

ORIGINAL ARTICLE

Safeners for chlorsulfuron on maize (*Zea mays* L.)

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Keywords

acetolactate synthase (ALS); chlorsulfuron (CS); herbicide safener; maize.

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Abstract

Introduction Chlorsulfuron inhibits acetolactate synthase (ALS), a key enzyme in plants needed in the biosynthesis of the branched amino acids isoleucine, leucine and valine. Maize is a susceptible crop to the action of this herbicide. **Objectives** This article examines the synthetic compound 1-(4-fluorophenylthiocarbamoyl)-4-methylpiperazine as a possible safener for chlorsulfuron in maize (*Zea mays* L. cv. Kneja 530). **Methods** Commercial herbicide safener, 1,8-naphthalic anhydride, was used as a standard. Maize seeds were impregnated with 1-(4-fluorophenylthiocarbamoyl)-4-methylpiperazine or naphthalic anhydride by soaking for 5 h in aqueous solutions, followed by a herbicide treatment for 5 h. Plants were grown as water cultures. **Results** Changes in growth and in specific activity of acetolactate synthase were determined 8 and 12 days after the treatment. Shoot length and fresh weight of maize plants treated with 10^{-5} M chlorsulfuron were inhibited 59% and 52%, respectively, compared with untreated plants, whereas pretreatment with 5×10^{-4} M 1-(4-fluorophenylthiocarbamoyl)-4-methylpiperazine and naphthalic anhydride reduced these inhibitions. Moreover, the decrease in the root growth caused by chlorsulfuron alone was almost completely reversed by 1-(4-fluorophenylthiocarbamoyl)-4-methylpiperazine. The specificity of naphthalic anhydride and 1-(4-fluorophenylthiocarbamoyl)-4-methylpiperazine's action on acetolactate synthase was supported by the lack of effect on the enzyme activity *in vitro*. However, pretreatment of seeds with both safeners overcomes chlorsulfuron-induced inhibition of acetolactate synthase activity in leaves and roots, 8 and 12 days after treatment. **Conclusion** Our data showed that the synthetic compound 1-(4-fluorophenylthiocarbamoyl)-4-methylpiperazine was a more active herbicide safener in the roots whereas the naphthalic anhydride, in the leaves.

Introduction

Herbicide safeners are a group of structurally diverse synthetic chemicals with the unique ability to protect crop plants from injury by certain herbicides (Farago *et al.*, 1994). Safeners have been exploited in two ways: to improve tolerance of target crops with limited selectivity to herbicides and to extend the use of herbicides on additional (susceptible) crops or on varying environmental conditions.

The selective sulfonylurea herbicide, chlorsulfuron (1-(2-chlorophenylsulfonyl)-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea), represents a major advancement in weed

control technology by combining high herbicidal activity, safety to several small grains cereals such as wheat, barley, oat, and rye, and very low mammalian toxicity (Tranel & Wright, 2002). Sulfonylurea herbicides kill plants by preventing synthesis of branched-chain amino acids through inhibition of acetolactate synthase (ALS, EC 4.1.3.18), the first enzyme in the biosynthetic pathway (Umberger, 1978; Chaleff & Mauvias, 1984; Ray, 1984). Because of their high activity and broad spectrum of weed control, sulfonylureas would be a particularly good group of herbicides to develop safeners against (Devlin & Zbiec, 1990). Nevertheless, research on the effect of safeners on herbicidal activity of sulfonylureas is limited.

Maize, one of the most important crops, is often rotated with small grain cereals. Maize was used as a test plant because it is quite susceptible to soil residues from chlorsulfuron-treated crops to the preceding chlorsulfuron-tolerant crops.

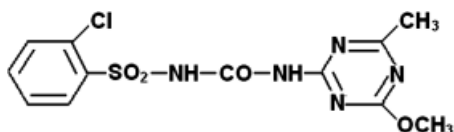
Parker *et al.* (1980) first observed a safening effect on sulfonylurea herbicides when they showed that naphthalic anhydride could partially protect maize from chlorsulfuron. There is an acceptance of the phenomenon of chemical protection of maize from chlorsulfuron injury, but a less agreement on the protective mechanisms. Naphthalic anhydride decreased the chlorsulfuron half-life by > 50% in experiments by Sweetser (1985) but did not increase the rate of chlorsulfuron metabolism in the experiments by Frear *et al.* (1987).

