PLANTA DANINHA
SOCIEDADE BRASILEIRA DA CIÊNCIA DAS PLANTAS DANINHAS
SBCPD
<http://www.sbcpd.org>
ISSN 0100-8358 (print)
1806-9681 (online)

Article

Enzymatic Properties and Ryegrass Resistance Mechanism to Iodosulfuron-Methyl-Sodium Herbicide

Propriedades Enzimáticas e Mecanismo de Resistência de Azevém ao Herbicida Iodosulfurom-Metílico Sódio

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ABSTRACT - Ryegrass (*Lolium multiflorum*) is one of the most worrisome species with regard to herbicide resistance. This study aimed at characterizing the acetolactate synthase (ALS) enzyme activity of resistant biotypes (NC and AR) and susceptible biotypes to iodosulfuron-methyl-sodium (iodosulfuron). Different concentrations of pyruvate (1, 10, 20, 40, 60, 80 and 100 mM), of iodosulfuron (0 to 100 µM for the resistant biotypes and 0 to 0.5 µM for the susceptible biotype) and concentrations of valine, leucine and isoleucine (0, 0.001, 0.01, 0.1, 1, 10 and 100 mM) were used for the in vitro characterization. According to the results, the Michaelis constant (K_m) values (pyruvate) for the assessed biotypes were similar. With iodosulfuron, resistant biotypes needed 395 to 779 times more herbicide in order to inhibit 50% of the enzyme activity, compared to the susceptible biotype. This confirmed a sensitivity modification of the ALS enzyme in the resistant biotypes. The resistant biotypes were less sensitive to the inhibition of the enzyme activity in the presence of the three amino acids. It was possible to conclude that biotype resistance was due to the enzyme insensitivity to iodosulfuron.

Keywords: acetolactate synthase (ALS), *Lolium multiflorum*, amino acids, sulfonylurea.

*RESUMO - O azevém (**Lolium multiflorum**) é uma das espécies mais preocupantes em relação à resistência aos herbicidas. Este estudo objetivou caracterizar a atividade da enzima acetolactato sintase (ALS) de biótipos resistentes (NC e AR) e suscetível de azevém ao herbicida iodosulfurom-metílico sódio (iodosulfurom). Diferentes concentrações de piruvato (1, 10, 20, 40, 60, 80 e 100 mM) e iodosulfurom (0 a 100 µM para biótipos resistentes e 0 a 0,5 µM para o biótipo suscetível) e concentrações de valina, leucina e isoleucina (0, 0,001, 0,01, 0,1, 1, 10 e 100 mM) foram utilizadas para a caracterização in vitro. De acordo com os resultados, os valores da constante de Michaelis (K_m) (piruvato) para os biótipos avaliados foram semelhantes. Na presença do iodosulfurom, os biótipos resistentes necessitaram de 395 e 779 vezes mais herbicida para inibir 50% da atividade da enzima, comparado com o biótipo suscetível. Isso confirmou uma modificação na sensibilidade da enzima ALS nos biótipos resistentes. Os biótipos resistentes foram pouco sensíveis à inibição da atividade da enzima na presença dos aminoácidos. Conclui-se que a resistência dos biótipos decorre da insensibilidade da enzima ao herbicida iodosulfurom.*

Palavras-chave: acetolactato sintase (ALS), *Lolium multiflorum*, aminoácidos, sulfonilureia

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Received: September 16, 2016
Approved: April 20, 2017

Planta Daninha 2019; v37:e019169383

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Doi: 10.1590/S0100-83582019370100001

