BIOLOGY, MANAGEMENT AND BIOCHEMICAL/GENETIC CHARACTERIZATION OF WEED BIOTYPES RESISTANT TO ACETOLACTATE SYNTHASE INHIBITOR HERBICIDES

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ABSTRACT: *Bidens pilosa* and *Amaranthus quitensis* are major weeds infesting soybean [*Glycine max* L (Merrill)] fields in Brazil and Argentina. The repetitive use of acetolactate synthase (ALS EC 4.1.3.18) inhibiting herbicides in São Gabriel do Oeste, MS, Brazil and in the provinces of Córdoba and Tucumã, Argentina, has selected for resistant (R) biotypes of these weeds. Research work was developed to study the management, growth, biochemistry, and genetics of these R weed biotypes. In a field experiment it was found that chlorimuron-ethyl and imazethapyr at recommended rates (both ALS inhibitor herbicides), did not control R *B. pilosa*, but the alternative lactofen, fomesafen and bentazon were effective, either sprayed alone or mixed with the ALS inhibitor herbicides. Greenhouse studies confirmed the cross-resistance of both R biotypes to the imidazolinone and sulfonylurea herbicides, and these alternative herbicides, when sprayed alone or mixed with the ALS inhibitor, efficiently controlled both R and S populations. A growth analysis of the R and S biotypes of these weeds, under non-competitive conditions, indicated that there is no adaptive cost to the R biotypes (pleiotropic effect). A quick bioassay using ALS and ketoacid reductoisomerase (KARI) inhibitors showed that the resistance of the R biotypes to herbicides is related to a lack of sensitivity of the ALS enzyme to the herbicides. On the other hand, the sequencing of the gene that codifies the ALS resistance in R *A. quitensis* did not present any mutation in the A Domain region, suggesting that other positions of the gene that confer insensitivity of the ALS to sulfonylurea and imidazolinone herbicides could have mutated.
Key words: *Amaranthus quitensis, Bidens pilosa,* chlorimuron-ethyl*, Glycine max* L. Merrill, imazethapyr

BIOLOGIA, MANEJO E CARACTERIZAÇÃO BIOQUÍMICA E GENÉTICA DE BIÓTIPOS RESISTENTES AOS HERBICIDAS INIBIDORES DA ACETOLACTATO SINTASE

RESUMO: *Bidens pilosa* e *Amaranthus quitensis* são as principais plantas daninhas infestantes na cultura de soja [*Glycine max* L (Merrill)] no Brasil e Argentina, respectivamente. O uso repetitivo de herbicidas inibidores da acetolactato sintase (ALS EC 4.1.3.18) em São Gabriel do Oeste (MS - Brasil) e nas províncias de Córdoba e Tucumã (Argentina), selecionaram biótipos resistentes (R) destas plantas daninhas. Esta pesquisa foi desenvolvida para estudar o manejo, crescimento, a bioquímica e genética destes biótipos resistentes. Em um experimento de campo concluiu-se que chlorimuron-ethyl e imazethapyr (inibidores da ALS), aplicados nas doses recomendadas, não controlaram o biótipo R de *B. pilosa*, mas os herbicidas alternativos lactofen, fomesafen e bentazon foram eficientes quando aplicados sozinhos ou em mistura com os herbicidas inibidores da ALS. Estudos em casa-de-vegetação confirmaram a resistência cruzada para os biótipos de ambas espécies aos herbicidas dos grupos químicos das imidazolinonas e sulfuniluréias e os herbicidas alternativos sozinhos ou em mistura com os inibidores da ALS controlaram eficientemente populações resistentes e suscetíveis. Análises de crescimento dos biótipos R e S destas plantas daninhas em condições não competitivas mostraram que não existe um custo adaptativo para os biótipos R (efeitos pleiotrópicos). O bioensaio rápido usando inibidores da ALS e ketoacid reductoisomerase (KARI) indicaram que a resistência decorre da insensibilidade da enzima ALS aos herbicidas. Por outro lado, o seqüenciamento do gene que codifica a ALS em R *A. quitensis* não mostrou mutação no Domínio A, sugerindo que outras posições do gene poderiam estar sofrendo mutações que conferem a insensibilidade da ALS a sulfuniluréias e imidazolinonas.
Palavras-chave: *Amaranthus quitensis, Bidens pilosa,* chlorimuron-ethyl*, Glycine max* L. Merrill, imazethapir

INTRODUCTION

Nine weed species have developed resistance against herbicides in Brazil: *Bidens pilosa* L. (hairy beggarticks), *Bidens subalternans* (beggarticks), *Euphorbia heterophylla* (wild poinsettia), *Cyperus difformis* (smallflower umbrella sedge), *Fimbristyles miliacea* (globe fringerish), and *Sagittaria montevidensis*

(california arrowhead) to acetolactate synthase inhibitor (ALS) herbicides; *Brachiaria plantaginea* (alexandergrass) to acetyl coenzyme A carboxylase (ACCase) inhibitor herbicides; *Echinochloa crus-galli* (barnyardgrass), and *Echinochloa crus-pavonis* (gulf cockspur) to synthetic auxin herbicides (Heap, 2002). Around the world, the number of resistant biotypes is 272 (Heap, 2002). Herbicide resistance is a trait shown by a species (as intact plants or plant cells in culture) to withstand higher concentrations of a herbicide than the wild type of the same plant species (Maxwell & Mortimer, 1994).

