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Properties of the enzyme acetolactate synthase in herbicide resistant canola

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ABSTRACT: Mutations in the gene that encodes the enzyme acetolactate synthase (ALS), conferring plant resistance to ALS inhibitor herbicides, can reduce or inhibit enzymatic function. The objective of this work was to determine the functionality and kinetic characteristics of the enzyme ALS and evaluate the action of the herbicides imazapic (imidazolinone) and metsulfuron-methyl (sulfonylurea) on the activity of the enzyme ALS in canola hybrid Hyola 571CL (imidazolinone resistant), compared to hybrids Hyola 555TT (triazine resistant) and Hyola 61 (conventional). The plants were grown in a greenhouse, and leaves were collected to perform ALS activity analysis. The concentration of pyruvate to provide initial velocity equal to half the maximum reaction rate (K_m) and the maximum reaction rate (V_{max}) was determined, as well as the enzyme's activity in the presence of imazapic and metsulfuron-methyl inhibitors. There was no change in the K_m and V_{max} in the hybrid Hyola 571CL in relation to Hyola 61. The hybrid Hyola 555TT presented V_{max} higher than the other hybrids. In the hybrid Hyola 571CL, the activity of ALS was reduced only by the application of metsulfuron-methyl, and the functionality of the enzyme did not change in relation to the other hybrids. The hybrid Hyola 571CL is resistant only to the imidazolinone group herbicide and this resistance does not cause adaptive cost in relation to the other hybrids.

Key words: *Brassica napus* L., enzyme functionality, metsulfuron-methyl, imazapic.

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INTRODUCTION

The enzyme acetolactate synthase (ALS) is present in plants on the biosynthesis route of branched-chain amino acids, catalyzing the condensation of two molecules of pyruvate to form acetolactate (precursor of valine and leucine) and the condensation of pyruvate and α-ketobutyrate, forming acetohydroxybutyrate (precursor of isoleucine) (Chipman et al. 1998; Endo et al. 2013). In susceptible plants, ALS enzyme-inhibiting herbicides bind to a domain that crosses the entrance of the catalytic site, preventing the substrate from connecting to it, interrupting the synthesis of amino acids (McCourt et al. 2006).

Imidazolinone-resistant canola cultivars were developed from two mutants, called PM1 and PM2, which were obtained by induced mutagenesis of microspores (Krato et al. 2012). The PM1 mutant, with a serine to asparagine substitution at codon 653, is resistant to imidazolinones only, and PM2, with a codon 574 modification, from tryptophan to leucine, is resistant to imidazolinones and sulfonylureas (Krato et al. 2012).

The mutation in ALS genes, which causes resistance to ALS inhibitor herbicides through lower affinity of the enzyme with the herbicide, may lead to loss or reduction of enzyme function. Studies on the activity of the ALS enzyme in different concentrations of the substrate (pyruvate) provide important information about the kinetic characteristics of the enzyme (Dal Magro et al. 2010). When the mutation causes damage to the enzyme, it may represent an adaptive cost to the plants. In addition, tests with different concentrations of herbicides on the enzyme ALS allow us to calculate the amount of herbicide necessary to inhibit 50% of enzyme activity (I_{50}) and to adjust enzyme inhibition curves by the herbicide.

In Brazil, there are two canola hybrids resistant to imidazolinones registered (MAPA 2016) and one commercial product registered for use on these hybrids, which has imazamox as it's active ingredient (MAPA 2017). Imazamox is one of the imidazolinone compounds which can be used in imidazolinone resistant crops, along with imazapyr, imazapic, and imazethapyr (Tan et al. 2005). The registration of these imadozolinone resistant hybrids enables canola cultivation in areas infested with dicotyledonous weeds, since it allows the selective post emergence control of the culture. This will contribute to the rotation of winter crops and of herbicide action mechanisms, reducing the selection pressure of resistant weed biotypes.

