemenschneider *et al.* 2005a; Hesse *et al.* 2004a; Hesse and Hoefgen 2003; Matthews 1999; Hell and Rennenberg 1998; Hell 1997; Miflin and Lea 1990) or catabolic processes to balance pool sizes (Galili and Höfgen 2002).

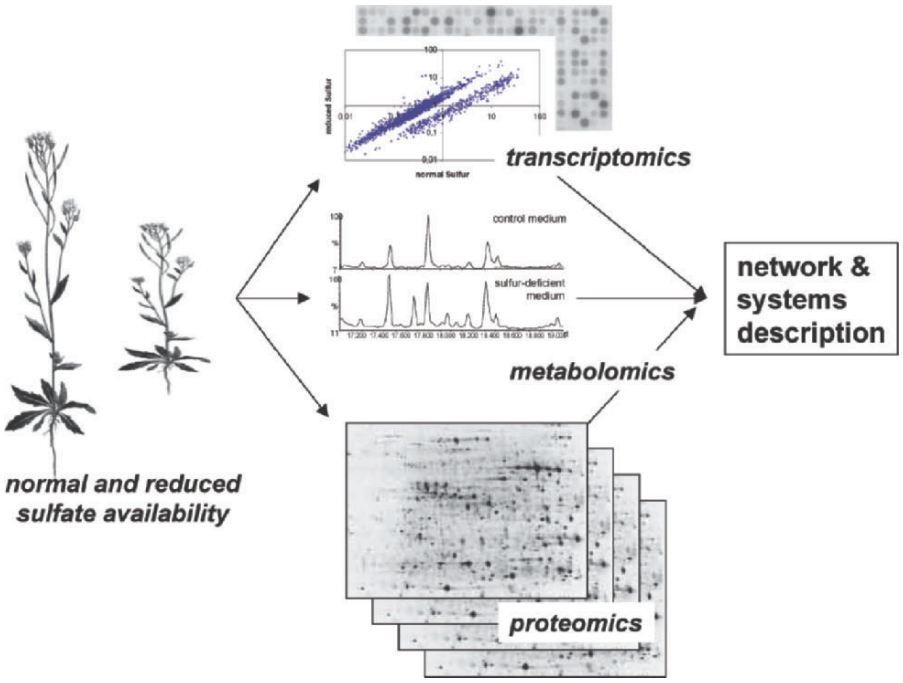


*Figure 3.* High-throughput analytical tools allow resolution of the multifaceted response of plant metabolism to alterations in sulfate nutrient availability. Transcriptomics allows scoring for changes in gene expression levels, metabolomics investigates the steady state pools of cellular compounds, i.e. essentially the result of adaptation processes to external conditions or developmental programs, and proteomics analyses reports changes in protein abundance as a result of altered transcript availability or alterations in protein stability and turnover. These data will allow a description of the systems response to sulfate in the environment.

When the regulatory capacities of the enzymatic machinery can no longer cope with the accumulating disbalances due to enduring starvation, the induction of further adaptation mechanisms involving alterations in gene expressions is observed (Saito 2000, 2004; Nikiforova *et al.* 2003, 2004, 2005a; Kutz *et al.* 2002; Hirai *et al.* 2003; Maruyama-Nakashita *et al.* 2003). Recently, a sulfur-related cis-element in the promoter of some sulfur responsive genes has been identified (Maruyama-Nakashita *et al.* 2005). In the case of sulfate deprivation of *Arabidopsis* plants in hydroponic cultures, changes in gene expression seem to be triggered about 2 days after onset of starvation and with continuing starvation the

number of induced genes further rises and increasingly involves pathway unrelated genes (Nikiforova *et al.* 2004). The accumulation of "downstream" effects is probably caused by cross influencing "linked" pathways due to the lack of metabolites or accumulation of pathway intermediates, eventually resulting in the induction of downstream processes in a snowball-like effect (Nikiforova *et al.* 2003, 2004, 2005a; Hirai and Saito 2004). Thus, the analysis of the response at the gene expression level (transcriptome analyzes using array technologies) in response to sulfate availability provides insights in the response mechanisms (Nikiforova *et al.* 2003, 2004; Saito 2004; Hesse *et al.* 2004b; Hirai *et al.* 2003; Maruyama-Nakashita *et al.* 2003; Kutz *et al.* 2002).

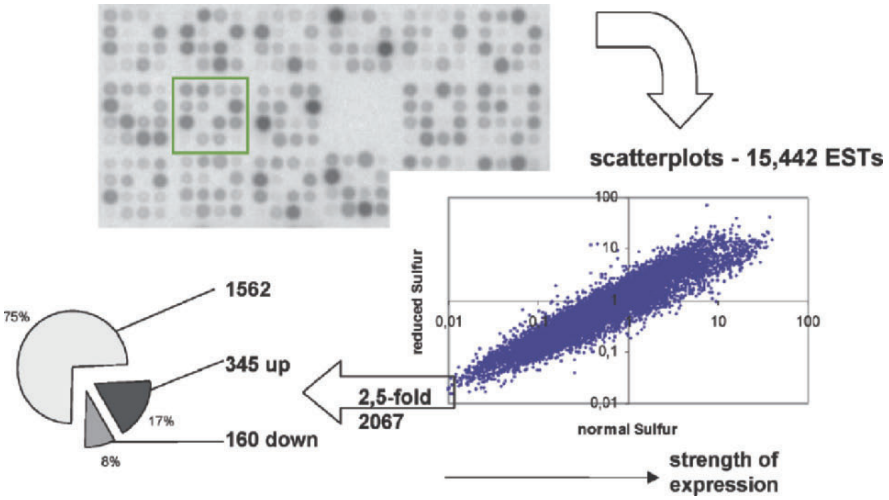


*Figure 4.* Transcriptomics allows the comparison of the transcriptome, the sum of all expressed genes, between plants or tissues under varying conditions. Here a comparison of plants under sulfate starvation with controls on sufficient sulfate supply provides a signature of expressed genes under both conditions depicted as hybridization patterns or as scatter plots of both conditions. Application of thresholds accounting for the natural occurring variability in gene expressions (fluctuation and noise) result in a set of genes showing significant diversity of expression levels between both states. The number of genes and the ratios of their expression differences depend on the time, strength, and nature of the challenging treatment. Here sulfate starvation resulted in 1,562 genes showing differential expression of which 345 and 160 were significantly up-or downregulated, respectively.

*Proteome analysis of* Arabidopsis thaliana *in response to sulfur deprivation*

Proteome analysis would provide information on changes in protein abundance in response to, here, sulfate starvation. However, currently there are no systematic proteome studies reported on plants subjected to changing environmental sulfate conditions. Detailed targeted studies have been performed using antibodies against specific proteins, performing enzyme activity assays or providing local and not proteome wide information, for example in plasma membranes (Hawkesford and Belcher 1991). Studies which have been executed on agar grown seedlings on sulfate starvation (Nikiforova *et al.* 2003) resulted in a general reduction of the total protein content, however, only in marginal changes in the content of single proteins, despite the observed significant changes in transcripts and metabolites (R. Hoefgen, personal communication).

*Metabolome analysis of* Arabidopsis thaliana *in response to sulfur deprivation*

Metabolomics intends to qualitatively and quantitatively determine the levels of as many low-molecular weight compounds as possible through ideally unbiased analytical techniques. Determination of ions and elements is sometimes termed ionomics (Lahner *et al.* 2003; Salt 2004), but is subsumed here into the wider term metabolomics. Metabolomics intends to provide the complete metabolic profile of the cell, the metabolome, comprising the steady state of metabolic and physiological processes. Thus, measurements of the metabolome in different physiological states are likely to be more indicative for the purposes of systems-oriented studies than transcriptome analyzes (Hesse and Hoefgen 2006). Eventually, this would ideally lead to a nearly complete molecular picture of the state of a particular biological system at a given time. The current status of metabolomics is summarized in several reviews (e.g. Fiehn 2002; Sumner *et al.* 2003; Stitt and Fernie 2003). Profiling schemes for *Arabidopsis* and other plants have been developed in recent years (Roessner *et al.* 2000; 2001; Fiehn *et al.* 2000; Wagner *et al.* 2003). However, major technological limitations still need to be overcome. For instance, the chemical diversity of the metabolome necessitates the use of different analytical techniques to cover different polarities and molecular sizes. Further, annotation of identified metabolite peaks is still incomplete. In this study for example of the detected peaks in GC-MS 43% and of the LC-MS 5% could be assigned to specific compounds. Experimental tools in use are element analysis via ICP-AES, ion analysis via HPLC or CE, specific HPLC analyses as, e.g. for amino acids and thiols, and highly

random, high-throughput approaches mainly based on mass spectrometry combined with various prior separation tools such as GC-MS, GC-TOF, LC-MS (Roessner *et al.* 2000; Fiehn 2002; Fiehn and Weckwerth 2003; Wagner *et al.* 2003) or others as NMR techniques (Ward *et al.* 2003; Ott *et al.* 2003; Defernez and Colguhoun 2003; Le Gall *et al.* 2003). Furthermore, the coupling of electrospray ionization (ESI) MS with CE (Soga *et al.* 2002) and hydrophilic interaction chromatography (Tolstikov and Fiehn 2002) has been successfully applied to metabolomics problems. First efforts have been made by the plant metabolomics community to agree on conventions for data formats and the description of metabolomics experiments (Bino *et al.* 2004; Jenkins *et al.* 2004). Furthermore, a platform for mass spectral and retention time indices has been established and will be extended (MSRI, www.csbdb.mpimp-golm.mpg.de/gmd.html; Schauer *et al.* 2005).

The response of the metabolome of plants to sulfur starvation has been studied in various independent approaches, mainly in *Arabidopsis*. Metabolite pools have been determined by GC-MS or LC-MS based metabolite profiling (Nikiforova *et al.* 2003, 2005b; Fiehn *et al.* 2000, 2001; Hirai *et al.* 2003; Maruyama-Nakashita *et al.* 2003, 2004, 2005). The reduced sulfate availability provides a block for cysteine synthesis as insufficient amounts of sulfide are provided through the uptake and sulfate reduction pathway (Figure 2). This leads to a reduction of the immediate products, cysteine and GSH, while the precursors, *O*-acetylserine and serine accumulate (Nikiforova *et al.* 2005b; Riemenschneider *et al.* 2005b). As serine is linked closely to glycine formation (Li *et al.* 2003; Bauwe and Kolukisaoglu 2003) the concurrent accumulation of glycine is following expectations. Cysteine itself serves as precursor of methionine through a transsulfuration reaction (Hesse *et al.* 2004a; Hesse and Hoefgen 2003). Unexpectedly though, methionine levels are not significantly reduced but kept relatively constant with minor reductions over a wide time range of sulfate and thus cysteine reduction (Nikiforova *et al.* 2005b). The main endpoints of methionine synthesis are on the one hand incorporation into proteins and on the other hand the biosynthesis of the main plant C1-metabolism methyl donor, SAM. Usually SAM is recycled and resynthesized. Both, protein and SAM are significantly reduced upon *S*-starvation which we hypothesize to be the main effector of the various downstream pleiotropic effects. Sulfur deprived plants show an accumulation of phenolic compounds as is typical for all nutrient starvations and various other environmental stresses such as cold, drought, or high light and is easily observable through changes of the leaf color (Noctor and Foyer 1998; Nikiforova *et al.* 2003, 2004, 2005a,b; Hirai *et al.* 2003, 2005; Hirai and Saito 2004). These phenolic compounds, e.g. anthocyanin, are

derived from the aromatic amino acids, tyrosine, phenylalanine, and tryptophan. Under sulfur stress a slight increase of the common precursor shikimate and of the aromatic amino acids is observed which is in concordance with the accumulation of their downstream, secondary products. While sulfate assimilation is impaired, nitrogen assimilation continues (Hesse *et al.* 2004b; Kopriva and Rennenberg 2004). The relative ratio of N to S is shifted toward an excess of N. Reduced nitrogen is bound to asparagine and glutamine seemingly buffering an excess of reduced nitrogen under sulfur limiting conditions.

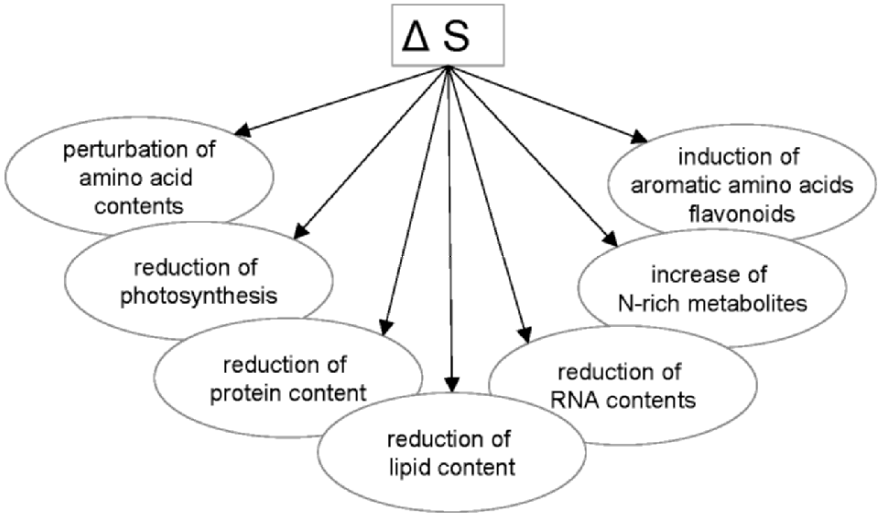


*Figure 5*. Metabolite profiling provides information on metabolites significantly altered in amount between various conditions. Hundreds of metabolites might be affected when primary environmental inputs propagate through the plant system and lead to downstream, often termed pleiotropic, effects. Under prolonged sulfate starvation a complex pattern of responses can be determined and is summarized as depicted.

In summary, metabolites directly dependent on supply of reduced sulfur are decreasing, while precursors accumulate. Upon depletion of affected precursor pools, the plant is forced into a response cascade resulting in an adjustment of the enzyme composition better suited to the altered environmental conditions (Figure 5). After sensing imbalances in nutrients or metabolites, alterations in gene expression are triggered. The transcriptional and enzymatic response of the system is eventually transmitted into alterations of metabolite pool sizes and/or metabolite

fluxes. These alterations are corresponding to an adjustment of the metabolite pool sizes and a rebalancing of the resources in adaptation to a nutrient limitation. Hence, metabolite pool sizes represent the integration of disturbed biosynthetic pathways, altered gene expression levels, and altered enzyme abundances and activities and are thus a good indicator for the response of the entire system.

*Bioinformatics: merging expressional and metabolite data*

Modeling of physiological processes is the ultimate goal of systems biology. This should greatly rationalize our attempts to understand plants. For example, genes with similar responses at the level of expression over a range of conditions are often functionally clustered together and assumed to be under the control of common transcription factors. Modeling of higher plant physiology will be especially challenging because of the differential responsiveness of various cell types/organs to a given perturbation. Collation of comprehensive data needed for modeling might initially be most successful using single-cell microorganisms or higher plant cells grown in defined liquid cultures. The modeling of *Escherichia coli* and yeast is already under way (Jönsson *et al.* 2003; Stelling *et al.* 2002; Lee *et al.* 2002) which should be a blueprint the modeling of other organisms. To model plant response accurately, a multitude of software programs of the type widely used by engineers (e.g. parameter optimization, flux balance analysis, systems analysis, and computer model simulations, to name a few) need to be adapted. The derived *in silico* models then need to be tested *in vivo* with mutant systems or refined analyses. Bioinformatic tools allow biologists to move beyond cataloguing and simple linear interpretations to increase our understanding of how network components interact (Fiehn *et al.* 2000; Girke *et al.* 2000, 2003; Jasny and Ray 2003; Bray 2003; Alon 2003; Stitt and Fernie 2003). Statistical tools are available or being established to exploit, extract, and mine raw data to perform correlation analyses and deduce matrices and networks. Furthermore, as both data sets rely on ratios between an experimental and a control state it is possible to fuse metabolome and transcriptome databases (Figure 6). Combined analyses have been performed, however mainly with only a few metabolites or on pairwise correlations (Askenazi *et al.* 2003; Urbanczyk-Wochniak *et al.* 2003). Analytical tools to analyze with distinct statistical methods the perturbation of a system in transcript and metabolic data are for example: MetaGeneAlyse (http://metagenealyse.mpimp-golm.mpg.de; Daub *et al.* 2003), MapMan (Thimm *et al.* 2004), Ara-Cyc (http://www.*Arabidopsis*.org/tools/aracyc/).

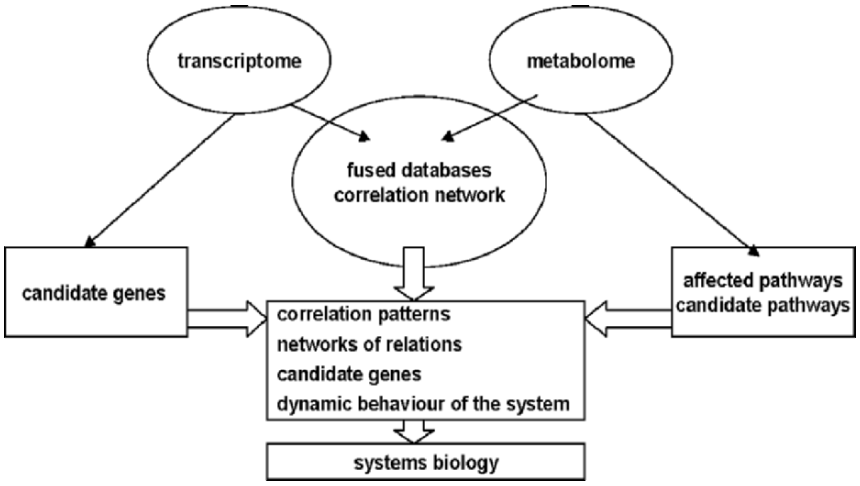


*Figure 6.* Bioinformatic approaches help to order and analyze the vast amount of high-throughput data produced. Transcriptome analyses alone provide information on candidate genes, while metabolomics provides information on affected pathways and, hence, groups of genes. Fusing these data and treating them in a combined matrix allows deduction of further contextual information not provided through either of the individual data sets. This, eventually, provides a basis for a systems level understanding.

The reconstruction of a response network is based on similarities of the patterns of the coherent behavior of the individual elements (Kitano 2002) (Figure 7). From this, the network features and elements will be deduced (Bray 2003). Such a network does not any longer mirror biochemical pathways *per se* (though it might in part) but rather describes families of cobehaving (coherent) elements (vertices, nodes) and their correlation via connecting lines (edges) (Jasny and Ray 2003). Typically, biological networks are expected to show inhomogeneous connectivity patterns distinct from a random network (Bray 2003) with elements of highest connectivity (hubs), while other elements remain lowly connected (Figure 7). These hubs will be points of high interest for further investigations and often do not appear among the usually selected genes or metabolites with highest ratios for alteration. This will allow deduction of functional relations from the network. Furthermore, this approach can be easily applied to other nutrient and environmental stresses challenging the ability of a plant to adapt, or also to investigations of plant developmental programs.

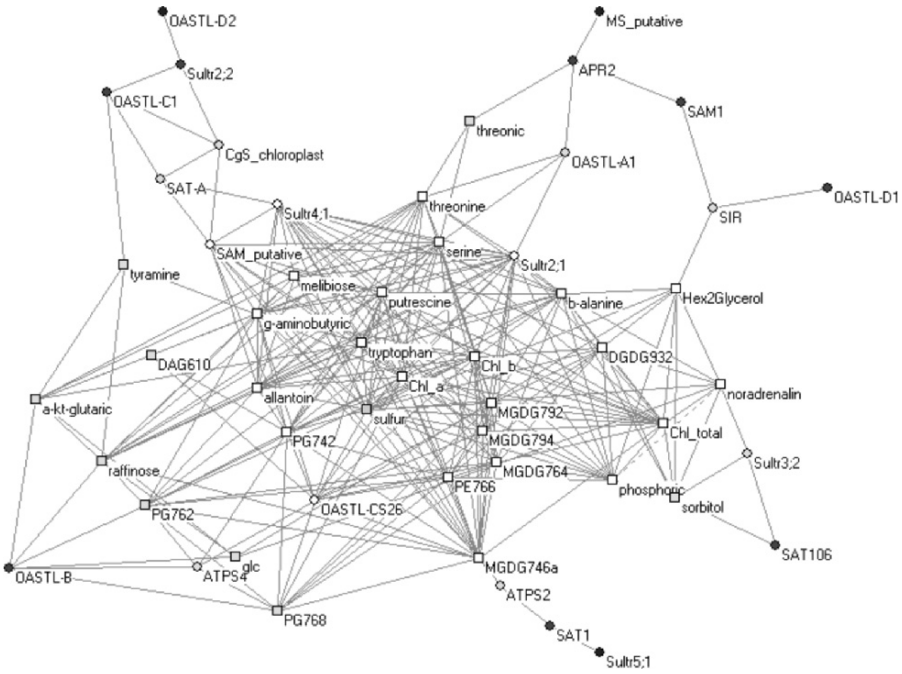


*Figure 7.* Relations between matrix elements based on item cobehavior and correlation analyses can be visualized in network representations. Sulfate starvation data (8,000 elements) on joint transcriptome and metabolome changes thus resulted in a scale free network of 600 elements after extensive selection of significant values. Such a network comprises elements with either high or low connectivity and rather visualizes correlative relationships rather than pathways, though, as depicted here by genes and metabolites of the sulfate pathway, pathway elements might still be connected as they often respond coordinately (adapted from Nikiforova *et al.* 2005a).

Such bioinformatical tools have been employed on transcriptome and metabolome data of sulfur metabolism in *Arabidopsis* to describe in a holistic way the biochemical, molecular, and physiological response of a plant to nutrient starvation (Hirai and Saito 2004; Nikiforova *et al.* 2005a; Hirai *et al.* 2005, Figure 7). It was possible to show that genes and metabolites involved in glucosinolate metabolism were coordinately modulated (Hirai and Saito, 2004). Thus, by understanding such gene to metabolite networks it was possible to identify gene function of three genes encoding sulfotransferases with previously unknown function, as

being involved in glucosinolate biosynthesis (Hirai *et al.* 2005). Compilation of various analyses such as GC-MS and LC-MS based determination of metabolites (Nikiforova *et al*. 2005b; Fiehn *et al.* 2000, 2001) and unbiased screenings using array technologies (Nikiforova *et al.* 2003, 2005a) provides a first description of the system response. Some of the results are obviously consistent with the expectations or corroborate previous findings, such as SAT induction and OAS accumulation and serine/glycine accumulation coupled to serine hydroxymethyltransferase (SHMT) induction. However, even far more reaching explanations appear possible. Folates are refueling the SAM-C1 transfer cycle through methylation of homocysteine to methionine (Zeh *et al.* 2002; Hesse and Höfgen 2001). As SAM levels are decreasing accompanied by a reduction in at least one isoform of the MS, folates might accumulate and might be speculated to have a feedback effect on their own synthesis, again making the accumulation of the folate (and cysteine) precursors serine and glycine likely. The cysteine depletion also results in an induction of OAS-TL. Furthermore, S-starvation resulting in SAM depletion induces genes of SAM synthesis and recycling to reconvert the demethylated SAM back to methionine and, eventually, SAM (Nikiforova *et al.* 2005b). The network reconstruction corroborates previous findings but also provides new conclusions which need to be tested in *in vivo* systems, i.e. mutants. Thus, already at this level of systems analysis hypothesis generation is fostered.

## CONCLUSIONS

In order to approach a real systems description, integration of mRNA, proteomic and metabolomic data of continuous models is required (Gill *et al.* 2002). Such data will lead to substantial improvements of the transcriptional and translational data interpretations in order to achieve a better understanding of cellular mechanisms (Hesse and Hoefgen 2006; Sweetlove *et al.* 2003; Bray 2003; Minorsky 2003; Alon 2003). The amount, the variability of the data, and the incomparability of experimental conditions provides a challenge for the analytical procedures and the data analysis using bioinformatics (Katagiri 2003). The goal, eventually, will be to describe the wiring scheme of metabolic and physiological processes in plants (Chong and Ray 2002; Quackenbush 2003) or even across species (Stuart *et al.* 2003). Through this, responses of plants to genetic manipulations and environmental perturbations will become increasingly predictable. This will make systems biology attractive as a tool to create hypothesis.

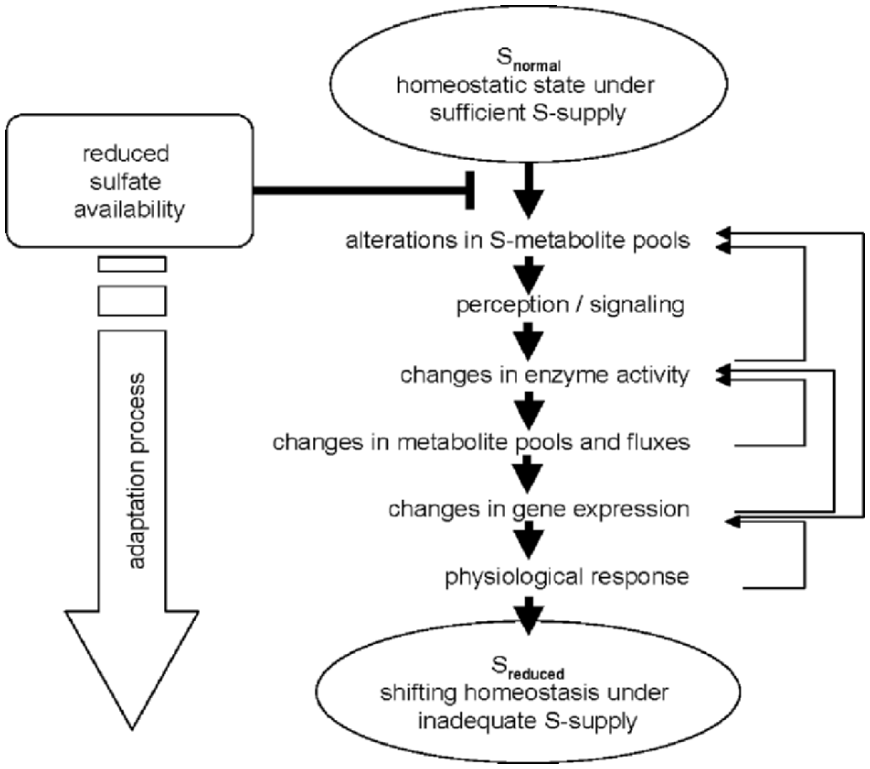


*Figure 8.* As a result of the genomics study, a first response scheme of the order of events can be provided which describes the order of events how plants react and adapt to sulfate starvation conditions and of the various putative control loops and elements. This model can now be challenged and eventually refined using, for example, mutant analysis. When a robust state of the model is achieved allowing predictions, the model can be widened to include other biosynthetic pathways, environmental conditions, or ecological interactions.

