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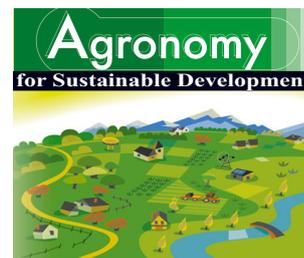
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Research article

Festuca arundinacea grass and herbicide safeners to prevent herbicide pollution

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Abstract – Buffer strips are uncultivated zones left along the boundaries of crops. Buffer strips are used to eliminate or reduce the environmental impact of herbicides. As the efficiency of buffer strips is improved by the presence of non-crop vegetation, we studied the possible role of growing the perennial grass *Festuca arundinacea*. We investigated the activity in festuca of glutathione S-transferase (GST), which is an enzyme very active in metabolising herbicides. Our results evidence GST activity, which is enhanced by the following compounds: benoxacor, cloquintocet-mexyl, fenchlorazol-ethyl, fenclorim, fluxofenim and oxabtrinil. These synthetic compounds are named herbicide safeners because they protect crop plants against injury from some herbicides without reducing the action of herbicides against the target weeds. The increases in GST activity were found to be concomitant with changes in V_{\max} and K_M , that are kinetic constants related directly to the enzyme concentration in the protein “pull” and inversely to the substrate–enzyme affinity, respectively. In particular, V_{\max} increase with K_M decrease was observed in response to benoxacor, V_{\max} increases were found in response to fenchlorazol-ethyl, fenclorim, fluxofenim and oxabtrinil, and K_M decrease was observed in response to cloquintocet-mexyl. The GST activity was also found to be enhanced by the safeners when it was tested toward the herbicides terbuthylazine and butachlor as substrates. In particular, the increases in GST toward terbuthylazine ranged in the following decreasing order: 154.6%, 91.7%, 89.2%, 88.3%, 82.5% and 30.8% in response to fluxofenim, fenchlorazol-ethyl, fenclorim, oxabtrinil, benoxacor and cloquintocet-mexyl, respectively. The increases in GST toward butachlor ranged in the following decreasing order: 77.0%, 71.2%, 59.0%, 41.0% and 33.1% in response to oxabtrinil, benoxacor, fenclorim, fluxofenim and fenchlorazol-ethyl, respectively. A further test, performed to evaluate the relevance of the above effects on a macro-scale level, evidenced 10.1% and 32.7% increased amounts of metabolised terbuthylazine and butachlor, respectively, in response to the addition of benoxacor safener to the herbicide treatments. 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.

Festuca arundinacea / glutathione S-transferase / terbuthylazine/ butachlor / safeners / detoxification / buffer strips

1. INTRODUCTION

The use of herbicides to protect crops is a current practice and its costs are fully compensated for by the increase in production. On the negative side of these benefits, there is the risk of soil and water pollution, which creates problems for the environment and for the safety of agricultural and food production. A strategy designed and tested to eliminate or reduce the environmental impact of herbicides is the use of so-called buffer strips (Lacas et al., 2005). Buffer strips can also have a positive effect on insect and plant biodiversity (De Cauwer et al., 2006a, b). Buffer strips are uncultivated zones, or zones vegetated with no crop species, left along the boundaries of crops to reduce losses of sediments and the contamination of surface runoff by various pollutants, herbicides included,

by trapping rain or irrigation waters. The efficiency of buffer strips is improved by the presence of non-crop vegetation with widespread root development and adapted to a high-water or pluviometric system (Delgado et al., 1995; Patty et al., 1997; Vianello et al., 2004). Due to its large root development and to its ability to grow in a large variety of soil types and climatic conditions, the perennial grass *Festuca arundinacea* might be able to adsorb herbicides moving with sediments and surface water and to metabolise them, so preventing or reducing the pollution of the soil-water system.

The capacity of plants to metabolise herbicides can be ascribed to the adaptation of certain enzymes to exogenous substrates such as herbicides. Some of the transferase enzymes, in particular the glutathione S-transferases (EC 2.5.1.18; named GSTs), have proved to be very active. These are a family of enzymes able to conjugate the tripeptide glutathione with a variety of endogenous and exogenous electrophilic

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compounds by a nucleophilic substitution reaction. By virtue of this reaction, the GST enzymes are implicated in the detoxification of many herbicides in plants. In fact, plant GSTs have been found to be involved in the conjugation of chloroacetanilides, chloro *s*-triazines, thiocarbamates, triazinone sulphoxides, solfonylureas, aryloxyphenoxypropionates, diphenylethers, thiodiazolidines and sulphonamides, to give less or no toxic derivatives (Timmerman, 1989; Jablonkai and Hatzios, 1991; Scarponi et al., 1991; Edwards, 1995; Edwards et al., 2000; Deng and Hatzios, 2002a; Scarponi et al., 2003).