This study had as an objective to determine if there is a difference in the functionality and kinetic characteristics of the enzyme ALS in canola of the hybrid Hyola 571CL (resistant to imidazolinones), compared to the other hybrids, Hyola 555TT (resistant to triazines) and Hyola 61 (conventional). The study also aimed to evaluate the action of the herbicides imazapic and metsulfuron-methyl, belonging to the group of imidazolinones and sulfonylureas, respectively, on the activity of the enzyme ALS in canola of hybrid Hyola 571CL, compared to hybrids Hyola 555TT and Hyola 61.

MATERIAL AND METHODS

Seeds of Hyola 571CL canola (resistant to herbicides of the imidazolinone group), Hyola 555TT (resistant to herbicides of the triazine group) and Hyola 61 (without herbicide resistance) were used. In Brazil, the hybrids Hyola 571CL and Hyola 61 are registered (MAPA 2016) and the hybrid Hyola 555TT is undergoing experimentation.

The sowing was carried out in pots with a capacity of 2 dm^3, containing commercial peat substrate (Plantas Garden Plus Turfa Fértil) and, after thinning, four canola plants were maintained per pot. The pots were kept in a greenhouse during the whole experiment, being irrigated manually. At 37 and 64 days after emergence, canola leaves were collected for ALS activity assays at different concentrations of the pyruvate substrate and at different concentrations of herbicides, respectively. The difference in age of trials plants is due to their separation in time, since the plants were sown on the same date.

For leaves collection, the pots containing canola plants were taken to a laboratory and leaves were collected randomly from the four plants in each pot, until the required amount to carry out the proposed assays was obtained. Leaves were cut and chopped using scissors, removing the main vein, in order to increase the amount of protein extracted from each sample. Immediately after, the chopped fresh tissue was placed in a mortar, and liquid nitrogen was added to facilitate the maceration process.

Extraction of the ALS enzyme

The enzymatic extraction method proposed by Singh et al. (1988) was used, with modifications. The canola leaves were macerated with liquid nitrogen in a mortar until a fine →

powder was obtained. Homogenization was achieved by a potassium phosphate extraction buffer (100 mM, pH 7.5) at 4 °C, in the proportion of 1:10 m/v (1 g of macerated fresh tissue : 10 mL of extraction buffer). For the preparation of the buffer, 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) were used.

The solution was kept under stirring for 20 minutes at 4 °C and then filtered through two layers of gauze to discard the solid waste. Subsequently, centrifugation was performed at 5800 xg for 80 minutes at 4 °C. The supernatant removal was carried out and the solid residue was discarded.

Bioassay *in vitro* with the ALS enzyme

In the bioassay with the enzyme ALS, the experimental design was completely randomized, with three technical replicates, in which each test tube per treatment was considered as a repetition. In test tubes, reactions were prepared so that each tube received 500 µL of enzyme solution and the reaction buffer with different concentrations of the pyruvate substrate (0; 0.5; 2.0; 4.0; 8.0; 16.0; 32.0; 64.0; and 100.0 mM) or with different concentrations of the metsulfuron-methyl (0.01; 0.10; 0.50; 5.0; 10.0; 50.0; and 500.0 µM) or imazapic (0.001; 0.50; 1.0; 5.0; 10.0; 50.0; and 100 µM) herbicides. These concentrations of pyruvate and herbicides were based on another study, which evaluated the ALS enzyme activity of weed biotypes (Dal Magro et al. 2010).

The final reaction volume in each test tube was 1 mL. To make up the control sample, 50 µL of the 3 M sulfuric acid solution (H_2SO_4) was added in order to prevent enzyme activity. The absorbance was read in a spectrophotometer (Femto, Model 800 XI) at 530 nm and the control values were used to discount values of the readings in the other treatments.

For the reaction, it was used 80 mM potassium phosphate buffer, pH 7.0, by the addition of 20 mM $MgCl_2$; 200 mM sodium pyruvate; 2 mM TPP; 20 µM FAD, according to methodology proposed by Yu et al. (2010), with modifications. After preparation of the reaction, the samples were incubated for 60 minutes at 35 °C for the formation of acetolactate. The reaction was then stopped by the addition of 50 µL of 3 M H_2SO_4 solution in the test tubes, except for the control, which already contained the acid. The second incubation for 15 minutes at 60 °C was performed for the formation of acetoin by the reaction of the sulfuric acid with the acetolactate previously formed. 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 2.5 M NaOH, were added. After mixing, the samples were again incubated for 15 minutes at 60 °C for color development. The tubes were then cooled to room temperature and the absorbance was read.

