Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9275
ISSN on-line: 1807-8621
Doi: 10.4025/actasciagron.v39i3.32521

Mutation of Trp-574-Leu ALS gene confers resistance of radish biotypes to iodosulfuron and imazethapyr herbicides

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ABSTRACT. Acetolactate synthase inhibitors are the main group of herbicides used in winter crops in Southern Brazil where their intensive use has selected for herbicide-resistant biotypes of radish. The resistance affects the efficacy of herbicides, and identifying the resistance mechanism involved is important for defining management strategies. The aim of this study was to elucidate the resistance mechanism of radish biotypes by quantifying the enzyme activity, ALS gene sequencing and evaluating the response of biotypes to iodosulfuron and imazethapyr herbicide application after treatment with a cytochrome P_{450} monooxygenase inhibitor. The susceptible (B_1) and resistant (B_4 and B_{13}) biotypes were from wheat fields in the Northwest of Rio Grande do Sul State. The results demonstrated that the enzyme affinity for the substrate (K_M) was not affected in biotypes B_4 and B_{13} but that the V_{max} of the resistant biotypes was higher than that of biotype B_1. The resistant biotypes showed no differential metabolic response to iodosulfuron and imazethapyr herbicides when inhibited by malathion and piperonyl butoxide. However, gene sequencing of ALS showed a mutation at position 574, with an amino acid substitution of tryptophan for leucine (Trp-574-Leu) in resistant biotypes.

Keywords: *Raphanus sativus*, mechanism of resistence, ALS enzyme activity, gene mutation, metabolism.

Mutação da Trp-574-Leu do gene ALS confere resistência de biótipos de nabo ao herbicida iodosulfurom e imazetapir

RESUMO. Os inibidores da acetolactato sintase são o principal grupo de herbicidas usados em culturas de inverno do Sul do Brasil onde seu uso intenso selecionou biótipos resistentes de nabo. A resistência afeta a eficácia dos herbicidas, e a identificação do mecanismo de resistência envolvido é importante na definição de estratégias de manejo. O objetivo deste estudo foi elucidar o mecanismo de resistência em biótipos de nabo através da quantificação da atividade da enzima, sequenciamento do gene ALS e avaliar a resposta dos biótipos à aplicação do herbicida iodosulfurom e imazetapir após tratamento com inibidores do metabolismo da citocromo P_{450} monooxigenase. Os biótipos suscetível (B_1) e resistentes (B_4 e B_{13}) eram de lavouras de trigo da região Noroeste do Estado do Rio Grande do Sul. Os resultados demonstraram que a afinidade da enzima pelo substrato (K_M) não foi afetada nos biótipos B_4 e B_{13}, porém o V_{max} dos biótipos resistentes foi superior quando comparado ao biótipo B_1. Os biótipos resistentes não apresentaram resposta metabólica diferencial ao herbicida iodosulfurom e imazetapir quando inibidos pelo malathion e butóxido de piperolina. Entranto, o sequenciamento do gene ALS evidenciou mutação na posição 574 com uma substituição do aminoácido triptofano por leucina (Trp-574-Leu) nos biótipos resistentes.

Palavras-chave: *Raphanus sativus*, mecanismo de resistência, atividade da enzima ALS, mutação gênica, metabolismo.

Introduction

Raphanus sativus L. (radish) is a dicotyledonous weed species that is found in wheat, barley and canola fields of southern Brazil, where it causes yield reduction (Rigoli, Agostinetto, Schaedler, Dal Magro, & Tironi, 2008). Acetolactate synthase (ALS) is the most common enzyme in the branched-chain amino acid biosynthetic pathway and produces leucine, isoleucine and valine (McCourt & Duggleby, 2006). ALS inhibitor herbicides are essential for a variety of crops due to their selectivity, low effective dosage, reduced toxicity to animals and high potential for inhibiting the ALS enzyme (Yu, Han, & Vila-Aiub, 2010; Endo, Shimizu, Fujimori,

Yanagisawa, & Toki, 2013). Iodosulfuron and imazethapyr are the main herbicides used in wheat and canola where their intensive use has favored the selection of herbicide-resistant radish biotypes (Pandolfo, Presotto, Poverene, & Cantamutto, 2013).

