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Benoxacor Induction of Terbuthylazine Detoxification in *Zea mays* and *Festuca arundinacea*

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The action of safener benoxacor on the detoxification of terbuthylazine (TBA) in *Zea mays* and *Festuca arundinacea* was ascertained by the investigation of the effects of benoxacor on the activity of glutathione-*S*-transferases (GSTs) in the shoots of the two plant species. TBA treatment generally reduced GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) in corn and did not affect the enzyme activity in festuca. When applied alone, benoxacor stimulated GST activity in both plants; however, when it was applied in mixture with TBA, generally, an enhancement of the enzyme activity was found in corn but not in festuca in comparison with the respective TBA-treated samples. The enhancement of GST activity in response to the benoxacor treatment in both corn and festuca resulted to be concomitant with decreases in apparent K_M in both the plant species, with V_{max} unaffected, and with an increased expression of proteins having molecular masses in the characteristic range of plant GSTs. After the benoxacor treatment, increased GST activity toward TBA as a substrate was observed in both corn and festuca. As a consequence, lesser amounts and persistence of TBA residues were found in shoots of corn and festuca treated with the TBA and benoxacor mixture compared to TBA-only-treated samples. Therefore, benoxacor enhances TBA detoxification in both corn and festuca; the induction of detoxifying activity in a nondomesticated grass is discussed in view of its use in vegetating buffer strips around crops to prevent TBA pollution.

KEYWORDS: Benoxacor; terbuthylazine detoxification; *Zea mays*; *Festuca arundinacea*; glutathione-*S*-transferase; buffer strips

INTRODUCTION

A group of structurally different synthetic compounds, named herbicide safeners, has been recognized as being able to protect crop plants against injury from certain herbicides. The majority of these compounds are typically active in cereal crops, including corn, sorghum, wheat, and rice (*1*−*7*). Many studies have attempted to understand the biochemical mechanism by which specific improvement of herbicide tolerance is conferred to plants by safeners. Literature reports evidence of the ability of many safeners to induce the activity of glutathione-*S*-transferases (GST; EC 2.5.1.18), which are a family of enzymes involved in the conjugation of glutathione (GSH) with a variety of endogenous and exogenous electrophilic compounds by a nucleophilic substitution reaction. In fact, plant GST results are composed of isoforms having varying degrees of specificity for different herbicides and herbicide classes; therefore, they are implicated in the detoxification of many herbicides, thus becoming responsible for herbicide persistence and selectivity in some plant species (*4, 8*−*13*).

The GSTs involved in herbicide detoxification in corn have been characterized as a group of dimer isoenzymes, distinguished on the basis of the composition of their subunits, which can detoxify a range of herbicides including alachlor, metolachlor, atrazine, and fluorodifen (*14*−*16*). GST subunits have been found to be induced by safeners, and they also have been identified and characterized, namely, in corn and wheat (*15, 17*−*19*).

The safener benoxacor [(±)-2,2-dichloro-1-(3,4-dihydro-3-methyl-2H-1,4-benzoxazin-4-yl)ethanone] is known to be very effective in inducing GST isoenzymes in corn, which are active against a chloroacetanilide such as metolachlor (*17, 20*−*22*). In contrast, triazine, a chloro-*s*-triazine that also undergoes GST-mediated detoxification, is not safened in corn even though GSTs active in metabolizing the herbicide are enhanced by treatment with the safener dichlormid (*15*). Although no other clear information exists on safeners able to protect corn from chloro-*s*-triazines, the possibility of benoxacor to induce the detoxification of these herbicides in corn should not be underestimated.

Terbuthylazine (TBA; 2-tert-buthylamino-4-chloro-6-ethylamino-1,3,5-triazine) is a chloro-*s*-triazine herbicide increasingly used in corn weeding instead of the more persistent atrazine, which can cause soil and water pollution with repeated treatments (such as occurred during the early 1990s in Italy). Therefore, one aim of the research was to ascertain the

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possibility of benoxacor to enhance TBA detoxification in corn so as to protect it from herbicide injury and also to reduce the risk of pollution from herbicide persistence.

