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Target-site is the main mechanism of resistance to *ALS*-inhibitor herbicides in a rice flatsedge population from Southern Brazil

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Abstract: Background: Overuse of ALS-inhibiting herbicides in rice paddy fields has resulted in the selection of *Cyperus iria* L. (rice flatsedge) with a high level of resistance to this herbicide group.

Objective: This study aimed to identify mutations endowing *ALS* resistance (target-site resistance) and the involvement of a metabolic-mediated resistance mechanism of *C. iria* to ALS herbicides.

Methods: Dose-response experiments were performed to estimate GR_{50} values (GR_{50} denotes the rate at with a herbicide reduces growth by 50%). Experiments I and II were conducted in a greenhouse, with a factorial arrangement comprised of two *C. iria*populations, CYPIR-S and CYPIR-R (with and without malathion treatment), which were susceptible and resistant to ALS-inhibiting herbicides, respectively; ALS-inhibiting herbicides (bispyribac-sodium, imazapyr + imazapic, penoxsulam, and pyrazosulfuron-ethyl); and herbicide doses above and below the maximum field rates. Genomic DNA was extracted from CYPIR-S and CYPIR-R for partial sequencing of the *ALS* gene.

Results: The GR_{50} values of CYPIR-R were 400 to > 4,000 times higher than those of CYPIR-S, indicating a high level of resistance to all herbicides evaluated. Tests with bispyribac-sodium plus malathion showed that metabolism might be involved. However, dose-response curves demonstrated that a specific mutation in the *ALS* gene is the main resistance mechanism. DNA sequencing electropherogram analysis for CYPIR-R showed different nucleotide changes at Trp_{574} in the first [thymine (T) to adenine (A)] and second bases [guanine (G) to T], which can result in changes from tryptophan (TGG) to arginine (AGG), to leucine (TTG), and/or to methionine (ATG), respectively.

Conclusions: Target-site resistance was involved in the high level of resistance to ALS-inhibiting herbicides in the rice flatsedge, due to a nucleotide change resulting in an amino acid substitution at the Trp_{574} *ALS* gene.

Keywords: Acetolactate synthase, bispyribac-sodium; *Cyperus iria*, imazapyr + imazapic; penoxsulam; pyrazosulfuron-ethyl

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1. Introduction

Rice flatsedge (*Cyperus iria* L.) is an annual plant with a short cycle, tolerant to flooding (Mann et al., 2007), and can coexist with rice crops throughout the growing period (Nunes et al., 2018). This weed is a polyploid species, and some cytotypes have 14x (tetradecaploid) and 16x (hexadecaploid) genomes with n = 56, and n = 64, respectively (Bir et al., 1992). Its polyploid genome causes complex effects of the enlarged copies of each genome, which increases genetic diversity and versatility in many environmental conditions (Fox et al., 2020). Sedges can cause reduction of up to 90% in rice yields (Agostinetto et al., 2016). Among them, *C. iria* is one of the main troublesome species (Ulguim et al., 2019), requiring control measures.

Acetolactate synthase (ALS) inhibitors are the main herbicides used to control sedges in paddy fields (Schaedler et al., 2013), especially after the introduction of the Clearfield® Production System. ALS is the crucial enzyme in the biosynthetic pathway of essential branched-chain amino acids (isoleucine, valine, and leucine), and its inhibition by herbicides causes mortality of most sensitive plants (Duggleby et al., 2008). However, the selection pressure imposed by the constant use of herbicides inhibiting the same target site is decisive in the evolution of herbicide resistance (Norsworthy et al., 2012). This begins with the survival of individuals within a population that are not killed by the recommended rate of the herbicides.

Overuse of ALS-inhibiting herbicides in rice paddy fields has led to selection of *C. iria* with high levels of cross-resistance (resistance factor > 10-fold), making chemical management difficult and increasing production costs (Ulguim et al., 2019; Yu, Powles, 2014). High levels of resistance to ALS inhibitors has already been reported in *C. iria* populations for bispyribac-sodium (pyrimidinyl benzoate - PB), imazamox/imazethapyr (imidazolinone - IMI), penoxsulam (triazolopyrimidine, type 2 - TP) and halosulfuron/ pyrazosulfuron-ethyl (sulfonylurea - SU) (Riar et al., 2015; Chiapinotto et al., 2017). Cross-resistance, which is defined as resistance to different herbicides caused by one gene or one mechanism (Délye et al., 2013), is

an important type of weed resistance to herbicides (Yu, Powles, 2014). This high level of resistance is often caused by some specific mutations at the site of action (Target-Site Resistance – TSR) (Yu, Powles, 2014).

ALS is vulnerable to single nucleotide polymorphisms (SNPs), in which a single nucleotide change resulting in a non-synonymous alteration can cause amino acid substitution, altering the enzyme's structure and preventing herbicide binding (Ntoanidou et al., 2016). Apart from target-site point mutations, which can lead to cross-resistance, Non-Target-Site Resistance (NTSR), along with accelerated metabolism, is also a prominent mechanism related to herbicide resistance (Riar et al., 2012; Iwakami et al., 2013; Busi et al., 2017). Cytochrome P_{450} monooxygenase proteins (P_{450}) can metabolize ALS herbicides, reducing the amount of herbicide reaching the site of action (Iwakami et al., 2013). When this NTSR is involved, the resistance factor that controls the resistant biotype is lower (< 10-fold) than some mutations at *ALS* target sites that confer high levels of resistance (Heap, Duke, 2017). Thus, *in vivo* assays are required to determine the dose to control (C_{50}) or cause growth reduction (GR_{50}) by 50% in relation to the resistant and sensitive biotypes (Yu, Powles, 2014).

