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Late Watergrass (*Echinochloa phyllopogon*): Mechanisms Involved in the Resistance to Fenoxaprop-*p*-ethyl

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Fenoxaprop-*p*-ethyl (FE), 2-{4-[(6-chloro-2-benzoxazolyl)oxy]phenoxy} propanoate, ethyl ester (*R*), is an aryloxyphenoxypropionate herbicide for postemergence control of annual and perennial grasses in paddy fields; its site of action is acetyl-coenzyme A carboxylase (ACCase), an enzyme in fatty acids biosynthesis. The possible mechanism(s) of resistance to FE in a resistant biotype of *Echinochloa phyllopogon* was examined, namely, absorption, translocation, and metabolism of FE and ACCase susceptibility to fenoxaprop acid (FA). Studies of the in vitro inhibition of ACCase discounted any differential active site sensitivity as the basis of resistance to FE. There were differences in absorption rates between biotypes from 3 to 48 h after application (HAA). Biotypes did not differ in either the amounts or the rates of FE translocated; 98% of applied [^{14}C]FE remaining in the treated leaf. However, there was a good correlation between the rate of herbicide metabolism and the plant resistance. The R biotype produced 5-fold less FA and approximately 2-fold more nontoxic (polar) metabolites 48 HAA than the S biotype. Moreover, the higher rate of GSH conjugation in the resistant biotype as compared to the susceptible one indicates that GSH and cysteine conjugation is the major mechanism of resistance of the R biotype against FE toxicity.

KEYWORDS: Absorption; ACCase; fenoxaprop-*p*-ethyl (FE); GST; metabolism; *Echinochloa phyllopogon*

INTRODUCTION

Late watergrass [*Echinochloa phyllopogon* (Stapf) Kossenko] is a noxious weed of rice in the Sacramento valley of California. In 1997, late watergrass biotypes putatively multiple-resistant to bispyribac-sodium, fenoxaprop-*p*-ethyl (FE), molinate, and thiobencarb were found in rice fields scattered throughout the Sacramento Valley (*1*, *2*). It was suggested that these originated from a single biotype, because of their similarities in morphological and amplified fragment-length polymorphism traits, establishing that resistance moved by spikelet dispersal, not by independent mutation events (*3*). FE, 2-{4-[(6-chloro-2-benzoxazolyl)oxy]phenoxy} propanoate ethyl ester, is an aryloxyphenoxy-propionate (AOPP) herbicide for the postemergence control of annual and perennial grasses such as graminaceous weed species of the genera *Avena*, *Digitaria*, *Panicum*, *Setaria*, *Sorghum*, and *Echinochloa* in soybeans, rice, wheat, and turf (*4*). FE induces cessation of growth and inhibition of lipid biosynthesis in susceptible grass species. The primary mechanisms for herbicidal selectivity in grasses involve metabolic detoxification in tolerant species (*5*).

Like other AOPPs and cyclohexanedione (CHD) herbicides, the site of action of FE is acetyl-CoA carboxylase (ACCase, EC 6.4.1.2), an enzyme catalyzing the first committed step in de novo fatty acid biosynthesis (*6*). ACCase is located in both cytosol and chloroplasts (*7*) and catalyzes the ATP-dependent conversion of acetyl-CoA to malonyl-CoA (*8*−*10*). Up to date, five amino acid residues within the CT domain of grass chloroplastic ACCase are known to be responsible for target site resistance if they are replaced (Ile_{1781}, Trp_{2027}, Ile_{2041}, Asp_{2078}, and Gly_{2096}) (*11*). Mechanisms other than a target site modification can be summarized as nontarget site resistance and can be endowed with an enhanced metabolism, a decrease in certain enzyme functionalities (i.e., esterases deactivation, *12*), compartmentation, or the active exclusion of the herbicide from the site of action and reduced herbicide absorption or translocation (or both) (*13*).

Plants can metabolize herbicides and thus reduce the amounts of those xenobiotics that can reach their action sites. The first phase involves oxidative reactions, such as hydroxylation, and the second phase involves conjugation with glutathione or other compounds (*14*). Cytochrome P450 monooxygenases (CYPs, EC 1.14.14.1) are membrane-bound and heme-iron-containing

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enzymes and are well-known as key enzymes in the initial detoxification of herbicides (*15*). CYPs could confer tolerance in cereals (*16*, *17*) and legumes (*18*) or resistance in weed species such as *Alopecurus myosuroides* (*19*), *Lolium rigidum* (*20*), or *E. phyllopogon* (*2*, *21*). Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are homo- or heterodimer, multifunctional enzymes located in the cytosol found in all eukaryotes, which catalyze the conjugation of synthetic electrophilic compounds with the tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine or γ-Glu-Cys-Gly, GSH) (*22*), and are found in relatively high concentrations in most plant tissues (*23*). GSTs in plants were first studied because of their ability to detoxify herbicides and impart herbicide selectivity in several plant species (*24*). Many herbicide families, including sulfonylureas, aryloxyphenoxypropionates, triazinone sulfoxides, and thiocarbamates, are susceptible to GSH conjugation (*24*). Furthermore, there is a positive correlation of both GSH levels and the activity of specific GST enzymes with herbicide conjugation and detoxification rates (*25*). Resistance of weeds to herbicides has been widely reported in the involvement of GST in herbicide conjugation (*26*). Up to date, no target-site resistance to ACCase inhibitors in *E. phyllopogon* has been reported; this is related to a differential absorption and metabolism, e.g., cyhalofop-butyl (*12*), bispyribac-sodium (*2*), and bensulfuron-methyl (*2*), suggesting the involvement of cytochrome P450 in herbicide detoxification. The mechanisms of resistance to FE in late watergrass have not yet been described, although many studies have suggested the role of GSTs in FE conjugation (*14*, *27*).