The survival of biotypes can occur due to factors that may be related to the herbicide target site or non-target-site (Yuan, Tranel, & Stewart, 2007). The occurrence of DNA mutations in the gene sequence and the overexpression of the ALS enzyme are possible factors resulting in reduced sensitivity to the herbicide due to insufficient or excessive levels of biosynthetic product (Duggleby, McCourt, & Guddat, 2008; Han et al., 2012). Mutations in the ALS gene can affect enzyme structure and function, thereby reducing the enzyme activity and herbicide affinity with the target site (Han et al., 2012). In *Raphanus raphanistrum* L., ALS gene mutations affecting the efficacy of ALS inhibitor herbicides were identified for proline (Pro_{197}), aspartate (Asp_{376}), tryptophan (Trp_{574}) and alanine (Ala_{122}); (Tan & Medd, 2002; Yu, Hashem, Walsh, & Powles, 2003; Yu, Han, Purba, Walsh, & Powles, 2012; Han et al., 2012).

Non-target-site herbicide resistance mechanisms can occur due to increased metabolism and compartmentalization, a reduction in absorption or the differential translocation of the herbicide molecule (Powles & Yu, 2010; Délye, Jasieniuk, & Le Corre, 2013). These mechanisms of resistance are also characterized by higher rates of herbicide detoxification due to increased glutathione-s-transferase, cytochrome P_{450} monooxygenase or glycosyltransferase activities (Délye et al., 2013). Nevertheless, both mechanisms affect herbicide efficacy and should be evaluated due to the possibility of their coexistence (Ahmad-Hamdani et al., 2013, Brosnan et al., 2016).

Therefore, elucidating the resistance mechanism in radish biotypes is important for determining alternative weed management strategies and for reducing the herbicide-resistance selective pressure. The aim of this study was to elucidate the resistance mechanism of radish biotypes by quantifying ALS enzyme activity, ALS gene sequencing and evaluating the response of biotypes to iodosulfuron and imazethapyr herbicide application after treatment with a cytochrome P_{450} monooxygenase inhibitor.

Material and methods

Seeds from radish plants that survived application of iodosulfuron herbicide were collected in wheat fields in the Northwest region of Rio Grande do Sul State. To screen for resistant biotypes, plants grown from these seeds were sprayed with 5 g a.i. ha^{-1} of iodosulfuron and 106 g. i.a. ha^{-1} of imazethapyr; and dose response studies were then carried out for biotypes B_1, B_4 and B_{13}. The B_4 and B_{13} biotypes were from Três de Maio and Boa Vista do Cadeado, Rio Grande do Sul State municipalities, respectively, and demonstrated cross resistance to these herbicides and a high level of resistance to the iodosulfuron herbicide. Biotype B_1, from Três de Maio, Rio Grande do Sul State, was susceptible to the iodosulfuron and imazethapyr herbicides (Cechin et al., 2016).

ALS enzyme activity and in vitro assays with the herbicides

The enzymatic extraction method was adapted from methods described by Singh, Stidham, and Shaner (1988). Seven grams of young plant leaves was collected, frozen in liquid nitrogen (N_2) and ground to a fine powder. Then, 70 mL (1:10 p/v) of 100 mM phosphate extraction buffer (pH = 7.5) containing 0.5 mM magnesium chloride ($MgCl_2$), 10 mM sodium pyruvate, 0.5 mM thiamine pyrophosphate (TPP), 10 μM flavin adenine dinucleotide (FAD), 10% glycerol, 1 mM dithiothreitol and 5% polyvinylpolypyrrolidone (PVPP) was added. The material was homogenized for 20 minutes at 4°C, and the mixture was filtered to remove solid sediments. The liquid portion was centrifuged at 12,000 rpm for 20 minutes, the supernatant was collected and the solid residue was discarded.