At low sulfate supply, the adaptive processes of biosynthesis of metabolites allow plants to readjust homeostasis and to remain viable and produce seeds for dispersal (Figure 8). The integrity of the biosynthesis system is kept, although shifted from a normal to an adapted state. In case of continued starvation, such as the artificial zero sulfate supply situation imposed in experimental conditions, disturbances accumulate and propagate through the system by triggering further downstream reactions leading finally to plant death, when vital components fail to be synthesized at all. This response is governed by three main processes. First, the lack of sulfate and thus reduced sulfide provision leads to a halt in cysteine

biosynthesis and its downstream sulfur-containing derivatives such as GSH and SAM and an accumulation of precursors of cysteine synthesis, *O*-acetylserine, serine, and glycine. These metabolic changes obviously impair a number of physiological processes, forcing the plant to shift to alternative strategies to remain viable. Second, continued carbon backbone provision and nitrate assimilation coupled to reductions in protein synthesis and further biosynthetic processes such as a reduced C1-metabolism, chlorophyll, and lipid biosynthesis (Nikiforova *et al*. 2005b) lead to a relative imbalance of nitrogen over sulfur content. Excess nitrogen then triggers processes to dump reduced nitrogen into various N-rich sink molecules as glutamine, asparagine, and ureides. It can be speculated that these strategies help to eventually prevent ammonia intoxication. Third, a lack of cysteine leads to reduced GSH levels and a disturbance of the central cellular active oxygen scavenging system, the GSH–ascorbate cycle (Noctor and Foyer 1998). Probably in order to maintain the ability of plants to deal with stresses (Riemenschneider *et al.* 2005b; Bloem *et al.* 2004; Haneklaus *et al.* 2003) the biosynthesis of aromatic secondary compounds is induced which might functionally substitute the GSH–ascorbate cycle.

Yet, currently data acquisition mainly relies on describing pool sizes which is currently the closest approximation to determine the state of a biological system in response to environmental conditions or developmental programs. It will be of increasing importance to measure and describe fluxes in order to understand the regulatory properties of plants with regard to resource and energy management strategies as well as investment priorities. Only then will it be possible to model a plant system and to understand how plants sustain, and steer growth and propagation under certain environmental parameters.

The aim is to describe the molecular, biochemical, and physiological level, even more complex interactions, such as plant associations, or whole ecosystems. For this challenging aim it will be necessary to merge biogeochemistry, ecology, and plant systems biology.

## ACKNOWLEDGMENTS

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Chapter 7

# SULFUR IN RESISTANCE TO ENVIRONMENTAL STRESSES

Kathryn A. North and Stanislav Kopriva

## INTRODUCTION

It is essential for all organisms to be able to cope with changes in their external environment in order to grow and reproduce. Whereas animals are able to move away from unfavorable conditions, plants must endure and adapt and as such have developed complex survival mechanisms. The abiotic stress conditions which plants are exposed to can vary widely; these include physical factors such as extremes in temperature, high light, and drought. Chemical factors including air pollutants, salinity, and heavy metals can also cause abiotic stress. These conditions may be seasonal, permanent, or transient, so it is important for plants to be able to respond to variable conditions that are ever changing.

A common feature of both biotic and abiotic stresses in plants is the occurrence of oxidative stress, from the generation of reactive oxygen species (ROS). ROS can be both positive and negative for living organisms. On the one hand ROS can provide cells with essential signaling information conveying messages for cellular function and survival, but on the other hand they are highly reactive and potentially damaging. Cells must find the balance between beneficial ROS and oxidative stress. Central to the mechanisms controlling this balance between benefit and stress is glutathione (GSH). GSH, a tripeptide antioxidant with low molecular weight, is present at mM concentrations within cells. GSH is an end product of sulfur assimilation and is the major nonprotein thiol in plants (Buchanan *et al*. 2000; Mullineaux and Rausch 2005). GSH synthesis and its protective function will be discussed later in this chapter; first it is



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important to understand more about ROS, and their role in causing oxidative stress.

## REACTIVE OXYGEN SPECIES

ROS include hydrogen peroxide ($H_2O_2$), superoxide ($O_2^-$) and singlet oxygen ($^1O_2$), and may be derived from external factors such as exposure to ozone, but mostly they are generated within cells as a consequence of the oxygenic environment. ROS are able to oxidise other, less oxidising compounds, often disrupting molecular bonds and generating toxic by-products and free radicals. For example, the accumulation of superoxide can be cytotoxic as although it is only moderately reactive, it can generate the more reactive hydrogen peroxide and hydroxyl radicals ($OH^-$, Halliwell and Gutteridge 1999). In addition to hydrogen peroxide generation from dismutation of superoxide, it can also be formed from the reaction of ozone with water. Hydrogen peroxide is freely able to cross biological membranes, which enhances its properties as a signaling molecule, but also its potential to cause damage (Halliwell and Gutteridge 1999). The damage that can be caused by ROS effects cells on many levels; damage to proteins and enzymatic function can occur via disruption of bonds, such as the disulfide bridges, that play an important role in protein folding and structure. When ROS react with membranes the effects can be twofold, membrane integrity may be lost and toxic products of lipid peroxidation can be formed, which may in turn cause cellular damage. Furthermore, DNA degradation can occur as a result of ROS action. The overall effects of oxidative stress in plants are therefore a reduction in photosynthesis and productivity, electrolyte leakage, accelerated senescence, and necrosis (Marrs 1996; Sharma and Davis 1997; Edwards *et al*. 2000).

## ANTIOXIDANTS

The balance of ROS is maintained by a complex network of antioxidants including GSH, ascorbate, and α-tocopherol as well as by antioxidant enzymes such as superoxide dismutase (SOD), ascorbate and glutathione peroxidase (APX and GPX), glutathione *S*-transferases (GST), glutathione reductase (GR), and catalase. Ascorbate is the primary ROS scavenger, whereas tocopherol is the major antioxidant in membranes. In addition, GSH can directly detoxify ROS via the reaction catalyzed by GPX. However, its major antioxidant role is in the regeneration of ascorbate

from its reduced form, dehydroascorbate (DHA), in the Halliwell–Asada pathway, also known as the ascorbate–GSH cycle (Figure 1). During the interaction with ROS ascorbate oxidises to monodehydroascorbate (MDA). This is reduced back to ascorbate by NADPH dependent monodehydroascorbate reductase (MDAR), or disproportionates nonenzymatically to ascorbate and DHA. DHA is reduced back to ascorbate by dehydroascorbate reductase (DHAR) using electrons from reduced GSH. The oxidised glutathione (GSSG) is then converted back to GSH by GR, using NADPH, and thus completing the cycle (Halliwell and Gutteridge 1999; Apel and Hirt 2004).

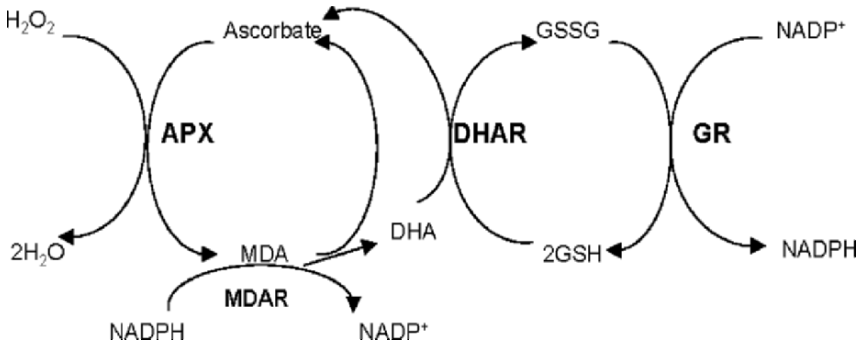


*Figure 1.* The ascorbate–glutathione cycle.

The involvement of GSH in plant stress defence however goes beyond ascorbate regeneration. GSH can also conjugate to toxic electrophilic compounds that are usually hydrophobic, and form nontoxic peptide derivatives in reactions catalyzed by GSTs. Glutathione-conjugated products can be removed from the cytosol by ATP-binding cassette (ABC) transporter proteins (Dixon *et al*. 1998). It appears that the removal of these products into the vacuole plays a major part in their detoxification (Coleman *et al.* 1997). The GSTs themselves form a large and diverse family of proteins, with five distinct classes in plants (Dixon *et al.* 2002), in fact there are 48 different GST genes in *Arabidopsis thaliana* alone, and 41 of these are known to be expressed (Dixon *et al*. 2002). Given the large number of GSTs in plants, and their functional diversity ranging from detoxification to cell signaling and regulation, it is clear that glutathione is very important in its function as a cosubstrate for GSTs (Dixon *et al*. 2002). In addition, GSH is used directly or indirectly, as substrate for synthesis of phytochelatins, involved in protection against heavy metals.

It is clear, therefore, that GSH is central in plant defence against oxidative stress.

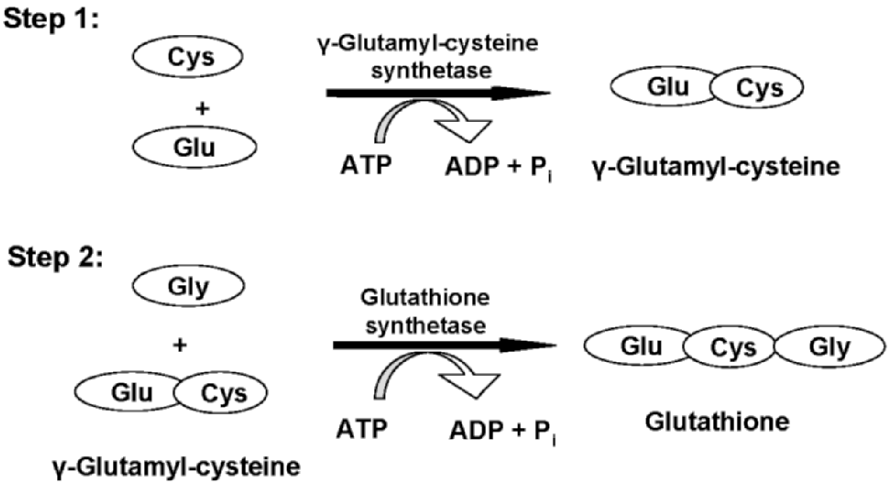


*Figure 2*. The glutathione biosynthetic pathway.

## GLUTATHIONE SYNTHESIS

The building blocks of g GSH are the amino acids cysteine, glutamate, and glycine. Cysteine contains the sulfhydryl (-SH) group that leads to the reducing properties of GSH. The synthesis of GSH is a two-step ATP dependent process (Figure 2), whereby cysteine and glutamate are first converted into γ-glutamylcysteine (γ-EC) in a reaction catalyzed by γ-glutamylcysteine synthetase (γ-ECS). The second step is the addition of glycine to γ-glutamylcysteine by glutathione synthetase (GSHS) to produce GSH. The rate of synthesis of GSH is controlled by the supply of its constituent amino acids and by regulation of γ-ECS (Kopriva and Rennenberg, 2004). Most of our knowledge on regulation of GSH synthesis is derived from experiments with poplars overexpressing bacterial enzymes of GSH synthesis, which clearly demonstrated that γ-ECS possesses far higher control of the pathway than GSHS (Strohm *et al.* 1995; Noctor *et al*. 1996). This control is exerted by transcriptional and post-transcriptional regulation of the gene and by feedback inhibition of the enzyme by GSH. GSH levels are increased upon exposure to salicylic

acid and abscisic acid (ABA), although the mechanisms are not known. Higher demand for GSH synthesis leads to increased consumption of cysteine and consequently to a higher rate of sulfate assimilation.

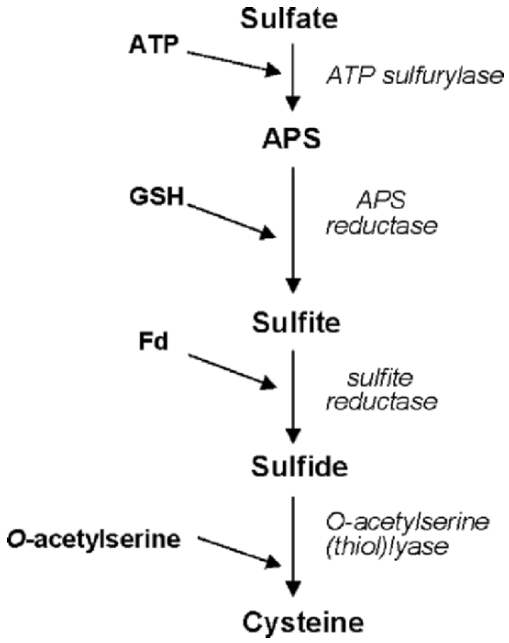


*Figure 3.* The sulfur assimilation pathway.

## SULFATE UPTAKE AND ASSIMILATION

There is considerable evidence that availability of cysteine, the end product of sulfate assimilation, has a large influence on control of GSH levels (Noctor *et al.* 2002; Kopriva and Rennenberg 2004; Mullineaux and Rausch 2005). By limiting the supply of sulfur there is a knock-on effect on the supply of cysteine which in turn restricts the γ-ECS catalyzed reaction of GSH biosynthesis; this in turn results in a decrease in the GSH content of shoots and roots (Lappartient and Touraine 1997; Hirai *et al.* 2003; Nikiforova *et al.* 2003; Kandlbinder *et al.* 2004). Initial uptake of sulfate into plants and its transport throughout the plant occur via a series of sulfate transporters, as has already been discussed in Chapter 1. Once sulfate has entered plants the sulfate assimilation pathway provides a further level of controlling cysteine synthesis. The pathway of assimilatory

sulfate reduction is the major route for uptake of inorganic sulfate into plants (Kopriva and Koprivova 2004). In the pathway, sulfate is first activated by adenylation to adenosine 5′-phosphosulfate (APS) by ATP sulfurylase, this is then reduced to sulfite, in a reaction catalyzed by APS reductase (APR), and sulfite is in turn reduced to sulfide by sulfite reductase. The final reaction of the pathway is that of sulfide with *O*-acetylserine, catalyzed by *O*-acetylserine(thiol) lyase, which results in the formation of cysteine (Figure 3, Kopriva and Koprivova 2004). APR is a key enzyme in the regulation of this pathway. Under normal conditions the pathway is repressed, however it has been demonstrated that plants growing in conditions of limiting sulfur supply can upregulate the mRNA abundance and activity of APR (Brunold *et al.* 1987; Gutierrez-Marcos *et al.* 1996). In addition, feeding with cysteine and glutathione downregulated APR, but not ATP sulfurylase and significantly reduced the flux through the pathway (Vauclare *et al*. 2002), thus demonstrating that it is APR that possesses the major control over sulfate assimilation. Plants undergoing oxidative stress also increase APR activity, which may be necessary to provide sufficient amounts of reduced sulfur for increased GSH production to increase the capacity to cope with ROS.

## PHYSICAL FACTORS CAUSING OXIDATIVE STRESS

### *High light*

Light intensity varies naturally depending on the time of day and the season; however factors such as cloud cover and shading may also play a role in short-term changes in light intensity. The effects of light on plants have been studied extensively, in particular with regard to photosynthesis and the production of ROS. Under normal conditions ROS are a natural by-product of photosynthesis, but are produced at levels sufficiently low to be scavenged before oxidative stress occurs. At higher light intensities, however, photosynthesis is saturated and the excess electrons are transferred to oxygen, leading to ROS accumulation, damage of photosynthetic apparatus and finally to photoinhibition (May *et al.* 1998). Damage to photosystem II by immediate maximal excitation of chlorophyll molecules caused by a change to high light may inhibit photosynthetic electron transport and result in the overproduction of electrons and release of more ROS, leading to oxidative stress (Karpinski *et al.* 1997; 1999). Vital to the detoxification of these reactive intermediates is the ascorbate–GSH cycle, components of which are located in the chloroplast close to the photosynthetic apparatus. The involvement of

glutathione in plant responses to high light has been studied extensively. When light intensity undergoes a sudden change from low to high, there is an initial decrease in the glutathione redox state, followed by an increase up to 2.5-fold (Karpinski *et al.* 1997; Muller-Moulé *et al.* 2003). High light treatment has been demonstrated to increase mRNA levels and activity of APR, indicating a higher rate of sulfate assimilation (North *et al*. 2005). Experiments with two mutants of γ-ECS, *rax1* and *cad2*, revealed that the biosynthesis of glutathione acts in several different ways to influence stress mechanisms. There was ≥ 50 % lowered foliar glutathione levels in *rax1*, and under nonstressed conditions the mutant did not have altered hydrogen peroxide levels, lipid peroxidation products, or altered ascorbate or glutathione redox states compared to wild-type plants (Ball *et al.* 2004). In *cad2*, glutathione levels were similar to those in *rax1* but γ-EC was not detectable and cysteine was increased up to 70%. In *rax1* an elevated level of *APX2* mRNA was found in nonstressed conditions, although this transcript is normally not detectable in nonstressed wild-type plants but induced by high light treatment. In fact, high light treatment caused *APX2* mRNA to accumulate above wild-type levels in the *rax1* and in *cad2*. However, no difference in hydrogen peroxide level was seen, nor was the pattern of change in glutathione redox state in response to high light different in the mutants compared to wild type. *Rax1* and *cad2* were no more affected by photooxidative stress at a whole plant level than wild-type (Ball *et al.* 2004). The constitutive expression of APX2 and the higher induction of APX2 in the γ-ECS mutant(s) indicate a direct link between glutathione biosynthesis and stress gene expression. A further 12 stress associated genes were investigated which were influenced by excess light-stressed *rax1* and *cad2*. Six of these were affected in nonstressed conditions in the mutants but six were not; this shows that GSH must act in several ways to influence stress response mechanisms (Ball *et al.* 2004).

*Temperature extremes*

Low temperatures also result in a reduction in photosynthetic capacity, therefore in combination with high light intensities, chilling is capable of causing photoinhibition and oxidative stress, such as the generation of hydrogen peroxide (Prasad *et al.* 1994). Sensitivity to chilling is species-dependent, and can differ between cultivars, for example maize is a chilling-sensitive species but different cultivars are more resistant to chilling than others. The chilling tolerance of different maize cultivars may correlate with the capacity for increasing GSH concentration and GR activity under stress conditions (Leipner *et al.* 1999). Indeed, at low temperatures, GSH content and reduction state are higher in chilling tolerant compared to

chilling sensitive genotypes of maize (Kocsy *et al.* 1996). When GSH content was increased in chilling sensitive maize by treatment with herbicide safeners, the chilling induced injury was significantly reduced (Kocsy *et al.* 2001), whereas reduction of GSH by inhibiting its synthesis with buthionine sulfoximine (BSO) in a chilling tolerant genotype resulted in increased leaf injury at low temperature (Kocsy *et al.* 2000). Consequently, in maize, chilling induces foliar thiol levels and activities of APR, γ-ECS, and GSHS (Brunner *et al.* 1995). Total GSH content and the activities of APR and GR are increased in chilling tolerant maize genotypes compared to a sensitive one even at standard growth conditions (Kocsy *et al.* 1997; Kopriva *et al.* 2001).

High temperatures have a complex impact on plants, leading to changes in membrane properties, enzyme activity, oxidative stress, and ultimately cell death. The major mechanism of defence is the synthesis of heat shock proteins with a plethora of functions (Larkindale *et al.* 2005). However, antioxidants also play a role in plant defence against excessive heat. GSH content and synthesis rate increased significantly in maize roots exposed to 40°C (Nieto-Sotelo and Ho 1986). Mutants deficient in biosynthesis of antioxidants, such as ascorbate or GSH exhibited reduced thermotolerance, again showing the important role GSH plays in plant defence against abiotic stress (Larkindale *et al.* 2005). This was further corroborated by the demonstration that tobacco overexpressing GST/GPX were more tolerant to high-temperature and high-salt treatments (Roxas *et al.* 2000).

### *Drought and osmotic stress*

Exposure of plants to extended drought leads to water loss from the tissues. Leaf $CO_2$ is depleted due to stomatal closure, which in high light, a condition that usually accompanies drought, results in photoinhibition and oxidative stress (Munns 2002). It is therefore not surprising that the activities of ROS detoxifying enzymes including DHAR and GR increase in these conditions (Boo and Jung 1999). Drought caused an oxidation of the GSH pool in barley or pine (Smirnoff 1993; Tausz *et al.* 2001) and increased its total content in wheat leaves (Bartoli *et al.* 1999). During desiccation of lichens, the GSH pool becomes completely oxidized. The rate of its reduction upon rehydration however, seems to be the major determinant of desiccation tolerance (Kranner 2002). During desiccation of the moss *Tortula ruralis* GR, GPX, and GST activity were increased as well as the oxidation state of GSH (Dhindsa 1991).

Similar to drought, in affecting the plant water status, is osmotic stress. In nature this can be caused by high-salt concentration in the soil, in the laboratory it is usually achieved by incubation with mannitol or

polyethyleneglycol. Plants subjected to osmotic stress react similarly to plants treated with drought or salinity: the oxidation state of antioxidants is increased and the activities of ROS detoxifying enzymes are also induced. The tolerance to osmotic stress is genetically determined, as for example different wheat genotypes differ in their tolerance of osmotic stress (Kocsy *et al.* 2004). This tolerance can again be attributed to the variation in the capacity of the genotypes to induce GSH synthesis upon the stress treatment. During osmotic stress the tolerant genotypes incorporated more $^{35}S$ from [$^{35}S$] sulfate into thiols than the sensitive ones (Kocsy *et al.* 2004). The general involvement of GSH in defence against abiotic stress is best documented by the fact that the osmotic stress tolerant genotypes were selected on the basis of their freezing tolerance.

## CHEMICAL FACTORS CAUSING OXIDATIVE STRESS

### *Ozone*

Some chemicals to which plants are exposed are ROS themselves, for example the air pollutant ozone ($O_3$). It is well known that ozone has beneficial effects in the stratosphere, 15–50 km above the Earth's surface, as it absorbs damaging ultraviolet (UV) radiation. On the other hand, in the troposphere, up to 15 km above the surface of the Earth, it is the major secondary gaseous air pollutant (Colls 2002). Tropospheric ozone occurs naturally by exchange with the stratosphere and is present at background concentrations of 10–80 parts per billion (ppb) (Mauzerall and Wang 2001; Colls 2002). However, it can also be formed photochemically from the action of UV photons on nitrogen oxides such as nitric oxide (NO) and nitrogen dioxide ($NO_2$). In an unpolluted troposphere ozone will readily react with NO to form $NO_2$ and $O_2$, maintaining low and stable ozone concentrations. However increased NO pollution, for example from transport emissions, and unburnt hydrocarbons in the atmosphere contribute to the formation of ozone and therefore tropospheric concentrations are increased (Halliwell and Gutteridge 1999; Colls 2002). The tropospheric ozone concentration varies depending upon environmental conditions such as light intensity and levels of other pollutants. In addition, although episodes of high ozone are seasonal, a general increase in ozone pollution has been observed over the past century. This is projected to increase further with the continued burning of fossil fuels and use of $NO_x$ emitting fertilisers (Chameides *et al.* 1994; Mauzerall and Wang 2001; Colls 2002). Ozone pollution is not restricted to urban areas, which has implications for

agriculture and natural ecosystems at some distance from the sources of air pollution (Krupa and Manning 1988; Colls 2002).

Plant responses to ozone are varied and complex, and in general ozone exposure causes a reduction in plant growth and productivity by inhibiting photosynthesis, initiating premature senescence and can lead to localised cell death and necrotic lesions (Sen Gupta *et al.* 1991; Rao *et al*. 2000; Saitanis and Karandinos 2002). Ozone enters plants via the stomata on the surface of leaves. It has been proposed that ozone immediately reacts in the apoplast to form other ROS such as hydrogen peroxide and hydroxyl radicals (Mehlhorn *et al.* 1990; Kanofsky and Sima 1995; Runeckles and Vaartnou 1997; Heath and Taylor 1997). In addition to ozone-degradation-derived ROS, ozone itself may also reach the plasma membrane. This is because the rate of reaction of ascorbate, the most abundant antioxidant present in the apoplast, is only moderate and this is likely to carry out most of the primary scavenging of ozone-derived ROS (Moldau and Bichele, 2002). The generation of DHA from ROS detoxification requires processing through the ascorbate–GSH cycle to return ascorbate back to its reduced form. This reaction depends on GSH availability so it is important for plants to have sufficient glutathione for the detoxification process.

In addition to the direct production of ROS from the reactions of ozone, it has been demonstrated that ozone can initiate the active production of ROS. For example, active generation of hydrogen peroxide (Schraudner *et al*. 1998, Pellinen *et al.* 1999), and superoxide (Overmyer *et al*. 2000; Rao and Davis 1999) has been detected during and after ozone exposure. This leads to the hypothesis that acute ozone may mimic an elicitor of plant pathogen interactions at whole plant level, including the hypersensitive response (HR; Kangasjarvi *et al.* 1994; Sandermann *et al.* 1998; Schraudner *et al.* 1998; Rao and Davis 2001). The HR involves a rapid, massive and transient activation of oxidative metabolism resulting in accumulation of ROS following incompatible plant–pathogen interactions (Lamb and Dixon 1997; Langebartels *et al.* 2002). An example of the active production of ROS similar to the HR in response to ozone was seen in birch leaves. Hydrogen peroxide accumulation at the plasma membrane with ozone exposure and was later seen in the cytoplasm, mitochondria, and peroxisomes. The ROS producing cell wall peroxidases and NADPH oxidase were proposed sources of the hydrogen peroxide (Pellinen *et al*. 1999). In *A. thaliana* superoxide is the main ROS produced with ozone exposure, and cell death appears to correlate with the accumulation of superoxide rather than hydrogen peroxide (Rao and Davis 1999; Wohlgemuth *et al.* 2002). Ozone exposure induced a biphasic increase in ROS in *A. thaliana* and the ROS signal was propagated by NADPH oxidases located in stomatal guard cells (Joo *et al.* 2005). The potential for

ozone-induced oxidative stress in plants is therefore twofold, both as a result of direct reactions of ozone and a plant-derived oxidative burst. As such it is no surprise that ozone is an inducer of plant defence-related enzymes and antioxidants.