In order to reduce the phytotoxicity of herbicides to plants, some chemical agents, named herbicide safeners, have been developed for major crops such as maize, rice, sorghum and wheat, and they are applied by mixing with herbicide formulations (Davies and Caseley, 1999; Hatzios, 2004). The herbicide safeners are a group of structurally different synthetic compounds capable of protecting crop plants against injury from certain herbicides, without reducing their action against the target weed species (Davies and Caseley, 1999; Abu-Qare and Duncan, 2002). The predominant mechanism of safener action is an enhancement of herbicide metabolism and, therefore, an improved herbicide detoxification. This involves the induction of the activity of some enzymes, such as cytochrome P450-dependent monooxygenases, glucosyl transferases and, namely, the GST enzymes (Gaillard et al., 1994; Deng and Hatzios, 2002b; Scarponi et al., 2003; Scarponi and Del Buono, 2005). The majority of herbicide safeners are typically active in cereal crops; however, a few papers indicate that some non-crop species are responsive to safeners (De Ridder et al., 2002; Edwards et al., 2005).

The first aim of this research was to ascertain the inducibility of festuca GST by some herbicide safeners diffused in cereal protection, such as: benoxacor, cloquintocet-mexyl, fenclorazol-ethyl, fenclorim, fluxofenim and oxabetrinil. Another related aim was to ascertain the existence of a safener enhancement of the activity of festuca GST toward the herbicides butachlor and terbuthylazine as exogenous substrates. These were chosen as representative compounds of the α -chloroacetanilide and *s*-triazine groups, which are largely used in wheat and maize weeding. In order to consider the feasibility of utilising the *Festuca arundinacea* to vegetate buffer strips and safeners to reduce herbicidal pollution of the soil/plant system, a further aim of the research was to ascertain the consequence, on a macro-scale level, of the above effects by evaluating the amounts of persistent and metabolised herbicides in festuca tissues as well as in its growth substrate in response to herbicide treatments with and without addition of an adequate safener.

2. MATERIALS AND METHODS

2.1. Plant material and growth conditions

Festuca seeds (*Festuca arundinacea*, hybrid *Villageoise*) (40 g) were germinated in plastic flats (0.08 m²) containing quartz sand pre-washed with a solution of hydrochloric acid (10%, v/v) and sterilised with a solution of NaClO

(5%, w/v). Seedlings were grown in the dark at 23 °C (relative humidity 80%). After two days the seedlings were submitted to day-night conditions (12 h of light at 21 °C, light intensity 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and 12 h of darkness at 23 °C) and watered daily. When the seedlings were 12 days old, they were treated with one of the following herbicide safeners: benoxacor, cloquintocet-mexyl, fenclorazol-ethyl, fenclorim, fluxofenim or oxabetrinil; the treatments were performed by adding to the plastic flats 100 mL of a water solution (0.028 mM fenclorim, 0.14 mM for the other safeners). Three trays were used for each herbicide safener treatment and three trays were left untreated as controls. Shoots were collected at 24, 48 and 72 h after the treatment for GST extraction and purification.

2.2. Glutathione S-transferase extraction and purification

The extraction of GST was carried out according to the procedure of Cummins et al. (1997). Shoots of festuca (3.0 g) were powdered in liquid nitrogen using a mortar and pestle. The powders were suspended in extraction buffer (1/5, w/v), composed of 100 mM Tris-HCl (pH 7.5), containing 2 mM EDTA, 1 mM dithiothreitol and 1.5% (w/v) polyvinylpyrrolidone. After filtration through two layers of muslin, the homogenate was centrifuged at 15 000 rpm for 20 min and the supernatant was adjusted to 80% saturation of (NH₄)₂SO₄ to precipitate the proteins (4 °C for 3 h). The resulting suspension was centrifuged at 15 000 rpm for 10 min and the protein pellets collected and stored at -20 °C. The pellet was dissolved in 20 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol and applied onto a Sephadex G-25 for desalting (enzymatic extract).

All the extraction steps were carried out at 4 °C and the amount of protein was determined according to Bradford (1976).