Bidens pilosa is an important weed associated to soybean production in Brazil. Populations of this weed have survived recommended rates of ALS herbicides in field tests (Christoffoleti et al., 1997; Ponchio, 1996; Monquero, 1999). A more reliable way to confirm resistance is to use curve studies. Christoffoleti et al. (1997) reported the first use of growth rate inhibition of 50% (GR_{50}) for confirmation of a herbicide-resistant biotype in Brazil. Several experiments were conducted under greenhouse conditions, using R and S biotypes of *B. pilosa*, at increasing rates of ALS inhibitor herbicides. The GR_{50} values for imazethapyr, 2-[4,5,-dihydro-4-methyl-4-4(1—methylenthyl)5-oxo-1*H*-imidazol-2-yl]-5-ethyl-3-pyridine carboxylic acid; nicosulfuron, 2-(4,6-dimethoxy pyrimidine-2-ylcarbamoylsulfanoyl)-N, N-dimethylnicotinamide; metsulfuron, 2—(4-methoxy-6-methyl-1,3,5-triazin-2-ylcarbamoylsulfanoyl)benzoic acid; and chlorimuron-ethyl, 2-[[[[(4-chloro-6-methoxy-2-pyrimidine) amino]carbonyl]amino]sulfonyl]benzoic acid were 370, 39, 26 and 12 times higher for R biotypes, as compared to S biotypes. Monquero (1999) confirmed the resistance of *A. quitensis* to ALS inhibitor herbicides showing insensitivity of the ALS to nicosulfuron, metsulfuron, imazethapyr, and chlorimuron-ethyl.

The resistance mechanism to ALS inhibitor herbicides, in most cases, results from an alteration of the ALS enzyme at the site of action of the herbicide, which makes it insensitive. Alteration of the ALS gene sequence that confers resistance is frequently located at the A domain region of the DNA that coded the enzyme (Boutsales et al., 1999). Guttieri et al. (1995) sequenced the gene that codes the ALS enzyme in R plants and found a point mutation in region 173 of the A domain, with threonine, serine, arginine, leucine, glutamine, or alanine substituting proline in the R biotypes. Several other researchers have found substitutions in the A domain region (Bernasconi et al., 1995; Foes et al., 1999; Wright & Penner, 1998; Ott et al., 1996).

Research carried out on herbicide resistance in weeds provides evidence that, in some cases, resistance is accomplished at a pleiotropic cost of fitness at the whole plant level (Holt & Thill, 1994). This principle is most clearly shown for weeds resistant to triazine herbicides in which the mechanism of resistance is a target site mutation, which impairs the normal function at that site. Nevertheless, in the field, selection occurs for traits unrelated to resistance, which may attenuate detrimental effects of the resistance mutation (Holt & Thill, 1994). Christoffoleti (1992) demonstrated that the R biotype of *Kochia scoparia* has the same competitive ability of the S biotype in either competitive or non-competitive situations.

The objectives of this research were to determine herbicide alternatives for the management of ALS R biotypes of *B. pilosa* and *A. quitensis*; to measure the relative growth of R and S biotypes; to identify any pleiotropic effect of the R biotype; to characterize the biochemical sensitivity of the ALS enzyme to herbicides imidazolinone and sulfonylurea in the R biotype; and to sequence the DNA of the ALS gene that codes the A domain region of the R and S biotypes.

MATERIAL AND METHODS

Field experiment

The experiment was initiated in São Gabriel do Oeste, MS, Brazil (19º23'43"S and 54º33'59"W) on December 22, 1996 and continued through 1997, using a cultivated soybean field, where broadleaf weed management had been done during the previous eight years primarily with imazaquin, imazethapyr and chlorimuron-ethyl. *Bidens pilosa* was the most common weed and its control had been lower than expected in the few preceding years. The experiments consisted of a randomized complete block design (n = 4), using 24-m^2 plots with eight soybean rows spaced 0.5 m. Herbicide treatments and rates are presented in Table 1. Treatments were sprayed using an experimental backpack sprayer, pressurized by CO_2 at 240 kPa, adapted to an eight-nozzle boom with XR 11002 tips, and a spray volume of 200 L ha^{-1}. The atmospheric conditions during herbicide application were 29ºC and 56%, for air temperature and relative humidity, respectively. *B. pilosa* was the predominant weed at the time of application (85% in 1996 and 75% in 1997) with 4-5 pairs of leaves. *B. pilosa* control evaluations were made 14 and 21 days after herbicide application (DAA), by visual observation, with of rating 0 for no control and 100% for total control. Data were analyzed by means of analysis of variance, and the Tukey test was used to compare significant treatment means. Since there was no significant interaction between years, data obtained for both years were pooled for statistical analysis purposes.

Alternative herbicides

Another greenhouse experiment was carried out in Piracicaba, SP, Brazil, with R and S biotypes of *B. pilosa* and *A. quitensis*, using R and S seeds collected from soybean production areas of São Gabriel do Oeste

and of the Province of Córdoba, Argentina (31°21'S and 64°05'W). Resistant seeds were collected in São Gabriel do Oeste from several plants that survived ALS inhibitor herbicide treatments at the field experiment described previously. Resistant seeds of *A. quitensis* were harvested in Argentina from plants that survived treatments with ALS inhibitor herbicides, in an area that had been sprayed with imidazolinones for more than seven years. Susceptible seed samples of *Bidens pilosa* and *Amaranthus quitensis* were collected from surrounding areas that had never been sprayed with ALS inhibitor herbicides. Seeds were planted in 200 mL plastic pots over mixture of soil and organic matter, 3:1 ratio. Uniform plants (4 plants per pot) having 2 to 4 pairs of leaves were selected and sprayed with the herbicides (Table 2). The herbicides were applied using a laboratory sprayer operating at 40 psi, with a 11002E tip and a spray volume of 300 L ha^{-1}. Weed control was evaluated 14 DAA, using a visual rating scale ranging from 0 to 100%, as described. Data were analyzed by analysis of variance and the means were compared by the Tukey test.

Rate-response curves

A third experiment was conducted at Piracicaba, SP, Brazil, using methodology described by Saari et al. (1992), with some modifications. R and S seeds of *B. pilosa* and *A. quitensis* were seeded in plastic pots as described previously. Plants were sprayed at the four-leaf stage, and treatments were multiples of the recommended rates of the herbicides chlorimuron-ethyl at 0; 3; 6; 30; 60; 300; 3000, and 6000 (x rate of 15 g ha^{-1}); imazethapyr at 0; 0.04; 0.4; 0.8; 4; 8; 40; and 80 (x rate of 80 g ha^{-1}), and nicosulfuron at 0; 0.0015; 0.03; 0.3; 3; 15, and 30 (x rate of 60 g ha^{-1}). The herbicides were sprayed using a laboratory sprayer and plants were visually rated 14 DAA, as described. Dose-response curves were constructed based on the log-logistic model for *B. pilosa* (Seefeldt et al., 1995); however, other non-linear models had a better fit for *A. quitensis*.