One of the acclimation responses of plants during exposure to ozone is an increase in the ROS scavenging capacity by increasing expression and activity of various antioxidants (Kangasjarvi *et al.* 1994; Conklin and Last 1995; Sharma and Davis 1997; Tamaoki *et al*. 2003). Changes in antioxidants in response to ozone are well documented. For example, the expression of *GST*, *APX*, and *Cu/Zn SOD* increased upon ozone treatment (Conklin and Last 1995; Price *et al.* 1990; Clayton *et al.* 1999). Microarray analysis found that the expression of one *GST* was upregulated 37-fold, and three putative *GSTs* were upregulated up to sevenfold following ozone exposure (Tamaoki *et al.* 2003). In addition, cytosolic *O*-acetylserine lyase was increased 3.5-fold, and APR activity increased upon ozone exposure (Bick *et al.* 2001) suggesting an increase in demand for cysteine which was potentially destined for GSH synthesis. Additionally, an ozone-induced increase in total glutathione and a large decrease in the ratio of reduced to oxidised glutathione was observed in *Populus* (Sen Gupta *et al.* 1991). All of these findings indicate a greater need for detoxification processes including the ascorbate–GSH cycle and the detoxification of hydrophobic electrophilic substances that can be associated with lipid peroxidation, both of which involve glutathione (Sharma and Davis, 1997). Furthermore, treatment with hydrogen peroxide increased the expression and activity of *GST* (Price *et al*. 1994; Levine *et al.* 1994; Wagner *et al.* 2002; Rentel and Knight 2004). Since hydrogen peroxide is a breakdown product of ozone, and can accumulate during and following ozone exposure, this again points toward GSH-mediated detoxification processes in response to ozone. Recent evidence suggests that ascorbate provides much of the protection against ozone in *A. thaliana*. This was found using transgenic lines impaired in DHAR function (Yoshida *et al* 2006). These plants contained roughly the same levels of ascorbate, an increase in total glutathione and a lower ascorbate to DHA ratio. The transgenic line was more susceptible to ozone-induced damage, presumably due to less abundant reduced ascorbate (Yoshida *et al.* 2006). The reduction of DHA to ascorbate in the ascorbate–GSH cycle (Figure 1) is emerging as being important in protection against ozone, and again points toward a vital function for glutathione as a necessary cofactor for the activity of DHA reductase. It is possible that the increase in total GSH content in the *dhar* transgenic lines could be an attempt by the plant to increase the rate of reduction of ascorbate. If glutathione availability is normally rate limiting in the ascorbate–GSH cycle, an increase in GSH content may be one

method adopted by plants to adapt to stress. In these plants, the increase in glutathione would not help since it was the enzyme rather than the cofactor that was limiting.

### *Other air pollutants*

Other air pollutants causing oxidative stress to plants include the oxides of nitrogen and sulfur, and hydrogen sulfide. The sulfur-containing volatiles can be easily taken up by plant leaves and enter the pathway of sulfate assimilation. Hydrogen sulfide can support growth and compensate for inadequate sulfate supply through the roots (De Kok 1990, Chapter 5). In line with the demand driven control of sulfate assimilation, sulfate uptake and reduction are reduced (Westerman *et al.* 2000, 2001). However at higher $H_2S$ concentrations thiols accumulate and symptoms of toxicity, including necrotic lesions and limitation of growth occur (De Kok 1990). Sulfur dioxide is a common atmospheric pollutant which is a by-product from the combustion of fossil fuels. Although it can also be partially assimilated by plants via sulfate assimilation, it causes oxidative stress and cell death. Indeed, cysteine content increases in $SO_2$ fumigated plants, even though the ATP sulfurylase activity decreases (Brunold *et al.* 1983). The primary root of $SO_2$ detoxification, however, is its oxidation to sulfate by sulfite oxidase (Hänsch et al. 2006).

Oxides of nitrogen are another class of common air pollutants. Nitrogen dioxide contributes to ROS production; therefore at higher concentrations it causes leaf damage. Plants can use $NO_2$ as an additional nitrogen source so that $NO_2$ fumigated plants produce higher biomass than controls at moderate nitrate supply (Takahashi *et al.* 2006). The capacity of plants to cope with $NO_2$ is linked with nitrite reductase activity (Takahashi *et al.* 2001). The other widespread nitrogen gas is NO, which is a pollutant but has been recently discovered as an important component of signaling in the plant–pathogen response (Delledonne 2005). The action of NO as a signaling molecule depends on factors such as its rate of production and diffusion and the redox status of the cell. NO acts in tandem with hydrogen peroxide in incompatible plant–pathogen interactions. It prevents the accumulation of thylakoidal APX thereby slowing the detoxification of hydrogen peroxide and promoting cell death (Murgia *et al.* 2004), NO can also act as a posttranscriptional regulator of proteins by binding to amino acids. Irreversible modification of proteins and a loss of function can occur from the nitration of tyrosine and the oxidation of methionine. Reversible modification that can control protein function is brought about by nitrosylation of cysteine, through the action of nitrosothiol (*S*-nitrosoglutathione; GSNO) or by transfer of an NO group by another *S*-nitrosylated protein

(Delledonne 2005). It has been proposed that the potential targets of for *S*-nitrosylation could include stress-related proteins, adding another mechanism of control to stress responses. *S*-nitrosoglutathione seems to be the transport form of NO, revealing another important role for glutathione in plant stress response.

### *Salinity*

Among the chemically induced abiotic stresses, high salinity represents a major environmental problem. High salt levels are found in a third of the world's cropland resulting in suboptimal growth and reduced yields. Over 6% of land throughout the world is adversely affected by salt either by salinity or the associated condition of sodicity (Food and Agricultural Organization UN, 2005). Salt has both osmotic and salt-specific effects on plants (Munns 2002), which impact at different times. Salinity reduces water uptake, which rapidly reduces growth rate, accompanied by metabolic changes similar to those caused by drought. These mechanisms are related to water stress, as cellular ion concentrations remain below toxic levels (e.g. Hu and Schmidhalter 1998). Later, excessive salt uptake results in premature senescence thus reducing the photosynthetic area to below critical levels. In addition high salt disrupts nutrient uptake, which takes somewhat longer to damage the plant. As with other types of abiotic stress, high salt treatment resulted in upregulation of antioxidative systems in tomato (Mittova *et al.* 2004). Accordingly, GSH and cysteine levels were increased in canola under salt stress (Ruiz and Blumwald 2002). However, increased antioxidants are not the most efficient way of coping with salt stress, as transgenic canola overexpressing a $Na^+/H^+$ antiporter became significantly more salt tolerant than wild-type and the previously observed increase in thiol accumulation did not occur (Ruiz and Blumwald 2002).

The increased demand for GSH synthesis leads to upregulation of enzymes of assimilatory sulfate reduction. This was demonstrated for the cytosolic isoform of *O*-acetylserine(thiol) lyase, which increased significantly after 24 h of salt treatment (Romero *et al.* 2001). This regulation is dependent on ABA, as the mRNA was not induced in *A. thaliana aba1* or *abi2* mutants deficient in ABA synthesis or signaling (Barroso *et al.* 1999). A search of available microarray data in the Genevestigator database (Zimmermann *et al.* 2005) revealed increases in mRNA levels for other genes involved in sulfate assimilation: ATP sulfurylase and APR. Indeed, APR mRNA level and activity increased at least twofold in roots within 5 h of NaCl treatment (Koprivova A, personal communication).

### *Heavy metals and xenobiotics*

Heavy metals represent another source of danger for plants due to their interaction with proteins and cell walls and potential for ROS production. Some of them are however essential micronutrients for plants (Cu, Zn, and Mn) therefore precise mechanisms to keep the metals in the right concentration range to avoid both toxicity and starvation have to be in place. Sulfate assimilation is enormously important for plant defence against heavy metals and the major player in this process is GSH, either as direct ligand of the metals or as a precursor of phytochelatins, as will be described in Chapter 8.

Plants are also exposed to variety of man-made compounds from industrial action or agriculture. Many of these compounds are toxic and therefore used as herbicides, but they accumulate in the food chain and present a potential problem for animal and human health. The detoxification mechanism of xenobiotics in plant cells usually starts with phase one reactions introducing active groups in otherwise inert molecules by e.g. cytochrome P-450s or diverse oxidases, at the same time making the compounds more hydrophilic (Morant *et al.* 2003). These groups are than attacked by phase 2 reactions conjugating glutathione or sugars via the action of GSTs or glycosyltransferases. The conjugates are than transported into vacuoles where the detoxification is completed (Edwards *et al.* 2000). Differences between plant ecotypes and varieties in their capacity to cope with xenobiotics, e.g. herbicides, are again often linked to the presence of a particular GST or a general capacity of the plant to supply sufficient GSH. It is thus not surprising that growth of transgenic poplars overexpressing γ-ECS in the cytosol or in the chloroplast was less reduced upon treatment with chloroacetanilide herbicides than that of the wild-type (Gullner *et al.* 2001). Induction of GSH synthesis by herbicide safeners in crops is a valuable mechanism for weed control in agriculture. Treatment of maize with dichloroacetamide safeners increases cysteine and GSH content due to upregulation of assimilatory sulfate reduction and thus prevents the damage by subsequent treatment with thiocarbamate and chloroacetanilide herbicides (Farago and Brunold 1990).

## GSH AND ROS IN SIGNALING

Although ROS cause serious cellular damage they are also beneficial for plants. For example, root growth is dependent on production of ROS by NADPH oxidase (Foreman *et al.* 2003). GSH has also been tightly linked with root growth as a mutation in γ-ECS, causing only 1–3% GSH levels in the mutant compared to the wild type, leading to a root meristemless phenotype (Vernoux *et al.* 2000). The involvement of ROS and GSH in the regulation of growth may be functional, by affecting properties of cell walls and proteins or may be due to involvement in the signaling cascades. Indeed, it has been postulated that it is ROS-signaling rather than oxidative stress *per se* that contributes to cell death resulting from oxidative stress (Foyer and Noctor 2005). ROS themselves may act as signals, for example, hydrogen peroxide can act as part of a systemic signal conferring acclimatory responses to systemic leaves in plants undergoing high light stress (Karpinski *et al*. 1999). Singlet oxygen has also been attributed a role in signaling following high light treatment, as demonstrated using the *flu* mutant of *A. thaliana*. The FLU protein is a nucleus-encoded chloroplast protein that plays an important role during the negative feedback control of chlorophyll biosynthesis. Inactivation of FLU in the mutant leads to the overaccumulation of free protochlorophyllide which, when excited by light, causes singlet oxygen generation. This activates stress response genes, different to those activated by superoxide and hydrogen peroxide (Op den Camp *et al.* 2003). The actual redox status of glutathione may be an important factor in signaling, as has been described for stress response to high light (Creissen *et al*. 1999; Ball *et al.* 2004), ozone (Evans *et al.* 2005), and in relation to calcium signaling (Gomez *et al.* 2004; Evans *et al.* 2005). Redox potential is important in the salicylate signaling cascade involving NPR1, a regulator of systemic acquired resistance in plantpathogen interactions, which may also play a role in abiotic stress responses. Conversion of NPR1 into a monomeric form by a change in cellular reduction potential allows it to migrate to the nucleus where it can activate defence gene expression (Mou *et al.* 2003). In addition, as already discussed, GSH interacts with NO signaling and a direct role for GSH in stress signaling was demonstrated by characterization of the *A. Zthaliana rax1* mutant (Ball *et al.* 2004). Unfortunately, despite the importance of learning about the molecular mechanisms of stress responses we still know very little of the exact nature of the signaling cascades and involvement of GSH. A schematic representation of the central role that glutathione plays in responses to abiotic stress is

shown in Figure 4, showing the links between signaling, gene expression, and detoxification processes.

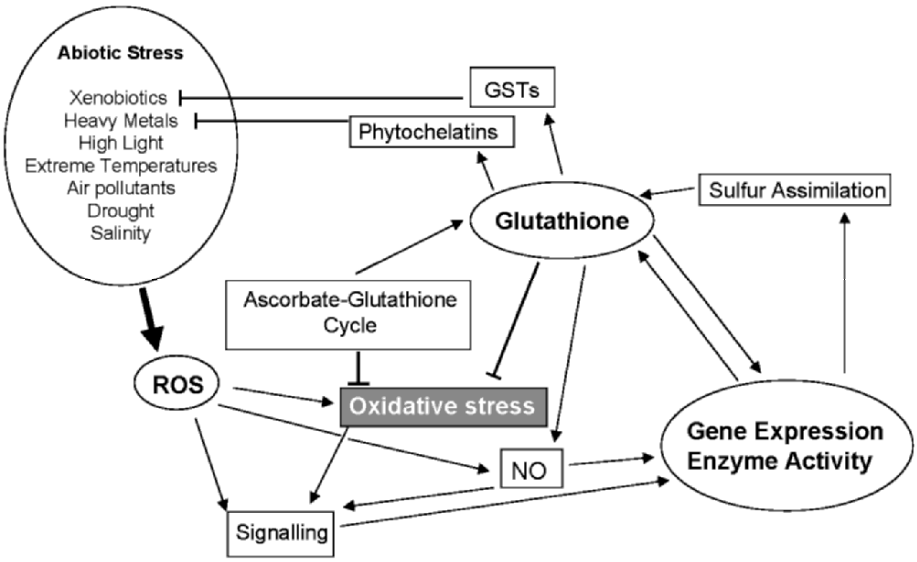


*Figure 4.* Overview of the role of glutathione as a central mediator in responses to abiotic stresses.

## ACCLIMATION TO STRESS THROUGH GLUTATHIONE

From the discussion above it is evident that glutathione is involved in protection of plants against a variety of abiotic stress conditions. On many occasions the difference between stress tolerant and sensitive genotypes can be pinpointed to differences in their capacity to induce GSH synthesis. It would seem therefore, that an increase of steady state GSH levels would protect plants against the various stress conditions. Consequently, many attempts have been made to increase resistance to environmental stress by changing glutathione metabolism. However, overexpression of γ-ECS or GSHS in transgenic poplar did not increase resistance to the herbicide paraquat or to ozone, although this was enhanced in wild-type poplar upon feeding with GSH (Strohm *et al.* 1999; Will *et al.* 2001). Tolerance to heavy metals was not increased in these plants (Kopriva *et al.* 2001), however, they were less damaged by herbicides (Gullner *et al.* 2001). Chloroplast targeted overexpression of GR in poplar leading to increased

foliar GSH content and reduction state did not improve tolerance to paraquat exposure. The same transgenic poplar plants, however, showed a higher resistance to photoinhibition (Foyer *et al.* 1995). In contrast, increased ascorbate peroxidase and GR activity in transgenic cotton overexpressing MnSOD in chloroplasts (Payton *et al.* 1997), and overexpression of GR in the chloroplasts of tobacco resulted in a slightly increased resistance to high light and paraquat triggered damage (Foyer and Rennenberg 2000). It seems, therefore, that the capacity to regenerate GSH by enhanced GR activity may be more important for the protection against oxidative stress than enhanced foliar GSH concentration (Foyer *et al.* 1995).

As an example of a successful approach to improving stress tolerance by manipulating GSH, overexpression of a GST with GPX activity increased the tolerance of tobacco to different stresses (Roxas *et al.* 2000). The seedlings had higher MDHAR activity, higher GSH and ascorbate content and the GSH pools were more oxidised. In wild-type plants, treatment with high and low temperatures and high salinity inhibited growth, caused lipid peroxidation and reduced metabolic activity. Oxidative damage in response to the same stresses was lower in the transgenic plants, growth was not reduced and lipid peroxidation did not increase. It was suggested that the reduction in oxidative damage was due to an increase in GSH-dependent detoxification of ROS and alterations in GSH and ascorbate metabolism which allowed the seedlings to maintain growth under stressful conditions (Roxas *et al.* 2000). However, changing GSH metabolism has not always resulted in positive effects. For example, whereas genetic manipulation to increase the ascorbate–GSH cycle enzymes in chloroplasts increased resistance to chilling-related photooxidative stress in cotton plants under laboratory conditions (Payton *et al*. 2001), resistance to chilling in these lines was not seen in field trials (Logan *et al.* 2003). Furthermore, manipulation of GSH content in tobacco plants overexpressing γ-ECS resulted in a threefold increase in GSH, and a parallel increase in oxidative stress (Creissen *et al.* 1999). This was attributed to a failure in the redox sensing process in the chloroplast. It is evident that the balance between GSH levels and redox sensing is important, and increased tolerance may not be achieved purely by increasing the GSH level or shifting the redox state enzymatically. This was also highlighted when feeding with GSH increased rather than reduced oxidative stress in rice caused by high light treatment (Xu *et al.* 2000), whereas in catalase deficient mutants, sensitive to high light due to reduced hydrogen peroxide scavenging capacity, the exogenous application of GSH protected against oxidative stress (Dat *et al.* 2003). Another complexity in the role of GSH in stress defence was added by finding that glutathionylation of proteins is increased during oxidative

stress, and that several stress defence proteins are targets of this modify-cation (Dixon *et al.* 2005). Altogether it seems that the effects of GSH are so complex in plants, that it is very difficult to know its precise roles in individual cells.

## CONCLUSIONS

Glutathione is central to plant defence in abiotic stress. Its function in detoxification of ROS, xenobiotics, and heavy metals is indisputable and well characterised. Its involvement in signaling and in interplay with ROS and other signals in the regulation of various aspects of plant stress response and metabolism is beginning to emerge. However the role of GSH in stress defence is complicated by the dynamics of the system and by compartmentalization, which have seldom been taken into account during interpretation of physiological and/or molecular studies. The biggest hurdle however is to translate the knowledge obtained in controlled experiments into strategies to improve plant stress tolerance in the field. Here, the real breakthrough has not yet been achieved. Transgenic approaches so far have not been entirely satisfactory, so we may expect to see an increased use in the exploitation of natural variation in investigating the mechanisms of stress defence. Glutathione metabolism will certainly remain central to these future approaches.

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Chapter 8

# MOLECULAR LINKS BETWEEN METALS IN THE ENVIRONMENT AND PLANT SULFUR METABOLISM

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## INTRODUCTION

*Toxic metals in environment*

Soil, surface water, and ground water contamination with organic and inorganic compounds poses an increasing environmental problem worldwide. Toxic mineral elements enter ecosystems by both natural and anthropogenic processes (He *et al.* 2005). Most metals and metalloids are present naturally in the Earth crust at various levels; some soils, for example that developed from basaltic igneous rocks, contain a high background of metals such as copper (Cu), zinc (Zn), chromium (Cr), cobalt (Co), and nickel (Ni). Anthropogenic sources of contamination with toxic metals are the metal-smelting industry, mining and burning fossil fuels and waste, as well as some pesticides and fertilizers used in agriculture. An excess of metals in the soil results in soil quality degradation, impacts on crop yield production and causes a poor quality of the agricultural product. A significant hazard to humans, animals, and ecosystems are posed by metals and metaloids as arsenic (As), cadmium (Cd), Cr, Cu, lead (Pb), mercury (Hg), Ni, selenium (Se), silver (Ag), Zn, and less common aluminum (Al), cesium (Cs), Co, manganese (Mn), molybdenum (Mo), strontium (Sr), and uranium (U) (Yang *et al.* 2005).

*Mineral nutrition and metal ion homeostasis in plants*

Plants require mineral nutrients for survival and the transfer of abiotic elements to the biotic components of ecosystems is mainly performed by



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plants. Uptake of mineral elements by plants is essential not only for plant growth, but also for human health and nutrition and for plant-based bioremediation of environments contaminated with inorganic pollutants (Guerinot and Salt 2001; Pilon-Smits 2005; White and Broadley 2005). The mineral elements present in plants can be classified according to at least three criteria: (i) on the basis of their essentiality for plants as macronutrients (N, P, S, K, Mg, Ca), micronutrients (Fe, Mn, Zn, Cu, B, Mo, Cl, Ni), and "beneficial" elements (Na, Si, Co) (Marshner 2002); (ii) on the basis of their physiological and biochemical function as structural (N, S, P), coenzymatic (Mg, Ca, Mn, Zn, Fe, Cu, Mo, Mn) or affecting membrane potential (Cl, Na, K); and (iii) on the basis of their chemical properties as nonmetals (N, P, S, Cl, B), alkali and alkaline earth metals (K, Ca, Mg, Na), and heavy metals (Fe, Cu, Mn, Zn, Ni).

The mineral nutrients that are necessary for a plant to complete its life cycle are defined as essential. An essential element is either a constituent of an essential metabolite or is needed for an enzymatic function. Many essential micronutrients, including heavy metals, are present in plants at low concentration and, therefore, they can be defined as plant trace elements. Genomic scale profiling of nutrients and trace elements in plants clearly indicated that the levels of various ions within an organism are coordinately regulated (Lahner *et al.* 2003; Rea 2003). The development and application of modern molecular biological techniques and completion of genomic sequences of some plant species has accelerated progress in describing and understanding nutrient homeostasis in plants and introducing the concept of the ionome in addition to the terms transcriptome, proteome, and metabolome (Salt 2004). Two elements are essential for a control of cellular and subcellular concentration of a nutrient by plant: (i) sensing of its concentration in the cellular and extracellular space and (ii) control of the expression and activity of the appropriate transporters, ligands, and target molecules. A knowledge of the plant ion homeostasis network is crucial to a full understanding of the integrative metabolism of organic and inorganic compounds.

*Toxic metals and plant metabolism*

Living organisms require trace amounts of some metals and metalloids, including Co, Cu, Mn, Mo, V, Sr, Se, Ni, and Zn. On the other hand excessive levels can be detrimental to the organism, and some of these ions pose a particular challenge because of the fine balance between the required and the toxic concentration. Other metals such as Hg, Pb, and Cd have no known essential or beneficial effect on organisms, and their accumulation over time in the bodies of mammals can cause serious

illness. The uptake and accumulation of toxic metals and metalloids by plants play a key role in their entry to terrestrial food chains.

The molecular mechanisms of heavy metal accumulation by plants and a possibility of using plants for phytoremediation has been the subject of several recent excellent reviews (Clemens *et al.* 2002; Pilon-Smits 2005; Yang *et al.* 2005). Accumulation of essential micronutrients and nonessential elements in plants is a complex phenomenon and involves several common steps: (i) transport to the roots; (ii) xylem loading and a long distance transport to the shoots; and (iii) detoxification and sequestration. The situation is complicated by the fact that many metal ions are transported by more than one transporter family and many transporter families transport more than one metal ion (Hall and Williams 2003; Reid and Hayes 2003; Yang *et al.* 2005). Different ligands are used for storage and long-distance translocation of toxic metal ions, however, the general mechanism for detoxification of harmful metals in plants is either their distribution to the apoplast or sequestration in the vacuoles after complexation with ligands. All of the above steps may be subject to specific regulation that might influence metal accumulation potential and metal tolerance of a given plant species.

As was thoroughly discussed for Cd (Sanita di Toppi and Gabbrielli 1999), the toxic effects of heavy metals in plants are mostly related to their strong reactivity resulting in inhibiting enzyme activity and oxidative damage to the cell. For these reasons, heavy metal ions are present in the cytoplasm mostly in a bound form. Interestingly, competing effects of Cd for the uptake, translocation, and/or metabolism of Fe, an essential microelement, have been recently proposed (Kim *et al.* 2006; Yoshihara *et al.* 2006).

## CHEMICAL SPECIATION OF ACCUMULATED METALS AND METAL CHELATORS

Most metal ions present in the shoots and roots of plants are bound to low molecular mass ligands or to proteins (Marmiroli *et al.* 2005; Salt *et al.* 2002). Chemical speciation of metals accumulated by plants has been mostly determined either by X-ray absorption spectroscopy (XAS) or by nuclear magnetic resonance (NMR) spectroscopy (Ueno *et al.* 2005). A method based on chromatography and capillary electrophoresis with parallel element-specific (inductively coupled plasma mass spectrometry) and molecule-specific (electrospray mass spectrometry) detection has been used for Ni speciation (Vacchina *et al.* 2003). According to XAS data, in

nonaccumulating plants Cd seems to be preferably bound to S-ligands rather than to O- and N-ligands; Zn and Ni seem to prefer O- and N-ligands; As(III) forms preferably complexes with S-ligands (Salt *et al.* 2002) and Pb with plant ligno-cellulose structure (Marmiroli *et al.* 2005). In the leaves of a hyperaccumulator, *Thlaspi caerulescens* Cd was coordinated mainly with malate and most probably stored in the vacuoles (Ueno *et al.* 2005).

The ligands that have been reported to play a role in sequestering, transport, and storage of the metals in plants are discussed below. The association constants of possible metal complexes with some of the ligands are shown in Table 1.

*Table 1.* Association constants (lg *K*) of metal complexes with selected ligands. Modified from Callahan *et al*. (2006).

| Ligand | $Fe^{2+}$ | $Fe^{3+}$ | $Co^{2+}$ | $Ni^{2+}$ | $Cu^{2+}$ | $Zn^{2+}$ |
|---|---|---|---|---|---|---|
| Nicotinamine | 12.1 | 20.6 | 14.8 | 16.1 | 18.6 | 14.7 |
| | 12.8 | | | | | 15.4 |
| Citric acid | 4.4 | 11.5 | 4.1 | 5.4 | 5.9 | 5.0 |
| Histidine | 5.9 | 4.7 | 6.9 | 8.7 | 10.2 | 6.6 |
| Cysteine | | | | 9.8 | 7.0 | 9.2 |

*Sulfur-free ligands*

S-free ligands include free amino acids (histidine, asparagine, and alanine), organic carboxylic acids (citric, malic etc.) and phytosiderophores (nicotinamine (NA), mugineic acid, avenic acid). The N-donor ligand, histidine is assumed to play a role in hyperaccumulators; histidine has an especially high association constant for Ni and it has been suggested that Ni-hyperaccumulation relies on histidine-dependent root-to-shoot translocation of Ni (Kerkeb and Kramer 2003; Sharma and Dietz 2006). Histidine may form complexes with Zn in roots of *T. caerulescens* (Salt *et al.* 1999). Asparagine and alanine may be involved in making complexes in xylem with Ni (Bhatia *et al.* 2005) and Cu (White *et al.* 1981). Amino acids do not bind Zn efficiently at acidic pH, therefore, organic acids are the predominant ligands at low pH (Sharma and Dietz 2006; White *et al.* 1981).

The carboxylic acids that are present at high concentration in plant vacuoles include citric, isocitric, oxalic, tartaric, malic, malonic, and aconitic acids. Many studies suggest roles in metal hyperaccumulation (Callahan *et al.* 2006 and references within). Additionally, it has been

suggested that in younger tissues Zn and Cd require stronger ligands (*S*-ligands) while in older tissues the metal-O bonds dominate (Kupper *et al.* 2004). Organic acids do not bind metals strongly enough to extract them from the soil (Table 1) and are unlikely to act as a long-distance transporters.

NA is formed by the condensation of three *S*-adenosyl-methionine molecules and is linked to Fe homeostasis both in monocots and dicots and apparently involved in translocation of Ni and possibly other metals (Callahan *et al.* 2006; Hell and Stephan 2003; Sharma and Dietz 2006).

*S-containing ligands*

The major types of S-containing ligands potentially involved in metal binding include metallothioneins (MTs), phytochelatins (PCs), and glutathione (GSH), although also a complexation of Co ions with free cysteine has been reported (Oven *et al.* 2002). Extensive reviews have been published on MT and PCs and for the details and other references the reader is referred to two of them (Clemens 2006; Cobbett and Goldsbrough 2002).

