Herbicide Safeners : Recent Advances and Biochmical Aspects of Their Mode of Action

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Abstract

Herbicide safeners are a group of chemically diverse compounds with the ability to improve the crop selectivity of certain herbicides. Several agrochemical companies have commercially developed safeners for thiocarbamate, chloroacetanilide, and aryloxyphenoxypropionate herbicides in major monocotyledonous crops including maize, small grain cereals, rice, and grain sorghum. Safeners are employed to extend the use of available herbicides on additional crops and to exploit new herbicides with marginal crop selectivity. There is considerable evidence that safeners act by accelerating herbicide metabolism and detoxification in crop plants. Metabolic reactions that are enhanced include oxidation (hydroxylation, oxidative dealkylation) and conjugation to endogenous moieties such as glucose and glutathione. A number of enzymes involved in these metabolic pathways, such as glutathione S-transferase isozymes and various forms of cytochrome P 450-dependent monooxygenase activities, have been shown to be induced by safeners. Safeners have also been reported to increase the biosynthesis and accumulation of glutathione, and to enhance the process of compartmentation of herbicide conjugates. The botanical specificity of safener protection has been investigated in the light of the recently developed aryloxyphenoxypropionate herbicide and safener combination, clodinafopropargyl and cloquintocet-mexyl (Topik®). The herbicide exhibits different metabolic routes in wheat and in target weeds, and only crop specific herbicide metabolism is enhanced by the safener.
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Key words: safener (glutathione S-transferase, glutathione, cyt P-450 monooxygenase), Topik

Introduction

Weed management in modern agriculture requires efficient weed control technologies that are safe to the crop. The search for environmentally compatible herbicides with high biological activity and crop tolerance is a challenge for agrochemical research. Insufficient crop tolerance is one of the major constraints in the development of new herbicides and in the use of existing herbicides in particular crops or under unfavourable environmental conditions. Based on the available knowledge of the mechanisms of herbicide selectivity, the chemical optimization of lead structures for crop tolerance will in the foreseeable future depend more on broad-based synthesis and testing of herbicidal molecules rather than on "rational design" (Brown *et al*., 1991). Recent efforts are thus aimed at protecting crops from herbicidal injury by means of selection or genetic engineering of herbicide-tolerant crop cultivars (Hinchee *et al*., 1993) Another approach is to improve crop tolerance to new and existing herbicides by using herbicide safeners.

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The safener concept

Herbicide safeners are a group of chemically diverse compounds with the unique ability to protect crop plants from injury by certain herbicides without impairing weed control efficacy. The safener concept has been established by the pioneering work of O. L. Hoffmann (Stephenson and Yancoby, 1991). After the initial discoveries of naphthalic anhydride (naphthalene-1, 8-carboxylic anhydride) and dichlormid (N, N-diallyl-2, 2-dichloroacetamide) as herbicide safeners, a number of other safeners have been developed for all major monocotyledonous crop species including maize, small grain cereals, rice and grain sorghum. In most crop-weed associations, selective improvement of crop tolerance can be achieved with safeners applied as mixed formulations with the herbicide; however, seed-treatment with safeners is also currently used, mainly in grain sorghum, to protect only the crop and not botanically closely related weeds. Safeners have been exploited in two ways: to improve the tolerance of newly developed herbicides with limited selectivity on target crops, and to extend the use of available herbicides on additional crops.

In the past, the search for safeners has been most successful for pre-plant soil incorporated and pre-emergence herbicides of the thiocarbamate and chloroacetanilide classes in maize, and for chloroacetanilides in sorghum and wet-sown rice. More recently, safeners have also been developed for post-emergence grass weed control in cereals in combination with aryloxyphenoxypropionate herbicides. Protection by various safeners has also been reported for members of several other herbicide classes such as the sulfonylureas, imidazolinones, cyclohexanediones, and isoxazolidinones.

Mechanism of action of herbicide safeners

Several hypotheses have been advanced for the mechanism (s) of the protective action of herbicide safeners. It has been suggested that safeners may reduce herbicide uptake or translocation to sensitive site (s) within the plant, or increase the rate of herbicide metabolism and detoxification. Alternatively, safeners may prevent binding of the herbicide to the cellular target site, or antagonize herbicidal effects at the physiological level. Finally, a combination of several of these factors may be encountered. Understanding of herbicide-safener interactions is hampered by a general lack of exact knowledge of the herbicidal mechanisms ultimately leading to plant injury. However, there is a large body of evidence that the hitherto commercialized safeners protect crop plants by enhancing herbicide metabolism and detoxification.