The objectives of this research were to examine the degree of safening by pretreatments of maize seeds with naphthalic anhydride and with the newly developed safener, 1-(4-fluorophenylthiocarbonyl)-4-methylpiperazine (FPTU), against chlorsulfuron injury and to explain the observed difference between unsafened and protected plants by the effects of safeners relative to the ALS activity.

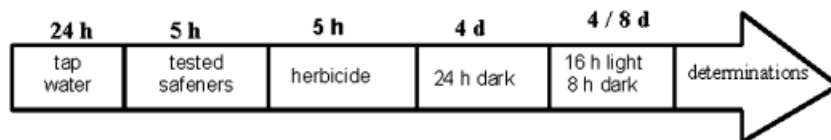
Materials and methods

Chemicals

Chlorsulfuron – 1-(2-chlorophenylsulfonyl)-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea,



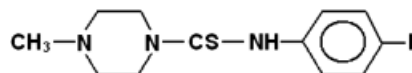
A commercially formulated product chlorsulfuron (Glean, 75% w/w a.i., DuPont, Wilmington, DA, USA) was used. The



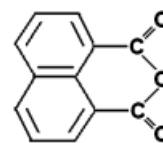
concentration causing 50% inhibition of shoot growth of maize was 10^{-5} M (Stoilkova & Yonova, 2007). Thus, chlorsulfuron was applied at 10^{-5} M in all the following experiments.

One protecting compound used in this research, FPTU, has been synthesized and characterized in our laboratory. The chemical purity was > 95% as determined by high-pressure liquid chromatography analysis.

FPTU,



1,8-naphthalic anhydride was used as a standard,



Naphthalic anhydride used in this research was obtained commercially from Merck Schuchardt OHG (Hohenbrunn, Germany).

The concentration of safeners chosen for studies was 5×10^{-4} M because this is the concentration that provided some protection against chlorsulfuron injury in maize. Pyruvate, 1,4-dithiotreitol, flavin adenine dinucleotide (FAD), 1-naphthol, and creatine monohydrate were purchased from Merck Schuchardt OHG. Thiaminepyrophosphate (TPP) was obtained from Sigma (Sigma, Munich, Germany).

Plant material and growth conditions

Maize was used as a model system. Seeds were soaked in tap water for 24 h. Imbibed seeds were soaked consecutively in aqueous solutions of potential protecting agents, FPTU or naphthalic anhydride, for 5 h followed by chlorsulfuron for 5 h. Control seeds were soaked in distilled water for 10 h. The treated seeds of all plants were rolled in moist filter paper bands. The rolls were put into cups with distilled water for germination in darkness (27°C) for 4 days, and then the maize seedlings were transferred to a growth chamber ($160 \mu\text{mol m}^{-2} \text{s}^{-1}$, 16 h light and 8 h dark, $24 \pm 1^\circ\text{C}$). After 4 and 8 days, 10 plants were selected for

measurements. Their shoot and root lengths, and fresh weights (FW) were determined. Treatment of the seeds is presented in the following scheme:

Estimation of chlorophyll content

Chlorophyll (*a+b*) was extracted with 80% acetone and estimated according to Arnon (1949).

ALS extraction

Plant tissue was cut in small pieces and suspended in ice-cold extraction buffer (buffer: plant material, 4:1, v/w) that contained 100 mM potassium phosphate (pH 7.5), 10% (v/v) glycerol, 0.5 mM MgCl₂, 10 mM sodium pyruvate, 0.5 mM TPP, 0.5 mM pyruvate, 1,4-dithiothreitol, and 10 μM FAD. Soluble polyvinylpyrrolidone (5%, w/v) was added. All subsequent operations were conducted at 0–4 °C. The homogenate was centrifuged at 14 000 × g for 30 min at 4 °C. The supernatant fraction was brought to 50% saturation with ammonium sulfate and allowed to stand for 30 min. Then the mixture was centrifuged at 14 000 × g for 30 min and the supernatant was discarded. The final pellet was suspended in the extraction buffer (3.5 mL g⁻¹ fresh material). A small amount of insoluble matter was removed by centrifugation at 14 000 × g for 15 min, before the enzyme was desalted on a Sephadex G-25 mini column equilibrated with an extraction buffer (GE Healthcare, Munich, Germany).

