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Scientific Article

Try574 leu mutation confers cross-resistance to ALS-inhibiting herbicides in wild radish

Mutação Try574 conferindo resistência cruzada a herbicidas inibidores da ALS em nabiça

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ABSTRACT - Understanding how weeds resist herbicides, their resistance mechanisms, and alternative control methods are crucial for managing herbicide-resistant weeds. This study aims to unravel the resistance mechanism of a *Raphanus raphanistrum* biotype to acetolactate synthase (ALS) inhibitors. To this end, dose-response studies, DNA sequencing, and metabolic pathway verification were conducted. ALS-inhibiting herbicides showed low efficacy in controlling this biotype, confirming cross-resistance. Sequencing of the ALS enzyme revealed the presence of the previously reported Try-574-Leu mutation, known to confer cross-resistance to this mode of action. However, the metabolization verification assay demonstrated that this mechanism did not contribute to the observed resistance. Chemical control studies with alternative herbicides yielded promising results, indicating the potential for effective management of the resistant biotype. Our findings showed that the wild radish biotype used exhibits cross-resistance to ALS-inhibiting herbicides due to the presence of the Try-574-Leu mutation in the target enzyme. Notably, herbicides with alternative mechanisms of action prove highly effective in controlling this resistant biotype, offering valuable options for weed management strategies.

Keywords: Single-nucleotide polymorphism. *Raphanus raphanistrum*. Wheat. Weed Control.

RESUMO - A caracterização da resistência de plantas daninhas a herbicidas, bem como os estudos sobre os mecanismos de resistência e as alternativas para o controle químico são fundamentais para o manejo de plantas daninhas resistentes a herbicidas. Desta forma, este trabalho teve como objetivo reportar e elucidar o mecanismo de resistência de um biótipo de *Raphanus raphanistrum* a inibidores da acetolactato-sintase (ALS). Foram desenvolvidos trabalhos de dose resposta, sequenciamento do gene ALS, verificação de metabolização e alternativas para o controle químico. Ocorreu baixa eficiência de controle de herbicidas inibidores de ALS a esse biótipo, sendo confirmada a resistência cruzada. O sequenciamento gene ALS demostrou que o biótipo apresentou a mutação Try-574-Leu, já reportada na literatura e que confere resistência cruzada a este mecanismo de ação. O ensaio para verificação de metabolização provou que esse mecanismo não está envolvido na resistência. Já o estudo de controle químico indicou que existem alternativas para o controle do biótipo resistente. Conclui-se que o biótipo de nabiça apresentou resistência cruzada aos herbicidas inibidores de ALS, com a mutação Try-574-Leu na enzima alvo e que herbicidas de outros mecanismos de ação promovem elevada eficiência de controle.

Keywords: Polimorfismo de nucleotídio simples. *Raphanus raphanistrum*. Trigo. Controle de plantas daninhas.

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INTRODUCTION

Acetolactate synthase (ALS)-inhibiting herbicides function by blocking the production of branched-chain amino acids like valine, leucine, and isoleucine in plants. These herbicides are categorized into five chemical classes: imidazolinones, sulfonylureas, triazolopyrimidines, pyrimidiniothio(or oxy)-benzoates, and sulfonylamino-carbonyltriazolinones (MENDES et al., 2020). The issue of acetolactate synthase resistance is not new to Brazilian agriculture, as numerous reports spanning various states and agricultural systems have emerged since the 1990s (HEAP, 2023).

Costa and Rizzardi (2014) confirmed the resistance of a *Raphanus raphanistrum* biotype from northern Paraná to metsulfuron-methyl. Similarly, Cechin et al. (2017) documented resistance in an *R. sativus* biotype from Três de Maio (Rio Grande do Sul State) to iodosulfuron, and in another study, these researchers demonstrated cross-resistance to ALS inhibitors in *R. sativus* from northwestern Rio Grande do Sul. More recently, Costa et al. (2021) affirmed resistance in *R. sativus* and *R. raphanistrum* biotypes from Cafelândia in Paraná State, and from Júlio de Castilhos and Cruz Alta in Rio Grande do Sul State.

A survey conducted by Owen, Martinez and Powles (2015) on *R. raphanistrum* resistance to herbicides revealed that only 18% of biotypes from Western Australia, a major wheat-producing state in Australia, remained susceptible to ALS inhibitors of the sulfonylurea chemical group. Additionally, 50% of populations were found to be susceptible to imidazolinone and triazolopyrimidine herbicides.

In this context, *R. raphanistrum* emerges as a particularly challenging weed to control, especially in winter cereals like wheat, oats, and barley. Instances of resistance to ALS-inhibiting herbicides (OWEN; MARTINEZ; POWLES, 2015), photosystem II inhibitors (BECKIE; BUSI; ZHANG, 2020), synthetic auxins (WALSH et al., 2004), phytoene desaturase inhibitors (LU et al., 2020a), HPPD inhibitors (LU et al., 2020b), AGCML inhibitors (BECKIE; BUSI; ZHANG, 2020), and multiple herbicide resistance have been documented in Western Australia (OWEN; MARTINEZ; POWLES, 2015). This historical data underscores the rapid evolution of resistance within this species.

In most cases, resistance to ALS inhibitors is attributed to mutations in the ALS gene, resulting in a single nucleotide change that replaces an amino acid at the herbicide binding site on the enzyme (TRANEL; WIGHT; 2002). To date, 28 amino acid substitutions conferring resistance to ALS inhibitors have been reported, primarily at sites Pro197 (Ala, Arg, Asn, Gln, His, Ile, Leu, Lys, Met, Ser, Thr, Trp, and Tyr), Ala122 (Thr, Tyr, and Val), Ala205 (Val), Asp376 (Glu), Arg377 (His), Trp574 (Arg, Leu, Gly, and Met), Ser653 (Asn, Ile, and Thr), and Gly654 (Glu and Asp), in various weed species (TRANEL; WRIGHT; HEAP, 2021). Gene amplification, linked to increased ALS enzyme gene expression, is a less common resistance mechanism, identified only in one corn lineage and *Sisymbrium orientale* (FORLANI et al., 1991).