Metallothioneins (MT) are small, cysteine-rich, metal binding proteins that are ubiquitous in many organisms. The family of higher plants MT is large, complex and can be divided into at least four types. Recently it has been shown that plant type 1 MT are stabilized by Cd binding and to a lesser extent by As and Cu. Moreover, the MT1 knockdown lines of *Arabidopsis thaliana* were more sensitive to Cd and accumulated less Cd, Zn, and As than wild-type plants (Zimeri *et al.* 2005).

The most important difference between MT and PCs is that the former are gene-encoded, while the latter are enzymatically synthesized by phytochelatin synthase (PCS). PCs contain only three amino acids with a general formula $(\gamma GluCys)_nGly$ where $n$ is between 2 and 11, most often between 2 and 5. It is generally believed that PC-based sequestration of Cd, Cu, Zn, Ni, and Co is a major mechanism functioning in nonaccumulators, while metal-accumulators use other ligands for metals complexation (Schat *et al.* 2002).

## SULFUR METABOLISM AND REGULATION OF SULFUR-RELATED GENE EXPRESSION UNDER CADMIUM STRESS

Most of the targetted work addressing transcriptional regulation of the genes encoding proteins involved in S-assimilation and metabolism of S-containing compounds has been focussed on the response of these genes to

S-deficit. Expression of the genes encoding specific sulfate transporters, ATP sulfurylase, APS reductase, some isoforms of serine acetyltransferase (SAT) and *O*-acetylserine(thiol)lyase (OAS-TL) is induced during sulfur limitation. This regulation is either directly or indirectly mediated by OAS, cysteine, and GSH. Several papers, including the most recent by Hawkesford and De Kok (2006), review the regulatory aspects of sulfur metabolism in plants. The expression of sulfur starvation-induced genes during exposure of plants to heavy metals has not been fully investigated. Nevertheless, in many cases an increased expression of some genes of the pathway, including the genes encoding sulfate transporters (Heiss *et al.* 1999; Nocito *et al.* 2002), ATP sulfurylase and APS reductase (Harada *et al.* 2002; Heiss *et al.* 1999), OAS-TL (Domínguez-Solis *et al.* 2001), SAT (Howarth *et al.* 2003; Kawashima *et al.* 2005), γ-glutamylcysteine synthetase (Schafer *et al.* 1998; Xiang *et al.* 2001), glutathione synthase (Harada *et al.* 2002; Xiang *et al.* 2001) and PCs (Clemens *et al.* 1999; Heiss *et al.* 2003; Lee and Korban 2002) was reported for various plant species in response to heavy metal exposure. Plant responses to toxic concentrations of Cd are the most frequently studied (Sanita di Toppi and Gabbrielli 1999). A comparative review of plants, yeast, and protists responses to Cd stress, with a particular focus on S-related genes and metabolites, was recently published (Mendoza-Cozatl *et al.* 2005). The reaction to Cd is best characterized in yeast where a coordinated response of the transcriptome and metabolome to Cd stress seems to exist (Fauchon *et al.* 2002; Jamieson 2002). Multiple yeast factors participating in regulation of transcription of genes involved in S-metabolism in response to S nutrition and Cd exposure were identified (Dormer *et al*. 2000; Vido *et al.* 2001). The main transcriptional activator of sulfate assimilation pathway, Met4 plays an essential role not only in response to S nutrition but also to Cd exposure. Unfortunately, plant transcriptional factors involved in regulation of gene expression in response to either S nutrition or Cd stress are not yet characterized.

A theoretical kinetic modeling of GSH and phytochelatin synthesis in plants under control conditions and in plants exposed to Cd has been recently performed in an attempt to determine the mechanisms controlling flux of these compounds (Mendoza-Cozatl and Moreno-Sanchez 2006). The general conclusions of the theoretical modeling are in agreement with the previously proposed role of γ-ECS as a rate-limiting step for GSH and phytochelatin synthesis (Noctor *et al.* 1998; Zhu *et al.* 1999a,b). The kinetic model of the pathways clearly showed that at low GSH demand (control conditions) the activity of γ-glutamylcysteine synthetase is not limiting for GSH concentration, whilst at high GSH demand (exposure to Cd) the GSH concentration is affected by both, GSH synthesis

(γ-glutamyl-cysteine synthetase activity) and glutathione-consuming enzymes (PCs activity and GSH *S*-transferases activity). Therefore, for maximal GSH accumulation or for maximal phytochelatin production, optimal results will be achieved not only by the increase of limiting synthesis activities but also by the simultaneous decrease of the branching GSH flux.

## METAL HYPERACCUMUALATION AND SULFUR COMPOUNDS

Metal hyperaccumulation is a rare phenomenon that occurs in plants and has been known since the 19th century. The term hyperaccumulator is used for plants which accumulate very high concentrations of a metal in their aerial tissues in their natural habitats. These habitats are soils with high metal concentrations, either naturally due to mineralization, or as a result of human activities such as mining and smelting. Threshold values of tissue metal concentrations that are two or three orders of magnitude higher than in plant species growing on uncontaminated soils have been used to define metal hyperaccumulation. Currently, the accepted metal concentrations in shoots of hyperaccumulator plants are 1.0% for Zn and Mn; 0.1% for Co, Cu, Ni, As, and Se; and 0.01% for Cd, on dry weight basis (Baker *et al.* 2000).

To date, over 400 plant species have been identified as natural metal hyperaccumulators, currently less than 0.2% of all angiosperms, however the list is increasing. The majority of the hyperaccumulators are endemic to metalliferous soils and can be regarded as strict metallophytes and others are facultative metallophytes, with populations on both metal-rich and normal soils. A number of the plants belong to *Brassica*ceae such as *Alyssum*, *Thlaspi*, *Arabidopsis* species and *Brassica juncea*, whilst others belong to the Violaceae or Leguminosae (Reeves and Baker 2000, Figure 1). Examples of hyperaccumulators that have received attention are the well-known Zn/Cd hyperaccumulator *T. caerulescens* and also the Zn/Cd hyperaccumulator *A. halleri,* the Cd hyperaccumulator *B. juncea,* the Ni hyperaccumulators *T. goesingense* and *Alyssum lesbiacum* (Baker *et al.* 2000; Reeves and Baker 2000) and the As hyperaccumulator fern *Pteris vittata* (Ma *et al.* 2001).

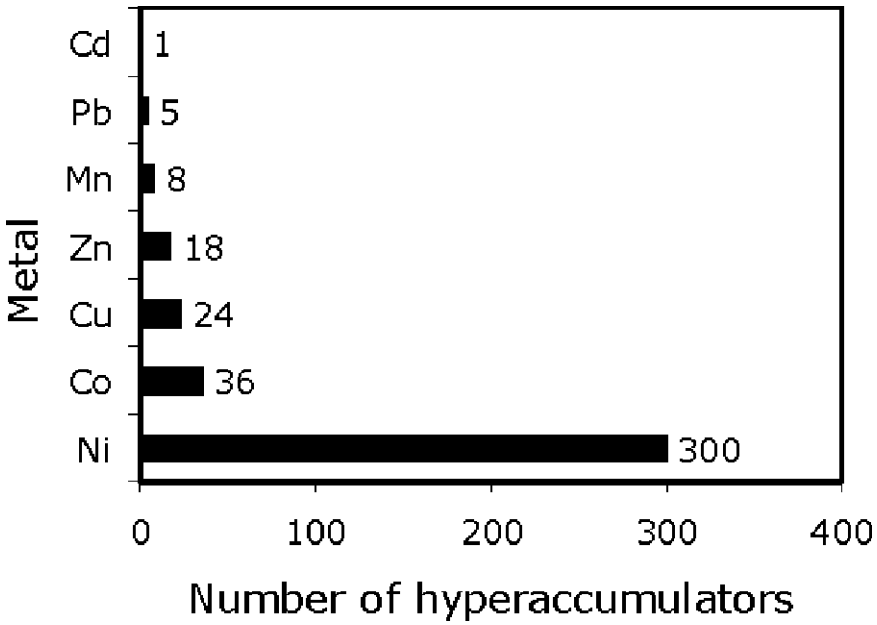


*Figure 1.* Angiosperm families that hyperaccumulate metals. The families dominating are Asteraceae, *Brassica*ceae, Caryophyllaceae, Cyperaceae, Cunouniaceae, Fabaceae, Flacourtiaceae, Lamiaceae, Poaceae, Violaceae, and Euphobiaceae. The term hyperaccumulator is used for plants, which accumulate a metal at concentrations two or three orders of magnitude higher than plant species growing in their natural habitats.

*General mechanisms underlying hyperaccumulation*

A hyperaccumulator plant is necessarily a tolerant plant, not only able to grow in the presence of high concentrations of the metal without showing toxicity symptoms or reduction in root or shoot dry weights, but also highly metal tolerant at the tissue and cellular level. The steps involved in hyperaccumulation are the same as involved in nonaccumulating plants: uptake of the metal by root cells, xylem loading and translocation, detoxification and sequestration in cellular locations. In recent years, substantial efforts have been devoted to identify genetic traits underlying hyperaccumulation that would facilitate molecular engineering approaches (for reviews see: Clemens *et al.* 2002; Eapen and D'Souza 2005; McGrath and Zhao 2003; Pollard *et al.* 2002). Although the scope of this chapter is to describe the role of sulfur compounds in plant metal hyperaccumulation, other determinants of the hyperaccumulation are exemplified below.

Important insights have been obtained by comparisosn between hyperaccumulators and closely related nonaccumulators. Thus, the overexpression of the Zn transporter ZNT1 is an important component of the Zn hyperaccumulation trait in *T. caerulescens* when compared to the nonaccumulator *T. arvense* (Pence *et al.* 2000). A heavy metal-translocating ATPase has been also suggested to play a key role in the mechanisms underlying metal hyperaccumulation in *T. caerulescens*, by functioning in metal xylem loading (Papoyan and Kochian 2004). Constitutively elevated expression of the cation diffusion facilitator, *MTP1*, in *T. goesingense* was suggested to play a role in Ni hyperaccumulation, compared to nonaccumulators *T. arvense*, *A. thaliana*, and *B. juncea* (Persans *et al.* 2001), but later studies have shown no differences between these plant species for Zn accumulation (Kim *et al.* 2004). However, in *A. halleri* leaves, the total *MTP1* transcript levels were substantially higher than in the closely related nonaccumulators *A. lyrata* and *A. thaliana*, and it was suggested that MTP1 proteins mediate the detoxification of Zn in the cell vacuoles of the hyperaccumulator (Drager *et al.* 2004).

Hyperaccumulation necessarily involves complexation of the metals and storage within the shoots, mainly vacuoles. With regard to complexation, a comparison between *Thlaspi* species showed that Ni is localized in vacuoles of the hyperaccumulator *T. goesingense* as a Ni-organic acid complex (Kramer *et al.* 2000). In *A. lesbiacum*, however, histidine biosynthesis is the major determinant of Ni tolerance, both through chelation with the metal and facilitating the xylem loading (Ingle *et al.* 2005a). In addition, NA plays an important role in detoxification of extravacuolar Ni in hyperaccumulator plants. Constitutive overexpression of NA in *T. caerulescens* and *A. halleri* supports the conclusion of NA chelating as one mechanism underlying Ni hyperaccumulation (Becher *et al.* 2004; Weber *et al.* 2004). In the case of Zn, the metal is predominantly complexed to malate in leaves of *A. halleri* while in the nonaccumulator *A. lyrata*, Zn is sequestrated with phosphate, similarly to crop species (Sarret *et al.* 2002).

*Evidence for the roles of sulfur-compounds in metal hyperaccumulation*

There is strong evidence suggesting that PCs are the major ligands for complexion of different heavy metals, particularly Cd and As, in nonaccumulator plants. However, naturally selected heavy metal hypertolerance found in plant populations from Cd-, Zn-, or Cu-toxic environments, does not seem to be associated with enhanced phytochelatin biosynthesis. In a comparative study of the role of PCs in heavy metal tolerance in hyperaccumulator and nonaccumulator metallophytes, it was concluded that PC-mediated

sequestration is not essential for constitutive hypertolerance to Cd, Co, Cu, Ni, and Zn (Schat *et al.* 2002). The role of PCs was also examined in the hyperaccumulator *T. caerulescens* and the related nonaccumulator *T. arvense*, and although these peptides were produced by both species in response to Cd, they do not appear to be involved in metal tolerance in the hyperaccumulator. While synthesized in response to Cd exposure, PCs were generally present at lower levels in *T. caerulescens* than in the nonaccumulator (Ebbs *et al.* 2002). Thus, PCs seem not to be involved in the detoxification of excessively accumulated metals, but they may have other functions, like metal micronutrient homeostasis under nontoxic conditions, as the plants contain PCs at low concentrations. From an energetics point of view, it is likely more economical for the plant to pump the metal into the vacuole where they may be stored weakly bound to organic acids, rather than investing energy for synthesizing the large amounts of PCs that would be required for binding high concentrations of the metal in the case of an hyperaccumulator plant. Differences in the complexation of Cd depending on the tissue or age of the plant were observed in *T. caerulescens*, where oxygen ligands dominated in mature leaves and sulfur ligands in young leaves. Furthermore, it was observed that sulfur ligands were not involved in the Zn resistance of hyperaccumulator plants (Kupper *et al.* 2004).

Detoxification of arsenate involves reduction to arsenite followed by complexation with PCs in nonaccumulator species. In the arsenic hyperaccumulator *P. vittata*, the arsenate reductase and PCs have been described, which may suggest a similar detoxification mechanism in the hyperaccumulator (Dong 2005; Duan *et al.* 2005). However, there are several lines of evidence indicating that the main storage form of As is uncomplexed arsenite. In *P. vittata*, it was concluded that arsenate is taken up via the phosphate transporters, reduced to arsenite, and sequestered in the fronds primarily as As (III) (Wang *et al.* 2002), and only a small proportion of As was complexed with PCs (Zhao *et al.* 2003). In the As-tolerant grass *Holcus lanatus* and the As hyperaccumulator *P. cretica*, As was predominantly in nonbound inorganic forms, although a minor fraction was present as the As(III)-$PC_3$ complex in *H. lanatus* and as a mixed glutathione-As(III)-$PC_2$ complex in *P. cretica* (Raab *et al.* 2004).

Metallothioneins bind metal ions in metal-thiolate clusters, predominantly Zn and Cu in nonaccumulator plants, where roles in metal homeostasis or detoxification have been suggested. In hyperaccumulator plants different types of metallothionein genes have been identified, but a direct link between their gene expression and metal tolerance has not been conclusively demonstrated (Roosens *et al.* 2004; van Hoof *et al.* 2001).

Excess of metals are known to induce oxidative stress in plants, leading to lipid peroxidation and loss of membrane integrity. To avoid this oxidative damage, plants contain various antioxidant defense systems, enzymatic and non-enzymatic, designed to control the concentration of reactive oxygen species (ROS) tightly (Schutzendubel and Polle 2002). GSH plays a central role in the antioxidant defense systems and recent evidence indicates a correlation between elevated GSH concentrations and metal tolerance of hyperaccumulator plants. In various *Thlaspi* hyperaccumulators, and in nonaccumulator relatives, the concentrations of GSH and its precursors, cysteine and *O*-acetylserine (OAS), strongly correlated with the ability to hyperaccumulate Ni in shoot tissues. Detailed analysis of *T. goesingense* revealed that constitutively high activities of both SAT and glutathione reductase are responsible of the high levels of OAS, cysteine, and GSH. These enhanced cysteine and GSH biosynthesis and accumulation coincided not only with the ability to hyperaccumulate Ni but also to resist oxidative damage as significantly less lipid peroxidation and ROS generation was observed (Freeman *et al.* 2004). This mechanism of Ni tolerance in *Thlaspi* hyperaccumulators seems to represent a general mechanism, as it has been identified in various species of hyperaccumulator and nonaccumulator plants. Enhanced sulfur assimilation associated with Ni hyperaccumulation seems to be a constitutive trait, in contrast to the nonaccumulators that induce their response in the presence of metal as Cd-mediated PC biosynthesis. Further studies have shown that constitutively elevated levels of salicylic acid (SA) observed in *Thlaspi* hyperaccumulators signal the GSH-mediated Ni tolerance mechanism, through posttranslationally upregulated SAT activity (Freeman *et al.* 2005). SA is a molecule known to be involved in signaling pathogen defence responses in plants, and the studies described above suggest a cross talking between signaling pathways in plant responses to abiotic and biotic stresses.

The antioxidant responses to As have been studied in the As hyperaccumulator *P. vittata*, showing that both enzymatic and nonenzymatic antioxidants play significant roles in As detoxification and hyperaccumulation (Cao *et al.* 2004). While the activities of enzymatic antioxidants increased at low levels of As exposure, but decreased at high As concentration, the contents of the nonenzymatic antioxidant GSH significantly increased at high levels of As exposure, suggesting a correlation with metal hyperaccumulation. Other evidence suggests that superior antioxidant defences may play an important role in the metal hyperaccumulation phenotype and should be considered important genetic traits underlying hyperaccumulation (Boominathan and Doran 2003; Gratao *et al.* 2005).

*Molecular engineering approaches*

The use of plants to clean up contaminated soils is a technique known as phytoremediation that offers a less expensive alternative to stripping pollutants directly from the soil, and it has received increased attention in recent years. Plants ideal for phytoremediation of metal-contaminated soils should fulfill some requirements: fast growing, high biomass, extensive root system, easy to harvest, ability to tolerate and accumulate a range of different heavy metals in their harvestable parts. Most naturally occurring hyperaccumulator plants have small biomass and are slow growing, with the exception of the As-hyperaccumulator ferns. Furthermore selective hyperaccumulation of metals may be required, whereas the soils often contain multiple contaminant elements. Thus, to allow remediation within a reasonable period, dramatically improved plant species would be required, which may be developed by transgenic approaches. Numerous studies indicate that manipulation of relevant plant features involved in metal tolerance is a realistic possibility.

Substantial progress has been made in elucidating the molecular mechanisms of homeostasis and detoxification of metals in nonaccumulator plants and the mechanisms of metal tolerance and hyperaccumulation in the hyperaccumulator plants. Based on this knowledge, diverse molecular engineering approaches have been attempted with the aim of maximizing the capacity of plants for the phytoremediation process. Many of these genetic manipulations have been performed in the model plant *A. thaliana*, which does not have a direct phytoremediation application but presents the advantage of the available molecular tools and resources. In this way, the successful genetic manipulation of *Arabidopsis* for improving its metal tolerance or/and accumulation is considered an initial step prior to performing the same manipulations in plant species suitable for phytoremediation. Furthermore, the enormous understanding of aspects of metal metabolism and detoxification in nonplant systems has allowed the development of ingenious strategies for the manipulation of plants using genetic traits from *Escherichia coli* or yeast. One example of these strategies was the successful introduction of the modified bacterial genes, mercuric reductase *merA* and organomercurial lyase *merB*, to generate plants extremely valuable for Hg phytoremediation (Bizily *et al.* 2000; Rugh *et al.* 1998).

*Table 2.* Genes that have been introduced into plant species for metal tolerance (T) and/or accumulation expressed on tissue weight basis (A).

| Gene | Product | Source | Plant | Maximum effect | Reference |
|---|---|---|---|---|---|
| *APS1* | ATP sulfurylase | *A. thaliana* | *B. juncea* | (T) 50 μM Se (A) twofold higher | (Pilon-Smits *et al.* 1999) |
| *TgSAT-m* | SAT | *T. goesingense* | *A. thaliana* | (T)100 μM Ni | (Freeman *et al.* 2004) |
| *Atcys-3A* | OAS-TL | *A. thaliana* | *A. thaliana* | (T) 400 μM Cd, (A) 1.8-fold higher | (Domínguez-Solis *et al.* 2001, 2004) |
| *CSase* | OAS-TL | Spinach | Tobacco | (T) 300 μM Cd, 250 μM Se, 500 μM Ni; (A) twofold higher | (Kawashima *et al.* 2004) |
| *cysK* | OAS-TL | *E. coli* | Tobacco | (T) 300 μM Cd | (Sirko *et al.* 2004) |
| *RCS1* | OAS-TL | Rice | Tobacco | (T) 100 μM Cd | (Harada *et al.* 2001) |
| *gshI* | γ-EC synthetase | *E. coli* | *B. juncea* | (T) 250 μM Cd , (A) 1.9-fold higher | (Zhu *et al.* 1999a) |
| *GSH1* | γ-EC synthetase | *A. thaliana* | *A. thaliana* | (T) no increase | (Xiang *et al.* 2001) |
| *arsC* *gshI* | Arsenate reductase γ-EC synthetase | *E. coli* | *A. thaliana* | (T) 200 μM As, (A) threefold higher | (Dhankher *et al.* 2002) |
| *gshI* | γ-EC synthetase | *E. coli* | Poplar | (T) 2 mM Cd, (A) threefold higher | (Koprivova *et al.* 2002) |
| *gshII* | GSH synthetase | *E. coli* | *B. juncea* | (T) 200 μM Cd, (A) 1.4-fold higher | (Zhu *et al.* 1999b) |
| *GR* | GSH reductase | *E. coli* | *B. juncea* | (T) 100 μM Cd | (Pilon-Smits *et al.* 2000) |
| *AtPCS1* | PC synthase | *A. thaliana* | *A. thaliana* | Sensitivity to Cd | (Lee *et al.* 2003a,b) |
| *AtPCS1* | PC synthase | *A. thaliana* | Tobacco | (T) 100 μM Cd, (A) twofold higher | (Pomponi *et al.* 2006) |
| *cysE* *gshI* *PCS* | SAT γ-EC synthetase PC synthase | *E. coli* *E. coli* *S. pombe* | Tobacco | (A) Cd 1.5-fold higher in roots, no difference in shoots | (Wawrzynski *et al.* 2006) |
| *TaPCS1* | PC synthase | Wheat | *N. glauca* | (T) 0.8 mM Pb, (A) 1.8-fold higher | (Gisbert *et al.* 2003) |
| *MT-I* | Metallothionein | Mouse | Tobacco | (T) 200 μM Cd | (Pan *et al.* 1994) |
| *MT-II* | Metallothionein | Human | *B. napus* | (T) 100 μM Cd | (Misra and Gedamu 1989) |
| *CUP1* | Metallothionein | *S. cerevisiae* | *B. oleracea* | (T) 400 μM Cd | (Hasegawa *et al.* 1997) |
| *MT* | Metallothionein | *N. glutinosa* | Tobacco | (T) 200 μM Cd | (Suh *et al.* 1998) |
| *PsMTA* | Metallothionein | Pea | *A. thaliana* | (A) Cu sixfold higher | (Evans *et al.* 1992) |

*Engineering different steps of the S-compound biosynthesis*

Different engineering approaches have been performed to improve the ability of plants to tolerate and accumulate heavy metals, many of which are have enhanced metal uptake and vacuolar compartmentalization. Within the scope of this book, the present focus is on genetic strategies which attempt to enhance sulfur assimilation and the conversion to downstream metabolites. A summary of the engineered genes involved in sulfur-compound biosynthesis and the effects of their expression on metal tolerance and accumulation in plants is given in the Table 2. The most upstream enzyme of the sulfur assimilation that has been engineered is ATP-sulfurylase, overexpression of which in *B. juncea* increased Se tolerance and accumulation (Pilon-Smits *et al.* 1999). This enhanced tolerance is due to a fact that Se and S are nutrients with very similar chemical properties and their uptake and assimilation proceed through common pathways (see Chapter 10).

The last steps of the sulfur assimilation pathway resulting in cysteine biosynthesis, catalyzed by serine SAT and OAS-TL have been also proposed to be involved in metal tolerance, and genetic manipulations of SAT and OAS-TL have been performed. Overproduction of SAT from *T. goesingense* in the nonaccumulator *A. thaliana* mimicked the biochemical characteristics observed in the Ni hyperaccumulator and produced a five-fold increase in shoot Ni resistance. In this transgenic *Arabidopsis*, GSH concentrations strongly correlated with increased Ni resistance, but no significant differences were observed in shoot Ni accumulation (Freeman *et al.* 2004). However, the positive correlation found between GSH concentrations and Ni hyperaccumulation may suggest that at least in Ni hyperaccumulators collected from Ni-enriched environments, a link between Ni tolerance and Ni accumulation should exist, and that the genetic traits underlying metal accumulation should be different between nonaccumulator *Arabidopsis* and the hyperaccumulator *T. goesingense*. Several other strategies have focussed on the manipulation of OAS-TL gene expression to increase cysteine availability. *Arabidopsis*-transformed plants overexpressing the most abundant cytosolic OAS-TL isoform showed an increased tolerance to Cd, allowing the transgenic plants to survive under severe heavy metal stress conditions. This Cd tolerance was due to an enhanced Cd accumulation in leaves, with trichomes as the main location of the heavy metal accumulation (Domínguez-Solis *et al.* 2001, 2004). Similar Cd tolerance and/or accumulation were observed when spinach or *E. coli* OAS-TL were overproduced in tobacco plants, showing the highest resistance in plants where the OAS-TL was overexpressed in the cytosol (Kawashima *et al.* 2004; Sirko *et al.* 2004). Furthermore,

tobacco plants transformed with a cytosolic isoform of OAS-TL from rice exhibited greater tolerance than wild-type plants (Harada *et al.* 2001). These results may indicate that the cysteine biosynthesis in the cytosol could be a limiting step for metal tolerance, at least in nonaccumulator species.

Another approach attempted by several groups to enhance heavy metal tolerance and accumulation in plants, has been to increase the rate of biosynthesis of the PC-precursor, GSH. In Indian mustard (*B. juncea*), the two steps involved in GSH biosynthesis have been genetically engineered by overexpressing the *E. coli gshI* and *gshII* genes encoding γ-glutamylcysteine (γ-EC) synthetase and GSH synthetase, respectively. Both transgenic plants showed enhanced production of GSH and PCs and improved Cd tolerance and accumulation, suggesting that enhanced GSH biosynthesis appears to be a promising strategy for the production of plants with superior phytoremediation capacity (Zhu *et al.* 1999a,b). The phytoremediation potential of these transgenic Indian mustard plants was examined in metal-contaminated mine tailings, and an enhanced metal phytoextraction was observed (Bennett *et al.* 2003). Furthermore, overexpression of *E. coli* GSH reductase in *B. juncea* targeted to the chloroplast gave enhanced Cd tolerance (Pilon-Smits *et al.* 2000). However, the elevation of GSH levels by overproduction of γ-EC synthetase in *Arabidopsis* did not increase metal resistance as a consequence of only a modest increase in GSH concentration and PC formation (Xiang *et al.* 2001). These controversial results may suggest that the limiting step in the Cd tolerance process could be different between species.