ALS activity

ALS activity was assayed as described by Forlani *et al.* (1991), by determining indirectly the amount of acetolactate produced. ALS was decarboxylated to acetoin under acidic conditions.

The reaction mixture in a 0.5 mL total volume contained 20 mM potassium phosphate (pH 7.5), 1 mM MgCl₂, 40 mM sodium pyruvate, 0.1 mM TPP, 0.01 mM FAD, and enzyme. Incubation in darkness continued for 60 min at

37 °C, and the enzyme reaction was terminated by adding 50 μL of 9.75 M sulfuric acid. Incubation continued at 37 °C for 30 min to decarboxylate the acetolactate to acetoin, and then 1 mL of colorimetric mixture (109 mg creatin in 20 mL distilled water and 1090 mg 1-naphthol in 20 mL 5 N NaOH) was added. Samples were incubated at 60 °C for 15 min, centrifuged at 10 000 × g for 10 min, and then read at 520 nm against an appropriate blank. ALS activity was expressed as μmol acetoin mg⁻¹ protein h⁻¹, using the extinction coefficient $\epsilon = 15 \text{ mM}^{-1} \text{ cm}^{-1}$.

The *in vitro* effect of the tested compounds on ALS activity (isolated from 8-day-old untreated plants) was measured by adding the compounds to the reaction medium to give a final concentration of 10 μM for chlorsulfuron and 500 μM for safeners FPTU and naphthalic anhydride. Stock solutions were prepared in 100 mM potassium phosphate buffer, pH 7.5 before adding to the reaction mixture.

The *in vivo* effect of tested safeners was studied by extracting ALS from the leaves and roots of maize whose seeds were treated as described above.

Estimation of protein

Protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Statistics

The data presented are means of three independent experiments with three replications each. The data were analyzed

Table 1 Effect of 1-(4-fluorophenylthiocarbonyl)-4-methylpiperazine (FPTU) and naphthalic anhydride (NA) as safeners against inhibition of shoot and root growth induced by chlorsulfuron (CS) in maize (means ± SE, *n* = 6)

CS(M)	Safeners (M)	Shoots				Roots			
		Length		Fresh weight		Length		Fresh weight	
		mm	% control	mg plant ⁻¹	% control	mm	% control	mg plant ⁻¹	% control
0	0	181 ± 0.691	100	433 ± 2.123	100	140 ± 0.365	100	210 ± 0.254	100
10 ⁻⁵	0	74 ± 0.204	41	209 ± 1.569	48	70 ± 0.258	50	80 ± 0.011	38
0	FPTU (5 × 10 ⁻⁴)	139 ± 0.026	77	385 ± 0.236	89	147 ± 1.145	105	317 ± 0.654	151
10 ⁻⁵	FPTU (5 × 10 ⁻⁴)	107 ± 1.145	59	277 ± 0.512	64	122 ± 1.247	87	250 ± 1.006	119
0	Naphthalic anhydride (5 × 10 ⁻⁴)	167 ± 0.204	92	433 ± 0.956	100	68 ± 0.698	49	147 ± 0.921	70
10 ⁻⁵	Naphthalic anhydride (5 × 10 ⁻⁴)	139 ± 0.561	77	364 ± 0.543	84	79 ± 0.214	57	97 ± 0.123	46
LSD	5%	6.436		2.30		5.621		3.125	
	1%	8.547		3.317		7.465		4.337	

statistically and the least significant difference was used to evaluate differences between the treatments.

Results

Effect of FPTU and naphthalic anhydride as safeners of maize growth and chlorophyll (a + b) content to chlorsulfuron inhibition

Chlorsulfuron applied alone caused a significant inhibition in the growth of shoots of maize plants – in length by 59% and in FW by 52% of the control (Table 1). There was no significant change in FW, in the aboveground parts of maize, after the application of naphthalic anhydride compared with

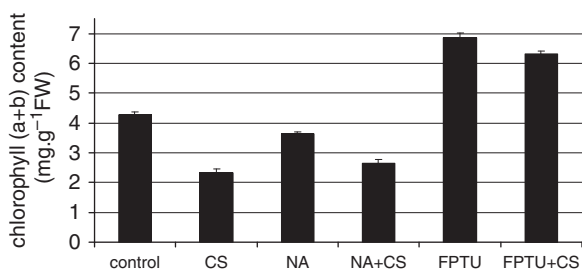


Figure 1 Effect of chlorsulfuron (CS), 1-(4-fluorophenylthiocarbamoyl)-4-methylpiperazine (FPTU), and naphthalic anhydride on chlorophyll (a + b) content of maize (means \pm SE, $n = 6$).