INTRODUCTION

The first enzyme in the branched-chain amino acid biosynthesis is acetolactate synthase (ALS), also known as acetohydroxyacid synthase (AHAS). The enzyme catalyzes the condensation of two pyruvate molecules to form acetolactate (precursor of valine and leucine) and the condensation of pyruvate and ketobutyrate to form acetohydroxybutyrate (precursor of isoleucine) (Singh and Shaner, 1995; Duggleby et al., 2008). The activity of this enzyme is regulated by the final concentration of amino acids and it is sensitive to inhibition by ALS inhibiting herbicides, such as the sulfonylureas and imidazolinones chemical group (Chipman et al., 1998). There are more than 30 chemical substances of these herbicides registered to be used use worldwide (Mccourt et al., 2006). They are considered essential to control broad spectrum weeds in major agricultural crops, and their advantages involve low toxicity to animals and the fact that they are highly selective and require low application concentrations (Endo et al., 2013).

The evolution of weed resistance to these herbicides has become a concern worldwide, with the highest number of cases compared to other mechanisms of action (Heap, 2014). Among the species, there are ryegrass (*Lolium multiflorum*), an important winter weed, which has evolved to resist ALS inhibitors in several countries, such as Argentina, Brazil, Chile, Denmark, Italy and the United States (Heap, 2017).

Most cases of ALS inhibitor resistance occur due to the change in the action site of the herbicide (Tan et al., 2007, Yu et al., 2008, Délye et al., 2009, Kaloumenos et al., 2012, Kaundun et al., 2012). In this case, the substitution of one or more amino acids changes the conformation of the enzyme, preventing or limiting the binding of the herbicide (Duggleby et al., 2008). Most studies have shown that there is no change in the affinity (K_m) of the enzyme for the substrate in resistant biotypes, but they change the sensitivity of the ALS enzyme to inhibition by the feedback of the branched-chain amino acids, resulting in their accumulation (Eberlein et al., 1997; Preston et al., 2006; Ashigh and Tardif, 2007).

The catalytic activity of the ALS enzyme is regulated by the final concentration of biosynthesis products: the previously mentioned amino acids (Duggleby et al., 2008). However, the sensitivity of the enzyme in the feedback regulation may be influenced by mutations that occur in plants that are resistant to the inhibitor (Duggleby and Pang, 2000). Insensitivity by inhibition through the feedback of some or all three amino acids can be affected by the type of substitution that gives resistance (Eberlein et al., 1997). As a consequence, this change in the amino acid concentration may alter the metabolism during its plant life cycle (Ashigh and Tardif, 2007), as well as representing a cost of adaptation, which keeps mutant alleles at low frequencies in untreated populations (Preston et al., 2006).

The quantification of pleiotropic effects on resistant weeds is necessary for the strategic control of these species (Menchari et al., 2008). *Lolium rigidum* biotypes, each with a specific mutation in the enzyme, present plant growth and enzyme functionality with few changes, and these, when they occur, depend on the type of substitution involved in the resistance (Yu et al., 2010). However, the main form of ryegrass control remains the chemical one, with the rapid evolution of resistance. Since there is no prediction of the launch of new mechanisms of action as an alternative control method, the management of resistant plants, as well as the understanding of their resistance mechanism, is important to prevent and/or decelerate the evolution of resistance.

The hypotheses of this research are that *Lolium multiflorum* biotypes that are resistant to iodosulfuron do not differ from the susceptible biotype as for the kinetic characteristics of ALS, and that the mechanism of resistance comes from the insensitivity of the enzyme to the herbicide. Thus, the objective of this work was to characterize the activity of the acetolactate synthase enzyme (ALS) of ryegrass biotypes that are resistant (NC and AR) and susceptible to iodosulfuron-methyl sodium.

MATERIAL AND METHODS

Plant material

The experiments were conducted at the Weed Science/Herbicide Chemistry of Soil and Crop Science Department laboratory, at the Texas A&M University - U.S. Seeds of resistant

ryegrass biotypes from North Carolina (NC) and Arkansas (AR) were used, together with one susceptible (S) to iodosulfuron methyl sodium. Seeds were sown on Fafard 2 MIX® substrate in 2 L capacity pots. Plants were grown in a growth chamber under controlled conditions with 12 hours of photoperiod, temperature of 22/18 °C and 250 µmol photons m^{-2} s^{-1} of light intensity. Plants were irrigated manually, whenever necessary. Each pot contained 12 plants, which, having reached the 2 to 3 leaf stage, were collected and immediately placed in liquid nitrogen, to proceed with the enzymatic test.

