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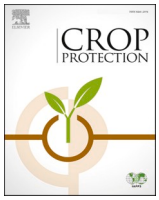

Application of ALS inhibitors at pre-emergence is effective in controlling resistant barnyardgrass biotypes depending on the mechanism of resistance

Guilherme Menegol Turra [a], Luan Cutti [a,1], Filipi Mesquita Machado [a], Gabriel Machado Dias [a], André Andres [b], Catarine Markus [a], Aldo Merotto Jr. [a,*]

[a] Crop Science Department, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Brazilian Agricultural Research Corporation, Pelotas, RS, Brazil

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ABSTRACT

Echinochloa species are one of the most troublesome weeds in rice crops, and their control is hampered due to herbicide resistance. The aim of this study was to identify the cross-resistance pattern and the differential resistance level for pre- and post-emergence applications of imazethapyr and penoxsulam in populations of barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.] with different *ALS* gene mutations. Of 26 biotypes, 23 were imazethapyr-resistant, and 10 were cross-resistant to penoxsulam. The resistance index (RI) to imazethapyr was 5.7–19.5 for Ser653Asn, 26.7–68.3 for Ala122Thr and Ala205Asn, and 70.9–252.9 for Trp574Leu. Only Trp574Leu also resulted in resistance to penoxsulam. The double ALS mutation Ala122Asn + Trp574Leu resulted in a RI for imazethapyr and penoxsulam higher than 2800. The ED_{50} for penoxsulam applied at pre-emergence was three and six times lower than at post-emergence for the susceptible and resistant biotypes, respectively. The application of imazethapyr at pre-emergence was more effective than at post-emergence only for the biotypes with low resistance level mutation Ser653Asn. The efficacy of the herbicide quinclorac was similar for the application at pre- and post-emergence for susceptible and resistant biotypes. The ALS mutations Ala205Asn and Ala122Asn + Trp574Leu are reported for the first time in barnyardgrass. The use of ALS inhibitors at pre-emergence should be prioritized over post-emergence in fields with resistant barnyardgrass and in need of using these products to control other non-resistant weeds.

1. Introduction

Herbicide inhibitors of the acetolactate synthase (ALS, HRAC group 2) enzyme (EC 4.1.3.18) are among the world's most important products for weed control. Unfortunately, herbicide resistance has been the Achilles' heel of these compounds (Tranel and Wright, 2002). In rice, the first ALS inhibitor available for grass control was bispyribac-sodium, a pyrimidinyl benzoate, in the 90s, followed by penoxsulam, a triazolopyrimidine-type 2 (Bond et al., 2007) and several other sulfonylurea compounds most frequently applied at post-emergence. The use of ALS inhibitors in rice increased with the development of imidazolinone-resistant rice varieties in the early 2000s (Goulart et al., 2012). In this system, imidazolinones are recommended at both pre- and post-emergence to control broadleaf and grasses, but the use of these herbicides were prioritized at post-emergence due to ease of application facilities. Another common herbicide applied at post-emergence in paddy fields is quinclorac, which belongs to the quinoline-carboxylate group and inhibits cellulose synthesis (HRAC group 29) in grasses. Although these herbicides are effective in several paddy weeds, their most important targets are species of the genus *Echinochloa*, the most troublesome weeds in rice crops.

Echinochloa comprises at least 40 species, including the harmful weeds *E. crus-galli* (L.) Beauv., *E. colona* (L.) Link, and *E. phyllopogon* (Stapf) Koss. (synonymy *E. oryzicola* Vasinger) (Hoste and Verloove, 2022), commonly known as barnyardgrass, junglerice, and late watergrass, respectively. The first two species are hexaploid and have three copies of the *ALS* gene, whereas the last is tetraploid (Wu et al., 2022). These species can cause severe rice yield losses, and their control is highly dependent on herbicide applications (Rao, 2021). The reliance on herbicides has led to a rapid evolution of herbicide resistance in these

* Corresponding author. Crop Science Department, Federal University of Rio Grande do Sul, 7712 Bento Gonçalves Avenue, PO Box 15100, Porto Alegre, RS, 91501-970, Brazil.
E-mail address: merotto@ufrgs.br (A. Merotto Jr.).
[1] Current address: Plant, Soil and Microbial Sciences Department, Michigan State University, Michigan, United States of America.

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three species, which continues to increase in rice fields around the world (Damalas and Koutroubas, 2023). The largest number of herbicide-resistant cases in grasses occur in *Lolium* spp., *Echinochloa* spp., and *Alopecurus myosuroides* Huds., but the complexity of resistance is larger in *Echinochloa* spp., as they occur in heavy infestations in both tropical and temperate areas and are polyploid. Herbicide resistance in weeds is a major concern for crop management (Hulme, 2023), and new studies are required to mitigate its consequences for food production and security.

Herbicide resistance can evolve in weeds through a series of strategies related to target site (TS) or non-target site (NTS) mechanisms (Gaines et al., 2020). As of this date, TS ALS inhibitor resistance can be caused by mutations that result in substitutions in amino acid positions Ala122, Pro197, Ala205, Phe206, Asp376, Arg377, Trp574, Ser653, and Gly654 (Fang et al., 2022; Murphy and Tranel, 2019) [positions based on *Arabidopsis thaliana* (L.) Heynh. ALS sequence]. Among them, single amino acid substitutions in positions Ala122 (Riar et al., 2013), Pro197 (Löbmann et al., 2021), Ala205 (Fang et al., 2019a), Phe206 (Fang et al., 2022), Asp376 (Löbmann et al., 2021), Trp574 (Panozzo et al., 2013), and Ser653 (Matzenbacher et al., 2014) are reported to cause ALS-inhibitor herbicide resistance in *Echinochloa* spp. Additionally, NTS mechanisms, especially metabolism-based, can cause resistance to ALS inhibitors and quinclorac in *Echinochloa* species, although quinclorac resistance mechanisms are not fully understood (Chayapakdee et al., 2020; Fang et al., 2019b; Iwakami et al., 2019; Yang et al., 2021). Furthermore, some populations present both TS and NTS resistance mechanisms to ALS inhibitors (Fang et al., 2019b; Feng et al., 2022).

Integrated weed management strategies, such as rotation of herbicide mode of action and non-chemical control, are fundamental to preventing and controlling herbicide-resistant weeds. It is well known that the different amino acid substitutions associated with TS resistance to ALS inhibitors cause different resistance levels and cross-resistance patterns among the five classes of ALS inhibitors (Löbmann et al., 2021). These herbicides are considered modern compounds due to low mammalian toxicity and environmental risk, use at low doses, and a broad spectrum of weed control (Jeschke et al., 2019). Therefore, it is unreasonable to eliminate all ALS inhibitors in situations where a certain biotype evolved herbicide resistance because susceptibility can still exist to products of the same or different classes of ALS inhibitors (Merotto et al., 2010). In addition, the site of uptake related to application at pre- or post-emergence may differentially affect the availability of the herbicide and the survival of herbicide-resistant plants. Herbicide application at post-emergence occurs ideally in plants at the three-to four-leaf stage, resulting in a unique time-point interaction with the target plant. Otherwise, application at pre-emergence affects young emerging tissues and results in the continuous availability of the herbicide. Observations in rice fields in southern Brazil indicated that, in some fields, the application of ALS inhibitors at pre-emergence resulted in satisfactory control of *Echinochloa* spp. populations resistant to these compounds applied at post-emergence. However, the same procedure did not lead to the same results in other fields that were also infested with *Echinochloa* spp. resistant to ALS inhibitors. This introduces the present study hypothesis, whereby the variability of the effect of ALS inhibitors applied at pre- or post-emergence may be related to the resistance level, and the high frequency of ALS-inhibiting herbicide resistance in *Echinochloa* species in southern Brazil is associated with not only one but different *ALS* gene mutations. Therefore, this study aimed to identify the *ALS* gene mutations and corresponding herbicide resistance patterns to imazethapyr and penoxsulam applied at pre- and post-emergence in biotypes of *E. crus-galli*.