untreated plants. In contrast, pretreatment of seeds with naphthalic anhydride reduced the growth inhibition caused by chlorsulfuron by 36% for each one of the parameters; a similar effect, but expressed in a lower degree, was observed when the seeds were pretreated with the synthetic compound FPTU. The decreases in the shoot length and the FW provoked by herbicide were offset by FPTU to 18% and 16%, respectively.

Root growth of maize was inhibited by 50% in length and 67% in FW after the herbicide treatment (Table 1). The reduction of the root growth (length and FW) of plants treated with naphthalic anhydride was significant compared with untreated plants. The percentage reduction in length and FW of roots were 51% and 30%, respectively (Table 1). When treated in combination with the herbicide naphthalic anhydride the injurious effect of chlorsulfuron (by 7% in length and 8% in FW) was slightly reduced. The treatment with FPTU alone induced a 5% increase in root length and a 51% increase in FW for maize compared with the untreated plant. This synthetic compound, applied before chlorsulfuron, diminished significantly the herbicide-induced reduction in root elongation. FPTU treatment before the herbicide treatment led to a 19% increase in root FW compared with the control values.

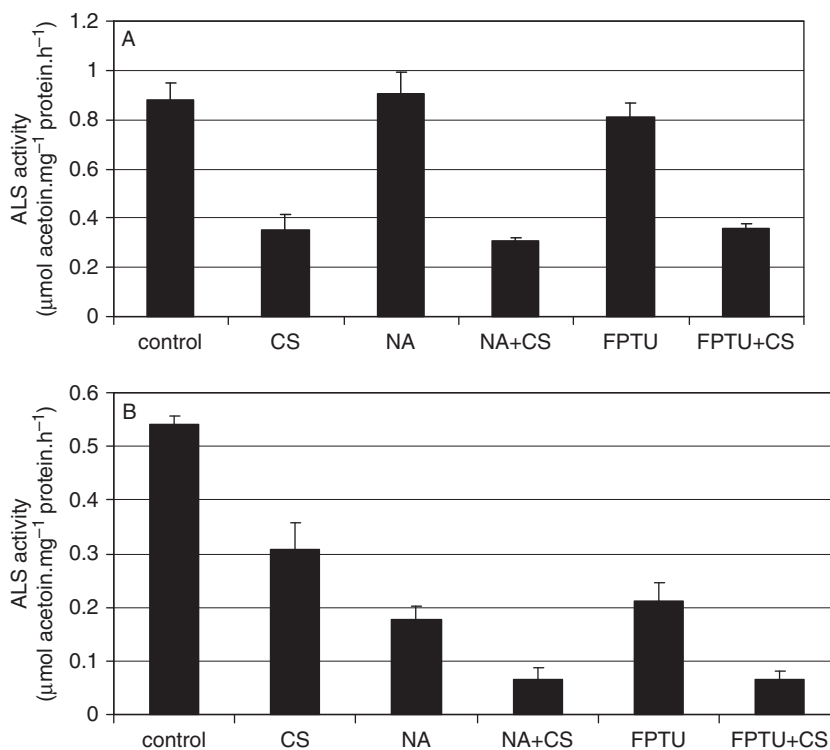


Figure 2 *In vitro* sensitivity of crude ALS extract to chlorsulfuron (CS), 1-(4-fluorophenylthiocarbamoyl)-4-methylpiperazine (FPTU), and naphthalic anhydride (NA). ALS was extracted from leaves (A) and roots (B) of 8-day-old untreated maize plants (means \pm SE, $n = 6$).