Among the strategies designed to reduce the environmental impact of herbicides, the use of buffer strips is increasing popular. These are uncultivated zones left along the boundaries of crops to reduce the contamination of surface runoff by various pollutants, herbicides included (*23*, *24*). The efficiency of buffer strips is improved by the presence of no crop vegetation with widespread root development and capable of adapting to high-water or pluviometric systems (*25*). The perennial grass *Festuca arundinacea* is a very suitable species for vegetating buffer strips by virtue of its large root development, potentially able to adsorb xenobiotic compounds, and of its ability to grow under a large range of climatic conditions as well as in a large variety of soil types. Because GSTs capable of detoxifying herbicides have been recognized in a variety of no crop species (*13*, *26*, *27*), a further aim of the research was to ascertain the possible ability of festuca GSTs to act on TBA as a substrate and of benoxacor to induce TBA detoxification in festuca. This is in view of the utilization of festuca to vegetate buffer strips to enhance their potentiality in environmental protection against TBA pollution.

MATERIALS AND METHOD

Plant Material and Growth Conditions. Corn (*Zea mays*, hybrid *Belgrano*) and festuca (*Festuca arundinacea*, hybrid *Villageoise*) seeds were used. Seeds were germinated in plastic flats (0.08 m^2) containing sand quartz prewashed with a solution of hydrochloric acid (10%, v/v) and sterilized with a solution of NaClO (5%, w/v). Seedlings were grown in the dark at 23 °C (relative humidity at 80%). After 4 days, the seedlings were submitted to day−night conditions (12 h of light at 26 °C, light intensity of 300 μmol m^{-2} s^{-1}, and 12 h of darkness at 21 °C) and watered daily. When the seedlings were 7 and 12 days old for corn and festuca, respectively, the plants were divided into four groups: one group was left as the control; the second was treated with TBA at 12 mg/flat; the third was treated with TBA (12 mg/flat) together with benoxacor at 4 mg/flat; and the last group was treated with benoxacor only (4 mg/flat). Treatment rates were based on recommended field application rates. Shoots were collected at 12, 24, 48, 72, 96, 120, 144, 168, and 192 h after treatment, rinsed with water to remove nonadsorbed chemicals, dried by blotting with paper, and subjected to TBA and benoxacor residue analyses, GST activity determinations, and electrophoretic analyses.

GST Extraction and Purification. GST extraction was carried out according to the procedure of Cummins et al. (*18*). Shoots of corn and festuca (4.0 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) polyvinylpolypyrrolidone. After filtration through the two layers of muslin, the homogenate was centrifuged at 15 000 rpm for 20 min and the supernatant was adjusted to 80% saturation with respect to $(NH_4)_2SO_4$ 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 were 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 of the extraction steps were carried out at 4 °C.

GST Assays. The spectrophotometric procedure described by Edwards and Owen (*28*) was used to determine GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB). The GST activity was determined by adding 25 μL of 40 mM CDNB to a solution containing 900 μL of 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 6.5), 25 μL of enzymatic extract, and 50 μL of 0.1 M GSH (pH 7.0). The amount of conjugate formed by the reaction between GSH and CDNB 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 (nonenzymatic reaction) was then subtracted. The GSTs activity was expressed as nmol of GSH−CDNB formed sec^{-1} (mg of $protein^{-1}$) employed for the assay.

To determine GST constants V_{max} and K_M against CDNB, the GSH cosubstrate concentration was kept constant at 5 mM, while the concentration of CDNB was varied from 0.1 to 2 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.

1D and 2D SDS−PAGE Analyses. Enzymatic extracts were loaded on a glutathione-agarose affinity column equilibrated with a buffer composed of 100 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 1 mM dithiothreitol, and then the GSTs were eluted with the same buffer containing 10 mM GSH (*6*).

The 1D SDS−PAGE analyses were performed according to the Laemmli procedure (*29*). A solution of cold acetone (80%, v/v) was added to precipitate and to clean the protein extracts from soluble interfering substances. The resulting suspension was centrifuged, and then the pellets were resuspended, washed with cold acetone (80%, v/v), and centrifuged until the supernatant was colorless. Finally, the cleaned protein extracts were dried at room temperature, resuspended with the Laemmli buffer, and denatured at 95 °C for 5 min. The same amounts of proteins for each sample was submitted to 1D SDS−PAGE analyses using a resolving gel containing 12.5% acrylamide and a stacking gel containing 4% acrylamide. The proteins were resolved under a constant current of 15 mA in an Amersham Hoefer miniVE Vertical Electrophoresis System. All of the gels were stained with Coomassie Brilliant Blue R-250 and Silver Staining. The 1D images were processed using Gel Dool 2000 (Bio-Rad).

