RESISTANCE TO ACCASE INHIBITORS IN *Eleusine indica* FROM BRAZIL INVOLVES A TARGET SITE MUTATION[1]

*Resistência aos Inibidores de ACCase em **Eleusine indica** do Brasil Envolve uma Mutação na Enzima Alvo*

OSUNA, M.D.[2], GOULART, I.C.G.R.[3], VIDAL, R.A.[3], KALSING, A.[3], RUIZ SANTAELLA, J.P.[4], and DE PRADO, R.[4]

ABSTRACT - *Eleusine indica* (goosegrass) is a diploid grass weed which has developed resistance to ACCase inhibitors during the last ten years due to the intensive and frequent use of sethoxydim to control grass weeds in soybean crops in Brazil. Plant dose-response assays confirmed the resistant behaviour of one biotype obtaining high resistance factor values: 143 (fenoxaprop), 126 (haloxyfop), 84 (sethoxydim) to 58 (fluazifop). ACCase in vitro assays indicated a target site resistance as the main cause of reduced susceptibility to ACCase inhibitors. PCR-generated fragments of the ACCase CT domain of the resistant and sensitive reference biotype were sequenced and compared. A point mutation was detected within the triplet of aspartate at the amino acid position 2078 (referred to EMBL accession no. AJ310767) and resulted in the triplet of glycine. These results constitute the first report on a target site mutation for a Brazilian herbicide resistant grass weed.

Keywords: goosegrass, cross-resistance, enzyme, PCR, herbicide.

*RESUMO - **Eleusine indica** (ELEIN) é uma espécie monocotiledônea, diploide. No Brasil, ela desenvolveu resistência aos inibidores da ACCase durante os últimos dez anos, devido ao uso intensivo e frequente desses graminicidas para controlar plantas daninhas em lavouras de soja. Experimentos de dose-resposta realizados com a planta confirmaram a resistência de um biótipo. Houve elevada tolerância aos herbicidas, com fatores de resistência da ordem de 143 (fenoxaprop), 126 (haloxyfop), 84 (sethoxydim) e 58 (fluazifop). Ensaios com a enzima ACCase in vitro indicaram a insensibilidade desta como a principal causa de suscetibilidade reduzida a esses herbicidas. Fragmentos de PCR gerados do domínio CT da enzima ACCase dos biótipos resistente e sensível de referência foram sequenciados e comparados. Foi detectada uma mutação dentro do tripleto de asparagina na posição do aminoácido 2078 (referente ao acesso número AJ310767 no EMBL), que resultou no tripleto de glicina. Esses resultados constituem o primeiro caso de uma mutação em ACCase em uma espécie daninha gramínea do Brasil.*

Palavras-chave: capim-pé-de-galinha, resistência cruzada, enzima, PCR, herbicidas.

INTRODUCTION

Eleusine indica Gaertn (goosegrass) is a species originated from Asia and represents one of the most important grass weeds in crops from tropical and temperate regions of the world. It is an annual species and the average production per plant is approximately 40,000

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[2] Centro de Investigacion "Finca La Orden-Valdesequera", Departamento de Hortofruticultura, 06187 Guadajira, Badajoz (Spain); [3] Universidade Federal do Rio Grande do Sul, Faculdade de Agronomia, Dep. de Plantas de Lavoura, Av. Bento Gonçalves, 7712, Porto Alegre-RS, Brasil; [4] Universidad de Córdoba, Dep. de Química Agrícola, 14004 Córdoba (Spain).

seeds when in pure stands (Kissman & Groth, 1991).The soybeans mass was reduced 12, 19 and 25% due to root, shoot, and both shoot/root competition with *E. indica*, respectively (Xia et al., 1997).

During the past three decades grass weeds were controlled in soybean predominantly with herbicides inhibitors of the enzyme acetyl-CoA carboxylase (ACCase). The herbicides that affect ACCase enzyme include the chemistries aryloxyphenoxypropionates (AOPP), cyclohexanediones (CHD), and pyrazolines. In Brazil, an *E. indica* biotype was diagnosed with resistance to this group of herbicides in 2005 in soybean crops located in the central part of Mato Grosso state (Vidal et al., 2006).