An example of introducing novel pathways into plants has been the genetic strategy developed for As tolerance and accumulation in *A. thaliana*. The *E. coli* arsenate reductase, which reduces arsenate to arsenite using GSH as electron donor, was overproduced in *Arabidopsis* combined with the *E. coli* γ-EC synthetase. The bacterial *arsC* gene product was directed to leaf and stem tissues, conferring to the plants the potential to trap more As in aboveground tissue as arsenite–thiol complexes. The other co-expressed transgene resulted in an increased production of thiol-peptide compounds in the PC pathway for binding the produced arsenite and potentially contribute to the As tolerance and accumulation. Each transgene complemented their activities resulting in increased As resistance and accumulation in the aerial parts (Dhankher *et al.* 2002). This is the first example of multigene strategy in plants with phytoremediation potential.

Engineering of GSH biosynthesis for phytoremediation purposes has been also attempted in poplar trees. Poplar was the first tree species to besuccessfully transformed and transgenic trees seem to be more suitable for phytoremediation because of their higher biomass, extensive root systems, and longer life spans. Poplars overexpressing bacterial γ-EC synthetase were able to accumulate significantly more Cd than wild type. Furthermore, there was an increased allocation of Cd to the young leaves, which represented a potential advantage for the phytoremediation process as the same plants could be used over several vegetation periods (Koprivova *et al.* 2002). Evaluation of these transgenic trees in field experiments demonstrated their high capacity for phytoremediation purposes with a high accumulation of Cu (Peuke and Rennenberg 2005).

Another logical engineering approach has been to improve the heavy metal tolerance and accumulation by increasing the production of metal-chelating molecules. Several groups have attempted to enhance PC synthesis by overexpressing PC synthase, with contradictory results. The *Arabidopsis* PC synthase gene was overexpressed to increase PC production and curiously the increased capacity of PC synthesis did not lead to Cd tolerance, but on the contrary to Cd hypersensitivity. This hypersensitivity was also observed for Zn but not for Cu, and disappeared when GSH was supplemented in the medium. It was proposed that the phenotype of these transgenic lines was due to the toxicity of PCs, as they existed at supraoptimal levels when compared to GSH levels (Lee *et al.* 2003a). Only the transgenic lines showing slight increases in *AtPCS1* gene expression and PC content exhibited enhanced Cd tolerance and accumulation compared to wild type (Lee *et al.* 2003b). In an independent study, overexpression of PC synthase in *Arabidopsis* gave rise to the same Cd hypersensitivity phenotype. However, these plants were more resistant to As but did not result in increased aboveground As accumulation when compared to wild type (Li *et al.* 2004). When the same *Arabidopsis* gene (*AtPCS1*) was overexpressed in tobacco plants, Cd tolerance and accumulation was increased. However, the amount of Cd accumulated was higher in roots than in shoots, suggesting that the PC synthase overproduction did not enhance long distance root–shoot Cd translocation (Pomponi *et al.* 2006). Furthermore in tobacco plants, a simultaneous overexpression of three genes supposed critical for the efficient production of PCs also resulted in enhanced Cd accumulation in roots but not in shoots (Wawrzynski *et al.* 2006). Thus, Cd translocation in tobacco seems not to be linked to PCs. The differences observed in the effects of PC synthase overproduction between *Arabidopsis* and tobacco could be species-specific. Similarly to tobacco plants, overexpression of the PC

synthase gene in *Nicotiana glauca* increased Pb tolerance and accumulation (Gisbert *et al.* 2003).

Several groups have introduced MTs from different sources into plants with different purposes, for example to reduce metal accumulation in shoots by chelating it in the roots or to enhance metal tolerance. Overexpression of *MT* genes from mouse, human, and yeast in different plant species conferred Cd tolerant phenotypes, demonstrating the functionality of these genes in plants (Hasegawa *et al.* 1997; Misra and Gedamu 1989; Pan *et al.* 1994). *MT* genes isolated from plants also increased plant tolerance to specific metals, for example *N. glutinosa MT* overexpressed in tobacco enhanced Cd resistance (Suh *et al.* 1998), and pea *MT* enhanced a significant Cu accumulation in roots when overproduced in *A. thaliana* (Evans *et al.* 1992). In shoots, metal accumulation was only slightly increased in a few cases, which limits the phytoremediation application of MTs.

## THE "OMICS" TECHNOLOGIES APPLIED TO METAL RESPONSES LINKED TO SULFUR

The recent development of multiparallel, highly sensitive and high throughput techniques is allowing the understanding of the interacting metabolic networks within the plant system (see Chapter 6). These technologies are directed toward obtaining profiles of transcripts, proteins, metabolites, ions, etc., at a given state or condition, and the integration of all the data unravels complex plant systems biology. The application of these profiling approaches to metal hyperaccumulator plants will facilitate insights into metal homeostatic networks in metal tolerant plant species.

Two research groups have very recently performed the first "omics" study independently aiming to identify genes with a potential involvement in metal accumulation in shoots and roots of the Zn hyperaccumulator *A. halleri* (Becher *et al.* 2004; Weber *et al.* 2004). Comparative transcriptomic analysis between *A. halleri* and its relative nonaccumulator *A. thaliana* was performed using *A. thaliana* gene chips, demonstrating that this is a valuable tool for the elucidation of phenotypic differences between such species. Upon Zn exposure, transcript abundance of several genes was found to be substantially higher in *A. halleri* shoots compared to *A. thaliana* (Becher *et al.* 2004). These genes encoded proteins involved in Zn uptake and a NA synthase involved in the synthesis of metal chelators. Similarly, when the *Arabidopsis* gene chips were used to identify genes more active in roots of *A. halleri* compared to *A. thaliana* under control

conditions, the two genes showing the highest expression also encoded a NA synthase and a Zn uptake system (Weber *et al.* 2004). In addition transcript levels of an OAS-TL-like gene were significantly higher in roots and shoots of *A. halleri* relative to *A. thaliana* (Becher *et al.* 2004; Weber *et al.* 2004) This gene encodes a truncated putative cytosolic OAS-TL in *A. thaliana,* which does not contribute significantly to the total cystosolic OAS-TL transcript levels and enzyme activity, but it remains to be investigated whether this protein is functional in *A. halleri.* In fact, the stop codon proposed to result in a truncated protein in *A. thaliana* was not present at the corresponding position in a partial *A. halleri* cDNA. However, no significant differences in either OAS-TL protein amount or enzyme activity were detectable for *A. halleri* compared to *A. thaliana*. Interestingly, cysteine, the metabolite synthesized by OAS-TL, is a precursor of NA, suggesting a molecular link between both metabolites that deserves to be investigated. Other S-related genes expressed at higher levels in *A. halleri* compared to *A. thaliana* under Zn exposure were putative glutaredoxin and GSH *S*-transferase genes that could be involved in increased antioxidant defences.

Comparative transcript profiling has been also performed in Zn-treated *T. caerulescens* plants of two accessions originating from metalliferous and nonmetalliferous soils. Analysis with microarrays containing about 1900 cDNAs from *T. caerulescens* roots revealed some genes with unknown functions with strong induction or repression, which appeared to be unique features of the hyperaccumulator plant. In addition, the induction of enzymes involved in the pathway of NA biosynthesis, including methionine synthase and *S*-adenosylmethionine synthase, was found (Plessl *et al.* 2005). Furthermore, genes from *B. juncea* with altered transcript expression upon Cd treatment were identified using an approach that could be considered a "semi-omics" technology, the cDNA-amplified fragment length polymorphism (cDNA-AFLP) technique. In this study, 3,000 cDNA fragments were visualized on the gels, 100 were found to be Cd regulated, including a gene encoding for OAS-TL enzyme and two putatitve GSH *S*-transferases (Fusco *et al.* 2005).

The first proteomic approach performed has been applied to the Ni hyperaccumulator *A. lesbiacum* to identify proteins playing a role in its metal accumulation phenotype. 2D gels showed very few polypeptides with altered abundance upon Ni exposure in root tissues. The majority of these polypeptides were identified to be involved in S metabolism, consistent with a reallocation of S toward cysteine, or a downstream compound such as GSH. A cytosolic OAS-TL isoform increased in abundance while a cytosolic methionine synthase isoform decreased, the

combination of both altered abundances producing the shift of S away from methionine and toward cysteine. Furthermore, the observed increase in abundance of the cytosolic isoform of serine hydroxymethyltransferase that catalyzes the conversion of the photorespiratory glycine to serine, also might contribute to reallocation to cysteine, as serine is a precursor of cysteine. Other proteins potentially involved in protection against oxidative stress increased in abundance, including a GSH *S*-transferase (Ingle *et al.* 2005b).

Metabolomics is an emerging tool which has been used to reveal insights into plant responses to nutritional alterations. NMR-based metabolomic approaches has been applied to investigate the metabolic responses of *Silene cucubalus* following Cd exposure, allowing the identification of increases in malic acid and acetate, and decreases in glutamine and branched amino acids, but no changes in S metabolites was observed (Bailey *et al.* 2003).

At the present, as described above there have been few applications of "omics" technologies to unravel the plant responses to heavy metals. However, some coincidences related to S metabolism have been observed, such as the flux of S to cysteine biosynthesis and the induction of antioxidant S-related defences.

## CONCLUSIONS

Excess of various metals, both essential and nonessential, can be harmful to plants. Exposure to toxic levels of heavy metals affects plant redox homeostasis and results in extensive changes of the metabolome and transcriptome, including the induced expression of the genes involved in S-metabolism and biosynthesis of thiol-containing compounds (GSH, PCs, MT). These observations suggest that, at least in some cases, an increased S nutrition and an enhanced S metabolism could result in higher plant tolerance and accumulation of toxic metals. Indeed, in many cases an increased level of S-containing metabolites positively correlated with an increased metal tolerance, however, the S-containing compounds are only a small part of a sophisticated mechanism whose appropriate functioning is needed for growth in contaminated soils. Heavy metal resistance and accumulation is not related to the presence of only one particular compound or of only one enzyme activity. It is a result of a complex regulation at the genetic and enzymatic levels of several processes: sequestration, long-distance transport, storage, and protection of plants against the harmful effects of the stored ions. An in-depth understanding of

the networks of nutritional ions homeostasis is necessary for engineering plants with changed capacity to accumulate toxic metals.

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Chapter 9

# SULFUR IN BIOTIC INTERACTIONS OF PLANTS

Rüdiger Hell and Cordula Kruse

## INTRODUCTION

Most plants are sessile and have to deal *in situ* with biotic and abiotic factors that act on them such as light, temperature, water availability, animals that distribute their seeds and herbivores. All these factors are not single events, rather they interact with one another. In order to tackle environmental fluctuations and to prevent invasion by pathogens, plant metabolism must be flexible and dynamic (Noctor and Foyer 1998). Sulfur is found in the amino acids cysteine and methionine as an essential component of peptides and proteins, as well as in iron–sulfur clusters, cofactors and sulfolipids, and as such is involved in many stress response reactions.

S-metabolic pathways are essential for a variety of S-containing secondary metabolites, which often play important roles in defence against pathogens and herbivores. Examples are the sulfur-rich protein classes of thionins and defensins, glucosinolates and phytoalexins (Schnug *et al*. 1995; Bloem *et al.* 2004; Rausch and Wachter 2005). In addition, the low molecular weight thiol, glutathione (GSH), plays an important role in response to various biotic and abiotic stresses as it is a major thiol-disulfide redox buffer in plant cells (May *et al.* 1998; Schafer and Buettner 2001). GSH synthesis is dependent on the availability of cysteine, which in turn is dependent on sulfate assimilation. Measurements of total GSH and GSSG levels have been made to estimate the redox environment in plants after pathogen attack (Vanacker *et al.* 2000).



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## POSITIVE AND NEGATIVE BIOTIC INTERACTIONS IN PLANTS

Any given plant is usually not susceptible to most of the pathogens in its environment and development of a successful infection is not the rule, but the exception., This is because either the prospective host plant does not provide the prerequisites for survival of the microorganism, or passive, constitutive barriers like the cuticle are sufficient to prevent invasion. Sometimes the beginning of infection structures can be found, but in general, basic defence mechanisms are sufficient to restrict them. All these cases of resistance are polygenically determined and are referred to as nonhost resistance or horizontal resistance (Keller *et al.* 2000). A proof-of-concept of nonhost resistance is provided by an elegant mutant screen using *Arabidopsis thaliana*. *Arabidopsis* is a host to the powdery mildew *Erysiphe cichoracearum* and nonhost to *Blumeria graminis* f. sp. *hordei*, the powdery mildew pathogenic on barley (*Hordeum vulgare*). Screening for *Arabidopsis* mutants deficient in resistance to barley powdery mildew identified mutant plants with independent genetic loci that permitted both increased invasion into epidermal cells and initiation of hyphae by B. g. *hordei*, thus breaking the nonhost resistance of this plant–pathogen couple (Collins *et al.* 2003).

Plants, like animals, have evolved both innate and acquired immunity to counter attacks by microbial pathogens (Dangl and Jones 2001). When the preformed structural and biochemical resistance factors that protect a plant from the attack by most potentially phytopathogenic microorganisms have failed, a second line of defence is activated, including induced structural and biochemical resistance reactions. Penetration of pathogens can occur at different sites, at open sites like stomata and wounds, at the border between two cells or directly through the cuticle. As a structural response in some plants, a newly developed meristem may produce a new layer of cells which may become suberized or lignified. In other cases, the cell walls of attacked plant cells may be fortified by the incorporation of new cell wall components, often accompanied by the apposition of new cell wall material in the form of a papilla (Moerschbacher and Mendgen 2001). Biochemical responses of a penetrated host cell may involve the accumulation of phytoalexins as well as pathogenesis and defence related proteins that inhibit or at least delay pathogen growth. One trigger for cell wall fortification could be the generation of reactive oxygen species (ROS) in response to microbial pathogen attack (the oxidative burst). This is an ubiquitous early part of the resistance mechanisms of plant cells (Bolwell *et al.* 2002), which often leads to a hypersensitive response (HR), the most

familiar form of plant programmed cell death associated with successful plant immune responses (Epple *et al.* 2003). In an extreme reaction, the cytoplasm collapses and thus pathogen proliferation is prohibited due to this localized induced cell death at the site of infection. One model for the genetic basis of HR-mediated disease resistance was given by Flor (1956, 1971), who established the gene-for-gene hypothesis of plant–pathogen interactions. According to this theory, the establishment of a compatible or incompatible interaction depends on the presence of cognate paired genes of avirulence genes in the pathogen and corresponding resistance (*R*) genes in the host. Resistance is only expressed when a plant that contains a specific *R* gene recognizes a pathogen that has the corresponding avirulence gene (incompatible interaction). Other combinations of genes lead to lack of recognition by the host, resulting in disease (compatible interaction). Each plant genome encodes hundreds of resistance (R) proteins that allow the plant to recognize specific pathogen-derived molecules known as avirulence (avr) factors. In addition to programmed cell death and production of ROS, this R-avr recognition triggers not only HR, but involves synthesis of antimicrobial compounds at the site of infection, leading to resistance against the respective pathogen (Dangl and Jones 2001).

The oxidative burst is an early plant reaction that can occur in compatible as well as in incompatible interactions. The differences are revealed by analysis of the kinetics. In tests with transgenic tomato cell suspension cultures, only in the presence of the corresponding R-avr gene pair, could two distinct phases of the oxidative burst be observed: a rapid first burst followed by a slower and more prolonged second burst. Otherwise either no burst or only a first burst was observed, indicating that the second burst is correlated with disease resistance (Chandra *et al.* 1996). Compatible or incompatible interactions are therefore a consequence of genetically determined recognition and subsequent kinetics and intensity of the early reaction.

The local accumulation of ROS during the oxidative burst is really a special situation for the plant: on the one hand it takes advantage of the direct antimicrobial effects of ROS like superoxide anion and hydrogen peroxide production, as well as involving its signal function in cell-to-cell communication. The increased levels of ROS activate defence gene expression as part of protective responses to both biotic and abiotic stimuli (Karpinski *et al.* 1999; Grant and Loake 2000; Fryer *et al.* 2002; 2003; op den Camp *et al.* 2003). On the other hand the plant has to ensure that the oxidative damage remains constricted to the site of infection, so a scavenger system is of extreme importance.

The thiol compound GSH is probably a key player in this situation, since it is a component of the antioxidant network consisting of low molecular weight antioxidants, enzymes that keep them reduced and ROS-scavenging enzymes (Karpinski *et al.* 1997; Asada 1999). Recent results suggest that GSH is probably not only important to restrict ROS to a narrow area of a few cells at the site of pathogen attack or wounding by herbivores, but seems to be linked to the signal pathways that lead to plant defence mechanisms (Ball *et al.* 2004). Considering GSH is a major determinant of cellular redox state, it may have an influence on many fundamental cellular processes by interference with thiol-disulfide exchange reactions (Cooper *et al.* 2002). This may be a way to link the regulation of gene expression to the redox state of cells or specific subcellular compartments (Schafer and Buettner 2001; Noctor *et al.* 2002).

In plants, the number of regulatory processes in plant–microbe interactions that are known to be potentially influenced by the levels or redox state of cellular GSH pools is small. Not many examples exist that have confirmed the possibility of GSH redox-mediated control of nuclear-located defence gene expression. GSH may activate the regulatory proteins NPR1 (for nonexpressor of PR genes) and possibly protein phosphatase 2C (*ABI2* locus), important in salicylic acid (SA) and abscisic acid (ABA) signaling, respectively (Meinhard *et al.* 2002; Mou *et al.* 2003). Plants with reduced GSH levels, like the *cad2-1* or the *rax1-1* mutant, were shown to be more susceptible to metal stress, light stress and avirulent bacteria (May *et al.* 1996; Ball *et al.* 2004). These plants are affected in the *GSH1* gene coding for the plastidic enzyme γ-ECS (Figure 1).

The role of sulfur metabolism and the influence of sulfur-containing metabolites on plant–pathogen interactions is multifaceted. Examples of the involvement could be found in all three phases of active defence: (1) signal perception, (2) signal transduction and the realization of (3) the defence reaction itself.

The specific recognition of a pathogen by the host (signal perception) is often triggered by elicitors. It was shown that β-1,3 glucan sulfate, but not β-1,3 glucan, induces the SA signaling pathway in tobacco and *Arabidopsis*. In tobacco leaves the laminarin sulfate PS3, but not laminarin, caused electrolyte leakage, triggered SA accumulation and the expression of ethylene- and SA-dependent PR proteins. In *Arabidopsis*, PS3-induced PR1 expression was also NPR1 dependent. In tobacco PS3 induced immunity against tobacco mosaic virus infection, whereas laminarin induced only a weak resistance (Menard *et al.* 2004). The likely role of GSH in signal transduction via the redox state of the cell was discussed above. Sulfur containing metabolites as part of the defence

reaction can be found in preformed (phytoanticipins) as well as in induced substances (phytoalexins). Among them are compounds like glucosinolates and the indole camalexin, but also some sulfur-containing amides like sinharine and cyclooctasulfur (VanEtten *et al.* 1994; Osbourn 1996, Mansfield 2000). Camalexin, the only known phytoalexin of *Arabidopsis*, was shown to be important for resistance toward the necrotrophic fungi *Alternaria brassicicola*, *Botrytis cinerea*, and *Leptosphaeria maculans* (Thomma *et al.* 1999; Ferrari *et al.* 2003; Bohman *et al.* 2004).

The role of sulfur compounds has to be extended to plant–herbivore interactions. For instance glucosinolates can serve as a defence against some generalist herbivores and pathogens. But there are also interactions in which an insect takes advantage of defence strategies originally evolved to ensure survival of the plant. For example, glucosinolates can serve as feeding and oviposition stimulants to specialist herbivores (Reichelt *et al.* 2002). Some insects developed coevolutionary "plant"-like enzymes, such as an aphid which contains a myrosinase, presumably to access the nitrogen- and sulfur-containing aglucone part of the glucosinolates (MacGibbon and Beuzenberg 1978 cited in Pontoppidan *et al.* 2001).

## SULFUR-RICH PROTEINS AND THEIR ROLE IN PLANT DEFENCE

Thionins, defensins, and several other classes of peptides together form the group of sulfur-rich defence proteins. Their role in pathogen defence is generally accepted, but far from clear in terms of precise functions in the plant and mechanisms of toxicity against microbial pathogens. The formation of several thionins and defensins has been shown to be induced by environmental factors, particularly in response to pathogen attack. While the role of sulfur-rich proteins for survival of plants in natural environments has hardly been assessed, attempts are underway to improve the resistance of crops by transfer of thionin or defensin genes (Kruse *et al.* 2005a, b).

The common features of sulfur-rich defence proteins are several disulfide bridges (2–6), their relatively small size of 4–11 kDa, and antimicrobial activity *in vitro.* Mature thionins and defensins are 45–54 amino acids in length, contain 3–4 disulfide bridges and are processed from larger preproteins. The group includes thionins, defensins, lipid-transfer proteins, and snakins according to their primary amino acid sequences (Garcia-Olmedo *et al.* 1998). All show a mostly polar amino acid composition and a rather compact tertiary structure. While thionins are found

ubiquitously throughout the plant kingdom, plant defensins had first been termed γ-thionins. Only later it was realised that these proteins are structurally related to mammalian defensins. In fact, defensins are widespread in phyla including insects and molluscs (Thomma *et al.* 2002).

Sulfur-rich proteins may have more than one precise physiological function *in vivo* in addition to defence (Florack and Stiekema 1994). The suggestion of a role of defensins in zinc hyperacculumation and tolerance in *A. halleri* may lead to the discovery of new physiological tasks of these proteins (Mirouze *et al.* 2006). However, their most important role appears to be in the battle against pathogens as shown by genetic evidence (Kruse *et al.* 2005a, b). Originally they were discovered as protein components of seeds: thionins were found in barley endosperm and defensins in the seed coat of radish (Florack and Stiekema 1994; Thomma *et al.* 2002). These defence compounds were found in the cell wall, in many cases preferentially within the surface cell layers of the plant organ. Indeed, accumulation of inducible leaf cell wall thionins has been observed around the infection sites in the case of the barley and powdery mildew interaction (Ebrahim-Nesbat *et al.* 1989; Apel *et al.* 1990). The sequence of all of the *Arabidopsis* thionin proteins and most of the thionins from other plant species contain amino-terminal domains with signatures for transport via the endoplasmic reticulum to the apoplast. Occasionally vacuolar localization, for example in some barley thionins, has been observed (Reimann-Philipp *et al.* 1989). Furthermore, many of the defensin genes carry a predicted signal peptide for secretion into the cell wall. Thus, most but not all of these proteins are localized to the primary infection sites, a prerequisite for the efficacy of phytoanticipins. In addition, some of these sulfur-rich proteins function as phytoalexins. Pathogen infection induces the defensin gene *PDF1.2* in *Arabidopsis* in several developmental stages via the jasmonic acid (JA) and ethylene (ET) signaling pathways (Thomma and Broekaert 1998; Da Silva Conceicao and Broekaert 1999), while the thionin gene *THI2.1* is inducible by pathogens, wounding and chemicals via the JA pathway (Epple *et al.* 1995; Bohlmann *et al.* 1998). Their importance for pathogen defence is best defined by analyses of genetic mutants. Mutants with defects in these signal transduction pathways are susceptible to *B. cinerea* due to the lack of expression of inducible defensins (Thomma *et al.* 1998). Constitutive overexpression of THI2.1 leads to enhanced resistance of *Arabidopsis* to *Fusarium oxysporum* infection (Epple *et al.* 1997). The inducibility of thionins and defensins may help to save resources in the absence of pathogens. Since the abundance of sulfur-rich protein formation upon infection are not known, it is not yet possible

to evaluate the importance of optimized sulfur status for the defence potential of an attacked plant.

Thionins, thionin-like proteins and defensins have been intensively investigated with respect to their toxicity. They show a wide range of antifungal activity, some members of the groups even being active against Gram-positive bacteria, yeasts, insects, or nematodes, but not against mammals. The exact mechanism of toxicity of sulfur-rich proteins has long been debated (Florack and Stiekema 1994; Garcia-Olmedo *et al.* 1998). The most detailed proposed mechanism is based on *in vitro* assays with artificial lipid bilayer membranes and mammalian cell lines (Hughes *et al.* 2000). Electrophysiological measurements using a β-purothionin from wheat flour demonstrated the formation of cation-selective ion channels upon interaction of purothionin with plasmalemma components. This effect may cause the dissipation of ion concentration gradients that are essential for cellular function. However, this model was not tested with authentic pathogenic fungi.

Experiments with the model fungi *Neurospora crassa* and *Saccharomyces cerevisiae* show the permeabilization of membranes by defensins at low concentrations. Defensins were applied and their effect monitored using uptake of a fluorescent dye into fungal cells (Thevissen *et al.* 1999). A direct peptide–phospholipid interaction that can be suppressed by cations in the medium was concluded. It is possible that the conformation of the binding site is changed by ions and so successful permeabilization leads to the inhibition of fungal growth.

Although the mechanism of toxicity of defensins seems to be different from that of thionins, both components appear to confer a broad range of resistance. Such a broad resistance is of general interest for breeding for plant resistance. Expression of efficient thionins or defensins either by using transgenic overexpression or by marker-assisted introgression into elite lines could potentially improve the resistance of economically relevant crops. In contrast, approaches based on *R*-genes in gene-for-gene interactions (Flor 1956) run the risk of failure due to their mono- or oligogenic conferred high host–pathogen specificity. Broad resistance by thionins and defensins would imitate the success of insecticidal proteins from *Bacillus thuringiensis* showing toxicity against pathogenic fungi but being harmless against animal and human cells. As thionins and defensins are naturally occurring plant proteins there is a good chance that this approach has no impact on environment and will have a higher acceptance in society.

A number of experiments using transgenic plants have shown the enhancement of resistance to pathogens by overexpression of sulfur-rich

proteins. However despite strong antimicrobial activities of such proteins in bioassays, several experiments have also failed to confer resistance (Florack and Stiekema 1994; Broekaert *et al.* 1995; De Bolle *et al.* 1996; Epple *et al.* 1997). Furthermore, plants such as barley contain more than 80 known thionin genes (Bohlmann and Apel 1991). An improvement of pathogen tolerance by the presence of additional sulfur-rich proteins may be difficult to achieve. Nevertheless, the earliest transgenic expression of a sulfur-rich protein refers to an α-thionin from barley (Carmona *et al.* 1993). The gene was transformed into tobacco under control of a constitutive promoter. Enhanced resistance toward two strains of *Pseudomonas syringae* was correlated with the amount of α-thionin protein detectable in the different transgenic tobacco lines.