The specific objectives of the present study were to (i) confirm resistance to FE in *E. phyllopogon* and (ii) determine the mechanism(s) of resistance by evaluating differential absorption, translocation, metabolism of [^{14}C]FE, and target site by examining multifunctional ACCase subunit sensitivity to fenoxaprop acid (FA).

MATERIALS AND METHODS

Chemicals. The following herbicides and reagents were used in this study: [^{14}C]fenoxaprop-ethyl ([U-^{14}C]chlorophenyl, specific activity, 1838 kBq μmol^{-1}) and technical grade FA, 2-{4-[(6-chloro-1,3-benzoxazol)-2-yloxy]phenoxy}propionic acid (*R*). A commercial herbicide formulation (FE, 6.59% w/v EC) was used for dose−response assays, and all other reagents were obtained at analytical grades.

Plant Material. Seeds from the resistant (R) and susceptible (S) *E. phyllopogon* biotypes were obtained after two successive generations (*3*) and were originally collected in paddy fields of the Sacramento Valley in California (*1*). The R biotype had shown resistance to FE, bispyribac-sodium, molinate, and thiobencarb (*1*, *2*).

Growth Conditions. Seeds of R and S biotypes of *E. phyllopogon* were germinated on moistened filter paper in Petri dishes. Seedlings were planted in pots (five plants per pot) containing peat and sandy loam potting mixture (1/2, v/v) in a growth chamber at 28/18 °C (day/night) in a 16 h photoperiod under 350 μmol m^{-2} s^{-1} photosynthetic photon-flux density and 80% relative humidity.

Dose−Response Assays. Treatments were applied to plants at the 3−4 leaf stage, using a laboratory track sprayer equipped with a Tee Jet 80.02E VS flat-fan nozzle delivering a spray volume of 300 L ha^{-1} at 200 kPa. FE was applied at the rates of 0, 55, 110, 165, and 220 g of ai ha^{-1} for the R biotype and 0, 5.5, 8.25, 11, and 16.5 g of ai ha^{-1} for the S biotype. Above-ground fresh weight per pot was determined 21 days after spraying, and data were expressed as percentage of the untreated control. Herbicide rates to inhibit plant growth by a 50% decrease in growth with respect to the untreated control (ED_{50}) were determined, and the R/S ratio was computed as $ED_{50}(R)/ED_{50}(S)$. Treatments were replicated three times and were arranged in a completely randomized design with four replications per dose. Each replication consisted of three plants per pot. Data were pooled and fitted to a nonlinear, log−logistic regression model

$$Y = c + \{(d - c)/[1 + (x/g)^b]\}$$

where Y is the fresh above-ground weight expressed as a percentage of the untreated control, c and d are the coefficients corresponding to the lower and upper asymptotes, b is the slope of the line, g is the herbicide rate at the point of inflection halfway between the upper and the lower asymptotes, and x (independent variable) is the herbicide dose. Regression analysis was conducted using the Sigma Plot 8.0 statistical software (*12*).

Table 1. R_f Values for FE and Metabolites for the Different Solvent Systems Used in the Chromatographic Assays (*27*, *28*)

compound[a]	(A) toluene/ethyl acetate/acetic acid/water (50:50:1:0.5) (v/v/v/v)	(B) chloroform/methanol/water (65:25:1) (v/v/v)	(C) butanone/acetic acid/water (10:1:1) (v/v/v)
FE	0.94	0.98	0.97
FA	0.14	0.53	0.97
CDHB	0.55	0.98	0.93
HPP	0.14	0.24	ND
HQ	0.64	0.86	ND
CDHB-GSH	0.00	ND	0.08
CDHB-GlyCys	0.00	ND	0.39
CDHB-Cys	0.00	ND	0.62

[a] HQ, Cys, *S*-(6-chlorobenzoxazole-2-yl) cysteine; Cys, cysteine; Gly, glycine. [b] ND, not determined.

