Chapter 1

General introduction

Sulfur metabolism

Amongst other elements sulfur is present in plant tissue in minor quantities only; its content strongly varies between species and ranges from 0.03 to 2 mmol g⁻¹ dry weight (0.1 to 6 %; Tabatabai, 1986; Schnug, 1998; Pedersen et al., 1998). Plants generally utilize sulfate taken up by the roots as sulfur source for growth and prior to its assimilation sulfate needs to be reduced to sulfide, before it is metabolized into organic sulfur compounds (Fig. 1). Roots contain all enzymes necessary to reduce sulfate to sulfide, although the chloroplast appears to be the primary site for the reduction of sulfate to sulfide and its subsequent incorporation into cysteine (Brunold, 1990, 1993; Davidian et al., 2000). Cysteine is the sulfur donor for most other organic sulfur compounds in plants (Fig. 1). The predominant proportion of the sulfur is present in proteins, as cysteine and methionine residues, wherein it is highly significant in the structure, conformation and function. Sulfur is also required for the synthesis of various other compounds, as thiols (glutathione), sulfolipids and secondary sulfur compounds (alliins, glucosinolates, phytochelatins), which play an important role in the physiology of plants and in the protection and adaptation of plants against stress and pests. The uptake and assimilation of sulfur and nitrogen are strongly interrelated and dependent upon each other (Brunold, 1993). Proteins contain both sulfur and non-sulfur amino acids and for this reason the availability of nitrogen and sulfur interacts with the utilization of nitrogen and sulfur for proteins and plant growth. The organic N/S ratio of plants and seeds reflects the sulfur status of the plant. At a sufficient sulfur supply the organic N/S ratio is generally around 20 (Dijkshoorn and Van Wijk, 1967; Brunold, 1993; De Kok et al., 2000). Sulfur deficiency will result in loss of plant fitness, plant’s resistance to environmental stress and pests and in decreased food quality and safety (De Kok et al., 2002c).

Under normal conditions the rate of uptake and assimilation of sulfur will be in tune with the plant’s sulfur requirement for growth, which can be defined as the rate of sulfur uptake and its assimilation considered necessary per gram plant biomass produced with time. When a plant is in the vegetative growth stage, it can be calculated as follows (De Kok et al., 2000):

\[
\text{Sulfur}_{\text{requirement}} \left( \mu\text{mol g}^{-1} \text{ plant day}^{-1} \right) = \text{RGR} \left( \text{g g}^{-1} \text{ day}^{-1} \right) \times \text{Sulfur}_{\text{content}} \left( \mu\text{mol g}^{-1} \text{ plant} \right)
\]

where RGR represents the relative growth rate and Sulfur\text{content} the total plant tissue sulfur content. The sulfur requirement for growth may vary at different developmental stages.
(vegetative growth period, seed production) and largely differs between species. At optimal growth conditions the sulfur requirement (equivalent to sulfur flux) of different crop species ranges from 2 to 10 µmol g⁻¹ plant fw day⁻¹ (0.08 to 0.4 µmol g⁻¹ plant fw h⁻¹, Fig. 1).

Plants contain a variety of sulfate transporters with specific functions in the uptake of sulfate by the roots, its transport to the shoot and its subcellular distribution (Hawkesford and Smith, 1997; Hawkesford, 2000, 2003; Hawkesford and Wray, 2000; Hawkesford et al., 2003a,b). The sulfate transporters gene family has been classified in up to five different groups according to their cellular and subcellular expression and possible functioning (Davidian et al., 2000; Hawkesford 2000; Hawkesford et al., 2003a,b; Buchner et al., 2004a,b). Regulation and expression of the majority of sulfate transporters are controlled by the sulfur nutritional status of the plants. The root-expressed transporters are highly regulated and induced when sulfur supply is limited and repressed in the presence of excess sulfur, which is illustrated by a higher and lower sulfate uptake capacity, respectively (Hawkesford and Smith, 1997; Smith et al., 1997; Davidian et al., 2000; Hawkesford, 2000; Hawkesford and Wray, 2000; Westerman et al., 2000a). Sulfate itself, O-acetylserine (OAS) or metabolic products of the sulfate assimilation, such as cysteine or glutathione may be involved in the mechanism of the negative feedback regulation of the sulfate uptake and its transport to the shoot (Cram, 1990; Clarkson et al., 1993; Hell and Rennenberg, 1998; Davidian et al., 2000; Hawkesford, 2000; Hawkesford and Wray, 2000).