1. Enhancement of herbicide metabolism

Differences in herbicide metabolism and detoxification between tolerant and susceptible plant species have long been recognized as one important mechanism contributing to the selective action of many herbicides (Owen, 1987). Biotransformations of herbicides in plants generally include oxidation, hydrolysis, reduction (rarely), and conjugation to e.g. glutathione, glucose, or amino acids. To date, two different metabolic pathways have been related to the mode of action of herbicide safeners. The first represents the conjugation of chloroacetanilide and sulfoxidized thiocarbamate herbicides with glutathione; the second includes hydroxylation and subsequent glucose conjugation. The latter pathway appears to be important predominantly in safener protection to aryloxyphenoxypropionate, sulfonylurea, and imidazolinone herbicides.

2. Glutathione conjugation and glutathione S-transferases

Conjugation of herbicides *via* the thiol function of reduced glutathione (γ-glutamylcysteinylglycine) is well established as one of the major detoxification and selectivity factors in plants (Lamoureux *et al.*, 1991). Though glutathione conjugations can proceed non-enzymatically at appreciable rates with some substrates, the reactions are usually accelerated through catalysis by glutathione S-transferase enzymes (EC 2.5.1.18). The rate of glutathione conjugation of herbicides in plants may be regulated, in principle, by both glutathione S-transferase level and activity as well as by glutathione availability.

Chloroacetanilide herbicides are initially metabolized in plants through glutathione conjugation by nu-

cleophilic displacement of chlorine from the chloroacetyl side chain, without the need for a preceding metabolic step to increase electrophilicity. Fürst and Gronwald (1986) have shown that protection of sorghum from metolachlor injury by oxabetrinil and other safeners is closely correlated with their ability to accelerate metolachlor metabolism in shoot tissues. Maize exhibits a reduced tolerance to metolachlor and other chloroacetanilide herbicides under certain adverse growing conditions such as high soil moisture and low soil temperature before seedling emergence. Effects of soil temperature have been related in part to a slower rate of herbicide metabolism, as well as to greater herbicide exposure due to slower seedling emergence (Viger *et al.*, 1991). The safener benoxacor protects maize from metolachlor injury under a wide range of environmental conditions (Peek *et al.*, 1988). The predominant site of uptake of pre-emergence applied chloroacetanilides is the coleoptile of germinating grass species, while the primary anatomical sites affected are the enclosed developing leaves and apical and intercalary meristems (LeBaron *et al.*, 1988). After shoot application of metolachlor a comparatively small proportion of the herbicide moved into the enclosed developing leaves, and most of the absorbed herbicide was retained in the coleoptile and was metabolized there *via* glutathione conjugation (Kreuz *et al.*, 1989). The developing leaves, however, were found to be relatively slow to metabolize metolachlor as compared to the coleoptile. They also exhibited the lowest glutathione S-transferase activity of all seedling tissues examined. Benoxacor significantly reduced the concentration of unmetabolized metolachlor mainly in the developing leaves, but also in the coleoptile and mesocotyl, as a consequence of enhanced metabolism. Activity of glutathione S-transferase accepting metolachlor as substrate was increased fivefold in seedling shoots upon benoxacor treatment (Kreuz *et al.*, 1989; Viger *et al.*, 1991). Similar results were reported from studies with metazachlor and the safener BAS 145138 (1-dichloroacetylhexahydro-3,3,8 α-trimethylpyrrolo [1, 2 α] -pyrimidin-6-(2 H)-one) in maize (Fuerst and Lamoureux, 1992).