Enzyme extraction

The enzymatic extraction method followed the proposed methodology (Singh et al., 1988), with modifications. The weight ratio of the sample (plants) in relation to the used extraction buffer was 1/10 (w/v); different ratios were previously tested. Each sample of plant material was composed of several plants, enough to obtain the desired weight. Plants that were frozen in liquid nitrogen were macerated in a mortar until reaching a fine powder and were homogenized with a 100 mM potassium phosphate extraction buffer, pH 7.5, at 4 °C. The buffer was prepared with: 0.5 mM magnesium chloride ($MgCl_2$); 10 mM sodium pyruvate; 0.5 mM thiamine pyrophosphate (TPP); 10 µM flavin adenine dinucleotide (FAD); 10% v/v glycerol; 1 mM dithiothreitol; and 5% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was kept under stirring for 20 minutes at 4 °C and then filtered in two layers of gauze in order to remove and dispose of solid wastes. It was then centrifuged at 5,800 *xg* for 80 minutes at 4 °C. After centrifugation, the supernatant was removed, and the solid residue was discarded.

***In vitro* bioassay with ALS enzyme**

The experiments were conducted in a completely randomized experimental design with three replications. For *in vitro* bioassays, reactions were prepared in test tubes, where each tube received 200 µL of the enzyme solution, and the reaction buffer had different substrate concentrations (experiment I), different concentrations of the herbicide (experiment II) and different concentrations of amino acids (experiment III), both for a final reaction volume of 1 mL. To the zero treatment (control treatment), 50 µL of H_2S0_4 3 M solution were added, in order to prevent enzyme activity. The absorbance values of the control treatment were used to discount the reading values of the others.

The potassium phosphate buffer (75 mM, pH 7.5) for the reaction was prepared, and the following components were added: 20 mM $MgCl_2$; 100 mM sodium pyruvate; 2 mM thiamine pyrophosphate (TPP); and 20 µM flavin and adenine dinucleotide (FAD), according to methodology proposed by Yu et al. (2010), with modifications.

After preparing the reaction, the samples were incubated for 60 minutes at 35 °C for the formation of acetolactate. The reaction was stopped by adding 50 mL of the H_2S0_4 3 M solution into each test tube, except for the zero control, which contained the acid before the incubation. The second incubation was carried out for 15 minutes at 60 °C to allow the formation of acetoin from the reaction of sulphuric acid with the acetolactate formed during the first reaction. The following step was performed to form the colored complex, where 1 mL of creatine solution (0.5% w/v) and 1 mL of α-naphtol (5% w/v), prepared in NaOH 2.5 M. After mixing, the samples were incubated for 15 more minutes at 60 °C for color development. The tubes were then cooled to room temperature, and the absorbance was read in a spectrophotometer (Beckman Coulter, Model DU-520) at 530 nm.

Values referring to the activity of the ALS enzyme were standardized by protein concentration, quantified through the Bradford method, and presented by the amount of produced acetoin (µmol mg^{-1} h^{-1}) determined by the acetoin standard curve.