TSR, and in particular a $Trp_{-574-}Leu$ mutation in the *ALS* gene, has previously been identified as the mechanism conferring high cross-resistance to ALS-inhibiting herbicides in *C. iria* in the middle-southern USA (Riar et al., 2015). In addition, no evidence of enhanced metabolism was found. In another study, a precursor to the current one, Chiapinotto et al. (2017) also reported high cross-resistance in a *C. iria* population, indicating the presence of a possible mutation in the *ALS* gene. However, mutations in some species of the Cyperaceae family are not easily identifiable, requiring sequencing analysis (Scarabel et al., 2010; Tehranchian et al., 2014). Furthermore, TSR and NTSR mechanisms can sometimes coexist (Brosnan et al., 2016), in which case it is essential to implement alternative management strategies. This study aimed: 1) to evaluate herbicide metabolism as a mechanism of resistance to ALS inhibitors in a *C. iria* population in southern Brazil with confirmed resistance to this herbicide group; and 2) to partially sequence the *ALS* gene, to further understand the underlying mechanisms of *C. iria* resistance to ALS inhibitors.

2. Material and Methods

2.1 Plant Material

Resistant (CYPIR-R – 28°55'42"S, 56°11'18"W) and sensitive (CYPIR-S – 29°09'43"S, 56°33'06"W) *C. iria* biotypes from a paddy field in southern Brazil were used. In a previous study, Chiapinotto et al. (2017) determined the resistance pattern of this population, demonstrating a high level of cross-resistance (> 10-fold) of CYPIR-R to the herbicides bispyribac-sodium (pyrimidinyl benzoate - PB), imazethapyr (imidazolinone - IMI), penoxsulam (triazolopyrimidine, type 2 - TP) and pyrazosulfuron-ethyl (sulfonylurea - SU). The two biotypes were identified and voucher specimens were deposited in the herbarium of the Department of Biology, Federal University of Pelotas (Herbário Pel), Rio Grande do Sul, Brazil, under accession numbers 27,049 (CYPIR-S) and 27,050 (CYPIR-R).

2.2 Assays with ALS-inhibiting herbicides and interaction with malathion

Two greenhouse experiments were performed. The experimental design was completely randomized, with four replicates. The factorial arrangement comprised: 1) CYPIR-S and CYPIR-R biotypes – both with and without malathion, which was used as an indicator to detect metabolic resistance (Riar et al., 2012); 2) ALS-inhibiting herbicides: bispyribac-sodium (Nominee, 400 g a.i. L^{-1}, SC); imazapyr + imazapic (Kifix, 525 + 175 g a.i. Kg^{-1}, WG); pyrazosulfuron-ethyl (Sirius, 250 g a.i. L^{-1}, SC) and penoxsulam (Ricer, 240 g a.i. L^{-1}, SC), with addition to each herbicide of the respective recommended adjuvant; and 3) Herbicide doses of: 0, 0.0039, 0.0078, 0.0156, 0.0312, 0.0625, 0.125, 0.25, 0.5 and 1x for CYPIR-S; and 0, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128x for CYPIR-R (imazapyr + imazapic); and 0, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8 and 16x for the other herbicides. The maximum recommended field rates (dose X) for control of *C. iria* by each herbicide are presented in Table 1.

In October 2018, CYPIR-R and CYPIR-S seeds were placed in 0.05 L plastic pots containing a commercial substrate for germination. When the seedlings were at the one-leaf stage, they were transplanted into 0.5 L pots (two plants per pot) containing sieved soil classified as "haplic planosol" (identified as a silty loam soil type according to texture). When the plants reached 3-4 leaves, herbicides

Table 1 - ALS-inhibiting herbicides according to active ingredient (a.i.), chemical family (acronyms in parentheses), trade name, dose x (g a.i. ha^{-1}), and adjuvants (v/v)

Active ingredient	Chemical family	Trade name	Dose x[1]	Adjuvant
Bispyribac-sodium	Pyrimidinyl benzoate (PB)	Nominee	50	Iharol 0.25%
Imazapyr + imazapic	Imidazolinone (IMI)	Kifix	73.5 + 24.5 (98)[2]	Dash 0.5%
Pyrazosulfuron-ethyl	Sulfonylurea (SU)	Sirius	20	Iharol 0.25%
Penoxsulam	Triazolopyrimidine (TP)	Ricer	36	Veget oil 1%

[1] The maximum field rate recommended for the control of *Cyperus iria*. [2] Dose x is the sum of imazapyr + imazapic (73.5 g a.i. ha^{-1} + 24.5 g. a.i. ha^{-1} = 98 g a.i. ha^{-1}).

