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1. Introduction

Herbicide safeners, formerly referred to as herbicide antidotes, are chemical agents that increase the tolerance of monocotyledonous cereal plants to herbicides without affecting the weed control effectiveness. The use of safeners offer several benefits to agricultural weed control. Safeners may allow: (1) the selective chemical control of weeds in botanically related crops; (2) the use of nonselective herbicides for selective weed control; (3) the counteraction of residual activity of soil-applied persistent herbicides such as triazines in crop rotation systems; (4) an increase in the spectrum of herbicides available for weed control in “minor” crops; (5) an expansion and extension of the uses and marketability of generic herbicides; (6) the elucidation of sites and mechanism by serving as useful biochemical tools [1]. The commercial viability of safener concept is indicated by the growing number of herbicide-safener products available on the pesticide market. With the use of safeners, difficult weed control problems can be addressed and without safeners, many herbicidally active substances could have never been applied for weed control [2].

The concept to enhance crop tolerance to nonselective herbicide by using chemical agents was introduced by Otto Hoffman. In the late 1940s Hoffmann serendipitously found that no herbicide injury symptoms were developed in tomato plants previously treated with 2,4,6-T, an inactive analogue of herbicide 2,4-D when plant were exposed accidentally to vapors of 2,4-D due to the malfunction of the ventilation system of the greenhouse [3]. Following this observation Hoffmann reported later the antagonistic effects of 2,4-D against herbicidal injury by barban after foliar treatments of wheat plants [4]. Research and development in finding new safeners as well as subsequent commercialization proceeded very intensively in the 1970s. Since the patent application of the safening properties of 1,8-naphthalic anhydride (NA) intensive research on discovery of new safeners resulted in compounds with diverse chemis-
tries (Table 1) successfully applied to alleviate injury symptoms by various classes of herbicides in cereal crops.

NA patented by Hoffmann [5] has been considered as the most versatile safener showing less botanical and chemical specificity than other safeners developed later. NA protected cereals as seed treatments against various herbicide chemistries [6]. NA was reported to be mildly phytotoxic to maize (chlorosis and growth inhibition) under some growing conditions. One problem in treating seeds with safeners prior to planting is that phytotoxicity can increase as the time the safener is exposed to the seed increases. With NA, the phytotoxicity to the crop increases with increased time the safener is in contact with the seed during storage. This problem has thus far prevented NA from being introduced to the commercial market [7].

The introduction of dichloroacetamide derivatives developed as safeners against thio carbamates and chloroacetanilides was a breakthrough in the history of the safeners since these compounds can be applied to the soil in preplant incorporated (PPI) or preemergence (PRE) technology in prepackaged tank mixture with the herbicide. Generally, prepackaged herbicide-safener mixtures offer several advantages over seed safeners. First of all, the manufacturer controls all components of the formulation, secondly, the farmers buy and use a single and reliable product which allows a wider selection of crop cultivars. Dichlormid exhibited a remarkable degree of chemical and botanical specificity in protection of maize against thio carbamates such as EPTC, butylate, vernolate but the safener was less protective to maize against chloroacetanilides. In addition to dichlormid, a number of dichloroacetylated amine derivatives were marketed. Among them AD-67, a spiro-oxazolidine compound was commercialized to protect maize plants against acetochlor while benoxaxor can be used to safen S-metolachlor or racemic metolachlor in maize. Furilazole, in addition to providing protection against acetochlor, has a very good safening effect on sulfonylureas particularly halosulfuron. The dichloromethyl-1,3-dioxolane MG-191 the most active member of dichloroacetal and ketal derivatives, protects maize against thio carbamates and chloroacetanilide injuries. MG-191, similarly to dichlormid, is more effective against thio carbamates than chloroacetanilides.

The oxime ethers such as cyometrinil, oxabetrinil, and fluxofenim were marketed as seed treatment safeners to protect sorghum plants against chloroacetanilides, in particular, metolachlor. Flurazole, a 2,4-disubstituted 5-thiazolecarboxylate is also a seed safener allowing the safe use of alachlor in sorghum. The Phenylpyrimidine safener fenclorim was introduced against pretilachlor in rice and can be used in tank mixture formulated together with the chloroacetanilide herbicide.

The urea type dymron and the thio carbamate dimepiperate are actually herbically active compounds that possess safening activity against pretilachlor [8] and bensulfuron [9] in rice. Trends toward post-emergence herbicide treatments and the use of high-activity herbicide molecules have led to the development of safeners with post-emergence application in winter cereals. A new era in safener research began with the discovery of 1,2,4-triazolcarboxylates and fenchlorazole-ethyl was developed as a post-emergence safener against ACCase inhibitor fenoxaprop-ethyl in wheat in a tank mixture with the herbicide. Similarly, the dihydropyrazol dicarboxylate mefenpyr-diethyl was used against ACCase inhibitors including fenoxaprop-
ethyl as well as mesosulfuron and iodosulfuron in a variety of cereals. The main application of 8-quinolinoxy-acetate cloquintocet-mexyl is against clodinafop-propargyl in wheat. Dihydroisoxazole-carboxylate isoxadifen-ethyl can safen herbicides of various mode of action. First, it was applied in maize in combination with foramsulfuron but mixture with foramsulfuron and iodosulfuron-methyl is also in use. In rice, it can be used with fenoxapop-P-ethyl and ethoxysulfuron. The arylsulfonyl-benzamide, cyprosulfamide is the latest achievement in safener research. It protects maize against isoxaflutole pre-emergence and can also be used in maize with isoxaflutole plus thiencarbazone in pre-emergence and early post-emergence applications [10].

Interestingly, no successful safeners have been developed for broad-leafed crops. Recently, the non-phytotoxic microbial inhibitor dietholate (O,O-diethyl-O-phenyl phosphorothioate) [11] used to inhibit soil microbes that degrade thiocarbamate herbicides was patented as a Table 1 safener for cotton plants against injuries by clomazone [12].

Despite large amount of information published on the activity, mode of action and uses of safeners during the 50-year history of these herbicide antagonists this overview will focus on several less addressed topics such as a) relationships between the molecular structure and the safening properties; b) basis for differential chemical selectivity; and c) safener effects on detoxifying enzymes in crop plants and weeds.

