UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE AGRONOMIA
PROGRAMA DE PÓS-GRADUAÇÃO EM FITOTECNIA

Epidemiologia da resistência a herbicidas em espécies do gênero *Conyza* e avaliação da aplicação exógena de RNA para silenciamento gênico

Paula Sinigaglia Angonese
Engenheira Agrônoma/UFRGS
Mestre em Fitotecnia/UFRGS

Tese apresentada como um dos requisitos
à obtenção do Grau de Doutor em Fitotecnia
Área de Concentração: Sistemas de Produção Vegetal

Porto Alegre (RS), Brasil
Maio de 2026

CIP - Catalogação na Publicação

Angonese, Paula Sinigaglia
Epidemiologia da resistência a herbicidas em espécies do gênero Conyza e avaliação da aplicação exógena de RNA para silenciamento gênico / Paula Sinigaglia Angonese. -- 2026.
155 f.
Orientador: Aldo Merotto Jr..

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Agronomia, Programa de Pós-Graduação em Fitotecnia, Porto Alegre, BR-RS, 2026.

1. Conyza spp.. 2. resistência a herbicidas. 3. marcadores moleculares. 4. RNAi. 5. silenciamento pós-transcricional. I. Merotto Jr., Aldo, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

PAULA SINIGAGLIA ANGONESE
Engenheira Agrônoma - UFRGS
Mestre em Fitotecnia - UFRGS

TESE

Submetida como parte dos requisitos

para obtenção do Grau de

DOUTORA EM FITOTECNIA

Programa de Pós-Graduação em Fitotecnia

Faculdade de Agronomia

Universidade Federal do Rio Grande do Sul

Porto Alegre (RS), Brasil

Aprovado em: 14 de maio de 2026
Pela Banca Examinadora

ALDO MEROTTO JR.
Orientador
Universidade Federal do Rio Grande do Sul

SIMONE MUNDSTOCK JAHNKE
Coordenadora do Programa de Pós-Graduação em Fitotecnia

CATARINE MARKUS
Universidade Federal do Rio Grande do Sul

DIECSON RUY ORSOLIN DA SILVA
Universidade Federal de Santa Maria

PAULO VITOR DUTRA DE SOUZA
Diretor da Faculdade de Agronomia

FELIPE KLEIN RICACHENEVSKY
Universidade Federal do Rio Grande do Sul

Dedico à minha família

Meus pais

Minha irmã

AGRADECIMENTOS

Ao professor Aldo Merotto Jr. pela orientação acadêmica, oportunidades, ensinamentos, amizade, motivação, compreensão e principalmente por seu exemplo durante todos esses anos.

À professora Catarine Markus pelos ensinamentos, amizade e apoio.

Ao professor Todd Gaines e colegas do Weed Research Laboratory por me receberem durante o período de Doutorado Sanduíche.

Aos meus pais Iane Sinigaglia Angonese e Paulo Angonese e à minha irmã Liana Sinigaglia Angonese por todo carinho, incentivo e por sempre acreditarem em mim.

À nona Inez Sinigaglia e toda minha família pelo carinho e incentivo.

Aos amigos e colegas da pós-graduação do GUIHE, Guilherme Menegol Turra, Luan Cutti, Estéfani Sulzbach e Eduardo Rudell, pelo companheirismo e ensinamentos.

Aos amigos do GUIHE, Othon, Filipi, Vinícius, Gabriel, Fernando, Fernanda, Pablo, William, Leonardo, Lucas, Eduarda, Matheus, Antônio, Davi, Inácio, Bruna, Wallace e Natália, pela amizade e ajuda nas atividades.

Aos funcionários da Universidade Federal do Rio Grande do Sul e aos servidores técnico-administrativos do Departamento de Plantas de Lavoura e do PPG Fitotecnia pelo auxílio.

Aos professores Catarine Markus, Diecson Ruy Orsolin da Silva e Felipe Klein Ricachenevsky por aceitarem compor a banca de avaliação e contribuir com o trabalho.

À CAPES pela bolsa de estudos durante o período do Doutorado e ao CNPq pela bolsa de estudos durante o Doutorado Sanduíche.

EPIDEMIOLOGIA DA RESISTÊNCIA A HERBICIDAS EM ESPÉCIES DO GÊNERO *CONYZA* E AVALIAÇÃO DA APLICAÇÃO EXÓGENA DE RNA PARA SILENCIAMENTO GÊNICO[1]

Autora: Paula Sinigaglia Angonese
Orientador: Aldo Merotto Jr.

RESUMO

Espécies do gênero *Conyza* (buva) estão entre as plantas daninhas mais problemáticas nos sistemas de produção de soja no Brasil devido à sua ampla distribuição, alta capacidade adaptativa e rápida evolução de resistência a múltiplos mecanismos de ação de herbicidas. O objetivo desta tese foi caracterizar a distribuição de diferentes mecanismos de resistência a herbicidas em espécies de buva no Brasil e avaliar metodologias de silenciamento gênico para o controle dessas espécies. Estudos de resistência foram conduzidos utilizando populações coletadas em diferentes regiões agrícolas do país. A resistência a herbicidas inibidores da ALS foi amplamente distribuída, sendo que, em cerca de 54% das populações, ocorreu devido a mutações no gene *ALS*, principalmente em Pro197 (Pro197Ser, Pro197Arg e Pro197His), além de Ala122Thr e Trp574Leu. Em 17% das populações, a resistência esteve associada ao aumento no número de cópias e/ou na expressão do gene *ALS*, enquanto em 25% o mecanismo não foi elucidado. Marcadores moleculares foram desenvolvidos para rápida identificação das mutações. A resistência a herbicidas auxínicos foi menos frequente, com identificação de resistência a 2,4-D, dicamba e triclopir. O fenótipo de rápida necrose ocorreu em 44% e 62% das populações em resposta a 2,4-D e triclopyr, respectivamente, mas não foi consistentemente associado à sobrevivência. A expressão do gene *PLP2* foi associada a essa resposta, enquanto genes transportadores ABC foram sugeridos na translocação diferencial ou no sequestro do herbicida. A frequência de resistência ao glifosato foi de 90%, com níveis de resistência influenciados pela temperatura. Múltiplos mecanismos foram identificados, incluindo mutações no *EPSPS*, variação no número de cópias e expressão diferencial entre cópias, com maior contribuição de cópias presentes no cromossomo 4 para a expressão total. Três populações apresentaram rápida necrose induzida por glifosato, sugerindo a atuação de mecanismos de resistência não relacionados ao local de ação, possivelmente envolvendo transportadores. Por fim, abordagens baseadas em RNA foram avaliadas através da aplicação exógena de siRNA e dsRNA direcionados ao gene *PDS* de buva. Ensaios de absorção indicaram baixa penetração foliar, sugerindo a presença de barreiras morfológicas e bioquímicas. Embora não tenham sido observados sintomas visuais de branqueamento, infiltração de siRNA reduziu os níveis de transcritos de *PDS*, e resultados semelhantes foram observados com misturas de dsRNA. Análises *in silico* indicaram baixa similaridade de sequência com organismos não alvo. Esses resultados evidenciam limitações relacionadas à entrega de RNA e fornecem base metodológica para o desenvolvimento de tecnologias de controle baseadas em RNA. De forma integrada, os dados demonstram que a resistência a herbicidas em buva é amplamente distribuída e complexa, sendo determinada pela interação de múltiplos mecanismos relacionados e não relacionados ao local de ação, que variam entre classes de herbicidas e condições ambientais. Esses resultados fornecem uma base para o monitoramento e manejo da resistência no Brasil e identificam limitações e oportunidades para o desenvolvimento de estratégias de controle baseadas em RNAi.

[1]Tese de doutorado em Fitotecnia, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (155f.). Maio, 2026.

HERBICIDE RESISTANCE EPIDEMIOLOGY IN *CONYZA* SPECIES AND EVALUATION OF EXOGENOUS RNA APPLICATION FOR GENE SILENCING[1]

Author: Paula Sinigaglia Angonese
Adviser: Aldo Merotto Jr.

ABSTRACT

Species of the genus *Conyza* are among the most problematic weeds in soybean production systems in Brazil due to their widespread distribution, high adaptive capacity, and rapid evolution of resistance to multiple herbicide modes of action. The objective of this thesis was to characterize the distribution of different herbicide resistance mechanisms in *Conyza* species in Brazil and to evaluate gene silencing methodologies for their control. Resistance studies were conducted using populations collected from diverse agricultural regions across the country. Resistance to ALS-inhibiting herbicides was widely distributed, with approximately 54% of populations with resistance due to mutations in the *ALS* gene, primarily at Pro197 (Pro197Ser, Pro197Arg, and Pro197His), as well as Ala122Thr and Trp574Leu. In 17% of populations, resistance was associated with increased *ALS* gene copy number and/or expression, while in 25%, the mechanism was not elucidated. Molecular markers were developed for the rapid identification of these mutations. Auxinic herbicide resistance was less frequent, with resistance detected to 2,4-D, dicamba, and triclopyr. The rapid necrosis phenotype was observed in 44% and 62% of populations in response to 2,4-D and triclopyr, respectively, but was not consistently associated with plant survival. Expression of the *PLP2* gene was strongly associated with this response, whereas ABC transporter genes were suggested to play a role in herbicide translocation. Glyphosate resistance was highly prevalent in 90% of populations, with resistance levels influenced by temperature. Multiple mechanisms were identified, including mutations in the *EPSPS* gene, variation in gene copy number, and differential expression among copies, with copies located on chromosome 4 contributing more to total expression. Three populations exhibited rapid necrosis following glyphosate application, suggesting the involvement of non-target-site resistance mechanisms, possibly mediated by transporters. Finally, RNA-based approaches were evaluated through the exogenous application of siRNA and dsRNA targeting the *PDS* gene. Uptake assays indicated limited foliar penetration, suggesting morphological and biochemical barriers. Although no visible bleaching symptoms were observed, infiltration of siRNA reduced *PDS* transcript levels, and similar results were observed for dsRNA mixtures. *In silico* analysis indicated low sequence similarity with non-target organisms. The results highlight key limitations associated with RNA delivery while providing a methodological basis for the development of RNA-based technologies for weed control in *Conyza*. Together, these data demonstrate that herbicide resistance in *Conyza* is widespread and mechanistically complex, driven by multiple target-site and non-target-site mechanisms across herbicide classes and environmental conditions. These findings provide a foundation for resistance monitoring and management in Brazil and identify limitations and opportunities for RNAi-based control strategies.

[1]Doctoral Thesis in Plant Science, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (155p.). May 2026.

SUMÁRIO

Página

1. INTRODUÇÃO 1

1.1 Objetivo geral 3

1.2 Objetivos específicos 3

1.3 Hipóteses 3

2. REVISÃO BIBLIOGRÁFICA 5

2.1 Caracterização das espécies de buva 5

2.2 Epidemiologia da resistência aos herbicidas 7

2.3 Mecanismos de resistência aos herbicidas 8

2.3.1 Inibidor da EPSPS 10

2.3.2 Inibidores da ALS 12

2.3.3 Herbicidas auxínicos 13

2.4 RNAi no controle de plantas daninhas 15

2.5 Referências 17

3. ARTIGO 1 - Mapping target-site resistance mechanisms to ALS-inhibiting herbicides in *Conyza bonariensis* and *Conyza sumatrensis* in Brazil 25

3.1 Introduction 26

3.2 Material and Methods 29

3.2.1 Plant material 29

3.2.2 ALS-inhibiting herbicides resistance screening 30

3.2.3 *ALS* gene sequencing 30

3.2.4 Mutation markers 31

Página

3.2.5 *ALS* gene copy number variation and overexpression 32
3.2.6 P450 inhibitor assay 33
3.2.7 Data analysis 33
3.3 Results and Discussion 34
3.3.1 ALS-inhibiting herbicides resistance frequency 34
3.3.2 *ALS* gene sequencing 35
3.3.3 Identification of *ALS* gene mutations through SNAP markers 38
3.3.4 *ALS* gene copy number variation and overexpression 39
3.3.5 P450 inhibition does not alter the resistant response 42
3.3.6 Epidemiologic approach 42
References 45
Supplementary Material 49
4. ARTIGO 2 - Mapping auxinic herbicide resistance in *Conyza* populations in Brazil and investigating candidate resistance gene expression 51
4.1 Introduction 53
4.2 Materials and Methods 55
4.2.1 Plant material 55
4.2.2 Auxinic herbicides resistance screening 55
4.2.3 Auxinic herbicide mixtures and combinations with glyphosate for controlling resistant populations 55
4.2.4 Expression of candidate resistance genes associated with rapid necrosis symptoms in response to different auxinic herbicides 57
4.2.5 Expression of candidate resistance genes associated with rapid necrosis symptoms in response to auxinic herbicide mixtures and combinations with glyphosate 58
4.3 Results and Discussion 58

Página

4.3.1 Auxinic herbicides resistance frequency 58

4.3.2 Auxinic herbicide mixtures and combinations with glyphosate for controlling resistant populations 64

4.3.3 Expression of candidate resistance genes associated with rapid necrosis symptoms in response to different auxinic herbicides 66

4.3.4 Expression of candidate resistance genes associated with rapid necrosis symptoms in response to auxinic herbicide mixtures and combinations with glyphosate 69

4.4 Conclusion 73

References 73

5. ARTIGO 3 - Complex *EPSPS* copy architecture, subgenome-specific expression, and non-target-site mechanisms shape glyphosate resistance levels in Sumatran and hairy fleabane 77

5.1 Introduction 79

5.2 Material and Methods 81

5.2.1 Plant material 81

5.2.2 Glyphosate resistance screening 82

5.2.3 Glyphosate dose-response curves under different temperature conditions. 82

5.2.4 *EPSPS* gene sequencing 83

5.2.5 *EPSPS* gene copy number variation 85

5.2.6 *EPSPS* global and copy-specific expression 86

5.2.7 Investigation of an unusual necrotic phenotype and expression of candidate genes 87

5.3 Results and Discussion 88

5.3.1 Glyphosate resistance frequency and distribution 88

5.3.2 Low temperature reduces glyphosate resistance levels 89

Página

5.3.3 Multiple *EPSPS* copies and SNP diversity in Sumatran and hairy fleabane....91

5.3.4 Variation in *EPSPS* copy number across populations 96

5.3.5 Global and copy-specific *EPSPS* expression 97

5.3.6 Unusual glyphosate-induced necrotic phenotype and expression of ABC transporter genes 99

5.4 Conclusion.... 105

References.... 105

Supplementary Material.... 110

6. ARTIGO 4 - Exogenous RNAi targeting phytoene desaturase in Sumatran fleabane: methodological evaluation and limitations 118

6.1 Introduction 119

6.2 Materials and Methods 122

6.2.1 siRNA and dsRNA design and synthesis.... 122

6.2.2 siRNA and dsRNA uptake by Sumatran fleabane leaves 124

6.2.3 *PDS* siRNA and dsRNA efficacy in Sumatran fleabane 125

6.3 Results and Discussion.... 127

6.3.1 Low foliar uptake of siRNA and dsRNA in Sumatran fleabane.... 127

6.3.2 siRNA and dsRNA oligonucleotides specificity 130

6.3.3 Limited *PDS* gene silencing following siRNA oligonucleotide application in Sumatran fleabane 131

6.3.4 Limited *PDS* gene silencing following exogenous dsRNA application in Sumatran fleabane.... 133

6.4 Conclusion.... 135

References.... 136

7. CONSIDERAÇÕES FINAIS 140

RELAÇÃO DE TABELAS

Página

ARTIGO 1

1. Primers developed for sequencing, markers, copy number variation, and overexpression studies of the *ALS* gene of *C. bonariensis* and *C. sumatrensis*…….32

2. Injury (%) following malathion and imazethapyr or cloransulam-methyl application 21 days after treatment in Sumatran and hairy fleabane populations……....42

S1. Efficacy of control 28 days after herbicide treatment in 70 *Conyza* spp. populations from Brazil with four ALS-inhibiting herbicides at 50% and 100% of the label rate……...…….…...49

ARTIGO 2

1. Auxinic herbicides and combinations with glyphosate and application doses evaluated in Sumatran fleabane populations……..……..……..……..……56

2. Efficacy of control (%) at 28 days after herbicide treatment in 75 *Conyza* spp. populations from Brazil with four auxin herbicides at 50% and 100% of the label rate…….……...……...60

ARTIGO 3

1. Primers developed for sequencing, copy number variation, and expression studies of the *EPSPS* gene from hairy (Cb) and Sumatran fleabane (Cs)……...……..84

2. ED_{50} values, resistance factor (RF), and temperature-response ratio (TRR) for 29 putatively glyphosate-resistant populations and two susceptible populations of Sumatran and hairy fleabane, evaluated at high (30/25°C) and low (15/12°C) day/night temperatures…….……..….90

S1. Efficacy of control 28 days after herbicide treatment in 70 *Conyza* spp. populations from Brazil with glyphosate at 50% and 100% of the label rate……...……..110

ARTIGO 4

1. siRNA oligonucleotide sequences with corresponding modifications for the *PDS* gene of Sumatran fleabane……..……..…….123

2. dsRNA oligonucleotide sequences for the *PDS* gene of Sumatran fleabane……..…124

3. Query coverage and percent identity of siRNA and dsRNA oligonucleotides targeting the *PDS* gene of Sumatran fleabane across non-target species genomes……..130

RELAÇÃO DE FIGURAS

Página

ARTIGO 1

1. Resistance patterns to ALS-inhibiting herbicides among 70 *Conyza* spp. populations (A) and their distribution across Brazil (B). In panel A, numbers represent the number of populations across categories, with percentages shown below……35

2. Partial alignment of *ALS* gene sequence of *A. thaliana* (X51514.1), Sumatran fleabane (MZ816701.1, MZ816700.1, and MZ816699.1), and hairy fleabane (BLAST in CM112843.1, CM112844.1, and CM112845.1) reference genomes, with C31 (Thr122 and His197), G14 (Ser197) (Sumatran fleabane), T1 (Arg197), and T2 (Leu574) (hairy fleabane) populations (A) and with *C. canadensis* (HM067014.1), *A. artemisiifolia* (KX870184.1), *P. rhoeas* (AJ577316.1) and *S. vulgaris* (KR024410.1) (B)..…….37

3. Melt curve and amplification plots for the Ala122, Ser197, Arg197, His197, and Leu574 markers validated in populations (C31, C63, T2, C25, and T3) with mutations identified through *ALS* sequencing and S populations as control……..38

4. Resistance (red) and susceptible (green) patterns to chlorimuron-ethyl (CHL), imazethapyr (IMA), and cloransulam-methyl (CSL), presence of *ALS* mutations as indicated by molecular markers, relative copy number (orange bars), and relative expression (purple bars) in resistant hairy and Sumatran fleabane populations. Two susceptible populations (C7 and G8) are shown for comparison…….....…41

5. *Conyza* species *ALS* mutations (A) and target-site resistance mechanisms (B) distribution across Brazil……...……..43

6. Resistance (red) and susceptibility (green) patterns to chlorimuron-ethyl (CHL), imazethapyr (IMA), and cloransulam-methyl (CSL), mutation marker, and sequencing results for 16 resistant hairy and Sumatran fleabane populations without mutations identified in the marker analysis…...…….....…….44

ARTIGO 2

1. Resistance patterns to auxinic herbicides in 75 *Conyza* spp. populations (A) and distribution of the RN phenotype following 2,4-D and triclopyr application (B) in Brazil…….……..59

2. Injury (%) following application of auxinic herbicides applied alone or in mixtures in Sumatran fleabane populations with contrasting resistance and RN phenotypes: C2 - susceptible to all auxinic herbicides without RN (A), C7 - susceptible with RN to 2,4-D (B), G5 - resistant to 2,4-D with RN to 2,4-D at 2 h and triclopyr at 4 h (C), G10 - resistant to 2,4-D with RN to both herbicides at 4 h (D), and C22 - resistant to 2,4-D without RN to 2,4-D but with RN to triclopyr (E)……..……...…64

Página

3. Relative expression of the *PLP2* gene in four Sumatran fleabane populations: susceptible (C3, A), RN 2,4-D 2 h (G5, B), RN 2,4-D 4 h (C25, C), and no RN (C22, D), following treatment with 2,4-D, triclopyr, dicamba, and halauxifen at 5, 30, and 60 min after treatment……..……...……67

4. Relative expression of the *ABCB4* gene in four Sumatran fleabane populations: susceptible (C3, A), RN 2,4-D 2 h (G5, B), RN 2,4-D 4 h (C25, C), and no RN (C22, D), following treatment with 2,4-D, triclopyr, dicamba, and halauxifen at 5, 30, and 60 min after treatment…….…….……68

5. Relative expression of the *ABCC3* gene in four Sumatran fleabane populations: susceptible (C3, A), RN 2,4-D 2 h (G5, B), RN 2,4-D 4 h (C25, C), and no RN (C22, D), following treatment with 2,4-D, triclopyr, dicamba, and halauxifen at 5, 30, and 60 min after treatment……..……69

6. Heatmaps showing the relative expression (log_2 fold change, log_2FC) of *PLP2* (A) and *ABCC3* (B) genes, and the visual control (%) of auxinic herbicides and their mixtures (C), in five Sumatran fleabane populations (C2 - susceptible, C7 - susceptible with RN to 2,4-D, G5 - resistant to 2,4-D with RN to 2,4-D at 2 h and triclopyr at 4 h, G10 - resistant to 2,4-D with RN to both herbicides at 4 h, and C22 - resistant to 2,4-D without RN to 2,4-D but with RN to triclopyr)……..……...……...71

ARTIGO 3

1. Resistance to glyphosate in 70 *Conyza* spp. populations (A) and their distribution across Brazil (B)……..……...88

2. Dose-response curves for hairy fleabane (A) and Sumatran fleabane (B) in one susceptible population and two representative resistant populations per species (hairy fleabane: G2 (S), G3, G1; Sumatran fleabane: G8 (S), G16, G13), evaluated at high (30/25 °C) and low (15/12 °C) day/night temperatures……90

3. Region encompassing codons 100-106 of all identified copies of the *EPSPS* gene in hairy fleabane (Cb) and Sumatran fleabane (Cs) (A). Representative sequencing electropherograms from populations without SNPs (B), and with the mutations Ala100Ser and Pro106Thr (C), Thr102Ile (D), and Gly101Ala (E)……...92

4. Resistance (red) and susceptible (green) patterns to glyphosate, *EPSPS* relative copy number (orange bars), and relative global expression (purple bars) in resistant hairy and Sumatran fleabane populations……...…….96

5. Relative *EPSPS* expression at the copy-specific level compared with global expression in six Sumatran fleabane populations (C10-S, G14-R, G19-R, C40-R, G5-R, G6-R) measured by qPCR analysis……..……..…..……..…..98

6. Relative *EPSPS* expression at the copy-specific level in two Sumatran fleabane populations (G5-R, G6-R), with three replicates, measured by RNASeq analysis……...99

7. Sumatran fleabane plants without symptoms (A) and showing atypical necrosis symptoms (B) at 3 days after application (DAT) of glyphosate diammonium salt at 1,920 g ha^{-1}. Sumatran fleabane plants treated with glyphosate potassium salt at 1,080 g ha^{-1} and maintained at 30 °C and 15 °C, evaluated at 5 DAT (C) and 21 DAT (D)..100

Página

8. Injury in three Sumatran fleabane populations (G5, C2 and C4) exhibiting unusual necrotic symptoms under four temperature regimes before/after glyphosate potassium salt application (high/high, low/high, high/low, and low/low) (A); dose-response curves of population G5 under the same temperature regimes with glyphosate potassium salt (B); and dose-response curves of population G5 conducted at 12 °C and 25 °C using glyphosate diammonium salt (C) and potassium salt (D)……..……...101

9. Relative expression of putative genes associated with glyphosate-induced necrosis in resistant (R) and susceptible (S) populations under low (15 °C) and high (30 °C) temperature conditions…….…….……..…..102

ARTIGO 4

1. Fluorescence content in wash-off and in-leaf (a) and in-leaf only (b), following foliar application of fluorescently labeled siRNA in Sumatran fleabane using spraying (SP), infiltration (INF), and incubation (IC) at 0, 1, and 5 μM concentrations. Representative confocal microscopy images (35 μm depth, 120 ms exposure time) of leaf sections showing siRNA fluorescence in the tissue (c)……..……..……..….127

2. Fluorescence content in the wash-off solution and in-leaf tissue in non-injured and injured leaves (a) following foliar application of fluorescently labeled dsRNA to Sumatran fleabane by spraying (SP) and infiltration (INF) at concentrations of 0, 1, and 2 μM. Representative confocal microscopy images (35 μm depth, 800 ms exposure time) of leaf sections showing dsRNA fluorescence in the tissue (b)…….....128

3. Relative expression of the *PDS* gene in Sumatran fleabane 24 h after application of four siRNA oligonucleotides by foliar spraying at 2 μM (a), infiltration at 2 μM (b), infiltration at 10 μM (c), and 48 h after infiltration at 2 μM (d)……..……....132

4. Agarose gel electrophoresis of the synthesized dsPDS1, dsPDS2, and dsPDS3 fragments (a) and representative images of Sumatran fleabane leaves three days after treatment application (b)…….…….....133

5. Relative expression of the *PDS* gene in Sumatran fleabane 24 h after application of three dsRNA oligonucleotides by foliar spraying at 3 μM (a); 72 h after the last application of the three dsRNAs applied individually or in combination post-synthesis (mix) at 5 μM in two applications (b); and 24 h after application of 2 μM for three consecutive days of the three dsRNAs applied individually, as a mixture during synthesis (mix 1) or as a post-synthesis mixture (mix 2) (c)……..…134

1. INTRODUÇÃO

A resistência a herbicidas possui elevado impacto na agricultura e promove aumento nos custos de produção e na necessidade de mudanças no manejo devido à dificuldade de controle das plantas daninhas resistentes (Délye, Jasieniuk e LeCorre, 2013). Atualmente existem 548 casos únicos de resistência de plantas daninhas a herbicidas no mundo, definidos como combinações entre espécie e mecanismo de ação do herbicida, sendo 61 casos identificados no Brasil (Heap, 2026). No país, estima-se a ocorrência de resistência a herbicidas em 20,1 milhões de hectares no sistema de produção de soja, o que resulta em custo médio anual de R$ 9 bilhões, entre custo de manejo e perdas devido à resistência (Adegas, Vargas e Gazziero, 2017). O controle de plantas resistentes é dificultado pela baixa disponibilidade de novos ingredientes ativos, pela baixa eficiência de herbicidas alternativos e pela proibição de uso ou pela retirada do mercado de herbicidas por problemas toxicológicos ou questões comerciais. A resistência a herbicidas pode ser conferida por diferentes mecanismos relacionados ao metabolismo da planta ou alterações em genes-alvo (Délye, Jasieniuk e LeCorre, 2013; Gaines, 2020).

O monitoramento da evolução e da distribuição dos casos de resistência associados ao seu mecanismo de resistência é um passo fundamental para adoção de práticas de manejo eficientes no controle das plantas daninhas resistentes de ocorrência em cada local (Comont *et al.*, 2019). O desenvolvimento e a aplicação de ferramentas rápidas para a detecção de mecanismos de resistência em nível populacional, como testes bioquímicos, marcadores moleculares, diagnósticos baseados em expressão gênica ou análises metabólicas, são essenciais para permitir diagnósticos precoces e em larga escala (Burgos *et al.*, 2013; Sulzback *et al.*, 2024). Estudos focados na epidemiologia da resistência são necessários para analisar de forma mais abrangente e em larga escala a ocorrência de populações resistentes e adequar o manejo da resistência a nível de propriedade. Essa abordagem permite a identificação de padrões espaciais e temporais de dispersão e a antecipação de cenários de risco devido à seleção por herbicidas (Hicks *et al.*, 2018).

Espécies de buva (*Conyza* sp.) estão dentre as principais plantas daninhas dos cultivos agrícolas no mundo devido às suas características de elevada prolificidade, fácil dispersão e alto potencial competitivo (Dan *et al.*, 2013). Falhas no controle de plantas de buva resultam em perdas de produtividade de 48 a 90% em soja (Byker *et al.*, 2013; Gazziero *et al.*, 2010; Soltani, Shropshire e Sikkema, 2022) e 32% em milho (Soltani, Shropshire e Sikkema, 2021). A evolução de populações de buva para resistência a herbicidas de diferentes mecanismos de ação dificulta o controle e limita as opções de manejo (Dan *et al.*, 2013). Atualmente, é registrada resistência de espécies de buva a herbicidas de seis mecanismos de ação, sendo eles inibidores dos fotossistemas I e II, da enzima 5-enolpiruvilshikimate-3-fosfato sintase (EPSPS), da enzima acetolactato sintase (ALS), da enzima protoporfirinogênio oxidase (PPO) e a herbicida mimetizador de auxinas, totalizando 116 casos únicos de resistência em buva no mundo (Heap, 2026).

Diante do cenário de limitado desenvolvimento de novas moléculas herbicida e da rápida evolução da resistência a esses compostos, o desenvolvimento de outros métodos de controle é fundamental no manejo de espécies de plantas daninhas nos cultivos agrícolas. Nesse sentido, o RNA de interferência (RNAi) é uma ferramenta de silenciamento gênico utilizada em estudos de expressão e função gênica (Dalakouras *et al.*, 2020; Kumaran *et al.*, 2020), e possui potencial de uso no controle de plantas daninhas através de aplicação exógena para compor o manejo integrado dessas espécies (Zabala-Pardo *et al.*, 2022). Estudos indicam que genes-alvo de silenciamento por RNAi podem incluir os mesmos genes que codificam as enzimas-alvo de herbicidas, genes associados à resistência, como aqueles envolvidos na detoxificação, bem como genes relacionados ao florescimento, esterilidade, dormência e degrane de sementes (Panozzo *et al.*, 2025; Zabala-Pardo *et al.*, 2022).

A seleção do gene-alvo é determinante para a especificidade do RNAi, podendo resultar em efeitos seletivos ou não seletivos em relação à cultura. Essa característica define o posicionamento da tecnologia no manejo, permitindo sua aplicação tanto em pós-emergência da cultura quando há seletividade, quanto em dessecação pré-semeadura, em aplicações não seletivas (Zabala-Pardo *et al.*, 2022). Em ambos os cenários, o RNAi apresenta potencial para integrar programas de manejo, especialmente como ferramenta complementar na rotação de mecanismos de ação, contribuindo para a mitigação da pressão de seleção para evolução de resistência a herbicidas. Embora avanços recentes tenham demonstrado o uso potencial de RNAi no controle de plantas daninhas (Castro *et*

al., 2026; Cintra *et al.*, 2025), desafios importantes ainda limitam sua aplicação prática, incluindo identificação e validação de genes-alvo, desenvolvimento de sistemas eficientes de entrega, como nanopartículas ou formulações que aumentem a absorção e estabilidade, persistência das moléculas em condições ambientais e a viabilidade econômica em escala de campo (Panozzo *et al.*, 2025).

1.1 Objetivo geral

Caracterizar a distribuição da ocorrência de diferentes mecanismos de resistência a herbicidas em espécies de *Conyza* no Brasil e avaliar metodologias de silenciamento gênico para controle dessas espécies.

1.2 Objetivos específicos

i. Determinar a frequência de ocorrência dos mecanismos de resistência aos principais herbicidas em espécies do gênero *Conyza* no Brasil.
ii. Desenvolver marcadores moleculares para a detecção rápida de resistência a herbicidas inibidores da ALS ocasionada por mecanismos relacionados ao local de ação em espécies de *Conyza*.
iii. Caracterizar a arquitetura de cópias do gene *EPSPS* e a contribuição de mecanismos não relacionados ao local de ação na modulação dos níveis de resistência ao glifosato em espécies de *Conyza*.
iv. Avaliar estratégias de aplicação de dsRNA e siRNA para indução de silenciamento gênico em *Conyza sumatrensis*.

1.3 Hipóteses

i. A resistência ao glifosato e aos herbicidas inibidores da ALS em espécies de buva pode ser identificada por meio de marcadores moleculares específicos associados a mutações no local-alvo, incluindo Pro106Ser no gene *EPSPS* e Pro197Ser, Pro197Ala, Trp574Leu e Ala205Val no gene *ALS*.
ii. A resistência ao glifosato predomina nas populações de buva no Brasil, associada principalmente a mutações no gene-alvo e a múltiplas origens independentes,

seguida pela resistência cruzada a herbicidas inibidores da ALS. A resistência aos herbicidas auxínicos é mais recente e possivelmente apresenta origem única.

iii. A epidemiologia da resistência a herbicidas permite caracterizar a frequência, distribuição geográfica e mecanismos de resistência em espécies de buva no Brasil, possibilitando prever a evolução da resistência e planejar estratégias de manejo e prevenção.

iv. Genes-alvo de herbicidas e genes associados aos mecanismos de resistência constituem alvos promissores para aplicação exógena de RNAi visando ao silenciamento gênico e ao controle de populações resistentes de buva, tornando essa tecnologia uma alternativa potencial para o manejo da resistência.

2. REVISÃO BIBLIOGRÁFICA

2.1 Caracterização das espécies de buva

O gênero *Conyza* (Asteraceae) possui aproximadamente 50 espécies, conhecidas no Brasil como buva ou voadeira (Kissmann e Groth, 1999). Dentre estas, destacam-se *Conyza bonariensis* (L.) Cronquist, *Conyza canadensis* (L.) Cronquist e *Conyza sumatrensis* (Retz.) E. Walker, amplamente distribuídas em sistemas agrícolas em diferentes regiões do mundo, e consideradas espécies invasoras de elevada adaptabilidade (Kalsing *et al*., 2024b). Essas espécies são originárias das Américas, sendo *C. bonariensis* e *C. sumatrensis* originárias da América do Sul (Kissmann e Groth, 1999; Thebaud e Abbott, 1995) e *C. canadensis* nativa da América do Norte (Frankton e Mulligan, 1987).

As espécies de *Conyza* apresentam ciclo predominantemente anual, podendo *C. canadensis* comportar-se como anual ou bienal, conforme as condições ambientais (Regehr e Bazzaz, 1979). As plantas são autógamas, com reprodução exclusivamente por sementes, apresentando elevada produção, que pode variar de 200 mil a 800 mil sementes por planta (Kaspary *et al.*, 2017; Shields *et al.*, 2006), o que contribui para o rápido estabelecimento e disseminação das populações. As sementes são pequenas, leves e com presença de papus, o que favorece a dispersão pelo vento, principalmente a curtas distâncias, embora eventos de dispersão a longa distância também possam ocorrer (Andersen, 1993; Dauer, Mortensen e Vangessel, 2006; Shields *et al.*, 2006).

A germinação das sementes de buva é favorecida por temperaturas amenas, cerca de 20 °C (Vidal *et al.*, 2007), e ocorre na presença de luz, sendo as sementes fotoblásticas positivas (Nandula *et al.*, 2006). Dessa forma, a emergência dessas espécies ocorre predominantemente em períodos mais frios, como outono e inverno, especialmente em sistemas de plantio direto, onde as sementes permanecem na superfície do solo (Vargas *et al*., 2007). Apesar da alta produção, as sementes apresentam baixa longevidade no solo,

resultando em um banco de sementes transitório e dependente de reposição anual (Vargas *et al.*, 2018). As sementes apresentam baixa dormência e possuem capacidade de germinar prontamente após a dispersão da planta quando as exigências de temperatura, luz e umidade são atendidas (Vargas *et al.*, 2018; Wu *et al.*, 2007).

Em relação à ploidia, *C. canadensis* é diploide (2n = 18), enquanto *C. bonariensis* e *C. sumatrensis* possuem genoma hexaploide (2n = 54) (Gomes *et al.*, 2025; Thebaud e Abbott, 1995). A poliploidia nessas espécies tem origem alopoliploide, possivelmente derivada de diferentes combinações de espécies ancestrais, sem indícios de participação recente de *C. canadensis* nesse processo (Thebaud e Abbott, 1995). A condição poliploide em espécies vegetais está associada ao aumento da variabilidade genética e da plasticidade fenotípica, características que favorecem sua adaptação a diferentes ambientes e sistemas de manejo (Rutland, Hall e McElroy, 2021).

O estabelecimento das plantas de buva ocorre principalmente em ambientes com perturbações (Tremmel e Peterson, 1983), como áreas agrícolas, pomares e margens de estradas, onde apresentam elevada capacidade competitiva. Em culturas como a soja, infestações podem causar reduções expressivas na produtividade, especialmente quando o estabelecimento ocorre antes da semeadura (Gazziero *et al.*, 2010; Trezzi *et al.*, 2014). A combinação de elevada produção de sementes, dispersão eficiente, estabelecimento favorecido em sistemas perturbados e variabilidade genética contribui para o sucesso dessas espécies como plantas daninhas em sistemas agrícolas. Esses fatores também estão associados à elevada capacidade adaptativa da espécie e à frequente evolução de resistência a herbicidas de diferentes mecanismos de ação (Kalsing *et al*., 2024c).

Os primeiros relatos de resistência a herbicidas em espécies do gênero *Conyza* datam da década de 1980, com a identificação de biótipos de *C. canadensis* e *C. sumatrensis* resistentes ao paraquat (inibidor do fotossistema I) na Ásia (Fuerst, 1985), seguidos por *C. bonariensis* resistente à simazina (inibidor do fotossistema II) na Europa (Shaaltiel e Gressel, 1987). Na década de 1990, foram descritos os primeiros casos de resistência múltipla, envolvendo herbicidas com mecanismos de ação distintos, como inibidores do fotossistema II e da ALS (Heap, 2026). A partir dos anos 2000, a resistência ao glifosato (inibidor da EPSPS) tornou-se amplamente disseminada, inicialmente nos Estados Unidos e, posteriormente, no Brasil, onde foi registrada a partir de 2005 (Vangessel, 2001; Vargas *et al.*, 2007). No contexto brasileiro, a partir de 2011, foram reportados biótipos com resistência múltipla ao glifosato e a inibidores da ALS, o que

intensificou o uso de herbicidas de outros mecanismos de ação, como mimetizadores de auxina, inibidores da glutamina sintetase (GS) e da PPO (Santos *et al.*, 2014; Soares *et al.*, 2012). Recentemente, foram reportados casos de resistência múltipla envolvendo diferentes mecanismos de ação herbicida em *C. sumatrensis*, evidenciando a elevada capacidade de adaptação da espécie sob pressão de seleção (de Pinho *et al*., 2019).

2.2 Epidemiologia da resistência aos herbicidas

A evolução da resistência a herbicidas em plantas daninhas é resultado da pressão de seleção imposta pelo uso contínuo de herbicidas com o mesmo mecanismo de ação, o que reduz a eficiência de controle e limita as opções de manejo (Délye, Jasieniuk e LeCorre, 2013; Norsworthy *et al.*, 2012). A epidemiologia da resistência a herbicidas refere-se ao estudo da distribuição, frequência e dinâmica temporal e espacial de biótipos resistentes, assim como dos fatores que influenciam sua evolução e dispersão em sistemas agrícolas (Comont e Neve, 2021). A resistência pode ser conferida por diferentes mecanismos nas plantas, originados da variabilidade genética presente nas populações, e pode surgir independentemente em diferentes locais ou a partir de uma origem comum, seguida de dispersão via fluxo gênico por pólen ou sementes (Délye, Jasieniuk e LeCorre, 2013; Kreiner *et al.*, 2022).

Atualmente, são registrados mais de 500 casos únicos de resistência a herbicidas em espécies de plantas daninhas distribuídas globalmente (Heap, 2026), configurando um processo de evolução recorrente em ambientes de cultivo (Comont e Neve, 2021). Nesse contexto, abordagens epidemiológicas são empregadas para avaliar, em escala regional ou nacional, a distribuição da resistência, os níveis de suscetibilidade das populações e sua relação com as práticas de manejo. Esses estudos têm demonstrado elevada variabilidade na resposta das populações a herbicidas e evidenciam a associação entre a intensidade de uso de determinados mecanismos de ação e a seleção de biótipos com sensibilidade reduzida a determinados herbicidas (Comont *et al.*, 2019; Mascanzoni *et al.*, 2018; Ravet *et al.*, 2021).

Diferentemente de abordagens tradicionais, focadas na caracterização pontual de populações resistentes, a epidemiologia da resistência permite identificar padrões espaciais e temporais de seleção e fatores de risco associados à evolução da resistência,

como sistemas de monocultivo e uso recorrente dos mesmos herbicidas (Beckie *et al.*, 2008; Hicks *et al.*, 2018). A integração de dados epidemiológicos com informações sobre os mecanismos de resistência permite compreender a prevalência e a distribuição desses mecanismos em diferentes regiões, o que é essencial para o desenvolvimento de estratégias de manejo mais eficientes e sustentáveis.

2.3 Mecanismos de resistência aos herbicidas

A resistência a herbicidas ocorre por meio de mecanismos relacionados ao local de ação (RLA) e não relacionados ao local de ação (NRLA) dos herbicidas (Powles e Yu, 2010). Os mecanismos RLA envolvem alterações na enzima-alvo do herbicida e incluem mutações pontuais que modificam aminoácidos envolvidos na interação herbicida-enzima, reduzindo a afinidade da molécula pelo sítio ativo (Gaines *et al.*, 2020). Essas mutações estruturais podem conferir desde níveis elevados de resistência, como observado na substituição tripla TAP-IVS na *EPSPS* de *Amaranthus hybridus* (Perotti *et al.*, 2019; Sulzbach *et al.*, 2024), até níveis baixos ou moderados, como a mutação Pro106Thr na *EPSPS* de *Eleusine indica, Digitaria insularis* e *Lolium rigidum* (de Carvalho *et al*., 2012; Ng *et al*., 2004; Wakelin e Preston, 2006). Em alguns casos, as alterações estruturais da enzima podem acarretar custos adaptativos às plantas, afetando seu crescimento mesmo na ausência do herbicida (Zangeneh *et al.*, 2018). Em *E. indica*, por exemplo, a mutação dupla TIPS em *EPSPS* foi associada a custos adaptativos expressos pela redução da capacidade reprodutiva das plantas resistentes (Han *et al*., 2017).

Além das mutações, a superexpressão do gene-alvo constitui outro importante mecanismo RLA. O aumento da quantidade da enzima-alvo, devido à amplificação gênica ou ao aumento da transcrição, reduz a eficácia do herbicida em inibir a enzima a níveis letais para a planta (Gaines *et al.*, 2010; Galeano *et al.*, 2016). Até o momento, nove mecanismos de ação herbicida possuem casos documentados de resistência RLA, sendo eles inibidores de ACCase (acetil-coa carboxilase), ALS, EPSPS, GS, PPO, FSII, tubulina, PDS (fitoeno desaturase) e herbicidas auxínicos (Murphy e Tranel, 2019). Em geral, a resistência RLA apresenta caráter monogênico e é específica para herbicidas do mesmo mecanismo de ação (Gaines *et al.*, 2020).

Os mecanismos NRLA englobam alterações fisiológicas e bioquímicas que reduzem a quantidade de herbicida que atinge o local de ação, sem implicar modificações na enzima-alvo (Rigon *et al.*, 2020). Esses mecanismos incluem redução da absorção foliar, alterações no transporte e na translocação do herbicida, processos de compartimentalização e aumento da capacidade de metabolização para formas não tóxicas (Gaines *et al.*, 2020). A absorção reduzida é menos frequentemente relatada como mecanismo primário de resistência, mas pode atuar de forma complementar, como descrito em *Amaranthus tuberculatus* resistente ao glifosato, no qual alterações cuticulares contribuíram juntamente com resistência RLA (Nandula *et al.*, 2013).

A alteração da translocação é um mecanismo NRLA bem caracterizado para os herbicidas paraquat e glifosato. Em diversas espécies resistentes, a limitação do transporte está associada ao sequestro vacuolar, impedindo que o herbicida alcance tecidos meristemáticos (Jugulam e Shyam, 2019; Powles e Yu, 2010). Esse mecanismo já foi descrito em *Lolium perenne*, *L. rigidum*, *C. bonariensis*, *C. canadensis*, *A. tuberculatus*, *Ambrosia trifida* e *Amaranthus palmeri* (Jugulam e Shyam, 2019; Moretti e Hanson, 2017). Transportadores da família ABC e de poliaminas têm sido propostos como mediadores desse sequestro, embora os mecanismos moleculares ainda não estejam totalmente elucidados. Para herbicidas auxínicos, como o 2,4-D, alterações na abundância do transportador ABCB19 foram relatadas em *Raphanus raphanistrum*, sugerindo que maior atividade de transporte possa reduzir a concentração do herbicida em tecidos sensíveis (Goggin *et al.*, 2020). Em *C. sumatrensis*, mecanismos envolvendo transportadores ABC também foram sugeridos para explicar a resposta de rápida necrose ao 2,4-D (Queiroz *et al.*, 2022).

Outro mecanismo NRLA frequentemente reportado é o aumento da metabolização dos herbicidas, que pode envolver a ação coordenada de enzimas citocromo P450 monooxigenase (CytP450), glutationa-S-transferases (GST), glicosiltransferases (GTs) e transportadores ABC (Gaines *et al.*, 2020; Powles e Yu, 2010). A detoxificação ocorre em três fases: modificações da molécula por oxidação, redução ou hidrólise mediadas por CytP450; conjugação dos metabólitos por GSTs ou GTs; e compartimentalização vacuolar ou deposição na parede celular por transportadores ABC (Rigon *et al.*, 2020). Esse mecanismo pode conferir resistência metabólica múltipla, pois as enzimas envolvidas podem atuar sobre herbicidas de diferentes mecanismos de ação (Délye, Jasieniuk e LeCorre, 2013). A resistência metabólica já foi descrita para herbicidas

inibidores de ACCase, ALS, FSII, PPO, tubulina, PDS, HPPD (hidroxifenil piruvato dioxigenase), DOXP (deoxi-d-xilulose fosfato sintase) e para herbicidas auxínicos (Gaines *et al.*, 2020). Em diversas espécies, o aumento da atividade de CytP450 e GST está associado à resistência a herbicidas dos grupos ACCase, ALS e FSII, destacando seu papel central na resistência metabólica em plantas daninhas (Rigon *et al.*, 2020).

2.3.1 Inibidor da EPSPS

A enzima 5-enolpiruvilshikimate-3-fosfato sintase (EPSPS) atua na via do ácido chiquímico, responsável pela biossíntese dos aminoácidos aromáticos fenilalanina, tirosina e triptofano, essenciais para o crescimento vegetal. O glifosato inibe a EPSPS ao competir com o fosfoenolpiruvato pelo sítio ativo da enzima, interrompendo a síntese desses aminoácidos e levando ao acúmulo de metabólitos tóxicos e à morte da planta. Alterações estruturais na enzima, como mutações no gene *EPSPS*, podem reduzir a afinidade do herbicida pelo sítio de ligação, conferindo resistência (Duke e Powles, 2008).

A resistência ao glifosato em espécies de *Conyza* é amplamente documentada e envolve mecanismos RLA e NRLA, cuja contribuição varia entre biótipos e regiões geográficas. Entre os mecanismos RLA, destaca-se a superexpressão do gene *EPSPS*. Em biótipos resistentes de *C. canadensis* e *C. bonariensis* provenientes dos Estados Unidos e da Espanha, foram observados níveis basais de expressão da *EPSPS* duas a três vezes superiores aos de plantas suscetíveis, indicando que o aumento da atividade enzimática contribui para a resistência e pode refletir uma base genética comum à evolução desse fenótipo nessas espécies (Dinelli *et al.*, 2006, 2008). Embora a amplificação gênica de *EPSPS* seja um mecanismo consolidado em outras espécies, como *A. palmeri*, que pode apresentar desde dezenas até mais de cem cópias adicionais do gene (Gaines *et al.*, 2010), sua ocorrência ainda é pouco explorada para o gênero *Conyza*.

Outro mecanismo RLA envolve mutações pontuais no gene *EPSPS*, principalmente na posição Pro106. A substituição Pro106Ser foi identificada em biótipos resistentes de *C. canadensis* no Canadá e nos Estados Unidos (Beres *et al.*, 2020; Page *et al.*, 2018), conferindo altos níveis de resistência, com relatos de sobrevivência a doses até 40 vezes superiores à recomendada (Beres *et al.*, 2020). Entretanto, como observado para outras espécies, como *E. indica* e *A. tuberculatus*, mutações na posição Pro106 geralmente

resultam em resistência de nível baixo a moderado *(*Baerson *et al*., 2002; de Carvalho *et al*., 2012; Nandula *et al*., 2013). Assim, a contribuição isolada dessas mutações possivelmente não explica o alto nível de resistência observado em determinados biótipos de *Conyza* e indica a provável contribuição de mecanismos NRLA.

A resistência atribuída a mecanismos NRLA é reconhecida como predominante em *Conyza* (Jugulam e Shyam, 2019). Entre esses mecanismos, a redução da translocação do glifosato, frequentemente associada ao sequestro vacuolar, constitui o processo mais caracterizado. Em *C. canadensis*, biótipos resistentes apresentaram movimentação preferencial do herbicida do citosol para o vacúolo, padrão não observado em plantas suscetíveis, sugerindo o envolvimento de sistemas de transporte especializados (Ge *et al.*, 2010). De modo semelhante, biótipos resistentes de *C. bonariensis* demonstram influência ambiental na expressão do fenótipo resistente, com reversão quase completa da resistência em condições de baixa temperatura (12°C), atribuída à limitação da translocação e ao aumento do sequestro vacuolar (Dalazen *et al.*, 2019). Esses resultados confirmam que a resistência ao glifosato em *Conyza* depende não apenas de fatores genéticos, mas também do efeito ambiental dos processos de transporte intracelular.

Estudos transcriptômicos fornecem evidências adicionais sobre a característica multigênica dos mecanismos NRLA em espécies de *Conyza*. Em *C. bonariensis*, análises de expressão revelaram que biótipos resistentes podem apresentar resistência decorrente de regulação diferencial de vários genes associados à absorção, translocação, compartimentalização e metabolismo de xenobióticos (Hereward *et al.*, 2018), reforçando a complexidade dos mecanismos NRLA. Em adição a isso, em determinados biótipos, há evidências da atuação simultânea de múltiplos mecanismos. Em *C. canadensis*, foi descrita a superexpressão coordenada do gene *EPSPS* e de genes transportadores ABC, principalmente da subfamília MRP-like, após a aplicação de glifosato (Tani, Chachalis e Travlos, 2015). Os transportadores ABC constituem uma das maiores superfamílias de proteínas em plantas, envolvidas no transporte ativo e no sequestro vacuolar de diversas moléculas (Theodoulou, 2000), e cada vez mais estão sendo associados a NRLA de resistência a herbicidas (Pan *et al*., 2021; Yuan *et al*., 2010). A atuação conjunta entre o aumento da expressão de *EPSPS* e a atividade de transportadores ABC representa um exemplo de resistência multifatorial, frequentemente observado em populações com elevada sobrevivência ao glifosato.

A resistência de espécies de *Conyza* ao glifosato caracteriza-se pela combinação variável de mecanismos RLA e NRLA, com predominância destes últimos, sendo frequentemente associada ao sequestro vacuolar, limitação da translocação, alterações na expressão gênica e alterações no gene alvo. A complexidade do processo de resistência contribui para a elevada adaptabilidade dessas espécies e favorece a rápida disseminação de biótipos resistentes em sistemas agrícolas.

2.3.2 Inibidores da ALS

A enzima acetolactato sintase (ALS) catalisa a primeira etapa da biossíntese dos aminoácidos de cadeia ramificada valina, leucina e isoleucina. Herbicidas inibidores da ALS bloqueiam essa via metabólica ao se ligarem à enzima, interrompendo a divisão celular e o crescimento das plantas. Mutações no gene *ALS* podem alterar a conformação do sítio de ligação do herbicida, reduzindo sua afinidade pela enzima e conferindo resistência (Tranel e Wright, 2002).

A resistência aos herbicidas inibidores da ALS em espécies de *Conyza* é relatada com frequência em áreas agrícolas devido ao uso recorrente desses herbicidas desde a década de 1990, especialmente em sistemas de soja (Merotto *et al.*, 2022). Levantamentos regionais recentes indicam que a resistência está amplamente distribuída, sendo que 35% de 1.184 populações e 51% de 461 populações amostradas no Brasil apresentaram resistência múltipla a clorimuron-etílico e glifosato (Lorenzetti *et al.*, 2024; Mendes *et al.*, 2021). Embora esses estudos caracterizem a distribuição espacial da resistência, os mecanismos envolvidos raramente são avaliados em larga escala, e as informações disponíveis são geradas em análises pontuais de populações específicas. Em *Conyza*, a resistência aos herbicidas inibidores da ALS é frequentemente associada a mutações no gene *ALS*. Em outras espécies de plantas daninhas, mecanismos adicionais como aumento da metabolização (Iwakami *et al.*, 2014), variação no número de cópias (Iwakami *et al.*, 2017) e superexpressão do gene *ALS* (Panozzo *et al.*, 2021) já foram descritos, mas esses mecanismos não foram relatados para *Conyza*.

Diferentes mutações no gene *ALS* foram documentadas em espécies de *Conyza*, relacionadas a diferentes grupos químicos de inibidores da ALS. Nos Estados Unidos, populações resistentes de *C. canadensis* apresentaram resistência cruzada aos grupos

químicos sulfonilureias, triazolopirimidinas e pirimidiniltio-benzoatos devido às substituições Pro197Ser e Pro197Ala, enquanto outra população, com a mutação Asp376Glu, exibiu resistência adicional a imidazolinonas (Zheng *et al.*, 2011). Em Israel, foram identificadas diferentes mutações em *C. canadensis*, incluindo Trp574Leu, responsável por resistência cruzada a imidazolinonas, sulfonilureias e pirimidinil-benzoatos, Ala205Val, conferindo resistência a imidazolinonas e pirimidinil-benzoatos, e Pro197Ser, associada à resistência a sulfonilureias e pirimidinil-benzoatos (Matzrafi *et al.*, 2015). No Brasil, a mutação Pro197Ser foi identificada em *C. sumatrensis*, conferindo resistência cruzada a sulfonilureias e triazolopirimidinas (Vital Silva *et al.*, 2022). Esses resultados corroboram a predominância do mecanismo RLA baseado em mutações no gênero *Conyza*.