Absorption and Translocation Studies. [^{14}C]FE was mixed with commercially formulated FE to prepare an emulsion with a specific activity of 0.83 kBq μL^{-1} (both absorption and translocation studies) and a FE concentration of 0.275 g ai L^{-1} (corresponding to 82.5 g ha^{-1} at 300 L ha^{-1}). The labeled herbicide was applied to the mid of the adaxial surface of the second leaf of each plant in four 0.5 μL droplets using a microapplicator (Dispenser, Hamilton PB 6000 TA, Hamilton Co., Reno, NV). A total of 1.66 kBq were applied on each plant. Plants of both biotypes at the 3−4 leaf stage of growth were harvested in batches of three plants at several time intervals after herbicide application (3, 6, 12, 24, 48, 72, and 120 h) and separated into treated leaves, upper leaves, root, and the remainder of the shoots. Unabsorbed [^{14}C]FE was removed from the leaf surface by washing the treated area with 3 mL of methanol 80% (v/v). Washes from each batch were pooled and analyzed by liquid scintillation spectrometry (LSS) (Scintillation counter, Beckman LS 6500 TA, Beckman Instruments Inc., Fullerton, CA). The plant tissue was dried at 55 °C for 72 h and combusted in a sample oxidizer (Oxidizer, Tri Carb model 307, Packard Instrument Co., Downers Grove, IL). The $^{14}CO_2$ evolved was trapped and counted in a 10 mL mixture of Carbo-Sorb E and Permafluor E$^+$ (3/7, v/v) (Perkin-Elmer, Packard Bioscience BV, Groningen, The Netherlands). The radioactivity was quantified by LSS and expressed as a percentage of the recovered radioactivity, according to the following formula: % absorption = [KBq in combusted tissue/(KBq in combusted tissue + KBq in leaf washes)] × 100. The experiment was repeated three times.

In the translocation studies, the treated plants were removed from pots at the same times. Roots were rinsed, and whole plants were sampled 3, 6, 12, 24, 48, 72, and 120 h and oven-dried (50 °C, 4 days), pressed against a 25 cm × 12.5 cm phosphor storage film during 6 h, and scanned (Cyclone, Perkin-Elmer, Packard Bioscience BV) for radiolabel dispersion. Means and standard errors (of the mean) were computed for all parts of plants, and means were tested for group differences and compared using an analysis of variance (ANOVA) and a Tukey HSD posthoc test.

Metabolism Studies. The metabolism of [^{14}C]FE was examined in the second leaf tissue in R and S biotypes of *E. phyllopogon* plants at the 3−4 leaf stage of growth. The labeled herbicide was applied to the mid of the adaxial surface of the second leaf in four 0.5 μL droplets using a microapplicator. A total of 1.66 kBq were applied on each plant. Plants of both biotypes were sampled 3, 6, 12, 24, 48, 72, and 120 h after treatment. Treated leaves were washed as above. An aliquot

Table 2. Parameters of the Log–Logistic Equation[a] Used To Calculate the Herbicide Dose Required for 50% Reduction of the Fresh Weight (ED_{50}) and/or the Herbicide Concentration Required for Reduction of the ACCase Activity (I_{50}) of R and S Biotypes of *E. phyllopogon*[b]

E. phyllopogon	herbicide	D	C	B	value	pseudo r^{2c}	*P*	R/S
R	FE	100	27	3.412	ED_{50} [110[d] (±18)]	0.97	<0.0001	10
S	FE	100	17	1.997	ED_{50} [11[d] (±3.2)]	0.96	<0.0001	
R	FA	100	19	3.211	I_{50} [0.58 (±0.15)]	0.94	<0.0001	1.14
S	FA	100	17	1.854	I_{50} [0.51 (±0.03)]	0.94	<0.0001	

[a] $Y = c + \{(d - c)/[1 + (x/g)^b]\}$, where Y is the fresh aboveground weight expressed as percentage of the untreated control, x (independent variable) is the herbicide dose, c and d are the coefficients corresponding to the lower and upper asymptotes, b is the slope of the line, and g is the herbicide rate at the point of inflection halfway between the upper and the lower asymptotes. For $C = 0$ and $D = 100$, g becomes the I_{50}. [b] Data were pooled and fitted to a nonlinear regression model. Data are means of three experiments. ED_{50} ± confidence interval. [c] Approximate coefficient of determination for nonlinear models with a defined intercept calculated as pseudo $r^2 = 1 -$ (sums of squares of the regression/corrected total sums of squares). [d] There were significant differences between R and S biotypes according to a Tukey HSD posthoc test.

of the leaf wash solution was assayed for radioactivity, and the remaining solution was stored at −20 °C until analysis. The treated plants were separated into roots and shoots. The shoots from each plant were ground in a mortar using 4 mL of 100% acetone, and the homogenate was centrifuged at 20000*g* for 10 min. The pellet was washed with 100% acetone until [^{14}C] was no longer extracted and then oven-dried at 60 °C for 48 h and combusted in a sample oxidizer. The supernatants were combined, evaporated to dryness at 40 °C under a stream of N_2 at 10 kPa, and redissolved in 500 μL of 100% acetone. The FE and its metabolites in the supernatant were identified by two-dimensional thin-layer chromatography (TLC) on 20 cm × 20 cm, 250 μm silica gel plates (Silica gel 60, Merck, 64271 Darmstadt, Germany) using a toluene/ethyl acetate/acetic acid/water (50:50:1:0.5, v/v/v/v) mobile phase (**Table 1A**) (*27*) for separating FE, FA, and metabolites and a chloroform/methanol/water (65:25:1, v/v/v) mobile phase (**Table 1B**) (*28*) for separating FA and 2-(4-hydroxyphenoxy)propionate (HPP). Because the only signal found corresponded to FA in all cases, an additional study was conducted to differentiate the conjugates formed. Thus, metabolites were scraped off and separated in a butanone/acetic acid/water (10:1:1, v/v/v) mobile phase (**Table 1C**) (*28*). The radioactive zones were detected with a radiochromatogram scanner (Berthold LB 2821, 7547 Wildbald, Germany), and their chemical nature was identified by comparing their R_f values with those shown in **Table 1**. For quantitative determinations, the radioactive spots were scraped off, extracted with 100% acetone, and counted by LSS. The experimental design was completely randomized and was repeated three times.