<table>
<thead>
<tr>
<th>Chemical class</th>
<th>Name</th>
<th>Structure</th>
<th>logP</th>
<th>Herbicide</th>
<th>Crop</th>
<th>Appl. method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anhydride</td>
<td>1,8-Naphthalic anhydride (NA)</td>
<td><img src="image" alt="Structure" /></td>
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<td>Thiocarbamates</td>
<td>Maize</td>
<td>Seed-treatment</td>
</tr>
<tr>
<td>Dichloridic</td>
<td>Dichlormid</td>
<td><img src="image" alt="Structure" /></td>
<td>1.84</td>
<td>Thiocarbamates</td>
<td>Chloroaet-anilides</td>
<td>Maize</td>
</tr>
<tr>
<td>Dichloro–acetamide</td>
<td>Furfazole</td>
<td><img src="image" alt="Structure" /></td>
<td>2.12</td>
<td>Acetochlor</td>
<td>Halosulfuron-methyl</td>
<td>Maize</td>
</tr>
<tr>
<td></td>
<td>AD–67</td>
<td><img src="image" alt="Structure" /></td>
<td>2.32</td>
<td>Acetochlor</td>
<td>Maize</td>
<td>PRE</td>
</tr>
<tr>
<td></td>
<td>Benoxacin</td>
<td><img src="image" alt="Structure" /></td>
<td>2.69</td>
<td>Metolachlor</td>
<td>Maize</td>
<td>PRE</td>
</tr>
<tr>
<td>Chemical class</td>
<td>Name</td>
<td>Structure</td>
<td>logP</td>
<td>Herbicide</td>
<td>Crop</td>
<td>Appl. method</td>
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<tr>
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<tr>
<td>Oxime ether</td>
<td>Cyometrinil</td>
<td><img src="image1" alt="" /></td>
<td>1.56</td>
<td>Chloroacet-anilides (metolachlor)</td>
<td>Sorghum</td>
<td>Seed-treatment</td>
</tr>
<tr>
<td></td>
<td>Oxabetrinil</td>
<td><img src="image2" alt="" /></td>
<td>2.76</td>
<td>Chloroacet-anilides (metolachlor)</td>
<td>Sorghum</td>
<td>Seed-treatment</td>
</tr>
<tr>
<td></td>
<td>Fluxofenim</td>
<td><img src="image3" alt="" /></td>
<td>2.90</td>
<td>Chloroacet-anilides (metolachlor)</td>
<td>Sorghum</td>
<td>Seed-treatment</td>
</tr>
<tr>
<td>Thiazole carboxylic acid</td>
<td>Flurazole</td>
<td><img src="image4" alt="" /></td>
<td>3.64</td>
<td>Alachlor</td>
<td>Sorghum</td>
<td>Seed-treatment</td>
</tr>
<tr>
<td>Dichloromethyl-ketal</td>
<td>MG–191</td>
<td><img src="image5" alt="" /></td>
<td>1.35</td>
<td>Thiocarbamates Chloroacet-anilides</td>
<td>Maize</td>
<td>PRE</td>
</tr>
<tr>
<td>Phenyl–pyrimidine</td>
<td>Fenclorim</td>
<td><img src="image6" alt="" /></td>
<td>4.17</td>
<td>Pretilachlor</td>
<td>Rice</td>
<td>PRE</td>
</tr>
<tr>
<td>Urea</td>
<td>Dymron</td>
<td><img src="image7" alt="" /></td>
<td>2.70</td>
<td>Pyributicarb Pretilachlor Pyrazosulfuron-ethyl</td>
<td>Rice</td>
<td>PRE, POST</td>
</tr>
<tr>
<td>Piperidine–1–carbothioate</td>
<td>Dimepiperate</td>
<td><img src="image8" alt="" /></td>
<td>4.02</td>
<td>Sulfonyureas</td>
<td>Rice</td>
<td>POST</td>
</tr>
<tr>
<td>8–Quinolinoxy-carboxylic esters</td>
<td>Cloquintocet-mexyl</td>
<td><img src="image9" alt="" /></td>
<td>5.03</td>
<td>Clodinafop-propargyl</td>
<td>Cereals</td>
<td>POST</td>
</tr>
<tr>
<td>1,2,4-Triazole-carboxylate</td>
<td>Fencloazol-ethyl</td>
<td><img src="image10" alt="" /></td>
<td>4.52</td>
<td>Fenoxyaprop-ethyl</td>
<td>Cereals</td>
<td>POST</td>
</tr>
</tbody>
</table>
Table 1. Structure, logP and application of some important safeners.

<table>
<thead>
<tr>
<th>Chemical class</th>
<th>Name</th>
<th>Structure</th>
<th>logP</th>
<th>Herbicide</th>
<th>Crop</th>
<th>Appl. method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydropyrazole–dicarboxylate</td>
<td>Mefenpyr–diethyl</td>
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<td>3.83</td>
<td>ACCase inhibitors</td>
<td>Wheat, Rye, Triticale, Barley</td>
<td>POST</td>
</tr>
<tr>
<td>Dihydroisoxazole–carboxylate</td>
<td>Isoxadifen–ethyl</td>
<td><img src="image" alt="Isoxadifen-Ethyl" /></td>
<td>3.88</td>
<td>ACCase inhibitors</td>
<td>Maize, Rice</td>
<td>POST</td>
</tr>
<tr>
<td>Arylsulfonyl–benzamide</td>
<td>Cypro–sulfamide</td>
<td><img src="image" alt="Cypro-Sulfamide" /></td>
<td>2.09</td>
<td>Isoxaflutole</td>
<td>Maize</td>
<td>PRE, POST</td>
</tr>
</tbody>
</table>

*Safeners used as racemic mixtures are indicated by R/S in their structures.

Log P values unavailable were calculated by ALOGPS 2.1 program available online at www.vclab.org/articles/cite.html.

2. Structure–safening activity relationships

Structure-activity correlations are very important in the search for biological activity because they provide useful information about chemical substituents that are necessary for the required bioactivity. Published structure-activity correlation studies with safeners and analogous compounds have been limited.