2.3.3 Herbicidas auxínicos

Herbicidas auxínicos atuam como análogos sintéticos da auxina natural (ácido indolacético, IAA), promovendo desregulação do crescimento e do desenvolvimento vegetal (Peterson *et al.*, 2016). O mecanismo de ação desses herbicidas está relacionado à interação com o complexo receptor TIR1/AFB, que promove a degradação de proteínas repressoras da família Aux/IAA. Em condições normais, essas proteínas limitam a atividade dos fatores de resposta à auxina (ARFs), regulando a expressão de genes responsivos à auxina. Após a aplicação de herbicidas auxínicos, ocorre ativação excessiva dessa via, liberando os ARFs e induzindo respostas que resultam na morte da planta (Grossmann, 2000; Kubeš e Napier, 2019). Além disso, a absorção, redistribuição e translocação desses herbicidas dependem da atividade de transportadores de membrana, incluindo proteínas das famílias AUX1/LAX, responsáveis pelo influxo celular, e PIN e ABCB, associadas ao efluxo e à redistribuição polar da auxina entre tecidos (Kubeš e Napier, 2019).

Os primeiros casos de resistência a herbicidas auxínicos foram reportados em 1957 para o 2,4-D (Hilton, 1957; Switzer, 1957) e atualmente são registrados 96 casos de resistência a herbicidas com este mecanismo de ação (Heap, 2026). Apesar do número crescente, a frequência de resistência a auxínicos permanece baixa quando comparada a outros grupos herbicidas, o que reforça sua importância no manejo de plantas daninhas resistentes (Peterson *et al.*, 2016). No entanto, o uso recorrente desses herbicidas em

sistemas agrícolas intensivos tem resultado na evolução de populações resistentes principalmente por mecanismos NRLA, embora casos recentes de RLA também tenham sido relatados (Moreno-Serrano, Gaines e Dayan, 2024).

Mecanismos RLA a herbicidas auxínicos foram identificados em poucos casos, envolvendo mutações pontuais ou deleções em diferentes membros da família gênica Aux/IAA. Alterações estruturais que reduzem a interação entre proteínas Aux/IAA, herbicidas auxínicos e receptor TIR1 impedem a ubiquitinação mediada por TIR1, conferindo resistência a esses herbicidas (Figueiredo *et al*., 2022b). Alterações pontuais em *IAA16* conferiram resistência ao dicamba em *Kochia scoparia* (LeClere *et al.*, 2018) e *Chenopodium album* (Ghanizadeh *et al.*, 2024), enquanto mutações em *IAA34* foram relacionadas à resistência ao 2,4-D em *Sisymbrium orientale* (Qi *et al.*, 2025). Além disso, deleções gênicas observadas em *IAA2* de *S. orientale* (Figueiredo *et al*., 2022b) e em *IAA20* de *Sonchus oleraceus* (Krishnan *et al.*, 2025), também resultaram em resistência a herbicidas auxínicos, incluindo 2,4-D e dicamba. Para o gênero *Conyza*, ainda não foram identificadas alterações nos genes-alvo associadas à resistência a herbicidas auxínicos, como mutações e deleções em genes *Aux/IAA* descritas em outras espécies.

A maioria dos casos de resistência a herbicidas auxínicos ocorre por mecanismos NRLA, principalmente redução da translocação e aumento da metabolização dos herbicidas. Redução da translocação de 2,4-D foi relatada em *Papaver rhoeas* (Rey-Caballero *et al.*, 2016) e *R. raphanistrum* (Goggin, Cawthray e Powles, 2016), embora genes específicos ainda não tenham sido identificados. Metabolização aumentada do 2,4-D, possivelmente mediada por citocromos P450, foi reportada em *A. tuberculatus* (Figueiredo *et al*., 2022a). Plantas de *C. sumatrensis* resistentes ao 2,4-D com o fenótipo de rápida necrose também apresentaram translocação alterada (Queiroz *et al.*, 2020; Souza *et al.*, 2023). Estudos identificaram genes candidatos com maior expressão em plantas resistentes tratadas com 2,4-D. Entre eles, *PATATIN-LIKE PROTEIN 2* (*PLP2*) foi associado aos sintomas de necrose, enquanto os transportadores *ABCB4, ABCG1, ABCC15* e *ABCC3* provavelmente atuam na redistribuição diferenciada do herbicida na planta, permitindo o rebrote de meristemas (Angonese, 2022). Até o momento, não há evidências de outros mecanismos de resistência a herbicidas auxínicos em espécies de *Conyza*, embora já tenham sido relatados, em condições de campo, casos de resistência a dicamba e triclopir nessas espécies (Kalsing *et al*., 2024a).

2.4 RNAi no controle de plantas daninhas

A aplicação exógena de RNA de interferência (RNAi) constitui uma estratégia de silenciamento gênico transcricional e pós-transcricional capaz de induzir efeitos de silenciamento sem introduzir modificações herdáveis no genoma da planta (Dalakouras *et al.*, 2020; Mai *et al.*, 2021). Nessa abordagem, moléculas de RNA fita dupla (dsRNA) ou pequenos RNAs interferentes (siRNAs) pré-processados são aplicados aos tecidos vegetais. Quando dsRNA está presente no citoplasma, ele é processado por enzimas ribonucleases DICER-LIKE (DCL) em siRNAs de 21-23 nucleotídeos (Liu, Feng e Zhu, 2009). Alternativamente, siRNAs aplicados de forma exógena podem contornar essa etapa de processamento e ser diretamente incorporados ao complexo indutor de silenciamento por RNA (RISC). Em ambos os casos, a fita guia do siRNA é incorporada ao RISC, direcionando o corte específico do mRNA-alvo e resultando no silenciamento do gene-alvo (Dubrovina e Kiselev, 2019).

Nos últimos anos, estudos utilizando a aplicação exógena de RNAi para controle de plantas daninhas têm aumentado. Esse avanço demonstra o crescente interesse no método como alternativa e/ou complemento ao uso de herbicidas, principalmente em cenário de expansão da resistência a herbicidas (Zabala-Pardo *et al.*, 2022). Moléculas de dsRNA ou siRNA podem ser desenhadas para conduzir ao silenciamento de genes essenciais à sobrevivência ou ao desenvolvimento da planta, resultando em sua inativação funcional e, consequentemente, na morte do organismo. Além disso, genes associados à resistência, principalmente monogênica, podem ser utilizados como alvo, de modo que seu silenciamento restaure a suscetibilidade aos herbicidas e potencialize a eficácia das ferramentas químicas de manejo (Dalakouras *et al.*, 2020; Panozzo *et al.*, 2025).

A aplicação exógena de moléculas de RNA apresenta desafios relacionados à absorção, estabilidade e eficiência de entrega aos tecidos e às células-alvo para indução de silenciamento efetivo (Panozzo *et al.*, 2025; Zabala-Pardo *et al.*, 2022). Barreiras como a cutícula, parede celular e atividade de nucleases limitam a absorção e a persistência do RNA na planta após a aplicação (Bennett *et al.*, 2020). Para superar essas limitações, diferentes estratégias de entrega, incluindo nanopartículas, adjuvantes anfifílicos e métodos alternativos de aplicação, têm sido investigadas para melhorar a estabilidade, absorção e movimentação sistêmica do RNA nas plantas (Basso *et al.*, 2025; Shivashakarappa *et al.*, 2025). Evidências obtidas no manejo de insetos e doenças

demonstram a viabilidade dessa abordagem, uma vez que moléculas de RNA aplicadas externamente podem ser absorvidas pelas plantas e subsequentemente ingeridas por insetos ou absorvidas por patógenos, resultando em silenciamento gênico efetivo desses organismos (Cagliari *et al.*, 2019; Vetukuri *et al.*, 2021). Além disso, estudos ambientais indicam que moléculas de RNA são degradadas rapidamente em condições de campo, sem resíduos detectáveis após poucos dias, reforçando a segurança ambiental dessa tecnologia (Parker *et al.*, 2019).

Estudos recentes relataram algum nível de silenciamento gênico em *Mikania micrantha* (Mai *et al.*, 2021), *Oryza sativa* (Polito *et al.*, 2024), *D. insularis* (Cintra *et al.*, 2025) e *Setaria viridis* (Castro *et al.*, 2026) por meio da aplicação exógena de moléculas de dsRNA ou siRNA, apesar de não ter sido relatada morte das plantas. Em *M. micrantha*, observou-se redução na expressão de genes que codificam proteínas ligantes de clorofila a/b, resultando em amarelecimento e murcha das plantas aos 14 dias após a pulverização (Mai *et al.*, 2021). Em *O. sativa*, a aplicação de siRNA reduziu a expressão do gene *PDS* e induziu necrose foliar nas áreas tratadas, sem efeitos nas regiões não tratadas da planta (Polito *et al.*, 2024). Em *D. insularis*, houve redução na expressão do gene *EPSPS* e diminuição de 44% no crescimento das plantas após aplicação de dsRNA (Cintra *et al.*, 2025). Em *S. viridis*, observou-se redução não significativa na expressão do gene *fumarase*, envolvido no metabolismo de carbono, 48 h após o tratamento com dsRNA entregue por nanopartículas à base de quitosana (Castro *et al.*, 2026). Apesar da eficiência ainda limitada e da ausência de controle letal consistente das plantas, os resultados obtidos nesses ensaios, ainda restritos a condições controladas e sem validação em campo, representam um passo inicial importante e evidenciam o potencial da indução de silenciamento gênico via aplicação exógena de RNA como estratégia para o manejo de plantas daninhas.

2.5 Referências

ADEGAS, F. S.; VARGAS, L.; GAZZIERO, D. L. P. Status of herbicide weed resistance in Brazil. **Proceedings of the Global Herbicide Resistance Challenge**, Denver, p. 165, 2017.

ANDERSEN, M. C. Diaspore morphology and seed dispersal in several wind-dispersed Asteraceae. **American Journal of Botany**, Columbus, v. 80, p. 487–492, 1993.

ANGONESE, P. S. Efeito de inibidores metabólicos e análise do transcriptoma *de Conyza sumatrensis* para caracterização do mecanismo de resistência ao herbicida 2,4-D relacionado à rápida necrose. Dissertação (Mestrado). Porto Alegre: Universidade Federal do Rio Grande do Sul, maio 2022.

BAERSON, S. R. *et al.* Glyphosate-resistant goosegrass. Identification of a mutation in the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase. **Plant Physiology**, Rockville, v. 129, n. 3, p. 1265–1275, 2002.

BASSO, M. F. *et al.* Progress and Opportunities of In Planta and Topical RNAi for the Biotechnological Control of Agricultural Pests. **Agronomy**, Basel, v. 15, n. 4, p. 859, 2025.

BECKIE, H. J. *et al.* Weed Resistance Monitoring in the Canadian Prairies. **Weed Technology**, Champaign, v. 22, n. 3, p. 530–543, 2008.

BENNETT, M. *et al.* Barriers to Efficient Foliar Uptake of dsRNA and Molecular Barriers to dsRNA Activity in Plant Cells. **Frontiers in Plant Science**, Lausanne, v. 11, p. 531649, 2020.

BERES, Z. T. *et al.* Target-site *EPSPS* Pro-106-Ser mutation in *Conyza canadensis* biotypes with extreme resistance to glyphosate in Ohio and Iowa, USA. **Scientific Reports**, London, v. 10, n. 1, 2020.

BURGOS, N. R. *et al.* Review: confirmation of resistance to herbicides and evaluation of resistance levels. **Weed Science**, Champaign, v. 61, n. 1, p. 4–20, 2013.

BYKER, H. P. *et al.* Glyphosate-resistant Canada fleabane [*Conyza canadensis* (L). Cronq.]: Dose response to glyphosate and control with postemergence herbicides in soybean in Ontario. **Canadian Journal of Plant Science**, Lausanne, v. 93, n. 6, p. 1187–1193, 2013.

CAGLIARI, D. *et al.* Management of Pest Insects and Plant Diseases by Non-Transformative RNAi. **Frontiers in Plant Science**, Lausanne, v. 10, 2019.

de CARVALHO, L. B. *et al.* Pool of resistance mechanisms to glyphosate in *Digitaria insularis.* **Journal of Agricultural and Food Chemistry**, Washington, v. 60, n. 2, p. 615–622, 2012.

CASTRO, C. G. *et al.* Chitosan-dsRNA nanoparticles for targeted gene in the model C4 species *Setaria viridis*: synthesis, characterization, and preliminary assessment of fumarase gene silencing. **Plant Nano Biology**, Amsterdam, v. 15, 100243, 2026.

CINTRA, L. A. *et al.* RNAi spray-induced gene silencing of *EPSPS* by topical application of dsRNA in the weed *Digitaria insularis*. **Frontiers in Plant Science**, Lausanne, v. 16, p. 1688755, 2025.

COMONT, D. *et al.* Evolutionary epidemiology predicts the emergence of glyphosate resistance in a major agricultural weed. **New Phytologist**, London, v. 223, n. 3, p. 1584–1594, 2019.

COMONT, D.; NEVE, P. Adopting epidemiological approaches for herbicide resistance monitoring and management. **Weed Research**, Oxford, v. 61, n. 2, p. 81–87, 2021.

DALAKOURAS, A. *et al.* Genetically modified organism-free RNA interference: Exogenous application of RNA molecules in plants. **Plant Physiology**, Rockville, v. 182, n. 1, p. 38–50, 2020.

DALAZEN, G. *et al.* Low temperature reverses the resistance to glyphosate in hairy fleabane (*Conyza bonariensis*). **Journal of Plant Protection Research**, Poznań, v. 59, n. 4, p. 433–440, 2019.

DAN, H. A. *et al.* Histórico da Infestação de Buva Resistente a Herbicidas no Mundo e no Brasil. In: CONSTANTIN, J. *et al.* (Ed.). **Buva**: Fundamentos e recomendações para manejo. Curitiba: Omnipax, 2013. cap. 2, p. 5-9.

DAUER, J. T.; MORTENSEN, D. A.; VANGESSEL, M. J. Temporal and spatial dynamics of long-distance *Conyza canadensis* seed dispersal. **Journal of Applied Ecology**, Oxford, v. 44, n. 1, p. 105–114, 2006.

DÉLYE, C.; JASIENIUK, M.; LECORRE, V. Deciphering the evolution of herbicide resistance in weeds. **Trends in Genetics**, Cambridge, v. 29, n. 11, p. 649–658, 2013.

DINELLI, G. *et al.* Physiological and molecular insight on the mechanisms of resistance to glyphosate in *Conyza canadensis* (L.) Cronq. biotypes. **Pesticide Biochemistry and Physiology**, New York, v. 86, n. 1, p. 30–41, 2006.

DINELLI, G. *et al.* Physiological and molecular bases of glyphosate resistance in *Conyza bonariensis* biotypes from Spain. **Weed Research**, Oxford, p. 257–265, 2008.

DUBROVINA, A. S.; KISELEV, K. V. Exogenous RNAs for Gene Regulation and Plant Resistance. **International Journal of Molecular Sciences**, New York, v. 20, n. 9, 2019.

DUKE, S. O.; POWLES, S. B. Glyphosate: A once-in-a-century herbicide. **Pest Management Science**, Malden, v. 64, n. 4, p. 319–325, 2008.

FIGUEIREDO, M. R. A. *et al.* Identification of a Novel 2,4- D Metabolic Detoxification Pathway in 2,4- D -Resistant Waterhemp (*Amaranthus tuberculatus*). **Journal of Agricultural and Food Chemistry**, Washington, v. 70, p. 15380–15389, 2022a.

FIGUEIREDO, M. R. A. *et al.* An in-frame deletion mutation in the degron tail of auxin coreceptor *IAA2* confers resistance to the herbicide 2,4-D in *Sisymbrium orientale*. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 119, n. 9, p. e2105819119, 2022b.

FRANKTON, C.; MULLIGAN, G. A. **Weeds of Canada**. Toronto: NC Press, 1987.

FUERST, E. P. Paraquat resistance in *Conyza*. **Plant Physiology**, Rockville, v. 77, p. 984-989, 1985.

GAINES, T. A. *et al.* Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 107, n. 3, p. 1029–1034, 2010.

GAINES, T. A. The quick and the dead: a new model for the essential role of ABA accumulation in synthetic auxin herbicide mode of action. **Journal of Experimental Botany**, Oxford, v. 71, n. 12, p. 3383–3385, 2020.

GAINES, T. A. *et al.* Mechanisms of evolved herbicide resistance. **Journal of Biological Chemistry**, Bethesd, v. 295, n. 30, p. 10307–10330, 2020.

GALEANO, E. *et al.* EPSPS variability, gene expression, and enzymatic activity in glyphosate-resistant biotypes of *Digitaria insularis*. **Genetics and Molecular Research,** Ribeirao Preto, v. 15, n. 3, 2016.

GAZZIERO, D. L. P. *et al*. Interferência da Buva em áreas cultivadas com soja. *In:* CONGRESSO BRASILEIRO DA CIÊNCIA DAS PLANTAS DANINHAS, 2010, Ribeirão Preto. **Anais**. Londrina: Sociedade Brasileira da Ciência das Plantas Daninhas, 2010. p. 1555-1558.

GE, X. *et al.* Rapid vacuolar sequestration: The horseweed glyphosate resistance mechanism. **Pest Management Science**, Malden, v. 66, n. 4, p. 345–348, 2010.

GHANIZADEH, H. *et al.* A novel mutation in *IAA16* is associated with dicamba resistance in *Chenopodium album*. **Pest Management Science**, Malden, v. 80, n. 7, p. 3675–3683, 2024.

GOGGIN, D. E.; CAWTHRAY, G. R.; POWLES, S. B. 2,4-D resistance in wild radish: Reduced herbicide translocation via inhibition of cellular transport. **Journal of Experimental Botany**, Oxford, v. 67, n. 11, p. 3223–3235, 2016.

GOGGIN, D. E. *et al.* Plasma membrane receptor-like kinases and transporters are associated with 2,4-D resistance in wild radish. **Annals of Botany**, Oxford, v. 125, n. 5, p. 821–832, 2020.

GOMES, A. C. *et al.* Chromosome-level assemblies of the allohexaploid genomes of *Conyza sumatrensis* and *Conyza bonariensis*. **Genome Biology and Evolution**, Oxford, v. 17, n. 4, evaf065, 2025.

GROSSMANN, K. Mode of action of auxin herbicides: a new ending to a long, drawn out story. **Trends in Plant Science**, Oxford, v. 5, n. 12, p. 506–508, 2000.

HAN, H. *et al*. A double *EPSPS* gene mutation endowing glyphosate resistance shows a remarkably high resistance cost. **Plant, Cell & Environment, Oxford**, v. 40, n. 12, p. 3031–3042, 2017.

HEAP, I. International Herbicide-Resistant Weed Database. Disponível em: <http://www.weedscience.org/>.

HEREWARD, J. P. *et al.* Gene expression in response to glyphosate treatment in fleabane (*Conyza bonariensis*) – glyphosate death response and candidate resistance genes. **Pest Management Science**, Malden, v. 74, n. 10, p. 2346–2355, 2018.

HICKS, H. L. *et al.* The factors driving evolved herbicide resistance at a national scale. **Nature Ecology and Evolution**, London, v. 2, n. 3, p. 529–536, 2018.

HILTON, H. W. Herbicide tolerant strains of weeds. **Hawaiian Sugar Planters Association Annual Report**, Honolulu, p. 69–72, 1957.

IWAKAMI, S. *et al.* Cytochrome P450 CYP81A12 and CYP81A21 are associated with resistance to two acetolactate synthase inhibitors in *Echinochloa phyllopogon*. **Plant Physiology**, Rockville, v. 165, n. 2, p. 618–629, 2014.

IWAKAMI, S. *et al.* Copy number variation in acetolactate synthase genes of thifensulfuron-methyl resistant *Alopecurus aequalis* (shortawn foxtail) accessions in Japan. **Frontiers in Plant Science**, Lausanne, v. 8, p. 250064, 2017.

JUGULAM, M.; SHYAM, C. Non-Target-Site Resistance to Herbicides: Recent Developments. **Plants**, Basel, v. 8, n. 10, 2019.

KALSING, A. *et al*. Dispersion of 2,4-D Resistance in *Conyza* Spp. And Evidence of Cross-Resistance to Synthetic Auxins Across Soybean Cropping Regions and Seasons in Brazil and Paraguay. **SSRN Electronic Journal**, 2024a.

KALSING, A. *et al.* Taxonomic resolution of fleabane species (*Conyza* spp.) based on morphological and molecular markers and their dispersion across soybean-cropping macroregions and seasons in Brazil. **Weed Science**, Champaign, p. 1–31, 2024b.

KALSING, A. *et al.* The population genomics of *Conyza* spp. in soybean macroregions suggest the spread of herbicide resistance through intraspecific and interspecific gene flow. **Scientific Reports**, London, v. 14, n. 1, p. 19536-, 2024c.

KASPARY, T. E. *et al.* Growth, phenology, and seed viability between glyphosate-resistant and glyphosate-susceptible hairy fleabane. **Bragantia**, Campinas, v. 76, n. 1, p. 92–101, 2017.

KISSMANN, K. G.; GROTH, D. **Plantas infestantes e nocivas**. 2. ed. São Paulo: BASF, 1999.

KREINER, J. M. *et al.* Repeated origins, widespread gene flow, and allelic interactions of target-site herbicide resistance mutations. **eLife**, Cambridge, v. 11, 2022.

KRISHNAN, M. *et al.* A novel mutation in *SoIAA20* confers cross-resistance to 2,4-Dichlorophenoxyacetic acid and other auxinic herbicides in *Sonchus oleraceus*. **Pest Management Science**, Malden, v. 81, n. 1, p. 141–148, 2025.

KUBEŠ, M.; NAPIER, R. M. Non-canonical auxin signalling: fast and curious**. Journal of Experimental Botany**, Oxford, v. 70, n. 10, p. 2609-2614, 2019.

KUMARAN, N. *et al.* Gene technologies in weed management: a technical feasibility analysis. **Current Opinion in Insect Science**, London, v. 38, p. 6–14, 2020.

LECLERE, S. *et al.* Cross-resistance to dicamba, 2,4-D, and fluroxypyr in *Kochia scoparia* is endowed by a mutation in an *AUX/IAA* gene. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 115, n. 13, p. 2911–2920, 2018.

LIU, Q.; FENG, Y.; ZHU, Z. Dicer-like (DCL) proteins in plants. **Functional & Integrative Genomics**, Heidelberg, v. 9, n. 3, p. 277–286, 2009.

LORENZETTI, J. B. *et al.* Identification, mapping, and chemical control of fleabane resistant to glyphosate, chlorimuron, paraquat, and 2,4-D. **Weed Technology**, Champaign, v. 38, 2024.

MAI, J. *et al.* Study on RNAi-based herbicide for *Mikania micrantha*. **Synthetic and Systems Biotechnology**, Beijing, v. 6, n. 4, p. 437–445, 2021.

MASCANZONI, E. *et al.* Epidemiology and agronomic predictors of herbicide resistance in rice at a large scale. **Agronomy for Sustainable Development**, Versailles, v. 38, n. 6, 2018.

MATZRAFI, M. *et al. Conyza* species: distribution and evolution of multiple target-site herbicide resistances. **Planta**, Heidelberg, v. 242, n. 1, p. 259–267, 2015.

MENDES, R. R. *et al.* Monitoring glyphosate-and chlorimuron-resistant *Conyza* spp. Populations in Brazil. **Anais da Academia Brasileira de Ciências**, Rio de Janeiro, v. 93, n. 1, 2021.

MEROTTO, A. *et al.* Herbicide use history and perspective in South America. **Advances in Weed Science**, Champaign, v. 40(Spec1), p. e020220050, 2022.

MORENO-SERRANO, D.; GAINES, T. A.; DAYAN, F. E. Current Status of Auxin Mimic Herbicides**. Outlooks on Pest Management**, Burnham, v. 35, n. 3, p. 105–112, 2024.

MORETTI, M. L.; HANSON, B. D. Reduced translocation is involved in resistance to glyphosate and paraquat in *Conyza bonariensis* and *Conyza canadensis* from California. **Weed Research**, Oxford, v. 57, n. 1, p. 25–34, 2017.

MURPHY, B. P.; TRANEL, P. J. Target-Site Mutations Conferring Herbicide Resistance. **Plants**, Basel, v. 8, n. 10, 2019.

NANDULA, V. K. *et al.* Factors affecting germination of horseweed (*Conyza canadensis*). **Weed Science**, Champaign, v. 54, p. 898-902, 2006.

NANDULA, V. K. *et al.* Glyphosate Resistance in Tall Waterhemp (*Amaranthus tuberculatus*) from Mississippi is due to both Altered Target-Site and Nontarget-Site Mechanisms. **Weed Science**, Champaign, p. 374–383, 2013.

NG, C. H. *et al.* Glyphosate resistance in *Eleusine indica* (L.) Gaertn. from different origins and polymerase chain reaction amplification of specific alleles. **Crop & Pasture Science**, Collingwood, v. 55, n. 4, p. 407–414, 2004.

NORSWORTHY, J. K. *et al.* Reducing the Risks of Herbicide Resistance: Best Management Practices and Recommendations. **Weed Science**, Champaign, p. 31–62, 2012.

PAGE, E. R. *et al.* Target and Non-target site Mechanisms Confer Resistance to Glyphosate in Canadian Accessions of *Conyza canadensis*. **Weed Science**, Champaign, v. 66, n. 2, p. 234–245, 2018.

PAN, L. *et al.* An ABCC-type transporter endowing glyphosate resistance in plants. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 118, n. 16, 2021.

PANOZZO, S. *et al.* Target-Site Mutations and Expression of *ALS* Gene Copies Vary According to *Echinochloa* Species. **Genes**, Basel, v. 12, n. 11, 2021.

PANOZZO, S. *et al.* RNAi technology development for weed control: all smoke and no fire? **Pest Management Science**, Malden, v. 81, n. 7, p. 3430–3436, 2025.

PARKER, K. M. *et al.* Environmental Fate of RNA Interference Pesticides: Adsorption and Degradation of Double-Stranded RNA Molecules in Agricultural Soils. **Environmental Science & Technology**, Washington, v. 53, n. 6, p. 3027–3036, 2019.

PEROTTI, V. E. *et al.* A novel triple amino acid substitution in the *EPSPS* found in a high-level glyphosate-resistant *Amaranthus hybridus* population from Argentina. **Pest Management Science**, Malden, v. 75, n. 5, p. 1242–1251, 2019.

PETERSON, M. A. *et al.* 2,4-D Past, Present, and Future: A Review. **Weed Technology**, Champaign, v. 30, n. 2, p. 303–345, 2016.

de PINHO, C. F. *et al.* First evidence of multiple resistance of Sumatran Fleabane (*Conyza sumatrensis* (Retz.) E.Walker) to five-mode-of-action herbicides. **Australian Journal of Crop Science**, Lismore, v. 13, n. 10, p. 1688–1697, 2019.

POLITO, R. A. *et al.* Precise transcript targeting using RNAi for weed control. **Advances in Weed Science**, Champaign, v. 42, p. e020240014, 2024.

POWLES, S. B.; YU, Q. Evolution in Action: Plants Resistant to Herbicides. **Annual Review of Plant Biology**, Palo Alto, v. 61, n. 1, p. 317–347, 2010.

QI, Y. *et al.* A point mutation in *IAA34* confers resistance to the auxin herbicide 2,4-D in *Sisymbrium orientale*. **Pest Management Science**, Malden, v. 81, n. 7, p. 3523–3531, 2025.

QUEIROZ, A. R. S. *et al.* Rapid necrosis: A novel plant resistance mechanism to 2,4-D. **Weed Science**, Champaign, v. 68, n. 1, p. 6–18, 2020.

QUEIROZ, A. R. S. *et al.* Rapid necrosis II: Physiological and molecular analysis of 2,4-D resistance in Sumatran fleabane (*Conyza sumatrensis*). **Weed Science**, Champaign, v. 70, n. 1, p. 36–45, 2022.

RAVET, K. *et al.* Genomic-based epidemiology reveals independent origins and gene flow of glyphosate resistance in *Bassia scoparia* populations across North America. **Molecular Ecology**, Oxford, v. 30, n. 21, p. 5343, 2021.

REGEHR, D. L.; BAZZAZ, F. A. The population dynamics of *Erigeron canadensis*, a successional winter annual. **Journal of Ecology**, Oxford, v. 67, n. 3, p. 923–933, 1979.

REY-CABALLERO, J. *et al.* Unravelling the resistance mechanisms to 2,4-D (2,4-dichlorophenoxyacetic acid) in corn poppy (*Papaver rhoeas*). **Pesticide Biochemistry and Physiology**, New York, v. 133, p. 67–72, 2016.

RIGON, C. A. G. *et al.* Metabolism-based herbicide resistance, the major threat among the non-target site resistance mechanisms. **Outlooks on Pest Management**, Burnham, v. 31, n. 4, p. 162–168, 2020.

RUTLAND, C. A.; HALL, N. D.; MCELROY, J. S. The Impact of Polyploidization on the Evolution of Weed Species: Historical Understanding and Current Limitations. **Frontiers in Agronomy**, Lausanne, v. 3, 2021.

SANTOS, G. *et al.* Multiple resistance of *Conyza sumatrensis* to chlorimuron-ethyl and to glyphosate. **Planta Daninha**, Viçosa, v. 32, n. 2, p. 409–416, 2014.

SHAALTIEL, Y.; GRESSEL, J. Kinetic Analysis of Resistance to Paraquat in *Conyza*: Evidence that Paraquat Transiently Inhibits Leaf Chloroplast Reactions in Resistant Plants. **Plant Physiology**, Rockville, v. 85, n. 4, p. 869–871, 1987.

SHIELDS, E. J. *et al.* Horseweed (*Conyza canadensis*) seed collected in the planetary boundary layer. **Weed Science**, Champaign, v. 54, p. 1063-1067, 2006.

SHIVASHAKARAPPA, K. *et al.* Nanoparticle-mediated gene delivery techniques in plant systems. **Frontiers in Nanotechnology**, Lausanne, v. 7, p. 1516180, 2025.

SOARES, D. J. *et al.* Control of glyphosate resistant hairy fleabane (*Conyza bonariensis*) with dicamba and 2,4-D. **Planta Daninha**, Viçosa, v. 30, n. 2, p. 401–406, 2012.

SOLTANI, N.; SHROPSHIRE, C.; SIKKEMA, P. H. Control of glyphosate-resistant horseweed (*Conyza canadensis*) with tiafenacil mixes in corn. **Weed Technology**, Champaign, v. 35, n. 6, p. 908–911, 2021.

SOLTANI, N.; SHROPSHIRE, C.; SIKKEMA, P. H. Control of glyphosate-resistant horseweed with Group 4 herbicides in soybean. **Weed Technology**, Champaign, v. 36, n. 5, p. 643–647, 2022.

SOUZA, A. S. *et al.* Nontarget-site resistance due to rapid physiological response in 2,4-D resistant *Conyza sumatrensis*: reduced 2,4-D translocation and auxin-induced gene expression. **Pest Management Science**, Malden, v. 79, n. 10, p. 3581-3592, 2023.

SULZBACH, E. *et al.* Smooth pigweed (*Amaranthus hybridus*) and unresolved *Amaranthus* spp. from Brazil resistant to glyphosate exhibit the *EPSPS* TAP-IVS substitution. **Weed Science**, Champaign, v. 72, n. 1, p. 48–58, 2024.

SULZBACK, J. R. M. *et al.* Optimizing molecular assays for glyphosate and ALS-inhibitor resistance diagnostics in four weed species. **Canadian Journal of Plant Science**, Ottawa, v. 104, n. 6, p. 540–554, 2024.

SWITZER, C. M. The existence of 2,4-D-resistant strains of wild carrot. **Proceedings of the Northeastern Weed Control Conference**, Washington, v. 11, p. 315–318, 1957.

TANI, E.; CHACHALIS, D.; TRAVLOS, I. S. A Glyphosate Resistance Mechanism in *Conyza canadensis* Involves Synchronization of *EPSPS* and ABC-transporter Genes. **Plant Molecular Biology Reporter**, New York, v. 33, n. 6, p. 1721-1730, 2015.

THEBAUD, C.; ABBOTT, R. J. Characterization of Invasive *Conyza* Species (Asteraceae) in Europe: Quantitative Trait and Isozyme Analysis. **American Journal of Botany**, Columbus, v. 82, p. 360–368, 1995.

THEODOULOU, F. L. Plant ABC transporters. **Biochimica et Biophysica Acta - Biomembranes**, Amsterdam, v. 1465, n. 1–2, p. 79–103, 2000.

TRANEL, P. J.; WRIGHT, T. R. Resistance of weeds to ALS-inhibiting herbicides: what have we learned? **Weed Science**, Champaign, v. 50, n. 6, p. 700–712, 2002.

TREMMEL, C. D.; PETERSON, K. M. Competitive subordination of a piedmont old field successional dominant by an introduced species. **American Journal of Botany**, Columbus, v. 70, n. 8, p. 1125–1132, 1983.

TREZZI, M. *et al.* Impact of *Conyza bonariensis* density and establishment period on soyabean grain yield, yield components and economic threshold. **Weed Research**, Oxford, v. 55, p. 34–41, 2014.

VANGESSEL, M. J. Glyphosate-resistant horseweed from Delaware. **Weed Science**, Champaign, v. 49, p. 703–705, 2001.

VARGAS, A. A. M. *et al.* Longevity of Horseweed Seed Bank Depending on the Burial Depth. **Planta Daninha**, Viçosa, v. 36, p. e018172073, 2018.

VARGAS, L. *et al. Conyza bonariensis* biotypes resistant to the glyphosate in Southern Brazil. **Planta Daninha**, Viçosa, v. 25, n. 3, p. 573–578, 2007.

VETUKURI, R. R. *et al.* Spray-induced gene silencing: an innovative strategy for plant trait improvement and disease control. **Crop Breeding and Applied Biotechnology**, Viçosa, v. 21, n. Special Issue, p. e387921S11, 2021.

VIDAL, R. A. *et al.* Impacto da temperatura, irradiância e profundidade das sementes na emergência e germinação de *Conyza bonariensis* e *Conyza canadensis* resistentes ao glyphosate. **Planta Daninha**, Viçosa, v. 25, n. 2, p. 309-315, 2007.

VITAL SILVA, V. *et al.* A Target-Site Mutation Confers Cross-Resistance to ALS-Inhibiting Herbicides in *Erigeron sumatrensis* from Brazil. **Plants**, Basel, v. 11, n. 4, 2022.

WAKELIN, A. M.; PRESTON, C. A target-site mutation is present in a glyphosate-resistant *Lolium rigidum* population. **Weed Research**, Oxford, v. 46, n. 5, p. 432–440, 2006.

WU, H. *et al.* Germination, persistence, and emergence of flaxleaf fleabane (*Conyza bonariensis* [L.] Cronquist). **Weed Biology and Management**, Carlton, v. 7, n. 3, p. 192–199, 2007.

YUAN, J. S. *et al.* Functional Genomics Analysis of Horseweed (*Conyza canadensis*) with Special Reference to the Evolution of Non–Target-Site Glyphosate Resistance. **Weed Science**, Champaign, v. 58, n. 2, p. 109–117, 2010.

ZABALA-PARDO, D. *et al.* RNAi as a tool for weed management: Challenges and opportunities. **Advances in Weed Science**, Londrina, v. 40, n. spe1, 2022.

ZANGENEH, H. S. *et al.* Clodinafop-Propargyl Resistance Genes in *Lolium rigidum* Guad. Populations Are Associated with Fitness Costs. **Agronomy**, Basel, v. 8, n. 7, 2018.

ZHENG, D. *et al.* Cross-resistance of horseweed (*Conyza canadensis*) populations with three different ALS mutations. **Pest Management Science**, Malden, v. 67, n. 12, p. 1486–1492, 2011.

3. ARTIGO 1

Mapping target-site resistance mechanisms to ALS-inhibiting herbicides in *Conyza bonariensis* and *Conyza sumatrensis* in Brazil*

*Artigo a ser submetido para a Revista Weed Science

Title: Mapping target-site resistance mechanisms to ALS-inhibiting herbicides in *Conyza bonariensis* and *Conyza sumatrensis* in Brazil

Authors: Paula Sinigaglia Angonese[1], Guilherme Menegol Turra[1], Luan Cutti[1], Eduardo Carlos Rudell[1], Fernanda Pinto Marques[1], Luiz H. Zobiole[1], Catarine Markus[1], Aldo Merotto Jr.[1]

[1]Crop Science Department, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

Abstract

ALS-inhibiting herbicides have been widely used for *Conyza* control in soybean since the late 1980s, but their use increases mainly at burndown application for controlling glyphosate-resistant populations. After that, a large variability in efficacy among ALS-inhibiting herbicides had been observed, probably associated with the occurrence of different herbicide resistance mechanisms. This study aimed to evaluate the frequency of *Conyza* resistance to ALS-inhibiting herbicides across major agricultural regions of Brazil and to identify the predominant target-site resistance mechanisms. An initial study revealed the *ALS* gene substitutions Ala122Thr, Pro197Ser, Pro197Arg, Pro197His, and Trp574Leu for which molecular markers were developed. The evaluation of 70 populations indicated that the substitutions at position 197 were the most common, with Pro197Ser occurring in 25% of the populations. Thirty-four populations also showed increased gene copy number and/or expression. No evidence of a major contribution of metabolic resistance was observed. Interestingly, the Ser653Ala substitution, previously associated with herbicide resistance in other species, was detected in all resistant and susceptible *Conyza* populations, suggesting that it is not associated with resistance in this species. The molecular markers developed in this study enable large-scale resistance monitoring and support the management of populations that are still controlled by ALS-inhibiting herbicides.

Keywords: *ALS* mutation; copy number variation; overexpression; resistance distribution

3.1 Introduction

Herbicide resistance occurs in almost all major weeds and herbicides used, reflecting the early stages of a global epidemic (Comont *et al.*, 2019). Herbicide resistance can significantly increase soybean production costs by approximately US$50 ha^{-1}

(Adegas *et al*., 2017), and weed interference in soybean can lead to yield losses of up to 52.1% (Soltani *et al*., 2022). Most herbicide resistance assessments are based on individual cases, and the resistance mechanisms are typically identified in specific populations (Beckie *et al.*, 2008). Resistance can occur through different mechanisms within the same species and can have substantial environmental impacts (Gaines *et al.*, 2020). Therefore, studies focusing on the epidemiology of herbicide resistance are essential to better understand the large-scale occurrence and variation of resistance mechanisms (Hicks *et al.*, 2018). However, such studies remain limited (Cutti *et al.*, 2026; Mendes *et al.*, 2021). For widely distributed species such as *Conyza* spp., weedy rice (*Oryza sativa* L.), and *Amaranthus* spp. this approach is especially useful due to the large occurrence worldwide (García *et al*., 2020; Mendes *et al*., 2021; Ruzmi *et al*., 2020).

Conyza species are among the most problematic weeds in crop fields due to high genetic variability, prolific seed production, and the ability to adapt to diverse agricultural environments, and cause significant impacts on crop profitability, particularly soybeans (Bajwa *et al.*, 2016). In Brazil, Sumatran fleabane (*Conyza sumatrensis*) is widely spread in soybean fields, while hairy fleabane (*C. bonariensis*) is sparsely dispersed, mainly in the southern region (Kalsing *et al*., 2024). Both species are hexaploid (n=27) (Montgomery *et al.*, 2024), which can confer adaptive advantages, including greater genetic variability and phenotypic plasticity, potentially contributing to herbicide resistance (Rutland *et al*., 2021). Globally, these two *Conyza* species have exhibited resistance to herbicides with six different modes of action (Heap, 2026). The first case of resistance was reported in Sumatran fleabane in 1980 in Taiwan to paraquat (Fuerst, 1985), and in hairy fleabane in 1987 in Spain to simazine (Shaaltiel and Gressel, 1987). In Brazil, the first case of resistance was reported in 2005, a case of hairy fleabane resistant to glyphosate (Vargas *et al.*, 2007), and since then, populations of Sumatran fleabane have been identified as resistant to glyphosate, chlorimuron-ethyl, paraquat, saflufenacil, diuron, and to 2,4-D, with rapid necrosis being the novel plant resistance mechanism for this weed (Mendes *et al*., 2021; de Pinho *et al*., 2019; Queiroz *et al*., 2020; Vital Silva *et al*., 2022). Resistance to paraquat, saflufenacil, and diuron is rare, and resistance to 2,4-D is a growing problem.

Numerous cases of resistance to ALS-inhibiting herbicides have been reported in the field due to the large use of these herbicides for pre- and post-emergence control of *Conyza* weeds, particularly in soybean crops since the early 90s (Merotto *et al.*, 2022).

Some studies have aimed to assess the frequency of resistant populations across sampled regions. For example, recent surveys found that 35% (Mendes *et al.*, 2021) and 51% (Lorenzetti *et al.*, 2024) of 1,184 and 461 sampled *Conyza* populations, respectively, across Brazil exhibited multiple resistance to chlorimuron-ethyl and glyphosate. However, the mechanism of resistance of these populations was not assessed. Typically, such information is obtained for specific cases of resistance in a given species to a particular herbicide (de Pinho *et al*., 2019; Queiroz *et al*., 2020; Vital Silva *et al*., 2022). In *Echinochloa crus-galli,* recent studies with 233 populations from different regions of Brazil identified the most important mechanisms associated with ALS-inhibiting herbicides resistance, and based on that, indicated the feasibility of using certain ALS-inhibiting herbicides depending on the mechanism of resistance occurring in certain populations (Cutti *et al.*, 2026; Rudell *et al.*, 2025).

Resistance to ALS-inhibiting herbicides in *Conyza* species has been associated with mutations in the *ALS* gene, a target-site mechanism. In other species, resistance to these herbicides has been related to herbicide metabolism (Iwakami *et al.*, 2014), *ALS* copy number variation (Iwakami *et al.*, 2017), and *ALS* overexpression (Panozzo *et al.*, 2021; Wang *et al.*, 2023), but such mechanisms have not been reported in *Conyza* yet. In the United States, two horseweed (*C. canadensis*) populations from Indiana were identified with resistance to sulfonylureas, triazolopyrimidines, and pyrimidinylthio-benzoates herbicides due to Pro197Ser and Pro197Ala mutations, while another population with the Asp376Glu mutation was resistant to imidazolinones as well (Zheng *et al.*, 2011). In Israel, two horseweed populations exhibited cross-resistance to imidazolinones, sulfonylureas, and pyrimidinylthio-benzoates due to the Trp574Leu mutation; one population to imidazolinones and pyrimidinylthio-benzoates due to Ala205Val, and another population to sulfonylureas and pyrimidinylthio-benzoates due to Pro197Ser (Matzrafi *et al.*, 2015). In Brazil, the Pro197Ser mutation was identified in one population of Sumatran fleabane, conferring cross-resistance to sulfonylureas and triazolopyrimidines (Vital Silva *et al.*, 2022). All the studies described above were based on DNA sequencing without the knowledge of the composition of the three specific copies of the *ALS* gene of Sumatran and hairy fleabane. The recent availability of the genome sequence at chromosome-level contiguity for these species (Gomes *et al.*, 2025) allows more precise DNA sequencing targeting individual copies, decreasing potential false negative errors, and providing precise identification of *ALS* gene mutations

associated with herbicide resistance. This precision on the SNP identification in *Conyza* increases the feasibility of using molecular markers for large-scale studies related to the occurrence and identification of the mechanisms of resistance in a larger number of populations.

The control of herbicide-resistant weeds can be improved through the identification of the resistance mechanisms (Gaines *et al.*, 2020). The rapid diagnosis of these mechanisms through molecular markers (Sulzback *et al.*, 2024) can contribute to understanding the evolution of herbicide resistance and maintain the use of certain herbicide molecules. Often, all active ingredients of ALS-inhibiting herbicides are ruled out for controlling resistant *Conyza* populations, although some herbicides may still be effective depending on the specific mutation conferring resistance. An epidemiological approach to herbicide resistance can provide valuable insights into its evolutionary dynamics (Hicks *et al.*, 2018), whether resistance arises from a single origin and spreads via gene flow or emerges independently in multiple populations (Kreiner *et al.*, 2022). In the field, this dynamic is intensified by the occurrence of multiple resistance mechanisms, diverse life cycles and propagation strategies among weed species, varying infestation densities, and interactions with environmental factors, all of which can complicate the application of evolutionary-based management approaches. The epidemiological perspective based on the landscape analysis of the distribution of the mechanisms of resistance can contribute to more effective management strategies tailored to the specific type of resistance occurring in each field (Comont *et al.*, 2019). This study aimed to evaluate the frequency of *Conyza* resistance to ALS-inhibiting herbicides in agricultural regions across Brazil and to associate these cases with the predominant target-site resistance mechanisms. Molecular markers were developed to enable an epidemiological approach to these cases.

3.2 Material and Methods

3.2.1 Plant material

Sumatran and hairy fleabane populations were originally collected from herbicide escapes in agricultural fields across Brazil between 2016 and 2024 (Table S1). For all experiments, recently produced seeds obtained through population maintenance under controlled cultivation conditions were used. Seeds from each population were sown in pots filled with commercial substrate to germinate. Plants with four leaves were

transplanted and grown in 200 ml pots filled with a mixture of argisol and commercial substrate. The plants were grown in a greenhouse at 28 ± 5°C and irrigated daily.

3.2.2 ALS-inhibiting herbicides resistance screening

Plants at 6-8 leaf stage were screened with three ALS-inhibiting herbicides: chlorimuron-ethyl (sulfonylureas), imazethapyr (imidazolinones), and cloransulam-methyl (triazolopyrimidines) at 50 and 100% the label rate of each herbicide, which were 10 and 20 g ha^{-1} of chlorimuron-ethyl (Classic, 250 g Kg^{-1}, WG, Corteva Agriscience) + adjuvant Veget'Oil (EC, Oxiquímica Agrociência LTDA) at 0.05% v/v; 53 and 106 g ha^{-1} of imazethapyr (Zaphir, 106 g L^{-1}, SL, UPL) + adjuvant Dash (EC, Basf) at 0.5% v/v; 17 and 34 g ha^{-1} of cloransulam-methyl (Pacto, 840 g Kg^{-1}, WG, Corteva Agriscience) + adjuvant Dash 0.5% v/v. The herbicides bispiribac-sodium (Nominee, 400 g L^{-1}, SC, IHARA) from the pyrimidinylbenzoate group and flucarbazone-sodium (Everest, 200 g L^{-1}, SC, UPL) from the sulfonylaminocarbonyltriazolinone group were initially evaluated in 25 populations to represent the herbicides from the other two ALS chemical groups. However, due to their low efficacy in Sumatran and hairy fleabane, they were not evaluated in all populations. The application was performed with an automated spray chamber (Generation III, Devries manufacturing) at a spray volume of 200 L ha^{-1}. Each treatment had five biological replicates. The control efficacy was assessed on a visual scale at 28 days after treatment (DAT), where 0% were not injured, and 100% were dead plants. Plants that survived and exhibited less than 85% control efficacy compared with untreated and known susceptible controls were identified as candidates for resistance characterization.

3.2.3 *ALS* gene sequencing

Initially, 20 Sumatran and hairy fleabane populations (four susceptible and 16 resistant) were used for the *ALS* gene isolation and sequence. Three sets of primers were designed to overlap all known regions with mutations related to herbicide resistance - Ala122, Pro197, Ala205, Phe206, Asp376, Arg377, Trp574, Ser653, and Gly654. Since these species are hexaploid and have three copies of the *ALS* gene on chromosome 2 in hairy fleabane and on chromosomes 3, 22, and 23 in Sumatran fleabane, the primers were designed to amplify all *ALS* copies in both species (Gomes *et al.*, 2025). Total DNA was

extracted from the leaf tissue of all evaluated populations using the cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). DNA was diluted to 50 ng μL^{-1} for sequencing and marker studies. PCR reactions were performed with a final concentration of 1 x PCR buffer, 0.2 mM dNTP, 2 mM $MgCl_2$, 0.5 U Taq DNA polymerase (Invitrogen), 0.2 µM primer, 50 ng μL^{-1} DNA, and DNAse-free water to complete a 20 µL final volume. PCR conditions were 95 °C for 5 min; 35 cycles of 95 °C for 30 s, annealing temperature (Ta) (Table 1) for 45 s, and 72 °C for 10 min. The PCR products were verified in agarose gel (1.5%), purified with ExoSAP-IT™ (Applied Biosystems) protocol, and sequenced using Sanger sequencing. The sequences were aligned with the *Arabidopsis thaliana ALS* gene as reference (X51514.1). Chromatograms were analyzed with SnapGene® (https://www.snapgene.com/).

3.2.4 Mutation markers

The SNAP-type markers (single-nucleotide amplified polymorphism) were developed for the five mutations Ala122Thr, Pro197Ser, Pro197Arg, Pro197His, and Trp574Leu on both *Conyza* species identified in the *ALS* sequencing study described above. The markers were subsequently screened across the 59 resistant populations identified in the screening assay. The markers have the last base corresponding to the substitution of the changed amino acid and an additional changed base to improve the specificity. PCR reactions were performed with 5 µL PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, ThermoFisher Scientific) + primer and 5 µL DNA. PCR conditions were 95 °C for 2 min; 40 cycles of 95 °C for 15 s, annealing temperature (Ta) (Table 1) for 1 min in a QuantStudio 3 Real-Time PCR system (Applied Biosystems, ThermoFisher Scientific, Waltham, USA). For each population, three technical replicates were used. The mutation corresponding to each marker was considered present in the population when amplification occurred, and a specific melt curve was observed, which was absent in the no-template controls and susceptible populations.

Table 1. Primers developed for sequencing, markers, copy number variation, and overexpression studies of the *ALS* gene of *C. bonariensis* and *C. sumatrensis*.

Analysis	Primer	Sequence 5'-3'	Fragment size (bp)	Ta (°C)	Position
Sequencing study	ALS_4F	TTCCACCACCTTCATTCA	594	54	Ala122, Pro197, Ala205, Phe 206
	ALS_4R	ACCTAGATAAATAACCAGGAA			
	ALS_2F	TGTGTGCCTAAATGGGATGA	699	60	Asp376, Arg377
	ALS_5R	AAACTGAGCAGCCCACATCT			
	ALS_7F	TTCAGGTGGACTAGGGGCTA	585	60	Trp574, Ser653, Gly654
	ALS_7R	TCCAACAAACTGCTCACACC			
Markers study	Thr122_2F	CTTCGCTTACCCTGGTTGC**A**	151	60	p122
	Ala122_1R	TATACAGACGCCGGGGAGAC			
	Ser197_2F	GCCATCACCGGCCAAGGT**T**	165	60	p197
	Arg197_2F	GCCATCACCGGCCAAGCTC**G**		56	
	His197_1F	GCCATCACCGGCCAAGTGC**A**		60	
	Pro197_1R	ACCCGACCCGGCAAGATAAA		56-60	
	Leu574_1F	CATTTAGGTATGGTGGTTCAGT**T**	93	56	p574
	Trp574_1R	TATTTCAGCCTCATTGGTTGG			
CNV and overexpression	ALS_6F	CGTGGAGGACAGAGTTGGAT	170	60	-
	ALS_5R	AAACTGAGCAGCCCACATCT			

Ta - annealing temperature; CNV - copy number variation; the bold base represents the SNP (single nucleotide polymorphism) of the mutation, and the underlined base represents the additional SNP.

3.2.5 *ALS* gene copy number variation and overexpression

The *ALS* copy number variation (CNV) and *ALS* overexpression were investigated through RT-qPCR analysis using a primer pair that targets the three *ALS* genes of *Conyza* species (Table 1). DNA was used for the CNV study, and cDNA was used for overexpression analysis. RNA extraction from each population's not-treated leaf tissue was performed with Concert™ Reagent (Invitrogen, ThermoFisher Scientific, Waltham, USA), and cDNA was synthesized using M-MLV Reverse Transcriptase (Invitrogen). The PCR reactions were a final concentration of 1 x PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTP, 0.1 µM ROX Reference Dye, 0.1 x SYBR™ Green, 0.5 U Platinum™ Taq DNA Polymerase (Invitrogen), 0.2 µM primer, 50 ng μL^{-1} DNA or cDNA, and DNAse-free water to complete a 10 µL final volume. PCR conditions were 50 °C for 1 min, 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s in a QuantStudio 3 Real-Time PCR system. For each population, three biological replicates and three technical replicates were used. *ALS* gene CNV and *ALS* gene overexpression were calculated relative to the susceptible populations using the $2^{-\Delta\Delta Ct}$ method (Dussault and Pouliot, 2006). The housekeeping *HSP70* (*heat shock protein-70*) gene was used as a

calibrator (Moretti *et al*., 2017). The data were compared according to ANOVA, and when significant ($p \leq 0.05$), the averages were compared according to Tukey 5%.

3.2.6 P450 inhibitor assay

The cytochrome P450 enzyme inhibitor, malathion, was applied to 14 populations for which the resistance mechanism had not been identified, to evaluate the potential involvement of detoxification processes in herbicide resistance, and to one susceptible population as a control. Plants at the 6-8 leaf stage were grown in a growth chamber (14 h light/10 h dark; 25 °C day/20 °C night). Malathion (Malathion 1000 g L^{-1}, EC, FMC) was applied at 2000 g ha^{-1}, 2 h before herbicide application (Cutti *et al*., 2026). Populations were treated with the ALS-inhibiting herbicide to which resistance had previously been identified. Accordingly, 13 populations were treated with imazethapyr at 53 and 106 g ha^{-1} (Zaphir, 106 g L^{-1}, SL, UPL) + the adjuvant Dash (EC, BASF) at 0.5% v/v, while one population was treated with cloransulam-methyl at 17 and 34 g ha^{-1} (Pacto, 840 g kg^{-1}, WG, Corteva Agriscience) + Dash at 0.5% v/v. The susceptible population was treated with both herbicides. Each treatment had five biological replicates. The control efficacy was assessed on a visual scale at 21 DAT. Populations in which herbicide control increased to >85% after malathion application, relative to the herbicide applied alone, were considered indicative of the potential involvement of cytochrome P450-mediated detoxification in herbicide resistance.