The same amounts of proteins for each sample were submitted to 2D SDS−PAGE analysis according to the procedure of O'Farrel (*30*). Samples of proteins extracted from corn shoots were cleaned as described above, and they were dissolved with a rehydratation buffer containing 8 M urea, CHAPS (0.5%, w/v), Pharmalyte (0.2%, v/v), and bromophenol blue (0.002%, w/v). IEF was performed in 7-cm long pH 4−7 Immobiline DryStrips at 200 V for 1 min, followed by a progressive increase from 200 to 3500 V for 90 min and a final constant step at 3500 V for 90 min. After IEF, the strips were briefly washed with water and saturated with an equilibration buffer of 50 mM Tris-HCl (pH 8.8), containing 6 M urea, glycerol (30%, v/v), SDS (2%, w/v), and bromophenol blue (0.002%, w/v). Dithiothreitol and iodoacetamide were added to the strips to preserve the fully reduced state of the proteins and to prevent their reoxidation. The strips were placed on Excel Gel Homogeneous SDS 12.5% acrylamide, and the analyses were performed using an Amersham Multiphor II at 120 V for 20 min followed by 70 min at 600 V. The gels were stained with Coomassie Blue R-250 and Silver Staining.

Assay of GST toward TBA and Benoxacor. The activity of GST toward TBA and benoxacor was determined according to the procedure of Hatton et al. (*26*). Herbicide (2.0 mM; 25 μL), dissolved in acetone, was added to 325 μL of 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 6.5), 50 μL of 10 mM glutathione (pH 7.0), and 50 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 HCl. The solution was centrifuged at 10 000 rpm for 2 min and then frozen at −20 °C. The samples (20 μL) were then analyzed by HPLC to quantify enzymatic activity from the difference between the initial amount of herbicide added and the herbicide residue found at the end of the reaction (*26*). The amount of herbicide lost in the nonenzymatic reaction, determined in a reaction mixture with enzymatic extract substituted by the reaction buffer, was subtracted from this result. The GST activity was expressed as nmol of herbicide consumed h^{-1} (mg of $protein^{-1}$) employed for the assay.

Determination of TBA and Benoxacor Residues. The determination of residues of TBA and benoxacor was performed following the GC−MS procedure of Vischetti et al. (*31*) with slight modifications. Shoots of corn and festuca (2 g) 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), preactivated 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 a ethyl acetate/*n*-hexane (2:3, v/v) solution. The recovered fraction was evaporated to dryness, rinsed with 1 mL of methanol, and

Table 1. Extractable GST Activity of Untreated, TBA-, Benoxacor-, and TBA and Benoxacor-Treated Corn and Festuca Shoots[a]

	GST(CDNB) activity [nmol sec^{-1} (mg of protein^{-1})]			
hours after treatment	8	24	48	72
	Corn			
untreated samples	2.33 ± 0.05a	2.66 ± 0.30a	2.49 ± 0.10a	2.11 ± 0.20a
TBA-treated samples	1.86 ± 0.10b	2.99 ± 0.30a	2.10 ± 0.25b	1.80 ± 0.07b
benoxacor-treated samples	2.04 ± 0.04a,b	4.39 ± 0.20b	2.90 ± 0.10c	2.73 ± 0.2c
TBA and benoxacor-treated samples	2.80 ± 0.40c	4.81 ± 0.20c	2.73 ± 0.10c	2.52 ± 0.20c
	Festuca			
untreated samples	2.50 ± 0.50a	2.49 ± 0.04a	3.12 ± 0.50a	3.05 ± 0.40a
TBA-treated samples	1.47 ± 0.20b	2.71 ± 0.30a,b	3.01 ± 0.30a	3.27 ± 0.15a
benoxacor-treated samples	2.07 ± 0.30a	2.94 ± 0.20b	4.32 ± 0.20b	3.90 ± 0.0.50b
TBA and benoxacor-treated samples	2.45 ± 0.22a	2.56 ± 0.20a	2.70 ± 0.10a	3.35 ± 0.20a,b

[a] The data represent the means of triplicate determinations. Means within the same column followed by the same letter are not significantly different at a 5% level using the *t* test.