Growth analysis of R and S biotypes

Individual R and S plants of *B. pilosa* and *A. quitensis* were grown in 2-L plastic pots in the greenhouse. Starting 14 days after seeding, when plants were

Table 1 - Control of *Bidens pilosa* in the field experiment, 14 and 21 days after herbicide application (DAA).

Herbicide	Rates		Control (%)	
	g a.i.ha^{-1}	L ha^{-1}	14 DAA	21 DAA
Chlorimuron ethyl	20	0.08	42.5	21.6
Chlorimuron ethyl + lactofen	12.5 + 120	0.05 + 0.5	53.8	63.8
Chlorimuron ethyl + fomesafen	12.5 + 150	0.05 + 0.6	72.5	72.5
Chlorimuron ethyl + bentazon	12.5 + 384	0.05 + 0.8	47.5	67.8
Chlorimuron ethyl + imazethapyr	12.5 + 50	0.05 + 0.5	37.5	17.2
Lactofen	192	0.8	80.0	80.5
Fomesafen	225	0.9	82.6	85.3
Bentazon	480	1.0	66.7	72.6
Imazethapyr	100	1.0	0.3	0.0
Check	---	---	0.0	0.0
LSD (0.05)			10.80	11.13

a.i. = active ingredient

Table 2 - Percentage of control of R and S biotypes of *B. pilosa* and *A. quitensis* 14 days after herbicide application.

Herbicide	Rates	*B. pilosa*		*A. quitensis*	
	g a.i. ha^{-1}	R	S	R	S
Chlorimuron-ethyl	20	39.1	75.0	11.0	78.9
Chlorimuron-ethyl + fomesafen	20 + 250	78.6	83.5	90.0	74.1
Chlorimuron-ethyl + lactofen	20 + 192	86.7	90.0	83.5	90.0
Chlorimuron-ethyl + imazethapyr	20 + 100	31.3	76.1	18.1	68.5
Chlorimuron-ethyl + bentazon	20 + 480	84.3	84.3	63.8	65.4
Lactofen	192	90.0	86.7	85.5	82.1
Fomesafen	250	82.1	78.9	78.7	79.6
Bentazon	480	78.7	78.7	58.0	58.4
Check	---	0.0	0.0	0.0	0.0
LSD (0.05)		18.4	19.8	15.0	25.9

at the four-leaf stage, six replications of single plants of each biotype were harvested and the shoot dry biomass and leaf area were measured. Data were analyzed by the Richards asymptotic polynomial function (RF).

$$\ln W = \ln A - \frac{1}{N} \ln [1 \pm e^{-k(T-T0)}]$$

Parameter W is biomass yield; *A* represents the asymptotic maximum at an infinite time; N is related to the shape of the curve and determines the position of the inflection point; *K* is the rate at which *A* is approached, T is the time and T_0 estimates the time when the curve first rises above zero.

Bioassay testing ALS sensitivity to herbicides

This experiment was based on the methodology of Gerwick et al. (1993), with some modifications. Seeds of R and S biotypes of *B.pilosa* and *A.quitensis* were seeded in plastic trays and when plants had 3 to 4 pairs of true leaves, they were sprayed with a manual sprayer according to the treatments: 1,1-cyclopropanodicarboxylic acid (CPCA), chlorimuron-ethyl + CPCA, imazethapyr + CPCA, and check. The CPCA inhibits ketoacid reductoisomerase (KARI), and ALS is inhibited either by sulfonylurea or imidazolinone. These enzymes catalyze the reaction in the biosynthesis of the amino acids valine, leucine and isoleucine. If a resistant plant is sprayed with both ALS and KARI inhibitors, creatine is formed as a consequence of the accumulation of the formed acetolactate. On the other hand, a susceptible plant with sensitive ALS enzyme does not form creatine, since the ALS enzyme is inhibited.

During the biosynthesis of the branched chain amino acids, pyruvate is converted to acetolactate, in the presence of ALS and co-factors. This reaction is halted by the addition of H_2SO_4 which converts the acetolactate into acetoin, which in turn reacts with creatine and naphtol, producing a red complex.

DNA sequence that codifies the A domain region of the ALS of *A. quitensis*

The R and S biotypes of *A. quitensis* were isolated and induced to self-pollination. The F_1 seeds were seeded in plastic pots using the substrate Promix, side in a greenhouse. When plants had four pairs of true leaves, the meristematic portion of the plant was used for DNA extraction, based on the methodology described by Murray & Thompson (1980). ALS gene fragment amplification in the R and S biotypes of *A. quitensis* was made using primers obtained from known sequences in several plants: *Arabidopsis thaliana*, *Kochia scoparia* Stapf, *Nicotiana tabacum* L., *Xanthium* spp., and *Amaranthus rudis* L., analyzed through the NCBI site (http://www.ncbi.nlm.nih.gov). After the nucleotide homology analysis, the region close to the A Domain of the ALS gene was chosen for primer synthesis described as follows, and used to amplify the DNA segment sequencing with no restriction sites:

ALS P1

5'GT(A/C/G/T) TT (((C/T)GC(G/T)TA(C/T)CC(A/T)GG(A/C/T)GG(A/C/T)GC 3'

ALS P2

5'CC(A/T)CG(A/C/T)CA(A/T)GA(A/G)CA(A/G)GG(C/G/T)G 3'

ALS P3

5'GG(A/G/T)ATGCA(C/T)GG(G/T)AC(C/G/T)GT(G/T)TA(C/T) 3'

ALS P4

5'C(A/G)TA(A/C)AC(A/C/G)GT(A/C)CC(A/G)TGCAT(A/C/T)CC 3'