Non-target-site-based resistance (NTSR) mechanisms hinder herbicides from reaching their intended site of action. These mechanisms, usually controlled by multiple genes, involve a series of enzymes and processes that reduce the herbicide's phytotoxic effects on the plant (DÉLYE, 2012).

ALS inhibitors hold the record for the highest number of confirmed resistance cases in Brazil. However, most studies have focused on confirming resistance and developing alternative chemical control methods for these biotypes. Therefore, investigations into the mechanisms underlying herbicide resistance are crucial for enhancing our understanding of resistance evolution in Brazil.

Based on the above, our goal is to confirm the resistance of an *R. raphanistrum* biotype to ALS inhibitors, investigate its resistance mechanism, and assess chemical control alternatives.

MATERIAL AND METHODS

Plant material

Seeds of the *Raphanus raphanistrum* biotype used were sourced from the municipality of Catanduvas (25°21′26″ S, 53°08′51″ W, and 752 m), in western Paraná State (Brazil). These seeds were collected by harvesting ten siliques from nine plants that had not been subjected to control measures following exposure to metsulfuron-methyl herbicide (2.0 g ha^{-1}). The collection site was a commercial field with a history of soybean cultivation in the first season and wheat in the second season, as part of a succession cropping. The collected siliques were brought to the laboratory for threshing, cleaning, and identification, ultimately constituting the biotype with suspected resistance (R).

The susceptible (S) biotype was collected in the Center of Agricultural and Veterinary Sciences (27°47′36″ S, 50°18′08″ W, and 914 m), in the municipality of Lages, Santa Catarina State (Brazil). The collection site was a non-agricultural area with no prior history of herbicide application, where weed control was limited to mowing. In this case, we collected mature siliques from ten plants.

Whole-plant dose-response experiment

A greenhouse-based experiment was conducted in the municipality of Lages. Each experimental unit comprised a plant in a 0.4 dm^3 plastic pot filled with commercial inert potting soil, for both F1 and F2 generations. Seeds were previously treated with gibberellic acid at 5% to break dormancy. Two seeds were initially sown per experimental unit, with subsequent thinning to retain one plant per unit.

The surviving plants of the F1 generation from both biotypes were retained for seed production, resulting in the F2 generation. Putative resistant plants were isolated and manually fertilized. Following manual fertilization, the plants were maintained in a greenhouse with a controlled environment. This environment maintained a temperature range of 15 to 30°C and an air humidity of 30 to 60%. Additionally, all wild radish plants within a 100-meter radius were eliminated. Plant maintenance under these controlled conditions facilitated the acquisition of a higher quantity of seeds.

Experimental treatments involved two biotypes (R and S), three herbicides (metsulfuron-methyl [sulfonylurea], imazethapyr [imidazolinone], and pyroxsulam [triazolopyrimidine]), and varying application doses according to the biotype. For the F1 generation (corresponding to the field-collected generation), the following doses were applied: 0, 1, 2, 4, 8, 16, and 32 times the recommended dose for each herbicide (D). For the biotype S, the doses were 0.000, 0.125, 0.250, 0.500, 1.000, 2.000, and 4.000 times the D. In the F2 generation, the doses for both biotypes were 0.000, 0.001, 0.010, 0.100, 1.000, 10.000, and 100.000 times the D.

Following the label instructions for each commercial product, the D values for wild radish control were 3.96, 106.00, and 15.30 g ha^{-1} for metsulfuron-methyl, imazethapyr, and pyroxsulam herbicides, respectively. Three replications were conducted for each treatment.

The plants were subjected to intermittent irrigation until treatment application. Herbicide applications were performed when plants were at an optimal control stage, typically with two to four true leaves. The applications were carried out using a CO_2-pressurized sprayer equipped with a boom containing four XR 110 02 nozzles, spaced at 0.5 meters, and positioned 50 cm above the target, delivering 200 L ha^{-1}, pressurized at 220 KPa.

Visual control assessments were performed 28 days after application (DAA) based on a control scale ranging from 0 to 100%, wherein 0 signified no control and 100% indicated plant death.

DNA extraction and ALS gene sequencing

For DNA extraction, ten plants from each biotype were carefully selected. Approximately 10 grams of fresh tissue from each plant was finely ground in liquid nitrogen. DNA extraction was performed using the Wizard Genomic DNA Purification Kit.

Subsequently, the extracted DNA samples underwent polymerase chain reaction (PCR) to amplify the ALS gene. The amplification of the ALS gene employed primers previously described by Tan and Medd (2002) and Han et al. (2012), which are deposited in the National Center for Biotechnology Information (NCBI) Gene Bank (Table 1). These primers were specifically designed to amplify and sequence fragments of the ALS gene, which includes five highly conserved regions where mutations associated with resistance to ALS inhibitors have previously been identified.

Table 1. Primers used for ALS gene sequencing in populations of *Raphanus raphanistrum* susceptible and resistant to ALS inhibitors.

Primer[1]	Product size (bp)	Melting temperature (ºC)	Sequence 5'-3'
WR 376 F	363	60	TTGCGAGTACTTTGATGGGG
WR 376 R	363	60	GCTTCTGCTCGCTCAATTCAC
WR 122 F	1751	55	TCTCCCGATACGCTCCCGACG
WR 205 R	527	60	GCAAGCTGCTGCTGAATATCC
WR 574 F	504	55	TTGTCATCATCAGGCCTTGGA
WR 653 R	1751	55	TCAGTACTTAGTGCGACCATC

[1]Primers cited by Tan and Medd (2002), Han et al. (2012), and Yu et al. (2012).
F: forward primer.
R: reverse primer.