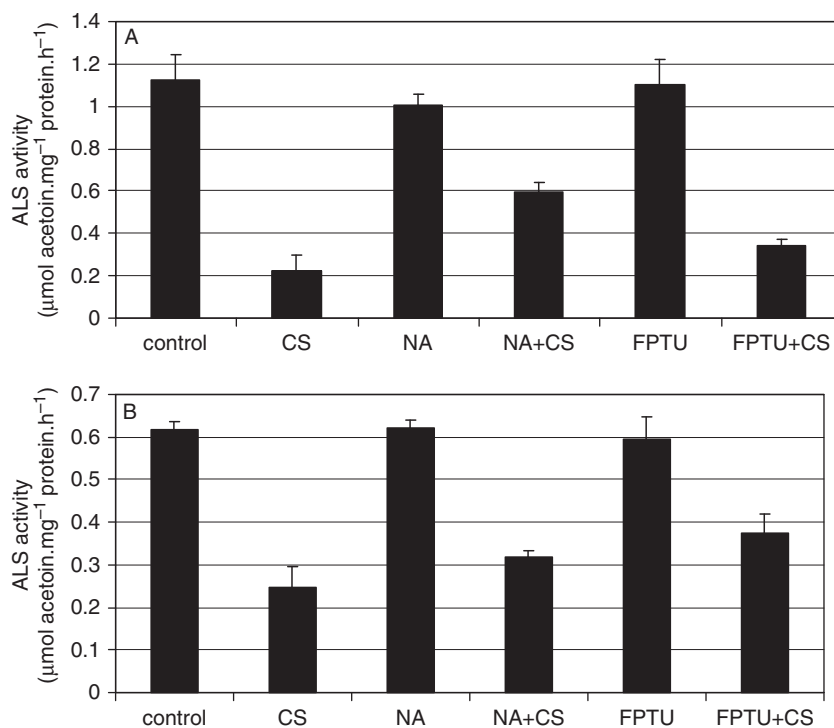


Figure 3 ALS activity in leaves (A) and roots (B) of maize plants 8 days after treatments of the seeds with 1-(4-fluorophenylthiocarbonyl)-4-methylpiperazine (FPTU), naphthalic anhydride (NA), and chlorsulfuron (CS) (means \pm SE, $n = 6$).

Chlorsulfuron caused a decrease in chlorophyll ($a+b$) level by a 45% as compared with the control (Figure 1). The data demonstrate that both safeners prevent the degradation of chlorophyll ($a+b$) content (naphthalic anhydride only by 7% points and FPTU by 93% points) induced by herbicide treatment.

***In vitro* effect of FPTU and naphthalic anhydride on chlorsulfuron-induced ALS-inhibition in maize**

To check the ability of both safeners to act as antagonists of herbicide, *in vitro* ALS assays were carried out (Figure 2). The obvious result of the *in vitro* ALS assay was that the specific activity of ALS preparations was lower in maize roots than in leaves. The ALS activity in leaves was $0.881 \mu\text{mol acetoin mg}^{-1} \text{ protein h}^{-1}$ while the activity in roots was $0.54 \mu\text{mol acetoin mg}^{-1} \text{ protein h}^{-1}$.

Chlorsulfuron caused a reduction in activity of ALS extracted from roots by 60% and leaves by 43% compared with the control.

FPTU or naphthalic anhydride applied alone to the reaction medium *in vitro* did not cause a change in ALS activity extracted from leaves compared with the control (Figure 2). However, both safeners reduced the activity of ALS extracted from roots with respect not only with the

control (naphthalic anhydride by 67% and FPTU by 61%) but to plants treated with chlorsulfuron (CS) as well (naphthalic anhydride by 24% and FPTU by 18%). Applied in combination with the herbicide, both compounds did not change the enzyme activity extracted from leaves and additionally augmented the CS-induced decrease in this activity from roots with respect to the CS treatment.

Effect of FPTU and naphthalic anhydride on chlorsulfuron-induced ALS inhibition in maize after seed treatment

Inhibition of ALS by chlorsulfuron-treated maize seed was very significant in the leaves – 80% at 8 days after treatment (Figure 3) and 40% at 12 days after treatment (Figure 4). Enzyme activities in the leaves and roots, 8 days after herbicide treatment (Figure 3) were inactivated much more by CS compared with the *in vitro* measurement (Figure 2). However, 12 days after chlorsulfuron treatment in maize seed, the ALS activity was increased compared with that at 8 days (Figures 3 and 4).