The suitability of defensin expression was shown by transformation of rice by *Agrobacterium tumefaciens* with the Wasabi defensin that originates from Japanese Radish (*Wasabia japonica*; Kanzaki *et al.* 2002). Wasabi is one of the spices traditionally served with Sushi, but its pungent taste is derived from glucosinolate breakdown products. The Wasabi defensin is especially toxic against rice blast disease, a worldwide fungal pathogen which causes severe damage and reduced yield. The best transgenic rice lines reached resistance levels comparable to rice cultivars carrying the true blast resistance gene according to leaf lesion tests. The resistance was stable over several generations, giving rise to the hope of durable and wide-spectrum resistance against various rice blast races in the field.

Further options to improve the efficacy of sulfur-rich proteins and to assure environmental safety refer to protein engineering. Active proteins with broad specificity against different microbes but reduced toxicity against mammalian cells could be selected in bioassay guided mutagenesis approaches. An alternative approach to the biotechnical transformation of single genes could be the selection of resistant plant genotypes using sulfur-rich proteins as a target, supported by marker-assisted breeding. However, if such approaches were successful, biosafety assessments would have to be comprehensive at least for outcrossing crops such as oilseed rape to avoid unwanted transfer of this advantageous trait to populations of wild relatives.

## GLUCOSINOLATES AND ALLIINS: PLANT-BASED PEST CONTROL

The genus *Allium* and members of several other plant taxa have evolved a system that potentially combines storage of reduced C, N, and S with plant defence. An in-depth review with emphasis on pathways of biosynthesis and degradation has recently been provided by Jones *et al.* (2004). A large variety of cysteine and GSH derived S-alk(en)yl sulfoxide precursors accumulate in all *Allium* organs, but particularly the overwintering bulbs of many *Allium* species which may carry up to 1-5% of dry weight of these compounds (Lancaster and Kelly 1983). In their function in defence against pests and pathogens they thus belong to the category of phytoanticipins. They may represent an example for the principle of evolutionary development of nonprotein amino acids for purposes of chemical defence in many organisms, such as synthesis of L-canavanine from arginine as toxic agent against insects (Rosenthal 1992).

The cysteine derivatives of this group are present in cultivated *Allium* species and thus familiar for culinary reasons. *S*-allylsulfoxide (alliin) predominates in garlic, *S*-propylcysteine sulfoxide (isoalliin) and trans-*S*-1-propenyl cysteine sulfoxide (propiin) are the abundant components of onion, while *S*-methylcysteine sulfoxide (methiin) is found in *Allium* and *Brassica*ea species. In addition, γ-glutamyl peptides derived from GSH are abundant in the *Allium* species. They are conjugated with *S*-alk(en)yl sulfoxides and may form intermediates from a biosynthetic pathway or form additional variants of precursors (Lancaster and Shaw 1991). Substantial amounts of these precursors apparently accumulate in the cytosolic compartment. To initiate the formation of active compounds with respect to antimicrobial activity, odor, pungency, and pharmacological activity they have to be cleaved by the enzyme alliinase. Since alliinase is a vacuolar enzyme, destruction of the cells or transport of either precursors or the enzyme into the appropriate compartment is required for biological activity. Unless an unknown mechanism operates, this would also be mandatory for controlled degradation if they function as storage compounds. Alliinase is a glycosylated protein, cleaves a C–S bond and releases pyruvate, ammonia, and a thiosulfinate (Nock and Mazelis 1987). This is further processed by spontaneous or enzyme catalyzed reactions to yield the active end products. The lacrimatory factor of onion, propanthial-S-oxide, is such an end product that requires at least one additional enzymatic step for formation (Imai *et al.* 2002). Apart from culinary aspects, the mixture of cysteine sulfoxide precursors and products are known for their health promoting activities for humans. This includes

positive effects of cholesterol contents in blood plasma and inhibitory effects on thrombocyte (platelet) aggregation. However, tissue disruption and processing have deterrent effects on feeding organisms due to pungency and antimicrobial effects, which may prevent secondary infection on, wound sites by phytopathogenic bacteria. While the toxicity of crude extracts has been verified *in vitro* (Ankri and Mirelman 1999), little is known about *in vivo* action of this group of natural plant products. Thus, the functions of cysteine sulfoxides in plants with respect to pathogen defence are still not fully elucidated. *Allium* species are also known to release a number of volatile sulfur compounds, the simplest being methanethiol. These are common among the genus and often known to function as insect attractants (Städler 2000).

In contrast, the role of glucosinolates as part of the defence system of the *Brassica*ceae and some other plant families is more thoroughly investigated. In-depth reviews have addressed their biosynthesis and biological functions in deterrence of generalist herbivores and pathogens as well as attraction of specialized herbivores (Bones and Rossiter 1996; Wallsgrove *et al.* 1999; Wittstock and Halkier 2002; Grubb and Abel 2006; Halkier and Gershenzon 2006). Glucosinolates are amino acid-derived secondary metabolites consisting of a thioglucose moiety, a sulfonated aldoxime, and a side chain derived from either aliphatic or aromatic amino acids. When plant tissues are disrupted the glucosinolates are hydrolyzed by the highly active plant enzyme myrosinase, a thioglucosidase. *In planta* myrosinase is stored in a different compartment. The cleavage of the glucose thioester linkage produces an unstable intermediate that rearranges into biologically active thiocyanates, isothiocyanates, nitriles and oxalidine-2-thiones, depending on reaction conditions and presence of additional proteins (Lambrix *et al.* 2001). These products are chemically very reactive and may interfere with proteins and free amino acids. They are generally caustic, carcinogenic, and potentially toxic, hence their antimicrobial activities in plant defence. Although the glucosinolates also occur in a number of other plant families, the economic importance of oilseed rape, mustard, and the cabbage subspecies spurred interest in the biochemistry and molecular biology of glucosinolates biosynthesis and degradation. This led to the discovery of an intricate system of storage, transport, breakdown and toxicity, commonly termed the "mustard oil bomb", a topic of great interest for chemical ecology.

Moreover, some glucosinolates (i.e. their breakdown products) benefit human health. Sulforaphane (4-methylsulfinyl-butylisothiocyanate), a breakdown product of glucoraphanine, has been ascribed potent cancer-preventive potential according to epidemiological studies (but not for all

cancer types). A prominent example is the reduced risk of prostate cancer when broccoli forms a significant but small part of long-term diets (Giovannucci *et al.* 2003). Glucoraphanine is the major glucosinolate in broccoli sprouts (about 47%), but is also abundant in other cabbage vegetables. It induces the so-called phase 2 enzymes in the human cells such as GSH *S*-transferase and quinone reductase that initiate detoxification and metabolism of procarcinogenic compounds. Crop management strategies have been developed to improve the quantity and composition of phytochemicals in *Brassica* species (Schreiner 2005). In addition wild relatives with much higher glucosinolates are included in breeding strategies to elevate glucosinolates contents in cultivated broccoli (currently 50–100 mg in 100 g fresh broccoli sprouts).

Apart from culinary aspects, oil production by rapeseed (*Brassica napus* and *B. campestris* cultivars) is of major economic interest as reflected by millions of hectares of oil seed and canola field world wide. These huge agroecosystems are even increasing to the application of genetic engineering to increase yield. The genetically modified (GM) plants mainly carry insect resistance conferred by the *B. thuringiensis* toxin and herbicide resistance traits. Further demand is created by the suitability of oilseed rape to produce biodiesel as a renewable resource. The huge amounts of processed seeds leave the seed meal after pressing, containing valuable protein and carbohydrates that can be used as feed. However, the toxicity of glucosinolate breakdown products prevented or reduced the applicability of seed meal for a long time. A well-known problematic effect on animals is the inhibitory activity of a degradation product of 2-hydroxy-3-butenyl glucosinolate, a major glucosinolate in oilseed rape. The product, 5-vinyl-oxazolidine, causes the goitre disease in cattle fed with too much seed meal. Selective breeding of oilseed rape led to the so-called double-low varieties. These combine low erucic acid content (another toxic plant compound) with low seed glucosinolates. This provides a more useful seed meal after oil extraction as an animal feed. Some of the early double-low varieties were obviously more susceptible to pests and pathogens. Presumably they contained lowered profiles of glucosinolates not only in seeds but also leaves. The currently used double-low varieties contain only lowered seed glucosinolates levels and are equally resistant or susceptible than the single low lines they had replaced (Wallsgrove *et al.* 1999).

Glucosinolates and myrosinases have been detected in all major plant organs. While glucosinolates have been detected in vacuoles early on (Matile 1980), the cellular organization to separate them from myrosinase is still not entirely clear and differ between plant organs and species. The

existence of myrosin cells that are scattered in a given tissue but appear to be glucosinolate free seems clear. Sulfur-rich cells presumably containing glucosinolates have been identified between the phloem and endodermis of the *Arabidopsis* flower stalk that are neighbored by myrosinase cells (Wittstock and Halkier 2002). However, in order to provide an effective defence system, the presence of glucosinolates in most cell types of the leaf and other organs should be expected and is indeed supported by high concentrations found in certain tissues (Wallsgrove *et al.* 1999; Brown *et al.* 2003). Biosynthesis and degradation are always under developmental control. They are additionally affected by nitrogen and sulfur nutrition on one hand (Blake-Kalff *et al.* 1998) and by pathogen infection as well as wound and defence signals such as JA and SA on the other hand (Figure 1) (Wallsgrove *et al.* 1999). When sulfate is withdrawn from growing oilseed rape, free sulfate but not glucosinolates is the major source of sulfur in leaves (Blake-Kalff *et al.* 1998). However, long-term sulfur limitation results in strongly reduced glucosinolates contents in *Arabidopsis* leaves (C. Kruse, M. Reichelt, R. Hell, unpublished). In general, the measured concentrations are high in young tissues and decreasing in mature tissues. The pattern of differences found in *Arabidopsis*, meanwhile the best investigated plant glucosinolate metabolism, fits with their function in defence against herbivores and pathogens. The reproductive organs including seeds, flowers, and fruits show the highest contents and younger leaves have more glucosinolates than older leaves. Interestingly, this seems not to be a result of decrease during growth of older leaves. Rosette leaves appear to have fixed concentrations during expansion, but this fixed level is much higher in leaves that are formed late in development as compared to the contents in early initiated leaves (Brown *et al.* 2003). Glucosinolate contents decrease strongly in senescent leaves, presumably by a combination of transport via the phloem to developing inflorescences and catabolism. Glucosinolates are potentially excellent energy sources containing glucose and reduced nitrogen and sulfur. This additional function is supported by decreasing glucosinolate contents in germinating seeds (Brown *et al.* 2003), but appears not to apply to sulfur deficiency in vegetative stages as outlined above. Very little is known about glucosinolates catabolism apart from the myrosinase system.

During coevolution of plants and their herbivores some alterations in plant ecotypes and generalist and specialist feeders developed. A definitive link between plant defence genotypes and insect herbivory phenotypes was shown by the feeding behavior of the larvae of the generalist lepidopteran herbivore *Trichoplusia ni*. Using polymorphisms in the *Arabidopsis* ecotypes Columbia and Landsberg *erecta*, a quantitative trait locus for a

distinct pattern of glucosinolates breakdown products was mapped (Lambrix *et al.* 2001). Columbia produces mainly the toxic isothiocyanates, whereas Landsberg produces mainly the less toxic nitriles upon tissue disruption. Consequently, *T. ni* feeding rates are higher on the Landsberg ecotype. The underlying gene was identified as epithiospecifier protein (ESP). ESP had been biochemically known from studies with *B. napus*, but was now recognized as cofactor of myrosinase. ESP alone is not functional in the absence of myrosinase, but myrosinase activity is not absolutely dependent on ESP. *In vitro* ESP from *Arabidopsis* directs the formation of myrosinase products strongly toward formation of the less toxic simple nitriles instead of isothiocyanates (Lambrix *et al.* 2001). However, if *T. ni* prefers nitriles over isothiocyanates, it is unclear why a plant would develop a protein that favors generalist feeding herbivores. One possible answer could be that nitriles might be more effective to other pests or might attract natural enemies of the herbivorous insects.

The cabbage white butterfly (*Pieris rapae*) is biochemically adapted to the binary glucosinolate–myrosinase system of the crucifers. Its caterpillar possesses a midgut protein that prevents the formation of toxic isothiocyanates when fed with *Arabidopsis*, *Sinapis alba*, or *B. oleracea* leaves (Wittstock *et al.* 2004). This nitrile specifier protein redirects glucosinolates breakdown by myrosinase from isothiocyanates to less toxic nitriles that are excreted with the faeces. The larval protein itself has no hydrolyzing activity on glucosinolates in the absence of myrosinase and appears to be the key biochemical component of adaptation of *P. rapae* to the mustard oil bomb.

A different strategy has been developed by the diamondback moth (*Plutella xylostella*), another *Brassica* feeding specialist and major worldwide pest on many cabbage species, rapeseed, and mustard that developed frequent resistances to chemical pest management strategies. Faeces of this larvae contain only desulfoglucosinolates after feeding on leaves of *Arabidopsis* and other crucifers. The larvae circumvent the chemical defence system by secretion of a sulfatase into the gut. The enzyme recognizes a broad range of glucosinolates and by cleaving the sulfate moiety masks the precursors from myrosinase and thus prevents production of the toxic isothiocyanates (Ratzka *et al.* 2002).

In fact, all the herbivorous insects that are specialized on crucifers respond not only with increased feeding, but with increased oviposition (egg laying) to the volatile isothiocyanates. Nonadapted herbivores avoid feeding and egg laying on *Brassica*ea because of the glucosinolates. In specialized herbivores however, chemoreceptor neurons have been identified that respond to volatile or contact glucosinolate metabolites

(Städler 2000). Some specialized herbivores are not only attracted by the preformed defence compounds but also by specific sulfur-containing phytoalexins that are induced upon damage or infection. In addition to the brassinins to which camalexin belongs, the cabbage root fly (*Delia radicum*) is stimulated to lay more eggs by a "cabbage identification factor", another sulfur-containing indole derivative (Städler 2000). These mechanisms of chemical coevolution are apparently advantageous to the insects, most likely by reduced loss of eggs to other enemies and less feeding competitors for the larvae.

## NEW SULFUR COMPOUNDS AS FACTORS OF PLANT DEFENCE: ELEMENTAL SULFUR AND SULFIDE

Elemental sulfur was probably the first fungicide applied in agriculture. It is still in use for foliar applications, not the least because it combines efficacy with minimal ecological impact. Elemental sulfur can be used as a preventive fungicide against powdery mildew, rose black spot, rusts, and other diseases. $S^0$ is also effective against the plant pathogens *Blumeria*, *Cladosporium*, *Colletotrichum*, *Verticillium*, and *Fusarium* (Williams and Cooper 2004). It inhibits spore germination and therefore must be applied before development of disease symptoms because established populations of plant pathogens mostly show little response to any chemical or organic agent. Formulations of elemental sulfur such as fine powder or sprayed with colloidal suspensions are commercially distributed as commodities worldwide and are as such of importance for many agroecosystems. Fungicides of high specificity often lose much of their effectiveness within a few years due to interaction with a single fungal enzyme. Thus, a single mutation may confer resistance on the fungus. When fungicidal activity is due to interaction with many proteins, as with a fungicide of low specificity, resistance may last longer. Elemental sulfur containing fungicides have effectively retained their effectiveness through over 100 years of use (Carlile *et al.* 2001).

Interestingly, elemental sulfur has no toxic effect on bacteria, but apparently has unspecific effects on many cell constituents and processes of fungi. Mammals or oomycetes such as *Peronospora* species are unaffected, but certain groups of insects (mites) are sensitive. The precise mechanisms of action on susceptible organisms are still unclear and have been subject of many speculations (Williams and Cooper 2004). It seems most accepted that $S^0$ is permeable to membranes and thus taken up into the cytoplasm. In mitochondria it may interfere with the respiratory chain

accepting electrons from cytochrome *b* and interfering with electron transport and the supply of energy available to the fungal cell. In this way $S^0$ may become reduced to the very toxic hydrogen sulfide. A remarkable coincidence has been noted by Cooper and Williams (2004) who observed that $S^0$ resistant organisms like mammals and oomycetes contain cholesterol as major plasmalemma sterol whereas $S^0$ sensitive organisms like fungi and mites have ergosterol as dominant membrane sterol. Substances that differentially target these sterols may provide the clue to understanding of toxicity of elemental sulfur.

Only 10 years ago the existence of elemental sulfur was demonstrated in cocoa plants and linked to pathogen defence (Cooper *et al.* 1996). The observation was confirmed by the finding of sulfur in the $S^0$ oxidative stage in diverse plant families (Williams and Cooper 2003). It was therefore concluded that plants actually possess "man's oldest fungicide" (Williams and Cooper 2004). In some species $S^0$ is locally produced in defence to vascular pathogens, in the *Brassica* family it is preformed and reaches concentrations of 1–6 μg $g^{-1}$ fresh weight in leaves (*A. thaliana*). Its accumulation may also be induced in other plants and then is found in the vasculature, probably to prevent the spread of fungal infections via the plant's transport routes. When tomato genotypes with increased and lowered susceptibility against *Verticillium dahliae* were compared, local concentrations of 10 μg $g^{-1}$ fresh weight in excised xylem were sufficiently fungitoxic (Williams *et al.* 2002). In this context it is puzzling how the water-insoluble droplets of $S^0$ interfere with the fungal invader. Interestingly, transient increases in concentrations of sulfate, cysteine, and GSH were observed in vascular tissues of *Verticilllium* resistant tomatoes (Williams *et al.* 2002). However, the biosynthetic pathway leading to $S^0$, possibly either from the oxidation states of sulfate or sulfide, is still a mystery. It seems clear that a compartmental separation from reductive sulfate assimilation in the plastids and sulfite oxidation in the peroxisomes (Hänsch *et al.* 2006) is required. The durable success of elemental sulfur as a fungicide in plant production fits with the wide evolutionary distribution in plant taxa. The elucidation of the pathway leading to production of $S^0$ in plants and the mechanism of action in the vasculature are therefore of great interest for basic and applied research.

Most recently the volatile sulfur compound $H_2S$ has been implicated with plant pathogen defence. Local release of $H_2S$ might reduce the growth of fungal hyphae and prevent germination of spores. Sulfide at certain concentrations is believed to be fungitoxic, whereas plants are able to tolerate substantial concentrations. In fact, it has been demonstrated that $H_2S$ can fully replace sulfate as sulfur source for prolonged growth of

*B. oleracea* (Westerman *et al.* 2001, Chapter 5). $H_2S$ is assumed to be membrane permeable and to be incorporated into metabolism by the activity of *O*-acetylserine(thiol) lyase (OAS-TL) which forms cysteine from *O*-acetylserine (OAS) and $H_2S$. The same enzyme can catalyze a partial backward reaction, cleaving cysteine into $H_2S$ and pyruvate. OAS-TL thus can produce $H_2S$, but due to reduced substrate affinities this backward reaction is unlikely to operate under *in vivo* conditions (Wirtz *et al.* 2004). On the other hand, plants are known to continuously release volatile sulfur compounds to the atmosphere such as $H_2S$, carbonyl sulfide, carbon disulfide, dimethyl sulfide, or methylmercaptan (Schröder 1993, Chapter 5). A survey of $H_2S$ release has been provided by Bloem *et al.* (2005). $H_2S$ is probably produced by cysteine desulfydrases of which D- and L-form specific enzymes exist in plants. The report about reduced hydrogen sulfide releasing capacity in transgenic potato plants with downregulation of either cytosolic or plastidic OAS-TL is probably based on the saturating conditions used to assay $H_2S$ production from cysteine, thus including the backward activity of OAS-TL (Riemenschneider *et al.* 2005).

It has been speculated that fungal attack could be encountered by hydrogen sulfide production. Initial evidence for this hypothesis comes from field experiments with *B. napus* and infection by leaf blight, *Pyrenopeziza brassicae* (Bloem *et al.* 2004). The degree of infection correlated with the levels of cysteine and GSH and increased together with total activity of L-cysteine desulfydrase. The authors therefore concluded that *B. napus* plants were able to react to a fungal infection with a greater potential to release $H_2S$. Enhanced fertilization on the other hand increased the levels of sulfur compounds but decreased L-cysteine desulfydrase activity (Bloem *et al.* 2004). A major problem for the proper assessment of sulfide production during fungal infection is the difficult determination of $H_2S$ *in situ*. At present there is neither a clear correlation nor direct evidence for a functional relationship between sulfur nutrition, $H_2S$ release, and resistance to fungi.

## INTEGRATION OF SULFUR METABOLISM AND SULFUR-ENHANCED DEFENCE

From an ecological perspective sulfur covers two essential biological functions: it is an essential macroelement (usually as sulfate) for plant life with numerous cellular functions (Leustek *et al.* 2000), and many sulfur-containing substances contribute to the management of environmental

influences via redox reactions, detoxification of heavy metals and xenobiotics, metabolism of secondary products, etc. (Saito 2000). GSH plays a crucial role in this context as major component of redox homeostasis (Meyer and Hell 2005) and stress tolerance. While functions in abiotic stress situations have been recognized early on, its immediate connection of GSH to biotic stress defence is only beginning to emerge (Rennenberg and Brunold 1994; Mou *et al.* 2003; Senda and Ogawa 2004). These observations together with results from field experiments drew attention additionally to the sulfur-containing defence compounds discussed previously in this chapter. The simplified conclusion from these considerations is that optimized management of sulfur nutrition may improve the tolerance of plants against pathogens in natural and especially in agroecosystems (reviewed in detail by Bloem *et al.* 2004; Hell *et al.* 2005; Rausch and Wachter 2005).

Agronomic evidence for the importance of sulfur supply came from field observations. Intensive farming had greatly increased the demand for sulfur (and other nutrients) in crop production in the last decades. Beginning with the 1980s, filtering of industrial fumes in Europe decreased the atmospheric deposition of sulfur dioxide that had been released by the burning of S-containing fossil fuels. Consequently, in areas where the soil contains less available sulfur, the successful reduction of atmospheric sulfur dioxide pollution caused enhanced problems of disease susceptibility in sulfur-demanding crop plants in northern Europe. This limited sulfur supply led to a decrease in crop yield and a lower food quality (Dämmgen *et al.* 1998). Systematic multifactorial field trials with different sulfur fertilization, fungicide application, and oilseed rape genotypes suggested a correlation between sulfur supply to the plant and tolerance to fungal infection (Schnug *et al.* 1995). These observations were intriguing because of the multiple infection situation (although apparently dominated by the necrotrophic fungus *P. brassicae*, leaf blight) and the known requirement of *B. napus* for sulfur due to its high glucosinolates contents. This susceptibility was therefore attributed to suboptimal formation of sulfur-containing defence compounds under fungal pathogen pressure and led to the concept of sulfur-induced resistance (SIR) (Schnug *et al.* 1995). Further support for this concept came from experiments with phytopathogenic bacteria. A significant effect of soil-applied sulfur on the reduction of infection of grapes with *Uncinula necator*, and potato tubers with *Rhizoctonia solani* (Bourbos 2000 cited in Schnug *et al.* 1995; Bloem *et al.* 2004; Klikocka 2005) also suggested that sulfur metabolites are involved in disease resistance.

The SIR concept raises several questions: (1) Is a genetic and metabolic activation of sulfur metabolism required to fight off pathogens more successfully? (2) Is sulfate availability the limiting factor? (3) Is an optimal sulfate supply the basis to stimulate genetic and biochemical processes in primary and secondary metabolism? (4) Will the SIR concept hold for all plant–pathogen interactions or is it only applicable to certain plant species/pathogen combinations and environmental situations? The SIR concept is certainly of quantitative and multifactorial nature and not based on resistance in a pathobiological sense as defined in the gene-for-gene model (Flor 1956). This latter model of host–parasite interactions is, however, oversimplified, because it disregards examples of polygenic or recessive resistance. Furthermore the model did not consider the influence of environment and neglected the contribution that genetic background can have on gene expression (Holub 2006).

To address the open question and to shed light on the molecular mechanisms underlying the field-derived SIR concept, approaches under defined laboratory conditions were initiated. As a result of these investigations the concept of interaction of sulfur metabolism and plant defence under nonfield conditions was modified to sulfur-enhanced defence (SED; Hell *et al.* 2005; Kruse *et al.* 2005; Rausch and Wachter 2005). Along these lines Dubuis *et al*. (2005) compared the susceptibility of oilseed rape grown under inadequate sulfur supply with fertilized plants to the blackleg fungus *L*. *maculans*, to the generalist necrotroph *B*. *cinerea* and to the oomycete *Phytophthora brassicae*. Sulfur-deficient plants were more susceptible and metabolite extracts from these plants carried no or reduced antifungal activities *in vitro*. This effect was attributed to the strong decrease in phytoanticipins of the glucosinolate class due to sulfur limitation.

Jost *et al*. (2003) analyzed the influence of sulfate supply on the defence response of *A*. *thaliana* to an *A*. *brassicicola* infection in an axenic culture system which allowed the control of nutrient supply as well as the precise inoculation with one defined pathogen. This allowed the comparison of sufficiently sulfur-supplied plants without visible phenotype with optimally sulfur-supplied plants of the same relative growth rate. Seven days after infection the free sulfate pools in sufficiently supplied plants were depleted whereas optimally supplied plants still contained high sulfate levels. At the same time the expression of the *Thi2.1* gene encoding a sulfur-rich thionin was induced more strongly in the optimally supplied *Arabidopsis* plants, suggesting an effect of nutritional status of gene expression (Jost *et al.* 2003). Using the same pathosystem, Kruse *et al*. (2005) showed an increase of cysteine and GSH contents during the first

week of infection. Furthermore, infection of *Arabidopsis* with virulent and avirulent *P. syringae* strains also resulted in elevated cysteine contents (Kruse *et al.* 2005a, b). Increases in thiol contents after fungal infection had also been reported for *B. napus* and tomato after fungal infections, although under quite different conditions (Bloem *et al.* 2004; Cooper and Williams 2004; Kruse *et al.* 2005; Salac 2005). The axenic *Arabidopsis* pathosystems furthermore allows microscopical, enzymatical, and metabolical analyses during the infection process. Synthesis of such defence metabolites takes place in reply to the signal transduction of a perceived pathogen attack by signaling molecules like SA and JA as well as ET (Glazebrook *et al.* 2003). Therefore, the efficient and rapid synthesis of sulfur-related defence compounds upon infection requires cross talk between the networks of pathogen response and sulfur metabolism (Figure 1, Hell *et al.* 2005).