Table 2 - Estimated parameters *b*, *d*, GR_{50} (g a.i. ha^{-1}) and resistance factor (RF)[1] by the non-linear mixed-effect equation[2], based on shoot dry weight (% of the untreated control), for resistant (CYPIR-R with and without malathion treatment) and sensitive (CYPIR-S with and without malathion treatment) *Cyperus iria* biotype, 28 days after herbicides treatment

Herbicide	Biotype			
Bispiribac-sodium	**CYPIR-R**	**CYPIR-R + Mal**	**CYPIR-S**	**CYPIR-S + Mal**
b	2.41 ± 0.67**	2.05 ± 0.63**	2.08 ± 0.41***	1.89 ± 0.45**
d	95.47 ± 4.64***	91.33 ± 4.72***	101.04 ± 4.17***	99.99 ± 3.52***
GR_{50}	116.90 ± 6.99***	50.81 ± 2.29***	0.27 ± 0.07**	0.13 ± 0.07*
RF	-	-	432.96	-
Imazapyr + imazapic[(3)]	CYPIR-R	CYPIR-R + Mal	CYPIR-S	CYPIR-S + Mal
b	0.70 ± 0.22**	0.55 ± 0.17**	2.04 ± 0.37***	1.30 ± 0.20***
d	112.36 ± 3.50***	110.26 ± 4.00***	100.41 ± 3.73***	99.42 ± 6.06***
GR_{50}	> 25088.00[ns]	> 25088.00[ns]	6.16 ± 0.67***	1.05 ± 0.17***
RF	-	-	> 4072.72	-
Penoxsulam	CYPIR-R	CYPIR-R + Mal	CYPIR-S	CYPIR-S + Mal
b	4.09 ± 1.13**	2.76 ± 0.76**	2.19 ± 0.61**	2.13 ± 0.62**
d	100.62 ± 4.64***	100.11 ± 4.54***	103.64 ± 4.79***	100.08 ± 4.54***
GR_{50}	335.75 ± 16.53***	311.42 ± 14.27***	0.26 ± 0.06**	0.13 ± 0.06*
RF	-	-	1291.35	-
Pyrazosulfuron-ethyl	CYPIR-R	CYPIR-R + Mal	CYPIR-S	CYPIR-S + Mal
b	1.26 ± 0.50*	1.37 ± 0.50*	2.09 ± 0.48***	2.10 ± 0.60**
d	103.40 ± 5.47***	103.75 ± 4.57***	99.91 ± 1.88***	99.96 ± 1.80***
GR_{50}	117.76 ± 18.93***	105.00 ± 14.65***	0.06 ± 0.02**	0.04 ± 0.02*
RF	-	-	1962.67	-

[1] RF = GR_{50} CYPIR-R/ GR_{50} CYPIR-S; [2] Y = d/ (1+ exp[b(log(x) – log (e))]; [3] The GR_{50} value is the sum of dose x of imazapyr + imazapic (73.5 + 24.5 = 98 g a.i. ha^{-1}); *(p. < 0.1); **(p < 0.01); *** (p < 0.001).

were applied. Malathion treatment (1,000 g a.i. ha^{-1}) was applied 30 minutes before the herbicides (Zhang et al., 2017). All chemical treatments were applied with a CO_2 pressurized sprayer equipped with flat fan nozzles (XR 110.015 model, 0.5 m apart). The application was performed at 250 kPa working pressure, delivering 150 L ha^{-1} of carrier volume. Nozzles were positioned at 0.5 m from the top of the leaves. During application the average temperature was 18.7 °C, relative humidity (RH) was 68.2%, and wind speed was 2.52 km h^{-1} (monitored with a digital Kestrel® 4500 Weather Meter).

In February 2019, the experiment was repeated, following the same factorial scheme and procedures. In order to obtain a better fit of the dose-response curves, and mainly the GR_{50} value of the herbicide imazapyr + imazapic, herbicide doses were adjusted for the CYPIR-R to 0, 1, 2, 4, 8, 16, 32, 64, 128 and 256x for all herbicides. The average temperature was 20.5 °C, RH was 82.3%, and average wind speed was 5.04 Km h^{-1}.

In each experiment, a visual evaluation of control was performed 28 days after treatment (DAT), based on a percentage scale, where 0% indicated no effect and 100% denoted total weed death (data not shown). The remaining plants were cut close to the ground and placed in a forced-air oven at 60 °C until they reached a constant weight to determine the shoot dry weight (SDW).

2.3 ALS Gene Sequencing

Genomic DNA (gDNA) was extracted from 100 mg of leaf tissue from six plants of CYPIR-R and one plant of CYPIR-S, using the Wizard Genomic DNA Purification Kit (Promega) – measured by DeNovix (Wilmington, Delaware). Polymerase Chain Reactions (PCR) were performed in 25.0 µL samples containing 2.0 µL of template DNA (approximately 40 ng), 5.0 µL of 5X GoTaq buffer, 0.2 µL of 5 U $µL^{-1}$ GoTaq G2 Hot Start Polymerase (Promega), 0.5 µL of 10 mM dNTPs, 0.5 µL of each forward and reverse primer at 10 µM, 1.5 µL of 25 mM $MgCl_2$, and 14.8 µL of nuclease-free water. The PCR cycling was performed with a T100 Thermal Cycler (Bio-Rad) and was composed of an initial denaturing step of 95 °C for 2 min, followed of 40 cycles at 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, with a final extension step at 72 °C for 5 min. Amplified fragment size was verified on 1% agarose gel using a 100 base pairs (bp) molecular weight (ladder, Sinapse).