Experiment I. determinations of K_m and V_{max}

The kinetic parameters of the enzyme, such as the Michaelis constant (K_m) (substrate concentration at which the rate of the enzymatic reaction is half of the maximum reaction rate)

and the maximum reaction rate (V_{max}), were used to describe the affinity of the enzyme with the substrate. In order to obtain K_m and V_{max}, tests were performed with different concentrations of the substrate. In this case, pyruvate was not used in the extraction. Seven final pyruvate concentrations were used in the reaction: 1, 10, 20, 40, 60, 80 and 100 mM for both biotypes (resistant and susceptible). K_m and V_{max} values were initially determined from the Michaelis-Menten equation ($p \leq 0.05$) (Nelson and Cox, 2006), as follows:

$$y = V_{max} * [S] / K_m + [S]$$

where y = activity of the ALS enzyme (μmol mg^{-1} h^{-1}); V_{max} = maximum reaction rate; S = substrate concentration (pyruvate); and K_m = substrate concentration (pyruvate) which provides an initial rate equal to half the maximum reaction rate. To better represent the collected data, the Michaelis-Menten equation was linearized, resulting in the Lineweaver-Burke or double reciprocal plot, with the interception point between the straight line and the ordinate axis equivalent to $1/V_{max}$, and the interception point between the line and the abscissa axis, equivalent to $-1/K_m$, represented by the equation:

$$1/V = K_m + [S]/V_{max*}[S] = K_m/V_{max} * 1/[S] + 1/V_{max}$$

where V = reaction speed; K_m = Michaelis-Menten constant; V_{max} = maximum reaction rate; and [S] = concentration of the substrate (pyruvate). Data were submitted to analysis of variance, using the statistical program SAS 9.3, and the means were compared by the Tukey's test at 5% probability.

Experiment II. *in vitro* test of the ALS enzyme with herbicide

The reaction procedures were identical to those described in the section "*in vitro*" bioassay with ALS enzyme. From the 1,000 μM stock solution of the iodosulfuron-methyl herbicide, dilutions were made at the desired working concentrations. One hundred μl of the herbicidal solution were added to each vial at a final concentration of 0, 0.01, 0.1, 0.25, 0.5, 1, 10 and 100 μM for resistant biotypes (AR and NC), and of 0, 0.0001, 0.001, 0.0025, 0.005, 0.1, 0.025 and 0.05 μM for susceptible biotype. The bioassays had two standard treatments without herbicide, represented by 0% and 100% of activity, in which the former received 50 μL of 3 M sulphuric acid solution at the beginning of the bioassay, and the latter consisted of the standard treatment, corresponding to the control treatment without the inhibiting herbicide.

Absorbance values were corrected by subtracting the zero control value. The obtained values were used to calculate the I_{50}, which represents the inhibitor amount required to inhibit 50% of the enzyme activity, using the non-linear logistic regression model (Seefeldt et al., 1995). The resistance factor (RF) was also determined; it was calculated by dividing the I_{50} of the resistant biotype by its correspondent in the susceptible biotype. The RF expresses the number of times the dose required to inhibit 50% of the enzyme activity in the resistant biotype is greater than the dose inhibiting 50% of the susceptible biotype (Hall et al., 1998).