2. Material and methods

2.1. Seed source and plant material

Seeds from 26 *Echinochloa* biotypes (Table S1) were collected from paddy fields that had historically used ALS inhibitors in southern Brazil between the 2010/11 and 2019/20 summer seasons. The biotypes were self-pollinated for at least two generations to obtain a high homozygosity level. Seeds were stored in paper bags at 4 °C until use. *Echinochloa* species were identified based on morphological traits (Tabacchi et al., 2006). The experiments were conducted in a greenhouse at the Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil (30°4′29.66 ″S, 51°7′59.94 ″W). The greenhouse was maintained at 25 ± 5 °C, air humidity over 80%, and light-supplemented with sodium-vapor lamps delivering between 500 μmol m^{-2} s^{-1} and 600 μmol m^{-2} s^{-1} in a 14/10h (light/dark) cycle. All the experiments were conducted in a completely randomized design with four replicates and were repeated twice, except for the primary screening evaluation.

2.2. Primary whole-plant screening

Approximately 30 seeds of each biotype were directly sown into round plastic pots (500 mL) containing a clay loam soil/potting mix (10:1 ratio), fertilized with 240 kg ha^{-1} of N and 200 kg ha^{-1} of P_2O_5 and K_2O. After germination, seedlings (±2 cm) were individually transplanted to plastic pots (200 mL) containing the same soil/potting mix substrate. The herbicides imazethapyr (Zaphir, UPL, 100 g L^{-1}) at 50 and 100 g ha^{-1} with Dash (BASF, 0.5% v/v), penoxsulam (Ricer, Corteva, 240 g L^{-1}) at 36 and 72 g ha^{-1} with Veget'Oil (Oxiquímica, 0.5% v/v), and quinclorac (Facet, BASF, 500 g kg^{-1}) at 187.5 and 375 g ha^{-1} with Assist (BASF, 0.5% v/v) were sprayed when plants were at the three-to four-leaf stage. The doses correspond to half and full-field recommended Brazilian doses, respectively. For each biotype, plants were conducted without herbicide application, which corresponds to the untreated control. Additionally, a parallel experiment was carried out to test the effect of malathion (Malathion 1000 EC, FMC, 1000 g L^{-1}) applied at 1000 g ha^{-1} 2 h prior to the application of imazethapyr at the same doses described above. The plants maintained as untreated controls were applied with malathion alone. Treatments were applied using an automated spray chamber (Generation III, Devries manufacturing) equipped with a TP9502EVS (TeeJet) nozzle and calibrated to deliver 200 L ha^{-1} at a constant pressure of 40 psi. Each treatment had four replicates. The plants were kept flood-irrigated in the greenhouse.

The evaluation of barnyardgrass control efficacy (%) was done visually 21 days after treatment (DAT), considering a 0–100 scale comparative to the untreated control, where zero means no visible symptoms and 100 means plant death. The aboveground dry biomass (g) was carried out by the harvest of fresh biomass 21 DAT. The biomass was dried at 60 °C until reaching constant weight. Statistical analyses were performed using R (R Core Team, 2021). The Shapiro-Wilk and Levene's tests were performed to check the assumption of normal distribution of residuals and constant variance of errors, respectively, for both control efficacy and aboveground dry biomass data. These data were subjected to analysis of variance (ANOVA), adopting a linear mixed model with replicates as random effects and treatments (biotypes and herbicides) as fixed effects. If treatment effects were significant, the means were separated by the Skott-Knott's test at $p \leq 0.01$ with *fat2.dic* command in *ExpDes.pt* package (Ferreira et al., 2014).

2.3. ALS gene sequencing

Plant leaf tissue (0.1 g $sample^{-1}$) was sampled from two plants of each biotype. DNA was extracted using the CTAB method (Doyle and Doyle, 1987) and diluted to a final concentration of 50 ng μL^{-1}. Three primer pairs (Table 1) were used to amplify the *ALS* gene region, which covers the codons of amino acids positions Ala122, Pro197, Ala205, Phe206, Asp376, Arg377, Trp574, Ser653, and Gly654. The primers were designed based on conserved regions of the three copies of *E. crus-galli ALS* gene sequences (NCBI LC006058.1, LC006059.1, LC006061.1) (Iwakami et al., 2015) to cover all *E. crus-galli* subgenomes, using Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3p

Table 1
Code, nucleotide sequence, melting temperature, annealing temperature, expected fragment size, and cover positions of the primers used for *E. crus-galli* and *E. colona ALS* gene sequencing.

Primer code	Sequence (5′-3′)	Tm (°C)	Ta (°C)	Fragment (bp)	Covered position[b]
EcALS2F	CATCATTGCCACTGGTGTTG	61.3	60	574	Trp574, Ser653, Gly654
EcALS1R	ATACACGGTCCTGCCATCAC	63.5			
EcALS4F	GAGTCTGCCGGGGTACATT	63.0	60	415	Asp376, Arg377
EcALS4R	CACATGTGGCTGCTTGTTCT	62.7			
EcALS6F	CGACGTCTTCGCCTACCC	63.8	69[a]	538	Ala122, Pro197, Ala205, Phe206
EcALS5R	CACCTGCTCAAGCAATTCAG	61.0			

Tm: melting temperature; Ta: annealing temperature; bp: base pairs.
[a] Initial temperature with −1.0 °C each cycle.
[b] Based on the *Arabidopsis thaliana ALS* gene.

lus/primer3plus.cgi).

PCR reactions were prepared for a final concentration of 1 x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.5 U *Taq* DNA polymerase (Invitrogen), 0.2 μM each primer, 50 ng μL^{-1} genomic DNA, and q.s. 20 μL DNAse free water. For the EcALS6F-EcALS5R primer pair, 1 x PCRx Enhancer (Invitrogen) was added to the reaction. The PCR was carried out in a thermocycler (Bio-Rad C1000) at 95 °C for 10 min; 40 cycles of 95 °C for 30 s, 60 °C for 45 s, and 72 °C for 60 s; 72 °C for 5 min for primer pairs EcALS4F-EcALS4R and EcALS2F-EcALS1R. For the EcALS6F-EcALS5R primer pair, the PCR followed the touchdown protocol of 95 °C for 10 min; 15 cycles of 95 °C for 30 s, 69 °C for 30 s with minus 1.0 °C each cycle, and 72 °C for 30 s; 25 cycles of 95 °C for 30 s, 50 °C for 45 s, and 72 °C for 60 s; 72 °C for 5 min. The PCR products were verified in agarose gel (2%) and purified using ExoSAP-IT™ (Applied Biosystems) protocol before sequencing. Sequencing was performed in an ABI 3730 equipment (Applied Biosystems) using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The sequences were aligned with the *A. thaliana ALS* gene as reference (NCBI AY124092.1), and chromatograms were analyzed using SnapGene® (V. 6.2.1, Dotmatics, https://www.snapgene.com/).