The previously published forward F22 (TGTTCCAACTCAGGTGCAGA) and reverse R22 (ATCATATCCTTGAATGCTCCTC) primers (Riar et al., 2015) were used for amplification of a 1,055 bp ALS fragment (Figure 1). PCR products were purified (ExoSAP-IT™ Express reagent – Thermo Fisher), 10X diluted, and 1 µL was used in the sequencing reactions. These reactions were performed with the BigDye cycle sequencing terminator kit (Thermo Fisher), and sequencing was performed with an Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher). The partial *ALS* sequences of CYPIR-S and CYPIR-R were uploaded to the GenBank of the National Center for Biotechnology Information (NCBI) under accession numbers OP503617 and OP503618, respectively. All sequence electropherograms were analyzed and aligned with the gene sequences encoding the *ALS* enzyme of *Arabidopsis thaliana* L. (X51514) and *Cyperus brevifolius* (Rottb.) (AB719979.1), using the open-source bioinformatics software UGENE (Unipro, 2012).

A sequence of 966 bp was amplified in resistant and sensitive samples. All seven samples from CYPIR-R and CYPIR-S plants were in alignment with the gene encoding the *ALS* enzyme in *A. thaliana*, indicating that sequencing covered the amino acids (Aas) from 390 to 599, where the domains β (Aas 281-451) and γ (Aas 463-639) are partially found (McCourt et al., 2006). Within these domains is a conserved region named B [Aas 573-576], covering the position Trp_{574}, where mutations related to resistance to ALS-inhibiting herbicides occur (Riar et al., 2015).

2.4 Statistical Analysis

The dose-response SDW data are reported as percentage in relation to the SDW of the untreated control, and were evaluated using the R program (R Core Team, 2019). The *medrc* and *drc* packages were used to create dose-response curves with the non-linear mixed-effects model, assuming a three-parameter log-logistic equation with a separate set of *b*, *d*, and *e* parameters for each of the biotypes (Equation 1). Individual assay effects were included on these parameters to model the between-assay variability using the metadrm function, with the arguments cid2 (as a factor identifying individual curves) and ind (as a factor containing the between-curve grouping of the data).

$$Y = \frac{d}{1 + \exp\{b(\log(x) - \log(e))\}} \quad \text{(Equation 1)}$$

where Y is the SDW reduction (%); *e* is the GR_{50} value (the dose that causes 50% SDW reduction); *d* is the upper limit; and *b* indicates the slope around *e* (Ritz et al., 2019).

The metadrm (using drm and summary functions) estimated the parameters and their standard errors, where p-values ($p < 0.05$) indicated significance of the parameters. GR_{50} and the resistance factors (RF = GR_{50} CYPIR-R/ GR_{50} CYPIR-S) were estimated by the ED and EDcomp functions (95% confidence intervals). We fit a joint model based on the entire dataset including both experiments to create the figures and to test the lack of fit of the model (when not significant, the regression analysis was accepted as describing the variation of data similar to ANOVA).

3. Results and Discussion

3.1 Whole plant assays

The assays detected differences between CYPIR-R and CYPIR-S populations regarding all evaluated herbicides. For the CYPIR-R, the doses needed to reduce SDW by 50% (GR_{50}) were 2, > 256, 9, and 6 times above the maximum field rate for bispyribac-sodium, imazapyr + imazapic, penoxsulam and pyrazosulfuron-ethyl, respectively. The GR_{50} values of CYPIR-R were approximately 400 to > 4,000 times higher than those of CYPIR-S (Table 2; Figures 2a-d). These results demonstrate high levels of cross-resistance (Yu, Powles, 2014; Heap, Duke, 2017).

In the absence of herbicides, treatment with malathion alone did not reduce the SDW compared to the untreated control, either for the CYPIR-R or CYPIR-S biotypes (data not shown), which is consistent with the results of Zhao et al. (2017). The GR_{50} values for CYPIR-S + malathion decreased only for imazapyr + imazapic in

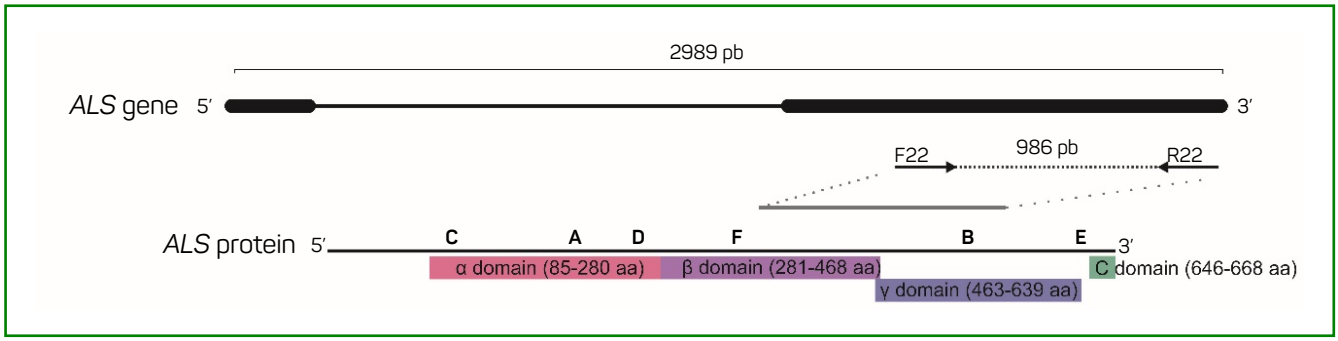


Figure 1 - Strategy for partial sequencing of the coding gene for the *ALS* enzyme, with forward (F22) and reverse (R22) primers used to amplify and sequence the enzyme in *Cyperus iria L.* biotypes sensitive and resistant to ALS-inhibiting herbicides. Scheme adapted from Riar et al. (2015)

comparison with CYPIR-S (Figure 2b). Similar results were obtained by Owen et al. (2012). Furthermore, malathion reduced the dose for CYPIR-R control by bispyribac-sodium. The GR_{50} value for CYPIR-R was 117 g a.i. ha^{-1}, while the value for CYPIR-R + malathion was 51 g a.i. ha^{-1} (Table 2; Figure 2a).