Treatment of maize seeds with both safeners (NA and FPTU) did not cause a considerable change in the ALS activity from leaves compared with the control 8 and 12 days after applications (Figures 3 and 4). The combination of

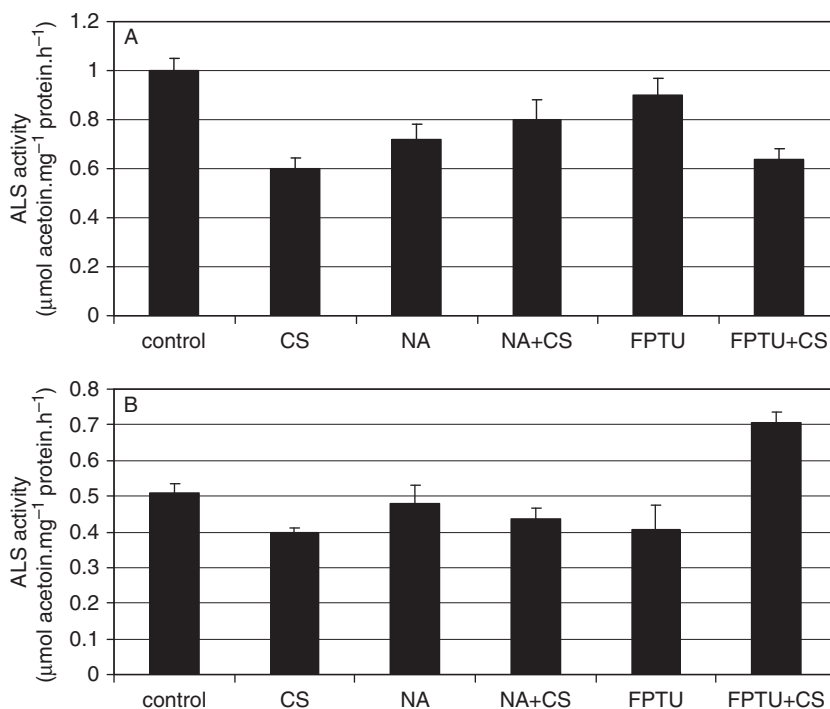


Figure 4 ALS activity in leaves (A) and roots (B) of maize plants 12 days after treatments of the seeds with 1-(4-fluorophenylthiocarbonyl)-4-methylpiperazine (FPTU), naphthalic anhydride (NA), and chlorsulfuron (CS) (means \pm SE, $n = 6$).

chlorsulfuron and safeners decreased the enzyme activity compared with the untreated plants at the 8 days after treatment. However, the measured ALS activities on the 8th day were increased compared with chlorsulfuron-treated plants alone.

Discussion

The ALS-inhibiting herbicides accumulate in meristematic regions of the plant and the herbicidal effects are first noted there. Symptoms include plant stunting, chlorosis (yellowing), and tissue necrosis (death) and are evident 1–4 weeks after the herbicide application.

Growth analysis allows preliminary evaluation of the degree of damage induced by a herbicide on the general physiological processes of the treated plants. As expected, CS strongly inhibited the length of shoots and roots and their FW compared with the control. The new synthetic compound FPTU was a more effective safener against CS injury in roots than in shoots, while the standard naphthalic anhydride more effectively protected the aboveground parts of maize. However, the effect of FPTU on the chlorophyll content was more visible than in that of the standard.

The level of ALS inhibition by CS may be related to the activity of ALS present in the different plant tissues. The fact

that a higher portion of the enzyme isolated from leaves was more sensitive to *in vitro* inhibition by CS than in that isolated from roots was not surprising, because the results agreed with a previous report for maize (Rubin & Casida, 1985; Royuela *et al.*, 1991). A possible explanation for these results may be the presence of two forms of ALS that differ in their sensitivity to chlorsulfuron. The highly sensitive form accounted for 40–50% of total activity in roots and 70–80% in shoots (Rubin & Casida, 1985).

Despite several reports, relatively little is known about the mechanisms of action of herbicide safeners leading to a reduction of damage induced by herbicides. According to Hatzios (1988) safeners may act either as ‘bioregulators’ influencing the amount of herbicide that reaches its target site in an active form or as ‘antagonists’ of herbicidal effects at a common site of action.

To check the ability of safeners to act as antagonists of herbicide, an *in vitro* ALS assay was carried out (Figure 2). NA and FPTU did not act *in vitro* to change the leaf and root enzyme response to tested herbicide. Previously, Milhomme and Basdtide (1990) also showed that NA did not protect maize against chlorsulfuron inhibition *in vitro*. Our results give us reason to propose that both safeners tested in this study did not compete with chlorsulfuron for the active site of the ALS enzyme in leaves of maize.