ALS activity values were standardized by the concentration of bovine serum albumin (BSA), quantified by the Bradford method (Bradford 1976), and expressed by the amount of acetoin produced per minute of incubation per milligram of protein (enzyme) (µmol·min^{-1}·mg^{-1} protein), determined by the standard curve of acetoin (data not shown).

Activity of the ALS enzyme as a function of pyruvate concentrations

Analyzes were performed containing concentrations of the pyruvate substrate in order to obtain the kinetic parameters of Michaelis constant (K_m), which represent the concentration of the substrate at which the rate of the enzymatic reaction is half the maximum reaction rate, and the maximum reaction rate (V_{max}), which inform the affinity of the enzyme for the substrate. In this case, pyruvate was not used in enzyme extraction.

The experiment was conducted using a completely randomized design, with three replications, in a 3×9 two-factor scheme (three hybrids x nine pyruvate concentrations). The following final concentrations of pyruvate were used: 0; 0.5; 2.0; 4.0; 8.0; 16.0; 32.0; 64.0; and 100.0 mM, for all hybrids. The values of K_m and V_{max} were initially determined from the Michaelis Menten equation ($p \leq 0.05$) (Nelson and Cox 2014), as shown in Eq. 1:

$$y = V_{max} \cdot [S] / (K_m + [S]) \quad (1)$$

where y = activity of the enzyme ALS (µmol·mg^{-1}·h^{-1}); V_{max} = maximum rate of reaction; S = substrate concentration (pyruvate); K_m = substrate concentration (pyruvate), which provides initial velocity equal to half the maximum reaction rate.

To better represent the data collected, the Michaelis Menten equation was linearized resulting in the Lineweaver Burk or double reciprocal plot, the intercept point between →

the straight line and the ordinate axis being $1/V_{max}$, and the intercept point between the straight line and the abscissa axis equivalent to $-1/K_m$, represented by Eq. 2:

$$1 / V = (K_m + [S]) / V_{max} * [S] = K_m / V_{max} * 1 / [S] + 1 / V_{max} \quad (2)$$

where V = is the reaction rate, K_m = is the Michaelis Menten constant, V_{max} = is the maximum rate of the reaction and [S] = the concentration of the substrate (pyruvate).

Activity of the ALS enzyme as a function of herbicide concentrations

Two experiments were carried out. The first one consisted of the use of the herbicide imazapic [2-((RS)-4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-5- methylnicotinic acid] and the second of the use of metsulfuron-methyl [methyl 2-(4-methoxy-6-methyl-1,3,5-triazin-2-ylcarbamoylsulfamoyl) benzoate] herbicide. The sulfonylurea herbicide was used to confirm that imidazolinone resistant canola does not have cross-resistance to this chemical group and to be a positive control in the enzymatic assay, inhibiting ALS enzyme activity. The herbicides used were those suitable for use in laboratory analysis, with purity of approximately 99% and without inert ingredients.

Both consisted of a 3×7 two-factor scheme (three hybrids x seven concentrations of herbicide) in the completely randomized design, with three replications. For the herbicide imazapic, the final concentrations of 0.001; 0.50; 1.0; 5.0; 10.0; 50.0; and 100 µM were used and for metsulfuron-methyl herbicide, the final concentrations were 0.01; 0.10; 0.50; 5.0; 10.0; 50.0; and 500.0 µM.

For the assay, the reaction procedures mentioned above were followed. To prepare the concentrations of the herbicides imazapic and metsulfuron-methyl, a stock solution of 1000 µM was used, and the dilution was carried out at the desired concentrations. In each test tube, 100 µL of the herbicidal solution was added to the final concentration of 0 to 100 µM for the herbicide imazapic and 0 to 500 µM for the metsulfuron-methyl herbicide.