Experiment III. *in vitro* test of the ALS enzyme with amino acids

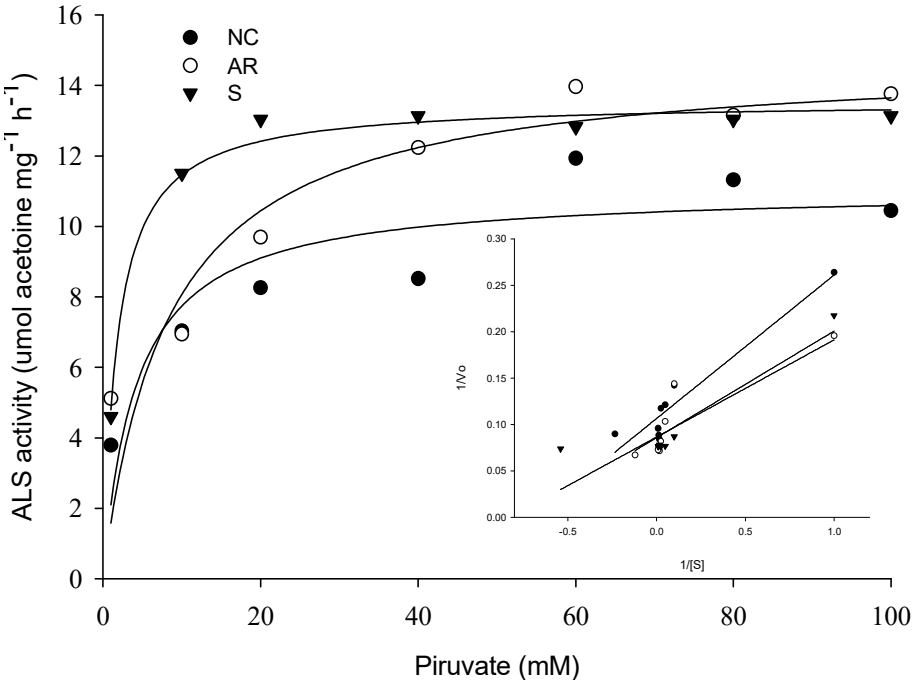
In order to evaluate the inhibition of the enzyme through amino acid feedback, the activity analysis was performed with each amino acid (valine, leucine and isoleucine). The procedures were identical to those previously mentioned in "*in vitro*" bioassay with ALS, and each tube received different concentrations of valine, leucine or isoleucine. The final concentrations of the respective amino acid in each tube were 0.001, 0.01, 0.1, 1, 10 and 100 mM. The data obtained by the treatment with each amino acid were submitted to statistical analysis separately, and were presented as percentage in relation to the treatment without the respective amino acid.

RESULTS AND DISCUSSION

K_m and V_{max}

K_m (pyruvate) values for the evaluated biotypes were similar to each other (Figure 1 and Table 1). When resistance was associated with the change in the action site of the herbicide, K_m

may or may not represent a change in the functionality of the enzyme, resulting in unchanged, insufficient or excessive biosynthesis product (Vila-Aiub et al., 2009). The magnitude of the alteration depends on the type of mutation in the enzyme. Certain altered amino acids may cause adverse impacts on substrate binding and thus alter the functionality of the ALS enzyme (Yu et al., 2010). As for the V_{max} parameter of the enzyme, the AR and NC biotype values were similar to those of the susceptible biotype (Figure 1 and Table 1). These results discard the hypothesis of probable resistance by the overexpression mechanism of the target enzyme.



Texas A&M University, U.S., 2012.

Figure 1 - Activity of the enzyme acetolactate synthase (ALS) of resistant (NC and AR) and susceptible (S) ryegrass biotypes to the iodosulfuron methyl sodium herbicide, expressed as acetoin production, according to different concentrations of substrate (pyruvate) and inverse of activity and concentration values, representing the reciprocal double.

Table 1 - K_m (concentration of pyruvate giving the initial rate equal to half of the maximum reaction rate) and V_{max} (maximum reaction rate) of resistant (NC and AR) and susceptible (S) ryegrass biotypes to the iodosulfuron-methyl sodium herbicide

Biotype	K_m (mM)	V_{max} (µmol mg^{-1} h^{-1})
S	1.20	11.62
AR	1.35	11.76
NC	1.45	9.43