Hoffmann’s original patent for NA against EPTC in maize claimed only a few NA analogs such as alkyl esters, barium and tin salts as well as \(N,N’\)-diallyl naphthalene-1,8-dicarboxylic acid, \(N,N’\)-diallyloxamide, \(N,N’\)-dipropynyloxamide, \(N,N,N’,N’\)-tetrapropyloxamide and dipropynylmalonamide [5]. In addition to the original patent, the effects of other structural analogs of NA were tested against EPTC in maize as seed dressing [13]. The presence of the dicarboxylic anhydride group and at least one aromatic ring attached directly to the anhydride appeared to be essential for the protective activity of NA structural analogues. Derivatives such as acenaphthylene-1,2-dione, benzoisoquinoline-1,3-dione, 4-amino-NA, naphthalic-dianhydride, phtalic anhydride as well as diphenic anhydride showed safening effects while chlorinated NA, 2-phenylglutaric anhydride and phenalene-1-one were toxic to maize.

Detailed structure-activity correlations were conducted mainly with various amide safeners that protect maize from thiocarbamate injury. Studies with several hundred of amides revealed that the most effective safeners were \(N,N\)-disubstituted acetamides [14] or substituted \(N\)-acetyl-1,3-oxazolidines [15, 16]. Structure-activity studies with dichloroacetamides revealed that \(N,N\)-disubstituted derivatives were more effective than monosubstituted amides. A
variety of substituents on the nitrogen atom including alkyl, haloalkyl, alkenyl and heterocyclic groups impart various degrees of protective activity. Nevertheless, mono- and trichloroacetamides exhibited less safening activity than dichloro analogues [17, 18]. Based on these SAR studies similarities between the chemical structure of the herbicide and its safener, the possible competitive antagonism between the thiocarbamate and the safener molecules for a common target site has been postulated [19]. Computer-aided molecular modeling (CAMM) studies supported this theory [20]. Superimposing of the structures of dichlorimid and EPTC revealed that the two chlorine atoms of the safener do not superimpose over any functional group of the EPTC. If structure of EPTC sulfoxide, the very phytotoxic EPTC metabolite, and the dichloromid were superimposed, the two compounds were similar with functional groups in the same location on both molecules. Comparative three-dimensional quantitative structure-activity relationship studies using comparative molecular field analysis (CoMFA) also supported the competitive antagonism theory and predicted a structure of \(N\)-allyl-\(N\)-methoxyethyl dichloroacetamide as a potent highly effective safener [21].

Structure-safening activity studies with oxime ether derivatives revealed that the safening activity is affected by the number of nucleophilic sites present in the molecule. An oxime ether with two nucleophilic sites was more effective than those with only one. In addition to cyometrinil, oxabetrinil and fluoxafenim pyridin-2-aldoxime O-ethers such as benzyl and phenylethyl ethers were protective to grain sorghum in seed treatments against metolachlor. The oxime and aldehyde derivatives tested, in terms of decreasing safening effectiveness, were dimethyglyoxime > benzophenone oxime > pyridine-2-aldoxime > benzoin-oxime > methyl thioacetoxyhydroxamate > pyridine-2-aldoxime methiodide > 5-nitro-furanacarboxyaldehyde [22]. CAMM evaluations of the oxime ether analogues cyometrinil, oxabetrinil and fluoxafenim revealed that as the effectiveness of the safener increases so does its molecular similarity to metolachlor [20].

Structure-safening activity relationships for thiazol-5-carboxylic acids against acetamide herbicides were described for 60 derivatives in the original patent [23]. Thiazolecarboxylates substituted by a trifluoromethyl in the 4-position are clearly superior to those substituted in the 4-position by methyl in reducing herbicidal injury to sorghum. Another preferred group of thiazolecarboxylates contained a halogen atom at position 2 preferably chlorine.

A structure-activity relationship study to safen maize against acetochlor was carried out with the herbicide safener MG-191 and its acetal and ketal analogues at preemergence application [24-26]. Open chain acetals formed from 1,1-dichloroacetdehyde exhibited only marginal safening efficacy. Diallyl ketals of 1,1-dichloroacetone showed increasing effectiveness up to 3 carbon length of the alkyl group with further increases in carbon atoms resulted in loss of activity. The 5-, 6- and 7-membered 1,3-dioxacycloalkanes prepared from dichloroacetaldehyde had hardly detectable safening activity. However, introducing alkyl or aryl substitution at the 2-position of the 1,3-dioxacycloalkane ring remarkably increased the safening activity. Regarding ring size the highest activity observed was for 2-dichloromethyl-2-methyl-1,3-dioxepane. Replacing an oxygen in the 1,3-dioxolane ring for nitrogen resulted in oxazolidines with reduced safening activities but alkyl or aryl substitution on the nitrogen increased the safening activity of compounds. Replacement of oxygens by sulfur atoms leads to less active
derivatives among which 1,3-dithiolane derivative showed higher activity than the oxathiolanes. Various 1,3-dioxolane-4-ones provided significant protection against the acetochlor. Benzo[1,3]-dioxoles were ineffective while benzo[1,3]dioxin-4-ones were protective in safening maize. 5-Dichloromethyl-3-substituted-isoxazoles were also active safeners.

Unfortunately, no publication has been reported for the other chemistry of safeners. However, no unifying structural motifs for compounds to be safeners can be predicted from these studies.

3. Chiral safeners

The importance of the chirality in the biological activity has long been recognized. Since biochemical processes in the cells take place in chiral environment and most enzymatic pathways are stereoselective, a high degree of enantiomeric and enantiotopic selectivity can be obtained when chiral or prochiral molecules are introduced into biological systems. About one fourth of the presently available pesticides are chiral, existing as two mirror images called enantiomers. These stereoisomers generally possess identical physico-chemical properties but widely different biological activities, such as toxicity, mutagenicity, and carcinogenicity [27]. The active enantiomer of the chiral pesticide would have the desired effect on target species while the other may be inactive [28].

Among the commercially available safeners, four such as benoxacor, furilazole, cloquintocet-mexyl, and mefenpyr-diethyl are chiral compounds but used exclusively as racemic (R/S) mixtures in herbicidal compositions and no information accessible on the safening efficacy of the individual enantiomers. In one recent patent, the R enantiomer of furilazole is described in a herbicidal mixture as a safener [29].