ALS P5

5'CCATC(A/C/G/T)CC(A/G)TC(A/G)AT(A/GA/G)C(A/C/T)AC(A/C/T)AC 3'

ALS P6

5'CAT(A/C/G)GGCA(A/G)(A/C)AC(A/G)TG(C/T)TC 3'

The following combinations of the following primers were tested: P1-P4 (348 pb), P2-P5 (612 pb), P2-P5 (780 pb), and P1-P5 (1227pb). The PCR reactions were prepared using 100 ng of the total DNA, 10 pmols of the primers, 0.5 unit of Taq DNA Polymerase (Promega 18038-018), 1.5 mmol L^{-1} $MgCl_2$ and 200 nmoles dNTPs and PCR buffer reaction. The PCR product (50 μL) was placed in 0.8% agarose gel for separation of PCR fragments according to size and net electric charge. The band size, corresponding to the combination of primers, was isolated and purified by the Qiaex Kit containing the DNA, and then ressuspended in 20 μL water.

For cloning of the PCR fragment of interest onto vector pUC18, a Sure Clone Ligation Kit (Pharmacia) was used. The reaction was prepared and maintained for 4 hours at 16°C, followed by the transformation of the DH5a line bacteria. The bacteria was colonized on agar + ampiciline medium. After colony growth, inoculation in LB + ampiciline medium was made. After plasmidial DNA isolation through a DNA miniprep, which contained the cloned fragment, it was sequenced using the reverse and forward primers. The obtained sequences were analyzed by the NCBI's BLAST software, to study the degree of homology with the ALS gene sequences of different organisms already sequenced and described in the gene bank.

RESULTS AND DISCUSSION

Field experiment

Neither chlorimuron-ethyl nor imazethapyr controlled *B. pilosa*, confirming the suspicion that it was a

herbicide resistant case for ALS inhibitor herbicides (Table 1). When ALS inhibitor herbicides were sprayed mixed with alternative herbicides such as fomesafen and lactofen (PROTOX inhibitors) and bentazon (PSII inhibitor - P_{680}), the weed control efficacy was excellent. When the mechanism of herbicide resistance is not multiple, mixing recommended herbicides with different modes of action is supposed to be an alternative for weed management (Powles & Holtum, 1994). This strategy can delay the manifestation of herbicide resistance, since the initial resistance frequency of the alleles for resistance is very low for each herbicide in the mixture, and when mixed, the probability of getting plants that are resistant to all herbicides in the mixture is even lower. Herbicide mixtures with different modes of action are important tools in preventing and managing self-pollinated plants, since cross-pollinated species that present individual alleles conferring resistance can cross among themselves and thus increase the probability that progenies will be resistant to both herbicides (Gould, 1995).

The alternative herbicides, when sprayed alone, effectively controlled the resistant population of *B. pilosa,* indicating the importance of avoiding the continued use, in the same area, of the same herbicides or herbicides with the same mechanisms of action. Herbicide and crop rotations are important agricultural practices that can be used by growers to prevent or manage herbicide resistance. Christoffoleti et al. (1994) stated that crop rotation allows the sequence of herbicides used to be changed, contributing to reduce the selection pressure in the agroecosystem.

Alternative herbicides

Chlorimuron-ethyl and imazethapyr, when sprayed alone or together, did not effective control R biotypes of *B. pilosa* and *A. quitensi*. However, a satisfactory control was achieved for the S biotypes (Table 2). According to Lorenzi (1994), the herbicides chlorimuron-ethyl and imazethapyr are recommended to control those weeds in early post-emergence in soybean fields.

The mixture of ALS inhibitor herbicides with the alternative herbicides bentazon, fomesafen, and lactofen, resulted in satisfactory control of both S and R biotypes (Table 2). In the same way, the alternative herbicides sprayed alone, were efficient against both biotypes of *B. pilosa* and *A. quitensis*; however, in spite of this, treatments lactofen, chlorimuron ethyl + lactofen, and chlorimuron-ethyl + fomesafen provided better control of *B. pilosa* and *A. quitensis*. According to Powles & Holtum (1994), when biotypes are resistant to only one class of herbicide, the results aimed by herbicide resistance management can be achieved by spraying alternative herbicides. The success of this strategy is, however, maintained only over a short interval, because if alternative herbicides are used very intensively, there is also risk of the development of herbicide resistance to these herbicides. Herbicide resistance cases have increased very rapidly and are not restricted to one or a few herbicide classes, but are associated to the repetitive use of herbicides with the same action mechanism, and to monoculture. Therefore, it is imperative to train growers and agricultural consultants on strategies that prevent or delay the manifestation of resistances, such as spraying alternative herbicides, mixing herbicide sequences with different mechanisms of action, and the rational use of herbicides, including rotation of herbicide action mechanisms.

Rate-response curves

The curve model segment that refers to the R biotype is significantly lower than the S biotype of *B. pilosa*, indicating lower control of R biotypes by the ALS inhibitor herbicides, even at rates higher than the recommended (Figure 1). The GR_{50} values clearly show the difference between the R and S biotypes of both weed species (Table 3). The R biotype demanded much higher rates than the recommended, for all three herbicides to attain 50% control. However, the S biotype had a much lower GR_{50}. For the R and S biotypes of A. *quitensis,* represented in Figure 2*,* the results were similar to *B. pilosa*. The curve model segment that refers to the R biotype is lower than the S biotype and the GR_{50} values show that the R biotype demanded much higher rates than the S biotypes.

The results of this experiment show cross-resistance of R and S biotypes of *B. pilosa* and *A. quitensis* to sulfonylurea and imidazolinone. Powles & Howat (1990) described that cross-resistance occur when an R biotype is resistant to different classes of herbicides, but with the same action mechanism. Cross-resistance to ALS inhibitor herbicides is caused by an ALS enzyme insensitive to herbicides of different chemical classes, but with the same mode of action. However, this experiment did not prove the enzyme to be insensitive.

Growth analysis of R and S biotypes

Growth analysis of shoot dry weight and leaf area of the R and S biotypes of *B. pilosa,* in a non-competi-

Table 3 - GR_{50} and resistance rate (R/S) of the R and S biotypes of *B. pilosa* and *A. quitensis*.