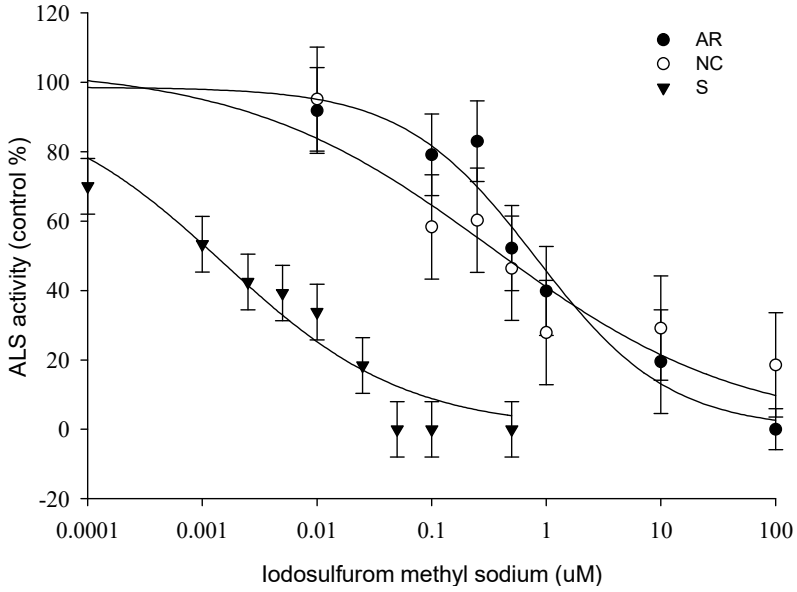
Texas A&M University, U.S., 2012.

Sulphonylureas and imidazolinones obstruct the substrate binding to the active site of the enzyme. Any change in the active site prevents herbicide binding, maintaining enzyme compliance and its functionality (Mccourt et al., 2006). The occurrence of changes in the ecology and physiology of plants occurs in a few types of mutations, but they are less likely to occur (Yu et al., 2010; Li et al., 2013). This also explains the rapid evolution of plants that are resistant to ALS inhibitors, since small changes are required for the resistance to herbicides without, therefore, representing an adverse effect on plants (Yu et al., 2010).

In vitro assay of the ALS enzyme with iodosulfuron

In vitro assays with the ALS enzyme, conducted to evaluate the enzymatic activity, demonstrated that a 0.001 µM dose of iodosulfuron is required to inhibit 50% of the enzyme

activity (I_{50}) in the susceptible biotype (Figure 2). Compared to resistant biotypes, doses were 0.779 and 0.395 µM for RA and NC, respectively, resulting in a ratio of 779 and 395 fold higher compared to the susceptible biotype (Table 2). These results confirm the altered sensitivity of the ALS enzyme in resistant biotypes.



Texas A&M University, U.S., 2013.

Dots represent the average values and the vertical bars represent the confidence intervals.

Figure 2 - *In vitro* inhibition of the activity of the acetolactate synthase ALS enzyme in a susceptible biotype (S) and two resistant biotypes (NC and RA) by iodosulfuron-methyl sodium.

Table 2 - Logistic sigmoidal equation, determination coefficient (R^2), iodosulfuron methyl sodium dose that inhibits 50% of the activity of the acetolactate synthase (ALS) enzyme (I_{50}) and ratio between resistant and susceptible (R/S) biotypes *in vitro*, in resistant and susceptible ryegrass biotypes (NC and AR) to iodosulfuron methyl sodium, submitted to different doses of the herbicide

Biotype	Equation	R^2	I_{50} (µM)	R/S
S	$y=95.82/[1+(x/0.0015)^{0.54}]$	0.94	0.001	-
AR	$y=98.56/[1+(x/0.81)^{0.75}]$	0.95	0.779	779
NC	$y=104.33/[1+(x/0.33)^{0.39}]$	0.88	0.395	395