Nevertheless, only a few molecules have been reported as safeners in enantiomerically pure form. The optical isomers of 4-(dichloromethylene)-2-[N-α-(methylbenzyl)imino]-1,3-dithiolane hydrochloride were synthesized and were tested against triallate in wheat [30]. The R enantiomer exhibited high safening activity and its activity exceeded that of the S and the racemic compound. The monoterpene R-carvone was found more effective than the S enantiomer to safen maize against acetochlor injury [31]. 2-Dichloromethyl-2-methyl-[1,3]oxathiolane 3-oxide, a structural analogue of the MG-191 safener, was prepared and the enantiomers were separated by chiral HPLC [32]. The more polar diastereomeric pair was as effective as MG-191 while the other exhibited only marginal protection against acetochlor. Inducibility of ZmGSTF1-2 from roots was more enhanced by the stereoisomers with higher safening efficacy while only one of these enantiomers was effective in shoots. The findings indicated the importance of the stereochemistry in the protective effectiveness. The safener (S)-3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine was found to induce the GSH content and GST activity in root and shoot of maize seedlings but the effect of the R form was not reported in these experiments [33]. As a future prospect, the needs for broad application of the green technology in the sustainable agriculture will probably induce a shift in the use and development of enantiomerically pure safeners.
4. Prosafeners and natural compounds with safening activity

The term prosafeners refers to molecules with safening activity undergoing biotransformation to the actual safening agent prior to exhibiting their safening effect. Substituted N-phenylmaleamic acids and their progenitors N-phenylmaleimides and N-phenylisomaleimides exhibited safening activity against alachlor in sorghum at preemergence application [34]. Simple hydrolytic ring-opening reaction of N-phenylmaleimides and N-phenylisomaleimides results in the N-phenylmaleamic acid derivatives with safening activity. Two thiazolidine derivative L-2-oxathiazolidine-4-carboxylic acid (OTC) [35] and thioproline (L-thiazolidine-4-carboxylic acid) [36] have been reported to safen sorghum against tridiphane injury. OTC is converted by 5-oxoprolinase to S-carboxy-L-cysteine which spontaneously decarboxylates to yield L-cysteine. The conversion of thioproline to cysteine takes place in two steps, first proline oxidase yields N-formyl-L-cysteine from which cysteine is forming by hydrolysis. Either source of cysteine elevates the glutathione level in plants and therefore enhance herbicide detoxication.

Safening activities of natural cyclic hydroxamic acids (DIMBOA, DIBOA, and MBOA) as well as synthetic analogues such as 1,4-benzoxazin-3-ones and 1,3-benzoxazolidin-2-ones were prepared and tested to safen maize against acetochlor and EPTC injuries [37]. Cyclic hydroxamic acids were supposed to act as safeners by catalyzing hydroxylation of herbicides containing reactive chlorine in their structure and they are ineffective against herbicides not possessing leaving groups. While no safening activities of natural hydroxamic acids were detected, the synthetic analogues exhibited low to moderate activity.

Metabolism of the herbicide safener, fenclorim resulting, in a semi-natural product with safening activity has recently been described in Arabidopsis thaliana cell cultures [38]. The metabolism of fenclorim mediated by GSTs yielded S-(fenclorim)-glutathione conjugate that was sequentially catabolized to S-(fenclorim)-cysteine then to 4-chloro-6-(methylthio)phenylpyrimidine (CMTP). Although the fenclorim conjugates tested showed little GST inducing activity in Arabidopsis, the formation of CMTP resulted in metabolic reactivation, with the product showing enhancing activity similar to that of parent safener. In addition, CMTP safened rice plants and induced rice GSTs. The formation of CMTP by metabolic bioactivation can contribute to the longevity of safener action since it was found stable 8 – 24 h after application.

Oxylipins constitute a family of oxygenated natural products which are formed from fatty acids. Safeners and reactive electrophilic oxylipins (RES oxylipins) have a common biological activity in that they both strongly induce the expression of defence genes and activate detoxification responses in plants [39, 40]. Surprisingly, the application of oxylipin A has been found to reduce the herbicidal injury [41].

5. Interaction of safeners and herbicides on the absorption and translocation

Published results on how safeners affect the herbicide absorption are rather contradictory and, therefore, no general conclusion can be drawn. In an excellent summary the effect of 15 safeners
toward various herbicides was reviewed [2]. Interestingly the majority of papers published report safener-enhanced herbicide uptake followed by no effect then reduced uptake results. According to a recent study mefenpyr-diethyl had no effect on the uptake of either mesosulfuron-methyl or iodosulfuron-methyl-sodium [10]. These results suggest that the influence of safeners on the herbicide uptake may not be a decisive factor in the protective action. However, the knowledge of absorbed amounts of safeners and herbicides by crops may help to determine the optimal herbicide/safener ratios applicable in the agricultural practice. In addition, determination of the site of safener and herbicide uptake can contribute to prepare the most selective herbicide-safener mixture. A suitable placement of soil-applied herbicides to roots or the emerging shoots is of great practical importance in achieving the most effective weed control and the least injury to crop plants.

Studies on how maize can differentiate in the absorbtion of herbicides and safeners were conducted with radiolabeled EPTC, acetochlor and MG-191 [42, 43]. Time-dependent uptake of root-applied $^{14}$C EPTC reached a maximum after 6h and decreased up to 3 days (Figure 1). The first measurable shoot growth inhibition appeared just after 1-day-exposition to the herbicide and 38% shoot length inhibition was observed 3 DAT. In general, the MG-191 safener had no influence on the herbicide uptake except for 1 DAT when the safener enhanced the herbicide uptake by 1.5-fold as compared to that in the unsafened plants. Nevertheless, the safener conferred a complete protection to maize throughout the study. The highest amount of herbicide uptake was 65 μg/g fresh weight.

As a comparision, the amount of root-absorbed $^{14}$C acetochlor was continuously increased up to 3 days (Figure 2). As a result of increasing uptake the first detectable shoot length inhibition occurred 6h after treatment. At 3 DAT 28% shoot and 52% root (data not shown) growth inhibition by the herbicide occurred. Addition of the MG-191 safener did not affect the acetochlor absorption by maize seedlings but completely antagonized the herbicide shoot

![Figure 1. Influence of MG-191 safener on uptake and shoot length inhibition of root-applied $^{14}$CEPTC.](http://dx.doi.org/10.5772/55168)
growth inhibition. The maize seedlings absorbed much higher amounts of acetochlor (377 μg/g fresh weight).
to the roots of maize plants than the less water-soluble acetochlor (log P 4.14). The higher logP of acetochlor also supports its higher uptake as compared to MG-191.