Sulfate needs to be activated to adenosine 5'-phosphosulfate (APS) prior to its reduction, a reaction catalyzed by ATP sulfurylase (Fig. 1). The reduction of APS occurs in two steps. In the first step APS is reduced by APS reductase (APR) to sulfite (Fig. 1) and this enzyme is only present in the plastids (Leustek and Saito, 1999). APS reductase is believed to be a prime regulation point in the sulfate reduction pathway and its activity changes rapidly in response to sulfur starvation or exposure to reduced sulfur compounds (Brunold, 1990; Leustek and Saito, 1999; Hawkesford and Wray, 2000). Sulfite is reduced to sulfide by sulfite reductase with reduced ferredoxin as reductant (Fig. 1). The incorporation of sulfide into cysteine is catalyzed by O-acetylserine(thiol)lyase (OAS-TL), with sulfide and OAS as substrates (Fig. 1). The synthesis of OAS is catalyzed by serine acetyltransferase (SAT) and together with OAS-TL it is associated as enzyme complex named cysteine synthase (Droux et al., 1998; Hell, 2003). The remaining sulfate in plant tissue is transferred into the vacuole. The remobilization and redistribution of the vacuolar sulfate reserves appear to be rather slow and sulfur-deficient plants may still contain detectable levels of sulfate (Cram 1990; Davidian et al., 2000; Hawkesford, 2000). Symptoms of sulfur deficiency include a reduction in growth, yellowing of the youngest leaves, a decrease in sulfur containing metabolites and an increase in nitrogen containing metabolites due to an imbalance in protein synthesis (De Kok et al., 1997; Stuiver et al., 1997).
Fig. 1. An overview of sulfate reduction and assimilation in plants (APS, adenosine 5′-phosphosulfate; Fd\textsubscript{red}, Fd\textsubscript{ox}, reduced and oxidized ferredoxin; RSH, RSSR, reduced and oxidized glutathione) and the rates of sulfate uptake by the roots and its reduction and assimilation in the shoots of a variety of plant species grown under optimal sulfur supply (adapted from De Kok et al., 2002a).