The methodology used for the *in vitro* biossay with the herbicide was adapted from the method of Gerwick (Gerwick, Mireles, & Eilers, 1993). The assay was performed in test tubes using three replicates with a factorial treatment design, where factor A was the different biotypes (B_1, B_4 and B_{13}) and factor B consisted of different concentrations of iodosulfuron or imazethapyr (zero, 0.001, 0.01, 0.1, 1.0, 10, 100, and 1,000 μM). Each tube received 600 μL of enzyme solution, 100 μL of herbicide solution and 300 μL of 80 μM phosphate reaction buffer (pH = 7.0) containing 20 mM de magnesium chloride, 200 mM sodium piruvate, 2 mM thiamine pyrophosphate and 20 μM flavin adenine dinucleotide. The assay had two standard treatments without herbicide to measure zero and 100% enzyme activity. The zero activity standard received 50 µL sulfuric acid (H_2SO_4 - 3 M) at the start of assay to prevent enzyme activity, and the 100% activity standard received 100 µL milli-Q water instead of the herbicide solution. The absorbance values for the zero activity control were subtracted from the

values read in other treatments. After preparation of the reaction, samples were incubated for 60 minutes at 30°C. The reactions were stopped with 50 μL of 3 M sulfuric acid for all treatments other than the zero activity control treatment, where the reaction had been stopped initially. Next, the tubes were incubated for 15 minutes at 60°C to create acetoin from the reaction of sulfuric acid with acetolactate. Then, 0.5% creatin (1,000 µL) and 0.5% 1-naphtol (1,000 µL) were prepared in 2.5 M sodium hydroxide (NaOH) and added to produce a colored complex. The final reaction was incubated for 15 minutes at 60°C, and the absorbance at 530 nm was read in a spectrophotometer. The ALS enzyme activity (µM acetoin $min.^{-1}$ mL^{-1}) was determined by the amount of acetoin produced. For the standard curve, three repetitions were used; each tube receveid 1000 µL with different concentrations of solution acetoin (0, 10, 20, 40, 60, 80, 100, 200, and 400 µM). The colored complex was obtained by adding creatine, 1-naphtol and NaOH and incubated as described above.

The kinetic parameters of enzyme activity (K_M and V_{max}) were obtained using ten pyruvate concentrations (zero, 0.5, 1, 2, 4, 8, 16, 32, 64, and 100 mM). The substrate concentrations (pyruvate) were obtained by diluting phosphate reaction buffer (80 μM, pH = 7.0) with 100 mM pyruvate solution. The buffer contained 20 mM magnesium chloride, 2 mM thiamine pyrophosphate and 20 μM flavin adenine dinucleotide. The values of K_M and V_{max} were determined using the Michaelis-Menten equation: $y = V_{max} \star X / K_M + X$ (Nelson & Cox, 2008), where y = ALS enzyme activity (μmol $min.^{-1}$ mL^{-1}); V_{max} = maximum reaction velocity; X = substrate concentration (pyruvate); and K_M = substrate concentration, where the initial velocity is equal to half of the maximum reaction velocity. The data obtained were analyzed for normality (Shapiro-Wilk test) and submitted to analysis of variance ($p \leq 0.05$), where the K_M and V_{max} values of biotypes were compared by a Duncan Test ($p \leq 0.05$).

The absorbance values were corrected with the zero standard, and I_{50} (amount of herbicide to inhibit 50% enzyme activity) was calculated using the logistic non-linear regression model: $y = a / [1 + (x / x_{I50})]^b$ (Seefeldt, Jensen, & Fuerst, 1995), where y = ALS enzyme activity (%); a = maximum point; x = dose of iodosulfuron or imazethapyr (µM); x_{I50} = dose of iodosulfuron or imazethapyr that corresponds to a 50% inhibition of the ALS enzyme; and b = curve declivity. The resistance factor (RF) was calculated dividing the I_{50} of the resistant biotype by values from the susceptible biotype (Hall, Stromme, & Horsman, 1998). The level of total protein was obtained using the Bradford method (Bradford, 1976).