In maize, sorghum and rice, herbicide safeners increase the extractable glutathione S-transferase activities as evaluated with the model substrate 1-chloro-2, 4-dinitrobenzene, chloroacetanilide herbicides, and EPTC-sulfoxide (maize only). Constitutive and safener-induced glutathione S-transferases from sorghum and maize have been distinguished based on chromatographic elution characteristics and differential activities towards various substrates (Dean *et al.*, 1990; Fuerst *et al.*, 1993). Maize contains at least two major constitutive glutathione S-transferase isozymes accepting metolachlor as substrate and whose activities were enhanced by treatment of seedlings with benoxacor. A third such isozyme was found to be absent constitutively and highly induced by benoxacor (Fuerst *et al.*, 1993). Complementary DNAs for two maize glutathione S-transferases have been cloned and the deduced amino acid sequences were shown to possess some similarity to each other as well as to animal glutathione S-transferases (reviewed by Timmerman, 1989). Safener induction of glutathione S-transferase activity is associated with a net accumulation of enzyme protein and requires *de novo* protein synthesis. The steady-state levels of messenger RNA coding for a particular isozyme subunit were increased in maize treated with dichlormid or flurazole (benzyl 2-chloro-4-trifluoromethylthiazole-5-carboxylate), which suggests that regulation of enzyme synthesis by safeners is exerted at the level of gene transcription.

3. Regulation of glutathione levels and biosynthesis

Safeners have repeatedly been shown to increase the tissue concentration of reduced glutathione in plants, yet the significance of elevated glutathione levels for safener action is still uncertain. A weak correlation has been found between the increase in glutathione content of sorghum shoots and the degree of protection from metolachlor injury conferred by a particular safener (Gronwald *et al.*, 1987). On the other hand, decreased glutathione contents of maize shoots due to treatment with buthionine sulfoximine, an inhibitor of γ-glutamylcysteine synthetase, are correlated with increased metolachlor susceptibility (Farago *et al.*, 1993). In roots of maize seedlings, dichlormid and benoxacor increased the contents of free cysteine and glutathione and enhanced the biosynthesis of these thiols from inorganic sulfate (Farago and Brunold, 1990; 1994). This was attributable to an increase in the extractable activities of adenosine 5'-phosphosulfate sulfotransferase and ATP-sulfurylase (EC 2.7.7.4) two key regulatory enzymes of assimilatory sulfate reduction and γ-glutamylcysteinesynthetase. Glutathione reductase activity (EC 1.6.4.2) has also been reported to be enhanced in the shoots of safener-treated maize seedlings (Komives *et al.*, 1985).

4. Oxidative metabolism and cytochrome P 450 monooxygenases

Oxidation and subsequent glucose conjugation constitute a very important pathway responsible for herbicide selectivity that has recently also been associated with safener action. Oxidation appears not always to afford complete herbicide detoxification, but is frequently a necessary and rate-limiting step for subsequent glucose conjugation. Safeners have been shown to stimulate oxidative metabolism of herbicides belonging to the groups of sulfonylureas, imidazolinones and aryloxyphenoxypropionates in plants *in vivo* (Hatzios, 1991).

There is accumulating evidence that cytochrome P 450-dependent monooxygenases (EC 1.14.14.1) play a pivotal role in the oxidation of many herbicides in plants. The cytochromes P 450 found in plants are like the well-characterized ones in the endoplasmic reticulum from mammalian liver, membrane-bound haemoproteins of approximately 55 kDalton molecular mass (Donaldson and Luster, 1991). They require molecular oxygen for catalytic activity, NADPH, and a second protein component, the flavoprotein NADPH-cytochrome P 450 reductase. Reactions mediated by plant cytochrome P 450-monooxygenases on herbicide substrates include aryl and alkyl hydroxylations and oxidative N-and O-demethylations. Conclusive evidence for the involvement of cytochrome P 450-monooxygenases in plant herbicide metabolism has been obtained for 2, 4-D, diclofop, bentazone, flumetsulam (N-(2, 6-difluorophenyl)-5-methyl-1, 2, 4-triazolo [1, 5-α] pyrimidine-2-sulfonamide), metolachlor and members of the phenylurea and sulfonylurea herbicide classes (Moreland *et al.*, 1993 and references cited therein ; Frear *et al.*, 1993). The criteria that have been employed to demonstrate cytochrome P 450 monooxygenase catalysis in those reactions include spectral evidence, photoreversible inhibition of these reactions by carbon monoxide, requirement for molecular oxygen and NADPH, sensitivity to known cytochrome P 450 inhibitors and involvement of NADPH-cytochrome P 450 reductase activity. Despite considerable experimental difficulties, e.g. low constitutive enzyme activity levels, apparent instability of the enzymes and presence of endogenous inhibitors in crude microsomal preparations, much effort has been devoted in recent years to elucidate the putative multiplicity of cytochrome P 450 isoforms and the regulation implicated in safener action. These studies have revealed that microsomes isolated from plants treated with herbicide safeners or other xenobiotics, such as ethanol and phenobarbital, contain elevated cytochrome P 450 monooxygenase activities towards particular substrates (Fonne-Pfister *et al.*, 1990 ; Frear *et al.*, 1991 ; Moreland *et al.*, 1993). Studies in grain sorghum have shown that microsomal cytochrome P 450-linked metabolism of bentazone (hydroxylation), diazinon (desulfuration and oxidative dearylation) and lauric acid (in-chainhydroxylation) is stimulated to varying degrees by a number of safeners applied to the seed prior to planting (Moreland *et al.*, 1993). Cytochrome P 450-mediated oxidation of several herbicides and its induction by naphthalic anhydride, ethanol, or phenobarbital has been demonstrated in wheat (Frear *et al.*, 1991). In this system, increases in monooxygenase activities as determined with diclofop, chlorsulfuron and triasulfuron as substrates ranged from five to twentyfold, depending on the particular substrate and on the inducer employed. Interestingly, strong evidence has been obtained that in wheat diclofop aryl hydroxylase is identical with lauric acid (ω-1) hydroxylase which, for the first time, links a herbicide-metabolizing monooxygenase activity to a particular cytochrome P 450 isoform that participates in a defined physiological reaction (Zimmerlin and Durst, 1992). Both enzyme activities exhibited similar induction patterns with naphthalic anhydride and phenobarbital.