The PCR samples from R11 and R20 biotypes presented two double peaks in the target codon. These samples were cloned into a vector to isolate *ALS* copies from different *E. crus-galli* subgenomes. The TOPO TA cloning Kit (Invitrogen) was used following the manufacturer's protocol. One Shot™ TOP10 chemically competent *Escherichia coli* kit (Invitrogen) was used to transform the vectors into *E. coli*, following the manufacturer's protocol. The *E. coli* were incubated in Lysogeny broth (LB) agar plates with kanamycin at 37 °C for 24 h to select effective transformants. Twelve colonies of each biotype were used to perform another PCR reaction and sequencing, as described before.

2.4. Dose-response experiments at pre- and post-emergence

The biotypes R1, R11, R12, R13, R14, R15, R16, R19, and R20 were selected based on multiple- and cross-resistance patterns and ALS amino acid substitution, and the S1 biotype was selected as a susceptible check. Only *E. crus-galli* biotypes were selected for this study due to their higher frequency than *E. colona*. These biotypes were submitted to dose-response curves with imazethapyr, penoxsulam, and quinclorac in pre- and post-emergence applications, conducted twice in the greenhouse. For pre-emergence application, 10 seeds of each biotype were direct seeded in plastic pots (200 mL) containing a loamy haplic gley soil with 16% clay (0–20 cm), pH 5.5, 1.3% organic carbon, collected from paddy field and fertilized with 240 kg ha^{-1} of N and 200 kg ha^{-1} of P_2O_5 and K_2O. The soil was watered previously the seeding, and the seeds were lightly pressed against the soil to provide seed-soil contact. Herbicide treatments occurred as described in 2.2 at one day after seeding. The irrigation was done using a mist sprinkler once a day. For post-emergence application, the biotypes were conducted and treated as described in 2.2. For both application methods, imazethapyr doses ranged from 12.5 g ha^{-1} to 25,600 g ha^{-1} for resistant biotypes and 0.78 g ha^{-1} to 100 g ha^{-1} for the susceptible biotype. Penoxsulam doses were from 72 g ha^{-1} to 47,520 g ha^{-1} for resistant biotypes and from 0.072 g ha^{-1} to 72 g ha^{-1} for the susceptible one. Quinclorac doses ranged from 750 g ha^{-1} to 48,000 g ha^{-1} for resistant biotypes and from 0.375 g ha^{-1} to 375 g ha^{-1} for the susceptible one. A treatment without herbicide application, representing the dose zero, was included as an untreated check for all biotypes. The aboveground dry biomass was evaluated at 28 DAT for pre- and 21 DAT for post-emergence applied herbicides, as detailed in the 2.2 section.

To assess the differences among experiment repetitions, the data were verified based on homogeneity of variance using Levene's test ($p \leq 0.05$) of the *car* package (Fox et al., 2022) in R (R Core Team, 2021), considering the variable aboveground dry biomass in the function of two repetitions for each dose-response curve. The test indicated that the variances of the two experimental runs were homogeneous for both pre- and post-emergent assays; then, barnyardgrass aboveground dry biomass data were pooled ($n = 8$) and subjected to ANOVA using *ExpDes.pt* package in R. When the interaction between biotype and doses was significant ($p \leq 0.05$), the pooled data were fitted to the three-parameter log-logistic non-linear regression model using the *drc* (Ritz and Streibig, 2005) package in R, as follows: $y = b/1 + (x/e)^d$; where y is the aboveground dry biomass, x is the herbicide dose, b is the curve slope at the inflection point, d is the upper limit, and e is the inflection point, representing the relative effective dose (ED_{50}) that causes 50% of growth reduction. The resistance index (RI) was calculated based on the ED_{50} ratio between resistant and susceptible biotypes, and the relative potency of application (RPA) was determined for each biotype by the ED_{50} ratio between post- and pre-emergence application. Comparisons and standard errors were obtained using the command *EDcomp* in the *drc* package considering relative ED_{50} and the Delta method. The graphics were designed using SigmaPlot (v14.0, Systat Software, Palo Alto, USA).

3. Results

3.1. Biotype screening

Of the 26 evaluated biotypes, 23 were resistant to imazethapyr, 10 were resistant to penoxsulam, and seven were resistant to quinclorac. The biotypes R15, R19, R20, and R22 were resistant to all tested herbicides, and S1, S2, and S3 were susceptible to the three applied herbicides. The herbicide resistance pattern for all biotypes is presented in Fig. 1A. With the recommended dose treatment, the susceptible plants showed at least 91.7% of aboveground dry biomass reduction, whereas the mass reduction for resistant barnyardgrass reached 78.9% maximum (Fig. S1). Barnyardgrass biotypes with control efficacy at the recommended dose higher than 95% were considered susceptible (Table S2). The application of malathion resulted in no differences of control efficacy and aboveground dry biomass between untreated control plants and the two doses of imazethapyr (data not shown).

A				B								
Biotype	Herbicide resistance			122 Ala (GCC)	197 Pro (CCC)	205 Ala (GCC)	206 Phe (TTC)	376 Asp (GAT)	377 Arg (CGT)	574 Trp (TGG)	653 Ser (AGC)	654 Gly (GGT)
	IMA	PEN	QUI									
S1	S	S	S	Ala	Pro	Ala	Phe	Asp	Arg	Trp	Ser	Gly
S2	S	S	S	Ala	Pro	Ala	Phe	Asp	Arg	Trp	Ser	Gly
S3	S	S	S	Ala	Pro	Ala	Phe	Asp	Arg	Trp	Ser	Gly
R1	R	S	R	Ala	Pro	Ala	Phe	Asp	Arg	Trp	**Asn (AAC)**	Gly
R2	R	S	S	Ala	Pro	Ala	Phe	Asp	Arg	Trp	**Asn (AAC)**	Gly
R3	R	R	S	Ala	Pro	Ala	Phe	Asp	Arg	**Leu (TTG)**	Ser	Gly
R4	R	S	S	Ala	Pro	Ala	Phe	Asp	Arg	Trp	**Asn (AAC)**	Gly
R5	R	S	S	Ala	Pro	Ala	Phe	Asp	Arg	Trp	**Asn (AAC)**	Gly
R6	R	S	S	Ala	Pro	Ala	Phe	Asp	Arg	Trp	**Asn (AAC)**	Gly
R7	R	R	S	Ala	Pro	Ala	Phe	Asp	Arg	**Leu (TTG)**	Ser	Gly
R8	R	S	S	Ala	Pro	Ala	Phe	Asp	Arg	Trp	**Thr (ACC)**	Gly
R9	R	S	S	Ala	Pro	Ala	Phe	Asp	Arg	Trp	**Asn (AAC)**	Gly
R10	R	R	S	Ala	Pro	Ala	Phe	Asp	Arg	**Leu (TTG)**	Ser	Gly
R11	R	S	R	Ala	Pro	**Asn (AAC)**	Phe	Asp	Arg	Trp	Ser	Gly
R12	R	S	R	Ala	Pro	Ala	Phe	Asp	Arg	Trp	**Asn (AAC)**	Gly
R13	R	S	S	**Thr (ACC)**	Pro	Ala	Phe	Asp	Arg	Trp	Ser	Gly
R14	R	R	S	Ala	Pro	Ala	Phe	Asp	Arg	**Leu (TTG)**	Ser	Gly
R15	R	R	R	Ala	Pro	Ala	Phe	Asp	Arg	**Leu (TTG)**	Ser	Gly
R16	R	S	S	Ala	Pro	Ala	Phe	Asp	Arg	Trp	**Asn (AAC)**	Gly
R17	R	S	S	Ala	Pro	Ala	Phe	Asp	Arg	Trp	**Asn (AAC)**	Gly
R18	R	R	S	Ala	Pro	Ala	Phe	Asp	Arg	**Leu (TTG)**	Ser	Gly
R19	R	R	R	Ala	Pro	Ala	Phe	Asp	Arg	**Leu (TTG)**	Ser	Gly
R20	R	R	R	**Asn (AAC)**	Pro	Ala	Phe	Asp	Arg	**Leu (TTG)**	Ser	Gly
R21	R	R	S	Ala	Pro	Ala	Phe	Asp	Arg	**Leu (TTG)**	Ser	Gly
R22	R	R	R	Ala	Pro	Ala	Phe	Asp	Arg	**Leu (TTG)**	Ser	Gly
R23	R	S	S	**Thr (ACC)**	Pro	Ala	Phe	Asp	Arg	Trp	Ser	Gly