Elevated levels of a target enzyme can provide the basis for the protecting effect of herbicide safeners to certain ALS-inhibiting herbicides. Rubin and Casida (1985) reported that pretreatment of maize with dichlormid elevated the ALS activity contributing partially to protection of maize against chlorsulfuron injury. However, Polge *et al.* (1987) found that while NA and dichlormid enhanced the ALS activity in treated maize seedlings, the enzyme extracted from safened plants was more sensitive to CS inhibition. Further, Barrett (1989) failed to detect measurable effects of the safeners NA, oxabenztril, dichlormid, and flurazole on ALS activity in shoots or roots of maize seedlings (Muhitch *et al.*, 1987; Royuela *et al.*, 1991).

The effect of ALS-inhibiting herbicides cannot be detected by measuring the extractable ALS activity because the strength of the enzyme-inhibitor complex is most likely insufficient to remain bound throughout the enzyme extraction procedures. Thus, the determination of ALS activity after seed treatment allows for the most accurate assessment of this activity in plants. This enables to assess the magnitude of inhibition status. The enzyme activities measured in leaves and in roots 8 days after seed treatment with herbicide were considerably inactivated by CS (Figure 3) compared with the *in vitro* determination (Figure 2). However, 12 days after chlorsulfuron was applied to seeds, ALS activity was increased (by 167% in leaves and by 62% in roots, respectively) compared with the 8th day (Figures 3 and 4). These results are not surprising because Muhitch *et al.* (1987) showed that ALS-inhibiting herbicides bind slowly but tightly to the target enzyme. However, some evidence suggests that the interaction between these herbicides and ALS is a reversible process (Muhitch *et al.*, 1987; Gaston *et al.*, 2002).

Treatments of maize seeds with either safener, naphthalic anhydride, or FPTU, did not cause a considerable change in the ALS activity from leaves compared with the control 8 and 12 days after applications (Figures 3 and 4). The same values were obtained for ALS activity from leaves of control maize plants and those treated with FPTU or naphthalic anhydride, indicating that while elevating the ALS activity the safeners did not change the proportion of sensitive and insensitive forms or isozymes (Figures 3 and 4). Applied in combination with herbicide these tested safeners decreased the enzyme activity with respect to the untreated control, which was more evident on the 8th day than on the 12th day after treatments. However, the measured ALS activities in these cases were increased compared with plants treated with herbicide. The fact that safeners applied alone did not increase the ALS activity is reason enough to suggest that the

antidote action of both compounds do not involve an effect on enzyme synthesis.

FPTU alone did not provoke much change of enzyme activity in both *leaves and roots* compared with the control, but applied in combination with CS overcame completely the CS-induced inhibition of ALS activity in roots, especially 12 days after seed treatments.

It is well known that the maize is one of the most important crops and is often rotated with small grain cereals. However, maize is quite susceptible to chlorsulfuron residues in soil arising from treatments to the preceding CS-tolerant crops (barley, wheat, and soybean). Residues may persist in soil for several years. So, we suggest that maize could be safened with FPTU against the phytotoxic effects of residual amounts of sulfonylurea herbicide chlorsulfuron in fields where wheat/maize, barley/maize, and soybean/maize rotations take place.

Conclusion

Summarizing, naphthalic anhydride and FPTU have a unidirectional influence on ALS activity, but the different extent of their effect may determine the observed specific character of the antidote action, i.e. naphthalic anhydride is more effective as a safener aboveground whereas FPTU is more effective in roots of maize plants. Because of our poor understanding of the mechanisms of the action of herbicide safeners and the limited information available on metabolic detoxification of chlorsulfuron in susceptible plants, the observed interactions between tested safeners and chlorsulfuron are difficult to explain. Both safeners (NA and FPTU) have a significant effect for protecting maize against CS injury. Based on our data, it is safe to conclude that a safening-induced enhancement of ALS activity does not appear to play a major role in the safening action of both compounds. It could be presumed that the safening effect of naphthalic anhydride and FPTU was due to stimulation of the oxidation metabolism of CS and/or induction of the enzymes involved in detoxification of this herbicide. Probably both naphthalic anhydride and FPTU act as 'regulators' influencing the amount of CS that reaches its target site in an active form.

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