3.2.7 Data analysis

Statistical analyses were performed in R v.4.2.1 (R Core Team, 2022) using RStudio 2025.05.1. Data normality was assessed using the Shapiro–Wilk test. Gene expression and malathion inhibitor assay data were subjected to analysis of variance (ANOVA), and means were compared using Tukey's test at the 5% significance level.

3.3 Results and Discussion

3.3.1 ALS-inhibiting herbicides resistance frequency

Herbicides from three ALS-inhibiting chemical classes were screened across 70 hairy and Sumatran fleabane populations. The herbicides bispiribac-sodium (pyrimidinylbenzoate group) and flucarbazone-sodium (sulfonylaminocarbonyltriazolinone group) presented low control, including susceptible populations, and were not considered in the resistant frequency analysis (data not presented). These two herbicides were considered to evaluate all five ALS chemical groups, although they are not recommended for *Conyza* control. Only 16% of the populations were susceptible to ALS-inhibiting herbicides (Figure 1A, Table S1). Resistance patterns showed that 21% of the populations exhibited cross-resistance to chlorimuron-ethyl, imazethapyr, and cloransulam-methyl, whereas resistance to at least two ALS-inhibiting herbicide groups occurred in 45% of the populations. Additionally, resistance to sulfonylureas, imidazolinones, and triazolopyrimidines, either alone or in combination with other ALS-inhibiting herbicide groups, was observed in 51%, 68%, and 31% of the populations, respectively. ALS resistance was widely distributed across the Brazilian regions evaluated (Figure 1B). However, a cluster-specific pattern of resistance was observed in a particular region of Brazil. Predominance of resistance only to imazethapyr was detected in samples collected from the state of Mato Grosso do Sul. In Paraná, about half of the sampled populations were resistant to all evaluated herbicides (Figure 1B). Some localized patterns were noted, such as in Toledo/PR, where sampled populations were resistant only to imazethapyr, suggesting a possible single origin of resistance followed by subsequent spread. In Rio Grande do Sul, resistance to all herbicides was predominant, particularly in populations of hairy fleabane (Figure 1B).

The frequency of resistance to chlorimuron-ethyl, imazethapyr, and cloransulam-methyl found in Figure 1A is associated with the different use patterns of these herbicides. After the availability of glyphosate-resistant soybean, chlorimuron-ethyl and imazethapyr are primarily used at burndown and at the pre-emergence application of soybean cultivation, while cloransulam-methyl is mainly applied in post-emergence (Merotto *et al.*, 2022). In Brazil, chlorimuron-ethyl and other ALS-inhibiting herbicides, mainly metsulfuron-methyl, were largely used in mixture with glyphosate for controlling *Conyza* populations resistant to this herbicide until early 2010'. After that, due to the occurrence

of resistance, the use of these herbicides decreased and was surpassed by auxinic herbicides. However, the utilization of imazethapyr was maintained mainly at pre-emergence application in mixture with other residual herbicides. This use pattern explains the large occurrence of *Conyza* resistant to imazethapyr in comparison with chlorimuron-ethyl and cloransulam-methyl (Figure 1A).

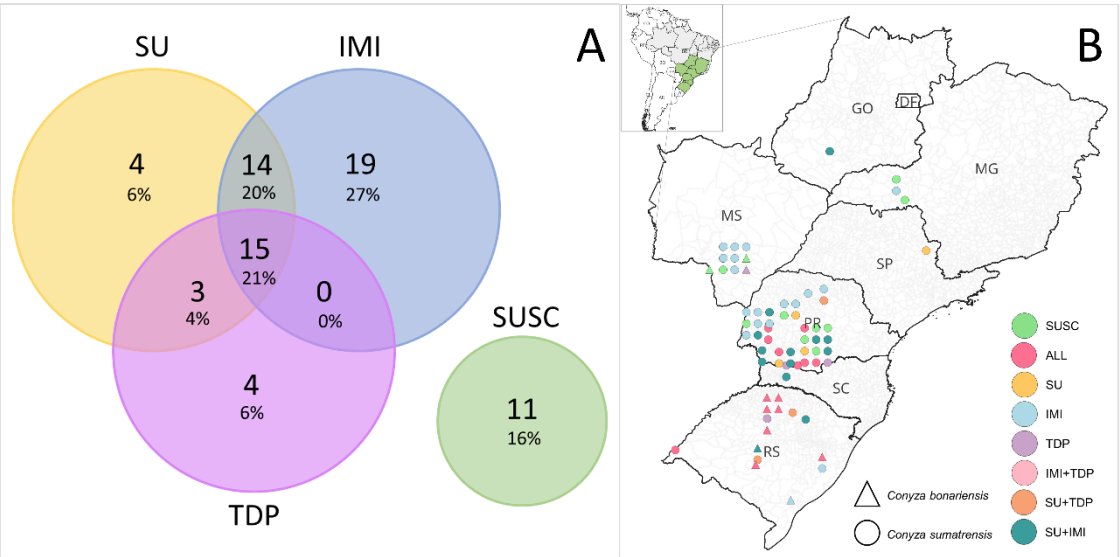


Figure 1. Resistance patterns to ALS-inhibiting herbicides among 70 *Conyza* spp. populations (A) and their distribution across Brazil (B). In panel A, numbers represent the number of populations across categories, with percentages shown below. SUSC = susceptible; SU, IMI, and TDP indicate resistance to sulfonylureas, imidazolinones, and triazolopyrimidines, respectively.

3.3.2 *ALS* gene sequencing

The primers used to sequence the *ALS* gene were designed based on the *ALS* sequence of the Sumatran and hairy fleabane, targeting conserved regions to cover all three copies of the gene located in subgenomes A, B, and C for both species. Of the 20 populations sequenced, two were Sumatran fleabane susceptible, two were hairy fleabane susceptible, 11 were Sumatran fleabane resistant, and five were hairy fleabane resistant. The sequences were aligned with the *ALS* gene from Sumatran fleabane (MZ816701.1, MZ816700.1, and MZ816699.1), hairy fleabane (BLAST in CM112843.1, CM112844.1, and CM112845.1), and *Arabidopsis thaliana* (X51514.1) (Figure 2A). Chromatogram analysis revealed overlapping peaks corresponding to nucleotide substitutions, consistent with the hexaploid genomic structure of Sumatran and hairy fleabane. Triple peaks were also observed at positions unrelated to herbicide resistance-conferring mutations, confirming that the primers amplified all three *ALS* copies (Figure 2C). The substitutions

Thr122, Ser197, Arg197, His197, and Leu574 were identified in 1 (C31), 2 (G14, C63), 1 (T1), 3 (C25, C31, C50) and 3 (T2, T3, T4) populations sequenced, respectively (Figure 2A).

The C31 Sumatran fleabane population showed two amino acid substitutions at positions 122 and 197 (Ala122Thr + Pro197His). Since the primers used were universal for all three *ALS* copies, it was not possible to determine whether the two mutations were present in the same subgenome or different subgenomes. This population showed resistance to the three herbicide classes evaluated. Double mutations in the *ALS* gene have also been identified in *Amaranthus palmeri* (Singh *et al.*, 2019) and *E. crus-galli* (Turra *et al.*, 2023), resulting in cross-resistance to imazetapyr, flumetsulam, primisulfuron-methyl, pyrithiobac-sodium, and trifloxysulfuron; and imazetapyr and penoxsulam, respectively.

Interestingly, all samples of Sumatran fleabane and hairy fleabane sequenced exhibit the Ser653Ala substitution, resulting from a base change from AGT to GCT (Figure 2A). Mutations in this position are frequently associated with ALS-inhibiting herbicide resistance, primarily imidazolinones, in several other weed species, such as *Amaranthus hybridus* (García *et al.*, 2020), *E. crus-galli* (Turra *et al.*, 2023), and weedy rice (Ruzmi *et al*., 2020). This substitution is also present in reference sequences from Sumatran and hairy fleabane and all susceptible and resistant sequenced populations, from both species (Figure 2A). In the species *C. canadensis* (diploid), *Papaver rhoeas* (diploid), *Senecio vulgaris* (tetraploid), and *Ambrosia artemisiifolia* (diploid), the presence of this substitution has also been identified in plants susceptible to ALS-inhibiting herbicides (Figure 2B), but it does not appear to be associated with resistance (Délye *et al.*, 2020; Rey-Caballero *et al.*, 2017). In this case, the presence of both codons AGT and GCT in the *ALS* gene maintains the enzyme's susceptibility to ALS-inhibiting herbicides in different plant species. This particularity, related to the absence of resistance caused by the Ser653Ala in species of the Asteraceae and Papaveraceae families in comparison with the effect of resistance in Poaceae species, even causing high levels of resistance, is unknown. Studies using the molecular docking approaches based on the custom definition of the ALS enzyme and the affinity with different ALS-inhibiting herbicides (Kumar *et al.*, 2025; Sun *et al.*, 2024) may shed light on the variability of the effect of the mutation Ser653Ala in different species.

In addition to the unexpected presence of the Ser653Ala substitution related to herbicide resistance in susceptible plants, the composition of the reference genome is also important. In the reference genome A of hairy fleabane, threonine is present at position 197 instead of the expected proline, due to a codon substitution from CCC to ACC (Figure 2A). This suggests that the plant used for the reference genome sequencing (Gomes *et al.*, 2025) is resistant to ALS-inhibiting herbicides. This is particularly important in studies that will use the standard reference genome as an alignment basis for *ALS* mutation in comparison with the target potential ALS-resistant populations.

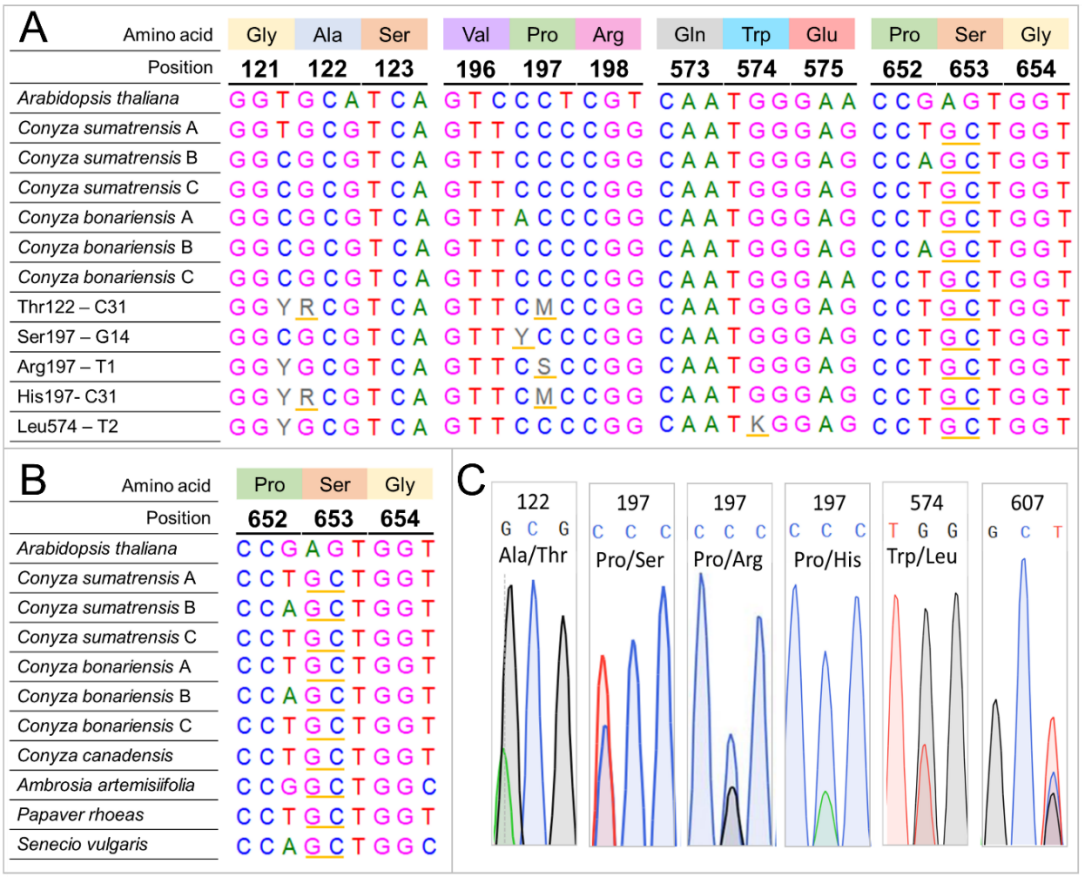


Figure 2. Partial alignment of *ALS* gene sequence of *A. thaliana* (X51514.1), Sumatran fleabane (MZ816701.1, MZ816700.1, and MZ816699.1), and hairy fleabane (BLAST in CM112843.1, CM112844.1, and CM112845.1) reference genomes, with C31 (Thr122 and His197), G14 (Ser197) (Sumatran fleabane), T1 (Arg197), and T2 (Leu574) (hairy fleabane) populations (A) and with *C. canadensis* (HM067014.1), *A. artemisiifolia* (KX870184.1), *P. rhoeas* (AJ577316.1) and *S. vulgaris* (KR024410.1) (B). Chromatograms with double peaks at the positions of the Ala122Thr, Pro197Ser/Arg/His, and Trp574Leu mutations, and a triple peak indicating amplification of all three genomes (C). Amino acid positions based on *A. thaliana ALS* gene (X51514.1). Bases highlighted represent the base change. Y (C or T), R (A or G), M (A or C), S (C or G), and K (G or T) letters represent the degenerate bases due to species hexaploidy.

3.3.3 Identification of *ALS* gene mutations through SNAP markers

Based on the identification of the five mutations found in the sequencing of the 20 populations, SNAP markers were developed to identify these mutations in the large-scale population study. These markers were designed to cover all subgenomes of Sumatran and hairy fleabane. The markers were initially validated in the 10 populations where the mutations were identified, using two susceptible populations from each species as controls. Marker specificity was assessed through the melting curve and amplification plots (Figure 3). Plants without mutation or with a mutation different from the one being evaluated did not show amplification. After establishing the protocols, the five markers were tested using RT-PCR in the 59 resistant populations of *Conyza*. The Ala122, Ser197, Arg197, His197, and Leu574 markers were amplified in one, fifteen, six, seven, and four populations, respectively, totaling 33 populations (Figure 4). However, 26 resistant populations did not amplify for any of the mutations tested, indicating that resistance in more than 44% of the resistant populations evaluated in this study is likely caused by other mutations or mechanisms of resistance.

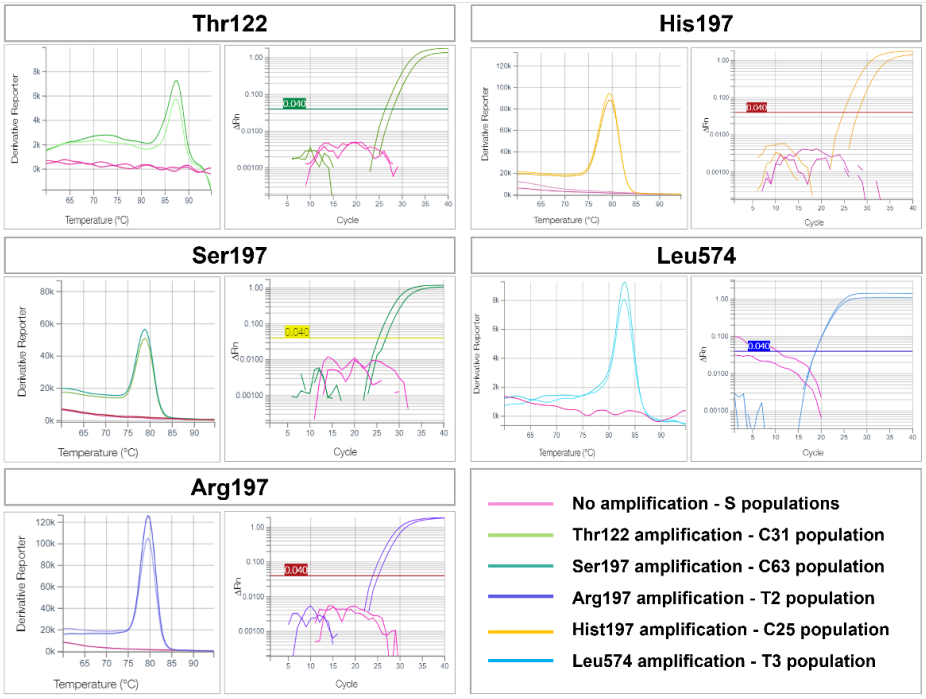


Figure 3. Melt curve and amplification plots for the Thr122, Ser197, Arg197, His197, and Leu574 markers validated in populations (C31, C63, T2, C25, and T3) with mutations identified through *ALS* sequencing and S populations as control.

After sequencing 20 populations and analyzing markers in 59 resistant populations, the overall mutation frequencies across both species were 25% for Ser197, 12% for His197, 10% for Arg197, 7% for Leu574, and 2% for Thr122 (Figure 4). When analyzed by species, in hairy fleabane, only the mutations Arg197 and Leu574 were found in one and three populations, respectively. The other mutations were found only in Sumatran fleabane plants. Previously, only one study had identified a mutation in one Sumatran fleabane population as the cause of resistance to ALS-inhibiting herbicides, reporting the Ser197 mutation, which confers cross-resistance to sulfonylureas and triazolopyrimidines (Vital Silva *et al.*, 2022). And recently, a study identified the mutations Arg197 and Leu574 in one and three hairy fleabane populations, respectively (Kaspary *et al.*, 2025). The mutations Ser197 and Leu574 were previously reported in *C. canadensis*, even as Ala197, Val205, and Glu376 (Matzrafi *et al.*, 2015; Sulzback *et al.*, 2024; Zheng *et al.*, 2011). It is important to emphasize that *C. canadensis* was not present in the studied region, similarly to previous identifications in other study (Kalsing *et al*., 2024). The mutations His197 and Thr122 were not previously reported in *Conyza* species, but were reported for many other species, like *P. rhoeas* and *Raphanus raphanistrum* for His197, and *Amaranthus retroflexus*, *A. palmeri*, and *A. hybridus* for Thr122 (Tranel *et al*., 2026).

Crossing the results from the resistance phenotypic analysis and the mutations identified, we observed that in general, the mutations Thr122, His197, and Leu574 confer resistance to all sulfonylureas, imidazolinones, and triazolopyrimidines groups. In contrast, the results for Ser197 and Arg197 are higher variables, probably because of the contribution of other resistance mechanisms in the resistance response. In cases where populations are resistant to only one or two ALS herbicide groups, herbicides from the remaining groups can still effectively control plants with these specific mutations. This is the case of populations C45, C51, and C62, which are resistant to clorimuron-ethyl and imazethapyr due to the mutation Arg197, that can still be controlled with cloransulam-methyl, or populations C13 and C59 that are resistant only to cloransulam-methyl due to the Ser197 mutation (Table S1). This information is valuable for guiding the strategic use of each herbicide group in specific situations and at the local scale.

3.3.4 *ALS* gene copy number variation and overexpression

Complementary to the sequencing of the *ALS* gene, target-site resistance was assessed by evaluating the number of gene copies and gene expression. Approximately

50% of the resistant populations exhibited the same copy number as the susceptible populations (Figure 4). Meanwhile, 38%, 10%, and 2% showed 2-, 3-, and 4-fold higher copy numbers, respectively. Regarding gene expression, 54% of the populations had the same expression level as the susceptible populations. In contrast, 30%, 2%, 10%, and 4% of the populations showed -2, -3, -4, and 6-fold higher *ALS* expression, respectively. *ALS* gene amplification, although less common than *EPSPS* amplification, has been reported in resistant *Alopecurus aequalis* accessions carrying additional *ALS* copies and a Pro197 substitution (Iwakami *et al.*, 2017). In the present study, most populations that showed an increased *ALS* gene copy number compared to susceptible populations also carried a mutation in the gene. The populations G7, G9, G10, G15, G19, G23, C4, and C44, without a detected mutation but exhibiting a slightly higher gene copy number, were predominantly resistant to imazetapir (Figure 4). In such cases, other mechanisms may contribute to resistance, such as enhanced herbicide metabolism mediated by cytochrome P450 enzymes, as previously reported in *E. crus-galli* (Dalazen *et al.*, 2018) and *A. palmeri* (Shyam *et al.*, 2021). The elevated *ALS* expression observed in our *Conyza* populations may be attributed to the increased gene copy number.

The association of the increased copy number and expression of the *ALS* gene is not solid, mainly considering that most of these populations had one mutation related to herbicide resistance (Figure 4). Several recent studies claimed the occurrence of multiple mechanisms of herbicide resistance by the identification of variability of translocation, or target and non-target site gene expression, in addition to mutation on the target site gene (Vázquez-García *et al.*, 2021; Xu *et al.*, 2025; Zhu *et al.*, 2023). However, this variability may be intrinsic to the species, and the actual resistant mechanism may be only related to the mutation on the target site gene. Dedicated studies based on the isolation of each potential mechanism in the same genetic plant background may clarify the occurrence of multiple resistant mechanisms. Moreover, herbicide-induced upregulation of the *ALS* gene cannot be excluded, as the populations in this study were evaluated in the absence of herbicide application. Therefore, our analysis would only capture constitutive increases in gene expression, potentially missing inducible responses.

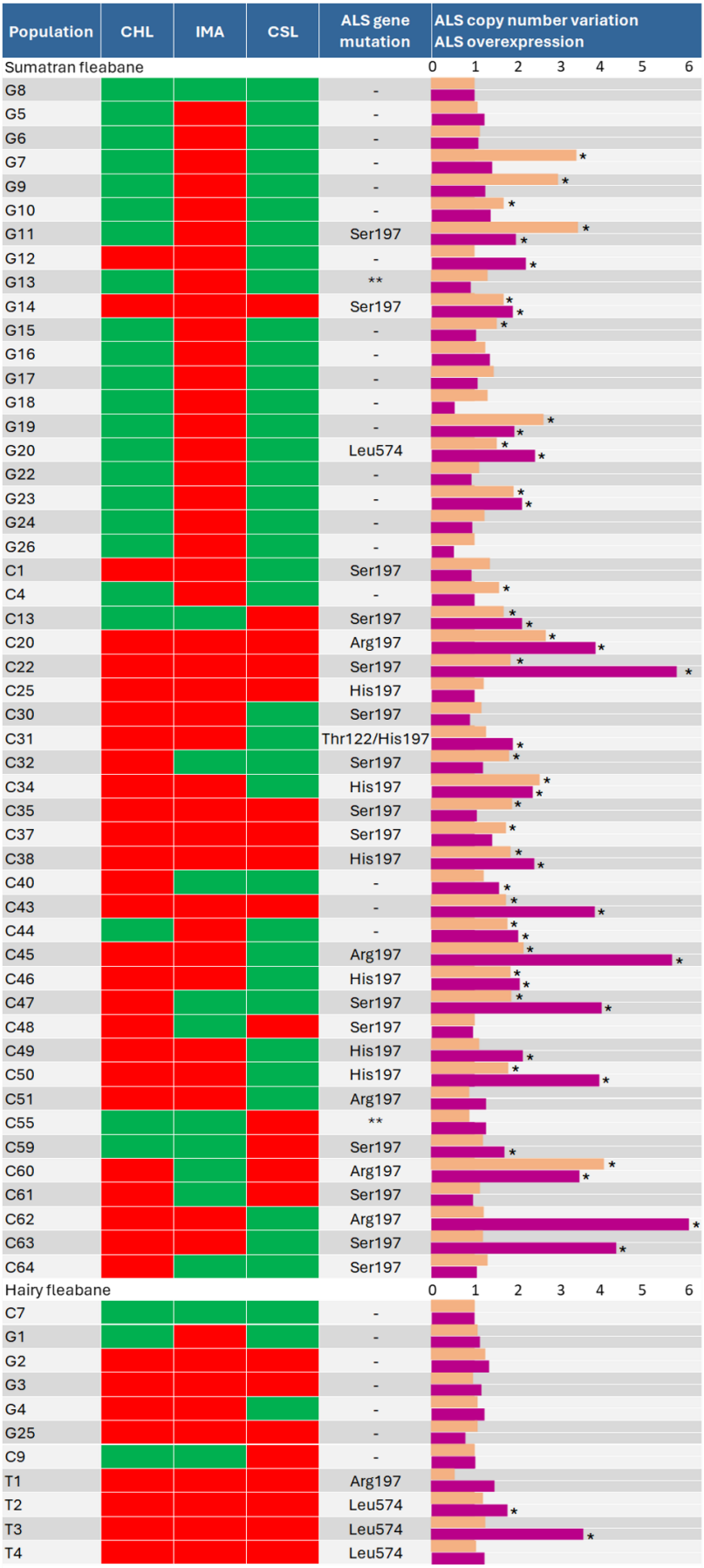


Figure 4. Resistance (red) and susceptible (green) patterns to chlorimuron-ethyl (CHL), imazethapyr (IMA), and cloransulam-methyl (CSL), presence of *ALS* mutations as

indicated by molecular markers, relative copy number (orange bars), and relative expression (purple bars) in resistant hairy and Sumatran fleabane populations. Two susceptible populations (C7 and G8) are shown for comparison. *indicates significant differences compared to susceptible populations (Tukey's test, $p < 0.05$). **indicates that the mutations were subsequently identified by sequencing.

3.3.5 P450 inhibition does not alter the resistant response

The effect of a cytochrome P450 inhibitor was evaluated in the resistant populations for which target-site mechanisms had not been identified. A slight increase in control was observed only for populations C9 at both doses and G6 at the higher dose (Table 2). Overall, these results indicate that it is unlikely that resistance in these populations is primarily driven by enhanced herbicide metabolism mediated by P450 enzymes; however, the involvement of metabolic resistance cannot be fully excluded.

Table 2. Injury (%) following malathion and imazethapyr or cloransulam-methyl application 21 days after treatment in Sumatran and hairy fleabane populations.

Treatments	Injury (%) by population														
	C2 (S)	C9	G1	G2	G3	G4	G5	G6	G16	G17	G18	G22	G24	G25	G26
Untreated-control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Malathion	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dose 1	86	74	69	68	71	55	61	55	68	65	61	75	65	60	45
Dose 1 + Malathion	88	96*	73	76	73	50	65	54	74	63	65	75	70	65	47
Dose 2	100	76	76	75	81	58	85	63	76	76	79	100	98	76	75
Dose 2 + Malathion	100	100*	88	78	79	60	98	81*	83	74	70	83	89	64	80

*means a significant difference between treatment with and without malathion at the same dose. Dose 1: 53 g ha^{-1} of imazethapyr or 17 g ha^{-1} of cloransulam-methyl. Dose 2: 106 g ha^{-1} of imazethapyr or 34 g ha^{-1} of cloransulam-methyl. Cloransulam-methyl was tested only in population C9 and the susceptible population, while imazethapyr was evaluated for the other populations.

3.3.6 Epidemiologic approach

Based on the development of molecular markers to identify the mechanisms related to target site resistance, it was found that only 54% of the resistant populations exhibited mutation as the resistance mechanism, among those evaluated (Figure 5A). Additionally, 17% showed only higher relative copy number and/or higher relative expression of the *ALS* gene, but the participation of these in the resistance process is unknown. However, in more than 25% of the populations, the resistance mechanism could not be identified

through the molecular markers (Figure 5B). To confirm that resistance in these populations was not conferred by any mutation not detected by the markers, these populations were sequenced, and no additional mutations were found, corroborating the results indicated by the markers (Figure 6). However, the mutations Leu574 and His197 were found in populations G13 and C55, respectively (Figure 6). This finding suggests that resistance in the remaining populations may be conferred by enhanced herbicide metabolism. To evaluate this possibility, the effect of prior malathion application in combination with the herbicides was assessed in the 14 populations lacking target-site mutations. Nevertheless, no synergistic response to malathion was observed, suggesting that P450-mediated metabolism was not strongly involved in the resistant phenotype under the conditions evaluated.

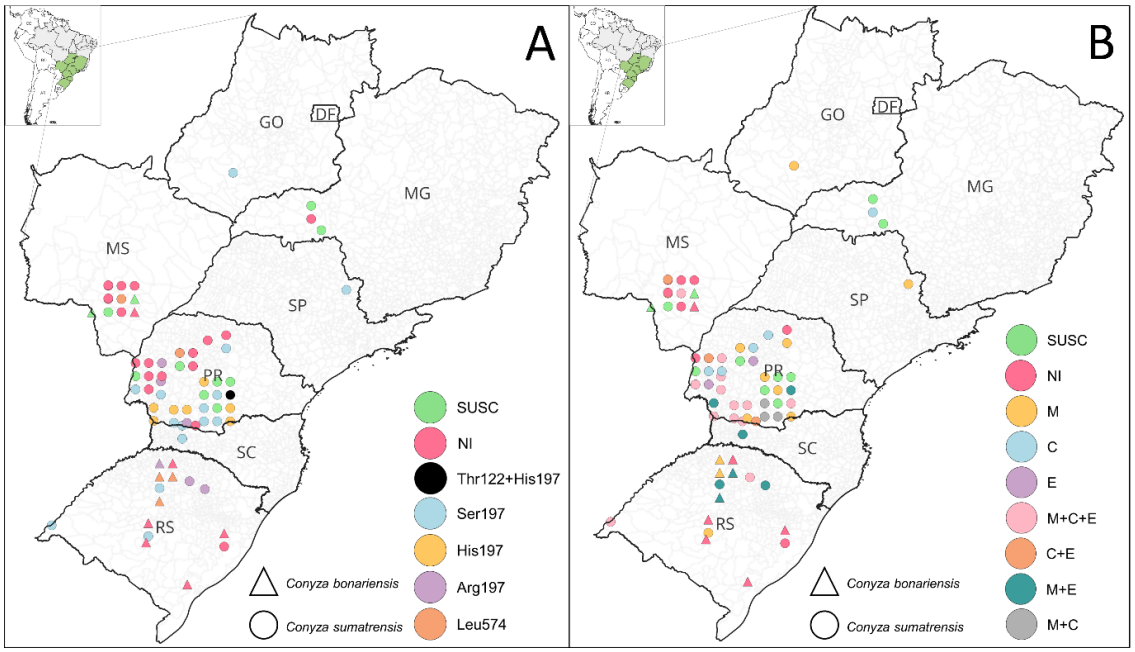


Figure 5. *Conyza* species *ALS* mutations (A) and target-site resistance mechanisms (B) distribution across Brazil. SUSC - no resistance; NI - mutation or resistance mechanism not identified; M - *ALS* mutation; C - *ALS* copy number increase; E - *ALS* overexpression.

Considering the different number of samples collected from each state, we observe that there is a predominance of hairy fleabane populations resistant to all evaluated ALS-inhibiting herbicide groups in Rio Grande do Sul. In contrast, in Paraná and Mato Grosso do Sul, Sumatran fleabane populations are more prevalent, with a high frequency of populations resistant only to imazetapir observed in Mato Grosso do Sul. In these cases,

no known resistance mechanism was identified, indicating an independent origin of resistance in this region. The large-scale study of ALS-inhibiting herbicides resistance in Sumatran and hairy fleabane in Brazil indicates high variability of the mechanism of resistance, resulting in difficulties for predicting opportunities of using specific ALS-inhibiting herbicides at the regional level. Due to the low cost and necessity of use for controlling susceptible species, several ALS-inhibiting herbicides are still being used in Brazil without a rational selection. However, local identification of the predominant mutation associated with herbicide resistance using the molecular markers developed in this study may support the reasoned choice of ALS-inhibiting herbicides and the strategic inclusion of herbicides with alternative mechanisms of action, improving the control of Sumatran and hairy fleabane and optimizing herbicide use during burndown operations in no-till systems.

Population	CHL	IMA	CSL	Markers					Sequencing
				Thr122	Ser197	His197	Arg197	Leu574	
G1				-	-	-	-	-	-
G2				-	-	-	-	-	-
G3				-	-	-	-	-	-
G4				-	-	-	-	-	-
G5				-	-	-	-	-	-
G6				-	-	-	-	-	-
G13				-	-	-	-	-	Leu574
G16				-	-	-	-	-	-
G17				-	-	-	-	-	-
G18				-	-	-	-	-	-
G22				-	-	-	-	-	-
G24				-	-	-	-	-	-
G25				-	-	-	-	-	-
G26				-	-	-	-	-	-
C9				-	-	-	-	-	-
C55				-	-	-	-	-	His197

Figure 6. Resistance (red) and susceptibility (green) patterns to chlorimuron-ethyl (CHL), imazethapyr (IMA), and cloransulam-methyl (CSL), mutation marker, and sequencing results for 16 resistant hairy and Sumatran fleabane populations without mutations identified in the marker analysis.

The study assessed the occurrence of resistance to ALS-inhibiting herbicides and the target-site resistance mechanisms associated with hairy and Sumatran fleabane species on a large scale across crop regions in Brazil. The findings of this work highlight the contribution of multiple resistance mechanisms to ALS-inhibiting herbicides, variable levels of cross-resistance, and the potential for utilizing herbicides from other ALS chemical groups in resistant Sumatran and hairy fleabane populations. *ALS* gene mutation

at the Pro197 position resulted in resistance in 47% of resistant *Conyza* populations. The most frequent mutation was Ser197, found in 25% of populations. *ALS* gene mutations, copy number, and expression resulted in large variations in resistance levels and cross-resistance patterns.

Acknowledgement

The authors thank Corteva Agriscience (São Paulo, SP, Brazil) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship to PSA, and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the fellowship to AMJ.

References

Adegas FS, Vargas L, Gazziero DLP. Status of herbicide weed resistance in Brazil. Proceedings of the Global Herbicide Resistance Challenge 2017:165.

Bajwa AA, Sadia S, Ali HH, Jabran K, Peerzada AM, Chauhan BS. Biology and management of two important *Conyza* weeds: a global review. Environ Sci Pollut Res Int 2016;23:24694–710. https://doi.org/10.1007/S11356-016-7794-7.

Beckie HJ, Leeson JY, Thomas AG, Brenzil CA, Hall LM, Holzgang G, et al. Weed Resistance Monitoring in the Canadian Prairies. Weed Technology 2008;22:530–43. https://doi.org/10.1614/wt-07-175.1.

Comont D, Hicks H, Crook L, Hull R, Cocciantelli E, Hadfield J, et al. Evolutionary epidemiology predicts the emergence of glyphosate resistance in a major agricultural weed. New Phytologist 2019;223:1584–94. https://doi.org/10.1111/NPH.15800.

Cutti L, Turra GM, Machado FM, Sulzbach E, Angonese PS, Markus C, et al. Subgenomic Distribution and Herbicide Cross-Resistance of *ALS* Gene Mutations in Allohexaploid *Echinochloa crus-galli*. Plant Direct 2026;10:e70168. https://doi.org/10.1002/pld3.70168.

Dalazen G, Pisoni A, Rafaeli RS, Merotto A. Degradation Enhancement as the Mechanism of Resistance to Imazethapyr in Barnyardgrass. Planta Daninha 2018;36:e018179504. https://doi.org/10.1590/S0100-83582018360100119.

Délye C, Michel S, Pernin F, Gautier V, Gislard M, Poncet C, et al. Harnessing the power of next-generation sequencing technologies to the purpose of high-throughput pesticide resistance diagnosis. Pest Manag Sci 2020;76:543–52. https://doi.org/10.1002/ps.5543.

Dussault AA, Pouliot M. Rapid and simple comparison of messenger RNA levels using real-time PCR. Biol Proced Online 2006;8:1–10. https://doi.org/10.1251/BPO114.

Fuerst EP. Paraquat resistance in Conyza. Plant Physiol 1985;77:984–9.

Gaines TA, Duke SO, Morran S, Rigon CAG, Tranel PJ, Küpper A, et al. Mechanisms of evolved herbicide resistance. Journal of Biological Chemistry 2020;295:10307–30. https://doi.org/10.1074/JBC.REV120.013572.

García MJ, Palma-Bautista C, Vazquez-Garcia JG, Rojano-Delgado AM, Osuna MD, Torra J, et al. Multiple mutations in the *EPSPS* and *ALS* genes of *Amaranthus hybridus* underlie resistance to glyphosate and ALS inhibitors. Scientific Reports 2020 10:1 2020;10:1–11. https://doi.org/10.1038/s41598-020-74430-0.

Gomes AC, Montgomery J, Araujo ALS, Morran S, Cutti L, Patterson E, et al. Chromosome-level assemblies of the allohexaploid genomes of *Conyza sumatrensis* and *Conyza bonariensis*. Genome Biol Evol 2025. https://doi.org/10.1093/GBE/EVAF065.

Heap I. International Herbicide-Resistant Weed Database 2026. http://www.weedscience.org/.

Hicks HL, Comont D, Coutts SR, Crook L, Hull R, Norris K, et al. The factors driving evolved herbicide resistance at a national scale. Nat Ecol Evol 2018;2:529–36. https://doi.org/10.1038/s41559-018-0470-1.

Iwakami S, Endo M, Saika H, Okuno J, Nakamura N, Yokoyama M, et al. Cytochrome P450 CYP81A12 and CYP81A21 are associated with resistance to two acetolactate synthase inhibitors in *Echinochloa phyllopogon*. Plant Physiol 2014;165:618–29. https://doi.org/10.1104/PP.113.232843.

Iwakami S, Shimono Y, Manabe Y, Endo M, Shibaike H, Uchino A, et al. Copy number variation in *acetolactate synthase* genes of thifensulfuron-methyl resistant *Alopecurus aequalis* (shortawn foxtail) accessions in Japan. Front Plant Sci 2017;8:250064. https://doi.org/10.3389/FPLS.2017.00254/BIBTEX.

Kalsing A, Nunes FA, Gotardi GA, Bueno JC, Schneider AA, Tropaldi L, et al. Taxonomic resolution of fleabane species (*Conyza* spp.) based on morphological and molecular markers and their dispersion across soybean-cropping macroregions and seasons in Brazil. Weed Sci 2024:1–31. https://doi.org/10.1017/WSC.2024.3.

Kaspary TE, Cutti L, Turra GM, Angonese PS, dos Santos OD, Merotto A. *Conyza bonariensis* resistance to glyphosate and ALS inhibitors involves target and non-target site resistance. Pestic Biochem Physiol 2025;213:106501. https://doi.org/10.1016/J.PESTBP.2025.106501.

Kreiner JM, Sandler G, Stern AJ, Tranel PJ, Weigel D, Stinchcombe JR, et al. Repeated origins, widespread gene flow, and allelic interactions of target-site herbicide resistance mutations. Elife 2022;11. https://doi.org/10.7554/ELIFE.70242.

Kumar P, Bishnoi R, Priyadarshini P, Chhuneja P, Singla D. Understanding the structural basis of *ALS* mutations associated with resistance to sulfonylurea in wheat. Sci Rep 2025;15:1–14. /https://doi.org/10.1038/s41598-025-91379-0.

Lorenzetti JB, Danilussi MTY, Albrecht AJP, Barroso AAM, Albrecht LP, Silva AFM, et al. Identification, mapping, and chemical control of fleabane resistant to glyphosate, chlorimuron, paraquat, and 2,4-D. Weed Technology 2024;38. https://doi.org/10.1017/wet.2024.10.

Matzrafi M, Lazar TW, Sibony M, Rubin B. *Conyza* species: distribution and evolution of multiple target-site herbicide resistances. Planta 2015;242:259–67. https://doi.org/10.1007/s00425-015-2306-4.

Mendes RR, Takano HK, Netto AG, Junior GJP, Cavenaghi AL, Silva VFV, et al. Monitoring glyphosate-and chlorimuron-resistant *Conyza* spp. Populations in Brazil. An Acad Bras Cienc 2021;93. https://doi.org/10.1590/0001-3765202120190425.

Merotto A, Gazziero LP, Oliveira MC, Scursoni J, Garcia MA, Figueroa R, et al. Herbicide use history and perspective in South America. Advances in Weed Science 2022;40(Spec1):e020220050. https://doi.org/10.51694/AdvWeedSci/2022;40:seventy-five010.

Montgomery J, Morran S, MacGregor DR, McElroy JS, Neve P, Neto C, et al. Current status of community resources and priorities for weed genomics research. Genome Biol 2024;25. https://doi.org/10.1186/s13059-024-03274-y.

Moretti ML, Alárcon-Reverte R, Pearce S, Morran S, Hanson BD. Transcription of putative tonoplast transporters in response to glyphosate and paraquat stress in *Conyza bonariensis* and *Conyza canadensis* and selection of reference genes for qRT-PCR. PLoS One 2017;12:e0180794. https://doi.org/10.1371/journal.pone.0180794.

Panozzo S, Mascanzoni E, Scarabel L, Milani A, Dalazen G, Merotto AJ, et al. Target-Site Mutations and Expression of *ALS* Gene Copies Vary According to *Echinochloa* Species. Genes (Basel) 2021;12. https://doi.org/10.3390/genes12111841.

de Pinho CF, Lourenço Leal JF, dos Santos Souza A, de Oliveira GFPB, de Oliveira C, Langaro AC, et al. First evidence of multiple resistance of Sumatran Fleabane (*Conyza sumatrensis* (Retz.) E.Walker) to five-mode-of-action herbicides. Aust J Crop Sci 2019;13:1688–97. https://doi.org/10.21475/ajcs.19.13.10.p1981.

Queiroz ARS, Delatorre CA, Lucio FR, Rossi CVS, Zobiole LHS, Merotto A. Rapid necrosis: A novel plant resistance mechanism to 2,4-D. Weed Sci 2020;68:6–18. https://doi.org/10.1017/wsc.2019.65.

Rey-Caballero J, Menéndez J, Osuna MD, Salas M, Torra J. Target-site and non-target-site resistance mechanisms to ALS inhibiting herbicides in *Papaver rhoeas*. Pestic Biochem Physiol 2017;138:57–65. https://doi.org/10.1016/J.PESTBP.2017.03.001.

Rudell E, Cutti L, Turra GM, Sinigaglia Angonese P, Tasca V, Santos O, et al. Variability and Spatial Distribution of Als-Inhibitor Resistance Mechanisms in Brazilian *Echinochloa Crusgalli*. Plant Physiol. Biochem. 2025;228. https://doi.org/10.1016/j.plaphy.2025.110237.

Rutland CA, Hall ND, McElroy JS. The Impact of Polyploidization on the Evolution of Weed Species: Historical Understanding and Current Limitations. Frontiers in Agronomy 2021;3. https://doi.org/10.3389/fagro.2021.626454.

Ruzmi R, Ahmad-Hamdani MS, Mazlan N. Ser-653-Asn substitution in the *acetohydroxyacid synthase* gene confers resistance in weedy rice to imidazolinone herbicides in Malaysia. PLoS One 2020;15:e0227397. https://doi.org/10.1371/JOURNAL.PONE.0227397.

Shaaltiel Y, Gressel J. Kinetic Analysis of Resistance to Paraquat in *Conyza*: Evidence that Paraquat Transiently Inhibits Leaf Chloroplast Reactions in Resistant Plants. Plant Physiol 1987;85:869–71. https://doi.org/10.1104/PP.85.4.869.

Shyam C, Borgato EA, Peterson DE, Dille JA, Jugulam M. Predominance of Metabolic Resistance in a Six-Way-Resistant Palmer Amaranth (*Amaranthus palmeri*) Population. Front Plant Sci 2021;11:614618. https://doi.org/10.3389/FPLS.2020.614618.

Singh S, Singh V, Salas-Perez RA, Bagavathiannan M V., Lawton-Rauh A, Roma-Burgos N. Target-site mutation accumulation among ALS inhibitor-resistant Palmer amaranth. Pest Manag Sci 2019;75:1131–9. https://doi.org/10.1002/PS.5232.

Soltani N, Shropshire C, Sikkema PH. Control of glyphosate-resistant horseweed with Group 4 herbicides in soybean. Weed Technology 2022;36:643–7. https://doi.org/10.1017/WET.2022.61.

Sulzback JRM, Borgato EA, Cutti L, Hill EC, Burns EE, Patterson EL. Optimizing molecular assays for glyphosate and ALS-inhibitor resistance diagnostics in four weed species. Canadian Journal of Plant Science 2024;104:540–54. https://doi.org/10.1139/CJPS-2024-0020.

Sun P, Niu L, He P, Yu H, Chen J, Cui H, et al. Trp-574-Leu and the novel Pro-197-His/Leu mutations contribute to penoxsulam resistance in *Echinochloa crus-galli* (L.) P. Beauv. Front Plant Sci 2024;15:1488976. https://doi.org/10.3389/FPLS.2024.1488976/BIBTEX.

Tranel PJ, Wright TR, Heap IM. Mutations in herbicide-resistant weeds to Inhibition of Acetolactate Synthase - Database. 2026.

Turra GM, Cutti L, Machado FM, Dias GM, Andres A, Markus C, et al. Application of ALS inhibitors at pre-emergence is effective in controlling resistant barnyardgrass biotypes depending on the mechanism of resistance. Crop Protection 2023;172:106325. https://doi.org/10.1016/J.CROPRO.2023.106325.

Vargas L, Bianchi MA, Rizzardi MA, Agostinetto D, Dal Magro T. *Conyza bonariensis* biotypes resistant to the glyphosate in Southern Brazil. Planta Daninha 2007;25:573–8.

Vázquez-García JG, Alcántara-De La Cruz R, Rojano-Delgado AM, Palma-Bautista C, De Portugal Vasconcelos JM, De Prado R. Multiple Herbicide Resistance Evolution: The Case of *Eleusine indica* in Brazil. J Agric Food Chem 2021;69:1197–205. https://doi.org/https://doi.org/10.1021/acs.jafc.0c03999.

Vital Silva V, Mendes R, Suzukawa A, Adegas F, Marcelino-Guimaraes F, Oliveira R. A Target-Site Mutation Confers Cross-Resistance to ALS-Inhibiting Herbicides in *Erigeron sumatrensis* from Brazil. Plants 2022;11. https://doi.org/10.3390/plants11040467.

Wang R, Sun Y, Lan Y, Wei S, Huang H, Li X, et al. *ALS* gene overexpression and enhanced metabolism conferring *Digitaria sanguinalis* resistance to nicosulfuron in China. Front Plant Sci 2023;14:1290600. https://doi.org/10.3389/fpls.2023.1290600

Xu H, Cheng J, Leng Q, Cao R, Su W, Sun L, et al. Characterization of acetolactate synthase genes and resistance mechanisms of multiple herbicide resistant *Lolium multiflorum*. Plant Physiology and Biochemistry 2025;219:109324. https://doi.org/10.1016/J.PLAPHY.2024.109324.

Zheng D, Kruger GR, Singh S, Davis VM, Tranel PJ, Weller SC, et al. Cross-resistance of horseweed (*Conyza canadensis*) populations with three different *ALS* mutations. Pest Manag Sci 2011;67:1486–92. https://doi.org/10.1002/ps.2190.

Zhu G, Wang H, Gao H, Liu Y, Li J, Feng Z, et al. Multiple Resistance to Three Modes of Action of Herbicides in a Single Italian Ryegrass (*Lolium multiflorum* L.) Population in China. Agronomy, 2023;13:216. https://doi.org/10.3390/AGRONOMY13010216.

Supplementary Material

Table S1. Efficacy of control 28 days after herbicide treatment in 70 *Conyza* spp. populations from Brazil with four ALS-inhibiting herbicides at 50% and 100% of the label rate. ERIBO - *Conyza bonariensis*, ERISU - *Conyza sumatrensis**.

Population	Species	Local	Chlorimuron-ethyl		Imazethapyr		Cloransulam-methyl	
			0.5x	1x	0.5x	1x	0.5x	1x
G1	ERIBO	Capão do Leão/RS	74	90	41	63	100	100
G2	ERIBO	Vila Nova/RS	84	70	75	75	69	73
G3	ERIBO	Dois Irmãos das Missões/RS	78	76	72	75	73	83
G4	ERIBO	Santa Maria/RS	58	83	56	58	100	100
G5	ERISU	Maripá/PR	91	98	37	54	100	100
G6	ERISU	Londrina/PR	73	91	37	46	100	100
G7	ERISU	Marialva/PR	99	100	50	66	96	100
G8	ERISU	Toledo/PR	100	100	87	88	100	100
G9	ERISU	Toledo/PR	100	100	70	80	96	86
G10	ERISU	Toledo/PR	67	92	72	64	100	100
G11	ERISU	Toledo/PR	97	100	38	47	100	100
G12	ERISU	Toledo/PR	99	81	48	58	100	100
G13	ERISU	Campo Mourão/PR	77	97	40	38	75	88
G14	ERISU	Uruguaiana/RS	42	55	48	79	63	63
G15	ERISU	Itambé/PR	100	100	50	46	59	96
G16	ERISU	Dourados/MS	100	100	40	81	89	100
G17	ERISU	Dourados/MS	100	100	42	80	92	98
G18	ERISU	Dourados/MS	100	100	42	78	100	100
G19	ERISU	Dourados/MS	100	98	40	45	100	100
G20	ERISU	Dourados/MS	100	100	47	51	94	98
G21	ERISU	Rio Brilhante/MS	100	100	42	88	100	100
G22	ERISU	Rio Brilhante/MS	100	100	63	68	100	100
G23	ERISU	Maracaju/MS	85	100	47	75	95	100
G24	ERISU	Maracaju/MS	84	93	50	53	100	100
G25	ERIBO	Porto Alegre/RS	64	78	60	64	65	69
G26	ERISU	Porto Alegre/RS	67	96	43	79	100	100
C1	ERISU	Rio Verde/GO	77	83	42	85	-	-
C2	ERISU	Uberaba/MG	100	100	98	100	100	100
C3	ERISU	Uberlandia/MG	100	100	100	100	100	100
C4	ERISU	Uberlandia/MG	91	100	96	82	74	100

C7	ERIBO	Ponta Porã/MS	99	100	99	97	100	100
C9	ERIBO	Rio Brilhante/MS	100	100	94	91	61	75
C10	ERIBO	Dourados/MS	53	100	50	92	100	100
C13	ERISU	Abelardo Luz/SC	93	95	98	97	61	66
C20	ERISU	Cascavel/PR	41	43	41	54	52	42
C22	ERISU	Corbelia/PR	9	34	44	37	71	44
C25	ERISU	Guarapuava/PR	46	42	33	33	20	46
C26	ERISU	Guarapuava/PR	100	100	65	87	-	-
C27	ERISU	Guarapuava/PR	100	100	92	89	-	-
C28	ERISU	Guarapuava/PR	100	100	76	100	-	-
C30	ERISU	Guarapuava/PR	40	54	59	66	-	-
C31	ERISU	Guarapuava/PR	38	36	35	31	-	-
C32	ERISU	Guarapuava/PR	46	70	65	100	-	-
C33	ERISU	Guarapuava/PR	100	100	63	92	-	-
C34	ERISU	Guarapuava/PR	41	61	36	57	-	-
C35	ERISU	Guarapuava/PR	41	60	43	76	65	70
C37	ERISU	Turvo/PR	73	65	78	81	63	75
C38	ERISU	Itapejara d'Oeste/PR	69	43	51	60	59	78
C39	ERISU	Juranda/PR	100	94	77	89	100	100
C40	ERISU	Juranda/PR	78	84	71	100	100	100
C43	ERISU	Mariópolis/PR	57	75	59	55	66	74
C44	ERISU	Ouro Verde do Oeste/PR	55	100	58	73	-	-
C45	ERISU	Ouro Verde do Oeste/PR	49	56	38	37	-	-
C46	ERISU	Pato Branco/PR	65	66	68	63	66	92
C47	ERISU	Pato Branco/PR	38	71	82	88	-	-
C48	ERISU	Londrina/PR	57	71	49	95	45	52
C49	ERISU	Planalto/PR	20	49	39	34	-	-
C50	ERISU	Santa Izabel do Oeste/PR	40	45	32	44	85	90
C51	ERISU	São João/PR	50	80	78	84	92	99
C55	ERISU	Turvo/PR	100	100	42	88	63	76
C59	ERISU	Cruz Alta/RS	63	96	87	100	60	68
C60	ERISU	Passo Fundo/RS	40	65	48	88	59	65
C61	ERISU	Santa Maria/RS	53	39	80	100	74	73
C62	ERISU	São Domingos/SC	31	45	30	37	90	100
C63	ERISU	Xanxerê/SC	14	47	39	53	-	-
C64	ERISU	Casa Branca/SP	62	67	52	91	87	85
T1	ERIBO	Chapada/RS	41	46	60	65	20	45
T2	ERIBO	Santa Bárbara do Sul/RS	21	60	16	28	15	23
T3	ERIBO	Novo Barreiro/RS	10	20	15	43	17	23
T4	ERIBO	Novo Bareiro/RS	24	43	19	43	41	38

*ERIBO and ERISU are Bayer codes derived from the nomenclature *Erigeron bonariensis* and *Erigeron sumatrensis*, respectively. Species are referred to as *Conyza bonariensis* and *Conyza sumatrensis* throughout this study.

4. ARTIGO 2

Mapping auxinic herbicide resistance in *Conyza* populations in Brazil and investigating candidate resistance gene expression*

*Artigo a ser submetido para a Revista Weed Technology

Title: Mapping auxinic herbicide resistance in *Conyza* populations in Brazil and investigating candidate resistance gene expression

Authors: Paula Sinigaglia Angonese[1], Estéfani Sulzbach[1], Fernando Lacerda Cappellari[1], Fernanda Pinto Marques[1], Gabriel Machado Dias[1], Leonardo Vicente Ellert Kroth[1], Catarine Markus[1], Aldo Merotto Jr.[1]

[1]Crop Science Department, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

Abstract

Auxinic herbicide resistance has been increasingly reported in *Conyza* populations in Brazil. The rapid necrosis phenotype is frequently associated with resistance to 2,4-D. Genes involved in cell death and transport processes may contribute to this response, but the effects of other auxinic herbicides on their expression remain poorly understood. This study aimed to assess the frequency of resistance to auxinic herbicides in *Conyza* populations from Brazil and to determine its association with the rapid necrosis phenotype and the expression of candidate resistance genes after application of different auxinic herbicides, applied alone or in mixtures. Resistance to 2,4-D, dicamba, and triclopyr was observed in 32%, 4%, and 8% of the populations, respectively. Rapid necrosis symptoms were present in 44% and 62% of the populations after 2,4-D and triclopyr application, respectively; however, these symptoms were not always associated with plant survival. Mixtures with auxinic herbicides improved the control of 2,4-D-resistant populations. The *PLP2* gene, previously associated with the necrosis response, was expressed in populations exhibiting these symptoms, mainly following application of 2,4-D and triclopyr, consistent with the occurrence of rapid necrosis. In contrast, the expression of ABC transporter genes was not directly associated with the necrosis phenotype and may be related to differential herbicide translocation. Although resistance to auxinic herbicides is still relatively limited in Brazil, understanding how resistance mechanisms respond to different auxinic herbicides is important for the development of effective management strategies and the selection of alternative control options.