2.3. Glutathione S-transferase assays toward 1-chloro-2,4-dinitrobenzene

The spectrophotometric procedure described by Edwards and Owen (1986) was used to determine GST activity toward 1-chloro-2,4-dinitrobenzene. The activity of GST toward 1-chloro-2,4-dinitrobenzene was determined by adding 25 μL of 40 mM of the substrate to a solution containing 900 μL of 0.1 M KH₂PO₄ / K₂HPO₄ buffer (pH 6.5), 25 μL of enzymatic extract and 50 μL of 0.1 M glutathione (pH 7.0). The amount of conjugate formed by reaction between glutathione and 1-chloro-2,4-dinitrobenzene was evaluated spectrophotometrically at 340 nm and 35 °C. From this result the amount of conjugate formed in a reaction mixture in which the enzymatic extract was substituted by the buffer (non-enzymatic reaction) was then subtracted. The GST activity was expressed as nmol of conjugate formed by reaction between 1-chloro-2,4-dinitrobenzene and glutathione by the $\text{s}^{-1} \text{mg protein}^{-1}$ employed for the assay.

To determine the kinetic constants V_{\max} and K_M of GST toward 1-chloro-2,4-dinitrobenzene, the glutathione co-substrate concentration was kept constant at 5.0 mM, while the concentration of 1-chloro-2,4-dinitrobenzene was varied from 0.1 to 2.0 mM using 50 μg of protein. The kinetic parameters were determined from linear regression analysis of $1/V$ versus $1/S$ according to double reciprocal plots.

2.4. Assays of glutathione S-transferase toward terbuthylazine and butachlor

The activity of GST against terbuthylazine and butachlor was determined according to the procedure of Hatton et al. (1996). Terbuthylazine or butachlor (2.0 mM; 25 μL), dissolved in acetone, was added to 325 μL of 0.1 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 6.5), 50 μL of 10 mM glutathione (pH 7.0) and 25 μL of enzymatic extract. The mixture was incubated at 35 °C for 2 h, and the reaction was stopped by adding 10 μL of 3.6 M hydrochloric acid. The solution was centrifuged at 5000 rpm for 1 min and then frozen at -20 °C. The samples (20 μL) were then analysed by HPLC. The enzymatic activity was evaluated from the difference between the initial amount of herbicide added and the herbicide residue found at the end of the reaction. The amount of herbicide lost in non-enzymatic reaction, determined in a reaction mixture with the enzymatic extract replaced by the reaction buffer, was subtracted from this result. The GST activity was expressed as nmol of herbicide consumed by the $\text{min}^{-1} \text{mg}^{-1}$ protein employed for the assay. Each sample preparation and determination was run in triplicate.

2.5. Determination of terbuthylazine and butachlor residues

To achieve the mass balance of terbuthylazine and butachlor, their residues were determined in the shoots of festuca as well as in the growth substrate. For this experiment festuca seeds (10 g) were germinated in plastic flats (0.02 m^2) as described in Section 2.1. When the seedlings were 12 days old the plastic flats were divided into five groups: two groups were treated with terbuthylazine or butachlor at the recommended field rates of 350 and 750 g/ha (corresponding to 3.0 mg/flat and 6.0 mg/flat, respectively); two groups were submitted to the same treatment in combination with the safener benoxacor (1.7 mg/flat); one group was left untreated as a control. Two further groups of plastic flats were prepared without festuca seeds and they were submitted to terbuthylazine or butachlor treatment (reference tests). The residue determinations were performed 72 h after the treatment following the gas chromatography-mass spectrometry procedure (GC-MS) of Scarponi and Del Buono (2005) with slight modification. Total roots and shoots of festuca contained in each plastic flat (6.5 ± 0.4 g and 8.5 ± 0.5 g, respectively) were powdered in liquid nitrogen using a mortar and pestle and extracted with methanol (1:5, w/v). After filtration, the samples were dried under a vacuum and redissolved with 2 mL of n-hexane. The

solution was charged in a Florisil column (1000 mg/6 mL, 170 μm , 80 A), pre-activated with 15 mL of n-hexane, and then washed with 5 mL of n-hexane prior to recovery of the herbicide with 4 mL of an ethyl acetate/n-hexane (2:3, v/v) solution. The recovered fraction was evaporated to dryness, rinsed with 1 mL of methanol, and subjected to GC analysis. A Perkin-Elmer Auto System XL gas chromatograph equipped with an OV17 capillary column (0.53 mm \times 30 m length, inside diameter of 0.5 μm) was employed. The injector temperature was 100 °C. Column temperature was 100 °C for 2 min and then raised at 25 °C min^{-1} to 230 °C. Cleanup temperature was at 230 °C for 10 min, and gas carrier flow (helium) was 5 mL min^{-1} . The herbicide residues in the growth substrate were determined by collecting all the quartz sand contained in each plastic flat (300 g). The quartz sand samples were extracted with water (5 \times 100 mL) and the collected water extracts were filtered and submitted to extraction with n-hexane (3 \times 40 mL). The recovered organic fraction was evaporated to dryness, rinsed with 2 mL of methanol and submitted to GC-MS analysis as described above.