Fig. 1. Herbicide resistance screening of 26 *Echinochloa* spp. biotypes from southern Brazil. (A) Resistance pattern to post-emergence applied imazethapyr (IMA), penoxsulam (PEN), and quinclorac (QUI). (B) The amino acid substitution in the ALS sequence for each biotype. R (in red): resistant; S (in green): susceptible; Ala: alanine; Arg: arginine; Asn: asparagine; Asp: aspartate; Gly: glycine; Leu: leucine; Phe: phenylalanine; Pro: proline; Ser: serine; Thr: threonine; Trp: tryptophan; A: adenine; T: thymine; G: guanine; C: cytosine.

3.2. ALS gene mutations

Missense mutations that cause amino acid substitutions were found in the positions Ala122, Ala205, Trp574, and Ser653 in 23 of the 26 biotypes evaluated (Fig. 1B). A substitution from alanine to threonine was found in position 122 in biotypes R13 and R23. Only biotype R11 presented substitution in residue 205, which changed an alanine to an asparagine. In residue 574, the substitution from tryptophan to leucine was detected in 10 biotypes. The shift from serine to asparagine in position 653 was detected in nine biotypes, while the substitution to threonine was found in one biotype. Furthermore, biotype R20 revealed a double mutation with the change from alanine to asparagine in residue 122 and the substitution from tryptophan to leucine in position 574.

Among the mutations found, Ala122Thr and Ser653Asn were derived from a G-A transition in the first and second nucleotides of the codons, respectively. In contrast, the Ser653Thr was due to a G-C transversion in the second nucleotide of the codon. The Ala122Asn and Ala205Asn (GCC to AAC) substitutions were caused by both a G-A transition and a C-A transversion in the first nucleotide of the codon. All Trp574Leu substitutions were originated by a G-T transversion in the second nucleotide of the codon. All the sequencing chromatograms showed a double peak in the mutated positions (Fig. 2).

3.3. Post-emergence herbicides effect

The ED_{50} for post-emergent applied imazethapyr varied from 18.9 to 5085.9 g ha^{-1} (Fig. 3A, Table S3). The S1 biotype needed only 1.7% of the recommended imazethapyr dose to reduce 50% of aboveground dry biomass. The RI for imazethapyr applied as post-emergent varied according to the ALS substitution (Fig. 4A). Biotypes with substitution Ser653Asn had RI lower than 20 (Fig. 3D, Table S3). The biotypes with a single amino acid substitution in the positions Ala122 and Ala205 had RI between 26 and 36. The Trp574Leu mutation resulted in a RI for post-emergent applied imazethapyr higher than 70. The R20 biotype, with the double mutation Ala122Asn + Trp574Leu, had an RI higher than 2800. Penoxsulam applied at post-emergence resulted in ED_{50} from 2.9 to 31.7 times the recommended field dose (Fig. 3B, Table S3) for biotypes R14, R15, and R19, whereas it was only 1.5% of the recommended dose for S1 biotypes. The RI for these three penoxsulam-resistant biotypes was significant ($p \leq 0.05$) and higher than 194.5 (Fig. 3E, Table S3). The R20 biotype showed an ED_{50} higher than the maximum tested dose of penoxsulam at post-emergence, indicating that the aboveground dry biomass did not achieve 50% reduction. All penoxsulam-resistant biotypes had the Trp574Leu mutation. The RI for post-emergent applied penoxsulam in the biotype with the double mutation Ala122Asn + Trp574Leu was at least 20 times higher than the Trp574Leu isolated. The biotypes resistant to quinclorac presented an ED_{50} for post-emergence application of at least 5.6 times higher than the field recommended dose, and the S1 had an ED_{50} 256.8 times lower than the recommended dose (Fig. 3C, Table S3). The RI for quinclorac applied at post-emergence was significant ($p \leq 0.05$) and varied from 1438.3 to 4016.5 (Fig. 3F).

3.4. Pre-emergence herbicides effect

The ED_{50} for imazethapyr applied at pre-emergence ranged from 7.3 to 5419.4 g ha^{-1} for resistant biotypes (Fig. 3A, Table S3). The resistance level to imazethapyr in pre-emergence, related to the ALS substitutions, followed the same pattern as the post-emergence application (Fig. 4B). The RI for imazethapyr at pre-emergence was higher than at post-emergence for the biotypes R11, R13, R14, R19, and R20 (Fig. 3D, Table S3). The ED_{50} for penoxsulam at pre-emergence for the biotypes R14 and R15 was lower than the field-recommended dose of 72 g ha^{-1} (Fig. 3B, Table S3). The biotype R20 showed ED_{50} over the maximum dose tested. The S1 biotype only needed 0.5% of the recommended dose of penoxsulam to reach 50% of biomass reduction. The RI for penoxsulam as pre-emergent (Fig. 3E) was at least half the RI in post for the biotypes R14, R15, and R19. Quinclorac applied as pre-emergent resulted in variable ED_{50}, depending on the resistant biotype (Fig. 3C, Table S3), and the RI ranged between 614 and 3045 (Fig. 3F, Table S3). The S1 biotype reached 50% of dry biomass reduction with 0.33% of the field-recommended dose of quinclorac.

3.5. Relative potency of application (RPA) of pre- in relation to post-emergence application

The applications of imazethapyr at pre-emergence were significantly ($p \leq 0.05$) more effective than at post-emergence for the biotypes R1, R12, and R16 (Figs. 3G, Fig. 5A–C). The RPA for these biotypes ranged from 1.45 to 4.66. For penoxsulam, the application at pre-emergence resulted in significant RPA, which was higher than 6.0 for the resistant biotypes R14, R15, and R19 and 3.0 for the susceptible S1 (Figs. 3H, Fig. 5D–G). The application of penoxsulam at pre-emergence resulted in complete control of the resistant biotypes R14 and R15 at higher tested doses, while at post-emergence, the same dose reached a maximum of 84% of aboveground dry biomass reduction (Fig. 5D and E). Quinclorac resulted in significant RPA for the biotypes R1, R11, R12, R15, and R19, ranging from 1.6 to 3.0 (Fig. 3I). The higher biomass reduction of quinclorac applied at pre-emergence occurred only at doses superior to 12 times the recommended field dose (Fig. 5H-L). The R20 biotype, which has the double mutations Ala122Asn + Trp574Leu, showed no differences between the pre- or post-emergence application of imazethapyr, penoxsulam, and quinclorac (Fig. 3G–I).