Herbicide	GR 50 - *B. pilosa*		R/S
	R	S	
Chlorimuron-ethyl	1.49	0.15	9.90
Nicosulfuron	1.27	0.14	9.07
Imazethapyr	20.08	0.74	27.03
	GR 50 - *A. quitensis*		R/S
	R	S	
Chlorimuron - ethyl	6.80	0.15	45.30
Nicosulfuron	23.54	0.13	181.08
Imazethapyr	2.45	0.10	24.55

tive situation, is represented in Figure 3. The R *B. pilosa* yielded more shoot dry weight relative to S biotype 21 days after planting (DAP); however, equation parameters A, N, K and T_o did not differ ($P < 0.05$) from the S biotype (Table 4). Leaf area parameters, on the other hand, differed between the two biotypes (Figure 3), indicating higher competitive ability of the S biotype.

The *A. quitensis* growth analysis indicated an accumulation of greater shoot dry weight biomass in the S biotype (Figure 4); however, S plants are leafier. The hypothesis that resistant plants are less adapted than susceptible plants in the absence of herbicide selection pressure is based on the fact that mutations that confer resistance imply in deleterious or pleiotropic effects, affect-

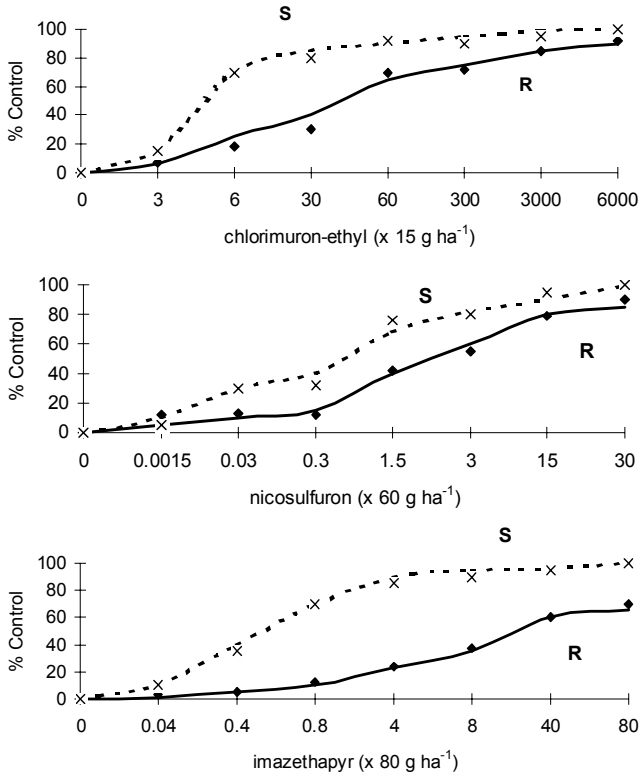

Figure 1 - Rate-response curves of R and S biotypes of *B. pilosa* to chlorimuron-ethyl, nicosulfuron and imazethapyr.

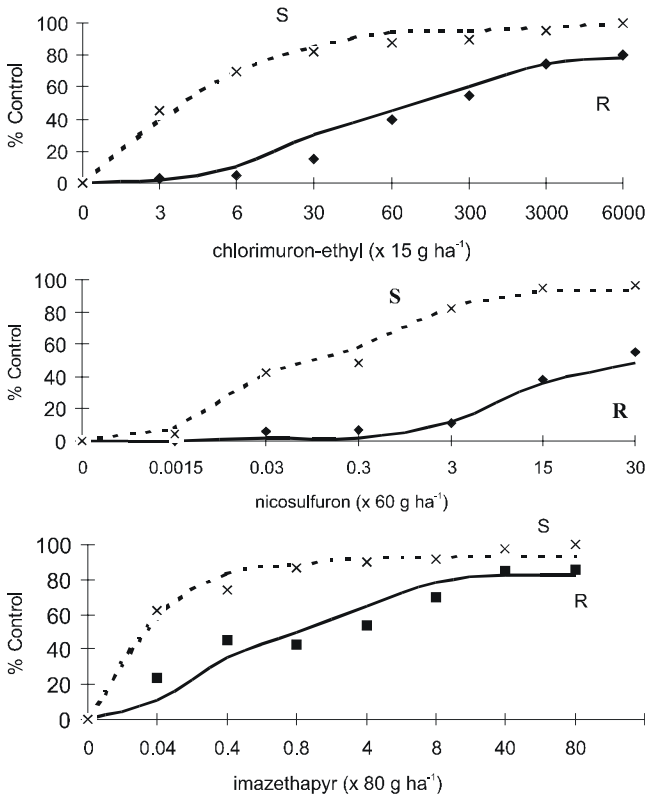

Figure 2 - Rate-response curves of R and S biotypes of *A.quintesis* to chlorimuron-ethyl, nicosulfuron and imazethapyr.

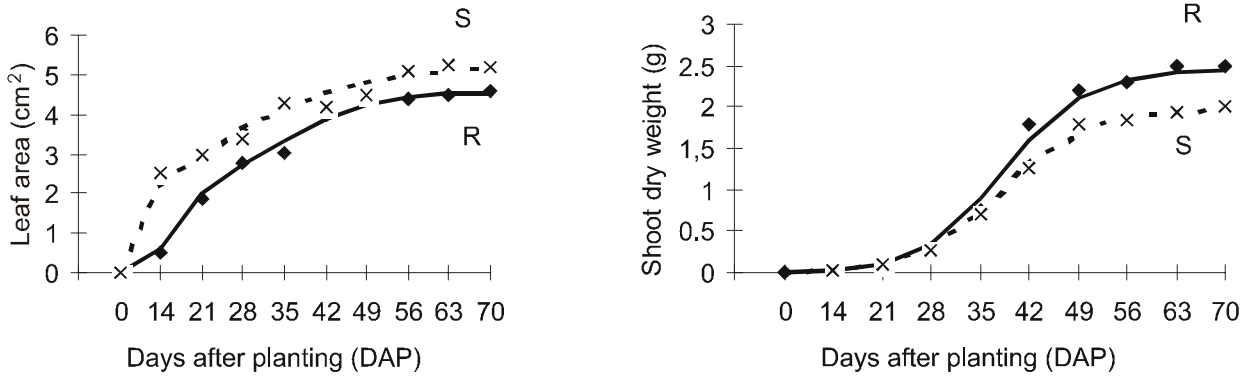

Figure 3 - Shoot dry weight and leaf area of R and S biotypes of *B. pilosa*.