It is also difficult to put the results of safener affected translocation of absorbed herbicides in perspective. Reduction of translocation of herbicides such as acetochlor, methazachlor, and imidazolinones from roots of maize to the shoots following treatments with dichlormid, BAS 145,138 and NA is likely a consequence of the safener-enhanced herbicide metabolism to more polar and less mobile products [45-49]. On the other hand, no effect of MG-191 on EPTC and acetochlor translocation has been observed [42, 44]. It is interesting to note that safener MG-191 and the herbicide acetochlor exhibit different translocation patterns (Figure 4). While the majority of the absorbed radiolabel from [14C] acetochlor was found in the roots and coleoptiles of maize seedlings (Figure 4a), the root-applied [14C]MG-191 distributed evenly within the plants (Figure 4b) showing similar mobility and distribution as EPTC (data not shown). This may be further evidence for the higher protective efficacy of this safener against EPTC as compared to acetochlor. The similar translocation pattern of the herbicide and the safener may be a prerequisite for the high level of saening activity.

6. Action of safeners on the glutathione-mediated detoxification of herbicides

Various chemistries of safeners were found to enhance the herbicide detoxification in the safened plants by elevating the activity of the mediating enzymes such as glutathione S-transferases (GSTs), cytochrome P450 mixed function oxidases (CYPs), glycosyltransferases (UGTs) and ATP-binding cassette (ABC) transporter proteins as well as a cofactor endogenous glutathione (GSH) involved in detoxification of herbicides [2, 50-52]. The best studied group

Figure 3. Time-course uptake of root-applied [14C]MG-191 by 5-day-old maize seedlings and the influence of EPTC.
of plant enzymes involved in herbicide metabolism is the GSTs that mediate the conjugation of the major cellular thiol tripeptide, GSH with herbicide substrates. GSTs are multifunctional enzymes, each composed of two subunits which catalyze conjugation of a broad range of electrophilic substrates with GSH [53]. Herbicides known to conjugate with GSH include thiocarbamates, chloro-s-triazines, triazinone sulfoxides, chloroacetanilides, diphenylethers, some sulfonylureas, aryloxyphenoxypropionates, thiazolidines, and sulfonamides [54, 55]. Plant GSTs comprise a large and diverse group, with 54 GST genes encoded by the Arabidopsis genome, and have been classified on sequence similarity, genomic organization and functions into several distinct subclasses [56]. In plants, phi (F) and tau (U) classes are the most prominent GSTs involved in herbicide detoxification [57-59]. In addition to up-regulating GST expression, safeners also enhance the activity of enzymes involved in sulfate assimilation and GSH biosynthesis thereby elevating the level of GSH [50, 60].

Only two studies are available in the literature on how the safener structure affects the expression of GST isoforms. The herbicide safener MG-191 (2-dichloromethyl-2-methyl-1,3-dioxolane) and its less effective structural analogue dichloromethyl-dioxolanone (NO-17; 2-dichloromethyl-2,5-dimethyl-1,3-dioxolane-4-one) were reported to differentially enhance the expression of members of the GSTs in maize [61]. None of these safener molecules had influence on the expression of ZmGSTF1-2 (Figure 5a and b). However, MG-191 and, to a lesser extent NO-17 selectively enhanced the expression of tau class ZmGSTU1 in both root and shoot tissues after 1 day of treatment (Figure 5c and d). Addition of cycloheximide to the treatment solutions suppressed the enhancement of expres-
The expression of ZmGSTU1 only in the roots. ZmGSTU1 has previously been shown to play a key role in metabolism of nitrodiphenyl ether type herbicides [54].

Figure 5. Western blots of crude GST extracts from maize roots and shoots (a) Analysis of GSTs from maize shoots using the anti-ZmGSTF1-2 serum. (b) Analysis of GSTs from maize roots using the anti-ZmGSTF1-2 serum. (c) Analysis of GSTs from maize shoots using the anti-ZmGSTU1-2 serum. (d) Analysis of GSTs from maize roots using the anti-ZmGSTU1-2 serum.

Analysis of isoenzyme profile of maize GSTs revealed that phi class of GSTs predominate, with ZmGSTF1 as the major subunit which is present constitutively and shows high specificity to 1-chloro-2,4-dinitrobenzene (CDNB) substrate [62]. A second phi type GST termed ZmGSTF2 accumulates following treatments with herbicide safeners. These subunits can dimerise together to form ZmGSTF1-1 and ZmGSTF2-2 homodimers as well as ZmGSTF1-2 heterodimer. In addition to these three phi GST isoenzymes a phi type GST ZmGSTF3 and three tau class GSTs ZmGSTU1, ZmGSTU2 and ZmGSTU3 are present in lower amounts [63, 64]. While the expression of ZmGSTF2 was enhanced by auxins, herbicides, the herbicide safener dichlormid and glutathione, the ZmGSTU1 subunit was induced more selectively, only accumulating significantly in response to dichlormid treatment [63]. Although ZmGSTF2 has been consid-
ered more active in detoxifying metolachlor and alachlor than ZmGSTF1 it is far less abundant [65]. The importance of de novo synthesis of the isoenzyme ZmGSTU1 in its safening action is difficult to explain. Nevertheless, these results indicate that dichloromethyl-dioxolane type MG-191 is a more specific inducer of maize GSTs than other compounds commonly used to safen thiocarbamate or chloroacetanilide herbicides in maize.

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Table 2. Safening activity and inducibility of shoot GSH content and GST activities by acetics, ketals and amides in maize

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* based on shoot length; protection (%) = 100 x [(herbicide + safener)] / [control - herbicide]; shoot lengths 14 DAT: control, 27.9±5.3 cm, acetochlor, 3.1±0.3 cm;

b GSH content relative to that of untreated control; GSH contr.: 0.55±0.09 μmol/g fresh weight;

c GST(CDN) activity as compared to that of untreated control; GST contr.: 3.87±0.33 nkat/mg protein;

d GST(Ac) activity as compared to that of untreated control; GST contr.: 8.26±1.68 pkat/mg protein

In other, structure and GST isoform expressing ability studies with acetal and ketal analogues of MG-191 as well as mono- and dichloroacetamides (Table 2) demonstrated that the safener structure affects the specific expression of GSTs mediating the detoxication of acetochlor (Matola et al., 2003). Nevertheless, no correlation was found between the degree of induction of GSH and GSTs and the safening activity as related to the structure. A higher inducibility of these GST isoforms was observed in root tissues (Figure 6a and c). In shoots, when the heterodimer ZmGSTF1-2 was used the expression of the constitutive ZmGSTF1 and inducible ZmGSTF2 was enhanced only by 2f (MG-191) and its analogue 2g having a 6-membered ring (Figure 6b). These molecules and also 2h were the most potent inducers of the expression of tau class ZmGSTU1 in shoot tissues (Figure 6c). ZmGSTU1 has previously been shown to play a key role in metabolism of nitrodiaryl ether herbicides [54]. These results confirm previous findings that dichloromethyl-ketal safeners are more specific inducers ZmGSTU1-2 than other compounds commonly used to safen thiocarbamate and chloroacetanilide herbicides in maize [61].