3. RESULTS AND DISCUSSION

3.1. Activity of glutathione S-transferase of *Festuca arundinacea* and effect of herbicide safeners

As a preliminary test, the activity of GST enzymes of festuca toward the 1-chloro-2,4-dinitrobenzene as a standard substrate was ascertained over a 24–72 h period; the same tests were also performed in festuca samples treated with each herbicide safener. GST activity was found in the unsafened festuca which ranged during the experimental period from 2.10 to 2.75 $\text{nmols}^{-1} \text{mg protein}^{-1}$ (controls). In comparison with the unsafened controls, all the safener treatments enhanced the enzyme activity; these enhancements showed the greatest duration in response to benoxacor, followed in a decreasing order of duration and efficacy by oxabetrinil and fenclorim, and lastly by fluxofenim, fenchlorazol-ethyl and cloquintocet-mexyl (Tab. I). In particular, the response to each safener was as follows: benoxacor produced increases of 18.6%, 17.8% and 11.8% 24, 48 and 72 h after the treatment, respectively; cloquintocet-mexyl produced a 18.2% increase in enzyme activity 48 h after the treatment; fenclorazol-ethyl produced a 31.6% increase in enzyme activity 72 h after the treatment; fenclorim produced increases ranging from 59.0% to 18.9% during the 24–48 h period after the treatment; fluxofenim produced a 45.6% increase in enzyme activity 72 hours after the treatment; oxabetrinil produced increases from 57.1% to 13.1% during the 24–48 h period after the treatment. These results evidence the presence in festuca of a GST activity naturally expressed and inducible by various herbicide safeners. In order to clarify the nature of this induction, the GST kinetic constants, V_{\max} and K_M , were determined: the first being proportional to the enzyme concentration in the protein “pull” and the second inversely related to the substrate–enzyme affinity. These tests were carried out by using enzymatic extracts from the unsafened and safened shoots, collected at the times

Table I. Activity of glutathione S-transferase (GST) of *Festuca arundinacea* toward the standard substrate 1-chloro-2,4-dinitrobenzene in the absence (controls) and in the presence of herbicide safeners: the data show that all the safeners enhanced the enzyme activity during a 72 h period after the treatments.

Time after the treatment	GST activity (nmol s ⁻¹ mg protein ⁻¹)		
	24 h	48 h	72 h
Controls	2.10 ± 0.25	2.75 ± 0.02	2.37 ± 0.02
Benoxacor	2.49 ± 0.02*	3.24 ± 0.25*	2.65 ± 0.02*
Cloquintocet-mexyl	1.94 ± 0.07	3.25 ± 0.15*	2.22 ± 0.17
Fenchlorazol-ethyl	2.24 ± 0.23	2.84 ± 0.06	3.12 ± 0.05*
Fenclorim	3.34 ± 0.02*	3.27 ± 0.13*	2.42 ± 0.15
Fluxofenim	2.16 ± 0.20	2.80 ± 0.16	3.45 ± 0.07*
Oxabetrinil	3.30 ± 0.11*	3.11 ± 0.07*	2.43 ± 0.22

The data represent the means of triplicate determinations ± standard deviation. Mean figures within the same column followed by * are significantly different from the untreated sample, at the level of 5% using the *t* test.

corresponding to the maximum enhancement of GST activity toward 1-chloro-2,4-dinitrobenzene by each safener treatment. From the data it is apparent that the safeners significantly changed one or both the above constants, with respect to those of the unsafened control (Tab. II). In particular, benoxacor produced an 18.3% increase in V_{max} and a 31.2% decrease in K_M ; cloquintocet-mexyl produced a 52.1% decrease in K_M without any effect on the V_{max} value; fenchlorazol-ethyl, fenclorim, fluxofenim and oxabetrinil produced V_{max} increases of 77.7%, 42.6%, 115.7% and 21.3%, respectively, and did not affect the K_M values. Benoxacor, which gave rise to the most prolonged enhancement of the GST activity toward 1-chloro-2,4-dinitrobenzene, was also the only safener able to determine V_{max} increase coupled with K_M decrease. The increases in V_{max} values without changes in K_M values in response to fenchlorazol-ethyl, fenclorim, fluxofenim and oxabetrinil suggest an effect of the above safeners on the overall expression of GST enzymes. The decrease in K_M in response to cloquintocet-mexyl could result from an induction of GST isoforms endowed with more affinity to the 1-chloro-2,4-dinitrobenzene substrate, without involvement of the overall expression of the enzyme. The above hypotheses are supported by some literature reports which evidence that safeners mediate enhancement of GST activity in plants as a result of an increased level of constitutively expressed GST enzymes and of the induction of specific GST isoenzymes (Holt et al., 1995; Fuerst et al., 1995; Cummins et al., 1997; Riechers et al., 1997; Pascal and Scalla, 1999; Edwards et al., 2005).