Highly conserved regions were amplified using a reaction mixture consisting of 34.75 µL of nuclease-free water (dH_2O), 10 µL of 5X Buffer, 1 µL of Deoxynucleotide Triphosphates (dNTPs), 1 µL of forward (F) primers (3.2 µM), 1 µL of reverse (R) primers (3.2 µM), 0.25 µL of Taq DNA Polymerase, and 2 µL of DNA from each biotype (~10 ng/µL). This resulted in a final volume of 50 µL.

The PCR was performed using the Applied Biosystems® Veriti® 96-Well Thermal Cycler. The PCR protocol was identical for both forward and reverse primers and involved the following steps: an initial denaturation at 94 °C for 4 minutes, followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 5 minutes, concluding with a temperature hold at 4 °C.

After amplification, the PCR products were visualized through gel electrophoresis. This involved mixing 5 µL of the PCR product with 1 µL of bromophenol blue and 1 µL of red gel. The mixture was subjected to electrophoresis on a 1% agarose gel, and the electrophoresis process was allowed to run for approximately 40 minutes.

After obtaining PCR products and confirming amplification success through visualization, they were subjected to sequencing at ACTGENE ANÁLISES MOLECULARES LTDA, located in Alvorada, Rio Grande do Sul State (Brazil). Before sequencing, the samples were meticulously prepared, and their concentrations were assessed to determine the appropriate quantity of PCR product for sequencing. The sequencing result consisted of a total volume of 1 µL, comprising 1 µL of the forward primer, 1 µL of the reverse primer, and 2 µL of nuclease-free water (dH_2O).

The sequences were determined and compared using DNA Baser® software. Different nucleotide sequences were visualized and compared against the nucleotide sequence of *R. raphanistrum* deposited in GenBank to identify variations in both nucleotides and amino acids.

P450 inhibitor experiment

To investigate herbicide metabolization by cytochrome P450, we utilized malathion, an insecticidal compound that inhibits enzymes within the P450 system. This experiment took place in a greenhouse, comprising 40 experimental units, divided into 10 treatments. Each experimental unit consisted of a plant in a plastic pot with a volumetric capacity of 0.4 dm^3, filled with potting soil.

The experimental design employed a completely randomized design (CRD) with treatments arranged in a factorial scheme (4 x 2) + 2. Factor A encompassed herbicides from the ALS inhibitors chemical group, including metsulfuron-methyl, imazethapyr, imazamox, and penoxsulam, each applied at doses of 3.96, 106, 91, and 15.3 g ha^{-1}, respectively. Factor B pertained to the presence or absence of malathion at a rate of 2,000 g ha^{-1}. Malathion was applied two hours before the herbicides. Two additional treatments included an untreated control and a malathion-only check (VARANASI; BRABHAM; NORSWORTHY, 2018).

Evaluation of control and shoot dry mass (SDM) occurred 28 days after application (DAA). This involved cutting the plants close to the ground and subsequently drying them in an oven at 65°C for 72 hours until a constant weight was achieved. The SDM was quantified using a semi-analytical scale (0.001 g).

Alternative herbicide experiment

This experiment took place in a greenhouse, employing a completely randomized design. Each experimental unit was represented by a plant in a plastic pot with a volumetric capacity of 0.4 dm^3. For this experiment, only the R biotype was utilized.

The herbicides were applied in two modalities: pre-emergence and post-emergence. The pre-emergence herbicides included pendimethalin (1820 g ha^{-1}), flumioxazin (75 g ha^{-1}), and clomazone (432 g ha^{-1}). On the other hand, the post-emergence herbicides consisted of 2,4-D (564.2 g ha^{-1}), MCPA (732 g ha^{-1}), dicamba (720 g ha^{-1}), bentazon (576 g ha^{-1}), glyphosate (2095.65 g ha^{-1}), metribuzin (144 g ha^{-1}), saflufenacil (49 g ha^{-1}), glufosinate (400 g ha^{-1}), paraquat (400 g ha^{-1}), triclopyr (1360 g ha^{-1}), and flumioxazin (50 g ha^{-1}). Consequently, a total of 15 treatments were

evaluated, encompassing 14 herbicides and an untreated control.

The evaluations were conducted similarly to those in the P450 inhibitor experiment, with the only difference being in the assessments for pre-emergence applications. In this case, the number of emerging plants was evaluated per experimental unit, expressed as a percentage of surviving plants relative to the untreated control.

Statistical analysis

The data obtained from the dose-response experiment were initially subjected to analysis of variance using the F-test ($p<0.05$). If the results were found to be significant, non-linear regression analysis was subsequently performed.

For the regression analysis of the control data, the two-parameter exponential model was employed:

$$y = \alpha(1 - e^{-bx})$$

where y represents the percentage of control, x denotes the herbicide dose, and a and b are the curve coefficients, with a corresponding to the maximum point of the curve, while b indicates the slope of the curve.

To confirm resistance, the lethal dose 50 (LD_{50}) data was utilized, which signifies the dose required to control 50% of the population. The LD_{50} parameter was calculated using the inverse equation. LD_{50} data allowed for the determination of the resistance factor (RI), indicating the quantity needed to be applied to the resistant biotype to achieve the same level of control as the susceptible biotype. The RI_{50}, calculated for 50% control, is the more commonly used metric.