The exact mechanism of the safener-mediated enhancement of GST activity is not completely understood. GSTs are induced by a diverse range of chemicals and accompanied by the production of active oxygen species. Thus the connection between safener-mediated protection of crops and oxidative stress tolerance has been suggested [66]. Many GSTs are effective not only in conjugating electrophilic substrates but also function as glutathione peroxidases. Safeners may induce GST expression by mimicking oxidative insult [67]. Our results indicate
that safener structure plays a decisive role in specific expression of GSTs mediating the detoxication of chloroacetamide herbicides. Since no correlation between the degree of induction of levels of GSH and GST isoforms and the safener activity was found, the mode of action of safeners is a more complex process than simply promoting the metabolism of herbicides.

Figure 6. Western blots of crude GST extracts from maize roots and shoots; (a) and (b) analysis of GSTs using the anti-ZmGSTF1-2 serum from maize roots and shoots; (c) and (d) analysis of GSTs using the anti-ZmGSTU1-2 serum from maize roots and shoots.

7. Effect of safeners on herbicide detoxification enzymes in weeds

Studies on the mechanism of action of safeners revealed that herbicide safeners improve crop tolerance to herbicides by regulating the expression of genes involved in herbicide metabolism [68]. It is widely accepted that safeners selectively protect crop plants against herbicide injury by stimulating the plant detoxifying mechanism at herbicide rates required for effective weed control. Nevertheless, only a few papers were published on the safener effect of GSTs and cytochrome P450 monooxygenases of various weed species. To a better understanding on why safeners do not provide protection to weeds it is essential to explore the safener action on detoxification enzymes of weeds.

7.1. Effect of safeners on weed glutathione (GSH) content and glutathione S-transferase (GSTs) activities

Safeners such as MG-191, dichlormid, AD-67, BAS-145138, and flurazol were reported to reduce phytotoxicity of EPTC in grassy weeds [69]. MG-191, BAS-145138 and flurazole offered
moderate safening to *Bromus secalinus* (brome grass) and flurazole was also moderately protective in *Setaria glauca* (yellow foxtail) at sublethal rate of EPTC. Safener-induced elevation of GSH contents and GST activities is widely considered as key element for increased tolerance to thiocarbamates and chloroacetanilides of safened plants [50]. Tolerance of plant species such as maize, soybean and several weeds to acetochlor has been correlated with their glutathione and homoglutathione content [70]. It was also apparent that a relationship exists between the relative GST activities toward alachlor and metolachlor in maize and various weed species ([71]. GST activities toward metolachlor were found to correlate well with the selectivity of the herbicide toward the broadleaf weeds but not toward the grass weeds [72]. However, there was no correlation between total activity of cysteine biosynthesis from serine (CBS) and susceptibility to metolachlor of sorghum, maize, and various grassy weeds [73]. GST isozymes involved in herbicide metabolism is cell suspension culture of a grass weed *Setaria faberri* (giant foxtail) exhibited a similar level of complexity to those from maize cell cultures [74]. Nevertheless, much less is known about GSH or other non-protein thiol contents and GST activities of different weed species following treatments by herbicides and safeners. In order to explain differential physiological and biochemical responses of monocot and dicot weeds to these herbicides, non-protein thiol levels and GST activities were studied in selected mono- and dicot weeds species [75]. The most sensitive *Echinochloa crus-galli* (ECHCR, barnyardgrass) contained higher level of non-protein thiols than less sensitive dicot seedlings (Figure 5). Nevertheless, thiol contents in the most tolerant maize and in the least sensitive moncotyledonous *Bromus secalinus* (BROSE, cheatgrass) were comparable. In general, either herbicide or safener pretreatments did not alter thiol contents substantially. *Abutilon theophrasti* (ABUTH, velvetleaf) was the only exception because 1 μM acetochlor and 10 μM AD-67 resulted in remarkable increases (73% and 87%, respectively) in the levels of non-protein thiols.

![Figure 7. Effect of treatments on non-protein thiol contents of mono- and dicot weed species.](http://dx.doi.org/10.5772/55168)
Glutathione S-transferase (GST) activities using CDNB substrate were not correlated with herbicide susceptibility of the selected weed species (Figure 6a). The GSTs extracted from monocot seedlings exhibited much higher activities than from dicot seedlings. GSTCDNB activity detected in *Avena fatua* (AVEFA, wild oats) exceeded that in maize. In general, elevation of GSTCDNB activities following pretreatments with both herbicides and safeners were more pronounced (2- to 10-fold of controls) in the highly sensitive *Echinochloa crus-galli* and *Amaranthus retroflexus* (AMARE, redroot pigweed) compared to less sensitive species.

**Figure 8.** Effect of treatments on glutathione S-transferase activities of selected weed species. a) GSTCDNB activities; b) GSTacetochlor activities of untreated and treated 6-day-old etiolated seedlings.
With $^{14}$C-acetochlor substrate, GST$_{acetochlor}$ activities of both mono- and dicot seedlings were in the same range except for velvetleaf (ABUTH) (Figure 6b). Regardless of treatment, extractable GSTs from velvetleaf did not show specificity for acetochlor. Nevertheless, GST$_{acetochlor}$ activities in all weed species were less expressed than in maize. No correlation was found between enzyme activity and acetochlor susceptibilities of these weed species. In monocot seedlings higher enzyme inductions (up to 2-fold increase) were observed as compared to those in dicots following safener treatment. Nevertheless, GST$_{acetochlor}$ activity of the maize seedlings exceeded those of weed species which may indicate that the higher detoxication capability of crop plant is closely related to the herbicide tolerance. It is also noteworthy that both GSH and cysteine conjugates of chloroacetamides were found inhibitory to GSTs from maize, Avena fatua, and Echinochloa crus-galli suggesting that GSH conjugation in crops and weeds takes place in a complex manner [76].