3.2. Activity of glutathione S-transferase of *Festuca arundinacea* toward the herbicides terbuthylazine and butachlor as substrates

In order to confirm the ability of festuca GST to act toward herbicides, tests of enzyme activity toward terbuthylazine

Table II. K_M and V_{max} kinetic constants of glutathione S-transferase of *Festuca arundinacea* toward the standard substrate 1-chloro-2,4-dinitrobenzene in the absence (control) and in the presence of herbicide safeners: the data show that all the safeners changed V_{max} and/or K_M constants.

Samples	K_M (mM)	V_{max} (nmol min ⁻¹)
Control	0.635 ± 0.050	19.7 ± 0.7
Benoxacor	0.437 ± 0.013*	23.3 ± 0.2*
Cloquintocet-mexyl	0.304 ± 0.023*	18.8 ± 0.8
Fenchlorazol-ethyl	0.719 ± 0.051	35.0 ± 2.5*
Fenclorim	0.707 ± 0.030	28.1 ± 0.8*
Fluxofenim	0.708 ± 0.032	42.5 ± 1.1*
Oxabetrinil	0.642 ± 0.012	23.9 ± 1.1*

The data represent the means of triplicate determinations ± standard deviation. Mean figures within the same column followed by * are significantly different from the untreated sample, at the level of 5% using the *t* test.

Table III. Activity of festuca glutathione S-transferase (GST) of *Festuca arundinacea* toward the herbicides terbuthylazine and butachlor as substrates in the absence (controls) and in the presence of herbicide safeners: the data show that the safeners enhanced the enzyme activity, with the exception of cloquintocet-mexyl on GST toward butachlor.

	GST activity toward terbuthylazine (nmol h ⁻¹ mg protein ⁻¹)	GST activity toward butachlor
Controls	2.40 ± 0.50	1.39 ± 0.01
Benoxacor	4.38 ± 0.10*	2.38 ± 0.50*
Cloquintocet-mexyl	3.14 ± 0.30*	1.38 ± 0.20
Fenchlorazole-ethyl	4.60 ± 0.50*	1.85 ± 0.04*
Fenclorim	4.54 ± 0.53*	2.21 ± 0.24*
Fluxofenim	6.11 ± 0.44*	1.96 ± 0.43*
Oxabetrinil	4.52 ± 0.10*	2.46 ± 0.24*

The data represent the means of triplicate determinations ± standard deviation. Mean figures within the same column followed by * are significantly different from the untreated sample, at the level of 5% using the *t* test.

(GST-terbuthylazine) and butachlor (GST-butachlor), in substitution of 1-chloro-2,4-dinitrobenzene as substrate, were performed. For these tests enzymes extracted from festuca shoots were used, collected at the times corresponding to the maximum enhancement of GST activity toward glutathione and 1-chloro-2,4-dinitrobenzene, following safener treatments. The data of the safened samples, compared with those of the corresponding unsafened ones, show that the safeners strongly enhanced GST activity toward the two herbicides, with the only exception of GST-butachlor in response to cloquintocet-mexyl (Tab. III). In particular, the increases in GST-terbuthylazine activity ranged in the following decreasing order: 154.6%, 91.7%, 89.2%, 88.3%, 82.5% and 30.8% in response to fluxofenim, fenchlorazol-ethyl, fenclorim, oxabetrinil, benoxacor and cloquintocet-mexyl, respectively; the increases in GST-butachlor ranged in the following decreasing order: 77.0%,

Table IV. Terbutylazine residues (mg) 72 h after the herbicide treatment in the not sown and sown growth substrate and in the roots and shoots of unsafened and benoxacor-safened *Festuca arundinacea*: the data show an enhancement of metabolised herbicide in the benoxacor-safened samples.