4. Discussion

4.1. Cross-resistance pattern according to ALS gene mutation

All mutations resulted in a high RI (>10 fold) for imazethapyr. A large variation was found for ED_{50} and RI among the resistant biotypes treated with imazethapyr at both pre- or post-emergence, and a small variation occurred among the biotypes with the same ALS mutation (Figs. 3A and 4). It is well known that the cross-resistance pattern for each plant species depends on the mutated amino acid position and the specific amino acid substitution (Yu and Powles, 2014). In that regard, the variation of ED_{50} and RI after imazethapyr application found in our study was mostly explained by the ALS substitutions found. Although no NTS resistance mechanism was investigated, some level of herbicide metabolization could explain the differences in RI among biotypes with equal mutations. Furthermore, only the Trp574Leu mutation conferred cross-resistance to penoxsulam. We did not evaluate other ALS inhibitor herbicides from different chemical families because they have little or no utilization in rice production.

The Ala122Thr substitution resulted in an intermediary resistance level for imazethapyr and susceptibility for penoxsulam in barnyardgrass. Contrary to our results, the mutation Ala122Thr was reported to cause low to moderate penoxsulam resistance levels in *E. crus-galli* based on an ALS in vitro assay (Riar et al., 2013). The Ala205Asn mutation resulted in susceptibility to penoxsulam and had an intermediary resistance level to imazethapyr. To our knowledge, this is the first report of an Ala205Asn substitution identified in an herbicide-resistant weed. This specific mutation was described for the first time in artificial-generated mutant yeast and resulted in sulfonylurea resistance, but the effect of other chemical groups of ALS inhibitors was not evaluated (Bedbrook et al., 1991). In plants, only the mutations Ala205Val and Ala205Phe were reported for this amino acid residue (Tranel et al., 2023). The first one (Ala205Val) conferred low RI to imidazolinones and moderate RI to penoxsulam in *E. crus-galli* (Fang et al., 2019a) and resistance to imidazolinones, but susceptibility to triazolopyrimidines in eastern black nightshade (*Solanum ptychanthum* Dun.) (Ashigh and Tardif, 2007). The second one (Ala205Phe) resulted in resistance to imidazolinones and triazolopyrimidines in annual bluegrass (*Poa annua*

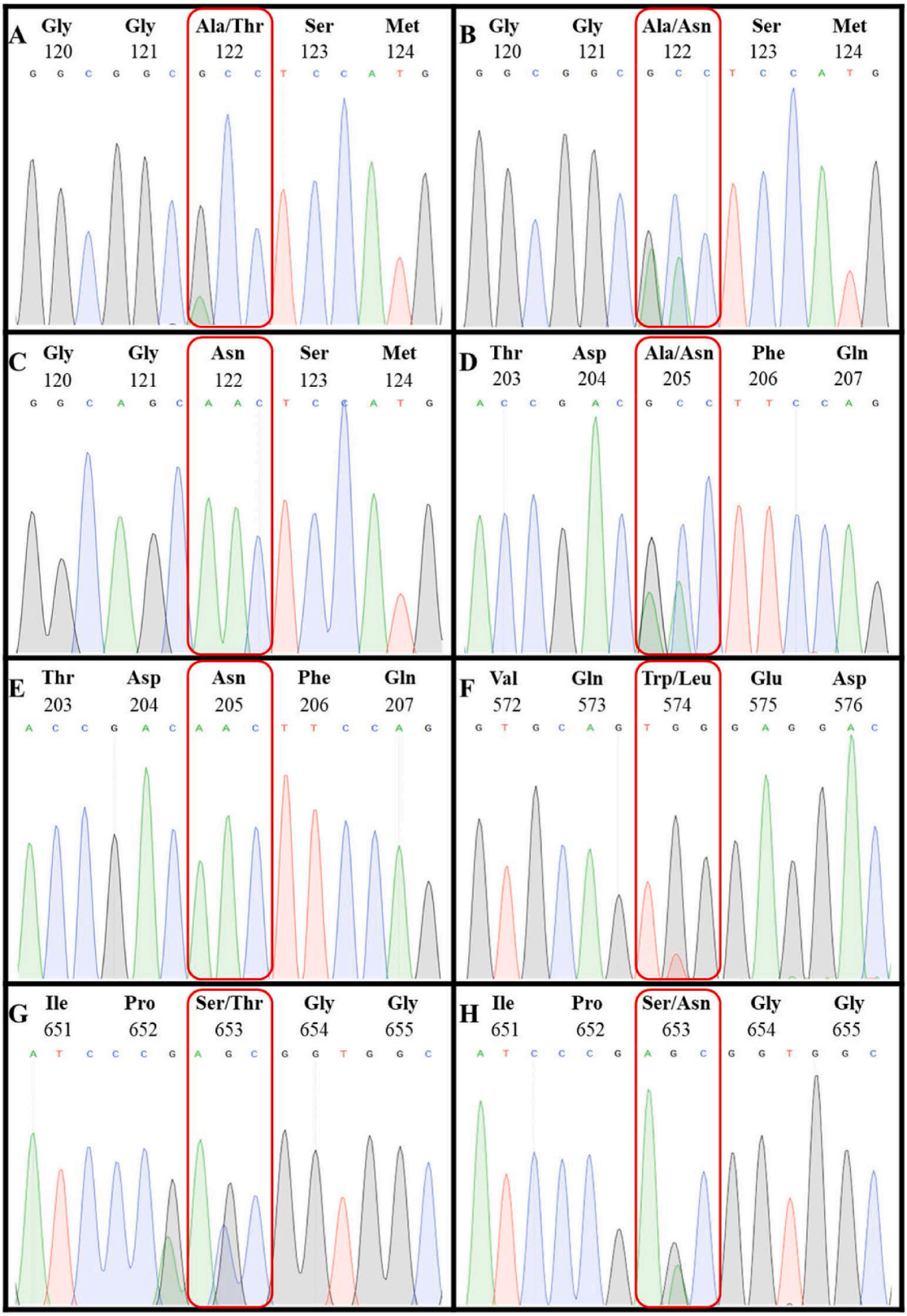

Fig. 2. Chromatograms of the barnyardgrass *ALS* gene sequence. The codons are numbered according to the *A. thaliana ALS* sequence, and the target codons are marked in red boxes. Mutations are recognized as double peaks when at least one gene copy has the wild-type codon because of the hexaploidy. Ala122Thr from R13 (A); Ala122Asn from R20 with double peaks in the first and second position of the codon (B); Ala122Asn from R20 after cloning (C); Ala205Asn from R11 with double peaks in the first and second position of the codon (D); Ala205Asn from R11 after cloning (E); Trp574Leu from R20 (F); Ser653Thr from R8 (G); Ser653Asn from R12 (H). Ala: alanine; Arg: arginine; Asn: asparagine; Asp: aspartate; Gln: glutamine; Glu: glutamate; Gly: glycine; Ile: isoleucine; Leu: leucine; Met: methionine; Phe: phenylalanine; Pro: proline; Ser: serine; Thr: threonine; Trp: tryptophan; Val: valine; A: adenine; T: thymine; G: guanine; C: cytosine.