Keywords: 2,4-D, screening, rapid necrosis, cell death, ABC transporters

4.1 Introduction

C*onyza* species are among the most problematic weeds worldwide due to their high prolificacy, high dispersal capacity, genetic variability, and numerous cases of herbicide resistance (Bajwa *et al.*, 2016). To date, 116 cases of resistance have been reported globally in *Conyza* species (Heap, 2026). In Brazil, Sumatran fleabane (*Conyza sumatrensis*) and hairy fleabane (*C. bonariensis*) are the main species of this genus, occurring predominantly in soybean fields (Kalsing *et al*., 2024b) with reported resistance cases since 2005, particularly to glyphosate and chlorimuron (Kaspary *et al.*, 2025; Mendes *et al.*, 2021). In 2019, the first case of resistance to five modes of action was confirmed in Sumatran fleabane, glyphosate, paraquat, diuron, saflufenacil, and 2,4-D (de Pinho *et al*., 2019).

The first cases of resistance to auxinic herbicides were reported for 2,4-D in 1957, simultaneously in *Commelina diffusa* in the United States and *Daucus carota* in Canada (Hilton, 1957; Switzer, 1957). Currently, 73 cases of resistance to herbicides with this mechanism of action have been recorded in broadleaf weed species (Heap, 2026). Even so, auxinic herbicides have demonstrated relatively low resistance frequencies compared to other herbicide groups, which reinforces their value as a tool in herbicide resistance management strategies (Peterson *et al.*, 2016). However, the recurrent use of auxinic herbicides in recent years has led to the evolution of resistant weed populations through non-target site resistance (NTSR) mechanisms, and also, some target site resistance (TSR) cases are being reported (Moreno-Serrano *et al*., 2024).

Target-site mechanisms to auxinic herbicides were identified in five cases involving point mutations or deletions in different members of the *Aux/IAA* gene family. Alterations that disrupt the interaction of Aux/IAA proteins with auxinic herbicides and the TIR1 receptor prevent TIR1-mediated ubiquitylation, thereby conferring herbicide resistance (Figueiredo *et al*., 2022b). Resistance to 2,4-D was reported in *Kochia scoparia*, associated with point mutations in *IAA16* (LeClere *et al.*, 2018); in *Sisymbrium orientale*, associated with point mutations in *IAA34* (Qi *et al.*, 2025) and base deletions in *IAA2* (Figueiredo *et al*., 2022b); and in *Sonchus oleraceus*, associated with base deletions in *IAA20* (Krishnan *et al.*, 2025). In contrast, resistance to dicamba was reported in *Chenopodium album*, due point mutations in *IAA16* (Ghanizadeh *et al.*, 2024).

Cases involving NTSR to auxinic herbicides have been mainly associated with reduced herbicide translocation and enhanced herbicide metabolism (Moreno-Serrano *et*

al., 2024). Resistance to 2,4-D has been related to reduced translocation in *Papaver rhoeas* (Rey-Caballero *et al.*, 2016) and *Raphanus raphanistrum* (Goggin *et al*., 2016), although specific genes have not yet been identified. Additionally, enhanced 2,4-D metabolism has been described in *Amaranthus tuberculatus*, possibly due to the action of cytochrome P450 monooxygenases (Figueiredo *et al*., 2022a). Sumatran fleabane plants resistant to 2,4-D showed an unusual symptom, rapid necrosis (RN), and the mechanism of resistance is unknown (Queiroz *et al*., 2020; Souza *et al*., 2023). Some candidate genes were identified in previous studies, showing overexpression in resistant plants treated with 2,4-D. Among these, *PATATIN-LIKE PROTEIN 2* (*PLP2*) may be involved in necrosis symptoms, while the transporter genes *ABCB4*, *ABCG1*, *ABCC15*, and *ABCC3* are likely related to differential herbicide transport (Angonese, 2022). But their response to other auxinic herbicides is unknown.

Auxinic herbicides have been used for the selective control of broadleaf weeds since the introduction of 2,4-D in 1945 (Peterson *et al.*, 2016). In recent years, the use of these herbicides has increased in importance, mainly for controlling glyphosate-resistant broadleaf weeds in soybean fields (Garcia *et al.*, 2025). The use of auxinic herbicides also increased to control biotypes resistant to ALS-inhibiting herbicides, particularly in *Conyza* species (Merotto *et al.*, 2022). The utilization of auxinic herbicides is fundamental in the no-till systems. This cropping system is important for soil conservation and environmental protection. In the last decade, the necessity of using auxinic herbicides in the burndown operation in no-till areas increased, and consequently, the selection pressure resulted in the evolution of herbicide-resistant weeds. In addition, the recent commercialization of crops resistant to auxinic herbicides enabled their application for pre-sowing burndown and post-emergence applications (Moreno-Serrano *et al*., 2024), which also contributes to increasing the selection pressure for herbicide resistance. This study aimed to assess the frequency of auxin herbicide resistance in *Conyza* populations in agricultural regions of Brazil and to associate these cases with the occurrence of RN symptoms and the expression of candidate resistance genes in response to the application of various auxinic herbicides, either alone, in mixtures, or in combination with glyphosate.

4.2 Materials and Methods

4.2.1 Plant material

Seeds from 75 populations of Sumatran and hairy fleabane were collected from herbicide escape on agricultural fields in Brazil between 2016-2024 (Table 2). For all experiments, recently produced seeds obtained through population maintenance under controlled cultivation conditions were used. Seeds from each population were sown in pots filled with commercial substrate. Plants with four leaves were transplanted and grown in 200 ml pots filled with a mixture of argisol and commercial substrate. The plants were grown in a greenhouse at 28 ± 5°C and irrigated daily.

4.2.2 Auxinic herbicides resistance screening

Plants at the 6-8 leaf stage were screened with four auxin herbicides: 2,4-D (phenoxycarboxylates), dicamba (benzoates), triclopyr (pyridyloxycarboxylates), and halauxifen-methyl (arylpicolinates) at 50 and 100% the label rate of each herbicide, which were 502 and 1005 g ha^{-1} of 2,4-D (DMA, 670 g L^{-1}, SL, Corteva Agriscience), 360 and 720 g ha^{-1} of dicamba (Atectra, 480 g L^{-1}, SL, Basf), 360 and 720 g ha^{-1} of triclopyr (Garlon, 480 g L^{-1}, EC, Corteva Agriscience), and 3.6 and 7.3 g ha^{-1} of halauxifen-methyl (Elevore, 71.4 g L^{-1}, SC, Corteva Agriscience). The application was carried out with an automated spray chamber (Generation III, Devries manufacturing) at a spray volume of 200 L ha^{-1}. Each treatment had five biological replicates. The number of populations evaluated was 75 for 2,4-D, and 50 for dicamba, triclopyr, and halauxifen-methyl. The plant injury was assessed on a visual scale at 28 days after treatment (DAT), where 0% were not injured, and 100% were dead plants. Plants that survived and exhibited less than 85% control efficacy compared with untreated and known susceptible controls were identified as candidates for resistance characterization. The occurrence of RN symptoms associated with resistance to the herbicide 2,4-D was evaluated based on the time required for symptom observation after application and by visual injury at 2 DAT.

4.2.3 Auxinic herbicide mixtures and combinations with glyphosate for controlling resistant populations

Selected Sumatran fleabane populations with contrasting responses to auxinic herbicides were chosen for evaluation in mixtures and in combination with glyphosate.

These included populations 1) susceptible to all auxinic herbicides without RN (C2), 2) susceptible with RN to 2,4-D (C7), 3) resistant to 2,4-D with RN to 2,4-D at 2 hours after treatment and triclopyr at 4 hours after treatment (G5), 4) resistant to 2,4-D with RN to both 2,4-D and triclopyr at 4 hours after treatment (G10), and 5) resistant to 2,4-D without RN to 2,4-D but with RN to triclopyr (C22). These populations were treated with the auxinic herbicides 2,4-D, dicamba, triclopyr, and halauxifen-methyl, applied alone and in mixtures with each other or with glyphosate (Zapp Qi®, 620 g L^{-1}, SL, BASF) in doses shown in Table 1.

The herbicides were applied at 1.6 times the recommended label rate (Table 1) to ensure sufficient treatment intensity for subsequent gene expression analyses, thereby enabling direct comparison with previously reported results (Angonese, 2022). The plants were grown as previously described and, at the 6-8 leaf stage, were transferred from the greenhouse to a growth chamber (BDR16, Conviron) set at 25 °C with a 13-hour photoperiod and a light intensity of 400 µmol m^{-2} s^{-1} for 48 hours before treatment application. The treatment application was carried out with an automated spray chamber as described previously. The control efficacy was assessed on a visual scale at 21 DAT.

Table 1. Auxinic herbicides and combinations with glyphosate and application doses evaluated in Sumatran fleabane populations.

Herbicides	Mode of action	Dose (g ha^{-1})
Halauxifen-methyl (Hal)	auxin herbicide	11
2,4-D (24D)	auxin herbicide	1608
Triclopyr (Tri)	auxin herbicide	1152
Dicamba (Dic)	auxin herbicide	685
Halauxifen-methyl + 2,4-D	auxin herbicide	11 + 1608
Halauxifen-methyl + triclopyr	auxin herbicide	11+1152
Halauxifen-methyl + dicamba	auxin herbicide	11+685
Halauxifen-methyl + glyphosate (Gly)	auxin herbicide + EPSPS inhibitor	11+1080
2,4-D + triclopyr	auxin herbicide	1608+1152
2,4-D + dicamba	auxin herbicide	1608+685
2,4-D + glyphosate	auxin herbicide + EPSPS inhibitor	1608+1080
Triclopyr + glyphosate	auxin herbicide + EPSPS inhibitor	1152+1080
Dicamba + glyphosate	auxin herbicide + EPSPS inhibitor	685+1080

4.2.4 Expression of candidate resistance genes associated with rapid necrosis symptoms in response to different auxinic herbicides

In parallel with the screening evaluation, four populations with different responses to 2,4-D were selected for tissue collection and analysis of the expression of genes potentially related to the RN resistance mechanism to 2,4-D (Angonese, 2022). The populations were classified as susceptible (C3), resistant with RN symptoms within 2 hours after treatment (population G5) and after 4 hours (population C25), or resistant without RN symptoms (population C22) following treatment with 2,4-D. Plants at the 6-8 leaf stage were transferred from the greenhouse to a growth chamber (BDR16, Conviron) set at 25 °C with a 13-hour photoperiod and a light intensity of 400 µmol m^{-2} s^{-1} for 48 hours before treatment application. Treatments consisted of the application of the herbicides 2,4-D, dicamba, triclopyr, and halauxifen-methyl by depositing four 0.65 µL drops per leaf using a micropipette, following previous methodology studies (Angonese, 2022). The herbicides were applied at 1.6 times the recommended label rate, corresponding to 1608 g ha^{-1} of 2,4-D, 685 g ha^{-1} of dicamba, 1152 g ha^{-1} of triclopyr, and 11g ha^{-1} of halauxifen-methyl. Plant tissue was sampled at 5, 30, and 60 minutes after treatment by collecting six leaf discs of 11 mm diameter from the treated area per sample. Treatments were collected and analyzed in triplicate.

RNA extraction from leaf tissue was performed with Concert™ Reagent (Invitrogen, ThermoFisher Scientific, Waltham, USA), and cDNA was synthesized using M-MLV Reverse Transcriptase (Invitrogen). The qPCR reactions were a final concentration of 1 x PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTP, 0.1 µM ROX Reference Dye, 0.1 x SYBR™ Green, 0.5 U Platinum™ Taq DNA Polymerase (Invitrogen), 0.2 µM primer, 50 ng $µL^{-1}$ DNA or cDNA, and DNAse-free water to complete 10 µL final volume. PCR conditions were 50 °C for 1 min, 95 °C for 10 min; 40 cycles of 95 °C for 15 s, annealing temperature for 30 s, and 72 °C for 30 s in a QuantStudio 3 Real-Time PCR system. For each population, three biological replicates and three technical replicates were used.

The expression was evaluated for *PATATIN-LIKE PROTEIN 2* (*PLP2*) and ATP Binding-Cassette (ABC) transporter genes: *ABCB4* and *ABCC3*. The primers used are described in (Angonese, 2022). Gene expression was calculated relative to the untreated susceptible populations using the $2^{-\Delta\Delta Ct}$ method (Dussault and Pouliot, 2006). The *HSP70* (*heat shock protein-70*) gene was used as the housekeeping reference gene (Moretti *et al*.,

2017). The data were compared according to ANOVA, and when significant ($p \leq 0.05$), the averages were compared according to Tukey 5%.

4.2.5 Expression of candidate resistance genes associated with rapid necrosis symptoms in response to auxinic herbicide mixtures and combinations with glyphosate

In a simplified approach, the expression of two putative genes associated with 2,4-D resistance was also evaluated in the Sumatran fleabane populations C2, C7, G5, G10, and C22 under herbicide mixture treatments. The plants treated for the mixture control assay were collected for the expression analysis 60 min after the herbicide application. The genes *PLP2* and *ABCC3* were analyzed, using *HSP70* as the endogenous reference gene. All other experimental procedures, including plant growth conditions, tissue collection, RNA extraction, cDNA synthesis, and RT-qPCR analysis, were performed as described in the previous section.

4.3 Results and Discussion

4.3.1 Auxinic herbicides resistance frequency

Auxinic herbicides from four different chemical groups were screened across hairy and Sumatran fleabane populations. Among the 75 populations evaluated for 2,4-D, 24 populations survived the recommended dose, representing 32% of the populations assessed (Table 2). For dicamba, triclopyr, and halauxifen-methyl, 50 populations were evaluated, with survival rates of 4% and 8% observed for dicamba and triclopyr, respectively, at the higher dose (Table 2). No resistance to halauxifen-methyl was observed, however, some plants did not show complete control, possibly due to the growth stage at the time of application.

Three populations were identified as resistant to both 2,4-D and triclopyr, and one population was identified as resistant to both 2,4-D and dicamba (Table 2). No populations were identified as resistant to all three auxinic herbicides. The evaluation of all populations for all herbicides was not possible due to low germination rates in some seed lots, and because of that, the evaluations prioritized 2,4-D due to its history of use in the areas where the populations were collected. Resistance to 2,4-D was predominantly identified in populations collected in the states of Paraná and Mato Grosso do Sul. For

dicamba, the two resistant populations were collected in Paraná. In the case of triclopyr, three resistant populations originated from Dourados and one from Rio Brilhante, both located in Mato Grosso do Sul (Figure 1A).

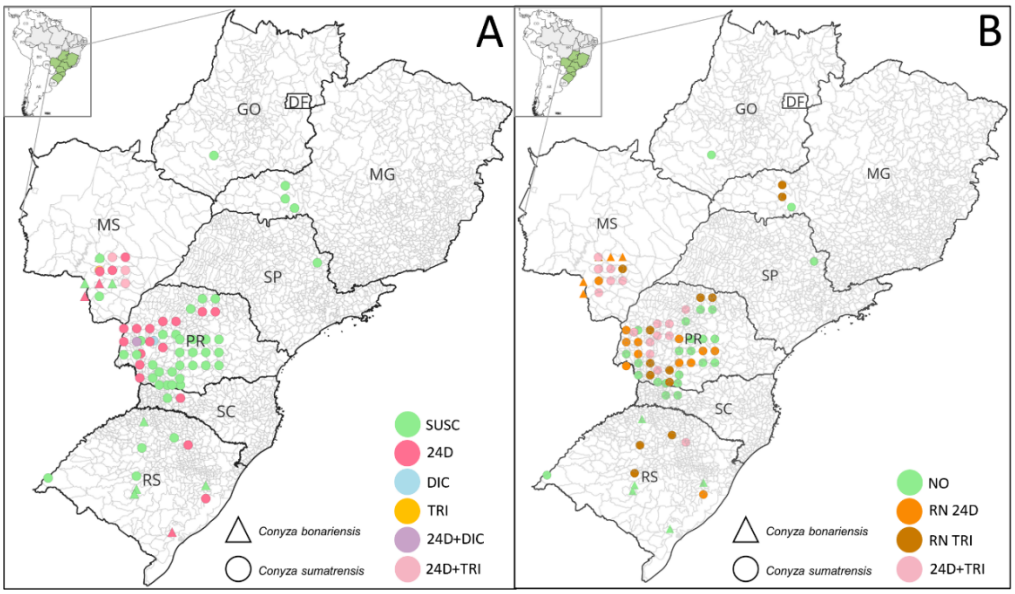


Figure 1. Resistance patterns to auxinic herbicides in 75 *Conyza* spp. populations (A) and distribution of the RN phenotype following 2,4-D and triclopyr application (B) in Brazil. SUSC: susceptible; 24D, DIC, and TRI: resistance to 2,4-D, dicamba, or triclopyr, respectively; 24D+DIC or 24D+TRI: cross resistance; NO: no RN; RN 24D, TRI, or 24D+TRI: RN to 2,4-D, triclopyr, or to both, respectively.

Symptoms of RN following 2,4-D application have been reported in Sumatran fleabane under field conditions (Kalsing *et al*., 2024a; Queiroz *et al*., 2020). In the present study, these symptoms were observed in 44% of the populations treated with 2,4-D and in 62% of the populations treated with triclopyr, whereas no RN was observed after dicamba or halauxifen-methyl application (Table 2). Among the populations exhibiting RN, only four populations treated with 2,4-D and one treated with triclopyr were identified as hairy fleabane, whereas the majority of populations showing this phenotype corresponded to Sumatran fleabane. For both herbicides, the RN cases were concentrated in Paraná and Mato Grosso do Sul, with a few populations collected in Rio Grande do Sul, Minas Gerais, and Santa Catarina (Figure 1B).

Overall, RN symptoms following triclopyr application occurred later, approximately 6 hours after treatment, and only at the higher dose. In contrast, RN symptoms following 2,4-D application developed generally within 2 to 4 hours after treatment and were observed at both tested doses (data not shown). Comparison between

RN symptoms and survival data indicated that some populations exhibiting RN did not survive the herbicide application, probably because of variations in the size, physiological status, and effective herbicide dose received by the plant.

A survey of 382 *Conyza* accessions collected from Brazil and Paraguay reported that approximately 79% of the accessions were susceptible to all evaluated auxinic herbicides, whereas about 20% exhibited resistance to 2,4-D (Kalsing *et al*., 2024a). Resistance to other auxinic herbicides was less frequent, with only one accession resistant to triclopyr and four resistant to dicamba, and 12 accessions showing cross-resistance to two or more auxinic herbicides (Kalsing *et al*., 2024a). A similar pattern was observed in the present study, where resistance was most frequent to 2,4-D, while resistance to dicamba and triclopyr occurred more locally, and cross-resistance was uncommon. Although the overall frequency of resistance to auxinic herbicides remains relatively low compared to the widespread resistance to glyphosate and ALS-inhibiting herbicides in *Conyza* spp. across Brazil (Mendes *et al.*, 2021), their use has increased substantially. This trend is driven by the need to control resistant populations and the adoption of auxinic herbicide-resistant crop technologies, such as Enlist® and Xtend®. This increased reliance is expected to intensify selection pressure and may contribute to the gradual evolution and spread of resistance, highlighting the importance of early detection and proactive resistance management strategies.

A survey of 461 populations from Paraná and Mato Grosso do Sul, Brazil, identified multiple resistance to glyphosate, chlorimuron, and 2,4-D in 13% of populations, and 5% showing resistance also to paraquat. Rapid necrosis symptoms were also reported (Lorenzetti *et al.*, 2024). Similarly, multiple resistance was identified in populations evaluated in the present study, including a population simultaneously resistant to ALS inhibitors, glyphosate, 2,4-D, triclopyr, paraquat, and fomesafen (data not shown). The occurrence of resistance to herbicides with different mechanisms of action is particularly concerning, as it severely limits available control options and increases the risk of control failure under field conditions.

Table 2. Efficacy of control (%) at 28 days after herbicide treatment in 75 *Conyza* spp. populations from Brazil with four auxin herbicides at 50% and 100% of the label rate. ERIBO - *Conyza bonariensis*, ERISU - *Conyza sumatrensis**, and the occurrence of rapid necrosis symptoms.

Population	Species	Local	Efficacy of control (%)								Rapid necrosis symptoms			
			2,4-D		dicamba		triclopyr		halauxifen-methyl					
			0.5x	1x	0.5x	1x	0.5x	1x	0.5x	1x	2,4-D	dicamba	triclopyr	halauxifen-methyl
G1	ERIBO	Capão do Leão/RS	82	83	92	95	87	93	93	94	no	no	no	no
G2	ERIBO	Vila Nova/RS	100	100	95	100	86	98	87	87	no	no	no	no
G3	ERIBO	Dois Irmãos das Missões/RS	100	100	100	100	100	100	77	80	no	no	no	no
G4	ERIBO	Santa Maria/RS	100	100	100	100	100	100	77	80	no	no	no	no
G5	ERISU	Maripá/PR	48	73	78	100	80	100	75	81	yes	no	yes	no
G6	ERISU	Londrina/PR	100	100	100	100	100	100	94	100	no	no	no	no
G7	ERISU	Marialva/PR	100	100	100	100	100	100	100	100	no	no	no	no
G8	ERISU	Toledo/PR	90	100	74	100	96	100	71	91	yes	no	yes	no
G9	ERISU	Toledo/PR	100	100	100	100	100	100	95	98	yes	no	no	no
G10	ERISU	Toledo/PR	27	31	100	100	92	92	97	88	yes	no	yes	no
G11	ERISU	Toledo/PR	70	82	79	68	92	92	94	98	no	no	no	no
G12	ERISU	Toledo/PR	84	69	100	100	100	100	100	100	yes	no	no	no
G13	ERISU	Campo Mourão/PR	54	55	78	91	76	86	74	75	yes	no	yes	no
G14	ERISU	Uruguaiana/RS	100	100	96	100	90	100	90	90	no	no	no	no
G15	ERISU	Itambé/PR	59	71	72	87	69	94	80	77	yes	no	yes	no
G16	ERISU	Dourados/MS	31	53	85	97	76	77	85	80	yes	no	yes	no
G17	ERISU	Dourados/MS	35	32	85	100	90	96	96	93	yes	no	yes	no
G18	ERISU	Dourados/MS	82	93	92	100	73	82	89	86	no	no	yes	no
G19	ERISU	Dourados/MS	55	62	100	100	92	100	93	91	yes	no	no	no
G20	ERISU	Dourados/MS	50	74	85	92	56	73	74	85	yes	no	yes	no
G21	ERISU	Rio Brilhante/MS	31	79	74	90	72	83	73	77	yes	no	yes	no
G22	ERISU	Rio Brilhante/MS	80	88	100	100	100	100	100	100	yes	no	yes	no
G23	ERISU	Maracaju/MS	44	87	91	100	75	92	82	78	yes	no	yes	no
G24	ERISU	Maracaju/MS	31	30	100	100	88	100	95	100	yes	no	yes	no

G25	ERIBO	Porto Alegre/RS	100	100	98	100	82	95	65	74	no	no	no	no
G26	ERISU	Porto Alegre/RS	28	71	-	-	-	-	-	-	yes	-	-	-
C1	ERISU	Rio Verde/GO	100	100	-	-	-	-	-	-	no	-	-	-
C2	ERISU	Uberaba/MG	100	100	96	98	100	100	100	100	no	no	no	no
C3	ERISU	Uberlandia/MG	100	100	90	100	100	100	96	90	no	no	yes	no
C4	ERISU	Uberlandia/MG	100	100	100	100	99	100	98	100	no	no	yes	no
C7	ERIBO	Ponta Porã/MS	93	87	100	100	77	95	96	95	yes	no	no	no
C8	ERIBO	Ponta Porã/MS	63	60	-	-	-	-	-	-	yes	-	-	-
C9	ERIBO	Rio Brilhante/MS	84	100	-	-	-	-	-	-	yes	-	-	-
C10	ERIBO	Dourados/MS	95	93	100	100	79	100	96	91	no	no	yes	no
C11	ERIBO	Rio Brilhante/MS	26	72	-	-	-	-	-	-	yes	-	-	-
C13	ERISU	Abelardo Luz/SC	100	100	-	-	-	-	-	-	no	-	-	-
C14	ERISU	Ampere/PR	58	62	-	-	-	-	-	-	no	-	-	-
C19	ERISU	Cascavel/PR	62	95	-	-	-	-	-	-	yes	-	-	-
C20	ERISU	Cascavel/PR	100	100	100	80	92	98	91	90	no	no	no	no
C21	ERISU	Clevelândia/PR	70	91	-	-	-	-	-	-	no	-	-	-
C22	ERISU	Corbelia/PR	52	87	100	100	76	95	99	79	no	no	yes	no
C24	ERISU	Dois Vizinhos/PR	87	100	100	100	95	100	88	100	no	no	yes	no
C25	ERISU	Guarapuava/PR	97	100	-	-	-	-	-	-	yes	-	-	-
C26	ERISU	Guarapuava/PR	98	91	-	-	-	-	-	-	no	-	-	-
C27	ERISU	Guarapuava/PR	100	100	-	-	-	-	-	-	no	-	-	-
C28	ERISU	Guarapuava/PR	100	96	-	-	-	-	-	-	no	-	-	-
C29	ERISU	Guarapuava/PR	83	100	-	-	-	-	-	-	no	-	-	-
C30	ERISU	Guarapuava/PR	100	100	-	-	-	-	-	-	no	-	-	-
C31	ERISU	Guarapuava/PR	96	99	-	-	-	-	-	-	yes	-	-	-
C32	ERISU	Guarapuava/PR	100	99	-	-	-	-	-	-	yes	-	-	-
C33	ERISU	Guarapuava/PR	100	100	-	-	-	-	-	-	yes	-	-	-
C34	ERISU	Guarapuava/PR	100	96	-	-	-	-	-	-	yes	-	-	-
C35	ERISU	Guarapuava/PR	100	100	100	100	100	100	85	94	no	no	no	no

C37	ERISU	Turvo/PR	100	100	87	93	73	94	92	85	no	no	no	no
C38	ERISU	Itapejara d'Oeste/PR	86	89	83	98	92	98	85	86	yes	no	yes	no
C39	ERISU	Juranda/PR	93	95	100	100	100	96	100	100	yes	no	yes	no
C40	ERISU	Juranda/PR	85	95	100	100	100	100	100	100	yes	no	yes	no
C41	ERISU	Juranda/PR	57	30	100	100	100	100	100	100	yes	no	yes	no
C43	ERISU	Mariopolis/PR	100	100	98	100	91	86	94	93	no	no	no	no
C44	ERISU	Ouro Verde do Oeste/PR	26	46	-	-	-	-	-	-	yes	-	-	-
C45	ERISU	Ouro Verde do Oeste/PR	66	78	-	-	-	-	-	-	yes	-	-	-
C46	ERISU	Pato Branco/PR	100	100	84	98	86	93	96	100	no	no	yes	no
C47	ERISU	Pato Branco/PR	100	100							no	-	-	-
C48	ERISU	Londrina/PR	97	100	100	100	100	100	86	89	no	no	yes	no
C49	ERISU	Planalto/PR	71	72	-	-	-	-	-	-	no	-	-	-
C50	ERISU	Santa Izabel do Oeste/PR	100	94	93	96	100	100	80	88	no	no	yes	no
C51	ERISU	São João/PR	100	100	89	95	100	100	84	91	no	no	yes	no
C52	ERISU	Sertanopolis/PR	-	-	97	95	100	100	93	95	-	no	yes	no
C54	ERISU	Tamarana/PR	95	87	-	-	-	-	-	-	no	-	-	-
C55	ERISU	Turvo/PR	100	100	-	-	-	-	-	-	no	-	-	-
C59	ERISU	Cruz Alta/RS	100	100	100	100	92	100	97	98	no	no	yes	no
C60	ERISU	Passo Fundo/RS	100	98	93	95	100	100	97	94	no	no	yes	no
C61	ERISU	Santa Maria/RS	100	100	100	100	100	100	100	100	no	no	yes	no
C62	ERISU	São Domingos/SC	80	77	85	96	100	100	100	100	yes	no	yes	no
C63	ERISU	Xanxerê/SC	78	75	-	-	-	-	-	-	no	-	-	-
C64	ERISU	Casa Branca/SP	97	100	100	100	98	100	100	100	no	no	no	no

*ERIBO and ERISU are Bayer codes derived from the nomenclature *Erigeron bonariensis* and *Erigeron sumatrensis*, respectively. Species are referred to as *Conyza bonariensis* and *Conyza sumatrensis* throughout this study.

4.3.2 Auxinic herbicide mixtures and combinations with glyphosate for controlling resistant populations

Representative populations from different response groups were evaluated with mixtures of auxinic herbicides and glyphosate to determine their effect on control. Plants were visually assessed at 21 DAT. The susceptible population (C2) showed ≥85% control with all auxinic herbicides applied alone, reaching 100% control with 2,4-D and dicamba, and near-complete control with most mixtures (Figure 2A). Similarly, the susceptible population expressing RN (C7) remained effectively controlled by auxinic herbicides, with 87% control from 2,4-D and 100% from dicamba and triclopyr (Figure 2B). Although RN symptoms were observed, plants did not regrow after necrosis, in contrast to resistant populations.

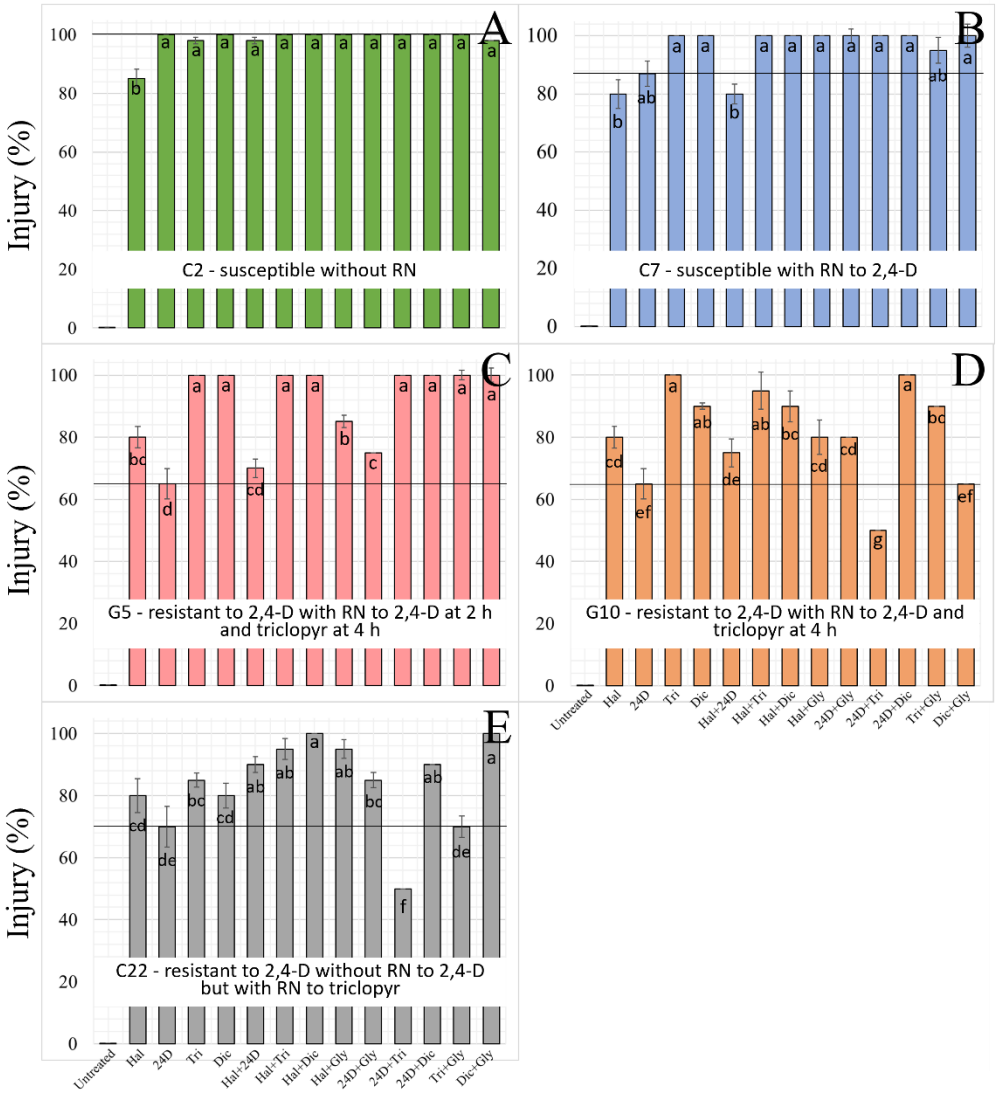


Figure 2. Injury (%) following application of auxinic herbicides applied alone or in mixtures in Sumatran fleabane populations with contrasting resistance and RN phenotypes: C2 - susceptible to all auxinic herbicides without RN (A), C7 - susceptible

with RN to 2,4-D (B), G5 - resistant to 2,4-D with RN to 2,4-D at 2 h and triclopyr at 4 h (C), G10 - resistant to 2,4-D with RN to both herbicides at 4 h (D), and C22 - resistant to 2,4-D without RN to 2,4-D but with RN to triclopyr (E). Error bars represent the standard deviation of the mean. Different letters indicate statistically significant differences among treatments according to Tukey's test at 5% significance level. The horizontal line indicates the control provided by 2,4-D applied alone in each population as a reference.

In contrast, populations resistant to 2,4-D showed consistently reduced control with this herbicide applied alone, ranging from 65 to 70%, frequently followed by plant regrowth after RN. The G5 and G10 populations also exhibited reduced control with mixtures containing 2,4-D, such as halauxifen-methyl + 2,4-D (70-75%) and 2,4-D + glyphosate (75-80%), indicating that combining these herbicides did not overcome resistance (Figures 2C and 2D). Similarly, the C22 population showed only 70% control with 2,4-D and 50% control with the mixture 2,4-D + triclopyr (Figure 2E).

Auxinic herbicides remain important tools for the control of *Conyza* populations resistant to ALS-inhibiting herbicides and glyphosate. However, the evolution of resistance to these herbicides, as observed in the present study, reinforces the need for alternative strategies, including the use of herbicide mixtures and combinations with different modes of action. In this study, mixtures of 2,4-D with other auxinic herbicides generally provided effective control of resistant populations, although some limitations were observed depending on the population resistance pattern.

Halauxifen-methyl is a relatively new synthetic auxin herbicide used for broadleaf weed control in preplant burndown applications at low use rates and exhibits an alternative binding affinity at the site of action (Epp *et al.*, 2016). It was included in this study as a potential alternative for the management of populations resistant to other auxinic herbicides, and to evaluate its performance in mixtures. Overall, halauxifen-methyl contributed to effective control in several treatments; however, reduced control was observed in some mixtures, particularly with auxinic herbicides to which the populations were already resistant. These limitations may also be associated with the plant developmental stage at application.

Previous studies have demonstrated that halauxifen-methyl is highly effective at low doses, particularly when applied to smaller plants. For example, glyphosate-resistant horseweed (*C. canadensis*) was effectively controlled by halauxifen-methyl applied alone or in mixtures, achieving up to 90% control (Zimmer *et al*., 2018). In addition, dose-

response studies have shown that halauxifen-methyl requires substantially lower doses to achieve similar growth reduction compared to 2,4-D and dicamba, especially at early growth stages (McCauley and Young, 2019). For instance, combinations including contact herbicides such as glufosinate or PPO inhibitors have been shown to enhance control of *Conyza* spp., especially when used in sequential applications or targeting larger plants (Cassol *et al.*, 2024; Lorenzetti *et al.*, 2024).

4.3.3 Expression of candidate resistance genes associated with rapid necrosis symptoms in response to different auxinic herbicides

The expression of genes potentially associated with RN and resistance to 2,4-D was evaluated in response to four auxinic herbicides in populations with contrasting responses to 2,4-D. For *PLP2*, gene expression was assessed at 5, 30, and 60 min after treatment. In the susceptible population (C3), *PLP2* expression remained relatively stable across treatments, with only a slight increase in response to halauxifen-methyl at 60 min (Figure 3A). In contrast, the G5 population, which exhibits RN symptoms within 2 h after 2,4-D application, showed early induction of *PLP2* expression (Figure 3B). Increased expression was observed at 5 min following 2,4-D treatment, at 5 and 30 min in response to dicamba, and at 30 and 60 min following triclopyr application. In contrast, halauxifen-methyl induced minimal variation, with expression levels comparable to those observed in the susceptible population.

The C25 population, which develops RN symptoms 4 h after treatment, exhibited a stronger induction pattern, with increased *PLP2* expression at all evaluated time points following 2,4-D treatment, particularly at 60 min (Figure 3C). Elevated expression was also observed in response to triclopyr at 30 and 60 min and to dicamba at 5 and 30 min. Similar to the susceptible population, halauxifen-methyl induced only minor changes in expression. In contrast, the resistant population without RN symptoms (C22) exhibited limited variation in *PLP2* expression across herbicides and time points, with expression profiles similar to those of the susceptible population (Figure 3D).

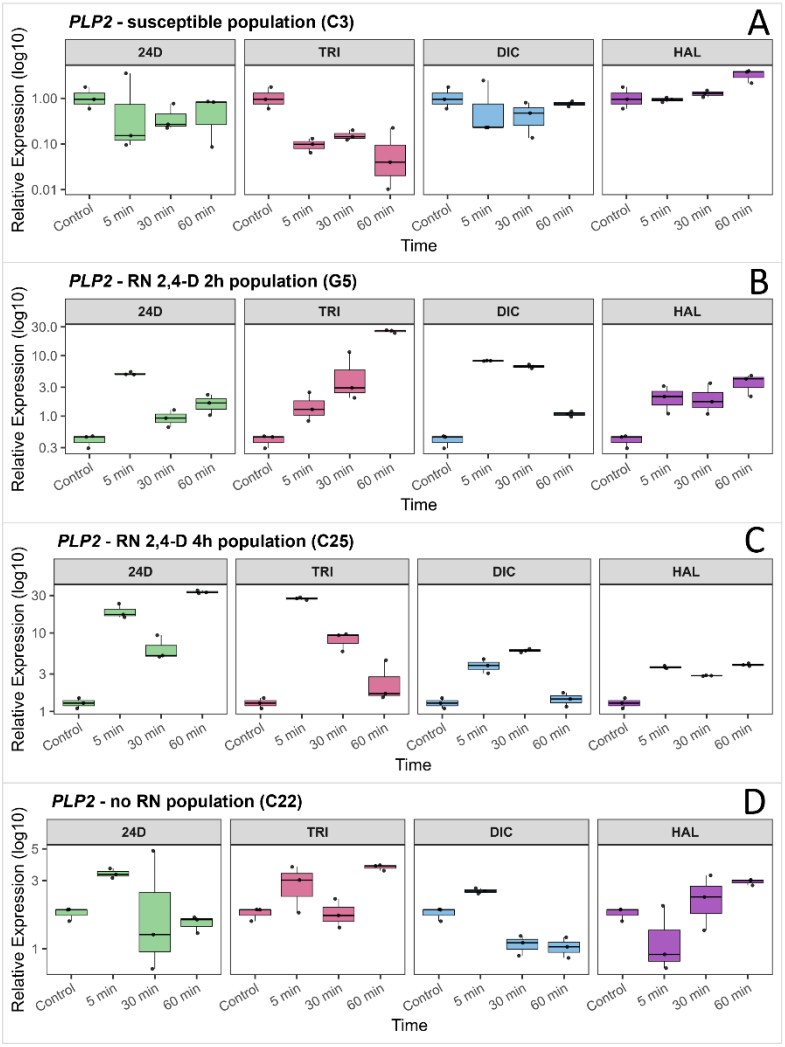


Figure 3. Relative expression of the *PLP2* gene in four Sumatran fleabane populations: susceptible (C3, A), RN 2,4-D 2 h (G5, B), RN 2,4-D 4 h (C25, C), and no RN (C22, D), following treatment with 2,4-D, triclopyr, dicamba, and halauxifen at 5, 30, and 60 min after treatment. Panels are presented using different y-axis scales.

The expression of ABC transporter genes was evaluated at 30 and 60 min after treatment. For *ABCB4*, increased expression relative to the untreated control and the susceptible population (C3) was detected in the G5 population (RN 2,4-D 2 h), particularly at 60 min following 2,4-D and triclopyr treatments and at 30 min in response to dicamba (Figure 4B). In contrast, the remaining populations showed limited variation in expression across herbicides and time points (Figure 4A, C, D). A similar expression profile was observed for *ABCC3*, with induction in the G5 population at 60 min following 2,4-D and triclopyr application and at 30 min in response to dicamba (Figure 5B). In contrast, expression patterns in the other populations were variable and did not show a consistent induction trend (Figure 5A, C, D).

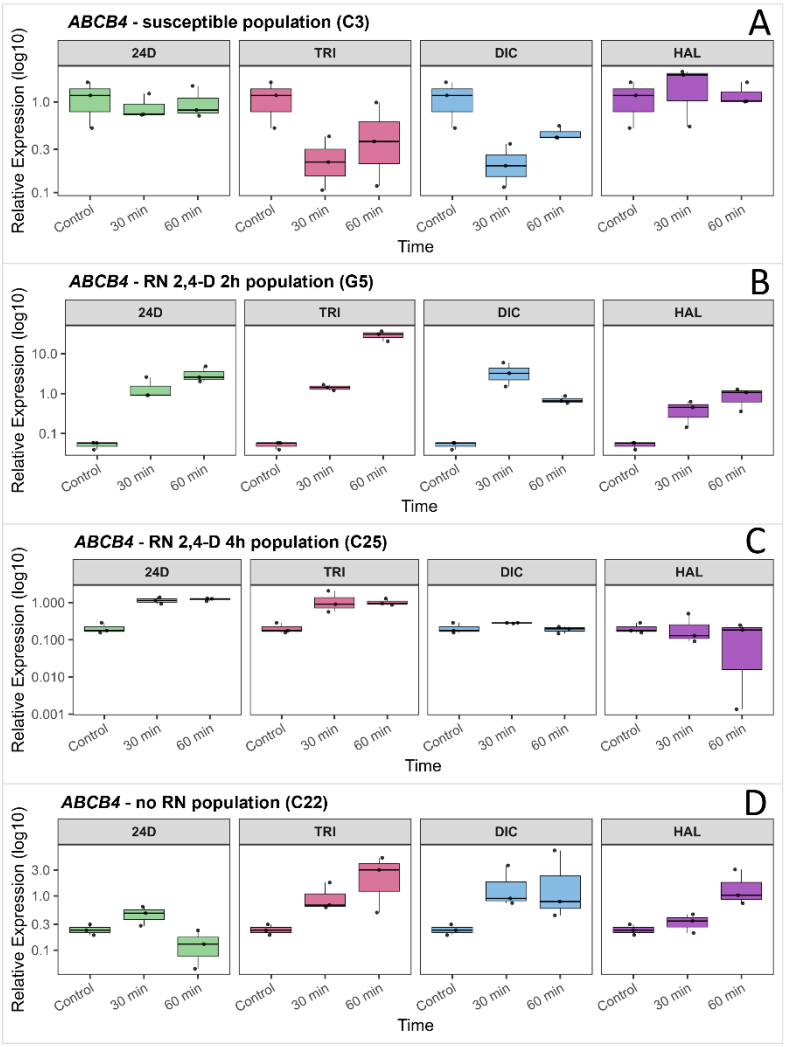


Figure 4. Relative expression of the *ABCB4* gene in four Sumatran fleabane populations: susceptible (C3, A), RN 2,4-D 2 h (G5, B), RN 2,4-D 4 h (C25, C), and no RN (C22, D), following treatment with 2,4-D, triclopyr, dicamba, and halauxifen at 5, 30, and 60 min after treatment. Panels are presented using different y-axis scales.

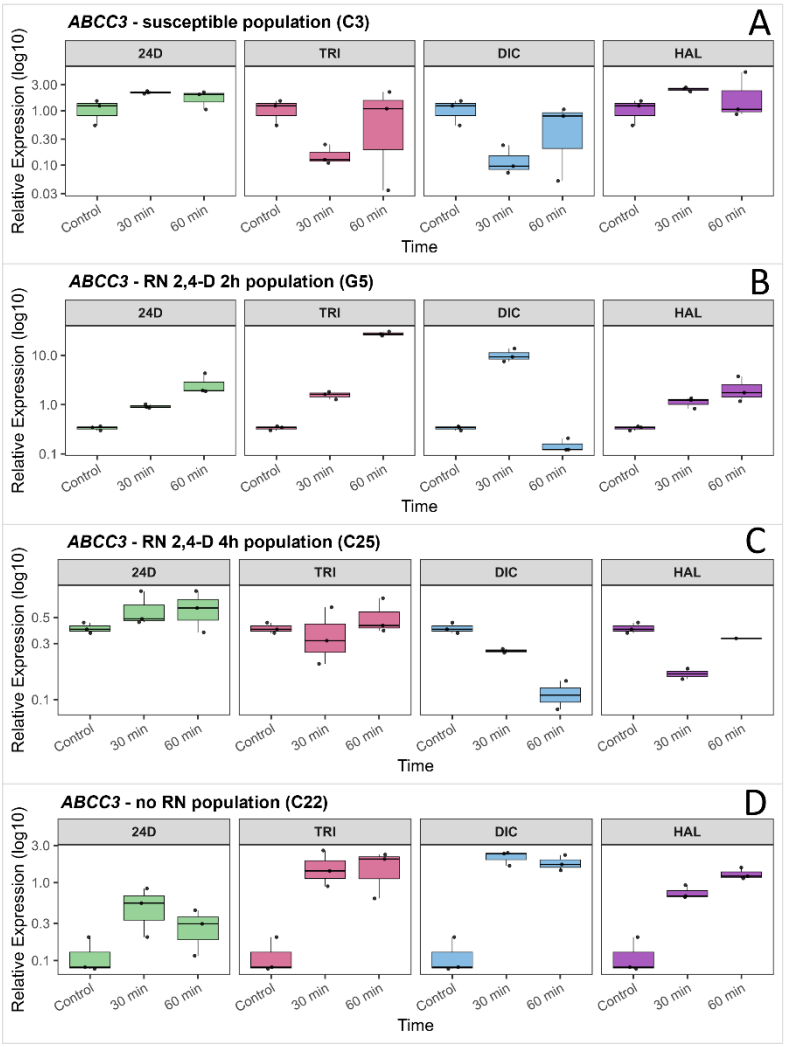


Figure 5. Relative expression of the *ABCC3* gene in four Sumatran fleabane populations: susceptible (C3, A), RN 2,4-D 2 h (G5, B), RN 2,4-D 4 h (C25, C), and no RN (C22, D), following treatment with 2,4-D, triclopyr, dicamba, and halauxifen at 5, 30, and 60 min after treatment. Panels are presented using different y-axis scales.

4.3.4 Expression of candidate resistance genes associated with rapid necrosis symptoms in response to auxinic herbicide mixtures and combinations with glyphosate

The expression of *PLP2* and *ABCC3* genes was evaluated in response to auxinic herbicides applied alone or in mixtures, at 60 minutes after treatment, in five populations with different responses to 2,4-D herbicide: susceptible without RN (C2), susceptible with RN to 2,4-D (C7), resistant to 2,4-D with RN to 2,4-D at 2 h and triclopyr at 4 h (G5), resistant to 2,4-D with RN to both 2,4-D and triclopyr at 4 h (G10), and resistant to 2,4-D without RN to 2,4-D but with RN to triclopyr (C22).

For *PLP2*, fold change values revealed clear differences in expression patterns among populations (Figure 6A). The susceptible population (C2) and the resistant population without RN to 2,4-D (C22) exhibited minimal changes in expression across all treatments, indicating little or no transcriptional response to the herbicides evaluated (Figure 6A). In contrast, populations showing RN (C7, G5, and G10) exhibited a consistent induction pattern of *PLP2*. Among these, G10 showed the highest fold change values, particularly in treatments containing 2,4-D, either applied alone or in mixtures with dicamba, glyphosate, triclopyr, and halauxifen-methyl. The populations G5 and C7 also showed increased expression in response to these treatments, although at lower magnitudes compared to G10. Additional induction was observed in response to triclopyr applied alone and to mixtures involving halauxifen-methyl, although these responses were more variable among populations.

For *ABCC3*, fold change values indicated a more intense induction pattern across treatments (Figure 6B). Populations expressing RN (C7, G5, and G10) showed increased expression in response to most herbicides, both applied alone and in mixtures. The highest expression levels were consistently associated with treatments containing 2,4-D alone or in combinations with dicamba, glyphosate, triclopyr, and halauxifen-methyl. The visual control patterns varied among populations and herbicide treatments (Figure 6C). Susceptible populations (C2 and C7) generally exhibited higher control levels, whereas resistant populations (G5, G10, and C22) showed reduced control, particularly in response to treatments containing 2,4-D applied alone or in mixtures.

The expression patterns observed for *PLP2*, *ABCB4*, and *ABCC3* suggest that RN and plant survival in response to auxinic herbicides are governed by distinct but interconnected processes. Importantly, these responses were not exclusive to 2,4-D, as similar patterns of gene induction and RN symptoms were observed following treatment with triclopyr and some for dicamba, indicating that multiple auxinic herbicides may trigger this mechanism. This conserved response across different auxinic classes suggests that RN and associated gene expression are linked to a common signaling pathway downstream of auxin perception, rather than to an herbicide site of action-specific effect. The mode of action of auxinic herbicides involves the deregulation of auxin signaling through the interaction with the TIR1/AFB receptor complex, leading to the degradation of AUX/IAA transcriptional repressors and downstream transcriptional reprogramming (Hagen and Guilfoyle, 2002; Kubeš and Napier, 2019). Differences in herbicide structure

and receptor affinity may result in distinct signaling outputs, which could explain the variation in RN intensity and gene expression observed among herbicides.

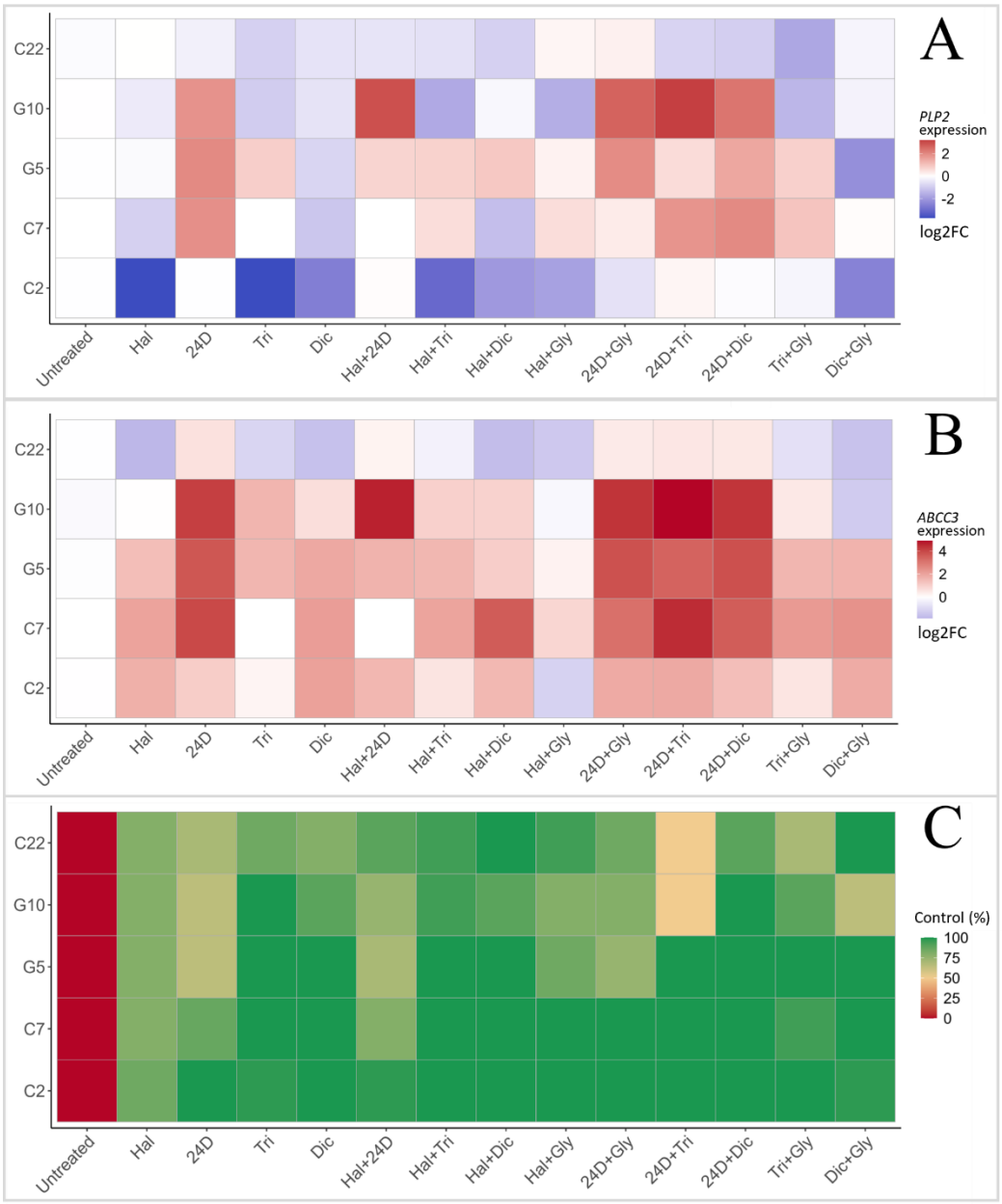


Figure 6. Heatmaps showing the relative expression (log_2 fold change, log_2FC) of *PLP2* (A) and *ABCC3* (B) genes, and the visual control (%) of auxinic herbicides and their mixtures (C), in five Sumatran fleabane populations (C2 - susceptible, C7 - susceptible with RN to 2,4-D, G5 - resistant to 2,4-D with RN to 2,4-D at 2 h and triclopyr at 4 h, G10 - resistant to 2,4-D with RN to both herbicides at 4 h, and C22 - resistant to 2,4-D without RN to 2,4-D but with RN to triclopyr). Expression values were calculated relative to the untreated control of each population. In panels A and B, red indicates upregulation, blue indicates downregulation, and white indicates no change ($log_2FC = 0$). In panel C, red indicates lower control, green indicates higher control, and intermediate colors represent intermediate control levels.