Weed resistance to herbicides is defined as the inheritable ability of individuals to survive after repeated use of a compound that controls the original population (Gherekhloo et al., 2012). Since 1975, about ten new herbicide resistant biotypes are documented yearly. Cross-resistance is referred as resistance to several herbicides that affect one target enzyme, and usually is endowed by a single gene (Délye, 2005). Herbicide cross-resistance on many plant species have been documented due to ACCase enzymes with amino acid substitutions at the positions: 1781 (White et al., 2005), 1999 (Liu et al., 2007); 2027 (Délye et al., 2003; Liu et al., 2007; Gherekhloo et al., 2012); 2041 (Délye et al., 2003; Zhang & Powles, 2006; Liu et al., 2007); 2078 (Délye et al., 2005; Kaundun, 2010; Hochberg et al., 2009); at 2088 (Yu et al., 2007), and 2096 (Délye et al., 2005). The choice of herbicide to control a resistant population is complicated by the fact that the specific amino acid substitutions in one enzyme may give different patterns of cross resistance to the different herbicides that affect it (Powles &Yu, 2010).

This work was designed to test the hypotheses that a) there is cross-resistance of *E. indica* to several ACCase inhibitors, b) the ACCase enzyme is insensitive to this group of herbicides on this biotype, and c) a mutation on the ACCase gene is the molecular basis of the resistance on a population from Brazil.

MATERIALS AND METHODS

Seed source

Eleusine indica seeds from two biotypes were collected from at least 1000 plants. The biotype suspect of resistance to ACCase inhibitors (R) was collected from plants growing on a farm located in the county of Lucas do Rio Verde, in Mato Grosso state, Brazil. This farm has been cropped with soybeans during the last 20 years with at least one post-emergence spray of sethoxydim at 230 g ha^{-1} per year. The biotype susceptible to herbicides (S), used as a reference population, was collected in the state of São Paulo, Brazil, in places never treated with herbicides for the past 15 years.

Dose-response experiments at plant level

Dose-response curves were generated in eight experiments to evaluate the resistance to several ACCase inhibitors (butroxydim, clethodim, cyhalofop, fenoxaprop, fluazifop, haloxyfop, quizalofop, sethoxydim) on both biotypes. Seeds of *E. indica* from each biotype were placed in a 2.0 L capacity trays containing potting mix (50% soil / 50% sand (v/v)) as substrate. The trays were kept irrigated and when seedlings were at the two-leaf growth stage they were transplanted to 0.3 L pots with potting mix as already described. Plants were kept on a greenhouse with day/night temperature of 29 ± 5 e 20 ± 6 °C, respectively. When plants were at the six to eight-leaf growth stage, the herbicides were sprayed with a CO_2 hand-held sprayer delivering a total of 220 L ha^{-1} at 200 kPa.

A hierarchic factorial arrangement of the treatments was used for each experiment, using a completely random design and three replicates for each treatment. The first factor was the biotype (R and S); and the second factor was the herbicide rates, which included 0, 25, 35, 50, 70, 100, 200, 400, 800, 1,600, and 3,200% of the labeled rate. The herbicide labeled rates were: butroxydim 375 g ha^{-1}, clethodim 108 g ha^{-1}, cyhalofop-butyl 57 g ha^{-1}, fluazifop-P-butyl 125 g ha^{-1}, haloxyfop-P-methyl 60 g ha^{-1}, sethoxydim 230 g ha^{-1}. Visual evaluations of plant injury were collected at

21 days after herbicide spray (DAT), using the 0 to 100 scale described by Frans et al. (1986), where 0 represents no effect and 100 represents plant death.

Data were subjected to the analysis of variance (ANOVA). When significant herbicide rate x biotype interaction was detected, curve fitting techniques were used to adjust dose-response curves for each biotype. The data was fitted to a nonlinear, log-logistic three-parameters regression model

$$Y = a / [1 + (x/ ED_{50})^{b}]\},$$

where Y is the dependent variable (expressed as 100 - plant injury), a corresponds to the upper asymptote; b is the slope of the curve; and ED_{50} is the effective dose needed for 50% plant injury. The resistant factor (RF) was calculated by dividing the ED_{50} of the resistant biotype by the ED_{50} of the susceptible biotype.

ACCase enzyme purification and assay

The ACCase enzyme was isolated using procedures adapted from (De Prado et al., 2000). Leaves (6 g fresh weight) of R and S biotypes of *E. indica* 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 (pH7.5), 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 (24,000 g, 30 min, 4 °C). The supernatant was fractioned with ammonium sulfate and centrifuged (12,000 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.