5. Glucose conjugation

Metabolism of herbicides to derivatives containing free hydroxy groups is generally followed by extensive carbohydrate conjugation, with O-β-D-glucosides representing the most common group of these conjugates (Lamoureux *et al.*, 1991). Glucose conjugation seems in some cases necessary to complete herbicide detoxification ; since free hydroxylated metabolites do not commonly accumulate to significant levels in plants, glucosylation appears *prima facie* not an important site for safener action. During the metabolism of chlorimuron-ethyl in maize, however, hydroxylation at the 5-position of the pyrimidine ring yielded the major metabolite, 5-hydroxychlorimuron-ethyl (Lamoureux and Rusness, 1992). Chlorimuron-ethyl causes injury to maize that can be alleviated by BAS 145138. This safener increased the capacity for 5-pyrimidyl-O-glucoside formation from chlorimuron-ethyl, and feeding experiments with 5-hydroxychlorimuron-ethyl revealed that the *in vivo* rate of glucosylation was indeed accelerated. The

glucose conjugate was less inhibitory *in vitro* towards the target enzyme, acetolactate synthase, as compared to the free hydroxychlorimuron-ethyl. Interestingly, the safener BAS 145138 increased, in addition to glucose conjugation, the capacity for pyrimidine-ring hydroxylation as well as glutathione conjugation of chlorimuron-ethyl in maize. Evidence for a safener-induced increase in the rate of glycosylation has also been obtained with a hydroxylated aryloxyphenoxypropionate herbicide in wheat (Kreuz *et al.*, 1991).

6. Secondary metabolism of herbicide conjugates and compartmentation processes

Glutathione conjugates of herbicides in plants usually undergo extensive processing to e.g. cysteine or thiolactic acid derivatives and conjugates thereof with malonate, to name but a few (Lamoureux *et al.*, 1991). Simple glucose conjugates are frequently subject to secondary conjugations to carbohydrate or malonyl residues. Terminal products may be stored as soluble metabolites, presumably in the vacuole, or deposited as "bound residues" into cell wall components. Formation of soluble secondary metabolites and bound residues from the initial glutathione conjugates of propachlor and metolachlor in maize was only marginally influenced by the safener BAS 145138 and therefore appeared to be of minor significance for safener action (Khalifa and Lamoureux, 1990).