Table 4 - Parameter estimate for Richards growth analysis function of R and S biotypes of *B. pilosa* and *A. quitensis*.

Parameter	*B. pilosa*				*A. quitensis*			
	Shoot dry weight		Leaf area		Shoot dry weight		Leaf area	
	R	S	R	S	R	S	R	S
A	1.80	1.98	95.95	201.42	2.32	3.02	175.22	201.00
N	0.60	0.80	1.46	0.46	0.82	1.26	1.09	0.46
K	0.10	0.16	0.15	0.07	0.08	0.15	0.13	0.07
To	26.89	36.18	38.67	30.13	51.5	47.12	40.0	30.13

ing survival and/or reproductive ability. However, little evidence of this phenomenon for the R biotypes of *B. pilosa* and *A. quitensis* was observed. Several reports have indicated that R biotypes resistant to ALS inhibitor herbicides are not necessarily less productive than S biotypes of the same weed species. Christoffoleti (1992) did not observe differences in the ecological adaptability of R and S kochia biotypes.

Gurjeet et al. (1996) did not find any differences in the ecological adaptability of R and S biotypes to herbicides, nor in seed germination speed and shoot dry weight biomass of R and S *Lolium rigidium* biotypes. Biotypes resistant to ALS inhibitor herbicides of certain weed species present high initial germination speed in relation to S biotypes (Dyer et al., 1993), as related to a higher content of the amino acids leucine and valine in the seeds of the R biotypes.

An exact estimation of the ecological adaptability of R and S biotypes in the absence of the herbicide is very difficult. However, it is very useful in prediction models, verify to if the initial S population can be reestablished in the absence of the herbicide application, since it is more adapted ecologically and thus can eliminate the R biotype through competition. The three major problems in determining ecological adaptability are: (1) good selection of the S biotype, (2) experimental conditions where the comparison is made, and (3) the parameters measured must provide good, comparable results, such as shoot dry biomass, leaf area, plant vigor, and seed viability (Christoffoleti, 1992).

Bioassay testing ALS sensitivity to herbicides

Acetoin accumulation by R and S *B. pilosa* and *A. quitensis*, as related to the effect of CPCA, is shown in Table 5. Chlorimuron-ethyl and imazethapyr did not inhibit the ALS R biotypes in any of the species. There was no accumulation of acetolactate in the solution. These results are confirmed by the spectrophotometer absorbance readings, as compared to the check.

Gerwick et al. (1993), using CPCA for KARI inhibition, clearly told R from S biotypes of *Xanthium strumarium* to the herbicide imazaquin. In the same way, Simpson et al. (1995) found acetolactate accumulation in the leaves of *Glycine max* (L) Merrill, *Zea mays* L., *Amaranthus hybridus* L., *Xanthium strumarium* L. and *Sorghum bicolor* (L) Moench, after foliar application of CPCA at 766 g a.i. ha^{-1}, and observed the accumulation of acetolactate in young soybean leaves. *In vitro* bioassay using ALS by differential accumulation of acetoin is a practical and efficient method of diagnosing resistance to ALS inhibitor herbicides.

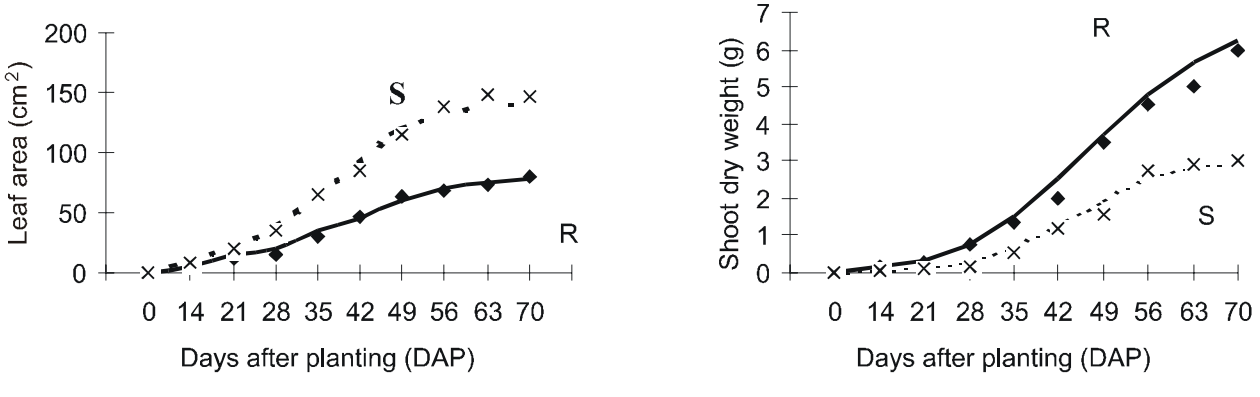

Figure 4 - Shoot dry weight and leaf area of R and S biotypes of *A. quitensis*.

Table 5 - Accumulation of acetolactate by the R and S biotypes of *B. pilosa* and *A. quitensis*.