The assays had two standard treatments without herbicide, called zero (0) and 100% enzymatic activity, in which the first one received 50 µL of 3 M sulfuric acid at the beginning and the second one consisted of the standard treatment, without addition of an inhibitor. The absorbance values were corrected, subtracting from them the value of the zero standard treatment (with 0% of enzymatic activity), and the results were expressed as a percentage of the 100% standard treatment (with 100% of enzymatic activity). The amount of herbicide required to inhibit 50% of enzyme activity (I_{50}) was determined, according to the equations generated for each hybrid.

The data were submitted to analysis of variance ($p \leq 0.05$) by the statistical program CoStat (Costa and Castoldi 2009), in a two-factor scheme (hybrids x concentrations of pyruvate or herbicide), and curves were adjusted for the enzymatic activity of ALS in relation to the concentrations of pyruvate or the herbicides imazapic and metsulfuron-methyl when there was significance.

RESULTS AND DISCUSSION

Activity of the ALS enzyme as a function of pyruvate concentrations

The enzymatic activity at the different concentrations of the pyruvate substrate was similar between the Hyola 571CL and Hyola 61 hybrids, according to the Michaelis Menten equation (Fig. 1). At higher concentrations (≥ 8.0 mM), greater enzymatic activity was observed for the hybrid Hyola 555TT, and in the concentration of 100 mM, the average enzyme activity was 33.3 and 34.5% higher than the values found for the hybrids Hyola 61 and Hyola 571CL, respectively.

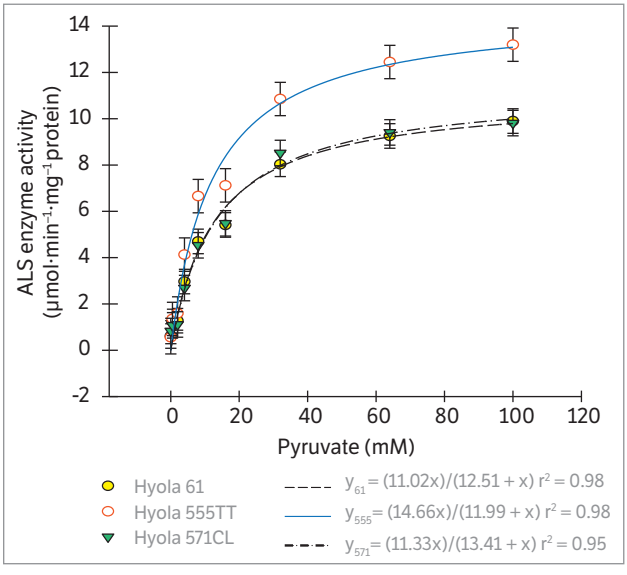

Figure 1. Activity of the enzyme acetolactate synthase (ALS) in the different concentrations of the pyruvate substrate in Hyola 571CL (571), Hyola 61 (61) and Hyola 555TT (555) canola hybrids. Passo Fundo, RS, 2016. Vertical bars represent the standard error of the mean.

Due to the difficulty in the experimental determination of V_0 in high concentrations of pyruvate, that is, in the extrapolation of the hyperbolic curve to obtain an exact value of V_{max}, by means of the Michaelis Menten equation, linear transformations such as Lineweaver Burk are commonly used (Wilson and Walker 2010). The Lineweaver Burk graph, as seen in Fig. 2, allows us to identify two specific values, $1/V_{max}$, when the line intercepts the *y*-axis ($x = 0$), and $-1/K_m$, when the line intercepts the *x*-axis ($y = 0$). Since this graph represents the inverse of the values, the line that intersects the *y*-axis at a lower point than the others, indicating that its V_{max} is larger, making it easier to interpret the results, as well the more accurate determination of its value in this type of graph.

Based on Fig. 2, it is observed that the values of $1/V_{max}$ and $-1/K_m$ are similar for the Hyola 571CL and Hyola 61 hybrids. The hybrid Hyola 555TT presents greater difference between the hybrids, mainly with respect to the value of $1/V_{max}$ and, therefore, V_{max}.