ALS gene sequencing

RNA was extracted from leaf tissue (100 mg) in biotypes (B_1, B_4, and B_{13}) using 500 µL of extraction buffer (reagent Kit PureLink™ Plant RNA) according to the manufacturer's instructions. The quality and quantity of RNA were verified by electrophoresis gel and spectrophotometry, respectively. cDNA was synthesized from 2 µg RNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen™ - USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) products were obtained using the *primers* WR122F, WR653R, WR205R, WR376R, and WR574F (Han et al., 2012) and the ALS gene sequence of *R. raphanistrum* L. (AJ344986) that was deposited in GenBank of National Center for Biotechnology Information (NCBI).

PCR was conducted in a final volume of 25 µL containing 1.25 µL cDNA, 0.25 µM each of the *forward* (F) and *reverse* (R) *primers*, 12.5 µL 2x GoTaq™ Green Master Mix (Promega™) and nuclease-free water. cDNA denaturation was conducted at 94°C for 4 minutes, with 40 cycles of 94°C for 30 seconds. The annealing of *primers* occurred at 55°C for 30 seconds, and sequence extension was performed at 72°C for 30 to 120 seconds (Han et al., 2012). *Amplicons* were purified using the PCR Purification Combo Kit (Invitrogen™ - USA) and sequenced using the ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences from the various biotypes were aligned using the Bioedit version 7.2.5 software and compared with the sequence of *R. raphanistrum* L. (AJ344986) deposited at GenBank (www.ncbi.nlm.nih.gov/genbank).

Metabolization

The metabolization of radish biotypes was analyzed in two additional experiments that were performed in a greenhouse with a completely randomized design and four replications. Seeds were sown in plastic trays, and two days after emergence, each seedling was transplanted to plastic pots with volume capacities of 0.75 L that contained soil and PlantMax substrate at a 2:1 ratio.

The treatments were arranged in a factorial design, where factor A was the radish biotypes (B_1, B_4 and B_{13}) and factor B were the cytochrome P_{450} monooxygenase inhibitors malathion and piperonyl butoxide. Spraying occurred when plants were at the three to four leaf stage by using a CO_2 backpack

sprayer calibrated to deliver 120 L ha^{-1}. The metabolism inhibitors were sprayed 30 minutes before herbicide application at a dose of 500 g a.i. ha^{-1} for malathion and 525 g a.i. ha^{-1} for piperonyl butoxide (PBO). The doses of iodosulfuron and imazethapyr herbicides sprayed were 3.5 and 106 g a.i. ha^{-1}, respectively.

The control and shoot dry matter (SDM) were evaluated at 28 days after application (DAA). A percentage scale for control was adopted, in which zero (0) and one hundred (100) corresponded to the absence of damage and complete death of the plants, respectively (Frans & Crowley, 1986). The SDM was determined by drying the vegetable material in kiln with circulated forced air at 60°C for 72 hours and expressed as grams per plant.

The obtained data were analyzed for normality (Shapiro-Wilk test), which did not require data transformation, and were then submitted to analysis of variance ($p \leq 0.05$). When statistical significance was observed, the data were submitted to the Duncan test ($p \leq 0.05$).

Results and discussion

ALS enzyme activity and in vitro assay with the herbicides

The K_M values (pyruvate) of resistant biotypes (B_4 and B_{13}) did not show statistically significant differences in enzyme affinity for the substrate, and their V_{max} was higher than that of the the susceptible biotype (Figure 1). However, the K_M values of the B_4 and B_{13} biotypes were 20 and 25% higher, respectively, than that of the susceptible biotype (Table 1). Further, the V_{max} values were 7.78, 5.73, and 1.87 µM acetoin mg^{-1} protein h^{-1} for B_4, B_{13} and B_1 biotypes, respectively (Table 1). Other studies have demonstrated that biotypes that are resistant to ALS inhibitor herbicides do not present changes in the kinetic parameters (K_M and V_{max}) compared to susceptible biotypes (Ashigh & Tardif, 2007; Dal Magro et al., 2010), which suggests that resistance does not affect pyruvate binding. Similar results were found in *Cyperus difformis* L. biotypes resistant to pirazosulfuron-ethyl herbicide, in which the affinity of enzyme was not affected and the V_{max} was 52% greater (Dal Magro et al., 2010).