Treatment	Growth substrate	Roots	Shoots	Metabolised
terbutylazine (not sown growth substrate)	2.28 ± 0.05 ^a (100)			
terbutylazine	0.44 ± 0.04 (19.3)	0.77 ± 0.01 0.77 ± (33.8)	0.24 ± 0.03 (10.5)	0.83 ± 0.02 (36.4)
terbutylazine + benoxacor	0.51 ± 0.04 (22.4)	0.52 ± 0.01* 0.52 ± (22.8)	0.19 ± 0.02 (8.3)	1.06 ± 0.02* (46.5)

^a Reference test, taken as 100% in the mass balance of the herbicide.

The data represent the means of triplicate determinations (the % variations in brackets) ± standard deviation.

Means within the same column followed by * are significantly different at the 5% level using the *t* test.

71.2% 59.0%, 41.0% and 33.1% in response to oxabtrinin, benoxacor, fenclorim, fluxofenim and fenclorazol-ethyl, respectively. A significant enhancement of GST activity toward 1-chloro-2,4-dinitrobenzene, coupled with a relevant increase in GST proteins in response to benoxacor, has been ascertained in *Arabidopsis thaliana* (Edwards et al., 2005). Therefore, the results of Table III suggest that GST induction could occur in festuca in response to the safeners, similarly to what happened in *Arabidopsis* in response to benoxacor.

As was expected, the substitution of 1-chloro-2,4-dinitrobenzene with terbutylazine and butachlor as substrates also changed the levels of GST enhancement induced by safeners. It is known that the wide spectrum of conjugative activity of GSTs toward different chemicals as substrates is attributable to the structural characteristics of these enzymes. In fact, it is known they form dimeric proteins by combination of different subunits; each subunit shows an independent active site consisting of two functional components: a glutathione binding site and a second site which binds xenobiotic hydrophobic substrates with different affinity (Reinemer et al., 1996; Dixon et al., 1997; Van Eerd et al., 2003). From this, the different activity exhibited by festuca GST toward 1-chloro-2,4-dinitrobenzene, terbutylazine and butachlor could be explained, as well as the different enhancement of the enzyme activity in response to the various safeners.

3.3. Terbutylazine and butachlor residues and their mass balance

In order to evaluate, on a macro-scale level, the effect of the safener induction of GST on the fate of terbutylazine and butachlor given to festuca, the amount and the mass balance of residues of the two herbicides were performed 72 h after the herbicide treatments with or without addition of benoxacor. This safener was chosen for this experiment because of its efficacy in enhancing the activity of GST-terbutylazine and GST-butachlor (Tab. III). The residues of the two herbicides were determined in the shoots and roots of festuca as well as in the total growth substrate contained in each plastic flat. In order to leave out of the mass balance possible losses of herbicides by volatilisation, chemical and/or photochemical degra-

dation and by other physical and chemical factors, reference tests were also performed by giving each of the herbicide treatments to the growth substrate without festuca seedlings. Taking as 100 the amount of herbicides present at sampling time in the respective reference tests, the total herbicide that disappeared from the system constituted by shoots and roots of festuca and by growth substrate was considered as metabolised herbicide.

In response to benoxacor addition to the terbutylazine treatment a significant decrease in herbicide residues from 0.77 to 0.52 mg was found in roots; no significant variation was observed in the residues present in shoots and growth substrate (Tab. IV). Taking into consideration the terbutylazine present in the reference test (2.28 mg), considered as 100, an increase from 36.4% to 46.5% in the metabolised terbutylazine was deduced; this means a 10.1% increase in metabolised herbicide in response to the safener addition to terbutylazine treatment.

In response to benoxacor addition to the butachlor treatment significant decreases in herbicide residues from 2.23 to 1.32 mg in roots, from 0.54 to 0.25 mg in shoots and from 0.97 to 0.57 mg in the growth substrate were found (Tab. V). Taking into consideration the butachlor present in the reference test (4.90 mg), considered as 100, an increase from 23.7% to 56.4% in metabolised butachlor was deduced; this means a 32.7% increase in metabolised herbicide in response to the safener addition to butachlor treatment. The results of Table V evidence 5.9% and 18.6% diminutions of residual herbicide in shoots and roots, respectively, in response to benoxacor addition to the herbicide treatment. The relevant decrease in butachlor in roots appears to have promoted herbicide uptake from the growth substrate, in which a 8.2% decrease in butachlor was found.

These results evidence the ability of benoxacor to enhance the detoxification of the two herbicides by conjugation with glutathione via GST enzymes; the greater efficacy exhibited toward butachlor rather than toward terbutylazine suggests benoxacor to be more active in inducing GST activity toward the α -chloroacetanilide derivative butachlor rather than toward the *s*-triazine derivative terbutylazine.