Enzyme Purification. Leaves (6 g fresh weight) of R and S biotypes of *E. phyllopogon* were harvested from plants in 3−4 leaf stages and ground in liquid N_2 in a mortar and then added to 24 mL of extraction buffer [0.1 M Hepes-KOH (pH 7.5), 0.5 M glycerol, 2 mM EDTA, and 0.32 mM PMSF]. The homogenate was mixed for 3 min with a magnetic stirrer and filtered sequentially through four layers of cheesecloth and two of Miracloth (Miracloth, Calbiochem, San Diego, CA). The crude extract was centrifuged (24000*g*, 30 min, 4 °C). The supernatant was fractionated with ammonium sulfate and centrifuged (12000*g*, 10 min, 4 °C). Material precipitating between 35 and 45% $(NH_4)_2SO_4$ saturation was resuspended in 1 mL of S400 buffer [0.1 M Tricine-KOH (pH 8.3), 0.5 M glycerol, 0.05 M KCl, 2 mM EDTA, and 0.5 mM DTT]. The clarified supernatant was applied to a desalting column (PD-10 columns, Sephadex G-25 M, Amersham Biosciences AB, SE-751 84, Uppsala, Sweden), previously equilibrated with 25 mL of S400 buffer. ACCase enzyme was eluted from the column in 2 mL of S400 buffer.

ACCase Assays. The enzyme activity was assayed by measuring the ATP-dependent incorporation of NaH[^{14}C]O_3 into an acid-stable [^{14}C]product. The reaction product had been previously shown to be [^{14}C]malonyl-CoA (*29*). Assays were conducted in 7 mL scintillation vials containing 0.1 M Tricine-KOH (pH 8.3), 0.5 M glycerol, 0.05 M KCl, 2 mM EDTA, 0.5 mM DTT, 1.5 mM ATP, 5 mM $MgCl_2$, 15 mM NaH[^{14}C]O_3 (1.22 MBq μmol^{-1}), 50 μL of enzyme fraction, and 5 mM acetyl-CoA in a final volume of 0.2 mL. The activity was assayed for 5 min at 34 °C, and the reaction was stopped after 5 min by adding 30 μL of 4 N HCl. A piece of filter paper was added to the reaction vial, and samples were dried at 40 °C under a stream of air. After drying, ethanol/water (1:1, v/v, 0.5 mL) was added to the vial, followed by the addition of 5 mL of scintillation cocktail (Ready safe, Beckman Instruments Inc.). Radioactivity was determined by LSS. Background radioactivity, measured as acid-stable counts (dpm) in the absence of acetyl-CoA, was subtracted from each treatment. One unit of ACCase activity was defined as 1 μmol malonyl CoA formed min^{-1}. FA concentrations resulting in a 50% inhibition of enzyme activity (I_{50}) were determined in crude extracts. Data were pooled and fitted to the log−logistic model described before. Experiments were repeated three times. A blank was included and consisted of all of the ingredients of the reaction with the exception of acetyl coenzyme A, and this value was subtracted from the rest of the measurements obtained in the assay.

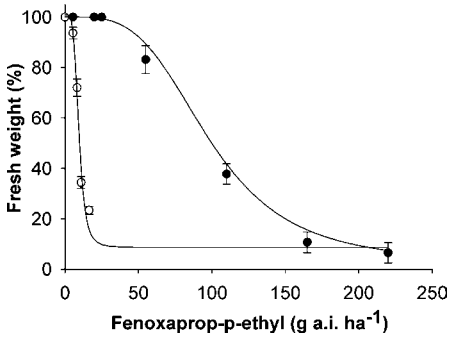

Figure 1. Dose–response assays of S (○) and R (●) biotypes of *E. phyllopogon* to FE. The plant fresh weight was determined 21 DAT, and data are expressed as percentage (%) of the mean control; each point is the mean ± standard errors (SE) of three experiments.

Protein Determination. Protein was estimated by the Bradford assay (*30*) using γ-globulin as a standard.