The ALS inhibitor herbicides act by blocking the channel that leads to the active site of the enzyme; therefore, any changes in this channel can impede the binding of the herbicide while maintaining the conformation and function of the enzyme (McCourt, Panf, King-Scott, Guddat, & Duggleby, 2006). In *Lolium rigidum* (Gaudin) biotypes resistant to sulfometuron and imazapyr herbicides, a mutation of tryptophan to leucine in position 574 (Trp_{574}-Leu) did not change the K_M, and V_{max} was 287% greater in resistant biotypes (Yu et al., 2010). The results obtained for in vitro assays with herbicide treatment demonstrated that 0.043 µM iodosulfuron and 3.2 µM imazethapyr inhibited 50% of the ALS enzyme activity (I_{50}) in the susceptible biotype. For the resistant biotypes, the I_{50} was 0.65 and 0.82 µM for iodosulfuron and 718 and 425 µM for imazethapyr in B_4 and B_{13} biotypes, respectively (Figure 2, Table 2).

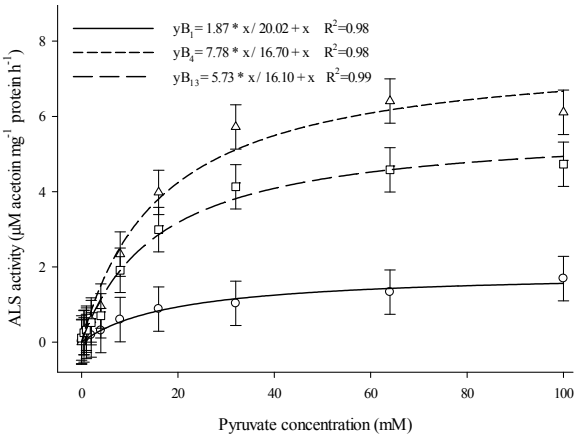

Figure 1. ALS activity (µM acetoin mg^{-1} protein h^{-1}) of susceptible (B_1) and resistant (B_4 and B_{13}) radish biotypes to iodosulfuron and imazethapyr herbicides subjected to differential pyruvate concentrations (mM). Points represent the mean values and bars represent least significant difference ($p < 0.05$).

Table 1. Kinetic parameters K_M (mM) and V_{max} (µM acetoin mg^{-1} protein h^{-1}) of susceptible (B_1) and resistant (B_4 and B_{13}) radish biotypes in response to iodosulfuron and imazethapyr herbicide treament.

Biotypes	K_M (mM)	V_{max} (µmol mg^{-1} protein h^{-1})
B_1 (Susceptible)	20.02^{ns}	1.87 C
B_4 (Resistant)	16.70	7.78 A
B_{13} (Resistant)	16.10	5.73 B
V.C. (%)		6.44

*means followed by the same uppercase letter (column) do not differ by Duncan's test ($p \leq 0.05$). ^{ns} = not significant ($p > 0.05$).

These values of I_{50} for biotypes B_4 and B_{13} resulted in a resistance factor (RF) of 15 and 19 to iodosulfuron herbicide and a RF of 224 and 133 to imazethapyr herbicide, respectively (Figure 2, Table 2). High enzyme inhibition was observed in *R. raphanistrum* L. where the I_{50} value in resistant and susceptible biotypes to clorosulfuron herbicide was 1.55 and 0.009 µM, respectively (Yu et al., 2012). The results demonstrated that B_4 and B_{13} biotypes present high levels of resistance to the herbicide imazethpayr (Table 2). Similar results were reported for radish biotypes resistant to ALS inhibitor herbicides, for which the I_{50} was higher for imazethapyr than for flumetsulam and metsulfuron-methyl herbicides (Yu et al., 2012; Pandolfo et al.,

2016). Different levels of resistance to herbicides may be related to the position of the mutation sites in relation to the herbicide-coupling site (Yu et al., 2012; Pandolfo et al., 2016).

