

REVIEW

Sulfur assimilation in plants and weed control: Potential targets for novel herbicides and action sites of certain safeners

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Sulfur is an indispensable element for plants. It is found in sulfur-containing amino acids, cysteine and methionine, and in various other important biochemical components and processes. Inhibitors of sulfur assimilation, or cysteine and methionine synthesis, could be potential herbicides. In the present paper, the sulfur assimilation pathway in plants is described, followed by the introduction of several compounds (inhibitors and safeners) acting on this pathway. Uptake of inorganic sulfate through the roots is the first step of sulfur assimilation in plants. Sulfate is reduced mainly in chloroplasts to sulfide by a multistep process, and sulfide is then incorporated into cysteine. Cysteine is converted to cystathionine, homocysteine and methionine. Cysteine is incorporated into glutathione (GSH) by γ -glutamylcysteine synthetase and GSH synthetase. Three enzymes involved in cysteine and methionine biosynthesis, cysteine synthase, cystathionine γ -synthase and cystathionine β -lyase, have been investigated as target sites for herbicides. Several inhibitors of these enzymes (e.g. rhizobitoxine and propargylglycine) were also phytotoxic, suggesting that the synthetic pathway of sulfur-containing amino acids could be a new target site for herbicides. Some safeners for herbicides were found to act on the sulfur assimilation pathway and on GSH synthesis to increase GSH, which can be involved in herbicide metabolism and detoxification. Several safeners elevate GSH levels by increasing the activities of enzymes involved in sulfur assimilation and GSH synthesis. Further studies on plant sulfur metabolism may lead to the discovery of new herbicides and to the comprehensive understanding of the mode of action of safeners.

Keywords: cysteine, glutathione, methionine, safener, sulfur assimilation, weed control.

INTRODUCTION

A wide variety of agrochemicals have been developed and used for weed control. These chemicals include herbicides and safeners. Herbicides control weeds by disrupting essential biochemical or physiological plant processes, and safeners protect crops from herbicide action mainly by enhancing herbicide detoxification. Biosynthetic pathways of amino acids are excellent herbicide targets. Indeed, various classes of herbicides that inhibit enzymes in amino acid biosynthesis pathways

have been developed and used in many crops. These target sites include acetolactate synthase (Muhitch *et al.* 1987; Brown & Cotterman 1994), EPSP synthase (Amrhein *et al.* 1980; Bradshaw *et al.* 1997) and glutamine synthetase (Wild & Wendler 1990). In addition, other enzymes involved in amino acid biosynthesis, such as isopropylmalate dehydrogenase in the leucine pathway (Wittenbach *et al.* 1994) and imidazoleglycerol phosphate dehydratase in the histidine pathway (Mori *et al.* 1995), have been explored as new herbicide target sites.

Similarly, inhibition of sulfur assimilation could be lethal to plants. Sulfur is an essential macronutrient for plant growth, and is contained in cysteine (Cys), methionine (Met) and a variety of essential metabolites derived from these two amino acids (Hell 1997; Leustek & Saito 1999;

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Saito 1999; Saito 2000). Several studies on the metabolic pathway of sulfur-containing amino acids for new molecular target sites of herbicide have been conducted (Datko & Mudd 1982; Hirase & Molin 2001a), and several inhibitors have been identified that have whole-plant activity (Datko & Mudd 1982).

Herbicide safeners are chemicals that enhance selectivity between crops and weeds (Hatzios 1989a). In certain instances the phytotoxicity of herbicides on crops can be greatly reduced with safeners, and weeds that are closely related to certain crops can be controlled selectively. Safeners might be used to expand the marginal selectivity of new chemistries, thereby allowing new herbicides to be developed, or to expand the use of established herbicides. Safeners can be applied to crops before planting as a seed dressing and to soil or crops as a package mixture with herbicides. Safeners are most commonly used in sorghum (*Sorghum bicolor* L.), corn (*Zea mays* L.), rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.). Safeners change the sensitivity of crops to herbicides by various mechanisms involving detoxification, altered transport, and herbicide antagonism. An increase in the rate of herbicide detoxification in safener-treated crops may be due to enhanced synthesis or activation of herbicide detoxifying enzymes. One of the well-known detoxification processes, which is enhanced by certain safeners, is glutathione (GSH) conjugation (Gronwald 1989; Hatzios 1989b). GSH conjugation is the key metabolic pathway contributing to the selectivity of thiocarbamate, chloroacetanilide, s-triazine, and other classes of herbicides between tolerant crops and susceptible weeds (Edwards & Dixon 2000; Hatzios 2001). The degree of protection from metolachlor injury conferred by safeners was strongly correlated with their ability to enhance glutathione S-transferase (GST), suggesting that GST is closely related to the action of safeners (Gronwald *et al.* 1987). In this case, sufficient GSH must be supplied for the conjugation. Sulfur assimilation seems to be important for GSH conjugation because GSH has the sulfur-containing amino acid, cysteine, and GSH content was increased in corn and sorghum treated with safeners such as dichlorimid and flurazole (Lay & Casida 1976; Gronwald *et al.* 1987).