The resistance factor (F) was derived from the LD_{50} values, representing the ratio between the LD_{50} of the resistant biotype and the LD_{50} of the susceptible biotype. The F factor ($RI=LD_{50}R/LD_{50}S$) quantifies how many times the dose required to control 50% of the resistant biotype exceeds the dose needed for 50% control of the susceptible biotype. This parameter serves as an indicator of the resistance level.

Regarding the data from the alternative control and P450 inhibitor experiments, an analysis of variance was conducted using the F-test ($p<0.05$). If significance was observed, mean comparisons were carried out using Tukey's test ($p<0.05$) to identify statistically significant differences.

RESULTS AND DISCUSSION

Whole-plant dose-response results

The analysis of variance revealed significance in the control variable, with a notable interaction between herbicides and doses for both biotypes in both generations. Consequently, non-linear regression analysis was undertaken to calculate the LD_{50} and RI parameters. Table 2 provides an overview of the estimated parameters of the logistic regression model along with the coefficient of determination.

Table 2. Logistic model parameters for the herbicides metsulfuron-methyl, imazethapyr, and pyroxsulam in F1 and F2 generations of *Raphanus raphanistrum* biotypes R and S.

Metsulfuron-methyl		
Fitted model		
Generation	Resistant	R^2
F1	$y = 58.2873(1 - e^{(-0.0764X)})$	0.8756
F2	$y = 54.7111(1 - e^{(-0.0217X)})$	0.9874
Susceptible		
F1	$y = 97.3582(1 - e^{(-7.4614X)})$	0.9982
F2	$y = 100.5805(1 - e^{(-9.2240X)})$	0.9998
Imazethapyr		
Fitted model		
Generation	Resistant	R^2
F1	$y = 32.6105(1 - e^{(-0.0029X)})$	0.8604
F2	$y = 86.0913(1 - e^{(-0.0011X)})$	0.9612
Susceptible		
F1	$y = 97.2521(1 - e^{(-0.2285X)})$	0.9947
F2	$y = 94.8772(1 - e^{(-0.0508X)})$	0.9848
Pyroxsulam		
Fitted model		
Generation	Resistant	R^2
F1	$y = 26.5691(1 - e^{(-0.0114X)})$	0.8745
F2	$y = 81.8039(1 - e^{(-0.0019X)})$	0.9961
Susceptible		
F1	$y = 100.2858(1 - e^{(-0.8129X)})$	0.9970
F2	$y = 98.6014(1 - e^{(-0.3526X)})$	0.9881

Differences in sensitivity to metsulfuron-methyl between the resistant and susceptible biotypes were observed through the dose-response curve (Figure 1). Non-linear regression analysis was employed to calculate essential parameters, including LD, RI, and C_{max} (Table 3). Notably, significant differences in LD were apparent between the two biotypes.

For F1, LD and RI values based on achieving 50% control could not be determined since the herbicides pyroxsulam and imazethapyr did not achieve 50% control for the biotype R. However, for F2, the calculated RI reached an astonishing 1516, clearly indicating a prominent level of resistance to metsulfuron-methyl in the biotype R.

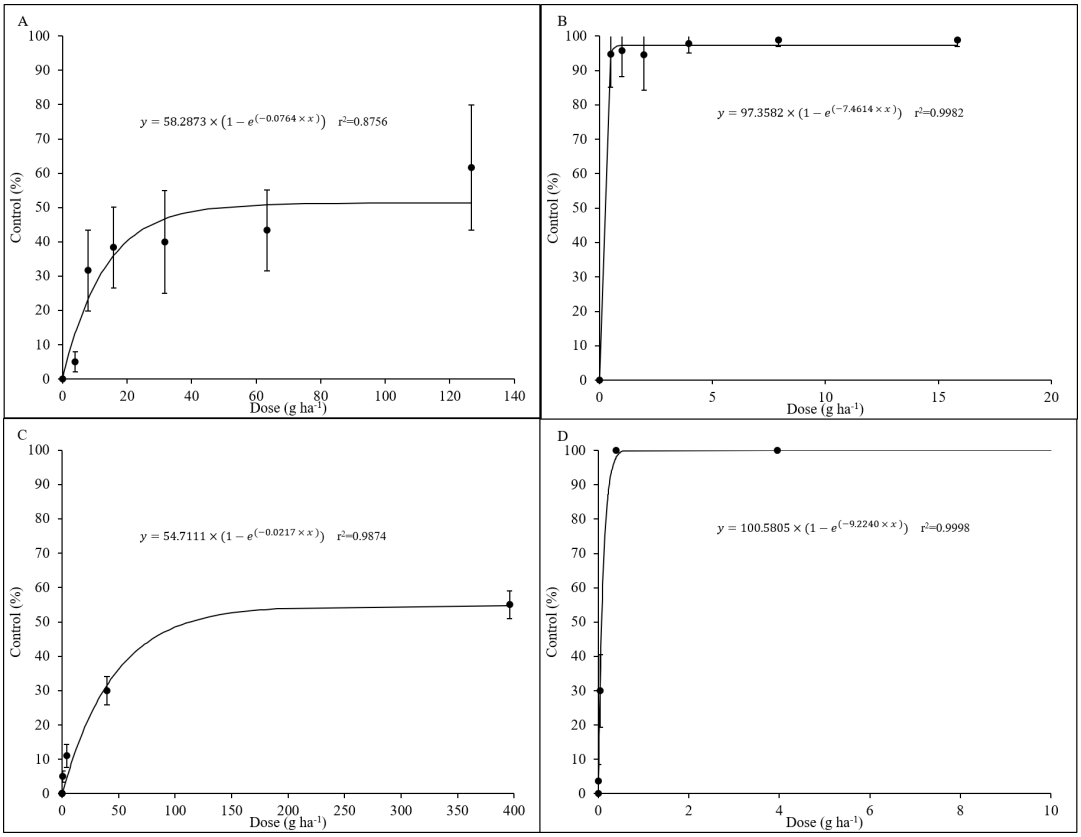

Figure 1. Dose-response fitted model for the herbicide metsulfuron-methyl. A: F1 of biotype R, B: F1 of biotype S, C: F2 of biotype R, and D: F2 of biotype S.