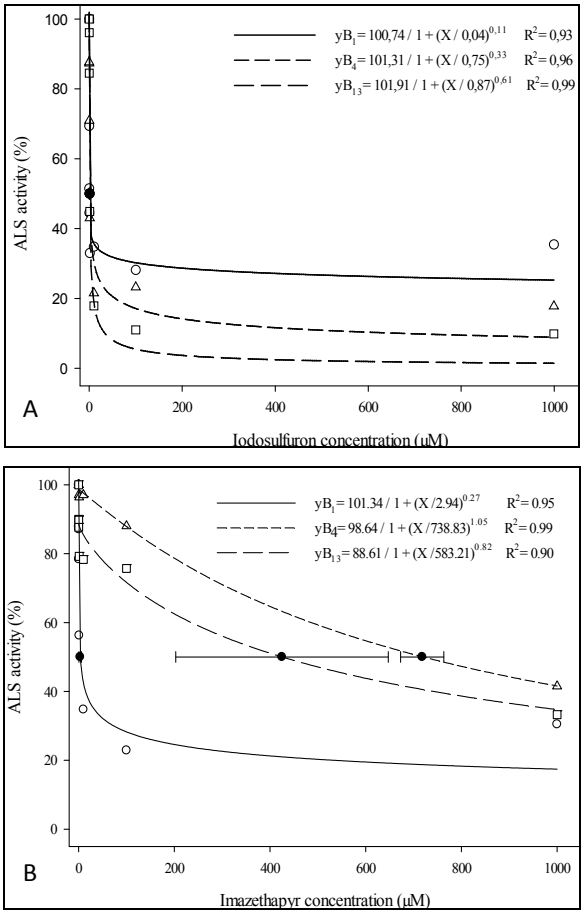

Figure 2. In vitro inhibition of ALS activity (%) of susceptible (B_1) and resistant (B_4 and B_{13}) radish biotypes subjected to different concentrations of iodosulfuron (A) and imazethapyr (B) herbicides (µM). Points represent the mean values of repetitions in each biotype.

Table 2. I_{50} values with confidance intervals (95% CI) and resistance factors (RF) of susceptible (B_1) and resistant (B_4 and B_{13}) radish biotypes subjected to different concentrations of iodosulfuron and imazethapyr herbicides.

Biotypes	I_{50}[1]		Resistance fator[2]
	M	95 CI	(FR)
Iodosulfuron (B_1)	0.043	0.09 - (-)0.004	-
Iodosulfuron (B_4)	0.65	1.05 – 0.25	15
Iodosulfuron (B_{13})	0.82	1.00 – 0.64	19
Imazethapyr (B_1)	3.2	5.8 - 0.6	-
Imazethapyr (B_4)	718	763.04 – 672.96	224
Imazethapyr (B_{13})	425	646.94 – 203.06	133

[1]I_{50} = dose required to inibit 50% of ALS enzyme activity.
[2]RF obtained by division I_{50} of the resistant biotype by I_{50} of the susceptible biotype.

ALS gene sequencing

A 1758 bp fragment of the ALS gene with five conserved regions was sequenced from the cDNA of susceptible (B_1) and resistant (B_4 and B_{13}) radish biotypes. This region includes all domains with mutation points that were previously identified in biotypes of *R. raphanistrum* L. resistant to ALS inhibitor herbicides (Tan & Medd, 2002; Yu et al., 2003; 2012; Han et al., 2012). The partial sequence presented a single nucleotide change of TGG to TTG, which led to a Trp-574-Leu substitution in resistant biotypes (Figure 3).

This mutation was identified in different weeds resistant to ALS inhibitor herbicides, including a recent discovery in biotypes of *R. sativus* L. (Pandolfo et al., 2016). Mutation of the ALS gene can compromise the herbicide coupling to the target site of the enzyme and affect the weed control with ALS inhibitor herbicides (McCourt et al., 2006). The Trp-574-Leu substitution is considered the most relevant mutation because it confers resistance to all five chemical groups of the ALS inhibitor herbicides (Pandolfo et al., 2016). However, this mutation has not been reported in resistant biotypes of *R. sativus* L. in Brazil; this can be considered the first identified case. In biotypes of *R. raphanistrum* L., the most frequent mutations involve a proline at amino acid position 197 (Pro_{197}), which confers resistance to the chemical groups of sulfonylureas and triazolopyrimidines (Tan & Medd, 2002; Yu et al., 2003). However, the levels of cross-resistance will depend on the position where the mutation occurred and the modified amino acid (Yu et al., 2012; Han et al., 2012).