In the past few years, remarkable progress has been made in comprehending the mechanism of sulfur assimilation and synthesis of sulfur-containing amino acids (Saito 2000). In the present review, sulfur assimilation pathways in plants are described as possible molecular target sites for herbicides and as a physiological process increasing herbicide selectivity by safeners.

SULFUR ASSIMILATION IN PLANTS

Sulfur assimilation in plants begins with the uptake of inorganic sulfate through the root cell plasma membranes by way of an active uptake mechanism. Sulfate is the major form of sulfur transport. Sulfate is reduced to sulfide in chloroplasts primarily by a multistep process, and sulfide is then incorporated into Cys. Cys may be converted to Met by three enzymatic steps, or into GSH by an alternate two-step process (Fig. 1).

Sulfate uptake and transport

Sulfate transporters localized in the membranes mediate the uptake of sulfate from the soil into the symplastic system. Transport is activated by a proton gradient main-

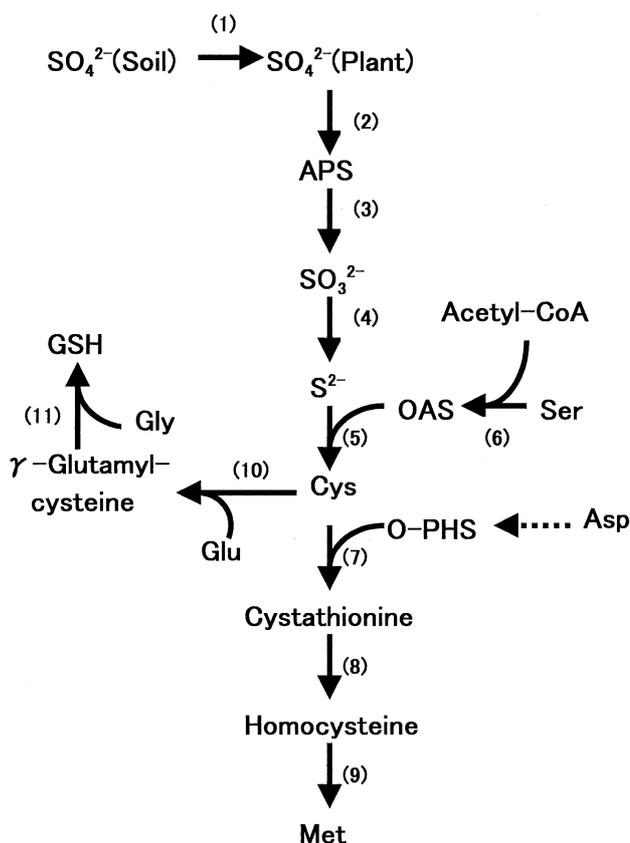


Fig. 1. Sulfur assimilation pathway in plants. Enzymes involved in this pathway are: (1) sulfate transporter; (2) ATP sulfurylase; (3) adenosine 5'-phosphosulfate (APS) reductase; (4) sulfite reductase; (5) cysteine synthase (CS); (6) Ser acetyltransferase (SAT); (7) cystathionine γ -synthase (CGS); (8) cystathionine β -lyase (CBL); (9) Met synthase; (10) γ -glutamylcysteine synthetase (GGCS); (11) glutathione (GSH) synthetase. OAS, O-acetylserine; O-PHS, O-phosphohomoserine.

tained across the plasma membrane by a proton-pumping ATPase. If the proton pump activity decreases, diminishing the proton gradient, then sulfate uptake is reduced or inhibited. Sulfate uptake is also inhibited by sulfite, selenate and molybdate. These anions compete with sulfate for binding to the transporter (Crawford *et al.* 2000).

Reduction of sulfate to sulfide

Sulfate reduction to sulfide, an energy-consuming process, requires 732 kJ mol^{-1} , and an adequate supply of ATP and reductant derived from photosynthesis (Crawford *et al.* 2000). In non-photosynthetic tissues, the reductant seems to be provided by the oxidative pentose phosphate pathway and respiration. Sulfate is first activated by the enzyme, ATP sulfurylase, to form adenosine 5'-phosphosulfate (APS) and pyrophosphate (PP_i). The equilibrium constant for ATP sulfurylase favors the reverse reaction; the forward reaction is facilitated by PP_i hydrolysis by a highly active pyrophosphatase and APS removal by APS kinase and APS reductase. Two isoforms of ATP sulfurylase were purified from spinach (*Spinacia oleracea* L.). The major isoform, which accounts for 85–90% of total ATP sulfurylase activity, exists in chloroplast, and the minor one is cytoplasmic. Both isoforms consist of four subunits, and each of these subunits is 49–50 kDa. Transgenic Indian mustard (*Brassica juncea* L.) over-expressing ATP sulfurylase accumulated more GSH than a normal plant (Pilon-Smit *et al.* 1999). Conversely, tobacco (*Nicotiana tabacum* L.) cells, which over-express ATP sulfurylase, did not show any enhancement of influx or sulfate content compared with a wild-type (Hatzfeld *et al.* 1998).