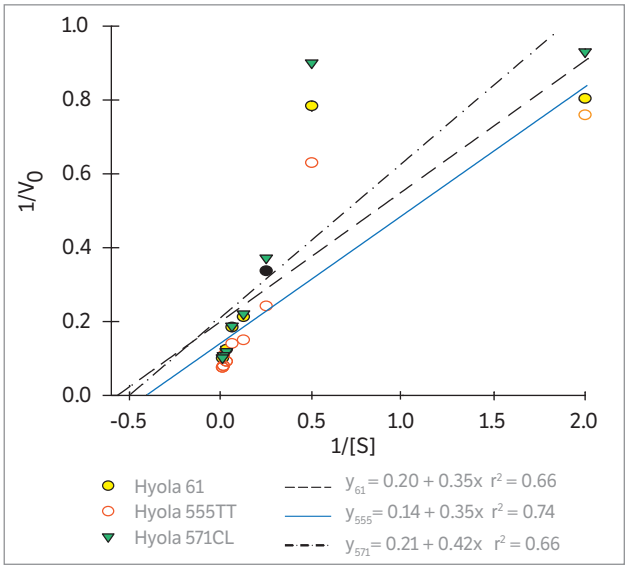

Figure 2. Lineweaver Burk graph (double reciprocal) with the inverse values of the acetolactate synthase (ALS) enzyme activity ($1/V_0$) and of the pyruvate concentration (1/[S]) in Hyola 571CL (571), Hyola 61 (61) and Hyola 555TT (555) canola hybrids. Passo Fundo, RS, 2016.

The K_m parameter was similar among all canola hybrids analysed, ranging from 11.99 mM for Hyola 555TT to 13.41 mM for Hyola 571CL, according to the Michaelis Menten equation, and from 1.77 to 2.45, according to Lineweaver Burk's equation (Table 1). If the mutation conferring resistance to the herbicide, associated with the change in the target site, causes a change in the functionality of the enzyme (lower enzyme activity, lower affinity to the substrate or inhibition by altered feedback) there may be a cost associated with the resistance, which results in insufficient or excessive biosynthesis of the product (Vila-Aiub et al. 2009). This phenomenon, however, was not observed.

Herbicides that inhibit ALS do not compete with substrate (pyruvate), as they do not connect to the catalytic site of the enzyme. Furthermore, several mutations for resistance do not modify the functionality of the enzyme (Powles and Yu 2010). The active site of the ALS is formed at the interface of two monomers in a deep channel in the crystallographic structure, and the imidazolinones and sulfonylureas herbicides do not connect directly to the active site but close to it, inhibiting enzyme activity by blocking substrate access to it (Yu and Powles 2014).

Both the Michaelis Menten equation and the Lineweaver Burk equation show a higher V_{max} value for the Hyola 555TT hybrid than the Hyola 571CL and Hyola 61 hybrids, representing values 29.4 and 33.0% higher when using the equation of Michaelis Menten and 48.8 and 41.4% higher when using the Lineweaver Burk equation, respectively (Table 1).

The maximum rate of reaction is reached when all enzyme molecules are in the form of the enzyme+substrate complex and the free enzyme concentration is small (Nelson and Cox 2014). There is no absolute value of V_{max} and its value depends on the amount of enzyme used for its experimental determination (Wilson and Walker 2010). The amount of

Table 1. Michaelis constant (K_m) and maximum reaction rate (V_{max}) of the enzyme acetolactate synthase (ALS) of Hyola 555TT, Hyola 571CL and Hyola 61 canola hybrids according to the equations of Michaelis Menten and Lineweaver Burk. Passo Fundo, RS, 2016.

Hybrid	Michaelis Menten equation		Lineweaver Burk equation	
	K_m (mM)	V_{max} (umol·mg^{-1}·min^{-1})	K_m	V_{max}
Hyola 555TT	11.99 (± 1.20*)	14.66 (± 0.44)	2.45 (± 0.14)	7.07 (± 0.14)
Hyola 571CL	13.41 (± 2.01)	11.33 (± 0.53)	2.00 (± 0.20)	4.75 (± 0.20)
Hyola 61	12.51 (± 1.24)	11.02 (± 0.33)	1.77 (± 0.17)	5.00 (± 0.17)

*The values in parentheses represent the standard error of the mean

enzyme present in the experiments conducted in the present work was estimated by the amount of protein present in the samples. As a result, the hybrid Hyola 555TT may have presented higher amount of enzyme in the sample, resulting in a higher value of V_{max} in relation to the other hybrids.