The enzyme activity was assayed by measuring the ATP-dependent incorporation of $NaH[^{14}C]O_3$ into [^{14}C]malonyl-CoA. 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 $MgCl_2$, 15 mM $NaH[^{14}C]O_3$ (1.22 MBq μmol^{-1}), 50 µL of enzyme fraction, 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 4N 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 (Redy safe, Beckman Instruments Inc.). Radioactivity was determined by LCC. Background radioactivity measured as acid-stable counts (dpm) in the blank treatment, which consisted of all ingredients of the reaction but acetyl-CoA, was subtracted from each treatment. One unit of ACCase activity was defined as 1 µmol malonyl CoA formed min^{-1}. Herbicide concentrations resulting in a 50% ihibition of enzyme activity (EC_{50}) were determined in crude extracts. Herbicide and range of concentrations tested consisted of: cyhalofop acid from 0 to 300 µM, fenoxaprop acid from 0 to 30 µM, sethoxydim from 0 to 2000 µM, and tepraloxydim from 0 to 30 µM. Experiments were repeated three times. Data were submitted to analysis of variance and fitted to the logistic equation as described before.

Molecular studies

Total genomic DNA was extracted from fresh plants at the four-leaf stage by using the Qiagen DNA Extraction Kit from 100 mg of frozen leaf material. In all cases, DNA was quantified with NanoDrop then immediately used for PCR reactions or stored at -80 ºC until its use.

Primers were designed to amplify regions in the CT domain know to be involved in sensitivity to ACCase herbicides. Two sets of primers (Table 1) covering all known mutation sites, were designed based on the chloroplastic ACCase sequences of other grass weeds, *Alopecurus myosuroides* (accession no. AJ632096), *Lolium rigidum* (AY995232), *Avena fatua* (AF231335) and *Phalaris paradoxa* (AM745339) (Table 1). Ten individual plants from each population were genotyped.

Table 1 - Primers designed to amplify regions in the CT domain know to be involved in sensitivity to ACCase herbicides

Primer	Sequence
ELEIN_1781F	GCGTGCTGCTGGGCTGAAT
ELEIN_1781R	CCGGTCAAAATAATGGGCTGGTC
ELEIN_2027_f	AATGGCTGGGTGGTATGTTTGAC
ELEIN_2027_r	ATCCTCCGCCGTAATCTCTTGTAG

PCR was conducted in a 25 µL volume that consisted of about 300 ng of genomic DNA, 0.5 µM of each primer, and 12.5 µL of 2X GoTaq Green Master mix (Promega). The PCR was run in an Eppendorf Master Gradient Thermo cycler Model 96 programmed for an initial denaturation step of 94 °C of 4 min followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C. A final extension step for 5 min at 72 °C was also included. The PCR products were directly purified or purified from agarose gel with Wizard SV gel and PCR Clean-up system (Promega), and were sequenced by the central facilities of the University of Córdoba.

RESULTS AND DISCUSSION

Dose-response experiments at plant level

For all herbicides tested, a significant ($P<0.01$) biotype by rate interaction was detected with the F test (data not shown). The data of plant injury from the R and S biotypes were fitted to the logistic dose-response curves with significant ($P<0.01$) level of significance of the nonlinear model (Table 2). For all herbicides, and for both biotypes, with the increase of the herbicide rate the level of plant injury increased following a typical sigmoidal dose-response curve. Cross resistance to ACCase inhibitor herbicides was confirmed in the *E. indica* biotype originated from Mato Grosso.

For the resistant biotype, the herbicide rate necessary to promote 50% plant injury (ED_{50}) ranged from 185 g ha^{-1}, for haloxyfop-P-methyl, to >1,800 g ha^{-1}, for cyhalofop-butyl (Table 2). The ED_{50} determined for the resistant *E. indica* biotype with the herbicides clethodim and cyhalofop-butyl was superior to the highest herbicide rate tested in the experiments, indicating that at the tested rates the level of plant injury had not reached 50%.

For the susceptible biotype, however, the ED_{50} ranged from 11 g ha^{-1}, for haloxyfop-P-methyl, to 190 g ha^{-1}, for butroxydim (Table 2). This indicates that for all herbicides, the level of plant injury evaluated on the susceptible biotype was high at very low herbicide rates.