APS reductase catalyzes the conversion of APS to sulfite (Bick & Leustek 1998). This enzyme, which used to be called APS sulfotransferase (Suter *et al.* 2000), requires GSH for its catalytic activity (Bick *et al.* 1998). Activity of this enzyme is increased with sulfur starvation and is repressed by reduced forms of sulfur. Expression of this enzyme is also regulated by light and its activity follows diurnal cycles (Kopriva *et al.* 1999).

Sulfite reductase is responsible for the reduction of sulfite to sulfide (Bork *et al.* 1998; Yonekura-Sakakibara *et al.* 1998). This enzyme, which is found in plastids, is ferredoxin-dependent and adds six electrons to free sulfite to form sulfide. Ferredoxin is reduced directly by photosynthesis in leaves and indirectly by NADPH in roots (Yonekura-Sakakibara *et al.* 2000). The mRNA concentration encoding sulfite reductase in plants is not affected by sulfur starvation or reduced sulfur treatment (Crawford *et al.* 2000). Activity of this enzyme might be

maintained in excess to scavenge sulfite because sulfite is toxic and would damage cells if accumulated.

Cysteine synthesis

Cys synthesis is a key step in the pathway of sulfate assimilation. In this reaction, a sulfide moiety is incorporated into the β-position of alanine, and the carbon skeleton is derived from serine. Cys biosynthesis proceeds via two enzymatic reactions (Crawford *et al.* 2000). Serine acetyltransferase (SAT) catalyzes the acetylation of serine by acetyl-CoA and then Cys synthase (CS) catalyzes the reaction of O-acetylserine (OAS) with sulfide to yield Cys.

SAT and CS have been isolated and characterized separately from several plants (Droux *et al.* 1992; Ruffet *et al.* 1995). However, several studies have suggested that SAT and CS exist in an enzyme complex in chloroplasts (Ruffet *et al.* 1994; Droux *et al.* 1998; Crawford *et al.* 2000). The SAT-CS complex could function in the metabolic channeling of the intermediate OAS (Saito *et al.* 1995), thereby contributing to more efficient Cys formation (Bogdanova & Hell 1997). OAS concentrations in plants are assumed to be very low and OAS readily converts to N-acetylserine at alkaline pH (Ikegami *et al.* 1993). In addition, CS exhibits a relatively low affinity for OAS (Hirase & Molin 2001a). A high concentration of OAS can be maintained at the active site of CS by a SAT-CS complex (Droux *et al.* 1992). CS concentration in chloroplasts is, however, much higher than that of SAT, suggesting that a large amount of CS is present as a free form. The free CS may be responsible for the actual catalytic function, and the bound form of CS may modulate the SAT activity in the enzyme complex (Droux *et al.* 1998). CS requires pyridoxal-5'-phosphate (PLP) as a cofactor (Ikegami *et al.* 1993; Hirase & Molin 2001a).

Unlike the reduction of sulfate to sulfide, which takes place mainly in chloroplasts, SAT and CS are located in cytosol, mitochondria and chloroplasts (Lunn *et al.* 1990; Ruffet *et al.* 1995; Takahashi & Saito 1996; Noji *et al.* 1998; Hesse *et al.* 1999). A feedback regulation on cytosolic SAT by Cys was observed, but plastidic and mitochondrial SAT is not feedback inhibited (Noji *et al.* 1998). SAT activity was important in regulating the content of Cys and other thiols in plant tissue because the transgenic potato (*Solanum tuberosum* L.) plants expressing SAT gene from *Escherichia coli* increased the level of SAT activity 20-fold (Harms *et al.* 2000). Elevated levels of Cys and GSH in leaves were found in this transgenic potato (Harms *et al.* 2000). Although various kinds of sulfur-containing molecules are synthesized directly or

indirectly from Cys, only Met and GSH are considered in the present paper.

Methionine synthesis

Three enzymes catalyze Met biosynthesis from Cys; cystathionine γ -synthase (CGS), cystathionine β -lyase (CBL) and Met synthase (Azevedo *et al.* 1997; Matthews 1999). The first step in this pathway is catalyzed by CGS, which produces cystathionine from Cys and O-phosphohomoserine (O-PHS) through the sulfur linkage. O-PHS is derived from aspartate. Following this, the C₃ skeleton of Cys is cleaved by CBL so that the sulfur is attached to the homoserine carbon skeleton, forming homocysteine, pyruvate and ammonium. The final step in Met synthesis is catalyzed by Met synthase, which converts homocysteine to Met by tetrahydrofolate-mediated methylation. This process serves not only *de novo* Met synthesis but also the recycling of S-adenosylhomocysteine produced by methyl-transfer of S-adenosylmethionine (SAM).