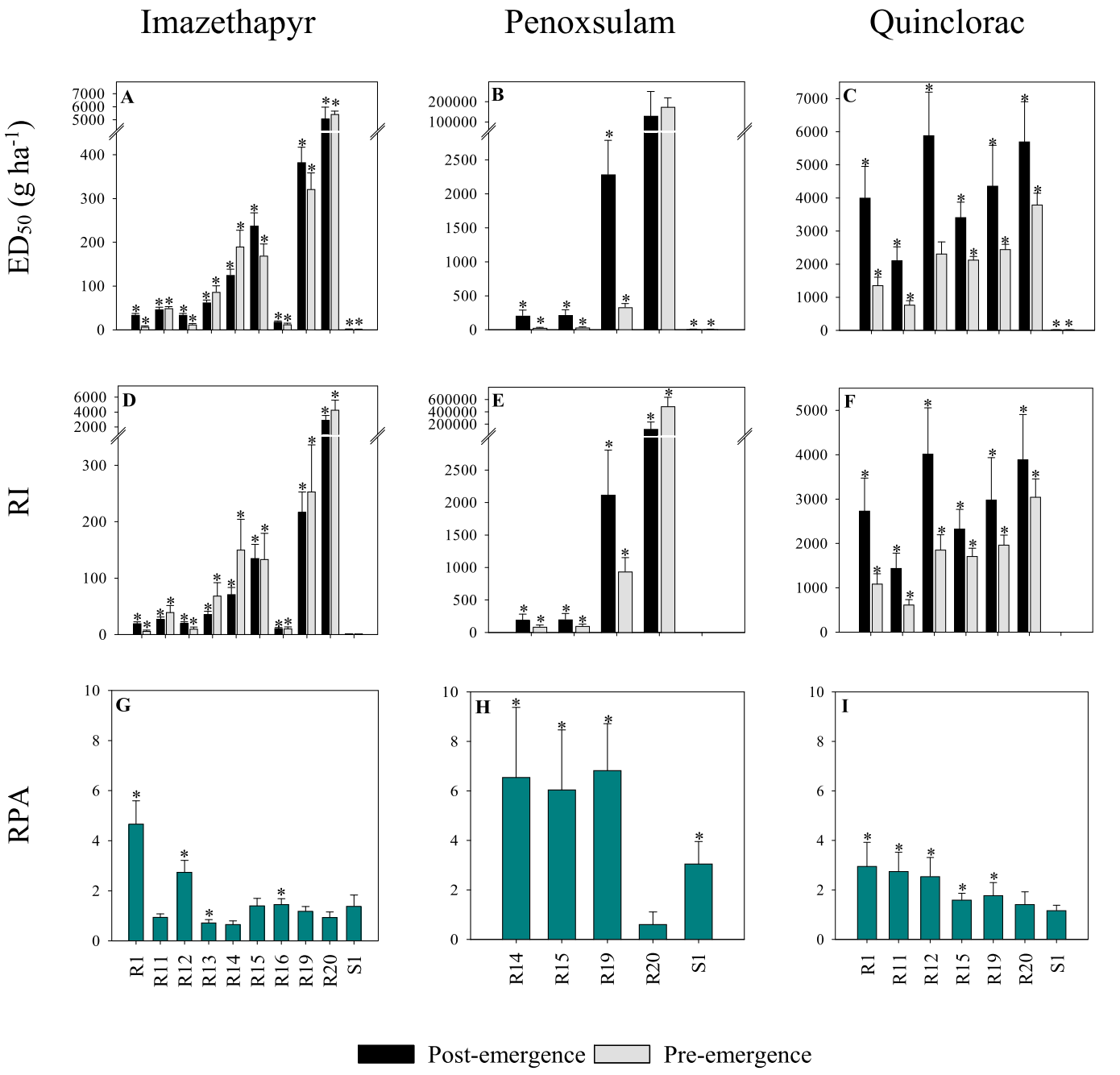

Fig. 3. Effective dose for 50% of growth reduction (ED_{50}) (A, B, and C), resistance index (RI) (D, E, and F), and the relative potency of application (RPA) (G, H, and I) for the herbicides imazethapyr (A, D, and G), penoxsulam (B, E, and H), and quinclorac (C, F, and I). Vertical bars represent the standard error of the mean (n = 8). * represents statistical significance ($p < 0.05$). For ED_{50}, the significance was based on least squares estimation. For RI and RPA, the significance of the comparison between resistant/susceptible biotypes and between applications at post-/pre-emergence was based on the Delta method.

L.) (Brosnan et al., 2016).

The Trp574Leu had the highest resistance level among the isolated substitutions found. This substitution was previously attributed to a high resistance level to imazethapyr and penoxsulam in barnyardgrass (Panozzo et al., 2013) and other species (Kaloumenos et al., 2013; Sun et al., 2021; Tehranchian et al., 2019). The Trp574Leu is the most common substitution worldwide and results in barnyardgrass resistance for all ALS inhibitor's chemical families (Tranel et al., 2023). The substitution Ser653Asn conferred the lowest RI among the studied mutations. Another study also reported imazethapyr resistance in *E. crus-galli* due to this substitution, but penoxsulam was not evaluated (Matzenbacher et al., 2014). Similar to our results, this substitution in *Amaranthus tuberculatus* (Moq.) Sauer resulted in imazethapyr resistance and susceptibility to triazolopyrimidines (Patzoldt and Tranel, 2007). In contrast, the Ser653Asn mutation endowed triazolopyrimidine and imidazolinones resistance in *Bromus tectorum* L. (Kumar and Jha, 2017). The differences among species and studies of cross-resistance patterns and resistance levels for a certain *ALS* mutation may be related to herbicide detoxification capacity, herbicide doses used, plant stage at application, and growing conditions.

Unexpectedly, we found a double mutation in the plants of biotype R20, which resulted in a very high resistance level for imazethapyr and penoxsulam at both pre- and post-emergence (Fig. 3D and E). This is the first reported case of a double mutation involving the substitutions

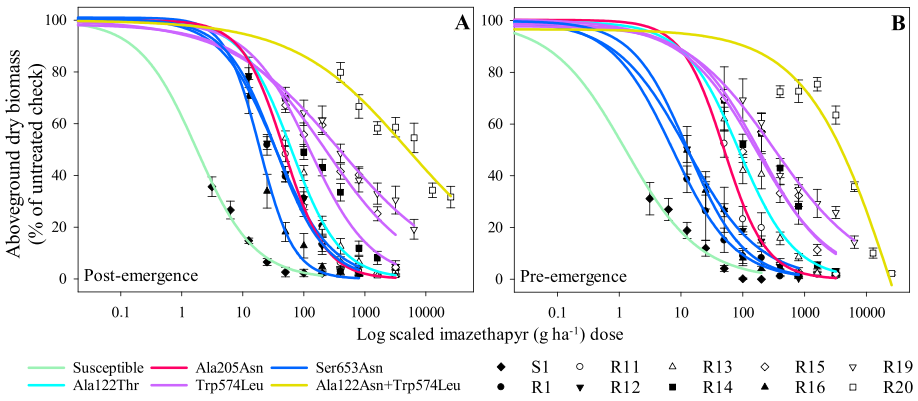

Fig. 4. Dose-response curves for aboveground dry biomass after application of imazethapyr at post-emergence (A) and pre-emergence (B). The ALS substitutions are grouped by color. The dots represent the mean (n = 8), and the vertical bars are the confidence interval (α = 0.05).