The dose-response curve indicated a high and broad cross-resistance of CYPIR-R to ALS-inhibiting herbicides belonging to the chemical groups of SU, IMI, triazolopyrimidine (TP – type 2), and PB. Similar results were reported by Chiapinotto et al. (2017). Malathion was used as an indicator to detect metabolic resistance in the resistant biotype (Riar et al., 2012) and may or may not have an effect on the sensitive biotype (Owen et al., 2012; Zhao et al., 2017). Malathion is an organophosphate insecticide used as an indicator of enhanced metabolism in NTSR (Wang et al., 2018), since it inhibits P_{450} activity (Riar et al., 2012; Iwakami et al., 2013). Malathion's chemical structure has a phosphorus atom covalently linked to sulfur or oxygen, and reduces herbicide doses due to competition in P_{450}-mediated reactions and oxidative desulfurization (Busi et al., 2017).

Adding malathion to bispyribac-sodium, imazethapyr and penoxsulam in *Echinochloa crus-galli* increased the efficiency of the control of the resistant biotypes (Riar et al., 2012). In *Echinochloa glabrescens*, pretreatment with malathion also reduced the control dose of the penoxsulam-resistant biotype (Yan et al., 2019). In the present study, malathion reduced the control dose of bispyribac-sodium, indicating that its metabolism might be involved. However, studies are needed to assess whether there is overexpression of the transcription of genes coding for P_{450} enzymes and/or the identification of herbicide metabolites (Yasuor et al., 2009; Iwakami et al., 2013). The absence of plant growth reduction with application of the other herbicides plus malathion could have been due to the presence of P_{450} isozymes (Riar et al., 2015). However, when only