CGS is localized in plastids in spinach leaves and *Arabidopsis thaliana* L. (Ravel *et al.* 1995a; Ravel *et al.* 1998a) and two isoforms of CBL have been found in plastids and cytosol in spinach (Droux *et al.* 1995). The plastidic isoform of CBL seems to contribute to the Met biosynthesis more efficiently (Ravel *et al.* 1995b; Ravel *et al.* 1998b; Turner *et al.* 1998). Both CGS and CBL are PLP dependent (Kreft *et al.* 1994; Ravel *et al.* 1996). Met synthase is located in cytosol (Ravel *et al.* 1998b). Met is incorporated into protein and is converted into SAM, which is a methyl donor in the synthesis of plant components such as lignin, choline and pectin. SAM is also a precursor of ethylene and polyamines.

Glutathione synthesis

GSH is synthesized from Cys in two steps. First, γ -glutamylcysteine synthetase (GGCS) forms a peptide bond between the γ -carboxyl group of glutamate and the α -amino group of cysteine, producing γ -glutamylcysteine. Next, GSH synthetase catalyzes the formation of another peptide bond between the cysteinyl carboxyl group of γ -glutamylcysteine and the α -amino group of glycine, forming GSH (Hell & Bergmann 1988, 1990; May *et al.* 1998). These two reactions are accompanied by ATP hydrolysis. Both enzymes are localized in plastids and cytosol, but apparently not in mitochondria (Hell & Bergmann 1988, 1990; Rügsegger & Brunold 1993).

GGCS is generally regarded as the rate-limiting enzyme in GSH biosynthesis, and the regulation is conducted

through feedback inhibition of GGCS by GSH. GGCS is inhibited by reduced GSH *in vitro* (Hell & Bergmann 1990), however, synthesis can continue even when the levels of GSH exceed the K_i of GGCS *in vivo*. Cys availability is the limitation factor for GSH levels in plants through kinetic restriction of GGCS. Overexpression of GGCS increased GSH in transgenic poplar hybrid (*Populus tremula* \times *Populus alba*), suggesting that the enhancement of the activity removes a major limitation over the rate of GSH synthesis (Strohm *et al.* 1995; Noctor *et al.* 1996). In contrast, GSH synthetase does not seem to limit GSH formation because a transgenic poplar overexpressing bacterial GSH synthetase did not increase GSH.

POTENTIAL TARGET SITE FOR HERBICIDES ON SULFUR ASSIMILATION PATHWAY

Three of the enzymes involved in the sulfur assimilation pathway have been investigated as target sites for herbicides. They are CS, CGS and CBL, all of which are located between sulfide and Met. This pathway does not occur in animals, therefore the enzymes catalyzing the reactions in this pathway are considered to be attractive targets for the development of herbicides. Possible inhibitors (Table 1, Fig. 2) of these enzymes were prepared, and their inhibitory activities and phytotoxicity were examined.

Cysteine synthase

Cys plays important roles in most organisms as a precursor of protein Cys, GSH, and the sulfur moiety of Met. CS is considered to detoxify toxic levels of sulfide or sulfite by incorporating them into Cys (Saito *et al.* 1994; Youssefian *et al.* 1993). Inhibition of CS may cause injury in plants because of the starvation of these amino acids or disturbance of sulfur assimilation process. Indeed, the inhibition of CS gene expression in plant seedlings results in severe developmental abnormalities (Kloti *et al.* 2002). Cys is not an essential amino acid but does not exist in animals, which can synthesize Cys from Met. Multi-heterocyclic compounds (Compounds A, B and C, Fig. 2) inhibited CS isolated from spinach leaves (Imanaka 1991). The K_i values of Compounds A, B and C were 16 nM, 2.8 μ M and 22 nM, respectively. Phytotoxic activity of Compound B was examined in tobacco cell culture. It inhibited cell growth at 1 p.p.m. and completely killed the tobacco cells at 10 p.p.m. Several naphthalenecarboxylic acids also inhibited CS (Imanaka & Hiramatsu 1991). Compound D, a representative compound of this class, inhibited CS isolated