Table 3. Summary of lethal dose (LD), resistance index (RI), and maximum control (C_{max}) data for F1 and F2 generations of resistant and susceptible wild radish.

Metsulfuron-methyl (SU)						
Biotype	F1			F2		
	LD_{50}	RI_{50}	C_{max}	LD_{50}	RI_{50}	C_{max}
WR-R	49.02	505	50	113	1516	77.5
WR-S	0.097	-	100	0.075	-	100
Imazethapyr (IMI)						
Biotype	F1			F2		
	LD_{50}	RI_{50}	C_{max}	LD_{50}	RI_{50}	C_{max}
WR-R	>3392	>1073	41	870.0	58.98	88
WR-S	3.16	-	100	14.75		100
Pyroxsulam (TP)						
Biotype	F1			F2		
	LD_{50}	RI_{50}	C_{max}	LD_{50}	RI_{50}	C_{max}
WR-R	>489.6	>562	30	498	247.7	77.5
WR-S	0.87	-	100	2.01	-	100

The dose-response curves for imazethapyr revealed varying sensitivity between the resistant (Figures 2A and 2C) and susceptible biotypes (Figures 2B and 2D). Calculations of the LD_{50}, C_{max}, and RI for imazethapyr in the F1 generation indicated values exceeding 3392 g ha^{-1}, 41%, and >1073 for the R biotype. In the F2 generation, these values were determined to be 870 g ha^{-1}, 88%, and 58.98, respectively (Table 3). These results confirm the presence of resistance to imidazolinone herbicides in both generations, highlighting the reduced sensitivity of the resistant biotype to imazethapyr.

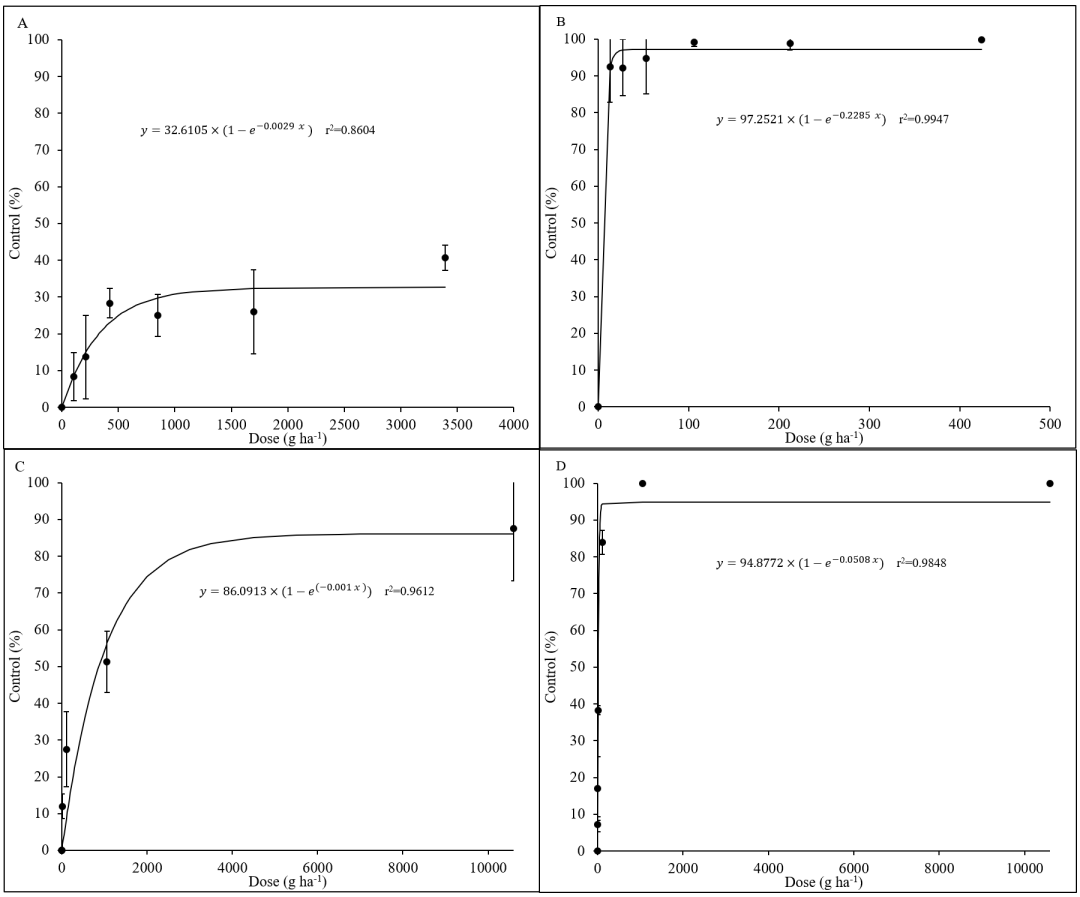

Figure 2. Dose-response fitted model for the herbicide imazethapyr. A: F1 of biotype R, B: F1 of biotype S, C: F2 of biotype R, and D: F2 of biotype S.