Ala122Asn and Trp574Leu in the *E. crus-galli*. Double mutations were already reported for *E. crus-galli* (Löbmann et al., 2021), but it was unclear if the mutations occurred in the same plant. Additionally, double *ALS* mutations were also identified in *Lolium rigidum* Gaud. (Pro197Ser and Trp574Leu) (Yu et al., 2008) and *Raphanus raphanistrum* L. (Pro197Ala and Trp574Leu) (Yu et al., 2012). The occurrence of the double ALS mutation Ala122Asn + Trp574Leu in the R20 biotype may be considered as a stacking herbicide resistance. In this case, the origin of the mutations may be related as a simultaneous event, which is very rare. Most likely, the first mutation occurs, and the second mutation is added in the same individual originated from gene flow or of an independent mutation event caused be the continuous herbicide selection pressure. Probably the last possibility occurs in the R20 biotype, where initially occurred the mutation Ala122Asn. In the region where the barnyardgrass biotypes where collected, farmers had continuously increased imidazolinone herbicides doses in the last 15 years. This may result in a second selection pressure event, which result in the evolution and stacking of the mutation Trp574Leu associated with high resistance level. The Ala122Asn isolated caused high resistance to penoxsulam and other ALS inhibitors from the imidazolinones, sulfonylureas, and pyrimidinyl benzoate chemical groups in barnyardgrass (Panozzo et al., 2017). Unfortunately, we could not evaluate and assign the RI at pre- and post-emergence or the RPA of the Ala122Asn mutation because it was not identified separately from Trp574Leu in any of the studied biotypes.

NTS resistance mechanisms are of significant concern in weeds, particularly considering the increasing likelihood of multiple herbicide resistance evolution. The reported cases of NTS resistance to ALS inhibitors in barnyardgrass involve mainly penoxsulam and may be associated with metabolic degradation (Pan et al., 2022). NTS resistance was only superficially investigated in the present study, as mutations were identified as the primary driver of ALS herbicide resistance in barnyardgrass, which has been supported by several reports (Kaloumenos et al., 2013; Fang et al., 2019a; Damalas and Koutroubas, 2023). It is noteworthy that all imazethapyr and penoxsulam-resistant biotypes examined in our study presented a TS resistance mechanism (Fig. 1). Moreover, the obtained results indicated that malathion was not effective for decreasing the resistance level of all biotypes. Malathion has been used as an indication of degradation enhancement caused by Cytochrome P450 enzymes (Cutti et al., 2021; Torra et al., 2021). In studies where no TS mutation is identified, the positive result of malathion is inferred as an indicative of NTS resistance. It is important to emphasize that the precise identification of mutations related with the TS resistance is fundamental, mainly in polyploid species. Furthermore, the variability of detoxification occurs largely in plants, and the effect of malathion should be used as a preliminary result and followed by additional studies. The selection of populations with NTS resistance mechanisms could be attributed to the utilization of low herbicide doses and control at advanced growth stages. The application of herbicides at pre-emergence targets plants with lower levels of resistance, which may imply in effective control even for biotypes exhibiting degradation enhancement. This approach may hamper the evolution of NTS resistance to imazethapyr and penoxsulam at the field level.

4.2. *Penoxsulam applied at pre-emergence is more effective in controlling barnyardgrass*

The ED_{50} was lower for the application of penoxsulam at pre-emergence than at post-emergence for both susceptible and resistant biotypes (Fig. 3B). Pre-emergent herbicides are fundamental in dry-seeded rice systems (Marchesi and Chauhan, 2019). Penoxsulam has historically been used as a post-emergent herbicide to control weeds in Brazil (Agostinetto et al., 2011) and other rice areas worldwide. This herbicide has recently been applied at pre-emergence mainly based on field observations about the large efficacy on populations identified as penoxsulam-resistant at post-emergence applications. The pre-emergent application of penoxsulam constantly delivers the herbicide dose for seeds at the beginning of the germination process, where a lower amount of ALS and detoxification enzymes are present compared with post-emergence applications that interact with a fully developed plant. Besides, penoxsulam remains active in the soil for at least 21 days after application under aerobic conditions (Lewis et al., 2016), which provide the herbicide effect on germinating seeds within this period.

The resistant barnyardgrass biotypes R14, R15, and R19 had an RI at least halved by the penoxsulam application at pre-emergence compared to post-emergence (Fig. 3E). Barnyardgrass is a hexaploid species with three *ALS* copies (Iwakami et al., 2015). At least one of these copies was not mutated in the biotypes analyzed in the present study, as observed by the presence of the wild-type codon in the sequencing chromatograms (Fig. 2), and must be completely inhibited by the herbicide. This inhibition implies a lower amount of the amino acids valine, leucine, and isoleucine. It may reduce plant metabolism due to partial amino acid depletion, particularly noted at the beginning of barnyardgrass germination in pre-emergence applications. Nevertheless, the ALS enzyme containing the Trp574Leu substitution was reported to cause a fitness cost of less development in *Raphanus sativus* L. plants (Vercellino et al., 2021) and may contribute to the effects of penoxsulam at pre-emergence in resistant *E. crus-galli*.

4.3. *Improvement of barnyardgrass control based on knowledge of herbicide resistance patterns and resistance mechanisms*

The dose needed to control the S1 biotype of imazethapyr, penoxsulam, and quinclorac at pre- or post-emergence was lower than 2% of the recommended dose of each herbicide (Fig. 3A–C). These herbicides are potent tools for controlling susceptible biotypes of barnyardgrass,