RESULTS AND DISCUSSION

Dose−Response Assays. Resistance was confirmed in the R biotype of *E. phyllopogon* (ED_{50}, 110 g of ai ha^{-1}), this being 10-fold less sensitive than the S biotype (ED_{50}, 11 g of ai ha^{-1}) (**Table 2**). FE reduced the fresh weight of S biotype at low doses, whereas in the R biotype the same dose had little effect on the fresh weight. The R biotype was highly resistant to FE (R/S = 10) and displayed a high level of resistance to bispyribac-sodium, molinate, and thiobencarb (*1*, *2*). The symptomology in the R biotype suggests degradation enhancement as a mechanism for resistance, since plants treated at low herbicide rates initially exhibit some growth retardation followed by a recovery of growth 6 days after application. The growth of susceptible plants was drastically suppressed at very low herbicide rates (**Figure 1**).

ACCase Assays. ACCase-specific activities (±SE) for R [27.47 ± 2.73 pKatal formed product (FP) mg^{-1} prot] and S (28.81 ± 3.95 pKatal FP mg^{-1} prot) biotypes of *E. phyllopogon* show that ACCase overproduction does not contribute to

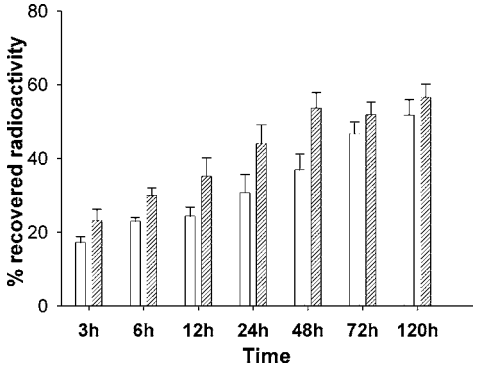

Figure 2. Absorption of [^{14}C]FE in R (open bar) and S (hatched bar) biotypes of *E. phyllopogon*. Values are means of three experiments, and vertical bars represent standard errors (SE).

resistance in these species. Overproduction of the normal susceptible ACCase has only been reported as a mechanism of resistance in corn (*Zea mays* L.) cell lines and in a biotype of johnsongrass (*Sorghum halepense*) resistant to AOPP and CHD herbicides (*31*, *32*). In vitro assays showed that the target site (ACCase) was similarly susceptible to FA in both R and S biotypes (**Table 2**). Therefore, resistance cannot be attributed to an altered target enzyme. Currently, there are no records of mutations on the carboxyl transferase (CT) domain on the chloroplastic ACCase of *Echinochloa* species although a target site resistance has been widely reported in other grass species that confer resistance to AOPP and CHD (*11*).

Absorption and Translocation. There were significant differences in the absorption of [^{14}C]FE in R and S biotypes of *E. phyllopogon* (**Figure 2**) from 3 to 48 HAA. Three HAA absorption was low for both biotypes, ranging from an average of 21% of the applied radioactivity absorbed by up to 52% at 120 HAT for the R biotype vs 24 and 54%, respectively, for the S biotype. The greater differences were obtained from 12 HAA (28%) to 48 HAA (38%) in the R biotype vs 36 and 45%, respectively, for the S biotype. Quantitative absorption results gave significant differences between times in both biotypes (**Table 3**) or between biotypes (from 3 to 48 HAA) (**Table 3**), according to a Tukey test, obtaining the maximum absorption at 120 HAA.

In the translocation study, the recovered radioactivity of both biotypes revealed a similar distribution of [^{14}C]FE (or any [^{14}C]-metabolite formed thereafter), in the treated leaves (almost 99% of the recovered radioactivity), with no appreciable acropetal or basipetal herbicide translocation (or both) in R and S biotypes from 3 to 120 HAA (**Table 3**) while only approximately less than 1% was translocated out of the treated leaf to the upper leaves (**Table 3**). These results clearly indicate the low mobility of FE inside the biotypes examined. No differences were found in the translocation studies, either between biotypes or between times (**Table 3**). These findings, which are consistent with earlier studies (*33*), suggest that only a small amount of the applied herbicide is needed for phytotoxic action in the meristematic region. Although the translocation increases with time, it does not constitute a differential mechanism of resistance in the R biotype.

Metabolism. Generally, FE is rapidly de-esterified by hydrolysis in plants to yield FA, which is the active herbicide (*34*), although both compounds are phytotoxic, and is followed by the formation of water-soluble metabolites. Metabolism is a major mechanism for species selectivity (*34*). FA is metabolized in rice to major nontoxic metabolites via glutathione (GSH) or cysteine-mediated ether bond cleavage to yield nonphytotoxic GSH or cysteine conjugates of benzoxazolone (CDHB) (*35*). Other polar metabolites, presumed to be carbohydrate conjugates, have been found (*27*, *36*). The FE metabolism pattern was different between R and S biotypes of *E. phyllopogon* in terms of the differential rate of FE hydrolysis and FA metabolism to form nonactive metabolites. The FE de-esterification rate was fairly similar in both biotypes from 3 to 12 HAA with approximately 35% of absorbed [^{14}C]FE remaining 12 HAA, being critical at 24 HAA, where the S biotype had 2-fold more (34%) than the R biotype (17%), as after that a similar percentage of from 48 to 120 HAA remained (**Table 4** and **Figure 3**). FA, which is the active herbicide, was metabolized to presumably nontoxic compound polar metabolites significantly faster in the R biotype. The FA percentage was fairly similar in both biotypes only at 3 HAA; it was quickly metabolized in the R biotype to nontoxic metabolites [S-(6-chlorobenzoxazole-2-yl)glutathione (CDHB-GSH), S-(6-chlorobenzoxazole-2-yl)glycylcysteine (CDHB-GlyCys), and S-(6-chlorobenzoxazole-2-yl)cysteine (CDHB-Cys)]. Thus, 26% of the recovered radioactivity remained as acid 6 HAA, decreasing after that to 9% after 120 HAA. In the S biotype, the FA percentage was constant from 12 to 48 HAA (approximately