The biotype R required 500 and 1500 g ha^{-1} of pyroxsulam to control wild radishes by 26% and 81% (Figures 3A and 3C). Conversely, only 10 g ha^{-1} of pyroxsulam proved sufficient to achieve complete control (100% and 98%) of the biotype S in both generations. In this sense, analysis of LD, RI, and C_{max} values further illustrated the notably elevated level of resistance exhibited by the biotype R to pyroxsulam (Table 3). These results unequivocally demonstrate that this biotype displays a resistance level significantly exceeding 10 for all three herbicides, with control levels remaining below 80% even at the recommended dose. Additionally, this inheritable resistance was maintained in the F2 generation. Consequently, the investigation highlights the presence of cross-resistance to ALS inhibitors within the chemical groups of sulfonylurea, imidazolinones, and triazolopyrimidine in the biotype R. In contrast, the biotype S remained susceptible to all three herbicides.

The occurrence of resistance factors as high as those identified in this study is relatively uncommon, with RI values exceeding 10 being considered indicative of remarkably high resistance levels. Consequently, this biotype exhibits profound insensitivity to ALS-inhibiting herbicides. For instance, in the study by Costa and Rizzardi (2014) involving wild radish resistant to ALS inhibitors, they reported an RI of 267. These authors also documented LD_{50} values of 98 and 0.36 for the resistant and susceptible biotypes, respectively. Notably, the LD_{50} of the resistant biotype in their study closely resembles our findings, whereas the LD_{50} of the sensitive biotype was higher. These variations may be attributed to differences in susceptibility between populations, emphasizing the existence of intraspecific variation. Similarly, in the research conducted by Cechin et al. (2017) on the resistance of *R. sativus* to iodosulfuron (sulfonylurea), RI values of 89 and 252 were obtained for two populations from Rio Grande do Sul.

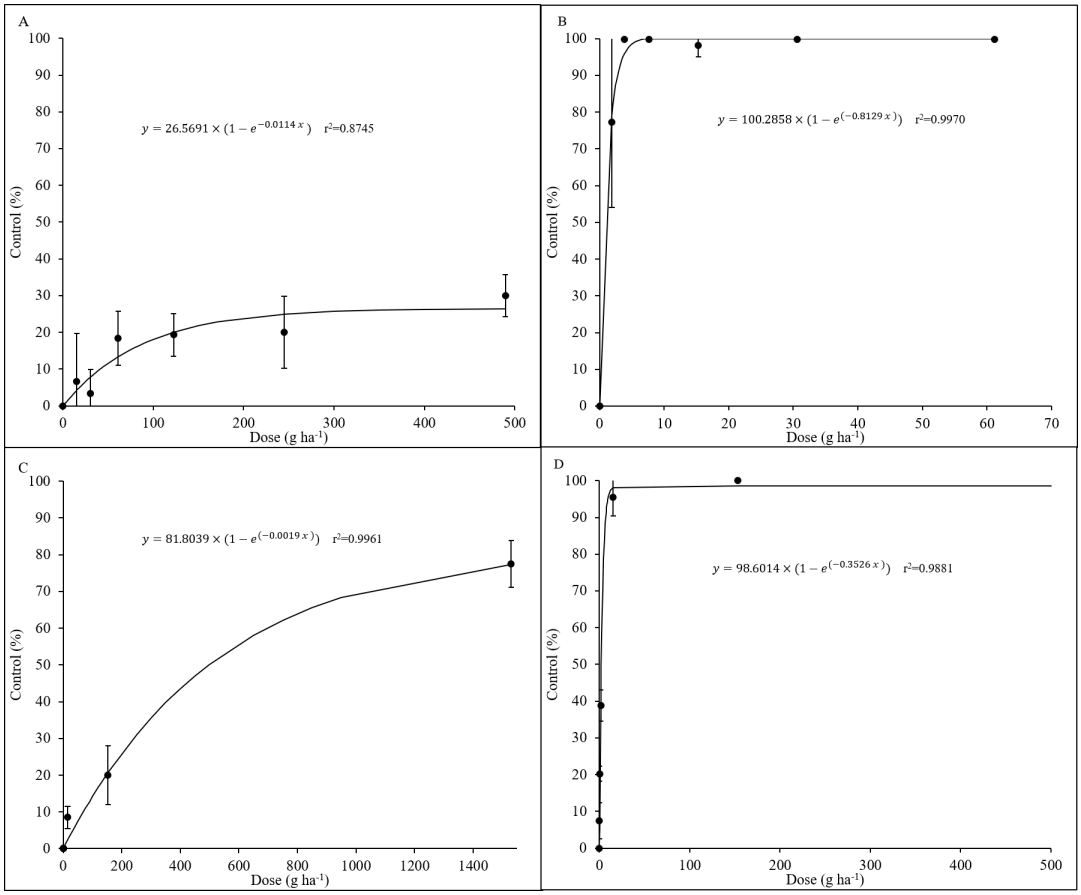

Figure 3. Dose-response fitted model for the herbicide pyroxsulam. A: F1 of biotype R, B: F1 of biotype S, C: F2 of biotype R, and D: F2 of biotype S.

ALS gene sequencing

The genomic DNA amplification generated three DNA fragments, encompassing all the regions where most amino acid modifications that confer resistance to ALS inhibitors typically occur (TAN; MEDD, 2002; TRANEL; WRIGHT; HEAP, 2021).

Partial ALS gene sequencing unveiled a notable change in the resistant biotype. Specifically, the base guanine (G) was substituted by thymine (T) in the ALS gene (Table 4). This base alteration corresponds to the replacement of the amino acid tryptophan at position 574 by leucine (Trp-574-Leu) within the ALS gene (Table 4). Importantly, no mutations were detected in other regions known to confer resistance to ALS inhibitors (Table 4).

Table 4. Nucleotide sequences of the ALS enzyme obtained from wild radish resistant and susceptible to ALS inhibitors.