Table 2. K_M and V_{max} Values of GST(CDNB) Extracted from Safened and Unsafened Corn and Festuca Shoots[a]

	corn		festuca	
	V_{max} (nmol min^{-1})	K_M (mM)	V_{max} (nmol min^{-1})	K_M (mM)
unsafened	44.0 ± 5.0a	1.79 ± 0.035a	25.0 ± 1.0a	0.541 ± 0.001a
safened	38.0 ± 1.0a	1.34 ± 0.040b	23.0 ± 1.0a	0.268 ± 0.022b

[a] The data represent the means of triplicate determinations. Means within the same column followed by the same letter are not significantly different at a 5% level using the *t* test.

Table 3. Percentage of Proteins, with the Molecular Masses of Corn Subunits of GSTs, Extracted from Benoxacor- and TBA-Treated Corn Samples, Compared to Untreated Corn Samples Left as Controls (100% of Protein Amounts)[a]

	time after the treatment (h)					
corn samples	24		48		72	
kDa	benoxacor	TBA	benoxacor	TBA	benoxacor	TBA
28.60 ± 0.78	NS	NS	NS	NS	NS	NS
26.98 ± 0.98	210%	NS	122%	NS	NS	NS
26.07 ± 0.77	136%	NS	135%	118%	150%	135%

[a] The relative percentages were determined using Gel Dool 2000. Each determination was run in triplicate, and the samples giving a *t* test with $p < 0.15$ were considered significant. The molecular masses are reported ±SD.

subjected to GC analysis. A Perkin−Elmer Auto System XL gas chromatograph equipped with an OV17 capillary column (0.53 mm × 30 m length, inside diameter of 0.5 μm) was employed. 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}.

RESULTS

Effect of TBA and Benoxacor Treatments on the Activity of GST(CDNB) in Corn and Festuca Shoots. As an initial experiment, the activity of GST toward CDNB in corn and festuca shoots was assayed over a 72 h period after treatments with TBA alone, benoxacor alone, or a combined treatment (**Table 1**).

In comparison to the untreated controls, TBA treatment generally reduced the enzyme activity in corn with the exception at 24 h, and the decreases ranged from 14.7 to 20.2% during the experimental period. Benoxacor treatment generally increased the enzyme activity for extents ranging from 16.4 to 65.0%. In comparison to the benoxacor-alone-treated shoots of corn, the TBA and benoxacor mixture caused 37.2 and 9.6% increases of the enzyme activity 8 and 24 h after treatment, respectively; thereafter, the mixture did not modify the enzyme activity exhibited by the benoxacor-alone-treated shoots.

In festuca, TBA did not affect the GST(CDNB) activity, compared to the untreated controls, with the exception of a 41.2% decrease 8 h after the treatment. In contrast, benoxacor stimulated the enzyme activity, starting from 24 h after treatment, and the increases ranged from 18.1 to 38.5% during the subsequent experimental period. Such an effect was not exerted by benoxacor when applied with TBA; therefore, the TBA and benoxacor mixture generally did not determine significant changes in the enzyme activity in comparison to the TBA-treated shoots of festuca during the entire experimental period.

Kinetic Parameters and Electrophoretic Analyses of Corn and Festuca GST. To clarify the nature of the induction of GST(CDNB) activity in response to benoxacor treatment in corn and festuca shoots, the kinetic constants, V_{max} and K_M, were determined using the enzyme extracts from the shoots. The shoots were harvested at 24 h (corn) and 48 h (festuca) after treatment, corresponding to the times of maximum enhancement of GST(CDNB) activity by benoxacor in comparison with the respective untreated controls. The protein extracts were also subjected to SDS−PAGE analyses.

In both corn and festuca, the V_{max} values were unaffected by the benoxacor treatment, whereas the K_M values were decreased by 25.1% in corn and 50.4% in festuca (**Table 2**).