Activity of the ALS enzyme as a function of herbicide concentrations

For the herbicide imazapic, logistic sigmoidal curves were adjusted for the activity of the ALS enzyme in relation to the concentration of the herbicide for hybrids Hyola 555TT and Hyola 61 (Fig. 3). According to regression analysis, no curve was significant for the hybrid Hyola 571CL. The enzyme activity values, using the herbicide imazapic, did not differ between the concentrations for the hybrid Hyola 571CL, as observed by means of the standard deviation of the measurements (data not shown).

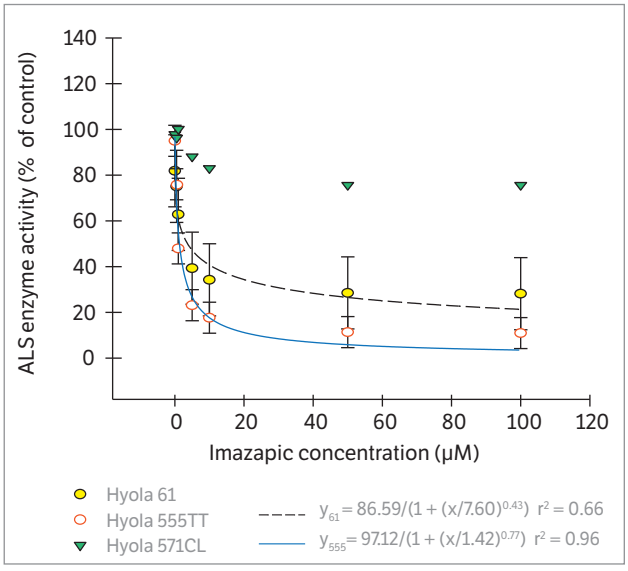

Figure 3. *In vitro* inhibition of the activity of the enzyme acetolactate synthase (ALS) in Hyola 555TT (555), Hyola 571CL (571) and Hyola 61 (61) canola hybrids by the imazapic herbicide. Passo Fundo, RS, 2014.Vertical bars represent the standard error of the mean.

The hybrid Hyola 555TT showed a greater reduction in the activity of the enzyme ALS in high concentrations of the herbicide imazapic, reaching the value of 3.5% in the enzymatic activity (reduction of 96.5%), while Hyola 61 presented a value of 21.5% (reduction of 78.5%) in relation to the standard control, without herbicide. However, the Hyola 555TT and Hyola 61 hybrids did not differ in the concentrations evaluated, as it can be observed by the mean standard error represented in Fig. 3.

Exponential decrease curves were adjusted for the ALS activity data in relation to the metsulfuron-methyl herbicide concentration (Fig. 4). At the lower concentrations, there was a drastic reduction in the activity of the enzyme with the increase of the concentration of the metsulfuron-methyl herbicide for the evaluated hybrids.

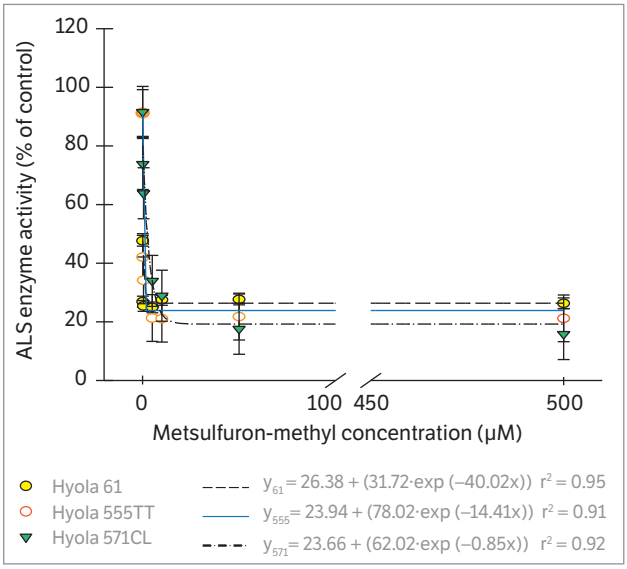

Figure 4. *In vitro* inhibition of the activity of the enzyme acetolactate synthase (ALS) in Hyola 555TT (555), Hyola 571CL (571) and Hyola 61 (61) canola hybrids by the metsulfuron-methyl herbicide. Passo Fundo, RS, 2014. Vertical bars represent the standard error of the mean.