Biotype	Nucleotide sequence 5'-3'							
	122	197	205	339	360	376	574	653
WR-S	GCT	CCT	GCG	TAT	GCT	GAT	TGG	AGT
	Ala	Pro	Ala	Tyr	Ala	Asp	Trp	Ser
WR-R	GCT	CCT	GCG	TTC	TCT	GAT	TTG-	AGT
	Ala	Pro	Ala	Phe	Ser	Asp	Leu	Ser

The remarkable resistance values identified in this study can be attributed to the high efficiency of the observed mutation in preventing herbicide binding. Notably, this mutation has been previously identified in various weed species resistant to ALS inhibitors (POWLES; YU, 2010; YU; POWLES, 2014), including *R. raphanistrum* (TAN; MEDD, 2002; YU et al., 2003; HAN et al., 2012; YU et al., 2012). The initial report of the Trp-574-Arg replacement emerged in *Digitaria sanguinalis* in China, and subsequent research has established that alterations at the Trp-574 site often confer broad cross-resistance to ALS inhibitors (MURPHY; TRANEL, 2019).

Cechin et al. (2017) conducted investigations into *Raphanus sativus* biotypes from Rio Grande do Sul and similarly identified the Trp-574-Leu mutation. This same mutation has been previously documented by Costa et al.

(2021) and Cechin et al. (2017), who worked with *R. raphanistrum* and *R. sativus* biotypes, thereby confirming cross-resistance across distinct species.

As per Mendes et al. (2020), the mutation at position Trp-574-Leu induces an increase in channel volume, primarily because the leucine side chain occupies a significantly smaller space compared to tryptophan. This channel serves as the primary binding site for herbicides with the ALS enzyme. Consequently, the mutation hinders the herbicide's entry into the channel, resulting in reduced ALS inhibition and, herbicide resistance.

Studies involving ALS inhibitor-resistant *R. raphanistrum* have identified multiple mutations in the ALS enzyme. These mutations include Pro197-His/Thr/Ser/Ala (YU et al., 2003), Asp-376-Glu (YU et al., 2012), and Ala-122-Tyr (HAN et al., 2012). In the fragments of the ALS gene sequenced in this study, additional mutations were identified that have not been previously reported in the literature as causing resistance to ALS-inhibiting herbicides. Specifically, these mutations were Ala-360-Ser and Tyr-339-Phe. Nonetheless, several mutations in the ALS gene cause no problems to plants or are associated with herbicide resistance. Notably, the Ala-360-Ser mutation was described only in a wild radish biotype from Cafelândia (Paraná State) in a study conducted by Costa et al. (2021), while the Tyr-339-Phe mutation has not been reported in prior research.

Interestingly, some studies have highlighted the presence of double mutations in response to ALS inhibitors. For instance, Zeineb et al. (2021) identified a plant in a *Glebionis coronaria* population from Tunisia with Pro-197-Arg and Asp-376-Glu mutations, in addition to a mutation at position 574, with no other mutations being detected. Similarly, Singh et al. (2019) discovered double mutations at position 574 and other locations in *Amaranthus palmeri* biotypes from Arkansas, USA. These studies have indicated that the resistance factor is higher when a biotype carries a double mutation compared to a single mutation at position 574.

P450 inhibitor experiment results

The results obtained in this study provide compelling evidence that the resistant biotype does not employ mechanisms related to herbicide metabolization through cytochrome P450. This conclusion is supported by the absence of any significant difference wasdifferences observed in the control evaluations and shoot dry matter (Table 5). These findings suggest that the growth and development of the biotype remain unaffected by the inhibition of cytochrome P450 enzymes, which play a crucial role in the initial phase of herbicide detoxification, known as the activation phase.

Table 5. Control (%) and aboveground biomass (g plant^{-1}) at 28 DAA in resistant wild radish with or without application of malathion and herbicides.

Treatment	Dose (g ha^{-1})	Control	Aboveground biomass
Untreated	0	0 ^{ns}	0.82 ^{ns}
Malathion	2000	0	0.91
Metsulfuron-methyl	3.96	0	1.06
Imazethapyr	106	0	1.44
Pyroxsulam	15.3	0	1.48
Imazamox	91	0	1.21
Metsulfuron-methyl + Malathion	3.96 + 2000	0	0.75
Imazethapyr + Malathion	106 + 2000	0	1.15
Pyroxsulam + Malathion	15.3 +2000	0	0.92
Imazamox + Malathion	91 + 2000	0	1.13

ns = p>0.05.

In contrast to target-site-based resistance (TSR), non-target-site-based resistance (NTSR) mechanisms are uncommon when it comes to resistance involving ALS inhibitors. Currently, only a few biotypes are known to employ this resistance mechanism. Cases of metabolization as a resistance mechanism are seldom documented in the plant class Magnoliopsida (VELDHUIS et al., 2011). This suggests that the significance of NTSR mechanisms for ALS inhibitors may be underestimated in weeds belonging to this class, possibly because site-of-action mechanisms are more commonly observed (DÉLYE, 2012).

Our findings unequivocally demonstrate the absence of a metabolism-related mechanism involving enzymes of the P450 complex. Similarly, Costa et al. (2021) reported no increase in metabolization in wild radish biotypes originating from different regions of Brazil. It is worth noting that resistance mechanisms other than site-of-action mutations for ALS inhibitors are relatively uncommon. However, among these mechanisms, metabolic resistance via the P450 monooxygenase complex is the most frequently observed (YU; POWLES, 2014). Instances of the accumulation of two resistance mechanisms (alteration in the site of action and metabolization by the P450 complex) have been documented in plant species like *Lolium rigidum* and *Alopecurus myosuroides* (POWLES; YU, 2010).

Interestingly, *R. raphanistrum* has been diagnosed with the NTSR mechanism, albeit for PDS inhibitors. This mechanism involves the action of cytochrome P450 enzymes. In a related study, Lu et al. (2020a) employed a method similar to that used in this study and observed an increase in susceptibility to PDS inhibitors when cytochrome P450 was inhibited. Consequently, this mechanism was found to be

responsible for herbicide resistance in that biotype. The resistance factors measured were 4.9 for the herbicide diflufenican and 11.2 for fluridone.