The 1D SDS−PAGE analyses (**Table 3**) showed some significant increases of 27- and 26-kDa proteins in corn in response to benoxacor and TBA treatments. In particular, the increases of 27-kDa proteins ranged from 110 to 22% during

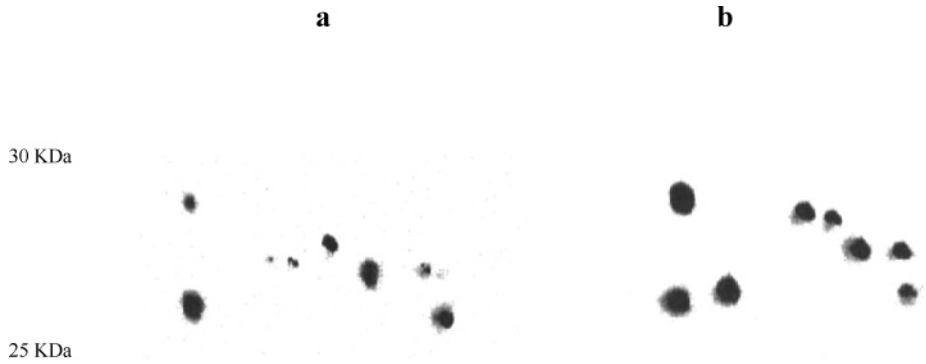

Figure 1. Two dimensional SDS–PAGE analysis of proteins extracted from unsafened (a) and safened (b) maize samples. The proteins were stained for analyses with Coomassie Blue R-250 and Silver Staining.

Table 4. Extractable GST Activities toward TBA and Benoxacor from Shoots of Corn and Festuca Untreated and Benoxacor and TBA Treated[a]

	GST(TBA) [nmol h^{-1} (mg of $protein^{-1}$)]		GST(benoxacor) [nmol h^{-1} (mg of $protein^{-1}$)]	
	corn	festuca	corn	festuca
untreated	2.28 ± 0.15a	0.91 ± 0.17a	6.30 ± 0.20a	3.82 ± 0.20a
benoxacor treated	4.21 ± 0.30b	1.36 ± 0.10b	11.20 ± 0.50b	3.70 ± 0.24a
TBA treated	2.72 ± 0.15c	1.09 ± 0.10a	4.90 ± 0.20c	2.60 ± 0.10b

[a] The data represent the means of triplicate determinations. Means within the same column followed by the same letter are not significantly different at a 5% level using the *t* test of Student.

the 24−48 h period after benoxacor treatment, while no effects were observed in response to TBA treatments; the increases of 26-kDa proteins in corn ranged from 35 to 50% during the 24−72 h period after benoxacor treatment and from 18 to 35% during the 48−72 h period after TBA treatment.

An increase of 26-kDa proteins in festuca was also observed, ranging from 24 to 300% and from 20 to 188% in response to benoxacor and TBA treatment, respectively, which occurred during the 24−72 h period following treatment (data not reported).

2D SDS−PAGE analyses were not performed on the enzyme extracts of festuca because the GSTs of festuca have not been characterized. Therefore, the analyses were focused on the typical range of molecular masses (25−30 kDa) and on isoelectric point (pI) values characteristic of GST proteins of corn. The results showed an increased number and intensity of the spots in benoxacor-treated samples compared to untreated controls (**Figure 1**).

GST Activity toward TBA and Benoxacor in Response to the Herbicide and Safener Treatments. The activity of GST toward TBA [GST(TBA)] and toward benoxacor [GST(benoxacor)] was assayed in the corn and festuca extracts from shoots collected respectively 24 and 48 h after the treatments, when maximum GST(CDNB) activity was found (**Table 4**).

The results show that benoxacor treatment increased GST(TBA) activity in corn and festuca by 84.6 and 49.4%, respectively, while TBA treatment increased GST(TBA) activity by 14.0% in corn and was found to be ineffective in festuca. Benoxacor treatment increased GST(benoxacor) activity in corn by 77.8%, while it did not affect GST(benoxacor) in festuca; in contrast, TBA treatment decreased GST(benoxacor) in both corn and festuca respectively by 22.2 and 32.0%.