The RF calculated for the R biotype indicates three group of cross-resistance. A low level resistance (~3 to 6) for butroxydim and fluazifop-P-butyl; a medium level of resistance (10 to 16) for clethodim and haloxyfop-P-methyl; and a high level of resistance (>20) for cyhalofop-butyl and sethoxydim (Table 2).

ACCase enzyme assay

Two herbicides representing the AOPP and two representing the CHD were used to test the sensitivity of the ACCase enzyme of both R and S biotypes. For all herbicides tested, a significant ($P<0.01$) biotype by concentration interaction was detected with the F test (data not shown). The data of plant injury from the R and S biotypes were fitted to the logistic dose-response curves with significant ($P<0.01$) level of significance of the nonlinear model (Table 3). For all herbicides, and for both biotypes, with the increase of the herbicide rate the activity of the ACCase enzyme decreased following a typical sigmoidal dose-response curve.

For the resistant biotype, the herbicide concentration necessary to promote 50% enzyme inhibition (EC_{50}) ranged from 12 µM for fenoxaprop acid to 1169 µM for sethoxydim (Table 3). For the susceptible biotype the EC_{50} ranged from 0.13 µM for fenoxaprop acid to 104 µM for sethoxydim (Table 3). Like the results at the plant level, the ACCase enzyme activity on the susceptible *E. indica* biotype was affected significatively at very low herbicide concentrations.

Molecular studies

Ten individuals of each population were sequenced. Then, the sequences were aligned to each other and to the chloroplastic

Table 2 - Parameters of the equation[1/] used to calculate the herbicide dose required for 50% plant injury (ED_{50}) of resistant (R) and susceptible (S) biotypes of *Eleusine indica*

Herbicide [2/]	Biotype	a	b	$ED_{50} \pm SE$ [3/]	Pseudo r^2 [4/]	P[5/]	RF[6/]
BU	R	99.88	1.84	432.36 ± 68.45	0.93	< 0.01	2.3
	S	93.62	1.97	190.36 ± 54.25	0.79	0.01	-
CB	R	99.75	0.98	3027.22 ± 229.78	0.98	<0.01	94.1
	S	99.78	5.68	32.17 ± 3.41	0.85	0.02	-
CL	R	100.89	1.06	737.10 ± 184.17	0.80	0.01	10.7
	S	97.59	4.51	68.70 ± 9.55	0.81	0.01	-
FB	R	97.55	2.47	202.22 ± 8.47	0.98	<0.01	6.7
	S	100.00	58.41	30.22 ± 5.93	0.99	<0.01	-
HM	R	102.40	0.96	185.31 ± 50.38	0.86	<0.01	16.8
	S	100.02	2.73	11.02 ± 1.74	0.98	<0.01	-
SE	R	102.57	3.07	521.20 ± 21.80	0.99	0.01	21.5
	S	99.99	1.58	24.20 ± 3.56	0.99	<0.01	-

[1/] Logistic equation $Y = a / [1 + (x/ED_{50})^b]\}$, where Y is the percentage of plant injury, x (independent variable) is the herbicide rate (g ha^{-1}), a is the the upper asymptote, b is the slope of the line, and ED_{50} is the effective rate required for 50% plant injury. Data were pooled and fitted to nonlinear regression model. Data are means of four replicates. [2/] BU=butroxydim, CB=cyhalofop-butyl, CL=clethodim, FB=fluazifop-P-butyl, HM=haloxyfop-P-methyl, SE=sethoxydim. [3/] SE=standard error of the value. [4/] Approximate coefficient of determination of nonlinear models with a defined intercept calculated as pseudo $r^2 = 1$ - (sums of squares of the regression/corrected total sums of squares). [5/] Probability level of significance of the nonlinear model. [6/] RF = resistant factor = ED_{50} of resistant / ED_{50} of susceptible biotype.

Table 3 - Parameters of the equation[1/] used to calculate the herbicide concentration required for 50% reduction of ACCase activity (EC_{50}) of resistant (R) and susceptible (S) biotypes of *Eleusine indica*

Herbicide [2/]	Biotype	a	b	$ED_{50} \pm SE$ [3/]	Pseudo r^2 [4/]	P[5/]	RF[6/]
CA	R	100.26	1.01	112.4 ± 1.4	0.99	<0.01	55.4
	S	99.91	0.56	2.03 ± 0.1	0.99	<0.01	-
FA	R	98.42	1.07	12.3 ± 0.9	0.99	<0.01	94.5
	S	100.00	0.52	0.1 ± 0.04	0.99	<0.01	-
SE	R	100.70	0.58	1168.9 ± 423.3	0.96	<0.01	11.2
	S	11.10	0.85	104.7 ± 10.9	0.99	<0.01	-
TP	R	99.69	2.31	23.0 ± 0.1	0.99	0.01	15.26
	S	99.71	1.22	1.5 ± 0.05	0.99	<0.01	-