Table V. Butachlor residues (mg) 72 h after the herbicide treatment in the not sown and sown growth substrate and in the roots and shoots of unsafened and benoxacor-safened *Festuca arundinacea*: the data show an enhancement of metabolised herbicide in the benoxacor-safened samples, coupled with a decrease in herbicide residue in the growth substrate.

Treatment	Growth substrate	Roots	Shoots	Metabolised
butachlor (not sown growth substrate)	4.90 ± 0.04 ^a (100)			
butachlor	0.97 ± 0.05 (19.8)	2.23 ± 0.09 (45.5)	0.54 ± 0.15 (11.0)	1.16 ± 0.03 (23.7)
butachlor + benoxacor	0.57 ± 0.09* (11.6)	1.32 ± 0.21* (26.9)	0.25 ± 0.11* (5.1)	2.76 ± 0.02* (56.4)

^a Reference test, taken as 100 in the mass balance of the herbicide.

The data represent the means of triplicate determinations (the % variations in brackets) ± standard deviation.

Means within the same column followed by * are significantly different at the 5% level using the *t* test.

4. CONCLUSION

This research evidences the presence in *Festuca arundinacea* of active naturally-expressed glutathione *S*-transferase, which is responsive to various herbicide safeners. It is also evident that festuca GST is able to act toward the herbicides terbutylazine and butachlor as substrates; therefore safener addition to herbicide treatments resulted in a greater detoxification of herbicides in the plant. In particular, the relevant increase in butachlor detoxification in response to benoxacor addition to herbicide treatment resulted in a substantial diminution of residual herbicide in the growth substrate. These findings assume particular relevance in relation to the possibility to potentiate the efficacy of buffer strips in the prevention of herbicide pollution. In fact, they indicate that herbicide diffusion following the runoff of surface waters and loss of sediments can be prevented or significantly reduced by vegetating buffer strips with festuca and taking advantage of a combination of herbicide and a suitable safener. Such a possibility must obviously be validated by field experiments

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REFERENCES

- Abu-Qare A.W., Duncan H.J. (2002) Herbicide safeners: uses, limitations, metabolism, and mechanisms of action, *Chemosphere* 48, 965–974.
- Bradford M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72, 248–254.
- Cummins I., Cole D.J., Edwards R. (1997) Purification of multiple glutathione transferases involved in herbicide detoxification from wheat (*Triticum aestivum* L.) treated with the safener fenchlorazole-ethyl, *Pestic. Biochem. Phys.* 59, 35–49.
- Davies J., Casey J.C. (1999) Herbicide safeners. A review, *Pestic. Sci.* 55, 1043–1058.
- De Cauwer B., Reheul D., Nijs I., Milbau A. (2006a) Effect of margin strips on soil mineral nitrogen and plant biodiversity, *Agron. Sustain. Dev.* 26, 117–126.
- De Cauwer B., Reheul D., De Laethauwer S., Nijs I., Milbau A. (2006b) Effect of light and botanical species richness on insect diversity, *Agron. Sustain. Dev.* 26, 35–43.
- De Ridder B.P., Dixon D.P., Beussman D.J., Edwards R., Goldsbrough P.B. (2002) Induction of glutathione *S*-transferases in *Arabidopsis* by herbicide safeners, *Plant Physiol.* 130, 1497–1505.
- Delgado A.N., Periago E.L., Siqueira F.D.F. (1995) Vegetated filter strips for wastewater purification. A review, *Bioresource Technol.* 51, 13–22.
- Deng F., Hatzios K.K. (2002a) Purification and characterization of two glutathione *S*-transferase isozymes from indica-type rice involved in herbicide detoxification, *Pestic. Biochem. Phys.* 72, 10–23.
- Deng F., Hatzios K.K. (2002b) Characterization and safener induction of multiple glutathione *S*-transferases in three genetic lines of rice, *Pestic. Biochem. Phys.* 72, 24–39.
- Dixon P.D., Cole D.J., Edwards R. (1997) Characterisation of multiple glutathione transferases containing GST I subunits with activities towards herbicide substrates in maize (*Zea mays*), *Pestic. Sci.* 50, 72–82.
- Edwards R. (1995) Glutathione transferases in plant involved in herbicide detoxification, *Proc. Brighton Crop Protect. Conf.-Weeds*, pp. 823–832.
- Edwards R., Del Buono D., Fordham M., Skipsey M., Brazier M., Dixon D.P., Cummins I. (2005) Differential induction of glutathione transferases and glucosyltransferases in wheat, in maize and *Arabidopsis thaliana* by herbicide safeners, *Z. Naturforsch. C* 60, 307–316.
- Edwards R., Dixon D.P., Walbot V. (2000) Plant glutathione *S*-transferases enzymes with multiple functions in sickness in health, *Trends Plant Sci.* 5, 193–198.
- Edwards R., Owen W.J. (1986) Comparison of glutathione *S*-transferases of *Zea mays* responsible for herbicide detoxification in plants and suspension cultured cells, *Planta* 169, 208–215.
- Fuerst E.P., Irzyk G.P., Miller K.D., McFarland J.E., Eberlein C.V. (1995) Mechanism of action of the herbicide safener benoxacor in maize, *Pestic. Sci.* 43, 242–244.
- Gaillard C., Dufaud A., Tommasini R., Kreuz K., Amrhein N., Martinoia E. (1994) A herbicide antidote (safener) induces the activity of both the herbicide detoxifying enzyme and of a vacuolar transporter for the detoxified herbicide, *FEBS Lett.* 352, 219–221.
- Hatton P.J., Dixon D., Cole D.J., Edwards R. (1996) Glutathione transferase activity and herbicide selectivity in maize and associated weed species, *Pestic. Sci.* 46, 267–275.
- Hatzios K.K. (2004) Metabolism-based herbicide resistance: regulation by safeners, *Weed Sci.* 52, 454–467.