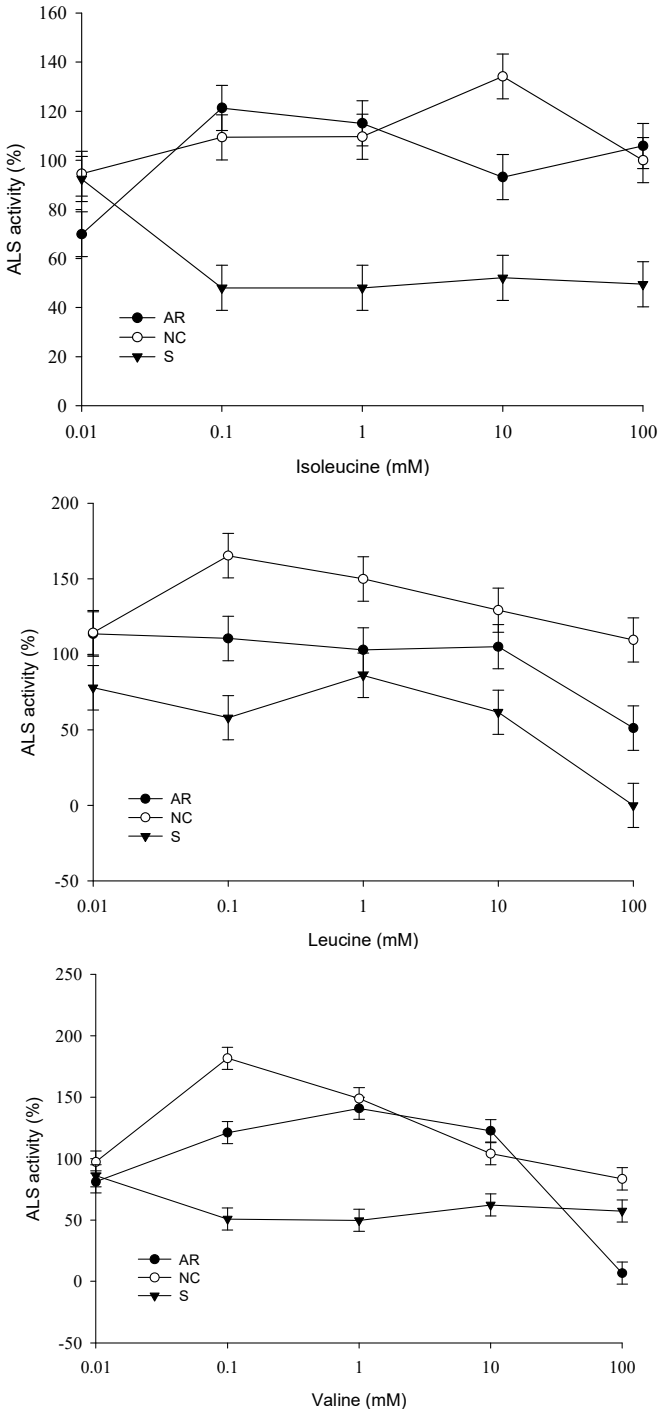
Texas A&M University, U.S., 2013.

The application of iodosulfuron on plants from the tested biotypes, grown in a greenhouse, highlighted the need for doses of more than 200 times, compared to the susceptible biotype, to reduce 50% of the dry matter (Mariani, 2014). When populations of *Lolium rigidum*, isolated with different types of amino acid substitutions, were evaluated as for enzyme activity, it was possible to observe relations as for the I_{50} between resistant (R) and susceptible (S), ranging from 95 to >1333 times, depending on the location and the altered amino acid type (Yu et al., 2010).

In vitro assay of the ALS enzyme with amino acids

There was an interaction between biotypes and concentration of amino acids; however, data about the three amino acids did not fit the mathematical model. Resistant biotypes (NC and AR) show low sensitivity to the inhibition of the enzyme activity in the presence of amino acids. In the presence of isoleucine (100 mM), the activity for resistant biotypes was higher than the one of the control treatment without amino acids, with a 31% inhibition at the concentration of

0.01 mM for the AR biotype (Figure 3). In the presence of leucine (100 mM), the AR biotype decreased the activity by 50% and the reduction behavior for the NC biotype was similar when in the presence of isoleucine, with a higher enzymatic activity than the control treatment (Figure 3). The highest activity inhibition was observed with valine (100 mM), with a 94% decrease in RA activity and 17% for NC (Figure 3). The susceptible biotype decreased activity by 50% at the concentration of 0.1 mM of the three amino acids, reaching 100% with leucine (100 mM).