Table 1. Inhibitors of sulfur assimilation enzymes

Enzyme	Inhibitor	Reference
Cysteine synthase	Multi-heterocyclic compounds (Compounds A, B and C in Fig. 2)	Imanaka (1991)
	Naphthalenecarboxylic acids (Compound D in Fig. 2)	Imanaka & Hiramatsu (1991)
Cystathionine γ -synthase	Amino-oxyacetate, hydroxylamine	Hirase & Molin (2001b)
	Amino-oxyacetate, hydroxylamine	Kreft <i>et al.</i> (1994)
	Propargylglycine	Clausen <i>et al.</i> (1999), Kreft <i>et al.</i> (1994)
	3-(phosphonomethyl)pyridine-2-carboxylic acid (PPCA)	Clausen <i>et al.</i> (1999)
	4-(phosphonomethyl)pyridine-2-carboxylic acid	Clausen <i>et al.</i> (1999), Kreft <i>et al.</i> (1994)
	Z-3-(2-phosphonoethen-1-yl)pyridine-2-carboxylic acid	Clausen <i>et al.</i> (1999), Kreft <i>et al.</i> (1994)
Cystathionine β -lyase	DL-E-amino-5-phosphono-3-pentenoic acid (APPA)	Clausen <i>et al.</i> (1999), Kreft <i>et al.</i> (1994)
	5-carboxymethylthio-3-(3'-chlorophenyl)-1,2,4-oxadiazol (CTCPO)	Steebhorn <i>et al.</i> (2001)
	Rhizobitoxine	Giovanelli <i>et al.</i> (1971)
γ -Glutamylcysteine synthetase	L-aminoethoxyvinylglycine (AVG)	Ravanel <i>et al.</i> (1996)
	β -cyanoalanine	Giovanelli <i>et al.</i> (1971)
	Buthionine sulfoximine (BSO)	Hell & Bergmann (1990)

from spinach leaves by 81% at 40 μ M. It inhibited the growth of tobacco cultured cells at 10 p.p.m.

Because CS requires PLP as a cofactor, the effect of inhibitors of PLP dependent enzymes on CS was examined. Amino-oxyacetate (AOA) and hydroxylamine, non-specific inhibitors of PLP-dependent enzymes, inhibited CS extracted from leaves of barnyardgrass (*Echinochloa crus-galli* L.), and the degree of inhibition increased with the incubation period of the enzyme and inhibitors (Hirase & Molin 2001a, 2001b). These two compounds inhibited shoot growth of barnyardgrass in a whole-plant bioassay. In the same experiment, allylglycine, β -chloroalanine and 3-bromopropionate slightly inhibited CS and had low phytotoxicity *in vivo* (Hirase & Molin 2001b).

Cystathionine γ -synthase

CGS and CBL are important enzymes in Met biosynthesis. Inhibition of these enzymes may cause plant growth inhibition by Met starvation. In addition to being a protein constituent, Met plays important roles in the initiation of mRNA translation as a terminal amino acid, and serves as a precursor of the methylating agent of SAM, polyamines and the plant hormone, ethylene. The antisense suppression of CGS encoding gene resulted in severe growth reduction, which is restored by the exogenous addition of Met

(Ravanel *et al.* 1998b; Kim & Leustek 2000). This finding suggests that CGS is a potential target site for herbicides.

CGS, like CS, is PLP-dependent. The enzyme extracted and purified from wheat was inhibited by AOA and hydroxylamine (Kreft *et al.* 1994). Propargylglycine, produced by *Streptomyces* (Scanell *et al.* 1971), inhibits CGS from tobacco and wheat (Kreft *et al.* 1994; Clausen *et al.* 1999). In this case, CGS is inhibited by propargylglycine in a time-dependent manner, and the inactivation followed pseudo first-order kinetics. This inactivation appears to be irreversible (Clausen *et al.* 1999; Kreft *et al.* 1994). Propargylglycine produced rapid and very severe growth inhibition of *Lemna paucicostata*. Inclusion of Met or cystathionine in the growth medium of *L. paucicostata* completely prevented these effects (Datko & Mudd 1982).

The effect of substrate analogs on CGS was examined. Analogs of O-PHS, such as 3-(phosphonomethyl)pyridine-2-carboxylic acid (PPCA), 4-(phosphonomethyl)pyridine-2-carboxylic acid, Z-3-(2-phosphonoethen-1-yl)pyridine-2-carboxylic acid and DL-E-amino-5-phosphono-3-pentenoic acid (APPA), were found to be reversible inhibitors of CGS (Kreft *et al.* 1994; Clausen *et al.* 1999). In tobacco, the inhibition by these four compounds was competitive with O-PHS ($K_i = 0.2, 0.3, 0.45$ and 0.027 mM, respectively), and