- Holt D.C., Lay V.J., Clarke E.D., Dinsmore A., Jepson I., Bright S.W.J., Greenland A.J. (1995) Characterization of the safener-induced glutathione S-transferase isoform II from maize, *Planta* 196, 295–302.
- Jablonkai I., Hatzios K.K. (1991) Role of glutathione and glutathione-S-transferase in the selectivity of acetochlor in maize and wheat, *Pestic. Biochem. Phys.* 41, 221–231.
- Lacas J.-G., Voltz M., Gouy V., Carluet N., Gril J. (2005) Using grassed strips to limit pesticide transfer to surface water: a review, *Agron. Sustain. Dev.* 25, 253–266.
- Pascal S., Scalla R. (1999) Purification and characterization of a safener-induced glutathione S-transferase from wheat (*Triticum aestivum*), *Physiol. Plant.* 106, 17–27.
- Patty L., Real B., Grill J.J. (1997) The use of grassed buffer strips to remove pesticides, nitrate and soluble phosphorous compounds from runoff water, *Pestic. Sci.* 49, 243–251.
- Reinemer P., Prade L., Hof P., Neufeind T., Huber R., Zettl R., Palme K., Schell J., Koelln I., Bartunik H.D., Bieseler B. (1996) Three-dimensional structure of glutathione S-transferase from *Arabidopsis thaliana* at 2.2 angstrom resolution: Structural characterization of herbicide-conjugating plant glutathione S-transferases and a novel active site architecture, *J. Mol. Biol.* 255, 289–309.
- Riechers D.E., Irzyk G.P., Jones S.S., Fuerst E.P. (1997) Partial characterization of glutathione-S-transferases from wheat (*Triticum* spp.) and purification of a safener-induced glutathione-S-transferase from *Triticum tauschii*, *Plant Physiol.* 114, 1461–1470.
- Scarponi L., Del Buono D. (2005) Benoxacor induction of terbuthylazine detoxification in *Zea mays* and *Festuca arundinacea*, *J. Agr. Food Chem.* 53, 2483–2488.
- Scarponi L., Del Buono D., Vischetti C. (2003) Persistence and detoxification of pretilachlor and fenclorim in rice (*Oryza sativa*), *Agronomie* 23, 147–151.
- Scarponi L., Perucci P., Martinetti L. (1991) Conjugation of 2-chloroacetanilide herbicides with glutathione: Role of molecular structures and of glutathione-S-transferase enzymes, *J. Agr. Food Chem.* 39, 2010–2013.
- Timmerman K.P. (1989) Molecular characterisation of corn glutathione-S-transferase isozymes involved in herbicide detoxification, *Physiol. Plantarum*, 77, 465–471.
- Van Eerd L.L., Hoagland R.E., Zablotowicz R.M., Hall J.C. (2003) Pesticide metabolism in plants and Microorganisms, *Weed Sci.* 51, 472–495.
- Vianello M., Vischetti C., Scarponi L., Zanin G. (2004) Herbicide losses in runoff events from a field with a low slope: role of vegetative filter strip, *Chemosphere* 61, 717–725.