The association between *PLP2* expression and RN suggests that this gene is involved in the early signaling events leading to cellular damage (Angonese, 2022). Patatin-like proteins are known to participate in lipid hydrolysis, providing precursors for oxylipin biosynthesis, which act as signaling molecules involved in plant defense and programmed cell death (Blée, 2002; La Camera *et al*., 2005). Thus, the induction of *PLP2* in resistant populations may reflect the activation of a defense-like response, in which auxinic herbicides are perceived as stress signals or elicitors, triggering localized cell death (Angonese, 2022). This RN may act to restrict herbicide translocation, limiting its movement to meristematic tissues. The results obtained in the present study are consistent with this hypothesis.

Notably, the expression of *PLP2* segregated according to the RN phenotype across populations, with strong induction observed only in populations expressing RN, regardless of their resistance status. In contrast, populations without RN showed minimal variation in *PLP2* expression. This clear association reinforces the role of *PLP2* as a putative molecular marker associated with the necrotic response. However, rapid necrosis alone does not necessarily result in plant survival in this study. The ability of some populations to recover and regrow after exhibiting RN indicates that additional mechanisms modulate survival after herbicide treatment. In this study, the induction of ABC transporter genes, particularly *ABCB4* and *ABCC3*, supports their involvement in these survival responses. The expression pattern of *ABCB4*, which was more transient and population-specific, suggests a role in the redistribution of auxinic herbicides within plant tissues. Members of the ABCB subfamily are associated with auxin transport and cellular efflux (Cho and Cho, 2013), and their induction may contribute to reducing herbicide accumulation in sensitive tissues, such as the meristem. This process could facilitate the movement of the herbicide away from active growth regions toward more mature tissues.

The intense and consistent induction of *ABCC3* across treatments suggests a role in detoxification processes, such as vacuolar sequestration. ABCC transporters are commonly associated with the compartmentalization of xenobiotics, reducing their cytotoxic effects (Hwang *et al.*, 2016). Increased expression of *ABCC3* may therefore enable resistant plants to tolerate herbicide exposure by limiting its bioavailability at the cellular level.

Together, these results support a model in which auxinic herbicides trigger a dual response: a rapid, localized necrotic response mediated by PLP2 and associated lipid signaling pathways, which may restrict herbicide translocation, and a systemic detoxification and redistribution response mediated by ABC transporters, which reduces herbicide toxicity and enables plant recovery. The balance between these processes may determine whether plants die or survive following herbicide application.

4.4 Conclusion

This study evaluated the distribution of resistance to auxinic herbicides in populations of hairy and Sumatran fleabane across Brazil. Resistance to 2,4-D with the symptom RN was predominant among 2,4-D-resistant populations; however, six populations were resistant to this herbicide without exhibiting RN. Although relatively few populations were classified as resistant to triclopyr, a high frequency of RN symptoms was observed in response to this herbicide, indicating that this phenotype is widespread and may represent an early indicator or distinct manifestation of altered auxinic herbicide response. Auxinic herbicide mixtures were effective for the control of resistant populations. The independent occurrence of *PLP2* overexpression from ABC transporter gene expression suggests that RN is driven by distinct molecular mechanisms that are consistently induced by different auxinic herbicides. These findings highlight that RN is a conserved response across auxinic herbicides and may play a key role in resistance expression, while survival and regrowth likely depend on additional mechanisms such as herbicide transport. Understanding these processes is essential for improving resistance management and optimizing control strategies for *Conyza* populations in Brazil.

References

Angonese PS. Efeito de inibidores metabólicos e análise do transcriptoma de *Conyza sumatrensis* para caracterização do mecanismo de resistência ao herbicida 2,4-D relacionado à rápida necrose. MSc dissertation, Universidade Federal do Rio Grande do Sul, 2022.

Bajwa AA, Sadia S, Ali HH, Jabran K, Peerzada AM, Chauhan BS. Biology and management of two important *Conyza* weeds: a global review. Environ Sci Pollut Res Int 2016;23:24694–710. https://doi.org/10.1007/S11356-016-7794-7.

Blée E. Impact of phyto-oxylipins in plant defense. Trends Plant Sci 2002;7:315–22. https://doi.org/10.1016/S1360-1385(02)02290-2.

La Camera S, Geoffroy P, Samaha H, Ndiaye A, Rahim G, Legrand M, et al. A pathogen-inducible patatin-like lipid acyl hydrolase facilitates fungal and bacterial host colonization in *Arabidopsis*. Plant J 2005;44:810–25. https://doi.org/10.1111/J.1365-313X.2005.02578.X.

Cassol M, Albrecht AJP, Albrecht LP, Silva AFM, Larini WF, Galvão FGC. Mixtures of herbicides for the control of multiple resistant *Conyza sumatrensis* in soybean pre-sowing burndown. Agrária - Revista Brasileira de Ciências Agrárias 2024;19:e3719–e3719. https://doi.org/10.5039/agraria.v19i3a3719.

Cho M, Cho H. The Function of ABCB Transporters in Auxin Transport. Plant Signal Behav 2013;8:e22990.

Dussault AA, Pouliot M. Rapid and simple comparison of messenger RNA levels using real-time PCR. Biol Proced Online 2006;8:1–10. https://doi.org/10.1251/BPO114.

Epp JB, Alexander AL, Balko TW, Buysse AM, Brewster WK, Bryan K, et al. The discovery of ArylexTM active and RinskorTM active: Two novel auxin herbicides. Bioorg Med Chem 2016;24:362–71. https://doi.org/10.1016/J.BMC.2015.08.011.

Figueiredo MRA, Barnes H, Boot CM, De Figueiredo ABTB, Nissen SJ, Dayan FE, et al. Identification of a Novel 2,4- D Metabolic Detoxification Pathway in 2,4- D -Resistant Waterhemp (*Amaranthus tuberculatus*). J Agric Food Chem 2022a;70:15380–9. https://doi.org/10.1021/acs.jafc.2c05908.

Figueiredo MRA, Küpper A, Malone JM, Petrovic T, de Figueiredo ABTB, Campagnola G, et al. An in-frame deletion mutation in the degron tail of auxin coreceptor *IAA2* confers resistance to the herbicide 2,4-D in *Sisymbrium orientale*. Proc Natl Acad Sci 2022b;119:e2105819119. https://doi.org/10.1073.

Garcia NS, Dayan FE, Camargo ER, Ceolin BC, Deuner S, de Avila LA. Auxin-mimic herbicides dilema: Their benefits and limitations. Pest Manag Sci 2025. https://doi.org/10.1002/PS.8913.

Ghanizadeh H, He L, Griffiths AG, Harrington KC, Carbone V, Wu H, et al. A novel mutation in IAA16 is associated with dicamba resistance in *Chenopodium album*. Pest Manag Sci 2024;80:3675–83. https://doi.org/10.1002/PS.8071.

Goggin DE, Cawthray GR, Powles SB. 2,4-D resistance in wild radish: Reduced herbicide translocation via inhibition of cellular transport. J Exp Bot 2016;67:3223–35. https://doi.org/10.1093/jxb/erw120.

Hagen G, Guilfoyle T. Auxin-responsive gene expression: genes, promoters and regulatory factors. vol. 49. Auxin Molecular Biology; 2002.

Heap I. International Herbicide-Resistant Weed Database 2026. http://www.weedscience.org/.

Hilton HW. Herbicide tolerant strains of weeds. Hawaiian Sugar Planters Association Annual Report 1957:69–72.

Hwang JU, Song WY, Hong D, Ko D, Yamaoka Y, Jang S, et al. Plant ABC Transporters Enable Many Unique Aspects of a Terrestrial Plant's Lifestyle. Mol Plant 2016;9:338–55. https://doi.org/10.1016/j.molp.2016.02.003.

Kalsing A, Fernandes AV, Rossi CVS, Nunes FA, Pereira GR, Velini ED, et al. Dispersion of 2,4-D Resistance in *Conyza* Spp. And Evidence of Cross-Resistance to Synthetic Auxins Across Soybean Cropping Regions and Seasons in Brazil and Paraguay 2024a. https://doi.org/10.2139/ssrn.4817079.

Kalsing A, Nunes FA, Gotardi GA, Bueno JC, Schneider AA, Tropaldi L, et al. Taxonomic resolution of fleabane species (*Conyza* spp.) based on morphological and molecular markers and their dispersion across soybean-cropping macroregions and seasons in Brazil. Weed Sci 2024b:1–31. https://doi.org/10.1017/WSC.2024.3.

Kaspary TE, Cutti L, Turra GM, Angonese PS, dos Santos OD, Merotto A. *Conyza bonariensis* resistance to glyphosate and ALS inhibitors involves target and non-target site resistance. Pestic Biochem Physiol 2025;213:106501. https://doi.org/10.1016/J.PESTBP.2025.106501.

Krishnan M, Petrovic T, Schwerdt JG, Merriam AB, Hereward JP, Preston C. A novel mutation in *SoIAA20* confers cross-resistance to 2,4-Dichlorophenoxyacetic acid and other auxinic herbicides in *Sonchus oleraceus*. Pest Manag Sci 2025;81:141–8. https://doi.org/10.1002/PS.8413.

Kubeš M, Napier R. Non-canonical auxin signalling: Fast and curious. J Exp Bot 2019;70:2609–14. https://doi.org/10.1093/jxb/erz111.

LeClere S, Wu C, Westra P, Sammons RD. Cross-resistance to dicamba, 2,4-D, and fluroxypyr in *Kochia scoparia* is endowed by a mutation in an AUX/IAA gene. Proc Natl Acad Sci U S A 2018;115:E2911–20. https://doi.org/10.1073/pnas.1712372115.

Lorenzetti JB, Danilussi MTY, Albrecht AJP, Barroso AAM, Albrecht LP, Silva AFM, et al. Identification, mapping, and chemical control of fleabane resistant to glyphosate, chlorimuron, paraquat, and 2,4-D. Weed Technology 2024;38. https://doi.org/10.1017/wet.2024.10.

McCauley CL, Young BG. Differential response of horseweed (*Conyza canadensis*) to halauxifen-methyl, 2,4-D, and dicamba. Weed Technology 2019;33:673–9. https://doi.org/10.1017/wet.2019.56.

Mendes RR, Takano HK, Netto AG, Junior GJP, Cavenaghi AL, Silva VFV, et al. Monitoring glyphosate-and chlorimuron-resistant *Conyza* spp. Populations in Brazil. An Acad Bras Cienc 2021;93. https://doi.org/10.1590/0001-3765202120190425.

Merotto A, Gazziero LP, Oliveira MC, Scursoni J, Garcia MA, Figueroa R, et al. Herbicide use history and perspective in South America. Advances in Weed Science 2022;40(Spec1):e020220050. https://doi.org/10.51694/AdvWeedSci/2022;40:seventy-five010.

Moreno-Serrano D, Gaines TA, Dayan FE. Current Status of Auxin Mimic Herbicides. Outlooks on Pest Management 2024;35:105–12. https://doi.org/10.1564/V35_JUN_04.

Moretti ML, Alárcon-Reverte R, Pearce S, Morran S, Hanson BD. Transcription of putative tonoplast transporters in response to glyphosate and paraquat stress in *Conyza*

bonariensis and *Conyza canadensis* and selection of reference genes for qRT-PCR. PLoS One 2017;12:e0180794. https://doi.org/10.1371/journal.pone.0180794.

Peterson MA, McMaster SA, Riechers DE, Skelton J, Stahlman PW. 2,4-D Past, Present, and Future: A Review. Weed Technology 2016;30:303–45. https://doi.org/10.1614/wt-d-15-00131.1.

de Pinho CF, Lourenço Leal JF, dos Santos Souza A, de Oliveira GFPB, de Oliveira C, Langaro AC, et al. First evidence of multiple resistance of Sumatran Fleabane (*Conyza sumatrensis* (Retz.) E.Walker) to five-mode-of-action herbicides. Aust J Crop Sci 2019;13:1688–97. https://doi.org/10.21475/ajcs.19.13.10.p1981.

Qi Y, Krishnan M, Tucker M, Preston C. A point mutation in *IAA34* confers resistance to the auxin herbicide 2,4-D in *Sisymbrium orientale*. Pest Manag Sci 2025;81:3523–31. https://doi.org/10.1002/PS.8720.

Queiroz ARS, Delatorre CA, Lucio FR, Rossi CVS, Zobiole LHS, Merotto A. Rapid necrosis: A novel plant resistance mechanism to 2,4-D. Weed Sci 2020;68:6–18. https://doi.org/10.1017/wsc.2019.65.

Rey-Caballero J, Menéndez J, Giné-Bordonaba J, Salas M, Alcántara R, Torra J. Unravelling the resistance mechanisms to 2,4-D (2,4-dichlorophenoxyacetic acid) in corn poppy (*Papaver rhoeas*). Pestic Biochem Physiol 2016;133:67–72. https://doi.org/10.1016/J.PESTBP.2016.03.002.

Souza A dos S, Leal JFL, Montgomery JS, Ortiz MF, Simões Araujo AL, Morran S, et al. Nontarget-site resistance due to rapid physiological response in 2,4-D resistant *Conyza sumatrensis*: reduced 2,4-D translocation and auxin-induced gene expression. Pest Manag Sci 2023. https://doi.org/10.1002/PS.7541.

Switzer CM. The existence of 2,4-D-resistant strains of wild carrot. Proceedings of the Northeastern Weed Control Conference 1957;11:315–8.

Tan X, Calderon-Villalobos LIA, Sharon M, Zheng C, Robinson C V, Estelle M, et al. Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature 2007;446:640–5. https://doi.org/10.1038/NATURE05731.

Zimmer M, Young BG, Johnson WG. Weed Control with Halauxifen-Methyl Applied Alone and in Mixtures with 2,4-D, Dicamba, and Glyphosate. Weed Technology 2018;32:597–602. https://doi.org/10.1017/wet.2018.48.

5. ARTIGO 3

Complex *EPSPS* copy architecture, subgenome-specific expression, and non-target-site mechanisms shape glyphosate resistance levels in Sumatran and hairy fleabane*

*Artigo a ser submetido para a Revista Plant Physiology and Biochemistry

Title: Complex *EPSPS* copy architecture, subgenome-specific expression, and non-target-site mechanisms shape glyphosate resistance levels in Sumatran and hairy fleabane

Authors: Paula Sinigaglia Angonese[1], Guilherme Menegol Turra[1], Luan Cutti[1], Othon Dias dos Santos[1], Vinícius Ferrari Tasca[1], Filipi Mesquita Machado[1], Catarine Markus[1], Todd Adam Gaines[2], Aldo Merotto Jr.[1]

[1]Crop Science Department, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

[2]Departament of Agricultural Biology, Colorado State University, Fort Collins, US

Abstract

Glyphosate resistance in *Conyza* species is widespread and geographically distributed across Brazil. However, little is known about the population-level distribution of resistance mechanisms and the genetic regulation underlying glyphosate resistance in this hexaploid weed complex. This study aimed to characterize phenotypic levels of glyphosate resistance and to investigate how *EPSPS* copy architecture and subgenome-specific expression interact with potential non-target-site mechanisms to shape resistance in Sumatran and hairy fleabane. Reduced control with glyphosate was observed in 67% and 91% of the 12 hairy and 58 Sumatran fleabane evaluated populations, respectively. In 27 evaluated populations, glyphosate resistance levels were strongly reduced under low-temperature conditions, with temperature-response ratios (TRR = ED_{50} high / ED_{50} low temperature) ranging from 2 to 122. In Sumatran fleabane, the substitutions Ala100Ser (GCA/TCA) and Pro106Thr (CCA/ACA) were identified in an *EPSPS* copy located on chromosome 4, subgenome B; however, this copy showed minimal expression relative to total *EPSPS* expression. Similarly, hairy fleabane showed the same Pro106Thr (CCA/ACA) mutation at the same copy. Variation in *EPSPS* copy number was also detected among populations, reaching up to a fourfold increase compared with susceptible plants. Differential expression among gene copies was observed, with copies located on chromosome 4 generally contributing most to total *EPSPS* expression. Three populations also exhibited atypical necrotic symptoms after glyphosate application under low-temperature conditions, which could be associated with increased expression of the putative transporter gene *ABCC3*, indicating the involvement of NTSR mechanisms. Together, these results suggest that glyphosate resistance in *Conyza* populations is not driven by a single mechanism but reflects a complex interaction between target-site variation, *EPSPS* copy number and expression, and potential NTSR mechanisms.

Keywords: gene duplication, temperature response, multiple mechanisms, *Conyza* sp.

5.1 Introduction

Species of the genus *Conyza* are highly problematic in cropping systems due to their high dispersal capacity, adaptation to diverse environmental conditions, and widespread evolution of herbicide resistance (Heap, 2026; Lazaroto *et al*., 2008). In Brazil, Sumatran fleabane (*Conyza sumatrensis*) is widely distributed in soybean fields, whereas hairy fleabane (*C. bonariensis*) occurs less frequently and is mainly restricted to the southern region (Kalsing *et al*., 2024). Both species are hexaploid (*n*=27) (Montgomery *et al.*, 2024), a genomic characteristic that may enhance adaptive potential by increasing genetic redundancy and regulatory complexity, thereby facilitating the evolution of herbicide resistance (Rutland *et al*., 2021). Worldwide, *Conyza* species are also reported as important weeds in Argentina, USA, Canada, and Europe (Bajwa *et al.*, 2016).

Since the early 2000s, glyphosate-resistant populations of *Conyza* species have emerged worldwide as a consequence of the intensive and repeated use of this herbicide as a primary weed management tool (Heap, 2026). In Brazil, glyphosate resistance was first reported in hairy fleabane and horseweed (*C. canadensis*) in 2005 (Vargas *et al.*, 2007), followed by Sumatran fleabane in 2010 (Santos *et al.*, 2014). Subsequent surveys have documented the widespread distribution of resistant populations across the country (Kaspary *et al*., 2025; Lamego and Vidal, 2008; Mendes *et al*., 2021; Moreira *et al*., 2007), highlighting the agronomic relevance of understanding the mechanisms underlying resistance in this genus.

Glyphosate resistance in *Conyza* spp. has been associated with both target-site resistance (TSR) and non-target-site resistance (NTSR) mechanisms. A TSR mechanism identified in horseweed and hairy fleabane is the overexpression of the *EPSPS* gene (Dinelli *et al.*, 2006, 2008). Resistant biotypes from the United States and Spain exhibit two- to threefold higher basal *EPSPS* expression than susceptible plants, suggesting that enhanced *EPSPS* expression contributes to resistance and may reflect a conserved genetic basis across species (Dinelli *et al.*, 2006, 2008). Gene copy number variation represents another important TSR mechanism to glyphosate. In *Amaranthus palmeri*, resistant populations had approximately 5- to over 160-fold higher *EPSPS* copy number than susceptible plants, and increased copy number is strongly associated with elevated *EPSPS*

expression and resistance levels (Gaines *et al.*, 2010), with amplified copies later shown to occur as extrachromosomal circular DNA (eccDNA) in some cases (Koo *et al.*, 2018).

Point mutations in the *EPSPS* gene were also identified as a TSR mechanism. Substitutions at position Pro106, such as Pro106Ser, have been identified in resistant horseweed populations in Canada and the United States (Beres *et al.*, 2020; Page *et al.*, 2018), and can confer high resistance levels, with survival reported at up to 40 times the recommended glyphosate dose (Beres *et al.*, 2020). However, the contribution of NTSR mechanisms to these high resistance levels remains unclear, as NTSR was not evaluated in studies focusing on the Pro106 mutation (Beres *et al.*, 2020), although NTSR mechanisms have been investigated in other resistant populations (Page *et al.*, 2018). Similar Pro106 substitutions have been reported in several weed species, including *Eleusine indica*, *Amaranthus tuberculatus*, *Digitaria insularis*, and *Lolium rigidum*, generally conferring low to moderate resistance (Baerson *et al*., 2002; Bell *et al*., 2013; de Carvalho *et al*., 2012; Nandula *et al*., 2013; Yu *et al*., 2007).

In *Conyza* species, glyphosate resistance had been associated with NTSR mechanisms (Jugulam and Shyam, 2019), particularly reduced herbicide translocation mediated by cellular transport processes that sequester glyphosate into vacuoles or redistribute it within the plant (Feng *et al.*, 2004; Shaner, 2009). In resistant horseweed, preferential movement of glyphosate from the cytosol to the vacuole has been observed (Ge *et al.*, 2010). Environmental conditions can affect resistance expression. Resistant hairy fleabane biotypes exhibited a substantial reduction in resistance under low temperature, with control increasing from 20% at 25°C to 91% at 12°C (Dalazen *et al.*, 2019), likely reflecting temperature-dependent effects on herbicide translocation and sequestration. Transcriptome-wide studies in hairy fleabane have also demonstrated that resistance may occur in the absence of *EPSPS* mutations or overexpression, instead involving the coordinated regulation of multiple genes associated with NTSR pathways (Hereward *et al.*, 2018).

Multiple resistance mechanisms may act simultaneously within a single biotype (Gaines *et al.*, 2020). In resistant horseweed, coordinated overexpression of *EPSPS* and *ABC* transporter genes has been reported shortly after glyphosate application (Tani *et al*., 2015), suggesting that enhanced target-site activity and active herbicide transport can act synergistically. ABC transporters form a large and diverse superfamily responsible for

the transport and sequestration of a wide range of compounds in plants (Kang *et al*., 2011; Theodoulou, 2000), and their involvement in herbicide resistance is increasingly recognized (Pan *et al*., 2021; Tani *et al*, 2015; Yuan *et al*., 2010).

Despite extensive reports of TSR glyphosate resistance, the recent availability of reference genomes for hairy and Sumatran fleabane (Gomes *et al.*, 2025) provides a new opportunity to investigate the genomic architecture underlying resistance in these polyploid species. Emerging evidence indicates that NTSR mechanisms may play a significant and sometimes complementary role in shaping resistance phenotypes, suggesting that target-site alterations alone may not fully explain observed variation (Hereward *et al.*, 2018). Genome-resolved approaches now enable the characterization of *EPSPS* copy structure and the assessment of subgenome-specific expression patterns, offering new insight into how polyploid genome organization influences resistance. Most studies investigating herbicide resistance mechanisms in *Conyza* have been based on one or a limited number of populations, constraining inferences about variability among populations and across broader geographic scales (Beres *et al.*, 2020; Dinelli *et al.*, 2006). Therefore, this study aimed to characterize the variability in glyphosate resistance levels and to investigate how *EPSPS* copy architecture and subgenome-specific expression interact with potential NTSR mechanisms to shape resistance in Sumatran and hairy fleabane.

5.2 Material and Methods

5.2.1 Plant material

Sumatran and hairy fleabane seeds from 70 populations were collected from herbicide escapes in soybean fields in Brazil between 2016 and 2024 (Table S1). For all experiments, recently produced seeds obtained through population maintenance under controlled cultivation conditions were used. Seeds from each population were sown in pots filled with commercial substrate to germinate. Plants with four leaves were transplanted and grown in 200 ml pots filled with a mixture of argisol and commercial substrate. The plants were grown in a greenhouse at 28 ± 5°C and irrigated daily.

5.2.2 Glyphosate resistance screening

Plants at the 6-8 leaf stage were screened with glyphosate (Roundup Original Mais, SL, 480 g L^{-1}, diammonium salt, Bayer) at 960 and 1920 g ha^{-1}. The application was performed with an automated spray chamber (Generation III, Devries manufacturing) using a spray nozzle TJ8002E (TeeJet) calibrated to spray a volume of 200 L ha^{-1}. Each treatment had five biological replicates. Control efficacy was assessed on a visual scale at 28 days after treatment (DAT), with 0% injured and 100% dead plants. Plants that survived and exhibited less than 85% control efficacy compared with untreated and known susceptible controls were identified as candidates for resistance characterization.

5.2.3 Glyphosate dose-response curves under different temperature conditions

Dose-response curves were conducted with two susceptible reference populations and 29 putatively resistant populations of Sumatran fleabane and hairy fleabane that represented all the levels of control efficacy obtained in the screening study. Experiments were performed in growth chambers under two temperature conditions, 30/25°C or 15/12°C (day/night), and plants were maintained at their respective temperatures throughout the experiment. The growth chamber conditions were 14 h/10 h light/dark, and 400 μmol m^{-2} s^{-1} of light intensity. Glyphosate (Zapp QI, SL, 620 g ha^{-1}, potassium salt, Syngenta) was applied at 0, 125, 250, 500, 1,000, 2,000, 4,000, and 8,000 g ha^{-1} as described above. Each treatment had five replicates. Control efficacy and fresh biomass were evaluated at 21 DAT. The data was submitted to the analysis of variance (ANOVA), and when the interaction between populations and doses was significant ($p \leq 0.05$), the data was fitted to the four-parameter log-logistic nonlinear regression model using the drc package in R (Ritz and Strebig, 2016), as follows: $y = c + (d - c) / [1 + (x/e)^b]$, where y is the response variable (control efficacy or biomass), x is the herbicide dose, b is the slope of the curve around the inflection point, c and d are the lower and upper asymptotes, respectively, and e is the inflection point corresponding to the effective dose required to cause 50% response (ED_{50}). The resistance factor (RF) was calculated as the ratio between the ED_{50} of resistant and susceptible populations. The temperature-response ratio (TRR) was obtained as the ratio of the ED_{50} at high to low temperature for each population.

5.2.4 *EPSPS* gene sequencing

Hairy and Sumatran fleabane are hexaploid species with their genomes recently sequenced and annotated (Gomes *et al.*, 2025). Based on searches performed in these genome assemblies (GCA_049639985.1 and GCA_047495845.1), seven putative *EPSPS* gene copies were identified in hairy fleabane: Chr03B (1386 bp), Chr03C (1560 bp), Chr04A (1665 bp), Chr04B1 (1563 bp), Chr04B2 (1172 bp), Chr04C (1560 bp), and Chr07A (1560 bp). In Sumatran fleabane, seven putative *EPSPS* copies were also identified: Chr03A (1386 bp), Chr03B (1539 bp), Chr03C (1560 bp), Chr04A (1572 bp), Chr04B1 (1662 bp), Chr04B2 (1623 bp), and Chr04C (1560 bp). These identified copies were used as the basis for the analyses conducted in this study. However, the existence of additional, structurally distinct, truncated, or highly divergent *EPSPS* copies cannot be excluded, as such variants may not have been detected in the current genome assemblies.

Copy-specific and universal (all copies) *EPSPS* primers were designed for gene amplification and sequencing (Table 1). Genomic DNA and complementary DNA (cDNA) were used in the analyses. Total DNA was extracted from the leaf tissue of all evaluated populations using the cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1987) and was diluted to 50 ng μL^{-1}. RNA extraction from not-treated leaf tissue was performed with Concert™ Reagent (Invitrogen, ThermoFisher Scientific, Waltham, USA), and cDNA was synthesized using M-MLV Reverse Transcriptase (Invitrogen).

PCR reactions were performed with a final concentration of 1 x PCR buffer, 0.2 mM dNTP, 2 mM $MgCl_2$, 0.5 U Taq DNA polymerase (Invitrogen), 0.2 µM primer, 50 ng μL^{-1} DNA or cDNA, and DNAse-free water to complete a 20 µL final volume. PCR conditions were 95 °C for 5 min; 35 cycles of 95 °C for 30 s, annealing temperature (Ta) (Table 1) for 45 s, and 72 °C for 10 min. The PCR products were verified in agarose gel (1.5%), purified with ExoSAP-IT™ (Applied Biosystems) protocol, and sequenced using Sanger sequencing. The sequences were aligned with the horseweed *EPSPS* genes as reference (FR872820, FR872821, AY545668). Chromatograms were analyzed with SnapGene® (https://www.snapgene.com/).

Table 1. Primers developed for sequencing, copy number variation, and expression studies of the *EPSPS* gene from hairy (Cb) and Sumatran fleabane (Cs).

Analysis	Primer	Sequence 5'-3'	Ta (°C)	Fragment size (bp)	*EPSPS* genes
Sequencing study	EPSPS4F	ATCGGATCCTCCTTCTTGCT	60	357	all copies, both species
	EPSPS4R	AGGCCTCCACTTCCAACTACA			
	EPSPS7F	AATGTTGAAGAAAATGGTGCAA	60	150	all copies, both species
	EPSPS9F	CCAAGTCTTTATCTAATCGGATC	60	-	all copies, both species, except CsChr04B2
	Chr03A	ATTTACCCGGGTCCAAGTCG	64	287; 693	CsChr03A
	Chr03B	GTACGGTTAATTTACCCGGC	64	296; 702	CsChr03B
	Chr03C	TTGCAACCCATTAAAGAAATA	64	321; 727	CsChr03C
	Chr04A	TTCAAGAAATTTCGGGTACGGTC	64	311; 717	CsChr04A
	Chr04B1	CCATTCAAGAAATTTCGGGC	64	314; 720	CsChr04B1
	Chr04B2	GATTCTCCTTCTTGCTGCA	64	256; 662	CsChr04B2
	Chr04C	GAGAAGCCATCAAAAGCACCT	64	354; 760	CsChr04C
	EPSPS7R	AGCAGTAACGGCAGCAGTC	-	-	both species
	EPSPS8R	CATAAGCATTTCCAGGTGAC	-	-	both species
Cloning sequencing	EPSPSclF1	GGCCTCTAGAATGGCAGCTACTCACATTAACA	64	1538	all copies, only Cs
	EPSPSclR1	AACCGTCGACTTAATGCTTGGCAAATCTTTGAAG			
	EPSPSclF2	GGCCTCTAGAGTTCATTACATGCTTGGAGCT	64	230	all copies, only Cs
	EPSPSclR2	AACCGTCGACCGAGGAACGCCATCTAGTA			
CNV and global expression	EPSPS_6F	GCTGAGGTCCTTGGACAAAT	60	164	all copies, both species
	EPSPS_3R	AGGGCAACCACAGCAA			
Copy-specific expression	EPSPS3A_F	ATTTACCCGGGTCCAAGTCG	58	287	CsChr03A
	EPSPS3B_F	GTACGGTTAATTTACCCGGC	58	296	CsChr03B
	EPSPS3C_F	TTGCAACCCATTAAAGAAATA	58	321	CsChr03C
	EPSPS4A_F	TTCAAGAAATTTCGGGTACGGTC	64	311	CsChr04A
	EPSPS4B1_F	CCATTCAAGAAATTTCGGGC	60	314	CsChr04B1
	EPSPS4B2_F	GATTCTCCTTCTTGCTGCA	60	256	CsChr04B2
	EPSPS4C_F	GAGAAGCCATCAAAAGCACCT	64	354	CsChr04C
Housekeeping	HSP70_F	CTTGCAAAGCTCCTTTCCGT	64	150	-
	HSP70_R	AAGTGTATGGAGCCCGTTGA			

Ta - annealing temperature; CNV - copy number variation.

As a complementary approach to genomic sequencing, *EPSPS* gene copies from Sumatran fleabane were cloned into an *Escherichia coli* system to enable separation and individual sequencing of distinct copies. Two primer pairs were designed to amplify all *EPSPS* copies, one pair covering the full-length coding region (~1,538 bp) and a second pair amplifying a 230 bp fragment covering known glyphosate resistance mutation sites and diagnostic single-nucleotide polymorphisms (SNPs) that distinguish individual copies. Both primer pairs included restriction sites for XbaI and SalI enzymes (Table 1).

The *EPSPS* gene from populations C3 (susceptible) and G15 (resistant) was amplified by PCR in 50 µL reactions containing 5× PCR buffer, 10 mM dNTPs, 0.5 µM of each primer, 0.5 U high-fidelity DNA polymerase (SuperFi, Thermo Fisher Scientific), 50 ng cDNA template, and nuclease-free water. PCR conditions consisted of 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 64 °C for 10 s, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. PCR products were separated on a 1.5% agarose gel, purified using the Monarch DNA Gel Extraction Kit (New England Biolabs), and digested with XbaI and SalI along with the pUC19 vector. Digested inserts and vector were ligated using T4 DNA ligase (New England Biolabs) at a 2:1 insert-to-vector ratio.

Ligation products were transformed into chemically competent *E. coli* TOP10 cells by heat shock at 42 °C for 30 s, followed by recovery in SOC medium for 30 min at 37 °C. Cells were plated on LB agar containing ampicillin and incubated overnight at 37 °C. Individual colonies were screened by colony PCR using *EPSPS* primers, and DNA from positive colonies (n = 14 for the full-length fragment and n = 6 for the 230 bp fragment) was submitted for Oxford Nanopore long-read sequencing. Sequences were aligned to identify copy-specific polymorphisms.

5.2.5 *EPSPS* gene copy number variation

EPSPS copy number variation (CNV) was analyzed by quantitative PCR (qPCR) using genomic DNA as template and a primer pair designed to amplify the seven putative *EPSPS* copies identified in Sumatran fleabane and hairy fleabane (Table 1). The PCR reactions were a final concentration of 1 x PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTP, 0.1 µM ROX Reference Dye, 0.1 x SYBR™ Green, 0.5 U Platinum™ Taq DNA Polymerase (Invitrogen), 0.2 µM primer, 50 ng μL^{-1} DNA, and DNAse-free water to complete 10 µL final volume. PCR conditions were 50 °C for 1 min, 95 °C for 10 min; 40 cycles of 95 °C

for 15 s, 60 °C for 30 s, and 72 °C for 30 s in a QuantStudio 3 Real-Time PCR system. For each population, three biological replicates and three technical replicates were used. *EPSPS* gene CNV was calculated relative to the susceptible populations using the $2^{-\Delta\Delta Ct}$ method (Dussault and Pouliot, 2006). The housekeeping *HSP70* (*heat shock protein-70*) gene was used as a calibrator (Table 1) (Moretti *et al*., 2017). The data were compared using ANOVA, and when significant ($p \leq 0.05$), the averages were compared using Tukey's test at 5%.

5.2.6 *EPSPS* global and copy-specific expression

EPSPS gene expression was analyzed both for all copies (global) in hairy and Sumatran fleabane resistant populations and at the individual copy level for six Sumatran fleabane selected populations (C10, C40, G14, G19, G5, G6) using qPCR. Individual copy-level expression was not assessed in hairy fleabane due to the unavailability of a reference genome at the time of the study. Global expression was assessed using a primer pair designed to amplify all identified *EPSPS* copies in both species (Table 1), whereas expression of each *EPSPS* copy was evaluated using primers targeting unique SNPs specific to each copy in Sumatran fleabane (Table 1). cDNA was synthesized from RNA extracted from untreated plant tissue. qPCR reactions were performed and analyzed under the same conditions described for the CNV analysis.

Copy-specific *EPSPS* expression was also evaluated in two untreated resistant populations of Sumatran fleabane (G5 and G6) using RNA-seq data. Sequencing reads were aligned to the reference genome of Sumatran fleabane (GCA_047495845.1) using STAR. Gene-level read counts were obtained with featureCounts using a SAF annotation file. Paired-end reads were counted without multi-mapping or multi-overlapping assignments, so only uniquely assigned read pairs were included in the analysis. Count data were normalized using DESeq2 and $\log_2$-transformed before analysis. A heatmap was generated in R to visualize expression patterns among *EPSPS* copies. Three biological replicates were used per population.

5.2.7 Investigation of an unusual necrotic phenotype and expression of candidate genes

During the initial screening, atypical necrotic symptoms were observed in populations C2, C4, and G5 within 48 h after application of glyphosate diammonium salt when plants were maintained in a greenhouse, particularly under conditions of reduced light and temperature following application. A similar response was observed in population G5 during dose-response experiments with glyphosate potassium salt when plants were maintained at low temperature. To further investigate this response, complementary experiments were conducted using glyphosate potassium salt in populations C2, C4, and G5 at 1,000 g ha^{-1} under four temperature regimes: (i) greenhouse conditions before and after application (high/high), (ii) greenhouse before and 15 °C after application (high/low), (iii) 15 °C before and after application (low/low), and (iv) 15 °C before and greenhouse conditions after application (low/high). Low-temperature treatments were conducted in growth chambers maintained at 15 °C, 14 h/10 h light/dark, and 400 μmol m^{-2} s^{-1} of light intensity. Greenhouse conditions were 28°C ± 5, 14 h/10 h light/dark, and 500-600 μmol m^{-2} s^{-1} of light intensity.

Dose-response curves were conducted for population G5 using glyphosate potassium salt (Zapp QI) at doses ranging from 0 to 8,000 g ha^{-1} under the same four temperature regimes. In addition, to compare glyphosate salt formulations, independent dose-response experiments were conducted in population G5 using glyphosate potassium salt (Zapp QI; 0-8,000 g ha^{-1}) and glyphosate diammonium salt (Roundup Original Mais; 0-5,760 g ha^{-1}). In these experiments, plants were maintained under controlled temperatures of 12 °C or 25 °C in growth chambers before and after application. Herbicide application procedures and plant evaluations were performed as described above for the dose-response experiments.

To complement the analyses, the expression of putative genes potentially involved in these responses was evaluated. Leaf tissue was collected 24 h after treatment with 2,000 g ha^{-1} of glyphosate potassium salt at 15 °C and 30 °C from the resistant population G5 and a susceptible population (G8), as well as from untreated control plants. Candidate genes included the putative gene associated with the auxinic rapid necrosis response, *PLP2* (F: CATCAGCAGCCCCAACTTAT; R: AAACCGGCCATACTCTGTTG; Ta: 60 °C), and three ABC transporters identified in *Conyza* and named based on homology with transporters from related species potentially involved in herbicide transport and

resistance responses: *ABCC3* (F: TTCATCTATCCCCAGCGAAC; R: ACAAGGCGGAAGAGTGTTTG; Ta: 64 °C) and *ABCB4* (F: CAAGTGGCTCAGAATGCAAA; R: GCACTCCTCTTTCCCCTACC; Ta: 60 °C), previously associated with response to 2,4-D in *Conyza* (Angonese, 2022), and *ABCC8* (F: TGTTGTTCTTCAGGCAGCAG; R: GGAGGCTTTTAATCCCAAAA; Ta: 60 °C), identified as a homolog of a transporter reported in *Echinochloa colona* (Pan *et al*., 2021). RNA extraction, cDNA synthesis, qPCR reactions, and gene expression analysis were performed as described previously.

5.3 Results and Discussion

5.3.1 Glyphosate resistance frequency and distribution

Glyphosate (diammonium salt) applied at 960 and 1920 g ha^{-1} was evaluated in 12 populations of hairy fleabane and 58 populations of Sumatran fleabane. At the highest rate tested, 67% and 91% of the hairy and Sumatran fleabane populations, respectively, showed less than 85% control (Figure 1A, Table S1). Of the 70 populations evaluated, only seven were classified as susceptible to glyphosate. Glyphosate resistance was widely distributed across the evaluated regions, except in Minas Gerais, where all three populations assessed were susceptible to glyphosate (Figure 1B).

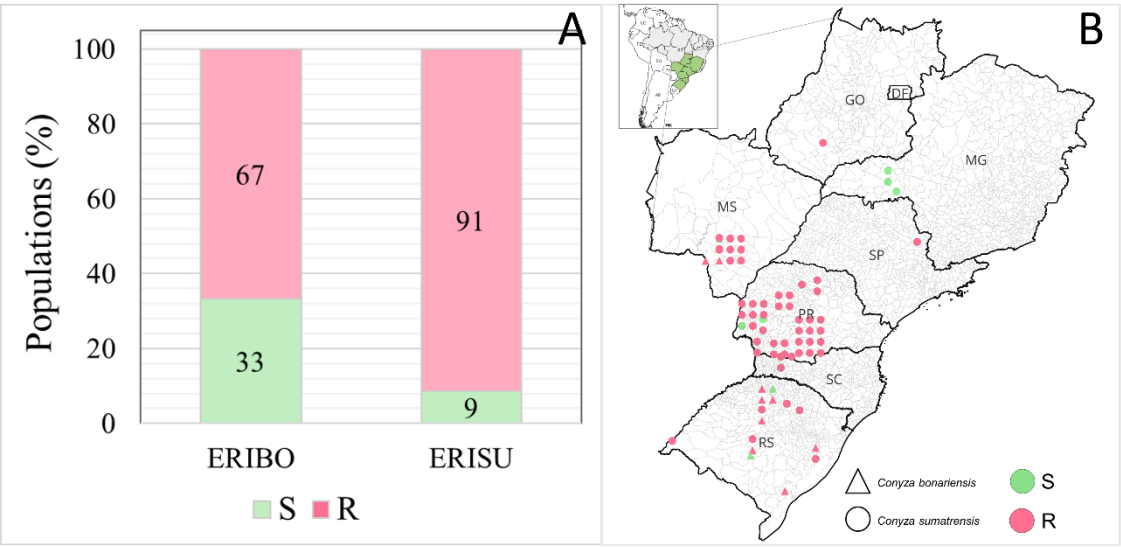


Figure 1. Resistance to glyphosate in 70 *Conyza* spp. populations (A) and their distribution across Brazil (B). S - susceptible (control > 85%); R - resistant (control < 85%); ERIBO - *Conyza bonariensis*; ERISU - *Conyza sumatrensis*.

The high frequency of glyphosate resistance and its widespread distribution across the main grain-producing regions of Brazil observed in this study was expected based on field reports. A larger-scale monitoring study conducted across nine Brazilian states between 2014 and 2018 evaluated 1,184 *Conyza* populations and reported that approximately 71% of the populations were resistant to glyphosate, confirming that glyphosate resistance is already broadly established in Brazilian agricultural systems (Mendes *et al.*, 2021). The high prevalence of glyphosate resistance in *Conyza* is largely associated with the strong selection pressure imposed by the intensive use of glyphosate in cropping systems based on glyphosate-tolerant crops. In many regions of Brazil, glyphosate is repeatedly applied during fallow periods, for pre-sowing burndown and for post-emergence weed control, often as the primary weed control strategy. This management practice promotes the selection and rapid increase of resistant biotypes, contributing to the widespread occurrence of glyphosate-resistant populations across large agricultural areas (Lucio *et al.*, 2019; Merotto *et al.*, 2022).

5.3.2 Low temperature reduces glyphosate resistance levels

Four hairy fleabane populations and 25 Sumatran fleabane populations previously identified as putatively glyphosate-resistant were selected to characterize resistance levels under high (30 °C) and low (15 °C) temperature conditions. For hairy fleabane, resistance factors (RF) were low, ranging from 1.4 to 2.1 at high temperature, and generally decreased under lower temperature conditions (Figure 2A, Table 2). The ED_{50} values ranged from 1,125.5 to 2,807.1 g ha^{-1} at 30 °C, compared with 829 g ha^{-1} for the susceptible population, and decreased to a maximum of 236.1 g ha^{-1} at 15 °C. The TRR ranged from 5.2 to 24.7, indicating that glyphosate sensitivity increased substantially under lower temperature conditions. In Sumatran fleabane, a wide range of resistance levels was observed among populations. Resistance factors ranged from 1.2 to 32.4 at 30 °C. A general reduction in resistance was also observed under lower temperature conditions. For example, in population G13, the ED_{50} decreased from 26,948 g ha^{-1} at 30 °C to 220.1 g ha^{-1} at 15 °C (Figure 2B, Table 2). This evident temperature effect was reflected in TRR values ranging from 2.0 to 122.4 among populations from both species.

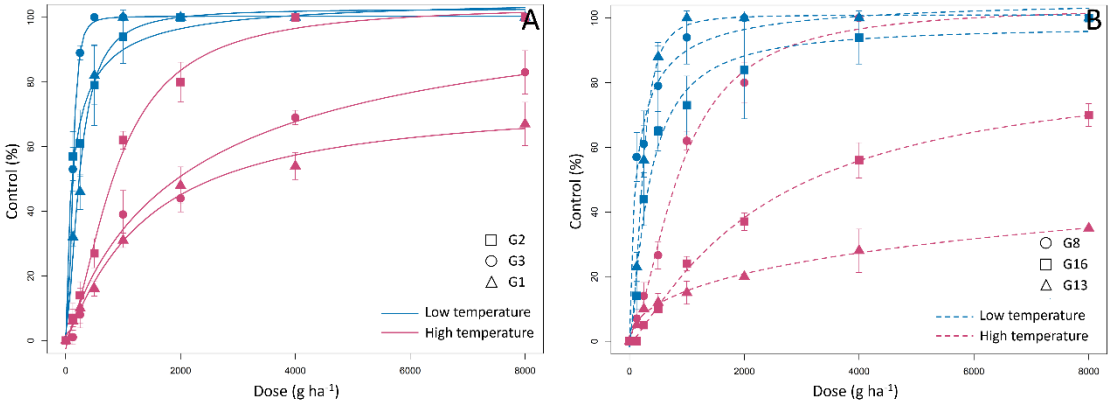


Figure 2. Dose-response curves for hairy fleabane (A) and Sumatran fleabane (B) in one susceptible population and two representative resistant populations per species (hairy fleabane: G2 (S), G3 (R), G1(R); Sumatran fleabane: G8 (S), G16 (R), G13(R)), evaluated at high (30/25 °C) and low (15/12 °C) day/night temperatures.

Table 2. ED_{50} values, resistance factor (RF), and temperature-response ratio (TRR) for 29 putatively glyphosate-resistant populations and two susceptible populations of Sumatran and hairy fleabane, evaluated at high (30/25°C) and low (15/12°C) day/night temperatures.

Population	High temperature		Low temperature		TRR
	ED_{50}	RF	ED_{50}	RF	
Sumatran fleabane					
G8 (S)	831.3	-	118.8	-	7.0
G5	1689.7	2.0	133.3	1.1	12.7
G6	1341.7	1.6	135.8	1.1	9.9
G7	5432.1	6.5	276.5	2.3	19.6
G10	10483.0	12.6	564.8	4.8	18.6
G11	5931.7	7.1	278.3	2.3	21.3
G12	4531.0	5.5	221.0	1.9	20.5
G13	26948.0	32.4	220.1	1.9	122.4
G14	8223.2	9.9	430.8	3.6	19.1
G15	3204.6	3.8	143.6	1.2	22.3
G16	3191.4	3.8	348.1	2.9	9.2
G17	9230.0	11.1	119.8	1.0	77.1
G18	3322.4	4.0	187.2	1.6	17.8
G20	1038.1	1.2	55.7	0.5	18.6
G21	2053.3	2.5	93.4	0.8	22.0
G22	3756.5	4.5	201.7	1.7	18.6
G23	4359.2	5.2	354.3	3.0	12.3
G24	2197.2	2.6	116.7	1.0	18.8
G26	605.9	1.3	67.0	0.8	9.0
C35	3035.0	6.7	337.5	4.3	9.0
C37	1584.8	3.5	197.0	2.5	8.0
C38	9065.9	20.0	1924.1	24.3	4.7
C40	1169.2	2.6	44.2	0.6	26.4

C43	2127.4	4.7	125.6	1.6	16.9
C55	776.4	2.3	382.0	1.5	2.0
C60	3943.8	11.7	304.2	1.2	13.0
Hairy fleabane					
G2 (S)	829.1	-	127.4	-	6.5
G3	1780.6	2.1	120.1	1.0	14.8
G1	2807.1	3.4	236.1	2.0	11.9
G4	1125.5	1.4	45.5	0.4	24.7
G25	189.6	0.2	36.2	0.3	5.2

For most hairy and Sumatran fleabane populations evaluated in this study, resistance factors decreased when glyphosate was applied under lower temperature conditions. A similar response was previously reported in hairy fleabane, where resistance was evaluated at 12 and 25 °C. In that study, the resistance factor was reduced more than 9-fold, and resistance was completely reversed in plants maintained at 12 °C (Dalazen *et al.*, 2019). These results indicate that temperature can significantly impact the level of glyphosate resistance in *Conyza* species. From a practical perspective, this response suggests that herbicide applications performed during colder periods of the day may improve the control of glyphosate-resistant *Conyza* populations under field conditions. Although the mechanisms involved in this response were not investigated, they may be associated with temperature-dependent physiological processes such as herbicide translocation or sequestration in the vacuole.

Previous studies on hairy and Sumatran fleabane have shown that glyphosate resistance can be associated with reduced herbicide translocation; however, these studies did not evaluate the influence of temperature on this process (Moretti and Hanson, 2017). In Sumatran fleabane, lower temperatures were shown to increase glyphosate absorption and translocation, suppressing the resistance mechanism and enhancing herbicide efficacy (Palma-Bautista *et al.*, 2019). In horseweed, higher glyphosate accumulation in the vacuole of resistant plants has also been reported, suggesting that vacuolar sequestration may play an important role in resistance (Ge *et al.*, 2010). However, the genetic mechanisms involved in this process remain unclear.

5.3.3 Multiple *EPSPS* copies and SNP diversity in Sumatran and hairy fleabane

Seven *EPSPS* gene copies were identified in the Sumatran fleabane reference genome (GCA_047495845.1). Within the region containing known glyphosate

resistance-associated mutations, the substitutions Ala100Ser (GCA→TCA) and Pro106Thr (CCA→ACA) were identified in the CsChr04B2 copy, whereas all other copies maintained the wild-type sequence at these positions (Figure 3A, Supplemental Material). For hairy fleabane (GCA_049639985.1), the Pro106Thr (CCA→ACA) was also identified in the CbChr04B2 copy (Figure 3A, Supplemental Material).

Several sequencing approaches were used to evaluate *EPSPS* sequences in the evaluated *Conyza* populations. Using the EPSPS4FR primer pair, which covers exonic regions, cDNA from Sumatran fleabane populations G8 (susceptible) and G6, G19, and G22 (resistant) was sequenced. No amino acid substitutions were detected at positions Ala100, Gly101, Thr102, Ala103, Met104, Arg105, or Pro106 (Figure 3B).

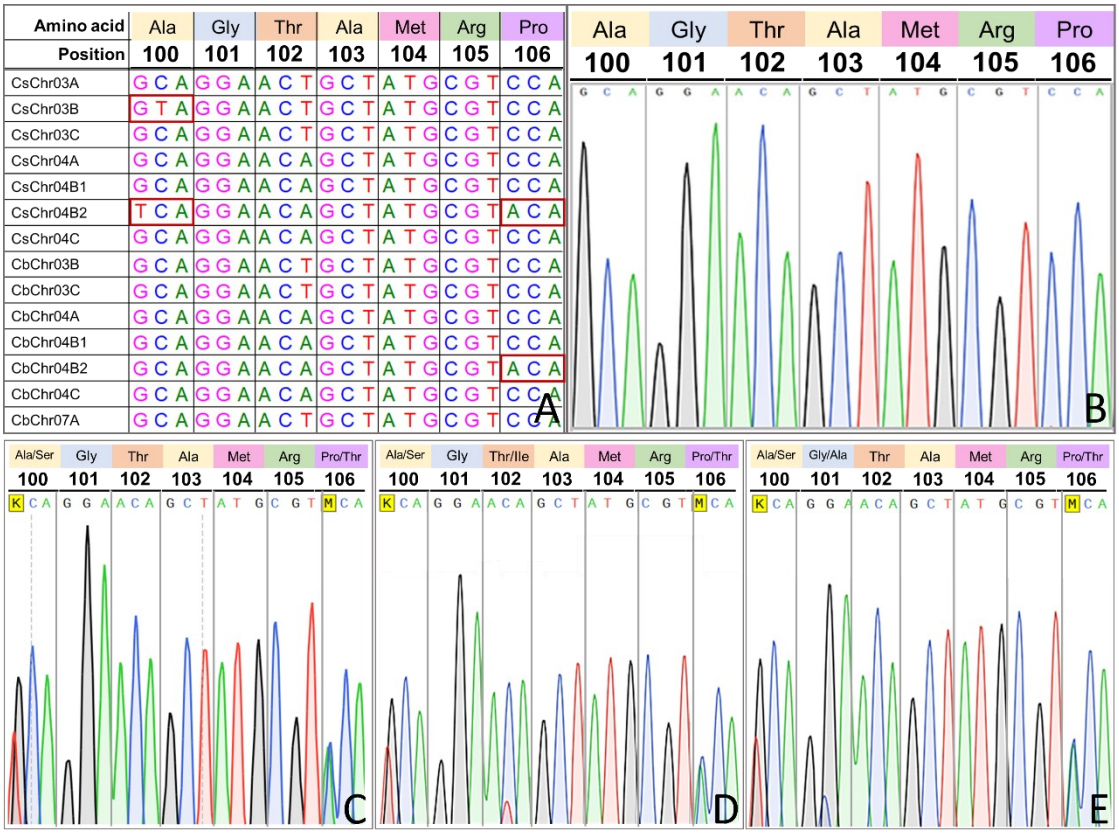


Figure 3. Region encompassing codons 100-106 of all identified copies of the *EPSPS* gene in Sumatran fleabane (Cs) and hairy fleabane (Cb) (A). Representative sequencing electropherograms from populations without SNPs (G8) (B), and with the mutations Ala100Ser and Pro106Thr (G8) (C), Thr102Ile (T3) (D), and Gly101Ala (T4) (E). Double peaks indicate the presence of two bases at the same position.

The EPSPS7FR primer pair, designed within an exonic region to capture all gene copies, was used to amplify genomic DNA from hairy fleabane populations G2

(susceptible) and G1, T1, T2, T3, and T4 (resistant), and from Sumatran fleabane populations G8 (susceptible) and G14, G16, G17, and G19 (resistant) (Figure 3C). Double peaks were observed at codons 100 and 106, corresponding to the substitutions Ala100Ser and Pro106Thr, consistent with the sequence of the Chr04B2 copy. This response was detected in both resistant and susceptible populations for both species, indicating that these mutations could be copy-specific and not uniquely associated with glyphosate resistance. Additionally, population T3 exhibited a Thr102Ile substitution (Figure 3D), and population T4 showed a Gly101Ala substitution (Figure 3E).