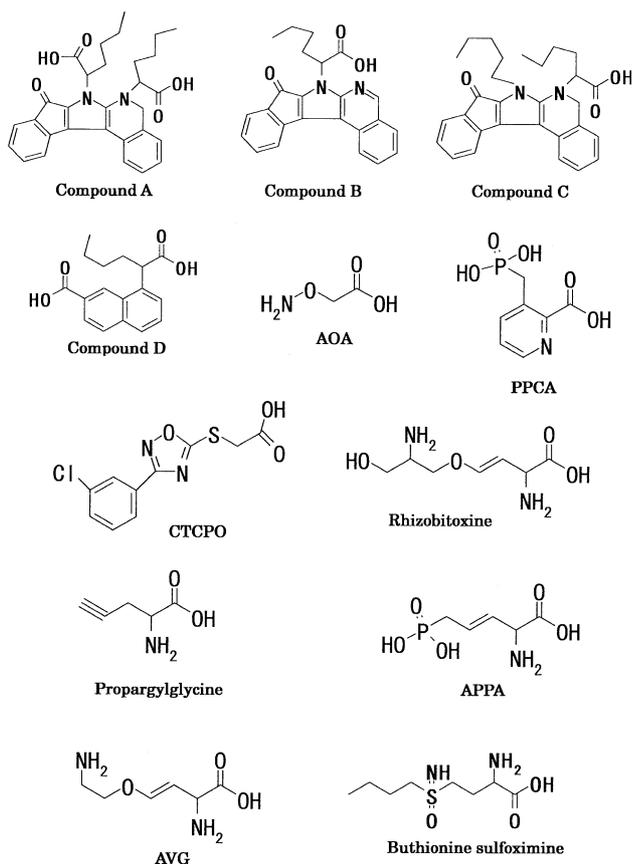


Fig. 2. Chemical structures of inhibitors acting on sulfur assimilation and the sulfur-containing amino acid synthesizing pathway.

non-competitive with L-Cys ($K_i = 0.46, 0.51, 0.96$ and 0.042 mM, respectively; Clausen *et al.* 1999).

An oxadiazol compound, 5-carboxymethylthio-3-(3'-chlorophenyl)-1,2,4-oxadiazol (CTCPO), was identified as a competitive inhibitor of CGS with a K_i value of $2 \mu\text{M}$ (Steegeborn *et al.* 2001). The affinity of this compound for CGS is therefore higher than those of substrate analogs mentioned earlier. CTCPO represents a novel class of CGS inhibitors that only have a carboxyl group in common with the substrate O-PHS. This class of compounds has been patented as herbicides (Laber *et al.* 2001). The interaction between PPCA, APPA and CTCPO with CGS was studied in detail using crystal structures of enzyme-inhibitor complexes (Steegeborn *et al.* 2001).

Cystathionine β -lyase

Rhizobitoxine (Fig. 2) inactivated CBL purified from spinach leaves *in vitro* (Giovanelli *et al.* 1971) and it par-

tially inhibited the enzyme of spinach and corn seedlings *in vivo* (Giovanelli *et al.* 1973). In the latter case, treatment of corn seedlings with rhizobitoxine leads to accumulation of increased amounts of cystathionine (Giovanelli *et al.* 1973). Rhizobitoxine is an enol-ether amino acid (Owens *et al.* 1972) produced by a root nodule bacterium, *Rhizobium japonicum*. This amino acid passes from the bacteria in the nodule to the leaves of the host soybean (*Glycine max* L.) plant, and inhibits greening of new leaves (Owens & Wright 1965b). Rhizobitoxine isolated from the culture medium (Owens & Wright 1965a) is phytotoxic to soybean and other higher plant species (Owens 1973). This compound also inhibits the growth of green alga *Chlorella* (Owens 1969) and *Salmonella typhimurium* (Owens *et al.* 1968). Growth inhibition of *S. typhimurium* was prevented by the supplementation of homocysteine or Met, but not by serine, homoserine or cystathionine. Inactivation of CBL seems to be irreversible (Giovanelli *et al.* 1971).

CBL of *Arabidopsis thaliana* expressed in *Escherichia coli* was isolated, and found to be inhibited by L-aminoethoxyvinylglycine (AVG). This inactivation seemed to be irreversible (Ravel *et al.* 1996). AVG produced a progressive inhibition on the growth of *Lemma paucicostata*, and the inclusion of Met in the medium almost completely prevented these effects and permitted continued growth (Datko & Mudd 1982). In addition, β -cyanoalanine is thought to be a competitive inhibitor of spinach CBL (Giovanelli *et al.* 1971).

γ -Glutamylcysteine synthetase

Buthionine sulfoximine (BSO), known as a potent inhibitor of GGCS from mammalian cells (Griffith & Meister 1979), was found to be an effective inhibitor of GGCS from tobacco cell suspension cultures (Hell & Bergmann 1990). GGCS does not seem to be a target site for herbicides because BSO did not impair plant growth (Farago *et al.* 1993). However, GSH levels in plant tissue are important for herbicide selectivity. Inhibitors of GSH biosynthesis may enhance the efficacy of herbicides that are detoxified by GSH conjugation. Indeed, when metolachlor and various levels of BSO were applied together, the growth of corn shoots was inhibited with increasing concentrations of BSO. This growth inhibition significantly correlated with the decreased GSH content (Farago *et al.* 1993). Therefore, it is suggested that GGCS inhibitors could act as non-phytotoxic additives able to induce herbicide synergy by the inhibition of GSH synthesis (Farago *et al.* 1993).