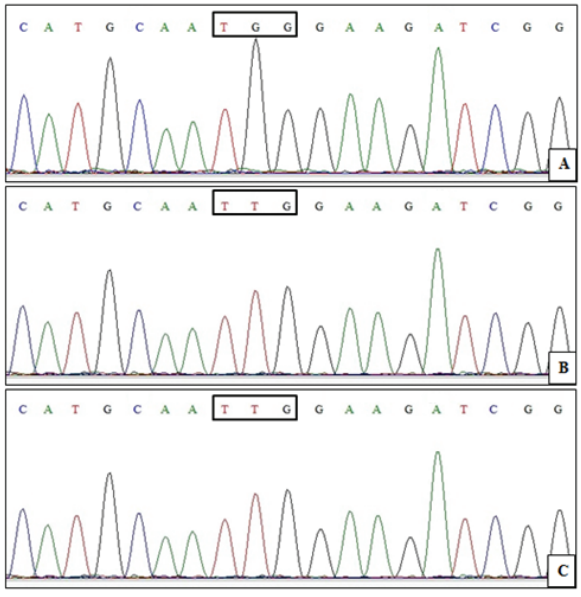

Figure 3. Comparison of partial ALS gene sequence of *Raphanus sativus* L. where the TGG codon for Trp-574 is the susceptible biotype (A) and the TTG codon for Leu-574 is present in biotypes resistant to iodosulfuron and imazethapyr herbicides (B and C).

Furthermore, all biotypes presented silent mutations at the 122 position that contains an alanine (Ala_{122}), where the codon GCC was substituted for GCT (data not shown) without resulting in a modification to the amino acid sequence. Amino acid substitutions at Ala-122 are more unlikely due to the necessity of two nucleotide alterations to modify the amino acid (Yu et al., 2012). However, in *R. raphanistrum* biotypes, the substitution of Alanine 122 to Tyrosine (Ala-122-Tyr) conferred resistance to three chemical groups of ALS inhibitors herbicides (Han et al., 2012).

The mutation diagnosed here (Trp-574-Leu) is one of the most important because it confers resistance to all chemical groups of ALS inhibitor herbicides, thus reducing the control options for resistant biotypes (Pandolfo et al., 2016; Cechin et al., 2016).

Metabolization

The results demonstrated that the application of cytochrome P_{450} monooxygenase inhibitors followed by the application of iodosulfuron or imazethapyr herbicides did not efficiently control the resistant biotypes (Table 3). The isolated spraying of cytochrome P_{450} monooxygenase inhibitors did not cause phytotoxicity in any of the radish biotypes (data not showed).

Table 3. Control (%) in susceptible (B_1) and resistant (B_4 and B_{13}) radish biotypes subjected to the application of iodosulfuron or imazethapyr herbicides alone or preceded by cytochrome P_{450} monooxygenase inhibitors at 28 days after application (DAA).

Treatment	28 DAA					
	B_1		B_4		B_{13}	
Iodosulfuron	99	aA	5.0	bA	0.0	bB
Malathion + iodosulfuron	100	aA	4.0	cA	21	bA
PBO + iodosulfuron	99	aA	2.0	bA	4.0	bB
Control	0.0	^{ns}B	0.0	A	0.0	B
V.C. (%)	18.00					
Imazethapyr	97	aA	1.0	bA	1.0	bA
Malathion + imazethapyr	97	aA	0.0	bA	0.0	bA
PBO + imazethapyr	98	aA	2.0	bA	1.0	bA
Control	0.0	^{ns}B	0.0	A	0.0	A
V.C. (%)	6.95					

*means followed by the same lowercase letter (line) and the same uppercase letter (column) do not differ by Duncan's test ($p \leq 0.05$). ^{ns} = not significant ($p > 0.05$).