At the highest concentration (500 µM) of metsulfuron-methyl, enzyme activity values were close to 25% for all hybrids, and the reduction in enzyme activity was 76.3; 73.6; and 76.1% for hybrids Hyola 571CL, Hyola 61 and Hyola 555TT, respectively. In this concentration, the values did not differ among the evaluated hybrids, as observed by the standard error of the mean.

The I_{50} for the herbicide imazapic was 1.32 µM for the hybrid Hyola 555TT and 3.70 µM for the Hyola 61, and these values correspond to the herbicide dose required to inhibit 50% of the activity of the ALS enzyme (Table 2). For the highest tested concentration, 100.0 µM, the hybrid Hyola 571CL showed enzymatic activity 6.9 times higher than Hyola 555TT and 2.7 times higher than Hyola 61 (data not shown). For metsulfuron-methyl herbicide, the I_{50} was 1.01 µM for the hybrid Hyola 571CL, 0.08 µM for Hyola 555TT and 0.01 µM for Hyola 61 (Table 2).

Based on the results obtained in the present work, the resistance in the Hyola 571CL hybrid occurs only to herbicides of the imidazolinones group, possibly associated to the mutation at the codon 653, from serine to asparagine. Replacement of serine at codon 653 for asparagine does not modify the connection of →

Table 2. Adjusted equations for the activity of the enzyme acetolactate synthase (ALS) of Hyola 555TT, Hyola 571CL and Hyola 61 canola hybrids as a function of concentrations of the herbicides imazapic and metsulfuron-methyl, coefficient of determination (r^2), and amount of herbicide required to inhibit 50% of enzyme activity (I_{50}), in an *in vitro* assay. Passo Fundo, RS, 2014.

Herbicide	Hybrid	Equation	r^2	I_{50} (µM)
Imazapic	Hyola 555TT	y = 97.12/(1 + (x/1.42)^0.77)	0.96	1.32
	Hyola 571CL	no equation adjusted	-	-
	Hyola 61	y = 86.59/(1 + (x/7.60)^0.43)	0.66	3.70
Metsulfuron-methyl	Hyola 555TT	y= 23.94 + (78.02·exp(-14.41x))	0.91	0.08
	Hyola 571CL	y = 23.66 + (62.02·exp(-0.85x))	0.92	1.01
	Hyola 61	y = 26.38 + (31.72·exp(-40.02x))	0.95	0.01

the sulfonylureas in the enzyme since the side chain remains correctly oriented, however, it prevents the connection of imidazolinone herbicides since it obstructs the space in which the aromatic ring is located (McCourt et al. 2006).

Most studies on plant resistance through mutation in the ALS gene demonstrated no adaptive cost associated with resistance (Yu and Powles 2014). The absence of a significant adaptive cost by ALS resistant alleles at the whole plant level is consistent with the absence of an adaptive cost at the enzyme level, which is expected because the interaction between ALS and inhibitor occurs at a distance from the catalytic center of the enzyme (Yu and Powles 2014).

The results of inexistence of adaptive cost in hybrid Hyola 571CL in relation to the other hybrids studied is fundamental to subsidize the choice of canola hybrids for cultivation. Thus, in a field with dicotyledonous weed infestation, the hybrid with imidazolinones resistance can be used without impairing the growth and development of the plants due to genetic mutation for resistance.

CONCLUSION

The Hyola 571CL canola hybrid does not present alteration in functionality of the ALS enzyme in relation to hybrids Hyola 555TT and Hyola 61.

The herbicide imazapic does not reduce the activity of the ALS enzyme in the Hyola 571CL canola hybrid.

The activity of the ALS enzyme is reduced by the application of the metsulfuron-methyl herbicide in the Hyola 571CL, Hyola 555TT and Hyola 61 canola hybrids.

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