EFFECT OF SAFENERS ON THE SULFUR ASSIMILATION PATHWAY

Safeners are compounds that protect crops from injury by herbicides (Hatzios 1989a) and many studies suggest that this protection is mediated by enhancing herbicide detoxification (Hatzios 1989a, 1989b). Herbicides, such as chloroacetanilides, can be detoxified by direct or GST catalyzed conjugation with GSH. If conjugation of herbicides with GSH is a major mechanism of detoxification, then sufficient GSH must be available for conjugation. Indeed, GSH content was increased in plant tissues by several safeners (Lay & Casida 1976; Gronwald *et al.* 1987). These safeners may promote sulfur assimilation and subsequent GSH biosynthesis. In this section, sulfur assimilation and GSH biosynthesis processes that are influenced by safeners (Figs 3 and 4) are introduced.

Sulfate uptake and its assimilatory reduction to cysteine

Dichlormid and R29148 (3-dichloroacetyl-2,2,5-trimethyloxazolidine) slightly increased the uptake of [³⁵S]sulfate in corn roots at p.p.m. levels (Adams *et al.* 1983). Content of free sulfate in roots, however, was decreased by dichlormid, and the decrease was closely associated with an increase in Cys and GSH levels, sug-

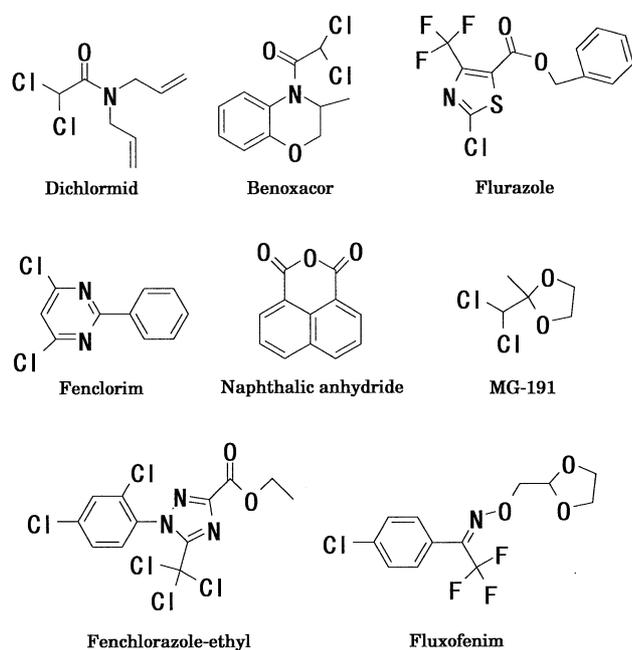


Fig. 3. Chemical structures of safeners acting on sulfur assimilation and the glutathione (GSH) synthesis pathway.

gesting that the safener acts at a step in the conversion of sulfate to Cys (Adams *et al.* 1983). Indeed, dichlormid added to the culture medium at 3 p.p.m. increased extractable activity of ATP sulfurylase, the first enzyme of assimilatory sulfate reduction, in corn shoots (Adams *et al.* 1983). In another study, dichlormid and benoxacor at 1 and 0.01 mM, respectively, elevated Cys and GSH levels in corn seedlings by increasing the activities of ATP sulfurylase and APS reductase (Farago & Brunold 1990). In this case, both safeners affected APS reductase more than ATP sulfurylase activity. This result is consistent with other results from various systems, all of which showed that ATP sulfurylase is less susceptible to regulatory signals than APS reductase (Brunold & Schmidt 1976; Brunold *et al.* 1981).

When flurazole was applied to sorghum seeds at 1.25 g kg⁻¹ seed, the extractable CS activity in the shoots increased (Hirase & Molin 2001c). A dose-response between flurazole treatment and CS activity was found. The increase in extractable CS activity was also caused