Texas A&M University, U.S., 2013.

Dots represent the average values and the vertical bars represent the confidence intervals.

Figure 3 - Activity of the acetolactate synthase (ALS) enzyme with different concentrations of the amino acids isoleucine, leucine and valine in relation to the control treatment without amino acids, in resistant (NC and AR) and susceptible (S) ryegrass biotypes to iodosulfuron methyl sodium.

When there is a change in the structure of the enzyme, imbalance in amino acid production may occur (Yu et al., 2010). For resistant biotypes, higher concentration of amino acids may be required for a total inhibition. These results confirm that there was an alteration in the conformation of the enzyme in resistant biotypes, since they have a lower sensitivity to the amino acid feedback inhibition, compared to the susceptible biotype. The differential response of biotypes to feedback inhibition may be due to the specific alteration that caused the resistance, and, as a consequence of the lower sensitivity to inhibition, the accumulation of amino acids occurred (Tanaka, 2003). The activity of the resistant biotype of *Lactuca serriola*, with the substitution of proline 197 for histidine in the A domain, is less sensitive to the inhibition by valine, leucine and isoleucine, compared to the susceptible biotype (Eberlein et al., 1997).

The ALS enzyme is divided into two subunits: a catalytic one and a regulatory one. The regulatory subunit stimulates the activity of the catalytic subunit and gives sensitivity to the inhibition by branched-chain amino acid feedback (Duggleby et al., 2008). In bacteria, valine is the most powerful inhibitor; in plants, leucine acts synergistically with valine or isoleucine (Duggleby et al., 2008). In *Arabidopsis thaliana*, the ALS enzyme is inhibited with leucine and valine concentration of 336 and 231 mM, respectively, but the inhibition decreases to 12.3 mM when both are used together (Lee and Duggleby, 2001).

There are two separate regulatory sites: one for leucine and one for valine and isoleucine. In bacteria, when the subunits were separately tested, the catalytic subunit was insensitive to valine inhibition, whereas when combined with the regulatory subunit, activity was stimulated 7 to 10 times, making it susceptible to inhibition (Pang and Duggleby, 2001). In plants, enzyme inhibition by amino acids generally occurs in the order of potency leucine>valine>isoleucine (Pang and Duggleby, 2001). In a study with rice plants with alteration in the catalytic subunit of the ALS enzyme, a high degree of resistance and also resistance to the inhibition of the enzyme by amino acid feedback was observed (Endo et al., 2013); the result was possibly due to the mutation in the subunit catalytic activity, which changes the conformation of the enzyme and inhibits the binding of the amino acids in the regulatory subunit.

The high resistance degree of the biotypes to the iodosulfuron methyl sodium herbicide, demonstrated in the tests, advises about being careful with resistance management; it is probable that the increase in the doses used on the field will not be effective in controlling the respective biotypes. In addition, studies with ryegrass that are resistant to ALS inhibitors have shown that the resistance gene has a dominant characteristic, transmitted by both the male and female parent (Mariani et al., 2015), helping the rate of resistance dissemination when measures that decrease the selection pressure are not adopted. Studies investigating the molecular bases of ryegrass resistance, comparing the sequenced ALS gene of resistant and susceptible biotypes, will be useful and complementary to help understanding the evolution of resistance.

It is possible to conclude that the resistance of *Lolium multiflorum* biotypes (NC and AR) comes from the insensitivity of the enzyme to the iodosulfuron methyl sodium herbicide, with no changes in the affinity parameters of the enzyme with the substrate, with little sensitivity to the inhibition by the feedback of the amino acids valine, leucine and isoleucine.

ACKNOWLEDGEMENTS

To CNPq, for granting the scholarship to carry out the sandwich doctorate. To the Department of Soil and Crop Science - Texas A&M University, for the infrastructure and the support to conduct the experiments during the sandwich doctorate.

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