One step in unraveling the connecting factors is provided by the work of Jost *et al*. (2005) who showed the comprehensive induction of genes of the primary and secondary sulfur assimilation pathway in *Arabidopsis* after methyl jasmonate treatment. The expression of genes encoding adenosine-5′-phosphosulfate-reductases (APRs), serine acetyltransferases (SATs) and thionins began to increase already after 3–6 h after treatment using close to *in vivo* concentrations of the inducer homolog methyl jasmonate (Jost *et al.* 2005). These finding were corroborated by longer incubations with extremely high methyl jasmonate concentrations that also resulted in increased levels of cysteine and GSH in these plants (Sasaki-Sekimoto *et al.* 2005). Taken together, these finding strongly support the concept of SED. Not only is sulfur metabolism important for defence against several pathogens in phytoanticipins and phytoalexins, but also the activation of the metabolic pathway is required to achieve a maximum of tolerance against pathogen attack. The role of sulfur nutrition seems less evident in these experiments. One reason is the definition of sufficient and optimal sulfur supply: when is enough really enough? The comparison of phenol-typically sulfur-deficient plants with well-supplied plants allows a clearer distinction, but implies an obvious result at least with respect to preformed defence compounds and possibly reduced fitness of the host plant.

The dissection of SED is further complicated by the different lifestyle of pathogens and the different signal transduction pathways used. It has been suggested that the SA-dependent pathway should ensure effective defence against biotrophic pathogens and a different set of defence responses activated by JA and ET signaling should provide protection to the plant toward necrotrophic pathogens (Glazebrook 2005). While this model seems generally correct, there are of course exceptions and

additional complexities, not the least due to different definitions and classification of certain pathogens (Glazebrook 2005; Howlett 2006).

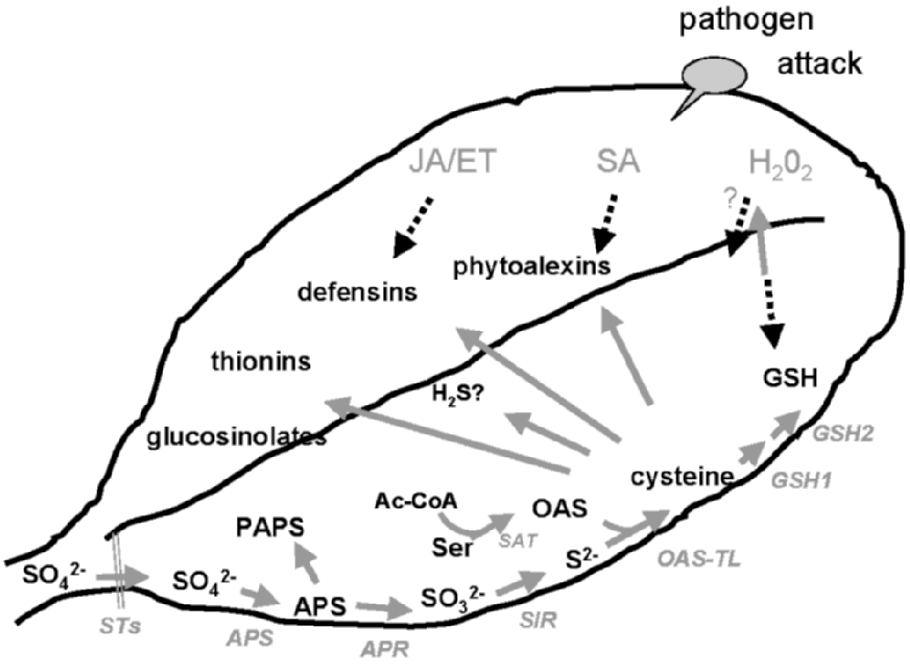


*Figure 1*. The signaling pathway of pathogen response leads to induction of defence metabolites. Many of these are directly or indirectly derived from primary sulfur metabolism. Efficient and rapid synthesis of sulfur-related defence compounds upon infection requires cross talk between the networks of pathogen response and sulfur metabolism (Hell 2005). Abbreviations: APS, adenosine 5′-phosphosulfate; PAPS, phosphoadenosine 5′-phosphosulfate; Ac-CoA, acetyl-coenzyme A; Ser, serine; ST, sulfate transporter; ATPS, ATP sulfurylase; SIR, sulfite reductase; GSH2, glutathione synthetase.

Broadly accepted definitions are as follows: biotrophs rely on living plant tissue, whereas necrotrophs kill plant cells to derive nutrition. Hemibiotrophs usually have an initial biotrophic phase, then become necrotrophic. The typical answer of plants toward many pathogens, the programmed cell death, arrests pathogen growth, in particular of that of biotrophs. In contrast, necrotrophic pathogens benefit from host cell death, so they are not confined by cell death and SA-dependent defences, but are

able to avoid host defence responses such as the HR to derive nutrition from the dead host tissue (Howlett 2006).

These differences have to be considered carefully when the impact of a primary pathway in such a complex interaction like the dependence of relationship between pathogen and host on the nutritional conditions is examined. It is known that biotrophs stimulate primary metabolism whereby necrotrophs enhance plant cell death. Therefore, parameters for defence, like accumulation of ROS and necrosis, have to be combined with phenotypical analysis and quantification of pathogen growth. The pure activation of single gene expression, an enzyme activity or metabolite content, is only one prerequisite to conclude a positive or negative role in disease resistance. Further aspects are kinetics of key parameters and local reaction of infected tissue, combined with quantification of the proliferating pathogen therein. Time and place are probably the decisive elements of a successful defence in the SED concept.

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Chapter 10

# SELENIUM AND ITS RELATIONSHIP WITH SULFUR

Philip J. White, Martin R. Broadley, Helen C. Bowen, and Sarah E. Johnson

## INTRODUCTION

Selenium (Se) is placed in Group VIA of the Periodic Table. Its chemistry is similar to sulfur (S) and Se occurs naturally in one of four oxidation states: –2 (selenide), 0 (elemental Se), +4 (selenite), and +6 (selenate). The Se concentrations in most soils are low (0.01–2.0 mg Se $kg^{-1}$), but concentrations up to 1200 mg Se $kg^{-1}$ occur in soils associated with particular shales, sandstones, limestones, slate, and coal series, such as those formed in Cretaceous, Jurassic, Triassic, Carboniferous, Ordovician, and Permian periods (Dhillon and Dhillon 2003; Fordyce 2005). In some regions of the world, soil Se concentrations are sufficiently high to be toxic to many plants, and these regions support a unique flora (Rosenfield and Beath 1964; Shrift 1969; Dhillon and Dhillon 2003). Seleniferous soils are widespread in the Great Plains of the USA, Canada, South America, Australia, India, China, and Russia. In agricultural soils, S concentrations generally lie between 0.1 and 4 g S $kg^{-1}$, and the S/Se quotient approximates 500–3000 g S $g^{-1}$ Se (Bisbjerg 1972).

Anthropogenic inputs of Se to the environment, like those of S, arise from the combustion of fossil fuels, metal processing, applications of fertilizers, lime and manure, and the disposal of sewage sludge (Fordyce 2005; Broadley *et al.* 2006). In the UK, atmospheric deposition of Se has been estimated to be between 2.2 and 6.5 g Se $ha^{-1}$ $year^{-1}$ and Se concentrations in rainwater range from 0.01 to 1 μg Se $l^{-1}$ (Fordyce 2005). The serendipitous input of Se to agricultural soils from inorganic fertilizers can also be significant (Bisbjerg 1972). For example, ammonium sulfate



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fertilizers contain up to 36 mg Se $kg^{-1}$, phosphate rocks contain up to 55 mg Se $kg^{-1}$, and single superphosphate fertilizers contain up to 25 mg Se $kg^{-1}$ (Bisbjerg 1972). The replacement of single superphosphate fertilizers with triple superphosphate fertilizers, which contain less than 4 mg Se $kg^{-1}$, has reduced the serendipitous fertilizer inputs of Se to agricultural soils in the recent past, but the use of specialist Se-containing fertilizers, such as Selcote and Top Stock, is becoming commonplace on low Se agricultural soils because of the importance of Se for animal health (see later; Dhillon and Dhillon 2003; Broadley *et al.* 2006).

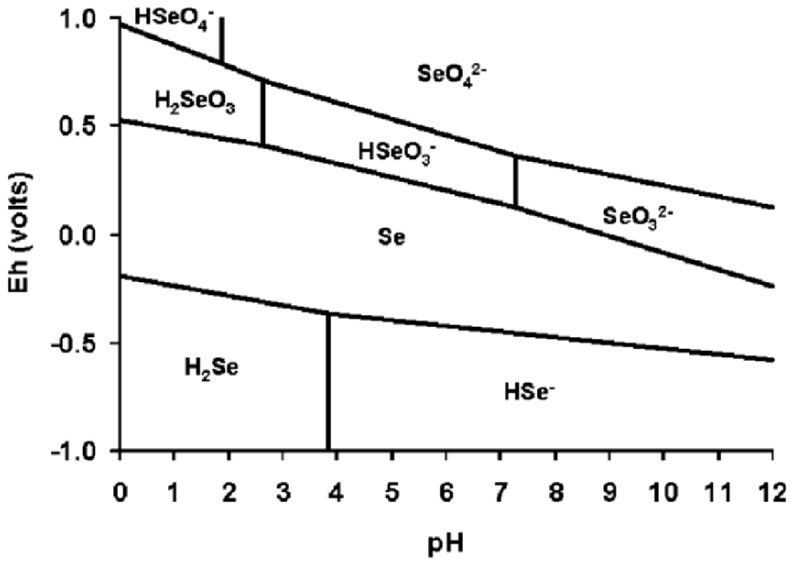


*Figure 1.* The effects of redox potential (Eh) and pH on selenium speciation in an aqueous system containing 1 μM Se. Each line in the diagram represents an equilibrium between the oxidized form written above the line and the reduced form written below it, or the protonated form written on the left and the unprotonated form written on the right of the line. (Adapted from Mikkelsen *et al.* 1989.)

The chemistry of Se in the soil solution is dominated by the anions, selenate ($SeO_4^{2-}$) and selenite ($SeO_3^{2-}$), although elemental Se is also stable over a wide pH range under reducing conditions (Figure 1, Bisbjerg 1972; Mikkelsen *et al.* 1989). In contrast to S, the +6 oxidation state (selenate) is less stable than the +4 oxidation state (selenite). Selenate is highly mobile in the soil solution, but selenite is strongly absorbed by hydrous secondary

iron oxides and, to a lesser extent, by clays and organic matter. Selenate predominates in soil solutions under high-redox conditions (pe + pH > 15), but selenite predominates under milder redox conditions (pe + pH = 7.5 to 15). Selenide species appear under low-redox conditions (pe + pH < 7.5).

## SELENIUM UPTAKE, ASSIMILATION, AND ACCUMULATION BY PLANTS

Plants acquire Se from the soil solution. The uptake of Se by plant roots is influenced by the chemical form and concentration of Se in the soil solution, soil redox conditions, the pH of the rhizosphere, and the presence of competing anions such as sulfate and phosphate (Mikkelsen *et al.* 1989; Blaylock and James 1994; Dhillon and Dhillon 2003; Liu *et al.* 2004; Wu 2004). Plant roots can take up Se as selenate, selenite, or organoselenium compounds. Roots take up selenate faster than selenite at the same concentration (Hurd-Karrer 1935; Rosenfeld and Beath 1964; Ulrich and Schrift 1968; Bisbjerg and Gissel-Nielsen 1969; Gissel-Nielsen 1973; Asher *et al.* 1977; Smith and Watkinson 1984; Mickelsen *et al.* 1987; Bañuelos and Meek 1989; Arvy 1993; de Souza *et al.* 1998; Zayed *et al.* 1998; Hopper and Parker 1999; Pilon-Smits *et al.* 1999a; Shanker and Srivastava 2001; Montes-Bayón *et al.* 2003; Cartes *et al.* 2005; Zhao *et al.* 2005), but aquire organoselenium compounds, such as selenocysteine (SeCys) and selenomethionine (SeMet), most avidly (Rosenfeld and Beath 1964; Montes-Bayón *et al.* 2003). Roots cannot take up colloidal elemental selenium or metal selenides (Hurd-Karrer 1935; Peterson and Butler 1966). Once it has been taken up by roots, Se is converted to selenate and transported to the shoot via the xylem (Asher *et al.* 1977; Smith and Watkinson 1984; Gissel-Nielsen 1987; Arvy 1993; de Souza *et al.* 1998; Wu 1998; Zayed *et al.* 1998; Hopper and Parker 1999). Selenium is then assimilated into organoselenium compounds and redistributed within the plant, from the shoot to the root or from senescing to younger leaves, in a manner analogous to S (Rosenfeld and Beath 1964; Cooper and Clarkson 1989).

Selenate enters root cells through sulfate transporters in the plasma membranes (Terry *et al.* 2000; White *et al.* 2004; Sors *et al.* 2006; Broadley *et al.* 2006, Chapter 1). A $H^+$-coupled symport, with a stoichiometry of one anion to three protons has been proposed, which accounts for (i) the accumulation of sulfate and selenate against their electrochemical gradients (Brown and Shrift 1982; Smith *et al.* 2000) and (ii) the increased uptake of both anions when the rhizosphere is acidified (Leggett and

Epstein 1956; Ulrich and Shrift 1968; Vange *et al.* 1974). The presence of sulfate in the rhizosphere inhibits selenate uptake and accumulation, suggesting direct competition between selenate and sulfate for transport (Figure 2, Hurd-Karrer 1935; Bisbjerg 1972; Gissel-Nielsen 1973; Pratley and McFarlane 1974; Mikkelsen *et al.* 1988; Mikkelsen and Wan 1990; Bell *et al.* 1992; Barak and Goldman 1997; Wu 1998; Zayed *et al.* 1998; Hopper and Parker 1999; Pezzarossa *et al.* 1999; Grieve *et al.* 2001; Vickerman *et al.* 2002; White *et al.* 2004; Wang *et al.* 2005; Lyi *et al.* 2005), but paradoxically, increasing selenate concentration in the rhizosphere often increases shoot S concentrations (Bisbjerg 1972; Smith and Watkinson 1984; Mikkelsen *et al.* 1988; Mikkelsen and Wan 1990; Bell *et al.* 1992; Kopsell and Randle 1997; Takahashi *et al.* 2000; Feist and Parker 2001; Yoshimoto *et al.* 2002; Suarez *et al.* 2003; White *et al.* 2004; Lyi *et al.* 2005; Lyons *et al.* 2005b). The latter observation has been interpreted as the consequence of either selenate or organoselenium compounds interfering with the regulation of sulfate uptake by plant S status (White *et al.* 2004). Indeed, changes in the root transcriptome in response to selenate in the rhizosphere mimic those observed during S starvation (Van Hoewyk *et al.* 2005). Sulfate uptake is regulated at the level of gene transcription (Hawkesford 2005; Hawkesford and De Kok 2006), and both the downregulation of sulfate transport capacity by sulfate, cysteine, or glutathione, and the upregulation of sulfate transport capacity by increased *O*-acetylserine or decreased sulfide concentrations, have been proposed (Hawkesford and Smith 1997; Bolchi *et al.* 1999; Smith *et al.* 2000; Takahashi *et al.* 2000; Terry *et al.* 2000; Vauclare *et al.* 2002; Hirai *et al.* 2003, 2005; Hopkins *et al.* 2005; Hawkesford and De Kok 2006).

The genome of the model plant, *Arabidopsis thaliana*, contains 14 genes encoding sulfate transporters, and a similar number are present in the genomes of other plant species (Hawkesford 2005; Hawkesford and De Kok 2006). The overexpression of genes encoding high-affinity sulfate transporters (HASTs) in roots of transgenic plants increases the uptake of both sulfate and selenate (Terry *et al.* 2000) and mutants lacking HASTs show reduced uptake of sulfate and selenate (Shibagaki *et al.* 2002). Historically, it was observed that sulfate and selenate competed for influx to plant roots (Leggett and Epstein 1956; Pettersson 1966; Ulrich and Shrift 1968; Shennan *et al.* 1990), and exhibited similar Michaelis constants for high-affinity transport ($K_m$ = 15–20 μM). However, when plants are supplied with mixtures of sulfate and selenate, the Se/S concentration ratio in shoot tissues is rarely identical to the Se/S concentration ratio in the rhizosphere (Hurd-Karrer 1937; Bell *et al.* 1992;

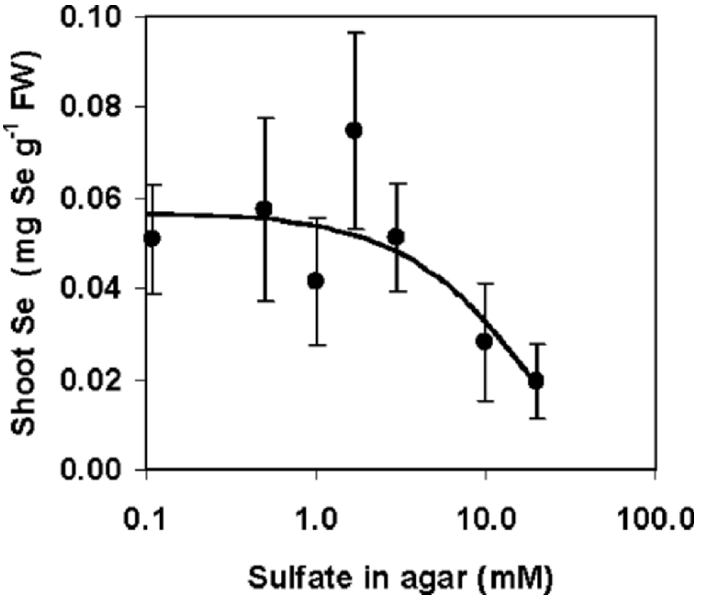


*Figure 2.* Sulfate reduces selenate uptake. The relationship between the shoot Se concentration of 21-day old *Arabidopsis* (Columbia *gl1*) plants and the sulfate concentration in an agar medium containing a complete mineral complement plus 100 μM selenate. Data are expressed as mean ± SE from 4 or 5 replicate experiments (adapted from White *et al.* 2004).

Barak and Goldman 1997; Feist and Parker 2001; Ellis and Salt 2003; Suarez *et al.* 2003; White *et al.* 2004). Indeed, there is often no correlation between the shoot Se and S concentrations of different plant species (or even ecotypes of the same species) growing in the same environment (Rosenfeld and Beath 1964; Feist and Parker 2001), although strong correlations between shoot Se and S concentrations have been reported when the analysis is limited to Se nonaccumulator crop plants (e.g. Hurd-Karrer 1937). The Se/S ratio in shoots of plants, such as *Astragalus bisulcatus* and *Stanleya pinnata*, which appear to be specialized to seleniferous soils, is higher than that in the rhizosphere (Bell *et al.* 1992; Feist and Parker 2001; Ellis and Salt 2003), whereas the Se/S ratio in shoot tissues of most plants, including *A. thaliana*, *Brassica juncea*, *B. napus, B. nigra*, *B. oleracea* and *Lesquerella fendleri*, is lower than that in the rhizosphere (Hurd-Karrer 1937; Bell *et al.* 1992; Barak and Goldman

1997; Kopsell and Randle 1997; 1999, Feist and Parker 2001; Grieve *et al.* 2001; Suarez *et al.* 2003; White *et al.* 2004). This suggests that the transporters responsible for the uptake and/or translocation of these anions within the plant are selective for either selenate, in Se-accumulator plants, or sulfate, in Se nonaccumulator plants (White *et al.* 2004). In *Arabidopsis*, several transporters with contrasting sulfate/selenate selectivities appear to facilitate the uptake of selenate and sulfate, and their relative abundance and/or activities vary between plants of contrasting nutritional status (White *et al.* 2004). The Se/S accumulation ratio is increased by S supply, suggesting that the sulfate transporters induced by S deficiency are more selective for sulfate than the sulfate transporters present constitutively. Nevertheless, the HASTs induced by S starvation in *Arabidopsis* roots (AtSultr1:1 and AtSultr1:2) appear to catalyze the influx of both sulfate and selenate (Takahashi *et al.* 2000; Terry *et al.* 2000; Shibagaki *et al.* 2002; Yoshimoto *et al.* 2002; Maruyama-Nakashita *et al.* 2003). Taken together, these observations suggest that several sulfate transporters, with contrasting anionic selectivities, facilitate the uptake of sulfate and selenate by plant roots, and that the complement of these is determined genetically and may be regulated by plant nutritional status. However, the structural basis of the anionic selectivity of different sulfate transporters is unknown.

Following uptake by root cells, S and Se are converted to sulfate and selenate, which are then loaded into the xylem and transported to the shoot, where they are assimilated into organic compounds. Plants appear to be developmentally programmed to direct these anions to towards leaves approaching full expansion (Hawkesford and De Kok 2006). It is thought that Se is assimilated into organic compounds through the S-assimilation pathway. This metabolic pathway has been described in detail (Hawkesford 2005; Hawkesford and De Kok 2006) and the regulation of S assimilation by plant S status, through both enzyme and kinetic (Wirtz *et al.* 2004; Nikiforova *et al.* 2005) and transcriptional (Hirai *et al.* 2003, 2005; Maruyama-Nakashita *et al.* 2003; Nikiforova *et al.* 2003; 2005) mechanisms has been well documented.

Most sulfate assimilation occurs in the shoot, and the enzymes responsible are generally encoded by extensive gene families whose products are directed to different intracellular compartments (Hawkesford 2005; Hawkesford and De Kok 2006). An increase in the expression of genes encoding these enzymes is commonly observed during S starvation (Hirai *et al.* 2003, 2005; Maruyama-Nakashita *et al.* 2003; Nikiforova *et al.* 2003, 2005).

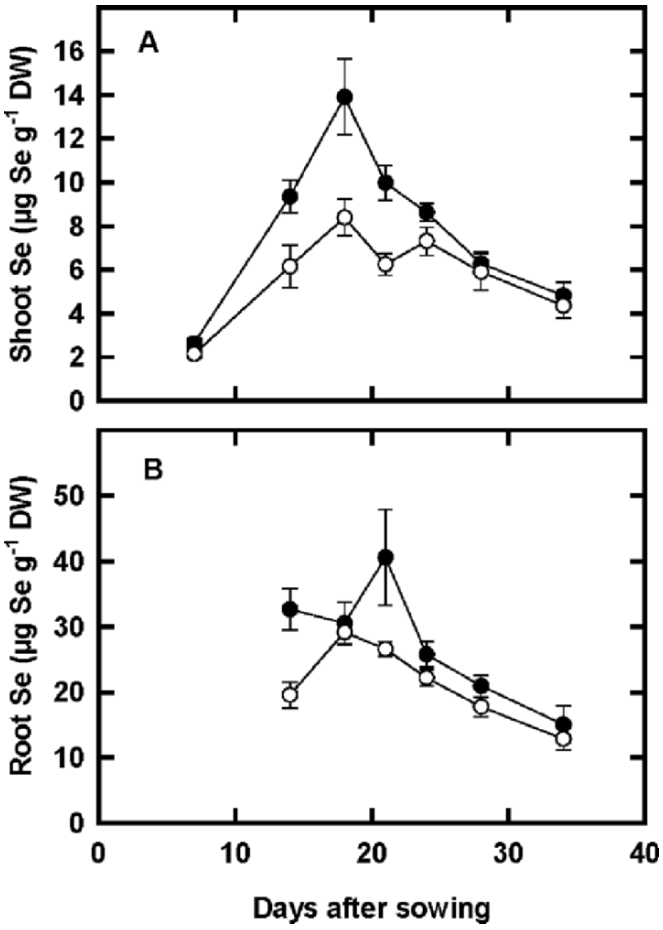


*Figure 3.* Selenium concentrations in shoots and roots of *Arabidopsis* change during plant development. Changes in (**A**) shoot Se concentration and (**B**) root Se concentration with plant age of *Arabidopsis* plants (Columbia accession, *filled circles*; Landsberg *erecta* accession, *open circles*) grown in a medium containing 0.3 μM selenate. Data show the mean ± SE for 3 replicate experiments (S.E. Johnson, M.R. Broadley and P.J. White, unpublished data).

Selenate appears to be converted to organoselenium compounds by the S assimilation pathway (Brown and Shrift 1982; Terry *et al.* 2000; Dhillon and Dhillon 2003; Ellis and Salt 2003; Sors *et al.* 2006). In plants, Se is present in diverse organoselenium compounds including selenoamino acids (SeCys and SeMet) and selenoproteins (Fordyce 2005). However, although obligate selenoproteins, in which UGA codons are recognized by

the SeCys-tRNA through a translational reprogramming mechanism, play essential roles in prokaryotes, archaebacteria, and eukaryotes (Driscoll and Copeland 2003; Castellano *et al.* 2004; Romero *et al.* 2005), no obligate selenoproteins have been found in higher plants despite their possession of SeCys-tRNAs that recognize the UGA codon. Nevertheless, homologs of selenoproteins do occur in plants, in which SeCys is replaced by cysteine, and nonspecific replacement of cysteine by SeCys can occur (Ellis and Salt 2003; Castellano *et al.* 2004). The replacement of cysteine by SeCys and methionine by SeMet may alter protein stability and functional activity and is thought to account for Se toxicity in non-accumulator plants (Brown and Shrift 1982).

The conversion of selenate to organoselenium compounds proceeds through adenosine 5′-phosphoselenate, selenite, and selenide. Selenide is then converted to SeCys, from which SeMet is synthesized via selenocystathionine and homoselenocysteine. Both SeCys and SeMet can be incorporated into proteins or methylated. For example, Se-methylselenocysteine (SeMSeCys), γ-glutamyl-SeMSeCys and Se-methylselenomethionine (SeMSeMet) are characteristic Se assimilation products of species in the genera *Allium* and *Brassica* (Hamilton 1975; Grant *et al.* 2004; Kahakachchi *et al.* 2004; Shah *et al.* 2004; Sugihara *et al.* 2004; Lyons *et al.* 2004; Lyi *et al.* 2005; Ogra *et al.* 2005; Montes-Bayón *et al.* 2003, 2006). Transgenic plants overexpressing ATP sulfurylase and/or genes involved in glutathione synthesis (Pilon-Smits *et al.* 1999b; Bañuelos *et al.* 2005a) have increased Se accumulation and Se tolerance. Interestingly, the expression of a gene encoding homocysteine *S*-methyltransferase (SMT = SeCys methyltransferase) appears to be upregulated in plants exposed to selenate, resulting in increased SeMSeCys accumulation, but this response is antagonized by the presence of sulfate and/or sulfite in the rhizosphere (Lyi *et al.* 2005). The effect of sulfate on selenate-induced SMT expression may be an indirect consequence of reduced selenate uptake. Methylated selenoamino acids can also be converted to dimethylselenonium propionate (Grant *et al.* 2004) and the volatile selenocompounds dimethylselenide (DMSe) and dimethyldiselenide (DMDSe; Ellis and Salt 2003). Selenium accumulation appears to be determined by the production of sink metabolites (Sors *et al.* 2005). The accumulation MCys, SeMCys, and total Se in shoots of *Astragalus* species with contrasting abilities to accumulate Se was correlated with SMT activity (Sors *et al.* 2005) and shoot Se concentrations are greater in transgenic plants overexpressing SMT (Montes-Bayón *et al.* 2003; Ellis *et al.* 2004; LeDuc *et al.* 2004). Whereas, the accumulation of Se in *Astragalus* species with contrasting abilities to accumulate Se was not

correlated with the activities of ATP sulfurylase, APS reductase or serine acetyltransferase (Sors *et al.* 2005).