Interestingly, Arabidopsis plant cultures were more responsive to induction by safeners than either maize or wheat [77]. Enhancement of GST$_{CDNB}$ activity was greatest with fenclorim however treatment with flurazol, CMPI and benoxacor also offered significant increases. O-Glucosyltransferase and N-glucosyltransferase activities were also stimulated but to a lesser extent. Safeners mefenpyr diethyl and fenchlorazole-ethyl enhanced fenoxaprop-ethyl tolerance of weed Alopecurus myosuroides (black-grass) [78]. In black-grass, these detoxification pathways were only slightly enhanced by safeners, suggesting that metabolism alone was unlikely to account for increased herbicide tolerance. Instead, it was determined that safening was associated with an accumulation of glutathione and hydroxymethylglutathione and enzymes with antioxidant functions including phi and lambda glutathione transferases, active as glutathione peroxidases and thiol transferases respectively. In addition to enhanced glutathione metabolism safener treatment resulted in elevated levels of flavonoids in the foliage of black-grass plants, notably flavone-C-glycosides and anthocyanins. Safening of grass weeds was concluded as a mechanism associated with an inducible activation of antioxidant and secondary metabolism. The ability of safeners to induce GSTs of grassy weeds can be exploited in phytoremediating herbicide-contaminated soils. In recent studies safener benoxacor was used to enhance GSTs of the perennial grass Festuca arundinacea to establish a basis for preventing environmental herbicide pollution [79]. Further studies revealed that in addition to benoxacor cloquintocet-ethyl, fenchlorazol-ethyl, fenclorim, fluxofenim and oxabetrinil were also able to enhance GST activity in Festuca [80]. These results indicate that herbicide diffusion following the runoff of surface waters can be prevented or significantly reduced by vegetating buffer strips with Festuca and by the combination of herbicide and a suitable safener. By this way, the application of safeners can be extended by using non crop-species in phytoremediating contaminated soils.

### 7.2. Interaction of safeners on weed cytochrome P450 monooxygenases

The involvement of cytochrome P450 monooxygenases in herbicide detoxication and selectivity has been well demonstrated [81, 82]. The role of cytochrome P450 monooxygenases in enhanced metabolism of resistant weed species has also been documented [83, 84]. Neverthe-
less, only a few examples can be found in the literature as to cytochrome P450-dependent monooxygenase system in weed species [85].

Monocotyledonous (Avena fatua, Bromus inermis, Echinochloa crus-galli) and dicotyledonous (Amaranthus retroflexus, Abuthilon theophrasti, Xantium strumarium) weeds were used to study the interaction of safeners, herbicides metabolized by cytochrome P450 enzymes, and P450 inhibitors on herbicide phytotoxicity and P450 levels of weeds and maize [86]. The safener NA was slightly protective to all monocots at the reduced rate (50 g/ha) of nicosulfuron and also exhibited safening effects on dicots against all herbicides. MG-191 reduced growth inhibition of EPTC in A. fatua and E. crus-galli.

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<td>X. strumarium</td>
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<td>Maize&lt;sup&gt;e&lt;/sup&gt;</td>
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<sup>a</sup> 0.5 %w/v; <sup>b</sup> 1 μM; <sup>c</sup> 7-day-old etiolated weed seedlings; <sup>d</sup> ND not detectable; <sup>e</sup> 4-day-old etiolated maize seedlings.

Table 3. Cytochrome P450 contents of mono- and dicot weeds and influence of treatment with the safener NA and P450 inhibitor ABT.

Weed microsomal cytochrome P450 enzymes were found less stable than those from maize. Carbon-monoxide difference spectra for B. inermis and X. strumarium could not be recorded probably due to dark colors of microsomal preparations and difficulties in resuspending the microsomes. Cytochrome P450 content in the microsomal membrane fraction of A. fatua was 2.4-fold greater than in E. crus-galli (Table 3). Among dicotyledonous plants, A. theophrasti contained 5.1-fold higher level of the enzyme as compared to that of A. retroflexus. However, the P450 level was higher in maize than in weeds.

It is difficult to evaluate changes in the enzyme contents of weed species pretreated with the safener NA or the P450 inhibitor ABT due to the high values of standard deviation of the data. Following treatments with NA, a stimulating tendency could be observed for weeds except E. crus-galli. With maize the NA treatment had no enhancing effect on the enzyme content. However, a significant increase (43%) was found when maize seedlings treated with ABT but the P450 inhibitor was ineffective on weed P450s.

For further characterization of in vivo interaction of the combination of the herbicides with safeners and inhibitors microsomes isolated from etiolated maize seedlings were used (Figure 7). Treatment of maize seedlings with nicosulfuron resulted in 30% elevation in P450 level while no effect of EPTC was found. The combination of NA with either bentazon or nicosul-
Furon decreased P450 levels by about 50% as compared to the untreated control. Interestingly, without herbicide pretreatment with NA had no influence on maize P450. The inhibitory effect of NA in vitro on maize P450 was reported by the formation of an enzyme-NA Type I complex [87]. Pretreatments with the combination of MG-191 and all herbicides yielded slight increases in the enzyme concentration. It is interesting to note that no binding of MG-191 to P450 was detected [88] which may indicate why MG-191 was not inhibitory to P450. The P450 inhibitor PBO simultaneously applied with bentazon and nicosulfuron substantially reduced P450 levels while the ABT was less inhibitory.

These results demonstrate that safeners can marginally protect weed species by stimulating the herbicide detoxifying enzymes but the lower level of these enzymes in weeds as compared to those in crops provide a basis for the botanical selectivity of safeners.

8. Mechanism of safener action

The mechanism by which safeners act is currently unknown despite the widespread agricultural use and the substantial experimental evidence accumulated on the biochemical basis of action. Safeners appear to induce a set of genes that encode enzymes and biosynthesis of cofactors involved in the herbicide detoxication [50, 52, 89, 90].