**Characteristics of *Allium* species**

*Allium cepa* L. (onion) was possibly one of the first domesticated vegetables by man and it was already cultivated by the ancient Egyptians. Nowadays, it has the second largest acreage of all vegetables (after tomato) and it has important functions in food flavor and in phytopharmaceutics (Griffiths et al., 2002). The allyl group in its secondary sulfur
compounds is derived from its genus name and is characteristic for all 400 *Allium* species. *Allium* species viz. onion, garlic, leek and chive, contain a variety of secondary sulfur compounds: \(\gamma\)-glutamyl peptides and alliins (S-alk(en)ycysteine sulfoxides). The content of these secondary sulfur compounds is strongly dependent on stage of development of the plant, temperature, water availability and the level of nitrogen and sulfur nutrition (Randle et al., 1993, 1995; Randle, 2000; Randle and Lancaster, 2002; Coolong and Randle, 2003a,b). They form a potential sink for reduced sulfur, since in onion bulbs their content may account for up to 80% of the organic sulfur fraction (Schnug, 1993). Less is known about the content of secondary sulfur compounds in the seedling stage of the plant. It is assumed that alliins are predominantly synthesized in the leaves, from where they are subsequently transferred to the attached bulb scale (Lancaster et al., 1986). The biosynthetic pathways of synthesis of \(\gamma\)-glutamyl peptides and alliins are still ambiguous. \(\gamma\)-Glutamyl peptides are formed from cysteine (via \(\gamma\)-glutamylcysteine or glutathione) and can be metabolized into the corresponding alliins via oxidation and subsequently hydrolization by \(\gamma\)-glutamyl transpeptidases (Lancaster and Boland, 1990; Randle and Lancaster, 2002). However, other possible routes of the synthesis of \(\gamma\)-glutamyl peptides and alliins can not be excluded (Granroth, 1970; Lancaster and Boland, 1990; Edwards et al., 1994; Randle and Lancaster, 2002). The alliins and their breakdown products (e.g. allicin) are the flavor precursors for the odor and taste of *Allium* species. Flavor is only released when plant cells are disrupted and the enzyme alliinase from the vacuole is able to degrade the alliins, yielding a wide variety of volatile and non-volatile sulfur-containing compounds (Lancaster and Collin, 1981; Block, 1992), which are mainly responsible for the specific odor and taste of *Allium* species and for their health benefits (Griffiths et al., 2002; Haq and Ali, 2003). The physiological function of \(\gamma\)-glutamyl peptides and alliins is rather unclear (Schnug, 1993). These compounds may have significance in chemical defense against insects and pathogens and in the storage of nitrogen and sulfur (Lancaster and Boland, 1990). It has been suggested that in onion bulbs, \(\gamma\)-glutamyl peptides may be the main storage form of nitrogen and sulfur and they might be rapidly hydrolyzed to alliins during sprouting and germination (Lancaster and Shaw, 1991; Randle and Lancaster, 2002).

**Hydrogen sulfide**

The toxic effects of atmospheric H\(_2\)S are well established, although there is a wide variation in susceptibility between species to H\(_2\)S. It may negatively affect growth at atmospheric levels of 0.03 \(\mu\)l l\(^{-1}\) and higher and may even cause visible injury and defoliation at \(\geq 0.3 \mu\)l l\(^{-1}\) (De Kok et al., 1998, 2000, 2002b). The mechanisms of toxicity are, however, still largely unclear. The internal sulfide concentration and its accessibility to the meristem appear to be the determining factors of H\(_2\)S phytotoxicity (De Kok, 1989; De Kok et al., 1989, 2002b; Stulen et al., 2000) through the reaction of sulfide with enzymes and
membranes, causing a disturbed metabolism (De Kok, 1990). Contrary to its detrimental effects, foliarly absorbed \( \text{H}_2\text{S} \) can also be utilized as sulfur source for plants and may even be beneficial when the sulfur supply to the roots is limited (De Kok et al., 2000, 2002c; Westerman et al., 2000a). \( \text{H}_2\text{S} \) is taken up via the stomates, metabolized with high affinity into cysteine and subsequently into other sulfur compounds (De Kok et al., 1998; Stuiver and De Kok, 2001). The rate of uptake is determined by both the stomatal conductance and the internal (mesophyll) resistance towards \( \text{H}_2\text{S} \) (De Kok and Tausz, 2001; De Kok et al., 2002b).

In *Brassica oleracea* L. there was a direct interaction between foliar \( \text{H}_2\text{S} \) deposition and the uptake and metabolism of pedospheric sulfate (De Kok et al., 2000; Westerman et al., 2000a,b, 2001a,b). \( \text{H}_2\text{S} \) exposure resulted in a negative feedback regulation of the uptake and assimilation of pedospheric sulfate. Within one or two days after the onset of the exposure, \( \text{H}_2\text{S} \) exposure induced a reduction of sulfate uptake by the roots (Westerman et al., 2000a, 2001b). Furthermore, \( \text{H}_2\text{S} \) exposure resulted in a substantial decrease in the activity of APS reductase, the rate-limiting enzyme in the sulfate reduction pathway (Westerman et al., 2001a), in shoots of *Brassica oleracea*. As a result, the total sulfur content and the total N/S ratio of both shoot and roots of *Brassica oleracea* L. were hardly affected upon prolonged \( \text{H}_2\text{S} \) exposure, even not at atmospheric levels higher than 0.2 µl l\(^{-1}\), which have been established to exceed the sulfur requirement of this species (Stulen et al., 1998; De Kok et al., 2000, 2002b; Westerman et al., 2001b). Brassicaceae are known to contain a variety of secondary sulfur compounds, namely glucosinolates, which might act as storage for excessive sulfur (Schnug, 1990, 1993). However, glucosinolates only form a small fraction of the total sulfur content and when *Brassica oleracea* was exposed to 0.2 µl l\(^{-1}\) \( \text{H}_2\text{S} \), an increase in glucosinolate content could not be observed (Westerman et al., 2001a).