Herbicide	Rates*	R		S	
		Reaction color	Acetoin	Reaction color	Acetoin
		B. pilosa			
CPCA	0.5	Red	2.09	Red	1.88
CPCA + chlorimuron-ethyl	0.50 + 0.20	Red	1.53	Brown	0.2
CPCA + imazethapyr	0.50 + 0.25	Pink	1.17	Brown	0.1
Check	0.0	Brown	0.0	Brown	0.0
		A. quitensis			
CPCA	0.5	Pink	1.63	Pink	0.72
CPCA + chlorimuron-ethyl	0.50 + 0.20	Pink	1.42	Brown	0.24
CPCA + imazethapyr	0.50 + 0.25	Pink	1.69	Brown	0.30
Check	0.0	Brown	0.0	Brown	0.0

*rates in mL or g 100 mL^{-1} H_2O

DNA sequence that codes for the A domain region of the ALS in *Amaranthus quitensis*

DNA segments were obtained through PCR from several *A. quitensis* plants. This is an *in vitro* method for sequencing specific DNA parts, by using two oligonucleotides (primers) that replicate the DNA using the enzyme polymerase mixed with dNTPs, at the region of interest of the DNA target. A repetitive series of 35 cycles involving denaturation at 94°C, primers ringing at 72°C and extension of these DNA polymerases at 55°C, resulted in an exponential accumulation of the specific fragment, whose separation success could be seen in agarose gel 0.8%. It was then possible to purify all fragments obtained with Qiaex and cloned with vector pUC18 (Sure Clone Kit) before sequencing. A higher frequency of amplification of the DNA segments was obtained by using the degenerated primers (ALS-P1 and P6). After several attempts, amplifications were obtained with the following primer combinations: P2-P4: *Amaranthus quitensis* R and S; P1-P5: *Amaranthus* q*uitensis* R and S; and, P1-P6: *Amaranthus quitensis* S.

The P2-P4 combination, with specific homology, was cloned in vector pUC18 (Sure Clone Kit). After cloning restriction-site SmaI segments of the pUC18 vector, transformed colonies were analyzed in the presence of the insertion fragment using restriction enzymes SstI and HindIII. When clones presenting a fragment of the corresponding PCR product size were obtained, the clone was sequenced. For *A. quitensis*, similarity with *A. rudis* already sequenced was verified through BLAST-N (similarity of nucleotides) (Altschul et al., 1997), using the information displayed on the NCBI web site (http://www.Ncbi.nlm.nih.gov). R and S *A. quitensis* showed alignment with the sequence of nucleotides of the ALS gene. *Amaranthus quitensis* had an E-value of 0.0 for *Amaranthus* sp. as expected, e-109 for *Kochia scoparia*, 7e-48 for *Arabidopsis thaliana*, 3e-28 for *Xanthium* sp., 2e-26 for *Brassica napus*, 3e-19 for *G. hirsutum*, and 1e-18 for *N. tabacum*.

DNA sequence in the A Domain:

A Domain

A. rudis	**{GCC**	**ATT**	**ACT**	**GGG**	**CAA**	**GTT**	**CCT**	**CGG**	**CGT**	**ATG**	**ATT**	**GGT**
S-A	-	-	-	-	-	-	CCT(C/T)	-	-	-	-	
R-A	-	-	-	-	-	-	-	-	-	-	-	
A. rudis	**ACT**	**GAT**	**GCT}**	TTT	CAA	GAG	ACA	CCT	ATA	GTT	GAG	
S-A	-	-	-	-	-	-	ACA(T)	CCT(A)	ATA(T)	-		
R-A	-	-	-	-	-	-	-	-	-	-		

Five plants of each *A. quitensis* biotype were sequenced. There was no change among the biotypes in the A domain region, where a mutation, in most cases, causes resistance to ALS inhibitor herbicides in weeds and cultivated plants. There were modifications in some of the regions, such as occurred with two plants of the R biotype in the 497 position, sequence TCA (Serine), instead of CCA (Proline). Regions other than the A domain must have suffered mutation, in addition to the mutation in the A domain region. Guttieri et al. (1995) compared one region consisting of 86 base pairs (bp) of the ALS gene among 10 biotypes of *Kochia scoparia* R to chlorsulfuron and three susceptible biotypes. They concluded that in seven of the resistant plants there was a point mutation in position 173 of the A domain, where proline was replaced by threonine, serine, arginine, leucine, glutamine, or alanine. The resistance mechanism was determined by the R biotypes that did not present mutation in the A domain. The resistance mechanism was determined for those R biotypes that did not present a point mutation in the A domain region. All biotypes presented resistance as a consequence of ALS insensitivity, what indicates that at least one mutation had occurred after the A domain region; similarly, R plants in the species *A. quitensis* showed an ALS that is insensitive to herbicide action, presuming that mutations had occurred in the non-A domain region.

The analysis of various plants resistant to ALS inhibitor herbicides, allowed several generalizations: substitutions by $Alanine_{114}$ or $Serine_{649}$ resulted in a high level of resistance to the imidazolinone group, but low or no resistance to sulfonylurea. Substitution of $Proline_{189}$ causes a high level of resistance to sulfonylurea and very low to imidazolinone; substitution of $Alanine_{197}$ causes moderate resistance to both herbicide groups, and substitution of $Triptofane_{570}$ causes a high degree of resistance to both groups (Wright & Penner, 1998).

The population of *B. pilosa* infesting soybean in São Gabriel do Oeste, MS - Brazil is resistant to ALS inhibitor herbicides, and so is *A. quitensis* from Argentina. These weed biotypes are cross-resistant to both sulfonylurea and imidazolinone herbicides. However, they do not express multiple resistance to the photosystem II inhibitor (bentazon) and to PROTOX inhibitor (lactofen and fomesafen). The resistance of the R biotypes of *B. pilosa* and *A. quitensis* to ALS inhibitor herbicides is related to insensitivity of the ALS to these herbicides; this is caused by an alteration in the DNA sequence of the R biotype; however, this alteration is very likely located outside of the A domain region of the DNA. No pleiotropic effects have occurred in the R resulting from the mutation that confers resistance, since the comparative growth analysis between R and S biotypes is not significantly different.

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REFERENCES

ALTSCHUL, S.F.; MADDEN, T.L.; SHCAEFFER, A.A.; ZHANG, J.; ZHANG, A.; MILLER, W.; LIPMAN, D.J. Grapped BLSAST and PSI-BLAST: a new generation of protein database search programs. **Nucleic Acids Research**, v.25, p.3389-3402, 1997.