At 28 DAA, spraying of iodosulfuron and imazethapyr herbicides (isolated or combined with P_{450} inhibitors) did not result in any significant differences in control for B_1, B_4, and B_{13} biotypes (Table 3). However, for B_{13} biotype, the application of iodosulfuron herbicide after the use of malathion as a P_{450} inhibitor resulted in 21% control, in contrast to the other treatments (Table 3). The use of the cytochrome P_{450} inhibitor in *Lolium rigidum* (Gaudin) biotypes before clorosulfuron herbicide showed a synergistic effect compared to the isolated application of the herbicide (Yu & Powles, 2014). However, spraying imazethapyr herbicide either alone or in combination with PBO or malathion resulted in less than 2% control, suggesting that there is no differential metabolism by cytochrome P_{450} monooxygenase in these biotypes.

The results for SDM did not show significant differences in dry matter accumulation for biotypes B_1, B_4, and B_{13} when imazethapyr herbicide was sprayed (Table 4). Nevertheless, the use of malathion or PBO in the resitant biotype B_{13} resulted in 66% and 48% reductions in SDM, respectively, compared with the isolated spraying of iodosulfuron (Table 4). Similar results were found in *Echinochloa phyllopogon* (Stapf) biotypes resistant to penoxsulan herbicide, where the use of malathion before herbicide application reduced 60% of the SDM content at a dose of 10 g a.i ha^{-1} of penoxsulam (Yasuor et al., 2009).

Table 4. Shoot dry matter (SDM) in susceptible (B_1) and resistant (B_4 and B_{13}) radish biotypes subjected to the application of iodosulfuron or imazethapyr herbicides alone or preceded by cytochrome P_{450} monooxygenase inhibitors at 28 days after application (DAA).

Treatment	SDM					
	B_1		B_4		B_{13}	
Iodosulfuron	0.43	bB	1.74	aAB	1.84	aA
Malathion + iodosulfuron	0.33	bB	1.58	aB	1.11	aB
PBO + iodosulfuron	0.25	bB	1.45	aB	1.24	aB
Control	1.88	^{ns}A	2.01	A	1.87	A
V.C. (%)	20.18					
Imazethapyr	0.26	bB	3.55	bA	4.05	aA
Malathion + imazethapyr	0.15	bB	4.09	aA	4.33	aA
PBO + imazethapyr	0.21	cB	3.36	bA	4.31	aA
Control	3.52	^{ns}A	3.51	A	3.89	A
V.C. (%)	11.36					

*means followed by the same lowercase letter (line) and the same uppercase letter (column) do not differ by Duncan's test ($p \leq 0.05$). ^{ns} = not significant ($p > 0.05$).

However, in an experiment with ALS herbicide-resistant biotypes of *Poa annua* L., an increase in control by bispyribac-sodium herbicide was observed when malathion was used as an inhibitor of P_{450} monooxygenase. However, these results were not observed for other ALS enzyme inhibitors, indicating that the mechanism of resistance involved cannot be attributed to differential metabolism by P_{450} monooxygenase (Brosnan et al., 2016).

The differences found among the biotypes for control and SDM when malathion or PBO inhibitors where used indicate cytochrome P_{450} may be involved in metabolic resistance, which may be specific to a given herbicide.

Therefore, the results of control and SDM observed in B_4 and B_{13} biotypes did not indicate the involvement of cytochrome P_{450} enzyme in resistance of radish to iodosulfuron and imazethapyr herbicides.

Conclusion

The kinetic parameters of ALS enzyme were not affected in resistant biotypes. A mutation was detected at position 574 of the ALS gene, which resulted in a substitution of tryptophan to leucine (Trp-574-Leu) in resistant radish biotypes. The results demonstrated that there was no differential metabolism of iodosulfuron and imazethapyr herbicides in resistant biotypes when cytochrome P_{450} was inhibited by malathion and piperonyl butoxide.

Acknowledgements

We thank the coordination of the Higher Education Personnel Training (CAPES) for the scholarship to the first author and also the Embrapa/Monsanto Partnership.

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Received on July 2, 2016.
Accepted on October 18, 2016.