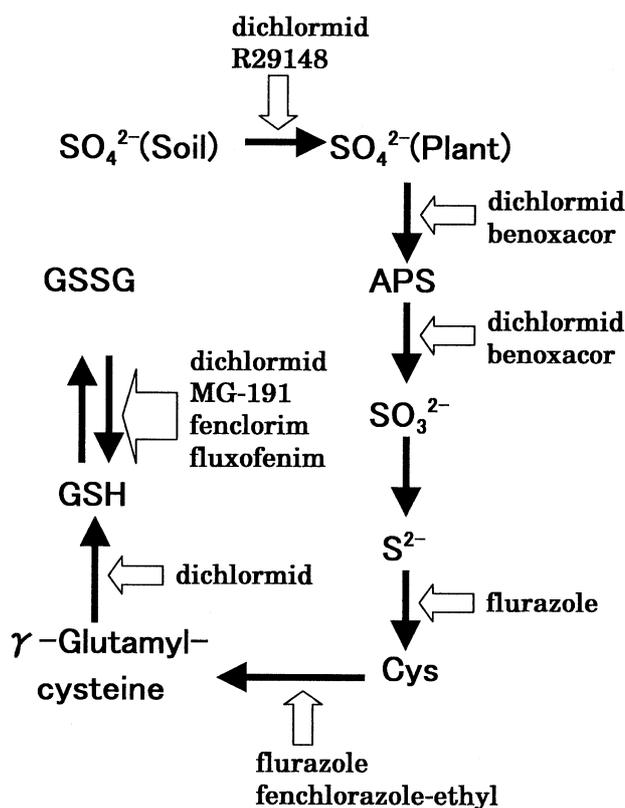


Fig. 4. Action site of safeners in sulfur assimilation and GSH biosynthesis pathway. APS, adenosine 5'-phosphosulfate; Cys, cysteine; GSH, glutathione; GSSG, oxidized GSH.

by seed treatments of fluxofenim, naphthalic anhydride, benoxacor and dichlormid in sorghum (Hirase & Molin 2001c). CS substrate, OAS, is produced by SAT from serine and acetyl-CoA. *In vitro* assay procedures for measuring the activity of Cys biosynthesis from serine (CBS), which is a coupled reaction catalyzed by SAT and CS, were developed using crude extracts from sorghum shoots. Flurazole seed treatment enhanced CBS activity and non-protein thiol content, which presumably contributed to herbicide tolerance in sorghum (Hirase & Molin 2002).

Glutathione synthesis

Corn roots pretreated with dichlormid showed no significant increase or decrease in GSH synthetase activity, but *in vitro*, the activity of this enzyme was increased by dichlormid at 5–500 nM (Carringer *et al.* 1978). These data indicate a possible allosteric modification of the enzyme that increases the rate of GSH synthesis.

In corn, GSH conjugate of flurazole is one of the initially detected metabolites (Breux *et al.* 1989). The GSH conjugate may interfere with the normal regulation of GSH biosynthesis in corn. Regulation is made through feedback inhibition of GGCS by GSH (Noctor *et al.* 1996). GSH conjugates of xenobiotics can bind to GGCS and override the feedback inhibition caused by GSH (Kondo *et al.* 1984). Thus, the GSH conjugate of flurazole may bind to GGCS, thereby circumventing the feedback regulation of the GSH biosynthesis pathway (Breux *et al.* 1989).

Fenclorazole-ethyl has been shown to eliminate the phytotoxicity of fenoxaprop-ethyl in crops such as wheat and barley (*Hordeum vulgare* L.) and it also increased the rates of herbicide conjugation with GSH (Tal *et al.* 1993). Seed treatment of fenclorazole-ethyl increased the levels of GSH and decreased those of Cys in wheat and barley (Tal *et al.* 1995). This safener may also interfere with the normal feedback inhibition of GGCS and thus increase GSH production.

Exogenous Cys or precursors of Cys may increase the levels of GSH, and protect plants from herbicide injury. Indeed, exogenous Cys supplied to poplar leaf discs increased GSH content, suggesting that GSH synthesis is limited by Cys availability (Noctor *et al.* 1996). Applications of L-2-oxothiazolidine-4-carboxylic acid (OTC) increased GSH levels in mouse livers and protected the animals against the toxic effects of acetaminophen which conjugates with GSH (Williamson *et al.* 1982). OTC is enzymatically converted to S-carboxy-L-cysteine, which spontaneously decarboxylates to yield Cys. Cys is then

incorporated into GSH. Addition of OTC to intact and excised corn roots increased levels of GSH. OTC also partially circumvented the growth inhibition of corn, wheat and sorghum caused by tridiphane (Hilton & Pillai 1986).

Glutathione reductase

Additionally, safeners may increase GSH levels in treated plants indirectly by enhancing the activity of glutathione reductase (GR). GR is a NADPH-dependent enzyme that reduces oxidized glutathione (GSSG) to GSH. Increases in GR activity will maintain a high GSH/GSSG ratio in safener-treated plants to utilize GSH for the conjugation. GR activity was stimulated by dichlormid and MG-191 in corn (Kömives *et al.* 1985), by fenclorim in rice (Han & Hatzios 1991), and by fluxofenim in sorghum (Yenne & Hatzios 1990).

CONCLUSION

Sulfur assimilation and synthesis of sulfur-containing amino acids are very important for plant growth, along with various other physiological and biochemical steps. Inhibition of sulfur metabolism causes plant injury and such inhibitors seem to be potential herbicides. Further studies are anticipated to discover new target sites for herbicides acting on sulfur metabolism. Sulfur assimilation is also related to the mode of action for safeners that protect crops by GSH conjugation. Several steps from sulfate uptake through GSH synthesis have been revealed as action sites for safeners, but many other steps should be examined to depict the whole mechanism of action for safeners acting through GSH conjugation.

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