Table 3. Distribution of Radioactive Compounds (Expressed as Percentage of Total Radioactivity Applied) after [^{14}C] FE Application in the Second Leaf of *E. phyllopogon*[a]

	mean ± SE						
	3 h	6 h	12 h	24 h	48 h	72 h	120 h
				Biotype S			
nonabsorbed	74 e ± 6	66 de ± 5	54 cd ± 5	48 bc ± 5	46 ab ± 5	44 a ± 7	41 a ± 9
treated leaf	24 a,a ± 3	31 ab,a ± 3	36 bc,a ± 7	42 c,a ± 6	45 cd,a ± 5	51 e,a ± 2	54 de,a ± 3
upper leaves	9×10^{-3} a ± 3×10^{-4}	5×10^{-4} a ± 4×10^{-4}	5×10^{-4} a ± 2×10^{-4}	2×10^{-3} a ± 10^{-3}	3×10^{-3} a ± 2×10^{-4}	2×10^{-3} a ± 2×10^{-3}	8×10^{-3} b ± 3×10^{-4}
total	98 ± 9	97 ± 8	90 ± 12	90 ± 11	91 ± 10	95 ± 9	95 ± 12
				Biotype R			
nonabsorbed	76 e ± 11	74 de ± 6	70 cd ± 4	63 bc ± 5	53 ab ± 3	50 a ± 7	46 a ± 7
treated leaf	21 a,b ± 2	23 ab,b ± 4	28 bc,b ± 5	34 c,b ± 5	38 cd,b ± 6	48 e,a ± 2	52 de,a ± 3
upper leaves	3×10^{-4} a ± 7×10^{-5}	5×10^{-4} a ± 9×10^{-5}	4×10^{-4} a ± 10^{-4} 10^{-3} a ± 7×10^{-4}	10^{-3} a ± 2×10^{-4}	10^{-3} a ± 2×10^{-4}	4×10^{-3} b ± 2×10^{-4}	
total	97 ± 13	97 ± 10	98 ± 9	97 ± 10	91 ± 9	98 ± 9	98 ± 10

[a] Data are means of three experiments; SE, standard error. For each quantitative character of each biotype, mean values of different times with the same letters do not differ significantly according to a Tukey HSD test ($p < 0.05$) (first letter; x, y). In addition, for each time, the means of treated leaves of both biotypes were compared using ANOVA and a Tukey posthoc test and mean values with the same letters do not differ significantly according to a Tukey HSD test ($p < 0.05$) (second letter; x, y).

Table 4. Percentage of Radioactive Compounds Found by TLC (Expressed as Percentage of Total Radioactivity Recovered) after [^{14}C]FE Application in the Second Leaf in *E. phyllopogon*[a]

	3 h	6 h	12 h	24 h	48 h	72 h	120 h
				Biotype R			
FE	70 ± 8	53 ± 4.6	33 ± 2.7	17 ± 3.9	13 ± 3.6	4 ± 2	2 ± 2
FA	21 ± 3.4	26 ± 1.9	12 ± 4.3	10 ± 1.6	9 ± 2.7	10 ± 1.2	9 ± 3.3
CDHB	not found						
HPP	not found						
HQ	not found						
CDHB-GSH	5 ± 0.5	15 ± 1.5	30 ± 4.1	45 ± 5.9	50 ± 4.7	45 ± 3.9	45 ± 5.4
CDHB-GlyCys		6 ± 1.2	20 ± 3.8	21 ± 5.8	15 ± 4	23 ± 3.4	26 ± 4.9
CDHB-Cys				6 ± 0.8	5 ± 1.2	14 ± 2.3	17 ± 3
				Biotype S			
FE	69 ± 6.8	60 ± 7.3	37 ± 4.4	34 ± 2.8	9 ± 1.3	4 ± 0.8	2 ± 0.2
FA	23 ± 4.4	35 ± 2.8	41 ± 5.6	42 ± 6	44 ± 3.7	35 ± 5.9	32 ± 3
CDHB	not found						
HPP	not found						
HQ	not found						
CDHB-GSH			4 ± 0.5	12 ± 2.4	29 ± 3.4	32 ± 4	29 ± 3.3
CDHB-GlyCys				7 ± 1.1	9 ± 2.3	15 ± 2.7	19 ± 4.5
CDHB-Cys						6 ± 1.3	

[a] Data are means of three experiments ± SE.