Accumulation and Persistence of TBA in the Unsafened and Benoxacor-Treated Shoots of Corn and Festuca. The procedure for identifying and determining TBA and benoxacor residues was validated in corn and festuca shoots: recovery tests ≥ 90% and detection limits ≤ 0.05 ppm made the analytical procedure satisfactory for the detection of TBA and benoxacor residues.

Data concerning the accumulation and persistence of TBA in the unsafened and benoxacor-treated corn and festuca shoots show that the herbicide residues in both the plants were generally lower and less persistent in the safened than in the unsafened shoots (**Table 5**). In particular, TBA residues in corn reached maximum levels of 2.90 and 1.20 ppm 12 h after treatment in the unsafened and safened shoots, respectively, and became negligible (<0.05 ppm) in the respective samples 192 and 144 h after treatment. The TBA residues in festuca reached the maximum levels of 1.00 and 0.41 ppm 48 h after the treatment in the unsafened and safened shoots, respectively, and became negligible (<0.05 ppm) in the respective samples 168 and 120 h after treatment.

The persistence of the safener in the shoots was found to be of little relevance; in fact, the benoxacor residues reached levels of 0.15 ppm in corn and 0.12 ppm in festuca, and they became negligible (< 0.05 ppm) 12 and 24 h after treatment, respectively.

DISCUSSION

The enzyme assays performed with the "standard substrate" CDNB evidenced the ability of TBA and benoxacor to interfere in GST activity in corn and festuca. In fact, the data in **Table 1** show that, for corn, TBA treatment generally inhibited GST(CDNB) activity, whereas benoxacor treatment stimulated it with a strong effect at 24 h after the treatment; the benoxacor addition to TBA treatment largely counterbalanced the inhibiting effect of TBA, so that the mixture had a stimulating effect on the enzyme activity. With festuca, TBA treatment did not affect the GST(CDNB) activity with the exception of the first 8 h after the treatment, while benoxacor treatment stimulated it starting from 24 h after the treatment; however, contrary to corn, it did not exhibit this ability in combination with TBA. Therefore, the TBA and benoxacor mixture was found to be generally ineffective on enzyme activity, similarly to the TBA-alone treatment.

Further information on the role of benoxacor in GST induction in corn and festuca was obtained from the V_{max} and

Table 5. Residual Amount of TBA in Corn and Festuca Shoots Treated with TBA and with TBA and Benoxacor Mixture[a]

	TBA residual amount (ppm)			
	corn		festuca	
hour after treatment	TBA	TBA and benoxacor	TBA	TBA and benoxacor
12	2.90 ± 0.07a	1.20 ± 0.050b	*b*	*b*
24	1.90 ± 0.21a	0.80 ± 0.2b	1.00 ± 0.24a	0.41 ± 0.01b
48	1.10 ± 0.10a	0.45 ± 0.06b	0.50 ± 0.01a	0.39 ± 0.16a
72	0.80 ± 0.02a	0.60 ± 0.01b	0.35 ± 0.05a	0.19 ± 0.03b
96	0.60 ± 0.02a	0.32 ± 0.01b	0.36 ± 0.02a	0.06 ± 0.01b
120	0.26 ± 0.02a	0.26 ± 0.04b	0.06 ± 0.01a	*b*
144	0.25 ± 0.02a	*b*	0.05 ± 0.02a	*b*
168	0.07 ± 0.02a	*b*	*b*	*b*
192	*b*	*b*	*b*	*b*

[a] The data represent the means of triplicate determinations ± SD. Means within a line followed by the same letter are not significantly different at a 1% level using the *t* test. The statistical analyses must be considered only for the same plant. [b] Not detectable (<0.05 ppm).

K_M parameters: in response to benoxacor treatment, V_{max} values were unaffected, while K_M values were significantly reduced. Because V_{max} is proportional to the enzyme concentration and K_M is inversely related to the substrate−enzyme affinity, it can be hypothesized that benoxacor treatment did not modify the GST content in the protein "pull" of corn and festuca shoots, while it induced GST forms endowed with a greater affinity to the substrate.