[1/] Logistic equation $Y = a / [1 + (x/EC_{50})^b]\}$, where Y is the percentage of enzyme inhibition, x (independent variable) is the herbicide concentration (µM), a is the the upper asymptote, b is the slope of the line, and EC_{50} is the effective concentration required for 50% enzyme inhibition. Data were pooled and fitted to nonlinear regression model. Data are means of three replicates. [2/] CB=cyhalofop acid, FA=fenoxaprop acid, SE=sethoxydim, TP=tepraloxydim. [3/] SE=standard error of the value. [4/] Approximate coefficient of determination of nonlinear models with a defined intercept calculated as pseudo $r^2 = 1$ - (sums of squares of the regression/corrected total sums of squares). [5/] Probability level of significance of the nonlinear model. [6/] RF = resistant factor = EC_{50} of resistant / EC_{50} of susceptible biotype.

ACCase genes of other grass weeds (Figure 1). The nucleotide sequences of the B region for the resistant biotype differed from that of the susceptible one by a single nucleotide substitution (G/C), consequently yielding an Asp 2078 to Gly change in the resistant population.

The data from this work, confirmed the hypothesis that a target site mutation is the mechanism of resistance to herbicides in this ELEIN biotype from Brazil. Only recently the understanding of the mechanisms involved on ACCase inhibitors cross resistance was elucidated. So far, six variable amino acids in

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                                2020       2030       2040       2050       2060       2070       2080       2090       3000
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S A. myosuroides (AJ310767)  REGLPLEILANWRGFSGGQRDLFEGILQAGSTIVENLRTYNQPAFVYIPKAAELRGGAWVVIDSKINPDRIECYAERTAKGNVLE
S T. aestivum (AJ310767)     ...............................................................F...........
S L. rigidum (AJ519781)      ...............................................................F..T........
S A. fatua (AF231334)        ..................................................................T........
S P. minor (AY196481)        ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
S P. paradoxa (AM745339)     ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
R E. indica (Brazil)         ...............................................G..... ..... .....
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Figure 1 - Alignment of partial chloroplastic sequences from resistant (R) and susceptible (S) grasses. ACCase genes from the following species are presented: *Alopecurus myosuroides, Triticum aestivum, Lolium rigidum, Avena fatua and Phalaris minor, P. paradoxa* and *Eleusine indica*. Positions refer to the *Alopecurus myosuroides* ACCase gene (AJ310767).

the ACCase protein may confer different degrees of sensitivity to the herbicides because of difficulties in their access to and/or the fit inside the enzyme active site (Délye et al., 2005; Zhang & Powles, 2006). Furthermore, the high level of resistance verified to sethoxydim is a strong indicator of target site-based resistance because this compound is not exposed to metabolism-based resistance (Moss et al., 2003). As expected, on the ACCase enzyme assay, the RF calculated for the R biotype ranged between 11 and 94 (Table 3). These high values for the RF factors are strong evidence of a target site mechanism of resistance in the *E. indica* biotype from Mato Grosso, Brazil.

Further molecular studies have indicated an Asp-2078-Gly substitution in the R population endowed resistance to both APP and CHD herbicides (Figure 1). The same mutation has been reported in other grass weeds (Délye et al., 2005; Liu et al., 2007; Yu et al., 2007; Hochberg et al., 2009) and endows resistance of these to ACCase-inhibitors.

It is probable that few grass herbicide options for the soybean crop in Brazil and lack of crop rotation may have aided the selection of target site mutations in *E. indica*, and consequent resistance to ACCase inhibitors. This complicates the design of adequate weed management strategies. Herbicides from alternative modes of action other than ACCase inhibitors would be recommended strategy to deal with the *E. indica* resistance. However, caution should be taken to avoid selection pressure from only one herbicide option, because worldwide there have been reported evolved resistant *E. indica* populations to glyphosate (Ng et al., 2003; Baerson et al., 2002; Kaundun et al., 2011), to metribuzin (Brosnan et al., 2008), and to glufosinate and paraquat (Seng et al., 2010).

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