The EPSPS7F8R primer pair was also used with cDNA from the same populations, and the Ala100Ser and Pro106Thr substitutions were again detected. The EPSPS9F7R primer pair was designed to amplify all *EPSPS* copies except Chr04B2, based on a C/T SNP at the 3′ end of the primer binding site. Using this primer pair, population G19 (resistant) showed no substitutions at positions 100-106. However, populations G10 (resistant) and C3 (susceptible) exhibited double peaks corresponding to Ala100Ser and Pro106Thr, indicating incomplete primer specificity and possible co-amplification of the Chr04B2 copy.

As universal *EPSPS* primers did not allow clear resolution of individual copy sequences, copy-specific primers were designed based on unique SNP regions to distinguish each Sumatran fleabane *EPSPS* copy. Population G19 (resistant) was sequenced using cDNA. Amplification of most copies produced clean sequences without double peaks, confirming primer specificity. However, the fragment amplified with the Chr04B1-specific primer, although showing no double peaks, contained the substitutions Ala100Ser (TCA) and Pro106Thr (ACA), corresponding to the sequence previously identified for the Chr04B2 copy. To further investigate this result, the Chr04B-targeting primer was used with cDNA from populations C2 (susceptible), G19, G5, and C40 (resistant). Population G19 showed minor secondary peaks corresponding to the mutant codons at positions 100 and 106, whereas populations C2 and C40 showed clean sequences containing the mutant codons. In contrast, population G5 showed a clean sequence corresponding to the wild-type Chr04B copy. These results indicate inconsistent discrimination between Chr04B-related copies, suggesting occasional co-amplification of closely related paralogs.

Overall, primers designed for the other *EPSPS* copies showed high specificity. Importantly, the presence of the Ala100Ser and Pro106Thr substitutions in both susceptible and resistant populations supports that these mutations are copy-specific rather than resistance-specific, suggesting that mutations in the Chr04B-related copies alone may not be sufficient to confer glyphosate resistance. The inconsistent amplification and sequence overlap between Chr04B1 and Chr04B2 suggest high sequence similarity between these loci. This may reflect closely related paralogs, allelic variants, or potential assembly redundancy in the reference genome.

Cloning of *EPSPS* copies from Sumatran fleabane in *E. coli* was performed in an attempt to better separate individual paralogs, particularly Chr04B1 and Chr04B2. All 14 sequenced colonies (seven each population), obtained using the full-coverage primer and cDNA as template, showed single chromatogram peaks, confirming the isolation of individual amplicons. However, all sequences corresponded exclusively to copy Chr03C. Although the number of colonies analyzed was limited, the consistent recovery of Chr03C may indicate that this copy is more highly expressed, which would have favored its amplification when using cDNA. Alternatively, the primer used in the initial cDNA amplification may have preferentially amplified Chr03C due to higher binding affinity. Direct sequencing of the initial PCR product revealed diagnostic SNPs corresponding to copies Chr03A, Chr03B, and Chr03C, confirming the presence of all three chromosome 3 copies, as well as SNPs indicating the presence of at least three *EPSPS* copies located on chromosome 4 in the cDNA pool. Cloning using a shorter amplicon (230 bp) resulted in the same result, with all sequenced colonies corresponding exclusively to Chr03C. These findings suggest that, despite the transcription of multiple *EPSPS* copies, amplification and/or cloning bias favored the recovery of Chr03C, preventing the isolation of other copies such as Chr04B1 and Chr04B2.

Mutations in the *EPSPS* gene are widely reported as a TSR mechanism to glyphosate in several weed species. Substitutions at codon Pro106 have been identified in resistant populations of hairy fleabane (Kaspary *et al.*, 2025), Sumatran fleabane (Palma-Bautista *et al.*, 2020), and horseweed (Beres *et al.*, 2020; Page *et al.*, 2018), and can confer variable levels of resistance. Similar target-site mutations have also been described in other species, including the double mutation Thr102Ile + Pro106Ser (TIPS) in *E. indica* (Yu *et al.*, 2015) and the triple mutation Thr102Ile + Ala103Val + Pro106Ser (TAP-IVS) in *Amaranthus hybridus* (Perotti *et al.*, 2019; Sulzbach *et al.*, 2024),

conferring high levels of glyphosate resistance. However, resistance levels associated with single Pro106 substitutions are often moderate and may occur together with NTSR mechanisms (Page *et al.*, 2018).

In the present study, substitutions in the region encompassing codons 100-106 were detected in both susceptible and resistant populations for both Sumatran and hairy fleabane, indicating that these variants are not uniquely associated with glyphosate resistance in the evaluated populations. Genome analysis revealed seven *EPSPS* copies in both species, with the substitutions Ala100Ser (only in Sumatran fleabane) and Pro106Thr (in both species) restricted to a specific copy (Chr04B2). The occurrence of these substitutions in both resistant and susceptible plants suggests that they represent copy-specific polymorphisms rather than resistance-conferring mutations. Similar observations were reported in hairy fleabane, where the Pro106Thr mutation was detected in both susceptible and resistant lines, and different *EPSPS* copies showed variable expression levels (Hereward *et al.*, 2018). The polyploid characteristic of *Conyza* further complicates the interpretation of target-site mutations, as transcriptomic analyses have indicated the presence of multiple *EPSPS* copies consistent with the allohexaploid genome structure of these species. In these genomes, mutations occurring in a single copy may have limited phenotypic impact due to the presence of several functional wild-type copies and potential differences in expression among paralogs (Hereward *et al.*, 2018).

The interpretation of *EPSPS* mutations in *Conyza* species may also be influenced by the coexistence of multiple resistance mechanisms. In several weed species, high levels of glyphosate resistance have been associated with the combination of target-site mutations and NTSR processes (Sammons and Gaines, 2014). Therefore, even when *EPSPS* mutations are present, their contribution to the resistance phenotype may depend on the relative expression of the mutated copy and on the presence of additional NTSR mechanisms (Hereward *et al.*, 2018). Additionally, most previous studies investigating *EPSPS* mutations in *Conyza* were conducted before reference genomes for these species became available. As a result, primers were typically designed to amplify conserved regions of the gene, which may have simultaneously amplified multiple paralogous copies or excluded some. The availability of reference genomes now allows a more precise evaluation of *EPSPS* copy diversity and suggests that some mutations previously interpreted as resistance-associated may instead represent variation among paralogous copies.

5.3.4 Variation in *EPSPS* copy number across populations

Eleven hairy fleabane populations and 55 Sumatran fleabane populations were evaluated for *EPSPS* gene copy number relative to the susceptible reference population of each species. Approximately 9% of hairy fleabane and 24% of Sumatran fleabane populations exhibited higher *EPSPS* copy numbers than their respective susceptible populations, ranging from 1.5- to 4-fold increases (Figure 4).

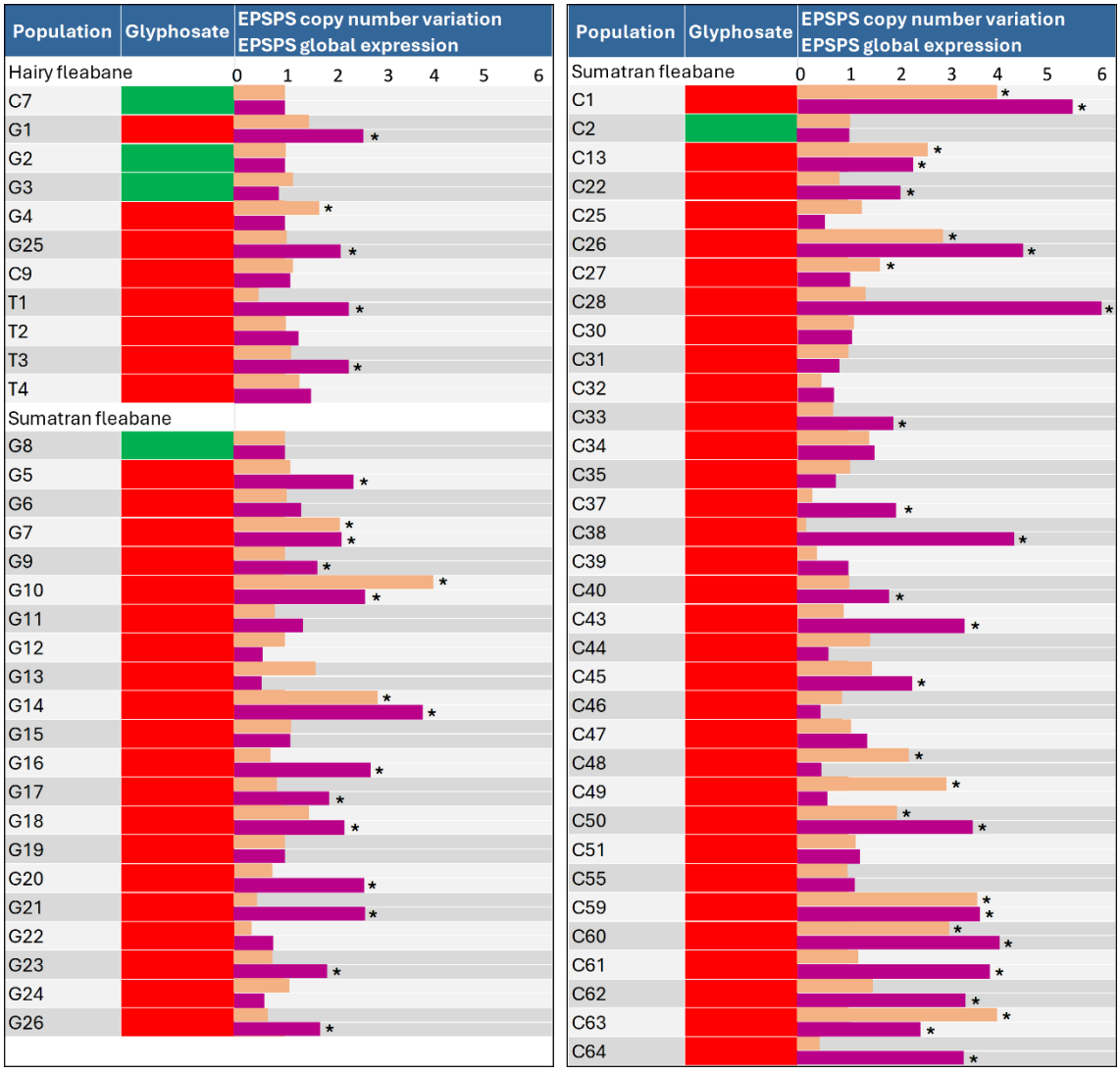


Figure 4. Resistance (red) and susceptible (green) patterns to glyphosate, *EPSPS* relative copy number (orange bars), and relative global expression (purple bars) in resistant hairy and Sumatran fleabane populations. *indicate significant differences compared to susceptible populations (Tukey's test, $p < 0.05$).

Gene amplification as a mechanism of herbicide resistance was reported in different weed species, such as *A. palmeri*, with increases ranging from 5- to 160-fold (Gaines *et al.*, 2010), *A. tuberculatus*, with up to 12-fold amplification (Chatham *et al.*, 2015), and *E. indica*, with up to 28-fold amplification (Chen *et al.*, 2015). In *A. palmeri*, amplified *EPSPS* copies were later shown to occur as autonomously replicating extrachromosomal circular DNA molecules (eccDNAs), which can be transmitted during mitosis and meiosis and may facilitate rapid adaptive evolution to glyphosate selection (Koo *et al.*, 2018). This mechanism is associated with glyphosate resistance because additional copies of the target-site gene produce higher transcript levels and, consequently, greater amounts of the target-site protein (Gaines *et al.*, 2010). The increase in the target protein pool requires higher herbicide concentrations to effectively inhibit the enzyme and disrupt the associated biochemical pathway. In the present study, resistant populations exhibited only a fourfold increase in gene copy number compared with susceptible populations. Although it remains unclear whether this level of amplification alone is sufficient to confer resistance, it may contribute to the resistant phenotype associated with *EPSPS* gene mutations and/or NTSR mechanisms.

5.3.5 Global and copy-specific *EPSPS* expression

The same populations evaluated for *EPSPS* copy number were also evaluated for relative global *EPSPS* expression levels. Approximately 36% of hairy fleabane and 55% of Sumatran fleabane populations exhibited higher expression levels than their respective susceptible populations, ranging from 1.5- to 6-fold (Figure 4).

Copy-specific *EPSPS* expression was further evaluated in one susceptible and three resistant Sumatran fleabane populations using copy-specific primers and compared with total *EPSPS* expression levels. Expression patterns varied among populations for each copy (Figure 5). Most copies showed population-dependent variation in their relative contribution to total *EPSPS* expression, indicating differential regulation among *EPSPS* copies. In contrast, the Chr04B2 copy consistently exhibited minimal expression across all populations. Aggregated expression by chromosome revealed that copies on chromosome 4 generally contributed more to total *EPSPS* expression.

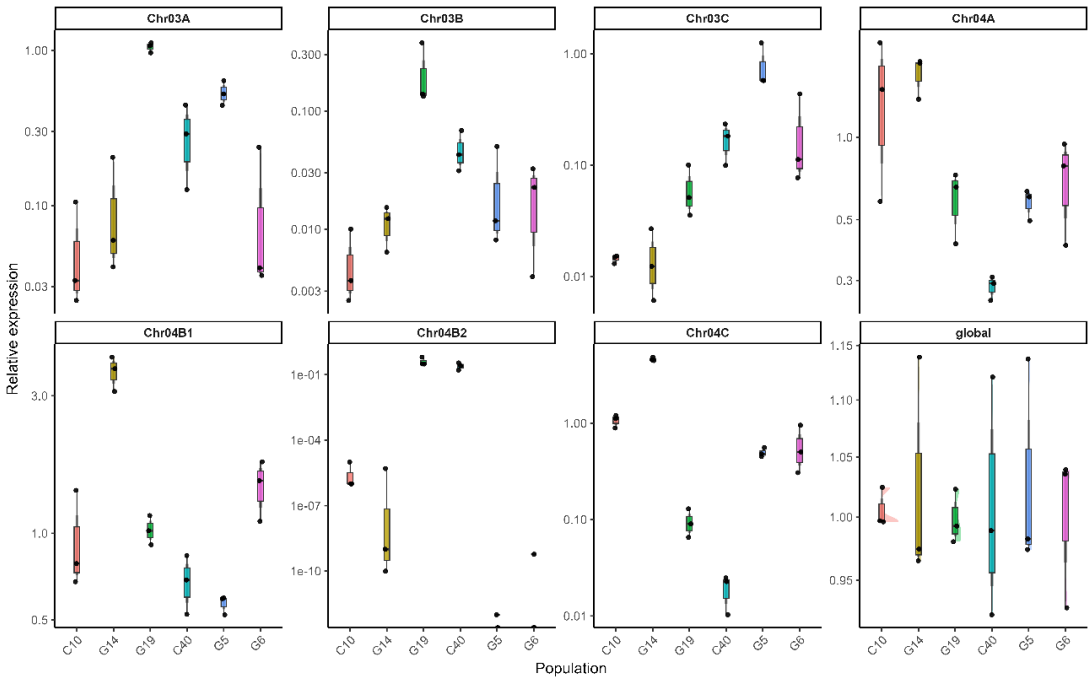


Figure 5. Relative *EPSPS* expression at the copy-specific level compared with global expression in six Sumatran fleabane populations (C10-S, G14-R, G19-R, C40-R, G5-R, G6-R) measured by qPCR analysis. Panels are presented using different y-axis scales.

RNA-seq data from two resistant populations were also used to evaluate copy-specific *EPSPS* expression relative to RNA obtained from untreated leaf tissue. This analysis revealed variation in expression among *EPSPS* copies, with Chr03C, Chr04A, Chr04B1, and Chr04C generally showing higher expression levels, although some variation among populations was observed (Figure 6). In contrast, the Chr04B2 and Chr03B copies consistently exhibited low expression. These results were consistent with the expression patterns observed by qPCR.

Unequal expression among duplicated genes is commonly observed in polyploid species and may reflect subgenome dominance or differential gene regulation. For example, in *Triticum aestivum*, an *ALS* mutation in subgenome D conferred higher herbicide resistance than the same mutation in subgenomes A or B (Chen *et al*., 2021), while an *ACCase* mutation in subgenome A resulted in greater resistance than mutations in subgenomes D or B (Ostlie *et al.*, 2014). A similar pattern has been reported in *Echinochloa crus-galli*, where *ALS* mutations were most frequently detected in subgenome A, whereas the *ALS* copy from subgenome C showed the highest expression levels. In that species, *ALS* genes from subgenomes A and C contribute approximately 13% and 50%, respectively, to the total ALS transcript pool (Cutti *et al.*, 2026).

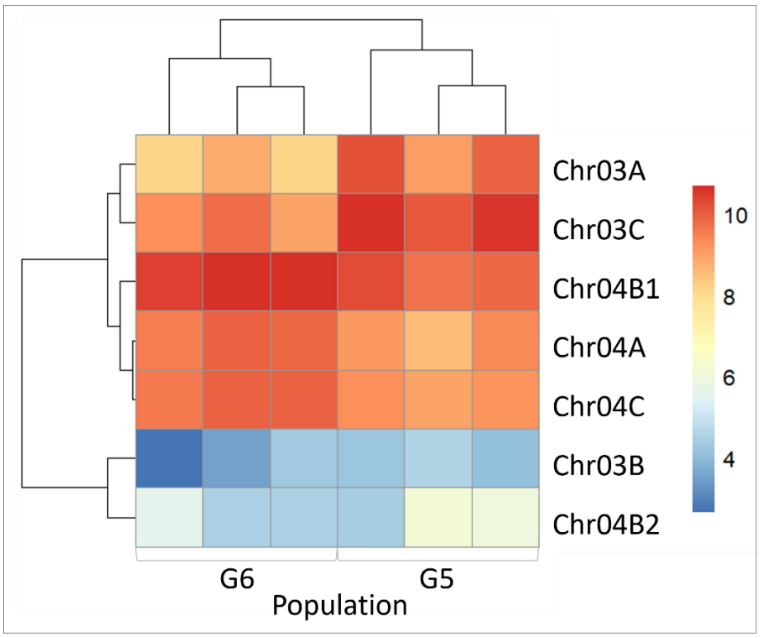


Figure 6. Relative *EPSPS* expression at the copy-specific level in two Sumatran fleabane populations (G5-R, G6-R), with three replicates, measured by RNASeq analysis.

A similar pattern may occur in *Conyza*, where the Chr04B2 copy, which contains glyphosate resistance-associated substitutions, showed minimal expression and was detected in both susceptible and resistant populations. The high sequence similarity and difficulty in distinguishing Chr04B-related copies raise the possibility that these loci represent allelic variants or assembly redundancy rather than independent functional gene copies. Together, these results suggest that target-site mutations in this copy are unlikely to play a major role in glyphosate resistance.

5.3.6 Unusual glyphosate-induced necrotic phenotype and expression of ABC transporter genes

During the initial screening, atypical necrotic symptoms were observed in populations C2, C4, and G5 within 48 h after application of glyphosate diammonium salt in conditions of low temperature and luminosity (Figure 7B), compared to plants with regular symptoms (Figure 7A). A similar response was observed in population G5 during the dose-response study with glyphosate potassium salt when plants were kept at low temperature (Figure 7C, D). In several cases, the necrotic lesions that developed within 48 h after treatment were followed by plant death.

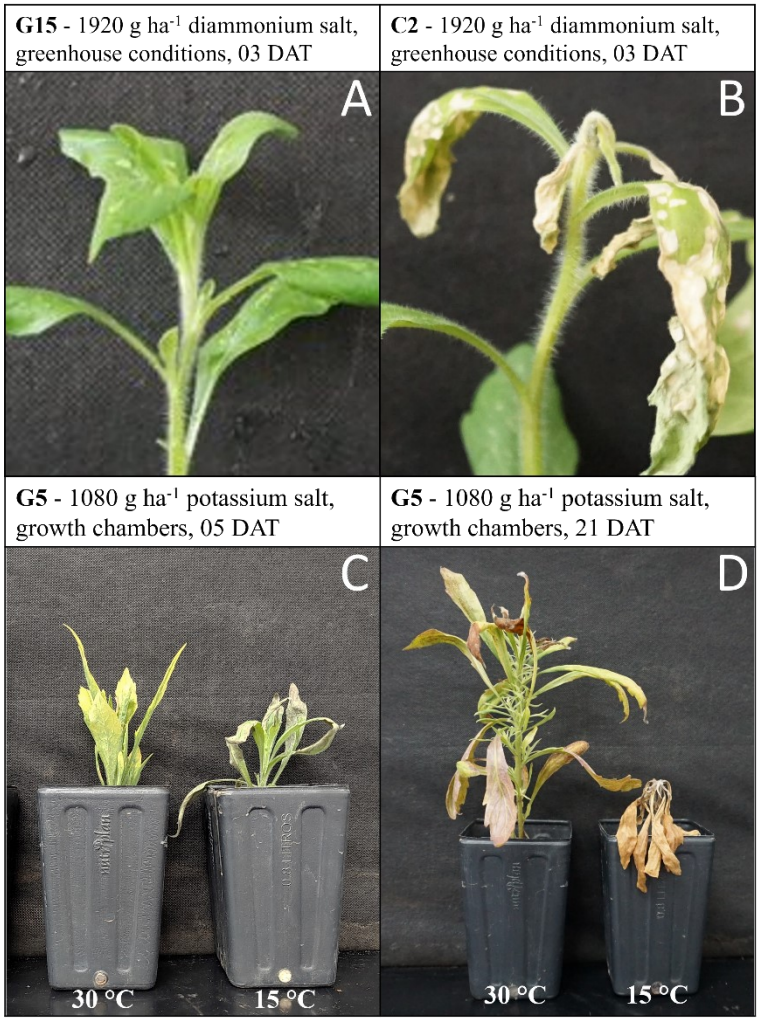


Figure 7. Sumatran fleabane plants without symptoms (A) and showing atypical necrosis symptoms (B) at 3 days after application (DAT) of glyphosate diammonium salt at 1,920 g ha^{-1}. Sumatran fleabane plants treated with glyphosate potassium salt at 1,080 g ha^{-1} and maintained at 30 °C and 15 °C, evaluated at 5 DAT (C) and 21 DAT (D).

Across the three populations evaluated under the four temperature regimes, increased injury was observed when plants were maintained at low temperature after diammonium salt glyphosate application, regardless of the temperature conditions before application (Figure 8A). Necrotic symptoms were consistently observed in plants exposed to low temperature following treatment. Population G5 was further evaluated under the same temperature regimes in dose-response experiments with glyphosate potassium salt. Necrotic spots were again observed, beginning 24 to 48 h after treatment in plants maintained at low temperature after application, regardless of the temperature before application. Under these conditions, the ED_{50} values were 176, 181, 6,500, and 10,500 g ha^{-1} for plants maintained at low/low, high/low, low/high, and high/high temperature regimes before/after application, respectively (Figure 8B). These results confirm a strong

reduction in resistance when plants were exposed to low temperatures after glyphosate application.

Dose-response experiments conducted with two glyphosate salt formulations showed a similar response pattern. In population G5, the ED50 decreased from 1,064.01 to 120.98 g ha^{-1} for the diammonium salt and from 1,876.36 to 134.60 g ha^{-1} for the potassium salt under high and low temperature conditions, respectively (Figure 8C, D). Necrotic symptoms were observed within 48 h in plants maintained at low temperature for both salt formulations.

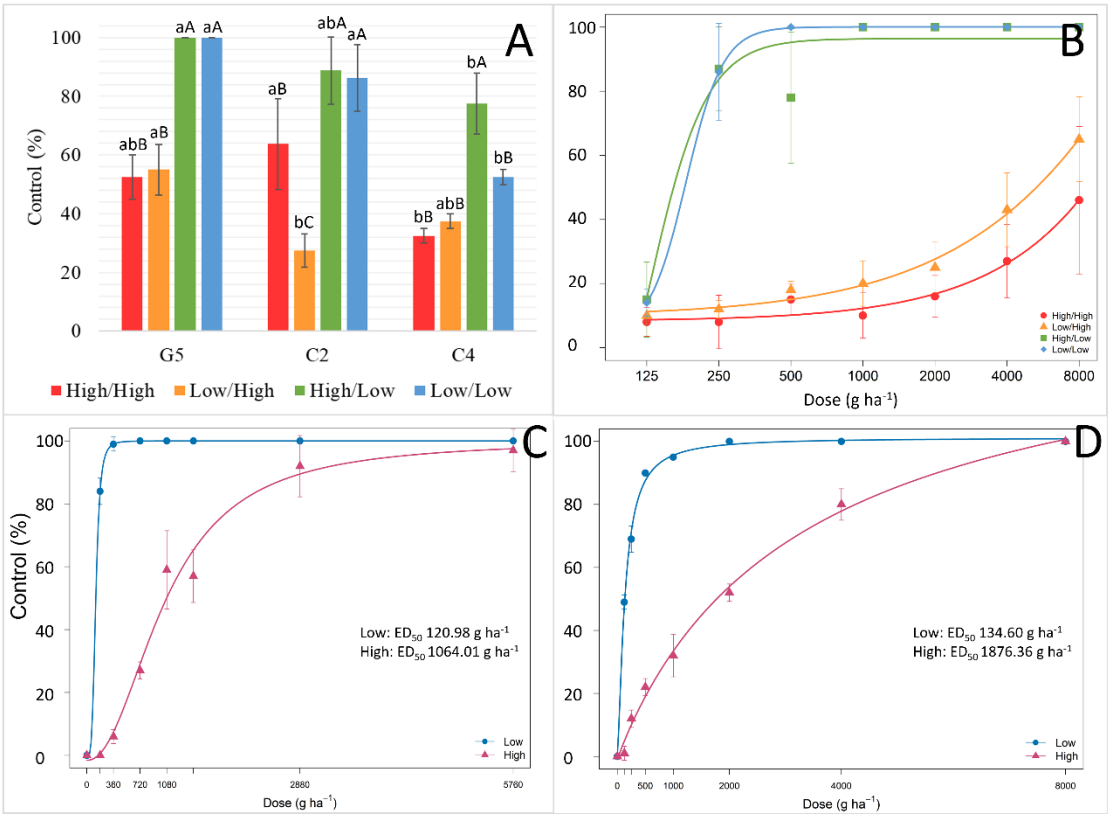


Figure 8. Injury in three Sumatran fleabane populations (G5, C2 and C4) exhibiting unusual necrotic symptoms under four temperature regimes before/after glyphosate potassium salt application (high/high, low/high, high/low, and low/low) (A); dose-response curves of population G5 under the same temperature regimes with glyphosate potassium salt (B); and dose-response curves of population G5 conducted at 12 °C and 25 °C using glyphosate diammonium salt (C) and potassium salt (D). Lowercase letters indicate comparisons among populations within the same temperature regime, whereas uppercase letters indicate comparisons among temperature regimes within the same population (Tukey's test, $p \leq 0.05$).

Among the putative genes evaluated in this response, increased expression was observed only for *ABCC3* in the resistant population (G5) at low temperature (15 °C) compared with the non-treated control (Figure 9). In contrast, no differences in expression were detected between resistant and susceptible populations for *PLP2*, a putative gene previously associated with herbicide-induced necrosis in Sumatran fleabane, or for the putative herbicide transporter genes *ABCB4* and *ABCC3* under the conditions evaluated (Figure 9).

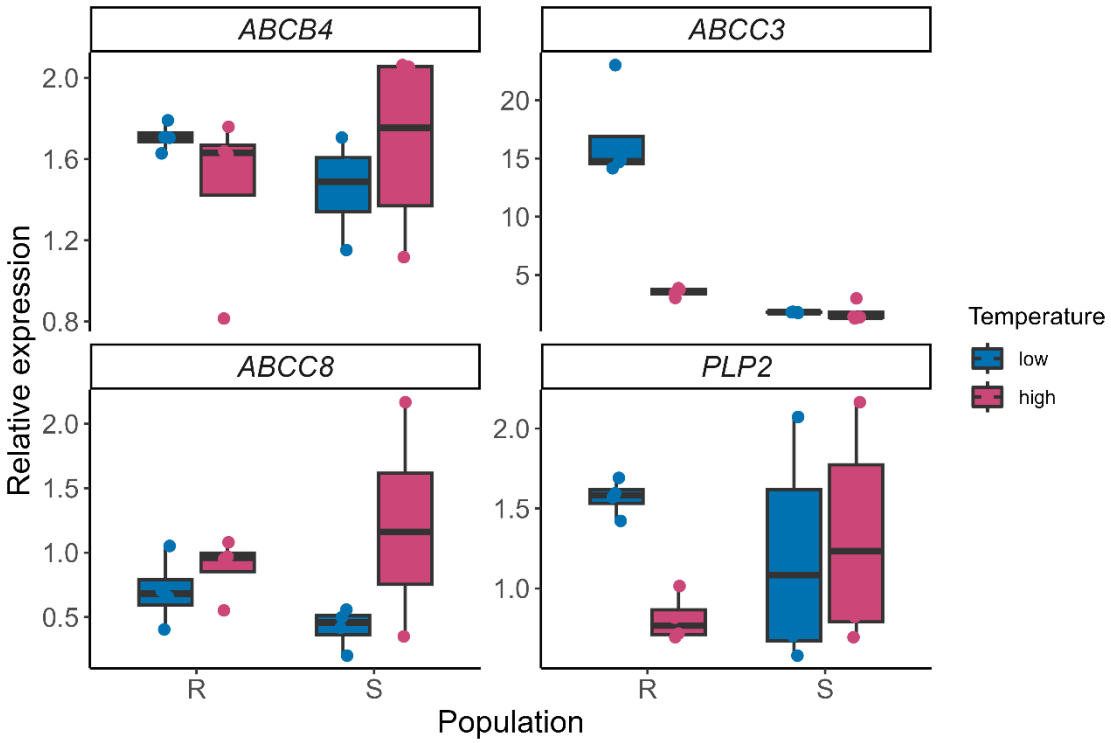


Figure 9. Relative expression of putative genes associated with glyphosate-induced necrosis in resistant (R) and susceptible (S) populations under low (15 °C) and high (30 °C) temperature conditions. Panels are presented using different y-axis scales.

The occurrence of atypical necrotic symptoms shortly after glyphosate application under low temperature conditions indicates that environmental factors can strongly influence the resistance phenotype in these populations. The rapid development of necrotic lesions and the marked reduction in ED_{50} values suggest that resistance mechanisms operating in these plants may be compromised when plants are exposed to low temperatures following herbicide application.

Temperature can influence glyphosate efficacy by affecting herbicide absorption, translocation, and intracellular compartmentalization (Dalazen *et al.*, 2019; Guo *et al.*,

2023). In *Conyza* species, resistance to glyphosate has been associated with reduced translocation and vacuolar sequestration mechanisms that limit herbicide movement to sensitive tissues (Ge *et al*., 2010; Moretti and Hanson, 2017). However, environmental conditions can significantly interfere with these physiological responses. For example, a study showed that low temperatures increased glyphosate absorption and translocation in glyphosate-resistant Sumatran fleabane, reducing resistance compared with plants grown under higher temperatures. In that study, the resistance factor decreased from 5.5 under high temperature to 1.6 under low temperature, indicating that the resistance mechanism was partially suppressed under colder conditions (Palma-Bautista *et al.*, 2019).

The results observed in the present study are consistent with these findings. Plants exposed to low temperature after glyphosate application showed rapid necrotic symptoms and exhibited lower ED_{50} values compared with plants maintained at higher temperatures. This response suggests that the resistance mechanism occurring in these populations may depend on processes that are sensitive to environmental conditions, such as glyphosate transport or sequestration. The rapid necrotic response may therefore result from increased glyphosate accumulation in metabolically active tissues when resistance mechanisms are disrupted under low temperature conditions.

A similar atypical response has been reported in glyphosate-resistant giant ragweed (*Ambrosia trifida*), in which rapid foliar necrosis developed within 2 h after glyphosate application and was preceded by hydrogen peroxide accumulation as early as 0.5 h after treatment (Jeffery, 2014). Interestingly, necrosis occurred only in mature leaves, whereas immature tissues and meristems remained unaffected, even when directly treated with glyphosate. Resistant plants also exhibited reduced herbicide translocation compared with susceptible biotypes, although no differences in *EPSPS* sequence or copy number were detected (Van Horn *et al*., 2018; Moretti *et al*., 2018). Furthermore, resistance in this species was not associated with vacuolar sequestration, target-site alteration, or *EPSPS* amplification, suggesting the involvement of alternative physiological mechanisms (Van Horn *et al*., 2018; Moretti *et al*., 2018). To our knowledge, this is the first report of a rapid necrotic response following glyphosate application in resistant *Conyza* spp., a phenotype previously described only in *A. trifida*.

Among the putative genes evaluated, increased expression of *ABCC3* was detected only in the resistant population under low temperature conditions. ABC transporters have

been increasingly implicated in NTSR to herbicides in several weed species. In *Echinochloa colona*, the transporter gene *ABCC8* was upregulated in glyphosate-resistant plants and was proposed to act as a membrane transporter capable of effluxing glyphosate, representing one of the first reports linking an ABCC transporter to glyphosate transport in a resistant weed population (Pan *et al*., 2021). Similarly, transcriptomic analyses in *E. indica* identified *ABCC4* as a putative candidate gene associated with glyphosate resistance (Deng *et al.*, 2022). The *ABCC3* gene has recently been proposed as a potentially conserved NTSR gene in *Lolium* species, where overexpression of *ABCC3* in transgenic rice increased glyphosate resistance (Luo *et al.*, 2025). In *Alopecurus myosuroides*, several ABCC transporters contribute to enhanced metabolic resistance to other herbicides, rather than glyphosate, with evidence suggesting that these proteins can transport herbicides and their metabolites into the vacuole (Goldberg-Cavalleri *et al.*, 2023).

Although the functional role of *ABCC3* was not directly demonstrated in the present study, a temperature-dependent expression pattern was observed. Together with the response detected in the dose-response experiments, these results suggest that environmental conditions may influence herbicide resistance through effects on transport or sequestration mechanisms.

Taken together, the results of this study carried out with 61 resistant populations highlight the complex genetic and physiological basis of glyphosate resistance in *Conyza* species. Although *EPSPS* mutations have been widely reported as a target-site mechanism of resistance, our findings indicate that mutations detected in specific *EPSPS* copies, the Ala100Ser and Pro106Thr substitutions identified in the Chr04B2 copy, are unlikely to independently explain the resistance phenotype observed in these populations. Instead, resistance appears to be influenced by multiple interacting factors, including variation in *EPSPS* copy number, differential expression among paralogous gene copies, and probably the contribution of NTSR mechanisms involving temperature response. A more complete resolution of *EPSPS* copy-specific sequences and expression patterns will be required to determine whether additional mutations in other paralogous copies contribute to glyphosate resistance in hairy and Sumatran fleabane.

5.4 Conclusion

Isolated *EPSPS* target-site mutations are unlikely to explain glyphosate resistance in the evaluated populations. Instead, resistance appears to result from the combined contribution of *EPSPS* copy number variation, differential gene expression, and probably NTSR mechanisms that may be influenced by environmental conditions. These findings indicate that glyphosate resistance in *Conyza* is a complex trait shaped by multiple interacting mechanisms rather than a single genetic change.

References

Angonese PS. Efeito de inibidores metabólicos e análise do transcriptoma de *Conyza sumatrensis* para caracterização do mecanismo de resistência ao herbicida 2,4-D relacionado à rápida necrose. MSc dissertation, Universidade Federal do Rio Grande do Sul, 2022.

Baerson SR, Rodriguez DJ, Tran M, Feng Y, Biest NA, Dill GM. Glyphosate-resistant goosegrass. Identification of a mutation in the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase. Plant Physiol 2002;129:1265–75. https://doi.org/10.1104/pp.001560.

Bajwa AA, Sadia S, Ali HH, Jabran K, Peerzada AM, Chauhan BS. Biology and management of two important *Conyza* weeds: a global review. Environ Sci Pollut Res Int 2016;23:24694–710. https://doi.org/10.1007/S11356-016-7794-7.

Bell MS, Hager AG, Tranel PJ. Multiple Resistance to Herbicides from Four Site-of-Action Groups in Waterhemp (*Amaranthus tuberculatus*). Weed Sci 2013;61:460–8. https://doi.org/10.1614/ws-d-12-00166.1.

Beres ZT, Giese LA, Mackey DM, Owen MDK, Page ER, Snow AA. Target-site *EPSPS* Pro-106-Ser mutation in *Conyza canadensis* biotypes with extreme resistance to glyphosate in Ohio and Iowa, USA. Sci Rep 2020;10. https://doi.org/10.1038/s41598-020-64458-7.

de Carvalho LB, Alves PLDCA, González-Torralva F, Cruz-Hipolito HE, Rojano-Delgado AM, De Prado R, et al. Pool of resistance mechanisms to glyphosate in *Digitaria insularis*. J Agric Food Chem 2012;60:615–22. https://doi.org/10.1021/jf204089d.

Chatham LA, Wu C, Riggins CW, Hager AG, Young BG, Roskamp GK, et al. *EPSPS* Gene Amplification is Present in the Majority of Glyphosate-Resistant Illinois Waterhemp (*Amaranthus tuberculatus*) Populations. https://doi.org/10.1614/wt-d-14-00064.1.

Chen Jingchao, Huang H, Zhang C, Wei S, Huang Z, Chen Jinyi, et al. Mutations and amplification of *EPSPS* gene confer resistance to glyphosate in goosegrass (*Eleusine indica*). Planta 2015;242:859–68. https://doi.org/10.1007/s00425-015-2324-2.

Chen Z, Wang Z, Heng Y, Li J, Pei J, Cao Y, et al. Generation of a series of mutant lines resistant to imidazolinone by screening an EMS-based mutant library in common wheat. Crop J 2021;9:1030–8. https://doi.org/10.1016/j.cj.2020.11.001.

Cutti L, Turra GM, Machado FM, Sulzbach E, Angonese PS, Markus C, et al. Subgenomic Distribution and Herbicide Cross-Resistance of *ALS* Gene Mutations in Allohexaploid *Echinochloa crus-galli*. Plant Direct 2026;10:e70168. https://doi.org/https://doi.org/10.1002/pld3.70168.

Dalazen G, Pisoni A, Menegaz C, Merotto A. Low temperature reverses the resistance to glyphosate in hairy fleabane (*Conyza bonariensis*). J Plant Prot Res 2019;59:433–40. https://doi.org/10.24425/jppr.2019.131267.

Deng W, Duan Z, Li Y, Peng C, Yuan S. Multiple Resistance Mechanisms Involved in Glyphosate Resistance in *Eleusine indica*. Plants 2022, Vol 11, 2022;11. https://doi.org/10.3390/plants11233199.

Dinelli G, Marotti I, Bonetti A, Catizone P, Urbano JM, Barnesà J. Physiological and molecular bases of glyphosate resistance in *Conyza bonariensis* biotypes from Spain. Weed Res 2008:257–65.

Dinelli G, Marotti I, Bonetti A, Minelli M, Catizone P, Barnes J. Physiological and molecular insight on the mechanisms of resistance to glyphosate in *Conyza canadensis* (L.) Cronq. biotypes. Pestic Biochem Physiol 2006;86:30–41. https://doi.org/10.1016/j.pestbp.2006.01.004.

Dussault AA, Pouliot M. Rapid and simple comparison of messenger RNA levels using real-time PCR. Biol Proced Online 2006;8:1–10. https://doi.org/10.1251/BPO114.

Feng PCC, Tran M, Chiu T, Sammons RD, Heck GR, CaJacob CA. Investigations into glyphosate-resistant horseweed (*Conyza canadensis*): retention, uptake, translocation, and metabolism. Weed Sci 2004;52:498–505. https://doi.org/10.1614/ws-03-137r.

Gaines TA, Duke SO, Morran S, Rigon CAG, Tranel PJ, Küpper A, et al. Mechanisms of evolved herbicide resistance. Journal of Biological Chemistry 2020;295:10307–30. https://doi.org/10.1074/JBC.REV120.013572.

Gaines TA, Zhang W, Wang D, Bukun B, Chisholm ST, Shaner DL, et al. Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. Proc Natl Acad Sci 2010;107:1029–34. https://doi.org/10.1073/pnas.0906649107.

Ge X, André d'Avignon D, Ackerman JJH, Douglas Sammons R. Rapid vacuolar sequestration: The horseweed glyphosate resistance mechanism. Pest Manag Sci 2010;66:345–8. https://doi.org/10.1002/ps.1911.

Goldberg-Cavalleri A, Onkokesung N, Franco-Ortega S, Edwards R. ABC transporters linked to multiple herbicide resistance in blackgrass (*Alopecurus myosuroides*). Front Plant Sci 2023;14:1082761. https://doi.org/10.3389/fpls.2023.1082761.

Gomes AC, Montgomery J, Araujo ALS, Morran S, Cutti L, Patterson E, et al. Chromosome-level assemblies of the allohexaploid genomes of *Conyza sumatrensis* and *Conyza bonariensis*. Genome Biol Evol 2025. https://doi.org/10.1093/GBE/EVAF065.

Guo W, Zhang C, Wang S, Zhang T, Tian X. Temperature influences glyphosate efficacy on glyphosate-resistant and -susceptible goosegrass (*Eleusine indica*). Front Plant Sci 2023;14:1169726. https://doi.org/10.3389/fpls.2023.1169726.

Heap I. International Herbicide-Resistant Weed Database 2026. http://www.weedscience.org/.

Hereward JP, Werth JA, Thornby DF, Keenan M, Chauhan BS, Walter GH. Gene expression in response to glyphosate treatment in fleabane (*Conyza bonariensis*) – glyphosate death response and candidate resistance genes. Pest Manag Sci 2018;74:2346–55. https://doi.org/10.1002/ps.4804.

Jeffery T. Investigation into the molecular and biochemical mechanisms of resistance in two biotypes of glyphosate resistant giant ragweed. Guelph (ON): University of Guelph; 2014.

Jugulam M, Shyam C. Non-Target-Site Resistance to Herbicides: Recent Developments. Plants, 2019;8. https://doi.org/10.3390/plants8100417.

Kalsing A, Nunes FA, Gotardi GA, Bueno JC, Schneider AA, Tropaldi L, et al. Taxonomic resolution of fleabane species (*Conyza* spp.) based on morphological and molecular markers and their dispersion across soybean-cropping macroregions and seasons in Brazil. Weed Sci 2024:1–31. https://doi.org/10.1017/WSC.2024.3.

Kang J, Park J, Choi H, Burla B, Kretzschmar T, Lee Y, et al. Plant ABC Transporters. The Arabidopsis Book / American Society of Plant Biologists 2011;9:e0153. https://doi.org/10.1199/TAB.0153.

Kaspary TE, Cutti L, Turra GM, Angonese PS, dos Santos OD, Merotto A. *Conyza bonariensis* resistance to glyphosate and ALS inhibitors involves target and non-target site resistance. Pestic Biochem Physiol 2025;213:106501. https://doi.org/10.1016/J.PESTBP.2025.106501.

Koo DH, Molin WT, Saski CA, Jiang J, Putta K, Jugulam M, et al. Extrachromosomal circular DNA-based amplification and transmission of herbicide resistance in crop weed *Amaranthus palmeri*. Proc Natl Acad Sci U S A 2018;115(13):3332-3337. https://doi.org/10.1073/pnas.1719354115.

Lamego FP, Vidal RA. Resistance to glyphosate in *Conyza bonariensis* and *Conyza canadensis* biotypes in Rio Grande do Sul, Brazil. Planta Daninha 2008;26:467–71. https://doi.org/10.1590/s0100-83582008000200024.

Lazaroto CA, Fleck NG, Vidal RA. Biologia e ecofisiologia de buva (*Conyza bonariensis* e *Conyza canadensis*). Ciência Rural 2008;38:852–60.

Lucio FR, Kalsing A, Adegas FS, Rossi CVS, Correia NM, Gazziero DLP, et al. Dispersal and Frequency of Glyphosate-Resistant and Glyphosate-Tolerant Weeds in Soybean-producing Edaphoclimatic Microregions in Brazil. Weed Technology 2019;33:217–31. https://doi.org/10.1017/wet.2018.97.

Luo Z, Zeng Y, Qian H, Zhao H, Yu Q, Bai L, et al. Conjoint Genome−Transcriptome Analysis to Identify the Conserved *ABCC3* Gene Involved in Glyphosate Resistance in *Lolium* spp. Cite This: J Agric Food Chem 2025;73. https://doi.org/10.1021/acs.jafc.5c10416.

Mendes RR, Takano HK, Netto AG, Junior GJP, Cavenaghi AL, Silva VFV, et al. Monitoring glyphosate-and chlorimuron-resistant *Conyza* spp. Populations in Brazil. An Acad Bras Cienc 2021;93. https://doi.org/10.1590/0001-3765202120190425.

Merotto A, Gazziero LP, Oliveira MC, Scursoni J, Garcia MA, Figueroa R, et al. Herbicide use history and perspective in South America. Advances in Weed Science

2022;40(Spec1):e020220050. https://doi.org/10.51694/AdvWeedSci/2022;40:seventy-five010.

Montgomery J, Morran S, MacGregor DR, McElroy JS, Neve P, Neto C, et al. Current status of community resources and priorities for weed genomics research. Genome Biol 2024;25. https://doi.org/10.1186/s13059-024-03274-y.

Moreira MS, Nicolai M, Carvalho SJP, Christoffoleti PJ. Resistência de *Conyza canadensis* e *C. bonariensis* ao herbicida glyphosate. Planta Daninha 2007;25:157–64. https://doi.org/10.1590/S0100-83582007000100017.

Moretti ML, Alárcon-Reverte R, Pearce S, Morran S, Hanson BD. Transcription of putative tonoplast transporters in response to glyphosate and paraquat stress in *Conyza bonariensis* and *Conyza canadensis* and selection of reference genes for qRT-PCR. PLoS One 2017;12:e0180794. https://doi.org/10.1371/journal.pone.0180794.

Moretti ML, Hanson BD. Reduced translocation is involved in resistance to glyphosate and paraquat in *Conyza bonariensis* and *Conyza canadensis* from California. Weed Res 2017;57:25–34. https://doi.org/10.1111/wre.12230.

Moretti ML, Van Horn CR, Robertson RR, Segobye K, Weller SC, Young BG, et al. Glyphosate resistance in *Ambrosia trifida*: Part 2. Rapid response physiology and non-target-site resistance. Pest Manag Sci 2018;74(5):1081-1088. https://doi.org/10.1002/ps.4563.

Nandula VK, Ray JD, Ribeiro DN, Pan Z, Reddy KN. Glyphosate Resistance in Tall Waterhemp (*Amaranthus tuberculatus*) from Mississippi is due to both Altered Target-Site and Nontarget-Site Mechanisms. Weed Sci 2013:374–83. https://doi.org/10.1614/WS-D-12-00155.1.

Ostlie M, Haley SD, Anderson V, Shaner D, Manmathan H, Beil C, et al. Development and characterization of mutant winter wheat (*Triticum aestivum* L.) accessions resistant to the herbicide quizalofop. Theoretical and Applied Genetics 2014;128:343–51. https://doi.org/10.1007/s00122-014-2434-4.

Page ER, Grainger CM, Laforest M, Nurse RE, Rajcan I, Bae J, et al. Target and Non-target site Mechanisms Confer Resistance to Glyphosate in Canadian Accessions of *Conyza canadensis*. Weed Sci 2018;66:234–45. https://doi.org/10.1017/wsc.2017.69.

Palma-Bautista C, Alcántara-de la Cruz R, Rojano-Delgado AM, Dellaferrera I, Domínguez-Martínez PA, De Prado R. Low temperatures enhance the absorption and translocation of 14C-glyphosate in glyphosate-resistant *Conyza sumatrensis*. J Plant Physiol 2019;240:153009. https://doi.org/10.1016/j.jplph.2019.153009.

Palma-Bautista C, Tataridas A, Kanatas P, Travlos IS, Bastida F, Domínguez-Valenzuela JA, et al. Can control of glyphosate susceptible and resistant *Conyza sumatrensis* populations be dependent on the herbicide formulation or adjuvants? Agronomy 2020;10. https://doi.org/10.3390/agronomy10101599.

Pan L, Yu Q, Wang J, Han H, Mao L, Nyporko A, et al. An ABCC-type transporter endowing glyphosate resistance in plants. Proc Natl Acad Sci 2021;118. https://doi.org/10.1073/pnas.2100136118.

Perotti VE, Larran AS, Palmieri VE, Martinatto AK, Alvarez CE, Tuesca D, et al. A novel triple amino acid substitution in the *EPSPS* found in a high-level glyphosate-resistant

Amaranthus hybridus population from Argentina. Pest Manag Sci 2019;75:1242–51. https://doi.org/10.1002/ps.5303.

Ritz C, Strebig J. Package 'drc' - Analysis of Dose-Response Curves 2016.

Rutland CA, Hall ND, McElroy JS. The Impact of Polyploidization on the Evolution of Weed Species: Historical Understanding and Current Limitations. Frontiers in Agronomy 2021;3. https://doi.org/10.3389/fagro.2021.626454.

Sammons RD, Gaines TA. Glyphosate resistance: State of knowledge. Pest Manag Sci 2014;70:1367–77. https://doi.org/10.1002/ps.3743.

Santos G, Oliveira RS, Constantin J, Francischini AC, Osipe JB. Multiple resistance of *Conyza sumatrensis* to chlorimuron-ethyl and to glyphosate. Planta Daninha 2014;32:409–16. https://doi.org/10.1590/S0100-83582014000200019.

Shaner DL. Role of Translocation as A Mechanism of Resistance to Glyphosate. Weed Sci 2009;57:118–23. https://doi.org/10.1614/ws-08-050.1.

Sulzbach E, Turra GM, Cutti L, Kroth LVE, Tranel PJ, Merotto A, et al. Smooth pigweed (*Amaranthus hybridus*) and unresolved *Amaranthus* spp. from Brazil resistant to glyphosate exhibit the *EPSPS* TAP-IVS substitution. Weed Sci 2024;72:48–58. https://doi.org/10.1017/wsc.2023.70.

Tani E, Chachalis D, Travlos IS. A Glyphosate Resistance Mechanism in *Conyza canadensis* Involves Synchronization of *EPSPS* and ABC-transporter Genes. Plant Mol Biol Report 2015. https://doi.org/10.1007/s11105-015-0868-8.

Theodoulou FL. Plant ABC transporters. Biochim Biophys Acta 2000;1465:79–103. https://doi.org/10.1016/S0005-2736(00)00132-2.

Van Horn CR, Moretti ML, Robertson RR, Segobye K, Weller SC, Young BG, et al. Glyphosate resistance in *Ambrosia trifida*: Part 1. Novel rapid cell death response to glyphosate. Pest Manag Sci 2018;74(5):1071-1080. https://doi.org/10.1002/ps.4561.

Vargas L, Bianchi MA, Rizzardi MA, Agostinetto D, Dal Magro T. *Conyza bonariensis* biotypes resistant to the glyphosate in Southern Brazil. Planta Daninha 2007;25:573–8.

Yu Q, Cairns A, Powles S. Glyphosate, paraquat and ACCase multiple herbicide resistance evolved in a *Lolium rigidum* biotype. Planta 2007;225:499–513. https://doi.org/10.1007/s00425-006-0364-3.

Yu Q, Jalaludin A, Han H, Chen M, Douglas Sammons R, Powles SB. Evolution of a double amino acid substitution in the 5-enolpyruvylshikimate-3-phosphate synthase in *Eleusine indica* conferring high-level glyphosate resistance. Plant Physiol 2015;167:1440–7. https://doi.org/10.1104/pp.15.00146.

Yuan JS, Abercrombie LLG, Cao Y, Halfhill MD, Zhou X, Peng Y, et al. Functional Genomics Analysis of Horseweed (*Conyza canadensis*) with Special Reference to the Evolution of Non–Target-Site Glyphosate Resistance. Weed Sci 2010;58:109–17. https://doi.org/10.1614/ws-d-09-00037.1.

Supplementary Material

Table S1. Efficacy of control 28 days after herbicide treatment in 70 *Conyza* spp. populations from Brazil with glyphosate at 50% and 100% of the label rate. ERIBO - *Conyza bonariensis*, ERISU - *Conyza sumatrensis**.