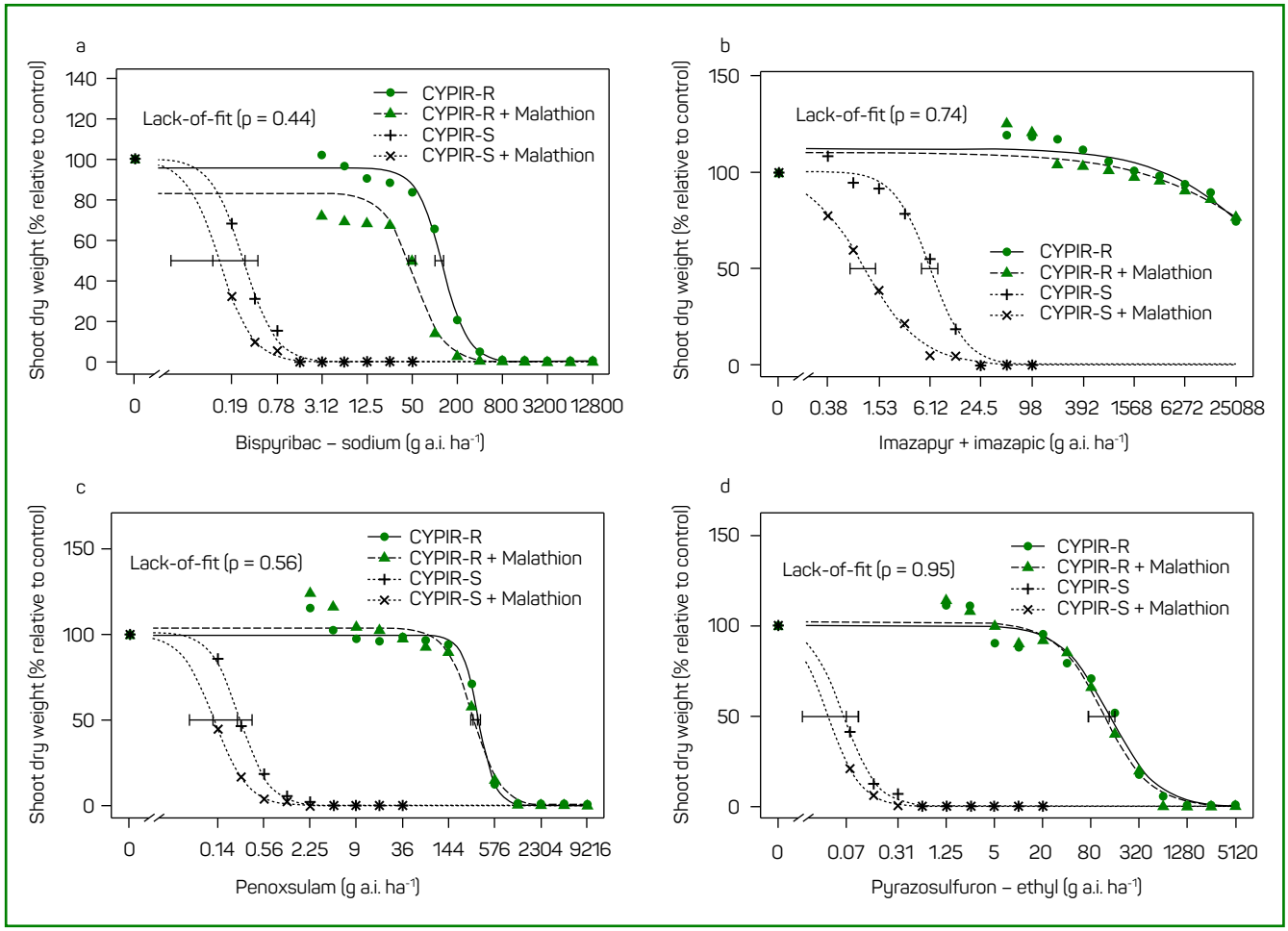


Figure 2 - Average of observed and adjusted values of shoot dry weight (% relative to untreated control) of resistant (with and without malathion pretreatment – Δ CYPIR-R + Malathion and • CYPIR-R, respectively) and sensitive (with and without malathion pretreatment - + CYPIR-S and x CYPIR-S + Malathion, respectively) *Cyperus iria L.* biotypes, at 28 days after treatment with bispyribac-sodium (a), imazapyr + imazapic (b), penoxsulam (c) and pyrazosulfuron-ethyl (d) - by the joint model based on the entire dataset. The horizontal bar represents the 95% confidence interval to obtain 50% shoot dry weight reduction (GR_{50})

enhanced metabolism is involved, the resistance factor for controlling the resistant population is lower (<10-fold) than some mutations at the *ALS* target site that confer high levels of resistance (Heap, Duke, 2017).

3.2 ALS gene sequencing

Alterations in the Trp_{574} position were detected in three samples of the CYPIR-R biotype (Figure 3). DNA electropherogram analysis of the three samples of CYPIR-R showed the presence of double peaks at position Trp_{574}. This overlap demonstrates changes in the first [thymine (T) to adenine (A)] and/or second nucleotide bases [guanine (G) to T]. Thus, different nucleotide changes are present at position 574 of the *ALS* gene and can result in changes from tryptophan (TGG) to arginine (AGG), to leucine (TTG), and/or to methionine (ATG) – Trp_{-574-}Arg, Trp_{-574-}Leu, and Trp_{-574-}Met, respectively (Figure 4). However, this study did not distinguish whether the mutation found was an accumulation of the single nucleotide substitution codons of AGG and TTG, or an accumulation of the double nucleotide substitution codon ATG in the wild-type codon TGG. *C. iria* may be a hexadecaploid species (Bir et al., 1992). Thus, even using cloning techniques (Lamego et al., 2009), precise determination of allelic variants that endow resistance is very difficult.

High doses of ALS-inhibiting herbicides applied in rice fields tend to select weed plants with mechanisms that confer elevated resistance (Ulguim et al., 2019). High cross-resistance (> 10-fold), like the CYPIR-R, is a result of mutations at the site of action (TSR) (Yu, Powles, 2014). The herbicides do not bind directly to the active site of the ALS enzyme, but are able to block access to the substrate (Duggleby et al., 2008). The substitution of amino acids in conserved regions maintains the enzyme's functionality without pleiotropic effects but compromises the binding of herbicides, conferring high cross-resistance (Yu, Powles, 2014).

Single nucleotide substitution is common and can change the amino acid sequence of ALS (Panozzo et al., 2013), but double nucleotide substitution can also occur (Brosnan et al., 2016; Tehranchian et al., 2015; Palma-Bautista et al., 2022). In *Echinochloa crus-galli*, *Papaver rhoeas*, *Cyperus difformis*, *Poa annua*, *Lolium perene,* and *Cyperus iria*, alterations in Ala_{-122-}Asn (Panozzo et al., 2017), Pro_{-197-}Phe (Palma-Bautista et al., 2022), Pro_{-197-}His (Tehranchian et al., 2015), Ala_{-205-}Fen (Brosnan et al., 2016), Asp_{-376-}Glu (Menegat et al., 2016) and Trp_{-574-}Leu (Riar et al., 2015), respectively, caused broad and high cross-resistance to ALS-inhibiting herbicides.