Genes encoding enzymes involved in S and Se uptake and assimilation are differentially expressed during plant development. Using experiments described by Schmid *et al.* (2005) and abstracting raw data from NASC arrays (Craigon *et al.* 2004), the expression of 42 of the 50 genes (14 sulfate transporters (At4g08620, At1g78000, At1g22150, At5g10180, At1g77990, At3g51900, At4g02700, At1g23090, At3g15990, At5g19600, At5g13550, At3g12520, At1g80310, At2g25680), 4 ATP sulfurylases (At3g22890, At1g19920, At4g14680, At5g43780), 4 APS kinases (At2g14750, At4g39940, At3g03900, At5g67520), 3 APS reductases (At4g04610, At1g62180, At4g21990), sulfite reductase (At5g04590), 10 *O*-acetylserine(thiol) lyases (At4g14880, At2g43750, At3g59760, At3g22460, At1g55880, At3g03630, At3g04940, At3g61440, At5g28020, At5g28030), 4 serine acetyltransferases (At1g55920, At2g17640, At3g13110, At5g56760), 2 cystathionine γ-synthases (At1g33320, At3g01120), 1 cystathionine β-lyase (At3g57050), 5 SAM lyases (At3g17390, At4g01850, At1g02500, At2g36880, At5g16450) and sulfite oxidase (At3g01910)) involved in S and Se uptake and assimilation analyzed in the entire vegetative rosette differed ($P < 0.05$) between *Arabidopsis* grown on soil for 7, 14, and 21 days. There was a general decline in the expression of all genes involved in S and Se uptake and assimilation, with the exception of an OAS-TL (At3g03630) and a SAM synthase (At4g01850). In 17-day old *Arabidopsis* plants grown in continuous light, the expression of many genes (27 of 50 genes) involved in S and Se uptake and assimilation differed in at least one of the leaves ($P < 0.05$). The expression of many genes was lower in younger leaves than in older leaves, except for a gene encoding an OAS-TL. Assuming that gene expression does not change in a particular leaf, these observations imply that S and Se assimilation will occur predominantly in the first leaves that the plant produces. These changes in gene expression are consistent with an initial increase in shoot Se concentrations followed by a gradual decline as plants age. This trend has been observed in *Arabidopsis* (Figure 3), *A. bisulcatus* and other plants (Rosenfeld and Beath 1964; Xue *et al.* 2001; Turakainen *et al.* 2004), whose shoot Se concentrations increase to a maximum during seedling growth, then decline prior to, or upon, flowering. It would be interesting to determine the Se species present in *Arabidopsis* leaves of contrasting ages. In *A. bisulcatus* the Se in older leaves was predominantly selenate, whereas that in new leaves and roots was present as organoselenium compounds (Pickering *et al.* 2000,

2003). These observations suggest the effective translocation of organo-selenium compounds from older to younger leaves.

## ECOLOGICAL ASPECTS TO SELENIUM ACCUMULATION BY PLANTS

Angiosperm species differ markedly in their ability to assimilate and accumulate Se, and are divided into three groups: "Se nonaccumulator", "Se-indicator" and "Se-accumulator" plants (Rosenfeld and Beath 1964; Brown and Shrift 1982; White *et al.* 2004). Most angiosperms are Se nonaccumulators and cannot tolerate tissue Se concentrations above about 10–100 μg Se $g^{-1}$ dry matter, although the exact value depends critically upon their tissue Se/S quotient (Rosenfeld and Beath 1964; White *et al.* 2004). They tolerate increased Se concentrations in the rhizosphere by restricting Se uptake and movement to the shoot, but cannot colonize seleniferous soils (Wu 1998). Likewise, *Arabidopsis* mutants lacking root sulfate transporters survive higher Se concentrations in the rhizosphere than wild-type plants (Shibagaki *et al.* 2002). Several plant species can grow adequately in both seleniferous and nonseleniferous soils, and can accumulate up to 1,000 μg Se $g^{-1}$ dry matter in their shoot tissues without consequence. Such species include members of the genera *Aster*, *Astragalus*, *Atriplex*, *Castilleja*, *Comandra*, *Grayia*, *Grindelia*, *Gutierrezia*, *Machaeranthera*, *Mentzelia,* and *Sideranthus* (Rosenfeld and Beath 1964; Rodriguez *et al.* 2005). These species are termed Se-indicator plants. A few plant species, termed Se-accumulator plants, can contain over 15,000 mg Se $g^{-1}$ dry matter when sampled from seleniferous environments, for example, in the Great Plains of the USA (Rosenfeld and Beath 1964). These species include members of the Fabaceae (*A. bisulcatus*, *A. racemosus*), Asteraceae (*Aster occidentalis*, *Machaeranthera ramosa*) and *Brassica*ceae (*S. pinnata*). Fruits of the Lecythidaceae, such as *Bertholletia excelsa* (Brazil nut), *Lecythis zabucaja* (Paradise nut), *L. ollaria* (Coco de Mono) and *L. elliptica* (Sapucaia nut), are also renowned for their accumulation of Se (Broadley *et al.* 2006). The Se-accumulator species are capable of colonizing seleniferous soils, but they are rarely observed in nonseleniferous areas (Brown and Shrift 1982). It has been speculated, but never proven, that Se might be required for the growth of these plants. The evolution of the flora of seleniferous soils has not been studied in detail, but, since Se-accumulator species occur in many unrelated genera (Brown and Shrift 1982; White *et al.* 2004), it is unlikely to have evolved from a single Se-accumulating ancestor. It is more likely

that Se accumulation and tolerance arose by convergent evolution of appropriate biochemical pathways in disparate angiosperm clades (Brown and Shrift 1982). The ability of plants to colonize such a hostile ecological niche as seleniferous soils has a clear evolutionary advantage (Brown and Shrift 1982) and Se accumulation has been shown to protect plants against fungal infection and herbivorous insects (Vickerman *et al.* 2002; Hanson *et al.* 2003, 2004). In addition, improved Se nutrition may provide protection against oxidative stresses in higher plants (Cartes *et al.* 2005; Djanaguiraman *et al.* 2005; Hartikainen 2005; Kong *et al.* 2005). However, despite the advantages to Se accumulation and tolerance, it has been speculated that present-day Se-accumulator plants might be the remnants of an ancient flora that evolved during periods when seleniferous soils were more widespread, but that the biochemistry required for Se accumulation and tolerance became relatively disadvantageous when soil Se concentrations declined (Brown and Shrift 1982).

Despite remarkable differences in Se accumulation between plant species, and unlike the case for some essential mineral elements, such as Ca, Mg, Zn, and K (Broadley *et al.* 2001, 2003, 2004), there is no evidence for systematic differences in shoot Se concentrations between angiosperm orders (White *et al.* 2004). Over 95% of the variation in shoot Se concentration can be attributed to within-order variance, which is likely to reflect species-specific adaptations (White *et al.* 2004). Interspecies differences in Se accumulation are most pronounced within genera containing Se-accumulator or Se-indicator plants, such as *Astragalus* and *Brassica* (White *et al.* 2004; Sors *et al.* 2005). Differences in the ability of plant species to tolerate high tissue Se concentrations are thought to be a consequence of differences in their Se metabolism (Shrift 1969; Brown and Shrift 1982; Terry *et al.* 2000; Dhillon and Dhillon 2003; Ellis and Salt 2003; Sors *et al.* 2006). The assimilation of Se into organoselenium compounds is thought to compete with S assimilation and, since both SeCys and SeMet can be incorporated into proteins, it is proposed that impaired activities of selenoproteins could contribute significantly to Se toxicity (Brown and Shrift 1982). In particular, the replacement of Cys with SeCys will impair the formation of disulfide bridges, which are critical for protein structure (Brown and Shrift 1982). In Se-indicator and Se-accumulator plants, the accumulation of SeCys appears to be restricted, and SeCys is converted to compounds such as MSeCys, SeMSeCys, γ-glutamyl-SeMSeCys, selenocystathionine, and dimethyl-selenonium propionate (Shrift 1969; Hamilton 1975; Brown and Shrift 1982; Neuhierl *et al.* 1999; Pickering *et al.* 2000, 2003; Terry *et al.* 2000; Ferri *et al.* 2004; Grant *et al.* 2004; Kahakachchi *et al.* 2004; LeDuc *et al.* 2004; Shah *et al.*

2004; Sugihara *et al.* 2004; Lyi *et al.* 2005; Ogra *et al.* 2005; Sors *et al.* 2005; Montes-Bayon *et al.* 2003, 2006). Interestingly, SeMet and SeMSeMet appear to be present at low concentrations in some Se-accumulator plants (Shrift 1969), but are abundant in many Se-indicator and nonaccumulator plants grown in the presence of Se (Shrift 1969; Stadlober *et al.* 2001; Vonderheide *et al.* 2002; Montes-Bayón *et al.* 2003; Kannamkumarath *et al.* 2005; Dumont *et al.* 2006). The incorporation of selenoamino acids into proteins also appears to be lower in Se-accumulator plants than in Se nonaccumulator plants. However, it should be noted that the form of Se in Se-accumulator and Se-indicator plants grown on seleniferous soils varies greatly between plant species, with some species, such as *Astagalus bisulcatus* and *S. pinnata*, accumulating Se almost exclusively in organic forms, whilst others, such as *A. occidentalis* or *Atriplex nuttallii*, accumulate Se predominantly as selenate (Rosenfeld and Beath 1964).

Although excessive Se accumulation is toxic to most plants, the critical tissue Se concentration, defined as the tissue Se concentration at which yield is reduced by 10%, varies both with plant species and S nutrition (Hurd-Karrer 1935; Rosenfeld and Beath 1964; Mikkelsen *et al.* 1989; White *et al.* 2004). It is thought that Se-accumulator plants tolerate higher tissue Se concentrations compared to other plants because of their ability to synthesize non-toxic Se metabolites, and it has been observed that most plants will tolerate higher tissue Se concentrations when they are S-replete (White *et al.* 2004). The latter phenomenon has been attributed to a competition between Se and S for a biochemical process, such as assimilation into essential proteins. The toxicity of selenate in the rhizosphere will be reduced by the addition of sulfate because increasing sulfate concentrations in the rhizosphere compete with and inhibit selenate uptake (see above), thereby reducing the tissue Se/S quotient and (presumably) the relative incorporation of SeCys and SeMet into proteins. In *Arabidopsis*, growth (expressed as the shoot FW obtained in the presence of selenate divided by the shoot FW obtained in the absence of selenate at the same sulfate concentration) was found to be approximately linearly related to the shoot Se/S concentration ratio (Figure 4, White *et al.* 2004), which is consistent with the hypothesis that Se inhibits growth through its interactions with S metabolism.

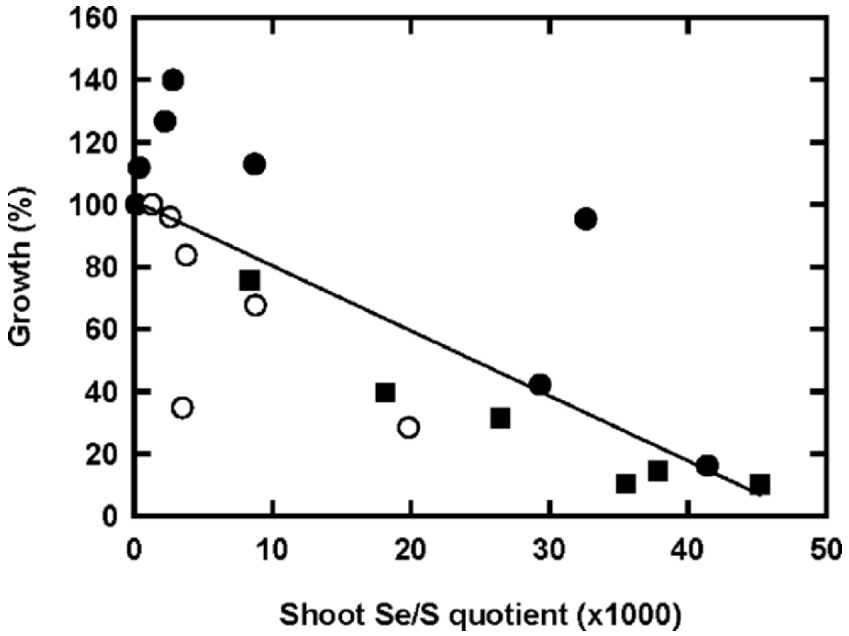


*Figure 4.* Selenate toxicity in *Arabidopsis* is related to the shoot Se/S quotient. The relationship between the growth of *Arabidopsis* (Columbia *gl1*) plants (expressed as the shoot FW obtained in the presence of selenate divided by the shoot FW obtained in the absence of selenate at the same sulfate concentration) and the shoot Se/S quotient (g Se $g^{-1}$ S). Data were derived from the mean values for 4 or 5 replicate experiments in which plants were grown on agar in the presence of sulfate at either 1.73 mM (•) or 50 μM (o) and various selenate concentrations, or in the presence of selenate at 100 μM (■) and various sulfate concentrations (Figure 2). Data from conditions yielding the lowest growth were excluded. (Adapted from White *et al.* 2004.)

## PHYTOREMEDIATION OF SOILS CONTAMINATED BY SELENIUM

The consumption of feed containing over 1–5 mg Se $kg^{-1}$ dry matter may result in Se toxicity in animals (Gissel-Nielsen 1998; Dhillon and Dhillon 2003). Plants growing on seleniferous soils contain tissue Se concentrations of this magnitude and eating them may cause chronic and acute selenosis in animals (Dhillon and Dhillon 2003; Ellis and Salt 2003). Most seleniferous soils are of geological origin, but activities such as the combustion of coal, the disposal of sewage sludge, coal ash or mine

tailings, the irrigation of fields in seleniferous areas, and/or the domestic release of Se containing products to waste water, such as antidandruff shampoo, have led to Se enrichment of agricultural soils (Dhillon and Dhillon 2003; Broadley *et al.* 2006). Whatever their origin, whether natural or anthropogenic, seleniferous soils must be managed to reduce the likelihood of Se toxicities occurring in the food chain.

Many anthropogenically created seleniferous soils would be highly productive, but Se toxicity in crops and the bioenrichment of Se in the food chain makes them unsuitable for agricultural use. However, there are several strategies that may enable crops to be grown on these areas. The first strategy is to grow crops that do not accumulate Se readily, but can tolerate the Se concentrations in the soil. The second strategy is to reduce the uptake of selenate by plants by increasing sulfate in the rhizosphere, through the application of S-fertilizers. The third strategy is to convert soil Se into forms that are unavailable to plants. This could be achieved through microbial innoculates that either volatilize Se (Frankenburger and Arshad 2001) or convert selenate and selenite to elemental Se or selenide (Dhillon and Dhillon 2003). Unfortunately, the latter approach could reduce Se concentrations in edible produce sufficiently to impact on the nutritional requirements of animals. The fourth strategy is to remove the Se-enriched soils. This could be achieved by replacement, or mixing, of seleniferous soil with nonseleniferous soil for immediate agricultural production, but phytoremediation is the favored option for natural landscapes (Wu 1998; Terry *et al.* 2000; Dhillon and Dhillon 2003). Phytoremediation can be achieved using high-biomass plants that grow rapidly and accumulate high tissue Se concentrations. These plants can be harvested and the Se removed from the seleniferous site. It has been suggested that, rather than burying these plants, plants with high tissue Se concentrations could be cut into animal feed or used as a "green manure" in areas with inadequate soil Se concentrations (Terry *et al.* 2000; Dhillon and Dhillon 2003).

Recently, considerable research effort has been devoted to identifying, or genetically engineering, plants for phytoremediation of seleniferous soils (Dhillon and Dhillon 2003; LeDuc and Terry 2005; Pilon-Smits 2005). This has included searching for rapidly growing, high-biomass, Se-accumulating plants and/or the genetic engineering of enhanced Se accumulation in transgenic plants. Unfortunately, natural Se-accumulator plants appear to be slow growing, unresponsive to fertilizers, and susceptible to pests and diseases (Dhillon and Dhillon 2003). The ideal plant for the phytoremediation of seleniferous soils would grow rapidly, accumulate high tissue Se concentrations and/or volatilize Se efficiently,

and tolerate both biotic and abiotic stresses (Terry *et al.* 2000). Extensive screening of diverse plant species and their ecotypes has identified various *Brassica* species, such as Indian mustard (*B. juncea*) and canola (*B. napus*), as candidates for the phytoremediation of seleniferous soils (Van Mantgem *et al.* 1996; Bañuelos *et al.* 1997; Terry *et al.* 2000; Goodson *et al.* 2003; Bañuelos and Lin 2005; Bañuelos *et al.* 2005a). These species not only tolerate high tissue Se concentrations and accumulate biomass rapidly, but also volatilize Se effectively (Zayed *et al.* 1998; de Souza *et al.* 1998; Pilon-Smits *et al.* 1999a; Terry *et al.* 2000; Bañuelos *et al.* 2005b). Volatilization removes Se permanently from both the soil and the food chain, as the (relatively) nontoxic gases DMSe and DMDSe. Rates of volatilization can exceed 2.5 mg Se $kg^{-1}$ $d^{-1}$ when plants are grown in solutions containing 20 μM selenate (Pilon-Smits *et al.* 1999a; Terry *et al.* 2000). The rate of selenate reduction and cystathionine-γ-synthase activity appear to limit Se volatilization (de Souza *et al.* 1998; Van Huysen *et al.* 2003). Cattails (*Typha* spp.), bulrushes (*Scirpus* spp.), duckweeds (*Lemna* spp.), hydrilla (*Hydrilla verticillata*), iris-leaved rush (*Juncus xiphioides*) and parrot's feather (*Myriophyllum brasiliense*) are promising plant species for wastewater treatment in constructed wetlands (Pilon-Smits *et al.* 1999a; Carvalho and Martin 2001; Shardenu *et al.* 2003).

In addition to the selection of plant species and ecotypes with improved Se phytoremediation potential, a better understanding of the physiological mechanisms underpinning Se accumulation, coupled with the use of modern molecular–genetic techniques, has enabled the genetic engineering of transgenic plants with improved Se accumulation and/or Se tolerance. For example, (1) overexpressing ATP sulfurylase, γ-glutamyl-cysteine synthetase or glutathione synthetase increased shoot Se accumulation and Se tolerance in transgenic *Brassica juncea* (Pilon-Smits *et al.* 1999b; Van Huysen *et al.* 2004; Bañuelos *et al.* 2005a); (2) overexpressing a SeCys methyltransferase gene from *A. bisulcatus* increased Se accumulation, Se volatilization and selenite tolerance in transgenic *Arabidopsis* and *B. juncea* (Montes-Bayón *et al.* 2003; Ellis *et al.* 2004; LeDuc *et al.* 2004); (3) overexpressing cystathionine-γ-synthase increased Se volatilization and selenite tolerance in transgenic *B. juncea* (Van Huysen *et al.* 2003); (4) expressing a mouse SeCys lyase or the chloroplast protein CpNifS, which both convert SeCys to elemental Se and alanine, increased tissue Se concentrations and selenate tolerance in transgenic *Arabidopsis* (Garifullina *et al.* 2003; Pilon *et al.* 2003; Van Hoewyk *et al.* 2005); and (5) overexpressing a selenium-binding protein (AtSBP1) increased selenite tolerance in transgenic *Arabidopsis* (Agalou *et al.* 2005).

## BIOFORTIFICATION OF CROPS WITH SELENIUM

In addition to being toxic when consumed in large quantities, Se is also an essential mineral element for all animals, including humans (Rayman 2000, 2002, 2004; Dhillon and Dhillon 2003; Finley 2005a; White and Broadley 2005). The recommended dietary allowance in the USA is 55 μg Se $d^{-1}$ and the reference nutrient intake in the UK is 60–75 μg Se $d^{-1}$ (Rayman 2004; White and Broadley 2005). Selenium deficiency in humans is associated with cardiovascular disorders, hypothyroidism, a weakened immune system, male infertility, and increased incidence of various cancers (Gupta and Gupta 2002; Rayman 2000, 2002; Johnson 2004; Whanger 2004; Finley 2005b). It is estimated that about 15% of the world's population are Se deficient (Rayman 2000, 2002; White and Broadley 2005), which is a consequence of consuming crops grown on Se-deficient soils.

To address Se deficiency in the human diet, agronomists and plant breeders are pursuing two complementary strategies to produce crops with greater Se concentrations (White and Broadley 2005; Broadley *et al.* 2006). The first strategy employs selenate fertilizers to increase Se accumulation by crops, and this strategy has been used successfully in both Finland and New Zealand (Eurola *et al.* 1989, 1991, 2004; Lyons *et al.* 2003; Hartikainen 2005). The second strategy involves the development of novel crop genotypes that accumulate more Se, by either conventional breeding and/or genetic engineering. This strategy is exemplified in the screening of germplasm collections for genotypes with high yield and tissue Se concentrations (Graham *et al.* 2001; Lyons *et al.* 2003, 2005a).

Agronomic biofortification of pastures or forages with Se through the application of Se fertilizers to the soil or in foliar sprays has been widely demonstrated, and has been show to have beneficial effects on animal health and to deliver Se to the human diet (Gissel-Nielsen 1998; Gupta and Gupta 2002; Broadley *et al.* 2006). Selenium fertilizers are generally applied to pastures and forages at a rate of 5–10 g Se $ha^{-1}$ $year^{-1}$ and, although soluble selenate salts such as $Na_2SeO_4$ or $K_2SeO_4$ provide an immediate source for Se uptake by plants, in the years following Se fertilization, selenite or a less soluble selenate salt such as $BaSeO_4$ provide longer-lasting Se availability. Selenium concentrations in food crops can also be increased through Se fertilization (Gissel-Nielsen 1998; Gupta and Gupta 2002; Broadley *et al.* 2006). This strategy has been pioneered in Finland, where the addition of Se to fertilizer formulations has been mandatory since 1984 and, currently, fertilisers containing 10 mg Se $kg^{-1}$

are applied to all crops, including food crops. Mandatory Se fertilization in Finland has increased the Se concentrations in many indigenous food items over tenfold (Eurola *et al.* 1989, 1991, 2004) and, simultaneously, has increased Se intake by Finns and their serum Se concentrations (Aro *et al.* 1995; Varo *et al.* 1988; Wang *et al.* 1998; Rayman 2002; Hartikainen 2005). To improve the Se status of human populations worldwide, it may be necessary to target crops that contribute significantly to the human diet. Staple crops include wheat, maize, rice, common bean, cassava, and potato. The Se concentrations in all these crops can be increased by Se fertilization (Eurola *et al.* 1989, 1991; Chen *et al.* 2002; Gupta and Gupta 2002; Lyons *et al.* 2003, 2004, 2005b; Turakainen *et al.* 2004). However, since the dominant organoselenium compounds differ between plant species (see previous sections), it is noteworthy that, although vegetables and fruit often deliver small proportions of minerals to the diet (Eurola *et al.* 1989, 1991), some vegetables contain high concentrations of organoselenium compounds that are particularly beneficial to human health. For example, *Allium* and *Brassica* accumulate SeMSeCys, which can be converted into methyl selenol, a bioactive substance that may protect against cancer (Ip *et al.* 2002; Whanger 2004).

Increasing the Se concentrations in produce through the application of Se fertilizers can be complemented by breeding crops with an increased ability to acquire and accumulate Se. There appears to be sufficient, heritable genetic variability in Se accumulation within crop species, or their close relatives, to make this strategy feasible (Graham *et al.* 2001; Lyons *et al.* 2003, 2005a). For example, although there appears to be little variation in grain Se concentrations between cultivars of modern bread and durum wheats, the wild wheats (*Triticum dicoccum*, *T. spelta*) and their relatives (*Aegilops tauschii*) have significantly higher Se concentrations than cultivated wheat (Lyons *et al.* 2005a). Variation in Se accumulation between genotypes of soybean (Yang *et al.* 2003; Zhang *et al.* 2003) broccoli (Robbins *et al.* 2005), and tomato (Shennan *et al.* 1990; Pezzarossa *et al.* 1999) has also been reported. Ultimately, breeding for genotypes with higher Se concentrations will benefit from knowledge of the genes that impact on Se accumulation. These may be approached through quantitative trait (QTL) analyses using genetic mapping populations available for both model plants, such as *Arabidopsis* (Zhang *et al.* 2006), and crop plants (Vreugdenhil *et al.* 2005). However, before the potential of any candidate genes can be realized in crop plants, either through marker-assisted breeding or genetic engineering, a deeper understanding of the physiological consequences of their manipulation should be obtained.

## CONCLUSIONS

Selenium concentrations in natural soils are determined by geology, and vary widely. In some areas Se concentrations can be so high that many plants are unable to grow. These areas support a unique flora, composed of plant species that can accumulate and tolerate high tissue Se concentrations. Plant species that grow solely on seleniferous soils often have extremely high tissue Se concentrations and are termed "Se-accumulator plants", whereas plant species that colonize both seleniferous and non-seleniferous soils generally have lower tissue Se concentrations and are termed "Se-indicator" plants. Plant species that cannot tolerate high tissue Se concentrations are termed "Se nonaccumulator" plants. It is thought that differences in Se metabolism, in particular the ability to exclude SeCys from proteins, could account for the contrasting abilities of plant species to tolerate and accumulate high tissue Se concentrations.

Plant roots take up Se from the soil solution as selenate, selenite, and organoselenium compounds. Sulfate and selenate compete for uptake through sulfate transporters in the plasma membrane of root cells and, following uptake, Se is distributed and assimilated within the plant through the S transport and assimilation pathways. In Se-accumulator and Se-indicator plants, SeCys is often converted to methylated compounds. There is considerable interest in developing plants that can accumulate more Se, either to remediate sites that have been contaminated through anthropogenic Se inputs or to develop crops that can accumulate more Se from soils with low Se concentrations. Plant species suitable for remediation of Se-contaminated soils have been identified, and knowledge of plant Se metabolism has allowed the genetic engineering of transgenic plants with enhanced Se uptake, accumulation, and volatilization capacities.

Selenium is an essential element for all animals, including humans. It is estimated that about 15% of the world's population are Se deficient, which is a consequence of consuming crops grown on soils with low Se concentrations. To address this problem, agronomists and plant breeders are pursuing two complementary strategies to increase Se concentrations in crop plants. The first strategy is to increase Se concentrations in edible portions through the application of Se fertilizers. This strategy has been demonstrated successfully in Finland. The second strategy, which has yet to produce results, is to select, or breed, crop genotypes with an enhanced ability to acquire and accumulate Se. Together, these strategies will deliver adequate Se to the diet with minimal Se fertilization.

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