Population	Species	Local	Glyphosate	
			0.5x	1x
G1	ERIBO	Capão do Leão/RS	20	13
G2	ERIBO	Vila Nova/RS	100	100
G3	ERIBO	Dois Irmãos das Missões/RS	92	100
G4	ERIBO	Santa Maria/RS	55	84
G5	ERISU	Maripá/PR	40	49
G6	ERISU	Londrina/PR	61	37
G7	ERISU	Marialva/PR	25	30
G8	ERISU	Toledo/PR	90	96
G9	ERISU	Toledo/PR	48	40
G10	ERISU	Toledo/PR	25	19
G11	ERISU	Toledo/PR	38	77
G12	ERISU	Toledo/PR	32	30
G13	ERISU	Campo Mourão/PR	25	37
G14	ERISU	Uruguaiana/RS	32	50
G15	ERISU	Itambé/PR	31	36
G16	ERISU	Dourados/MS	28	41
G17	ERISU	Dourados/MS	32	39
G18	ERISU	Dourados/MS	47	61
G19	ERISU	Dourados/MS	39	47
G20	ERISU	Dourados/MS	39	44
G21	ERISU	Rio Brilhante/MS	33	59
G22	ERISU	Rio Brilhante/MS	40	35
G23	ERISU	Maracaju/MS	26	29
G24	ERISU	Maracaju/MS	38	65
G25	ERIBO	Porto Alegre/RS	64	81
G26	ERISU	Porto Alegre/RS	45	55
C1	ERISU	Rio Verde/GO	25	27
C2	ERISU	Uberaba/MG	66	97
C3	ERISU	Uberlandia/MG	60	90
C4	ERISU	Uberlandia/MG	61	98
C7	ERIBO	Ponta Porã/MS	58	94
C9	ERIBO	Rio Brilhante/MS	43	28
C10	ERIBO	Dourados/MS	33	88
C13	ERISU	Abelardo Luz/SC	58	68
C20	ERISU	Cascavel/PR	45	96
C22	ERISU	Corbelia/PR	33	45
C25	ERISU	Guarapuava/PR	31	35

C26	ERISU	Guarapuava/PR	51	50
C27	ERISU	Guarapuava/PR	34	36
C28	ERISU	Guarapuava/PR	38	54
C30	ERISU	Guarapuava/PR	36	43
C31	ERISU	Guarapuava/PR	29	55
C32	ERISU	Guarapuava/PR	54	52
C33	ERISU	Guarapuava/PR	52	51
C34	ERISU	Guarapuava/PR	45	36
C35	ERISU	Guarapuava/PR	35	32
C37	ERISU	Turvo/PR	60	52
C38	ERISU	Itapejara d'Oeste/PR	33	40
C39	ERISU	Juranda/PR	54	48
C40	ERISU	Juranda/PR	53	40
C43	ERISU	Mariópolis/PR	42	64
C44	ERISU	Ouro Verde do Oeste/PR	14	39
C45	ERISU	Ouro Verde do Oeste/PR	65	66
C46	ERISU	Pato Branco/PR	59	39
C47	ERISU	Pato Branco/PR	62	59
C48	ERISU	Londrina/PR	43	63
C49	ERISU	Planalto/PR	14	39
C50	ERISU	Santa Izabel do Oeste/PR	33	39
C51	ERISU	São João/PR	16	22
C55	ERISU	Turvo/PR	10	37
C59	ERISU	Cruz Alta/RS	60	58
C60	ERISU	Passo Fundo/RS	17	36
C61	ERISU	Santa Maria/RS	35	38
C62	ERISU	São Domingos/SC	28	32
C63	ERISU	Xanxerê/SC	11	16
C64	ERISU	Casa Branca/SP	39	41
T1	ERIBO	Chapada/RS		
T2	ERIBO	Santa Bárbara do Sul/RS		
T3	ERIBO	Novo Barreiro/RS		
T4	ERIBO	Novo Bareiro/RS		

*ERIBO and ERISU are Bayer codes derived from the nomenclature *Erigeron bonariensis* and *Erigeron sumatrensis*, respectively. Species are referred to as *Conyza bonariensis* and *Conyza sumatrensis* throughout this study.

Sumatran fleabane – EPSPS copies

>ConSuChr03Ag259220
ATGTTTAATAGTTTCTGCCGTTGCCACCACCGAGAAACCGTCAACGGTGCCGGAAATTGTGTTACAACCC
ATTAAAGAAATCTCGGGTACGGTTAATTTACCCGGGTCCAAGTCGTTGTCTAATCGGATCCTCCTCCTTG
CTGCGCTTGCTGAGGGAACGACCATTGTTGACAACTTACTCAACAGTGATGATGTTCATTACATGCTTGG
AGCTTTAAGAACTCTAGGGCTAAACGTTGAGGAGGATGTTGCAATTAAAAGGGCAATTGTGGAAGGTTGT
GGCGGTGTGTTTCCTGTGGGTAAAGAAGCTAAAGATGACATACAGCTTTTTCTTGGGAAT**GCA**GGAACTG
CTATGCGT**CCA**TTGACTGCCGCAGTTACTGCTGCTGGTGGTAATTCAAGCTACGTACTAGATGGCGTTCC
TCGTATGAGAGAGAGACCAATAGGTGATTTGGTCACGGGTCTTAAGCAGCTTGGGGCAGATGTTGACTGT
TCTCTCGGGACGAACTGCCCTCCTGTGCGTGTAGTTGGTGGAGGTGGTCTCCCTGGAGGAAAGGTTAAGT
TGTCGGGATCTATTAGTAGTCAATACCTTACTGCGCTGCTTATGGCTTCTCCCCTTGCCCTTGGGGACGT
GGAAATTGAAATCATAGATAAACTAATTTCCATACCATATGTCGAGATGACACTGAAATTAATGGAACGG
TTCGGCGTCTCGGTAGAACATAGTGATAGTTGGGACCAGTTCTTTATTCGAGGCGGCCAAAAGTACAAGT
CACCTGGAAATGCTTATGTAGAAGGTGATGCGTCAAGTGCGAGTTACTTCTTGGCTGGTGCTGCCATAAC
CGGAGGCACCATCACCGTTGAAGGCTGCGGAACAAGTAGTCTGCAGGGTGATGTGAAGTTTGCGGAGGTA
CTTGGACAAATGGGTGCGGAAGTAACATGGACTGAGAACTCAGTCACAGTTAAGGGCCCACCAAGGGATT
CTTCTGGAAGGAAACATTTACGTGCTGTTGATGTGAACATGAACAAGATGCCTGATGTTGCCATGACTCT
TGCTGTGGTCGCTCTTTATGCTGATGGCCCTACAGCCATTAGAGATGTTGCTAGCTGGAGAGTTAAAGAA
ACCGAAAGGATGATTGCCATTTGCACAGAACTTAGAAAGTTGGGAGCAACAGTTGAAGAAGGTCCGGACT
ATTGTGTGATCACTCCGCCAGAGAAGTTAAACGTGACAGCAATAGACACATATGATGATCACAGGATGGC
CATGGCTTTCTCGCTTGCCGCTTGTGCAGATGTTCCTGTGACCATTAAGGATCCTTCTTGCACTCGTAAG
ACATTTCCTGATTACTTTGAAGTTCTTCAAAGATTTGCCAAGCATTAA
>ConSuChr03Bg296380
ATGGCAGCTACTCACATTAACACCACCAACATTGCCCACAATCTCCAAGCGACCACCAGTCTTTCCAAAA
CCCAAACCCCATCAATAAATTCACAACCTTTTTTATATTTTGGGCCAAAACACAAAAACCCAATTGCCCA
TTTCTCTGTTTCTTCTTCCAATAATAAAAATCTTGGAAAAAAATCCACCACCGAGAAACCATCAACGGTG
CCGGAAATTGTGTTGCAACCAATTAAAGAAATCTCGGGTACGGTTAATTTACCCGGCTCCAAGTCTTTGT
CTAATCGGATCCTCCTCCTTGCTGCGCTTGCTGAGGGAACGACCGTTGTTGACAACTTACTCAACAATGA
TGATGTTCATTACATGCTTGGAGCTTTAAGAACTCTAGGGCTAAACGTTGAGGAGGATGGTGCAATTAAA
AGGGCAATTGTGGAAGGTTGTGGCAGTGTGTTTCCTGTGGGTAAAGAAGCTAAAGATGACATACAACTTT
TTCTTGGGAAT**GTA**GGAACTGCTATGCGT**CCA**TTGACTGCCGCAGTTACTGCTGCTGGTGGTAATTTAAG
CTACGTACTAGATGGCGTTCCTCGTATGAGAGAGAGACCAATAGGTGATTTGGTCACGGGTCTTAAGCAG
CTTGGGGCAGATGTTGACTGTTCTCTTGGGACGAACTGCCCACCCGTGCGTGTAGTTGGAGGAGGTGGTC
TCCCTGGAGGAAAGGTTAAGTTGTCGGGATCTATTAGTAGTCAATACCTTACTGCTCTGCTTATGGCTTC
TCCCCTTGCCCTTGGGGATGTGGAGATTGAAATCATAGATAAACTAATTTCCATACCATATGTCGAGATG
ACACTGAAATTAATGGAACGGTTCGGCGTCTCGGTAGAACATAGTGATAGTTGGGACCGGTTCTTTATTC
GAGGCGGCCAAAAGTACAAGTCACCTGGAAATGCTTATGTAGAAGGTGATGCATCAAGTGCGAGTTACTT
CTTGGCTGGTGCCTCCATAACTGGAGGCACCATCACCGTTGAGGGCTGCGGAACAAGTAGTTTGCAGGGT
GATGTGAAGTTTGCGGAGGTACTTGGACAAATGGGTGCGGAAGTAACATGGACTGAGAACTCAGTCACAG
TTAAAGGCCCACCAAGAGATTCTTCTGGAAGGAAACATTTACGTGCTGTTGATGTGAACATGAACAAGAT
GCCTGATGTTGCCATGACTCTTGCTGTGGTCGTTCTTTATGCTGATGGCCCTACAGCCATTACAGATGTT
GCTAGCTGGAGAGTTAAAGAAACCGAAAGGATGATTGCCATTTGCACAGAACTCAGAAAGTTGGGAGCAA
CAGTTGAAGAAGGTCCGGACTATTGTGTGATCACTCCGCCAGAGAAGTTAAACGTGACTGCAATAGACAC
ATATGATGATCATAGGATGGCCATGGCTTTCTCTCTTGCTGCTTGTGCAGATGTTCCTGTGACCATTAAG
GACCCTTCTTGCACTCGTAAGACGTTTCCTGATTACTTTGAAGTTCTTCAAAGATTTGCCAAGCATTAA
>ConSuChr03Cg309970
ATGGCAGCTACTCACATTAACACCACCAACATTGCCCATAATCTCCAAGCTACCACCAGTCTTTCCAAAA
CCCAAACCCCATCAATAAAGTCACAACCTTTTTTATCTTTTGTGCCAAAACACACAAACCCAATTGCCCA
TTTCTCTGTTTCTTCTAATAATAACAGAAATCTTGGAAAAAAATGTTTAATTGTTTCTGCCGTTGCCACC
ACCGAGAAACCATCAACGGTGCCGGAAATTGTGTTGCAACCCATTAAAGAAATATCGGGTACGGTTAATT
TACCCGGGTCCAAGTCTTTGTCTAATCGGATCCTCCTCCTTGCTGCGCTCGCCGAGGGAACGACCATTGT
TGACAACTTACTCAACAGTGATGATGTTCATTACATGCTTGGAGCTTTAAGAACTCTAGGGCTAAACGTT
GAGGAGGATGGTGCAATTAAAAGGGCAATTGTGGAAGGTTGTGGCGGTATGTTTCCTGTGGGTAAAGAAG
CTAAAGATGACATACAGCTTTTTCTTGGGAAT**GCA**GGAACTGCTATGCGT**CCA**TTGACTGCCGCAGTTAC
TGCTGCTGGTGGTAATTCAAGCTACGTACTAGATGGCGTTCCTCGTATGAGAGAGAGACCAATAGGTGAT
TTGGTCACGGGTCTTAAGCAGCTTGGGGCAGATGTTGACTGTTCTCTCGGGACGAACTGCCCACCCGTAC
GTGTAGTTGGAGGAGGTGGTCTCCCTGGAGGAAAGGTTAAGTTGTCGGGATCTATTAGTAGTCAATACCT
TACTGCTCTGCTTATGGCTTCTCCCCTTGCCCTTGGGGATGTGGAGATTGAAATCATAGATAAACTAATT
TCCATACCATATGTCGAGATGACTCTGAAATTAATGGAATGGTTCGGCGTCTCGGTAGAACATAGTGATA
GTTGGGACCGCTTCTTTATTCGAGGCGGCCAAAAGTACAAGTCACCTGGAAATGCTTATGTAGAAGGTGA

TGCATCAAGTGCGAGTTACTTCTTGGCTGGTGCTGCCATAACTGGAGGAACCATCACCGTTGAAGGCTGC
GGAACAAGTAGTTTGCAGGGTGATGTGAAGTTTGCGGAGGTACTTGGACAAATGGGTGCGGAAGTAACAT
GGACTGAGAACTCAGTCACAGTTAAGGGCCCACCAAGGGATTCTTCTGGAAGGAAACATTTACGTGCTGT
TGATGTGAACATGAACAAGATGCCTGATGTTGCCATGACTCTTGCTGTGGTCGCTCTTTATGCTGATGGC
CCTACAGCCATTAGAGATGTTGCTAGCTGGAGAGTTAAAGAAACCGAAAGGATGATTGCCATTTGCACAG
AACTTAGAAAGTTGGGAGCAACAGTTGAAGAAGGTCCAGACTATTGTGTGATCACTCCGCCAGAGAAGTT
AAACGTGACAGCAATAGACACATATGATGATCATAGGATGGCCATGGCTTTCTCTCTTGCTGCTTGTGCA
GATGTTCCTGTGACCATTAAGGACCCTTCTTGCACTCGTAAGACGTTTCCTGATTACTTTGAAGTTCTTC
AAAGATTTGCCAAGCATTAA
>ConSuChr04Ag361620
ATGGCAGTTCACATCAACAACTCCAACATACCCATTTTCAACACTTCCAATCTCACAACCCAAAACCCAT
CTTCAAAGTCATCATCTTTTTTATCTTTTGGATCCAACTTCAAAAACCCATTAAAAAACAATAATAACAA
TAATTATACCTCTGTTTCTTGTAATGTGAAAAACAACAAAAACCCATTTAAAGTATCAGCTTTCTCTGCC
ACTTCCACCAAAGAGAAGCCATCTAAAGCTCCAGAAGAAATTGTGTTGAAACCCATTCAAGAAATTTCGG
GTACGGTCCATTTACCCGGATCCAAGTCTTTGTCTAATCGGATCCTCCTTCTTGCTGCCCTGTCTGAGGG
GACTACTGTTGTAGACAACTTGTTAAACAGTGATGATGTTCATTACATGCTTGGAGCTTTAAGAGTTCTA
GGGTTAAATGTTGAAGAAAATAGTGCAATTAAAAGAGCAATCGTAGAAGGTTGTGGTGGTGTATTTCCCG
TGGGTAAAGAAGCCAAGGATGAAATCCAGCTTTTTCTTGGAAAT**GCA**GGAACAGCTATGCGT**CCA**TTGAC
TGCTGCCGTTACTGCTGCCGGTGGAAACTCAAGCTACATACTAGATGGTGTTCCTCGAATGAGGGAGAGG
CCAATTGGTGATCTGGTCACCGGTCTAAAACAGCTTGGTGCAAATGTTGATTGTTCTCTCGGTACAAACT
GCCCACCGGTTCGTGTAGTTGGAAGTGGAGGCCTTCCTGGTGGAAAGGTGAAATTGTCAGGATCTATAAG
TAGCCAATACTTGACTTCTTTGCTTATGGCGGCTCCTCTTGCACTGGGAGACGTAGAGATAGAAATTGTA
GATAAATTGATCTCTGTACCATATGTGGAGATGACACTTAAGTTGATGGAGCGGTTTGGGGTTTCAGTAG
AACACAGTGATACTTGGGACAGATTCCATGTCCGAGGCGGTCAAAAGTACAAGTCACCTGGAAATGCTTA
TGTGGAAGGTGATGCTTCAAGTGCGAGTTACTTCTTAGCTGGTGCTGCCATCACTGGCGGAACTGTCACC
GTGGAAGGTTGCGGGACAAGCAGTTTACAGGGTGATGTAAAATTTGCTGAGGTCCTTGGACAAATGGGTG
CTGAAGTAACCTGGACAGAGAACTCTGTCACGGTGAAGGGTCCGCCAAGGAATTCTTCCGGAAGGGGACA
CTTGCGTCCAGTAGATGTGAACATGAACAAAATGCCGGATGTTGCGATGACTCTTGCTGTGGTTGCCCTT
TATGCTGATGGCCCCACTGCCATTAGAGACGTGGCTAGCTGGAGAGTAAAAGAAACGGAAAGGATGATTG
CCATCTGCACAGAACTAAGAAAGTTGGGAGCAACAGTCGAAGAAGGTCCAGATTATTGTGTGATCACTCC
ACCAGAGAAATTGAATGTGACAGCAATCGACACATACGATGATCACAGAATGGCCATGGCTTTCTCGCTT
GCCGCCTGTGCAGAGGTTCCTGTCACCATTAAGGACCCGGGTTGCACCCGTAAGACCTTCCCCGACTACT
TTGAAGTTCTTGAAAGATACGCTAAGCATTAA
>ConSuChr04B1g385180
ATGGCGGTTCACATCAACAATACAAACATACCCAATTTCAACACTTGCAATCTCACAACCCAAAAGCAAT
CTTCAAAGTCATCATCTTTTTTATCTTTTGGATCCAACTTCAAAAACCCATTAAAAACCAATAATAATAA
TTATTATTCCTCTGTTTCTTGTAATGTGAAAAACAACCCATTTAAAGTATCAGTTTTCTCTGCCACTACC
ACCAAAGAGAAGCCATCAAAAGCTCCAGAAGAAATTGTGTTGAAACCCATTCAAGAAATTTCGGGCACGG
TTCATTTACCCGGATCCAAGTCTTTATCTAATCGGATCCTCCTTCTTGCTGCCCTTTCTGAGGGGACTAC
TGTTGTAGACAACTTATTAAACAGTGATGATGTTCATTACATGCTTGGAGCTTTAAGAGCTCTAGGGTTA
AATGTTGAAGAAAATGCTGCAATTAAAAGAGCAATCGTAGAAGGTTGTGGTGGTGTATTTCCTGTGGGTA
AAGAGGCCAAGGATGAAATCCAGCTTTTTCTTGGAAAT**GCA**GGAACAGCTATGCGT**CCA**TTGACTGCTGC
CGTTACTGCAGCTGGTGGAAATTCAAGCTACGTACTAGATGGTGTTCCTCGAATGAGAGAGAGGCCAATT
GGTGATCTGGTCACCGGTCTAAAACAACTTGGTGCAAATGTTGACTGTTCTCTAGGTACAAACTGCCCAC
CAGTTCGTGTAGTTGGAAGTGGAGGCCTTCCTGGTGGAAAGGTGAAATTGTCAGGATCTATAAGTAGTCA
ATACTTGACTTCTTTGCTCATGGCAGCTCCCCTTGCACTGGGAGACGTAGAGATAGAAATTATAGATAAA
TTGATCTCTGTACCATATGTAGAGATGACACTGAAGTTGATGGAACGGTTTGGGGTTTCAGTAGAACACA
GTGATACTTGGGACAGATTCTATGTCCGAGGCGGTCAAAAGTACAAGTCACCTGGAAATGCTTATGTGGA
AGGTGATGCTTCAAGTGCGAGTTACTTCTTAGCTGGTGCTGCCATCACTGGCGGAACTGTCACCGTGGAA
GGTTGCGGGACAAGCAGTTTACAGGGTGATGTAAAATTTGCTGAGGTCCTTGGACAAATGGGTGCTGAAG
TAACCTGGACAGAGAACTCTGTCACAGTGAAGGGTCCACCAAGGAATTCTTCCGGAAGGGGACACTTGCG
TCCAGTAGATGTGAACATGAACAAAATGCCAGATGTTGCGATGACTCTTGCTGTGGTTGCCCTTTATGCT
GATGGCCCCACTGCCATCAGAGACGTGGCTAGTTGGAGAGTAAAAGAAACAGAAAGGATGATTGCCATTT
GCACTGAACTAAGAAAGTTGGGAGCAACAGTGGAAGAAGGTCCAGATTATTGTGTGATCACTCCACCAGA
GAAATTAAATGTGACAGCAATTGACACATACGATGATCACAGAATGGCCATGGCTTTCTCGCTTGCCGCC
TGTGCAGAGGTTCCTGTCACCATTAAGGACCCAGGTTGCACCCGTAAGACCTTCCCCGACTACTTTGAAG
TTCTTGAAAGATACACTAAGCATTAA
>ConSuChr04B2g385170
ATGGAATTTAGCTGGAATCAAACACACCCACAATGGCAGTTCACAACACCAATGGCAGTTCACTTCAACA
ACACCAATTTCACCGCTTCCAATCTCATAACCCAAAAGGCATCTTCAAAGTCATCATCTTTTTTATCTTT
TGGATCCAACTTCAAAAACCCATTAAAAACCaataatactaataataataatTCCTCTTTTTCTTTTTCT

TGTAATGTGAAAAACAACAAAAACCCATTTAAAGTGTCAGCCTTTTCTGCCACTACCACCAAAGAGAGGC
CATCTAAAGCTCCAGAAGAAATTGTGTTGAAACCCATTCAAGAAATTTCGGGTACGGTTCATTTACCCGG
ATCCAAGTCTTTATCTAATCGGATTCTCCTTCTTGCTGCACTTTCTGAGGGGACTACTGTTGTAGACAAC
TTATTAAACAGTGATGATGTTCATTACATGCTTGGAGCTTTAAGAGCTCTAGGGTTAAATGTTGAAGAAA
ATGCTGCAATTAAAAGAGCAATCGTAGAAGGTTGTGGTGGTGTATTTCCTGTGGGTAAAGAAGCCAAGGA
TGAAATCCAGCTTTTTCTTGGAAAT**TCA**GGAACAGCTATGCGT**ACA**TTGACTGTTGCCATTACTGCTGCT
GGTGGAAATTCAAGCTACGTACTAGATGGTGTTCCTCGAATGAGAGAGAGGCCAATTGGTGATCTGGTCA
CCGGTCTAAAACAACTTGGTGTAAATGTTGATTGTTCTCTAGGTACAAACTGCCCACCAGTTCGTGTAGT
TGGAAGTGGAGGCCTTCCTGGTGGAAAGGTGAGATTGTCAGGATCTATAAGTAGTGTATACTTGACTTCT
TTGCTCATGGCAGCTCCCCTTGCACTGGGAGACGTAGAGATAGAAATTATAGATAAATTGATCTCTGTAC
CATATGTACAGATGACACTGAAGTTGATGAAACAGTTTGGGGTTTCAGTAGAACACAGTGATACTTTGGA
CAGATTCCATGTCCGAGGTGGTCAAAAGTACAAGTCACCTGGAAATGCTTATGTGGAAGGTGATGCTTCA
AGTGCGAGTTACTTCTTAGCTGGTGCTGCCATCACTGGCGGAACTGTCACCGTGGAAGGTTGCGGGACAA
GCAGTTTACAGGGTGATGTAAAATTTGCTGAGGTCCTTGGACAAATGGGTGCTGAAGTAACCTGGACAGA
GAACTCTGTCACAGTGAAGGGTCCGCCAAGGAATTCTTCCGGAAGGGGACACTTGCGTCCAGTAGATGTG
AACATGAACAAAATGCCAGATGTTGCGATGACTCTTGCTGTGGTTGCCCTTTATGCTGATGGTCGCACTG
CCATTAGAGACGTGGCTAGCTGGAGAGTAAAAGAAACAGAAAGGATGATTGCCATTTGCACAGAACTAAG
AAAGTTGGGAGCAACAGTGGAAGAAGGTCCAGATTATTGTGTGATCACTCCACCAGAGAAATTGAATGTG
ACAGCAATTGACACATACGATGATCACAGAATGGCCATGACTTTCTCGCTTGCTGCCTGTGCAGACGTTC
CTGTCACCATTAAGGACCCAGGTTGCACCCGTAAGAGCTTCCCCGACTACTTTGAAGTTCTTGAAAGATA
CACTAAGCATTAA
>ConSuChr04Cg430010
ATGGCAGTTCACATCAACACCACCAATTTCACCACTTCCAATCTCATTACTCAAAACCCATCTTCAAAGT
CATCATCTTTTTTATCTTTTGGATCCAACTTAAAAAACCCATTAAAAACCCATAATAATAATTATTCTTC
TGTTTCTTGTAATTTGAAAAACAACAAAAACCCATTTAAAGTATCAGCTTTCTCTGCCACTACCACCAAA
GAGAAGCCATCAAAAGCACCTGAAGAAATTGTGTTGAAACCCATTCAAGAAATTTCGGGTACGGTTCATT
TACCCGGATCCAAGTCTTTATCTAATCGGATCCTCCTTCTTGCTGCCCTTTCTGAGGGGACTACTGTTGT
AGACAACTTATTAAACAGCGATGATGTTCATTACATGCTTGGAGCTCTAAGAGCTCTAGGGTTAAATATT
GAAGAAAATGGTGCAATTGAAAGAGCAATCGTAGAAGGTTGTGGTGGTGTATTTCCTGTGGGTAAAGAAG
CCAAGGATGAAATCCAGCTTTTTCTTGGAAAT**GCA**GGAACAGCTATGCGT**CCA**TTGACTGCTGCCGTTAC
TGCTGCTGGTGGAAATTCAAGCTACGTACTAGATGGTGTTCCTCGAATGAGAGAGAGGCCAATTGGTGAT
CTGGTCACTGGTCTAAAACAACTTGGTGCAAATGTTGACTGTTCTCTAGGTACAAACTGCCCACCAGTTC
GTGTAGTTGGAAGTGGAGGCCTTCCTGGTGGAAAGGTGAAATTGTCAGGATCTATAAGTAGTCAATACTT
GACTTCTTTGCTCATGGCAGCTCCCCTTGCACTGGGAGACGTAGAGATAGAAATTATAGATAAATTGATC
TCTGTACCATATGTAGAGATGACACTGAAGTTGATGGAACGGTTTGGGGTTTCAGTAGAACACAGTGATA
CTTGGGATAGATTCTATGTCCGAGGCGGTCAAAAGTACAAGTCACCTGGAAATGCTTATGTGGAAGGTGA
TGCTTCAAGTGCGAGTTACTTCTTAGCTGGTGCTGCCATCACTGGCGGAACTGTCACCGTGGAAGGTTGC
GGGACAAGCAGTTTACAGGGTGATGTAAAATTTGCTGAGGTCCTTGGACAAATGGGTGCTGAAGTAACCT
GGACAGAGAACTCTGTCACAGTGAAGGGTCCGCCAAGGAATTCTTCCGGAAGGGGACACTTGCGTCCAGT
AGATGTGAACATGAACAAAATGCCAGATGTTGCGATGACTCTTGCTGTGGTTGCCCTTTATGCTGATGGC
CCCACTGCCATTAGAGACGTGGCTAGCTGGAGAGTAAAAGAAACAGAAAGGATGATTGCCATTTGCACAG
AACTAAGAAAGCTGGGAGCAACAGTTGAAGAAGGTCCAGATTATTGTGTGATCACTCCACCAGAGAAATT
GAATGTGACAGCGATTGACACATATGATGATCACAGAATGGCCATGGCTTTCTCGCTTGCCGCCTGTGCA
GAGGTTCCTGTCACCATTAAGGACCCAGGTTGCACCCGTAAGACCTTCCCCGACTACTTTGAAGTTCTTG
AAAGATATACTAAGCATTAA

Hairy fleabane – EPSPS copies

>ConBoChr03Bg331930
ATGAAGTCACATCCTTTTTTATCTTTTGGGCCAAAACACAAAAACCCAATTGCCCATTTCTCTGTTTCTT
CTTCCAATAATAAAAATCTTGGAAAAAAATCCACCACCGAGAAACCATCAACGGTGCCGGAAATTGTGTT
GCAACCAATTAAAGAAATCTCGGGTACGGTTAATTTACCCGGTTCCAGGTCTTTGTCTAATCGGATCCTC
CTCCTTGCTGCGCTTGCTGAGGGAACGACCGTTGTTGACAACTTACTCAACAGTGATGATGTTCATTACA
TGCTTGGAGCTTTAAGAACTCTAGGGCTAAACGTTGAGGAGGATGGTGCAATTAAAAGGGCAATTGTGGA
AGGTTGTGGCAGTGTGTTTCCTGTGGGTAAAGAAGCTAAAGATGACATACAGCTTTTTCTTGGGAAT**GCA**
GGAACTGCTATGCGT**CCA**TTGATTGCCGCAGTTACTGCTCTGATTATTTTTTCCAGCTACGTACTAGATG
GCGTTCCTCGTATGAGAGAGAGACCAATAGGTGATTTGGTCACGGGTCTTAAGCAGCTTGGGGCAGATGT
TGACTGTTCTCTTGGGACGAACTGCCCACCCGTACATGTAGTTGGAGGAGGTGGTCTCCCTGGAGGAAAG
GTTAAGTTGTCGGGATCTATTAGTAGTCAATACCTTACTGCTCTGCTTATGGCTTCTCCTCTTGCCCTTG
GGGATGTGGAGATTGAAATCATAGATAAACTAATTTCCATACCATATGTCGAGATGACACTGAAATTAAT
GGAACGGTTCGGCGTCTCGGTAGAACATAGTGATAGTTGGGACCGGTTCTTTATTCGAGGCGGCCAAAAG

TACAAGTCACCTGGAAATGCTTATGTTGAAGGTGATGCATCAAGTGCGAGTTGCTTCTTGGCTGGTGCTG
CCATAACTGGAGGCACCATCACCGTTGAGGGCTGTGGAACAAGTAGTTTGCAGGGTGATGTGAAGTTTGC
GGAGGTACTTGGACAAATGGGTGCGGAAGTAACATGGACTGAGAACTCAGTCACAGTTAAGGGACCACCA
AGAGATTCTTCTGGAAGGAAACATTTACGTGCTGTTGATGTGAACATGAACAAGATGCCTGATGTTGCCA
TGACTCTTGCTGTGGTCGTTCTTTATGTTGATGGCCCTACAGCCATTACAGATGTTGCTAGCTGGAGAGT
TAAAGAAACCGAAAGGATGATTGCCATTTGCACAGAACTCAGAAAATTGGGAGCAACAGTTGAAGAAGGT
CCGGACTATTGTGTGATCACTCCGCCAGAGAAGTTAAACGTGACTTCAATGGACACATATGATGATCATA
GGATGGCCATGGCTTTCTCTCTTTGCTGCTTGTGCAGATGTTCCTGTGACCATTAA
>ConBoChr03Cg347080
ATGGCAGCTACTCACATTAACACCACCAACATTGCCCATAATCTCCAAGCTACCACCAGTCTTTCCAAAA
CCCAAACCCCATCAATAAAGTCACAACCTTTTTTATCTTTTGTGCCAAAACACACAAACCCAATTGCCCA
TTTCTCTGTTTCTTCTAATAATAACAGAAATCTTGGAAAAAAATGTTTAATTGTTTCTGCCGTTGCCACC
ACCGAGAAACCATCAACGGTACCGGAAATTGTGTTGCAACCCATTAAAGAAATATCGGGTACGGTTAATT
TACCCGGGTCCAAGTCTTTGTCTAATCGGATCCTCCTCCTTGCTGCGCTCGCCGAGGGAACGACCATTGT
TGACAACTTACTCAACAGTGATGATGTTCATTACATGCTTGGAGCTTTAAGAACTCTAGGGCTAAACGTT
GAGGAGGATGGTGCAATTAAAAGGGCAATTGTGGAAGGTTGTGGCGGTATGTTTCCTGTGGGTAAAGAAG
CTAAAGATGACATACAGCTTTTTCTTGGGAAT**GCA**GGAACTGCTATGCGT**CCA**TTGACTGCCGCAGTTAC
TGCTGCTGGTGGTAATTCAAGCTACGTACTAGATGGCGTTCCTCGTATGAGAGAGAGACCAATAGGTGAT
TTGGTCACGGGTCTTAAGCAGCTTGGGGCAGATGTTGACTGTTCTCTCGGGACGAACTGCCCACCCGTAC
GTGTAGTTGGAGGAGGTGGTCTCCCTGGAGGAAAGGTTAAGTTGTCGGGATCTATTAGTAGTCAATACCT
TACTGCTCTGCTTATGGCTTCTCCCCTTGCCCTTGGGGATGTGGAGATTGAAATCATAGATAAACTAATT
TCCATACCATATGTCGAGATGACTCTGAAATTAATGGAATGGTTCGGCGTCTCGGTAGAACATAGTGATA
GTTGGGACCGCTTCTTTATTCGAGGCGGCCAAAAGTACAAGTCACCTGGAAATGCTTATGTAGAAGGTGA
TGCATCAAGTGCGAGTTACTTCTTGGCTGGTGCTGCCATAACTGGAGGAACCATCACCGTTGAAGGCTGC
GGAACAAGTAGTTTGCAGGGTGATGTGAAGTTTGCGGAGGTACTTGGACAAATGGGTGCGGAAGTAACAT
GGACTGAGAACTCAGTCACAGTTAAGGGCCCACCAAGGGATTCTTCTGGAAGGAAACATTTACGTGCTGT
TGATGTGAACATGAACAAGATGCCTGATGTTGCCATGACTCTTGCTGTGGTCGCTCTTTATGCTGATGGC
CCTACAGCCATTAGAGATGTTGCTAGCTGGAGAGTTAAAGAAACCGAAAGGATGATTGCCATTTGCACAG
AACTAAGAAAGTTGGGAGCAACAGTTGAAGAAGGTCCAGACTATTGTGTGATCACTCCGCCAGAGAAGTT
AAACGTGACAGCAATAGACACATATGATGATCATAGGATGGCCATGGCTTTCTCTCTTGCTGCTTGTGCA
GATGTTCCTGTGACCATTAAGGACCCTTCTTGCACTCGTAAGACGTTTCCTGATTACTTTGAAGTTCTTC
AAAGATTTGCCCAGCATTAA
>ConBoChr04Ag390530
ATGGCAGTTCACATCAACAACTCCAACATACCCATTTTCAACACTTCCAATCTCACAACCCAAAACCCAT
CTTCAAAGTCATCATCTTTTTTATCTTTTGGATCCAACTTCAAAAACCCATTAAAAAACAATAATAACAA
TAATTATACCTCTGTTTCTTGTAATGTGAAAAACAACAAAAACCCATTTAAAGTATCAGCTTTCTCTGCC
ACTTCCACCAAAGAGAAGCCATCTAAAGCTCCAGAAGAAATTGTGTTGAAACCCATTCAAGAAATTTCGG
GTACGGTCCATTTACCCGGATCCAAGTCTTTGTCTAATCGGATCCTCCTTCTTGCTGCCCTGTCTGAGGG
GACTACTGTTGTAGACAACTTGTTAAACAGTGATGATGTTCATTACATGCTTGGAGCTTTAAGAGTTCTA
GGGTTAAATGTTGAAGAAAATAGTGCAATTAAAAGAGCAATCGTAGAAGGTTGTGGTGGTGTATTTCCCG
TGGGTAAAGAAGCCAAGGATGAAATCCAGCTTTTTCTTGGAAAT**GCA**GGAACAGCTATGCGT**CCA**TTGAC
TGCTGCCGTTACTGCTGCCGGTGGAAACTCAAGCTACATACTAGATGGTGTTCCTCGAATGAGGGAGAGG
CCAATTGGTGATCTGGTCACCGGTCTAAAACAGCTTGGTGCAAATGTTGATTGTTCTCTCGGTACAAACT
GCCCACCGGTTCGTGTAGTTGGAAGTGGAGGCCTTCCTGGTGGAAAGGTGAAATTGTCAGGATCTATAAG
TAGCCAATACTTGACTTCTTTGCTTATGGCGGCTCCTCTTGCACTGGGAGACGTAGAGATAGAAATTGTA
GATAAATTGATCTCTGTACCATATGTGGAGATGACACTTAAGTTGATGGAGCGGTTTGGGGTTTCAGTAG
AACACAGTGATACTTGGGACAGATTCCATGTCCGAGGCGGTCAAAAGTACAAGTCACCTGGAAATGCTTA
TGTGGAAGGTGATGCTTCAAGTGCGAGTTACTTCTTAGCTGGTGCTGCCATCACTGGCGGAACTGTCACC
GTGGAAGGTTGCGGGACAAGCAGTTTACAGGGTGATGTAAAATTTGCTGAGGTCCTTGGACAAATGGGTG
CTGAAGTAACCTGGACAGAGAACTCTGTCACGGTGAAGGGTCCGCCAAGGAATTCTTCCGGAAGGGGACA
CTTGCGTCCAGTAGATGTGAACATGAACAAAATGCCGGATGTTGCGATGACTCTTGCTGTGGTTGCCCTT
TATGCTGATGGCCCCACTGCCATTAGAGACGTGGCTAGCTGGAGAGTAAAAGAAACGGAAAGGATGATTG
CCATCTGCACAGAACTAAGAAAGTTGGGAGCAACAGTCGAAGAAGGTCCAGATTATTGTGTGATCACTCC
ACCAGAGAAATTGAATGTGACAGCAATCGACACATATGATGATCACAGAATGGCCATGGCTTTCTCGCTT
GCCGCCTGTGCAGAGGTTCCTGTCACCATTAAGGACCCGGGTTGCACCCGTAAGACCTTCCCCGACTACT
TTGAAGTTCTTGAAAGATACACTAAGCATTAA
>ConBoChr04B1g441030
ATGGCGGTTCACATCAACAATACAAACATACCCAATTTCAACACTTGCAATCTCACAACCCAAAAGCAAT
CTTCAAAGTCATCATCTTTTTTATCTTTTGGATCCAACTTTAAAAACCCATTAAAAACCAATAATAATAA
TTATTCCCCTGTTTCTTGTAATGTGAAAAACAACCCATTTAAAGTATCAGTTTTCTCTGCCACTACCACC
AAAGAGAAGCCATCAAAAGCTCCAGAAGAAATTGTGTTGAAACCCATTCAAGAAATATCGGGCACGGTTC

ATTTACCCGGATCCAAGTCTTTATCTAATCGGATCCTCCTTCTTGCTGCCCTTTCTGAGGGGACTACTGT
TGTAGACAACTTATTAAACAGTGATGATGTTCATTACATGCTTGGAGCTTTAAGAGCTCTAGGGTTAAAT
GTTGAAGAAAATGGTGCAATTAAAAGAGCAATCGTAGAAGGTTGTGGTGGTGTATTTCCTGTGGGTAAAG
AGGCCAAGGATGAAATCCAGCTTTTTCTTGGAAAT**GCA**GGAACAGCTATGCGT**CCA**TTGACTGCTGCCGT
TACTGCAGCTGGTGGAAATTCAAGCTACGTACTAGATGGTGTTCCTCGAATGAGAGAGAGGCCAATTGGT
GATCTGGTCACCGGTCTAAAACAACTTGGTGCAAATGTTGACTGTTCTCTAGGTACAAACTGCCCACCAG
TTCGTGTAGTTGGAAGTGGAGGCCTTCCTGGTGGAAAGGTGAAATTGTCAGGATCTATAAGTAGTCAATA
CTTGACTTCTTTGCTCATGGCAGCTCCCCTTGCACTGGGAGACGTAGAGATAGAAATTATAGATAAATTG
ATCTCTGTACCATATGTAGAGATGACGCTGAAGTTGATGGAACGGTTTGGGGTTTCAGTAGAACACAGTG
ATACTTGGGACAGATTCTATGTCCGAGGCGGTCAAAAGTACAAGTCACCTGGAAATGCTTATGTGGAAGG
TGATGCTTCAAGTGCGAGTTACTTCTTAGCTGGTGCTGCCATCACTGGTGGAACTGTCACCGTGGAAGGT
TGCGGGACAAGCAGTTTACAGGGTGATGTAAAATTTGCTGAGGTCCTTGGACAAATGGGTGCTGAAGTAA
CCTGGACAGAGAACTCTGTCACAGTGAAGGGTCCGCCAAGGAATTCTTCCGGAAGGGGACACTTGTGTCC
AGTAGATGTGAACATGAACAAAATGCCAGATGTTGCGATGACTCTTGCTGTGGTTGCCCTTTATGCTGAT
GGCCCCACTGCCATTAGAGACGTGGCTAGTTGGAGAGTAAAAGAAACAGAAAGGATGATTGCCATTTGCA
CTGAACTAAGAAAGTTGGGAGCAACAGTCGAAGAAGGTCCAGATTATTGTGTGATCACTCCACCAGAGAA
ATTAAATGTGACAGCAATTGACACATACGATGATCACAGAATGGCCATGGCTTTCTCGCTTGCCGCCTGT
GCAGAGGTTCCTGTCACCATTAAGGACCCAGGTTGCACCCGTAAGACCTTCCCCGACTACTTTGAAGTTC
TTGAAAGATACACTAAGCATTAA
>ConBoChr04B2g441040
ATGCTTGGAGCTTTAAGAGCTCTAGGGTTAAATGTTGAAGAAAATGCTGCAATTAAAAGAGCAATCGTAG
AAGGTTGTGGTGGTGTATTTCCTGTGGGTAAAGAAGCCAATGATGAAATCCAGCTTTTTCTTGGAAAT**GC**
AGGAACAGCTATGCGT**ACA**TTGACTGCTGCCGTTACTGCTGCTGGTGGAAATTCAAGCTACGTACTAGAT
GGTGTTCCTCGAATGAGAGATAGGCCAATTGGTGATCTGGTCACCGGTCTAAAACAACTTGGTGTAAATG
TTGATTGTTCTCTAGGTACAAACTGCCCACCAGTTCGTGTAGTTGGAAGTGGAGGCCTTCCTGGTGGAAA
GGTGAAATTGTCAGGATCTATAAGTAGTGTATACTTGACTTCTTTGCTCATGGCAGCTCCCCTTGCACTG
GGAGACGTAGAGATAGAAATTACAGATAAATTGATCTCTGTACCATATGTACAAATGACACTGAAGTTGA
TGAAACAGTTTGGGGTTTCAGTCGAACACAGTGATACTTTGGACAGATTCCATGTCCGAGGCGGTCAAAA
GTACAAGTCACCTGGAAATGCTTATGTGGAAGGTGATGCTTCAAGTGCGAGTTACTTCTTAGCTGGTGCT
GCCATCACTGGCGGAACTGTCACAGTGGAAGGTTGCGGGACAAGCAGTTTACAGGGTGATGTAAAATTTG
CTGAGGTCCTTGGACAAATGGGTGCTGAAGTAACCTGGACAGAGAACTCTGTCACAGTGAAGGGTCCGCC
AAGGAATTCTTCCGGAAGGGGACACTTGCGTCCAGTAGATGTGAACATGAACAAAATGCCAGATGTTGCG
ATGACTCTTGCTGTGGTTGCCCTTTATGCTGATGGTCGCACTGCCATTAGAGACGTGGCTAGCTGGAGAG
TAAAAGAAACAGAAAGGATGATTGCCATTTGCACAGAACTAAGAAAGTTGGGAGCAACAGTCGAAGAAGG
TCCAGATTATTGTGTGATCACTCCACCAGAGAAATTGAATGTGACAGCAATTGACACATACGATGATCAC
AGAATGGCCATGACTTTCTCGCTTGCTGCCTGTGCAGACGTTCCTGTCACCATTAAGGACCCAGGTTGCA
CCCGTAAGAGCTTCCCCGACTACTTTGAAGTTCTTGAAAGATACACTAAGCATTAA
>ConBoChr04Cg462900
ATGGCAGTTCACATCAACACCACCAATTTCACCACTTCCAATCTCATTTCCCAAAACCCATCTTCAAAGT
CATCATCTTTTTTATCTTTTGGATCCAACTTCAAAAACCCATTAAAAACCCATAATAATAATTATTCTTC
TGTTTCTTGTAATTTGAAAAACAACAAAAACCCATTTAAAGTATCAGCTTTCTCTGCCACTACCACCAAA
GAGAATCCATCAAAAGCACCTGAAGAAATTGTGTTGAAACCCATTCAAGAAATTTCGGGTACGGTTCATT
TACCCGGATCCAAGTCTTTATCTAATCGGATCCTCCTTCTTGCTGCCCTTTCTGAGGGGACTACTGTTGT
AGACAACTTATTAAACAGTGATGATGTTCATTACATGCTTGGAGCTCTAAGAGCTCTAGGGTTAAATGTT
GAAGAAAATGGTGCAATTGAAAGAGCAATCGTAGAAGGTTGCGGTGGTGTATTTCCTGTGGGTAAAGAAG
CCAAGGATGAAATCCAGCTTTTTCTTGGAAAT**GCA**GGAACAGCTATGCGT**CCA**TTGACTGCTGCCGTTAC
TGCTGCTGGTGGAAATTCAAGCTACGTACTAGATGGTGTTCCTCGAATGAGAGAGAGGCCAATTGGTGAT
CTGGTCACTGGTCTAAAACAACTTGGTGCAAATGTTGACTGTTCTCTAGGTACAAACTGCCCACCAGTTC
GTGTAGTTGGAAGTGGAGGCCTTCCTGGTGGAAAGGTGAAATTGTCAGGATCTATAAGTAGTCAATACTT
GACTTCTTTGCTCATGGCAGCTCCCCTTGCAATGGGAGACATAGAGATAGAAATTATAGATAAATTGATC
TCTGTACCATATGTAGAGATGACACTGAAGTTGATGGAACGGTTTGGGGTTTCAGTAGAACACAGTGATA
CTTGGGATAGATTCTATGTCCGAGGCGGTCAAAAGTACAAGTCACCTGGAAATGCTTATGTGGAAGGTGA
TGCTTCAAGTGCGAGTTACTTCTTAGCTGGTGCTGCCATCACTGGCGGAACTGTCACCGTGGAAGGTTGT
GGGACAAGCAGTTTACAGGGTGATGTAAAATTTGCTGAGGTCCTTGGACAAATGGGTGCTGAAGTAACCT
GGACAGAGAACTCTGTCACAGTGAAGGGTCCGCCAAGGAATTCTTCTGGAAGGGGACACTTGCGTCCAGT
AGATGTGAACATGAACAAAATGCCAGATGTTGCGATGACTCTTGCTGTGGTTGCCCTTTATGCTGATGGC
CCCACTGCCATTAGAGACGTGGCTAGCTGGAGAGTAAAAGAAACAGAAAGGATGATTGCCATTTGCACAG
AACTAAGAAAGCTGGGAGCAACAGTTGAAGAAGGTCCAGATTATTGTGTGATCACTCCACCAGAGAAATT
GAATGTGACAGTGATTGACACATATGATGATCACAGAATGGCCATGGCTTTCTCGCTTGCCGCCTGTGCA
GAGGTTCCTGTCACCATTAAGGACCCAGGTTGCACCCGTAAGACCTTCCCGGACTACTTTGAAGTTCTTG
AAAGATATACTAAGCATTAA

>ConBoChr07Ag669780
ATGGCAGCTACTCACATTAACACCACCAACATTGCCCACAATCTCCAAGCTACCACCAGTCTTTCCAAAA
CCCAAACCCCATCAATAAAGTCACAACCTTTTTTATCTTTTGGGCCAAAACACAAAAACCCAATTGCCCA
TTTCTCTGTTTCTTCTAATAATAATAGAAATCTTGGAAAAAAATGTTTAATAGTTTCTGCCGTTGCCACC
ACCGAGAAACCGTCAACGGTGCCGGAAATTGTGTTACAACCCATTAAAGAAATCTCGGGTACGGTTAATT
TACCCGGGTCCAAGTCGTTGTCTAATCGGATCCTCCTCCTTGCTGCGCTTGCTGAGGGAACGACCATTGT
TGACAACTTACTCAACAGTGATGATGTTCATTACATGCTTGGAGCTTTAAGAACTCTAGGGTTAAACGTT
GAGGAGGATGTTGCAATTAAAAGGGCAATTGTGGAAGGTTGTGGCGGTGTGTTTCCTGTGGGTAAAGAAG
CTAAAGATGACATACAGCTTTTTCTTGGGAAT**GCA**GGAACTGCTATGCGT**CCA**TTGACTGCCGCAGTTAC
TGCTGCTGGTGGTAATTCAAGCTACGTACTAGATGGCGTTCCTCGTATGAGAGAGAGACCAATAGGTGAT
TTGGTCACGGGTCTTAAGCAGCTTGGGGCAGATGTTGACTGTTCTCTCGGGACGAACTGCCCTCCCGTGC
GTATAGTTGGTGGAGGTGGTCTCCCTGGAGGAAAGGTTAAGTTGTCGGGATCTATTAGTAGTCAATACCT
TACTGCGCTGCTTATGGCTTCTCCCCTTGCCCTTGGGGACGTGGAAATTGAAATCATAGATAAACTAATT
TCCATACCATATGTCGAGATGACACTGAAATTAATGGAACGGTTCGGCGTCTCGGTAGAACATAGTGATA
GTTGGGACCGGTTCTTTATTCGAGGCGGCCAAAAGTACAAGTCACCTGGAAATGCTTATGTAGAAGGTGA
TGCGTCAAGTGCGAGTTACTTCTTGGCTGGTGCTGCCATAACCGGAGGCACCATCACCGTTGAAGGCTGC
GGAACAAGTAGTCTGCAGGGTGATGTGAAGTTTGCGGAGGTACTTGGACAAATGGGTGCGGAAGTAACAT
GGACTGAGAACTCAGTCACAGTTAAGGGCCCACCAAGGGATTCTTCTGGAAGGAAACATTTACGTGCTGT
TGATGTGAACATGAACAAGATGCCTGATGTTGCCATGACTCTTGCTGTGGTCGCTCTTTATGCTGATGGC
CCTACAGCCATTAGAGATGTTGCTAGCTGGAGAGTTAAAGAAACCGAAAGGATGATTGCCATTTGCACAG
AACTTAGAAAGTTGGGAGCAACAGTTGAAGAAGGTCCGGACTATTGTGTGATCACTCCGCCAGAGAAGTT
AAACGTGACAGCAATAGACACATATGATGATCACAGGATGGCCATGGCTTTCTCTCTTGCCGCTTGTGCA
GATGTTCCTGTGACCATTAAGGATCCTTCTTGCACTCGTAAGACGTTTCCTGATTACTTTGAAGTTCTTC
AAAGATTTGCCAAGCATTAA

6. ARTIGO 4

Exogenous RNAi targeting *phytoene desaturase* in Sumatran fleabane: methodological evaluation and limitations*

*Artigo a ser submetido para a Revista Weed Research

Title: Exogenous RNAi targeting *phytoene desaturase* in Sumatran fleabane: methodological evaluation and limitations

Authors: Paula Sinigaglia Angonese[1], Fatemeh Abdollahi[2], Guilherme Menegol Turra[1], William Augusto Ellert Kroth[1], Inácio João Barbosa[1], Catarine Markus[1], Todd Adam Gaines[2], Aldo Merotto Jr.[1]

[1]Crop Science Department, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

[2]Departament of Agricultural Biology, Colorado State University, Fort Collins, US

Abstract

Sumatran fleabane is a problematic weed in soybean cropping systems, with resistance reported to six modes of action of herbicides, limiting available control options. RNA-based technologies have emerged as a promising alternative for weed management. This study evaluated the foliar uptake of siRNA and dsRNA oligonucleotides and their ability to silence the *PDS* gene in Sumatran fleabane. Uptake assays using fluorescently labeled oligonucleotides showed limited delivery into leaf tissue across all application methods. In silico off-target analysis indicated high sequence similarity with the other plant species *Conyza canadensis*, *Zea mays*, and *Glycine max*, while low similarity was observed with *Apis mellifera* and *Homo sapiens*. None of the oligonucleotide treatments induced foliar bleaching. However, infiltration of siPDS1 and siPDS2 at 2 μM resulted in an approximately 50% reduction in *PDS* expression 24 h after application. Similar, application of dsRNA oligonucleotides in mixtures, either combined during synthesis or mixed immediately before application, led to a reduction in *PDS* transcript levels compared with the control. Foliar uptake of siRNA and dsRNA by Sumatran fleabane was low, likely due to morphological and biochemical barriers. Although effective *PDS* silencing and bleaching symptoms were not obtained, these results represent a first step toward the development of RNA-based control strategies for *Conyza*, a species with a hexaploid genome and significant foliar barriers.

Keywords: siRNA, dsRNA, post-transcriptional silencing, *PDS*, *Conyza sumatrensis*

6.1 Introduction

Herbicide resistance is a major threat and concern in modern agriculture. There are currently 548 unique cases of herbicide resistance involving 21 herbicide modes of action

across 275 weed species (Heap, 2026), which represents a global epidemic (Comont and Neve, 2021). This problem reduces the number of herbicides available for weed control, which increases the costs of managing the plants, as it requires changes in the management approach and can lead to high yield losses (Powles and Yu, 2010).

Sumatran fleabane (*Conyza sumatrensis*), a hexaploid species (*n*=27) (Montgomery *et al*., 2024), is an agronomically important weed in soybean cultivation. The estimated genome size of Sumatran fleabane is approximately 2.15 Gb, with 90,322 predicted protein-coding genes, reflecting extensive gene duplication and genome expansion associated with polyploidization events (Gomes *et al*. 2025). This genomic complexity can contribute to intricate gene regulatory interactions, including functional redundancy, subfunctionalization, and differential gene expression among homeologs, which may enhance the adaptive capacity of this species under environmental and chemical stresses (Adams and Wendel, 2005). In addition, genome duplication poses challenges for gene silencing approaches, as multiple homeologous gene copies may need to be simultaneously targeted to achieve effective knockdown. This requirement, combined with potential sequence divergence among homeologs and functional compensation, may lead to incomplete or variable silencing, ultimately reducing the efficiency of RNAi-based strategies (Travella *et al*., 2006).

There are currently 22 documented cases of resistance involving six different herbicide modes of action (Heap, 2026): EPSPS inhibitor (Group 9), ALS inhibitors (Group 2), PSI inhibitor (Group 22), PSII inhibitor (Group 5), PPO inhibitor (Group 14), and auxin mimics (Group 4). A single population has been reported to be resistant to five of these herbicide groups (de Pinho *et al.*, 2019), which underscores the difficulty of controlling this species. Because of the widespread resistance to the main post-emergence herbicides used to control broadleaf weeds and the lack of new herbicide modes of action, effective management of Sumatran fleabane requires the use of pre-emergence herbicides, mixtures of different modes of action, integration of multiple weed management strategies, and the development of new control approaches (Beckie *et al*., 2019).

The exogenous application of RNA interference (RNAi) is a transcriptional and post-transcriptional gene silencing strategy capable of inducing silencing effects without introducing heritable modifications to the plant genome (Dalakouras *et al.*, 2020; Mai *et*

al., 2021). In this approach, either double-stranded RNA (dsRNA) or pre-processed small interfering RNAs (siRNAs) are delivered to plant tissues. When dsRNA is present in the cytoplasm, it is processed by DICER-LIKE (DCL) ribonucleases into siRNAs of 21-23 nucleotides (Liu *et al*., 2009). Alternatively, exogenously applied siRNAs can bypass this processing step and be directly incorporated into the RNA-induced silencing complex (RISC). In both cases, the guide strand of the siRNA is added into RISC, where it directs sequence-specific cleavage of the target mRNA, resulting in the target gene silencing (Dubrovina and Kiselev, 2019).

In recent years, studies focusing on weed control through the exogenous application of RNAi have increased, indicating a growing trend toward this approach as an alternative and/or complement to herbicide use (Mai *et al.*, 2021; Polito *et al.*, 2024; Zabala-Pardo *et al.*, 2022). dsRNA and siRNA oligonucleotides can be designed to target genes essential for plant survival, and their silencing can result in plant death. Resistance genes identified in specific cases may also be targeted, as silencing these genes could restore herbicide susceptibility in the plant (Dalakouras *et al.*, 2020).