Alternative herbicides

In the pre-emergence phase, the herbicides flumioxazin and clomazone were particularly effective in weed control, achieving survival rates of 0% and 17.5%, respectively, when compared to the untreated control (Table 6). Notably, flumioxazin and clomazone nearly eliminated the production of dry mass in the weeds. On the other hand, pendimethalin exhibited lower control efficiency, resulting in higher shoot dry mass production (Table 6).

In the post-emergence phase, the majority of the herbicides demonstrated control rates exceeding 80%, and all of them exhibited significant differences compared to the untreated control. The only exceptions were metribuzin (67%) and flumioxazin (77.5%), which achieved control rates slightly below 80% (Table 6). Despite these differences in control rates, all herbicides led to a reduction in shoot dry mass, with no significant variations among them (Table 6).

Table 6. Plant survival (%), control (%), and aboveground biomass (g pot^{-1}) of wild radish resistant to ALS-inhibiting herbicides for alternative herbicides in the pre-or post-emergence application at 28 DAA.

Pre-emergence	Dose (g ha^{-1})	Survival	Control	Aboveground biomass
Check no treated		100 a	-	0.05 ab
Pendimethalin	2000	75 ab	-	0.1 b
Flumioxazin	75	0 c	-	0.0 a
Clomazone	432	17.5 bc	-	0.005 a
Post-emergence				
Check no treated		-	0 a	2.63 a
2,4-D	564.2	-	100 c	0 b
MCPA	732	-	100 c	0 b
Dicamba	720	-	88.7 bc	0.35 b
Bentazon	720	-	100 c	0 b
Glyphosate	2095.6	-	100 c	0 b
Metribuzin	144	-	67 b	0.74 b
Saflufenacil	49	-	99.5 c	0.05 b
Glufosinate	400	-	100 c	0 b
Paraquat	400	-	100 c	0 b
Triclopyr	1360	-	100 c	0 b
Flumioxazin	50	-	77.5 bc	0.7 b

Means followed by the same letter do not differ from each other by the Tukey's test ($p>0.05$).

Our evaluation of alternative methods to control the resistant biotype revealed that pendimethalin yielded unsatisfactory results, with only a 75% survival rate for wild radish plants. This deficient performance can be attributed to the high affinity of this molecule for non-polar compounds and the specific characteristics of the soil used, which had a high organic matter content (45 g kg^{-1}) and a significant clay component (650 g kg^{-1}). These factors limit the availability of the herbicide in the soil solution.

In a study by Costa and Rizzardi (2014), a similar wild radish biotype from northern Paraná State with metsulfuron-methyl resistance was examined, and it produced comparable results. Glyphosate, bentazon, and 2,4-D achieved control rates close to 100%. Cechin et al. (2017) also demonstrated the effectiveness of alternative herbicides to control ALS inhibitor-resistant wild radish in corn and soybeans, including glufosinate, paraquat, diuron + paraquat, glyphosate, saflufenacil, fomesafen, mesotrione, tembotrione, and atrazine.

Notably, not all herbicides are selective in their application. Glyphosate, glufosinate, paraquat, flumioxazin, dicamba, and triclopyr can be used for desiccation or fall management. Among pre-emergence treatments, pendimethalin is selective and registered for use in wheat. While flumioxazin has demonstrated selectivity (ASSUNÇÃO et al., 2017), it is not registered for this crop. Likewise, clomazone, though unregistered, shows selectivity for wheat when used with the safener dietholate (SCHMITZ et al., 2018).

For post-emergence, the options are narrowed down to auxin-mimicking herbicides (2,4-D and MCPA) and photosystem II inhibitors (bentazon and metribuzin). Saflufenacil can also be used in post-emergence but poses a risk of causing significant phytotoxicity to the wheat crop (MALDANER; SCHNEIDER, 2019). Our findings emphasize that the primary chemical tool for managing wild radish is desiccation, offering a range of action mechanisms. However, in post-emergence control, only three mechanisms are viable: auxin mimics, photosystem II inhibitors, and protox inhibitors. The use of auxin mimics requires precise timing, before wheat plant stalk elongation, and careful application (AGOSTINETTO et al., 2016) due to resistance issues observed in Australia, including multiple resistance (LU et al., 2020b). PSII inhibitors are wheat-selective but demand

precise control timing due to their limited mobility. Metribuzin, for example, should be applied to reach the roots effectively (PIASECKI et al., 2017). PPO inhibitor saflufenacil, while highly efficient, can cause phytotoxicity in wheat (PIASECKI et al., 2017). Still, the biotype R did not show multiple resistance to herbicides tested in our study. Nevertheless, it is crucial to adopt different strategies to prevent the development of multiple resistance, even with these effective herbicides.

CONCLUSIONS

The wild radish biotype from Catanduvas, western Paraná State, exhibited cross-resistance to ALS enzyme inhibitors, characterized by a high resistance factor, poor control even at recommended doses, and a hereditary nature of resistance. This cross-resistance is attributed to a specific mutation at position 574 in domain B of the ALS enzyme, where tryptophan is replaced by leucine. Notably, the increased herbicide metabolism via cytochrome P-450 does not contribute to wild radish resistance to ALS inhibitors.

For effective control of the wild radish biotype with cross-resistance to ALS inhibitors, pre-emergence application of herbicides flumioxazin and clomazone, as well as post-emergence application of 2,4-D, MCPA, dicamba, bentazon, glyphosate, saflufenacil, glufosinate, paraquat, and triclopyr, proved to be efficient solutions.

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