The exact mechanism of safener-mediated enhancement of GST activity is not completely understood. GSTs are induced by a diverse range of chemicals and accompanied by the production of active oxygen species. Thus the connection between safener-mediated protection of crops and oxidative stress tolerance has been suggested [66]. Many GSTs are effective not only in conjugating electrophilic substrates but also function as glutathione peroxidases. Safeners may induce GST expression by mimicking oxidative insult [67]. Herbicide safeners
increase herbicide tolerance in cereals but not in dicotyledonous crops. The reason(s) for this difference in safening is unknown. Treatment of Arabidopsis seedlings with various safeners resulted in enhanced GST activities and expression of GSH-conjugate transporters such as AtMRP1-4 [91]. Safeners also increased GSH content of Arabidopsis seedlings. However, treatment of Arabidopsis plants with safeners had no effect on the tolerance of seedlings to chloroacetanilide herbicides. Immunoblot analysis confirmed that AtGSTU19 was induced in response to several safeners. These results indicate that, although Arabidopsis may not be protected from herbicide injury by safeners, at least one component of their detoxification systems is responsive to these compounds.

Concerning the location of safener binding site(s) of plants, few studies have been conducted. A high-affinity cytosolic-binding site for the dichloroacetamide safener (R,S)-3-dichloroacetetyl-2,2,5-trimethyl-1,3-oxazolidine was found in etiolated maize seedlings ([92]. The binding was highest in the coleoptiles and lowest in the leaves. A good correlation was shown between the safener effectiveness. Chloroacetanilide and thiocarbamate herbicides were effective inhibitors of safener binding at low concentrations. The inhibition by alachlor and EPTC was shown to be competitive. The safener binding protein (SafBP) was purified to homogeneity having a molecular mass of 39 kDa [93]. Based on the peptides obtained from proteolytic digests of SafBP a cDNA encoding SafBP was cloned and expressed in E. coli. The predicted primary structure of SafBP was related to a phenolic O-methyltransferase but SafBP did not catalyze O-methylation of catechol or caffeic acid. It was concluded that SafBP may not be the primary site of action of the dichloroacetamide safeners. Supporting the participation of O-methyltransferases in the safener action, treatment of wheats (Triticum aestivum L.) with cloquintocet-mexyl resulted in an accelerated depletion of flavone C-glycosides and a selective shift in the metabolism of endogenous phenolics [94]. Changes in phenolic content were associated with an increase in O-methyltransferase and C-glucosyltransferase activity toward flavonoid substrates.

Proteomic methods were used to identify herbicide safener-induced proteins in the coleoptile of Triticum tauschii [95]. The herbicide safener, fluxofenim, dramatically increased protein abundance in the molecular weight range of 24 to 30 kDa as well as a few higher molecular weight protein and overall 20 proteins were identified. Among the eighteen inducible proteins 15 were glutathione S-transferase subunits that fall into three subclasses: eight proteins were from the tau subclass, six proteins were from phi subclass, and one was from the lambda class. Another three safener inducible proteins showed homology to the aldo/keto reductase family with proteins that have roles in glycolysis and the Krebs cycle. One of the two constitutively expressed proteins showed the highest homology to the dehydroascorbate reductase subclass of GSTs while the other to an ascorbate peroxidase. Results indicated that the induced proteins were associated with herbicide detoxication and with general stress response. In another study with cloquintocet-mexyl safener and dimethenamid herbicide 29 safener-induced and 10 herbicide-regulated proteins were identified in Triticum tauschii seedlings [39]. Surprisingly, mutually exclusive sets of proteins were identified following herbicide or safener treatment suggesting a different signaling pathway for each chemical. Safener-responsive proteins were mostly involved in
xenobiotic detoxication whereas herbicide-regulated proteins belonged to several classes involved in general stress responses. Quantitative RT-PCR revealed that multidrug resistance-associated protein (MRP) transcripts were highly induced by safeners and two MRP genes were differently expressed.

Figure 10. Suggested safener-mediated signalling pathway for regulation of defense genes and activation of detoxification pathways in plants by Riechers et al. [52]. Dashed lines indicate possible but unproven signaling pathways while solid lines indicate known signaling pathways. ODPA: 12-oxo-phytodienoic acid; OPRs: ODPA-reductases; PPA1: A1-type phytoprostanes; JA: jasmonic acid; TGA: TGA transcription factor; Nrf2: nuclear factor (erythroid-derived 2)-like 2; CoI1: coronative insensitive protein 1; JAZ: transcriptional repressor protein; TF: transcriptional factor/activator.

Safeners were suggested to trigger an unidentified, preexisting signaling pathway for detoxification of endogenous toxins or xenobiotics [96]. According to a new hypothesis, safeners may be utilizing an oxidized lipid-mediated (oxylipins) or cyclopentenone-mediated signaling pathway which subsequently leads to the expression of GSTs and other proteins involved in detoxification and plant defense [52]. Some possible safener-mediated signaling pathways for the regulation of defense genes and activation of detoxification pathways have been suggested (Figure 8). Safeners may tap into a RES oxylipin-mediated signaling pathway and up-regulate TGA transcription factors, an Nrf2-Keap1-mediated as well as jasmonic acid-mediated signaling pathways. Safeners and oxylipins as reactive electrophilic species (RES oxylipins) have a common biological activity since both strongly induce the expression of defense genes and activate detoxification responses in plants [39, 40].
9. Conclusions

Fifty-year of herbicide safeners research and use confirms that these molecules offered new ways to improve herbicide selectivity. Although this technology now competes with herbicide-tolerant, genetically-modified or naturally-selected crops, safeners still comprise an important part of the herbicide market in maize, cereals and rice [10]. Many of the commercial safeners are in off-patent status offering a chance for the generic manufacturers to enter the market together with off-patent herbicides. In contrast, recent herbicide mixture patents with new herbicides still allow their exclusive usage by the patent holder [10].

Although safeners do not improve herbicide tolerance in dicot plants, but the utilization of biotechnology tools may help in extending the safener response from monocot to dicots. It was found, however that Arabidopsis transgenic plants did not respond to safeners at whole-plant level despite the increase of the expression of tau class protein in the roots [91]. Additionally, knowledge of critical regulatory elements in the promoters or untranslated regions of genes encoding detoxification enzymes, or a comprehensive understanding how gene expression is up-regulated by safeners might lead to the precise manipulation of transgene expression of plants [52].

The use of safeners to enhance tolerance of plants to organic pollutants such as herbicides, heavy metals or oils in the environment (soil, water) could also be a promising application of these chemicals. Phytoremediation studies with soils contaminated with oils and heavy metals and safener-treated wheat seeds have recently been reported [97]. While untreated seeds were unable to germinate on the contaminated soil, safener treatments resulted in seedlings briefly growing before succumbing to the pollutants.

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References