The complexity and diversity of the corn GST enzyme family was clearly demonstrated: at least five distinct genes encoding GSTs have been supposed to be contained in corn (*32*). Several GSTs have been characterized, such as GST I, a homodimer of 29-kDa subunits; GST II, a heterodimer of 27- and 29-kDa subunits; GST III, a homodimer of 26-kDa subunits; and GST IV, a homodimer of 27-kDa subunits (*33*). Furthermore, it was ascertained that a least three GST isoenzymes are induced in corn by the safener benoxacor (*17*). On the basis of SDS−PAGE analyses, a similar behavior seems to have occurred in our experiment. In fact, an induced expression of proteins of 27- and 26-kDa magnitude was exhibited in response to benoxacor treatment to corn, and furthermore, the induction of the above proteins was shown to be concomitant with 84.6 and 77.8% increases of GST(TBA) and GST(benoxacor) activity, respectively (**Table 4**). The unaffected V_{max} values for CDNB substrate following benoxacor treatment (**Table 2**), despite the increased number and intensity of the protein spots following the safener treatment (**Figure 1**), might be attributable to GST subunits sensitive to TBA and benoxacor.

In regards to festuca, the benoxacor-induced expression of 26-kDa proteins was found to be concomitant with a 49.4% increase of GST(TBA) activity. Even though the GSTs are not known for festuca as they are for corn, this result suggests that an induction of GSTs in response to benoxacor treatment in festuca could have occurred as it did for corn.

Following TBA treatment of corn, a 19.2% increase in GST-(TBA) activity was found (**Table 4**), whereas an inhibiting effect on GST(CDNB) activity was generally exhibited in response to the same treatment (**Table 1**). Therefore, it seems that corn may activate against TBA, a defense mechanism consisting of the induction of GST isoforms able to detoxify the herbicide. A similar effect was previously found to occur in corn, soybean, and broad bean in response to treatment with some chloroacetanilide herbicides (*34*−*36*). Such a response of plants to herbicide exposure confirms that plant GST can be induced in response to diverse stimuli including xenobiotics (*37*). Such an ability was not found to be present in festuca.

The consequence of the benoxacor activation of corn and festuca GSTs is evidenced by the data on the accumulation and persistence of TBA and benoxacor residues in the unsafened and benoxacor-safened shoots of corn and festuca (**Table 5**). In fact, in comparison with the corresponding samples treated with TBA alone, the treatment with the TBA and benoxacor mixture caused an average reduction of 45.3 and of 51.9% in the TBA residues in corn and festuca, respectively, as well as a 48 h reduction of their persistence in the shoots. Moreover, although benoxacor residues in the corn and festuca shoots were rather low and not very persistent, the benoxacor activation of corn GST(benoxacor) made the safener less persistent in corn than in festuca.

In conclusion, while it is known that benoxacor is a safener that protects corn from chloroacetanilide herbicides by enhancing their conjugation with GST, there is no information in the literature about the ability of safeners to effectively safen chloro-*s*-trazines. Nevertheless, the results of this research demonstrate that the corn safener benoxacor enhances GST(TBA) activity in corn and festuca and that this effect was found to be associated with a more rapid removal of TBA in both the safened plants. Therefore, benoxacor seems to be able to enhance TBA detoxification in corn apparently by the same mechanism found on chloroacetanilide herbicides. This hypothesis appears to be supported also by the increased expression of proteins having molecular masses in the range of corn GSTs in response to benoxacor treatment, and a similar behavior seems to occur in festuca.

Although *Festuca arundinacea* is not a cultivated species, its increased tolerance to TBA in response to benoxacor exposure likewise may assume relevance in relation to the practices of the soil−water system protection. In fact, because of its morphological and physiological characteristics, festuca is a very suitable plant to vegetate buffer strips. Therefore, the benoxacor-induced ability of festuca to metabolize the herbicide can result in improved buffer strip efficiency in protecting the soil-surface water environment against TBA pollution.

ABBREVIATIONS USED

CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione; GST, glutathione-*S*-transferases; IEF, isoelectric focusing; pI, isoelectric point; 1D SDS−PAGE, monodimensional sodium dodecyl sulfate−polyacrylamide gel electrophoresis; 2D SDS−PAGE, two-dimensional sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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