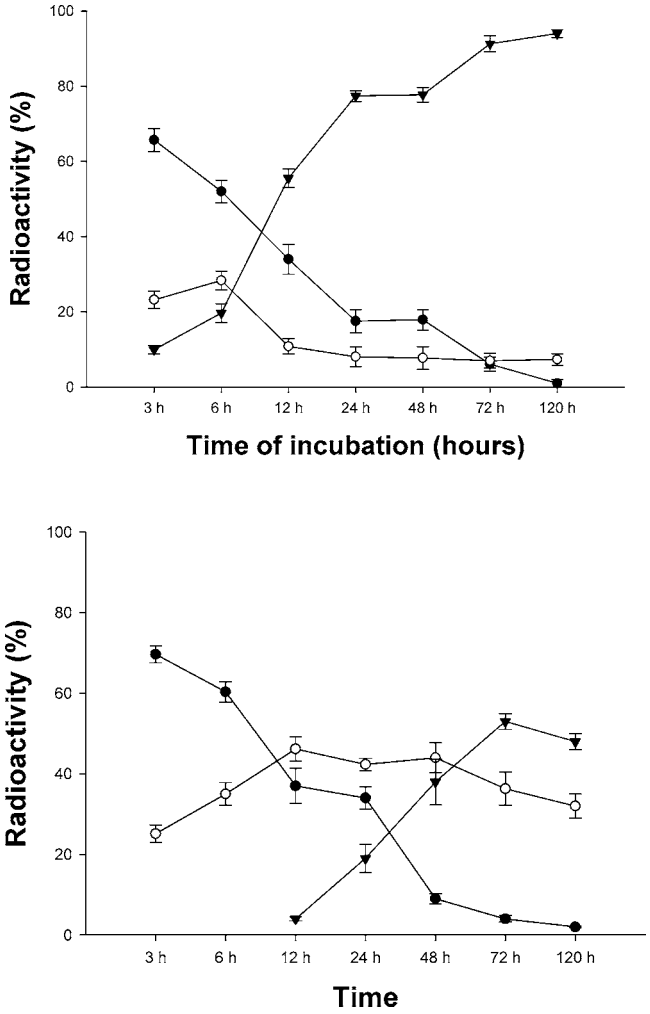

Figure 3. Percentage of absorbed radiolabel found as (●) FE, (○) FA, and (▼) polar metabolites (CDHB-GSH, CDHB-GlyCys, and CDHB-Cys) in leaf extracts of R (top) and S (bottom) biotypes of *E. phyllopogon* during a 120 h period after treatment with [^{14}C]FE. Values are mean of three experiments. Bars represent standard errors.

42%) decreasing to 32% of the recovered radioactivity at 120 HAA (**Table 4**).

There was 5 times less FA and approximately 2 times more polar metabolites (CDHB-GSH, CDHB-GlyCys, and CDHB-Cys) in the R biotype 48 HAA than in the S biotype (**Table 4**). By 120 HAA, 9% of the total FE absorbed remained as FA and 88% as polar metabolites in the R biotype vs 32 and 48%, respectively, for the S biotype (**Table 4**). The R biotype started the metabolism of FA much faster than the S biotype. A detailed comparison of metabolite profiles revealed quantitative but not qualitative differences between the biotypes examined (**Table 4**), suggesting a common route for FE metabolism in *E. phyllopogon* (**Figure 4**). After FE de-esterification, three different molecules can be formed (**Table 4** and **Figure 4**), CDHB (6-chloro-2,3-dihydro-benzoxazol-2-one) and 4-hydroxyphenoxy-propanoic acid, as a consequence of the breaking down of the ether bond by hydrolases. FA can be conjugated directly with GSH (mediated by GSTs) (**Figure 4**). TLC studies detected three signals of the recovered radioactivity of conjugates corresponding to the CDHB-GSH, CDHB-GlyCys, and CDHB-Cys. Although 4-hydroxyphenoxy-propanoic acid and CDHB (6-chloro-2,3-dihydrobenzoxazol-2-one) were not detected by TLC (**Table 4**), the last one of these is vital for conjugating with cysteine, so that more accurate techniques are necessary (high-performance liquid chromatography) to detect it. The first conjugate formed is GSH conjugate, which accounted for 45% of the recovered radioactivity only 24 HAA (**Table 4**). CDHB-GlyCys and CDHB-Cys were found, too, but in lower percentages. Although the qualitative pathway followed in both biotypes was similar (**Figure 4**), the S biotype started conjugation later, with minimal percentages of conjugates during the first 24 h, starting the conjugation from 48 to 120 HAA but in lower percentages (**Table 4**). Many pesticides in numerous plant species have been shown to be converted to GSH conjugates either by GSTs enzymes (*37*) or by nonenzymatic conjugation (*38*). Nonenzymatic GSH conjugation is considered when GST activity is very low but has been reported to be important for the metabolism of several herbicides (*39*) such as wheat where increased GSH concentrations protected it from fenoxaprop injury (*14*).