**Aim and outline of the thesis**

In *Allium* species a substantial amount of the total sulfur may be present in the secondary sulfur compounds fraction as \( \gamma \)-glutamyl peptides and alliins (Schnug, 1993; Randle and Lancaster, 2002). These compounds are presumably pre-dominantly formed during the bulbing stage of the plant, and their content is dependent on the sulfur nutritional status of the plant (Randle et al., 1993, 1995; Randle and Lancaster, 2002). In view of the current recognition that these compounds might have significance as phytopharmaceutics, a better understanding of their occurrence and synthesis in other growth stages and plant parts is necessary. Studies on the possible interaction between atmospheric and pedospheric sulfur nutrition have shown to be helpful as tools to get insight into the regulation of sulfate uptake and sulfur assimilation and the dissection of the signal transduction pathways involved. Foliarly absorbed \( \text{H}_2\text{S} \) is directly incorporated into cysteine and subsequently into \( \gamma \)-glutamylecysteine and glutathione, which most likely are the direct precursors for
the synthesis of γ-glutamyl peptides and alliins. The general aim of this study was to get a better understanding of the regulation of sulfur metabolism in plants and specifically to investigate the significance of secondary sulfur compounds as possible sink for foliarly absorbed sulfur in onion and how this may affect the metabolism of H$_2$S and the uptake and assimilation of sulfate. Furthermore it might lead to a better understanding of the pathways of synthesis of secondary sulfur compounds in Allium.

Chapter 2 represents the used materials and methods. In Chapter 3 H$_2$S uptake kinetics were studied in onion. The impact of H$_2$S on growth and sulfur metabolism was studied in relation to the sulfur requirement. Long-term exposure revealed a strong impact of H$_2$S on sulfur metabolism, which was further examined in a comparison between different species and cultivars of Allium. In Chapter 4 short-term H$_2$S exposure and its impact on sulfur metabolism were studied in detail. The role of secondary sulfur compounds (γ-glutamyl peptides and alliins) as sink for reduced sulfur was discussed. The interaction between atmospheric and pedospheric sulfur nutrition was studied in order to investigate the regulation of sulfur metabolism and to get a better understanding of the fate of the incorporated H$_2$S. The role of H$_2$S as sulfur source for growth in sulfate-deprived plants is dealt with in Chapter 5. In Chapter 6 the metabolism of atmospheric sulfur gases was studied with emphasis on the toxicity of H$_2$S, the role of sulfolipids as sink pool for atmospheric H$_2$S and differences in metabolization between H$_2$S and SO$_2$. In Chapter 7 the impact of H$_2$S exposure and sulfate deprivation on sulfate uptake was investigated and the impact of H$_2$S exposure on the enzymes of sulfur assimilation was discussed. The decrease in accumulated sulfur compounds was studied in H$_2$S-exposed plants after cessation of the exposure in order to investigate the nature of the accumulated compounds and their impact on sulfur metabolism. In the general discussion in Chapter 8 the metabolism of H$_2$S in onion, the significance of secondary sulfur compounds as sink for reduced sulfur and the interaction between atmospheric and pedospheric sulfur nutrition was evaluated.