BERNASCONI, P.; WOODWORTH, A.R.; ROSEN, B.A.; SUBRAMANIAN, M.V.; SIEHL, D.L. A naturally occurring point mutation confers broad range tolerance to herbicides that target acetolactate synthase. **Journal of Biological Chemistry**, v.270, p.17381-17385, 1995.

BOUTSALES, P.; KHARTOUM, J.; POWLES, S.B. Molecular basis of resistance of acetolactate synthase-inhibiting herbicides in *Sisymbrium orientale* and *Brassica tourneorti*. **Pesticide Science**, v.55, p.507-516, 1999.

CHRISTOFFOLETI, P.J. Growth competitive ability, and fitness of sulfonylurea resistant and susceptible *Kochia scoparia*. Fort Collins: Colorado State University, 1992. 198p. (Dissertation - Ph.D)

CHRISTOFFOLETI, P.J; VICTORIA FILHO, R.; SILVA, C.B. Resistência de plantas daninhas aos herbicidas. **Planta Daninha**, v.12, p.13-20, 1994.

CHRISTOFFOLETI, P.J; VICTORIA FILHO, R.; MONQUEIRO, P.A. Resistência cruzada e herbicidas alternativos de controle de biotipos de *B. pilosa* resistentes aos herbicidas inibidores da ALS. In: CONGRESSO BRASILEIRO DA CIÊNCIA DAS PLANTAS DANINHAS, 21.,Caxambú, 1997. **Abstract.** Caxambú: SBPD, 1997. p.64.

DYER, W.E.; CHEE, P.W.; FAY; P.K. Rapid germination of sulfonylurea resistant *Kochia scoparia* accessions is associated with elevated seed levels of branched chain aminoacids. **Weed Science**, v.41, p.18-22, 1993.

FOES, M.J.; LIU, L.; VIGUE, G.; STOLLER, E.W.; WAX, L.M.; TRANEL, P.J. A Kochia (*Kochia scoparia*) biotype resistant to triazine and ALS - inhibiting herbicides. **Weed Science**, v.47, p.20-27, 1999.

GERWICK, B.C.; MIRELES, L.C.; EILERS, R.J. Rapid diagnosis of ALS/AHAS inhibitor herbicide resistant weeds. **Weed Technology,** v.7, p.519-524, 1993.

GOULD, F. Comparisons between resistance management strategies for insects and weeds. **Weed Technology,** v. 9, p.830-839, 1995.

GURJEET, S.G.; COUSENS, R.D.; ALLAN, M.R. Germination, growth and development of herbicide resistant and susceptible populations of rigid ryegrass (*Lolium rigidum*). **Weed Science,** v.44, p.252-256, 1996.

GUTTIERI, M.J.; EBERLEIN; C.V.; THILL, D.C. Diverse mutations in the synthase gene confer chlorsulfuron resistance in kochia (*Kochia scoparia*) biotypes. **Weed Science**, v.43, p.175-178, 1995.

HEAP, I. International survey of herbicide resistant weeds - HRAC (Herbicide Resistance Action Committee). http://www.weedscience.com. 2002. (10/03/2003).

HOLT, J.; THILL, D.C. Growth and productivity of resistant plants. IN: POWLES, S.B.; HOTUM, J.A.M. (Ed.) **Herbicide resistance in plants:** biology and biochemistry. Boca Raton: Lewis Publishers, 1994. Chap.11, p.299-316.

LORENZI, H.J. **Plantas daninhas do Brasil**. 4.ed. Nova Odessa: Plantarum, 1994. 440p.

MAXWELL, B.; MORTIMER, A.M. Selection for herbicide resistance. IN: Powles, S.B.; HOTUM, J.A.M (Ed.) **Herbicide resistance in plants:** biology and biochemistry. Boca Raton: Lewis Publishers, 1994. chap.1, p.1-26.

MONQUERO, P.A. Biologia, manejo e caracterização bioquímica e genética de biótipos resistentes de plantas daninhas aos inibidores da acetolactato sintase. Piracicaba: USP/ESALQ, 1999. 94p. (Dissertação - Mestrado)

MURRAY, M.G.; THOMPSON, W.F. Rapid isolation of high molecular weight plant DNA. **Nucleic Acids Research,** v.8, p.4321-4325, 1980.

OTT, K.H.; KWAGH, J.G.; STOCKTON, G.W.; SIDORV, V.; KAKEFUDA, G. Rational molecular design and genetic engineering of herbicide resistant crops by structure modeling and site-directed mutagenesis of acetohydroxyacid synthase. **Journal of Molecular Biology,** v.263, p.359-368, 1996.

PONCHIO, J.A. Resistência de *Bidens pilosa* L. aos herbicidas inibidores da enzima acetolactato sintase. Piracicaba: USP/ESALQ, 1996. 138p. (Tese - Doutorado)

POWLES, S.B.; HOWAT, P.D. Herbicide resistance weeds in Australia. **Weed Technology,** v.4, p.178-185, 1990.

POWLES, S.; HOLTUM, J. **Herbicide resistance in plants:** biology and biochemistry. Boca Raton: Lewis Publishers, New York: CRC Press. 1994. 353p.

SAARI, L.L.; COTTERMAN, J.C.; SMITH, W.F.; PRIMIANI, M.M. Sulfonylurea resistance in common chickweed perennial ryegrass and russian thistle. **Pesticide Biochemistry and Physiology,** v.42, p.110-116, 1992.

SEEFELDT, S.S.; JENSEN, J.E.; FUERST, P. Log-logistic analysis of herbicide dose response relationships. **Weed Technology**, v.9, p.218-227, 1995.

SIMPSON, DM.; STOLLER, E.W.; WAX, L.M. An *in vivo* acetolactate synthase assay. **Weed Technology**, v.9, p.17-22, 1995.

WRIGHT, T.R.; PENNER, D. Classification of acetolactate synthase (ALS) resistance mutations. **Weed Science**, v.38, p.11–15, 1998.

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