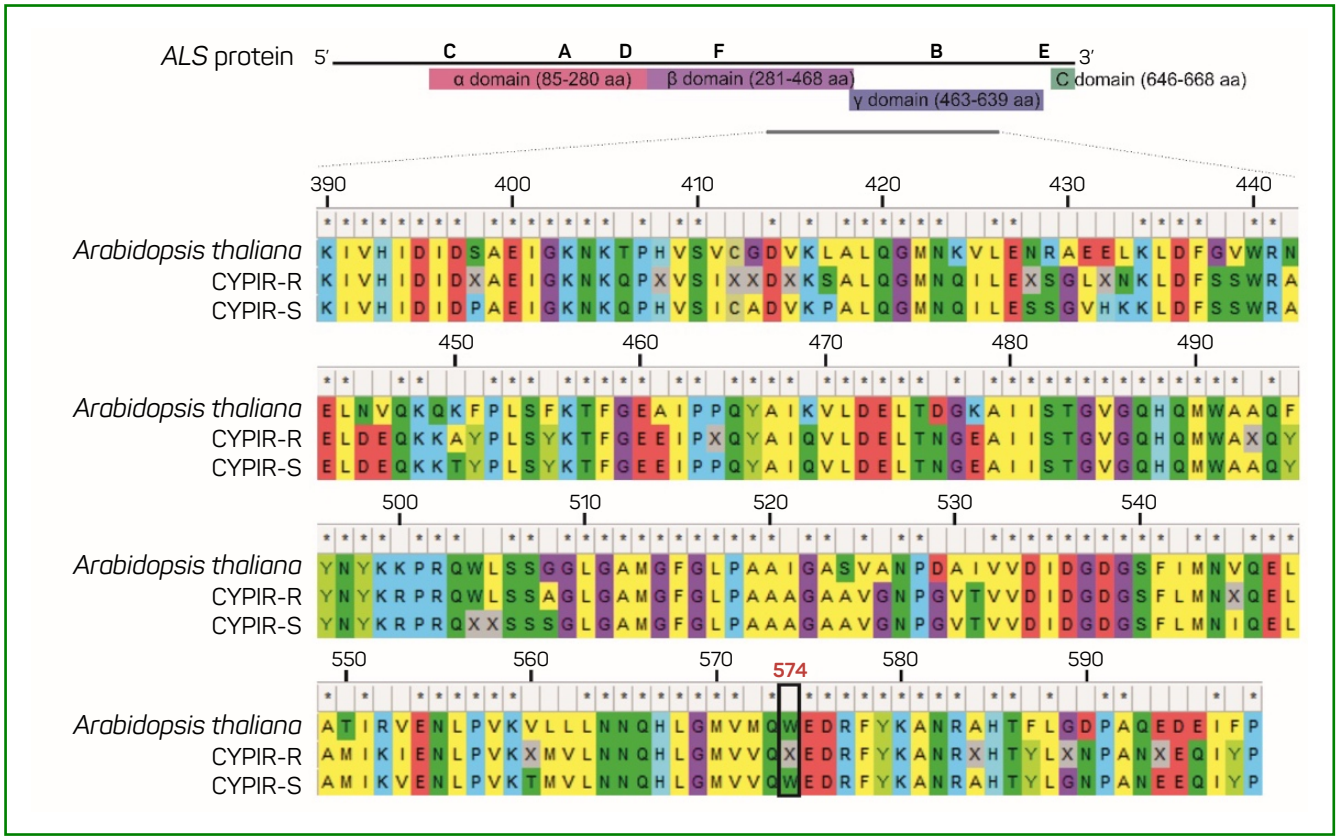


Figure 3 - Alignment of the partial amino acid sequence of *Cyperus iria L. ALS* protein resistant (CYPIR-R – OP503618) and sensitive (CYPIR-S – OP503617) biotypes to ALS-inhibiting herbicides with *Arabidopsis thaliana* L. (X51514). Alignment cover gene position where resistance-associated mutations occur: Trp_{574}. The CYPIR-R showed alteration at Trp_{574}, resulting in changes from W (tryptophan) to X (arginine, leucine, and/or methionine)

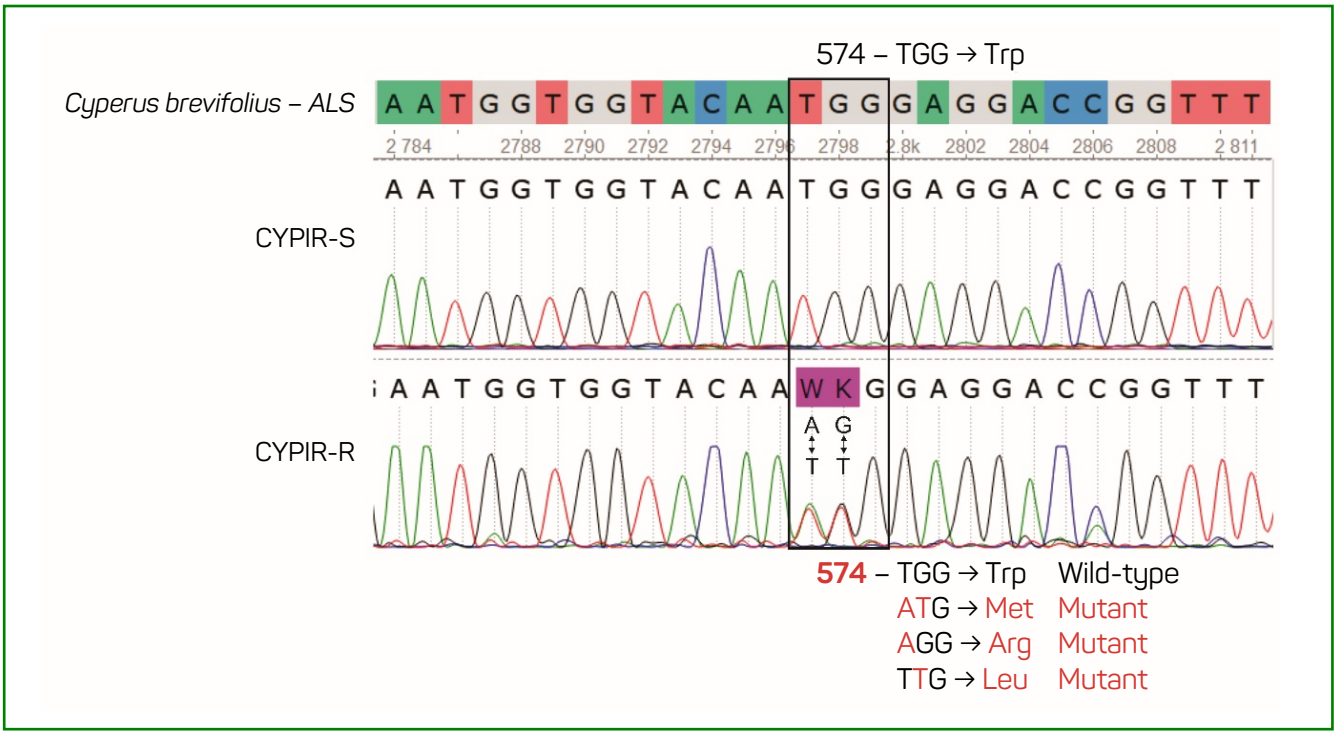


Figure 4 - Resistant and sensitive *Cyperus iria L* DNA electropherogram for the *ALS* gene. The overlapping of peaks in CYPIR-R at position Trp_{574} shows changes in the first (thymine to adenine) and in the second (guanine to thymine) nucleotide base, that is, changing tryptophan (TGG) to leucine (TTG), to arginine (AGG), and/or to methionine (ATG)