Circumstantial evidence indicates a reduced mobility in the plant of herbicide conjugates as compared to the parent herbicides (see e.g. Fuerst and Lamoureux, 1992). This is conceivably due to the lower membrane permeability of hydrophilic conjugates, but specific compartmentation processes have also been inferred. The commonly observed addition of a malonyl residue to initially formed glucose conjugates or glutathione-derived cysteine and thiolactic acid conjugates has been proposed to be a mechanism to facilitate transport into the vacuole (Lamoureux *et al.*, 1991). However, vacuolar localization of herbicide conjugates has rarely been demonstrated, and transport processes have apparently not been investigated as yet. Only recently, active transport of the glutathione conjugates of metolachlor and other xenobiotics into the plant vacuole has been discovered and shown to be mediated by an ATP-dependent carrier in the tonoplast membrane (Martinoia *et al.*, 1993). This vacuolar carrier showed a striking resemblance to the glutathione S-conjugate export pump in the canalicular membrane of mammalian liver. It is not yet known whether such transport processes into plant vacuoles are influenced by herbicide safeners.

7. Specificity of safener action

Safeners used as a tank-mixture or prepackaged formulation with the herbicide act specifically with respect to the plant species that are protected from herbicidal injury. Fenchlorazole-ethyl, a compound developed for use in conjunction with the aryloxyphenoxypropionate herbicide, fenoxaprop-ethyl, has been reported to act as both a safener on wheat and as a synergist of herbicidal action on *Digitaria ischaemum* (Yaancoby *et al.*, 1991). In both plant species, fenchlorazole-ethyl stimulated deesterification of fenoxaprop-ethyl to the herbicidally active free acid. Further metabolism and detoxification of the herbicide, however, were only enhanced in wheat but not in *D. ischaemum*. More recently, specific safener action of cloquintocet-mexyl (5-chloro-8-quinolinoxyacetic acid-1-methylhexyl ester) for the herbicide clodinafop-propargyl (2-propynyl-R-2- [4-(5-chloro-3-fluoro-2-pyridinyloxy)-phenoxy] propionate) on wheat (Amrein *et al.*, 1989) could be explained on the basis of the qualitatively different metabolic pathways of the herbicide in wheat and in susceptible target weeds. Metabolism of clodinafop-propargyl in wheat proceeded through deesterification to the herbicidally active acid, followed by hydroxylation and ether cleavage to yield 2-[4-(6-hydroxy-5-chloro-3-fluoro-2-pyridinyloxy) phenoxy] propionic acid and 2-(4-hydroxyphenoxy) propionic acid, respectively. All metabolites were subject to carbohydrate conjugation. In excised wheat leaves, rapid deesterfication occurred, while all subsequent metabolic steps where significantly enhanced by the safener cloquintocet-mexyl (Kreuz *et al.*, 1991). In leaves of *Alopecurus myosuroides* and *Lolium rigidum*, the readily formed free acid of clodinafop-propargyl was slowly converted to a major metabolite that was identified by mass spectrometry ^{1}H-NMR and chemical synthesis as the ester conjugate of the herbicide acid with malate (Kreuz *et al.*, unpublished results). No oxidative metabolism of clodinafop-propargyl was detected in these weed species. The rates of deesterification of clodinafop-propargyl and re-esterification with malate were not influenced in these weeds by the safener cloquintocet-mexyl. Thus, the metabolic pathway conferring moderate herbicide tolerance to wheat in the absence of cloquintocet-mexyl and which is enhanced by the safener to confer full tolerance is completely

absent in these susceptible weeds.

Conclusion

Herbicide safeners act at multiple sites of herbicide metabolism and detoxification pathways in plants by enhancing oxidative reactions, glucose conjugation, glutathione conjugation, and glutathione biosynthesis. There are indications that induction of metabolism is exerted at the level of transcription of genes coding for herbicide-metabolizing enzymes. Safeners appear to enhance, in a particular plant species, metabolic pathways that are already expressed at a certain constitutive level, rather than to induce qualitatively different reactions. The molecular mechanisms involved in these induction processes, however, still remain elusive and require further research.

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Discussion

Jong Yeong Pyon (Korea) : Why are safeners mostly effective on monocot crops only ?

Answer : I do not know.

Kwon, Y.W. (Korea) : Under paddy conditions, unlike under dry land conditions, the safener may not be effective presumably due to differences in the molecules and may not be available when the crop needs it. This phenomenon could also be related to the movement of water. What would be the quantity of the product to be added ?

Answer : Allard, J.L. (CIBA-GEIGY, Switzerland) : Uptake of fenclorim as a safener of pretilaclor is not affected by the soil conditions in wet-seeded rice because fenclorim is absorbed by the roots out of the paddy water.