RNA-based molecules exogenous application has several challenges related to uptake, stability, and efficient delivery to target tissues and cells to induce effective gene silencing (Zabala-Pardo *et al.*, 2022). Barriers such as the plant cuticle, cell wall, and nuclease activity limit RNA absorption and persistence after application (Bennett *et al.*, 2020). To overcome these limitations, different delivery strategies, including nanoparticles, adjuvants, and alternative application methods, have been investigated to enhance RNA stability, uptake, and systemic movement in plants (Basso *et al.*, 2025; Shivashakarappa *et al.*, 2025). Evidence from insect and plant disease management demonstrates the viability of this approach, as externally applied RNA molecules can be absorbed by plants and subsequently ingested by insects or taken by pathogens, resulting in effective gene silencing (Cagliari *et al.*, 2019; Vetukuri *et al.*, 2021). Additionally, environmental studies indicate that RNA-based molecules degrade rapidly under field conditions, with no detectable residues within a few days after application, supporting the environmental safety of RNA-based technologies (Parker *et al.*, 2019).

Recent studies have reported gene silencing through the exogenous application of dsRNA or siRNA oligonucleotides in diploid species such as *Mikania micrantha* (Mai *et al.*, 2021), *Oryza sativa* (Polito *et al.*, 2024), and *Setaria viridis* (Castro *et al.*, 2026), and

in tetraploid species *Digitaria insularis* (Cintra *et al.*, 2025). In *M. micrantha*, a reduction in the expression of genes encoding chlorophyll a/b-binding proteins was observed, resulting in leaf yellowing and plant wilting at 14 days after dsRNA spraying (Mai *et al.*, 2021). In *O. sativa*, siRNA spraying promoted reduced expression of the *PDS* (*phytoene desaturase*) gene and induced leaf necrosis symptoms in the treated areas, with no effects observed in untreated parts of the plant (Polito *et al.*, 2024). In *D. insularis*, reduced expression of the *EPSPS* (*5-enolpyruvylshikimate-3-phosphate synthase*) gene and a 44% reduction in plant growth were observed after dsRNA application (Cintra *et al.*, 2025). The study conducted in *S. viridis* observed a modest, non-significant 2.6-fold reduction in the expression of *fumarase* gene, which is involved in carbon metabolism, 48 h after treatment with dsRNA delivered using chitosan-based nanoparticles (Castro *et al.*, 2026). Despite the incomplete gene silencing and the absence of plant death, the results obtained across different species represent an important initial step and indicate the potential of gene silencing induction via exogenous RNA application as a strategy for weed control.

PDS is a key enzyme in the carotenoid biosynthesis pathway, and loss of its function results in a bleaching phenotype due to impaired carotenoid production. This easily detectable phenotype makes *PDS* a widely used target for gene silencing studies (Qin *et al.*, 2007). In this study, we aimed to evaluate the absorption of siRNA and dsRNA oligonucleotides in Sumatran fleabane leaves and their effect on silencing the *PDS* gene.

6.2 Materials and Methods

6.2.1 siRNA and dsRNA design and synthesis

The coding sequence of the *PDS* gene from *Arabidopsis thaliana* (NM_117498.4) was used as a query against the Sumatran fleabane genome (assembly GCA_047495845.1) to identify homologous sequences for this gene. Two matches were identified on chromosomes 2A (CM104895.1) and 7C (CM104912.1), with E-values of 2e-36 and 5e-39, respectively, and percent identities of 80.95% and 82.14%. These sequences were used to design siRNA and dsRNA oligonucleotides targeting both gene copies to silence the *PDS* gene in Sumatran fleabane plants. No homologous copy was identified on chromosomes corresponding to the B genome, based on the current genome assembly.

Four 21-bp siRNAs (siPDS1, siPDS2, siPDS3, and siPDS4) were designed to target different regions of the coding sequence of the Sumatran fleabane *PDS* gene (Table 1). Conserved regions between the two gene copies, with low potential for secondary structure formation, were selected using MEGA (https://www.megasoftware.net/) for sequence alignment and the RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) for RNA secondary structure prediction. The siDirect 2.0 tool (http://sidirect2.rnai.jp/design.cgi) was used to assist in selecting siRNA sequences with optimal seed-duplex stability, GC content, and to avoid sequences with four or more identical nucleotides in sequence. The empirical criteria proposed by Amarzguioui and Prydz (2004), Reynolds *et al*. (2004), and Ui-Tei *et al*. (2004) were also considered in the design for effective gene silencing. Each siRNA consisted of 21 nucleotides, 19 complementary to the target sequence and 2-nucleotide 3′ overhangs (Elbashir *et al.*, 2001). To enhance the association with the RISC complex, the siRNAs were modified with a 5′ phosphate group (Schwarz *et al.*, 2003), and a 2′-O-methylation was introduced at the 3′ ribose to increase siRNA stability (Ji and Chen, 2012). These modifications were effective in inducing gene silencing in *O. sativa* (Polito *et al.*, 2024). The siRNAs were synthesized in vitro by Sigma-Aldrich with HPLC-grade purification.

Table 1. siRNA oligonucleotide sequences with corresponding modifications for the *PDS* gene of Sumatran fleabane.

Oligonucleotide	passenger 5'-3' (sense)			
	5' modification	Sequence	3' modification	Overhang
siPDS1	3' Phosphorylation	GGAAUUUGGGCUAUCUUGA	-	GG
siPDS2	3' Phosphorylation	GCAAUUCCUUACUUCAAGA	-	AG
siPDS3	3' Phosphorylation	CCAUUAGAGGGAUUCUAUU	-	UA
siPDS4	3' Phosphorylation	GGAAUUUGGGCUAUCUUGA	-	UU
Oligonucleotide	guide 5'-3' (antisense)			
	5' modification	Sequence	3' modification	Overhang
siPDS1	3' Phosphorylation	UCAAGAUAGCCCAAAUUCC	2′-O-methyl	AU
siPDS2	3' Phosphorylation	UCUUGAAGUAAGGAAUUGC	2′-O-methyl	UU
siPDS3	3' Phosphorylation	AAUAGAAUCCCUCUAAUGG	2′-O-methyl	AG
siPDS4	3' Phosphorylation	UCAAGAUAGCCCAAAUUCC	2′-O-methyl	UU

Three dsRNA sequences (dsPDS1, dsPDS2, dsPDS3) targeting the *PDS* gene were synthesized using the MEGAscript (ThermoFisher) according to the manufacturer's instructions. PCR templates for in vitro transcription were generated from cDNA of Sumatran fleabane using primers containing the T7 RNA polymerase promoter sequence (Table 2). The amplified fragments were confirmed by electrophoresis on a 1.5% agarose gel. The dsRNA sequences were designed following the same principles as siRNA, ensuring coverage of both gene copies and targeting regions with minimal predicted secondary structure. Fragments ranging from 150 to 300 bp were prioritized to maximize dsRNA yield.

Table 2. dsRNA oligonucleotide sequences for the *PDS* gene of Sumatran fleabane.

Oligonucleotide	T7 sequence + Forward 5'-3'	T7 sequence + Reverse 5'-3'	Size (bp)
dsPDS1	**TAATACGACTCACTATAGGGAGA** TGATATTATTGACGCTACGATGA	**TAATACGACTCACTATAGGGAGA** GTAATGGACGGCAAGGTTCACA	158
dsPDS2	**TAATACGACTCACTATAGGGAGA** GTTATAAACGTTCATATATGG	**TAATACGACTCACTATAGGGAGA** CGGAAATAGTCTTGAAAGTTC	237
dsPDS3	**TAATACGACTCACTATAGGGAGA** AGGTCTCGCTGGTTTATCAAC	**TAATACGACTCACTATAGGGAGA** TGGGAAATCAAACCGACTAAA	280

The specificity of all siRNA and dsRNA oligonucleotides was evaluated in silico using NCBI BLAST (https://www.ncbi.nlm.nih.gov/) against *Conyza canadensis*, *Glycine max*, *Zea mays*, *Apis mellifera*, and *Homo sapiens* genomes.

6.2.2 siRNA and dsRNA uptake by Sumatran fleabane leaves

No studies have been reported on the uptake of siRNA and dsRNA by *Conyza* leaves. Therefore, before evaluating the efficacy of oligonucleotides targeting the *PDS* gene, preliminary evaluations of fluorescent siRNA and dsRNA uptake in Sumatran fleabane leaves were conducted. For siRNA evaluation, the MISSION® siRNA Fluorescent Universal Negative Control #1, 6-FAM (Sigma-Aldrich) was used. For dsRNA, fluorescently labeled dsRNA molecules were synthesized incorporating 20% fluorescent UTP (Cy3-UTP, APExBIO) using the MEGAscript™ in vitro transcription kit (Thermo Fisher Scientific), following the manufacturer's instructions.

Fluorescent siRNA was applied at concentrations of 1 and 5 µM using three delivery methods: handheld airbrush spraying (SP), foliar infiltration (INF), and incubation (IC). For spray application, 100 µL of siRNA solution was applied per leaf to the adaxial surface of Sumatran fleabane plants at the 6-8 leaf stage using a handheld airbrush connected to compressed air at 15 psi, positioned approximately 5 cm from the leaf surface, until uniform coverage was achieved. For foliar infiltration, 100 µL of siRNA solution was infiltrated into the abaxial leaf surface using a needleless syringe with gentle pressure until the infiltrated area became visibly saturated. Incubation (IC) was evaluated using detached mature leaves incubated in 1 µM fluorescent siRNA solution for 12 h in the dark at 24 °C. For dsRNA evaluation, 100 µL per leaf was applied at concentrations of 1 and 2 µM by spraying on both adaxial and abaxial leaf surfaces or by foliar infiltration in Sumatran fleabane plants at the 6-8 leaf stage. Following all treatments, plants or detached leaves were maintained in the dark for 24 h after application to minimize RNA degradation. Leaves were then collected for foliar fluorescence quantification using a SpectraMax iD3 microplate reader (Molecular Devices) and for imaging analysis with an ImageXpress Pico Automated Cell Imaging System (Molecular Devices).

6.2.3 *PDS* siRNA and dsRNA efficacy in Sumatran fleabane

As none of the siRNA or dsRNA application methods resulted in efficient oligonucleotide absorption by Sumatran fleabane leaves, plants were treated with four siRNA oligonucleotides (siPDS1, siPDS2, siPDS3, and siPDS4) and three dsRNA oligonucleotides (dsPDS1, dsPDS2, and dsPDS3) using different application methods to evaluate their efficacy. For siRNA, applications were performed using airbrush spraying at a concentration of 2 µM, and by foliar infiltration at concentrations of 2 µM and 10 µM, with a total volume of 100 µL per leaf in plants at the 6-8 leaf stage on the adaxial leaf surface. Leaf tissue was collected 24 hours after oligonucleotide application for RNA extraction and gene expression analysis. For the infiltration treatment with 2 µM oligonucleotide concentration, the plants were also sampled 48 hours after treatment.

For dsRNA, applications were performed exclusively by foliar spraying at concentrations of 2, 3, and 5 µM on both the adaxial and abaxial leaf surfaces, using a volume of 100 µL per leaf. The 3 µM dsRNA treatment was applied with the adjuvant

Silwet^{TM} at 0.05%, and leaf tissue was collected 24 h after application. The dsRNA treatment at 5 µM was applied in a solution containing 1% polyethylene glycol (PEG) and 5 mM phosphate buffer and was reapplied three days after the initial application. Leaf tissue was collected 72 h after the final treatment. The dsRNA treatment at 2 µM combined with 1% PEG was applied during three consecutive days to the same plants, and leaf tissue was collected 24 h after the final application. For some treatments, the three oligonucleotides were mixed either during synthesis or immediately before spraying to evaluate their combined effect.

All assays included a control treatment consisting of the respective spray solutions without RNA-based molecules. For all siRNA and dsRNA evaluations, following application, plants were incubated in the dark for 24 h and subsequently monitored for 21 days to assess the development of bleaching symptoms characteristic of *PDS* gene silencing. A foliar application of clomazone (Gamit® 500 g L^{-1}) at a rate of 2 L ha^{-1} was included as a positive control for bleaching symptoms.

RNA extraction from the collected leaves was performed with Direct-zol RNA Miniprep Plus (Zymo Research), and cDNA was synthesized using ProtoScript® II First Strand cDNA Synthesis Kit (New England BioLabs). A set of primers (PDS_1F: TTTGCACCTGCAGAAGAATG and PDS_1R: GGCAAGGTTCACAGTCAGGT) covering both Sumatran fleabane *PDS* copies was designed for expression analysis. The fragment size was 187bp, and the annealing temperature was 60°C.

qPCR reactions were carried out in a final volume of 10 µL, containing 5 µL of cDNA (50 ng μL^{-1}), 0.2 µL of each primer (forward and reverse) at a final concentration of 0.2 µM, and 4.6 µL of PerfeCTa SYBR® Green SuperMix (QuantaBio). qPCR conditions were 95 °C for 3 min; 35 cycles of 95 °C for 10 s, Ta for 30 s, 65 °C for 5s and 72 °C for 30 s in CFX96 Real-time System (Bio-Rad). For each treatment, three biological replicates and three technical replicates were used. Gene expression was calculated relative to the untreated samples using the $2^{-\Delta\Delta Ct}$ method (Dussault e Pouliot, 2006). The housekeeping *HSP70* (*heat shock protein-70*) gene was used as a calibrator (Moretti *et al*., 2017). The data were compared according to ANOVA, and when significant ($p \leq 0.05$), the averages were compared according to Tukey 5%.

6.3 Results and Discussion

6.3.1 Low foliar uptake of siRNA and dsRNA in Sumatran fleabane

The uptake of fluorescently labeled siRNA oligonucleotides by Sumatran fleabane leaves was evaluated using different application methods. The treatments with higher siRNA concentrations, SP 5 µM, INF 5 µM, and IC 1 µM, resulted in greater fluorescence detected in the leaf wash solution, indicating lower uptake by the leaves (Figure 1A). In contrast, internal leaf fluorescence was generally low across treatments. Incubation with 1 µM and infiltration with 1 µM and 5 µM siRNA resulted in the highest internal fluorescence levels (Figure 1B). Confocal microscopy further confirmed minimal variation in internal fluorescence among treatments with fluorescence and the no-siRNA control (0 µM) (Figure 1C).

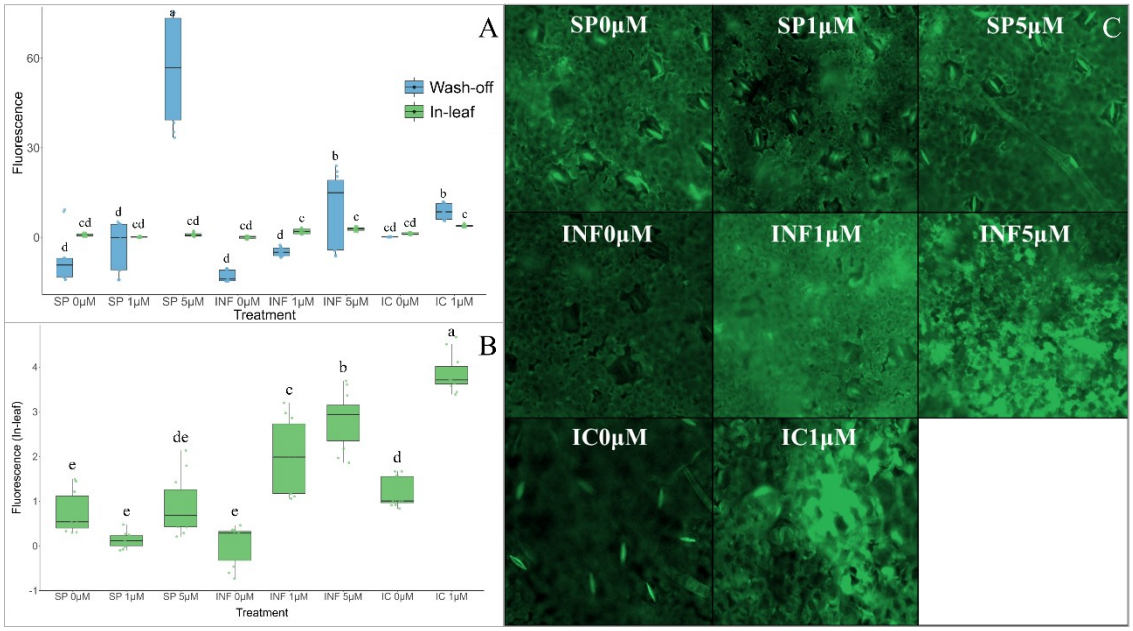


Figure 1. Fluorescence content in wash-off and in-leaf (A) and in-leaf only (B), following foliar application of fluorescently labeled siRNA in Sumatran fleabane using spraying (SP), infiltration (INF), and incubation (IC) at 0, 1, and 5 µM concentrations. Representative confocal microscopy images (35 µm depth, 120 ms exposure time) of leaf sections showing siRNA fluorescence in the tissue (C). Different letters indicate statistically significant differences among treatments according to LSD's test at 5% significance level.

The foliar uptake of fluorescent-labeled dsRNA oligonucleotides in Sumatran fleabane leaves was also low compared with the amounts recovered in the wash solutions

following spray application and infiltration at 1 µM (Figure 2A). However, in leaves that were slightly injured by spray pressure and in which part of the leaf trichomes was removed using adhesive tape, higher fluorescence levels were detected within the leaf tissue following spray application at 2 µM (Figure 2A). Fluorescent signals were observed inside treated leaves, although their distribution was not uniform (Figure 2B).

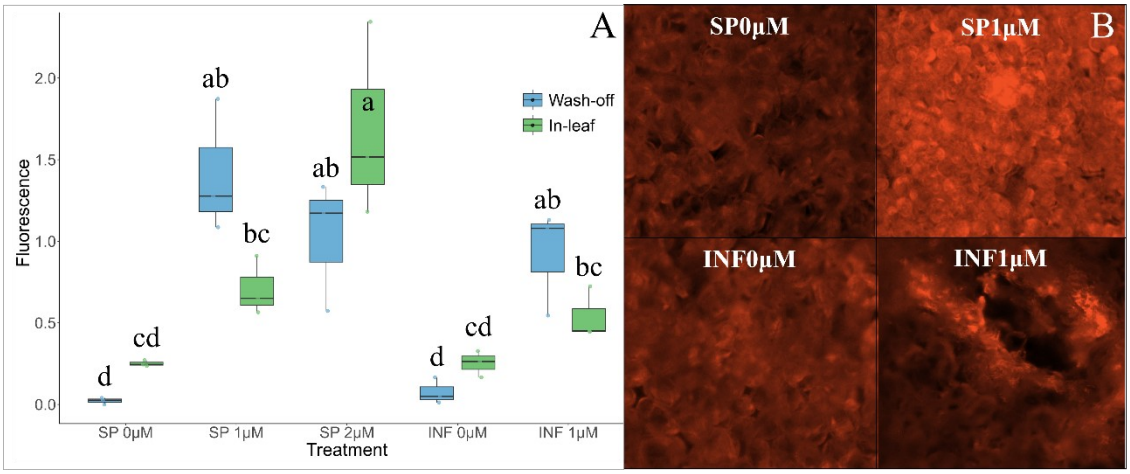


Figure 2. Fluorescence content in the wash-off solution and in-leaf tissue in non-injured and injured leaves (A) following foliar application of fluorescently labeled dsRNA to Sumatran fleabane by spraying (SP) and infiltration (INF) at concentrations of 0, 1, and 2 µM. Representative confocal microscopy images (35 µm depth, 800 ms exposure time) of leaf sections showing dsRNA fluorescence in the tissue (B). Different letters indicate statistically significant differences among treatments according to LSD's test at the 5% significance level.

Conyza species exhibit high trichome density and a thick cuticle on both abaxial and adaxial leaf surfaces, with a particularly high frequency of trichomes on the adaxial surface (Baccin *et al.*, 2023; Silva *et al.*, 2025). Trichomes are specialized epidermal structures that act as a physical barrier and can intercept and retain herbicides and similar compounds, thereby influencing their absorption and, consequently, their effectiveness in plant control (Liu *et al*., 2023). It is possible that a similar mechanism occurred with dsRNA and siRNA molecules following foliar application; however, to date, no studies have reported this effect in *Conyza* species.

The plant cuticle is the first external chemical barrier of the leaf. It has a predominantly lipophilic composition that prevents excessive water loss, acts as a physical barrier, and reduces the absorption of water-soluble compounds (Arya *et al.*, 2021). RNA-based molecules are water-soluble and, because of the composition of the

cuticle, may face limitations in penetrating this barrier. A study in *Amaranthus palmeri* evaluated the uptake of fluorescently labeled siRNA (Cy3) through the adaxial leaf surface using the adjuvant Silwet L-77 at 0.5%. Most of the applied Cy3-siRNA was retained on the leaf surface, given its molecular size and relatively high-water solubility (Bennett *et al.*, 2020), which is consistent with the results observed in Sumatran fleabane. In contrast, studies conducted with monocotyledonous species have shown that the use of Silwet at concentrations of 0.01% and 0.08% was enough to enhance the uptake of dsRNA and siRNA oligonucleotides (Cintra *et al.*, 2025; Polito *et al.*, 2024). In the present study, this adjuvant was also tested in preliminary evaluations; however, it caused phytotoxic injury even at low concentrations (0.005% and 0.01%) (data not shown) and was not included in subsequent experiments.

To enhance cuticle penetration of siRNA, the induction of slight leaf injury has been evaluated in some studies. Spraying abrasive particles with sizes >2 μm, applied together with or following siRNA application, resulted in improved siRNA penetration through the cuticle (Bennett *et al.*, 2020). Similarly, mechanical abrasion using sandpaper enhanced siRNA penetration and RNAi silencing after adaxial foliar application (Bennett *et al.*, 2020; Huang *et al*., 2018). In the present study, leaves injured by spray pressure and leaves in which part of the trichomes were removed using adhesive tape exhibited higher fluorescence levels within the leaf tissue, suggesting increased penetration of the applied molecules; however, this increase was not sufficient to deliver a biologically effective amount of RNA-based molecules into the leaf.

Nuclease activity on the leaf surface may also have affected molecule absorption in Sumatran fleabane. The presence of extracellular nucleases in plants suggests that RNA degradation can limit the ability of foliar-applied siRNAs to access plant cells, as demonstrated in *Nicotiana benthamiana* leaves (Bennett *et al.*, 2020). In that study, siRNA applied together with a nuclease inhibitor or polybrene, which binds and protects double-stranded RNA, remained detectable up to 24 h after application. While siRNA applied in the absence of a nuclease inhibitor was no longer detectable as early as 6 h post-application (Bennett *et al.*, 2020).

6.3.2 siRNA and dsRNA oligonucleotides specificity

Off-target effects of RNA-based molecules are primarily elicited by complementary base pairing between siRNAs derived from si/dsRNA and unintended mRNA targets. To evaluate this possibility, we analyzed the degree of sequence complementarity between the si/dsRNA molecules and potential off-target in other species. BLASTn analysis revealed that all siRNAs had 100% identity and coverage with *C. canadensis* and *G. max,* and also matched *Z. mays* with high identity (Table 3). Potential off-target matches were also detected in *A. mellifera* and *H. sapiens*, but with lower alignment coverage and/or sequence identity compared with the target plant species. BLASTn analysis of the designed dsRNAs showed that all fragments showed 100% identity with *C. canadensis*, and also displayed high similarity to *G. max* and *Z. mays*. Low matches were detected in *H. sapiens* and *A. mellifera*, suggesting minimal off-target potential in these non-target organisms (Table 3).

Table 3. Query coverage and percent identity of siRNA and dsRNA oligonucleotides targeting the *PDS* gene of Sumatran fleabane across non-target species genomes.

Oligonucleotide	*Conyza canadensis*		*Glycine max*		*Zea mays*		*Apis mellifera*		*Homo sapiens*	
	query cover	per ident	query cover	per ident	query cover	per ident	query cover	per ident	query cover	per ident
siPDS1	100%	100%	100%	100%	100%	100%	79%	100%	100%	95%
siPDS2	100%	100%	100%	100%	89%	100%	100%	68-95%	89-95%	100%
siPDS3	100%	100%	100%	100%	100%	100%	89%	95%	100%	100%
siPDS4	100%	100%	100%	100%	100%	100%	79%	100%	100%	95%
dsPDS1	100%	100%	100%	90-100%	78-91%	95-100%	65-91%	100%	86-87%	95-100%
dsPDS2	100%	100%	90-100%	95-100%	90-100%	90-100%	90%	95-100%	86-100%	95-100%
dsPDS3	100%	100%	86-95%	90-95%	81-100%	94-100%	76-100%	100%	71-100%	95-100%

There is concern regarding off-target effects of RNA-based molecules on non-target organisms, which may be detrimental to crops, insects, and humans. To address this risk, initial in silico analyses are commonly performed to assess the potential complementarity of si/dsRNA molecules to the genomes of non-target species. A review evaluated in silico the degree of sequence similarity between published dsRNA molecules and the genome

of *A. mellifera* and identified 101 insecticidal RNAs sharing high sequence similarity with genomic regions in this species. The authors also reported that off-target sequence similarity increased significantly as dsRNA length increased (Mogren and Lundgren, 2017). Other studies conducted with different insect species have shown that dsRNAs with >80% sequence identity to target genes efficiently triggered RNAi, and that dsRNAs containing ≥16 bp segments of perfectly matched sequences or >26 bp segments of near-perfect matches with one or two sparsely distributed mismatches were also capable of inducing RNAi (Chen *et al.*, 2021). In our study, the designed siRNA and dsRNA molecules showed high similarity with the evaluated plant genomes and low similarity with *A. melifera* and humans. However, sequence similarity alone does not necessarily imply that RNAi will occur in the non-target organism.

6.3.3 Limited *PDS* gene silencing following siRNA oligonucleotide application in Sumatran fleabane

No bleaching symptoms were observed with any of the siRNA oligonucleotides or application methods. At 24 h after application, no significant differences in *PDS* gene expression were observed between the no-siRNA control and siRNA treatments when applied by foliar spraying at 2 µM (Figure 3A) or by infiltration at 10 µM (Figure 3C). In contrast, infiltration at 2 µM with siPDS1 and siPDS2 resulted in an approximately 50% reduction in *PDS* expression compared with the other treatments at 24 h after siRNA application (Figure 3B). A similar reduction was observed for siPDS3 at 48 h after infiltration at 2 µM, however, at this time point, *PDS* expression in plants treated with siPDS1 and siPDS2 did not maintain the silencing pattern observed at 24 h (Figure 3D).

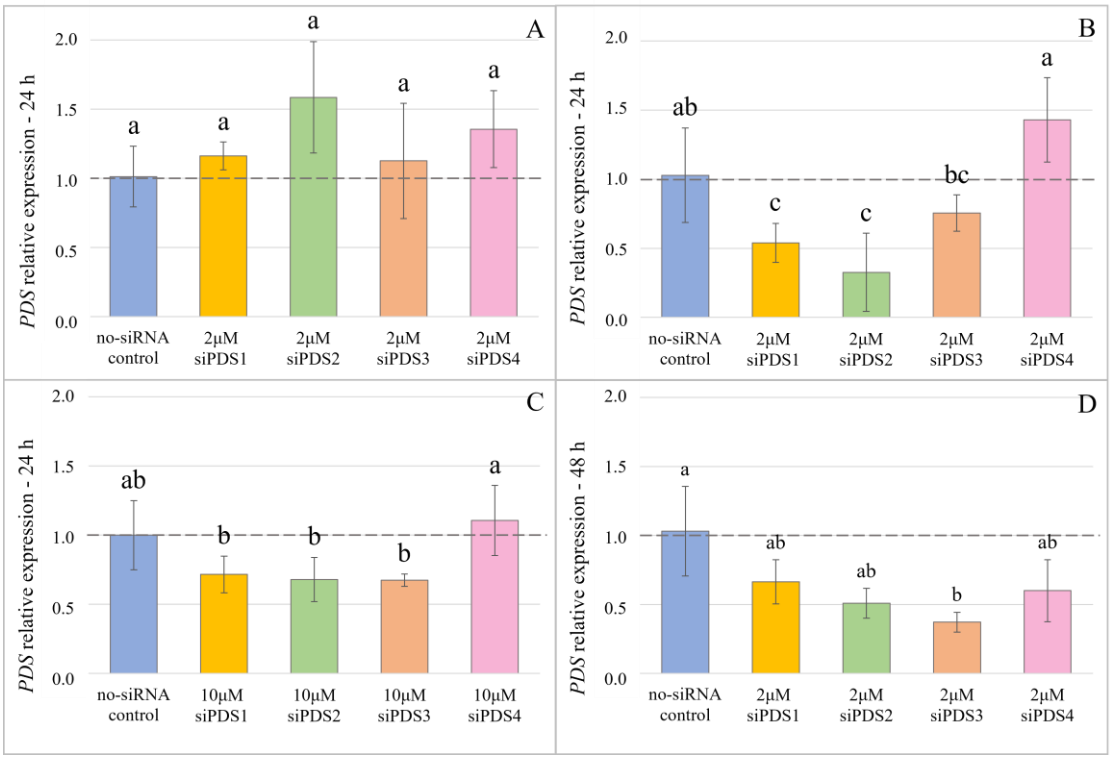


Figure 3. Relative expression of the *PDS* gene in Sumatran fleabane 24 h after application of four siRNA oligonucleotides by foliar spraying at 2 µM (A), infiltration at 2 µM (B), infiltration at 10 µM (C), and 48 h after infiltration at 2 µM (D). Different letters indicate statistically significant differences among treatments according to Tukey's test at 5% significance level.

Oligonucleotides of siRNA designed to target the *PDS* gene induced foliar necrosis symptoms in *O. sativa* plants following partial gene silencing, but bleaching symptoms were not observed (Polito *et al.*, 2024). Different from *O. sativa*, a diploid monocotyledonous species, Sumatran fleabane is a dicotyledonous species with a hexaploid genome, containing six sets of chromosomes. In the present study, only two *PDS* gene copies were identified in Sumatran fleabane based on the more complete whole genome sequencing available (Gomes *et al.*, 2025), nevertheless, the presence of an additional copy cannot be ruled out. If the designed siRNA oligonucleotides did not effectively target all existing *PDS* copies, residual activity of the PDS protein could still occur, preventing complete gene silencing and the manifestation of bleaching symptoms. Notably, even in *O. sativa*, a species with a simpler genome, partial *PDS* silencing was insufficient to induce bleaching and cause plant death (Polito *et al.*, 2024). Furthermore, the limited foliar uptake of siRNA molecules observed in Sumatran fleabane likely resulted in low intracellular concentrations, which may have been insufficient to induce the level of gene silencing required to trigger visible phenotypic effects. Taken together,

genomic redundancy associated with polyploidy and inefficient delivery of siRNA into leaf tissues may explain the absence of bleaching symptoms.

6.3.4 Limited *PDS* gene silencing following exogenous dsRNA application in Sumatran fleabane

The dsPDS1, dsPDS2, and dsPDS3 fragments, with lengths of 158, 237, and 280 bp, respectively, were efficiently synthesized in the laboratory, with final concentrations ranging from 700-900 ng μL^{-1} and showing a single band of the expected size on agarose gel electrophoresis (Figure 4A).

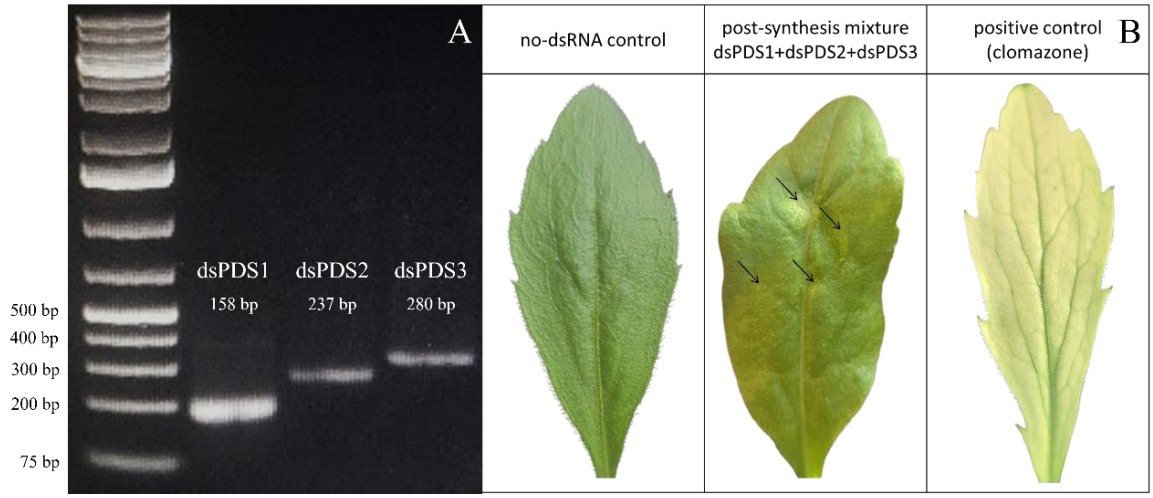


Figure 4. Agarose gel electrophoresis of the synthesized dsPDS1, dsPDS2, and dsPDS3 fragments (A) and representative images of Sumatran fleabane leaves three days after treatment application (B).

Application of dsRNA oligonucleotides also did not result in bleaching symptoms in any of the evaluated treatments. No differences in *PDS* gene expression were observed between the no-dsRNA control and the treatments with 3 µM or 5 µM dsRNA at 24 h after application (Figure 5A) or at 72 h after the last dsRNA application when plants were treated twice (Figure 5B). However, in the assay in which treatments were applied to the same plants for three consecutive days at a concentration of 2 µM, variation in *PDS* gene expression was observed. When dsPDS1, dsPDS2, and dsPDS3 were applied individually, gene expression was equal to the no-dsRNA control. In contrast, application of the three dsRNA oligonucleotides in mixture, either combined during synthesis (mix

1) or mixed immediately before application (mix 2), resulted in a decrease in *PDS* gene expression relative to the control (Figure 5C).

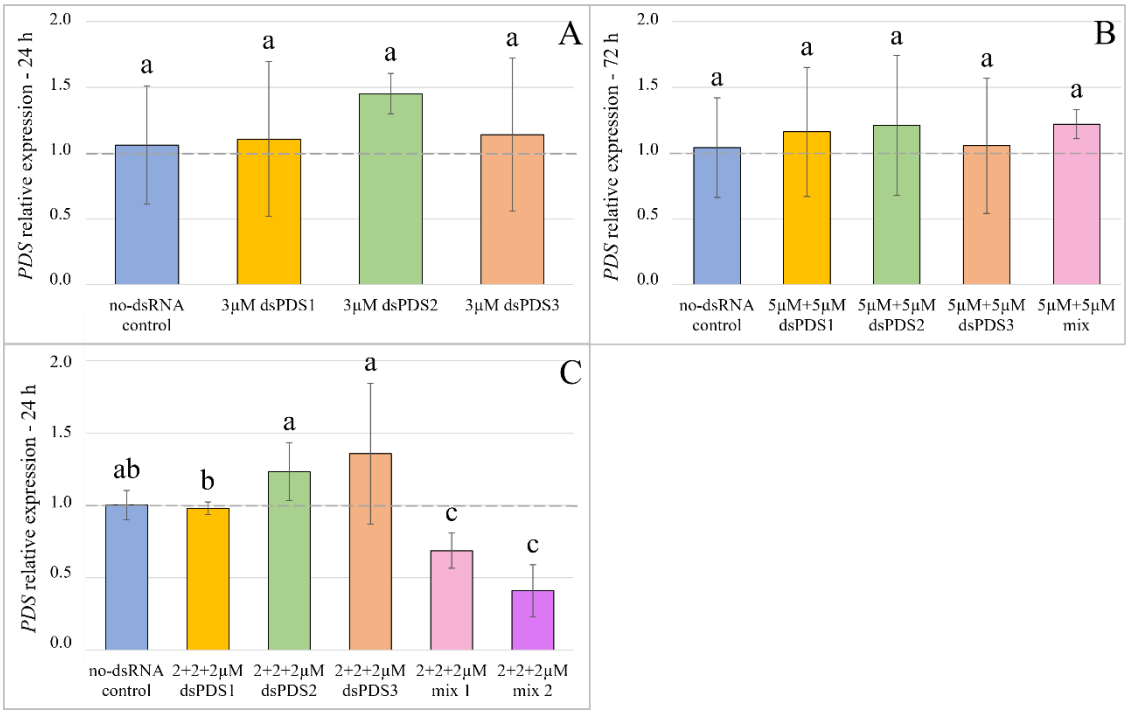


Figure 5. Relative expression of the *PDS* gene in Sumatran fleabane 24 h after application of three dsRNA oligonucleotides by foliar spraying at 3 µM (A); 72 h after the last application of the three dsRNAs applied individually or in combination post-synthesis (mix) at 5 µM in two applications (B); and 24 h after application of 2 µM for three consecutive days of the three dsRNAs applied individually, as a mixture during synthesis (mix 1) or as a post-synthesis mixture (mix 2) (C). Different letters indicate statistically significant differences among treatments according to Tukey's test at the 5% significance level.

Similar to the approach used in the present study, dsRNA was applied via foliar spraying to *D. insularis* plants to silence the *EPSPS* gene, resulting in reduced plant biomass and partial silencing of the target gene (Cintra *et al.*, 2025). In that study, *EPSPS* dsRNA was synthesized through expression in *Escherichia coli* using a cloning vector, allowing the production of high concentrations of the oligonucleotide. In contrast, for Sumatran fleabane, we synthesized dsRNA exclusively by in vitro amplification of the *PDS* gene using primers containing T7 promoters to generate double-stranded RNA. This approach resulted in relatively low dsRNA concentrations, ranging from 500 to 800 ng μL^{-1}, which limited the application of higher volumes and concentrations to the plants and restricted the evaluation of alternative delivery methodologies. Therefore, dsRNA

synthesis using a bacterial expression system may represent a promising alternative for generating larger quantities of oligonucleotides for functional evaluation.

In *S. viridis*, dsRNA uptake was significantly improved through the use of chitosan-dsRNA nanoparticles, and the molecules were efficiently detected within leaf tissues. However, consistent silencing of the *fumarase* gene and clear phenotypic effects were not observed (Castro *et al.*, 2026). RNAi induces a gradual depletion of the target protein through mRNA degradation, a process that may require days to weeks to result in visible phenotypic effects, depending on the role of the target protein. In the case of PDS, phenotypic responses are generally expected to occur more rapidly due to its direct role in carotenoid biosynthesis; however, limited uptake may still constrain the magnitude of the silencing response. Therefore, the use of delivery systems such as nanoparticles represents a promising strategy to enhance RNA uptake and potentially improve the effectiveness of exogenous RNAi approaches in Sumatran fleabane.

The use of both dsRNA and siRNA in the present study aimed to evaluate whether differences in molecular form would result in distinct silencing outcomes in Sumatran fleabane. Exogenously applied dsRNA (200-600 nt) requires cellular uptake followed by processing by DCL ribonucleases to generate multiple siRNAs, which can promote a more sustained silencing response (Dalakouras *et al.*, 2020; Dubrovina and Kiselev, 2019). In turn, siRNAs are already in their functional size range (21-23 nt) and can bypass DCL processing, being directly loaded into the RISC, which is generally associated with a faster onset of silencing (Li and Rana, 2012). Due to their smaller size, siRNAs were expected to exhibit improved penetration through leaf surface barriers compared with dsRNAs, particularly in species with thick cuticles and high trichome density (Bennett *et al.*, 2020). However, despite these theoretical differences, both RNA forms exhibited limited foliar uptake in Sumatran fleabane, indicating that delivery efficiency rather than RNA structure was the primary constraint under the experimental conditions tested.

6.4 Conclusion

The results obtained in this study indicate low foliar absorption of siRNA and dsRNA oligonucleotides by Sumatran fleabane leaves, likely due to the high trichome density, the barrier imposed by the cuticle, and nuclease activity on the leaf surface. Under single-application conditions, the concentrations of siRNA and dsRNA were insufficient

to enable consistent *PDS* gene silencing or the development of visible bleaching symptoms. However, recurrent applications resulted in a measurable reduction in *PDS* expression, indicating that gene silencing can be achieved under sustained exposure conditions, although at limited efficiency and dependent on sufficient RNA availability.

Overall, these findings represent an important first step toward the development of RNA-based control strategies for *Conyza*, a species with a hexaploid genome and foliar barriers to the uptake of exogenous molecules. The results highlight the need for more efficient delivery methods that can overcome the physical barriers imposed by trichomes and the cuticle, as well as improved synthesis approaches for siRNA and dsRNA, which enable the application of higher oligonucleotide concentrations to plants.

Acknowledgement

The authors thank Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS; Project RITEs 22/2551-0000394-0), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Project 442144/2023-7), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support.

References

Adams KL, Wendel JF. Polyploidy and genome evolution in plants. Curr Opin Plant Biol 2005;8:135–41. https://doi.org/10.1016/j.pbi.2005.01.001.

Amarzguioui M, Prydz H. An algorithm for selection of functional siRNA sequences. Biochem Biophys Res Commun 2004;316:1050–8. https://doi.org/10.1016/j.bbrc.2004.02.157.

Arya GC, Sarkar S, Manasherova E, Aharoni A, Cohen H. The Plant Cuticle: An Ancient Guardian Barrier Set Against Long-Standing Rivals. Front Plant Sci 2021;12:663165. https://doi.org/10.3389/FPLS.2021.663165.

Baccin LC, Albrecht AJP, Araújo L da S, Dotta MA, Pedroso RM, Victoria Filho R. Glyphosate-resistant hairy fleabane (*Conyza bonariensis*) exhibits a larger number of trichomes and altered stomatal density relative to the susceptible counterpart. Bioscience Journal 2023;39:e39047–e39047. https://doi.org/10.14393/BJ-V39N0A2023-64343.

Basso MF, Vásquez DDN, Campos-Pinto ER, Pinheiro DH, Cruz B, Maktura GC, et al. Progress and Opportunities of In Planta and Topical RNAi for the Biotechnological

Control of Agricultural Pests. Agronomy 2025;15:859. https://doi.org/10.3390/AGRONOMY15040859/S1.

Beckie HJ, Ashworth MB, Flower KC. Herbicide Resistance Management: Recent Developments and Trends. Plants 2019, Vol 8, Page 161 2019;8:161. https://doi.org/10.3390/PLANTS8060161.

Bennett M, Deikman J, Hendrix B, Iandolino A. Barriers to Efficient Foliar Uptake of dsRNA and Molecular Barriers to dsRNA Activity in Plant Cells. Front Plant Sci 2020;11:531649. https://doi.org/10.3389/FPLS.2020.00816/BIBTEX.

Cagliari D, Dias NP, Galdeano DM, dos Santos EÁ, Smagghe G, Zotti MJ. Management of Pest Insects and Plant Diseases by Non-Transformative RNAi. Front Plant Sci 2019;10. https://doi.org/10.3389/FPLS.2019.01319/FULL.

Castro CG, Duarte KE, dos Reis RA, Moreira VA, Lombello RA, Castro JC, et al. Chitosan-dsRNA nanoparticles for targeted gene in the model C4 species *Setaria viridis*: synthesis, characterization, and preliminary assessment of fumarase gene silencing. Plant Nano Biology 2026;15:100243. https://doi.org/10.1016/J.PLANA.2025.100243.

Chen J, Peng Y, Zhang H, Wang K, Zhao C, Zhu G, et al. Off-target effects of RNAi correlate with the mismatch rate between dsRNA and non-target mRNA. RNA Biol 2021;18:1747–59. https://doi.org/10.1080/15476286.2020.1868680.

Cintra LA, Rosa J, Assis R, Marin SRR, Adegas FS, Rech EL, et al. RNAi spray-induced gene silencing of *EPSPS* by topical application of dsRNA in the weed *Digitaria insularis*. Front Plant Sci 2025;16:1688755. https://doi.org/10.3389/FPLS.2025.1688755/BIBTEX.

Comont D, Neve P. Adopting epidemiological approaches for herbicide resistance monitoring and management. Weed Res 2021;61:81–7. https://doi.org/10.1111/wre.12420.

Dalakouras A, Wassenegger M, Dadami E, Ganopoulos I, Pappas ML, Papadopoulou K. Genetically modified organism-free RNA interference: Exogenous application of RNA molecules in plants. Plant Physiol 2020;182:38–50. https://doi.org/10.1104/pp.19.00570.

Dubrovina AS, Kiselev K V. Exogenous RNAs for Gene Regulation and Plant Resistance. International Journal of Molecular Sciences 2019, Vol 20, 2019;20. https://doi.org/10.3390/IJMS20092282.

Dussault AA, Pouliot M. Rapid and simple comparison of messenger RNA levels using real-time PCR. Biol Proced Online 2006;8:1–10. https://doi.org/10.1251/BPO114.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001;411:494–8. https://doi.org/10.1038/35078107;KWRD=SCIENCE.

Gomes AC, Montgomery J, Araujo ALS, Morran S, Cutti L, Patterson E, et al. Chromosome-level assemblies of the allohexaploid genomes of *Conyza sumatrensis* and *Conyza bonariensis*. Genome Biol Evol 2025. https://doi.org/10.1093/GBE/EVAF065.

Heap I. International Herbicide-Resistant Weed Database 2026. http://www.weedscience.org/.

Huang S, Iandolino A, Peel G. Methods and Compositions for Introducing Nucleic Acids into Plants. U. 20180163219A1, 2018.

Ji L, Chen X. Regulation of small RNA stability: methylation and beyond. Cell Research 2012 22:4 2012;22:624–36. https://doi.org/10.1038/cr.2012.36.

Li Z, Rana TM. Molecular Mechanisms of RNA-Triggered Gene Silencing Machineries. Acc Chem Res 2012;45:1122. https://doi.org/10.1021/AR200253U.

Liu Q, Feng Y, Zhu Z. Dicer-like (DCL) proteins in plants. Funct Integr Genomics 2009;9:277–86. https://doi.org/10.1007/S10142-009-0111-5.

Liu Y, Hanati A, Lan H. Characterization of leaf trichomes and their influence on surface wettability of *Salsola ferganica*, an annual halophyte in the desert. Physiol Plant 2023;175. https://doi.org/10.1111/PPL.13905.

Mai J, Liao L, Ling R, Guo X, Lin J, Mo B, et al. Study on RNAi-based herbicide for *Mikania micrantha*. Synth Syst Biotechnol 2021;6:437–45. https://doi.org/10.1016/j.synbio.2021.11.005.

Mogren CL, Lundgren JG. In silico identification of off-target pesticidal dsRNA binding in honey bees (*Apis mellifera*). PeerJ 2017:e4131. https://doi.org/10.7717/PEERJ.4131/SUPP-1.

Montgomery J, Morran S, MacGregor DR, McElroy JS, Neve P, Neto C, et al. Current status of community resources and priorities for weed genomics research. Genome Biol 2024;25. https://doi.org/10.1186/s13059-024-03274-y.

Moretti ML, Alárcon-Reverte R, Pearce S, Morran S, Hanson BD. Transcription of putative tonoplast transporters in response to glyphosate and paraquat stress in *Conyza bonariensis* and *Conyza canadensis* and selection of reference genes for qRT-PCR. PLoS One 2017;12:e0180794. https://doi.org/10.1371/journal.pone.0180794.

Parker KM, Barragán Borrero V, Van Leeuwen DM, Lever MA, Mateescu B, Sander M. Environmental Fate of RNA Interference Pesticides: Adsorption and Degradation of Double-Stranded RNA Molecules in Agricultural Soils. Environ Sci Technol 2019;53:3027–36. https://doi.org/10.1021/ACS.EST.8B05576.

de Pinho CF, Lourenço Leal JF, dos Santos Souza A, de Oliveira GFPB, de Oliveira C, Langaro AC, et al. First evidence of multiple resistance of Sumatran Fleabane (*Conyza sumatrensis* (Retz.) E.Walker) to five-mode-of-action herbicides. Aust J Crop Sci 2019;13:1688–97. https://doi.org/10.21475/ajcs.19.13.10.p1981.

Polito RA, Viana VE, Camargo ER, Avila LA. Precise transcript targeting using RNAi for weed control. Advances in Weed Science 2024;42:e020240014. https://doi.org/10.51694/AdvWeedSci/2024;42:00016.

Powles SB, Yu Q. Evolution in Action: Plants Resistant to Herbicides. Annu Rev Plant Biol 2010;61:317–47. https://doi.org/10.1146/annurev-arplant-042809-112119.

Qin G, Gu H, Ma L, Peng Y, Deng XW, Chen Z, et al. Disruption of *phytoene desaturase* gene results in albino and dwarf phenotypes in *Arabidopsis* by impairing chlorophyll, carotenoid, and gibberellin biosynthesis. Cell Res 2007;17:471–82. https://doi.org/10.1038/CR.2007.40.

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. Rational siRNA design for RNA interference. Nat Biotechnol 2004;22:326–30. https://doi.org/10.1038/NBT936,.

Schwarz DS, Hutvágner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the Assembly of the RNAi Enzyme Complex. Cell 2003;115:199–208. https://doi.org/10.1016/S0092-8674(03)00759-1.

Shivashakarappa K, Marriboina S, Dumenyo K, Taheri A, Yadegari Z. Nanoparticle-mediated gene delivery techniques in plant systems. Frontiers in Nanotechnology 2025;7:1516180. https://doi.org/10.3389/FNANO.2025.1516180/FULL.

Silva PV da, Medeiros ES de, Schedenffeldt BF, Salmazo PAV, Borges RPN, Munaro FC, et al. Evaluation of leaf surface structure and control of *Conyza* spp. by herbicides applied in soybean pre-plant burndown. Brazilian Journal of Biology 2025;85:e295543. https://doi.org/10.1590/1519-6984.295543.

Travella S, Klimm TE, Keller B. RNA Interference-Based Gene Silencing as an Efficient Tool for Functional Genomics in Hexaploid Bread Wheat. Plant Physiol 2006;142:6. https://doi.org/10.1104/PP.106.084517.

Ui-Tei K, Naito Y, Takahashi F, Haraguchi T, Ohki-Hamazaki H, Juni A, et al. Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res 2004;32:936. https://doi.org/10.1093/NAR/GKH247.

Vetukuri RR, Dubey M, Kalyandurg PB, Carlsson AS, Whisson SC, Ortiz R. Spray-induced gene silencing: an innovative strategy for plant trait improvement and disease control. Crop Breeding and Applied Biotechnology 2021;21:e387921S11. https://doi.org/10.1590/1984-70332021V21SA24.

Zabala-Pardo D, Gaines T, Lamego FP, Avila LA. RNAi as a tool for weed management: Challenges and opportunities. Advances in Weed Science 2022;40. https://doi.org/10.51694/ADVWEEDSCI/2022;40:SEVENTY-FIVE006.

7. CONSIDERAÇÕES FINAIS

Em relação aos herbicidas inibidores da ALS, os resultados demonstram que a resistência está amplamente distribuída em populações de buva no Brasil, sendo predominantemente associada a mutações no gene *ALS*, especialmente na posição Pro197. A ocorrência de diferentes substituições, aliada à variação no número de cópias e na expressão gênica, resulta em níveis distintos de resistência e em padrões variáveis de resistência cruzada. Esses resultados evidenciam a contribuição conjunta de múltiplos mecanismos de resistência relacionados ao local de ação e reforçam a importância do uso estratégico de herbicidas de diferentes grupos químicos dentro dos inibidores da ALS, associados a herbicidas com mecanismos de ação alternativos, para o manejo de populações resistentes.

Quanto aos herbicidas auxínicos, a resistência ainda é menos frequente. A rápida necrose foi predominante em populações resistentes ao 2,4-D, mas também foi observada em populações que morreram após a aplicação. Esse fenótipo também foi verificado em resposta ao triclopir. A expressão do gene *PLP2* mostrou-se associada a esse fenótipo em diferentes populações de forma independente da expressão de transportadores ABC. Estes transportadores podem estar associados a resposta de rebrote após a aplicação dos herbicidas através de transporte diferencial de moléculas. O uso de misturas de herbicidas auxínicos e combinação com glifosato foi eficaz no controle de populações resistentes.

Em relação ao glifosato, os resultados evidenciam que a resistência em buva não é explicada por mutações isoladas no gene *EPSPS*. As substituições identificadas em cópias específicas do gene não são suficientes para explicar o fenótipo observado, indicando que a resistência resulta da interação entre múltiplos fatores, incluindo variação no número de cópias, expressão diferencial entre cópias gênicas e, possivelmente, mecanismos não relacionados ao local de ação com influência de condições ambientais. Esses resultados

demonstram que a resistência ao glifosato em buva é um caráter complexo, determinado pela interação de múltiplos mecanismos, e não por uma única alteração genética.

Por fim, a avaliação de abordagens baseadas em RNA representa um passo inicial para o desenvolvimento de estratégias de controle em buva, uma espécie com genoma hexaploide e barreiras foliares à absorção de moléculas exógenas. Os resultados evidenciam a limitação da absorção foliar, associada a estruturas como tricomas e cutícula, e indicam a necessidade de avanços em métodos de entrega e na síntese de siRNA e dsRNA, de forma a viabilizar a aplicação de maiores concentrações e aumentar a eficiência do silenciamento gênico.

De forma integrada, os resultados demonstram que a resistência a herbicidas em espécies de buva é amplamente distribuída e resulta da interação de múltiplos mecanismos genéticos e fisiológicos, com variação entre herbicidas, populações e condições ambientais. Esses padrões refletem também a dinâmica epidemiológica da resistência no campo, evidenciando sua dispersão espacial e heterogeneidade regional no Brasil. Esses resultados reforçam a necessidade de estratégias de manejo baseadas na compreensão dessa complexidade epidemiológica e molecular, bem como no uso de ferramentas moleculares para monitoramento e suporte à tomada de decisão.

Uso de ferramentas de inteligência artificial

Ferramentas de inteligência artificial, como ChatGPT e Grammarly, foram utilizadas pontualmente como apoio à revisão textual e à clareza da escrita científica.