Changes in the positions mentioned above explain the high cross-resistance of CYPIR-R. Among these, the Trp_{-574-}Leu mutation occurs the most and results in a high cross-resistance to all chemicals in this group evaluated in this study. It has already been associated with cross-resistance in *C. iria* (Riar et al., 2015). Furthermore, in *Digitaria sanguinalis* (Li et al., 2017), a non-synonymous mutation caused the change from tryptophan 574 to arginine. In *Apera spica-venti* (Hamouzová et al., 2014), a double nucleotide change caused the alteration from tryptophan 574 to methionine, respectively, leading to ALS resistance.

In some species of the Cyperaceae family, including the *Cyperus* genus, mutations are not easily identifiable due to the level of ploidy, the occurrence of additional genes, or the presence of alleles (Scarabel et al., 2010; Tehranchian et al., 2014; Riar et al., 2015). In *C. esculentus*, no mutation was identified in DNA sequencing (Tehranchian et al., 2014). However, in the electropherogram analysis, double peaks were observed at the second base of codon Trp_{574}, indicating a change from G to T, changing tryptophan to leucine. The results showed heterozygosity or gene duplication (Tehranchian et al., 2014). Similar results were found in polyploid *Echinochloa crus-galli* (Panozzo et al., 2013), *Poa annua* (McElroy et al., 2013) and *Alopecurus japonicus* (Zhao et al., 2019). Thus, at the position of the mutant nucleotides, there are double peaks in the resistant biotype but single ones in the sensitive biotype.

Double peaks were observed in the first and second nucleotide bases of CYPIR-R. A similar result was found in glyphosate-resistant polyploid *E. colona* plants with double nucleotide peaks at the *EPSPS* gene, indicating that mutations can occur (e.g., Pro_{-106-}Leu, Pro_{-106-}Thr and Pro_{-106-}Ile) (Han et al., 2016). Considering the complexity of the *C. iria* genome, reported as a polyploid species with biotypes showing 14x and 16x genomes with n = 56, and n = 64 (Bir et al., 1992), that variation in chromosome number may be associated with the differences among biotypes, intraspecific variation (Bir et al., 1992), and natural hybridization among *C. iria* and other *Cyperus* species (Chozin, Yasuda, 1991). Also, many *Cyperus* species have been reported to have cross-pollination reproduction (Ihenetu et al., 2021) and agmatoploidy that occurs in species that have so-called holocentric chromosomes in which chromosomal breaks are recurrent (Arias et al., 2011). The *C. iria* genome has not been sequenced yet. Thus, there is no information related to copy number of the *ALS* gene in the genome. Its polyploid genome leads to the complex effects of the enlarged copies of each genome, which improves genetic diversity and versatility in many environmental conditions (Fox et al., 2020).

Polyploid species can accumulate more than one mutation at the same position. In *Capsella bursa-pastoris* (L.) Medik., two mutations at position 197 (Pro_{-197-}Ser and Pro_{-197-}His) were identified (Wang et al., 2019). According to the level of ploidy, certain species have a variable number of *ALS* genes and may have mutations in different positions. In *Schoenoplectus mucronatus*, three genes encoding ALS were identified. Mutations that confer resistance were related to ALS1, with alterations in Pro_{-197-}His and

Trp_{-574-}Leu (Scarabel et al., 2010). Similar results were also found in *S. juncoides*, with two genes coding for ALS, each responsible for a mutation associated with resistance (Yamato et al., 2013). Thus, it is not possible to rule out the presence of mutations in positions not covered by the CYPIR-R sequencing, corroborating the results found in this study.

4. Conclusions

TSR was involved in the high and broad cross-resistance of the *Cyperus iria* population, due to nucleotide changes that can result in an amino acid substitutions at the Trp_{574} of *ALS* gene. This is the first report of the resistance mechanism to ALS-inhibiting herbicides in a *C. iria* population occurring in paddy rice fields in Brazil. Practices such as reducing selection pressure, using herbicides with different mechanisms of action or herbicide mixtures, as well as crop rotation are recommended for management of the species.

Authors' contributions

All authors read and agreed to the submitted version of the manuscript. DMC, LAA, CES, SMM, CO, VEV, ERC: conceptualization of the manuscript and development of the methodology. DMC, CES, SMM, CO, VEV: data collection and curation. DMC, CES, SMM, CO, VEV: data analysis. LAA, ERC: data interpretation. DMC, LAA, CES, SMM, CO, VEV, ERC: funding acquisition and resources. DMC, LAA, CES, SMM, CO, VEV, ERC: project administration. LAA, CES, ERC: supervision. DMC: writing the original draft of the manuscript. DMC, LAA, CES, SMM, CO, VEV, ERC: writing, review and editing.

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