The very low conversion of FA to water-soluble metabolites in susceptible plants could be apparently due to insufficient enzymes and/or substrate levels for the detoxification of the herbicide. Although GSH concentrations vary during plant

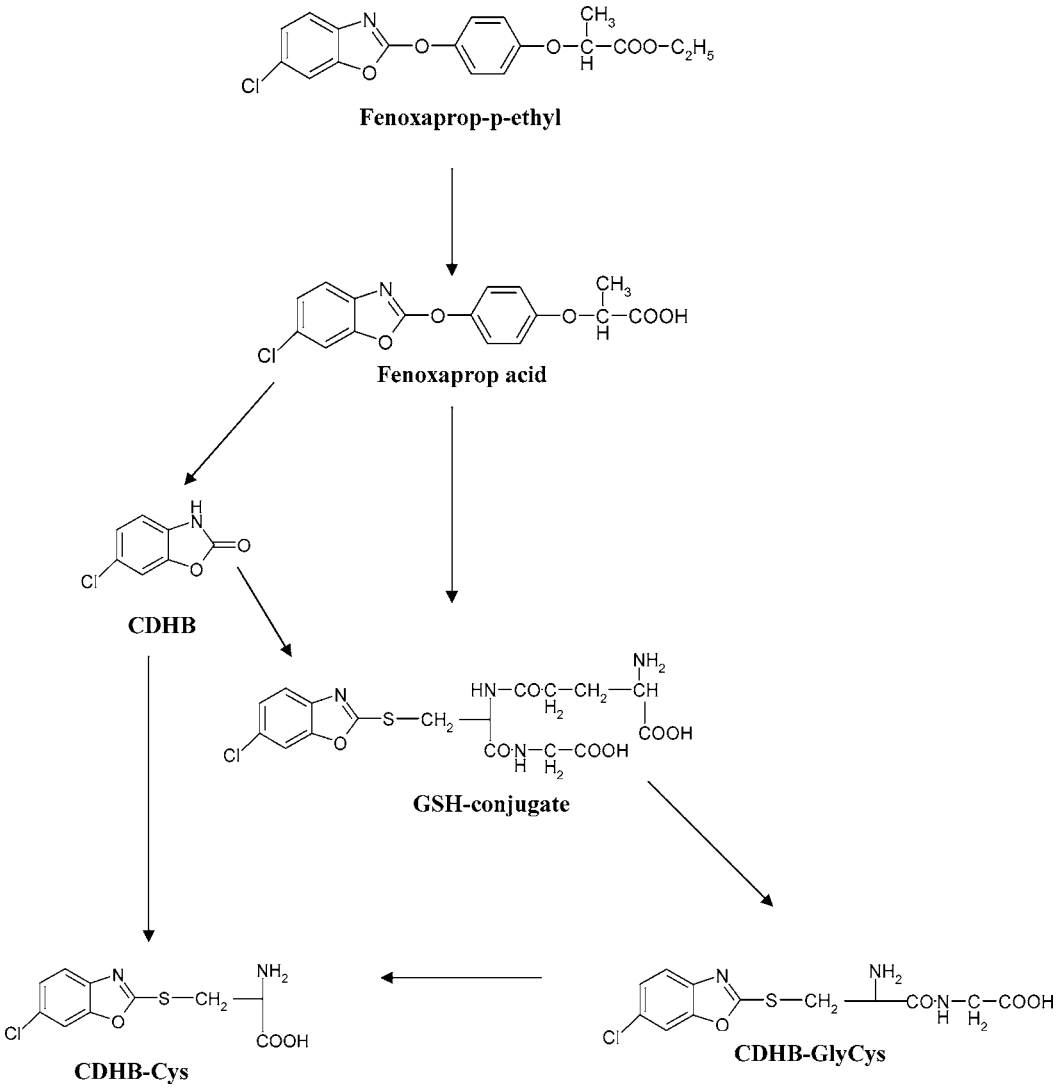

Figure 4. Proposed primary initial metabolic pathway of FE.

development (*23*, *40*, *41*), GSH is found in relatively high concentrations in most plant tissues (*23*). Generally, GSH synthesis is limited by the availability of cysteine and, hence, by the concentration of sulfate ions. Up to date, many herbicide families, including sulfonylureas, aryloxyphenoxypropionates, triazinone sulfoxides, and thiocarbamates, are susceptible to GSH conjugation (*24*). Furthermore, there is a positive correlation of both the GSH levels and the activity of specific GST enzymes with the rate of herbicide conjugation and detoxification (*25*). For example, the resistance of a velvetleaf (*Abutilon theophrasti* Medicus) biotype to atrazine was the result of an enhanced rate of GSH conjugation (*42*, *43*). In the present study, the higher rate of GSH conjugation in resistant as compared to susceptible biotypes indicates that GSH and cysteine conjugation is the major mechanism of resistance of the R biotype against FE toxicity although it is a complex mechanism and has not been completely identified. Evidence suggests that more than one mechanism is involved in the detoxification of FE in the R biotype of *E. phyllopogon* (*44*). This could be in accordance if we take into account that the resistance in *E. phyllopogon* was selected for the continuous application of thiocarbamates, in whose metabolism are involved two different enzyme systems: monoxydation mediated by cytochrome P450 and conjugation mediated by GSTs (*45*).

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