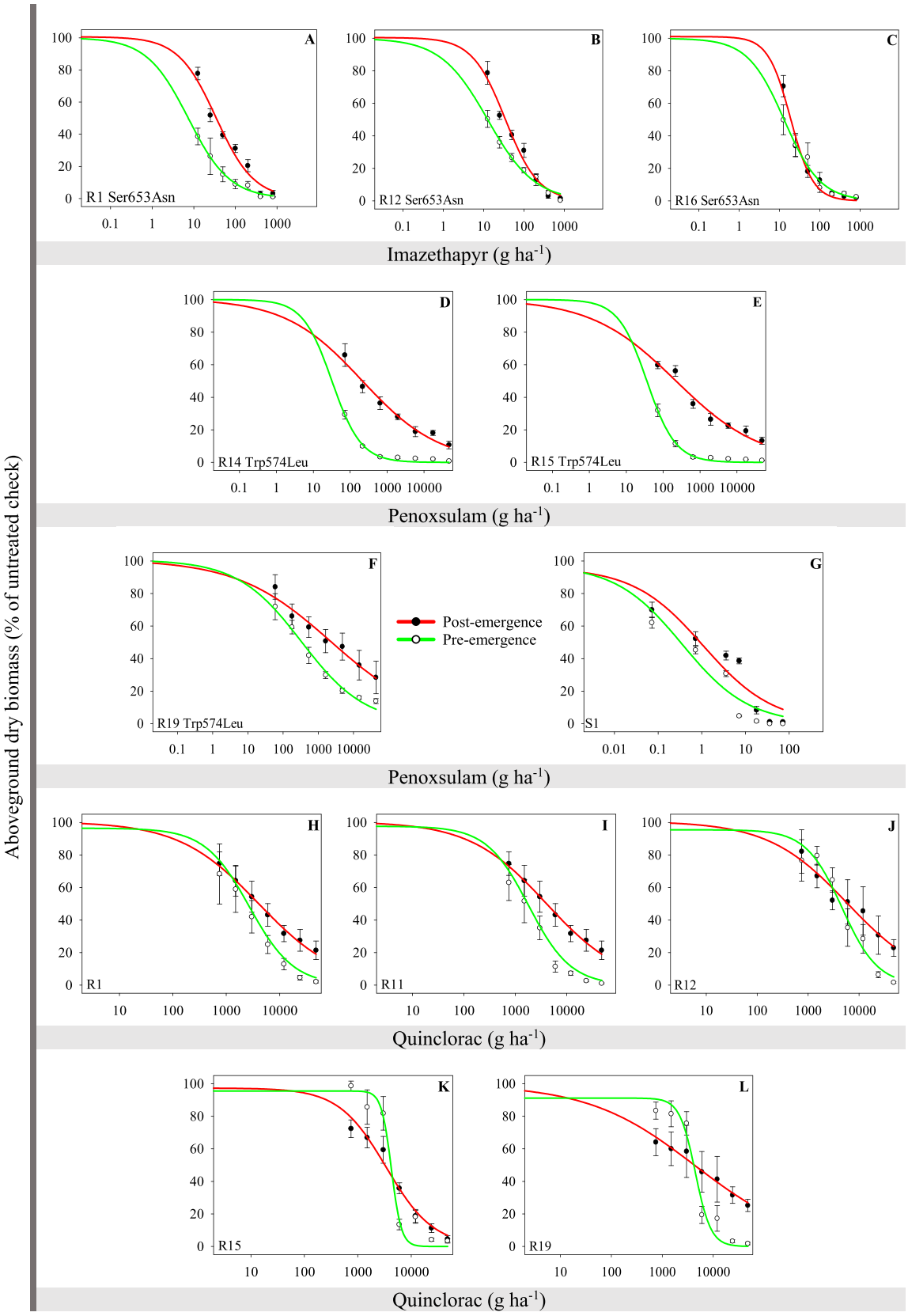

Fig. 5. Dose-response curves for aboveground dry biomass after application of imazethapyr (A–C), penoxsulam (D–G), and quinclorac (H–L) at post-emergence (red line, black dot) and pre-emergence (green line, white dot). The biotype is indicated in each plot. The dots represent the mean (n = 8), and the vertical bars are the confidence interval (α = 0.05).

and similar efficacy occurs for other non-resistant weeds. Moreover, the barnyardgrass biotypes containing the substitutions Ala122Thr, Ala205Asn, and Ser653Asn are resistant to imazethapyr but could be easily controlled by the application of penoxsulam at pre- or post-emergence. Recently, *Echinochloa* was listed as the second most problematic weed on rice fields in southern Brazil, and herbicide-resistant weeds, including *Echinochloa* species, were pointed out as the major reason for yield losses (Silva et al., 2021). Although the application at pre-emergence could not completely control barnyardgrass-resistant plants at the recommended field dose, all resistant biotypes, except the one with double mutation, had less development due to the imazethapyr, penoxsulam or quinclorac action. Some biotypes reduced 50% of aboveground dry biomass with the herbicide dose lower than the recommended (Fig. S1). Therefore, a second application at post-emergence could be used to control these resistant biotypes. It is well known that the best recommendation for the prevention and control of herbicide-resistant weeds is to rotate the herbicide mechanism of action, and in that scenario, the adjustment related to application at pre-emergence is not acceptable. However, weed control strategies should be considered not only for the species that evolved herbicide resistance, such as barnyardgrass. In most rice-growing areas, other weed species also occur at large infestations, such as *Aeschynomene* spp., *Cyperus* spp., *Fimbristilis miliacea* (L.) Vahl, *Heteranthera* spp., *Ischaemum rugosum* Salisb., and *Sagittaria montevidensis* Cham. & Schltdl., which are effectively controlled by ALS inhibitors in most cases. Therefore, these herbicides are still necessary in the rice fields, and optimization of their application at pre-emergence may be prioritized in comparison with post-emergence. However, in areas where resistance to ALS inhibitors has evolved across multiple weed species, it becomes crucial to use herbicides other than ALS inhibitors or explore alternative strategies beyond chemicals.

Among the 26 biotypes evaluated, only seven were resistant to quinclorac (Fig. 1A). This herbicide has a limited control spectrum, indicated just for *Echinochloa* and *Aeschynomene* spp. in Brazilian rice fields (SOSBAI, 2018). The first reported case of quinclorac resistance in *E. crus-galli* from Brazil was in 1999 (HEAP, 2023). For these reasons, its use has been limited in the rice production system. Additionally, the development of imidazolinone-tolerant rice varieties, which resulted in effective barnyardgrass control, made the use of quinclorac minimal. However, after the first cases of ALS inhibitors resistance, quinclorac returned as an alternative tool to control imazethapyr- and penoxsulam-resistant barnyardgrass biotypes. Although quinclorac is recommended only for post-emergence application (SOSBAI, 2018), no differences between pre- and post-emergence were observed for the susceptible biotype in our study (Fig. 3C, I). The mode of action of quinclorac in grasses is not fully understood, but the herbicide may be absorbed by roots and germinating seeds (Fipke et al., 2016), which ensures its action as pre-emergent.

Quinclorac resulted in significant RPA for the resistant biotypes but not for the susceptible ones (Fig. 3I). The herbicide resistance mechanism for this herbicide in *Echinochloa* is uncovered but may be associated with the ethylene biosynthesis route (Qiong et al., 2019; Yang et al., 2021) or cyanide detoxification (Gao et al., 2017). The resistant plants may have a smaller pool of detoxification enzymes in the early stages of development, which implies better herbicide action. However, at the recommended field dose, the aboveground dry biomass for resistant biotypes after pre-emergent applied quinclorac was equal to or higher than post-emergent applied, and the differences between application times were just expressed at high doses (Fig. 5H-L). For these reasons, quinclorac-resistant populations should be managed with other strategies rather than application time.

The knowledge of cross- and multiple-resistance patterns and herbicide resistance mechanisms is important for defining the best management strategies as well as using alternative herbicides, different application times, and other integrated weed management practices. Using physical and cultural methods of weed control is also desired. For example, the combined effect of pre-emergent herbicides and early flooding efficiently reduced plant density and increased the control of barnyardgrass under field conditions, even for herbicide-resistant biotypes (Turra et al., 2023). The integrated weed management should be redesigned based not only on the current weed control problem regarding the species type and infestation but also based on the characteristics of the herbicide resistance problem. Non-chemical weed control practices are being successively developed but are still challenging to use in large-scale production systems (Moss, 2019). In addition, new herbicide mechanisms of action with a large weed spectrum are still far from being commercialized. Therefore, integrated weed management practices should consider adjustments of the currently available herbicides, such as time of application, doses, and sequential applications, mainly for herbicides with environmental and agronomic advantages, such as ALS inhibitors.

5. Conclusions

Several mutations on the *ALS* gene were associated with resistance in barnyardgrass and resulted in different herbicide resistance level and cross-resistance patterns for applications at pre- or post-emergence. The application of penoxsulam at pre-emergence was more effective at controlling susceptible and ALS inhibitor-resistant barnyardgrass than at post-emergence. Imazethapyr applications at pre-emergence were more effective than at post-emergence only for biotypes with the low resistant level mutation Ser653Asn. The application of imazethapyr and penoxsulam at pre-emergence should be prioritized in fields with resistant barnyardgrass and where these herbicides are necessary to control other non-resistant weeds. Quinclorac could be effectively utilized at both pre- and post-emergence to control some biotypes with imazethapyr or penoxsulam resistance. Optimization of existing herbicides for which weeds are already resistant must be considered in the weed control planning, with complementation of other integrated weed management strategies such as rotation of herbicide mode of action and non-chemical control.

Author contributions

GMT: conceptualization, formal analysis, investigation, writing - original draft, writing - review & editing. LC: formal analysis, investigation. GMD: investigation, visualization. FMM: investigation, visualization. AA: conceptualization, writing - review & editing. CM: supervision, funding acquisition, writing - review & editing. AM: conceptualization, project administration, supervision, funding acquisition, writing - review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2023.106325.

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